BIOLOGICAL STUDIES ON HEAT-KILLED MYCOBACTERIUM VACCÆ

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ABSTRACT

A preparation of heat-killed *Mycobacterium vaccae* (SRL-172) is being investigated as a potential immunotherapeutic in mycobacterial infections, allergies, cancers, malaria, vascular disease, psoriasis, periodontal disease and other conditions. The potential effects of this agent upon specific lymphocyte subsets *in vitro* and humoral responses to the 60/65 kDa heat shock protein (hsp60/65) family *in vivo* were investigated.

Flow cytometric assessment of proliferation, activation marker expression (CD25, HLA-DR, CD69, CD80 and CD86) and production of the cytokines IFN-γ and IL-4 was performed after the incubation of freshly isolated peripheral blood mononuclear cells with dilutions of heat-killed *M. vaccae*. γδ T lymphocytes were assessed in experiments incorporating incubation with isopentenyl pyrophosphate, PPD and exogenous IL-2. Results demonstrated an influence on proliferation, CD25, CD69 and CD86 expression and cytokine production in γδ lymphocytes and proliferation, CD25 and CD69 expression in CD56+ NK lymphocytes. No significant effects on (CD56+ CD3+) NKT cells were demonstrated.

The majority of the gene for the *M. vaccae* hsp65 molecule was sequenced (for comparison with the human and known mycobacterial homologues, specifically with relation to epitopes implicated in autoimmune and other conditions) and the antibody titres to the human hsp60 molecule (including fragments there-of) and the *M. bovis* hsp65 protein were assessed in both rats and humans that had received placebo or combinations of immunisations including SRL-172. DNA sequence comparisons failed to highlight specific epitopes that may be implicated in the efficacy of SRL-172, but ELISA assays demonstrated a relative increase in IgG2a titres to the human (but not the mycobacterial) hsp60/65 protein in rats that had received SRL-172 prior to hsp65 immunisation, and a correlation between aortic contractility and IgG2a titres to human hsp60 in rodents that received both hsp65 and high-dose SRL-172 vaccination.

This work has increased the knowledge of the immunology behind this promising immunotherapeutic and suggested several areas for further research.
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1 GENERAL INTRODUCTION

1.1 Mycobacterium vaccae

*Mycobacterium vaccae* (*M. vaccae*) is one of about 60 named species within the genus *Mycobacterium*. It is rapidly growing, pigmented yellow and normally occurs as an environmental saprophyte.

The strain of *M. vaccae* used in the formulation of SRL-172 was first isolated from Uganda in 1971. Originally it contained both rough and smooth colonies and was designated R877, but a morphologically stable rough colony (R877R) was subsequently deposited and registered with the National Centre for Type Cultures as NCTC 11659.

Interest in the immunological potential of killed *M. vaccae* began with observations in the 1970s by Professor John Stanford that protein preparations from *M. vaccae* produced skin test responses in leprosy patients similar to those of *M. leprae* and also apparently lacked both type I and type III mycobacterial antigens (found in slow- and fast-growing species respectively) in a precipitin analysis. These properties appeared unique and suggested, at the time, that *M. vaccae* may have the ability to prime protective and not pathogenic responses to mycobacterial infections (J Stanford, personal communications). Initially preparations of killed *M. vaccae* were assessed in the treatments of mycobacterial infections but now a preparation of heat-killed *M. vaccae*, SRL-172, is also being studied for efficacy in cancers, allergies and other conditions.

1.2 SRL-172

The immunotherapeutic preparation, SRL-172, is produced to Good Manufacturing Practice from conventionally cultured *M. vaccae*. The organisms
are grown on Sauton’s medium solidified with 1.5% agar and then killed by autoclaving at 121°C/151b for 15 minutes. The heat-killed product is then suspended in borate buffered saline (pH 8.0; comprising sodium tetraborate, boric acid, sodium chloride, Tween80 and purified water) and stored, protected from the light, at 2 to 8°C.

The final sterile product comprises 10mg (wet weight)/ml, equivalent to approximately $10^9$ bacilli of *M. vaccae* per 0.1ml. It contains orange-yellow particles that are easily resuspended, resulting in a turbid solution.

The usual dose in human studies is 0.1ml injected intradermally over the upper third of the deltoid muscle with a 26 gauge needle.

### 1.3 Known Immunology of SRL-172

*Type 1/Type 2 Balance and Cytokine Profiles:*

In 1986 it was reported that cloned murine helper T cells could be divided into two main subsets on the cytokines that they produced (Mosmann et al., 1986). Th1 cells produced interferon-gamma (IFN-γ) and also interleukin-2 (IL-2), and Th2 cells produced mainly IL-4, but also IL-5, IL-6 and IL-10. The cytokine profiles of these clones correlated with functional properties - Th1 CD4 lymphocytes promote cell-mediated immune responses and Th2 CD4 lymphocytes aid antibody production and the humoral arm of the adaptive immune response. Within a few years evidence was accumulating for a similar dichotomy within human T lymphocytes (Romagnani, 1991).

It soon became apparent however that the complexity of an immune response could not be fully explained or contained within a model of this simplicity. Cytokines were discovered that promoted cellular responses (such as IL-12) that were not produced by T lymphocytes, other cell types were found to secrete Th1- or Th2-type cytokines, Th1 cells were found to promote some humoral responses (e.g. the production of IgG2a in the mouse) and many human CD4+ T cells could
not be categorised as either Th1 or Th2 as they produced cytokines associated with both sub-types or only a single, non-defining cytokine. Though flawed this model has provided many insights into protective and mal-adaptive immune responses to infections, malignancies, allergies and inflammatory conditions. The terminology can be shifted to the use of the terms type-1 and type-2 cytokines to encompass cells such as CD8+ T lymphocytes, monocyte-macrophages, dendritic cells, natural killer cells, mast cells, eosinophils and B cells that can produce cytokines that fit within this general pattern.

Several lines of experimental data illustrate an effect of heat-killed *M. vaccae* on such cytokine balances. Vaccination with $10^7$ killed organisms has been shown to elicit a type 1 dominated response in murine splenocytes, whilst doses equivalent to $10^9$ organisms tended to induce a mixed type 1/type 2 profile (with increased IL-4 production) (Hernandez-Pando and Rook, 1994). Other supportive evidence for an influence on Th1/Th2 balance comes from studies on the pathogenesis of tuberculosis in mice pre-immunised with SRL-172, where animals receiving an equivalent of $10^7$ killed bacilli were partially protected and maintained a high IL-2/IL-4 ratio within the lung (compared to controls), but mice that received a high dose of SRL-172 (equivalent to $10^9$ organisms) died more rapidly with a massive pneumonia, correlating with a mixed cytokine response (Hernandez-Pando et al., 1997).

Separately, a decrease in a Th2 response as a result of immunisation with SRL-172 *in-vivo* was illustrated in a murine allergy model utilising ovalbumin (OVA) (Wang and Rook, 1998). BALB/c mice received two immunisations with OVA in incomplete Freund’s adjuvant and were confirmed to have developed a type 2 response by the demonstration of elevated levels of serum IgE and the secretion of IL-5 (but not IL-2) by splenocytes exposed to OVA *in-vitro*. Later injections of SRL-172, at doses equivalent to $10^7$, $10^8$ and $10^9$ killed bacilli, suppressed this allergen-induced IL-5 secretion and decreased the serum IgE level.

It is also interesting to note that it is a type-1 cytokine response that has been utilised as a potency assay for SRL-172 in its manufacturing. THP1 cells, a human
monocyte cell line, secrete IL-12 when cultured in-vitro with SRL-172, and ELISA estimates of this cytokine are used to compare batches of the product.

**Active Components:**
The Genesis Research and Development Corporation in New Zealand have approached the basic science and immunology of heat-killed *M. vaccae* from a different direction (Tan et al., Patent Document). In an attempt to identify specific active components within the heat-killed product they produced 28 recombinant proteins from *M. vaccae* and analysed these in terms of their ability to induce either proliferation or IFN-γ secretion in mouse splenocytes and human peripheral blood mononuclear cells.

Several proteins appeared to induce proliferation and IFN-γ secretion in both humans and mice - the heat shock protein 65 (hsp65) molecule, the Ag85 complex, an ABC transporter, a histone-like homologue, a fibronectin attachment homologue and a molecule of unknown function (GV7). They further analysed the abilities of the recombinant proteins to act as adjuvants, evaluating the induction of IL-12 secretion by macrophages and influences upon a cytotoxicity assay (involving simultaneous immunisation with OVA followed by assessment of the cytotoxic response to OVA-transfected syngeneic tumour cells). It was found that the Ag85 complex, the ABC transporter and a separate molecule of unknown function (GV22B) were all potent inducers of macrophage IL-12 production and that hsp65, the ABC transporter and the histone-like homologue all significantly increased the lysis of the transfected target cells. However they failed to identify a specific protein, or combination, that matched the activity of whole heat-killed *M. vaccae* in their assays.

Therefore they adopted an alternative approach and adapted autoclaved *M. vaccae* by washing, solvent extractions and ethanol refluxing (removing soluble components, neutral lipids and extractable glycolipids respectively). The resultant product, termed pVac, was then utilised in clinical studies and has shown activity in psoriasis but not in asthma. In-vitro it has enhanced ability (when compared to untreated autoclaved *M. vaccae*) to induce macrophage production of IL-12 and may increase cytotoxicity in the above model. In humans it is able to induce a
local DTH response on intradermal injection, induce proliferation and CD69 expression on T lymphocytes and promote the secretion of the cytokines IL-1β, IL-12, TNFα, IFN-γ and IL-10 by peripheral blood mononuclear cells.

It was also shown to enhance CD40 and CD80 expression on dendritic cells.

**Effects On Antigen Presenting Cell Surface Markers:**

As mentioned above, pVac, the de-lipidated version of heat-killed *M. vaccae*, enhanced the expression of both CD40 and CD80 on dendritic cells. SRL-172 itself has been studied for effects on the human monocytic cell line THP1 where it too up-regulated the surface expression of CD40 and CD80, and also CD86 and ICAM-1 (Armenika Etemi, personal communication).

### 1.4 Putative Efficacies of SRL-172

Immunotherapy with SRL-172 is being actively investigated in several diseases. These conditions provide the basis for the directions of much of the basic science studies being performed on this product, including those contained within this thesis. The data supporting the putative efficacy in each of these settings will be discussed in this section.

#### 1.4.1 Tuberculosis

The immunotherapeutic SRL-172 was first designed as a potential therapy for tuberculosis. To date, despite multiple trials, it has yet to be generally accepted and incorporated into treatment strategies. This is partly due to conflicting results from several animal and human studies.

**1.4.1.1 Animal Models**

Initial studies in animals failed to show any effect of vaccination with heat-killed *M. vaccae* (though doses approximating $10^9$ bacilli were used) on either relapse of infection in the Cornell mouse model (Dhillon and Mitchison, 1994) or prevention of experimental *M. bovis* infection in cattle (Buddle et al., 1995). However later...
studies showed that heat-killed *M. vaccae* afforded some limited protection from tuberculosis in other mouse models (which was abrogated if the *M. vaccae* was genetically altered to express the 19kDa lipoprotein of *M. tuberculosis*) (Abou-Zeid et al., 1997), and further that doses of $10^7$ killed organisms were protective but this effect was reversed (with increased mortality) at doses approximating $10^9$ killed bacilli (Hernandez-Pando et al., 1997). Recent studies have demonstrated that therapeutic vaccination with SRL-172 in mice infected via the trachea with *M. tuberculosis* restored a type-1 cytokine pattern, increased IL-1α and TNF-α expression and encouraged a switch from pneumonia to granuloma within the lungs (Hernandez-Pando et al., 2000).

Splenocytes from mice that have received heat-killed *M. vaccae* have been shown to proliferate and secrete IFN-γ when exposed to live *M. tuberculosis* *in-vitro*. CD8+ lymphocytes from these animals, isolated at 2 weeks, were able to kill syngeneic macrophages infected with *M. tuberculosis*. After 3 weeks these cells could no longer be isolated, but became re-apparent after stimulation with either killed *M. vaccae* or *M. tuberculosis* *in-vitro* suggesting the development of a memory phenotype (Skinner et al., 1997).

The only primate data to date involved *M. tuberculosis* infection of cynomologus monkeys who received intra-dermal or intra-tracheal heat-killed *M. vaccae*. The product, by either route, increased survival to levels comparable to vaccination with live BCG (Genesis Corporation, personal communication).

In other animal models it has been reported that heat-killed *M. vaccae* demonstrated statistically significant protection from tuberculosis in a rat pneumoconiosis model (Yang et al., 1997), but failed to demonstrate any effect in a small study of cavitatory tuberculosis in rabbits (Converse et al., 1998). Vaccination has however been shown to protect certain species of duck from natural acquisition of tuberculosis due to *M. avium* (Cromie et al., 2000).

### 1.4.1.2 Human Studies

The majority of trials of the potential efficacy of killed *M. vaccae* in tuberculosis have been performed in humans.
Early studies from Kuwait on vaccination with radiation-killed *M. vaccae* failed to exhibit a bacteriological benefit but did demonstrate improved weight gain and immunological parameters such as lymphocyte proliferation and antibody responses to mycobacterial antigens (Stanford et al., 1990). This preparation was then compared with various others, including autoclaved (heat-killed) *M. vaccae*, and the latter was shown to improve cavitory healing on chest X-ray films and was adopted for future work (Bahr et al., 1990).

Other preliminary studies, not performed to Good Clinical Practice (GCP) guidelines, have demonstrated significant effects on survival and bacteriological cure when heat-killed *M. vaccae* was added onto poor or failing treatment regimens, however not when added to adequate conventional regimens. Examples of the former are a trial in Nigeria with patients receiving sub-optimal and incomplete regimens, where SRL-172 vaccination was shown to lead to earlier sputum conversion, an increased proportion of bacteriological cure, improved weight gain and decreased ESR levels (Onyebujoh et al., 1995), and Romanian patients with chronic or relapsing disease (and therefore, by implication, in receipt of sub-optimal therapy) who demonstrated improved bacteriological cure, weight gain, chest X-ray film changes and ESR (Corlan et al., 1997a). The experience of the use of SRL-172 immunotherapy in patients with multi-drug resistant tuberculosis has recently been published and suggested some potential efficacy when compared to historical controls (Stanford et al., 2001).

An example of a study with adequate drug therapy demonstrated a trend to better recovery in newly diagnosed Romanian patients that failed to reach statistical significance (Corlan et al., 1997b). Such trials with good conventional treatment have however shown significant improvements in secondary end-points, such as the weight gain, ESR, time to resolution of fever, radiology and sputum conversion (as well as decreases in serum levels of IL-4, IL-10 and TNF-α with increases in serum IFN-γ) in Argentinian HIV sero-negative patients (Dlugovitzky et al., 1999).

Two studies have been performed to GCP standard. No apparent effect of SRL-172 immunotherapy was seen in a large placebo-controlled trial from Durban, South Africa, that enrolled both HIV sero-positive and HIV sero-negative
tuberculosis patients (Durban Immunotherapy Trial Group, 1999), but there was an increased sputum conversion rate at 1 month and improved radiological appearances at 6 and 12 months in a trial enrolling 120 HIV sero-negative Ugandan patients with newly diagnosed tuberculosis (Johnson et al., 2000). The improved sputum conversion rates were no longer significant at 2 months and thereafter, however the majority of patients exhibited bacteriological cure by this stage.

Several trials are still on-going or are due to commence shortly. A study of the use of SRL-172 in over 1500 Zambian and Malawian patients with newly diagnosed tuberculosis is due to report in 2001, a trial examining prophylactic vaccination with SRL-172 in HIV-positive individuals (with the incidence of disseminated mycobacterial disease a primary end-point) is funded and due to commence shortly in sub-Saharan Africa and a study of multi-drug resistant tuberculosis is planned for the near future in Estonia and the Baltic States.

1.4.2 Leprosy
The other major mycobacterial illness for which vaccination with SRL-172 has been investigated is infection with \textit{M. leprae}.

1.4.2.1 Animal Models
Due to the lack of adequate models for this disease there have been few animal experiments aimed at addressing the protective response of killed \textit{M. vaccae} in leprosy. The only data available showed a lack of protection afforded by killed \textit{M. vaccae} in a murine leprosy challenge model (Singh et al., 1989).

1.4.2.2 Human Studies
In humans a combination of killed \textit{M. vaccae} and BCG has been shown to be more efficacious at stimulating skin-test positivity for leprosin A than either BCG alone or a combination of BCG and killed \textit{M. leprae} (Ganapati et al., 1989). Vaccination with killed \textit{M. vaccae} alone in people who had previously received BCG also increased the response to leprosin A on skin testing 8 years later
(Stanford et al., 1989). This effect has also been demonstrated in Argentinean contacts of leprosy cases (Bottasso et al., 1998).

The use of a combined BCG/M. vaccae vaccination demonstrated a trend towards a decrease in leprosy incidence when compared to BCG alone that failed to reach statistical significance (though follow-up was reported to be difficult) (Ghazi Saidi et al., 1989).

1.4.3 Asthma

Recent studies have provided better supportive data for the use of SRL-172 in asthma.

Asthma and allergies were initially investigated as potential diseases in which SRL-172 may have a therapeutic role due to the immunological data suggesting a role in Th2/Th1 switching (see earlier section). More recently the evolution of the ‘Hygiene Hypothesis’ has furthered the impetus to investigate the role of vaccination with killed M. vaccae in these diseases. Briefly this hypothesis states that the immune system, analogously to the developing brain, requires ‘information’ input and without it is deprived of essential fine-tuning in its functions and responses (Rook and Stanford, 1998). Thus, though it is still capable of essential physiological processes, it may not function appropriately at the more subtle responses, for example the correct cytokine and regulatory reaction to an immunogen (Rook et al., 2000). This hypothesis can be utilised to explain the apparent increases in both allergic and autoimmune diseases encountered in the increasingly ‘clean and sterile’ developed world. Mycobacteria are ubiquitous within the environment and exposure to them has been part of mankind’s evolutionary history for millennia. As a result they may be one factor utilised by the immune system for ‘fine tuning’ and an immunisation with a heat-killed environmental mycobacterium, such as M. vaccae, may provide some of the required input whose lack might otherwise lead to, or perpetuate, atopies and other diseases.
1.4.3.1 Animal Models
The efficacy of SRL-172 in decreasing pulmonary allergic responses has been assessed in mice sensitised to ovalbumin (OVA) and challenged with methacholine. Vaccination with SRL-172 three weeks prior to the first OVA immunisation reduced local and systemic eosinophilia and decreased airway hyper-responsiveness to inhaled methacholine (Zuany-Amorim et al., 2001). Also, as discussed in the previous section, a murine model of induced allergy to ovalbumin (OVA) with later vaccination with SRL-172 demonstrated an inhibition of the IgE and IL-5 responses to OVA (Wang and Rook, 1998) and in a similar mouse atopy model pre-immunisation with a killed *M. vaccae*/killed BCG combination at birth decreased the later induction of an OVA allergic response as measured by total serum IgE in both female and male animals (as opposed to females only with killed BCG alone) (Tukenmez et al., 1999).

Further murine asthma model studies are presently planned.

1.4.3.2 Human Studies
In a placebo controlled study of hay fever sufferers 3 doses of SRL-172 were given at monthly intervals just prior to the start of the hay fever season. Diary cards illustrated, in those patients with a wheezing component to their illness, a significant decrease in chest symptoms, bronchodilator use and an improvement in peak expiratory flow rate measurements over the season (Hopkin et al., 1998).

A placebo controlled phase I/II GCP study of the use of intradermal SRL-172 in 24 mild to moderate allergic asthmatics has been performed (personal communication, G Rook). On allergen challenge there was a 30% decrease in FEV\textsubscript{1} changes during the late airway response (p=0.019) and a reduced maximal fall in FEV\textsubscript{1} (p=0.005). There was also a significant gradual decrease in allergen-induced *in-vitro* production of IL-5 (p=0.046), but only a trend to a decrease in total serum IgE levels. However a separate study comparing placebo, heat-killed *M. vaccae* and de-lipidated *M. vaccae* failed to demonstrate any effects of either product on clinical or immunological outcomes (including eosinophil count, IgE levels and T cell proliferative and cytokine responses) in 43 house dust mite allergic, stable, moderately severe asthmatics (Shirtcliffe et al., 2001).
1.4.4 Cancer

As with asthma and allergies the initial impetus to investigate a role of vaccination with SRL-172 in oncology came from the immunological data suggesting an ability to boost Th1 and downregulate Th2 responses, thereby potentially increasing cell-mediated immune responses to neoplasia. The majority of studies are in human patients and are presently on-going.

1.4.4.1 Animal Models

In rat prostate cancer models SRL-172 has been shown to result in improved survival and reduced tumour growth when combined with autologous cancer cells, but not on its own or in prevention models (Hrouda et al., 1998b). Preparations of *M. vaccae*, though not heat-killed, have been investigated in several other animal and *in-vitro* models. A sonicate of *M. vaccae* has been shown to inhibit the *in-vitro* growth of murine ascitic plasmacytomas (Moras et al., 1985), and other workers have identified an antineoplastic water-soluble protein-polysaccharide complex derived from *M. vaccae* (Tian and Groves, 1999; Tian et al., 1999). This complex, extracted from boiled *M. vaccae*, was shown to be active in an *in-vivo* murine sarcoma model and to activate murine peritoneal macrophages *in-vitro*.

1.4.4.2 Human Studies

1.4.4.2.1 Prostatic Carcinoma

Patients with hormone-refractory prostatic carcinoma, who had a poor prognosis, have been vaccinated with SRL-172 at regular intervals and 2 out of 10 in a published (non placebo-controlled) series had some clinical response as assessed by a PSA decrease (which correlated with improved IL-2 production by peripheral blood mononuclear cells) (Hrouda et al., 1998a).

1.4.4.2.2 Melanoma

In a phase I/II study of advanced stage IV malignant melanoma, immunisation with SRL-172 improved survival (relative to historical controls). This improved
prognosis correlated with increased intracellular IL-2 staining of peripheral blood lymphocytes (as assessed by flow cytometry) (Maraveyas et al., 1999).

1.4.4.2.3 Lung Carcinoma and Mesothelioma

SRL-172, in combination with chemotherapy, is being used in the treatment of small cell lung cancer and, though the study is still on-going, interim results have shown an increase in median survival from 8.5 months (chemotherapy alone) to 13.8 months (chemotherapy + SRL-172) \( p=0.07 \) (G. Bowen, personal communication).

In a study of the use of SRL-172 in 28 patients with inoperable non-small cell lung carcinoma or mesothelioma there were trends to improved response rates (54% vs. 33%), median survival (9.7 months vs. 7.5 months) and one year survival (42% vs. 18%) in the combined arm compared to chemotherapy alone (O'Brien et al., 2000). This was associated with a statistically significant improvement in appetite but no apparent changes in serum IFN-γ or TNF-α levels.

Intra-tumoral SRL-172 is also being investigated, in combination with chemotherapy, for the treatment of mesothelioma. 15 patients received either of 4 differing doses (from 1μg to 1mg) of SRL-172 into their tumours at 3 weekly intervals, with intradermal standard dose SRL-172 being given 4 weekly to all patients. Symptomatic responses were recorded in 8 of the 14 assessable patients, with the lower intra-tumoral doses potentially being more active than the 1mg dose regimen. All patients demonstrated a decrease in IL-4 producing T lymphocytes and an increase in activated NK cells, though these parameters did not correlate with clinical response.

A pan-European 420 patient phase III study in symptomatic non-small cell lung cancer is due to formally report in 2001, but information presently available indicates no significant difference between treatment groups.

1.4.4.2.4 Renal Carcinoma

There is no data to date in this condition though a 60 patient UK/France phase II study is due to report late in 2001.
1.4.5 Vascular Disease

Immunisation with heat-killed *M. vaccae* has been investigated in several models of vascular disease. The rationale behind these studies have been two-fold. Clinically it has been observed that patients with chronic leprosy treated with SRL-172 developed warmer hands, with increased capillary blood flow confirmed by Doppler ultrasound (J Stanford, personal communication), and immunologically there is increasing evidence that the 60-65kDa heat shock protein family may be involved in the aetiology of arteriosclerosis and it has been hypothesised that SRL-172 may alter immune reactivity to this family of molecules (referenced and discussed in section 4.2). As a result rat models of aortic contractility, post-angioplasty stenosis and aortic transplantation have been studied.

Results illustrated a significant effect of pre-immunisation with SRL-172 on aortic contractility and a significant inhibition of the intimal thickening seen in aortic allografts and in native arteries after angioplasty (C.Berwanger & G.Rook, personal communications).

1.4.6 Psoriasis

Chance observations of improvements in the psoriasis of a leprosy patient after receipt of SRL-172 (personal communication, J Stanford) supported the investigation of possible immunotherapy with heat-killed *M. vaccae* for this disease. This rationale was aided by suggestions that psoriasis is secondary to a dysregulated response to the heat shock protein, hsp60/65 (Rambukkana et al., 1992; Rambukkana et al., 1993), which might be influenced by immunisation with SRL-172 (see section 4.2).

No animal models have been investigated, however two studies have been performed with heat-killed *M. vaccae* in humans - both demonstrating efficacy. In the first, 21 patients with chronic plaque psoriasis received SRL-172 (with a further 14 subjects receiving placebo) (Lehrer et al., 1998). The SRL-172 group showed a statistically significant improvement in disease scores, with the effect
from a single injection lasting up to 6 months, and the receipt of a further injection increasing this to 1 year.

In the second, a 2-dose vaccination regimen of heat-killed *M. vaccae* resulted in marked improvements in the majority of 24 patients with moderate to severe psoriasis (5 patients demonstrating complete clearance of lesions at 6 months) (Balagon et al., 2000). However this study did not include a placebo arm.

Phase II GCP studies are presently being performed in this area by Corixa, Seattle, USA.

1.4.7 Malaria

The work on malaria comes from both *in-vitro* and *in-vivo* animal studies on the use of SRL-172 as an adjuvant to malarial vaccines. This work is as yet unpublished but demonstrates that vaccinating mice subcutaneously with a combination of SRL-172 and a merozoite glycolipid extract, followed by boosting with the merozoite extract 2 weeks later and challenging with *Plasmodium yoelii* after a further 3 weeks, resulted in a significantly decreased parasitaemia and improved survival. Unvaccinated controls demonstrated 100% mortality, and mice in receipt of the merozoite extract only (no heat-killed *M. vaccae*) showed improved survival and parasite levels but not to the degree of the combined vaccination protocol (B deSouza, personal communications).

In a separate protocol vaccinating with SRL-172 intravenously 4 days before infective challenge (and with no malarial extracts) also significantly improved parasitaemia and prognosis. Work in progress is implicating both NK and γδ lymphocytes in this improved response (possibly by early cytokine secretion) (B deSouza, personal communications).

There have been, as yet, no studies on the use of heat-killed *M. vaccae* in human malaria.

1.4.8 Periodontal Disease

Inflammation of the gums, induced by oral micro-organisms, may either be non-destructive (gingivitis) or destructive (periodontitis). This dichotomy correlates with the local cytokine secretion profiles - the destruction being associated with a
shift from a Th1 to a Th2 or Th0 profile (Tokoro et al., 1997). In a rat model of ligature-induced periodontal disease pre-immunisation with SRL-172 was shown to markedly diminish the destruction of the tooth-supporting tissues, potentially correlating with its ability to down-regulate Th2 responses (Breivik and Rook, 2000). Immunotherapy with SRL-172 has now also demonstrated efficacy in a treatment model of periodontitis (G Rook, personal communication).
2 AIMS & OBJECTIVES

The principle aim of this research is the investigation of the immunological effects of SRL-172, a preparation of heat-killed *M. vaccae* NCTC 11659, which is being investigated as a potential immunotherapeutic agent for mycobacterial infections, cancers, asthma and other conditions.

The research investigates two main areas that, in theory, may explain certain of the known efficacies and immunological effects of SRL-172:

1. Effects upon lymphocyte subsets *in-vitro*, specifically:
   - γδ T cells
   - Natural Killer (NK) cells
   - NKT cells

2. Effects on the *in-vivo* humoral responses to the heat shock protein hsp60/65 family in both humans and rats. As part of this investigation the gene for the hsp65 molecule of *M. vaccae* NCTC 11659 has been amplified and sequenced to allow comparisons of specific epitopes.

The overall objective was to increase the knowledge of the basic immunology behind this promising immunotherapeutic agent.
3 IMMUNE CELL ACTIVATION

3.1 Flow Cytometry

The development of flow cytometry (also known as fluorescent activated cell scanning/FACS) has provided a useful method of rapidly and easily assessing multiple parameters, including cytokine production and phenotypic markers, for large numbers of cells. This technique was therefore employed to assess the effects of heat-killed \textit{M. vaccae} on immune cell activation \textit{in-vitro}.

3.1.1 Principle

Flow cytometry is a technique that can provide information on the relative size (by forward scatter of laser light), relative granularity (by side scatter of the same light) and relative fluorescence intensities (at several wavelengths) of individual cells. Fluorochromes can be used as stains (usually by being conjugated to specific antibodies) which can be detected by their emission spectra after excitation with laser light. The relative fluorescence intensity detected is proportional to the number of binding sites for the stain, i.e. proportional to the amount of cellular constituent in question.

The flow cytometer consists of three main systems. The first is a fluidics system for introducing and focusing the cells (allowing them to be analysed individually), the second an optical system for generating and collecting light signals, and the third a system of electronics to convert the optical signals into proportional electronic signals and to digitalize them for computer analysis.

The machine used for the experiments contained in this thesis was the FACSCaliber (Becton Dickinson) which contains a 15mW 488nm air-cooled argon-ion laser and a 635nm red diode laser. The detectors FL1, 2, 3 and 4 detect light within wavelength ranges 500-560nm, 560-627nm, $\geq$670nm and 645-677nm respectively due to a system of optical filters. FL1, 2 and 3 are used with the
488nm laser and FL4 with the 635nm laser. The resultant data are analysed with CellQuest software (Becton Dickinson).

Several fluorochromes are available for use in flow cytometry. The 488nm laser can excite fluorescein isothiocyanate (FITC) (and the similar carboxyfluorescein (CFS)), phycoerythrin (PE) and peridinin chlorophyll protein (PerCP) which emit maximally at 520nm, 576nm and 677nm respectively. A fluorochrome which is a conjugate of a cyanine dye and phycoerythrin (PE-Cy5) can also be used - the light at 488nm excites the PE molecule which then passes the majority of the resultant energy to the Cy5 molecule, which emits at 670nm. The FACSCaliber cytometer second laser (635nm) is able to excite allophycocyanin (APC) which then emits maximally at 660nm.

As the fluorochromes often have overlapping emission spectra the detector primarily set up to detect one dye may also detect small amounts of another dye’s emission spectrum. The software enables the user to compensate the various detectors, effectively eliminating this cross-over of detected spectra.

An important evolution in flow cytometry was the development of protocols to stain intracellular targets. This depends on techniques that fix and permeabilise the cell membranes allowing antibodies to access the internal cellular components. Most protocols use detergents (principally Tween20 or saponin) or alcohols (ethanol or methanol) to permeabilise the cell membrane. Another requirement for the detection of cytokines produced by the cell is to encourage their intracellular accumulation (to reach detectable levels). This is achieved by the use of agents that inhibit their secretion from the cell (principally monensin and brefeldin A).

3.1.2 Standardisation

The basic protocol for flow cytometric estimation of intracellular cytokine production was obtained from Becton Dickinson and the conditions and timings of each stage were assessed and adjusted to provide optimal results as follows:

3.1.2.1 Blood Collection

Blood on drawing was placed in either sterile laboratory tubes with added sterile preservative-free Na⁺ heparin, Vacutainers containing lithium heparin either filled
or with only 2ml blood, Vacutainers containing EDTA and plain Vacutainers with added preservative-free Na\(^+\) heparin. The added heparin was used at 5-10\(\mu\)g/ml blood. Tubes were left overnight and then stimulated (with PMA and ionomycin) and stained for CD4 (FITC-labelled, Becton Dickinson) and IL-2 (PE-labelled, Becton Dickinson).

Vacutainers with EDTA, as expected, showed very low IL-2 levels (the calcium chelating action of EDTA interfering with the stimulation by ionomycin). Of the other containers the laboratory tube with Na heparin and the lithium heparin Vacutainers (either full or with 2ml blood) were comparable, but the plain Vacutainer with added Na heparin showed less IL-2 positive cells. The reason for this is not clear but may relate to preservatives within the plain Vacutainers.

Plain laboratory tubes with added Na heparin were therefore chosen for ease, cost and availability.

### 3.1.2.2 Stimulation Stage

For the assessment of the cytokine profiles of peripheral blood lymphocytes some kind of stimulation is required. This may be antigen based, in which case only antigen-specific cells will be expected to respond. However if an assessment of the overall bias in lymphocyte cytokine production is to be assessed (e.g. whether there is a Th1 or Th2 profile) then most or all lymphocytes must be stimulated.

Activation of T lymphocytes requires at least activation of protein kinase C and an increase in cytosolic free calcium (both of which normally occur upon TCR ligation). Anti-CD3 molecules can partially produce these effects. Phorbol esters, such as PMA (phorbol 12-myristate 13-acetate), are able to activate protein kinase C, and calcium ionophores, such as ionomycin, allow influx of \(Ca^{2+}\) from extracellular pools. The combination of PMA and ionomycin is therefore commonly used.

The effect of varying the length of stimulation was assessed by determining IL-2 and IFN-\(\gamma\) production by lymphocytes from freshly drawn venous blood. Standard doses of PMA (final concentration of 25 ng/ml) and ionomycin (final concentration of 1 \(\mu\)g/ml)(both Sigma) were used, with monensin (Sigma) at 3 \(\mu\)M to promote the intracellular accumulation of the cytokines. The blood was incubated for the
stimulation step at 37°C and 5% CO₂ and anti-IL-2 PE (Becton Dickinson) and anti-IFN-γ CFS (R&D) were used following the standard protocol (see later). Results are shown in figure 3.1.2.2 a and demonstrate a significant increase in the detected proportion of cytokine producing cells with increased time of stimulation (over the tested time-course).

It is however known that, in mice, PMA alone may cause a transient loss of CD4 expression on the surface of T lymphocytes, which is accentuated by the addition of ionomycin (also leading to a partial decrease in surface CD8). In humans PMA alone is known to induce significant loss of surface CD4 and partial loss of CD8, with no synergism from the addition of ionomycin (Anderson and Coleclough, 1993). Therefore the length of stimulation may significantly affect the reliability of surface staining for these glycopeptides.

This was assessed on fresh whole blood stimulated with PMA and ionomycin (at 25ng/ml and 1μg/ml respectively and monensin at 3μM) (all Sigma) for 0, 1, 2, 3, 4, 5 and 6 hours and stained with PerCP-labelled anti-CD4 (Becton Dickinson) and PE-labelled anti-CD8 (Becton Dickinson). The results for CD4 staining are shown in figure 3.1.2.2.b.
Figure 3.1.2.2 a: Effect of length of incubation with stimulants PMA and ionomycin on staining for IL-2 and IFN-gamma.

Figure 3.1.2.2.b: Effect of Length of PMA/Ionomycin Stimulation on CD4 Surface Staining
As shown there was significant downregulation of surface CD4 from 4 hours onwards. Though at the 4 hour time-point CD4 positive and CD4 negative cells were still easily discernible, stimulation times longer than this produced CD4 downregulation to a degree where the positive and negative groups could no longer be confidently separated. There did not appear to be any significant downregulation of surface CD8 during the 6 hour time-course of this experiment (data not shown).

As a result 4 hours was chosen as the standard length of stimulation with these stimulants. A separate conclusion from these experiments is that the stimulation times must be kept strictly constant between experiments whose results are to be directly compared.

3.1.2.3 Cell Surface Staining
The timing of the cell surface staining stage may obviously affect the degree of staining achieved but the temperature of the incubation step is also potentially important as 4°C could theoretically decrease the internalisation of cross-linked surface molecules and higher temperatures would theoretically encourage the association of the antibody and antigen.

Experiments were performed using FITC-labelled anti-human CD4 (Becton Dickinson) on freshly drawn normal volunteer blood. 15min vs. 30min and 4°C vs. RT were directly compared. Geometric means of fluorescent intensity and percentage of cells in the resultant stained population were used for comparisons between groups. Results showed that 15min at RT, 30min at RT and 30min at 4°C were comparable and superior to 15min at 4°C (data not shown). 15min at RT was therefore adopted for convenience.

3.1.2.4 Stains
New stains, whether for surface molecules or intracellular cytokines, were assessed before first use to determine the optimal dose and variability of response
produced by small variations in dose. The establishment of the doses of stain that produced a plateau in the percent of 'positive' cells and the geometric mean of fluorescence intensity of these positively staining cells provide a graphical way of determining both factors. The dose range analysed is typically the manufacturers' recommended to a log₁₀ decrement below. Optimal doses would be those that produced degrees of staining well onto the plateau.

Some stains were rejected as not being suitable or robust, e.g. where a plateau is not achieved over the dose range suggested and any potential minor variation in dose or conditions may produce differing results.

3.1.2.5 Controls

Various types of controls can be used in flow cytometry. The more commonly used are isotype-matched, ligand-blocked, unconjugated-antibody-blocked and unstimulated controls.

Isotype-matched immunoglobulin of irrelevant specificity can be used to stain target cells at the same concentration as the principal antibody. The underlying hypothesis is that the degree of non-specific binding to the target cell is mainly dependent on the isotype of the stain and therefore will be comparable between the two antibodies. Extensive screening of potential isotype controls is generally performed by the manufacturers to ensure that there is no unusual or specific binding, though the suitability should be assessed with each new isotype purchased in a similar manner to that of the stains themselves (see above). To be a suitable control the geometric means of fluorescence intensity and histogram profiles should match those of the 'negative' cells with an equivalent dose of stain over the potential dose range.

Ligand-blocked controlling consists of pre-incubating the antibody with an excess of the ligand of interest, before adding to the target cells. Any staining is therefore theoretically non-Fab mediated. This control is particularly suitable for demonstrating the specificity of the stain. The main drawback however is the extra cost involved in purchasing the ligand.

Unconjugated-antibody-blocked controlling involves the pre-incubation of target cells with unlabelled antibody (of exactly the same type as the stain) prior to
staining with the fluorochrome-conjugated antibody. Again the main drawbacks are the increased costs of reagents and the increased length of assays.

For some assays an unstimulated control is suitable and cheaper than many of the alternatives. Here the usual fluorochrome-conjugated antibody is used to stain the target cells of interest and also cells that have been unstimulated (either by antigen or mitogens). The increase in target ligand seen in the stimulated cells above that in the normal, unstimulated cells can therefore be easily calculated. This control therefore removes non-specific binding and also background, resting levels of the target ligand. The major theoretical problem with this control is that stimulated and unstimulated cells may not necessarily be comparable in terms of non-specific binding, though in practice this appears to be only theoretical and not of significance for lymphocytes.

3.1.2.6 Effects of Delays in Staining/Reading

The effects of delays between blood drawing and staining, and delays between staining and analysing were assessed. Blood was drawn on day 0 and stained on days 0, 1, 2, 3 and 4 having been kept in the dark at 4°C. Samples stained were analysed on the same day and consecutive days. Anti-IL-2 (PE-labelled, Becton Dickinson) and anti-IFN-γ (FITC-labelled, Pharmingen) were used with relevant isotype controls.

Overall results (combining those of IL-2 and IFN-γ staining) were as follows.

Delays between drawing blood and staining: It was not possible to assess the loss of positively-staining cells due to a delay in staining over the first 24 hours as there was sub-optimal stimulation on the first day. However loss in positive staining from 24 to 48 hours after the drawing of blood averaged 27%, with a further 20% loss over the following 24 hours.

Delays in analysis after staining: The average losses of positive staining were 8% over the first 24 hours, a further 14% over the next 24 hours and a further 20% during the next 24 hours.
Therefore the effects of delays in either staining the cells or analysing them were significant. Ideally samples should be stained and analysed on the day of blood drawing, however if this is not possible, and results are to be compared, then care must be taken to ensure an equivalent delay for all the relevant samples.

3.1.2.7 Reproducibility of Technique
This was assessed for a whole blood assay with fresh venous blood aliquoted equally into 5 FACS tubes which were stimulated and stained with FITC-labelled anti-CD4 (Becton Dickinson) and PE-labelled anti-IL-2 (Becton Dickinson) in parallel. One of these tubes was then analysed sequentially a further 3 times. Each analysis was performed on 5000 cells. Data was thus obtained on inter-tube variability and variability within the FACSCaliber.

The mean and standard deviations were calculated as:

<table>
<thead>
<tr>
<th></th>
<th>CD4</th>
<th>IL-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parallel Tubes</td>
<td>CD4: mean= 44.92 standard deviation=0.47</td>
<td>IL-2: mean= 22.96 standard deviation=0.59</td>
</tr>
<tr>
<td>Repeated Tube</td>
<td>CD4: mean= 43.93 standard deviation=0.59</td>
<td>IL-2: mean= 21.98 standard deviation=0.53</td>
</tr>
</tbody>
</table>

This assessment of variability for intracellular staining was also performed on isolated peripheral blood mononuclear cells (separated by density-gradient centrifugation) stimulated in a similar manner, using anti-IL-2 PE (Becton Dickinson) and anti-IFN-γ CFS (R&D) in 7 tubes stained in parallel, and one tube run through the FACSCaliber flow cytometer 6 times consecutively. Again only 5000 cells within the lymphocyte gate (on forward- and side-scatter) were acquired.

The means and standard deviations were calculated as:

<table>
<thead>
<tr>
<th></th>
<th>IL-2 Staining:</th>
<th>IFN-γ Staining:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parallel tubes</td>
<td>mean= 17.9</td>
<td>mean= 51.9</td>
</tr>
<tr>
<td>(n=7)</td>
<td>standard deviation= 1.0</td>
<td>standard deviation= 2.4</td>
</tr>
<tr>
<td>Consecutive Runs</td>
<td>IL-2 Staining:</td>
<td>mean= 16.0</td>
</tr>
<tr>
<td>(n=6)</td>
<td>IFN-γ Staining:</td>
<td>mean= 39.2</td>
</tr>
<tr>
<td></td>
<td>standard deviation= 1.0</td>
<td>standard deviation= 2.4</td>
</tr>
</tbody>
</table>
Thus the inter-tube variability appears to be very small with the variability present being effectively attributable to errors of the flow cytometry analysis stage.

The errors at the FACSCaliber stage may be a result of the low numbers of cells analysed (5000), in which case the degree of variability would be expected to decrease with an increase in sample size. This was assessed by analysing larger samples of stimulated PBMCs (with 4 analyses at each level). Staining was with PE-conjugated IFN-γ stain (Becton Dickinson).

The statistics for the percentages of positively staining cells within the lymphocyte gate, for each size of gate, were:

<table>
<thead>
<tr>
<th>Cells</th>
<th>Mean</th>
<th>Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>5000</td>
<td>26.64</td>
<td>0.47</td>
</tr>
<tr>
<td>10000</td>
<td>26.26</td>
<td>0.20</td>
</tr>
<tr>
<td>20000</td>
<td>26.10</td>
<td>0.09</td>
</tr>
<tr>
<td>40000</td>
<td>26.08</td>
<td>0.09</td>
</tr>
</tbody>
</table>

Therefore the main variability appears to be due to sampling error due to small sample sizes in the previous examples, and not due to interpretation errors of the flow cytometer.

3.1.3 Resultant FACS Staining Protocol

For Whole Blood:

**Materials:**
- Falcon 5ml Round-Bottomed Polystyrene FACS Tubes (Becton-Dickinson)
- RPMI 1640 (GibcoBRL)
- Monensin (Sigma)
- PMA (phorbol 12-myristate 13-acetate) (Sigma)
- Ionomycin (calcium salt) (Sigma)
- PBS - 1 litre H₂O
  + 8g NaCl
  + 0.2g KH₂PO₄
  + 1.135g Na₂HPO₄·2H₂O
  + 0.2g KCl (all from BDH).

Staining Buffer - PBS
+ 0.1% Sodium Azide (BDH)  
+ 1% BSA (Bovine Serum Albumin)  

(Sigma)  

Lysing Solution (Becton Dickinson)  
diluted 1:10 with water  

Permeabilizing Solution (Becton Dickinson)  
diluted 1:10 with water  

Fixing Buffer - PBS  
+ 1% paraformaldehyde (Sigma)  

Stains - doses established by dose-response experiments (see above)  

Media: RPMI 1640 + 2mM L-glutamine  

Stimulation Medium with:  
3µM Monensin  
25ng/ml PMA  
1µg/ml Ionomycin  
(final concentrations)  

Non-Stimulation Medium with:  
3µM Monensin  

1. In each FACS tube place 50µl blood (anticoagulated with sodium heparin 5-10u/ml) and 50µl medium (stimulation or non-stimulation)  
2. Place in incubator at 37°C, 5%CO₂ for 4 hours  
3. Flick tube (to resuspend cells)  
4. Add surface stain, vortex gently and place in dark at RT for 15min  
5. Add 2ml lysing solution, mix gently and leave in dark at RT for 10min  
6. Centrifuge at 2000g for 5min. Pour off and discard supernatant and then flick tube hard to resuspend cell pellet  
7. Add 0.5ml permeabilizing solution, vortex gently and leave in dark at RT for 10min  
8. Wash: Add 2ml staining buffer and centrifuge at 2000g for 5min. Discard supernatant and flick tube hard to resuspend cell pellet  
9. Add intracellular stain, vortex gently and leave in dark at RT for 30min
10. Wash: Add 2ml staining buffer and centrifuge at 2000g for 5min. Discard supernatant and flick tube hard to resuspend cell pellet.

11. Add 0.1-0.2ml fixing buffer and vortex gently. Keep in dark and at 4°C (if analysis is to be delayed more than 1 hour)

For Cell Culture:
As above, but transfer the culture well contents into FACS tube (with no extra culture medium added) and proceed directly to step 4.
A stimulation step generally is not required (as the cells have usually been antigen stimulated) but monensin/brefeldin-A must be added for a period prior to transfer from wells if cytokine analysis is to be performed (see next section).

For Surface Staining Only:
Proceed to step 4 as above but then proceed directly to stage 10.

3.1.4 Adaptations
3.1.4.1 Golgi-block
For antigen stimulated (rather than phorbol ester and ionophore driven) stimulation it is preferable to use pharmacological ‘Golgi blocking’ for an extended period, e.g. overnight, as the rate and degree of cytokine production is significantly decreased. As shown for the four hour phorbol ester and ionophore stimulation above, monensin at a final concentration of 3μM is generally used. An alternative agent is brefeldin-A, commonly used at a concentration of 10μg/ml. They both result in intracellular accumulation of secreted proteins by interfering with processing and packaging of proteins for export at the Golgi apparatus. The molecular target for brefeldin is not known but may involve guanine nucleotide exchange proteins acting on ADP-ribosylation factor 1. The target of monensin is similarly not identified but it appears to affect the exchange of protons for Na⁺ which leads to osmotic swelling of post-Golgi endosomal structures and Golgi subcompartments (Dinter and Berger, 1998).
Both agents used for extended periods are toxic to metabolically active cells and therefore a combination of both at varying doses was examined to determine an optimal combination in terms of cytokine accumulation and lack of toxicity.

The method of assessing toxicity chosen was propidium iodide (PI) staining. PI is excluded from cells with an intact membrane barrier but in those that are undergoing death can enter the cytoplasm and when exited by the 488nm laser of the FACSCaliber machine emits widely over the red spectrum (thus being detectable on the FL2 and FL3 detectors of the FACSCaliber).

Intracellular accumulation of IFN-γ was assessed with 16 hours of exposure to combinations of monensin (Sigma) and brefeldin-A (Sigma) varying between 0-3μM and 0-10μg/ml respectively.

Initial experiments used freshly isolated peripheral blood mononuclear cells (PBMCs) incubated for 6 days with Mycobacterium tuberculosis sonicate as the antigen (at a final concentration of 1μg/ml), however the cytokine levels achieved were too small to reliably estimate the effects of the Golgi-blocking agents. Therefore the experiments were repeated using overnight stimulation (16 hours) with PMA and ionomycin at final concentrations of 25ng/ml and 1μg/ml respectively. These are the concentrations usually employed for the 4 hour stimulation of fresh blood and were chosen as they would be expected to produce significant IFN-γ production from freshly isolated PBMCs and would also be expected to be significantly toxic over a 16 hour exposure.

A stock solution of propidium iodide (Sigma) of 50μg/ml was utilised (10μl added to cell suspension, incubated at RT for 30 min and then cell suspension washed 3 times with staining buffer (see section 3.1.3)).

Results are illustrated in the Figures 3.1.4.1.a and b. PE-labelled anti-IFN-γ (Becton Dickinson) was utilised.

As can be seen the highest intracellular levels of IFN-γ were detected when a combination of monensin 1μM and brefeldin-A 1μg/ml was utilised. PI staining showed relatively low levels of positive staining except when brefeldin-A was not utilised. In this case the toxicity was unexpectedly increased. It is possible that, in
view of the particularly toxic nature of the stimulants used, the brefeldin-A was protective by preventing some of the excessive metabolic strain imposed by the phorbol ester and ionophore. The result of this particular experiment established the optimal dose combination for 16 hour 'Golgi-block' of PBMCs to be monensin 1µM and brefeldin-A 2µg/ml.
Figure 3.1.4.1 a: Effect of Differing 16 Hour Brefeldin/Monensin Combinations on IFN-gamma Staining.

Figure 3.1.4.1 b: Effect of Differing 16 Hour Brefeldin/Monensin Combinations on PI Staining.
3.1.4.2 Autofluorescence

A significant problem when assessing low degrees of positivity, especially in cells staining positive for two markers, is the presence of autofluorescing cells. It appears that some cells, especially those that are dying or are dead, will fluoresce as they run through the flow cytometer (even in the absence of stain) and thus are detected as positively stained.

Simple experiments using unstained PBMCs that were several days old (and therefore contained a significant degree of dead and dying cells) found that the same cells were detected autofluorescing on detectors FL1, FL2 and FL3 of the FACSCaliber, but there was negligible autofluorescence detected with the FL4 detector. Therefore it appeared that autofluorescence was excited by light at 488nm (the laser used for FL1,2&3), but not by light at 635nm (the laser used for the 4th colour). As a result, by leaving one of the fluorochromes detected by FL1, 2 or 3 unused when staining, and establishing good compensation of the detectors during acquisition of data, the software (CellQuest, Becton Dickinson) could be used to backgate and exclude the autofluorescent cells (which would show positive fluorescence on the ‘unused’ detector) from analysis. This method was utilised in the majority of the analyses in this thesis.

3.1.4.3 Assessment of Proliferation or Cell Division

A significant problem with the interpretation of much of flow cytometry is that it generally deals with percentages of cells and not absolute numbers. As a result it is possible for the percentages of a certain cell type or subtype to alter as a result of a large change in the size of another cell type or subtype also gated, even though the absolute numbers of the first have not changed. Two methods were assessed to circumvent this problem – the incorporation of BrdU into proliferating cells, and the identification of cells that had undergone division with CFSE.

3.1.4.3.1 BrdU Staining

BrdU, or bromodeoxyuridine, is a thymidine analogue that is incorporated into DNA during the S phase of the cell cycle. A specific monoclonal antibody can
bind BrdU and thus be used to detect cells that have been through S phase (though, as it can only bind to single-stranded DNA, in-vitro treatment of the cells with DNase must generally be first employed).

The usual technique for permeabilisation of cells for intracellular staining is not adequate for BrdU staining. The literature contains several alternative protocols. Some are based on the use of 0.1 to 0.5% Tween20 solutions for the permeabilisation but produced variable results. Ultimately therefore a protocol using ethanol (Tough and Sprent. 1994) was adapted.

BrdU Staining Protocol:

Briefly:
Cell suspension was transferred from the culture well to 5ml FACS tubes (Falcon, Becton Dickinson) and centrifuged at 2000g for 5 minutes. Supernatant was discarded, surface staining and then washing were performed as per normal FACS protocol. After resuspension in 0.5ml ice-cold 0.15M NaCl, 1.2ml ice-cold 95% ethanol was added dropwise, followed by incubation at 4°C for 30 minutes. The cells were then washed by the addition of 2ml PBS and centrifugation at 5500g for 5 minutes. The cells were fixed by incubation (for 30 minutes at RT) with 1ml 1%(w/v) paraformaldehyde and 0.01%(v/v) Tween20 in PBS. After a wash the cells were resuspended in 1ml DNase I solution (50 Kunitz units/ml DNase I (Sigma) in 4.2mM MgCl₂ / 0.15M NaCl, pH 5) and incubated for 10 minutes at RT. After a wash the cells were stained with anti-BrdU (Pharmingen) (with incubation for 30 minutes at RT), then washed, resuspended in PBS and analysed.

However, having established this technique within the laboratory and whilst performing dose-response experiments for the pulsing with BrdU (Sigma), it became apparent that there could be variable quenching/removal of surface staining during this procedure which could potentially negate results. Therefore an alternative method, CFSE staining, was investigated.
3.1.4.3.2 CFSE Staining

5-(and 6-) carboxyfluorescein diacetate succinimidyl ester (CFSE) is an amine reactive dye that easily traverses cell membranes and forms very stable bonds with a variety of intracellular proteins. The key factor to its use in the assessment of cell proliferation is that each time a cell divides half of the CFSE (bound to cellular proteins) remains in each of the daughter cells. Thus, with this sequential halving of fluorescent staining at each division step, progeny from multiple rounds of cell division can be identified (Lyons and Parish, 1994).

Simple dose-response experiments were performed for staining peripheral blood mononuclear cells (PBMCs) after separation and before plating (data not shown). Results demonstrated that staining with 2μM CFSE (Molecular Probes) at 37°C for 5 minutes (followed by two washes) provided staining that was at the upper level of detection on FL1 of the FACScaliber (when set for lymphocyte analysis) and therefore was adopted for the experiments contained within this thesis.
3.2 Gamma Delta Cells

Effects of heat-killed *M. vaccae* upon T lymphocytes bearing the γδ T cell receptor would be able to explain many of the putative efficacies and known immunological characteristics of SRL-172, and therefore the influence of this immunotherapeutic agent on these cells was investigated.

3.2.1 General discussion

The γδ T cell receptor was first identified by Brenner and colleagues in 1986 after analysing lymphocytes that expressed the T cell marker, CD3, but not the recognised αβ heterodimeric complex (Brenner et al., 1986). With an estimated evolutionary history of 400-500 million years, γδ T cells are found in all jawed vertebrates from the cartilaginous fish upwards. The numbers of Vy and Vδ genes however vary markedly in different species. In humans there appear to be six functional Vy genes whilst chickens probably possess over 30, and similarly there seem to be eight to ten human Vδ genes whilst mice possess an estimated 16 and chickens approximately 20 to 30.

γδ cells are present in the peripheral blood, however their predominant distribution in all animals tested is within the tissues, especially mucosal surfaces (in particular the intestine where they may constitute the majority of intra-epithelial lymphocytes). They are only very rarely found in lymph nodes, spleen, Peyer’s patches or thymus, implying that they may not require professional antigen presentation for stimulation (as this is only known to significantly occur within such lymphoid and secondary lymphoid tissues). This localisation of γδ cells was one of the factors that led to their putative role in the ‘first line of defence’ hypothesis, where a major function is the surveillance of epithelial surfaces (which are the points of entry of most infectious agents) (Janeway et al., 1988).

In humans the Vy2Vδ2 subset (also called Vy9Vδ2 in a separate nomenclature (Bukowski et al., 1998)) is predominant in the peripheral circulation, whilst the other subsets, particularly the Vδ1, are predominant in tissues.
3.2.1.1 γδ Specific Antigens

Phosphoantigens: The discovery of the γδ cell stimulatory properties of the non-peptide prenyl pyrophosphates such as isopentenyl pyrophosphate (IPP) (an intermediate in steroid metabolism and required for the prenylation of proteins) (Tanaka et al., 1995), joined the description of many so-called γδ phosphoantigens that are either organic phosphoesters or nucleotide-conjugated compounds (Constant et al., 1994; Pfeffer et al., 1990; Schoel et al., 1994; Tanaka et al., 1994). Most phosphomonoesters require concentrations in the high micromolar range to significantly stimulate γδ cells, IPP in the low micromolar range, and there are poorly characterised compounds isolated from mycobacteria that appear active in the nanomolar range.

Antigen uptake and presentation are not required for phosphoantigens as fixed APCs are adequate, and MHC class I or II, CD1 and TAP are not required (Morita et al., 1995). Cell-cell contact is however required (Lang et al., 1995) and there is strong evidence that phosphoantigen recognition requires the TCR – monoclonal antibodies to the γδ TCR can block recognition, only Vy2/Vδ2 expand to these antigens (Davodeau et al., 1993; Tanaka et al., 1994) and transfection of TCR-loss mutants of the αβ Jurkat cell line with Vy2/Vδ2 cDNA conferred responsiveness to phosphoantigens (whilst transfection with Vy1/Vδ1 did not) (Bukowski et al., 1995). In fact the Vy2 region appears to contain the critical region for recognition (Bukowski et al., 1998).

Aminobiphosphonates, such as pamidronate, have also been recently reported to activate γδ cells, but interestingly, unlike the above phosphoantigens, appear to require presentation by monocyte-lineage cells (Miyagawa et al., 2001).

Alkyl Amines: Recently alkylamines (particularly straight or branched-chain alkyl phosphates or primary alkylamines of two to five carbons) have been identified as γδ cell antigens able to induce proliferation of the Vy2/Vδ2 subset (Bukowski et al., 1999). These antigens can reach millimolar concentrations in many bacterial supernatants and are present in many living organisms, including edible plants. Their recognition similarly appears to be TCR dependent.
MICA, MICB and MHC Molecules: In contrast to human systemic γδ cells, intra-epithelial γδ cells seem to principally recognise markers of stress in autologous epithelial cells. These include the MHC class I related molecules MICA and MICB that are up-regulated by infection, malignant transformation and other stresses (Groh et al., 1998).

Also several γδ cell hybridomas have been demonstrated to recognise MHC class Iβ gene products that are up-regulated on activated cells (Bonneville et al., 1989; Weintraub et al., 1994), and separately recognition of MHC class II products has been recorded (Hampl et al., 1999).

CD1: The CD1 family of surface glycoproteins are non-MHC encoded antigen presenting molecules. They show some structural similarity to MHC class I and associate with β2-microglobulin, but progress through an endocytic pathway similarly to MHC class II. They are principally of interest due to their ability to present non-peptide antigens, such as lipids and glyco-lipids, to lymphocytes. In humans CD1a, b and c form a sub-group and are expressed on professional antigen-presenting cells. Mice have no homologues of these proteins, however CD1d has murine homologues and is abundantly expressed on intestinal epithelium.

Non-peptide antigens can be recognised (in association with CD1) by a range of lymphocytes, including γδ cells and CD4-CD8- αβ T lymphocytes (DN cells). A sub-group of Vδ1 γδ cells has been shown to recognise CD1c molecules directly, in the absence of foreign antigen, and lyse expressing cells (Spada et al., 2000).

Heat Shock Proteins: Both murine (Vγ1) and human (Vγ2Vδ2) γδ cells may recognise mycobacterial and mammalian homologues of hsp60/65 (Fisch et al., 1990). Another heat shock protein, grp75, has been shown to present immunoglobulin determinants from B cell lymphomas to human γδ cells (Kim et al., 1995).
3.2.1.2 Effector Functions

γδ cells may be cytolytic via either the perforin, granulysin, granzyme M (Sayers et al., 2001) or Fas pathways but also appear to have a role in the coordination of immune responses through the secretion of cytokines. The cytokine profiles can be diverse with Vγ2Vδ2 T cells being capable of producing IL-2, IL-3, IL-4, IL-5, IL-6, TNF-α, IFN-γ, GM-CSF and TGF-β, though seeming to produce mainly type 1 cytokines in response to nonpeptide bacterial antigens. However γδ cells produce only low amounts of IL-2 themselves and, as IL-2 is a required growth factor for these cells, require exogenously added cytokine or CD4+ cell 'help' to expand in-vitro. Generally IL-1 and IL-7 strongly activate γδ cells (Skeen and Ziegler, 1993) and the Vγ2Vδ2 response to nonpeptide antigen is also enhanced by IL-12 and IL-15 and weakly augmented by IL-4. IL-10 inhibits this response (probably by inhibiting CD4+ help) (Marx et al., 1997; Wesch et al., 1997). More recently CD30 co-stimulation on γδ cells has been shown to potentiate the Ca^{2+} fluxes induced by CD3 cross-linking and increase the expression of IFN-γ and IL-4 (but not IL-10) on γδ lymphocytes (Biswas et al., 2000).

γδ T cells may also co-ordinate immune responses through effects on adhesion, chemotaxis and growth/maturation - secreting certain chemokines (such as MIP1α, MIP1β, RANTES and lymphotaxin) (Boismenu et al., 1996) and growth factors (such as keratinocyte growth factor) (Boismenu and Havran, 1994).

3.2.2 Rationale for Investigation

γδ T cells have been shown to be involved in the immune responses (whether protective or pathogenic) to tuberculosis, many neoplasia, asthma and atopy, malaria and periodontal disease – conditions for which SRL-172 has putative efficacies.

3.2.2.1 γδ Lymphocytes and Tuberculosis

γδ T cells are known to have a role in the human and murine responses to tuberculosis infection.
3.2.2.1 *In-Vitro* Studies

There are many antigens derived from *M. tuberculosis*, both protein and non-protein, that have been shown to induce proliferation and cytokine secretion in the main human peripheral blood γδ cell subset, Vγ2Vδ2 (Batoni et al., 1998), and *in-vitro* studies have demonstrated cytotoxic potential and IFN-γ secretion when γδ cells are exposed to *M. tuberculosis* infected macrophages (Tsukaguchi et al., 1995; Dieli et al., 2000b). Indeed, in this situation, γδ cells appear to be more efficient producers of IFN-γ than CD4+ T cells (Tsukaguchi et al., 1999).

3.2.2.1.2 Animal Models

γδ cells are known to accumulate in experimental lesions (Fulton et al., 2000) but most knowledge of the roles of γδ T lymphocytes in tuberculosis has been gained from the use of gene-knockout mice. They appear to play an important role in protection from high-dose systemic *M. tuberculosis* inocula but are less important in protection from small aerosol challenges (D'Souza et al., 1997; Ladel et al., 1995). In the latter case they may have a more regulatory role as γδ-deficient mice have a higher initial bacterial burden and develop a more pyogenic and destructive response, possibly confirmed by the development of abnormal granulomas in mice depleted of a subset of γδ cells expressing WC-1 (Smith et al., 1999).

*M. bovis* pulmonary infection of calves is associated with an infiltrate of WC1+ γδ cells in the pulmonary lesions during the first 3 weeks of illness (Cassidy et al., 2001). γδ lymphocytes from the peripheral blood of such cattle showed higher degrees of activation on culture with *M. bovis* derived antigens than other T cells, and demonstrated significant proliferation but only minimal IFN-γ secretion (Smyth et al., 2001).
3.2.2.1.3 Human Studies

Children who have tuberculosis, or show skin test responses to PPD, have an increased γδ response to non-peptidic antigens compared to uninfected, PPD skin-test negative children (Dieli et al., 2000a), and these responses (in the Vγ2Vδ2 subset) are augmented in acute primary infection (Poccia et al., 1999) and in both the cerebrospinal fluid and blood of children with tuberculous meningitis (Dieli et al., 1999).

The peripheral blood γδ cells from tuberculous patients appear phenotypically activated (with up-regulation of ICAM-1 and MHC class II) (Behr-Perst et al., 1999) but until recently there has been controversy as to their overall numbers in both patient blood and broncho-alveolar lavage fluid (Balbi et al., 1993; Li et al., 1996). This has largely been resolved by the demonstration of rapid up-regulation and surface expression of CD95-L on γδ cells and consequent prompt activation-induced death (Li et al., 1998; Manfredi et al., 1998). The timing and conditions of such analyses is therefore vital.

It is possible that Vγ2Vδ2 responses to mycobacterial antigens are increased in individuals that have received BCG vaccination, or in subjects with evidence of prior sensitisation to mycobacterial lysates (i.e. prior infection with mycobacteria other than tuberculosis) (Hoft et al., 1998). This may have relevance to the use of a mycobacterial product, such as SRL-172, in immunotherapy for tuberculosis.

3.2.2.2 γδ Lymphocytes and Cancers

γδ cells have important anti-tumour activities. Neoplasia can be recognised by a variety of different mechanisms, including MHC class I detection by NK receptors (such as CD94/NKG2) (Poccia et al., 1997), and tumour specific and non-specific antigens. Examples of the later are γδ cells that recognise the stress and malignancy induced antigens MICA and MICB (Groh et al., 1999) and Vδ1 cell lines developed from patients with colorectal cancer that have been shown to lyse not only autologous and allogenic colorectal cancer cells, but also cells from renal cancers, pancreatic cancers, and freshly isolated explants of human intestine.
Several potential anti-tumour effector functions may be employed by \( \gamma\delta \) cells. They are known to possess granule-dependent cytotoxic mechanisms (Dieli et al., 2000b) and may also induce apoptosis via Fas (CD95) (Huber, 2000; Troye-Blomberg et al., 1999b), as discussed above. In some situations however they may function to inhibit other anti-tumour responses, such as those by NK and CD8+ T lymphocytes, possibly via secretion of the cytokines IL-10 and TGF-\( \beta \) (Seo et al., 1998; Seo et al., 1999).

Specifically in relation to neoplasia for which SRL-172 is currently being investigated there is some published data supporting activity of \( \gamma\delta \) cells in melanoma, prostate cancer and renal cell carcinoma.

In necrotising melanoma it has been noted that survival correlated with the number of infiltrating \( \gamma\delta \) lymphocytes (Bialasiewicz et al., 1999) and \( \gamma\delta \) lymphocytes may recognise prostate cancer cells via their expression of MICA and MICB (Groh et al., 1999). Renal cell carcinoma is characterised by the presence of infiltrating \( \gamma\delta \) cells which are activated (Kowalczyk et al., 1996) and often express \( \gamma\delta \) (with an under-representation of \( \alpha\beta \) T cells that are predominately activated by this tumour (Lahn et al., 1999a). A more recent study has also demonstrated an increased number of (oligoclonal) \( \gamma\delta \) cells in patients with renal cell carcinoma, and this increase correlated with tumour stage and decreased after surgery (Kobayashi et al., 2001).

Many other neoplasms have been associated with infiltrations of \( \gamma\delta \) lymphocytes, from hepatocellular carcinomas (Chin et al., 1995) to dysgerminomas, seminomas, pancreatic carcinomas and breast cancers. In the latter tumours the lytic
capabilities of γδ lymphocytes for neoplastic cells have also been demonstrated (Bank et al., 1993; Kitayama et al., 1993; Zhao et al., 1995).

3.2.2.3 γδ Lymphocytes and Asthma

Increasingly data is accumulating of regulatory and other roles for γδ cells in asthma and allergies.

3.2.2.3.1 Animal Models

The most commonly utilised animal model of asthma is an ovalbumin (OVA)-sensitised mouse subjected to inhaled OVA. Acute challenge with OVA in this model has been shown to lead to the formation of clusters of γδ cells in lung tissue (Yiamouyiannis et al., 1999). Also in this model the marked pulmonary inflammation (with airway eosinophilia and peribronchial lympho-plasmocytic infiltration) and the elevated serum IgE seen in the wild-type mice are attenuated in both TCRdelta(-/-) knockout animals and mice treated with anti-TCRγδ monoclonal antibodies - with associated decreases in specific IgE (and IgG1), pulmonary IL-5 production, eosinophil and T lymphocyte infiltration and airway responsiveness to aerosolised methacholine (Schramm et al., 2000). Many of these effects could be counteracted by administration of exogenous IL-4 during primary immunisation, suggesting that early IL-4 production may be a major function of γδ cells in this model (Zuany-Amorim et al., 1998).

The converse, a more protective putative role, may be seen in other models. In rats repeated inhalational OVA challenges induce a state of antigen-specific tolerance which can be adoptively transferred by small numbers of γδ cells (producing high levels of IFN-γ) (McMenamin et al., 1995) and adoptive transfer of small numbers of γδ cells was also able to selectively suppress Th2-dependent IgE production (without affecting parallel IgG responses) in mice (McMenamin et al., 1994). Direct effects on airway hyper-responsiveness have recently been demonstrated with δ knockout mice exhibiting an exaggerated response despite lower apparent inflammatory changes (Lahn et al., 1999b).
Due to the general lack of access to lung tissue from asthmatics some research has been performed on nasal γδ cells in patients with allergic rhinitis, and conclusions extrapolated to pulmonary γδ lymphocytes in asthmatic subjects.

**Allergic Rhinitis:** Patients with allergic rhinitis have markedly increased proportions of γδ cells within their nasal mucosa (25-30% of CD3+ cells, as compared with 5-10% in normal nasal mucosa) (Pawankar et al., 1996) and in mite-allergic patients nasal Vγ1Vδ1 lymphocytes have been shown to proliferate to the mite-derived antigen Derf2 but not unrelated antigens. Many of these nasal γδ cells secrete IL-4 and IL-13 and have been shown to induce IgE synthesis in B cells (Pawankar et al., 1995). Usually no changes are seen in the peripheral blood γδ population, and the mucosal γδ lymphocytes are thought to be organ-specific and oligoclonally expanded under specific antigenic stimulation (Pawankar, 2000).

**Asthma:** Analogous to the allergic human nasal mucosa, there are increases in the proportions of Th2 cytokine secreting, allergen-specific CD4+ γδ lymphocytes obtained in broncho-alveolar lavage (BAL) fluid from untreated patients with allergic asthma (Spinozzi et al., 1995), the majority expressing Vδ1 and being steroid sensitive (Spinozzi et al., 1996). Assessments of peripheral blood γδ cells have shown either decreases (potentially related to therapies) or no changes in absolute levels (Chen et al., 1996; Krejsek et al., 1998). Pulmonary γδ cells may be locally expanded as it has been demonstrated that BAL γδ lymphocytes express a different Vγδγ gene usage from those in the peripheral blood (Molfino et al., 1996).
3.2.2.4 γδ Lymphocytes and Malaria

3.2.2.4.1 Animal Models

In many murine models of malaria γδ T cells appear to be involved in the successful and rapid removal of parasites or the containment of infection. αβ T cell deficient mice can be successfully immunised against *P. yoelii* with (predominately pre-erythrocytic) protection being abrogated if γδ lymphocytes were removed, and a γδ cell clone derived from such a mouse was able to adoptively transfer protection (Tsuji et al., 1994). In separate studies of murine *P. yoelii* infection both IFN-γ and TNF-α were found to be important in the early control of parasitaemia, and both γδ and NK lymphocytes contributed to their production (Choudhury et al., 2000). In *P. chabaudi* infection mice deficient in, or depleted of, γδ lymphocytes demonstrated an exacerbation of the early parasitaemia, and a significantly higher chronic parasite level (Seixas and Langhorne, 1999). Other studies in murine *P. c. adami* infection confirmed that γδ lymphocyte deficient mice could not contain infection and established that most of their activity was independent of antibody or B lymphocytes (van der Heyde et al., 1995). Some of these protective activities may be mediated via recognition of the 60kDa and 70kDa heat shock proteins (Kopacz and Kumar, 1999b).

Other models however fail to demonstrate a significant role for γδ lymphocytes in malarial infection (Sayles and Rakhmilevich, 1996), or suggest that they may impede a protective immune response (Kopacz and Kumar, 1999a) and note their accumulation in the cerebral lesions of ordinarily resistant mice made susceptible to *P. yoelii* infection (by IL-2 infusion) (Haque et al., 2001).

3.2.2.4.2 In-Vitro Studies

It has been established that lysates of *P. falciparum* can expand Vy2Vδ2 cells *in vitro* (Behr and Dubois, 1992) (probably via a phosphoantigen released from schizonts (Pichyangkul et al., 1997)) and that antigen derived from the asexual blood stages, or asexual parasites themselves, preferentially stimulate proliferation of γδ over αβ T cells in malaria naïve and experienced individuals (Behr and Dubois, 1992; Goerlich et al., 1991; Goodier et al., 1992; Goodier et al., 1993).
\text{Vγ2Vδ2} cells may be responsible for a significant proportion of the T cell derived cytokine response, particularly IFN-γ and TNF-α (Goodier et al., 1995), and human \text{Vγ2Vδ2} clones have been shown to inhibit the replication of \textit{P. falciparum} \textit{in-vitro} by activity against extracellular merozoites (Elloso et al., 1994). Vδ1 cells also showed some, though lesser, inhibitory activity in this study and other workers have confirmed that T cells bearing either Vδ1 or Vδ2 were able to inhibit \textit{in-vitro} growth of the asexual blood stages of \textit{P. falciparum}, whilst \text{αβ} T cells were not (Troye-Blomberg et al., 1999a).

3.2.2.4.3 Human Studies

Most human \textit{in-vivo} studies have demonstrated expansions of peripheral blood γδ cells in malarial infection. Paroxysms of \textit{P. vivax} infection (Perera et al., 1994) and acute \textit{P. falciparum} infection (Ho et al., 1990; Roussilhon et al., 1990) have been associated with increased numbers of circulating γδ lymphocytes. \text{Vγ2Vδ2} are the predominant subset usually responding (Schwartz et al., 1996), though some groups have reported expansion of Vδ1 subsets (Worku et al., 1997). One such was a study of semi-immune children in Ghana where an increase in (predominately highly activated, IFN-γ secreting, Vδ1) γδ cells to as high as 50% of all T lymphocytes was noted following the initiation of treatment for \textit{P. falciparum} malaria (Hviid et al., 2001).

Other studies of children from endemic areas have however failed to show the same increased frequency of γδ cells in their peripheral blood (Hviid et al., 1996) and in at least one study of experimental adult \textit{P. falciparum} infection γδ cell percentages remained within the normal ranges (Rzepczyk et al., 1996), though this study did show that the peripheral blood γδ cells were phenotypically activated with increased surface HLA-DR and CD69 expression (which were later shown to return to normal levels after 3-6 months (Rzepczyk et al., 1997)).

γδ T cells have also been demonstrated to be markedly increased in number in the spleens of patients who died of cerebral malaria, a finding experimentally confirmed in rhesus monkeys and rodents (Nakazawa et al., 1994; van der Heyde et al., 1993).
3.2.2.5 **γδ Lymphocytes and Periodontal Disease**

Abnormal proportions of peripheral blood γδ T cells occur in patients with periodontal disease, with both increased and decreased levels seen (Nagai et al., 1993). Locally, within gingival tissue, there is a significant increase in γδ cells (as a proportion of all T cells) in periodontitis biopsies with significant inflammatory infiltrates (Gemmell and Seymour, 1995). Clones of lymphocytes derived from such tissues that proved to be reactive to *Porphyromonas gingivalis* were demonstrated to be γδ TCR positive (Gemmell et al., 1996), and another periodontal pathogen, *Fusobacterium nucleatum*, was found to stimulate a γδ cell response when injected into the peritoneal cavities of mice (Saito et al., 1997).

3.2.3 **Materials, Methods and Experimental Results**

3.2.3.1 **In-Vitro Model**

The proliferative response to phosphoantigens was used as a model to assess the effects of SRL-172 on γδ T cells. The assay established was based on that of Wesch et al. which analysed the proliferative response of γδ cells to isopentenyl pyrophosphate (IPP) in the presence of IL-2, IL-15 or CD4 cells activated by tetanus toxoid, alloantigen or superantigens (Wesch et al., 1997). The activated CD4 cells are thought to provide 'help' for γδ proliferation via secretion of IL-2 and/or IL-15.

In the following experiments isopentenyl pyrophosphate (IPP) (Sigma) was utilised as the phosphoantigen and initially CD4 help was stimulated by the addition of purified protein derivative (PPD) (Evans Medical). Peripheral blood mononuclear cells (PBMCs) were separated by density gradient centrifugation of freshly drawn heparinised venous blood using Lymphoprep (Nycomed).
3.2.3.1.1 PBMC Separation Protocol

1. Fresh heparinised blood was centrifuged at 6000g for 8 minutes.
2. The plasma was removed and saved.
3. RPMI 1640 culture medium (GibcoBRL) was added to produce a final volume twice that of the original volume drawn.
4. 20ml of this suspension was then carefully layered above 9ml Lymphoprep, and centrifuged at 3500g for 28 minutes.
5. The PBMCs accumulate in a layer between the Lymphoprep and culture medium and were withdrawn with a sterile Pasteur pipette.
6. The cells were then washed in RPMI 1640 (involving centrifugation at 2750g for 15min) and were resuspended in a known volume of culture medium before counting on a haemocytometer.
7. The cell suspension was then spun at 1500g for 9 minutes and resuspended in RPMI 1640 (GibcoBRL) + 2mM L-glutamine (Sigma) + 100u/ml benzyl penicillin (Britannia) / 10% autologous plasma to the required cell density.

Experiments were performed in 24 well flat-bottomed plates (Nunclon delta, Nunc) and the cells were incubated (with or without antigens and other additives as appropriate) for 6 days at 37°C, 5%CO₂ before staining (as per usual FACS surface staining protocol, section 3.1.3).

Initial experiments were performed as follows:

3.2.3.1.2 Dose/Response of IPP

1x10⁶ PBMC in a total volume of 1ml were prepared and plated (as single wells) as above. PPD was used at 10µg/ml and IPP at dose range of 1 to 20µg/ml. The cells were incubated as above and then stained with anti-γδ-1 FITC (Becton Dickinson). Results are shown in figure 3.2.3.1.2.

The resultant usual IPP concentration utilised in the following 6 day γδ stimulation experiments was 20µg/ml.
3.2.3.1.3 Dose/Response of PPD

A similar experiment to that of the IPP dose/response above was performed with IPP at a fixed concentration of 20μg/ml and a PPD dose range of 5 to 100μg/ml in single wells. Results are shown in figure 3.2.3.1.3.

As a result of this experiment the usual PPD concentration utilised in the 6 day IPP/PPD γδ stimulation experiments was 20μg/ml.
Figure 3.2.3.1.2: Dose-Response for IPP in Gamma Delta Assay

Figure 3.2.3.1.3: Dose-Response for PPD in Gamma Delta Assay
3.2.3.1.4 Initial Assessments of Effects of SRL-172 on γδ Proliferation

1x10^6 PBMC in a total volume of 1ml culture medium were prepared and plated as above (as single wells). SRL-172 was added to relevant wells at 0.001 to 100μg/ml having been serially diluted with RPMI 1640 (so 10μl volume added to each well). The plates were incubated and stained as above. Results are shown in figure 3.2.3.1.4.a

The results from this experiment suggested that SRL-172 may downregulate the proliferative response of γδ cells to IPP when this is assessed at 6 days. However this was an initial experiment assessing the utility of the assay and had several potential faults. The dose range of SRL-172 was broad with only a few points, single wells were set up and in SRL-172 the heat-killed M. vaccae is suspended in M/15 borate buffer but in this experiment the serial dilutions were performed with RPMI culture medium resulting in differing concentrations of borate buffer being added to the differing wells.

A more extensive dose/response of SRL-172 was therefore performed but with serial log10 dilutions of the heat-killed M. vaccae performed in M/15 borate buffer. The control wells had no borate buffer added, but a borate buffer control was included (10μl of stock buffer).

The results are shown in figure 3.2.3.1.4.b.

The conclusions drawn from this experiment were that M/15 borate buffer, in the concentrations used, appears to down-regulate the proliferative response of γδ cells to IPP, and that the addition of SRL-172 may partially correct this down-regulation or stimulate γδ cell proliferation.
Figure 3.2.3.1.4.a: Initial Experiment Assessing Effect of Serially Diluted SRL-172 in Gamma Delta Assay. The addition of SRL-172 (in borate buffer) appears to inhibit the response to IPP&IL2.

Figure 3.2.3.1.4.b: Experiment Assessing Effect of Serially Diluted SRL-172 in Gamma Delta Assay with Borate Buffer Control. The borate buffer appears to be inhibitory to the effects of SRL-172 in this assay and is probably the cause of the decrease in gamma delta percentage at 10mcg/ml SRL-172.
The effect of borate buffer was potentially interesting. At high concentrations borate ions are known to interfere with cis-hydroxyl groups and perhaps RNA metabolism and ion transportation (Blevins and Lukaszewski, 1994). But it was however possible that this buffer, at the concentrations used, was having a specific effect on γδ cells themselves or the presentation of IPP to them. Neither effect had previously been described. As discussed in the preceding introduction, little is known of the mechanics and dynamics of phosphoantigen presentation to γδ lymphocytes. Antigen presentation is required, but not by specialised antigen presenting cells, and antigen uptake and processing appear not to be necessary. The presenting molecules (if any) have not been identified, but it is known that cell-cell contact is needed and that the recognition of phosphoantigens is TCR-dependent.

The influence of borate buffer in this assay was therefore investigated as it was thought that it might potentially provide insights into the process of phosphoantigen recognition by γδ T cells and/or their consequent proliferative response, and also to help understand and remove the confounding effect of this buffer on future assays.

3.2.3.1.5 Effect of Borate Buffer on IPP/γδ and Other Assays

The laboratory stock of M/15 borate buffer was used, consisting of an autoclaved solution comprising:

\[
\begin{align*}
\text{Na}_2\text{B}_4\text{O}_7\cdot10\text{H}_2\text{O} & \quad 18.25\text{g} \\
\text{H}_3\text{BO}_3 & \quad 5.25\text{g} \\
\text{NaCl} & \quad 6.19\text{g} \\
\text{Tween}80 & \quad 0.0005\%
\end{align*}
\]

per litre H₂O, with pH adjusted to 8.0.

A dose/response of borate buffer serially diluted (in RPMI 1640) from the usual working concentration to 1:1000 was performed. The results are shown in figure 3.2.3.1.5.a.
A similar experiment was performed with the separate constituents of M/15 borate buffer at dilutions between the usual working concentration and 1:100. Results are shown in figure 3.2.3.1.5.b. Both experiments were performed with single wells.

It can be seen that both borax (Na$_2$B$_4$O$_7$) and boric acid (H$_3$BO$_3$) appear to have an inhibitory effect on the γδ T cell proliferative response to IPP (when assessed as a percentage of the lymphocyte gate at 6 days) that is comparable to that induced by the complete M/15 buffer.

In the original buffer the molarities of borax and boric acid are 47.85mM and 84.9mM respectively. Thus in 1ml culture wells with 10μl of borate buffer added, the concentrations became 478.5μM and 849μM respectively. These concentrations had no demonstrable effects on the pH of the culture medium (data not shown).

To assess if this effect of borate was specific to γδ cell proliferation, or a non-specific inhibitory effect on biological systems, M/15 borate buffer was added to a separate assay.

**TB Sonicate/CD30 Assay:**
Exposure to *M. tuberculosis* sonicate increases the surface expression of CD30 on lymphocytes and this effect was utilised to assess the influence of M/15 borate buffer.

1x10$^6$ PBMC in 1ml RPMI 1640 + 2mM L-glutamine + 100u/ml benzyl penicillin / 10% autologous plasma were plated in a 24-well flat-bottomed plate (Nunclon delta, Nunc). 50μg/ml H37Rv *M. tuberculosis* sonicate and 10μl M/15 borate buffer were added as appropriate. After 7 days incubation at 37°C and 5% CO$_2$, the cells were stained as per usual FACS surface staining protocol with anti-CD30 FITC (Dako). Results are shown in figure 3.2.3.1.5.c.

From this separate assay it appears that the inhibition seen with borate buffer is a non-specific biological effect and not specific to IPP stimulation of γδ T cells.
Similar trends were seen in PMA/ionomycin and SRL-172 driven IFN-γ production by PBMCs (data not shown).

Therefore to remove the influence of this variable on further γδ cell experiments a preparation of heat-killed *M. vaccae* suspended in sterile PBS (rather than M/15 borate buffer) was prepared, and named SRL-PBS.
Figure 3.2.3.1.5.a: Effect of Serial Dilutions of M/15 Borate Buffer on Gamma Delta Assay
A decrease in percentage staining gamma delta positive is seen at higher concentrations than a tenth of the normal buffer concentration utilised.

Figure 3.2.3.1.5.b: Effect of Constituents of M/15 Borate Buffer on Gamma Delta Assay.
Both borax and boric acid appear to be inhibitory and mimic the complete buffer.

Figure 3.2.3.1.5.c: Effect of M/15 Borate Buffer on TB Sonicate Induced CD30 Expression.
Borate buffer is demonstrated to be inhibitory in this *in-vitro* model also.
3.2.3.1.6 Preparation of SRL-PBS

*M. vaccae* (NCTC 11659) was grown on Sauton’s medium for approximately 1 month and a sample placed in sterile PBS. The suspension was then exposed to shear stress by drawing into a syringe, and expelling whilst pushed against the base of a universal container. This was repeated 10 times, the suspension diluted to a concentration of 10mg/ml with sterile PBS and autoclaved at 121°C for 15 minutes under 15lb pressure.

3.2.3.1.7 Replacement of PPD with IL-2

To further refine these assays and potentially remove some variability, PPD-induced CD4 help was now replaced by the addition of exogenous IL-2 (Proleukin, Chiron) (suspended in sterile PBS/0.1% human serum albumin (Sigma)).

A dose/response was performed in experiments of identical design to those previously, with IPP at a fixed concentration of 20µg/ml and IL-2 ranging from 0.1 to 30ng/ml final concentration. Results are shown in figure 3.2.3.1.7. Error bars are standard deviations of triplicate wells (IL2&IPP and IPP control only).

A concentration of IL-2 of 20ng/ml was chosen for further experiments to provide sufficient γδ lymphocyte proliferation to detect any influence of SRL-PBS whilst avoiding any plateau in the proliferative response.
Figure 3.2.3.1.7: Dose Response for IL-2 in Gamma Delta Assay. Error bars = standard deviations.
3.2.3.1.8 Final γδ Cell Assay Protocol

PBMCs were separated and suspended in culture medium as per section 3.2.3.1.1 and plated at densities of 1x10^6/ml, 1ml per well in a 24 well flat-bottomed plate (Nunclon delta, Nunc).

IPP (at 20μg/ml), IL-2 (at 20ng/ml) and SRL-PBS serially log10 diluted in RPMI were added to relevant wells.

Plates were incubated (wrapped in plastic) at 37°C, 5%CO₂ for 6 days prior to staining as per usual FACS protocol (see section 3.1.3). For the assessment of cytokine production brefeldin-A and monensin (at 2μg/ml and 1μM respectively) were added to wells 16 hours prior to harvesting (as per section 3.1.4.1).

3.2.3.2 Effect of SRL-PBS on γδ Cell Assay

Responses of γδ cells to SRL-PBS in the above protocol (section 3.2.3.1.8) were assessed in terms of proliferation (as gauged by the percentage of the lymphocyte gate staining for γδ TCR), activation markers and cytokine production. For these analyses 20000 cells within the lymphocyte gate were acquired on the flow cytometer and autofluorescent cells were excluded from analysis (as per section 3.1.4.2).

3.2.3.2.1 γδ Cells as a Percentage of Lymphocyte Gate

Staining with FITC-conjugated anti-human γδ-1 TCR (Becton Dickinson) or APC-conjugated anti-human γδ (Pharmingen) was utilised to determine, by flow cytometry, the proportion of lymphocytes expressing the γδ TCR. The results from representative experiments are shown in figures 3.2.3.2.1a-d.

Five separate experiments examining the response in IPP&IL2 stimulated cells are shown in figure 3.2.3.2.1.a. Error bars shown (experiment c) are standard
deviations from quadruplicate wells. Experiments a and e were performed with
duplicate wells, and experiments b and d with single wells.

Four separate experiments demonstrating the response in IPP stimulated cells are
shown in figure 3.2.3.2.1.b, and 4 separate experiments of the response in IL-2
stimulated cells in figure 3.2.3.2.1.c. Experiments d in both graphs were
performed in duplicate. Figure 3.2.3.2.1.d shows the response to SRL-PBS alone
in 3 separate experiments. Experiment c was performed with duplicate wells. The
other experiments illustrated were performed with single wells.

The addition of SRL-PBS appears to have had a significant effect upon
proliferation of γδ cells as assessed by the percentage of cells within the
lymphocyte gate that stained for the γδ TCR. One donor, subject 1, was used to
illustrate that in all experiments the percentage of γδ staining cells increased when
10 or 100μg/ml SRL-PBS was added with IL-2 & IPP (p<0.0001 for both doses,
paired t-test) (figure 3.2.3.2.1.a) and with either IL-2 or IPP alone at the higher
SRL-PBS dose (100μg/ml) (p=0.025 and p=0.019 respectively, paired t-test)
(figures 3.2.3.2.1.b and c). No effect of SRL-PBS was seen when there was no IL-
2 or IPP present.
Figure 3.2.3.2.1.a: Effect of SRL-PBS on Gamma Delta Cells as a Percentage of Lymphocytes (IL2&IPP Stimulation)
Subject 1 (5 Separate Experiments a-e).
Significant increases in gamma delta cells as a proportion of the lymphocyte gate demonstrated at SRL-PBS 10mcg/ml (p<0.0001) and 100mcg/ml (p<0.0001).
Error bars = standard deviations.

Figure 3.2.3.2.1.b: Effect of SRL-PBS on Gamma Delta Cells as a Percentage of Lymphocytes (IL2 Stimulation)
Subject 1 (4 Separate Experiments a-d).
Significant effect demonstrated at 100mcg/ml SRL-PBS (p=0.025).
Figure 3.2.3.2.1.c: Effect of SRL-PBS on Gamma Delta Cells as a Percentage of Lymphocytes (IPP Stimulation)
Subject 1 (4 Separate Experiments a-d).
Significant increase demonstrated at 100mcg/ml SRL-PBS (p=0.019).

Figure 3.2.3.2.1.d: Effect of SRL-PBS on Gamma Delta Cells as a Percentage of Lymphocytes (SRL-PBS Only Stimulation)
Subject 1 (3 Separate Experiments a-c).
No effect of addition of SRL-PBS demonstrated.
3.2.3.2.2 Activation Markers on γδ Cells

The above experiments assessed the effects of heat-killed *M. vaccae* on γδ cell proliferation as assessed by the proportion of lymphocytes gated that express the γ δ TCR. However it is known that γδ T lymphocytes can express surface markers such as CD25 and HLA-DR upon activation and therefore the above model of γδ cell stimulation by IPP and IL-2 was used to analyse any effects of SRL-PBS on these, and other, markers.

All the following experiments utilised either FITC-conjugated anti-human γδ-1 (Becton Dickinson) or APC-conjugated anti-human γδ (Pharmingen).

3.2.3.2.2.1 CD25

CD25, the receptor for IL-2, is composed of α, β and γc chains and intracellular signalling results from heterodimerization on ligand binding. The β and γc chains are sufficient for such signalling but the α chain markedly increases the affinity of the receptor for IL-2. It is a well-recognised marker of activation on T lymphocytes and the influence of SRL-PBS on its surface expression on γδ cells was therefore investigated. FITC-conjugated or PE-conjugated anti-human CD25 (both Becton Dickinson) were utilised as stains.

Figure 3.2.3.2.2.1.a shows the CD25 expression on γδ staining cells that have been stimulated with both IL-2 and IPP (with or without SRL-PBS) from 4 different healthy donors. Two separate experiments on donor 1 are shown, 1a was performed with duplicate wells and 1b with triplicate. The experiment on subject 2 was performed with quadruplicate wells and for subjects 3 and 4 duplicate. Error bars shown are standard deviations. Figures 3.2.3.2.2.1.b and c show the more detailed responses with varying stimulations for 2 of the healthy donors, subjects 1 and 4. These assays were performed with single wells only.

Most subjects analysed had almost maximal staining for CD25 (>90% of γδ cells) when incubated with IPP & IL-2 (see figure 3.2.3.2.2.1.a). In subject 4, whose
level of CD25 expression with just IPP & IL2 was significantly lower than the other donors, an increase in CD25 expression was noted with increasing concentrations of SRL-PBS (p=0.0007 for 100μg/ml, p=0.008 for 10μg/ml, p=0.009 for 1μg/ml and p=0.008 for 0.1μg/ml of SRL-PBS as compared to control, t-test).

SRL-PBS does appear however to have effects on CD25 staining when incubated with IPP alone, IL-2 alone and PBMCs alone (figures 3.2.3.2.2.1.b and c). It is shown to increase the expression of this surface molecule with increasing concentration (though with only a modest effect on PBMCs alone, in the absence of IL-2 and IPP). These experiments were performed in single wells only and formal statistical analyses were therefore not performed.
Figure 3.2.3.2.1.a: Effect of SRL-PBS on CD25 Staining in Gamma Delta Assay (IPP&IL2 Stimulation).
Statistically significant increases in CD25 staining demonstrated in subject 4 with SRL-PBS at 0.1 mcg/ml and greater. No significant effects seen on the other series which demonstrate almost maximal staining throughout.
Error bars = standard deviations.
Figure 3.2.3.2.2.1.b: Effect of SRL-PBS on CD25 Staining in Gamma Delta Assay (Subject 1). Formal statistics not performed due to the use of single wells only.

Figure 3.2.3.2.2.1.c: Effect of SRL-PBS on CD25 Staining in Gamma Delta Assay (Subject 4). Formal statistics not performed due to the use of single wells only.
3.2.3.2.2.2 HLA-DR

HLA-DR, one of the 3 classical MHC class II molecules, is expressed on antigen presenting cells such as macrophage/monocytes, B cells, dendritic cells and thymic epithelial cells but also on activated T cells. It's surface expression on IPP & IL-2 stimulated γδ cells, and the influence of SRL-PBS upon this, were therefore investigated. PE-conjugated anti-human HLA-DR (Pharmingen) was used.

Figure 3.2.3.2.2.2 demonstrates the effect of SRL-PBS on HLA-DR staining on γδ cells derived from the PBMCs of 2 healthy donors, subjects 3 and 4. The responses in IPP&IL-2 stimulated cells from both donors were assessed in duplicate, whilst the responses to IPP, IL-2 and SRL-PBS alone in subject 4 were analysed using single wells.

These graphs demonstrate, analogously to CD25, that SRL-PBS has little effect upon the almost maximal HLA-DR expression induced by IPP & IL-2 in certain subjects (specifically subject 3 in this case) but there is a trend (that fails to reach statistical significance) in a subject with lesser HLA-DR expression to an increase in expression of this molecule when SRL-PBS (though only at the highest concentration investigated (100 μg/ml)) is combined with IPP & IL-2. This is potentially also seen in combination with IL-2, IPP or in PBMCs alone.

3.2.3.2.2.3 CD69

CD69 is an early activation marker for T and B lymphocytes and also is expressed on activated macrophages. It is a member of the Ca^{2+}-dependent lectin superfamily of type II transmembrane receptors. The ligand that binds to CD69 in-vivo has yet to be identified. The surface expression of this antigen on γδ cells was assessed utilising PE-labelled anti-CD69 (Pharmingen).

The analyses of CD69 expression on IPP&IL-2 stimulated γδ cells after 6 days incubation for healthy donor 1 (two separate experiments) and donor 3 are shown

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in figure 3.2.3.2.3.a. Duplicate wells were analysed for subject 3 and single wells for both experiments on subject 1.

Figure 3.2.3.2.3.b shows the analyses from single wells on CD69 expression at days 4 and 6 on cells derived from subject 1 stimulated with IPP&IL-2, IPP, IL-2 (with or without SRL-PBS) and SRL-PBS alone.

It is apparent from figure 3.2.3.2.3.a that concentrations of SRL-PBS of 10 μg/ml and above, when combined with IPP & IL-2, result in a decrease in surface staining for this molecule when assessed at 6 days in all subjects tested (p=0.007 for 100μg/ml and p=0.043 for 10μg/ml SRL-PBS, paired t-test). This effect is probably less prominent at 4 days (though this differential effect failed to reach statistical significance in any of the groups). IL-2 (without IPP) induces high levels of CD69 expression that are stable between 4 and 6 days and similar to the levels seen with IPP & IL-2 in combination with lower concentrations (or the absence of) SRL-PBS. SRL-PBS in the absence of IPP or IL-2 has no effect on the much lower background levels of CD69 expression, but in combination with IPP appears to induce increased CD69 surface staining at concentrations of 10 or 100μg/ml which is stable between 4 and 6 days (but performed in single wells only).
Figure 3.2.3.2.2: Effect of SRL-PBS on HLA-DR Staining in Gamma Delta Assay.
Trends, failing to reach significance, are seen for HLA-DR staining with increasing SRL-PBS concentrations in subject 4, but no effect is demonstrated on the almost maximal staining seen in subject 3.
Figure 3.2.3.2.2.3.a: Effect of SRL-PBS on CD69 Staining in Gamma Delta Assay (6 Days IPP&IL2 Stimulation).
Decreased staining for CD69 is demonstrated at 10mcg/ml (p=0.043) and 100mcg/ml (p=0.007) SRL-PBS at 6 days incubation.

Figure 3.2.3.2.2.3.b: Effect of SRL-PBS on CD69 Staining in Gamma Delta Assay at 4 and 6 Days (Subject 1).
A trend to less CD69 downregulation is seen at 4 days (compared to 6) in IPP&IL2 wells with 10 and 100mcg/ml SRL-PBS. IL-2 induces high CD69 staining which is stable between 4 and 6 days. There is a trend to increased CD69 staining in IPP wells with higher SRL-PBS concentrations (formal statistics not performed). No effect is demonstrated in the absence of IPP and IL2.
3.2.3.2.2.4 CD80 & CD86

Antigen-unrestricted co-stimulatory signals are required for the optimal activation of T cells, and one of the major such signals is mediated via CD80/86 binding of CD28. CD80 (also known as B7-1) and CD86 (B7-2) are immunoglobulin-like membrane proteins that are expressed mainly on antigen-presenting cells. Their principle ligand is CD28, present on both resting and activated T cells, but they may also bind CTLA-4, present on activated T lymphocytes. Binding of the latter results in a negative signal, inhibiting activation. Neither CD80 nor CD86 are usually expressed on resting T cells, but can be induced to do so following activation. Generally CD86 is expressed more rapidly and at higher levels than CD80. The expression of both these B7 molecules on the surfaces of γδ cells in the above assay was investigated and results are shown in figures 3.2.3.2.2.4.a-c.

Figure 3.2.3.2.2.4.a shows the results of CD80 staining on subjects 1 and 3, the former being performed in single and the latter in duplicate wells.

Figure 3.2.3.2.2.4.b illustrates the results of analyses of CD86 staining on IPP & IL-2 stimulated γδ cells from subjects 1 (2 separate experiments) and 3. Experiment 1a was performed in single wells, experiment 1b with triplicate and the experiment on donor 3 in duplicate. Error bars shown are standard deviations.

Figure 3.2.3.2.2.4.c demonstrates the results of CD86 staining on IPP & IL-2, IPP, IL-2 (with or without SRL-PBS) and SRL-PBS stimulated γδ lymphocytes from subject 1 (in single wells).

Figure 3.2.3.2.2.4.a examines the CD80 expression levels on γδ lymphocytes from 2 subjects. Subject 3 appears to have high level expression on cells incubated with IL-2 & IPP and this is not altered by the addition of SRL-PBS. For subject 1 there are much lower levels of expression and the wells containing IL-2 without IPP appear to contain more CD80 expressing γδ cells than wells that contain both IL-2 and IPP. There is apparent up-regulation of CD80 expression on γδ cells from wells containing IL-2 (with or without IPP) with the addition of higher
concentrations of SRL-PBS. Formal statistical analyses were inhibited by the use of single or duplicate wells only in these analyses.

Figures 3.2.3.2.4.b and c examine the expression of CD86. Again there is some variability. In two of the experiments in figure 3.2.3.2.4.b there is an increased expression of CD86 on γδ cells from wells combining IL-2 and IPP, but in the other experiment (using cells from one of the same donors but at a different time) this effect is not seen. Overall however these effects reach statistical significance for SRL-PBS at 100μg/ml (p=0.028, paired t-test) and 10μg/ml (p=0.014, paired t-test). Assessments of the effects of SRL-PBS on IPP only, IL-2 only and unstimulated wells are shown in figure 3.2.3.2.4.c. There is possibly an increase in CD86 surface staining with higher SRL-PBS concentrations in the IPP or IL-2 containing wells, and potentially a decrease in the unstimulated wells, however formal statistical analyses were inhibited by the use of only single wells.
Figure 3.2.3.2.4.a: Effect of SRL-PBS on CD80 Staining in Gamma Delta Assay.  
Formal statistical analyses not performed as single or duplicate wells only used.
Figure 3.2.3.2.2.4.b: Effect of SRL-PBS on CD86 Staining in Gamma Delta Assay (IPP&IL-2 Stimulation).
Significant increases in CD86 staining demonstrated for all series combined at 10mcg/ml ($p=0.014$) and 100mcg/ml ($p=0.028$) SRL-PBS. Error bars = standard deviations.

Figure 3.2.3.2.2.4.c: Effect of SRL-PBS on CD86 Staining in Gamma Delta Assay (Subject 1).
Formal statistical analyses not performed as single wells only.
3.2.3.2.3 Cytokine Production by Gamma Delta Cells

Similarly the IPP and IL-2 model was used to assess any influence heat-killed *M. vaccae* had upon the cytokine secretion profile of γδ T lymphocytes. The cytokines chosen for evaluation were IFN-γ and IL-4, as these are known to be produced in detectable quantities by γδ cells and are representatives of the type-1 and type-2 cytokine families respectively.

The above 6 day *in-vitro* model was used with the addition of monensin and brefeldin-A (at 1μM and 2μl/ml respectively) for the final 16 hours of incubation to prevent the export of cytokines from the cells and allow their intracellular staining by the previously described protocols (section 3.1.4.1).

3.2.3.2.3.1 IFN-γ

PE-conjugated anti-human IFN-γ (Becton Dickinson) was utilised. Results of experiments performed on cells derived from subject 1 are shown in figure 3.2.3.2.3.1 (triplicate wells for IPP & IL-2 stimulation, single otherwise). Error bars shown are standard deviations.

3.2.3.2.3.2 IL-4

FITC-conjugated anti-human IL-4 (Pharmingen) was utilised. Results of experiments performed on cells derived from subject 1 are shown in figure 3.2.3.2.3.2 (triplicate wells for IPP & IL-2 stimulation, single otherwise). Error bars shown are standard deviations.

SRL-PBS increases the production of both cytokines at concentrations of 10 and 100μg/ml in IPP & IL-2 stimulated cells (p=0.0004 and p=0.0012 for IFN-γ and p=0.006 and p=0.002 for IL-4 with 100μg/ml and 10μg/ml SRL-PBS respectively, t-test). In IPP stimulated and IL-2 stimulated cells there appears to be a similar effect but this is only minimal in otherwise unstimulated cells. For both cytokines there is an apparent plateau of the stimulatory effect between 10 and 100μg/ml.
Figure 3.2.3.2.3.1: Effect of SRL-PBS on IFN-gamma Staining in Gamma Delta Assay.
Statistically significant effects are seen on IFN staining in IPP&IL2 wells containing 10mcg/ml (p=0.0012) and 100mcg/ml (p=0.0004) SRL-PBS. Formal statistical analyses were not performed for IPP, IL2 or nil-added wells (single wells only). Error bars = standard deviations.

Figure 3.2.3.2.3.2: Effect of SRL-PBS on IL-4 Staining in Gamma Delta Assay.
Statistically significant effects are seen on IL-4 staining in IPP&IL2 wells containing 10mcg/ml (p=0.002) and 100mcg/ml (p=0.006) SRL-PBS. Formal statistical analyses were not performed for IPP, IL2 or nil-added wells (single wells only). Error bars = standard deviations.
3.2.4 Discussion

The potential influence of SRL-172 on γδ T lymphocytes was investigated as it seemed theoretically plausible, in view of the known immunology and putative clinical efficacies of this product, that they may be involved in the vaccine’s mechanisms of action. The only well established models studying such cells have been the assessment of responses, predominately proliferative, to antigens such as isopentenyl pyrophosphate. Such a model was therefore adapted, providing an assessment of the percentage of lymphocytes bearing a γδ TCR and therefore a measure of the proliferative response to the γδ-specific antigen, and allowing the assessments of surface activation markers and cytokine production.

γδ Cells as a Percentage of Lymphocyte Gate:
The addition of SRL-PBS appears to have had a significant effect upon proliferation of γδ cells as assessed by the percentage of cells within the lymphocyte gate that stained for the γδ TCR. This is not a direct measure of proliferation as the final percentage may obviously depend on changes in other cellular populations also gated, however this is the readout used in several papers (such as Wesch et al., 1997b), and the marked alterations in percentage levels seen should reflect changes in proliferation itself.

In all experiments the percentage of γδ staining cells gated increased with increasing concentrations of SRL-PBS (significantly at 10-100μg/ml for IL-2&IPP stimulated cells and at 100μg/ml for cells stimulated with IPP or IL2 alone) except in the absence of either IPP or IL-2 (when no effect was seen).

The reasons for this increased proliferation may be multifactorial. SRL-PBS may be having direct effects upon γδ cells but these findings may be a feature of an effect of SRL-PBS on CD4 lymphocytes (and other cells) that themselves then affect the γδ cells. An example of this type of action might be the stimulation of IL-2 production in such bystander cells (analogously to the initial use of PPD in this assay). This increased IL-2 would be expected to increase the proliferative
response of γδ cells whether combined with IPP or with a background level of exogenous IL-2 itself (as potentially shown by the dose response for IL-2 in figure 3.2.3.1.7). Also this IL-2 release might be relatively sustained and could be particularly relevant if the exogenous IL-2 was either fully utilised or degraded. It is probable that SRL-PBS is unable to induce sufficient IL-2 production to cause proliferation of γδ cells when not added onto a significant background level of this cytokine (as shown by the lack of effect in figure 3.2.3.2.1.d). IL-15 would be an alternative cytokine that might be induced by SRL-PBS and have such effects. Another factor to consider is that SRL-PBS is a preparation of a heat-killed mycobacterium and such organisms are known to contain a variety of γδ antigens (see previous discussion). Therefore an increase in proliferation could be due to an increase in specific antigens without any alteration in cytokine and growth factor levels.

CD25 Expression:
Gamma Delta cells are known to express CD25 on stimulation with IPP (Wesch et al., 1997a). Most subjects analysed in this thesis appeared to have almost maximal staining for CD25 (>90% of γδ cells) when incubated with IPP&IL-2 (figure 3.2.3.2.1.a). It may therefore be that the presence of SRL-PBS is unable to increase this level further in these donors. In separate donors who have a lower expression of CD25 in the presence of IPP&IL-2 a significant effect of SRL-PBS on increasing CD25 expression is however shown.
SRL-PBS does appear to have effects (in both subjects studied) on CD25 staining when incubated with IPP alone, IL-2 alone and PBMCs alone (figures 3.2.3.2.1.b and c). Formal statistical analysis is however difficult as these particular experiments were performed with single wells only.
It therefore appears that the higher concentrations of the heat-killed M. vaccae preparation can partially substitute for either IL-2 or IPP in this assay (in terms of inducing CD25 expression), but only in the presence of the other. It does not appear to be able to induce this response (to statistically significant levels) in the absence of both.
As above these may be direct effects on γδ cells or alternatively may be attributable to an influence on other cell types and act by increasing the levels of IL-2 (for example) within the media. In-vitro exposure to IL-2 has indeed been shown to increase expression of this activation marker on γδ cells (Kjeldsen-Kragh et al., 1993).

HLA-DR Expression:

γδ cells may express HLA-DR on activation (illustrated in Vγ2Vδ2 cells in HIV infection (Grottrup-Wolfers et al., 1997a; Jouen-Beades et al., 1996) and in tumour-infiltrating γδ cells (Yu et al., 1999)). In the subjects examined in this thesis, analogously to CD25, SRL-PBS has little effect upon the almost maximal HLA-DR expression induced by IPP & IL-2 in one subject but in a subject with lesser HLA-DR expression a potential trend to an increase is noted when SRL-PBS is combined with IPP&IL-2, and possibly when combined with IL-2, IPP and PBMCs alone (figure 3.2.3.2.2.2).

Though direct effects of SRL-PBS can be hypothesised these trends fail to reach significance and may be artefactual or, even if validated, may again be explained by increases in factors such as IL-2 and a bystander effect. Indeed exogenous IL-2 has also been shown to increase the expression of this surface molecule on γδ lymphocytes in-vitro (Kjeldsen-Kragh et al., 1993).

CD69 Expression:

CD69 is an early activation marker on T cells and it's expression on γδ lymphocytes has been noted to be important in reactivity to mycobacterial antigens (Gonzalez-Amaro et al., 2000) and to be up-regulated in HIV infection (Grottrup-Wolfers et al., 1997b) and other conditions such as Behcet’s Disease (Freysdottir et al., 1999).

Concentrations of SRL-PBS of 10 μg/ml and above result in a significant decrease in surface staining for CD69 in wells with combined IPP & IL-2, and this effect is probably more prominent at 6 days than at 4 (though the difference fails statistical significance). It is possible therefore that the maximal stimulation induced by IPP
IL-2 in combination with high concentrations of SRL-PBS results in rapid up-regulation of this early activation marker and that levels are already decreasing by 4 days and are even lower by day 6. IL-2 (without IPP) induces high levels of CD69 expression that are stable between 4 and 6 days and similar to the levels seen with IPP & IL-2 in combination with lower concentrations (or the absence of) SRL-PBS. SRL-PBS in the absence of IPP or IL-2 has no effect on the much lower background levels of CD69 expression, but in combination with IPP does appear to result in increased CD69 surface staining at concentrations of 10 or 100 μg/ml (though formal statistical analysis of these findings is difficult as only single wells were analysed). These effects could again be explained by increased IL-2 (or other factor) secretion by other cell populations. As for CD25 and HLA-DR it has been shown that exposure to exogenous IL-2 in-vitro up-regulates the surface expression of this activation marker on γδ cells (Kjeldsen-Kragh et al., 1993).

CD80 and CD86 Expression:
SRL-PBS appears to have little influence on the expression of CD80 on γδ lymphocytes, except for a potential non-significant trend for high concentrations of SRL-PBS to increased levels on γδ cells also incubated with IL-2 - though only in one donor (who demonstrated lower CD80 expression overall). This assay however appeared to show some degree of variability and further conclusions cannot be drawn.

Assays examining CD86 expression again show significant variability. In two of the experiments there appears to be increased expression of CD86 on γδ cells from wells combining IL-2 and IPP, but in the other experiment (using cells from one of the same donors but at a different time) this effect was not seen. There is probably some increase in CD86 surface staining with higher SRL-PBS concentrations in the IPP or IL-2 containing wells, and potentially a decrease in the unstimulated wells, however again there is a degree of variability making these and further conclusions difficult.
Cytokine Staining:
SRL-PBS does appear to increase the production of both cytokines studied (IFN-γ and IL-4) at concentrations of 10 and 100μg/ml (and possibly, though non-significantly, at 1μg/ml) in IPP&IL-2 stimulated cells, and potentially in IPP stimulated and IL-2 stimulated cells, but has only a minimal, if any, effect on otherwise unstimulated cells. For both cytokines there is an apparent plateau of the stimulatory effect between 10 and 100μg/ml.

The percentage of cells staining for IFN-γ in the presence of SRL-PBS and IL-2 apparently did not alter upon the presence or absence of IPP (though the later analyses were with single wells only). Therefore it appears that this phosphoantigen may not be required for the stimulation of production of this cytokine in the presence of these substances and does not increase the level of IFN-γ production further. A similar effect is seen for IL-4 staining.

The high level of IL-4 production in the γδ cells was unexpected. It has been confirmed in a separate experiment on the same donor (data not shown) but levels this high would ordinarily be unusual. Confirmation of this finding will require further assays with alternative donors and different stains. It would also be of interest to establish whether individual γδ cells were producing both cytokines (and therefore had a mixed type-1 and type-2 profile) or whether most γδ cells produced either one or the other (though due to the high percentages at least some must be dually produced). Double staining of the same cells for cytokines was not performed in these experiments.

The explanations for the effects on cytokine profiles are not clear. It is unlikely to be solely a bystander effect of IL-2 production by CD4 lymphocytes, though this cytokine is a growth factor for both type-1 and type-2 cells. The plateau between SRL-PBS concentrations of 10 and 100μg/ml is also not a feature generally seen on the activation marker assays above. These issues cannot however be resolved with the data presently available and require further investigation.

As discussed in the relevant sections the effects upon proliferation and the activation markers above might easily be explained by effects upon IL-2
production (or possibly other factors such as IL-15) by CD4 or other cells. As such the proliferative or phenotypic changes seen could be bystander effects and not direct actions of SRL-PBS. This issue could be resolved by assessing the levels of these cytokines in the culture supernatants during incubation and at harvesting by techniques such as ELISA. PBMCs could then be incubated with equivalent concentrations of these cytokines and similar phenotypic changes sought. An alternative would be to separate γδ lymphocytes from the other cells within the PBMC population before the addition of IL-2 and IPP and incubation. This is technically more difficult to achieve to sufficient levels of purity and may have other untoward effects upon the assay (for instance the presentation of IPP to γδ cells might be influenced). Alternatively cell lines could be employed. However no well described γδ cell lines, that have been shown to behave analogously to freshly isolated cells in such IPP assays, are available.

Alternatively these responses might be secondary to the presence of γδ antigens within SRL-PBS. This possibility is harder to resolve as it would not be technically feasible to remove such antigens.

An approach, which might help address these issues, is to evaluate other killed mycobacteria prepared in similar fashions. These would also be expected to contain such γδ antigens (though potentially at a differing concentration) and be expected to activate CD4 cells etc. Therefore if any of these phenotypic or proliferative changes were absent or markedly attenuated in heat-killed M. smegmatis (for example) then it would strongly hint that the effect was specific to SRL-PBS and unlikely to be secondary to bystander activation or increased antigenic load.

Other issues related to the assay developed in this thesis are problems of variability, the assessment of activation and relevance. Firstly marked variability was sometimes encountered in these experiments. This was multifactorial. The γδ cells were sometimes sensitive to the positioning of the wells within the plate – wells at the periphery (and therefore more susceptible to temperature and humidity shifts during incubation) often showed poorer up-
regulation of activation markers. Also dilutions of SRL-PBS were prepared freshly from 10mg/ml stock for each experiment and the product is particulate. It was therefore not possible to ensure that the dilutions were equivalent (though due to the log_{10} dilutions used this effect was hopefully minimised). Finally the IPP preparation purchased was suspended in methanol and as such the concentration might vary slightly due to evaporation during the set-up of large assays.

Secondly, though a broad selection of activation markers was assessed, there are many more potential phenotypic indicators of activation, stimulation or inhibition that could be utilised and may ultimately be shown to be of relevance.

Thirdly the γδ cells predominately analysed here were of the Vγ2Vδ2 subset. All the γδ antigens commercially available (IPP, ethylamine etc.) stimulate these cells but, as discussed in earlier sections, this is the predominant peripheral blood subtype and many of the γδ lymphocytes active in diseases such as cancers are of other subsets. This assay may therefore have been designed to investigate the wrong γδ subsets. Also the induction of proliferation and activation by optimal doses of IPP and IL-2 will not parallel any in-vivo situation and though IL-2 only, IPP only and unstimulated wells were included for each of the assays these similarly may have no meaning in-vivo.

Many of these factors are presently unavoidable however and this assay has been optimised as far as possible. It remains to be seen whether the changes seen secondary to SRL-PBS in γδ lymphocytes are simply due to increased IL-2 or increased antigenic load and whether they have relevance to the clinical efficacies of heat-killed M. vaccae.
3.3 NK and NKT Cells

Two other, partially overlapping, subsets of lymphocyte that theoretically might explain some of the known immunology and clinical efficacies of SRL-172 are Natural Killer (NK) cells and NKT cells.

3.3.1 NK Cells

Natural Killer cells, a population of large granular lymphocytes, comprise 5-10% of circulating lymphocytes. Their most studied activities are against tumours and virally infected cells, but increasingly regulatory roles (through the secretion of cytokines) are becoming recognised.

NK cells lack a T cell receptor and also lack CD4, however approximately half of human NK cells express CD8α/α. They constitutively express the β and γ subunits of the IL-2 receptor (as do γδ cells), which are shared by the IL-15 receptor. Indeed NK cells are activated by both these cytokines, and also by IL-18.

Two surface molecules are generally utilised for identifying NK cells, CD56 (a form of neural cell adhesion molecule 1) and CD16 (an IgG receptor).

NK recognition of target cells is complex. The most characterised method involves the 'missing self' hypothesis (Karre et al., 1986), where inhibitory receptors fail to signal when their ligands, MHC class I molecules, are absent or have been downregulated on the cell surface (as may commonly occur, possibly as a result of selection pressure, in malignancies and viral infections). These inhibitory receptors for MHC class I may either be Ig-like (the KIR family) or lectin-like (such as the CD94 and NKG2A complex). However there are members of the KIR and lectin-like families that appear to be activating, not inhibitory, though it is assumed that they are normally superseded by the inhibitory receptors.

A separate member of the NKG2 lectin family of receptors, NKG2D, which is expressed on NK cells as well as some γδ and CD8 lymphocytes, recognises not
MHC class I molecules but the related stress-induced molecule MICA (Bauer et al., 1999).

The most studied effector functions of NK cells have involved lysis of neoplastic or virally infected cells. NK cells can be cytotoxic through the perforin pathway and are known to express granzymes A and M (Sayers et al., 2001), but also may induce apoptosis through activation of TNF-family death receptors, with activated NK cells expressing FasL (CD95L) (Bossi and Griffiths, 1999) and even unactivated NK cells expressing TNF-related apoptosis-inducing ligand (TRAIL) (Zamai et al., 1998).

NK cells are also able to secrete a variety of cytokines, including IFN-γ, GM-CSF, TNF-α, TGF-β, IL-3, IL-5, IL-10, and IL-13, and thus a major function may be in the regulation and orchestration of other immune cells.

An important cellular subset express NK markers but also the T cell receptor and CD3 and are known as NKT cells. These cells appear to have very different features to NK cells and are discussed separately later in this section.

3.3.1.1 Rationale for Investigation of NK Cells

3.3.1.1.1 Cancer

NK cells were first identified in oncology models and are a predominant cellular subset (with CD8 lymphocytes) in the prevention and control of malignancies (Brittenden et al., 1996; Whiteside et al., 1998). Neoplasia are recognised by a variety of mechanisms (including the lack of inhibitory signals through the MHC class I-detecting receptors) and NK lymphocytes may kill tumour cells either directly (via the perforin pathway) or by the induction of apoptosis (discussed above).

3.3.1.1.2 Tuberculosis

CD16+ NK cells have been shown to proliferate in-vitro when exposed to live M. tuberculosis in monocyte-depleted cultures (Esin et al., 1996). They can activate
M. tuberculosis-infected human monocytes to kill their intracellular bacilli (via soluble products) (Yoneda and Ellner, 1998), and are also directly lytic to such cells (an activity augmented by IL-12 (Denis, 1994) but not associated with IFN-γ production, cytotoxic granule exocytosis or Fas-FasL interactions (Brill et al., 2001)). MHC-independent cytotoxicity towards several mycobacterial antigens has been illustrated for CD16+CD56+ NK cells (Ravn and Pedersen, 1994).

In murine models, DTH responses to PPD have been adoptively transferred to anergic mice by BCG-activated NK cells (Falcone et al., 1993) and in-vivo depletion of NK cells produces an increased susceptibility to infection with M. avium (Harshan and Gangadharam, 1991).

In human patients there appears to be reduced NK function in multi-drug resistant (as compared to sensitive) tuberculosis (Ratcliffe et al., 1992), and in tuberculosis compared to controls (Restrepo et al., 1990), though not all studies have confirmed this (Morikawa et al., 1989; Onwubalili and Scott, 1985). Patients with tuberculous pleuritis, known for a strong cell-mediated immune response, demonstrate NK-mediated cytotoxicity towards crude M. tuberculosis antigen and the 70kDa heat shock protein (Arruda et al., 1998).

3.3.1.1.3 Asthma
In the rodent OVA sensitisation and inhalation asthma model, increased numbers of NK cells are seen in the lung parenchyma and BAL of rats, and in mice depletion of NK cells prior to immunisation inhibited the pulmonary eosinophilia, the increased bronchoalveolar lavage fluid levels of IL-4, IL-5, and IL-12, and the elevated allergen-specific IgE seen in controls (Korsgren et al., 1999).

In the peripheral blood increased NK cell numbers were seen in asthmatic adults (Harmaneci et al., 1998), but no changes were apparent in asthmatic children in either their number, cytotoxicity or IFN-γ production (Chou et al., 1999). Within the sputum of asthmatics the proportion of CD16+ natural killer (NK) cells has been reported to be reduced (Louis et al., 1997).
3.3.1.4 Malaria

In mice the role of NK cells appears to depend on the model. In *P. berghei* infection, depletion of NK cells affected neither parasitaemia nor splenocyte IFN-γ production (Yoneto et al., 1999), however in the *P. chabaudi* AS murine model the early secretion of IFN-γ (and possibly TNF-α) by NK cells seemed to play a major role in blood-stage protective immunity (Mohan et al., 1997), and NK cells are also involved in early IFN-γ and TNF-α production in infection with *P. yoelii* (De Souza et al., 1997; Choudhury et al., 2000). There is increased mortality of mice depleted of NK cells in *P. chabaudi* infection (Kitaguchi et al., 1996).

In humans a reduction in peripheral blood NK numbers has been seen in acute infections with *P. vivax* (Worku et al., 1997) and *P. falciparum*, where both CD16+ and CD56+ NK cells have been shown to lyse erythrocytic schizonts (Orago and Facer, 1991).

3.3.1.5 Vascular

NK cells are known to be present in arteriosclerotic lesions within the coronary arteries of heart transplant recipients (Cramer et al., 1992), though rodent models of arterial transplantation arteriosclerosis fail to show any involvement of NK cells (Shi et al., 1996) and immunosuppressants that prevent arteriosclerosis in this model fail to affect NK cells (Lernstrom et al., 1996).

3.3.1.6 Periodontal disease

Killed oral bacteria can stimulate NK cells *in-vitro* (Lindemann et al., 1988), as can *Porphyromonas gingivalis*, one of the bacteria involved in the pathogenesis of periodontitis (Champaiboon et al., 2000).

There is local accumulation of NK cells in severe forms of periodontal disease (Fujita et al., 1992), and a significant increase in CD56+ NK cells has been seen in the peripheral blood of patients (Afar et al., 1992; Celenligil et al., 1990).

The influence of SRL-172 on NK cells - principally effects on surface markers, proliferation and cytokine secretion – was therefore investigated.
3.3.1.2 Methods and Results

Freshly isolated PBMCs from normal donors (separated as per section 3.2.3.1.1) were suspended in culture medium (RPMI 1640+2mM L-glutamine / 10% autologous serum) at $1 \times 10^6$ cells/ml, and 1ml plated into the relevant wells of 24-well flat-bottomed tissue culture plates (Nunclon delta, Nunc). $\log_{10}$ dilutions of SRL-172 (in culture medium) were added as appropriate and the cells incubated for 7 days at $37^\circ C/5\%CO_2$. Concentrations of SRL-172 exceeding $10\mu g/ml$ were avoided due to the compounding effects of borate buffer (discussed in $\gamma\delta$ lymphocyte section).

NK cells were identified by surface staining with APC- or FITC-labelled anti-CD56 (Pharmingen and Becton Dickinson respectively). The usual FACS protocols were followed (sections 3.1.3 and 3.1.4.1), with autofluorescent cells excluded (as per section 3.1.4.2). For the following analyses 20000 cells were gated on the flow cytometer.

3.3.1.2.1 NK Cells as a Percentage of Lymphocyte Gate

After 7 days incubation the proportion of cells contained within the lymphocyte gate on the flow cytometer that stained for CD56 was assessed. Results of 5 separate experiments on healthy donor 1 and an experiment on donor 3 are shown in figure 3.3.1.2.1. Experiments 1d and 1e were performed with quadruplicate wells, 1c in triplicate and the remainder in duplicate. Error bars shown are standard deviations.

No effect on NK cells as a percentage of the lymphocyte gate was demonstrated.
Figure 3.3.1.2.1: Effect of SRL-172 on CD56+ Cells as Percentage of Lymphocyte Gate.
No significant effects were demonstrated.
Error bars = standard deviations.

Figure 3.3.1.2.2: Effect of SRL-172 on CFSE Fluorescence of CD56+ Cells.
Significant increases in the proportion of CD56+ cells with diminished CFSE fluorescence are seen for 1 mcg/ml (p=0.0012) and 10 mcg/ml (p<0.0001) and at both these doses this was significantly increased upon that seen for the lymphocyte gate as a whole.
Error bars = standard deviations.
3.3.1.2.2 Proliferation

Proliferation was assessed by pre-staining the PBMCs with CFSE prior to plating and incubation (see previous protocol, section 3.1.4.3.2). After 7 days incubation the percentages of CD56+ cells that showed evidence of having undergone cell division (and hence had decreased CFSE fluorescence) in relation to the overall percentage of cells within the lymphocyte gate that had decreased CFSE fluorescence are shown in figure 3.3.1.2.2. Quadruplicate wells were analysed and error bars shown are standard deviations.

An effect of SRL-172 upon the proliferation of CD56 staining cells is illustrated. When compared to controls (with no added SRL-172) there were significant increases in the proportion that showed decreased CFSE fluorescence with 10μg/ml (p<0.0001, t-test) and 1μg/ml (p=0.0012, t-test) SRL-172. Similar increases were noted amongst the gated cells as a whole (p=0.0011 and p<0.0001 for 10 and 1μg/ml respectively, t-test). However there were significantly more CD56 staining cells showing evidence of proliferation at both these doses of SRL-172 (p=0.027 and p=0.043 respectively, t-test) than for the cells gated as a whole. Therefore these cells show a statistically significant increase in the level of cell division at the higher SRL-172 concentrations used (1 and 10μg/ml) but not at the lower concentration (0.1μg/ml). This effect is more marked than that upon the lymphocyte gate as a whole and implies a differential, increased, effect upon cell division on CD56 staining cells.

3.3.1.2.3 Activation Markers

3.3.1.2.3.1 CD25

After 7 days incubation the percentages of CD56+ cells that demonstrated surface staining for CD25 are shown in figure 3.3.1.3.3.1. PE or FITC labelled anti-CD25 (both Becton Dickinson) were used.
The experiment on donor 2 involved quadruplicate wells, and single wells were used for donor 3. Error bars shown are standard deviations.

Increased surface staining for CD25 is shown on CD56+ cells with increasing concentration of SRL-172 in both donors (p=0.004 and p=0.0042 for 10 and 1μg/ml SRL-172 respectively, t-test).

3.3.1.2.3.2 CD69

After 7 days incubation the percentages of CD56+ cells that demonstrated surface staining for CD69 are shown in figure 3.3.1.2.3.2. PE-labelled anti-human CD69 (Pharmingen) was used.

2 separate experiments were performed on cells derived from donor 1 (experiment 1a with triplicate wells and 1b in duplicate), and an experiment using single wells for subject 3. Error bars shown are standard deviations.

The first experiment on subject 1 (experiment 1a) and that performed on subject 2 demonstrate an increased surface expression of CD69 on cells incubated with concentrations of SRL-172 exceeding 0.1μg/ml (increased CD69 expression with increased SRL-172 over the concentrations utilised). This effect was not as pronounced in the second experiment on donor 1 (experiment 1b) though a similar trend (at higher SRL-172 concentrations) may be seen. For all the experiments taken together there is a significant increase in surface CD69 staining at an SRL-172 concentration of 10μg/ml (p=0.035, t-test) but for experiment 1a the increase is significant both at 10 and 1μg/ml (p<0.0001 and p=0.0137 respectively, t-test).
Figure 3.3.1.2.3.1: Effect of SRL-172 on CD25 Staining of CD56+ Cells. Statistically significant increased CD25 staining is seen at both 1mcg/ml (p=0.004) and 10mcg/ml (p=0.004) for subjects taken together. Error bars = standard deviations.

Figure 3.3.1.2.3.2: Effect of SRL-172 on CD69 Staining of CD56+ Cells. Significantly increased CD69 staining is seen in series 1a with SRL-172 of 1mcg/ml (p=0.0137) and 10mcg/ml (p<0.0001), and for all series taken together at 10mcg/ml (p=0.035). Error bars = standard deviations.
Figure 3.3.1.2.3.3: Effect of SRL-172 on HLA-DR Staining of CD56+ Cells. No significant effect was demonstrated.
Figure 3.3.1.2.3.4.a: Effect of SRL-172 on CD80 Staining of CD56+ Cells
No significant effect was demonstrated.

Figure 3.3.1.2.3.4.b: Effect of SRL-172 on CD86 Staining of CD56+ Cells
A trend to an increase in CD86 staining is seen in subject 1 with increasing SRL-172 but this fails to reach statistical significance.
3.3.1.2.3.3 HLA-DR
After 7 days incubation the percentages of CD56+ cells that demonstrated surface staining for HLA-DR are shown in figure 3.3.1.2.3.3. PE-labelled anti-HLA-DR (Pharmingen) was used.
The experiments, on subjects 1 and 2, were performed with duplicate wells.
No significant effect of SRL-172 on HLA-DR surface staining was demonstrated.

3.3.1.2.3.4 CD 80 & CD 86
After 7 days incubation the percentages of CD56+ cells that co-stained with PE-conjugated anti-human CD80 or CD86 (both Pharmingen) are shown in figures 3.3.1.2.3.4.a and b respectively. Experiments on subject 1 were performed in single wells, whilst those on subject 2 were in duplicate.
An increase in CD80 staining is seen at the higher concentration of SRL-172 utilised (10µg/ml) in donor 1 but not donor 2. Similarly an increase in CD86 surface staining is demonstrated for donor 1 (at SRL-172 concentrations of 0.1µg/ml and above) but none for donor 2. None of these effects reaches statistical significance.

3.3.1.2.4 Cytokine Production
Staining for IFN-γ and IL-4 was performed having exposed the cells for the final 16 hours of their 7 day incubation to monensin 1µM and brefeldin-A 2µg/ml (both Sigma). PE-labelled anti-IFN-γ (Becton Dickinson) and PE-labelled anti-IL4 (Becton Dickinson) were used.
Results of duplicate analyses are shown in figure 3.3.1.2.4.
A trend to an increase in staining (above controls) for IFN-γ is shown (p=0.054, t-test) and potentially a trend (non-significant) to an increase in IL-4 staining, at the higher SRL-172 concentrations.
Figure 3.3.1.2.4: Effect of SRL-172 on Cytokine Staining of CD56+ Cells (Subject 1)
Trends for an increase in staining for both cytokines fail to reach statistical significance.
3.3.1.3 Discussion

In contrast to the findings with γδ lymphocytes, heat-killed *M. vaccae* has no demonstrable effect on the proportion of CD56 staining cells within the lymphocyte gate. When proliferation is assessed by CFSE staining however, there is an apparent differential stimulation for proliferation of CD56+ cells than for the lymphocyte population gated as a whole. It appears that such NK cells are preferentially stimulated to proliferate by SRL-172 at the higher concentrations examined. As discussed in previous sections the use of the former assay is limited by the influence of increases/decreases in the numbers of other cell populations within the gated population, but the CFSE assay overcomes some of these limitations. Therefore the overall data demonstrate that SRL-172 does stimulate the proliferation of this subset of NK cells.

CD69 and CD25 are known to be expressed by NK cells after stimulation of PBMC populations with both non-pathogenic and pathogenic bacteria (Haller et al., 2000). CD69 has been recorded as being expressed on 80% of NK cells after 72 hours of stimulation (Werfel et al., 1997) and similarly the low resting levels of CD25 can be markedly up-regulated within 72 hours of stimulation (Hodge et al., 2000). In the subjects assessed in this thesis CD25 is significantly increased on the surface of CD56+ cells when incubated with 10μg/ml and 1μg/ml of SRL-172. CD69 is similarly significantly increased at an SRL-172 concentration of 10μg/ml when the experiments are taken as a whole, and also at 1μg/ml in experiment 1a.

There appears to be no significant effect upon HLA-DR staining, though it is known that these cells can express this activation marker (it is HLA-DR positive NK cells that express TNF-α in response to MAI) (Michelini-Norris et al., 1991). The explanation for a lack of response as determined by HLA-DR staining is unclear. It may be that insufficient cells and insufficient donors were assessed or alternatively heat-killed *M. vaccae* may fail to influence HLA-DR expression in contrast to the other activation markers assessed.
Two donors were assessed for CD80 and CD86 expression on CD56 staining cells after incubation with SRL-172. One showed non-significant increases in the expression of both B7 molecules at the higher SRL-172 concentrations tested but the other failed to show any effects. To draw conclusions on the effects of this heat-killed mycobacterium on CD80 or CD86 expression on CD56+ cells will require further assays on more donors.

Assessments of cytokine profiles showed increases in IFN-γ staining with the higher concentrations of SRL-172 (just failing to reach statistical significance) and potentially a modest, but non-significant, elevation in IL-4 staining. This latter finding could, if shown in future to be significant, be of interest as NK cells are not generally recognised to secrete this cytokine at appreciable levels. However it does raise the suspicion that the trends seen here are artefactual only.

Similarly to the γδ lymphocyte assay described in the previous section it is possible that these changes in activation markers (and potentially cytokine production) are bystander effects due to increases in the local IL-2 (or other cytokine, such as IL-12) levels and not due to direct effects of heat-killed *M. vaccae* upon NK cells themselves. It is known that CD69 expression on NK cells can be induced by IL-2 (Borrego et al., 1999) and blocked in in-vitro PBMC stimulation experiments by anti-IL-2 (Werfel et al., 1997) and that IL-12 can up-regulate the surface expression of CD25, HLA-DR and CD69 on NK cells (Rabinowich et al., 1993). The levels of such cytokines and similar factors could be assayed in the culture media at various time-points by techniques such as ELISA, however this would not prove a direct causal relation and has not yet been done. Incubating PBMCs with similar levels of such cytokines, though potentially technically difficult, could provide supportive evidence for a bystander explanation for these changes if similar alterations in activation markers etc. are seen.

It is however possible that these changes are a direct effect of SRL-172 on NK cells. Preparations of heat-killed *M. vaccae* would be expected to contain many so-called danger signals for the innate and adaptive immune responses (such as
heat shock proteins), and it is teleologically plausible that a cell type involved in the cross-over of such innate and adaptive responses, NK cells, would respond to such products directly. This question could be further addressed by performing such assays on NK cell lines or purified NK cells.

Overall however it may be that the wrong type of assay is being performed. Examining phenotypic changes such as activation markers, and potentially cytokine responses, may have little relation to *in-vivo* effects on NK cells. Also the time point examined, 7 days, may not be optimal or correlate with any *in-vivo* efficacies. Other options include direct effector assays such as cytotoxicity towards appropriate target cells (e.g. tumour cell lines labelled with $^{51}$Cr). These are either being performed or are planned by co-workers, but no results are presently available.

A separate issue is that of whether the correct subset of NK cells are being identified in the above assays. CD56 staining was utilised to identify such cells but this is not the only marker of such cells and is not present on all such cells. Indeed the relative expressions of CD16 and CD56 on human NK cells vary with location of the cell and to some degree activation – CD56hi cells are often CD16lo and have lower levels of cytotoxicity (Nagler et al., 1989). Other alternatives include 2B4, a surface molecule present on most human and mouse NK cells, however it is also present on some activated T cells and human monocytes and therefore not suitable for the assays used in this thesis. A recently identified surface molecule, NKp44, is present on activated NK cells and might be suitable for future investigations when commercial antibody stains become readily available (Vitale et al., 1998).

Due to doubt of their likeness to freshly isolated cells, NK cell lines were not utilised in this thesis. However the above effects (and any others substantiated in further work) could be easily assessed in these cells with similar techniques.
3.3.2 NK T Cells

These cells that express NK markers but also CD3 appear to recognise very different targets and have very different effector functions from NK cells. Most share the same T cell receptor α chain (Vα14-Jα281 in the mouse and Vα24-JαQ in the human) and they respond to glycolipid antigens presented in the context of the non-classical MHC class I molecule, CD1d (Exley et al., 1997; Naidenko et al., 1999). They are therefore potentially of interest in the immunology of killed *M. vaccae* as depletion of glycolipids from the heat-killed product seems to remove certain efficacies, e.g. that in asthma.

The predominant functions of NKT cells appear to be regulatory. By the secretion of IFN-γ or IL-4 they are able to skew a developing immune response towards either the Th1 (Kawamura et al., 1998) or Th2 (Yoshimoto et al., 1995) ends of the spectrum. In fact roles for NKT cells in the regulation of autoimmune diseases have been established for diabetes (Wilson et al., 1998) and systemic sclerosis (Sumida et al., 1995) in humans, and diabetes (Hammond et al., 1998; Lehuen et al., 1998) and SLE (Mieza et al., 1996) in mice, and a role has been hypothesised in the maintenance of tolerance in sites of immune privilege (Sonoda et al., 1999). They may however also have lytic capabilities and have been shown to express granzyme M (Sayers et al., 2001).

### 3.3.2.1 Rationale for Investigation of NKT Cells

Overall there is little data available for the roles of NKT cells in many conditions, and that for diseases for which SRL-172 has putative efficacy is sparse.

In mice it has been shown that BCG infection alters the cytokine secretion profile of CD4+ NKT cells from IL-4 to IFN-γ (probably through IL-12 induction) allowing these cells to secrete IFN-γ prior to the more conventional Th1 cells (Emoto et al., 1999). In oncology models NKT cells, activated by a CD1d-specific antigen α-galactosylceramide *in-vivo* or *in-vitro*, can induce non-specific tumour cell lysis and prevent metastasis (Kawano et al., 1998b; Toura et al., 1999). In OVA asthma models NKT cells have been shown to down-regulate the specific
IgE and Th2 response (Cui et al., 1999), and CD4-CD8- NKT cells were noted to be diminished in patients with asthma (Oishi et al., 2000). Certain murine malarias induce an increase in the proportion of NKT cells, many of which have an activated phenotype (with increased surface expression of CD69), and these cells have some inhibitory effect upon hepatocyte stage parasite development (Pied et al., 2000). NKT cells have been identified in psoriatic lesions, associated with the over-expression of CD1d on keratinocytes, and in vitro NKT cells cluster around such cells, secrete large amounts of IFN-γ but do not proliferate (Bonish et al., 2000).

The effects of SRL-172 upon this lymphocyte subset in-vitro were investigated using a similar model to that above (for NK cells).

3.3.2.2 Methods and Results

Freshly isolated PBMCs from normal donors were suspended in culture medium (RPMI 1640 + 2mM L-glutamine / 10% autologous serum) at 1x10^6 cells/ml, 1ml/well in 24-well flat-bottomed tissue culture plates (Nunclon delta, Nunc) (as per section 3.2.3.1.1). Log_{10} dilutions of SRL-172 (in culture medium) were added as appropriate and the cells incubated for 7 days at 37°C/5%CO₂. Concentrations of SRL-172 exceeding 10μg/ml were avoided due to the compounding effects of borate buffer (discussed in γδ lymphocyte section).

NKT cells were identified by surface staining with either APC-labelled anti-CD56 and PerCP-labelled anti-CD3 (Pharmingen and Becton Dickinson respectively) or FITC-labelled anti-CD56 with APC-labelled anti-CD3 (both Becton Dickinson). The usual FACS protocols were followed (sections 3.1.3 and 3.1.4.1), with autofluorescent cells excluded (as per section 3.1.4.2). 20000 cells within the lymphocyte gate were acquired for each analysis.
3.3.2.2.1 NKT Cells as a Percentage of Lymphocyte Gate

After 7 days incubation the proportion of cells within the lymphocyte gate on the FACScaliber that double stained for CD56 and CD3 was assessed. Results are shown in figure 3.3.2.2.1 for subjects 1 (performed with triplicate wells), 2 (in quadruplicate) and 3 (in duplicate). Error bars shown are standard deviations. The experiment on donor 2 demonstrates a significant effect of SRL-172 (at the higher concentration utilised) upon the percentage of NKT cells within the lymphocyte gate (p=0.0009, t-test) but the identical experiments on donors 1 and 3 showed no such effect.

3.3.2.2.2 Proliferation

PBMCs were pre-stained with CFSE prior to plating and incubation (see previous protocol, section 3.1.4.3.2). After 7 days incubation the percentages of cells double staining for CD56 and CD3 that showed evidence of having undergone cell division (i.e. showed decreased CFSE fluorescence) were assessed in relation to the overall percentage of cells gated that demonstrated diminished CFSE fluorescence and the results shown in figure 3.3.2.2.2. Duplicate wells were analysed.

No significant effect of SRL-172 on the division of CD3+CD56- cells was demonstrated for this single donor. Overall these cells appeared to divide in-vitro less frequently than the lymphocyte population as a whole (analysis of all SRL-172 doses combined p=0.0002, paired t-test).

3.3.2.2.3 HLA-DR, CD69

Only small numbers of NKT cells could be acquired in the analyses. The percentages of such cells that co-expressed CD56, CD3 and either HLA-DR or CD69 after 7 days incubation with dilutions of SRL-172 are shown in figure 3.3.2.2.3. Duplicate wells were analysed.

CD69 surface staining was modestly increased at the highest SRL-172 concentration studied (10μg/ml) (p=0.048, t-test) but no effect on HLA-DR was demonstrated.
3.3.2.2.4 CD25, CD80, CD86 and Cytokine Staining

Attempts were made to perform these analyses however, due to the small number of CD56+CD3+ cells within the flow cytometry gates, results could not be interpreted.
Figure 3.3.2.2.1: Effect of SRL-172 on NKT Cells as Percent of Lymphocyte Gate
No significant effects demonstrated for subjects 1 and 3. Significant effect demonstrated for subject 2 at 10 mcg/ml SRL-172 (p=0.0009) only. Error bars = standard deviations.

Figure 3.3.2.2.2: Effect of SRL-172 on CFSE Fluorescence of NKT Cells
NKT cells appear to divide in-vitro less frequently than the lymphocyte population as a whole (p=0.0002)
Figure 3.3.2.2.3: Effect of SRL-172 on CD69 & HLA-DR Staining of NKT Cells
CD69 staining increased (p=0.048) at 10mcg/ml SRL-172. No significant effect on HLA-DR staining demonstrated.
3.3.2.3 Discussion

CD69 is known to be expressed on antigen-activated NKT cells (Nishimura et al., 2000), as are CD25 and HLA-DR (D'Andrea et al., 2000), and these cells are also known to be capable of producing IFN-γ and IL-4 (Hammond et al., 1999). These markers of activation were therefore sought in the above in-vitro model, as well as effects upon proliferation.

There appeared to be some increase in the proportion of CD3+CD56+ cells within the lymphocyte gate in one of the three donors assessed at the higher SRL-172 concentrations studied. The two other donors however fail to illustrate such an effect. On one of these donors CFSE staining was performed (subject 1) and this failed to show any influence of SRL-172 on NKT cell proliferation. Unfortunately CFSE staining was not performed on subject 2 who potentially showed some effect on the total percentage of cells double staining for CD3 and CD56 that were gated. It is also important to note that in the CFSE staining experiment insufficient cells were harvested in wells incubated with 10µg/ml SRL-172 and therefore results for this concentration (which had the predominant effects in CD56+ NK cells (above)) are not available. Overall the NKT cells analysed in the CFSE experiments show only a low level of division when compared to the cells within the lymphocyte gate as a whole.

Repeats of these assays and further assessments upon NKT cells in this model were hampered by similarly small numbers of CD3+CD56+ cells harvested from the wells. This fact negated the evaluation of CD25, CD80/86 and cytokine levels (though these assays were attempted). No apparent effect was seen on HLA-DR staining but an increase, just reaching significance, was seen in CD69 staining, however these findings need much larger sample sizes to evaluate further and draw any conclusions.

Two main alternatives are available to increase the yield of CD3+CD56+ cells and permit such assessments. The first is the use of larger numbers of PBMCs within a well or combining multiple wells at harvesting. This was not performed as the
quantity of venous blood drawn was already large for the existing assay. A second alternative is to develop an assay analogous to that utilised for the investigation of γδ cells – i.e. the investigation of the effects of SRL-172 on antigen-driven NKT cell proliferation and activation. An antigen that would permit such assays (analogous to the use of IPP in the γδ lymphocyte assays) is α-galactosylceramide (Kawano et al., 1998a).

In the assay as it presently stands no conclusions on the effect of SRL-172 upon NKT cells (as identified by CD56 and CD3 double staining) can be made.

3.4 Overall Conclusions on Immune Cell Activation/Stimulation

Alterations in activation marker expression on γδ lymphocytes, CD56 expressing NK cells and possibly NKT cells have been demonstrated in the above assays. As commented in the relevant sections each assay protocol has it’s limitations and direct extrapolation of the results to in-vivo situations is not presently permissible.

A further limitation for all the assays at present is the inability to assess whether the effects shown are due to direct influences of the preparations of heat-killed M. vaccae used upon the cell subsets or bystander effects due to the stimulation of other cells and their elaboration of factors (such as cytokines) that then influence the activation and cytokine secretion status of the lymphocyte subset being investigated. This factor does not however negate the potential relevance of these findings to the in-vivo efficacies of SRL-172 as biological systems are complex and the stimulation of these cells by whichever route (direct, indirect or other) could result in effector lymphocytes that have beneficial activities in the disease states being investigated.

More definitive conclusions of the relevance of these cell types to the in-vivo efficacies of these vaccines could be obtained by the use of systems such as gene knock-out mice (which could be engineered to lack specific cells). However a biological readout of the vaccine’s efficacy would be required to perform these experiments and this is, at present, lacking.
In conclusion therefore it has been shown that preparations of heat-killed *M. vaccae*, specifically SRL-172 and SRL-PBS, can stimulate γδ lymphocytes and CD56+ NK cells as assessed by proliferation, surface activation markers and cytokine secretion profiles *in-vitro*. The relevance of this stimulation, and whether it is direct or indirect, needs further investigation. It is currently unclear, on the basis of work presented here, whether these vaccine preparations have any effects upon NKT cells.
4 HSP60/65

4.1 General Introduction

The heat shock response was first described in the fruit fly, *Drosophila melanogaster*, after an incubator’s thermostat settings were accidentally elevated and the transcription of several genes in the fly were found to be up-regulated (Ritossa, 1996). The products of these genes became known as the heat shock proteins and were classified according to their molecular size. It was later recognized that many types of stress, including oxidative, mechanical and ischaemic/hypoxic, induce the transcription of these proteins. They are highly conserved, from basic prokaryotes to the higher mammals, and collectively constitute the most abundant group of molecules in living organisms. Many are involved in escorting proteins and regulating their folding within the cell and hence earned the name chaperonin, but many also appear to be involved in diverse immunological reactions.

4.1.1 The hsp60/65 Family

One of the major families of heat shock proteins comprises molecules of approximately 60 to 65 kDa, and is known as the hsp60/65 family. The most studied is the homologue of *E. coli*, known as GroEL. In combination with a smaller heat shock protein, GroES, these molecules form large, double-chambered structures in which protein folding occurs. Initially polypeptides are bound to hydrophobic sites within the chamber but with ATP hydrolysis the structure changes conformation and the polypeptide becomes surrounded by hydrophilic residues allowing its correct folding (Saibil, 2000). Correct protein folding appears to be the major function of all homologues of the hsp60/65 family.

In prokaryotes the hsp60/65 molecule is contained free within the cytoplasm but in eukaryotic cells it appears to be normally located within the mitochondrion.
4.1.2 hsp60/65 Immunology

The hsp60/65 molecule is of particular interest not solely for this apparent ubiquitous and essential protein folding function but also for the diverse immunological properties that have been associated with it.

As discussed above a wide variety of cellular stresses up-regulate the transcription of hsp60 and the other hsps. Teleologically it has therefore been suggested that heat shock proteins are a logical choice for immunodominant target antigens in that they are highly conserved in all living creatures and, by their nature, would be expressed on both stressed host cells (for instance in malignant transformation or infection) and on stressed microbes themselves. They may therefore act as ‘danger signals’ that activate the immune system (Matzinger, 1994).

Indeed hsp60/65 seems to directly stimulate several components of the innate and the adaptive immune systems. hsp60/65 molecules may be directly recognised by subsets of γδ and other lymphocytes, and exogenous hsp60 can induce TNF-α, IL-12 and IL-15 production by macrophages and IL-6 secretion from endothelial cells, macrophages and smooth muscle cells (Chen et al., 1999; Kol et al., 1999). hsp60 homologues can increase endothelial cell adhesion molecule expression (particularly E-selectin, ICAM-1, and VCAM-1) and activate the transcription factor NF-κB (Kol et al., 1999). It is possible that some of these effects are mediated through CD14 (analogously to lipopolysaccharide) (Kol et al., 2000).

Contrary to its potential role as a ‘danger signal’, hsp60/65 has also been shown to regulate and diminish certain immune responses. Examples include the decrease in incidence and severity of diabetes after vaccination with hsp60/65 (or the adoptive transfer of T cells reactive to this heat shock protein) in a murine model (Ablamunits et al., 1999), an amelioration or resistance to adjuvant arthritis in the rat in response to certain hsp60/65 epitopes and epitope spreading (Bloemendal et al., 1997; Moudgil, 1998) and the regulation of disease activity and cytokine production in humans with arthritis (Prakken et al., 1996; Prakken et al., 1997; van Roon et al., 1997).

However the mechanisms by which these effects occur are poorly understood.
Overall, associations with immune reactivity to hsp60/65 have been found in a wide variety of conditions. In humans both abnormal T and B cell reactivity have been associated with various arthritides, particularly rheumatoid arthritis, reactive arthritis and juvenile oligo-arthritides (Domeika et al., 1997; Gaston, 1998; MacHt et al., 2000; Mertz et al., 1998; Mertz et al., 2000; Rudolphi et al., 1997; de Graeff-Meeder et al., 1995; Prakken et al., 1996; Prakken et al., 1997). Associations have also been reported for diabetes mellitus (Abulafia-Lapid et al., 1999), recurrent oral ulceration (Hasan et al., 1995), Behcet's disease (Direskeneli et al., 1996; Kaneko et al., 1997; Tanaka et al., 1999), primary biliary cirrhosis (Vilagut et al., 1997; Yamaguchi et al., 1994), multiple sclerosis (Birnbaum and Kotilinek, 1997; Prabhakar et al., 1994), glaucoma (Wax et al., 1998) and possibly Crohn's disease (Stevens et al., 1992). In rodent models reactivity to hsp60/65 has also been associated with diabetes (in the NOD mouse) (Birk et al., 1996; Feili-Hariri et al., 2000) and various arthritides (Barker et al., 1996; Moudgil, 1998; van Eden et al., 1998).

4.2 Rationale For Investigation

Of particular relevance to the potential immuno-modulatory properties of SRL-172 are the associations between hsp60/65 and arteriosclerosis, tuberculosis, cancers, malaria, psoriasis, periodontal disease, asthma and allergies.

4.2.1 Arteriosclerosis

A group in Innsbruck, headed by Georg Wick, have pioneered much of the work into the association between reactivity to hsp60/65 and arteriosclerosis. In a rabbit model they demonstrated that immunization with hsp65 induced arteriosclerotic lesions in the aortic intima (Xu et al., 1992) and that the hsp60 expressed on endothelial and smooth muscle cells in human aortic and carotid biopsies induced the infiltration of both αβ and γδ T lymphocytes (Kleindienst et al., 1993). These cells proved to be hsp65-responsive in the rabbit (Xu et al., 1993a). Further, in
humans with confirmed carotid atherosclerosis or coronary artery disease, an increased titre of anti-hsp65 antibodies was demonstrated (Hoppichler et al., 1996; Xu et al., 1993), these antibodies cross-reacted with human hsp60 (Xu et al., 1993b) and the relevant antibody epitopes were found to comprise two N-terminal sequences, aa 97-109 and aa 179-187, and one C-terminal sequence, aa 504-512 on the hsp65 molecule (Metzler et al., 1997). These anti-hsp60 antibodies were shown to promote complement-mediated lysis of hsp60-expressing rat endothelial cells (Xu et al., 1994) and complement-mediated and antibody-dependent cellular cytotoxicity on stressed human umbilical vein endothelial cells (Schett et al., 1995) and heat-stressed human macrophages (Schett et al., 1997).

Separate groups have demonstrated the development of atherosclerosis in LDL-receptor deficient mice with hsp65 immunisation (Afek et al., 2000) and in other mice immunised with hsp60 in association with high cholesterol diets (Mori et al., 2000). hsp65 immunisation induced less fatty streak formation in IL-4 knock-out, as compared to wild-type, mice and this correlated with decreased anti-hsp65 serum antibodies but no differences in cellular immune responses to this molecule (George et al., 2000).

Raised serum antibody titres to hsp65 have also been found in humans with borderline hypertension, a risk factor for arteriosclerosis (Frostegard et al., 1997). This has led many to hypothesise that hsp60/65 induction, and the immune response induced, provides a unifying explanation for many of the risk factors for arterial disease including the possible infectious co-associations such as *chlamydia* and *helicobacter* (Birnie et al., 1998; Kol et al., 1999; Kol and Libby, 1999; Mosorin et al., 2000).

As a consequence of this association of reactivity to hsp60/65 and vascular disease, co-workers investigated the effect of vaccination with SRL-172 in several rat models, including the assessment of aortic contraction, post-angioplasty restenosis and the rejection of transplanted aortae (discussed previously). The first and third groups of animals provided plasma at sacrifice for hsp60/65 antibody determination and these studies are discussed in the later section on ELISA determination of rat plasma samples (section 4.4.4.3).
4.2.2 Tuberculosis

The immunodominant nature of hsp65 antigens in many pathogens has been well established, and this is particularly true for humans and animals with tuberculosis (Lorgat et al., 1992; Ottenhoff et al., 1988; Silva and Lowrie, 1994). Indeed much work is now being performed into DNA vaccination as a therapeutic and preventative approach to tuberculosis and the gene for hsp65 is one of the potential agents (Lowrie et al., 1997; Silva, 1999; Silva et al., 1999). Such vaccination has been shown to induce protection to subsequent challenge with *M. tuberculosis* (Lowrie et al., 1997; Tascon et al., 1996) and to induce sterilising immunity when used as a post-exposure vaccine (Lowrie et al., 1999). hsp65-specific T cell clones can adoptively transfer protection in murine models and such cells can either lyse target cells through granule or Fas-FasL pathways (though the former has significantly more sterilising effect) (Silva & Lowrie, 2000). However studies have also demonstrated increased lung damage and no protection from hsp65 protein and DNA vaccination in guinea pigs (Turner et al., 2000).

4.2.3 Cancer

Published research supports some roles for hsp60/65 in cancer immunology, though there is little available data of associations between hsp60/65 and prostate, renal and lung cancers – neoplasia for which SRL-172 has some putative efficacies. In melanoma, however, a cell line resistant to lysis secondary to very low surface MHC expression did develop significantly increased levels of MHC class I after hsp65 transfection, and was then effectively lysed by alloreactive T cells (Wells et al., 1997). Mice immunized with these cells thence became resistant to further challenge with the wild-type melanoma cells. In human primary prostate cancers there was no consistent correlation shown between hsp60 expression and phenotypic behaviour (Cornford et al., 2000).

Several human tumours have been shown to express hsp60, including Hodgkin's lymphoma Reed-Sternberg cells (Hsu and Hsu, 1998), gastric lymphomas
(Kobayashi et al., 1998) and ovarian carcinomas. For ovarian neoplasms it has further been demonstrated that the tumours expressing hsp60 have an improved prognosis compared to those with no detectable hsp60 (Schneider et al., 1999), though another study showed that low (but detectable) expression was associated with a better outcome than high level expression (Kimura et al., 1993).

Transfection of cells with hsp60 has been attempted in several neoplasia, including a macrophage tumour cell line in-vitro (Lukacs et al., 1993) and in-vivo (Lukacs et al., 1997) where loss of tumourigenicity, resistance to further challenge with wild-type tumour cells and regression were illustrated.

Some patients with cancer (and certain animal oncology models) appear to possess increased humoral and cellular responses to hsp60/65. Gastric epithelial cell binding antibodies, present in the serum of patients with gastric mucosa-associated lymphoid tissue (MALT) lymphomas, predominately bind hsp60 (Kawahara et al., 1999), and osteosarcoma patients have significantly increased anti-hsp60 antibodies (Trieb et al., 2000). It is hsp60 that is recognized by γδ lymphocytes on Daudi Burkitt’s lymphoma cells (Kaur et al., 1993), oral cancer cells (Laad et al., 1999) and oesophageal tumours (where they show lytic ability) (Thomas et al., 2000). CD4+ T cells recognizing hsp60 on a mouse fibrosarcoma line have been demonstrated to induce cytostasis and tumour-specific cytotoxic T lymphocytes (Harada et al., 1993).

4.2.4 Malaria

In a Plasmodium yoelii murine model a γδ cell response was noted and these γδ cells proliferated when presented with P. falciparum hsp60 (Kopacz and Kumar, 1999). In a similar P. yoelii model hsp65 was found to be expressed on splenic macrophages and this correlated closely with protection against infection (Zhang et al., 1999). Vaccination of mice with recombinant P. yoelii hsp60 (in association with GM-CSF) has demonstrated immunogenicity and some, though variable, protective efficacy on challenge with P. yoelii (Sanchez et al., 2001).
In Thai malarial patients ELISA assays demonstrated an increased IgA titre to hsp65, but no significant elevation of the other antibody classes (Zhang et al., 2001).

4.2.5 Psoriasis
Rambukkana and colleagues have published data correlating chronic psoriasis with humoral reactivity to mycobacterial hsp65, including a correlation with disease score (Rambukkana et al., 1993). This is potentially related to molecular mimicry with human epidermal cytokeratin 1/2 (Rambukkana et al., 1992). However other groups have only found a correlation when there was a potential infective focus (Izaki et al., 1996).

4.2.6 Periodontal Disease
Many of the pathogens associated with periodontal disease are known to up-regulate expression of their hsp60 homologues at the gingival surface (Ando et al., 1995), and patients with periodontal disease have been demonstrated to show a diminished PBMC proliferative response to both human hsp60 and mycobacterial hsp65 when compared to controls with gingivitis (Petit et al., 1999). The authors concluded that poor reactivity to hsp60/65 might be a susceptibility factor for destructive periodontal disease.
Interestingly a murine model of atheroma induction by hsp60 immunisation and high cholesterol diets also demonstrated periodontal inflammation (Mori et al., 2000).

4.2.7 Asthma
IgA antibodies to the hsp60 homologue of *Chlamydia pneumoniae* have been shown to be significantly elevated in patients with recently active asthma, with FEV1 measurements being negatively correlated with titre (Huittinen et al., 2001), but otherwise little research has been done in this area.
The hsp65 molecule of *M. vaccae* is known to be one of the major immunogenic components of the heat-killed preparation – inducing proliferation and IFN-γ secretion in human peripheral blood mononuclear cells and mouse splenocytes, and promoting lysis of OVA-transfected cells after simultaneous immunisation with OVA (see Chapter 1).

It was hypothesised that a molecule that is a markedly immunogenic part of SRL-172, and that appears to be involved in the immune response in a variety of disease states (including those where the vaccine has a putative utility), may be involved in the efficacy of this vaccine. It was therefore decided to sequence the gene for the hsp65 homologue of *M. vaccae* NCTC 11659 and examine the humoral responses to this family of proteins in animals and humans who have received SRL-172.

### 4.3 hsp65 Sequencing

#### 4.3.1 DNA Extraction

The DNA extract of the vaccine strain of *M. vaccae* (NCTC 11659) used for the following sequencing was the laboratory stock obtained by the Puregene system (Gentra) (involving cell lysis, treatment with RNase A, protein precipitation and DNA precipitation with isopropanol).

#### 4.3.2 Primers

The sequences for two primer pairs that amplified sections of the hsp65 molecules of most mycobacteria were obtained from the published literature: TB11 & TB12 (Telenti et al., 1993) and TB13 & 65-1400 (Steingrube et al., 1995).

Further primers were designed to amplify the remainder of the hsp65 gene in a step-wise and piggy-back fashion. For the majority of the sequence this was achieved by obtaining the known hsp65 DNA sequences for *M. tuberculosis, M. paratuberculosis, M. leprae* and other mycobacteria from Entrez at the National Centre of Biotechnology Information, USA (www.ncbi.nlm.nih.gov/entrez/), and assessing regions of homology with Align (www2.igh.cnrs.fr/bin/align-guess.cgi).
or BLAST (www.ncbi.nlm.nih.gov/blast/) - the rationale being that such conserved regions would probably also be conserved in *M. vaccae*.

The primers thus obtained and utilised were:

- **TB11**  
  ACCAACGATGGTGTTGCCCAT
- **TB12**  
  CTTGTGAACCGCATACCT
- **TB13**  
  GGCTACATCTCGGGGTACTTC
- **65-1400**  
  CTCGGTGAGGTCAACAACAGC
- **AU1**  
  ATGGCCAAGACAATTTCG
- **AU-2**  
  CGAGGAGAGCAACACCTTCGG
- **1-21**  
  AGTGCTAGGTCGCGCAGGCTGG
- **1825**  
  CCTTCCTCCGGGCTTGTCGGC
- **US-1**  
  GCGAGCTCATCGCCGAGGCA
- **US-2**  
  GTCTCGAGCGACAGGCGACC
- **US-E**  
  CAGAAGTCCATGCCACCCATG

Obtained from Oswel DNA Services.

Different approaches were required to design primers to amplify the 5' and 3' ends of the gene, and these will be discussed in following sections (4.3.6.1 & 4.3.6.2).

**4.3.3 Polymerase Chain Reaction**

Polymerase chain reaction product was obtained for sequencing by the addition of DNA extract and primers to 45µl pre- aliquoted PCR Mastermix (Advanced Biotechnologies) containing:

- 1.5mM MgCl₂
- 1.25µM Taq polymerase
- 75mM Tris-HCL (pH 8.8 at 25°C)
- 20mM (NH₄)₂SO₄
- 0.01%(v/v) Tween 20
- 0.2mM dATP, dCTP, dGTP, dTTP.
To the Mastermix was added 2µl of each of two primers (adjusted to 25µM), and 1µl of the DNA extract (either neat or 1:10 diluted with molecular grade water). The total volume for the PCR reaction was therefore 50µl.

All these additions were performed on ice, the mixtures then vortexed and pulsed in the microfuge.

Initial PCR reactions were performed in a Hybaid Thermal Reactor, however later reactions utilised a Phoenix thermal cycler (Techne Cambridge Ltd.).

The PCR cycle used depended on the primer combination but was based on the following structure:

<table>
<thead>
<tr>
<th>Denaturing</th>
<th>Annealing</th>
<th>Extension</th>
<th>Number of Cycles</th>
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<tr>
<td>95°C for 5min</td>
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<td>1</td>
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<tr>
<td>95°C for 1min</td>
<td>$T_a$ for 1min</td>
<td>72°C for 1min</td>
<td>35</td>
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<td></td>
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<td>72°C for 5min</td>
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Where $T_a$ was either 60°C or adjusted according to the calculated melting temperature (Tm) of the primers (using the formula $T_m = 2(A+T) + 4(G+C)$, where A,T,G&C are the numbers of the respective bases within the oligonucleotide). Generally an annealing temperature a few degrees below the lower primer melting temperature was utilised.

Resultant product was then stored at 4°C.

4.3.4 Gel Preparation and Running

Resulting product was then separated by electrophoresis on an agarose gel and visualised under ultra-violet light with ethidium bromide staining.

The agarose gels were prepared from electrophoresis grade agarose (GibcoBRL) and TBE buffer.

10xTBE buffer was prepared as follows: 1litre $H_2O$

+ 107g Tris base
+ 55g Boric acid
+ 7.46g NaEDTA.2H₂O

and diluted with ultra-pure water at usage.

1.5% agarose (weight/volume) was added to TBE buffer, heated in a microwave oven for approximately 5min with frequent shaking until fully dissolved. This was allowed to cool after the addition of ethidium bromide (3μl per 50ml of agarose solution), and the resultant mixture poured into a clean PCR tray (with comb) and left to set at room temperature.

The set gel was immersed in TBE electrophoresis buffer (prepared from 10x stock solution with ultra-pure water) within the PCR apparatus. 5μl PCR product was loaded into the wells of the gel having been mixed with 1μl 6x loading buffer (40% glycerol, 0.25% bromo-phenol blue). Appropriate commercial DNA ladders were used.

Electrophoresis was performed with either a BioRad 3000Xi electrophoresis power supply (at a constant 100mA for 30 minutes to 1 hour) or with an EPS 301 power supply (Amersham Pharmacia Biotech) (at a constant 95V for 30 minutes to 1 hour) depending on the gel size.

Polaroid film was used to record the appearance of the gel under ultra-violet light.

4.3.5 Sequencing

Sequencing was performed by a technician at the Photosynthesis Laboratory, UCL, on an ABI Prism 310 Genetic Analyser (Applied Biosystems) with Big Dye Terminator Cycle Sequencing Chemistry.

4.3.6 5' and 3' Ends of hsp65 Gene

The above methods were not sufficient to obtain product that included the terminal ends of the hsp65 gene. This was for several reasons. Firstly few sequences from other mycobacteria were initially obtainable that contained sequence flanking the transcribed section of the gene. Therefore it was difficult to align sequences to obtain candidate conserved primer binding regions that could be used to amplify the terminal sections. Secondly the candidate primers that were so designed proved to be either non-specific or poor at amplifying the M. vaccae DNA.
Therefore different approaches were employed.

4.3.6.1 5' End

Some DNA sequences became available in the public domain during the course of this thesis that included sequence data preceding the transcribed section of the gene. These were aligned and potential candidate primer binding regions were assessed. Rational primer designing was employed on the following principles:

- Primer pairs would be designed such that their estimated melting temperatures were within 1°C and the annealing temperature of the PCR cycle adjusted to be optimal for the pair.
- The 3' base of each primer would preferentially be a cytosine or guanine as these bases bind to their complementary base by 3, not 2, hydrogen bonds thereby increasing the specificity and strength of binding.
- Primers would be assessed for any potential intra- or inter-primer complex formation (to decrease primer-dimer formation and increase specificity). Polypurine or polypyramidine stretches and other unusual sequences would usually be avoided.
- The uniqueness of the specificity of the primers (for both known mycobacterial sequences and any other known, potentially contaminating DNA (e.g. human)) would be assessed via searches of the BLAST database (www.ncbi.nlm.nih.gov/blast).

As a result, of several potential primers, AH-2 and AH-3 were designed and utilised:

AH-2: GCGAGCACGCTGGCGGTGG
AH-3: CGTCCGTCGCGGGCACTGC

Obtained from MWG Biotech.
These were adjusted to 10μM concentrations and 1μl of each was added to the Mastermix (with 3μl DNA extract).

Initial PCR amplification experiments produced only poor quality, relatively non-specific product and therefore the procedure of touchdown PCR was employed.

4.3.6.1.1 Touchdown PCR

As the annealing temperature of the PCR cycle increases, the specificity of primer binding increases, but often the sensitivity of the amplification decreases. To increase the specificity whilst still maintaining sufficient sensitivity, touchdown PCR can be employed. This involves commencing the PCR reactions using an elevated annealing temperature, and gradually dropping this temperature with consecutive cycles until it reaches the usual optimal annealing temperature (generally a few degrees below the calculated melting temperatures for the primers). The first few cycles amplify the specific product and though, as the temperature drops, the primers may bind less specifically the specific product will now have accumulated and will out-compete other products.
A touchdown cycle for primers AH-2 and AH-3 was employed as follows:

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<td>95°C for 1min</td>
<td>70°C for 1min</td>
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<td>69°C for 1min</td>
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<td>72°C for 5min</td>
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Good quality product was obtained that was successfully sequenced.

4.3.6.2 3' End

Few databases within the public domain contained sequence data for the region flanking the 3' ends of mycobacterial hsp65 genes. Those that did showed no regions of sufficient homology to permit primer design. Therefore the method of inverse PCR was utilised in an attempt to obtain product spanning this region.

4.3.6.2.1 Inverse PCR

This technique, allowing the amplification of DNA flanking a region of known sequence, was described in 1988 (Ochman et al., 1988). The basic principle is that of cutting the DNA with a restriction enzyme and then allowing intra-molecular ligation of the resultant ends to produce circular DNA molecules. Primers designed to extend outwards from the known core DNA sequence can then be used to amplify a linear fragment that incorporates the unknown flanking region, and this product can be sequenced.

In applying this technique to sequence the 3' end of the hsp65 gene of *M. vaccae* NCTC 11659 the previously obtained sequence approaching the 3'end was
analysed, with the known sequences of several other mycobacterial hsp65 genes, for two primer binding sites in close proximity to each other. Primers INV-1 (and later rINV-4) and INV-2 were thus designed (sequences listed later). This known terminal sequence was then assessed (using MAPSORT, at www.hgmp.mrc.ac.uk) for potential restriction enzyme cleavage sites. The optimal restriction enzyme would cleave near to the 5' end of the binding site of INV-1/rINV-4 (thereby preventing the incorporation of excessive known sequence in the resultant amplified product), would not cleave the DNA between the binding sites of INV-1/rINV-4 and INV-2 (which would negate the procedure) but would also be expected to cleave DNA at fairly frequent intervals (to prevent an excessively long resultant circular DNA fragment which would not permit adequate amplification and consequent sequencing). Two candidate enzymes that fulfilled these criteria were SacII and MspAI.

4.3.6.2.1.1 Restriction Enzyme and DNA Ligation Protocols
DNA digests with SacII and MspAI (both Promega) were performed as follows: 1µg M. vaccae NCTC 11659 DNA was incubated with 5u of the restriction enzyme, 0.2µl BSA and 2µl 10xRE buffer (adjusted to a total volume of 20µl) at 37°C for 2.5 hours. The resultant product was then heated to 68°C for 30 minutes to remove the restriction enzyme activity.

Ligation of the resultant DNA fragments was then performed with T4 DNA ligase (Promega) whereby either 1µl or 10µl of the digest product was incubated with 1u ligase and 5µl 10xbuffer (in a total volume of 50µl) at room temperature either overnight or for 3 hours.

Amplification of potentially circularised DNA was then attempted with primers INV-2 and INV-1 (and later rINV-4) using the basic PCR cycle (above) with the annealing temperature set at 55°C. In the later amplifications, for adequate specific product, the technique of hot-start PCR was employed.
4.3.6.2.2 Hot-Start PCR

The basic principle behind this technique is that Taq polymerase is maintained in an inactive form and only becomes activated when heated to 95°C. As a result DNA amplification can only commence once this temperature has been reached when, theoretically, all DNA strands will be separated. Thus problems of primer dimerisation and non-specific annealing at lower temperatures are diminished.

The Qiagen system (HotStarTaq) was utilised for this work, and Q solution (provided with the kit) was also utilised to decrease inhibition and the effects of electrolyte imbalances. Manufacturer’s instructions were followed.

Primers utilised:

INV-1  CGGCGTTGAGGCCGTGAC
INV-2  CGTCGCCGACCCGGTG

Obtained from Oswel DNA Services.

rINV-4  GCAGGTCTCGTACTCAC

Obtained from MWG Biotech.

Initial attempts at inverse PCR using primers INV-1 and INV-2 provided only poor quality sequence results but by comparing 5’-3’ and 3’-5’ sequences from duplicate samples a small area of homology was found that permitted the design of the primer INV-3 (GGTCCGAACCGAGTGATC, MWG Biotech).

PCR was then performed on raw *M. vaccae* NCTC 11659 DNA extract (not restriction digested and ligated) using INV-3 and INV-2 in an attempt to obtain good sequence data adjoining the known sequenced region.

The inverse PCR procedure was also repeated from initial restriction digesting utilising primers rINV-4 and INV-1.
4.3.6.23 Results

The sequence data obtained from primers INV-2 and INV-3 respectively, when matched on duplicate samples, were as follows:

GTTGACCCCAACACAGGCTCCACGGGCAAAGTATCTTCTACCTCTTGG
TCCGAGTCTATCCGGTAAACCCCAATGAACTCGTGGAGACTGCGCC
GACAAGTGCGCTCTTCGGTCTGCAATTGTGCCGAGTACTACGAC
GGGCTCGGTGATCTGGGAGATCGGGCCGCTGCAACGTGCGCCGAC
ATCGAAGACGCCTTCAACGCTCCAGGGCAACGTCTATACGTCCGAC
CCCTTGATCACTCGGTT

ACGTTCGCCCGCTGAAGCGTTAACCCTTCTTCCGATATCGGGCGCCCGC
AGCACCCTTTCGGTCGATCTGCCAGATCCACGAGCCGCGTGGTACTCCG
GCACAATTGCGAGACGAGCTCGACCGACCTTGTCCGCGACGCTCTCC
CACCGGTTTATTTGCGTCAAACCCGGAATGACTCCGGAAGAGTAGAACG
GTACTTTTGCGGCGTGGAGCCTGTGTGGGGTCAACGGCTGACGGGAC
ATNGCGCAAGGTACACAGCCGACCGGTCCGGACG

The sequence data obtained from the use of rINV-4 and INV-1 on duplicate samples (sequenced with rINV-4) were of poor quality:

NTCGNGCCANACCNAANGNANGAGATCTGNTGNANNCTGCAATGTGAT
CCTGNAGGTATACAAGGCTGAGACCCNGCAGGGGGCTGAGCTGTTG
CANACCTTGGTACGTTCTGCTTNTGCTCANACNGTNTCANNGGA
CATNNGAATACGTGACATGTGNCGGNTCTGTGGATGCGTGNCCANG
CANCNTGTGCGGACACCTCTCTCTGCGACTTGGACACNNCTACTCGATGCT
GANNAGACGANCNGCNCTGNTNGCCNGCNCNGCCGACAACATGAAATT
CGATCGNACAGCCTTGGCAACNGTGAATGGAACATGNTGACGCCACGC
TGCACGCTNGCAANCATTNGNNNTGNCGAANGNCGCNCAGCCACTCCAT
CAACCTGNGCNNTNGNGCANGNTAANGCGACNCNATGAGCG
GGACAACCNTNTAAACCTGNTGNNNGCAATAACGACNCTNANACTCCN
ANNCACGGGAAACANGCGACANCNGCNNNGGGNGACATGNCANNA
CGCCACANNANACNTACTGAATGCTACTNGTANAAACACCAGGTTTGC
ATNGANGCCGAACCCGACAGGTGTTGCGAATNCACGCC
Where N was an undetermined base.

From this sequence data however several potential primers were designed, one of which was rINV-8 (GTGCGAGGAAGGAGGTC, MWG Biotech).

Attempts were then made to join the known, previously obtained, sequence with the new fragments sequenced via inverse PCR. To this end two further primers:

- INV-5 (TCAAGCAGATCGCCTTCAACG)
- INV-6 (GCATCGAGGACGCCGTC)

(both from MWG Biotech) were designed within the known, previously obtained hsp65 sequence.

PCR reactions utilising INV-3 with either INV-5 or INV-6 failed repeatedly to provide product. However rINV-8 with INV-6 provided strong product that provided the following sequence:

CGGGTGTGTGGTGACGATGCCGATCCATCAGCGTGCCCGGATCCTCA

However this sequence failed to join with the known, previously obtained sequence and when submitted to BLAST (www.ncbi.nlm.nih.gov/blast) failed to match significantly with any known mycobacterial sequences. It therefore had to
be concluded that the inverse PCR process in this case had probably resulted in the amplification of a separate part of the *M. vaccae* genome, unconnected with the hsp65 gene. Further attempts were therefore abandoned and the 3' end of the gene presently remains unsequenced.

4.3.7 Discussion

4.3.7.1 Interpretation of the Sequencing Results

Each sequence obtained is inaccurate at the extreme ends of the sequencing product. This potential problem was diminished in two ways. Firstly the actual sequencing result was examined with EditView (Version 1.0, Applied Biosystems 1996) and the terminal regions of poor signal identified and removed. Secondly sequences were obtained which overlapped to significant degrees and several separate PCR products from a particular combination of primers were sequenced. Also most products were sequenced in both directions (5'-3' and 3'-5'). The resultant sequences were aligned against the known sequence of *Mycobacterium paratuberculosis* hsp65 and nucleotides and regions that were non-homologous between *M. vaccae* sequences were identified and either verified or discarded.
The final sequence, built-up in this way, was:

ATGGCCAAGACAATTGCGTATGACGAAGAGGCCCGTCGCGGCCTCGAGCGGGGCCTCAACGCCCTCGCCGACGCGGTAAAGGTGACGTTGGGCCC
CAAGGGCCAGCATCGTCTTGGAGAAGAATGGGGCGCCCTCCCACCTCGACATGGTGTGTCCATCGCCAAGGAGATCGAGCTGGAGGACC
CCTACGAGAAGATCGGCGCAGAGCTGGTCAAGGAAGTCGCCAAGAAGACCGACGACGTCGCGGGTGACGGCACCACCACCGCCACCGTGCTGGCC
CAGGCGCTCGTGCAGAGGCTCGCAACGTCGCCGCCGGCGCCAACCGCTCGGCCTGAAGCGCGGCATCGAGAAGGCCGTCGCCAAGGTCACC
GAGACCCTGCTGCTGCCTGGCCCAAAAAGAGGGTCGAGACCCAGAAGGAGCAT
CGCTGCCCAAGCTCGCCGCTCGCCGATCTCGGCCGCGGTGACAGCCAGATCGGCGAGCT
CATCGGCCAGGGCATGCAAGGTCGGCAACGAGGGTGTCATCACCGTGAGGAGAGCAACACCTTCGGCCTGCAGCTCGAGCTCACCGAGGGCAT
GCTGGCCAAGCTGGCCGGCGGTGTTGCGGTGATCAAGGCCGGAGCTGCCACCGAGGTGGAGCTCAAGGAGCGCAAGCACCGCATCGAGGACGCC
GTCCGCAACGCCAAGGCTCGCCCGTGCAAGAGGGCATCGTCCGGCCGGTGCCAGGAGCAGCTGCAGGACATGGCGATCCTCACCGGTGGCCAGGTCGTCAGCGAAGAGGTCGGCCTGTCGCTCGAGACCGCCGACGTCTCGCTGCTGGGCAAGGCGC
CGCAAGGCGCTGCTGCTCCGACTCCGACGCCATCGCCGGCCGCGTCTCGCAGATCCGCGCCG
GATCGAAGACAGCAGCAGCTCGCAATACGACCCCGAAGCTGCAGGAGCGCTGCCTGGCCAAGCTGGCCGGCGGTGTTGCGGTGATCAAGGCCGGAGCTGCCACCGAGGTGGAGCTCAAGGAGCGCAAGCACCGCATCGAGGACGCC
GTCCGCAACGCCAAGGCTCGCCCGTGCAAGAGGGCATCGTCCGGCCGGTGCCAGGAGCAGCTGCAGGACATGGCGATCCTCACCGGTGGCCAGGTCGTCAGCGAAGAGGTCGGCCTGTCGCTCGAGACCGCCGACGTCTCGCTGCTGGGCAAGGCGC
CGCAAGGCGCTGCTGCTCCGACTCCGACGCCATCGCCGGCCGCGTCTCGCAGATCCGCGCCG
GATCGAAGACAGCAGCAGCTCGCAATACGACCCCGAAGCTGCAGGAGCGCTGCCTGGCCAAGCTGGCCGGCGGTGTTGCGGTGATCAAGGCCGGAGCTGCCACCGAGGTGGAGCTCAAGGAGCGCAAGCACCGCATCGAGGACGCC
GTCCGCAACGCCAAGGCTCGCCCGTGCAAGAGGGCATCGTCCGGCCGGTGCCAGGAGCAGCTGCAGGACATGGCGATCCTCACCGGTGGCCAGGTCGTCAGCGAAGAGGTCGGCCTGTCGCTCGAGACCGCCGACGTCTCGCTGCTGGGCAAGGCGC
CGCAAGGCGCTGCTGCTCCGACTCCGACGCCATCGCCGGCCGCGTCTCGCAGATCCGCGCCG
GATCGAAGACAGCAGCAGCTCGCAATACGACCCCGAAGCTGCAGGAGCGCTGCCTGGCCAAGCTGGCCGGCGGTGTTGCGGTGATCAAGGCCGGAGCTGCCACCGAGGTGGAGCTCAAGGAGCGCAAGCACCGCATCGAGGACGCC
GTCCGCAACGCCAAGGCTCGCCCGTGCAAGAGGGCATCGTCCGGCCGGTGCCAGGAGCAGCTGCAGGACATGGCGATCCTCACCGGTGGCCAGGTCGTCAGCGAAGAGGTCGGCCTGTCGCTCGAGACCGCCGACGTCTCGCTGCTGGGCAAGGCGC
CGCAAGGCGCTGCTGCTCCGACTCCGACGCCATCGCCGGCCGCGTCTCGCAGATCCGCGCCG
GATCGAAGACAGCAGCAGCTCGCAATACGACCCCGAAGCTGCAGGAGCGCTGCCTGGCCAAGCTGGCCGGCGGTGTTGCGGTGATCAAGGCCGGAGCTGCCACCGAGGTGGAGCTCAAGGAGCGCAAGCACCGCATCGAGGACGCC
GTCCGCAACGCCAAGGCTCGCCCGTGCAAGAGGGCATCGTCCGGCCGGTGCCAGGAGCAGCTGCAGGACATGGCGATCCTCACCGGTGGCCAGGTCGTCAGCGAAGAGGTCGGCCTGTCGCTCGAGACCGCCGACGTCTCGCTGCTGGGCAAGGCGC
CGCAAGGCGCTGCTGCTCCGACTCCGACGCCATCGCCGGCCGCGTCTCGCAGATCCGCGCCG
GATCGAAGACAGCAGCAGCTCGCAATACGACCCCGAAGCTGCAGGAGCGCTGCCTGGCCAAGCTGGCCGGCGGTGTTGCGGTGATCAAGGCCGGAGCTGCCACCGAGGTGGAGCTCAAGGAGCGCAAGCACCGCATCGAGGACGCC

With the first 9 bases preceding the transcribed gene.
MAKTIAYDEEARRGLERGLNALADAVKVTLPGRNVVLEKKWGAPTT
NDGVSIAKEIELEDPYEKIGAEVLKEEVAKKTDDVAGDGGTTATVLAQALV
REGLRNVAAGANPLGLKRGIKEAVAKVETTLLASAKEVEKSEQIAATAISS
AGDSQIGELIAEAMDKVNGEVTVEESNTFGQLLELTEGMRFDKGYISGY
FVTDAERQAEAVLEDPYIIYSSKVSTVKKLDLLNEKVIQSGKPLLIAEDVE
GEAALSTLVAWVKIRGTFKSVAVKAPGFDREKAMLQDMALTGGQVVSEE
VGLSLETADVSSLGKARKVTVTKDETITVEGAGDSDAIAAGRVSQRAEIEN
SDSYDREKLRLEKLAGGVAVAGAATKEVKERKHIEDAVRNKAAVEEGIVAGGGVALLQSAPSLLELKTGDEATGANSRVALSAPLKVQIAF
NGGLEPGVVEKVTNSPAGTGLNAATGHEYEDLLKAGVADPVKVVT

As discussed above this sequence is incomplete. The last 132 bases, encoding 44 amino acids (if the molecule is of the same length as *M. paratuberculosis*) have not, as yet, been sequenced.
4.3.7.2 Comparisons with Other Sequences

The nucleotide and amino acid sequences were compared with known mycobacterial, human and rat hsp60/65 sequences using Emboss (www.ebi.ac.uk/emboss/align).

Results of overall degrees of nucleotide homology were:

<table>
<thead>
<tr>
<th>Organism</th>
<th>Sequence Reference</th>
<th>Nucleotide Homology (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. tuberculosis</em></td>
<td>3261527</td>
<td>88.6</td>
</tr>
<tr>
<td><em>M. avium paratuberculosis</em></td>
<td>438180</td>
<td>92.0</td>
</tr>
<tr>
<td><em>M. avium</em></td>
<td>9652215</td>
<td>92.1</td>
</tr>
<tr>
<td><em>M. bovis BCG</em></td>
<td>149933</td>
<td>89.6</td>
</tr>
<tr>
<td><em>M. leprae</em></td>
<td>4154036</td>
<td>86.5</td>
</tr>
<tr>
<td><em>M. vaccae</em> (partial cds = 360)</td>
<td>602240</td>
<td>90.3</td>
</tr>
<tr>
<td>Genesis Corporation <em>M. vaccae</em></td>
<td></td>
<td>92.4</td>
</tr>
<tr>
<td>Human</td>
<td>184411</td>
<td>61.3</td>
</tr>
<tr>
<td>Rat</td>
<td>1778211</td>
<td>66.3</td>
</tr>
</tbody>
</table>

Results of overall degrees of amino acid homology (including conservative mutations) were:

<table>
<thead>
<tr>
<th>Organism</th>
<th>Sequence Reference</th>
<th>Homology (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. tuberculosis</em></td>
<td>2909515</td>
<td>96.2</td>
</tr>
<tr>
<td><em>M. avium paratuberculosis</em></td>
<td>438181</td>
<td>97.6</td>
</tr>
<tr>
<td><em>M. avium</em></td>
<td>9652216</td>
<td>98.0</td>
</tr>
<tr>
<td><em>M. bovis BCG</em></td>
<td>149934</td>
<td>96.2</td>
</tr>
<tr>
<td><em>M. leprae</em></td>
<td>149924</td>
<td>96.4</td>
</tr>
<tr>
<td><em>M. vaccae</em> (partial aa = 120)</td>
<td>602241</td>
<td>97.5</td>
</tr>
<tr>
<td>Genesis Corporation <em>M. vaccae</em></td>
<td></td>
<td>97.6</td>
</tr>
<tr>
<td>Human</td>
<td>306890</td>
<td>69.6</td>
</tr>
<tr>
<td>Rat</td>
<td>1778213</td>
<td>69.6</td>
</tr>
</tbody>
</table>
For some putative disease specific protein epitopes identified on the *M. tuberculosis* hsp65 molecule comparisons were made using Blast-2-sequences (www.ncbi.nlm.nih.gov/gorf/bl2.html).

<table>
<thead>
<tr>
<th>Association</th>
<th>Epitopes</th>
<th>Reference</th>
<th>Homology</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atheroma</td>
<td>97-109</td>
<td>(Metzler et al., 1997)</td>
<td>10/13</td>
</tr>
<tr>
<td></td>
<td>179-187</td>
<td></td>
<td>9/9</td>
</tr>
<tr>
<td></td>
<td>504-512</td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td>Adjuvant Arthritis</td>
<td>180-188</td>
<td>(Anderton et al., 1994; Hogervorst et al., 1992; Thompson et al., 1998; van Noort et al., 1994)</td>
<td>9/9</td>
</tr>
<tr>
<td></td>
<td>216-225</td>
<td></td>
<td>10/10</td>
</tr>
<tr>
<td></td>
<td>226-235</td>
<td></td>
<td>10/10</td>
</tr>
<tr>
<td></td>
<td>261-270</td>
<td></td>
<td>10/10</td>
</tr>
<tr>
<td></td>
<td>376-408</td>
<td></td>
<td>19/33</td>
</tr>
<tr>
<td>Rheumatoid Arthritis</td>
<td>241-255</td>
<td>(Gaston et al., 1991; Quayle et al., 1992)</td>
<td>14/15</td>
</tr>
<tr>
<td></td>
<td>251-265</td>
<td></td>
<td>15/15</td>
</tr>
<tr>
<td>Diabetes Mellitus</td>
<td>422-445</td>
<td>(Feili-Hariri et al., 2000)</td>
<td>18/23</td>
</tr>
<tr>
<td>Behcet’s Disease</td>
<td>111-125</td>
<td>(Kaneko et al., 1997; Pervin et al., 1993)</td>
<td>14/15</td>
</tr>
<tr>
<td></td>
<td>154-172</td>
<td></td>
<td>16/19</td>
</tr>
<tr>
<td></td>
<td>311-325</td>
<td></td>
<td>14/15</td>
</tr>
<tr>
<td>Recurrent Oral Ulceration</td>
<td>91-105</td>
<td>(Hasan et al., 1995)</td>
<td>6/15</td>
</tr>
</tbody>
</table>

NS = not sequenced.
4.3.7.3 **Overall Discussion**

Despite many efforts the final bases of the hsp65 gene for *M. vaccae* NCTC 11659 could not be sequenced. It is interesting to note that Genesis Corporation, who now are predominately a genomics company, also failed to obtain the final section of the sequence for their strain of *M. vaccae*. It is unclear why this section of the gene proved so difficult. It may be possible that the flanking DNA sequence is similar to the flanking sequence within the *M. tuberculosis* genome – a PPE gene. These genes are known to be highly polymorphic and contain many (sometimes inverted) repetitive sequences. Indeed several groups are investigating PPE genes to aid molecular typing of *M. tuberculosis* strains. As illustrated above the main approach utilised in this thesis was inverse PCR and though some good quality sequencing results were obtained it proved impossible to join this sequence data with that already obtained, and the sequences demonstrated no homology with known mycobacterial sequences. It may therefore be possible that the technique amplified a separate section of the *M. vaccae* genome, unrelated to hsp65. This is currently being investigated.

The *M. vaccae* (NCTC 11659) sequence that was obtained however was of good quality and multiply verified as described above. It therefore allowed comparisons with equivalent regions of publicly available mycobacterial hsp65 gene sequences, particularly *M. tuberculosis*, *M. avium*, *M. avium paratuberculosis*, *M. bovis* BCG and *M. leprae*. A comparison was also made with the only GeneBank deposited sequence of *M. vaccae* (a 360bp segment amplified by primers TB11 and TB12), the sequence obtained by the Genesis Corporation and with the human and rat hsp60 sequences (as these were the species that have received vaccination with SRL-172 in the studies related to this thesis). Many putative disease-related epitopes have been described and some of these were also evaluated in the *M. vaccae* NCTC 11659 sequence.

One of the most interesting results from these comparisons was that there is significant variation between the strain of *M. vaccae* sequenced here (NCTC 11659) and that sequenced by the Genesis Corporation. Both these strains have
been used to prepare heat-killed products, the former SRL-172, and mostly they appear to have similar clinical efficacies. Indeed it is interesting to note that the degree of nucleotide homology between the two *M. vaccae* strains (92%) is only slightly greater than the degree of homology between the sequence derived here and that known for *M. avium paratuberculosis*. In comparing the amino acid sequences there is a similar degree of variability between the sequence derived here and that for the Genesis *M. vaccae* strain, *M. avium* and *M. avium paratuberculosis*. Further strain typing is presently being undertaken (specifically examining the 16s ribosomal subunit).

Comparisons with the known disease epitopes are difficult to interpret at present. As further clinical uses of vaccination with heat-killed *M. vaccae* are investigated, and possibly the *in-vivo* immune responses to overlapping short peptides examined, then this data may become more relevant.

To investigate the potential influence of immunisation with heat-killed *M. vaccae* on the immune responses to particular epitopes within the hsp60/65 molecule, the antibody responses to overlapping fragments of human hsp60 (and to the complete *M. bovis* hsp65 molecule) were investigated in two series of rats and one of humans who received vaccinations with SRL-172. It was hoped that data thus obtained could be correlated with the sequence data above to narrow down specific regions of interest and permit the further identification of important epitopes.
4.4 Humoral Responses to hsp60/65

4.4.1 ELISA Assay

Humoral responses to proteins can be determined by a variety of methods. The most popular and useful method presently available is the ELISA or enzyme-linked immunosorbant assay (developed in 1971 by Engvall and Perlman).

One variation of ELISA is the indirect assay. This technique was used to assess the antibody response to human and mycobacterial hsp60/65 in sera from series of humans and rats that had received vaccination with SRL-172 or placebo.

The analysis of different sera and of differing antibody isotypes required indirect ELISA assays adapted from a basic protocol as follows:

4.4.1.1 Basic Indirect ELISA Protocol

Materials:

Flat bottomed 96-well plates are used (Maxisorb/Immunosorb, Nunc).

Antigen(Ag) - Complete and deletion fragments of human hsp60 and hsp65 of M. bovis (see below).

Coating Buffer - 100ml sterile water (sterile water for irrigation, Baxter)
+ 0.159g Na₂CO₃ (BDH)
+ 0.293g NaHCO₃ (BDH)

(Resultant pH>9.5).

PBS - 1litre H₂O
+ 8g NaCl
+ 0.2g KH₂PO₄
+ 1.135g Na₂HPO₄.2H₂O
+ 0.2g KCl  (all from BDH).

Washing Buffer - PBS + 0.05% Tween 20
(polyoxyethylene-sobitan monolaurate, Sigma).

For stages pre-blocking (see below) sterile water for irrigation (Baxter) is used to make PBS, for those stages after blocking (see below) ultra-pure water is used.
Blocking Buffer - Dependent on protocol, though usually containing
BSA (bovine serum albumin, Sigma) or
Skimmed milk (dried).

Conjugates - Streptavidin (Peroxidase conjugated) (Dako)
Extravidin (Alkaline phosphatase conjugated) (Sigma)

Chromogens - TMB (3,3',5,5'-tetramethylbenzidine, Sigma)
pNPP tablets (p-nitrophenyl phosphate, Sigma)

Wet Box - Watertight box with moistened tissue at base
(to decrease evaporation from wells during assay).

1. Coating: Ag is Suspended to 1μg/ml in coating buffer and then 50μl is added
to the well. The plate is left overnight at 4°C.

2. Wash x1: The liquid within the wells is thrown out and the plate is then
banged against clean paper towels to remove further fluid. 200μl washing
buffer is then added to each well and left for approximately 3min. The liquid is
then thrown out and the plate banged on towels again.

3. Block: 200μl blocking buffer is added to each well and the plate placed in
an incubator at 37°C for a period of time dependent on protocol.

4. Wash x1: As above.

5. Serum: 50μl of serum (pre-diluted in blocking buffer) is added to each well
and the plate placed in an incubator at 37°C. Degree of dilution and duration of
incubation dependent on protocol.

6. Wash x3: As above but 3 consecutive times.

7. Antibody: 50μl of secondary antibody (prediluted in blocking buffer to degree
dependent on antibody and protocol) is added to the wells and the plate is
placed in an incubator at 37°C for a period of time dependent on protocol.

8. Wash x3: As above.

9. Conjugate (if required): 50μl Streptavidin/Extravidin (prediluted in blocking
buffer to degree dependent on protocol) is added to the wells and the plate is
placed in an incubator at 37°C for 1 hour.

10. Wash x3: As above.
11. Chromogen: 50\(\mu\)l TMB or pNPP are added to each well and the plate is left at room temperature (time dependent on protocol). If required 25\(\mu\)l 0.5M H\(_2\)SO\(_4\) added to TMB as a 'stop solution'.

12. Reading: Performed on Dynatech MR5000, after 20sec shake, with filters as follows:

<table>
<thead>
<tr>
<th>Filter</th>
<th>Wavelength (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMB</td>
<td>630</td>
</tr>
<tr>
<td>TMB + H(_2)SO(_4)</td>
<td>450 (with reference filter 570nm)</td>
</tr>
<tr>
<td>pNPP</td>
<td>405</td>
</tr>
</tbody>
</table>

4.4.2 Antigens

Both the human hsp60 protein and the hsp65 protein of Mycobacterium bovis were used as antigens.

The human hsp60 protein and deletion fragments there-of were obtained from Dr Gaston, Dept. Rheumatology, Addenbrooke's Hospital, Cambridge. They were prepared from expressed recombinants of the human hsp60 molecule in E. coli.

The fragments were arranged along the whole hsp60 molecule as follows:

<table>
<thead>
<tr>
<th>Fragment</th>
<th>Length (aa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>h7</td>
<td>27-573</td>
</tr>
<tr>
<td>a1</td>
<td>138-573</td>
</tr>
<tr>
<td>d2</td>
<td>243-573</td>
</tr>
<tr>
<td>g3</td>
<td>359-573</td>
</tr>
<tr>
<td>h4.5</td>
<td>466-573</td>
</tr>
<tr>
<td>h5.8</td>
<td>27-465</td>
</tr>
</tbody>
</table>

with the mature human polypeptide being aa 27-573 (preceded by a 26aa signal).

All mutants also had RS.HHHHHHH at the C-terminus.

The M. bovis hsp65 protein was obtained from Dr M Singh (Braunschweig, Germany). The second series of rat serum ELISAs (aortic transplantation model) utilised recombinant human hsp60 purchased from StressGen, as the stock of h7 had become low.
4.4.3 Serum Samples

Serum samples utilised were obtained from two series of rats at sacrifice, the St. Mary's and NPH experiments. Sera were also obtained from human volunteers in a hay fever trial prior to and at the completion of the protocol.

St. Mary's Rats:

In the St. Mary's experiment, primarily investigating aortic contractility and intimal/medial thickening, dark Agouti male rats, 200g±20g, were treated according to 8 different protocols:

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment Abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Placebo</td>
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<tr>
<td>B</td>
<td>hsp65 alone</td>
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<tr>
<td>C</td>
<td>hsp65 &amp; low SRL-172</td>
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<td>D</td>
<td>hsp65 &amp; hi SRL-172</td>
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<td>E</td>
<td>OVA</td>
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<tr>
<td>F</td>
<td>Low SRL-172 x2</td>
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<td>G</td>
<td>Low SRL-172 x2 &amp; hsp65</td>
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<td>H</td>
<td>Low SRL-172 x2 &amp; OVA</td>
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Unfortunately the exact treatment protocols are unknown (the investigator performing these studies died just after their completion), but what is known is that:

Group A received no specific treatment but were sacrificed at day 70.
Group B were immunised with hsp65 and sacrificed on either day 42 or day 60.
Group C received an immunisation with hsp65 on day 0 and SRL-172 (at a dose equivalent to $10^7$ organisms) on day 14 and were sacrificed on either day 42 or day 70.
Group D received an immunisation with hsp65 on day 0 and SRL-172 (at a dose equivalent to $10^9$ organisms) on day 14 and were sacrificed on either day 42 or day 70.
Group E received an immunisation with ovalbumin (exact dose/concentration and
Group F received two immunisations with SRL-172 (at a dose equivalent to $10^7$ organisms) (exact timings of injections and sacrifice not known).

Group G received two immunisations with SRL-172 (at a dose equivalent to $10^7$ organisms) followed by an immunisation with hsp65 (exact timings of injections and sacrifice not known).

Group H received two immunisations with SRL-172 (at a dose equivalent to $10^7$ organisms) followed by an immunisation with ovalbumin (exact timings of injections and sacrifice not known).

The hsp65 immunisation used comprised 100μg *M. bovis* hsp65 in 100μl saline, emulsified with 100μl Freund’s incomplete adjuvant. The injection was given subcutaneously into the tail base.

The SRL-172 was made up to a volume of 100μl with saline and injected subcutaneously over the neck.

Blood was obtained by intracardiac puncture at sacrifice and separated serum aliquoted directly.

**NPH Rats:**

In the NPH experiment, primarily investigating intimal/medial thickening in aortic grafts, dark Agouti rats were treated according to 7 different protocols:

Group A comprised isograft controls that received no pharmacological interventions.

Group B comprised allograft controls that received no pharmacological interventions.

Group C received isografts and were immunised with SRL-172.

Group D received allografts and cyclosporin A.

Group E received allografts and were immunised with SRL-172.

Group F received allografts and cyclosporin A, and were immunised with SRL-172.

Group G received cryopreserved allografts and were immunised with SRL-172.

The rats treated with cyclosporin A (CyA) received 2.5mg/kg of this agent and
also 2mg/kg azathioprine and 0.1mg/kg prednisolone orally daily for days 1-15, alternate days for days 16-30 and every third day for days 31-60.

Rats treated with SRL-172 received a subcutaneous injection with the equivalent of $10^8$ organisms on day -10 (pre-transplant) and day 28.

The donor aortic graft was obtained from rats anaesthetised with fentanyl, fluanisone and diazepam. A midline incision was performed and a 16mm segment of the infra-renal aorta dissected out with ligation (or cauterisation) of the lumbar branches. This was then washed and subsequently stored in saline (and cryopreserved as appropriate for the protocol).

Cryopreservation was performed by suspending the graft in Krebs-Henseleit solution with 0.1M sucrose and 10% DMSO and decreasing the temperature by approximately 1°C/min to -70°C and thence storing the aorta in liquid nitrogen. Thawing was performed at room temperature with washing in Krebs-Henseleit solution.

The recipient rat was similarly anaesthetised and the infra-renal aorta dissected and clamped proximal and distal to an approximate 12mm segment which was then removed.

A matched length of the donor aorta was prepared and inserted by a continuous ethilon suture, the clamps removed, patency confirmed and the incision closed.

At harvesting removal of the aorta was performed with identical anaesthesia and a similar midline approach.

Blood was obtained at harvesting from the proximal inferior vena cava and separated serum aliquoted directly.

**Human Hayfever Trial:**

In a double-blind study, 40 adult patients with grass-pollen allergy (some of whom also had asthma) received three 0.1ml intradermal injections of SRL-172 (or placebo) at monthly intervals just prior to the start of the hay fever season (local ethics committee approval had been received). Serum samples were obtained just prior to the first vaccination and at the end of the hay fever season (5 months later).
4.4.4 Rat ELISA

4.4.4.1 Methods

Sera was frozen (at -20°C) on sacrifice from the above series of rats and these were analysed for antibody titres to human hsp60 (and fragments there-of) and *M. bovis* hsp65 in a blinded fashion. Protocols were developed by analysing random representative sera with chequer-board variations in timings/concentrations of the relevant stages.

All the following analyses, except when stated, were performed with triplicate wells positioned in different regions of the same ELISA plate (thereby decreasing variability due to local plate conditions (e.g. evaporation or speed of temperature change)). All plates contained a control serum to allow inter-plate comparisons and standardisation (ensuring that OD measurements were taken on the dynamic part of the curve and thence allowing measurements to be mathematically adjusted such that the control serum OD was equivalent on all plates).

4.4.4.1.1 IgG2a Rat ELISA

It became apparent that the rats, though analysed in a blinded fashion, either possessed moderate or low titres of IgG2a antibodies to hsp60/65 and therefore two protocols were developed – the ‘high’ and ‘low’ titre protocols described below.

4.4.4.1.1.1 High Titre IgG2a Protocol

As basic ELISA protocol with:

- Blocking buffer = washing buffer + 1% BSA.
- Blocking stage = 1 hour at 37°C.
- Sera = diluted 1:200 with blocking buffer.
- Serum stage = 1.5 hours at 37°C.
- Secondary antibody = biotinylated mouse anti-rat IgG2a (Zymed) diluted 1:2000 with blocking buffer.
Secondary antibody stage = 1 hour at 37°C.
Conjugate = streptavidin diluted to 1:5000 with blocking buffer.
Conjugate stage = 1 hour at RT.
Chromogen = TMB 30min at RT.
Reading was performed at 630nm.
On each plate a control serum (B10 from the aortic contractility study) was included.

4.4.4.1.1.2 Low Titre IgG2a Protocol
For low IgG2a titre sera a more sensitive ELISA was designed.
As basic ELISA protocol with:
Blocking buffer = 1% BSA in PBS:0.05% Tween 20.
Blocking stage = 1 hour at 37°C.
Sera = diluted 1:100 with blocking buffer.
Serum stage = 2 hours at 37°C.
Secondary antibody = biotinylated anti-rat IgG2a (Zymed) antibody diluted 1:1000 with blocking buffer.
Secondary antibody stage = 1 hour at 37°C.
Conjugate = streptavidin diluted 1:1000.
Conjugate stage = 1 hour at 37°C.
Chromogen = TMB with the reaction stopped at 1 hour with 0.5M H2SO4.
Reading was performed at 450nm (with reference filter 570nm).
On each plate a control serum (from a separate study) was included.

4.4.4.1.2 IgG1, IgG2b, IgA, IgM ELISA
4.4.4.1.2.1 IgG1
As basic ELISA protocol with:
Blocking buffer = washing buffer +1% BSA.
Blocking stage = 1.5 hours at 37°C.
Sera = 1:50 in blocking buffer.
Serum stage = for 2 hours at 37°C.
Secondary antibody = biotinylated mouse anti-rat IgG1 (Zymed) at 1:1000 in blocking buffer.
Secondary antibody stage = 1 hour at 37°C.
Conjugate = streptavidin at a dilution of 1:2000 with blocking buffer.
Conjugate stage = 1 hour at 37°C.
Chromogen = TMB at room temperature with addition of 25μl 0.5M H₂SO₄ at 1 hour.
Reading was performed at 450nm (with reference filter 570nm).
On each plate a control serum (B10) was included.

4.4.4.1.2.2 IgG2b and IgA
No significant titres of these classes of antibody could be detected to h7 (using biotinylated mouse anti-rat IgG2b and anti-rat IgA (both from Zymed)) despite attempts at increasing the sensitivity and optimising the assays.

4.4.4.1.2.3 IgM
Optimisation of the IgM titre assay (with biotinylated mouse anti-rat IgM (Zymed)) was difficult due to the high background colour changes seen with the rat sera as sensitivity was increased - potentially obscuring any positive results. Different blocking agents, based on PBS with combinations of BSA (from 1-5%(w/v)), Tween 20 (from 0.05-0.1%(v/v)) and skimmed milk (from 1-5%(w/v)) as well as several commercial agents were tested. The optimum result was finally obtained with PBS + 2% skimmed milk, however there was a suggestion that the very low titles detected were due to cross-reactivity with the proportionally much higher IgG2a titres and therefore this assay was abandoned.
4.4.4.2 Results

4.4.4.2.1 St Mary's Rats- Aortic Contractility Study

It was apparent whilst performing the ELISA tests that some rats possessed easily detectable titres whilst some did not (the assays were performed blinded). Therefore two separate IgG2a ELISAs were developed, the high titre and low titre protocols, as described above. Once the rat treatment groups were unblinded it became apparent that those rats with 'high' IgG2a titres were those that had received immunisations with mycobacterial hsp65, whilst those that had not received hsp65 required the low titre protocol to obtain meaningful results.

The high titre protocol was performed with duplicate wells, whilst the low titre protocol utilised triplicate wells positioned in differing areas of the plate.

The IgG2a titre results to the whole and fragments of human hsp60 for the aortic contractility study rats are illustrated in appendix A (figures StM a-d for the low titre rats and figures StM e-h for the high titre rats) with group means shown here in figures 4.4.4.2.1.a and b. The relative IgG2a titres to *M. bovis* hsp65 and the whole human hsp60 molecule are shown in appendix A (figures StM i-p) with group means shown here as figure 4.4.4.2.1.c.

The data forming the graphs have been corrected for background OD change (by subtracting the mean OD of the control-buffer-only wells from each individual OD measurement before further analysis) and for inter-plate variation by standardising the resultant OD measurements with the plate control. The error bars shown are the standard deviation for the data group.
Figure 4.4.4.2.1.a: St. Mary’s Rats- Low Titre IgG2a to hsp60 Fragments - Group Means.
A statistically significant effect was seen on the titre to g3 with the receipt of OVA (p=0.0022). No other significant effects were demonstrated.

Figure 4.4.4.2.1.b: St. Mary’s Rats- High Titre IgG2a to hsp60 Fragments - Group Means.
No statistically significant effects of treatment received were demonstrated. Titres to fragment h4.5 were significantly lower than those to a1, d2 and g3.
Figure 4.4.4.1.c: St Mary's Rats- IgG2a Titres to bovis hsp65 and Human hsp60 - Group Means.
A significant effect (p<0.005) on differential titre to hsp60/hsp65 (due to relatively increased titre to human hsp60) was demonstrated in the low SRL-172 + hsp65 group. No other significant effects seen (apart from differential titres between rats that did and did not receive immunisation with hsp65).

Figure 4.4.4.1.d: St Mary's Rats- Correlation of IgG2a Titres to Human hsp60 and Aortic Contraction for Group D Rats (hsp65 + Hi SRL-172).
A correlation (p=0.0045) was found for this group but no other.
Statistical analyses comparing the IgG2a titres between rats that did not receive hsp65 as part of their vaccination protocols (placebo (GrpA), OVA (GrpE), low SRL-172 x2 (GrpF) and low SRL-172 x2 & OVA (GrpH)) demonstrated a significant increase (p=0.0022, one-way ANOVA with Bonferroni’s post-test) in titres to hsp60 fragment g3 in those animals that received OVA immunisation compared to those that received placebo only. No other significant effects of treatment received on IgG2a to hsp60 (and fragments there-of) were seen.

Figure 4.4.4.2.1.b demonstrates that the presence or absence of SRL-172 in the vaccination protocols of those rats that did receive hsp65 vaccination produced no qualitative or quantitative change in the serum IgG2a antibody levels to hsp60 (and fragments there-of).

Though the degree of antibody responses between those rats that did not (figure 4.4.4.2.1.a) and did (figure 4.4.4.2.1.b) receive hsp65 immunisation cannot be directly correlated (as different ELISA protocols were followed), it is apparent that the levels of anti-hsp60 antibody in the sera of those rats that had received hsp65 were many fold higher than in those that had not.

A qualitative difference between those rats that did and did not receive hsp65 immunisation is also apparent. Amongst those that did receive hsp65 there is an apparent decrease in recognition of the h4.5 fragment when compared to the other fragments and the whole hsp60 molecule (statistically significant when comparing fragments a1 (p<0.01), d2 (p<0.001) and g3 (p<0.001) with titres to h4.5, one-way ANOVA with Tukey’s multiple comparison test). This is not apparent in those rats that did not receive hsp65 in their vaccination protocols (which demonstrates a borderline significant relative decrease in titre to fragment d2 compared to fragment g3 (p<0.05) only, one-way ANOVA with Tukey’s multiple comparison test).

Figure 4.4.4.2.1.c illustrates the mean IgG2a antibody responses to mycobacterial hsp65 and human hsp60 in these rats. As above a qualitative and quantitative difference can be seen between those rats that did and those that did not receive mycobacterial hsp65 immunisation. Those that were immunised with this protein showed a higher IgG2a response to the mycobacterial than to the human
homologue. Those that did not receive hsp65 showed no significant difference between recognition of these two homologues. Overall there was a significant correlation between the IgG2a titres to the *M. bovis* and human hsp60/65 homologues (*p*=0.009, Pearson).

Statistical analyses comparing the differential IgG2a titre results to the *M. bovis* hsp65 and the human hsp60 molecule show a significant (*p*<0.005, one-way ANOVA with Bonferroni’s post-test) difference between group G (low dose SRL-172 and hsp65) and the other groups that received hsp65 as part of their vaccination protocol. Examination of figure 4.4.4.2.1.c illustrates that this difference is due to the relatively increased titre to the human homologue (hsp60) whilst IgG2a titres to the bovis hsp65 homologue remain constant between groups. Otherwise no significant differences between groups were seen in IgG2a titres to hsp65.

Correlating the IgG2a titres with the other clinical parameters obtained in this study demonstrated a significant correlation between aortic contractility measurement and IgG2a titres to the whole human hsp60 in rats from group D only (*p*=0.0045, Spearman) (see figure 4.4.4.2.1.d). These were the animals immunised with hsp65 followed by vaccination with high dose SRL-172 (equivalent to $10^9$ organisms). The correlations with the hsp60 fragments and in the other rat treatment groups failed to reach significance. No significant correlations were discovered with intimal/medial wall thickness and the titres of antibodies to hsp60/65.

IgG1 titres to hsp60 appeared to be random. Only two rats showed relatively high titres (rats G2 and B2.7), several showed moderate titres (rats G6, B2.3 and A2.6) and the remainder showed either no significant titres or very low levels (data not shown). Assessments of the IgG1 titres to the fragments of hsp60 were not performed.

No significant IgG2b, IgA or IgM titres were detected above background in these rats (data not shown).
4.4.4.2.2 NPH Rats- Aortic Transplantation Study

The results of the IgG2a antibody titres to human hsp60 (and the fragments thereof) are illustrated in appendix A (figures NPH a-g) with the mean results of each treatment group illustrated here in figure 4.4.4.2.2.a. The relative IgG2a titres to \textit{M. bovis} hsp65 and the whole human hsp60 molecule are shown in appendix A (figures NPH h-n) with the mean group results shown in figure 4.4.4.2.2.b. The low titre protocol was utilised.

The data forming these graphs have been corrected for background OD change (by subtracting the mean OD of the control-buffer-only wells from each individual OD measurement before further analysis) and for inter-plate variation by standardising the resultant OD measurements with the plate control. The error bars shown are the standard deviation for the data group.

Examination of the results for individual rats (appendix A) demonstrates that the error bars (standard deviations) are small, indicating that the ELISA estimates of IgG2a antibodies to hsp60/65 are of good quality with little variability between the triplicate wells.
Figure 4.4.4.2.2.a: NPH Rats- Mean IgG2a Titres to hsp60 Fragments by Treatment Group.
No significant effects apart from decreased titres in rats that received the CyA protocol were demonstrated.

Figure 4.4.4.2.2.b: NPH Rats- IgG2a Titres to Human hsp60 & bovis hsp65 Group Means.
Titres to hsp65 were significantly greater (p=0.0013) but there was good correlation between IgG2a titres to the two hsp homologues (p=0.0001). No significant effects of treatment received (apart from decreased titres with CyA protocol) were demonstrated.
There were no apparent significant qualitative or quantitative differences between the IgG2a antibody responses to the fragments of hsp60 as a result of the receipt of SRL-172 in the treatment protocol. Similarly there were no significant differences as a result of the use of iso-, allo- or cryopreserved grafts.

There was however a trend to decreased corrected OD measurements in all the treatment protocols that included the receipt of cyclosporin A (CyA). This reached statistical significance in the titres to \textit{M. bovis} hsp65 for those rats that received CyA compared to those that did not (p=0.022, t-test), and borderline significance (p=0.045, one-way ANOVA with Tukey's multiple comparison test) for IgG2a titres to fragment a1 between rats that received isografts and those that received allografts with CyA.

Taking all treatment groups together the pattern of IgG2a responses to the hsp60 fragments showed significantly increased titres to fragments g3, h4.5 and h5.8 when compared to the whole hsp60 molecule and fragments a1 and d2 (all p<0.001, one-way ANOVA with Tukey's multiple comparison test). Also titres to fragment h5.8 were significantly greater than those to fragment g3 (p<0.05, one-way ANOVA with Tukey's multiple comparison test).

Overall there was again good correlation between the IgG2a titres to human hsp60 and \textit{M. bovis} hsp65 for the individual rats (p<0.0001, Pearson) with the titres to hsp65 being significantly greater (p=0.0013, paired t-test).

Estimates of the IgG1 titres to hsp60 and hsp65 showed only low, background levels (corrected OD values of less than 0.01) with the exception of IgG1 antibodies to \textit{M. bovis} hsp65 in a single rat, G12, which received a cryopreserved isograft (corrected OD of 0.824).

Due to the lack of significant IgG2b, IgA and IgM titres seen in the St Mary's aortic contractility study rats these antibody titres were not determined in these rats.
4.4.4.3 Discussion

Several immunoglobulin class responses were of interest in these studies. It is known that a type-1 response in rodents principally induces IgG2a antibodies, whilst a type-2 response is mostly associated with the IgG1 class. IgG2a antibodies are also known to be complement fixing in the rat which is of particular interest in that it was such antibodies that were shown to exhibit effects on vasculature in the studies of Georg Wick (see section 4.2.1). IgM antibody is associated with primary immune responses, IgA is the predominant antibody in external secretions and IgG2b responses appear to be similar to, though less common than, IgG2a responses.

4.4.4.3.1 St Mary's Rats- Aortic Contractility Study

No significant titres of IgG2b, IgA or IgM to mycobacterial hsp65 or human hsp60 were detected in the rats from this aortic contractility model. Assessment of IgG1 titres demonstrated only a few significant titres and these in rats from varying treatment groups. It was therefore thought probable that these few titres were secondary to factors outside the experimental protocol (for instance a sub-clinical infection) and IgG1 titres were consequently not pursued further.

Significant IgG2a titres to hsp60/65 were however detected. Both the degree and pattern of the IgG2a antibody response to human hsp60 appeared to depend on the presence of M. bovis hsp65 within the vaccination protocol. Rats that received hsp65 had significantly higher IgG2a titres to the hsp60 fragments (permitting a separate ELISA protocol) and a different pattern of response. Indeed the main humoral reactivity to this molecule was preserved within the ELISA assays to all hsp60 fragments excepting h4.5. This implies that the main IgG2a epitopes within the hsp60 molecule in rats that have received immunisation with hsp65 are not between amino acids 466 and 573, and most probably the main epitopes are contained within amino acids 359 to 465. The borderline decrease in IgG2a titres to fragment d2 when compared to g3 in those rats that did not receive hsp65 immunisation is difficult to explain in terms of the design of the hsp60 deletion
fragments and possibly may be the result of aberrant folding of the peptides (discussed in section 4.4.3.2).

Between the different vaccination protocols that included hsp65 (hsp65 alone (GrpB), hsp65 + low SRL-172 (GrpC), hsp65 + high SRL-172 (GrpD) and low SRL-172 x2 + hsp65 (GrpG)) there were no significant differences in either the degree or pattern of IgG2a response. Between the different treatment groups that did not receive a vaccination of hsp65 (placebo (GrpA), OVA (GrpE), low SRL-172 x2 (GrpF) and low SRL-172 x2 + OVA (GrpH)) there were no differences in either the degree or pattern of the IgG2a response apart from a differential reactivity in titres to the g3 fragment of hsp60 between those rats that received placebo and those that received OVA vaccination. Immunological correlates and explanations for this finding are hard to identify and assess, and this may well be a spurious result (though a relatively, but non-significant, higher titre is also seen to fragment g3 in those rats that received OVA with low dose SRL-172).

Of particular relevance to this study was the effect of SRL-172 on the antibody response to hsp60/65. Vaccination with SRL-172 did not have a significant quantitative or qualitative effect upon the IgG2a titres in those rats that did not receive hsp65 immunisation. This later point is of relevance to safety issues relating to the use of SRL-172 in humans. As discussed previously abnormal responses to hsp60/65 have been implicated in many autoimmune and other conditions. The fact that immunisation with heat-killed \textit{M. vaccae} fails to induce a significant antibody response to hsp60/65 is therefore an important safety point.

No significant effect on the IgG2a responses to the fragments of hsp60 could be seen with either low, high or preceding vaccination with SRL-172 in rats that received hsp65 as part of their treatment protocol. However rats that received immunisation with low dose SRL-172 prior to hsp65 immunisation (group G) appeared to have a relatively increased IgG2a response to the human hsp60 molecule (compared to the other groups that received hsp65 immunisation) in the assays comparing reactivity to the bovis and human homologues (a similar trend,
not reaching significance, was seen in the assays examining the IgG2a titres to the hsp60 fragments). Why pre-immunisation with heat killed *M. vaccae* should have this differential effect on the human (but not the mycobacterial) hsp60/65 homologue is not clear. In theory antibodies to hsp60/65 may bind species-specific epitopes or common epitopes. It is possible that pre-immunisation with SRL-172 is priming for an increased IgG2a reactivity to one or more of these shared epitopes on the mycobacterial and human homologues. Alternatively, but less plausibly, it may be increasing reactivity to human-only epitopes. There is no evidence of this potential latter effect in rats that did not receive hsp65 in their vaccination protocols and the mechanism by which a preparation of killed mycobacteria could stimulate an immune response to epitopes that it itself does not possess (or expose) is not clear. Therefore the former possibility is presently preferred, but which epitopes (as no specific differential IgG2a reactivity to the fragments of hsp60 was demonstrated) and by what mechanism is far from clear. Potentially the IgG2a titres to the mycobacterial hsp65 molecule may have reached a plateau, or the small increase that could be attributable to SRL-172 is obscured by the already high titres. This may warrant further investigation.

Correlating the IgG2a titres obtained with the results of aortic contractility provided for the individual rats only showed a correlation with IgG2a to human hsp60 in the group D rats (those that received high dose SRL-172 and hsp65 immunisations). IgG2a antibodies are known to be complement fixing in rats and therefore this finding is of potential interest as such antibodies have been implicated in vascular disease by Wick and colleagues in humans and SRL-172 has putative efficacy in such conditions (see previous discussion).

Overall it appears that the antibody response in these rats was only significant for the IgG2a subclass, and this response depended on the receipt of hsp65 immunisation both quantitatively and qualitatively. Statistically significant effects were seen with OVA immunisation on IgG2a titre to fragment g3 of hsp60, and with SRL-172 pre-immunisation on the reactivity to human hsp60 (but not mycobacterial hsp65). A significant correlation between aortic contractility and
IgG2a titres to hsp60 was also demonstrated in those rats that received vaccination with the higher dose SRL-172 (and mycobacterial hsp65).

4.4.4.3.2 NPH Rats- Aortic Transplantation Study
Low IgG2a titres were apparent for fragments a1 and d2 and the whole hsp60 molecule (h7), whilst relatively higher titres were detected for fragments g3, h4.5 and h5.8. This is hard to explain as there is no single epitope or region contained within all these later fragments that these antibodies may be binding to. The most likely explanation of this finding is differential folding of the peptides revealing differing epitopes for antibody binding. These deletion fragments of hsp60 were originally designed for cellular assays (utilising antigen presenting cells) and so this issue had not previously arisen. Though several computer-based peptide folding programmes were utilised to assess this potential, none demonstrated different tertiary structures for the conserved sections of the differing fragments. This does not however exclude this possibly occurring in-vitro.

The receipt of cyclosporin A and the immunosuppressive regimen was seen to decrease the IgG2a responses to the proteins assessed. This compound is thought to predominately act by inhibiting calcineurin and thus may inhibit cytokine production by T cells (Bentin 1995; Halloran et al., 1998). Therefore, with diminished CD4 cell help, a decrease in B cell antibody production might be expected and would produce these results. Prednisolone and azathioprine, also given as part of the immunosuppressive protocol, are also known to have similar effects.

In the assessment of differential IgG2a titres to the human hsp60 molecule and M. bovis hsp65 several rats have elevated titres to the mycobacterial protein though not a significant elevation to the human homologue. This appears to be independent of treatment group though diminished anti-M. bovis hsp65 antibodies are seen in those rats receiving cyclosporin A (discussed above). These rats were not grown and kept in pathogen-free conditions and it is interesting to hypothesise that those with elevated antibodies to mycobacterial hsp65 may have been infected
at some stage with bacteria (perhaps even mycobacteria) that were able to elicit such an immune response, whilst the others have either not encountered these organisms or have failed to mount a similar antibody response. The fact that the rats with elevated hsp65 antibodies did not show similar elevations in antibody titres to the human homologue hsp60 may indicate the closer relationship of the antibody-inducing pathogen to mycobacteria than to mammals and further illustrates that though there is remarkable conservation of amino acid sequence between the hsp60/65 molecules of all living organisms, group- and pathogen-specific epitopes do exist.

Significant IgG1 titres to hsp65 were only seen in a single rat, G12 (which received a cryopreserved isograft), and therefore it was concluded that none of the treatment modalities used significantly elevated or altered these antibody levels (or those to human hsp60). The fact that no other rat showed any significant IgG1 titre to hsp60/65 illustrates that these antibodies are uncommon in rats generally and in rat G12 were perhaps associated with a past or present bacterial infection (as above). These rats were sacrificed at the end of the study and it is not possible to further investigate this possibility.

Overall it appears that treatment with SRL-172 fails to significantly alter the IgG2a or IgG1 antibody titres to human hsp60, fragments of human hsp60 and *M. bovis* hsp65 in this aortic transplantation atheroma model.
4.4.5 Human ELISA

Sera was frozen (at -20°C) both before and after the course of SRL-172 vaccinations from the hay fever patients discussed above. Similarly to the rat sera these were analysed for antibody titres to human hsp60 (and fragments there-of) and *M. bovis* hsp65 in a blinded fashion, with protocols developed by analysing random representative sera with chequer-board variations in timings/concentrations of the relevant stages. All the following analyses were similarly performed with triplicate wells positioned in different regions of the same ELISA plate (thereby decreasing variability due to local plate conditions (e.g. evaporation or speed of temperature change)). All plates contained a control serum to allow inter-plate comparisons and standardisation (ensuring that OD measurements were taken on the dynamic part of the curve and thence allowing measurements to be mathematically adjusted such that the control serum OD was equivalent on all plates).

4.4.5.1 Methods

4.4.5.1.1 IgG Protocol

As basic ELISA protocol with:

Blocking buffer = 2% dried skimmed milk in PBS

Blocking Stage = 1.5 hour at 37°C

Sera = Diluted 1:200 with blocking buffer

Serum Stage = 1.5 hours at 37°C

Secondary Antibody = HRPO conjugated goat anti-human IgG (Harlan) diluted 1:4000 with blocking buffer

Secondary Antibody Stage = 1 hour at 37°C

Chromogen = TMB 30min at RT

Reading was performed at 630nm.

On each plate a control serum (from a separate study) was included.
4.4.5.1.2 IgG Subclass Assays

Attempts were made to analyse the IgG4 and IgG1 titres (which correspond to a predominant type 2 and type 1 response \textit{in-vivo} respectively). However despite increasing the concentrations of the secondary antibodies (biotinylated mouse anti-human IgG4 and mouse anti-human IgG1 (both from Sigma)), using alternative antibodies (biotinylated anti-human IgG4 and anti-human IgG1 (The Binding Site)), increasing the concentrations of the sera, increasing the timings of the stages (including overnight secondary antibody and chromogen stages) and using streptavidin/TMB or extravidin/pNPP combinations, no significant titres above background could be detected.

To eliminate the possibility that the total IgG titres observed were due to predominant IgG2 and/or IgG3 subtypes, ELISAs were performed with biotinylated mouse anti-human IgG2 and IgG3 secondary antibodies (both from Sigma). No significant titres were demonstrated.

4.4.5.1.3 IgM Assay

Initial optimisation of a human IgM titre ELISA was performed, however no significant titres were demonstrated and the assay was abandoned.

4.4.5.2 Results

Humans with hay fever form a very heterogeneous group in many respects. This includes their exposures to infections and other conditions which might be associated with elevations/alterations in antibody levels to the hsp60/65 family. In this study plasma from two time-points, before commencement and after the course of immunotherapy with SRL-172, was available for antibody estimation by ELISA. Therefore, to attempt to remove much of the confounding factors, analyses were performed on the changes in titres to hsp60/65 between the two time-points.

The ELISA assays were of good standard with only very small differences between triplicate wells (data not shown). As with the rat ELISAs, results were
corrected for background (by subtracting the mean coating-buffer-control OD reading from each value) and standardised between plates with the inter-plate control.

The data has been analysed by treatment modality received (SRL-172 or placebo) and subgroups (patients who had, or had not, a wheezing component to their illness). The changes in IgG titres to human hsp60 (and fragments there-of) are shown in appendix A (figures Hay a-f). The mean effects of receipt of placebo or SRL-172 on the IgG titres to the hsp60 fragments are shown in figure 4.4.5.2.a, and by patient subgroup in figure 4.4.5.2.b. The changes in the responses to M. bovis hsp65 and the whole human hsp60 molecules (tested in parallel) are shown in figure 4.4.5.2.c. Error bars shown are standard deviations of the relevant data groups.
**Figure 4.4.5.2.a**: HayFever- Changes in IgG Titres to hsp60 Fragments by Group. No significant effects were demonstrated.

**Figure 4.4.5.2.b**: HayFever- Mean Changes in IgG Titre to hsp60 Fragments by Subgroup. Non-wheezers (combined) demonstrated a significant decline in titres ($p=0.0299$), particularly to h4.5 and h5.8. No other significant effects were demonstrated.
Figure 4.4.5.2.c: HayFever- Changes in Titres to hsp60(h7) and bovis hsp65 by Subgroup.
No significant differences were demonstrated.
It can be seen that there is no influence on IgG titre responses to any of the fragments of hsp60 as a result of receipt of SRL-172. Indeed over the trial period there was no significant change in the titres of either those that received SRL-172 or placebo when analysed as a group. Similar analysis of the patient subgroups also demonstrated no significant changes in hsp60 fragment IgG titres over the course of this trial. A trend however was seen in a decrease in IgG response to most fragments, especially h4.5 and h5.8, in the non-wheezers that received SRL-172, and in IgG titres generally, but especially to fragments d2 and g3, in the non-wheezing placebo recipients. Overall non-wheezers demonstrated a statistically significant decrease in IgG titres to fragment h5.8 compared to wheezers (p=0.0299, t-test), but the other trends failed to reach significance.

There are no apparent differential responses seen in IgG titres to either the human or mycobacterial hsp60/65 homologue in any of the patient subgroups.

As mentioned above, in the determination of IgG subtype titres problems were encountered. Despite many attempts at increasing the sensitivity of the assays no significant titres of IgG subtypes (above background) could be detected. A screen of the sera for positive IgM titres to hsp60/65 also failed to show any results above background and this assay was therefore abandoned.

4.4.5.3 Discussion

As can be seen from the figures in appendix A there is marked variability in changes in IgG titres to hsp60/65 over the trial period between the subjects. Overall this appears unrelated to treatment received (SRL-172 or placebo) or to patient sub-group. As mentioned previously a very large variety of infections and other conditions might be expected to influence and alter these titres and therefore it may be concluded that, by most analyses, either SRL-172 has no effect upon these antibody levels or it is only a small influence that is obscured by the variation due to other sources. There is however a trend to a decreased IgG antibody response to the whole human hsp60 molecule and certain fragments in
those subjects that were non-wheezeers and received SRL-172. This is shown in experiments directly comparing IgG titres to h7 and \textit{M. bovis} hsp65 and in experiments (performed separately) assessing antibodies to the human hsp60 fragments. However it fails to reach statistical significance and it is also worth noting that there is a similar trend seen in non-wheezeers that received placebo. In fact it is only the combined group of non-wheezeers (including both those that received placebo and those in receipt of SRL-172) that achieved statistically significant decreases in IgG titre to hsp60 compared to the combined group of wheezeers. Importantly however it was the patients that \textit{did} have a wheezing component to their illness that showed benefit from injections with SRL-172 and no significant clinical effect was seen in non-wheezeers. Therefore it was concluded that this probably reveals nothing about the underlying mode of action of heat-killed \textit{M. vaccae} as an immunotherapeutic agent and it is, at present, not possible to correlate this finding with the known immunology of seasonal rhinitis.

Determination of IgG subtype titres proved impossible in these samples. In humans \textit{type-1} orientated responses consist predominately of the IgG1 subtype and \textit{type-2} responses of the IgG4 subtype. No significant anti-hsp60/65 titres of either these classes were found. Though it was not expected that the IgG titres seen would comprise significant IgG2 or IgG3 levels this possibility was excluded by failing to detect these subtypes. The discrepancy between positive total IgG titres to hsp60/65 and no significant IgG subtype titres probably rests with the secondary antibodies used. The anti-total IgG secondary antibody utilised was polyclonal whilst the variety of anti-IgG subtype secondary antibodies investigated were all monoclonal in nature. The anti-total IgG antibody would be expected to bind to several sites on IgG with resultant increased sensitivity. This loss of sensitivity in using monoclonal IgG subtype antibodies could not be overcome despite markedly increasing the lengths of the ELISA steps and the concentrations of reagents. Utilising different reagents, such as pNPP, similarly failed to overcome this decrease in sensitivity.
No significant IgM titres were discovered on a screen of the plasmas. Overall IgM usually comprises 5-10% of the total serum immunoglobulin and is the first Ig class produced in a primary response. It was not expected that significant alterations in anti-hsp60/65 IgM would be detected in this study as data from the rat studies illustrate that SRL-172 probably does not induce significant antibody responses to this molecule and also most hsp60/65 responses in adults would not be expected to be primary in nature (as we are challenged almost continuously with pathogens expressing this immunodominant antigen).

IgA and IgE titres were not investigated in this study. Potentially IgA titres would be of interest as this antibody class comprises the predominant Ig in external secretions including those of the respiratory tract (and these patients suffered from hay fever). However the plasma was in short supply and it was not thought likely that significant changes in serum anti-hsp60/65 IgA would be detected. IgE antibodies mediate hypersensitivity reactions and, though these patients were atopic in nature, anti-hsp60/65 IgE has not been implicated in hay fever or other allergies and it was not thought that their investigation would be likely to provide interesting data.
4.4.6 Overall Conclusions On ELISA Assays

The principal conclusion for the ELISA assays in the three studies discussed above is that there is no strong evidence that SRL-172 acts through alteration in immune recognition of hsp60/65 molecules. The only relevant, statistically significant, effects were the receipt of SRL-172 pre-immunisation resulting in a relative increase in the IgG2a titres to the human (but not the mycobacterial) hsp60/65 homologue, and a correlation between aortic contractility and IgG2a titres to hsp60 in rats that received both high dose SRL-172 and hsp65 immunisations.

Other statistically significant findings were the relative increase in IgG2a titres to hsp60 fragment g3 in those St. Mary's rats that received OVA vaccination (which may be spurious), and a decrease in IgG titres to hsp60 fragment h5.8 in those hay fever sufferers who did not have a wheezing component to their illness. Neither of these findings were dependent on the receipt of SRL-172 vaccination.

A final conclusion relevant to vaccination with SRL-172 is that no evidence emerged in any of the studies that this preparation of heat-killed *M. vaccae* (which would be expected to contain a significant proportion of hsp65) induced a humoral immune response either to mycobacterial hsp65 or the human homologue hsp60. This is of relevance for the potential safety profile of this agent as such humoral responses have been associated with several autoimmune diseases (see earlier).

One issue that has relevance to the above assays, but only became apparent after the ELISA assays had been completed, is the presence of soluble hsp60 in the serum. This has been described by Pockley (Pockley et al., 1999) and has recently also been confirmed by other workers (G. Rook, personal communications). This soluble protein may well complex with the soluble antibody and potentially block recognition of the antibodies to hsp60/65 in the above assays. Thus the data above may illustrate only the free serum anti-hsp60/65 antibody and, without also analysing the concentration of soluble hsp60 protein, the total antibody titre is
potentially unknown. If enough serum remains from the above studies the concentration of soluble hsp60 may be analysed in the future.


5 DISCUSSION

Due to the only limited basic science knowledge behind SRL-172 it is difficult to establish fruitful directions of research into this product. The approach adopted here involved the collation of the present knowledge base in both the immunology and clinical efficacies of this preparation of heat-killed *M. vaccae* and the development of hypotheses based upon these that could then be tested. Such hypotheses studied in this thesis were effects on less well understood lymphocyte subsets, especially γδ TCR cells, NK cells and NKT cells, and the effects on the humoral responses to the heat shock protein hsp60/65. The latter sub-study also involved the amplification and sequencing of the hsp65 gene of the vaccine strain of *M. vaccae*. Other approaches that were investigated, but have not been included in this thesis, were potential effects on cross presentation of exogenous antigen onto MHC class I molecules by dendritic and other antigen-presenting cells, effects on double negative (DN) T cells (negative for CD4, CD8, γδTCR and CD56 but positive for the T cell marker CD3), and the effects of *in-vivo* vaccination in healthy volunteers on cytokine profiles of peripheral blood mononuclear cells (as assessed by RT-PCR).

The overall results of the work contained in this thesis can be summarised as the demonstration of alterations in proliferation and activation markers on γδ T cells (in particular effects upon CD25, CD69, CD86 and cytokine production), and on CD56+ NK cells (principally effects upon proliferation and the activation markers CD25 and CD69, with trends to increased cytokine production) as a result of exposure to heat-killed *M. vaccae*. The relevance of these findings to the *in-vivo* activities of SRL-172 is to be further investigated. Some effects of SRL-172 immunisation on the humoral responses to the 60kDa heat shock protein family were also demonstrated. Principally these were the relative increase in IgG2a titres to the human (but not the mycobacterial) hsp60/65 homologue in rats that received SRL-172 prior to hsp65 immunisation, and the apparent correlation between aortic contractility and IgG2a titres to hsp60 in those rodents that received both hsp65
and high-dose SRL-172 vaccination. The relevances of these results to the in vivo efficacies of this vaccine have yet to be fully established.

There are several different ways that investigation into the basic immunological mechanisms of action of heat-killed M. vaccae may be taken forward. One such, that is presently being set up within the laboratory, is the investigation of differential gene induction by SRL-172. Differential display RT-PCR (DD-PCR) is a technique that permits the identification of such up- and down-regulated genes and, though it frequently produces false positive results, has the advantages of relative simplicity and the use of established techniques. Other methods, such as selective subtractive hybridisation, might also be used. These techniques would be expected to highlight certain genes, and thereby potentially certain cell types, that then merit further investigation. Also, as more stringent and larger clinical trials and basic immunological studies are completed, further information will be gained on efficacies and other parameters that might suggest further, different approaches.
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Lehrer, A., A. Bressanelli, V. Wachsmann, O. Bottasso, M. L. Bay, M. Singh, C. Stanford,


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Appendix A - ELISA Data

The following figures illustrate the data obtained for the ELISA assays of anti-hsp60/65 antibodies on the sera discussed in section 4.4.3. Summary figures of these data are included within the thesis (figures 4.4.4.2.1.a-d, figures 4.4.2.2.a-b and figures 4.4.5.2.a-c).
Figure S8.m: St Mary's Rats- lgG2a Titres to bovis hsp65 and Human hsp60:

- hsp65 Immunised
- hsp65 + Low SRL-172
- hsp65 + Hi SRL-172

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**Figure StMa.p:** St Mary's Rats- IgG2a Titres to bovis hsp66 and Human hsp60: Low SRL-172 + hsp65
Figure NPH.m: NPH Rats- IgG2a Titres To Human hsp60 & bovis hsp65
Allografts & SRL-172 & CyA

Figure NPH.n: NPH Rats- IgG2a Titres To Human hsp60 & bovis hsp65
Cryopreserved isografts
Figure Hay.g: Hayfever - Changes in IgG Titres to hsp60/hsp65
Placebo/Wheezers

Figure Hay.h: Hayfever - Changes in IgG Titres to hsp60/hsp65
Placebo/Non-Wheezers
Appendix B - Manufacturer Information

Advanced Biotechnologies
Units B1-2 Longmead Business Centre
Blenheim Road
Epsom
Surrey KT19 9QQ.
UK.

Amersham Pharmacia Biotech AB
SE-751 84
Uppsala
Sweden.

Applied Biosystems
7 Kingland Grange
Woolston
Warrington
Cheshire WA1 7SR.
UK.

BDH
BDH Laboratory Supplies
Poole
Dorset BH15 1TD.
UK.

Becton Dickinson
Becton Dickinson Immunocytometry Systems
2350 Qume Drive
San Jose
CA 95131-1807
USA.

The Binding Site Ltd.
P.O.Box 4073
Birmingham B29 6AT.
UK.

Bio-Rad Laboratories
Alfred Nobel Drive
Hercules
CA 94547
USA.
Chiron Corporation
4560 Horton Street
Emeryville
CA 94608-2916
USA.

Dako
Dako AS
DK-2600 Glostrup
Denmark.

Dynatech
Acterna
Portland House
Aldermaston Park
Aldermaston
Berkshire RG7 4HR.
UK.

Evans Medical Ltd.
Regent Park
Leatherhead KT22 7PQ.
UK.

Gentra
13355 10th Avenue N
Suite 120
Minneapolis
MN 55441
USA.

GibcoBRL
Life Technologies
3 Fountain Drive
Inchinnan Business Park
Paisley.
UK.

Harlan Sera-Lab
Dodgeford Lane
Loughborough
Leicestershire LE12 9TE.
UK.
Hybaid Ltd.
Action Court
Ashford Road
Ashford
Middlesex TW15 1XB.
UK.

MWG Biotech AG
Anzinger Str 7
D-85560 Ebersberg
Germany.

Molecular Probes
4849 Pitchford Av.
Eugene
OR 97402-9165
USA.

Nunc
Nalge Nunc International Corp.
75 Panorama Creek Drive
P.O.Box 20365
Rochester
NY 14602-0365, USA.

Nycomed
Nycomed Pharma AS
Diagnostics
P.O.Box 4220 Torshov
N-0401 Oslo
Norway.

Oswel
Oswel DNA Service
Medical & Biological Sciences Building
University of Southampton
Boldrewood
Bassett Crescent East
Southampton SO16 7PX.
UK.

Pharmingen
10975 Torreyana Road
San Diego
CA 92121
USA.
(now a Becton Dickinson Company).
Promega
Promega Corporation
2800 Woods Hollow Road
Madison
WI 53711-5399
USA.

Qiagen Ltd.
Boundary Court
Gatwick Road
Crawley
W.Sussex RH10 9AX.
UK.

R&D
R&D Systems Europe Ltd.
4-10 The Quadrant
Barton Lane
Abingdon OX14 3YS.
UK.

Sigma
Sigma-Aldrich Co LTD.
The Old Brickyard
Gillingham
Dorset SP8 4XT.
UK.

Stressgen
Stressgen Biotechnologies Inc.
409 2nd Avenue
Collegeville
PA 19426-2655
USA.

Techne Cambridge Ltd.
Duxford
Cambridge
CB2 4PZ.
UK.

Zymed
Zymed Laboratories Inc.
458 Carlton Court
So. San Francisco
CA 94080
USA.
Appendix C - Statistical Analyses

These were performed using Graphpad Prism 3.0 software.

Paired t-tests, t-tests (unpaired), Pearson correlation and ANOVA analyses were performed as indicated in the text.

For ANOVA analyses post-tests were performed as indicated:
Bonferroni’s post-test was applied when 4 or fewer groups were compared.
Tukey’s multiple comparison test was applied for 5 or larger number of groups.

Statistical significance was defined as a p value ≤ 0.05.