Therapeutic effect of Interleukin-4 and Interleukin-1 receptor antagonist in *Actinobacillus pleuropneumoniae* challenged pigs

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ABSTRACT

Immunological stressors, in the form of clinical and sub-clinical disease are currently controlled using both prophylactic antibiotics in-feed, and therapeutic antibiotic treatment. Respiratory disease, primarily *Actinobacillus pleuropneumoniae* (App) infection, is recognised as a major factor causing reduced productivity in pigs. This thesis reports investigations into the use of novel immunomodulators in particular Interleukin 4 (IL-4) and Interleukin 1 receptor antagonist (IL-1ra) as alternatives to antibiotics to treat App infection. Immunological and molecular biological assays were used to investigate and accumulate data. An *in vitro* study undertaken to find potential anti-inflammatory substances, revealed that Interleukin 8 (IL-8) mRNA production stimulated by PMA or LPS in whole pigs' blood was suppressed by IL-4. IL-1ra also suppressed stimulated IL-8 mRNA production by heat killed App bacteria (KB) *in vitro*. An acute LPS challenge in pigs *in vivo* however, showed no variation in illness or weight loss between pigs treated prophylactically with anti-inflammatory substance (IL-4 and IL-1ra) and saline treated pigs. The use of plasmids as a delivery system for anti-inflammatory substance did not show promise since it did not enhance growth or prolong the expression of the substances in the pigs. However, in the chronic App challenge model IL-4 and IL-1ra administered prophylactically *in vivo* showed an ability to improve growth. The therapeutic administration of IL-4 and IL-1ra to App challenged pigs showed no difference in pigs' growth, regardless of the treatment or control administered. To conclude, IL-4 and IL-1ra showed promise when administered prophylactically and improved growth and abrogated disease under conditions of App challenge. However when IL-4 and IL-1ra where administered therapeutically they did not perform as well. Moreover these compounds

have potential as a commercial application to reduce the growth reduction caused by disease such as App.

STATEMENT OF ORIGINALITY

The work presented in this thesis is, to the best of my knowledge, original and entirely my own, unless stated otherwise. No part of this thesis has been submitted for a degree at this or any other university.

Citation of references in the text and the list of literature cited follow the Instructions to Contributors from the Journal of Veterinary Immunology.

All procedures were approved by the University of Sydney Animal Ethics Committee, in compliance with the NSW Animal Research Act, 1985.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

Ad-5/IL-10	IL-10 attached to Adenovirus-5
App	*Actinobacillus pleuropneumoniae*
APSB	Alkaline Phosphatase Substrate Buffer
CD	Cluster Designation
cDNA	Single chain deoxyribonucleic acid
Con A	Concanavalin A
CRP	C-reactive Protein
DEPC	Diethyl pyrocarbonate
DNA	Deoxyribonucleic Acid
ELISA	Enzyme Linked Immunosorbent Assay
EMAI	Elizabeth Macarthur Agricultural Institute
EtBr	Ethidium bromide
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
HE	Dihydroethidium
HRP	Horse Radish Peroxidase
ICAM-1	Intercellular Adhesion Molecule 1
IFNγ	Interferon γ
Ig	Immunoglobulin
IgA	Immunoglobulin A
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL-1	Interleukin 1
IL-10	Interleukin 10
IL-13	Interleukin 13
IL-1ra	Interleukin 1 receptor antagonist
IL-1α	Interleukin-1α
IL-1β	Interleukin-1β
IL-2	Interleukin 2
IL-4	Interleukin 4
IL-4ra	Interleukin 4 receptor antagonist
IL-5	Interleukin 5
IL-6	Interleukin 6
IL-8	Interleukin 8
KB	Heat killed App Bacteria
LPS	Lipopolysaccharide
LTB4	Leukotriene B_4
LTC4	Leukotriene C_4
mRNA	Messenger RNA
NSAID	Non Steroidal Anti-inflammatory Drug
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate-buffered saline
PBS-T	Phosphate-buffered saline plus Tween 20
PCR	Polymerase Chain Reaction
PHA	Phytohemagglutinin
PMA	Phorbol 12-myristate 13-Acetate
PMN	Polymorphonuclear Cells
PRDC	Porcine Respiratory Disease Complex
RNA	Ribonucleic Acid
RT	Reverse Transcriptase

RT-PCR	Reverse Transcription Polymerase Chain Reaction
SSC	Saline Sodium Citrate
TGF-β	Transformation Growth Factor
Th	T helper cells
TNF	Tumour Necrosis Factor
TNF-α	Tumour Necrosis Factor α
WBC	White Blood Cells

CHAPTER 1
INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

What is stress? Stress is defined as "a disturbing physiological or psychological influence which produces a state of severe tension in an individual" (Macquarie Dictionary, 1998).

The effects of stress can manifest as a range of illnesses such as increased susceptibility, infection, eating disorders and mental breakdown (Bartrop *et al.,* 1977). Furthermore, Solomons *et al.,* (1993) implicated environmental stress, particularly that caused by recurring infections, in the poor growth rates of children in underdeveloped countries and their inability to reach genetic adult stature.

Stress can be referred to as physiological or psychological disturbances, which can originate primarily from the environment. In this Chapter the environmental stress factors that reduce growth in animals (pigs) will be examined, this will aim to give some perspective on the relationship between different types of stress factors and their influence on growth. In particular the immunological mechanisms of infection and methods of reducing its inhibiting effect on growth will be examined.

Chapter 1 examines the relationship of different environmental stress factors and their influence on growth in pigs.

1.2 Stress and its impact on growth

Previous studies have shown that stress factors, which have a deleterious effect on growth and/or health, are mainly environmental. The most significant environmental factors relating to pigs are group housing (Hyun *et al.,* 1998),

temperature and climate changes (Scheepens *et al.,* 1994), air quality (Torremorell *et al.,* 1997) and infection (Bartrop *et al.,* 1977; Perez *et al.,* 1995; Solomons *et al.,* 1993).

1.2.1 Group housing

Group housing (an environmental stress) is a consequence of the industrialisation of animal production. In recent years, pigs have been penned from weaning to market weight in groups of 10-30 (McGlone *et al.,* 1994). However the escalating pressure to increase the output of stock has caused pig group sizes to increase 50, 100 or even more (McGlone *et al.,* 1994). Bustamante *et al.,* (1996) found that there was no difference in the performance of pigs who were fed and housed individually or in groups. This allowed farmers to maximise housing use and minimise housing cost, with increased overall profitability.

Contrary to this, many experiments have reported a negative outcome on growth due to group housing (Hyun *et al.,* 1998). For example, Tonn *et al.,* (1985) observed a higher growth rate in boars (male pigs) individually penned as compared to those in group pens. Hyun *et al.,* (1998) examined the effect of crowding on growth rate and also found that crowding decreased growth by 9.6%. Randolph *et al.,* (1993) similarly demonstrated that as floor space decreased the daily feed intake of the pigs reduced and in turn daily live weight gain was also reduced.

In addition Edwards *et al.,* (1988) illustrated that when floor space was decreased to less than $0.034m^2$ per kg body weight, the pigs' wellbeing and growth was affected. The different findings between Bustamante *et al.,* (1996) and Edwards *et al.,* (1988) may be the result of different floor space allowances per animal.

Growth is influenced by group size because as group size increases the incidence of respiratory infection also increases. Studies have found the risk of respiratory disease can be controlled by depopulation, segregation of cohorts (a group of pigs at the same stage of life) and the use of an "all-in/all-out system". All-in/all-out production refers to a housing strategy whereby an entire group of pigs enter an empty shed at the same time. Later, they exit the shed together for the next phase of production. Torremorell *et al.,* (1997) and Ekkel *et al.,* (1995) showed that mixing of pigs, in addition to crowding, increased the transmission of respiratory infection. Straw *et al.,* (1999) found that the greater the number of pigs housed together, the higher the risk of respiratory disease. It seems large herds with increased levels of mixing between individuals are more prone to respiratory disease outbreaks than smaller herds.

Infectious bacteria can be spread by nose rubbing, coughing, sneezing and vomiting. Lack of space means the infection can be transmitted through the herd quickly decreasing the chance to isolate or quarantine the sick animals before the disease is spread. Age and site segregation in rearing has been used to control *Actinobacillus pleuropneumoniae* (App) (Straw *et al.,* 1999). Management strategies have positive effects by reducing the risk of respiratory disease, especially if pigs are weaned at or before 21 days of age (Veenhuizen, 1998; Leman *et al.,* 1992).

A system of all-in/all-out has also shown positive results for reducing infectious outbreaks from overcrowding (Straw *et al.,* 1999). Torremorell *et al.,* (1997) confirmed that a reduction in airborne bacterial load can be achieved when the "all-in/all-out system" is applied. However, the "all-in/all-out system" can cause a loss of important bloodlines. The opposite system to all-in/all-out is a "continuous-flow management system". Here, animals at different stages of life are moved in and out

of the pens accordingly. This system maintains the bloodlines of animals, however the sheds are not cleaned completely since they are never empty.

It would appear that stress from overcrowding has a greater negative implication on the pigs and outweighs the benefits. Respiratory infection seems to be perpetuated by overcrowding.

1.2.2 Temperature and climate

Temperature and climate can exert stress upon animals in various ways. For example Schoenbaum *et al.*, (1990) found that a low humidity of 55% and a low temperature of 4^oC increased the spread of airborne particles. Therefore climatic conditions and the risk of respiratory illness seem to be related. Scheepens *et al.,* (1994) explored the effect of unpredictable draughts daily or intermittent on weaners and found that their health status was reduced and their growth was impaired.

A reduction in Tumor necrosis factor α (TNF-α) and Interleukin-6 (IL-6) cytokine production in the whole blood of humans stimulated with LPS was observed in autumn as compared to the other seasons. Anti-inflammatory cytokine Interleukin-10 (IL-10), Tumor necrosis factor receptor 1 (TNF-RI) and Tumor necrosis factor receptor 2 (TNF-RII) production was also reduced in human whole blood during summer and autumn (Myrianthefs *et al.,* 2003)

Studies on pigs exposed to hot and cold temperature fluctuations show negative immune response both *in vivo* and *in vitro* (Kelly 1980; Scheepens *et al.,* 1994).

Minton *et al.,* (1988) observed that a 3 to 4 week nursery phase of pigs exposed to fluctuating temperatures increased significantly in insulin levels (>3.35μU/mL), neutrophils (>100 cells/μl) and eosinophils (>338 cells/μl) as compared to those pigs

exposed to constant ambient temperature. Studies by Creshaw *et al.,* (1986) showed no effect on immune system by fluctuating temperatures, but an effect reduction on growth as observed. That being so, it could be concluded that cold draughts, humidity and large temperature fluctuations increase susceptibility to disease, and reduce growth rate (Kelly, 1980).

1.2.3 Air quality

Poor air quality is a further aspect implicated in growth rate and reduced health in livestock (pigs). Curtis *et al.,* (1975) and Leman *et al.,* (1992) found that as ammonia levels increased, normal mucocilliary function was damaged immunocompromising the animal. Some studies found that recycling air within an animal housing facility could contribute to the spread of disease (Straw *et al.,* 1999). In addition, this recirculation coupled with insufficient ventilation contributes to a high concentration of microbes, dust and dander, chronically stimulating the immune system to produce Interleukin-1 (IL-1) (Straw *et al.,* 1999). Similarly, Jobert *et al.,* (2000) found recirculation of air detrimental to pigs. His experiment contained 2 housing units; one with pigs infected with *Actinobacillus pleuropneumoniae* (App) and one with uninfected pigs. Six days post infection the units were connected, by a rectangular opening and the air was circulated from the infected pigs' unit to the uninfected pigs' unit. The infection spread rapidly and App was isolated from the pigs' tonsils and lungs. It was also isolated from the experimental unit's air. This concluded that over a distance of at least 2.5m App was readily transmitted through aerosol, thereby impeding the health and growth of the pigs. Solomons *et al.,* (1993) and Roura *et al.,* (1992) found that dirty environments with poor air quality suppressed the growth of chickens and increased plasma IL-1 production. Since IL-1 producing lymphocytes

were found *in vitro* this showed the chickens' immune system was stimulated. Under commercial conditions (dirty environment) infectious disease was found to be spread from animal to animal or from farm personnel to animal via aerosols, and airborne droplets (Straw *et al.,* 1999). Torremorell *et al.,* (1997) similarly showed that App was transmitted by air over a distance of 1m. Healthy pigs were exposed to air from a pen containing App infected pigs for 2 to 7 weeks (Torremorell *et al.,* 1997). Most pigs died 12 days after initiation of the experiment from the airborne exposure to App infection. Therefore an unsanitary environment, in this case pathogen contaminated air, can cause health and growth problems and even pig mortality. The "all-in/all-out system" may reduce the problems associated with poor air quality, as can be seen with Davos *et al.,* (1981) who found a 53% decrease in airborne bacteria in the "all-in/all-out system" compared to the "continuous-flow management system".

1.2.4 Viral disease and Opportunistic pathogens

The final deleterious effect on health and growth discussed in this Chapter is microbial infection (viral disease and pathogens). These are obviously considered a major problem in the farming community. According to Leman *et al.,* (1992) pigs seldom reach slaughter weight without contracting a respiratory lesion.

Nutritional problems related to diet and feed intake have often been assumed to be the cause of poor growth. However, Solomons *et al.,* (1993) found environmental factors such as airborne pathogens and airborne microbes to strongly influence growth. Poultry reared in unsanitary conditions (high number of airborne microbes, high levels of ammonia and seldom cleaned living areas) were examined. Results showed that chickens did not achieve maximum growth potential. Unsanitary conditions

mimic commercial conditions or environments where livestock are raised under intensive farming conditions.

Similarly, Hill *et al.,* (1952) found that chickens raised in sanitary environments attained a higher growth rate (15%) and greater skeletal muscle accretion than those raised in unsanitary environments. In-feed antibiotics made no difference to the weight gain of those chicks in ideal conditions. Whereas antibiotic-assisted chickens in the unsanitary environment reached a similar growth status as those raised in the ideal environment (Hill *et al.,* 1952). Furthermore, Solomons *et al.,* (1993) stated that "antibiotics are often considered growth permitters or promoters in that they permit birds (chickens) kept under practical husbandry conditions to realise the rate of growth that occurs normally when sanitation is excellent." The literature shows that by decreasing a stress factor such respiratory disease, normal growth rate will return.

Solomons *et al.,* (1993) refer to recurrent infection of respiratory and gastrointestinal tracts of people as not only a problem but also as a factor in reducing animal growth. Solomons *et al.,* (1993) demonstrated a relationship between frequency of respiratory infection in children and growth reduction in epidemiological studies, confirming the association.

Respiratory disease, primarily App infection, is a major immunological stress in the form of clinical and sub-clinical disease in pigs. Immunological, environmental and social stressors negatively influence growth performance of commercially housed pigs.

It has been argued that a possible reason for growth reduction is that infection redirects nutrients towards host defences instead of physiological processes (Solomons *et al.,* 1993). Metabolic changes in response to immunological stresses

such as disease, cause partitioning of dietary nutrients away from growth and skeletal muscle accretion in favor of metabolic processes that support immune response and disease resistance (Solomons *et al.,* 1993).

The mechanics of the so-called metabolic changes requires further scrutiny. Studies have found that TNF-α (Beutler *et al.,* 1988), primary monokines IL-1 and Interleukin-6 (IL-6) are produced during infection (Baarsch *et al.,* 1995). Accumulated data from chickens, rats and mice showing that these cytokines are involved in infection will be discussed in Section 1.4.2 'Over-Production of Pro-inflammatory cytokines'.

1.3   Respiratory disease caused by opportunistic pathogens like *Actinobacillus pleuropneumoniae (App)*

App respiratory disease is one of the most important diseases in swine production with a large impact on animal welfare and production economics (Angen *et al.,* 2001). App bacterium produces an immunological stress in the form of clinical and sub-clinical disease which negatively influences growth.

App respiratory disease is a contagious, fibrinous haemorrhagic and necrotizing disease, which causes septicemia, lung lesions, poor growth performance and even mortality (Straw *et al.,* 1999; Goethe *et al.,* 2000). This disease is most commonly known as pleuropneumonia. App is a gram negative capsulated rod (Pohl *et al.,* 1983) with coccibacillary morphology, with haemolytic activity on sheep blood agar and a negative result for urease (Goethe *et al.,* 2000). Clinical signs include fever, anorexia, diarrhoea, vomiting, increase in pulse rates, cardiac and circulatory failure,

coughing, blueness, pleurisy, mouth breathing and loss of appetite (Sebunya, *et al.*, 1983; Jobert *et al.,* 2000; Straw *et al.*, 1999).

Wallgren *et al.,* (1994) and Beskow *et al.,* (1989) found that at slaughter 8% of pigs showed signs of pleuritis. Most lesions were found in the respiratory tract. They are caused by the infiltration of neutrophils, macrophages and platelet activation (Liggett *et al.,* 1987; Bertram, 1986 and 1985,) which are linked to pigs' inflammatory responses to the invading bacteria (reviewed by Standiford 2000).

The App bacterium lives for a short time outside its former host. However, if the bacterium is encompassed in mucus or other organic matter the survival period and potential for re-infection is increased (Straw *et al.,* 1999). Sebunya *et al.,* (1983) found that pigs that survived had a reduced growth rate and became asymptomatic carriers with the infection found in necrotic lung lesions, tonsils and the nasal cavity. These carriers play a major role in the initiation of App respiratory disease outbreaks resulting in respiratory disease. Furthermore, the impact of subclinical infection on growth productivity is not currently addressed in farming practice. Researchers have isolated App bacterium from seemingly healthy looking pigs (ie. no clinical signs), (Heidt *et al.,* 1982; Kielstein *et al.,* 2001) which demonstrates that as long as the herd is in good health the disease may not manifest itself. These findings conflict with Leman *et al.,* (1992) who only found App bacteria in sick or compromised animals. However, Heidt *et al.,* (1982) also isolated App bacteria from lung lesions of pigs suffering pneumonia. Therefore, it is possible that Heidt *et al.,* (1982) isolated the App microbe from carrier pigs with subclinical infection rather than truly healthy pigs (Heidt *et al.,* 1982).

1.3.1 The implications of App

As earlier stated (reviewed by Solomons *et al.,* (1993); Hill *et al.,* (1952)) viral disease and opportunistic pathogens may contribute to reduced growth productivity in animals such as chickens and humans, due to respiratory disease. Most clinically significant diseases are seldom from one infection alone. During the early fattening period Wallgren *et al.,* (1994) found that *Mycoplasma hyopneumoniae* was gained which causes lesions containing App. This is an example of a pathogen reducing the animals' defences thereby allowing another pathogen, such as App bacteria, to infect the host.

App respiratory disease has been referred to as the most common cause of respiratory disease in pigs (Angen *et al.,* 2001; Wallgren *et al.,* 1994). Healthy pigs are colonised by *Mycoplasma flocculare*, *Mycoplasma hyorhinis, Haemophilus parasuis* and other microbes. These bacteria live as commensals while respiratory defences neutralise their pathogenicity (Leman *et al.,* 1992). However, at the first sign of reduction in the host's wellbeing the microbe reproduces and makes the animal sick. Respiratory tract infections by these pathogens may help the development of diseases such as pleuropneumonia.

The pathogen App causes respiratory disease and is one of the most significant environmental factors affecting growth of pigs (Beskow, *et al.,* 1989).

App is a bacterium with high pathogenicity. It is inhaled from the environment, and typically infects the host after influenza or Aujeszky's disease (Straw *et al.,* 1999). App and *M. hypopneumoniae* were only found in compromised pigs; it is usually associated with a subclinical infection (Straw *et al.,* 1999). The presence of *M.*

hypopneumoniae decreases the host's resistance against App bacteria (Leman, *et al.,* 1992), which explains why they commonly occur together. Therefore App seems to be implicated in respiratory disease which is mainly caused by another stress factor such as another pathogen. These pathogens are acquired through the host suffering environmental stresses eg. group housing, temperature fluctuations and bad air quality. All these factors seem to work synergistically to reduce the host's defences and increase susceptibility to the App bacteria and hence cause respiratory disease suffered by the pigs.

1.3.2 Treatment of App

In the past Ampicillin, and Sulfonamisde have been used to treat App respiratory infection. However, due to prolonged use of antibiotics, the App bacteria has developed resistance to Ampicillin and Sulfonamisdes (Straw *et al.,* 1999). These antibiotics do not affect serovar 1, 3, 5, and 7 greatly (Straw *et al.,* 1999; Hirsh *et al.,* 1982). Antibiotics kill the App bacteria but have no effect on the toxins released by the App bacteria (Straw *et al.,* 1999; Hirsh *et al.,* 1982; Veenhuizen 1998). The success of therapy depends on early detection of clinical signs and rapid therapeutic intervention (Straw *et al.,* 1999; Veenhuizen 1998). Antibiotics must be given in high dosages, subcutaneously or intramuscularly, and in repeated treatments to ensure effectiveness. (Straw *et al.,* 1999).

Veenhuizen, (1998) showed that in-feed medication such as antibiotics reduced porcine respiratory disease complex (PRDC) and improved growth performance of pigs. PRDC is produced by a combination of factors including; viruses, bacteria and environmental stresses. *Mycoplasma hyopneumoniae, Pasteurella multocida,* and *Actinobacillus pleuropneumonia* are the three most important bacterial respiratory

pathogens that contribute to PRDC (Veenhuizen 1998). However, Veenhuizen (1998) did not do follow-up experiments regarding in-feed medication or observed if the disease recurred and if the antibiotics were still able to control the disease. Huang *et al.,* (1998) found current vaccines and bacterins from commercial sources contained one or a combination of secreted toxins. In the experiments of Huang *et al.,* (1998) the severity of lung lesions and clinical disease were reduced. However, some vaccinated animals still suffered acute and chronic disease from serovars that they were not vaccinated against and therefore became carriers of App.

Fenwick *et al.,* (1994) established that various purified and partially purified antigens of App used as a potential vaccine reduced mortality but did not prevent the initial infection and therefore gave only part protection.

Huang *et al.,* (1998) showed that secreted components from host cell surfaces act synergistically in the pathogenesis of porcine App. Huang *et al.,* (1998) found animals that recovered from natural infection became immune to subsequent infections. Therefore more focus is needed on host defences as a treatment or preventative of this disease since the defences do not change but disease strains do.

In contrast, antigens from outer membrane lipoproteins of App (serotype 2 and 9) grown under iron restriction conditions showed an effective protection against clinical signs of App (serotype 2) disease. Unfortunately large-scale production of this as a vaccine is problematic and may not provide cross protection from serotype to serotype (Goethe *et al.,* 2000).

1.3.3 Pathology

Rioux, (2000) set out to show that capsular polysaccharides of App play a role in the pathogenicity of the disease. However, a mutant strain was found without capsular polysaccharides and which adhered better to the lungs but was more susceptible to host defences. It was concluded that outer membrane components were masked by the capsular polysaccharides. The capsular polysaccharides protect the bacterium from the host defences. Similarly, Dimango (1995) found binding specific genes (pillin and flagellin) produced by *Pseudomonus aeruginosa* were necessary for the host to produce cytokines. Dimango (1995) found non-pilated and non-adherent isogenic mutants produced less cytokines in lung epithelial cells. Therefore the host did not have to produce a vigorous attack since these mutants were more susceptible to host defences. Therefore the host defences may play a role in the disease outcome. The immunological mechanisms of the infection needs further investigation, especially the disease relationship to the host defences, so to reduce the negative effect of the disease on growth and health.

1.4 Immunological response to infection with App

As mentioned at the beginning of this review, environmental factors primarily cause stress and stimulate physiological disturbances such as loss of weight, muscle protein degradation, fever, lipolysis, infection and possibly death (Straw *et al.,* 1999). Microbial infection (an environmental stress factor) produces an immunological response, which then produces a physiological response. The physiological response to App respiratory disease in pigs was discussed earlier when the clinical signs of the disease were described.

App respiratory infection, as stated earlier, is a secondary infection which may occur as a result of another stress (eg. mycoplasma) (Caruson and Ross 1990). Once the mycoplasma infection is established it allows the App bacteria to take hold by gaining entry as airborne particles. The moist warm conditions in the lungs allow rapid multiplication (Veenhuizen 1998) and production of toxins (cytotoxic factors) and waste product (Huang *et al.,* 1999). App bacteria have many secreted and non-secreted cytotoxic factors such Apx I, II and III (Huang *et al.,* 1998) and LPS (Idris *et al.,* 1993; Udeze *et al.,* 1987) which stimulate host responses. Resting monocytes, macrophages and eosinophils are activated to aggregate to the infected area (reviewed by Hazlett 2002). The inability of pulmonary alveolar monocytes to kill App bacteria is believed to lead to rapid neutrophil infiltration. The App toxins degenerate neutrophils, which release lysosomal components, which damage vascular tissue, cause necrosis and pleural adhesions (Veenhuizen 1998). Macrophages become phagocytic, release lysosomal enzymes to counter the bacteria and therefore the macrophages produce cytokines (Tizard 1996; Brostoff 1991). The death of the pulmonary alveolar monocytes may increase the inflammatory response which causes the influx of neutrophils to the infection site leading to necrosis and lesion formation (Liggett *et al.,* 1987). In response to infection with App bacteria and the subsequent activation and infiltration of immune cells, a cascade of cytokines is produced with profound effects on the pig. In particular the influx of neutrophils produces IL-8 and thereby has a feedback in recruiting more neutrophils (Strieter *et al.,* 1992) causing further necrosis and lesion formation. Of concern are the pro-inflammatory cytokines, implicated in the pathology of App and poor growth of livestock.

1.4.1 Cytokines

Cytokines are a heterogeneous group of unrelated molecules unified by their ability to influence the behavior of other cells (Klein *et al.,* 1997; Tizard 1996). In other words they are immune messangers and act via cell receptors on target cells to influence local or systemic effects. Cytokines can interact with other systems such as the endocrine axis and the central nervous system.

Pro-inflammatory cytokines mobilise host defences and are responsible for recruitment of immune cells to inflammatory sites (Khair 1996). The cytokines produced by the activated monocytes and macrophages are IL-1, IL-6, Interleukin-8 (IL-8) and TNF-α (Klein 1997; Tizard 1996). These pro-inflammatory cytokines are present at various levels in an inflamed or injured area.

The need for pro-inflammatory cytokines has been demonstrated by Laichalk *et al.,* (1996) and Standiford *et al.,* (2000). They showed that TNF-α present in the lung of mice has antibacterial properties (Laichalk *et al.,* 1996; Standiford *et al.,* 1999), which have been overlooked due to the damage it (TNF-α) can cause when over produced. Another pro-inflammatory, MIP-2 (homolog of human IL-8), is needed by the host (mouse) to recruit neutrophils (reviewed by Standiford *et al.,* 2000) and to isolate invading bacteria (Brostoff *et al.,* 1991; Tizard 1996).

TNF-α on the other hand induces lipolysis and affects serum triglycerides which after chronic exposure may cause weight loss and body protein depletion (Mullin 1990).

IL-1 enhances fatty acid synthesis and secretes very low density lipoproteins. (reviewed by Johnson 1997). Lipoproteins are detrimental to the modern meat

industry since increased fat is now considered undesirable because of links between dietary fat in-take and cardiovascular disease in humans.

Cytokines play an essential role in the development of humoral immune responses to infection. Epithelial cells in the respiratory tract produce cytokines such as IL-8, IL-6, TNF-α and Granulocyte Macrophage Colony Stimulating Factor (GM-CSF) (Khair *et al.,* (1996); Levine 1997) for protection in response to bacterial challenge. Hakansson *et al.,* (1996) showed that IL-6 and IL-8 were produced by airway epithelial cells in response to the attachment of *Haemophilus influenzae*. In addition, DiMango *et al.,* (1995) and Massion *et al.,* (1994) reported an increase in cytokines secreted by bronchial epithelia *in vitro* exposed to *Pseudomonus aeruginosa.* These studies showed that pro-inflammatory cytokines like IL-6, IL-8 and TNF-α in mucosal tissue are essential for host defences against pathogen stresses.

The first cytokine released into the circulation 4-6 hours after an antigen is detected by macrophages is IL-1 (Tizard 1996; reviewed by Johnson 1997). IL-1 binds to receptors on T helper lymphocytes and activates a cascade of immune responses (Tizard 1996).

Kroemer *et al.,* (1993) stated that monocytes and macrophages play a major role in the activation of lymphocytes through the cytokines that they secrete.

Antibodies are produced as a result of the cytokine cascade (within 10-14 days post-infection) to recognise the infection and help in recovery and protection against re-infection. Cytokines such as IL-6 and IL-1 stimulate T helper 2 lymphocytes to secrete Interleukin 4 (IL-4) and Interleukin 5 (IL-5), which stimulate eosinophils and B cells to produce immunoglobulins (reviewed by Johnson 1997; Tizard 1996;

Saleem *et al.,* 1998). IL-6 promotes the production of IL-4 (an anti-inflammatory cytokine), and enhances the production of Immunoglobulin A (IgA).

IL-8 recruits neutrophils at the site of infection (reviewed by Murtaugh *et al.,* 1996). Since neutrophils and eosinophils are most mobile of the blood leukocytes they arrive at the damaged or infected area first (Brostoff *et al.,* 1991). The function of neutrophils is to capture and destroy foreign material through phagocytosis (Brostoff *et al*., 1991; Klein *et al.,* 1997). When an antigen or foreign matter is too large for one cell (macrophages or neutrophils) to ingest, cells aggregate and surround the antigen. The same occurs when an antigen persists (Roitt 1994; Brostoff *et al.,* 1991). This behavior (infiltration) stops the antigen from spreading but it is detrimental to the surrounding tissue as it causes the development of lesions when this occurs in the lung. Lesions can lead to breathing difficulties and lethargy. The aggregation of cells is not desirable since the lung is a soft, spongy and filtering organ (Tizard 1996) and cellular infiltration inhibits oxygen exchange. Morrison *et al.,* (2000) found that IL-1 and TNF-α were implicated in massive lung injury.

As alluded to earlier, pro-inflammatory cytokines mediate immunological responses and inflammation but over-production can exacerbate the inflammation or disease and end in death (reviewed by Sharma *et al.,* 1997). An optimal outcome of immunological activation depends on the balance between inflammatory cytokines and the release of immunosuppressors or anti-inflammatory activity.

1.4.2 Over Production of Pro-inflammatory cytokines

The over-expression of pro-inflammatory cytokines such as IL-1, IL-8 and TNF-α can be detrimental to the (pig) host (Baarsch *et al.,* 1995). The detrimental effects of pro-inflammatory cytokines occurs in most animals not just pigs. Olsson (1995) realised that elevated levels of TNF-α play a role in Multiple Sclerosis (humans), and since levels of IL-1α, IL-1β and IL-6 were elevated in systemic juvenile chronic arthritis (humans). Arend *et al.,* (1990), came to a similar conclusion that elevated pro-inflammatory cytokines are detrimental. Overproduction of IL-6 is known to cause toxic shock/endotoxic shock (Mire-sluis and Thorpe 1998; Tizard 1996). IL-6 also has been found at an elevated level in patients suffering Crohns disease (Mire-Sluis and Thorpe 1998). This pro-inflammatory cytokine (IL-6) has been associated with fever (Tizard 1996) and is implicated in auto immune conditions (Mire-Sluis and Thorpe 1998). IL-6 when not over-produced can be protective against Listeria bacteria in the mouse (Lui and Cheers 1992) and protective against *Pseudomonas aeruginosa* corneal infection in the mouse (Cole 2001).

Huang *et al.,* (1999) recorded an elevation in TNF-α and IL-1 in the serum, which coincided with the onset of acute clinical disease (App) in pigs. Neutrophil chemoattractants (IL-8) rather than the App bacterial components are the major factors causing acute lung inflammation (Huang *et al.,* 1999).

The detrimental effect of the over production of pro-inflammatory cytokines, such as TNF-α or IL-1β, exacerbate disease caused by bacterial infection (Morrison *et al.,* 2000). Further studies (Baarsch 1995) showed the presence of IL-8 and IL-1 in the pigs' lungs after App infection, and concluded that these inflammatory cytokines are

associated with the development of pleuropneumonia and contribute to the severity of the disease. Over production of pro-inflammatory cytokines can exacerbate the inflammation or disease and end in death (reviewed by Sharma *et al.,* 1997).

1.5 *Prevention of (App) Pathogen stress in Pigs*

Factors which contribute negatively to the physiological state of the pigs should be removed in order to prevent or reduce poor growth. From this review and others (reviewed by Johnson 1997) it can be seen that all stress factors contribute to the poor growth of animals such as pigs, chickens and humans. In fact, these stress factors, (i.e. large group size, temperature and climate fluctuation, poor air quality and pathogens) act synergistically to activate the endocrine-immune axis (reviewed by Johnson 1997). Preventing or decreasing the stress factors will help increase growth. Reducing stocking rate and group size in pigs showed a positive effect on growth (Tonn *et al.,* 1985). However, this is not commercially viable and may not be cost effective.

Crowding and air quality are related as overcrowding causes an increase in microbial load and an increase in respiratory disease with elevated pig numbers (Curtis *et al.,* 1975). Increasing ventilation causes a reduction in ammonia, carbon dioxide, microbes and dust and thus increases the health and performance (Jobert *et al.,* 2000) of pigs; again this is a costly project and calls for the rebuilding of farm sheds.

Currently, App and similar clinical and sub-clinical diseases are controlled using prophylactic antibiotics in-feed and therapeutic antibiotic treatment (Straw *et al*., 1999). Several components of the host immune system (eg. immunosuppressors and

anti-inflammatory cytokines) have shown promise as prophylactics and therapeutics to abrogate disease under App challenge and hence improve growth.

1.5.1 Anti-inflammatory cytokines as a therapeutic and/or prophylactic

The increase in severity of App by pro-inflammatory cytokines could be reduced by the use of anti-inflammatory cytokines. IL-10 is an anti-inflammatory which usually down regulates the balance of pro-inflammatory cytokines. For example, Muller (1998) found that the absence of IL-10 prolonged and enhanced systemic juvenile chronic arthritis (humans). Morrison *et al.,* (2000) also demonstrated that IL-10 reduced the acute disease caused by App infection and decreased lung damage significantly (pigs). IL-10 and Transformation growth factor-β (TGF-β) are associated with Multiple Sclerosis patients going into remission (Tian *et al.,* 1998). IL-10 also suppressed the activity of neutrophils therefore reducing necrosis. Since anti-inflammatory cytokines such as IL-10 have positive effects on disease, it would seem to be a good idea to use gene therapy to reduce the over-production of pro-inflammatory cytokines and thereby reduce the severity of the disease.

Van Roon *et al.,* (1996) found that IL-4 could reduce the ratio of type 1 and 2 T cells by acting as a growth factor for type 2 T cells, causing an increase in anti-inflammatory cytokines such as IL-10. IL-4 also reduced cartilage degradation in rheumatoid arthritis (humans) and hence IL-4 is another anti-inflammatory cytokine which should be observed in an immunocompromised system (Isomaki 1996). Other researchers also observed that IL-4 and IL-10 inhibited the synthesis of pro-inflammatory cytokines (reviewed by Bendtzen *et al.,* (1994) and reviewed by Mosmann (1994)).

Although IL-1 is considered a pro-inflammatory cytokine and can intensify inflammation, it can be reduced or even inhibited. Arend *et al.,* (1993) found IL-1β and α were inhibited by Interleukin 1ra (IL-1ra) which binds to receptor sites of IL-1 and stops signal transduction. Morrison *et al.,* (2000) showed upregulation of IL-1ra therefore helping in the reduction of pneumonic disease in pigs. Anti-inflammatory cytokines decrease or even inhibit disease. Research showed that Interleukin 13 (IL-13) inhibited IL-8 release and stimulated IL-1ra synthesis in blood mononuclear cells (Seitz *et al.,* 1996). IL-4 was also found to have anti-inflammatory effects on blood mononuclear cells. Zhou *et al.,* (1994) found that IL-4 suppresses inflammatory cytokines (IL-1, IL-8, TNF-α) in alveolar macrophages at a transcriptional level in pigs. With further study a therapeutic application may be established.

Saleem *et al.,* (1998) also observed that IL-4 suppresses neutrophil influx and therefore limits tissue damage. IL-4 also suppresses IL-8 production (Hazlett 2002) by neutrophils and therefore the feed-back system (Strieter 1992) is halted.

Inflammatory cells producing pro-inflammatory cytokines (TNF-α, IL-1β and IL-8) found in the lungs of App infected pigs (Baarsch *et al.,* 1995) could be reduced or halted by anti-inflammatory cytokines such as IL-4. IL-4 stimulates Interleukin 4 receptor antagonist (IL-4ra) while inhibiting IL-1 and TNF-α production by monocytes. IL-4 inhibits Intercellular Adhesion Molecule 1 (ICAM-1) mRNA in mice and therefore blocks neutrophils from adhering to lung endothelium or epithelium (Saleem *et al.,* 1998).

The exacerbation of App respiratory disease by pro-inflammatory cytokines, eg. IL-8, can be reduced by anti-inflammatory cytokines, such as IL-4 and IL-1ra as stated earlier, restoring the immunological activity balance. Neutrophil infiltration produced

by IL-8 over-production causes pathogenic lesions which can be reduced or stopped by stimulating the production of IL-4 or IL-1ra. The potential of IL-1ra and IL-4 as therapeutic or prophylactic alternatives to antibiotics, needs further investigation in the App model and may have subsequent commercial application.

The main aim of this thesis is to investigate novel immunomodulators in particular Interleukin 4 and Interleukin 1 receptor antagonist as alternatives to antibiotics to treat the infectious stress of App respiratory disease which is detrimental to health and growth

CHAPTER 2
GENERAL MATERIALS AND METHODS

2.1 Animals

Male pigs (Landrace X large white from Westmill Piggery, Young, NSW) were nasal swabbed and confirmed to be free of App and mycoplasma by PCR (NSW Agriculture).

Animals were maintained in a piggery with a controlled environment at Elizabeth Macarthur Agricultural Institute (EMAI, Menangle, NSW) for the duration of the trials. The relative humidity was maintained at 60% with a temperature of $24^{o}C$ and fresh air circulated constantly. The floor was fully slatted concrete and the sub-floor area was flushed twice daily. Pigs were acclimated to their surroundings for at least 2 weeks before initiation of each experiment. Pigs were fed a commercial grower diet in pellet form and water was provided ad libitum via a nipple dispenser. Pigs were randomly allocated into groups, rooms and pens according to the experimental protocols. Weight was measured using a walk in cage balance, at which time anal temperatures were taken as required.

2.2 Blood samples

Animals were restrained using a nasal snare, and blood was collected by venipuncture from the jugular vein. Blood was collected into Vacutainer™ tubes (Becton Dickinson, Franklin Lakes, NJ, USA) and stored on ice for transport.

For the collection of serum, blood was collected in Vacutainer™ SST II tubes with a clotting accelerant and centrifuged at 2000g for 10 minutes at 4°C. Serum was removed using a pipette and aliquoted into tubes for storage at -20°C.

For the collection of whole blood, the blood was collected into Vacutainer™ tubes lined with lithium heparin. The blood was stored at 4°C prior to use.

2.3 Infection Protocol

Pigs were restrained using a nasal snare and injected intravenously with xylazine (0.1mL/10kg) and ketamine (1mL/10kg) anaesthetic prior to intra-tracheal inoculation. A laryngoscope was used to enable proper placement of an endotracheal tube. The *Actinobacillus pleuropneumoniae* was isolated from pig lungs and grown on PPLO agar (2%w/v heart infusion, 3%w/v yeast enriched peptone, 1.6%w/vNaCl, 4.6% Agar, pH7.8) then transferred to PPLO broth and grown to mid log phase. App serovar 1, strain Hs54 was maintained, cultured and stored (-80°C in 70% glycerol) at EMAI, and the appropriate dose delivered according to each experimental protocol.

2.4 RT-PCR

Preliminary investigations revealed that compared to conventional methods such as chloroform extractions, the Promega SV RNA total extraction kit (Promega, Madison, USA) yielded a greater amount of RNA over a minimal amount of time. Therefore it was used to extract RNA from porcine blood and tissue samples. A standard amount of RNA between 0.2μg/μL to 0.4μg/μL was used to synthesise cDNA.

2.4.1 RNA Extraction Method

Whole blood was collected in Vacutainer™ tubes and mixed well. Red blood cells interfere with the extraction therefore they were removed using 900μL SV RNA Red Blood Lysis solution (Promega, Madison, USA) added to 500μL of whole blood in a 1.5μL microfuge tube. Samples were left to incubate for 15 minutes at room temperature, during which time they were inverted twice.

The samples were then centrifuged at 20,000g for 20 seconds to produce a white pellet. The supernatant was discarded and the pellet lysed using 175μL SV RNA lysis buffer (Promega, Madison, USA) to release nucleotides.   Preparation of tissue samples (1cm3 lung and lymph nodes samples) involved crushing them in a 1.5μL microfuge tube using a microfuge pestle in 175μL SV RNA lysis buffer (Promega, Madison, USA). The remaining components of the SV RNA kit (Promega, Madison, USA) were used according to the manufacturer's instructions for both blood and tissue (Appendix 4). However, the obtained yield of approximately 0.22μg/μL RNA required the use of 50μL of water to elute the RNA from the column, which was an exception to the manufacturer's instructions.

2.4.2 RT cDNA Synthesis

The total RNA concentration was determined using a Spectromax 250 plate reader (Molecular Devices, Sunnyvale, CA, USA). A dilution of 1 in 200 for each RNA sample was made and 200μL of each sample was aliquoted into a 96 well quartz plate (Hellma GMBh & Co KG, Müllheim, Germany) which was placed on ice. The plate was placed in the Spectromax 250 and read at 260nm and 280nm. The 260nm result was used to calculate the concentration of RNA (RNAμg/μL =

A260nm/0.025*dilution factor/1000) and the ratio of the 260nm result over the 280nm result gave the purity of the sample (1.8 to 2.0 optimum).

Total RNA was reverse transcribed using a reverse transcriptase system (Promega, Madison, USA) to produce cDNA; $0.2\mu g/\mu L$ RNA was used for each sample.

2.4.3 PCR

The whole procedure was performed on ice to control reactions until all the cDNA samples were added, allowing the direct comparison of samples. A master mix was prepared and aliquoted into tube strips or 384 well plates (Nunc, Service Life Science, Rockilde, Denmark) adding all components except the cDNA (Appendix 3).

The master mix was prepared containing 0.05units/μL Taq polymerase (Sigma-Aldrich, St Louis, USA), 1.5mM $MgCl_2$, $100\mu M$ dNTP (Promega, Madison, USA), 1X reaction buffer (10X reaction buffer; 100mM Tris-HCl, pH 8.3, 500mM KCl, 11mM $MgCl_2$ and 0.1% gelatin) (Sigma-Aldrich, St Louis, USA) and 10pmol specific primers. Approximately $1\mu L$ neat cDNA was used; this volume was previously determined to be optimum via titration. The specific paired primers used were IL-8, IL-6, (created by Dr Nicola Kingsford using Primer3 web program (Whitehead Institute, Cambridge, MA, USA)) IL-1β, TNF-α; β-actin (Sequences for paired primers found in Dozois, et al., 1997).

The IL-8 primer pair was derived from the porcine IL-8 nucleotide (Genbank accession M86923) and the IL-6 primer pair was derived from the porcine IL-6 nucleotide (Genbank accession M86722). The primer sequences and product sizes are listed in Appendix 1.

The cycling conditions consisted of initial denaturation at 95°C for 2 minutes, followed by 94°C for 5 seconds; annealing was performed at 56°C for 5 seconds and extension at 72°C for 24 seconds for 34 cycles and final extension for 2 minutes at 72°C. Cycling was performed on a PTC-200 DNA Engine thermal cycler (MJ Research Inc. Waltham, MA, USA). A positive control (master mix plus cloned known positive cDNA) and negative control (master mix plus water) were run with each plate or strip reaction. PCR sample (10μL) were migrated on a 1-2% TBE (0.089M Tris base, 0.089M Boric acid, 2mM EDTA, pH 8.3) agarose gel. The gel was stained in 250mL TBE containing 5μl EtBr (stock 10mg/mL) and was shaken for 20 minutes at room temperature. The gel was transferred to the destaining container, which contained milliQ water and was destained by shaking for 20 minutes at room temperature. The gels were photographed using a CCD Kodak digital camera (Kodak, Rochester, NY, USA) and the images were subsequently analysed using Quantity One (Biorad, California, USA). For analysis the above staining technique provided a clearer gel image rather than incorporation of EtBr into the gel prior to running the samples. The ratio of the density of the cytokine product over the corresponding density of the internal standard (β-actin) product was used to calculate relative band intensity for individual samples.

2.5 Cloning

PCR products were purified and cloned to create positive controls, for running with PCR products from porcine samples, on gels. Approximately 50μl of PCR solution was found to be the optimal amount for purification. A wizard PCR Prep Purification Kit (Promega Corporation, Madison, USA) was used to purify the PCR product

according to the manufacturers instructions (Appendix 5). The purified sample was

stored at –20°C until the next step.

2.5.1 DNA Ligation

Ligation was done overnight according to manufacturer's instructions using pGEM-T easy vector system (Promega Corporation, Madison, USA). Ligation entailed placing the purified PCR product inside a plasmid to enable insertion into a cell for replication.

2.5.2 Transformation

Transformation was done by inserting a plasmid vector into a cell for replication. The cell in this case was an Escherichia coli cell line JM109 (Gibco, Maryland, USA) which was stored at $-70°C$.

Ampicillin LB plates (LB medium, 1.5% Agar, 100μg/mL ampicillin) were prepared by making LB medium (1% Tryptone, 5% Yeast extract, 0.5% NaCl) (Appendix 7). Plates were taken out of the fridge to equilibrate to room temperature; 80μg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) and 0.5mM IPTG were added to plates following manufacturer's instructions for the pGEM-T easy vector system. The white colonies containing the vector were then grown up in Ampicillin LB broth (LB medium, 100μg/mL Ampicillin) to obtain one single clone.

2.6 White Blood Cell counts

White blood cell (WBC) counts were obtained from heparinised blood samples and analysed using a Sysmex analyser (Toa Medical Electronics Co. Ltd., Japan).

2.7 Differential white blood cell counts

White blood cell differentials were counted manually. Heparinised blood was inverted in the Vacutainer ™ tubes to obtain a homogeneity of cells. About 5 drops of

blood was smeared on a microscope slide and air-dried overnight. Slides were stained with Diff-Quick, a modified Wright stain (Lab Aids Pty Ltd, NSW, Australia). A total of 200 cells were counted and tallies of each cell type were recorded for the determination of cell population percentages. The number of each cell type per milliliter was obtained by multiplying the percentage by the total WBC count.

2.8 Enumeration of Lymphocyte Subsets via Flow Cytometry

2.8.1 Preparation and Staining of Lymphocytes

Heparinised blood was dispensed (100μl) into Falcon tubes (Becton Dickinson, Franklin Lakes, NJ, USA). Blood samples to be stained for the B cell marker immunoglobulin (Ig) were washed twice, in 2mL Potassium Buffered Saline (PBS) (0.8% NaCl, 0.02% KCl, 0.115% Na_2HPO_4, 0.02% KH_2PO_4 pH7.5) and centrifuged at 850g and 4°C for 5 minutes to remove immunoglobulin in the serum. Each subsequent wash and centrifuge was done using the same parameters as stated above.

The optimal dilutions of primary antibodies were determined previously by titration. The primary antibodies were monoclonal mouse antibodies specific for porcine CD3, CD4, CD8 and immunoglobulin (Ig light chain). Primary antibodies were diluted in PBS. Mouse anti-porcine Ig (Serotec, Raleigh, USA) antibody was diluted 1:10, mouse anti-porcine CD8 (Serotec, Raleigh, USA) was diluted to 1:20, mouse anti-porcine CD4 (VMRD, Washington, USA) was diluted to 1:200 and mouse anti-porcine CD3 (Veterinary Medical Research and Development, Washington, USA) diluted to 1:100. Samples were washed, and incubated at 4°C for 20 minutes with FITC-conjugated goat anti-mouse IgG (ICN, Costa Mesa, CA, USA). Samples were mixed and incubated at 4°C for 20 minutes. Red blood cells were lysed by the addition of 2mL of FACS Lyse (Becton Dickinson, Franklin Lakes, NJ, USA).

Samples were incubated at room temperature in the dark for 10 minutes. The tubes were centrifuged at 400g at 4^oC for 5 minutes and the supernatant was discarded. The remaining pellets of white blood cells were washed and centrifuged at 400g at 4^oC for 5 minutes. The pellets were resuspended in 1% paraformaldehyde (200µL) and stored in the dark at 4^oC prior to analysis.

2.8.2 Flow Cytometric Procedure

The fluorescence in each sample was quantified using a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). The resulting cell plots were analysed using CellQuest software version 3.0.1f (Becton Dickinson, Franklin Lakes, NJ, USA). The lymphocyte population was identified on the basis of their granularity and size, and this population was gated to exclude dead cells and other cell types. The fluorescence of the gated population was recorded, from which the background fluorescence of a negative control sample containing no primary antibody was subtracted.

2.9 Neutrophil function assay

The phagocytic capacity of neutrophils was adapted from the method of Perticarari et al., (1991).

Heparinised blood was aliquoted (100µL) into Falcon tubes (Becton Dickinson, Franklin Lakes, NJ, USA). Six 100µL aliquots were taken from a randomly chosen blood sample and dispensed into the Falcon tubes. These tubes were numbered

Control 1 to 6. All samples along with controls 2, 4 and 5 received 1μg of HE in 10μL. Other controls received an equal volume of PBS. All tubes were mixed and incubated at 37°C for 15 minutes. Approximately 100μL serum opsonised FITC-zymosan (Appendix 9) was added to samples and controls 2 and 3. The remaining controls received 100μL PBS. PMA (Phorbol 12-Myristate 13-Acetate) (10ηg/μL) (Sigma-Aldrich, St Louis, USA) was added to samples and controls 5 and 6, with other controls again receiving an equal volume (40μL) of PBS (or Killed App bacteria (KB) at $1x10^7$cfu/mL was added to appropriate tubes instead of PMA). Tubes were mixed and incubated at 37°C for 45 minutes. Phagocytosis was then stopped by placing the tubes on ice.

Red blood cells were lysed with FACSlyse (2mL) (Becton Dickinson, Franklin Lakes, NJ, USA) and tubes were incubated at room temperature for 10 minutes in the dark. The tubes were centrifuged at 300g for 5 minutes and the pellet was resuspended in 2mL of PBS. Tubes were centrifuged at 300g for 5 minutes and the pellets were resuspended in 500μl PBS. The tubes were stored on ice and immediately prior to analysis, trypan blue (1mg/mL) was added to tubes to quench auto-fluorescence. Fluorescence was quantified using a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) and analysed using CellQuest (Becton Dickinson, Franklin Lakes, NJ, USA).

Neutrophils were gated according to their size and granularity on the light scatter plot and the fluorescence on the red and green channels recorded. Green fluorescence indicated the amount of FITC-zymosan that had been phagocytosed and internalized. Red fluorescence indicated the oxidative burst had commenced.

2.10 Lymphocyte proliferation assay in whole blood

The lymphocyte proliferation assay was adapted from the method of Fletcher et al., (1987).

2.10.1 Preparation of Culture Plates

Culture media (100mL RPMI (ICN, Australia), 2μg/mL L-glutamine and 0.01unit/mL penicillin, 1μg/mL streptomycin) was prepared and added with stimulatant into some wells and without stimulants into other wells of a 96 well round bottom plates (Nunc Service Life Science, Rockilde, Denmark). Columns 1-4 were filled with 100μl culture media only (controls). Columns 5-8 were either filled with 100μl of killed App bacteria (KB) to a final concentration of $1x10^7$cfu/mL in culture media or with 100μl Con A at 10ug/mL in culture media. Columns 9-12 were filled with 100μl of PHA (8μg/mL) in culture media. Plates were stored at -20°C. The optimal concentrations of KB, Con A and PMA had previously been determined by titration.

2.10.2 Assay

Prepared plates were thawed prior to use. Heparinised blood was diluted 1:5 with culture media then 100μL was aliquoted into 12 wells across 1 entire row of each plate and each sample was placed in 4 control wells, 4 KB wells and 4 PHA wells. Thus, 8 blood samples filled a pre-prepared plate. Plates were incubated for 54 hours in a tissue culture incubator at 37°C, 85% humidity and 5% CO_2.

2.10.3 Pulsing

Tritiated [^{3}H] thymidine (Amersham, Buckinghamshire, England) was diluted 1:100 with culture media then 25μL was added to each well and plates were incubated for 18 hours. Plates were wrapped in aluminium foil and stored at -20°C to stop further incorporation of thymidine.

2.10.4 Harvesting and Analysis

Cells were harvested from each well using a Skatron combi-harvester (Molecular devices, Sunnyvale, CA, USA) and placed onto glass fiber filter paper. Incorporated radioactivity was measured using a liquid scintillation counter (Beckman, Fullerton, CA, USA). Stimulation indices were calculated by dividing the mean counts per minute of stimulated wells by the mean counts per minute from non-stimulated (control) wells.

2.11 Enzyme-Linked Immunosorbent Assay (ELISA)

Antibody levels in pig antisera were determined using a sandwich ELISA. Mouse anti-pig IgG light chain antibodies (Serotec, Raleigh, USA) were diluted 1:500 in 0.6M carbonate buffer, pH 9.6, and applied (100μL/well) to a 96 well flat bottom microtitre plate (Nunc, Rockilde, Denmark). This was incubated at 4°C overnight. Unbound antibodies were removed by inverting and flicking the plates. The plates were washed three times with PBS-T (0.15M PBS, pH 7.2, containing 0.05% Tween-20 (Sigma-Aldrich, St Louis, USA)). The plates were inverted and flicked to remove the excess PBS-T. Consecutive washes were performed in a similar manner unless stated otherwise. Gelatin (1% in carbonate buffer) was applied (150μL/well) and

incubated for 1 hour at 37°C to block reactive sites on the polystyrene. The plates were inverted and flicked before washing to remove unbound gelatin.

Porcine IgG (Serotec, Raleigh, USA) was used as a standard. It was titrated from a concentration of 500μg/mL in doubling dilutions and applied to the first 2 columns (100μL/well) in duplicate. The serum samples were diluted 1:200 with PBS-T and applied in duplicate to each plate (100μL/well). Plates were incubated for 2 hours at 37°C, and then washed with PBS-T. Horse radish peroxidase (HRP) conjugated goat anti-porcine IgG heavy and light chain (Serotec, Raleigh, USA) was diluted 1:2500 with PBS-T and 100μL applied to each well. Plates were incubated for 1 hour at 37°C followed by 10 washes with PBS-T. A working substrate was made using 10mg of 2-2 azino-di (3-ethyl-benzthiazoline sulphonic acid) (ABTS) (Sigma-Aldrich, St Louis, USA) in 33mL 0.05M citric acid, pH 4 plus 125μL 30% hydrogen peroxide (100μL/well). The degree of hydrolysation of the added substrate was measured by reading plates at 405nm using a Spectromax 250 plate reader (Molecular Devices, Sunnyvale, CA, USA).

2.12 TNF-α Bioassay

This method was modified from the methods of Baarsch et al., (1991), Blagosklonny et al., (1993), Jeffes et al., (1991) and performed across 3 days.

Day 1

Supernatant from the flasks containing L929 cells was removed and 4mL trypsin/EDTA (0.25% trypsin/EDTA) (Sigma-Aldrich, St Louis, USA) was added and returned to the incubator for a further couple of minutes. When the cells detached, 30mL media (RPMI-1640 (ICN, Australia), 100U/mL penicillin, 100μg/mL

streptomycin, 2mM L-glutamine, 10% v/v heat inactivated Fetal calf serum (Sigma-Aldrich, St Louis, USA)) was added to neutralise the trypsin/EDTA. Cells were transferred from the flask into a 50mL centrifuge tube and centrifuged at 500g, for 5 minutes at room temperature. Pellet was resuspended in 10mL of media. Cells were stained with trypan blue and counted using a haemocytometer (Sigma-Aldrich, St Louis, USA) to determine cells/mL. Cells were diluted to a final concentration of $4x10^6$ cells in 10mL. Approximately a 100μL of cell suspension (ie. $4x10^4$ cells/well) was added to each well (96 well plate (Nunc, Rockilde, Denmark)). The plates were then incubated overnight at 37^oC, 85% humidity and 5% CO_2.

Day 2

Plates with L929 cells were removed from the incubator. Standards were prepared using recombinant porcine TNF-α stock (R&D systems Inc. Minneapolis. MN. USA) from 1500pg and double diluted to a final contraction of 25pg. The supernatant was removed from the wells of each plate and discard using a multichannel pipette so as not to disrupt the monolayer. Approximately 50μL of each standard from 1500pg to 25pg was added in duplicate to the first 2 columns of the plates. Serum from pigs (50μL) was applied neat in duplicate. Actinomycin D (Sigma-Aldrich, St Louis, USA) solution at 8μg/mL was prepared in media and 50μL was added to every well. The plates were returned to the incubator for a further 18 hours at 37^oC, 85% humidity and 5% CO_2.

Day 3

The plates were removed from the incubator and the supernatant was removed using multi-channel pipette so as not to disturb the monolayer. Approximately 50μL of

crystal violet solution (0.5%w/v crystal violet, 20%v/v ethanol) was added to each

well and the plates were left to incubate at room temperature for 10 minutes. The

stain was rinsed under tap water without washing cells off the plate. Water was

flicked off and the plates were dried by resting on absorbent paper overnight.

Approximately 100μl of methanol was added to each well. The plates were shaken for

20 seconds and the read at 595nm absorbance. Samples were compared with the

standard curve to determine TNF-α concentration in sample.

CHAPTER 3

DEVELOPMENT OF METHODOLOGIES TO MEASURE THE PRODUCTION OF PRO-INFLAMMATORY CYTOKINES *IN VITRO*

3.1 Introduction

Stimulation of cells with mitogens is a common method to measure non-specific responses of immune cells. A mitogen is a substance or agent that triggers or induces mitosis such as lectins, phorbol esters and endotoxins.

It was necessary to develop a method which demonstrated that the production of pro-inflammatory cytokines could be mitogenically stimulated in whole blood, and subsequently measured. Ideally, this method would allow for the selection of the most potent mitogens and their optimal concentrations for *in vitro* stimulation of cytokines. Such a method would provide a useful tool for screening the ability of substances to inhibit or reduce the production of pro-inflammatory cytokines, and for the selection of potential treatments for future *in vivo* experiments.

Such an assay would use whole pig blood instead of a more conventional method of separating out the white cells (Polymorphonuclear Cells (PMN), lymphocytes, monocytes) before stimulation with mitogen. Most assays use blood separated by centrifugation on density gradient medium such as ficoll-hypaque resolving medium (Huang et al., 1998 used ficoll-hypaque resolving medium). A whole blood method was preferable as it permits the processing of large sample numbers. Most importantly whole blood may give a more accurate reflection of what could occur *in vivo* since plasma, serum, cells and other components are present. Such components in whole blood may interact when stimulated by mitogens. Furthermore, separation techniques are not efficient in obtaining all the white

blood cells, which is important for observations of the interactions of pro-inflammatory cytokines. A whole blood assay seemed to be the simplest, most cost effective and time effective assay to develop.

From the four pro-inflammatory cytokines of interest; IL-1, IL-6, IL-8 and TNF-α, IL-8 was chosen since it has been associated with the pathogenicity of App disease (Baarsch *et al.,* 1995) and the mRNA of IL-8 was easily detectable. The neutrophil chemotactic ability of IL-8 is known to be associated with the influx of neutrophils (Zhou *et al.,* 1994) leading to the development of lung lesions in pigs infected with App (reviewed by Standiford *et al.,* 2000). The robustness of the IL-8 RT-PCR assay was proven in other studies run previously by this Laboratory. cDNA transcription of samples were done according to the methods described in Section 2.4.2. IL-8 paired primers were used to perform PCR described in Section 2.4.3. The ease of established primers was another reason IL-8 mRNA was detected in these *in vitro* experiments.

The mitogens to be used included; Phorbol 12-Myristate 13-Acetate (PMA), Phytohemagglutinin (PHA), Concanavalin A (Con A), lipopolysaccharides (LPS) and Killed *Actinobaccillus pleuropneumoniae* bacteria (KB). PHA is a lectin which is isolated from kidney beans. It is also a glycoprotein, which binds to carbohydrate receptors on the surface of lymphocytes (Di Sabato, *et al*. 1987). It has the capacity to stimulate proliferation of lymphocytes (B cells, T cells). Con A is a lectin which activates T cells (Gunther, *et al.* 1973) and has similar properties to PHA . PMA is a phorbol ester which activates T cells and polyclonal B cells. It is a direct agonist of protein kinase C which plays a role in B cell activation (Schmidt and Hecker 1975). The rapid expression of cytokines is due to the

presence of regulatory sequence elements in the PMA protein (Schmidt and Hecker 1975). It mimics the effect of various cytokines by inducing the production of cytokines (Schmidt and Hecker 1975).

LPS is an endotoxin found in gram negative bacteria cell walls. LPS is a B cell stimulator and functions as a polyclonal B cell activator. It also activates macrophages and complements cascades. It is a physiological stimulus for the synthesis of pro-inflammatory cytokines.

KB (heat killed App bacteria) has LPS in the cell wall (Idris *et al.,* 1993 and Udeze *et al.,* 1987) and other secreted and non-secreted toxins which may give an indication of potential stimulation of pro-inflammatory cascades resulting from bacterial infection *in vivo*.

The purpose of this Chapter was to develop an assay which could be used to estimate the amount of mitogen needed to produce a pro-inflammatory cytokine response in whole blood. A further purpose was to identify substances which could inhibit or reduce this inflammatory response.

3.2 Experiment 1: Titration of mitogens and killed App for the stimulation of IL-8 production in porcine whole blood.

3.2.1 Objectives

The aim of this experiment was to discover which of the four mitogens (PMA, PHA, Con A and LPS) and KB samples produced the greatest immune response measured as the production of pro-inflammatory cytokines.

Further investigations were required to determine optimal concentrations of mitogens for the stimulation of pro-inflammatory cytokines.

3.2.2 Experiment 1 Materials and Methods

3.2.2.1 Experiment 1a Whole blood Stimulation

The blood was taken by venipuncture from a male pig with no observable illness according to the method described in Section 2.2. The animal was housed at the University of Sydney Piggery (May Farm), Camden. Blood was collected into lithium heparin Vacutainer ™ tubes and put on ice.

Heparinised blood was then aliquoted (2mL) into 16 sterile plastic Falcon test tubes (Becton Dickinson, Franklin Lakes, NJ, USA). The proliferation method using microtitre plates as outlined in Section 2.10 was modified for use in test tubes, to enable removal of samples from each test tube over time. The test tubes were separated into 5 groups of 3 test tubes. Saline was added to the 16th test tube (control). Each group was treated with a stimulant at 3 different concentrations described as low, medium and high (Table 1). The tubes were incubated in 5% CO_2 at 37°C and 85% relative humidity; 300μL aliquots were removed prior to stimulation, and 2 hours, 4 hours and 24 hours after stimulation for total RNA extraction.

Table 3.1: The final concentration of mitogens per mL of whole blood, used in the test tube based proliferation assay, experiment 1a.

Stimulant	Concentration		
	Low	Medium	High
PMA	1ug/mL	5ug/mL	10ug/mL
PHA	1ug/mL	5ug/mL	10ug/mL
ConA	1ug/mL	5ug/mL	10ug/mL
KB-App	$3x10^5$cfu/mL	$3x10^7$cfu/mL	$3x10^8$cfu/mL
LPS	1ug/mL	10ug/mL	50ug/mL

3.2.2.2 Experiment 1b

A volume of 1.8mL of blood was aliquoted into 27 sterile plastic Falcon test tubes (Becton Dickinson, Franklin Lakes, NJ, USA). Samples were separated into 5 groups with 5 concentrations of mitogens (Table 3.2) and a control each done in duplicate. The control blood samples were given 200μL sterile injectable saline (1.5mMNaCl, AstraZeneca, North Ryde, Australia). All saline treated blood samples in the future had been given 200μL, so as to compare between *in vitro* experiments. RNA was extracted for cytokine analysis of IL-8, TNF-α and IL-1β.

Blood was analysed for cytokine production before stimulation to observe any baseline constitutive cytokine presence. Another reason to measure this is that individual differences between animals and their baseline cytokine levels may occur. One could easily make an assumption of up-regulation that may only be the base level of pro-inflammatory cytokines normally circulating in peripheral blood.

The 5 mitogen groups used in the first part of this experiment (Table 3.1) were expanded in concentration (Table 3.2). After 200μl of mitogen was added, each tube contained a final volume of 2mL. The tubes were incubated with shaking in 5% CO_2 at 37°C and 85% relative humidity. Aliquots of 300μL were removed prior to stimulation, 2 hours and 24 hours after stimulation. The control blood samples were stimulated with saline and the un-stimulated blood sample was not processed in any way.

Table 3.2: Variable concentrations of mitogens used to stimulate whole blood in experiment 1b.

PMA	Tube 1 20μg/mL	Tube 2 10μg/mL	Tube 3 1μg/mL	Tube 4 500ηg/mL	Tube 5 100ηg/mL
PHA	Tube 6 20μg/mL	Tube 7 10μg/mL	Tube 8 1μg/mL	Tube 9 500ηg/mL	Tube 10 100ηg/mL
ConA	Tube 11 20μg/mL	Tube 12 10μg/mL	Tube 13 1μg/mL	Tube 14 500ηg/mL	Tube 15 100ηg/mL
KB	Tube 16 $2x10^9$cfu/mL	Tube 17 $1x10^9$cfu/mL	Tube 18 $1x10^4$cfu/mL	Tube 19 $1x10^2$cfu/mL	Tube 20 $1x10^1$cfu/mL
LPS	Tube 21 20μg/mL	Tube 22 10μg/mL	Tube 23 1μg/mL	Tube24 500ηg/mL	Tube 25 100ηg/mL

Total RNA was isolated using SV Total RNA Isolation System (Promega, Madison, USA) according to the manufacturer's instructions, and as outlined in Section 2.4.1.

RNA samples were transcribed to cDNA in a 10μL reaction according to the methods described in Section 2.4.2. PCR was performed using IL-8, TNF-α, and IL-1β paired primers as described in Section 2.4.3.

3.2.3 Experiment 1 Results

3.2.3.1 Experiment 1a Results

Saline treatment of porcine whole blood did not stimulate the production of any detectable IL-8 mRNA and was therefore considered to be the baseline production of IL-8 mRNA for that particular pig, and an appropriate negative control. Unfortunately, at 24 hours the blood samples were found to be coagulated, possibly due to the plastic tubes used to perform the assay.

The stimulation of whole blood by the 5 mitogens at all 3 concentrations produced IL-8 mRNA above the baseline with the exception of KB at $3x10^8$cfu/mL.

KB at $3x10^7$cfu/mL stimulated the most IL-8mRNA production at 4 hours.

All 3 concentrations of PMA stimulated production of IL-8 mRNA after 2 hours of stimulation in a dose dependent manner (Figure 3.1a). There was little difference between IL-8 mRNA stimulation by the different concentrations of PMA at 4 hours. It remains to be seen whether IL-8 mRNA production would increase, decrease or plateau after 4 hours of stimulation with PMA.

An IL-8 mRNA response, similar to that produced by PMA, was stimulated by all 3 concentrations of Con A (Fig 3.1c). IL-8 mRNA production peaked at 2 hours and remained relatively constant until 4 hours. PMA and Con A stimulation showed similar trends for the production of IL-8 mRNA at high (10μg/mL) and medium (5μg/mL) concentrations (Figure 3.1a, Figure 3.1c).

The high concentration (3×10^{8}cfu/mL) of KB failed to stimulate production of IL-8 mRNA (Fig 3.1d). The medium concentration of KB stimulated IL-8 mRNA production at 2 hours and this level was further increased at 4 hours (Figure 3.1d). At the low concentration, KB stimulated IL-8 mRNA at 2 hours and again at 4 hours (Figure 3.1d).

In the case of LPS stimulation, all 3 concentrations stimulated similar levels of IL-8 mRNA production after 2 hours (Figure 3.1e). The low concentration of LPS showed no stimulation of IL-8 mRNA production at 4 hours. Production of mRNA for IL-8 increased throughout the study for the high concentration of LPS, while there was little change between 2 hours and 4 hours for the medium concentration.
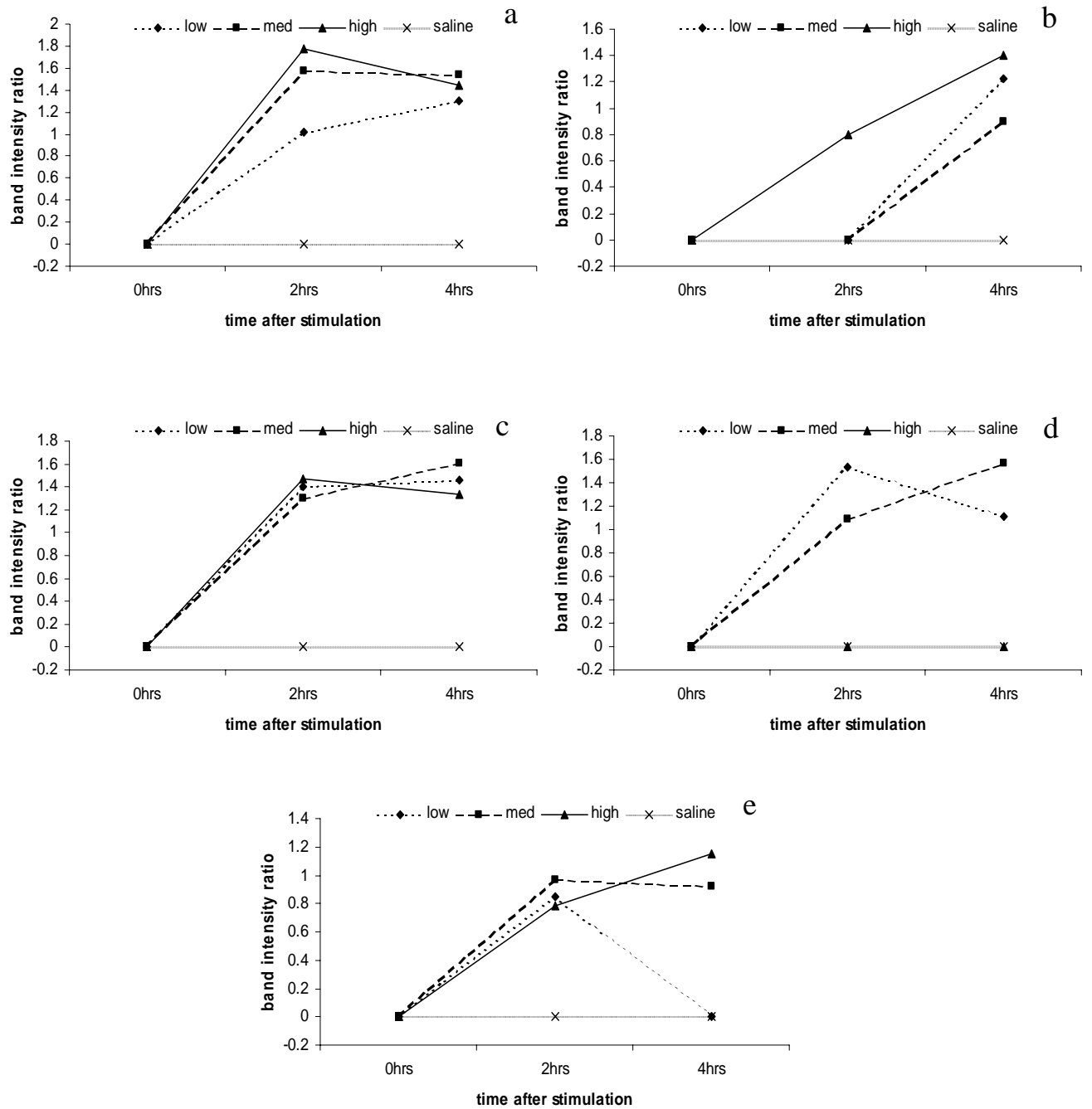
Figure 3.1: The temporal expression of IL-8 mRNA, measured by RT-PCR, in porcine whole blood after stimulation with a) PMA, b) PHA, c) Con A, d) KB, and e) LPS, applied at low medium and high concentrations. Control whole blood sample was stimulated with saline. Data from Experiment 1a.

3.2.3.2 Experiment 1b Results

Results were only obtained for blood prior to stimulation and at 2 hours after stimulation, as the blood coagulated by 24 hours after the addition of mitogens.

Whole blood samples with saline were found to produce IL-8 mRNA, resulting in a relative band intensity of 0.7. This was considered to be a constitutive level of expression in this pig and was presented as a background line in Figure 3.2. Therefore, anything above the line was considered to be up regulated.

All 3 PMA concentrations showed the presence of pro-inflammatory cytokine IL-8 mRNA prior to stimulation (Figure 3.2a). PMA concentrations between 0.1µg/mL and 20µg/mL showed negligible difference in their ability to stimulate the production of IL-8 mRNA in whole blood.

PHA at the 1µg/mL concentration stimulated the greatest production of IL-8 mRNA in whole blood (Figure 3.2b). However, there was little difference in the results obtained for concentrations < 0.5 µg/mL. No production of IL-8 or internal standard β-actin was detected when blood was stimulated with PHA at 0.1µg/mL (Figure 3.2b).

Con A at 0.1µg/mL stimulated IL-8 mRNA production with a band intensity of 1.3 (Figure 3.2c) which was much larger than the mRNA produced at 0.5µg/mL and 20µg/mL. Again, there was little difference in the IL-8 mRNA levels stimulated by 0.1µg/mL, 1µg/mL and 10µg/mL. At 20µg/mL Con A did not stimulate any IL-8 mRNA production.

LPS at 10μg/mL performed very well at stimulating increased levels of IL-8 mRNA production (Figure 3.2e) in whole blood. The remaining concentrations of LPS also stimulate IL-8 mRNA production but to a lower level than that seen with 10μg/mL LPS (Figure 3.2e).

The production of IL-8 mRNA by KB was at the greatest when the blood was stimulated with KB at concentrations of $1x10^9$cfu/mL and $2x10^9$cfu/mL (Figure 3.2d).

Pro-inflammatory cytokines such as TNF-α mRNA and IL-1β mRNA were also produced by the stimulation of whole blood by LPS or PMA (Figure 3.3).

The production of TNF-α mRNA and IL-1β mRNA in whole blood after stimulation with saline resulted in a relative band intensity of ~0.5 (Figure 3.3). This was considered existing baseline production of TNF-α and IL-1β mRNA in the whole blood obtained from that particular pig. Therefore anything above baseline (~0.5 band intensity) was considered up-regulation.

PMA stimulation of whole blood at all concentrations produced IL-1β and TNF-α mRNA above baseline and therefore was considered to up-regulate these cytokines.

LPS at all concentrations except 1μg/mL stimulated the production of IL-1β mRNA in whole blood above baseline. At 0.5μg/mL to 10μg/mL LPS stimulated the production of TNF-α mRNA above baseline.

In Figure 3.3, PMA at 20μg/mL stimulated the greatest production of TNF-α mRNA, however at concentrations between 1μg/mL and 10μg/mL a stable pro-

inflammatory cytokine production profile was produced. PMA stimulation of whole blood generally produced more IL-1β than TNF-α mRNA.

LPS at 10μg/mL produced the most stimulation of IL-1β mRNA in whole blood, while the highest level of TNF-α occurred at 1μg/mL LPS stimulation. This level was not much higher than the TNFα mRNA level produced by LPS at 10μg/mL concentration.

At 10μg/mL stimulation with both PMA and LPS resulted in considerable increases in the production of mRNA for IL-8, TNF-α and IL-1β in porcine whole blood in vitro. It would seem these concentrations of mitogens would produce a pro-inflammatory profile which would be sufficient for the testing in assays to screen for substances able to inhibit or reduce pro-inflammatory cytokine production since up-regulation was achieved above baseline.
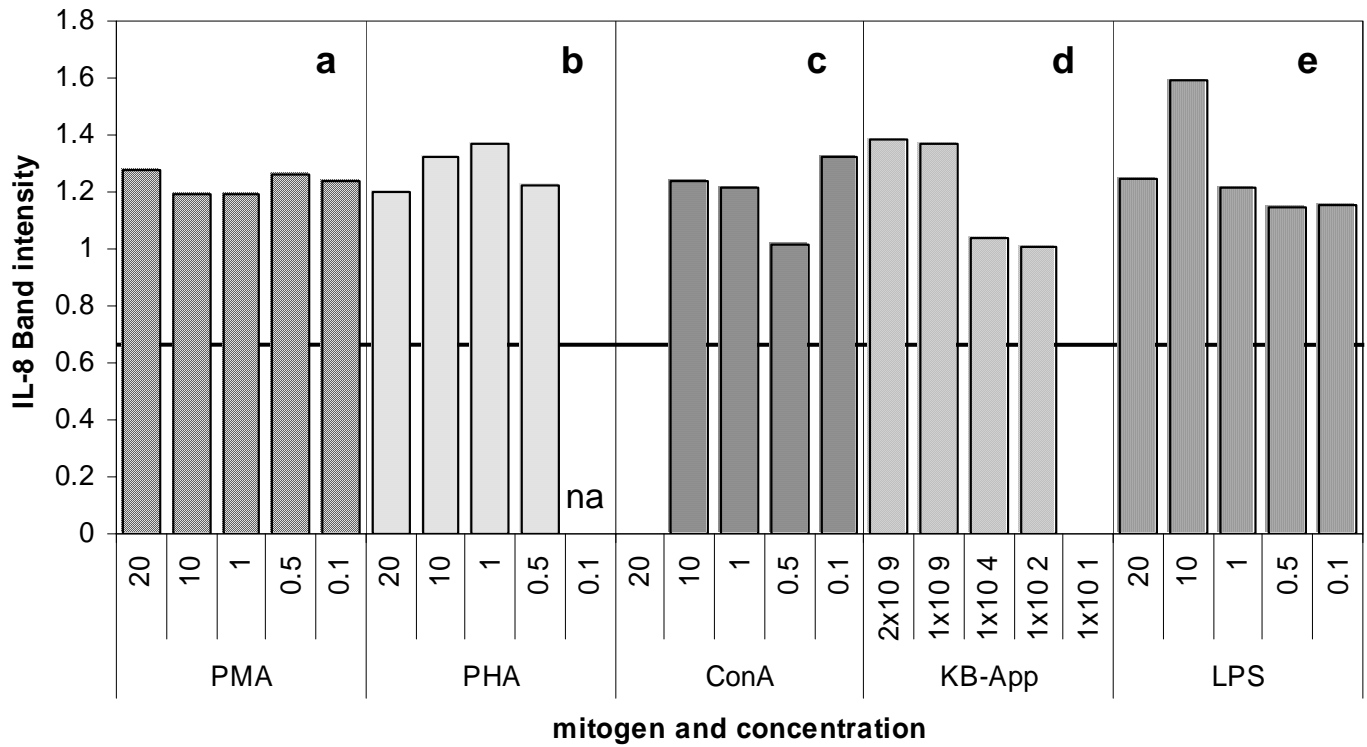
Figure 3.2: The expression of IL-8 mRNA measured by RT-PCR, in porcine whole blood after 2 hours stimulation with a) PMA, b) PHA, c) Con A, d) LPS, and e) KB, applied at varying concentrations; na indicates not assessable. Black line in the middle and in the background indicates baseline IL-8 mRNA production. Data from Experiment 1b.
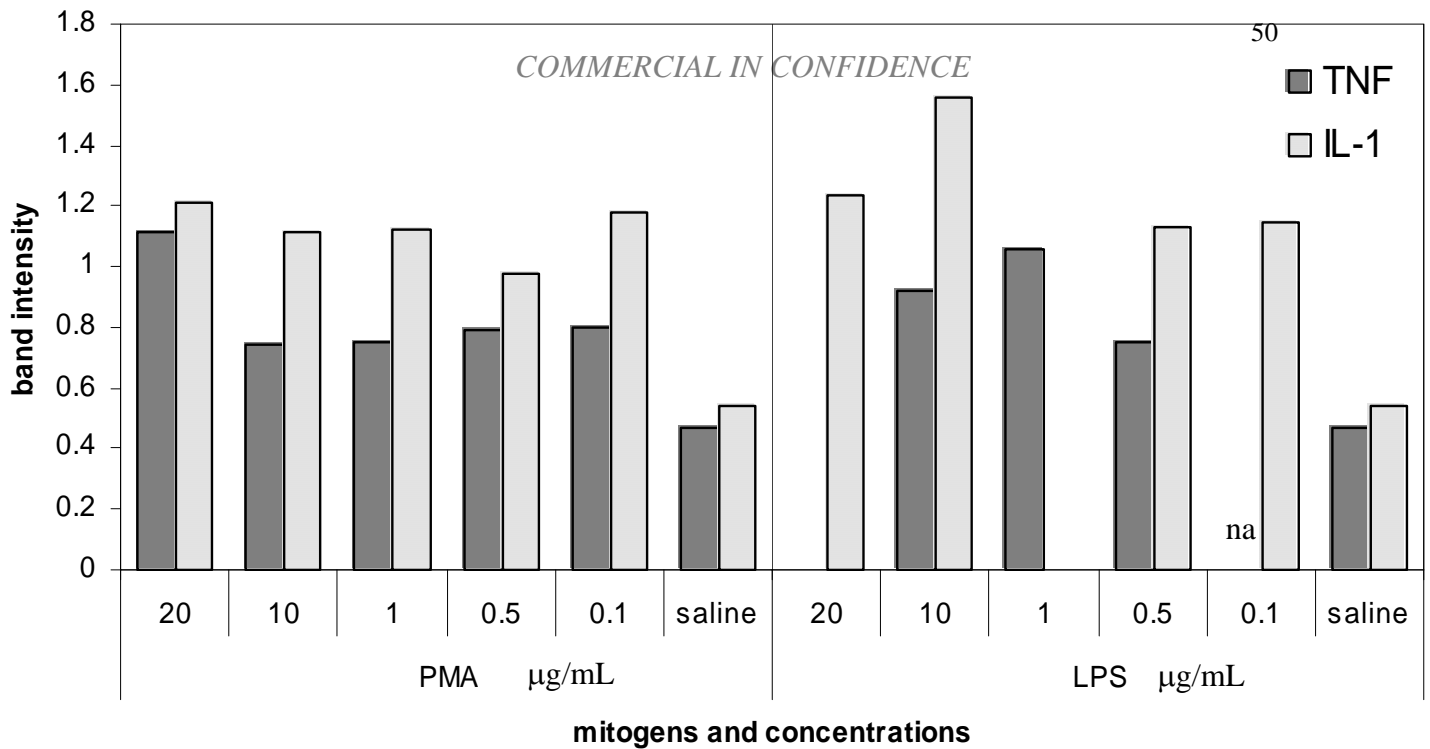
Figure 3.3: The expression of mRNA for IL-1β and TNFα measured by RT-PCR, in porcine whole blood after 2 hours stimulation with PMA and LPS at varying concentrations; na indicates not assessable. The zero concentration on the x-axis represents saline stimulated blood producing IL-1β and TNFα mRNA which was considered baseline. Production of cytokines above this level was considered up-regulation. Data from experiment 1b.

3.2.4 Experiment 1 Summary

- PMA, LPS and KB were the best performing mitogens in terms of increasing the production of IL-8 mRNA in these preliminary titration studies.

- PMA was chosen because PMA at all the concentrations stimulated IL-8 mRNA as compared to PHA and Con A. Con A stimulation resulted in a similar level of IL-8 mRNA as that found for PMA in experiment 1a which for this reason Con A was excluded.

- The optimal concentrations of PMA and LPS were determined to be 10μg mitogen/mL whole blood.

- Since the next *in vitro* experiment was a time course to 24 hours and $3x10^7$cfu/mL stimulated the most IL-8mRNA at 4 hours (1.6 band intensity) which was higher than the IL-8mRNA (1.4 band intensity) produced by $2x10^9$cfu/mL at 2 hours, the concentration of KB chosen to continue with was $1x10^7$cfu/mL.

- The abovementioned mitogens and their optimal concentrations were selected for use in further in vitro assays.

3.3 Experiment 2: The temporal effect of selected mitogens on IL-8 production in porcine whole blood

3.3.1 Objectives

The optimal concentrations of KB, PMA and LPS established from the previous experiment were used to determine whether stimulation of IL-8 mRNA production occurred in a repeatable fashion between individual pigs. Mitogen stimulation was also measured across time to confirm the ideal duration of stimulation for in vitro assays.

3.3.2 Experiment 2 Materials and Methods

3.3.2.1 Whole Blood Stimulation

From the results of experiments 1a and 1b, PMA (10μg/mL), LPS (10μg/mL), and KB ($1x10^7$cfu/mL) were selected for the stimulation of pro-inflammatory cytokines in this experiment. The whole blood was treated in a similar manner to Experiment 1 except glass test tubes were used and blood was collected from 4 different pigs. As in Experiment 1a, 1.8mL was aliquoted into 16 sterile glass test-tubes. Blood from each pig was treated with PMA, LPS, KB, saline or left untreated (un-stimulated control). The IL-8 mRNA produced by the saline

stimulated and un-stimulated whole blood was considered baseline IL-8 mRNA. Once again any IL-8 mRNA produced above this level was considered up-regulation.

All samples were shaken in 5% CO_2 at $37^{\circ}C$ and 85% relative humidity. A volume of 300μL blood was removed from each test tube prior to stimulation, and 2 hours, 4 hours and 24 hours after stimulation for extraction of RNA and subsequent RT-PCR. Extraction of RNA and RT-PCR were conducted according to the methods outlined in Sections 2.4.2 and 2.4.3 respectively.

3.3.3 Experiment 2 Results

Mitogens stimulated IL-8 mRNA production to a similar level at all 3 sampling times (Figure 3.4).

The production of IL-8 mRNA by the saline and un-stimulated blood was significantly less ($p<0.05$) than that produced by the mitogens at 2 hours and 24 hours post stimulation (Figure 3.4). The 4 hour time point appeared highly variable with high standard errors confounding any trends.

PMA, LPS and KB stimulation of IL-8 mRNA in whole blood showed no significant difference between each other at all time points post stimulation (Figure 3.4). KB increased IL-8 mRNA production significantly at 24 hours compared to 4 hours (1.124 vs 0.953 P< 0.05).

There was no difference in IL-8 mRNA production between the un-stimulated blood and the saline stimulated blood. Thus, either un-stimulated blood or saline stimulated blood were suitable controls demonstrating the constitutive baseline levels of IL-8 mRNA expression in pig whole blood samples. The 24 hour time

point produced little variation between samples stimulated with mitogens (mean ±

sem) and as such, 24 hours may be considered the optimal stimulation time for in

vitro cytokine production assays in whole blood.
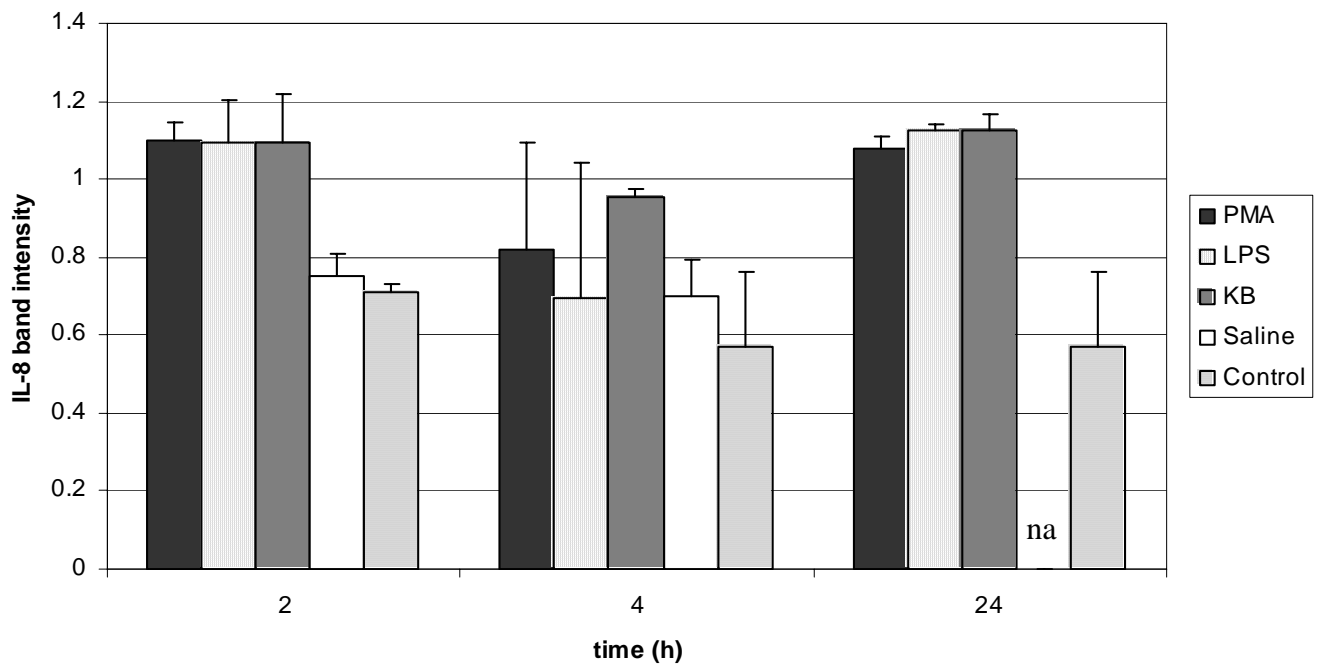
Figure 3.4: The temporal expression of mRNA for IL-8 as measured by RT-PCR, in porcine whole blood after stimulation with PMA (10μg/mL), LPS (10μg/mL), killed App (KB, $1x10^7$ cfu/mL), saline, and un-stimulated blood (control). na indicates not assessable. Bars refer to mean (n=4), and standard error.

3.3.4 Experiment 2 Summary

- Stimulation of porcine whole blood with LPS, PMA and KB resulted in similar levels of IL-8 mRNA production across individual pigs, as illustrated by low variability.

- Variability between animals producing IL-8 mRNA in response to mitogens was the lowest at 24 hours after stimulation, and this time point was thus the ideal duration of stimulation for such in vitro assays.

- These results confirm the robustness of this assay system for producing consistent results across individual blood samples.

3.4 Experiment 3.: Screening of anti-inflammatory agents on mitogenic stimulation of pro-inflammatory cytokines, to inhibit the production of these cytokines in porcine whole blood

3.4.1 Objectives

Results described in the previous 2 experiments showed the optimal concentrations of a series of mitogens and KB and the optimal stimulation time for the stimulation of pro-inflammatory cytokine production in porcine whole blood. These concentrations have been selected for use in an inhibition assay to examine the effect of putative anti-inflammatory compounds on the production of pro-inflammatory cytokines. It is envisaged that compounds which inhibit the production of pro-inflammatory cytokines in response to mitogens and KB *in vitro* may have potential as therapeutics for the treatment of inflammatory syndromes in pigs in vivo.

3.4.2 Experiment 3 Materials and Methods

PMA, LPS and KB were administered at the confirmed concentrations outlined in experiment 2. Anti-inflammatory cytokines IL-4, IL-1ra and Flunix (a non steroidal anti-inflammatory drug (NSAID)) were applied at 3 concentrations; low, medium and high in order to reduce the production of pro-inflammatory cytokines.

The low concentration of anti-inflammatory cytokines and NSAID was applied at 10μg/mL for all the substances. The medium concentration for Flunix and IL-1ra was 50μg/mL, while IL-4 was applied at 25μg/mL. The high concentration was 100μg/mL for both Flunix and IL-1ra, and 50μg/mL for IL-4.

Whole blood (1.7mL) was placed in glass test tubes, to which 100μL of stimulant and 200μL of anti-inflammatory compounds was applied to make a final volume of 1mL.

Un-stimulated whole blood was also treated with all anti-inflammatory compounds all at 50μg/mL.

Saline controls were again incorporated in order to determine the animal constitutive level of IL-8 mRNA. Tubes were then placed in 5% CO_2 and 85% relative humidity and agitated for 24 hours at 37^oC.

3.4.3 Experiment 3 Results

The un-stimulated controls and the saline control samples showed a baseline production of IL-8 mRNA at a band intensity of 0.7 indicated by the black lines in Figure 3.5. Once again the lines represent the constitutive amount of IL-8 mRNA in the whole blood and any production of IL-8 mRNA exceeding these lines was believed to have been caused by immunological stimuli. Production of IL-8

mRNA less than baseline was indicated as a percentage reduction. Flunix showed promising results in limited *in vivo* tests in pigs for reducing fever (Knowles personal communication).

The production of IL-8 mRNA stimulated by KB was suppressed 100% by a high concentration of Flunix (Figure 3.5). IL-1ra at medium concentration also reduced KB stimulated IL-8 mRNA by 100% (Figure 3.5). However, the production of IL-8 mRNA in whole blood stimulated by KB was not reduced or inhibited by the 3 concentrations of IL-4.

Flunix at all 3 concentrations (low, medium and high) did not reduce the production of IL-8 mRNA by the stimulation of PMA (Figure 3.5). The whole blood stimulated with PMA and treated with a high concentration of IL-1ra yielded no result since the house keeping gene β-actin could not be detected in this sample. Whole blood stimulated by PMA and subsequently treated with IL-4 at a low concentration reduced IL-8 mRNA production by 100% (Figure 3.5).

Whole blood stimulated by LPS and treated with Flunix at a high concentration showed a 100% reduction in IL-8 mRNA production. Unfortunately, LPS stimulation of IL-8 mRNA and treatment with IL-1ra at a low concentration did not yield any data since the house keeping gene β-actin again could not be detected. However the whole blood stimulated by LPS and subsequently treated with a low dose of IL-4 reduced IL-8 mRNA production 100% below baseline (Figure 3.5).

Saline, Flunix, and 2 anti-inflammatory cytokines (IL-1ra and IL-4) were applied to un-stimulated blood at the same concentration (Figure 3.6). The saline

stimulated blood produced IL-8 mRNA at a band intensity of 0.7 consistent with

baseline IL-8 production from Figure 3.5. Flunix and IL-1ra reduced baseline

constitutive IL-8 mRNA to zero. Conversely IL-4, up-regulated the production of

IL-8 mRNA in un-stimulated porcine whole blood (Figure 3.6).
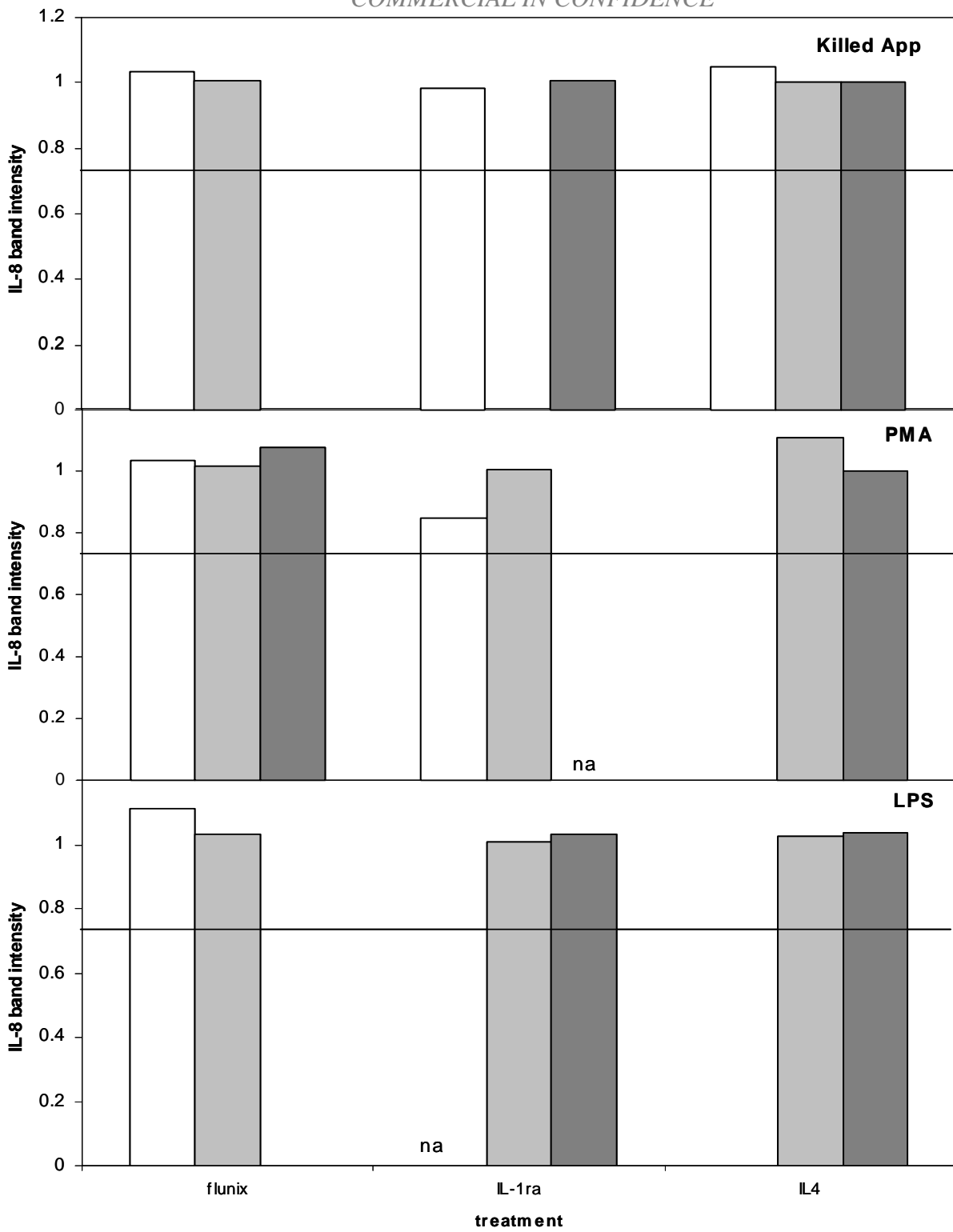
Figure 3.5: Mitogenic stimulation of IL-8 mRNA in porcine whole blood, co-incubated with Flunix, IL-4 and IL-1ra at varying concentrations. The black lines in the middle of the graphs indicate the baseline constitutive IL-8 mRNA for this particular pig.
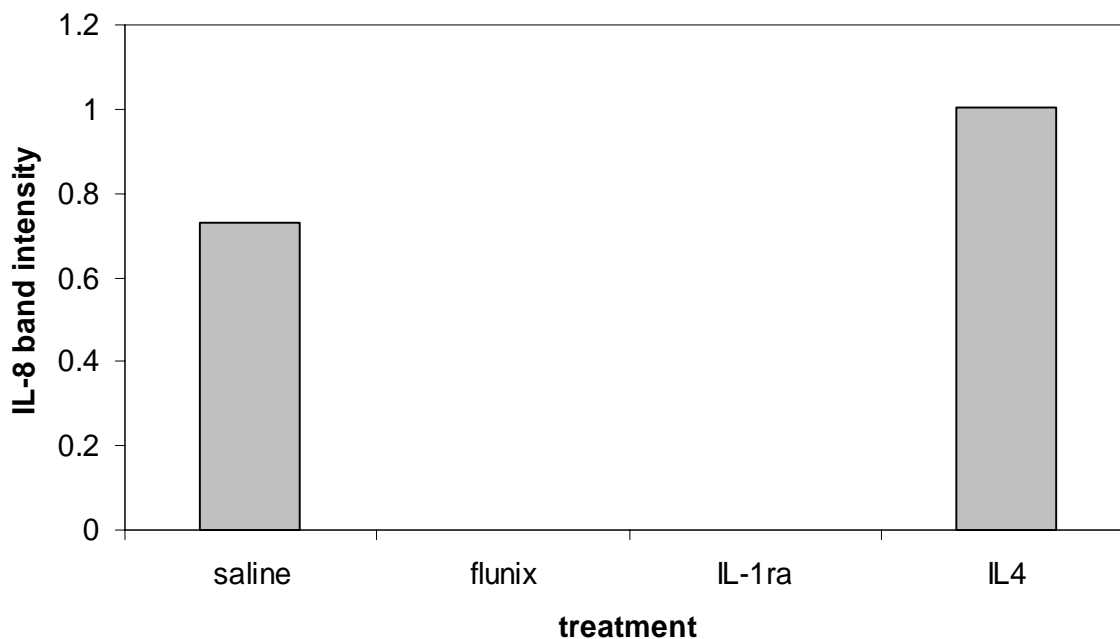
Figure 3.6: The effect of saline, Flunix (50μg/mL), IL-1ra (50μg/mL) and IL-4 (50μg/mL) on the production of IL-8 mRNA in porcine whole blood which has not been stimulated by mitogens. Bars show relative band intensity of IL-8 mRNA compared with β-actin mRNA.

3.4.4 Experiment 3 Summary

- IL-8 mRNA produced in whole blood stimulated by KB after 24 hours was inhibited by the high (100μg/mL) concentration of Flunix. This concentration of Flunix also inhibited the production of IL-8 mRNA produced by the stimulation of LPS after 24 hours.

- The stimulation of IL-8 mRNA by KB was inhibited 100% by the medium concentration (50μg/mL) of IL-1ra.

- The stimulation of IL-8 mRNA by both PMA and LPS was inhibited by the low (10μg/mL) concentration of IL-4.

- Baseline IL-8 mRNA produced by un-stimulated whole blood was 100% inhibited by Flunix and IL-1ra, while IL-4 up-regulated the production of IL-8 mRNA.

3. 5. Discussion

The main objective of the experiments described in this Chapter was to develop a method which demonstrated that the production of mRNA encoding for the pro-inflammatory cytokines could be mitogenically stimulated in whole blood. This method was developed to provide a useful tool for screening of substances which may inhibit or reduce the production of pro-inflammatory cytokines in vitro.

Larsson et al., (1999) found constitutive levels of IL-8 in epithelial cells while Strieter (1992) found constitutive levels of IL-8 produced by neutrophils, it would seem reasonable to assume that cells present in whole blood (particularly neutrophils and monocytes) also produce a constitutive level of IL-8.

In Experiment 1, five stimuli (PMA, PHA, Con A, LPS and KB) were tested to observe which stimuli delivered at which concentration would produce the greatest pro-inflammatory response. IL-8 was of most interest since it is a neutrophil chemotactic factor and is implicated in respiratory disease pathology caused by App (Baarsch, *et al.,* 1995). Baarsch *et al.,* (1995) found pro-inflammatory cytokines such as IL-1β and IL-8 were associated with pleuropneumonia and may contribute to the severity of the disease.

The high concentration of KB (Figure 3.1d) used to stimulate the production of IL-8 mRNA in porcine whole blood may have been too high and instead of

stimulating the production of IL-8 mRNA, toxins like LPS present in KB. (Idris et al., 1993 and Udeze et al., 1987) may have killed the cells. KB may also have other toxins present since App has secreted proteins and non-secreted components such as ApxI, II and III which act synergistically to cause damage to lung tissue (Huang et al., 1998). However this is mere speculation until this assay is repeated with blood from several different pigs which was done in Experiment 2.

However, assumptions made about KB toxins at high concentrations ($3x10^8$cfu/mL) damaging or busting the mononuclear cells in Experiment 1a were dispelled with the resulting production of IL-8 mRNA by KB at $>1x10^9$cfu/mL in Experiment 1b. KB in Experiment 1a stimulated the greatest production of IL-8 mRNA at $3x10^5$cfu/mL at 2 hours with a resulting band intensity of 1.5. In Experiment 1b an IL-8 mRNA band intensity of 1.4 was produced by the stimulation of KB at $2x10^9$cfu/mL (Figure 3.2d). Therefore the best concentration of KB for the stimulation of IL-8 mRNA production is somewhere between $3x10^5$cfu/mL and $2x10^9$cfu/mL, possibly around $7.5x10^5$cfu/mL.

IL-8 mRNA was not detected when blood was stimulated with PHA at 0.1µg/mL (Figure 3.2b). β-actin (the internal standard) was not detectable. It can be assumed that the RNA extraction failed or the RNA degraded before synthesis to cDNA.

The IL-8 mRNA levels (0.7 band intensity) produced by the saline treated blood was much higher than that produced when the whole blood in Experiment 1a was treated with saline (zero band intensity), which showed individual differences in the pigs constitutive levels of pro-inflammatory cytokines present in peripheral blood.

From the 5 stimuli (4 mitogens and KB) applied to whole pigs' blood, PMA, LPS and KB produced the most IL-8 mRNA in Experiment 1b.

Whole blood was stimulated by LPS and PMA in Experiment 1b to produce other pro-inflammatory cytokines of interest such as IL-1 and TNF-α above baseline levels. LPS has, in other studies, stimulated the production of IL-1 and TNF-α, in white cells, epithelial and endothelial cells (reviewed by Surendran 1999). Unlike Experiment 1a, only the 2 hour time point was sampled due to clotting of the 4 hour and 24 hour samples. Clotting was reduced by agitating the tubes and also by switching from plastic tubes to glass which was implemented in Experiment 2.

PMA in Experiment 1a produced a similar IL-8 mRNA profile to Con A, while PMA, Con A and PHA showed similar stimulation of IL-8 mRNA profiles in experiment 1b.

Of these 3 mitogens, PMA was selected for future use based on its consistency in stimulation of IL-8, TNF-α and IL-1β

LPS, PMA and KB all stimulated the production of IL-8 mRNA in whole blood to a similar level (Figure 3.2) and this stimulation was consistent across time (Figure 3.4).

Unstimulated blood and saline stimulated blood produced a constitutive level of IL-8 mRNA production which was significantly less than that produced by porcine whole blood stimulated with mitogens. Variation in production of IL-8 mRNA by individual animals in response to mitogens was minimal in Experiment 3, suggesting the results seen with the animals' blood in Experiments 1 and 2 are repeatable.

In Experiment 3 the pro-inflammatory cytokine IL-8 mRNA was up-regulated by IL-4 in un-stimulated whole blood. This up-regulation could have been due to IL-4 which enhances or activates in some cases the Th 1 cytokine responses (Nuntaprasert et al., 2005). Nuntaprasert et al., 2005 also found that LPS and IL-4 given at the same time to alveolar macrophages suppressed the secretion of IL-8, TNF-α, IL-6 and IL-1α however when IL-4 was given before LPS challenge in an in vivo system (pigs) it increased severity of respiratory failure and endotoxic shock.

The ability of IL-4 to inhibit or reduce IL-8 mRNA in LPS stimulated whole blood seen in Experiment 3 is supported by the results of Zhou et al., (1994). He found that IL-8 mRNA induced by LPS could be suppressed by IL-4 in vitro in pig alveolar macrophages.

IL-4 performed the best in the in vitro Experiment 3 and reduced the production of IL-8 mRNA in pig's whole blood stimulated by LPS or PMA (Figure 3.5). IL-1ra also performed well in the in vitro Experiment 3 and reduced KB stimulated IL-8 mRNA production to zero (Figure 3.5).

It was hoped that the influx of neutrophils and inflammatory cells found by other authors in App infection could be reduced or halted by delivery of IL-4 in vivo. As outlined in the data from the experiments in this Chapter IL-4 successfully reduced IL-8 mRNA production in LPS stimulated porcine whole blood at a transcriptional level, IL-8 recruits and activates cells such as neutrophils to inflammatory sites (reviewed by Standiford et al., 2000; Murtaugh et al., 1996). Furthermore, neutrophils, macrophages and platelets make up the lesions found in the respiratory tract of pigs suffering App infection (Liggett, et al., 1987).

LPS is known to stimulate the production of IL-1β, TNF-α, IL-8 and IFNδ in white cells and epithelial cells (reviewed by Surendran et al., 1999, and by Murtaugh et al., 1996). In this Chapter the production of IL-1β and TNF-α mRNA in whole blood by the stimulation of LPS established similar results in whole blood. It was observed that IL-1ra suppressed IL-8 mRNA production by KB (Figure 3.5). Chen et al., (1998) also found that IL-1ra inhibited the production of IL-8 by IL-1α stimulation in an in vitro system (squamous cell carcinoma human cell line).

IL-1ra inhibits IL-Iβ and α by binding to receptor sites of IL-1 thereby stopping signal transduction (Arend et al., 1993). IL-1β and IL-8 are associated with the severity of pleuropneumonia (Baarsch et al., 1995) and IL-1β and α also stimulate the production of IL-8 by macrophages (Tizard, 1996), thus IL-1ra may be able to reduce not only IL-1β, but other over produced pro-inflammatory cytokines such as IL-8.

These in vitro experiments have given an insight into the production of pro-inflammatory cytokines in response to immunological stimuli, and to what extent anti-inflammatory cytokines can inhibit the production of pro-inflammatory cytokines. A whole blood model has been established to test the inhibitory properties of potential anti-inflammatory substances. These selected substances have potential as treatments for future in vivo experiments to reduce the impact of pro-inflammatory cytokines which result from infections with bacterial pathogens such as App. The illness and weight loss experienced by pigs in response to App challenge may also be reduced or inhibited by delivery of anti-inflammatory substance such as IL-1ra, IL-4 and Flunix. Chapter 4 executes these investigations.

COMMERCIAL IN CONFIDENCE

CHAPTER 4

PROPHYLACTIC DELIVERY OF ANTI-INFLAMMATORY AGENTS FOR THE PREVENTION OF SYMPTOMS CAUSED BY INFLAMMATORY EFFECTORS IN PIGS

4.1 Introduction

Pro-inflammatory cytokines such as IL-8, IL-6, IL-1 and TNF-α have been implicated in the poor growth rate (Morrison *et al.,* 2000 and Baarsch *et al.,* 1995) of livestock under commercial conditions. In Chapter 3, I performed *in vitro* experiments which revealed that, a reduction or inhibition at a transcriptional level has been demonstrated by anti-inflammatory compounds such as Flunix (non-steroidal anti-inflammatory drug), IL-4 (anti-inflammatory cytokine) and IL-1ra (Interleukin 1 receptor antagonist). The experiment in this Chapter was performed to demonstrate whether the illness and weight loss experienced by pigs infected with App may be reduced or inhibited by the prophylactic delivery of these anti-inflammatory substances. Two challenge models were attempted; an acute challenge model with LPS, followed by a chronic infection with App.

Flunix is used therapeutically in horses to reduce and control fever due to bacterial infections. Flunix is a cyclo-oxygenase inhibitor, potent antipyretic and anti-inflammatory (MIMS 1988, IVS annual). It inhibits the production of prostaglandins and other chemicals which stimulate inflammatory responses in the body.

IL-4 was selected as it performed the best in the *in vitro* experiment of Chapter 3 by reducing the production of IL-8 mRNA. IL-4 successfully reduced LPS stimulated IL-8 mRNA production in porcine whole blood at a transcriptional level (Chapter 3 and Zhou *et al.,* 1994). IL-8 recruits and activates cells such as neutrophils to

inflammatory sites (reviewed by Standiford *et al.,* 2000 and reviewed by Murtaugh *et al.,* 1996). It has been found that lesions in the respiratory tract of pigs suffering the App infection consist of neutrophils, macrophages and platelet activation (Bertram 1986, Bertram 1985, Liggett *et al.,* 1987). Given the activity of IL-4 against IL-8 *in vitro*, it was hoped that the influx of neutrophils and inflammatory cells could be reduced or halted by IL-4 *in vivo*.

IL-1ra was used since it too performed well in the *in vitro* experiment of Chapter 3 and reduced KB stimulated IL-8 mRNA production to zero. LPS is known to stimulate the production of IL-1β, TNF-α, IL-8 and IFN-δ in white cells and epithelial cells (reviewed by Surendran *et al.,* 1999, and Murtaugh *et al.,* 1996). The production of IL-1β and TNF-α mRNA in whole blood by the stimulation of LPS in Chapter 3 showed the same trends seen in other studies reviewed by Surendran *et al*., 1999. Arend *et al.,* (1993) found IL-1ra to inhibit IL-1β and α by binding to receptor sites of IL-1 and stopping signal transduction. Since IL-1β is associated with the severity pleuropneumonia (Baarsch *et al.,* 1995), and IL-1β and α also stimulate the production of IL-8 by macrophages (Tizard 1996). Therefore IL-1ra may be able to reduce not only IL-1β, but other over produced pro-inflammatory cytokines such as IL-8. Chen *et al.,* 1998 also found IL-1ra can directly influence the production of IL-8 since IL-1ra inhibited IL-8 in an *in vitro* system (squamous cell carcinoma human cell line).

The aim of this Chapter was to examine whether compounds with known anti-inflammatory activity could reduce the severity of App and the deleterious effects of this infection on growth. In addition investigation into whether deliveries of recombinant cytokines or plasmid cytokines were effective was examined.

4.2 Experimental Protocol LPS Challenge

Male pigs at an average weight of 52kg were weighed and were randomly allocated into 7 treatment groups as described in table 4.1.

Table 4.1: Summary of treatment groups and of treatment schedule in relation to LPS challenge

Treatment groups	Dose rate	Treatment applied (days before challenge)
Saline	50μL/kg	-1 days
Plasmid control	2μg/kg	-7 days
Flunix	2.2mg/kg	-1 day
Recombinant IL-4	2μg/kg	-1 days
Recombinant IL-1 ra	2μg/kg	-1 days
Plasmid IL-4	2μg/kg	-7 days
Plasmid IL-1 ra	2μg/kg	-7 days

Each pen contained 9 pigs, one pig from each treatment group plus 2 spaces and the pen was repeated in 4 replicates. The plasmid groups were given the corresponding plasmid 7 days before LPS challenge. The recombinant cytokine groups received the corresponding cytokine 1 day before LPS challenge.

Plasmids were given intra-muscularly in the upper hind leg while recombinants and saline were given behind the ear subcutaneously. Flunix was administered as a 2mL dose (2.2mg/kg) and according to the manufacturer's instructions. Recombinants and plasmids were provided by CSIRO laboratories in Geelong by Dr. David Strom.

The weights of the animals were recorded at allocation, 1 day before challenge, 6 days post challenge and finally at the conclusion of the LPS challenge. The pigs were challenged with 5μg/kg LPS intravenously. Animals were restrained using a nasal

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snare, and whole blood was collected by venipuncture from the jugular vein as described in Section 2.2. Blood was collected before LPS challenge and 1 day after LPS challenge. Pigs were observed after each challenge and throughout the duration of the experiment to obtain clinical scores and to check that the animals were not in distress.

4.2.1 Assays performed

4.2.1.1 Immunoassays

Lymphocyte stimulation, neutrophil function, T lymphocytes and B lymphocyte stimulation, TNF-α bioassays, differential and white blood cell (WBC) counts were performed according to the methods outlined in Chapter 2.

In order to show a complete set of cluster designation (CD) receptor data, obtained from the T lymphocyte assay, data was supplied as both percentage and cells/mL (Table 4.4, 4.6-4.7). Due to unforeseen circumstances (machine error) the WBC (Table 4.8) for the App challenge model at 24 hours was not obtained, therefore cells/mL for this sample time was not calculated. The missing data is displayed by na which indicates not assessable.

4.2.1.2 Measurement of Pro-inflammatory Cytokines and CRP by RT-PCR

RNA was extracted from whole blood samples as outlined previously in Section 2.4.1.

RT-PCR was performed as described in Sections 2.4.2 and 2.4.3, using paired primers for (IL-8, IL-6, IL-1β, TNF-α and C-reactive protein (CRP)). Minor modifications to the methods included IL-8 and TNF-α PCR increased cDNA template changes to 3μl and the remainder IL-6, IL-1β and CRP to 2μl.

Additives used with some primers are documented in Appendix 3. Data from all assays were analysed using ANOVA.

4.3 Results of LPS Challenge Model

4.3.1 Growth, in response to LPS Challenge

The 3 week LPS challenge was not stringent enough to show a difference between administrated treatments and controls in terms of weight gain (Figure 4.1).
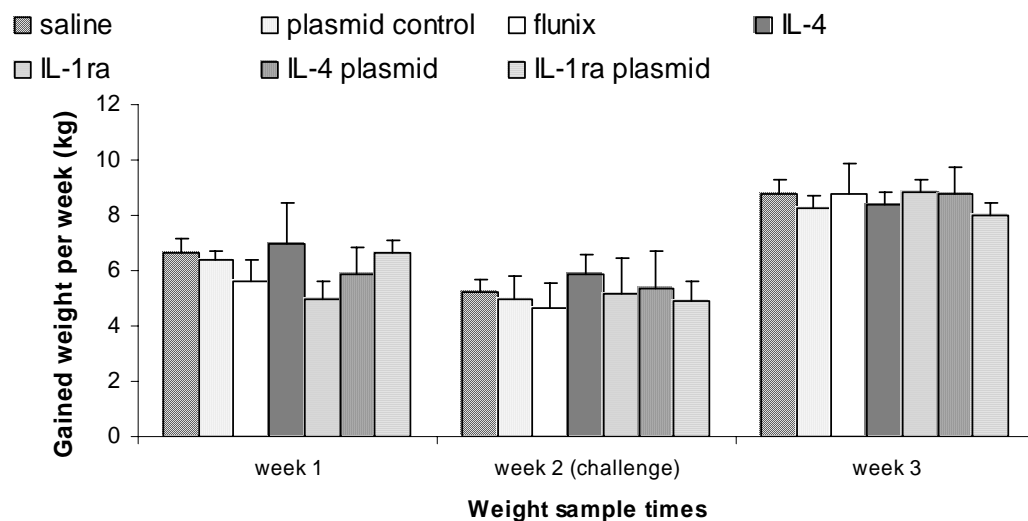
Figure 4.1: Average weight (kg) gain in pigs at one week prior to LPS challenge (week 1), the week of challenge (week 2) and one week after challenge. Pigs were treated with saline, Flunix, recombinant cytokines, plasmid cytokines prior to LPS challenge. Bars represent mean±SEM, n=5.

Weight gain was not changed in response to LPS challenge. All groups showed a similar level of weight gain the week after challenge (week 3).

4.3.2 Immunological and Molecular Techniques LPS

Table 4.2: Results from immunoassays on blood from pigs before and after challenge with LPS. Pigs were treated with saline, Flunix, recombinant cytokines or plasmid anti-inflammatory cytokines and subsequently challenged with LPS. Data represented as mean±SEM, n=5.

Assay	saline	Plasmid control	Flunix	IL-4	IL-1ra	IL-4 plasmid	IL-1ra plasmid
PHA Lymphocyte Proliferation Pre LPS % stimulated cells	99.02+/-49.72	124.80+/-47.39	98.33+/-14.03	30.34+/-11.09	46.18+/-24.45	72.82+/-45.10	110.71+/-30.10
Post LPS % stimulated cells	156.28 +/- 29.73	157.41 +/- 36.87	143.43 +/- 25.37^	311.34 +/- 99.93*#	173.80 +/- 74.21+	76.09 +/- 5.77	140.87 +/- 17.34
Con A Lymphocyte proliferation Pre LPS % stimulated cells	185.37 +/- 89.46	204.12 +/- 72.44	146.88 +/- 40.99	84.93 +/- 8.88	119.56 +/- 25.36	133.86 +/- 63.67	161.34 +/- 38.90
Post LPS % stimulated cells	345.63 +/- 51.13	544.93 +/- 287.13	259.88 +/- 54.13	492.90 +/- 110.56	387.27 +/- 103.53	198.62 +/- 66.89	149.90+/-26.36
Phagocytosing neutrophils Pre LPS % phagocytosis	18.43+/-2.06	18.99+/-0.55	28.56+/-2.36	19.48+/-1.43	25.58+/-2.77	18.74+/-3.34	19.18+/-1.68
Post LPS % phagocytosis	44.60+/-2.70^	48.32+/-4.26&	37.35+/-3.90	28.92+/-0.89	52.07+/-3.47	36.59+/-3.85	33.1+/-3.56
B lymphocytes Pre LPS % cells	20.42+/-3.13	22.56+/-3.42	24.64+/-3.07	22.12+/-1.98	18.83+/-2.85	26.53+/-2.97	26.81+/-4.15
Post LPS % cells	11.37+/-3.74	11.85+/-3.22	7.08+/-2.66	4.15+/-0.57#	7.21+/-2.08	10.13+/-0.90	13.80+/-3.19
WBC Pre LPS WBCx10^6cells/mL	24.18+/-2.04	20.10+/-1.21	23.58+/-1.40	24.32+/-2.30	21.12+/-3.52	19.26+/-1.69	22.96+/-2.24
Post LPS WBCx10^6cells/mL	21.74+/-1.57	22.72+/-1.69	23.20+/-2.19	23.46+/-2.58	20.78+/-2.27	18.78+/-1.34	20.28+/-0.88

+ IL-1ra pre LPS vs IL-1ra post LPS p<0.05, * IL-4 pre LPS vs IL-4 post LPS p<0.05, ^ saline vs IL-4, Flunix p<0.05, & plasmid control vs IL-4 plasmid and IL-1ra plasmid p<0.05, # IL-4 vs saline post LPS p<0.05.

The pigs' whole blood from the LPS challenge model showed no difference in lymphocyte proliferation after Con A stimulation between the treated pigs or between sample times. However the whole blood from the LPS challenge model stimulated with PHA showed that pigs treated with IL-1ra produced significantly greater (p<0.05) lymphocyte proliferation after LPS challenge as compared to the

proliferation produced by the same treatment group pre LPS challenge (Table 4.2). The whole blood from the IL-4 treated pigs at post LPS challenge, after stimulation with PHA had 10 fold greater lymphocyte proliferation than that of the same pigs prior to challenge, and the saline control-treated pigs ($p<0.05$).

Neutrophil phagocytic capacity generally increased for all treatments after challenge with LPS (Table 4.2). Compared to saline controls IL-4 and Flunix treated pigs had significantly ($p<0.05$) lower phagocytic capacity of neutrophils after LPS challenge. IL-4 plasmid and IL-1ra plasmid treated pigs had significantly less ($p<0.05$) phagocytic capacity of neutrophils after LPS challenge as compared to the plasmid control.

The immunoglobulin Ig receptor found on the cell surface of B lymphocytes was enumerated using flow cytometry. There was a significant decrease in B lymphocyte numbers in all the treatments and control group after LPS challenge (Table 4.2). After LPS challenge the pigs treated with IL-4 had significantly ($p<0.05$) less B cells than the blood from saline-treated pigs after LPS challenge (Table 4.2).

The number of total white blood cells in the pigs' whole blood was not different between treated pigs or between sampling time (Table 4.2).

Table 4.3 mRNA production of pro-inflammatory cytokines and C reactive protein was established by semi-quantitative RT-PCR. Changes in pro-inflammatory cytokines were normalised against β-actin. Serum production of pigs TNFα protein was also measured by bioassay to determine the translation, if any, of mRNA present as protein. bnd int = band intensity. Data represented as mean±SEM, n=5.

Assay	saline	Plasmid control	Flunix	IL-4	IL-1ra	IL-4 plasmid	IL-1ra plasmid
IL-6 mRNA bnd int Pre LPS	0+/-0	0+/-0	0+/-0	0+/-0	0+/-0	0+/-0	0.24+/-0.15@
Post LPS	0.29+/-0.18	0.12+/-0.12	0.07+/-0.07	0.38+/-0.16*	0+/-0	0+/-0	0+/-0
IL-8 mRNA bnd int Pre LPS	0+/-0	0+/-0	0+/-0	0+/-0	0+/-0	0+/-0	0.12+/-0.12
Post LPS	0.26+/-0.16	0.50+/-0.20	0.53+/-0.21	0.33+/-0.20	0.44+/-0.23	0.36+/-0.22	0.72+/-0.18
IL-1β mRNA bnd int Pre LPS	0+/-0	0.79+/-0.20	0.25+/-0.22	0.71+/-0.21	1.03+/-0.03	0.67+/-0.20	0.59+/-0.24
Post LPS	0.93+/-0.03	0.60+/-0.24	0.81+/-0.21	0.77+/-0.19	0.39+/-0.24+	0.49+/-0.25	0.37+/-0.23
CRP mRNA bnd int Pre LPS	0+/-0	0.70+/-0.18	0+/-0	0.46+/-0.19	0.50+/-0.19	0.49+/-0.25	0.86+/-0.07
Post LPS	0.67+/-0.10^	0.52+/-0.22	1.03+/-0.08	0.80+/-0.20	0.12+/-0.12	0.24+/-0.15	0.18+/-0.18
TNF-α mRNA bnd int Pre LPS	0.42+/-0.25	0.70+/-0.17	0+/-0	0+/-0	0.24+/-0.21	0.81+/-0.06	0.43+/-0.17
Post LPS	0.89+/-0.03	0.80+/-0.037	0.94+/-0.01	0.55+/-0.22#	0.59+/-0.24	0.87+/-0.03	0.64+/-0.06
TNF protein Pre LPS pg/mL	0+/-0	0+/-0	0+/-0	0+/-0	0+/-0	0+/-0	0+/-0
Post LPS pg/mL	14.06+/-4.50	9.69+/-4.76	14.10+/-2.79	16.77+/-5.17	14.77+/-4.53	35.22+/-8.51	43.78+/-10.01

* IL-4 pre LPS vs IL-4 post LPS p<0.05, + IL-1ra pre LPS vs IL-1ra post LPS p<0.05, ^ saline vs IL-1ra p<0.05, # saline vs IL-4 p<0.05, @ IL-1ra plasmid pre LPS vs IL-1ra plasmid post LPS p<0.05.

The pigs treated with IL-1ra plasmid showed decreased in IL-6 mRNA production to zero after the LPS challenge. The remaining treated pigs showed increased IL-6 mRNA after LPS challenge with the exception of recombinant IL-1ra and IL-4 plasmid treated pigs, which remained at an undetectable level (Table 4.3).

IL-8 mRNA production was significantly increased (p<0.05) after LPS challenge in all groups (Table 4.3). The pigs treated with IL-4 showed the least IL-8 mRNA production as compared to all the treatments except for the saline-treated pigs after LPS challenge (not significant) (Table 4.3).

Pigs treated with IL-1ra had significantly decreased (p<0.05) IL-1β mRNA levels after LPS challenge as compared to levels before challenge (Table 4.3).

After LPS challenge saline and Flunix treated pigs showed increased C-reactive protein (CRP) mRNA from an undetectable level prior to challenge to a detectable level (Table 4.3). The production of CRP mRNA in pigs treated with IL-4 plasmid, IL-1ra and IL-1ra plasmid was significantly less (p<0.05) than that of saline-treated pigs after challenge (Table 4.3).

Pigs treated with IL-4 showed the least production of TNF-α mRNA (p<0.05) in whole blood after LPS challenge as compared to the pigs treated with saline (Table 4.3). After challenge Flunix and IL-4 treated pigs showed increased TNF-α mRNA from an undetectable level prior to LPS challenge (Table 4.3) to a detectable level.

Protein production of TNF-α was undetectable in all treated pigs prior to LPS challenge however, it significantly increased (p<0.05) in the IL-4 and IL-1ra plasmid treated pigs (Table 4.3).

Table:4.4 Cluster designation (CD) lymphocyte data obtained in whole blood from pigs treated with saline, Flunix, recombinants or plasmid anti-inflammatory cytokines before and after pigs were stimulated by an LPS challenge. Data presented as mean±SEM, n=5.

Assay	saline	Plasmid control	Flunix	IL-4	IL-1ra	IL-4 plasmid	IL-1ra plasmid
CD 3 Pre LPS $x10^6$ cells/mL	6.88+/-1.09	6.74+/-0.46	6.99+/-0.86	4.60+/-0.50	6.46+/-0.73	6.14+/-1.40	5.33+/-1.60
Post LPS $x10^6$ cells/mL	7.80+/-1.49	7.75+/-0.74	11.10+/-2.08	7.23+/-2.67	9.28+/-1.02	5.33+/-0.57	9.03+/-1.88
CD 4 Pre LPS $x10^6$ cells/mL	2.40+/-0.48	2.39+/-0.16	2.78+/-0.22	2.13+/-0.15	2.74+/-0.34	2.60+/-0.49	2.10+/-0.55
Post LPS $x10^6$ cells/mL	1.86+/-0.23	1.38+/-0.34	2.24+/-0.48	4.24+/-1.89[#]	2.07+/-0.27	1.68+/-0.27	1.60+/-0.12
CD 8 Pre LPS $x10^6$ cells/mL	3.31+/-0.66	3.38+/-0.22	4.10+/-0.40	2.87+/-0.14	3.50+/-0.27	3.55+/-0.72	3.54+/-1.02
Post LPS $x10^6$ cells/mL	2.41+/-0.28	2.22+/-0.43	3.20+/-0.59	2.09+/-0.23	3.12+/-0.78	1.87+/-0.39	2.45+/-0.58
CD 4:8 Pre LPS % cells	0.77+/-0.14	0.72+/-0.07	0.70+/-0.07	0.75+/-0.06	0.78+/-0.07	0.77+/-0.07	0.64+/-0.08
Post LPS % cells	0.82+/-0.14	0.62+/-0.16	0.72+/-0.09	1.85+/-0.73[#]	0.75+/-0.09	0.92+/-0.05	0.83+/-0.24

saline vs IL-4 p<0.05.

CD3 is the T cell receptor (TCR) on the surface of all T lymphocytes, therefore antibodies against CD3 can help quantify the number of total T cells present in a sample. The blood from pigs that were treated with Flunix, IL-1ra or IL-1ra plasmid, showed increased T cells numbers after LPS challenge (p<0.05) (Table 4.4).

T cells can be separated into two populations; those with T cell receptors 1 (TCR1) and those with T cell receptors 2 (TCR2). T cells with TCR2 make up 95% of T cell population (Roitt, 1994). T cells with TCR2 receptors can be further separated into T cells with a CD4 marker or T cells with a CD8 marker.

T cells with a CD4 marker are T helper cells, which recognise antigens in association with major histocompatibity complex class II (MHC II) and T cells with a CD8 marker are cytotoxic or suppressor cells, which recognise antigens in association with

MHC I (Roitt, 1994). T cells with the CD4 receptors can be further split into T helper 1 and T helper 2 cells (Roitt, 1994). Pigs treated with IL-4 showed significant increased ($p<0.05$) CD4 marked cells after the LPS challenge compared with the number of CD4 cells the saline-treated pigs showed (Table 4.4).

There was no difference in CD8 T cells produced by all treatments after LPS challenge (Table 4.4).

When the number of cells with the CD4 receptor/marker are divided by the number of cells with the CD8 receptors/marker to produce a ratio, the resulting value can be a guide to the kind of immune response produced. For example, it is known that viral infections can result in a high CD4:CD8 (Roitt 1994) (Table 4.4).

There was an increase in the CD4:CD8 ratio after LPS challenge in the pigs treated with IL-4, and this was significantly higher ($p<0.05$) than the ratio produced by the pigs treated with saline (Table 4.4).

4.4 LPS acute Challenge Model Summary

- Growth of all the treated pigs, was not impeded by the LPS challenge

- mRNA of pro-inflammatory cytokines mainly increased at post LPS however there was little distinction between the saline treated pigs and pigs treated with anti-inflammatory compounds.

- Lymphocyte proliferation in whole blood from saline treated pigs showed variation compared to the pigs treated with IL-4 at post LPS challenge.

- The inability of this model to induce inappetance and weight loss made it difficult to drawn conclusions.

4.5 App Challenge Model

4.5.1 Experimental Protocol App Challenge

Table 4.5: Summary of treatment groups and of treatment schedule in relation to App challenge.

Treatment groups	Dose rate	Treatment applied (days before challenge)
Saline	50μL/kg	-2days and challenge day
Plasmid control	2μg/kg	-10days
Flunix	2mg/kg	-2days and challenge day
recombinant IL-4	2μg/kg	-2days and challenge day
recombinant IL-1 ra	2μg/kg	-2days and challenge day
Plasmid IL-4	2μg/kg	-10days
Plasmid IL-1 ra	2μg/kg	-10days

This challenge was considered a chronic challenge since infection was of 2 weeks duration (after previous 3 week acute LPS challenge). The App trial commenced when the plasmids were given to corresponding plasmid groups, 10 days prior to App challenge (end of week 2 of LPS challenge). Pigs remained in their allocated pens from LPS challenge. Plasmid groups were given the corresponding plasmid 10 days before App challenge. The recombinant cytokine groups received the corresponding cytokine 2 day before App challenge and at challenge. Weight of the animals was recorded at 2 days before App challenge. The pigs weights were recorded again at 7 days post App challenge and just before euthanasia at 13 days post App challenge. 7.5×10^5 cfu/mL of App intra-tracheal was administered at App challenge (Refer to Section 2.3 Infection Protocol). Pigs were observed after App challenge and throughout the duration of the experiment to obtain clinical scores and to check that the animals were not in distress. Blood was taken 30 minutes before App challenge, 1 day after App challenge and the day before the animals were sacrificed. Animals were restrained using a nasal snare, and blood collected by venipuncture from the

jugular vein as per Section 2.2. Delivery of both plasmids and cytokines were given in the same manner as the LPS Challenge (Section 4.2). At 14 days the animals were euthanased by first sedating them with an overdose of barbiturates and then exsanguination.

4.5.2 Assays performed

As performed in Section 4.2.1.

4.6 Results of App Challenge Model

4.6.1 Growth, Production and Clinical Signs of App Challenge Pig Model

As previously stated in the App challenge experimental protocol, weight of the pigs were taken 2 days prior to the App challenge, 7 days after challenge and just before euthanasia.   Weight gain for week 1 was the weight gained in the week of challenge and week 2 was the weight gained after App challenge.   The growth of the IL-4 treated pigs was significantly greater (P<0.05) than that of the saline treated pigs in the week during the challenge (Figure 4.1).   The remaining pigs treated with recombinant cytokines and plasmid delivered cytokines also increased in growth during this week (Figure 4.1). Pigs treated with saline, plasmid control and Flunix decreased in weight during the challenge week.

At the end of the trial the, pigs treated with recombinant IL-4 and IL-1ra gained the most weight (Figure 4.2b) compared to the other treatment groups and control.
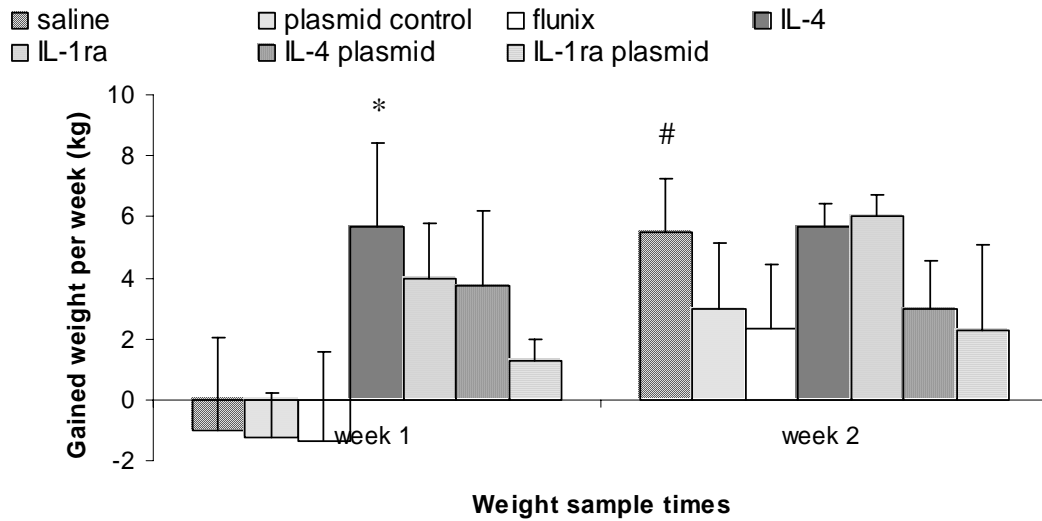
Figure 4.2: Average weight (kg) gain in pigs at week 1 and 2 post App challenge. Pigs were treated with saline, Flunix, recombinant cytokines or plasmid cytokines during chronic challenge with App. * IL-4 vs saline $p<0.05$, # saline at week 1 vs saline week 2. Bars represent mean±SEM, n=5.

Weight gain after 2 week showed no significant difference between the treatment groups when compared to their controls. The pigs treated with IL-4 increased in weight after 1 week compared to the pigs treated with saline (Figure 4.2).
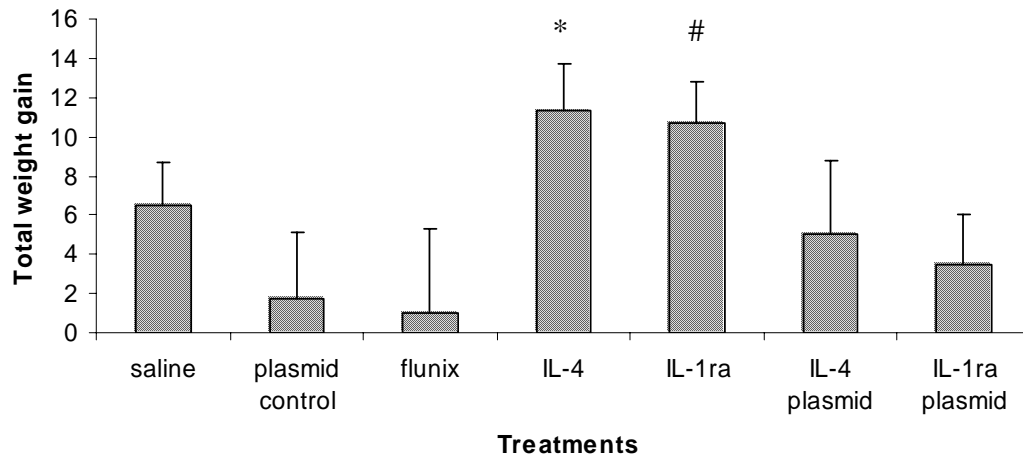
Figure 4.2b: Total weight (kg) gain at euthanasia. Pigs were treated with saline, Flunix, recombinant cytokines, plasmid cytokines prior to App challenge. * IL-4 vs saline $p<0.05$, # IL-1ra vs saline $p<0.05$,. Bars represent mean±SEM, n=5.

Prophylactic delivery of IL-4 and IL-1ra resulted in pigs which out-performed the saline controls by gaining the most weight by slaughter (Figure 4.2b). The plasmid pig groups performed the poorest overall in terms of growth.
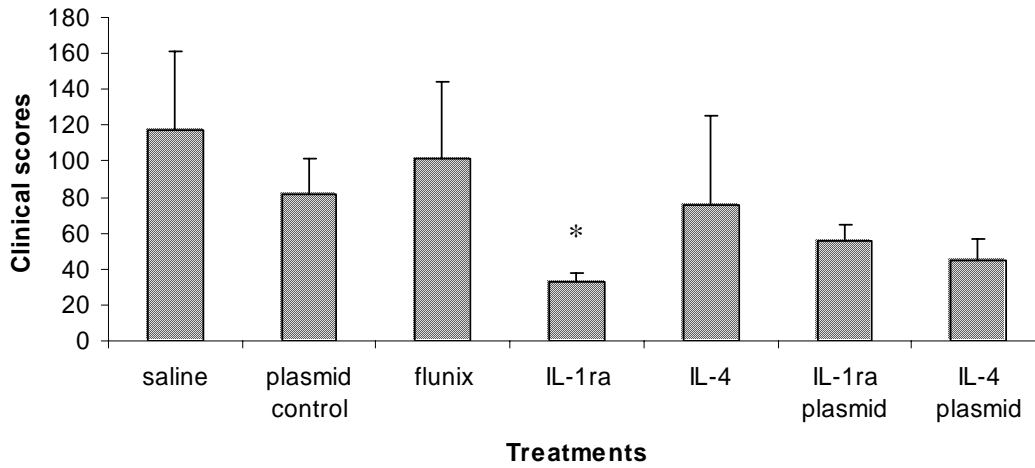
Figure 4.3: Clinical scores obtained by observation of pigs after infection with App. Clinical summary of treatment groups on a per visit basis. Total visits =30 and each pig was scored from 0-8 (maximum score 240) for each visit. A maximum of 8 indicating death of the animal.

Scoring criteria for the Clinical assessment was as follows:- 0 = no clinical signs, 1 = coughing, 2 = anorexia, 3 = fever, 4 = vomiting, 5 = increased pulse rate, 6 = blueness, 7 = cardiac and circulatory failure and 8 = death.

The IL-1ra treated pigs produced significantly less ($p < 0.05$) clinical scores when compared to the saline control (Figure 4.3).

4.6.2 Immunological and Molecular Assays
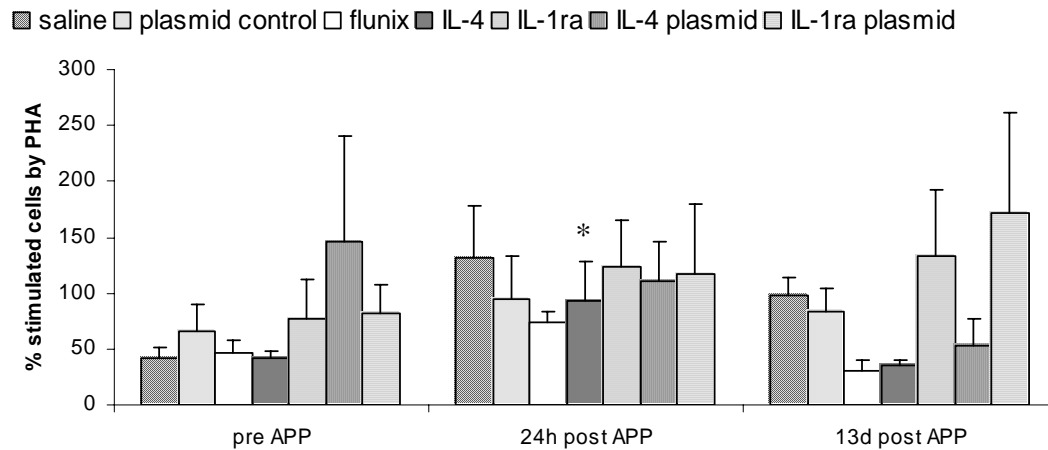
Figure 4.4: Stimulation of lymphocyte proliferation by PHA in whole blood from pigs treated with saline, Flunix, recombinant cytokines or plasmid cytokines and subsequently challenged with App. Whole blood from each pig cultured in media for 54 hours then pulsed with H^3 and read on a β counter. Bars represent mean±SEM, n=5.

The whole blood from pigs treated with IL-4 and stimulated with PHA, showed significantly (p<0.05) increased lymphocyte proliferation 24 hours after App challenge as compared to the lymphocyte proliferation in the whole blood of the same pig group recorded prior to App challenge (Figure 4.4). The whole blood from pigs treated with IL-4 and stimulated with Con A, showed decreased lymphocyte proliferation compared to blood from the saline-treated pigs 13 days after App challenge. There was no significant difference between treatments at pre App and 13 days post App which was why this data was not discussed.
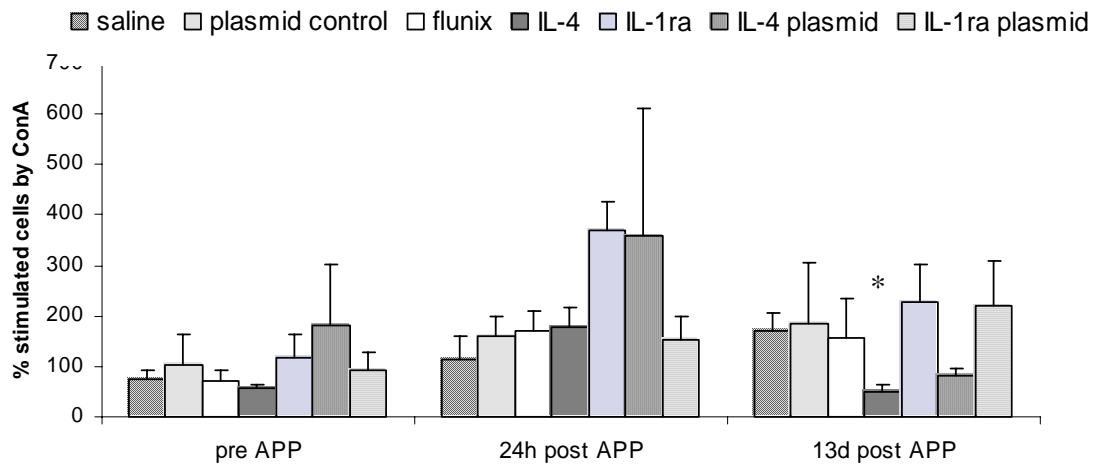
Figure 4.5: Stimulation of lymphocyte proliferation by Con A. Whole blood from pigs treated with saline, Flunix, recombinants or plasmid anti-inflammatory cytokines and exposed to App challenge. Blood cultured in media for 54 hours then Pulsed with H^3 and read on a β counter. * IL-4 at 24h post App vs IL-4 at 13d post App. Bars represent mean±SEM, n=5.

The lymphocyte proliferation in blood from IL-4 treated pigs 13 day after challenge was also reduced compared to blood taken from the same treated pig group at 24 hours after App challenge (Figure 4.5).
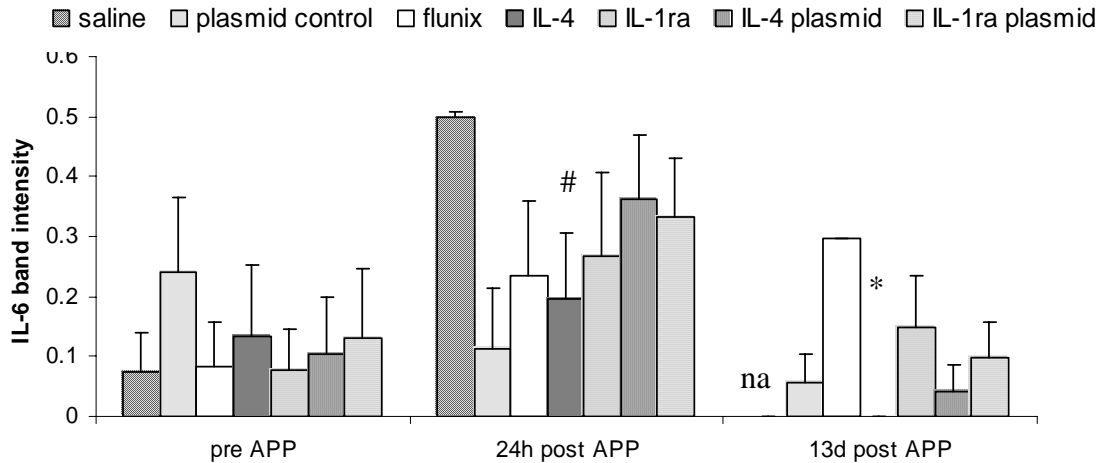
Figure 4.6: Levels of IL-6 mRNA in peripheral whole blood, measured by RT-PCR. Blood from pigs treated with saline, Flunix, recombinants or plasmid anti-inflammatory cytokines and exposed to App challenge. PCR products were migrated on an agarose gel which was photographed and quantified by densitometry. Changes in IL-6 were normalised against β-actin. na indicates not assessable. Bars show group mean±SEM, n=5. * IL-4 13d post App vs IL-4 24h post App $p<0.05$, # IL-4 24h post App vs saline 24h post App.

RNA extracted from the blood taken 13 days after challenge from pigs treated with saline was degraded and therefore it was unable to be transcribed (Figure 4.6).

The whole blood from pigs treated with IL-4 showed reduced IL-6 mRNA production 13 days after App challenge ($p<0.05$) (Figure 4.6). All treatment groups showed a trend in the reduction of IL-6 mRNA production in whole blood sampled at 13 days post App (Figure 4.6) with the exception of the Flunix treated pigs (Figure 4.6). At 24 hours after challenge all the treated pigs produced less IL-6 mRNA compared to the pigs treated with saline. Furthermore, the pigs treated with IL-4 reduced IL-6 mRNA production significantly ($p<0.05$) compared to the saline control treated pigs 24 hours after App challenge.

The whole blood sampled before App challenge showed no significant difference in IL-6 mRNA levels between treated pigs and control pigs. At the conclusion of the

trial the pig treated with IL-4 had the least (p<0.05) IL-6 mRNA production (Figure

4.6).
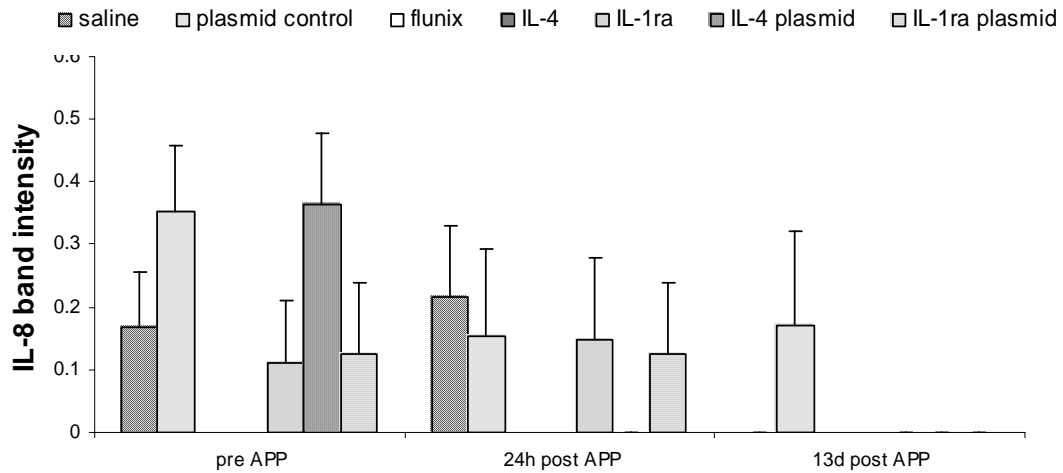
Figure 4.7: Levels of IL-8 mRNA in peripheral whole blood, measured by RT-PCR. Blood from pigs treated with saline, Flunix, recombinants or plasmid anti-inflammatory cytokines and exposed to App challenge. Products were migrated on an agarose gel which was photographed and quantified by densitometry. Changes in IL-8 were normalised against β-actin. Bars represent mean±SEM, n=5.

Whole blood from pigs treated with IL-4 and Flunix produced no IL-8 mRNA prior

to, during, or after the App challenge (Figure 4.7). The whole blood obtained from

the pigs treated with saline produced a constant level of IL-8 mRNA, however, at day

13 it reduced to an undetectable level (Figure 4.7). Levels of IL-8 were not different

between treatment groups.
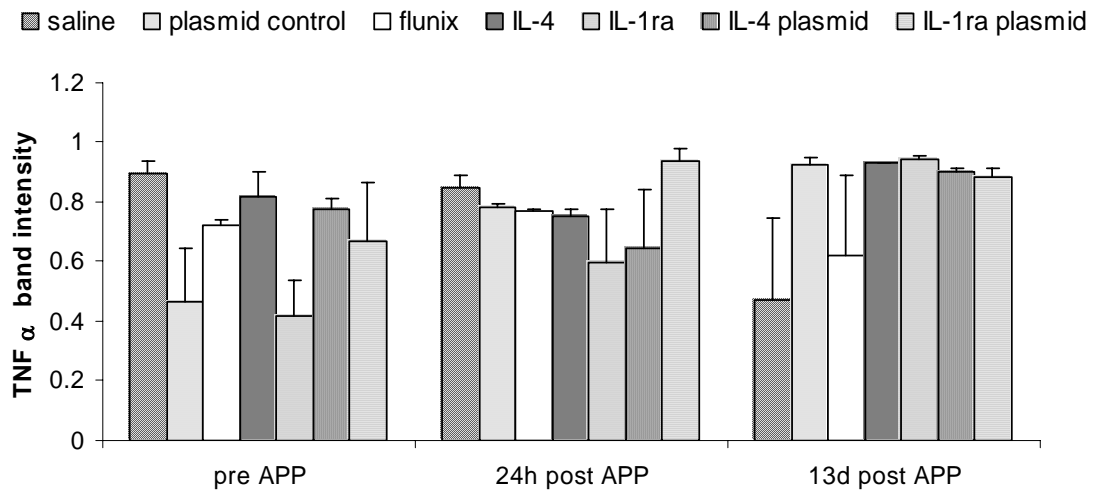
Figure 4.8 Levels of TNFα mRNA production in peripheral whole blood, measured by RT-PCR. Blood from pigs treated with saline, Flunix, recombinants or plasmid anti-inflammatory cytokines and exposed to App challenge. PCR products were migrated on an agarose gel which was photographed and quantified by densitometry. Changes in TNFα were normalised against β-actin. Bars represent mean±SEM, n=5.

The production of TNF-α mRNA in the whole blood, taken at 3 sample times from pigs infected with App and subsequently treated with anti-inflammatory compounds, showed no change between sampling time or treatment application (Figure 4.8).
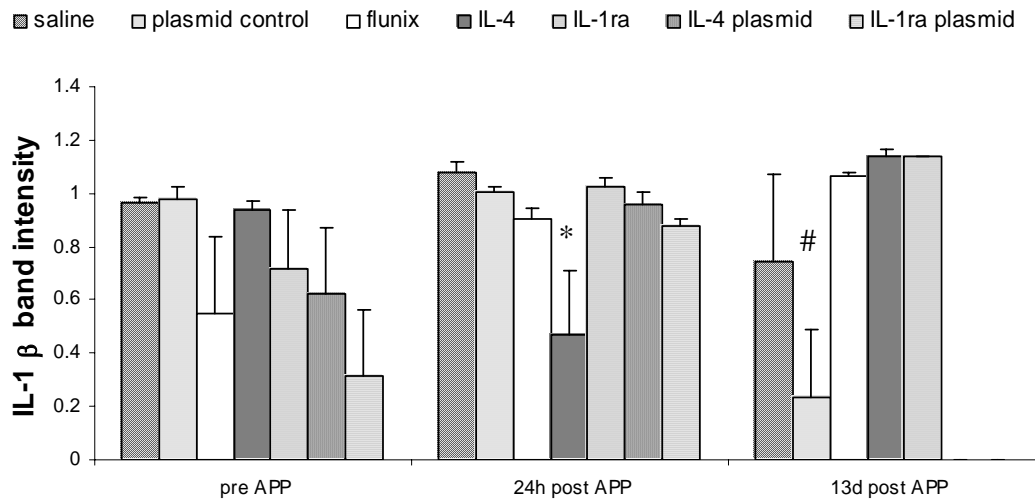
Figure 4.9: Levels of IL-1β mRNA in peripheral whole blood, measured by RT-PCR. Blood from pigs treated with saline, Flunix, recombinants or plasmid anti-inflammatory cytokines and exposed to App challenge. Products were migrated on an agarose gel which was photographed and quantified by densitometry. Changes in IL-1β were normalised against β actin. * IL-4 vs saline 24h post App, # plasmid control 13d post App vs plasmid control 24h post App. Bars represent mean±SEM, n=5.

The production of IL-1β in whole blood taken from the pigs treated with IL-4 showed a significant decline (p<0.05) at 24 hours post App challenge (Figure 4.9). This reduction was significantly lower (p<0.05) than the production of IL-1β mRNA levels in blood from saline treated pigs 24 hours after App challenge.

The pigs treated with plasmid IL-4 and IL-1ra had no detectable IL-1β mRNA in their whole blood 13 days after App challenge.

The whole blood from the pigs treated with the plasmid control (delivery vector) also showed a significant reduction (p<0.05) at 13 days post challenge as compared to the blood taken from the same group at 24 hours post App challenge (Figure 4.9).
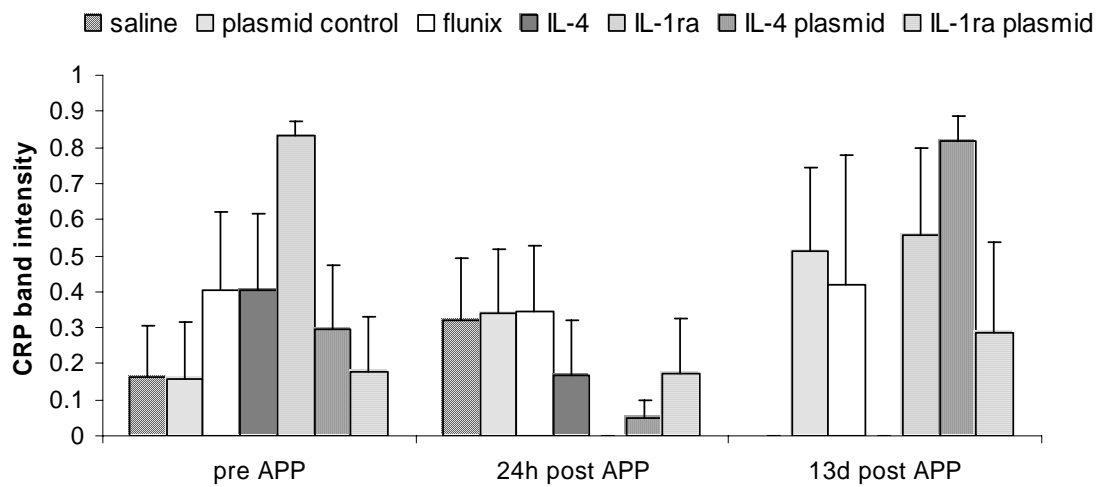
Figure 4.10 Levels of CRP mRNA in peripheral whole blood, measured by RT-PCR. Blood from pigs treated with saline, Flunix, recombinants or plasmid anti-inflammatory cytokines and exposed to App challenge. Products were migrated on an agarose gel which was photographed and quantified by densitometry. Changes in CRP were normalised against β-actin. Bars represent mean±SEM, n=5.

The blood from pigs treated with IL-4 recombinant showed a trend in decreasing in C-reactive protein mRNA (CRP) production across time to zero at euthanasia. The production of CRP mRNA in the peripheral blood of pigs treated with IL-1ra reduced to an undetectable level 24 hours after App infection.
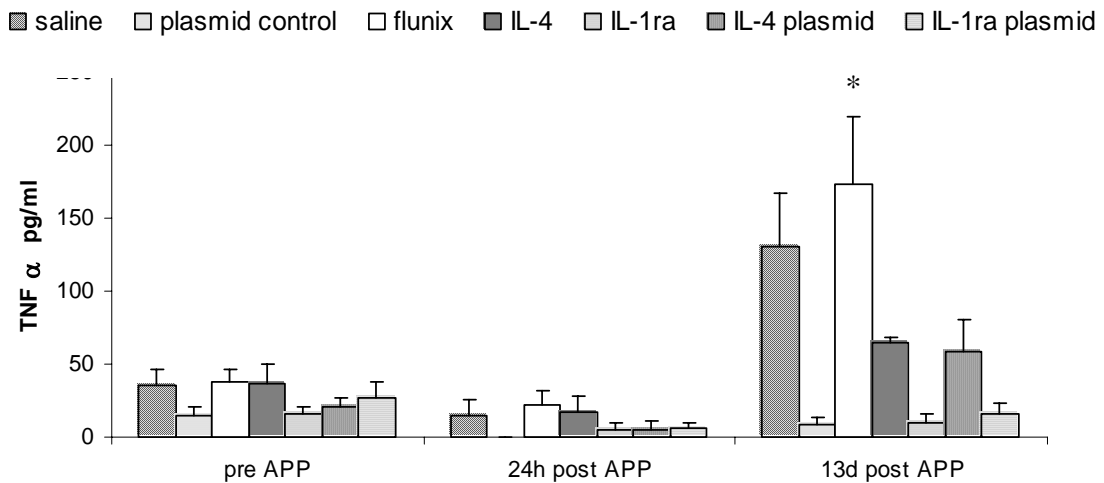
Figure 4.11: TNFα levels in serum detected by bioassay. The serum was from pigs treated with saline, Flunix, recombinants or plasmid anti-inflammatory cytokines and exposed to App challenge. Bars present the mean±SEM, n=5.
* Flunix 13d post App vs Flunix 24h post App.

The presence of TNF-α protein in the serum 13 days after App challenge was significantly higher (p<0.05) in the pigs treated with Flunix as compared to TNF-α produced by Flunix treated pigs at 24 hours post App challenge. The remaining treatments showed inhibited TNF-α production as compared to the saline treated pigs at 13 days post App.

Table:4.6: Cluster designation (CD) lymphocyte data obtained from pigs' whole blood during a 13 day challenge with App. Blood was taken from pigs treated with saline, Flunix, recombinants or plasmid anti-inflammatory cytokines. na indicates not assessable. Data is presented as the mean±SEM, n=5.

CD	saline	Plasmid control	Flunix	IL-4	IL-1ra	IL-4 plasmid	IL-1ra plasmid
CD 3 Pre App % cells	75.17+/- 1.70	71.93+/- 2.70	66.14+/- 2.41	69.16+/- 0.61	72.99+/- 1.43	70.43+/- 2.05	66.29+/- 10.23
24h Post App % cells	47.62+/- 3.45	49.45+/- 2.25	52.27+/- 6.20	54.55+/- 7.69	63.65+/- 2.81[#]	46.58+/- 7.84	58.04+/- 5.08
13d Post App % cells	67.76+/- 2.62	70.37+/- 3.40	72.72+/- 1.68	52.94+/- 17.85	71.92+/- 0.85	66.10+/- 2.38	75.84+/- 2.71
CD 3 Pre App $x10^6$ cells/mL	11.05+/- 0.60	10.50+/- 0.32	10.96+/- 1.08	10.77+/- 1.51	9.73+/- 1.03	8.55+/- 1.42	5.99+/- *
24h Post App $x10^6$ cells/mL	na	na	na	na	na	na	na
13d Post App $x10^6$ cells/mL	10.38+/- 2.94	12.47+/- 1.78	12.90+/- 1.08	10.93+/- 1.47	9.97+/- 0.54	6.96+/- 1.90	9.71+/- 2.06
CD 4 Pre App % cells	19.95+/- 2.03	19.82+/- 1.35	16.93+/- 2.17	21.13+/- 3.79	20.37+/- 1.67	23.17+/- 3.14	12.49+/- 3.55
24h Post App % cells	18.21+/- 1.51	18.41+/- 1.71	16.32+/- 1.24	20.99+/- 5.49	20.81+/- 1.65	18.83+/- 3.01	16.40+/- 2.68
13d Post App % cells	19.91+/- 1.91	18.51+/- 0.51	17.59+/- 1.55	20.81+/- 5.04	18.32+/- 1.43	20.49+/- 2.20	15.97+/- 0.94
CD 4 Pre App $x10^6$ cells/mL	2.90+/- 0.26	2.88+/- 0.13	2.72+/- 0.21	3.04+/- 0.23	2.68+/- 0.31	2.86+/- 0.76	3.18+/- *
24h Post App $x10^6$ cells/mL	na	na	na	na	na	na	na
13d Post App $x10^6$ cells/mL	2.82+/- 0.50	3.31+/- 0.55	3.14+/- 0.45	3.02+/- 0.47	2.53+/- 0.22	2.22+/- 0.70	2.03+/- 0.41

IL-1ra vs saline at 24h post App, * SEM could not be calculated due to insufficient data.

There was no significant difference in the production of T cells with the CD3 marker present in the whole blood from treated pigs collected across the 13 day challenge period (Table 4.6) (observed in percentage or cells/mL data). The CD3 T cells in the blood from pigs treated with IL-1ra remained at a high number through the 13 day

challenge. Pigs treated with IL-1ra were significantly higher (p<0.05) in CD3 T cells at 24 hours after challenge than saline treated pigs (Table 4.6).

There was no difference between the CD4 T helper cells in the whole blood of pigs' produced by the different treatments at any time during the 13 day App challenge (Table 4.6). Presenting this data as percentage cell or cells per mL produced the same observations (Table 4.6).

Table:4.7: Continuing cluster designation (CD) lymphocyte data obtained from pigs' whole blood during a 13 day challenge by App. B cell numbers were obtained using antibody specific flow cytometry to quantify Ig receptor presence. Blood from pigs treated with saline, Flunix, recombinants or plasmid anti-inflammatory cytokines. na indicates not assessable. Data is presented as the mean±SEM, n=5.

CD	saline	Plasmid control	Flunix	IL-4	IL-1ra	IL-4 plasmid	IL-1ra plasmid
CD 8 Pre App % cells	22.79+/-2.75	27.46+/-2.22	22.58+/-1.79	23.31+/-2.76	27.12+/-1.13	29.50+/-5.12	25.32+/-2.27
24h Post App % cells	23.30+/-2.72	29.39+/-1.42	20.64+/-2.03	23.25+/-4.08	25.11+/-2.72	20.90+/-5.36	23.41+/-2.90
13d Post App % cells	30.82+/-2.53	34.00+/-5.23	36.29+/-3.33 #	21.07+/-7.55	27.47+/-1.35	29.34+/-2.70	31.29+/-3.70
CD 8 x10^6 cells/mL Pre App	3.34+/-0.45	3.98+/-0.22	3.72+/-0.43	3.44+/-0.14	3.58+/-0.30	3.77+/-1.29	5.01+/-*
24h Post App x10^6 cells/mL	na	na	na	na	na	na	na
13d Post App x10^6 cells/mL	4.46+/-0.99	6.35+/-1.79	6.49+/-0.98	4.23+/-0.49	3.79+/-0.20	3.26+/-1.14	4.19+/-1.19
CD4:8 Pre App % cells	0.90+/-0.09	0.73+/-0.04	0.74+/-0.06	0.89+/-0.06	0.75+/-0.05	0.81+/-0.08	0.47+/-0.13
24h Post App % cells	0.79+/-0.03	0.62+/-0.03	0.81+/-0.10	0.86+/-0.11	0.86+/-0.04	1.04+/-0.24	0.78+/-0.24
13dPost App % cells	0.65+/-0.03	0.59+/-0.10	0.49+/-0.00	0.74+/-0.13	0.66+/-0.03	0.71+/-0.08	0.54+/-0.09
B lymphocytes Pre App % cells	13.77+/-1.56	18.46+/-3.24	17.49+/-1.93	18.64+/-4.64	16.03+/-2.22	20.95+/-1.76	18.26+/-1.54
24h Post App % cells	10.13+/-2.25	20.10+/-2.53	18.70+/-5.91	20.32+/-5.83	11.41+/-2.80	14.08+/-2.12	12.05+/-2.92
13d Post App % cells	22.28+/-6.36	23.31+/-4.78	25.68+/-3.26	21.03+/-5.02	14.12+/-3.85	23.58+/-3.86	29.68+/-1.11
B lymphocytes Pre App x10^6 cell/ml	2.00+/-0.22	2.82+/-0.77	2.82+/-0.24	2.90+/-1.04	2.05+/-0.21	2.47+/-0.33	3.15+/-*
24h Post App x10^6 cell/ml	na	na	na	na	na	na	na
13d Post App x10^6 cell/ml	2.79+/-0.05	3.92+/-0.70	4.69+/-1.10	3.15+/-0.83	1.86+/-0.38	2.71+/-1.14	3.79+/-0.79

Flunix 24h vs Flunix 13d post App, * SEM could not be calculated due to insufficient data.

The production of cytotoxic T cells in the whole blood from pigs treated with Flunix was significantly higher 13 days after the App challenge as compared to 24 hours

after the App challenge (Table 4.7) when data was presented as a percentage. The whole blood from the remaining pig groups showed no difference between sampling time or treatment administration, when data was presented as a percentage (Table 4.7). However all the treated pigs' blood revealed an increasing trend of cytotoxic cells at 13 days post App challenge.

There was no significant difference in the CD4:8 ratio of cells between the sampling time and the treatment administration across the App challenge.

B cells showed a decreasing trend in the blood from pigs treated with IL-1ra and its plasmid counterpart at 24 hours after challenge (not significant) (Table 4.7).

Table:4.8: Neutrophil phagocytic function and WBC counts from pigs treated with saline, Flunix, recombinant cytokines or plasmid cytokines and subsequently challenged with App. na indicates not assessable. Data is presented as the mean±SEM, n=5.

Assay	saline	Plasmid control	Flunix	IL-4	IL-1ra	IL-4 plasmid	IL-1ra plasmid
%Phagocytosing neutrophils Pre App	28.84+/-0.86	32.27+/-4.83	28.30+/-1.60	29.41+/-1.62	35.96+/-1.87	29.93+/-1.05	33.79+/-4.50
24h Post App % phagocytosis	33.00+/-3.57	33.96+/-2.38	34.01+/-4.69	32.07+/-4.50	29.33+/-2.83	31.37+/-1.52	26.86+/-2.57
13d Post App % phagocytosis	57.09+/-3.56	60.93+/-3.59	56.32+/-3.28	64.06+/-5.94	61.46+/-2.65	65.70+/-1.86	66.59+/-3.66
WBC Pre App $x10^6$ cell/ml	19.88+/-0.69	19.28+/-1.95	22.35+/-1.40	21.10+/-2.28	17.63+/-1.90	17.53+/-1.44	21.50+/-*
24h Post App $x10^6$ cell/ml	na	na	na	na	na	na	na
13d Post App $x10^6$ cell/ml	27.97+/-5.43	29.28+/-3.18	29.33+/-3.17	22.70+/-2.21	22.68+/-1.43	22.65+/-2.46	24.75+/-2.69

* SEM could not be calculated due to insufficient data

The proportion of neutrophils undergoing phagocytosis increased in whole blood of all the treated pigs after 13 days App challenge.

Unfortunately as earlier stated the machine which counts total whole blood cells did not function to produce a result for blood taken 24 hours after App challenge. From the results obtained the number of white blood cells was reduced in the blood taken at 13 days post App challenge from pigs treated with IL-4 and IL-1ra as compared to the saline-treated pigs (not significant)(Table 4.8)

4.6.3 Pathology of the App Challenge Model
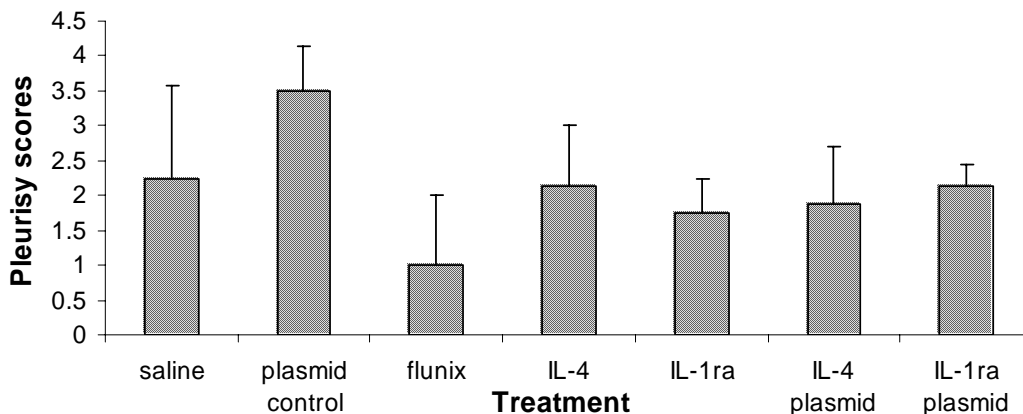
Figure 4.12 Degree of pleurisy at post-mortem expressed as pleurisy score (0-5) in pigs treated with saline, Flunix, recombinant cytokines or plasmid cytokines and subsequently challenged with App. Bars represented as mean±SEM. n=5.

Pigs were euthanased at the end of the trial and their lungs were examined and scored by a veterinarian for severity of pleurisy and App lesions. Pleurisy was scored from 0-5. Pigs treated with Flunix ($p<0.05$), IL-1ra, plasmid IL-4 and plasmid IL-1ra had less pleurisy than the saline controls, while pigs treated with the plasmid control had the highest levels of pleurisy (Figure 4.12).
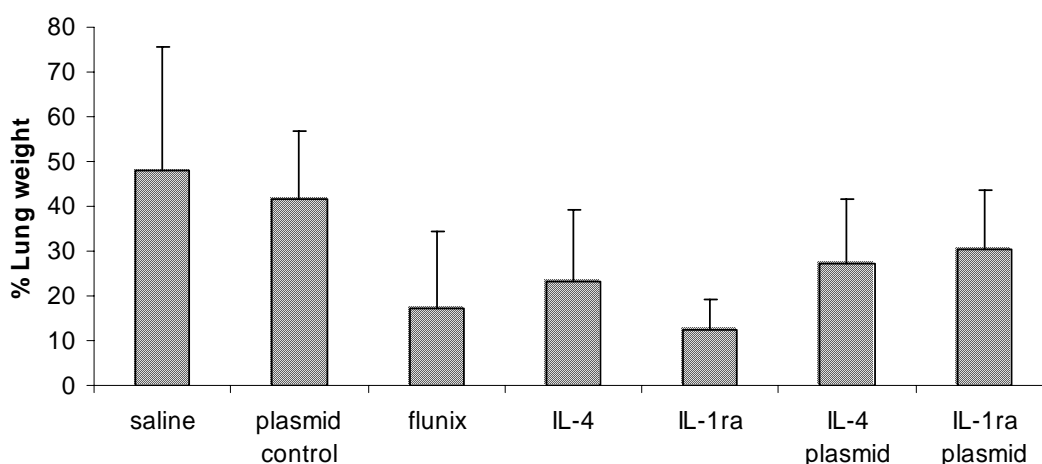
Figure 4.13: Percentage of lung affected by pleuropneumonia lesion. Pigs were treated with saline, Flunix, recombinant cytokines or plasmid cytokines and subsequently challenged with App. Bars represented as mean±SEM n=5.

The percentage of the affected lung was determined by removing and weighing the whole lung then measuring the weight of the affected part of the lung. This showed degree of pleuropneumonia as a percentage of the whole lung. Saline treated controls and plasmid controls had the highest affected lung weight, with greater than 40% of their lung weight consisting of App lesions (Figure 4.13). Pigs treated with Flunix, IL-4, IL-1ra and their plasmid counterparts had considerably less affected lung than saline controls (Figure 4.13).

4 7 Discussion

Although the pigs exhibited symptoms of severe fever and lethargy after the administration of LPS, they showed no significant weight loss between the week prior to challenge and the week during challenge (Figure 4.1). This could be due to the acute nature of the LPS challenge or the quick recovery of the pigs appetite. It can be concluded that the LPS challenge model did not produced significant enough negative growth effects in large pigs to enable an accurate assessment of the efficacy of treatments to increase growth.

The immunological and molecular assays utilized in the LPS acute challenge showed interesting responses to the challenge. The pigs treated with IL-4 showed significantly more ($p < 0.05$) lymphocyte proliferation in their whole blood compared to the saline treated pigs and the remaining treatment groups when stimulated with PHA.

Prior to the LPS challenge the constitutive amount of IL-8 mRNA detected in the whole blood of all the treated pigs was zero with the exception of pigs treated with IL-1ra plasmid. This group had minimal levels of IL-8 mRNA prior to the LPS

challenge. Prior to the App challenge the pigs treated with Flunix and IL-4 levels of IL-8 mRNA returned to an undetectable amount.

The pigs treated with IL-4 produced significantly less ($p<0.05$) phagocytosing neutrophils after LPS challenge (Table 4.2). It is known that IL-8 is a neutrophil recruiter (Tizard 1996) and hence if there is reduced IL-8 there is usually reduced neutrophils. Even though IL-8 mRNA was low in the blood of the pigs treated with IL-4 after LPS challenge, it was not lower than the saline treated pigs (not significant). The low levels of IL-8 mRNA could be due to IL-4 administration since Nuntaprasert *et al.,* (2004) also found administration of IL-4 at the same time as LPS inhibits the stimulation IL-8, production in pig alveolar macrophages. The pigs treated with IL-4 also produced significantly less ($p<0.05$) TNF-α mRNA than the saline treated pig group. The production of low neutrophil phagocytic capacity and low TNF-α mRNA in blood from pigs treated with IL-4 may be associated since Dubravec *et al.,* (1990) also found that polymorphonuclear neutrophils produced TNF-α mRNA after LPS stimulation. It was interesting to find that the constitutive levels of IL-6 mRNA were below detectable limit in all the treated pigs and controls except the pigs treated with IL-1ra plasmid, since human blood samples have a baseline constitutive level of IL-6 mRNA. However, Sipos *et al*., 2004 found IL-6 mRNA weakly or not detectable in whole blood samples of some control pigs when carrying out a cytokine profile of pigs suffering from natural post-weaning multi-systemic wasting syndrome.

The analysis of the immunological and molecular assays utilized in the App chronic challenge showed interesting responses to the challenge as well. The pigs treated with IL-4 and IL-1ra increased in weight compared to the controls over the 2 week period

of App challenge (Figure 4.2b). Flunix was the poorest performing treatment in terms of growth (Figure 4.2).

There was a reduction in pro-inflammatory cytokines such as IL-1β mRNA in whole blood taken from pigs treated with IL-4 24 hours after App challenge. This trend in reduced pro-inflammatory cytokines was observed again in the reduction of IL-8 mRNA 13 days after App challenge in the same treatment group. Interestingly, Flunix did not follow this trend instead it did not inhibit the production of serum TNF-α, which may explain the poor growth observed in this group. The pigs treated with Flunix did not reduce IL-6 mRNA production in their whole blood either (Figure 4.6). Morrison *et al.,* (2000) observation that excessive TNF-α can cause massive lung injury in pigs. Olsson (1995) also found that elevated levels of TNF-α play a role in Multiple Sclerosis (humans). This may explain the association between poor growth and TNF-α, since animals in poor health rarely achieve normal growth. Huang *et al.,* (1999) also recorded an elevation in TNF-α and IL-1 in the serum, which coincided with the onset of acute clinical disease (App) in pigs.

Pigs treated with IL-4 had significantly less (p<0.05) IL-6 mRNA at 13 day post App and the most growth as total weight gain (Figure 4.2b). Pigs treated with Flunix and IL-4 had an undetectable level of IL-8 mRNA in their peripheral blood prior to the App challenge, which was suppressed across the 13 day App challenge perhaps by the administration of these treatments (Figure 4.7). Zhou *et al.,* (1994) also found that IL-4 administration stopped the production of IL-8 mRNA at a transcriptional level however, it was in a *in vitro* system. The production of IL-8 mRNA 24 hours after App challenge was detected in the blood of pigs treated with IL-1ra (Figure 4.7) at the same quantity as that found in the control pig groups.

Pigs treated with IL-1ra had low clinical scores (Figure 4.3) and their affected lung was low (Figure 4.13). Phagocytosing neutrophils in the blood gives some perspective of neutrophils traveling to infection site (lung). Pigs treated with IL-1ra produced a similar number of phagocytosing neutrophils as the saline treated pig group (Table 4.8), however, the administration of IL-1ra showed an association with the reduction of lung damage in this group (Figure 4.13).

The suppression of IL-8 mRNA is produced 24 hours after App challenge (Figure 4.7) by pigs treated with IL-4. Perhaps IL-4 stops IL-8 at a transcriptional level (Zhou, *et al.,* 1994). IL-1ra can also influence IL-8 production since it was found by Chen *et al.,* (1998) that IL-1ra inhibited IL-8 which was stimulated by IL-1α in an *in vitro* system (squamous cell carcinoma human cell line).

The production of IL-8 mRNA by the pigs treated with saline and plasmid control, IL-1ra and its plasmid counterpart was present prior to, and 24 hours after the App challenge. This was interesting since the plasmid control and saline treated pig groups also produced the most lung lesions (Figure 4.13) and showed the greater clinical signs, yet the pigs treated with IL-1ra and IL-1ra plasmid did not show the same trends. However, the pigs treated with IL-1ra and IL-1ra plasmid differed from the saline and plasmid control treated pigs in that each group produced higher numbers of T cells with CD3 (data presented as percentages) receptors 24 hours after App challenge (Table 4.6). Furthermore increased TNF-α protein production showed this association with poor growth in pigs treated with Flunix. The reduction in weight gain of Flunix treated pigs were puzzling since the group had low pleurisy, low affected lung, and low clinical scores. Furthermore pigs treated with Flunix at the end of the trial had significantly higher T cells with the CD8 marker (data as percentages)

in their blood, as compared to 24 hours after the App challenge. Perhaps these suppressor/cytotoxic cells were compensating for the increase in TNF-α in the serum. The relationship between pro-inflammatory cytokines and impaired growth needs further study.

The production of IL-1β mRNA in pigs treated with IL-4 was reduced 24 hours after App challenge (Figure 4.9). IL-4 was found to stop the production of IL-1β mRNA at a transcirptional level (Zhou *et al.,* 1994). IL-1β is known to activate macrophages to produce IL-6 and IL-8 (Tizard 1996). However, there must be another inducer of IL-6 and IL-8 production or other cells producing these cytokines such as lymphocytes, fibroblasts, and endothelial cells or infiltrating neutrophils (Tizard 1996), since they are present in the peripheral blood.

Pigs treated with IL-1ra had suppressed TNF-α protein in serum over the 13 day App challenge (Figure 4.11). Although there was IL-1β mRNA present in pigs treated with IL-1ra, this does not necessarily mean it had been transcribed into protein (Figure 4.9). Therefore if there was no IL-1β protein in the blood of the pigs treated with IL-1ra then there would be reduced CRP mRNA (Figure 4.10) produced since as earlier stated CRP is an acute phase protein in the early stages of infection and mediated by IL-1 (Klein 1997). However the acute phase response is not induced by a single mediator (Taylor et al 1990). This may shed some light on the suppression of CRP mRNA 24 hours after App challenge in the pigs treated with IL-1ra however, the assumption that no IL-1β mRNA was transcribed to protein is mere speculation. Unfortunately time did not permit the analysis by ELISA of serum to obtain IL-1β protein profiles.

The pigs treated with IL-4 showed a reduction to zero in CRP mRNA (Figure 4.10) over the 13 day App challenge period, which also coincided with the suppression of IL-1β at 24 hours post infection (Figure 4.9).

Even though the anti-inflammatory cytokine treatments reduced the levels of pro-inflammatory cytokines in the circulation and increased growth in some treated pigs. The relationship between pro-inflammatory cytokines and impaired growth needs to be well defined by ongoing experiments into the role of pro-inflammatory cytokines and growth.

The lymphocyte proliferation profiles of the treated pigs blood stimulated by Con A (Figure 4.4) in the App challenge is almost identical to the lymphocyte proliferation profile stimulated by PHA (Figure 4.3) therefore, either can be used in the next experiment.

The production of TNF-α protein in the serum of pigs treated with Flunix may be secreted by the T cells with CD4 markers however, it seems unlikely since they are at the same numbers of cells as the remaining treated pig groups. Macrophages and other cells may also be producing the TNF-α in the serum of these pigs. As stated earlier the pigs treated with IL-1ra produced the most T cells with a CD3 marker present (Table 4.6) at 24 hour after App challenge. This large production does not mean all these T cells are active since IL-1ra binds to T helper 2 cells and inactivates the cell from producing IL-1, which is a precursor to other pro-inflammatory cytokines like IL-6 and IL-8 (Figure 4.6-4.7) (Arend *et al.,* 1993).

Since IL-1ra can stops IL-1β by binding to receptors on T helper 2 cells and blocking IL-1 from signal transduction (Arend *et al.,* 1993) therefore stopping the secretion of

IL-5 or IL-4 which stimulate B cells proliferation and growth perhaps could give an explanation to the low number of B cells. The fact that pigs treated with IL-1ra did not produce a large amount of B cells demonstrates that this treated pig group displayed better health. The decrease in clinical signs (Figure 4.3) and increase in weight gain in the pigs treated with IL-1ra reflected the improved health of this group (Figure 4.2b).

Pigs treated with IL-4 and IL-1ra performed better than the remaining treatment groups in terms of their clinical scores (Figure 4.3) and affected lung (Figure 4.13).

There was no significant difference between the treatment groups in terms of pleurisy, with the exception of the pigs treated with Flunix which produced less pleurisy scores than the pigs treated with plasmid control (Figure 4.12).

IL-1ra plasmid reduced the amount of lung lesions caused by App as compared to the saline and plasmid controls. Nevertheless recombinant IL-1ra dramatically improved the pathology of the pigs' lung. IL-1 is a precursor to pro-inflammatory cytokines like TNF-α which are implicated in lung damage (Morris *et al.,* 2000), along with IL-8 which causes acute lung inflammation (Huang *et al.,* 1999). TNF-α is not totally inhibited since this can also affect the pig negatively. Pigs treated with saline and plasmid controls had a similar amount of lung lesions and affected lung (Figure 4.13).

The pigs treated with IL-1ra produced significantly less ($p<0.05$) clinical scores (Figure 4.3) with saline and Flunix produced the most. Thus it can be said that in addition to improving the growth of pigs, some cytokines also improve the health of pigs especially those at risk to disease. Similarly Muller (1998), Mosmann (1994)

observed that IL-4 inhibited the synthesis of pro-inflammatory cytokines and therefore reduced rheumatoid arthritis in humans.

Some cytokines in addition to improving growth can boost the health of pigs exposed to disease such as App. For example IL-4 and IL-1ra treated pigs had reduced affected lungs was similar to the reduced IL-6 and IL-8 mRNA (Figure 4.13, 4.6, 4.7), (Zhou, *et al.,* 1994).

It may be concluded that plasmids are not useful as a delivery system for anti-inflammatory cytokines like IL-4, and IL-1 receptor antagonist since they did not differ in growth or health from the pigs treated with saline.

The NSAID (Flunix) performed as well as IL-4 and IL-1ra however, Flunix in large doses can be toxic. The prophylactic delivery of the cytokines and the non-steriodal drugs had a positive affect on pathological stress and hence on growth.

4.8   App chronic Challenge Model Summary

- During the first week of App challenge recombinant IL-4 and IL-1ra increased the growth of pigs compared to the saline control group. The pigs treated with Flunix had the lowest growth over the 13 day challenge period.

- At euthanasia the pigs treated with recombinant IL-4 and IL-1ra were 4kg heavier than their saline counterparts

- The pigs treated with Flunix, IL-1ra and IL-4 had the reduced pleurisy at post-mortem. These treatments and their plasmid counterparts reduced the percentage of lung affected by App lesions.

- The pigs treated with Flunix and IL-4 suppressed IL-8 mRNA production across the 13 day App challenge.

- Delievery of anti-inflammatory compounds through plasmids proved to be ineffective as a delivery system.

- IL-1ra and IL-4 administered as a prophylactic has demonstrated an ability to improve growth however, do these anti-inflammatory cytokines have the ability to improve growth when given as a therapeutic. This will be investigated in the next Chapter.

COMMERICAL IN CONFIDENCE

CHAPTER 5

THERAPEUTIC DELIVERY OF IL-4 AND IL-1RA FOR THE TREATMENT OF SYMPTOMS ASSOCIATED WITH APP IN PIGS

5.1 Introduction

Since the prophylactic use of IL-4 and IL-1ra showed promising results in the previous Chapter, the therapeutic potential of IL-4 and IL-1ra required investigation. The prophylactic characteristics of IL-4 and IL-1ra for reducing the production of pro-inflammatory cytokines such as IL-6, IL-8 and increasing weight gain was dramatic. Furthermore, the ability of IL-4 and IL-1ra to reduce App associated pathology in lungs of the pigs showed that these substances may also have use as therapeutics. A large trial examining therapeutic (ie, post-challenge) delivery of IL-4 and IL-1ra, was therefore conducted with a multitude of assays (including whole blood counts, T cell counts using CD markers, lymphocyte proliferations and RT-PCR).

The overall aim of this experiment was to observe if IL-4 and IL-1ra had a therapeutic affect to treat App infection. A commercially available medication (Excenel) for pigs suffering App infection was also included for comparison with IL-4 and IL-1ra.

Excenel is a broad spectrum antibiotic which has been shown to inhibit the cell wall synthesis of bacteria *in vitro* (IVS annual). It binds to inflammatory proteins in the lung and also works in extracellular fluid where pathogens (e.g. pneumonia) live.

5.2 Materials and Methods

5.2.1 Experimental Protocol

As in the previous experiment the pigs were weighed and randomly allocated into 5 treatment groups and 1 control group. Fifty four pigs at a mean weight of 34kg were introduced into the Elizabeth Macarthur Agricultural Institute (EMAI).

Each pen consisted of 6 pigs 1 from each of the 6 treatment groups. This pen design was replicated 3 times, in 3 separate rooms. The treatments included IIL 4 low concentration, IL-4 high concentration, IL-1ra low concentration, IL-1ra high concentration, Excenel (Pfizer AH, NY, USA) and saline.

Table 5.1: Summary of the treatment groups and of the treatment schedule in relation to challenge

Treatment groups	Dose rate	Treatment applied (post challenge)
Saline	10mg/pig	24h, 48h,1week
IL-4 low	2mg/kg	24h, 48h,1week
IL-4 high	10mg/kg	24h, 48h,1week
IL-1ra low	2mg/kg	24h, 48h,1week
IL-1 ra high	10mg/kg	24h, 48h,1week
Excenel	3mg/kg	Daily for 3 days according to manufacturer's instructions.

The pigs were challenged with $7.5x10^5$ cfu/mL App intra-tracheal at day zero(as described in Chapter 4 and section 2.3) and starting weights were recorded. Pigs were observed for 24 hours after App inoculation and then examined daily for clinical signs such as fever, apathy, lethargy and labored breathing. Weights were measured at 24 hours, 7 days and 2 weeks. Jugular blood samples were taken at 0 hour, 24 hours, 48 hours, 7 days and 2 weeks after infection for immunological and molecular analyses..

At 2 weeks after infection the animals were euthanasied and the blood and lungs were examined for analysis of pathological change due to pleuropneumonia.

5.2.3 Immunoassays Performed

Lymphocyte proliferation, neutrophil function, T and B lymphocyte number, immunoglobulin levels, TNFα levels, differential and whole blood counts were determined according to the methods outlined in, Sections 2.6-2.10 and 2.12.

Antibodies in the serum of treated pigs such as IgA and IgG were quantified using sandwich ELISA. The limit of detection was 15pg/mL, and data was expressed as μg/mL of immunoglulin present. IgG is a major immunoglobulin synthesized in the body (Roitt 1994).

Cluster designation (CD) receptor data, obtained by flow cytometry, was supplied as both percentages and cells/mL (Table 5.5-5.6). Chapter 4 has an incomplete set of data presented as cells/mL due to a machine breakdown. Therefore a full set of percentage lymphocyte stimulation data in this Chapter can be used to compare to the percentage CD cell count data in Chapter 4 (Table 4.6-4.7).

5.2.4 Measurement of Pro-inflammatory Cytokines by RT-PCR

RNA was extracted from whole blood samples as outlined in Section 2.4.1. RT-PCR was performed as described in Sections 2.4.2 and 2.4.3, using mRNA primers for β-act, IL-8, IL-6, TNFα, IL-1β and CRP (refer to the table in Appendix 1 for sequences). Minor modifications to the methods described previously included cDNA template changes and additives used with some primers which is documented in Appendix 3.

5.2.5 Paraffin Tissue Sections

Lung samples measuring <1cm3 were fixed in 10% formalin overnight. The tissue samples were then trimmed to a smaller size for embedding and placed in a transfer cassette and stored in 70% alcohol overnight. The cassettes were placed in an automatic processor which passed through an alcohol gradient, and were then infiltrated with molten paraffin. The samples were embedded in wax paraffin blocks and cut to 5μm sections. Sections were attached to clean glass slides from water bath and placed in an oven at 37°C until dry.

Prior to use, sections were dewaxed in two changes of xylol for 3 minutes each, and then rehydrated through an ethanol gradient (twice 100%, 95%, and 70%) for 3 minutes each. Slides were soaked in picric acid for 15 minutes to remove excess blood, and then washed under running tap water for 15 minutes. The slides were counter stained in haematoxylin (Sigma-Aldrich, St Louis, USA) for 3 minutes and washed for 2 minutes in running water, and stained in Scott's blueing solution for 2 minutes. Slides were washed again in running tap water and stained with eosin (Sigma-Aldrich, St Louis, USA) for 30 seconds. Slides were then passed through an

ethanol gradient (twice 70%, 95%, 100% and 100%). The slides were passed through a solution of 50% ethanol in xylol three times and once in 100% xylol. Sections were mounted in Depex and coverslip placed on top, and left to dry.

5.2.6 In situ Hybridization

5.2.6.1 Sample Collection and Cutting Frozen Sections

Tissue samples from pig lungs measuring approximately $1cm^3$ were placed in cryotubes and snap frozen in liquid nitrogen. Tissues were embededed in O.C.T compound (Tissue-Tek, Bayer, Leverkusen, Germany) and frozen. Sections of $8\mu m$ were cut using a cryomicrotome (International Equipment Company, CA, USA) and were mounted on silane-coated slides (Sigma-Aldrich , St Louis, USA).

5.2.6.2 IL-8 Probe

Cloned IL-8 cDNA was prepared as described in Section 2.4. PCR was performed on known IL-8 positive sample and the resulting PCR product was used to make the IL-8 probe following the manufacturer's instructions for the DIG-label kit (Roche, Postfach, Switzerland). A positive RNA probe (IL-8 anti-sense probe) and a negative RNA probe (IL-8 sense probe) were made, and $5\mu l$ of each was run on a 1.2% agarose gel to confirm probe production. Probes were purified by alcohol precipitation and the purity of the probe was confirmed by reading at 260nm on a Spectromax 250 (Molecular Devices, Sunnyvale, CA, USA) spectrophotometer. Alcohol precipitation was performed by adding 1:10 volume 3M $C_2H_3O_2Na$ and 2 volumes of ethanol to the probe volume and incubated at -20^oC overnight. This solution was then centrifuged at $15000g$ for 10 minutes at 4^oC. Supernatant was decanted and the pellet was washed with $200\mu L$ 70% ethanol and centrifuged again at $15,000g$ for 10 minutes at 4^oC.

Supernatant was decanted and the pellet air dried. The probes were diluted to 200ηg/mL, aliquoted and stored at -20°C prior to use.

5.2.6.3 Pre-Hybridization

Slides were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) pH 10, containing 0.1% active diethyl pyrocarbonate (DEPC) for 30 minutes. The slides were then soaked twice for 15 minutes in PBS containing 0.1% active DEPC. A volume of 150μl of 5x SSC (0.75M NaCl and 0.075M Na$_3$C$_6$H$_5$O$_7$ in DEPC water) was dispensed onto each tissue and the slides were placed in a hybridizing chamber at room temperature for 15 minutes. The SSC was removed by tilting the slides. Pre-hybridization solution (50% formide, 5x SSC and 40μg/mL unlabled cDNA), 150μl, was dispensed onto each tissue and the slides were returned to the hybridizing chamber. Parafilm was used as a coverslip to stop evaporation and the chamber was set at 58°C for 2 hours.

5.2.6.4 Hybridization

Slides were again tilted to removed pre-hybridization solution and either 400ηg/mL of positive probe or negative probe in fresh hybridization solution was added to each tissue. The slides were heated to 95°C for 2 minutes to denature the probes. Slides were then returned to the hybridization chamber at 58°C with parafilm coverslip attached and left to hybridize overnight.

5.2.6.5 Blocking

Slides were soaked in two changes of TB-S (0.1M Tris-HCL and 0.15M NaCl with 0.1% TritonX-100, pH 7.6) at 65°C for 1 hour each. The slides were returned to the chamber with 150 µl of blocking solution (10% heat inactivated foetal calf serum and 3% bovin serum albumin into TB-S) on each tissue and kept at room temperature for 30 minutes. Buffer was removed by tilting the slides, alkaline phosphatase-conjugated anti-DIG (Roche, Postfach, Switzerland) antibodies was diluted to 1:500 in blocking buffer and added to tissues. Slides were placed back into the chamber at a temperature of 37°C for 2 hours.

5.2.6.6 Staining

Alkaline phosphatase substrate buffer (APSB) 0.1M Tris-HCL pH9.5, (0.1M NaCl, 50mM $MgCl_2$) was used to wash slides in a container with constant stirring. Slides were washed twice for 30 minutes at room temperature. Slides were removed and stained for 20 minutes at room temperature with alkaline phosphatase substrate containing 0.33mg/mL nitroblue tetrazolium, 0.175mg/mL bromochloroindolyl phosphate and 3 mM levamisole in APSB. Colour development was stopped using distilled water. Slides were air-dried and coverslipped with Kaiser Jelly (gelatin w/v 7.7%, glycerol v/v 53.8%, sodium azide w/v 0.13%, phenol w/v 7.7% in distilled water.

5.2.6.7 Statistics

Data from all assays were analysed using ANOVA.

5.3.Results

5.3.1 Growth, Production and Clinical Signs

Change in weight was considered to be the most important parameter in determining treatment efficacy; however, there were no significant differences between groups at the end of the trial (Figures 5.2). Unlike the previous experiment in Chapter 4 all groups gained weight in the 2 weeks after App infection (Figure 5.1). IL-4 lo, IL-4 hi and IL-1ra lo treatment groups experienced similar weight gain after 2 weeks; the dramatic increase in weight of the IL-4 and IL-1ra treated pig groups observed in Chapter 4 (Figure 4.2) did not occur in this current experiment. After the first week

□ saline   □ IL-4 lo   □ IL-4 hi   □ IL-1ra lo   □ IL-1ra hi   □ excenel
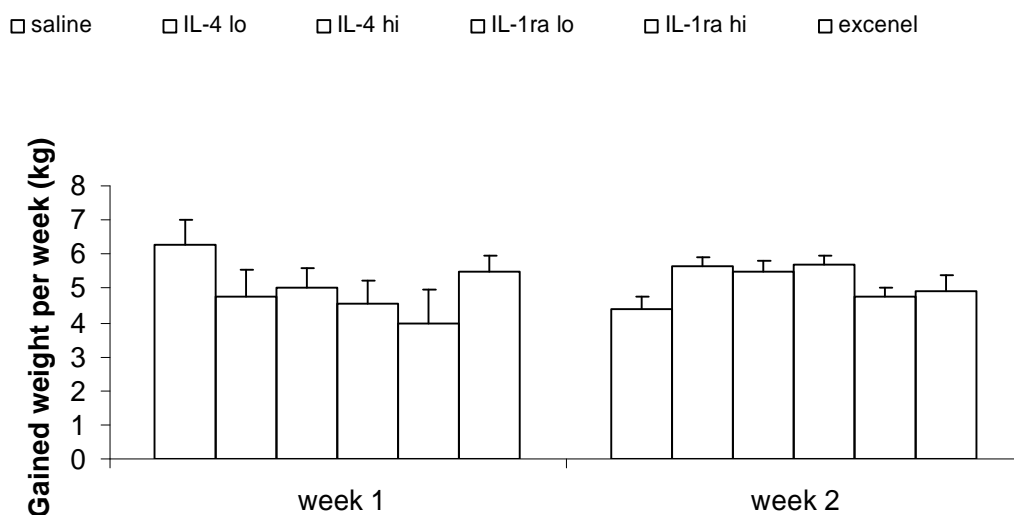
Figure 5.1: Weight gain of pigs challenged with App and subsequently treated with saline, Excenel, or recombinant cytokines at high and low concentrations at 1 and 2 weeks after challenge. Bars represent mean±SEM, n=9.
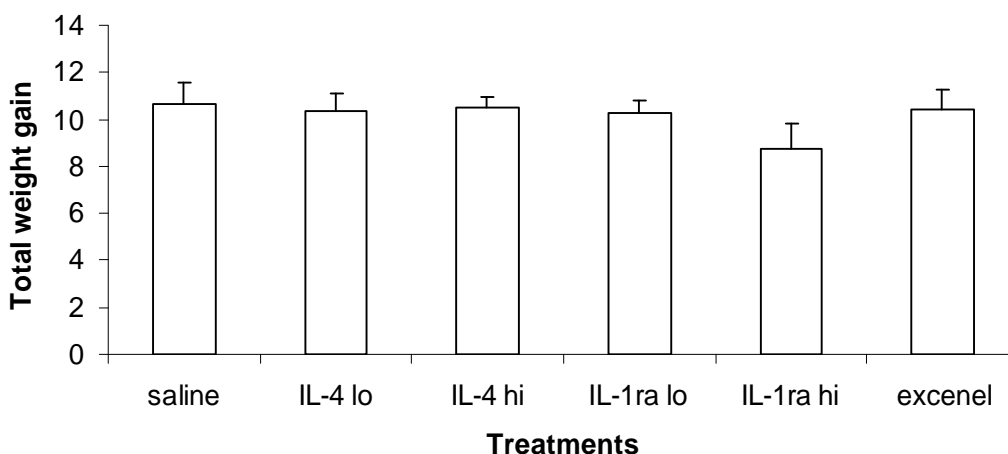
Figure 5.2: Mean total weight gain of pigs challenged with App and subsequently treated with saline, Excenel, or recombinant cytokines at high and low concentrations, at the conclusion of the trial. Bars represent Mean±SEM, n=9.

Regardless of the treatment administered there was no difference between treatment groups in the amount of weight gained at the conclusion of the trial (Figure 5.2).
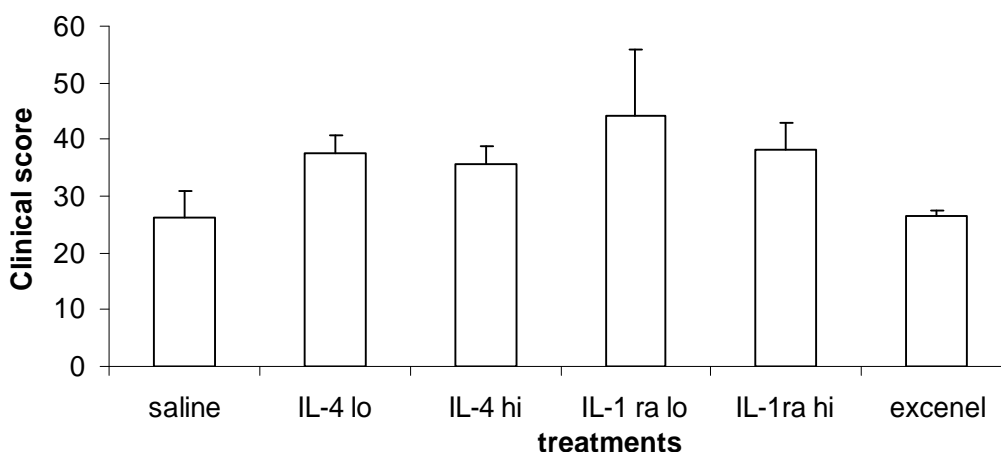
Figure 5.3: Clinical summary of pigs challenged with App and subsequently treated with saline, Excenel, or recombinant cytokines at high and low concentrations on a per visit basis. The pigs had 30 visits and each pig was scored from 0-8 (maximum score 240) for each visit. Bars represent mean ±SEM, n=9. * = IL-1ra hi vs excenel treated groups p<0.05.

There were significantly less clinical signs observed in the pigs treated with Excenel as compared to the pigs treated with IL-1ra at a high dose. Other than that there were no differences between the groups as far as clinical signs were concerned.

5.3.3 Immunological and Molecular Techniques

5.3.3.1 Lymphocyte Proliferation and Neutrophil phagocytosis

Whole blood from pigs treated with IL-4 lo had significantly increased lymphocyte proliferation in response to Con A, 48 hours after App infection as compared to the blood from pigs treated with saline at the same sample time (Table 5.2). All of the treatment groups slightly increased lymphocyte proliferation after stimulation with Con A at 48 hours post-infection compared to pre-challenge samples (Table 5.2). KB were also used to stimulate lymphocyte proliferation as outlined in Section 2.10.1, which caused an increase in proliferative response in the whole blood from all the treated pig groups (Table 5.2). The whole blood sampled at 24 hours and stimulated with KB showed decreased lymphocyte proliferation responses in all the treatment groups. Blood sampled at 48 hours post App from pigs treated with IL-1ra lo and stimulated with KB, showed an significant increase in lymphocyte proliferation compared to the blood from the pigs treated with IL-1ra hi however, there was no significant difference between these groups and saline-treated pigs (Table 5.2). Generally, the proliferation of lymphocytes stimulated by Con A and KB showed no significant differences between treatments or sample time.

Table:5.2 Lymphocyte proliferative responses to KB or ConA in whole blood from pigs infected with App and subsequently treated with saline, Excenel, or recombinant cytokines at high and low concentrations. Data presented as mean±SEM, n=9.

Assay	saline	IL-4 lo	IL-4 hi	IL-1ra lo	IL-1ra hi	Excenel
KB Lymphocyte proliferation 0h % stimulated cells	38.29+/- 5.51	40.31+/- 4.46	26.59+/- 6.86	41.96+/- 4.87	44.44+/- 6.54	33.71+/- 7.05
24h % stimulated cells	22.87+/- 4.46	24.75+/- 4.52	24.73+/- 4.07	30.37+/- 8.81	33.24+/- 6.65	21.24+/- 4.38
48h % stimulated cells	27.78+/- 8.71	28.28+/- 8.18	24.53+/- 8.88	41.90+/- 8.66#	19.72+/- 4.17	31.77+/- 5.50
1 week % stimulated cells	31.95+/- 4.46	22.49+/- 3.65	22.55+/- 3.33	25.56+/- 8.55	28.23+/- 6.75	22.89+/- 5.32
2 weeks % stimulated cells	35.59+/- 5.20	24.33+/- 4.98	28.94+/- 5.53	22.45+/- 5.78	31.49+/- 7.47	29.74+/- 5.22
ConA Lymphocyte proliferation 0h % stimulated cells	0.93+/- 0.09	0.97+/- 0.14	0.92+/- 0.17	1.12+/- 0.13	1.10+/- 0.16	1.07+/- 0.20
24h % stimulated cells	1.03+/- 0.12	1.10+/- 0.06	1.05+/- 0.07	1.03+/- 0.12	1.04+/- 0.05	1.22+/- 0.07
48h % stimulated cells	1.21+/- 0.13	1.76+/- 0.28*	1.41+/- 0.14	1.31+/- 0.08	1.47+/- 0.08	1.33+/- 0.09
1 week % stimulated cells	0.86+/- 0.08	0.71+/- 0.09	1.03+/- 0.09	1.05+/- 0.08	1.06+/- 0.08	0.83+/- 0.15
2 weeks % stimulated cells	1.24+/- 0.12	1.03+/- 0.18	1.63+/- 0.26	1.15+/- 0.12	1.17+/- 0.19	1.30+/- 0.19

* IL-4 lo vs saline at 48h, # IL-1ra lo vs IL-1ra hi at 48h

There was no difference between treatment groups in the ability of neutrophils to phagocytose zymosan or KB (Table 5.3). with the exception of the pigs treated with IL-1ra lo, which had a significantly higher proportion of phagocytosing neutrophils compared to IL-1ra hi treated pigs 48 hours after App challenge.

Table:5.3 Proportion of neutrophils phagocytosing zymosan or KB from pigs infected with App and subsequently treated with saline, Excenel, or recombinant cytokines at high and low concentrations. Data presented as mean±SEM, n=9.

Assay	saline	IL-4 lo	IL-4 hi	IL-1ra lo	IL-1ra hi	Excenel
Phagocytosis zymosan stimulated neutrophil 0h % phagocytosis	46.24+/-2.80	50.24+/-3.63	55.96+/-2.10	56.27+/-4.72	51.74+/-4.67	56.91+/-3.06
24h % phagocytosis	55.90+/-3.29	55.58+/-2.36	57.67+/-3.86	61.42+/-1.87	57.66+/-3.51	58.88+/-2.81
48h % phagocytosis	41.70+/-3.34	42.15+/-4.38	45.03+/-3.84	52.68+/-4.91[#]	37.59+/-4.22	48.06+/-2.40
1 week % phagocytosis	50.02+/-3.93	51.03+/-2.64	42.59+/-4.29	47.23+/-3.10	47.11+/-3.89	52.10+/-1.90
2 weeks % phagocytosis	65.36+/-3.66	62.56+/-2.69	59.62+/-3.82	61.81+/-3.65	61.15+/-2.27	69.79+/-2.64
Phagocytosis KB stimulated neutrophils 0h % phagocytosis	70.75+/-3.18	74.89+/-1.94	76.72+/-2.01	76.11+/-2.13	65.16+/-8.24	75.07+/-2.62
24h % phagocytosis	60.55+/-3.20	59.08+/-3.66	61.47+/-4.03	59.75+/-0.94	56.92+/-2.96	57.06+/-2.32
48h % phagocytosis	60.79+/-5.39	61.04+/-5.45	61.44+/-3.14	72.23+/-5.06	60.73+/-4.59	73.01+/-6.87
1 week % phagocytosis	84.53+/-1.23	80.72+/-2.82	80.34+/-1.93	82.20+/-2.52	81.98+/-2.41	83.16+/-1.51
2 weeks % phagocytosis	72.86+/-2.92	71.98+/-3.21	74.23 2.19	69.11+/-4.23	60.50+/-3.42	57.24+/-11.31

IL-1ra lo vs IL-1ra hi at 48h p<0.05

5.3.3.2 Semi-quantitative RT-PCR

IL-8 mRNA production in peripheral blood in all the treated pigs varied little between sample times (Table 5.4). However, 24 hours after the App challenge a significantly larger level of IL-8 mRNA was found in the pigs treated with IL-4 hi as compared to the pigs treated with IL-1ra lo. IL-8 mRNA production decreased to an undetectable amount between 48 hours and 1 week post-infection in the pigs treated with IL-4 hi but not in other treatments (Table 5.4).

IL-8 mRNA production in the peripheral blood of pigs treated with IL-4 hi is significantly higher than that produced by pigs treated with saline. In all treatment

groups the amount of TNFα mRNA in peripheral blood was high before the App infection was administered (Table 5.4). The saline treated pig group had a reduction in TNFα mRNA at 24h post App infection which increased slightly until euthanasia. TNFα mRNA levels decreased in pigs treated with IL-4 hi by more than half, 24 hours after App challenge and subsequently decreased to an undetectable amount followed by a slight increase at 2 weeks post App infection. Similar trends were seen for pigs treated with IL-1ra lo.

Pigs treated with IL-4 lo had decreased TNFα mRNA 24 hours after App after which the TNFα mRNA levels remained constant till euthanasia (Table 5.4). TNFα mRNA was reduced in Excenel treated pigs 24h post App further reduced to undetectable until the end of the trial. TNF-α mRNA from pigs treated with saline was reduced across time less than the pigs treated with IL-1ra and IL-4 (Table 5.4).Interestingly there was an elevation of IL-1β mRNA with a corresponding TNFα mRNA decrease (Table 5.4)

With the exception of the pigs treated with IL-1ra hi and Excenel, all the treated pigs had undetectable levels of IL-1β mRNA at the time of App challenge. There was significantly less IL-1β mRNA in the IL-1ra lo treated pigs as compared to the IL-4 hi and IL-4 lo treated pigs at 24 hours post App (Table 5.4). The production of IL-1β mRNA in all the blood of all the treated pigs increased with App challenge and was greater at the end of the trial. IL-6 mRNA production was undetectable in the blood of all treated pigs at all sample times over the 13 day challenge. the variability in cytokine production is due to individual difference among pigs and the difference in IL-6 mRNA baseline between experiments in Chapter 4 and 5 may be due to the previous LPS challenge pigs were exposed to the first experiment in Chapter 4.

Table:5.4 mRNA levels for pro-inflammatory cytokines was established by semi-quantitative RT-PCR. Changes in pro-inflammatory cytokine mRNA were normalised against β-actin. Whole blood from pigs infected with App and subsequently treated with saline, Excenel, or recombinant cytokines at high and low concentrations. Data presented as mean±SEM, n=9, bnd int = band intensity.

Assay	saline	IL-4 lo	IL-4 hi	IL-1ra lo	IL-1ra hi	Excenel
IL-8 mRNA bnd int 0h	0.14 +/- 0.14	0.33+/- 0.21	0.19+/- 0.19	0.25+/- 0.16	0.07+/- 0.07	0.37+/- 0.17
bnd int 24h	0.56+/- 0.18	0.85+/- 0.11	0.88+/- 0.18*	0.36+/- 0.18	0.41+/- 0.19	0.39+/- 0.19
bnd int 48h	0.45+/- 0.17	0.38+/- 0.18	0.00+/- 0.00	0.42+/- 0.16	0.70+/- 0.15	0.48+/- 0.15
bnd int 1 week	0.37+/- 0.23	0.25+/- 0.17	0.00+/- 0.00	0.63+/- 0.20	0.43+/- 0.25	0.57+/- 0.15
bnd int 2 weeks	0.18+/- 0.18	0.29+/- 0.19	0.74+/- 0.13#	0.20+/- 0.13	0.38+/- 0.12	0.00+/- 0.00
IL-1β mRNA bnd int 0h	0.00+/- 0.00	0.00+/- 0.00	0.00+/- 0.00	0.00+/- 0.00	0.17+/- 0.11	0.43+/- 0.20
bnd int 24h	0.28+/- 0.14	0.59+/- 0.15	0.57+/- 0.18	0.09+/- 0.09^	0.36+/- 0.17	0.34+/- 0.17
bnd int 48h	0.72+/- 0.16	0.30+/- 0.19	0.23+/- 0.15	0.47+/- 0.18	0.73+/- 0.16	0.62+/- 0.20
bnd int 1 week	0.36+/- 0.22	0.36+/- 0.17	0.56+/- 0.23	0.46+/- 0.20	0.71+/- 0.24	0.46+/- 0.18
bnd int 2 weeks	0.47+/- 0.21	0.28+/- 0.18	0.53+/- 0.19	0.11+/- 0.11	0.17+/- 0.12	0.13+/- 0.13
IL-6 mRNA bnd int 0h	0.00+/- 0.00	0.00+/- 0.00	0.00+/- 0.00	0.00+/- 0.00	0.00+/- 0.00	0.00+/- 0.00
bnd int 24h	0.00+/- 0.00	0.00+/- 0.00	0.00+/- 0.00	0.00+/- 0.00	0.00+/- 0.00	0.00+/- 0.00
bnd int 48h	0.00+/- 0.00	0.00+/- 0.00	0.00+/- 0.00	0.00+/- 0.00	0.00+/- 0.00	0.00+/- 0.00
bnd int 1 week	0.00+/- 0.00	0.00+/- 0.00	0.00+/- 0.00	0.00+/- 0.00	0.00+/- 0.00	0.00+/- 0.00
bnd int 2 weeks	0.00+/- 0.00	0.00+/- 0.00	0.00+/- 0.00	0.00+/- 0.00	0.00+/- 0.00	0.00+/- 0.00
TNFα mRNA bnd int 0h	1.30+/- 0.09	0.53+/- 0.24	1.44+/- 0.08	0.90+/- 0.20	0.95+/- 0.21	1.14+/- 0.23
bnd int 24h	0.35+/- 0.18	0.22+/- 0.14	0.56+/- 0.25	0.56+/- 0.18	0.68+/- 0.24	0.41+/- 0.20
bnd int 48h	0.49+/- 0.24	0.20+/- 0.20	0.00+/- 0.00	0.00+/- 0.00	0.16+/- 0.16	0.00+/- 0.00
bnd int 1 week	0.61+/- 0.38	0.22+/- 0.14	0.00+/- 0.00	0.00+/- 0.00	0.00+/- 0.00	0.00+/- 0.00
bnd int 2 weeks	0.60+/- 0.27	0.17+/- 0.17	0.15+/- 0.15	0.11+/- 0.11	0.13+/- 0.13	0.00+/- 0.00

^ IL-1ra lo vs IL-4 hi and IL-4 lo at 24h p<0.05, * IL-4 hi vs IL-1ra lo at 24hp<0.05,#IL-4 hi vs sline p<0.05

5.3.3.3 Cell Counts and Differential White Cell Counts

Table:5.5 Total white blood cell and differential counts of blood smears from pigs' infected with App and subsequently treated with saline, Excenel, or recombinant cytokines at high and low concentrations. Data presented as mean±SEM, n=9.

Assay	saline	IL-4 lo	IL-4 hi	IL-1ra lo	IL-1ra hi	Excenel
WBC 0h cell/ml	19.29+/-1.03	17.46+/-0.59	18.00+/-0.94	17.73+/-1.38	20.68+/-2.02	18.30+/-0.82
24h cell/ml	30.28+/-2.35*	30.70+/-2.61	30.09+/-2.00	28.80+/-2.47	34.11+/-3.59	30.59+/-1.61
48h cell/ml	22.92+/-1.68	23.54+/-1.69	22.67+/-1.75	17.56+/-1.96	25.57+/-2.75	18.94+/-1.44
1 week cell/ml	24.57+/-2.71	24.14+/-1.62	26.31+/-1.42	24.14+/-1.12	26.47+/-2.59	22.02+/-1.30
2 weeks cell/ml	14.50+/-1.46	16.79+/-1.83	16.76+/-2.00	16.14+/-0.95	20.17+/-3.02	14.76+/-1.04
Neutrophil count 0h cell/mL	6.33+/-0.45	6.47+/-0.59	6.70+/-0.60	6.01+/-0.56	7.12+/-1.07	4.91+/-0.66
24h cell/mL	15.53+/-1.74	19.04+/-2.47	16.96+/-1.72	18.88+/-2.13	21.59+/-2.24	19.26+/-0.94
48h cell/mL	12.31+/-1.52	14.66+/-1.55	13.17+/-1.48	7.26+/-0.89*	14.97+/-2.52	9.41+/-1.66
1 week cell/mL	10.53+/-1.75	13.17+/-1.19	11.92+/-2.21	10.15+/-1.42	13.89+/-2.31	8.13+/-1.29
2 weeks	3.04+/-0.60	3.76+/-0.53	4.66+/-0.63	5.00+/-0.62	6.28+/-1.79	3.00+/-0.43
Lymphocyte count 0h cell/mL	10.45+/-0.85	8.32+/-0.22	8.85+/-0.76	9.23+/-0.79	10.69+/-1.52	11.19+/-0.37
24h cell/mL	11.58+/-1.57	9.29+/-0.75	10.62+/-1.69	8.21+/-0.49	9.74+/-1.32	9.37+/-1.04
48h cell/mL	8.64+/-0.64	7.17+/-0.60	7.70+/-0.97	6.67+/-0.78	8.43+/-0.91	9.89+/-0.97
1 week cell/mL	11.97+/-1.64	9.26+/-0.56	12.83+/-1.43	11.94+/-0.46	10.51+/-1.00	12.20+/-0.97
2 weeks cell/mL	10.44+/-1.08	11.64+/-1.81	10.52+/-1.54	9.87+/-0.43	12.47+/-1.21	10.61+/-0.81

* IL-1ra lo vs saline at 48h p<0.05

There was an increase in WBC 24 hours after App challenge in all the treatment groups which subsequently decreased over time (Table 5.5).

Lymphocyte population in whole blood of treated pigs were relatively constant across time (Table 5.5). Neutrophils in all the treatment groups increased markedly 24 hours after App challenge then decreased across time. IL-1ra lo treated pigs produced the

least amount of circulating neutrophils at 48 hours post infection (Table 5.5) and this

level was significantly lower than the saline treated pigs (p<0.05).

Table:5.6 Differential counts from blood smears of pigs' infected with App and subsequently treated with saline, Excenel, or recombinant cytokines at high and low concentrations. Data presented as mean±SEM. n=9, * saline vs all treatments except Excenel at 2 weeks p<0.05.

Assay	saline	IL-4 lo	IL-4 hi	IL-1ra lo	IL-1ra hi	Excenel
Monocyte count cell/mL 0h	2.40+/-0.24	2.51+/-0.28	2.12+/-0.38	2.21+/-0.34	2.58+/-0.46	1.98+/-0.19
24h	2.58+/-0.53	2.05+/-0.27	2.12+/-0.31	1.35+/-0.23	2.32+/-0.37	1.64+/-0.12
48h	1.57+/-0.18	1.57+/-0.21	1.55+/-0.20	1.22+/-0.13	1.69+/-0.25	1.29+/-0.17
1 week	1.71+/-0.27	1.42+/-0.13	1.32+/-0.14	1.69+/-0.14	1.48+/-0.29	1.49+/-0.20
2 weeks	0.92+/-0.11	1.09+/-0.14	1.04+/-0.10	0.92+/-0.06	1.17+/-0.16	0.94+/-0.07
Eosinophil count cell/mL 0h	0.11+/-0.03	0.16+/-0.04	0.33+/-0.05	0.27+/-0.07	0.29+/-0.07	0.21+/-0.08
24h	0.59+/-0.10	0.31+/-0.09	0.39+/-0.20	0.36+/-0.13	0.46+/-0.18	0.33+/-0.17
48h	0.40+/-0.05	0.15+/-0.07	0.24+/-0.08	0.47+/-0.12	0.26+/-0.11	0.50+/-0.09
1 week	0.37+/-0.10	0.30+/-0.08	0.24+/-0.06	0.37+/-0.07	0.58+/-0.09	0.21+/-0.05
2 weeks	0.09+/-0.03*	0.30+/-0.07	0.36+/-0.10	0.53+/-0.16	0.24+/-0.04	0.20+/-0.06

No basophils were found (results not shown) , ^ Excenel vs saline at 48h p<0.05

Even though there was not a significant difference between the treatment groups, there was a trend of reduction in monocytes across time in the whole blood of all treated pig groups. Pigs treated with IL-1ra lo, saline and Excenel showed increased eosinophil numbers (not significant) as compared to the remaining treatments 48 hours after App infection (Table 5.7). Eosinophil numbers in whole blood of all the treated pigs were significantly higher (p<0.05) 2 weeks after App challenge than that of the saline treated pigs with the exception of the Excenel treated pigs.

Table:5.7 Quantity of cells with IgG and IgG_1 receptors present in whole blood from pigs infected with App and subsequently treated with saline, Excenel, or recombinant cytokines at high and low concentrations. Data presented as mean±SEM. n=9.

Assay	saline	IL-4 lo	IL-4 hi	IL-1ra lo	IL-1ra hi	Excenel
B cell IgG 0h cell/mL	2.77+/-0.54	1.92+/-0.23	1.85+/-0.27	1.90+/-0.23	2.19+/-0.24	2.18+/-0.31
24h cell/mL	2.86+/-0.74	2.21+/-0.28	2.52+/-0.39	1.29+/-0.19*	2.01+/-0.30	1.87+/-0.35
48h cell/mL	3.56+/-0.44	2.19+/-0.38	2.51+/-0.23	1.63+/-0.34^#*	3.48+/-0.61	3.00+/-0.34
1 week cell/mL	3.21+/-0.87	2.32+/-0.39	3.28+/-0.27	2.67+/-0.39	2.63+/-0.37	2.92+/-0.28
2 weeks cell/mL	1.76+/-0.30	1.52+/-0.17	1.57+/-0.41	1.55+/-0.33	1.64+/-0.31	1.73+/-0.19
B cells IgG_1 0h cell/mL	0.45+/-0.09	0.30+/-0.09	0.40+/-0.10	0.48+/-0.15	0.38+/-0.08	0.40+/-0.12
24h cell/mL	0.36+/-0.09	0.38+/-0.09	0.34+/-0.09	0.17+/-0.03	0.29+/-0.08	0.22+/-0.09
48h cell/mL	1.79+/-0.46&	0.81+/-0.17	0.89+/-0.21	1.10+/-0.23	1.24+/-0.26	1.47+/-0.34
1 week cell/mL	0.60+/-0.18	0.33+/-0.08	0.72+/-0.18	0.64+/-0.12	0.63+/-0.17	0.53+/-0.09
2 weeks cell/mL	0.11+/-0.04	0.17+/-0.04	0.16 0.06	0.29+/-0.11	0.20+/-0.06	0.14+/-0.03

* saline vs IL-1ra lo p<0.05, & saline vs IL-4 lo and IL-4 hi at 48h p<0.05, ^ IL-1ra hi vs IL-1ra lo p<0.05, # Excenel vs IL-1ra lo at 48h p<0.05.

In all the treated pigs' blood the number of B cells expressing IgG remained relatively constant 24 hours after App challenge, with the exception of the pigs treated with IL-1ra lo which had significantly reduced B cells (p<0.05). The pigs treated with IL-1ra lo had B cell numbers reduced by almost half that of the B cells produced by pigs treated with IL-1ra hi, saline and Excenel 48 hours after App challenge (Table 5.7). Cells expressing IgG decreased 2 weeks after App challenge in all treatment groups. There was little difference between B cells treatment groups for expressing IgG_1 at most sample times, with the exception of the pigs treated with IL-4 lo and IL-4 hi,

who had the lowest numbers of IgG_1 producing B cells 48 hours after App challenge

($p < 0.05$) (Table 5.7).

5.3.3.5 T cells counts

Table:5.8: T cell counts enumerated by flow cytometry on whole blood from pigs infected with App and subsequently treated with saline, Excenel, or recombinant cytokines at high and low concentrations. Data is presented as the mean±SEM, n=9.

Assay	saline	IL-4 lo	IL-4 hi	IL-1ra lo	IL-1ra hi	Excenel
CD 3 % 0h	72.89+/-	73.63+/-	69.87+/-	72.37+/-	74.41+/-	74.94+/-
24h	69.11+/- 1.94	69.37+/- 1.65	63.50+/- 2.40	71.13+/- 1.34	70.83+/- 2.24	69.48+/- 2.48
48h	67.24+/- 1.25	65.02+/- 1.39	59.92+/- 1.35	64.43+/- 2.13	66.91+/- 1.34	62.29+/- 8.15
1 week	67.42+/- 1.73	69.85+/- 1.69	64.55+/- 2.66	58.44+/- 7.89	67.85+/- 2.27	71.07+/- 2.53
2 weeks	46.90+/- 8.91	56.17+/- 7.44	57.59+/- 2.86	48.52+/- 9.31	64.93+/- 3.06	64.08+/- 2.93
CD 3cells/mL 0h	7.60+/- 0.64	6.00+/- 0.29	6.25+/- 0.65	6.77+/- 0.56	8.06+/- 1.27	8.22+/- 0.29
24h	7.90+/- 1.02	6.44+/- 0.55*	6.81+/- 1.18	5.80+/- 0.27	6.96+/- 1.10	6.57+/- 0.83
48h	5.68+/- 0.43	4.68+/- 0.41	4.66+/- 0.66	4.26+/- 0.49	5.64+/- 0.62	6.39+/- 1.14
1 week	7.97+/- 1.02	6.52+/- 0.53	8.39+/- 1.16	7.81+/- 0.40	7.16+/- 0.78	8.80+/- 0.94
2 weeks	4.60+/- 1.00	7.61+/- 1.50	6.05+/- 0.87	6.15+/- 0.32	8.09+/- 0.86*	6.92+/- 0.77
CD4 % 0h	28.28+/- 1.74	28.69+/- 1.69	31.92+/- 1.56	31.67+/- 2.11	31.21+/- 2.19	26.36+/- 1.70
24h	26.54+/- 1.94	29.60+/- 1.80	30.24+/- 1.73	28.79+/- 1.79	29.54+/- 2.57	26.12+/- 1.35
48h	22.01+/- 3.29	25.77+/- 1.24	26.82+/- 1.43	26.13+/- 1.07	27.66+/- 2.03	23.62+/- 1.12
1 week	27.19+/- 1.35	27.11+/- 1.86	28.61+/- 1.23	24.43+/- 3.25	27.14+/- 1.89	24.64+/- 1.90
2 weeks	11.39+/- 1.92	13.10+/- 2.52	14.60+/- 2.56	15.54+/- 3.23	16.11+/- 2.04	9.30+/- 2.27
CD4 cells/mL 0h	2.89+/- 0.23	2.51+/- 0.15	2.78+/- 0.21	2.82+/- 0.16	3.53+/- 0.82	2.93+/- 0.15
24h	2.87+/- 0.26	2.71+/- 0.22	3.31+/- 0.66	2.33+/- 0.14	3.07+/- 0.79	2.45+/- 0.30
48h	1.82+/- 0.31	1.83+/- 0.15	2.01+/- 0.21	1.72+/- 0.18	2.37+/- 0.35	2.31+/- 0.22
1 week	3.17+/- 0.36	2.47+/- 0.17	3.65+/- 0.39	3.28+/- 0.20	2.92+/- 0.43	2.92+/- 0.24
2 weeks	1.14+/- 0.25^	1.81+/- 0.57	1.71+/- 0.51	1.98+/- 0.20	2.03+/- 0.37	0.94+/- 0.19#

* saline vs IL-1ra hi $p<0.05$, # excenel vs all treatment except saline $p<0.05$, ^ saline vs IL-4 lo and hi $p<0.05$.

The results for T cell counts are presented as percentages as well as cells per mL so that comparisons could be made between this data and that described in the previous Chapter (Table 5.8-5.9).

Pigs treated with IL-1ra hi had significantly higher numbers of T cells 2 weeks after challenge compared with saline-treated pigs (Table 5.8). When described as percentages, T cells decreased 48 hours after App challenge in all treatment groups. This trend was also seen in the T cells with the CD4 and CD8 receptors (data as percentages) in the whole blood of all the treated pigs (Table 5.8-5.9). The decreasing trend of T cells with the CD4 receptor, 48 hours after App infection which occurred in all the treatment groups (presented as percentages in Table 5.8) is also observed in the data presented as cells/mL. At the conclusion of the trial the saline control and Excenel treated pigs produced the least (data as cell/mL) T helper cells (Table 5.8).

T cells expressing CD8 markers, were reduced 48 hours after challenge (data presented as cell/mL) as compared to 24 hours post App with the exclusion of the groups treated with IL-1ra hi (Table 5.9).

The T cell counts by flow cytometry is a method for determining the percentages of different subpopulations of cells in whole blood using the technique of flow cytometry". The raw data is given as % of cells gated which is then multiplied by the total Lymphocytes counted (which is obtained by using a haemocytometer to count Lymphocyte in a 200 total of cells then multiply by WBC) which then gives you a value of cells per mL. The data was presented as both % and cells per mL to allow comparison between experiments and Chapters due to a machine error the WBC (Table 4.8) for the App challenge model at 24 hours was not obtained, therefore cells/mL for this sample time was not calculated.

Table 5.9: Cluster designation (CD) lymphocyte data obtained from whole blood from pigs' infected with App and subsequently treated with saline, Excenel, or recombinant cytokines at high and low concentrations. Data is presented as the mean±SEM, n=9.

Assay	saline	IL-4 lo	IL-4 hi	IL-1ra lo	IL-1ra hi	Excenel
CD 8 % 0h	37.80+/- 2.25	39.25+/- 4.44	42.15+/- 2.34	41.73+/- 3.19	42.37+/- 2.59	38.56+/- 2.51
24h	36.76+/- 3.09	39.91+/- 2.14	42.90+/- 3.81	43.13+/- 4.16	39.89+/- 4.15	35.97+/- 2.84
48h	30.56+/- 1.82	30.96+/- 1.34	31.97+/- 2.36	33.84+/- 2.47	32.44+/- 1.67	30.18+/- 1.68
1 week	31.74+/- 1.66	33.05+/- 1.61	35.68+/- 0.75	33.36+/- 4.93	33.26+/- 1.98	32.40+/- 1.29
2 weeks	30.11+/- 5.06	29.52+/- 5.88	28.86+/- 5.82	22.02+/- 5.20	29.83+/- 3.64	24.47+/- 1.68
CD 8 cells/mL 0h	3.88+/- 0.32	3.20+/- 0.44	3.76+/- 0.41	3.82+/- 0.34	4.54+/- 0.74	4.19+/- 0.24
24h	3.94+/- 0.33	3.78+/- 0.43	4.44+/- 0.70	3.54+/- 0.44	4.13+/- 1.06	3.47+/- 0.56
48h	2.66+/- 0.26	2.25+/- 0.27	2.45+/- 0.34	2.28+/- 0.30	2.80+/- 0.37	2.86+/- 0.32
1 week	3.73+/- 0.51	3.05+/- 0.22	4.57+/- 0.52	4.45+/- 0.34	3.57+/- 0.52	3.91+/- 0.29
2 weeks	3.21+/- 0.82	4.37+/- 1.41	3.48+/- 1.23	2.82+/- 0.48	4.00+/- 0.92	2.58+/- 0.26
CD 4:8 % 0h	0.76+/- 0.04	0.87+/- 0.19	0.78+/- 0.07	0.77+/- 0.05	0.75+/- 0.05	0.69+/- 0.04
24h	0.74+/- 0.05	0.76+/- 0.07	0.80+/- 0.14	0.70+/- 0.06	0.77+/- 0.07	0.76+/- 0.07
48h	0.73+/- 0.10	0.85+/- 0.06	0.89+/- 0.10	0.79+/- 0.05	0.87+/- 0.07	0.80+/- 0.04
1 week	0.87+/- 0.06	0.82+/- 0.05	0.80+/- 0.04	0.75+/- 0.05	0.83+/- 0.06	0.75+/- 0.05
2 weeks	0.41+/- 0.07	0.48+/- 0.07	0.49+/- 0.08	0.77+/- 0.09*	0.51+/- 0.09	0.37+/- 0.08

Prior to App challenge and until 1 week after challenge, there was no difference in CD4:8 ratio across all treatment groups. However, 2 weeks after challenge, pigs treated with IL-1ra lo still had a significantly higher CD4:8 ratio ($p < 0.05$) while this ratio decreased in all other groups (Table 5.9). At the 2 week time point the saline and Excenel treated pigs had the lowest CD4:8 ratio.

5.3.3.4 ELISA

Table:5.10 Immunoglobulin G and Immunoglobulin A concentration in serum from pigs' infected with App and subsequently treated with saline, Excenel, or recombinant cytokines at high and low concentrations. Data presented as mean±SEM. n=9.

Assay	saline	IL-4 lo	IL-4 hi	IL-1ra lo	IL-1ra hi	Excenel
serum IgA 0h µg/mL	1316+/-269	945+/-209	1515+/-207	1289+/-305	1626+/-463	836+/-176
24h µg/mL	1015+/-282	1104+/-278	1912+/-331@	982+/-265	1666+/-463	785+/-175
48h µg/mL	1711+/-477	1402+/-463	1812+/-221	1243+/-227.16	1410+/-275	1421+/-194
1 week µg/mL	2156+/-233	1477.67+/-92.00	1391.96+/-193.53	1607.48 358.58	1542.74+/-153.06	1924+/-344
2 weeks µg/mL	774+/-285	328+/-42&	1446+/-293@	687+/-164	604+/-155	1249.05+/-317.41
serum IgG 0h µg/mL	4995+/-	5387+/-	5876+/-	5389+/-	7417+/-	7018+/-
24h µg/mL	1964+/-726	958+/-354	1861+/-871	423+/-177	920+/-621	2859 1077
48h µg/mL	3559+/-1023	3648+/-1331	3904+/-1046	1295+/-649*	1552+/-851	3993+/-1168
1 week µg/mL	2203.16+/-858.72	1300.76+/-772.46	2705.13+/-1015.98	991.20+/-315.50	1321+/-439	1973+/-393
2 weeks µg/mL	1514+/-359	938+/-256	2303+/-456	1106+/-373	1862+/-1053	1148+/-277

[@] saline vs IL-4 hi $p<0.05$, [&] saline vs IL-4 lo at 2wks $p<0.05$

The amount of serum IgA found in the IL-4 hi treated pigs was significantly greater ($p<0.05$) at 24 hours post infection than that found in the saline treated pigs (Table 5.10). Pigs treated with IL-4 hi also had the largest level of serum IgA at 2 weeks post infection as compared to the saline-treated group, while the low dose IL-4 treated pigs had the lowest serum IgA ($p<0.05$) (Table 5.10) 2 weeks after App challenge. Pigs treated with IL-1ra lo produced the least amount of serum IgG protein 48 hours after App challenge as compared to the IgG produced by the saline control pigs. At 2 week post App all the treatment groups showed decreased serum IgG (Table 5.10) compared to baseline levels prior to App challenge.

5.3.4 Pathology
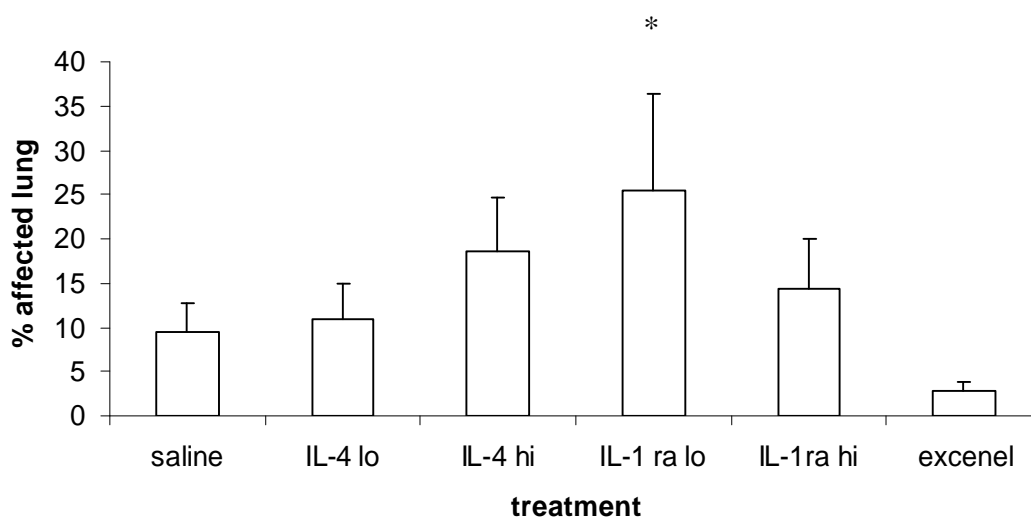
Figure 5.4: Degree of pleuropneumonia expressed as the percentage of affected lung by weight. Lungs obtained after euthanasia of pigs infected with App and subsequently treated with saline, Excenel, or recombinant cytokines at high and low concentrations. Bars show group mean±SEM, n=9, * IL-1ra lo vs Excenel $p < 0.05$.

The Excenel treated pigs had a significantly lower percentage of affected lung as compared to IL-1ra low ($p < 0.05$) and comparatively less than the other treatment groups and the saline control group (Figure 5.4).

There appeared to be a positive relationship between the percentage affected lung and clinical scores (Figure 5.5). Pigs that had higher clinical scores during the challenge period tended to have a greater proportion of their lung affected by App lesions.
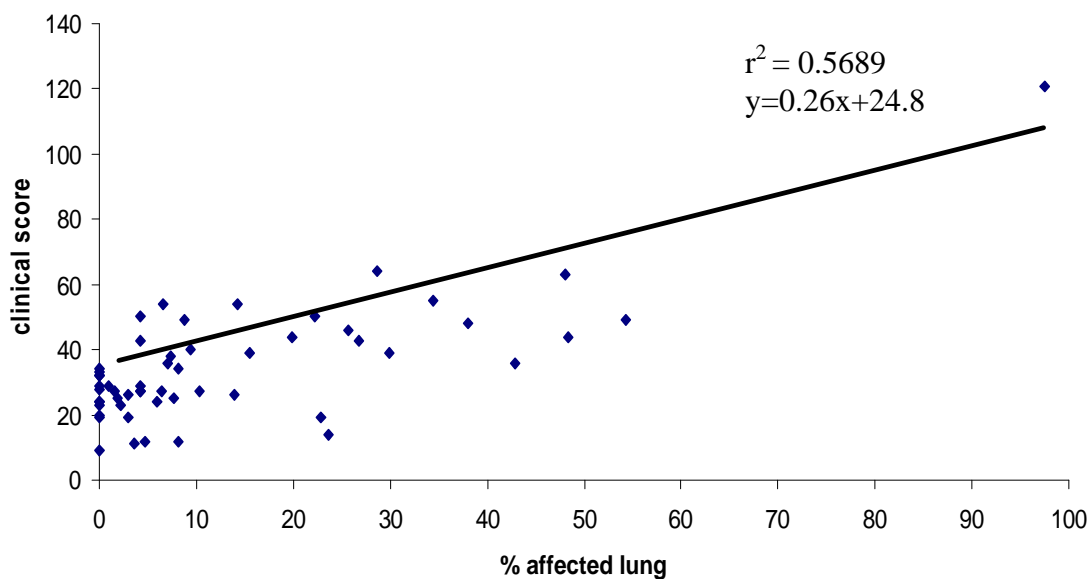
Figure 5.5: Affected lung plotted against clinical scores from pigs infected with App and subsequently treated with saline, Excenel, or recombinant cytokines at high and low concentrations.
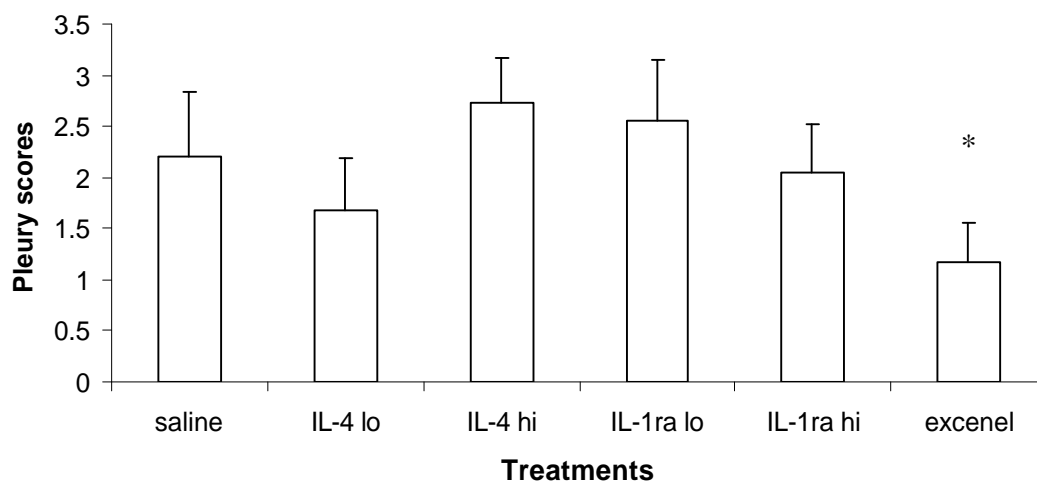
Figure 5.6: Degree of pleurisy expressed as pleurisy score (0-5) in pigs infected with App and subsequently treated with saline, Excenel, or recombinant cytokines at high and low concentrations. Bars show group mean±SEM, n=9, * Excenel vs IL-4 hi p<0.05.

Pigs treated with Excenel had the lowest mean pleurisy scores compared to the saline control group (Figure 5.6). Excenel treated pigs showed the lower pleurisy than the pigs treated with IL-4 high,IL-1ra lo and IL-1ra hi.

Figure 5.7 compares the appearance of App-affected lungs and clean lungs apparently free of obvious App lesions. Figure 5.7a is from a pig treated with saline while the healthy lung in Figure 5.7b was from a pig treated with IL-1ra hi.
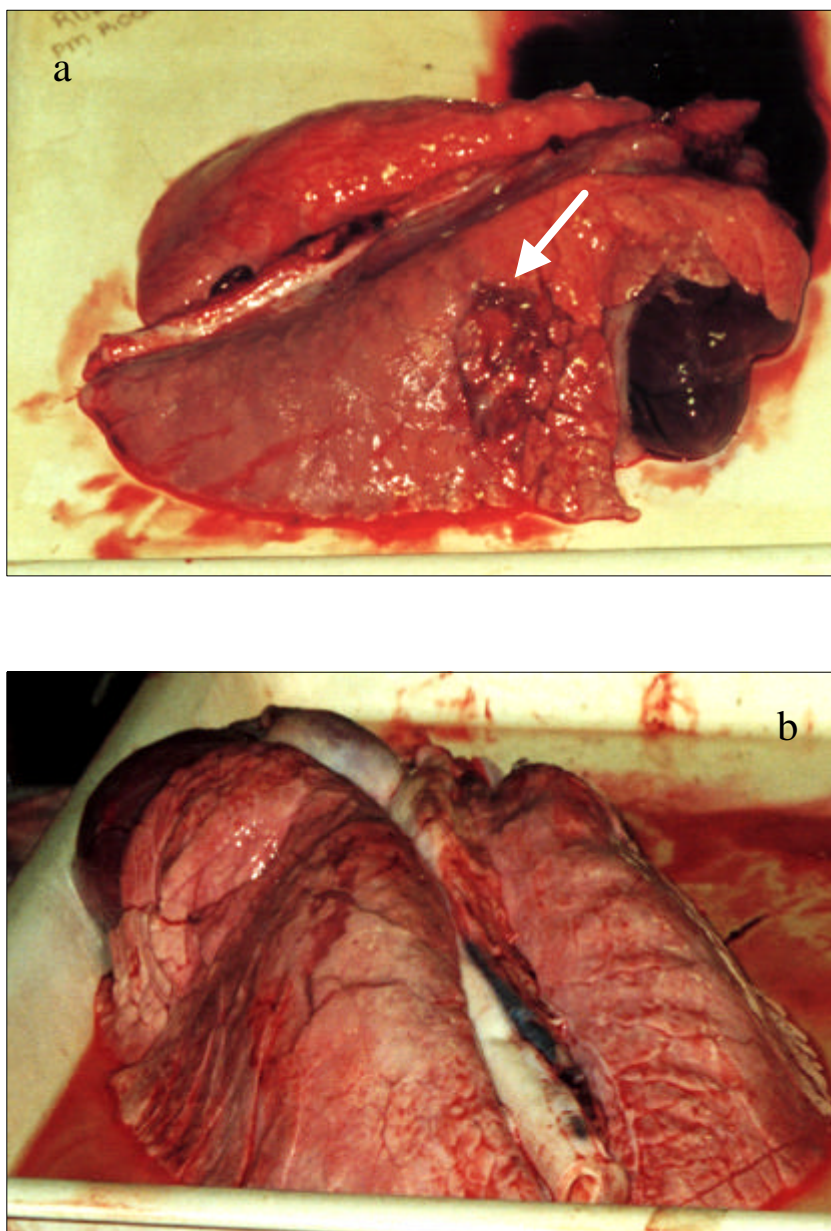
Figure 5.7 Lung from pigs challenged with App and subsequently treated with saline (a) and IL-1ra hi (b). Post-mortem was performed 2 weeks after challenge. The arrow indicates a gross App lesion.
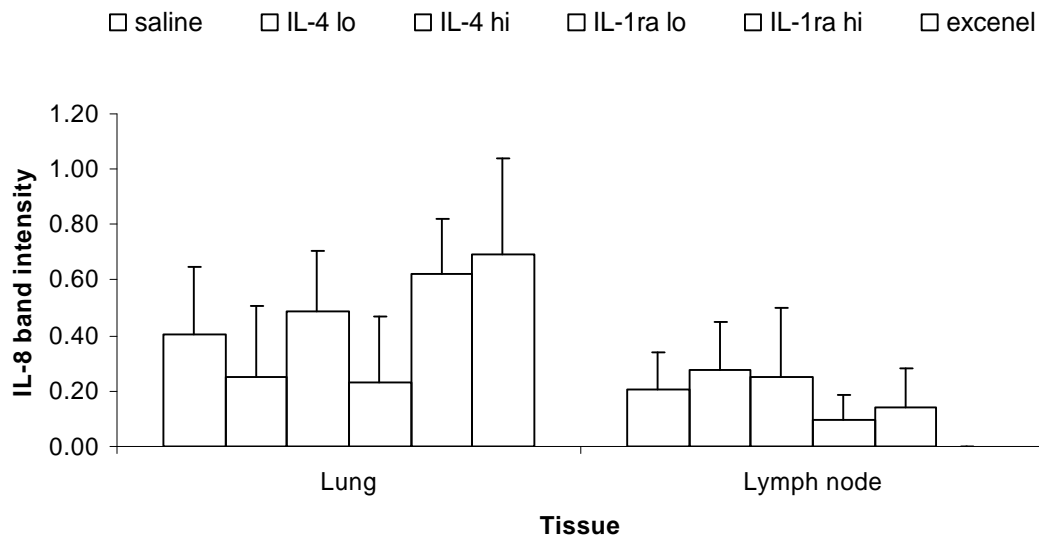
Figure 5.8: Levels of mRNA for pro-inflammatory cytokine IL-8 determined by RT-PCR in lung and lymph node tissue post-mortem. Tissue taken from pigs infected with App and subsequently treated with saline, Excenel, or recombinant cytokines at high and low concentrations. The results were normalised by presenting results as a ratio of the cytokine over β actin mRNA expression. Bars show group mean±SEM.

RT-PCR performed on lung and lymph node tissue confirmed the presence of localised IL-8 mRNA (Figure 5.8). IL-8 mRNA was detected in all the lung and lymph tissue at a similar level regardless of the treatment administered. The pigs treated with IL-4 lo and IL-1ra lo produced slightly less IL-8 mRNA than the saline control treated pigs (not significant).
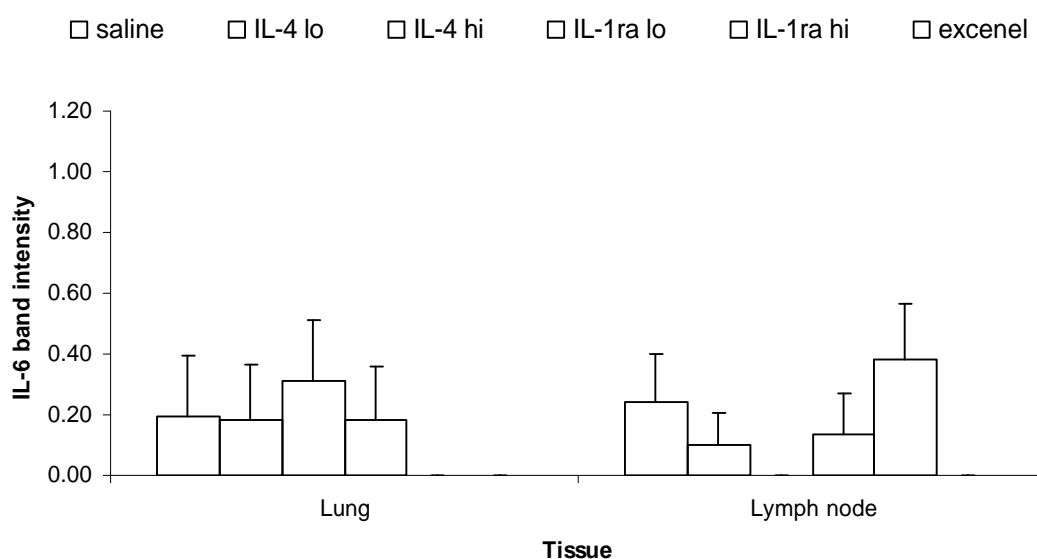
Figure 5.9: Levels of mRNA for pro-inflammatory cytokine IL-6 determined by RT-PCR in lung and lymph node tissue post-mortem. Tissue taken from pigs infected with App and subsequently treated with saline, Excenel, or recombinant cytokines at high and low concentrations. The results were normalised by presenting results as a ratio of the cytokine over β actin mRNA expression. Bars show group mean±SEM.

There was no IL-6 mRNA found in the lungs of pigs treated with IL-1ra hi and Excenel (Figure 5.9). Pigs treated with IL-4 hi produced slightly more IL-6 mRNA than the other treatments (not significant). However, IL-6 mRNA was not detected in the lymph nodes of pigs treated with IL-4 hi (Figure 5.9).

The *in situ* hybridization was performed 3 times on 5 sections of each treatment group.
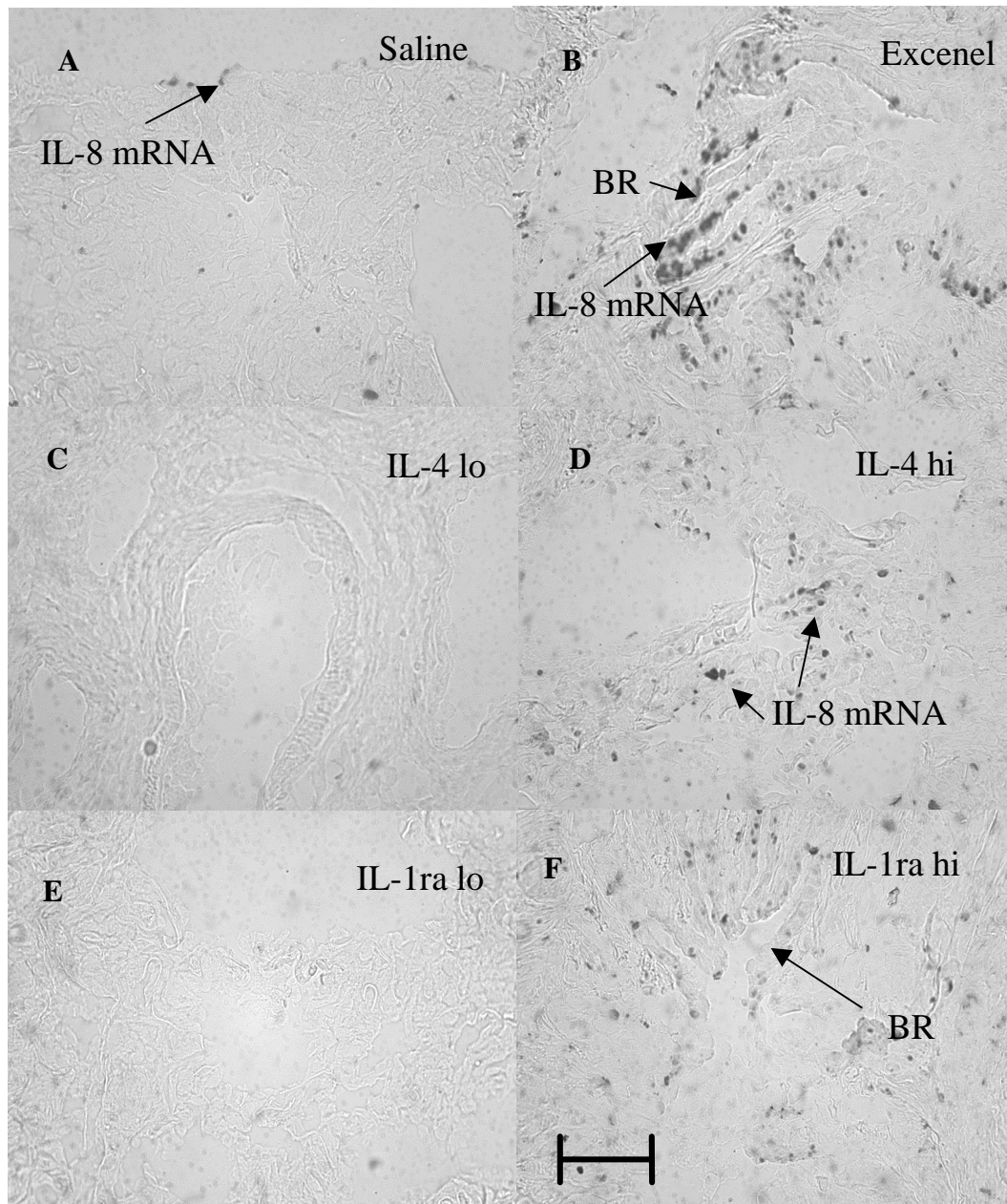
Figure 5.10: *In situ* hybridisation of IL-8 antisense oligonucleotide probe (positive probe) on lung tissue sections from pigs challenged with App and subsequently treated with saline, Excenel, or recombinant cytokines at high and low concentrations.

The dark spots indicate cells producing IL-8 mRNA. Bar represents 80μm. (Magnification x 400). BR – bronchus.
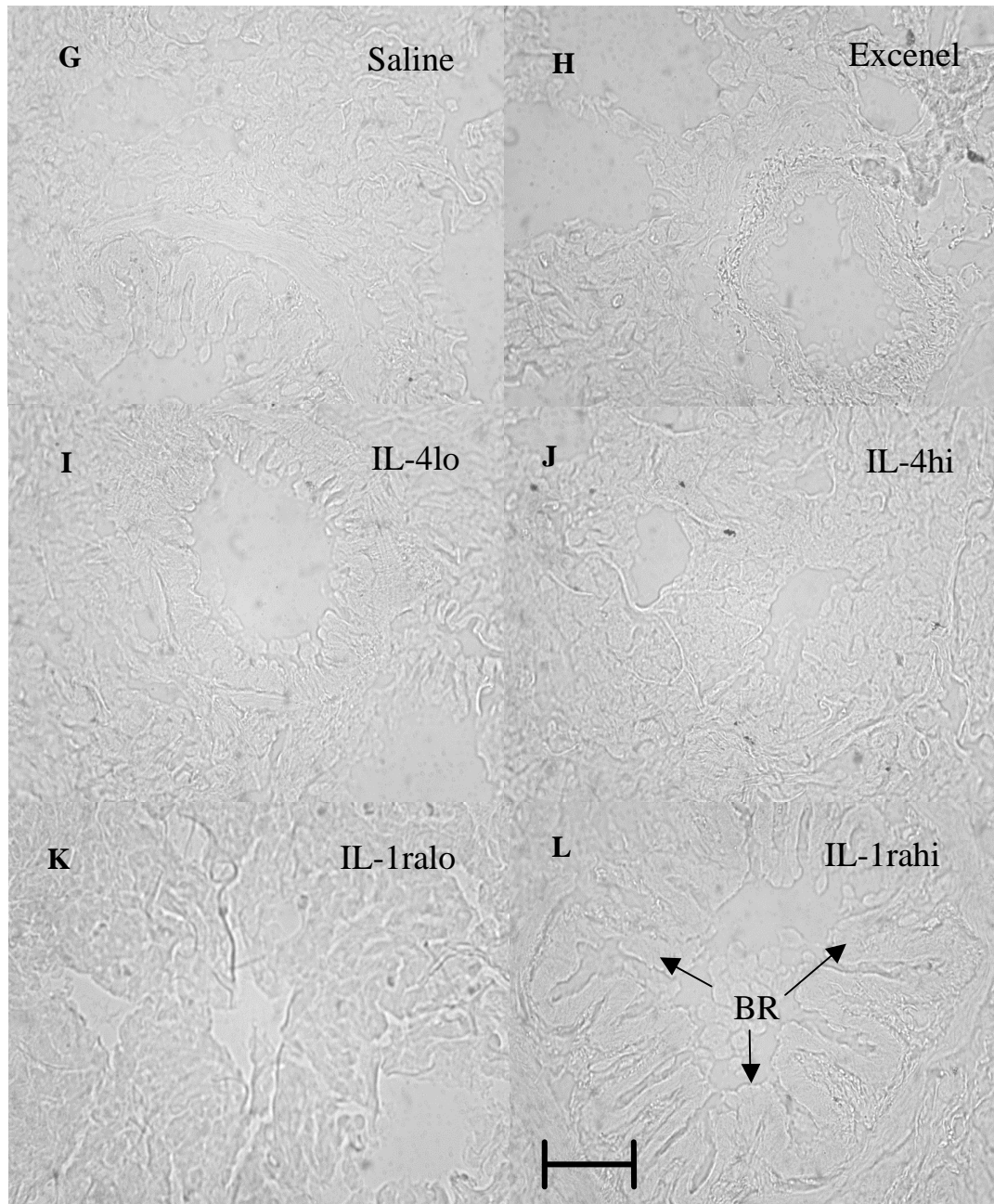
Figure 5.11: *In situ* hybridisation of IL-8 sense oligonucleotide probe (negative probe) on lung tissue sections from pigs challenged with App and subsequently treated with saline, Excenel, or recombinant cytokines at high and low concentrations. Bar represents 80µm. (Magnification x 400). BR – bronchus.

The expression of IL-8 mRNA was investigated using *in situ* hybridization in order to localise cytokine production in the lung (Figure 5.10). The lung tissue at post-mortem showed IL-8 mRNA near the lung lesions in some treatment groups. The spotting seen in Figure 5.10 appeared to be associated with macrophages and neutrophils. The bronchiolus of pigs treated with Excenel were found to contain large amounts of IL-8 mRNA since they stained heavily by *in situ* hybridization, while only a scattering of the cells in the saline control treated pigs were stained. Lungs from pigs treated with IL-4 hi and IL-1ra hi showed positive staining for IL-8. There was little or no IL-8 mRNA detected in lungs of pigs treated with IL-4 lo or IL-1ra lo. The trends in IL-8 mRNA levels detected by *in situ* hybridization (Figure 5.10) reflect those seen in RT-PCR on lung tissue (Figure 5.8). The IL-8 mRNA sense probe (negative control probe) did not hybridize to any lung tissue of any group (Figure 5.11).

A haematoxylin and eosin (H and E) stain was performed on a selection of lung tissue from each treatment group and a representative is shown (Figure 5.12). There is an increase in polymorphonuclear cells around the broncholi of lung tissue from pigs treated with IL-1ra lo (Figure5.12). The pigs treated with IL-4 hi also showed large numbers of polymorphonuclear cells in its lung tissue. Lung tissue from the saline, IL-4 lo and IL-1ra hi treatment pigs had normal morphology (Figure 5.12).
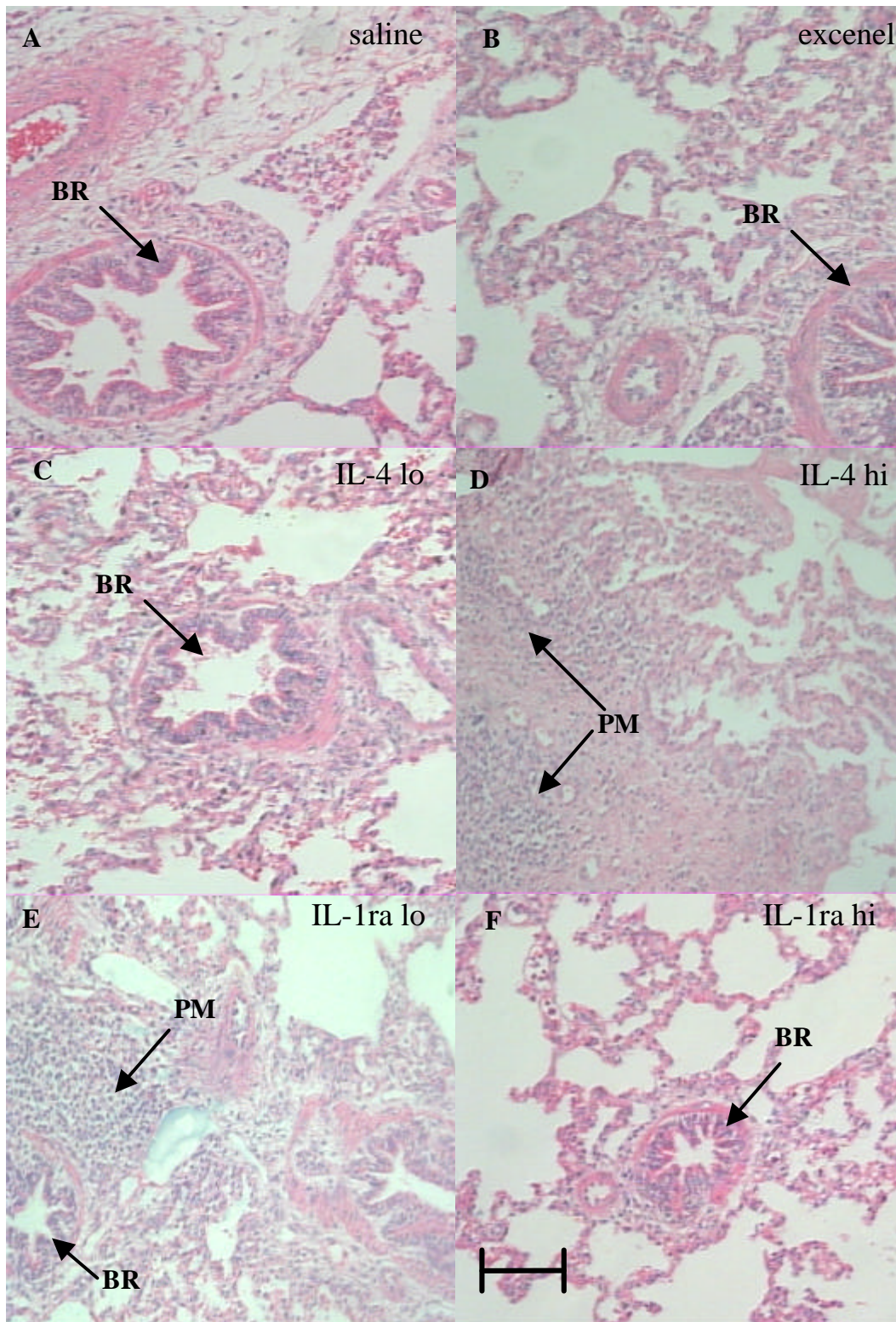
Figure 5.12. Sections of lungs from pigs challenged with App and subsequently treated with saline, Excenel, or recombinant cytokines at high and low concentrations; stained with H&E (Magnification x 400). A) Saline treatment. B) Excenel treatment. C) IL-4 treatment, low dose. D) IL-4 treatment, high dose. E) IL-1ra treatment, low dose. F) IL-1ra treatment, high dose. PM - lesions of abnormal morphology made up of polymorphonuclear cells. BR – bronchus. The bar represented 80μm.

5.4. Discussion

The dramatic weight increase of pigs prophylactically treated with IL-4 and IL-1ra as compared to the saline treated pigs reported in Chapter 4 unfortunately was not observed in this experimental trial examining therapeutic delivery of these treatments. No real weight difference was observed between the treatment groups in this trial. Clinical scores showed no difference between the pigs treated with saline and the remaining treated pigs with the exception of pigs treated with Excenel, which were significantly lower ($p<0.05$) in clinical scores compared to IL-1ra lo treated pigs (Figure 5.3). This trend was also observed in the percentage of affected lung (Figure 5.4).

The App infection was given at the same dosage of 7.5×10^5 cfu/mL in both the prophylactic trial (Chapter 4) and the therapeutic trail (Chapter 5). Even so, the pigs treated with saline in the prophylactic trial had approximately 3 times greater clinical symptoms and lung damage compared to the pigs treated with saline in the therapeutic trial.

Perhaps in the therapeutic trial the lack of weight variation between control treated pigs and cytokine-treated pigs may be due to the timing of infection and treatments, or the inability of treatments to deal with an already established infection (Figure 5.2). As the saline-treated pigs in the therapeutic trial failed to exhibit as severe an infection as they did in the prophylactic trial, it may be possible that the virulence of the inoculating bacteria was not maintained between trials. sub-culturing was done PPLO agar then transferred to PPLO broth and frozen at -80°C in 70% glycerol. The preparation of the inoculum was done by research staff at EMAI since we were working in collaboration. Retrospective enumeration of the inoculum would have

stopped questions like viability of the inoculums in Chapter 4 and 5. The pigs immunity or resistance to App may have come into question since they may have been exposed to this very bacteria before participating in this trial.

When comparing the cluster designation data (data as percentages) between the two trials, there is a decreasing trend in both trials in terms of T cell data. The therapeutic trail (Chapter 5) shows a decreasing trend 48 hour after App challenge in all the treated pigs T cells with the CD3, CD4 and CD8 receptor. The decreasing trend in the prophylactic trial (Chapter 4) however, occurred 24 hours after App challenge in all the treated pigs T cells with CD3, CD8 receptors. This may be related to the virulence of the bacteria between the trials. The T-lymphocytes may be mobilized to other areas such as the lung and leave the blood steam in response to infection. Since the prophylactic trial produced a decrease in peripheral T cells earlier in the infection perhaps the virulence of the bacteria used in this challenge was higher than that given to the pigs in the therapeutic trial.

Immunological and molecular biological assays showed an interesting perspective of the therapeutic App infection. The reduction in IL-8 mRNA in peripheral blood at 24 to 48 hours post infection found in the IL-4 hi treated pigs (Table 5.4) could be due to the ability of IL-4 to stop IL-8 production at a transcriptional level (Zhou *et al.,* 1994).

This reduction did not take place in the pigs treated with a low dose of IL-4. Perhaps the dose of IL-4 lo was too low. A similar dose given in the LPS prophylactic trial did not reduce the levels of IL-8 mRNA 24 hours post challenge, however the time lapse between the LPS challenge in Chapter 4 and the administration of IL-4 two days before App challenge reduced the production of IL-8 mRNA to an undetectable amount.

It is interesting to compare between the two trials. However it must be taken into consideration that there are many variables such as the virulence of bacterial challenge, number of pigs per group, blood sampling time and mainly the timing of administration of treatments, that differ between the 2 trials.

The absence of TNFα mRNA in the peripheral blood of pigs treated with IL-1ra lo, IL-4 hi and Excenel 48 hours and 1 week after App challenge (Table 5.4) could be associated with pathology of App, since these 3 treated pig groups exhibited the highest amount of affected lung and pleurisy. Interestingly, in support of this, Morrison *et al.,* (2000) found TNFα and IL-1β in moderate levels effectively controls lung infection of pigs. It was also found that lack of TNFα or the neutralization of TNFα can impair bacterial clearance from the lung of mice infected with a bacterial pneumonia infection (Laichalk *et al.,* 1996 and Standford *et al.*, 1999).

Pigs treated with IL-1ra lo produced lower levels of IL-1β mRNA in their peripheral blood at 24 hours post infection (Table 5.4) and also had high levels of App-associated pathology (Figure 5.4), which further support the argument that the absence of IL-1β and TNFα may contribute to pathology in the lung.

IL-6 mRNA was not detected in the lung of the pigs treated with IL-1ra hi and Excenel (Figure 5.9). IL-6 mRNA production is very difficult to detect as this cytokine is short-lived in the tissue and localized (Mire-Sluis and Thorpe 1998) which may have contributed to the absence of IL-6 in the lung of IL-1ra hi and Excenel treated pigs.

Neutrophil chemoattractant IL-8 was the major factor associated with of acute lung inflammation rather than App bacterial components (Huang *et al.,* 1999). The over

production of IL-8 may exacerbate disease caused by bacterial infection. Slocombe *et al.,* (1985) found from his research that animals with depleted neutrophils had normal lungs, whereas those that did not, suffered lung lesions after infection with *Pseudomonas haemolytica.* IL-8 recruits neutrophils to inflammatory sites (reviewed by Standiford *et al.,* 2000 and Murtaugh *et al.,* 1996) and lesions found in the respiratory tract of pigs suffering App infection are made up largely of neutrophils, macrophages and platelets (Liggett *et al.,* 1987). The IL-8 mRNA was probably produced by the alveoli macrophage and infiltrating neutrophils (Strieter 1992) in the lung. Therefore, IL-8 may be a significant player in inflammatory lung disease such as App infection.

Excenel, a commercially available broad spectrum antibiotic used for pigs suffering App infection is thought to bind to App-produced protein in the lung. The Excenel treated pigs produced a large amount of IL-8 mRNA in the lung (Figure 5.8). Excenel compound lyse the cell walls of the App bacteria. Pigs treated with Excenel had undetectable amounts of IL-8 mRNA in their peripheral blood at euthanasia. Pigs treated with Excenel did not produce any IL-6 mRNA in their lungs or lymph nodes at post-mortem or in their peripheral blood at any time.

In situ hybridization detected no IL-8 mRNA in the lung tissue of pigs treated with IL-1ra lo (Figure 5.10). However, it was detected at low levels when RT-PCR was performed (Figure 5.8). This could be due to the fact that PCR only required one signal to amplify while *in situ* hybridization needs more copies for detection. *In situ* hybridization showed (Figure 5.10) that IL-8 mRNA was being produced by alveolar macrophages as expected (Hakansson *et al.,* 1996; Khair *et al.,* 1996) and that infiltrating neutrophils were also producing IL-8 mRNA (Strieter 1992).

Inflammatory cells such as neutrophils and macrophages were found within alveoli interlobular spaces and the airway of animals in the IL-1ra lo treated group. The association between App lung infection and inflammatory cytokines such as IL-8 seems to suggest IL-8 is a direct cause of the pathology of the lung infection (Huang *et al.,* 1999; Baarsh *et al.,* 1995). IL-1ra and IL-4 given as a prophylactic (Chapter 4) reduced IL-8 mRNA production in the lung, however when given as a therapeutic did not have the same effect.

Pigs treated with IL-1ra hi produced significantly ($p<0.05$) more T lymphocytes (with the CD3 receptor) as compared to the saline control treated pigs. This suggest an elevated immune response is occurring with this treatment. Interestingly, this treatment group had no Polymorphonuclear Cells (PMN) present in the lung tissue at post-mortem despite IL-8 mRNA being present in the lung tissue and peripheral blood.

The lack of IL-6 in the lung tissue in this study could be explained in the findings of Scamurra *et al.,* (1996) who showed alveolar macrophages from pigs produced differing amounts of IL-6 which was stimulated by LPS. Scamurra *et al.,* (1996) found different pigs had different IL-6 (stimulated by LPS) ranging from low to high. Perhaps IL-1ra hi-treated pigs just produced an undetectable amount of IL-6 in the lungs as compared to the other treatment groups.

Pigs treated with saline and Excenel produced least T helper cells (CD4 receptor present) at conclusion of the trial.

IgG is the major immunoglobulin synthesized in the body. It diffuses into the extravascular body space where it neutralizes bacterial toxins (Roitt 1994). This

immunoglobulin is produced by B cells and therefore the more Ig protein present the more antigens from the infection are present. IgG_1 is a subclass of IgG, it makes up the largest class at 65% component of all the IgG_1-IgG_4. Significantly less B cells with IgG_1 were present in pigs treated with IL-4hi and IL-4 lo 48 hours after App as compared to the saline treated pig group. It is known that the growth and differentiation of B cells is stimulated by the secretion of IL-4 and IL-5 by Th2 lymphocytes (Tizard *et al.,* 1996). Perhaps IL-4 is stimulating Th 1 cytokine production instead of Th 2 lymphocyes cytokines. Nuntaprasert *et al.,* (2004) found that depending on administration of the IL-4 , it can cause either stimulate or inhibit pro-inflammatory cytokines like IL-8, TNFα, IL-6and IL-1α in pigs challenged with LPS.

IgG and IgA were detected by ELISA in the serum of treated pigs. IgA defends the exposed external surfaces eg. lung, gut, and nasal area from micro-organisms attack. It is synthesized locally and is in abundance in times of infection. Therefore it can be used as a tool to detect infection (Roitt 1994) serum levels of IgA may be indicative of IgA levels present in the lung.

As earlier stated IL-5 and IL-4 produced by Th 2 lymphocytes which stimulate B cells to grow and differentiate which then in turn produce IgG, IgA, IgM and Ig E (Tizard 1996). However in this study IgA production was significantly less in the serum of pigs treated with IL-4 lo as compared to the serum from saline control treated pigs 2 weeks after challenge (Table 5.10).

PMN are needed to isolate infection, however, too many neutrophils can cause even more lung damage in App infection such as necrosis (Liggett 1987). Pigs treated with IL-1ra lo had significantly less neutrophils at 48 hour post App and a reduced

numbers at 1 week which could contribute to the lung damage (Table 5.5). Pigs treated with IL-1ra lo also had the highest T lymphocyte CD4:8 ratio at the conclusion of the trial. Since PMN were found in the lung of IL-1ra lo treated pigs, neutrophils may have already crossed the blood tissue barrier and proceeded to the lung resulting in depleted neutrophils in the periphery. IL-8 mRNA in the peripheral blood was low and IL-8 mRNA was undetected in the lung tissue, *in situ,* of treated pigs treated with IL-1ra lo, since IL-8 is thought to recruit neutrophils (Standiford *et al.,* 2000) perhaps the PMN found in the lungs of this group were not made up of neutrophils but rather eosinophils and basophils. Eosinophils are known to produce tissue damage in the lung of asthmatics through its major protein eosinophil derived neurotoxin (Nicola 1997).

Another explanation is that PMN have neutrophils present in the lung of the pig treated with IL-1ra lo which could be recruited through by Leukotriene B_4 (LTB_4) through a different pathway (arachadonic pathway) (Taki, Iwata *et al.,* 1988; Atkins, Valenzano *et al.*, 1989; Pace, Profita *et al.*, 2004; VanderMeer, Menconi *et al.,* 1995).

None of the treatments delivered therapeutically after App infection increased weight gain. It is apparent in this study that clinical scores were considerably lower than those from the previous Chapter and the animals did not get as ill as the previous trial in Chapter 4. Due to infection already being established when treatments were given or differences in bacterial virulence between the trials.

5.5 *Experiment Summary*

- There was no difference in growth found between the treated pigs and saline (control) treated pigs.

- Saline and Excenel treated pigs had significantly less clinical signs and suffered less lung damage compared to the remaining treated pigs.

- Pigs treated with IL-1ra at a low dose suffered the most lung damage compared to the remaining treated pigs.

- IL-1ra lo, IL-4 hi and Excenel treated pigs produced no TNFα mRNA at 48 hours post infection.

- Polymorphonuclear cells were found in lung tissue from pigs treated with IL-1ra lo, IL-4 hi and Excenel treated pigs.

- The lung tissue of saline, Excenel, IL-4 hi and IL-1ra hi treated pig showed presence of IL-8 mRNA.

- IL-8 mRNA in pigs treated IL-4 hi reduced to an undetectable level at 24 and 48 hours post infection.

- In the lymphocyte proliferation assay killed bacteria (KB) was a better stimuli in whole blood than ConA, increasing lymphocyte proliferation (Table 5.2).

- IL-6 was not detected in the peripheral.

- IL-1ra and IL-4 administrated therapeutically did not have the ability to improve growth

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CHAPTER 6
GENERAL DISCUSSION

6.1 Discussion

Immunological mechanisms of infection associated with the inhibition of growth in pigs were examined in a series of in vivo studies in which App was the infection model. Both local and systemic effects of infection were examined.

The lung, along with the stomach, urinogenital tract and eye are mucosal surfaces with little if any natural bacterial flora, yet on infection these surfaces develop a cytokine response to bacterial pathogens (Svanborg *et al.,* 1993). Svanborg *et al.,* (1993) went on to state that stimulation of cytokines is influenced by the amount of interaction between bacteria and epithelium. Agace *et al.,* (1993), Cubitt *et al.,* (1995) and Eckmann *et al.,* (1993) found epithelial cells of mucosal surfaces can express and secrete pro-inflammatory and chemotatic cytokines in response to bacterial infection.

Epithelial cells in the respiratory tract produce cytokines such as IL-8, IL-6, TNF-α and GM-CSF (Khair *et al.,* 1996; Levine 1995) in response to bacterial challenge. In the therapeutic experimental trial (Chapter 5) IL-8 and IL-6 were found in the lung tissue at post-mortem.

Pro-inflammatory cytokines such as TNF-α, IL-1β, IL-8 and IL-6 have been identified as being associated with poor growth of pigs (Morrison *et al.,* 2000; Baarsch *et al.,* 1995). Other studies have found an association with lung damage in pigs and over-production of cytokines (TNF-α and IL-1β) which leads to poor health (Morrison *et al.,* 2000) and

growth.   It has also been noted that IL-1β and IL-8 were associated with pleuropneumonia severity in pigs (Baarsch *et al.,* 1995). The chemoattractant IL-8 has been linked to acute lung inflammation in pigs by Huang *et al.,* (1999). IL-1β and IL-6 were also found by Arend *et al.,* (1990) to be elevated in systemic juvenile chronic arthritis in humans. For all of the above reasons these pro-inflammatory cytokines were of interest and were measured in this study.

There are several examples of IL-6 and IL-8 production after infection with bacteria. Hakansson *et al.,* (1996) showed IL-6 and IL-8 are produced by airway epithelial cells due to the attachment of *Haemophilus influenzae*. Increased cytokine secretion has been shown by bronchial epithelia exposed to *Pseudomonas aeruginosa in vitro* (Dimango *et al.,* 1995; Massion *et al.,* 1994).

Over production of pro-inflammatory cytokines has been associated with the exacerbation of infection and reduced growth. To gain greater understanding of the relevant immune responses *in vitro*, it was necessary to find a mitogen which produced maximal stimulation of pro-inflammatory cytokines. This would help identify potential targets of interest and to test potential therapeutics to treat their effects.

Small *in vitro* trials were run (Chapter 3) and once the best inflammatory agent was determined, anti-inflammatory cytokines, receptor antagonists and anti-inflammatory drugs were tested for their ability to inhibit the production of pro-inflammatory cytokines.

The mitogens used were PMA, PHA, Con A and LPS and the optimal concentrations of these mitogens for maximum stimulation of pro-inflammatory cytokine mRNA were determined to be 10μg/mL, while 1x 10^7cfu/mL of KB was found to be the optimum concentration to use for further experiments.

In vitro experiments in Chapter 3 showed that blood taken from different pigs had varying amounts of baseline cytokines. Constitutive amounts of cytokines in the blood and tissue have long been established (Mayringer *et al.,* 2000). The experiments in Chapter 3 showed that of the mitogens tested PMA, LPS and KB performed the best by stimulating the most pro-inflammatory cytokines. Thus, if an anti-inflammatory cytokine or another substance inhibits or reduces the production of pro-inflammatory cytokines in blood stimulated by any of these mitogens, then it has potential as a therapeutic or prophylactic. The inhibitory properties demonstrated by anti-inflammatory cytokine IL-4 and the cytokine receptor antagonist IL-1 (IL-1ra) on the production of IL-8 mRNA in Chapter 3 Experiment 3 showed promise.

In Experiment 3 Flunix or IL-1ra or IL-4 were added to un-stimulated whole blood to check if these anti-inflammatory substances inhibited constitutive baseline levels of IL-8 mRNA. Flunix and IL-1ra were found to inhibit constitutive levels, interestingly IL-4 was found to up-regulate IL-8 mRNA production. This was interesting since the stimulation of IL-8 mRNA in whole blood by PMA and LPS was inhibited by IL-4 (low dose) to zero. The medium and high concentrations of IL-4 however, did not inhibit the stimulation of IL-8 mRNA by PMA and LPS. This up-regulation of IL-8 without mitogen stimulation by IL-4 can be explained by Nuntaprasert *et al.,* (2005) who found

that IL-4 when given with LPS to alveolar macrophages suppressed the secretion of TNF-α, IL-1α, IL-6 and IL-8. However when IL-4 was administered before LPS challenge in pigs, it increased the severity of respiratory failure and endotoxic shock and increased pro-inflammatory cytokines (Nuntaprasert *et al.,* 2005). Thus IL-4 has the ability of to activate or suppress pro-inflammatory cytokines depending on the concentration and time of administration.

The *in vitro* experiments performed in Chapter 3 and the above studies showed that IL-4 and IL-1ra needed further *in vivo* exploration.

The therapeutic treatments, high dose of IL-4, low dose of IL-1ra and Excenel reduced TNF-α mRNA to an undetectable level after 48 hours of App challenge (Chapter 5) which may be associated with increased pleurisy and lung damage from polymorphonuclear cells aggregating in lung tissue. The increase in pleurisy and lung damage may in turn be associated with the reduced growth performance of the pigs treated with the above mentioned treatments.

In contrast, prophylactic delivery of IL-4 and IL-1ra treatment (Chapter 4) did not reduce TNF-α mRNA production in pigs challenged with App, yet pleurisy and lung damage were reduced greatly below the saline control. Morrison *et al.,* (2000) found that the over production of IL-1β and TNF-α by macrophages in the lung of pigs suffering from an App infection was the main factor associated with massive lung injury. Lung damage and clinical scores in pigs treated prophylactically with IL-4 and IL-1ra were greatly reduced (Chapter 4). Pigs treated prophylactically with IL-4 and IL-1ra also produced

the fewest cytotoxic cells (CD8) and had reduced mRNA pro-inflammatory cytokines IL-8 and IL-6. Baarsch, *et al.,* (1995) found that pro-inflammatory cytokines, particularly IL-1 and IL-8, were associated with the development of pleuropneumonia and may contribute to disease severity. A reduction in IL-8 mRNA levels could explain why pigs prophylactically treated with IL-4 or IL-1ra had reduced in lung damage and increased growth. IL-8 is known to be a neutrophil recruiter to inflammatory sites (reviewed by Standiford *et al.,* 2000; Murtaugh *et al.,* 1996). Lesions in the respiratory tract of pig suffering App are made up of neutrophils, macrophages and platelets (Liggett *et al.,* 1987).This study found white blood cells in the lung and around bronchus of infected pigs suffering lesions. The reduction in IL-8 mRNA by IL-4 or IL-1ra may suppress the neutrophil influx into the lung and hence decrease lung damage. However the magnitude of IL-4 and IL-1ra must be accurate to produce this affect. Especially since pigs given a high concentration of IL-4 showed presence of IL-8 mRNA producing white cells in their lungs. Lung damage and clinical scores in pigs treated prophylactically with IL-1ra were reduced (Chapter 4). This could be due to the reduction in IL-8 mRNA exhibited in this group. It is known that IL-1ra can inhibit IL-1β by binding to receptor sites on the Th 2 lymphocytes, thereby stopping signal transduction (Arend *et al.,* 1993). In view of the fact that IL-1β and IL-8 are associated with the severity pleuropneumonia (Baarsch *et al.,* 1995), and IL-1β and α also stimulate the production of IL-8 by macrophages (Tizard 1996). Hence IL-1ra may be able to not only reduce IL-1β, but also IL-8. IL-1ra can also directly influence the production of IL-8 since it was found by Chen *et al.,* 1998 that IL-1ra inhibited IL-8 in an *in vitro* system (squamous cell carcinoma human cell line).

Prophylactically delivered IL-1ra and IL-4 not only reduced the production of IL-1β mRNA in the pigs peripheral blood, they also inhibited or reduced the production of TNF-α mRNA in peripheral blood. Lee *et al.,* 1995 also observed IL-4 ability to down regulate the amount of IL-1β, TNF-α and IL-6 mRNA expression by human peripheral blood mononuclear cells (PBMC) stimulated with LPS and IL-2. Prophylactic delivery of IL-4 to pigs similarly showed a reduction in TNF-α mRNA after LPS challenge (Chapter 4). Pigs treated prophylactically with IL-4 and IL-1ra also reduced IL-8 and IL-6 mRNA in peripheral blood in response to the App challenge (Chapter 4). Similarly Lee *et al.,* (1995) found that IL-4 stimulates IL-4ra while inhibiting the production of IL-1 and TNF-α by monocytes. In Chapter 4, both IL-1ra and IL-4 reduced the production of IL-1β mRNA 24 hours after LPS challenge as compared to the saline control. Although it appeared that the LPS challenge did not produce an effect on growth, it did have an affect on cytokine levels.

Prophylactic delivery of IL-4 and IL-1ra resulted in pigs which out-performed the saline controls by gaining the most weight by slaughter (Chapter 4). The plasmid pig groups performed the poorest overall in terms of growth and it would seem that this delivery system is not suited to this animal model.

Pigs with App, when treated therapeutically did not increase in weight compared to the saline controls (Chapter 5). This could be due to the App infection being established before the recombinant cytokine (IL-4) and receptor antagonist (IL-1ra) could produce their effect. By giving these treatments at a later stage, the pro-inflammatory cytokines were stimulated before they could be suppressed.

COMMERICAL IN CONFIDENCE

Even though there were no differences between the therapeutically treated pig groups in terms of weight gain there were differences in the leucocyte numbers, pro-inflammatory cytokine production and secreted immunoglobulins (Chapter 5).

The pig group given therapeutic IL-1ra at a low dose performed the worst and these animals had low IL-8 mRNA in their peripheral blood as compared to the other pig groups by 2 weeks post-App challenge. Very small amounts of TNF-α and IL-1β mRNA were found in IL-1ra (low dose) treated pigs' peripheral blood as compared to all other treatment groups including the saline control. A review by Standiford *et al.,* 2000 found the inhibition of TNF-α can stop bacterial clearing thus presence of TNF-α is essential in the control of bacterial pneumonia in mice. The reduction in IL-8 and TNF-α mRNA occurred concurrently with this groups' lung tissue and lymph nodes producing IL-6 and IL-8 mRNA which may have resulted in the very high mean affected lung and pleurisy (Chapter 5).

In response to the App infection undetectable to small amount of TNF-α mRNA were produced by pigs treated with IL-1ra (low dose) over time of (48 hours to 2 weeks). The production of IL-8 mRNA also reduced 2 weeks after App challenge (Chapter 5). It would be expected that this group (low dose IL-1ra) would perform well and increase in health and therefore growth since a reduction in IL-8 (neutrophil chemotactic) implies a reduction in neutrophil infiltration to inflamed site (reviewed by Standiford *et al.,* 2000).

A reduction in TNF-α has been shown to reduce lung damage (Morrison *et al.,* 2000). However, too little cytokines can be as damaging as too much. Standiford *et al.,* 1999

found that bacterial clearance and improved survival of *Klebsiella pneumoniae* challenged mice depended on the dose of TNF-α given to the animals. TNF-α is critical to antibacterial host defences of mice suffering *Klebsiella pneumoniae* infection (Laichalk *et al.,* 1996). Therefore the reduction in IL-8 and TNF-α may have been associated with the poor health and thus growth performance observed in the current studies.

Immunological stress such as disease cause partitioning of dietary nutrients away from growth and skeletal muscle accretion in favor of metabolic processes that support immune response and disease resistance (Solomons *et al.,* 1993). By increasing health of pigs using IL-4 and IL-1ra as done in these studies, dietary nutrients from feed in-take may be directed to growth as seen in Chapter 4. The negative effects of App infection were reduced by IL-4 and IL-1ra and growth was increased. However this was only successful if the pigs were given these substances as a prophylactic. Therefore it can be said that these substances delivered as prophylactics reduced the negative effect of disease on growth and health and have potential as alternatives to antibiotics.

The potential for anti-inflammatory treatments to address the deleterious effects of App on pig growth were successfully explored. The data provided in this thesis have implications for the future in the development of a product which could be delivered prior to housing animals. The potential also exist for the development of a viral delivery system for future application.

BIBLIOGRAPHY

Agace, W., S. Hedges, et al. (1993). "Selective cytokine production by epithelial cells following exposure to Escherichia coli." Infection & Immunity. 61(2): 602-9.

Angen, O., P. M. Heegaard, et al. (2001). "Isolation of Actinobacillus pleuropneumoniae serotype 2 by immunomagnetic separation." Veterinary Microbiology 79(1): 19-29.

Arend, w. (1993). "Interleukins and arthritis: IL-1 anatagonism in inflammatory arthritis."

Arend wp, D. j. (1990). "Cytokines and cytokine inhibitors or antagonists in rheumatoid arthritis." Arthritis & Rheumatism 33(3): 305-315.

Atkins, P. C., M. Valenzano, et al. (1989). "Identification of leukotriene B4 as the neutrophil chemotactic factor released by antigen challenge from passively sensitized guinea pig lungs." Journal of Allergy & Clinical Immunology. 83(1): 136-43,.

Baarsch, M. J., R. W. Scamurra, et al. (1995). "Inflammatory cytokine expression in swine experimentally infected with actinobacillus pleuropneumoniae." Infection & Immunity 63(9): 3587-3594.

Baarsch, M. J., M. J. Wannemuehler, et al. (1991). "Detection of tumor necrosis factor alpha from porcine alveolar macrophages using an L929 fibroblast bioassay." Journal of Immunological Methods **140**(1): 15-22.

Bartrop, R. W., E. Luckhurst, et al. (1977). "Depressed lymphocyte function after bereavement." Lancet 1(8016): 834-6.

Bendtzen, K. (1994). "Cytokines and natural regulators of cytokines." Immunology Letters. 43(1-2): 111-23.

Bertram, T. A. (1985). "Quantitative morphology of peracute pulmonary lesions in swine induced by Haemophilus pleuropneumoniae." Veterinary Pathology 22(6): 598-609.

Bertram, T. A. (1986). "Intravascular macrophages in lungs of pigs infected with Haemophilus pleuropneumoniae." Veterinary Pathology 23(6): 681-91.

Beskow, P., O. Soderlind, et al. (1989). "Actinobacillus (Haemophilus) pleuropneumoniae infections in swine: serological investigations and vaccination trials in combination with environmental improvements." Zentralblatt Fuer Veterinaermedizin Reihe B 36(7): 487-94.

Beutler, B. and A. Cerami (1988). "Tumor necrosis, cachexia, shock, and inflammation: a common mediator." Annual Review of Biochemistry 57: 505-18.

Blagosklonny, M. V. and L. M. Neckers (1993). "Sensitive and simple bioassay for human tumour necrosis factor-alpha." European Cytokine Network **4**(4): 279-83.

Brostoff, J. and e. al (1991). "Clinical Immunology." London; New York: Gower Medical Pub, ; Philadelphia: Lippincott.

Bustamante, M., G. W. Jesse, et al. (1996). "Effects of individual vs group penning on the performance of weanling pigs." Journal of Animal Science 74(7): 1457-1461.

Caruson, J. P. and R. F. Ross (1990). "Effects of Mycoplasma hyopneumoniae and Actinobacillus (Haemophilus) pleuropneumoniae infections on alveolar macrophage functions in swine." American Journal of Veterinary Research. 51(2): 227-31.

Chen, Z., I. Colon, et al. (1998). "Effects of interleukin-1-alpha, interleukin-1 receptor antagonist, and neutralizing antibody on proinflammatory cytokine expression by human squamous cell carcinoma lines." Cancer Research 58(16): 3668-3676.

Cole, N., M. Krockenberger, et al. (2001). "Effects of exogenous interleukin-6 during Pseudomonas aeruginosa corneal infection." Infection & Immunity **69**(6): 4116-9

Crenshaw, T. D., M. E. Cook, et al. (1986). "Effect of nutritional status, age at weaning and room temperature on growth and systemic immune response of weanling pigs." Journal of Animal Science 63(6): 1845-53.

Cubitt, C. L., R. N. Lausch, et al. (1995). "Differences in interleukin-6 gene expression between cultured human corneal epithelial cells and keratocytes." Investigative Ophthalmology & Visual Science. 36(2): 330-6.

Curtis, S. E., C. R. Anderson, et al. (1975). "Effects of aerial ammonia, hydrogen sulfide and swine-house dust on rate of gain and respiratory-tract structure in swine." Journal of Animal Science 41(3): 735-9.

Davos, D. E., C. F. Cargill, et al. (1981). "Outbreak of brucellosis at a South-Australian abattoir. 2. Epidemiological investigations." Medical Journal of Australia 2(12-13): 657-60.

Dimango, E., H. J. Zar, et al. (1995). "diverse Pseudomonas aeruginosa Gene Products stimulate respiratory epithelial cells to produce IL-8." Journal of Clinical Investigation. 96(5): 2204-10.

Di Sabato, G., J. M. Hall, et al. (1987). "T cell mitogens and polyclonal B cell activators." Methods in Enzymology **150**: 3-17.

Dozois, C. M., E. Oswald, et al. (1997). "A reverse transcription-polymerase chain reaction method to analyze porcine cytokine gene expression." Veterinary Immunology & Immunopathology 58(3-4): 287-300.

Dubravec, D. B., D. R. Spriggs, et al. (1990). "Circulating human peripheral blood granulocytes synthesize and secrete tumor necrosis factor alpha." Proceedings of the National Academy of Sciences of the United States of America 87(17): 6758-61.

Eckmann, L., M. F. Kagnoff, et al. (1993). "Epithelial cells secrete the chemokine interleukin-8 in response to bacterial entry." Infection & Immunity 61(11): 4569-74.

Edwards, S. A. and F. S.J. (1988). "The effects of straw in crated farrowing systems on peripartal behaviour of sows and piglets." British Veterinary Journal 144(2): 139-46.

Ekkel, E. D., C. E. A. Vandoorn, et al. (1995). "The specific-stress-free housing system productivity, health, and has welfare positive effects on of pigs." Journal of Animal Science 73(6): 1544-1551.

Fenwick, B. and S. Henry (1994). "Porcine pleuropneumonia." Journal of the American Veterinary Medical Association. 204(9): 1334-40.

Fitzgerald, K. A., L. A. J. O'Neill, et al. (2001). "The Cytokine Facts Book." Academic Press(2nd Edition): 21-94, 475-480.

Fletcher, M. A., G. C. Baron, et al. (1987). "Use of whole blood methods in assessment of immune parameters in immunodeficiency states." Diagnostic & Clinical Immunology 5(2): 69-81.

Goethe, R., O. F. Gonzales, et al. (2000). "A novel strategy for protective Actinobacillus pleuropneumoniae subunit vaccines: detergent extraction of cultures induced by iron restriction." Vaccine 19(7-8): 966-975.

Gunther, G. R., J. L. Wang, et al. (1012). "Concanavalin A derivatives with altered biological activities." Proceedings of the National Academy of Sciences of the United States of America **70**(4): 1012-6.

Hakansson, A., I. Carlstedt, et al. (1996). "Aspects on the interaction of Streptococcus pneumoniae and Haemophilus influenzae with human respiratory tract mucosa." American Journal of Respiratory & Critical Care Medicine. 154(4 Pt 2): S187-91.

Hazlett, L. D. (2002). "Pathogenic mechanisms of P. aeruginosa keratitis: a review of the role of T cells, Langerhans cells, PMN, and cytokines." DNA & Cell Biology. 21(5-6): 383-90.

Heidt, M. and R. Weiss (1982). "Occurrence of bacterial pathogens in pathologic anatomically changed lungs of swine with special reference to the genus Hemophilus. 2. Biochemical studies on Hemophilus strains." Berliner und Munchener Tierarztliche Wochenschrift. 95(23): 453-8.

Hill , D. H., Branion, H.D, Slinger, J.S, and Anderson ,G.W (1952). "Influence of environment on the growth response of chicks to Penicillin." Poultry Science 32: 462-466.

Hirsh, D. C., L. D. Martin, et al. (1982). "Plasmid-mediated antimicrobial resistance in Haemophilus pleuropneumoniae." American Journal of Veterinary Research 43(2): 269-72.

Huang, H., A. A. Potter, et al. (1999). "Pathogenesis of porcine Actinobacillus pleuropneumonia, part II: roles of proinflammatory cytokines." Canadian Journal of Veterinary Research 63(1): 69-78.

Huang, H. S., A. A. Potter, et al. (1998). "Pathogenesis of porcine actinobacillus pleuropneumonia - part i - effects of surface components of actinobacillus pleuropneumoniae in vitro and in vivo." Canadian Journal of Veterinary Research - Revue Canadienne de Recherche Veterinaire 62(2): 93-101.

Hyun, Y., M. Ellis, et al. (1998). "Effects of feeder type, space allowance, and mixing on the growth performance and feed intake pattern of growing pigs." Journal of Animal Science 76(11): 2771-8.

Idris, U. E., B. G. Harmon, et al. (1993). "Pulmonary lesions in mice inoculated with Actinobacillus pleuropneumoniae hemolysin and lipopolysaccharide." Veterinary Pathology. 30(3): 234-41.

Isomaki, P., R. Luukkainen, et al. (1996). "Interleukin-10 functions as an antiinflammatory cytokine in rheumatoid synovium." Arthritis & Rheumatism. 39(3): 386-95.

Jeffes, E. W., 3rd, K. Schmitz, et al. (1991). "A simple nonisotopic in vitro bioassay for LT and TNF employing sodium fluoride-treated L-929 target cells that detects picogram quantities of LT and TNF and is as sensitive as TNF assays done with ELISA methodology." Lymphokine & Cytokine Research **10**(1-2): 147-51.

Jobert, J. L., C. Savoye, et al. (2000). "Experimental aerosol transmission of Actinobacillus pleuropneumoniae to pigs." Canadian Journal of Veterinary Research 64(1): 21-6.

Johnson, R. W. (1997). "Inhibition of growth by pro-inflammatory cytokines: an integrated view." Journal of Animal Science 75(5): 1244-55.

Kelly , K. W. (1980). "Stress and Immune Function: A bibliographic review." Annual Rearch Veternary 11: 445-478.

Khair, O. A., R. J. Davies, et al. (1996). "Bacterial-induced release of inflammatory mediators by bronchial epithelial cells." European Respiratory Journal. 9(9): 1913-22.

Kielstein, P., H. Wuthe, et al. (2001). "Phenotypic and genetic characterization of NAD-dependent Pasteurellaceae from the respiratory tract of pigs and their possible pathogenetic importance." Veterinary Microbiology 81(3): 243-55.

Klasing, K. C. (1988). "Nutritional aspects of leukocytic cytokines." Journal of Nutrition 118(12): 1436-46.

Klein, J. and V. Horejsi (1997). "Immunology." Oxford; Malden, M.A.: Blackwell Science 2nd ed(2nd Edition): Chapter 10.

Koyama, S., E. Sato, et al. (2000). "The potential of various lipopolysaccharides to release IL-8 and G-CSF." American Journal of Physiology Lung Cellular & Molecular Physiology **278**(4).

Kroemer, G., I. Moreno de Alboran, et al. (1993). "Immunoregulation by cytokines." Critical Reviews in Immunology. 13(2): 163-91.

Laichalk, L. L., S. L. Kunkel, et al. (1996). "Tumor necrosis factor mediates lung antibacterial host defense in murine Klebsiella pneumonia." Infection & Immunity. 64(12): 5211-8.

Larsson, B. M., K. Larsson, et al. (1999). "Gram positive bacteria induce IL-6 and IL-8 production in human alveolar macrophages and epithelial cells." Inflammation. 23(3): 217-30.

Lee, J. D., K. Rhoades, et al. (1995). "Interleukin-4 inhibits the expression of tumour necrosis factors alpha and beta, interleukins-1 beta and -6 and interferon-gamma." Immunology & Cell Biology. 73(1): 57-61.

Leman, A. D., B. E. Straw, et al. (1992). "Diseases of swine." Ames,IA,Iowa State University Press 7th edition.: 401-408.

Levine, S. J., T. Wu, et al. (1997). "Extracellular release of the type i intracellular IL-1 receptor antagonist from human airway epithelial cells - differential effects of IL-4, IL-13, ifn-gamma, and corticosteroids." Journal of Immunology 158(12): 5949-5957.

Liggett, A. D., L. R. Harrison, et al. (1987). "Sequential study of lesion development in experimental haemophilus pleuropneumonia." Research in Veterinary Science. 42(2): 204-12.

Liu, Z., R. J. Simpson, et al. (1992). "Recombinant interleukin-6 protects mice against experimental bacterial infection." Infection & Immunity **60**(10): 4402-6

Massion, P. P., H. Inoue, et al. (1994). "Novel Pseudomonas product stimulates interleukin-8 production in airway epithelial cells in vitro." Journal of Clinical Investigation. 93(1): 26-32.

Mayringer, I., M. Reindl, et al. (2000). "A critical comparison of frequently used methods for the analysis of tumor necrosis factor-alpha expression by human immune cells." Journal of Immunological Methods 235(1-2): 33-40.

McGlone, J. J., Newby B.E (1994). "Space requirements for finishing pigs in confinement:behavior and performance while group size and space vary." Applied Animal Behaviour Science 39: 331-338.

MIMS (1988). "IVS Annual." Crows Nest, NSW: MIMS Publishing.

Minton, J. E., D. A. Nichols, et al. (1988). "Fluctuating ambient temperature for weaned pigs: effects on performance and immunological and endocrinological functions." Journal of Animal Science 66(8): 1907-14.

Mire-Sluis, A. and R. Thorpe (1998). "Cytokines." Academic Press: 1-167, 335-360.

Morrison, D. F., D. L. Foss, et al. (2000). "Interleukin-10 gene therapy-mediated amelioration of bacterial pneumonia." Infection & Immunity 68(8): 4752-4758.

Mosmann, T. R. (1994). "Properties and functions of interleukin-10." Advances in Immunology. 56: 1-26.

Muller, K., E. B. Herner, et al. (1998). "Inflammatory cytokines and cytokine antagonists in whole blood cultures of patients with systemic juvenile chronic arthritis." British Journal of Rheumatology 37(5): 562-9.

Mullin, J. M. and K. V. Snock (1990). "Effect of tumor necrosis factor on epithelial tight junctions and transepithelial permeability." Cancer Research **50**(7): 2172-6.

Murtaugh, M. P., M. J. Baarsch, et al. (1996). "Inflammatory cytokines in animal health and disease." Veterinary Immunology & Immunopathology 54(1-4): 45-55.

Myrianthefs, P., S. Karatzas, et al. (2003). "Seasonal variation in whole blood cytokine production after LPS stimulation in normal individuals." Cytokine. 24(6): 286-92.

Nicola, N. A. (1994). "Guidebook to Cytokines and Their Receptors." Oxford; New York: Oxford University Press.

Nuntaprasert, A., Y. Mori, et al. (2005). "The effect of recombinant swine interleukin-4 on swine immune cells and pro-inflammatory cytokine productions in pigs." Comparative Immunology, Microbiology & Infectious Diseases. 28(2): 83-101.

Olsson, T. (1995). "Cytokine-producing cells in experimental autoimmune encephalomyelitis and multiple sclerosis." Neurology. 45(6 Suppl 6): S115.

Pace, E., M. Profita, et al. (2004). " LTB4 is present in exudative pleural effusions and contributes actively to neutrophil recruitment in the inflamed pleural space." Clinical & Experimental Immunology. 135(3): 519-27.

Perez, L. and D. T. Lysle (1995). "Corticotropin-releasing hormone is involved in conditioned stimulus-induced reduction of natural killer cell activity but not in conditioned alterations in cytokine production or proliferation responses." Journal of Neuroimmunology 63(1): 1-8.

Pohl, S., H. V.Bertschinger, et al. (1983). "Transfer of Haemophilus pleuropneumoniae and Pasteurella haemolytica-like organisms causing porcine necrotic pleuropneumonia to the genus Actinobacillus (Actinobacillus pleuropneumoniae) on the basis of phenotypic and deoxyribonucleic acid relatedness." Int. J. Syst. Bacteriol. 33: 510-514.

Randolph, J. H., Cromwell, G.L, Stably, T.S (1993). "Effects of gtoup size allowance and performance and behaviour of swine." Journal of Animal Science 53: 922-927.

Rioux, S., C. Galarneau, et al. (2000). "Isolation and characterization of a capsule-deficient mutant of Actinobacillus pleuropneumoniae serotype 1." Microbial Pathogenesis 28(5): 279-289.

Roitt, I. (1994). "Essential Immunology." Blackwell Scientific Publications(8th Edition).

Roura, E., J. Homedes, et al. (1992). "Prevention of immunologic stress contributes to the growth-permitting ability of dietary antibiotics in chicks." Journal of Nutrition 122(12): 2383-90.

Saleem, S., Z. Dai, et al. (1998). "IL-4 is an endogenous inhibitor of neutrophil influx and subsequent pathology in acute antibody-mediated inflammation." Journal of Immunology. 160(2): 979-84.

Scamurra, R. W., C. Arriaga, et al. (1996). "Regulation of interleukin-6 expression in porcine immune cells." Journal of Interferon & Cytokine Research 16(4): 289-296.

Scheepens, C. J. M., M. J. C. Hessing, et al. (1994). "Effect of climatic stress on the immunological reactivity of weaned pigs." Veterinary Quarterly 16(3): 137-143.

Schmidt, R. and E. Hecker (1375). "Autoxidation of phorbol esters under normal storage conditions." Cancer Research **35**(5): 1375-7.

Schoenbaum, M. A., J. J. Zimmerman, et al. (1990). "Survival of pseudorabies virus in aerosol." American Journal of Veterinary Research. 51(3): 331-3.

Sebunya, T. N. and J. R. Saunders (1983). "Haemophilus pleuropneumoniae infection in swine: a review." Journal of the American Veterinary Medical Association. 182(12): 1331-7.

Seitz, M., P. Loetscher, et al. (1996). "Opposite effects of interleukin-13 and interleukin-12 on the release of inflammatory cytokines, cytokine inhibitors and prostaglandin e from synovial fibroblasts and blood mononuclear cells." European Journal of Immunology 26(9): 2198-2202.

Sharma, H. S. and D. K. Das (1997). "Role of cytokines in myocardial ischemia and reperfusion." Mediators of Inflammation 6(3): 175-183.

Sipos, W., J. C. Duvigneau, et al. (2004). "Systemic cytokine profile in feeder pigs suffering from natural postweaning multisystemic wasting syndrome (PMWS) as determined by semiquantitative RT-PCR and flow cytometric intracellular cytokine detection." Veterinary Immunology & Immunopathology **99**(1-2): 63-71

Slocombe, R. F., J. Malark, et al. (1985). "Importance of neutrophils in the pathogenesis of acute pneumonic pasteurellosis in calves." American Journal of Veterinary Research. 46(11): 2253-8.

Solomons, N. W., M. Mazariegos, et al. (1993). "The underprivileged, developing country child: environmental contamination and growth failure revisited." Nutrition Reviews 51(11): 327-32.

Standiford, T. J., W. C. Tsai, et al. (2000). "Cytokines as targets of immunotherapy in bacterial pneumonia." Journal of Laboratory & Clinical Medicine. 135(2): 129-38.

Straw, B. E., W. L. Mengeling, et al. (1999). "Diseases of swine." Oxford; Malden, M.A., USA: Blackwell Science(8th Edition).

Strieter, R. M., K. Kasahara, et al. (1992). "Cytokine-induced neutrophil-derived interleukin-8." American Journal of Pathology. 141(2): 397-407.

Surendran, M. and K. Gunasegaran (1999). "Cytokines and chemokine receptors on infectious disease." Immunology & Cell Biology. 77(6): 469-475.

Svanborg, C., W. Agace, et al. (1993). "Bacterial adherence and epithelial cell cytokine production." Zentralblatt fur Bakteriologie. 278(2-3): 359-64.

Taki, F., M. Iwata, et al. (1988). "Migration of neutrophils in experimental asthma." Annals of Allergy. 60(6): 508-12.

Taylor, A. W., N. O. Ku, et al. (1990). "Regulation of cytokine-induced human C-reactive protein production by transforming growth factor-beta." Journal of Immunology **145**(8): 2507-13.

Tian, W. Z., V. Navikas, et al. (1998). "Linomide (roquinimex) affects the balance between pro- and anti-inflammatory cytokines in vitro in multiple sclerosis." Acta Neurologica Scandinavica 98(2): 94-101.

Tizard, I. R. (1996). "Veterinary immunology: an introduction." Philadelphia: Saunders(5th Edition): Chapters 3 and 4.

Tonn, S. R., D. L. Davis, et al. (1985). "Mating behavior, boar-to-boar behavior during rearing and soundness of boars penned individually or in groups from 6 to 27 weeks of age." Journal of Animal Science 61(2): 287-96.

Torremorell, M., C. Pijoan, et al. (1997). "Airborne transmission of actinobacillus pleuropneumoniae and porcine reproductive and respiratory syndrome virus in nursery pigs." American Journal of Veterinary Research 58(8): 828-832.

Udeze, F. A., K. S. Latimer, et al. (1987). "Role of haemophilus pleuropneumoniae lipopolysaccharide endotoxin in the pathogenesis of porcine Haemophilus pleuropneumonia." American Journal of Veterinary Research. 48(5): 768-73.

Van Roon, J. A., J. L. van Roy, et al. (1996). "Prevention and reversal of cartilage degradation in rheumatoid arthritis by interleukin-10 and interleukin-4." Arthritis & Rheumatism. 39(5): 829-35.

Veenhuizen, M. F. (1998). "Three bacterial pathogens in the porcine respiratory disease complex." Compendium on Continuing Education for the Practicing Veterinarian 20(1 Suppl S): S 11 ff.

Wallgren, P., P. Beskow, et al. (1994). "Porcine lung lesions at slaughter and their correlation to the incidence of infections by Mycoplasma hyopneumoniae and Actinobacillus pleuropneumoniae during the rearing period." Zentralblatt Fuer Veterinaermedizin Reihe B 41(7-8): 441-52.

Zhou, Y. L., G. F. Lin, et al. (1994). "Interleukin-4 suppresses inflammatory cytokine gene transcription in porcine macrophages." Journal of Leukocyte Biology 56(4): 507-513.

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APPENDIX

APPENDIX 1- PRIMER SEQUENCES

Primer	Gene/Accession	Product size (bp)	Sequence 5'→3'	CDNA Cycles & concentration
IL-1β upper	IL-1β M86725	285	aaaggggacttgaagagag	29cycles
IL-1β lower	IL-1β X74568	285	ctgcttgagaggtgctgatgt	2μl cDNA
IL-6 240	IL-6 M86722	760	catcctcggcaaaatctctg	29cycles
IL-6 980	IL-6 M86722	760	cctggctctgaaacaacaag	2μl cDNA
IL-8 U2	IL-8 M86923	565	caccccaaatttatcaaggaactg	29cycles
IL-8 L2	IL-8 M86923	565	ttggggataaagaagaaactgagg	2μl cDNA
β-actin upper	U07786	233	ggacttcgagcaggagatgg	29cycles
β-actin lower	U07786	233	gcaccgtgttggcgtagagg	1μl cDNA
TNFα upper	M29079	351	atcggcccccagaaggaagag	29cycles
TNFα lower	X54857	351	gatggcagagaggaggttgac	1μl cDNA
CRP upper	c-reactive protein	420	ctcccgccatrrwctcaccaca	29cycles
CRP lower	c-reactive protein	420	actgccvgcrtcrccatag	3μl cDNA

R=a+g
V=a+c+g
W=a+t

APPENDIX 2-Synthesis of cDNA (RT)

Using the Reverse Transcription System by Promega

1. Prepare reactions on ice.

2. Prepare 10 μl reactions by adding following components (can be scaled up or down, but maintain the concentrations).

MASTER MIX for 10ul reaction

Component	Amount/sample	Final concentration
MgCl$_2$ (25 mM)	2 μl	5 mM
Reverse transcription 10X buffer	1 μl	1X
10 mM dNTP mixture	1 μl	1 mM dNTP
RNase Inhibitor	0.25 μl	1 U/μl
AMV reverse transcriptase	0.3 μl (15 U)	15 U/μg
Oligo(dT) primer	0.5 μl (0.5 μg)	0.5μg/μg RNA
RNA	0.5 μg	
Nuclease Free Water to a final volume of 10 μl.		

Everything is constant – regardless of RNA concentration, every sample receives 5.05ul MasterMix. The only components that vary are RNA and water. Water is required to make up the final volume to 10ul.

3. Use Excel table to determine RNA concentration as ug/ul. The ul RNA required for RT reaction = 0.5ug x1ul/RNA concentration (the excel table will calculate it for you)

4. Incubate reactions at 42 °C for 1 hr, followed by 5 min at 95 °C. (program 18 on the Omnigene thermal cycler)

5. Remove tubes/plate to 20oC.

6. Once cool, dilute the reaction 1/5 in Nuclease Free Water. We do 10ul reactions, so add 40ul water. Eg:

7. Store tubes in –20 until used in PCR.

APPENDIX 3-PCR COMPONENTS

Perform this procedure on ice in the PCR hood.

Make mastermix:

For a 25 μl reaction place the following components per tube (use PCR omnistrips or 384 well plate):

Component	Amount per reaction	Final concentration
RedTaq 10Xbuffer/ MgCl$_2$ (15 mM)	2.5 μl	1.5 mM
PCR mix (10 mM dNTP)	0.5 μl	100 μM
RedTaq Polymerase	1.25ul	
Upper primer*	1 μl	50 pmol total
Lower primer*	1 μl	50 pmol total
H$_2$O	Add to make up to 25ul	
cDNA	approx 1 μl	

*When using CRP, add primers at 0.5ul/reaction and thus, increase water by 1ul.

Additives:

Certain PCR reactions require the following additives (to adjust for this the amount of water added is changed).

Primers	Additive	Amount	Percentage in reaction	Amount of water in reaction
IL-1β	4% Glycerol	1.56μl	0.0624%	change accordingly
HAP	DMSO	2.5 μl	10%	

cDNA:

Primer name	Primer volume (μL)	cDNA volume (μL)
β-act	1	1
IL-8	1	3
IL-6	1	2
IL-1β	1	2
TNFα	1	3
CRP	1	2

Water was adjusted to have a final volume of 25μL

APPENDIX 4-RNA Isolation

Using the SV RNA Isoation kit by Promega

Before you start, turn water bath to 70°C.

1. Add 500μl blood to 1.5ml eppendorf tube
2. Add 900μl red blood cell lysis solution (RBC Lysis) and mix by inverting.
3. Incubate at room temp for 10 mins (invert tubes at 5 mins)
4. Centrifuge at 20,000 g for 20 seconds; discard supernatant by tipping the tube. You should see a white spot in the middle of a red pellet.
5. Add 175μl of SV RNA lysis buffer and mix
6. Add 350μl SV RNA dilution buffer (blue) and mix 3-4 times.
7. Put into water bath for *exactly* 3 mins (exceeding this time causes RNA degradation).
8. Put tubes on ice for 1 min.
9. Centrifuge at 20,000 g for 10 mins. Transfer the supernatant to a new 1.5m eppendorf using a P200. Pipette slowly and carefully so that you do not disturb the protein pellet. The supernatant may be yellow at this stage which is OK.
10. Add 200μl cold 95% ethanol to each tube (containing supernatant). Mix by inverting.
11. Remove a 'column assembly' from the packet and add to the column (the top bit) the supernatant from step 11.
12. Centrifuge at 13,000 g for 1min.
13. Disconnect column from collection tube – discard the liquid in the collection tube.
14. Reattach the column to the collection tube. Add 600μl SV RNA wash solution to the column.
15. Centrifuge at 13,000 g for 1 min.
16. Make up DNase solution fresh: (per sample requirements)
     40μl of Yellow Core Buffer,
     5μl 0.09M MnCl$_2$
     5μl DNase-1 enzyme
17. Remove columns from centrifuge. Discard the liquid in the collection tube.
18. Add 50μl DNase solution to each column.
19. Incubate at room temp for 15 mins.
20. Add 200μl DNase stop solution.
21. Centrifuge at 13,000 g for 1 min. (do not empty collection tube)
22. Add 600μl SV RNA wash solution to the column and centrifuge at 13,000 g for 1 min.
23. Discard the liquid in the collection tube.
24. To the column, add 250μl SV RNA wash solution.
25. Centrifuge at 15,000g for 2 min.
26. Attach the column to a labelled 1.5ml eppendorf (elution tube in the kit). You can now discard the original collection tube.

27. Add 100μl DEPC water to the column. Spin at 20,000 g for 1 min.
28. Remove the column and throw it away. Your RNA is in the eppendorf.

APPENDIX 5-PCR product purification

Using Wizard PCR prep from Promega

This method is to purify PCR products for sequencing, or to create pure PCR positive controls for running on gels.

Perform the PCR in room 220 wearing gloves and coat. Then, perform the purification in the main lab, wearing gloves and coat. Use baked/autoclaved labwear.

Use at least 30µl of a PCR reaction for purification (generally use a 50 µl reaction, ie., double the volume of a normal PCR).

1. Run PCR.

2. Transfer the PCR reaction to a 1.5 ml eppendorf tube. Add 100 µl **Direct Purification Buffer** and vortex briefly.

3. Add 1 ml of **Resin** and vortex briefly 3 time over a period of 1 min.

4. Prepare a Wizard PCR prep column/Syringe barrel assembly (from the Wizard Miniprep kit). Insert column into the vacuum manifold (the blue pig).

Reminder!!
Don't forget to take your tips and pipettes!
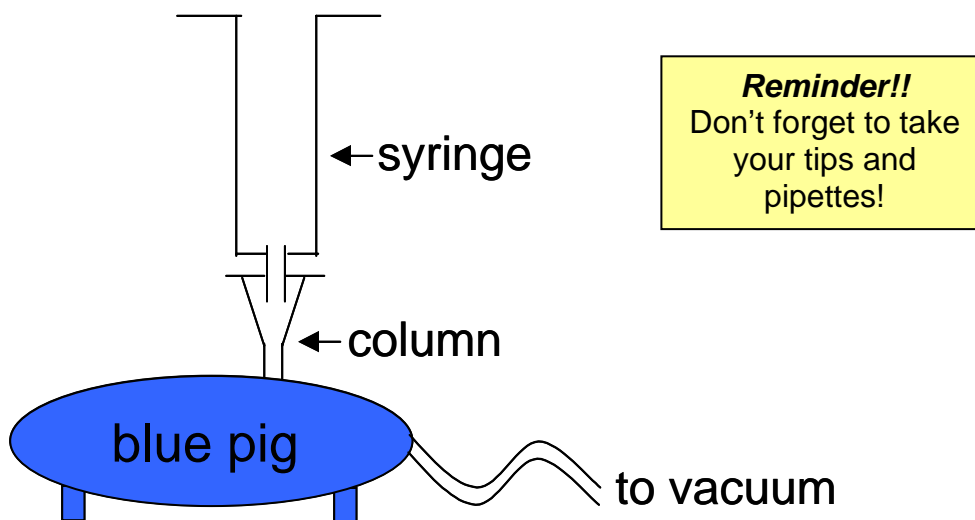
5. Pipette the resin/PCR mix into the syringe barrel. Apply vacuum to draw the resin/PCR mix into the Minicolumn.

6. Break the vacuum to the Minicolumn by separating the tube connection to the vacuum pump. Pipette 2 mls of 80% isopropanol to the syringe barrel and reapply the vacuum until the isopropanol has been drawn through the Minicolumn. Continue to apply vacuum for exactly 30 seconds *(use a timer!!!)* after the isopropanol has been drawn though.

7. Transfer the Minicolumn to a clean 1.5 ml eppendorf tube (you will need to snip off the lid of the epp). Centrifuge at 10 000 G for 2 mins. Discard the tube.

8. *Elution:* Transfer the Minicolumn to a clean 1.5 ml eppendorf tube with lid. Add 50 μl of DEPC water to the top of the column. Wait for at least 1 min (not longer then 30 mins) and then centrifuge at 10 000 G for 20 secs to elute the DNA. DNA is left in the tube.

9. Store the DNA at -20°C.

APPENDIX 6-DNA ligation

using the pGEM-T easy Vector System II by Promega

This is a method to stick PCR products (or other sources of DNA) into plasmids.

1. Set up ligation reaction as follows

T4 DNA Ligase 10X buffer	1 µl
Vector DNA (Plasmid)	50 ng (1 µl pGEM®-T Easy Vector)
Insert DNA (eg PCR product)	X µl
T4 DNA Ligase	1 µl
DEPC Water	To a final volume of 10 µl

2. Mix reaction by pipetting up and down and incubate overnight at 4°C.

APPENDIX 7-Transformation

Transformation refers to inserting the plasmid vector into a cell for replication.

Perform this method in the molecular lab (room 220). Ensure the plates are prepared beforehand.

Before you start:
1. Equilibrate 3 LB/ampicillin plates to room temperature per ligation. (if pGEM®-T Easy ligation then the plates are LB/ampicillin/IPTG/X-gal. Add 100µl IPTG and 20µl X-gal to each plate.) If using pQE vector, you don't need to add IPTG/X-gal). Leave to absorb at 37°C for 30mins.

2. Remove JM109 cells from -70°C and thaw on ice (about 5 mins). Flick tube to mix cells. Heat water bath to 42°C.

Transformation:
3. Centrifuge the tube containing the ligation reaction. Add 2µl of the reaction to a 1.5µl eppendorf tube on ice.

4. Add 50µl of JM109 cells to the eppendorf.

5. Flick the tube and place on ice for 20 mins.

6. Heat shock the cells by placing at exactly 42°C for 40-50 seconds in water bath.

7. Return tubes to ice for 2 mins.

8. Add 900µls of SOC medium.

9. Incubate for 1.5 hrs at 37°C with shaking (150 g).

10. Plate out 5 µl, 100 µl and the rest of the transformation on the plates prepared in step 1.

11. Incubate plates at 37°C overnight. (if using pGEM®-T Easy vector the white colonies contain the vector with the insert).

12. Pick up a colony (using a loop/toothpick) and grow up overnight in 10 ml of LB broth containing 20µl of 50 mg/ml ampicillin (final concentration =100ug/ml).

Screen for the presence of insert by PCR:
- add 5 µl of culture to a 50 µl PCR reaction.

Alternative:
- Purify the plasmid by running the culture through a Miniprep.
- Restriction digest the plasmid with enzyme/s that cut out the insert.
- Run on an agarose gel.

- If the insert is cut out, you will get more than 1 band in the gel. The target should occur around its usual PCR product size.

APPENDIX 8-Buffer Recipes - Transformation

IPTG stock solution
1.2g IPTG, made up to 50ml with sterile milliQ water.
Filter sterilise.
Store at 4°C.

LB Medium (1L)
10g bacto-tryptone
5g bacto-yeast extract
5g NaCl
pH 7.0 and autoclave

LB plates with Ampicillin
Add 15g agar to 1L LB medium. Allow the medium to cool to 50°C.
Add ampicillin to a final concentration of 100µg/ml.
Pour 30-35ml of medium per 85mm petri dish and allow to set.
Store at 4°C up to 1 month.

2M Mg^{2+} stock
$MgSO_4.7H_2O$ 2.46g
$MgCl_2.6 H_2O$ 2.033g
make up to 10ml with sterile water and filter

SOC medium
- 2g bacto-tryptone
- 0.5g bacto-yeast extract
- 1ml 1M NaCl
- 0.25ml 1M KCl

Add to 97ml distilled water. Stir, autoclave and return to room temp.

- 1ml 2M Mg^{2+} stock
- 1ml 2M glucose

make up to 100ml with autoclaved water
check that pH = 7.0
sterile filter through 0.2µm filter

APPENDIX 9- Zymosan - FITC labeling and serum opsonization

FITC-labelling zymosan

100mg zymosan
10ml carbonate buffer, pH 9.5 (30ml 1M Na_2CO_3, 70mls 1M NaHCO3, 900mls dH2O)
300μg FITC (ie 30μg FITC/ml)

Mix zymosan and buffer on vortex. Check pH with litmus paper. Add FITC and roll/mix for 2hrs.
Wash 2x in buffer, 1200rpm , 5mins, room temp.
Wash 2x in PBS.
Dilute to 200ml in PBS.
Check fluorescence under the fluorescent microscope.
Aliquot into 20ml lots in 50ml centrifuge tubes wrapped in foil.

(0.5mg zymosan/ml is approximately 1500 $X10^6$ particles/ml)

Opsonising FITC-labelled Zymosan

NB: Maximum amount of zymosan opsonised is 5mg/ml of serum

20 ml tube of FITC-zymosan at 0.5mg/ml
Add 10ml serum
Roll/agitate for 45 min at 37°C. Keep dark/in foil.
Spin at 800g (2000 rpm) for 10 min at room temperature. Discard the supernatant.
Resuspend the pellet to 20 ml in PBS. Retain as   serum opsonised FITC-zymosan   and aliquot in 5ml and 1ml lots. Store at -20°C.

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