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The use of cytokines to enhance vaccine efficacy in poultry

Analysis of the therapeutic and adjuvant properties of cytokines in Marek's disease virus infection

A report for the Rural Industries Research and Development Corporation

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Foreword

Marek's disease (MD), caused by the oncogenic herpes virus, Marek's disease virus (MDV), is a highly contagious lymphoproliferative disease of poultry with significant economic impacts for the world poultry industry. MDV infection has in the past been controlled by the extensive use of live vaccines. These vaccines are commonly administered either *in ovo*, at around 18 days of embryogenesis (E18), or to chicks at about the time of hatch. Although these vaccines have achieved a reduction in losses from disease, correspondingly, these vaccines have also been implicated in the selection of increased virulence as the MDV overcomes vaccine induced immunity. To cope with this development of hyper-virulent strains of MDV, new vaccines have been introduced, however, this leads to a vicious circle of the virus counteracting each new vaccine with strains of increased virulence. Therefore, in order to maintain the highest possible levels of animal health, welfare and productivity, it is vital that safe alternative vaccination strategies and therapeutics are developed.

Cytokines are naturally produced immune protein messages that direct and control the immune response during infection. Employing this activity of cytokines to augment vaccine action or for use as naturally occurring therapeutic represents an excellent alternative strategy to combat infection. The recent release of the chicken genome has lead to great advances in the identification of the chicken orthologues of many of the mammalian cytokines. However, at present there are very few of these cytokines that have been expressed in a recombinant form practical for use in experimental systems. With this in mind, the aim of this project was to assess the suitability of a number of recently developed recombinant cytokines as therapeutics during MDV challenge, as well as to examine their possible use as an adjuvant to enhance vaccine efficacy.

This report outlines the analysis of the immune response during MDV infection and the development of reagents used, and assessed, as potential cytokine therapeutics and vaccine adjuvants. Analysis of cytokine use in poultry trials has shown that treatment with chicken interleukin-2 (IL-2) appeared to be associated with reduced relative expression of the *Meq* gene of MDV. This evaluation of cytokine activity provides evidence for the rational use of chicken cytokines as therapeutics for disease.

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This report is an addition to RIRDC's diverse range of over 1500 research publications. It forms part of our Chicken Meat R&D program, which aims to support increased sustainability and profitability in the chicken meat industry by focusing research and development on those areas that will enable the industry to become more efficient and globally competitive and that will assist in the development of good industry and product images. Funding was also provided by the RIRDC Egg Program, now the Australian Egg Corporation Limited.

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Peter O'Brien
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Abbreviations

CAM	chorioallantoic membrane
CD	clusters of differentiation
cDNA	DNA complementary to mRNA
ConA	concanavalin A
cMGF	Chicken myelomonocytic growth factor
COS	*Cercopithecus aethiops* monkey kidney cell line
cpm	counts per minute
CSIRO	Commonwealth Scientific and Industrial Research Organisation
CTL	cytotoxic T lymphocyte
DMEM	Dulbecco's modified Eagle's medium
DNA	deoxyribonucleic acid
E	embryonic day
E. coli	*Escherichia coli*
FCS	fetal calf serum
G	guage
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HD11	chicken macrophage-like cell line
HSV	herpes simplex virus
HVT	herpes virus of turkeys
IFN	interferon
IL	interleukin
kDa	kilodalton(s)
LPS	lipopolysaccharide
M	molar
MD	Marek's disease
MDV	Marek's disease virus
M_r	relative molecular weight
Ni-NTA	nickel-nitrilotriacetic acid
NK	natural killer
nm	nanometers
OD	optical density
p	plasmid
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pfu	plaque forming unit
qPCR	quantitative polymerase chain reaction
RNA	ribonucleic acid
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SPF	specific pathogen free
TLR	Toll-like receptor
Th1	T helper 1
Th2	T helper 2
vv	very virulent
^{3}HT	methyl ^{3}H-thymidine
6x His	6x Histidine tag
7TD1	mouse IL-6 dependant cell line

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Executive Summary

MDV is a herpes virus that causes a lymphoproliferative disease of chickens. The virus causes infections in poultry world-wide and the disease is characterised by mononuclear cellular infiltrates in various tissues, T lymphomas and intense immunosuppression. MDV are categorised into 3 serotypes of virus: serotype 1 includes strains with oncogenic potential, serotype 2 includes non-oncogenic MDV strains, and serotype 3 is limited to herpes virus of turkeys (HVT). Fortuitously, serotype 2 and serotype 3 strains can be utilised as vaccines to protect against the more devastating effects of the serotype 1 strain. The most widely used MDV vaccine is HVT as it is efficient and inexpensive to manufacture, however, several other vaccines are also in use. MDV vaccines are effective when administered by *in ovo* vaccination at E18, or to chicks at hatch as the early development of an immune response is essential to the development of a protective response. Prior to the introduction of vaccines, infections from MDV presented a severe monetary risk to the poultry industry. Nevertheless, even in the face of the current vaccine approaches MDV remains a concern, particularly with the instability of vaccine induced protection and the development of more virulent strains of MDV. Furthermore, this may lead to the recurrence of MDV as a serious economic threat and, moreover, the emergence of hyper-virulent strains of MDV. This concern compels research towards the improvement of current vaccines and the development of new therapeutic approaches.

In the commercial situation it is generally thought that chicks are exposed to the virus through the inhalation of infected dander containing cell free virus. Although the pathogenesis of MDV infection is not fully understood, it appears these birds become infected as the virus makes its way into the lungs. This initiation of infection at the mucosal surface then triggeres the preliminary phases of the immune response against this virus. The ability to recognise and eliminate pathogens is essential for the survival of all animals. The diversity and number of infectious pathogens in the environment makes this a significant undertaking. The immune system comprises innate and acquired immunity that work together to eliminate invading organisms, thus protecting the host from infections. The innate immune response is essential for survival as it not only detects the presence of invading pathogens but acts to constrain the growth and spread of infection. Furthermore, the activation of innate immune cells, such as macrophages, heterophils and antigen presenting cells results in the production of cytokines that mediate ensuing responses and modulate the adaptive response. Consistent with the present models of pathogenesis, the immune cells lining the respiratory surface appear to be the target cells for the virus. Indeed, the phagocytic protective cells of the innate immune system have a peculiar role to play in the development of this disease. It is clear that macrophages are important in protection against MDV infection. *In vitro* experiments have shown that macrophages from MDV-infected chickens or activated macrophages inhibit MDV replication. Furthermore, depletion of macrophages *in vivo* during the infection results in an increased incidence of tumours. However, it seems that macrophages in their efforts to defend the mucosal surface happen to become the first victims of the virus. Furthermore, there is evidence to suggest that the immunosuppression observed during MDV infection may be mediated by macrophages. With this in mind, it is clear that the early immune response to the virus is critical in the control of the infection and conversely the development of immunosuppression. It is postulated that the infected macrophages move the virus from the respiratory system systemically to the lymphoid organs. In the periphery the virus changes its trophism and targets the effector cells of the adaptive immune response, the lymphocytes.

Acquired immunity is characterised by specific responses, which are controlled by T and B cells. As the infection proceeds the virus works its way along the immune cell types directed towards B cells in the lytic phase of infection. Subsequently, the infection changes from the cytolytic stage to a latent phase in which T cells become the objective of the virus. Activated T cells become the intended cell type for neoplastic transformation. This results in the development of lymphomas in various organs. Furthermore, at this stage the virus is delivered by the infected lymphocytes to the skin where a fully productive infection occurs in the feather follicle epithelium and infectious cell-free virus is shed. This progression of the virus through cells of the innate response, followed by the B cells of the humoral

response, then onto T cells of the cell mediated immune response, directs the acute incapacity of the immune system and marked immunosuppression during infection with MDV. In its various phases of infection the virus targets and tampers with the immune system, therefore, strategies directed at enhancing the immune response may help protect against infection. Activating the immune response may be beneficial in MDV-infected chickens as it might act to reduce initial viral replication and slow down early immunosuppression. The therapeutic use of recombinant chicken cytokines might provide one means to mediate such an effect. Furthermore, the concerns over the ability of current live vaccines to protect against emerging hyper-virulent strains of virus necessitate the need for alternative vaccine strategies such as the use of adjuvants to enhance their activity. Selection of an appropriate adjuvant is of the upmost importance as it is crucial that an adjuvant directs the immune response towards an appropriate protective response. If an adjuvant inadvertently skewed the response to a vaccine in the contrary direction it could have dire consequences with regard to infection.

Presently, there is a paucity of suitable, cost effective adjuvants for use in both the broiler and egg industries. As cytokines are proteins that control the immune responses following infection or vaccination they represent excellent, naturally occurring therapeutics and potential adjuvants. The efficacy of cytokine therapy and use as vaccine enhancers has been demonstrated in several human and animal studies, particularly in the treatment of virally induced immunodeficiencies - this type of virally induced immunosuppression somewhat akin to MDV infection. The recent release of the chicken genome information and the identification of a number of chicken orthologues of mammalian cytokines has provided the means to access a number of chicken cytokine reagents for testing. Furthermore, the practical use of these cytokines is becoming more feasible with the establishment of commercially feasible methods of delivery. However, there are many different types of cytokines that each perform different functions and although it may be postulated that these will function identically between chickens and mammals, the low levels of sequence homology may indicate functional differences. With this in mind, it is critical that the role of chicken cytokines be characterised and assessed for the identification of those most favourable.

To institute a proof-of-principal of the utility of chicken cytokines as therapeutics and adjuvants in MDV infection, we have assessed a number of recently identified chicken cytokines: chicken myelomonocytic growth factor (cMGF), IL-6, IL-18, cytokines connected with the early innate immune response, as well as interferon-γ (IFN-γ), IL-2 and IL-15, cytokines that are associated with cell mediated immunity. Each of these cytokines has its own particular role to play in the development of an immune response with the capacity to modulate the immune response to viral infection. We have previously shown that treatment with one of these cytokines, IFN-γ, when co-administered with antigen produced a prolonged secondary antibody response in specific pathogen free (SPF) birds that persisted at higher levels and for longer periods compared to antigen injected alone. Furthermore, this cytokine showed the capability to enhance the growth performance of broilers following a coccidiosis infection. However, this is just one potential cytokine and it is important to assess the prospective success of other newly discovered cytokines.

The overall objective of this research project was to assess the ability of chicken cytokines enhance disease resistance and increase vaccine efficacy in the face of MDV infection. Therefore, the first objective was to develop a model of MDV infection and analyse the cytokine response during infection. The second objective was to produce recombinant cytokines and use these in trials to assess their ability enhance vaccine efficacy and improve disease resistance. In this undertaking we have developed a model of MDV infection and assessed changes to the lymphoid organs across the course of infection. Further to this, we have assessed the cytokine profile of the spleen during infection and observed that during the early stages of infection (day 4 post-infection) a number of different cytokines are elevated, in particular IL-6. In mammals the immune response to a pathogen is often described as being a T helper 1 (Th1) type response when the predominant cytokine produced is IFN-γ, and often associated with cell-mediated immunity. In contrast, the immune response to other pathogens is often described as being a T helper 2 (Th2) type response when the predominant cytokine produced is IL-4, and often associated with humoral immunity. Intriguingly, during MDV infection, there was a relative increase in production of both IFN-γ and IL-4.

To assess the potential of administered cytokines to enhance disease resistance we developed several expression systems for the production of recombinant cytokine proteins. The recombinant cytokine products were expressed and purified from an *Eschericia coli* (*E. coli*) protein expression system and their bioactivity was validated in a number of *in vitro* cell culture systems. These recombinant cytokines were then assessed in a number of animal trials. In order to assess the impact of cytokine injection *in ovo*, E18 embryos were administered with various doses of cytokine and hatchability rates were determined. All of the cytokines tested had little impact on chick hatchability.

To determine the therapeutic potential of *in ovo* administered cytokines against MDV infections, the various cytokines were injected into the egg than hatched birds were challenged with MDV. Although analysis of lymphoid organ weights showed little statistically significant differences, trends in variation in thymic lobe size were observed in that IL-2, IL-6 and IL-18 all appeared to impact on thymic lobe size. When an analysis of the relative abundance of the viral *Meq* gene was made from trial bird spleens, a reduced relative expression was observed particularly for IL-2 treated chickens. When the cytokines were administered *in ovo* in combination with the HVT vaccine and hatched birds challenged with MDV, as previously observed, little statistically different changes were observed for lymphoid tissue weights, except for IL-18 which showed decreased splenomegaly. Observations of thymic lobe size suggested that IL-2 treated eggs showed a promising resistance to MDV induced atrophy. Taken together, these results suggested that the likely candidate cytokines to take into larger scale pen trials were IL-2 and IL-6. A pen trial to investigate the impact of cytokine administration to broiler bird performance reared under commercial conditions and challenged with MDV was carried out. Under these conditions no statistical difference was observed between the groups. There was, however, the observation that for IL-2 treat birds the mean score for spleen weight was reduced and the bursa mean weight was maintained. These trends might suggest some utility in the use of IL-2, however, it is apparent that futher work with the MDV model in broilers is required and a larger trial with greater numbers of animals is necessary to determine a clearer picture of this cytokines ability.

The results from this project have implications for the Australian poultry industry. Based on overseas experience with regard to the vaccine breaks with hyper-virulent forms of the MDV there is a need to investigate alternative strategies before a similar situation develops in Australia. By taking a proactive approach to investigation the potential of novel therapeutics to immunoenhance against viral infections the Australian poultry industry is enhancing their MDV preparedness. Future investigations may show that cytokine mediated enhancement of the immune system leads to decreased pathogen loads, resulting in healthier and more productive birds.

The aim of this study was to define the effects of various chicken cytokines on the development of MDV infection.

To best make use of the outcomes of this project, the following recommendations are made for further work required in this area:

1. Further evaluation of the nature of the Th1 against Th2 type response cytokines during infection with MDV.
2. Immune gene analysis of the protective response engendered by vaccination, HVT compared to Maravac.
3. Development of optimal methods for the safe and efficient delivery of therapeutics under commercial conditions.
4. Assessment of cytokine activity in the face of more virulent wild-type MDV strains in field trials.

1. Introduction

1.1 Marek's disease

MD is a highly infectious, lymphoproliferative disease of chickens with great economic importance to the poultry industry in most countries (Nazerian, 1973; Gimeno et al., 2004; Silva et al., 2004). MD is characterised by lymphoid infiltration in the peripheral nerves and development of tumours in the visceral organs, muscles and skin. MD was first described in 1907 by Hungarian veterinarian József Marek, presenting as a neuropathic polyneuritis disease of chickens (Kato and Hirai, 1985), as the consequences of this MD included paralysis, it was originally known as 'fowl paralysis'. In 1967, Churchill and Biggs identified a cell associated herpes virus as the causative agent of MD (Churchill and Biggs, 1967). Initially, it was defined as a member of the γ-herpes virus sub family, due to its tropism for lymphocytes. However, it was later found that MDV has the genomic structure of an α-herpes virus (Buckmaster et al., 1988). With this in mind, a new sub family, Marek's disease-like viruses was created (Baaten, et al., 2004). MDV is extremely contagious and an entire flock may be infected before the first birds show symptoms (Zander, 1972). In fact, it has been suggested that in commercial production many flocks may be infected with MDV at some stage in the process, however, most show little clinical evidence of the disease (Zander et al., 1972). This is evidenced by the presence of precipitating antibodies in flocks despite low disease incidence (Chubb and Churchill, 1968).

Until brought under control by vaccination, MDV was the major cause of mortality, carcase contamination and economic loss to the poultry industry in many countries (Payne et al., 1976). Moreover, vaccines, such as HVT, have been used to successfully control MDV for many years (Witter, 1987). However, it appears more virulent strains of MDV are developing and the current vaccines may no longer offer protection (Kaufman and Wallny, 1996). This, of course, could have a huge economic impact on the world poultry industry (Witter, 1997). The causes for the selection for hyper-virulent forms of MDV are unknown, however, it is speculated that it may at least be partly due to current vaccination strategies (Witter, 1998; Gimeno et al., 2004). It may be presumed that the use of increasingly more virulent strains of MDV vaccines are exerting selective pressure for more virulent strains of wild type infective MDV (Karaca et al., 2004). An example of this may be the isolation of very virulent serotype 1 MDV from Australian flocks vaccinated with a bivalent vaccine (De Laney et al., 1995; McKimm Breschkin et al., 1990). Hence, it is a developing fear that current vaccines may soon not offer optimal protection, which could in turn lead to decreased production performance. This impact is already evident in many of the world's poultry markets as even though many farms are vaccinated against MDV, epidemics still occur frequently enough to be a concern (Kaufman and Wallny, 1996).

1.2 The MDV infection process

Natural infection begins by inhalation of feather follicle epithelium which is of major significance in the natural transmission of MDV (Calnek et al., 1970; Reddy et al., 2000). However, this natural mode of infection has not been well replicated and most studies have involved intra-abdominal inoculation of cell-associated virus. With this in mind, little is known about the early responses to MDV infection in natural infection. Nevertheless, the response to MDV infection has been somewhat characterised in these intra-abdominal models (Figure 1.1). It is postulated that phagocytic cells in the lung take up viral particles and within 24-48 h the virus begins replicating in these cells. These infected cells then travel via lymph or blood, transferring the MDV to lymphoid tissues such as the spleen, bursa of Fabricius and thymus (Addinger and Calnek, 1973; Dalgaard et al., 2003; Schat, 1991; Kaufman and Wallny, 1996). The resulting cytolytic infection can be observed 3-6 days post-infection in B cells, which are presumed to be the primary target for viral replication (Calnek et al., 1982; Schat et al., 1981; Shek et al., 1983). In response to the B cells infection, activated T cells (Ia+) are recruited to the infection and are themselves made a target for the virus (Schat et al., 1991; Schat, 1991). Following this, at about 1 week post infection when viral levels peak, the MDV infection switches from cytolytic to a latent infection (Calnek et al., 1984; Buscaglia et al., 1988; Schat, 1991; Schat and Xing, 2000). The development of this latency is thought to be influenced by the immunosuppression generated by MDV infection (Buscaglia et al., 1988). Latently infected T cells carry MDV to the feather follicles

where a fully productive infection occurs from about 10 days onwards (Calnek et al., 1970). This infection of the feather follicle epithelium allows horizontal transmission by shedding infectious cell free MDV with flakes of skin into the environment. Non-infected birds can become infected by inhaling this cell free virus, thus starting the infection process again (Beasley et al., 1970; Calnek et al., 1970).

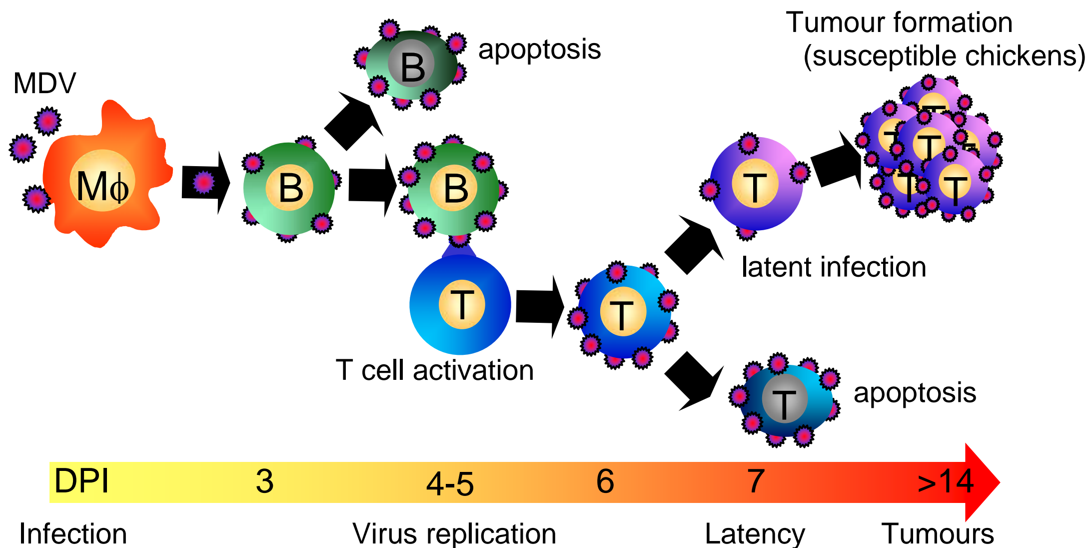

Figure 1.1. Progression of experimental intra abdominal MDV infection. Within 24-48 h after intra abdominal infection, replication of MDV is initiated in B cells and macrophages. This progresses into a cytolytic infection in lymphoid organs, which is observable between 3-6 dpi. In response to the B cell infection T cells are activated, leaving them susceptible to infection. By ~7 dpi, MDV enters latency and from 14 days onwards tumours develop in susceptible chickens.

After latency has been established there are two outcomes: lymphomas may develop leading to regression or death, or, in most cases infection remains latent without the development of any signs of disease (Nazerian, 1973). The development of lymphomas occurs several weeks or months after the establishment of latency and is influenced by factors such as genetics, age at infection and strain of virus. These lymphomas appear in the ovary, testes, kidney, liver, lung and heart (Nazerian, 1973; Kaufman and Wallny, 1996). Central nervous system lesions have also been reported as a consequence of MDV infection (Gimeno et al., 1999). Other symptoms MDV infections can include transient paralysis, acute transient paralysis, persistent neurologic disease and late paralysis (Gimeno et al., 1999). These symptoms are greatly influenced by the genetic strain of chicken and virulence of the virus (Gimeno et al., 1999).

1.3 MDV serotypes and oncogenicity

MD is grouped in 3 serotypes as first described by von Bulow et al., 1975. These serotypes of MDV are: serotype 1, which is made up of pathogenic virus strains with variable oncogenicity; serotype 2, made up of non-pathogenic strains of MDV which are generally naturally occurring and non-oncogenic; and serotype 3, the non-oncogenic HVT. The non-pathogenic strains, like HVT and serotype 2 strains, can be used as vaccines against serotype 1 strains. As virulence and oncogenicity is associated with serotype 1 MDV these strains are classified from mild through to very virulent plus depending on their oncogenicity and virulence. Although the oncogenicity of these strains has been shown to differ, no antigenic differences between the isolates have been found (von Bulow et al.,

1975). The difference in the pathogenicity of MDV strains is thought to be influenced by a number of factors, including spread rate of virus and immunogenicity (Calnek et al., 1977). It has been postulated that the more efficient early replication observed in the serotype 1 viruses, labelled as very virulent plus (vv+) MDV, may allow the virus to infect a greater number of cells and cause more damage before the chickens immune system can respond and establish protective immunity (Miles et al., 2001).

1.4 Immune response to MDV

As with any viral infection, MDV provokes a complex immune response. This response begins with an innate response followed by the ensuing humoral and cell mediated responses (Xing and Schat, 2000). It appears the early innate response of macrophages is important in control of the virus as in birds with depleted macrophages there was increased disease prevalence (Gupta et al., 1989; Akira and Hemmi, 2003). Moreover, macrophages appear to have a role in the suppression of MDV viral replication but intriguingly they also appear to be the primary target for the virus (Kodama et al., 1979; Haffer et al., 1979). Other aspects of the innate response, such as Natural Killer (NK) cells, also appear to play a role in the immune response to MDV infection. This NK cell activity may be influenced by the susceptibility of the chicken line as levels of NK cells have been shown to decline in susceptible chickens, whereas non-susceptible lines have elevated NK levels (Sharma, 1981). This may be due to the establishment of immunosuppression in susceptible lines, which correlates, with the development of a second or late viral cytolytic phase of infection and the development of lymphomas (Uni et al., 1994). Additionally, NK cells are thought to play a role in immune surveillance of tumours (Sharma, 1981). Further to the innate response, the adaptive immune response to MDV is clearly important in constraining the infection (Barton and Medzhitov, 2002). However, as MDV is cell associated, the role of MDV-specific antibodies may be minimal (Markowski-Grimsrud and Schat, 2002). Chickens infected with MDV develop precipitating antibodies within the first weeks of infection. Most of these antibodies are not relevant for a protective response, as they are directed against non-structural, non-envelope viral proteins. The presence of antibody does not prevent the establishment of virulent virus in the circulation (Churchill et al., 1969), however, the presence of maternal antibodies reduces the cytolytic infection and can also reduce the efficacy of low titres vaccines. With regard to the T cell adaptive response, it appears cytotoxic T lymphocytes (CTL) are involved in the anti-MDV response. reticuloendotheliosis virus-transformed cell lines transfected and expressing MDV genes were shown to be targets for CTL from infected and vaccinated chickens. These effector cells developed around 7 days post-infection and were characterized as typical CTL expressing CD3 and CD8. However, the immunosuppressive activity of MDV infection does impact on both humoral and cell-mediated immunity. This immunosuppression leads to reduced antibody responses and alterations in T cell activity.

As a part of both the innate and adaptive immune response, cytokines play an important part in the immune response to virus. Cytokines are a family of low molecular weight proteins that mediate and orchestrate the complex events in the immune response (Zekarias et al., 2002). Cytokines regulate the amplitude and duration of the immune response by controlling the numbers, lineages and functional activation of immune cells together with their recruitment to the sites of immune localisation and infection (Balkwill, 1988; Kelso, 1989; Arai et al., 1990). Generally, cytokines can be grouped into 2 broad categories: those that act to stimulate what are termed Th1 type immune responses, which mainly protect against intracellular viral, bacterial, fungal and protozoan pathogen (IFN-γ and IL-12) and those that stimulate Th2 type responses involved in protection against extracellular infections such a Helminths (IL-4, IL-5, IL-10 and IL-13)(Banyer, et al., 2000). In the initial stage of infection innate immune cells produce proinflammatory cytokines such as IL-1, IL-6, IL-12, IFN and chemokines to give rise to the inflammatory immune response (Djeraba et al., 2000). Additionally, IL-1 and IL-18 are key regulators of the innate and adaptive immune response to viral infection (Harte et al., 2003).

1.5 Cytokines in MDV infection

Until recently little was known about the variously roles of cytokines in the immune response to MDV. However, recent publications have expanded this area of research. Early studies of MDV infection had suggested that IFN were induced in resistant chickens. Moreover, it has become clear that early in the immune response to MDV changes in interferon expression occur. IFN-γ may be of

prime importance in the early stages of Marek's disease when the cytolytic phase of viral multiplication occurs (Djeraba et al., 2000). IFN-γ may act indirectly by stimulating macrophages to produce nitric oxide Djeraba *et al.,* found that when chicken macrophages were activated *in vitro* with IFN-γ, these cells were able to limit MDV replication. This anti-viral activity was presumably through nitric oxide dynamically inhibiting the division of cells infected with MDV (Djeraba et al., 2000). It has been shown that MDV infection results in up regulation of IFN-γ transcription as early as 3 days post-infection, however, the up regulation of IFN-α could not be demonstrated. Oral administration of IFN-α has been shown to decrease the number of virally infected splenocytes in chickens (Jarosinski et al., 2001). While *in vitro* studies on chick kidney cells cultures have shown IFN-α inhibits replication of MDV in a dose dependent manner (Jarosinski et al., 2001). Nevertheless, the role of IFN-α in protective immunity is somewhat unclear particularly with the lack of up regulation during infection.

Additionally the cytokines IL-6 and IL-18 have been linked to MDV resistance. Increased transcript levels were observed for both cytokines in splenocytes of susceptible genotypes, but not in resistant chickens (Kaiser et al., 2003). The increase in IL18 in susceptible chickens is puzzling as IL-18 has been shown to be potentially protective to a range of virus infections including the herpes viruses, such as herpes simplex virus (HSV) and murine cytomegalovirus (Baaten, et al., 2004). Furthermore, IL-18 has an ability to induce IFN-γ and enhance NK cell activity, which is not consistent with the results observed after MDV infection (Baaten, et al., 2004). Similarly, the increase in IL-6 in susceptible birds is somewhat intriguing as IL-6 has been show in other systems to contribute to resistance against the herpes virus HSV-1 (Paludan and Mogensen, 2001).

1.6 Vaccines and control of MDV

As with many other infectious viral diseases control of MD has involved a variety of approaches including genetic selection, sanitation and isolation of young chickens (Okazaki et al., 1970; Zander, 1972; Gavora and Spencer, 1979; Witter, 1998). These approaches have improved resistance to MDV infection, however, widespread use of vaccines produced a dramatic and direct reduction from losses. Effective use of HVT, a non-pathogenic herpes virus isolated from turkeys, as a vaccine decreased mortality from MDV infection (Goan et al., 1972), therefore, when HVT was introduced as a vaccine in broiler and layer chickens, losses due to MD were dramatically reduced (Witter, 1998; Witter, 1987). Similarly, it was found that low pathogenic strains of MDV isolated from healthy flocks were effective in protecting susceptible chicks against MDV challenges with virulent virus (Rispens et al., 1972a). However, further to this Rispens *et al*., (1972b) recognised that the inoculation of all day old chicks may not be a final answer to MDV control, particularly as this approach may be too expensive and time consuming (Rispens et al., 1972b). Due to the high cell association requirements of the vaccine viruses, more practical means of vaccination have not been forthcoming, nonetheless, administration of vaccine has improved to some extent with the introduction of *in ovo* injection.

The apparent reduction of vaccine efficacy and the continuing emergence of increasingly virulent viral strains have prompted understandable concern with regard to MDV vaccination strategies. One suggestion may be that immunoenhancement might provide an indispensable accessory for a successful vaccination approach.

1.7 Adjuvants

For a vaccine to be successful it must be able to induce defined immune responses that will confer protection against a specific pathogen. In spite of this, the low level immunogenicity of some vaccines means that adjuvants are commonly required for use with vaccines to enhance the specific immune response (Weeratna et al., 2000). Nevertheless, there are few adjuvants available that give the appropriate immunostimulatory activity, are safe and currently approved for use (Weeratna et al., 2000). Therefore, the lack of appropriate existing adjuvants suggests a need for the development of alternative forms.

The immunostimulatory potential of an adjuvant is its ability to induce the relevant and protective cytokines of the immune response against the pathogen that the vaccine is directed against. Cytokines, such as the interferons and proinflammatory cytokines, are crucial in immunogenicity, antigen

presentation and in the down stream clonal expansion of the adaptive immune response (Schijns, 2001). These immunostimulatory cytokines can be administered with a vaccine to enhance the development of protective immunity. The chicken cytokine cMGF when administered via fowl pox virus vector has been shown to modify tumour development in chickens infected with highly virulent MDV (Djeraba et al., 2002). Moreover, cMGF was shown to reduce tumour burden when administered in conjunction with HVT (Djeraba et al., 2002).

The current development in identification, cloning and expression of chicken cytokines has meant that a broad range of cytokines and other immunomodulatory molecules are increasingly being made available to investigate immunological responses against MDV. Furthermore, the exploitation of these new cytokines could lead to the development of improved vaccine strategies to play a key role in the continuing battle against MDV infection.

2. Objectives

The overall objective of this research project was to assess the ability of chicken cytokines enhance disease resistance and increase vaccine efficacy in the face of MDV infection.

These objectives involved:

- to develop a model of MDV infection and analyse the cytokine response during infection
- assessed the cytokine profile of the spleen during MDV infection
- to produce recombinant cytokine for use these in trials
- to assess the impact of cytokine injection *in ovo*
- to determine the therapeutic potential of *in ovo* administered cytokines against MDV infections
- to determine the adjuvant effect of cytokines in HVT vaccinated, MDV challenged birds
- to extend the therapeutic and adjuvant experiments into broiler bird trials

3. Methodology

3.1 Animals

SPF chickens were raised in flexible plastic isolators and fed fumigated feed and acidified water *ad libitum*. For commercial pen trials, broiler chicks (Ross strain) were obtained as day 18 embryo from Barter Pty Ltd and housed on deep litter in floor pens at the CSIRO Werribee animal facility. Broiler birds were maintained on a commercial feed (Ridleys Pty Ltd, Australia) regime consisting of 0.5 kg ration per bird of broiler starter between days 0-14, 1 kg of grower from days 15-28. Drinking water was provided by commercial nipple drinkers. All experimental procedures were approved by the CSIRO Animal Health Animal Experimental Ethics Committee.

3.2 Production of recombinant chicken cytokines

Chicken cytokines were produced in an *E. coli* expression system. Recombinant cytokines were cloned into expression vectors: pQE9 vector for IL-6, pET vector for IL-15 and pQE30 for cMGF, IL-18, IFN-γ, IL-2. These vectors were then transformed into the *E.coli* and expression cultures were set up. Log phase cultures were induced with 0.02 mM isopropyl-β-D-thiogalactopyranoside then incubated for 3 h at 37°C. Bacterial cell pellet were resuspended Ni-NTA phosphate buffer or Talon tris buffer, then sonicated and supernatants collected for purification. Recombinant cytokines were purified using either Ni-NTA resin or TALON™ Metal Affinity Resin. Solubilised protein samples were mixed with resin then allowed to bind. The cytokines were then eluted with elution buffer and analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western Blot.

3.3 Assay for chicken cytokine bioactivity

3.3.1 cMGF and IL-6 bioassays

The bioactivity of cMGF and IL-6 were determined using an IL-6 dependent mouse hybridoma cell line, 7TD-1. One ml of log phase cells were cultured with 6 µl of chicken IL-6 for 48 h, then cells starved of IL-6 for a further 48 h. Cells at 1×10^4 viable cells/ml were cultured in the presence of serial dilutions of recombinant cMGF or IL-6. After incubation at 37°C for 48 h in a humidified environment with 5% CO_2 (Forma Scientific, USA), proliferation was assessed by methyl ^{3}H-thymidine (^{3}HT, tritiated thymidine) incorporation.

3.3.2 IL-18 bioassays

Splenic mononuclear cells from 4-week-old SPF chickens at 2.5×10^6 cells/ml were cultured with serial dilutions of IL-18 or appropriate controls in the presence of a constant amount of concanavalin A (ConA) (Sigma) mitogen (5µg/ml). Assays were conducted at 37 °C for 48 h in a humidified environment with 5% CO_2. IL-18 activity was then measured by either cell proliferation (tritiated thymidine incorporation) or IFN-γ production.

3.3.3 IFN-γ bioassays

HD11, a chicken macrophage-like cell line, cells at 2×10^6 cells/mL were cultured with serial dilutions of samples for 12 h in Dulbecco's modified Eagle's medium (DMEM)/10% Fetal calf serum (FCS) and 20ng/mL of lipopolysaccharide (LPS) at 37 °C in a humidified environment with 5% CO_2. 50µL of culture supernatants were added to 100 µl of Griss reagent and the optical density (OD) measured at 540 nm using a Titertek multiscan plate reader.

3.3.4 IL-2 and IL-15 bioassays

72 h ConA blasts were made by stimulating chicken spleen cells ($5x10^6$ cells/ml) with 10 µg/ml ConA (Sigma, Germany) in DMEM/10% FCS at 37°C in a humidified environment with 5% CO_2. The blast cells were harvested and ConA inactivated by incubation of the cells with 0.1 M methyl α-D-mannopyranoside (α-MM; Sigma, Germany) for 30 min. Viable cells at $1.25x10^5$ cells/ml were cultured with serially diluted samples and appropriate controls for 37°C for 48 h. Cultures were pulsed with 1.0 µCi per well of ^{3}HT for an additional 6 h. Thymidine incorporation was measured using Betaplate Scint Liquid Scintillation (Wallac, Finland) and Luminsescence Counter (Wallac, Finland) according to manufacturer's instructions.

3.4 *In ovo* administration of cytokines

E18 SPF hybrid white leghorn eggs were purchased from SPAFAS (Woodend, Victoria, Australia). Eggs were candled and the air sac and the centre of the air sac marked, swabbed with iodine and a hole punched directly in the centre of the air sac. Cytokines, were administered at doses of 0.5, 5 and 50 µg/egg (made up in 200 µL of phosphate buffered saline (PBS)) into the amnion using a 25 guage (G), 2.5 cm needle. The hatchability of the eggs was determined and the chickens raised in flexible plastic isolators in the CSIRO Livestock Industries small animal facility in Geelong, and were fed fumigated feed and acidified water for 7 days. Chickens were then euthanased by CO_2 asphyxiation, body weight taken, blood and organs harvested.

3.5 Models of MDV infection

Several trials were performed in order to develop and utilise an MDV infection model. Trials were established in negative pressure isolators in the CSIRO Livestock Industries small animal facility in Geelong. Briefly, these trials entailed:

3.5.1 Trial 1

A MDV challenge strain MPF57/1:W7B3S was obtained from Greg Tannock, RMIT, Australia. Specific pathogen-free chickens were challenged at 7 days old with 200 µL of 500 plaque forming units (pfu)/mL virus in DMEM with 10% FCS. Birds were inoculated intra abdominally with a 21 G needle. These chickens were raised in negative pressure plastic isolators in the CSIRO Livestock Industries small animal facility in Geelong, and were fed fumigated feed and acidified water. To examine disease progression chickens were euthanased by CO_2 asphyxiation at 4, 7, 14 and 21 days post infection. At this time bodyweight was measured and spleen, bursa and the second thymus lobe on the right were removed and stored in RNAlater.

3.5.2 Trial 2

In addition to the cytokines, half the eggs also received the MDV vaccine, Poulvac HVT CF Vaccine (Fort Dodge, Australia) following the manufactures instructions. In short, ≥1000 pfu in 100 µL of Poulvac sterile diluent was injected into E18 eggs in the same method as the cytokines. Birds where challenge with 200 µL of 500 pfu/mL MPF57/1:W7B3S virus from Greg Tannock, RMIT, Australia in DMEM with 10% FCS. Birds were inoculated intra abdominally with a 21 G needle. Specific pathogen-free chickens were challenged at 7 days old. These chickens were raised in negative pressure plastic isolators in the CSIRO Livestock Industries small animal facility in Geelong, and were fed fumigated feed and acidified water. To examine disease progression chickens were euthanased by CO_2 asphyxiation at 4, 7, 14 and 21 days post infection. At this time bodyweight was measured and spleen, bursa and the second thymus lobe on the right were removed and stored in RNAlater.

3.6 Quantitative real time PCR (qPCR) analysis of chicken cytokines genes during MDV infection

3.6.2 qPCR

qPCR was performed using TaqMan 2 x universal master mix (Applied Biosystems, USA) along with 20x gene expression assay mix (Applied Biosystems, USA) for various genes of interest. For normalization of the results the endogenous gene GAPDH was used. The probes were labelled with 5' reporter dye FAM and the 3' quencher TAMRA. The reactions were performed in 96 well optical reaction plates. Every reaction contained: 12.5 µL TaqMan 2x universal master mix, 1.25 µL 20x gene expression assay mix and 11.25 µL of cDNA diluted 1/50. Each sample was tested in duplicate. The reactions were run on the ABI PRISM™ 770 sequence detection system (Applied Biosystems). The amplification program was: 50°C for 2 min, 95°C for 10 min, (95°C for 15 sec, 60°C for 1 min) x 40 cycles.

3.6.2 Relative expression

Relative expression of genes of interest (ΔΔCt method) was represented as level of gene expression in the test sample compared to relevant reference samples, normalised against the expression of an endogenous house keeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The comparative Ct method is similar to the standard curve method, except it used arithmetric formulas to achieve the same result for relative quantitation. The amount of target, normalised to an endogenous reference and relative to a calibrator is give by $2^{-\Delta\Delta Ct}$ as described in the ABI PRISM 7700 sequence Detection System user bulletin #2. Threshold cycle numbers (Ct) were determined with Sequence Detector Software (version 1.6; Applied Biosystems) and transformed using the ΔCt or ΔΔCt methods as described by the manufacturer using GAPDH as the calibrator gene (Zarember and Godowski, 2002).

3.7 Microarray analysis of gene expression during MDV infection

A two-colour cDNA microarray platform with 2500 genes (printed by Joan Burnside's laboratory, USA) was used to examine the differences between MDV infected chickens with uninfected chickens (controls). A total of six spleens (3 infected and 3 control) were collected from the chickens four days post MDV infection and mononuclear cells (lymphocytes) were isolated. Control and infected lymphocytes were directly compared to each other across four individual arrays (Figure 3.1).

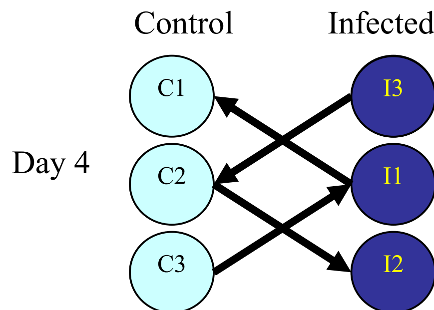

Figure 3.1 – Experimental design of MDV microarray experiment. Circles indicate samples used and black arrows indicate all arrays performed in the experiment.

Two fluorescent dyes were used (Alexa 555 and Alexa 647) to label the samples prior to hybridisation. Dye-swaps were performed to reduce the effect of dye bias in the experiment. Array results were analysed using BRB-Array Tools (version 3.2.2). All arrays were normalised and quality control methods based on intensity signals were applied. Student T-tests were then applied to detect significant differential gene expression between the control and infected samples.

3.8 Assessment of cytokines during challenge trials

Several trials were performed in order to assess the ability of the various cytokines to enhance the disease resistance or to assess their potential to augment the performance of chickens vaccinated against MDV.

3.8.1 Trial 3

Groups of 12 or more recived the cytokines cMGF, IL-6, IL-18, IFN-γ, IL-2 and IL-15 at the optimised dose of 50 μg/egg, injected into E18 eggs. In addition to the cytokines, half of each group also received the MDV vaccine, Poulvac HVT CF Vaccine (Fort Dodge, Australia) as described in section 3.1.2. The hatchability of the eggs was determined and the chickens raised in hard plastic negative pressure isolators in the CSIRO Livestock Industries small animal facility in Geelong, and were fed fumigated feed and acidified water. At 7 days of age the chickens where challenged with MDV as described in section 3.5.1. At 21 days post challenge the chickens were euthanased by CO_2 asphyxiation, body weights measured, and organs harvested.

The bodyweight of the chickens was measured and their spleen and second thymus lobe on the right was removed and weighed. All samples were stored in RNAlater at 4 °C until the RNA extracted.

3.8.2 Trial 4

The cytokines IL-6, IL-18 and IL-2 at 50 μg/egg were injected into E18 eggs. In addition to the cytokines, half of each group also received the MDV vaccine, Poulvac HVT CF Vaccine (Fort Dodge, Australia) as described in section 3.1.2. The hatchability of the eggs was determined and the chickens raised in hard plastic positive pressure isolators in the CSIRO Livestock Industries small animal facility in Geelong, and were fed fumigated feed and acidified water. At 7 days of age the chickens where challenged with MDV as described in section 3.5.1. At 21 days post challenge the chickens were euthanased by CO_2 asphyxiation, body weight measured, and organs harvested.

The bodyweight of the chickens was measured prior and their spleen and second thymus lobe on the right being removed. These samples were weighed and stored in RNAlater at 4 °C until the RNA was extracted.

3.8.3 Trial 5

Two groups of 66 broiler E18 eggs were vaccinated as described in 3.1.2. in conjunction with cytokines IL-2 and IL-6 at the optimised dose of 50 μg/egg as per section 3.4. 20 eggs where vaccinated only and a further 20 eggs where injected with only PBS.

The hatchability of the eggs was determined and the chickens raised as if in a commercial setting. All birds were fed as per normal commercial feed regime. At 7 days of age the chickens where challenged as described in section 3.5.1. At 21 days post challenge the chickens were euthanased by CO_2 asphyxiation, body weight measured, and organs harvested.
10 chickens where randomly selected for spleen and second thymus lobe on the right collection and bursa from all birds was harvetsed. The collected organs where weighed and stored for later use.

3.9 Statistical analysis

Data means and standard errors where calculated using the program Excel (Microsoft, USA). Further statistical analysis was performed using the program GraphPad Prism version 3.03 (GraphPad Software, USA). Two-tailed Mann-Whitney 'U' tests were used to determine differences between two groups of data. Kruskal-Wallis tests were used to compare multiple groups and where differences were found, a Dunn's multiple comparison post test was used to compare all pairs of groups. Experimental values differing significantly from control values are indicated by different levels of significance: $*p<0.05$, $**p<0.01$, $***p<0.001$.

4. Results

4.1 Establishment of an MDV infection model

4.1.1 Trial 1

Trials were performed to establish and develop a model of MDV infection. In this model birds were injected intra-abdominally with MDV (MPF57) and their body weight and tissues assessed 21 day post-infection. Although there was little body weight differences between control and infected birds (Figure 4.1A), there was however noticeable changes in spleen, thymus and bursa size and weight. Splenomegaly was seen across the course of infection (Figure 4.1B) and trends towards thymic and bursal atrophy were similarly seen (Figure 4.1C, D).

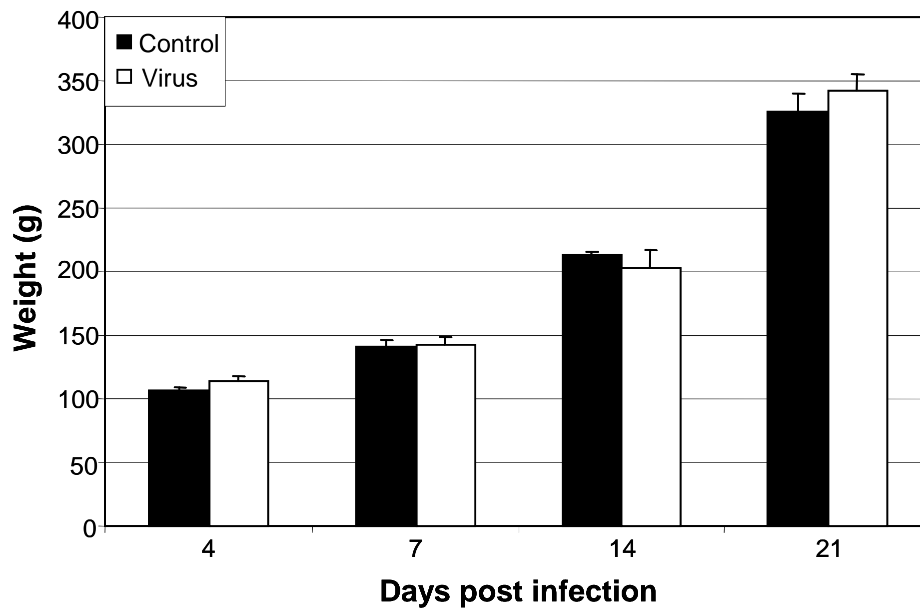

Figure 4.1A Effect of MDV on body weight over time. The graph shows the body weight of infected and uninfected chickens was measured at 4, 7, 14 and 21 days post infection. SPF chickens were infected intra-abdominally with MDV (MPF57) at 7 days post hatch. Values are mean ± SE (n=10).

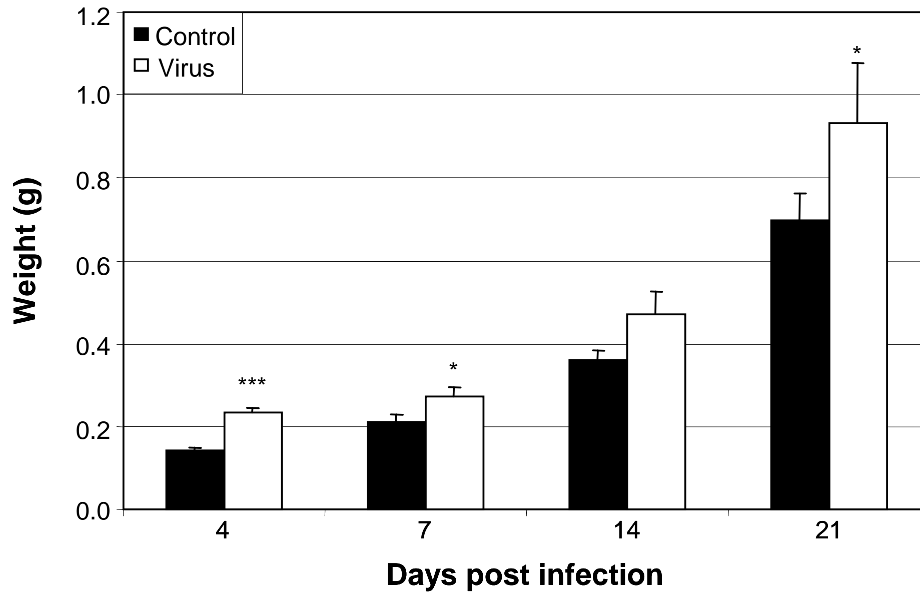

Figure 4.1B Effect of MDV on spleen size over time. Spleen weights of infected and uninfected chickens at 4, 7, 14 and 21 days post infection are shown within the graph. SPF chickens were infected intra abdominally with MDV (MPF57) at 7 days post hatch. Statistical significance between infected and uninfected groups using a Mann-Whitney U test is indicated: *(p<0.05);**(p<0.001); ***(p<0.0001). Values are mean ± SE (n=10).

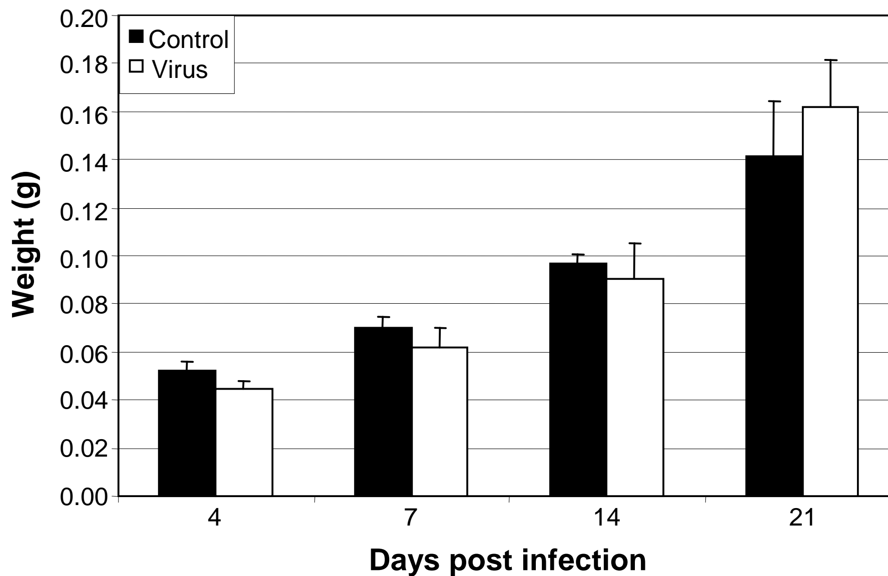

Figure 4.1C Effect of MDV on thymus size over time. The graph displays the thymus weights of infected and uninfected chickens at 4, 7, 14 and 21 days post infection. SPF chickens were infected intra abdominally with MDV (MPF57) at 7 days post hatch. Values are mean ± SE (n=10).

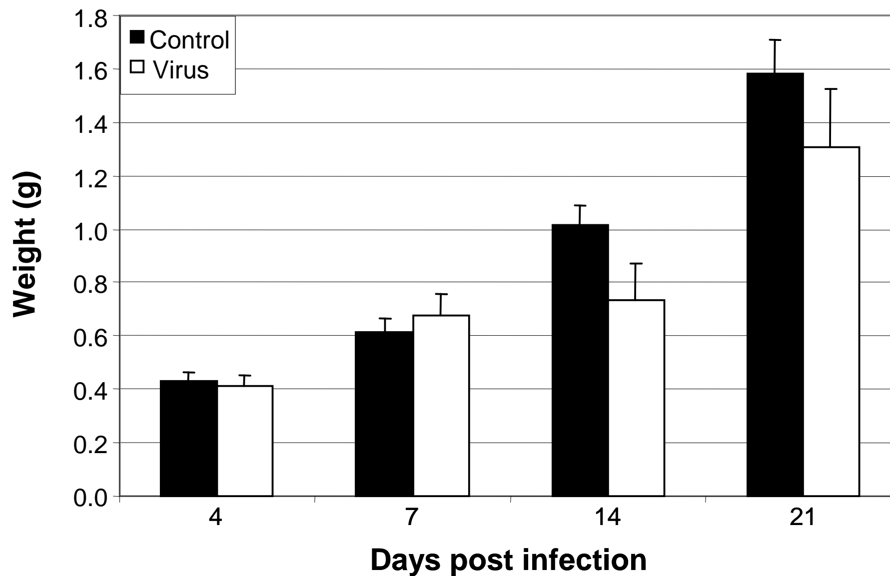

Figure 4.1D Effect of MDV on bursa size over time. Bursa weights of infected and uninfected chickens at 4, 7, 14 and 21 days post infection are shown on the graph. SPF chickens were infected intra abdominally with MDV (MPF57) at 7 days post hatch. Values are mean ± SE (n=10).

4.2 Assessment of cytokine profiles during MDV infection

To build on our understanding of how cytokines control the immune system and gain further insight on how to optimise immune responses to MDV vaccination we carried out an anlysis of the cytokine response during MDV infection. With the growing accessibility to a number of avian cytokine genes, and the recent development of Real Time quantitative PCR (qPCR) technology, cytokine profiles can now be measured during the course of an infection. To assess the nature of the cytokines produced in our model of MDV infection, qPCR was carried out and an evaluation of the levels of cytokine expression in test infected birds was compared to the relative levels expressed to control chickens. Although a number of cytokines were observed to show some level of variation in expression, IL-6 appeared to show increased levels (Figure 4.2A). When analysed across the time course of this infectious model, it appeared that this higher level of IL-6 response was generally associated with the earlier stages of infection (Figure 4.2B).

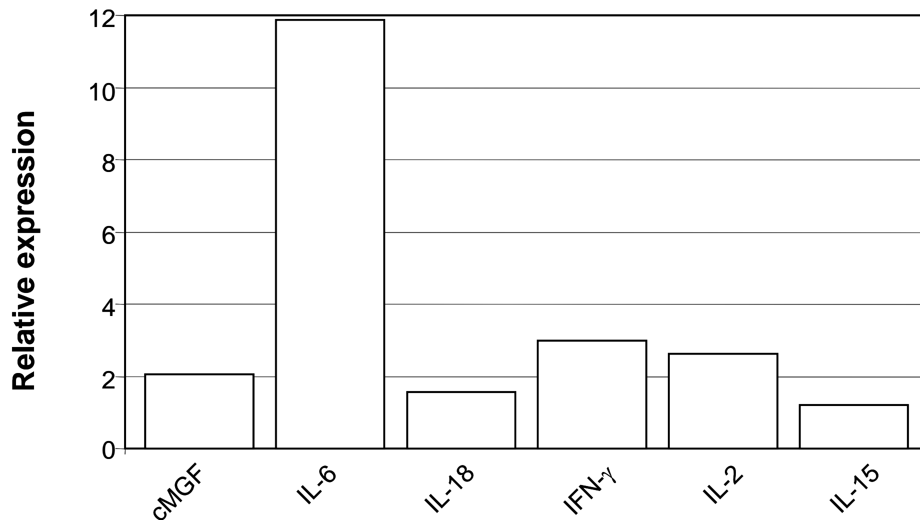

Figure 4.2A. Relative cytokine levels at day 4 of MDV infection. The graph shows the qPCR analysis of the various cytokines and their relative expression in the spleens of SPF chickens infected intra-abdominally with 200 pfu of MDV (MPF57). GAPDH was used as a housekeeping gene to standardised results. The graph shows expression in infected birds relative to age match controls.

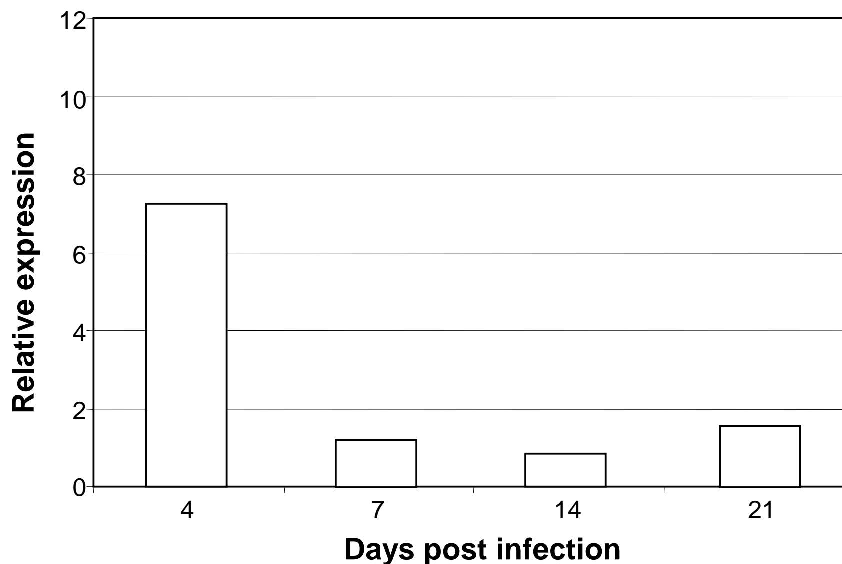

Figure 4.2B. Relative IL-6 cytokine levels during MDV infection. Spleens were removed from infected and age match controls at 4, 7, 14 and 21 days post infection and cDNA prepared from the RNA extracted from the spleens. The graph shows qPCR analysis of IL-6 relative expression levels. GAPDH was used as a housekeeping gene to standardised results. The graph shows expression in infected birds relative to age match controls.

Acquired protection against pathogens in mammals generally falls into one of two types - cell mediated or antibody mediated often described as Th1 and Th2 type responses, respectively. However, in chickens it has not been established whether the same Th1/Th2 paradigm exists as it does in mammals. To assess the relationship between the cytokines IFN-γ and IL-4 expression during MDV infection, qPCR was carried out to determine the relative levels of expression. Intriguingly, IL-4 levels appeared to be generally

expressed at higher levels throughout the course of infection, however, these were quite variable between birds (Figure 4.2C).

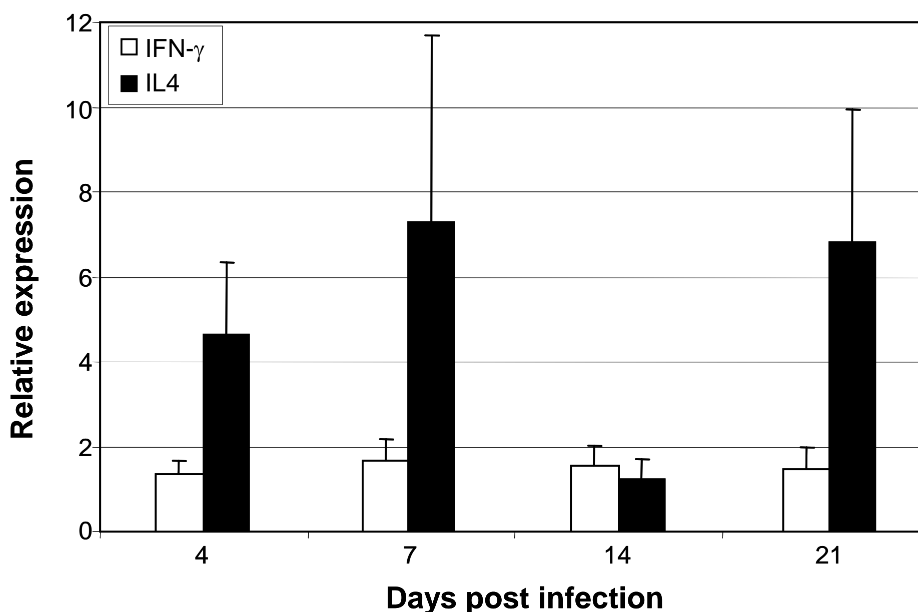

Figure 4.2C. Relative IFN-γ and IL4 cytokine levels during MDV infection. Spleens were removed from infected and age match controls at 4, 7, 14 and 21 days post infection and cDNA prepared from the RNA extracted from the spleens. The graph shows qPCR analysis of IFN-γ, IL4 relative expression levels. GAPDH was used as a housekeeping gene to standardised results. The graph shows expression in infected birds relative to age match controls.

4.3 Expression of biologically active cytokines

To study the activity and action of the chicken cytokines, cMGF, IL-6, IL-18, IFN-γ, IL-2 and IL-15, we have developed and expressed these recombinant proteins and have compared their level of biological activity. The expression of these cytokines required the development and optimisation of the protein expression systems to suit the characteristics of the individual cytokines. Expression vector culture, protein expression, protein purification, including solubilisation and protein refolding, were all developed to optimise the protein expression and biological activity of each the cytokines used in this project. Figure 4.3A shows a Western blot analysis of the purified recombinant cytokines. Each of the purified cytokines was produced in quantities useful for further *in vitro* and *in vivo* studies. The produced cytokines were then tested in a series of biological assays to determine their levels of activity. All of the cytokines stimulated activity within their various bioassays in a dose dependant manner (Figure 4.3B), except IL-15, which continually showed a less than favourable level of activity.

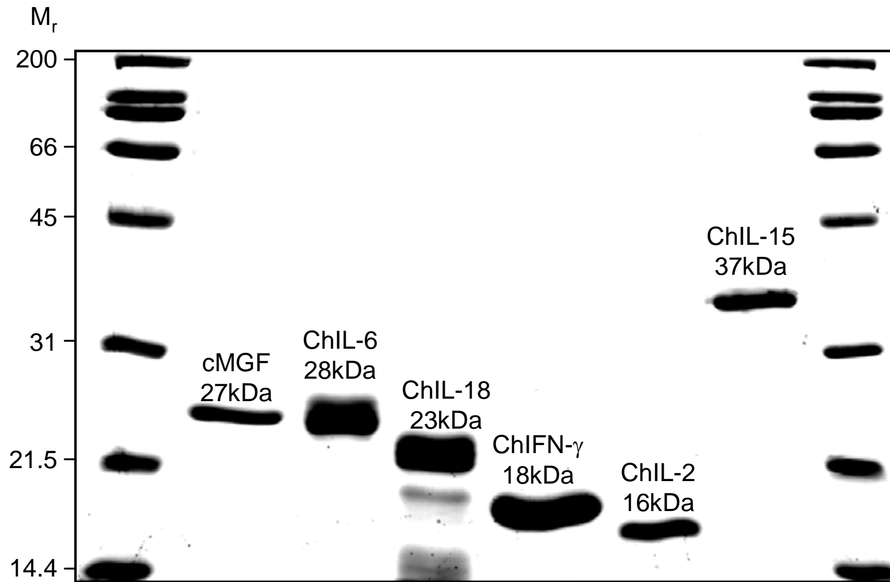

Figure 4.3A Western Blot analysis of recombinant chicken cytokines. Western blot analysis of SDS-PAGE showing the *E. coli* produced His-tagged recombinant cytokine proteins (cytokine and estimated M_r indicated). Marker proteins and their molecular sizes are shown in the far left and right of gel.

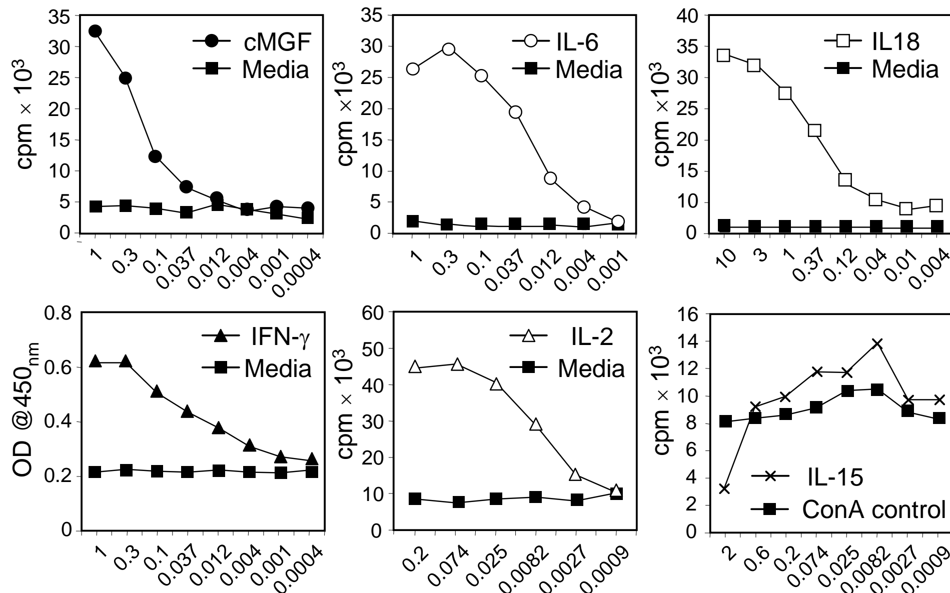

Figure 4.3B Bioactivity of recombinant chicken cytokines. Representative graphs show the activity of each of the various cytokines with regard to their individual bioassays (as described in the methods). Assays show the response to various dilutions (µg/ml) of *E. coli* expressed cytokines as compared to controls.

4.4 Assessment of the impact of *in ovo* administration of recombinant cytokines

Before embarking on the analysis of the potential for administered cytokine to immunoenhance during MDV infection it was important to establish some safe parameters for the administration of these

cytokines *in ovo*. With this in mind, cytokines were administered a various concentrations (50µg/egg, 5µg/egg and 0.5µg/egg) and an analysis of chick hatchability made. The hatch rates for eggs treated with the various cytokines remained around 80% for all concentrations tested which was similar to no injection and PBS control injected eggs (Figure 4.4A). Similarly, there were no apparent detrimental effects on body weight or spleen size in these tests (Figure 4.4B, 4.4C).

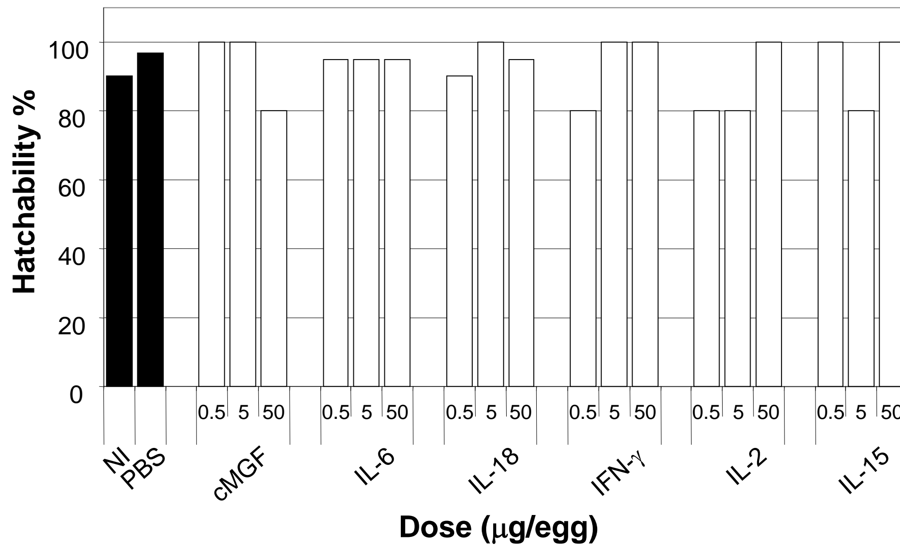

Figure 4.4A Effect of cytokines on hatchability. The graph demonstrates the effect on hatchability of *in ovo* administered cytokines into SPF E18 eggs. Values are total hatched per group expressed as a percentage of eggs for that treatment. (n≥5). Data is expressed as a percentage of hatchability for treatment groups.

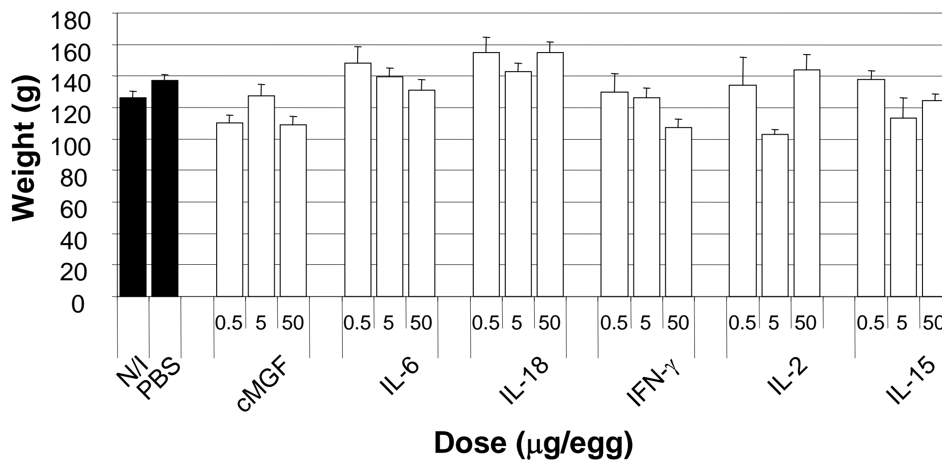

Figure 4.4B Effect of cytokines on body weight. The effect on body weight of *in ovo* administered cytokines into SPF E18 eggs is shown in the graph. Values are average weight per group expressed with SE bars.

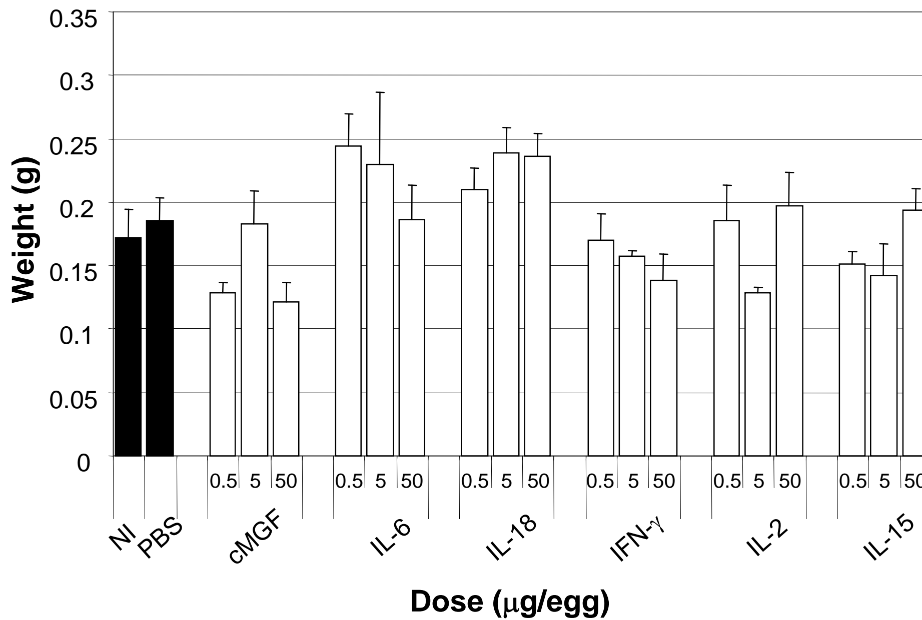

Figure 4.4C Effect of cytokines on spleen weight. The effect on spleen weight of *in ovo* administered cytokines into SPF E18 eggs is displayed in the graph. Values are average weight per group expressed with SE bars.

4.5 Development of a vaccine and challenge model

4.5.1 Trial 2

Trials were performed to establish and develop a model of vaccination, followed by MDV infection. In this model birds were treated at E18 with MDV vaccine, HVT or Maravac, then at 7 days post-hatch challenged intra-abdominally with MDV (MPF57). At days 4, 7, 14 and 21 following challenge the birds body and tissue weights were assessed. In most cases there was little difference in the body weight or organ size between the two types of vaccine. Body weights appeared similar across the various time points tested (Figure 4.5A) and so did spleen weights (Figure 4.5B), however, at 14 days post-infection HVT vaccinated birds showed a trend towards larger spleen weights, this was reflected in a difference in the spleen to body weight ratio (Figure 4.5C). Thymus lobe weight appeared similar between the vaccines (Figure 4.5D), and intriguingly, bursal weight was similar except for the day 14 analysis where Maravac treated birds appeared to have a lower bursal weight than HVT treated (Figure 4.5E).

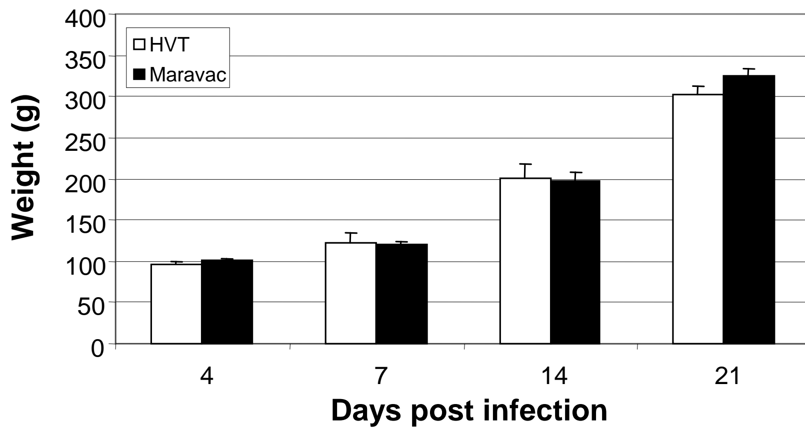

Figure 4.5A Comparison of body weight between HVT and Maravac vaccinated chickens. The graph shows the body weight of HVT and Maravac vaccinated SPF chickens at 4, 7, 14 and 21 days post infection with MDV (MPF57). Values are mean ± SE (n≥6).

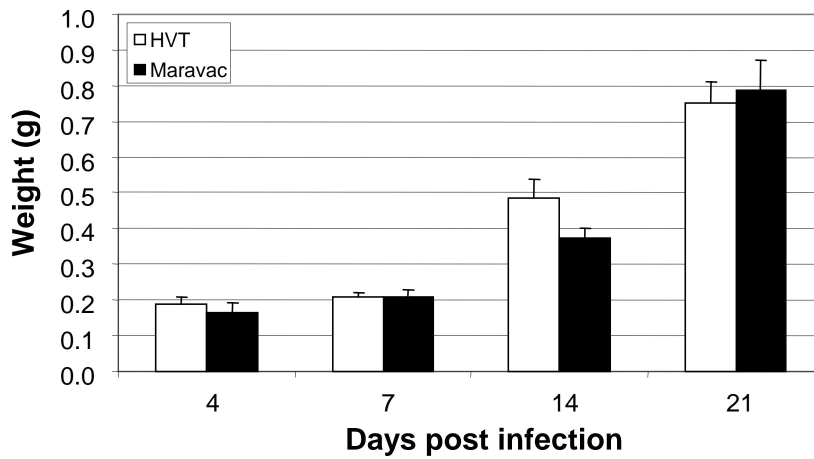

Figure 4.5B Spleens size comparison between HVT and Maravac vaccinated chickens. The graph highlights the comparison of spleen weight of HVT and Maravac vaccinated SPF chickens at 4, 7, 14 and 21 days post infection with MDV (MPF57).

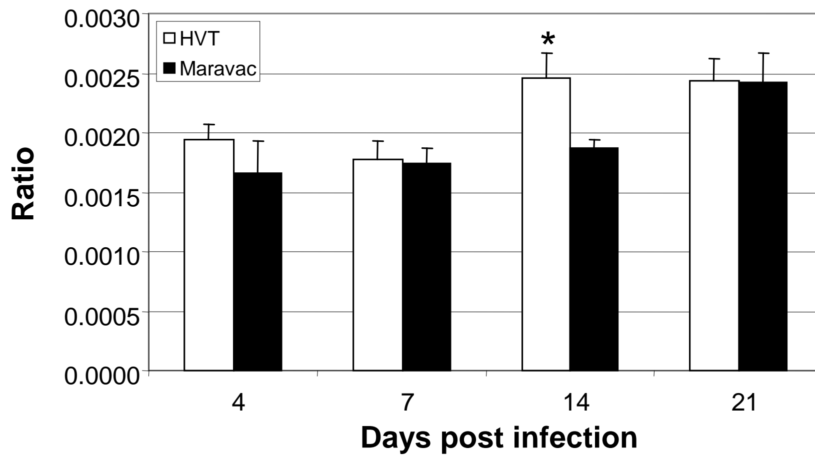

Figure 4.5C Spleen to body weight ratio comparison between HVT and Maravac vaccinated chickens. The graph shows a comparison of spleen:body weight ratio of HVT and Maravac vaccinated SPF chickens at 4, 7, 14 and 21 days post infection with MDV (MPF57). Values are mean ± SE (n ≥ 6).Mann-Whitney P values < 0.05 indicated (*).

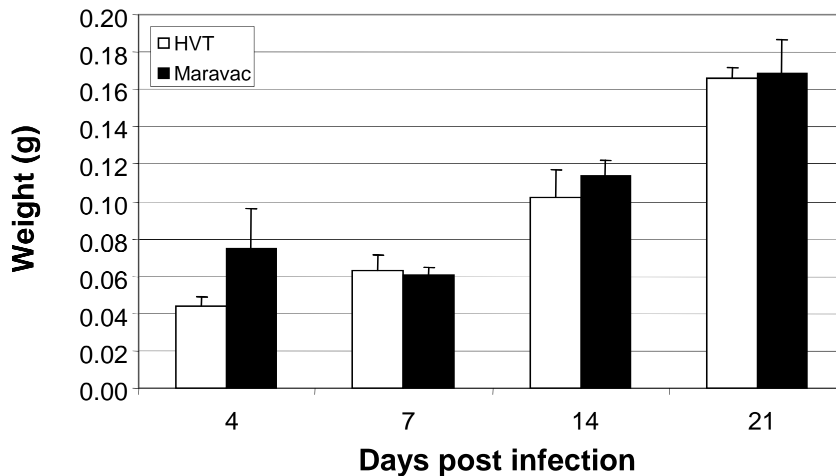

Figure 4.5D Thymic lobe size comparison of HVT or Maravac vaccinated chickens. A comparison of thymic lobe weight of HVT and Maravac vaccinated SPF chickens at 4, 7, 14 and 21 days post infection with MDV (MPF57) is shown in the graph. Values are mean ± SE (n ≥ 6).

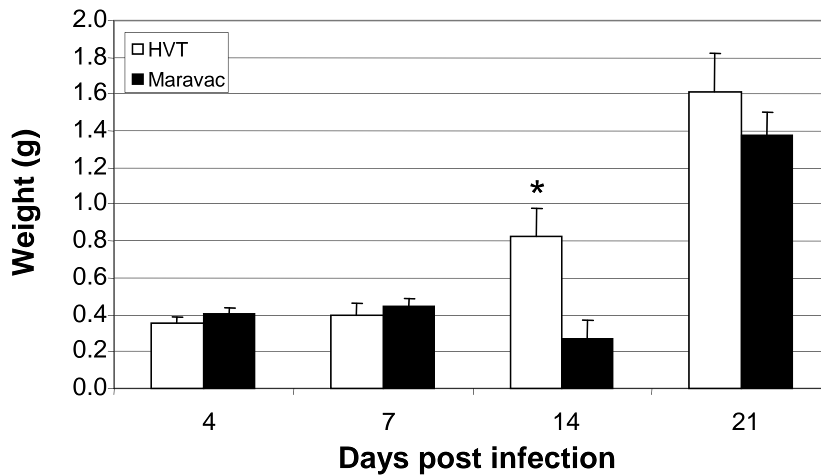

Figure 4.5E Bursal weight comparison between HVT and Maravac vaccinated chickens. The graph shows a comparison of bursa weight of HVT and Maravac vaccinated SPF chickens at 4, 7, 14 and 21 days post infection with MDV (MPF57). Values are mean ± SE (n ≥ 6). Mann-Whitney P values <0.05 indicated (*).

4.6 Assessment of the effects of cytokine administration on the outcomes of MDV infection

Several trials were performed in order to assess the ability of the chicken cytokines to enhance the immune performance of birds during MDV infection.

4.6.1 Trial 3

In this cytokine immunoenhancement trial, all the cytokines: cMGF, IL-6, IL-18 and IFN-γ, IL-2, and IL-15 were tested. An assessment was made of the combined effects of both the cytokine and vaccine administration on the hatchability of treated eggs. In this trial the eggs supplied by SPAFAS were not of the highest grade and therefore this impacted on the hatch rate of all groups, as observed for no injection and PBS injected controls. However, most treatments showed a hatch rate similar to the conrols, although, cMGF and IFN-γ showed somewhat reduced levels (Figure 4.61A).

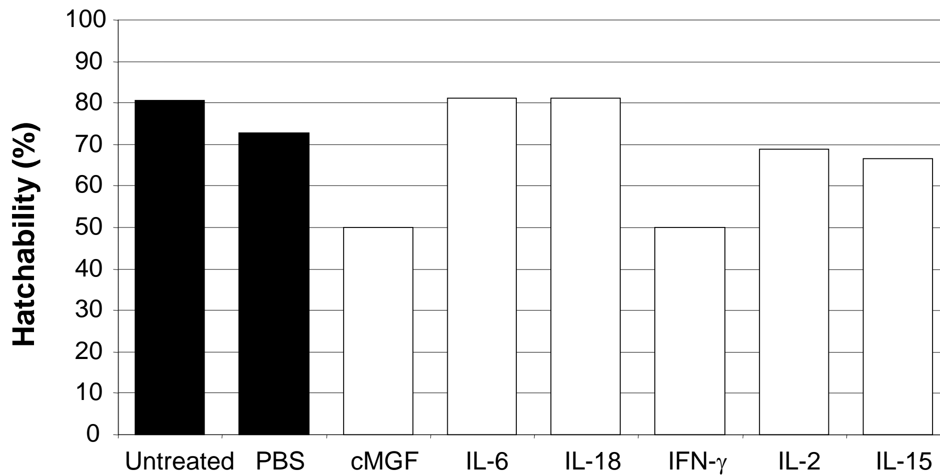

Figure 4.61A Effect of cytokines on hatchability. The graph displays the effect on hatchability of *in ovo* administered cytokines (50μg/dose) into SPF E18 eggs. Values are total hatched per group expressed as a percentage of eggs for that treatment.

To analyse the immunoenhancing potential of *in ovo* cytokine treatment followed by MDV challenge, 50μg of cytokine was administered E18 *in ovo*, then on day 7 post-hatch birds were challenged with MDV (MPF57). Twenty-one days later birds were assessed to determine the impact of the cytokine treatment. Figure 4.61B shows that the mean weight of cMGF treated birds appeared to show a trend towards maintaining a higher body weight. Similarly, cMGF showed a trend towards higher spleen weights, as did IFN-γ treated birds (Figure 4.61C). This trend was also observed for thymus weights between the groups (Figure 4.61D).

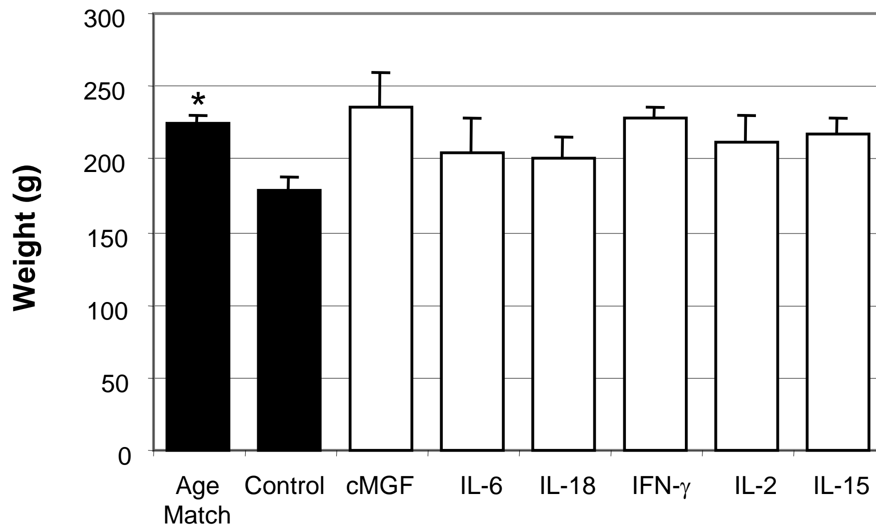

Figure 4.61B Comparison of body weights of cytokine treated unvaccinated chickens. The body weights of unvaccinated SPF chickens at 21 days post infection with MDV (MPF57), treated with cytokines *in ovo* are shown in the graph. Cytokines were administered at 50 μg/dose in 200 μL of PBS. Values are mean ± SE (n ≥ 3 except IL-2 and PBS where only 2 birds survived to this time point). Mann-Whitney P values < 0.05 indicated by *. All comparison made to irrelevant cytokine control.

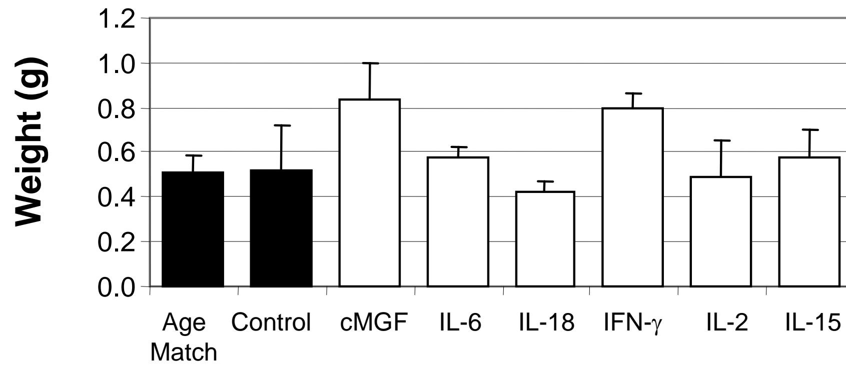

Figure 4.61C Comparison of spleens from cytokine treated, unvaccinated chickens. The graph shows a comparison of spleen weights from cytokine treated, unvaccinated SPF chickens at 21 days post infection with MDV (MPF57). Values are mean ± SE (n ≥ 3 except IL-2 and PBS where only 2 birds survived to this time point). All comparison made to irrelevant cytokine control.

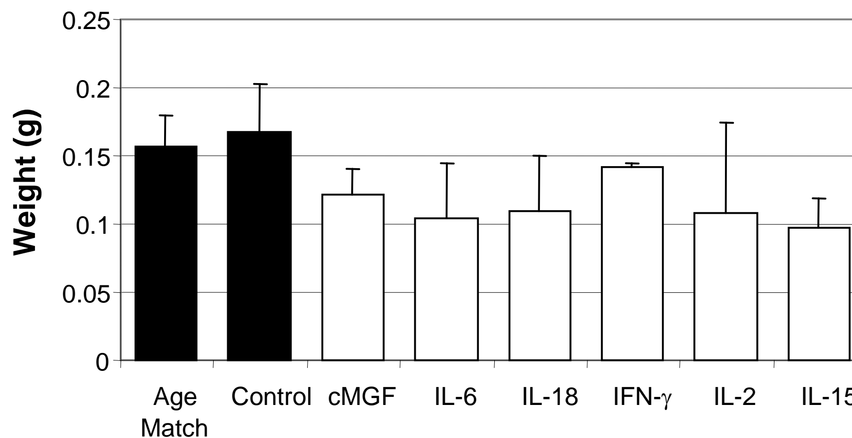

Figure 4.61D Comparison of thymus from cytokine treated, unvaccinated chickens. A comparison of thymus weights from cytokine treated unvaccinated SPF chickens at 21 days post infection with MDV (MPF57) is shown in the graph. Values are mean ± SE (n ≥ 3 except IL-2, IFN-γ and PBS where only 2 birds from these groups survived to this time point). All comparison made to irrelevant cytokine control.

An analysis of the vaccine enhancing potential of *in ovo* cytokine treatment involved the co-administration of HVT vaccine with 50µg of cytokine also administered E18 *in ovo* was made. On day 7 post-hatch birds were challenged with MDV (MPF57) and 21 days later these birds were assessed to determine the impact of the combined cytokine and vaccine treatment. Figure 4.61E shows the effect of *in ovo* co-administration of cytokine and HVT on hatch rates and, as previously observed, cMGF again showed a reduced hatch rate. As in the cytokine alone trial, trends toward higher mean body weights were seen in cMGF and IFN-γ groups (Figure 4.61F). With regard to splenic weights, intriguingly, IL-18 showed a significant decrease in spleen weight and a trend towards decreased thymus weight (Figure 4.61G, 4.61H).

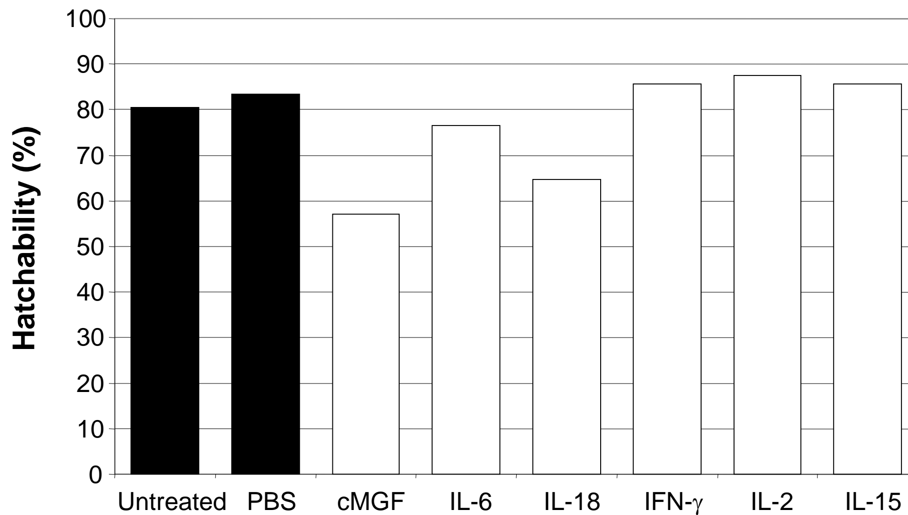

Figure 4.61E Combined effect of cytokines and HVT vaccine co-administration on hatchability. The effect on hatchability of *in ovo* administered cytokines (50µg/dose) and HVT vaccine into SPF eggs (E18) is shown in the graph. Values are total hatched per group expressed as a percentage of eggs for that treatment.

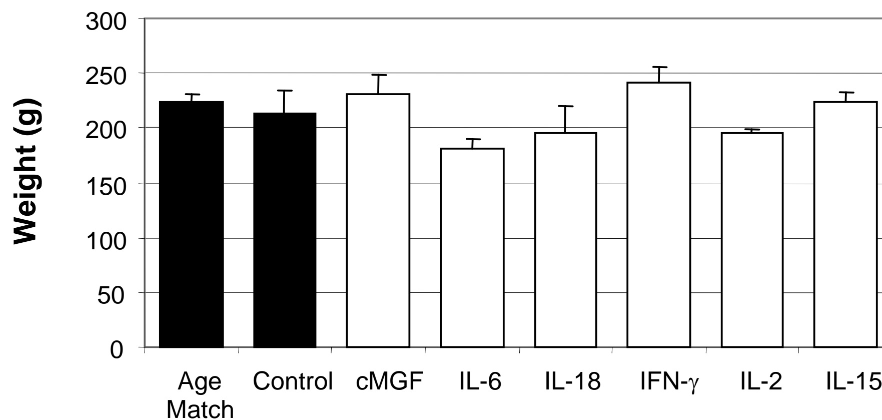

Figure 4.61F Comparison of body weights from combined cytokine and HVT treated MDV challenged chickens. The graph highlights the body weights as measured from birds co-administered with cytokine and HVT at 21 days post infection with MDV (MPF57). Cytokines were administered at 50 µg/dose in 200 µL of PBS. Values are mean ± SE (n ≥ 3). All comparison made to irrelevant cytokine control.

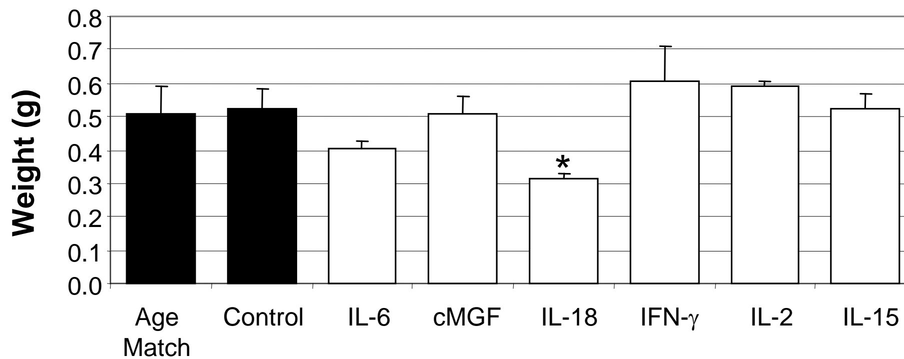

Figure 4.61G Spleen weight comparison between cytokine treated, vaccinated groups. The graph displays a comparison of spleen weights from cytokine treated, HVT vaccinated SPF chickens at 21 days post infection with MDV (MPF57). Values are mean ± SE (n ≥ 3). Mann-Whitney P values < 0.05 indicated by *. All comparison made to irrelevant cytokine control.

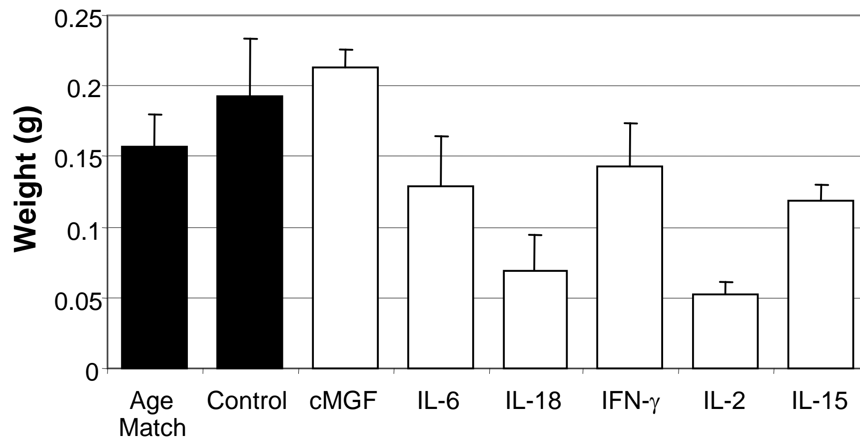

Figure 4.61H Comparison of thymic lobe weights from cytokine treated, HVT vaccinated chickens. The graph depicts the thymus weights from cytokine treated, HVT vaccinated SPF chickens at 21 days post infection with MDV (MPF57). Values are mean ± SE (n ≥ 3). Mann-Whitney P values < 0.05 indicated by *. All comparison made to irrelevant cytokine control.

4.6.2 Trial 4

In this cytokine immunoenhancement trial, the cytokines IL-6, IL-18 and IL-2 were further tested to assess their immunoenhancing potential. To analyse *in ovo* cytokine treatment followed by MDV challenge, 50µg of cytokine was administered E18 *in ovo*, then on day 7 post-hatch birds were challenged with MPF57. Twenty-one days later birds were assessed to determine the impact of the cytokine treatment. Figure 4.62A shows that although no statistical difference in body weights was observed, the mean weight of IL-2 treated birds appeared to show a trend towards maintaining a higher body weight. Similarly, spleen and thymus weights were similar between the groups (Figure 4.62B, 4.62C). However, the thymic lobe size from each group showed a great deal of variability (Figure 4.62D).

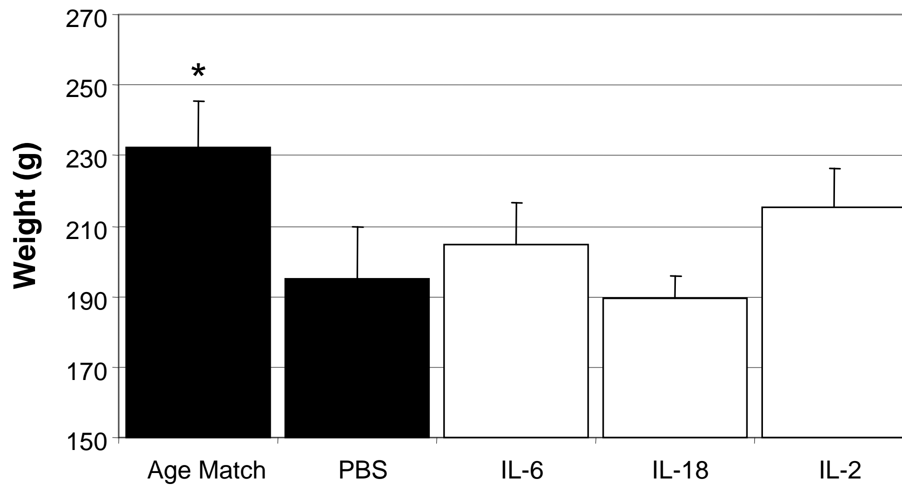

Figure 4.62A Body weight analysis of cytokine treated MDV challenged chickens. The graph shows the body weights of *in ovo* cytokine treated SPF chickens at 21 days post infection with MDV (MPF57). Cytokines were administered at 50 µg/dose in 200 µL of PBS. Values are mean ± SE (n ≥ 3). Mann-Whitney P values < 0.05 indicated by *. All comparison made to PBS control.

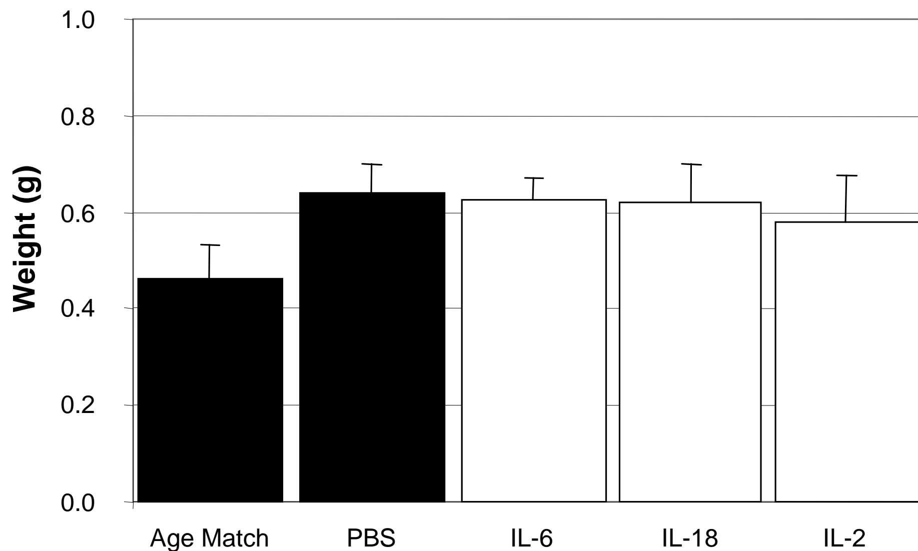

Figure 4.62B Comparison of spleens from cytokine treated, MDV challenged chickens. The graph highlights a comparison of spleen weights from cytokine treated unvaccinated SPF chickens at 21 days post infection with MDV (MPF57). Values are mean ± SE (n ≥ 3).

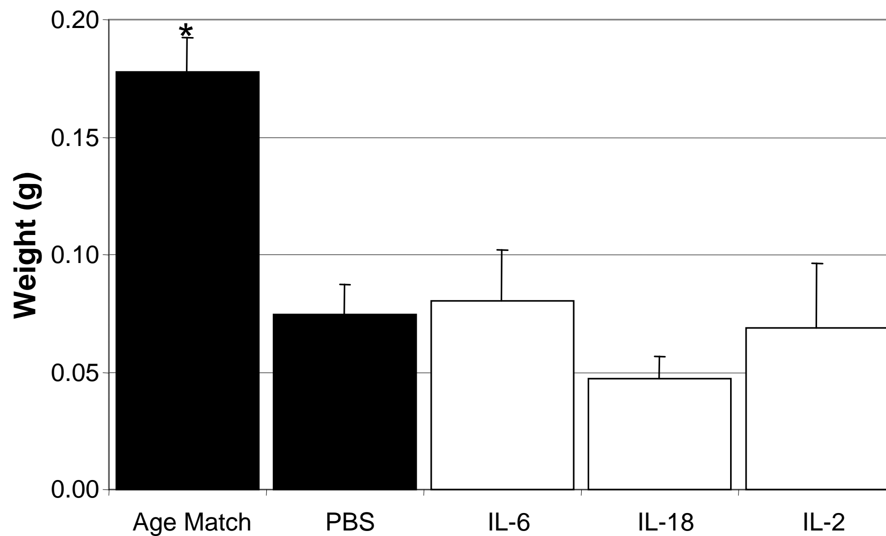

Figure 4.62C Comparison of thymus from cytokine treated, unvaccinated chickens. The graph shows a comparison of thymus weights from cytokine treated unvaccinated SPF chickens at 21 days post infection with MDV (MPF57). Values are mean ± SE (n ≥ 3). Mann-Whitney P values < 0.05 indicated by *. All comparison made to PBS control.

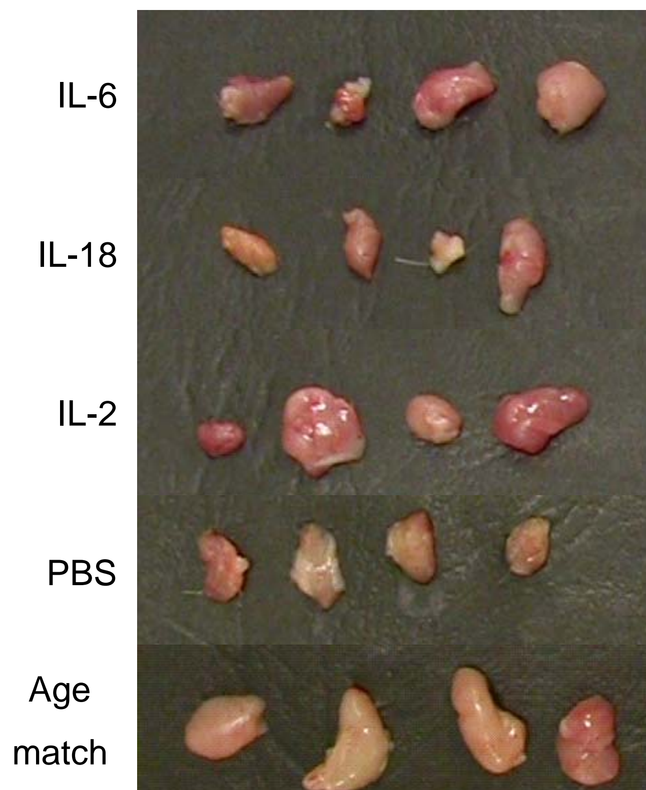

Figure 4.62D Relative thymic lobe size for cytokine treated MDV challenged birds. The photographs show the relative thymic lobe size of cytokine treated MDV (MPF57) challenged SPF chickens.

When qPCR was carried out to determine the relative abundance of the MDV *Meq* gene on cytokine treated groups the IL-2 group appeared to have the lowest relative level of viral gene expression (Figure 4.62E).

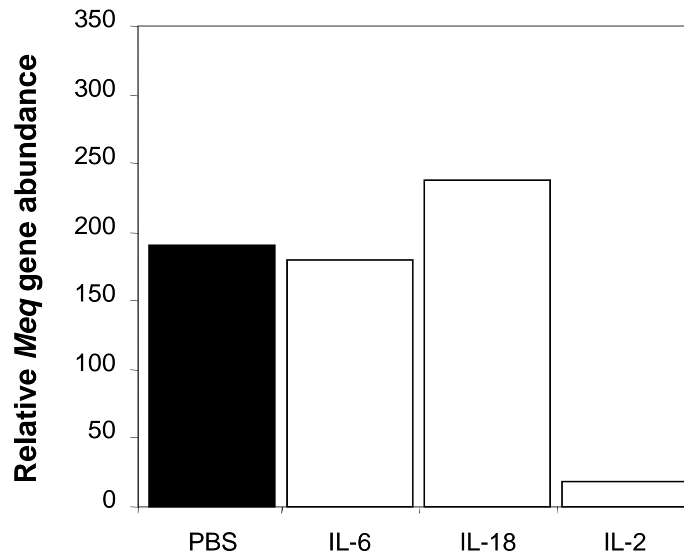

Figure 4.62E The relative expression of the *Meq* gene in MDV challenged cytokine treated chickens. The graph shows a comparison of the relative *Meq* gene expression in MDV challenged cytokine treated chickens at 21 days post infection with MDV (MPF57).

In the analysis of the vaccine enhancing potential of *in ovo* cytokine treatment involved the co-inoculation of HVT vaccine with 50μg of cytokine was administered E18 *in ovo*. On day 7 post-hatch birds were challenged with MPF57 and 21 days later these birds were assessed to determine the impact of the combined cytokine and vaccine treatment. Again the body weights of birds from all challenged groups appeared similar, however, the IL-18 treated group appeared to show a trend towards lower body weights (Figure 4.62F). Similarly, the spleen and thymic lobe weights were similar between groups and IL-18 showed a trend towards decreased mean lobe weight (Figure 4.62G, 4.62H, 4.62I).

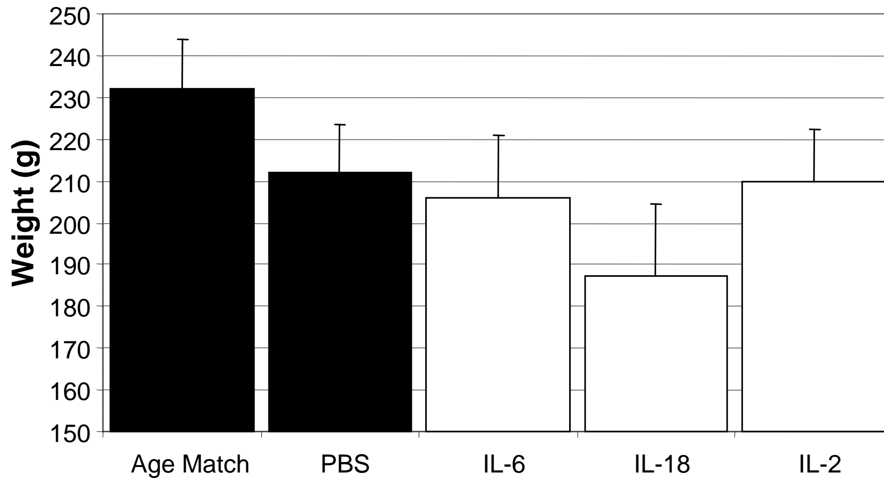

Figure 4.62F Comparison of body weights of cytokine treated vaccinated chickens. The graph shows the body weights of HVT vaccinated SPF chickens at 21 days post infection with MDV (MPF57) treated with cytokines *in ovo*. Cytokines were administered at 50 μg/dose in 200 μL of PBS. Values are mean ± SE (n ≥ 3).

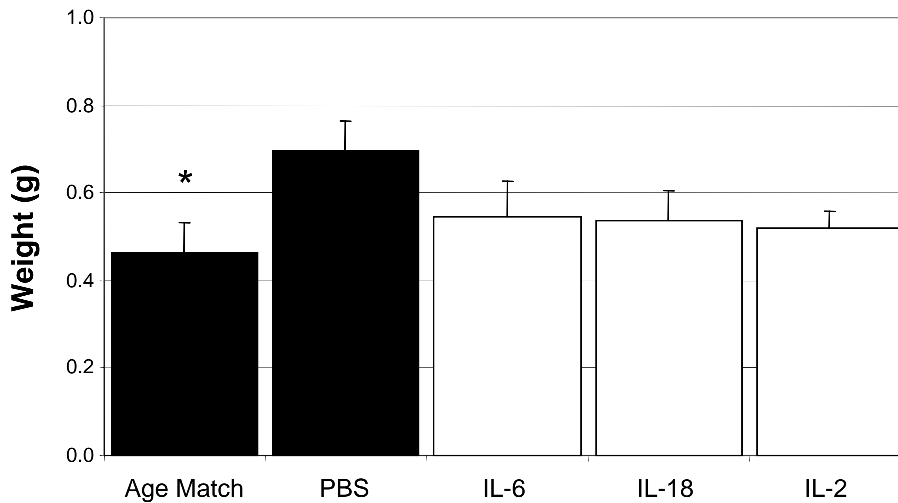

Figure 4.26G Comparison of spleens from cytokine treated, vaccinated chickens. The graph shows a comparison of spleen weights from cytokine treated, HVT vaccinated SPF chickens at 21 days post infection with MDV (MPF57). Values are mean ± SE (n ≥ 3). Mann-Whitney P values < 0.05 indicated by *. All comparison made to PBS control.

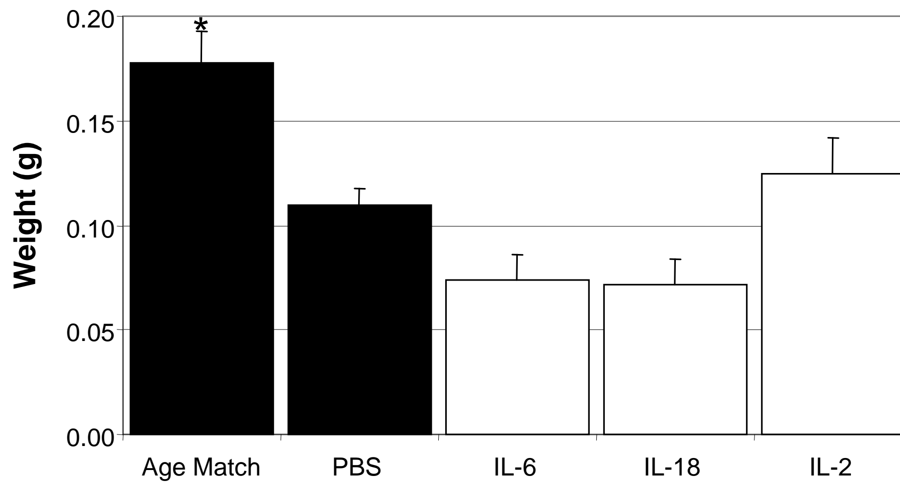

Figure 4.62H Comparison of thymus from cytokine treated, vaccinated chickens. A comparison of thymus weights from cytokine treated, HVT vaccinated SPF chickens at 21 days post infection with MDV (MPF57) is shown in the graph. Values are mean ± SE (n ≥ 3). Mann-Whitney P values < 0.05 indicated by *. All comparison made to PBS control.

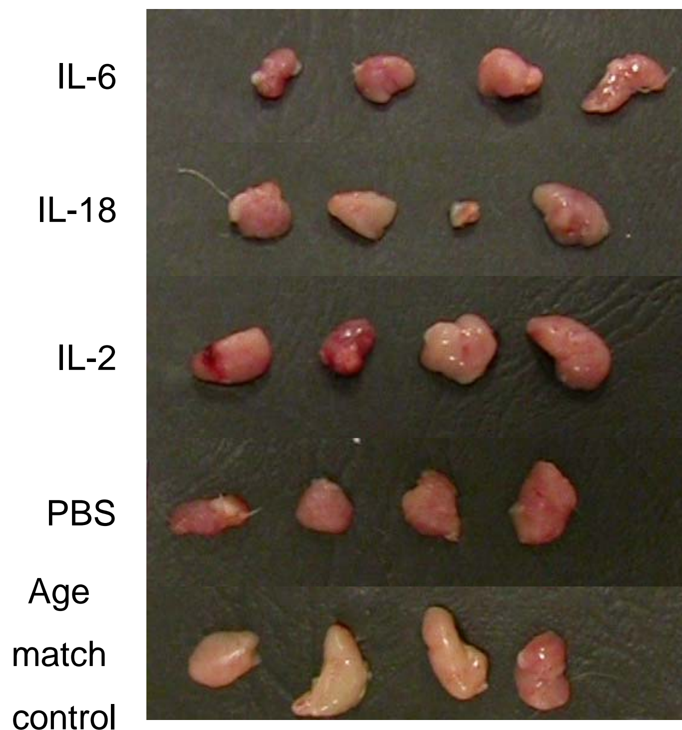

Figure 4.62I Relative thymic lobe size from cytokine co-administered with HVT then MDV challenged birds. Photographs show the relative size of thymic lobes from SPF chickens co-administered with cytokines and HVT vaccine then challenged with MDV (MPF57).

When qPCR was carried out to determine the relative abundance of the MDV *Meq* gene on cytokine and vaccine treated groups, all groups showed reduced relative levels of expression, however, the IL-2 group appeared to have the lowest relative level of viral gene expression (Figure 4.62J).

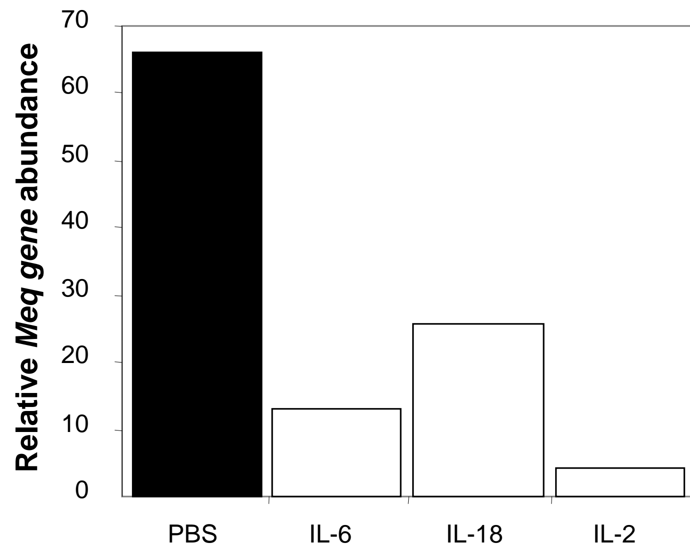

Figure 4.62J The relative abundance of *Meq* gene expression in chickens co-administered with cytokines and HVT then challenged with MDV. The graph shows a comparison of the relative *Meq* gene expression levels in MDV challenged cytokine/HVT treated chickens at 21 days post infection with MDV (MPF57) as compared to age match controls.

4.7 Assessment of the effects of cytokine administration on the outcomes of MDV infection in pen trials

Since previous trials were carried out under laboratory conditions utilising the SPF line of chickens trials the next trial was performed to assess the ability of the cytokines IL-6 and IL-2 to enhance the immune performance of broilers reared under commercial conditions.

4.7.1 Trial 5

An assessment was made of the combined effects of both the cytokine and vaccine administration on the hatchability of treated broiler eggs. The treatments with the cytokines IL-6 and IL-2 showed a greater than 80% hatch rate, however, HVT alone showed somewhat reduced levels (Figure 4.7A). This trial involved the analysis of the vaccine enhancing potential of *in ovo* cytokine treatment of broiler birds under commercial conditions. This involved co-administration of HVT vaccine with 50µg of cytokine was dispensed E18 *in ovo*. On day 7 post-hatch broiler birds were challenged with MPF57 and 21 days later these birds were assessed to determine the impact of the combined cytokine and vaccine treatment. Although no statistical difference was observed between the groups, the HVT treatment alone group appeared to have a higher mean body weight (Figure 4.7B). With regard to organ weights, the spleen, thymic lobe and bursal weights were similar between groups (Figure 4.7C, 4.7D, 4.7E).

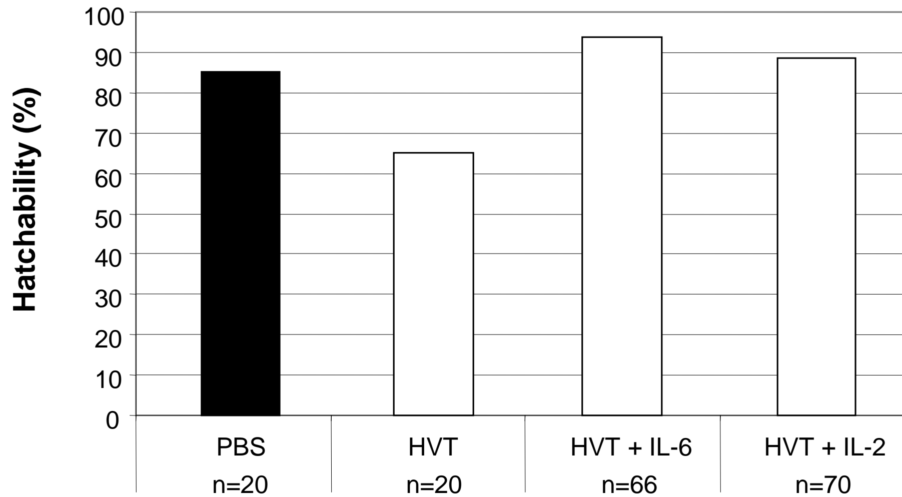

Figure 4.7A Effect of cytokines and HVT vaccine on broiler chicken hatchability rates. The graph shows the effect on hatchability of *in ovo* administered cytokines (50µg/dose) and HVT vaccine into broiler E18 eggs. Values are total hatched per group expressed as a percentage of eggs for that treatment.

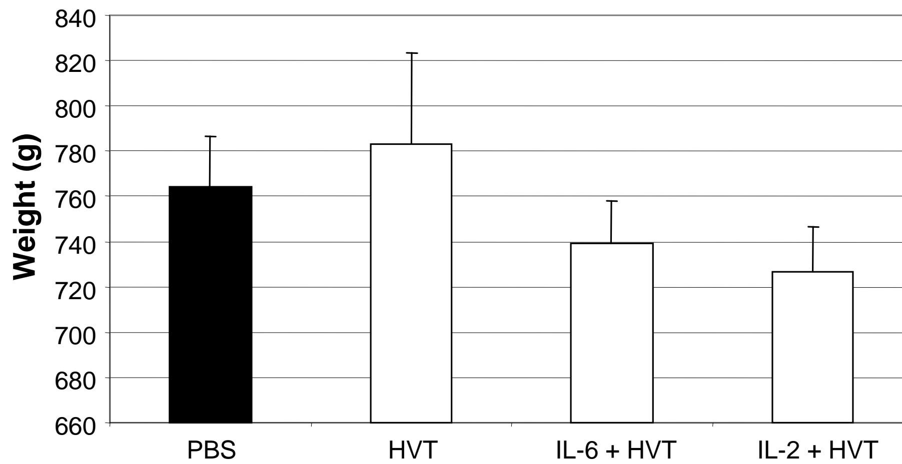

Figure 4.7B Comparison of body weights of cytokine treated chickens.
The graph shows an analysis of the body weights of broiler chickens at 21 days post infection with MDV (MPF57) treated with cytokines and HVT *in ovo*. Cytokines were administered at 50 µg/dose in 200 µL of PBS. Values are mean ± SE (n ≥ 13). All pairs analysed by Mann-Whitney 2-tailed t test with no statistical significance found.

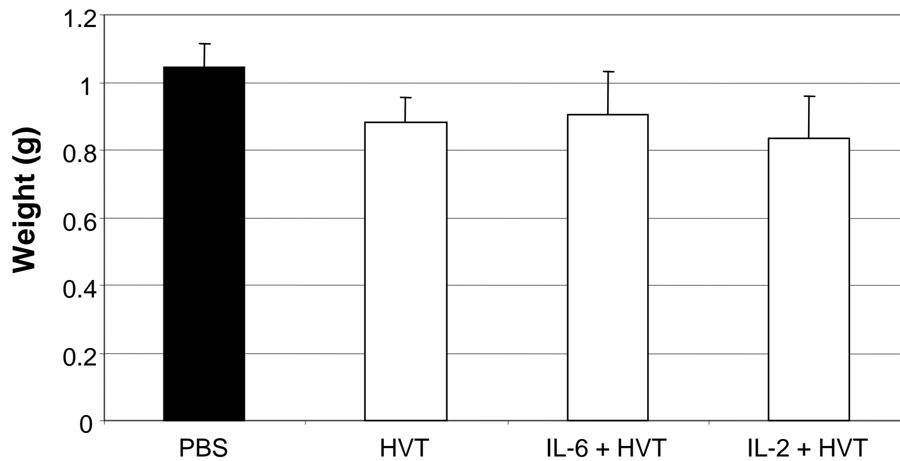

Figure 4.7C Comparison of the spleen weight from cytokine treated HVT vaccinated then MDV challenged broiler chickens. A comparison of spleen weights from broiler chickens co-administered with cytokine and HVT at 21 days post infection with MDV (MPF57) is shown in the graph. Values are mean ± SE (n =10).All pairs analysed by Mann-Whitney 2-tailed t test with no statistical significance found.

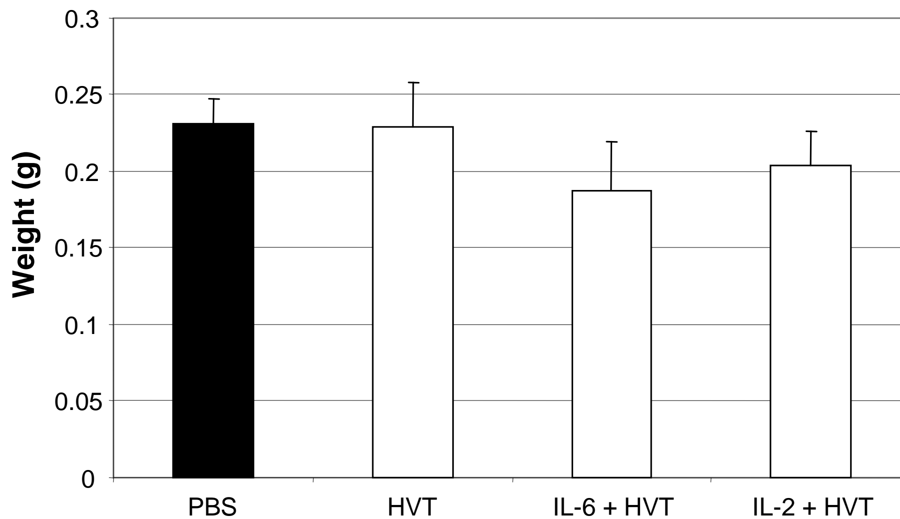

Figure 4.7D. Comparison of thymic lobe weights from broiler chickens co-administered with cytokine and HVT then MDV challenged. The graph shows a comparison of thymus weights from cytokine treated broiler chickens at 21 days post infection with MDV (MPF57). Values are mean ± SE (n =10). All pairs analysed by Mann-Whitney 2-tailed t test with no statistical significance found

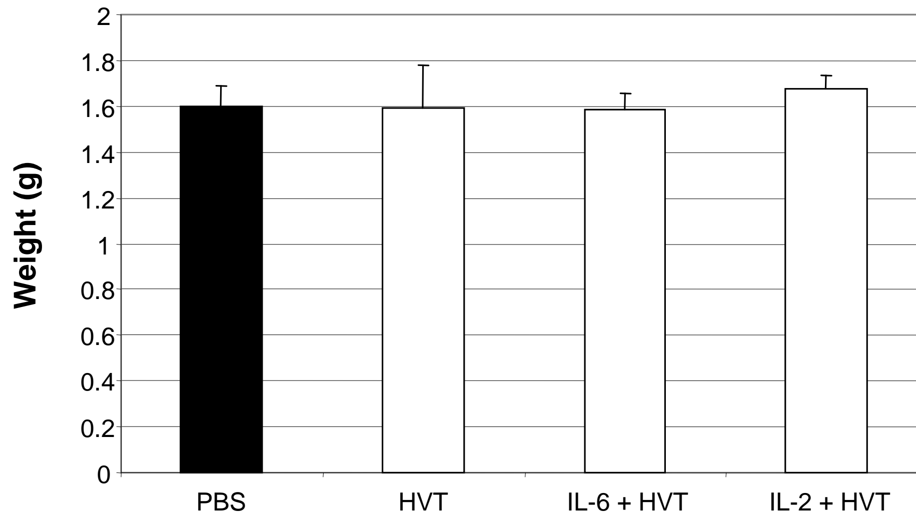

Figure 4.7E Comparison of bursa weight from cytokine and HVT co-administered, MDV challenged broiler chickens. The graph displays a comparison of bursa weights from cytokine and HVT treated broiler chickens at 21 days post infection with MDV (MPF57). Values are mean ± SE (n ≥ 13). All pairs analysed by Mann-Whitney 2-tailed t test with no statistical significance found.

4.8 Microarray studies on MDV infected tissue

Initial experiments have been carried out to analyse alterations in splenocyte gene expression levels during the course of MDV infection. A comparison of the genes expressed during infection against control uninfected samples was made employing a 2500 clone lymphoid gene enriched cDNA array. Scatter plot analysis indicated that a number of genes were both up and down regulated in MDV infected birds in comparison to controls. Analysis of these genes showed that many of the differentially regulated genes were from spots that were not yet fully annotated on the chip. A number of genes were then selected as having some relevance to the immune response and these are listed in Table 4.1. Furthermore, many of these selected genes are not fully annotated and their identified sequence homologue is indicated.

Table 4.1: Genes identified by microarray as differentially expressed in MDV infection

Gene chip locator number	Gene product name as annotated for gene chip	Expression level change*
2542	Chicken capping protein alpha 2 isoform (CapZ) mRNA complete cds	-37.9
6111	Chicken jun oncogene complete cds	25.3
1445	G. gallus mRNA for ubiquitin-ribosomal protein fusin protein	-150.3
5973	G. gallus mRNA for integrin beta3	-39.9
5214	G. gallus ODC mRNA for ornithine decarboxylase	-33.3
3529	G. gallus cysteine protease caspase-2 (ich-1) mRNA partial cds	-74.3
59	G. gallus L10 ribosomal protein gene partial cds	-54.4
4324	Homo sapiens clone hRPK.70_A_1 complete sequence	-67.8
5557	Homo sapiens 12q BAC RP11-76E16	-10.6
4963	Homo sapiens alanyl-tRNA synthetase (AARS) mRNA	-29.9
44	Homo sapiens ATP synthase H+ transporting mitochondrial F1F0 subunit d	-95.3
208	Homo sapiens cdk inhibitor p27KIP1 mRNA complete cds	1.0
2832	Homo sapiens CGI-147 protein (LOC51651) mRNA	-58.2
1550	Homo sapiens chromosome 16 clone RP11-5A3 complete sequence	-108.9
4717	Homo sapiens DKFZP564M082 protein (DKFZP564M082) mRNA	-56.5
2589	Homo sapiens F-box only protein 2 (FBXO2) mRNA	-70.2
3286	Homo sapiens genomic DNA chromosome 11q23.2 clone:KB4H11	25.2
51	Homo sapiens HSPC038 protein (LOC51123) mRNA	-2.3
4799	Homo sapiens HSPC297 mRNA partial cds	-74.6
3533	Homo sapiens hypothetical protein (FLJ20591) mRNA	-0.8
1354	Homo sapiens M-phase phosphoprotein 6 (MPHOSPH6) mRNA	-36.7
1157	Homo sapiens mRNA for exportin (tRNA)	-10.4
6035	Homo sapiens mRNA for KIAA0689 protein partial cds	-78.1
1952	Homo sapiens nemo-like kinase (LOC51701) mRNA	-45.5
2735	Homo sapiens NF-kB-activating kinase NAK mRNA complete cds	-56.4
3215	Homo sapiens PRO2207 mRNA complete cds	-31.6
52	Homo sapiens protease cysteine 1 (legumain) (PRSC1) mRNA	-0.3
3531	Homo sapiens ribosomal protein S6 kinase 70kD polypeptide 1	-46.5
1749	Homo sapiens serine/threonine protein kinase sgk mRNA complete cds	-154.0
4797	Homo sapiens small nuclear RNA activating complex polypeptide 5	-24.4
2141	Human clone A9A2BRB5 (CAC)n/(GTG)n repeat-containing mRNA	-70.7
1155	Human DNA sequence from clone 462O23 putative CpG island	-69.5
5915	Human DNA sequence from clone RP13-178D16 novel pseudogene	-33.2
3732	Human DNA sequence from clone RP3-365O19 glutamate receptor metabotropic	-134.8
2537	Human leptin receptor (LEPR) gene exon 17	-91.6
3420	Human PAC clone RP3-404F18 from Xq23 complete sequence	-146.1
41	Human RSU-1/RSP-1 mRNA complete cds	-0.8
1948	Human thymosin beta-4 mRNA complete cds	-95.7
4790	Mus musculus FK506 binding protein 5 (51 kDa) (Fkbp5) mRNA	-65.1
5979	Mus musculus macrophage activation 2 (Mpa2) mRNA	-77.6
4982	Mus musculus ribosomal protein S18 (Rps18) mRNA	-81.6
4983	Mus musculus tumor necrosis factor alpha-induced protein 3 (Tnfaip3) mRNA	-34.6
3928	Rat GAP-associated protein (p190)	-58.0
4801	Rat mRNA for ribosomal protein L31	-25.3

* a negative value indicates down regulation of the gene in MDV infection, a positive value indicates up regulation in MDV samples.

5. Discussion

5.1 The immune response during MDV infection

Until it was brought under control by vaccination, MDV was the major cause of mortality, carcase contamination and economic loss to the poultry industry of many countries (Payne et al., 1976; Witter, 1997; Baaten, et al., 2004). Nevertheless, there is a paucity of information on the exact mechanisms that govern vaccine-induced immune responses against MDV infection. Vaccines, such as HVT, have been used to successfully control MDV (Witter, 1987), however, virulent strains are emerging. Moreover, virus replication in the face of vaccination implies a breakdown in immune control. Therefore, it is crucial to fortify our understanding of the mechanisms associated with the establishment and maintenance of protective immunity to MDV. The current advances with regard to the identification and cloning of chicken cytokine genes coupled with technological advances in methods to analyse these genes has meant a more complete analysis of the immune response to infection can be made. With this in mind, we used qPCR and microarray analysis to investigate immune gene activity during MDV infection. The innate immune response is essential for establishing protective immunity as, not only does it serve as the first line of defence it also directs the subsequent adaptive response (Barton and Medzhitov, 2002; Akira and Hemmi, 2003; Ulevitch, 2004). To ascertain the relative contribution of particular cytokines to the initial response against MDV infection we assessed the relative expression of a number of cytokines in the spleens of MDV infected birds at day 4 post-infection. Within our laboratory model of MDV infection the relative expression of all the cytokines we assessed, cMGF, IL-6, IL-18, IFN-γ, IL-2, and IL-15, varied somewhat. IL-6 levels were observed to increase to the greatest degree with only minor changes in the other cytokines (Figure 4.2A). This finding is of relevance as IL-6 is known to contribute to resistance against the herpes virus HSV-1 (Paludan and Mogensen, 2001). This observation supports recent results from Kaiser *et al*., (2003), who also observed increased levels of IL-6 expression in response to MDV infection. Increases were seen in IL-18 and IFN-γ, however, contrastingly, Kaiser *et al*., (2003) reported large expression increases for these cytokines, as did Xing and Schat (2000) for IFN-γ. The observed differences may be due to variation in the immune inducing natures of the different challenge strains of MDV. Furthermore, as Kaiser *et al*., (2003) clearly demonstrated, different lines of birds respond with varying degrees of cytokine production and the observed difference may also be attributed to this. An intruiging observation was the observed increase in IL-4 production during the course of infection (Figure 4.2C). ***In mammals, cytokines are often grouped into 2 broad categories: those associated with Th1 type immune responses, such as IFNγ, and those that stimulate Th2 response, like IL-4 (Banyer et al., 2000). As the discovery of IL-4 and Th2 type cytokines in the chicken has only been recent, little is know about the role of this cytokine in immune responses or with regard to Th2 type responses in the chicken. It will be very important in future studies to readdress this observation and to make a more in-depth study of the relationship between the expression of Th1 type cytokines and Th2 type cytokines during the course of MDV infection. The recent identification of further Th2 associated cytokines, such as IL-10, in the chicken will provide a more complete panel of Th cytokines for analysis. Since the production of Th2 cytokines is often associated with immunoregulatory responses and the immunosuppression of Th1 type responses, it may be of some use to further study these Th2 cytokines and ascertain their role in the observed immunosuppression associated with MDV infection.*** It would be of great interest to investigate the levels of cytokines, such as IL-4 and IL-10 during the infection, and determine if down regulating the expression of these cytokines any benefit towards overcoming MDV has induced immunosuppression. Microarray analysis was carried out assessed the relative expression of genes in the spleens of MDV infected birds at day 4 post-infection in an effort to compare this to qPCR results. As with other publications that have utilised microarray to investigate the response to MDV and HVT, a large number of genes are observed to be varied between infected and non-infected samples (Lui et al. 2001; Morgan et al, 2001; Karaca et al., 2004). Quite often these genes are associated with cell signalling pathways and for many others their function is at present unknown. Table 4.1 highlights a number of the genes shown to be altered between the samples, however, we have restricted the analysis to clones that may have some relevance to the immune response. Clearly the area of microarray analysis in the

chicken has a long way to progress. The development of new array with a greater coverage of the genome will provide a far superior recourse for whole genome analysis. Of most value will be improvements to the annotation of the representative genes on the array, however, without ease of bioinformatic analysis this process of analysis will remain less practical.

An increased knowledge of the nature of the cytokine response associated with immune response to viral infection may assist in the rational choice of cytokines that may act as immunoenhancer to MDV infection. The cloning and expression of these cytokines allows their practical use to determine the potential for their application.

5.2 Cytokine production use

5.2.1 Cytokine expression

Cytokines are a diverse group of regulatory proteins secreted by a variety of cells, all crucial in the control of the immune system (Belardelli and Ferrantini., 2002). They are the major mediators of host defence, orchestrating the complex events in the immune response, both at the innate and adaptive levels of the immune response (Zekarias et al., 2002). These cytokines regulate the amplitude and duration of the immune response by controlling the numbers, lineages and functional activation of immune cells together with their recruitment to the sites of immune localisation and infection (Balkwill, 1988; Kelso, 1989; Arai et al., 1990). Therefore, it is clear that these cytokines intimately regulate the nature of the immune response and by controlling these cytokines the immune response may be modified.

There are several cytokines that have a variety of functions that are equally important in terms of regulating immune responses. In the initial stages of infection innate immune cells produce proinflammatory cytokines, such as IL-6 and IL-18, that give rise to the inflammatory immune response (Djeraba et al., 2000; Harte et al., 2003). Furthermore, whilst proinflammatory cytokines are crucial in immunogenicity and antigen presentation other cytokines, including IL-2, IL-4 and IFN-γ, may act more down stream during clonal expansion and differentiation of T and B cells (Schijns, 2001). At the outset of this project the chicken genome sequence had not yet been released and a restricted number of cytokines had been identified. To investigate the potential of these identified cytokines, we cloned, expressed and purified the proteins for use (Figure 4.2A, B). To determine the therapeutic potential these proteins, they must first be produced in sufficient quantities to allow appropriate experimentation. For the production of recombinant cytokines there are many expression systems available as reviewed by Geisse et al., (1996). E. coli expression systems are commonly employed as they yield a high quantity of active protein. Many recombinant cytokines have been produced in this manner and have been utilised as effective therapeutics (Geisse et al., 1996; Schneider et al., 2000; Schneider et al., 2001). For the expression of the various recombinant cytokines pQE-9 (IL-6), pET (IL-15) and pQE-30 (cMGF, IL-18, IFN-γ and IL-2) expression plasmids were used. The recombinant cytokines were expressed, purified and analysed on a Western blot and the observed size of proteins was consistent with previous reports. Bradford assays were used to determine the protein concentration which was typically between 0.5-1mg/ml, this is typically expected for E. coli expressed proteins (Geisse et al., 1996). The bioactivity of the cytokines was tested in appropriate assays and the activity of the proteins was confirmed. However, the recombinant IL-15, by comparison to the other cytokines, showed lower stimulation indices in its assays. In an effort to increase the stimulatory activity of IL-15 a number of different approaches were taken, however, these showed little improvement. The expression of the recombinant in a COS cell eukaryotic expression system, effort to optimise protein expression and protein refolding, all resulted in minimal changes to the activity of the cytokine. Lillehoj et al., (2001) were able to produce active COS cell produced recombinant protein, so it may well be that further development of this system for the expression of recombinant IL-15 is required for future studies. The production of these cytokines as purified recombinant proteins allows for an assessment of their therapeutic potential in ovo. In livestock, cytokine therapy has been shown to be effective in several animal models. It has been shown, in cattle, that the administration of recombinant bovine IL-2 or IFN-α and -γ greatly reduces mortality and the severity of clinical disease and illness in vaccinated calves when challenged with bovine herpes virus-1 (Reddy *et al*., 1989;

Babiuk *et al*., 1991). Similarly, (Nash *et al*., 1993) shows that IL-1-α and β, in sheep, acts as an adjuvant to significantly enhance the secondary humoral immune response to an experimental antigen. Furthermore, the use of cytokines as therapeutics has also been shown to be effective in the chicken. It has been shown that the administration of the cytokine chicken IFN-γ enhanced weight gain in broiler chickens and elevated antibody titres (Lowenthal *et al*., 1998), showing that IFN-γ has the potential as a growth promoting and as a therapeutic.

5.2.2 *In ovo* administration of cytokines

Cytokines are biologically active, immune modulating proteins (Belardelli and Ferrantini, 2002) and as for any biologically active protein, they function at an optimal concentration in their environment. To determine the effect a biologically active protein has on a system it is usually administered in a titrated manner, incorporating a broad range of concentrations. Usually therapeutics are administered as a dose per kilogram of bodyweight. In vivo, chicken IL-6 has been administered at 10μg/kg, in 8 week-old chickens (Schneider et al., 2000), similarly IL-2 has been administered in ovo at a concentration of 0.1mg in 200μl (100μg/egg), which equates to about 6μg/kg (Ford et al., 2002). Other studies showed that IL-2 administered at 0.5μg/kg (in cattle) and 30-100μg/kg (in mice) show increases in immune response, indicating that under certain circumstances cytokines function at doses 0.1-100μg/kg (Hughes, 1998). The results of the bioassay reported herein suggest that functional concentrations seen in the recombinant cytokines in vitro fall within the 0.1-100μg/kg activity range. With this in mind, to analyse the in ovo potential, these cytokines were diluted to concentrations of 0.5, 5 and 50μg in 200μl. These administrative doses have been shown to effect immune characteristics and so the concentrations are similar, incorporating these previous findings. As with any new therapeutic toxicity trials must be carried out. With the delivery of cytokines in ovo, a key measurement of dose toxicity on an embryo is hatchability and alterations in gross morphology relative to appropriate controls. Following the in ovo administration of the recombinant cytokines hatchability, body weight and spleen weights were taken. These three characteristics are key morphological parameters incorporating survival, development and impact on an immune regulatory organ. Significant alterations in these parameters in test birds relative to controls may suggest an adverse effect of the treatment. Hatchability was measured and characterised as a chick that hatched and survived, with the expected rate of hatchability for chickens bred and grown under SPF and commercial broiler conditions is between 90-100% (Ruiz and Lunam, 2002). The rates acheived were similar to this, however, for a number of experiments the quality of the supplied eggs was not first grade and, therefore, lower hatch rates were observed. In all cases and at all cytokine concentrations the rate of hatchability was at least similar to no injection controls confirming that the in ovo administration of the recombinant cytokines had little effect on the embryo's hatchability. This is consistent with previously published data on the in ovo administration of an IL-2-like culture supernatant appeared to have no effect on the hatchability (Ford et al., 2002), indicating that doses used may be considered practical.

5.2.3 Cytokines as therapeutics

Cytokine administration, *in ovo*, allows the potential for an up-regulation of the immune system prior to hatch boosting the immune response post-hatch. Eggs are formed with a set level of antibodies given by the hen, these antibodies protect the chicken for the first few days after hatching, after which time the immune response takes over. This set level maternal antibodies given by the mother declines from hatch over the first 2 weeks and, due to an immature immune system any response to infection will be sub-optimal (Lowenthal *et al*., 1994). *In ovo* delivery of therapeutics could potentially provide the means to boost an embryos immune system prior to hatch, thereby, decreasing susceptibility to viral infection in the chicken in the first 2 weeks post hatch. The *in ovo* administration of vaccine therapeutics involves the direct injection of the therapeutic into either the embryo, amniotic sac, the chorioallantois (CAM), the allantoic sac (Sharma and Burmester, 1982) or intra-venously. Evidently, each of these administration approaches will impact on the delivery of the cytokine to the developing embryo and therefore on the observed outcomes.

We examined the immunoenhancing potential of the various cytokines by *in ovo* treatment followed by MDV challenge and analysed the outcomes at 21 days post-infection. Although there was little

statistical differences between groups some trends were observed with regard to body and organ weight changes. The mean weight of cMGF treated birds appeared to show a trend towards maintaining a higher body and spleen weight. Alterations in the response to MDV with cMGF treatment has been previously observed (Djeraba et al., 2002). The use of cMGF, administered via fowl pox virus vector, has been previously shown to modify the response of chickens during MDV infection as tumour development was reduced in chickens infected with highly virulent MDV (Djeraba et al., 2002). Similarly, IFN-γ treated birds showed some observed weight differences. It has been well established that the interferons have powerful antiviral activity and plays a critical role in modulating immune responses to virus (Jarosinski et al., 2001). IFN-γ has been shown to significantly impact on the innate response and may be of prime importance in the early stages of Marek's disease (Djeraba et al., 2000). The mean weight of IL-2 treated birds appeared to show a trend towards maintaining a higher body weight. The *in ovo* administration of IL-2 has also been reported to increase body weight while increasing relative thymus, bursa and liver weights in broiler chickens (Ford et al., 2002) indicating potential use as a growth promoter and an immunotherapeutic. However, in this publication this was not necessarily a purified form of IL-2, but rather supernatants from Con A activated cells which, although presumably rich in IL-2, would contain a variety of cytokines and other molecules. This observation may not have been due to IL-2 alone, however, it might support a synergistic effect of cytokines. When qPCR was carried out to determine the relative abundance of the MDV *Meq* gene on cytokine treated groups the IL-2 group appeared to have the lowest relative level of viral gene expression (Figure 4.62E, 4.62J). This observation supports the further investigation of immunoenhancing potential of IL-2 in MDV infection.

As we build on our understanding of how cytokines control the immune system, we will gain further insight on how to optimise immune responses to vaccination.

5.2.4 Cytokines as adjuvants

Many of the current issues relating to vaccination revolve around the continual quest for the development of safe vaccines that provide an appropriate level of immunogenicity to elicit wide-ranging and persistent immune responses. The addition of adjuvants to vaccine formulas can help to induce this, however, there are few adjuvants available that can safely provide this (Weeratna et al., 2000). Oil-based adjuvants induce adverse site reactions resulting in decreased meat quality and animal discomfort, therefore, currently there is a lack of suitable, cost effective adjuvants for use in poultry, particularly broilers. Recent advances in our understanding of the immune response to pathogens have identified that the control of the direction of the immune reaction is critically dependant on the nature of the cytokine response. This information then supports the strategy of employing cytokines as adjuvants in an effort to influence the immune response in the appropriate direction to generate a protective response. Vaccines must be able to induce immune responses that will confer protection against a specific pathogen and this in many cases requires both humoral and cell mediated immunity (Weeratna et al., 2000). An appropriate adjuvant can direct the type of immune response, toward the generation of cell-mediated immunity (Th1) or the stimulation of the production of specific antibodies (Th2). Additionally, if an inappropriate adjuvant is used it may skew the immune outcome away from a protective response, say, towards a Th2 response when in fact a Th1 response is required (Weeratna et al., 2000). Therefore, it is critical to select an appropriate adjuvant for the desired outcome.

In the analysis of the vaccine enhancing potential of in the co-inoculation of HVT vaccine with the cytokines, only minor variation was observed between the groups, except for the IL-18 treated group appeared to show a trend towards lower body weights (Figure 4.62F). Similarly, the spleen and thymic lobe weights were similar between groups and IL-18 showed a trend towards decreased mean thymic lobe weight (Figure 4.62G, 4.62H, 4.62I). The observed decrease was somewhat unexpected as IL-18 has been shown to induce T cell proliferation (Schneider et al*.,* 2000), however, *in vivo* administration of IL-18 to monkeys showed to decrease lymphocyte counts by 50% (Herzyk *et al*., 2002). Intriguingly, there was no augmentation of the response in IFN-γ treated eggs. IFN-γ is one cytokine that has been assessed for its ability to enhance antibody responses. When co-administered to birds with antigen, recombinant chicken IFN-γ produced a prolonged secondary antibody response that

persisted at higher levels and for longer periods compared to antigen injected alone (Davidson *et al.*, 2002). It may well be that the nature of the *in ovo* delivery of the IFN-γ may not establish the same conditions that are required or that the augmentation of antibody by IFN-γ does little to enhance protection.

Quantitative PCR was carried out to determine the relative abundance of the MDV *Meq* gene on cytokine and vaccine treated groups. IL-2 showed the greatest difference in the relative abundance of the viral *Meq* gene, however, IL-6 and IL-18 also showed reduction. The lower levels observed suggested that IL-2 may be the candidate showing the most promise. With this in mind, an assessment was made of the combined effects of both the cytokine and vaccine administration on the hatchability of treated broiler eggs. Broiler eggs were treated with the cytokines IL-6 and IL-2 as they had previously shown the greatest difference in relative *Meq* levels. Co-administration experiments of HVT vaccine with either IL-2, or IL-6 in broiler birds showed little distinction between the groups. In fact, the HVT treatment alone group appeared to have a higher mean body weight (Figure 4.7B). It would appear that under these conditions and with a challenge to broiler birds the HVT vaccine alone represents a suitable vaccine. The addition of the cytokines appeared to have no real value particularly since the HVT alone provided adequate protection. Future experiments may require the use of a more virulent strain of MDV to challenge the vaccine efficacy and determine if there is any augmentation to the vaccine by cytokine co-administration.

5.3 Opportunities and obstacles

Cytokines offer a natural approach to therapeutics particularly in relation to the enhancement of protective immune responses produced by vaccines. With the escalating number of chicken cytokines being cloned, only time will tell just how important these regulatory immune proteins will be for the poultry industry. By the commencement of this project relatively few chicken orthologues of mammalian cytokines had been identified. Therefore, a simple approach was to clone and express these known cytokines and determine their potential to immunoenhance the immune response to MDV infection. However, during the course of this project information relating to the chicken genome was widely disseminated and subsequently, the number of chicken orthologues of mammalian cytokines has grown dramatically. One of the remaining challenges involves a closer understanding of the nature of protective immune responses. Acquired protection against pathogens in mammals generally falls into one of two types - cell mediated or antibody mediated. In chickens it has not been established whether the same Th1/Th2 paradigm exists as it does in mammals. In order to rationally design therapeutics for a particular disease it is critical to first understand the nature of the protective immune response and then replicate that response during a vaccination strategy. This involves studying the cytokines produced during infection by the pathogen in question as well as the immune cell populations affected. With the growing accessibility to a number of avian cytokine genes, and the recent development of Real Time qPCR cytokine profiles can now be accurately measured during the course of an infection. Of particular importance is that by this process the cytokines that are traditionally associated with a Th2 response in mammals have been identified and cloned. In this project we have initiated some preliminary analysis of the relative expression of Th2 type responses in the course of infection. However, there is now a great opportunity to investigate the role of these Th2 type cytokines in MDV infection particularly as the Th2 type cytokines have functions associated with immunosuppression. As we build on our understanding of how cytokines control the immune system, we will gain further insight on how to optimise immune responses to vaccination.

This evaluation has identified some key steps of the immune response to MDV and assessed cytokines as alternative adjuvants and therapeutics in this disease. Continued work in this area may provide the opportunity for the rational design of new vaccine approaches to control of MDV infection. Such vaccine tactics could contain the immunogenic elements of the virus as immunomodulatory molecules to direct and enhance immune responses so providing improved protection. The recent identification of the Toll-like receptors (TLR) and their role in the sensing of pathogens through pathogen recognition molecules highlights this opportunity. By identifying the particular features of a virus that may stimulate the TLR, such as its production of double stranded RNA, and mimicking this activity, this can be utilised to active cytokine pathways. Such that, rather than administering the cytokine directly,

the immunostimulatory elements of the virus may be used to trigger the appropriate cytokine release by the cells. Therefore, TLR ligands may be good adjuvants for vaccines (Jiang and Koganty, 2003). With this in mind, targeting the TLR3, TLR4 and TLR9 pathways allows for an enhanced response to certain viral infections (Ulevitch, 2004) and this, therefore, offers a new direction in utilising cytokine activity for immunoenhancement. Whatever the course of action selected involves, the overriding importance of future schemes is the design of vaccine approaches that are sustainable and do not select for MDV strains with ever-increasing virulence.

Following the trends observed overseas with regard to the selective pressure exerted by vaccination approaches on MDV, this development of the virus is continuing and the drift is unlikely to be checked by the present strategies. Importantly, the processes observed with MDV represent an example of the progression that may occur with other viruses that are economically important to the poultry industry. The MDV example suggests the need for caution and the development of sustainable vaccination strategies for the future.

6. Implications

It has been over 30 years since the Australian poultry industry first instituted the use of MD vaccines and this approach has in the past proven to be a successful strategy. However, the overseas experience has shown that MDV can break through many formerly successful vaccine programs posing a potential threat to poultry production. If the selection of more virulent strains of MDV is allowed to continue at the present rate then MDV may again emerge as a major economic problem for the Australian commercial layer chickens and broiler breeder flocks. Therefore, the concerns over the ability of current live vaccines to protect against emerging hyper-virulent strains of virus necessitate the need for alternative vaccine strategies such as the use of adjuvants to enhance their activity. The therapeutic and vaccine immunoenhancement activity of recombinant chicken cytokines might provide a solution to this possible threat . Selection of an appropriate adjuvant is of the upmost importance as it is crucial that an adjuvant directs the immune response towards an appropriate protective response. If an adjuvant inadvertently skewed the response to a vaccine in the contrary direction this could have dire consequences with regard to infection.

The results from this project have implications for the Australian poultry industry with regards to MDV infection. By taking a proactive approach to investigation the potential of novel therapeutics to immunoenhance against viral infections the Australian poultry industry is boosting their MDV preparedness. Future investigations may show that cytokine mediated enhancement of the immune system leading to decreased pathogen loads, resulting in healthier and more productive birds.

7. Recommendations

Within this project area we have identified some important steps in the evaluation of the immune response to MDV and with regard to chicken cytokines as alternative adjuvants and therapeutics in this disease. The next steps for the best use of the outcomes of this project are:

1. Further evaluation of the nature of the Th1 against Th2 type response cytokines during infection with MDV.

At the initiation of this project only a few cytokines had been identified and cloned, most of these were not related to cytokines that are generally associated with what is defined in mammalian systems as a Th2 type response. However, throughout the course of this project information relating to the chicken genome was publicly released allowing a fuller analysis of the existence of various cytokines. Subsequently, a number of cytokines that are traditionally associated with a Th2 response have been identified and cloned. We have carried out some preliminary analysis of the relative expression of IL-4, a cytokine associated with Th2 type responses, and observed a relative increase in its expression throughout the course of infection. As the Th2 type cytokines have functions associated with immunosuppression it may be of importance to further study the Th2 family of cytokines in regard to MDV infection to determine if they may play a role in the observed virally induced immunosuppression.

2. Immune gene analysis of the protective response engendered by vaccination, HVT compared to Maravac.

With regard to vaccination and MDV one of the remaining challenges is comprehension the nature of protective immune responses, our current understanding of the mechanisms of vaccine-induced immunity to MDV needs to improve. Since HVT and Maravac show differences in the development of a protective response and in the nature of protection they engender, an analysis of the immune response genes to these vaccines may shed light on the nature of the mechanism of protection afforded by either of these vaccines. One of the major aims of future research would be to strengthen our understanding of the mechanisms of protective immune responses against the virus.

3. Development of optimal methods for the safe and efficient delivery of therapeutics under commercial conditions.

Given that administration of recombinant cytokines by injection, even *in ovo*, will not be feasible in commercial poultry, an enduring obstacle to the utilisation of cytokine therapies in poultry involves the delivery of these therapeutics. We have previously shown that viral vectors, such as fowl adenovirus, may provide an effective commercial delivery system. However, future research may allow the MDV vaccines themselves to be a vector for cytokines. This type of approach will be important for the practical use of cytokines.

4. Assessment of cytokine activity in the face of more virulent wild-type MDV strains in field trials.

In these experiments the challenge strain of MDV was MPF57, however, since the major concern at present is the development of MDV strains that can break through vaccination future trials should be developed with this in mind when an outbreak virulent wild type MDV is isolated and characterised.

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9. Plain English Compendium Summary

Project Title:	
RIRDC Project No.:	CSA-26J
Researcher:	Dr Andrew Bean
Organisation:	CSIRO Livestock Industries Private Bag 24, Geelong VIC 3220
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Objectives	To enhance disease resistance and vaccine efficacy in poultry by administering therapeutic doses of chicken interferon-gamma to commercial broilers and layers
Background	Marek's disease is caused by the highly contagious oncogenic herpes virus and has significant economic impacts for the world poultry industry. Even in the face of the current successful vaccine approaches MDV remains a concern, particularly with the development of more virulent strains of MDV. If this situation continues it may lead to in the recurrence of MDV as a serious economic threat. Therefore, in order to maintain the highest possible levels of animal health, welfare and productivity it is vital to be proactive in the development of safe alternative vaccination strategies and therapeutics.
Research	Previous studies have identified the potential for cytokines to act as therapeutics and vaccine adjuvants. In this project, chicken cytokines were evaluated for their immunoenhancing capacity during Marek's disease virus infection.
Outcomes	This report outlines the analysis of the immune response, with particular emphasis on the cytokine response, during MDV infection. Further to this, biologically active recombinant chicken cytokines were produced from an *E. coli* expression system and used to assess their potential therapeutics and vaccine adjuvants during MDV infection. It was found that the cytokines IL-2 and IL-6 show immunostimulatory activity and with this in mind this evaluation of cytokines activity provides evidence for the rational use of chicken cytokines as therapeutics for disease
Implications	Continued interest will see the further development of this technology to the Australian poultry industry to alleviate some of the concerns associated with vaccination against MDV infection. These results test the feasibility of using cytokines as natural therapeutics and adjuvants and these cytokines are now available for a similar type of assessment treatments for other immunosuppressive viral infections such as infectious bursal disease virus, chicken infectious anaemia virus and infectious bronchitis.
Publications	M Asif, K A Jenkins, L S Hilton, W G Kimpton, A G.D Bean and J W Lowenthal. Cytokines as adjuvants for avian vaccines (2004). Immunol. Cell. Biol, 82, 638-643. R. J. Moore, T.J. Doran, T.G. Wise, S. Riddell, K. Granger, T.M. Crowley, K.A. Jenkins, A.J. Karpala, A.G.D. Bean and J. W. Lowenthal. Chicken functional genomics, Australian Journal of Experimental Agriculture. 2005. Invited presentations (at meetings): Lowenthal J.W., Bean A.G.D., Scott G. Tyack, Louise S. Hilton, Manija Asif, Kristie A. Jenkins, and Michael A. Johnson. Oral delivery of novel therapeutics. World's Poultry Congress, Istanbul, Turkey. 2004 Schat K.A., Bean A.G.D., Broadway M., Asif M, Thomas J. and Lowenthal J.W. *In vitro* propagation of dendritic-like cells from chicken spleens. Avian Immunology Research Meeting, Munich, 2004 Bean, A.G.D., Thomas, J.D., Bruce, M.P., Asif. M., Broadway, M.M., O'Neil, T.E., Jenkins, K.A., and Lowenthal, J.W. Immune system enhancement following *in ovo* adminstration of chicken cytokines, AIRG, Munich, 2004 Bean, A. G. D., Jenkins, K. A., Asif, M., Thomas, J., Hilton, L. S., O'Neil, T. E., Lowenthal, J. W. Innate Immunity: recognition and response, APSS, Sydney, Australia, 2003. Bean, A. G. D., Asif, M., Jenkins, K. A., Hilton, L. S., O'Neil, T. E., Lowenthal, J. W. Evaluation of the immunoenhancing capability of innate cell

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