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Researcher Contact Details
Dr Andrew Bean
CSIRO Private Bag 24
Geelong, Vic, 3220
Phone: 03 5227 5000
Email: andrew.bean@csiro.au

In submitting this report, the researcher has agreed to the Poultry CRC publishing this material in its edited form.

Poultry CRC Ltd Contact Details
PO Box U242
University of New England
Armidale, NSW, 2351

Phone: 02 6773 3767
Fax: 02 6773 3050
Email: admin@poultrycrc.com.au
Website: http://www.poultrycrc.com.au

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Executive Summary

A major problem faced by the both the world and Australian poultry industry is reduced productivity due to disease. Clearly, vaccination strategies have a central role to play in alleviating the impacts of infections, nevertheless, there is a continuing need to develop and produce vaccination strategies that keep pace with new and emerging isolates of pathogens. In order to rationally design adjuvants and vaccine strategies for a particular disease it is critical to first understand the nature of the protective immune response and then replicate that response during a control strategy. Newly identified candidate adjuvants, such as the interferons (IFNs), can then be assessed for their ability to act as immune enhancers, vaccine adjuvants or therapeutics. Major vaccine companies have indicated a need for such approaches and see this as a viable step in the development of improved vaccine strategies. Of particular relevance the interest in developing new vaccines, adjuvants and therapeutics for in ovo delivery.

We have recently identified interferon-λ (IFNλ) in the chicken and shown it to be a factor involved in antiviral activity. With this in mind, the aims of this project were to investigate IFNλ as an adjuvant in poultry vaccine formulations for better protection against disease. The project included an investigation of IFNλ as an adjuvant to augment the antibody response to commercial vaccines for use in poultry. As a part of this, it also included an investigation of the optimisation of IFNλ protein expression for large scale commercial purposes and identified that IFNλ has a range of antiviral-related activities in the chicken. An analysis of the adjuvant enhancing potential of IFNλ with commercial vaccine delivered in ovo was made. We have carried out experiments that have shown the adjuvant ability of IFNλ.

Productivity increases in the poultry industry are becoming more difficult and the safe and effective delivery of vaccines and therapeutics is a key challenge faced by the poultry industry. Investigations such as these are vital to making rational decisions about future vaccination strategies. These new insights into the early

processes of immune responses will offer improved vaccination and control strategies for a wide range of pathogens.

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Introduction

The increasing worldwide demand for “clean and green” poultry products, particularly in the face of the impacts of climate and population growth, has escalated the pressure to develop natural, non-chemical alternative strategies to manage infectious diseases in poultry. Adding to this dilemma is the observation that for many diseases the current vaccines offer incomplete protection and, in the case of newly emerging pathogens, there may an urgent requirement to develop new protective strategies. This, coupled with changes in production approaches, including reduced reliance on antibiotics and increased use of free-range systems, has increased the strain on existing protection strategies. However, meeting these demands requires innovative approaches which take into account the need to enhance quality, maintain health and food safety. Cytokines can be used as new forms of anti-viral agents or as vaccine adjuvants. With this in mind, the use of recombinant cytokines as therapeutics and adjuvants in poultry is attracting considerable attention.

Adjuvants play an important role in the efficacy of vaccines increasing the antibody response to a vaccine by boosting the response. However, the difficulty with using many of the existing commercially available adjuvants is that to help stimulate an immune response to a vaccine they also cause inflammation and cell damage. It is now understood that many of these existing chemical adjuvants stimulate the production of cytokines when they cause their inflammation and tissue damage. Clearly then, by using the appropriate cytokine as an adjuvant this boosting effect may be seen in the absence of dramatic cell damage which would be beneficial, particularly in areas such as livestock production. Therefore, the use of recombinant cytokines as adjuvants is attracting extensive attention as they provide an alternative to existing adjuvants. Furthermore, stimulating the correct immune response is a must when selecting an adjuvant to use for a new vaccine. Since one adjuvant alone is rarely optimal for all antigens, it is critical to have a selection of different types of adjuvants that function through

immunomodulation and are able to modify the cytokine network. A process that often involves up-regulating certain cytokines and down-regulating other cytokines. Therefore, the use of cytokines in the administration of vaccines has a unique value in obtaining the appropriate immune response and in ensuring a protective outcome. With our recent discovery of interferonλ (IFNλ) in the chicken, studies have been performed to assess its cytokine adjuvant activity. We have shown that IFNλ protein increased antibody responses in chickens when administered with a vaccine antigen, approximately 5-fold on the primary response and over 10-fold in the secondary response, when administered compared to vaccination with antigen alone. This remarkable observation supports the concept that IFNλ may have the potential to augment an immune response when used as an adjuvant with a variety of vaccines. Similarly, this boosting effect may have the potential to allow a lower dose of antigen to be used, which may result in more cost effective use of vaccines. These observations support IFNλ as having adjuvant activity and being used in vaccine formulations for better protection against disease.

The poultry industry is growing in size, with an annual production of approximately 40 billion birds worldwide. One of the major problems faced by the poultry industry is a loss of productivity due to disease, requiring prudent health management[1]. Birds are reared under intensive conditions that contribute to infection by pathogens; therefore, prevention of infection through effective vaccination is vital to minimize infectious disease in poultry. However, vaccines, particularly for poultry, must be cost-effective, particularly when they are required on a large scale of tens of billions of doses annually. Therefore, it is critically important to develop strategies to improve vaccine efficacy and extend the use, and reduce the dose of vaccine required. One method for enhancing vaccine efficiency is the use of an adjuvant. Therefore, veterinary adjuvants must be effective to augment the response to the vaccine and reduce to amount of costly vaccine required to induce protection against disease.

Currently, the most common adjuvants found in licensed veterinary vaccines are aluminium salts and oil emulsions[2, 3]. Aluminium hydroxide (alum) adjuvants have been commonly used in many veterinary and human vaccines because of their safety[2]. However, comparative studies in humans and animals show that alum is a weak adjuvant for the induction of antibody responses to recombinant protein vaccines. It has been shown in mice to bias towards Th2 (less inflammatory, targets extracellular pathogens) rather than Th1 (pro inflammatory, targets intracellular pathogens) responses[4]. Alum poorly induces cell-mediated immunity, particularly cytotoxic T-cell responses[5], which is a significant drawback for its use in vaccines against intracellular pathogens and viruses. The use of oil-based adjuvants, in contrast, are limited by induction of side-effects and adverse site reactions[6, 7, 8]. Complete freund's adjuvant (CFA) is a typical example, having been shown to induce inflammation and ulceration at the site of injection as well as fever and sensitivity reactions[9, 10]. This is an important point to note when considering an adjuvant for veterinary use. Choosing the right adjuvant is important in terms of industry production requirements and is critically important to animal welfare. The poultry industry strongly relies on meat quality; site reactions which lead to carcass damage affects the meat quality, resulting in a loss of productivity. Site reactions induced by adjuvants, such as alum or CFA, also lead to animal distress and discomfort which compromises the welfare of chickens. As these issues are of major importance to the consumer, it is vital to producers that site reactions be avoided. Poultry producers require effective adjuvants that promote protection and that do not cause any pain or distress to birds when administered with a vaccine. However, the currently used adjuvants do not fulfil these requirements. With this in mind, there is an increased need for effective, better vaccine adjuvants that avoid animal discomfort.

An adjuvant is an agent that increases the antigenic response of an antigen and when incorporated into a vaccine formulation, adjuvants act to accelerate, extend or enhance the magnitude of a specific immune response to the vaccine antigen[11]. The mechanism underlying adjuvant activity was somewhat poorly understood until the discovery and functional analysis of both interferons and

cytokines and their role in the resulting in the activation of innate and adaptive immunity[12, 13]. Adjuvants are crucial in triggering an interferon and cytokine response which then activates both innate and adaptive immunity, and enhances the response to a vaccine. Adjuvants act as “danger” signals (since molecules such as mineral oil and Alum are unexpected in normal tissue the immune system senses these as danger and makes a strong reaction) that stimulate an interferon and cytokine response. Nevertheless, as previously indicated, in the process of endeavouring to initiate an interferon and cytokine response these danger signals have deleterious outcomes for the immunisation site. Therefore, if the aim of the adjuvant process is to enhance vaccination by stimulating interferon and cytokine production, one approach to achieving this may be to “short-circuit” the process and directly use recombinant interferons and cytokines rather than stimulate their production with harsh oil and Alum.

The use of recombinant interferons and cytokines as adjuvants is attracting extensive attention [14, 15]. Interferons and cytokines are naturally derived proteins that play a crucial role in controlling the immune system, and are produced in response to infection. In mammals, they provide signals that help to direct the immune response towards either an antibody-mediated or a cell-mediated response[16, 17]. Both interferons and cytokines have been shown to be effective adjuvants in several studies[14, 15, 18, 19]. These studies highlight the use of interferons and cytokines as adjuvants and therefore open up the avenue to assess such activities in non-mammalian species such as chickens.

We have recently identified chicken IFNλ and shown it to be a factor involved in antiviral activity. Further to this, we have cloned and expressed biologically active recombinant IFNλ (r IFNλ) and identified that r IFNλ has a range of antiviral-related activities in the chicken. From this we have filed the International Patent Application No. PCT/AU2008/001390: “Novel avian cytokines and genetic sequences encoding same". In this we have carried out pilot experiments that have shown the adjuvant ability of r IFNλ when chickens were injected with SRBC with or without IFNλ. Treatment with IFNλ not only enhanced the primary

antibody response to an antigen 5-fold but also boosted the secondary response to greater than 10-fold. With this in mind, the aims of this project are to investigate IFNλ as an adjuvant in poultry vaccine formulations for better protection against disease.

Objectives

The objectives of this project was to investigate r IFNλ as an adjuvant to augment the antibody response to commercial vaccines for use in poultry. The aims included:

(i) An investigation of the optimisation of r IFNλ protein expression for large scale commercial purposes;
(ii) Analysis of the adjuvant enhancing potential of r IFNλ and the optimisation of the formulation;
(iii) Analysis of the adjuvant enhancing potential of r IFNλ with both live and killed commercial vaccine (Zoetis commercial NDV vaccines), including dosing, route of administration and immunisation schedule, in chickens; and
(iv) Analysis of the adjuvant enhancing potential of r IFNλ with both live and killed commercial vaccine (Zoetis commercial NDV vaccines) in ovo.

Methodology

2. MATERIALS AND METHODS

2.1. Ethics

Where this project involved the use of animals the experiment was conducted in accordance with the approvals and in observance of the regulations of the CSIRO-AAHL Animal Ethics Committee under Permit No. 1668.

2.2. Isolation of lymphocytes

Spleens were harvested from 4-week-old specific pathogen free (SPF) chickens and single cell suspensions of splenocytes were prepared from individual spleens by dispersal through a 70-µm strainer into a 50 mL Falcon tube containing complete DMEM (10% fetal calf serum (FCS), 2 mM glutamine, penicillin (100 U/mL), and streptomycin (100 µg/mL)). Blood was taken from a heart bleed from exsanguinated animals using a 25 gauge needle and a 1 mL syringe and then diluted 1:2 in PBS. Both cell suspensions were layered over equal volume of Lymphoprep™ (Stemcell Technologies, Australia) and centrifuged for 20 min at 1000 $\underline{g}_{max}$ in an Allegra X-12R centrifuge (Beckmann Coulter, USA) at room temperature with no brake. The interphase was collected, transferred to a 15 mL Falcon tube and washed in 10 mL complete DMEM media followed by centrifugation for 5 min at 400 x $\underline{g}_{max}$ in the Heraeus megafuge 1.0 centrifuge (Thermo Scientific, USA). The pellet was resuspended in 5 mL complete DMEM media.

2.3. Cell thawing and culture

Primary chicken embryonic fibroblasts (CEF) as well as chicken fibroblast (DF1), chicken macrophage like (HD11) and quail cell (CEC 511) lines and isolated splenocytes were used. All cells were retrieved from liquid nitrogen and thawed by placing them in a 37^{o} C water bath until the last ice crystal melted and the samples were transferred to a 15 mL tube containing 10 mL complete DMEM

and centrifuged for 5 min at 400 x $\underline{g}_{max}$ in the Heraeus megafuge 1.0 (Thermo Scientific, USA). The pellet was resuspended in 5 mL DMEM. The cells were counted using a Haemocytometer (Bright-line, Hausser Scientific, USA).

2.4. Freezing of cells

CEF, DF1, HD11 and CEC 511 cells were cultured by incubating each cell type in complete DMEM in a new T75 flask (Sigma-Aldrich, USA) at 37ºC supplied with 5% CO_2. Following culture expansion the cells were stored in liquid nitrogen. Briefly, cells were retrieved from tissue culture flasks by adding 2 mL trypsin and incubating for 5 min at 37ºC with 5% CO_2 and then 5 mL fresh DMEM was added to stop the trypsin reaction. Media containing cells and trypsin was then transferred to a 10 mL Falcon tube followed by centrifugation for 5 min at 400 x $\underline{g}_{max}$ in the Heraeus megafuge 1.0 (Thermo Scientific, USA). Supernatant was discarded and 5 mL complete DMEM was added and were diluted with an equal amount of freshly prepared freeze mix (40% DMEM, 40% FCS and 20% dimethyl sulfoxide (DMSO, Sigma-Aldrich, USA)) and cells were aliquoted into 1.5 mL cryotubes (Sarstedt, Germany). The tubes were immediately transferred to a freezing container Nalgene® Mr. Frosty (Sigma-Aldrich, USA) containing isopropanol, which was initially stored at -80oC for 24 h and later transferred to liquid nitrogen for long term storage.

2.5. Cytokines and mitogens

IFNλ was produced in our laboratory by Tim Adams (CSIRO-AAHL). IFNλ was stored at 4°C, -20°C, -80°C and in lyophilised form at room temperature for three different time points (1 week, 4 weeks and 12 weeks). IFNλ was lyophilized using FreeZone® Triad™ Freeze Dry System (Fischer Scientific, USA) following cycles of pre-freeze at -72°C for 7 h followed by two stage process, in both stages vacuum pump was switched on. Stage 1 comprised of primary drying for 9 h with shelf temp set at -45°C and collector temp at -82°C and in stage 2; secondary drying last for 3 h, shelf temp was at 23°C and collector temp at -82°C. Nucleic acid was stored at -80°C and cytokines were stored at 4°C unless otherwise

stated. The synthetic dsRNA analogue poly (I:C) (Invivogen, USA) was prepared and stored as per manufacturer's instructions and 50 µg/mL was used as immuno-stimulant.

2.6. Measuring gene expression using Real time qPCR

2.6.1. Isolation of RNA

Total RNA was harvested from cell and tissue samples using RNeasy® Mini Kit (Qiagen, Netherlands) according to manufacturer's instructions. Briefly, 350µL of RLT-lysis buffer was added to each samples. An additional equal volume of 70% ethanol was added to each sample and the total sample applied to a spin column. Following several washes with different washing buffers the RNA was eluted from column with 25µL RNase free water as elution buffer. RNA concentration and purity was determined by NanoDrop (Thermo Fisher Scientific, USA).

2.6.2. cDNA sysnthesis

Extracted RNA was reverse transcribed into complementary DNA (cDNA) using the SuperScript® III First-Strand Synthesis SuperMix (Thermo Fisher Scientific, USA). Briefly, 6 µL of extracted RNA, 1 µL annealing buffer and 1 µL primer oligoDT were incubated together at 65^{o}C for 5 min. 2X First strand reaction mix (10 µL) and SuperScript® III enzyme mix (2 µL) were added and incubated in T100 thermal cycler (Bio-Rad, USA) for 50 min at 50^{o}C, 5 min at 85^{o}C and then stored at -20^{o}C.

2.6.3. Real time quantitative PCR

In an MicroAmp® Optical 96-Well Reaction plate (Applied Biosystems®; 403012, USA) we combined 2 µL template cDNA, 10 µL 2x TaqMan PCR master mix (Applied Biosystems, USA), 1 µL primer mix (Table 2.1) and 7 µL of nuclease free water was added in a final volume of 20 µL. The plate was covered with a MicroAmp® Optical Adhesive Film Kit (Applied Biosystems®; 4313663, USA). The qPCR protocol consisted of a holding stage of 2 min 50^{o}C followed by denaturation at 95^{o}C for 10 min and a cycling stage of 15 sec 95^{o}C, 1 min at

60ºC. The machine used was an AB Applied Biosystems® Step-one Plus Real Time PCR system and analysed by the software StepOne™ Software v.2.0.

2.7. Vaccine

The live NDV vaccine (NDV V4) was provided by Zoetis (Netherlands). The vaccine is based on the avirulent V4 strain belonging to genotype I of NDV. The stock vaccine titre was $10^{9.25}$ EID50 diluted in phosphate-buffered saline (PBS) to give 10^6, 10^3 and 10^1 EID50 dose in 50 µL.

2.8. In ovo vaccination and sample collection

A total of 7 experimental groups containing 10 SPF eggs (AusSPF) per group were used for the in ovo trial, a total dose of vaccine, 10^1, 10^3 and 10^6 EID50, was administered with and without IFNλ in a total volume of 0.2 mL as well as a PBS alone group. Using a 25 gauge needle the vaccine was administered into the amniotic cavity at 18th embryonic day in eggs. Eggs were then allowed to hatch in the incubator and the number of eggs that hatched was recorded. The number of chicks that survived to day 7 was recorded. Finally, whole blood, serum and spleen samples were collected at day 8 and stored appropriately. Serum was stored at -20°C, whole blood and spleen were stored in RLT lysis buffer at -20°C. Serum was scored for NDV-specific humoral immunity by haemagglutination inhibition (HI) test.

2.9. Haemagglutination inhibition (HI) assay

Briefly, two fold serial dilution of 25 µL serum was made with PBS in U-bottomed Nunc™ MicroWell™ 96-Well Microplates (Thermo Fisher Scientific, USA) up to the tenth well. Using 25 µL of 4 haemagglutinating (HA) units of NDV were added till the eleventh well. The plates were kept at room temperature for at least 30 minutes to facilitate antigen antibody reaction. Then 50µl of 0.5% (v/v) chicken RBC suspension was then added to every well. The eleventh well contains antigen and RBCs as the positive control and the twelfth well contains just RBCs as the negative control. After gentle mixing, the RBCs were allowed to settle at

4^{o}C for 40 minutes and agglutination was evaluated by tilting the plates. The samples showing central button shaped settling of RBCs were recorded as positive and maximum dilution of each sample causing haemagglutination inhibition was considered as the end point, which was utilized to evaluate the HI titer. The HI titer of every serum test was evaluated as corresponding of the serum dilution.

2.10. NDV TCID50

The virus was tested for its viability in DF1 cells. DF1 at 2 x 10^5 cells/mL were seeded in Nunc™ MicroWell™ 96-Well Microplates (Thermo Fisher Scientific, USA) into quadruplicate wells for 48 h. NDV virus dilutions were prepared in complete DMEM starting from 1:5, then 1:10 serial dilutions were followed until the 8th row down the plate. Cells were then infected for 1 h and the virus was replaced with fresh DMEM and the cells were incubated at 37^{o}C for 96 h. Cytopathic effect (CPE) was observed at each dilution and the TCID50 was calculated according to the number of virus particles present at each dilution.

2.11. Detection of NDV in cultured cells

NDV infected DF1 cells were observed in bright-field microscope (EVOS® FL, Thermo Fisher Scientific, USA) at 10x and 40x objective. Cell death and syncytia were recorded as the measure for CPE. Further, cells were fixed and fluorescently-labelled antibodies were then used to detect the NDV virus. Fixing buffer comprising of 4% paraformaldehyde (PFA) in PBS was used to fix the cells. For 2 x 10^6 DF1 cells, spent media was replaced by 200 µL of the PBS buffer in the Nunc™ MicroWell™ 96-Well Microplates (Thermo Fisher Scientific, USA). Cell were then incubated in 100 µL 0.1% Triton X 100 (Sigma-Aldrich, USA) for 10 min. To block the non- specific binding the wells were incubated with 100 µL of 0.5% BSA (Sigma-Aldrich, USA) in PBS for 30 min. The cells were then incubated for 1 h with 50 µL/well of primary mouse monoclonal antibody i.e. NDV QV4 (CSIRO, Australia) (1/50 dilution), diluted in 0.5% BSA in PBS, followed by three 5 min washes with 100 µL PBS. Species specific secondary

antibody used was goat anti-mouse IgG Ab (1/250 dilution) diluted in 0.5% BSA in PBS and conjugated with Alexa fluor® 488 (Life Technologies, USA) was allowed to incubate for 1 h in dark on Rocking Platform Mixer (Ratek Instruments, Australia). Cells were washed two times with 5 min incubation in 100 µL PBS and followed by two rinses with 100 µL tissue culture (TC) water. Nuclei were labelled with freshly prepared DAPI (Sigma-Aldrich, USA) (1/2000) for 10 min in the dark and then rinsed twice with 100 µl TC water. The plate was imaged using the CellInsight Personal Image Cytometer (Thermo Fisher Scientific, USA) at a magnification of 10 x, 49 fields/well representing the entire well for the detection of CPE.

2.12. Antibody labelling for FACS

Approximately 1 x 10^6 peripheral blood mononuclear cells (PBMC) or splenocytes were aliquoted in 96 well Round-bottom microtiter plates (Thermo Fisher Scientific, USA). The plates were centrifuged for 3 min at 400 x g_{max} in the Heraeus megafuge 1.0 (Thermo Scientific, USA) and supernatant was discarded. All the antibodies used in FACS analysis are listed in Table 2.2. The antibodies were diluted in cold FACS buffer (2% FCS and 0.01% Sodium Azide in PBS) and 50 µL of antibody cocktail was added to the cells. The plate was kept for incubation for 30 min at 4^{o}C in the dark. Following 30 min incubation, the antibody-cell cocktail was washed using 100 µL FACS buffer and the plate was centrifuged for 3 min at 400 x g_{max} in the Heraeus megafuge 1.0 (Thermo Scientific, USA). The supernatant was discarded and the cells were resuspended in 150 µL of FACS buffer in preparation for flow cytometric analysis in BD LSR II (BD Biosciences, USA).

2.13. Statistical analysis

Data means and standard error (SE) were calculated utilizing Microsoft Excel (Microsoft Office Excel, 2013). Further statistical analysis was performed using GraphPad Prism version 5 (GraphPad Software, USA). ANOVA (one-way analysis of variance) technique was used to distinguish the contrast between two

or more groups of data, a post non-parametric test, Kruskal-Wallis test was additionally performed where required. Unless generally expressed the obtained statistical values were viewed as significant from control values when their probability was under 0.05 ($p<0.05$).

Table 2.1 PCR primers used in this study.

Target gene		**Primer sequence**
GAPDH	Forward	CCCCAATGTCTCTGTTGTTGAC
	Reverse	CAGCCTTCACTACCCTCTTGAT
Mx	Forward	GTCCAAGAGGCTGAATAACAGAGAA
	Reverse	GGTCGGATCTTTCTGTCATATTGGT
IFNλR1	Forward	GGATCTCCACCAGATGTGTTGTAC
	Reverse	GGAACCTTTATCCATTTGTCCATACG
TLR7	Forward	ATGACAAATCTTTCAGAGG
	Reverse	CTAAACAGTTTCCTGGAGAAG

Table 2.2 Antibodies used in this study.

Antibody	Conjugate	Dilution	Source
CD45	Allophycocyanin (APC)	1:100	Southern Biotech, USA
CD3	Spectral Red (SR)	1:200	Southern Biotech, USA
CD25	Fluroroscein Isothiocyanate (FITC)	1:200	Southern Biotech, USA
Bu-1	Phycoerythrin (PE)	1:400	Southern Biotech, USA
MHC II	Alexa Fluor® 700 (AF700)	1:800	Southern Biotech, USA

Results

Currently used poultry vaccine adjuvants can have associated unwanted site reactions causing a downgrading of the meat quality and ultimately a loss in production margins. This research project proposed the development of IFNλ protein as a potential novel adjuvant for augmenting vaccine efficacy for poultry vaccines. IFNλ, a type III IFN, has been reported in other species to generate a more tempered immune response when compared to the type I IFNs, such as IFNα. This combined with our data showing that IFNλ is capable of stimulating antibody responses to viral antigen suggests that it may provide an appropriate immunological adjuvant for poultry vaccines. With this in mind, we first sought to understand the mechanisms of the natural IFNλ response to aid in experimental vaccination design and in order to optimise the utility of IFNλ treatment, particularly with regard to any potential immunotoxicity.

We first aimed to investigate the potential of the natural antiviral IFNλ response in chickens, to determine if, like in other species, the strength of response is moderate when compared to type I IFNs. To achieve this we stimulated chicken splenocytes with poly I:C, a viral mimic, and measured the production of both IFNα and IFNλ using real time (RT) PCR (figure 1). Our data showed that the production of IFNλ transcript was significantly lower than that of IFNα. Increases in IFNλ transcript were between 5-10 fold higher than the untreated control. Similarly, IFNα transcript increased, however, it was between 20-1000 fold over the untreated control. The IFNα appeared to increase in a dose dependant manner with regard to poly I:C stimulation. Contrasting this, IFNλ response plateaued at the 1µg/mL concertation. Further analysis of the magnitude and duration of the IFN response showed that for splenocytes stimulated with either poly I:C or LPS both IFNλ and IFNα are upregulated in response to poly I:C stimulation with the peak of transcript expression being between 1.5 and 3h for both IFNs. Neither IFN was upregulated in response to the LPS (figure 2a and b).

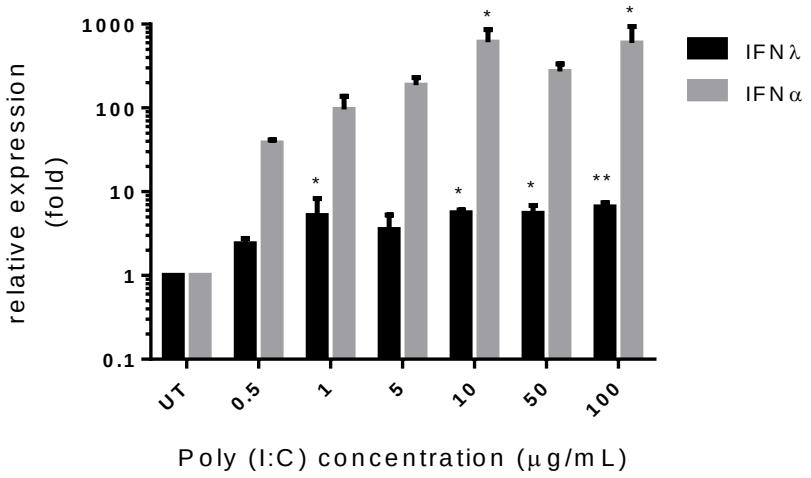


Figure 1: Dose response of chicken IFNs to poly (I:C)

Expression of IFNα and IFNλ mRNA in purified chicken splenocytes from SPF chickens stimulated with poly (I:C) at the concentrations indicated. The bars represent the mean fold change of 3 chicken spleens with the standard error of the mean (SEM) compared to the untreated sample normalized against the housekeeping gene GAPDH (* p value < 0.05 using a one-way ANOVA test with Fischer's uncorrected LSD test).

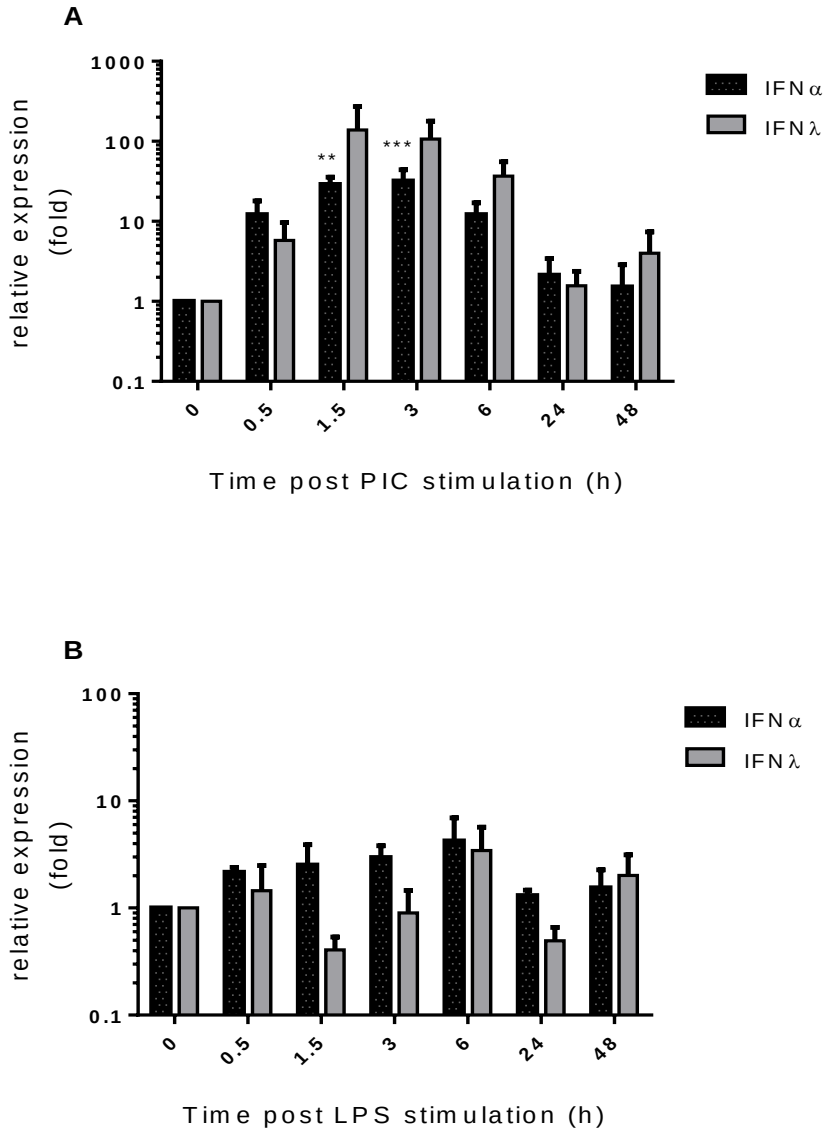


Figure 2: Time dependent stimulation of chicken IFNs

Expression of IFNα and IFNλ mRNA in purified splenocytes from three SPF chickens stimulated with 50 µg/mL PIC (A) and 10 µg/mL LPS (B) the indicated time course. The bars represent the mean fold change of 3 chicken spleens with the standard error of the mean (SEM) compared to the untreated sample normalized against the housekeeping gene GAPDH (* p value < 0.05, *** p value < 0.001 using a one-way ANOVA test with Fischer's uncorrected LSD test).

An investigation of the response of chicken immune cells to IFN stimulation was made. Splenocytes were stimulated with recombinant chicken IFNα

Figure (figure 3a) and recombinant chicken IFNλ (figure 3b) and levels of IFN measured. IFNα stimulation was able to induce IFNα but not IFNλ mRNA expression at early time points (0.5-6 hours). IFNλ treatment in contrast upregulated IFNλ much later at the 48 h time point where a 3000-fold increase was observed. IFNλ stimulation caused IFNα mRNA levels to decrease until 24h, but with a later upregulation to 350-fold at 48 h.

We investigated the role of IFNλ and IFNα on the upregulation of chicken interferon stimulated genes (ISGs) over time. We showed that IFNλ (figure 4a) was able to upregulate Mx and Viperin at a later time point than IFNα (figure 4a) which was able to also upregulate PKR and Zap. The peak of the response was earlier for IFNα (3-6 h) compared to IFNλ.

For IFNλ to be used as a commercial adjuvant requires the optimisation of IFNλ protein production. We compared several protein expression systems, including both bacterial and mammalian. Similarly, an in vitro assay to measure the biological activity of the IFNλ protein produced was developed. We investigated four different cell types for their ability to respond to IFNλ stimulation. The four cell lines chosen were DF1, CEC, HD11 and CEFs. DF1s, HD11s and CEFs all expressed the transcript for IFNλ receptor, however CEC cells did not (figure 5). The HD11 cells showed the highest level of transcript followed by both DF1 and CEFs. Further, stimulation of each of these cell types with IFNλ did not substantially increase IFNλ receptor (figure 6). Alternatively, the induction of ISGs may provide a suitable marker. Therefore, we chose to investigate the suitability of Mx as a measure of biological activity. We showed that in response the IFNλ the DF1, HD11 and CEF cells all had a corresponding upregulation of Mx, whereas the CEC cells did not (figure 7). Intriguingly, the highest fold induction of Mx came from the CEFs, with an equivalent response in the DF1 and HD11 cells, despite the highest level of receptor transcript being in the HD11s.

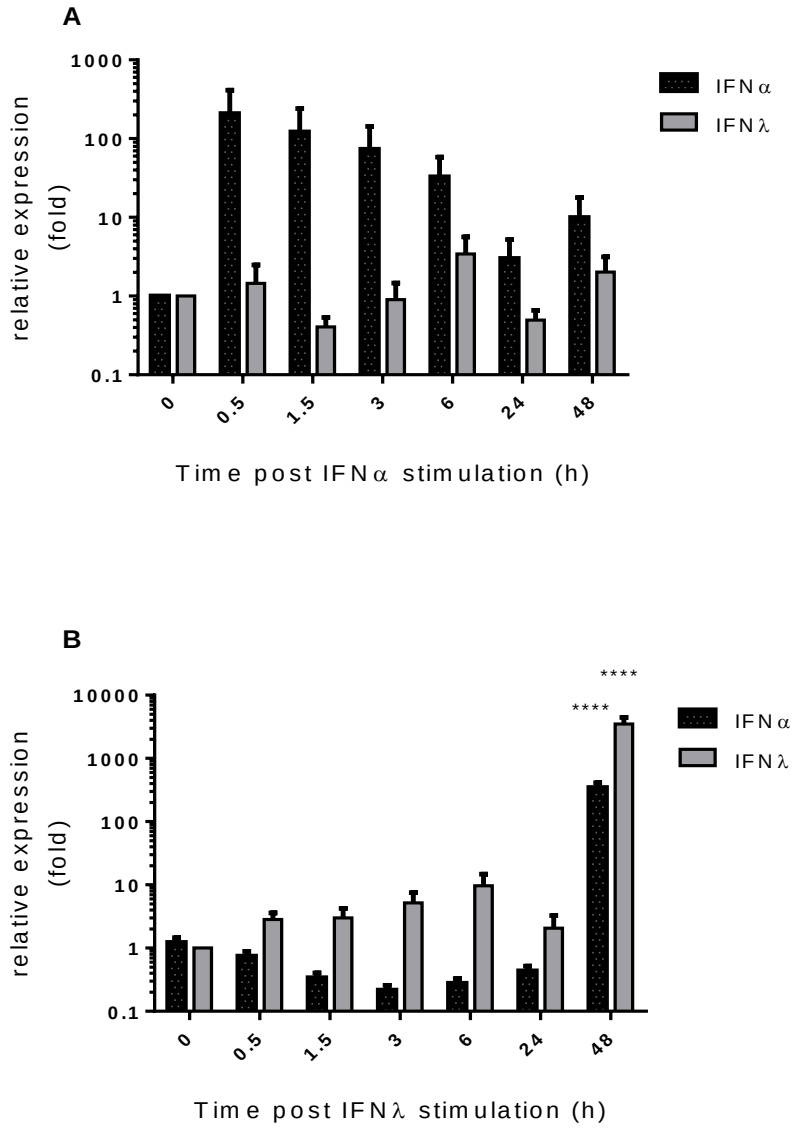


Figure 3: Time dependent IFN mediated induction of chicken IFN expression

Expression of IFNα and IFNλ mRNA in purified splenocytes from three SPF chickens stimulated with 500 ng/mL of IFNα (A) and 50 µg/mL of IFNλ (B) over the indicated time course. The bars represent the mean fold change of 3 chicken spleens with the standard error of the mean (SEM) compared to the untreated sample normalized against the housekeeping gene GAPDH (*** p value < 0.001 using a one-way ANOVA test with Fischer's uncorrected LSD test).

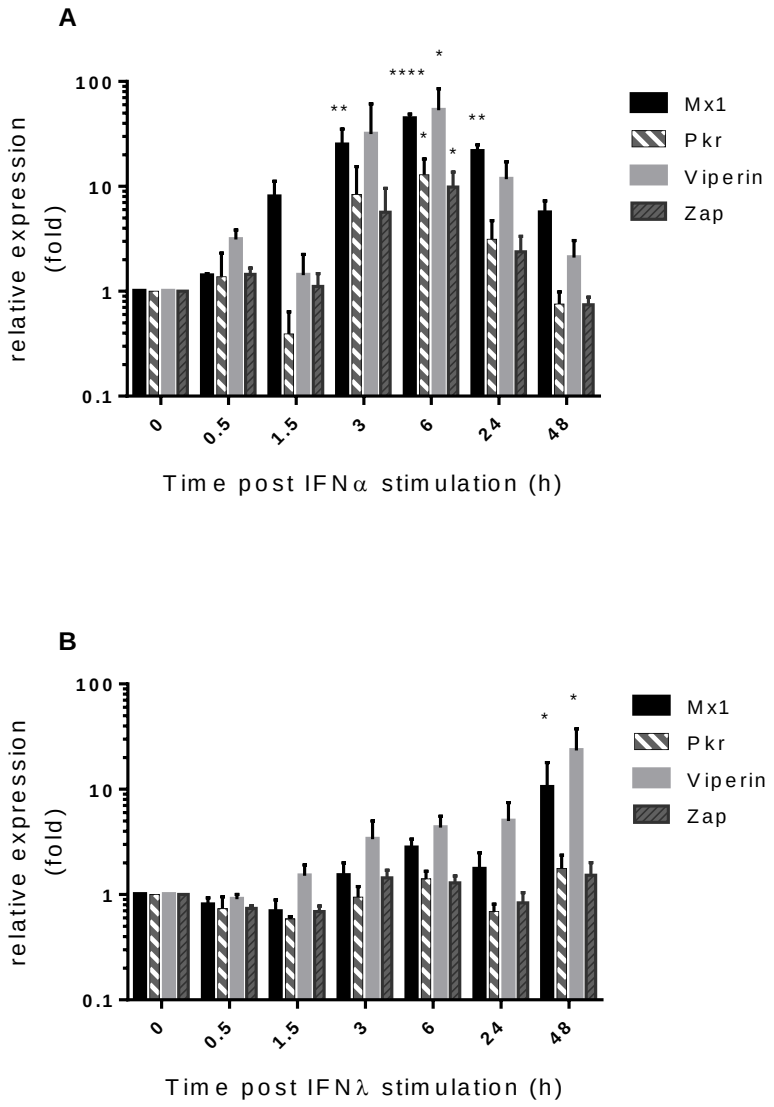


Figure 4: Characterisation of the type I/III mediated ISG response in primary chicken cells

Expression of Mx1, PKR, Viperin and Zap in purified splenocytes from three SPF chickens stimulated with 500 ng/mL of IFNα (A) and 50 µg/mL of IFNλ (B) over the indicated time course. The bars represent the mean fold change of 3 chicken spleens with the standard error of the mean (SEM) compared to the untreated sample normalized against the housekeeping gene GAPDH (* p value < 0.05, **** p value of <0.0001 using a one-way ANOVA test with Fischer's uncorrected LSD test).

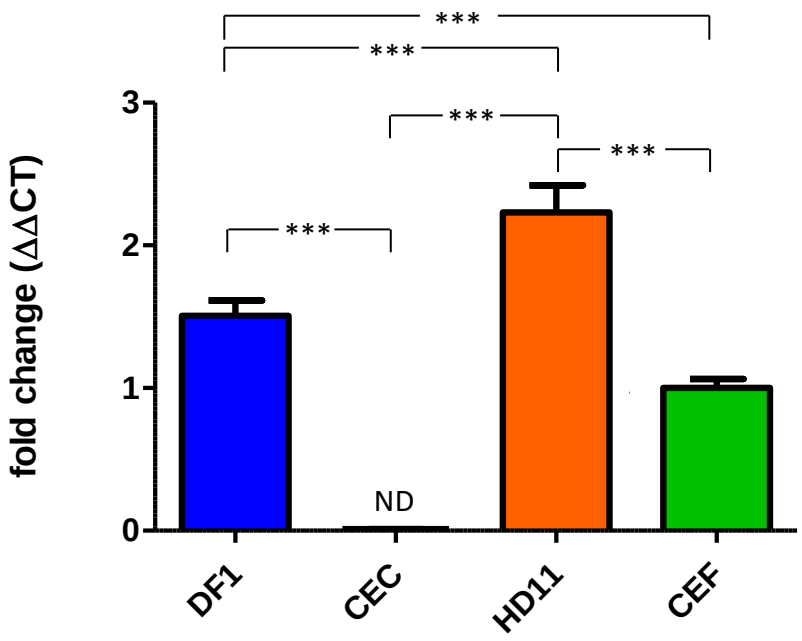


Figure 5: Chicken fibroblasts and macrophages express IFNλR1 receptor transcripts. The bar graph shows the expression of IFNλR1 receptor in chicken fibroblasts (DF-1 & CEF), macrophages (HD-11) and quail cell line (CEC 511) relative to unstimulated CEF as control. Real time qPCR was performed for IFNλR1 gene expression using GAPDH as housekeeping gene. The values are expressed as mean + SE; n=3 $p<0.05$.

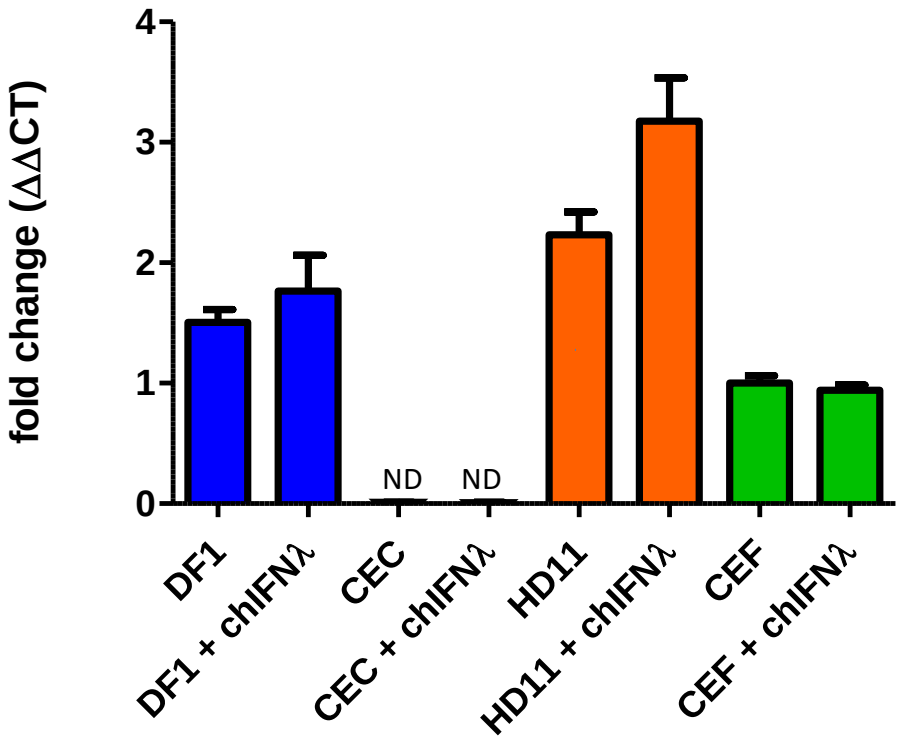


Figure 6: Chicken fibroblasts and macrophages express IFNλR1 receptor transcripts.

The bar graph shows the expression of IFNλR1 receptor in chicken fibroblasts (DF-1 & CEF), macrophages (HD-11) and quail cell line (CEC 511) relative to unstimulated CEF as control. Real time qPCR was performed for IFNλR1 gene expression using GAPDH as housekeeping gene. The values are expressed as mean + SE; n=3 $p<0.05$.

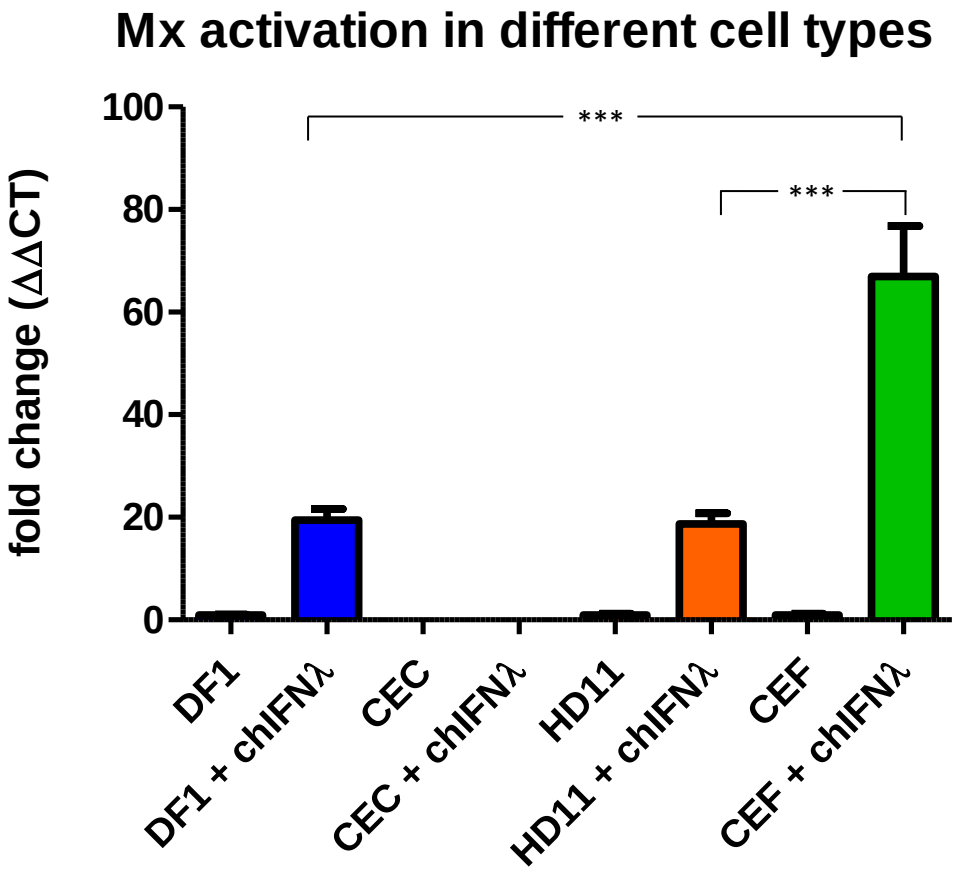


Figure 7: Chicken embryonic fibroblasts showed highest Mx gene expression.

The bar graph displays the induction of Mx transcripts in DF1, CEC, HD11 and CEF relative to their unstimulated control respectively. Extracted RNA was converted to cDNA to perform qRT-PCR. GAPDH was used as a housekeeping gene. The values are expressed as mean + SE; n=3, $p<0.05$.

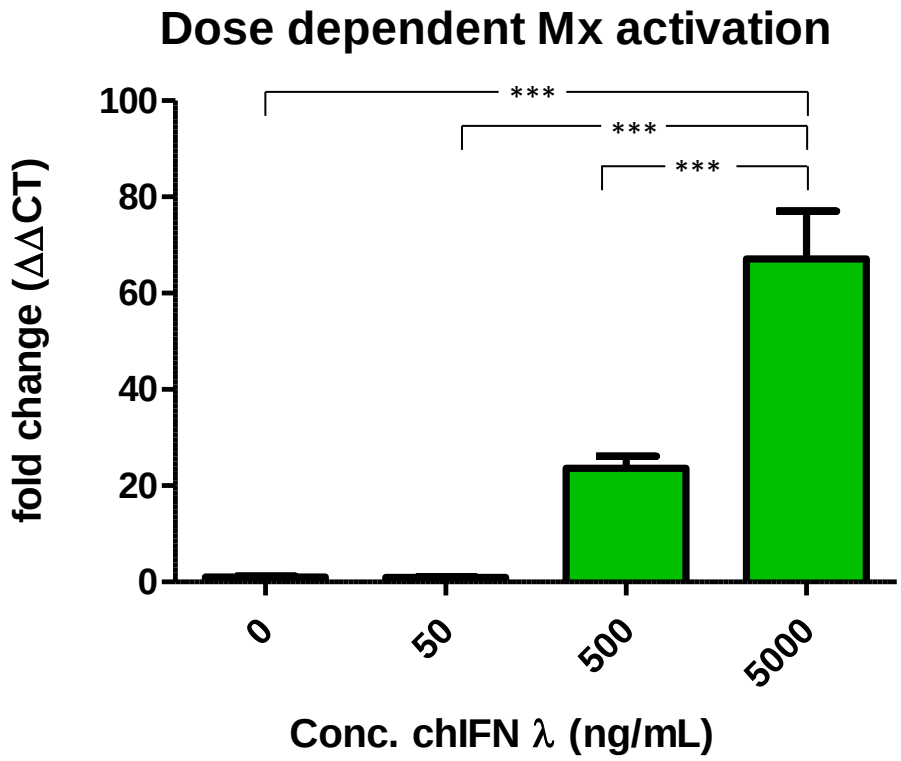


Figure 8: Dose dependent Mx gene expression in CEF.

CEF cells cultured with IFNλ (50, 500 and 5000 ng/mL) for 6 h, the total RNA was isolated and subjected to qRT-PCR to quantify the mRNA expression levels, normalized to GAPDH relative to unstimulated as control. The values are expressed as mean + SE from 3 different biological replicates, $p<0.05$.

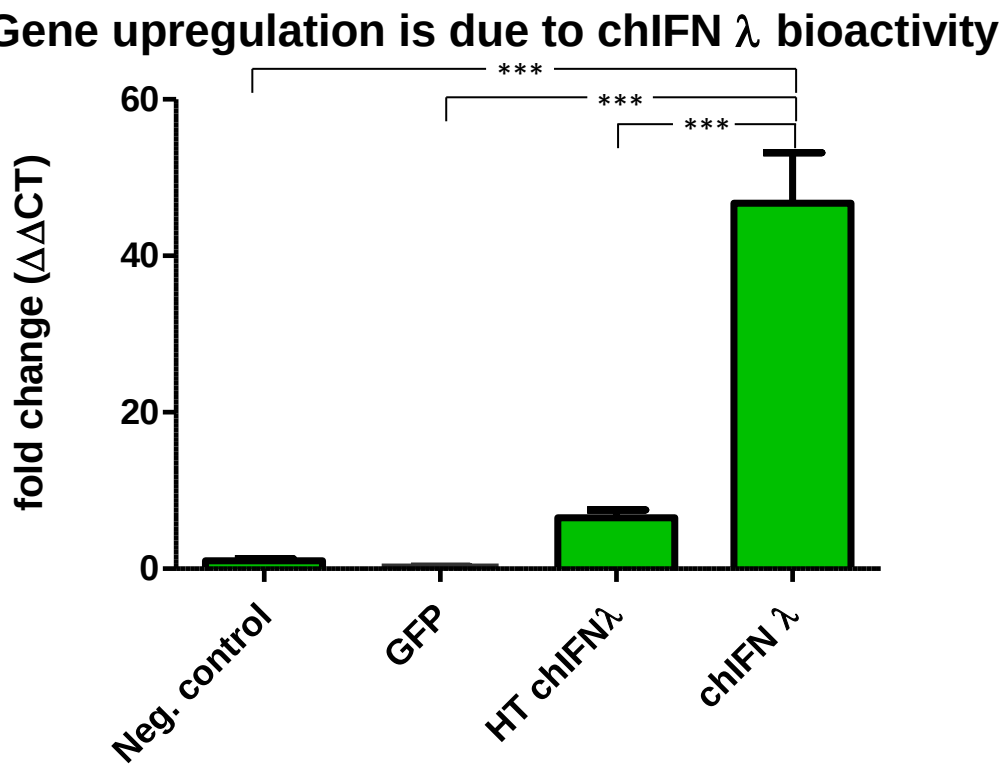


Figure 9: Mx gene upregulation is due to bioactivity of IFNλ.

The bar graph shows the expression of Mx gene was increased by IFN λ and not by, if present, any other contaminants in the protein. Total RNA was extracted from cultured CEF and mRNA expression was measured in qRT-PCR keeping GAPDH as housekeeping gene. The values are expressed as mean + SE; n=3 $p<0.05$.

Based on this data we chose to continue to investigate the CEF cells as a possible bioassay for the optimization of IFNλ protein production. To confirm that the response seen in Mx levels is due to specific stimulation of IFNλ and determine the optimal concentration of IFN to use for our bioassay we performed a dose titration of IFNλ on CEF cells. Under the stimulation conditions used we demonstrated a significant increase in Mx transcription in response to IFNλ in CEFs for 500 and 5000 ng/mL concentrations (figure 8). The Mx response was most robust at 5000 ng/mL and the fold induction observed was adequate for our assay. To further confirm the induction of Mx was due specifically to the IFNλ, we performed our bioassay and included the additional controls of an irrelevant protein (GFP) that was made in the same expression system, a sample of IFNλ that had been heat treated (90^{o}C for 10 minutes) to inactivate the protein and a negative control (assay buffer only) (figure 9). This experiment shows that only the intact IFNλ is able to produce a significant increase in Mx in our bioassay.

Having developed and confirmed a suitable biological assay we were able to proceed to the optimization of IFNλ protein production. The first step in the protein production optimization was to generate a codon optimized sequence. The first optimisation was for preferential codon usage by E. coli. The algorithm generated a new DNA sequence that was 78.98% identical to the original, meaning that 21.02% had been modified (figure 9). We also performed the same analysis to optimize the protein expression within human cells as we planned to undertake mammalian expression studies in HEK293T cells. This analysis showed a 77.01% homology to the original sequence indicating a 22.99% change (figure 10). We then synthetically generated the appropriate sequences and inserted them into a range of appropriate expression vectors (data not shown).

In order to produce the most biologically active product in high quantities at low cost, we undertook a comprehensive optimization of protein production in E. coli and in human HEK293T cells. For optimization of E. coli based production we

inserted the codon optimized sequence into three different expression vectors (pET22b – clone 1, pD444-SR – clone 2 and D864 – clone 3), all of which have a different potential for protein production. Our first optimization step was to undertake a small scale expression using the pET22b and pD444 vectors in 12 different bacterial cell lines. We also undertook these studies at two different temperatures (20^{o}C and 28^{o}C). These data are summarized in figure 11 where the total expression level is categorized as high (+++), medium (++), low (+) and negative (-). Both vectors produced the IFNλ protein to varying levels. Our data suggests that the IFNλ expression in E. coli was most efficient at 20^{o}C not 28^{o}C. We also observed a clear preference for expression in particular cells for each vector. The pET22b vector performed strongest in the BL21 Star (DE3) cell line, whereas the pD444 vector had the highest expression in the BL21 (DE3) C41 cells. An SDS-PAGE analysis on a subset of samples from clone 1 (figure 12) and clone 2 (figure 13) determined the size and purity of each of the production methods. The SDS-PAGE analysis of clone 1 (pET22b) clearly showed the production of an appropriate size band in lanes 7 and 9 which relates to the 20^{o}C production of the BL21 (DE3) and to a greater extent the BL21 (DE3) star cells. This suggests that the best production from the pET22b vector with regards to quantity was in the BL21 (DE3) star cells. The purity of each preparation varied between the different conditions with again the BL21 (DE3) star cells having the least quantity of contaminating proteins. Figure 13 showed that lanes 5, 9 and 11 have a similar band at the appropriate size, however, these appear to be of lower concentration compared to the clone 1 (pET22b) vector and additionally appear to have more contaminating bands. Although the expression profile of our optimization trials has demonstrated a band of the approximate correct size we needed to confirm the bands were a product of the expression vectors. To do this we performed Western blot analysis on selected protein production preparations (figure 14). The blot showed the detection of the His tag attached to the appropriate sized band in lanes 4 and 10 from clone 1 (pET22b) which related to the BLR (DE3) and BL21 (DE3) star, respectively.

Figure 9: Codon optimization for protein production in bacterial cells

The alignment shows the identical (*) and non-homologous nucleic acids between the original and E. coli codon optimized sequences as aligned by ClustalW. The sequences were optimized using Integrated DNA Technologies Codon Optimization Tool.

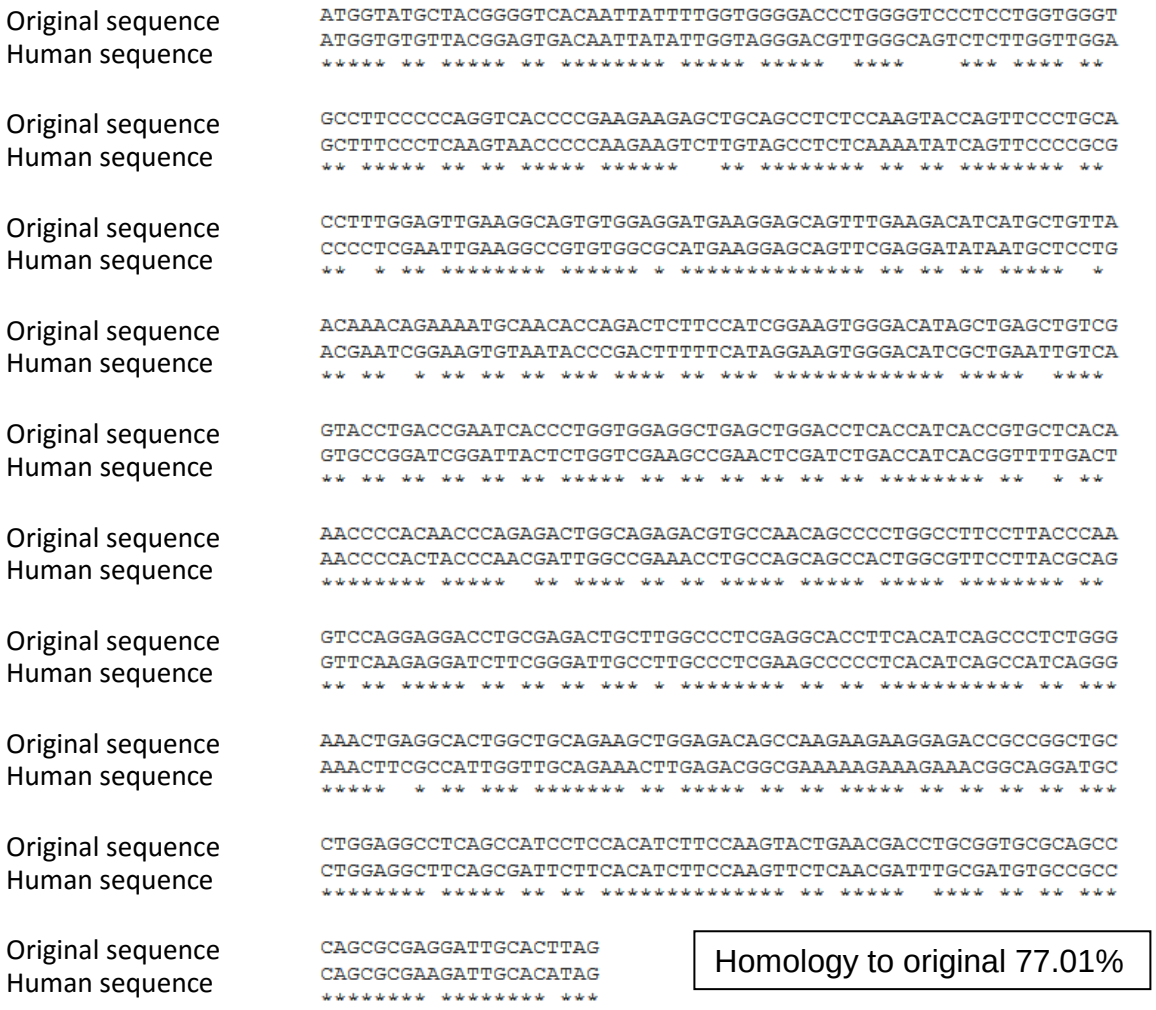


Figure 10: Codon optimization for protein production in human cells

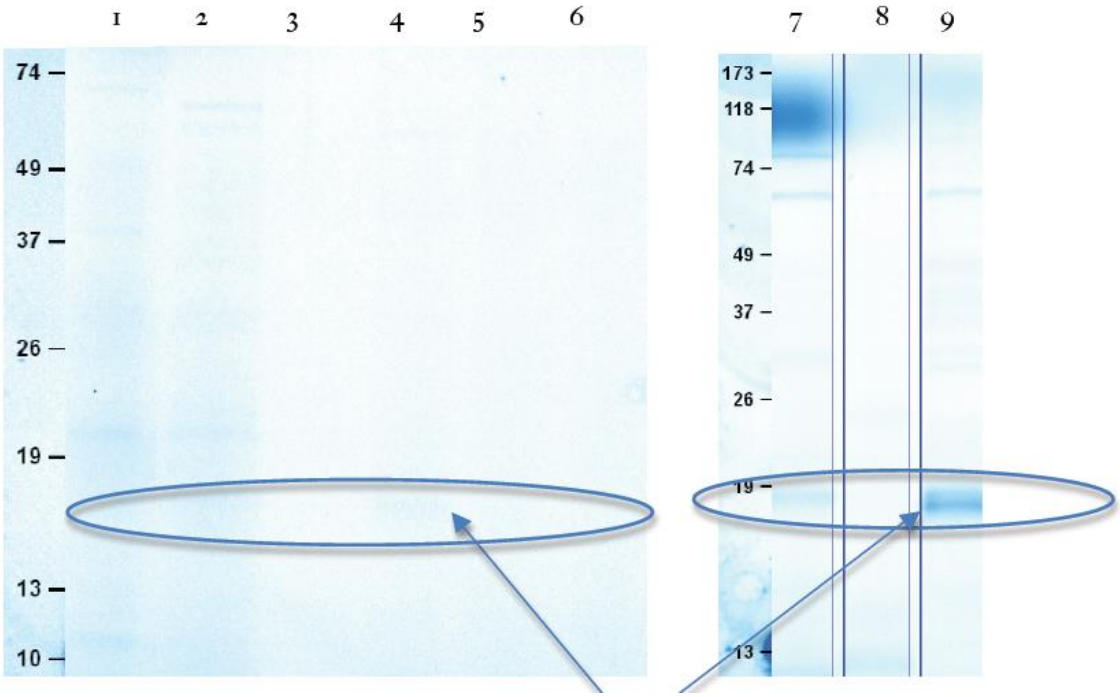
The alignment shows the identical (*) and non-homologous ( ) nucleic acids between the original and Human codon optimized sequences as aligned by ClustalW. The sequences were optimized using Integrated DNA Technologies Codon Optimization Tool.

CELL LINE	pET22b-IFNλ		pD444-SR-IFNλ	
	AT 28°C	AT 20°C	AT 28°C	AT 20°C
BL21(DE3) C43	–	–	–	–
BL21(DE3) C41	–	–	+	+++*
BL21(DE3) RIL	–	–	–	–
ROSETTA GAMI (DE3)	–	–	–	–
ROSETTA GAMI B (DE3)	–	–*	–	–
Rosetta 2 (DE3)	–	–*	–	+*
ROSETTA BLUE (DE3)	–	–*	+	+*
BLR (DE3)	+*	++	+	+*
NiCo21 (De3)	–	–*	–	+*
BL21 Star (DE3)	+*	+++*	–	–
SG13009	–	–	–	+
WK6	–	–	–	–

* Expression confirmed by Western blot analysis

Figure 11: Optimisation of IFNλ production in E. coli cells with pET22b and pD444-SR

We compared expression of the pET22b and pD444-SR vectors containing the codon optimized IFNλ sequence in 12 different strains of E. coli at two different temperatures. The results are summarized by level of expression expressed as negative (-), low (+), medium (++) and high (+++).



1=Rosetta 2 (DE3) grown at 28°C Clone 1

2=BLR(DE3) grown at 28°C clone 1

3=NeB NiCO21 (DE3) grown at 28°C Clone 1

4=BL21 (DE3) Star grown at 28°C- clone 1

5=Sg13009 (DE3) grown at 28°C clone 1

6= WK6 (DE3) grown at 28°C -Clone 1

7= BLR (De3) grown at 20°C- clone 1

8= NeB NiCO21 (DE3) grown at 20°C Clone 1

9= BL21 (DE3) Star grown at 20°C- clone 1

Figure 12: Optimised E. coli expression: Clone 1 (pET22b)

SDS-PAGE analysis shows molecular weight and relative quantity of selected protein production conditions. The gels were stained with Coomassie blue strain and molecular weight markers were used to estimate size, measured in kDa.

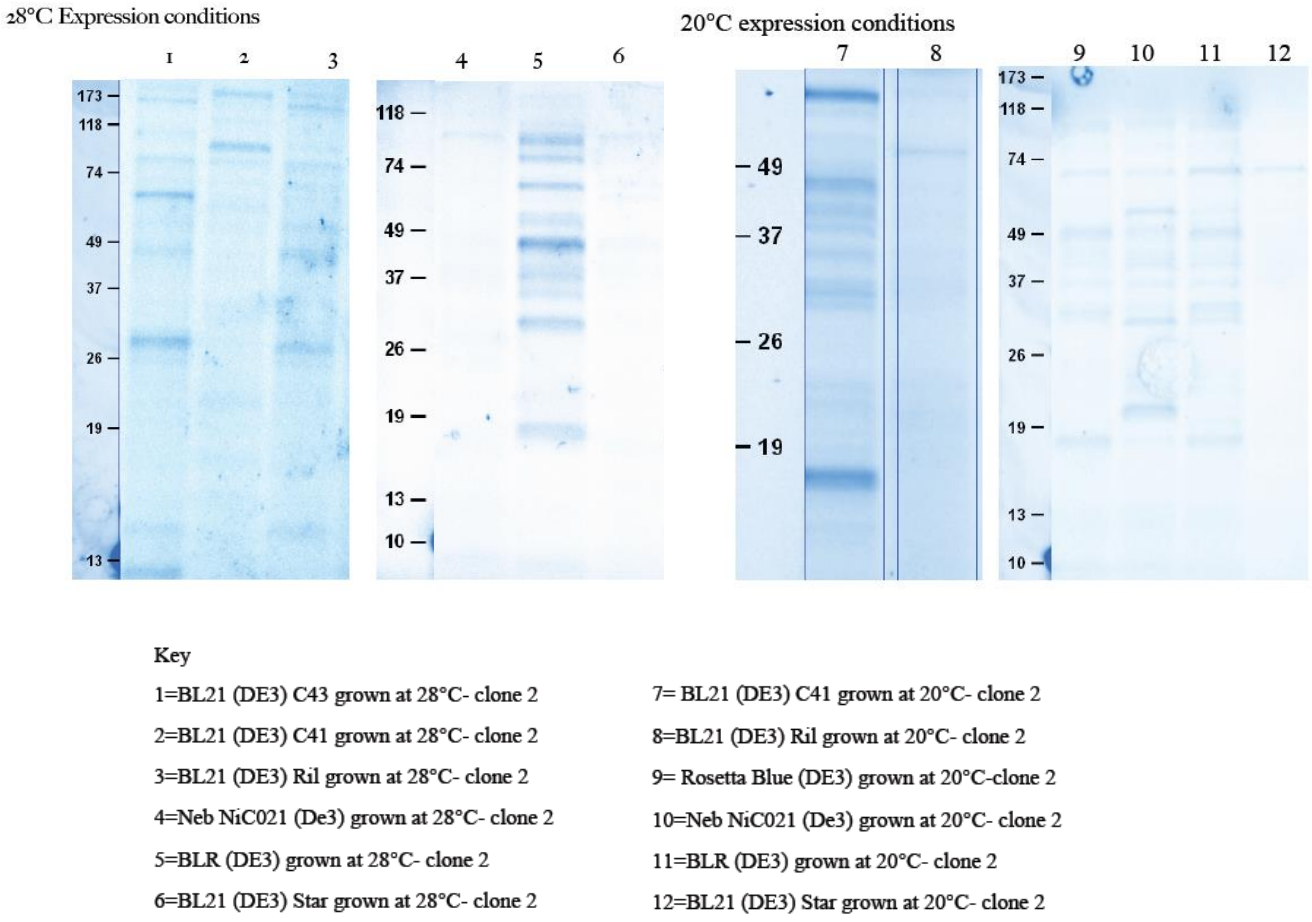
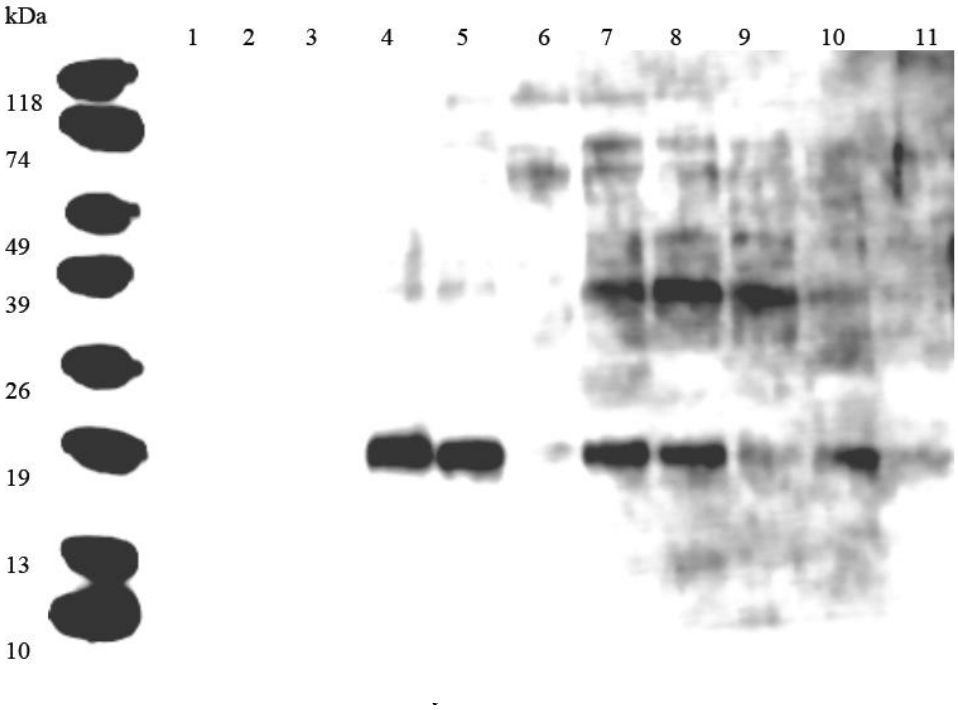


Figure 13: Optimised E. coli expression: Clone 2 (p444)

SDS-PAGE analysis shows molecular weight and quantity of selected protein production conditions. The gels were stained with Coomassie blue strain and molecular weight markers were used to estimate size, measured in kDa.

Detection of the His tag was also observed in Figure 14, lanes 5, 7, and 8 which relates to clone 2 (pD444-SR) with minor bands in lanes 6 and 9. Additional, larger bands were seen in lanes 7, 8 and 9. This larger band was approximately double the size of the other detected band, suggesting this may be a dimer of the IFNλ. We then focused on optimization of the D864 vector which relies on a specific cell line, the Lemo21 (DE3) cells. We focused on optimizing a cell line additive, rhamnose, at different concentrations to achieve the highest concentration using this vector (figure 15). This optimization showed the highest level of expression was observed a concentration of 1mM. We also compared a short rhamnose induction (6h) to a long induction (18h) at a concentration of 2mM, the longer timeframe appeared to be advantageous over the shorter. This experiment was followed again by SDS-PAGE and Western blot to analyse the purity and confirm the product of interest was produced (figure 16a, 16b). The SDS-PAGE gel showed a high proportion of contaminating bands in each preparation and that the increasing concentrations of rhamnose lead to an upregulation of all proteins in the preparation not just the IFNλ band observed at 19kDa. The Western blot analysis confirmed that the protein observed at 19kDa was the His tagged IFNλ and that the optimal concentration of rhamnose is 1mM, however, a purification step was likely to be required. We then performed a larger scale production and purification using the optimized conditions for both the pET22b vector as well as the D864 vector (data not shown).

As mammalian expression systems are well described for the production of biologically active proteins, we also investigated the production of the IFNλ in HEK293T cells using the pCAGGS vector. No optimization step was required for this production as the systems has been well established to optimal conditions in our laboratory, we did however undertake two separate production runs to ensure consistency. We performed an SDS-PAGE analysis of the purified protein and compared it to the E. coli expressed protein as well as a control protein (GFP) made in the same system (figure 17a).



1= Rosetta gami B (DE3) grown at 20°C Clone 1
2= Rosetta 2 Blue (DE3) grown at 20°C Clone 1
3= Rosetta Blue (DE3) grown at 20°C Clone 1
4= BLR(DE3) grown at 20°C Clone 1
5= BL21 (De3) C41 grown at 20°C Clone 2
6= Rosetta 2 (De3) grown at 20°C Clone 2
7= Rosetta Blue (DE3) grown at 20°C Clone 2
8= BLR(DE3) grown at 20°C Clone 2
9= NeB NiCO21 (DE3) grown at 20°C Clone 2
10= Bl21 (De3) Star grown at 28°C Clone 1
11= BLR(DE3) grown at 28°C Clone 1

Optimised 14: E. coli expression: Clone 1 (pET22b) and Clone 2 (pD444)

Western blot analysis of selected preparations from protein production methods under different conditions shows binding of an anti-His antibody bound to the 6-His tag on the recombinant protein. The size is estimated as compared to a molecular weight marker measured in kDa.

Concentration of l-rhamnose (mM)	Time (hrs)	Expression Level
0	6	+
0.1	6	+++
0.5	6	+++
1	6	++++
2	6	++
2	18	+++

Figure 15: Optimisation of IFNλ production in E. Coli cells with D864 vector

Soluble nickel affinity expression levels at different time points and concentration of l-rhamnose used to induce the protein expression in Lemo21 (DE3) cells using the D864-IFNλ vector at 30°C.

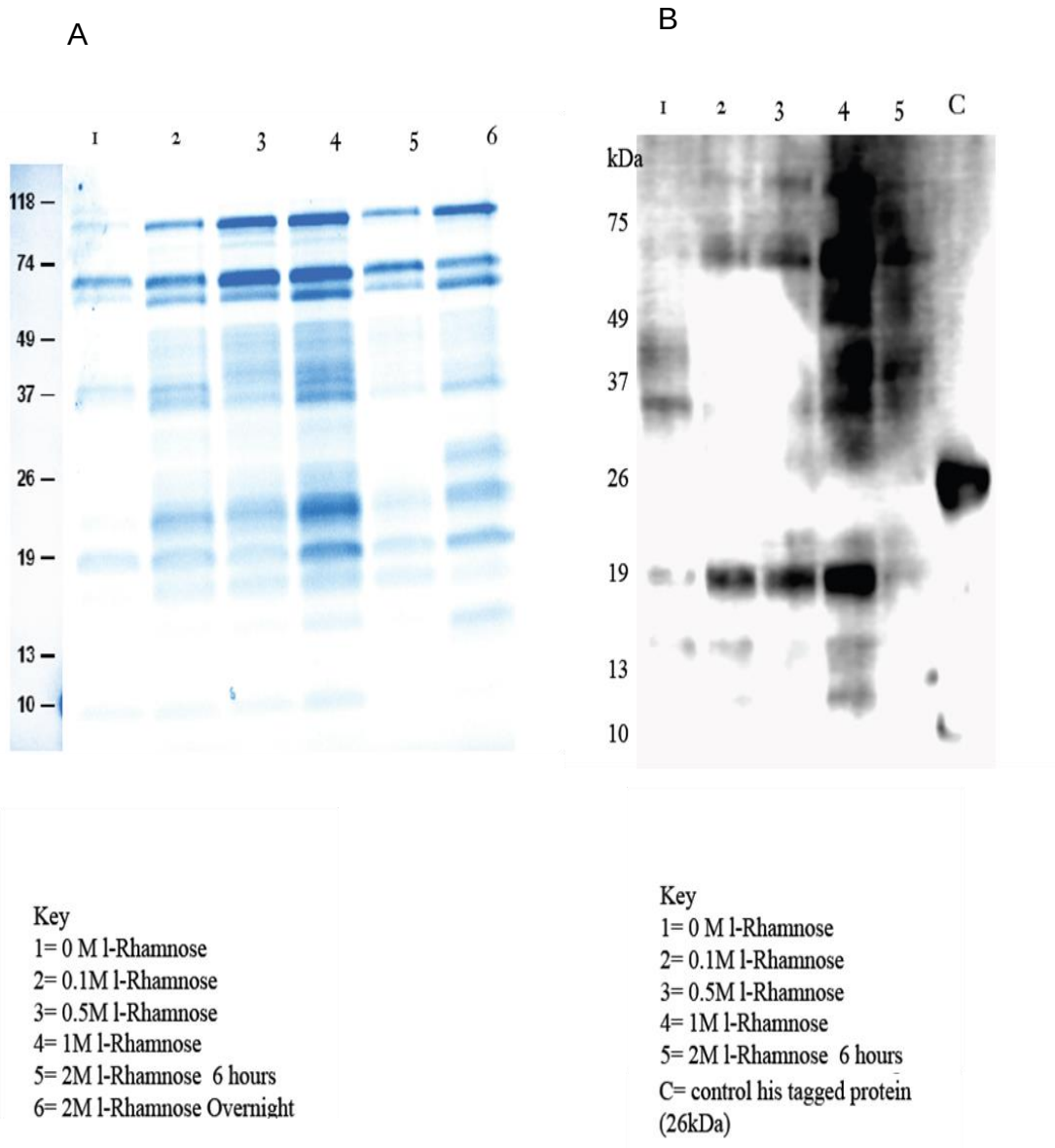
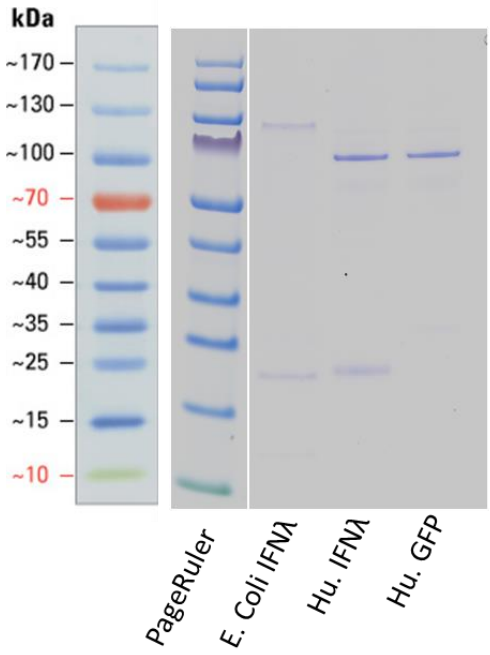


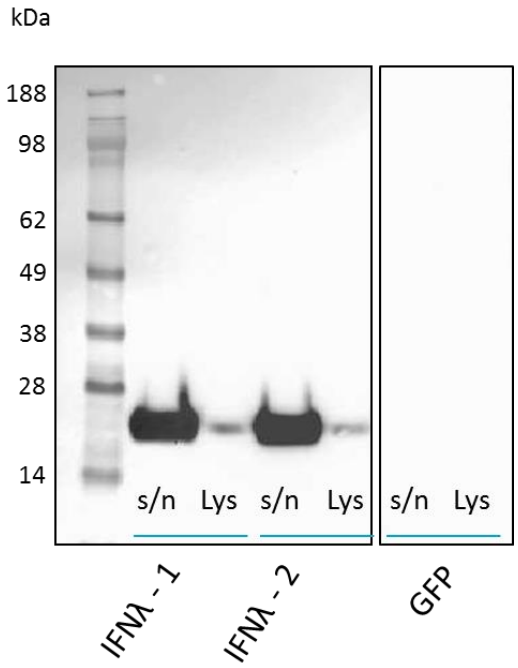
Figure 16: Increasing concentrations of rhamnose increases IFNλ expression using the D864 vector.

The figure shows the SDS-PAGE gel (a) and Western blot (b) analysis of the protein production preparations of the D864 vector when using increasing concentrations of rhamnose to induce protein production. The Western blot is detected using an anti-His tag antibody. Molecular weight markers are run on each to estimate size in kDa.

A



B



- Figure 17: Mammalian expressed IFNλ is secreted to the supernatant during production. (A) The SDS-PAGE gel shows the production of IFNλ by mammalian cells as compared to *E Coli.* Produced recombinant protein. The size is estimated compared to a molecular weight marker. (B) The Western blot analysis show detection of the mammalian express protein via FLAG antibody detection from either the supernatant (s/n) or the lysate (Lys).

The mammalian expressed IFNλ was slightly larger than the E. coli expressed protein possibly due to glycosylation, which is not part of the post translation modification in bacterial cells. When analysed by Western blot, this time using an anti-flag antibody, we were able to detect the mammalian expressed IFNλ predominantly expressed in the supernatant and to a lesser extent with in the cell lysate (figure 17b). In constructing the mammalian vector we including the native signal peptide to increase the likelihood that the protein would be excreted. No binding of the antibody to the control protein GFP was observed.

Two of the E. coli produced proteins were tested in a bioassay, one from the pET22b vector and one from the D864, in addition to the mammalian expressed protein from HEK293T cells. These proteins were compared for their ability to upregulate the ISG, Mx, at a range of different concentrations as shown in figure 18 (0.5, 1 and 2 µg/mL) and compared to a positive control (IFNα). The E. coli expressed proteins had a very limited ability to stimulate Mx expression, with a 4-5 fold increase. On the other hand, the mammalian expressed protein was able to stimulate the Mx gene to increase to greater than 700 fold increase using 2 µg/mL. At the 1 µg/mL concentration the fold increase was approximately half that of the 2 µg/mL, and minimal activity was observed at 0.5 µg/mL. From this observation we decided to continue our studies using the mammalian system.

Previously, the use of biological, as opposed to chemical or synthetic adjuvants, has been hampered by the need for cold chain storage and the ongoing losses of biological activity over time. To address this we aimed to determine the stability of IFNλ at a range of different temperature conditions, including as a lyophilized powder at room temperature. To achieve this we aliquoted a single batch of IFNλ into a number of different vials and stored multiple vials at 4^{o}C, -20^{o}C, -80^{o}C and lyophilized at RT. We then undertook to determine the biological activity of each condition after 1 week, 1 month and 3 months of storage (figure 19). As this assay requires the use of freshly isolated cells, leading to assay to assay variation, we were unable to compare directly between the time points.

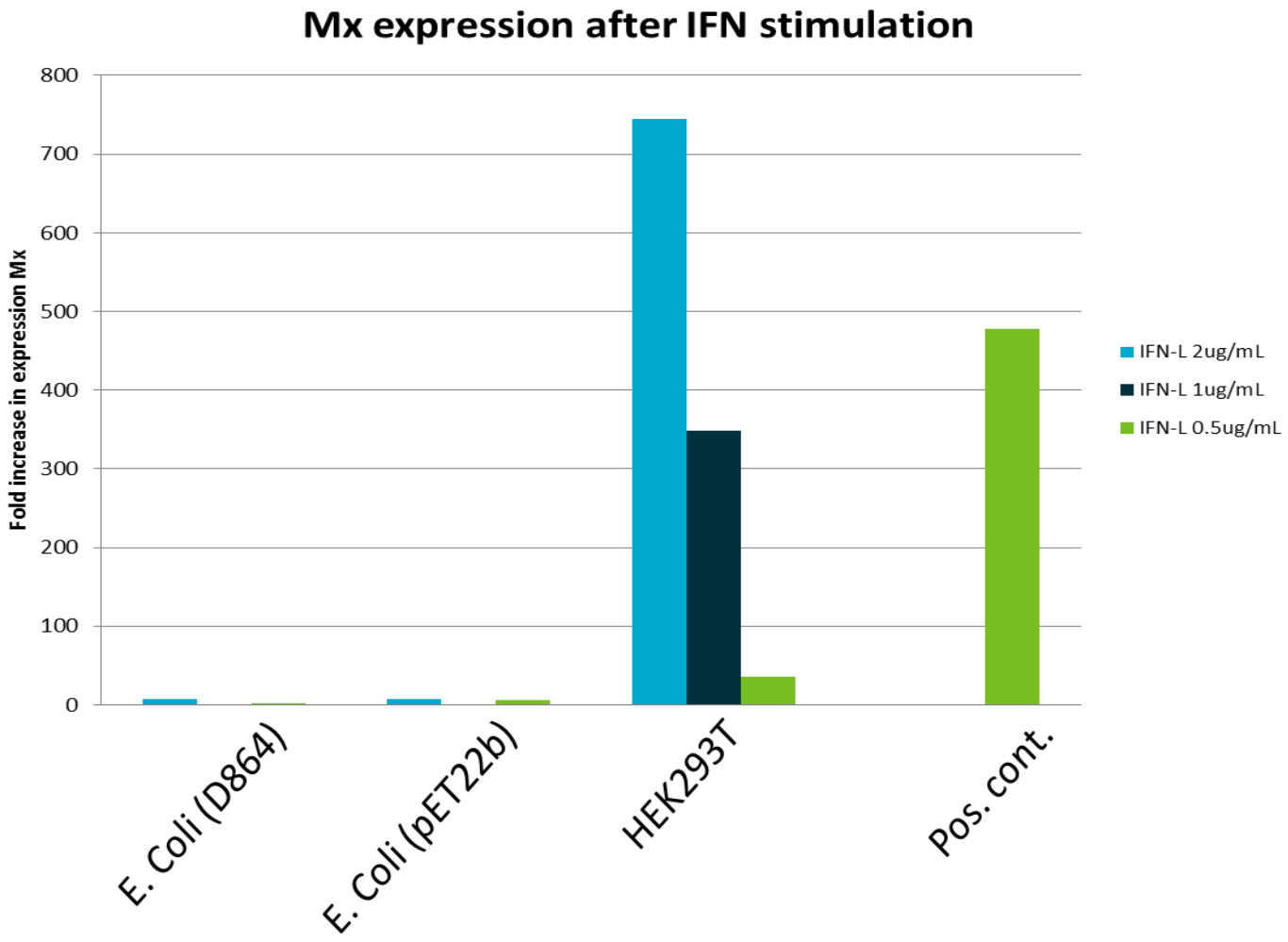


Figure 18: Mammalian expressed IFNλ had a significantly increased bioactivity over E. coli.

The bar graph shows the fold induction of Mx following stimulation with IFNλ from different protein preparations. Various concentrations were used ranging from 0.5 – 2 µg/mL. IFNα at 0.5µg/mL was used as a positive control.

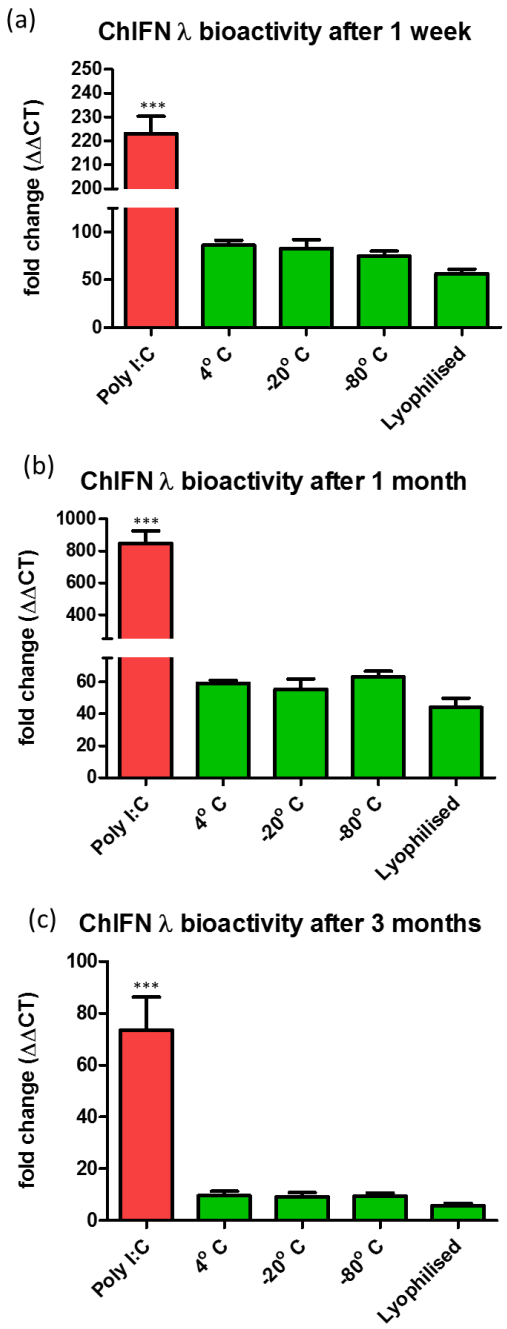


Figure 19: IFN λ maintained its bioactivity when stored in different conditions. IFNλ was tested for its stability after storing at 4° C, -20° C, -80° C and as lyophilized for (a) 1 week, (b) 1 month and (c) 3 months and tested in a CEF bioassay. Poly I:C was used as positive control and unstimulated cells as negative control (not shown). Extracted RNA was converted to cDNA to perform qRT-PCR. GAPDH was used as a housekeeping gene. The values are expressed as mean + SE; n=3, $p<0.05$.

The results showed that at all of the time points tested there was no loss of activity irrespective of the storage temperature and importantly, the biological activity was maintained after lyophilization. There did appear to be a drop in fold induction between 1 month and 3 months.

Having optimized the production, stability and biological activity of IFNλ we then moved to in ovo studies to assess the applicability of IFNλ as an adjuvant for NDV. Our first goal was to assess the applicability as a live bird adjuvant to replace the current adjuvant. In these studies we compared IFNλ as an adjuvant for NDV (LaSota strain) to the current adjuvant Montanide. Additionally, we administered a combination of both adjuvants, given together and separately (Montanide at one site and the IFNλ given at another) to determine if there was a systemic effect of the IFNλ. IFNλ was also given on its own to measure any immune impact only attributed of the adjuvant. We performed these studies on two separate occasions and combined the hemaglutination inhibition titer data of both studies (figure 20). Previous data has shown that an average HI titer of 2^3 is high enough to protect a flock of birds. Montanide was an effective adjuvant, providing an immune titer of 2^8 which well exceeded the 2^3 cut off. IFNλ on its own did not produce a protective response as expected, however, IFNλ with NDV gave an average immune titer greater than 2^4, which passed the protective threshold. Intriguingly, the Montanide combined with IFNλ, either at a single site or separate sites, gave a moderate but not statistically significant increase in HI titer compared to the Montanide alone.

We also performed assays on the immune cells following vaccination to elucidate the impact the IFNλ was having on the immune compartment. The white blood cells within the spleen and blood were counted as a representative measure of cell proliferation, migration or depletion (figure 21). When compared to Montanide alone the addition of IFNλ, either with or without Montanide led to a significant increase in total cellularity in the spleen (figure 21a). Interestingly, the cell counts with in the blood showed the converse result (figure 21b), whereby the IFNλ

containing vaccinations led to a decrease in PMBC total cellularity. This, however, was not true of the IFNλ with Montanide (single site) group. We further investigated the mechanism of action of IFNλ by measuring the specific subsets of cells within the spleen to understand the cause of increased cellularity. We performed flow cytometry on the lymphocyte populations, which are the predominant proportion of the spleen. We showed there was no significant change in the B cells with in the spleen (data not shown), however, the T cells (CD3+) mirrored the pattern seen for both spleen and PBMCs (figure 22). This was also true of both the CD4+ and CD8+ subsets of T cells.

Having shown that IFNλ is an effective adjuvant in vivo we investigated the utility of this adjuvant in ovo. Previous attempts to vaccinate eggs with NDV have resulted in low egg hatch rates and high 1 week mortality in birds that did hatch when a high virus titer was used. When lower virus titers were used the birds did not develop a protective immune response. Our aim was to investigate the used of IFNλ as an immune stimulant to increase the protection from virus during the in ovo stage as well as increase the antibody response to protective levels. We first investigated the role of IFNλ in viral in ovo infections. We therefore compared the impact of viral infection, in this instance influenza, on IFNα and IFNλ at the transcript level. Influenza was chosen due to the short timeframe and known infection kinetics. IFN transcripts in three tissues from the eggs (CAM, brain and muscle) were measured after 24 and 48 h of infection (figure 23). We then performed further in ovo studies to test our hypothesis, that IFNλ may reduce hatchability losses. In these studies we initially chose two doses of NDV (10^1 and 10^3 EID_{50}) as previous data had shown significant losses of eggs at 10^3 EID_{50} and at 10^1 EID_{50} the losses were not as great, however, the birds did not have a protective titer. Our aim was to determine if the addition of IFNλ would either reduce losses and/or increase titer. Therefore, we performed studies in two independent experiments. In our initial study, we observed no reduction in hatch rate nor a change in 1 week mortality when compared to the PBS control. This was unexpected as we had data from Zoetis which had shown a different result.

Together with Zoetis we decided to add a higher dose group (10^6 EID_{50}) the results of which are combined in figure 24 and figure 25.

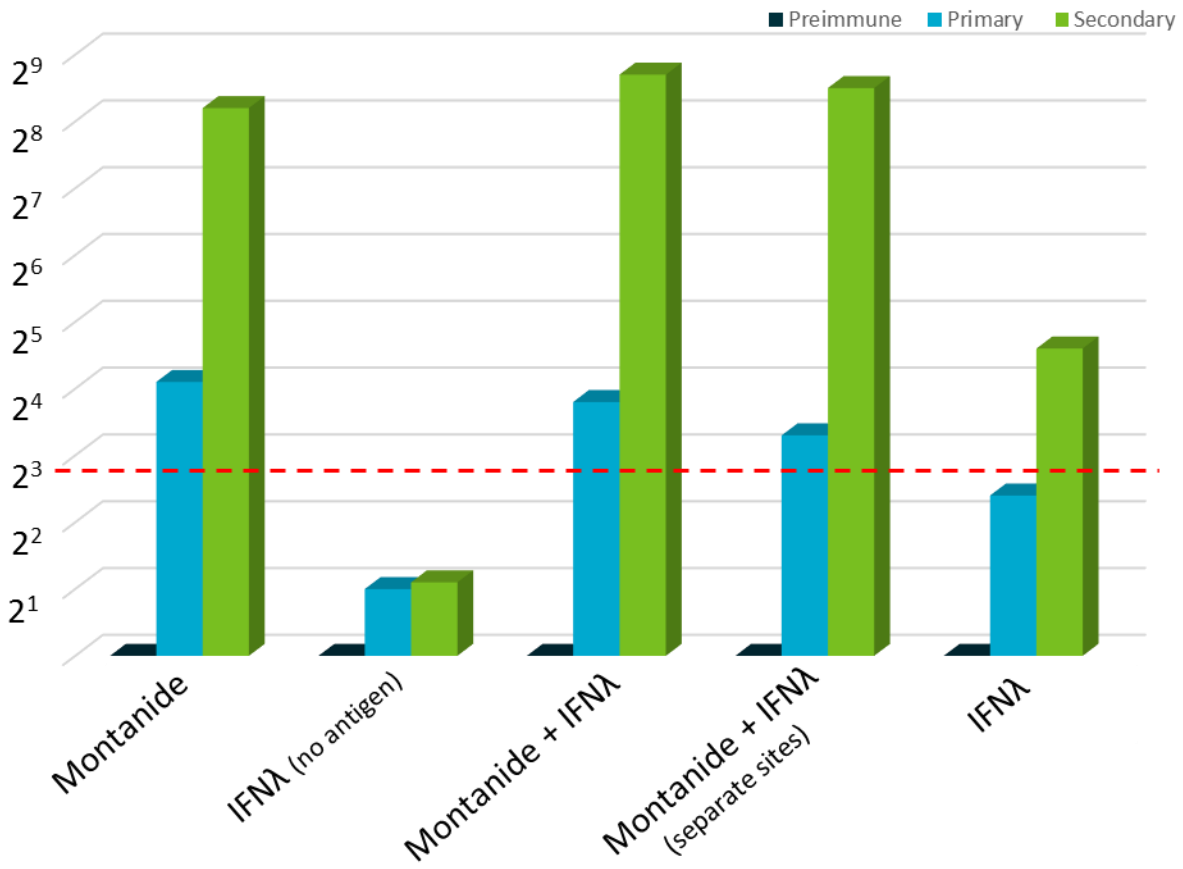


Figure 20: In vivo vaccination with LaSota strain with different adjuvants
Birds vaccinated with the LaSota strain of NDV in combination with the stated adjuvant were bled and serum extracted following the primary and secondary vaccination. The serum was subjected to a HI assay and the log_2 HI titer is presented in the bar graph. (n=10)

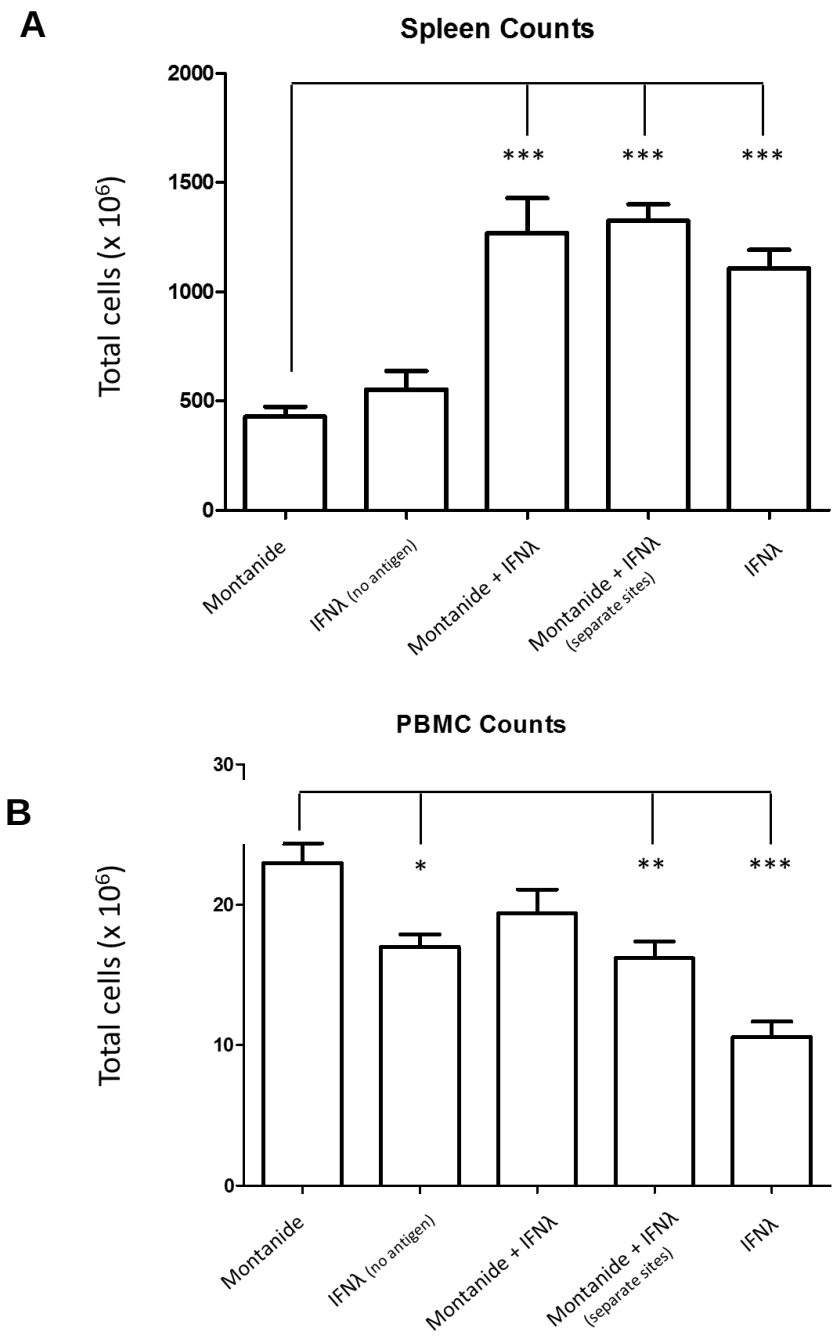


Figure 21: Spleen and blood white blood cell counts after LaSota vaccination.

White blood cells were isolated from spleen (a) and blood (b) using Lymphoprep density gradient isolation and counted for each vaccinated group. Error bars represent SEM (n=10). * = $p<0.05$, ** = $p<0.01$ and *** = $p<0.001$ as analysed by One way Anova with Dunnetts post test.

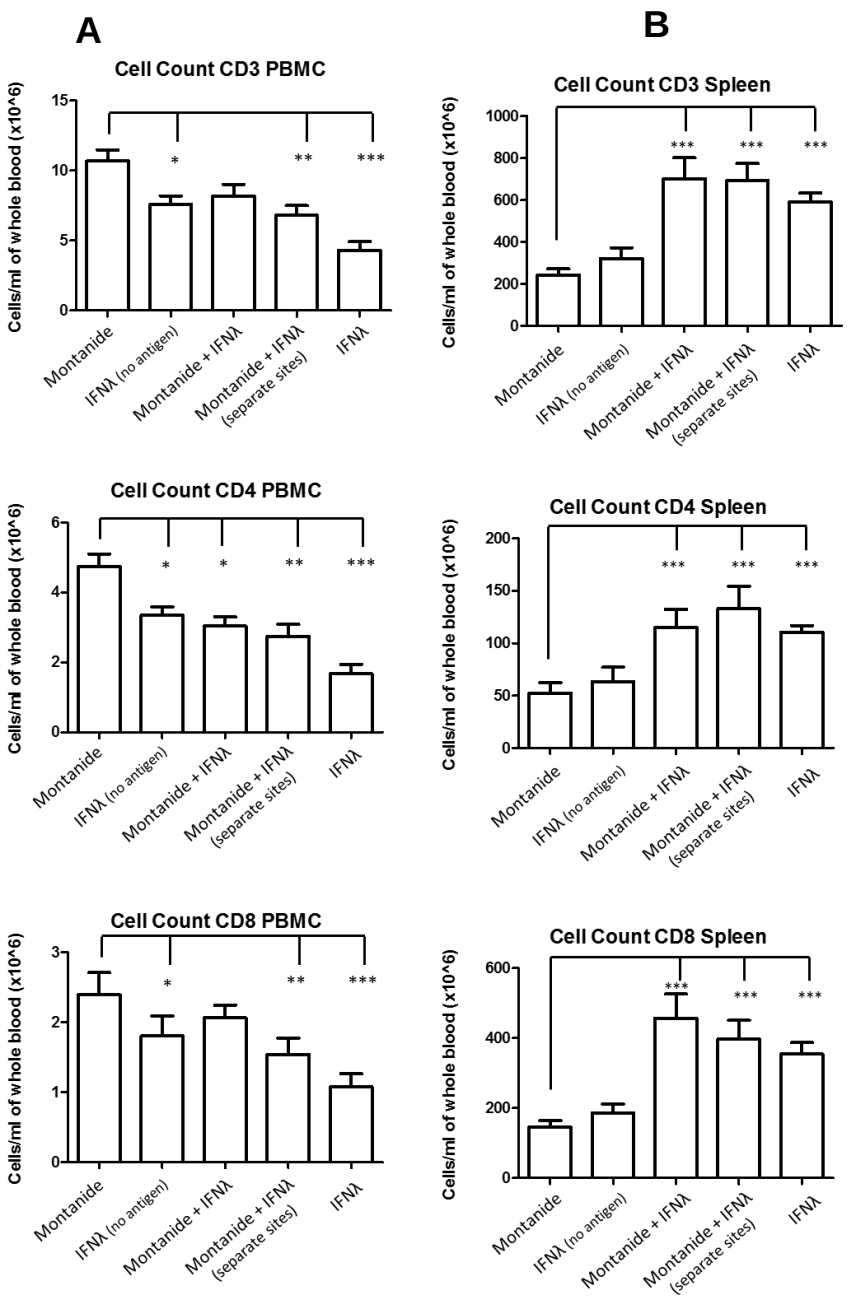


Figure 22: The changes in cellularity observed in spleen and blood are due to changes in T cells.

The bar graphs show flow cytometry analysis of the pan T cell marker CD3 as well T cell subsets CD4 and CD8 in either PBMC (a) or spleen (b). Error bars represent SEM (n=10). * = $p<0.05$, ** = $p<0.01$ and *** = $p<0.001$ as analysed by One way Anova with Dunnetts post test.

When comparing the hatch rate of the different groups we saw no difference ($p<0.05$) between the hatch rate of the PBS control group and any of the other groups, independent of whether IFNλ was added or not (figure 24). There did appear to be a trend toward a reduced hatch in the 10^1 and 10^6 EID_{50} groups. We then combined the numbers of birds that did not hatch with those that did not survive past 7 day (figure 25) and we did see a significant decrease between the control group and the 10^6 EID_{50} group irrespective of the presence of IFNλ, however again we saw no difference in the 10^1 EID_{50} or 10^3 EID_{50} groups. We further investigated the impact of NDV ± IFNλ vaccination in ovo by investigating the impact on the levels of Mx transcript present in the blood cells of vaccinated chicks (figure 26). Surprisingly, the addition of NDV ± IFNλ led to a significant decrease in the level of Mx transcription, independently of the presence of IFNλ. We again performed flow cytometry on cells from the vaccinated birds to determine the effect vaccination and /or IFNλ was having on the cellular compartment of the immune system. We analysed several markers including CD45 (detects all white blood cells), CD3 (detects all T cell), CD25 (detects activated lymphocytes), Bu1 (detects all B cell) and MHCII (detects antigen presenting cells). We again saw an increase in the number of T cells in the 10^6 EID_{50} group and an increase in the total leucocyte proportions in the 10^1 EID_{50} + IFNλ group (figure 27). Upon examination of HI titer from birds in the 10^6 EID_{50} at 2 weeks after vaccination we observed a $>2^3$ HI titer with or without IFNλ (figure 28a). At 8 weeks post vaccination the birds vaccinated with or without IFNλ had a HI titer of $>2^5$ (figure 28b). Interestingly, control birds (vaccinated with PBS) that had been co-housed with vaccinated birds also developed a protective titer by the 8 week time point. This suggested that the virus was still alive and able to be horizontally transmitted to the unvaccinated birds.

To determine the protective ability of the in ovo vaccination we vaccinated birds with 10^3 EID_{50} NDV V4. We observed a different result from our previous experiments as all vaccinated groups showed only 40% of eggs hatched whereas 100% of PBS administered eggs hatched (figure 29a). A further 20% of

birds died within the first 7 days post hatch in the NDV + IFNλ group whilst all birds in the PBS group survived (figure 29b).

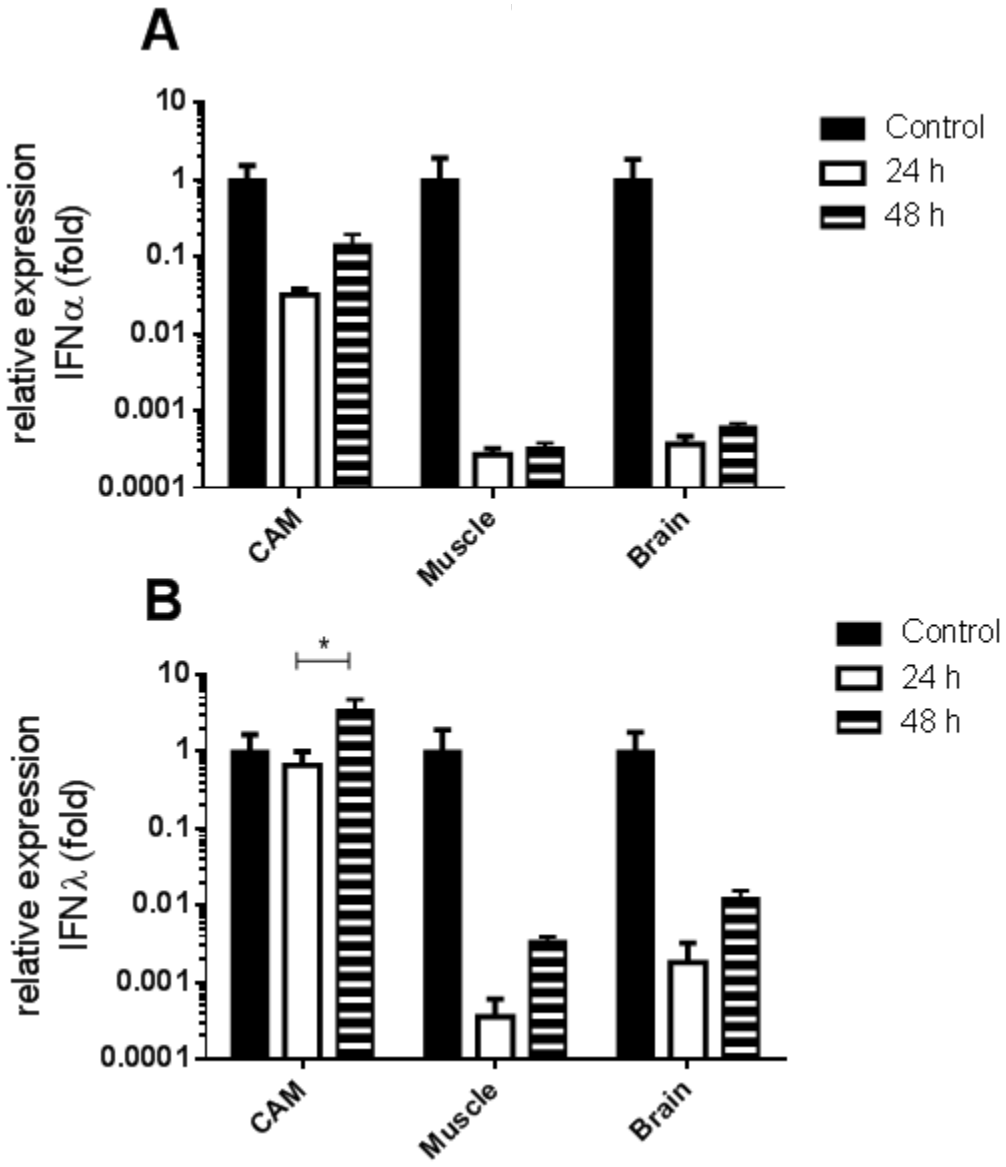


Figure 23: Confirmation of in ovo IFN expression in acute Influenza infection.

Expression of IFNα (A) and IFNλ (B) mRNA in tissues harvested from infected chicken eggs at time points indicated. Bars represent fold change of each stimulated tissue sample compared to uninfected egg of the same age normalized against the housekeeping gene GAPDH. (* p value < 0.05, ** p value < 0.01, *** p value < 0.001 using a two-way ANOVA with Dunnetts multiple comparisons test).

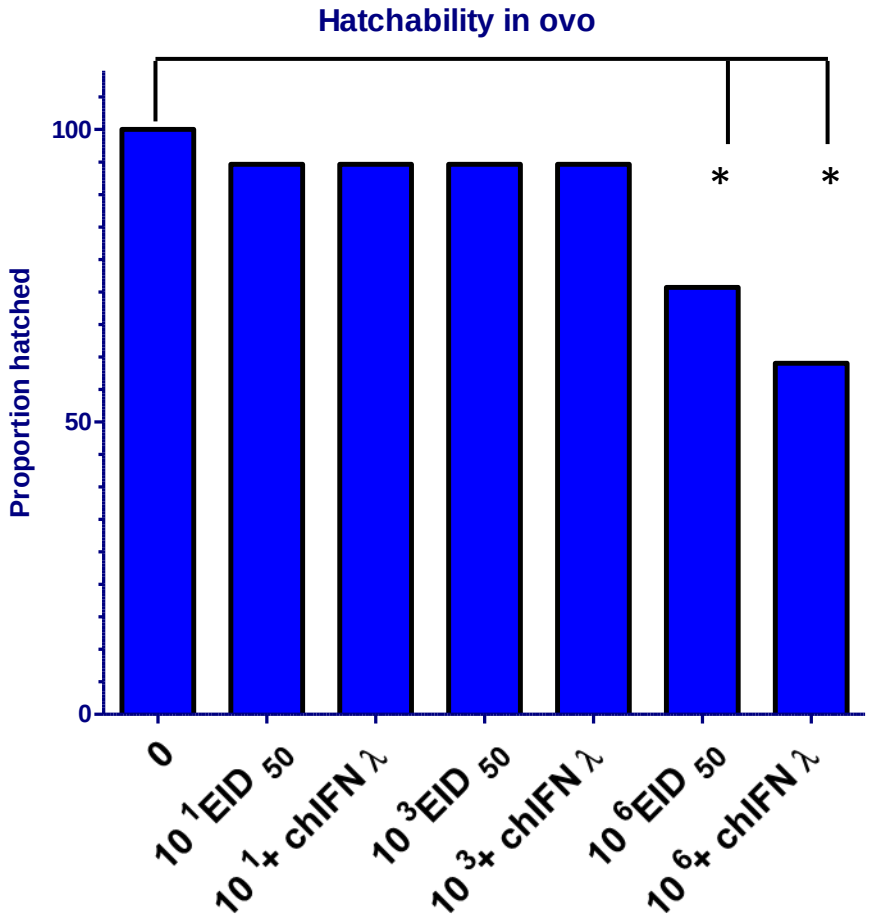


Figure 24: Effect of NDV +/- IFNλ given as vaccine on hatchability of SPF chickens. NDV V4 vaccine was administered at different EID_{50} concentrations (10^1, 10^3 and 10^6) with or without IFN λ at 18 embryonic day. The bars show the proportion of eggs hatched. Negative control group was PBS alone. The values are expressed as mean, n=40.

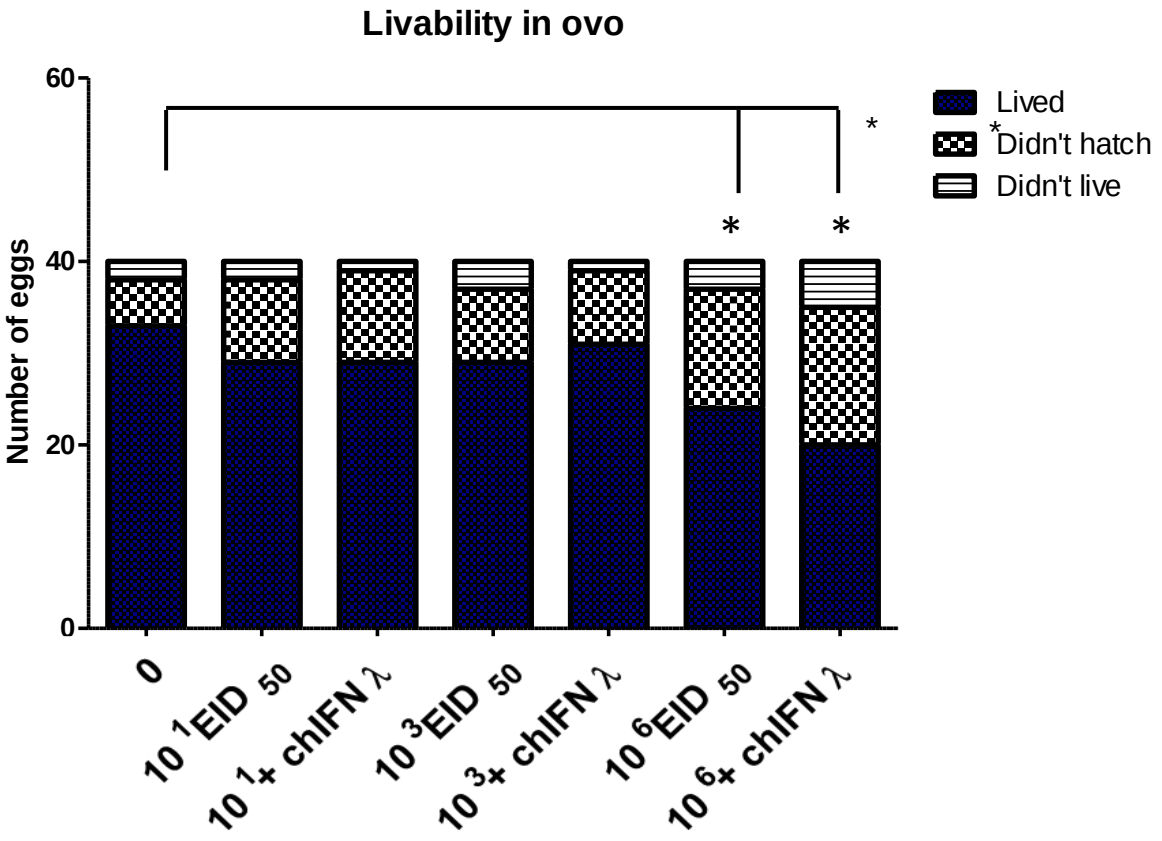


Figure 25: Effect of NDV +/- IFNλ given as vaccine on livability of SPF chickens. NDV vaccine was administered at different EID_{50} concentrations (10^1, 10^3 and 10^6) with or without IFN λ at 18 embryonic day. The bar shows the number of chicks that lived, died and didn't live for 1 week. Negative control group was PBS alone. The values are expressed as mean; n=40.

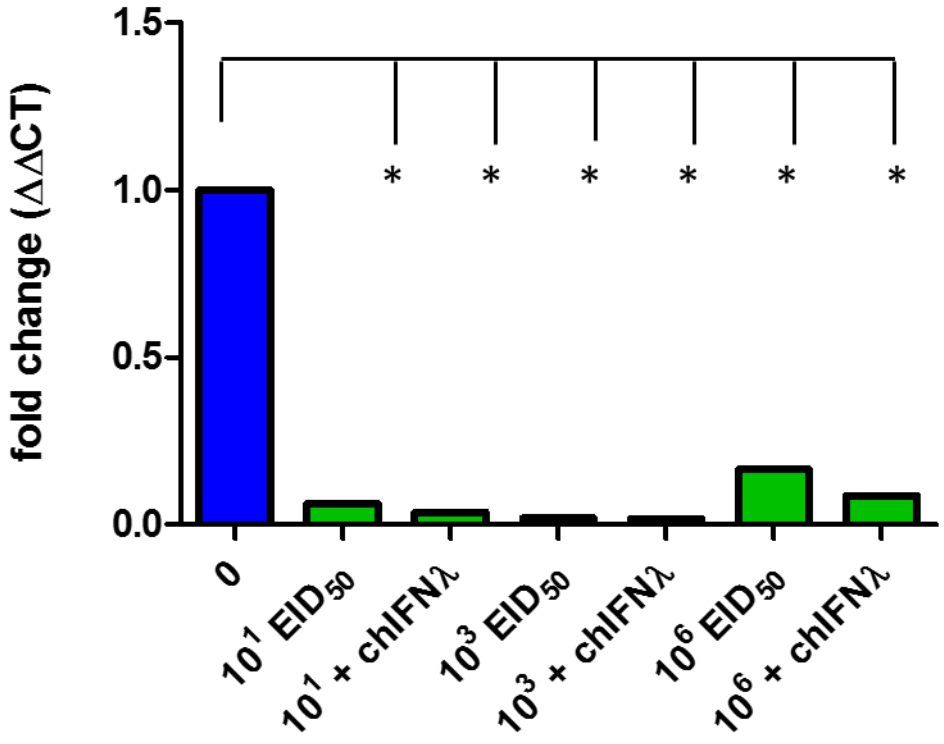


Figure 26: Mx gene expression in chicken whole blood following NDV vaccine +/- IFNλ inoculation. Mx mRNA levels were measured in the chicken whole blood after administering NDV vaccine with/without IFNλ in ovo using qRT-PCR and the expression is shown relative to PBS as control. GAPDH was used as a housekeeping gene to standardize results. The values are expressed as mean + SE; n=40, $p<0.05$.

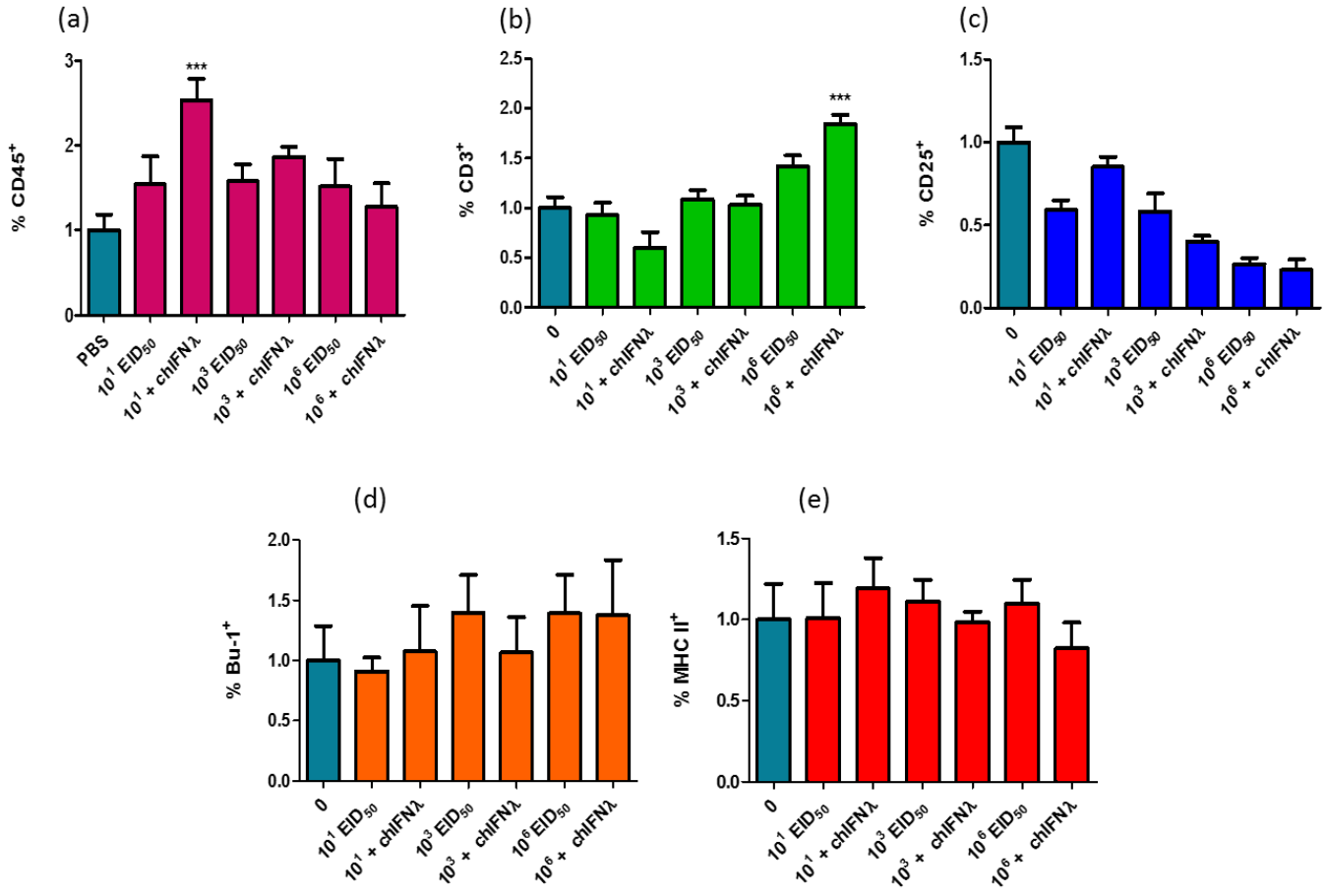


Figure 27: Proportion of subsets of immune cells in the blood of vaccinated chickens

FACS was performed to detect the changes in immune cell population levels in the whole blood of chickens after NDV vaccination w/wo IFNλ. (a) Bu-1$^+$, (b) CD3$^+$, (c) CD25$^+$, (d) CD45$^+$ and (e) MHC II$^+$. The values are expressed as mean + SE; n=40, $p<0.05$.

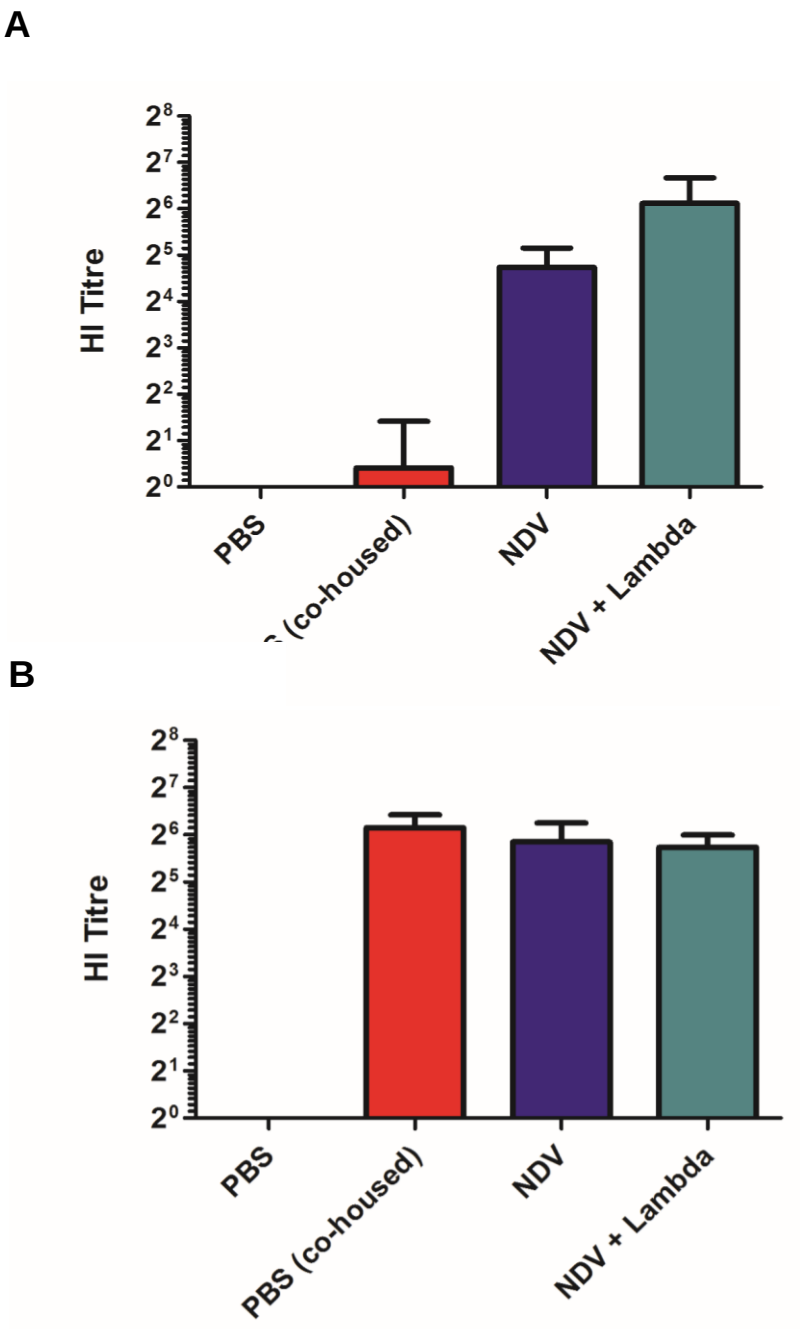


Figure 28: Serum titer post in ovo vaccination

The bar graph shows the HI titer of birds that were vaccinated with either PBS (white), PBS/co-housed (red), NDV (blue) and NDV + IFNλ (green) at either two weeks post vaccination (a) or 8 weeks post vaccination (b). Error bars indicate SEM. n=40.

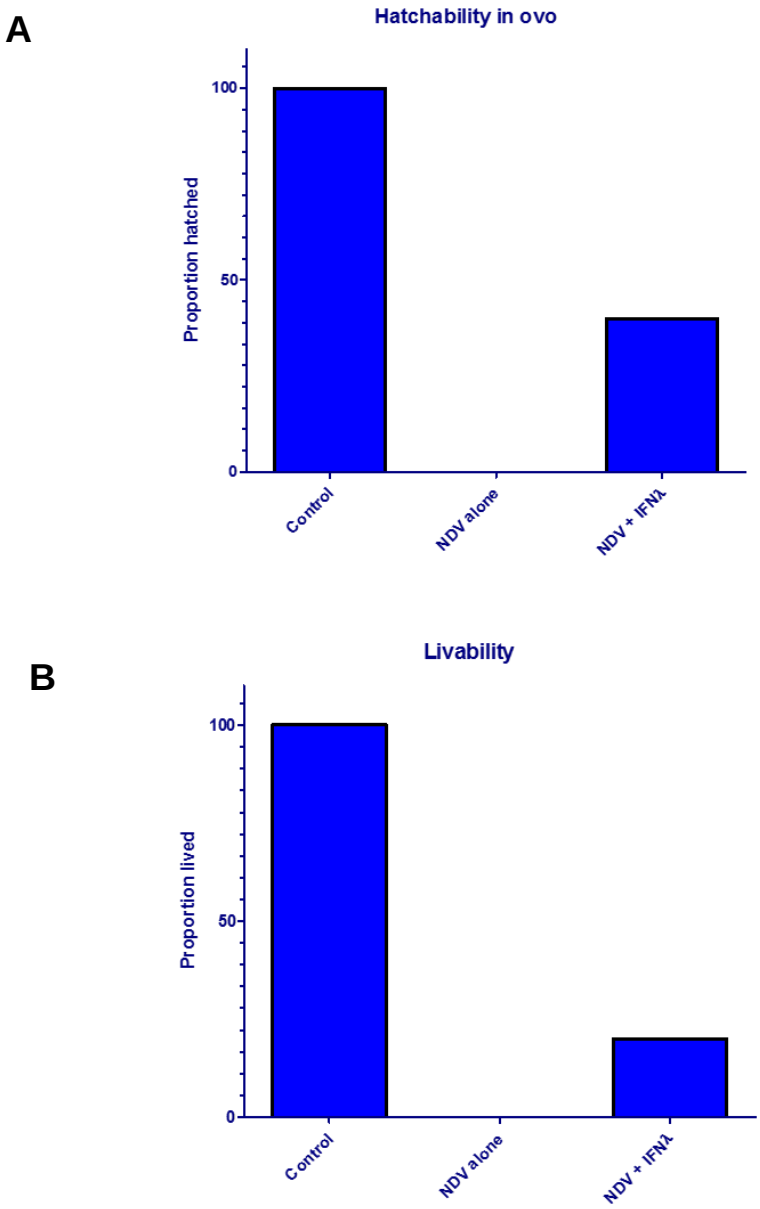


Figure 29: Hatchability and liveability of eggs vaccinated with 10^3 EID_{50} NDV V4

The bar graphs show the proportion of eggs that hatched (a) and lived past 1 week (b) after in ovo vaccination with 10^3 EID_{50} NDV V4 compared to control (PBS) vaccinated eggs and ± IFNλ.

Discussion of Results

The use of recombinant cytokines as adjuvants has attracted considerable attention and their potential role as adjuvants in mammals has been addressed by several studies. As cytokines are regulatory proteins secreted by a variety of cell types that play a crucial role in controlling the immune response, cytokines provide a useful candidate for adjuvant studies. Although IFN was first described in 1950's, it was a further three decades before the first IFN gene was cloned. However, in contrast to this, in recent times a number of chicken cytokines have been identified. An important class of cytokines produced in response to virus are the IFN. This family of cytokines have pronounced and diverse effects which extend to almost every aspect of the immune response. IFN, named for their ability to interfere with virus replication.

An adjuvant is a substance that functions to augment the extent of a specific immune response to a vaccine. The most prevalent adjuvants found in licensed veterinary vaccines are aluminium salts and oil emulsions. Alum adjuvants have been commonly used in many veterinary vaccines, nevertheless, alum is considered a weak adjuvant. Additionally, alum adjuvants have a tendency to induce IgE mediated immune responses and may promote IgE mediated allergic reactions. On the other hand, Freund's complete adjuvant, has limited use because of the induction of side effects and adverse site reactions. It has been shown to induce inflammation and ulceration at the site of injection as well as, fever and hypersensitivity reactions. This is an important point to note when considering an adjuvant for veterinary use as choosing the right adjuvant is important in terms of industry production requirements and critically important in the area of animal welfare. The poultry industry relies on meat quality, therefore, site reactions which lead to carcass damage affects the meat quality resulting in a loss of productivity. Site reactions induced by adjuvants like alum or FCA also lead to animal distress and discomfort, which compromises the welfare of

chickens. As these issues are of major importance to the consumer, it is vital to producers that site reactions be avoided. Poultry producers require effective adjuvants which promote protection and that do not cause any pain or distress in birds when administered with a vaccine. However, the currently used adjuvants do not fulfil these requirements. With this in mind, there is an increased need for effective, better vaccine adjuvants that avoid animal discomfort. Therefore, the objectives of this project was to investigate the hypothesis that IFNλ can enhance the antibody response to both vaccines in chickens when delivered in ovo. As such, we first sought to understand the activity of the IFNλ to develop an experimental vaccination protocol and to optimise the use of IFNλ.

In response to an IFN stimulator, poly I:C, the production of IFNλ transcript was significantly lower than that of IFNα even when multiple concentrations (0.5-50μg/mL) of poly I:C were used. On average, the increases in IFNλ transcript were between 5-10 fold higher than the untreated control, whereas the IFNα transcript increased between 20-1000 fold over the untreated control. This would suggest that IFNλ in the chicken presents a more moderate response than the type I IFNs. Interestingly, the IFNα appeared to increase in magnitude with increasing concentrations of poly I:C up to 10μg/mL, however the IFNλ response plateaued at the 1μg/mL concertation. This would suggest that stimulation of IFNλ production may become saturated more easily than IFNα and may explain a possible mechanism by which the IFNλ system maintains a moderate response to pathogens. Another important feature of the magnitude of the IFN response is the speed at which it is triggered and the duration of the response. Additionally, the specificity of the IFN response to virus and not other stimuli is important in the context of use as an adjuvant. We again used splenocytes stimulated with viral (poly I:C) in addition to bacterial (LPS) mimics to investigate the stimulation of IFNα and IFNλ over the course of 48h (figure 2a and b, respectively). Our data shows that both IFNλ and IFNα are upregulated in response to poly I:C stimulation with the peak of transcript expression being between 1.5 and 3h for both IFNs. Although previously thought to also be involved some bacterial

responses, neither IFN was upregulated in response the bacterial cell wall product LPS.

Having shown that chicken IFNλ acts in a similar manner in response to viral stimulation when compared to mammalian species in terms of magnitude and duration we then sought to investigate the response of chicken immune cells to IFN stimulation as this is the intended adjuvant pathway. Many IFNs are known to generate self-induction and/or positive feedback loops in the IFN system of mammals, where they play an important role in signal amplification, this feature is important to our studies into IFNλ as an adjuvant. Splenocytes from SPF chickens were exposed to recombinant chicken IFNα
Figure (figure 3a) and recombinant chicken IFNλ (figure 3b) and levels of IFN mRNA transcript was quantified. IFNα stimulation was able to induce IFNα but not IFNλ mRNA expression at early time points (0.5-6 hours). IFNλ treatment in contrast upregulated IFNλ much later at the 48 h time point where a 3000-fold increase was observed. IFNλ stimulation caused IFNα mRNA levels to decrease until 24 h, but with a later upregulation to 350-fold at 48 h. This again showed a distinction between the type I and type III IFNs in chickens, where by onset of the IFNλ response is much later than that of IFNα.

The downstream impact of IFN stimulation relies on upregulation of IFN stimulated genes (ISGs). These ISGs control the immune signalling and response and are a critical part of an antiviral vaccine response. We investigated the role of IFNλ and IFNα on the upregulation of chicken ISGs over time. We showed that IFNλ (figure 4a) was able to upregulate two of the tested ISGs (Mx and Viperin) again at a later time point than IFNα (figure 4a) which in addition to Mx and Viperin was able to upregulate two other ISGs, PKR and Zap. The peak of response of the ISG stimulation was again much earlier for IFNα (3-6 h) than the IFNλ. Taken together, these data indicate that as in mammals, chicken IFNλ produces a more moderate immune response than the type I IFN, IFNα. Type I IFNs have previously been shown to induce cellular damage due to an excessive

response. Our data indicates that IFNλ via its more moderate response may not be associated with cell damage.

The next step in the evaluation of IFNλ as a poultry adjuvant requires the optimisation of IFNλ protein production as the cost of goods is a key aspect to its commercial implementation. We aimed to compare and contrast several protein expression systems, including both bacterial and mammalian vector systems and within each of those systems optimise the amount of biologically active protein produced. To do this we first needed to develop a robust in vitro assay to measure the biological activity of the IFNλ protein produced by each system. We investigated four different cell types for their ability to respond to IFNλ stimulation, as splenocytes, which we have previously used, would not provide a consistent cell source throughout these studies (because as a primary cell culture there is a limited supply from the donor), would not be consistently available throughout these studies. We showed that the DF1s, HD11s and CEFs all expressed the transcript for IFNλ receptor, however the quail cell line CEC did not (figure 5). The induction of ISGs, which ultimately generate the enhanced immune response required to have an adjuvant effect may be a suitable measure. Of the two ISGs we have demonstrated to be upregulated by IFNλ (Mx and Viperin) we chose to investigate the suitability of Mx as a measure of biological activity. We showed that in response the IFNλ the DF1, HD11 and CEF cells all had a corresponding upregulation of Mx, whereas the CEC cells did not (figure 7). Interestingly, the highest fold induction of Mx came from the CEFs, with an equivalent response in the DF1 and HD11 cells, despite the highest level of receptor transcript being in the HD11s. We demonstrated, there was a significant increase in Mx transcription in response to IFNλ in CEFs when we compared 500 and 5000 ng/mL concentrations were used but not with 50 ng/mL (figure 8). Indicating that the Mx response is due to a component of the protein production preparation and that the most robust response was seen at 5000 ng/mL. The fold induction observed at 5000 ng/mL was adequate for our bioassay and therefore we did not investigate higher concentrations.

In order to produce the most biologically active product in high quantities at low cost, we undertook a comprehensive optimization of protein production in E. coli and the human HEK293T cells. The most critical aspect of protein production for adjuvant development is the ability for the resulting protein to trigger the intended immune response. Using our previously defined bioassay we compared the biological activity of three different preparations of protein. Two of the E. coli produced proteins were tested, one from the pET22b vector and one from the D864, in addition to the mammalian expressed protein from HEK293T cells. A stark observation was made, showing that the E. coli expressed proteins had a very limited ability to stimulate Mx expression, with a 4-5 fold increase. Conversely, the mammalian expressed protein was able to stimulate the Mx gene to increase to greater than 700 fold increase. This would imply that the mammalian expressed protein had a far higher concentration of biologically active protein compared to the same total mass of E. coli expressed. This is not surprising as the mammalian system is known to have a greater capability for native refolding of proteins, a critical element of generating a function IFN. Although it is possible to artificially refold bacterially expressed proteins, we decided to continue our studies using the mammalian system.

When using cytokines as a therapeutic or as adjuvants in livestock, special consideration must be taken into account with regards to delivery. The poultry industry relies on cost effective methods of vaccine and adjuvant delivery. The use of in ovo vaccination has increased in recent years and may eventually be the preferred means of immunization in poultry, due to reduced labor costs and less animal handling. In ovo administration involves the injection of vaccine/adjuvant into the egg. Many studies have shown a number of vaccines can be safely administered in ovo. Therefore, the development of automated systems for vaccine and adjuvant administration on a commercial scale introduces a new method of delivery of cytokines as vaccine adjuvants for future research. Automated machines can safely inject large numbers of eggs ensuring

accurate dosage and increased consistency. With this in mind, from an industry point of view, cytokines have the possibility to be administered to chickens in ovo along with other vaccines. Having optimized the production, stability and biological activity of IFNλ we then assess the applicability as a live bird adjuvant to replace the current adjuvant, which has a propensity to cause a site reaction that downgrade the meat quality and overall profit of production. In these studies we compared IFNλ as an adjuvant for NDV (LaSota strain) to the current adjuvant Montanide. Furthermore, although adjuvants can be used in a hatch bird situation, it should be remembered that for in ovo injection purposes many of the current adjuvants, such as Montanide, are not suitable and have severe implications for the developing bird. Montanide was an effective adjuvant, and IFNλ with NDV gave an average immune titer greater than 2^4, which passed the protective threshold. In ovo therapeutic administration has been shown to reduce expenses associated with individually injecting newly hatched chickens. However, for this system to be viable within the poultry industry, large scale cytokine production and delivery must be taken into consideration. Cytokines delivered as adjuvants in ovo could reduce the cost of vaccines by increasing the effectiveness of the vaccine, possibly reducing the dose needed and reducing labour costs. Therefore, in ovo administration provides a simple and effective delivery method and can be used to deliver cytokines to study their therapeutic potential in chickens. Having shown that IFNλ is an effective adjuvant in vivo we compared the impact of viral infection, in this instance influenza, on IFNα and IFNλ at the transcript level. Influenza was chosen due to the short timeframe and known infection kinetics. We measured the IFN transcripts in three tissues from the eggs (CAM, brain and muscle) after 24 and 48 h of infection (figure 23). Although it has been well established that viral infection leads to an upregulation of IFN production, we only observed an increase in IFNλ expression in the CAM at 48 h. All other measurements and time points saw a decrease in IFN transcription. This may suggest a possible differential regulation of the IFN system during development of the embryo. This too may play a role in the

previous low hatch, high mortality rates seen with NDV. We then performed further in ovo studies to test that IFNλ may reduce hatchability losses.

In our initial study, we observed no reduction in hatch rate of vaccinated eggs nor a change in 1 week mortality when compared to the PBS control. This was unexpected as we had data from Zoetis which had shown a different result, therefore, with Zoetis we decided to add a higher vaccine dose group (10^6 EID_{50}). When comparing the hatch rate of the different groups we saw no difference ($p<0.05$) between the hatch rate of the PBS control group and any of the other groups, independent of whether IFNλ was added or not (figure 24). There did appear to be a trend toward a reduced hatch in the 10^1 and 10^6 EID_{50} groups. We then combined the numbers of birds that did not hatch with those that did not survive past 7 day (figure 25) and we did see a significant decrease between the control group and the 10^6 EID_{50} group irrespective of the presence of IFNλ, however again we saw no difference in the 10^1 EID_{50} or 10^3 EID_{50} groups. We further investigated the impact of NDV ± IFNλ vaccination in ovo by investigating the impact on the levels of Mx transcript present in the blood cells of vaccinated chicks (figure 26). Surprisingly, the addition of NDV ± IFNλ led to a significant decrease in the level of Mx transcription, independently of the presence of IFNλ. Upon examination of HI titer from birds in the 10^6 EID_{50} we saw that 2 weeks after vaccination we observed a $>2^3$ HI titer with or without IFNλ (figure 28a). At 8 weeks post vaccination the birds vaccinated with or without IFNλ had a HI titer of $>2^5$ (figure 28b). Interestingly, birds vaccinated with PBS that had been co-housed with vaccinated birds also developed a protective titer by the 8 week time point. This suggested that the virus was still alive and able to be horizontally transmitted to the unvaccinated birds.

Our final aim for this study was to determine the protective ability of the in ovo vaccination in a challenge study. We vaccinated birds with 10^3 EID_{50} NDV V4 as from our previous studies the least impact on hatch rate. We included a PBS control group as well as NDV ± IFNλ. Our expectation was that ~90% of eggs

would hatch, however we observed a distinctly different result from our previous studies. Although all eggs pipped (broke the shell) in all groups only 40% of eggs hatched with IFNλ and no eggs hatched in the NDV alone group, despite having 100% hatch in the PBS alone group (figure 29a). A further 20% of birds died within the first 7 days post hatch in the NDV + IFNλ group, leaving only 20% surviving in this group (figure 29b). All birds in the PBS alone group survived past 7 days. The challenge study was abandoned at this point as dictated by our ethics approval due to an inability to perform the study as designed. We undertook additional studies on the vaccinate to ensure no changes had occurred between previous studies where very high proportions of eggs survived and we concluded there was no difference in vaccine titre (data not shown) and that the difference in result may be due to different cohorts of SPF eggs used. Using different cohorts has implications for variations in MHC expression, and as such, the potential immune susceptibility and resistance of a flock. This then has further ramifications for how the birds will respond and will influence the variability of an experimental trial.

IFN-λ, and its observed activity provides an opportunity for alternative IFN therapy in viral management. IFN biology has significantly contributed to our understanding of virus-host interactions and its use to control viral infection in mammals highlights its importance. Although, IFNλ may potentially be effective for overcoming virus infection or as an adjuvant in the chicken, nevertheless, further investigations in regards to their mechanisms of control and the impacts on the host immune responses are required.

Implications

The increasing demand for poultry products has increased the pressure to develop natural alternative strategies to manage infectious diseases in poultry. This, coupled with changes in production approaches, including reduced reliance on antibiotics and a trend towards free-range systems, has put further strain on existing protection strategies. Compounding this is the observation that for many diseases the current vaccines offer less than complete protection and, in the case of newly emerging pathogens, there may no vaccination strategies available.

There is a great emphasis on the development of new approaches to enhance existing vaccine approaches to provide long-term protection and to meet the industry demands of enhanced quality, health and food safety. However, existing adjuvants can have deleterious side-effects, such as inflammation, which may result in the down-grading of meat quality and a subsequent reduction in profits. In addition, the availability of these novel immunomodulatory molecules provides the poultry health area with products will increase Australia's preparedness against disease outbreak risks. This then assists in solving the major challenge of achieving sustainable, ethical poultry production and maintaining a supply of healthy and welfare conscious poultry products.

The results from this project have implications for the Australian poultry industry with regards to new approaches to dealing with infection. By taking a proactive approach to investigation the potential of novel adjuvants to immunoenhance vaccines the Australian poultry industry is boosting their preparedness and seeking alternative approaches. Since the move away from prolonged potential use of antibiotics for growth promotion in poultry, there has been a need to develop adjuvants to augment vaccines to support this approach. This requires characterising the function and activity of these cytokines and a study of their

impact on vaccination to determine their potential and to make a rational choice with regard to their use.

Cytokines delivered as adjuvants in ovo could reduce the cost of vaccines by increasing the effectiveness of the vaccine and reducing labour costs associated with post-hatch vaccination. Furthermore, the use of the natural cytokines as therapeutics or vaccine adjuvants has the potential to enhance Australia's image when it comes to sustainable food production. In addition, the availability of these novel immunomodulatory molecules provides the poultry health area with products will increase Australia's preparedness against disease outbreak risks. This then assists in solving the major challenge of achieving sustainable, ethical poultry production and maintaining a supply of healthy and welfare conscious poultry products.

Recommendations

That the future work in this important area includes:

- Extend proof of concept for IFNs to function as a commercial product and further assess in ovo delivery;
- An assessment of the manipulation of the immune response in an E18 embryo by cytokine use;
- An assessment of the immune system in an E18 embryo to determine its responsiveness to various adjuvants;
- With a commercial partner identify other vaccines that may be more amenable to in ovo vaccination.
- Work closely with a commercial partner to develop further develop in ovo adjuvants to enhance vaccination.

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POULTRY CRC

Plain English Compendium Summary

Sub-Project Title:	Development of IFN-λ as an adjuvant and immune enhancer for in ovo use.
Poultry CRC Sub-Project No.:	1.3.3
Researcher:	Andrew Bean
Organisation:	CSIRO
Phone:	03 5227 5000
Fax:	
Email:	andrew.bean@csiro.au
Sub-Project Overview	
Background	A major deficiency in managing poultry disease control involves the absolute requirement for a closer understanding of the nature of protective immune responses against pathogens and the development of new vaccine strategies. Further to this, currently existing adjuvants for poultry vaccines can have deleterious side-effects, such as inflammation, which may result in the down-grading of meat quality and a subsequent reduction in profits. Therefore, to enhance the use and effectiveness of vaccination, alternative adjuvants must be developed, particularly for in ovo use.
Research	At present there is an emphasis on the development of new therapeutics and the enhancement of existing vaccine approaches to provide long-term protection. Natural immunomodulators have the potential to be developed as novel therapeutics and vaccine adjuvants. Interferon lambda (IFNλ) is a newly identified natural immuno-enhancer that has health benefits such as anti-viral and vaccine adjuvant activity. It has the potential for in ovo use in the poultry industry.
Sub-Project Outcomes	We have shown that IFNλ is a potent adjuvant for poultry and that IFNλ protein increased antibody responses in chickens when administered with an antigen. This remarkable observation supports the concept that IFNλ may have the potential to augment an immune response when used as an adjuvant with a variety of vaccines. Similarly, this boosting effect may have the potential to allow a lower dose of antigen to be used, which may result in more cost effective use of

	vaccines. Our experiments have shown that the in ovo delivery of cytokines can enhance the immune response of the developing chicken and impact on the outcomes of infection. By working with a commercial partner a commercially viable delivery method of these cytokines needs to be developed. This type of approach will be important for the practical use of cytokines**.**
Implications	Within this project area we have identified some important steps in the evaluation of chicken cytokines as alternative adjuvants and therapeutics in disease. The first line of defence against viral infections is mediated by IFNs that are produced rapidly by the infected host. This project has investigated a new approach to vaccine enhancement for the Australian poultry industry. This approach is aimed at enhancing vaccination strategies and underpins a sustainable approach to poultry production. The outcomes of this project provide a catalyst to inventive resolutions to address significant poultry health and welfare issues.
Publications	During this project the research team has been highly successful in generating world class vaccine adjuvant research. The project has graduated 2 PhD students and trained a Post-Doctoral scientist. These researchers will be important future resources for the poultry industry.