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Refining the use of gG as an immune-enhancing vaccine adjuvant
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Executive Summary

This sub-project follows CRC sub-project 1.1.4 (*Testing a novel adjuvant to improve immune responses to Salmonella vaccination*), that investigated the use of recombinant glycoprotein G (gG, a chemokine binding protein) from infectious laryngotracheitis virus as a novel vaccine adjuvant to improve immune responses to *Salmonella* vaccines. In the previous sub-project we found that the addition of low doses of the baculovirus-expressed gG adjuvant (5 or 10 µg) to a live *Salmonella* vaccine, delivered sub-cutaneously or orally, significantly decreased the amount of *Salmonella* positive samples that were detected following challenge, indicating an improved protective immune response to vaccination. Importantly, the decreased proportion of *Salmonella* positive cloacal swabs and intestinal samples detected in the groups that received 5 or 10 µg of gG is likely to reflect levels of *Salmonella* contamination in the environment.

This current sub-project was intended to investigate if similar results can be obtained in a commercial breed of layer birds. This current study also aimed to test if refined forms of the protein adjuvant (i.e. smaller, biologically active forms of the protein adjuvant that can be produced more effectively using large-scale production methods) can result in similar improvements to *Salmonella* vaccine efficacy following oral delivery. These steps are necessary in order to determine if the protein adjuvant is likely to be suitable for commercial use in the future.

To this end full length and refined forms of the recombinant gG were expressed in a baculovirus and bacterial expression systems and purified. The chemokine binding activity of the purified protein was confirmed using ELISA. The protein adjuvant was added in different doses (doses equivalent to 0, 5 or 10 µg of full length protein per bird) to a live *Salmonella* vaccine delivered orally to 6-week old commercial layer chickens. The birds were boosted with identical vaccinations at 12 weeks of age and then challenged orally with live *Salmonella* at 16 weeks of age. Five birds per group were culled one week after challenge. All remaining birds were culled at 19 weeks of age.

After challenge, birds that received the vaccine and adjuvant formulations had a significantly lower proportion of *Salmonella* positive samples over the three weeks post challenge, as detected by using PCR applied to cloacal swabs and/or other tissues, compared with unvaccinated-challenged birds. The birds that received the vaccine alone (no adjuvant) also showed lower proportions of *Salmonella* positive samples over the three weeks after challenge, but this was not significantly different to unvaccinated-challenged birds. All vaccinated groups showed a significantly lower proportion of *Salmonella*-positive cloacal swabs three days after challenge (the time of peak shedding). Interestingly, a significantly smaller proportion of birds that received the peptide adjuvant and vaccine were positive for *Salmonella* in caecal tissues

(an important site of *Salmonella* carriage) compared to birds in the unvaccinated, challenged groups. This difference was not detected in the other vaccinated groups and potentially indicates that the peptide adjuvant may help to protect against *Salmonella* carriage becoming established in caecal tissue, however this needs further examination. Although immunological parameters were assessed in all birds (including examination of antibodies against *Salmonella* in serum and bile, and also lymphocyte sub-populations in blood and tissue) no clear immunological differences relating to the presence of absence of the adjuvant were detected. It is possible that differences may be elucidated as a component of on-going experiments that are characterising cytokine profiles in key tissues following challenge.

The results from this study indicate that the addition of the gG-derived adjuvants, including the peptide adjuvants, to the live, oral *Salmonella* vaccine, enables the birds to develop a level of protection against challenge that results in significantly fewer samples being positive for *Salmonella* over the three weeks following challenge, compared with unvaccinated-challenged birds. When the vaccine was administered alone under the same conditions there was no significant difference compared to unvaccinated-challenged birds over this time period. In this study the impact of the adjuvant was only small but given some of the difficulties associated with *Salmonella* studies such as this one, including oral delivery of the vaccine and adjuvant (most adjuvants are not highly active following oral delivery), the use of commercial birds that were already positive for *Salmonella* before vaccination, and the inability to induce a high level of *Salmonella* shedding and colonisation in the unvaccinated-challenged birds, the results suggest that this adjuvant may have the potential to improve control of *Salmonella*.

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1. Introduction

Salmonellosis is a leading cause of foodborne illness in many countries. Eggs and poultry are considered important vehicles of transmission. Although there are many points along the production chain where control strategies may be implemented to reduce the risk of human disease, risk assessments have concluded that reducing the prevalence of *Salmonella* in chickens results in a directly proportional (one-to-one) or a greater than one-to-one reduction in the expected risk of human illness (Anon, 2002).

Vaccination is one method that can be used to reduce the prevalence of *Salmonella* in chickens. Either live or killed *Salmonella* vaccines are available to increase the resistance of birds against *Salmonella* exposure and decrease shedding. None of the current *Salmonella* vaccines produce sterilising immunity in the intestine and so vaccinated chickens still shed S*almonella* but the level of faecal shedding is reduced with a subsequent reduction in contamination of the eggshell or carcass (Anon, 2004).

The mechanisms of immune protection against *Salmonella* infections in chickens are not well understood and this is a barrier to the rational design of improved *Salmonella* vaccines that could further reduce or eliminate intestinal colonisation and shedding of *Salmonella* in chickens (Anon, 2004; Withanage, 2005). One method of improving vaccines is to boost or enhance immune responses to the vaccine through the use of an adjuvant. Adjuvants can increase immune responses, extend the duration of immunity and enable fewer doses or single-dose applications of vaccines. Common adjuvants used in killed *Salmonella* vaccines are water-in-oil adjuvant and aluminium hydroxide. Despite these adjuvants being in long-term use their mechanisms of action are not well understood. This compounds the difficulties in designing rational and more efficacious *Salmonella* vaccines.

This project aims to use a newly characterised protein with immune-modulating properties as an adjuvant to improve the efficacy of *Salmonella* vaccines in poultry. The goal is to further reduce or eliminate *Salmonella* intestinal colonisation and shedding. Unlike many adjuvants, this protein (glycoprotein G; gG) has defined mechanisms of action and it is known to bind and inactivate chemokines (small cytokines) with subsequent effects on the ensuing innate and adaptive immune responses (Bryant, 2003; Devlin, 2006; Devlin, 2010). Chemokines are known to play important roles in development of immunity to *Salmonella* in chickens (Withanage, 2004; Withanage, 2005; Cheeseman, 2008) and other species (Zhang, 2003). Moreover, the importance of chemokines and chemokine-signalling in immune responses to other infectious agents means that this adjuvant has potential applications in improving other poultry vaccines.

In previous *in vivo* experiments that were undertaken in sub-project 1.1.4, a live attenuated *S*. Typhimurium vaccine (Vaxsafe® ST, Bioproperties) was administered to SPF chickens with birds receiving the live vaccine mixed with different amounts of recombinant gG from infectious laryngotracheitis virus (ILTV) (0, 5, 10 or 20 µg) delivered orally or subcutaneously. At 16 weeks of age, all birds (except one unvaccinated, unchallenged group) were orally challenged with 10^9 colony-forming units (cfu) of live *S*. Typhimurium. Cloacal swabs were collected from each bird one week before challenge, and then at 3, 7 and 14 days after challenge. Shedding of *S*. Typhimurium was measured by quantitative PCR of cloacal swabs. In that experiment we found that the addition of low doses of the gG adjuvant (5 or 10 µg) significantly decreased the number of chickens in which *Salmonella* was detected following challenge, indicating an improved protective immune response to vaccination, as well as likely reflecting levels of *Salmonella* contamination in the environment. These results indicated that the gG molecule may have potential as a vaccine adjuvant to improve the efficacy of *Salmonella* vaccination, however further research was considered to be necessary in order to better characterize this potential.

2. Objectives

This sub-project aimed to confirm if the novel adjuvant (glycoprotein G) was able to improve responses to a live *Salmonella* vaccine in a commercial layer chickens (Hy-line brown) when delivered by the oral route. Additionally, the project aimed to evaluate the effectiveness of a smaller fragment of the protein adjuvant. The focus of the study was on the ability of the adjuvant to decrease shedding of *Salmonella* following challenge. In addition, an assessment of the immune response to the different vaccine/adjuvant combinations was undertaken.

3. Methodology

3.1 Adjuvant design and production

The candidate protein adjuvant (ILTV gG) sequence was examined using THMM and SignalP (Krogh et al. 2001, Petersen et al. 2011), which identified the putative native signal sequence (1 - 25 aa) and the absence of a transmembrane domain. The gG protein sequence downstream of this signal sequence was used to design five peptides of 100 aa in length, with a 50 aa residue overlap between peptides, as well as a full length peptide (minus the native signal sequence) (Figure 1). Codon optimized sequences (for expression in *E. coli*) were cloned into pD444, with the addition of an N' ompA signal sequence and a C' polyhistadine tag, for the purposes of periplasmic purification as previously described (Van de Walle, 2008). The minimal binding region was identified using chemokine-binding ELISAs using these 100 aa peptides.

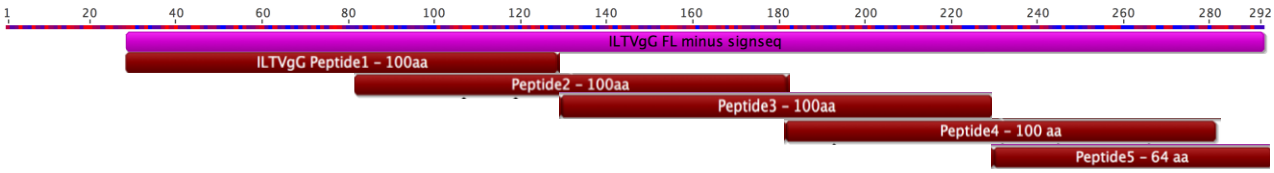


Figure 1. Schematic of *E. coli* expressed peptides (100 aa; red) spanning ILTV gG, as well as the full-length protein sequence, minus the native signal sequence (pink).

Large-scale expression and purification of the protein adjuvant was achieved using a baculovirus expression system as previously described (Devlin, 2010). Purified adjuvant was quantified using a colorimetric assay and cytokine-binding ELISAs were performed to confirm that the purified adjuvant was in an active form, as described previously and below (Baker, 2014). Purified adjuvant was stored at -80 °C in 200 µl aliquots and was added to vaccine inoculums on the day of vaccination.

Large-scale periplasmic and cytoplasmic purification of 100 aa proteins was outsourced to the Protein Production Unit at Monash University. Briefly, gG or fragments of this protein, expressed in the periplasmic space of *E. coli* strain BL21 (DE3) star were purified. Cells were lysed by osmotic lysis techniques or sonication to release his-tagged recombinant protein. Proteins were bound to a nickel affinity resin and eluted nickel affinity proteins were then further isolated using a gel filtration S75 column. Protein purification results are described in Table 1. Purified proteins were quantified using a colorimetric assay and stored in 200 – 400 ul aliquots at -80 °C, until required.

3.2 Chemokine binding assays

Chemokine binding ELISAs were optimized and performed using the *E. coli* expressed peptides, with the SF9-expressed full-length gG as a positive binding control. Briefly, two-fold dilutions of each peptide (20 – 2.5 μg/ml) were coated onto a 96-well microtitre plate and incubated overnight at 4 °C. Wells were blocked with 10 mg/ml bovine serum albumin (BSA) and 5% horse serum in PBS at 37 °C for 2 hrs. Recombinant peptide (mouse CCL5) at 3 μg/ml was added to wells and incubated for 60 mins at room temperature (RT), washed four times and then probed with anti-chemokine antibody (1:100) for 60 min at RT. Wells were washed four times and then an anti-goat antibody was added (1:400) and incubated for 45 mins at RT. Wells were again washed four times and 2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt (ABTS, ThermoFisher) was used as substrate. Wells were read at 405 nM. Diluent used was 5 mg/ml BSA and 2.5% horse sera in PBS with 0.05% Tween 20.

Binding assays on the 25 aa peptides were performed using nuclear magnetic resonance (NMR), as this method is well suited for the detection of interactions between small peptides. This was performed in conjunction with Monash Pharmaceutical Sciences Institute using an Oxford AS600 magnet.

3.3 Preparation of vaccine and challenge inoculum

For vaccination, a vial of Vaxsafe® ST (Batch number STM 152842, Bioproperties, Australia) containing live *Salmonella typhimurium* strain STM-1 was obtained from the manufacturer and the freeze-dried contents of the vial was re-suspended in 300 ml of sterile Marek's diluent (Merial Select INC, Gainesville USA) to make the working concentration equivalent to 1 label dose per bird. Separate aliquots of the suspension were prepared for each dose of the gG adjuvant to be tested and the purified adjuvant was added at the appropriate dose concentrations (a dose equivalent to 0, 5, or 10 μg of full length gG per bird) to the aliquots and mixed by inversion. All samples were then placed on ice until inoculation.

A *Salmonella typhimurium* phage type (PT) 44 isolate was used as a live challenge for the *in vivo* trial. This isolate was from a 2010 case of peritonitis in commercial birds. The isolate was streak plated onto Nutrient Agar (NA) plates containing 10 μg of lincomycin and 10 μg of

neomycin and incubated at 37 °C for 48 hours to confirm purity. Glycerol stocks (30% glycerol) were prepared using these plates and stored at -70 °C in 1 ml aliquots.

To estimate the challenge dose, the density of the suspension culture of *Salmonella typhimurium* PT44, was measured at OD_{600}. The recorded value of 1.0 corresponded to approximately 1.0 x 10^9 colony forming units (cfu)/ml, using previously prepared standard curves generated in our laboratory.

3.4 *In vivo* vaccine trial – study design

Approval to conduct the *in vivo* experiment was granted from the Animal Ethics Committee of the Faculty of Veterinary Science (Ethics #1613850).

The *in vivo* trial used a live *Salmonella* vaccine. A total of 105 six-week-old Hy-line brown layers were randomly divided into seven groups of 15 birds per group, wing-tagged and housed in isolator units or pens with (antibiotic free) food and water provided *ad libitum*. At six weeks birds were either mock-vaccinated with sterile diluent (groups 1 and 2), or were inoculated with the live *Salmonella* vaccine (Vaxsafe® ST) mixed with the equivalent of 0, 5 or 10 μg of the full length gG adjuvant (groups 3 to 7 respectively). All vaccines were delivered orally in a volume of 300 μl. A second identical (booster) vaccination was administered at 12 weeks of age. All birds, except those in group 1 (the negative control group), were orally challenged at 16 weeks of age with 1 x 10^9 cfu/ml of live *Salmonella* in a volume of 300 μl. Five birds per group were culled at 17 weeks of age, and all remaining birds were culled at 19 weeks of age.

During the experiment blood was collected from each bird immediately prior to each vaccination (at 6 and 12 weeks of age), prior to challenge (at 16 weeks of age), one week after challenge (at 17 weeks of age) and prior to euthanasia (at 19 weeks of age) to assess serum antibody levels to *Salmonella* using ELISA, or to assess lymphocyte sub-populations using flow cytometry. To assess bacterial shedding cloacal swabs were collected from each bird prior to vaccination, one week prior to challenge, three days after challenge, one week, two weeks and three weeks after challenge. At necropsy, intestinal sections, bile and small sections of spleen and ceca (containing both caecal tissue and contents) were collected for examination.

3.5 Sample processing and analysis

3.5.1 Detection and quantitation of *Salmonella*-specific antibodies levels in serum and bile by ELISA

An ELISA to assess IgG levels to *S. typhimurium* LPS was optimised and used to test the blood serum samples collected at selected time-points during the *in vivo* experiment. Briefly, wells were coated with 3 μg/ml of purified *S. typhimurium* LPS (Sigma-Aldrich) overnight at 4 °C. Blocking buffer [PBS with 10% BSA and 1% normal sheep serum (NSS)] was then applied to each well at 37 °C for 60 minutes. After blocking, the chicken sera were incubated (1:100) at RT for 60 mins, followed by goat anti-chicken HRP-conjugated antibody (1:2000) for 30 mins at RT. The diluent used for the primary and secondary antibody incubations was 5% BSA and 1% NSS in PBS. All wash steps were performed using PBS with 0.05% Tween 20. The substrate used was ABTS. For some serum samples, at selected time-points, endpoint antibody titres were determined by testing serial 10 fold dilutions of sera.

The ELISA used to assess IgA levels specific to *Salmonella typhimurium* LPS was optimised and used to test bile samples from 7 days after challenge, obtained at post mortem from the gall bladder. The ELISA was performed essentially as described above, except that the secondary antibody utilised a 1:4000 dilution of goat anti-chicken IgA HRP-conjugated antibody.

3.5.2 Detection of *Salmonella* in cloacal swabs and tissue samples by qPCR

The cloacal swabs were added to 800 ul of PBS and vortexed. The Invitrogen DNA/RNA Extraction Kit (Qiagen, Hilden, Germany) and a Corbett X-tractor Gene Robot (Qiagen) were used to extract DNA from 200 μl of each sample. Similarly, 1 cm sections from the ceca and jejunum were collected and added to 800 ul of RLT buffer for DNA extraction. The presence of *Salmonella* DNA was assessed using a qPCR and melting curve analysis (HRM) adapted from Pusterla et al., 2010, which targeted a 132 bp region of the *Salmonella* invasion gene A. For this, 500 nm of each primer (forward: 5'CATTTCTATGTTCGTCATTCCATTACC'3 and reverse: 5'AGGAAACGTTGAAAAACTGAGGATTCT'3) 50 μM of each dNTPs, 2.5 μM MgCl, 8 μM Syto9 (life technologies), 5 x Gotaq buffer (Promega) and 1 unit of Gotaq polymerase enzyme (Promega) were used per reaction. Cycling conditions were 40 cycles of 30 s at 95 °C, 30 s at 60 °C and 15 s at 72 °C. High resolution melt (HRM) analysis by measuring fluorescence within a temperature range of 72 °C to 95 °C and at a ramp of 0.15 °C, and gel electrophoresis of the PCR product in 1.5% agarose was used to confirm amplification of the correct product.

3.5.3 Analysis of lymphocyte sup-populations in blood and tissue by flow cytometry

Peripheral blood mononuclear cells (PBMCs) were prepared from blood samples collected pre and post challenge (16 weeks and 17 weeks of age, respectively) by layering 1 ml of blood onto 1 ml of ficoll-paque and centrifuging at 400 x g for 30 min with no brake. The cells were then washed twice with FACs buffer (PBS with 1% FCS). The cell pellet was resuspended in 1 ml of FACs buffer and 50 μl of cells were stained with mouse anti-CD3 antibodies (1:1500) to detect all T cells, anti-CD4 antibodies (1:1500 dilution) to detect T-helper cells, anti-CD8 antibodies (1:2000 dilution) to detect cytotoxic T cells, or anti-Bu1 antibodies (1:750 dilution) to detect B cells. Antibodies were conjugated to different fluorophores (CD3-AF647, CD4-RPE, CD8-SPRD and Bu1-FITC). Stained cells were resuspended in FACs buffer and cell populations were measured using flow cytometry (FACSVerse, Becton Dickson) and data analysed using FlowJo (FlowJo, LLC).

To process spleen and jejunum sections, the tissue samples (1 cm) were collected into PBS with 1% FCS and single cell suspensions were prepared using a 70 μm nylon mesh cell strainer before filtering the cells through sterile cotton to minimise debris and to bind and reduce red blood cells (RBCs). Approximately 1.0 x 10^6 cells per sample were then stained with the flurochrome-conjugated monoclonal antibodies, Bu1-FITC (1:750 dilution), CD3-AF647 (1:1500), CD4-PE (1:1500 dilution) and CD8-SPRD (1:2000 dilution) and the samples were processed and analysed as described for the PBMCs above.

3.6 Statistical analysis

Antibody titres and flow cytometry results were compared between groups using a 1way ANOVA followed by a Tukey's multiple comparisons post-test. *Salmonella* qPCR results were compared between groups using a Fisher's exact test (two-sided). Statistical significance was set at $p < 0.05$.

4. Adjuvant production

4.1 Purification, quantitation and activity

The various forms of recombinant ILTV gG adjuvant were expressed and purified from baculovirus and *E. coli* bacterial cultures (Tables 1 and 2). Purification of *E. coli* expressed full length ILTV gG protein was attempted both in-house and by outsourcing to the Protein Production Unit (Monash, Clayton). Both cytoplasmic and periplasmic purifications were attempted, as well as testing 6x His- and GST-tagged constructs, but all attempts were unsuccessful. Full length *E. coli* expressed gG was therefore excluded from the vaccine study. Quantitation of final protein adjuvants used in the *in vivo* trial is shown in Table 2 and Figure 2.

Table 1. Protein purification results for *E. coli* expressed 100 aa peptides and full-length (FL) protein adjuvant.

	FL	Peptide 1	Peptide 2	Peptide 3	Peptide 4	Peptide 5
Size (kDa)	29.6		11.4 - 12			8
Periplasmic 1[a]	None	420 µg	480 µg	600 µg	None	320 µg
Periplasmic 2 [a]	None	N/A	N/A	429 µg	None	277 µg
Cytoplasmic	None			2.6 mg	612 µg	2.3 mg

[a]Two attempts were made at periplasmic purifications, results of each are indicated. N/A= not attempted.

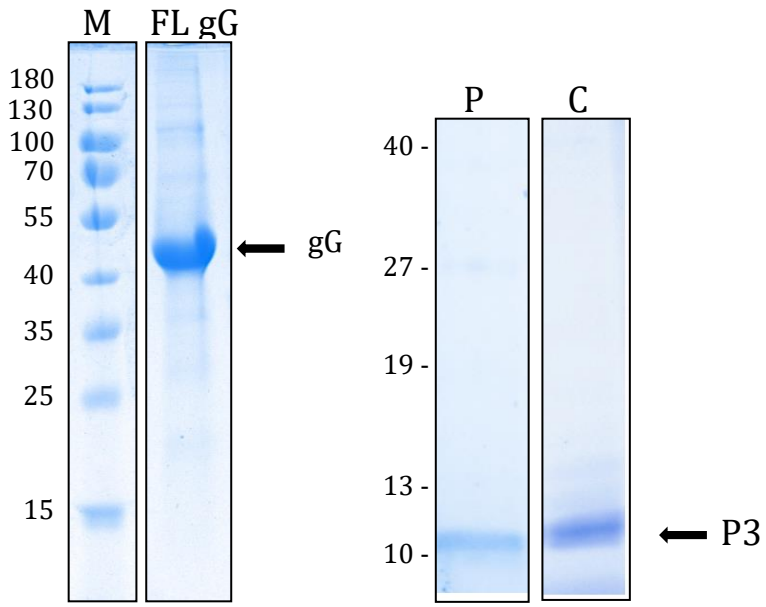
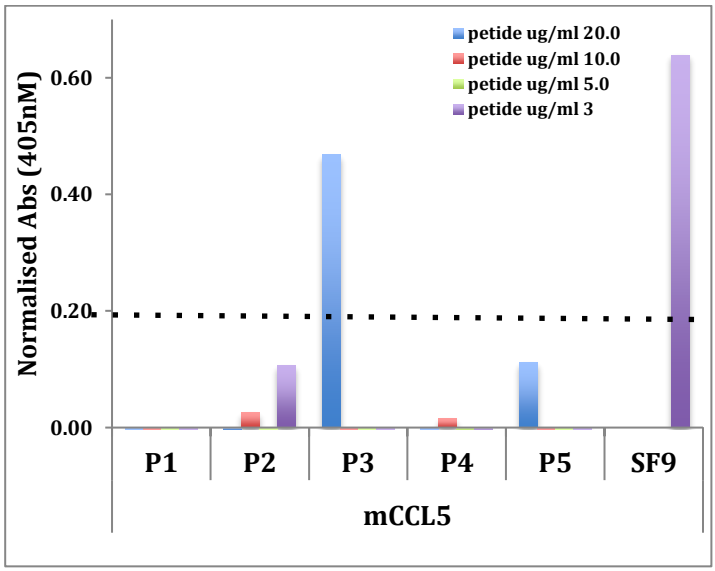


Figure 2. Coomassie brilliant blue stained acrylamide gel of purified protein adjuvants. A) Baculovirus-expressed full length protein adjuvant (FL gG) and B) *E. coli*- expressed periplasmic (P) and cytoplasmic (C) purified Peptide 3 (P3). The dominant band corresponding to ILTV gG is

approximately 45 kDa and the expected molecular weight of P3 is approximately 12 kDa. M = protein marker (PageRuler, Fermentas).

The chemokine-binding activity of the purified recombinant ILTV gG adjuvants was assessed by ELISA using the mouse chemokine CCL5 (Figure 3). Peptide 3 was capable of binding chemokine mCCL5 to a similar level as the positive control (full length baculovirus-expressed protein), indicating that the minimal binding domain was likely present between aa residues 129 - 228. Sequence alignments with the EHV-1 gG aa sequence indicated that the minimal binding domain of EHV-1 gG aligns with aa residues 207 to 246 of ILTV gG (Figure 4). This region overlaps with peptide 3 at residues 207 – 228 and thus a peptide 25 aa in length (peptide 10; P10) to cover this region was designed with the aim of further refining the minimal chemokine-binding domain.

A.



B.

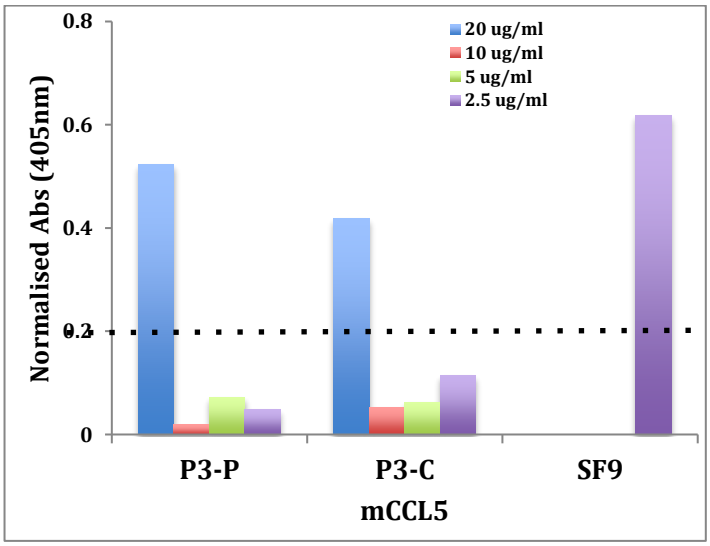


Figure 3. Chemokine binding ELISAs using mouse CCL5 against *E. coli* expressed adjuvant peptides. A) Most peptides tested, with the exception of P4, were periplasmically-derived.

Cytoplasmic P4 was tested, as periplasmic purification was unsuccessful for this peptide. B) Periplasmic (P3-P) and cytoplasmic (P3-C) purifications of P3 were tested and compared to the full-length baculovirus expressed ILTV gG (SF9; 3 μg/ml). Both preparations of P3 were found to bind mCCL5 at 20 μg/ml.

For comparison, small 25 aa peptides spanning the protein sequence surrounding the putative chemokine binding region were also designed for testing chemokine binding activity, with a 5 aa residue overlap (peptides 6 – 11; Figure 4). These peptides were sourced from Genscript. A 12 mg of each peptide was produced. A purity of > 95% was obtained for each peptide. Binding assays were performed using nuclear magnetic resonance (NMR) as this method is more suitable for the detection of interactions between small peptides. No binding to chemokines was detected with any of these smaller (25 aa) peptides. As a result, the minimal length where binding was detected was using the 100 aa P3 peptide, and this was the protein used for *in vivo* studies.

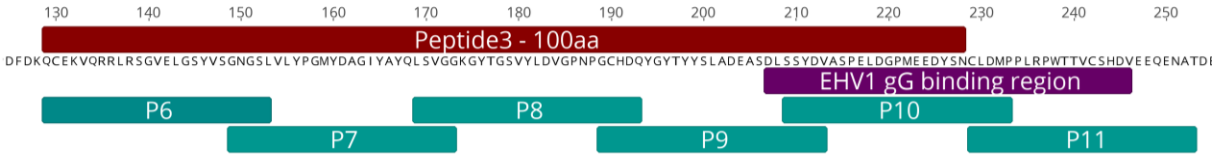


Figure 4. Schematic of synthesized peptides (25 aa; green) spanning the Peptide 3 region of ILTV gG (100 aa; red), as well as the segment of ILTV gG that aligns with the EHV1 gG minimal binding region (40 aa; purple).

As N-linked glycosylation could be required for *in vivo* chemokine binding activity (Van de Walle *et al.,* 2010), and as *E. coli* is incapable of providing such post-translational modifications, a baculovirus-expressed minimal length protein was initially proposed for inclusion in the *in vivo* studies. However, as the target binding-region (aa residues 200 - 250) was found not to include any N-linked glycosylation sites, a baculovirus-expressed minimal length protein was not further pursued.

Table 2. Quantity of protein adjuvants produced for the vaccine study.

Protein	Total quantity produced (mg)
Baculovirus expressed full-length gG	1.4
E. coli Peptide 3 (100 amino acids)	3.6

5. *Salmonella* vaccine study

5.1 Detection and quantitation of *Salmonella*-specific antibodies levels in serum and bile by ELISA

Serum antibody (IgG) levels to *Salmonella* LPS were quantified using ELISA. The results are summarised in Figure 5. Serum antibody levels to *Salmonella* LPS were further quantified in samples collected at 16 weeks of age (immediately before challenge) and at 17 weeks of age (1 week after challenge) by testing 10-fold serial dilutions of the sera by ELISA to determine end-point titres. These results are summarised in Figure 6.

Maximum absorbance values were first observed in week 12 in the groups given 10 μg of FL gG (G10) or no gG (G11). Greater than 50% of vaccinated birds were seropositive (Abs ≥ 0.2) by 16 weeks (Figure 5). By 17 weeks of age (1 week post-challenge) high ELISA absorbance values, at or around the maximum absorbance value, were recorded in serum from birds in all groups, except the negative control group. This slowed seroconversion was consistent with results from the orally vaccinated groups in sub-project 1.1.4.

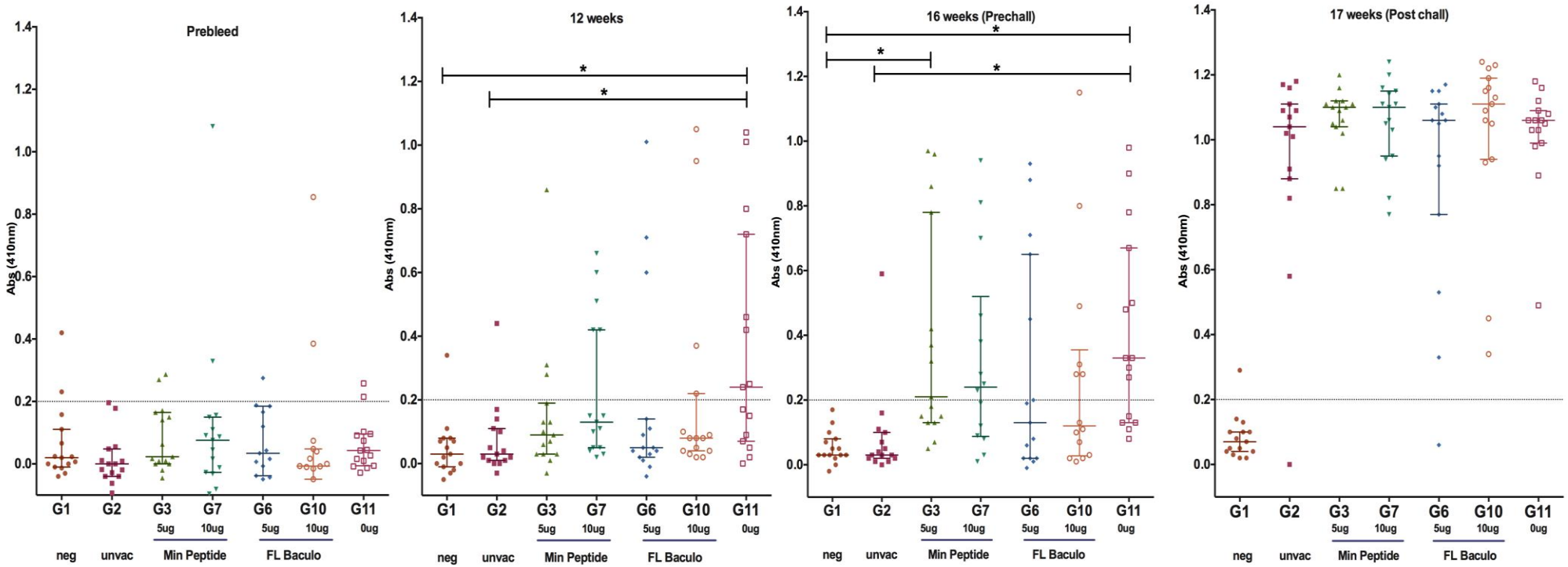


Figure 5. ELISA was used to assess serum antibody (IgG) levels, as indicated by absorbance values, to *Salmonella* LPS in negative control birds (neg), unvaccinated birds (unvac) or birds vaccinated with a live *Salmonella* vaccine (Vaxsafe® ST) in the absence of gG or in the presence of different amounts of ILTV gG (0, 5, 10 μg ILTV gG). Birds were vaccinated at 6 and 12 weeks of age and orally challenged with live *Salmonella* at 16 weeks of age, except for the negative control group. Pre-bleed = prior to first vaccination, performed at 6 weeks of age. Other bleeds were performed at 12, 16, and 17 weeks of age, respectively. Statistically significant differences (1way ANOVA, Tukey's post test) are indicated by asterisks. Scatter dot plots are shown, and error bars indicate median value and interquartile range.

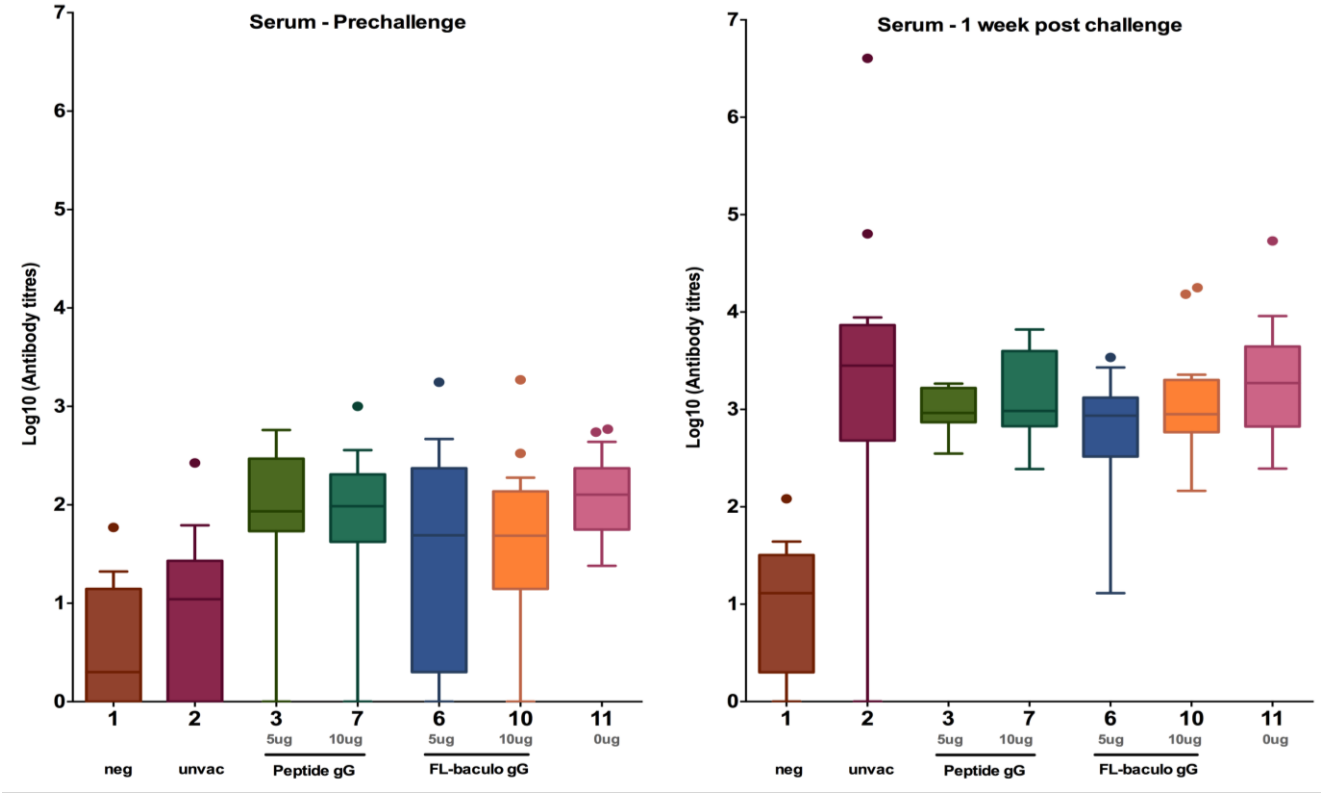


Figure 6. ELISA was used to assess serum antibody (IgG) levels to *Salmonella* LPS in negative control birds (neg), unvaccinated birds (unvac) or birds vaccinated orally with a live *Salmonella* vaccine (Vaxsafe® ST) in the absence of gG or in the presence of different amounts of peptide or full length baculovirus (FL-baculo) expressed ILTV gG (5, 10 μg ILTV gG). Endpoint antibody titers are shown. Birds were vaccinated at 6 and 12 weeks of age and orally challenged with live *Salmonella* at 16 weeks of age, except for the negative control group. Box and whisker plots are shown. No statistically significant differences were observed between vaccination groups at either time-point (1way ANOVA, Tukey's post test). Individual symbols indicate outlier values.

At 16 weeks of age, serum antibody titres were higher in groups of birds that were vaccinated, and by 17 weeks of age, all groups (except negative control) had seroconverted (Figure 6). These results are consistent with those obtained in sub-project 1.1.4. No statistically significant differences were identified between vaccination groups.

Absorbance values obtained from the ELISA used to detect IgA to *Salmonella* LPS in bile were similar between all *Salmonella* vaccinated groups at 17 weeks of age (Figure 7), and though lower levels were detected in the bile from birds vaccinated with 5 μg of FL gG this was not statistically significant.

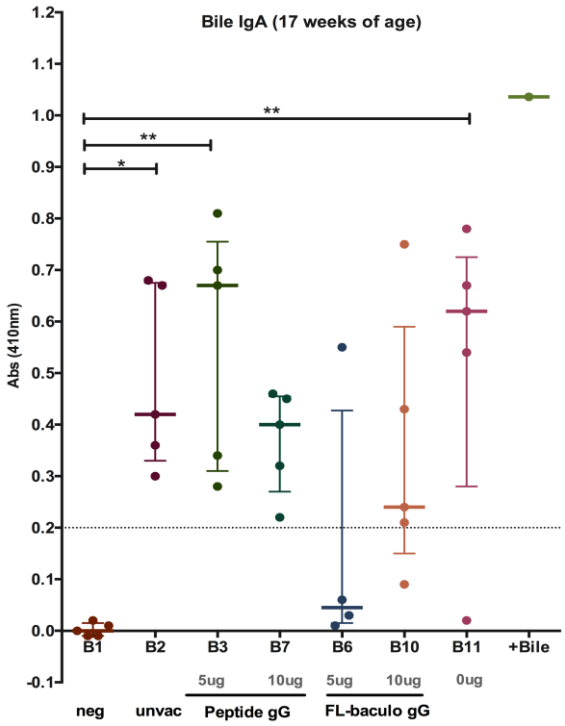


Figure 7. ELISA detection of IgA to *Salmonella* LPS in bile samples collected at one week post challenge. Neg = negative control, unvac = unvaccinated, ILTV gG at 0, 5 or 10 μg dose = indicated dose of gG + *Salmonella* vaccine. The effect of gG on IgA in bile one week after challenge is inconclusive due to the low sample size but appears to be reduced in the 5 μg baculovirus FL gG, compared to the vaccinated groups that received no adjuvant or that received gG peptide. Statistically significant differences (1way ANOVA, Tukey's post test) are indicated by asterisks. No statistically significant differences were observed between vaccination groups. Scatter dot plots are shown, and error bars indicate median value and interquartile range.

5.2 Detection of *Salmonella* in tissue and cloacal swabs by qPCR

Bacterial shedding was assessed using a *Salmonella*-specific qPCR that was applied to cloacal swabs collected from each bird pre-vaccination (6 weeks old), pre-challenge (15 weeks old) and 3, 7, 14 and 21 days after challenge, and also to individual tissue samples (ceca and intestine) collected at necropsy 7 and 21 days after challenge.

Table 3 summarises the results from PCR detection of *Salmonella* DNA in swabs and tissue samples after challenge. Detection of *Salmonella* DNA in cloacal swabs over time is shown in Figure 8. The PCR results for individual birds post-challenge are summarized in Figure 9.

Table 3: Detection of *Salmonella* by qPCR following challenge

Group	*Salmonella* positive cloacal swabs[a]	*Salmonella* positive caecum samples[b]	Total *Salmonella* positive samples[c]
Controls			
Non-vacc/ challenged	25/50	12/15	38/70
Non-vacc/ Non-challenged	5/50*	0/15*	5/70*
Vaccine only			
Vaxsafe® ST only	17/50	9/15	27/70
Peptide adjuvant + vaccine			
ILTV 5 μg	14/50*	4/15*	18/70*
ILTV 10 μg	15/50	4/15*	20/70*
Full length adjuvant + vaccine			
ILTV 5 μg	13/50*	10/15	25/70*
ILTV 10 μg	12/50*	9/15	24/70*

[a] The number *Salmonella* positive cloacal swabs following challenge (3, 7, 14 and 21 days after challenge); [b] The number of caecum samples that were *Salmonella* positive after challenge (7 and 21 days after challenge) [c] The number of total samples that were *Salmonella* positive after challenge (all samples at all time points). Asterisks indicate statistically significant differences when compared to the unvaccinated group (Fisher's exact test, two-sided). A significantly lower proportion of *Salmonella* positive cloacal swabs were also detected 3 days after challenge in all vaccinated groups, compared to the non-vaccinated challenged group (Fisher's exact test, two-sided)

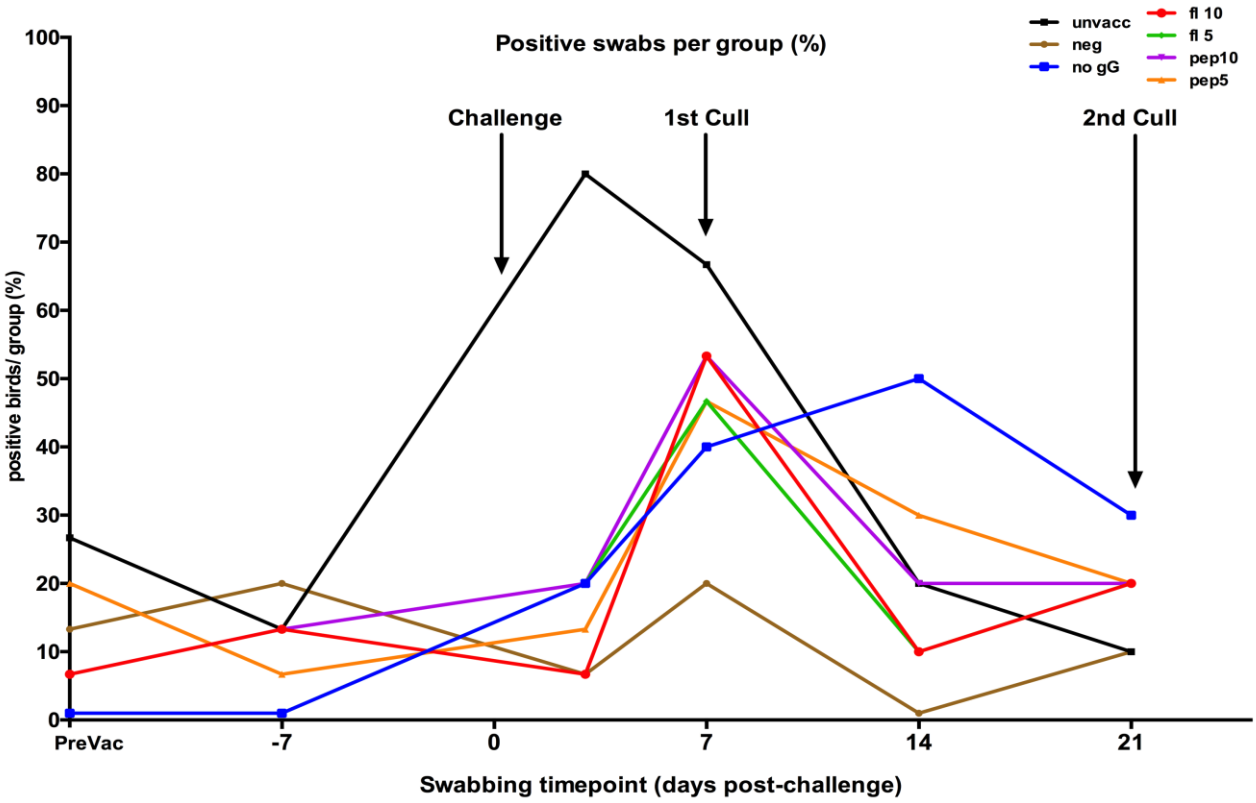


Figure 8. qPCR was used to assess *Salmonella* shedding in cloacal swabs before vaccination (6 weeks of age), prior to challenge (15 weeks of age) and then 3, 7, 14 and 21 days after challenge. The proportions of *Salmonella* positive birds are shown for each time point. Vaccination was performed with Vaxsafe® ST ± the indicated quantity of gG. Commercial Hy-line brown layers were orally vaccinated at 6 and 12 weeks of age and orally challenged with live *Salmonella* at 16 weeks of age (n = 15 birds until 7 days post challenge, n = 10 birds from 14 to 21 days).

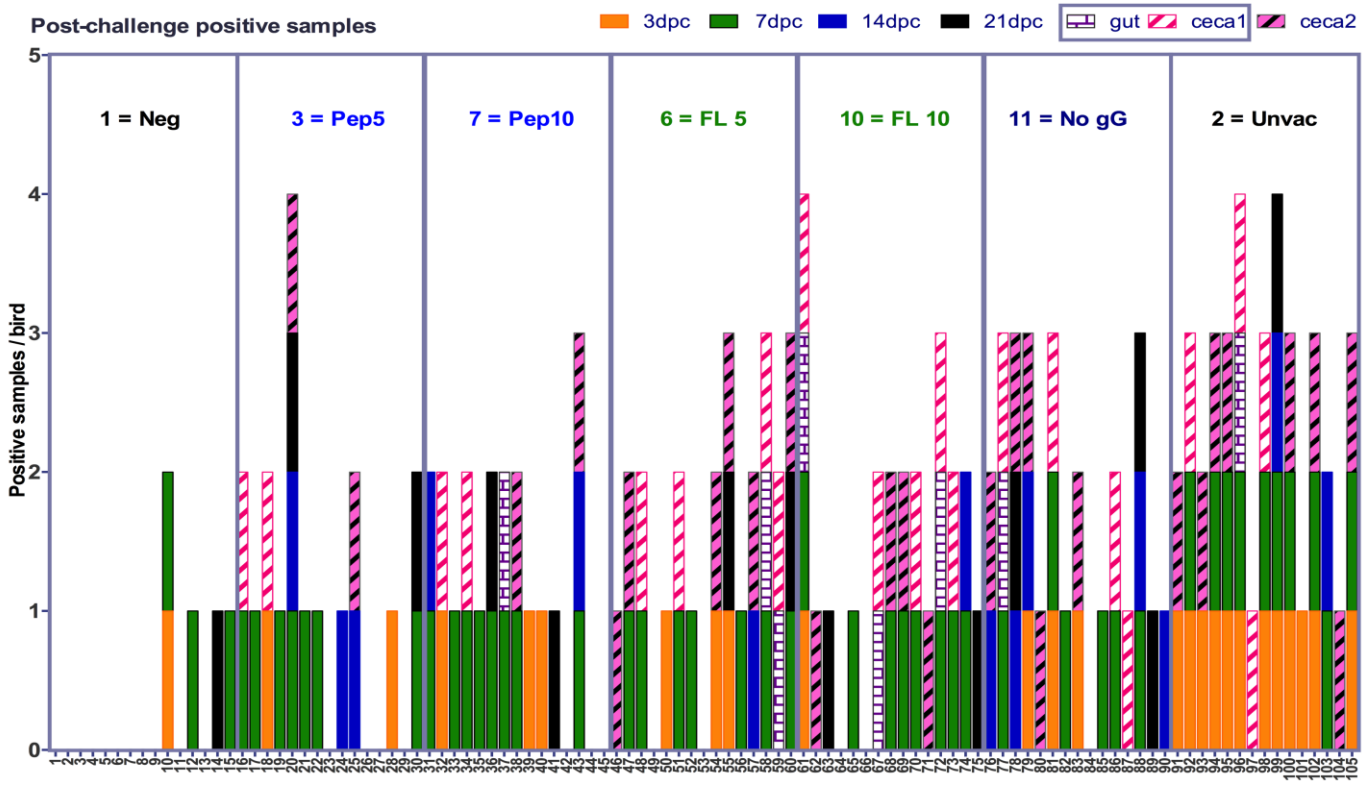


Figure 9. qPCR was used to assess *Salmonella* shedding in cloacal swabs at 3, 7, 14 and 21 days post challenge (dpc) and in ceca and intestine samples collected at 7 and 21 dpc. The number and type of positive swabs are displayed for individual birds in each of the experimental groups.

All birds in the unvaccinated-challenged group were positive for the presence of *Salmonella* on at least one occasion following challenge. Positive samples were also detected in the unvaccinated and unchallenged group, including before the first vaccination time point. This is likely to be a result of exposure to *Salmonella* at the time of hatch (as birds were brought to our facilities as day old chicks). Only four birds in the negative control group were positive for the presence of *Salmonella* DNA post-challenge. No *Salmonella* DNA was detected in any of the tissues tested from the negative control group at any time point.

5.3 Analysis of lymphocyte sub-populations in blood and tissue by flow cytometry

Our analysis of lymphocyte sub-populations used cell surface markers to identify different cell types that may play a role in generating a protective immune responses to *Salmonella.* These cell surface markers and the cells they are predicted to identify are summarised in Table 4.

Table 4: Summary of cell markers and predicted cell sub-populations examined in this study

Cell markers	Cell type
Bu1+	Mature B cells
CD3+	T cells
T Cell subsets	
CD3+CD4+CD8+	T Helper (αβ T cells)
CD3+CD4+CD8-	Monocytes → Macrophage/Dendritic cells
CD3+CD4-CD8+	Cytotoxic T cells
CD3+CD4-CD8-	γδ T cells (unknown function in chickens)
Other subsets	
CD3-CD4+CD8+	Unknown (potential pulmonary infection-associated cells)
CD3-CD4+CD8-	Uknown (potential lymphoid tissue inducer cells)
CD3-CD4-CD8+	Natural killer cells

The results from the analysis of lymphocyte sub-populations in blood at three key time-points (pre-challenge, 1 week post-challenge and 3 weeks post-challenge) are shown in Figures 10 – 12, respectively. Analysis of lymphocyte sub-populations in spleen and intestinal samples (n = 5) collected at 1 week post-challenge showed no statistical differences between groups for any cell sub-population tested (results not shown).

Although a number of statistically significant differences between groups were observed (Figures 10 – 12), there were only a small number of significant differences that were associated with the absence of the gG adjuvant, or its presence at different doses. In general, there was a large degree of variation in lymphocyte sub-populations within experimental groups, possibly reflecting the origin of the birds (commercial layers, rather than SPF chickens), which may have obscured the detection of significant differences between experimental groups. For those results showing a large degree of variation in lymphocyte sub-populations, the data in Figures 10 – 12 is presented using a logarithmic scale, rather than a linear scale. No significant differences were detected

between experimental groups for any of these lymphocyte sub-populations with a high degree of within-group variation.

Before challenge, peripheral blood lymphocytes from the group inoculated with 5 μg of gG peptide (G3) was found to contain statistically lower frequencies of CD3-CD8+ cells (possibly natural killer cells) compared with the group that received the vaccine only (Figure 10). No other differences were observed at this time-point.

One week after challenge, unvaccinated birds showed statistically higher frequencies of CD3-CD8+ cells compared to birds vaccinated with the full-length gG (G6 and G10) or no gG (G11). Increased B cells were also observed at this time in the unvaccinated group, particularly when compared to the groups inoculated with 5 μg of gG (both full length and peptide forms, G3 and G6). An increased frequency of the CD3+CD4-CD8- T cell subset was observed in G6, statistically higher than in the unvaccinated and 10 μg gG peptide (G7) groups (Figure 11). No other differences were observed at this time-point. The function of these cells are unknown in chickens, but they may have an overlapping function with natural killer cells (Gobel *et al*, 2001)

Three weeks after challenge, differences in the CD3-CD8+ subset of peripheral blood lymphocytes were again observed between vaccination groups (Figure 12). Specifically, an increased frequency in this subset of cells was observed in the group that received 10 μg of full-length gG (G10), particularly when compared to 5 μg (G6) and no gG (G11). Additionally, birds inoculated with 5 μg of gG peptide displayed lower frequencies of B cells in their peripheral blood lymphocytes at this time-point when compared to the unvaccinated and no gG groups (G2 and G11, respectively). No other differences were observed at this time-point.

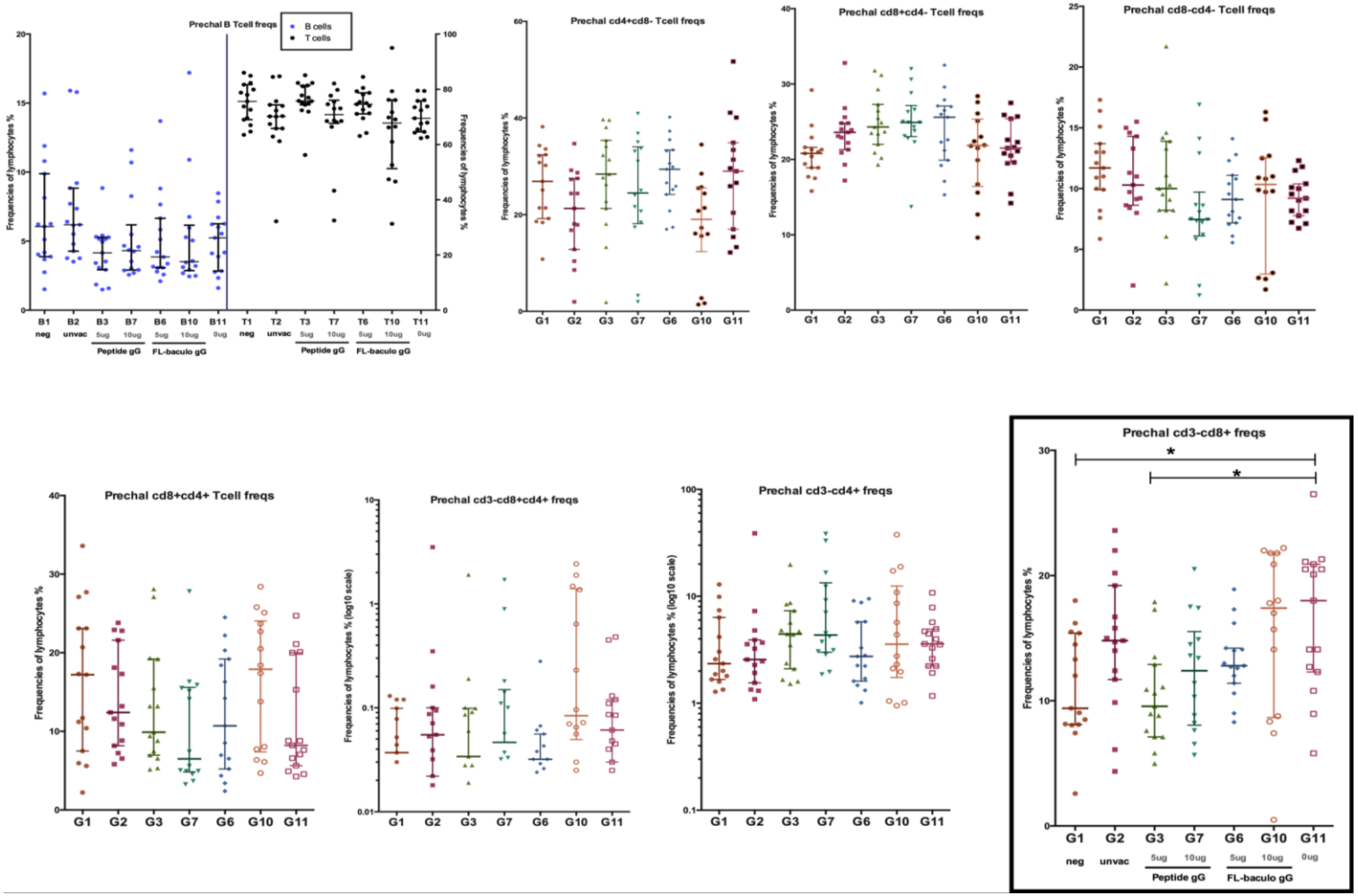


Figure 10. Lymphocyte sub-population frequencies in peripheral blood as assessed by flow cytometry. Pre-challenge blood samples were collected at 16 weeks of age, immediately prior to challenge. G1 (Neg) = negative control; G2 (unvac) = unvaccinated; 0, 5, or 10 μg dose = dose of gG administered with the live *Salmonella* vaccine, either in peptide (5 μg and 10 μg; G3 and G7) or full-length (5 μg and 10 μg; G6 and G10) form. Statistically significant differences (1way ANOVA, Tukey's post test) are indicated by asterisks in boxed plots. Scatter dot plots are shown, and error bars indicate median value and interquartile range.

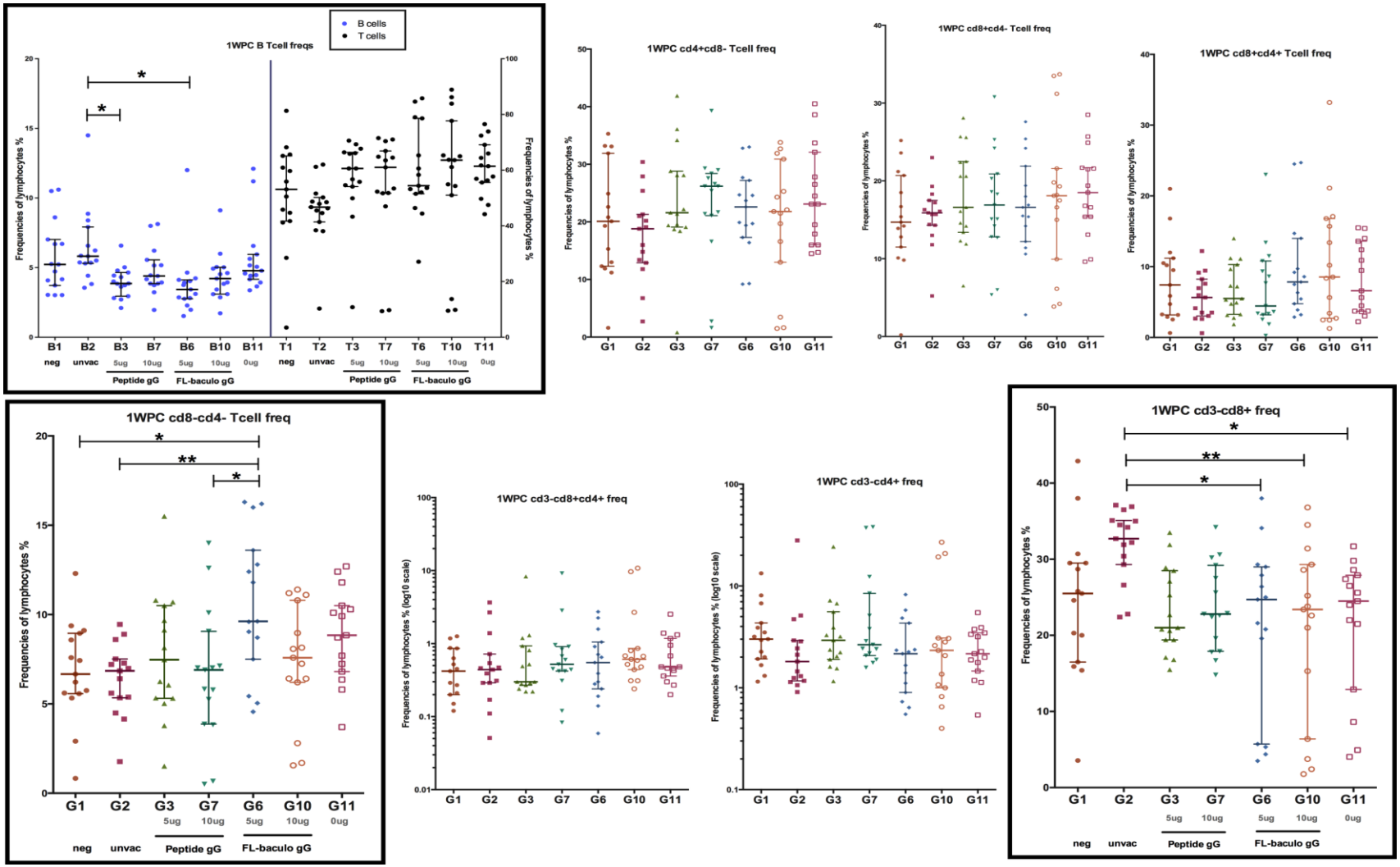


Figure 11. Lymphocyte sub-population frequencies in peripheral blood as assessed by flow cytometry. Post-challenge samples collected at 17 weeks of age, 1 week after challenge. G1 (Neg) = negative control; G2 (unvac) = unvaccinated challenged group (positive control); 0, 5, or 10 μg dose = dose of gG administered with the live *Salmonella* vaccine, either in peptide (5 μg and 10 μg; G3 and G7) or full-length (5 μg and 10 μg; G6 and G10) form. Statistically significant differences (1way ANOVA, Tukey's post test) are indicated by asterisks in boxed plots. Scatter dot plots are shown, and error bars indicate median value and interquartile range.

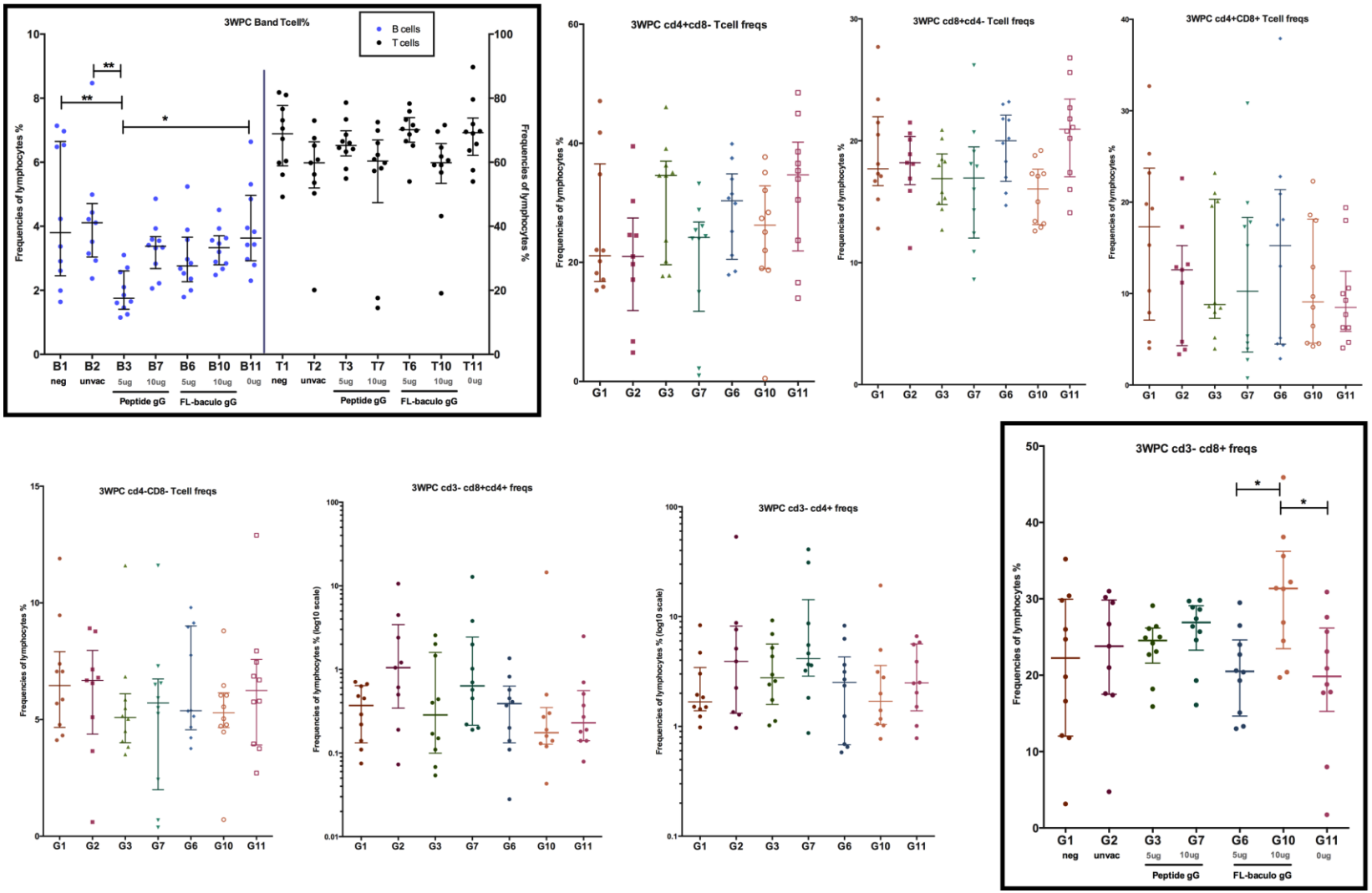


Figure 12. Lymphocyte sub-population frequencies in peripheral blood as assessed by flow cytometry. Post challenge blood samples collected at 19 weeks of age, 3 weeks after challenge. G1 (Neg) = negative control, G2 (unvac) = unvaccinated, 0, 5, or 10 μg dose = dose of gG administered with the live *Salmonella* vaccine, either in peptide (5 μg and 10 μg; G3 and G7) or full-length (5 μg and 10 μg; G6 and G10) form. Statistically significant differences (1way ANOVA, Tukey's post test) are indicated by asterisks in boxed plots. Scatter dot plots are shown, and error bars indicate median value and interquartile range.

6. Discussion of Results

This study identified a smaller, *E. coli*-expressed form of ILTV gG that was able to bind chemokines. This peptide (Peptide 3) was 100 amino acids in length. Smaller peptides (25 aa in length) spanning this 100 region did not show any chemokine-binding activity. The identification of this smaller, *E-coli* expressed form of the gG adjuvant is potentially useful because this represents a production system that is more suitable for large-scale production of protein for commercial purposes. The use of bacterial expression systems, instead of a baculovirus expression system, is associated with lower costs of production and therefore represents a more economically viable option for manufacture (Brondyk, 2008).

When used *in vivo* in commercial layer birds as a vaccine adjuvant, the peptide and full length forms of gG resulted in a significantly lower proportion of *Salmonella* positive samples, as detected by PCR applied to cloacal swabs and/or other tissues, compared with unvaccinated-challenged birds over the three weeks following challenge. There were no significant differences detected in the proportion of *Salmonella* positive samples between the groups of birds that received the vaccine with and without the adjuvant, however the birds that received the vaccine alone (no adjuvant) did not show significant differences in the detection of *Salmonella* over the three weeks after challenge compared to unvaccinated-challenged birds. All vaccinated groups showed a significantly lower proportion of *Salmonella*-positive cloacal swabs 3 days after challenge (the time of peak shedding). These results suggest that the impact of the adjuvant on protection following *Salmonella* challenge is small, but potentially important, and may effect the persistence of *Salmonella* shedding. Interestingly, a significantly smaller proportion of birds that received the peptide adjuvant and vaccine were positive for *Salmonella* in caecal samples, an important site of *Salmonella* carriage (Groves *et al.,* 2016), compared to birds in the unvaccinated, challenged groups. This difference was not detected in the other vaccinated groups and potentially indicates that the peptide adjuvant may help to protect against *Salmonella* carriage becoming established in the caecum, however this needs further examination.

Some features of the *in vivo* study used to assess the activity of the adjuvant may have made it difficult to detect significant differences between groups and demonstrate improvements in vaccine efficacy. These include oral delivery of the vaccine/adjuvant (most adjuvants show poor activity following oral delivery), the use of commercial birds that were already exposed to *Salmonella* at or shortly after hatch, and a challenge protocol that did not induce a high level of *Salmonella* shedding and colonisation in the unvaccinated-challenged birds (only approximately half the samples collected from birds in the positive control group were positive for *Salmonella*). Despite these experimental difficulties, the delivery of the adjuvant along with

the live vaccine did consistently induce statistically significant protection over the three weeks following challenge, compared to unvaccinated-challenged birds. Although the results from this study are not as striking as the results from sub-project 1.1.4 (which utilised SPF birds and examined both oral and sub-cutaneous delivery of the vaccine and adjuvant) the results are promising and indicate that the adjuvant may have some commercial potential, however this requires further consideration.

Although immunological parameters were assessed in all birds (including examination of antibodies against *Salmonella* in serum and bile, and also lymphocyte sub-populations in blood and tissue) no clear immunological differences relating to the presence of absence of the adjuvant were detected. A large variation in lymphocyte sub-populations was observed within experimental groups, which may have obscured the detection of significant differences between groups. This variation may reflect the origin of the birds (commercial layer birds, rather than SPF chickens). It is possible that further differences may be elucidated following characterisation of cytokine profiles in key tissues following challenge. This work is currently underway in our laboratories and, when completed, the results from these studies will be integrated with the results presented in this report in order to better understand the biological activity of the adjuvant in the context of *Salmonella* vaccination and challenge.

7. Implications

This project has identified a peptide adjuvant derived from ILTV gG that can improve protection against *Salmonella* challenge in commercial layer birds when delivered orally and combined with a live *Salmonella* vaccine. Few adjuvants are effective following oral administration and so this adjuvant has potential for future development, although it should be noted that the impact of the adjuvant in this current study was only small. Importantly the peptide adjuvant can be produced in bacterial expression systems (unlike the full length, baculovirus-expressed protein adjuvant) which is more suitable for large-scale, commercial production.

8. Recommendations

It is recommended that the commercial potential of the peptide adjuvant be considered in the context of *Salmonella* vaccines, and potentially other vaccines. This would require consideration of the costs associated with production of the adjuvant, as well as the anticipated benefits from its use. Some directions for future research include examining the efficacy of the adjuvant over a longer time period following challenge, potentially including the whole laying cycle, and also studies aimed at better understanding immune responses to this adjuvant. These latter studies may be best performed in SPF chickens, rather than commercial layers, in order to minimise variation in immunological parameters between birds within groups. Further development and refinement of the challenge model is also indicated and should ideally include sourcing only *Salmonella* negative birds (this was not possible in the timeframe of the current study) and also refinement of the challenge model to increase the number of unvaccinated, challenged birds that shed *Salmonella* following challenge. Future studies could also use quantitative PCR, rather than conventional PCR, to quantitate *Salmonella* shedding after challenge. Investigating alternative methods of delivering the adjuvant are also indicated and may include generating a vaccine candidate containing live, recombinant *Salmonella* that expresses the adjuvant protein.

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10. References

Anon (2002), Risk Assessments of *Salmonella* in Eggs and Broiler Chickens, In; Microbiological Risk Assessment Series 2. World Health Organization, Food and Agriculture Organization of the United Nations

Anon (2004), Opinion of the Scientific Panel on Biological Hazards on the requests from the Commission related to the use of vaccines for the control of Salmonella in poultry, The EFSA Journal, 114, 1-74

Baker, L., Chitas, A.M.L., Hartley, C.A., Coppo, M.J.C., Vaz, P.K., Stent, A., Gilkerson, J.R., Every, A,L., Devlin, J.M., (2014). Recombinant herpesvirus glycoprotein G improves the protective immune response to Helicobacter pylori vaccination in a mouse model of disease. PLOS ONE, 9(5): e96563.

Brondyk, W.H. (2008). Selecting an appropriate method for expressing a recombinant protein. Methods in Enzymology, 463, 131-147.

Bryant, N. A., Davis-Poynter, N., Vanderplasschen, A. & Alcami, A. (2003). Glycoprotein G isoforms from some alphaherpesviruses function as broad-spectrum chemokine binding proteins. EMBO J 22, 833–846

Cheeseman JH, Levy NA, Kaiser P, Lillehoj HS, Lamont SJ. (2008). Salmonella Enteritidis-Induced Alteration of Inflammatory CXCL Chemokine Messenger-RNA Expression and Histologic Changes in the Ceca of Infected Chicks. Avian Diseases 52(2): 229-234

Devlin, J.M., Viejo-Borbolla, A., Browning, G.F., Noormohammadi, A.H., Gilkerson, J.R., Alcami, A. and Hartley, C.A. (2010). Evaluation of immunological responses to a glycoprotein G deficient candidate vaccine strain of infectious laryngotracheitis virus. Vaccine. 28: 1325-1332

Devlin J.M., G.F. Browning, C.A. Hartley, N.C. Kirkpatrick, A. Mahmoudian, A.H. Noormohammadi and J.R. Gilkerson. (2006). Glycoprotein G is a virulence factor in infectious laryngotracheitis virus. Journal of General Virology, 87: 2839-47

Gobel T.W.F., Kaspers, B. and Stangassigner, M. (2001). NK and T cells constitute two major, functionally distinct intestinal epithelial lymphocyte subsets in the chicken. International Immunology. 13. 757-762.

Groves, P.J., Sharpe, S.M., Muir, W.I., Pavic, A. and Cox. (2016). Live and intactivated vaccine regimens against caecal Salmonella Typhimurium colonisation in laying hens. Australian Veterinary Journal. 94: 387 – 393.

Krogh, A., B. Larsson, G. Von Heijne, and E. L. Sonnhammer. (2001). Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. Journal of Molecular Biology 305: 567-580.

Petersen, T. N., S. Brunak, G. Von Heijne, and H. Nielsen. 2011. SignalP 4.0: discriminating signal peptides from transmembrane regions. Nat Meth 8: 785-786.

Pusterla, N., Byrne, B.A., Hodzic, E., Mapes, M., Jang, S.S. & Magdesian, K.G (2010). Use of quantitative real-time PCR for the detection of *Salmonella* spp. in fecal samples from horses at a veterinary teaching hospital. The Veterinary Journal, 186: 252-255.

Van de Walle GR, Kaufer BB, Chbab N, Osterrieder N (2010). Analysis of the herpesvirus chemokine-binding glycoprotein G residues essential for chemokine binding and biological activity. J Biol Chem 2009;284: 5968-5976.

Withanage, G. S., Kaiser, P., Wigley, P., Powers, C., Mastroeni, P., Brooks, H., Barrow, P., Smith, A., Maskell, D. & McConnell, I. (2004). Rapid expression of chemokines and proinflammatory cytokines in newly hatched chickens infected with Salmonella enterica serovar Typhimurium. Infect Immun 72, 2152–2159.

Withanage, G. S. K., Wigley, P., Kaiser, P., Mastroeni, P., Brooks, H., Powers, C., Beal, R., Barrow, P., Maskell, D. & McConnell, I. (2005). Cytokine and chemokine responses associated with clearance of a primary Salmonella enterica serovar Typhimurium infection in the chicken *an*d in protective immunity to rechallenge*.* Infect Immun 73*,* 5173*–5182.*

Zhang, S., R. A. Kingsley, R. L. Santos, H. Andrews-Polymenis, M. Raffatellu, J. Figueiredo, J. Nunes, R. M. Tsolis, L. G. Adams, and A. J. Baumler. (2003). Molecular pathogenesis of *Salmonella enterica* serotype Typhimurium-induced diarrhoea. Infect. Immun. 71:1-1