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Reduction of *Chlamydia pecorum* and Koala Retrovirus subtype B expression in wild koalas vaccinated with novel peptide and peptide/recombinant protein formulations

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ABSTRACT

Koalas are an endangered species under threat of extinction from several factors, including infections agents. *Chlamydia pecorum* infection results in morbidity and mortality from ocular and urogenital diseases while Koala Retrovirus (KoRV) infection has been linked to increased rates of cancer and chlamydiosis. Both *C. pecorum* and KoRV are endemic in many wild Australian koala populations, with limited treatment options available. Fortunately, vaccines for these pathogens are under development and have generated effective immune responses in multiple trials. The current study aimed to improve vaccine formulations by testing a novel peptide version of the *Chlamydia* vaccine and a combination *Chlamydia* – KoRV vaccine. Utilising a monitored wild population in Southeast Queensland, this trial followed koalas given either a 'Chlamydia only' vaccine (utilising four peptides from the chlamydial Major Outer Membrane Protein, MOMP), a combination 'Chlamydia and KoRV' vaccine (comprised of the chlamydial peptides plus a KoRV recombinant envelope protein (rEnv)) or no treatment. Clinical observations, *C. pecorum* and KoRV gene expression, serum IgG, and mucosal immune gene expression were assessed over a 17-month period. Overall, both vaccine formulations resulted in a decrease in chlamydiosis mortality, with decreases in *C. pecorum*, CD4, CD8β and IL-17A gene expression observed. In addition, the combination vaccine group also showed an increase in anti-KoRV IgG production that corresponded to a decrease in detected KoRV-B expression. While these results are favourable, the chlamydial peptide vaccine did not appear to outperform the established recombinant chlamydial vaccine and suggests that a combination vaccine formulated with recombinant MOMP plus KoRV rEnv could capitalize on the demonstrated benefits of both for the betterment of koalas into the future.

Introduction

The koala (*Phascolarctos cinereus)* is an endangered species under threat of extinction from several anthropogenic and natural risks, including disease related to infection with the intracellular bacterium *Chlamydia pecorum* [1,2]. Chlamydial infections are endemic throughout wild koala populations and, if left untreated, can progress to ocular and urogenital disease resulting in blindness, infertility, and death [2,3]. Treatment of chlamydial disease in koalas is limited to the use of antibiotics, which commonly results in gastrointestinal dysbiosis through the depletion of natural gut microbiota responsible for detoxifying and digesting their eucalyptus diet [2]. Fortunately, considerable effort has been placed on developing a chlamydial vaccine for koalas [4–14]. The current chlamydial vaccine formulation targets three endemic genotypes of *C. pecorum* (A, F and G) and has been proven to be 70 % effective in reducing disease presentation within wild koala populations [15,16]. However, this formulation utilises the full-length Major Outer Membrane Protein (MOMP) from *C. pecorum*, comprised of 390 amino acids and 16 transmembrane regions, recombinantly expressed and purified from *Escherichia coli* [14]. Generating this large, membrane-bound protein as a vaccine antigen is laborious, expensive and difficult to perform at large scale. If this antigen could be reduced to smaller synthetic peptides that retained the immunogenic properties of full-length MOMP, it would greatly reduce the burden of manufacturing and commercializing a chlamydial vaccine for koalas. Initial analysis of a two-peptide MOMP chlamydial vaccine in a captive koala population indicated anti-chlamydial immune responses could be induced [17]. Now, optimization and assessment of a chlamydial peptide vaccine is needed to determine if this formulation type can match the therapeutic

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and protective benefits of the recombinant formulation against chlamydial infection and disease in the wild.

In parallel, koalas are also under threat from a second infectious agent, Koala Retrovirus (KoRV). KoRV is also endemic throughout Australia, with all northern Australian koalas infected with the endogenous subtype KoRV-A and some combination of exogenous subtypes KoRV-B to KoRV-I [18]. KoRV infection in koalas has been linked to the development of cancers like lymphoma and increased susceptibility to chlamydial disease development [19–22]. With no treatment options available for KoRV, development of an anti-KoRV vaccine is underway [23–26]. As with the chlamydial vaccine, the initial formulation of the KoRV vaccine is as a recombinant protein vaccine, representing most of the envelope protein of KoRV-A (rEnv). Initial testing of the KoRV rEnv vaccine was done with a small number of southern koalas, where KoRV infection rates are low [23], and in a small number of captive northern koalas, which possess endogenous KoRV-A but were not exposed to *Chlamydia* [25]. These trials showed the KoRV rEnv vaccine to be safe, having no detectable adverse effect on any koala vaccinated [23,25]. Additionally, the vaccine induced humoral and cellular immune responses resulting in significant decreases in circulating virus particles [24–26]. With this foundation, the next step for KoRV vaccine development is to characterize the recombinant vaccine formulation performance in a larger, wild koala population with both endemic *Chlamydia* and KoRV.

This current study set out to advance the development of both the chlamydial and KoRV vaccines by trialling two novel formulations in a wild koala population with endemic *Chlamydia* and KoRV. The first formulation was an expanded four-peptide chlamydial vaccine ('Chlamydia only') while the second formulation was a combination of the four chlamydial peptides plus the KoRV rEnv vaccine ('Chlamydia and KoRV'). These vaccine formulations were given as a single dose and vaccinated koalas were compared to unvaccinated members of the population for chlamydial expression and disease states, KoRV expression patterns and humoral and cellular responses generated.

As both *C. pecorum* and KoRV are endemic in many koala populations, the goals of these wildlife vaccines are not necessarily to eradicate these agents from the population or completely prevent detectable infection within vaccinated animals. Instead, since chlamydial disease progression is associated with the load of detectable infection [27], the desired goal of these vaccines is to have a population-level effect by reducing the overall detectable expression levels of the target infectious agents in the vaccinated groups by promoting an overall appropriate immune response. With this goal in mind, this study reports the clinical findings, infectious agent gene expression, systemic IgG antibody responses and cellular immune gene responses of our vaccinated and unvaccinated animals as study groups rather than individuals. This approach reflects the need for koala disease management to be viewed from a population perspective.

Materials and methods

Vaccine preparation and procedure

This project utilised two vaccine formulations. The first vaccine formulation ('Chlamydia only') was comprised of four synthetic peptides as antigens, designed to represent highly immunogenic regions of the *C. pecorum* MOMP. These four peptides included two peptides representing amino acid (aa) sequences from the first (EGMSGDPCDPCATW (14 aa)) and forth (INYHEWQVGAALSYRLNMLIP (21 aa)) conserved regions of MOMP and have been tested in a previous study [17]. The other two peptides represent aa sequences from the leader sequence (KKLLKSAFLSAAFFAG (16 aa)) and the fifth (VLQIVSLQINKLKSRKACG (19 aa)) conserved region of MOMP and are novel to this vaccine formulation (Supplementary Fig. 1A). All four peptides were synthetised by Mimotopes Pty Ltd (Springvale Road, Mulgrave, Victoria, Australia) with a free amine located at the N-terminus and a free acid at the C-terminus, at a purity of > 70 % determined by High Pressure Liquid Chromatography.

The second vaccine formulation ('Chlamydia and KoRV') utilised the same combination of four synthetic peptides as the 'Chlamydia only' vaccine plus a recombinant protein representing the majority of the KoRV-A envelope protein (rEnv) as previously described [23] (Supplementary Fig. 1B).

Both vaccines ('Chlamydia only' and 'Chlamydia and KoRV') were combined with a three-component adjuvant, containing Poly I:C (250 μg), Host Defence Peptide-Innate Defence Regulator IDR-1002 (500 μg), and Polyphosphazene EP3 (250 μg) (VIDO-Intervac, University of Saskatchewan, Canada). Vaccine preparation involved mixing the Poly I:C and IDR-1002 and incubating for 15 min at room temperature, with gentle rocking. Next, EP3 and the appropriate antigens (either 50 μg of each chlamydia peptide or 50 μg of each chlamydia peptide plus 50 μg KoRV rEnv protein) were added, with the final mixture incubated for a further 15 min at room temperature, with gentle rocking, then stored at 4 °C until use.

Each vaccine was utilised in a total vaccine volume of 0.5 mL, prepared in a sterile endotoxin-free amber glass vial, and administered within 5 h of preparation. Each koala was vaccinated subcutaneously (while under anaesthesia) and visually monitored for several hours post-vaccination, prior to release.

Animal trial

A total of 69 koalas (32 male/37 female) with median age of 3.3 years (range of 1.1–12.0 years) were included in the trial. These koalas were monitored as part of a larger koala monitoring project conducted by Endeavour Veterinarian Ecology (EVE) as part of the Moreton Bay Rail (MBR) project. Koalas located in the Moreton Bay region, Queensland, Australia, were monitored utilising biotelemetry collars. All koalas selected for inclusion into the trial were deemed healthy at the baseline observation/sampling point, assigned to a study group, vaccinated (if appropriate) and released back into the wild for subsequent evaluations and sample collection. Assignment into each study group was mostly random, with consideration given to divide males and females approximately evenly between groups.

Project permits/approvals

The project was reviewed and approved by the University of the Sunshine Coast Animal Ethics Committee (approval number ANS1857). A scientific purposes permit was approved by the Queensland Government (permit number WA0020117). An *in-vivo* use permit was approved by the Australian government, department of Agriculture (approval number 2020/007). Finally, the project also followed the conditions set out in the Australian Pesticides and Veterinary Medicines Authority (APVMA) permit number PER7250.

Clinical examinations

As part of the monitoring program, koalas from this wild population underwent regular (6-monthly) standardised veterinary examinations. Examinations included a thorough physical examination, sonographic examination of the urogenital tract (including kidneys), and cytological examination of blood, bone marrow, peritoneal fluid and urine sediment. The minimum

requirement for the diagnosis of cystitis (urogenital chlamydial disease) was an inflammatory urine sediment cytological finding and for reproductive tract disease was a sonographic finding of reproductive cysts or other pathological changes associated with chlamydial reproductive tract disease.

Sampling procedures

Prior to vaccination and at all subsequent clinic visits, all koalas had two swab samples collected from the urogenital tract (urogenital sinus in females, penile urethra in males), with one swab placed in 500 μL of PBS and the other a 500 μL solution of RNAlater and stored at −20 °C. Each koala also had 3–5 mL of blood collected from the cephalic vein. The blood sample was centrifuged to separate serum and separated into two tubes, one containing 300 μL of RNA later and one with no additive and stored at −20 °C.

All examinations were performed under alfaxalone (Alfaxan, Jurox, Rutherford, NSW) sedation (dose rate 3–5 mg/kg intramuscular injection), supplemented with oxygen and isoflurane (Isoflo, Abbott, Botany, NSW) via face mask if required.

C. pecorum IgG ELISA

C. pecorum vaccine antigen-specific serum IgG was assayed utilising either vaccine peptides or recombinant MOMP G protein. For the peptide assay, biotin-labelled peptides (Mimotopes Pty Ltd, Springvale Road, Mulgrave, Victoria, Australia) representing each of the aa sequences utilised in the vaccine were pooled together and bound to a streptavidin-coated 96-well plate (Thermo Scientific) at a pooled concentration of 2 μg per well in 1xPBS tween-20 (0.01 %) and incubated at 4 °C overnight. For the recombinant protein assay, full-length MOMP G protein expressed and purified from *E. coli* (described in [14]) was bound to 96-well plates (Greiner Bio-One medium binding) at a concentration of 2 μg per well in carbonate-bicarbonate coating buffer and incubated at 4 °C overnight. The following day, each serum sample was diluted either 1:5 with sterile PBS tween-20 (0.01 %) (for the peptide assay) or 1:3 serially with dilutions starting at 1:50 (for the recombinant MOMP G assay) and assessed for IgG antibodies as previously described [14]. Briefly, plates bound with *Chlamydia* MOMP peptides/protein were washed four times using a Bio-Rad plate washer with 200 μL per well of PBS-Tween-20 (0.05 %). Post-washing, 100 μL of the individual diluted serum samples were added to the wells in duplicate, incubated and washed as before. The plates were then incubated with sheep anti-koala IgG (1:8000 in PBS-tween-20 (0.01 %)) (100 μL/well), incubated and washed as before. Plates were then incubated with HRP-labelled rabbit anti-sheep IgG (1:20,000 in PBS-tween-20 (0.01 %)) (100 μL/well), incubated as previously and washed five times with 200 μL per well with PBS. Finally, each well was incubated with 100 μL/well of tetramethyl-benzidine dissolved in phosphate citrate buffer containing sodium perborate dimethyl sulfoxide (1:10) and 30 % hydrogen peroxide (1:5000) at room temperature for 20 min, protected from light, after which 100 μL/well of 1 M sulphuric acid was added and the optical density measured at 450 nm (BioRad, 96-well plate reader). For end-point titres, calculations were done as per [12]. Samples from 67 study koalas were of sufficient quality to determine serum anti-IgG levels (23 'Chlamydia only' koalas [11 males/12 females], 22 'Chlamydia and KoRV' koalas [11 males/11 females] and 22 'Unvaccinated' koalas [10 males/12 females]).

KoRV IgG ELISA

KoRV antigen-specific serum IgG were assessed utilising KoRV rEnv protein (identical to the vaccine antigen), bound to a 96-well plate and subjected to ELISA as previously described [23]. Briefly, 96-well ELISA plates (Thermo Scientific) were coated with 1 μg of KoRV rEnv protein, incubated overnight at 4 °C then washed four times using a Bio-Rad plate washer with 200 μL per well of PBS-Tween-20 (0.05 %). Serum samples diluted 1:5 with sterile PBS tween-20 (0.01 %), with 100 μL added to each well in duplicate, incubated at 37 °C for one hour and washed as before. The following detection procedure was performed as per detection for anti-*C. pecorum* antibodies, described above. Samples from the same 67 study koalas were of sufficient quality to determine serum anti-KoRV IgG levels as for anti-*C. pecorum* above.

Expression analysis utilising Nanostring technology

Nanostring technology was utilised to assess the total expression of 14 different koala immune-related genes (MHC class II DAA exon 3, MHC class II DBA exon 3, CD4, CD8β, IL-1β, IL-4, IL-6, IL-8, IL-10, IL-17A, IL-18, IFN-γ, TNF-α and MID2), four specific KoRV genes (KoRV *pol*, KoRV-A *env* receptor binding domain (RBD), KoRV-B *env* RBD and KoRV-D *env* RBD), *C. pecorum* ompA gene and three koala specific housekeeping genes (ACTB, GAPDH and Hmg20a) at each collection point from cloacal swab samples stored in RNAlater. All samples were transported to Griffith University (Gold coast, Queensland, Australia), where they were extracted for total RNA, assessed for quality control, and processed utilising the nCounter Plexset 24 reagent pack with the Nanostring designed probes for each of the 22 genes selected. Probe sequences for each target are given in Supplementary Table S1. Expression data from the swabs of 65 study koalas were of sufficient quality after quality control and normalisation to be evaluated for differential gene expression (23 'Chlamydia only' koalas [10 males/13 females], 21 'Chlamydia and KoRV' koalas [10 males/11 females] and 21 'Unvaccinated' koalas [10 males/11 females]).

Statistical analysis

All ELISA results were averaged across duplicate results, blank adjusted and compared between baseline and months post-vaccination for each group ('Chlamydia only', 'Chlamydia and KoRV' and 'Unvaccinated'). Results were visualised as box and whisker plots with collections grouped into months post-vaccination and statistically assessed using a Kruskal-Wallis test. Analysis and visualisations were performed utilising the statistical program R studio on the package ggplot2 [28,29] and Microsoft Excel. All expression data were normalised, assessed for differential expression, and visualised utilising the statistical program R studio on the packages NanoStringNorm, NanoStringDiff and ggplots2 [28–31] and Microsoft Excel. The package NanoStringDiff utilizes a generalized linear model of the negative binomial family to characterize count data and allows for multi-factor design. NanoStrongDiff incorporates size factors, calculated from positive controls and housekeeping controls, and background level, obtained from negative controls, in the model framework so that all the normalization information provided by NanoStringNorm is fully utilized.

Results

Study population

This study recruited 69 koalas (32 male/37 female) into three trial groups: 'Chlamydia only' vaccine (24 koalas [11 males/13 females]); 'Chlamydia and KoRV' vaccine (22 koalas [11 males/11 females]) and 'Unvaccinated' (23 koalas [10 males/13 females]). Over a 17-month monitoring period, koalas were sampled an average of

four times each, with a total of 287 sample collection points obtained (Supplementary Fig. 2). Samples used for each analysis in this study, as well as the raw data for each analysis, are listed in Supplementary Table S2.

Clinical outcomes

In this study, koalas underwent comprehensive clinical examinations by experienced koala veterinarians at sample collection points. At the baseline observation/sampling point, all trial koalas were deemed healthy. Overall, the study population remained healthy throughout the trial period. There were no indications of adverse reactions to the vaccines, either at the time of administration or during the year and a half monitored follow-up period.

During the trial, three koalas were diagnosed with cystitis (urogenital chlamydial disease) and admitted to veterinary care for treatment: a 2.8-year-old female from the 'Unvaccinated' group at 2.0 months post-enrolment (Mamba), a 3.4-year-old male from the 'Chlamydia only' group at 5.5 months post-vaccination (Pandanus) and a 5.1-year-old male from the 'Chlamydia and KoRV' group at 8.0 months post-vaccination (Siva). In addition to severe cystitis, Mamba also presented with a cyst in her right uterus (characteristic of chlamydial reproductive disease). Following antibiotic therapy, Mamba developed oxalate nephrosis and despite treatment and supportive care, her condition deteriorated, and she was euthanized on humane grounds. Both Pandanus and Siva responded well to treatment, recovered, and were released back into the wild.

During the study period, there were also two mortality events unrelated to chlamydial disease: a 3.7-year-old male from the 'Chlamydia only' group died from severe dermatopathy, suspected to have been exacerbated by inclement weather (prolonged rain) at two months post-vaccination and a 2.4-year-old male from the 'Chlamydia only' group died from septicaemia secondary to pleuropneumonia, also suspected to be related to inclement weather (a cold, wet period) one-month post-vaccination. Necropsies found nothing to suggest this study or vaccination contributed to these mortalities.

C. pecorum ompA gene and KoRV pol and env gene expression levels

To determine the level of expression of *C. pecorum* and KoRV genes during this study, urogenital swabs were collected for RNA extraction and gene expression analysis. Probes to conserved regions within the *C. pecorum ompA* gene (coding for MOMP, the major protein constituent of cells which is constitutively expressed throughout growth [32]), KoRV polymerase (*pol*) gene (shared by all KoRV subtypes) and the KoRV receptor binding domains (RBDs) of subtypes A, B and D envelope (*env*) genes were used to determine the expression of each target at each sample collection point (Supplementary Table S1).

Detection of *C. pecorum ompA* gene expression from apparently healthy koalas in all three study groups throughout the trial confirmed that *C. pecorum* was endemic in this wild koala population (Fig. 1). Vaccination with either the 'Chlamydia only' or the 'Chlamydia and KoRV' vaccine resulted in a decrease in the number of koalas with detectable *C. pecorum* post-vaccination and a statistically significant decrease in the expression level of *ompA* in koalas still carrying *C. pecorum* (Fig. 1). This effect became more pronounced over time, with a consistent trend of lower detectable *ompA* expression up to the >6-months sampling point. Conversely, this trend was not seen in the 'Unvaccinated' group, where expression levels of *ompA* remained relatively consistent throughout the study, including at the>6-month sampling point.

Detectable, stable expression of the KoRV *pol* gene from all koalas at every sample point collected confirmed that KoRV was also endemic in this wild koala population (Supplementary Fig. 3). Based on the expression of subtype-specific targets, both KoRV-A and KoRV-D *env* genes were also detectable from all koalas sampled, with no differences detected in their gene expression within vaccine groups over the study period (Supplementary Fig. 3).

By comparison, KoRV-B *env* gene expression was only detected in a subset of koalas, with a trend of decreasing gene expression in 'Chlamydia and KoRV' vaccinated group over time (Fig. 2). This trend achieved statistical significance by > 6 months post-vaccination ($p < 0.001$; Fig. 2). In contrast, there was no consistent difference in KoRV-B *env* gene expression in the 'Chlamydia only' or 'Unvaccinated' groups over the study period.

C. pecorum and KoRV serum IgG levels

To obtain a measure of the humoral immune response induced by the trial vaccines, serum was collected to measure anti-*C. pecorum* and anti-KoRV circulating IgG. To first determine the pre-existing anti-*C. pecorum* MOMP antibody levels in the population, serum was tested against full-length recombinant MOMP (subtype G) protein to determine circulating IgG end point titres (EPTs) at baseline and again at ~ 6-months post-vaccination. All koalas in the study possessed detectable levels of anti-*C. pecorum* MOMP serum IgG throughout the monitoring period, with no significant differences in EPTs detected between vaccine groups at baseline or ~ 6-months post-vaccination (Supplementary Fig. 4).

The four-peptide chlamydial vaccine antigen component did not appear to induce a vaccine-specific anti-*C. pecorum* IgG response, with no differences in serum IgG levels detectable in any vaccine group over the study period to these four peptides (Fig. 3A). Conversely, the KoRV rEnv antigen component appeared to induce a significant change in anti-KoRV serum IgG levels in the 'Chlamydia and KoRV' vaccine group over the study period ($p < 0.001$), with increased levels detectable from one to five months post-vaccination (Fig. 3B). This increased anti-KoRV IgG response was not detectable in the 'Chlamydia only' and 'Unvaccinated' groups and did not appear sustained in the 'Chlamydia and KoRV' group beyond six months post-vaccination (Fig. 3B).

Mucosal cellular immune response gene expression

To obtain a measure of the mucosal cellular immune response induced by the trial vaccines, the same urogenital swabs analysed for pathogen expression were evaluated for the expression of two koala major histocompatibility complex (MHC) class II alleles (DAA and DBA), CD4 and CD8β, interleukin (IL)-1β, IL-4, IL-6, IL-8, IL-10, IL-17A, IL-18, interferon gamma (IFN-γ), tumour necrosis factor alpha (TNF-α) and koala viral host restriction factor MID2/TRIM1. From this dataset, only IL-17A, CD4 and CD8β gene expression patterns showed consistent and significant trends throughout the study period (Fig. 4). IL-17A expression had a decreasing trend in both the 'Chlamydia only' and 'Chlamydia and KoRV' vaccine groups, with this trend becoming statistically significant 3–6 months post-vaccination and more so > 6 months post-vaccination (Fig. 4A). Similarly, both CD4 and CD8β expression had decreasing trends, with > 6 months post-vaccination showing a statistically significant reduction in expression compared to baseline (Fig. 4B). These decreases were not observed in 'Unvaccinated' group during the study period.

Discussion

The goal of this study was to advance the development of koala vaccines against *C. pecorum* and KoRV by trialling two novel formulations in a wild koala population with endemic *Chlamydia* and

A

Vaccine group	Baseline	< 3 Months	3 – 6 Months	> 6 Months
Chlamydia only	3/17 (3.58)	2/14 (3.16)	2/14 (2.73)	1/15 (1.00)
Chlamydia & KoRV	9/18 (5.79)	4/11 (4.56)	5/16 (3.52)	4/11 (2.31)
Unvaccinated	5/18 (3.55)	6/14 (4.08)	2/14 (2.29)	1/10 (3.58)

B

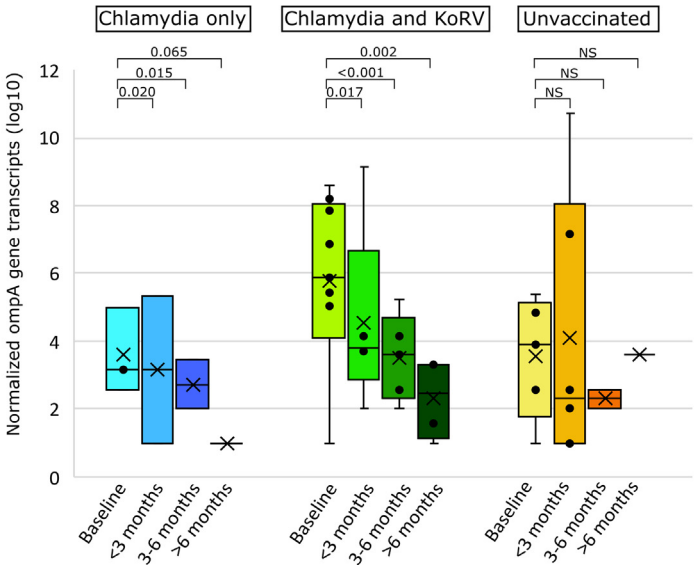

Fig. 1. Normalized *Chlamydia pecorum ompA* gene transcripts detected from koala urogential swabs. A) Number of *ompA* positive koalas/total number of koalas tested in that time frame (average number of *ompA* transcripts detected from positive koalas only, reported as log10). B) Box and whisker plots indicate average (X), median (line), inner sample points & outliers (circles) and interquartile ranges (box) of gene transcripts detected from positive koalas in each vaccine group. Statistical gene expression differences represent all koalas compared to their baseline (pre-vaccination) levels for the time frames indicated. NS = not significant.

A

Vaccine group	Baseline	< 3 Months	3 – 6 Months	> 6 Months
Chlamydia only	8/17 (6.19)	6/14 (8.42)	7/14 (6.67)	5/15 (8.28)
Chlamydia & KoRV	10/18 (6.00)	5/11 (5.03)	7/16 (4.59)	3/11 (4.93)
Unvaccinated	8/18 (3.65)	7/14 (3.90)	3/14 (2.29)	1/10 (2.32)

B

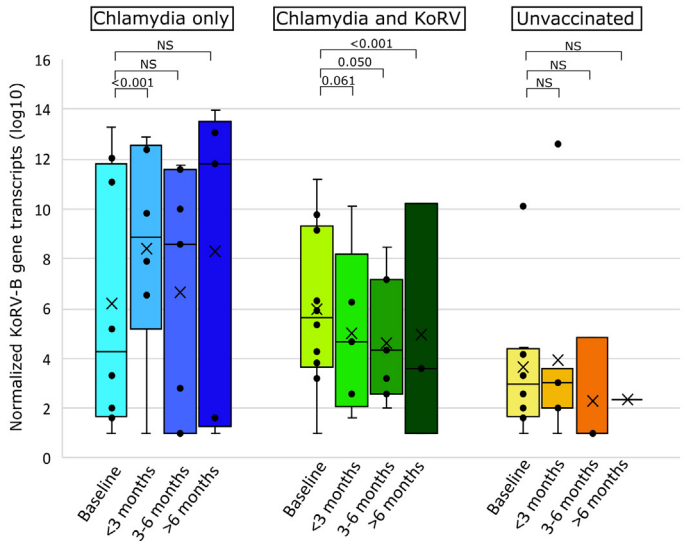

Fig. 2. Normalized Koala Retrovirus subtype B (KoRV-B) gene transcripts detected from koala urogential swabs. A) Number of KoRV-B positive koalas/total number of koalas tested in that time frame (average number of KoRV-B transcripts detected from positive koalas only, reported as log10). B) Box and whisker plots indicate average (X), median (line), inner sample points & outliers (circles) and interquartile ranges (box) of gene transcripts detected from positive koalas in each vaccine group. Statistical gene expression differences represent all koalas compared to their baseline (pre-vaccination) levels for the time frames indicated. NS = not significant.

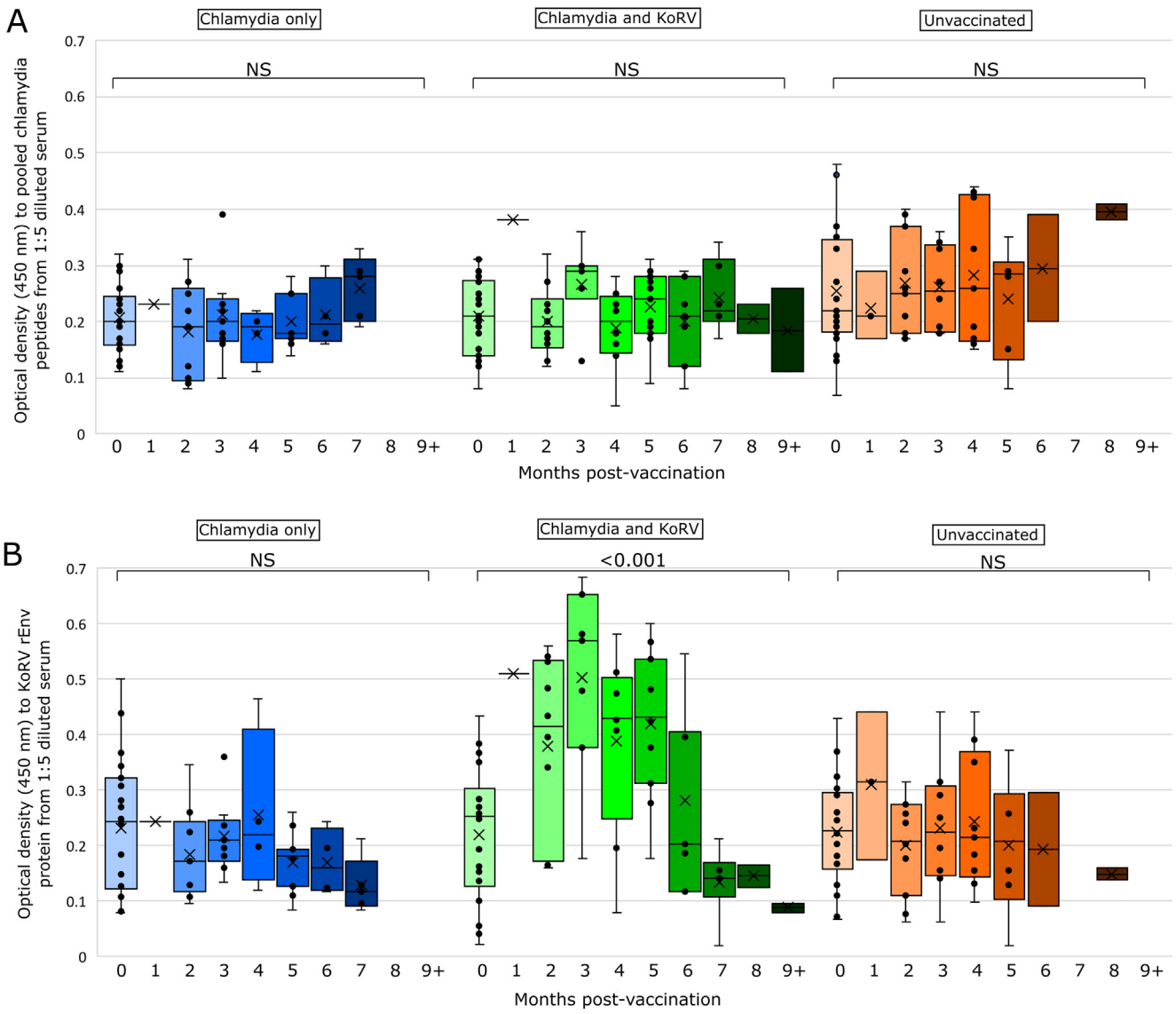

Fig. 3. Serum IgG antibody levels reported as optical density (OD450 nm) measurements from serum diluted 1:5 for each vaccine group by month post-vaccination. A) Anti-*Chlamydia pecorum* IgG targeting pooled chlamydia vaccine peptides. B) Anti-KoRV IgG targeting vaccine recombinant envelope protein. Box and whisker plots indicate average (X), median (line), inner sample points & outliers (circles) and interquartile ranges (box) of antibodies detected from koalas in each vaccine group.

KoRV. The first novel formulation tested the effect of reducing the established recombinant MOMP antigen to four immunogenic peptides for easier manufacture of a chlamydial vaccine while the second novel formulation combined the four chlamydia peptides with a KoRV recombinant envelope antigen to determine if a combination *Chlamydia*/KoRV vaccine could be safe and effective. Overall, no vaccinated koalas suffered from *C. pecorum*-associated koala mortality, while a single unvaccinated koala was euthanised due to complications associated with antibiotic treatment for chlamydial disease. Furthermore, in koalas with detectable *C. pecorum ompA* gene expression, *ompA* gene expression levels was lower post-vaccination in both vaccinated cohorts while the unvaccinated koalas showed no expression level changes. This reduction in chlamydial burden also corresponded in reductions of mucosal T cell markers and inflammatory cytokines. Additionally, the combination vaccine also induced anti-KoRV antibodies that correlated with a decrease in mucosal KoRV-B gene expression, suggesting a reduction in circulating viral load. From a disease management perspective, these were the desired outcomes and indicate that both vaccines can be considered successful.

The important additional value of this study is in comparing these vaccine outcomes to a decade of previous koala vaccine development research. The *C. pecorum* peptide antigens in this vaccine trial did not induce a detectable anti-*C. pecorum* vaccine antigen-specific IgG response while the KoRV recombinant protein was able to generate a KoRV vaccine antigen-specific IgG antibody response. When full-length *C. pecorum* MOMP recombinant antigens have been previously used as a vaccine antigen, vaccine-specific *C. pecorum* antibody responses have been regularly detected [5–7,9,12–14]. The lack of vaccine-induced circulating *C. pecorum* IgG was unexpected, as the peptides chosen for the vaccine antigen were based on IgG epitope mapping from the MOMP vaccine trials [6,9,14]. However, the process of antigen processing and presentation to B cells does not guarantee peptides will be presented the same as recombinant proteins, so replicating the MOMP antibody response with only four, short peptides was experimental and a key feature under investigation in this study.

Interestingly, successful reduction of *C. pecorum ompA* gene expression in vaccinated koalas was achieved without inducing an increased systemic *C. pecorum* IgG response, supporting the

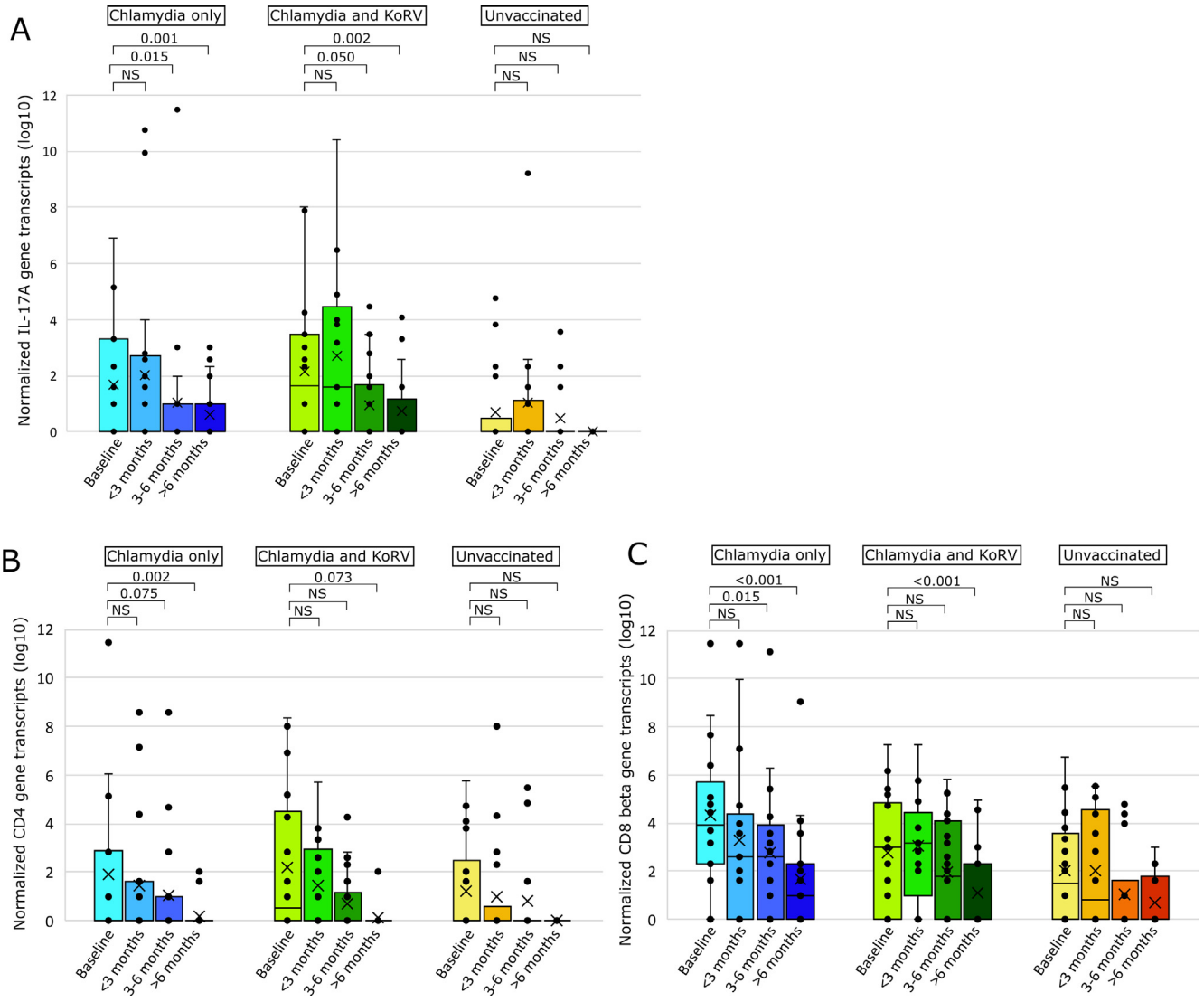

Fig. 4. Normalized (A) *IL-17A*, (B) *CD4* and (C) *CD8β* gene transcripts detected from koala urogenital swabs. Box and whisker plots indicate average (X), median (line), sample points & outliers (circles) and interquartile ranges (box) of gene transcripts detected from koalas in each vaccine group. Statistical gene expression differences represent all koalas compared to their baseline (pre-vaccination) levels for the time frames indicated. NS = not significant.

previously published body of evidence that B cells may not play a critical role in controlling *Chlamydia* infection. It has been experimentally shown that both B cell-deficient mice and antibody-depleted mice control primary *Chlamydia* genital infection as efficiently as wild type mice [33,34]. Additionally, while *Chlamydia* clearance in mice was slightly delayed following secondary infection in the absence of B cells [33,35], it has also been shown that CD4+ memory T cells could limit reinfection of *C. trachomatis* in B cell-deficient mice comparable to wild type [36]. Still, there is evidence that B cells and antibodies can contribute to *Chlamydia* clearance and protection [37] and it should be recognized that the koalas in this study did possess natural anti-*C. pecorum* IgG to full-length MOMP. The fact that the peptide version of the chlamydia vaccine did not induce additional anti-*C. pecorum* circulating IgG did not hinder the reduction of *C. pecorum ompA* gene expression detected in the vaccinated koalas studied. This suggests that perhaps, in this study, the chlamydial peptide vaccine acted in a more T cell-, as opposed to a B cell-, mediated manner. As there were no biological data available to direct the selection of MOMP T cell epitopes, it is possible that the *Chlamydia* peptides chosen represented T cell epitopes better than B cell epitopes.

Focusing on the T cell side of the vaccine response, three important cell-mediated immune markers showed a significant decrease post-vaccination that mirrored the reduction in *C. pecorum ompA* gene expression: *CD4, CD8 beta* and *IL-17A*. The importance of both $CD4^+$ and $CD8^+$ T cells for controlling *Chlamydia* infection has been well documented for over 20 years (reviewed by [38]). Activated CD4+ T cells are important producers of effector cytokines, such as IFN-γ for limiting *Chlamydia* replication, as well as critical activators of B cells and $CD8^+$ T cells [38]. In turn, $CD8^+$ T cells also produce effector cytokines, as well as possessing the ability to directly kill target cells as cytotoxic T lymphocytes [38]. IL-17, a hallmark cytokine of Th17 cells, has a pro-inflammatory function and plays an important defensive role against intracellular pathogens, including *Chlamydia* [39,40]. IL-17 can be detected in inflammatory tissues or at infection sites of almost all patients infected with Chlamydia [41], including during asymptomatic infection [42], and is believed to have an anti-Chlamydia role by up-regulating inducible nitric oxide synthase (iNOS) production, enhancing cooperation between nitric oxide (NO) and IFN-γ [43], and inducing type 1 T cell immunity by dendritic cells [40,44]. However, IL-17 can also cause tissue damage that leads to scarring and infertility, making

prolonged elevated levels detrimental to the host [44]. In the vaccinated groups in this study, as *C. pecorum*-infected koala numbers and *ompA* expression levels decreased, so did the detectable levels of expressed CD4, CD8β and IL-17A at the urogenital mucosal site. This correlation suggests that as *C. pecorum* infection resolved, lower levels of the cell-mediated immune markers were needed at this site.

On the KoRV side of the vaccine trial, this study continues to support the growing evidence that vaccination to an endogenous retrovirus can be safe in koalas. KoRV-A is endogenous in northern Australian koala populations and care must be taken when introducing a vaccine antigen to a host that may consider it a self-protein. In all the trials using KoRV-A rEnv protein as the vaccine antigen, including this one, no signs of adverse reactions or autoimmunity were detected in vaccinated koalas [23,25]. At the time of writing, it has been three years since the first KoRV antigen-containing vaccinations were given to this monitored koala population and wildlife veterinarians still report this population as healthy and growing. This provides additional evidence of the safety of KoRV vaccination using recombinant protein.

Beyond being a safe vaccine for koalas, the 'Chlamydia and KoRV' combination vaccine induced circulating anti-KoRV IgG antibodies and reduced the detection of KoRV-B envelope RNA at the urogenital site. Previous analysis found that the KoRV-A rEnv vaccine antigen induced neutralizing antibodies to KoRV and that the antibody response was associated with reduced circulating viral load [23,25]. As the KoRV-B subtype has specifically been linked to an increased rate of chlamydiosis and cancer in koalas in several studies [18,21], targeted reduction of its circulating load could have potential health benefits for koalas. Interestingly, while the vaccine antigen was designed to the KoRV-A envelope sequence, only KoRV-B showed a significant decrease in detected expression, with KoRV-A and KoRV-D expression levels remaining relatively stable across study groups over time. In this trial, all the koalas tested were identified with high levels of KoRV-A and KoRV-D envelope gene transcripts (average $\sim 10^{12}$ copies per sample) while only a subset of koalas (~50 %) had detectable levels of KoRV-B envelope transcripts at much lower levels (average 10^3–10^8 copies per sample). It is currently unknown why KoRV-B was the only subtype to show detectable differences post-vaccination, but this will be an area to monitor closely in future trials.

Conclusion

Considerable efforts are underway in Australia to protect and support endangered free-living koala populations, and vaccination has been recognized as an important component of these efforts. Foundational research over the last decade has created chlamydial and KoRV vaccine formulations that have been repeatedly demonstrated to be safe and effective. The challenge now is to take these research vaccines and transition them into commercial vaccines for use in wider koala disease management programs. This study found that, while a chlamydial peptide formulation can reduce chlamydial *ompA* expression in the wild, the previously tested recombinant protein formulation generates a more robust overall immune response. Combined with the robust, safe immune response generated from the KoRV recombinant protein formulation, there appears to be a clear path forward that involves a combination *Chlamydia*/KoRV vaccine composed of recombinant protein antigens. This continued progress towards better and more effective vaccines to protect koalas brings us another step closer to preserving these unique animals for generations to come.

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Data availability

All data has been attached as supplementary files.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

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