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Altered immune parameters associated with Koala Retrovirus (KoRV) and Chlamydial infection in free ranging Victorian koalas (*Phascolarctos cinereus*)

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Koala Retrovirus (KoRV) has been widely speculated to cause immune suppression in koalas (*Phascolarctos cinereus*) and to underlie the koala's susceptibility to infectious disease, however evidence for immunomodulation is limited. The aim of this study is to determine whether immunophenotypic changes are associated with KoRV infection in free ranging Victorian koalas. qPCR was used to examine mRNA expression for Th1 (IFNγ), Th2-promoting (IL6, IL10) and Th17 (IL17A) cytokines, along with CD4 and CD8 in whole blood of koalas (n = 74) from Mt Eccles and Raymond Island in Victoria, Australia, with and without natural chlamydial infection. KoRV positive koalas had significantly lower levels of IL17A (*p*`*0.023*) and IFNγ (*p* = *0.044*) gene expression along with a decreased CD4:CD8 gene expression ratio (*p* = *0.025*) compared to negative koalas. No effect of chlamydial infection or combined effect of KoRV and chlamydial infection was detected in these populations. The decreased expression of IFNγ could make KoRV infected koalas more susceptible to persistent chlamydial infection, and a decrease in IL17A could make them more susceptible to gram negative bacterial, fungal and mycobacterial infection; but more tolerant of chlamydial infection.

Koala Retrovirus (KoRV) is a gamma retrovirus that has been widely speculated to cause immune suppression in koalas (*Phascolarctos cinereus*) and to underlie the koala's susceptibility to infectious disease. The variant KoRV A, is endogenous and at 100% prevalence in the northern majority of the koala's range (the states of Queensland and New South Wales (NSW), where the koala is listed as Vulnerable)[1]. Although it is endogenous, it has open reading frames, is actively transcribed and all koalas are viraemic[2,3]. KoRV A is exogenous in the southern states of Victoria and South Australia, with variable prevalence[1,2,4] and a much lower proviral number per cell[1]. It has been postulated that this is an exogenous retrovirus that is currently in the process of endogenizing[2]. KoRV A is the only subtype so far detected in Victoria[4].

Evidence for an effect of KoRV on the immune system of koalas is limited and is largely based on the argument that the spectrum of diseases that northern koalas frequently suffer from, which include neoplasia (e.g. lymphoma, leukaemia, mesothelial and craniofacial tumours) and infectious diseases (e.g. *Chlamydia*, cryptococcosis, and a range of opportunistic infections), is similar to that seen in cats infected by the closely related gamma-retrovirus, feline leukaemia virus (FeLV)[5,6]. The role of KoRV as an immune modulator has previously been questioned as most koalas infected with endogenous KoRV A are healthy, and authors have suggested co-factors may be necessary to cause disease[3]. Recent studies have, however, reported an association between exogenous KoRV infection and wet bottom (fur staining associated with chronic urinary incontinence) and low body condition score in Victorian koalas[4] and an association between the exogenous subtype KoRV B, and

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chlamydial disease[7] in Queensland koalas. Immunological studies have potential to further validate such observations and provide information on mechanisms involved.

Experimental evidence for an effect of KoRV A on the immune system is limited to a single study in which human peripheral blood mononuclear cells (PBMC's) incubated with KoRV A increased expression of the Th2 associated cytokines Interleukin 6 (IL6) and Interleukin 10 (IL10)[8]. Such studies have not been performed on koala cells. KoRV does contain several structural elements that are associated with immune suppression by closely related gamma retroviruses such as FeLV and Murine Leukaemia Virus (MuLV). These include the viral transmembrane protein p15E[5], which is associated with a multitude of immune suppressive effects including inhibition of lymphocyte activation by mitogens and modulation of cytokine expression by peripheral blood mononuclear cells (PBMC's)[5]. CKS17, a 17 amino acid sequence that corresponds to a highly conserved portion of the hydrophilic region of p15E, is reported to down regulate production of Th1 cytokines, including Interferon gamma (IFNγ), Tumour Necrosis Factor alpha (TNFα), Interleukin 2 (IL2), and to up regulate Th1 inhibiting cytokines, including IL10, in rodents and humans (reviewed in[9]).

Chlamydia is considered to be the most important pathogen of koalas[10] and control of chlamydial disease has been identified as the key component in long term survival of some threatened koala populations in NSW and Queensland[11,12]. Based on our current understanding of chlamydial pathogenesis, we may expect that inhibition of Th1 pathways would be associated with persistence and increased pathogenesis of chlamydial disease in infected koalas. The immune response to *Chlamydia* has been studied extensively in humans and animal models and it is established that elimination of chlamydial infection is reliant upon Th1 cytokines, such as IFNγ, which promote cytotoxic T cell responses[13]. The immunological hypothesis of chlamydial pathogenesis states that Th2 cells generated in response to infection with *Chlamydia* spp. may down regulate the protective Th1-type immune responses and promote persistent infection[14,15]. Supporting this, a Th2-dominated response, characterised by increased Interleukin 4 (IL4) and promoted by IL10, is associated with persistence of infection (reviewed in Menon *et al.*[16]). Although population wide studies in humans are limited, researchers have consistently found that IL10 promoting polymorphisms are associated with increased ocular scarring and infertility in people with ocular and genital chlamydial infections[17,18].

Outside of the Th1-Th2 paradigm, the role of the Th17 immune profile appears to play an important role in chlamydial infection as the magnitude and duration of infection with *C. muridarum* in mice is significantly decreased in the absence of Interleukin 17 (IL17)[19]. In koalas, PBMC's from those with chlamydial disease had greater gene expression for IL17A, but not IL10, IFNγ or TNFα, when stimulated with inactivated *Chlamydia pecorum*[20], which may indicate that the Th17 response also plays an important role in immune-mediated chlamydial pathogenesis in that species.

Other factors proposed to influence the immune response of koalas include stress[21], fluctuations in dietary plant secondary metabolites[22,23], nutrient availability[24], genetic differences[25,26], sex[27], hormonal fluctuations, season and photoperiod[28]. Subclinical infections with other organisms such as recently discovered Trypanosomes[29–32], herpesviruses[33,34] or unknown pathogens could also play a role. Our previous work indicates that season has a marked effect on cytokine expression and the CD4:CD8 expression ratio in koalas[35].

A reduction in CD4:CD8 ratio is often used as a measure of progression of immunosuppression in individuals infected with retroviruses including HIV, FIV and, to a lesser extent, FeLV[36–40]. The impact of KoRV and chlamydial infection on the CD4:CD8 ratio in naturally infected free-ranging koalas is difficult to predict; in more closely related gammaretroviruses, such as most strains of FeLV, the ratio is often unchanged[41]. Our previous studies have shown that the CD4:CD8 mRNA ratio varies markedly with season in koalas but there was no significant difference in koalas infected with KoRV B and those not infected (all koalas were KoRV A positive)[35].

Immunological studies of wild animals are uncommon and are inherently difficult due to the greater variation (environmental, genetic, nutritional, parasitic, hormonal, microbiological etc.) that is present, relative to the highly controlled models used in laboratory immunology. However, it is only in the context of these wild settings that we can measure the association of immune phenotypes with host health and fitness and the likely interplay of environment[42]. This is a novel and exploratory approach to the examination of the immune system and although they are gaining momentum, the fields of wild immunology and eco-immunology are informing the significant challenges of transferring laboratory concepts to free-ranging populations of animals and, indeed, humans[42].

In this challenging context, the current study seeks to determine whether an association between KoRV infection and cytokine and CD4:CD8 gene expression can be detected in free-ranging koalas. We make use of natural infection by *Chlamydia spp*. to permit study of the immune system at rest and under a natural immune stimulus. We hypothesise that immune parameters will differ between KoRV positive and negative koalas, and *Chlamydia spp*. positive and negative koalas and that KoRV positive animals may respond differently to the immune challenge of chlamydial infection compared to KoRV negative animals. We examine resting IFNγ, IL6, IL10 and IL17A, CD4 and CD8 mRNA expression in koalas in two populations in Victoria (Mt Eccles and Raymond Island), in which KoRV A and *Chlamydia pecorum* infect koalas.

Results

The sample population consisted of 74 koalas, 47 from Mt Eccles (all female) and 27 from Raymond Island (16 female, 11 male). Multivariate linear mixed model detected no significant effect of collection site on overall cytokine gene expression, thus the data from Mt Eccles and Raymond Island (which are at similar latitudes and were collected within one month of each other) were combined for analysis. The KoRV and *Chlamydia* status of the populations is presented in Table 1. Sixteen animals had dual KoRV and *C. pecorum* infections, 13 were KoRV negative and *C. pecorum* positive, 12 were KoRV positive and *C. pecorum* negative and 33 were KoRV and *C. pecorum* negative. Reference gene cytokine mRNA levels could be quantified in 74 samples and, of those, IL17A mRNA was quantifiable in 68 samples, IL10 in 61, IFNγ in 55, IL6 in 52, CD4 in 42, CD8 in 51 and CD4:CD8 in 39.

Site	KoRV + *C. pecorum* −	KoRV − *C. pecorum* +	KoRV − *C. pecorum* +	KoRV − *C. pecorum* −	Total
Mt Eccles	11	6	8	22	47
Raymond Island	5	7	4	11	27
Total	16	13	12	33	74

Table 1. Koala retrovirus (KoRV) and *Chlamydia pecorum* PCR status of the koalas sampled.

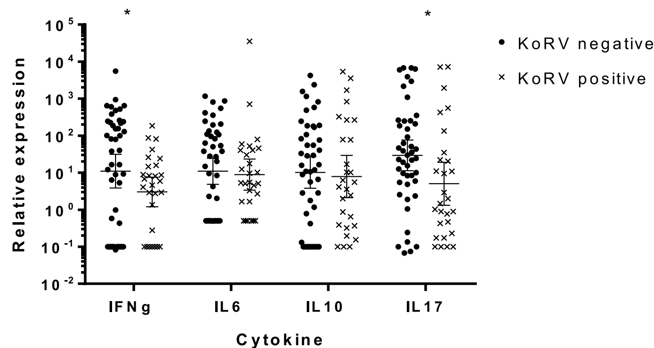

Figure 1. The effect of Koala retrovirus (KoRV) status on cytokine expression. Geometric mean and 95%CI of the geometric mean for relative expression of cytokine levels, compared to reference genes calculated using the ΔCq method[87]. IFNγ and IL17A were significantly decreased in KoRV positive koalas (*$p < 0.05$ Mann-Whitney U test).

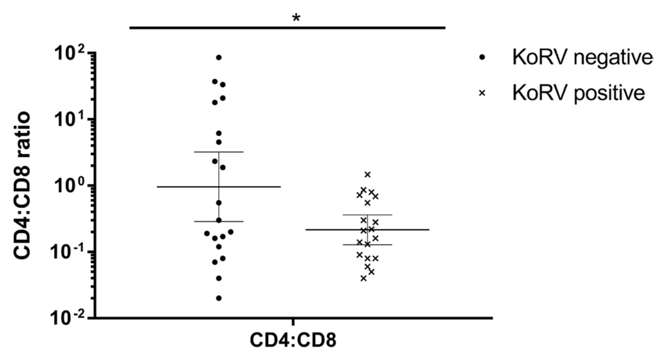

Figure 2. Effect of KoRV infection on CD4:CD8 expression ratio of KoRV negative (left, n = 20) vs KoRV infected (right, n = 19) koalas. Geometric mean (line) and 95% CI of the geometric mean for CD4:CD8 relative expression ratio calculated from CD gene expression relative to reference genes calculated using the ΔCq method[67]. The CD4:CD8 expression ratio is significantly lower in koala retrovirus (KoRV) positive koalas (*$p < 0.05$, T test with Welch's correction for uneven variance), and less variable.

There was a trend for overall cytokine gene expression to be lower in KoRV-positive koalas (multivariate linear mixed model; $p = 0.098$) and a significant effect of sex (multivariate linear mixed model; $p = 0.008$) on overall cytokine gene expression with males being higher than females. No overall effects of other variables, including *Chlamydia* infection status, age, abnormal health status, presence of pouch or back young were found. There was no overall interaction between KoRV and *Chlamydia* infection. There was no significant association between sex and KoRV (χ^2 test; $p = 0.77$) or *Chlamydia* status (χ^2 test; $p = 0.55$).

When the expression of genes was analysed individually, IL17A (Mann-Whitney; $p = 0.023$) and IFNγ (Mann-Whitney; $p = 0.044$) gene expression (Fig. 1) and CD4:CD8 gene expression ratio (t test; $p = 0.025$; Fig. 2) were significantly lower in KoRV positive koalas compared to negative koalas. Males had significantly higher resting expression of IL10 (Mann-Whitney; $p = 0.006$) than females. As males also had significantly higher resting expression of IL17A (Mann-Whitney; $p = 0.03$) (Fig. 3) analysis of KoRV effect on IL17 was repeated for females only and remained significant (Mann-Whitney; $p = 0.04$). There was no apparent effect of the presence of pouch or back young, abnormal health status or age on expression of any individual cytokine or on CD4:CD8 expression ratio.

There was a non-significant trend for an association between KoRV and chlamydial infection, as 68% of KoRV positive animals were also positive for *Chlamydia* compared with 42% of KoRV negative animals (χ^2 test $p = 0.19$). There was no effect of *Chlamydia* infection status or any additional effect of dual infection with KoRV and *Chlamydia* infection on cytokine expression.

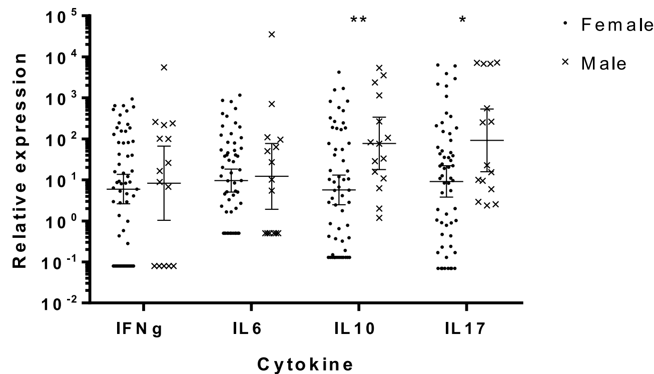

Figure 3. The effect of sex on cytokine expression. Geometric mean and 95%CI of the geometric mean for relative expression of cytokine levels, compared to reference genes calculated using the ΔCq method[67]. IL10 and IL17A were significantly greater in males ($*p < 0.05$, $**p < 0.01$ Mann-Whitney U test).

Discussion

This is the first report of immunophenotypic changes associated with KoRV infection and sex in free-ranging koalas. Although this study is unable to determine the biological impact of the changes observed, the lower resting expression of IFNγ, IL17A genes and lower CD4:CD8 gene expression ratio in KoRV-infected koalas provides a way forward to further explore mechanisms behind recently reported association of KoRV infection with disease in these populations[4]. To the authors' knowledge this is the first study of this type in koalas or marsupials in general and demonstrates that, although studies on wildlife immunology contain the inherent risks that potential variation caused by the disease of interest will be undetectable, this approach can provide valuable information about the immune response and infectious diseases in koalas and wildlife in general within the context of their normal environment. Sex-effects on cytokine gene expression will be important to consider in future immunological studies of koalas.

Studies examining the effect of infectious disease on cytokine expression in wild or semi wild animals are limited, as immunological methods are usually species specific. Most studies are recent and, similar to this study, use real time PCR for cytokine mRNA quantification. Even with the high sensitivity of these assays cytokine mRNA is often below quantifiable limits in non-stimulated cell samples[43] and some studies have found mostly inconclusive results[44]. The study of wildlife immunology is in its infancy and transfer of our knowledge of immunology, which is mostly derived from laboratory animals, to wild-living animals will be difficult but is expected to develop as technological (species specific reagents and annotated genomes) and statistical methods improve[42]. Koala populations are coming under increasing threat, especially in the northern part of their range, and studies such as this are an essential component of understanding the immune mechanisms underpinning this susceptibility to infectious disease and to help these threatened populations survive.

The present study has produced several findings that indicate follow-up flow cytometric studies using novel or cross-reactive antibodies to CD4[45], IFNγ[46] or IL17 would be worthwhile. The decision to use baseline gene expression in the present study, rather than cells preserved for flow cytometry, was driven by field constraints on sample storage and has its limitations; it will be important to determine whether reduced CD4:CD8 gene expression ratio and reduced expression of IFNγ and IL17 genes in the present study is related to gene down-regulation, or deletion of cells. Depletion of CD4+ cells has been reported with some fatal strains of the gammaretrovirus FeLV[38,40] and Th1 lymphocyte depletion can be induced by HIV[47,48]. The lower level of IFNγ gene expression observed in KoRV infected koalas in our study is suggestive of a switch from Th1 to Th2 CD4+ cell activity but this could not be directly examined as IL4, a key Th2 cytokine, was below quantifiable levels in our study.

It may be that KoRV infection is similar in pathogenesis to feline retroviral infections (FIV and FeLV), which are associated with a general cytokine dysregulation[49–51] rather than a clear Th1 to Th2 switch. The decrease seen in the CD4:CD8 gene expression ratio and IL17 and IFNγ gene expression in KoRV infected koalas is consistent with the effect of other retroviruses (HIV, FIV and to a lesser degree FeLV) in humans and felids[36–40] but the pattern is not clear cut in that, although the changes observed would be consistent with the up-regulation of IL10 observed in human lymphocytes incubated with KoRV *in-vitro*[8], no significant difference in IL10 expression was seen in association with KoRV in the present study.

Functional studies involving lymphocyte stimulation are also likely to add a valuable additional perspective. Human studies looking at both unstimulated and stimulated blood cytokine levels have found contrasting values (e.g. lower unstimulated but higher mitogen stimulated expression of IL6 and TNFα in diabetic patients compared to controls[52]). Additionally, our previous study that examined cytokine expression among KoRV A/B positive and KoRV A positive animals found KoRV B infection was associated with increased up-regulation of all measured cytokines (IFNγ, TNFα, IL4, IL6, IL10 and IL17A) in stimulated cells but in unstimulated cells a difference was only detected in IL10 gene expression[35].

The changes seen in association with KoRV infection in the current study are potentially relevant to the recent detection of an association between wet bottom (rump staining by urine, which is most commonly associated with chronic chlamydial disease) and KoRV infection in Victorian koalas[4]. As CD4+ cells play an essential role in the control of chlamydial infection in both humans and the murine model[46,53], depletion of CD4+ cells or reduction in CD4 expression could also play a role in making KoRV infected koalas more susceptible to chlamydial

infection and disease. The lower levels of IFNγ expression associated with KoRV infection in this study could also, in theory, make koalas more susceptible to chlamydial persistence, as IFNγ production is essential to eliminate or prevent the dissemination of infection[13]. Blocking production of IFNγ significantly prolongs chlamydial infection[54], exacerbates pathological lesions and increases chlamydial load[55]. There is also evidence in humans that haplotypes associated with reduced IFNγ production are associated with increased scarring secondary to ocular chlamydial infections[56]. If KoRV infected koalas have a reduced IFNγ response when exposed to chlamydial infection this could result in increased dissemination, higher pathogen burden, prolongation of disease and increase in pathological lesions in these animals. The relevance of reduced Th17 expression to chlamydial disease in koalas requires further investigation; while it is likely an important component of defence, elevated Th17 responses are also associated with chlamydial pathology[19] so limiting Th17 responses may either exacerbate pathogenesis by prolonging infection or moderate immune-mediated pathogenesis.

Although there was a tendency for association between KoRV and chlamydial infection in this cohort it was not statistically significant and there was no relationship between chlamydial infection and cytokine expression or CD4:CD8 expression ratio. This may be related to the inability of the chlamydial PCR assay to discriminate between animals with clinical and subclinical disease, which would be expected to have differing immunophenotypes. Similar to other southern Australian populations[10,57,58], the populations studied have high levels of sub-clinical chlamydial infection[59].

The effect of sex on immune parameters in the current study is a vital consideration for future immunological studies in this species. The greater expression of IL10 and IL17A genes in males, relative to females, is likely to reflect that, unsurprisingly, sex hormones influence the resting expression of these cytokines in koalas. These samples were taken during the koala mating season[60], and the greater IL10 expression in males may be associated with increased testosterone production, as testosterone is associated with increased IL10 production in humans and mice[61,62]. The sex hormone effect on IL17 production has been less well studied, however oestrogen has been found to inhibit Th17 cell differentiation in mice[63] and might explain the lower levels of IL17 in found in female koalas in this study.

Conclusion

This study presents the first evidence that exogenous KoRV A infection is associated with alterations in the immune system of koalas. Despite the heterogeneity of free ranging animals, a statistically significant reduction in resting IFNγ and IL17A levels and decrease in the CD4:CD8 gene expression ratio was found in koalas infected with KoRV A. The decreased expression of IFNγ could potentially make KoRV infected koalas more susceptible to disseminated chlamydial disease and could be an underlying mechanism behind the recently discovered association between KoRV infection and wet bottom in Victorian koalas[4]; however the current study does not confirm a role for KoRV in disease but rather reports associative relationships between KoRV infection and immune parameters that will be useful in targeting future *in vitro* and functional studies of causation and pathogenesis. Such studies would include examination of the cytokine response of koala cells (e.g. PBMC's) or cell lines to KoRV infection *in-vitro*, along with characterisation of the CD4+ and CD8+ cell responses in flow cytometric studies. It comprises a first step on the path to understanding the immune mechanisms underpinning the susceptibility to infectious disease in koala populations, which is essential to help these threatened populations survive. It also demonstrates that, while inherently risky and challenging, wildlife immunology studies can provide valuable information about diseases and immune responses in wild animals.

Materials and Methods

Ethics statement. All animal handling and sampling was conducted under a permit issued by the Victorian Department of Sustainability and Environment and Parks Victoria (permit no. 10005388). The University of Melbourne Animal Ethics Committee granted approval for physical examinations and sample collection on animals that were captured and restrained for routine management operations (ethics 1011687.1).

Koala sampling and clinical examination. The clinical examinations and sample collection for these koalas is described previously[59]. This occurred during routine population management operations conducted by the Victorian Department of Sustainability and Environment and Parks Victoria at Raymond Island (37.9088°S, 147.7545°E; October 2010) and Mt Eccles National Park (38.0667°S, 141.9167°E; September and October 2010).

Briefly, clinical examination included estimation of age from tooth wear, abdominal and lymph node palpation, scoring of "wet bottom" lesions (staining and maceration of rump epithelium and pelage), ocular or urogenital abnormalities, and body condition, along with noting the presence of pouch or back young. Teeth were graded as per the method of Martin[60] and McLean[64]; age class was defined as young (0–3.5 years, tooth wear class 0–1), mature (3.5–10 years, tooth wear class 2–7) or old (>10 years, tooth wear class 8–9). Ultrasound examination was performed on animals that were anesthetized and did not have pouch young ($n = 22$, all female). The wall and lumen of uteri, the urinary bladder, and the width of each kidney were measured. Any other urogenital abnormalities, including para-ovarian cysts, were also noted. The data from the physical examination were used to classify the animals as being in normal or abnormal health; abnormal health was based on palpation of reduced gut fill, enlarged lymph nodes, reduced body condition, the presence of wet bottom or any ocular, urogenital abnormalities or ultrasonographic abnormalities.

A minimum of 2 mL of blood was collected from the cephalic vein into EDTA anticoagulant. Following centrifugation, plasma was withdrawn and 0.5 mL buffy coat was collected from each sample using a micropipette and stored in 1 mL RNAlater (Sigma-Aldrich, St Louis, MO, USA) for cytokine mRNA analysis. Remaining sample was frozen at −20 °C and then at −70 °C for longer-term storage for KoRV testing.

KoRV and *chlamydia* testing. For KoRV provirus testing, DNA was extracted from 200 µL of each sample using DX Universal Liquid Sample DNA Extraction Kit (Qiagen, Doncaster, Vic, Australia) and a QIAextractor robot (Qiagen, Doncaster, Vic, Australia). Each 96 well plate had six negative control wells containing sterile water and one positive extraction control well containing diluted liquid culture of *E. coli* containing a portion of KoRV *pol* inserted into pGEM®-T (Promega) plasmid. Conventional PCR was used to screen clinical samples for the presence of KoRV proviral DNA. This PCR utilised primers targeting KoRV *pol* gene (5′TTGGAGGAGGAATACCGATTACAC- 3′ (sense) and 5′-GCCAGTCCCATACCTGCCTT-3′ (antisense))[65] and thus amplified all KoRV variants though, to date, no variant other than KoRV A has been detected in Victorian koalas[4]. qPCR was performed using a GoTaq Flexi DNA polymerase PCR kit (Promega, Madison, WI, USA). Each 25 µL reaction contained 5 µL of GoTaq Flexi Buffer, 10 mM of MgCl, 125 µM of each dNTP, 50 µM of each primer, 1.5 U of GoTaq DNA Polymerase, 6.7 µL of RNase-free treated water and 5 µL of template DNA. Cycling conditions (Icycler Thermal 45 Cycler, Bio-Rad, Hercules, CA, USA) were 95 °C for 1 min, followed by 35 cycles of 95 °C for 30 sec, 53 °C for 30 sec, 72 °C for 1 min. Each PCR included a positive control (diluted purified cloned KoRV *pol* DNA) and negative control (water). Following PCR amplification, all samples were subjected to agarose gel electrophoresis. To confirm specific amplification of KoRV DNA, band excision, gel DNA extraction using QIAquick gel extraction kit (Qiagen, Doncaster, Vic, Australia), and nucleotide sequencing (BDT3.1 Chemistry, Applied Genetic Diagnostics, Department of Pathology, The University of Melbourne) was performed on products of the expected size (110 bp).

Sampling and testing for *Chlamydia*, on ocular and urogenital/penile swabs, by qPCR was performed as previously described[59]. For positive samples, chlamydial species was determined by qPCR HRM curve analysis; only *C. pecorum* was identified[59].

Cytokine expression analysis. The mRNA was extracted from buffy coat in RNAlater using the RNAeasy minikit (Qiagen, Doncaster, Vic, Australia) following manufacturer's instructions. Extracted samples were treated with amplification grade DNAse 1 (AMPDI-1KT; Sigma Aldrich, MO, USA) to remove contaminating DNA, then cDNA was synthesised using the Revertaid first strand cDNA synthesis kit (Thermo Scientific, Lithuania). To control for contamination with genomic DNA 'no reverse transcriptase' (NRT) controls were made using the same protocol and omitting reverse transcriptase. The concentration and purity of RNA was assessed using a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

Reference gene (28 s and GAPDH) and cytokine/CD (IL6, IL10, IFNγ, CD4 and CD8) qPCR were performed in triplicate as described previously[66]. Conditions for IL17A qPCR[20] were re-optimised for our laboratory. Samples were excluded from analysis if the reference gene amplification fell below the limit of quantification[66].

Statistical analysis. Raw Cq values were transformed to 2^{-Cq} for analysis. Resting immune gene expression levels in samples were measured using the $2^{-\Delta Cq}$ method[67]. Cytokine expression was normalised against the geometric mean of the two reference genes 28 s and GAPDH. These have previously been found to be valid reference genes for koala cells[66].

The data were log transformed and then Shapiro-Wilk analysis was used to confirm normality and a χ^2 test used to confirm independence between sex, KoRV and chlamydial infection. Multivariate linear mixed models were then used to examine interactions across multiple cytokines and two-way ANOVA was used to examine interactions between cytokine expression and multiple independent variables (e.g. KoRV, *Chlamydia* status, or sex) (GenStat 17th edition, VSN International Ltd.). Cytokine results below quantifiable limits were included in calculations at a value equal to the lowest measurable value, to avoid bias associated with exclusion of results below quantifiable limits. Therefore, a conservative non-parametric approach was taken when conducting post-hoc analysis: post hoc *Mann-Whitney U t*ests (GraphPad Prism 7.02) were performed, and only on cytokines that appeared most likely to differ between two populations (e.g. KoRV positive and negative) based on visual examination of scatterplot data. A p value of <0.05 was considered significant.

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Author Contributions
I.M. and D.H. conceived of and planned the study. J.P., M.C. and J.D. planned and executed sample and field data collection as well as *Chlamydia* and KoRV testing. I.M. executed the immunology components and analysis under supervision of D.H. I.M. and D.H. wrote the manuscript and prepared the figures. All other authors reviewed and provided input to the manuscript.

Additional Information
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