Molecular characterisation and expression analysis of Interferon gamma in response to natural *Chlamydia* infection in the koala, *Phascolarctos cinereus*

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ABSTRACT

Interferon gamma (IFNγ) is a key Th1 cytokine, with a principal role in the immune response against intracellular organisms such as *Chlamydia*. Along with being responsible for significant morbidity in human populations, *Chlamydia* is also responsible for wide spread infection and disease in many animal hosts, with reports that many Australian koala subpopulations are endemically infected. An understanding of the role played by IFNγ in koala chlamydial diseases is important for the establishment of better prophylactic and therapeutic approaches against chlamydial infection in this host. A limited number of IFNγ sequences have been published from marsupials and no immune reagents to measure expression have been developed. Through preliminary analysis of the koala transcriptome, we have identified the full coding sequence of the koala IFNγ gene. Transcripts were identified in spleen and lymph node tissue samples. Phylogenetic analysis demonstrated that koala IFNγ is closely related to other marsupial IFNγ sequences and more distantly related to eutherian mammals. To begin to characterise the role of this important cytokine in the koala's response to chlamydial infection, we developed a quantitative real time PCR assay and applied it to a small cohort of koalas with and without active chlamydial disease, revealing significant differences in expression patterns between the groups. Description of the IFNγ sequence from the koala will not only assist in understanding this species' response to its most important pathogen but will also provide further insight into the evolution of the marsupial immune system.

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1. Introduction

The koala (*Phascolarctos cinereus*) occupies a unique status in Australian culture. Despite continued efforts by a variety of groups to conserve this species, wild koala numbers continue to decline, prompting the Australian Government to include the koalas of Queensland, New South Wales and Australian Capital Territory in the threatened species list (Koala species listing, 2012). Among the many factors that contribute to the decline of koala populations across different regions of Australia, habitat destruction is reported to have the most

wide reaching effects (Melzer et al., 2000). In order to stabilise these declining populations, however, modelling has shown that a 50% reduction in the incidence of disease would likely have the most success (Rhodes et al., 2011). The main aetiological agent responsible for morbidity in this marsupial species is *Chlamydia pecorum*, with a reported prevalence rate as high as 72–100% in some populations (Polkinghorne et al., 2013). Common manifestations of *C. pecorum* infection in koalas are cystitis, proliferative conjunctivitis and chronic, fibrotic disease of the urogenital tract leading to infertility and death (Polkinghorne et al., 2013). In attempting to understand chlamydial disease pathogenesis, we have previously shown that *C. pecorum* infectious load is semi-independent of chlamydial disease chronicity in the koala (Wan et al., 2011). Many koalas with high infectious loads display either no effects of infection or active, proliferative disease, while koalas with low infectious loads often suffer severe, chronic disease manifestations. The latter observation is consistent with studies of other *Chlamydia*-infected host species where, in the absence of high infectious loads of *Chlamydia*, cross-reactivity of the host response to infection has also been implicated in disease pathogenesis (Darville and Hiltke, 2010).

The ability to accurately measure the host immune response to chlamydial infection has been central to understanding the relationship between infection and disease. In this regard, in non-koala hosts, it has been shown that the activity of a key cytokine, interferon gamma (IFNγ), is required for the resolution and control of chlamydial infections (Loomis and Starnbach, 2002). IFNγ is a pleiotropic dimeric pro-inflammatory cytokine secreted by T cells and Natural Killer (NK) cells (Farrar and Schreiber, 1993). This cytokine influences both the innate and cell-mediated immune response, playing important roles not only in the activation of pathways to recognise and clear intracellular pathogens (Rottenberg et al., 2002) and tumours (Shankaran et al., 2001) but also in (i) immunoglobulin class switching, genesis of T-helper 1 (Th1) cells (Farrar and Schreiber, 1993); (ii) enhanced major histocompatibility class 2 (MHC-II) expression on mononuclear phagocytes and dendritic cells (Billiau and Matthys, 2009) and; (iii) differentiation of monocytes to macrophages (Delneste et al., 2003). Reflecting these important roles, it is not surprising to find that this gene has a wide distribution across most terrestrial and marine vertebrate lineages with a suspected evolutionary origin that dates back greater than 450 Ma ago (Savan et al., 2009). It is interesting to note that in spite of its role in immune response to pathogens and the low sequence similarity observed among the major lineages of subphylum vertebrate, evolutionary conservation of the core structural features important in the biological function of the molecule has been identified (Savan et al., 2009).

Our efforts to understand the koala immune response to chlamydial infection, including the IFNγ response, are restricted due to the lack of sequence information (and assays) for key koala cytokines. While we have been able to overcome some of these challenges using conventional PCR and degenerate primers based on mammalian orthologues (Mathew et al., 2013), this approach is challenging due to a low level of sequence conservation for IFNγ, and attempts to use this approach in other Australian marsupials have experienced mixed success (Harrison and Wedlock, 2000). The emergence of high-throughput methodologies to sequence coding genes from non-model organisms has revolutionised our ability to identify immune genes of interest from organisms with no previous genomic resources (Wong et al., 2006) and has allowed us to rapidly acquire and analyse the complete immune gene repertoires of an increasing number of non-model hosts (Hoffman et al., 2013; Papenfuss et al., 2012).

As a part of our ongoing efforts to characterise the transcriptome of the koala (unpublished data), we report the molecular analysis of a marsupial IFNγ. In an effort to understand the role of this cytokine in the koala host response to chlamydial disease, we designed koala-specific quantitative real time (qrtPCR) assay and performed preliminary analysis on the expression of this cytokine gene in healthy koalas as well as in animals with overt chlamydial disease. This analysis not only provides insight into the function of this gene in the koala but sheds light on the evolution of the Th1 response in marsupials.

2. Materials and Methods

2.1. Ethics statement

Queensland University of Technology Animal Ethics Committee (Approval No. 0700000845) approved the collection and subsequent analysis of the koala blood samples. Blood samples were collected by qualified veterinarians from koalas admitted to the Australia Zoo Wildlife Hospital, Beerwah, Queensland, Australia. Blood was collected from animals anesthetised for other clinical procedures.

2.2. Transcriptomic analysis of koala tissues

Total RNA was extracted from four immune related tissues; liver, lymph node, spleen and bone marrow, using a Trizol/Chloroform extraction. Paired-end sequencing of polyA enriched samples was performed on an Illumina HiSeq 2000, at Beijing Genomics Institute. For each tissue type, raw sequencing data was *de novo* assembled using CLC Genomics workbench version 6.0.2 using the following settings: kmer 24: mismatch cost 2; limit 8; insertion cost 3; deletion cost 3; length fraction 0.8; similarity 0.8; minimum distance 150; maximum distance 250. Assembled contigs for the different tissue libraries were used as BLASTx queries against the IFNγ protein sequence from *Monodelphis domestica* with a stringency of greater than 1×10^{-5}. Contigs that met these stringency requirements were used for downstream bioinformatic analysis.

2.3. Bioinformatics analysis of koala IFNγ sequence

Multiple sequence alignments were carried out using the Geneious alignment program in the Geneious Pro 5.6.5 software at a cost matrix of 65% similarity and GeneDoc version 2.7.000 software. Phylogenetic trees were constructed using Jukes Cantor, neighbour joining tree build method in Geneious Pro 5.6.5. IFNγ sequences for alignment and phylogenetic tree construction were obtained from GenBank and Ensembl: armadillo (DQ094083), buffalo (EU277737), camel (AB107657), cat (X86972), dog (FJ194478), elephant (EU000432), platypus (XM_001511108), human (BC070256), monkey (NM_001032905), chicken (AY501004), pigeon (DQ479967), common carp (AM168524), goldfish (EU909368), opossum (Wong et al., 2006), tammar wallaby (ENSMEUT00000007026) and Tasmanian devil (ENSSHAT00000017850). The koala IFNγ was uploaded to GenBank with accession number KC894757.

2.4. Koala sample collection

PBMCs were harvested from 10 koala blood samples for use in this study. 5–6 ml of blood was collected in 6 ml EDTA blood tubes from koalas brought into the hospital. Bloods were stored at 4 °C until further processing on the same day. Swabs for *Chlamydia* analysis were collected from the conjunctiva of the left eye, right eye, urogenital sinus (females) and urethra (males) using aluminium shafted cotton tipped swabs (Copan, Interpath Services, Melbourne). Current and previous *C. pecorum* infection status of these animals was determined using a previously described 16SrRNA *C. pecorum* species-specific qrtPCR assays (Wan et al., 2011) and western blots using *C. pecorum* His-tagged major outer membrane protein (MOMP) A, F and G (Kollipara et al., 2012).

2.5. IFNγ qrtPCR assay design and optimisation

The koala IFNγ mRNA sequence obtained from the lymph node transcriptome was used to design primers for IFNγ qrtPCR assay. Primers were designed to target a 167 bp product which spanned across exon 1 and exon 3 in order to avoid co-amplification of contaminating genomic DNA. The primers used for the IFNγ qrtPCR assay were as follows: F (5′-AGCTACCTCTTAGCATCC-3′) and R (5′-TCCTCTTTCCAACGATCC-3′). This 167 bp koala sequence was cloned in to a plasmid using the Promega pGEM-T Easy Vector Systems I as described in Mathew et al. (2013). The successfully cloned PCR product was then sequenced at the Australian Genome Research Facility using the AB 3730*xl* platform to confirm specificity of the primers used.

SYBR Green I based dye chemistry was used to optimise the IFNγ qrtPCR assay. Assay efficiency was determined using a standard curve constructed using serial dilutions of the 167 bp PCR product as previously described (Mathew et al., 2013). All reactions were carried out on a Corbett Rotor Gene 6000 real time PCR machine at a final volume of 25 μl, with 1.3 units of FastStart Taq Polymerase (Roche), 2.5 μl of 25 mM MgCl$_2$ (Roche), 2.5 μl of 10× buffer (Roche), 2.5 μl of 10 mm dNTPs (Roche), 3 μl of 1/10,000 SYBR Green and 1 μl each of 10 mM forward and reverse primers. After an initial incubation of 95 °C for 5 min, 35 cycles of 25 s at 95 °C, 30 s at 52 °C and 35 s of