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Further characterisation of cytokines in macropod marsupials: IL-10 and IL-10Δ3

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ABSTRACT

Interleukin-10 is an immunomodulatory cytokine that has been implicated, along with IFN-γ, in the disease sequelae of mycobacterial infection. In order to investigate the role of IL-10 in marsupial disease models we sequenced and characterised the *IL10* gene in the tammar wallaby (*Macropus eugenii*) and rufous hare-wallaby (*Lagorchestes hirsutus*). An isoform IL-10Δ3, in which an in-frame deletion of exon 3 occurs, was discovered in both macropod species. Analysis of wallaby and other reported marsupial IL-10 homologs suggests that while marsupial IL-10 is comparable to that of human IL-10, the predicted IL-10Δ3 protein may play a more complicated role in the modulation of IL-10-directed responses. Expression of the canonical gene and splicing variant was confirmed in both wallabies, and the rufous hare-wallaby showed differential expression across lymph node, spleen and liver, with isoform expression detected in the lymph node. This characterisation and expression of IL-10 in *de novo* tissues provides a basis for further study into the role of IL-10 in disease models in marsupials.

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1. Introduction

Metatherian mammals (marsupials) diverged from the eutherian line around 160 million years ago [1]. Abundant in Australia, this diverse group includes possums, wallabies, potoroos, the koala (*Phascolarctos cinereus)*, and the Tasmanian devil (*Sarcophilus harrisii*). The marsupial immune system is similar to other mammals [2,3], but distinctive features such as the T-cell Receptor μ chain [4], an expansion of antimicrobial peptides [5], and a novel family of Class I MHC genes [6] differentiate this system from that of their eutherian relatives.

Marsupials, including the family *Macropodidae*, have a reported vulnerability to intracellular pathogens such as *Mycobacterium* spp. [2,7]. In order to outline the marsupial response to these bacteria and others like it, the cytokine milieu and the cytokines themselves must first be characterised. The gene sequences and expression of several cytokines including IL-4 [8], IL1-β [9], IL-21 [10], IL-6 [11], and IFN-γ [12], have been established in the model macropod, the tammar wallaby (*Macropus eugenii*). More have been predicted *in silico* [13,14], which allows this information to be used as a platform for cytokine investigations into closely related, yet vulnerable species such as the rufous hare-wallaby (*Lagorchestes hirsutus*) [15]. To date, only IFN-γ has been reported in the rufous hare-wallaby, where high levels of similarity and identity between the tammar and rufous hare-wallaby and other marsupial species (the koala, gray short-tailed opossum (*Monodelphis domestica*), and Tasmanian devil), were also reported [12].

Interleukin-10 (IL-10), an immunomodulatory cytokine, functions as a homodimer in which each molecule has six α-helices in its tertiary structure, each molecule contributing three helices plus one from the dimeric chain to form the classic four alpha helical bundle [16]. It interacts sequentially with IL10R1 and IL10R2 to instigate signalling via the JAK-STAT pathway, resulting in the down-regulation of the expression of stimulatory cytokines such as TNF-α and IFN-γ. Regulation of IL-10 expression occurs primarily through epigenetic remodelling at the gene locus, while post-transcriptional regulation occurs through 3′UTR elements and microRNAs [17,18]. The genomic sequence has a highly conserved structure of five exons and four introns, and maps to chromosome one in both humans and mice [19]. It has been predicted in the tammar wallaby genome (Meug_1.0) in Ensembl v83 (www.ensembl.org) (ENSMEUG00000006779), fully sequenced in the brushtail possum (*Trichosurus vulpecula*) [20] and the gray short-tailed opossum [21], partially sequenced in the koala [22], and has been identified in the transcriptome of the Tasmanian devil [23].

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Interleukin-10 plays a regulatory role in both the innate and specific immune response. It has been implicated as a key factor in the control of the pathology of mycobacterial infection in humans [24,25] however, the effects of IL-10 on disease progression in mycobacterial infection is dependent on both the host species, and pathogen strain [17]. Research into the functional role of marsupial IL-10 in disease is limited, however its expression has been shown to be up-regulated in alveolar macrophages following LPS stimulation or *Mycobacterium bovis* infection in the brushtail possum [20]. More recently, up-regulation of IL-10 expression was demonstrated in both stimulated and actual chlamydiosis in koalas [26].

The expression of IL-10 has not yet been confirmed in a macropod species, nor has any known isoform [27–29] been reported within the marsupial group. This study reports the sequence, protein modelling and expression of the IL-10 gene (*IL10*) and its splicing variant, IL-10Δ3, in the tammar wallaby. Furthermore we report the expression of IL-10 and IL-10Δ3 mRNA in the closely related and vulnerable macropod, the rufous hare-wallaby.

2. Materials and methods

2.1. Tissue collection and preparation

2.1.1. Tammar wallaby

In order to confirm sequence and expression of the *IL10* gene in the context of macrophage response, we first obtained samples from the tammar wallaby as described previously [2,3,11,12]. Briefly, peripheral blood mononuclear cells (PBMC), lymph node leukocytes and PBMC enriched for adherent cells were prepared using density gradient medium as previously described. Cell preparations were cultured with serum-activated zymosan, PHA, and LPS respectively [3,30,31]. Total RNA was extracted using Tri-reagent (Sigma-Aldrich, Australia). Complementary DNA was synthesized using A3500 Reverse Transcription System (Promega, Australia), followed by the amplification of a 139 nt GAPDH product (primers from Daly, Lefévre, Nicholas, Deane and Williamson [32]) to verify RNA integrity.

2.1.2. Rufous hare-wallaby

Tissues including spleen, lymph node, kidney, liver, and muscle were collected opportunistically from a captive breeding population at Alice Springs Desert Park (NT, Australia) as previously described [31], and stored at −80 °C until further use. Total RNA was extracted following the methodology for the SV Total RNA Isolation Kit (Promega) using approximately 30 mg of tissue, with all samples analysed by Nanodrop for purity. Reverse Transcription was performed using GoScript Reverse Transcription (Promega), with subsequent cDNA amplification using GAPDH primers (Table 1).

2.2. Characterisation of cytokines

As the *IL10* gene prediction for the tammar wallaby was available in Ensembl v.83 (ENSMEUG00000006779) and appeared complete, this data was used to design gene specific UTR (untranslated region) primers that flanked the coding domain of the gene to confirm expression of the *IL10* transcript. Primers 10CDF and 10CDR (Table 1) were then used to obtain the full construct from cDNA of tammar wallaby leukocytes stimulated with zymosan. The full coding domain sequence of the rufous hare-wallaby was determined using cDNA prepared from spleen and lymph node tissues of two animals with the start primer and 3′UTR primer (Table 1). The primers 10CDF and 10CDR, located in the UTR of *IL10*, were unable to amplify any detectable product in the rufous hare-wallaby under the conditions used, with the 10CDF primer appearing selective for the tammar wallaby. In all cases, except for the initial screening, PCR was performed with Platinum Hi-Fi Taq (Invitrogen, Australia) at a 55 °C annealing temperature for 38 cycles with two replicates. Results were visualised under UV light using ethidium bromide gel electrophoresis and the amplicons were purified using Wizard SV Gel and PCR Clean-up System (Promega). The predicted amplicons were sequenced (Australian Genome Research Facility (AGRF), St Lucia, Australia) and compared by alignment to the annotated *IL10* gene in Ensembl v.83 to confirm its identity.

2.3. Expression of cytokines in the tammar wallaby

Expression and identification of IL-10 in the tammar wallaby was performed using the primers, MeIL10F and IL10R (Table 1), to screen stimulated tissue preparations of the tammar wallaby. These primers were designed within regions of highly conserved amino acid residues from an alignment of known mammalian sequences. The MyTaq HS polymerase (Bioline, Australia) was used with a 55 °C annealing temp for 35 cycles. We then used primers 10CDF and 10CDR (primers designed to incorporate marsupial sequence data available at that time; Table 1) to screen for IL-10 in control and zymosan-stimulated tammar wallaby leukocytes. The cDNA was normalised for GAPDH expression as described previously [11] and PCR performed using Platinum Hi-Fi Taq (Invitrogen, Australia) at a 55 °C annealing temperature for 35

Table 1
Primers used in this study.

Target	Primer	Location	Sequence	nt
Sequencing primers				
IL-10	10CDF	5′UTR	5′-GAAGACAAAGCCAAGCCCTA-3′	20
	MeIL10F	Start	5′-ATGCCTACCTTGATGCTGCTG-3′	20
	IL10R	Exon 5	5′-TCAAAATCCCCCATTGCTTTGTAGACTCC-3′	29
	10CDR	3′UTR	5′-CAGAGGCACCATCTTGGTTT-3′	19
Expression primers				
GAPDH	GapqF		5′-CTACAGTCCATGCCATTACTGCTAC-3′	25
	GapqR	–[a]	5′-CTGTGAGCTTCCTGTTCAGCTCAG-3′	24
IL-10	IL10qF	Exon 1–2	5′-GCAGTGTGAAGATATACTTTCAAATG-3′	25
	IL10qR	Exon 3–4	5′-GGAATCTGTGACAACGCTTGA-3′	21
IL-10Δ3	IL10q3F	Exon 1	5′-CTTGATGCTGCTGCTCTGC-3′	19
	IL10q3R	Exon 2–4	5′-GGGAGGAATCTGTGCTTTAATTCTTT-3′	25

[a] Denotes primer spanning the exonic boundary in GAPDH for which annotation of exons is not complete. Primer direction noted using F (forward) or R (reverse) as last letter of name.

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