

## Molecular Characterization of Interleukin-1Beta in the Tammar Wallaby (*Macropus eugenii*)

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**ABSTRACT.** Interleukin-1beta (IL-1 $\beta$ ) plays a significant role in the onset and pathogenesis of inflammation in mammalian hosts. Although well characterized in a range of vertebrate species, little is known about this important cytokine in marsupial mammals. We report here the molecular cloning and characterization of IL-1 $\beta$  in the tammar wallaby (*Macropus eugenii*). *M. eugenii* IL-1 $\beta$  has an open-reading frame of 813 nucleotides, coding for a putative protein of 270 amino acids to the termination codon. The IL-1 family motif and potential caspase cleavage site (necessary for production of the mature protein) is also present in the sequence. Molecular characterization of tammar wallaby IL-1 $\beta$  provides fundamental information necessary to progress the study of functional immune responses in this unique group of mammals.

**KEY WORDS:** cytokine, IL-1 $\beta$ , interleukin-1, *Macropus eugenii*, marsupial.

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Proinflammatory cytokines such as interleukin-1beta (IL-1 $\beta$ ) and tumor necrosis factor alpha (TNF- $\alpha$ ) play important roles in the pathogenesis of disease [3]. Well characterized for their roles in innate immunity and the inflammatory response [23], IL-1 $\beta$  and TNF- $\alpha$  have also been used as adjuvants to enhance host immunity [10]. More recently, the IL-1 and TNF family of molecules have been reported as important regulators of both adaptive and innate immune responses that include the development and differentiation of CD4+ T helper cell subsets [1, 28] and host defence to infections with atypical mycobacteria [25].

Marsupials (or metatherians) are mammals that diverged from their eutherian relatives over 100 million years ago [22]. Although the architectures of their immune systems are similar to those of eutherians, there are reported differences involving reduced T and B lymphocyte responses that have yet to be explained [26]. Captive marsupial populations are known to be susceptible to infections with atypical mycobacteria [8], a disease that has the potential to mitigate the success of captive-breeding populations of endangered marsupials in Australia [20]. The brushtail possum (*Trichosurus vulpecula*), the only marsupial species to date for which IL-1 $\beta$  has been characterized [24], is also an important reservoir for *Mycobacterium bovis* and is of economic concern in New Zealand. The tammar wallaby (*Macropus eugenii*), considered by some to be a model marsupial species, is susceptible to infection with hydatid cysts [5] and also has little or no inflammatory response to endoparasite infections such as coccidiosis [11]. Together with the apparent differences in adaptive immune responses, this susceptibility to infection and reduction of host protective

immunity suggests that an investigation of IL-1 is warranted. Here, we report the IL-1 $\beta$  coding sequence for the first macropod marsupial, the tammar wallaby, and describe the key features that support its identification as IL-1 $\beta$ .

The nucleotide sequence of the coding domain for tammar wallaby IL-1 $\beta$  was confirmed using a reverse transcriptase polymerase chain reaction (RT-PCR) strategy, followed by rapid amplification of cDNA ends (RACE) as previously described [13]. Consensus primers were designed to include regions of highest similarity after alignment of amino acid and nucleotide sequences for known mammalian IL-1 $\beta$  genes extracted from GenBank. In initial PCR experiments and for expression analysis, cDNA synthesized from Ficoll-isolated single-cell suspensions of lymph node cells stimulated with the plant mitogens phytohemagglutinin (PHA) and concanavalin A (ConA) were used as DNA sources, since stimulated lymphocytes are known to produce IL-1 $\beta$  [30]. Briefly, PHA (25  $\mu$ g/ml) and ConA (25  $\mu$ g/ml) were applied to  $7 \times 10^5$  cells/well in 96-well culture plates and incubated for 24 hr at 37°C and 5% CO<sub>2</sub> as previously described [26]. RNA was isolated from cells using Tri Reagent (Sigma-Aldrich, Australia) according to the manufacturer's protocol, before synthesis of cDNA using the Promega Reverse Transcription System as per the kit instructions (Promega, Australia). A 50- $\mu$ l reaction mix containing IL-1 $\beta$  consensus primers (IL-1 $\beta$  forward, 5'-tcttcaagaagaaccatc-3', and IL-1 $\beta$  reverse, 5'-ttgggaagtgctgatgtaccagttggg-3') was used to amplify a 464-base pair wallaby IL-1 $\beta$  amplicon. Gel-purified DNA was ligated into the pGEM-T Easy Vector (Promega, Australia) and transformed with JM109 competent cells. Mini-plasmid preparations were carried out using alkaline lysis according to the method of Zhou *et al.* [27], and plasmid DNA was sequenced in both directions at the Australian Genome Research Facility (AGRF; Brisbane, Australia). Data from both forward and reverse sequence reactions

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were aligned using ClustalW [21] to confirm the integrity of the sequence. The tammar wallaby IL-1 $\beta$  was identified using blastn (nucleotide basic local alignment search tool; [2]) with an E value of 5e-146. This product shared 90% and 83% identity at the nucleotide and amino acid levels respectively, with that of the brushtail possum between the area bounded by the forward and reverse primers.

The RACE technique [12] was used to obtain the nucleotide sequence for the remainder of the gene and 5' and 3' untranslated (UTR) regions. RACE DNA was prepared from a mammary associated lymph node of a tammar wallaby [13]. Outer and nested forward and reverse gene-specific RACE primers (GSP) for IL-1 $\beta$  were derived from the sequence returned from the RT-PCR product (5'-outer GSP, 5'-ccacgtattggtgggaatcgtc-3'; 5'- nested GSP, 5'-ccagttg-caggatgggcttctcgcacg-3'; 3'-outer GSP, 5'-ccagaactgt-gacgtttatgagctctgac-3'; 3'-nested GSP, 5'-tcggttggtatcctccaagattgcac-3'). Nested PCR was performed according to manufacturer's instructions (Clontech, Palo Alto, CA, U.S.A.). The 5' and 3' RACE amplicons produced under these conditions were 680 bp and 1324 bp, respectively.

RACE products were gel purified and cloned and sequenced as described earlier. A 1754-bp nucleotide product was constructed that extended through to the Poly-A tail. Preliminary gene identity as IL-1 $\beta$  was assigned based on blastn and blastx analyses [2], where the low E values ( $E < 0.001$ ) were indicative of the similarity of this sequence to other known IL-1 $\beta$  genes. With reference to the only other IL-1 $\beta$  cDNA marsupial sequence deposited in GenBank, that of the brushtail possum (accession number AF071539), the blastn and blastx E values were  $E=0$  and  $E=4e-131$ , respectively. The wallaby cDNA sequence shares 85% nucleotide identity with the brushtail possum sequence over its entire length. ClustalW [21] alignment with mammalian IL- $\beta$  sequences extracted from GenBank suggests that the most conserved regions of this gene are those that span exons 5 through to 7 in most mammalian species [7]. Conservation of the nucleotide sequence for the wallaby in this region is approximately 70%. This area falls within the coding domain for the mature protein and includes a sequence that codes for the  $\beta$ -trefoil structure that is common to the IL-1 gene family [30]. The nucleotide sequence reported in this paper was thus submitted to GenBank and assigned accession number GQ374410.

Mammalian IL-1 $\beta$  has 7 exons [16], and inspection of the wallaby IL-1 $\beta$  nucleotide sequence suggests that we have identified the exon 1 sequence in the wallaby gene, although it appears that similar to other mammals, this region remains untranslated [18]. A polyadenylation signal (AATAAA) was identified 15 nucleotides upstream of the poly(A) tail, and five 3' UTR ATTTA sequences and 2 consecutive 3' mRNA TA-rich elements (TTATTTAT) common to inflammatory mediators [9] were also present (see Fig. 1). The presence of these motifs, which are thought to be involved in regulating the stability of the mRNA [29], suggests that the sequence identified in this paper is neither a pseudogene

nor a result of truncated clones.

The translated wallaby IL-1 $\beta$  propeptide (GenBank accession number ACU65957) has 270 amino acids to the stop codon and a predicted molecular weight of 31 kDa. A number of conserved residues that are structurally and functionally relevant to known IL-1 $\beta$  gene products are present in the wallaby sequence. An InterProScan Signature Recognition Search through the ExPASy Proteomics Server [15] recognized the amino terminal end of the putative IL-1 $\beta$  protein as the IL-1 propeptide using Pfam (see Fig. 1). The IL1 family motif was also present as detected by PROSITE (see Fig. 2). Secondary structural analysis of the amino acid sequence using the PELE Protein Structure Prediction Tool [19] suggests that  $\beta$  sheets form the major structural elements of the mature peptide, which is characteristic of the  $\beta$ -trefoil fold found in IL-1 gene families [14] and is common to all mammals.

Although cytokines generally contain a clear signal sequence [3], the proinflammatory cytokine IL-1 $\beta$  does not. The mature protein is produced when the propeptide is cleaved by a caspase enzyme, resulting in a functional protein that is exported out of the cell [14]. Analysis of the hydrophobicity of the translated protein for the wallaby IL-1 $\beta$  precursor predicts the lack of a signal peptide as determined by SignalP 3.0 [6] and the Kyte-Doolittle hydrophobicity plot [17] (see Fig. 3). This is consistent with the human IL-1 $\beta$  homolog [4] and with vertebrate species studied to date [7]. There is, however, a conserved aspartic acid residue at position 117 in the translated wallaby sequence (see Figs. 1 and 2), which if functionally relevant, would result in a peptide with a predicted molecular weight of 17 kDa being secreted, which is similar to what is observed in other mammals. Commonly, the IL-1 $\beta$  propeptide is cleaved after this conserved aspartic acid residue by a caspase-1 enzyme, although elastase and granzyme A can also serve this function at sites close to the ICE (IL-1 $\beta$  converting enzyme) cut site [10]. Production of two forms of recombinant possum IL-1 $\beta$  using the predicted conventional aspartic acid cut site and an alternate arginine cut site resulted in functional peptides that nonetheless possessed reduced bioactivity when compared with a bovine homolog [24]. Whether or not this comparative difference in bioactivity is a function of the assay conditions used in that study or the intrinsic biological properties of possum IL-1 $\beta$  remains to be confirmed. The characterization of the tammar wallaby IL-1 $\beta$  nucleotide sequence here is the first step towards clarifying whether this bioactivity is a marsupial-specific attribute or unique to the possum.

Another functionally significant residue that is conserved in the wallaby sequence is Histidine 33, which is associated with receptor binding in the IL-1 $\beta$ /IL-1R1 complex and is involved in triggering signaling events associated with ligand/receptor binding [16]. Other residues present in the translated wallaby sequence that are generally conserved across mammals [16] and some vertebrate groups [7] and that are associated with functional IL-1 $\beta$  products are Arginine 4, Leucine 34, Phenylalanine 45, Lysine 104 and

1           ctgacactgaccaaagactcctatcacttctgccagcaaaggaatacaaaagtatc  
 57 atggcaatgggtgcctgaaatcacttgtgaggagggtgatttttatagggacaacgatgac  
 1 M A M V P E I T C E E V D F Y R D N D D 20  
 117 tgtcaattttatgaggctgatgggtcccaaccaaagaagggttacctccagcatcctgat  
 21 C Q F Y E A D G P N Q K K G Y L Q H P D 40  
 177 ctttgcctccaaagaagtcttctggaggaggatggcatccagctgaaattcaccagtcac  
 41 L C S K E S L L E E D G I Q L K F T S H 60  
 237 ccccatcaatttcatagaactgtgatggtagttgttgcaattgagaagatgaggcatctg  
 61 P H Q F H R T V M V V V A I E K M R H L 80  
 297 aatggagtctgctcccagctgttccaggataatgatttgatgaatatattcacaagcatc  
 81 N G V C S Q L F Q D N D L M N I F T S I 100  
 357 tttcaagaagaacacatcaccttccagaactgtgacgtttatgagtctgactccacgttt  
 101 F Q E E H I T F Q N C D V Y E S D S T F 120  
 417 cggttggatcctcccaagattgcaccattcaagacgcaaatacaaaatgcctgaccttg  
 121 R L V S S Q D C T I Q D A N Q K C L T L 140  
 477 tccaatgccagtgaaactgcaagctgtccatctcaatggacacacataagccaacaagt  
 141 S N A S E L Q A V H L N G H N I S Q Q V 160  
 537 attttcagcatgaagcgcctccttggtaaaataggatccaagaaaacacatggtgtcctg  
 161 I F S M K R L L G K I G S K K T H V V L 180  
 597 tgtatcaagaaaaataatctctatctgtcttgtgtgaagcgtggcgagaagcccatcctg  
 181 C I K K N N L Y L S C V K R G E K P I L 200  
 657 caactggagcaaattgacgatttccccaccaataacgtggaacacgattcatctttaac  
 201 Q L E Q I D D F P T N N V E T R F I F N 220  
 717 aagatggaattcaataatacaactgaatttgagtctgcagaataccctgactggatcacatc  
 221 K M E F N N T T E F E S A E Y P D W Y I 240  
 777 agcacttccaagtgatggatggcagcctgtcttctctggggaacatacgcgggggcaaggac  
 241 S T S Q V D G Q P V F L G N I R G G K D 260  
 837 ataactgacttctttttggatgatctctcttaa 869  
 261 I T D F F L D D L S \* 271  
  
 tcagcaaatctatgtatactgtgactctagaacctagacaaatgtgagaagaaaaggaat  
 ttgggagttcatatgtttgacatatgacatgtattcaacttaggccttaatatgttctttg  
 gaa**atttat**attgctaccattcagatactgactgtgctcagggaaacccccctgacctat  
 ttccagtgcctgactgagtagaatctgggaaatattgggtgatagtttcatattcaagggga  
 aagtttgacactggaaggcaggggagcattaggaggggggatttggaagaaagagaagatg  
 accatgaagtagaagggtaaaagagacctttgacctgccaattcagtaagggtatgacgt  
 aggaagaactgcgcgaatcaccatttcaagactcgcgatgcatgctgatcctaaacgggcca  
 ggctatgattgagccctgtcagctgtgaccgctcagaagtggtctttgcttttcccagatg  
 ccaagatgtggcctttctcgaatcgcccagaaccgtttccctaagcctgggtcatttcca  
 tgtcccccaactgccccttctgggttttttagcgaccgatcgtttgttttag**TTATTTATTT**  
**ATTTA**Ttatgacgtttgtttgtggacgctcatgtttgttaattttttgaaatgaaaaggcca  
 agaggaaccaaagtgcagtaagagagtaaagctttttgatgctctgtaaaactgaatta**attt**  
**at**attgttattgtttactcgaaagacctagaagggtactcacaatgtttttgaagggggaat  
**atttat**gaaagggaaacatgacctgataatttcagtgttaacacaataaaacttttggttaa  
 tatgaaaaaaaaaaaaaaaaaaaaaaaaaaaaa 1754

Fig. 1. Nucleotide and translated amino acid sequence of tammar wallaby interleukin 1 $\beta$ . The figure shows 1754 nucleotides (GenBank Accession No: GQ374410), and the nucleotide sequence is numbered for the predicted IL-1 $\beta$  open reading frame, 1–813. The polyadenylation signal sequence is underlined, and 3' mRNA TA-rich elements (*TTATTTAT*) and instability motifs (**attta**) are presented in upper case and bold, respectively. The translated peptide is 270 amino acids to the stop codon (GenBank Accession No: ACU65957). The IL1 propeptide is underlined, and the IL-1 family signature is boxed. An inverted black triangle ( $\blacktriangledown$ ) indicates the predicted ICE cut site (aspartic acid) characteristic of all mammalian IL-1 $\beta$  peptides.

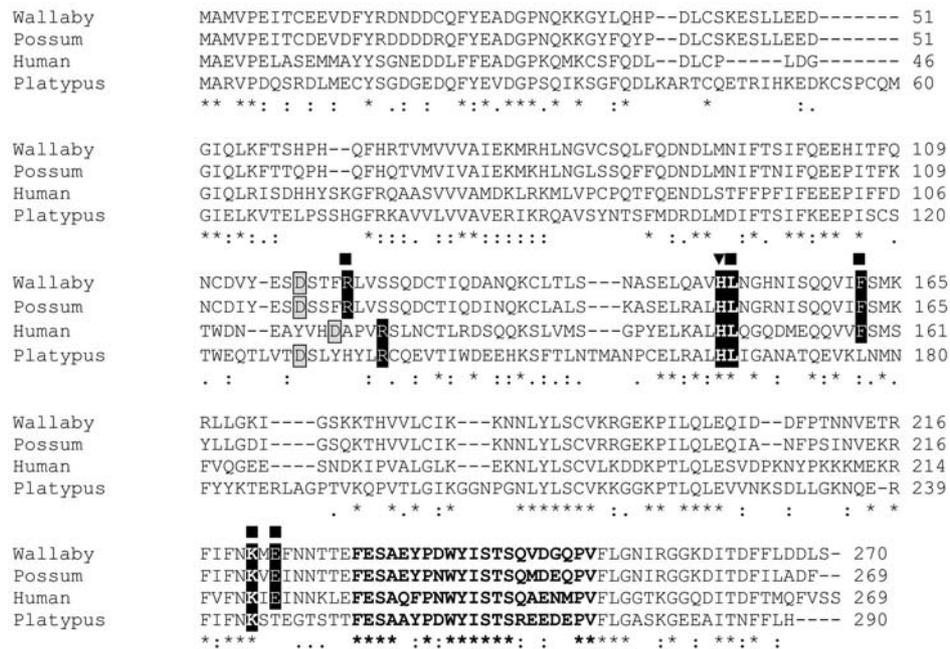


Fig. 2. Multiple sequence alignment of IL-1 $\beta$  peptide sequences in 3 extant mammalian groups (eutherians-human, prototherians (monotremes)-platypus, and metatherians (marsupials) - possum and tammar wallaby). Consensus of identical residues across all four mammals is shown by asterisks (\*) and conserved and semi-conserved substitutions are indicated by colons (: ) and periods ( . ), respectively. The IL-1 family signature is shown in bold, and a conserved Histidine (H33 of mature protein), which is important for biological function, is indicated by an inverted black triangle ( $\blacktriangledown$ ). Important receptor binding sites conserved across many IL-1 $\beta$  genes are indicated by a black square ( $\blacksquare$ ). The predicted IL-1 $\beta$  converting enzyme (ICE) cut site (Aspartic Acid Residue 117 [D117]) is shaded and boxed. The GenBank accession numbers for these sequences are AAD21871 for the brushtail possum, *Trichosurus vulpecula*, CAC80337 for the platypus, *Ornithorhynchus anatinus*, AAA36106 for human, *Homo sapiens*, and ACU65957 (this paper) for the tammar wallaby, *Macropus eugenii*.

Glutamate 106 (see Fig. 2). In summary, our characterization of the cDNA sequence reported in this study strongly suggests that IL-1 $\beta$  is conserved at both the molecular and structural level and is most likely similar in its function to other mammalian homologs. Despite the conservation of these important structural and functional elements, there are still differences in the peptide sequences across species that confirm that the wallaby gene has most in common with its mammalian counterparts. At the amino acid level, the tammar wallaby peptide shares 85%, 73%, 47%, 45%, 42% and 29% identity with the IL-1 $\beta$  gene products of the possum and the predicted sequence of the gray short-tailed opossum, cat, human, platypus and pufferfish, respectively (accession numbers: AF071539, XP\_001376779, P41687, P01584, CAC80337 and CAE00572).

Since it is unlikely that the high level of conservation in the wallaby predicted protein sequence can explain any differences in host protective responses, expression of IL-1 $\beta$  at the nucleotide level was investigated to determine if the classical IL-1 $\beta$  responses to mitogen and lipopolysaccharide stimulation were present. RT-PCR expression of tammar wallaby IL-1 $\beta$  (using the 3'-nested GSP and 5'-nested GSP

as the forward and reverse primers, respectively) was detected in both unstimulated and mitogen-stimulated lymph node cells in this study when the PCR conditions were optimized for detection of this molecule. However, when comparative expression was assessed against expression of the housekeeping gene, GAPDH (Accession number: EF654515), IL-1 $\beta$  was not readily detectable in unstimulated lymph node cells or peripheral blood mononuclear cells, but was detected in mitogen-stimulated lymph node cells treated as described earlier (see Fig. 4). Stimulation of the adherent-cell fraction of peripheral blood mononuclear cells with lipopolysaccharide (10  $\mu$ g/ml for 24 hr in serum-free medium) resulted in strong expression of this proinflammatory cytokine, consistent with IL-1 $\beta$  expression in the possum for similar cell populations [24]. This data suggests that wallaby IL-1 $\beta$  expression is consistent with typical mammalian responses, although a larger study may uncover temporal and quantitative effects that were not apparent here. Since IL-1 $\beta$  is only active in its secreted form [3], future studies on the function of the mature tammar wallaby protein are warranted in order to clarify the role of this inflammatory gene in marsupial immunobiology.

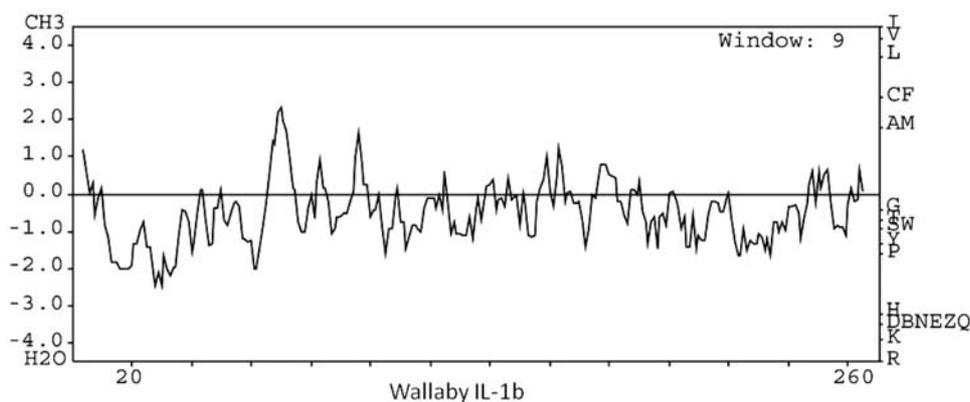


Fig. 3. Hydrophobicity plot of tamar wallaby IL-1 $\beta$  propeptide. Kyte-Doolittle plot of residues 1–270 showing the lack of hydrophobic residues at the start of the peptide sequence, which is consistent with the absence of a signal peptide. Positive values (to +4.0) represent increasing hydrophobicity. Negative values (to –4.0) indicate hydrophilic residues.

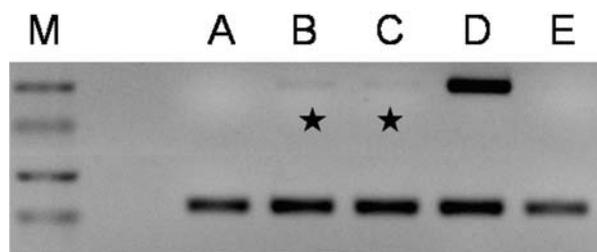


Fig. 4. Expression of IL-1 $\beta$  in tamar wallaby tissues. A 248-bp IL-1 $\beta$  amplicon was amplified in mitogen-stimulated lymph node lymphocytes (★) and the LPS-stimulated adherent cell population from tamar wallaby peripheral blood mononuclear cells (top panel, see text for details). Expression was detected after 32 cycles of PCR at an annealing temperature of 55°C and is shown against GAPDH expression in the same tissues (bottom panel). Molecular weight marker bands are 250 bp and 100 bp in each panel. Lane A: resting lymph node cells. Lane B: PHA-stimulated lymph node cells. Lane C: ConA-stimulated lymph node cells. Lane D: LPS-stimulated adherent peripheral blood mononuclear cells. Lane E: resting peripheral blood mononuclear cells.

Captive and some free-ranging species of marsupial readily succumb to disease caused by intracellular pathogens [8], so it is important to characterize key immune molecules in an attempt to understand the mechanisms of immunity available to these species. The brushtail possum is the only other marsupial IL-1 $\beta$  cDNA sequence elucidated thus far. We have previously reported the nucleotide sequence for TNF- $\alpha$  from the tamar wallaby [13] and now extend this work on characterizing the proinflammatory immune genes of this species to include IL-1 $\beta$ .

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