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CD14 and TLR4 are expressed early in tammar (*Macropus eugenii*) neonate development

Kerry A. Daly[1,2], Christophe Lefévre[2,3], Kevin Nicholas[2,3], Elizabeth Deane[4] and Peter Williamson[1,2,*]

[1]Centre for Advanced Technologies in Animal Genetics and Reproduction, Faculty of Veterinary Science, University of Sydney, NSW 2006, Australia, [2]Cooperative Research Centre for Innovative Dairy Products, Australia, [3]Department of Zoology, University of Melbourne, Parkville, Victoria 3010, Australia and [4]School of Environmental and Life Sciences, Macquarie University, Ryde, NSW 2109, Australia

*Author for correspondence (e-mail: peter_williamson@usyd.edu.au)

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SUMMARY

Marsupials are born in a relatively underdeveloped state and develop during a period of intensive maturation in the postnatal period. During this period, the young marsupial lacks a competent immune system, but manages to survive despite the potential of exposure to environmental pathogens. Passive immune transfer *via* the milk is one well-recognised strategy to compensate the neonate, but there also may be innate immune mechanisms in place. In this study, CD14 and Toll-like receptor 4 (TLR4), integral molecular components of pathogen recognition, were identified and characterised for the first time in a marsupial, the tammar wallaby (*Macropus eugenii*). Functional motifs of tammar CD14 and the toll/interleukin receptor (TIR) domain of TLR4 were highly conserved. The lipopolysaccharide (LPS) binding residues and the TLR4 interaction site of CD14 were conserved in all marsupials. The TIR signalling domain had 84% identity within marsupials and 77% with eutherians. Stimulation of adult tammar leukocytes resulted in the induction of a biphasic pattern of CD14 and TLR4 expression, and coincided with increased production of the pro-inflammatory cytokine TNF-α. Differential patterns of expression of CD14 and TLR4 were observed in tammar pouch young early in development, suggesting that early maturation of the innate immune system in these animals may have developed as an immune survival strategy to protect the marsupial neonate from exposure to microbial pathogens.

Key words: marsupial, innate immunity, microbial recognition, toll-like receptors, neonate.

INTRODUCTION

Marsupials are characterised by a unique reproductive strategy in which the young are born with only those systems essential for perinatal survival well developed. Organogenesis and physiological maturation in the young take place while suckling and are especially rapid in the immediate postnatal phase. As a consequence of this strategy, the newborn is exposed to environmental pathogens at a stage when it has no functional immune system; indeed, adaptive immunity is only evident in the 'switch phase', a period immediately before it first leaves the pouch (Basden et al., 1997). Prior to immune development, protection of the marsupial neonate is conferred by secretion of immune components in the milk of the mother (Adamski and Demmer, 1999; Adamski and Demmer, 2000; Demmer et al., 1999; Western et al., 2003), but there is also the possibility that the young are protected through innate immune mechanisms. However, there have been no studies that have examined the role of innate immune responses in the pouch young themselves during this vulnerable period, or at the molecular level in mature animals. Indeed, until recently there had been no studies of the central molecular components of the innate immune system in any marsupials. With recent developments in marsupial biology, experimental strategies to address these issues using molecular approaches are now possible (Daly et al., 2007a; Daly et al., 2007b; Lefevre et al., 2007; Belov et al., 2007; Daly et al., 2008a; Daly et al., 2008b).

Recognition of microbial non-self is a crucial initiating step in immune defence and surveillance. Of the membrane glycoproteins involved in pathogen recognition, the Toll-like receptor (TLR) family, molecules that are activated by the pathogen-associated molecular pattern (PAMP) of many microbes, and the co-receptor CD14 are essential. The TLR family comprises 11 members in mammals and all share a characteristic structure that includes a toll/interleukin receptor (TIR) intracellular domain, which initiates a complex intracellular signalling cascade that drives production of antimicrobial peptides, reactive oxygen and nitrogen intermediates, chemokines and pro-inflammatory cytokines (Akira and Takeda, 2004; Azuma, 2006; Kaisho and Akira, 2006). CD14, a membrane glycoprotein found predominantly on myelomonocytic cells, aids binding and sensitises TLR4 homodimers to lipopolysaccharide (LPS) (Fujihara et al., 2003). CD14 also interacts with TLR2 in binding gram-positive PAMP such as peptidoglycan and lipoteichoic acid (LTA) (Ellingsen et al., 2002; Iwaki et al., 2005; Muroi et al., 2002). In addition, CD14 may internalise and neutralise endotoxin (Ahmed-Nejad et al., 2002; Poussin et al., 1998), and it is involved in the clearance of apoptotic cells (Tobias, 2003).

In the present study, primary sequence, expression patterns and response to stimuli of CD14 and TLR4 from the tammar wallaby (*Macropus eugenii*) were characterised. We report that the functional motifs involved in LPS binding, signalling and homodimerisation of tammar CD14 and the TIR domain of TLR4 are highly conserved. Challenge of adult tammar leukocytes with LPS and LTA revealed that these PAMP induce expression of CD14 and TLR4 in a pattern that coincided with expression of the pro-inflammatory cytokine tumour necrosis factor-α (TNF-α). We also report the detection and

differential expression of CD14 and TLR4 in the pouch young throughout early development, a finding that suggests that innate immune defences are active prior to the maturation of the tammar wallaby immune system.

MATERIALS AND METHODS

Animals and collection of pouch young samples

Tammar wallabies (*Macropus eugenii* L.) were kept at the Macquarie University Fauna Park, Ryde, Australia, and in accordance with its management and husbandry procedures. Additional pouch young samples were also collected from tammars at the CSIRO Black Mountain Laboratories, Canberra, Australia, courtesy of Dr Lyn Hinds. Animals were fed *ad libitum* and had constant access to fresh water. Pouch young were killed by decapitation in accordance with Macquarie University Fauna Park management procedures, and Macquarie University Animal Ethics Committee and CSIRO ethics committee approval. Liver, spleen, cervical thymus, jejunum, bone marrow, lung, skin and kidney samples were collected from 42 pouch young aged from 5 to 120 days of age. Samples were immediately stored at –80°C until RNA extraction. Blood was collected from healthy adult tammars and stored at 4°C until leukocyte extraction for primary culture using ficoll-paque (Sigma-Aldrich, Castle Hill, NSW, Australia).

Culture of tammar leukocytes

Leukocytes were cultured in RPMI media (Invitrogen, Mount Waverly, Victoria, Australia) with added 10% FCS (fetal calf serum; Invitrogen) at 37°C and 5% CO_2. Tammar leukocytes were stimulated with either LPS or LTA (Sigma-Aldrich). LPS was derived from *Escherichia coli* and LTA from *Staphylococcus aureus*. LPS and LTA were added to tammar leukocytes at a dose of 5 μg ml^{-1} of media and cells were cultured for up to 24 h. Control groups of leukocytes were also cultured for up to 24 h but with neither stimulant.

Harvesting of RNA and quantitative RT-PCR

RNA was collected from stored samples using an RNeasy RNA extraction kit (Qiagen, Clifton Hill, Victoria, Australia) and following the manufacturer's guidelines. The concentration of RNA was measured using an Ultrospec 2000 spectrophotometer (Pharmacia Biotech, Cambridge, UK). RNA was converted to cDNA for use in quantitative PCR using MMLV reverse transcriptase (Invitrogen). PCR was performed using a master mix containing Taq DNA polymerase (Qiagen), primers for *CD14* or *TLR4* (Table 1) and Sybr green I (Molecular Probes, Eugene, OR, USA). This was designed to amplify a 109 base pair fragment from *CD14* and a 124 base pair fragment from *TLR4*. Tammar glyceraldehyde 3-phosphate dehydrogenase (GAPDH, GenBank accession number EF654515), 28S rRNA (GenBank accession number EF654517) and β-actin (GenBank accession number EF654516; Table 1) were amplified as housekeeping genes for *CD14*, *TLR4* and *TNF*-α, respectively, and their suitability for use was evaluated as described previously (Livak and Schmittgen, 2001). Primers were manufactured by Sigma-Genosys (Castle Hill, NSW, Australia). Quantitative PCR was performed using a Rotogene 6000 (Corbett Life Science, Mortlake, NSW, Australia). Samples were run in triplicate with conditions optimised to generate a single product; controls lacking template were run simultaneously. Melt curves were also performed to assess the accuracy of the PCR. A $2^{-\Delta\Delta Ct}$ relative expression analysis method (Livak and Schmittgen, 2001) was used to evaluate the expression of CD14 and TLR4 in the target tissues compared with the calibrator samples (i.e. control or non-lymphoid). Results were analysed by averaging mRNA relative expression levels in samples over 5 day periods and were graphed accordingly.

Isolation, alignment and comparison of sequences

CD14 was isolated from a tammar mammary gland cDNA EST library (Lefevre et al., 2007). *CD14* was compared with the *M. eugenii* WGS database on the NCBI website with a discontiguous megaBLAST (http://www.ncbi.nlm.nih.gov/BLAST/tracemb.shtml). Tammar *TLR4* (gnl/ti/1457595886) was isolated from the *M. eugenii* trace November 2006 release by tBLASTn. Tammar *TNF*-α had been previously characterised (accession number AF055915). Alignment for phylogeny and pairwise similarities was undertaken on amino acid sequences, which were deduced using the translate tool on the ExPASy site (http://au.expasy.org/tools/dna.html). Proteins were aligned using the ClustalW (Thompson et al., 1994) program available through BioManager by ANGIS. Pairwise similarities were calculated on aligned amino acid sequences using the OldDistances (GCG) program also available through BioManager. Monte Carlo scores for comparisons between sequences were calculated using PRSS (Pearson and Lipman, 1988) with BLOSUM50 scoring matrix, again available through BioManager. A score of above 0.1% was considered indicative of a poor alignment and these were discarded from the results. Kyte–Doolittle hydropathy plots were generated with GREASE (Pearson and Lipman, 1988) available through BioManager, using a frame size of nine. Sequences used in alignments and phylogeny are listed in Table 2.

Phylogenetic analysis

All trees were calculated based on alignments of the entire CD14 sequence or the TIR domain, in the case of TLR. Sequences for phylogeny were aligned used ClustalW (Thompson et al., 1994) with minor manual corrections. Distance method phylogenetic trees were constructed using PROTDIST in the PHYLIP package (Felsenstein, 1989) available through BioManager by ANGIS. The neighbour joining method using a Dayhoff PAM matrix, a George/Hunt/Baker categorisation of amino acids, universal genetic

Table 1. Primers for quantitative RT-PCR

Gene	Forward (5′–3′)	Reverse (5′–3′)	Fragment (bp)
CD14	ATTGCGATGTGCATTTCCG	GAAAGTCCACTGCTTTGCTCG	109
TLR4	TTATGTGGAGGGGAAAAGCAC	GGAGGCAGATTGGAATGAG	124
TNF-α	GGACATCACCAAACGACACC	CCCCAGCAAGAAGAAGGAAG	184
GAPDH	GGTAAGCTGTGGAGTGATGGGCG	TGGGAGTAGGAACACGGAAGGCC	118
23S rRNA	CGATGTCGGCTCTTCCTATC	TCCTCAGCCAAGCACATACA	205
β-Actin	GCGTCACCCACAACGTACCCATCT	GATGTCACGCACAATTTCCCGCTC	140

Primers used for quantitative PCR of CD14 and TLR4 expression in the organs of the pouch young. bp, base pairs. Primers were manufactured by Sigma-Genosys (Castle Hill, NSW, Australia).

Table 2. Sequences used in alignment and phylogeny

CD14	Human (*Homo sapiens*) – AAH10507, cow (*Bos taurus*) – BAA21517, water buffalo (*Bubalus bubalis*) – ABD97885, rabbit (*Oryctolagus cuniculus*) – BAA21770, horse (*Equus callabus*) – AAF08963, mouse (*Mus musculus*) – CAA32166, steppe mouse (*Mus spicilegus*) – BAB68587, sheep (*Ovis aries*) – CAJ90519, rat (*Rattus norvegicus*) – AAC35371, goat (*Capra hircus*) – ABE68725, big horned sheep (*Ovis canadensis*) – ABI95798, pig (*Sus scrofa*) – ABK32433 and short grey-tailed opossum (*Monodelphis domestica*) – XP_001377002.
TLR1	Human – CAG38593, fugu (*Takifugu rubripes*) – AAW69368, mouse – NP_109607, pig – NP_001026945, zebrafish (*Danio rerio*) – AAQ91305, cow – NP_001039969 and chicken (*Gallus gallus*) – NP_001007489.
TLR2	Fugu – AAW69370, zebrafish – AAQ91306, sheep – ABI58266, chicken – NP_989609, human – NP_003255, rat – NP_942064, mouse – NP_036035, cow – NP_776622 and pig – NP_998926.
TLR3	Fugu – AAW69373, human – NP_003256, mouse – NP_569054, rat – NP_942086, zebrafish – NP_001013287, cow – CAH19227, pig – ABB92547 and chicken – XP001231987.
TLR4	Tammar (*Macropus eugenii*) – gnl/ti/1457595886, human – AAI17423, rhesus monkey (*Macaca mulatta*) – AAX63196, orangutan (*Pongo pygmaeus*) – AAM18616, cow – ABH09760, water buffalo – ABH11655, sheep – ABI96901, goat – ABI96900, pig – AAT05612, horse – AAU95608 and cat (*Felis catus*) – NP_001009223.
TLR5	Fugu – AAW69374, human – AAI09119, mouse – AAI25248, cow – ABC68311, pig – BAD91800, chicken – NP_001019757 and zebrafish – ABD16185.
TLR6	Mouse – BAA78632, human – BAA78631, pig – NP_998925, cow – CAJ35083 and rat – NP_997487.
TLR7	Fugu – AAW69375, human – AAQ88659, mouse – NP_573474, chicken – NP_001011688, zebrafish – AAQ91312 and cow – NP_001028933.
TLR8	Fugu – AAW69379, human – AAQ88663, mouse – NP_573475, cow – NP_001029109 and pig – NP_999352.
TLR9	Fugu – AAW69377, human – AAQ89443, mouse – NP_112455, cow – NP_898904, pig – NP_999123 and rat – NP_937764.
TLR10	Human – AAQ88667, pig – NP_001025705 and cow – NP_001070386.

Accession numbers (GenBank) are given after each species name.

code and 1000 bootstrap replicates was used. Bootstrap values were calculated using Seqboot (Felsenstein, 1989) in BioManager – 75% was taken as the cut off for bootstrap values; however, branches with lower support than this were accepted if the division had been determined in previous studies. A maximum parsimony tree was also calculated using PROTPARS in the PHYLIP package (Felsenstein, 1989) in BioManager with no jumbles, outgroup or parsimony threshold selected. One-thousand bootstrap values were also calculated in Seqboot for the maximum parsimony tree. A maximum likelihood tree was drawn using PROTML (Adachi and Hasegawa, 1996) also in BioManager, with a JTT transition model and a quick add OTUS search strategy.

RESULTS

Isolation of CD14

The isolated tammar *CD14* (accession number EF654514) was 1395 nucleotides in length, which gave an open reading frame (ORF) of 380 residues. On comparison with eutherian *CD14* sequences, the

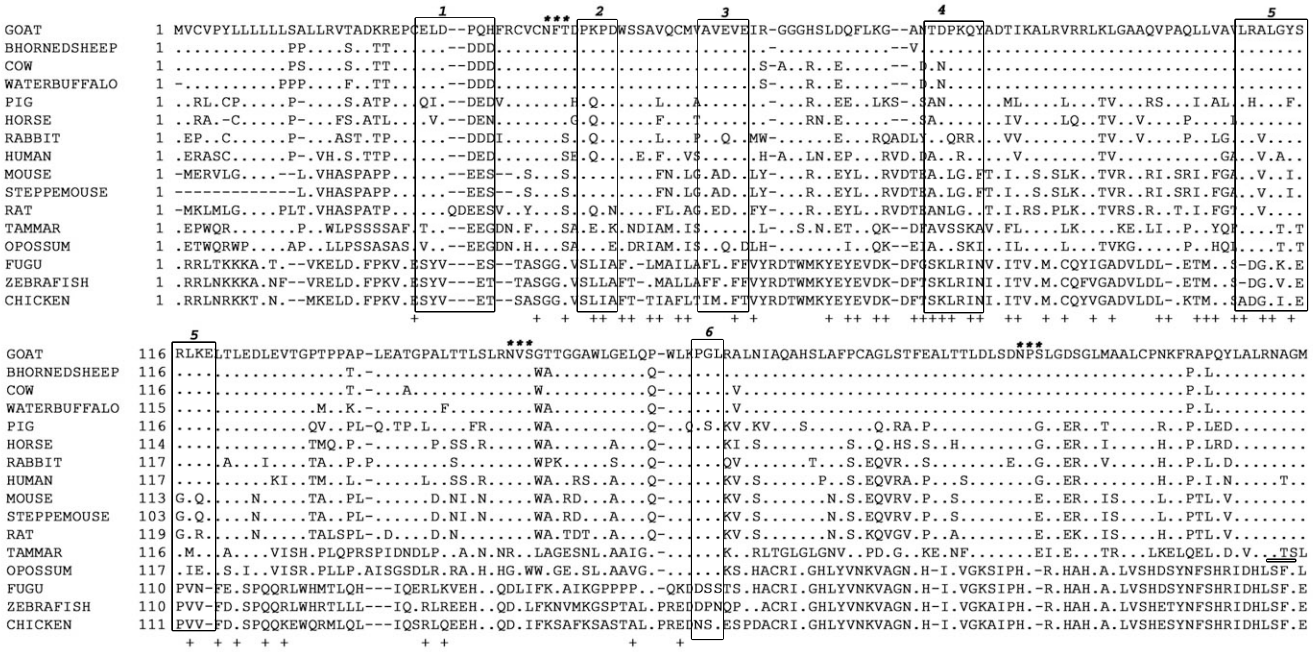

Fig. 1. Multiple alignment of the amino terminal end of CD14. Boxes 1–4 represent hydrophobic motifs that are part of the lipopolysaccharide (LPS) binding site. Boxes 5 and 6 represent motifs involved in Toll-like receptor 4 (TLR4) interactions. Asterisks indicate the shared potential N-linked glycosylation sites; marsupial-specific sites are underlined. Plus signs indicate the residues involved in the LPS binding pocket. Sequences used in the alignment are detailed in Table 2.

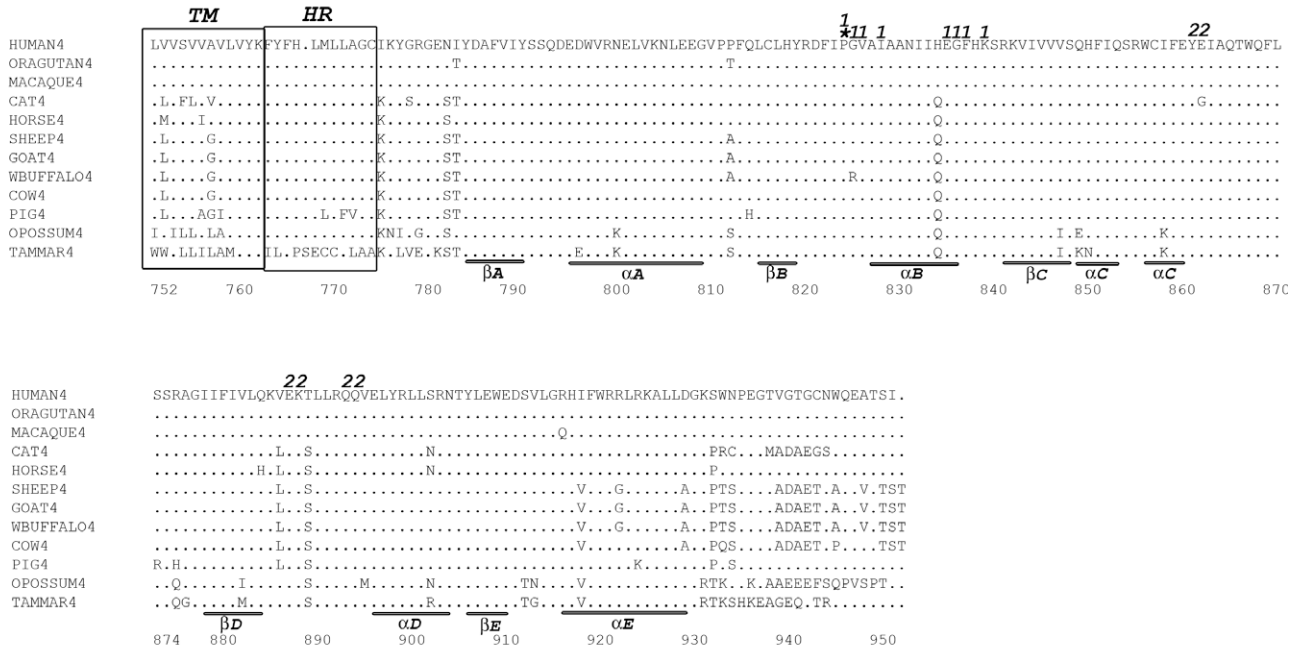

Fig. 2. Multiple alignment of the toll/interleukin receptor (TIR) domain of TLR4. The number suffix indicates the corresponding TLR of that species. The alpha helices and beta sheets are indicated beneath the alignment. TM, transmembrane region; HR, hydrophobic region; asterisk, site of LPS[d] mutation; 1, residues important in the BB protein interaction site; and 2, residues important in the DD loop protein interaction site. Sequences used in the alignment are detailed in Table 2.

tammar *CD14* sequence was found to be complete. Tammar CD14 had an average peptide percentage identity of 68% with the short grey-tailed opossum, 48% with eutherian sequences and 19% with lower order vertebrates. No other TLR4 accessory proteins could be isolated from either the tammar EST library or the latest tammar genome release.

Characterisation of CD14 and TLR4

A multiple alignment of the first 234 residues of CD14 demonstrated conservation of many motifs involved in LPS binding and TLR4 interaction (Fig. 1). Boxes 2–4 (Fig. 1) indicate motifs that all contribute to forming the hydrophobic LPS binding site (Cunningham et al., 2000; Juan et al., 1995; Stelter et al., 1999). These motifs are well conserved in the tammar, apart from box 4, which has low homology in most species. Residues previously identified as forming the LPS binding pocket (Kim et al., 2005) are indicated in Fig. 1 with plus signs. Boxes 1, 5 and 6 are involved in cellular signalling and or TLR4 interaction of CD14 (Juan et al., 1995; Muroi et al., 2002; Stelter et al., 1999). In box 1 (Fig. 1), the tammar has conservative substitutions apart from the non-polar glycine in place of an aspartic acid at residue 10. In the opossum, there is a non-conservative substitution of a valine at this position. Similar high levels of homology and conservative substitutions were seen in the 91–101 and 151–153 motifs (boxes 5 and 6, respectively, in Fig. 1) in all mammals. However, the replacement of a polar threonine with a non-polar glycine at residue 95 in the tammar CD14 sequence at the 91–101 motif is a non-conservative exchange. The tammar CD14 sequence has four potential N-linked glycosylation sites (common sites are marked with asterisks and marsupial-specific sites are underlined in Fig. 1). Two of these are shared with all eutherian sequences examined and the other two are unique to the tammar CD14 peptide.

Multiple alignment and phylogenetic analysis were used to confirm the subtype classification of TLR4. A multiple alignment of tammar TLR4 (Fig. 2) demonstrated the high degree of conservation seen in the TIR domain between TLR4 homologues. This corresponded with the peptide percentage identities, as the tammar TLR4 had average peptide percentage identities of 84% with other marsupials and 77% with eutherians. However, lower levels of homology were seen in the transmembrane and hydrophobic regions of the tammar TLR4 peptide (TM and HR boxed regions in Fig. 2), with a proline insertion in the hydrophobic region at position 778, but this region remained hydrophobic on a Kyte–Doolittle hydropathy plot (Fig. 3). Residues important in TLR4 homodimerisation, such as the BB loop (marked 1 on Fig. 2), were conserved in the tammar and all other species examined, with the exception of the water buffalo, which had an Arg^{726} instead of a glycine. A Pro^{725} at the site of

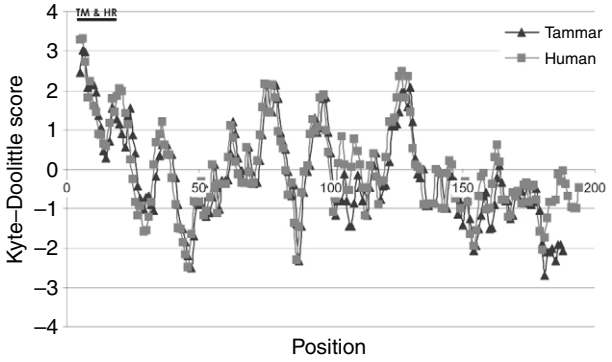

Fig. 3. Kyte–Doolittle hydrophobicity plots of the TIR domain of TLR4. Similar scores were seen over the entire domain, reflecting the high levels of conservation, including in the transmembrane (TM) and hydrophobic regions (bar).

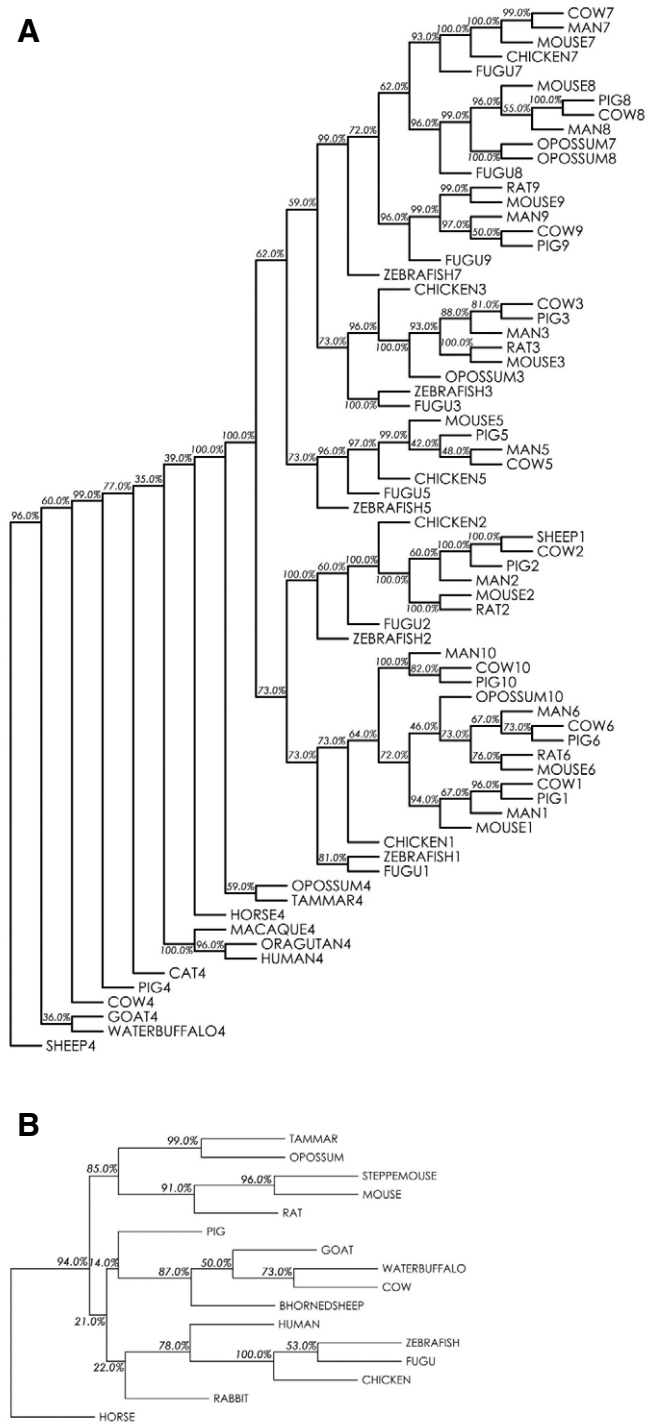

Fig. 4. Neighbour joining phylogenetic trees for TLR4 (A) and CD14 (B) calculated from protein sequences. Bootstrap values (1000 replicates) are indicated on each tree. In both trees, species are indicated by common names. In A, the number suffix indicates the corresponding TLR of that species.

the LPS^{d} mutation (Palsson-McDermott and O'Neill, 2004), which is crucial in TLR4 homodimerisation, was conserved in all species, including the tammar. Residues that form a potential protein interaction site in the DD loop (marked 2 on Fig. 2) were also extremely well conserved in most species, including the tammar.

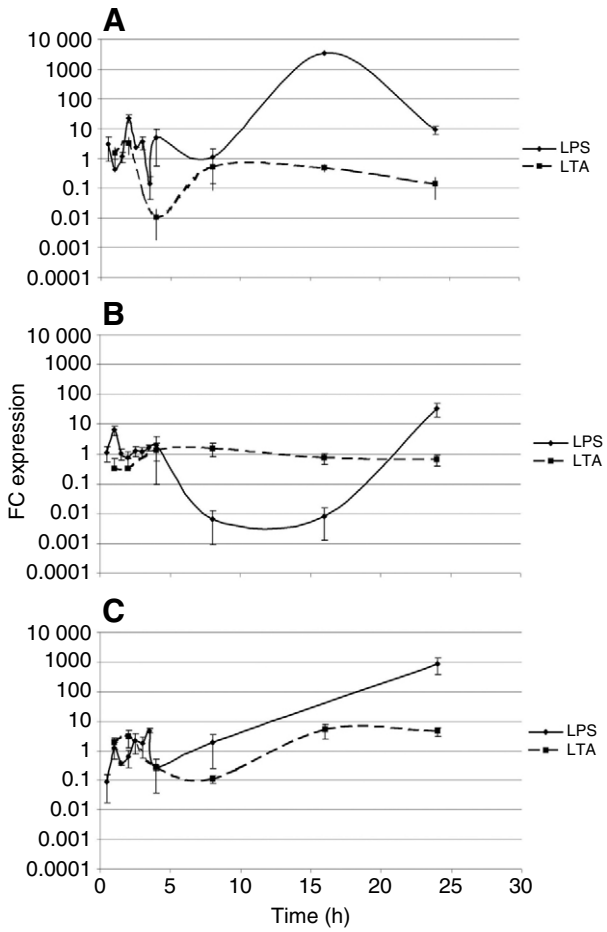

Fig. 5. Expression of CD14 (A), TLR4 (B) and TNF-α (C) in adult tammar leukocytes stimulated with either LPS (solid line) or LTA (broken line). FC expression, fold change in expression relative to the control non-stimulated tammar leukocytes. Relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). *Y* error bars indicate ±1s.d.

Phylogenetic analysis of CD14 and TLR4

Neighbour joining, maximum parsimony and maximum likelihood trees were generated for both CD14 and TLR4. The neighbour joining trees of CD14 and TLR4 are shown in Fig. 4. The tammar TLR4 sequence clustered with the TLR4 sequences of other species in all trees (bootstrap values 100% for neighbour joining and 91% for maximum parsimony; Fig. 4A). This clustering was seen for the other TLR included in all three trees. In all three phylogenetic trees for CD14 (Fig. 4B), the tammar CD14 sequence was found on a branch with the opossum (bootstrap values 35% for neighbour joining and 100% for maximum parsimony).

Expression of CD14, TLR4 and TNF-α in adult tammar leukocytes in response to challenge with LPS or LTA

LPS and LTA challenge generated different patterns of expression of CD14 and TLR4 in adult tammar leukocytes (Fig. 5). CD14 expression (Fig. 5A) was increased throughout most of the 24 h of LPS stimulation, with a peak at 16 h. In comparison, LTA stimulation resulted in lower levels of CD14 mRNA, with the highest expression around 2 h and 8–16 h, although only the former resulted in overall increased expression compared with control samples. TLR4 had a different pattern of expression (Fig. 5B). LPS-stimulated

leukocytes showed increased TLR4 mRNA expression around 2 h and later at 24 h. Expression of TLR4 in response to LTA challenge was not different from that of control tissue (i.e. fold change in TLR4 expression was around one for the entire 24 h). TNF-α mRNA expression (Fig. 5C) was increased early in LPS or LTA challenge (between 1 and 4 h), with levels increasing again later in the stimulation (16–24 h). TNF-α mRNA levels were higher in response to LPS than LTA challenge at 16 and 24 h.

Relative expression of CD14 and TLR4 in developing pouch young

CD14 and TLR4 mRNA were detected in all tissues examined. CD14 mRNA expression was increased relative to controls in most tissues in the first 3–4 weeks (Fig. 6A,B). CD14 mRNA expression also mildly to moderately increased compared with controls, in all tissues between 60 and 100 days, with levels decreasing after this time (Fig. 7A,B). CD14 mRNA in the lung demonstrated sharp peaks of expression around 60 and 90 days (Fig. 7B). TLR4 mRNA expression was also moderately elevated, compared with controls, in the first 3–4 weeks in the jejunum, lung, liver and to some extent in the bone marrow and thymus (Fig. 6C,D). All tissues had increased expression of TLR4 mRNA at around 70 days relative to controls (Fig. 7C,D). Elevated levels of TLR4 mRNA occurred in the bone marrow, cervical thymus, spleen, liver and gut from 90 to 105 days (Fig. 7C,D).

DISCUSSION

CD14 and TLR4 have fundamental roles in the recognition of pathogens and the initiation and direction of both innate and adaptive immune responses. This is the first study to examine these components of the innate immune system from any marsupial species. The tammar CD14 sequence had greater homology with eutherian CD14 than with CD14 from lower order vertebrates, as indicated by the percentage identities and close phylogenetic relationships. The amino terminal end of CD14 is important for LPS binding, interaction with TLR4 and signalling (Kim et al., 2005). Several hydrophobic pocket motifs, involved in the creation of the LPS binding pocket (Cunningham et al., 2000; Kim et al., 2005; Palsson-McDermott and O'Neill, 2004), were conserved in the tammar and all other species examined in this study. Four N-linked potential glycosylation sites were identified in the tammar sequence, two of which were specific to the tammar. These potential N-linked glycosylation sites are important in the formation of the glycosphosphatide anchor to the cell membrane (Muroi et al., 2002). Two non-conservative substitutions were also present in motifs involved in TLR interaction and signalling, although the majority of these motifs were well conserved. A non-polar glycine was substituted for an Asp^{10} and a polar threonine for $Glyc^{95}$. Mutations in these motifs are not crucial for LPS binding (Juan et al., 1995; Stelter et al., 1999) but they may affect cellular signalling of CD14 once LPS is bound. The transmembrane, hydrophobic regions and the TIR domain of TLR4 were also identified from tammar trace genome sequence releases. The TIR domain had a high percentage identity (77%) when compared with eutherian sequences, reflecting the importance of conserving the signal transduction machinery in this domain.

A biphasic expression pattern was noted for CD14 and TLR4 mRNA on challenge with LPS and was associated with the pro-inflammatory cytokine TNF-α. These results concur with the role of CD14 and TLR4 in the recognition of LPS, and the capacity of CD14 to neutralise endotoxin and bind different PAMP including LTA (Ellingsen et al., 2002; Takeuchi et al., 1999). This second

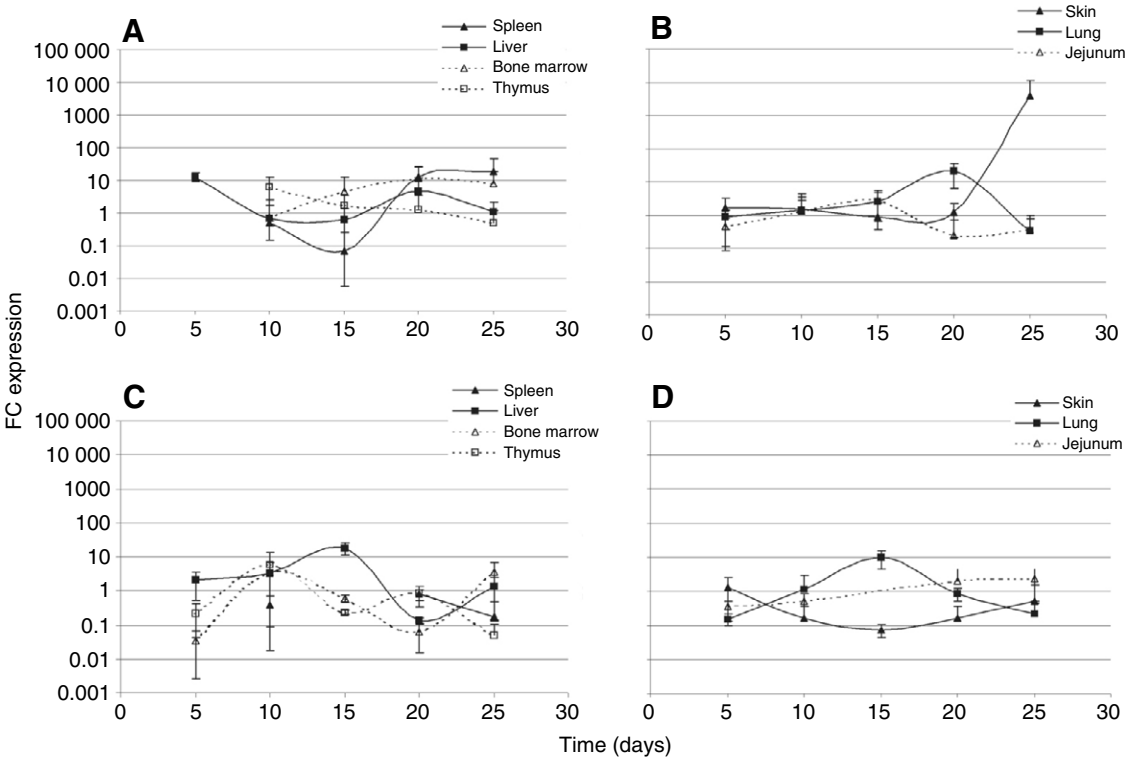

Fig. 6. Average expression of CD14 (A,B) and TLR4 (C,D) in organs of the tammar pouch young throughout the first 30 days of life. (A,C) Expression in cervical thymus, spleen, liver and bone marrow. (B,C) Expression in skin, lung and jejunum. Relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). *Y* error bars indicate ±1s.d.

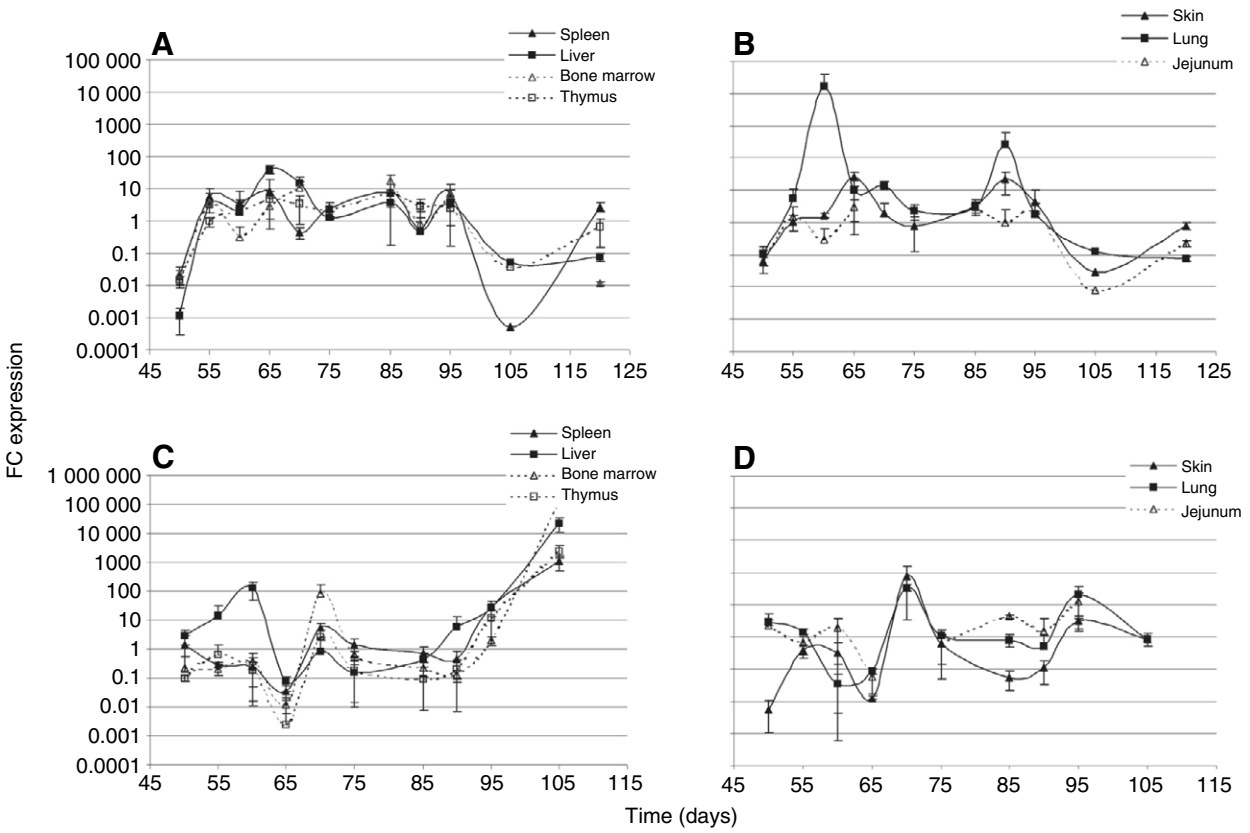

Fig. 7. Expression of CD14 (A,B) and TLR4 (C,D) in organs of the tammar pouch young up to 120 days of life. (A,C) Expression in cervical thymus, spleen, liver and bone marrow. (B,C) Expression in skin, lung and jejunum. Relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). *Y* error bars indicate ±1s.d.

phase increase of expression may reflect a feedback effect of other pro-inflammatory genes induced by the PAMP (Moller et al., 2005), or by the early increase in CD14 and TLR4 levels. This is supported by the TNF-α mRNA expression pattern in response to LPS stimulation. The induction of TNF-α mRNA by LTA appeared to be correlated with CD14 mRNA expression but was TLR4 independent in the tammar leukocytes. This finding correlates with a previous study in human polymorphonuclear leukocytes where the effect of LTA on cytokine induction was found to be CD14 dependent but TLR independent (Hattar et al., 2006).

Expression of both CD14 and TLR4 was identified in the pouch young in all tissues examined in the first month of life. At this time, the marsupial neonate is still developing an immune system and the finding suggests that an innate defence mechanism may be active at this early stage (Basden et al., 1997; Old and Deane, 2000). CD14 and TLR4 had distinct patterns of expression in early pouch life. CD14 mRNA levels were moderately increased from 60 to 100 days in all tissues. This time period coincides with the final maturation of organs, such as the spleen, lymph nodes and liver, and may also reflect a role of tissue macrophages in apoptosis, in which CD14 itself is known to play a part (Tobias, 2003). Increases in CD14 mRNA levels in the jejunum were severalfold lower than in most other organs, which may help to maintain low levels of inflammation in normal intestines as previously suggested in eutherians (Smith et al., 2001). While interstitial cells, secretory cells and sweat glands have been found to be weakly CD14 positive in the skin and lungs (Bordessoule et al., 1993), the increased expression in these organs seen during days 60–100 in the present study is more likely to be indicative of tissue macrophages, which have a much more dense surface expression of CD14.

Most pouch young tissues examined had increased expression of TLR4 mRNA around 70 days. These increases in the spleen, liver, cervical thymus and bone marrow in particular may reflect the increasing maturation of the immune system, as unique sets of TLR are also expressed on adult immune cells (Azuma, 2006) and mature lymphocytes are found in these tissues from 35 days *post partum* (Old and Deane, 2003). These increases also coincide with increased CD14 expression, further supporting the view that they may result from maturing immune cells. Inherent TLR expression on epithelial cells may also contribute to the increased expression in the skin, jejunum and lung. In the liver, expression of TLR4 mRNA drops sharply with the cessation of haematopoiesis (at 60 days) but inherent TLR4 expression (John and Crispe, 2005) may account for the much more moderate increase in TLR4 mRNA levels seen between 70 and 90 days. In addition, in the spleen, liver, cervical thymus, bone marrow and jejunum, TLR4 expression was elevated from 90 until 105 days. This coincides with the maturation of secondary immune tissues and adaptive immune responses (Basden et al., 1997; Old and Deane, 2000) and the start of the switch phase just prior to emergence. Hence, these increases may represent an immune strategy in preparation for exposure to new pathogens in the *ex marsupium* environment.

In summary, this work is the first to evaluate CD14 and TLR4 in any marsupial species. These important components of PAMP recognition are fundamental in the initiation and direction of both innate and adaptive immune responses. The findings of this study

have demonstrated a high degree of conservation of functional motifs in both CD14 and TLR4 and close phylogenetic relationships with eutherian orthologues. Further, the study has demonstrated that expression of CD14 and TLR4 increases in adult tammar leukocytes in response to certain PAMP, and that this correlates with the expression of the pro-inflammatory cytokine TNF-α. CD14 and TLR4 expression in pouch young, especially in the first month of life, suggests that innate immunity has a significant role in the altricial pouch young prior to the development of a competent adaptive immune system. Early maturation of the innate immune system may have developed as an immune survival strategy to protect the marsupial neonate from exposure to microbial pathogens.

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