Protection of marsupial young: Immune mechanisms which protect the developing tammar wallaby (*Macropus eugenii*)

Submitted by

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Declaration

Except where specific reference is made to other sources, the work presented in the thesis is the work of the author. It has not been submitted, in whole or in part, for any other degree.

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Melanie Jane Edwards

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Abstract

The newborn marsupial is born at a highly altricial stage of development when compared to eutherian newborn mammals. Interestingly, the marsupial young are born into a non-sterile environment and lack a developed immune system which prevents them from mounting an adaptive immune response.

Two reviews are presented in this thesis. The first review examined the complementary mechanisms which protect the developing marsupial young. Immune protection through forms of innate immunity and maternal contributions has been examined in marsupials. However, as there are many facets to immune protection, our understanding of the protection of the developing marsupial young is far from complete and much of the developing marsupial's innate immune system remains unexplored. Importantly, the review also recognised that the availability of marsupial genomes should be exploited to help direct further research on the protection of marsupial young.

The second review examined the role of the pouch in marsupial reproduction. While it is clear that the pouch provides immune protection to those species that have a pouch, the pouch cannot provide immune protection to the young of those species without a pouch. Thus, instead of using the pouch to explain immune protection of marsupial young, the review examined the role of the pouch in supporting highly altricial young and promoting reproductive success in various environments where a pouch may confer significant advantages over free hanging young.

The reviews set the direction for the experimental research presented in this thesis. The model species tammar wallaby (*Macropus eugenii*) was used to further examine innate immune genes. First, innate immune genes, mucin and lysozyme were identified or predicted in the tammar wallaby. The mucin and lysozyme genes were then physically

mapped to conserved regions of the tammar wallaby genome which facilitated the confirmation of the identification of the genes.

Second, the expression of immune genes was examined in the skin and lung of developing tammar wallabies considered immune-incompetent and immune-competent using transcriptome sequencing. There were 390 and 429 immune genes expressed in developing tammar wallaby skin and lung, respectively, suggesting that both tissues play a role in immune defense. Unexpectedly, mucin and lysozyme were not expressed in the skin and lung of wallabies considered to be immune-incompetent. Further, enrichment analysis of genes identified in Gene Ontology terms showed that specific immune genes, complement component 3, complement factor B and apolipoprotein A1, have increased expression in the skin and lung of wallabies considered immune-incompetent. The increased expression of the immune genes suggests that there may be specific innate immune system processes in play to compensate for the delayed development of the adaptive immune system in marsupial.

The research presented in this thesis identified gaps in the research of immune protection of marsupial young and identified, mapped and examined the expression of innate immune genes in the developing tammar wallaby. Thus, we are closer to understanding the complexity of the developing marsupial immune system. The identification of immune genes and their expression, lays the foundation for further functional research in marsupial developmental immunology.

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Chapter7. Synopsis and future work

List of abbreviations

AMOP	Adhesion associated domain in MUC4 and other proteins
AMP	Antimicrobial peptide
BAC	Bacterial artificial chromosome
cDNA	Complementary deoxyribonucleic acid
CSIRO	Commonwealth Scientific and Industrial Research Organisation
CK	Cysteine knot
EST	Expressed sequence tag
FDR	False discovery rate
FISH	Fluorescence in situ hybridisation
FPKM	Fragments per kilobase of exon per million fragments mapped
GIT	Gastrointestinal tract
GO	Gene Ontology
MEU	*Macropus eugenii* chromosome
MHC	Major histocompatibility complex
mRNA	Messenger ribonucleic acid
NCBI	National Center for Biotechnology Information
NIDO	Nidogen-like
NKC	Natural killer cells
PFAM	Protein families
PRR	Pattern recognition receptors
PS	Peptide sequences
PTS	Proline, threonine and serine
rDNA	Ribosomal deoxyribonucleic acid
SEA	Sea urchin sperm protein, enterokinase and agrin
VWD	Von Willebrand D

Species mentioned in this thesis

Aerobacter aerogenes	
Ambystoma mexicanum	Mexican salamander
Arctocephalus gazelle	Antarctic fur seal
Bos taurus	Cow
Chironectes minimus	Water opossum
Citrobacter freundii	
Dama dama	Fallow deer
Danio rerio	Zebrafish
Dasyurus hallucatus	Northern quoll
Didelphis albiventris	White-eared opossum
Didelphis virginiana	Virginia opossum
Escherichia coli	
Eubalaena glacialis	North Atlantic right whale
Homo sapiens	Human
Isoodon macrourus	Northern brown bandicoot
Macropus eugenii	Tammar wallaby
Macropus giganteus	Easter grey kangaroo
Macropus rufus	Red kangaroo
Macaca mulatta	Rhesus monkey
Mesocricetus auratus	Golden hamster
Monodelphis domestica	South American opossum
Mus musculus castaneus	House mouse
Nematostella vectensis	Starlet sea anemone
Notophthalmus viridescens	Eastern newt
Notorcytes typhlops	Marsupial mole
Ovis aries	Sheep
Petrogale xanthopus	Yellow-footed rock-wallaby
Phascolarctos cinereus	Koala
Pleuronectes americanus	Winter flounder

Potorus longipes	Long-footed potoroo
Pseudomonas aeruginosa	
Pseudocheirus peregrinus	Ringtail possum
Rana chensinensis	Chinese brown frog
Salmo salar	Atlantic salmon
Salmonella Newport	
Sarcophilus harrisii	Tasmanian devil
Setonix brachyurus	Quokka
Sminthopsis douglasi	Julia Creek dunnart
Staphylococcus albus	
Staphylococcus aureus	
Staphylococcus epidermidis	
Streptococcus faecalis	
Suncus murinus	Musk shrew
Trichosurus vulpecula	Brushtail possum
Vombatus ursinus	Common wombat
Xenopus laevis	African clawed frog

Genes and compounds mentioned in this thesis

AHSG	Apha-2-HS-glycoprotein
APOA1	Apolipoprotein A1
APOA2	Apolipoprotein A2
B6SF63_MACEU	
BACE1	Beta-site APP-cleaving enzyme 1
C2	Complement 2
C3	Complement 3
C4B	Complement 4b
CD14	Cluster of differentiation 14
CD81	Cluster of differentiation 81
CELA1	Chymotrypsin-like elastase family, member 1
CFB	Complement factor B
CFH	Complement factor H
Duox1	Dual oxidase 1
Duox2	Dual oxidase 2
EMCN	Endomucin
FCN3	Ficolin 3
FIMC1	Frog integumentary mucin C.1
FREM2	FRAS1 related extracellular matrix protein 2
GPX2	Glutathione peroxidase 2
HSPG2	Heparan sulfate proteoglycan 2
HPX	Hemopexin
IFNB1	Interferon beta 1
IFNG	Interferon gamma
Ig	Immunoglobulin
IGF2	Insulin-like growth factor 2
IL6	Interleukin 6
IL10	Interleukin 10
LTF	Lactoferrin
LYZ	Lysozyme
MaeuCath1 to *8*	Tammar wallaby cathelicidin 1 to 8
MCAM	Melanoma cell adhesion molecule

MCP1/CCL2	Monocyte chemoattractant protein 1
MMP7	Matrix metallopeptidase 7
MPO	Myeloperoxidase
MRPL23	Mitochondrial ribosomal protein L23
MUC1 to *21*	Mucin 1 to Mucin 21
MUC3A	Mucin 3A
MUC5AC	Mucin 5AC
MUC5B	Mucin 5B
OVGP1	Oviductal glycoprotein 1
PENK	Proenkephalin
PRDX1	Peroxiredoxin 1
SAA	Serum amyloid A
SIDT1	SID1 transmembrane family, member 1
SFTPA	Surfactant protein A
STAG1	Stromal antigen 1
TFF2	Trefoil factor 2
TGFB1	Transforming growth factor beta 1
TLR2 to *5*	Toll-like receptor 2 to 5
TNFA	Tumor necrosis factor alpha
VELP	Very early lactation protein
WAM1	Wallaby antimicrobial peptide derived from *MaeuCath1*

Chapter 1. Introduction

Background

In comparison to the broad altricial to precocial spectrum observed amongst different eutherian mammals at birth, marsupials are born at a developmental stage which marks the minimum onset of functionality for specific tissues (Hughes and Hall 1988). This stage of development can be visually compared to the developmental stage of a bird embryo, well before hatching, or to a eutherian mammal embryo *in utero*. The most visible characteristics of the marsupial at birth are the forelimbs, which the young uses to take hold of the mother's fur to propel itself from the mother's urogenital opening to a teat. The mouth parts are also well developed and have a very important role in fastening onto the mother's teat so the young can remain attached to the mother and obtain milk.

Remarkably, the undeveloped characteristics of marsupials outweigh the comparatively developed characteristics several-fold at birth. Many general physiological processes essential for adult survival, also remain immature until sometime after parturition. In particular, newborn marsupials have undeveloped immune tissue (Yadav *et al.* 1972; Cutts and Krause 1982; Basden *et al.* 1997) and insufficient numbers of circulating lymphocytes (Coutinho *et al.* 1995; Old and Deane 2003; Old *et al.* 2004) to mount an adaptive immune response.

Interestingly, the marsupial pouch contains a range of potentially pathogenic bacteria (Osawa *et al.* 1992; Old and Deane 1998; Deakin and Cooper 2004; Chhour *et al.* 2010) and this generates an immunologically challenging environment for the developing young as their immune tissues mature. Thus, evolutionary biologists and immunologists

are particularly interested in deciphering how these altricial young are immunologically protected in a non-sterile environment over such a critical period.

Direction

The overall aim of the research presented in this thesis was to further understand immune protection of developing marsupial young and to further contribute to the literature of marsupial developmental immunology. As the development of the immune tissues and the onset of adaptive immune protection have been well established in marsupials (Yadav *et al.* 1972; Cutts and Krause 1982; Coutinho *et al.* 1995; Basden *et al.* 1997; Old and Deane 2003; Old *et al.* 2004), the first aim was to review the literature examining complementary modes of immune protection in developing marsupial young. The review in Chapter 2 covers the exposure of developing marsupials to microbial organisms, maternal protection and innate immune protection of developing young.

Immune protection from the pouch was examined in the review in Chapter 3. However, as not all marsupials have pouches, protection from the pouch was difficult to comprehend, as some marsupial species have undeveloped young that attach to a teat on their mother's abdomen which are not enclosed by a pouch. Thus, the second aim was to review the role of the pouch in light of reproductive success and mammalian evolution. The review in Chapter 3 examines the characteristics of highly altricial young, the different types of pouches observed in marsupials and implications of the pouch for reproductive success and mammalian evolution.

The information generated from the first and second aim established the direction for the remaining aims. The reviews found that although the pouch has a number of important functions for the developing marsupial, not all marsupials have pouches (Russell 1982; Tyndale-Biscoe 2005), and therefore may not be an ideal starting point for examining immune protection in the developing marsupial. Additionally, many

researchers have already extensively examined maternal protection through the transfer of compounds with a potential role in immunity in milk.

As the mammalian innate immune system consists of many components, the remaining aims focused on innate immune genes using the model species tammar wallaby (*Macropus eugenii*). The third aim was to identify and physically map innate immune genes, mucin and lysozyme, in the tammar wallaby genome. Mucin and lysozyme genes are found in a broad range of species and play an important role in the protection of vertebrate neonates. The fourth aim sort to utilise the recently published tammar wallaby genome (Renfree *et al.* 2011) and advances in RNA-sequencing. A global assessment of the expression of innate immune genes in skin and lung of developing tammar wallabies was carried out on two tissues that are directly exposed to potentially pathogenic bacteria and environmental particulates. As RNA-sequencing permits the analysis of the expression of multiple genes in whole tissue, it was possible to venture away from compound specific analysis and use a holistic approach to determine which genes were expressed in developing tammar wallabies.

Specific aims

A summary of the aims include:

Aim 1: Review the literature examining microbial exposure and complementary modes of immune protection in developing marsupial young.

Aim 2: Review the role of the pouch in marsupial reproduction and mammalian evolution.

Aim 3: Identify and physically map innate immune genes, mucin and lysozyme, in the tammar wallaby genome.

Aim 4: Determine the expression of innate immune genes in the skin and lung of developing tammar wallabies that are considered immune-incompetent and immune-competent.

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Chapter 2. A review of complementary mechanisms which protect the developing marsupial pouch young

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Review

A review of complementary mechanisms which protect the developing marsupial pouch young

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ABSTRACT

Marsupials are born without a functioning adaptive immune system, into a non-sterile environment where they continue to develop. This review examines the extent of exposure of pouch young to microorganisms and describes the protective mechanisms that are complementary to adaptive immunity in the developing young. Complementary protective mechanisms include the role of the innate immune system and maternal protection strategies, such as immune compounds in milk, prenatal transfer of immunoglobulins, antimicrobial compounds secreted in the pouch, and chemical or mechanical cleaning of the pouch and pouch young.

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1. Introduction

The complexity of the mammalian immune system evolved before the divergence of the marsupial and eutherian lineages (Belov et al., 2007), thus, there are many similarities in the structure, function and genetic architecture of the innate and adaptive immune systems of marsupial and eutherian mammals (Siddle et al., 2010). However, and most interestingly, differences in the timing of the development and maturation of the immune system exist between the groups. In contrast to eutherians, marsupials are born remarkably undeveloped (Fig. 1). In particular, the newborn marsupial has undeveloped immune tissue (Basden et al., 1997; Cutts and Krause, 1982; Yadav et al., 1972b) and insufficient numbers of circulating lymphocytes (Coutinho et al., 1995; Old and Deane, 2003; Old et al., 2004) to mount an adaptive immune response.

Interestingly, the marsupial pouch contains a range of potentially pathogenic bacteria (Chhour et al., 2010; Deakin and Cooper, 2004; Old and Deane, 1998; Osawa et al., 1992) and this generates an immunologically challenging environment for the developing young as its immune tissues mature. Thus, evolutionary biologists and immunologists are particularly interested in deciphering how these altricial young are immunologically protected over such a critical period.

Over the past 20 years, research has focused on the exposure of pouch young to microorganisms, protective elements of milk and colostrum, antimicrobial activity in the pouch, production of

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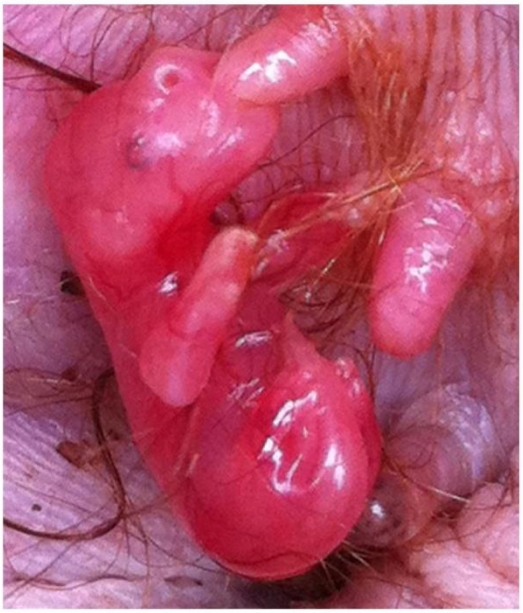

Fig. 1. A tammar wallaby weighing 350–400 mg, attached to a teat less than 24 h after birth.

antimicrobial compounds, and with the advent of new technologies and the sequencing of marsupial genomes, the identification and expression of important immune genes. The intent of this article is, therefore, to focus on (i) the extent to which pouch young are exposed to microorganisms to demonstrate that pouch young develop their immune tissue in a non-sterile environment, (ii) the complementary protective mechanisms to adaptive immunity, such as those that are provided by the mother, and (iii) the role of the innate immune system.

2. Microbial exposure

Although marsupial neonates are unable to mount an adaptive immune response at birth, they are born into a non-sterile environment. Determining microbial exposure is practical and informative. For example, knowing the microbial species that are present and the threat they pose in other species can provide an insight into the threat they may pose to marsupial young. Changes in microbial species over time may provide an insight into which species are harmful and selected against during the development of the fetus and the pouch young. Various sites of microbial colonisation have been explored in marsupials, including the urogenital opening, pouch and mouth of the mother and the gastrointestinal tract (GIT) of the pouch young (Charlick et al., 1981; Chhour et al., 2008, 2010; Deakin and Cooper, 2004; Old and Deane, 1998; Yadav et al., 1972a).

The marsupial neonate is born through the urogenital opening, which is separate from the anus, but located in a common chamber (see Fig. 1 in Chhour et al., 2008). In the tammar wallaby (*Macropus eugenii*), the female urogenital tract opening has been found to host at least 72 phylotypes of bacteria from five different phyla—Firmicutes, Actinobacteria, Fusobacteria, Proteobacteria and Bacteroidetes, while the anus just millimetres from the urogenital opening, has been found to host 50 phylotypes of bacteria, including species that were not present at the urogenital opening (Chhour et al., 2008). It is unknown if the bacterial community profile of the urogenital opening changes before and after birth to protect the neonate from potentially pathogenic strains.

The pouch microflora has been studied extensively over the past 40 years using various techniques, including culture-based biochemical tests and molecular-based methods. However, it is important to note that the methods used in the research described below are not nearly as sensitive as the current metagenomics approach which allows for the detection of a broader range of bacteria. Using culture-based methods, Old and Deane (1998) identified the greatest variety of bacteria in the tammar wallaby pouch when there were no young present in the pouch; this included 25 species, 15 of which were unique to pouches containing no young. Of interest were the Gram negative species of bacteria of which eight occurred in pouches with no pouch young, while three species occurred in pouches with young less than 3 weeks (Old and Deane, 1998). Chhour et al. (2010) also found that bacterial diversity was lowest in pouches containing a day old pouch young, while pouches containing no pouch young contained the greatest diversity of bacteria.

In the quokka (*Setonix brachyurus*) pouch, Gram negative bacilli and Gram positive *Corynebacterium*, identified using culture-based methods, were present during oestrus, but as the animal entered anoestrus the frequency of gram negative isolates decreased while the Gram positive species increased (Charlick et al., 1981). In another culture-based study in the quokka, there was a complete absence of pouch flora in one pregnant female (Yadav et al., 1972a). A culture-based study coupled with 16S rDNA identification found that during anoestrus, the pouches of female brushtail possums (*Trichosurus vulpecula*) also had a higher proportion of Gram positive cocci while females carrying a pouch young had fewer of these species. Females in oestrus, during gestation or carrying a pouch young had the highest occurrence of enteric Gram negative rods, such as *Escherichia coli* and *Klebsiella* species (Deakin and Cooper, 2004).

Collectively, the studies above show that different marsupial species harbour a diverse range of microorganisms suggesting that there is a selective pressure against Gram negative strains as birth approaches. Furthermore this suggests that maternal mechanisms may manipulate the pouch environment to favour or exclude particular strains of bacteria.

Females groom their pouch with their tongue in the period leading up to birth, which suggests that pouch young may also be in contact with the bacterial strains present in the mother's mouth. Using sequencing techniques to identify bacteria in the saliva of three different adult females, Chhour et al. (2010) found 48 different phylotypes belonging to five different phyla—Firmicutes, Fusobacteria, Actinobacteria, Bacteroidetes and Proteobacteria. However, only one phylotype present in the maternal saliva was also found in the pouch. It is likely that the pouch environment therefore did not permit the growth of bacterial strains found in the mother's saliva thereby limiting the exposure of marsupial neonates to microorganisms.

The GIT of a mammalian fetus is sterile and colonized at the time of birth and during subsequent development. The first source of bacteria to colonise the human GIT comes from the vagina and intestinal tract of the mother (Biasucci et al., 2008). It is likely that the bacteria that colonise the marsupial GIT are also from the mother's intestinal tract, other sites near the urogenital opening, and from fur and saliva, as the young makes its way from the urogenital opening to the pouch with its mouth and nostrils open (Tyndale-Biscoe, 2005). Once a young attaches to a teat, it remains firmly attached to the teat for up to 38–86% off pouch life (Tyndale-Biscoe and Janssens, 1988). Breast milk is likely to play a subsequent role; however, the microflora of marsupial milk is yet to be examined.

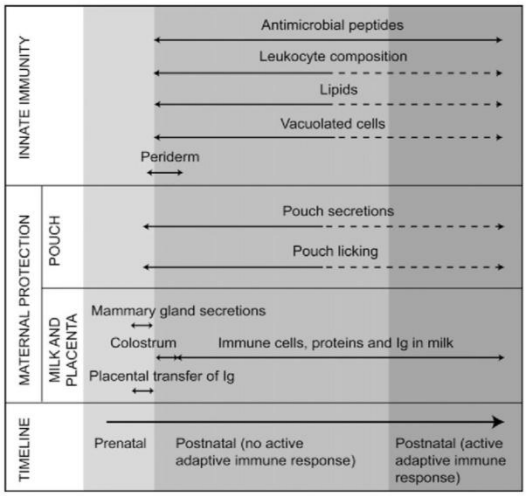

Fig. 2. A general diagram showing the complementary protective mechanisms for marsupial pouch young with respect to the occurrence of the adaptive immune response. Dashed lines indicate period of transition or reduction in importance/activity as young develops own adaptive immune response.

Few studies have identified specific species or numbers of species in the GIT of young marsupials. The quokka pouch young's intestine was found to be colonised with two commonly identified species, *E. coli* and *Streptococcus faecalis*, and with other organisms, such as *Aerobacter aerogenes*, *Klebsiella* species, *Salmonella newport*, *Pseudomonas aeruginosa* and *Staphylococcus albus* by Yadav et al. (1972a) using culture-based methods. A molecular approach showed that at 40 and 56 days postpartum, the GIT of the tammar wallaby pouch young contained at least 53 different phylotypes dominated by the *Enterococcus* and *Streptococcus* genera (Chhour et al., 2010). Chhour et al. (2010) also examined the microflora of the GIT of the tammar wallaby pouch young and compared it to the microbiome of different maternal sites, including the pouch, urogenital opening and saliva. Each site possessed its own unique microbiome and only nine out of 53 phylotypes were detected in both the pouch young GIT as well as a maternal site. The comparative study showed that the pouch microflora is unique, when compared to other maternal sites, suggesting that a mechanism may exist to control the pouch environment in favour of specific bacterial species.

Chhour et al. (2008) identified bacterial strains in the tammar wallaby and highlighted their pathogenicity using non-marsupial species. Although this is beyond the scope of this review, we include two studies which have linked, even if superficially, the threat microorganisms pose to the survival of marsupial pouch young. The first study showed that a non-sterile incision made in the skin of a young Virginia opossum (*Didelphis virginiana*) (less than 6 days old) failed to heal, becoming infected and resulting in the death of the animal (Block, 1960). A likely source of infection is bacteria found on the skin of the young opossum—as identified in Krause et al. (1978). In the second study, microorganisms were found in the pouches of four koalas (*Phascolarctos cinereus*), in which mortality of pouch young (one to 173 days postpartum) had occurred; although, autopsies were not carried out to confirm if the microorganisms were the cause of death (Osawa et al., 1992).

The phenomenon of how marsupial pouch young survive in a non-sterile environment without the ability to mount an immune response has received some attention. As a result, several hypotheses have arisen, and these can be divided into two groups—protection through maternal mechanisms and protection through innate immunity. These are discussed separately below; however, it is clear that both maternal and innate mechanisms play a substantial and essential role in the immune protection of marsupial pouch young. Fig. 2 illustrates general complementary protective mechanisms for marsupial pouch young and when they may occur with respect to the onset of the active adaptive immune response.

3. Maternal protection

Maternal protection strategies include the transfer of immunoglobulins (Igs) and other immune compounds in milk, prenatal transfer of Igs, antimicrobial compounds secreted in the pouch, and chemical or mechanical cleaning of the pouch and pouch young provided by maternal licking. Marsupial pouch young receive immune compounds passively from the mother through milk and colostrum, allowing them to achieve some protection against pathogens until their immune system has matured and they are able to elicit their own immune response (Old and Deane, 2000). In marsupials, the pattern of milk secretion is extremely complex and described using three to four phases, which are broadly correlated with major periods of growth and maturation of the young animal (Joss et al., 2009; Nicholas, 1988b). There are also two distinct periods where immune compounds are transferred via the mammary gland to the pouch young (Adamski and Demmer, 2000; Daly et al., 2007). The first distinct immune period (days 0–1 postpartum) is a colostral phase, which is thought to provide the new born with significant immune protection, and the second period occurs later during pouch life (from day 100 postpartum in brushtail possums; Adamski and Demmer, 2000) to provide additional protection to the young as it exits the pouch for the first time.

Several different compounds that can afford immune protection have been identified in marsupial milk, including Igs, lysozyme, transferrin, and immune cells, such as neutrophils and macrophages (Adamski and Demmer, 1999, 2000; Joss et al., 2009; Old and Deane, 2000; Piotte et al., 1997; Young et al., 1997). The roles of these compounds have been established in other mammals; however, their importance for the immune protection of marsupial young has not been assessed.

In the tammar wallaby, Joss et al. (2009) identified 14 proteins in the four phases of milk secretion that are known to have a primary function in host defence, including Igs, myeloid cathelicidin and complement B protein. In the young of other species IgA protects against pathogens in the respiratory and GIT mucus membranes of newborn animals, while IgG is transported across the gut epithelium into the circulatory system (Woof and Kerr, 2006). Likewise, Deakin and Cooper (2004) showed that maternal IgG antibodies to brushtail possum pouch bacteria were also present in the circulation of the developing young. Yadav (1971) showed that in the quokka and brushtail possum there was intestinal absorption of Ig until the young leave the pouch. Furthermore Igs were not detected in the serum of the Virginia opossum (Hindes and Mizell, 1976) or gray-short tailed opossum (*Monodelphis domestica*) (Samples et al., 1986) until they suckled for the first time, suggesting that for most species of marsupials there is only postnatal, not prenatal, transfer of Igs. However, the tammar wallaby is an exception, as IgG was found in fetal and neonatal serum (Deane et al., 1990; Renfree, 1973). The differences between prenatal transfer of Igs between marsupial species is unknown; however, prenatal transmission of IgG has not been identified in all mammals; for example, placental absorption by the young has been found in the dog (Carnivora) and human (Primates) but not in either the horse (Perissodactyla), cow, sheep or pig (Artiodactyla) (Baintner, 2007).

Other proteins with secondary immune function identified in the tammar wallaby, eastern grey kangaroo (*Macropus giganteus*) and brushtail possum milk include α-lactalbumin, β-lactoglobulin, haptocorrin, amphiphysin and very early lactation protein (VELP) (Godovac-Zimmermann and Shaw, 1987; Joss et al., 2009; Piotte et al., 1997, 1998; Trott et al., 2002). VELP has only been found in the milk of brushtail possums and tammar wallabies (Joss et al., 2007; Kuy et al., 2007) and is thought to have a similar role and function to mannose binding lectin protein, which is part of the classical complement pathway (Joss et al., 2009). Transferrin, fatty acid binding proteins, and haptoglobin have also been identified in the milk of tammar wallabies (Joss et al., 2007; Nicholas, 1988a). Transferrin and haptoglobin act as iron sequesters, making iron unavailable to pathogens, while fatty acid binding proteins prevent bacteria binding to mucosal surfaces and α-lactalbumin has bactericidal activity (reviewed in Joss et al., 2007).

Immune cells have also been identified in milk and colostrum providing another level of protection for the young. Neutrophils were identified in high numbers in the milk of tammar wallabies with young up to 105 days postpartum (Young et al., 1997) and a long-footed potoroo (*Potorous longipes*) feeding a very young animal of unknown age (Young and Deane, 2001). Neutrophils were not present in quokka milk until 20 days postpartum, while mononuclear cells were found in high numbers (Cockson and McNeice, 1980). Macrophages were found in high numbers in the milk of the tammar wallaby with young older than 105 days (Young et al., 1997) and in the milk of yellow-footed rock-wallaby (*Petrogale xanthopus*) with young ready to leave the pouch (Young and Deane, 2001). Small and large lymphocytes were present at 12 days postpartum in quokka milk (Cockson and McNeice, 1980), and in tammar wallaby milk with young up to 250 days postpartum (Young et al., 1997).

The research that has explored the composition of marsupial milk and when particular compounds or cells are secreted demonstrates the complexity of immune protection in marsupial species on just one level. However, another level of protection that occurs in the pouch appears equally as complex. The marsupial pouch is a fold of skin with an opening which creates a space and a physical barrier of protection in which marsupial young develop; however, not all marsupials have a pouch of this character. In some marsupial species there is no pouch, while in others folds of skin cover the young after it attaches to a teat (Tyndale-Biscoe, 2005). Recent studies have shown that the pouch is extremely complex and could also play a role in protecting marsupial young by providing an active site for immune compounds. For example, pouch secretions have been found to exhibit antimicrobial activity and also contain an array of proteins with potential antimicrobial activity (Ambatipudi et al., 2008; Bobek and Deane, 2002). The source of the antimicrobial activity is currently unknown; however, hypotheses are continuing to emerge to explain its origin.

Studies examining pouch washes (washes from inside the pouch) from koalas and tammar wallabies have documented antimicrobial activity against Gram negative *E. coli* but not Gram positive *Staphylococcus aureus* suggesting that specific compounds might be employed to manipulate the bacterial community profile and protect the pouch young. For example, pouch washes from the koala showed up to 63% inhibition of *E. coli* growth (Bobek and Deane, 2002), with the highest antimicrobial activity observed in pouch washes from female tammar wallabies in oestrus when compared to pouch washes from female tammar wallabies in pre and post-oestrous phases (Ambatipudi et al., 2008). In contrast, antibacterial activity was not observed from samples taken from tammar wallaby pouches from 2 days before birth to 6 weeks after birth (Baudinette et al., 2005). It is unknown why the results from the two studies differ; however, Baudinette et al. used swabs to collect pouch material while Ambatipudi et al. used whole pouch washes. It is likely that Ambatipudi et al. were able to collect more compounds from a larger area.

Further studies have examined compounds with potential immune function present in pouch washes, supporting the hypothesis that such compounds could potentially play a role related to the well being of the young (Ambatipudi et al., 2007, 2008). Proteins, in particular, have been shown to change over the life time of a female's pouch, with the greatest diversity of proteins seen in the pouch washes of mature reproductively active females (Ambatipudi et al., 2007). Proteins detected in pouch washes of tammar wallabies and common wombats (*Vombatus ursinus*) included β-lactoglobulin, α-lactalbumin, hornerin and dermcidin (Ambatipudi et al., 2007). Interestingly, β-lactoglobulin is an important source of biologically active peptides that play an important role in human health (Hernandez-Ledesma et al., 2008). In tammar wallaby pouch washes, β-lactoglobulins have been identified over a wide range of molecular weights (15–60 kDa) (Ambatipudi et al., 2008), including weights that are higher than those usually detected in the milk and gut, which suggests that β-lactoglobulin in the pouch may polymerise and become glycosylated (Ambatipudi et al., 2008). The significance of this difference is demonstrated by bovine β-lactoglobulin, which exists as a dimer under specific physiological conditions and polymerises to an octamer at low temperature and pH—Ambatipudi et al. (2008) suggested that a similar transition could occur in the marsupial pouch as pouch conditions change.

Antimicrobial activity and changes in microbial populations have been attributed to maternal licking, secretions from the pouch itself, secretions from mammary glands or secretions from the pouch young. Maternal licking immediately prior to birth has been observed in marsupials and is presumed to reduce bacterial flora through chemical action as there may be host defence compounds, such as salivary lysozymes or Igs which could be deposited during licking (Charlick et al., 1981). Several studies have examined compounds in marsupial saliva (Beal, 1987, 1990, 1992) but only from a physiological and dietary perspective, not in terms of the immunological role.

The pouch could also be a source of antimicrobial activity. Visible morphological changes have been found in the pouch during the reproductive life-time of the female, which could provide clues to the source or sources of antimicrobial activity. As a female tammar wallaby becomes sexually mature the teats evert and the pouch deepens (Nurse and Renfree, 1994), and as the female undergoes oestrous cycles and/or pregnancy during the breeding season the pouch skin becomes clean and moist (Ambatipudi et al., 2008). In the non-breeding season of a non-lactating female tammar wallaby the pouch is deep but the skin is dry and coated with a dried secretion (Ambatipudi et al., 2008). In another study the administration of oestrone triggered the development of the brushtail possum's pouch and a pigment was secreted within the pouch (Bolliger and Carrodus, 1938).

Pouch tissue contains sweat and sebaceous glands in the dermal layer, similar in appearance to skin. Sebaceous glands in other animals produce sebum that mainly consists of lipids that act as a water repellent and a surfactant of eccrine secretions. It has been suggested that sweat and sebaceous glands may be responsible for the antimicrobial activity in the pouch as *Corynebacterium* species, which are usually associated with these glands were found in high numbers in the pouch of the quokka during oestrus (Charlick et al., 1981).

Other hypotheses suggest that the antimicrobial compounds found in pouch washes could be derived from mammary gland secretions prior to attachment or as a digested product excreted by the pouch young. Ambatipudi et al. (2008) looked at mammary gland secretions before attachment and gut samples of young, and although their findings were inconclusive they suggested that

compounds, such as β-lactoglobulin, could be derived from the pouch, the mammary gland during pre-oestrus or from the gut of the neonate.

The histological appearance of the pouch epithelium over the reproductive period has both demonstrated and failed to demonstrate changes throughout oestrus and anoestrus. In the quokka, Waring and Cockson (unpublished data from Charlick et al., 1981), found that during the oestrous cycle sebaceous and apocrine glands developed in the pouch causing the lining epithelium to be moist. Further, during lactation the glandular component was more pronounced. In contrast to Waring and Cockson's observations, histological studies of the pouch skin of brushtail possums, as they entered an artificially induced oestrus after treatment with either oestradiol or follicle stimulating hormone followed by luteinizing hormone, failed to demonstrate any changes in the epithelial tissue beds reflective of increased secretory activity (Old et al., 2005).

4. Innate immune protection of developing young

While maternal mechanisms play a significant role in the protection of pouch young, the innate immune system also plays a substantial role. Innate immune protection is an extremely complex part of the immune system, and essentially comprises the forms of immune protection that young are born with. For the purpose of this review, the role of the different components of the innate immune system, including immune cells, antimicrobial compounds and physical and chemical barriers is examined. The extent to which these have been investigated in marsupial pouch young is examined below.

Innate immune cells that are employed by mammals include neutrophils, eosinophils, basophils, monocytes, macrophages, natural killer cells (NKC), mast cells and dendritic cells. Although it is tempting to presume that what the newborn marsupial lacks in adaptive immune cells it makes up for in innate immune cells, this is not the case, with the exception of one type of immune cell investigated so far—neutrophils.

Newborn Virginia opossums were found to have far fewer leukocytes (less than 600 cells/mm^3) than the Virginia opossum adult (approximately 18,300 cells/mm^3) (Cutts and Krause, 1980). Interestingly, the composition of leukocytes changed as the young developed, with neutrophils (which are capable of ingesting microorganisms or particles) comprising approximately 16% of the Virginia opossum's leukocytes at birth, approximately four times the number of neutrophils per mm^3 in adults (Cutts and Krause, 1980). A similar pattern was also found in the quokka, (Yadav, 1972) and tammar wallaby (Basden et al., 1996), suggesting that neutrophils have an important role in protecting the young marsupial. In contrast, the concentration of eosinophils, basophils and monocytes increased slightly over the first weeks after birth and then either decreased or remained constant (Cutts and Krause, 1980).

Examination of dendritic and mast cells in marsupials offered no further insight into the immune protection of the newborn (Coutinho et al., 1994; Santos et al., 2003). However, the identification of large lymphocytes in the quokka and Virginia opossum in the first days after birth (Cutts and Krause, 1980; Rowlands et al., 1964; Yadav et al., 1972b) may provide some clues. Although the lymphocytes were not further classified in the former studies, Parra et al. (2008) detected a T cell receptor transcript isoform in gray-short tailed opossum as young as 1 day old, suggesting that they at least have cells committed to becoming lymphocytes even though they may not be functionally mature at this stage. Alternatively, the large lymphocytes could be NKC—lymphocytes that kill different types of cancer and virus infected cells. NKC may emerge rapidly in young marsupials to kill altered cells which pose a threat to the young at such a critical time of development.

Immune cells have general characteristics which provide for another level of investigation into immune protection. For example, various immune cells have unique molecules on their surface for recognising pathogens and they also rely on messenger molecules for intercellular communication. In the tammar wallaby, the expression of two molecules used for surface recognition, CD14 and TLR4, have been identified in pouch young tissue (liver, skin, lung and jejunum) in animals as young as 5 days old (Daly et al., 2008b). While investigations of messenger molecules in marsupial young have not yet commenced, the identification of the genes for these compounds in marsupial genomes has laid the foundation for their detection (Belov et al., 2007; Siddle et al., 2010).

Numerous proteins have important roles in the innate immune system. Of particular interest are those that could be critical for immune protection in the developing young. Lactoferrin and lysozymes are particularly well-known for their antimicrobial properties and ubiquitous nature (Jollés and Jollés, 1984; Legrand and Mazurier, 2010). However, research examining the role of these compounds in marsupials is limited, with the exception of studies examining their occurrence in milk and colostrum (Joss et al., 2007, 2009; Kuy et al., 2007; Nicholas et al., 1989) and the mapping of two lysozyme genes to tammar wallaby chromosomes (Edwards et al., 2011). To date, the expression of genes encoding immune proteins in developing marsupials has included studies investigating the antioxidant, peroxiredoxin 1 (*PRDX1*) and antimicrobial peptides (AMPs). Although *PRDX1* has multiple functions, Daly et al. (2008c) showed that adult tammar wallaby leukocytes increased their expression of *PRDX1* after stimulation with toxicants from bacteria, suggesting that *PRDX1* has a role in immunity. The expression of *PRDX1* was demonstrated in tammar wallabies as young as 5 days old suggesting that it may also provide protection in the developing young.

AMPs have received a great deal of attention in other research areas, as they have the potential to serve as an alternative to antibiotics (Marshall and Arenas, 2003). AMPs are found in most organisms, from plants to mammals (Lehrer and Ganz, 1999; Marshall and Arenas, 2003), and are located in those parts of the organism that are most likely to come into contact with pathogens; for example, in the skin, ears, eyes and epithelial surfaces in animal species (Hancock and Scott, 2000). AMPs play a large a role in defence against microbes and are considered to be just as important to the host as antibodies and other specific immune cells (Hancock and Scott, 2000).

The two main mammalian AMPs include cathelicidins and defensins. Cathelicidins are especially crucial for neonatal survival and defence and have been detected in the skin of neonatal mice at levels much higher than seen in adults (Dorschner et al., 2003). Fourteen cathelicidin genes have been identified in tammar wallaby pouch young (Wang et al., 2011), with increased expression of MaeuCath1 in the skin, lung, jejunum, liver and spleen over the first 25 days postpartum (Daly et al., 2008a) and MaeuCath8 being expressed in the spleen and GIT of the tammar wallaby from 1 day after birth (Carman et al., 2009). Further, the synthesized cathelicidins were found to be effective in killing a broad range of bacteria, suggesting that the antimicrobials play a large role in protecting the pouch young before its adaptive immune system develops (Wang et al., 2011).

In the gray short-tailed opossum genome, 33 defensin genes have been identified, including a large opossum specific expansion area, which suggests that these genes could have a marsupial specific immunological role (Belov et al., 2007). While many other AMPs exist in various species, for example, magainin from the African clawed frog (*Xenopus laevis*) skin (Zasloff, 1987), protegrin from porcine neutrophils (Wessely-Szponder et al., 2010) and

pleurocidin from winter flounder (*Pleuronectes americanus*) skin (Cole et al., 1997), to date only defensins and cathelicidins have been identified in marsupials.

The skin epithelium provides a barrier which acts as the first line of defence against microbial pathogens through physical and chemical properties (Parham, 2009). Skin comprises many forms of protection, including Langerhans cells, which process microbial antigens to become antigen-presenting cells, and sebaceous glands, which produce sebum that acts as a pathogen inhibiting agent (Thibodeau and Patton, 2007). Peptides and lipids with antimicrobial activity are also secreted by the skin, and therefore constitute part of the innate immune system (Drake et al., 2008; Schröder, 1999).

Marsupial young are born with an outer layer of developing epidermis called the periderm, which is lost approximately 1 week after birth (Krause et al., 1978; Pralomkarn et al., 1990) as the epidermis increases in thickness (Buaboocha and Gemmell, 1997; Lyne et al., 1970). As mentioned previously, non-sterile cuts made into the periderm of Virginia opossums less than 6 days old resulted in the death of the animal (Block, 1960), showing that the periderm functions as an initial physical barrier of protection. Langerhans cells and sebaceous glands have been found to develop at different times in different marsupial species. They were evident at 23 and 59 days postpartum, respectively, in the quokka (Pralomkarn et al., 1990) and at 47 and 28 days, respectively, in the brushtail possum (Buaboocha and Gemmell, 1997). In contrast, Langerhans cells were detected in the newborn white-eared opossum (*Didelphis albiventris*) (Coutinho et al., 1995). While not traditionally thought to be a part of the immune system, Burkhart and Burkhart (2005) have proposed that melanocytes present in the epidermis produced substances with a range of biological activity including antimicrobial defence. In the brushtail possum, melanocytes are found in the epidermis from 2 to 100 days postpartum (Lyne, 1970), but not until 23 days postpartum in the quokka (Pralomkarn et al., 1990). The function of melanocytes, in the young marsupial, has not been examined.

A series of experiments investigating the development of the Virginia opossum GI and respiratory tract provides clues as to what cellular structures might be involved in protecting the developing young. The marsupial respiratory system is extremely undeveloped at birth and the conducting portion is lined by columnar epithelium which lack cilia and goblet cells (Krause and Leeson, 1973). At 9 days the trachea contains very few cilia and it is not until 60 days and the juvenile stage that the submucosal glands and goblet cells respectively form. Instead, vacuolated cells were found in earlier stages throughout the postnatal period of the Virginia opossum (Krause and Leeson, 1973) and at 10 days postpartum in brushtail possums (Buaboocha and Gemmell, 1997). In the rat colon, vacuolated cells released mucin (the main component of mucous) (Sakata and Engelhardt, 1981) and they could play a similar role in the respiratory tract of the developing marsupial. Recent research has identified mucin genes in the tammar wallaby; however their role in protecting marsupial pouch young has not been determined (Edwards et al., 2011).

An interesting and recurring observation in the stomach and small and large intestines is the occurrence of lipid droplets; furthermore, the lipid droplets tend to reduce or disappear a few weeks after birth (Krause et al., 1976, 1977; Waite et al., 2005). Lipids can also act as an antimicrobial agent (Thormar and Hilmarsson, 2007) and thus, may play a role in protection of the newborn marsupial GIT.

5. Conclusion and future directions

Developmental immunologists have been intrigued by the marsupial immune system for over 30 years, and have focused their attention on the immune properties of the mother's milk and the innate immune mechanisms, yet large gaps in our understanding still exist. Over the last 10 years research has focused on identifying microbial exposure and complementary protective mechanisms to adaptive immunity. Research examining the components of colostrum and milk has identified many cells and chemical compounds with potential protective roles, but studies examining their protective role are largely absent. Additionally, research examining the innate immune system, such as those that show that leukocytes are present at birth and that physical barriers can provide protection, suggest that the innate immune system is also playing a large role in protection; however, there is still much of the marsupial innate immune system that remains unexplored.

Research concerning the role of antimicrobial compounds has only just begun, with initial studies examining the two main AMPs in humans—cathelicidins and defensins. However, many antimicrobial compounds exist and given their large diversity, it is likely that all of the groups that occur in marsupials have not yet been identified. There are no studies which have examined antimicrobial lipids and it is likely that this area of research will remain uncharted while AMPs continue to be explored. Similarly, few researchers have examined the role of immune enzymes—most likely because there are so many enzymes and different environments in which they can act.

In their review of the developing marsupial immune system, Old and Deane (2000) concluded that the time was right for the metatherian immune system to be as well documented as that of eutherians. Since their review, the sequencing of marsupial genomes (Mikkelsen et al., 2007; Renfree et al., 2011) and the construction of an immunome database for marsupials (Wong et al., 2011) have facilitated a greater understanding of the metatherian immune system. However, our understanding of protection of developing pouch young is still far from complete. It is now time to exploit the immense amount of genetic data provided by the marsupial genomes, as well as the increasing number of transcriptomes, to direct future research. Currently, functional tests, like those conducted by Wang et al. (2011), are few and far between but are essential to provide stronger support for how marsupial pouch young are protected while their adaptive immune system matures during their development in a non-sterile pouch.

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Chapter 3. The marsupial pouch: implications for reproductive success and mammalian evolution

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The manuscript highlights the important role that the pouch has in the reproductive success of marsupials and suggests that the pouch may have also played an important role in the diversification of mammals by supporting the movement of marsupials into niches that are not supported by free hanging highly altricial young. The manuscript is to be published as part of a special issue dedicated to marsupial biologist Des Cooper.

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Review

The marsupial pouch: implications for reproductive success and mammalian evolution

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Abstract. Extant mammals are divided into sub- and infraclasses that are distinguished by their mode of reproduction. The monotremes lay eggs, the marsupials give birth to altricial young that typically develop in a pouch, and the eutherians have prolonged *in utero* development, resulting in well developed young at birth. The three groups exhibit what appears to be a nice progression of evolution towards the well developed newborn young of eutherian mammals. However, marsupials do not represent a step in the progression of producing well developed young, but maintain a reproductive strategy that has evolved to prosper in their specific niche. The production of undeveloped young with increased development in the pouch (or counterpart) provides specific advantages to those species living in diverse environments. The evolution of this reproductive strategy provides a clever solution to the uncertain and often adverse conditions encountered by many species, and the survival of the developing young in a pouch containing potentially harmful microorganisms is truly remarkable. In this review, we explore the unique features of the pouch, highlight the research questions that remain unanswered regarding this unique marsupial attribute and discuss the advantages of the marsupial reproductive strategy and the potential role of the pouch in mammalian diversification.

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Introduction

Many characteristics of reproduction are shared amongst the vertebrates. However, there are also a countless number of differences, demonstrating that different solutions have evolved independently to solve the various problems of reproduction (Lombardi 1998). The vertebrates not only display oviparous, viviparous and ovoviviparous reproductive strategies, but also exhibit extremely diverse developmental stages at birth, including the production of precocial, altricial and highly altricial offspring. The former two terms describe 'well developed', or sighted, covered young and 'undeveloped', or blind, naked young, respectively, while the latter term describes the most extreme form of altriciality. Furthermore, each strategy is not necessarily limited to a lineage, but is established in many lineages throughout the subphylum. For example, the snakes (suborder: Serpentes) include lineages of species that reproduce by the various 'viparous' or embryonic developmental strategies (Pope 1956) and the birds (class: Aves), although all oviparous, produce offspring that display a spectrum of altricial and precocial developmental stages (Starck and Ricklefs 1998).

Not surprisingly, there are similar variances in the reproductive strategies of mammals. All animals that produce milk to meet the nutritional requirements of their young are grouped into the class Mammalia and it is thought that lactation provides a specific advantage to mammals, enabling them to support the nutritional requirements of the young in any environment where adults are able to survive (Pond 1984). The extant mammals are divided into three major lineages that are distinguished by their modes of reproduction. The monotremes (or Prototheria – meaning 'first beasts') display a mixture of reptilian and mammalian features, as they lay eggs yet feed their young milk produced by mammary glands. Monotreme hatchlings are highly altricial. In contrast, 'placental' mammals (or Eutheria – meaning 'true beasts') produce developed young after a relatively long gestation time in which the developing foetus relies on a placenta for the exchange of factors between mother and foetus that are critical for survival, such as nutrients, gases and immune compounds. The eutherian mammals produce offspring varying from altricial to precocial; for example, even within the Leporidae, there are altricial rabbits and precocial hares (Trevathan 1987). The marsupials (or Metatheria – meaning 'behind true beasts') do not lay eggs but, like the monotremes, they produce highly altricial young that complete most of their development during a complex lactation phase rather than throughout gestation. Marsupials are born at a stage of development comparable to an 8–10-week-old human embryo or an 11–12-day-old mouse embryo (Block 1960; Smith 2001). Hence, much of the development that occurs in a mostly

pathogen-free environment in eutherian mammals takes place *ex utero* in marsupials and typically while the young are permanently attached to a teat within a pouch.

The unfortunate naming of these three groups of mammals – Prototheria, Metatheria and Eutheria – may suggest that monotremes and marsupials are evolutionary steps in the progression to eutherians, but a closer examination of the unique reproductive strategies adopted by each lineage shows that their modes of reproduction simply provide alternative solutions that have adapted under different conditions. The survival of the highly altricial young of marsupials is truly remarkable. Des Cooper demonstrated an interest in the uniqueness of the marsupial reproductive strategy, and was particularly interested in the survival of the altricial young in the pouch and even in the development of the pouch itself. Without Des Cooper's contribution, we would know little about the marsupial pouch, as the pouch has been largely overshadowed by another marsupial reproductive trait – the production of highly altricial pouch young. In this review we link highly altricial young to the pouch and make a case for the pouch in the role of marsupial reproductive success and mammalian evolution.

Highly altricial young

In comparison to the broad altricial to precocial spectrum observed amongst different eutherian mammals at birth, marsupials are born at a similar developmental stage, which marks the minimum onset of functionality for specific tissues (Hughes and Hall 1988). This stage of development can be visually compared with the developmental stage of a bird embryo, well before hatching, or to a eutherian mammal embryo *in utero*. Particularly, the most visually noticeable characteristics are the forelimbs, which the young uses to take hold of the mother's fur to propel itself from the mother's urogenital opening to a teat. The mouth parts are also well developed and have a very important role in fastening onto the mother's teat so the young can remain attached to the mother and attain colostrum and milk. Remarkably, the undeveloped characteristics of marsupials outweigh the comparatively developed characteristics several-fold at birth, with the undeveloped characteristics considered unnecessary for survival at this time of development.

Not only is the marsupial young visually undeveloped, but many general physiological processes essential for adult survival also remain immature until sometime after parturition. For example, the lungs are partly developed with partial gas exchange occurring through the skin. The degree of development differs between species; Julia Creek dunnarts (*Sminthopsis douglasi*), under approximately seven days, exhibit gas exchange through the skin which exceeds that through the lungs (Mortola *et al.* 1999), while the integument of the tammar wallaby (*Macropus eugenii*) is responsible for ~33% of gas exchange at birth, decreasing to 14% at six days post partum (MacFarlane *et al.* 2002).

Marsupial neonates are also ectothermic and exhibit a large surface area to weight ratio, which can cause rapid heat loss. However, the rate of heat loss reduces as the young develops. When the thermogenic response is initiated, the response is small and it is not until they are covered in body hair that the development of thermogenesis is complete (Hulbert 1988). Additionally, young are born with undeveloped immune tissue (Deane and Cooper 1988). For example, tammar wallaby young are not able to mount an adaptive immune response until 35 days post partum (Old and Deane 2003) and the maturation of their lymphoid tissue is determined to occur at ~90 days post partum (Basden *et al.* 1997).

The survival of highly altricial young appears to be a phenomenon when compared with the reduced survival rates of human neonates that are born prematurely. However, marsupials have evolved to produce young at a highly altricial developmental stage and exhibit specific traits that undoubtedly aid in the rearing of such undeveloped young. In particular, the pouch resolves many of the problems encountered by the production of highly altricial newborn young.

The pouch

Pouches are located ventrally but vary markedly between marsupial species; they can be shallow or deep and contain varying numbers of teats between species (Tyndale-Biscoe 2005). Russell (1982*b*) described six different pouch types. Figure 1 shows a phylogeny of the pouch and the pouch counterpart types for the major orders and suborders of marsupials. In Type 1 the mammary area is not covered, but folds of skin can develop in the breeding season; in Type 2 the mammary area is partially covered; in Type 3 the mammary area is covered and the teats are displayed in a circular arrangement with the pouch opening in the centre; in Type 4 the mammary area is covered and the teats are located in two pockets; in Type 5 the mammary area is covered and the pouch opens to the anterior; and in Type 6 the mammary area is covered and the pouch opens

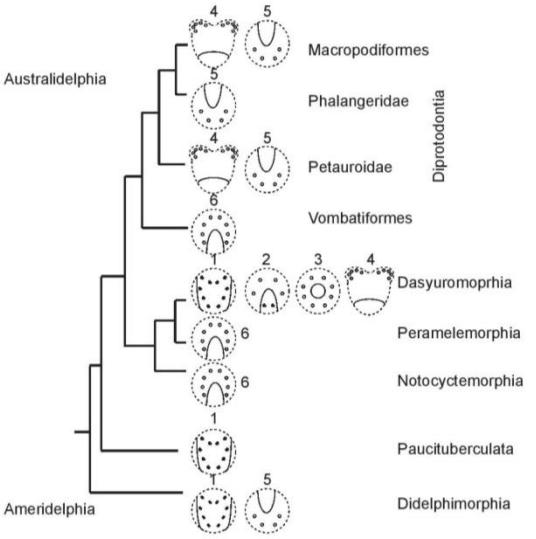

Fig. 1. A phylogeny showing different pouch types and pouch counterparts. Dark and light circles represent teats that are located exteriorly and interiorly, respectively. Solid and dotted lines represent the pouch opening and covered areas, respectively. Pouch types and diagrams are reported from Russell (1982*b*) and Tyndale-Biscoe and Renfree (1987).

to the posterior. Figure 2 shows external and internal images of a tammar wallaby pouch (Type 5), with and without a pouch young and with different degrees of pouch cleanliness (also known as 'pouch grot'), as described by Sharman and Calaby (1964) as brown to black scale for a 'dirty' pouch or clean and pinkish for a 'clean' pouch.

Russell (1982*b*) also identified three different patterns of parental care that are associated with different pouch types. Pattern A describes species with small pouches and large litters, whereby the mother leaves the young at an early developmental stage (with little fur, their eyes closed and no thermoregulation) in a nest after a period of teat attachment. Pattern B describes species with well developed pouches and fewer young, whereby the young remain in the pouch and are then left in a nest at a later developmental stage (when they are well furred, their eyes are open and they can thermoregulate). Pattern C describes species with large pouches and typically only one young, whereby the young remain in the pouch, as in Pattern B, and then leave the pouch but continue to follow the mother at foot. Gemmell *et al*. (2002) describes different methods, correlating to different pouch types, for newborn young to travel from the urogenital opening to the pouch; young may either climb upward to the pouch (e.g. the forward-facing pouch (Type 5) of the brushtail possum (*Trichosurus vulpecula*)) or the mother may place her urogenital opening above the pouch so the newborn can move down to the pouch (e.g. the backward-facing pouch (Type 6) of the bandicoot (*Isoodon macrourus*)).

Remarkably, there are still so many questions about the pouch that remain unanswered. Foremost of these is the trigger for pouch development. The pouch is not under hormonal control, but is thought to be under the control of a locus on the X chromosome, along with the scrotum and mammary glands (Shaw *et al*. 1989; Watson and Cooper 1995; Watson *et al*. 1997). The role of chemical protection in the pouch is also relatively unknown, although four genes for the antimicrobial peptide cathelicidin are expressed in tammar wallaby pouch skin (Wang *et al*. 2011). A haematoxylin and eosin stain of the pouch skin taken from within the tammar wallaby pouch also shows a very large active apocrine gland (Fig. 3); these are usually found in the axillary and genital areas (Morimoto and Saga 1995). Large sweat glands have also been identified in the red kangaroo (*Macropus rufus*) and brushtail possum pouches, suggesting that there may be active secretions into the pouch (Kubota *et al*. 1989; Old *et al*. 2005). Further work, possibly linking the glands to antimicrobial or mucosal activity needs to be completed (Edwards *et al*. 2011).

Although the pouch or marsupium gives the marsupials their name, not all marsupials have pouches. Instead, the position of their reproductive and excretory organs distinguishes them from the monotremes and the eutherians (Cooper and Hope 1989; Tyndale-Biscoe 2005). Species without a pouch instead form a tissue between the teat and the young so the young stay attached and do not become separated from the mother. To support the young the *ilio marsupialis* muscle passes through the mammary gland and up each teat (Griffiths and Slater 1988). It is surprising that a pouch is not associated with all marsupials as many challenges that are presented by producing highly altricial young appear to be overcome by the pouch. As a pouch is not necessary for the production of highly altricial young, below we examine the role of the pouch in reproduction and ultimately in mammalian evolution.

Implications of the pouch for reproductive success and mammalian evolution

The production of highly altricial young, and not the pouch, is usually the focus of discussion for marsupial reproduction. In the

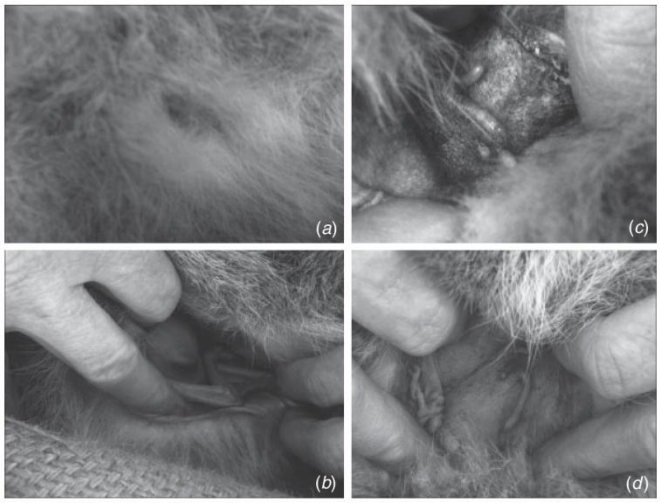

Fig. 2. An external view of a closed tammar wallaby (*Macropus eugenii*) pouch (*a*), a pouch young inside the pouch (*b*), and two images of an internal view of a tammar wallaby pouch without a pouch young (*c* and *d*). An elongated teat is evident in (*c*) and (*d*) and one pouch displays a large amount of 'pouch grot' (*c*).

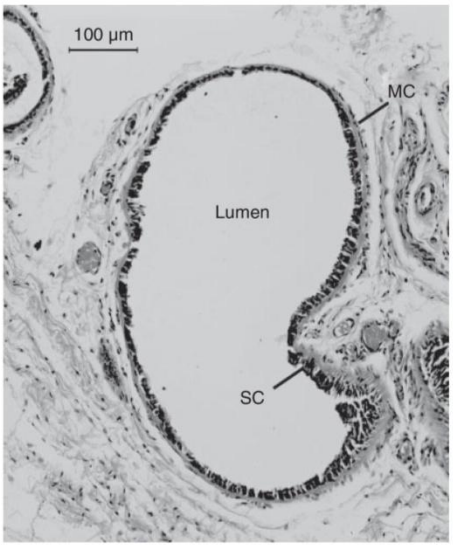

Fig. 3. An 8-µm section of a pouch skin biopsy stained with haematoxylin and eosin, taken from within the tammar wallaby (*Macropus eugenii*) pouch with a 2–3-day-old pouch young. An apocrine gland is evident with an extremely large lumen surrounded by secretory cells (SC) and myoepithelial cells (MC).

past, the notion that eutherian reproduction has a fundamental evolutionary advantage over marsupial reproduction was explored (Müller from Lillegraven 1975); however, Kirsch (1977*a*) highlighted that we should not be asking why marsupials lack a complex gestation, but asking whether a long gestation is necessary? Kirsch (1977*a*, 1977*b*), Parker (1977) and Low (1978) discussed that the difference between the reproductive strategies of marsupials and eutherians amounted from different selective environments. The support for the production of highly altricial young included that marsupials could terminate their investment in reproduction in response to unfavourable conditions (such as irregular drought episodes: Kirsch 1977*a*, 1977*b*; Parker 1977; Low 1978). Specifically, advantages were identified in terms of reproductive value and effort, and for marsupials these included: rapid birth (which reduced vigilance time), low cost associated with the foetus in terms of risk and energy (such as reduced vulnerability to predation and increased ability to forage), ability to resorb reproductive material, and low cost of the uterus when compared with finding or building a nest (Kirsch 1977*a*, 1977*b*; Parker 1977; Low 1978). However, Russell (1982*b*, 1982*a*), Lee and Cockburn (1985) and Cockburn (1989) examined the marsupial strategy further using additional examples of marsupial species and provided a comprehensive discussion that pointed to discrepancies in the 'unfavourable conditions' hypothesis.

Hopson (1973) proposed that the fundamental components of mammalian reproduction are selection for endothermy and small body size. As metabolic rate increases as body size decreases (creating an energetic dilemma), Hopson (1973) suggested that there is selection for the production of ectothermic young with low metabolic rate and increased parental care, including adaptations for creating warmth. Case (1978) presented a slightly different hypothesis to Hopson, and proposed that smaller young have a lower energetic requirement than larger young. Thus, parental costs are greater after birth and can be shared between parents, particularly when brooding and foraging cannot co-occur. Furthermore, the mammary gland and attachment to the teat increased maternal care and reduced the need for paternal care. Case (1978) also suggested that reproductive characteristics are shaped by aspects of a species' niche and habitat. For example, it may be beneficial for those species that spend a large amount of time and energy searching for food to produce small embryos as larger embryos may hinder their ability to forage.

Mostly, the pouch is disregarded in discussions on the marsupial reproduction strategy, although Hopson (1973) stated that pouches helped with trends to produce altricial young. It is likely that researchers question the role that the pouch has in the discussion of marsupial reproduction, as some marsupial species lack a pouch. However, when present, the pouch plays a major role in reproduction, thus it is necessary to examine the pouch explicitly.

The pouch has more than one role in the reproduction of many marsupial species and the large variation of pouches seen within the marsupials is likely to correspond to the varying levels of importance of the role of the pouch. First, direct physical protection from the external environment is provided by the pouch (Russell 1982*b*). In birds and monotremes, physical protection comes from the egg, while protection for eutherian mammals, at a comparable developmental stage, comes from the uterus and integument of the mother. The pouch may also provide protection on a chemical level, as pouch washes (obtained by flushing pouches with sterile water) contain proteins with antimicrobial activity and display antimicrobial activity when they are subjected to bacteria (Bobek and Deane 2001; Ambatipudi *et al*. 2007, 2008; Edwards *et al*. 2012). Second, the pouch may influence the humidity of the direct environment of the young (Kubota *et al*. 1989), which may aid in integumental gas exchange. Chemical protection and humidity control could come from secretions within the pouch (Kubota *et al*. 1989), or from the mother licking the pouch and depositing saliva (Charlick *et al*. 1981). Third, as the pouch is associated with the mother, direct contact between the mother and young allows the young to develop at a constant temperature that is comparable to that of the adult, thus resolving the issues of ectothermy in the developing young (Hulbert 1988). The ability to open and close the pouch may also provide an air-conditioning effect (Kubota *et al*. 1989). If a pouch provides a constant environment specific to the requirements of the young for protection, humidity, and warmth, it may have an increased chance of surviving and reproducing. For example, if the mother moves into or even through an environment in which conditions are unfavourable to the young, the pouch will keep the direct environment of the young constant.

Fourth, the pouch may make young inconspicuous to predators so that mothers may not be targeted while foraging, and, finally, the pouch evolved in unison with mammary glands. Although the pouch is always associated with mammary glands, the mammary glands are not always associated with a pouch (Shaw *et al*. 1989). Mammary glands provide the young with

essential nutrients and also pass on immune compounds to the developing animal (Deane *et al*. 1990; Young *et al*. 1997). In contrast to eutherian mammals, the supply of marsupial milk is multifaceted, as milk production occurs over three or four phases, which are associated with major periods of growth (Nicholas 1988; Joss *et al*. 2009).

It is clear that the pouch supports the undeveloped marsupial; however, the pouch also presents potential challenges. For example, the young must travel from the urogenital opening to the pouch before latching onto a teat. Additionally, the pouch is a non-sterile environment containing a range of bacteria that have the potential to harm the young (Yadav *et al*. 1972; Charlick *et al*. 1981; Old and Deane 1998; Deakin and Cooper 2004; Chhour *et al*. 2010).

The pouch not only provides insight into marsupial reproduction but may also shed light on mammalian diversification and evolution. Currently, the mammalian lineages are dominated by eutherian mammals, even though marsupials have been evolving for the same period (Bininda-Emonds *et al*. 2007). Müller (from Lillegraven 1975), Cooper and Steppan (2010), and Kelly and Sears (2011) suggested that the reproductive strategy of marsupials has contributed to limiting the marsupial's evolutionary potential when compared with eutherian mammals. The limitation comes from the need for developed forelimbs to travel from the urogenital opening to the teat, which may pose a functional constraint on the forelimb, limiting its morphological evolution. In other words, the marsupials must retain their forelimbs and cannot evolve wings, flippers or hooves, which have evolved in the eutherian clade. Kirsch (1977*b*) found the hypothesis unconvincing as claws in some marsupial species are deciduous.

Whether or not the forelimb of the marsupial has limited the evolutionary potential of the marsupials, we suggest that the pouch has positively influenced the range of extant marsupial species. The pouch provides a practical explanation that supports the movement of marsupials into niches that are not supported by free-hanging highly altricial young. For example, the water opossum (*Chironectes minimus*) has a strongly developed pouch muscle, the *pars pudenda*, which allows the pouch to close effectively enough to help protect the young from water (Enders 1937). The marsupial mole (*Notoryctes typhlops*) and other burrowing species have posterior-opening pouches that protect the young from sand and soil entering the pouch while they are burrowing (Johnson 1995). It is also likely that the pouch provides increased protection in jumping and gliding species (genus: *Macropus* and *Petaurus*) when the mother lands on successive trees or hops though scrubland, as it is difficult to see how free-hanging altricial young would survive in these types of situations.

The pouch, as a reproductive feature, fits Case's (1978) hypothesis, as it may be shaped by attributes of niche and habitat. Although we only provide examples of how a pouch might promote reproductive success in clearly different environments, it is likely that the presence or type of pouch is also going to be influenced by specific ecological traits such as mode of foraging or predator avoidance. Researchers are frequently fixed on explaining why there are not as many marsupials as there are eutherian mammals; perhaps turning the question around to ask why are there as many marsupials as there are, is just as interesting in evolutionary terms.

Conclusion

The marsupials, often referred to as Metatheria (or halfway mammals), have sometimes been thought of as being an intermediate group between the egg-laying monotremes, often referred to as Prototheria (or first mammals), and the comparatively more highly developed eutherians (or true mammals). However, it is important to remember that marsupials do not represent a step in the evolution of producing well developed young, but maintain an alternative mode of reproduction compared with the eutherian strategy that has evolved to prosper in their specific niche (Graves *et al*. 1989).

Much emphasis has been placed on the production of highly altricial young in the marsupial reproduction strategy, while the pouch is often disregarded. However, the pouch plays an extremely important role in the reproductive success of those species that have them. Additionally, the pouch may have played an important role in the diversification and evolution of mammals by supporting the movement of marsupials into niches that are not supported by free-hanging highly altricial young.

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Chapter 4. Physical mapping of innate immune genes, mucins and lysozymes, and other non-mucin proteins in the tammar wallaby (*Macropus eugenii*)

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Cytogenetic and Genome Research

Extent to which research is your own:

The research from this manuscript is my own, including sequence analysis, overgo design, library screening, FISH mapping and comparative analyses.

Your contribution to writing the paper:

I wrote the paper and received content and editorial comments from the other authors.

If paper not accepted, has the paper been rejected by any journals:

Not applicable.

Comments:

This manuscript identifies mucin and lysozyme genes in the tammar wallaby, maps these genes to tammar wallaby chromosomes and compares the gene locations with other species. It lays the foundation for further research conducted in Chapters 5 and 6.

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Physical Mapping of Innate Immune Genes, Mucins and Lysozymes, and Other Non-Mucin Proteins in the Tammar Wallaby *(Macropus eugenii)*

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Comparative mapping · Mucin · Lysozyme · Tammar wallaby

Abstract
Sequencing of the tammar wallaby *(Macropus eugenii)* genome has the potential to be an extremely valuable resource for investigating evolutionary and developmental aspects of the mammalian immune system. However, the tammar wallaby genome has only been sequenced to a 2-fold depth and consists of small contigs, leaving many sequence gaps, many putative orthologs unpredicted and the location of genes within the genome unknown. In the case of low sequenced genomes, physical maps of genes on chromosomes can help identify specific genes if they map to conserved regions. Genes corresponding to adaptive immunity have been mapped in the tammar wallaby; however, genes corresponding to the innate immune system have not been investigated. We predict 2 types of genes important to the innate immune system, mucins and lysozymes, in the tammar wallaby and compare the predicted peptide sequences and locations of the genes with the South American opossum *(Monodelphis domestica)* and human. We use fluorescence in situ hybridization to physically map the genes to tammar wallaby chromosomes, demonstrating the importance of identifying and mapping genes when genomes have low sequence coverage. As mucins and lysozymes play protective roles in young animals, we also propose that their immunological role in developing marsupials warrants further investigation.

The marsupial (or metatherian) immune system has been the focus of much research, from both an evolutionary and developmental perspective. Evolutionary interest stems from the separation of marsupials and eutherians, around 147 million years ago [Bininda-Emonds et al., 2007], which placed the marsupials in a prime location on the evolutionary tree for comparative studies with key eutherian species, such as mouse and human. Comparative studies have been invaluable in showing similarities in the genetic architecture of the innate and adaptive immune systems of marsupial and eutherian mammals [Siddle et al., 2010] and demonstrating that the evolution of complexity in the mammalian immune system occurred before the divergence of the marsupial and eutherian lineages [Belov et al., 2007].

Although the marsupial and eutherian immune systems share similarities in structure, function and genetic architecture, differences in the timing of their develop-

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ment exist, making the marsupial immune system also interesting from a developmental perspective. Marsupials are unable to mount a functional adaptive immune response until several weeks into their pouch life, as they are born with undeveloped immune tissue [Yadav et al., 1972; Cutts and Krause, 1982; Basden et al., 1997] and have insufficient numbers of mature lymphocytes [Coutinho et al., 1995; Old and Deane, 2003; Old et al., 2004]. The delayed development of their adaptive immune response is intriguing, as marsupial young are born into a nonsterile environment containing a range of potentially pathogenic bacteria [Osawa et al., 1992; Old and Deane, 1998; Deakin and Cooper, 2004; Chhour et al., 2010], yet they manage to survive while their immune tissues continue to mature. Determining how these altricial young are immunologically protected is of great interest, which has led to studies of the maternal contribution to both pre- and postnatal protection through such mechanisms as maternal milk [Adamski and Demmer, 1999, 2000; Joss et al., 2007, 2009] and pouch secretions [Ambatipudi et al., 2007, 2008]. Most recently there has been an especial focus on the innate immune system, including a number of antimicrobial peptides – primarily cathelicidins [Carman et al., 2008, 2009; Daly et al., 2008].

Two other important categories of compounds, mucins and lysozymes, are known to play an important role in innate immunity in eutherian mammals and form a primary defense against pathogenic microorganisms. Mucins are encoded by 17 different types of mucin *(MUC)* genes in human and their protein products are also known to play a role in hydration and lubrication [Hattrup and Gendler, 2008; Thornton et al., 2008; Dharmani et al., 2009; Voynow and Rubin, 2009]. Few studies have examined mucins in marsupials, with the exceptions exploring their association with Brunner's glands of the duodenum [Schumacher and Krause, 1995], fore-stomach maturation during cross-fostering [Kwek et al., 2009] and the coating of oocytes [e.g. Renfree and Lewis, 1996; Casey et al., 2002; Paris et al., 2005].

Lysozymes *(LYZ)* are encoded and categorized as distinct types [Jollés and Jollés, 1984]. Several studies have examined lysozymes in marsupials with particular attention paid to their occurrence in milk. Lysozymes have been detected in common ringtail possum *(Pseudocheirus peregrinus)*, brushtail possum *(Trichosurus vulpecula)* and tammar wallaby *(Macropus eugenii)* milk [Nicholas et al., 1989; Joss et al., 2007, 2009; Kuy et al., 2007], with research from the common ringtail possum demonstrating that it has similar enzyme activity to the c type (hen egg-white) lysozyme [Nicholas et al., 1989].

The sequencing of the tammar wallaby (2n = 16) genome has potential as an extremely valuable resource for investigations into evolutionary and developmental aspects of the mammalian immune system. However, the tammar wallaby genome, with a predicted size of 2.78 Gb, has only been sequenced to a 2-fold depth and consists of small contigs (http://www.ensembl.org/Macropus_eugenii/Info/Index), leaving many sequence gaps, many unpredicted putative orthologs and the location of genes within the genome unknown. Locating and physically mapping genes has demonstrated the amount of chromosomal rearrangement that has taken place between species and provided insights into their evolutionary history [Deakin et al., 2008]. Physical mapping of immunoglobulin and T cell receptor genes in the tammar wallaby (genes important to the adaptive immune response), demonstrated that there is consistent arrangement of these genes between marsupial species [Sanderson et al., 2009]. In stark contrast, MHC Class I genes in the tammar wallaby were found to be dispersed across the genome and not clustered like they are in most mammals [Deakin et al., 2007].

In this study, we predict lysozyme and mucin genes (as well as 2 other non-mucin protein genes, *OVGP1* and *MCAM*, previously known as *MUC9* and *MUC18*) in the tammar wallaby genome and determine the sequence similarities of the predicted genes, or specific domains within the genes, between tammar wallaby, South American opossum *(Monodelphis domestica)* and human. We map the predicted genes to tammar wallaby chromosomes to determine if they are found in similar genomic context in relation to the other mammalian species. The genomic information presented in this study demonstrates the importance of mapping genes when genomes have low sequence coverage and will contribute to the assembly of the tammar wallaby genome. The identification of these genes in tammar wallaby will also provide the foundation for future research into the development of the marsupial immune system.

Materials and Methods

In silico Gene Search and Overgo Design

Mucin and lysozyme gene sequences (and gene sequences for *OVGP1* and *MCAM*) were identified as predicted genes in the tammar wallaby genebuild (Meug_1.0) on Ensembl (*LYZ, MCAM, MUC2* and *MUC6*; v.57–58) [Flicek et al., 2010], or they were predicted by using cDNA sequences from the opossum *(MUC1, MUC3A, MUC4, MUC5B, MUC13* and *OVGP1)* or human *(MUC7, MUC12, MUC15, MUC16, MUC17, MUC19,MUC 20, MUC21* and *EMCN)* to BLASTN search the tammar wallaby genome assembly (Meug_1.0).

Table 1. Lysozyme, mucin and non-mucin genes with sequences that show high percent identity in tammar wallaby scaffolds, the overgo sequences (A and B) used to screen the tammar wallaby BAC library and the BACs identified after library screening

Gene	Wallaby scaffold	OvergoA	OvergoB	Isolated BACs
LYZ	23673	GAATGTAGTGTGTTCAGTGTGTGT	CACACCTGAACATTCTACACACAC	144I19, 134M3
LYZ	31416	AGCTCCACTTAAACATAGATTGGG	TGGAGGCCAACTCATTCCCAATCT	144I19, 108M7, 57C4, 61F19, 112G24
MUC1	416209	CTTGGTGTTGCTTGGGGGCACATA	TCATACTCATGGACGATATGTGCC	484D13, 68O10, 369K4
MUC2	9723	AGACCTCCAAAGCTTCTGGATGGT	CTTCCCTAACCATGGGACCATCCA	6H10, 107K14
MUC3A	18987	TATTCTGGAAGGTCCCGGGGCTTG	GAAGAGGCTGGAGGACCAAGCCCC	330N14
MUC4	78550	TTTCCAGGCCATCCTCACGACTGA	AAAAGAGCGGCTCCCATCAGTCGT	320O6
MUC5B	10142	CTTCACCATCCTGGGGGAGCTGCG	TGTCATGCCACACCTACGCAGCTC	350N20, 382N17, 291N13
MUC5B	20178	AGTGCCAAGACCCCCTGCCAACAG	GTGCAGTTGTGCAGGGCTGTTGGC	350N20, 334F2, 351C14, 327C17, 291N13
MUC6	8961	TAGGTAGACAGAGGTAAGTTCCTG	GCCTTGAACTCACATGCAGGAACT	6H10
MUC13	63531	TACCCTTCTGGACACAGGCAAAAG	CTCTGAACAATGAGTCCTTTTGCC	354D7, 301E20
MUC13	78826	CCAGTGCCTACCTGGCCACATCAG	ACAGGTTCCTTCCTTTCTGATGTG	354D7, 301E20, 317G15, 329D10
MCAM	2500	TACTGCGGAGTTGTTGGGGGAAAT	CTGGATTCCTTAGGTAATTTCCCC	35C13, 18D14, 65H21
OVGP1	127087	ATACAGTGGGCTGTTGTGCCCAGT	AAGTTGGGAAGTGGTCACTGGGCA	303O1, 492G15, 335M1, 305L3, 363J13

Overgos were designed for tammar wallaby sequence scaffolds, identified to contain the genes of interest (listed in table 1), using the Overgo Maker program downloaded from the Washington University Genome Sequencing Center (http://genome.wustl.edu/tools/software/overgo.cgi). When sequences from the opossum showed high identity to several different tammar wallaby scaffolds, overgos were designed for all of these scaffolds. The overgos were used to screen the tammar wallaby bacterial artificial chromosome (BAC) library for the predicted genes.

Library Screening

Overgos were labeled with ^{32}P-dATP and ^{32}P-dCTP (GE Healthcare UK Ltd., Little Chalfont, Bucks., UK) using the BACPAC hybridization protocol (http://bacpac.chori.org/overgohyb.htm). Unincorporated nucleotides were removed from the labeled probes using ProbeQuant™ G50 microcolumns (GE Healthcare) according to the manufacturer's instructions. Labeled overgo probes were denatured at 95°C, quenched on ice and pooled to screen 3 filters from the Me_KBa 11× coverage BAC library (Arizona Genomics Institute, Tucson, AZ, USA). The filters were hybridized overnight at 60°C, then subjected to a series of washes (1 mM EDTA, 1% SDS, 40 mM Na_2HPO_4 for 30 min; 1.5× SSC, 0.1% SDS for 20 min; 0.5× SSC, 0.1% SDS for 20 min) at 60°C and exposed to X-ray film (Amersham Hyperfilm™; GE Healthcare) at –80°C with intensifying screens from 1–8 days. The resulting positive BACs (listed in table 1) were further screened by dot blots using individual overgo pairs as described by Deakin et al. [2008].

Fluorescence in situ Hybridization

BAC DNA was extracted using the Promega WIZARD plus SV miniprep DNA purification system (Promega, Alexandria, N.S.W., Australia). Approximately 1 μg of DNA from each BAC was labeled with SpectrumOrange or SpectrumGreen dUTP (Abbott Molecular Inc., Des Plaines, Ill., USA) by nick translation. Labeled probes were precipitated overnight at –20°C with either sheared wallaby genomic DNA or wallaby C_0t-1 (1 μg) and glycogen (1 μl). The probes were hybridized to male tammar wallaby metaphase chromosome spreads overnight at 37°C as described by Alsop et al. [2005]. Slides were washed in 0.4× SSC, 0.3% (v/v) Tween® 20 (Sigma-Aldrich, Castle Hill, N.S.W., Australia) for 2 min at 60°C followed by 2× SSC, 0.1% (v/v) Tween 20 for 1 min at room temperature and mounted with DAPI in vectashield (1:3; Vectalab Inc., Burlingame, Calif., USA).

Slides were examined using a Zeiss Axioplan2 epifluorescence microscope. Images were captured using a SPOTRT monochrome charge couple device camera (Diagnostic Instruments Inc., Sterling Heights, Mich., USA) and merged using IPLab imaging software (Scanalytics Inc, Fairfax, Va., USA). At least 10 metaphase spreads were observed for each gene to determine their cytogenetic map location and FLpter value.

Comparison of Conserved Domains and Peptide Sequences

The coding sequences for each gene were obtained from Ensembl (v.57–59) or predicted using the cDNA sequences from opossum or human to BLASTN or TBLASTX search the tammar wallaby scaffolds. The sequences were confirmed to translate into a protein using MacVector (v.9.5.2). As mucins contain regions rich in proline, threonine and serine (PTS domains) that are poorly conserved in sequence, other specific mucin domains such as SEA (a domain found in sea urchin sperm protein, enterokinase and agrin), VWD (von Willebrand D), NIDO (nidogen-like), AMOP (adhesion-associated domain in *MUC4* and other proteins) and CK (cysteine knot) domains were used to compare sequence similarities with human and opossum. *MUC2, MUC5AC, MUC5B* and *MUC6* contain 3 VWD domains, *MUC1, MUC3A, MUC12, MUC13, MUC16* and *MUC17* contain SEA domains and *MUC4* contains NIDO, AMOP and VWD domains. The peptide sequence for the human mucin domains were obtained from The Mucin Biology Group Mucin Database (http://medkem.gu.se/mucinbiology/databases/) and were used to TBLASTN search the predicted coding sequences for tammar wallaby using NCBI/BLAST (v.2.2.24; http://blast.ncbi.nlm.nih.gov/Blast.cgi). The alignments were analyzed in PFAM (v.24.0; http://pfam.sanger.ac.uk/) [Finn et al., 2010] to confirm that the tammar wallaby alignments comprised the mucin domains. The peptide sequenc-

Table 2. Chromosome location of mucin and lysozyme genes in the human, South American opossum and tammar wallaby genomes. The tammar wallaby FLpter value, standard deviation of the mean and the number of metaphase chromosomes measured to determine these values

Gene	Human location	Opossum location	Wallaby expected location [Rens et al., 2003]	Wallaby location (this study)	Wallaby FLpter value	
					mean ± SD	n
MUC1	1q22	2	2q	2q3	0.735 ± 0.025	20
OVGP1	1p13.2	2	2q	2q3	0.580 ± 0.026	23
MUC4	3q29	4	5q	5q1	0.347 ± 0.025	20
MUC13	3q21.2	4	5q	5q1	0.357 ± 0.020	20
MUC3A	7q22.1	2	2q	2q1	0.244 ± 0.023	19
MUC2	11p15.5	Unknown	Unknown	2p1	0.046 ± 0.014	17
MUC5B	11p15.5	Unknown	Unknown	2p1	0.036 ± 0.010	20
MUC6	11p15.5	Unknown	Unknown	2p1	0.046 ± 0.014	17
MCAM	11q23.3	4	5q	5q2	0.462 ± 0.026	22
LYZ	12q15	8	3q	3q2.1	0.571 ± 0.024	20

Table 3. Percent peptide identity sequence between human, South American opossum and tammar wallaby for different PS (peptide sequences) and mucin domains

Gene	Coverage	Aligned length (amino acid)	Wallaby and opossum, %	Wallaby and human, %	Opossum and human, %
LYZ(1)	PS[a]	126	51.6	50.8	77.8
LYZ(2)	PS	148	62.3	59.5	75.0
MUC1	SEA[a]	86	72.9	33.7	36.0
MUC2	VWD1	146	85.5	67.1	68.5
	VWD2	155	–	80.0	–
	VWD3[a]	126	–	84.1	–
MUC3A	SEA[a]	51	51.0	31.4	31.4
MUC4	NIDO	86	80.0	60.5	57.0
	AMOP[a]	44	83.3	68.2	65.9
	VWD1[a]	92	83.5	67.4	70.7
MUC5B(1)	VWD1[a]	135	68.9	66.7	68.9
	VWD2	155	84.5	69.7	71.6
	VWD3	151	82.1	65.6	62.9
MUC5B(2)	VWD1[b]	149	55.0	55.0	67.8
	VWD2[b]	155	60.0	57.4	72.3
	VWD3[b]	151	61.6	55.0	62.9
MUC6	VWD1	147	–	63.4	–
	VWD2[a]	137	89.1	73.7	75.9
	VWD3	152	–	67.1	–
	CK[a]	32	–	59.4	–
MUC13(1)	SEA[a]	83	46.3	41.0	30.1
MUC13(2)	SEA	101	–	–	31.7
MCAM	PS[a]	612	84.3	62.5	62.1
OVGP1	PS[a]	282	53.7	43.6	57.8

SEA = Domain found in sea urchin sperm protein, enterokinase and agrin; VWD = von Willebrand D; NIDO = nidogen-like; AMOP = adhesion-associated domain in MUC4 and other proteins; CK = cysteine knot.

[a] Opossum and/or human sequences were truncated to compensate for gaps in the tammar wallaby sequence.

[b] Protein sequence gaps resulted in the identification of 2 VWD domains in tammar wallaby.

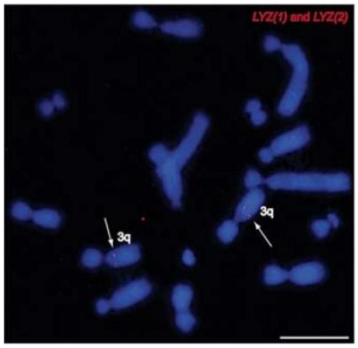

Fig. 1. FISH localization of BAC clone 144I19, containing *LYZ(1)* and *LYZ(2)*, to MEU3q. Scale bar = 10 μm.

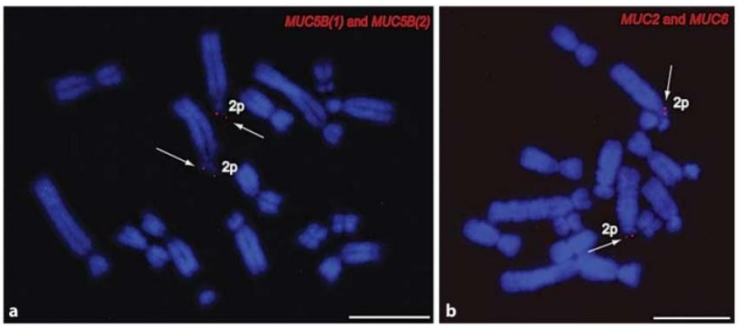

Fig. 2. Localization of **a** BAC clone 291N13, containing *MUC5B(1)* and *MUC5B(2)*, and **b** BAC clone 6H10, containing *MUC2* and *MUC6*, to MEU2p. Scale bar = 10 μm.

es for the opossum, human and tammar wallaby domains were aligned using ClustalW (MacVector v.9.5.2) to determine the sequence similarities between the species. In some cases it was necessary to truncate the opossum or human sequences if the domains spanned gaps in the tammar wallaby genome. The peptide coding sequence (in place of domains) was aligned with the opossum and human coding sequences for *LYZ*, *OVGP1* and *MCAM*.

Results and Discussion

We identified lysozyme, mucin and non-mucin genes in the tammar wallaby genome assembly and determined their percent sequence similarity to human and opossum using predicted peptide sequences for the gene or specific mucin domains. BACs were isolated for each gene and mapped to tammar wallaby chromosomes using fluorescence in situ hybridization.

The lysozyme genes, 2 of the 9 mucin genes *(MUC2, MUC6)* and *MCAM* were identified in Ensembl as predicted genes (http://www.ensembl.org/Macropus_eugenii/Info/Index), whereas the remaining 7 mucin genes *(MUC1, MUC3A, MUC4, MUC5B(1), MUC5B(2), MUC13(1), MUC13(2))* and *OVGP1* were identified by BLAST searching opossum sequences against the tammar wallaby database. The remaining genes *(MUC7, MUC12, MUC15, MUC16, MUC17, MUC19, MUC20, MUC21* and *EMCN)*, which were investigated by BLAST searching human sequences, were not identified in the tammar wallaby database. To confirm that the opossum mucin domains and lysozyme sequences corresponded to the genes of interest, the genes flanking the human genes of interest were compared to those flanking the opossum genes. Comparable flanking genes were identified for all genes except *MUC6* (data not shown), which is in the portion of the opossum genome presently unassigned to a chromosome.

The locations of genes in the tammar wallaby genome were predicted from cross-species chromosome painting, which has shown large regions of homology between the opossum and tammar wallaby, and the position of the opossum orthologs in the opossum genome assembly [Rens et al., 2003]. Table 2 lists the locations of the genes on the human, opossum and tammar wallaby genome, the locations predicted from cross-species chromosome painting and the tammar wallaby FLpter values. Three genes *(MUC2, MUC5B* and *MUC6)* have not been assigned to a chromosome in the opossum assembly and hence, their location in the tammar wallaby genome could not be predicted.

BACs containing 2 lysozyme genes mapped to *M. eugenii* chromosome (MEU) 3q (fig. 1) as predicted (table 2). The comparisons for the peptide sequences of the 2 lysozyme genes between opossum, human and tammar wallaby ranged from 50.8–77.8% (table 3). The tammar wallaby genes displayed 51.8% sequence identity (139 aa aligned length) to one another and both identified as type c lysozymes. This was not surprising as type c lysozyme from brushtail possum milk has been fully sequenced [Piotte et al., 1997] and is the major lysozyme in mammals [Jollés and Jollés, 1984]. The small number of lysozyme genes found in the tammar wallaby, a nonruminant foregut fermenter, is consistent with the results of Irwin and Wilson [1989], which demonstrated that nonrumi-

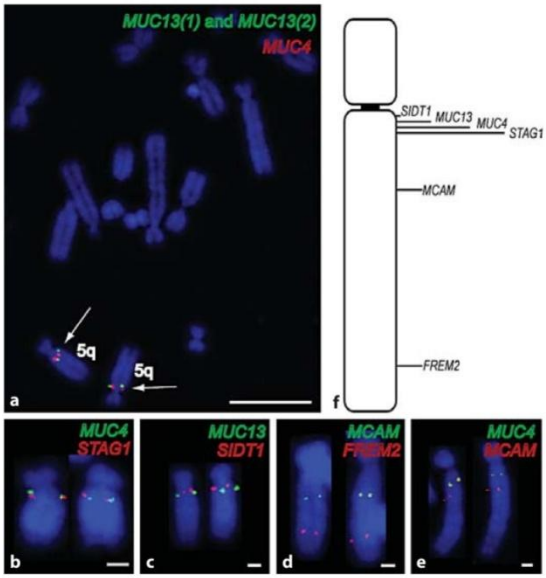

Fig. 3. FISH mapping of **a** BAC clones 320O6 and 301E20, containing *MUC4, MUC13(1)* and *MUC13(2)* respectively, to MEU5q. *MUC4, MUC13(1)* and *MUC13(2)* are located near the previously mapped genes **b** *STAG1*, and **c** *SIDT1*. **d** BAC clone 35C13, containing *MCAM*, was expected to map close to *FREM2*; however, *MCAM* mapped closer to **e** *MUC4, MUC13(1)* and *MUC13(2)*. **f** An ideogram of MEU5 showing the relative location of the genes on MEU5q mapped in this study. Scale bar = 10 μm (**a**). Scale bar = 1 μm (**b**–**e**).

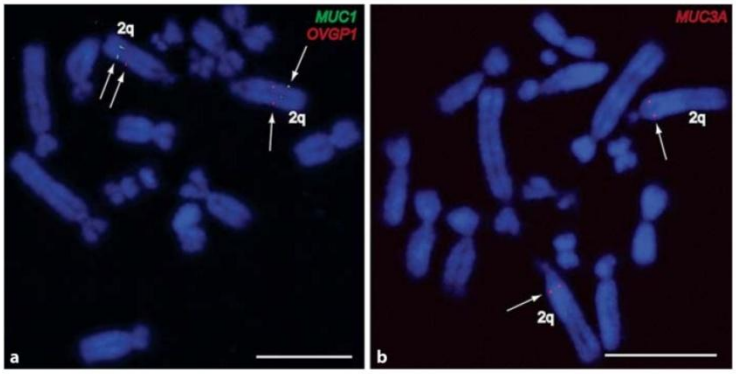

Fig. 4. FISH localization of **a** BAC clone 363J13, containing *OVGP1*, BAC clone 484D13, containing *MUC1*, and **b** BAC clone 330N14, containing *MUC3A*, to MEU2q. Scale bar = 10 μm.

nants (e.g. house mouse *(Mus musculus castaneus)*, rhesus monkey *(Macaca mulatta)* and human) have only 1 to a few lysozyme genes, while ruminants (e.g. cow *(Bos taurus)*, sheep *(Ovis aries)* and fallow deer *(Dama dama)*) have multiple lysozyme genes.

Mucins are often classified, based on their structure, as either gel-forming secreted *(MUC2, MUC5AC, MUC5B, MUC6, MUC7, MUC8, MUC19)* or membrane-bound *(MUC1, MUC3A, MUC4, MUC12, MUC13, MUC15, MUC16, MUC17, MUC20, MUC21* and *EMCN)* [Dharmani et al., 2009]. However, as the membrane-bound group of mucins includes splice variants that are secreted, Lang et al. [2007] suggested that mucins should be grouped by their characteristic domains (SEA, VWD and NIDO-AMOP-VWD). In this study we have used the latter classification, as our methodology included a comparison of the characteristic domains.

Marsupials display conservation of their arrangement of mucin genes when they are compared to the arrangement of mucin genes on human chromosomes. Genes *MUC2, MUC5B(1), MUC5B(2)* and *MUC6* (VWD domain mucins) localized to MEU2p (fig. 2), a region of the

genome for which chromosome painting failed to identify homology between the opossum and tammar wallaby (table 2). The VWD cluster, in tammar wallaby, also forms a cluster on human 11p15.5 and in other species such as mouse [Escande et al., 2004]. Prior to this study, only 3 genes *(IGF2, CD81* and *MRPL23)* have been mapped to MEU2p [Edwards et al., 2007]. These genes are also found on human 11p15.5, which suggests that MEU2p is equivalent to the human chromosome location 11p15.5.

Proteins with properties of the VWD mucins have been identified in the starlet sea anemone *(Nematostella vectensis)*, demonstrating that their evolutionary history can be traced to lower metazoa, while the remaining mucin genes examined here, arose in the vertebrate lineage [Lang et al., 2007]. We mapped *MUC4* (a NIDO-AMOP-VWD domain mucin) and *MUC13* (a SEA domain mucin; fig. 3a) to a conserved block comprising the genes *STAG1* and *SIDT1* on MEU5q (fig. 3b, c), that can also be found on human chromosome 3 and opossum chromosome 4 [Deakin et al., 2008]. Interestingly, 2 copies of *MUC13* were identified in the tammar wallaby; however, the SEA domain could not be identified on one of the scaffolds. *MUC13* originated from *HSPG2* and gave rise to *MUC3A* through a series of duplication events [Duraisamy et al., 2006], which suggests that one of the *MUC13* genes identified in this study may be a precursor to *MUC3A*; however, as *MUC3A* was also identified in this study, it is likely that the 2 *MUC13* genes in tammar wallaby are the result of an additional duplication event.

MCAM was predicted to be located on a conserved block comprising the genes *BACE1* and *FREM2* on MEU5q, that can also be found on human chromosome 13 and opossum chromosome 4 [Deakin et al., 2008]. However, *MCAM* mapped closer to the centromere than expected (fig. 3d) indicating that a small internal rearrangement may have taken place. Although located on the same chromosome, *MCAM* did not map near *MUC4* and *MUC13* (fig. 3e); the relative location of the genes on MEU5q is shown in figure 3f.

MUC1, *MUC3A* (the remaining SEA domain mucins) and *OVGP1* mapped to MEU2q (fig. 4). Unfortunately, MEU2 has not been mapped intensely like MEU5, so comparisons with nonmarsupial species were difficult. However, it was possible to distinguish that the genes did not cluster, which corresponds to those genes in human and opossum.

Sequence comparisons for the conserved mucin domains (VWD, SEA, NIDO, AMOP and CK) and peptide sequences for OVGP1 and MCAM, between opossum, human and tammar wallaby showed low to high conservation (30.1–89.1%; table 3) with SEA domains demonstrating lower sequence similarities compared to the VWD domains. The proline, threonine and serine (PTS) regions (a typical feature of mucins) were not used to compare sequences as they are not well conserved between species. Interestingly, many gaps were identified in tammar wallaby scaffolds where PTS regions were expected to be, based on their location relative to conserved neighboring mucin domains. This is likely a result of the repetitive nature of these sequences making them difficult to sequence and/or assemble.

The mapping data presented in this study will assist in the assembly of the tammar wallaby genome and demonstrates the importance of physically mapping genes when genomes have low sequence coverage, as it allows for a comparison of gene locations between species with conserved genomic regions. The identification of these genes also provides a foundation for future investigations in developmental marsupial immunology. Further research should focus on the role mucin and lysozyme genes play in protecting pouch young by determining the expression profile of these genes in various tissues of the developing neonate and the biological activity of these gene products on bacterial strains which are associated with the young.

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Chapter 5. Transcriptome analysis of the immune genes in the developing tammar wallaby (*Macropus eugenii*) skin

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Comments: This manuscript examines the transcriptome of the developing tammar wallaby skin, with particular reference to immune genes. The results suggest that specific innate immune mechanisms play a large role in the immune protection of marsupials before their immune tissues develop and until they can mount an adaptive immune response.

Transcriptome analysis of the immune genes in the developing tammar wallaby (*Macropus eugenii*) skin

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Key words

Marsupial; immune system; development; skin and transcriptome sequencing.

Abstract

Background: Marsupials are born with undeveloped immune tissue, yet at birth they must make their way from the sterile uterine environment to the teat located on the mother's abdomen. Thus, from birth, the newborn marsupial skin comes into direct contact with potentially pathogenic bacteria and must protect itself from immune challenges. As skin acts as a first line of defense against microorganisms, the main objective of this research was to examine the expression of immune genes in the skin of the developing tammar wallaby (*Macropus eugenii*). Transcriptome sequencing was used to obtain a holistic view of immune gene expression in the developing tammar wallaby skin. First, we identified the distribution of expression of different immune genes in immune related Gene Ontology (GO) terms. Second, we identified specific immune GO terms which included genes which were differentially expressed between immune-incompetent and immune-competent tammar wallabies. Finally, we examined genes previously identified or suggested to be expressed in the skin of developing tammar wallabies. **Results:** Several GO immune system process terms were represented by genes expressed in developing tammar wallaby skin, including activation and regulation of immune system processes, and leukocyte activation, homeostasis and migration, suggesting that the developing tammar wallaby skin plays a role as an immune defense organ. The genes with important immune functions with increased expression in the immune-incompetent group included two genes involved in complement, complement component 3 and complement factor B, two peroxidase genes, myeloperoxidase and glutathione peroxidase 2, and the lipoprotein component, apolipoprotein A1. Of the genes previously identified or suggested to be expressed in the skin of developing wallabies, three cathelicidin genes and peroxiredoxin 1 showed expression in skin; the tammar wallaby cathelicidin 1 and/or 2 genes showed increased expression in the immune-incompetent tammar wallabies compared to the immune-

competent tammar wallabies, while the gene for tammar wallaby cathelicidin 8 was highly expressed in both groups. **Conclusions:** The results suggest that specific innate immune protection mechanisms play a large role in the immune protection of marsupials before their immune tissues develop and until they can mount an adaptive immune response.

Background

Marsupials are born with undeveloped immune tissue [1-3] which develops as they suckle from the mother's teat. At birth, marsupial young are immunologically incompetent and cannot mount an adaptive immune response [4-6]. Instead, the young receives passively acquired immune protection from the mother through such mechanisms as milk [7-10] and pouch secretions [11-12]. The young also derives some protection from their own innate, non-specific immune system [13-18]; however, research in this field is lacking, and there are still many facets of immune protection in developing marsupials that require further examination [19]. Foremost is the immunological role of the pouch young skin, as young are directly exposed to potentially pathogenic bacteria as they make their way from the urogenital opening to the teat [20-23].

Mammalian skin acts as a first line of defense against microorganisms and commonly includes physical structures, chemical compounds and cells; including Langerhans (dendritic) cells, sebaceous glands (which secrete sebum) and antimicrobial peptides. The skin of the newborn marsupial comprises an outer layer of developing epidermis called the periderm, which is lost approximately one week after birth in the Virginia opossum (*Didelphis virginiana*) and Northern Quoll (*Dasyurus hallucatus*) [24,25]. The periderm functions as an initial and important physical barrier of protection, as early

research by Block (1960), found that non-sterile incisions which penetrated the periderm of the Virginia opossum resulted in the death of the animal [26].

To date, antimicrobial peptides, unique molecules located on immune cells which recognize pathogens, and an antioxidant have been investigated in the skin of the tammar wallaby (*Macropus eugenii*); however, research has been slow because compounds are usually examined individually and there are many potential immune compounds to investigate. Carman et al. [13,14] and Daly et al. [15] identified cathelicidins in the skin of tammar wallaby pouch young which were later found to be effective in killing a broad range of bacterial pathogens [18,27]. The expression of two molecules used for immune cell surface recognition, cluster of differentiation 14 (*CD14*) and toll-like receptor 4 (*TLR4*), have also been identified in tammar wallaby pouch young skin [16], as well as the gene for the antioxidant peroxiredoxin 1 (*PRDX1*), which has a possible role in immunity [17].

The extent of the role and importance of other forms of protection in the skin of the developing marsupial is unclear, as reported discrepancies in the timing of their first occurrence exist between species. For example, as summarized by Edwards *et al*. [19], Langerhans cells are found at birth in the newborn white-eared opossum (*Didelphis albiventris*) [4], while Langerhans cells and sebaceous glands are not evident until 23 and 59 days postpartum, respectively, in the quokka (*Setonix brachyurus*) [25] and immature Langerhans cells are evident at 10 days, with sebaceous glands evident at 28 days, in the brushtail possum (*Trichosurus vulpecula*) [28].

To date, research examining the expression of immune genes in skin has been compound specific and thus slow; however, with the advent of next generation sequencing it is now possible to venture away from compound specific research and to examine the expression of many genes involved in specific pathways or Gene Ontology

(GO) terms at one time. This research uses transcriptome sequencing to obtain a holistic view of immune gene expression in the developing tammar wallaby skin and aims to make use of the tammar wallaby genome, which has been sequenced to a 2-fold depth [29] to fill in research gaps associated with innate immunity and to highlight potential immune pathways that are relevant to future research for the protection of pouch young. Thus, before investigating potential new genes that may be protecting the pouch, we use information that is already available to achieve three main aims. First, we identify genes involved in specific immune GO terms which are expressed by pouch young. Second, we identify genes involved in specific immune GO terms which are differentially expressed between wallabies which are considered to be immune-incompetent and wallabies that are immune-competent. We consider tammar wallabies younger than 35 days to be immune-incompetent and wallabies older than 35 days to be immune-competent, based on histological studies, which imply that functional maturity of the adaptive immune system occurs by 35 days post partum, when mature T and B cells are found in lymphoid tissue beds [5]. However, as it is likely that there is a continuum of immune development, we examine animals that are younger or older than the 35 day time point. Finally, we also examine the expression of *TLR4*, *PRDX1*, cathelicidins, mucins and lysozymes, and where possible compare their expression with previous research, as these genes have previously been examined in developing tammar wallaby skin or have been suggested to play an immune role [13-19].

Results and discussion

Marsupials are born at an immune-compromised developmental stage, while their immune tissues are still developing. To date, the majority of immune protection research has come from maternal strategies, including studies of protection afforded by milk [19], while little research has been committed to examining the functioning aspects

of the young's own immune system. Thus, the focus of this study was to identify important immune genes that are expressed in the skin of the developing wallaby, an organ which is in direct contact with potentially pathogenic bacteria.

We performed transcriptome sequencing on skin samples obtained from four tammar wallabies aged 1.5 days (n = 1), 19 days (n = 1) and 57 days (n = 2) old. As tammar wallaby young do not appear to mount an adaptive immune response until 35 days post partum [5], 1.5 day and 19 day old pouch young wallabies (the younger group) represented an immune-incompetent group, while 57 day old pouch young (the older group) represented an immune-competent group.

Read mapping statistics

Previous research which has used RNA-sequencing to examine the skin transcriptome of other animals include studies on adult Atlantic Salmon (*Salmo salar)* [30] and the Antarctic fur seal (*Arctocephalus gazelle*) [31]; however, this is the first study to examine the skin transcriptome of a marsupial using RNA-sequencing. There were approximately 22 million reads obtained from the RNA-sequencing of developing tammar wallaby skin, after filtering for high quality sequenced reads (91.83 %; Additional file 1). TopHat was used to map 31.14 ± 2.22 % (mean ± SD) of the reads to the tammar wallaby genome (Additional file 1). As the tammar wallaby genome has only been sequenced to a 2-fold depth by Sanger Sequencing [29], it is likely that part of the genome has not been sequenced, which may account partly for the lower proportion of tags mapping to the genome. For further analysis the reads were normalized and filtered to include only those that made up the top 98 % of genes with the highest cumulative tag count (9 658 tammar wallaby genes) (Additional file 2). As long genes may generate more reads than equally expressed short genes, transcript length and fragments per kilobase of exon per million fragments mapped (FPKM) were

examined and found to display no bias (Additional file 3). The processed data file is available in Additional file 4 while the raw data can be downloaded from www.ncbi.nlm.nih.gov/sra [accession numbers SRR831709, SRR831710, SRR831711 and SRR836292] [32].

Distribution of immune gene expression across different immune system process terms

This is the first study to examine the immune transcriptome of developing wallaby skin and to identify genes associated with immune function in wallaby skin. Figure 1 shows the distribution of the expression of genes associated with immune system process GO terms, including the number of genes expressed in the skin for each GO term. There were 390 immune genes identified that are associated with the immune system process GO term. All samples showed similar expression of $\log_2$ normalized tag counts across immune system process child terms. Thus, it appears that the skin of the developing wallaby is an active site of the expression of genes involved in immunity and is likely to act as an immune defense organ. However, although this analysis identifies genes in immune system process terms, it is possible that not all genes are directly involved in immunity pathways, and instead are involved in other metabolic processes that may facilitate activation of immune response. Nevertheless, the GO analysis provides a comprehensive list of immune terms that lays the foundation for further and more specific immune gene analysis (Additional file 4).

Several previous studies have examined the expression of immune genes in the skin of various species. For example, Ierardi et al. [33] sequenced expressed sequence tags (ESTs), identifying 52 genes important in response to stress and immune challenges in the skin of the North Atlantic right whale (*Eubalaena glacialis*), while EST sequencing of Chinese brown frog (*Rana chensinensis*) skin identified 518 ESTs relevant to immunity and defense [34]. The skin of the zebrafish (*Danio rerio*) infected with

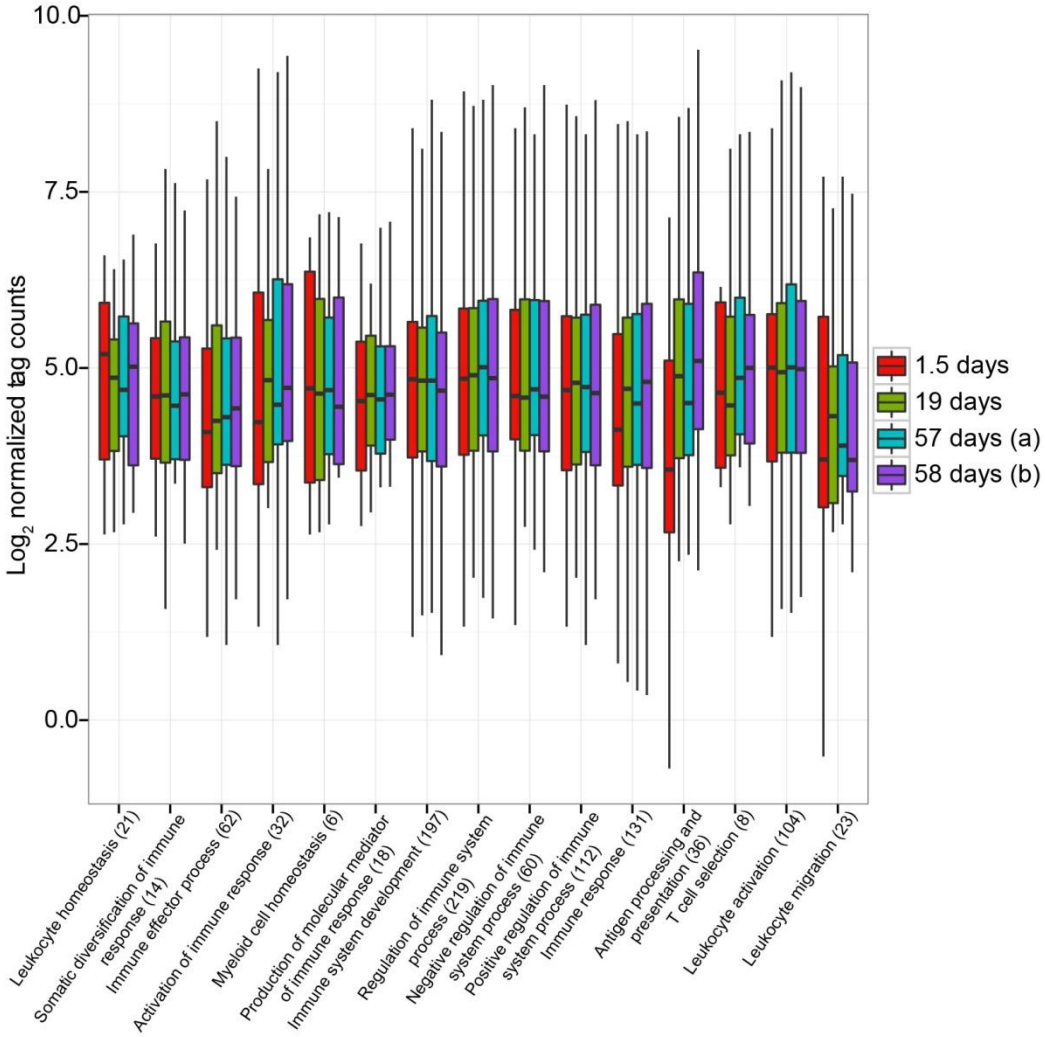

Figure 1. The expression of genes representing each immune system process child term in each sample that contained more than five genes. The upper, middle and lower ends of the box represent the upper quartile, median and lower quartile, while the upper and lower ends of the vertical lines represent the largest and smallest $\log_2$ tag count of the genes found within the immune system process child terms, respectively. The number of genes in each immune system process child term identified in the transcriptome is listed in brackets. Outliers (any data points above the 3rd quarter + 1.5 times the interquartile range and below the 1st quarter - 1.5 times the interquartile range) are not shown.

bacterium *Citrobacter freundii* was found to express 88 genes that were significantly involved with skin immunity including; complement activation and acute phase response, defense and immune response, response to stress and stimulus, antigen processing and presentation, cell adhesion and migration, platelet activation and coagulation factors and regulation of autophagy and apoptosis [35]. Next generation sequencing has also identified several immune relevant genes related to the adaptive and innate immune system in Atlantic salmon [30]. The studies examining immune gene expression in skin suggest that the skin of these species is also an active site of expression of genes involved in immunity.

Differential expression of immune genes between immune-incompetent and immune-competent groups

As reports suggest that there is a difference in the developmental stage of the immune system between marsupial young under 35 and young over 35 days old, we examined the differential expression of genes identified in the wallaby skin. First, a pairwise comparison of all samples was conducted to determine the relationship of variation between all samples (Additional file 5); with 75 % of the variation showing a relationship between the samples in the immune-incompetent group and 95 % of the variation showing a relationship between the samples in the immune-competent group. The lower score observed in the immune-incompetent group may be expected, compared to the higher score observed in the immune-competent group, as there is an age gap between the young in the immune-incompetent group. There were 229 genes (out of 9 658 filtered genes) which were identified as differentially expressed. In the immune-incompetent group, 131 genes were found to be more highly expressed than in the immune-competent group, while 98 genes were found to be more highly expressed

in the immune-competent group than in the immune-incompetent group (Additional file 6).

Genes that were significantly more highly expressed (P-value < 0.05, Benjamini-Hochberg false discovery rate (FDR) < 0.05) in the immune-incompetent group were used to perform GO term enrichment analysis by g:Profiler to detect significantly overrepresented GO terms compared to all genes. A total of 78 groups were identified as significantly overrepresented (P-value < 0.05), including 32 biological process terms, 18 cellular component terms and five molecular function terms (Additional file 4). Terms with immune function included five terms from the biological process term: humoral immune response, inflammatory response, regulation of inflammatory response, defense response, and regulation of defense response (Table 1). There was only one molecular function GO term, smoothened binding, identified as overrepresented in the immune-competent group when compared to the immune-incompetent group. It is not surprising that the younger group showed increased expression of genes in immune groups compared to the older group, as an organism faces new demands at birth as it moves from a sterile uterine environment to the external environment.

The genes from GO terms, which were significantly overrepresented in the immune associated term, with potential immune function were further examined to determine whether they were represented by both immune-incompetent individuals. The genes which were over expressed in the immune related GO terms by both individuals in the immune-incompetent group include, alpha-2-HS-glycoprotein (*AHSG*), apolipoprotein A1 (*APOA1*), complement component 3 (*C3*), complement factor B (*CFB*), glutathione peroxidase 2 (*GPX2*), hemopexin (*HPX*), and myeloperoxidase (*MPO*) (Figure 2a). Here we will focus on five genes which have potentially important immune roles that

Table 1. Significantly overrepresented Gene Ontology (GO) terms associated with immune related terms represented by highly expressed genes in the immune-incompetent wallabies.

GO term		Relative GO depth	Genes differentially expressed (no.)	P-value
Parent term	Child group			
Biological process	Defense response	1	12	0.00
	Regulation of defense response	2	7	0.05
	Inflammatory response	2	7	0.04
	Regulation of inflammatory response	3	6	0.01
	Humoral immune response	1	4	0.03

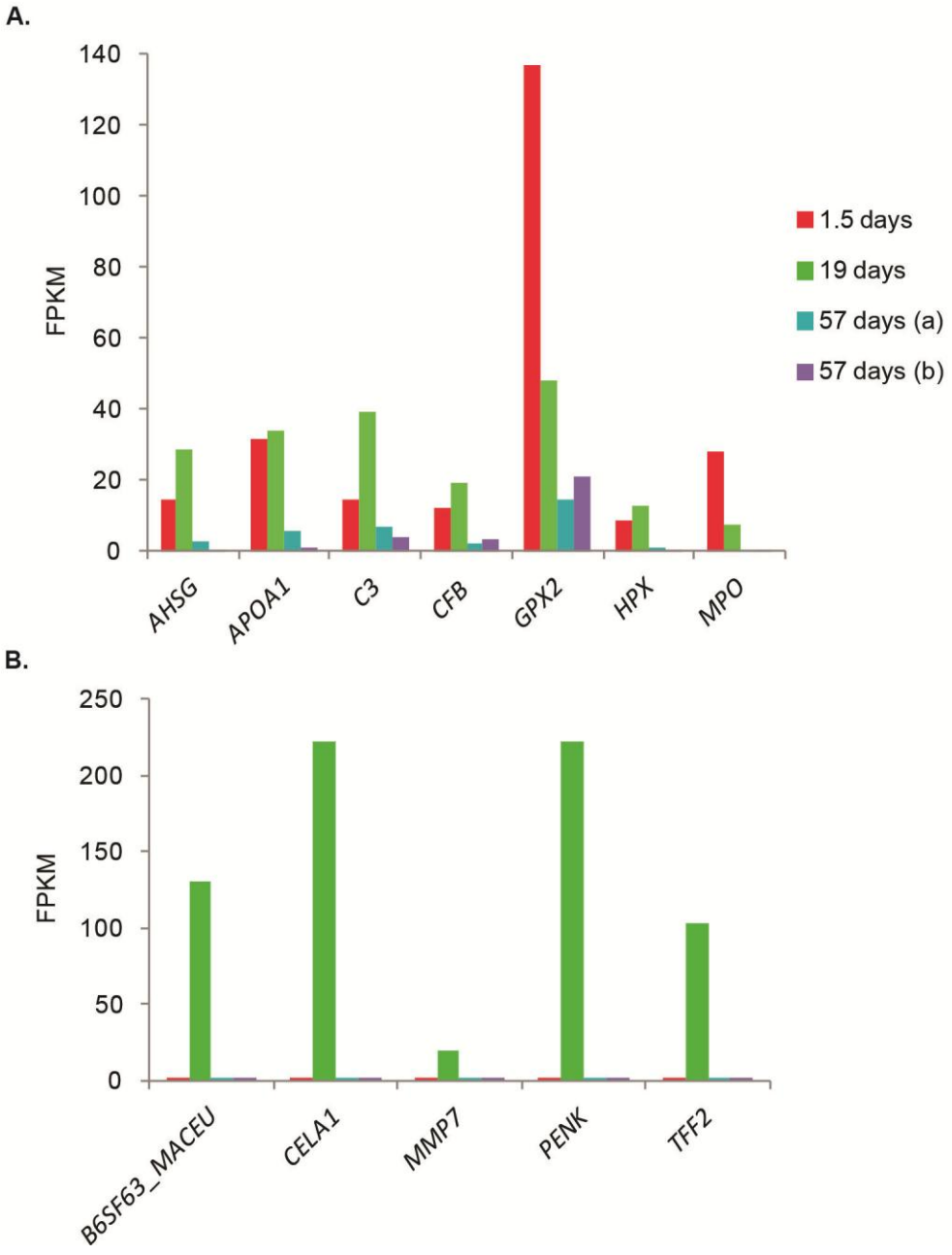

Figure 2. The expression (fragments per kilobase of exon per million fragments mapped (FPKM)) of genes which were identified in immune system process Gene Ontology (GO) terms that were significantly overrepresented in the GO term enrichment analysis in the immune-incompetent group. A. shows the genes that were expressed in the skin of both immune-incompetent individuals, while B. shows the genes that were highly expressed in the 19 day old individual.

can be extended to the skin, including the two genes involved in complement, *C3* and *CFB*, two peroxidase genes, *MPO* and *GPX2* and one lipid related term with potential immune function, *APOA1*.

Complement is one of the most ancient forms of immunity and provides an important role in defense as one of the first innate mechanisms awaiting pathogens that may penetrate the epithelial barrier [36]. In addition to roles in immunity, complement contributes to the growth and repair of tissue, induces growth factors and promotes restoration of tissue homeostasis [36]. The alternative pathway of complement activation can be triggered directly by microbial surfaces, thus the complement defense mechanism acts immediately after encountering a pathogen [37]. C3 is the central molecule to the alternative pathway and CFB protease binds to hydrolyzed C3 [36]. The occurrence of higher expression of *C3* in the younger marsupials is surprising as the complement system is underdeveloped in healthy term humans (38 to 42 weeks gestation), with circulating complement concentrations 10 to 80 % of adults [38]. By specifically examining C3, McGreal *et al.* [38] uncovered a large range of differences in complement levels in human neonates. For example, C3 can be found at concentrations 60 % that of the concentration of adults [39] which reaches the adult concentration between six days and six months old [40]; however, the concentration in newborns has also been observed at concentrations that are almost identical to adults [41], although, Shapiro *et al.* [41] report that their adult reference values are lower than those in other studies. McGreal *et al.* [38] also reports that preterm concentrations are much lower than those of term neonates; however, the research examining these concentrations compared them to the maternal serum concentrations which are usually higher than those of normal adults [42]. In contrast to the expression of *CFB* observed in developing tammar wallabies, with increased expression found in the immune-incompetent group

compared to the immune-competent group, in newborn humans, CFB serum concentrations are on average 54 % (36 to 73 %) that of normal adults [38].

Marsupials are born at a stage of development that is equivalent to a human at eight to 10 weeks gestation [26,43]. Although we are comparing the transcriptome analysis of complement in wallaby to the concentrations of complement in human serum, the results thus far suggest that complement activity is high in young wallabies and lacking in young humans. The complement system appears to be developing at a much earlier stage in marsupials when compared to eutherian mammals and it may be that the premature development compensates for the delayed development of their immune tissue and delayed ability to mount an adaptive immune response. Further research is warranted to examine this hypothesis.

Two peroxidase enzymes were also identified to be more highly expressed in the younger group. MPO has an important immunological function in the neutrophil's primary role of phagocytosis and destruction of microorganisms. MPO is released into the phagosome during degranulation or it is leaked outside of the cell before enclosure of the phagosome [44] and has an antimicrobial effect on microorganisms [45]. Skin contains blood vessels and leukocytes and hence, it is not surprising that a gene associated with the role of neutrophils would be expressed in tammar wallaby skin. MPO has been previously identified in the neutrophils of adult tammar wallaby serum [46]. Neutrophils make up the majority of leukocytes at birth in tammar wallaby [47], the quokka [48] and Virginia opossum [49]. A comparison of neutrophil composition in the leukocytes of Virginia opossum reduced from 89 % in newborns, to 23 % in adults [49]. Thus, the higher expression of *MPO* in younger versus older animals is not surprising if neutrophils comprise the largest proportion of the leukocyte population and

suggests that neutrophils are important to the immunological protection of the marsupial neonate.

The identification of *GPX2* in the skin was an unusual result as GPX2 is a major enzyme that reduces hydroperoxidase in the intestinal epithelium. Few studies have examined the immune role of GPX2; however, Chu *et al.* [50] found that GPX2 prevented ileocolitis and intestinal cancer by reducing oxidative stress caused by bacteria. As young are in contact with bacteria on their journey to the teat and within the pouch, GPX2 could play a similar role in the developing tammar wallaby skin.

In addition to the two complement components and the two peroxidase enzymes, an apolipoprotein gene was also identified to have higher expression in the skin of the younger group. APOA1 is a major apolipoprotein of high density lipoprotein, and may directly affect bacterial growth and promote self defense mechanisms of normal and immune-incompetent individuals, as a serum fraction containing APOA1 has shown antimicrobial activity against a gram positive bacteria *Staphylococcus epidermidis* [51]. In addition to the immune related GO terms, the enrichment analysis also identified many lipid related processes as overrepresented in the immune-incompetent group (Additional file 4). Apolipoprotein B (APOB), is a major structural component of low density and very low density lipoproteins, which plays a role in mediating the virulence of specific microorganisms. For example, APOB influences *agr* signaling in *S. aureus* which prevents the change in phenotype of *S. aureus* from colonizing to invasive [52]. Although the apolipoprotein analysis was conducted using serum, antimicrobial lipids are also found at the skin surface [53]. In a review of the complementary mechanisms that protect the developing marsupial pouch young, Edwards *et al.* [19] highlight that there are no studies which have examined antimicrobial lipids in developing marsupials

and that they are unlikely to be explored while there is still so much unknown about the antimicrobial proteins and peptides.

Differential expression of immune genes between 1.5 and 19 day old young

The aim of this research was to compare the skin of young considered to be immune-incompetent and the skin of young considered to be immune-competent; however, differences between the young in the immune-incompetent group were also observed. There were five genes that were over expressed in the immune related GO terms by the skin of the individual that was 19 days old, including *B6SF63_MACEU*, chymotrypsin-like elastase family, member 1 (*CELA1*), matrix metallopeptidase 7 (*MMP7*), proenkephalin (*PENK*) and trefoil factor 2 (*TFF2*) (Figure 2b). Particularly interesting is the over expression of *TFF2*, as *TFF2* codes for one of three mammalian TFF peptides. TFFs are synthesized in mucin producing epithelial cells and play a role protecting the epithelia [54]. Although normally found in the gastrointestinal tract of mammals, TFF peptides have also been detected in amphibian skin, with TFF domains forming a central part of frog integumentary mucin C.1 [55]. Mucins, the main component of mucus, often afford protection to the skin of other species, such as fish [56] and it has been suggested that they may play a similar role in the developing marsupial skin before the marsupial pelage develops. Previous research identifying and mapping mucin genes to the tammar wallaby genome proposed that mucins may play a role in the protection of developing marsupial young [57]. Reads corresponding to scaffolds which contain previously identified mucins were examined (data not shown), but were not identified to be expressed.

At the time of sample collection, the aim of the research was to examine the skin transcriptome of wallabies with and without immune competence. However, as there are differences in gene expression observed between the younger two samples further

studies examining the transcriptome of younger wallabies are warranted to determine if there is a difference between the ages of the wallabies or if the difference is due to individual variation.

Expression of immune genes previously detected in tammar wallaby skin

Previously, *TLR4* was identified in tammar wallaby skin from young aged five days with an increase in expression occurring at 70 and 90 days postpartum [16]. In this study, *TLR4* was identified in all samples but with an FPKM less than two, suggesting that other genes may play a larger role in immune protection of marsupial young than *TLR4*. *PRDX1*, however, was found to be represented across all samples with tag counts greater than 250 FPKM. Daly et al. [17] found that *PRDX1* was expressed in five day old tammar wallaby skin and that its expression decreased at around 25 days and then increased 70 to 90 days postpartum. Differential expression may not have been detected in this study as the oldest samples were 57 days old. Although there were reads that also mapped to the tammar wallaby lysozyme orthologues they were not included in the 98 % of cumulative tag counts retained for further analysis (Additional file 4), which suggests that lysozyme does not play a large role in the protection of marsupial young as hypothesized by Edwards et al. [19].

Reads corresponding to the tammar wallaby cathelicidin (MaeuCath) genes were also examined (Additional file 4). The reads mapping to *MaeuCath1* and *MaeuCath2* could not be differentiated but showed significantly higher expression in the immune-incompetent group compared to the immune-competent group (log fold change = 5.1, P-value and FDR value < 0.01), while *MaeuCath8* was expressed in all samples. The expression of *MaeuCath1* has previously been found to increase in the skin of developing wallabies in the first 25 days postpartum [15], and has also been detected along with *MaeuCath8* in tammar wallaby pouch young skin 20 to 120 days postpartum

[18]. *MaeuCath8* has been detected in the skin of tammar wallabies from seven days, but not at birth [14]. *MaeuthCath1*, splice variants of *MaeuCath1* and WAM1 (a wallaby antimicrobial peptide derived from *MaeuCath1*) possess antimicrobial activity against a range of microorganisms and are thought to provide the young with substantial immune protection during and after birth [18,27].

Conclusions

Our research examined the transcriptome of the developing wallaby skin and identified genes involved in several immune terms that were present in both the immune-incompetent and immune-competent group, but also genes involved in immune terms that were overrepresented in the immune-incompetent group, such as *C3*, *CFB*, *MPO*, *GPX2* and *APOA1*. Interestingly, complement components are usually found in much lower concentrations in human neonates than in human adults, suggesting that sections of the complement system develop faster in marsupials than in eutherian mammals and could possibly provide an immune compensation mechanism while the young's undeveloped immune tissue matures. We suggest that the skin of the developing marsupial is an active immune organ; however, further analyses are required to determine the specific function of these genes.

As research to date has been compound specific, we used transcriptome sequencing to identify genes in developing tammar wallaby skin which have already been annotated in the genome. Now that GO immune terms and immune genes have been identified further work examining these groups and processes would be highly beneficial. Additionally it would be interesting to compare neonatal skin of eutherian mammals to marsupials, or to do increased time series transcriptome studies with and without immune challenge. Marsupials provide for an interesting immunological case at birth and we are only just touching the surface of protective mechanisms. Not only can we

use information, such as annotated genomes and transcriptome sequencing to explore these protective mechanisms but there is also the potential for examining new compounds with large immunological roles.

Methods

Sample collection

Animal handling and sample collection were carried out at the Commonwealth Scientific and Industrial Research Organisation (CSIRO) Sustainable Ecosystems, Gungahlin, Canberra, Australia by methods approved by the Australian National University's Animal Experimentation Ethics Committee (Register no. R.CG.15.10) and the CSIRO Sustainable Ecosystems Animal Ethics Committee (Application no. 10-02). Animals were euthanized by decapitation. Skin tissue was obtained between the neck and tail on the dorsal side of four tammar wallaby pouch young, aged 1.5 days (n = 1), 19 days (n = 1) and 57 days (n = 2). The tissue samples were suspended in RNAlater® solution (Ambion) for at least 24 hrs, before they were stored at -80 °C or were immediately frozen at -80 °C following dissection.

RNA extraction, library construction and sequencing

Frozen tissue samples were homogenized in lysis solution with 2-mercaptoethanol. Total RNA was extracted using the GenElute™ Mammalian Total RNA Miniprep Kit (Sigma-Aldrich) with the protocol for fibrous tissue. Contaminating DNA was removed using an on-column DNase I digestion set (Sigma-Aldrich) during the RNA extraction or with DNA-*free*™ (Ambion) after the extraction, following the manufacturer's instructions. RNA integrity and concentration were determined using a Nano6000 chip on a 2100 Bioanalyser (Agilent Technologies). Each RNA sample was stabilized and stored in an RNAstable tube (Biometrica) and sent to the Beijing Genomics Institute,

Tai Po, Hong Kong for sequencing. cDNA libraries were generated using Illumina's mRNA Sample Preparation Guide (Cat # RS-930-1001); mRNA was enriched by oligo (dT) magnetic beads and fragmented to 200 basepairs using fragmentation buffer. First strand cDNA was synthesized by random hexamer-primer using the RNA fragments as templates. Buffer, dNTPs, RNase H and DNA polymerase were added to synthesize the second strand. The double strand cDNA was purified with QiaQuick PCR extraction kit and washed with EB buffer for end repair and single nucleotide adenine addition. Finally, sequencing adaptors were ligated to the fragments. The fragments were purified by agarose gel electrophoresis and enriched by PCR amplification. The library products were sequenced to a length of 49 nucleotides using the Illumina® HiSeq™ 2000 sequencing platform.

Quality assessment, read mapping and tag counting

Low quality sequenced reads were first filtered out using Trimmomatic [58] (version 0.25). Reads with an end nucleotide quality value less than three or reads with the mean quality value of the ast four nucleotides less than 20 were considered as low quality reads and removed from further analysis. Filtered high quality reads were visually assessed for their qualitative properties using FastQC [59]; data quality was deemed acceptable for further analysis.

The tammar wallaby genome sequence (Meug_1.0) and gene annotations were downloaded from Ensembl [60] (version 70). All reads were aligned to the wallaby genome reference sequence using TopHat (version 2.0.6) with default parameters. The alignments generated after running the TopHat command were further processed using Cufflinks [61] (version 2.0.2) to identify and assemble known and novel transcripts. Cuffmerge [61] was used to produce a non-redundant set of known and novel transcripts for our dataset. Read alignments were overlapped with gene annotations for the wallaby

genome using intersectBed [59] (BedTools version 2.17.0) and sequenced fragment counts were then generated for each gene in each sample using custom Perl scripts (Additional file 7). TopHat identified novel transcript isoforms of annotated genes or novel gene models overlapping with existing gene annotations. However, further analysis of the novel isoforms and gene models was beyond the scope of this study.

For further analysis, the sum of mapped tag counts for each sample was used as its effective library size. Read counts per gene were first normalized for library size and transcript length to obtain FPKM [62]. These counts were further normalized for total RNA output using the trimmed mean of M-values method [63]. Transcript length was compared to normalized FPKM to determine length versus expression bias.

Distribution of expression of immune system process terms

To determine the expression of immune genes across different immune system process terms, all genes associated with the child terms of the immune system process GO term (GO:0002376) were identified from Ensembl. The expression patterns for the genes associated with these GO terms were quantified using a Perl script (Additional file 7) implemented in the edgeR package [64] (version 2.6.12). GO terms, showing expression of less than six genes were excluded from the analysis.

Differential expression analysis

A pairwise comparison of all samples was first conducted to determine the relatedness between all samples. Differential expression analysis for genes between young (1.5 and 19 days) and old samples (57 days) was performed using the edgeR package [64] (version 2.6.12) following the author's recommendations. We selected all genes that contributed to the top 98 % of the cumulative counts in any one sample. Zero tag counts were adjusted to the mean value of the lowest normalized count and zero. For each

group of samples, dispersion estimates were obtained using the quantile adjusted conditional maximum likelihood method. Subsequently, differential expression tests were computed for each gene. FDR was calculated for multiple-testing using the Benjamini-Hochberg procedure. Genes were defined as differentially expressed if the P-value and FDR for differential expression was ≤ 0.05. A list of differentially expressed genes (up and down regulated) was sorted based on their log fold change. GO term enrichment analysis, to detect significantly overrepresented GO terms was performed in g:Profiler [65].

We also examined the expression of genes previously identified or suggested to be expressed in the skin of developing wallabies. *TLR4* [Ensembl: ENSMEUG00000009461], *PRDX1* [ENSMEUG00000006820] and lysozyme orthologues [ENSMEUG00000011846 and ENSMEUG00000015902] are annotated in the genome. However, cathelicidin genes [RefSEQ: gi_157042603; gi_157042605; gi_157042607; gi_157042609; gi_157042610; gi_157042612; gi_157042614; *MaeuCath8* [14]] and mucin genes [57] are not annotated in the genome. For genes not annotated in the genome we identified their locations using megablast and subsequently analyzed their tag counts or we counted tags for the scaffolds that are known to harbor these genes.

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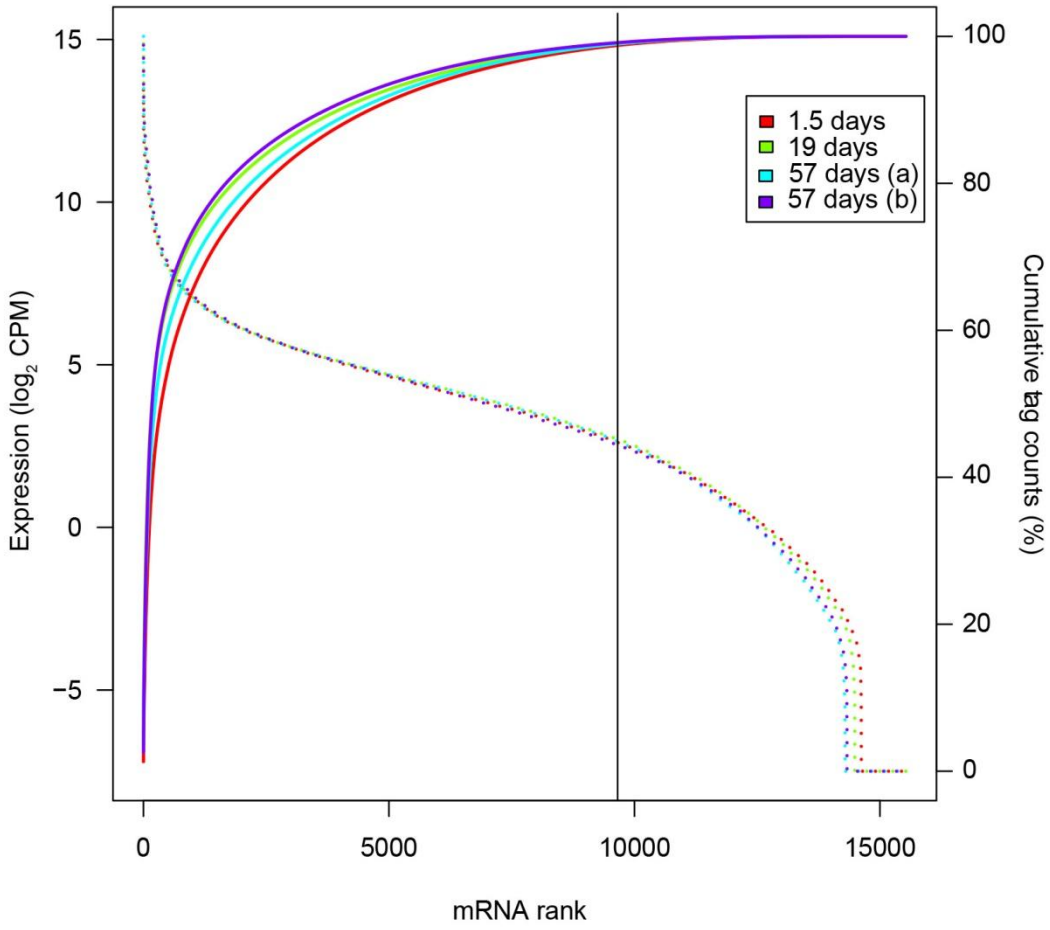
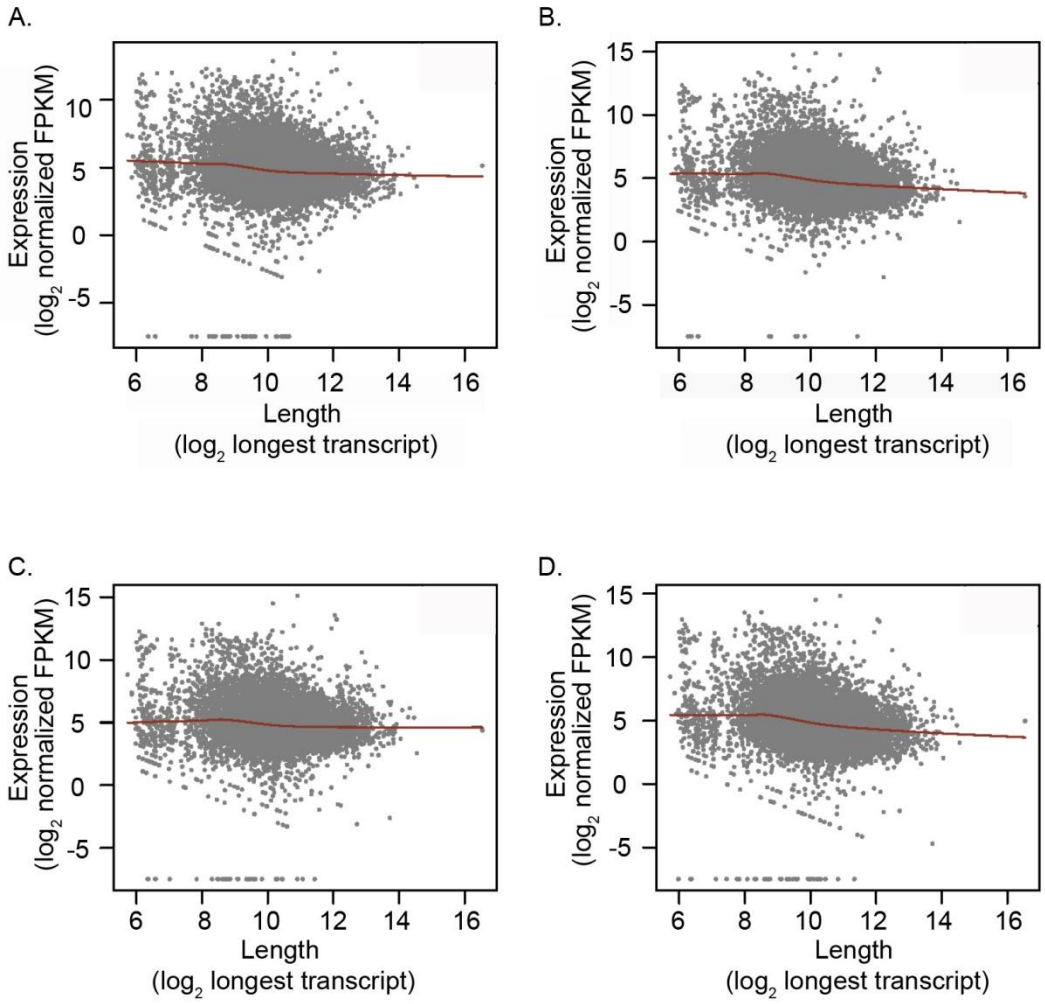
Additional File 1. Number of tag counts obtained from transcriptome sequencing, before and after quality value (Q.V.) filtering and number of tag counts successfully mapped to the tammar wallaby genome for tammar wallaby skin using TopHat.

Sample (days old)	Tag counts (raw)	Tag counts low Q.V. filter	Tag counts low Q.V. filter (%)	Tag counts low Q.V. filter mapped	Tag counts low Q.V. filter mapped (%)
1.5	24828905	22855751	92.05	6520026.00	28.53
19	24338153	22396985	92.02	7269031.00	32.46
57 (a)	24783405	22685259	91.53	7581735.00	33.42
57 (b)	23827686	21854875	91.72	6590673.00	30.16
Mean	24444537.25	22448217.50	91.83	6990366.25	31.14
Standard deviation	467044.96	438540.12	0.25	519084.35	2.22



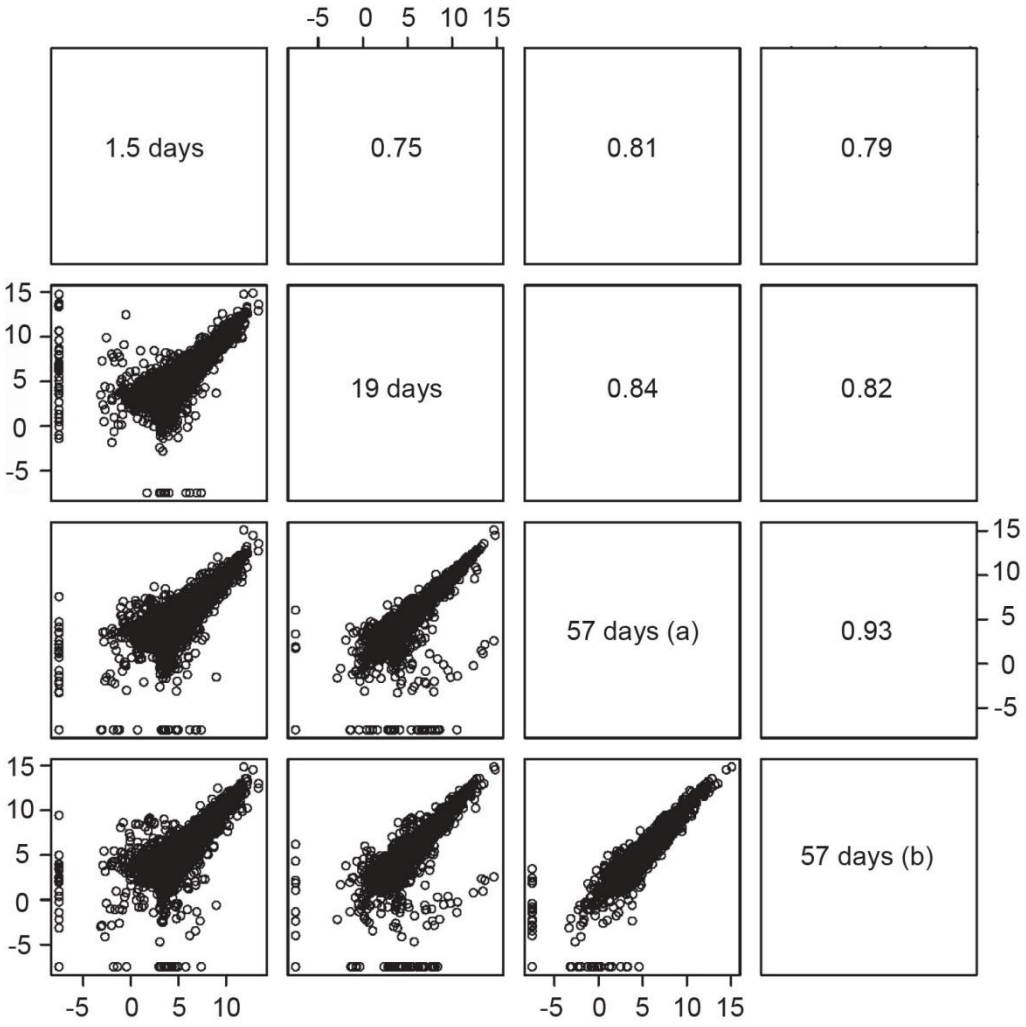
Additional file 2. The number of tag counts (log_2 counts per million (CPM)) for each gene (dotted lines) expressed in tammar wallaby skin and the cumulative proportion of tag counts for each gene (solid line). The black line represents the cut-off of genes which include genes containing 98 % of the cumulative tag counts.



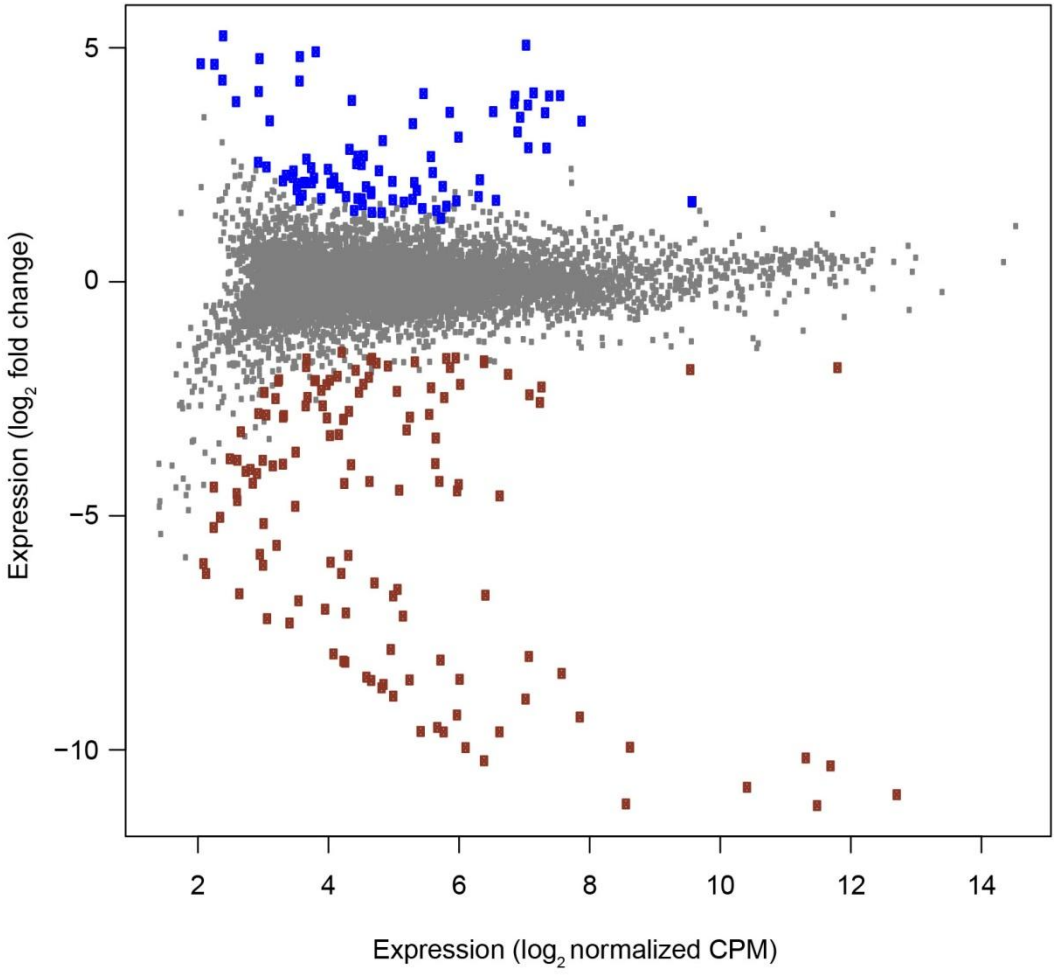
Additional file 3. The correlation between the FPKM (fragments per kilobase of exon per million fragments mapped) of transcripts expressed in tammar wallaby skin and the length of each transcript (A. 1.5 day old; B. 19 day old; C. 57 day old (a) and D; 57 day old tammar wallabies).

Additional file 4. Spreadsheet containing the data analyzed for the tammar wallaby developing skin transcriptome. The spreadsheet is available on the disk accompanying the thesis.

Sheet name	Description
RawCounts	Raw counts
FPK	Raw counts/base pair length
FPKM	FPKM/library size
NormFactors	Normalization factors
NormFPKM	FPKM/normalization factors
Retained	Genes making up 98 % of highest tag counts
Diffexp	Differential expression analysis results
NegLFC	Genes showing negative fold change between the immune-incompetent and immune-competent group
NegGOEnr	GO terms showing significant enrichment in the immune-incompetent group
PosLFC	Genes showing positive fold change between the immune-incompetent and immune-competent group
PosGOEnr	GO terms showing significant enrichment in the immune-competent group
Cathelicidin	Raw counts, NormFPKM, retained and Diffexp for cathelicidin analysis



Additional file 5. A pairwise comparison of genes expressed by tammar wallaby skin which shows correlation coefficients between each sample.



Additional file 6. Differential expression of genes from immune-incompetent and immune-competent tammar wallaby skin. Genes which are up regulated and down regulated in the immune-incompetent group compared to the immune-competent group are shown in blue and red respectively. The remaining points (grey) show genes which are not differentially expressed between the two groups.

Additional file 7. Perl script used to conduct tammar wallaby skin transcriptome analysis.

```
library(edgeR)
options(java.parameters = "-Xmx4000m")
library(xlsx)
source("Downloads/gProfileR/R/gProfileR.R")

sortcol <- function(x){sort(x,decreasing=TRUE)}
cumfreqcol <- function(x){cumsum((x/sum(x))*100)}
seltop <- function (x){slist<-cumsum(x[order(x,decreasing=TRUE)])/sum(x)<=0.98; bottom<-labels(slist)[slist==FALSE]; x[bottom]<-NA; return(x)}
panel.cor <- function(x, y){
    usr <- par("usr"); on.exit(par(usr))
    par(usr = c(0, 1, 0, 1))
    r <- abs(cor(x, y))
    txt <- format(c(r, 0.123456789), digits=2)[1]
    text(0.5, 0.5, txt)
}

a <- read.table("skin.FPKTagCounts.txt", header=TRUE, row.names=1)
rawcounts <- read.table("skin.rawTagCounts.txt", header=TRUE, row.names=1)
l <- read.table("skin.EffectiveGeneLength.txt", header=TRUE, row.names=1)
acpm <- cpm(a)
g <- c(1,1,2,2)
y <- DGEList(counts=acpm,group=g)
y <- calcNormFactors(y)
nc <- cpm(y, normalized.lib.sizes=TRUE)
nc[nc==0] = (range(nc[nc>0])[1]) / 2 ###adjust zero values to a value half-way between zero and the minimum value
```

```
ncs <- as.matrix(apply(nc,2,sortcol)) ####each column sorted

nccf <- as.matrix(apply(ncs,2,cumfreqcol)) ####cumulative proportion for each column

filt <- apply(nc, 2, seltop) ####select top 98% in at least one sample

retained <- filt[rowSums(filt,na.rm=TRUE)>0,]

retained <- data.frame(nc[rownames(retained),])

rl <- cbind(retained, l[rownames(retained),]) #####attach transcript lengths

y <- y[rownames(retained),] ###change DGE object for only top genes

y <- estimateCommonDisp(y)

y <- estimateTagwiseDisp(y)

et <- exactTest(y)

tt <- topTags(et,n=100000)

imp <- tt$table[tt$table$PValue <= 0.05 & tt$table$FDR <= 0.05,]

nimp <- tt$table[tt$table$PValue > 0.05 | tt$table$FDR > 0.05,]

impneg <- imp[imp$logFC < 0,]

impneg <- impneg[order(impneg$logFC),]

negenr <-
gprofileradv(organism="meugenii",ordered_query=1,significant=1,rownames(impneg),backgro
und=rownames(y$pseudo.alt))

imppos <- imp[imp$logFC > 0,]

imppos <- imppos[order(-imppos$logFC),]

posenr <-
gprofileradv(organism="meugenii",ordered_query=1,significant=1,rownames(imppos),backgro
und=rownames(y$pseudo.alt))


#####for a go term gene expression in four samples

go <- read.table("immunesystemprocess.goVSgene.txt", header=FALSE, sep="\t")

colnames(go) <- c("geneid", "goacc", "goname")

goexp <- merge(go, retained, by.x=1, by.y=0, all=FALSE)

dm <- melt(goexp, id.vars = c('geneid', 'goacc'), measure.vars=colnames(retained))

gocounts <- ddply(dm, c("goacc"), summarise, count=length(goacc)/ncol(retained))

rownames(gocounts) <- gocounts$goacc
```

```
gocounts$goacc <- NULL

dm$count<-gocounts[dm$goacc,]

dm$label = paste(dm$goacc, " (", dm$count,")", sep="")

dm$value <- log(dm$value,2)

bp <- boxplot(dm[dm$count>5,]$value ~
(dm[dm$count>5,]$label+dm[dm$count>5,]$variable))


#####write excel tables

write.xlsx(rawcounts, file="WallabyTranscriptome.xlsx",sheetName="RawCounts")

write.xlsx(a, file="WallabyTranscriptome.xlsx",sheetName="FPK", append=TRUE)

write.xlsx(acpm, file="WallabyTranscriptome.xlsx",sheetName="FPKM", append=TRUE)

write.xlsx(y$samples$norm.factors,
file="WallabyTranscriptome.xlsx",sheetName="NormFactors", append=TRUE)

write.xlsx(nc, file="WallabyTranscriptome.xlsx",sheetName="NormFPKM", append=TRUE)

write.xlsx(retained,
file="WallabyTranscriptomeAnalysis/WallabyTranscriptome.xlsx",sheetName="Retained",
append=TRUE)

write.xlsx(tt$table,
file="WallabyTranscriptomeAnalysis/WallabyTranscriptome.xlsx",sheetName="DiffExp",
append=TRUE)

write.xlsx(impneg,
file="WallabyTranscriptomeAnalysis/WallabyTranscriptome.xlsx",sheetName="NegLFC",
append=TRUE)

write.xlsx(negenr,
file="WallabyTranscriptomeAnalysis/WallabyTranscriptome.xlsx",sheetName="NegGOEnr",
append=TRUE)

write.xlsx(imppos,
file="WallabyTranscriptomeAnalysis/WallabyTranscriptome.xlsx",sheetName="PosLFC",
append=TRUE)

write.xlsx(posenr,
file="WallabyTranscriptomeAnalysis/WallabyTranscriptome.xlsx",sheetName="PosGOEnr",
append=TRUE)


#####Normalization factors

pdf("WallabySkinNormFactors.pdf")
```

```
barplot(y$samples$norm.factors, names.arg=rownames(y$samples))

dev.off()

#####pairwise comparison

pdf("WallabySkinPairwise.pdf")

pairs(log(retained,2), upper.panel=panel.cor)

dev.off()

#####Transcript Length plot

pdf("WallabySkinLengthvsFPKM.pdf")

par(mfrow=c(2,2))

for (i in 1:ncol(retained)){

  plot(log(rl[,(ncol(retained)+1)],2), log(rl[,i],2), pch=19, col="grey 50", cex=0.3,
xlab="log2(Longest Transcript Length)", ylab="log2(Normalized FPKM)")

  lines(lowess(log(rl[,(ncol(retained)+1)],2), log(rl[,i],2)), col="tomato 4")

  legend("topright", colnames(retained)[i], cex=0.8)

}

dev.off()

#####Differential Expression Graph

pdf("WallabySkin.DiffExp.pdf")

plot(nimp$logCPM, nimp$logFC, cex=0.4, col="grey 50", xlim=range(tt$table$logCPM),
ylim=range(tt$table$logFC), xlab="log2 (Normalized Counts/Million)", ylab="log2 (Fold
Change", main="Wallaby Skin Differential Expression Pattern")

points(imp[imp$logFC > 0,]$logCPM, imp[imp$logFC > 0,]$logFC, cex=0.8, col="blue",
pch=19)

points(imp[imp$logFC < 0,]$logCPM, imp[imp$logFC < 0,]$logFC, cex=0.8, col="tomato 4",
pch=19)

legend("right", c("Down", "Up"), fill=c("blue", "tomato 4"))

dev.off()

#####cumulative distribution plots

pdf("WallabySkin.CumDist.pdf")

par(mar = c(5, 4, 4, 4) + 0.3)  # Leave space for z axis

plot(log(ncs[,1],2), col="0000000", xlab= "mRNA Rank", main="Tag Count Distribution",
ylab="log2 CPM", ylim=range(log(ncs,2)), las=1)
```

```
for (i in 1:ncol(ncs)){
    lines(log(ncs[,i],2), pch=10, col=rainbow(ncol(ncs))[i], lty=3, lwd=2)
}
par(new=TRUE)
plot(nccf[,1], col="0000000", axes = FALSE, bty="n", xlab="", ylab="", ylim=c(0,100), las=1)
for (i in 1:ncol(ncs)){
        lines(nccf[,i], pch=10, col=rainbow(ncol(ncs))[i], lwd=2)
}
axis(side=4, at=pretty(c(1,100)))
mtext("% Cumulative Tag Counts", side=4, line=3)
legend("right", colnames(nc), fill=rainbow(ncol(nc)), cex=0.7, ncol=2)
abline(v=nrow(retained))
dev.off()
#####GO Term analysis
pdf("immunesystemprocess.pdf")
ggplot(dm[dm$count>5,], aes(x=label, y=value)) + geom_boxplot(aes(fill=variable),
position="dodge", outlier.shape=NA) + ylab("log2 (Normalized Tag Counts)") + xlab("Child
Terms of Immune System Process GO Term") + ylim(range(bp$stat)) + theme_bw() +
opts(axis.text.x=theme_text(angle=90))
```

Chapter 6. Transcriptome analysis of the immune genes in the developing tammar wallaby (*Macropus eugenii*) lung

Authors: Melanie J Edwards, Hardip R Patel, Lyn A Hinds, Elizabeth M Deane and Janine E Deakin

Current status of paper (circle as appropriate): Planned / **In Preparation** / Submitted / Under Revision / Accepted / Published

Date paper accepted for publication: Not applicable.

Name of journal: BMC Genomics

Extent to which research is your own: The research from this manuscript is my own, including sample collection and RNA extraction. Library construction and sequencing was carried out by the Beijing Genomics Institute, while read mapping and analysis was carried out with the assistance of the Genome Discovery Unit and RNA Biology Laboratory at the Australian National University.

Your contribution to writing the paper: I wrote the paper and received content and editorial comments from the other authors.

If paper not accepted, has the paper been rejected by any journals: Not applicable.

Comments: This manuscript examines the transcriptome of the developing tammar wallaby lung, with particular reference to immune genes. The results demonstrate the complexity of the developing marsupial immune system and suggest that specific innate immune genes may play a large role in the protection of newborn tammar wallabies while their immune tissues are still developing.

Transcriptome analysis of the immune genes in the developing tammar wallaby (*Macropus eugenii*) lung

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Key words

Marsupial; immune system; development; lung and transcriptome sequencing.

Abstract

Background: At birth, the marsupial lung is developmentally behind that of the newborn eutherian, and continues to develop after birth at a slower rate. The development of the immune system in the marsupial lung is particularly interesting as the largest part of development occurs postnatally, when young are exposed to an external environment where they must protect themselves from inhaling harmful environmental particulates. The intent of this research was to examine the immune system of the developing marsupial lung using the model species tammar wallaby (*Macropus eugenii*). We used RNA-sequencing to compare the transcriptome of the newborn wallaby, which is considered immune-incompetent when compared to older wallabies which are considered immune-competent. We used Gene Ontology (GO) classifications to identify which immune terms are represented by immune genes expressed in the lung and further examined specific immune genes which are highlighted in the literature of mammalian lung development. **Results:** We identified 429 immune related genes from a range of immune system process GO terms which suggests that the developing wallaby lung plays a role as an immune defense organ. We also identified five GO terms which were associated with immune function and significantly overrepresented in the immune-incompetent group compared to the immune-competent group, containing the genes *APOA1*, *APOA2*, *C2*, *C3*, *C4B*, *CFB* and *FCN3*. Surprisingly, there were no GO terms associated with immune function found to be overrepresented by the immune-competent group. We also examined the expression of specific immune genes which have been suggested to be expressed by marsupial lung and immune genes which are associated with the developing mammalian lung and found that several genes classified as antimicrobials, complement, cytokines and chemokines, and pattern recognition molecules had increased expression in the immune-incompetent group. **Conclusion:** The results demonstrate the complexity of the

developing marsupial immune system and suggest that specific innate immune genes may play a large role in the protection of newborn tammar wallabies while their immune tissues are still developing.

Background

The newborn marsupial is considered undeveloped at birth when compared to eutherian mammals, as the majority of a newborn marsupial's organs and physical structures are far from mature at birth [1]. In both clades, however, the postnatal lung is particularly undeveloped, and together, species representing the clades display a large range of developmental stages [2], depending on the growth and metabolic demands of the species [3]. The marsupial and eutherian lung developmental patterns correspond, yet the developmental stage of the newborn marsupial lung is generally behind that of the newborn eutherian and develops at a slower rate [2,4]. For example, the lung of the newborn marsupial gray short-tailed opossum (*Monodelphis domestica*) and the tammar wallaby (*Macropus eugenii*) are at the early terminal air sac stage of development and the first alveoli are found at 28 and 65 days post partum, respectively, while two newborn altricial eutherian species, the golden hamster (*Mesocricetus auratus*) and the musk shrew *(Suncus murinus*) are at the late stage of terminal air sac development, with the alveolar observed four days after birth in both species [4].

The development of the respiratory system has been intensely researched in marsupials, including research in relation to lung morphology and metabolic development [2,4], endothermy [5], mechanics [6], skin gas exchange [6,7] and immune defense [8]. At birth, most newborn marsupials have a poorly developed bronchial system and large terminal sacs, making respiration possible [2]. Thus, for most marsupial species at birth, the lung is structurally immature but functions as a gas exchange organ [9]. The largest part of marsupial lung development occurs postnatally [10], while the young resides

attached to the teat on the mother's exposed abdomen or in a partially covered to covered pouch. For example, in the tammar wallaby, during the first 70 days post partum there is tissue proliferation and septal development, terminal sac expansion and septal thinning; from 70 to 180 days, there is septal remodeling and microvascular maturation [11].

The development of the immune system in the marsupial lung is particularly interesting, as preterm eutherian young with immature lung development are extremely susceptible to respiratory infection [12,13], as a result of the immaturity of the innate immune components [14]. Yet the marsupial young with immature lung development are exposed to an external environment where they must protect themselves from harmful environmental particulates. Reviews on the innate immune system of the mammalian lung, including human and mouse, describe protection from leukocytes, epithelial cells and soluble constituents in airway fluid [15,16]. Alveolar macrophages are paramount as they account for 95 % of the airway leukocytes, with lymphocytes and neutrophils accounting for the other 5 % [15]. Airway epithelia play a role in the innate immune response in lung as ciliated epithelial cells move mucus and harmful particles out of lungs [16]. Toll like receptors (TLR), which recognize microbial products, are found on alveolar walls and the ciliated epithelium from the trachea to the terminal bronchi. Airway fluids may also contain compounds, including lysozyme, lactoferrin, immunoglobulin A (IgA), IgG, defensins, complement, surfactant and surfactant associated proteins which help to protect the lungs from invading particulates [15].

To date researchers have investigated only fragments of the marsupial lung immune defense mechanisms, which includes a poorly developed mucocilary blanket, pulmonary surfactant, alveolar macrophages, mucosal associated lymphoid tissue, Igs and antimicrobial peptides [8]. The newborn marsupial, however, lacks many structures

associated with the secretion and movement of immune compounds. For example, the newborn Virginia opossum (*Didelphis virginiana*) lack cilia and it is not until 60 days that the juvenile forms submucosal glands and goblet cells [17]. Goblet cells do not appear until 21 days partum and submucosal glands are only fully developed at 105 days postpartum in brushtail possums (*Trichosurus vulpecula*) [8]. Edwards *et al.* [18] reviewed the literature examining the structures of the developing marsupial respiratory tract which could be involved in protecting the developing young; the literature is sparse and includes specific compounds such as the pattern recognition molecules, cluster of differentiation 14 (CD14), toll-like receptor 4 (TLR4); the surfactant proteins SP-A and SP-B, an antioxidant enzyme peroxiredoxin 1 (PRDX1) and the antimicrobial peptides, cathelicidins [19-21] all which have been found or their genes have been found to be expressed in the lung of young developing tammar wallabies.

The intent of this research is to further examine the immune system of the developing marsupial lung using the model species tammar wallaby. We use RNA sequencing to compare the transcriptome of the newborn wallaby, which is considered immune-incompetent when compared to older wallabies which are considered immune-competent, as histology studies imply functional adaptive immune competence by 35 days postpartum [22]. Specifically we use Gene Ontology (GO) classifications to identify which immune terms are represented by immune genes expressed in the lung and further examine specific immune genes which are highlighted in the literature of mammalian lung development.

Results and discussion

Marsupials are well recognized for their short gestation period, and at birth, their reduced developmental stage and lack of developed immune tissue and cells that are important for mounting an adaptive immune response. However, the mechanisms of

immune protection are not well understood. The lung is typically continually exposed to pathogens, particulates and allergens found in inhaled air. Thus, to ensure normal lung function, an immune defense system is critical for defense against infection [23].

As there are many facets to the immune defense system of the lung, here we have examined the transcriptome of the developing tammar wallaby lung with specific reference to genes involved in immune system processes. First we examined the distribution of GO term pathways associated with the GO term immune system process. Second, we used enrichment analysis to determine if any GO terms are significantly overrepresented by differentially expressed genes in the immune-incompetent and immune-competent groups. Third, we examined the expression of specific immune genes which have been suggested to be expressed by marsupial lung and immune genes which are associated with the developing mammalian lung.

Read mapping statistics

This is the first study to examine the transcriptome of the developing lung of a marsupial using RNA-sequencing. Approximately 24 million reads were obtained from transcriptome sequencing (92 % of which were considered high quality reads; Additional file 1). Of the high quality reads, 31 % were mapped to the tammar wallaby genome by TopHat (Additional file 1). The reads were further filtered to include only those that made up the top 98 % of genes with the highest cumulative tag count (9 587 tammar wallaby genes; Additional 2). Transcript length and FPKM (fragments per kilobase of exon per million fragments mapped) were examined to show that there was no bias between the length of genes and number reads (Additional file 3). Additional file 4 contains the processed data while the raw data is available in Additional file 5.

Distribution of immune gene expression across different immune system process terms

From the 9 587 genes identified, 429 genes were associated with the GO term immune system process. To examine which immune system process terms were represented by genes expressed in the developing tammar wallaby lung, the distribution of the expression of genes for each term was examined and is shown in Figure 1. All samples showed similar expression of $\log_2$ normalized tag counts which suggests that the lung of the developing wallaby has immunological activity across a range of immune system process child terms. The immune system process child terms provides a comprehensive list of immune terms that lays the foundation for further and more specific immune gene analysis.

In three immune system process child terms; immune response, tolerance induction and T cell selection, the immune-incompetent wallabies contained genes with a tendency for reduced expression (Figure 1). Although not significant, the trend towards increased expression of the genes in the immune-competent wallabies is most likely the result of the development of immune tissue and competence, as the wallaby is considered to be immune-competent at 35 days post partum, which is well before the age of the individuals sampled in the immune-competent group.

Differential expression of genes in the immune-incompetent and immune-competent tammar wallaby lung

We identified 9 587 genes in the tammar wallaby developing lung. Of these, 663 genes were significantly upregulated in the immune-incompetent wallabies compared to the immune-competent tammar wallabies (P-value < 0.05; false discovery rate (FDR) value < 0.05), while 880 genes were significantly upregulated (P-value < 0.05; FDR value < 0.05) in the immune-competent compared to the immune-incompetent tammar

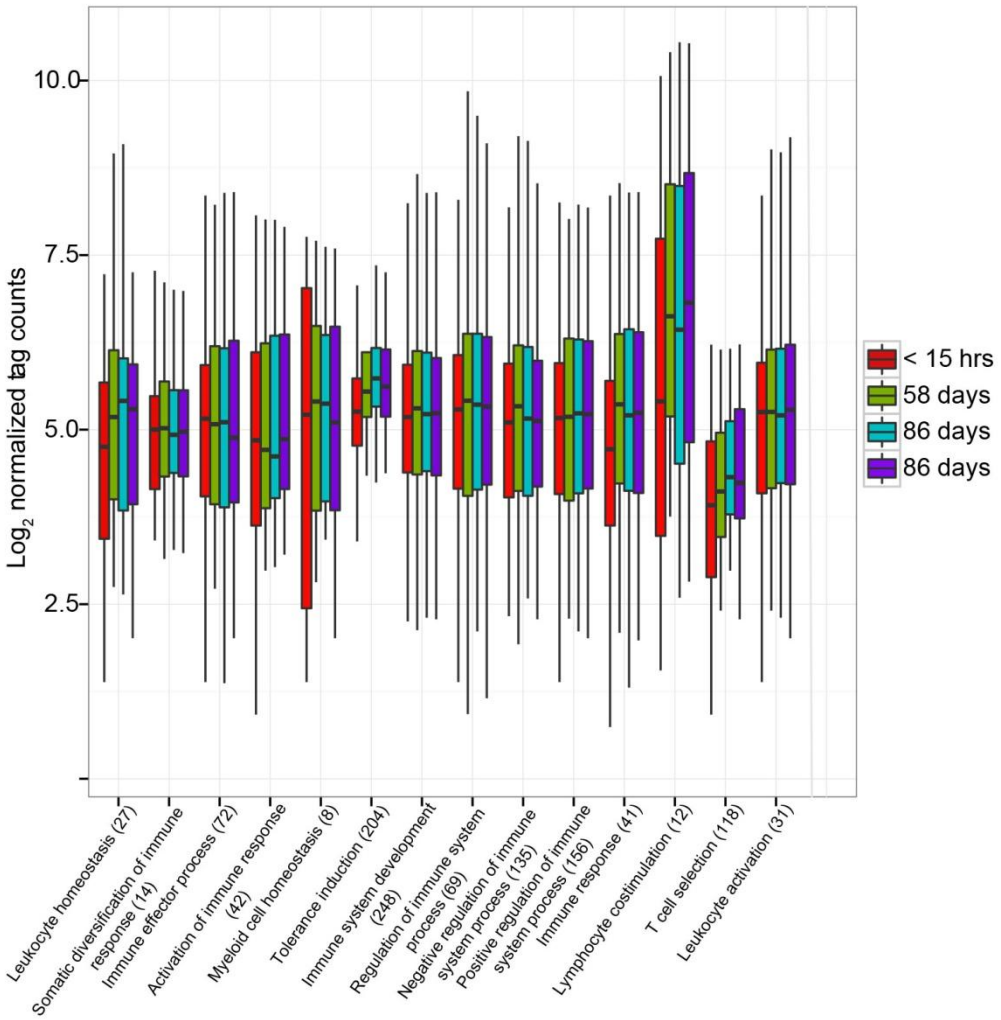

Figure 1. Immune system process child terms and the expression of genes involved in each term for each tammar wallaby lung sample. Terms which showed expression of less than six genes were removed. The upper, middle and lower ends of the box represent the upper quartile, median and lower quartile, while the upper and lower ends of the vertical lines represent the largest and smallest $\log_2$ tag count of the genes found within the immune system process child terms, respectively. The number of genes in each immune system process child term identified in the transcriptome is listed in brackets. Outliers (any data points above the 3rd quarter + 1.5 times the interquartile range and below the 1st quarter - 1.5 times the interquartile range) are not shown.

wallabies (Additional file 6). A pairwise comparison of all samples was conducted to determine the relationship of variation between all samples (Additional file 7). There was very little variation (with 95 to 96 % of the variation showing a relationship) between the samples in the immune-competent group (even with one sample 28 days younger than the remaining two). There was more variation (with 83 to 84 % of the variation showing a relationship) between the two groups.

By examining the differentially expressed genes using GO term enrichment analysis we found 125 biological process terms, 19 cellular component terms and 21 molecular function terms to be significantly overrepresented in the immune-incompetent groups compared to the immune-competent group (Additional material 4). There were five terms associated with immune function including complement activation; cytokine secretion involved in immune response; regulation of cytokine secretion involved in immune response; negative regulation of cytokine secretion; and negative regulation of cytokine secretion involved in immune response (Table 1). Figure 2 shows the differentially expressed genes involved in the GO terms associated with immune function and their FPKM, including genes identified in the tammar wallaby genome, apolipoprotein A1, (*APOA1*), apolipoprotein A2 (*APOA2*), complement component 2 (*C2*), complement component 3 (*C3*), complement component 4b (*C4B*), complement factor B (*CFB*) and ficolin 3 (*FCN3*). Surprisingly, GO term enrichment analysis did not identify significantly overrepresented GO terms in the immune-competent tammar wallaby lung.

Three genes, *APOA1*, *C3* and *CFB*, with increased expression in the lung of the immune-incompetent individuals, were also identified to have increased expression in the skin of immune-incompetent compared to immune-competent tammar wallabies (Edwards *et al.* in preparation), while there were four genes, *APOA2*, *C2*, *C4B* and

Table 1. Gene Ontology (GO) immune terms which were significantly overrepresented by genes up regulated in the immune-incompetent wallaby lung compared to the immune-competent wallaby lung.

GO term		Relative GO depth	Genes differentially expressed (no.)	P-value
Parent term	Child term			
Biological process	Complement activation	1	5	0.01
	Cytokine secretion involved in immune response	1	2	0.01
	Regulation of cytokine secretion involved in immune response	2	2	0.01
	Negative regulation of cytokine secretion	1	2	0.04
	Negative regulation of cytokine secretion involved in immune response	2	2	<0.01

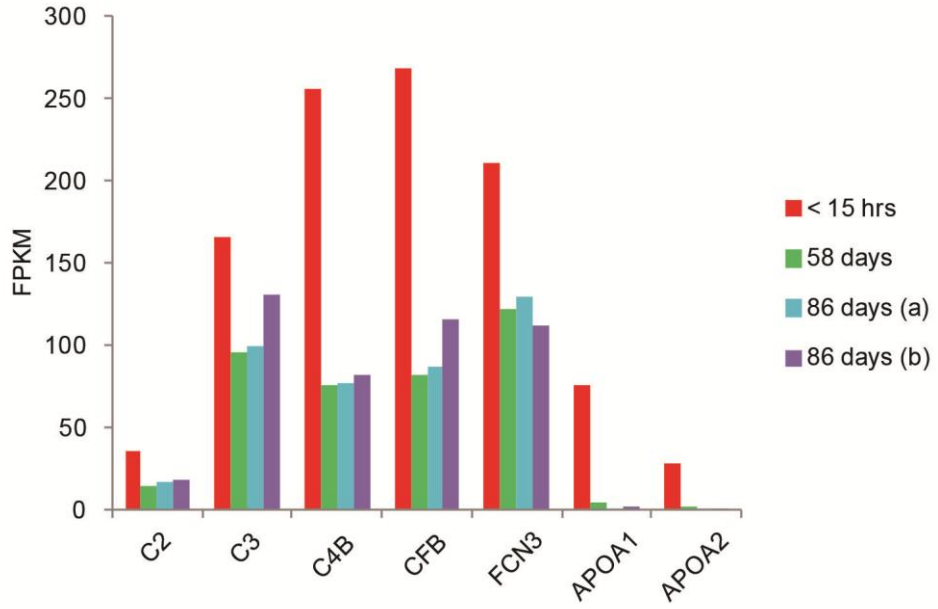

Figure 2. The expression of genes (fragments per kilobase of exon per million fragments mapped (FPKM)) which were identified in the Gene Ontology (GO) immune system process child terms, that were significantly overrepresented in the GO term enrichment analysis in the immune-incompetent group.

FCN3 that were identified in the lung of immune-incompetent compared to immune-competent tammar wallabies, not identified in the skin. C4b, C2 and FCN3 have significant immune roles in the complement system and will be discussed further. C2 is a protease which cleaves C3/C5 and is part of the classical and lectin pathway convertases, while C4b is a component of C4 which is also part of the classical and lectin pathway convertases [24]. FCN3 is also part of the complement cascade and acts as a pattern recognition molecule which recognizes carbohydrate patterns and initiates the lectin pathway [24].

The results from the skin transcriptome, identifying increased expression of *C3* and *CFB* provided the first indication that the complement system develops before, or very shortly after birth in marsupials (Edwards *et al.* in preparation). The increased expression of the complement components in immune-incompetent tammar wallabies is discussed in detail in Edwards *et al.* (in preparation); thus, here we will not repeat the discussion but reiterate that the increase in expression may represent part of the innate immune system which develops early in marsupials to compensate for the delayed development of the adaptive immune system. It is also important for us to reiterate that complement has been found to play a role in the development of organs that do not have an immune role [25]. For example, complement components have been found to play a role in the regeneration of limbs in the Mexican salamander (*Ambystoma mexicanum*) and the Eastern newt (*Notophthalmus viridescens*) [26] and in the liver of mice [27]. A study on the African clawed frog (*Xenopus laevis*) also found embryological expression of several components of the complement system in organs not always involved in immunity, however, their findings require further functional studies [28].

It is evident that further work is required to determine the role of complement in development versus immunity, especially in marsupials. Although further research to

determine the functional significance of complement was beyond the scope of this study, the detection of complement in the immune-incompetent individuals is interesting and provides the basis for further work to be carried out on these compounds. Experiments which directly compare the detection of complement between specific tissues from species from the marsupial and eutherian clades is warranted to determine if the complement system is developing earlier in marsupials compared to eutherians to compensate for the undeveloped adaptive immune system.

We also examined important innate immune genes that are annotated in the tammar wallaby genome which have previously been researched in the developing mammalian lung. The list of genes was mostly generated from genes identified in the developing ovine lung, as research has focused on the ovine lung, due to its similarity to the perinatal human lung [29]. The genes were grouped into antimicrobials, complement, cytokines and chemokines, and pattern recognition associated genes. The log fold change of expression for each gene is reported in Table 2. *C3*, *CFB*, an isoform of lactoferrin (*LTF*) and monocyte chemoattractant protein 1 (*MCP1/CCL2*), SP-A (*SFTPA*) and *TLR4* were all found to have increased expression in the immune-incompetent group. Complement factor H (*CFH*) and an isoform of *LYZ* were the only genes to show increased expression in the immune-competent group, while interleukin 6 (*IL6*), an isoform of *LTF*, two isoforms of *MCP1/CCL2*, transforming growth factor beta 1 (*TGFB1*), *TLR2* and *TLR3* were not differentially expressed. Dual oxidase 1 (*duox1*), dual oxidase 2 (*duox2*), interferon beta 1 (*IFNB1*), interferon gamma (*IFNG*), *IL10*, an isoform of *LYZ*, serum amyloid A (*SAA*), *TLR5* and tumor necrosis factor alpha (*TNFA*) were trimmed out of the analysis as they contained too few tag counts.

In an attempt to further understand what is contributing to the protection of immune-incompetent new born marsupials, here we focus on the genes with increased expression

Table 2. Upregulation, shown by log fold change, of specific immune genes found in typical mammalian lung in the lung of immune-incompetent (less than 15 hrs postpartum) and immune-competent wallabies (58 to 86 days postpartum).

Immune role	Gene symbol	Gene ID	Group up regulated	Log fold change	P-value	FDR
Antimicrobial	*LTF*	ENSMEUG00000009372	-	1.23	0.01	0.07
	LTF	ENSMEUG00000015994	Incompetent	1.37	<0.01	<0.01
	LYZ	ENSMEUG00000011846	Competent	2.71	<0.01	<0.01
Pattern recognition	*SFTPA*	ENSMEUG00000005512	Incompetent	1.60	<0.01	<0.01
	TLR4	ENSMEUG00000009461	Incompetent	0.91	<0.01	0.05
	TLR3	ENSMEUG00000015021	-	0.90	0.07	0.25
	TLR2	ENSMEUG00000008042	-	0.19	0.87	1
Cytokines and chemokines	*MCP1/ CCL2*	ENSMEUG00000000697	-	1.11	0.03	0.13
	MCP1/ CCL2	ENSMEUG00000005275	Incompetent	1.87	<0.01	<0.01
	MCP1/ CCL2	ENSMEUG00000009109	-	0.41	0.25	0.57
	TGFB1	ENSMEUG00000001194	-	0.32	0.10	0.33
	IL6	ENSMEUG00000012927	-	1.44	0.08	0.28
Complement	*C3*	ENSMEUG00000006258	Incompetent	0.60	<0.01	0.02
	CFB	ENSMEUG00000006029	Incompetent	1.49	<0.01	<0.01
	CFH	ENSMEUG00000002177	Competent	3.10	<0.01	<0.01

in the immune-incompetent group, an isoform of *LTF* and *MCP1/CCL2*, *SFTPA* and *TLR4*. LTF binds iron and is found on mucosal surfaces, in polymorphonuclear leukocytes, and in body fluids such as milk and saliva. LTF has antimicrobial activity, both by sequestering iron to make it unavailable to pathogens and as a direct bacteriocide [30]. MCP1/CCL2 also plays an important role in innate immunity as a chemoattractant cytokine which recruits monocytes, neutrophils and lymphocytes and induces chemotaxis. In particular, MCP1/CCL2 plays an essential role in regulating the migration and infiltration of monocytes and macrophages [31]. While transferrin, another compound of the family that contains lactoferrin has been examined in marsupials [32-34], LTF and MCP1/CCL2 have not yet been explored, but appear to be prime candidates for further examination of immune protection in developing marsupial lung.

Pattern recognition receptors (PPR) can include secretory PPRs (e.g. surfactant proteins) and signaling PPRs (e.g. TLRs) which are an essential part of the innate immune system for recognizing and processing pathogens [35]. SP-A is a member of the collectin family of C-type lectins which bind to the surface of microorganism promoting phagocytosis by innate immune cells such as macrophages and neutrophils. SP-A also poses direct bactericidal activity against bacteria and fungi [36]. In marsupials, SP-A has been detected in the lamellar bodies and cytoplasm of the alveolar type II cells and air spaces in newborn tammar wallabies using immunohistochemistry; however, Miller [19] found that the distribution of SP-A remained the same throughout development. *SFTPA* and *TLR4*, were also found to have increased expression in the immune-incompetent wallabies. Our results correspond to those of Daly *et al.* [21], which showed increased expression of *TLR4* in tammar wallaby young three to four weeks old, which peaked again at 70 days, a collection point just between that of the

samples used in the immune-competent group in this study of 56 days and 86 days post partum.

The increase in expression of these pattern recognition molecules may be important in newborn mammals, as *TLR4* increases several fold during the prenatal development and after birth in mice [37] and is also developmentally regulated in baboons [12]. In preterm baboons, reduced levels of SP-A is associated with incompetent host defense [38]. Although, at birth the marsupial lung is at a stage of development that corresponds to a preterm eutherian lung, which usually has reduced innate immune function, tammar wallabies have increased expression of innate immune genes compared to older tammar wallabies which have increased immune competence. These results suggest that marsupials may develop components of their innate immune system faster than eutherian mammals in order to compensate for their reduced maturation of immune tissue at this early stage of development, and to compensate for the different environment they are developing in—with limited protection *ex utero* as opposed to a protected environment *in utero* of the eutherian mammals.

Tammar wallaby cathelicidin (MaeuCath) genes were also examined for differential expression between the immune-incompetent and immune-competent groups (Additional file 4).Tags were mapped to each MaeuCath gene, however, there were too few genes mapping to *MaeuCath3* so it was not retained for further analysis. Although tags mapped to *MaeuCath4* , *MaeuCath5*, *MaeuCath6* and *MaeuCath7*, differential expression between the groups was not observed. The reads mapping to *MaeuCath1* and *MaeuCath2* could not be differentiated but showed significantly higher expression in the immune-incompetent group compared to the immune-competent group (log fold change = 1.9, P-value and FDR value < 0.01), while *MaeuCath8* showed significantly higher expression in the immune-competent group compared to the immune-

incompetent group (log fold change = 2.2, P-value and FDR value < 0.01). The expression of *MaeuCath2* has not been examined in developing tammar wallaby lung, however, the expression of *MaeuCath1* and *MaeuCath8* have been identified at five and one day/s postpartum, respectively [20,39]. Tammar wallaby cathelicidins have also previously displayed antimicrobial activity against a range of microorganisms [40,41].

Previous research has examined mucins in the tammar wallaby and suggests that mucins may play a large role in the protection of developing young [42]. Although tags mapped to three of the scaffolds that contain exons for mucin 4 (*MUC4*), the tag counts were too few and not retained for differential analysis. *MUC5AC* and *MUC5B* are common lung mucins in mammals, thus it was surprising that no tags were mapped to scaffolds containing mucin genes. The deficiency of tags mapped to mucin gene locations suggests that the developing tammar wallaby is also lacking another important component of the immune system at birth and during its time of development in the pouch. The results correspond to those examining the development of respiratory structures that are associated with mucin in the Virginia opossum and brushtail possum, whereby cilia is absent and it is not until well into pouch life that the juvenile forms submucosal glands and goblet cells [8,17].

Conclusions

We identified 429 genes that were associated with the GO immune system process term and expressed by the developing tammar wallaby lung, which suggests that the developing lung plays an important role as an immune defense organ in the tammar wallaby pouch young. In particular, GO term enrichment analysis identified *C2*, *C3*, *C4B* and *CFB* to have increased expression in the immune-incompetent tammar wallabies, while analysis of genes routinely associated with innate immunity in the developing mammalian lung also identified a range of innate immune genes with

increased expression in immune-incompetent tammar wallabies. Interestingly, preterm eutherian young generally have reduced expression of these innate immune genes, while tammar wallabies at a developmental stage resembling preterm eutherian mammals have increased expression of these innate genes when compared to older wallabies.

We propose that although marsupial lung development occurs at a slower rate than eutherian lung development, the expression of innate immune compounds of the lung develop earlier; most likely to compensate for the reduced and slow development of immune tissue and the difference in the environmental conditions in which development is taking place.

Methods

Animals, sample collection and RNA extraction

Whole lung (including bronchi to peripheral lung tissue) was dissected from six tammar wallaby pouch young, aged less than 15 hrs old (n = 3), 58 days old (n = 1) and 86 days old (n = 2). The tammar wallabies were maintained at the Commonwealth Scientific and Industrial Research Organisation (CSIRO), Sustainable Ecosystems, Gungahlin, Canberra, Australia and samples were collected by methods accepted by the Australian National University's Animal Experimentation Ethics Committee (Register no. R.CG.15.10) and the CSIRO Sustainable Ecosystems Animal Ethics Committee (Application no. 10-02).

Lung tissue was frozen at -80 °C or placed into RNAlater® solution (Ambion) for no less than 24 hrs and stored at -80 °C. The GenElute™ Mammalian Total RNA Miniprep Kit (Sigma-Aldrich) was used to extract total RNA from frozen whole lung homogenized in lysis buffer and 2-mercaptoethanol. Contaminating DNA was removed using an on-column DNase I digestion set (Sigma-Aldrich).

Library construction and sequencing

The RNA samples from the pouch young less than 15 hrs old were pooled as only a small amount of RNA was obtained from each lung. Each RNA sample was then checked for integrity and concentration on a 2100 Bioanalyser (Agilent Technologies) using a Nano6000 chip. The RNA was stabilized and stored in an RNAstable tube (Biometrica) and sequenced at the Beijing Genomics Institute, Tai Po, Hong Kong. cDNA libraries were generated using Illumina's mRNA Sample Preparation Guide (Cat # RS-930-1001) and the library products were sequenced using the Illumina® HiSeq™ 2000 Sequencing System.

Data preprocessing: read mapping, tag counting and normalization

Read quality assessment was carried out using Trimmomatic [43] (version 0.25). Reads with an end nucleotide quality value less than three or reads with the mean quality value of the last four nucleotides less than 20 were deemed low quality and filtered out for further analysis. The remaining reads were assessed using FastQC [44] and considered acceptable for further analysis.

Reads were then aligned to the tammar wallaby genome sequence (Meug_1.0) downloaded from Ensembl [45] (version 70) using TopHat (version 2.0.6) with default parameters. The alignments were assembled and identified using Cufflinks [46] (version 2.0.2) and a non-redundant set of known and novel transcripts was produced by Cuffmerge [46]. Read alignments were overlapped with gene annotations for the tammar wallaby downloaded from Ensembl using intersectBed [44] (BedTools version 2.17.0) and custom Perl scripts were used to count sequenced fragment counts for each gene (Additional file 8). Although TopHat identified novel transcript isoforms of annotated genes and novel gene models which overlapped with annotated genes, further analysis of novel isoforms and gene models was beyond the scope of this study.

The effective library size was determined by summing the mapped tag counts for each sample. Read counts per gene were first normalized for library size and transcript length to obtain counts per 1000 nucleotides per million mapped tags [47] and further normalized for total RNA output using the trimmed mean of M-values method [48]. Transcript length was compared to normalized FPKM to determine length versus expression bias.

Expression of immune system process terms

All genes associated with the child terms of the immune system process GO term (GO:0002376) were identified from Ensembl (version 70). The expression patterns for the genes associated with these GO terms were quantified using a Perl script (Additional file 8) implemented in the edgeR package [49] (version 2.6.12). GO terms showing expression of less than six genes were excluded from the analysis.

Differential expression analysis

We conducted a pairwise comparison of all samples to determine the relatedness of variation between samples. Differential expression analysis for genes between immune-incompetent (less than 15 hrs old) and immune-competent (greater than 58 days old) individuals was then performed using the edgeR package (version 2.6.12) according to the author's instructions [49]. All genes that contributed to the top 98 % of the cumulative counts in any one sample were selected for further analysis. As some genes produced tag counts of zero, these values were adjusted to the mean value of the lowest normalized count and zero. Dispersion estimates were obtained using the quantile adjusted conditional maximum likelihood method for each group of samples and differential expression tests were computed for each gene. FDR was determined for multiple-testing using the Benjamini-Hochberg test. Genes were defined as differentially expressed if the P-value and FDR for differential expression was less than

0.05. To detect significantly overrepresented GO terms, GO term enrichment analysis was performed in g:Profiler [50] and was conducted on the list of differentially expressed genes (up and down regulated) that were sorted based on their log fold change.

We also examined the expression of genes suggested to be expressed in developing wallabies. Genes annotated in the genome include lysozyme orthologues (*LYZ*) [ENSMEUG00000011846 and ENSMEUG00000015902] [42], while genes not annotated in the genome, include a range of mucin genes (*MUC1*, *MUC2*, *MUC3A*, *MUC4*, *MUC5AC*, *MUC5B*, *MUC6*, *MUC13*, *MCAM*, *OVGP1*) [42] and cathelicidin genes [RefSEQ: gi_157042603; gi_157042605; gi_157042607; gi_157042609; gi_157042610; gi_157042612; gi_157042614 and tammar wallaby cathelicidin 8 (*MaeuCath8*) [39]]. For genes not annotated in the Ensembl (version 70) gene build [45], we identified their locations using megablast and subsequently analyzed their tag counts or we counted tags for the scaffolds that are known to harbor these genes [42].

To identify and evaluate the expression of genes that are routinely expressed in the mammalian lung, we compiled a list of genes (Additional file 4) from the literature which has previously examined immune genes in the mammalian lung (Martin and Frevert 2005; Kawashima *et al.* 2006; Sow *et al.* 2009). The list was used to determine which of these genes were found to be expressed in the immune-incompetent and immune-competent groups.

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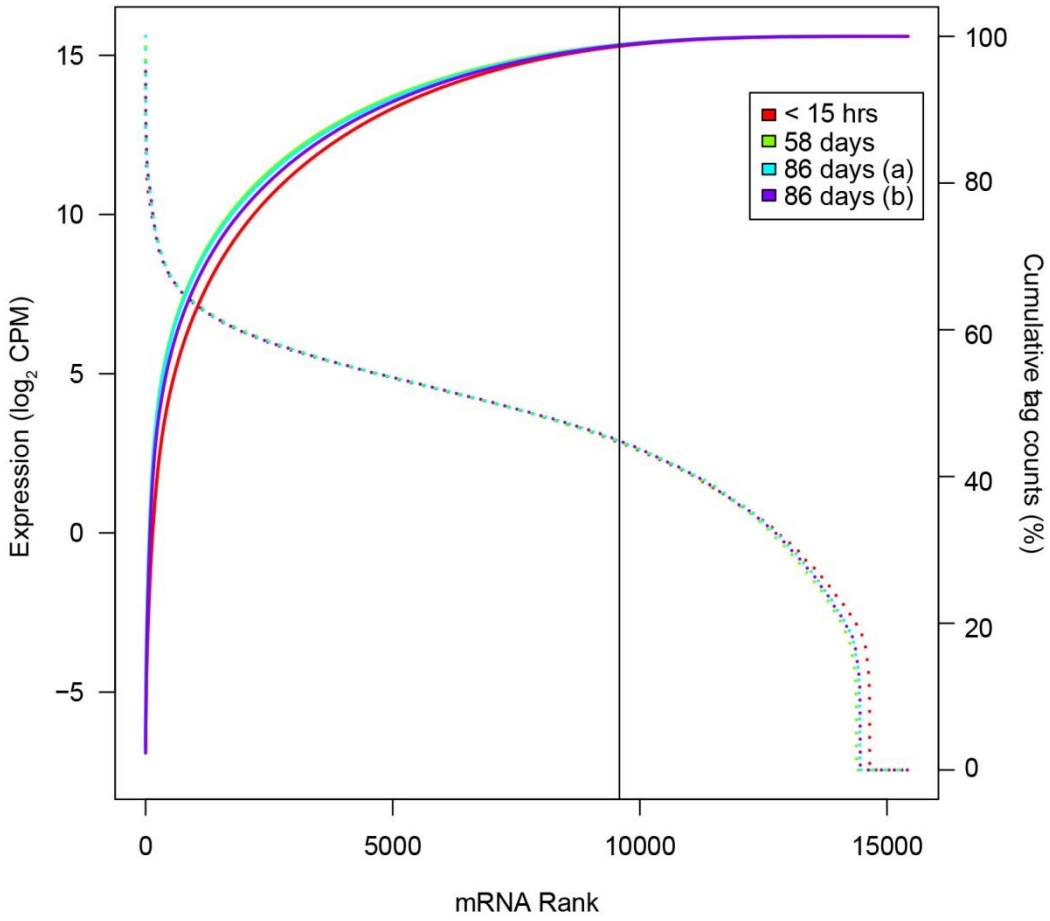
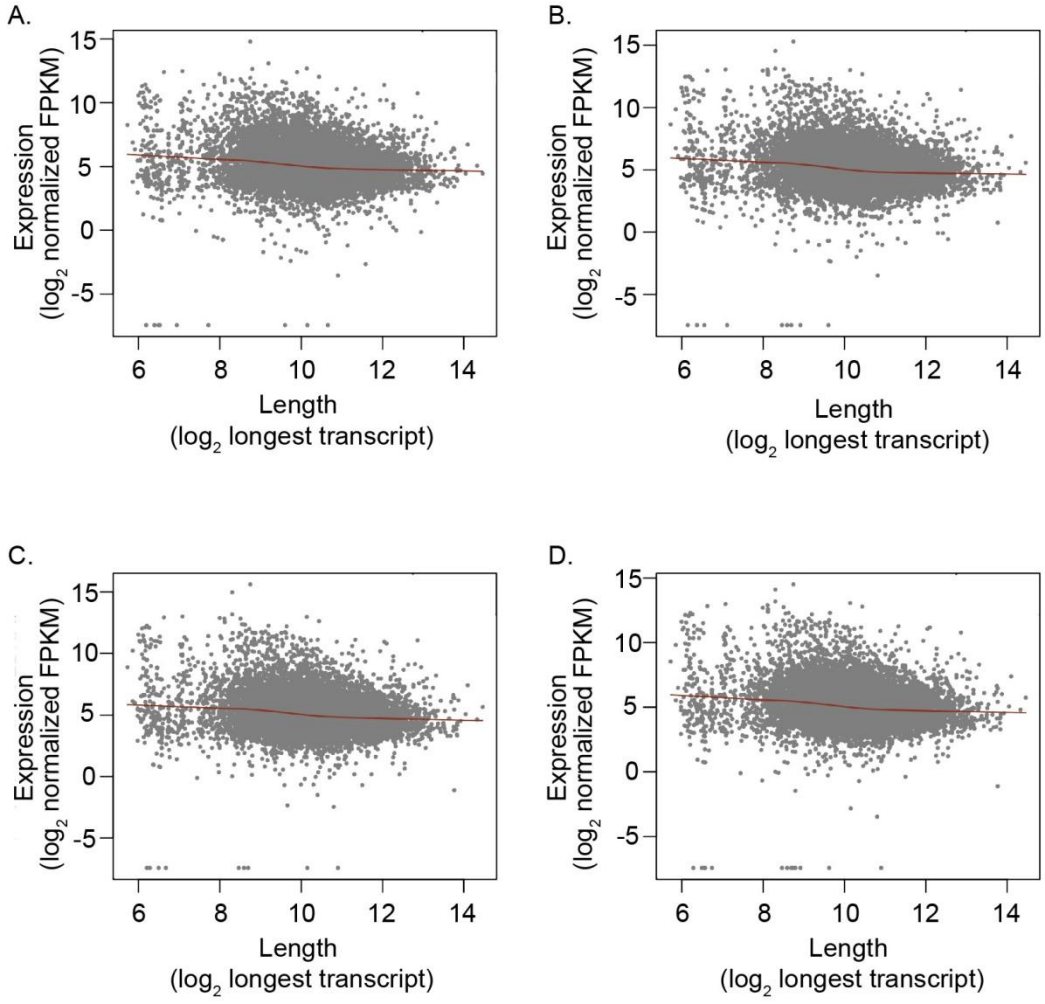
Additional File 1. Number of tag counts obtained from transcriptome sequencing, before and after quality value (Q.V.) filtering and number of tag counts successfully mapped to the tammar wallaby genome for tammar wallaby lung using TopHat.

Sample	Tag counts (raw)	Tag counts low Q.V. filter	Tag counts low Q.V. filter (%)	Tag counts low Q.V. filter mapped	Tag counts low Q.V. filter mapped (%)
< 15 hrs	22374809	20513718	91.68	6344510	30.93
59 days	23685629	21845905	92.23	6968583	31.90
86 days (a)	23918218	22090425	92.36	6892007	31.20
86 days (b)	24370263	21959014	90.11	6740448	30.70
Mean	23587229.75	21602265.50	91.59	6736387.00	31.18
Standard deviation	856804.29	732544.66	1.04	277918.84	0.52



Additional file 2. The dotted lines represent the number of tag counts (log_2 counts per million (CPM)) for each gene expressed in the tammar wallaby lung for each sample, while the solid lines represent the cumulative proportion of tag counts for each gene. The black line represents the cut-off of genes which include genes containing 98 % of the cumulative tag counts.

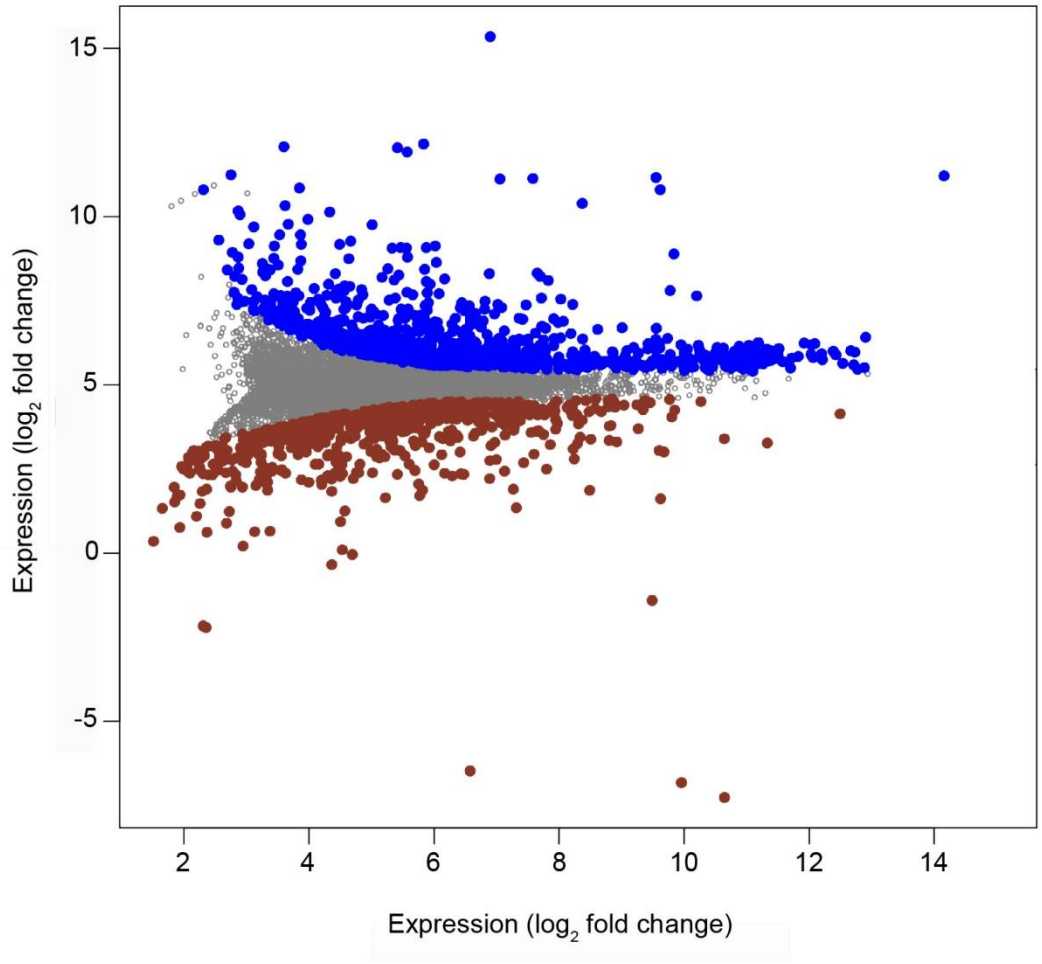


Additional file 3. The correlation between the length of each transcript and FPKM (fragments per kilobase of exon per million fragments mapped) of transcripts expressed in tammar wallaby skin (A. less than 15 hr old; B. 58 day old; C. 86 day old (a) and D; 86 day old tammar wallabies).

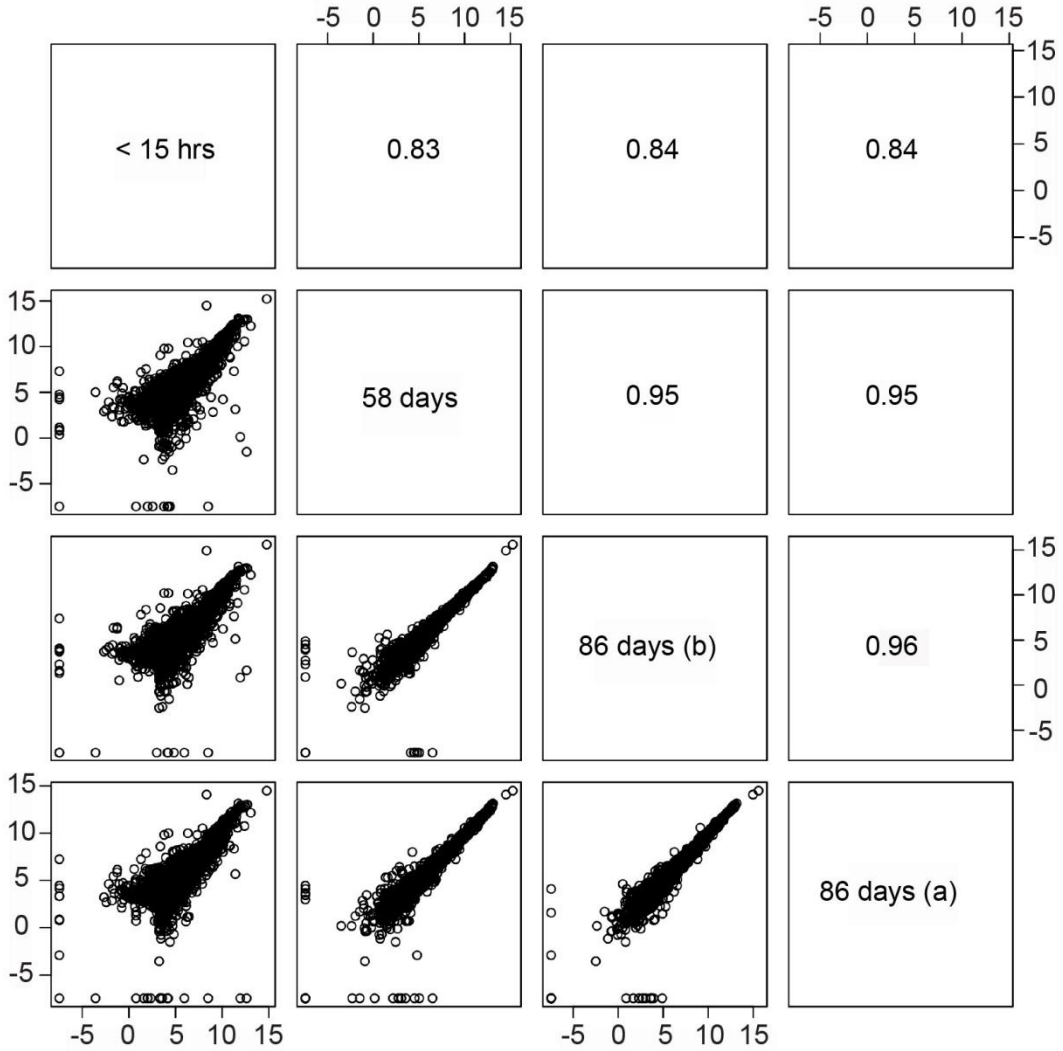
Additional file 4. The spreadsheet containing data analyzed for the tammar wallaby developing lung transcriptome. The spreadsheet is available on the disk accompanying the thesis.

Sheet name	Description
RawCounts	Raw counts
FPK	Raw counts/base pair length
FPKM	FPKM/library size
NormFactors	Normalization factors
NormFPKM	FPKM/normalization factors
Retained	Genes making up 98 % of highest tag counts
Diffexp	Differential expression analysis results
NegLFC	Genes showing negative fold change between the immune-incompetent and immune-competent group
NegGOEnr	GO terms showing significant enrichment in the immune-incompetent group
PosLFC	Genes showing positive fold change between the immune-incompetent and immune-competent group
PosGOEnr	GO terms showing significant enrichment in the immune-competent group
Cathelicidin	Raw counts, NormFPKM, retained and Diffexp for cathelicidin analysis
Specific lung genes	A list of genes from the literature which have previously been examined in the mammalian lung

Additional file 5. The raw read counts for the lung transcriptome analyses are available on the disk accompanying the thesis.



Additional file 6. Differential gene expression in immune-incompetent and immune-competent tammar wallaby lung. Up regulated genes in the immune-incompetent compared to the immune-competent group are shown in blue, and down regulated genes are shown in red. The grey points represent genes which are not differentially expressed between the immune-incompetent and immune-competent groups.



Additional file 7. A pairwise comparison of genes expressed by tammar wallaby lung (graphs located to the bottom and left of sample names) and correlation coefficients between each sample (boxes located to the top and right of sample names).

Additional file 8. Perl script used to conduct tammar wallaby lung transcriptome analysis.

```
library(edgeR)
options(java.parameters = "-Xmx4000m")
library(xlsx)
source("Downloads/gProfileR/R/gProfileR.R")

sortcol <- function(x){sort(x,decreasing=TRUE)}
cumfreqcol <- function(x){cumsum((x/sum(x))*100)}
seltop <- function (x){slist<-cumsum(x[order(x,decreasing=TRUE)])/sum(x)<=0.98; bottom<-labels(slist)[slist==FALSE]; x[bottom]<-NA; return(x)}
panel.cor <- function(x, y){
   usr <- par("usr"); on.exit(par(usr))
   par(usr = c(0, 1, 0, 1))
   r <- abs(cor(x, y))
   txt <- format(c(r, 0.123456789), digits=2)[1]
   text(0.5, 0.5, txt)
}

a <- read.table("lung.FPKTagCounts.txt", header=TRUE, row.names=1)
rawcounts <- read.table("lung.rawTagCounts.txt", header=TRUE, row.names=1)
l <- read.table("lung.EffectiveGeneLength.txt", header=TRUE, row.names=1)
acpm <- cpm(a)
g <- c(1,2,2,2)
y <- DGEList(counts=acpm,group=g)
y <- calcNormFactors(y)
nc <- cpm(y, normalized.lib.sizes=TRUE)
nc[nc==0] = (range(nc[nc>0])[1]) / 2 ###adjust zero values to a value half-way between zero and the minimum value
```

```
ncs <- as.matrix(apply(nc,2,sortcol)) ####each column sorted

nccf <- as.matrix(apply(ncs,2,cumfreqcol)) ####cumulative proportion for each column

filt <- apply(nc, 2, seltop) ####select top 98% in at least one sample

retained <- filt[rowSums(filt,na.rm=TRUE)>0,]

retained <- data.frame(nc[rownames(retained),])

rl <- cbind(retained, l[rownames(retained),]) #####attach transcript lengths

y <- y[rownames(retained),] ###change DGE object for only top genes

y <- estimateCommonDisp(y)

y <- estimateTagwiseDisp(y)

et <- exactTest(y)

tt <- topTags(et,n=100000)

imp <- tt$table[tt$table$PValue <= 0.05 & tt$table$FDR <= 0.05,]

nimp <- tt$table[tt$table$PValue > 0.05 | tt$table$FDR > 0.05,]

impneg <- imp[imp$logFC < 0,]

impneg <- impneg[order(impneg$logFC),]

negenr <-
gprofileradv(organism="meugenii",ordered_query=1,significant=1,rownames(impneg),backgro
und=rownames(y$pseudo.alt))

imppos <- imp[imp$logFC > 0,]

imppos <- imppos[order(-imppos$logFC),]

posenr <-
gprofileradv(organism="meugenii",ordered_query=1,significant=1,rownames(imppos),backgro
und=rownames(y$pseudo.alt))


#####for a go term gene expression in four samples

go <- read.table("immunesystemprocess.goVSgene.txt", header=FALSE, sep="\t")

colnames(go) <- c("geneid", "goacc", "goname")

goexp <- merge(go, retained, by.x=1, by.y=0, all=FALSE)

dm <- melt(goexp, id.vars = c('geneid', 'goacc'), measure.vars=colnames(retained))

gocounts <- ddply(dm, c("goacc"), summarise, count=length(goacc)/ncol(retained))

rownames(gocounts) <- gocounts$goacc
```

```
gocounts$goacc <- NULL

dm$count<-gocounts[dm$goacc,]

dm$label = paste(dm$goacc, " (", dm$count,")", sep="")

dm$value <- log(dm$value,2)

bp <- boxplot(dm[dm$count>5,]$value ~
(dm[dm$count>5,]$label+dm[dm$count>5,]$variable))


#####write excel tables

write.xlsx(rawcounts, file="WallabyTranscriptome.xlsx",sheetName="RawCounts")

write.xlsx(a, file="WallabyTranscriptome.xlsx",sheetName="FPK", append=TRUE)

write.xlsx(acpm, file="WallabyTranscriptome.xlsx",sheetName="FPKM", append=TRUE)

write.xlsx(y$samples$norm.factors,
file="WallabyTranscriptome.xlsx",sheetName="NormFactors", append=TRUE)

write.xlsx(nc, file="WallabyTranscriptome.xlsx",sheetName="NormFPKM", append=TRUE)

write.xlsx(retained,
file="WallabyTranscriptomeAnalysis/WallabyTranscriptome.xlsx",sheetName="Retained",
append=TRUE)

write.xlsx(tt$table,
file="WallabyTranscriptomeAnalysis/WallabyTranscriptome.xlsx",sheetName="DiffExp",
append=TRUE)

write.xlsx(impneg,
file="WallabyTranscriptomeAnalysis/WallabyTranscriptome.xlsx",sheetName="NegLFC",
append=TRUE)

write.xlsx(negenr,
file="WallabyTranscriptomeAnalysis/WallabyTranscriptome.xlsx",sheetName="NegGOEnr",
append=TRUE)

write.xlsx(imppos,
file="WallabyTranscriptomeAnalysis/WallabyTranscriptome.xlsx",sheetName="PosLFC",
append=TRUE)

write.xlsx(posenr,
file="WallabyTranscriptomeAnalysis/WallabyTranscriptome.xlsx",sheetName="PosGOEnr",
append=TRUE)


#####Normalization factors

pdf("WallabyLungNormFactors.pdf")
```

```
barplot(y$samples$norm.factors, names.arg=rownames(y$samples))

dev.off()

#####pairwise comparison

pdf("WallabyLungPairwise.pdf")

pairs(log(retained,2), upper.panel=panel.cor)

dev.off()

#####Transcript Length plot

pdf("WallabyLungLengthvsFPKM.pdf")

par(mfrow=c(2,2))

for (i in 1:ncol(retained)){

  plot(log(rl[,(ncol(retained)+1)],2), log(rl[,i],2), pch=19, col="grey 50", cex=0.3,
xlab="log2(Longest Transcript Length)", ylab="log2(Normalized FPKM)")

  lines(lowess(log(rl[,(ncol(retained)+1)],2), log(rl[,i],2)), col="tomato 4")

  legend("topright", colnames(retained)[i], cex=0.8)

}

dev.off()

#####Differential Expression Graph

pdf("WallabyLung.DiffExp.pdf")

plot(nimp$logCPM, nimp$logFC, cex=0.4, col="grey 50", xlim=range(tt$table$logCPM),
ylim=range(tt$table$logFC), xlab="log2 (Normalized Counts/Million)", ylab="log2 (Fold
Change", main="Wallaby Lung Differential Expression Pattern")

points(imp[imp$logFC > 0,]$logCPM, imp[imp$logFC > 0,]$logFC, cex=0.8, col="blue",
pch=19)

points(imp[imp$logFC < 0,]$logCPM, imp[imp$logFC < 0,]$logFC, cex=0.8, col="tomato 4",
pch=19)

legend("right", c("Down", "Up"), fill=c("blue", "tomato 4"))

dev.off()

#####cumulative distribution plots

pdf("WallabyLung.CumDist.pdf")

par(mar = c(5, 4, 4, 4) + 0.3)  # Leave space for z axis

plot(log(ncs[,1],2), col="0000000", xlab= "mRNA Rank", main="Tag Count Distribution",
ylab="log2 CPM", ylim=range(log(ncs,2)), las=1)
```

```
for (i in 1:ncol(ncs)){
    lines(log(ncs[,i],2), pch=10, col=rainbow(ncol(ncs))[i], lty=3, lwd=2)
}
par(new=TRUE)
plot(nccf[,1], col="0000000", axes = FALSE, bty="n", xlab="", ylab="", ylim=c(0,100), las=1)
for (i in 1:ncol(ncs)){
        lines(nccf[,i], pch=10, col=rainbow(ncol(ncs))[i], lwd=2)
}
axis(side=4, at=pretty(c(1,100)))
mtext("% Cumulative Tag Counts", side=4, line=3)
legend("right", colnames(nc), fill=rainbow(ncol(nc)), cex=0.7, ncol=2)
abline(v=nrow(retained))
dev.off()
#####GO Term analysis
pdf("immunesystemprocess.pdf")
ggplot(dm[dm$count>5,], aes(x=label, y=value)) + geom_boxplot(aes(fill=variable),
position="dodge", outlier.shape=NA) + ylab("log2 (Normalized Tag Counts)") + xlab("Child
Terms of Immune System Process GO Term") + ylim(range(bp$stat)) + theme_bw() +
opts(axis.text.x=theme_text(angle=90))
```

Chapter 7. Synopsis and future work

Introduction

Developmental immunologists have been intrigued by the marsupial immune system for over 50 years (e.g. Block 1960; Ashman and Papadimitriou 1975; Cutts and Krause 1980; Deane *et al.* 1990; Old and Deane 2000). Interest in the development of marsupial immunology stemmed from research examining the timing of the development of immune competence, which occurs up to weeks after the young are born—long after the young are exposed to the external environment (reviewed in Old and Deane 2000). What followed included research examining the exposure of the developing young to microorganisms (Yadav *et al.* 1972; Charlick *et al.* 1981; Old and Deane 1998; Deakin and Cooper 2004; Chhour *et al.* 2008; Chhour *et al.* 2010), along with potential alternative mechanisms to protection including those afforded by the mother (e.g. milk (Cockson and McNeice 1980; Nicholas 1988; Nicholas *et al.* 1989; Piotte *et al.* 1997; Young *et al.* 1997; Adamski and Demmer 1999; Adamski and Demmer 2000; Young and Deane 2001; Daly *et al.* 2007; Joss *et al.* 2007; Joss *et al.* 2009; Wanyonyi *et al.* 2011) and pouch secretions (Bobek and Deane 2002; Ambatipudi *et al.* 2007; Ambatipudi *et al.* 2008)) and the innate immune system of the young (Yadav 1972; Cutts and Krause 1980; Coutinho *et al.* 1994; Basden *et al.* 1996; Santos *et al.* 2003; Daly *et al.* 2008a; 2008b; 2008c; Carman *et al.* 2009; Wang *et al.* 2011).

The main objective of this thesis was to further examine the immune protection of marsupial young; first, to review the literature and to identify major gaps in our knowledge of the development of the immune system of the marsupial and second to begin to fill these gaps through experimental research. Specifically, the chapters include two reviews of the literature and three experimental studies. The first review examines

exposure to microorganisms and complementary protective mechanisms, while the second review examines the marsupial pouch and its implications for marsupial reproduction and mammalian evolution. The experimental chapters identify specific compounds involved in innate immunity in the tammar wallaby (*Macropus eugenii*) and examine the expression of genes that code for potential immune compounds in tissues of developing tammar wallabies that are readily exposed to microorganisms.

Protection of marsupial young

Over 10 years ago, Old and Deane (2000) reviewed the development of the immune system and immunological protection in marsupial pouch young. Therefore, the first aim of this thesis was to review the current literature examining the development and immunological protection of marsupial young. Since Old and Deane's (2000) review, research has focused on exposure of marsupial young to microorganisms, maternal protection and the innate immune system of the developing young.

Interestingly, only two studies have linked (even if superficially) the threat microorganisms pose to the survival of marsupial pouch young (Block 1960; Osawa *et al.* 1992); instead research examining the various sites of microbial colonisation in which marsupial young are exposed to microorganisms has dominated, as knowing the microbial species that are present and the threat they pose in other species can provide an insight into the threat they may pose to marsupial young. The sites examined include the urogenital opening, pouch and mouth of the mother and the gastrointestinal tract of the pouch young (Yadav *et al.* 1972; Charlick *et al.* 1981; Old and Deane 1998; Deakin and Cooper 2004; Chhour *et al.* 2008; Chhour *et al.* 2010). Thus, although the exact threat of microorganisms has not been established, the occurrence of microorganisms implies that an immune mechanism, elicited from the mother or the young, prevents the microorganisms from proliferating and causing harm to the developing young.

Protection provided to the pouch young by the mother has been examined extensively and largely includes through pouch secretions and milk. The review in Chapter 2 recognised that large gaps in our understanding of developmental marsupial immunology exists; and while research examining the components of colostrum and milk has identified many cells and chemical compounds with potential protective roles, experimental studies examining their protective role are largely absent (Cockson and McNeice 1980; Nicholas *et al.* 1989; Piotte *et al.* 1997; Young *et al.* 1997; Adamski and Demmer 2000; Young and Deane 2001; Joss *et al.* 2007; Joss *et al.* 2009). Additionally, elements of the innate immune system of the young have also been examined and suggest that the innate immune system also plays a large role in protection (Yadav 1972; Cutts and Krause 1980; Coutinho *et al.* 1994; Basden *et al.* 1996; Santos *et al.* 2003; Daly *et al.* 2008a; 2008b; 2008c; Carman *et al.* 2009; Wang *et al.* 2011). To conclude, the review recognises that the sequencing of marsupial genomes (Mikkelsen *et al.* 2007; Renfree *et al.* 2011; Murchison *et al.* 2012) facilitates a greater understanding of the metatherian immune system and can be exploited to increase our understanding of the protection of developing marsupial young.

The second aim of this thesis was to understand the role of the pouch. At first, immune protection from the pouch was difficult to comprehend, as some marsupial species have undeveloped young that attach to a teat on their mother's abdomen which are not enclosed by a pouch (Russell 1982; Tyndale-Biscoe 2005). It is clear that although the pouch may provide immune protection to those species that have them, the pouch cannot provide immune protection for those species with highly altricial young which do not have pouches. Thus, instead of using the pouch to explain protection of marsupial young, the review in Chapter 3 examines the role of the pouch in marsupial evolution in light of supporting highly altricial young and promoting reproductive success in various environments where a pouch may confer significant advantages over

free hanging young, as opposed to its immune protective role. It is likely that the pouch's immunological role arose to counteract a potential immune threat that the pouch itself may create, such as a humid environment where bacteria may thrive.

The first two review chapters helped to identify the direction for the remaining research reported in this thesis. Specifically, it was found that the innate immune system has received limited attention when compared to the vast amount of research that has been conducted on the role of milk and colostrum and that while the pouch may provide protection to those species that have them, it does not explain immune protection of developing marsupial young. Additionally, the sequencing of marsupial genomes provides a wealth of information that can be exploited to help address questions regarding the immunological development of marsupial young.

The remaining chapters exploited the marsupial genomes, particularly the genome of a model marsupial species, the tammar wallaby, to further examine the innate immune genes of developing marsupials. First, specific immune genes, mucins and lysozyme were identified and physically mapped to the tammar wallaby genome, and second, the transcriptome of two tissues of developing tammar wallaby young were sequenced to examine the difference in expression of innate immune genes between young that are considered immune-incompetent and young that are considered immune-competent.

Genome sequencing and its role in examining protection of developing marsupial young

Genome sequencing and the resulting genomes are becoming highly accessible to researchers, with three marsupial genomes; the gray short-tailed opossum (*Monodelphis domestica*) (Mikkelsen *et al.* 2007), tammar wallaby (Renfree *et al.* 2011) and the Tasmanian devil (*Sarcophilus harrisii*) (Murchison *et al.* 2012) publicly available. Thus, it is important that these genomes are exploited to their full potential, including to

facilitate further research, such as functional studies. Before starting the research presented in this thesis, it was evident that many researchers were selecting individual genes to explore immune protection of marsupial young based on their roles in other species; most of these studies also included the identification of the genes. Chapter 4, examining mucins and lysozymes, followed this pattern. However, as RNA-sequencing permits the analysis of gene expression in whole tissue, it was possible to venture away from individual genes or groups of genes and use a holistic approach to determine which genes were expressed in developing tammar wallabies. The research presented in this thesis has used a novel approach to examine the protection of marsupial young by exploiting the tammar wallaby genome. Although, the tammar wallaby genome has limitations, as it has only been sequenced to a 2-fold depth and consists of many scaffolds not anchored to chromosomes, it was still possible to identify several genes that are expressed in the skin and lung of developing tammar wallabies, which in turn, has raised several more questions relating to the immune protection of marsupial young.

Identification and physical mapping of innate immune genes, mucin and lysozyme

Mucins and lysozymes play an important role in innate immunity in eutherian mammals and form a primary defense against pathogenic microorganisms. As few studies have examined mucins in marsupials (Schumacher and Krause 1995; Renfree and Lewis 1996; Casey *et al.* 2002; Paris *et al.* 2005; Kwek *et al.* 2009), the research presented in Chapter 4 aimed to identify mucins and lysozymes in tammar wallabies. A preliminary examination of the genome found three putative mucin genes and two lysozyme genes predicted in the genome. The remaining mucin sequences were predicted by using cDNA sequences from the opossum or human to search the tammar wallaby genome. Of the 17 different types of mucin genes found in human, 11 mucin or mucin related genes and two lysozyme genes were identified in the tammar wallaby genome. The

mucin and lysozyme genes were mapped to tammar wallaby chromosomes to determine their location. The location of the genes showed that marsupials display conservation of their arrangement of mucin genes when they are compared to the arrangement of mucin genes on human and opossum chromosomes. Although examining the function of these genes was beyond the scope of this thesis, it is hypothesised that mucin and lysozyme play similar roles to those they play in innate immunity in eutherian mammals, as a primary defense against pathogenic microorganisms.

Identification and expression of immune genes from RNA-sequencing

The expression of the mucin and lysozyme genes were examined in the skin and lung of developing tammar wallabies in Chapters 5 and 6. In the skin transcriptome, reads mapped to the tammar wallaby lysozyme orthologues located on tammar wallaby scaffold 23673 and 31416, however, they were not included in the 98 % of cumulative tag counts retained for further analysis, suggesting that other genes play a larger role in immune protection of developing marsupial skin. In the lung, however, one lysozyme orthologue (located on scaffold 23673) also contained too few reads to warrant further analysis, while the other orthologue (located on scaffold 31416) showed increased expression in the immune-competent group. In the lung analysis, reads only mapped to the scaffolds of one mucin gene, *MUC4*, however the reads were too few to warrant further analysis and did not span all of the scaffolds containing the mucin gene. This was surprising as two mucin genes, *MUC5AC* and *MUC5B*, play an important immunological role in the mammalian lung (Thornton *et al.* 2008). However, the results correspond to those examining the development of respiratory structures that are associated with mucin in the Virginia opossum (*Didelphis virginiana*) and brushtail possum (*Trichosurus vulpecula*), whereby cilia is absent and it is not until well into pouch life that the juveniles form submucosal glands and goblet cells (Krause and

Leeson 1973; Cooke and Alley 2002). The deficiency of mucin gene expression suggests that the developing tammar wallaby is also lacking another important component of the immune system at birth and during its time of development in the pouch. However, in the young lung, immune protection may be acting in regions prior to the bronchi and peripheral lung, for example, mucin genes may be expressed and acting in the trachea, before bacteria reach the bronchi or peripheral lung.

Mucin gene expression was also not detected in the developing tammar wallaby skin. However, over expression of trefoil factor 2 (*TFF2*) was found in the individual aged 19 days old. TFFs are synthesized in mucin producing epithelial cells and play a role protecting the epithelia (Hoffmann *et al.* 2001). TFF peptides have also been detected in amphibian skin, with TFF domains forming a central part of frog integumentary mucin C.1 (Hauser and Hoffmann 1992). This result suggests that there may be other mucins or compounds similar to mucins that were not identified in Chapter 4 protecting the young.

In the past, research examining the innate immune system of developing marsupial young has been compound specific. However, with the advent of new technologies such as RNA-sequencing, it is now feasible to examine the whole transcriptome of cells and tissues. The research presented in Chapters 5 and 6 ventures away from the compound specific approach of examining immune genes in developing marsupials and uses RNA-sequencing to examine the expression of a collection of innate immune genes in two tissues in the developing tammar wallaby assumed to be readily exposed to bacteria from birth. This approach enables the exploitation of information, which is readily available, such as the tammar wallaby genome, and facilitates the informed identification of interesting immune compounds that can be selected for further functional research.

Skin was selected as a tissue of interest, as it acts as a first line of defense against microorganisms. RNA from the skin of developing tammar wallabies which are considered to be immune-incompetent and wallabies that are immune-competent was sequenced and examined to determine the variation in expression of innate immune genes. There were 390 immune genes from a range of immune related Gene Ontology (GO) groups identified in the skin of young, which suggests that the developing wallaby skin plays a role as an immune defense organ.

Several GO immune system process groups and genes which were differentially expressed between immune-incompetent and immune-competent individuals were also identified. The genes with important immune functions which were differentially expressed with increased expression in wallabies considered immune-incompetent compared to those that were considered immune-competent included two genes involved in complement, complement component 3 (*C3*) and complement factor B (*CFB*), two peroxidase genes, myeloperoxidase (*MPO*) and glutathione peroxidase 2 (*GPX2*), and a lipoprotein component, apolipoprotein A1 (*APOA1*).

The lung transcriptome of developing marsupials was also examined. The development of the immune system in the marsupial lung is particularly interesting as the largest part of development occurs postnatally, when young are exposed to an external environment where they must protect themselves from inhaling harmful environmental particulates. As with the skin analysis, GO classifications were used to identify which immune groups are represented by immune genes expressed in the lung. There were 429 immune genes from a range of immune related GO terms identified in the lung, which suggests that like skin, the lung of the developing wallaby also plays a role as an immune defense organ. There were also five GO terms associated with immune function that were significantly overrepresented in the immune-incompetent group compared to the

immune-competent group, containing the genes *APOA1*, apolipoprotein A2 (*APOA2*), complement component (*C2*), *C3*, complement component C4b (*C4B*), *CFB* and ficolin 3 (*FCN3*).

Further, specific immune genes which are highlighted in the literature of mammalian lung development were also examined and several genes classified as antimicrobials, complement, cytokines and chemokines, and pattern recognition molecules were found with increased expression in the immune-incompetent group. *C3*, *CFB*, an isoform of lactoferrin (*LTF*) and monocyte chemoattractant protein 1 (*MCP1/CCL2*), surfactant protein A (*SFTPA*) and toll-like receptor 4 (*TLR4*) were all found to have increased expression in the immune-incompetent group. Complement factor H (*CFH*) and *LYZ* were the only genes to show increased expression in the immune-competent group, while interleukin 6 (*IL6*), an isoform of *LTF*, two isoforms of *MCP1/CCL2*, transforming growth factor beta 1 (*TGFB1*), *TLR2* and *TLR3* were not differentially expressed. The results suggest that specific innate immune genes may play a large role in the protection of newborn tammar wallabies while their immune tissues are still developing.

In both the skin and lung of the developing tammar wallaby, *APOA1*, *C3* and *CFB* were found with increased expression in the immune-incompetent groups. Although direct comparisons with eutherian mammals cannot be made, complement components are usually found in much lower concentrations in human neonate serum than in human adult serum (Yonemasu *et al.* 1978; Strunk *et al.* 1979; McGreal *et al.* 2012). The increase in expression may represent part of the innate immune system which develops early in marsupials to compensate for the delayed development of the adaptive immune system. However, complement has been found to play a role in the development of organs that do not have an immune role (Mastellos and Lambris 2002). Thus, further

work is required to determine the role of complement in development versus immunity in marsupials. Although further research to determine the functional significance of complement was beyond the scope of this study, the detection of complement in the immune-incompetent individuals is interesting and provides the basis for further work to be carried out on these compounds.

APOA1 is a major apolipoprotein of high density lipoprotein, and may directly affect bacterial growth and promote self defense mechanisms of normal and immune-incompetent individuals, as a serum fraction containing APOA1 has shown antimicrobial activity against a gram positive bacteria *Staphylococcus epidermidis* (Tada *et al.* 1993). The review in Chapter 2 highlights that there are no studies which have examined antimicrobial lipids in developing marsupials and that they are unlikely to be explored while there is still so much unknown about the antimicrobial proteins and peptides.

Limitations

There were three main areas which presented limitations to this research. First, at the time of sampling which was limited and opportunistic, the success of RNA-sequencing of developing tammar wallaby skin or lung, in terms of achieving reads and then mapping the reads to the tammar genome, was unknown. Thus, in conjunction with the high cost of sequencing only a small number of samples (which sometimes comprised significant age gaps) were sequenced for the immune-incompetent and immune-competent groups.

Second, the tammar wallaby genome has only been sequenced to a 2-fold depth (Renfree *et al.* 2011) and therefore is likely to contain incomplete annotations, as is evident from Chapter 4 on mucin genes. Thus, general immune genes, along with marsupial specific immune genes, which have not been annotated in the genome will be

missing from the transcriptome analysis and the number of genes identified to be expressed or differentially expressed in the transcriptome analysis may be largely underestimated.

Finally, the methodology relating to the GO analysis may also contain limitations. It is possible that the genes associated with GO immune system process terms may not always be directly involved in immune pathways, and instead may be involved in other metabolic processes. Additionally, using GO term enrichment may mask the differential expression of important immune genes that are not found in specific GO terms or are found within terms that do not contain differential expression of the other genes. GO terms have also been developed based on the pathways of other species, thus, it is assumed that the immune genes of marsupials follows a similar pattern to those of other animals. As GO term enrichment identified genes that were differentially expressed between the immune-incompetent and immune-competent groups, the need to further identify interesting immune groups or pathways was also suppressed. Although the research comprises limitations, a baseline for research in marsupial developing immunology has been developed, whereby RNA-sequencing and the tammar wallaby genome can be utilised with some success.

Future work

The research presented in this thesis has identified gaps in the research of immune protection of marsupial young and has identified, mapped and examined the expression of innate immune genes in the developing tammar wallaby. Thus, we are closer to understanding the complexity of the developing marsupial immune system.

However, although the identification of immune genes and their expression implies an immune role, functional studies confirming the role of genes must be undertaken. For example, although an increase in expression of complement components is found in the

skin and lung of the individuals considered immune-incompetent compared to the individuals considered immune-competent, it is essential to determine that the function of these compounds is related to immunity. Increased time series transcriptome studies with and without immune challenge could help to determine the role of specific complement components or other immune related genes.

Further, to determine if specific immune compounds develop earlier in marsupials compared to eutherians to compensate for the undeveloped adaptive immune system it would be interesting to compare the results which showed increased expression of immune genes in immune-incompetent individuals to the expression of these immune genes in the same tissues at, or just after birth in eutherian species. Currently, the literature does not support research which allows this comparison to be made.

In the broader field of immunity, research tends to focus on antimicrobial peptides over antimicrobial lipids, as discussed Chapter 2. However, the results presented in this thesis, with increased expression of *APOA1* and *APOA2* in immune-incompetent compared to immune-competent individuals, supports the case to further examine antimicrobial lipids.

There is also a case for doing further age specific transcriptome analysis. At the time of skin sample collection, the aim of the research was to examine the transcriptome of wallabies with and without immune competence; however, as there are differences in gene expression observed between the immune-incompetent individuals, further studies examining the transcriptome of younger wallabies are warranted to determine when the majority of immune genes start to show differential expression, or if the differences are the result of individual variation.

Although mucins were not detected in the expression analysis of skin and lung, their absence means that another crucial part of the immune system is deficient from

marsupials at birth and while they are developing. However, it is possible that other mucins, which have not been identified yet, are acting in their place. Additional research is required to further examine mucins.

Importantly, with the transcriptome analysis of the developing marsupial lung and skin, it is now possible for researchers to use the information presented in this thesis to further seek out genes of interest. Now that genes are known to be present at specific developmental ages, researchers can make an informed decision to research these genes or compounds in the developing marsupial.

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