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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.

Contents

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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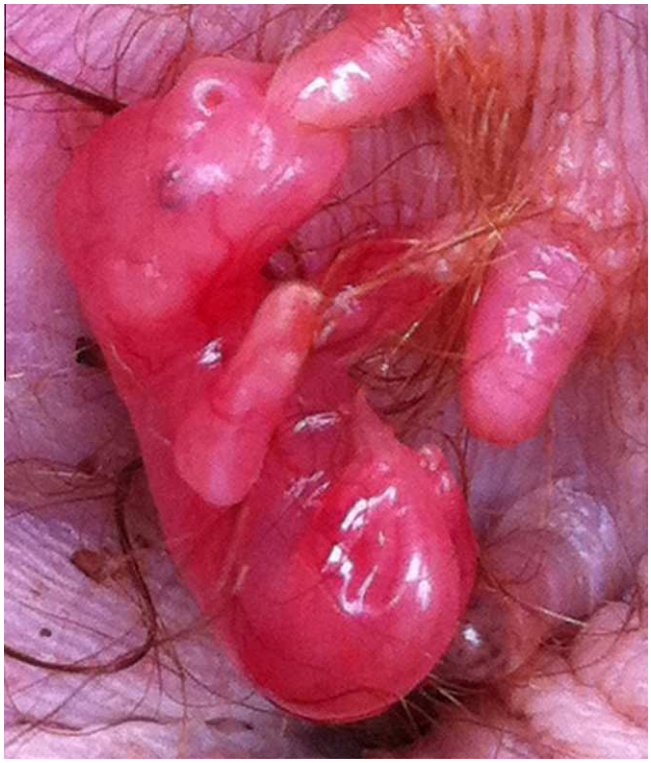

Fig. 1. A tammar wallaby weighing 350–400 mg, attached to a teat less than 24 h after birth.

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.

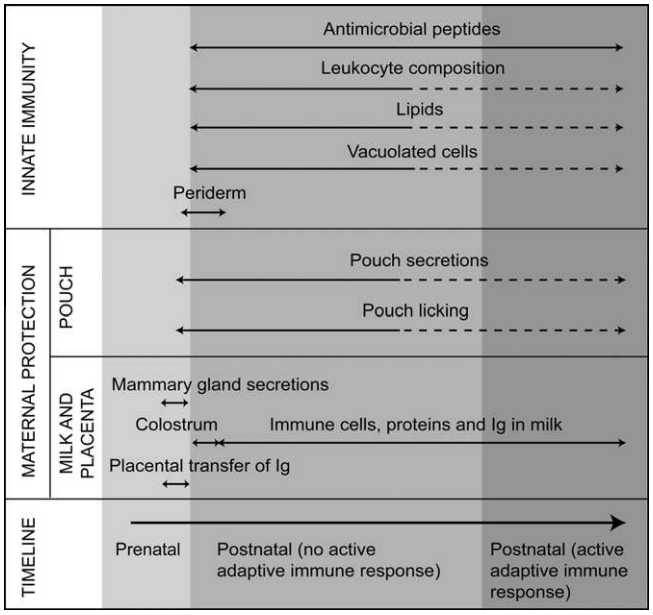

Fig. 2. A general diagram showing the complementary protective mechanisms for marsupial pouch young with respect to the occurrence of the adaptive immune response. Dashed lines indicate period of transition or reduction in importance/activity as young develops own adaptive immune response.

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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