REVIEW

The Development of the Immune Tissues in Marsupial Pouch Young

Casey R. Borthwick, Lauren J. Young, and Julie M. Old*

Native and Pest Animal Unit, School of Science and Health, Hawkesbury, University of Western Sydney, Locked bag 1797, Penrith, New South Wales 2751, Australia

ABSTRACT Current knowledge of the development of the marsupial immune system, particularly in the context of lymphoid tissue development and the appearance of lymphocytes, has been examined and limitations identified. While primary lymphoid tissues like the thymus have been extensively studied, secondary lymphoid tissues such as the spleen and lymph nodes have been examined to a lesser extent, partly due to the difficulty of macroscopically identifying these structures, particularly in very small neonates. In addition, little research has been conducted on the mucosal-associated lymphoid tissues; tissues that directly trap antigens and play an important role in the maturity of adaptive immune responses. Research on the development of the marsupial immune tissues to date serves as a solid foundation for further research, particularly on the mechanisms behind the development of the immune system of marsupials. With the recent sequencing and annotation of whole marsupial genomes, the current wealth of sequence data will be essential in the development of marsupial specific reagents, including antibodies, that are required to widen our specific knowledge of the complex marsupial immune system and its development. J. Morphol. 275:822–839, 2014. © 2014 Wiley Periodicals, Inc.

KEY WORDS: development; immunology; lymphocyte; lymphoid tissue; metatherian

INTRODUCTION

Marsupials as a group of mammals are notable for the birth of young at a very early developmental stage, followed by a lengthy period of further development in the maternal pouch (Smith, 2001). It is known that, anatomically, adult marsupials have primary and secondary lymphoid tissues like those of eutherian mammals (Old and Deane, 2000; Haynes, 2001). Adult marsupials also possess cellular immune system components similar to those seen in eutherian mammals; a number of key immunological molecules have been characterized, including major histocompatibility complex (MHC) Class I and II (Miska and Miller, 1999; Gouin et al., 2006), T-cell receptor (TCR) chains α, β, γ, and δ (Zuccolotto et al., 2000; Baker et al., 2001, 2005; Miller, 2010), and the immunoglobulins IgG, IgM, IgE, and IgA (Miller and Belov, 2000; Miller, 2010). However, at birth, these immunological features are not yet developed, as only the organs and systems essential for immediate survival and transit to the pouch are developed. Generally, the jaw is well developed to allow attachment to the teat and suckling, and the forelimbs to crawl and climb into the pouch (Smith, 2001; Keyte and Smith, 2010). At birth, only the very basic innate immune protection mechanisms are present, and it has been well established that at this time marsupials lack histologically mature lymphoid tissues and do not possess mature lymphocytes (Block, 1964; Basden et al., 1997; Baker et al., 1999; Cisternas and Armati, 1999; Old and Deane, 2003; Old et al., 2003, 2004a,b; Duncan et al., 2012). Some developmental milestones for selected species discussed here are shown in Table 1, for comparative purposes.

Two main complementary mechanisms are thought to compensate for the immunologically vulnerable newborn; passive maternal immune protection, and the rapid development of immunocompetence in the pouch-bound phase following birth. A number of maternal protection mechanisms have been suggested and were recently reviewed by Edwards et al. (2012). In the context of this review, we will focus on the immunological development of the young marsupial itself.

TABLE 1. Developmental milestones of selected marsupial species

Species	End fixed lactation	Eyes open	Pouch exit	Weaned	References
Antechinus	40 days	62 days	70 days	90 days	Wood, 1970; Westman et al., 2002
Quokka	70 days	140 days	180 days	300 days	Hayward et al., 2003
Brushtail possum	90 days	150 days	180 days	250 days	Gemmell and Hendrikz, 1993
Koala	90 days	155 days	230 days	350 days	Smith, 1979; Tobey et al., 2006
Tammar wallaby	100 days	140 days	250 days	300 days	Ryan, 2011

DEVELOPMENT OF LYMPHOID TISSUES AND THE APPEARANCE OF LYMPHOCYTES

It has been well documented that anatomically adult marsupials possess mature primary and secondary lymphoid tissues similar to eutherian mammals (Old and Deane, 2000), and it has long been suggested that the immunological responses of adult marsupials are broadly comparable to eutherian mammals (Ashman et al., 1975). However, it has become clear that lymphoid tissues are not histologically mature in marsupials at birth, unlike in eutherian mammals. Because of this lack of mature lymphocytes and mature lymphoid tissue, newborn marsupials are not able to mount specific immune responses. The maturation of the lymphoid tissues and lymphocytes occurs after birth, during the pouch-bound phase of life (Basden et al., 1997; Old et al., 2003). In a study of embryonic brushtail possums (*Trichosurus vulpecula*), Old and Deane (2003) did not detect any clearly defined lymphoid tissues or mature lymphocytes using histological techniques, providing further evidence that these tissues and cells mature after birth. In all of the marsupial species that have been examined to date, a period of very rapid lymphoid tissue development has been observed in the first few weeks following birth (Block, 1964; Basden et al., 1997; Old et al., 2000, 2003, 2004a,b).

Although there are clear differences in the timing of development between marsupials and other mammals, the order in which marsupial lymphoid tissues develop and mature is similar to that observed in eutherian species, with the thymus the first lymphoid tissue to develop (Old and Deane, 2000). As T-lymphocytes differentiate in the thymus before populating other sites of the body, lymphoid organs such as the spleen, lymph nodes, and gut-associated lymphoid tissues (GALT) develop into functional lymphoid tissue only after the thymus has reached maturity. In some species, such as the stripe-faced dunnart, *Sminthopsis macroura*, (Old et al., 2003) it has been shown that other immune tissues, including the secondary lymphoid tissues, reach maturation less rapidly than the thymus, and form a definitive structure complete with germinal centers at about the same stage as the young starts to leave the pouch and become less dependent on its mother. Although lymphoid tissues are easily distinguishable at the age of weaning, at this stage not all of these tissues have reached complete histological maturity (Old et al., 2003, 2004a).

Although lymphocytes have been observed in the developing lymphoid tissue of marsupials, the lack of specific antibodies has limited their phenotypic identification, however, immunohistochemistry has, to date, been used in a small number of marsupial species to document the appearance and localization of specific cells, including mature T- and B-cells. Immunohistochemistry is a valuable tool in marsupial developmental immunology, as it can be used to identify mature cells in the tissues, indicating when young marsupials develop mature immune cells, including T- and B-lymphocytes, plasma cells, and antigen presenting cells. As such, these techniques can be used to provide an indication of when the young marsupial would be able to mount an adaptive immune response against external pathogens.

Only a few commercially available anti-human antibodies against very highly conserved cell-surface peptide sequences are cross-reactive with marsupial tissues and are able to be used to stain specific cell populations. These cross-reactive anti-human antibodies include CD3 and CD5 for identification of T-cell populations, CD79a and CD79b for B-cell populations, the immunoglobulin IgA to identify plasma cell subpopulations and HLA-DR (MHC class II) to identify antigen presenting cells (Coutinho et al., 1993, 1995; Hemsley et al., 1995; Wilkinson et al., 1995; Canfield and Hemsley, 1996, 2000; Old and Deane, 2000, 2001).

Limited marsupial-specific antibodies have also been utilized. An anti-koala IgG antibody was used by Hemsley et al. (1995) and Canfield et al. (1996) to stain plasma cell populations in koala tissues, while Old and Deane (2002) successfully used a marsupial-specific TCRα-antibody to stain T-lymphocytes in the tammar wallaby. More recently, an anti-opossum (*Monodelphis domestica*) and an anti-tammar wallaby (*Macropus eugenii*) CD8α-antibody were designed by Duncan et al. (2012) to document the appearance and distribution of CD8α$^+$ T-cells in the mature lymphoid tissues of the gray short-tailed opossum and tammar wallaby, respectively, as well as examining CD8α localization in the developing lymphoid tissues of tammar wallaby pouch young.

TABLE 2.. Species, phylogeny and the types of thymus present in each

Species	Order	Group	Cervical thymus	Thoracic thymus
Stripe-faced dunnart	Dasyuromorphia	Polyprotodont	No	Yes
Brown antechinus	Dasyuromorphia	Polyprotodont	No	Yes
Dusky antechinus	Dasyuromorphia	Polyprotodont	No	Yes
Tasmanian devil	Dasyuromorphia	Polyprotodont	No	Yes
Virginian opossum	Didelphimorphia	Polyprotodont	No	Yes
Pouchless opossum	Didelphimorphia	Polyprotodont	No	Yes
White-eared opossum	Didelphimorphia	Polyprotodont	No	Yes
Northern brown bandicoot	Peramelemorphia	Polyprotodont	No	Yes
Koala[a]	Diprotodontia	Diprotodont	Yes	No
Tammar wallaby	Diprotodontia	Diprotodont	Yes	Yes
Quokka	Diprotodontia	Diprotodont	Yes	Yes
Brushtail possum	Diprotodontia	Diprotodont	Yes	Yes

[a]The koala is a diprotodont species, and like other diprotodonts has a cervical thymus, but unlike the other members of this group, does not have a thoracic thymus. (Haynes, 2001; Archer and Kirsch, 2006).

THYMUS

Due to the important role the thymus plays in the maturation of T-cells and in cell-mediated immunology, the thymus has been the most widely examined lymphoid tissue in marsupial species. Johnstone (1898) first described the anatomical location and basic features of the pouch young and adult marsupial thymus in a number of different marsupial species, while Yadav (1973) examined the mature thymi of 93 different marsupial species, and along with a more recent examination by Haynes (2001) reached a clear conclusion that marsupial thymi were fundamentally similar to those of eutherian mammals. Thymic lobes with cortices, medullae, and Hassall's corpuscles were evident in all marsupial species examined.

While there is debate over the precise role of Hassall's corpuscles in vivo, it has been observed in marsupials that there is a relationship between the maturity of Hassall's corpuscles in the thymus and the ability of an animal to produce antibodies in response to antigenic challenge (Block, 1964; Rowlands et al., 1964; Stanley et al., 1972). The quokka (*Setonix brachyurus*) first produces antibodies in response to sheep red blood cells (SRBC) at 10 days of age (Stanley et al., 1972), the same time as Hassall's corpuscles first appear in the cervical thymus. The removal of the cervical thymus, however, delays the onset of antibody production to SRBC to between 40 and 80 days postpartum (Stanley et al., 1972), and considering individual variation, this correlates with the appearance of Hassall's corpuscles in the thoracic thymus. A similar correspondence of the onset of humoral immune competence with the appearance of Hassall's corpuscles in the developing thymus also exists in the Virginian opossum (*Didelphis virginiana*; Block, 1964; Rowlands et al., 1964) and in humans (Norris, 1938; Solomon, 1971). Further, in those species where the allograft rejection response and mitogen proliferation assays of pouch young have been examined, neither response was detected before maturation of the thymus (La Plante et al., 1969; Ashman et al., 1977; Waring et al., 1978), which suggests that the maturation of the thymus corresponds to the onset of cellular immunity. In humans, Hassall's corpuscles have been found to produce the cytokine thymic stromal lymphopoietin (TSLP), a molecule that activates dendritic cells, which in turn induces the proliferation and differentiation of $CD4^+CD8^-CD25^-$ thymic T-cells into $CD4^+CD25^+FOXP3^+$ regulatory T-cells (Watanabe et al., 2005). As such, it has been proposed that Hassall's corpuscles have a critical role in dendritic cell-mediated secondary positive selection, leading to the generation of $CD4^+CD25^+$ regulatory T-cells within the thymus. A similar role of Hassall's corpuscles and TSLP could be expected in marsupials.

In marsupials, the anatomical location of the thymus differs between major family groups (Table 2.). Typically, the polyprotodont species possess only a thoracic thymus, while the diprotodont species possess both a thoracic and cervical thymus (Yadav, 1973; Deane and Cooper, 1988), with the exception of the koala (*Phascolarctos cinereus*), which has been found to possess a cervical thymus only (Yadav, 1973; Haynes, 2001). In marsupials with both types of thymus, the tissues appear to be histologically identical and to have the same function, but the cervical thymus is larger, and development of the thoracic thymus lags behind that of the cervical thymus (Stanley et al., 1972). Consistent with previous reports that in those animals that possess both cervical and thoracic thymi these tissues have identical histology and function, Wong et al. (2011) reported that the transcriptome of the cervical and thoracic thymus of the tammar wallaby both display gene expression profiles that are consistent with their roles in T-cell development and provides further evidence that the two thymi are functionally equivalent and both drive T-cell development. Further, all major TCR classes were expressed in both thymi, as well as RAG-1

and RAG-2 transcripts (Wong et al., 2011), genes that are involved in the rearrangement and recombination of T-cell receptors and immunoglobulin molecules.

In the polyprotodont species that have been examined to date, only a thoracic thymus is present (Yadav, 1973; Haynes, 2001), and while histologically immature at birth, it is the first lymphoid tissue to reach maturity in these animals (Old and Deane, 2000). In the Virginian opossum and the "pouchless" opossum, *Marmosa mitis*, on the first day of pouch life the thymus consisted of a sheet of epithelial cells, with large lymphocytes bordering the capillaries between lobules (Block, 1964), and this is similar to other marsupials at the same developmental stage (summarized in Table 3). Primitive Hassall's corpuscles were identified by day 4 in both the Virginian opossum and the pouchless opossum (Block, 1964; Bryant and Shifrine, 1974) and had matured in both species by day 6. At days 10 to 12, the entire thymus, except for the Hassall's corpuscles, was filled with small lymphocytes, with a very small, lymphocyte-poor edge also observed. The previously lymphocyte-poor lobular edges acquired the normal complement of mainly large and medium lymphocytes by days 12–14 (Bryant and Shifrine, 1974). By 23–32 days, the structure and morphology of the thymus resembled the fully developed thymus (Block, 1964).

In newborn [10-mm CRL (crown-rump length)] white-eared opossums (*Didelphis albiventris*), antibodies were used to more definitively identify cell types, and the thymus consisted of small clustered HLA-DR$^-$CD3$^-$ cells with round nuclei (Coutinho et al., 1995). Strongly stained HLA-DR$^+$ (antigen-presenting) cells were first observed in animals at 12-mm CRL, and by 14-mm CRL, weakly stained CD3$^+$ cells were observed in the thymic medulla. In 24-mm CRL animals, cortico-medullary differentiation was obvious, and HLA-DR$^+$ reticulo-epithelial cells were observed concentrated in the medulla, with some isolated cells seen in the cortex (Coutinho et al., 1995). The staining pattern and structure of the 24-mm CRL thymus persisted in juvenile animals, but in older animals, the lobular structure had diminished (Coutinho et al., 1995).

In *Antechinus* spp. *(A. stuartii* and *A. swainsonii)* within the first 2 weeks of life, the thymus was well formed with a definitive cortex and medulla (Poskitt et al., 1984a). Growth of the thymus was rapid and maximum size and maturity was reached at 14 weeks, at the same stage as the pouch young were being weaned (Poskitt et al., 1984a). A short time after exiting the pouch, at 16–17 weeks of age, the thymus of both *Antechinus* spp. started to involute (Poskitt et al., 1984a). Once initiated, the involution of the thymus was rapid, and by 33 weeks the cortex had been entirely eroded by fatty tissue, while only small islands of medullary tissue remained within the adipose tissue. Involution of the thymus was complete at 35 weeks, 2 months before sexual maturity, and was complete before the stress-related rise in plasma-free glucocorticoids that occurs in males of these species (Poskitt et al., 1984a). Thymic involution resulted in subsequent depletion of the T-lymphocyte regions of the spleen and lymph nodes, but not Peyer's patches. While thymic involution in *Antechinus* is more rapid than in eutherian mammals and other marsupials, it generally followed the same pattern, with a gradual loss of supportive cells, small lymphocytes and thymocytes until there were only small islands of thymic parenchyma in a mass of invasive fatty tissue (Poskitt et al., 1984a).

The thymus of the newborn Northern brown bandicoot (*Isoodon macrourus*) consisted of two small lobular masses of mesenchymal cells as well as a few epithelial cells (Cisternas and Armati, 1999). Early cortico-medullary differentiation was visible at day 3, and by the end of the first week, lobulation and cortico-medullary differentiation were more prominent (Cisternas and Armati, 1999). By day 13, a small number of developing Hassall's corpuscles were identified, and numerous septa were seen traversing the lobules (Cisternas and Armati, 1999). Lobulation was more distinct at this stage. At 21 days of age, numerous Hassall's corpuscles were observed in the medulla, while lobulation had further increased. By 60 days, tingible body macrophages, with a characteristically irregular shape, and pale eosinophilic cytoplasm were present in the cortex (Cisternas and Armati, 1999). In animals at 12 months of age, the lobes had the same structural appearance of those seen at 60 days. The thymus of adult animals was involuting, which was evident by the infiltration of adipose cells, and lobulation of the gland was now absent (Cisternas and Armati, 1999).

At birth, the thoracic thymus of the stripe-faced dunnart is made up of mainly stromal tissue, but by day 12, early cortico-medullary distinction of the thymus was visible and lymphocytes, including CD3$^+$ cells, were detectable (Old et al., 2003, 2004b). By day 40–45, the thymus had lobes with lobules, the tissue had a clearly defined cortex and medulla, and Hassall's corpuscles were clearly visible. In the majority of animals examined aged 45–50 days, adipose tissue had increased in the thymus; however, some samples did not contain adipose cells. Lobes of these animals were not as well defined as in younger animals (Old et al., 2003). CD5$^+$ cells were not detected in the thymus until day 50 (Old et al., 2004b).

The thymus of one 3-week old Tasmanian devil (*Sarcophilus harrisii*) pouch young was examined by Kreiss et al. (2009), along with tissues from

TABLE 3. Developmental milestones of the developing marsupial thymus

Species	Location	Lobulation first observed	Developing Hassall's corpuscles first observed	Initial cortico-medullary differentiation first observed	Mature Hassall's corpuscles first observed	Cortico-medullary differentiation complete	Mature structure reached	Involution first observed	References
Virginian opossum	Thoracic	Day 1	Day 5	Days 13–16	Days 13–16	Days 17–22	Days 23–32		Block, 1964
"Pouchless" opossum	Thoracic	Day 1	Day 4	Days 6–7	Day 6	Days 12–14	Day 15		Bryant and Shifrine, 1974
Quokka	Cervical	Day 1	Day 14	Day 10	Day 21	Day 30	Days 90–120		Ashman and Papadimitriou, 1975
Quokka	Thoracic	Day 7		Day 60	Day 60	Day 90	Day 120		Ashman and Papadimitriou, 1975
White-eared opossum	Thoracic			14-mm CRL		24-mm CRL	24-mm CRL		Coutinho et al., 1995, Stanley et al., 1972
Koala	Thoracic				8 months	8 months		5 years	Canfield et al., 1996
Tammar wallaby	Cervical				Day 21	Day 21	Day 38		Basden et al., 1997; Old and Deane, 2003; Duncan et al., 2012
Tammar wallaby	Thoracic	Day 1			Day 30		Day 60		
Brushtail possum	Thoracic	Day 1	Day 13		Day 25	Day 25	Day 25		Baker et al., 1999
Northern brown bandicoot	Thoracic			Day 3	Day 21	Day 21	Day 60		Cisternas and Armati, 1999
Antechinus spp.	Thoracic				Day 14	Day 14	Day 100	Day 112–120	Poskitt et al., 1984a
Stripe-faced dunnart	Thoracic			Day 12	Days 45–50	Days 45–50			Old et al., 2003, 2004b
Tasmanian devil	Thoracic		Day 21						Kreiss et al., 2009

older devils. Structures resembling Hassall's corpuscles were sparse in the medulla of the 3-week old pouch young, while they were more abundant in the same region of juvenile (∼1 year) animals. Fewer CD3$^+$ cells were detected in the thymus of the 3-week old pouch young compared to the juvenile (∼1 year) animals. These cells were located predominately in the medulla region, along with a few CD79b$^+$ cells (Kreiss et al., 2009). Unlike in juvenile devils, the pouch young thymus did not exhibit any infiltration of adipose cells. Only one pouch young was examined by Kreiss et al. (2009), however, structures resembling Hassall's corpuscles were identified, although to a lesser extent than in older animals, indicating that the thymus of the Tasmanian devil has started to mature by 3 weeks but has not yet completed its maturation.

In the cervical thymi of the youngest koala joeys (<80 days) examined by Canfield et al. (1996), lobules of lymphocytes in the cortical region were well developed but the distinction between cortex and medulla was poor. Nearly all of the lymphocytes that were present in these young animals were CD3$^+$, and a large majority of these cells were also CD5$^+$, while occasional cells in the medulla were CD79b$^+$ (Canfield et al., 1996). At 8 months of age, thymi exhibited distinct cortico-medullary differentiation, and Hassall's corpuscles were prominent in the medulla. Some sparse CD79b$^+$, MHC class II$^+$, and IgG$^+$ cells were seen. Histological findings in young koalas over 8 months of age were similar to those seen by 8 months of age (Canfield et al., 1996). Early stage involution of the thymus was evident in animals older than 5–6 years and was more pronounced and accelerated in animals that had experienced trauma (Canfield et al., 1996).

The quokka, unlike the other marsupials discussed to this point has two thymi; both a thoracic thymus and a cervical thymus. Within 24 h of birth, the cervical thymus of the quokka was lobulated and consisted of large epithelial cells, with some thin strands of connective tissue separating the lobules, while the thoracic thymus was not lobulated and only epithelial cells were present (Ashman and Papadimitriou, 1975). The proportion of large and medium lymphocytes decreased at this stage. At the end of the second week, small Hassall's corpuscles were found in the central zones of some cervical lobules, but did not appear in the lobules of the thoracic thymus until the second month of life. Division into cortex and medulla had begun by the end of the third week in the cervical thymus, and was distinct by the end of the first month. Thoracic cortico-medullary differentiation was observed in the second month of life and was well defined by the third month. In animals at 3–4 months of age, the cervical thymus very closely resembled the fully mature thymus, while the thoracic thymus reached mature morphology

TABLE 4. Developmental milestones of the marsupial bone marrow

Species	Haematopoiesis first observed	Primitive bone marrow first observed	Primary bone marrow first observed	Mature structure reached	References
Virginian opossum	Days 6–7	Day 5 (cranial half)	Day 5 (membranous bone)	Days 65–100	Block, 1964
Quokka	Day 7		Day 4 (cranial endochondral bone), Day 7 (caudal endochondral bone)		Ashman and Papadimitriou, 1975
Tammar wallaby	Day 14		Day 4		Basden et al., 1996
Stripe-faced dunnart			Day 11		Old et al., 2004a

by the end of 4 months (Ashman and Papadimitriou, 1975).

Like the quokka, the tammar wallaby also has both a cervical and thoracic thymus. Lymphocytes were first detected in the cervical thymus of the developing tammar wallaby at day 2 postpartum and in the thoracic thymus at day 6 (Basden et al., 1997). More recently, CD8α^+ cells were first observed in the tissue bed of the cervical thymus in the tammar wallaby at 4 days postpartum (Duncan et al., 2012). The first CD3$^+$ and CD5$^+$ T-cells were observed in the thoracic thymus at 12 days after birth (Old and Deane, 2003). By day 21, CD8α^+ cells were observed scattered through the distinct thoracic lobes (Duncan et al., 2012). In the cervical thymus of the tammar wallaby, by day 21 distinct areas of cortex and medulla were visible and Hassall's corpuscles were apparent. At this time the cervical thymus was inundated with CD8α^+ cells (Duncan et al., 2012). Scarce CD79b$^+$ B-cells were first detected at day 23 (Old and Deane, 2003). Hassall's corpuscles were detected in the thoracic thymus on day 30 (Basden et al., 1997). The cervical thymus reached complete histological maturity at day 38, with the thoracic thymus reaching maturity later, at day 60 (Basden et al., 1997).

Like the quokka and the tammar wallaby, the brushtail possum also possesses both a cervical and thoracic thymus (Haynes, 2001). However, only the thoracic thymus has been examined in a developmental sense. At day 1, the thoracic thymus was divided into distinct lobes (Baker et al., 1999), while CD3$^+$ cells were observed at day 2 postpartum, indicating that the thymus produces T-cells at or soon after birth. Thymic tissues from day 25 onward showed distinct regions of cortex and medulla, as in adult specimens. Hassall's corpuscles were observed at day 25 postpartum, and the thymus was fully populated with CD3$^+$ lymphocytes (Baker et al., 1999). Both the cortex and medulla of the adult thymus contained significantly greater numbers of CD3$^+$ cells than those observed in pouch young (Baker et al., 1999). CD79a$^+$ cells made up a significantly smaller proportion of cells than CD3$^+$. The numbers of CD79a$^+$ cells increased significantly from day 25 to 100. Peaks in CD79a$^+$ cells and IgG$^+$ cells were observed at 150 days postpartum, followed by a slight decrease of these cells in adulthood (Baker et al., 1999).

Ultimately, while these studies have provided a strong foundation for further studies of lymphoid tissue development in marsupials, more marsupial-specific antibodies are required to elucidate further information about the specific cell types present in the marsupial thymus, including when these cells first appear. This is particularly important to enable in-depth, specific studies of T-cell development in thymic tissues of marsupials and to further phenotype marsupial lymphocytes.

BONE MARROW

Bone marrow is an important component of the immune and lymphatic systems of mammals, as it is a major site of hematopoiesis, where blood cells, including red blood cells and the many different types of leukocytes differentiate from a common precursor, that is, hematopoietic stem cells. At birth, the endochondral bones of marsupial pouch young are solidly cartilaginous, and hematopoiesis is not evident (see Table 4), however, hematopoiesis was observed in all species examined before the end of the second week of life (Block, 1964; Ashman and Papadimitriou, 1975; Basden et al., 1996; Old et al., 2004a). This is not the case in eutherian mammals, where the bone marrow is hematopoietic at birth. Unlike the thymus, which has been widely examined, the development of bone marrow has only been examined in four marsupial species.

At day 5 in the Virginian opossum, primitive bone marrow was first observed in the diaphysis of endochondral bones in the cranial half, while primary bone marrow was present in membranous bone (Block, 1964). At days 6–7 mature eosinophil and neutrophil granulocytes were observed in the extravascular mesenchyme of endochondral bones (Block, 1964). The percentage of endochondral bone occupied by bone marrow had increased by days 8 and 9. The amount of bone marrow in

endochondral bones further increased on days 13–16 (Block, 1964). At days 23–32, the endochondral bones were filled with marrow except at the epiphyses, and the ratio of intravascular to extravascular hematopoiesis increased. At 33–45 days, there was an increase in the ratio of immature to mature granulocytes and occasional myelocytes were observed in membranous bone marrow (Block, 1964). By 65–100 days, the bone marrow was similar to that observed in adult animals (Block, 1964).

Although not hemopoietic at birth, the bone marrow of the stripe-faced dunnart exhibited hemopoietic activity shortly afterward (Old et al., 2004a). Bone marrow was first detected at day 11. By 50 days, numerous mature erythrocytes were observed in the marrow, as well as nucleated cells and occasional adipocytes, although no adipose tissue was present (Old et al., 2004a). At 57 days, adipose tissue was observed infiltrating the bone marrow. A variety of hematopoietic cells was also observed in the marrow, as well as mature erythrocytes. Megakaryocytes and platelets were observed. From 60 to 170 days, more numerous adipocytes were seen, while only small islands of hematopoiesis remained and the bone matrix was well developed (Old et al., 2004a).

In the quokka, primary bone marrow first appeared in the cranial endochondral bone on day 4 postpartum (Ashman and Papadimitriou, 1975), and consisted of myxoid tissue, in which a small number of sinusoids, mesenchymal cells, and histiocytes were observed. By the end of week 1, caudal endochondral bone also displayed primary bone marrow. At the end of week 2, there was an increase in the proportion of endochondral bone containing bone marrow. Cells including granulocytes, erythroblasts, and some large and medium lymphocytes were observed in sinusoids (Ashman and Papadimitriou, 1975). Some granulocyte precursors, as well as erythroblasts and megakaryocytes were observed in the extravascular space. Over the next 2 months, the amount of bone marrow increased, with intravascular erythroid precursors and megakaryocytes contributing to most of the bone marrow mass, while granulocytic precursors formed the major proportion of extravascular tissue (Ashman and Papadimitriou, 1975).

At day 4 postpartum, small areas of primary bone marrow were first detected in tammar wallaby pouch young (Basden et al., 1996), and erythroblasts were the most common cell type in the marrow. By day 8, megakaryocytes became apparent within the marrow, and by 2 weeks the diversity of cells, as well as the volume of cells had increased significantly (Basden et al., 1996). At 2 weeks postpartum, hematopoiesis was evident in tammar wallaby bone marrow (Basden et al., 1996). At the conclusion of the first month of pouch life, the bone marrow had taken over from the liver as the major site of hematopoiesis.

LYMPH NODES

Encapsulated lymph nodes occur only in marsupials and eutherian mammals and not in monotremes (Jurd, 1994). Relatively little is known about the lymphatic system of marsupials, and studies of the development of lymph nodes in marsupial species (summarized in Table 5) have been limited by the small size of the lymph nodes in these animals, particularly in pouch young, which has made these tissues difficult to locate and examine (Old et al., 2000). Difficulties in locating lymph nodes were reported by Azzali and Di Dio (1965) in both *Didelphis aurita* (formerly *D. azarae*) and the common opossum (*Didelphis marsupialis*). Poskitt et al. (1984b) reported difficulty in locating mesenteric lymph nodes in *Antechinus stuartii* and *A. swainsonii*, and Stone et al. (1996) also reported difficulties in locating lymph nodes in *Monodelphis domestica*. Old et al. (2003) further documented the extreme difficulty of macroscopically locating the lymph nodes of *Sminthopsis macroura*.

In the Virginian opossum, the first evidence of lymph nodes was observed on the fourth day postpartum and was located near the thymus (Block, 1964). On day 5, nodes were only present in the mediastinum, and at days 6 and 7, lymph nodes were only present between the cranial region and the diaphragm. On days 8 and 9, lymph nodes were found in the caudal region to the diaphragm (Block, 1964). On days 10 and 12, there was a recognizable cortex with dense lymphatic tissues, and a medulla with medullary cords. At 17–22 days, the number and size of the lymph nodes increased. There was a clear separation of cortex and medulla at 23–32 days of age (Block, 1964). Between 45 and 65 days, the cortex now formed a complete crescent separating the subcapsular sinus from the medulla. Nuclear debris was observed in both the cortex and medulla. There was a persistent and rapid increase in size up to 65–100 days of age, and at this stage, nodules with reaction and germinal centers were observed in the cortex. There was also an increase in the number of plasma and proplasma cells (Block, 1964).

In the pouchless opossum, the first evidence of lymph nodes was seen at 3 days of age (Bryant and Shifrine, 1974), when the first nodal anlagen developed, belonging to the inferior cervical lymph nodes, and within 24 h, 14 other primordial nodes, all anterior to the diaphragm, had developed. Bryant and Shifrine (1974) termed these the "early" anterior nodal group. The remaining nodes formed a "delayed" group that did not develop until at least 7 days of age. Differentiation of the cortex

TABLE 5. Developmental milestones of the marsupial lymph nodes

Species	First appearance of primitive node structure	Initial cortex/medulla appearance	Cortex-medulla differentiation complete	Follicles first appear	Nodules (no germinal centres) first appear	Nodules with germinal centres first appear	Mature structure reached	References
Virginian opossum	Day 4 (mediastinum), Days 8–9 (caudal)	Days 10–12	Days 45–65			Days 65–100		Block, 1964
"Pouchless" opossum	Day 3 (inferior cervical), Day 7 (caudal)	Day 20				Day 60	Day 60	Bryant and Shifrine, 1974
Quokka	Day 5 (cranial)	Day 14	Days 28–35		Day 63	Day 90	Day 120–150	Ashman and Papadimitriou, 1975
Tammar wallaby						Day 90	Day 150	Basden et al., 1997; Old and Deane, 2003
Brushtail possum			Days 48–53					Baker et al., 1999
Northern brown bandicoot	Day 1 (deep inferior cervical), Day 4 (inferior cervical and axillary)	Day 40	Day 60	Day 40		Day 60	Day 60	Cisternas and Armati, 1999
Antechinus								Poskitt et al., 1984a
Stripe-faced dunnart		Day 42 (splenic)	Day 31 (cervical and mammary)	Day 100 Day 50 (splenic)		Day 100	Day 31 (cervical and mammary)	Old et al., 2003, 2004b

and medulla began in the "early" nodal group at 20 days of age, and in the "delayed" nodal group at 25–40 days (Bryant and Shifrine, 1974). Germinal centers were present in the nodes by 60 days.

In the mesenteric lymph nodes of the white-eared opossum, HLA-DR^{+} and CD3^{+} cells were first observed once the animal had reached 75-mm CRL (Coutinho et al., 1995). In juvenile and weanling animals, HLA-DR^{+} antigen presenting cells (APC) were observed in the cords and sinuses. The germinal centers contained scattered CD3^{+} cells, but these cells were more concentrated in the paracortex. IgA^{+}, CD79a^{+}, and CD79b^{+} B-cells were seen in the peripheral regions of the follicles, as well as the cords and sinuses of the medulla (Coutinho et al., 1995).

In *Antechinus* spp. within the first 2 weeks of life, the lymph nodes were poorly developed, and while lymphocytes were abundant, there was no evidence of cortico-medullary differentiation (Poskitt et al., 1984a). At 14–15 weeks of age, when the *Antechinus* thymus was fully mature, the lymph nodes were densely cellular, with well defined B- and T-lymphocyte dependent regions and large secondary follicles and germinal centers were prominent in the cortex. As the thymus involuted, the T-lymphocyte dependent regions became depleted of lymphocytes, and the cortex and paracortex were greatly reduced (Poskitt et al., 1984a).

In the bandicoot, at birth there were no lymph nodes present, except for a small anlage of the deep inferior cervical lymph node that contained a number of small lymphocytes (Cisternas and Armati, 1999). By day 4, anlage of the superior deep cervical and axillary lymph nodes had started to form. Intercostal lymph nodes were initially observed as clusters of small lymphocytes that bordered the joining of the ribs to the spine, and no other lymph nodes were present at this stage (Cisternas and Armati, 1999). By day 9, the inferior cervical nodes had increased in size and at this stage the axillary, inguinal and superior lymph nodes contained medium and small lymphocytes, and the cranial mediastinal, lumbar and mesenteric lymph node anlage appeared. A thin connective capsule surrounded the axillary, inguinal, and mesenteric lymph nodes by day 13 (Cisternas and Armati, 1999). By day 21, the developing medullary sinuses and cords were apparent. At day 31, a thin capsule and an obvious subcapsular sinus were seen. Initial differentiation of the cortex and medulla was evident by 40 days of age, and had fully differentiated by day 60. The lymph nodes of adult bandicoots were morphologically similar to the node of pouch young at 60 days, with primary and secondary follicles with germinal centers identifiable within the cortex (Cisternas and Armati, 1999).

CD5^{+} cells were first detected in the lymph nodes of the stripe-faced dunnart at day 15 (Old

et al., 2004b), and histological maturity of some lymph nodes was reached by 31 days (Old et al., 2003). In one animal at 31 days of age, a cervical lymph node as well as a mammary-associated lymph node were identified. Both nodes had a surrounding capsule and cortico-medullary differentiation was evident. The cortex was observed to contain tightly packed lymphocytes, while the medulla was less dense in lymphocytes, and contained medullary cords and sinuses (Old et al., 2003). Two lymph nodes were detected in an individual at 42 days postpartum, a hepatic lymph node and a splenic lymph node (Old et al., 2003). The splenic node was similar to that identified in a 50-day old animal. The hepatic node was not differentiated but did contain numerous lymphocytes within the matrix. The hepatic node was enclosed by a thin capsule (Old et al., 2003). The splenic node displayed early cortico-medullary differentiation and had a large subcapsular space. The splenic lymph node in a 50 day animal was observed to contain primary follicles. CD3$^+$ T-cells, as well as B-cells were detected in the lymph nodes by 50 days (Old et al., 2004b).

In the quokka, on day 5 postpartum, the earliest rudiments of the lymph nodes were seen, and the cranial lymph nodes appeared before the caudal nodes (Ashman and Papadimitriou, 1975). A vague differentiation of cortex and medulla was observed at the end of the second week, and by the end of week 3, the differentiation of the cortex and medulla was obvious (Ashman and Papadimitriou, 1975). During the fourth and fifth week, there was a gradual increase in the size of the lymph nodes, with the cortex and medulla clearly differentiated and medullary cords well formed. In week 9, occasional small primary nodules without germinal centers were seen but were larger and more numerous in week 10. A rapid increase in size was observed into the third month of life. The increase was most pronounced in the cortex, with a relative decrease in the size of the medulla (Ashman and Papadimitriou, 1975). Primary follicles were present in small numbers, with many now containing germinal centers, and plasma cells were observed for the first time. Over the next 2 months, the number of primary nodules increased, and all follicles now had germinal centers. The paracortical and subcortical areas were now well developed, and plasma cells were regularly observed. By 4–5 months, the lymph nodes exhibited the anatomical features of the mature adult nodes (Ashman and Papadimitriou, 1975).

Lymphocytes were detected in tammar wallaby lymph nodes at day 4 (Basden et al., 1997). At day 21, CD8α^+ cells were identified throughout the undifferentiated tissue bed of the node (Duncan et al., 2012). At day 24, B- and T-cells were observed (Old and Deane, 2003). By day 60, CD8α^+ cells were concentrated in the paracortical region of the node, surrounding the lymphoid follicles (Duncan et al., 2012). Germinal centers were observed in the lymph nodes of tammar wallabies as early as 60 days, and nodules were found in lymph nodes by day 90. At day 144, the increase in CD8α^+ cells corresponded with the increase in tissue size (Duncan et al., 2012). Lymph nodes had reached adult structure by day 150 (Basden et al., 1997). Significant changes in the proportions of CD3$^+$CD8a$^-$ and CD3$^+$CD8a$^+$ cells were evident as the animals aged. Although both cell types were localized in the same region in both the neonatal and adult tissue, in the adult tissue CD3$^+$CD8a$^+$ cells were also found in the border between the mantle zone and germinal centers of the secondary follicles. In the neonate tammar wallaby, CD3$^+$CD8a$^-$ cells were present in greater numbers and appeared to outnumber the CD3$^+$CD8a$^+$ cells by 2 to 1. In the adult tissue, the CD3$^+$CD8a$^+$ cells had increased significantly and the number of CD3$^+$CD8a$^-$ and CD3$^+$CD8a$^+$ cells were very similar (Duncan et al., 2012).

In the brushtail possum, distinct areas of cortex and medulla were observed in mesenteric lymph nodes collected at 48 and 53 days, however, no differentiation into follicles containing germinal centers were observed (Baker et al., 1999). CD3$^+$ cells were observed throughout the nodes, while IgG$^+$ cells were observed mainly in the cortex of the tissue (Baker et al., 1999). Before the appearance of lymphocytes in Peyer's patches (90 days), both T- and B-cells were observed in the mesenteric lymph nodes (Baker et al., 1999).

SPLEEN

The spleen is an important lymphoid tissue that, like lymph nodes, acts to filter the blood (Mebius and Kraal, 2005), removing and recycling erythrocytes and efficiently removing blood-borne pathogens as well as cellular debris. The mature spleen contains regions of both white and red pulp. The white pulp contains large numbers of lymphocytes and antigen presenting cells, while the red pulp contains large numbers of cells, including red blood cells, granulocytes, platelets, and high numbers of undifferentiated monocytes (Swirski et al., 2009). In newborn marsupials examined to date, including the Virginian opossum, quokka, tammar wallaby, and bandicoot, the spleen generally consists of undifferentiated mesenchymal cells (see Table 6), but before the end of the first week of life, hematopoietic activity can be observed within the tissue (Block, 1964; Ashman and Papadimitriou, 1975; Basden et al., 1996; Cisternas and Armati, 1999).

In newborn Virginian opossums, the spleen consisted of dense mesenchyme (Block, 1964). On day 4, small foci of basophil erythroblasts as well and rare megakaryocytes were observed, and sinusoids

TABLE 6. Developmental milestones of the marsupial spleen

Species	Haematopoiesis observed	PALS first appearance	Capsule first appears	Trabeculae first appear	Red pulp/white pulp differentiation appears	Follicles (no germinal centres) first appear	Nodules with germinal centres first appear	Mature structure reached	References
Virginian opossum	Day 4			Days 10–12	Day 10–12		Days 65–100		Block, 1964
Quokka	Day 7				Day 60				Ashman and Papadimitriou, 1975
White-eared opossum		80-mm CRL							Coutinho et al., 1995
Tammar wallaby	Day 3	Day 7		Day 50	Day 60		Day 120	Day 120	Basden et al., 1996; Basden et al., 1997; Duncan et al., 2012
Brushtail possum				Day 48					Baker et al., 1999
Northern brown bandicoot	Day 7	Day 21				Day 31	Day 60		Cisternas and Armati, 1999
Antechinus spp.							Day 100		Poskitt et al., 1984a
Stripe-faced dunnart			Day 12	Day 42	Day 43			Day 74	Old et al., 2004a,b

were more prominent. On day 5, there were many discrete islands of erythroblasts and myelocytes, and on day 6, there was a significant increase in the number, size and maturation of the hematopoietic foci (Block, 1964). The increase in erythroblasts during days 13 to 16 resulted in rapid growth of the spleen. There was an increased ratio of white to red pulp between 45 and 65 days, with a further decrease in myeloid hematopoiesis in the red pulp. The white pulp is characterized by a densely lymphocytic core, and an outer, less lymphocyte rich rim. Differentiation of lymphatic nodules, with reactionary and germinal centers was observed between 65–100 days of life (Block, 1964).

In the pouchless opossum, splenic follicles began as a narrow periarterial lymphocytic zone on days 18–20 (Bryant and Shifrine, 1974). This zone became prominent and had expanded to the terminations of the arterial tree by day 23. The follicles did not acquire germinal centers or plasma cells before 60 days of age.

In the white-eared opossum, the first HLA-DR$^+$ cells in the spleen were observed surrounding the splenic arteries in animals at 80-mm CRL (Coutinho et al., 1995). At this stage, small arteries that branched out from the trabecular arteries had CD3$^+$ lymphocyte sheaths. The peripheral cells within the sheath were CD3$^-$, CD79a$^-$, CD79b$^-$, and IgA$^-$ (Coutinho et al., 1995). Some HLA-DR$^+$ APC cells were observed scattered between the CD3$^+$ cells. In juvenile and adult animals, HLA-DR$^+$ cells were seen scattered in the splenic cords of the red pulp and were also concentrated in germinal centers. CD3$^+$ lymphocytes were observed as isolated cells in the red pulp and were also seen in the thymus-dependent areas of the spleen. B-cells that were CD79a$^+$ or CD79b$^+$ were seen at the periphery of the follicles, within the blood vessels and also in the splenic cords and sinuses (Coutinho et al., 1995). Isolated IgA$^+$ cells were seen in the red pulp.

In Antechinus spp. within the first 2 weeks of life the spleen was poorly developed, and lacked white pulp (Poskitt et al., 1984a). At 14–15 weeks of age, when the Antechinus thymus was fully mature, the spleen was densely cellular, with well defined B- and T-lymphocyte dependent regions and large secondary follicles and germinal centers were prominent in the white pulp. At 17 weeks, as the thymus involuted, the T-lymphocyte dependent regions became depleted of lymphocytes (Poskitt et al., 1984a).

At birth, the bandicoot spleen appeared as a small rudiment of closely arranged mesenchymal cells (Cisternas and Armati, 1999). By day 21 there was an increase in the overall size of the spleen, and the PALS (periarterial lymphatic sheaths) were beginning to form. By day 31, the PALS increased in size and were now prominent.

Trabeculae and the capsule were also well developed. Primary follicles linked with the PALS and the marginal zone had also now developed (Cisternas and Armati, 1999). Secondary follicles developed toward the end of pouch life and possessed germinal centers (Cisternas and Armati, 1999). The spleen of adult bandicoots displayed trabeculae that radiated and extended from the capsule throughout the spleen parenchyma, and distinct white and red pulp areas were evident. The white pulp contained small lymphocytes that enclosed arterial vessels and their branches (PALS) and follicles surrounded by the marginal zone. Primary follicles and germinal centers were apparent (Cisternas and Armati, 1999). The red pulp surrounded the white pulp, and was penetrated by trabeculae.

The stripe-faced dunnart spleen was found to be undifferentiated at birth, and by day 12, while the majority of the cells present were mesenchymal, a thin capsule and a large subcapsular space were seen (Old et al., 2004a). By 40 days, the spleen was enclosed by the thin capsule, and some small trabeculae were observed on day 42, dissecting the matrix from the capsule. At day 43, red and white pulp areas were first observed, and CD79b^{+} cells and CD3^{+} cells were also observed at this stage (Old et al., 2004b). By 57 days, the red and white pulp areas were well defined. At 60 days, larger trabeculae were evident in the matrix and lymphocyte aggregations were observed separate from the areas of red pulp, while the white pulp was surrounded by loosely packed lymphocytes (Old et al., 2004a). By 74 days, there was a clear definition of the red and white pulp. Sinusoids were observed in the red pulp, and contained blood vessels carrying erythrocytes, neutrophils, and lymphocytes (Old et al., 2004a). CD5^{+} cells were observed at day 80 (Old et al., 2004b). The spleen at this stage was similar in appearance to that at 170 days and was histologically mature.

In the quokka, for the first 4 days of life, the spleen consisted primarily of mesenchymal cells (Ashman and Papadimitriou, 1975). By the end of the first week, sinusoids had increased in number, and discrete islands of erythroblasts and myelocytes were observed. The number and size of erythroblast and myelocyte islands increased in week 2 (Ashman and Papadimitriou, 1975). At the end of the first month, erythroblastic hemopoiesis was still prominent, but myelocytic hemopoiesis was now uncommon. During the second month, the number of medium and small lymphocytes increased and formed tightly packed aggregates, and as this white pulp region became prominent, the relative abundance of red pulp was reduced. Erythroblastic hemopoiesis occurred in the red pulp, but myelocytic hemopoiesis was rare (Ashman and Papadimitriou, 1975). As more lymphocytes aggregated during the third month of life,

the prominence of the white pulp increased further, and the red pulp continued to diminish. No reactive centers were observed in nodules. Islands of erythroblastic hemopoiesis became smaller and more infrequent. In subsequent months, the spleen increased in size, and follicles were observed to possess small reactive centers, while erythroblastic hemopoiesis continued to decline (Ashman and Papadimitriou, 1975).

Early in pouch life, the tammar wallaby spleen consists mostly of undifferentiated mesenchymal cells and by day 3, the spleen exhibits limited hemopoietic activity (Basden et al., 1996). At day 7, very rare CD8α^{+} cells were observed in the spleen (Duncan et al., 2012). CD3^{+}CD8a^{−} and CD3^{+}CD8^{+} cells were observed in the PALS surrounding the follicles. A number of resident CD8α^{+} cells were present in the tissue bed at both day 14 and 21 (Duncan et al., 2012). Both CD79b^{+} and CD3^{+} cells were first observed at 21 days (Old and Deane, 2003). At day 60, areas of red and white pulp were apparent. In animals at 60 days, CD8α^{+} staining was observed in the sheath surrounding the trabeculae of the white pulp (Duncan et al., 2012). Germinal centers were apparent in the spleen at day 120. By day 144, an increase in the number of CD8α^{+} cells was apparent, with the majority of these cells confined to the PALS surrounding the lymphoid follicles (Duncan et al., 2012). The observable ratio of white:red pulp increased as the pouch young aged, and by 4 months postpartum adult structure was achieved (Basden et al., 1996).

In the brushtail possum spleen, by day 25 numerous CD3^{+}, CD79a^{+}, and IgG^{+} cells were scattered throughout the spleen (Baker et al., 1999). On day 48, CD79a^{+} and CD3^{+} lymphocytes were identified in the follicles and parafollicular areas of the spleen (Baker et al., 1999). At this stage, the spleen was also penetrated by trabeculae, and numerous CD3^{+} cells were observed surrounding these trabeculae. There was a significant increase in the numbers of CD3^{+} cells from day 25 to 100. The number of these cells was also significantly higher in the adult than at 150 days postpartum (Baker et al., 1999). Numbers of observed CD79a^{+} cells in the spleen of brushtail possums did not differ significantly from CD3^{+} cells, and increased significantly from day 25 to 100. Numbers of CD79a^{+} cells observed in adults were also significantly higher than at day 150. IgG^{+} cells in the developing brushtail possum spleen gradually increased from day 25 to 100, with the numbers of cells at 150 days also being significantly lower than adult levels (Baker et al., 1999).

Although it is known that the marsupial spleen plays an important role in immunity and hematopoiesis, to date it has not been widely examined. Studies of the marsupial spleen would benefit from further marsupial-specific reagents, to

TABLE 7. Developmental milestones of the marsupial liver

Species	Haematopoiesis first observed	Decrease in haematopoiesis	Only occasional haematopoietic foci seen	Rare islands of haematopoiesis observed	Mature structure reached	References
Virginian opossum	Day 1	Days 8–9	Days 33–45	Days 45–65		Block, 1964
"Pouchless" opossum	Day 1	Day 13	Day 30			Bryant and Shifrine, 1974
Quokka	Day 1	Day 7	Day 30	Day 90		Ashman and Papadimitriou, 1975
Tammar wallaby	Day 1	Day 14			Day 120	Basden et al., 1996
Northern brown bandicoot	Day 1	Day 7	Day 21	Day 40	Day 60	Cisternas and Armati, 1999
Stripe-faced dunnart	Day 1	Day 12	Day 31	Day 40	Days 50–56	Old et al., 2004a

explicitly phenotype the many types of cells present, as well as their development and role within the splenic tissue. For example, antibodies to marsupial T- and B-cell subsets would be valuable in determining more precisely when pouch young marsupials first become immunocompetent and provide valuable insights into the more intricate details of the development of the marsupial immune system and disease states.

LIVER

Although not directly involved in immune responses, the liver is the main site of hemopoiesis during the first few weeks of pouch life in marsupials (Table 7). It has, therefore, been included in most studies on the development of the immune tissues in marsupials (Block, 1964; Basden et al., 1996; Old and Deane, 2000). In the case of eutherian mammals, the liver has ceased its hematopoietic role prior to birth (Payushina, 2012), and the bone marrow is the primary hematopoietic organ.

At day 1 after birth in the Virginian opossum, between 30 and 50% of the liver was hematopoietic (Block, 1964). At day 2, there was an increase in the hematopoietic tissue, and by day 5, hematopoiesis was more focal. The concentration of hematopoietic cells began to decrease from days 8 and 9, while there was an increase in the ratio of mature to immature hematopoietic cells (Block, 1964). Between days 23 and 32, there was a further reduction in the amount of hematopoietic tissue, and on days 33–45, only occasional hematopoietic foci were observed. Between days 45 and 65, only one small hematopoietic island was observed per 3 to 4 lobules, and at days 65–100 only one or two foci of erythroblasts and rare megakaryocytes remained (Block, 1964).

In the pouchless opossum, hepatic hematopoiesis was minimal at birth but increased rapidly, reaching maximum intensity at 8 to 12 days of age (Bryant and Shifrine, 1974). The ensuing gradual decline mirrored an increasing myeloid and splenic hematopoiesis. A few small erythroblastic foci were still lodged in the liver at 30 days.

The bandicoot liver at birth was undifferentiated (Cisternas and Armati, 1999). At day 4, numerous islands of hemopoiesis dominated by the production of red blood cells were scattered through the parenchyma. These hemopoietic islands were widespread in the tissue by day 7, but were less numerous than at day 4 (Cisternas and Armati, 1999). Hepatocytes had started to form lobular structures by day 21 (Cisternas and Armati, 1999). At this stage, hemopoiesis was not as prominent and was concentrated in the parenchyma periphery. On day 40, the sinusoids were surrounded by lobules of hepatic cells, and hemopoiesis was only present in small, discrete islands (Cisternas and Armati., 1999). By the conclusion of pouch life, the liver had fully differentiated, and little, if any, hemopoiesis was now evident.

In the stripe-faced dunnart, like in other marsupials examined, at birth the liver was found to be hematopoietic (Old et al., 2004a), but by day 12, there was a decrease in observed hemopoietic activity compared to younger animals, and the number of hepatocytes had increased significantly (Old et al., 2004a). At 31 days postpartum, only a few small isolated pockets of hemopoietic activity were seen, and by day 40 the liver had a more mature appearance, with only a few, very small islands of hemopoietic cells scattered in the periphery of the liver. At this stage, endothelial cells lined the central veins and blood vessels and bile ducts were now visible (Old et al., 2004a). A matrix of hepatocytes was observed; however, there were no defined hepatic cords or sinuses. By 50–56 days, no distinct hemopoietic areas were detected, and the liver was made up of several lobes. The liver was histologically mature, and hepatic cords and sinuses were observed (Old et al., 2004a).

In the quokka, the liver is the only functional hematopoietic tissue at birth and at day 1 postpartum, up to 50% of the liver was occupied by hemopoietic cells (Ashman and Papadimitriou, 1975).

TABLE 8. Developmental milestones of the marsupial GALT

Species	Peyer's patches first appear	Lymphocyte aggregates first appear	Primary follicles first appear	Germinal centres first appear	References
Quokka	Day 42	Day 42			Ashman and Papadimitriou, 1975
Stripe-faced dunnart		Day 57	Day 65		Old et al., 2003, 2004b
Antechinus spp.	Day 100		Day 100	Day 100	Poskitt et al., 1984b
Tammar wallaby		Day 120			Basden et al., 1997; Old and Deane, 2003; Duncan et al., 2012

Hemopoietic tissue was diffusely scattered throughout the liver, but became focal by day 3. At the end of the first week, the proportion of hemopoietic cells had decreased (Ashman and Papadimitriou, 1975). A further decline in hemopoietic tissue was seen in week 2, and islands of hemopoietic tissue were small and infrequent by the end of the first month and diminished over the following months, with only a few small islands remaining in the parenchyma by the third month (Ashman and Papadimitriou, 1975).

In tammar wallaby pouch young examined by Basden et al. (1996), the liver was the only hematopoietic site at birth, and at 2 weeks postpartum, hematopoiesis in the liver started to decline. Adult liver structure with lobular arrangement and complete absence of hematopoietic sites was observed at day 120 (Basden et al., 1996).

Although the pattern of liver development is similar in marsupials and eutherians, the timing in comparison to birth differs, and it appears that the liver is an essential hematopoietic component in the marsupial neonate, in contrast to the eutherian neonate, where the liver has ceased its hematopoietic role prior to birth. Further, more specific phenotyping of developing lymphocytes and other blood cells in the marsupial liver would be beneficial to gain a better understanding of early hematopoiesis in these species. Identification of hematopoietic stem cells would be beneficial, and studies investigating the movement of these cells from the liver to the primary and secondary immune tissues of marsupials would aid in determining when these immune tissues are initially infiltrated by early immune cell progenitors.

BLOOD

In a number of marsupial species studied to date, including the quokka (Yadav, 1972), Virginian opossum (Block, 1964), and tammar wallaby (Basden et al., 1996), the blood of very young animals was found to contain a significant number of neutrophils, and the proportion of these cells in the blood was significantly higher than in adults of the same species. These high levels of neutrophils persisted for 2 weeks postpartum, and as neutrophils confer nonspecific immunity, these high levels are suggested be important for the defence of the young animal against harmful pathogens during very early life (Basden et al., 1996). In the tammar wallaby, the first lymphocytes and monocytes were observed in blood circulation around 5–6 days postpartum, and by day 30 postpartum, blood films of pouch young were similar to those of mature animals (Basden et al., 1996). Further, studies characterizing which cell types are present in the neonatal blood of marsupials, and when these cell types first appear, will aid our understanding of the types of early, nonspecific protection mechanisms produced by these young, vulnerable animals.

MUCOSAL-ASSOCIATED LYMPHOID TISSUES

The mucosal-associated lymphoid tissues (MALT) are considered secondary lymphoid tissues rather than primary lymphoid tissues. Some examples of MALT include the tonsils, the GALT, the bronchus-associated lymphoid tissue (BALT), and the nasal-associated lymphoid tissue. Little is known about these lymphoid tissues in marsupials, and very little is known in terms of their development in pouch young. BALT was not observed in any stripe-faced dunnart pouch young examined by Old et al. (2003), and as such, only the GALT, including Peyer's patches, has been examined in developing marsupial pouch young (summarized in Table 8), although not extensively (Bryant and Shifrine, 1974; Ashman and Papadimitriou, 1975; Poskitt et al., 1984b; Baker et al., 1999; Old et al., 2003).

In the quokka, Peyer's patches were first detected at week 6, and at this stage constituted aggregates of small and medium lymphocytes (Ashman and Papadimitriou, 1975). During the following weeks, they were observed to become both larger and more numerous. GALT in the quokka is diffuse and Peyer's patches could not be found until well after the appearance of all other lymphoid tissue.

The small and large intestine of developing brushtail possums were examined by Baker et al. (1999). At day 1, only the small intestine was present, and CD3$^+$ cells were observed from 2 days

postpartum. By 28 days, numerous CD3$^+$ cells were observed in both the small and large intestine, however, few IgG$^+$ cells were observed. Only the occasional CD79a$^+$ cells were observed in either the small or large intestine (Baker et al., 1999). Peyer's patches were not observed in the intestines of any brushtail possum pouch young examined by Baker et al., (1999) (up to 73 days).

Lymphocytes were observed in the intestinal submucosa of stripe-faced dunnart pouch young at day 31. At day 40, no lymphocyte aggregates were observed. CD3$^+$ T-cells, as well as CD79b$^+$ B-cells were observed in GALT by day 50, with CD5$^+$ cells detected slightly later at day 57 postpartum (Old et al., 2004b). On day 57, a lymphocytic aggregate was observed in the intestinal wall, but no primary follicles were observed (Old et al., 2003). At 60 days, many lymphocytes were observed scattered throughout the submucosa (Old et al., 2003). At 65 days, a number of primary follicles were observed, with a large number of these observed by day 74, however, none of these follicles displayed germinal centres. At 2.5 months, lymphoid aggregates were identified; however, no follicle structures were identified.

At 14–15 weeks of age, when the thymus of *Antechinus stuartii* and *A. swainsonii* was fully mature, the Peyer's patches were densely cellular, with well defined B- and T-lymphocyte dependent regions. Large secondary follicles and germinal centers were prominent (Poskitt et al., 1984b). As the thymus involuted, the T-lymphocyte dependent regions, the interfollicular zones, remained densely cellular.

In the tammar wallaby, the first CD8α^+ cells were observed in the intestinal tissue of pouch young at day 3. From day 4 to 60, only a small number of CD8α^+ cells persisted in the intestine (Duncan et al., 2012). CD3$^+$ T-cells first appeared on day 12, while CD5$^+$ cells were not present until day 74 (Old and Deane, 2003). At day 100, the submucosa was found to be thin and mostly noncellular. Lymphocyte aggregates were first observed at day 120 (Basden et al., 1997). By day 144, a large number of CD8α^+ cells were observed to have infiltrated the intestinal tissue. CD8α^+ cells were found predominately within the mucosal layer of the intestine (Duncan et al., 2012). Juvenile animals (~11 months) displayed well-developed submucosa with lymphocytes scattered throughout. Larger, non-encapsulated lymphocyte aggregates were also observed (Basden et al., 1997).

In eutherian mammals, particularly humans, it is known that colonization of the gastrointestinal tract (GIT) by commensal bacteria can protect the host from infection and colonization by pathogenic bacteria (Goldszmid and Trinchieri, 2012), and a similar role of commensal microorganisms could be expected to be important for the immunological protection of marsupials. Marsupials demonstrate a lengthy lactation period, and the transfer of immune cells and other components via milk, and the highly efficient uptake of these molecules in the neonates GIT have been widely suggested to be a complementary mechanism for the protection of these vulnerable young animals. To date, very little research has been conducted on the GIT of young marsupials; however, there has been some initial examination of the bacterial colonization of the GIT of marsupial pouch young. Lentle et al. (2006) examined the GIT of tammar wallaby and brushtail possum pouch young and concluded that in both species, at no stage did the microbiome of the GIT in these animals exceed five different species. In sharp contrast to this finding, Chhour et al. (2010) constructed a clone library from two tammar wallaby pouch young, and found this library to represent 53 different phylotypes belonging to four different phyla, namely the Firmicutes, Proteobacteria, Actinobacteria, and Bacteroidetes. A phylotype was identified that represented 20.9% of the total number of clones and was found to be 99% identical to *Enterococcus faecalis*. In adult tammar wallabies, this phylotype represented less than 1% of the clones collected, indicating a change in bacterial colonization of the GIT at different life stages. In humans, strains of *E. faecalis* are known to reduce the possibility of subsequent colonization by pathogens and can inhibit *Staphylococcus aureus* colonization of the mucosal surfaces by production of bacteriocins or by lowering pH (Alomar et al., 2008). A further 9.3% of the clones isolated from tammar pouch young represented a phylotype that shared 99% identity with *Escherichia coli*. In a number of mouse and pig models (McFarland, 2000), commensal *E. coli* strains were shown to prevent colonization and infection by pathogenic enterotoxigenic strains of *E. coli*. The high presence of these *E. faecalis* and *E. coli* phylotypes in the GIT of tammar wallaby pouch young warrants further investigation, as these microorganisms could be expected to protect their host in a comparable manner to the protection elicited in eutherian species and are likely to aid in development of the normal GIT.

Immunological cells and molecules, including immunoglobulins and neutrophils that are provided to the pouch young via maternal milk during pouch life are also suggested to be important for protection of the young animal (Old and Deane, 2000) and normal GIT development. In the tammar wallaby, Young et al., (1997) found that the number and diversity of cells identified in the milk varied over time, and these changes reflected different developmental stages of the young in the pouch. Neutrophils were the predominant cell type in early milk and colostrum samples, while macrophages were observed in high numbers in postlactational mammary gland secretion samples.

Lymphocytes were not observed in the colostral phase, but were observed in early and late milk samples, and eosinophils were observed in all samples collected (Young et al., 1997).

SKIN

The skin is the largest protective organ and the very first line of defence against potential pathogens. The skin not only acts as a physical barrier to prevent entry of pathogens into the body but also produces other defence mechanisms, including the alteration of pH to limit the growth of pathogenic bacteria, as well as the production of a class of antimicrobial peptides, the primary members being the defensins and the cathelicidins.

In newborn white-eared opossums (10-mm CRL), rounded HLA-DR$^+$ cells were identified in the epidermis of the forelimbs and facial region (Coutinho et al., 1995). At 14-mm CRL, these HLA-DR$^+$ intraepithelial cells had short processes and were observed in the connective tissue of the dermis, as well as amongst the epithelial cells of the hair primordia. The dendritic aspects of the cells were well defined in older animals (Coutinho et al., 1995).

The expression of antimicrobial peptides in the skin of developing pouch young has been examined in the tammar wallaby (Carman et al., 2008, 2009; Daly et al., 2008; Wang et al., 2011). Antimicrobial peptides, including a class of molecules known as cathelicidins are an important part of the innate immune system of mammals (Scott and Hancock, 2000). These compounds can target potential pathogens, and stimulate adaptive immune responses to pathogens (Shinnar et al., 2003). Carman et al. (2008) used an anti-human cathelicidin LL37 antibody to locate tammar wallaby cells that expressed a similar compound to the human cathelicidin. In 8-day old pouch young, epithelial skin cells, lung, and GIT stained positively for LL37, epithelial skin cells also exhibited positive staining at day 26. These results indicate that pouch young tammar wallabies can synthesise and express an antimicrobial peptide similar to the human cathelicidin LL37. This compound is likely to be an important mechanism in the nonspecific immune protection of the pouch young at this early stage of development.

Expression of another cathelicidin, MaeuCath1, was examined by Daly et al. (2008) in the skin and lymphoid tissues of tammar wallaby pouch young. Expression was observed at days 45–55 in the skin, thymus, lung, liver, spleen, and bone marrow. MaeuCath1 expression stabilized in the skin by 90–120 days postpartum. Levels of expression in skin increased after day 90, but did not reach the same level of expression as the primary lymphoid tissues. The expression of MaeuCath1 during pouch life is also suggested to be a critical nonspecific protection mechanism.

Carman et al. (2009) identified a novel cathelicidin (MaeuCath8) in the tammar wallaby. Expression of this antimicrobial peptide was examined from birth to adulthood. MaeuCath8 expression was observed in the blood, GIT, and spleen less than 24 h after birth, and in the skin by 7 days postpartum, indicating that tammar wallaby pouch young are able to synthesise antimicrobial peptides soon after birth. MaeuCath8 presumably plays a crucial role in the protection of the vulnerable pouch young at early stages of development, particularly in terms of areas inclined to microbial colonization, particularly the GIT and skin, where expression is commonly observed in other young mammals (Marchini et al., 2002).

Wang et al. (2011) identified 14 cathelicidin genes in the tammar wallaby genome and 12 cathelicidin genes in the opossum genome. Expression analysis of selected cathelicidins in tammar wallaby pouch young skin was also examined. Four of the six cathelicidins examined by Wang et al. (2011) were expressed in the skin of pouch young at 20–40, 70–80, and 100–120 days. These four cathelicidins were also expressed in adult animals, while the two cathelicidins not expressed in pouch young were also not expressed in adults.

DISCUSSION

The studies of the marsupial immune system to date provide a solid foundation for further, more specific examination of immunological development, and the composition of the marsupial immune system. Additional research is required, particularly to examine the structure and development of the MALT, including the tonsils, GALT, and BALT, and alternative mechanisms or protocols are required to enable easier and more successful identification of lymph nodes in pouch young animals.

Early studies on some innate mechanisms, including the composition of the blood of pouch young (Block, 1964; Yadav, 1972; Basden, 1996), the composition of the commensal flora of the gut (Lentle, 2006; Chhour et al., 2010), expression of HLA-DR in the skin of newborn opossums (Coutinho et al., 1995) and the expression of antimicrobial peptides (Carman et al., 2008, 2009; Daly et al., 2008) has laid the foundation for our current understanding of the development of innate immune protection in pouch young marsupials. Further, research on the timing of the development of the innate immune system and early immune protection in marsupials is required.

The specific phenotype of lymphocytes in the marsupial immune system, and their mechanisms of development, including the maturation of leukocytes, including neutrophils and macrophages, and

the maturation of B-lymphocytes requires additional understanding. It is particularly interesting in the context of T-lymphocytes, and the potential of marsupials to exhibit the maturation of T-lymphocytes through the paradigm of double negative (DN) $CD4^-CD8^-$, double positive (DP) $CD4^+CD8^+$, and single positive (SP) $CD4^+$ or $CD8^+$ T-cells as seen in eutherian species. A comparison of the timing of development and the distribution of these different lymphocyte subsets in the different lymphoid tissues of various marsupial species requires examination. It is also important to establish the timing, appearance and distribution of other T-cell subsets in marsupials, including the αβ- and γδ-lymphocyte populations, as well as those T-cells that are positive for the unique receptor chain, TCRμ (Parra et al., 2007, 2009a,b).

These in depth, more specific studies require the development of marsupial-specific reagents, including antibodies to specific lymphocyte markers including the characteristic cell surface markers that enable definition of the DN-phenotype (CD44, CD25, and CD117), as well as CD4 in the case of examining DN to SP progression. Although Duncan et al., (2012) used a combination of a CD8α- and CD3-antibody to indirectly identify $CD4^+$ T-cells, in terms of positive identification of $CD4^+$ T-cells, particularly in early pouch life when they are potentially DP for CD4 and CD8, a specific CD4-antibody is essential. Antibodies are also required against the five TCR chains of marsupials (α, β, γ, δ, and μ) for examination of the αβ, γδ, and μ T, lymphocyte subsets. The development of these marsupial-specific reagents in turn requires the identification of these molecules and the genes that encode them in marsupial species. Identification and characterization of the coding sequences for these important immunological genes is currently lacking in the majority of marsupial species. However, with the recent sequencing and annotation of whole genomes for the gray short-tailed opossum (Mikkelsen et al., 2007), tammar wallaby (Renfree et al., 2011), and Tasmanian devil (Miller et al., 2011; Murchison et al., 2012), marsupial-specific sequence data is more accessible than ever before, enabling more simplified subsequent characterization of these molecules in other marsupial species.

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