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Review

Marsupial immunology bounding ahead

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Abstract. Marsupial immune responses were previously touted as 'primitive' but we now know that the marsupial immune system is complex and on par with that of eutherian mammals. In this manuscript we review the field of marsupial immunology, focusing on basic anatomy, developmental immunology, immunogenetics and evolution. We concentrate on advances to our understanding of marsupial immune gene architecture, made possible by the recent sequencing of the opossum, tammar wallaby and Tasmanian devil genomes. Characterisation of immune gene sequences now paves the way for the development of immunological assays that will allow us to more accurately study health and disease in marsupials.

Additional keywords: immunity, immunoglobulin, metatherian, MHC.

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Introduction

Marsupials provide excellent models for studying immunity. They occupy a key position on the vertebrate phylogenetic tree, having diverged from eutherian (placental) mammals ~148 million years ago (Bininda-Emonds *et al*. 2007). Once believed to have 'primitive' immune responses (Jurd 1994), we now know that the immune system of marsupials is just as intricate and complex as that of their eutherian counterparts. Moreover, their genomes provide a treasure trove of genetic information that allows us to gain insights into how the mammalian immune system evolved. By studying immune genes in different marsupial lineages we can see how different pathogens have shaped their immune systems. Understanding immune responses in marsupials also allows us to study health and disease in marsupials, many of which are threatened or endangered. Finally, marsupials are different to eutherians in that they give birth to altricial young without a developed adaptive immune system (Tyndale-Biscoe and Renfree 1987). At birth a newborn opossum is equivalent developmentally to an eight-week-old human foetus (Block 1964); therefore, they are excellent models for studying developmental immunology. Here we review the field of marsupial immunology, which has 'bounded' ahead in recent years with the advent of molecular genetics and the sequencing of several marsupial genomes. We pay tribute to the key role that Professor Des Cooper has played in the advancement of this field.

Marsupial immune tissues

The marsupial immune system develops in a similar way to that observed in eutherian mammals; however, the tissues develop and mature in a non-sterile environment (Deane and Cooper 1984; Basden *et al*. 1997; Old and Deane 2000), rather than a sterile uterus. Despite marsupials lacking mature immune tissues at birth (reviewed in Old and Deane 2000), they manage to survive in the hostile extrauterine environment by relying on maternal strategies such as immunoglobulins in milk, as shown by Deane and Cooper (1984), as well as other strategies (recently reviewed by Edwards *et al*. 2012). In this section we describe the cell and tissue architecture of the immune tissues of marsupials.

Research on development of immune tissues in marsupials has been limited to several species, the Virginian opossum (*Didelphis virginiana*) (Block 1964), Robinson's mouse opossum (previously called *Marmosa mitis* (Bryant and Shifrine 1974) but now referred to as a subspecies of *Marmosa robinsoni* (Bryant 1977)), quokka (*Setonix brachyurus*) (Ashman and Papadimitriou 1975*a*, 1975*b*), tammar wallaby (*Macropus eugenii*) (Basden *et al*. 1996, 1997), Brazilian white-bellied opossum (*Didelphis albiventris*) (Coutinho *et al*. 1995), striped-face dunnart (*Sminthospsis macroura*) (Old and Deane 2003; Old *et al*. 2004*a*, 2004*b*), brushtail possum (*Trichosurus vulpecula*) (Baker *et al*. 1999) and northern brown bandicoot (*Isoodon macrourus*) (Cisternas and Armati 1999). Readers are referred to Table 1 for a comprehensive summary of research papers in the field. All these marsupial species follow a similar pattern of development of immune tissues, which is similar to that of eutherians.

At birth, the marsupial liver is actively haematopoietic and a variety of cell types are visible at various stages of development including those of the erythrocytic, granulocytic and leucocytic lineages (Block 1964; Ashman and Papadimitriou 1975*a*, 1975*b*; Basden *et al*. 1996). Throughout pouch life haematopoiesis

declines in the liver and the bone marrow takes over the role, whilst the liver structure matures histologically and becomes largely restricted to gastrointestinal-related functions (Block 1964; Basden *et al*. 1996). The pattern of bone marrow development is similar to that of eutherians; however, the bone marrow in eutherians is actively haematopoietic at birth, and in metatherians the liver plays the main role in haematopoiesis at birth.

The thymus is the first lymphoid tissue to develop and become histologically mature in all marsupials. Johnstone (1898) was one of the first authors to note that some marsupials have only a thoracic thymus whereas others possess both a thoracic and a cervical thymus. Interestingly, he also included a description of the thymus from the now-extinct Tasmanian tiger (*Thylacinus cynocephalus*) and that from a rarely encountered marsupial mole (*Notoryctes* spp.), amongst others (Johnstone 1898). Since then, many investigators have described the thymus in a range of marsupials (Miller *et al*. 1965; Ashman *et al*. 1972; Turner *et al*. 1972; Yadav *et al*. 1972*a*, 1972*b*, 1974; Yadav 1973; Ashman and Papadimitriou 1975*a*, 1975*b*; Poskitt *et al*. 1984*a*; Canfield *et al*. 1996; Basden *et al*. 1997). In those marsupial species that have both cervical and thoracic thymuses the cervical thymus is larger and develops earlier (Yadav *et al*. 1972*a*, 1972*b*). Stanley *et al*. (1972) suggested, on the basis of thymectomy studies, that despite the cervical thymus becoming functional before the thoracic thymus, the cervical and thoracic thymus in the quokka had the same functional role (Stanley *et al*. 1972). More recently Haynes (2001) reviewed the location of the thymuses in several marsupial species and found that some also had aberrant thymic tissue present (Haynes 2001). A transcriptomic study by Wong *et al*. (2011*a*) compared gene expression patterns in the cervical and thoracic thymus of a juvenile tammar wallaby and found there to be no difference in the expression of key markers (Wong *et al*. 2011*a*). Hence, the reason for some marsupial species having one or two thymuses and/or aberrant thymic tissue, and others not, remains unexplained.

As in eutherians, the marsupial thymus is the key organ responsible for the maturation of T-cells, whereby immature cells migrate from the bone marrow to the thymic medulla, and then into the thymic cortex where they mature (Schuurman *et al*. 1997). The pattern of thymic development and T-cell maturation can be visualised in marsupials with the aid of a cross-species reactive antibody to a conserved region of the T-cell-specific CD3ε molecule (Jones *et al*. 1993; Old *et al*. 2001, 2006) and shown to cross-react in a range of marsupial species (Hemsley *et al*. 1995; Old and Deane 2002*a*; Old *et al*. 2006). T-cells in marsupials can also be identified with the use of an antibody to CD5 (Jones *et al*. 1993; Hemsley *et al*. 1995; Old and Deane 2001, 2003).

Thymus maturity is characterised by the appearance of a clearly defined cortex and medullary regions, and Hassall's corpuscles. Early thymectomy studies conducted in Virginian opossums at one week postpartum found a decrease in lymphocytes in the spleen, blood and lymph nodes, with the spleen exhibiting an increase in myeloid tissue. These studies confirmed that the thymus in marsupials plays a crucial role in the origin and maintenance of lymphoid tissue (Miller *et al*. 1965).

The secondary lymphoid tissues have also been described in marsupials, including the spleen, lymph nodes and mucosal-associated lymphoid tissues (MALT). Lymphocytes start to appear and mature in these tissues much later than in the thymus (reviewed in Old and Deane 2000). In adults, the spleen and lymph nodes, with characteristic follicles and germinal centres scattered throughout, are locations for B-cell proliferation and further development.

The mature splenic tissue in marsupials is similar to that described in eutherian mammals with areas of white and red pulp and follicles with germinal centres scattered throughout. Some differences have been noted in the size and number of sinuses, trabeculae and follicles, and appear to be due to differences in individual antigenic challenges or species-related differences (Hayes 1968; Cutts and Krause 1982; Poskitt *et al*. 1984*a*; Coutinho *et al*. 1995; Stone *et al*. 1996). Several authors have described the development of the spleen in marsupials (Block 1964; Cutts and Krause 1982; Basden *et al*. 1996; Baker *et al*. 1999; Old and Deane 2002*b*; Old *et al*. 2004*a*). In all species, the spleen develops from mesenchymal tissue that becomes populated by a range of early erythrocytes and megakaryocytes, followed by a range of granulopoietic cells and eventually develops adult splenic architecture (Cutts and Krause 1982).

The lymphatic system of marsupials has been studied in *Didelphis azarae* and *Didelphis marsupialis* (Azzali and Didio 1965) but is otherwise restricted to characterisation of lymph nodes in other species. Lymph nodes are unique to eutherian and marsupial mammals. Distinct lymph nodes are absent from monotremes and other vertebrate groups. The numbers and locations of lymph nodes vary among marsupial species (Bryant and Shifrine 1974), with many researchers noting the difficulty of locating lymph nodes, particularly in smaller species (Poskitt *et al*. 1984*a*; Stone *et al*. 1996; Old and Deane 2003). Regardless of their location, the anatomical structure of each node remains similar, as evidenced in several opossum species (Bryant and Shifrine 1974; Chiarini-Garcia and Pereira 1999), the tammar wallaby (Basden *et al*. 1997), *Antechinus* spp. (Poskitt *et al*. 1984*a*), fat-tailed and stripe-faced dunnarts (Haynes 1991), koala and brushtail and ringtail possums (Hemsley *et al*. 1996) and the rufous-hare wallaby (Young *et al*. 2003). Lymph nodes in marsupials, as in eutherians, have distinct cortical and medulla zones, medullary cords and sinuses, trabeculae, primary and secondary follicles, and germinal centres. Bryant (1974) described the development of the mesenteric lymph nodes in *Marmosa mitis*, whereby lymphocytes initially infiltrated the mesenchyme between an artery and the lymphatic space, after which time they quickly matured into the structures observed in adults (Bryant and Shifrine 1974).

More recently, effort has focussed on lesser-represented immune tissues, particularly two areas of mucosal-associated lymphoid tissues (MALT): gut-associated lymphoid tissues (GALT) and the bronchus-associated lymphoid tissues (BALT). There have also been specific studies on kowari tonsils (Nishikawa and Takagi 1988) and the oropharyngeal tonsils of the koala, and brushtail and ringtail possums (Hemsley *et al*. 1995, 1996).

GALT have been reported in marsupials by a few researchers yet relatively little functionality of the GALT in marsupials has

Table 1. Summary of characterised marsupial immune tissues

Species	Tissue examined	Reference
Brazilian white-bellied opossum, *Didelphis albiventris*	Thymus	Coutinho *et al*. 1995
	Spleen	Coutinho *et al*. 1995
	Lymph nodes	Coutinho *et al*. 1995; Chiarini-Garcia *et al*. 2000
	GALT	Coutinho *et al*. 1993, 1994
Brown antechinus, *Antechinus stuartii*	Lymph nodes	Poskitt *et al*. 1984*a*
	Thymus	Poskitt *et al*. 1984*b*, 1984*c*
	Spleen	Poskitt *et al*. 1984*b*
	GALT	Poskitt *et al*. 1984*b*, 1984*c*
	Thymus	Haynes 2001
Brushtail possum, *Trichosurus vulpecula*	Thymus	Johnstone 1898; Fraser and Hill 1916; Hemsley *et al*. 1995; Baker *et al*. 1999; Old and Deane 2003
	Liver	Old and Deane 2003
	Spleen	Hemsley *et al*. 1995; Baker *et al*. 1999; Old and Deane 2003
	Lymph nodes	Hemsley *et al*. 1995; Hemsley *et al*. 1996; Baker *et al*. 1999; Old and Deane 2003
	BALT	Cooke and Alley 2002; Old and Deane 2003
	Tonsils	Hemsley *et al*. 1995; Hemsley *et al*. 1996
	GALT	Hemsley *et al*. 1995; Hemsley *et al*. 1996; Baker *et al*. 1999; Old and Deane 2003
Common wombat, *Vombatus ursinus*	Thymus	Fraser and Hill 1916
Big-eared opossum, *Didelphis aurita*	Lymph nodes	Chiarini-Garcia and Pereira 1999
Didelphis azarae	Lymphatic system	Azzali and Didio 1965
Common opossum, *Didelphis marsupialis*	Lymphatic system	Azzali and Didio 1965
Didelphys murina	Thymus	Johnstone 1898
Didelphys pusilla	Thymus	Johnstone 1898
Eastern barred bandicoot, *Perameles gunni*	Thymus	Johnstone 1898
Eastern grey kangaroo, *Macropus giganteus*	Lymph nodes	Old and Deane 2001
	GALT	Old and Deane 2001
Eastern quoll, *Dasyurus viverrinus*	Thymus	Johnstone 1898
Fat-tailed dunnart, *Sminthopsis crassicaudata*	Lymph nodes	Haynes 1991
	Thymus	Haynes 2001
Feathertail glider, *Arcobates pygmaeus*	Thymus	Johnstone 1901
Agile gracile opossum, *Gracilinanus agilis*	Lymph nodes	Chiarini-Garcia and Pereira 1999
Gray short-tailed opossum, *Monodelphis domestica*	Thymus	Hubbard *et al*. 1991
Koala, *Phascolarctos cinereus*	Tonsils	Hemsley *et al*. 1995, 1996
	GALT	Hanger and Heath 1994; Hemsley *et al*. 1995, 1996
	Lymph nodes	Hanger and Heath 1994; Hemsley *et al*. 1995, 1996
	Thymus	Johnstone 1898; Fraser and Hill 1916; Hemsley *et al*. 1995; Canfield *et al*. 1996; Haynes 2001;
	Tonsils	Hemsley *et al*. 1995
Ringtail possum, *Pseudocheirus peregrinus*	Tonsils	Hemsley *et al*. 1995
Kowari, *Dasyuroides byrnei*	Tonsils	Nishikawa and Takagi 1988
Kultarr, *Antechinomys laniger*	Thymus	Johnstone 1898
Long-nosed bandicoot, *Isoodon nasuta*	Thymus	Fraser and Hill 1916
Marmosa mitis	Thymus	Bryant and Shifrine 1974
	Spleen	Bryant and Shifrine 1974
	Lymphatic system	Bryant and Shifrine 1974
	Lymph nodes	Bryant 1974; Bryant and Shifrine 1974
	Tonsils	Bryant and Shifrine 1974
	GALT	Bryant and Shifrine 1974
Marmosops incanus	Lymph nodes	Chiarini-Garcia and Pereira 1999
Macropus wilcoxii	Thymus	Johnstone 1898
Marsupial mole, *Notoryctes* spp.	Thymus	Johnstone 1898
Brown four-eyed opossum, *Metachirus nudicaudatus*	Lymph nodes	Chiarini-Garcia and Pereira 1999
Northern brown bandicoot, *Isoodon macrourus*	Liver	Cisternas and Armati 1999
	Thymus	Cisternas and Armati 1999; Haynes 2001
	Thymus	Cisternas and Armati 1999
	Spleen	Cisternas and Armati 1999, 2000
	Lymph nodes	Cisternas and Armati 1999, 2000; Old and Deane 2002*a*

(*Continued next page*)

Table 1. (*continued*)

Species	Tissue examined	Reference
	GALT	Old and Deane 2002*a*
	Thymus	Johnstone 1898
Numbat, *Myrmecobius fasciatus*	Thymus	Johnstone 1898
Long-nosed potoroo, *Potorous tridactylus*	BALT	Young 2012
Petaurus sp.	Thymus	Johnstone 1898
Gray four-eyed opossum, *Philander opossum*	Lymph nodes	Chiarini-Garcia and Pereira 1999
Long-footed potoroo, *Potorous longipes*	BALT	Young 2012
Quokka, *Setonix brachyurus*	Liver	Ashman and Papadimitriou 1975*a*, 1975*b*
	Thymus	Yadav and Papadimitriou 1969; Papadimitriou and Ashman 1972, Ashman and Papadimitriou 1975*a*, 1975*b*;Yadav 1973; Yadav *et al*. 1972*a*, 1972*b*, 1974; Stanley *et al*. 1972
	Bone marrow	Ashman and Papadimitriou 1975*a*
	Spleen	Ashman and Papadimitriou 1975*a*
	Lymph nodes	Ashman and Papadimitriou 1975*a*
	GALT	Ashman and Papadimitriou 1975*a*
Red-necked wallaby, *Macropus rufogriseus*	Thymus	Symington 1898
Red-tailed phascogale, *Phascogale calura*	Spleen	Old *et al*. 2006
	Lymph nodes	Old *et al*. 2006
	GALT	Old *et al*. 2006
	BALT	Old *et al*. 2006
Ringtail possum, *Pseudocheirus peregrinus*	Lymph nodes	Hemsley *et al*. 1996
	Tonsils	Hemsley *et al*. 1996
	GALT	Hemsley *et al*. 1996
Rufous hare-wallaby, *Lagorchestes hirsutus*	BALT	Young *et al*. 2003
	GALT	Young *et al*. 2003
	Spleen	Young *et al*. 2003
	Lymph nodes	Young *et al*. 2003
Southern brown bandicoot, *Isoodon obesulus*	Thymus	Haynes 2001
Southern hairy-nosed wombat, *Lasiorhinus latifrons*	Thymus	Haynes 2001
Striped-face dunnart, *Sminthopsis macroura*	Thymus	Old *et al*. 2004*a*, 2004*b*, 2003*a*, 2003*b*
	Liver	Old *et al*. 2004*a*, 2004*b*, 2003*a*
	Bone marrow	Old *et al*. 2004*a*, 2004*b*, 2003*a*
	Spleen	Old *et al*. 2004*a*, 2004*b*, 2003*a*
	Lymph nodes	Old *et al*. 2004*a*, 2003*a*, 2003*b*
	GALT	Old *et al*. 2004*a*, 2003*a*, 2003*b*
Tammar wallaby, *Macropus eugenii*	Thymus	Johnstone 1898; Basden *et al*. 1997; Old and Deane 2003; Duncan *et al*. 2012
	Bone marrow	Basden *et al*. 1996; Old and Deane 2003; Carman *et al*. 2008
	Spleen	Basden *et al*. 1996; Old and Deane 2002*b*, 2003; Duncan *et al*. 2012
	GALT	Basden *et al*. 1997; Old and Deane 2002*b*, 2003; Carman *et al*. 2008; Duncan *et al*. 2012
	BALT	Old and Deane 2002*b*, 2003
	Lymph nodes	Basden *et al*. 1997; Old and Deane 2002*b*, 2003; Carman *et al*. 2008; Duncan *et al*. 2012
	Liver	Basden *et al*. 1996; Old and Deane 2003
Tasmanian devil, *Sarcophilus harrisii*	Thymus	Haynes 2001
Thylacine, *Thylacine cynocephalus*	Thymus	Johnstone 1898
Virginian opossum, *Didelphis virginiana*	Thymus	Block 1964
	Bone marrow	Block 1964
	Spleen	Block 1964; Hayes 1968; Cutts and Krause 1982
	Lymph nodes	Block 1964
	GALT	Block 1964
	Liver	Block 1964
Western grey kangaroo, *Macropus fuliginous melanops*	Thymus	Haynes 2001
Yellow footed antechinus, *Antechinus flavipes*	Thymus	Haynes 2001
	Spleen	Poskitt *et al*. 1984*a*
	Lymph nodes	Poskitt *et al*. 1984*a*
	GALT	Poskitt *et al*. 1984*a*, 1984*b*
	Thymus	Poskitt *et al*. 1984*a*, 1984*c*

been uncovered. To date, research has largely been restricted to descriptions of the tissues, and in some studies has included the use of cross-species reactive antibodies to identify specific immunological cell types (Hemsley *et al*. 1996; Old *et al*. 2001, 2004*a*; Old and Deane 2002*a*; Young *et al*. 2003). Differences among marsupial GALT appearance have been demonstrated in many of these studies, whereby some have described scattered lymphocytes throughout the gastrointestinal tract, areas with lymphocytic aggregations and still others with clearly defined follicles and germinal centres. Peyer's patches and other lymphoid patches have been described in white-bellied opossums (Coutinho *et al*. 1993), *Antechinus* spp. (Poskitt *et al*. 1984*b*), possums and koalas (Hanger and Heath 1994; Hemsley *et al*. 1996), the tammar wallaby (Basden *et al*. 1997; Old and Deane 2003), rufous hare-wallaby (Young *et al*. 2003), eastern grey kangaroo (Old and Deane 2001) and northern brown bandicoot (Old and Deane 2002*a*). Perhaps one of the main reasons for the differences observed in GALT structure is that they include descriptions from different regions throughout the gastrointestinal tract. It may also be a reflection on the antigenic challenge faced by the animal, or may even be impacted by dietary differences among marsupials. It is interesting that similar differences in GALT organisation are also seen in domestic (eutherian) animals and are reviewed in Liebler-Tenorio and Pabst (2006).

A few researchers have also focussed on developmental aspects of the immune cells and tissues within the gut of the Brazilian white-bellied opossum (Coutinho *et al*. 1994), the tammar wallaby (Basden *et al*. 1997; Old and Deane 2003) and the stripe-faced dunnart (Old *et al*. 2004*a*).

Studies of adult marsupial BALT have increased recently, mostly due to the increased occurrence and recognition of tuberculosis infection, particularly among endangered and threatened marsupial populations (reviewed in Buddle and Young 2000), and the ongoing efforts in New Zealand to decrease tuberculosis transference from feral brushtail possums to livestock (Buddle *et al*. 2000). Cooke and Alley (2002), for example, have described the development of the lungs in the brushtail possum and described discrete lymphoid aggregates with lymphoid follicles and clearly defined germinal centres as well as occasional lymphoid aggregates beneath the pleura (Cooke and Alley 2002). Young *et al*. (2003) compared the lymphoid tissues, including BALT, in normal and mycobacterial-affected rufous hare-wallabies (*Lagorchestes hirsutus*) using histological and immunohistological techniques and found small aggregations of lymphocytes associated with the bronchi. In a later study of the lung tissues of potoroos (*Potorous longipes*, the long-footed potoroo, and *P. tridactylus*, the long-nosed potoroo) also exposed to this pathogen, BALT was located adjacent to bacterial sites and in areas consistent with airway exposure (Young 2012). Whether or not these animals develop BALT before any deliberate infection remains to be determined, but it is reasonable to assume that BALT arises as a consequence of mycobacterial disease in these species. It would be interesting, therefore, to assess the role of antigenic challenge on MALT development in marsupials, particularly BALT. It is interesting to note that in eutherians BALT is not constitutively present and is dynamic (Liebler-Tenorio and Pabst 2006). This also appears to be the case in marsupials.

Lymphocyte development and the ontogeny of marsupial immunocompetence

The altricial nature of marsupials at birth obviously raised questions regarding the timing of immune cell development in pre- and postnatal marsupial young. Successes with some available antibody reagents and the advent of gene sequencing and gene discovery (discussed in more detail in the following section) have facilitated immune cell ontogeny studies in marsupials and, more specifically, the timing of B- and T-cell development. $CD79b^{+}$ (a marker of B-cell lineage) lymphocytes were detected as early as postnatal Day 7 in tammar wallaby gut tissues using immunohistochemistry (Old and Deane 2003). Another study of B-cell lineage commitment in wallabies found CD79a transcription as early as postnatal Day 10, although that was the earliest time point investigated (Duncan *et al*. 2010). In the opossum, *M. domestica* commitment to the B lymphocyte lineage, based on CD79a and CD79b transcription was detectable earlier, in prenatal embryos (Wang and Miller 2012).

Based on the timing of gene rearrangements at the immunoglobulin heavy and light chains, the earliest timepoint that a newborn opossum would be able to generate endogenous antibody responses is still at least one week of age (Wang and Miller 2012; Wang *et al*. 2012*b*). Although Ig heavy-chain gene rearrangements can be detected within the first postnatal 24 h, the expression of the surrogate light chains and Ig light chain gene rearrangement is relatively delayed until near the end of the first postnatal week (Wang *et al*. 2012*a*, 2012*b*). The results are consistent with early studies on the ontogeny of antibody responses (Kalmutz 1962; La Via *et al*. 1963). This is not much different from studies of brushtail possum (*Trichosurus vulpecula*) pouch young where Ig heavy-chain transcripts could be detected at postnatal Day 10 and were likely there earlier (Belov *et al*. 2002*b*).

The diversity of heavy chains expressed early in ontogeny in the opossum is relatively restricted. This is most likely due to a limited number of B-cells rather than a bias in what genes are expressed (Wang *et al*. 2012*b*). Within the second postnatal week antibody diversity increases, mostly due to the diversity found in light chains (Wang *et al*. 2012*b*). Indeed, light-chain diversity appears to play a significant role in overall antibody diversity, an observation predicted by the complexity of the light-chain genes compared with heavy chains (discussed later in this review).

T-cell development is characterised by the appearance of CD3 by Day 12 postpartum before the appearance of CD5 at Day 50 postpartum in the dunnart thymus (Old *et al*. 2004*a*). However, in the tammar wallaby thymus both CD3 and CD5 are present by Day 12 (Old and Deane 2003). Baker *et al*. (1999) identified CD3+ cells in the brushtail possum thymus by Day 2 postpartum.

There is evidence of αβ T-cells within the first 24 postnatal hours in the opossum, suggesting that their differentiation also begins prenatally and before clear histological evidence of a thymus (Hubbard *et al*. 1991; Parra *et al*. 2009). What is unusual, for a mammal, is that αβ T-cell development appears to be initiated before that of the γδ T-cell lineage (Parra *et al*. 2009). In eutherians, γδ T-cells appear in the fetal thymus before αβ T-cells (reviewed in Hayday 2000). What significance this difference may have for the developing marsupial is not known.

One of the holy grails of marsupial immunology was the discovery of novel adaptations associated with their altricial birth. The discovery of a third major lineage of T-cells, called TCRμ$^+$ T-cells in marsupials raised the possibility of such novelties (Parra *et al*. 2007) (discussed in more detail later). An initial hypothesis regarding the function of TCRμ$^+$ T-cells centred on their being relevant to immune protection in early developing marsupials, a hypothesis supported by the absence of these cells from all eutherians analysed (Parra *et al*. 2008). This appears now not to be the case for two reasons. First, this hypothesis would predict the development of TCRμ$^+$ T-cells early in postnatal ontogeny; TCRμ$^+$ T-cells, however, are the last of the T-cell lineages to appear, at nearly two weeks of age (Parra *et al*. 2009). Second, TCRμ$^+$ T-cells or analogous T-cell subsets are found in several non-mammalian vertebrate lineages, suggesting that they are not a unique adaptation in marsupials, but rather an ancient lineage (Parra *et al*. 2010, 2012*b*).

Advancing our understanding of marsupial immunity using molecular genetics and comparative genomics

Rapid elucidation of marsupial immune genes began in the mid-1990s with the use of heterologous probes to isolate cDNA sequences from cDNA libraries, leading to the isolation of the relatively conserved immunoglobulin, T-cell receptor and MHC genes (described in detail below). Further advances were made after the release of the first marsupial genome in 2007. The sequencing of the opossum genome, a first for a marsupial (Mikkelsen *et al*. 2007), allowed elucidation of the composition and organisation of immune gene clusters and, following the sequencing of the tammar wallaby (Renfree *et al*. 2011) and Tasmanian devil (Murchison *et al*. 2012) genomes, provided an opportunity to study the evolution of these immune gene families. Key findings are summarised below.

Immunoglobulins

Elucidating the content and complexity of the genes encoding immunoglobulin (Ig) heavy and light chains revealed features that are both unique to marsupials as well as establishing time points for major evolutionary events in mammalian antibody evolution. In many ways the marsupial Ig genes are 'typically' mammalian in that they encode the IgM, IgG, IgE, and IgA heavy-chain isotypes (Aveskogh and Hellman 1998; Miller *et al*. 1998; Belov *et al*. 1998, 1999*a*, 1999*b*, 1999*c*). The discovery of IgG and IgE, in particular, in marsupials, and later monotremes, confirmed the early emergence of these uniquely mammalian antibody isotypes, through gene duplication (Aveskogh and Hellman 1998). The opossum Ig heavy (*Igh*) locus, however, is a minimal locus for a mammal. The genes for each of the IgM, IgG, IgE and IgA isotypes are single copy, and IgD is missing, apparently due to a deletion/replacement event involving retroelements (Wang *et al*. 2009). In the opossum there are only 19 functional VH genes and only two of the four JH genes are expressed. Hence, the overall contribution of heavy chains to antibody diversity is rather limited in the opossum. This is probably true for most or all marsupial species (Baker *et al*. 2005).

Like eutherians, marsupials have both the κ and λ Ig light chains (Lucero *et al*. 1998; Miller *et al*. 1999; Belov *et al*. 2001, 2002*a*). In contrast to the *Igh* locus, the genes encoding the antibody light chains are far more complex (Wang *et al*. 2009). This pattern of limited heavy-chain diversity, but complex light-chain diversity appears to be found across marsupials (Baker *et al*. 2005). This was an unexpected finding given that most other vertebrate lineages display a concerted or coevolving complexity for heavy and light chains (Sitnikova and Su 1998). So far, marsupials appear unique in appearing to rely on complex light-chain genes to compensate for limited germ-line heavy-chain diversity.

T-cell receptors

Arguably, the analyses of marsupial T-cell receptor (TCR) genes may have yielded one of the more novel discoveries in the marsupial immune system. T-cells are a critical cell type in the adaptive immune response of all vertebrates and a breakthrough in the understanding of T-cell biology and antigen recognition came in the early 1980s with the discovery of the αβ TCR. The discovery of the αβTCR was quickly followed by the discovery of a second receptor, the γδTCR, that defines a second lineage of T-cells associated primarily with epithelial sites (reviewed in Hayday 2000). By the mid-1990s it was clear that αβTCR and γδTCR were conserved across the jawed vertebrates. The loci encoding αβTCR and γδTCR chains have been characterised in the opossum in great detail and they are highly conserved with eutherian mammals both in content and organisation (Parra *et al*. 2008).

An analysis of the northern brown bandicoot (*Isoodon macrourus*) thymus transcriptome led to the discovery of a novel TCR chain that was ultimately named TCRμ (Baker *et al*. 2007; Parra *et al*. 2007). At first, TCRμ appeared to be a second TCRδ; however, its characteristics were sufficiently novel that it was given its own designation. Nonetheless, TCRμ clearly shares an evolutionary history with TCRδ and represented the first new TCR chain in a quarter century (Parra *et al*. 2007, 2012*a*). TCRμ also shares an evolutionary history with antibodies and current structural models suggest that it may bind antigen directly rather than antigen presented on MHC molecules like conventional TCR.

As stated earlier, it was first thought that TCRμ was novel to marsupials. It is now clear that TCRμ is an ancient TCR chain that is present in the platypus, and therefore was likely present in the last common ancestor of all living mammals, but was lost in the eutherian lineage (Wang *et al*. 2011*b*; Parra *et al*. 2012*a*). This is a rare example of discovery of novel genes and gene products in a marsupial that were not first discovered in mice or humans. Des Cooper would smile at that.

Major histocompatibility complex

Des Cooper was particularly interested in the marsupial Major Histocompatibility Complex (MHC) as a means to study immunological fitness of marsupial populations. MHC genes can be divided into three classes, with Class I and Class II genes involved in antigen presentation. Classical Class I genes are generally ubiquitously expressed on all nucleated cells and encode cell-surface molecules that present endogenously derived peptides to CD8+ cytotoxic T-cells. Classical Class II molecules present exogenously derived peptides to CD4+ helper T-cells and

are expressed on B-cells, dendritic cells and macrophages. The classical MHC Class I and Class II genes are known to be the most polymorphic genes in the vertebrate genome, with diversity concentrated in their peptide-binding regions. Antibodies against human MHC Class II (DRB) cross-react with Class II molecules in the white-eared opossum (Coutinho *et al*. 1995), koala (Canfield and Hemsley 2000) and red-tailed phascogale immune tissues (Old *et al*. 2006). However, gene characterisation reveals that marsupial Class II genes are paralogous to those found in eutherian mammals (Belov *et al*. 2004). Antibodies against eutherian Class I molecules do not cross-react with marsupial Class I molecules. Class I and Class II genes have been well characterised at the molecular level in the opossum, tammar wallaby, Tasmanian devil and brushtail possum. Identified genes are provided in Table 2, and readers are encouraged to seek additional details about methods of gene discovery and key findings about each gene from the references provided. It is important to note that functional studies of these molecules are only just beginning and it is premature to speculate on the likely functions of MHC Class I sequences that do not have clear features of classical molecules, i.e. ubiquitous expression, high polymorphism in the peptide-binding region.

Genomic organisation of the MHC in marsupials

Fluorescent *in situ* hybridisation has revealed that the opossum MHC is located on Chromosome 2q (Gouin *et al*. 2006), that of the tammar wallaby on 2q (Deakin *et al*. 2007) and that of the Tasmanian devil on 4q (Cheng *et al*. 2012*a*). Extensive research has been conducted on the gene content, gene organisation and evolution of the MHC regions of the opossum, tammar wallaby and Tasmanian devil (Belov *et al*. 2006; Siddle *et al*. 2011; Cheng *et al*. 2012*a*). These studies reveal that the ancestral marsupial MHC was likely a single cluster of genes, which was more similar in terms of gene organisation to that of non-mammals than to that of eutherian mammals. However, in the tammar wallaby MHC the classical Class I genes are not linked to the rest of the MHC and have spread throughout the genome (Deakin *et al*. 2007), possibly due to retroviral activity. Readers are referred to a recent review on the subject (Cheng and Belov 2012).

Diversity in the MHC

The area of research into marsupial MHC diversity was pioneered by Des Cooper, who together with Louise McKenzie published the first diversity study in 1994 (McKenzie and Cooper 1994). They used restriction fragment-length polymorphism analysis to measure MHC Class II β chain diversity in Garden Island and Kangaroo Island tammar wallabies and concluded that MHC diversity in these populations was low. It was not till exhaustive gene cloning and characterisation occurred that we were able, 15 years later, to overturn these results and show that MHC Class II DBB diversity in the Kangaroo Island tammars is actually high (Cheng *et al*. 2009). This was done using higher-resolution methods including sequencing of peptide-binding regions and use of MHC-linked microsatellites.

MHC diversity studies in marsupials are becoming more popular for a range of reasons, but are always initially hampered by the laborious gene characterisation required to develop locus-specific markers, a difficult feat as the MHC evolved rapidly through gene duplication and different species have stark differences in the number of gene copies in their MHC.

The study of MHC diversity in populations can provide an indirect measure of the immunological fitness of these populations. Populations with low MHC diversity are theoretically less likely to be able to mount appropriate responses to new disease threats. The Tasmanian devil provides a striking example of this. We have shown that MHC diversity in Tasmanian devils is critically low at both MHC Class I and MHC Class II (Siddle *et al*. 2007*a*; Cheng *et al*. 2012*b*), reducing their likelihood of being able to respond effectively to new disease threats. Interestingly, the pattern of MHC diversity in devils, compared with that of neutral markers, is reminiscent of a selective sweep that led to high frequencies of certain MHC alleles. Recent reports of a distempter-like disease in dasyurids at the turn of the century (Paddle 2012) may provide a possible

Table 2. Summary of known marsupial MHC genes

Species	Gene	No. of loci	Notes	References
Gray short-tailed opossum, *Monodelphis domestica*	Class I	11	UA1 – classical; UG – non-classical	Miska and Miller 1999; Miska *et al*. 2004; Belov *et al*. 2006; Gouin *et al*. 2006; Baker *et al*. 2009
	Class II	10	DAA, DAB; 2 × DBA, 2 × DBB; DCA, DCB; DMA, DMB	O'hUigin *et al*. 1998; Stone *et al*. 1999; Belov *et al*. 2006
Tammar wallaby, *Macropus eugenii*	Class I	15	UA, UB, UC – putative classical, UM, UK, UE, UO – putative non-classical	Siddle *et al*. 2006; Siddle *et al*. 2009
	Class II	~16	DAA, 7–10 DAB, 2 DBA, 4 DBB, DMA, DMB	Slade *et al*. 1994; Browning *et al*. 2004; Cheng *et al*. 2009; Siddle *et al*. 2011
Tasmanian devil, *Sarcophilus harrisii*	Class I	5	UA, UB, UC – putative classical UD, UK – putative non-classical	Cheng *et al*. 2012*a*
	Class II	4	DAA, 3 DAB	Cheng *et al*. 2012*a*
Brushtail possum, *Trichosurus vulpecula*	Class I	ND	UB	Lam *et al*. 2001*b*; Holland *et al*. 2008*a*
	Class II	~8	DAA, 3 × DAB, 2 DBA, 2 DBB	Lam *et al*. 2001*a*; Belov *et al*. 2004; Holland *et al*. 2008*b*

explanation for the genetic signatures that are visible now (Morris *et al*. 2013).

The low MHC diversity is also believed to have helped facilitate the spread of Devil Facial Tumour Disease, a contagious cancer that threatens Tasmanian devils with extinction (Siddle *et al*. 2007*a*). The cancer cells, which originated from a Schwann cell from a female devil (Murchison *et al*. 2010), would have had similar MHC-types to those of devils affected early in the disease epidemic in the north-east corner of Tasmania, allowing the cell to be passed between devils without encountering MHC barriers (Siddle *et al*. 2007*b*). Recent allograft experiments have demonstrated that allorecognition does occur in animals with minor differences in their MHC-types (Kreiss *et al*. 2011), suggesting that the tumour, which has now killed 85% of extant Tasmanian devils, must also be suppressing the immune responses. Further research is in progress to identify the mechanisms behind this.

The study of MHC diversity in non-model organisms can be challenging, due to the rapid rate of evolution of this gene family. MHC-linked microsatellites can act as proxies for diversity within the peptide-binding region. During her Ph. D. studies with Des Cooper, Mary Lam developed an MHC-linked microsatellite within an exon of the MHC Class II DAB gene of the brushtail possum (Lam *et al*. 2000). Banks *et al*. (2010) used this microsatellite as part of a larger study looking at genetic diversity in mountain brushtail possums (*Trichosurus cunninghami*) (Banks *et al*. 2010). Several associations were noted. Heterozygotes at the MHC-linked microsatellite had a greater survival than homozygotes. They also had a lower endoparasite faecal egg count. Interestingly, heterozygotes with phylogenetically similar alleles had the greatest survival probability. This study and others have shown that heterozygosity at the MHC can act as a measure of fitness.

Meyer-Lucht *et al.* (2008) sequenced MHC Class II DAB genes in two endemic South American mouse opossums. They observed high levels of MHC diversity within and among individual *Gracilinanus microtarsus* (Brazilian gracile mouse opossum) and much lower diversity in *Marmosops incanus* (gray slender mouse opossum). They suggested that diminished MHC diversity in *M. incanus* may be the result of a genetic bottleneck that resulted in lower genetic diversity across the entire genome. Further, they noted that the ground-dwelling *M. incanus* is more sensitive to habitat fragmentation than the canopy-dwelling *G. microtarsus* and that migration processes are likely less restricted in *G. microtarsus*, allowing MHC diversity to remain high.

An area of study that was of particular interest to Des Cooper was the impact that use of immunocontraceptive vaccines would have on feral brushtail possum populations in New Zealand (Deakin *et al*. 2005; Cooper and Larsen 2006). He argued that natural genetic variation in brushtail possum populations would mean that the vaccine would not be effective in a subset of animals and would ultimately result in selection for individuals resistant to the effects of the contraceptive vaccine. Indeed, considerable individual variation in response to any given immunocontraceptive vaccine was established (Duckworth *et al*. 2007). Olivia Holland sought to investigate the importance of the MHC in the variation to these responses during her Ph.D. She identified two MHC haplotypes with different responses to immunocontraceptive vaccines (Holland *et al*. 2009, 2010). This work substantiated Des Cooper's arguments about the genetic control of non-responsiveness and emphasised the importance of studying natural genetic diversity in any population before implementing control measures that may vary depending on host responses.

Natural Killer receptors

Key players in the innate immune response are the Natural Killer (NK) cells. They detect cancerous cells or cells infected by viruses using two major families of receptor molecules: the immunoglobulin superfamily and the C-type lectin superfamily. The immunoglobulin superfamily NK receptors are found as a cluster in the leukocyte receptor complex (LRC) while C-type lectin-like domain containing NK receptor genes are found clustered within the Natural Killer complex (NKC). The composition of the LRC and NKC differ markedly between species, as these gene families evolve quickly, with rapid lineage-specific expansions and contractions (reviewed in Kelley *et al*. 2005). Analogous functions are carried out by different genes within the two superfamilies in different eutherian lineages. These gene families have been characterised in two marsupials: the opossum and the Tasmanian devil (Belov *et al*. 2007; van der Kraan *et al*. 2013). The Tasmanian devil and opossum NKC are highly conserved, containing only six genes: CLEC4E, KLRK1, CLEC1A, CLEC1B, CLEC2-like and CD69. The NKC in these two marsupial lineages is much simpler than syntenic regions in eutherian mammals, where extensive lineage-specific gene expansions have occurred.

The LRC of the opossum and Tasmanian devil, however, contain lineage-specific expansions of Ig-domain containing NK receptors, with 45 NK receptors predicted in the genome of the opossum and 24 in the genome of the Tasmanian devil. This work paves the way for further research into the functional role of these molecules in immunity in marsupials.

Cytokines

We have established that the framework for a highly developed immune system is in place in marsupials, but there are signs that there are marsupial-specific features that may contribute to an as yet uncharacterised 'unique' immune response in these species. The discovery of TCRμ, the composition of NK receptors, and reports of CD1 as a pseudogene in the opossum (Baker and Miller 2007), all suggest that marsupials may have a distinctive niche from eutherian mammals with respect to antigen-recognition. Our current understanding of innate and adaptive immune responses in marsupials is restricted by a comparatively small number of laboratory experiments and even fewer *in vivo* reports. Outcomes of limited functional studies of marsupial T- and B-cells are inconclusive when directly compared with humans and mice and it still remains unclear whether reports of diminished and/or delayed archetypal T- and B-cell responses are typical of marsupials or are a function of suboptimal comparative assays. To more fully appreciate how these factors may influence the broader immune response, we need to be able to assess functional immunity in these species. This requires a thorough understanding of the communication

molecules that control the cellular response to antigen: the cytokines.

Cytokines are small immunoregulatory proteins that mediate cell-to-cell and intracellular signals that control immune function in mammals. They are principally (but not exclusively) secreted by leukocytes and signal through both autocrine and paracrine pathways. Essential biological processes such as haematopoiesis, development and maturation of immune tissues and the maternal/foetal interaction during vertebrate reproduction (Paulesu *et al*. 2008) are just some examples of the roles that these important signalling molecules play. Whether or not a host is able to appropriately recognise and reject foreign antigens is largely dependent on the cytokine composition of the local internal environment. This cytokine milieu dictates both the differentiation of immune cell populations and the 'signature' cytokines that these cells secrete (Zhu *et al*. 2010). Cytokines are therefore intimately involved in the establishment of infection and the effective control of disease, acting to determine the nature and duration of both innate and adaptive immune responses. To appreciate the context in which knowledge of marsupial cytokines has developed, it is pertinent to look at some of the early questions that arose concerning apparent differences between metatherian and eutherian immune systems.

Although there are no reports on the direct regulation of immune responses in marsupials, early observations of cell behaviour that are now known to be controlled by cytokines are found in the marsupial literature. Descriptions of *in vivo* phagocytic behaviour of splenic macrophages in the North American opossum (Marx *et al*. 1971) and chemotactic properties of possum lung macrophages (Moriarty and Thomas 1986) both suggested that modulation of immune responses was occurring in a manner similar to that observed for eutherian phagocytes. Although B-cell studies were (and still are) severely restricted by our inability to culture and maintain these cell lines, many of the foundation studies of the marsupial humoral immune response dealt with immunisation studies of responses to T-dependent antigens such as sheep red blood cells and bacteria (Croix *et al*. 1989). It is now well established in humans and mice that the interaction of T- and B-cells is mediated by cytokines, so these early marsupial experiments provided preliminary indications that a mechanism existed for marsupial lymphocyte populations to communicate with each other. This work was not without some controversy, though, as questions were being raised about the magnitude and timing of primary and secondary antibody responses in some marsupial species. At the same time, *in vitro* cell culture studies using plant lectins (ConA, PHA, PWM) and bacterial derivatives (LPS) began to pervade the literature, as mitogens are commonly used in mammals to stimulate immune cells and for assessment of cellular immune capacity (Kristensen *et al*. 1982). This work largely focussed on T-cell function – work that was challenging without the reagents to effectively separate marsupial lymphocyte subpopulations.

Following on from studies in humans on the elucidation of cell-secreted T-cell growth factor (now IL-2) (Mier and Gallo 1980), observations of cytokine-like factors were reported in the mitogen-stimulated cell culture supernatants of one American (*M. domestica*) and one Australian (*P. cinereus*) marsupial. A 15–17-kDa protein, presumed to be Interleukin-1, was produced by LPS-stimulated opossum macrophages (Brozek and Ley 1991) and Wilkinson *et al*. reported an Interleukin-2-like molecule secreted by spleen and peripheral blood lymphocytes of koala cultures (Wilkinson *et al*. 1992*a*). Some years later, TNF-α, IL-1β and IL-10 (Wedlock *et al*. 1996, 1998, 1999*a*, 1999*b*) would be identified in possum macrophage cultures, but this was not possible until RT–PCR gene-cloning techniques came into common use and recombinant cytokines were produced. Both stimulatory and inhibitory factors have also been observed in tammar wallaby cultures (Young and Deane 2007) but, to date, there have been no reports that specifically identify and assign bioactivity to individual cytokines of the wallaby or any marsupial species other than for inflammatory mediators of the brushtail possum previously mentioned.

What has been demonstrated, though, is that despite some differences in the degree of the responses, mitogen-driven assessment of metatherian T lymphocytes over the past 40 years has repeatedly confirmed T-cell immune capacity in both Australian [the quokka (Ashman *et al*. 1972); brushtail possum (Moriarty 1973; Baker *et al*. 1999), tammar wallaby (Ashman and Keast 1973; Young and Deane 2007), koala (Wilkinson *et al*. 1992*b*), red kangaroo (Montali *et al*. 1998), Tasmanian devil (Stewart *et al*. 2008)] and American [North American opossum (Fox *et al*. 1976), gray short-tailed opossum (Infante *et al*. 1991; Brozek *et al*. 1992)] marsupials. Cell-signalling pathways involved in stimulation and blastogenesis of immune cells (processes largely mediated by cytokines *in vivo*) are clearly intact in these species. Notwithstanding this evidence of functional T-cells, reports of poor or absent Mixed Lymphocyte Responses (assays that detect allogeneic recognition in eutherian mammals) in some of these species suggested that the mechanisms of cell-mediated immunity may not be the same as that of eutherian mammals. Since cell-mediated immunity is not only a function of T-cells, but also involves antigen-presenting cells, this assumption had specific implications for the susceptibility of possums (and captive marsupials) to mycobacterial disease and would also later influence our interpretation of the susceptibility of Tasmanian devils to transmissible facial tumours. In all cases, the lack of marsupial-specific reagents (due largely to the lack of sequence conservation across divergent immune genes) has prevented a detailed investigation and clarification of the mechanics of immune cell function in these and other marsupial mammals. Aside from a small number of antihuman antibodies that recognised intracytoplasmic domains of antihuman T- and B-cell-surface markers, there was very little experimental capacity to further this work and there was no capacity before our recent advances in genome sequencing to assess the role of the most divergent cytokines in any of the observations of marsupial immune cell behaviour.

It was not until the polymerase chain reaction and molecular cloning techniques became more widely used that we began to progress our direct knowledge of marsupial cytokines. Even then, only those orthologues that contained regions of high conservation within their coding domains (generally more than 60% conserved with eutherians at the nucleotide level) were discovered (for review see Harrison and Wedlock 2000). As previously outlined, the cDNA sequences and functional activities for recombinant peptides of TNF-α, IL-1β and IL-10 were identified in the brushtail possum. Expressed gene

sequences for TNF-α (Harrison *et al*. 1999) and Lymphotoxins –α and –β (Harrison and Deane 1999, 2000) were also reported during this time. Although many of the properties of these inflammatory mediators were predicted to be similar to those of eutherian mammals, the recombinant possum IL-1β peptide was found to have reduced species cross-reactivity, as did the IL-1 type factor derived from macrophages of the South American opossum (Brozek and Ley 1991). This question of comparative function is still unresolved and may be interesting since reports of susceptibility of the tammar wallaby to experimental infection with hydatid cysts (Barnes *et al*. 2007) and lack of inflammatory responses in coccidial infections (Duignan 2004) are consistent with reduced bioactivity of inflammatory mediators such as IL-1β. It may also point to differences in their modulation in these host environments. The nucleotide and predicted peptide sequence of the wallaby gene (Young and Harrison 2010) does not provide obvious clues to resolve this question and future investigations need to be undertaken to explain these observations.

Other conserved cytokines were amplified during the 10 years before data were available from the sequencing of the first marsupial genome. Interleukin-5 (eosinophil differentiation factor) in the tammar wallaby (Hawken *et al*. 1999) and Leukemia Inhibitory Factor (an IL-6 family member) in the brushtail possum (Cui and Selwood 2000) and fat-tailed dunnart (*S. crassicaudata*) (Cui *et al*. 2001) were both investigated at the cDNA level. Two Type I Interferons (IFN α and IFN β), intronless genes that have multifunctional roles that include immune modulation, and one (incomplete) chemokine gene (CCL28) in the tammar wallaby were also reported during this period (Harrison *et al*. 2003, 2004; Daly *et al*. 2007).

Despite these early successes, the more divergent genes now considered essential for effective cell-mediated immunity and T- and B-cell functions such as IL-2, IL-4 and the Type II IFN (IFN-γ), remained 'undiscovered'. Indeed, difficulties using molecular homology techniques for the detection of rapidly evolving cytokines such as IL-2 in marsupials and other evolutionarily distant species were reported (Harrison and Wedlock 2000; Zelus *et al*. 2000). By 2006, early sequence data for the opossum was publically available through the Ensembl browser and this paved the way for bioinformatic strategies and gene prediction models to progress the identification of immune genes in these species. *In silico* mining of the opossum genome identified full or partial gene sequences for 23 cytokine genes (IL-2, IL-4, IL-6, IL-12, IL-13 and six members of the IL-10 family), five of which were identified for the first time in marsupials (Wong *et al*. 2006). Further refinement of data-mining techniques, combined with manual annotations, later also identified an additional suite of opossum cytokines with representatives from all major cytokine families: interleukins, chemokines, interferons, transforming growth factors and tumour necrosis factors. The reader is referred to (Wong *et al*. 2011*b*) for details of this study, but it is salient to note that a further 36 chemokine genes (including what appears to be an expanded Macrophage Inflammatory Protein family), members of the IL-17 family (previously unrepresented in molecular reports of marsupial immune genes) and the first descriptions of IL-31 and IL-33 (Th-2 biased cytokines) outside of eutherian mammals were presented in this study. During this time, expression of several chemokine genes in tammar wallaby thymic tissue also complemented the findings from the predictions derived from the opossum (Wong *et al*. 2011*a*). Although there was still information missing from unsequenced regions in the genome and some untranslated regions of gene models, genome predictions of key T-cell development and differentiation markers not only provided the first step towards understanding the wider repertoire of regulatory molecules that are present in the genome of metatherian mammals, they have also confirmed the likely complexity and potential subtlety that may be involved in the modulation of marsupial immunity.

In addition to providing some clarity about the complexity of the immune response in marsupials, access to the genome sequences allowed researchers to renew their RT–PCR investigations at the laboratory bench. This time, with the ability to identify small areas of sequence corresponding to structural conservation within single gene families from a range of vertebrate lineages, we were able to finally detect the expression of the most elusive cytokines, IL-4 (Young 2011) and IL-2 (Young *et al*. 2012) in marsupial immune tissues.

Table 3. Marsupial cytokines identified by genomic (E) or cDNA studies

Molecules are reported and classified on the basis of their expression by and/or influence on the differentiation of major CD4 T-cell subsets in humans (Zhu *et al*. 2010). #E, Ensembl annotation (www.ensembl.org)

	Th1 (immunity to intracellular pathogens/ autoimmunity)	Th2 (protection against extracellular parasites/allergic responses)	Th17 (immunity to extracellular bacteria and fungi /organ-specific immune responses)	Treg (self-tolerance and immune modulation)
Tammar wallaby, *Macropus eugenii*	Lymphotoxins α and β (Harrison and Deane 1999, 2000), IFN-γ (E), IL12A and B (E), IL-18 (E)	IL-5 (Hawken *et al*. 1999), IL-4 (Young 2011)	IL-1β (Young and Harrison 2010), IL-17A and F (E), IL-6 (E), IL-21 (E), IL23α (E), TGF-β1 to 3(E)	TGF-β1 to 3(E)
Tasmanian devil, *Sarcophilus harrisii*	Lymphotoxins α and β (E), IFN-γ (E), IL-2 (E), IL-12A and B(E)	IL-4 (E), IL-5 (E), TSLP (E)	IL-1β (E), IL17A (E), IL-6 (E), IL-21 (E), IL23α (E), TGF-β1 to 3(E)	IL-2 (E), TGF-β1 to 3(E)
Gray short-tailed opossum, *Monodelphis domestica*	Lymphotoxins α and β (E), IL-12A and B (E)	IL-5 (E), IL-9 (E), TSLP (E)	IL-1β (E), IL-17F (E), IL-6 (E), IL-21 (E), IL-22 (E)	TGF-β1 to 3(E)

Of particular relevance to earlier marsupial immunology studies is that we are now in a strong position to investigate our earlier observations of lymphocyte behaviour. Many aspects of host immunity are controlled by CD4+ T-cells and we now know that individual cytokines are associated with the differentiation of four main subpopulations of naive Th cells in mammals (Zhu and Paul 2008). Th1 cytokines mediate immunity to intracellular pathogens and autoimmunity, Th2 cytokines are associated with protection against large extracellular parasites and are widely implicated in allergic responses. Organ-specific immune responses and immunity to extracellular bacteria and fungi are regulated by the Th17 subset. Regulatory T-cells (Tregs) are defined by their expression of the transcription factor, Foxp3, and play crucial roles in self-tolerance and immune regulation, including immune suppression. Examples of cytokines from each of these T-cell subsets have now been identified in marsupial genomes, and at least one gene from each group has been confirmed by gene expression (see Table 3 for a sample of cytokine genes identified in cDNA studies and annotated in published genome data www.ensembl.org). Readers are referred to previously cited references for exhaustive lists of cytokines and other marsupial immune gene families). In addition, cytokines associated with the development and differentation of these subsets from naïve lymphocytes (for extensive review, see Zhu *et al*. 2010) have also been reported in the sequenced marsupial genomes. Whilst there is still much to learn, we are now in a position to investigate the functional role of each of these molecules and the impact that they have on marsupial immune responses, an important period in the study of immunology of this diverse group of mammals.

Conclusion

For many years, sporadic reports of possible differences in the timing and complexity of T- and B-cell responses and an inexplicable absence or reduction of allograft responses in MLRs from a broad range of marsupials suggested that marsupials might have simple immune systems that explained the sluggish or diminished responses. It became increasingly important to more clearly understand the molecular workings of immunity in these species as marsupials were being used as models of biomedical research for studies of skin cancer (opossum), reproduction, development and aging (tammar wallaby and dunnarts) and sources of new antimicrobials (Selwood and Coulson 2006; Wang *et al*. 2011*a*). The role of the possum as a reservoir for bovine tuberculosis provided an important context for speculation about the susceptibility of marsupials to intracellular pathogens (Buddle and Young 2000) and, more recently, the significant impact of a contagious tumour that threatens population numbers of the Tasmanian devil (Belov 2012) has provided further reasons why research into the immunology of marsupials is urgent. In a new century where the majority of zoonoses responsible for emerging infectious disease arise from wildlife (Jones *et al*. 2008), the imperative to understand how infection is established and disease is controlled in marsupial species is arguably more important today than ever before. Sequencing of three marsupial genomes and continued investigation of the dynamics of antigen-receptor and recognition molecules has provided us with the tools to more fully understand the marsupial immune response. The next decade will see development of clinical assays to study health and disease in our native wildlife, an area of research that Des Cooper was passionate about.

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We pay tribute to Des Cooper, who inspired us with his brilliant mind, inquisitive spirit and love of science. He was a wonderful mentor and friend and will be deeply missed.

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