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Haematopoiesis in Marsupials

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ABSTRACT

Marsupials are a group of mammals that give birth to immature young lacking mature immune tissues at birth, and are unable to mount their own specific immune defence. Their immune tissues develop in a non-sterile ex-utero environment unlike that of eutherian mammals such as ourselves. Marsupials are therefore ideal models for studying the development of immune tissues, in particular haematopoiesis, yet relatively little has been investigated. Most studies have been restricted to histological or immunohistological studies, however some factors likely to be involved, based on eutherian studies in haematopoiesis, have been isolated and characterised, including a few key markers, and some cell signaling and regulation molecules, mostly involved in lymphocytopoiesis. However the role of many molecules in haematopoiesis is largely presumed. We currently lack much of the rudimentary information regarding time of appearance and expression levels of these molecules, and no functional studies have been conducted. This paper reviews our knowledge of marsupial haematopoiesis to date, and highlights the need for future research in marsupials to gain further insights into the evolution of haematopoiesis.

1. Introduction

Marsupials (metatherian mammals) are one of the three groups of mammals. They differ from their Prototherian counterparts (or monotremes) as they give birth to live young. Marsupials also differ from eutherian mammals, such as humans and mice, as they give birth to comparatively underdeveloped young, that lack histologically mature immune tissues (Ashman and Papadimitriou, 1975; Basden et al., 1996, 1997; Block, 1964; Old et al., 2003a, b), and are unable to mount specific immune defence (reviewed in Old and Deane, 2000).

As marsupials are born in such an underdeveloped state, immunologically, compared to eutherian mammals, they are excellent models for studying the development of the immune system and immunity in mammals. Studies describing the development of the immune tissues in marsupials have recently been reviewed and the gaps in our knowledge and understanding reported (Borthwick et al., 2014). One area that remains relatively elusive in terms of our knowledge is haematopoiesis. This paper is a review of the current knowledge of marsupial haematopoiesis and offers some avenues for future research in this relatively unexplored area of marsupial immunology.

Haematopoiesis in non-eutherian mammals (monotremes and marsupials) has been studied in only a few species to date (Table 1). In marsupials, the majority of these have focused on histological descriptions (Ashman and Papadimitriou, 1975; Basden et al., 1996; Block, 1964; Cisternas and Armati, 1999; Old et al., 2003a; 2003b; 2004a; Yadav, 1972). Even fewer studies on haematopoiesis in monotremes have been conducted. These studies are limited to histological descriptions of bone marrow and spleen from one adult platypus (*Ornithorhynchus anatinus*) (Tananka, 1986; Tanaka et al., 1988), and the histological and immunohistology of immune tissues with limited haematopoietic description in a further 15 adult platypuses (Connolly et al., 1999). There are no studies of the haematopoietic tissues of any echidna species or any developmental studies.

Histologically, marsupial haematopoiesis is similar to that of eutherian mammals, where it is initiated in the primitive yolk sac, and is continued by the liver, before being replaced by the bone marrow. As discussed earlier however, when compared to eutherian mammals, marsupials are born with no histologically mature immune tissues and the immune tissues must therefore develop externally in a non-sterile ex-utero environment. When born, unlike eutherian mammals, whereby the bone marrow is the active haematopoietic organ, in marsupials the liver is the active haematopoietic organ, and has been described in all marsupials studied (reviewed by Borthwick et al., 2014; and Old and Deane, 2000). The bones at birth in marsupials are limited to cartilaginous structures with medullary regions. Shortly after birth however the

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Table 1
: List of Marsupial and Monotreme species, their classification, and the haematopoietic tissues studied and methods used.

Species	Classification	Tissues studied	Method of study	References
Platypus (*Ornithorhynchus anatinus*)	Prototherian Ornithorhynchidae	Bone marrow Spleen	Histology; Immunohistology	Tanaka, 1986; Tanaka et al., 1988; Connolly et al., 1999
Virginian opossum (*Didelphis virginiana*)	Metatherian Polyprotodont Didelphidae	Liver Bone marrow Spleen	Histology	Block, 1964; Cutts et al., 1973; Cutts and Krause, 1982
Marmosa mitis	Metatherian Polyprotodont Didelphidae	Liver Bone marrow	Histology	Bryant and Shrifrine, 1974
White-bellied opossum (*Didelphis albiventris*)	Metatherian Polyprotodont Didelphidae	Spleen	Histology; Immunohistology	Coutinho et al., 1995
Gray short-tailed opossum (*Monodelphis domestica*)	Metatherian Polyprotodont Didelphidae	Liver Spleen	Specific gene isolation; Cell signaling and regulation gene isolation; *In silico* investigation	Aveskogh and Hellman, 1998; Aveskogh et al., 1999; Belov et al., 1999c; Miller et al., 1998; Lucero et al., 1998; Miller et al., 1999; Guth et al., 1998; Baker et al., 2005; Parra et al., 2009; Wang et al. 2012; Miller and Rosenberg, 1997; Wong et al., 2011a,b
Quokka (*Setonix brachyurus*)	Metatherian Diprotodont Macropodidae	Liver	Histology	Ashman and Papadimitriou, 1975
Tammar wallaby (*Macropus eugenii*)	Metatherian Diprotodont Macropodidae	Liver Bone marrow Spleen	Histology; Immunohistology; Specific gene isolation; Cell signaling and regulation gene isolation; *In silico* investigation	Basden et al., 1996; Harrison et al., 1999; Harrison and Deane, 1999; Zuccolotto et al., 2000; Baker et al., 2001; Harrison et al., 2003; Old and Deane, 2003; Parra et al., 2008; Young and Harrison, 2010; Wong et al., 2006; 2011a; 2011b; Young, 2011; Alsemgeest et al., 2013
Brushtail possum (*Trichosurus vulpecula*)	Metatherian Diprotodont Phalangeridae	Liver Spleen Lymph node Thymus Lung	Specific gene isolation; Cell signaling and regulation gene isolation	Belov et al., 1998, 2001, 2002a; 2002b; Wedlock et al., 1996; 1999a; 1999b; Young et al., 2012
Northern brown bandicoot (*Isoodon macrourus*)	Metatherian Polyprotodont Peramelidae	Liver	Histology; Immunohistology	Cisternas and Armati, 1999
Stripe-faced dunnarts (*Sminthopsis macrourus*)	Metatherian Polyprotodont Dasyuridae	Liver Bone marrow Spleen	Histology; Immunohistology	Old et al., 2003b; Old et al., 2004a; Old et al., 2004b

liver start to develop its mature structure with a decrease in haematopoietic cells until only a few haematopoietic foci are evident, before ceasing, at which time the liver becomes mature in appearance and no longer exhibits any haematopoietic activity.

At the same time the liver is decreasing its haematopoietic activity, the bones start to become ossified, a medullary cavity forms, and haematopoietic foci start to appear. In all marsupial species studied to date, haematopoiesis in the medullary cavity continues for sometime until involution starts to occur as the animal reaches a certain level of maturity, and virtually disappears, being replaced by yellow adipose tissue (reviewed by Borthwick et al., 2014; and Old and Deane, 2000).

Few studies have built on these histological investigations in marsupials. Old and Deane (2003) and Old et al. (2004b) have confirmed that the bone marrow of stripe-faced dunnarts (*Sminthopsis macrourus*) and tammar wallabies (*Macropus eugenii*) lack mature T and B-cells (based on immunohistochemical studies). However a lack of antibodies to immature immunological cell types, and other antibodies likely to cross-react with cells in the bone marrow are currently unavailable. Likewise, no genetic expression studies have focused on expression of any immunological markers in bone marrow samples. The descriptions of the haematopoietic cells in marsupials is therefore solely based on their histological appearance, these being primarily of the erythrocytic, leucocytic and granulocytic lineages (Ashman and Papadimitriou, 1975; Basden et al., 1996; Block, 1964).

The cell types that have been described in haematopoietic tissues of marsupials are similar to that described in eutherians and prototherians. As marsupials are born with no mature immune tissues and are unable to mount specific immune responses (reviewed by Old and Deane, 2000), it is not surprising that at birth high levels of erythropoiesis and granulocytopoiesis occur, with lymphocytopoiesis relatively absent until shortly after birth.

2. Histological description of haematopoiesis

2.1. Liver

In eutherians, the liver has essentially ceased haematopoiesis prior to birth, however the liver is actively haematopoietic at the time of birth in marsupials. In *Didelphis*, the liver diverticulum begins to form from the foregut (McCrady, 1938). The liver then enlarges and begins haematopoietic activity prior to birth. Block (1964) described haematopoiesis in the developing Virginian

opossum (*Didelphis virginiana*) from birth. On the first day after birth, around half the liver was haematopoietic and the dominant cells were erythroblasts, however early granulocytes, mostly early neutrophils, and occasional megakaryocytoblasts were also observed (Block, 1964). The bandicoot liver at birth is likewise undifferentiated with some nucleated erythrocytes, hepatocytes and neutrophil precursors present (Cisternas and Armati, 1999). Granulocytopoiesis, premyeloid megakaryocytopoiesis and erythropoiesis were confined to the sinuses of the liver, and haematopoiesis was starting to occur in *Marmosa mitis* liver at the time of birth but increased rapidly (Bryant and Shrifrine, 1974). On the day of birth the haematopoietic cells in the quokka (*Setonix brachyurus*) liver were of the granulocyte and erythrocyte lineages, with occasional megakaryocytes also seen (Ashman and Papadimitriou, 1975), and was similar to that of the tammar wallaby (Basden et al., 1996) and stripe-faced dunnart (Old et al., 2004a).

By day 3 postpartum the haematopoietic cells became aggregated and formed islands in the quokka liver (Ashman and Papadimitriou, 1975). In the Northern brown bandicoot (*Isoodon macrourus*) liver at day 4 after birth the haematopoietic islands were dominated by erythrocytes and their precursors, with a few promegakaryocytes (Cisternas and Armati, 1999). By day 4 in the Virginian opossum, haematopoiesis had increased and platelets had formed in some megakaryocytes (Block, 1964).

Haematopoiesis increased in the first week after birth and then decreased rapidly in the Virginian opossum (Cutts et al., 1973). By days 8–9 postpartum haematopoietic cells were starting to decrease however there was an increase in more mature haematopoietic cells throughout the liver (Block, 1964) and in *M. mitis* maximum haematopoiesis was occurring by days 8–12 postpartum in the liver (Bryant and Shrifrine, 1974). Haematopoietic islands had reduced in number by day 7 and by day 13 the prominent blood cell development occurring was erythropoiesis, with some granulocytopoiesis and thrombopoiesis in the bandicoot liver (Cisternas and Armati, 1999). Numbers of haematopoietic cells decreased but by week 2 the numbers of megakaryocytes had increased in the quokka and dunnart (Ashman and Papadimitriou, 1975; Old et al., 2004a). Myeloid megakaryocytopoiesis and erythrocytopoiesis occurs intravascularly and granulocytopoiesis extravascularly in the Virginian opossum (Block, 1964). Likewise in *M. mitis*, only a few islands of haematopoiesis were occurring in the liver at day 30 postpartum (Bryant and Shrifrine, 1974).

Over the following few weeks in all species haematopoiesis continued to decrease. By days 45–65 postpartum in the Virginian opossum only an occasional haematopoietic island remained in each of the hepatic lobules. These lobes mostly containing erythroblasts or a megakaryocyte, and by days 65–100 one or two islands and an occasional rare megakaryocyte remained (Block, 1964).

Throughout pouch life in the Virginian opossum as haematopoiesis decreased in the liver, the numbers of hepatic cells increased proportionately (Cutts et al., 1973). In the quokka and tammar wallaby haematopoiesis likewise continued to decrease and by the third month, only a few small haematopoietic islands remained (Ashman and Papadimitriou, 1975; Basden et al., 1996) and corresponded to the end of pouch life in the bandicoot (Cisternas and Armati, 1999). Adult marsupials lack haematopoietic areas in the liver (for example, Old and Deane, 2002; Old et al., 2003b).

2.2. Bone marrow

As stated previously no bone marrow or ossification occurs at birth in any marsupial, with the bones being cartilaginous (Ashman and Papadimitriou, 1975; Basden et al., 1996; Block, 1964; Old et al., 2004a). In the Virginian opossum primary marrow had appeared in the endochondral cranial bones by day 5, and by days 6 and 7 mature eosinophils and neutrophils were seen. The volume of marrow increased and by days 10–12 postpartum eosinophils, large lymphocytes and erythroblasts were observed. By days 23–32 the endochondral bones were filled with marrow and the numbers of immature to mature cells had increased. Erythropoiesis and megakaryocytopoiesis was more dominant than granulocytopoiesis by 45–65 days postpartum. Old et al. (2004a) likewise have described the appearance of megakaryocytes in the bone marrow of dunnarts as young as 12 days postpartum, and confirmed megakaryocytopoiesis occurs in marsupial bone marrow, as it does in eutherians.

Bone marrow appearance in quokkas (Ashman and Papadimitriou, 1975) and the tammar wallaby (Basden et al., 1996) was similar to that described in the Virginian opossum (Block, 1964), with its first appearance observed in the cranial endochondral bones on 4 days postpartum. In the tammar wallaby they were mainly erythroblasts and granulocytes (Basden et al., 1996). By day 5 some leucocytes were observed and by day 7 granulocytic precursors were evident in the quokka (Ashman and Papadimitriou, 1975). By day 8 postpartum megakaryocytes had appeared, and by two weeks the diversity and number of haematopoietic cells had increased in the tammar wallaby (Basden et al., 1996). Islands of haematopoiesis had started to appear in the dunnart by day 11 postpartum (Old et al., 2004a). By 14 days postpartum in the quokka, granulocytes, erythroblasts, a few large and medium lymphocytes, and a few megakaryocytes were observed (Ashman and Papadimitriou, 1975). Over the next two months marrow haematopoiesis increased in the quokka, with numbers of small and medium lymphocytes, erythroid precursors, and megakaryocytes increasing.

In adult marsupials, haematopoiesis in the bone marrow is essentially replaced by adipose tissue (for example, Old and Deane, 2002; Old et al., 2003b). In the dunnart this process of haematopoiesis reduction and replacement with adipose started to occur by 57 days postpartum with the appearance of adipocytes (Old et al., 2004a). In Virginian opossums by 65 days the bone marrow resembled that of adult animals (Block, 1964).

2.3. Spleen

The spleen is the last active erythropoietic immune tissue to mature in marsupials. The spleen in the Virginian opossum at birth was represented by avascular mesenchyme (Block, 1964; Cutts and Krause, 1982). Large lymphocytes appeared on day 2, with rare erythroblasts and very rare megakaryocytes present by day 3 postpartum (Block, 1964).

Megakaryocytes were however common in the tammar wallaby spleen from day 3 postpartum (Basden et al., 1996). The spleen by day 4 in the quokka was composed of mesenchymal cells with a few scattered myelocytes and erythroblasts (Ashman and Papadimitriou, 1975) and was similar to that of the tammar wallaby (Basden et al., 1996).

By days 6–7, haematopoiesis had increased and by days 10–12 postpartum erythropoiesis and granulocytopoiesis was rapidly increasing (Block, 1964). In the bandicoot lymphocytes, promegakaryocytes, erythroblasts and promyelocytes were observed in the spleen at the end of the first week postpartum (Cisternas and Armati, 1999). By the end of the first week in the quokka spleen, erythroblasts, myelocytes and occasional large lymphocytes were seen, and by the end of the second week, a few small and medium lymphocytes and occasional megakaryocytes were observed in the quokka spleen (Ashman and Papadimitriou, 1975).

By two weeks postpartum the Virginian opossum spleen had

increased in size and erythropoiesis was the dominant haematopoietic activity with some granulocytopoiesis occurring to a lesser extent (Cutts and Krause, 1982). On days 17–22 medium lymphocytes first appeared and an increase in erythropoiesis and to a lesser extent an increase in granulocytopoiesis and megakaryocytes had occurred in the Virginian opossum (Cutts and Krause, 1982). Erythroblasts and megakaryocytes increased and myelocytes decreased in the quokka spleen in the third week postpartum and by the end of the fourth week, myelocytic haematopoiesis was no longer common and erythroblastic haematopoiesis was the prominent haematopoiesis occurring (Ashman and Papadimitriou, 1975).

From around one month of age in the Virginian opossum, the amount of white pulp increased dramatically compared to red pulp, and by day 45 postpartum myeloid haematopoiesis had decreased (Block, 1964) and by day 60 granulocytopoiesis had ceased, but erythropoiesis continued and megakaryocytes persisted (Cutts and Krause, 1982). Between days 60–100 postpartum the splenic nodules and germinal centres appeared in the Virginian opossum (Block, 1964; Cutts and Krause, 1982), whereas in *M. mitis* follicles began to appear on days 18–20 after birth, but germinal centres did not appeared until day 60, and high levels of haematopoiesis was occurring by 120 days after birth (Bryant and Shrifrine, 1974). Comparatively at day 60 the tammar wallaby spleen had well defined areas of red and white pulp, and areas of white pulp increased until approximately four months postpartum (Basden et al., 1996). Erythropoiesis and megakaryocytes continues in adult marsupial spleens, for example, the Virginian opossum, even into old age (Cutts and Krause, 1982).

3. Lymphocytopoiesis

Granulocytopoiesis and erythropoiesis appears to occur mainly in the liver, bone marrow and to a lesser extent, spleen for granulocytes. Some lymphocytopoiesis is likely to occur but has not been studied in any depth in marsupials. Although we have discussed haematopoiesis in the spleen we have not yet focused on lymphocyte development. Early B-cell development occurs in the bone marrow of eutherians and presumably marsupials.

Later stages of B-cell development occur in the secondary immune tissues, primarily the follicles and germinal centres in eutherians, in association with antigen-presenting cells. B-cells presumably must travel to the secondary immune tissues to undergo further maturation in marsupials too. Follicles and germinal centres in secondary immune tissues have been described in many different marsupial species (for example Basden et al., 1996; Block, 1964; Old et al., 2003a, 2003b; 2004a), and are described as one of the maturational features of mature spleen and lymph node tissues. Follicles and germinal centres do form in marsupials and as such we have incorporated a brief description of haematopoiesis related to secondary immune tissue development in the spleen and lymph nodes. It must also be noted that in marsupials a broad range of secondary immune tissues do exist such as the gut-associated lymphoid tissues, bronchus-associated lymphoid tissues and tonsils, for example, but we have restricted our review to the spleen and lymph node as all these tissues contain follicles and germinal centres, and all secondary immune tissues presumably can act as sites for further B-cell development, in a similar matter to that observed in eutherian mammals. In the bandicoot, for example, Cisternas and Armati (1999) state B-lymphocyte development occurs in primary and secondary follicles. Old and Deane (2002) have likewise confirmed the presence of mature B-cells (those positively stained with anti-CD79b) in the mantles of germinal centres and throughout primary follicles in the spleen, lymph nodes, Peyer's patches in the gut and areas of bronchus-associated lymphoid tissue in the tammar wallaby. Coutinho et al. (1995) has also described mature B-cells ($CD79a^+$ and $CD79b^+$ cells) in the germinal centres, and cords and sinuses of the mature white-bellied opossum (*Didelphis albiventris*) spleen. An antibody to IgA also positively stained cells in the splenic cords, suggesting the presence of IgA-expressing plasma cells (Coutinho et al., 1995).

At birth in marsupials, the spleen is only present as a very rudimentary structure and no lymph nodes are present. In the Virginian opossum rudimentary lymph nodes have not been observed until day 4 after birth, and this was only in one of seven animals examined (Block, 1964). Erythroblasts appeared in the mesenchyme by day 5 postpartum, and on days 6–7 after birth small and medium lymphocytes, myelocytes and erythroblasts were visible in the cranial end of pouch young sinusoids. By days 10–12 postpartum there were areas that could be identified as medullary cords and cortex. Increases in small lymphocytes, and decreases in mainly myelocytes and megakaryocytes, but also erythroblasts, occur in the splenic cortex. The cortex and medulla were clearly defined by days 23–32, and by days 33–45 postpartum had increased in size mainly due to increased numbers of lymphocytes. Rare plasma cells were observed by days 45–65 and by days 65–100 postpartum the Virginian opossum lymph nodes had lymph nodules and germinal centres and the numbers of plasma cells had increased.

Lymph node development was similar to that in the spleen with no lymph nodes visible in the quokka until day 4 and only then as lymphatic vessels within mesenchymal connective tissue (Ashman and Papadimitriou, 1975). Rudimentary lymph nodes were visible on day 5, and by day 6 lymphocytic aggregates were more discrete, myeloid cells, erythroblasts a few megakaryocytes were seen. By day 9 myelocytic and erythroblastic cells decreased and the lymph nodes had become more mature in appearance. By the end of the third week the many mitotic lymphocytes were observed and the cortex and medulla were well defined. In the ninth week primary follicles were starting to appear. The lymph nodes continued to increase in size until around the third month postpartum and the number of lymphocytes had dramatically increased, and germinal centres had appeared.

As described previously, the spleen is very immature and is composed of mesenchymal tissue in marsupials at birth (for example, Cutts and Krause, 1982). Erythrocytes and megakaryocytes are some of the first cells to populate the developing spleen, followed by granulopoietic cells. As the spleen matures in histological appearance, representative areas of red and white pulp appear, in particular primary follicles appear, and as the tissue fully matures, germinal centres. Follicles and germinal centres are well recognised areas of B-cell maturation, differentiation, and proliferation in eutherians. These sites are key to maturation of B-cells into plasma cells and antibody synthesis. Staining using cross-species reactive anti-CD79b positively stains primary and secondary follicles as well as the mantle zones of germinal centres. The mantle regions of germinal centres are composed of mature B-cells in eutherians, and likewise the unstained centres of germinal centres contain immature B-cells.

A similar staining pattern of development occurs in lymph nodes to that in the spleen, whereby immunological cells, in this case lymphocytes, infiltrate the mesenchyme (Bryant and Shrifrine, 1974), and within a short timeframe the fully developed lymph node becomes apparent. The histological appearance of the follicles and germinal centres within mature lymph nodes is similar in appearance to those observed in marsupial spleen. Presumably, based on the similar distribution of mature T and B-cells in the spleen and lymph nodes, these cells undergo similar proliferation and maturation processes in marsupials, to that of eutherians. Similar follicles and germinal centres are also observed in the

marsupial gastrointestinal tract where they form part of the gut-associated lymphoid tissues (for example, Old and Deane, 2002; Old et al., 2003b), and sometimes in the respiratory tract (Cooke and Alley, 2002; Old et al., 2003b; Young et al., 2012; Young et al., 2003), although this appears to differ between individuals of the same species, as it does in eutherians, and is likely due to the level of antigenic challenge (Old et al., 2003b; Young et al., 2003).

T-lymphocytes, like in eutherians, and other animals, require further maturation and development before they can play active roles in immunity. Thymocytes presumably travel to the thymus where they undergo further development into mature T-cells. At birth the thymus in marsupials is rudimentary but maturation of this tissue occurs rapidly. Cisternas and Armati (1999) for example, have stated lymphopoiesis was extensive in the thymus in the first two weeks after birth in the bandicoot, and this is the case for all marsupials studied to date.

Although we have no confirmation that thymocytes travel from the bone marrow to the thymus in marsupials, we do know that mature T-cells first appear in the thymus in marsupials very shortly after birth (Baker et al., 1999; Coutinho et al., 1995; Old and Deane, 2002; Old et al., 2003b). Lymphocytes first appear in Virginian opossums (Block, 1964) on day 1 postpartum, primitive Hassall's corpuscles appear on day 5, medullary and cortical areas started to form on days 13–16 after birth and by day 23 the thymus had fully matured. In another opossum species (*M. mitis*) the thymus at birth was represented by a vascularized mesenchyme, and by day 2 a few lymphocytes were distributed throughout (Bryant and Shrifrine, 1974). Primitive Hassall's corpuscles appeared on day 4 and were mature by day 6, at which time cortico-medullary differentiation began, and had fully formed by day 8.

Throughout the development of the thymus, it becomes more evident that mature T-cells are located in the cortex of the thymus, whilst immature lymphocytes (presumably thymocytes) are present in the medulla of the thymus in marsupials, like that of eutherians (Schuurman et al., 1997). The distribution of mature and immature T-cells in marsupial thymuses has been observed in immunohistochemistry studies using a cross species cross-reactive CD3 antibody (eg. Old et al., 2003b) developed by Jones et al. (1993).

In the quokka, lobes were present in the cervical thymus during the first day after birth in the quokka (Ashman and Papadimitriou, 1975). By day 2 large lymphocytes were present in the medulla and by day 3 small and medium sized lymphocytes were visible. It must however be noted that some marsupials have two thymuses, the thoracic and the cervical. The thoracic thymus lagged behind the cervical thymus in terms of development in the quokka but both followed the same pattern of development (Ashman and Papadimitriou, 1975), and Wong et al. (2011a, b) has since found that there is no discernable difference in the genes expressed, and therefore presumably the function, of the thoracic and cervical thymus in the tammar wallaby.

B and T-cells are required to express essential components related to their immune response capacity. Whilst some have been identified in marsupials, there are still many that are yet to be isolated and described. Immunoglobulin A, E, G and M heavy chain genes (Aveskogh and Hellman, 1998; Aveskogh et al., 1999; Belov et al., 1998, 1999a, 1999b, 1999c; Miller et al., 1998), and λ and κ light chain genes (Belov et al., 2001, 2002a; Lucerno et al., 1998; Miller et al., 1999) have been isolated from gray short-tailed opossums and brushtail possums. Heavy chain D genes however appear to be lacking in marsupials.

The T-cell receptor has been isolated in several marsupials and includes TCRα, TCRβ, TCRδ, and TCRγ (Baker et al., 2001; Harrison et al., 2003; Parra et al., 2008; Zuccolotto et al., 2000), as well as the unique marsupial TCRμ (Baker et al., 2005). In the first 24 h after birth in the gray short-tailed opossum αβ T-cells appear, followed by γδ T cells and then around two weeks later T-cells expressing TCRμ appear (Parra et al., 2009). In eutherians, γδ T cells appear prior to αβ T cells in the fetal thymus (reviewed in Hayday, 2000).

Early B and T-cells undergoing somatic diversification necessary for immunoglobulin and T-cell receptor diversity, express terminal deoxynucleotidyltransferase (TdT) in eutherians (Civin and Gore, 1993). TdT has been isolated from a two month old the gray short-tailed opossum thymus (Guth et al., 1998), however its expression in bone marrow in marsupials remains unknown.

In the brushtail possum the first heavy chain immunoglobulin transcript has been detected at day 10 (IgM), and the first switched transcript (IgA) at day 18 postpartum using reverse transcription polymerase chain reaction (Belov et al., 2002a, b). IgG and IgE were however were not detected until day 103 postpartum, and corresponds to the time at which young possums lose the ability to absorb maternal immunoglobulins through the gut, and the time of first teat release (Belov et al., 2002a, b).

Three *variable preB* and the *λ5* surrogate light chain genes, essential for development of the pre-B-cell receptor, have been isolated from eutherian mammals. However, only the *VpreB3* surrogate light chain gene has been identified in all other non-eutherian vertebrates investigated, including from the spleen of an eight week old gray short-tailed opossum (Wang et al., 2012), suggesting VpreB1, VpreB2 and λ5 are unique to eutherian pre-B-cell receptor development.

Likewise, recombination activating genes (Rag) −1 and 2, responsible for gene recombination during development of the T-cell receptor, specifically, variable, diversity and joining segment recombination, has also been isolated from the gray short-tailed opossum (Miller and Rosenberg, 1997). In addition, the pre-TCRα chain has been identified using *in silico* techniques in the gray short-tailed opossum and the prototherian platypus (Smelty et al., 2010). In mice and humans the pre-TCRα chain controls early αβ-T cell development through ligand-independent self-oligomerisation (Fehling et al., 1995). However lagomorphs, the opossum, platypus and sauropods have a much shorter cytoplasmic tail and lack most of the critical residues necessary for self-oligomerisation of that present on the mouse and human pre-TCRα chain (Smelty et al., 2010). How the marsupial pre-TCRα chain functions with the shorter cytoplasmic tail and lack of critical residues is unknown. The function of the pre-TCRα chain in mice and humans, amongst other species, likely also warrants further investigation.

4. Cell signaling and regulation

As in other animals, the processes of cell proliferation, differentiation and maturation in marsupials is likely influenced by soluble factors such as cytokines, and other growth and transcription factors. Recent advances in genomics have resulted in the genomes of several species becoming publicly available and have led to some rapid advancements in marsupial immunology. For example, cytokines until relatively recently had been difficult to isolate due to a lack of sequence identity between eutherian and marsupial sequences with only a few cytokines isolated prior to the genomes becoming available (reviewed in Harrison and Wedlock, 2000). Marsupial immunologists have now identified and characterised several cytokines and it is largely assumed that these cytokines perform similar functions to those in eutherian mammals, however functional studies are lacking in marsupials at this time.

Therefore, whilst haematopoiesis and the processes involved in haematopoiesis have remained largely unexplored in marsupials, some cytokines important in eutherian haematopoiesis have been isolated and identified in marsupials. One of the first cytokines initially isolated prior to genomes becoming available was TNFα in

the (*Trichosurus vulpecula*) possum and tammar wallaby (Harrison et al., 1999; Wedlock et al., 1996, 1999a; 1999b). A strategy utilising "genomic walking" was then used to isolate LTα and LTβ (Harrison and Deane, 1999, 2000).

IL-1α and IL-1β have been identified in the tammar wallaby (Young and Harrison, 2010). Both forms bind to a common receptor and regulate haematopoiesis by inducing colony stimulating factors (granulocyte-macrophage, granulocyte and macrophage), and IL-6 in eutherians (Fibbe and Willemze, 1991). Opossum IL-2, IL-4 and IL-13 have been identified in the opossum using a manual synteny-based strategy, as automated annotation techniques had been unsuccessful (Wong et al., 2006). Opossum IL-6 was also identified in this study and found to have an amino acid identity to humans of 36.3%, highlighting the lack of conservation between some eutherian and marsupial cytokines (Wong et al., 2006). More recently Alsemgeest et al. (2013) was able to isolate IL-6 from the tammar wallaby as well as a splice variant. The splice variant (IL-6Δ2) is missing exon 2 and is predicted to maintain at least some of the function of IL-6. It was also the first report of a non-eutherian IL-6 isoform.

Wong et al. (2011a) identified further cytokines involved in eutherian haematopoiesis, IL-2, IL-12A and IL-12B, using genome mining. However, as a result of this more recent genome mining, and some transcriptomic studies (Wong et al., 2006, 2011a; 2011b), expressed cytokines with low amino acid sequence identity to their equivalent eutherian sequence have been able to be detected for the first time and include IL-4 in the tammar wallaby (Young, 2011) and IL-2 in the brushtail possum (Young et al., 2012). IL-4 was isolated from mitogen-stimulated lymphoid tissue (Young, 2011) and IL-2 from mitogen-stimulated lymph node cells (Young et al., 2012). Both IL-2 and IL-4 are important regulators of haematopoiesis in eutherians (Sonada, 1994).

The following molecules have been identified in the opossum genome (CD34, CD38 and GATA3) using the database created by Wong et al. (2011a, b). However the database was unable to be used to identify many of the haematopoietic markers and transcription factors of eutherians, due to database search limitations (eg. SCL, GATA2, CD45, VEGF, PU1, GM-CSF and M-CSF). In addition, where the database was able to be used to searched for some of these molecules, the search failed to identify the marsupial and monotreme equivalent (eg. CSF2, PAX5). A wider investigation into the identification of key cell signaling and regulation molecules as well as transcription factors involved in haematopoiesis needs to be conducted and will need to incorporate further genome databases.

5. Conclusions

The processes involved in haematopoiesis and further development of immunological cells are complex. Largely our understanding of haematopoiesis in marsupials is presumed based on our understanding of how the process occurs in eutherians. Clearly there is a great deal that requires further study and investigation regarding haematopoiesis in marsupials, and even more so in monotremes. In particular we are yet to identify many of the important haematopoietic markers involved in erythropoiesis and granulocytopoiesis, and the cell signaling and regulation molecules essential to haematopoiesis progression in eutherians. When these important molecules have been identified their appearance in the different tissues as haematopoiesis progresses and their effects on developing cells will need to be assessed and their function confirmed. By gaining a greater understanding of the process of haematopoiesis in marsupials, it will provide insights into the evolution of haematopoiesis and the immune system, of which marsupials are key.

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