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Cooperative Inflammation: The Recruitment of Inflammatory Signaling in Marsupial and Eutherian Pregnancy

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Abstract

The evolution of viviparity in therian mammals, *i.e.* marsupials and “placental” mammals, occurred by retention of the conceptus in the female reproductive tract and precocious “hatching” from the shell coat. Both eutherian embryo implantation and the opossum embryo attachment reaction are evolutionarily derived from and homologous to a defensive inflammatory process induced after shell coat hatching. However, both lineages, marsupials and placental mammals, have modified the inflammatory response substantially. We review the induction, maintenance, and effects of inflammation throughout pregnancy, with special attention to the role of prostaglandins and the mucosal inflammatory response, both of which likely had roles in early mammalian viviparity. We propose that the key step was not only suppression of the inflammatory response after implantation in placental mammals, but also the transfer of the inflammatory cell-cell communication network to a different set of cell types than in generic inflammation. To support this conclusion we discuss evidence that pro-inflammatory signal production in the opossum is not limited to maternal cells, as expected in *bona fide* defensive inflammation, but also includes fetal tissues, in a process we term *cooperative inflammation*. The ways in which the inflammatory reaction was independently modified in these two lineages helps explain major life history differences between extant marsupials and eutherians.

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Competing Interests

The authors have no competing interests to declare.

I declare that I participated in the study by assisting with conceptual development and literature review, writing the initial draft of the manuscript, and assisting with editing, and that I have seen and approved the final version. I have no conflicts of interest to declare.

Materials and Methods

No experiments were conducted explicitly for this review.

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Keywords

Inflammation; Implantation; Gene Expression Recruitment; *Monodelphis domestica*; Theria

1. Introduction

Stem therian mammals evolved viviparity from an ancestral egg-laying animal between 176 and 220 million years ago (Madsen, 2009). Pre-partum erosion of the eggshell barrier brought fetal and maternal tissues into direct physical contact, resulting in new cell-cell interactions from which the evolution of the placenta proceeded (Griffith and Wagner, 2017). Embryonic attachment to the uterus likely stimulated an inflammatory response due to tissue damage (Chavan *et al.*, 2017) and an adaptive immune response to paternally-derived fetal antigens (Medawar, 1953). How mammalian lineages addressed these challenges set the stage for their subsequent divergent reproductive evolution. This review focuses specifically on how maternal-fetal interactions following attachment were modified to reach their current state in marsupials and placental mammals.

Two extant clades of viviparous mammals diverged after the evolution of viviparity, the marsupials and the so-called “placental” mammals (Marshall, 1979; Killian *et al.*, 2001). Marsupials consist primarily of Australian endemic fauna such as kangaroos and koalas, as well as the opossums which remain in the New World. “Placental” mammals (hereafter referred to by their phylogenetic branch-based name, eutherians, to avoid confusion with the placental organ) include humans, mice, elephants, and all of the species that descend from their most recent common ancestor. Marsupial gestation is in most cases shorter than a sterile sexual cycle, and yields highly altricial young which must complete development attached to the mother’s nipple. On the other hand, crown eutherian lineages have evolved extended pregnancy longer than a single sexual cycle, which can yield precocial young (Chavan *et al.*, 2016).

Comparison of uterine gene expression during pregnancy suggests that this fundamental life history difference between marsupials and eutherians is due to independent modification of the inflammatory response that likely was ancestrally induced upon contact of fetal and maternal tissues (Griffith *et al.*, 2017). Invasive embryo implantation and placentation evolved in the eutherian stem lineage (Wildman *et al.*, 2006; Mess and Carter, 2006; Elliot and Crespi, 2009). It has been proposed that eutherians modified the ancestral inflammatory response into embryo implantation (Finn, 1986; Griffith *et al.*, 2017), and coupled it with a long anti-inflammatory period that extends pregnancy (Mor and Cardenas, 2010; Mor *et al.*, 2011; Chavan *et al.*, 2017). The decidual stromal cell type, responsible for decidualization of the endometrium to support an implanting embryo, also evolved in the stem of Eutheria (Mess and Carter, 2006). The original functional role of the decidual cells likely was suppression of inflammation caused by the attachment reaction (Chavan *et al.*, 2016). Early within the crown group of Eutheria, the major lineages independently evolved recognition of pregnancy (Chavan *et al.*, 2016).

Marsupials lack the anti-inflammatory period after embryo contact, and attachment of opossum embryos to the uterus coincides with the initiation of an inflammatory cascade that

is followed quickly by parturition (Griffith *et al.*, 2017; Hansen *et al.*, 2017), although whether there is a role for inflammation in the highly derived macropods (wallabies, kangaroos and their relatives) has not been investigated. The model marsupial for laboratory research, the gray short-tailed opossum *Monodelphis domestica*, retains a similar reproductive strategy to the reconstructed marsupial ancestral state (Zeller and Freyer, 2001; Freyer *et al.*, 2003). The therian common ancestor, too, likely underwent a gestation similar to that of the opossum (Chavan *et al.*, 2016).

Attachment-induced inflammation was therefore suggested to be a developmental constraint on the duration of marsupial gestation, which eutherians have overcome (Griffith *et al.*, 2017). In this review we come to the conclusion that in both, eutherians and marsupials, the attachment-induced inflammation underwent independent modifications by redistribution of signaling roles to different cell types to serve different purposes. In marsupials, this redistribution of signaling roles reinforces inflammatory processes and likely utilizes them to induce parturition, rather than suppressing them. We review evidence that the inflammatory processes in opossum pregnancy are not solely performed by maternal cells, as expected in a *bona fide* inflammatory response, but instead they depend upon the cooperation of fetal and maternal cells. The modification of the innate immune response into distinct physiological processes – induction of parturition in the case of marsupials, or embryo implantation in the case of eutherians – suggests that inflammation was not only a constraint to be overcome but also a source of evolutionary innovation.

2. Evolution of Viviparity

The three major mammalian clades, the monotremes, marsupials and eutherians, diverged at different points in the evolution of pregnancy. This is illustrated by their differential deposition and shedding of egg coats: the outer layers are deposited and maintained in monotremes until hatching, in marsupials they are deposited but shed within the body, and in eutherians the shell coat is absent except for the zona pellucida. The characteristic egg of the Amniota, which include reptiles and mammals, is surrounded by three acellular egg coats. The first layer, the zona (zona pellucida in mammals), is a glycoprotein matrix deposited by the ovarian follicular cells around the oocytes, and is present in all mammals (Hughes, 1977). Additional egg coats are secreted around the conceptus during passage through the reproductive tract. These consist of the mucoid coat, an acidic glycoprotein layer, and the proteinaceous shell coat comprised of ovokeratin, which is rich in disulfide bonds (Hughes, 1977; Selwood, 2000). The shell coat is primarily deposited in monotremes and marsupials within the upper uterus (Selwood, 2000; Zeller and Freyer, 2001). This part of the uterus is homologous to the shell gland which deposits calcified shell coats around bird and lizard eggs (Lombardi, 1998). Erosion of the shell coat during embryonic development evolved in the stem lineage of therian mammals, and in the eutherians it is no longer deposited at all.

Viviparity evolved in mammals by a heterochronic shift of shell coat hatching to earlier in development when the fetus is still inside the mother (Figure 1). Monotreme development involves an intrauterine egg incubation period of 15–24 days (Rismiller and McKelvey, 2000; Hawkins and Battaglia, 2009), followed by oviposition and around 10 days of external incubation, in a nest in the case of the platypus, or in the mother's skin pouch in the case of

the echidna (Griffiths *et al.*, 1969). This pattern is thought to resemble the reproductive mode of the egg-laying mammalian common ancestor. Marsupial gestation is ancestrally short as well, approximately the length of monotreme intrauterine gestation, with the opossum having a total gestation of 14 days (Harder *et al.*, 1993), although the macropods have evolved gestations of up to 38 days (Hughes, 1962; Poole, 1975). Breakdown of the shell coat in marsupials occurs at the somitogenesis stage of embryonic development (day 12 in the opossum; Zeller and Freyer, 2001). Marsupial neonates and monotreme hatchlings are altricial in similar ways, with underdeveloped hindlimbs but precociously formed forelimbs and claws necessary for maneuvering to the lactational areas (Griffiths, 1978:252; Hughes and Hall, 1998).

After shell coat hatching, embryo attachment and formation of the yolk sac placenta mark the beginning of the rapid growth phase characteristic of marsupial development, during which fetal size increases exponentially (Rose, 1989). The rapid growth phase involves increased nutrient transfer from maternal to fetal blood (Freyer *et al.*, 2006). This allows the marsupial fetus to complete the same amount of growth that would encompass the entire egg incubation period of monotremes in a fraction of the time, only the last two days of gestation in the opossum. The observation that this phase of development occurs under inflammatory conditions (Griffith *et al.*, 2017) suggests that certain components of inflammation may be beneficial to fetal growth rather than pathological or coincidental.

Eutherian embryos can implant into the endometrium and undergo extended gestation. Eutherian gestation ranges from less than a month in some rodents, including the golden hamster *Mesocricetus auratus* with a gestation as short as 16 days (Purdy and Hillemann, 1950), to humans at 9 months, and elephants at 22 months (Perry, 1953), unheard of for marsupials. Crucial to extended gestation is the ability of eutherian embryos to implant into the endometrium. While much of human pregnancy is considered anti-inflammatory, such a characterization applies only to the growth phase of eutherian pregnancy, which is evolutionarily derived (Griffith *et al.*, 2017; Chavan *et al.*, 2017). This anti-inflammatory phase is sandwiched between two stages, implantation and parturition, which are pro-inflammatory processes (Figure 1) (Mor and Cardenas, 2010). The evolution of extended gestation therefore involved two related evolutionary events, the modification of the pro-inflammatory attachment reaction into implantation to establish the fetus within the uterus, and the creation of an anti-inflammatory phase of pregnancy (Chavan *et al.*, 2017). The common ancestor of therian mammals likely experienced inflammation upon insemination (Paulesu *et al.*, 2008), embryo attachment (Finn, 1986; Finn, 1996; Griffith *et al.*, 2017) and parturition (Hansen *et al.*, 2017), but did not have sustained embryo invasion or an extensive anti-inflammatory growth phase as humans and other eutherian mammals do.

3. Inflammation Induced by Normal Pregnancy

The question of how viviparity could have evolved in the presence of an immune system that evolved to attack foreign bodies has long puzzled scientists (Billington, 2003). From an immunobiological perspective, loss of the shell coat barrier exposes the uterus to direct physical contact with the fetus, and by extension any tissue injury or alloantigen presence resulting from the apposition of an invasive semi-foreign body. An immune response to the

fetus would presumably have been induced as a consequence of this transition in the timing of hatching in the first viviparous mammals, as the immune system evolved long before viviparity. The version of the adaptive immune system known from humans is shared among gnathostomes, i.e. all vertebrates derived from the most recent common ancestor of humans and sharks (Litman *et al.*, 2010), whereas innate immune responses such as inflammation are of even more ancient origin, at least as old as metazoans [multicellular animals] (Rowley, 1996; Ashley *et al.*, 2012). While providing resilience against perturbations throughout life, such defenses also have consequences for the sustainability of a semi-allogeneic offspring within the mother. The anti-inflammatory phase of gestation does not simply involve temporary suppression of the immune response, as the compromise of host defense in such a critical time for organismal fitness would presumably carry an enormous fitness cost (Mor *et al.*, 2011). Rather, immune processes spatially specific to the uterus and temporally restricted to pregnancy must have diverged from the generic inflammatory response, and evolved along an independent evolutionary trajectory from normal defensive inflammation in order to enable viviparity. In the following sections we discuss points in therian mammal reproduction that might elicit immune or inflammatory responses, and whether these processes resemble or appear to be modified from normal host defense.

3.1. Antigen-Specific Immune Responses as a Consequence of Viviparity

The immune system responds to pathogenic non-self organisms or to tissue damage and stress. It acts through the antigen-specific responses of the adaptive immune system, as well as pattern recognition in the form of conserved non-self structural feature recognition, and detection of loss of homeostasis (damage) or danger through functional feature recognition (Iwasaki and Medzhitov, 2015). In the first viviparous vertebrates, the embryo may have induced the immune response through the same pathways as a non-self intruder. Here, we review potential non-self and damage induction mechanisms for the inflammatory response upon fetal-maternal contact as it likely took place in the first viviparous mammals (summarized in Table 1).

The question of maternal tolerance of the fetus has traditionally been posed in terms of the adaptive immune system's response to paternally-derived antigens, particularly cellular (T-cell mediated) immunity by analogy to graft-host interaction in tissue transplantation (Medawar, 1953). Such an allograft rejection-like reaction was posited to be a constraint responsible for the short duration of marsupial gestation (Moors, 1974; Lillegraven, 1975). In many marsupials the duration of the post-attachment intrauterine development, when the fetus establishes intimate contact with the uterus, is shorter than the experimentally determined onset of the graft rejection response suggesting that the marsupial gestation is constrained to be shorter than the time delay between antigen presentation and adaptive immune response. For example, in *Monodelphis domestica* skin graft rejection begins 19 days after transplantation and is complete at 31 days (Stone *et al.*, 1997), whereas the post-attachment period is only 2 to 3 days (Griffith *et al.*, 2017). In the Tasmanian devil *Sarcophilus harrisii*, the graft rejection occurs after 14 days (Kreiss *et al.*, 2011) but the post-attachment period is 2 to 7 days (Rose, 1989), and in the wallaby *Macropus eugenii* graft rejection takes 10–16 days whereas the post-attachment period is around 7.5 days (Walker and Tyndale-Biscoe, 1978; Renfree and Shaw, 2014). This timing is consistent with the

necessity for marsupials to give birth before the onset of an antigen-specific immune response, but not with such a response being the proximate cause for parturition. Furthermore, several predictions from the graft rejection model have not been supported. If the reason for marsupial short gestation is the onset of an antigen-specific reaction, it would be predicted that immunological memory from previous exposure to paternal antigens would cause complications or make parturition occur more quickly upon shell coat rupture in successive pregnancies, such as in multiple pregnancies with the same partner or under artificial conditions akin to vaccination. Attempts to induce graft rejection-like pregnancy failure by active immunization to paternal antigens have not been successful in marsupials (Rodger *et al.*, 1985), just as in eutherians (Lanman and Herod, 1965; Beer and Billingham, 1976; Wegmann *et al.*, 1979). Overall, the attachment reaction in marsupials appears to consist of non-antigen-specific inflammation, rather than an antigen-specific T cell-mediated response.

Another potential factor in the immune tolerance of the fetus is the presence of regulatory T cells, which are elevated during pregnancy in mice and humans (Somerset *et al.*, 2004). These consist of natural regulatory T cells produced in the thymus, and an induced regulatory T (iTreg) cell population that differentiates in peripheral tissues. Although iTreg cells have antigen-specific T cell receptors, upon activation through these receptors they dampen local immune responses through bystander suppression (Kahn and Baltimore, 2010). Outside of pregnancy, iTreg cells form a homeostatic counterbalance to pro-inflammatory Th17 cells, and aid in peripheral tolerance of commensal microorganisms in the gut-associated lymphoid tissues, among other functions (Littman and Rudensky, 2010). The generation of iTregs at the maternal-fetal interface is thought to be a derived trait only in eutherians, made possible by the insertion, in the stem eutherian lineage of eutherians, of a TGF β - and retinoic acid-response element CNS1 into the *FOXP3* locus, a gene crucial for Treg differentiation (Samstein *et al.*, 2012). The ablation of this promoter in mice leads to increased embryo resorption in allogeneic matings, but not complete sterility (Samstein *et al.*, 2012). The phylogenetic placement of this insertion coincides with the evolution of implantation and invasion, suggesting that Tregs may play important functions in implantation, even if their presence is not absolutely essential for successful pregnancy.

Several explanations for the lack of an adaptive immune response to the fetus in eutherians have been proposed. Classical major histocompatibility complex class I (MHC-I) proteins, expressed in most nucleated cells, present peptides to T cells, whereas non-classical MHC-I have immunomodulatory effects on uterine natural killer cells, CD8⁺ T cells, and dendritic cells at the fetal-maternal interface (Clark *et al.*, 2010). In humans, expression of classical MHC-I genes HLA-A and HLA-B by the trophoblast is suppressed, which may allow these cells to hide from allorecognition by reduced presentation of self peptides (Moffett and Loke, 2006). While human cytotrophoblast and syncytiotrophoblast are reported to lack MHC expression (Sunderland *et al.*, 1981; Tilburgs *et al.*, 2015), HLA-C as well as non-classical HLA-E and secreted HLA-G are expressed in extravillous trophoblast (Tilburgs *et al.*, 2015). There, these MHC molecules may play roles in suppression of natural killer cell cytotoxicity or CD8⁺ T cell activation (Fournel *et al.*, 2000), or act as positive regulators of inflammation that aid in implantation (Rajagopalan *et al.*, 2005; Trowsdale and Betz, 2006). However, these facts do not answer the question how immune tolerance first evolved

because extravillous trophoblast cells are only known to exist in great apes (Crosley *et al.*, 2013) and a somewhat similar invasive cell type in horses that ultimately forms the endometrial cups (Noronha and Antczak, 2010). In addition, both classical and non-classical MHC are expressed in the trophoblast of mice (Madeja *et al.*, 2011), of bovines in late pregnancy (Davies *et al.*, 2006) and of wallabies (Buentjen *et al.*, 2015), so this state likely represents the ancestral condition for therian mammals. Therefore, the suppression of HLA-A and HLA-B in trophoblast cells cannot explain the origin of extended gestation, but may instead be linked to traits such as deep invasion and the role of extravillous trophoblast in vascular remodeling.

Suppression of antigen-specific effector mechanisms is also thought to play a role in successful viviparity. The trophoblast produces various immunomodulatory factors. Placental prostaglandin E₂, among other functions, has been shown to inhibit proliferation of mouse cytotoxic T cells (Kvirkvelia *et al.*, 2002) and is associated with T cell anergy by defective phosphorylation of the CD3 chain of the T cell receptor (Volumenie *et al.*, 1997; Eblen *et al.*, 2002). Human T cell activity and antigen presentation following allotransplantation differ from T cell activity during fetal-maternal interaction (Collins *et al.*, 2009; Mor *et al.*, 2017). This led to the interpretation that local tolerance, rather than systemic suppression of the immune system, occurs during human pregnancy (Moffett and Loke, 2006), and that the fetal role is more akin to a tumor than an allograft due to its own contribution to tolerance (Beaman *et al.*, 2016; Mor *et al.*, 2017; Costanzo *et al.*, 2018). This distinction makes clear that the presence of an adaptive immune system with the capability to reject allografts does not preclude the evolution of viviparity. The pregnancy of viviparous sharks, for instance, can last from several months to 2 years, and yet their adaptive immune system rejects skin grafts in a matter of one month or less (Good and Finstad, 1964; Chaouat, 2016). As sharks and mammals are separated by substantial evolutionary divergence in both reproductive and immune biology, they achieved such prolonged gestation independently and likely through different mechanisms. Whether the lack of fetal rejection in these species is due to functions of the fetus that suppress it or because rejection is not induced in the first place has yet to be demonstrated.

Putting this work on maternal tolerance towards the semi-allogenic fetus into phylogenetic context suggests that the modulation of the adaptive immune response was a trait that evolved as the gestational length increased after the most recent common ancestor of eutherian mammals. The ancestral eutherian mammal was a small animal that gave birth to small neonates relative to the size of the mother, and thus likely had a short gestation period on the order of *Monodelphis* pregnancy (Chavan *et al.*, 2016). As noted above, the gestational length of basally branching marsupials, such as the opossum, is shorter (14 days) than the time it takes for an allograft rejection to develop in the same species (19 days), and the time of direct fetal-maternal contact is even shorter (2 days). The immunological processes in the uterus of ancestral therian and eutherian animals were thus more likely related to the activation of damage- and stress-induced inflammation than the activation of the adaptive immune response. To this issue we are turning in the next section.

3.2. Non-Antigen-Specific Responses as Consequences of Viviparity

A potential inducer of inflammation in viviparity is uterine tissue damage caused by direct contact with the fetal membranes, for instance due to continued secretion of proteases that play a role in hatching from the shell coat (Griffith *et al.*, 2007). Damaged and dying cells release damage-associated molecular patterns, or “hidden self,” which trigger inflammatory and immune responses (Matzinger, 1994; Kono and Rock, 2008). Danger cues produced by dead and dying cells include membrane depolarization and extracellular presence of DNA, ATP, and other compounds, and detection of these cues triggers inflammatory responses in a wide range of multicellular eukaryotes (Heil and Land, 2014). Contact between the fetal tissues and the uterus, accompanied by tissue turnover in the placenta, mean that the damage response could be a component of the immunological milieu of pregnancy (Rovere-Querini *et al.*, 2007), particularly the highly invasive pregnancy of humans, as tissue damage is more likely to result if the trophoblast is invasive. During human pregnancy, the tissue damage marker high mobility group box 1 protein (HMGB1) is released from the fetal membranes as well as from decidual cells (Holmlund *et al.*, 2007). HMGB1 is normally localized to the nucleus but released upon damage or necrosis, or actively secreted by monocytes and macrophages (Scaffidi *et al.*, 2002). HMGB1 binds to the receptor for advanced glycation end-products (RAGE), or toll-like receptors 2 and 4, and stimulates further inflammatory signaling (Park *et al.*, 2004). HMGB1 promotes angiogenesis when applied to the extraembryonic membranes of the chick (Mitola *et al.*, 2006), suggesting that this damage marker may also promote growth and vascularization of the placenta and the endometrium. The immune response to tissue damage promotes growth, and in doing so is suggested to nurture the fetus, rather than attacking it (Moffett and Loke, 2004).

Even in the opossum, tissue damage occurs upon fetal-maternal contact. Evidence for the invasive nature of the opossum trophoblast comes from the observation that shortly before birth, the trophoblast cells can be seen to penetrate the uterine epithelium and extend cytoplasmic processes into the endometrial stroma (Enders and Enders, 1969; Wagner, unpublished observation), in spite of the fact that opossum placentation is classified as epitheliochorial. Nevertheless, the invasive phenotype is only seen on the last day of pregnancy and certainly does not lead to sustained fetal implantation as in many eutherian mammals. Instead, we have proposed that this quasi-invasion may function, too, to accelerate parturition in the opossum (Griffith *et al.*, 2017).

Fetal protease secretion is another potential early inducer of inflammation. Secretion of proteases is a conserved feature of the therian trophoblast, and has been documented in human, rabbit and mouse blastocysts (Denker, 1977; Brosens *et al.*, 2014) and protease activity from the trophoblast has also been shown in marsupials such as the wallaby (Denker and Tyndale-Biscoe, 1986) and *Monodelphis* (Griffith *et al.*, 2017). We have previously proposed that serine protease secreted by the attaching blastocyst after hatching from the shell coat may be the ancestral trigger for the inflammatory attachment reaction (Griffith *et al.*, 2017). This protease could have induced inflammation through endometrial tissue damage, or by triggering pattern recognition. The hatching of eukaryotic parasite eggs also involves serine protease secretion (Young *et al.*, 1999), and as a consequence the mammalian innate immune system has evolved to recognize protease as a conserved pattern indicating

helminth presence and induce an anti-helminthic Th2 immune response (Sokol *et al.*, 2007). However, the anti-helminthic response is a type 2 immune response, whereas the inflammation induced after shell coat hatching in the opossum (Chavan *et al.*, 2017) or during implantation in the human (Mor and Cardenas, 2010) is type 1. Therefore, while serine proteases have the ability to cause pro-inflammatory tissue damage and to activate the type 2 immune response through pattern recognition, the lack of a type 2 profile in the post-attachment opossum suggests that has been lost in marsupials, which is a hint that the opossum post-attachment reaction may be evolutionarily modified.

Eutherians arrive in the uterus surrounded by the zona pellucida. While and after the blastocyst hatches from the zona pellucida the trophoblast of eutherians secretes serine protease. This has been shown in the mouse (Ruan *et al.*, 2012) and human (Brosens *et al.*, 2014). Serine proteases are thought to stimulate epithelial sodium channels (ENaC) in the uterine lumen, leading to calcium influx, prostaglandin production, and ultimately decidualization of the endometrial stromal fibroblasts. In humans, the ability of the embryo to secrete serine proteases has been suggested to function during embryo selection as a quality signal detectable by the endometrium (Brosens *et al.*, 2014; Macklon and Brosens, 2014). If so, this is likely a secondarily acquired function of protease secretion, with the original function having been disruption of the shell coat and the zona pellucida.

We thus suggest that the immunological problem for the evolutionary origin of eutherian pregnancy should be seen primarily from the perspective of an inflammatory damage response, while the dimensions of non-self exposure gained relevance only after the inflammatory challenge was overcome and gestational period could be extended.

Inflammatory signals have taken on physiological roles at several events throughout the reproductive cycle – post-copulation, implantation, and parturition. These events are of different phylogenetic ages and degrees of conservation across therian mammals, so they are considered separately. For each stage, the questions of whether inflammation is induced by the fetus or mother, and whether it represents an adaptation or a constraint, can be asked.

3.3. Inflammation Associated with Copulation

Uterine inflammation following copulation is reported across therian mammals, including horses, pigs, and cattle (Katila, 2012), as well as humans and mice (De *et al.*, 1991; Robertson *et al.*, 2003; Shima *et al.*, 2015), and opossums (Baggott *et al.*, 1987). A study of amphibians found that the cytokine IL-1 β and its receptor IL-1R1 are expressed more broadly in the uterine mucosa in species with internal fertilization compared to species with external fertilization (Jantra *et al.*, 2007). This inflammation recruits granulocytes such as neutrophils. In mice, neutrophil recruitment following insemination coincides with elevated expression of the mucosal inflammatory cytokine IL-17A, which is upstream of neutrophil chemoattractants (Song *et al.*, 2016). This IL-17A is thought to be derived from $\gamma\delta$ T cells, as seminal fluid has been shown to increase IL-17A production by $\gamma\delta$ T cells *in vitro* (Song *et al.*, 2016). Seminal fluid is a plausible inducer for inflammation after copulation, but it cannot be the only component. Inflammation, including recruitment of effector granulocytes to the uterus, also occurs in sterile sexual cycles in several species: neutrophils and eosinophils have been found to fluctuate throughout the estrous cycle in mice, peaking at

estrus and metestrus (Diener *et al.*, 2016), and in the opossum, granulocytes infiltrate the uterus at the time of copulation, but also during the times of maximum behavioral receptivity in sterile cycles (Baggott *et al.*, 1987). These findings suggest that the inflammation occurring after copulation is in part endogenously controlled, and may have an anticipatory role, i.e. activate the immune system in anticipation of the arrival of sperm and seminal fluid.

3.4. Inflammation and Implantation, Decidualization, and Post-Attachment Phases

Eutherian blastocyst implantation occurs by shedding of the zona pellucida, orientation, apposition, attachment, and invasion (Spencer *et al.*, 2004). In species with invasive placentation, the final invasion stage of the implantation process establishes the conceptus within the maternal tissues, although it has been suggested that perhaps a more appropriate term than invasion is “embedding,” due to the roles of both fetal and maternal tissues in orchestrating this interaction (Macklon and Brosens, 2014). In species with non-invasive placentation, such as pigs, and bovines and their relatives, as well as most marsupials, invasion does not occur.

Physiological roles for inflammation in normal pregnancy were first recognized in the late 19th century by noting similarities between the eutherian decidual reaction and inflammatory granulation (Creighton, 1878; summarized in Finn, 1986). In the majority of species with invasive placenta, decidualization transforms the endometrium in response to an implanting embryo. In menstruating species such as humans, decidualization is induced by maternal hormones, and occurs even in the absence of an embryo (Finn, 1998). However, phylogenetic inference suggests that decidualization was ancestrally induced by the fetus, as it still is in the majority of eutherian species (Emera *et al.*, 2011). As decidualization is a derived character that evolved in eutherian mammals (Mess and Carter, 2006), it likely arose as a modification of, the ancestral maternal reaction to the fetus (Finn, 1998; Erkenbrack *et al.*, 2018). The decidual reaction can be induced in the guinea pig uterus by physical damage to the endometrium (Loeb, 1907, 1908), or in the rat and mouse uterus with administration of an oil droplet or a plastic bead (Finn and Pope, 1991), suggesting that inflammation or stress is sufficient to induce decidualization. The decidua orchestrates the transition of implantation-associated inflammation into the Th2-dominated growth phase in eutherians whereas in opossums, embryo attachment initiates the rapid growth phase followed by parturition in short order (Chavan *et al.*, 2017).

The eutherian implantation reaction, complete with intrauterine embedding, is therefore proposed to have evolved from previously existing inflammatory pathways (Finn, 1986; Griffith *et al.*, 2017). In the opossum, the post-attachment phase involves an increase in inflammatory signaling, including type 1 cytokines *IL1A*, *IL6*, tumor necrosis factor (*TNF*), and neutrophil chemotactic factor (*CXCL8*) (Griffith *et al.*, 2017; Hansen *et al.*, 2017). Several of these genes, such as *IL6*, *TNF*, and *CXCL8*, are also expressed during implantation in humans (Mor *et al.*, 2011). Inflammatory marker expression in the opossum immediately follows the degradation of the shell coat, suggesting that inflammation is induced only upon attachment of the trophoblast to the uterus (Griffith *et al.*, 2017; Chavan *et al.*, 2017). Precocious shell coat hatching and induction of inflammation in response to

embryo attachment are reconstructed as traits shared between opossums and early viviparous mammals, as shared cytokine expression between eutherian implantation and marsupial attachment illustrates (Griffith *et al.*, 2017).

Furthermore, inflammation also modulates expression of adhesion molecules, which have important roles in placentation and implantation (Chaouat, 2013). The integrin- and L-selectin-mediated adhesion of the blastocyst to the endometrium resembles diapedesis, the process whereby leukocytes bind to endothelial cell adhesion molecules and transverse blood vessels during inflammation (Genbacev *et al.*, 2003; Dominguez *et al.*, 2005). Therefore, it has been argued that the machinery supporting transendothelial migration of leukocytes was evolutionarily coopted to support migration across the luminal epithelium during embryo implantation (Liu, 2018). Diapedesis, like implantation, is a cooperative reaction between the migrating cell or blastocyst and the tissue, and can only occur properly under inflammatory conditions: as a consequence, eutherian embryo implantation is dependent upon the presence of inflammatory signaling.

3.5. Inflammation at Parturition

Inflammatory signals play important roles at the end of gestation. In addition to acting as inflammatory mediators prostaglandins, particularly PGF_{2α}, induce “partition,” defined as either parturition/birth or oviposition, in a wide range of species. PGF_{2α} can be produced directly from PGH₂ (Kabututu *et al.*, 2009) or from PGE₂ (Phillips *et al.*, 2011). PGF_{2α} induces oviposition in oviparous reptiles (Guillette Jr. *et al.*, 1991; Hargrove and Ottinger, 1992; Kupittayanant *et al.*, 2009), and elicits myometrial contractions during labor in viviparous mammals (Karim, 1968; Challis *et al.*, 2000; McKeown *et al.*, 2000). PGF_{2α} binds to the prostaglandin F receptor, whose activation leads to release of intracellular calcium which in turn binds calmodulin; calcium-calmodulin then activates myosin light chain kinase to phosphorylate myosin light chains and cause muscle contraction (Challis *et al.*, 2000). This mechanism has been identified in bird oviposition, showing that it is not unique to viviparity (Kupittayanant *et al.*, 2009). PGF_{2α} surges at the time of parturition in the tammar wallaby (Renfree *et al.*, 1994) and is sufficient to induce birthing behavior in the opossum (Rose and Fadem, 2000) and tammar wallaby (Hinds *et al.*, 1990). These observations suggest that its role at parturition is shared among marsupials. Uterine PGF_{2α} also can elicit luteolysis in a wide range of amniotes, including turtles (Mahmoud *et al.*, 1988), squamates (Guillette Jr. *et al.*, 1984), tammar wallabies (Hinds *et al.*, 1990), and sheep (Douglas and Ginther, 1973). Thus, during the inflammatory end phase to gestation, prostaglandins promote expulsion of the fetus, after which inflammation can resolve.

Cytokines such as *IL6*, *IL1B* and *CXCL8* are upregulated in the human myometrium at labor (Bollopargada *et al.*, 2009). Labor also involves the cytokine-mediated recruitment of effector immune cells to the uterus: neutrophils are recruited at term (Kelly, 1996; Romero *et al.*, 2006) and in cases of pre-term labor (Elovitz and Mrinalini, 2004; Romero *et al.*, 2006; Goldenberg *et al.*, 2008). On the maternal side, granulocytes infiltrate the decidua and myometrium (Thomson *et al.*, 1999; summarized in Gomez-Lopez *et al.*, 2010). In mice, Fas ligand, which binds to and promotes apoptosis of Fas⁺ leukocytes, is expressed by uterine epithelia and stroma during early gestation, and by the trophoblast in late gestation, and in

its absence neutrophils and macrophages prematurely infiltrate the fetal-maternal interface, impairing fertility (Hunt *et al.*, 1997). Neutrophils infiltrate the uterus before parturition in various marsupials, including opossums (Chavan *et al.*, 2017), and several diprotodont species (Sharman, 1961). Recruited granulocytes produce pro-inflammatory signals such as TNF and eicosanoids, the group of compounds that includes prostaglandins (Cassatella, 1995; Gomez-Lopez *et al.*, 2010). They also express proteases that digest extracellular matrix such as neutrophil elastase, collagenase, and matrix metalloproteinase. On the fetal side, inflammation also occurs in the extraembryonic membranes during parturition (Halgunset *et al.*, 1994), and extracts from fetal membranes of women during labor have neutrophil chemotactic properties (Gomez-Lopez *et al.*, 2009).

This raises the question of what purpose neutrophils serve in the uterus during parturition. Neutrophils are specialized to carry out effector functions, primarily the removal of pathogens by phagocytosis and release of cytotoxic reactive oxygen species. The high cost of neutrophil recruitment due to collateral damage makes their recruitment beneficial only in specific circumstances: for this reason, there are no tissue-resident neutrophils, and the threshold for summoning them is high (Okabe and Medzhitov, 2016). The function of neutrophil recruitment in parturition, however, is not entirely clear. They may be part of the system activating uterine contractions, or may function afterwards in repair and cleanup (Keski-Nisula *et al.*, 2000; Timmons *et al.*, 2009; Shynlova *et al.*, 2013). If neutrophils promote expulsion of the fetus, then their presence following embryo attachment in marsupials but conspicuous absence in eutherian implantation (Chavan *et al.*, 2017) is noteworthy: prevention of neutrophil recruitment was a necessary prerequisite to the evolution of extended gestation. Hence neutrophil recruitment at parturition could be a phylogenetic rudiment of an ancestral acute inflammatory reaction that is suppressed during the growth phase in eutherians, and then gets released to aid in parturition.

4. Eutherian and Marsupial Inflammatory Signaling Networks during Gestation

Two of the pro-inflammatory events in eutherian pregnancy, implantation and parturition, are similar to and likely homologous to the inflammatory attachment reaction of marsupial pregnancy, and both have been proposed to be derived from the ancestral therian attachment-induced inflammatory response (Griffith *et al.*, 2017). While the marsupial (in particular the opossum) condition may approximate the therian ancestral state with its lack of eutherian- or macropod-specific extended gestation (Freyer *et al.*, 2003) more than 170 million years of independent evolution (Madsen, 2009) mean that the physiology of the opossum has likely diverged from that of the first viviparous mammals. Nevertheless, as no other extant mammalian lineage diverged after the evolution of viviparity and before the split of therian mammals, we must consider the ancestral inflammatory signaling network using insights from the opossum, as well as from eutherian mammals and defensive inflammation outside of the context of pregnancy.

4.1. A Hypothetical Early Viviparous Mammal Inflammatory Network

Two classes of inflammatory mediators relevant to immune responses at mucosal surfaces are eicosanoids (prostaglandins) and cytokines (particularly those in the interleukin 17 pathway). Prostaglandins have been discussed above.

IL-17-mediated immunity predates the evolution of the adaptive immune system in gnathostomes. IL-17 homologs have been discovered in lampreys, where they are expressed in VLR lymphocytes of the convergently evolved agnathan adaptive immune system (Guo *et al.*, 2009). IL-17 homologs are also expressed in the mucosal epithelial cells of echinoderms in response to extracellular bacteria (Buckley *et al.*, 2017), suggesting that IL-17 has deep evolutionary roots as a cell-autonomous inflammatory mediator, not unlike its expression in innate immune cells and mucosal epithelia in humans (Cua and Tato, 2010). If these functions are homologous, IL-17 functioned in mucosal epithelial defense before the divergence of deuterostomes. Thus, given resemblance of the embryo to an extracellular pathogen, IL-17 would have been a component of the original attachment-induced inflammatory response (Chavan *et al.*, 2017).

4.2. The Inflammatory Network in the Marsupial Post-Attachment Inflammation

There is little evidence for maternal recognition of pregnancy in marsupials outside of the macropods (Renfree, 2000), suggesting that ancestrally, marsupial pregnancy did not elicit systemic physiological changes in the mother. On the local scale of the uterus, local changes in response to the presence of fetuses do occur, such as the activation of uterine glands and replacement of the sub-epithelial stroma by a network of capillaries (Griffith *et al.*, 2019). That said, the inflammatory process is not solely maternal, but is the result of contributions from both the fetus and mother.

Expression of genes in the prostaglandin synthesis pathway are highly upregulated in the opossum uterus during the attachment reaction, e.g. *PTGS2* aka *COX2* (Griffith *et al.*, 2017). In the opossum, *PTGS2* is localized to the maternal uterine luminal epithelium, whereas the downstream enzyme for the production of PGE₂, *PTGES*, is localized to the trophoblast, i.e. expressed in cells of fetal origin (Figure 2b) (Griffith *et al.*, 2017). This suggests that PGE₂ synthesis during opossum pregnancy is cooperative: based on this expression pattern, in order for PGE₂ to be produced from arachidonic acid, both the fetal and maternal tissues would have to be in close enough proximity for the intermediate PGH₂ to travel between them, although such a shuttle mechanism of PGH₂ to neighboring cells has yet to be demonstrated. If this scenario is correct, it would constitute a mechanism of maternal recognition of pregnancy in the opossum, albeit not one where the embryo prolongs the function of corpus luteum, and if anything, one that brings about parturition. Furthermore, it suggests that the opossum fetus is not just incidentally setting off inflammatory signaling, but rather the fetus itself is instrumental in the production of inflammatory mediators, likely to effect its own birth.

After embryo attachment in the opossum, expression of *IL17A* is elevated, particularly during the last day before parturition (Griffith *et al.*, 2017; Hansen *et al.*, 2017). However, marsupial IL-17 signaling during pregnancy is not identical to typical mucosal defense

against an extracellular pathogen. We found that the source of *IL17A* expression at the end of opossum pregnancy is the trophoblast (Chavan *et al.*, in prep.). This suggests that IL-17 mediated inflammation, like prostaglandin metabolism, results from fetal as well as maternal components in opossum pregnancy. Production of *IL17A* by the opossum trophoblast suggests recruitment of expression of this inflammatory signal from maternal leukocytes or epithelia, the producers of *IL17A* in mucosal defense. Its expression, like prostaglandin synthesis, may contribute to expulsion of the embryo in parturition.

4.3. Modified Inflammatory Networks in Eutherian Pregnancy

Redistribution of expression of maternally-produced signals to the fetus and vice versa can be explained as a process of enhancing beneficial fetal-maternal interactions and pruning detrimental ones (Griffith and Wagner, 2017).

The evolution of extended gestation in eutherians was enabled by the gain of an anti-inflammatory period of pregnancy following implantation. Decidual stromal cells have been shown to suppress type 1 inflammation and wound healing pathways in the decidua until parturition (Nancy *et al.*, 2018). As the decidual stromal cell type is absent in marsupials (Kin *et al.*, 2014), these immunomodulatory functions represent divergent evolution in the eutherian lineage from the ancestral inflammatory response. However, the ancestral role of decidual stromal cells is reconstructed to have been at implantation, as their presence later in gestation is not universal within eutherians (Chavan *et al.*, 2016).

While the presence of prostaglandins in mammalian reproductive physiology is ubiquitous, the localization of enzymes in the prostaglandin pathway is variable within eutherians. Maternal prostaglandin production plays an important role in human implantation to increase receptivity of the endometrium (Achache *et al.*, 2010). In the mouse it has been shown that the pre-implantation blastocyst itself expresses *PTGS2* (Liu, 2018). When inflammation recurs at parturition, complementary expression of enzymes catalyzing different steps in the prostaglandin synthesis pathway is reported in humans (Pavli *et al.*, 2017). At term, the upstream enzyme involved in liberating arachidonic acid, phospholipase A2 (*PLA2G16*), is expressed in both fetal and maternal tissues, as is prostaglandin E synthase (*PTGES*) (Pavli *et al.*, 2017), although prostaglandin E synthase protein levels are elevated in the trophoblast relative to the decidua (Phillips *et al.*, 2014). On the other hand, other enzymes appear to be more exclusively maternal, such as prostaglandin F synthase (*FAM213B*) which is localized to the decidua (Pavli *et al.*, 2017). This suggests that after widespread PGH₂ synthesis, PGE₂ production, which increases blood flow and nutrient delivery to the fetus, is more under the control of the fetal membranes, whereas PGF_{2α} production, which more directly elicits contractions and parturition, occurs in the decidua and acts locally on the myometrium. Such a situation may reflect a legacy of gene expression recruitment.

5. Conclusions

5.1. Cooperative Inflammation as an Alternative Form of Viviparity

The brevity of marsupial pregnancy has provoked the theory that its duration is constrained by an overwhelming allograft rejection reaction (Moors, 1974; Lillegraven, 1975). Molecular studies (Griffith et al., 2017; Hansen *et al.*, 2017) revise the notion of immune rejection substantially to focus on inflammation, a component of the innate immune system, rather than the lymphocyte-mediated process of graft rejection, which in opossum has been shown to take 19 days, much longer than the duration of fetal-maternal attachment of two days. These all point to a model of developmental constraint by the induction of an ancestral defensive inflammatory response to the embryo that forces parturition (Figure 3a). In this model, marsupials respond to the fetus in the same way that they would to an extracellular pathogen, i.e. by inflammation and neutrophil recruitment.

In the light of findings that the expression of some inflammatory mediators is not limited to maternal cells, as expected in a *bona fide* inflammatory response, but instead results from both fetal and maternal cells, the constraint model needs to be amended. We propose that a fetal-maternal communication network has emerged out of a generic inflammatory response by gene expression recruitment to fetal cells. In marsupials, this has the effect of reinforcing the inflammatory response at the end of pregnancy, whereas in eutherians the outcome was the evolution of embryo implantation and the transition to an anti-inflammatory phase of pregnancy (Figure 3b). In both cases, *what was ancestrally an antagonistic response of host versus inflammatory inducer was modified into a cooperative fetal-maternal communication network*. In the case of marsupials, this cooperative network is pro-inflammatory and promotes parturition. We term this process *cooperative inflammation*. In the case of eutherians, fetus and mother cooperate to orchestrate the embedding of the fetal tissues within the mother, the process of blastocyst implantation. Both of these processes resulted from individualization of an uterine- or pregnancy-specific process from the generic inflammatory process, and the resulting individualized fetal-maternal networks each had their own evolutionary trajectory. Marsupials and eutherians have modified their inflammatory networks independently in ways that lead to divergent reproductive modes, implantation and cooperative inflammation, and predispose these clades to disparate life history patterns.

5.2. Clinical Implications

Understanding the ancestral female reproductive tract biology of therian mammals and how inflammatory networks were remodeled during the evolution of pregnancy has relevance to medical and veterinary practice. When exclusively viewed from the allograft perspective, the implication would be that suppression of inflammatory and immune responses would be beneficial to pregnancy. If, however, a good part of implantation and parturition is an evolutionarily modified inflammatory process then interventions affecting parts of this process are expected to be deleterious. Understanding the therian ancestral state as well as the independent modifications of the ancestral network in the marsupial lineage as compared to the human lineage should allow for better targeting of specific signaling interactions to

improve implantation success in the assisted reproduction clinic and treat implantation failure.

In addition, inflammation arising from bacterial infection during pregnancy increases the risk of preterm delivery, in part because inflammatory signaling has evolved to be integrated into the control of parturition, resulting in vulnerability to interferences from other sources of inflammatory stimuli. Better understanding of ways in which inflammation at parturition has been modified from defensive inflammation may help in the development of clinical interventions to prevent pre-term birth. The evolution of innate immune activation, maternal-fetal signaling and diversity between species is a promising field for reciprocal illumination between the basic and applied sciences.

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Abbreviations

HMGB1	high mobility group box 1 protein
MHC	major histocompatibility complex
MRCA	most recent common ancestor
PG	prostaglandin
PTGES	prostaglandin E synthase
PTGS	prostaglandin-endoperoxide synthase (cyclooxygenase)
Th2	type 2 helper T cell
TNF	tumor necrosis factor
Treg	regulatory T cell

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Highlights

- Inflammatory stages of pregnancy in therian mammals evolved by the transfer of a cell-cell communication network to a different set of cell types of both maternal and fetal origin.
- Recruitment of inflammatory signals into reproductive physiology likely occurred independently in marsupials and eutherian mammals.
- Marsupials evolved cooperative inflammation at term pregnancy which reinforced the ancestral inflammatory response induced by embryo attachment and likely elicits parturition.
- Eutherian mammals modified the inflammatory response induced by embryo attachment into the implantation process, and postponing the inflammation associated with parturition and resulting in prolonged gestation.

Figure 1.

Evolution of pregnancy within crown mammals. *Mammalian MRCA* The mammalian most recent common ancestor (MRCA) was likely oviparous with inflammatory internal insemination, deposition of a mucoid coat and leathery shell coat, oviposition, external egg incubation, and hatching. This condition is approximately maintained in living monotremes. *Therian MRCA* Shift of the shell coat hatching event from after partition to before partition resulted in the evolution of viviparity in therian mammals, and an inflammatory attachment stage. *Opossum* The rapid growth phase in marsupials is derived from the ancestral attachment-induced inflammatory reaction, but quickly leads to parturition. *Human* Eutherians evolved embryo implantation, fetal invasiveness, and the anti-inflammatory growth phase of pregnancy. Parturition remains an inflammatory process. Opossum silhouette © Sarah Werning CC BY 3.0 (<https://creativecommons.org/licenses/by/3.0>).

Figure 2.

Types of inflammatory signals produced at the maternal-fetal interface upon initial contact.

a. An inflammatory response was mounted to the fetus in the first viviparous mammals, possibly due to protease production during an early intrauterine hatching reaction.

Inflammatory signals are maternally produced: Tissue damage triggers inflammatory pathways such as prostaglandin production, type 17 mucosal inflammation recruits neutrophils to the uterus that degranulate, and ultimately the fetus is expelled. **b.**

Inflammatory signals are produced at the fetal-maternal interface of the opossum at embryo attachment, but the fetus plays a role in production of inflammatory mediators, such as IL-17A and PGE₂ production, in addition to initial induction of an immune response.

Figure 3.

Models for evolution of inflammation during pregnancy in therian mammals. **a.** In the constraint model, marsupial short gestation is a result of developmental constraint, stemming from the induction of an unmodified instance of the same inflammatory response that originally evolved to respond to mucosal damage or pathogens (mucosal inflammation, red) against the embryo. Eutherian embryo implantation evolved by the individualization and divergence of inflammation into a form specific to the pregnant uterus, which became embryo implantation (blue), enabling subsequent evolution of extended gestation. **b.** In the cooperative inflammation model (proposed in this paper), the first viviparous mammals reacted to the hatching of the fetus from the shell coat with defensive inflammation, but this physiological character individualized into a uterine-specific form and was modified independently in both the metatherian and eutherian lineages. In the metatherian lineage, inflammation was reinforced and expression of signals was redistributed to rely upon cooperation of the fetus and mother. Eutherian embryo implantation evolved by the modification proposed in (a). Opossum silhouette © Sarah Werning CC BY 3.0 (<https://creativecommons.org/licenses/by/3.0>).

Table 1.

Comments on potential immunogenic stimuli behind the inflammation observed upon embryo attachment in therian mammals.

Inducer	Potential Example	Ancestral Counterpart	Embryo Mimics	Comments
antigen (allograft)	alloantigen detected by T cell receptor	adaptive immunity	extracellular fungus or bacterium	internal fertilization should also induce reaction, adaptive immune response is too slow if shell coat hatching is a prerequisite
Pathogen-associated molecular pattern	evolved response to serine protease (allergy-like, Th2)	innate immunity, inflammation, detoxification	macro-parasite	mast cell response weak, anti-helminthic Th2 response is derived in eutherians
damage-associated molecular pattern	endometrial necrosis/apoptosis, downstream of invasion or protease	tissue repair/inflammation	tissue damage	likely therian ancestral state
signal	embryo hijacks maternal signaling (cytokines, hormones, prostaglandins)	maternal homeostasis, physiology, estrus	mother	proposed here as cooperative inflammation in a marsupial (<i>M. domestica</i>)