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The extension of mammalian pregnancy required taming inflammation: Independent evolution of extended placentation in the tammar wallaby

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In the first live-bearing mammals, pregnancy was likely short and ended with a brief period of inflammatory maternal–fetal interaction. This mode of reproduction has been retained in many marsupials. While inflammation is key to successful implantation in eutherians, a key innovation in eutherians is the ability to switch off this inflammation after it has been initiated. This extended period, in which inflammation is suppressed, likely allowed for an extended period of placentation. Extended placentation has evolved independently in one lineage of marsupials, the macropodids (wallabies and kangaroos), with placentation lasting beyond the 2 to 4 d seen in other marsupial taxa, which allows us to investigate the role of inflammation response after attachment in the extension of placentation in mammals. By comparing gene expression changes at attachment in three marsupial species, the tammar wallaby, opossum, and fat-tailed dunnart, we show that inflammatory attachment is an ancestral feature of marsupial implantation. In contrast to eutherians, where attachment-related (quasi-) inflammatory reaction is even involved in epitheliochorial placentation (e.g., pig), this study found no evidence of a distinct attachment-related reaction in wallabies. Instead, only a small number of inflammatory genes are expressed at distinct points of gestation, including *IL6* before attachment, *LIF* throughout placentation, and prostaglandins before birth. During parturition, a more distinct inflammatory reaction is detectable, likely involved in precipitating the parturition cascade similar to eutherians. We suggest that in wallaby, extended gestation became possible by avoiding an inflammatory attachment reaction, which is a different strategy than seen in eutherians.

marsupial | implantation | recognition of pregnancy | inflammation | placenta

Significance

Our data suggest that moderation of the inflammatory reaction to embryo attachment allows for extension of pregnancy in mammals. The ancestor of all mammals likely experienced an ancestral inflammatory reaction in response to embryo attachment. In contrast, eutherians and some marsupials, such as macropodids, have an extended period of fetal–maternal contact. During this period of placentation many inflammatory genes are silenced while a few others are still expressed. This moderated expression of inflammatory genes suggests that inflammatory mediators were coopted into establishing and maintaining the placenta. This challenges the perspective of inflammation as being detrimental to pregnancy, instead suggesting that fetal–maternal interactions are based on a modified inflammation response necessary for maintaining pregnancy over an extensive period of time.

In the first live-bearing mammals, pregnancy was short with only a brief period of maternal–fetal attachment (1). This period of attachment was likely characterized by an inflammation response termed the ancestral inflammatory reaction (1, 2). In eutherian pregnancy, there is an inflammatory reaction at implantation of the embryo, followed by the establishment of an anti-inflammatory environment necessary to sustain the fetal–maternal interface with the development of the placenta (3). The initial proinflammatory environment is necessary for implantation in early pregnancy but leads to termination or premature birth if it is sustained after implantation is complete (4, 5). A key innovation in eutherian pregnancy is the ability to switch off this inflammation after it has been initiated leading to a period of development and growth (1, 6). An anti-inflammatory environment is induced for most of the gestation period in eutherians, followed by a secondary inflammatory phase leading to parturition (7). This long anti-inflammatory period in the middle of pregnancy is characterized by the absence of proinflammatory cytokines and an increase in the concentration of anti-inflammatory cytokines such as IL-10 and TGFß (8–10). This extended phase in which inflammation is suppressed, likely allows for an extended period of placentation, which is a key difference between marsupial and eutherian reproduction (Fig. 1*A*).

During pregnancy of opossums, dunnarts, and other "basal" marsupials (but not macropodids, see below), placentation is brief with shell coat rupture and attachment of the embryo to the uterine epithelium occurring within the last 2 to 4 d of gestation (Fig. 1*A*, 15, 16). Phylogenetic evidence supports that opossums represent the more ancestral form of viviparous reproduction (17). Opossums (*Didelphidae*) have retained many of the reproductive features of the first live bearing mammals (17), having a short gestation and short period of fetal–maternal contact, development of a functional yolk sac placenta, and no systemic recognition of pregnancy (17, 18, 19). The gray short-tailed opossum, *Monodelphis domestica,* has a 14-d gestation, with endometrial recognition of pregnancy

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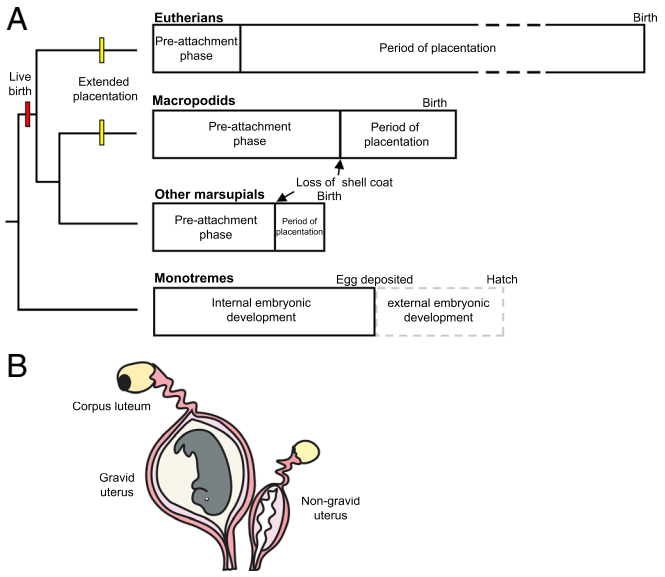

Fig. 1. (*A*) Comparison of the length of gestation and proportion of placentation during pregnancy across eutherians, macropodids, other marsupial taxa, and monotremes. In general, eutherians have longer gestations than marsupials with gestation extended beyond the length of the oestrus cycle and implantation occurring during early pregnancy. Macropodids have extended placentation compared to other marsupials with gestation almost the same length of time as the oestrus cycle. Most marsupials have shorter gestations than the oestrus cycle, with attachment of the early embryo and placentation occurring in the final 2 to 3 d of pregnancy. Monotremes have a period of internal embryonic development within the uterus before the eggs are laid into a pouch and external embryonic development continues supported by a complex milk. Yellow bars represent the independent evolution of extended placentation in macropodids and eutherians. The red bar represents the evolution of live birth that occurred once before the split of eutherian and marsupial mammals. (*B*) The unique reproductive anatomy of macropodids allows us to elucidate the direct effect of the embryo on the uterine environment and gene expression. All marsupials have two separate uteri with an accompanying ovary. The tammar wallaby is monovular with ovulation alternating between the two ovaries. This means that only one uterus becomes gravid each pregnancy (11). A postpartum oestrus and mating normally occurs within an hour of birth. The resulting early blastocyst is then kept in lactationally controlled embryonic diapause. Removal of pouch young leads to reactivation of the embryo from diapause and birth ~26.5 d later (12). Both uteri are under the same systemic endocrine conditions but experience different local, unilateral effects based on their proximity to the developing follicle or the corpus luteum (13). Differences between gravid and nongravid uteri reflect these local effects and the influence of the developing embryo in the gravid uterus (14).

and an inflammation reaction occurring at attachment on day 12 which intensifies through the short period of placentation (1, 19–21). Opossums lack the anti-inflammatory period after attachment seen in eutherians with an inflammatory cascade leading into parturition (22). The attachment induced inflammation reaction likely limits the length of placentation and therefore pregnancy in marsupials (1). This observation poses the question of whether an anti-inflammatory phase is required for the extension of placentation in mammalian pregnancy or whether the moderated inflammation response seen after implantation in eutherian pregnancy is just one method to establish a sustained fetal–maternal interface?

Wallabies offer a unique opportunity to identify how pregnancy and the period of placentation can be extended, in a lineage independent to eutherians. In comparison to other marsupial taxa, macropodids (wallabies and kangaroos) are considered more derived (17, 19). They are the only lineage of marsupials that have an extended period of placentation, greater than the 2 to 4 d seen in most other marsupial taxa with shell coat rupture occurring 8–10 d before birth in the tammar wallaby (*Notamacropus eugenii;* Fig. 1*A*, 23, 24). The maternal immune profile at attachment and throughout the extended period of placentation in this lineage of marsupials is unknown. The reproductive anatomy of macropodids means that only one uterus becomes gravid each pregnancy which allows us to elucidate the direct effect of the embryo on the uterine environment and gene expression (Fig. 1*B*, 11). To understand the nature of maternal–fetal interactions through extended placentation in the wallaby, we compared the histology of the endometrium during gestation and the gene expression in the uterus of the tammar wallaby from attachment through the late stages of pregnancy. We also compared the gene expression changes at attachment in the wallaby to two marsupial species with short periods of placentation, the gray short-tailed opossum and the fat-tailed dunnart (*Sminthopsis crassicaudata*). We found that the molecular environment at the maternal–fetal interface during maternal–fetal attachment in the tammar wallaby is homologous to that which occurs at attachment in the opossum and implantation in eutherian pregnancy. However, this proinflammatory state at attachment is followed by a uniquely moderated inflammatory profile with some key mediators of inflammation not expressed at all throughout tammar wallaby pregnancy. We argue that it is this moderation of an inflammation reaction at attachment and the subsequent suppression of inflammation that has facilitated extension of placentation in the macropodid lineage.

Results

The Tammar Wallaby Uterus Undergoes Extensive Remodeling During Placentation. Histology of the endometrium shows that some changes to the vasculature and gland structure are driven by maternal cycling due to similar changes occurring in both gravid and nongravid uteri at key timepoints of pregnancy (Fig. 2). Before attachment, the endometrium from both nongravid and gravid uteri has columnar epithelium with basally located nuclei. Capillaries are present underlying the luminal epithelium. After attachment, there is substantial glandular and vascular development in both gravid and nongravid uteri. The glandular epithelium is more cuboidal in structure with dilated gland openings and pink eosinophilic staining substances within the lumen. At this preattachment stage, there are similarities in morphology of gravid and nongravid uteri from the same timepoints of pregnancy. This supports the suggestion that some changes to the endometrium occur due to the systemic hormonal changes that occur during pregnancy rather than the presence of the fetus (25, 26, 27).

After attachment, the uterine environment is differentially remodeled in the presence of the fetus. The endometrium is greatly folded with regions closely interdigitated with fetal membranes (Fig. 2*D*). The luminal epithelial cells change structure by developing rounded apices. This rounded structure is a feature of cellular blebbing which can be caused by the detachment of adhesive structures along cell membranes (28). The domed shape and blebbing of the uterine epithelium are present throughout the entire period of attachment also seen in the final days of pregnancy (Fig. 2*F*). However, it is not seen in nongravid uteri (Fig. 2*E*) supporting the conclusion that it is the presence of the developing fetus and the placenta that influences this maternal recognition of pregnancy. The extent of blood vessel and glandular development is increased in gravid uteri compared to nongravid uteri at the same stage (13), suggesting that these changes facilitate the formation of the placenta.

Reproductive State and Timepoint of Pregnancy have Substantial Impacts on Uterine Gene Expression. In both the dunnart (*SI Appendix*, Fig. S1) (29) and the tammar wallaby (Fig. 3) (30), we saw substantial differences in uterine gene expression, between

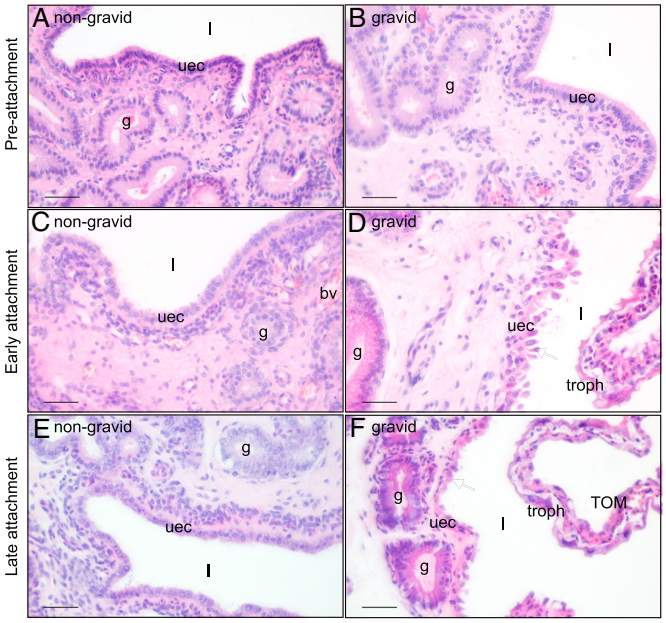

Fig. 2. Histological comparison of nongravid and gravid uterus throughout pregnancy in the tammar wallaby using hematoxylin and eosin staining. (*A*) Uterine sections from nongravid uteri and (*B*) gravid uteri at day 14: Preattachment of the blastocyst and loss of the shell coat (days 14 to 16, n = 2) show similar morphology of uterine epithelium. (*C*) and (*D*) Early attachment, after loss of the shell coat and formation of the placenta (days 20 to 23, n = 3), the uterine epithelium of gravid uteri has rounded apices and appears to be blebbing (arrow). (*E*) and (*F*) Late attachment, immediately before birth (days 25 to 26, n = 2) the uterine epithelium is short and rounded (arrow) in gravid uteri compared to nongravid. These morphological changes to the uterus through attachment in the tammar wallaby are similar to the short period of attachment in the opossum and implantation in eutherians. Staging is listed as days post copulation. The uterus is lined by luminal epithelium (uec) and the underlying stroma consists of connective tissue with uterine glands (g) embedded throughout. bv: blood vessel, l: uterine lumen, uec: uterine epithelial cells, g: gland, troph: trophoblast, TOM: trilaminar portion of the yolk sac. All scale bars are 50 μm.

reproductive stage and pregnancy state. For a detailed description of transcriptomic changes in the dunnart, see supplementary results. In tammar wallaby, a mean of 45.6 million reads were sequenced per sample with an average mapping rate of 92%. We compared differential gene expression analysis between nongravid and gravid uteri from several stages of pregnancy: preovulation (PO), preattachment (day 14), early attachment (day 20), late attachment (d25), and postpartum (PP). The transcriptomic data show that in the wallaby the stage of pregnancy (preattachment, postattachment, end of pregnancy) has the biggest impact on uterine gene expression followed by the reproductive state (gravid and nongravid uteri) (Fig. 3*A*). Pairwise comparisons show differentially expressed genes between nongravid and gravid uteri at each timepoint of pregnancy (Fig. 3*B* and Datasets S1–S8). Samples from the preattachment stage of pregnancy (day 14 gravid and day 14 nongravid) distinctly separate from the postattachment stages of pregnancy and postpartum uteri (Fig. 3*A*). There are thousands of significantly differentially expressed genes in pairwise comparisons when comparing samples from preattachment and postattachment stages of pregnancy (Fig. 3 *C* and *D*). There is distinct separation of samples from preattachment (day 14) and early attachment (day 20) gravid and nongravid uteri. The greatest separation is seen between gravid and nongravid uteri from late attachment (day 25). This suggests that the presence of the fetus has more influence on gene expression during the period of attachment and before birth than the preattachment stages of pregnancy. Nongravid samples from the latest stage of pregnancy (day 25 nongravid) cluster close to samples from preovulation uteri (PO; Fig. 3*A*) with under a hundred significantly differentially expressed genes in pairwise comparisons (Datasets S1–S8). Preovulation uteri samples are from the uterus contralateral to the postpartum uterus within 24 h of birth. Ovulation occurs from the ovary contralateral to the postpartum uterus within 40 h of birth, and the embryo enters the uterus ipsilateral to this ovary up to 2 d later.

We found 3,376 upregulated and 3,013 downregulated genes, when comparing early attachment on day 20 to preattachment on day 14. These changes are correlated with the loss of the shell coat and attachment, where the endometrium comes into direct contact with the choriovitelline placenta. This process causes endometrial remodeling and likely complex signaling/communication between mother and fetus. Gene Ontology (GO) analysis shows that at early attachment compared to preattachment, there is a significant enrichment of genes associated with cell adhesion and extracellular matrix organization with clear clustering of GO terms based on biological processes (Fig. 4*A*; Datasets S9–S21). This aligns with the reinforcement of cell adhesion and remodeling of the plasma membrane that occurs at embryo attachment with the development of an epitheliochorial placenta in this species (31, 32). There is a cluster of overrepresented gene ontology terms related to the regulation of cell migration which is interesting as there is no migration or invasion of uterine epithelial cells with noninvasive placentation in the tammar wallaby. It is more likely that these genes are associated with changes to the endometrial stroma. We found an upregulation of genes associated with tight junction and gap junction proteins (*TJP1*, *CLDN7*, *CLDN23*, and *GJA5*) and cadherin associated proteins (*CTNNA1*, *CDH3*, and *CDH7*) which play a role in maintaining the integrity of the uterine epithelium (reviewed in 33). There is also an upregulation of aquaporin (*AQP1*) and mucins (*MUC1* and *MUC7*), which play a role in maintaining polarization of uterine epithelium and movement of proteins during implantation in species with invasive placentation (34, 35). There is an upregulation of proteases expressed in the endometrium such as matrix metallopeptidases (*MMP7*, *MMP15,* and *MMP25*) which may play a role in the breakdown of the shell coat that occurs at attachment in the tammar wallaby or remodeling of the endometrium at this stage of pregnancy. There is also enrichment of genes associated with angiogenesis and regulation of vascular development (*VEGF*, *ESM1*) which supports

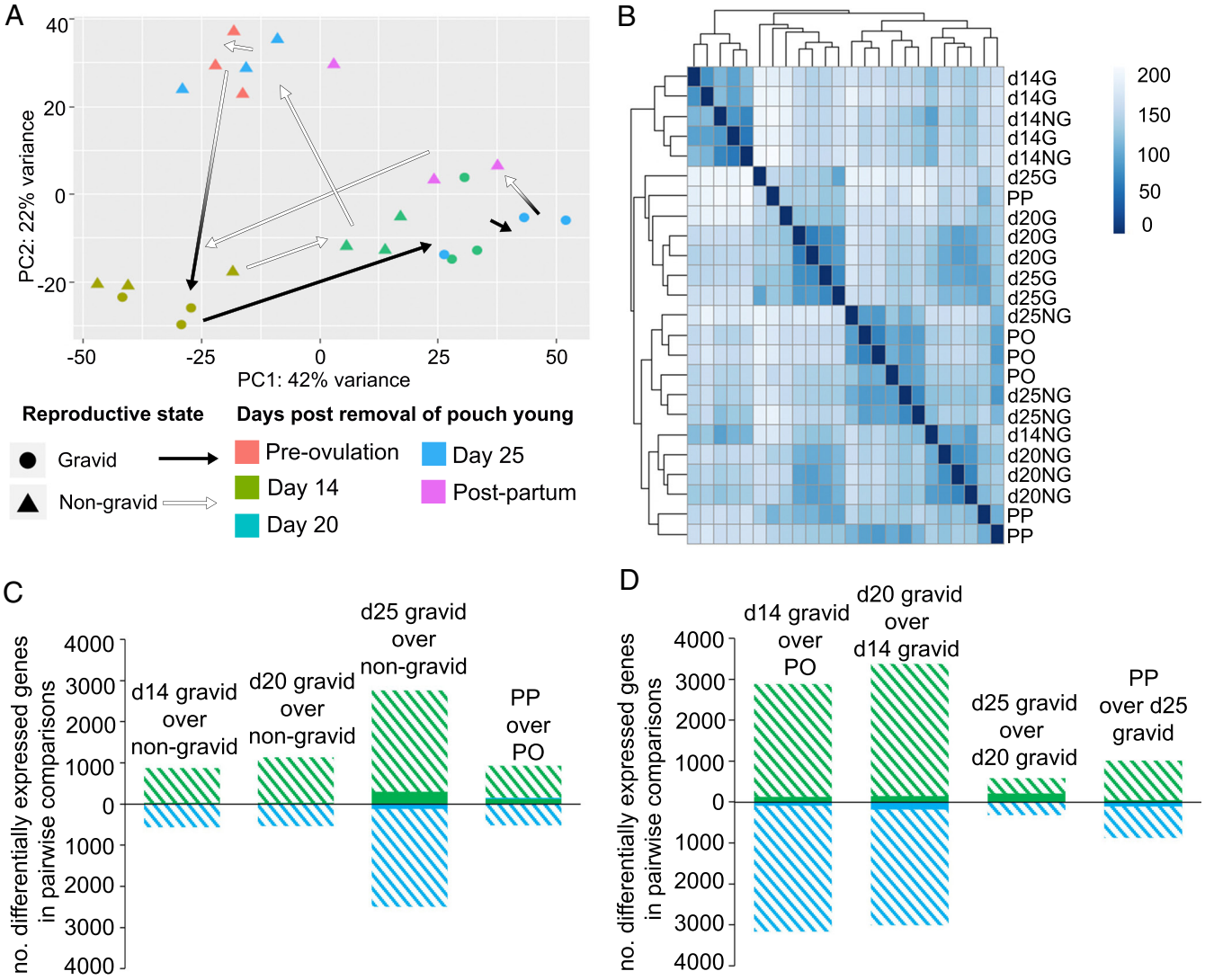

Fig. 3. Correlations of transcriptome data from uterine samples collected from different stages of pregnancy in the tammar wallaby. (*A*) Principal component analysis (PCA) of uterine transcriptomes of different reproductive states and timepoint during pregnancy. (*B*) Heatmap showing pairwise comparisons. (*C*) Number of differentially expressed genes in pairwise comparisons of gravid over nongravid uteri. Bars show the number of significantly upregulated (green) and downregulated (blue) genes in gravid compared to nongravid uteri from preattachment (d14), early attachment (d20), late attachment (d25), preovulation uterus (PO), and postpartum uterus (PP). (*D*) Number of differentially expressed genes in pairwise comparisons of preovulation (PO), preattachment (d14), postattachment (d20), late attachment (d25), and postpartum (PP) uteri. Hashed bars represent the total number of significantly differentially expressed genes, while the solid bars represent significantly differentially expressed genes with a fourfold difference in expression.

the substantial increase in vasculature that occurs throughout the endometrium during the later stages of pregnancy.

The late attachment timepoint of pregnancy, 1.5 to 2 d before birth (day 25) shows the greatest number of differentially expressed genes relative to the nongravid uterus. This correlates with the extensive morphological and physiological changes that occur to the endometrium at this timepoint. The most overrepresented GO terms (top 10 gene ontology terms ordered by p-value) upregulated when comparing the early and late attachment timepoints are associated with fibrinolysis, regulation of coagulation, and wound healing (Fig. 4*B*; Datasets S9–S21). Clustering of gene ontology terms is not as clear when comparing these timepoints suggesting that the time of placentation features many of the same changes in the gravid and nongravid uterus. At late attachment, the uterus is preparing for parturition, involving further substantial reorganization of the endometrium and detachment of the fetus at birth. Although placentation is noninvasive in macropodids, there is extensive vascular remodeling throughout the period of placentation (36) and tissue injury at the time of birth as suggested by the upregulation of genes associated with wound healing. Although there are thousands of differentially expressed genes throughout the period of attachment (upregulated on day 25 gravid compared to day 20), there are substantially fewer differences in gene expression between uteri from the final days of pregnancy (day 25) and postpartum (PP) suggesting that it takes longer than 24 h for the endometrium to transition back to a nongravid state. The preovulation uteri (PO) have thousands of differentially expressed genes when compared to gravid preattachment uteri (d14). This suggests that there are gene expression differences in early pregnancy before attachment while the developing fetus is surrounded by a shell coat. These early stages are characterized by minor changes to the morphology of the endometrium and no direct contact between the maternal endometrium and developing fetus.

Marsupial Attachment is Associated with Similar Gene Expression Changes to Eutherian Implantation. We investigated the expression of key implantation markers from eutherian pregnancy in the tammar wallaby. Expression of Heparin-binding

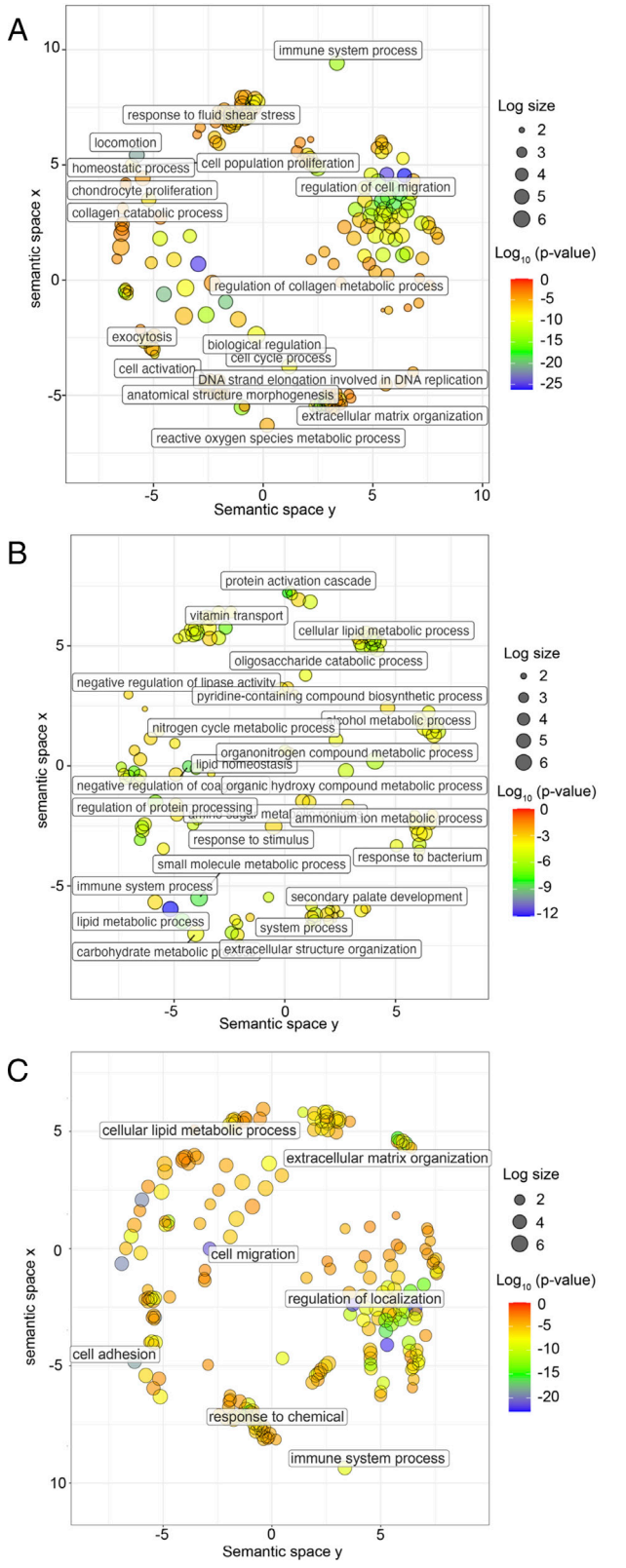

Fig. 4. GOrilla gene ontology (GO) enrichment analysis of differentially expressed genes from uterine samples collected at different stages of pregnancy in the tammar wallaby using scatterplots from REVIGO for biological processes. (*A*) Significantly overrepresented GO terms from the list of significantly upregulated genes at early attachment (d20) compared to preattachment (d14). (*B*) Significantly overrepresented GO terms from the list of significantly upregulated genes at late attachment (d25) compared to early attachment (d20). (*C*) Significantly overrepresented GO terms from the list of significantly upregulated genes in gravid uteri at late attachment (d25) compared to nongravid uteri from the same stage.

EGF-like growth factor (*HBEGF*) and Mucin 1 (*MUC1*) genes are upregulated after the loss of the shell coat and maintained throughout the period of attachment (d20 and d25; Fig. 5). *MUC1* expression increases further in postpartum uteri but *HBEGF* expression decreases after birth. *MMP7* expression is upregulated in tammar wallaby endometrium during the period of attachment. Immunohistochemistry shows that *MMP7* staining is localized almost exclusively in the nucleus of the uterine luminal and glandular epithelial cells which suggests that it has a gene regulatory function during late attachment (d25; Fig. 5*F* and *SI Appendix*, Fig. S2). In the fetal membranes, staining is both nuclear as well as cytoplasmatic. There is no staining in the uterine endometrium from the preattachment stage (d14) of pregnancy which correlates with the low expression levels at this reproductive stage (Fig. 5*E*).

There is an upregulation of expression of cathepsins in tammar wallaby endometrium during the period of attachment (*CTSZ*, *CTSC*, *CTSD*, and *CTSB*) along with Cystatin C (*CST3*). This expression pattern is similar to that which occurs in eutherian implantation (mice, sheep, and pig) where cathepsins are likely involved in remodeling of the endometrium (37–39).

There Is a Modified Inflammatory Gene Expression Profile as Pregnancy Progresses. To determine the inflammatory gene profile during pregnancy and how it is affected by the presence of a fetus, we focused on GO terms associated with an inflammatory response between nongravid samples and gravid samples at matching timepoints. There was no enrichment of GO terms related to an inflammatory response between gravid and nongravid samples before attachment (d14) suggesting that the presence of a shell coat around the developing fetus prevents a maternal inflammatory response in early pregnancy.

There is a significant overrepresentation of genes with GO terms related to an immune response before birth (d25) in the gravid uterus compared to the nongravid uterus at the same timepoint. This provides evidence for the influence of the fetus on endometrial gene expression after attachment inducing a moderated inflammatory response with some inflammatory genes being upregulated throughout the period of placentation while others are suppressed or show a modified expression. There is an overrepresentation of genes associated with an inflammatory response and immune system processes such as LIF—leukemia inhibitory factor, complement genes (*C2*, *C3*, and *C9*), Major histocompatibility complex, class i, a, (*HLA-A*), Interleukins and their receptors (*IL6*, *IL1RAP*, *IL1R1*, and *IL12A*), and genes associated with prostaglandin synthesis and signaling (*PTGS2*, *PTGES*, *PTGDR*, and *PTGFR*). There is also an increase in the expression of genes involved in neutrophil activation and neutrophil-mediated immunity such as proteases (*PRSS2*) and cystatins (*CSTB* and *CST3*).

There is an enrichment of genes associated with an immune effector process at early attachment (d20) compared to preattachment (d14; Fig. 4*A*). During early attachment, there is an enrichment of genes associated with an inflammatory response with an upregulation of acute phase proteins known to be involved in an inflammatory response such as Coagulation factor viii (*F8*) and interleukins (*IL34*). However, there is an upregulation of genes associated with fibrinogen (*FGG*, *FGA,* and *FGB*) at preattachment (d14), followed by suppression during early attachment (d20) and further upregulation at late attachment before birth (d25). This suggests that genes associated with mediators of inflammation (fibrinogen complex genes) show changes in expression through the period of attachment as the fetus grows and the fetal maternal interface develops.

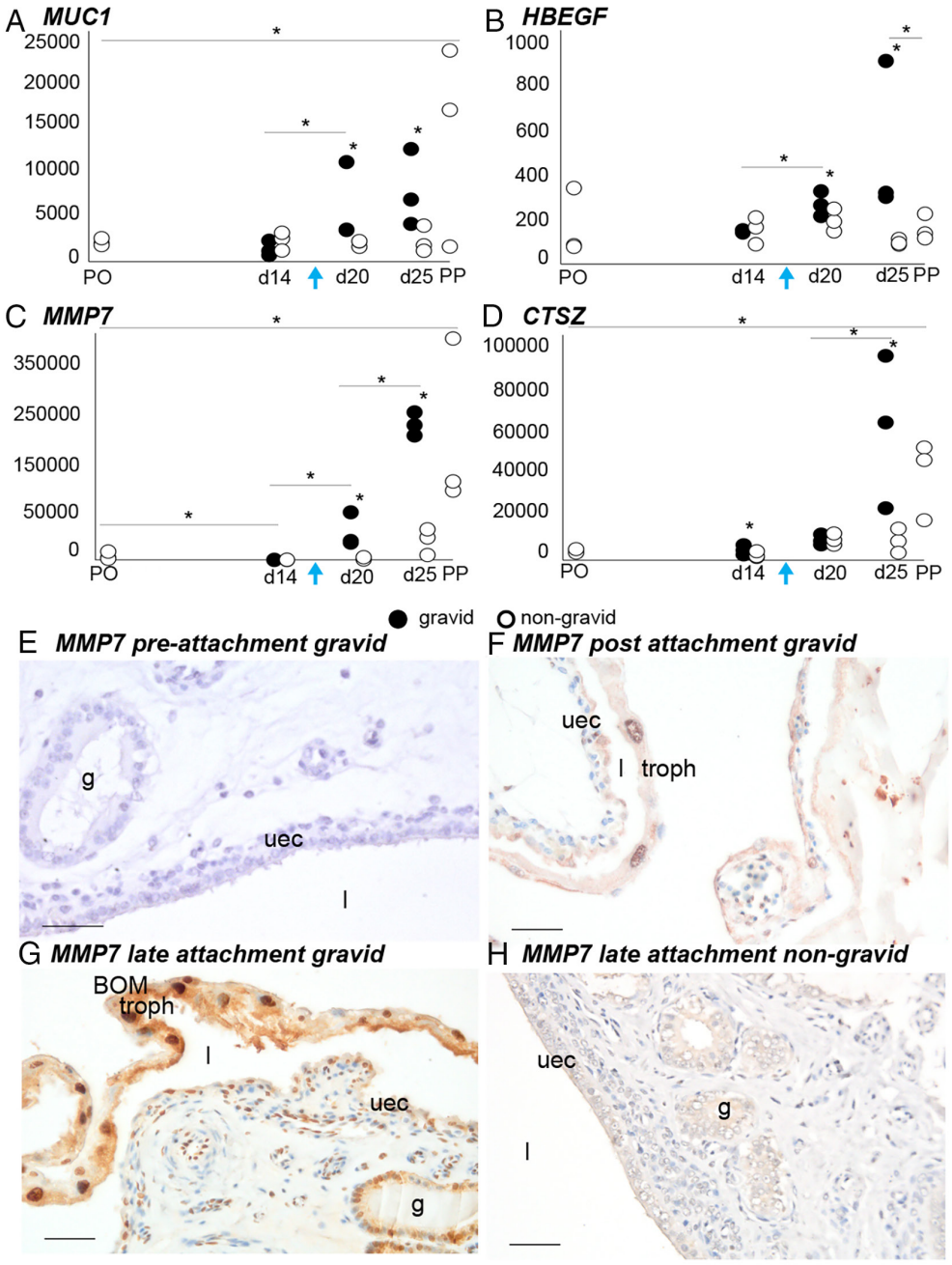

Fig. 5. Transcriptomic gene expression of implantation markers from uterine samples collected at different stages of pregnancy in the tammar wallaby with day of pregnancy on the x-axis and relative read counts on the y-axis. (*A*) MUC1, (*B*) HBEGF and proteases, (*C*) MMP7, and (*D*) Cathepsin Z in gravid and nongravid uteri at different timepoints during tammar wallaby pregnancy. Uteri from preovulation (PO), preattachment (d14), post attachment (d20), late attachment (d25), and postpartum (PP). Asterisks show significance ($P < 0.05$). Blue arrows indicate the timing of attachment and placentation. (*E–H*) There was no immunolocalization of the protease MMP7 in nongravid uterine epithelium and fetal membranes as well as in gravid uteri at preattachment (d14) and post attachment (d20). In comparison, there is localized staining to the nuclei of the uterine luminal and glandular epithelium at late attachment (d25). Nuclei are blue due to hematoxylin counterstaining and the immunostaining signal is brown due to 3,30-diaminobenzidine (DAB). l: uterine lumen, uec: uterine epithelial cells, g: gland, troph: trophoblast, BOM: bilaminar portion of the yolk sac. All scale bars are 50 μm.

To further test the hypothesis that an inflammatory reaction occurs at attachment in the tammar wallaby and is moderated throughout the period of placentation, we compared gene expression changes for the complete mucosal inflammatory cascade in the opossum, dunnart, and wallaby (Fig. 6). In the opossum and dunnart, almost all genes in the inflammatory cascade are up-regulated at attachment. In the tammar wallaby however, we found only a selection of the inflammatory genes are upregulated at key time points of pregnancy. Cytokines, such as IL6, are upregulated before attachment (d14) and birth (d25) and maintained in postpartum uteri (PP; Fig. 6*A*). *LIF* expression is upregulated after attachment (d20) and maintained during the period of placentation (d20 and d25; Fig. 6*B*). *LIF* expression is also maintained in both preovulation (PO) uteri and postpartum uteri (PP). The loss of expression of many of the key players in inflammation but maintenance of the expression of some genes, suggests that while a generalized inflammatory reaction does not occur during placentation in the tammar wallaby, some inflammatory genes may have been co-opted to facilitate functions of pregnancy.

In the tammar wallaby, genes associated with prostaglandin synthesis are upregulated leading up to birth which is known to facilitate parturition. *PTGS2* and *PTGES* expression in gravid

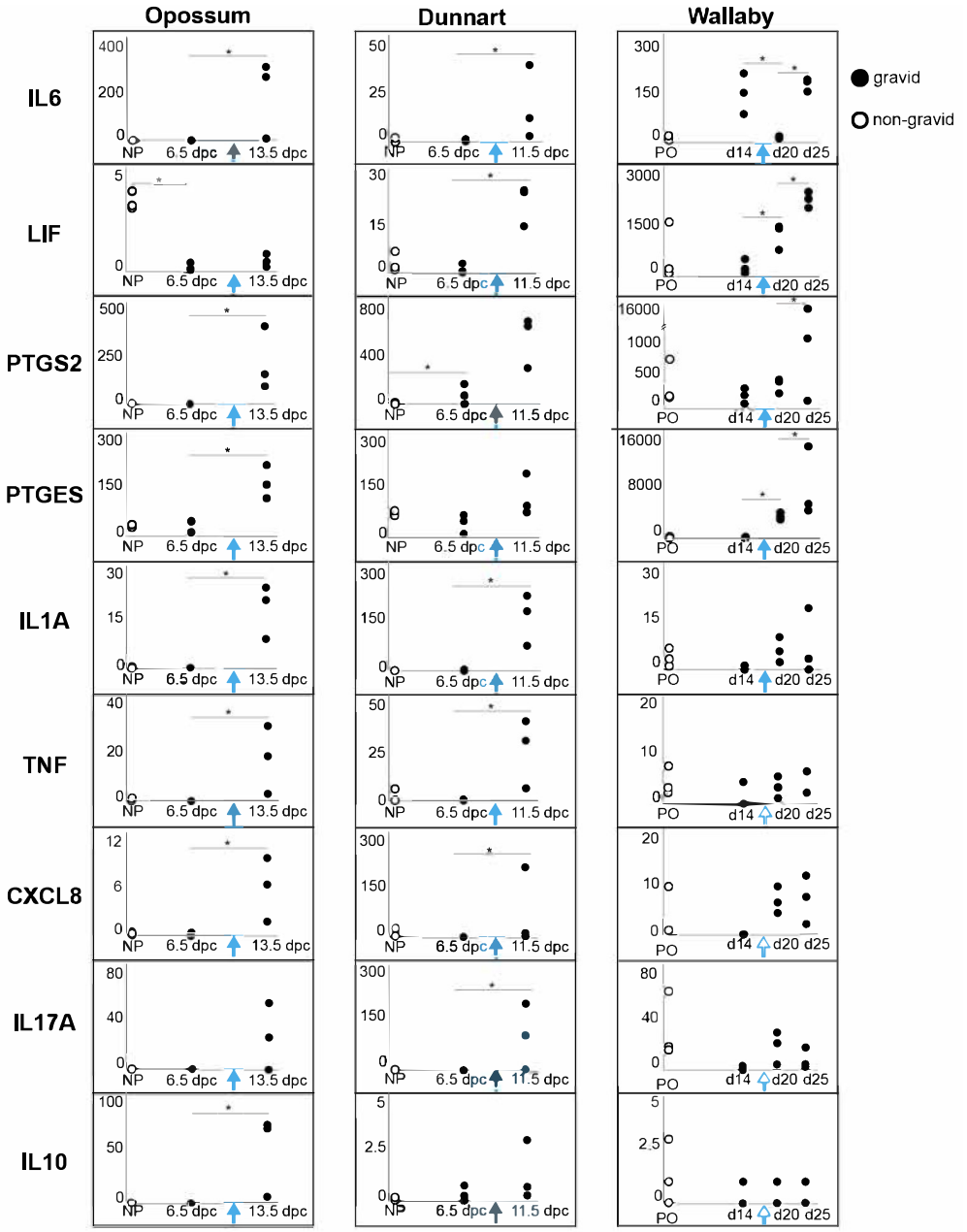

Fig. 6. Transcriptomic gene expression comparison of mediators of inflammation across marsupial species with invasive placentation: the gray short-tailed opossum and the fat-tailed dunnart and noninvasive placentation: the tammar wallaby. The day of pregnancy is on the x-axis and gene expression (in TPM for opossum and dunnart and relative read counts for wallaby) on the y-axis. Opossum uterine samples were collected from nonpregnant (NP), preattachment (6.5 dpc) and postattachment (13.5 dpc). Dunnart uterine samples were collected from nonpregnant (NP), preattachment (6.5 dpc), and postattachment (11.5 dpc). Wallaby uterine samples were collected from the preovulation (PO), preattachment (d14), early attachment (d20), and late attachment (d25) stage of pregnancy. Blue arrows indicate the timing of attachment and placentation in each species. Asterisks show significance ($P < 0.05$).

uteri is upregulated at late attachment, before birth (d25; Fig. 6 *C* and *D*) as prostaglandins are in the yolk sac and yolk sac fluid and circulation (40, 41). This gene expression pattern aligns with the spike in concentration of PGF2α, PGE2, and 6-keto-PGF1 seen at birth in tammar wallaby uterine and fetal tissues (40, 41). Circulating prostaglandins then drop dramatically within an hour of birth (41).

The differential expression of some inflammatory genes and not others throughout pregnancy suggests that a moderated inflammatory response occurs in the tammar wallaby. Despite the upregulation of known inflammatory markers, other key regulators of an inflammatory response such as *IL17A*, *IL10*, and *IL1A* show very low raw reads with no differential gene expression through pregnancy. The low expression of these key inflammatory genes suggests that only a partial inflammatory response occurs during attachment in the tammar wallaby and extension of pregnancy was enabled through the avoidance of a distinct inflammatory attachment reaction.

Discussion

In eutherian mammals, and some marsupials such as the opossum and dunnart, there is a highly conserved inflammatory cascade at the time of maternal–fetal attachment (Fig. 7). We show that during tammar wallaby pregnancy, there is no distinct inflammatory reaction, only a moderated expression of inflammatory mediators with some inflammatory genes expressed at key points of gestation. Proinflammatory cytokines, including *IL6*, are expressed before

embryo attachment, *IL1A* and *LIF* throughout the period of placentation and prostaglandins before birth. These results are consistent with specific inflammatory genes being co-opted into regulation of tammar wallaby pregnancy, while a generalized inflammatory response is not being induced. This lack of an attachment induced inflammation reaction differs to the ancestral attachment reaction seen in opossum and dunnart pregnancy and the inflammatory implantation reaction in eutherian pregnancy (Fig. 7). We thus conclude that in the wallaby lineage, the extension of pregnancy was achieved differently than in eutherians, namely by the avoidance of an inflammatory attachment reaction rather than its induction and subsequent down regulation.

The Period of Attachment is Homologous to Eutherian Implantation. Several morphological changes to the uterus through attachment in the tammar wallaby suggest that this period of close maternal–fetal apposition is homologous to the short period of attachment in the opossum and implantation in eutherians. Remodeling of the endometrium and gene expression patterns during the period of attachment and placentation mirror the changes seen during attachment in the opossum (1, 43, 44) and implantation in eutherian mammals (45, 46). Key implantation markers, Heparin-binding EGF-like growth factor (*HBEGF*) and Mucin 1 (*MUC1*) are upregulated after the loss of the shell coat and maintained throughout the period of placentation in the tammar wallaby. Similarly, in the opossum, there is a moderate increase in expression of *MUC1* following attachment and a further increase in expression through the final days of pregnancy (1). This increased expression pattern is also seen in eutherian species such as rodents and humans where *MUC1* is upregulated in uterine epithelium during the window of implantation. However, at the points of embryo attachment, its expression is lost in the maternal epithelium (47, 48). During eutherian implantation, an inflammatory gradient created by cytokines and chemokines facilitates the formation of a mucin layer and remodeling of the uterine epithelium which is necessary for apposition and adhesion of the blastocyst at implantation (reviewed in 3).

The process of attachment and implantation of the embryo is associated with remodeling of the endometrium. Many features of inflammation are shared with tissue remodeling and so may play a beneficial role in the process of attachment and implantation (49). For example, during pregnancy, mast cells are activated in response to sex steroids (estradiol and progesterone), to release mediators such as histamine, VEGF, proteases, and metalloproteinases (MMPs). These mediator proteins and compounds contribute to an inflammation response (50–53). MMPs also degrade the extracellular matrix of the endometrium and are important regulators of vascular and uterine remodeling (reviewed in 50, 54). High levels of MMP2 protein have been identified in uterine flushings from tammar wallabies in late pregnancy (55). There is an upregulation of matrix metallopeptidases (*MMP25*, *MMP7*, and *MMP15*) in tammar wallaby endometrium throughout placentation. The maternal

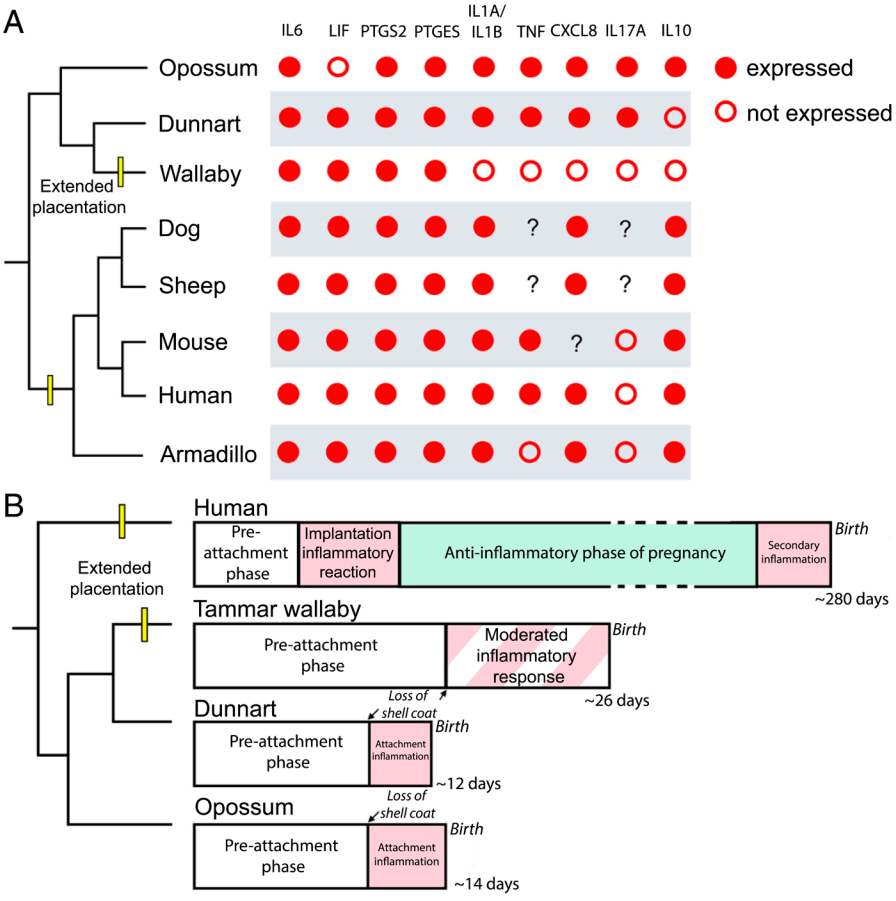

Fig. 7. Comparison of the inflammatory markers and the inflammation profile during pregnancy across Theria. (*A*) Comparison of key mediators of inflammation at attachment across Theria (reviewed in 2, 42). Proinflammatory cytokines such as IL17A, IL1A, IL1B, IL8, IL10, and TNF are expressed at low levels in tammar wallaby endometrium but show no differential expression throughout pregnancy. (*B*) Comparison of the length and maternal immune profile across eutherian and marsupial pregnancy showing the difference in immune profile across human, tammar wallaby, dunnart, and opossum pregnancy. Eutherian pregnancy is characterized by two proinflammatory phases at implantation and before birth. Macropodids such as wallabies have extended placentation compared to other marsupials with a moderated inflammation response occurring during the period of placentation. Adapted from (1). Yellow lines represent the independent evolution of extended placentation in macropodids and eutherians.

expression of MMP7 during tammar wallaby pregnancy with localization of Immunohistochemistry (IHC) staining to nuclei in the uterine luminal and glandular epithelium suggests that it has a gene regulatory function there. However, there is also strong staining in the cytoplasm of the trophoblast which suggests a different secretory function for embryonic tissues. Key markers of implantation, such as mucins and MMPs, show a moderated expression throughout the period of placentation in the tammar wallaby with similarities to eutherian species highlighting that this period likely is homologous to the period of implantation in eutherian pregnancy.

Only the Ghost of an Inflammation Response Persists in Tammar Wallaby Pregnancy. Opossums and dunnarts experience an acute inflammatory reaction at attachment, including neutrophil recruitment, which likely limits the extension of placentation beyond a few days. This is characterized by a progressive upregulation of genes involved in an inflammatory reaction such a proinflammatory cytokines (*IL6*, *TNF*, and *IL17A*) and prostaglandin synthases (*PTGES* and *PTGS2*; 1). The proinflammatory cytokines, *IL17A* and *IL6* are highly expressed by the trophoblast giant cells at embryo attachment (42). Eutherian implantation has the relatively full gamut of inflammatory signaling expected of a mucosal tissue but is missing IL17A signaling. The suppression of IL17A signaling is achieved by decidual stromal cells and results in downstream prevention of neutrophil recruitment to the endometrium, which is likely important for establishing placentation in eutherians (2, 42).

The inflammatory environment at implantation across eutherian lineages is mostly conserved (Fig. 7). Inflammatory cytokines LIF, IL6, IL1A, and IL1B and prostaglandins are expressed at the fetal–maternal interface at implantation across eutherian lineages including the more basally branching eutherian clades such as Xenarthra (which includes the armadillo; 2, 42, 56, 57). *LIF*, which is absent during placentation in the opossum, plays an important role in decidualization of endometrial stomal cells during eutherian implantation (58). Before implantation in eutherians, there is an upregulation of VEGF which is a potent inducer of increased vascular permeability associated with angiogenesis and inflammation (59, 60). This is followed by an estrogen-induced increase in blood flow resulting in uterine oedema which creates an optimal environment for endometrial remodeling and implantation (61). In eutherian pregnancy, the inflammatory environment preceding birth also promotes contraction of the uterus (6). Prostaglandins are key markers of acute inflammation playing a role in increasing vascular permeability (62). Some of them, such as PGF1a, also induce contraction of the myometrium contributing to the process of parturition in eutherian and marsupial species (63, 64, reviewed in 65). These features of an inflammation response suggest that they have been co-opted into important roles for pregnancy.

In contrast to eutherians, we found that some inflammatory genes are expressed throughout placentation in tammar wallaby pregnancy but not all. Genes that are expressed in the ancestral inflammatory attachment reaction such as IL17A, IL1A, and IL1B are not expressed during pregnancy in the tammar wallaby. The absence of key mediators of inflammation suggests that there is no activation of a complete inflammation cascade at attachment in the tammar wallaby. The loss of expression of particular inflammatory genes through the period of placentation likely allows for the extension of placentation in macropodids. In comparison to both the opossum and eutherian state, many of the downstream inflammatory genes are not differentially expressed at attachment in tammar wallaby pregnancy (Fig. 7). The genes that are differentially expressed are regulators of an inflammatory response including Interleukin-6 family cytokines. It is important to note, that our results cannot rule out inflammatory genes being co-opted to induce inflammatory changes in the tammar wallaby; however, our results demonstrate that the conserved inflammatory cascade which occurs in eutherian mammals and other studied marsupials does not occur. The expression of genes that have been retained during tammar wallaby pregnancy regulate vascular permeability, remodeling of the extracellular matrix, oedema, and tissue repair. This provides support for the hypothesis that some features of the inflammatory process might be beneficial to fetal development and being integrated into the physiology of implantation and attachment during pregnancy.

Building a General Model for the Evolution of Extended Placentation. Our findings suggest that the moderation of inflammation is a general requirement to extend the period of placentation in mammals. The short period of attachment experienced by the first live-bearing mammals was likely characterized by an inflammation response similar to what occurs during opossum pregnancy (1, 2). This inflammatory reaction at attachment limited the length of placentation due to a sustained influx of proinflammatory factors contributing to the onset of parturition after only a few days of maternal–fetal interaction.

During eutherian pregnancy, a short proinflammatory environment is necessary for embryo implantation but is switched off after implantation for most of placentation (4, 6). In macropodids, another mammalian lineage that has extended placentation, we show that they also moderate inflammation, by silencing key inflammatory genes throughout embryo attachment and the period of maternal–fetal contact. We propose that avoiding a distinct inflammatory attachment reaction is an alternative strategy for extending mammalian pregnancy. However, in either case, the eutherian and the wallaby pregnancy, prolonged fetal–maternal interaction is associated with and perhaps enabled by suppressing an extensive period of inflammation.

Methods

Sample Collection and Design. Tammar wallaby (*N. eugenii*) uterine tissue was collected from our breeding colony at the University of Melbourne. All experiments were approved by the University of Melbourne Animal Ethics Committees and followed the National Health and Medical Research Council (2013) guidelines. The endometrium was separated from the myometrium and fetal tissues by dissection, and samples were stored for RNA analysis, histology, and western blot analysis. Fat-tailed dunnart (*S.* crassicaudata) uterine tissue was collected from our breeding colony at Macquarie University. All experiments were approved by the Macquarie University Animal Ethics Committees and followed the National Health and Medical Research Council (2013) guidelines. Transcriptomic comparisons to opossum data used sequence data from refs. 1, 21 and some samples from nonpregnant and early pregnant dunnart used sequence data from ref. 66.

Histology and Immunostaining. Samples of gravid and nongravid uteri were collected for histological analysis at preattachment (days 14 to 16, n = 2), early attachment (days 20 to 23, n = 3), late attachment (days 25 to 26, n = 2), and postpartum (n = 2) stages of pregnancy based on the number of days after the removal of pouch young. Fixed tissues were embedded in paraffin, sectioned, deparaffinized, cleared, and then stained with hematoxylin and eosin following a standard protocol (67).

The expression of MMP7 was localized using immunohistochemistry. Uterine samples were fixed, embedded in paraffin, sectioned, deparaffinized, cleared, and then incubated in primary antibody specific to the protein of interest (rabbit anti-MMP7 antibody #NBP1-99123 (1.28 mg/mL; Novus Biologicals). Sections were washed and incubated with a secondary antibody (Polymer-HRP Anti-rabbit and DAB following kit protocol (DC EnVision + System, HRP; DAKO). Slides were counterstained with hematoxylin, dehydrated, and coverslipped for imaging using an Olympus BX53 with DP74 camera and cellSens Imaging Software (Olympus).

The specificity of the primary antibody was confirmed with Western blot analysis (*SI Appendix, Methods and Results*). Further immunohistochemistry analysis of key markers was not possible for the tammar wallaby as there are no species-specific antibodies available and have limited cross-reactivity with antibodies raised in eutherian species.

RNA Sequencing and Analysis. For RNA-seq analysis, we examined uterine tissue from nongravid and gravid uteri from pregnant females based on the number of days after the removal of pouch young. The stages included preovulation (sample collected from the uterus contralateral to the postpartum uterus within 24 h of birth; n = 2), preattachment (day 14; gravid n = 3, nongravid n = 2), early attachment (day 20; gravid n = 3, nongravid n = 2), late attachment (day 25; gravid n = 3, nongravid n = 3), and postpartum (sample collected within 24 h of birth; n = 2). Illumina sequencing libraries were generated by the Ramaciotti Center for Genomics (UNSW Sydney, Australia) using the TruSeq Stranded mRNA prep and sequenced with NovaSeq S1 100 bp sequencing. Raw reads were aligned to the tammar wallaby genome v3.0 using hisat2, and reads were counted using htseq-count. Hierarchical clustering and principal component analysis (PCA) were performed in the R *stats* package (68) to confirm that there were general patterns in gene expression between treatment groups. For the tammar wallaby, we compared differential gene expression analysis between nongravid PP, d14, d20, d25, and preovulation (PO), gravid d14, d20, d25 uteri using DeSeq2 default parameters (69, Datasets S1–S8). Gene ontology was analyzed using GOrilla and visualized with REVIGO (Datasets S9–S21, 70). For the dunnart, Illumina sequencing libraries were generated by the Ramaciotti Center for Genomics (UNSW Sydney, Australia) using the TruSeq Stranded mRNA prep and sequenced with NovaSeq S1 100 bp sequencing. We compared differential gene expression analysis between nonpregnant, early, and late pregnant uteri using DeSeq2 default parameters (Datasets S22 and S23). Gene ontology was analyzed using GOrilla and visualized with REVIGO (Datasets S24–S27).

Data, Materials, and Software Availability. Transcriptome data have been deposited in SRA (PRJNA987836, PRJNA1148679).

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