

Evolution of embryo implantation was enabled by the origin of decidual cells in eutherian mammals

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1 **Abstract**

2 Embryo implantation is the first step in the establishment of pregnancy in eutherian (Placental)
3 mammals. Although viviparity evolved prior to the common ancestor of marsupials and
4 eutherian mammals (therian ancestor), implantation is unique to eutherians. The ancestral therian
5 pregnancy likely involved a short phase of attachment between the fetal and maternal tissues
6 followed by parturition rather than implantation, similar to the mode of pregnancy found in
7 marsupials such as the opossum. Embryo implantation in eutherian mammals as well as embryo
8 attachment in opossum, induce a homologous inflammatory response in the uterus. Here, we
9 elucidate the evolutionary mechanism by which the ancestral inflammatory fetal-maternal
10 attachment was transformed into the process of implantation. We performed a comparative
11 transcriptomic and immunohistochemical study of the gravid and non-gravid uteri of two
12 eutherian mammals, armadillo (*Dasypus novemcinctus*) and hyrax (*Procavia capensis*); a
13 marsupial outgroup, opossum (*Monodelphis domestica*); and compared it to previously published
14 data on rabbit (*Oryctolagus cuniculus*). This taxon sampling allows inference of the eutherian
15 ancestral state. Our results show that in the eutherian lineage, the ancestral inflammatory
16 response was domesticated by suppressing a detrimental component *viz.* signaling by the
17 cytokine IL17A, while retaining components that are beneficial to placentation, *viz.*
18 angiogenesis, vascular permeability, remodeling of extracellular matrix. IL17A mediates
19 recruitment of neutrophils to inflamed mucosal tissues, which, if unchecked, can damage the
20 uterus as well as the embryo and lead to expulsion of the fetus. We hypothesized that the uterine
21 decidual stromal cells, which evolved coincidentally with embryo implantation, evolved, in part,
22 to prevent IL17A-mediated neutrophil infiltration. We tested a prediction of this hypothesis *in*
23 *vitro*, and showed that decidual stromal cells can suppress differentiation of human naïve T cells
24 into IL17A-producing Th17 cells. Together, these results provide a mechanistic understanding of
25 early stages of the evolution of the eutherian mode of pregnancy, and also identify a potentially
26 ancestral function of an evolutionary novelty, the decidual stromal cell-type.

27 Introduction

28 Embryo implantation is the process by which the blastocyst establishes a sustained fetal-maternal
29 interface for the maintenance of pregnancy. It begins with apposition of the blastocyst to
30 endometrial luminal epithelium, followed by its attachment via molecular interactions, and, in
31 many eutherian species, invasion of the endometrium to establish a direct contact with the
32 endometrial connective tissue and vasculature (Mossman 1937, Enders and Schlafke 1969,
33 Schlafke and Enders 1975, Ashary, Tiwari et al. 2018). Implantation is one of the most critical
34 steps in the establishment of a successful pregnancy, but it only occurs in eutherian mammals
35 (also known as Placental mammals). Mammalian viviparity originated before the common
36 ancestor of eutherian mammals and marsupials, i.e. in the stem lineage of therian mammals.
37 However, marsupial and eutherian pregnancies are different in many fundamental ways,
38 including embryo implantation.

39 Marsupial pregnancy is very short — in most cases shorter than the ovarian cycle (Renfree 1994,
40 McAllan 2011). For most of the duration of marsupial pregnancy, the embryo is present inside of
41 an eggshell (Selwood 2000, Griffith, Chavan et al. 2017) that precludes a direct physical contact
42 between the fetal and maternal tissues. Towards the end of the pregnancy, the eggshell breaks
43 down and the fetal membranes attach to the uterine luminal epithelium. The phase of attachment
44 lasts a short fraction of the length of gestation, and is soon followed by the birth of highly
45 altricial neonates. For instance, pregnancy in the South American marsupial, the grey short-tailed
46 opossum (*Monodelphis domestica*), lasts 14.5 days. Embryo attachment begins approximately on
47 the 12th day post-copulation (dpc) and induces an acute inflammatory response in the uterus
48 (Griffith, Chavan et al. 2017). This inflammation is presumably why embryo attachment
49 precipitates parturition rather than implantation (Chavan, Griffith et al. 2017, Hansen, Faber et
50 al. 2017).

51 Puzzlingly, in many eutherian mammals such as human, mouse, pig, and sheep, embryo
52 implantation also shows signs of an inflammatory reaction; some of these inflammatory
53 processes are in fact necessary and beneficial for a successful implantation (Keys, King et al.
54 1986, Barash, Dekel et al. 2003, Waclawik and Ziecik 2007, Plaks, Birnberg et al. 2008, Mor,
55 Cardenas et al. 2011, Dekel, Gnainsky et al. 2014, Robertson and Moldenhauer 2014, Chavan,
56 Griffith et al. 2017, Whyte, Meyer et al. 2017). Resemblance of the physiological process of
57 implantation to an inflammatory reaction appears paradoxical at first because inflammation in
58 later stages of pregnancy leads to the termination of pregnancy. However, analysis of the
59 evolutionary history of embryo implantation suggests that this resemblance is due to the
60 evolutionary roots of implantation in an inflammatory response to embryo attachment (Finn
61 1986, Griffith, Chavan et al. 2017). Griffith and colleagues (Griffith, Chavan et al. 2017,
62 Griffith, Chavan et al. 2018) argued, based on a comparison of embryo attachment in opossum to
63 implantation in eutherian mammals, that eutherian implantation and the inflammatory marsupial

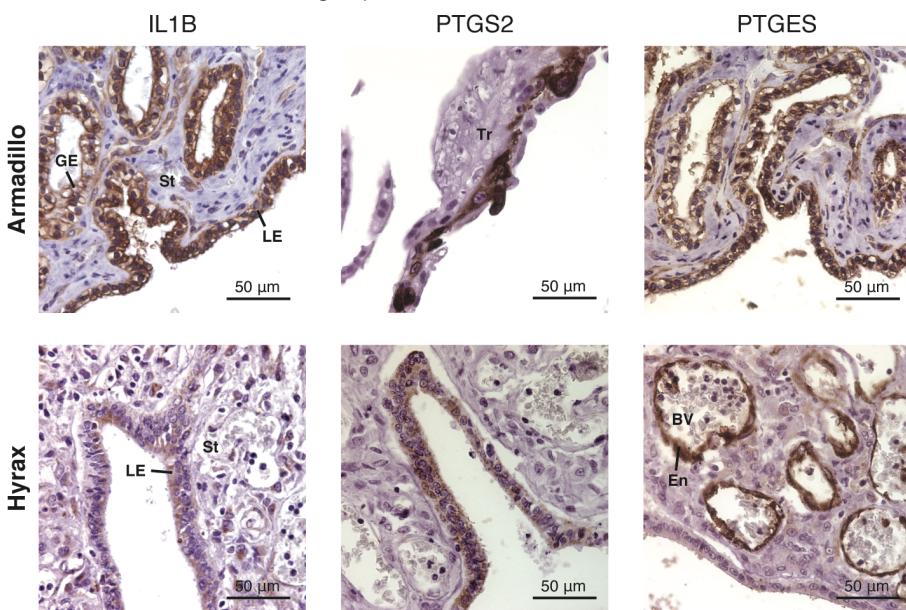
64 attachment reaction are homologous processes. That is, these processes evolved from an
65 inflammatory fetal-maternal attachment reaction that likely existed in the therian ancestor.
66 The difference between the fetal-maternal attachment in marsupials and eutherians is its
67 outcome. In the opossum the brief inflammatory attachment results in parturition, whereas in
68 eutherians it results in implantation and establishment of a sustained fetal-maternal interface.
69 Here, we elucidate the mechanism by which the ancestral attachment-induced inflammatory
70 response was transformed into the process of embryo implantation in the eutherian lineage. We
71 show that the origin of decidual stromal cells (DSC) — a novel eutherian cell type — was
72 integral to this transformation. First, we provide evidence to further support the homology
73 between opossum attachment reaction and eutherian embryo implantation. Then, we compare
74 gene expression in the uterus of opossum during attachment to that in two eutherians during
75 implantation, armadillo and rabbit. The key differences in gene expression suggest that embryo
76 implantation evolved through suppression of a specific module of the ancestral mucosal
77 inflammatory reaction — neutrophil recruitment mediated by the pro-inflammatory cytokine
78 IL17A. We hypothesized that the origin of DSC in the eutherian lineage (Mess and Carter 2006),
79 coincidentally with embryo implantation, was responsible for the suppression of IL17A in
80 members of this clade. A test of this hypothesis using human cells showed that secretions from
81 DSC inhibit the differentiation of Th17 lymphocytes, the primary producers of IL17A, by
82 inducing a non-standard type-1 interferon response that down-regulates their protein synthesis.

83 Results and Discussion

84 Inflammatory implantation is an ancestral eutherian trait

85 The inference of homology between the opossum attachment reaction and eutherian embryo
86 implantation is derived from comparison of opossum to species from Boreotheria (Griffith,
87 Chavan et al. 2017). Boreotheria is one of the three major clades that make up Eutheria and
88 includes primates, rodents, ungulates, carnivores, bats and their kin. However, Eutheria also
89 contains two other major clades, Xenarthra and Afrotheria (dos Reis, Inoue et al. 2012, Tarver,
90 dos Reis et al. 2016), for which data on inflammatory gene expression at implantation was
91 previously unavailable. Xenarthra includes armadillo, sloth, anteater, etc; and Afrotheria includes
92 elephant, hyrax, tenrec, aardvark, dugong, etc. See **Figure 1d** for the phylogenetic relationship
93 among eutherian species. To test the inference of homology, we investigated the hypothesis that
94 inflammatory implantation is a shared eutherian character; that is, it is not limited to Boreotheria,
95 but is also observed in Xenarthra and Afrotheria. We did this by assessing the expression of
96 marker genes of inflammation during embryo implantation in the nine-banded armadillo
97 (*Dasypus novemcinctus*) and rock hyrax (*Procavia capensis*), as representatives of Xenarthra and
98 Afrotheria, respectively.

(a) Inflammation mediators during implantation

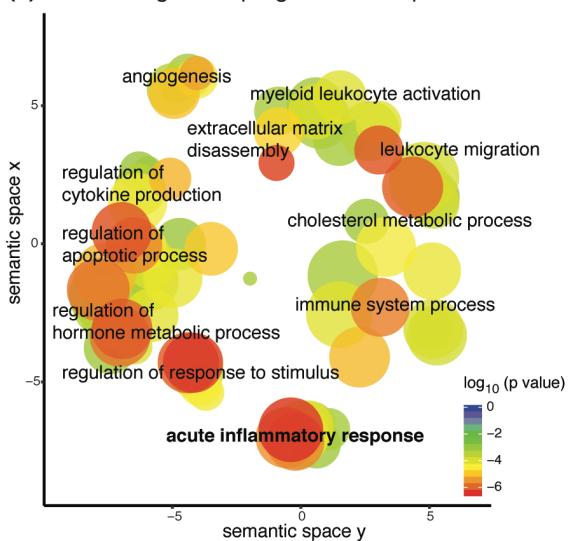


(b) Armadillo: cytokines (mRNA)

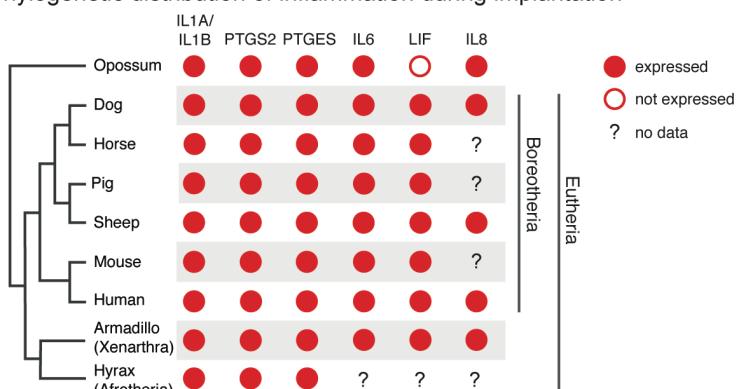
	Non pregnant	Peri implantation
IL1A	1.7	39.3
IL1B	0	2.2
TNF	0	0.5
IL1R1	6.1	31.4
IL6	2.3	6.4
LIF	0	115.4
PTGS2	2.4	14.6
PTGES	1.2	100.2
PTGER2	6.4	18.8
PTGER4	18.4	18.4
IL8	0.6	47.2
IL10	0	15.4

tpm

(c) Armadillo: genes upregulated at implantation



(d) Phylogenetic distribution of inflammation during implantation



100 **Figure 1** Inflammatory implantation is an ancestral eutherian character. **(a)** Immunohistochemistry for
101 inflammation marker genes IL1B, PTGS2, and PTGES at the fetal-maternal interface in armadillo and
102 hyrax. Nuclei are blue due to hematoxylin counterstaining and the immunostaining signal is brown due to
103 DAB (3,3'-diaminobenzidine). GE = glandular epithelium, LE = luminal epithelium, St = stroma, Tr =
104 trophoblast, BV = blood vessel, En = endothelium. **(b)** Abundance of mRNA transcripts (TPM =
105 Transcripts per Million) of key inflammatory genes in armadillo uterus in non-pregnant and peri-
106 implantation stage. **(c)** Enriched gene ontology (GO) categories among the genes that are upregulated at
107 least 10-fold in armadillo uterus in transition from non-pregnant to peri-implantation stage. GO categories
108 are clustered by semantic similarity. GO categories closer to each other are semantically similar; those
109 represented by red circles are more significantly enriched than those in blue. **(d)** Comparison of
110 expression of inflammatory genes during embryo attachment or implantation in therian mammals. Data
111 for human: cytokines (reviewed in Van Sinderen, Menkhorst et al. 2013), PTGS2 (Marions and
112 Danielsson 1999), PTGES (Milne, Perchick et al. 2001). Data for sheep, horse, pig, dog, and mouse:
113 (reviewed in Chavan, Griffith et al. 2017).

114 One of the earliest signals of inflammation, IL1B, is expressed during implantation in the
115 luminal epithelium of the endometrium in armadillo and hyrax (**Figure 1a**). PTGS2 and PTGES,
116 enzymes involved in the synthesis of prostaglandin E₂ (PGE₂), are also expressed during
117 implantation in both species, although the tissues in which these genes are expressed differ
118 between species. PTGS2 and PTGES are expressed on the two sides of the fetal-maternal
119 interface in armadillo — PTGS2 in the trophoblast, while PTGES in the endometrial luminal
120 epithelium. In hyrax, they are both expressed on the maternal side, with PTGS2 in the luminal
121 epithelium and PTGES in the endothelia within the endometrium.

122 Next, we used transcriptome data to test whether there is a broad signature of inflammation
123 during implantation in armadillo uterus. A variety of inflammatory genes are up-regulated during
124 armadillo implantation: cytokines such as *IL1A*, *IL1B*, *IL6*, *LIF*, *IL8* (*CXCL8*), and *IL10*;
125 cytokine receptor *IL1R1*; prostaglandin synthesis enzymes *PTGS2* and *PTGES*; and
126 prostaglandin receptors *PTGER2* and *PTGER4* (**Figure 1b**). None of the cytokines shown in
127 **Figure 1b** is expressed in the non-gravid uterus, i.e. their mRNA abundance is below the
128 operational threshold of 3 TPM (Wagner, Kin et al. 2013), but most of them are expressed highly
129 during implantation. Genes up-regulated more than 10-fold in the transition from non-pregnant
130 to implantation stage are significantly enriched in Gene Ontology (GO) categories related to
131 inflammation (**Figure 1c**), such as acute inflammatory process, immune system process,
132 leukocyte migration, regulation of cytokine production, and regulation of response to stimulus.

133 We summarized the above expression pattern of inflammation marker genes during implantation
134 on a phylogeny of therian mammals, along with the expression patterns from other representative
135 eutherian species, and from a marsupial, opossum, at the time of attachment between fetal and
136 maternal tissues at 13.5 days post-copulation (dpc) (**Figure 1d**). In all major clades of Eutheria,
137 the uterine changes during embryo implantation closely resemble an acute inflammatory
138 reaction. Parsimoniously, this suggests that embryo attachment-induced uterine inflammatory
139 signaling is a plesiomorphic trait of eutherian mammals, i.e. a trait that was inherited from an

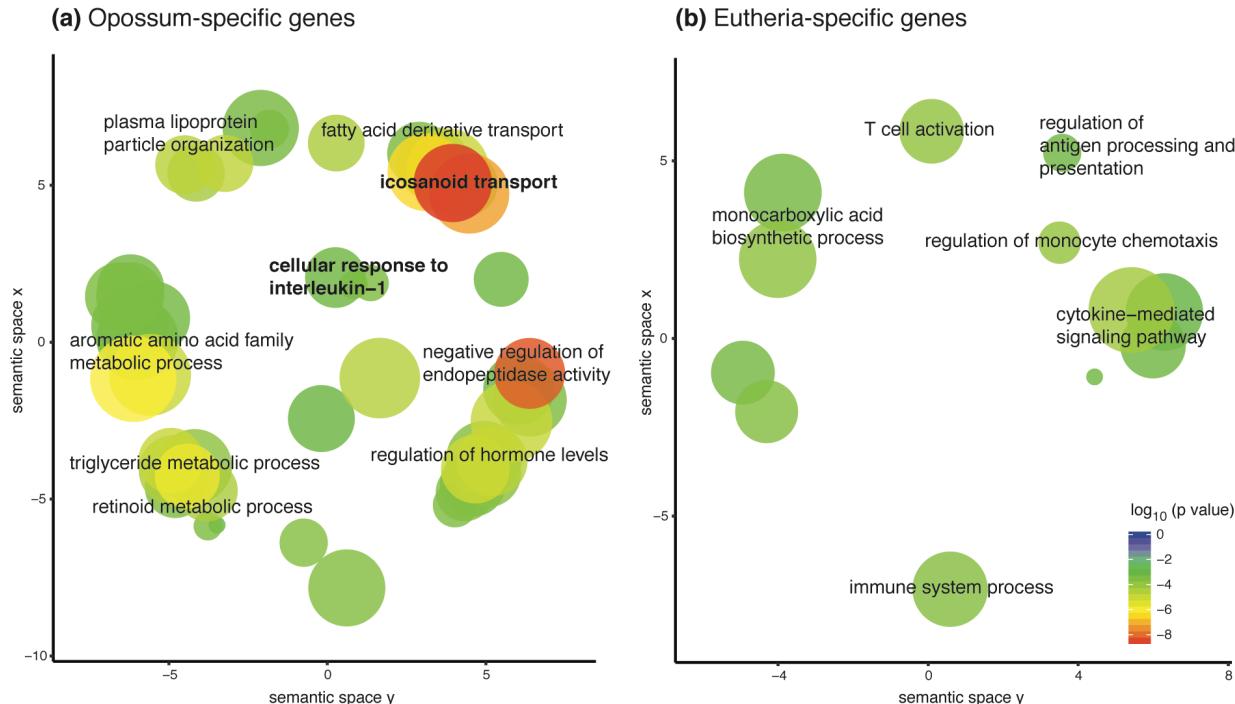
140 ancestral lineage and shared with the sister group, the marsupials, adding further support to the
141 argument that attachment-induced inflammation of marsupials is homologous to eutherian
142 embryo implantation (Griffith, Chavan et al. 2017, Griffith, Chavan et al. 2018, Liu 2018). In
143 other words, eutherian implantation likely evolved from, and through modification of, ancestral
144 attachment-induced inflammation.

145 **Differences in uterine gene expression between opossum and eutherians**

146 To understand *how* eutherian implantation evolved from the ancestral therian attachment induced
147 inflammation, we compared uterine gene expression during attachment-induced inflammation in
148 opossum (*Monodelphis domestica*) to that during implantation in two eutherian mammals,
149 armadillo (*Dasypus novemcinctus*) and rabbit (*Oryctolagus cuniculus*) (rabbit data from Liu,
150 Zhao et al. 2016). Gene expression patterns shared by armadillo and rabbit are likely to be shared
151 by eutherians in general since armadillo and rabbit are phylogenetically positioned so that their
152 common ancestor is the common ancestor of all extant eutherian mammals.

153 In the uterine transcriptomes of opossum, armadillo, and rabbit, we classified each gene as either
154 expressed or not expressed (see Methods). We then classified these genes as “opossum-specific”
155 if they are expressed in opossum but not expressed in armadillo and rabbit, and “Eutheria-
156 specific” if they are not expressed in opossum but are expressed in both armadillo and rabbit.
157 There are 446 and 456 genes in these categories, respectively, among the total of 11,089 genes
158 that have one-to-one orthologs in all three species.

159 First we identified enriched GO categories in the opossum-specific and Eutheria-specific lists of
160 genes (**Figure 2**). To increase the specificity of GO enrichment analysis, we used only the subset
161 of the opossum-specific expressed genes that have an at least 2-fold higher gene expression in
162 the attachment phase compared to the non-pregnant stage (204 genes). Such refinement of the
163 Eutheria-specific gene set was not possible since we do not have a transcriptome of non-pregnant
164 rabbit uterus. The opossum-specific set is enriched for genes related to lipid metabolism,
165 especially mobilization of fatty acids from cell membrane, and lipid transport. Lipid metabolism
166 genes are also upregulated in the pregnant uterus of another marsupial, fat-tailed dunnart
167 (*Sminthopsis crassicaudata*) (Whittington, O’Meally et al. 2018). These genes may have
168 functions related to nutrient transfer or steroid metabolism but lipid metabolism is also important
169 in inflammation: for example, the first step in the synthesis of prostaglandins is to break down
170 membrane triglycerides. The opossum-specific set is also enriched for GO category “cellular
171 response to Interleukin-1”, i.e. genes downstream of IL1A and IL1B. This suggests that although
172 inflammatory signaling is initiated by IL1A and/or IL1B upon embryo attachment in opossum as
173 well as in eutherians, their downstream targets are activated only in the opossum. This
174 observation recapitulates at the molecular level a phenomenon at the organismal level —
175 inflammatory signaling is observed in both, but has different outcomes of parturition and
176 implantation in opossum and eutherians respectively.



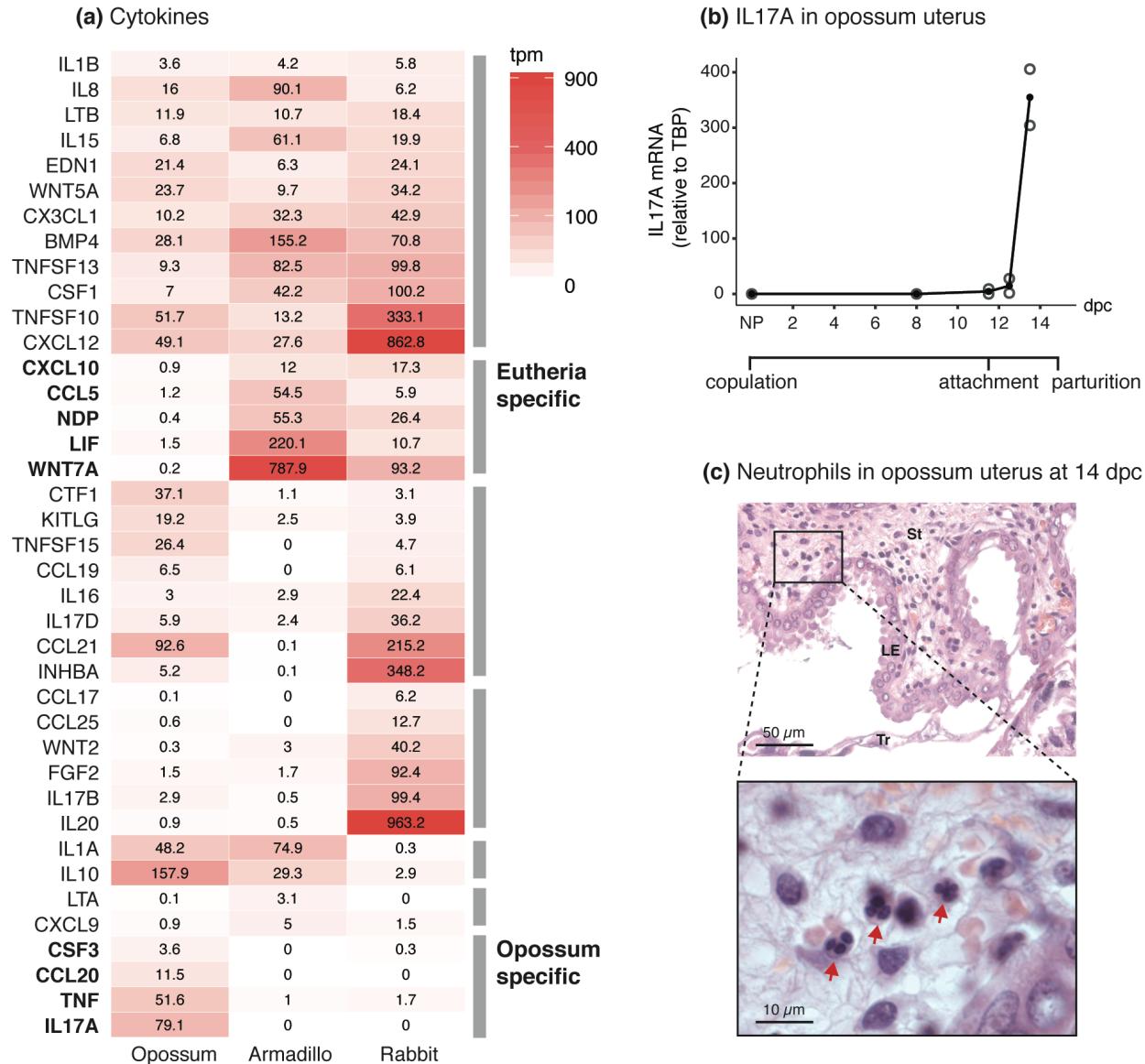
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178 **Figure 2** Transcriptomic differences between marsupial and eutherian attachment reaction. Genes were
179 classified as opossum-specific **(a)** and eutherian-specific **(b)**. GO categories enriched in each set are
180 shown in the figures, where semantically similar categories are clustered together in space.

181 **IL17A expression was suppressed in Eutheria**

182 IL1A and IL1B set off a cascade of inflammatory signaling events mediated by cytokine
183 molecules. Therefore, in order to identify the specific differences between opossum and
184 eutherians in response to interleukin-1, we looked for differences in the expression pattern of
185 cytokines (**Figure 3a**). Cytokines were identified as genes assigned to GO category “cytokine
186 activity” (GO:0005125).

187 Eutheria-specific expressed cytokines are *CXCL10*, *CCL5*, *NDP*, *WNT7A*, and *LIF*. The first two,
188 *CXCL10* and *CCL5* attract leukocytes such as T cells, NK cells, and dendritic cells to the sites of
189 inflammation (Schall 1991, Dufour, Dziejman et al. 2002). *NDP* and *WNT7A* are both members
190 of the Wnt signaling pathway, which is important for communication between implanting
191 blastocyst and endometrium (Wang and Dey 2006, Chen, Zhang et al. 2009, Sonderegger,
192 Pollheimer et al. 2010); inhibition of this process prevents successful implantation in mouse
193 (Mohamed, Jonnaert et al. 2005). *LIF* is a critical signaling molecule in eutherian mammals for
194 the differentiation of decidual stromal cells from endometrial stromal fibroblasts; and its
195 expression is therefore obligatory for successful implantation (Shuya, Menkhorst et al. 2011).
196 This set of genes represents cytokines and signaling molecules that were likely recruited within
197 the eutherian lineage to enable decidual cell differentiation, embryo-uterine crosstalk, regulation
198 of leukocyte traffic, and therefore implantation.



199

200 **Figure 3** IL17A signaling is suppressed in eutherians. **(a)** Gene expression of cytokines during embryo
201 attachment or implantation in opossum, armadillo, and rabbit uterus. Intensity of red is proportional to the
202 abundance of mRNA. Genes expressed below 3 TPM are considered unexpressed (Wagner, Kin et al.
203 2013). Rows are ordered by the gene expression patterns across species: expressed in all species,
204 expressed only in Eutheria, expressed in opossum and rabbit, rabbit-specific, expressed in opossum and
205 armadillo, armadillo-specific, and opossum-specific. Cytokines not expressed in any of these species are
206 not shown in the figure. **(b)** Expression of *IL17A* through the pregnancy of opossum, measured relative to
207 *TBP*, by qPCR. Embryo attachment begins around 11.5 days post-copulation (dpc), and pregnancy ends at
208 14.5 dpc. (number of biological replicates: NP = 3, 8 dpc = 3, 11.5 dpc = 2, 12.5 dpc = 2, 13.5 dpc = 2)
209 **(c)** Neutrophils infiltration in H&E stained opossum uterus at 14 dpc, indicated by red arrows in the
210 zoomed in micrograph. LE = luminal epithelium, St = stroma, Tr = trophoblast. TPM = Transcripts per
211 Million.

212 The inverse set, opossum-specific cytokines, includes *CSF3*, *CCL20*, *TNF*, and *IL17A*. *CSF3*,
213 also known as G-CSF (Granulocyte Colony Stimulating Factor) attracts granulocytes (Lieschke,
214 Grail et al. 1994, Panopoulos and Watowich 2008), such as neutrophils, and is positively
215 regulated by *IL17A* (Ye, Rodriguez et al. 2001, Onishi and Gaffen 2010). *CCL20* attracts
216 lymphocytes as well as neutrophils and also is a downstream gene of *IL17A* (Onishi and Gaffen
217 2010). This pattern is indicative of a mucosal inflammatory reaction, culminating with
218 recruitment of effector cells such as neutrophils.

219 Since *IL17A* is upstream of the other genes in the opossum-specific set, we then measured the
220 expression of *IL17A* in opossum uterus by qPCR to test whether it is induced in response to the
221 attaching embryo or expressed throughout pregnancy. **Figure 3b** shows that *IL17A* is not
222 expressed in non-pregnant and 8 dpc uteri, and only begins expression at 11.5 dpc, coincidental
223 with the egg-shell breakdown and beginning of fetal-maternal attachment. Its expression reaches
224 a very high level by 13.5 dpc; 79 TPM according to the transcriptomic data. This time-course
225 expression data suggests that *IL17A* is induced specifically in response to embryo attachment in
226 opossum, even though it is completely absent in armadillo and rabbit during implantation (0
227 TPM in both species). One of the hallmarks of *IL17A*-mediated inflammation — through the
228 action of cytokines like CXCL8, *CSF3*, *CCL20* — is neutrophil infiltration into the inflamed
229 tissue (Medzhitov 2007, Onishi and Gaffen 2010, Griffin, Newton et al. 2012, Pappu, Rutz et al.
230 2012, Flannigan, Ngo et al. 2016). Therefore, we tested whether *IL17A* expression in opossum is
231 also accompanied by neutrophil infiltration. Neutrophils are not seen in early stages of
232 pregnancy, but at 14 dpc, neutrophil infiltration is clearly seen histologically (**Figure 3c**).
233 Consistent with the pattern of *IL17A* expression, neutrophils are absent from the fetal-maternal
234 interface at the time of implantation in eutherian mammals (evidence reviewed in Chavan,
235 Griffith et al. 2017).

236 The absence of *IL17A* expression in eutherian mammals at implantation is likely to be due to its
237 loss in the eutherian lineage rather than its recruitment in the marsupial lineage. The discovery of
238 *IL17* homologs as the early-responding cytokines in the sea urchin larva during gut infection
239 (Buckley, Ho et al. 2017) suggests that it may be an ancient mucosal inflammatory cytokine at
240 least as old as deuterostomes. Since the endometrium is a mucosal tissue, *IL17A* — a key
241 mucosal inflammatory cytokine — is likely to have been expressed in the ancestral therian
242 endometrium during attachment-induced inflammation, and its expression was lost later during
243 evolution in the eutherian lineage.

244 Because *IL17A* is 1) the most highly expressed gene among opossum-specific cytokines, 2) an
245 important regulator of mucosal inflammation, 3) upstream of chemokines like *CSF3*, and 4) not
246 expressed in armadillo and rabbit at all, even in a leaky manner, we posited that the loss of *IL17A*
247 expression — and thus the loss of neutrophil infiltration — after embryo attachment was one of

248 the key innovations that transformed the ancestral inflammatory attachment reaction into embryo
249 implantation.

250 Contrary to the data from armadillo and rabbit, some IL17A expression has been reported at the
251 fetal-maternal interface in mouse and human, specifically in the $\gamma\delta$ T cells in the mouse (Pinget,
252 Corpuz et al. 2016). However, functional evidence suggests that even in mouse and human, an
253 induction of IL17A expression at the fetal-maternal interface is detrimental to pregnancy. The
254 strongest evidence perhaps comes from mouse, where injection of polyI:C to mimic viral
255 infection during pregnancy induces the expression of IL17A in the decidua. Maternal IL17A then
256 makes its way into the fetal circulation, interferes with brain development of the embryos, and
257 leads to behavioral abnormalities resembling Autism Spectrum Disorder (ASD) in pups (Choi,
258 Yim et al. 2016). Li and colleagues (Li, Qu et al. 2018) showed that in a mouse model of
259 spontaneous abortion, Th17 cell numbers are higher in the decidua relative to normal pregnancy.
260 In human, Th17 levels in peripheral blood are elevated in women with recurrent spontaneous
261 abortions compared to women with healthy pregnancy (Wang, Hao et al. 2010). Nakashima and
262 colleagues (Nakashima, Ito et al. 2010) found that while IL17A-positive cells were only
263 occasionally present in the deciduae of normal pregnancies, their numbers were significantly
264 elevated in pregnancies with first trimester spontaneous abortions. These studies clearly indicate
265 that IL17A signaling at the fetal-maternal interface is not conducive to successful pregnancy, and
266 the cases in human and mouse where IL17A expression is observed, the downstream signaling is
267 likely inhibited in some way.

268 Next, we sought to identify the mechanism by which IL17A signaling was suppressed in the
269 evolution of eutherian mammals.

270 **Decidual stromal cells suppressed IL17A expression at implantation in Eutheria**

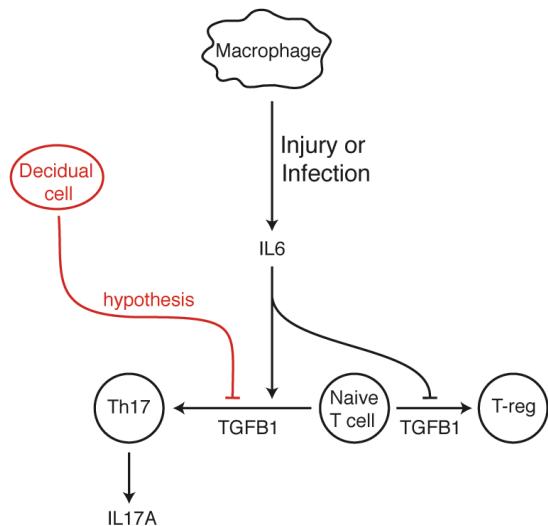
271 Decidual stromal cells (DSC) are a novel cell type that originated in the stem lineage of eutherian
272 mammals (Mess and Carter 2006, Wagner, Kin et al. 2014, Erkenbrack, Maziarz et al. 2018).
273 They differentiate from endometrial stromal fibroblasts (ESF) in many eutherian mammals
274 during pregnancy (Gellersen and Brosens 2014) and also during the menstrual cycle in primates,
275 some bats, the elephant shrew (Emera, Romero et al. 2011), and the spiny mouse (Bellofiore,
276 Ellery et al. 2017) in a process called decidualization. The evolution of DSC from ancestral
277 therian ESF was associated with modulation of expression of genes involved in the innate
278 immune response (Kin, Maziarz et al. 2016). DSC perform many functions critical to the
279 maintenance of pregnancy in human and mouse, for example, regulation of the traffic of
280 leukocytes into the endometrium during pregnancy (Nancy, Tagliani et al. 2012, Erlebacher
281 2013), production of hormones like prolactin, regulation of invasiveness of the trophoblast
282 (Gellersen and Brosens 2014), regulating communication between cell types at the fetal-maternal
283 interface (Pavlicev, Wagner et al. 2017). Outside of euarchontogliresian mammals (primates,
284 rodents and their relatives), however, DSC are not maintained throughout the pregnancy. In bats

285 (Laurasiatheria), hyrax, tenrec (Afrotheria), and armadillo (Xenarthra, also see **Supp. Figure 1**),
286 DSC differentiate around the time of implantation but are often lost soon after implantation. This
287 suggests that the ancestral function of DSC when they originated, was likely to have been limited
288 to the time of implantation (Chavan, Bhullar et al. 2016). Based on their inferred ancestral role at
289 the time of implantation (Chavan, Bhullar et al. 2016), their ability to regulate the immune
290 response during pregnancy (Erlebacher 2013), and because their origin in the eutherian stem
291 lineage (Mess and Carter 2006) coincides with the evolution of suppression of IL17A expression
292 (this study), we hypothesized that DSC played a role in the regulation of IL17A during
293 implantation.

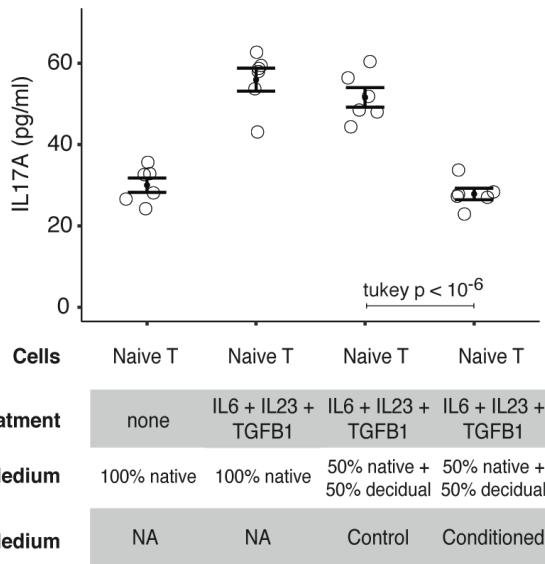
294 IL17A is typically expressed by Th17 cells, which differentiate from naïve T cells in the
295 presence of IL6 and TGFB1 (Bettelli, Carrier et al. 2006) (**Figure 4a**). We hypothesized that
296 DSC suppress IL17A expression by inhibiting the differentiation of naïve T cells into Th17 cells.
297 To test this hypothesis, we differentiated primary human naïve T cells into Th17, in the presence
298 of DSC-conditioned or control medium and measured IL17A secreted by T cells with ELISA.
299 Treatment of differentiating T cells with DSC conditioned medium decreases their IL17A
300 production significantly (2 fold, $p < 10^{-6}$), while treatment with unconditioned DSC medium has
301 no effect. The decreased level of IL17A is not statistically distinguishable from that in
302 unstimulated naïve T cells, suggesting that DSC-conditioned medium completely suppresses
303 upregulation of IL17A production during Th17 differentiation (**Figure 4b**; see **Supp. Figure 2**
304 for mRNA levels of *IL17A*).

305 Wu and colleagues (Wu, Jin et al. 2014) showed that Th17 cells are present in the human
306 endometrium during the first trimester and that they are recruited there by DSC, which appears to
307 contradict what we have shown in this study. However, the Th17 cells reported in the decidua by
308 Wu and colleagues are CD45RO+, i.e. they are memory Th17 cells that are already
309 differentiated, while we show that DSC inhibit the differentiation of Th17 cells from naïve T
310 cells.

(a) Regulation of Type 17 response



(b) Test of hypothesis

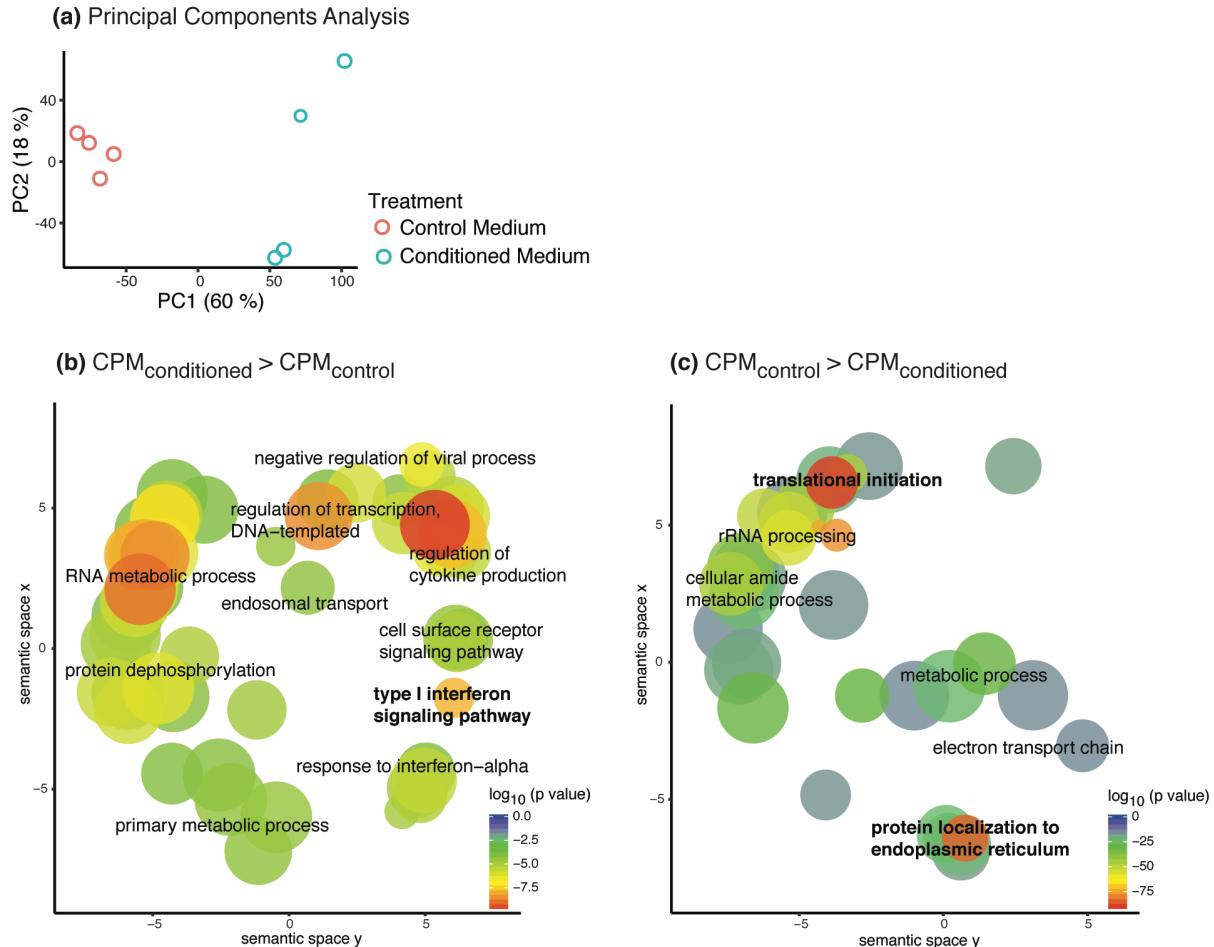


311

312 **Figure 4** Decidual stromal cells suppress Th17 cell differentiation. (a) Schematic showing how Th17
313 cells differentiate from naïve T cells, and the hypothesis for the role of DSC (b) Test of the hypothesis
314 using *in-vitro* differentiation of human naïve T cells into Th17 cells. IL17A secretion (pg/ml) by Th17
315 cells is shown (mean and standard error of the mean). The first two samples are reference points, where
316 Th17 cells were differentiated in their native medium, and the last two samples were differentiated in
317 DSC control or conditioned medium.

318 **DSC inhibit protein synthesis during Th17 differentiation**

319 To understand how DSC suppress differentiation of naïve T cells into Th17 cells, we sequenced
320 the transcriptomes of the differentiating T cells that were either treated with DSC conditioned
321 medium or control medium (conditions 3 and 4 from **Figure 4b**). Principal components analysis
322 of the transcriptome data shows that the DSC conditioned medium had a systematic effect on the
323 gene expression profile of the T cells since 60% of the variance between samples can be
324 explained by the first principal component along which the samples separate by treatment
325 (**Figure 5a**).



326

327 **Figure 5** RNA-seq of Th17 cells differentiated in control vs. DSC conditioned medium. **(a)** Principal
 328 components analysis of transcriptomes of the control and conditioned medium treated cells. Square root
 329 of CPM was used for this analysis. **(b)** and **(c)** respectively show GO categories enriched in genes that
 330 have higher expression in T cells treated with conditioned medium compared to the control medium, and
 331 vice versa. CPM = Counts per Million.

332 Genes significantly up-regulated, as assessed by EdgeR (Robinson, McCarthy et al. 2010), in T
 333 cells treated with DSC conditioned medium compared to T cells treated with control medium are
 334 enriched for GO categories related to defense of viral infection, such as the type-1 interferon
 335 signaling pathway, negative regulation of viral process, response to interferon alpha, RNA
 336 metabolic process (**Figure 5b**). The down-regulated genes are enriched for GO categories related
 337 to protein synthesis and secretion, e.g. rRNA processing, translation initiation, protein
 338 localization to endoplasmic reticulum. Genes of the electron transport chain are also down-
 339 regulated, suggesting that the cells down-regulated their ATP synthesis and became quiescent
 340 (**Figure 5c**).

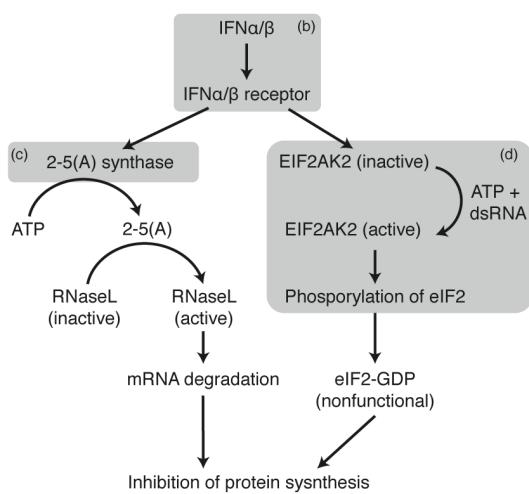
341 Then we looked more specifically at genes in the type-1 interferon signaling pathway (**Figure**
 342 **6a**). Cells infected with virus produce type-1 interferons (IFN- α and IFN- β), which regulate gene
 343 expression in an autocrine and paracrine manner via binding to type-1 interferon receptors

344 IFNAR1 and IFNAR2. Curiously, none of the IFN- α genes is expressed in either samples, and
345 IFN- β gene (*IFNB*) is barely expressed in both samples (<1 TPM) but is not differentially
346 expressed in response to DSC conditioned medium. Both IFN receptor genes, however, are
347 significantly up-regulated in samples treated with conditioned medium (**Figure 6b**). Importantly,
348 none of the type-1 interferons are expressed in DSC either (**Figure 6b**). This suggests that the
349 activation of type-1 interferon signaling in T cells is in response to a specific non-interferon
350 signal from DSC and not a genuine viral defense response.

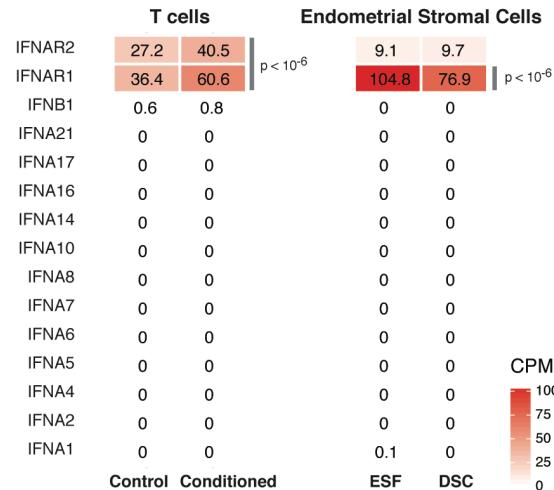
351 One of the prominent outcomes of type-1 interferon signaling is inhibition of protein synthesis
352 via mRNA degradation and inhibition of translation initiation machinery (Kindt, Goldsby et al.
353 2007). This ensures that viral nucleic acids do not replicate within the host cells and produce
354 more viral particles. In T cells treated with DSC-conditioned medium, three out of four 2'-5'
355 oligoadenylate synthase genes (*OAS1*, *OAS2* and *OAS3*) genes are up-regulated (**Figure 6c**).
356 These genes activate RNaseL, which in turn degrades mRNA. Most of the eukaryotic translation
357 initiation factors (EIFs) are also down-regulated and at the same time two negative regulators of
358 EIFs, *viz.* *EIF2AK2* and *EIF4EBP2* are up-regulated (**Figure 6d**), indicating decreased protein
359 synthesis.

360 Together these observations suggest that DSC suppress Th17 differentiation by inducing type-1
361 interferon signaling, which consequently decreases overall protein synthesis and ATP synthesis.
362 However, DSC secrete neither type-1 IFNs nor do they induce the expression of type-1 IFNs in T
363 cells, suggesting that DSC hijack the viral defense mechanism downstream of the IFN receptors
364 to inhibit protein synthesis in T cells. This is in contrast to the bovine pregnancy in which the
365 ruminant-specific type-1 interferon IFN- τ (*IFNT*) from the embryo suppresses *IL17A* expression
366 in maternal peripheral blood mononuclear cells (Talukder, Rashid et al. 2018). Signals from DSC
367 that inhibit protein synthesis in human T cells are unlikely to be generic molecules that can
368 induce type-1 IFN signaling, e.g. cell free RNA or DNA. First of all, such molecules would
369 induce type-1 IFN signaling in all cells at the fetal-maternal interface, which would be
370 detrimental to the maintenance of pregnancy, just as a viral infection is (Yockey and Iwasaki
371 2018, Yockey, Jurado et al. 2018). A generic signal would also suppress differentiation of naïve
372 T cells into not only Th17 cells but also other effector T cells. However, T-reg cells are
373 necessary for maintenance of pregnancy (Aluvihare, Kallikourdis et al. 2004), and their
374 differentiation should not be hindered by DSC. Potential signals from DSC that may be acting on
375 T cells are discussed in the supplementary material (**Supp. Figure 3**).

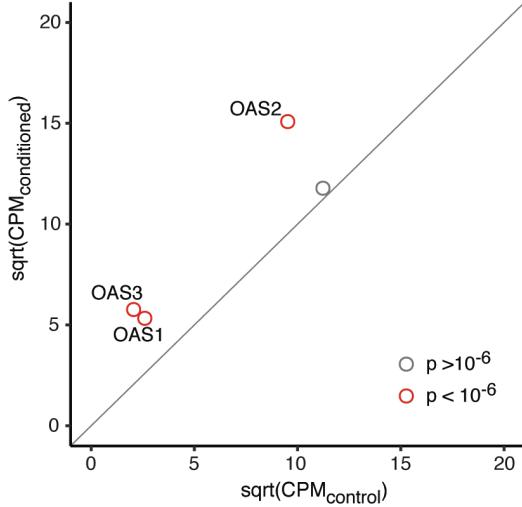
(a) Type 1 IFN signaling



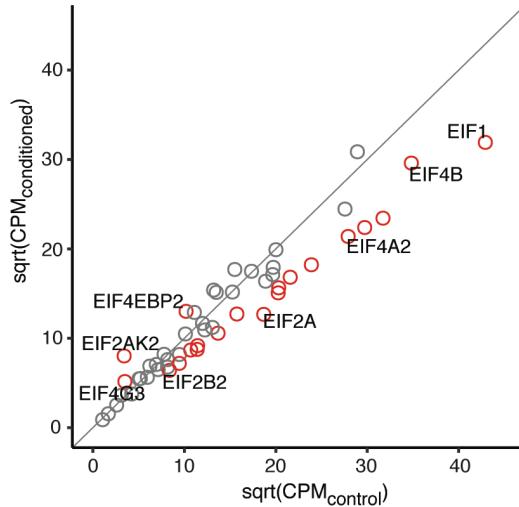
(b) Type 1 IFN genes and receptors



(c) 2'-5' Oligoadenylate synthases



(d) Translation initiation factors

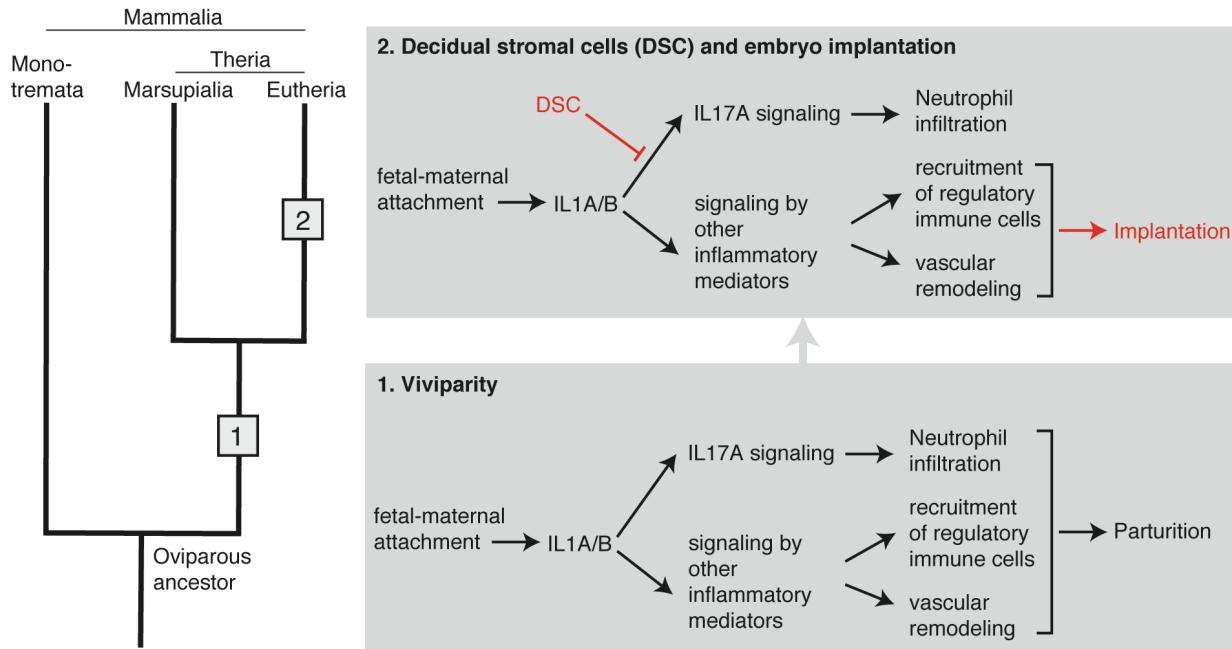


376

377 **Figure 6** Interferon signaling genes in T cells treated with DSC conditioned medium. **(a)** Schematic
378 showing how type-1 interferon signaling pathway inhibits protein synthesis, adapted from (Kindt,
379 Goldsby et al. 2007). Grey boxes show the sets of genes from this pathway whose gene expression is
380 shown in the following panels of the figure. **(b)** Type-1 interferon genes and their receptors in Th17 cells
381 differentiated with control or DSC conditioned medium, and in endometrial stromal cells. **(c)** 2'-5'
382 Oligoadenylate synthase genes. **(d)** Eukaryotic translation initiation factors and some of their regulators.
383 Red points in (c) and (d) represent genes significantly differentially expressed between control and DSC
384 conditioned medium treated T cells according to EdgeR (see Methods). CPM = Counts per Million.

385 **A model for the evolution of implantation**

386 Placing the results from this study in the context of previous studies, the following model for the
387 evolution of embryo implantation emerges (**Figure 7**).



388

389 **Figure 7** Model for the evolution of eutherian implantation from attachment-induced inflammation.
390 Evolutionary events in the therian and eutherian stem lineages are represented by numbers 1 and 2
391 respectively.

392 The ancestor of all mammals was an egg-laying amniote. Mammalian viviparity originated in the
393 stem lineage of therian mammals by early “hatching” of the embryo while it was still within the
394 uterus/shell gland, leading to a direct physical contact between fetal membranes and the uterine
395 endometrial lining. This novel tissue interaction, and potentially an irritation of the endometrial
396 lining from fetal proteases that help dissolve the shell (Griffith, Chavan et al. 2017), induced an
397 acute inflammatory response in the endometrium. An inflamed uterus, unable to maintain and
398 nourish a live embryo within it, expelled the embryo, i.e. parturition ensued as a consequence of
399 attachment-induced inflammation.

400 Hence we assume that the ancestral condition for therians was a typical mucosal inflammation in
401 response to embryo attachment. However, in the stem lineage of eutherian mammals, this
402 inflammatory response became evolutionarily modified such that the endometrium could
403 maintain and nourish the embryo, even though it initiated an inflammation-like response. One of
404 these modifications was the suppression of IL17A signaling, which resulted in the prevention of
405 recruitment of neutrophils to the endometrium during implantation. This may have been a crucial
406 modification to the inflammatory response since neutrophils are known to cause collateral tissue
407 damage from the digestive enzymes they secrete; and the attaching embryo also would likely
408 have not been spared from this tissue damage. Turning off IL17A early in the evolution of the

409 eutherian lineage may have allowed the endometrium to maintain the embryo for a prolonged
410 period. In contrast, many other aspects of the ancestral inflammation may have been beneficial to
411 the maintenance and nourishment of the embryo, e.g. increased vascular permeability and
412 angiogenesis may have helped nutrient transfer, therefore still maintained as a necessary part of
413 implantation. The ancestral attachment-induced inflammation induced a stress reaction in the
414 endometrial stromal fibroblasts (ESF), causing the death of most of these cells. In the eutherian
415 lineage, however, ESF evaded the stress-induced cell death (Kajihara, Jones et al. 2006, Leitao,
416 Jones et al. 2010, Muter and Brosens 2018) by evolving mechanisms to differentiate into a novel
417 cell type, decidual stromal cells (DSC) (Erkenbrack, Maziarz et al. 2018). It is this novel cell
418 type that likely brought about the suppression of IL17A by inhibiting the differentiation of
419 IL17A-producing cells at the fetal-maternal interface, and thus enabled the evolution of embryo
420 implantation and a sustainable fetal-maternal interface.

421 **Methods**

422 **Animals and tissue samples**

423 Nine-banded armadillos (*Dasypus novemcinctus*) were collected in Centerville, TX, USA, in
424 accordance with Yale University IACUC protocol #2014-10906. Two females were used in this
425 study — one non-pregnant and one at the peri-implantation stage of pregnancy where the fetal
426 membranes have begun invading the endometrium but no placental villi are yet formed. Rock
427 hyrax (*Procavia capensis*) samples were collected at Bar-Ilan University, Israel. One out of three
428 females collected was in the implantation phase of pregnancy as determined by histological
429 examination. The blastocyst was attached to the uterine lumen but had not started invading the
430 endometrium. Opossum (*Monodelphis domestica*) tissues were collected from the colony housed
431 at Yale University. Samples were fixed in 4% paraformaldehyde in phosphate-buffered saline
432 (PBS) for histology and immunohistochemistry, saved in RNAlater (Ambion) for RNA
433 extraction. For more information about armadillo samples, see (Chavan and Wagner 2016), and
434 about opossum samples see (Griffith, Chavan et al. 2017). A list of animals used in this study is
435 given in **Supp. Table 1**.

436 **Immunohistochemistry**

437 Formaldehyde-fixed tissues were dehydrated in ethanol, cleared in toluene, and embedded in
438 paraffin. Sections of 5 μ m thickness were made on a microtome and placed on poly-L-lysine
439 coated glass slides. Immunohistochemistry was performed by following the protocol in (Chavan
440 and Wagner 2016). Briefly, slides were incubated at 60 °C for 30 min and allowed to cool at
441 room temperature for 5 min. Paraffin was removed by de-waxing the slides in xylene. Slides
442 were then rehydrated by successive washes in 100% ethanol, followed by running tap water.
443 Sodium Citrate buffer (pH 6.0) was used for heat mediated antigen retrieval. After washing the
444 slides in PBS, they were blocked in a 0.1% solution of Bovine Serum Albumin (BSA) in PBS.

445 Endogenous peroxidases were suppressed with Peroxidase Block (DAKO). Optimized dilutions
446 of primary antibodies (see **Supp. Table 2**) were added to the slides and were incubated overnight
447 at 4°C in a humidification chamber. Secondary antibody was added after washing the primary
448 antibody off with PBS and 0.1% BSA-PBS, and incubated for 1 hour at room temperature, and
449 washed off with PBS and 0.1% BSA-PBS. Horseradish peroxidase (HRP) tagged secondary
450 antibodies were detected by either 3,3'-diaminobenzidine (DAB) and counterstained with
451 hematoxylin.

452 Cell culture experiments

453 DSC and DSC conditioned medium

454 Immortalized human endometrial stromal fibroblasts (hESF) from ATCC (ATCC; cat. no. CRL-
455 4003) were cultured in growth medium with the following contents per liter: 15.56 g DMEM
456 (D2906, Sigma-Aldrich), 1.2 g sodium bicarbonate, 10 ml sodium pyruvate (11360, Thermo
457 Fisher), 10 ml ABAM (15240062, Gibco), 1 ml ITS (354350, VWR), and 100 ml charcoal-
458 stripped FBS. ESF were differentiated into decidual stromal cells (DSC) in differentiation
459 medium with the following contents per liter: 15.56g DMEM (D8900, Sigma-Aldrich), 1.2g
460 sodium bicarbonate, 10 ml ABAM, 0.5 mM cAMP analog 8-Bromo-adenosine 3',5'-cyclic
461 monophosphate sodium salt (B7880, Sigma-Aldrich), 1 μM progesterone analog
462 Medroxyprogesterone 17-acetate (MPA; M1629, Sigma-Aldrich), and 20 ml FBS.

463 Cells were differentiated for 48 hours at 37 °C, conditioned medium was collected from at least 6
464 replicate flasks, filtered through sterile 0.45 μm filter to remove cell debris, aliquoted, and frozen
465 at -80 °C until used. For control samples, decidualization medium was incubated for 48 hours at
466 37 °C without any cells in at least 6 replicate flasks, processed in the same way as conditioned
467 medium, and frozen at -80 °C.

468 T cells

469 Human Peripheral Blood Mononuclear Cells (PBMC) were isolated from whole blood (70501,
470 StemCell Technologies) using Lymphoprep (07851, StemCell Technologies) and 50 ml SepMate
471 tubes (85450, StemCell Technologies). These PBMCs were used to isolate CD4+ CD45RO-
472 naïve T cells with EasySep Human Naïve CD4+ T Cell Isolation Kit (19555, StemCell
473 Technologies). Naïve T cells were resuspended at 10⁶ cells/ml in ImmunoCult-XF T Cell
474 Expansion medium (10981, StemCell Technologies) in the presence of ImmunoCult
475 CD3/CD28/CD2 T Cell Activator (10970, StemCell Technologies) for culture or Th17
476 differentiation. For Th17 differentiation the following were added to the above cell suspension:
477 20 ng/ml IL6 (78050, StemCell Technologies), 5 ng/ml TGFB1 (78067, StemCell Technologies),
478 and 50 ng/ml IL23 (14-8239-63, eBioScience). If conditioned medium was used, the above
479 suspension was made in 1:1 solution of conditioned or control medium and the T cell expansion
480 medium. Cell suspension was then transferred to 24-well plates, with 10⁶ cells per well. Samples
481 with different treatments were placed in a Latin square design on 24-well plates to prevent

482 systematic effects arising from the positions of the samples in the plate. Cells were incubated at
483 37 °C for 7 days for differentiation. Cells, which are in suspension, were spun down to collect the
484 supernatant and cell pellet. RNA was extracted from the cell pellets for RNA-seq (see below),
485 and secreted IL17A was measured in the supernatants with Quantikine ELISA kit for human
486 IL17A (D1700, R&D Systems).

487 **RNA-seq data**

488 Among the animal tissue collected for this study, armadillo samples were used for RNA-
489 sequencing. RNA was extracted from whole uteri. Sequencing library for RNA from non-
490 pregnant armadillo was prepared and sequenced at the Yale Center for Genome Analysis.
491 Library preparation and sequencing of the RNA from implantation stage armadillo was
492 performed at Cincinnati Children's Hospital Medical Center.

493 RNA from CD4+ naïve T cells differentiated into Th17 cells in the presence of DSC conditioned
494 medium was extracted using Qiagen RNeasy Micro Kit (74004, Qiagen). Libraries were
495 prepared and sequenced at the Yale Center for Genome Analysis.

496 Data for rabbit uterus (*Oryctolagus cuniculus*), grey short-tailed opossum (*Monodelphis
497 domestica*) and human endometrial stromal cells were downloaded from Gene Expression
498 Omnibus (GEO) (Barrett, Wilhite et al. 2013). GEO Accession numbers of the downloaded
499 datasets as well as those generated in this study are listed in **Supp. Table 3**.

500 **RNA-seq analysis**

501 RNA-seq data were aligned to the following Ensembl genomes using TopHat2 (Kim, Pertea et
502 al. 2013): GRCh37 for human, DasNov3 for armadillo, OryCun2 for rabbit, and MonDom5 for
503 opossum. Number of reads mapping to genes were counted with HTSeq (Anders, Pyl et al.
504 2015). Read counts were normalized to Transcripts per Million (TPM) (Wagner, Kin et al. 2012),
505 and 3 TPM was used as an operational threshold to call genes as expressed or unexpressed
506 (Wagner, Kin et al. 2013). Median length of all transcripts of a gene was used as its ‘feature
507 length’ for TPM normalization. Differential gene expression analysis for human T cell and
508 endometrial stromal cell data was performed on protein coding genes with the R package EdgeR
509 (Robinson, McCarthy et al. 2010). For these samples, read counts are normalized by EdgeR to
510 Counts per Million (CPM), and are presented as such.

511 In analyses involving multiple species, only those genes were included that have one-to-one
512 orthology among the species compared. Orthology data from Ensembl Compara database
513 (Herrero, Muffato et al. 2016) were used. In these analyses, read counts were re-normalized to
514 TPM using only the set of orthologous genes in order to make TPM values comparable between
515 species.

516 Enriched gene ontology (GO) categories in sets of genes were identified with the online tool
517 GOrilla (Eden, Navon et al. 2009). Lists of enriched GO categories were visualized on REViGO

518 (Supek, Bošnjak et al. 2011), which clusters semantically similar GO terms in space, simplifying
519 interpretation of long lists of redundant GO categories.

520 **qPCR**

521 Expression of IL17A through opossum pregnancy was measured using qPCR at a finer time
522 scale than the transcriptomic data. RNA from non-pregnant, 8 dpc, 11.5 dpc, 12.5 dpc, and 13.5
523 dpc uteri was extracted and reverse transcribed to cDNA using High Capacity Reverse
524 Transcriptase Kit (4368814, Thermo Fisher). Abundance of IL17A mRNA was measured
525 relative to TBP (Tata Binding Protein) mRNA with a standard curve approach using Power
526 SYBR Green PCR Master Mix (4367659, Applied Biosystems) on StepOne Plus Real Time PCR
527 System (Applied Biosystems). Primer sequences are in **Supp. Table 4**.

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531 **Competing Interests**

532 No competing interests to declare.

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