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1 **Protective adaptive immunity to *Chlamydophila abortus* infection and control of**
2 **ovine enzootic abortion (OEA)**

3

4

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23 **Keywords:** Cell mediated immunity (CMI), pregnancy, *Chlamydophila abortus*,
24 ovine enzootic abortion (OEA), sheep

25

25 **Abstract**

26 Ovine enzootic abortion (OEA) remains a major problem in sheep-rearing countries
27 despite the availability of protective vaccines. The causative agent, *Chlamydophila*
28 *abortus*, is a Gram-negative bacterium that can induce a persistent, subclinical
29 infection in non-pregnant sheep. The development of a new safe, effective and
30 practical vaccine requires a detailed understanding of host-pathogen interactions and
31 the identification of clear correlates of protection. Since disease (abortion) is only
32 observed during pregnancy, the nature of host immunity to *C. abortus* and the
33 specialised immunological features that permit maternal acceptance of the semi-
34 allogeneic fetus are central to the pathogenesis of OEA. We review the current
35 literature on persistence of *C. abortus*, host immunity to infection and mechanisms of
36 abortion. We identify the key outstanding questions surrounding OEA and discuss the
37 current knowledge gaps with a view to developing improved control strategies.

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75 **1. Introduction**

76 Ovine enzootic abortion (OEA) is caused by the Gram-negative bacterium
77 *Chlamydophila abortus* (Aitken and Longbottom, 2007). Despite the existence of
78 commercially-available protective vaccines, OEA is a major problem in sheep-rearing
79 countries and remains the single most common diagnosed cause of infectious abortion
80 in the UK (Longbottom and Livingstone, 2006). There is a clear need for improved
81 control strategies for OEA that include more effective diagnosis, prophylaxis and
82 farm management systems. The development and implementation of such strategies
83 depends on a greater understanding of the biology of *C. abortus* and its transmission,
84 host immunity and how the immunological and physiological changes associated with
85 pregnancy relate to disease pathogenesis. Here we review the current knowledge of
86 protective host immune responses to *C. abortus* infection in sheep and discuss how
87 this can be applied to future vaccine design.

88

89 **2. Pathogenesis of OEA**

90 Organisms belonging to the genera *Chlamydia*/*Chlamydophila* have the potential to
91 establish persistent infections in a wide range of natural hosts, often without overt
92 signs of disease. Sheep infected with *C. abortus* usually have no clinical signs of the
93 infection outwith pregnancy, during the early- to mid-stages of pregnancy, but then
94 abort most commonly in the last 2-3 weeks of gestation (Longbottom and Coulter,
95 2003). In addition to abortion, ewes may exhibit a vaginal discharge around the time
96 of abortion, but otherwise appear healthy. For these reasons, *C. abortus* infection
97 often goes unnoticed or undetected in sheep flocks until it is too late to implement
98 preventative measures during the lambing period (Aitken and Longbottom, 2007).
99 Following abortion, characteristic signs of OEA are thickened, necrotic placental

100 membranes and placental exudate. Aborted fetuses are usually well-developed and do
101 not have gross anatomical abnormalities, suggesting that placentitis is a key element
102 in the pathogenesis of OEA (Buxton et al., 2002).

103

104 **2.1 Questions surrounding OEA**

105 Primary infection with *C. abortus* is thought to occur mucosally, most likely via the
106 oro-nasal route. The source of infection for naïve ewes is contaminated birth material
107 (placental or fetal) or exudate from infected ewes that shed *C. abortus* at lambing
108 time. These newly-infected ewes are those that are likely to abort in the next season
109 (Entrican et al., 2001). This transmission and disease profile raises some key
110 outstanding questions regarding OEA. Firstly, how do sheep respond
111 immunologically to a primary mucosal infection that leads to subclinical persistence
112 of *C. abortus*? Understanding the nature of that immune response is important since it
113 is protective for the ewe so long as she is not pregnant, but is not protective in terms
114 of OEA. Secondly, where is the anatomical site of latency/persistence of *C. abortus*?
115 *C. abortus* is an obligate intracellular bacterium and neither the cell type nor tissue in
116 which the organism resides during a persistent phase in non-pregnant sheep has been
117 identified. Thirdly, what are the factors that dictate invasion of the placenta from the
118 site of persistence? Underlying this question is one of pathogen latency versus
119 persistence and how those terms are interpreted. For example, latency could be
120 regarded as a static phase of the pathogen that is asymptomatic for the host whereas
121 persistence could be either a static or productive phase of the pathogen that may or
122 may not be symptomatic. In this review we will use the term ‘persistence’ rather than
123 ‘latency’. It is not known if *C. abortus* recrudesces from a persistent phase in the ewe

124 at a particular stage during pregnancy, producing infectious, extracellular *C. abortus*
125 elementary bodies (EBs) that invade the placenta, or if EBs are constantly being
126 released at low levels during persistence and opportunistically enter the placenta from
127 maternal blood at a specific time during pregnancy. If the former is true, it suggests a
128 role for alterations in maternal immunity during pregnancy, if the latter is true it
129 suggests that physiological development of the placenta will be a defining factor. This
130 leads on the fourth question: why is the placenta a target for *C. abortus* and why does
131 it grow so well in placental trophoblast? The organism proliferates in the placenta and
132 ultimately causes disease indicating that the immune response mounted by the ewe
133 following primary infection is not adequate to control placental infection after
134 invasion has occurred. Finally, what constitutes a protective, adaptive immune
135 response to *C. abortus*? Ewes that have aborted as a result of OEA develop strong
136 protective immunity and successfully reproduce in subsequent seasons. For future
137 sub-unit vaccines to be effective, they need to mimic this protective state, which in
138 turn demands a clear understanding of the immune mechanisms involved.

139

140 There is no detailed literature on the adaptive immune response in sheep following
141 primary mucosal infection with *C. abortus*, nor has the site of persistence been
142 identified, so the first two questions cannot be answered. However, there are data on
143 host immunity to *C. abortus* in sheep and on the specialised immunological features
144 of mammalian pregnancy that can help to address the remaining three questions.
145 These will be the focus of this review.

146

147 **2.2 Mechanisms of persistence of *C. abortus* in sheep**

148 A role for the ovine host immune response in establishing and maintaining persistence
149 has been identified. This involves cytokine production and subsequent induction of
150 intracellular biochemical pathways that restrict chlamydial multiplication.
151 Experimental infection of ovine cells with *C. abortus* EBs results in growth of the
152 organism and subsequent cell death. However, if the cells are treated with
153 recombinant ovine interferon-gamma (IFN- γ) at the time of infection, growth of the
154 organism is restricted in a dose-dependent manner (Entrican et al., 1998). It was found
155 during extended studies that infected cells could be maintained in IFN- γ for many
156 weeks and that infectious EBs would cease to be detectable in the cell-free culture
157 supernates. However, subsequent removal of IFN- γ from these cultures resulted in the
158 re-appearance of EBs (Brown et al., 2001). The concentrations of IFN- γ used in those
159 experiments were physiologically-relevant, as demonstrated by the concentrations of
160 IFN- γ found in lymph collected *ex vivo* from an immune sheep challenged with *C.*
161 *abortus* (Graham et al., 1995). It was established that the anti-chlamydial effects of
162 IFN- γ were distinct from its antiviral effects, since treatment of infected cells with
163 type-I IFN matching the antiviral activity of the IFN- γ , did not restrict *C. abortus*
164 growth (Entrican et al., 1998). Many experiments have demonstrated that the
165 antichlamydial effects of recombinant ovine IFN- γ are directly associated with
166 induction of the enzyme indoleamine 2,3-dioxygenase (IDO) and tryptophan
167 deprivation (Brown et al., 2001; Entrican et al., 2002). Confirmation of the
168 dependency of *C. abortus* for host tryptophan came with the sequencing of the *C.*
169 *abortus* genome, conclusively demonstrating that the organism is auxotrophic for
170 tryptophan since it lacks a tryptophan synthase operon (Thomson et al., 2005).
171 Collectively, these data indicate that IFN- γ controls *C. abortus* growth in a reversible,
172 dose-dependant manner and allows it to become persistent in ovine cells. Any

173 alterations in the balance between IFN- γ production, IDO expression and tryptophan
174 availability could therefore alter the dynamics of a persistent *C. abortus* infection in
175 ovine cells. If such changes occur during pregnancy they could explain the disease
176 pattern of OEA. It is necessary to identify the site of persistence in sheep to
177 adequately address that question.

178

179 **2.3 Maternal immunity during pregnancy**

180 It was recognised over 50 years ago that pregnancy in eutherian (placental) mammals
181 presents a theoretical challenge to the self-nonsel model of immune activation
182 (Medawar, 1953). The self-nonsel model predicts that the maternal immune system
183 should reject a fetus that has inherited half of its genes from its father, a prediction
184 that does not hold up. Sir Peter Medawar proposed a several explanations for maternal
185 acceptance of the semi-allogeneic fetus within the framework of self-nonsel immune
186 discrimination. Among these was suppression of the maternal immune system
187 (Medawar, 1953). However, if this was the case, a logical consequence would be
188 increased maternal susceptibility to infections and tumours during pregnancy, a
189 phenomenon that is not generally observed.

190

191 This dichotomy has been resolved to a certain extent by the observation there is
192 regulated modulation, rather than suppression, of the maternal immune system
193 (Ragupathy, 1997, Sacks et al., 2003). A major feature of this modulation is the bias
194 of the maternal immune system towards a Th(helper)-2 type phenotype away from a
195 Th1-type phenotype, characterised by an increased ratio of IL-4 relative to IFN- γ

196 production. A large body of literature from studies on cytokine expression during
197 pregnancy in rodents and humans has led to the hypothesis that a predominance of
198 IFN- γ production is incompatible with successful pregnancy (Ragupathy, 1997). The
199 Th1/Th2 paradigm in pregnancy has been re-evaluated and it is now considered to be
200 an over-simplification of maternal immune modulation (Chaouat, 2003). Furthermore,
201 a role for regulatory T cells (Th3/Treg) in normal pregnancy has been identified
202 recently, adding to the complexity of cytokine interactions in the maternal periphery
203 (Aluvihare et al., 2004). Nevertheless, it is still recognised that alterations in cytokine
204 production do occur during normal pregnancy in humans, with a predominance of IL-
205 4 (Devereaux, 2006). What are the underlying mechanisms that influence the balance
206 of cytokine production? The answer may lie in the physiological signals essential for
207 the maintenance of pregnancy, namely hormones. Maternally- and fetally-produced
208 hormones such as progesterone, oestrogen and prostaglandins are fundamental in
209 supporting the pregnancy from implantation to its successful outcome.

210

211 The immunomodulatory effects of pregnancy-related hormones are multi-fold.
212 Progesterone is of particular interest with regards to cytokine profiles since it is
213 known to induce IL-4 production by human T cells and inhibit the cytotoxic activities
214 of T cells and NK cells (Piccini et al., 1995; Arck et al., 2007). Progesterone
215 concentrations increase markedly from around day 90 onwards in pregnant sheep
216 (Wooding and Flint, 1994). Could this cause a reduction in IFN- γ production and
217 therefore enhanced growth of *C. abortus* at the site of persistence, leading to placental
218 invasion? The effect of progesterone on IL-4 production by ovine lymphocytes has
219 not been studied since antibodies to measure ovine IL-4 have only recently become

220 available (Hope et al., 2005). Progesterone can inhibit mitogen-driven and allogeneic-
221 driven proliferation of ovine lymphocytes *in vitro* when used at concentrations of 10^{-6}
222 to 10^{-5} M. However, this is 100-1000 fold higher than the concentration of
223 progesterone observed in plasma during ovine pregnancy ($\sim 10^{-8}$ M) so the
224 physiological relevance of the *in vitro* observations on maternal immunity is debatable
225 (Hansen, 1998). In a recent study we have shown that antigen-driven production of
226 IL-4, IFN- γ and IL-10 by ovine lymphocytes appears to be unaltered during
227 pregnancy (Wattegedera et al., 2008). There are data indicating that delayed-type
228 hypersensitivity responses are reduced in sheep around the time of parturition, but the
229 underlying cytokine profiles have not yet been determined (Caroprese et al., 2006).
230 Consequently, and despite elevated plasma concentrations of progesterone in late
231 gestation, there is no evidence as yet that Th1/Th2 cytokine immune modulation
232 occurs in pregnant sheep in a manner analogous to that in pregnant rodents and
233 humans.

234

235 Such findings are not compatible with the hypothesis that placental infection is due to
236 recrudescence of *C. abortus* from a peripheral site of persistence during pregnancy as
237 a direct consequence of reduced maternal IFN- γ production. In fact, as will be
238 discussed later, T cells from pregnant sheep are capable of producing IFN- γ following
239 experimental *C. abortus* infection and the magnitude of IFN- γ production correlates
240 with protection against abortion (Fig 2). An alternative explanation is one of
241 accessibility of placental trophoblast by *C. abortus*. To explore this theory, we need to
242 consider the structure of the sheep placenta.

243

244 **2.4 Structure of the sheep placenta**

245 Sheep have a synepitheliochorial placenta as opposed to the hemochorial placenta of
246 humans and rodents (Wooding and Flint, 1994). It is characterised by contact, but not
247 fusion, between fetal chorionic membranes and maternal uterine epithelium. Unlike
248 the hemochorial placental, the trophoblast is not bathed in maternal blood. Instead
249 there is very restricted infiltration and invasion of the maternal uterine blood vessels
250 by trophoblast. This infiltration takes place in specialized placental structures called
251 placentomes, where interdigitating materno-fetal villi are responsible for exchange of
252 nutrients and waste products (Figure 1). At around 60 days of gestation (normal sheep
253 gestation is around 147 days), maternal haematomas develop at the materno-fetal
254 interface (in the hilus of each placentome) due to fusion between fetal trophoblast
255 binucleate cells and maternal cells (Meeusen et al., 2001). This results in trophoblast
256 invasion of the maternal uterine epithelium resulting in direct contact between
257 maternal blood and fetal trophoblast, representing a very localised area of intimate
258 materno-fetal contact that is analogous to the more generalised contact observed in
259 hemochorial placentation. Such physiological changes may have an important bearing
260 on OEA. Placental pathology is observed from around day 90 gestation, but *C.*
261 *abortus* antigen has been detected from around day 60 gestation, coinciding with the
262 first appearance of haematomas (Aitken and Longbottom, 2007). These
263 haematomas may allow *C. abortus* to invade the placenta from maternal blood in
264 persistently-infected ewes. The pathogenesis of OEA has been linked to the ability of
265 the ewe to control the levels of infectious EBs in blood, suggesting that both timing
266 and dose of placental infection are important factors (Sammin et al., 2005; 2006).
267 Thus, it may be simply be a case of opportunistic infection of trophoblast by *C.*
268 *abortus* when fetal cells come in direct contact with maternal blood. This could also

269 explain why *C. abortus* infection in pregnant women is so rapid and severe (Hyde and
270 Benirschke, 1997), given the relatively high exposure of trophoblast to maternal blood
271 compared to sheep.

272

273 **2.5 Placental infection with *C. abortus***

274 Once in the placenta, *C. abortus* grows efficiently and is controlled as it is during the
275 persistent phase in the ewe. It is not known why *C. abortus* has such affinity for
276 trophoblast or why it grows so freely in the placenta. There are several possible
277 explanations. The first is specialised receptor-ligand interactions on trophoblast. Such
278 interactions have been reported for strains of the protozoan parasite *Plasmodium*
279 *falciparum* that cause diseases in pregnant women by binding to placental chondroitin
280 sulphate A (Duffy, 2007). *C. trachomatis* serovar E utilises a component of the
281 estrogen receptor to bind to human endometrial cells (Davis et al., 2002), but this has
282 not been studied for *C. abortus*. Another factor could be the availability of nutritional
283 elements that facilitate *C. abortus* growth. For example the virulence of *Brucella*
284 *abortus* is linked to an ability to utilize erythritol (Essenberg et al., 2002), although it
285 is not exactly known how this relates to abortion or if *C. abortus* preferentially derives
286 nutrients from trophoblast. The third factor could be the highly-specialised nature of
287 immunological interactions at the materno-fetal interface to prevent fetal rejection
288 (Table 1). It is currently unknown if many of these mechanisms operate in sheep.

289

290 The uterine intraepithelial γ/δ T cells found in sheep are distinct sub-population from
291 the majority of the circulating γ/δ T cells, analogous to situation in humans where

292 decidual natural killer (NK) cells differ phenotypically from those found in the
293 circulation. Uterine γ/δ T cells do not express the surface marker WC1, have lower
294 expression of the TCR receptor and are no longer capable of proliferating (Fox et al.,
295 1999). They are found in inter-caruncular regions and it is thought they might be
296 involved in restricting fetal trophoblast invasion, but this remains to be proven. There
297 are currently no definitive markers for NK cells in sheep, precluding any attempts to
298 study their potential function in ovine reproduction. Equally, there are no reports for
299 Treg in sheep, but efforts are underway to develop tools to study this important
300 regulatory T cell subset. There are also no reports as yet of alternatively-activated
301 macrophages or tolerising dendritic cells (DC) in the pregnant ovine uterus (Blois et
302 al., 2004; Kammerer, 2005). However, immune regulation has been reported in the
303 sheep uterus, mediated by uterine serpin, a protein produced in the endometrium that
304 inhibits lymphocyte proliferation by reducing expression of the IL-2 receptor, CD25
305 (Peltier et al., 2000). Local administration of progesterone into the sheep uterus
306 promotes the survival of uterine xenografts at concentrations lower than those
307 required to suppress lymphocyte proliferation, arguing for a local immunomodulatory
308 effect (Majewski and Hansen, 2002). This indicates that there is at least one potential
309 immunosuppressive mechanism in sheep to facilitate maternal acceptance of the fetus.

310

311 **2.6 Mechanisms of abortion**

312 The main pathological feature of *C. abortus* infection in pregnant sheep is necrosis of
313 the endometrial epithelium and the fetal trophoblast. In pregnant ewes infected
314 subcutaneously with *C. abortus* at mid-gestation (day 75), *C. abortus* inclusions can
315 be detected as early as 14 days post infection (d.p.i.) in the mononuclear cells of the
316 maternal stroma and successively then in the epithelium next to the inter-caruncular

317 areas (Navarro et al., 2004). These inclusions, most likely of a haematogenous origin
318 (see Figure 1) are accompanied by a mononuclear and polymorphonuclear cell
319 infiltration. Focal necrosis and *C. abortus* colonization of the maternal epithelial cells
320 has occasionally been observed without concomitant trophoblast damage, although
321 maternal lesions are usually limited (Navarro et al., 2004). The fetal portion of the
322 placentome juxtaposing the maternal lesions presents more severe lesions 28 d.p.i.
323 with chorioallantoic oedema, arteritis, thrombosis and a mononuclear and
324 polymorphonuclear cellular infiltration accompanied by substantial multiplication of
325 *C. abortus* in the trophoblast cells and increased thickness of the intercotyledonary
326 membranes (Navarro et al., 2004; Buxton et al., 1990). Phenotypical characterisation
327 of aborted placentas has revealed that the inflammatory infiltrate consists mainly of
328 cells staining positive for CD14 or MHC class II, indicative of macrophages. Lower
329 numbers of T cells ($CD4^{+ve}$, $CD8^{+ve}$, γ/δ TCR^{+ve}) and B cells were also observed
330 (Buxton et al., 2002). The cellular infiltrate is accompanied by extensive production
331 of the pro-inflammatory cytokine tumour necrosis factor (TNF)- α , but not of the
332 protective host cytokine IFN- γ (Buxton et al., 2002). Abortion is therefore thought to
333 be the result of several factors, including vascular thrombosis induced by cytokines,
334 disruption of the chorionic epithelium due to uncontrolled *C. abortus* growth, loss of
335 endocrine function of the placenta and subsequent disruption in the balance of
336 pregnancy-maintaining hormones (Entrican, 2002).

337

338 **3. Protective adaptive immunity to *C. abortus* infection**

339 The experimental evidence presented so far suggests that once *C. abortus* invades the
340 sheep placenta, a cascade of events is set in motion. This cascade is linked to growth

341 of the organism in the chorionic epithelium and appears to accelerate after day 90
342 gestation with cell damage and inflammation almost inevitably leading to abortion.
343 Consequently, protection against OEA is likely to be highly dependant on the
344 maternal immune system preventing *C. abortus* reaching the placenta in the first
345 instance. Ewes that have experienced OEA do not repeatedly abort, so rational
346 vaccine design should aim to mimic the protective immunity of post-abortion ewes
347 (Entrican et al., 2001). There are two protective vaccines for OEA currently available.
348 One comprises live attenuated organisms, the other is whole killed organisms. The
349 relative advantages and drawbacks of these vaccines have been reviewed recently and
350 are not the focus of this review (Longbottom and Livingstone 2006). However, to
351 improve on these vaccines we need to identify the elements of protective host
352 immunity to OEA.

353

354 **3.1 Humoral immunity**

355 It is well established that ewes seroconvert following *C. abortus* infection
356 (Livingstone et al., 2005). Antibody is useful for diagnosis and is likely to play a
357 protective role against re-infection by either opsonizing EBs or preventing EB
358 attachment to target cells. However, the role of antibody in protection against OEA
359 once infection is established is debatable. Experimentally-infected sheep that
360 seroconvert to major outer membrane protein (MOMP) or to polymorphic outer
361 membrane proteins (POMPs) during pregnancy will still develop placental pathology
362 and abort (Livingstone et al., 2005).

363

364 3.2 Cellular immunity

365 Given that *C. abortus* is an obligate intracellular pathogen, it is not surprising that the
366 major interest has been the identification of cell-mediated immune responses,
367 particularly IFN- γ -producing T cells. The importance of IFN- γ in controlling *C.*
368 *abortus in vivo* has been confirmed in a mouse model (McCafferty et al., 1994).
369 Moreover, IFN- γ has been identified as a clear correlate of protection in sheep both by
370 its presence in lymph of immune sheep following *in vivo* challenge with EBs and by
371 its ability to inhibit *C. abortus* growth in ovine cells (Graham et al., 1995; Brown et
372 al., 2001). The source of IFN- γ is of great interest since vaccine delivery systems can
373 be designed and combined with various pathogen components to target different T
374 cell subsets to achieve the desired effect. An intraperitoneal challenge model of *C.*
375 *abortus* infection in mice depleted of different T cell subsets indicates a dominant role
376 for CD8^{+ve} T cells in protection compared to CD4^{+ve} T cells (Martinez et al., 2006).
377 How this translates to sheep is not yet known, although this could be addressed since
378 T cell subset depletions in lambs are feasible (Lloyd et al., 2000). Phenotypic studies
379 of efferent lymph collected from immune sheep after *in vivo* challenge infection
380 indicated blasting populations of CD4^{+ve}, CD8^{+ve} and γ/δ T cells as well as B cells,
381 and a predominance of CD8^{+ve} T cells at the time of resolution of infection (Huang et
382 al., 1991). It is therefore likely that multiple cell types are involved and it is their
383 interactions and cytokine profiles as well as their effector functions that determine
384 protection against OEA.

385

386 We have studied the cytokine profiles of peripheral blood mononuclear cells (PBMC)
387 collected from sheep experimentally infected at day 70 gestation with *C. abortus* and

388 re-stimulated *in vitro* with the T cell mitogen Concanavalin A (Con A). The
389 experimental protocol has already been published (Buxton et al., 2002). At the time of
390 doing those experiments there were limited tools to measure ovine cytokines. We
391 have since re-analysed the samples with newly-developed ELISAs that detect ovine
392 IL-4 and IL-10 (Hope et al., 2005; Kwong et al., 2002). Our data show that T cells
393 from infected sheep are primed *in vivo* to produce IFN- γ when re-stimulated *in vitro*
394 with Con A compared to uninfected controls ($p < 0.001$; Figure 2a). Interestingly,
395 infected ewes that did not abort in our experiment produced a higher early peak of
396 IFN- γ compared to those that did abort following the experimental subcutaneous
397 infection. The other noticeable pattern is the peak of IFN- γ production around the time
398 of abortion and lambing (later peak) in the infected ewes. We hypothesise that this
399 peak corresponds to strong antigenic challenge of the ewe due to extensive placental
400 infection with *C. abortus*, since many of the ewes that did not abort still showed signs
401 of placental infection, although this was not as severe as in the aborted placentas
402 (Buxton et al., 2002). These patterns are consistent with IFN- γ being a correlate of
403 protection against OEA. Levels of IL-4 expression remained low throughout
404 pregnancy with no clear patterns emerging between uninfected and infected ewes,
405 irrespective of abortion (figure 2b). There were also no significant differences in IL-
406 10 production between the groups (Figure 2c).

407

408 We have not identified the cellular sources of these cytokines, however, we believe
409 that they may be derived from phenotypically distinct cells based on the the paradigm
410 of the cytokine profiles of CD4^{+ve} T cell subsets (Th1/Th2/Th3/Treg/Th17) described
411 in mice (Bettelli et al., 2007). We have analysed the responding phenotypes of

412 lymphocytes from post-abortion (immune) ewes following *in vitro* re-stimulation with
413 LPS-depleted *C. abortus* EBs. The major responding population was not CD4^{+ve} T
414 cells as expected, but γ/δ T cells, closely followed by B cells (Figure 3a). This was
415 initially thought to be non-specific, but it can be seen that γ/δ T cells from naïve sheep
416 do not proliferate. Moreover, the proliferation is clearly demonstrable when combined
417 with carboxyfluorescein succinimidyl ester (CFSE) staining which shows the daughter
418 generations in the proliferating cells from an immune but not naïve sheep (Figure 3b).
419 The high percentage of proliferating γ/δ T cells may be reflective of the lower (and
420 therefore potentially less antigen-restricted) T cell receptor repertoire compared to
421 that of cells expressing the α/β T cell receptor which is regarded as more specific.
422 Nevertheless, γ/δ T cells have traditionally not been considered to be a component of
423 the adaptive immune response, acting more as a bridge between innate and adaptive
424 immunity. We were therefore unsure as to how to interpret these data since they
425 appear to show a selective recall response within the γ/δ T cell population in sheep
426 immune to *C. abortus*. However, a recent publication has identified immunological
427 memory residing in a γ/δ T cell population in cattle, induced by a killed *Leptospira*
428 vaccine (Blumerman et al., 2007). This has exciting possibilities for vaccine design
429 and delivery, particularly since γ/δ T cells are an important component of the
430 ruminant cellular immune system and capable of producing IFN- γ (Baldwin et al.,
431 2002; Hein and Griebel, 2003).

432

433 **3.3 Vaccine-induced immunity**

434 The *C. abortus* components that will eventually comprise a subunit vaccine remain to
435 be elucidated. As previously mentioned there is a wide body of literature on candidate

436 chlamydial vaccine antigens (Longbottom and Livingstone, 2006). Interestingly,
437 although the current vaccines are protective, we are not aware of any literature
438 defining aspects of cellular immunology in relation to their efficacy. We have studied
439 cellular responses in sheep immunised with Enzovax® (the live attenuated vaccine) to
440 solubilised components of *C. abortus* EBs. We have found that we can stimulate
441 antigen-driven proliferation and IFN- γ production in PBMC from vaccinated sheep
442 compared to control sheep (Figure 4). This mimics the situation observed with PBMC
443 from post-abortion (immune) sheep and re-inforces the concept that IFN- γ is a strong
444 correlate of protection against OEA.

445

446 **4. Conclusion**

447 *C. abortus* is very well adapted to its ovine host, establishing a persistent, sub-clinical
448 infection in non-pregnant ewes and causing abortion in late gestation, resulting in
449 large numbers of infectious EBs being released into the environment around lambing
450 time for transmission to susceptible animals. We are developing a good understanding
451 of the pathogenesis of OEA and the sequencing of the *C. abortus* genome is proving
452 to be an invaluable resource to study pathogen biology. However, several outstanding
453 questions remain, such as the site of persistence in non-pregnant sheep and the
454 development of immunity following mucosal infection. A role has been identified for
455 IFN- γ in maintaining persistence in ovine cells and therefore it can contribute to
456 disease pathogenesis. However, IFN- γ is also a strong correlate of protection in sheep,
457 indicating complex host-pathogen interactions that need to be defined for the effective
458 design of a future sub-unit vaccine. Current knowledge indicates that prevention of
459 placental infection is the key to success. Once *C. abortus* reaches the placenta, the

460 specialised environment at the materno-fetal interface is not conducive to
461 inflammatory immune responses and abortion is a likely outcome. Since repeat
462 abortions are rare, a vaccine that mimics the host immune response that is generated
463 after abortion (rather than the immune response generated after primary mucosal
464 infection) is the most likely to generate protection. The precise nature of that
465 protective response in sheep remains to be elucidated, including the cell population(s)
466 chiefly responsible for production of IFN- γ . Identifying those cells will not only
467 inform on pathogen components to be included in vaccine design, but will also
468 determine the choice of delivery system.

469

470 **Conflict of interest statement**

471

472 None of the authors (Mara Rocchi, Sean Wattegedera, Ilaria Meridiani and Gary
473 Entrican) has a financial or personal relationship with other people or organisations
474 that could inappropriately influence or bias the paper entitled "Protective adaptive
475 immunity to *Chlamydophila abortus* infection and control of ovine enzootic abortion
476 (OEA)"

477

478

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486 study cytokine biology in sheep. We also thank Jill Sales from Biomathematics and
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488

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642

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642 **Figure captions**

643

644 **Figure 1.** Schematic representation of a placentome in the sheep placenta.

645

646 **Figure 2.** Cytokine production by peripheral blood mononuclear cells (PBMC) from
647 pregnant sheep following *in vitro* re-stimulation with the T cell mitogen Concanavalin

648 A. Sheep were infected subcutaneously with *C. abortus* at week 10 of pregnancy
649 (Buxton et al, 2002) and PBMC were collected at 2-3 week intervals. PBMC

650 (2×10^6 /ml) were cultured for 96 hours in Con A (5 μ g/ml), cell-free supernates were
651 collected and analysed for interferon- γ (IFN- γ ; a), interleukin (IL)-4 (b) and IL-10 (c)
652 by ELISA according to previously published protocols (Wattegedera et al, 2007).

653 Uninfected ewes (open bars): n=5; infected, non-aborting ewes (grey bars): n=4;

654 infected, aborting ewes (hatched bars): n=3.

655

656 **Figure 3.** Proliferation of ovine peripheral blood mononuclear cells (PBMC)

657 following *in vitro* re-stimulation *C. abortus* elementary bodies (EBs). PBMC from
658 uninfected and post-abortion (immune) sheep were labelled with carboxyfluorescein

659 succinimidyl ester (CFSE), cultured for 120 hours with LPS-depleted *C. abortus* EBs

660 then stained with monoclonal antibodies specific for ovine CD4, CD8, γ/δ T cell
661 receptor and IgM (for B cells). The percentage cells undergoing proliferation in each

662 subset was calculated relative to the total number of each subset within the PBMC

663 population (a). Open bars: uninfected sheep (n=2); hatched bars: immune sheep (n=4).

664 Examples of the daughter populations of CFSE-labelled proliferating γ/δ T cells from

665 an uninfected sheep (left hand histogram) and an immune sheep (right hand

666 histogram) are shown in (b).

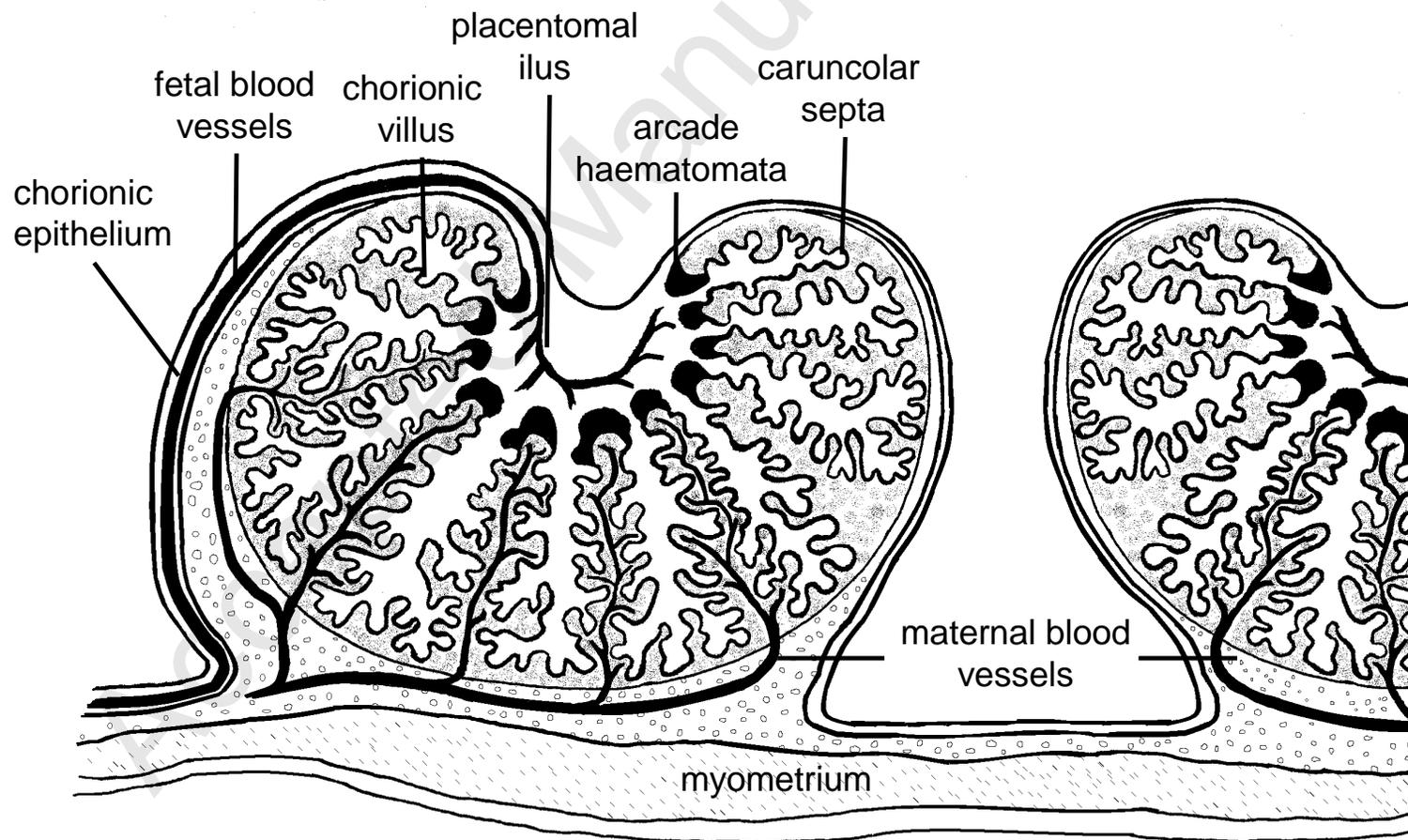
667

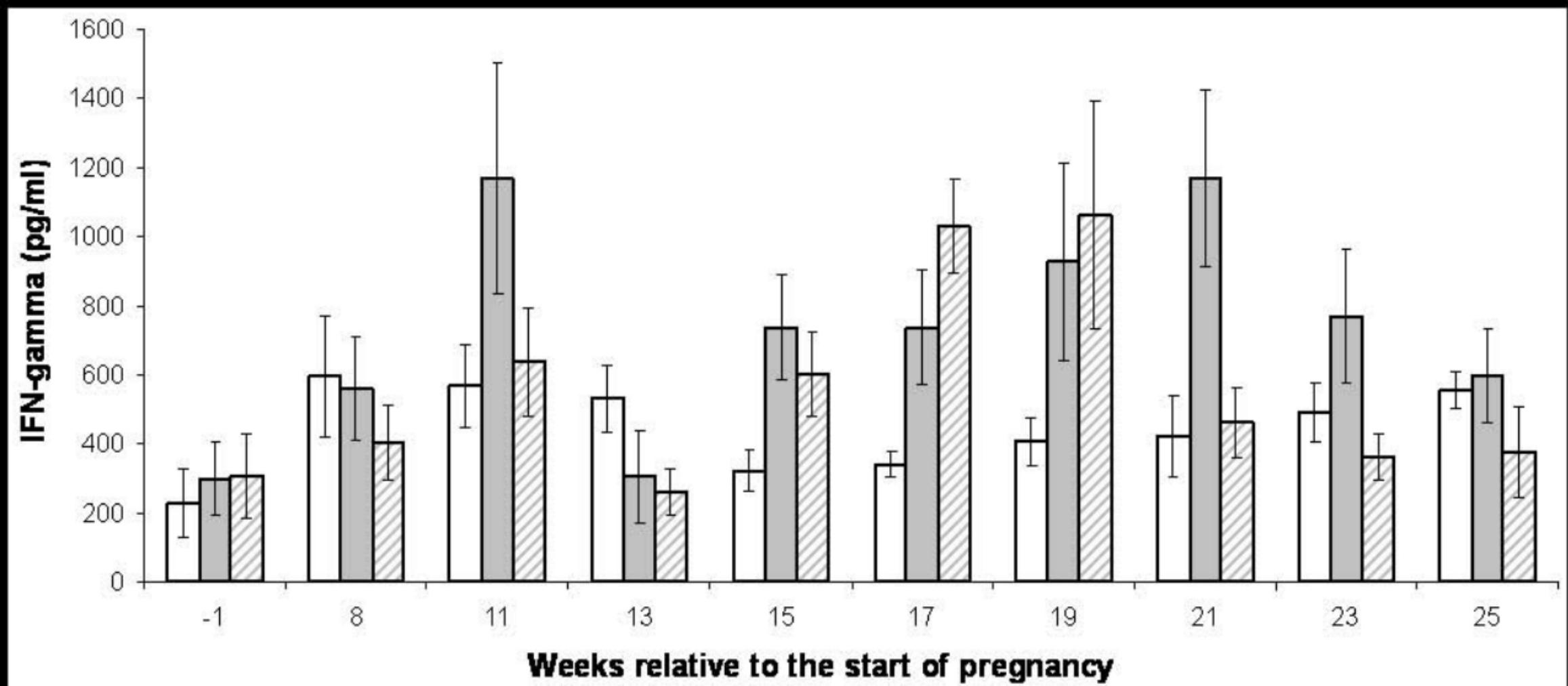
668 **Figure 4.** Proliferation and interferon- γ (IFN- γ) production by peripheral blood
669 mononuclear cells (PBMC) from vaccinated and control sheep in response to *in vitro*
670 re-stimulation with dilutions of a soluble fraction of *C. abortus* elementary bodies
671 (EBs). PBMC proliferation was measured by labelling with ^3H -thymidine for the final
672 18 hours of 120 hours culture. Proliferation is represented as counts per minute (cpm)
673 incorporated ^3H thymidine (shown as bars on figure). Open bars: control sheep (n=3);
674 hatched bars: sheep vaccinated with Enzovax[®] (n=3). Cell-free supernates were
675 harvested after 96 hours of culture and analysed for IFN- γ (shown as lines on figure)
676 by the commercial Bovigam ELISA. Continuous line: control sheep (n=3); dotted
677 line: sheep vaccinated with Enzovax[®] (n=3).

Table 1. Immunomodulatory mechanisms at the materno-fetal interface

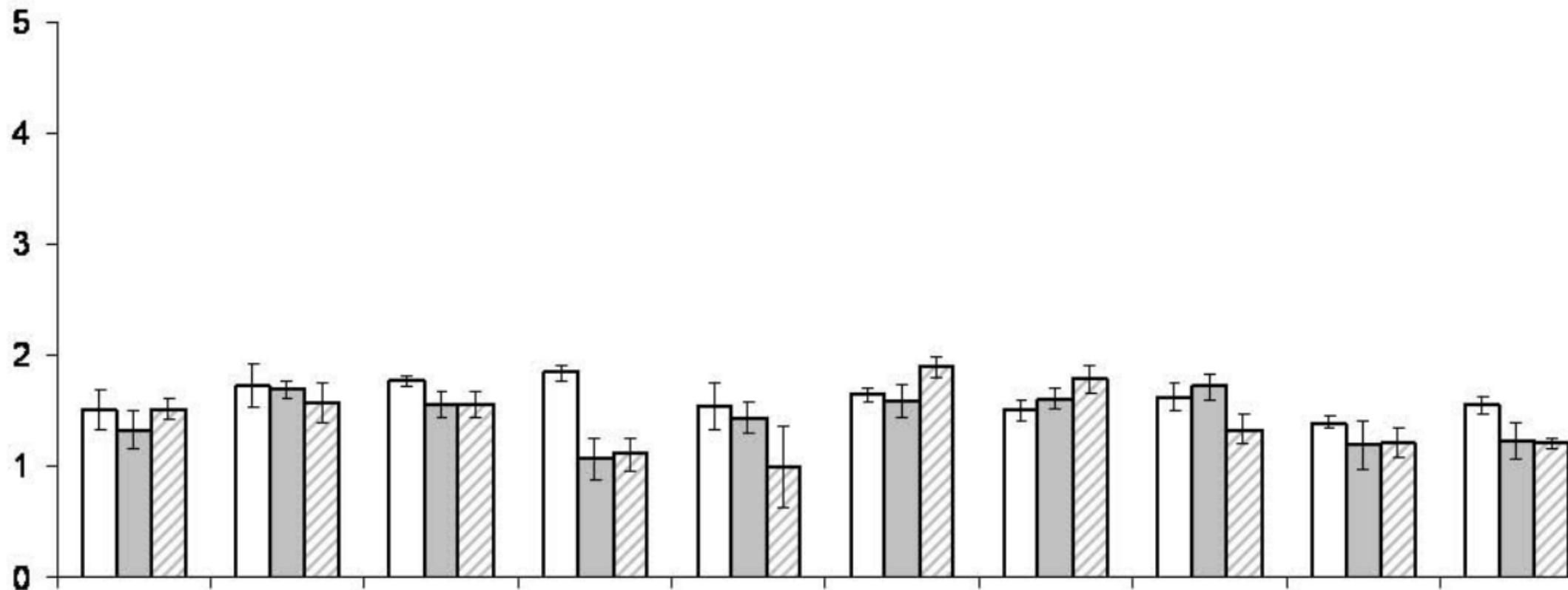
Cells, molecules and putative effects	
Cell populations	References
Decidual NK cells. Regulate angiogenesis and control trophoblast invasion into maternal tissue.	Moffett et al, 2004
Uterine intra-epithelial, inter-caruncular $\gamma\delta$ T cells. Regulate trophoblast invasion.*	Meeusen et al, 2001
Decidual DC. Production of regulatory cytokines and induction of Treg.	Blois et al, 2004; Kammerer, 2005
Maternal CD4 ^{+ve} Treg. Tolerance and production of regulatory cytokines	Habicht et al, 2007
Molecules	
Restricted expression of classical and non-classical MHC class I by fetal trophoblast. Interactions with NK cells, avoidance of maternal CD8 ^{+ve} T cell activation.	Moffett et al, 2006
Expression of IDO by fetal trophoblast. Tolerance of maternal CD8 ^{+ve} T cells.	Mellor and Munn, 2002
Fas ligand expression by fetal trophoblast. Apoptosis of activated T cells expressing Fas.	Hunt et al, 1997
Production of uterine serpin by endometrial cells. Inhibition of T cell proliferation.*	Peltier et al, 2000

Abbreviations: NK: natural killer; DC: dendritic cell; Treg: regulatory T cell; MHC: major histocompatibility complex. * Mechanisms identified in sheep.

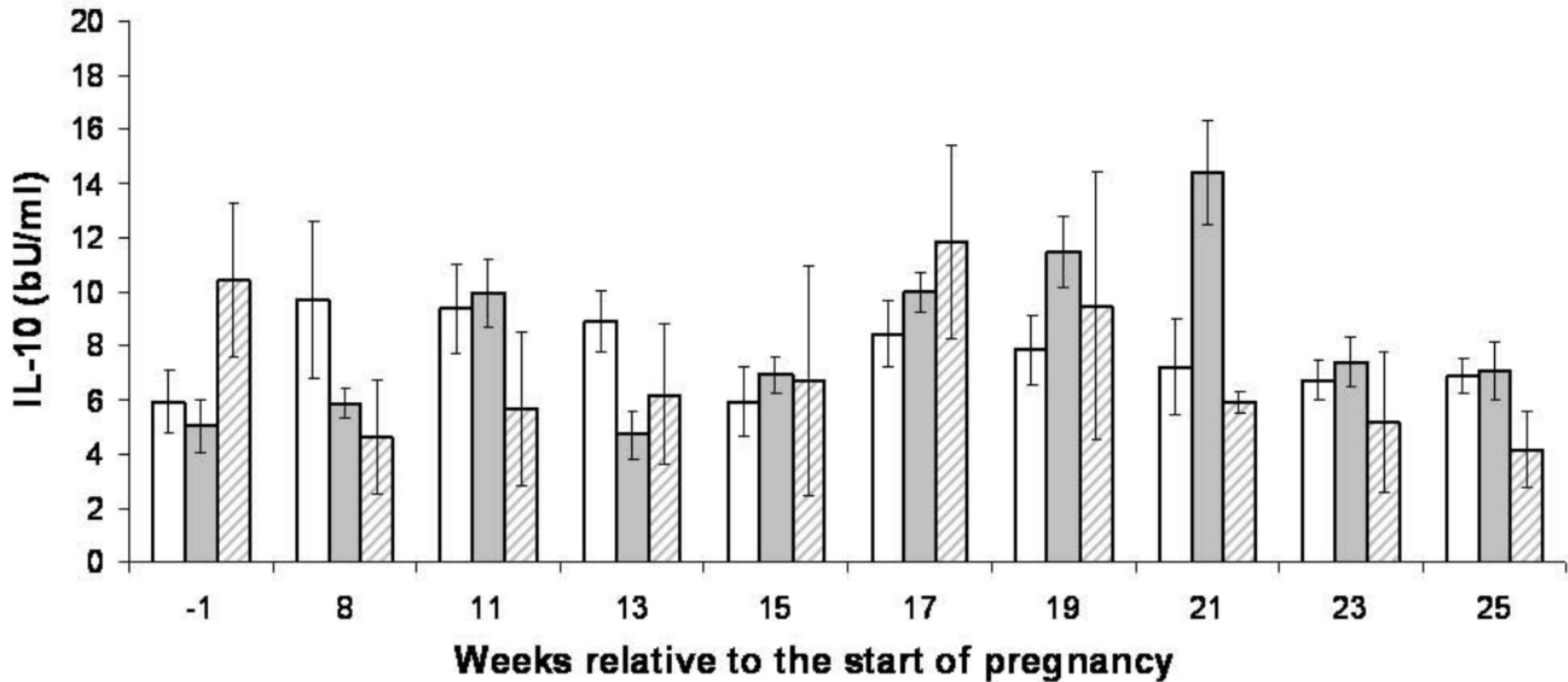




IL-4 (bU/ml)



Weeks relative to the start of pregnancy



**Percentage of subset-specific cells
undergoing proliferation**

