To drive or be driven: the path of a mouse model of recurrent pregnancy loss

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Abstract

This review is an example of the use of an animal model to try to understand the immune biology of pregnancy. A well-known model of recurrent spontaneous pregnancy loss is put in clinical, historical, and theoretical context, with emphasis on T cell biology.

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Introduction

The ‘problem of viviparity’ (Medawar 1954) remains among the most fundamental questions in biology. From an immunological standpoint, this problem stems from the conundrum presented by the fetus as a ‘semi-allograft’ and the mother’s need to both protect and tolerate the fetus. The implications of the relationship(s) between the mother, fetus, and immune system reach clinical significance with regard to emerging infectious disease, vaccinations, autoimmunity, specific disease of pregnancy, fetal development, and neonatal health. It has been our hypothesis that understanding of critical clinical and basic biological problems can result from the iterative use of good clinical/epidemiological data and well-understood animal models (Bonney 2013). Such an approach should lead to significant progress not only in our understanding of pregnancy but also in logical and successful clinical intervention.

Depending on one’s viewpoint, it seems that studies using animal models of pregnancy-related disease, as compared with study of other disease models, have failed to deliver. Why? As outsiders, we would posit that due to unrecognized subtle and not so subtle differences in human and animal physiology the models themselves take some of the blame (Bonney 2013) but not all of it. In addition, we propose that the theoretical framework which elicited the use of and interpretation of the data generated by these models, and, further, the experimental direction lead by this interpretation may have impeded the finding of solutions. To support this assertion, what follows is a focused discussion of one animal model wherein female mice of the CBA/J strain mated with male mice of the DBA/2 strain experience a high rate of reproductive failure. We think the theoretical context

that drives the use of this model is founded on classical immune theory of how the maternal immune system decides between activation and tolerance, namely the discrimination between self and nonself. We will discuss how this model appears to have driven thinking about the T cell biology of pregnancy, and how thinking about this model might be redirected in the context of an alternative to self/nonself determination theory (Matzinger 1994, Bonney 2007) to generate experiments leading to better understanding. Figure 1 illustrates the path of the model and our discussion.

A driver: the clinical problem of recurrent spontaneous pregnancy loss

It has long been recognized that there are couples who suffer from recurrent pregnancy loss (see Branch *et al.* (2010) for a recent review). The most concerning are those who never had a successful pregnancy and who at a similar time in each of several pregnancies suffered this loss. While some of this phenomenon was attributed to such factors as chromosomal defects (Kim *et al.* 1975), progesterone insufficiency (Shearman & Garrett 1963), uterine anomalies (Branch *et al.* 2010), or psychosocial factors (Weil & Tupper 1960), many of these factors were hotly debated and it soon became clear that there was a subset of couples for whom no cause could be found (Christiansen *et al.* 2008). Over many years, several lines of evidence (briefly summarized here) have suggested the involvement of the immune system in this disease. Earlier (e.g. McIntyre & Faulk (1983) and McIntyre *et al.* (1989)) and more recent studies have suggested a role for HLA and minor antigens (e.g. Nielsen *et al.* (2009) and Christiansen *et al.* (2012)) in

Figure 1 Immune theory and the path of the CBA×DBA model.

recurrent pregnancy loss. Moreover, the association between the generation of auto-antibodies, autoimmune disease (Firkín *et al.* 1980), disorders of complement regulation (Laitinen *et al.* 1991), and recurrent pregnancy loss then and now (Oku *et al.* 2009, Mohlin *et al.* 2013) supports the idea that the immune system plays a role in this disease. In addition, there exists evidence of the important role played by natural killer cells, both in normal pregnancy (Bulmer *et al.* 1991, Koopman *et al.* 2003) and in recurrent pregnancy loss, with the later thought to stem from lack of trophic supportive (e.g. growth and development) activity and/or overactive killer activity (Nakashima *et al.* 2012), although this remains controversial (Tang *et al.* 2011) and awaits further study (Tang *et al.* 2013). Earlier studies of distinct immune-reactive antigens expressed on the human placenta (e.g. Faulk *et al.* (1978)) suggested the possibility of alternative flavors of immune responses – some protective and some harmful to the fetal–placental unit (McIntyre & Faulk 1979, Ecker *et al.* 1993). Finally, there is evidence supporting an association between altered decidual T cell populations and recurrent pregnancy loss (Sasaki *et al.* 2004, Nakashima *et al.* 2012, Inada *et al.* 2013).

Immune theory, bolstered by years of basic experimental data, suggested that the immune system's primary and activating focus was to respond to that which was nonself. Pregnancy was considered an example of this theory (Medawar 1954), and successful pregnancy was considered critically dependent on suppression of maternal immunity. However, it had been observed that human (Faulk & McIntyre 1983, McIntyre *et al.* 1983) and animal females could make complex immune responses to fetal/placental antigens during normal pregnancy. An early, prominent report suggested that women with recurrent abortion who shared several HLA antigens with their partners could be 'immunized' by transfusion with allogeneic leukocytes (Taylor & Faulk 1981) to enhance fetal-protective immunity and therefore helped to have successful pregnancies. Although subsequent randomized trials and meta-analyses have cast some doubt on its use in most couples (Ober *et al.* 1999, Porter *et al.* 2006), the possibility of an immune-based therapy for recurrent abortion at that time supported the development of animal models to address the issue.

The origins of the 'CBA×DBA' model

History of the mouse strains

CBA/J has been maintained at Jackson Laboratories (Bar Harbor, ME, USA) since 1948 (Table 1). It was originally created in as an inbred strain in 1920 by Strong, by crossing a Bagg albino female with a DBA/2 male (see <http://jaxmice.jax.org>). CBA/J mice were selected for having a low incidence of mammary tumors. Based on this history, modern CBA/J and DBA/2 are expected to be genetically identical at half of all loci. Like all inbred strains, CBA/J has some specific features. In particular, CBA/J mice are homozygous for *Pde6b^{rd1}* (phosphodiesterase 6B, cGMP, rod receptor, and beta polypeptide), which is a mutation causing early retinal degeneration and blindness by the age of weaning. Thus, all CBA/J adult mice are blind. In addition, CBA/J mice are known to have a high incidence of tubulointerstitial renal lesions.

DBA mice are the oldest of all inbred strains and were originally developed by Little. DBA/1 and DBA/2 were established as sub-strains in 1929–1930. DBA/2 mice are homozygous for a mutation that results in progressive hearing loss. In addition, DBA/2 mice develop progressive glaucoma-like eye abnormalities, the inheritance of which involves at least two genes.

T cell antigens

These two strains were among those extensively used to gain understanding of major, minor, and 'super' antigens. CBA/J is (histocompatibility) H-2 haplotype *k*. The CBA/J H2-T23 (Qa1) and H2-T18 types are both *b* (Fischer Lindahl 1997). An important minor antigen presented as

a peptide in a histocompatibility molecule and capable of inducing tissue graft rejection by females is the male antigen, H-Y (Scott *et al.* 1997, Simpson *et al.* 1997). CBA/J is a 'non responder' to the male antigen H-Y (Gordon *et al.* 1975, von Boehmer *et al.* 1978), although CBA/J×C57BL/6 F1 female mice do respond to this antigen. Both CBA/J and DBA/2 mice express the mouse mammary tumor virus locus 7 allele originally called Mls-1^a. Strains carrying this allele delete T cells expressing specific V beta chains (e.g. V beta 8.1) from their peripheral T cell pool (Kappler *et al.* 1988). DBA/2 is H-2 haplotype *d*, its H2-T23 (Qa1) type is *b* (Fischer Lindahl 1997), and its H2-T18 type is *c*. DBA/2 mice have the unique characteristic that they do not express the NK cell surface receptor CD94/NKG2A, due to a deletion within the *Klrtd2* gene (Vance *et al.* 2002). Balb/c mice are H-2 *d*, their H2-T23 and H2-T18 types are the same as DBA/2, and their Mtv7 type is *b*. Balb/c mice do not respond to the male antigen H-Y (von Boehmer *et al.* 1978).

The pregnancy-loss phenotype

CBA/J mice have been housed and bred in several facilities around the world. The primary model comprises a comparison between CBA/J females mated to DBA/2 males and CBA/J females mated to males of other strains, including C3H, C57BL/6, and the most common, Balb/c. Early studies suggested a high rate of non-pregnancy in DBA/2-mated CBA/J females (Clark *et al.* 1980). However, mothers of related CBA/J strains, CBA/Ca and CBA/N, do not exhibit this phenomenon (Bobe & Kiger 1989). Investigators have noted that in DBA/2-mated CBA/J females, beginning around day 7 of

Table 1 Genetic and immunogenetic features of the CBA×DBA model.

Strain	Origin	MHC	H2-T23 (Qa1)	H2-T18	MMTV-7	Known features/ mutations	Response to the male antigen H-Y
CBA/J	Cross between Bagg female and DBA male in 1920; selected for low incidence of mammary tumors	H-2 haplotype <i>k</i>	<i>b</i>	<i>b</i>	<i>a</i>	Homozygous for the retinal degeneration allele <i>Pde6b^{rd1}</i> ; causes blindness High frequency of renal tubulointerstitial lesions	Nonresponder
DBA/2	Oldest of all inbred strains; produced by Little	H-2 haplotype <i>d</i>	<i>b</i>	<i>c</i>	<i>a</i>	Widely used strain; traits often contrasted with C57BL/6J Hearing loss CD94 deficient (DBA/2) Low incidence of atherosclerosis Eye abnormality used as model for glaucoma	Nonresponder
Balb/c		H-2 haplotype <i>d</i>	<i>b</i>	<i>c</i>	<i>b</i>	Well known for susceptibility to autoimmune encephalomyelitis (EAE) Widely used for the production of monoclonal antibodies	Nonresponder

gestation, there is a loss of cellular contact between decidual cells and cells of the ectoplacental cone (Gendron & Baines 1989). This is accompanied by an infiltration of natural killer and nonnatural killer leukocytes, including T and B cells (Gendron & Baines 1988, 1989). Later studies also suggest that resorbing tissues have an increased infiltration of mast cells (Zencussen *et al.* 2003). The pathology of these pregnancies progresses by day 8 to comprising increased tissue spaces and abnormal syncytium formation by day 9 (Gendron & Baines 1989). Finally, by days 10–12, there is evidence of embryonic loss and pronounced infiltration of polymorphonuclear leukocytes, CD8-positive and -negative T cells, and B cells. By day 12, there is a clear difference between ‘resorbing’ fetal placental units and normal units, whereby the resorbing units are dark, small, and necrotic, with indeterminate fetal and placental elements, while normal fetal placental units are pink and show overt evidence of a developed placenta separate from a fetus within an amniotic sac. From this discussion, it should be obvious that not every fetal–placental unit undergoes resorption. In addition, from time to time, fetal placental units resorb in all strains. The importance of specific factors that differentiate resorbing from non-resorbing fetal placental units in either ‘normal’ or ‘abnormal’ strain combinations appears to have been afforded less relevance in the study of this model.

Although it has been reported that the number of early implantation sites in this breeding is relatively normal (Chavez *et al.* 1987), ultimately, litter size in CBA/J×DBA/2 matings is variable, from two to eight pups per litter (Heine *et al.* 1989). Data on litter size are not as prevalent as data on what is called “resorption”. Pregnancy loss in the CBA/J×DBA/2 model is usually reported as the number (or percent) of resorptions/total number of implantations (R/T), pooling data from individual mice (Clark *et al.* 2008a) and this is deemed preferable to reporting median and range of resorbed units or to comparing data from individual mice. The percentage of resorbing fetal–placental units in this mating are highly variable, with some values as low as 10% and others upwards of 80% depending on several factors – in particular, the microbial barrier status of husbandry (Hamilton & Hamilton 1987, Clark *et al.* 2003). A large range in resorption rate can be seen within the same set of experiments (Clark *et al.* 1994). There also appears to be some variability with regard to second pregnancies in this mating, with most reports suggesting that second pregnancies decrease the resorption rate (Chavez *et al.* 1987, Clark *et al.* 1994, Ahmed *et al.* 2010). However, this has not been observed by all investigators (Baines *et al.* 1996). This later discrepancy may be related to the time-lapse between the first and second pregnancies. As in human populations, this mating combination experiences

increased pregnancy loss with increasing maternal age (Chavez *et al.* 1987, Ho *et al.* 1994).

Evolving views of immune suppression and the ‘CBA/J×DBA’ model

Inherent pregnancy-related suppression?

Clonal selection theory and the related self/nonself discrimination model of immune system activation (Burnet 1962) led immunologists to suggest model(s) wherein the maternal immune system was suppressed during normal pregnancy (Medawar 1954). Though some early studies (e.g. Clark & McDermott (1978)) documented the expansion rather than contraction of maternal lymphoid tissue during allogeneic pregnancy, this theoretical context, and studies in humans (Finn *et al.* 1972), and varied animal species (e.g. armadillos, Anderson & Benirschke (1964)) drove the search for immune-suppressive factors. It was thought that the expression of such factors in lymphoid and other tissues (e.g. placenta) would affect both systemic and local immunity of pregnant mice and support fetal tolerance. The CBA/J×DBA/2 model first arose in the context of delineating these putative suppressive factors (Clark *et al.* 1980) as the model was held up as an example of adverse pregnancy outcome (resorption) in the face of deficiency of such suppression (Clark *et al.* 1980). Continued study of the model was also a venue for examining the production of soluble suppressive factors produced in the placenta or decidua. For example, it was observed that a factor in normal fetal–placental units suppressed systemic NK cell activity, but that this factor was not in CBA/J×DBA/2 (Gendron *et al.* 1990). However, these factors were not tested on purified populations of local (decidual) NK cells. Another issue that deserved analysis was, given this suppressor factor, the mechanisms which led to the resorption of some fetal–placental units and not others.

Early investigations suggested that within the uterus there is an early infiltration of a CD8 T cell population with suppressive activity which later gives way to a non-T cell suppressor population (Clark *et al.* 1989). However, it was not clear the extent to which the infiltration of these cells is deficient in normal vs resorption-prone matings. Through examination of this model, another population of cells thought to provide site-specific suppressor activity was described as consistent with γ - δ T cells (Clark *et al.* 1997) and although depletion on days 8–9 of gestation increased the resorption rate (Arck *et al.* 1999), peri-implantation depletion of this T cell subset had no effect. Although apparently not available in the CBA/J strain background, generally available strains of mice deficient in this particular cell type are not known to have an overt pregnancy failure (see <http://jaxmice.jax.org>).

Is the model evidence of antigen-specific, T cell-mediated resorption?

CBA/J×DBA/2 pregnancies are 'semi-allogeneic', in that CBA/J are H-2 k and DBA/2 mice are H-2 d. A potential myriad of minor and other antigens might be relevant in the immune response of the CBA/J immune system against DBA/2 cells, yet the specific antigenic focus of the resorption-generating immune response is not known. Nor is it clear which cell is the primary effector. Histologic and other findings suggest that NK cells (de Fougerolles & Baines 1987, Gendron & Baines 1988), macrophages (Redecha *et al.* 2009), and neutrophils (Gendron & Baines 1989, Girardi 2011) play an important role in the pregnancy loss observed in this model. Over time, however, the drive to understand pregnancy loss in the context of self/nonself theory leads to an examination of T cell biology, and in particular defects in T cell tolerance as a major mechanism of pathology. Several studies suggested that pregnancy loss in the CBA/J×DBA/2 model was related to altered T cell function. For example, low but not high doses of cyclosporine A (Du *et al.* 2007, Zhou *et al.* 2008), blockade of T cell co-stimulation (Jin *et al.* 2004, Zhu *et al.* 2005), and overexpression of the T cell regulatory molecule CTLA4 (Li *et al.* 2009) decreased resorptions.

These studies led to examinations in other mating combinations and suggested a role for T cell-mediated resorption (Riella *et al.* 2013). One of these important studies in another mating combination investigated the role of the molecule indoleamine 2,3-dioxygenase (IDO), which catabolizes tryptophan and thus limits T cell proliferation (Munn *et al.* 1998, Mellor & Munn 2001). Although the presence of T cells and an allogeneic mating was required for pregnancy loss in response to inhibition of IDO, no T cell antigen specificity was demonstrated (e.g. specific attack of antigen-expressing fetal-placental units vs nonantigen expressing units in the same uterus), suggesting that the T cell response may have been a 'bystander' effect but not the primary mover of the immune response. It should also be noted that deficiency in IDO does not negate pregnancy in other strain combinations (Riella *et al.* 2013).

Although there is no direct evidence that inhibition of this molecule increases resorption in the CBA/J×DBA/2 model, the idea that altered IDO expression could be the basis for pregnancy loss in the CBA/J×DBA/2 model led to experiments showing that enhanced exposure to molecules such as CTLA4 correlates with decreased resorption and increased IDO expression (Li *et al.* 2009). Here again, however, while these models presume that it is an anti-allogeneic response which leads to resorption, the exact antigen has not been delineated and the specific response has not been demonstrated.

Generation of 'protective immunity' against pregnancy loss

Another critical element suggesting the role of immune modulation in the CBA/J×DBA/2 model was developed either concurrent to or in response to the finding that a history of recurrent miscarriage could be ameliorated by transfusion with allogeneic leukocytes (Taylor & Faulk 1981). Investigators discovered that while administration of DBA/2 or CBA/J spleen cells about 1 week before mating did not decrease the resorption rate, administration of 'third party' Balb/c spleen cells did (Chaouat *et al.* 1983, Bobe *et al.* 1986), as long as it occurred within 8 weeks before mating with a DBA/2 male (Baines *et al.* 1996). Pre-immunization with spleen cells from DBA/2 by Balb/c recombinant strains led to differing results, with some strains decreasing and others increasing the resorption rate (Kiger *et al.* 1985). Early studies suggested that exposure to castrated Balb/c males or even dirty Balb/c male bedding was protective for DBA/2-mated CBA/J females (see Table 2 for representative manipulations of the model and associated references). These studies were precursors to others which provided the observation that exposure to seminal plasma fluids and likely seminal plasmid-related antigenic peptides presented in the context of MHC mediate decreased resorption (Clark *et al.* 2013). Prior mating to a Balb/c male was also protective, as long as subsequent pregnancy with a DBA/2 male occurred within 6 weeks (Baines *et al.* 1996).

It appears that mostly male cells were given, although this is not always easily discernible, and in some cases it was observed that administration of female spleen cells did not decrease resorption (Kiger *et al.* 1985). Moreover, the number of spleen cells given varied, with some giving up to 50 million male spleen cells (Chaouat *et al.* 1995). A rather unique immunization protocol using C57BL/6 (not Balb/c) male spleen and thymus cells, and multiple immunizations both before pregnancy and up to day 5 of gestation, produced decreased resorptions in DBA/2-mated CBA/J females. However, continued exposure into a second pregnancy increased the resorption rate compared to that seen in unmanipulated CBA/J×DBA/2 pregnancies (Chavez *et al.* 1987). This again suggesting fluidity in the protective effect.

While attention was focused on finding the mechanisms leading to enhanced suppression of anti-DBA/2 immunity in CBA/J mothers, the results of pre-pregnancy immunization protocols led to differing findings in the peripheral tissues vs decidua. For example, very early sets of observations suggested that although pre-pregnancy immunization apparently decreased the rate of resorption, it did not correlate with suppression of maternal spleen T cell proliferation, or generation of anti-DBA/2 cytotoxic T cell activity *in vitro* in some cases (Bobe *et al.* 1986), whereas in others it apparently did (Chaouat *et al.* 1985). Moreover, while pre-pregnancy immunization of CBA/J females

Table 2 Representative manipulations of CBA×DBA matings (arranged roughly according to mechanism).

Baseline resorption rate* (%)	Manipulation (injection unless noted)	Resulting resorption rate* (%)	References
Decreased resorption rate			
31	Second pregnancy	1	Chavez <i>et al.</i> (1987)
30–35	Second pregnancy	15	Gendron <i>et al.</i> (1992)
23	Add normal Balb/c male to cage on day 6 of 1st pregnancy	4	Baines <i>et al.</i> (1994)
23	Add castrated Balb/c male to cage on day 6 of 1st pregnancy	9	Baines <i>et al.</i> (1994)
23	Add Balb/c male bedding to cage on day 6 of 1st pregnancy	9	Baines <i>et al.</i> (1994)
30	Specific pathogen-free room	8	Hamilton & Hamilton (1987)
20–35	Balb/c male spleen cells before mating [#]	5–15	Kiger <i>et al.</i> (1985)
35	Balb/c male spleen cells before mating	20	Clark <i>et al.</i> (2008b)
20–35	Balb/c male spleen cells before mating	10	Clark <i>et al.</i> (2013)
20–35	Balb/c spleen cells pre-incubated with Balb/c seminal plasma before mating	3	Clark <i>et al.</i> (2013)
20–35	DBA spleen cells pre-incubated with Balb/c seminal plasma before mating	10	Clark <i>et al.</i> (2013)
30	Spleen cells from Balb/c×DBA recombinant inbred strains K,N,C before mating	5–17	Kiger <i>et al.</i> (1985)
24	CBA bone marrow-derived dendritic cells pulsed with lysate of DBA male spleen cells	5	Blois <i>et al.</i> (2004)
24	Anti-CD11b antibody i.v. on day 6	12	Duclos <i>et al.</i> (1994)
27	Anti-CD8 and anti-CD86	9	Jin <i>et al.</i> (2005)
24	Anti-CD86	8	Zhao <i>et al.</i> (2007)
28	Anti-CD86	7	Zhu <i>et al.</i> (2005)
20	Cyclosporin A (5 mg/kg)	3	Du <i>et al.</i> (2007)
27	Cyclosporin A (5 mg/kg)	16	Li <i>et al.</i> (2009)
27–30	Adenovirus-driven overexpression of <i>Ctla4</i> on day 5	12	Li <i>et al.</i> (2009)
23	i. vag. injection TGFβ day 0.5	12	Clark <i>et al.</i> (2008b)
70	Interleukin 10 (IL10) from culture supernatant on days 6, 8, and 10	5	Chaouat <i>et al.</i> (1995)
35	Tumor necrosis factor (TNF) inhibitor (Pentoxifyllin) on days 6, 8, and 10	20	Chaouat <i>et al.</i> (1995)
35	Anti γ-interferon early in gestation	15	Chaouat <i>et al.</i> (1995)
35	TNF inhibitor + anti γ-IFN early in gestation	10	Chaouat <i>et al.</i> (1995)
46–55	IL3 on days 6.5–10.5	19–28	Chaouat <i>et al.</i> (1990)
35–40	Interferon-τ	5–10	Chaouat <i>et al.</i> (1995)
43	IL2 daily for 10 days from 4 days before mating	15	Chen <i>et al.</i> (2013)
31	C57Bl/6 male spleen and thymus cells	8	Chavez <i>et al.</i> (1987)
31	DBA male spleen and thymus cells	12	Chavez <i>et al.</i> (1987)
27	T cells from anti-B7-treated nonpregnant mice on day 4 of gestation	11	Jin <i>et al.</i> (2004)
18–20	CD4+ CD25+ T cells from day 14 CBA×Balb/c females on days 0–2 of pregnancy	~0	Zenclussen <i>et al.</i> (2005) and Wafula <i>et al.</i> (2009)
20	CD4+ CD25+ T cells from day 14 CBA×Balb/c females on days 0–2 of pregnancy + anti-CTLA4 on days 0, 3, 6, and 9 of pregnancy	~0	Wafula <i>et al.</i> (2009)
29	Fresh CD4+ CD25+ T cells from nonpregnant CBA females on days 1–4 of pregnancy	19–22	Yin <i>et al.</i> (2012)
29	<i>In vitro</i> expanded CD4+ CD25+ T cells from nonpregnant CBA females on days 1–4 of pregnancy	10–12	Yin <i>et al.</i> (2012)
30	3×10 ⁵ regulatory B cells on day 0 of pregnancy	~0	Jensen <i>et al.</i> (2013)
20	Lipopolysaccharide (LPS) 2 weeks before mating	9	Baines <i>et al.</i> (1996)
20	Complete Freund's adjuvant 2 weeks before mating	10	Baines <i>et al.</i> (1996)
43	Flt-3 ^S on day 6 before mating	14	Chen <i>et al.</i> (2013)
29	Anti-Crry Ig	10	Girardi <i>et al.</i> (2006)
29	Anti-C5a	10	Girardi <i>et al.</i> (2006)
29	C5a receptor antagonist peptide	10	Girardi <i>et al.</i> (2006)
30	Prevastatin	10	Redecha <i>et al.</i> (2009)
Increased resorption rate			
19	Second experiment, same publication (change housing)	37	Clark <i>et al.</i> (1994)
19	Third experiment, same publication (change housing)	45	Clark <i>et al.</i> (1994)
43	TNF ~day 5.5	52	Chaouat <i>et al.</i> (1990)
28	TNF ~day 7	98	Chaouat (1994)
43	TNF ~9.5	88	Chaouat <i>et al.</i> (1990)
43	TNF ~13.5	67	Chaouat <i>et al.</i> (1990)
28	γ-IFN ~day 7	75	Chaouat (1994)
30	IL2 ~day 7	65	Chaouat (1994)
50	Anti-IL10 on days 6, 8, and 10	80	Chaouat <i>et al.</i> (1995)
19	Anti-GMCSF on day 7.5	38	Clark <i>et al.</i> (1994)
?	LPS on day 0.5	18	Clark <i>et al.</i> (2008b)
38	LPS ~day 7	100	Chaouat <i>et al.</i> (1990)
28–30	LPS ~day 7	55–58	Chaouat (1994) and Clark <i>et al.</i> (2004)

Table 2 Continued.

Baseline resorption rate* (%)	Manipulation (injection unless noted)	Resulting resorption rate* (%)	References
24	Poly I:C	65	Duclos <i>et al.</i> (1994)
30	Balb/c × DBA recombinant inbred strains L,E	50–62	Kiger <i>et al.</i> (1985)
10–23	Sound stress for 24 h on day 5.5	44	Prados <i>et al.</i> (2011)
10	Sound stress for 24 h on day 5.5	30	Arck <i>et al.</i> (1999)
33	Anti-CD25 antibody on day 1	67	Chen <i>et al.</i> (2013)
10	Anti-T cell receptor antibody on day 5.5	~100	Arck <i>et al.</i> (1997)
11	Anti-γδ T cell antibody on day 8.5	44–48	Arck <i>et al.</i> (1999)
19	Anti-CD8 antibody on day 6.5	60	Clark <i>et al.</i> (1994)
45	Anti-CD8 antibody on day 6.5	78	Clark <i>et al.</i> (1994)
20	CD4+ CD25+ T cells from day 14 CBA × Balb/c females on days 0–2 of pregnancy + anti-PD1 on days 0, 3, 6, and 9 of pregnancy	~30	Wafula <i>et al.</i> (2009)
No change in resorption rate			
25	Prior delivery with Balb/c males 6 weeks before mating		Baines <i>et al.</i> (1996)
20	Second pregnancy with rapid re-mating (7 days) after delivery		Baines <i>et al.</i> (1996)
20–35	CBA male spleen cells 7 days before mating		Clark <i>et al.</i> (2013)
20–35	DBA male spleen cells 7 days before mating		Clark <i>et al.</i> (2013)
20–35	Nonpregnant female Balb/c spleen cells i.p. 7 days before mating		Kiger <i>et al.</i> (1985)
20–35	Male Balb/b or Balb/k spleen cells i.p. 7 days before mating		Kiger <i>et al.</i> (1985)
27	Balb/c spleen cells 8 weeks before mating		Baines <i>et al.</i> (1996)
20–35	Balb/c spleen cells pre-incubated with DBA seminal plasma		Clark <i>et al.</i> (2013)
11	Anti-Vgamma 1.1, anti-Vdelta 6 antibody on day 5.5		Arck <i>et al.</i> (1999)
30	Spleen cells from Balb/c × DBA recombinant inbred strains M,J,D		Kiger <i>et al.</i> (1985)

*Rounded to nearest percent. #Administration of Balb/c spleen cells works to decrease resorptions up to 4 days after mating. \$fms-related tyrosine kinase 3. ?LPS is used to “boost” the resorption rate in matings done in clear rooms but it is unclear what the baseline rate is.

before mating with DBA/2 males increased the apparent ability for placental cells to suppress NK cell-mediated lysis in this model, several normal mating combinations expressed similar low levels of such suppression to the unmanipulated CBA/J mothers (Chaouat *et al.* 1985). An additional finding related to the protective effect of immunization was the elaboration of an anti-paternal MHC antibody or serum protein that possessed a suppressive factor, underlying the complexity of the ‘protective’ effect of immunization (Chaouat *et al.* 1985).

T helper cytokine biology and the ‘CBA/J × DBA’ model

Very early studies of the CBA/J × DBA/2 model had suggested that two types of immune response were present in the placenta and decidua, one protective and the other harmful. The idea that successful pregnancy was dependent on the limitation of maternal immunity evolved to include the idea that pregnancy was dependent on regulating/suppressing the expression of T helper 1 (TH1)-type immunity and enhancing TH2-type immune responses (Lin *et al.* 1993, Krishnan *et al.* 1996). This idea was supported by several studies on the CBA/J × DBA/2 model where investigators observed deficiency in cytokines such as interleukin 4 (IL4) and IL10 in fetal–placental units of CBA/J × DBA/2 pregnancies compared with normal pregnancies in unrelated strains (Chaouat *et al.* 1995) and increased TH1-type systemic responsiveness of CBA/J maternal T cells (Jin *et al.* 2006). Moreover administration of TH1 cytokines alone, such as tumor necrosis factor (TNF) and γ-interferon, greatly increased

resorption rates (Chaouat 1994). Further, the investigators observed that post-implantation antibody-mediated depletion of IL10 resulted in increased pregnancy loss, while administration of exogenous IL10 had the opposite effect (Chaouat *et al.* 1995). Related studies have also suggested that enhanced expression of these cytokines is a mechanism by which pre-pregnancy immunization of CBA/J females decreases the resorption rate subsequently observed (Chaouat *et al.* 1995). The issue continued to be studied and yielded data suggesting that CBA/J × DBA/2 pregnancies, compared with ‘non-resorbing’ CBA/J × Balb/c, produced CD4 T cells with adhesion molecules consistent with a TH2-like phenotype (Jiang *et al.* 2009). Demonstration of normal pregnancy, despite immunization against paternal antigens in mice deficient in IL4 (Bonney 2001) and IL10 (Bonney & Onyekwuluje 2004) (among others), and several studies showing the importance of TH1-type cytokines in normal placental development and decidual function (e.g. Ashkar *et al.* (2000), Chaouat *et al.* (2002), and Monk *et al.* (2005)), suggested that a TH1/TH2 paradigm alone was too simplistic to explain resorption in the CBA/J × DBA/2 model in particular and maternal tolerance in general (Chaouat *et al.* 2004, Mas *et al.* 2008).

Alternative models of immune system activation and the ‘CBA/J × DBA’ model

Although the CBA/J × DBA/2 model delineated complexities of the cytokine milieu present at the maternal–fetal interface, use of the model continued to support the fundamental theory of self/nonself discrimination and the

critical dependence of successful pregnancy on the regulation, limitation, and deviation (toward certain classes of response) of local or systemic maternal immunity. The mid-1990s brought forth alternative theories about the role of tissues, antigens, and the immune system in the generation of T cell activation. One model (Matzinger 1994) suggested that it was not nonself recognition, but the presence of metabolic dysfunction, necrosis, or other fundamental tissue or cellular-level dysregulation that generated the signals to initiate the immune response. These 'Danger' signals in the course of this process could lead to activation of local cells whose primary purpose was processing and presentation of antigen (professional antigen-presenting cells (Matzinger 1994) and the generation of both primary and co-stimulatory signals used by T cells to achieve full activation. A critical assertion of the theory was that as long as a tissue was functioning normally, and not undergoing any stress, damage, or fundamental dysregulation, there was no need to suppress, limit, or deviate the immune system in order to prevent auto-reactive T cell-mediated damage. This theory suggested that this should be true for the fetus, even though the fetus might be 'semi-allogeneic' to the mother (Bonney 2007). An alternative to the Danger theory suggested that immune system activation was primarily generated through the recognition of evolutionary or infectious nonself (Janeway 1992) but kept many tenets of self/nonself discrimination theory. This later alternative posited that immune system activation occurred based on the recognition of molecular patterns that comprised bacterial products (Janeway *et al.* 1996). Investigators working with the CBA/J×DBA/2 model appeared to meld the specifics of the two models to explain some of the peculiar findings observed in DBA/2-mated CBA/J mice.

One such finding was the wide variation in resorption rate, which appeared to be increased in dirtier conventional housing vs 'clean' housing (Clark *et al.* 1997). It was further observed that injection of lipopolysaccharide (LPS) also induced resorption when given i.v. for 7 days after mating (Chaouat *et al.* 1990), and that injection of inflammatory cytokines, such as TNF, mimicked (Chaouat *et al.* 1990) and augmented (Chaouat 1994, Clark *et al.* 2004) the LPS response. However, this effect was also observed in nonabortion-prone matings, even if to a lesser extent (Chaouat *et al.* 1990). When it was later observed that the effect of LPS and other molecules on resorptions was indeed dependent on Toll-like receptor (TLR) signaling (Clark *et al.* 2003), this and related mechanisms were thought to comprise a 'third signal' or pathway by which resorptions could occur in this mating combination. The finding that immunization with Balb/c spleen could counteract resorption due to early LPS- or TNF-related administration, but not preterm delivery due to late administration of the molecules in DBA/2-mated CBA/J mice (Chaouat 1994), suggested to some that resorption was in part due to mechanisms that were

unique to 'Danger' and fell within the context of failed maternal tolerance in the classical (self/nonself) sense.

Another observation generated by this mating combination yet difficult to explain by classic immune theory was that exposure to sound stress for 24 h on day 5.5 of pregnancy increased the resorption rate (Arck *et al.* 1995). This was associated with the expression of inflammatory cytokines (Arck *et al.* 1995), adhesion molecules important in lymphocyte trafficking (Prados *et al.* 2011), and a decrease in suppressive molecules mediating T cell metabolism (Blois *et al.* 2005), and was ameliorated by administration of Balb/c spleen cells. As these studies developed, it was hypothesized that sound stress changed intestinal permeability and was similar to systemic injection with LPS (Clark *et al.* 2004). Although this was supported by other studies in the literature (Bijlsma *et al.* 2001), it was not definitively proven in this model. It could be said that the investigators using the CBA/J×DBA/2 model did not see these observations as strongly arguing against either self/nonself discrimination theory or its corollary, that lack of inherent suppression or limitation of the maternal immune system was the primary pathway by which resorptions occurred in this model. Work continued to find other mechanisms supportive of a suppressive process.

However, according to the 'Danger' theory, signals through TLR and other molecules recognizing pathogen-associated molecular patterns comprise a particular subset of the wide range of possible 'Danger' signals. Potentially any molecule that is deregulated in expression, location, or configuration (folding, Seong & Matzinger (2004)) could, in the right context during pregnancy (Bonney 2007), signal that 'Danger' is occurring in a cell or tissue and activate local antigen-presenting cells. Even complement itself could serve as a Danger signal (Kwan *et al.* 2012). From this perspective, the task of understanding the high resorption rate present in the CBA/J×DBA/2 model might be framed as having two components: i) the effort to incorporate existing data that suggest fundamental dysregulation in the critical tissues present at the maternal-fetal interface and ii) the search to find mechanisms by which this dysregulation occurs.

Published observations have noted abnormal decidual vascular modification (Dixon *et al.* 2006), altered expression of VEGF and its receptor (Girardi *et al.* 2006), decreased blood perfusion (Redecha *et al.* 2009), decreased trophoblast giant cells (Girardi *et al.* 2006), increased fibrin deposition (Redecha *et al.* 2009), increased tissue factor expression (Redecha *et al.* 2009), and increased thrombin activity dysregulation (Clark *et al.* 1998). Increased placental/decidual expression of complement component C3 is also an element of the model that is thought to promote increased influx of macrophages and neutrophils and local expression of TNF (Girardi *et al.* 2006). Many of these abnormalities suggest comparison with the innate immune response following ischemia-reperfusion injury (Zhang & Carroll 2007). Moreover, this

mating combination suffers from increased oxidative stress that can be remediated by overexpression of heme-oxygenase-I (Zenclussen *et al.* 2006). In addition, there is evidence suggesting a differential expression of a placental ATPase in this combination compared with normal or syngeneic Balb/c matings (Jaiswal *et al.* 2011), and this is correlated with altered macrophage function in the placenta. However, while LPS can cause this altered ATPase expression, it is not clear whether altered macrophage function drives or is driven by altered ATPase expression in this abortion-prone model (Jaiswal *et al.* 2011). Early (Muzikova & Clark 1995) and later studies (Brown *et al.* 2013) also suggest that abnormalities existing in the CBA/J decidua and its interaction with the developing embryo are the prime movers of the pathology related to these pregnancies, and this idea is supported by the fact that 'reverse' matings between DBA/2 females and CBA/J males are considered normal (Dixon *et al.* 2006). Finally, this mating combination suffers from a fundamental disorganization of DNA methylation (Brown *et al.* 2013). Thus the CBA/J×DBA/2 mating combination is abnormal on many levels potentially independent of primary immune system dysregulation.

Since the original description of increased pregnancy failure in CBA/J dams mated to DBA/2 males, most reports have focused on the pregnancy failure aspect of the model and have not commented about a maternal phenotype. A 2010 report (Ahmed *et al.* 2010) notes that DBA/2-mated CBA/J females develop significant proteinuria by day 12 of gestation, indicating some sort of pregnancy-induced renal damage. Light and electron microscopic analysis of the maternal kidneys of these dams showed evidence of endothelial injury and diminished blood flow, consistent with the proteinuria. Although DBA/2-mated CBA/J dams did not develop elevated blood pressure during pregnancy, they were markedly more sensitive to a challenge with angiotensin II than were control animals, indicating a generalized effect of pregnancy on the maternal vasculature. This was further corroborated by *ex vivo* studies showing a stronger response of the aortic ring to angiotensin II in DBA/2-mated CBA/J dams. These studies highlight a potential fundamental and systemic dysregulation underlying this mating.

Regulatory T cells: resurgence of immune modulation and the 'CBA/J×DBA' model

The resurrection of the suppressor T cell as a CD4+ CD25+ regulatory T cell expressing the Forkhead transcription factor *Foxp3* (Fontenot *et al.* 2003) opened a new avenue for those who sought to explain, in the context of self/nonself discrimination, why there was increased resorption in the CBA/J×DBA/2 model and why pre-pregnancy immunization with Balb/c spleen cells, exposure to Balb/c seminal plasma

(Clark *et al.* 2013), or administration of specific cytokines such as transforming growth factor β (TGF β) (Clark *et al.* 2008b) decreased the resorption rate.

Several lines of evidence suggested that 'naturally' occurring T regs were a specific lineage, bearing *Foxp3*, highly dependent on IL2, and expressing molecules such as CD25 and CTLA4. It is thought that these cells resulted from having a high affinity for and seeing 'self' antigen in the thymus. The now-named 'tT-reg' (Abbas *et al.* 2013) were thought to leave the thymus into the periphery as fully mature and functional cells, many expressing the memory cell markers (e.g. CD44^{hi}, CD62L^{lo}) that could suppress other subsets of self-reactive T cells (reviewed in Sakaguchi *et al.* (2008)). In addition, investigators have observed that exposure of naïve T cells in the periphery to antigen in the context of TGF β , IL2, retinoic acid, or signals via Fms-related tyrosine kinase 3 ligand, could produce a 'pT reg' (Abbas *et al.* 2013) T cell with regulatory function (Sakaguchi *et al.* 2008).

In contrast, exposure to antigen in the context of TGF β and IL6 could generate TH17 T cells. The stability of T reg and TH17 phenotypes may not be strong (Sakaguchi *et al.* 2013), as conversion can occur in the context of hormonal dysregulation (Li *et al.* 2013), severe infection (Rowe *et al.* 2012a, Zhang *et al.* 2012), and TLR signaling (Nyirenda *et al.* 2011). Understanding the various mechanisms by which thymic or peripheral T regs exert their effects on T cells is ongoing, and can include suppression of cytokine production and proliferation (Thornton & Shevach 1998), direct killing (Abdulahad *et al.* 2011), and modulation of function (Collison *et al.* 2009, Zelinsky *et al.* 2013).

Regulatory T cell dysfunction has been implicated for many years in mouse models of premature ovarian failure and autoimmune oophoritis and other reproductive track-related autoimmune diseases (Bonomo *et al.* 1995, Tung & Teuscher 1995). Investigators observed that administration of CD4+ CD25+ spleen and thymus cells from a day 14 normally pregnant (Balb/c-mated) CBA/J female to a DBA/2-mated CBA/J female on days 0–2 of pregnancy decreased the resorption rate (Zenclussen *et al.* 2005, Wafula *et al.* 2009), while antibody to CD25 (Chen *et al.* 2013) increased the rate of resorption and blockade of PD1 (Wafula *et al.* 2009) and abrogated the protective effect of T reg administration. These experiments did not delineate the specificity of the regulatory T cell pool, and indeed it has been shown that CD4+ CD25+ cells with regulatory capacity, potentially naturally occurring, thymus-derived T regs, expand during syngeneic as well as allogeneic pregnancies (Aluvihare *et al.* 2004, Teles *et al.* 2013). Moreover, like many of the experiments done on this model, the T regulatory administration was not done in a pregnancy where neither the role of the specificity nor the level of target paternal antigen expression (e.g. a CBA/J×(CBA/J×DBA/2 F1) backcross) on resorption could be assessed.

Experiments in this model have suggested that *in vitro* activated and expanded CD25+ CD4+ vs naïve T regs were effective in decreasing resorption (Yin *et al.* 2012). In other models, it has also been observed that shared expression of antigen in the maternal thymus and the fetus markedly enhances antigen-specific T reg proliferation in the uterine-draining lymph nodes (Chen *et al.* 2013). However, these observations do not prove that exposure to a specific antigen is directly related to the generation of T reg function and decreased resorptions specifically in antigen-expressing sites. Moreover, while expansion of such cells is thought to occur at the level of the uterine draining lymph nodes (Teles *et al.* 2013), it is not clear where and to what extent the transferred CD25+ CD4+ cells traffic in the process of decreasing resorptions in this model.

Does the presence of T regs and the effects of their modification prove the self/nonself theory, or that pregnancy is critically dependent on suppression, deviation, or limitation of the maternal immune system? Are T regs critical to successful semi-allogeneic pregnancy? While many users and proponents of the CBA/J×DBA/2 model may still hold to this thinking (Chen *et al.* 2013), there is an alternative. For example, it is known that T cells with regulatory function and memory are generated by antigen exposure in the right context (TGFβ, etc.) during pregnancy (Rowe *et al.* 2012b). It could be said that these cells are essentially another flavor of T cells which have been revealed by the experimental context generated. Moreover, they along with their partner TH17 cells represent a similar balance of reactivity dependent on tissue need and tendency (Matzinger & Kamala 2011) as that ascribed to the TH2–TH1 paradigm of some years ago. It is even possible, if not likely, that there are several flavors of T reg cells, perhaps ones that specifically regulate certain types of inflammation (Bizargity *et al.* 2009). These cells are not the ‘Sang Real’ of tolerance. Danger, for example, in the form of intracellular infection, can decrease the presence and activity of T regs and generate anti-fetal immunity (Rowe *et al.* 2012a). While such a situation is obviously harmful to the fetus, it protects the mother against infection and other losses associated with carrying a potentially abnormal fetus to term.

Another possibility, that includes naturally occurring T regs, is that these cells exist to limit ‘collateral damage’ (Thangavelu *et al.* 2013) or ‘bystander effect’ (Anderson 2006). In this vein, the original Danger model dealt with the generation of auto-reactive T cells in the context of an immune response to infection by suggesting that tissues, especially large and fast-growing tissues, could essentially ‘out run’ the effects of auto-reactive T cells (Matzinger 1994), while small, slow growing tissues could not (Anderson *et al.* 2001). It is possible then to see T regs, especially the naturally occurring ones which tend to be tissue specific (del Rio *et al.* 2011), as a possible mechanism by which specific tissues may

‘out run’ bystander auto-reactive T cell generation. Self/nonself theory may strongly depend on the existence of T regs. Although they are not critical, they can be incorporated into this alternative theory.

Driving where? The future of the ‘CBA/J×DBA’ model

The recent observation that there is a very pronounced maternal renal/vascular phenotype in the CBA/J×DBA/2 model (Ahmed *et al.* 2010) and that the model relates only to first pregnancies (Singh *et al.* 2011) has several important implications. First, the fact that the mothers are so profoundly affected by the abnormal pregnancies strongly implies that the CBA/J×DBA/2 model should not be interpreted as being a model for most human first trimester miscarriages. In the majority of human miscarriages, there is no evidence of any sort of maternal renal/vascular disease although pregnancy loss can be the first manifestation of disease (Yin *et al.* 2013). In fact, pregnancy failure secondary to severe maternal illness caused by the pregnancy itself is relatively rare, and the pregnancy ‘failures’ are usually the consequence of deliberate termination procedures. Secondly, the fact that the CBA/J×DBA/2 model relates almost entirely to first pregnancies makes one question its relevance to recurrent pregnancy loss in humans. The generally understood concept behind recurrent pregnancy loss is that there is some process at the maternal–fetal interface that similarly affects all pregnancies. If the pathophysiological mechanism in the CBA/J×DBA/2 model was similar to the human situation, then women who had had one pregnancy loss would be expected to have a diminished probability for a subsequent pregnancy to end in miscarriage. In fact, the evidence suggests that women who have had one miscarriage have a somewhat increased probability for loss in a subsequent pregnancy (Regan *et al.* 1989). On this basis, there may be concern regarding the potential to gain general understanding of most human recurrent miscarriages.

However, the observations generated in the CBA/J×DBA/2 model show the capacity for the maternal immune system to respond to signals presented by its environment. Taken together the observations that: i) T regs can be increased either through administration of third-party spleen cells or other exposure to seminal plasma antigens, ii) there is a significant amount of tissue-level metabolic and other dysregulation, and iii) resorption is associated with a significant innate immune response (neutrophils, macrophages, dendritic cells, inflammatory cytokines) suggests that a likely role for T cells with regulatory function in this model is as modulators of innate immunity. This is in keeping with what has been observed in the nonpregnant state (Maloy *et al.* 2003), suggested by studies of the regulation of LPS-induced preterm birth in a syngeneic

mouse mating (Bizargity *et al.* 2009), and inferred from studies of human preterm birth (Ito *et al.* 2010). In this light, the model could be very useful in understanding the intricate metabolic and developmental programming particular to the maternal–fetal interface, further delineation of the ‘Danger’ signal, or ‘signal 0’, and further understanding of the complex and intricate mechanisms by which adaptive immunity might exert feedback control of innate immunity. This model could also examine the role of the recently ‘re-recognized’ innate lymphoid cells, including natural killer T cells (Boyson *et al.* 2006) and the non-T cell receptor expressing counterparts of known TH cell subsets (Lane *et al.* 2012, Spits & Cupedo 2012). Moreover, recent data suggest that transfer of CD19+ CD5+ CD1d+ B cells i.v., with concomitant increase in such cells in the spleen, also decreases resorptions (Jensen *et al.* 2013), and further examination of this cell type is warranted.

The mid-1990s witnessed the use T cell receptor transgenic mice in the study of maternal tolerance (Tafari *et al.* 1995). Since then several investigators have produced evidence in these systems that the maternal T cells are ‘aware’ that the host is pregnant (Bonney *et al.* 2011), undergo proliferation and death (Norton *et al.* 2010), and are able to respond to fetal antigens (Erlebacher *et al.* 2007, Norton *et al.* 2010). For most mice, neither the increased presence of T cells specific for fetal antigen nor manipulations to increase fetal antigen-specific T cell proliferation or function has resulted in specific pregnancy loss (see review by Moldenhauer *et al.* (2010)). The CBA/J×DBA/2 model has perhaps encouraged the expanded use of TCR transgenic mice. In one particular model, baseline levels of the overall fetal resorption rate are low, despite a high frequency of maternal T cells specific for an antigen transgenically expressed on the fetus (Chen *et al.* 2013). Immunization against this antigen along with depletion of T regs with antibody to CD25 increased overall resorption several fold; however, the role of the level of fetal antigen expression in resorbed vs non-resorbed fetuses is yet to be delineated (Chen *et al.* 2013). It might be very useful to generate a TCR transgenic mouse model within the CBA/J (expressing maternal specific anti-fetal T cells), DBA/2 (expressing specific fetal antigen), and Balb/c (expressing fetal antigen) backgrounds. If there is a specific fetal antigen that drives the resorptions in the CBA/J×DBA/2 model, it needs to be found in order to do similar experiments based on responsiveness to that particular antigen.

More important than the generation of specific tools, however, is the willingness to engage in experimentation with the model in the context of evolving theory of how the immune system chooses between activation and tolerance. Although several theories that might be considered (Anderson *et al.* 2001, Zinkernagel & Hengartner 2004, Anderson 2006, Cohn 2013), we have herein give one possible interpretation of the data

derived from the CBA/J×DBA/2 model that would allow for alternatives to fundamental self/nonself discrimination theory as the basis for maternal tolerance. We posit that with minds open to this and other alternatives, we will be able to use the CBA/J×DBA/2 model to continue to produce observations that enhance our understanding of the critically important biology of successful pregnancy and its complications.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review.

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