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Review article

Embryotoxic cytokines—Potential roles in embryo loss and fetal programming

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ABSTRACT

Cytokines in the reproductive tract environment at conception mediate a dialogue between the embryo and maternal tissues to profoundly influence embryo development and implantation success. Through effects on gene expression and the cell stress response, cytokines elicit an epigenetic impact with consequences for placental development and fetal growth, which in turn affect metabolic phenotype and long-term health of offspring. There is substantial evidence demonstrating that pro-survival cytokines, such as GM-CSF, CSF1, LIF, HB-EGF and IGFII, support embryos to develop optimally. Less attention has been paid to cytokines that adversely impact embryo development, including the pro-inflammatory cytokines TNF, TRAIL and IFNG. These agents elicit cell stress, impair cell survival and retard blastocyst development, and at sufficiently high concentrations, can cause embryo demise. Experiments in mice suggest these so-called 'embryotoxic' cytokines can harm embryos through pro-apoptotic and adverse programming effects, as well as indirectly suppressing uterine receptivity through the maternal immune response. Embryotrophic factors may mitigate against and protect from these adverse effects. Thus, the balance between embryotrophic and embryotoxic cytokines can impart effects on embryo development and implantation, and has the potential to contribute to endometrial 'biosensor' function to mediate embryo selection. Embryotoxic cytokines can be elevated in plasma and reproductive tract tissues in inflammatory conditions including infection, diabetes, obesity, PCOS and endometriosis. Studies are therefore warranted to investigate whether excessive embryotoxic cytokines contribute to infertility and recurrent implantation failure in women, and compromised reproductive performance in livestock animals.

1. Introduction

After conception, the embryo traverses the oviduct before reaching the uterus to complete blastocyst development and implantation. In this free-floating phase the embryo is highly responsive to external signals that, through epigenetic mechanisms, contribute to setting the trajectory of the embryo's future developmental program. There is an evolutionary benefit of this sensitive stage – the embryo can sense and respond to local signals, in order to achieve a phenotype suited to the external environment. Embryo plasticity is thought to have evolved to provide the best chances of offspring survival and reproductive success to the organism – but there is a cost when the resulting phenotype constrains capacity to withstand health and environmental challenges after birth (Waterland and Jirtle, 2004).

An emerging view is that the female reproductive tract is not passive but plays an active role in the process driving plasticity prior to and during implantation (Robertson, 2010), and indeed can be a powerful force in selectively supporting or eliminating embryos (Teklenburg et al., 2010; Macklon and Brosens, 2014). This view is supported by data from several mammalian species indicating that uterine receptivity rather than ovulation and conception is rate-limiting in reproductive success (Foote and Carney, 1988). In women, it is estimated that around 30% of naturally conceived fertilised oocytes do not successfully implant (Norwitz et al., 2001), and implantation failure is even higher in assisted reproductive treatment cycles (Boomsma et al., 2009).

The complex, dynamic milieu of bioactive agents mediating maternal tract–embryo communication includes an array of cytokines and growth factors. These agents have paracrine cell–cell signalling actions that directly affect embryo viability and developmental potential (Hardy and Spanos, 2002; O'Neill, 2008; Richter, 2008), as well as regulating uterine receptivity (Dimitriadis et al., 2005). Cytokines permeate the zona pellucida and ligate specific cell surface receptors to activate intracellular signalling pathways, which in turn modulate gene expression and impact cellular functions including metabolism, secretory function, differentiation and the cell stress response. The maternal cytokine milieu is emerging as an important determinant of embryo

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survival and implantation competence, and can shape post-implantation development in both positive and negative ways (O'Neill, 2008; Robertson et al., 2015).

The balance of cytokines is responsive to systemic and local regulation by a wide range of maternal parameters and environmental exposures (Robertson et al., 2015), and provides a likely mechanism by which the consequences of these conditions are exerted on embryo development and implantation. Several health conditions and disease states have potential to alter reproductive tract cytokines, either by direct effects on the physiological function of the reproductive tissues, or by systemic elevation of circulating pro-inflammatory agents. Although preimplantation embryo development is well known to be susceptible to maternal health status, there has been poor understanding of the upstream signals that affect embryo survival, development and epigenetic modification. Some metabolic, chemical or physical insults might directly impact the cell stress response, apoptotic machinery and the epigenome, but this would require agents to access and act locally in the reproductive tract. It seems more biologically plausible that the effects of maternal conditions are communicated by local interlocutory mediators to influence embryonic survival and programming, as well as uterine receptivity.

There is a substantial literature on pro-survival embryotrophic factors secreted from female tissues and produced endogenously within the embryo (Dimitriadis et al., 2005; Thouas et al., 2015). In this review, we focus on their counterpart embryotoxic cytokines that exert adverse effects on preimplantation embryo development. These are pro-inflammatory cytokines that, in excessive concentrations, can harm embryo survival and development and/or induce altered developmental programming. Given the ethical and practical challenges of studying human tissues, we refer mainly to data from animal species, while drawing on human studies when possible. Collectively, the assembled evidence indicates that elevated pro-inflammatory cytokines contacting the developing embryo can contribute to the inhibitory effects on fertility of some common health conditions, and also exert effects on developmental programming to mediate intergenerational transfer of metabolic disease susceptibility.

2. Cytokines in the oviduct and uterus

Several cytokines are expressed in distinct spatial and temporal patterns over the course of the ovarian cycle. Synthesis is regulated primarily by ovarian steroid hormones, acting in concert with local endogenous factors, signals from the conceptus, the reproductive tract microbiome and constituents of the male partner's seminal fluid. Systemic factors such as nutritional status and micronutrient availability, neuroendocrine signals, circulating and local hormones, growth factors and microRNAs, and genetic polymorphisms in cytokine and cytokine receptor genes also influence the cytokine balance (Fig. 1).

The epithelial cells forming the luminal surface of the oviduct and endometrium, and the endometrial glands are a major source of maternal cytokines released into the luminal fluid. Amongst the best studied are colony stimulating factor-1 (CSF1, also known as M-CSF), granulocyte-macrophage CSF (GM-CSF, also known as CSF2), leukaemia inhibitory factor (LIF) and transforming growth factor β (TGFB) (Dimitriadis et al., 2005; Thouas et al., 2015). Epithelial cells also secret chemokines and vascular endothelial growth factors (VEGFs), that can influence embryos as well as regulating leukocyte infiltration and vascular remodelling (Hannan et al., 2011).

Epithelial cell cytokine synthesis is not constitutive, but is regulated over the reproductive cycle. In women, estrogen-regulated cytokines including CSF1, GM-CSF and tumor necrosis factor (TNF) increase over the proliferative phase and peri-ovulatory phase. Expression shifts during the luteal phase when increasing progesterone dampens pro-inflammatory cytokines, driving a shift towards anti-inflammatory cytokines such as CSF1, LIF and TGFB once implantation commences (Dimitriadis et al., 2005; Thouas et al., 2015). The embryo secretes

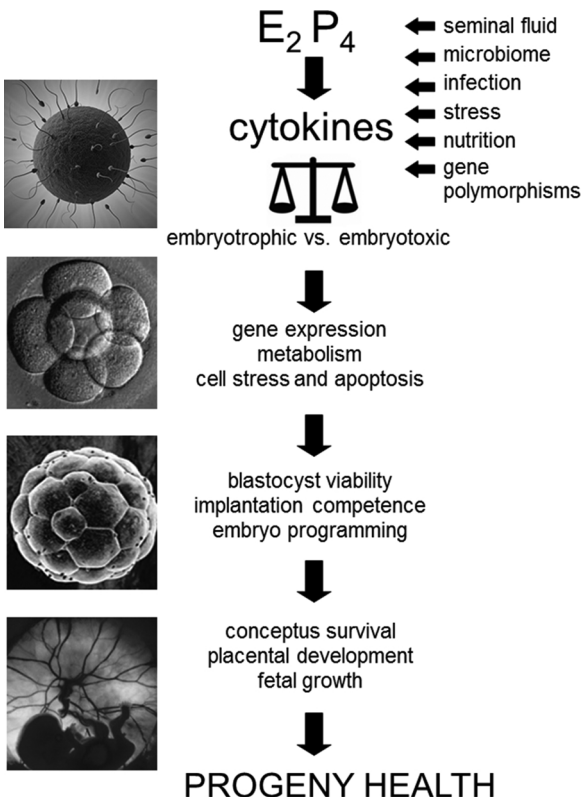

Fig. 1. Cytokine expression in the female reproductive tract is regulated by ovarian steroid hormones and a variety of intrinsic and extrinsic factors. The cytokine environment in the periconception period comprises both embryotrophic (pro-survival) and embryotoxic (pro-apoptotic) factors. Together these factors influence gene expression, metabolism, cell stress and apoptosis in the pre-implantation embryo to initiate downstream events impacting implantation, placental development and fetal growth, and ultimately the phenotype and health of progeny (updated from Robertson et al., 2015).

factors such as interleukin–1 B (IL1B) and human chorionic gonadotrophin (hCG) can regulate embryotrophic factor release from uterine epithelial cells, and this bidirectional communication synchronises embryo development with maternal tract receptivity (Thouas et al., 2015).

In many regards, the cytokine and immune cell response to embryo implantation reflects a controlled inflammatory response, akin to that seen in tissue injury and wound healing (Mor et al., 2017). Luteal phase progesterone is critical in the switch from a pro-inflammatory environment at conception, to a regulated and contained inflammation at implantation. This ensures the phenotype and balance of inflammatory mediators is consistent with continued development. When embryo implantation fails, or in a non-conception cycle, progesterone decline upon luteolysis causes a surge in pro-inflammatory cytokines such as TNF, IL6 and IL1 before menstruation commences (Salamonsen et al., 2002). A similar increase in TNF and IL8 expression is seen in Fallopian tubes after luteal phase administration of the progesterone antagonist RU486 (Li et al., 2004).

Comparable patterns are seen in the mouse but in this species there is additional regulation afforded by male seminal fluid contact. Effects of seminal fluid on cytokine release are well described in rodents and other species where seminal fluid accesses the uterine cavity to induce a surge in GM-CSF, IL6 and an array of chemokines from estrogen-primed uterine and oviductal epithelial cells after mating (Robertson, 2005; Bromfield et al., 2014). In women, seminal factors including TGFB and prostaglandin E2 (PGE_2) stimulate cervical epithelial cells to induce expression of IL8, GM-CSF, IL6 and various chemokines to in turn activate the immune adaptation required for pregnancy (Sharkey et al., 2012). Whether seminal fluid factors can impact cytokines in the

human endometrium or oviduct is unknown. Although *in vitro* studies imply cytokine production by endometrial epithelial cells can be regulated by male factors (Gutsche et al., 2003; Chen et al., 2014), and seminal fluid factors carried by sperm including TGFB (Chu et al., 1996) may reach the uterus and Fallopian tube (Bulletti et al., 1997), data to demonstrate an *in vivo* response is not yet available.

Large numbers of leukocytes reside in the mucosal surface of the endometrium and oviduct in all mammalian species. These macrophages, dendritic cells, granulocytes, lymphocytes and mast cells are all key agents in the endometrial decidual response, vascular remodelling and induction of immune tolerance required for embryo implantation. Cytokines released by immune cells might also access the luminal cavity and developing embryo. Their secretory profile is highly variable depending on activation state, and excessively pro-inflammatory phenotypes shift the cytokine balance towards inflammatory mediators. In particular, the cytokine secretion profile of uterine macrophages varies in response to ovarian hormones, as well as signals from the conceptus and semen, and microbial populations. In successful implantation, decidual macrophages synthesise anti-inflammatory and tolerance-inducing cytokines TGFB and IL10 (Chen et al., 1993; Heikkinen et al., 2003) and IL1 receptor antagonist (Tabibzadeh and Sun, 1992), reflecting an anti-inflammatory "M2" phenotype. If macrophages exhibit an aberrant M1 phenotype, increased TNF and IL1 B release will ensue.

Decidual lymphocytes are an important source of cytokines, particularly uterine natural killer (uNK) cells which can produce many cytokines that influence embryo development and implantation including GM-CSF, CSF1, IL10 and LIF, as well as TNF and interferon-γ (IFNG) (Croy et al., 2006). The profile of pro- and anti-inflammatory factors released reflects the phenotype of the uNK cells. Although sparse in the endometrium, T-lymphocytes also exhibit complex and variable patterns of cytokine production (Jokhi et al., 1994; Piccinni and Romagnani, 1996). A pivotal T-lymphocyte population essential for embryo implantation are CD4 + CD25 + T regulatory (Treg) cells, which synthesise copious amounts of TGFB and IL10 (Guerin et al., 2009), to limit inflammatory activation in macrophages and uNK cells.

3. Embryotrophic cytokines

Several cytokines synthesised by epithelial cells and local leukocytes support cleavage stage embryos and blastocyst development, protecting blastomeres from cell stress and apoptosis, to promote implantation competence. GM-CSF, CSF1, LIF, IL6, insulin-like growth factor-1 (IGFI) and IGFII are all reported to have beneficial or embryotrophic activity (Dimitriadis et al., 2005; Thouas et al., 2015).

Embryotrophic cytokines also influence embryo programming, such that withdrawal of cytokine support by ex vivo culture in simple culture media can cause cell stress and epigenetic changes linked with altered fetal development and altered growth trajectory after birth (Robertson et al., 2015). This principle is well demonstrated with GM-CSF in mouse (Sjoblom et al., 2005) and cattle (Hansen et al., 2014), where in both cases male and female embryos are differentially affected such that faster-growing male embryos are more adversely impacted by cytokine deficiency than females. GM-CSF alleviates the adverse impact of ex vivo embryo culture on fetal and post-natal growth, and protects adult progeny from disturbed metabolic function (Sjoblom et al., 2005). LIF also acts to increase cell number in blastocysts, generating fetuses with higher birth weights (Cheung et al., 2003).

The beneficial effect of embryotrophic cytokines is largely a consequence of protection from cell stress, such that in the absence of environmental stress, exogenous cytokines may be more dispensable (Chin et al., 2009). Recent data in leukocytes shows that the memory of exposure to pro-survival cytokines can be mediated by epigenetic modifications to the chromatin of target cells, such that phenotypes are retained in subsequent cell generations (Kanno et al., 2012; Kittan et al., 2013), but whether this occurs in embryos to explain protection from cell stress responses is yet to be determined.

4. Embryotoxic cytokines

There can be considerable variability in embryo development and implantation competence, but generally it has been assumed that this reflects a passive arrest and demise due to embryo intrinsic factors such as aneuploidy, lack of embryotrophic support or insufficient receptivity in female tissues. Recently, the concept of an endometrial 'biosensor' that can actively select and influence progression of embryos has emerged (Teklenburg et al., 2010). The existence of cytokines that impart cell stress and exert inhibitory effects on embryos suggests that active destruction by maternally-derived agents could contribute to this selection process and contribute to so-called 'hostile' reproductive tract conditions. Proinflammatory cytokines released from female tract tissues under specific circumstances can exert potent adverse effects on embryo viability and development. In particular, TNF, IFNG and TNF-related apoptosis-inducing ligand (TRAIL) act to suppress embryo development, inducing cell death when present at sufficiently high concentrations (Hill et al., 1987; Pampfer et al., 1994; Riley et al., 2004). Like the embryotrophic factors described above, these cytokines are responsive to induction and regulation in the female tract, and in appropriately controlled levels may contribute to normal implantation and development. However when expression is elevated and sustained due to infection, non-infectious inflammatory conditions or other causes, or when insufficient counter-balancing embryotrophic cytokines are present, this likely contributes to altered developmental programming or embryo demise (Fig. 2).

4.1. Tumour necrosis factor (TNF)

TNF is a pleiotropic cytokine and archetypal member of the TNF

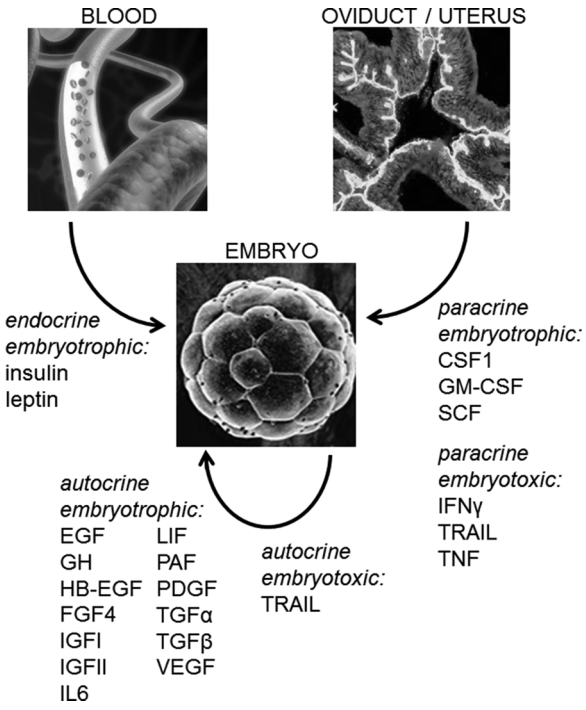

Fig. 2. A range of cytokines regulate development of the preimplantation embryo as it traverses the female reproductive tract prior to implantation. These cytokines can be classified according to their embryotrophic (pro-survival) or embryotoxic (pro-apoptotic) effects. Several cytokines are synthesised endogenously within the embryo and also are secreted by female tract tissues, to mediate both autocrine and paracrine regulation. Some cytokines, notably GM-CSF and CSF1 are not made by the embryo and are solely of maternal origin. Embryotoxic factors IFNG, TRAIL and TNF, are elevated in response to maternal health conditions or environmental exposures and can exert potent effects leading to embryo demise or altered developmental programming (updated from Robertson et al., 2015).

superfamily of cytokines. TNF is primarily associated with pro-inflammatory and apoptosis-inducing activity, and a range of different responses in many cell lineages (Ting and Bertrand, 2016). TNF ligation of its receptors TNFR1 or TNFR2 stimulates translocation of the nuclear factor kappa B (NFκB) transcription factors to the nucleus, triggering transcription of various inflammatory mediators such as IL6 and IL1A, and depending on environmental circumstances, cell survival or cell death may ensue (Ting and Bertrand, 2016). TNF is detectable in oviductal and uterine tissues throughout the preimplantation period in mice and in women (Sanford et al., 1992; Hunt, 1993). Uterine macrophages and uNK cells are a primary source of TNF in the reproductive tract, as well as oviductal epithelial cells (Hunt, 1993). At appropriate levels, local TNF may contribute to implantation success (Boomsma et al., 2009).

Preimplantation mouse embryo express TNFR1 and can be highly responsive to elevated TNF (Pampfer et al., 1997; Wuu et al., 1999; Whiteside et al., 2003). Cleavage stage mouse embryos can secrete TNF under some circumstances (Lachapelle et al., 1993). Addition of TNF to embryo in vitro culture medium increases the percentage of apoptotic blastomeres in mouse (Byrne et al., 2002; Fabian et al., 2007) and rat blastocysts, particularly the inner cell mass (Pampfer et al., 1997). Culture of flushed mouse blastocysts with sufficiently high concentrations of TNF significantly increases cell death and retards development in a dose- and time-dependent manner (Fabian et al., 2007). Bovine embryos exposed to TNF after fertilisation show reduced development to blastocyst stage, with the incidence of cell death increasing with elevated TNF concentration, due to caspase-9-mediated apoptosis (Soto et al., 2003; Loureiro et al., 2007).

TNF can attenuate responsiveness to other cytokines; for example, mouse trophectoderm outgrowths exposed to TNF downregulate CSF1 receptor expression (Ben-Yair et al., 1997). Direct effects of TNF on embryos as well as indirect effects through the immune response presumably contribute to the impact of TNF administration in early pregnancy, which impairs implantation and reduces litter size in mice and rats (Clark et al., 1998). Embryotrophic factors can influence susceptibility to TNF exposure − co-culture with stem cell factor (SCF), IGFI, IGFII or TGFA is protective against the inhibitory effects of TNF in embryos (Glabowski et al., 2005; Kawamura et al., 2007).

4.2. Interferon gamma (IFNG)

IFNG is a pro-inflammatory cytokine that plays important roles in diverse cellular processes, including activation of innate and adaptive immune responses, inhibition of cell proliferation and induction of apoptosis (Boehm et al., 1997). IFNG contributes to events that assist implantation, including initiation of endometrial vascular remodelling, angiogenesis at implantation sites, and maintenance of the decidual component of the placenta in normal rodent pregnancies (Murphy et al., 2009), but elevated production of this cytokine is detrimental to embryo survival.

The IFNG receptor is expressed on mouse oocytes and pre-implantation embryos (Truchet et al., 2001) and IFNG is inhibitory in mouse embryo culture (Haimovici et al., 1988; Hill et al., 1992). Altered expression of proteins required for embryo adhesion is also seen in response to IFNG (Fontana et al., 2004). Systemic administration of exogenous IFNG in the pre-implantation period prevents implantation and inhibits the maintenance of pregnancy (Chaouat et al., 1990; Martal et al., 1997). IFNG inhibits the secretion of GM-CSF, a key cytokine that promotes blastocyst growth and differentiation, in mouse and human reproductive tract epithelial cells (Glynn et al., 2004).

In cattle, IFNG is one of several proinflammatory cytokines reported to be differentially elevated in the endometrium of cattle with failed or retarded pre-implantation embryo development (Beltman et al., 2013). IL6, IL10 and IFNA were similarly over-expressed, while the anti-inflammatory cytokine TGFB was relatively under-expressed. Also in cattle, IFNG was reported to modify gene expression in blastocysts, inducing expression of non-classical MHC class I NC2, NC3 and N4, implying a possible role in embryo-induced immune tolerance (Al Naib et al., 2011).

4.3. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)

TRAIL is a cell death-inducing cytokine that belongs to the TNF superfamily (Wang and El-Deiry, 2003). Both TRAIL and TRAILR are expressed from the 1-cell through to the blastocyst stage of murine preimplantation embryo development, and embryos induced to express TRAIL or exposed to TRAIL in culture undergo apoptosis (Riley et al., 2004). We have recently reported that TRAIL is expressed constitutively in mouse oviducts, but in healthy pregnancy then becomes attenuated during the peri-conception period (Bromfield et al., 2014). Whether this is mediated by signals emanating from the preimplantation embryo, or as a consequence of seminal fluid contact, is yet to be determined.

There are unique features of TRAIL that implicate this factor as a key agent in embryo quality control. Importantly, unlike other death-inducing cytokines such as soluble Fas ligand (FasL), TRAIL does not damage normal tissues in the reproductive tract (Fluhr et al., 2009; Groebner et al., 2010), and requires a specific set of conditions to overcome protective mechanisms and enable death-inducing signal delivery. Several factors integrate to increase sensitivity, including (1) slowed rate of cell cycle and mitotic progression; (2) down-regulation of surface TRAIL decoy receptors DcR1 and DcR2; (3) limited availability of pro-survival factors signalling through NFKB and Akt, (4) elevated expression of endogenous TRAIL, and (5) activation of the intrinsic apoptosis pathway and reduced activity of apoptosis inhibitors cFLIP and Bcl-2 (Wang and El-Deiry, 2003; Falschlehner et al., 2009). When these conditions are met, TRAIL induces apoptosis by binding and cross-linking TRAILR, which then result in caspase activation. In vitro, a TRAIL-sensitive state can be replicated in embryos as in other cells by sensitization with actinomycin-D (Wang and El-Deiry, 2003; Riley et al., 2004).

Mouse embryos express the death-inducing TRAIL receptor, a homologue of human DR5 (mKILLER), and three decoy receptors (mDcTRAILR1 and mDcTRAILR2) (Riley et al., 2004). These decoy receptors attenuate TRAIL bioavailability in tissues under normal conditions, but can be down-regulated in cell stress (Almasan and Ashkenazi, 2003). Death signalling receptors exert their death-inducing capabilities upon binding TRAIL, causing TRAIL receptors to trimerise and form the death-inducing signalling complex (DISC). In turn this causes recruitment of intracellular adaptors such as Fas-associated death receptor (FADD) proteins to death domains present in the intracellular portion of the receptor. These adaptor proteins recruit more adaptors proteins to build a signalling cascade, which results in caspase activation and cell death via intrinsic or extrinsic pathways (Wang and El-Deiry, 2003). These different signalling options and network of regulatory interactions provides the opportunity for TRAIL to exert both positive and negative functions on embryo development, depending on the environmental circumstances.

Likewise, both death signalling receptors (DR4 and DR5/KILLER) and decoy receptors (TRAIL-R3, TRAIL-R4 and osteoprotegerin) for TRAIL are present in human reproductive tissues and embryos. In decidualised human endometrial stromal cells, TRAIL regulates cytokine expression (Fluhr et al., 2009), and TRAIL is one of several TNF superfamily ligands transcribed in placental trophoblasts and placental macrophages (Phillips et al., 2001). Trophoblasts can release TRAIL to induce smooth muscle cell death and facilitate invasion into maternal decidua (Keogh et al., 2007). Nitric oxide synthesis is an important regulator of TRAIL receptor expression and reduces susceptibility to TRAIL-induced apoptosis in human trophoblasts (Lumicisi et al., 2015).

5. Embryotoxic cytokines and embryonic programming

Several stressors can act in the reproductive tract environment at

conception including immunological, infectious, nutritional, metabolic and physiochemical perturbations to exert subtle but permanent alterations in the trajectory of fetal development and postnatal growth. This plasticity may be desirable to allow responsiveness and adaptation to resource constraints in the external environment (Hochberg et al., 2011). Plasticity can be exerted at several levels mediated by a variety of epigenetic adaptations, resulting in short and long-term changes to gene expression, cell number and cell lineage fate (Young, 2001; Morgan et al., 2005). Maternal tract cytokines are well-suited to provide a pathway through which inflammatory, infectious and chemical insults can converge and integrate, since all of these factors alter cytokine expression in the reproductive tract (Robertson et al., 2011). Experiments in mouse and other animal models show that reduced bioavailability of embryotrophic cytokines alters embryonic programming (Robertson et al., 2011). Elevated embryotoxic cytokines may be another signal through which embryos sense a less than optimal prevailing environment and adjust their developmental program accordingly.

Embryotoxic cytokines can affect at least three parameters of embryonic programming – (i) activation of the cell stress response which alters gene expression and has a lasting impact on developmental trajectory, (ii) permanent changes to behaviour and phenotype through epigenetic modification, and (iii) cell death by apoptosis, which reduces cell number. Even small perturbations in blastomere number and inner cell mass/trophectoderm allocation in the blastocyst influence later growth trajectory of the fetus and offspring (Thompson et al., 2002). Changes in the epigenetic modification of imprinted genes *Igf2*, *H19*, *Grb10* and *Grb7* occur when mouse embryos are cultured in the presence of serum (Khosla et al., 2001) or in sub-optimal culture media deficient in cytokines (Doherty et al., 2000) each of which are linked with later growth changes. Since expression of methyltransferases and other methylation machinery is cell cycle-regulated, disruption in their function is thought to result from slower kinetics of embryo development in culture (De Rycke et al., 2002). The presence of embryotoxic factors *in vivo* could exert a similar effect, whereas embryotrophic growth factors that protect blastomere survival and sustain cell division dynamics would oppose this. Cytokines can also exert direct effects on the transcription of genes regulating methylation (Zhao et al., 2005), but whether this occurs in embryos is unknown.

The effects of exposure to embryotoxic cytokines on later fetal development are not well defined. Embryo transfer experiments show that embryos surviving exposure to TNF during the pre-implantation period have fewer viable blastomeres at blastocyst stage, and the rate of apoptosis is elevated in the inner cell mass (Wuu et al., 1999). After transfer into recipient mice, blastocysts exposed to TNF implant at about the same rate as controls, but a significantly higher rate of resorption is found among fetuses after TNF exposure. In later gestation, the weight of surviving fetuses is significantly lower than for control fetuses (Wuu et al., 1999). This elegant work demonstrates that the legacy of exposure to embryotoxic cytokines can manifest much later in gestation, as poor fetal viability and fetal growth restriction.

6. Maternal health conditions and embryotoxic cytokines

Cytokines produced by the maternal tract can influence embryo survival and developmental competence, and impart programming information to the embryo. This raises the question of the physiological significance and specific health conditions that affect the balance of reproductive tract cytokines and impact cytokine-mediated maternal-embryo communication. Several diseases and health conditions linked with reduced fertility are associated with altered cytokine production. These include metabolic conditions such as diabetes, hyperglycaemia and obesity; immune conditions associated with infectious disease and autoimmune diseases; hormonal conditions such as endometriosis or polycystic ovarian syndrome; and exposure to smoking and environmental toxins.

6.1. Reproductive tract infection

Reproductive tract cytokine patterns are susceptible to interference from the pro-inflammatory effects of infection or dysbiosis. Chlamydia bind to Toll-like receptors (TLRs) expressed by epithelial cells in the rat and human uterus and oviduct, and alter the cytokine synthesis profile, notably inducing elevated TNF and IL1RA (Rasmussen et al., 1997; Kaushic et al., 2000; Buckner et al., 2013). Meta-analysis of inflammatory cytokine responses in various reproductive tract infections identifies known embryotoxic cytokines as induced locally and in peripheral blood by infection. A higher TNF response during *C. trachomatis* infection correlates positively with infertility, while *T. palladium* infection is particularly associated with elevated urogenital IFNG (Singer and Ouburg, 2016). The TLR-mediated response to bacterial and viral entities plays a vital role in recognising infection and eliciting protective immunity to limit viral replication and clear infection. However, excessive or prolonged stimulation of the TLR-induced pro-inflammatory pathway can override the normal hormone-regulated expression of endometrial cytokines.

Virus-mediated inflammation appears likely to operate through a similar mechanism. Cytokine networks induced after reproductive tract infection act in an amplifying cascade involving elevated IFNG and TNF production (Fichorova and Anderson, 1999). Notably, infection with cytomegalovirus (CMV) and other viruses (Sedger et al., 1999; Cummins and Badley, 2009; Falschlehner et al., 2009) elevates TRAIL expression in reproductive tract cells. Human uterine epithelial cells secrete TNF and IFNG in response to TLR3 activation (Schaefer et al., 2005) and in vivo, this would be expected to impair embryo development. Together these data imply that elevation of embryotoxic cytokine expression may be an under-appreciated pathway through which sexually transmitted infection interferes with fertility.

Systemic infections may also exert influence on inflammatory cytokines expressed in reproductive tissues. There is evidence that mastitis impairs fertility in lactating dairy cattle, with a longer interval and more inseminations required to achieve pregnancy. Higher embryonic mortality is one explanation for this observation, although anovulation, fertilisation failure and/or systemic hormone aberrations are also possible. Since mastitis is linked with elevated circulating TNF, IL1, IL6 and other proinflammatory cytokines, a mechanism by which inflammatory cytokines cause embryonic mortality, potentially amplified by nitric oxide and prostaglandin F2α, has been proposed (Hansen et al., 2004). This is consistent with studies showing bovine oviductal epithelial cells are sensitive to induction of TNF, IL1B and other proinflammatory cytokines in response to stimulation with low dose bacterial lipopolysacharide (LPS) (Marey et al., 2016).

In recent experiments, we have developed a model that mimics the impact of elevated inflammatory load due to chronic infection at the time of conception. *E.coli* or *S. typhimurium* LPS was administered i.p. to mated mice at approximately 12 h and 36 h after mating, at a range of doses. LPS caused upregulated expression of TNF, IFNG and TRAIL in oviduct and uterine tissues (Chin et al., in preparation). At high doses, pregnancy did not progress and this was associated with low rates of blastocyst development. At moderate doses, fewer blastocysts were recovered from tracts and these had reduced numbers of cells, particularly in the inner cell mass, consistent with high rates of apoptosis and cell stress. These effects were mediated indirectly and most likely by the elevated inflammatory cytokines, since co-culture experiments showed that LPS had no direct impact on embryo development. At low doses, LPS treatment before implantation caused little overt adverse impact on blastocyst development, but remarkably when mice were allowed to continue pregnancies, fetal and placental growth disturbances were evident in late gestation. We believe this is the result of fetal programming due to elevated embryotoxic cytokines, since normal embryos transferred into LPS-treated recipient mice showed the same adverse impact on development (Chin et al., 2010; Chin, 2014).

Altered fetal programming was also reported in studies where mice

were administered low to moderate doses of LPS on day 0.5 post coitum (Williams et al., 2011). Although female reproductive tract cytokines were not measured, serum pro-inflammatory cytokines including TNF, IL1B and IL12p40 were elevated, and associated with fewer viable inner cell mass cells in blastocysts. At the higher LPS doses, offspring conceived in inflammatory conditions showed elevated peripheral fat mass and altered immune function as adults (Williams et al., 2011). One interpretation of the less severe effects in this study is that inflammatory perturbations exert more profound effects when elicited in the preimplantation phase, than in the 24 h after mating, when reproductive tract proinflammatory cytokines are already elevated due to seminal fluid contact.

6.2. Metabolic and endocrine health disorders

As well as infectious stimuli, cytokine patterns are susceptible to interference from a range of sterile insults, including metabolic and endocrine disorders, or exposure to noxious agents. Chronic low-grade inflammation is a hallmark of obesity, and obesity and metabolic disorder are particularly linked with elevated plasma TNF and TRAIL (Tzanavari et al., 2010; Harith et al., 2013), that reasonably could access female reproductive tract tissues. Systemic TRAIL expression is also elevated by the sterile inflammation accompanying high alcohol intake (Vaculova et al., 2004; Plante et al., 2013). Polycystic ovarian syndrome is linked with reduced fertility, low-grade inflammation and elevated circulating inflammatory cytokines − although the overlap with obesity in this condition is a confounding factor (Duleba and Dokras, 2012). These conditions each impact on fertility and are linked with intergenerational transmission of metabolic dysfunction to offspring, consistent with TNF and TRAIL being key local signals for mediating embryo loss or adverse programming impact.

There is good evidence of a causal role for TNF in the embryopathy that accompanies diabetes, through effects on embryos as well as the maternal tract (Torchinsky and Toder, 2007). TNF is upregulated in the uterus of diabetic pregnant rats, and has been demonstrated to contribute to early embryopathy in this model, since the TNF inhibitor etanercept can reduce embryotoxicity (Pampfer, 2001). Additionally, the pregnancy rate in streptozytocin-induced diabetic mice is lower than that in non-diabetic females, but this is substantially normalized in TNF null mutant mice (Torchinsky et al., 2004). In recent studies, we have confirmed that streptozytocin-induced diabetes in mice is linked with elevated uterine and oviduct expression of TNF, and a trend to increased TRAIL and IFNG, accompanied by high rates of embryonic loss and poor blastocyst development (Brown et al., 2018). Further studies using mice with cytokine null mutations are required to determine whether these latter cytokines also contribute to embryopathy.

6.3. Infertility, miscarriage and endometriosis

A range of cytokines are differentially expressed in the endometrium of women with implantation failure and recurrent miscarriage, with down-regulation of several embryotrophic cytokines including CSF2, LIF and CSF3 and upregulation of IL1RA which would be expected to suppress embryo-derived IL1-mediated signalling (Ledee et al., 2011; Koot et al., 2012).

IFNG has been explored as contributing factor in recurrent spontaneous abortion in women and failure of in vitro fertilisation, as women suffering repeated miscarriage have high serum IFNG (Jenkins et al., 2000). Mouse embryos cultured with serum from peripheral blood of women with recurrent miscarriage undergo demise and IFNG is implicated as a key causative agent (Hill et al., 1992; Cameo et al., 1999). Elevated TRAIL is also detected in maternal serum in recurrent miscarriage (Rull et al., 2013). A key question is whether elevated embryotoxic cytokines in the reproductive tract precedes loss and so may contribute to causal mechanisms through eliciting cell stress in embryos before implantation.

Only a few studies have investigated TNF, TRAIL and IFNG in oviductal or uterine luminal fluids in infertile or subfertile women. In the condition of hydrosalpinx, elevated TNF has been detected in oviductal fluids (Bedaiwy et al., 2005) and likely interacts with other cytokines to mediate embryotoxicity (Bao et al., 2017). Endometriosis is also associated with poor embryo quality and reduced fertility, and higher levels of pro-inflammatory cytokines including TNF in peritoneal fluid and peripheral blood have been implicated as a potential mechanism (Stilley et al., 2012). Whether direct effects of elevated proinflammatory cytokines are reflected in higher embryo exposure to these factors and the extent to which this causally impacts fertility, is still to be determined.

7. Evolutionary significance of embryotoxic cytokines

7.1. Maternal tract quality control

The existence of hostile maternal cytokines implies that a balance between trophic and toxic cytokines exists and might comprise a physiological ‘quality control’ system. The regulatory networks governing cytokine balance would equip the system to integrate information on environmental, gamete and embryo parameters to effectively determine whether and which embryos survive and progress to implantation and pregnancy. Perturbations resulting from local or systemic inflammation, nutritional deficiency, or aberrant or insufficient signals from seminal fluid or the embryo would be expected to shift the balance towards embryotoxic cytokines. This would reduce the chances of pregnancy when the female tract is not adequately prepared or the immune or nutritional environment is unsuitable (Robertson, 2010).

Experimental evidence for embryotoxic cytokines mediating a quality control mechanism comes from several mouse models. Our studies using LPS in the pre-implantation phase provide evidence that an aberrant proinflammatory stimulus induces TNF, TRAIL and IFNG in the female reproductive tract, and a cytotoxic impact on embryos is evident since embryos are rescued by flushing from the tract and development ex vivo, or embryo transfer to a normal tract (Chin et al., 2010; Chin, 2014). In another model, we show that in conceptions sired by males rendered deficient in seminal plasma by seminal vesicle excision (SVX), embryos generally arrest at cleavage stage in vivo as a consequence of oviduct expression of elevated embryotoxic cytokines − again, this can largely be rescued by flushing 2 cell stage embryos and culture ex vivo, where upon the majority develop to blastocyst stage (Bromfield et al., 2014).

Embryos surviving exposure to embryotoxic cytokines later exhibit hallmark symptoms of altered developmental programming. In both the LPS and SVX models, exposed blastocysts go on to display restricted fetal growth, followed by accelerated growth after birth, and increased adiposity in adult life (Bromfield et al., 2014; Chin, 2014), with male offspring more profoundly affected. In both cases, embryo transfer experiments prove that the oviduct mediates the adverse effects − blastocysts from perturbed donors transferred to untreated recipients retain the programming impact of the oviduct environment (Bromfield et al., 2014; Chin, 2014).

On the basis of this data, we postulate that common maternal agents underpin both arrested embryo development and the programming insult, and that cell stress and apoptosis are involved. This is plausible since activation of apoptosis pathway is the culmination of the adaptive response to stress in embryos where it acts to remove damaged cells (Brison and Schultz 1997; Hardy, 1999). Whether or not cell stress results in cell death or recovery and survival, presumably depends on the severity of cell damage (Leese, 1998).

In bovine uterine epithelial cells, suppression of TNF and elevated release of anti-inflammatory cytokines after conception is mediated by effects of embryo-derived IFNτ (Talukder et al., 2017). The activity of embryo-derived factors in suppressing embryotoxic factors implies that embryo quality also impacts their expression, such that failure to down-

regulate female tract embryotoxic cytokines could identify embryos with poor developmental potential and thereby assist in maternal selection of high quality embryos.

The cytokine network is therefore a pathway through which environmental cues and intrinsic factors can converge to actively prevent embryos from implanting in unfavourable circumstances, and sparing maternal investment in less than optimal reproductive opportunities. This capacity of oviduct and endometrial cytokines to influence progression of pregnancy can be considered a form of 'cryptic female choice' (Eberhard, 2009) that presumably operates together with other mechanisms of female quality control mediated by the endometrial stroma and immune system (Robertson, 2010; Teklenburg et al., 2010). A role for differential cytokine expression in a stringent quality control process fits with the observations in humans of a high rate of embryo demise before or around implantation (Norwitz et al., 2001).

7.2. Paternal influence on embryotoxic cytokines

In several species, sperm and seminal fluid constituents act to regulate female reproductive tract cytokines (Robertson, 2005; Bromfield et al., 2014). If seminal fluid influences oviduct and uterine cytokine expression, an additional driver of maternal cytokine-mediated quality control might be the strength of seminal fluid signals. This would offer evolutionary value as a mechanism to assess paternal fitness as occurs in Drosophila and other invertebrate species (Gillott, 2003).

In mice, induction of oviduct embryotrophic cytokines GM-CSF, LIF and IL6 only occurs optimally when intact seminal plasma signals are received at mating, and conversely, the embryotoxic cytokine TRAIL is suppressed by intact seminal fluid exposure but not by seminal plasma or sperm alone (Bromfield et al., 2014). In seminal fluid deficient SVX males, a shift in the ratio between embryo survival and apoptosis-inducing factors causes embryo demise, demonstrating that seminal fluid is a key determinant of cytokine balance. Similar effects are demonstrated in bovine oviductal epithelial cells, where sperm act to down-regulate TNF and other proinflammatory cytokines (Marey et al., 2016). Seminal fluid regulation of oviduct and uterine cytokine expression occurs in all mammalian species examined (Schjenken and Robertson, 2014), although in many species expression of embryotoxic cytokines has not been evaluated. These observations raise the prospect that male-female seminal fluid signalling in mammals, as in invertebrates, has an evolutionary benefit in ensuring optimal female reproductive investment and maximal progeny fitness (Eberhard, 2009). Moreover, oviduct expression of embryotoxic cytokines that must be suppressed by seminal fluid signals would constitute an additional mechanism for cryptic female choice, through which seminal fluid quality impacts the cytokine balance to either progress or terminate reproductive investment.

An impact of sperm on cytokine expression in the oviduct and uterus offers potential for male factors to influence fertility in species such as human where seminal fluid is largely retained in the cervix. There is variation between men, and even within men over time, in the capacity for seminal fluid to induce gene expression in female reproductive tract cells (Sharkey et al., 2007; Sharkey et al., 2016), that can be impacted by environmental exposures and infection status (Lotti et al., 2011; Kafka et al., 2012). Seminal fluid capacity to alter female tract embryotoxic as well as embryotrophic cytokines could thus contribute, together with an impact on the female immune response (Robertson and Sharkey, 2016), to variation in fertility in men. Further studies are warranted to investigate how sperm and seminal fluid composition influences oviduct and endometrial cytokines in women.

8. Summary and conclusions

Secreted cytokines have an important role as agents for communication between the female reproductive tract and the embryo. In striving to better understand their properties and functions, it has become evident that secreted factors can exert not just beneficial trophic effects, but also adverse effects on embryo development. Agents including TNF, TRAIL and IFNG, at sufficient concentrations and under specific circumstances that allow death receptor-mediated signalling, cause cell stress and apoptosis. Depending on the strength of signal this can result in embryo demise and implantation failure, or irreversible impact on the developmental program in such a way as to alter the trajectory of fetal and postnatal development. This may be a useful adaptation to resource constraint, but can have adverse impact depending on the postnatal environment. In these ways, the existence of embryotoxic as well as embryotrophic cytokines can mediate a form of maternal quality control that would be evolutionarily beneficial for population health and species survival.

There is evidence that in certain health conditions associated with chronic inflammation, embryotoxic cytokines are elevated in women. If borne out by further studies, this insight will have practical application in assisted reproductive treatments, where embryotoxic cytokines may prove to be a key factor in unexplained infertility. Understanding the biological processes and pathophysiological conditions in which cytokines act to promote or to limit preimplantation development and to influence the phenotype of offspring will provide helpful tools in managing fertility and promoting and protecting reproductive health. More research is warranted to define the full biological significance of embryotoxic cytokines and the environmental and lifestyle factors influencing their synthesis and bioactivity. Future studies will likely explore diagnostic strategies and utility of pharmacological and lifestyle treatment interventions to suppress embryotoxic cytokine production. Additionally it will be important to define the significance of this nexus in embryo programming and as a pathway to intergenerational transmission of metabolic dysfunction and potentially other non-communicable disease conditions.

Conflict of interest

Sarah Robertson is an inventor on patents licenced to ORIGIO A/S.

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