A Novel In Vitro Model of Villitis of Unknown Etiology Demonstrates 1 Altered Placental Hormone and Cytokine Profile

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A Novel In Vitro Model of Villitis of Unknown Etiology Demonstrates Altered Placental Hormone and Cytokine Profile

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Keywords:	T cells, Placental dysfunction, Inflammation, VUE

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1 **A Novel *In Vitro* Model of Villitis of Unknown Etiology Demonstrates Altered Placental**
2 **Hormone and Cytokine Profile**

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7 **Running Title:** Placental inflammation *in vitro*

8 **Summary statement:** Co-culture of human placental explants with matched maternal T-cells
9 elicits an inflammatory reaction that mimics villitis of unknown etiology and detrimentally
10 affects placental function.

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Abstract

Problem: Placental dysfunction is present over 50% of cases of stillbirth and fetal growth restriction (FGR). Villitis of unknown etiology (VUE), an inflammatory condition of the placenta characterised by maternal T cell infiltrates in the villous stroma and dysregulation of inflammatory cytokines, is more frequent in FGR and stillbirth.

Method of Study: A novel *in vitro* model of placental inflammation was developed to test the hypothesis that inflammatory cells seen in VUE and/or cytokines impair placental function.

Results: Co-culture of placental explants with maternal leukocytes resulted in increased leukocytes in villous tissue and elevated concentrations of IL-1 β , IL-1Ra, IL-6, IL-10 and IFN- γ ($p \leq 0.05$). Human chorionic gonadotrophin secretion was reduced following co-culture with leukocytes ($p \leq 0.01$) and cytokines ($p \leq 0.05$).

Conclusions: These observations support the hypothesis that altered placental inflammation has deleterious effects on placental function. This model could be used to further understanding about the pathophysiology of VUE and to test potential therapies.

Keywords: VUE, Inflammation, Placental dysfunction, T cells

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32 **1 Introduction**

33 Placental dysfunction is cited as a cause of death in up to 65% of stillbirths (Flenady et al.,
34 2011; Helgadóttir, 2012). Until recently, investigations of the origins of placental dysfunction
35 have generally concentrated on the implications of failure of spiral artery remodelling as
36 seen in early onset pre-eclampsia and fetal growth restriction (FGR) (Burton et al., 2009).
37 However, interest has grown in the consequences of placental inflammation and its
38 contribution as a cause of stillbirth and FGR. Infectious agents such as cytomegalovirus or
39 toxoplasmosis can lead to placental inflammation, though such cases are usually in the
40 minority (Redline, 2007). More frequently, placental inflammation occurs in the absence of
41 infection (Girard et al., 2014). Villitis of unknown etiology (VUE) is one such inflammatory
42 condition of the villous tissue. As its name suggests, the origin of VUE is unknown, but it is
43 not attributable to infectious agents.

44 VUE occurs in approximately 15% of term placentas, with a higher incidence in FGR
45 (Derricott et al., 2013). Increased severity of inflammation has been linked to stillbirth
46 (Redline, 2007) and it has been reported that VUE may recur in subsequent pregnancies
47 (Redline and Abramowsky, 1985). VUE is characterised by infiltration of the placental villous
48 stroma by maternal CD4 and CD8 T-cells (Kapur et al., 2004; Kim et al., 2008) and expansion
49 of the placental macrophage (Hofbauer cell) population (Derricott et al., 2016; Kim et al.,
50 2008, 2009; Redline and Patterson, 1993; Tang et al., 2011). The mechanisms by which this
51 inflammatory infiltrate negatively affects placental function are unknown. We recently
52 demonstrated altered cytokine protein levels in VUE lesions, including reduced interleukin
53 (IL)-4 and elevated IL-2 and -12, indicating a localised pro-inflammatory environment in
54 these placenta, which could disrupt normal placental viability and function (Derricott et al.,
55 2016).

56 We hypothesised that the presence of maternal CD4 and CD8 T-cells and/or their related
57 cytokines in the villous stroma damages trophoblast, the key functional cell layer, leading to
58 impaired placental function. As VUE is diagnosed on histological examination of the placenta
59 after delivery it cannot be detected before birth. This limits the ability to assess the effects
60 of VUE on placental function using a traditional case-control or cohort study design. In the
current study we developed a novel *in vitro* model of VUE, whereby maternal T cells (CD4

and CD8) were co-incubated with, and infiltrated, villous tissue explants from matched placentas to assess the impact on placental function. We also utilised an established placental explant model to perturb specific cytokine levels, to reproduce the cytokine environment found in VUE *in vivo* (Abumaree et al., 2012; Audette et al., 2010; Benyo et al., 1997; Derricott et al., 2016; Heazell et al., 2008; Simán et al., 2001). In both cases a range of established placental analyses were used to assess the impact on trophoblast viability and placental function including: cell turnover, nutrient transport and endocrine function.

2 Materials and Methods

2.1 Sample Collection

Term (37-42 weeks) placentas (n=14), with matched maternal blood samples (n=8), were obtained within 30 minutes of delivery from women undergoing elective caesarean section. Tissue was donated with informed written consent in accordance with North West NHS REC approval (Ref: 08/H1010/55+5). Placental tissue was taken from uncomplicated pregnancies with the following exclusion criteria: maternal body mass index ≥ 30 , maternal disease (e.g. pre-eclampsia, hypertension, gestational diabetes mellitus), fetal anomalies, small for gestational age (SGA) infants (defined as individualised birthweight centile (IBC) $< 10^{\text{th}}$ centile), or multiple pregnancies.

2.2 Explant Culture with Cytokines

Full thickness villous biopsies of $\sim 1\text{cm}^3$ were randomly sampled from four different areas of placenta and dissected into $4\text{-}5\text{mm}^2$ fragments. An established method of placental explant culture that has been extensively utilised to assess placental viability and function was employed (Audette et al., 2010; Lager and Powell, 2012; Simán et al., 2001). Explants were cultured for 4 days in CMRL-1066 culture medium (Gibco, Paisley, UK) supplemented with 5% heat-inactivated FBS, 100 IU mL^{-1} penicillin, $100\text{ }\mu\text{g mL}^{-1}$ streptomycin, $1\text{ }\mu\text{g mL}^{-1}$ insulin, $0.1\text{ }\mu\text{g mL}^{-1}$ hydrocortisone, and $0.1\text{ }\mu\text{g mL}^{-1}$ retinyl acetate (Sigma Aldrich, Dorset, UK). On day 4, explants were cultured in reduced serum Opti-MEM[®] medium (Gibco, Paisley, UK) and treated with recombinant human IL-2 (rhIL-2, 10 ng mL^{-1}), recombinant human IL-12 (rhIL-12, 10 ng mL^{-1}) (Life Technologies, Paisley, UK) or IL-4 neutralising antibody ($1\mu\text{g/mL}$, R&D Systems, Oxford, UK) individually or all three in combination. Opti-MEM[®] alone served

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91 as the control. Conditioned culture medium was collected after 4 and 6 days of culture. On
92 day 6, explants were processed for analyses of placental viability, cell turnover, nutrient
93 transport, endocrine section and cytokine expression.

94 **2.3 Placental Explant and Maternal T Cell Co-Culture**

95 Villous tissue explants were prepared as above. To enhance the accessibility of villous
96 stroma to T cells, a mild trypsin treatment was carried out to lift areas of
97 syncytiotrophoblast (STB), the endocrine/nutrient transporting epithelium that surrounds
98 the villous stroma, without compromising tissue integrity and function.. Concentration of
99 trypsin and incubation times were optimised by modifying a previously described treatment
100 (Forbes et al., 2008) (see Derricott et al., 2015 for the full protocol). Following trypsinisation,
101 explants were cultured for 24 hours in RPMI 1640 medium (Gibco, Paisley, Scotland)
102 supplemented with 5% fetal bovine serum (FBS), 1 $\mu\text{g mL}^{-1}$ insulin, 100 $\mu\text{g mL}^{-1}$ streptomycin
103 sulphate, 100 IU mL^{-1} penicillin G, 0.1 $\mu\text{g mL}^{-1}$ retinol acetate, 25 $\mu\text{g mL}^{-1}$ L-alanine, 200 μg
104 mL^{-1} L-cysteine and 50 $\mu\text{g mL}^{-1}$ ascorbic acid. After 24 hrs, RPMI culture medium was
105 aspirated and isolated CD4 and CD8 T-cells were applied to each explant in 10 μL
106 suspensions (approximately 10^5 cells per explant). RPMI medium alone was applied to
107 control tissue. After 24 hours, 300 μL medium was added to each well, with a further media
108 change after a further 24 hours. Conditioned medium was collected on days 3 and 4. To
109 ensure T cells remained viable for the duration of the culture, explants were harvested on
110 day 4 and processed for analyses of placental function.

111 **2.4 CD4 and CD8 T Cell Isolation**

112 CD4 and CD8 T-cells were isolated from maternal blood samples with EasySep Human T Cell
113 Isolation Kit (STEMCELL Technologies, Cambridge, UK) following the manufacturers'
114 protocol. CD8 T cells were isolated from peripheral blood mononuclear cells (PBMCs) by
115 positive selection, followed by negative selection of CD4 T cells. Isolated CD4 and CD8 T-cells
116 were incubated for 24 hours on anti-CD3 (BD Pharmingen, Oxford, UK) coated 12-well plates
117 in RPMI 1640 medium with 1 $\mu\text{g mL}^{-1}$ anti-CD28 antibody (BD Pharmingen) and 10 ng mL^{-1}
118 recombinant IL-2 and IL-15 (Gibco, Paisley, UK) to activate and stimulate proliferation of T
119 cells (Derricott et al., 2015).

120 **2.5 CellTracker™ Fluorescence**

121 Cultured CD4 and CD8 T-cells were harvested after 24 hours by application of ice-cold
122 Dulbecco's PBS (without Ca^{2+} and Mg^{2+}) and gentle scraping. The manufacturer's protocol
123 for CellTracker™ fluorescence (Life Technologies, Paisley, UK) was followed to label CD4 T
124 cells red and CD8 T-cells green. Cell suspensions were re-suspended in RPMI supplemented
125 with rhIL-2 and rhIL-15 before application to placental explants. Intracellular fluorescence
126 was verified by viewing cells with a fluorescence microscope. Cell number and viability was
127 assessed by counting using a haemocytometer and staining with Trypan Blue (Sigma Aldrich,
128 Poole, UK).

129 **2.6 Cell Migration**

130 Explants that had been co-cultured with maternal CD4 and CD8 T-cells were fixed in 10%
131 NBF, embedded in OCT and frozen at -80°C . 10 μm sections were mounted with aqueous
132 DAPI-containing mountant and viewed with a Zeiss fluorescence microscope (Zeiss, Welwyn
133 Garden City, UK) to determine the presence of labelled CD4 and CD8 T-cells in the villous
134 stroma.

135 **2.7 Assessment of System A Amino Acid Transporter Activity**

136 Following the culture period, placental explants were incubated in buffer with the
137 radiolabelled amino acid analogue N-methylated aminoisobutyric acid (^{14}C -meAIB) at $0.5 \mu\text{Ci}$
138 mL^{-1} ($8.5 \mu\text{M}$) as previously described (Audette et al., 2010). The incubation buffer was either
139 control Tyrode's or Na^{+} -free Tyrode's (NaCl replaced with equimolar (135 mM L^{-1}) choline
140 chloride. Co-culture explants were incubated for 10, 30 or 60 minutes; explants cultured
141 with cytokines were incubated for 60 minutes only. Following incubation, explants were
142 lysed in dH_2O then denatured in 0.3 M NaOH for assessment of protein content by Bio-Rad
143 protein assay (Bio-Rad Laboratories, Hemel Hempstead, UK). The radioactivity of the explant
144 lysate was determined using a β -scintillation counter. System A is a Na^{+} -dependent nutrient
145 transporter and its activity was calculated by subtracting ^{14}C -meAIB uptake in Na^{+} -free
146 Tyrode's from uptake in control Tyrode's buffer, with adjustment for explant protein
147 content.

148 **2.8 hCG and PlGF Hormone Secretion**

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Conditioned culture medium was assayed for hCG and PlGF concentrations by ELISA (hCG: DRG Diagnostics, Marburg, Germany, PlGF: Duoset ELISA, R&D Systems, Oxford, UK) according to the manufacturers' protocol. Plates were read on a FLUOstar Omega microplate reader (BMG Labtech, Aylesbury, UK), values corrected for explant protein content and expressed as mIU/mL/mg protein (hCG) or pg/mL/mg protein (PlGF).

2.9 Proliferation, Apoptosis, STB Regeneration and Leukocytes in Explants

Proliferation and apoptosis were assessed in formalin-fixed explants using immunostaining and quantification of markers of proliferation (Ki67; Dako, Cambridge, UK) and apoptosis (M30, Roche, West Sussex, UK). STB regeneration was investigated by assessing area of immunostaining for cytokeratin 7 (CK7, Dako). The presence of leukocytes in explants was determined by immunostaining for the cell surface marker CD45 (leukocyte common antigen, (Dako)). A standardised laboratory protocol for immunostaining with colorimetric detection was followed, as described previously (Derricott et al., 2016; Hamilton et al., 2012). Primary antibodies were applied at the following concentrations: CK7 0.9 µg mL⁻¹, Ki67 0.4 µg mL⁻¹, M30 0.01 µg mL⁻¹, and CD45 0.46 µg mL⁻¹. Each tissue section had a corresponding negative control on which mouse IgG (Sigma Aldrich, Dorset, UK) was applied at the same concentration as the primary antibody.

2.10 Image Capture and Analysis

Explant sections were visualised using the x10 objective of an Olympus BX41 light microscope (Southend-on-Sea, UK). Images were captured with QIcam Fast 1394 camera (QImaging, BC, Canada) and Image Pro Plus 6.0 (Media Cybernetics Inc., MD, USA). Quantitative, unbiased quantification of staining area was completed using HistoQuest image analysis software (TissueGnostics, Vienna, Austria) (Chu et al., 2015; Derricott et al., 2016). Image pixels were converted to greyscale (0-256 scale) and assigned an arbitrary number following detection of DAB and haematoxylin and optimisation of staining intensity ranges. Results are presented as histograms or dot plot scattergrams to which staining area and/or intensity thresholds can be applied to differentiate between cell populations.

2.11 Cytokine Analysis in Culture Supernatant and Tissue Lysates (Bio-Plex Assay)

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3 177 Tissue lysates were prepared using a Bio-Plex cell lysis kit (Bio-Rad Laboratories, Hemel
4 178 Hempstead, UK). Tissue culture medium and tissue lysates were analysed for a panel of 10
5 179 inflammation-related cytokines by Bio-Plex assay (Bio-Rad Laboratories, Hemel Hempstead,
6 180 UK) following manufacturers' instructions. The cytokines selected for assay were IL-1 β , IL-
7 181 1Ra, IL-2, IL-4, IL-6, IL-10, IL-12A (p70), IL-17, IFN- γ and TNF- α . Cytokine levels were
8 182 extrapolated from fluorescence values based on supplied standards.

13 14 183 **2.12 Statistical Analysis**

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17 184 A power calculation using prior data on hCG secretion, system A activity and cytokine
18 185 production and secretion from culture in hypoxic conditions or with dexamethasone
19 186 determined that 4-6 samples per group would be required with an 80% power, $\alpha=0.05$
20 187 (Audette et al., 2010; Heazell et al., 2008). Statistical analyses were carried out using
21 188 GraphPad Prism 6.0 software (San Diego, CA, USA). Non-parametric continuous data were
22 189 analysed with Mann Whitney U-test or Kruskal-Wallis test with Dunn's post-hoc test.
23 190 Comparisons between groups of non-parametric data were analysed using Wilcoxon signed
24 191 rank test or Friedman test. Due to considerable variability in the basal values of control
25 192 placentas, ELISA and immunohistochemistry results are presented as fold change from
26 193 control.

27 28 194 **3 Results**

29 30 195 **3.1 An In Vitro Model of VUE Shows Altered Placental Function**

31 196 Representative images of the co-culture explants (n=6) illustrate CD4 and CD8 T-cells in the
32 197 intervillous space, the equivalent of the maternal blood space, and within the villous stroma
33 198 (Figure 1). Flow cytometry demonstrated that positive (CD8) and negative (CD4) antibody
34 199 selection techniques yielded T cell populations with ~91% and ~83% purity respectively. An
35 200 average of ~12,000 cells (range 800-24,500) was applied to each villous tissue fragment,
36 201 with the T cell yield dependent on blood volume obtained. Isolated cells had an average
37 202 viability of 97.9% (range 95.2-99.6%). Immunohistochemistry for CD45 demonstrated a
38 203 significant increase in leukocyte numbers in explants co-cultured with CD4/CD8 T-cells
39 204 compared to controls ($p\leq 0.05$; Figure 2A). Significant increases were detected in the
40 205 concentrations of IL-1 β ($p\leq 0.01$), IL-10 ($p\leq 0.05$), IFN γ ($p\leq 0.05$) and IL-1Ra ($p\leq 0.05$) secreted

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206 into culture medium from explants cultured with CD4/CD8 T-cells on day 3. These
207 differences were diminished by day 4 of culture (data not shown). Significantly greater
208 concentrations of IL-6 ($p \leq 0.01$) and IL-1 β ($p \leq 0.05$) were detected in the lysates of placental
209 explants cultured with T-cells compared to controls after 4 days of culture (Figure 2F,G). No
210 significant differences were detected in IL-2, -4, -12, -17 or TNF α in either culture
211 supernatant or tissue lysate samples.

212 There was a significant decrease in hCG concentrations in conditioned culture medium at
213 day 3 in the explants cultured with CD4/CD8 T-cells compared to those cultured in medium
214 alone ($p \leq 0.01$; Figure 3A). A similar trend was detected for PlGF; however, in this group
215 there was a limited sample size ($n=3$; Figure 3B). No significant differences were recorded in
216 hCG concentrations at day 4. There was no significant difference in system A activity of the
217 placental tissue or in proliferation, apoptosis or STB regeneration/area in the explants
218 cultured with CD4/CD8 T-cells compared to control (Figure 3D-F).

219 **3.2 Explant Culture with VUE-Associated Cytokines Alters Placental Function**

220 There was a significant decrease in human chorionic gonadotropin (hCG) concentrations in
221 conditioned culture medium at day 6 in the explants cultured with rhIL-2, rhIL-12 and IL-4
222 neutralising antibody in combination compared to control ($n=8$, $p < 0.05$) (Figure 4A). There
223 was a trend towards decreased placental growth factor (PlGF) in conditioned culture
224 medium at day 3 in the explants cultured with all treatments, but this did not reach
225 statistical significance ($p=0.16$; Figure 1B). Treatment with individual cytokines did not affect
226 hCG production.

227 Placental system A activity was not significantly affected by independent or combined rhIL-
228 2, rhIL-12 and IL-4 neutralising antibody treatment compared to control (Figure 1C). A
229 significant reduction in proliferation (Ki67 staining) was detected in the explants treated
230 with IL-2 ($p < 0.05$). No significant differences were seen in apoptosis or STB
231 regeneration/area in any of the experimental conditions compared to control (Figure 1D-F).
232 No significant differences were observed in cytokine levels in explant lysates or conditioned
233 culture medium under any of the treatment conditions (Supplementary Figure 1).

234 **4 Discussion**

VUE is a well-documented inflammatory condition of the placenta in late pregnancy and its association with poor pregnancy outcome (particularly FGR) is accepted (Aviram et al., 2010; Derricott et al., 2013; Vedmedovska et al., 2011). Despite these associations, little is known about the mechanisms by which VUE may cause placental insufficiency and adverse pregnancy outcome. Here we present new insights into the possible functional consequences of immune cell interaction with the placenta. This was possible due to the development of a novel *in vitro* model of placental inflammation involving co-culture of placental explants with matched T-cells obtained from maternal peripheral blood samples. We successfully introduced maternal immune cells into normal placental tissue and demonstrated induction of a localised pro-inflammatory response. Furthermore, both the presence of maternal immune cells within the villous core and treatment with VUE-associated cytokines detrimentally affect placental endocrine and/or syncytiotrophoblast function, as identified by the significant reduction in hCG secretion. As well as being a critical placental-derived hormone for the maintenance of pregnancy, hCG is a sensitive marker of syncytiotrophoblast renewal and viability (Evain-Brion and Malassine, 2003; Gude et al., 2004; Omata et al., 2013). These findings were supported by *in vitro* modulation of cytokine levels to mimic the altered cytokine profile detected in VUE lesions (Derricott et al., 2015). Deficiencies in hCG following inflammatory insult therefore indicate adverse effects on syncytiotrophoblast function or integrity. Consistent with this, hCG concentrations in maternal serum are reduced in pregnancy pathologies characterised by placental dysfunction, including intrauterine fetal demise and FGR (Dutton et al., 2012; Haddad et al., 1999; Londero et al., 2013). These findings support the hypothesis that VUE can adversely affect placental function, and provides a potential mechanistic link for the increasing susceptibility to FGR and/or stillbirth.

Increased expression and/or secretion of IL-1 β , IL-1RA, IL-6, IL-10 and IFN- γ were detected from explants co-cultured with maternal T-cells. These data are consistent with our identification of IL-1 as a central mediator of inflammatory-induced placental dysfunction (Girard et al., 2014). Women reporting a reduction in fetal movements (RFM) have a 3-fold increased risk of stillbirth or FGR (Heazell and Frøen, 2008; Warrander et al., 2012); the placentas from these pregnancies exhibit marked syncytiotrophoblast dysfunction, including reduced nutrient transport capacity, accompanied by altered trophoblast proliferation and

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apoptosis, and impaired endocrine function, including a reduction in hCG (Dutton et al., 2012; Warrander et al., 2012). We recently identified elevated placental inflammation in these pregnancies, including increased expression of placental IL-1 family members, in the absence of infection (Girard et al., 2014). In trophoblast, as in other cell types, IL-1 potently stimulates the expression of other pro-inflammatory cytokines, e.g. IL-6 and CCL2, highlighting a coordinating role in mediating placental inflammation (Girard et al., 2015; Kandere-Grzybowska et al., 2003; Parikh et al., 1997). Furthermore, in vitro treatment with IL-1 impairs trophoblast fusion, viability, hCG secretion and system A activity (Girard et al., 2015). In the co-culture model in the current study, elevated concentrations of IL-1Ra were detected in the culture medium; this supports that the elevated IL-1 β produced is functional, as IL-1Ra expression is stimulated as a consequence of IL-1R activation (Girard et al., 2014). These data, therefore, extend *in vivo* evidence of the contribution of placental inflammation to placental dysfunction and adverse pregnancy outcome.

Representative images of isolated T-cells in co-culture confirm that the cells were present in the intervillous space, and a proportion of T cells had migrated across the STB and infiltrated the placental villus. Chronic intervillitis of unknown etiology (CIUE), another inflammatory placental condition, describes the presence of lesions that are confined to the intervillous space (maternal blood space between placental villus structures) and comprise monocytes, CD4 and CD8 T cells of exclusively maternal origin (Capuani et al., 2013). Therefore, this in vitro model provides an overlapping model of VUE and CIUE and has functional relevance, as T-cells in the intervillous space and those in the villous stroma will have distinct interactions with placental cells. In CIUE, interactions will be between immune cells and the microvillous membrane of the syncytiotrophoblast, whereas in VUE immune cells within the villus core can interact with multiple cell types, including trophoblast, stromal cells and placental vasculature. The responses to these situations may vary depending on cell-cell interaction or cytokine receptor localisation on STB, stromal cells, endothelium, monocytes and macrophages. In VUE there is also the potential for interactions between maternal T-cells and fetal macrophages (Hofbauer cells). VUE has been likened to immune rejection; either the mother rejects the semiallogeneic placenta (host-versus-graft) (Redline, 2007; Rudzinski et al., 2013) or maternal T-cells infiltrate the placenta acting in a graft-versus-host manner (Hulthén Varli et al., 2012; Kim et al., 2006). What remains to be elucidated is the manner of

maternal-fetal cell interaction. It is not yet known whether maternal T-cells can be activated by fetal Hofbauer cells or whether they recognise fetal MHC as non-self.

It is perhaps unsurprising that exogenous treatment with VUE-associated cytokines did not completely mirror those changes detected in the co-culture experiments. Culturing explants with cytokines alone would not necessarily reflect a complex inflammatory phenomenon such as VUE. Moreover, the physical presence of maternal immune cells within the placental villus is likely to affect placental cell viability and function. It is highly likely that both the cell and cytokine milieu is ultimately responsible for a cascade of events resulting in placental dysfunction.

It should be noted that all of the placentas used in this study were from uncomplicated, healthy pregnancies in which it is likely that maternal tolerance of the feto-placental unit was well established. In addition, it was not possible to determine the Hofbauer cell population in the placentas prior to culture, a factor which may influence maternal T cell interactions in VUE (Kim et al., 2008). This may be a reason why experimental lesions were not as extensive as those seen in the high grade villitis described in the literature (Redline, 2007; Russell, 1980). Other factors that may have influenced the extent of T cell infiltration include the duration of the culture experiments compared to the duration of a pregnancy and the lack of blood flow in the *in vitro* model. The study was limited by the length of time the co-culture could be sustained. A culture period of 4 days was chosen in order that the viability of the isolated T-cells could be maintained. The cytokine culture experiments could be extended by using a cytotrophoblast culture model rather than an explant culture model, although this would be more representative of the effect of generalised inflammation on trophoblast rather than VUE on the placenta.

If chronic disruption of the syncytiotrophoblast had occurred, then a change in nutrient transport capacity via system A may have been expected. The absence of a significant alteration in system A activity is potentially related to the relatively short exposure to cells/cytokines compared to that *in utero*. In this study, system A was chosen because of its pivotal role in supporting fetal growth; placental system A activity is reduced in FGR pregnancies compared to normal (Glazier et al., 1997) and in pregnancy associated with

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RFM (Warrander et al., 2012). However, there are multiple other nutrient transporter systems present in the placenta that could have been affected.

This study provides the first evidence for functional consequences in the placenta of inappropriate maternal immune responses, by the development and use of a novel *in vitro* model of VUE. As VUE is a post-partum histopathological diagnosis, previous investigations have been limited to descriptive studies, thus the scope for functional interpretation has been limited. Furthermore, although VUE is reported to have an increased risk of recurrence, there is no proposed treatment. The co-culture of placental villous tissue with maternal T-cells demonstrates that the presence of maternal lymphocytes in the placenta, and/or their derived cytokines, adversely affects placental function, which builds on previous evidence highlighting that trophoblast fusion and syncytiotrophoblast renewal is particularly susceptible to placental inflammation. Further use and development of *in vitro* models of VUE and associated placental inflammation has the potential to determine the mechanisms underlying associations between VUE and FGR and stillbirth, and to provide an *in vitro* model to identify and test targets for therapeutic intervention.

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346 **Competing Interests:**

347 No competing interests declared.

348 **Author Contributions:**

349 HD, RLJ, SLG and AEPH conceived and designed the experimental concept. HD and SLG
350 carried out the preliminary studies, HD carried out the experimental work. HD compiled the
351 manuscript, RLJ, SLG and AEPH provided editorial advice.

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	Explant Co-Culture (n=8)	Explant Culture with Cytokines (n=6)
Maternal Age (years)	36.5 (34-45)	32.5 (26-38)
Gestational Age (weeks + days)	39 ⁺¹ (37 ⁺⁴ -39 ⁺²)	38 ⁺⁶ (38 ⁺⁰ -39 ⁺⁰)
Maternal BMI (kg/m ²)	25.59 (19.49-28.48)	26.11 (20.31-28.58)
Gravidity (n)	3 (2-4)	2 (1-3)
Primiparity (%)	62.5%	67%
Birthweight (grams)	3450 (2590-4000)	3292.5 (2840-3630)
IBC	52.55 (6.3-98.2)	55.15 (10.8-66.3)
Gender % male	75%	50%

Table 1: Demographic information of the study participants. Results are median (range).

IBC – individualised birthweight centile, BMI – body mass index at time of booking to antenatal care.

Figure 1: Representative images of explants at day 4 after culture with isolated T-cells. Red arrows highlight CD4 T-cells, green arrows highlight CD8 cells, A, C and D) nuclei stained blue with DAPI. A-C) Isolated maternal T-cells are visible in the villous stroma (V) and intervillous space (IVS). A-B) Scale bar represents 100 μ m, original magnification x200, C) Scale bar represents 20 μ m, original magnification x630 D) explant cultured without isolated cells, scale bar represents 20 μ m, original magnification x630. Dotted lines delineate the perimeters of villi in cross-section where these can be accurately discerned.

398x300mm (150 x 150 DPI)

Figure 2: Effect of explant co-culture with matched maternal CD4 and CD8 T-cells on leukocyte number, cytokine release and tissue cytokines. (A) increased area of DAB+ staining for CD45 leukocytes presented as fold change compared to control (p<0.05). Levels of IL-1 β (B), IL-1Ra (C), IL10 (D) and IFN γ (E) in culture medium were significantly elevated in the presence of CD4/CD8 T-cells compared to control. IL-1 β (F) and IL-6 (G) were significantly higher in the tissue lysates of explants co-cultured with T-cells compared to control. Wilcoxon signed rank test, n=8.

231x300mm (150 x 150 DPI)

Figure 3: The effects of explant co-culture with matched maternal CD4 and CD8 T-cells on placental function and cell turnover. (A) hCG concentration in culture medium, (B) PIGF concentration in culture medium (n=3), (C) system A activity (amino acid transporter). DAB+ staining area presented as fold change compared to control of (D) Ki67 (proliferation), (E) M30 (apoptosis) and (F) cytokeratin 7 (syncytiotrophoblast). Lines represent median. (A, C-E) Wilcoxon signed rank test, n=8 (B) no statistical analysis performed, n=3.

234x235mm (150 x 150 DPI)

Figure 4: The effects of placental explant culture with cytokines and/or neutralising antibodies. (A) hCG concentration in culture medium, (B) PlGF concentration in culture medium, (C) System A activity (amino acid transporter). DAB+ staining area presented as fold change from control: (D) Ki67 (proliferation), (E) M30 (apoptosis) and (F) cytokeratin 7 (syncytiotrophoblast). CTRL: no treatment, IL-2: treatment with 10ng/mL rhIL-2, IL-12: treatment with 10 ng/mL rhIL-12, ANTI-IL4: treatment with 1 mg/mL anti-IL4 neutralising antibody, ALL: treatment with 10 ng/mL IL-2, IL-12 and 1 mg/mL anti-IL4. Lines represent median. (A-B, D-F) Wilcoxon signed rank test, (C) Kruskal-Wallis test, n=6.

267x265mm (150 x 150 DPI)

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2 **Figure S1: The effects of placental explant culture with recombinant cytokines and/or**
3 **neutralising antibodies on cytokine concentrations in tissue lysate and culture**
4 **supernatant samples as measured by Bio-Plex assay. No significant differences were**
5 **observed in levels of (A-B) IL-1β in lysates or culture medium, (C-D) IL-1Ra in lysates or**
6 **medium, (E-F) IL-6 in lysates or medium. CTRL: no treatment, IL-2: treatment with 10 ng mL⁻¹**
7 **recombinant human IL-2, IL-12: treatment with 10 ng mL⁻¹ recombinant human interleukin-**
8 **12, ANTI-IL4: treatment with 1 mg mL⁻¹ anti-IL4 neutralising antibody, ALL: treatment with**
9 **10 ng mL⁻¹ IL-2, IL-12 and 1 ng mL⁻¹ anti-IL4. Friedman test, n=6.**