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Macrophage polarisation affects their regulation of trophoblast behaviour

R.J. Buckley, G.S. Whitley, I.E. Dumitriu, J.E. Cartwright

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1 Macrophage polarisation affects their regulation of trophoblast behaviour

2

3 RJ Buckley, GS Whitley, IE Dumitriu, JE Cartwright*

4 Cardiovascular and Cell Sciences Research Institute, St. George's, University of London, Cranmer
5 Terrace, London, SW17 0RE.

6

7 Author for correspondence: Prof. Judith Cartwright, jcartwri@sgul.ac.uk, 0208 725 0803

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29 Abstract

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31 Introduction: During the first trimester of human pregnancy, fetally-derived extravillous trophoblast
32 (EVT) cells invade into uterine decidua and remodel the uterine spiral arteries to ensure that
33 sufficient blood reaches the maternal-fetal interface. Decidual macrophages have been implicated in
34 the regulation of decidual remodelling and aberrant activation of these immune cells is associated
35 with pre-eclampsia.

36 Methods: The monocytic cell line THP-1 was activated to induce an M1 or M2 phenotype and the
37 conditioned media was used to treat the EVT cell line SGHPL-4 in order to determine the effect of
38 macrophage polarisation on trophoblast behaviour *in-vitro*. SGHPL-4 cell functions were assessed
39 using time-lapse microscopy, endothelial-like tube formation assays and western blot.

40 Results: The polarisation state of the THP-1 cells was found to differentially alter the behaviour of
41 trophoblast cells *in-vitro* with pro-inflammatory M1 conditioned media significantly inhibiting
42 trophoblast motility, impeding trophoblast tube formation, and inducing trophoblast expression of
43 caspase 3, when compared to anti-inflammatory M2 conditioned media.

44 Discussion: Macrophages can regulate trophoblast functions that are critical during decidual
45 remodelling in early pregnancy. Importantly, there is differential regulation of trophoblast function
46 in response to the polarisation state of these cells. Our studies indicate that the balance between a
47 pro- and anti-inflammatory environment is important in regulating the cellular interactions at the
48 maternal-fetal interface and that disturbances in this balance likely contribute to pregnancy
49 disorders associated with poor trophoblast invasion and vessel remodelling.

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52 Keywords: decidua; macrophage; extravillous trophoblast; polarisation; THP-1

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1 INTRODUCTION
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3 Human pregnancy represents a unique immunological paradigm; requiring tolerance of the semi-
4 allogeneic fetus, regulation of placentation, and maintenance of host-defence against pathogens.
5 During the first trimester of pregnancy, the uterine decidua changes significantly as fetally-derived
6 extravillous trophoblast (EVT) cells invade and remodel the uterine spiral arteries, ensuring a
7 sufficient blood supply to facilitate the transfer of nutrients across the maternal-fetal interface (1).
8 Pre-eclampsia is a complication of pregnancy typically characterised by gestational hypertension and
9 proteinuria, and clinically diagnosed after the 20th week of gestation (2). Pre-eclampsia is estimated
10 to affect 2-8% of pregnant women worldwide and is a leading cause of maternal and fetal morbidity
11 and mortality (3). Although the pathophysiology of pre-eclampsia is yet to be fully elucidated,
12 inadequate spiral artery remodelling and shallow trophoblast invasion during the first trimester are
13 associated with the condition (4-6).
14
15 Macrophages are large mononuclear phagocytic cells that predominantly function to clear
16 extraneous cellular material from the interstitial environment but also have a central role in innate
17 and adaptive immunity (7). Given the array of macrophage functions, considerable macrophage
18 diversity and plasticity exists (8). The extremes of activation state are represented by classically
19 activated (CA) macrophages which act as effector cells in immune responses, and alternatively
20 activated (AA) macrophages which are involved in immunosuppression and wound healing/tissue
21 repair. However, specific differentiation depends on the local tissue environment, with evidence that
22 macrophages can switch between activation states when exposed to pro- or anti-inflammatory
23 cytokines (9).
24
25 During the first-trimester of pregnancy, approximately 40% of all decidual cells are leukocytes, of
26 which 70% are decidual natural killer cells and 20-30% are decidual macrophages (10, 11).
27 Histological studies have shown that the population of decidual macrophages remains relatively
28 stable throughout pregnancy as opposed to the population of decidual natural killer cells, which
29 declines as pregnancy progresses (11). Furthermore, decidual macrophages are found in abundance
30 at the site of implantation, clustered around spiral arteries and in close proximity to invading EVT
31 (12-14), suggestive of an important role at the maternal-fetal interface.
32
33 Decidual macrophages have not been extensively characterised though microarray studies have
34 shown that they have a unique phenotype with expression of genes associated with both classical
35 and alternative activation. When compared to peripheral blood monocytes, the majority of
36 upregulated genes are found to be implicated in immune modulation and tissue remodelling,
37 reflecting the phenotype of an AA macrophages (15). DNA methylation profiling of decidual
38 macrophages has demonstrated hypermethylation of genes encoding classical markers of
39 macrophage activation and hypermethylation of genes encoding alternative activation (16).
40
41 However, decidual macrophages also express some genes associated with immune activation, and
42 secrete pro-inflammatory cytokines such as TNF-alpha, in addition to potent anti-inflammatory
43 cytokines such as IL-10 (17, 18). Recent studies have suggested that there may be sub-sets of
44 decidual macrophages characterised by CD11c expression, with a high CD11c expression associated

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45 with lipid metabolism and inflammation, and low CD11c expression associated with extracellular
46 matrix formation, muscle homeostasis, and tissue development (19). ICAM-3 expression has also
47 been correlated with the CD11c expressing sub-populations (20). The expression of genes associated
48 with alternative activation in addition to some genes associated with immune activation likely
49 reflects the need for a tolerogeneic environment to support successful pregnancy while maintaining
50 the potential for an effective inflammatory response against pathogens.
51
52 Aberrant decidual macrophage activation towards a more CA phenotype has previously been
53 associated with the pathology of pre-eclampsia. Term decidua from pre-eclamptic pregnancies has
54 significantly more pro-inflammatory CD86+ macrophages when compared with normal pregnancies
55 (21). In addition, a study of first trimester decidual tissue from chorionic villus sampling found a
56 lower ratio of regulatory CD206/CD86+ macrophages in the decidua of women who subsequently
57 developed pre-eclampsia compared to those with a normal pregnancy outcome. Moreover, there is
58 an increase in decidual macrophage mRNA expression of the pro-inflammatory cytokine IL-6 prior to
59 clinical signs of pre-eclampsia (22), and excess TNF-α production has been postulated to inhibit
60 normal EVT invasion in pre-eclampsia (23).
61
62 The aim of our study was to model the effects of differential macrophage polarisation on
63 trophoblast behaviour. The human acute monocytic leukemia cell line (THP-1) can be polarised to
64 generate macrophage phenotypes at the extreme ends of the polarisation spectrum and were used
65 to generate CA and AA macrophage-like cells. The effect of factors secreted by these cells on a
66 trophoblast cell line was analysed with respect to the motility, proliferation, apoptosis, and
67 formation of network structures.
68
69
70 MATERIALS AND METHODS
71 *Macrophage Differentiation*
72 THP-1 cells were differentiated into macrophage-like cells by adapting a previously described
73 method (24). Briefly, cells were treated with 100nM of phorbol 12-myristate 13-acetate (Sigma-
74 Aldrich, Dorset, UK) in phenol red free Roswell Park Memorial Institute (RPMI) 1640 medium
75 (Invitrogen, Paisley, UK) supplemented with 10% (v/v) fetal bovine serum (FBS), containing 2mmol/L
76 L-glutamine, 100 IU/mL penicillin, 100mg/mL streptomycin and 2.5µg/mL amphotericin B (THP-1
77 medium). After 6h 100ng/mL of lipopolysaccharide (LPS) and 20ng/mL of IFN-γ or 20ng/mL of IL-4
78 (PeproTech, Rocky Hill, NJ) and 20ng/mL of IL-13 (PeproTech) was added to generate an CA or AA
79 phenotype, respectively. The cells were cultured in the polarising media for 3 days and washed
80 thoroughly three times with PBS. The cells were then treated with fresh THP-1 medium containing
81 10% (v/v) FBS or no serum. The conditioned media (CM) was collected after 24h, centrifuged to
82 remove cellular debris, and stored at -80°C until used.
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85 *Characterisation of Macrophage Polarisation*

86 To assess polarisation, the pro-inflammatory cytokines TNF-α and IL-6 (CA markers), or the anti-
87 inflammatory cytokine transforming growth factor beta (TGF-β) (AA marker) were quantified in the
88 CM. For this purpose, Human Duo-set enzyme-linked immunosorbent assay (ELISA) kits (R&D
89 Systems, Abingdon, UK) were used according to the manufacturer's instructions.

90

91 *Trophoblast Cell Line culture*

92 SGHPL-4 cells were derived from primary human first trimester trophoblast and have been used
93 extensively as a model for EVT (25, 26). SGHPL-4 cells express HLA-G and have been shown to
94 respond in a manner similar to primary EVT (Cartwright et al 2002, Harris et al 2006). SGHPL-4 cells
95 were cultured in Hams F10 media supplemented with 2mM L-glutamine, 100 units/ml penicillin,
96 0.1mg/ml streptomycin and 10% (v/v) FBS (SGHPL-4 medium).

97

98 *SGHPL-4 Survival, Proliferation and Motility Assay*

99 To determine whether macrophage polarisation impacts on EVT behaviour, SGHPL-4 cells were
100 treated with polarised THP-1 CM and assessed using time-lapse microscopy. The SGHPL-4 cells were
101 serum-starved overnight (SGHPL-4 medium containing 0.5% (v/v) FBS) prior to treatment with
102 polarised THP-1 CM ($1x10^4$ cells/ml). An Olympus 1x70 inverted microscope (Olympus, Southend-on-
103 Sea, UK) with a Hamamatsu C4742-95 digital camera and motorised stage (Hamamatsu Protonics)
104 and Image-Pro Plus software (MediaCybernetics, Version 4.5) was used to image two positions in
105 each well every 15min for 48h. Forty cells from each treatment were chosen at random and tracked
106 using Image-Pro Insight software. To determine apoptotic cell death, the time frame at which
107 apoptotic morphology became apparent was recorded (a phase bright appearance followed by
108 membrane blebs or blisters (27)). To determine cell proliferation, the time frame at which a cell
109 divided was recorded.

110 SGHPL-4 motility was assessed using Image-Pro Insight software to track the individual trajectory of
111 20 cells chosen at random for each treatment. To explore a possible role for macrophage secreted
112 TNF-α in regulating trophoblast motility, TNF-α was neutralised in the CM using 5µg/ml of Mouse
113 Anti-Human TNF monoclonal antibody with mouse IgG1κ used as an isotype control (BD Pharmingen,
114 Oxford). Previous studies have shown that Mouse Anti-Human TNF monoclonal antibody is capable
115 of neutralising the bioactivity of TNF in CM when used as per the manufacturer's instructions (28).

116

117 *SGHPL-4 Western Blot Analysis*

118 A western blot analysis was undertaken to assess whether SGHPL-4 treatment with THP-1 CM affects
119 the levels of apoptotic marker cleaved caspase 3. SGHPL-4 cells were cultured in polarised THP-1 CM
120 for 24h ($1x10^5$ cells/ml). After 24h the cell lysate was collected and a western blot analysis
121 performed, using 1:1000 rabbit anti-cleaved caspase 3 (Cell Signaling, UK) and 1:10000 mouse anti-
122 α-tubulin (Abcam, Cambridge, UK). Western blots were scanned using an Odyssey Scanner and the

123 density of each band determined. Results are expressed as a ratio to the loading control within each
124 sample.

125

126

127 *SGHPL-4 Network-Formation Assay*

128 As EVT invade towards uterine spiral arteries, they alter their phenotype to become more
129 endothelial-like (29). This can be modelled by assessing the ability of SGHPL-4 to form endothelial-
130 like network structures (30). The effect of the polarised THP-1 CM on the ability of SGHPL-4 cells to
131 form endothelial-like networks on Matrigel™ (BD, Oxford, UK) was assessed using a µ-slide
132 Angiogenesis Assay (Ibidi, Planegg, Germany) according to the manufacturer's instructions. Images
133 were captured as above, and network formation was assessed by measuring the average branch
134 length and counting the total number of individual branches using Image-Pro Insight software.

135

136 *Statistics*

137 Statistical analysis was carried out using GraphPad Prism (Version 6.01). Paired T-tests or repeated
138 measures one-way ANOVAs with post-test multiple comparisons were carried out as stated and
139 statistical significance was assumed at $p<0.05$.

140

141 RESULTS

142 *THP-1 cells can be polarised to reflect an CA or AA phenotype*

143 To confirm THP-1 polarisation, secretion of pro- and anti-inflammatory cytokines by the CA and AA-
144 polarised THP-1 cells was assessed by ELISA (**Figure 1**). The CA CM had a significantly higher
145 concentration of the pro-inflammatory cytokines IL-6 and TNF-α than the AA cells ($p<0.05$). The CA
146 CM contained approximately 60-fold more IL-6 than the AA CM, where levels were only just within
147 the assay detection limit. TNF-α production was over two-fold higher in the CA CM than the AA CM.
148 Conversely, the AA CM contained approximately 5-fold more of the anti-inflammatory cytokine TGF-
149 β ($p<0.001$) than the CA CM.

150

151 *CA macrophages reduce SGHPL-4 motility*

152 SGHPL-4 motility in response to the polarised THP-1 CM was assessed by tracking individual SGHPL-4
153 cells over 48h (**Figure 2**). Cells treated with the CA CM had significantly lower cell motility when
154 compared to the media control and the AA CM ($p<0.01$ and $p<0.01$, respectively). Motility was
155 unaffected by the AA CM when compared with the control. In order to assess whether TNF-α
156 secretion was responsible for the differential effect of the CA and AA CM on the motility of the
157 trophoblast cell line, TNF-α was blocked from the CM but this had no effect when compared to the
158 control IgG1κ.

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159 *Both CA and AA macrophages inhibit SGHPL-4 proliferation*

160 To determine whether THP-1 polarisation state altered SGHPL-4 proliferation, SGHPL-4 cells were
161 monitored by time-lapse microscopy following treatment with CA and AA CM. Treatment with both
162 CA and AA CM almost completely abolished SGHPL-4 cell proliferation compared with the control
163 ($p<0.001$) (**Figure 3A, B**), with an average of 49.5% of the SGHPL-4 cells proliferating in the control
164 compared with 5.5 and 6% of the SGHPL-4 proliferating in the CA and AA CM, respectively.

165

166 *Secreted factors from CA polarised macrophages induce SGHPL-4 apoptosis*

167 Trophoblast apoptosis in response to polarised THP-1 CM was assessed by culturing SGHPL-4 cells
168 with CA or AA CM and monitoring morphological changes by time-lapse microscopy. A significant
169 difference in trophoblast apoptosis was not observed with either treatment ($p>0.05$) (**Figure 3C, D**).
170 However, upon visual inspection morphological differences were consistently observed between the
171 CA and AA treatments. Consequently, a western blot for the apoptotic protein cleaved PARP was
172 undertaken. The CA CM was found to significantly increase the levels of cleaved caspase 3 in SGHPL-
173 4 cells, over that of the control and the AA CM ($p<0.05$) (**Figure 4**).

174

175 *AA macrophages are more able to promote SGHPL-4 network-formation than CA macrophages*

176 The ability of SGHPL-4 to form endothelial-like networks in response to polarised THP-1 CM was
177 assessed. The AA CM promoted the formation of long tube-like structures that appeared to contain
178 many trophoblast cells, whereas the CA CM resulted in the formation of significantly shorter tube-
179 like structures containing fewer cells ($p<0.05$). Branching of the tube-like structures was observed
180 with both the CA and AA CM, although branching was more frequent with the CA CM resulting in the
181 formation of shorter but more numerous and branching tube-like structures ($p<0.01$), and a network
182 resembling a fine mesh of cells with a total length significantly higher than that of the control or AA
183 treatment ($p<0.001$). No, or very few, tube-like structures were observed when SGHPL-4 were
184 culture in the absence of THP-1 CM (**Figure 5**).

185

186

187 DISCUSSION

188 Impaired trophoblast invasion and spiral artery remodelling are associated with complications of
189 pregnancy such as pre-eclampsia. The aim of this study was to determine whether the polarisation
190 status of macrophages can impact upon trophoblast behaviour *in-vitro*. This was achieved by
191 activating and polarising the human leukemic cell line THP-1 to reflect either a mature CA or AA
192 macrophage phenotype and treating a trophoblast cell line with conditioned media (CM) from these
193 cells. The results of this study suggest that macrophages can alter the behaviour of trophoblast cells
194 *in-vitro* and that this behaviour may be differentially affected by polarisation state.

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The mechanisms involved in the regulation of trophoblast invasion and survival are poorly understood. However, when these important functions are impaired, inadequate placentation and remodelling of spiral arteries can occur and are implicated in complications of pregnancy such as pre-eclampsia (4-6). An inverse relationship between reduced trophoblast invasion and macrophage infiltration of the spiral arteries has been reported in pre-eclampsia (14, 21), however, the polarisation status of these macrophages was unknown. In this study, when we exposed a trophoblast cell line to CA CM a significant inhibition of motility was observed when compared to the AA CM or control medium. This suggests that decidual macrophage polarisation towards a more pro-inflammatory phenotype may contribute to impaired migration of trophoblast impacting on their ability to interact with cells of the spiral artery.

Macrophages are primary producers of TNF-α, a pro-inflammatory cytokine belonging to a superfamily of soluble TNF ligands with diverse functionality (31). TNF-α is known to have a role in cellular apoptosis, proliferation, and motility (32) and is implicated in the pathology of pre-eclampsia. For example, studies have shown that serum concentrations of TNF-α are significantly elevated in women with pre-eclampsia compared to normotensive controls (33). Furthermore, TNF-α has previously been shown to inhibit EVT migration and invasion as a result of plasminogen activator inhibitor (PAI)-1 induction (18, 34). Therefore, we investigated whether TNF-α was the factor responsible for the differential effect of the CA and AA CM on EVT motility. However, blocking TNF-α was not found to alter the CA effect suggesting that additional mechanisms may influence trophoblast motility in response to macrophage polarisation.

Decidual macrophages secrete a plethora of additional soluble factors some of which are known to influence trophoblast invasion and migration. For example, they are known to secrete vascular endothelial growth factor (VEGF) and IL-10 both of which are thought to have an inhibitory effect on trophoblast motility (35, 36). They also produce a number of soluble factors that have been shown to promote trophoblast motility such as IL-1β and IL-8 (37, 38). It is likely that a combination of inhibitory and promotional factors is responsible for macrophage regulation of trophoblast motility, and it is possible that the balance is tipped towards inhibition with CA polarisation.

Apoptosis is an important mechanism in normal placental development and decidual remodelling, although excessive apoptosis may play a role in the pathology of pre-eclampsia (39). When we investigated the effect of macrophage polarisation on EVT apoptosis by time-lapse microscopy no significant difference above basal levels was observed with either the CA or AA treatment. However, differences in SGHPL-4 morphology between the treatments were observed. When SGHPL-4 protein levels of cleaved caspase 3 were investigated, the CA treatment was found to significantly up-regulate this apoptotic marker. It is likely that these results reflect the stage at which apoptosis was assessed; caspase 3 is an intracellular protein with a central role in the apoptotic cascade whereas morphological analysis of membrane integrity through time-lapse assesses the terminal stage of apoptosis. This observation correlates with previous studies that have shown an association between the number of activated macrophages and apoptosis in other EVT cell lines (40).

During the first trimester of pregnancy, EVT migrate in columns from the placental villi into the decidua. EVT at the tips of these columns subsequently detach and differentiate to become either interstitial or endovascular trophoblast which, as they differentiate, exit the cell cycle and cease proliferating (41, 42). In our study treatment with both the CA and AA CM was found to abolish

237 SGHPL-4 proliferation. Given these observations, it is possible that macrophages within the decidua
238 are capable of influencing EVT differentiation towards a less proliferative, more invasive,
239 endovascular phenotype, irrespective of macrophage phenotype. It will be important to confirm this
240 with isolated primary first trimester trophoblast cells.

241 Spiral artery remodelling occurs during the first half of human pregnancy and results in replacement
242 of the endothelium and vascular smooth muscle cells by invasive endovascular EVT (41). This
243 requires endovascular EVT to adopt a vascular phenotype (29) and SGHPL-4 cells have previously
244 been shown to form tube-like networks when seeded on Matrigel™ (30, 43). Decidual macrophages
245 secrete a range of angiogenic growth factors including angiogenin, keratinocyte growth factor,
246 fibroblast growth factor B, vascular endothelial growth factor A, and angiopoietin-1 and -2 (44). Both
247 the CA and AA macrophage CMs were found to induce network formation however the AA CM
248 induced formation of significantly longer tube-like structures comprised of many cells in close
249 contact, whereas the CA CM produced shorter, branching structures, which were morphologically
250 less organised and contained fewer cells. AA macrophages have been shown to express fewer angio-
251 inhibitory cytokines than classically activated MØ (45). Our results may reflect a requirement for
252 tissue remodelling AA-like decidual macrophages during the co-ordinated changes to spiral arteries,
253 and suggests that the presence of inflammatory CA-like decidual macrophages may lead to
254 inefficient remodelling.

255 By using THP-1 cells to model the extremes of macrophage polarisation we have clearly shown that
256 macrophage phenotype can affect trophoblast behaviour. THP-1 cells are widely used to model
257 human monocytes and macrophages, and have been well characterised (46). PMA is commonly used
258 to activate THP-1 cells (47-49) and LPS and IFN-γ, and IL-13 and IL-4, frequently used to generate CA-
259 and AA-like cells, respectively, in order to mimic the extremes of macrophage polarisation (24, 50,
260 51). In future studies it would be interesting to determine the effect of different stimulatory
261 conditions on primary decidual macrophages and their interactions with trophoblast.
262
263 *In-vivo,* it is unlikely that decidual macrophages can be defined as either CA or AA with increasing
264 evidence that they have a unique phenotype. For instance, gene expression profiling has
265 demonstrated that first trimester decidual macrophages resemble AA macrophages when compared
266 to peripheral blood $CD14^+$ monocytes, with up-regulation of genes implicated in immune modulation
267 and tissue remodelling, but also upregulate genes corresponding to an CA phenotype (15).
268 Furthermore, it is possible that there are different macrophage populations within the decidua, in
269 response to the local microenvironment. For example, two distinct subsets of $CD14^+$ decidual
270 macrophages have been characterised by their level of CD11c expression (19) and intercellular
271 adhesion molecule 3 (ICAM-3) expression (20). Therefore, in future studies it will be important to
272 investigate whether decidual macrophage activation is location-specific and to determine how this
273 contributes to the regulation of neighbouring cells, in addition to addressing whether this is different
274 in pre-eclamptic pregnancies.
275
276 Although further *in-vivo* studies are required, our study demonstrates that macrophage polarisation
277 can affect the behaviour of a trophoblast cell line *in-vitro* and is likely to have an important role in
278 the regulation of placental development. Furthermore, macrophage polarisation towards a more
279 pro-inflammatory phenotype may be one of the mechanisms responsible for the shallow
280 placentation and impaired spiral artery remodelling observed in pre-eclampsia.

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282 *Author's roles*

283 R.JB., I.E.D., G.S.W. and J.E.C. designed the experiments. R.B. carried out all of the experiments. The
284 manuscript was prepared by R.B. and J.E.C. and all authors critically revised the manuscript and
285 approved the final version.

286

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1 **Figure 1. Quantification of IL-6, TNF-α and TGF-β1 in the CM of THP-1 cells exposed to PMA and**
2 **polarising factors.** THP-1 cells ($4x10^6$) were activated in 6ml of THP-1 medium with 100nM PMA and
3 classically or alternatively activated with 100ng/ml of LPS and 20ng/ml of IFN-γ or 20ng/ml of IL-4
4 and 20ng/ml of IL-13, respectively. After 72h the polarising media was replaced with fresh THP-1
5 medium without polarising factors. The CM was collected after 24h and the concentration of IL-6,
6 TNF-α and TGF-β assessed by ELISA and the treatments compared statistically using a paired T-test.
7 **A)** The concentration of IL-6 (pg/ml), n=9, *p<0.05. **B)** The concentration of TNF-α (pg/ml), n=9,
8 *p<0.05. **C)** The concentration of TGF-β1 (pg/ml), n=6, ***p<0.001. Data is presented as mean +
9 SEM.

10

11 **Figure 2. SGHPL-4 motility when treated with polarised THP-1 CM.** SGHPL-4 cells ($1x10^4$/ml) were
12 serum-starved with SGHPL-4 medium containing 0.5% (v/v) FBS for 18h and subsequently treated
13 with 1ml of CA or AA CM, or THP-1 medium as a control. The cells were imaged using time-lapse
14 microscopy for 48h. Motility is presented as an average of 20 cells per treatment in arbitrary units.
15 The treatments were compared statistically using a repeated measures ANOVA and Tukey's multiple
16 comparisons test**. A)** CA CM significantly decreased the motility of trophoblast, n=5 experimental
17 repeats, **p<0.01. **B)** THP-1 CM was treated with a neutralising antibody against TNF-α (anti-TNF) or
18 a control IgG1κ. TNF-a neutralisation had no effect on trophoblast motility, n=4 experimental
19 repeats, *p<0.05. Data is presented as mean + SEM.

20

21 **Figure 3. SGHPL-4 proliferation and cell death when treated with polarised THP-1 CM.** SGHPL-4
22 cells ($1x10^4$/ml) were serum-starved with SGHPL-1 medium for 18h and subsequently treated with
23 1ml of CA or AA CM, or THP-1 medium as a control. The cells were imaged using time-lapse
24 microscopy for 48h and cell morphology observed for division or apoptotic changes. The treatments
25 were compared statistically using a repeated measures ANOVA and Tukey's multiple comparisons
26 test, n=5 experimental repeats. **A)** A significant difference in proliferation was found between the
27 treatments and the control, ***p<0.001. **B)** Proliferation over time. **C)** No significant differences in
28 apoptosis were found between the treatments, p>0.05. **D)** Apoptosis over time. Data is presented as
29 mean + SEM.

30

31 **Figure 4. SGHPL-4 expression of cleaved caspase 3 (17kDa) when treated with polarised THP-1 CM.**
32 SGHPL-4 cells were incubated with CA CM, AA CM or control media for 24h, total protein collected,
33 and western blot analysis of cleaved caspase 3 undertaken. The treatments were compared
34 statistically using a repeated measures ANOVA and Tukey's multiple comparisons test, n=6
35 experimental repeats. **A)** Representative western blot analysis of cleaved caspase 3 and tubulin. **B)**
36 SGHPL-4 cell incubation with CA CM significantly upregulates expression of cleaved caspase 3
37 (17kDa) when compared with AA CM or THP-1 control medium, **p<0.01. Data is presented as
38 mean + SEM. **C)** Representative morphology of SGHPL-4 cells treated with CA CM, AA CM or control
39 media. Original magnification: 10X.

40

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41 **Figure 5. SGHPL-4 endothelial-like network formation when treated with polarised THP-1 CM.**
42 SGHPL-4 cells were cultured on Matrigel^{TM} in the presence of polarised THP-1 CM, or THP-1 medium
43 as a control, to induce endothelial-like network formation, and imaged after 24h. The treatments
44 were compared statistically using a repeated measures ANOVA and Tukey's multiple comparisons
45 test, n=4 experimental repeats. **A)** The total number of branches was significantly increased in the
46 CA CM compared with the AA CM and control (**$p<0.01$). **B)** The average length of branch structures
47 was significantly increased in response to the AA CM when compared with the CA CM and the
48 control (**$p<0.01$). Original magnification: 4X. Data is presented as mean + SEM.

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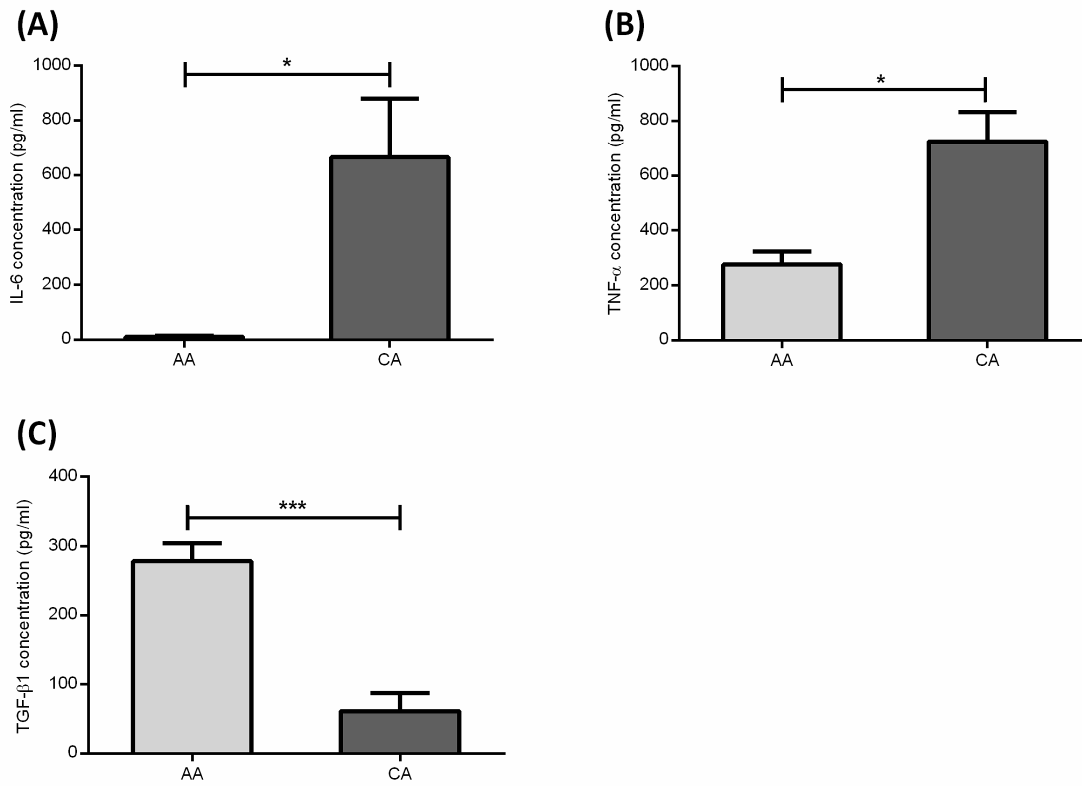

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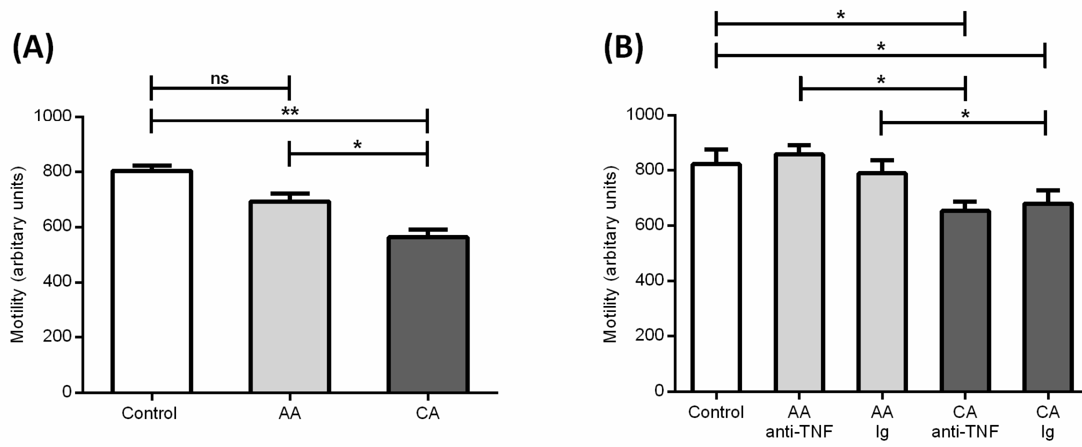

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Figure 3

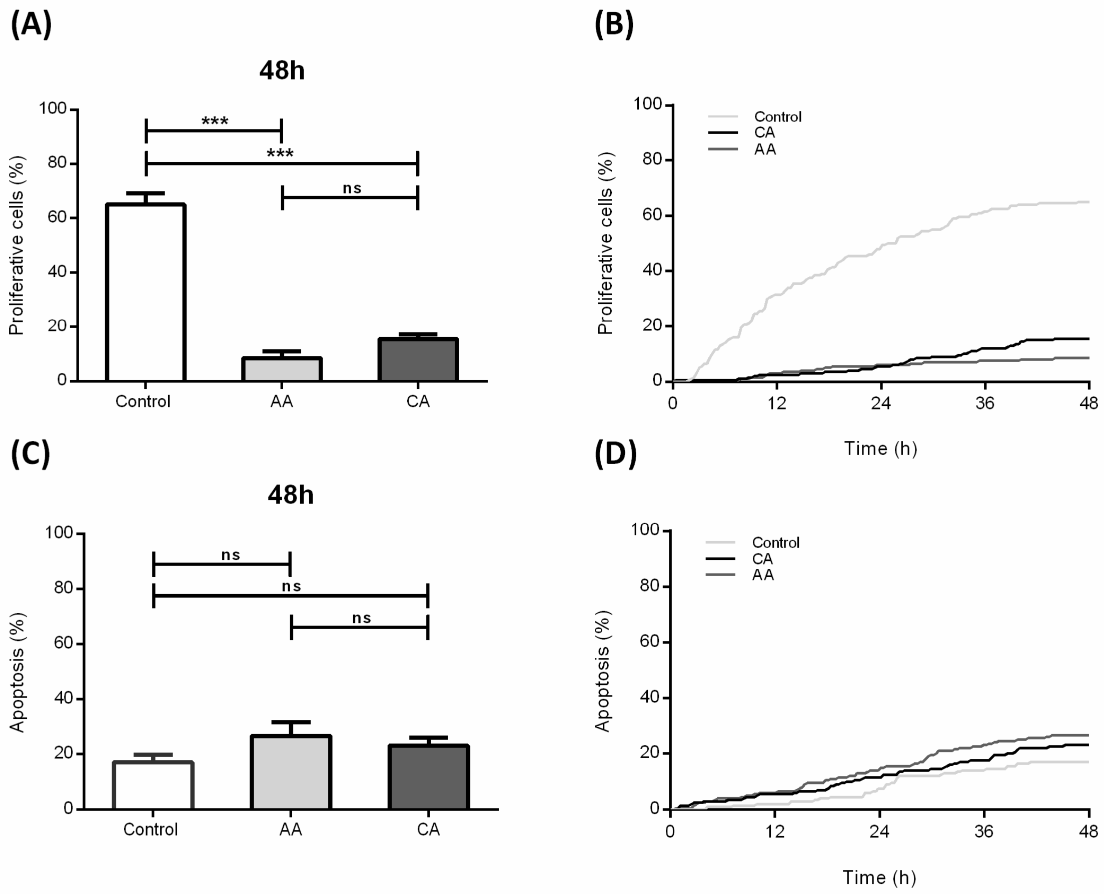

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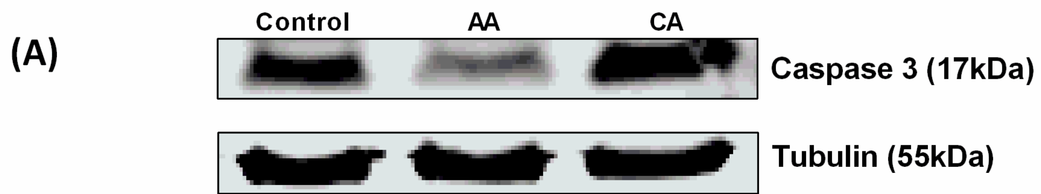
Figure 4

(A)



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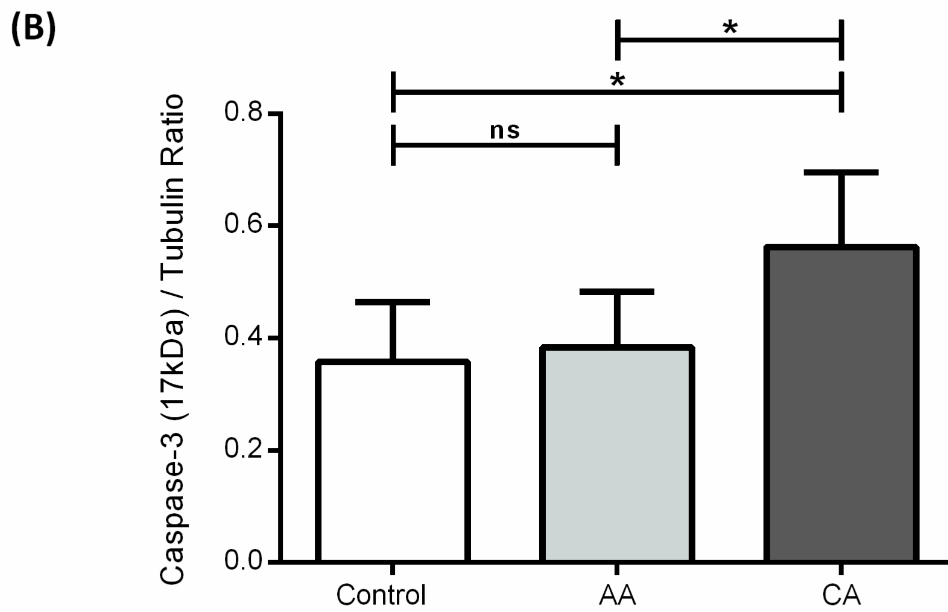

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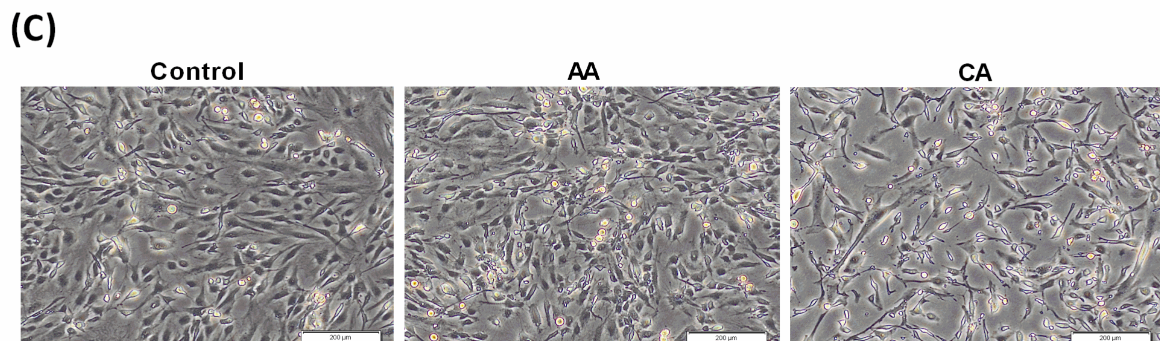

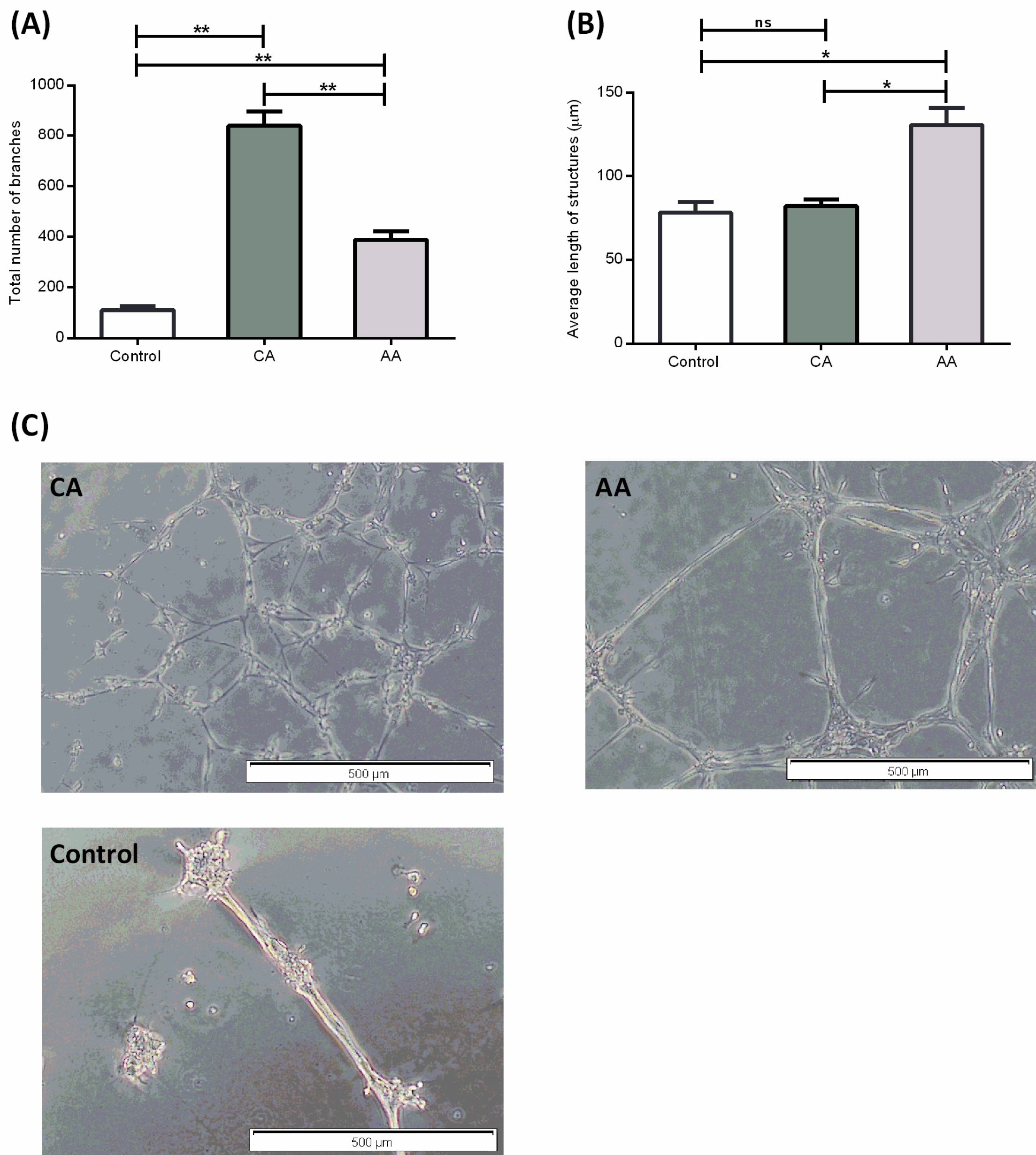
Figure 5
(A)
**
**
**
1000
800
600
400
200
0
Total number of branches
Control
CA
AA
(B)
ns
*
*
150
100
50
0
Average length of structures (µm)
Control
CA
AA
(C)
CA
500 µm
AA
500 µm
Control
500 µm

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Highlights

- Extravillous trophoblast motility is inhibited by classically activated macrophages.
- Alternatively activated macrophages support trophoblast network-formation.
- Macrophage polarisation can affect important trophoblast functions.
- Alterations in macrophage phenotype may impair trophoblast decidual remodelling.

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