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Increased placental macrophages and a pro-inflammatory profile in placentas and maternal serum in infants with a decreased growth rate in the third trimester of pregnancy

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Abstract

Problem: There is growing evidence for the role of placental inflammation in the pathophysiology of pregnancy complications including fetal growth restriction (FGR). This study aimed to characterize the inflammatory profile in the maternal circulation and the placenta of infants who were growth restricted and those that were small for gestational age (SGA).

Method of study: Placental villous tissue and maternal serum were obtained from pregnancies where infants were SGA at birth or who had a decreasing growth rate (≥25 centiles) across the third trimester. Immunohistochemical and histological analyses of placental samples were conducted for macrophage number, alongside vascular and cell turnover analysis. Inflammatory profile was analyzed in maternal and placental compartments via ELISAs and multiplex assays.

Results: There were significantly more $CD163^+$ macrophages in placentas of infants with a decreased growth rate compared to controls, but not in SGA infants (median 8.6/ nuclei vs 3.8 and 2.9, $P = .008$ and $P = .003$, respectively). Uric acid ($P = .0007$) and IL-8 ($P = .0008$) were increased in placentas, and S100A8 ($P < .0002$) was increased in maternal serum of infants with decreased growth rate. No changes in the maternal serum or placental lysates of SGA infants were observed.

Conclusion: The evidence of an altered inflammatory profile in infants with a decreasing growth rate, but not in those that were born SGA, provides further evidence that inflammation plays a role in true FGR. It remains unclear whether the increased placental macrophages occur as a direct result, or as a consequence of the pro-inflammatory environment observed in fetal growth restriction.

KEYWORDS

fetal growth restriction, inflammation, macrophage, placenta, serum

1 | INTRODUCTION

Placental macrophages, also termed Hofbauer cells in humans, are fetal in origin and are found in the stromal core of placental villi. Evidence to date has suggested that they are M2-like, or anti-inflammatory, a phenotype which would promote tissue repair.[1,2] Hofbauer cells are thought to be pro-angiogenic, through their production of VEGF and FGF2,[3] and are also proposed to play a role in clearing apoptotic cells.[4] The number of Hofbauer cells increases up to the middle of the second trimester, but then decreases in the third trimester.[5] There is a growing body of evidence which reports increased numbers of Hofbauer cells in pregnancy complications, including stillbirth, small for gestational age (SGA) infants with a birthweight <10th percentile,[6] HELLP syndrome,[7] women who are obese,[8] and women with diabetes mellitus.[9] These observations support a role for placental inflammation in the pathophysiology of late pregnancy complications.

Fetal growth restriction (FGR) describes the failure of a fetus to reach its growth potential.[10] However, definitions of FGR vary between clinical studies potentially affecting the observations obtained. Three commonly used definitions are a birthweight, or estimated fetal weight of ≤3rd, ≤5th or ≤10th percentile, often combined with evidence of abnormal uterine or umbilical artery blood flow as assessed by Doppler ultrasound.[11-13] However, in many studies, these definitions are based on birthweight in isolation and do not take into consideration the growth trajectory in utero. Thus, they do not distinguish those infants with growth restriction and those that are constitutionally small. Due to these considerations, various other criteria, such as decreased growth rate, have been described following a Delphi consensus exercise.[11]

Understanding the pathophysiology of FGR is important as it is the single strongest risk factor for stillbirth in high-income countries,[14] such as the UK, where the population attributable risk is 46%.[15] A better understanding of processes underlying FGR would inform initiatives to reduce perinatal mortality and potentially therapy. Aberrant cell turnover, placental dysfunction, and a pro-inflammatory phenotype have previously been identified in cases of SGA, FGR, reduced fetal movements (RFM), and stillbirth.[6,16-18] However, these data span definitions ranging from a birthweight ≤3rd to ≤10th percentile, which increases the likelihood of including infants who are constitutionally SGA rather than FGR. An inflammatory response has been characterized in the placenta without any sign of obvious infection.[19] Damage-associated molecular patterns (DAMPs), also known as alarmins, such as uric acid and HMGB1 are elevated in the placentas and maternal blood of pregnancies complicated with FGR and RFM.[17,19]

Thus, this study aimed to characterize placental and maternal inflammation in uncomplicated pregnancies, those in which the infant was SGA (defined by a specific percentile threshold) and infants with evidence of reduced fetal growth rate in the third trimester. We hypothesized that inflammation would be increased in both groups of fetuses with abnormal growth but would be most pronounced in those with evidence of reduced fetal growth rate.

2 | METHODS

To address this hypothesis, the following groups were studied: pregnancies ending in the birth of an infant with an individualized birthweight centile (IBC) ≤3rd percentile and pregnancies with evidence of a decreased growth rate defined as a fall in ≥25 percentiles between a third trimester estimated fetal weight by ultrasound scan and birth. As routine third trimester scanning is not performed in our maternity unit, samples were obtained from women who underwent ultrasound scanning for RFM and were found to have a decreased growth rate (n = 14). Consequently, two control groups were used as follows: i) women who presented with RFM but who were ±5 percentiles from their third trimester EFW (n = 12) and ii) those who had not presented with RFM (20th-90th IBC; n = 12).

For placental analysis, those with a ≤3rd IBC (n = 16) were compared to samples with similar demographic characteristics: maternal age, maternal BMI, maternal smoking status, maternal ethnicity, mode of delivery, and fetal sex to appropriate for gestational age (between 20 and 90th percentile) healthy infants (n = 18). Due to the availability of samples, maternal serum analysis used samples with ≤5th IBC (n = 23) which were matched using the characteristics above with healthy controls (n = 22). Samples from infants with a decreased growth rate (n = 12) were matched with those who presented with RFM but who were ±5 percentiles from their third trimester EFW (n = 8) and those who had not presented with RFM (n = 12).

All infants were live-born ≥37 weeks' gestation, and the mothers were free of other pregnancy complications such as diabetes mellitus and preeclampsia. All samples were free from signs of maternal infection. All samples were collected following written, informed consent in accordance with research ethics approvals (08/H1011/83, 11/NW/0650, 11/NW/0664, and 18/NW/0451).

2.1 | Histology and immunohistochemistry

Three randomly sampled sections of placenta were fixed, embedded into wax, and cut into 5 µm sections. Sections underwent histochemical or immunohistochemical staining following a standard protocol as previously described,[6,18] using antibodies raised to immune cell markers CD163 (EDHu-1, Bio-Rad, 5 µg/mL) and CD45 (2B11 + PD7/26, 1.85 µg/mL, Dako) and structural and morphological markers for apoptosis (M30 CytoDEATH 1.1 µg/mL, Roche), proliferation (Ki67 MIB-1, 184 ng/mL, Dako), vascularity (CD31 JC70A, 4.1 µg/mL, Dako), followed by DAB (Sigma-Aldrich) and counterstained with Harris Hematoxylin Solution (Sigma-Aldrich). Syncytial nuclear aggregates (SNAs) were stained for with hematoxylin and eosin (Sigma-Aldrich).

Slides were imaged using a Pannoramic 250 slide scanner (3D Histech) or 10 fields of view per section (CD31 and syncytial knot analysis). Analysis for CD163, CD45, Ki67, and M30 was performed using QuPath (0.1.2). CD163- and CD45-positive immune cells were calculated as a percentage of positive cells per number of nuclei.

TABLE 1 Patient demographics for IBC ≤3rd percentile, decreased growth rate, and controls for placenta sample analysis

Demographics	IBC ≤ 3rd percentile	Control	*P value*	Reduced fetal movement (RFM)			Decreased Growth Rate vs All Controls *P value*	RFM control vs normal control *P* value
				Decreased Growth Rate	Control	Control		
Number	16	18		n = 14	n = 12	n = 12		
Gestation (d) median (range)	267 (260-278)	273 (261-282)	.02	277.5 (265-292)	282.5 (267-290)	274.5 (264-285)	n/a	n/s
Maternal age (y) median (range)	28 (18-38)	29.5 (23-39)	n/s	29.5 (18-36)	31 (21-38)	25.5 (20-37)	n/s	n/s
Maternal BMI (kg/m^2) median (range)	23.77 (18.65-29.46)	24.79 (19.36-29.74)	n/s	23.(19.43-29)	24 (20-26)	23.7 (21-28.72)	n/s	n/s
Ethnicity								
Caucasian	10 (62)	12 (67)	n/s	9 (64)	8 (67)	8 (67)	n/s	n/s
Non-Caucasian	6 (38)	6 (33)		5 (36)	4 (33)	4 (3)		
Birthweight (g) median (range)	2155 (942-2470)	3317 (2840-3910)	.0001	3055 (2454-3420)	3598 (2988-4140)	3260 (2840-3910)	.0052	n/s
IBC median (range)	0.9 (0-2.8)	54.05 (20.2-80.4)	.0001	16.1 (3.5-59)	56.65 (25.6-89.7)	42.14 (29.68-80.6)	.0011	n/s
Smoking status								
Smokers	5 (31)	4 (22)	n/s	0 (0)	0 (0)	2 (17)	n/s	n/s
Sex								
Male	10 (62)	8 (44)	n/s	7 (50)	4 (33)	4 (33)	n/s	n/s
Female	6 (38)	10 (56)		7 (50)	8 (67)	8 (67)		
Mode of Delivery								
NVD	5 (31)	4 (22)	n/s	14 (100)	9 (75)	10 (83)	n/s	n/s
C/S	11 (69)	14 (78)		0 (0)	3 (25)	2 (17)		

Note: Statistics: Mann-Whitney or Kruskal-Wallis as appropriate. Non-categorical data Fisher's exact test.

Significance level $P \leq .05$.

Abbreviations: C/S, caesarean section; IBC, individualized birthweight centile; NVD, normal vaginal delivery.

TABLE 2 Patient demographics for IBC ≤ 5th percentile, decreased growth rate, and controls for maternal serum sample analysis

Demographics	IBC ≤ 5th Percentile	Control	*P* value	Reduced fetal movement (RFM)			Decreased growth rate vs all controls *P* value	RFM control vs Normal control *P* value
				Decreased Growth Rate	Control	Control		
Number	23	22		12	8	12		
Gestation (days) median (range)	273 (260-293)	283.5 (266-294)	.015	283 (266-293)	283.5 (267-289)	278.5 (271-294)	n/s	n/s
Maternal Age (yr) median (range)	35 (21-40)	28.5 (18-39)	.0366	27.65 (21-36)	31 (21-35)	28 (20-39)	n/s	n/s
Maternal BMI (Kg/m^2) median (range)	24.5 (19.1-30.47)	23.1 (19-30.49)	n/s	23.37 (19.43-28.2)	24.5 (21-26)	23.5 (21-27.5)	n/s	n/s
Ethnicity								
Caucasian	18 (78)	17 (77)	n/s	9 (75)	7 (87)	10 (83)	n/s	n/s
Non-Caucasian	5 (13)	5 (23)		3 (25)	1 (13)	2 (17)		
Birthweight (g) median (range)	2548 (1900-3190)	3415 (2850-3780)	<.0001	3064 (2454-3461)	3640 (3100-4140)	3300 (3060-3812)	.0017	n/s
IBC median (range)	3.047 (0.03-5.0)	38.15 (20.7-88.3)	<.0001	14.65 (3.5-48.2)	50 (26-89.7)	44.89 (20.7-80.57)	.0003	n/s
Smoking status								
Smokers	10 (44)	9 (41)	n/s	0 (0)	0 (0)	2 (17)	n/s	n/s
Sex n (%)								
Male	13(56)	11 (50)	n/s	6 (50)	3 (37)	4 (33)	n/s	n/s
Female	10 (44)	11 (50)		6 (50)	5 (63)	8 (67)		
Mode of Delivery								
NVD	16 (70)	20 (91)	n/s	12 (100)	6 (75)	11 (92)	n/s	n/s
C/S	7 (30)	2 (9)		0 (0)	2 (25)	1 (8)		

Note: Statistics: Mann-Whitney or Kruskal-Wallis as appropriate. Non-categorical data Fisher's exact test.

Significance level $P \leq .05$.

Abbreviations: C/S, caesarean section; IBC, individualized birthweight centile; NVD, normal vaginal delivery.

Ki67 and M30 were also calculated as a percentage of positive cells per number of nuclei.[6]

Vascular and SNA analyses were performed blinded to study group by either MS or HB. Vascularity was determined by calculating the average number of vessels, number of villi, number of avascular villi, and total percentage of avascular villi in 10 fields of view per placental section and averaged for each placenta.[18] Villi were excluded if they were too large to be intermediate or terminal villi. The definition used to identify SNAs was a group of 10 or more nuclei protruding from the villous surface, not in direct contact with adjacent villi and normalized to the villous area.[20]

2.2 | ELISA and multiplex assays

For the detection of both pro- and anti-inflammatory cytokines and DAMPs, ELISAs (DUOSET, R&D Systems, uric acid QuantiChrom, BioAssay Systems, and HMGB1, IBL International) or multiplex assay (BioPlex, Pro Human Cytokine Human Group 1 Panel 17-Plex; Bio-Rad) was performed on placental lysates and maternal serum samples. The complete set of ELISAs performed is in Table S1.

Placental lysates were made from snap-frozen placental tissue. Briefly, two 30mg pieces of villous tissue were homogenized (Stuart SHM1 Homogeniser, 5-20 mm generator, Bibby Scientific, Stone, UK) in Protease Inhibitor Cocktail (Set 1, Calbiochem, Merck Millipore). Homogenates were centrifuged at 12 500 *g* at 4°C for 10 minutes and the supernatants frozen at −80°C until use. The protein content of each sample was determined using a Pierce™ BCA Protein Assay (Thermo Scientific) according to the manufacturer's instructions.

For the Duoset ELISAs, 96-well plates were coated with the capture antibody and left overnight at room temperature. Plates were washed three times in PBS containing 0.05% Tween-20 (wash buffer). Plates were blocked in 1% BSA in PBS for two hours before three washes in wash buffer. Standard curves were added to each plate. Samples were diluted to fit on the standard curve and were added in duplicate, before incubating on a rocker overnight. The plates were thoroughly washed prior to applying detection antibody for two hours, followed by detection with Streptavidin-HRP and substrate solution. The plates were read on a microplate reader at 450 and 570 nm. HMGB1 and uric acid ELISAs were conducted following the manufacturer's instructions. Samples with undetectable levels of cytokines or DAMPS were assigned a value of half the lower limit of detection. ELISA results were corrected using calculated inter-plate calibrators, and the intra-assay coefficient of variation was <10% for all assays.

2.3 | Multiplex protocol

Multiplex assay (BioPlex, Pro Human Cytokine Human Group 1 Panel 17-Plex; Bio-Rad) was performed using a panel of 17 human cytokines (see Table S1) on maternal serum samples according to the manufacturer's instructions. Samples were diluted to fit onto the standard curve.

2.4 | Statistical analysis

Statistical analysis was conducted using GraphPad Prism (7.04). Normality tests were performed, and Mann-Whitney or Kruskal-Wallis, with Dunn's post hoc tests, was performed as appropriate for normally and non-normally distributed data. For non-categorical data, Fisher's exact test was used. Using data from previous studies which found increased numbers of $CD163^+$ macrophages in FGR compared to controls,[6] we calculated that 13 samples would be required to demonstrate a statistically significant difference for 80% power and $\alpha = .05$. The significance level was set at $P < .05$. To reduce the chance of a false-positive discovery by false-positive results (type 1 error) with measurements of multiple cytokines in placental lysates and maternal serum, a Bonferroni correction was used to reduce the p-value threshold due to the number of comparisons ($P < .004$ and $P < .003$, respectively).

3 | RESULTS

The full demographics of the participant's samples used for placental immunohistochemistry and cytokine and DAMPs analysis are in Table 1. There were no significant differences between maternal age, maternal BMI, ethnicity, smoking status, mode of delivery or whether labor was induced, between any of the growth-restricted infants and their controls. By design, the infants with an IBC ≤3rd percentile and those with a decreased growth rate had statistically lower birthweights ($P < .0001$ and $P = .005$) and IBCs ($P < .0001$ and $P = .001$) compared to their respective controls. Those with an IBC ≤3rd percentile were born at a lower median gestation than controls (267 vs 273 days, $P = .02$); however, this is within the range of a term birth (259-294 days).

The full demographics of participant's samples used for maternal circulating cytokine and DAMPs analysis are in Table 2. Infants with an IBC ≤5th percentile were born at an earlier gestation than controls (median 273 vs 283.5 days) ($P = .02$), although all were born ≥37 weeks' gestation which is considered a term birth (259-294 days). Additionally, the mothers of infants with an IBC ≤5th percentile were slightly older than the control women (median 35 vs 28.5, $P = .04$). By design, those with an IBC ≤5th percentile had significantly lower birthweights and IBC compared to controls (both $P < .0001$). Infants with a decreased growth rate had a significantly lower birthweight and IBC compared to controls ($P = .002$ and $P = .0003$, respectively).

3.1 | Macrophage number

There was no difference in percentage of $CD163^+$ cells/total nuclei in placentas of infants with an IBC ≤3rd percentile compared

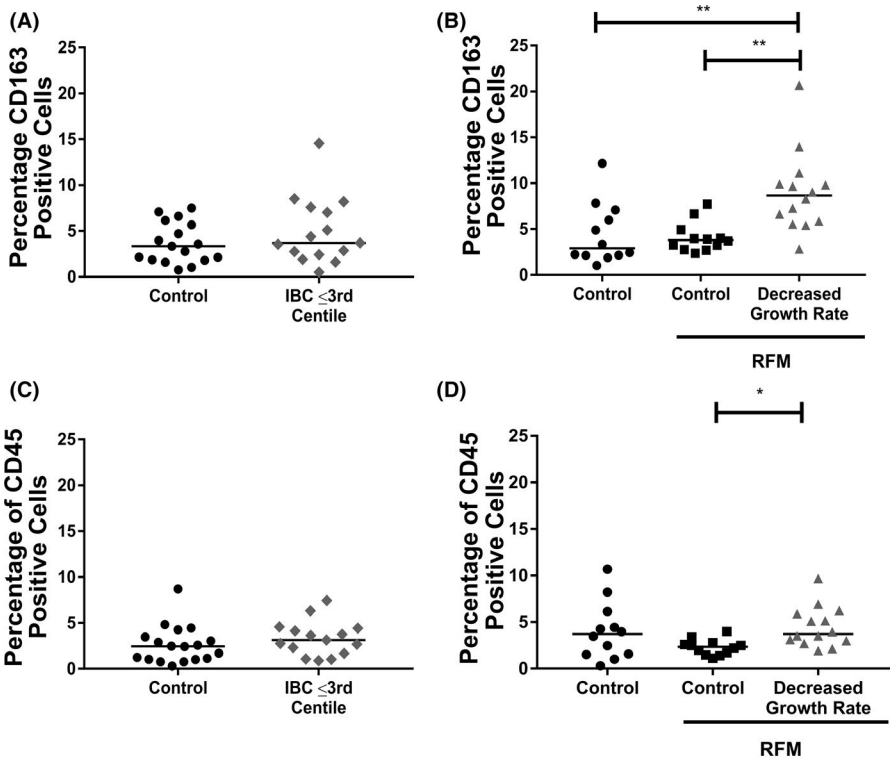
to controls (Figure 1A). In contrast, those fetuses with a decreased growth rate had an increased percentage of CD163$^+$ cells/nuclei compared to both control groups (P = .008 and .0003) (Figure 1B).

Analysis of CD45$^+$ cells was performed to determine whether the placentas had a widespread increase in leukocytes suggestive of infection which was not evident at delivery. None of the groups had a higher percentage of CD45$^+$ cells/nuclei compared to the percentage of CD163$^+$ cells/nuclei (non-significant). The increase in CD163$^+$ cells was deemed not to be of infective origin (Figure 1).

3.2 | Placental structure

There were no differences in the vascularity of placentas in those with a decreased growth rate compared to their controls (Figure 2). There were also no differences in the percentage of Ki67-positive cells/ nuclei (proliferation), percentage of M30-positive cells/nuclei (apoptosis), or syncytial nuclear aggregates (Figure 3).

The only difference in the placenta of infants with an IBC ≤3rd percentile was a decrease in the number of villi compared to controls (P = .003) (Figure 2E). There were no significant differences in the number of SNAs, percentage of Ki67-positive cells/nuclei, or



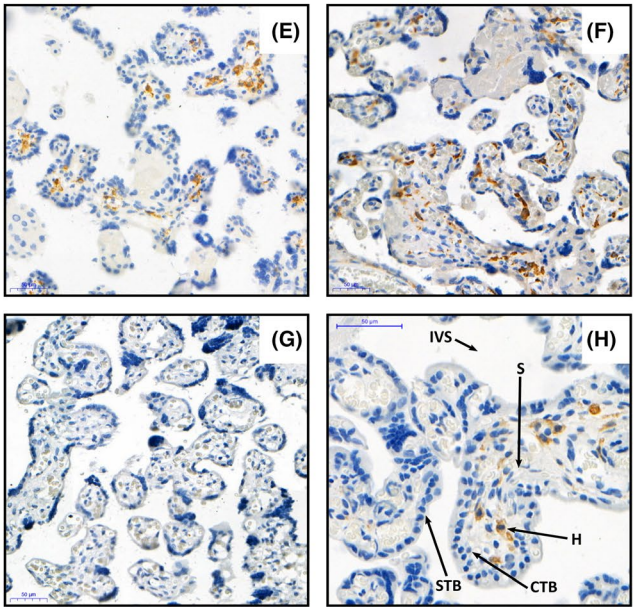


FIGURE 1 The percentage of CD163$^+$ macrophages and CD45$^+$ cells in placentas of infants IBC ≤3rd percentile and infants with a decreased growth rate. Immunostaining for either CD163 in (A) IBC ≤3rd percentile, (B) decreased growth rate placentas or CD45 in (C) individualized birthweight centile (IBC) ≤3rd percentile, and (D) decreased growth rate placentas. Data are the percentage of positive nuclei. Line represents the median. *P < .05, **P < .01 Kruskal-Wallis with Dunn's post hoc test or Mann-Whitney. Representative images of CD163$^+$ cells (E) IBC ≤3rd percentile, (F) decreased growth rate, (G) negative control, and (H) a labeled image. CTB, cytotrophoblast; H, Hofbauer cells; IVS, intervillous space; S, stroma; STB, syncytiotrophoblast. Images taken at 20X magnification in 3D Histech CaseViewer

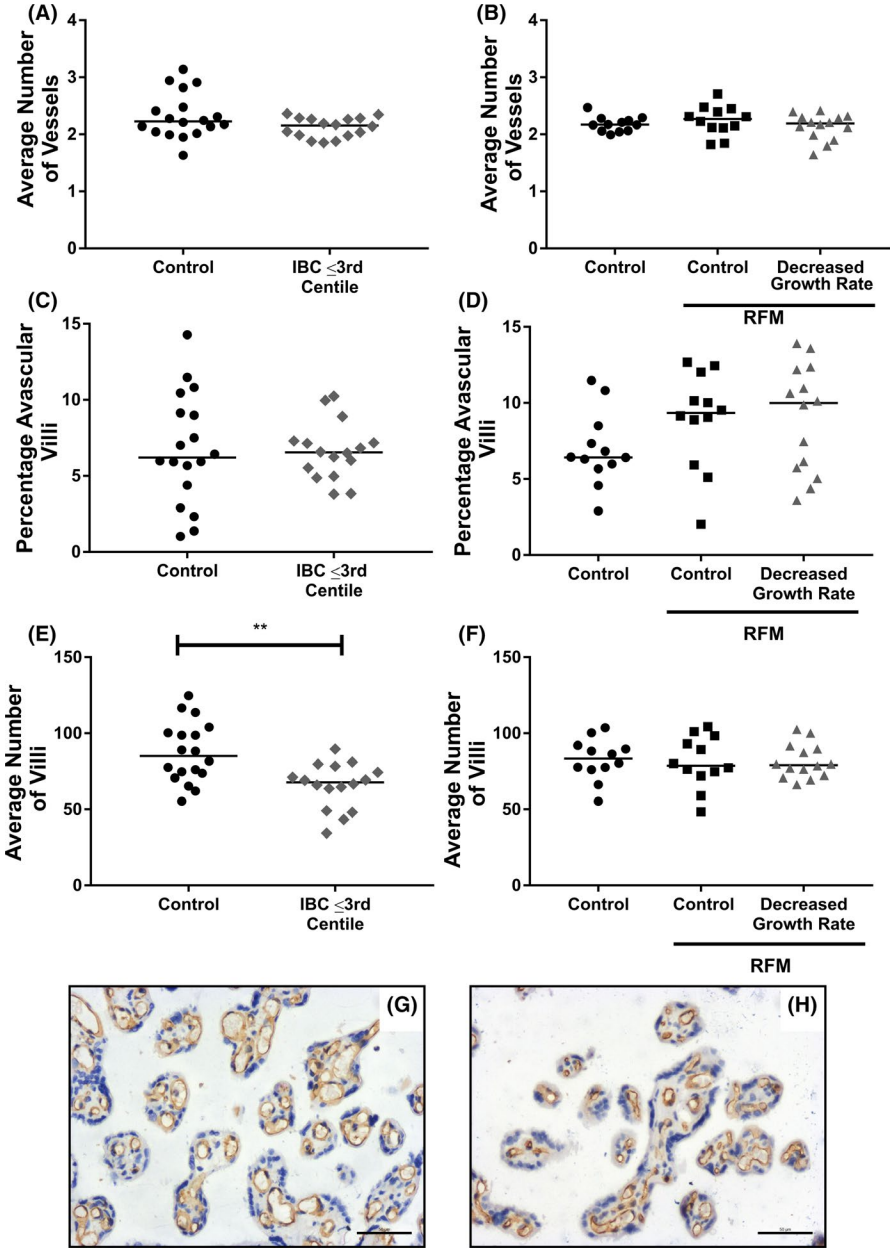


FIGURE 2 Vascularity in IBC ≤3rd percentile and decreased growth rate infants. Quantification of (A) the average number of vessels, (C) percentage of avascular villi, (E) average number of terminal villi in individualized birthweight centile (IBC) ≤3rd percentile, and (B) the average number of vessels, (D) percentage of avascular villi, or (F) average number of villi in decreased growth rate placentas. Line represents the median. Significance $P \leq .05$. $^{**}P < .01$ Kruskal-Wallis with Dunn's post hoc test or Mann-Whitney. Representative IHC images of (G) a decreased growth rate and (H) healthy control. Images taken at 200X magnification

percentage of M30-positive cells/nuclei in those with IBC ≤3rd percentile compared to controls (Figure 3).

3.3 | Inflammatory profile

3.3.1 | Placental lysates

In infants with an IBC ≤3rd percentile, there were no significant differences observed in any of the placental cytokines. However, there was a trend toward an increase in levels of IL-6 ($P = .006$) although this did not reach statistical significance with application of the Bonferroni correction.

Those infants with a decreased growth rate had significantly higher levels of IL-8 than infants who presented with RFM but who had a normal growth rate ($P = .0008$) (Table 3). In contrast to the results in infant's ≤3rd IBC, uric acid levels were significantly increased in women who presented with RFM ($P = .002$) but were most elevated in women with RFM and a decreased growth rate compared to healthy infants ($P = .0007$). Values for all the placental lysate ELISAs and accompanying P values can be found in Table S2.

3.3.2 | Maternal serum

Due to the availability of samples, maternal serum samples were collected from infants with an IBC ≤5th percentile rather than <3rd percentile. There were no significant differences in the levels of any cytokines or DAMPs compared to healthy controls (Table 4). In

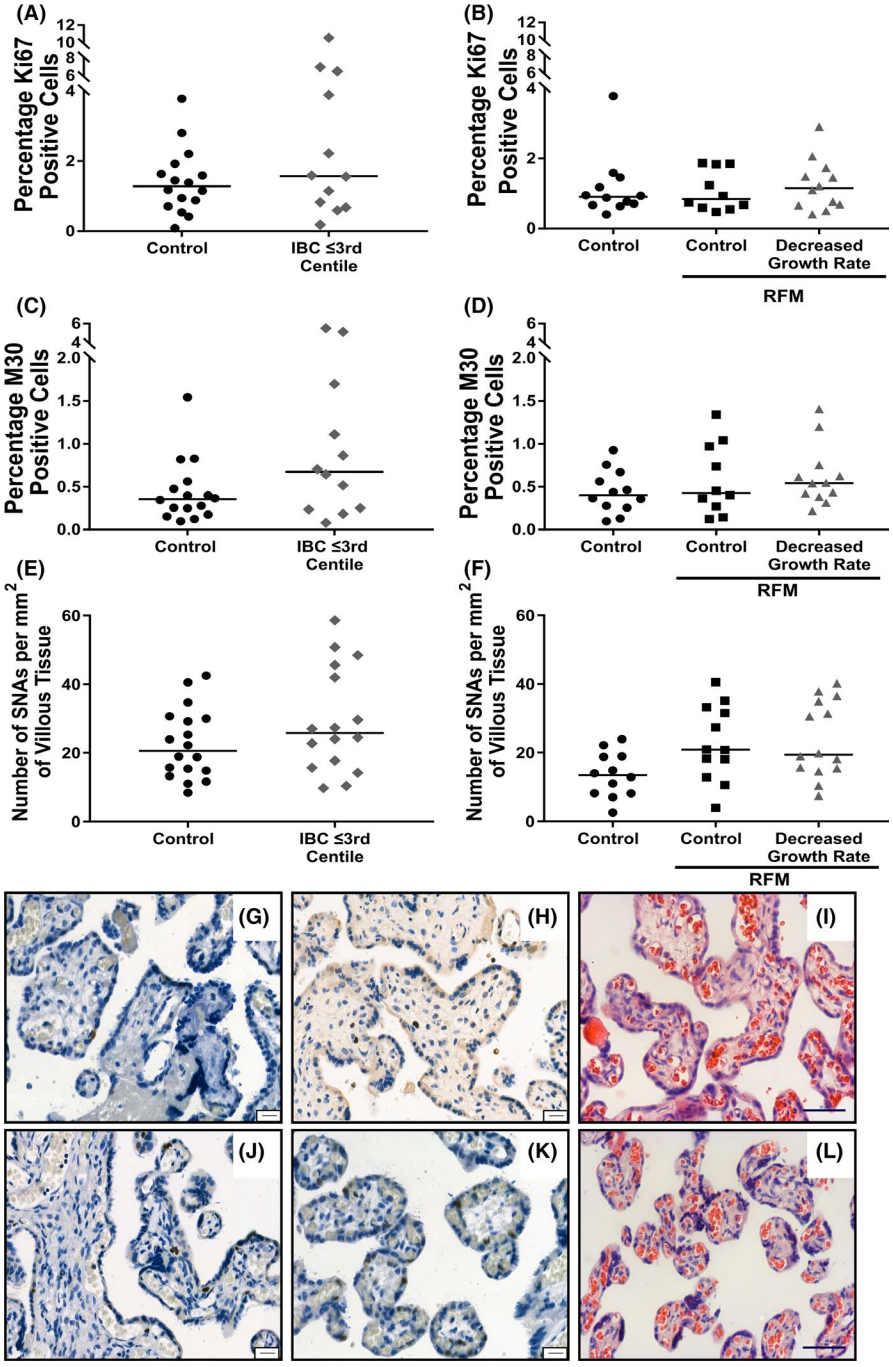


FIGURE 3 Cell turnover in IBC ≤3rd percentile and decreased growth rate infants. Analysis of (A) the percentage of Ki67-positive cells, (C) percentage of M30-positive cells, or (E) the number of syncytial nuclear aggregates (SNAs) in individualized birthweight centile (IBC) ≤3rd percentile placentas, and (B) the percentage of Ki67-positive cells, (D) percentage of M30-positive cells, or (F) SNAs in decreased growth rate placentas. Representative images of (G) Ki67 IBC ≤3rd percentile, (H) Ki67 decreased growth rate, (I) M30 IBC ≤3rd percentile, (J) M30 decreased growth, (K) H&E healthy control, and (L) H&E decreased growth rate. Mann-Whitney or Kruskal-Wallis as appropriate. Significance $P \leq .05$. Line is the median. Images of SNAs taken at 200X magnification, Images of Ki67 and M30 staining taken at 4 magnification in 3D Histech CaseViewer

infants with a decreased growth rate, there were significantly increased levels of S100A8 compared to healthy controls ($P < .0002$) (Table 4). There were significantly reduced levels of MIP-1β in women who presented with RFM and in those who had RFM with decreased growth rate ($P = .0015$ and $P = .0024$, respectively). IL-8 was only reduced in women with reduced growth rate ($P = .0029$). Cytokine results missing from Table 4 were below the level of detection in all samples. Values for all the maternal serum ELISAs and accompanying *P* values can be found in Table S3.

4 | DISCUSSION

These data demonstrate that placental macrophages are increased in infants with a decreased growth rate (loss of ≥25 centiles) in the third trimester, but not in infants who were classified as SGA based on having an IBC ≤3rd percentile. Similarly, there were no differences in maternal or placental cytokine expression in the SGA infants, whereas changes were observed in the decreased growth rate group consistent with a pro-inflammatory bias. As these

TABLE 3 Results of the cytokine and DAMPs found in placental lysates of infants IBC ≤ 3rd percentile and infants with a decreased growth rate

							Reduced fetal movements (RFM)								
	Control			IBC ≤ 3rd percentile			Control			Control			Decreased growth rate		
ELISA	Median	IQR	N	Median	IQR	N	Median	IQR	N	Median	IQR	N	Median	IQR	N
IL-1α/IL-1F1	3.8	2.4-5.9	17	3.2	2.3-4.4	15	3.5	2.2-5.7	10	4.4	2.4-7.6	12	3.9	1.2-7.4	11
IL-1β/IL-1F2	6.6	3.7-12.6	17	7.9	4.9-11.4	15	6.0	3.0-9.8	10	6.3	4.2-11.3	12	4.7	2.5-11.1	11
IL-1ra/IL-1F3	120.4	105.5-237.8	17	173.8	142.4-319	15	129.3	56.1-293	10	215.6	133.6-330.3	12	322.8	200.9-742.7	11
IL-6	14.9	12.9 - 21.9	17	24.2	17.9-38.5*	14	17.4	14.5-30.6	9	12.4	7.7-16.4	12	18.5	6.3-24.9	12
IL-8/CXCL8	5.5	3.3-12.7	16	5.4	3.4-8.1	15	1.7	0.4-4.8	10	0.4	0.3-0.9	12	5.1	**1.5-20.7**[a]	11
IL-4	2.8	1.5-9.5	17	3.2	2.2-4.6	15	4.8	2.6-12.7	10	7.8	5.7-23.1	11	10.5	7.2-25.8	11
TNF-a	2.2	0.8-4.2	17	3.2	2.2-5.1	15	1.2	0.7-2.8	10	1.7	1.2-2.3	12	2.1	1.2-6.9	11
TGF-β	29.4	18.1-41.2	17	23.2	18.9-40.8	15	31.3	21.9-62.5	10	46.6	28.7-127.2	12	49.1	35.6-104.9	11
IFN-γ	2.8	2.0-3.9	17	4.2	2.4-7.1	15	3.5	2.4-12.2	10	6.5	4.6-17.9	12	10.0	4.6-35	11
IL-10	502.1	144.3-801.4	16	926	317.3-1256	15	693.4	226-1143	10	588.8	298.2-821.4	11	584.1	445.6-902.8	11
HSP70/ HSPA1A	29 264	25 435-39 322	17	34 814	25 234- 45 486	15	41 146	26 929- 111 027	10	80 348	55 128- 172 187	12	92 294	34 271- 149 479	11
S100A8	126.8	90.0-309.2	17	216.7	92.2-358.4	15	155.4	74.8-326.9	10	517.8	144.9-1205	12	544.3	83.6-1088	11
HMGB1	57.4	47.1-75.7	17	48.4	37.7-51.9	15	48.5	22.0-90.1	10	38.1	23.3-100.7	12	79	44.1-89.4	11
CCL2/MCP-1	41.3	26.8-65.9	15	65.2	41.6-85.5	15	43.9	19.8-131.1	10	45.7	27.5-62.9	11	48.9	24.4-74.7	11
Uric Acid	23.9	18.9-47.0	15	19.2	16.0-25.3	15	9.9	5.5-14.4	9	26.8	**21.4-37.4**[c]	12	28.9	**20.8-43.7**[b]	12

Note: All measured in pg/mL/mg protein except for HMGB1 (ng/mL/mg protein) and uric acid (mg/dL/mg protein).

Mann-Whitney or Kruskal-Wallis, as appropriate.

Statistical significance set at $P \leq .004$ following Bonferroni correction.

[a]$P = .0008$ (decreased growth rate vs RFM control).

[b]$P = .0007$ (decreased growth rate vs normal control).

[c]$P = .002$(normal control vs RFM control).

*$P = .006$ (IBC < 3rd percentile vs control)

TABLE 4 Results of the cytokines and DAMPs found in maternal serum of IBC ≤ 5th percentile and infants with a decreased growth rate

							Reduced Fetal Movements (RFM)								
	Control			IBC ≤ 5th percentile			Control			Control			Decreased Growth Rate		
ELISA	Median	IQR	N	Median	IQR	N	Median	IQR	N	Median	IQR	N	Median	IQR	N
IL-1β	0.2	0.2-0.5	22	0.2	0.2-0.4	23	0.04	0.03-0.06	12	0.04	0.03-0.04	8	0.04	0.04-0.23	12
IL-6	1.1	0.7-2.7	22	1.2	0.05-4.4	23									
IL-8	1.7	1.0-2.8	22	2.0	1.5-2.8	23	0.1	0.1-0.3	12	0.09	0.07-0.1	8	0.055	0.04-0.1[a]	12
TNF-α	2.0	0.9-3.4	22	1.9	0.9-4.3	23	0.06	0.04-0.08	12	0.1	0.04-0.2	8	0.115	0.07-0.3	12
IL-10	1.3	0.8-5.2	22	1.5	0.7-3.8	23									
HSP70/HSPA1A	1216	771.5-1366	18	1038	857.9-1525	16	773.4	617.3-1183	12	1003	885.8-1346	8	1227	1037-1740	12
S100A8	325	65.3-903	18	746.2	192.9-1384	16	19.9	16.2-257.9	12	840.9	600.2-1354*	8	1194	727.3-2080[b]	12
HMGB1	1.0	0.8-1.8	16	1.1	1.0-1.4	13	1.8	1.1-2.6	12	2.2	1.4-2.6	8	2.108	1.8-2.4	12
Uric Acid	5.0	4.6-6.5	18	4.9	4.1-5.6	16	4.9	3.9-5.9	12	5.7	5.0-6.0	8	4.642	4.4-5.7	12
IL-12	1.2	0.8-2.6	22	1.2	0.7-2.6	23	0.3	0.3-0.9	12	0.3	0.3-0.3	8	0.34	0.3 - 0.4	12
G-CSF	5.7	3.2-8.2	22	5.5	2.2-12.1	23	2.2	1.4-3.3	12	1.5	0.9-1.8	8	1.805	1.4-3.0	12
MIP-1β	23.1	14.7-31.9	22	19.8	11.6-33.8	23	7.4	5.1-10.7	12	2.2	1.5-4.1[d]	8	2.375	1.8-3.8[c]	12

Note: All measured in pg/mL except for HMGB1 (ng/mL) and uric acid (mg/dL) All results missing were below the level of detection are not reported.

Mann-Whitney or Kruskal-Wallis, as appropriate.

Statistical significance set at $P \leq .003$ following Bonferroni correction.

[a]$P < .003$ (decreased growth rate vs normal control).

[b]$P < .0002$ (decreased growth rate vs normal control).

[c]$P < .001$ (decreased growth rate vs normal control).

[d]$P < .003$ (normal control vs RFM control).

*$P = .009$ (RFM control vs normal control).

changes are associated with elevated uric acid, this is thought to represent non-infectious inflammation, due to the absence of clinical signs of maternal, fetal, and placental infection. This observation provides further evidence of a role for inflammation in late-onset FGR.

4.1 | Strengths and limitations

As third trimester scanning is not routinely performed in the UK,[21] this study used an available cohort of infants who had a third trimester ultrasound scan performed for reduced fetal movements to derive a group of infants with decreased growth rate and a separate group who had SGA births.[22,23] This study was strengthened by the availability of sufficient meta-data to ensure that cases and controls had similar profiles of potential confounding factors (eg smoking[24,25] and obesity (as reviewed by Pantham et al[26]). We were also able to exclude women who had clinical conditions which may be associated with altered inflammatory phenotype, including preeclampsia (as reviewed by Harmon et al[27]) and gestational diabetes mellitus (as reviewed by Abell et al[28]). The study design also recognized that women with RFM may have altered placental phenotype; this was controlled for by the use of a control group which presented with RFM but in which the fetuses were normally grown. Importantly, these two control groups showed few differences between them, suggesting that RFM alone is unlikely to account for our observations. However, in the future, the use of a large scale, unselected cohort of women who had a third trimester EFW routinely performed should be considered to validate our findings.

Although we measured a large number of cytokines in both the maternal and placental compartments due to limited sample availability, it was not possible to correlate cytokine levels in the same participants. There is a notable lack of investigations of matched maternal and placental compartments in the literature, except for analysis in infective pathologies,[29] and there is limited information as to whether cytokines can cross the placenta.[30,31] A prospective study is required to collect matched samples to provide this important information. As the infants with a decreased growth rate were not considered growth restricted at birth, the placentas would not have undergone routine placental pathology examinations, and therefore, pathology reports cannot be obtained. Future studies investigating decreased growth rate in the third trimester should consider obtaining a pathology report to assess classical lesions such as distal villous hypoplasia, fetal thrombotic vasculopathy, advanced villous maturity, decidual vasculopathy infarction or acute fetal inflammation.

All infants included in this study were born at a gestation which would be considered a term delivery (37-42 weeks gestation). However, within these criteria, the gestational age at delivery was lower for the FGR ≤3rd and ≤5th IBC compared to their control groups (Tables 1 and 2). Additionally, due to the difficulty in obtaining maternal blood samples, those collected from infants with an IBC ≤5th percentile were collected earlier in gestation than the controls. The RFM infants with a normal growth rate had maternal samples collected later in gestation than the healthy controls. There is a paucity of research investigating the changes in cytokine levels over the final weeks of the third gestation, with studies usually reporting changes between trimesters.

In this study, the common leukocyte marker CD45 was used to indicate whether there was an increase in the number of immune cells in the placenta which could be suggestive of infection. No increases in CD45 over that of CD163 were observed in our samples, suggesting that infection was not the cause of the increased macrophage number. However, as a decrease in growth rate is a comparatively new way of defining fetal growth restriction, further placental investigations could be performed to assess the presence of inflammation. Chorioamnionitis, the presence of inflammation in the chorionic plate, can be identified clinically in the mother, or histologically in the placenta, through the presence of polymorphonuclear leukocytes, for example neutrophils.[32] The incidence of chorioamnionitis is between 3% and 10% in term deliveries, and it is elevated in preterm birth where the incidence is approximately 30%, but one study suggests the level of chorioamnionitis is unchanged in SGA births.[33-35] Neutrophil elastase is one marker that could be used in future studies to examine the presence of neutrophils in the chorionic plate of placentas of decreased growth rate infants.

4.2 | Context

Our observation of a pro-inflammatory shift in both maternal serum and placenta in pregnancies with FGR is consistent with previous observations.[6,16-18] By adding information about fetal growth rate, we were able to separate infants who were small for gestational age from those who had a significant reduction in growth velocity, that is who had true FGR. The fact that the increased macrophages and pro-inflammatory state was only evidence in decreased growth rate is an important observation, and suggestive of a putative involvement in the pathogenesis of FGR. The elevated numbers of macrophages, cytokines, and DAMPs in the absence of clinically detectable infection are consistent with non-infective inflammation.[36] However, it may also be indicative of senescence which may precede inflammation.

As well as being produced by macrophages, IL-8 is produced by senescent cells.[37] Senescence has been proposed as a mechanism of placental dysfunction associated with late-onset pregnancy complications such as preeclampsia (reviewed by Manna et al[38]) and stillbirth.[39] As well as producing pro-inflammatory cytokines as part of the senescence-associated secretory pathway (SASP), inflammation will induce senescence in previously healthy cells.[40] Activation of SASP recruits immune cells, including macrophages, resulting in their clearance. The use of specific markers, such as SA-β-Gal, or the senescence inducers p21 or p53,[41] some of which have previously been reported as elevated in FGR placentas[42] would add weight to this hypothesis.

Sterile inflammation occurs as a result of the release of DAMPs,[36] four of which were assessed here (uric acid, HMGB1,

HSP70, and S100A8). Our data suggest alterations in the profile of DAMPs in both maternal and placental compartments. Uric acid was increased in the placental lysates of pregnancies with a decreased growth rate. In addition to its role in inflammation, uric acid is a potent antioxidant and is upregulated in response to oxidative stress,[43] which is implicated in the pathogenesis of FGR.[44] Moreover, uric acid can contribute directly to declining fetal growth rate through decreasing system A amino acid transporter activity[45] and promoting apoptosis.[19] An increase in the DAMPs HSP70 and S100A8 was observed in the maternal serum in pregnancies with a decreased growth rate, both of which are associated with necrosis and cellular injury.[36] There is emerging evidence in animal models that administration of increasing amounts of HSP70 leads to an increase in neonatal mortality of up to 30%.[46] This may in part be attributed to the binding of HSP70 to TLR 4, which is expressed by syncytiotrophoblast and placental macrophages.[47-50] The increase of HSP70 observed in the maternal serum may have contributed to the decrease in fetal growth rate through this mechanism. Furthermore, a significant reduction in fetal weight has been observed following the administration of uric acid, accompanied by an increase in placental macrophages.[19] Although an increase in uric acid was not observed in the maternal serum, it was significantly higher in the placentas of infants with a decreased growth rate along with an increase in macrophage number. Increases in circulating DAMPs have been identified in pregnancy pathologies including preeclampsia and gestational diabetes mellitus.[51,52] The absence of any increase in DAMPs in the placentas and maternal serum of infants ≤3rd percentile suggests that a proportion of the infants are not growth restricted.

The lack of differences in the amount of apoptosis or proliferation in the placentas of both infants with a decreased growth rate and those with an IBC ≤3rd percentile differs from prior observations.[42] However, all samples analyzed here were from infants born ≥37 weeks' gestation whereas previous studies have included infants who were born at earlier gestations[16] which may reflect differences between early- and late-onset FGR. Nevertheless, the lack of difference in cell turnover suggests that the increased numbers of macrophages in placentas of infants with decreased growth rare are not elevated in response to increased apoptosis. However, this does not exclude a response to alternative patterns of cell death such as the caspase-independent necroptosis, which have been observed in placental pathologies.[53] The presence of alternative forms of cell death could provide evidence for cell type and location of the observed DAMP release and merit further exploration.

Levels of IL-8 in maternal circulation increase across gestation.[54] The observed decrease in IL-8 and MIP-1β in the maternal circulation of infants with a decreased growth rate could be the result of a failure to switch to a pro-inflammatory state toward the end of pregnancy.[55] Additional work should examine whether macrophages or syncytiotrophoblasts are the source of elevated IL-8 and IL-6 detected in placental lysates. Although the numbers of macrophages were quantified, the phenotypes of these cells were not examined. Macrophages can be broadly split into two categories of phenotype: the pro-inflammatory M1-like macrophages and M2-like which are anti-inflammatory.[56] In reality, it is likely that there is continuity between the two phenotypes, dependent on the expression level of markers. Future work should investigate whether phenotypic changes are present in the placentas of infants with a decreased growth rate. Interestingly, the increase in $CD163^+$ cells was higher than the $CD45^+$ cells in both infants with an IBC ≤3rd percentile and infants with a decreased growth rate (although only the latter group reached statistical significance). Although CD45 is a common leukocyte antigen, variable expression on macrophages has been noted, including on macrophages found in the brain (microglial cells),[57] where differences in expression level are associated with functional differences.

4.3 | Clinical implications

This study found no differences in the cytokine profile and placental dysfunction measurements in SGA infants. This suggests this group represents infants that are constitutionally small, rather than growth restricted. FGR is difficult to diagnose in late pregnancy, and this could underlie the discrepancies in the numbers of placental macrophages compared to those reported in other studies.[6] The recent Delphi consensus[11] included the definition of a change in ≥50 percentiles to define a reduction in growth rate. Given that we observed immunological and inflammatory changes using a more modest definition of ≥25 percentiles across the third trimester, we argue that even a smaller decrease in fetal growth rate may be pathological and occur in the presence of a pro-inflammatory state.

Villitis of unknown etiology (VUE) is diagnosed by the presence of large numbers of maternal lymphocytes, predominately $CD8^+$ and $CD4^+$ T cells, and macrophages in the placental villi, and has been identified as being present in up to 15% of placentas that are deemed appropriately grown at term.[58,59] Importantly, "appropriately grown" in these studies usually refers to infants with a birthweight >10th percentile (and ≤95th percentile). If the growth rate is unknown, some of these infants may have had an undiagnosed decrease in growth rate as defined by our or the Delphi criteria.[11] Thus, the assumption that it is normal to find the presence of inflammatory lesions such as VUE in a term, appropriately grown infant may be inaccurate. Ideally, longitudinal studies investigating the role of placental dysfunction on fetal growth should include information about the fetal growth rate.

5 | CONCLUSIONS

Our data show an increase in the number of placental macrophages in infants who have a decreased growth rate in the third trimester of pregnancy providing evidence for the role of placental inflammation in late-onset FGR. Although there is a shift toward a pro-inflammatory environment in both the maternal serum and placental compartment of these infants, it is unclear whether the increase seen is a cause, or the result, of the inflammation. Future studies are required

to determine whether there is also a shift in the phenotype and function of these macrophages. Additionally, the impact of the increased macrophages on placental function requires further investigation to determine whether modulation of this inflammatory response could enhance placental function, preventing the consequences of decreased fetal growth.

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CONFLICT OF INTERESTS

The authors do not have any conflicts of interest to declare.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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