IMMUNOPATHOLOGY AND INFECTIOUS DISEASES

Characterizing Villitis of Unknown Etiology and Inflammation in Stillbirth

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Villitis of unknown etiology (VUE) is an enigmatic inflammatory condition of the placenta associated with fetal growth restriction and stillbirth. Greater understanding of this condition is essential to understand its contribution to adverse outcomes. Our aim was to identify and quantify the cells in VUE in cases of stillbirth and to characterize immune responses specific to this condition. Immunohistochemistry was performed on placentas from stillborn infants whose cause of death was recorded as VUE to identify CD45⁺ leukocytes, CD163⁺ macrophages, CD4⁺ and CD8⁺ T cells, neutrophils, and proinflammatory and anti-inflammatory cytokines. Images were quantified with HistoQuest software. CD45⁺ leukocytes comprised 25% of cells in VUE lesions: macrophages (12%) and CD4 T cells (11%) being predominant cell types; CD8 T cells were observed in all lesions. Leukocytes and macrophages were increased throughout the placenta in stillbirths; pan-placental CD4⁺ and CD8⁺ T cells outside VUE lesions were increased in stillbirth with VUE. There was increased IL-2 and IL-12 and reduced IL-4 immunostaining in VUE lesions. Our results suggest VUE in stillbirth has a similar immune cell profile to live birth. Pan-placental macrophages, CD4 and CD8 T cells indicate a wider inflammatory response unrestricted to VUE lesions. The cytokine profile observed suggests a skew towards inappropriate Th1 immune responses. Full characterisation VUE lesion phenotype confirms its immunological origins and provides foundations to develop novel investigations. (*Am J Pathol* 2016, 186: 952–961; <http://dx.doi.org/10.1016/j.ajpath.2015.12.010>)

Stillbirth, the death of a baby after 20 weeks' gestation, places a sustained economic and emotional burden on both maternity services and the wider community. Despite significant effort, the stillbirth rate has decreased at a slower rate than neonatal or infant death.¹ In high-income countries stillbirth rates are variable. The United States and United Kingdom perform relatively poorly with rates of 1 in 160 and 1 in 212, respectively, whereas in Finland 1 in 400 pregnancies result in a stillborn infant.^{2–4}

Placental dysfunction as a cause of stillbirth has increasingly become a focus of research effort with recognition that placental disorders are the cause of death in up to 65% of stillbirths.⁵ However, the placental lesions associated with stillbirth are extremely varied and poorly defined, and their relation to pathologic processes leading to stillbirth is difficult to assess.^{6,7} Placental lesions associated with stillbirth include those with genetic,

environmental, infective, inflammatory, mechanical, metabolic, and vascular origins.⁶

Understanding of inflammatory disorders of the placenta remains relatively limited in comparison with vascular lesions. Two main inflammatory conditions have been linked to placental dysfunction in the absence of infection: villitis of unknown etiology (VUE) and chronic histiocytic intervillitis.⁸ VUE is an inflammatory condition of the placenta reported to occur in up to 15% of term placentas and more frequently in pregnancies resulting in poor outcome.^{9,10} The origin of VUE is unclear, although it is proposed to be maternal immune rejection of a semiallogeneic placenta.^{11–13} VUE is identified and characterized by the presence of elevated numbers of fetal macrophages (Hofbauer cells) and an infiltrate

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Table 1 Antibodies and Positive Control Tissues Used for Identifying Immune Cell Populations and Cytokines in Placental Villous Tissue

Antigen	Antigen retrieval	Supplier	Working concentration (µg/mL)	Ab type	Control tissue
CD45	Sodium citrate	Dako	0.5	M mAb	Tonsil
CD163	Sodium citrate	AbD Serotec	10	M mAb	Tonsil
CD4 (clone 4B12)	Tris-EDTA or sodium citrate	Dako	4.6	M mAb	Tonsil
CD8	Tris-EDTA	Dako	1.6	M mAb	Tonsil
NE	None	Dako	1.1	M mAb	PTLI
IL-2	Sodium citrate	Novus Biologicals	4.5	R pAb	Tonsil
IL-4	Sodium citrate	Thermo Scientific	2.5	R pAb	Tonsil
IL-6	Sodium citrate	Novus Biologicals	9.6	M mAb	Tonsil
IL-10	None	Abcam	5	M mAb	Liver (Kupffer cells)
IL-12	Sodium citrate	Sigma-Aldrich	2.5	R pAb	Trophoblast
TGF-β	Sodium citrate	Novus Biologicals	10	M mAb	Tonsil

M mAb, mouse monoclonal antibody; NE, neutrophil elastase; PTLI, preterm labor with infection; R pAb, rabbit polyclonal antibody; TGF, transforming growth factor.

of maternal T lymphocytes in the villous stroma. Areas of the placenta not affected by VUE lesions display apparently normal structural characteristics.⁹ The cell profile of VUE has previously been characterized in both appropriately grown and growth-restricted live born infants.^{12,14–16} Conflicting reports state either CD4⁺ or CD8⁺ T cells are the most common lymphocyte type in VUE lesions.^{12,14,17,18} The subtype of CD4⁺ cells (Th1/Th2) has not been examined; therefore, the type of immune response in VUE remains to be elucidated.

A thorough characterization of VUE has yet to be completed in stillbirth; it is not understood why it may cause fetal demise. In addition, the cytokine profile in VUE has not been described in either live births or stillbirths. Characterization of the cell types present and their cytokine profile will provide a greater understanding of the pathophysiology of VUE. We hypothesized that the immune cells in VUE lesions in stillbirth would be comparable with those described in live birth and that evidence of widespread inflammatory changes would be detectable in the placentas of stillborn infants with VUE. In this immunomorphologic study we aimed to accurately quantify the type and number of immune cells present in VUE lesions, assess leukocyte infiltration across the placenta, and analyze the expression of a range of proinflammatory and anti-inflammatory cytokines with the use of immunohistochemical approaches, coupled with an unbiased sensitive image analysis method. We compared leukocyte infiltration

and cytokine expression across the placenta to ascertain whether this was altered in dysfunctional placentas from stillbirth with and without VUE and fetal growth restriction (FGR) compared with healthy controls.

Materials and Methods

Study Population

Placental tissues from pregnancies resulting in stillbirth in which a contributory cause of death was recorded as VUE ($n = 12$) or FGR ($n = 12$) were obtained from the Pediatric Histopathology departments of Manchester Royal Children's Hospital and Edinburgh Royal Infirmary. Samples from stillborn growth-restricted infants had no evidence of VUE lesions recorded on postmortem examination. Consent for tissue use in research was obtained at the time of consent for postmortem; their use in this project was approved by Humber Bridge Research Ethics Committee (Ref. 13/YH/0176). Live born infants ($n = 11$) with an individualized birth weight centile less than the fifth were considered FGR infants; placental tissue was obtained from the Maternal and Fetal Health Research Group Biobank, University of Manchester (North West REC 08/H1010/55). Matched placental samples from women with uncomplicated pregnancies ($n = 12$) were selected from the Maternal and Fetal Health Research Group

Table 2 Demographic Information for the Women Included in the Study

Characteristic	Stillbirth with VUE ($n = 12$)	Stillbirth with FGR ($n = 12$)	Live birth with FGR ($n = 11$)	Normal pregnancy ($n = 12$)	<i>P</i>
Maternal age (years)	32 (21–36)	23.5 (19–42)*	32 (16–35)	35 (25–42)	0.019
Gestational age (weeks)	39 (20–41)	35.21 (28–38)	37.8 (27.7–42.1)	33.5 (30.0–41.7)	0.098
Gravidity	3 (1–7) ($n = 7$)	1 (1–4)	1 (1–9)	2 (1–18)	0.298
Parity	1 (0–3) ($n = 7$)	0 (0–2)	0 (0–3)	1 (0–3)	0.118
Birthweight (g)	NA	1061 (385–2000)***	2246 (747–3060)**	3430 (2900–3840)	<0.001
IBC	23 (3–90) ($n = 7$)	NA	3 (0–10)***	45 (12–93)	<0.001

Results are presented as median (range).

* $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ compared with normal pregnancy.

FGR, fetal growth restriction; IBC, individualized birth weight centile; NA, not available; VUE, Villitis of unknown etiology.

Figure 1 Serial images of a representative VUE lesion immunostained for immune cell markers. CD45 (A), CD163⁺ macrophages (B), CD8 cytotoxic T cells (C), CD4⁺ T helper cells (D), neutrophil elastase (E), and negative (F). Arrows highlight examples of positively stained cells; arrowheads highlight positively stained cells in the intervillous space. Scale bar = 50 μ m. Original magnification, $\times 100$. VUE, villitis of unknown etiology.

Biobank and used as controls. All placentas were taken from mothers who showed no evidence of conditions such as pre-eclampsia or diabetes. Detailed case note review showed no clinical, biochemical, hematologic, or microbiological evidence of infection was present. Multiple pregnancies, abnormal fetal heartbeat (live birth only), and fetuses with anomalies were excluded.

Sample Collection

Placentas from cases of stillbirth had two to seven randomly selected samples per placenta obtained at the time of post-mortem for histologic analysis. For live births three samples of placental villous tissue were taken, one each from the center, middle, and periphery of the tissue.¹⁹ Biopsies were fixed in neutral-buffered formalin for 24 hours before being processed and embedded in paraffin wax.

IHC

For immunohistochemistry (IHC), 5- μ m serial sections were cut and mounted onto slides coated with poly-L-lysine (Sigma-Aldrich, Poole, UK). Immunostaining with colorimetric detection was performed as described previously.²⁰ VUE lesions were initially identified by detecting clusters of leukocytes with the use of a mouse monoclonal antibody to CD45 (leukocyte common antigen). A VUE lesion was defined as an area of villous tissue with evidence of an

extensive CD45⁺ cell infiltrate that displayed the morphologic properties of macrophages and lymphocytes. Subsequent immunostaining for specific immune cells was performed to identify CD4⁺ T cells, CD8⁺ T cells, CD163⁺ macrophages, and neutrophils. Further immunostaining for cytokines IL-2, IL-4, IL-10, and IL-12 was performed on representative sections from all samples. Finally, staining for IL-6 and transforming growth factor (TGF)- β was performed on selected stillbirth samples with VUE ($n = 6$) and healthy pregnancies ($n = 6$). Heat-mediated antigen retrieval was performed for all antibodies except anti-neutrophil elastase and anti-IL-10 monoclonal antibodies. Primary antibodies were applied at the working concentrations detailed in Table 1. Each tissue section had a corresponding negative control on which mouse or rabbit IgG (Sigma-Aldrich) was applied at the same concentration as the primary antibody. Each staining run included a positive control tissue as described in Table 1.

Immunofluorescence

The phenotype of the macrophages and T cells in the placentas/lesions was investigated by dual immunofluorescence with combinations of immune cell markers CD4 and CD163 and cytokines IL-2, IL-12, or IL-4. Sections were fixed and processed as described in the previous section. Autofluorescence was quenched by 1-hour incubation in 0.25% ammonia (Sigma-Aldrich) in 70% ethanol. Primary antibodies were applied at the concentrations described in Table 1 at 4°C overnight. Secondary antibodies (goat anti-mouse Alexa 568 or goat anti-rabbit Alexa 488; Life Technologies, Paisley, UK) were applied at 5 μ g/mL and incubated for 30 minutes at room temperature. DAPI-containing aqueous mounting medium was used to apply coverslips.

Image Capture and Analysis

Tissue sections were visualized with the 10 $\times$ objective of an Olympus BX41 light microscope (Southend-on-Sea, UK). Images were captured with QIcam Fast 1394 camera (QImaging, Surrey, BC, Canada) and Image Pro Plus 6.0 (Media Cybernetics Inc., Rockville, MD). Quantitative, unbiased image analysis was completed with HistoQuest image analysis software version 3.5 (TissueGnostics, Vienna, Austria). The pixels of images viewed in this software are converted to grayscale (0 to 256 scale) and assigned an arbitrary number relating to staining intensity. The image analysis software differentiates hematoxylin⁺ and diaminobenzidine (DAB)⁺ cells or areas that are recorded as events (cells/nuclei) or area of staining. Results are presented as histograms or dot plot scattergrams to which staining area and/or intensity cutoffs can be applied to differentiate between cell populations. Analysis variables were optimized such that the number of DAB⁺ events and hematoxylin⁺ events were counted.

Two analyses were performed. First, for characterization of VUE lesions in stillbirth, inflammatory foci were identified in CD45-stained sections, then the remaining immunostains were

Figure 2 A–C: Screenshots from HistoQuest image analysis software demonstrating the selection of DAB⁺ events in sections stained for CD45 leukocyte common antigen (A and B) and cytotoxic T-lymphocyte marker CD8 (C). Positive events are highlighted in red; events encircled in green indicate hematoxylin positive/DAB[−] events. D: A representative scattergram from which staining intensity variables were ascertained. Scale bar = 50 μ m. Original magnification, $\times 100$. DAB, diaminobenzidine.

matched and captured in serial sections. In tissue sections with widespread/numerous inflammatory foci, representative lesions were selected ($n = 2$ to 5 per section).

To assess pan-placental inflammation in tissue, 10 random images (excluding lesions) were taken for each placental slide from all groups (stillbirth with VUE, stillbirth with FGR, live birth with FGR, and controls), immunostained for CD45, CD163 macrophage, CD4 T cell, and CD8 T cell markers. DAB⁺ cells and nuclei were quantified to ascertain whether there were any differences in leukocyte numbers between the study groups.

The presence of cytokines was determined by immunostaining for IL-2, IL-4, IL-6, IL-10, IL-12, and TGF- β . Images were analyzed quantitatively in sections of tissue from VUE lesions, areas of stillbirth with VUE placentas that were devoid of lesions, stillbirth with FGR placentas, live birth with FGR placentas, and controls ($n = 10$ images per placenta). Area and intensity of staining were recorded for each placental sample, and quantitative analysis was completed with HistoQuest image analysis software.

Fluorescent-stained sections were visualized with a Zeiss fluorescence microscope, AxioCam MRn camera with Zen pro image processing software version 20.12 (Zeiss, Welwyn Garden City, UK).

Statistical Analysis

We powered our analysis to determine the number of CD45⁺ and CD163⁺ cells in placentas from stillborn infants, assuming similar increases in these cells as seen in placentas from poor outcome pregnancies experiencing reduced fetal movements.²¹ No similar studies have been conducted with CD4 and CD8 T cells, so we were not able to take direct comparisons. For 80% power with an α value of 0.05, the sample size was calculated as 12. All statistical analysis

was performed with GraphPad Prism software version 6.0 (GraphPad Software, San Diego, CA). Nonparametric categorical data were analyzed with Fisher's exact test; nonparametric continuous data were analyzed with *U* test or Kruskal-Wallis test with Dunn's post hoc test.

Results

Participant Demographic Characteristics

The demographic data for the 48 women included in the study are shown in Table 2. A significant difference was found in maternal age between the stillbirth with FGR and normal pregnancy groups ($P = 0.02$). Contributory causes of death for the stillborn infant cohorts are detailed in Supplemental Table S1. By experimental design birth weight and individualized birth weight centile differences between stillbirth with FGR, live birth with FGR, and normal pregnancies were highly significant ($P < 0.001$ and $P < 0.01$, respectively). Average time between fetal death and delivery of infant was 3.5 days (range, 1 to 14 days). Comparisons of the grading/extent of VUE between tissue samples could not be drawn because this information was not recorded in postmortem reports (Supplemental Table S2).

Characterization of Focal Inflammatory Lesions

VUE lesions were identified in all placentas from the stillbirth with VUE cohort; a representative lesion is shown in Figure 1. In total 104 VUE lesions from 12 placentas were photographed and analyzed. The number of CD45⁺ cells in the lesions contributed 25% (median; range, 8% to 48%) to the total number of nuclei. Macrophages and CD4⁺ T lymphocytes were the most prevalent immune cells in the lesions, accounting for 12% (9% to 23%) and 11% (3% to

Figure 3 Quantification of pan-placental immune cells in normal and pathologic pregnancies. CD45 (**A**), macrophages (CD163, **B**), cytotoxic T cells (CD8, **C**), and T helper cells (CD4; **D**). Horizontal lines represent median. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, Kruskal-Wallis test with Dunn's post hoc test. LB FGR, live birth with fetal growth restriction; NP, normal pregnancy; SB FGR, stillbirth with fetal growth restriction; SB VUE, stillbirth with villitis of unknown etiology.

14%) of the total number of nuclei, respectively. CD8⁺ T lymphocytes were also present in VUE lesions, contributing 4% (0.3% to 11%) to the total number of nuclei. Neutrophil levels were low in all samples, accounting for 1% (0.37% to 2%) of total number of nuclei.

Differentiating Cell Populations

Cell populations were differentiated by applying staining area and/or intensity cutoffs to the results presented as histograms or dot plot scattergrams (Figure 2).

Quantification of Pan-Placental Immune Cells

Significant increases were detected in the number of pan-placental CD45⁺ leukocytes in all placentas from pregnancies from stillborn infants (with or without VUE) compared with normal pregnancies ($P < 0.001$) (Figure 3A). Significant elevation in macrophage numbers was detected in the placentas from stillborn infants with VUE ($P < 0.01$), stillborn infants with FGR ($P < 0.001$), and live born infants with FGR ($P < 0.01$) compared with normal pregnancy (Figure 3B). Placentas from the stillbirth with VUE group showed

Figure 4 Representative images of placental sections immunostained for proinflammatory and anti-inflammatory cytokines. **A**, **D**, and **G**: VUE lesions were stained for IL-2 (**A**), IL-12 (**D**), and IL-4 (**G**). **B**, **E**, and **H**: Cytokine staining in areas of placenta unaffected by lesions from stillborn infants with VUE were stained for IL-2 (**B**), IL-12 (**E**), and IL-4 (**H**). **C**, **F**, and **I**: Representative sections of placenta from pregnancies with a normal outcome were stained for IL-2 (**C**), IL-12 (**F**), and IL-4 (insets, negatives) (**I**). Insets show negative control slides using IgG isotypes. Arrows highlight staining associated with cells; arrowheads highlight staining associated with trophoblast. Scale bar = 50 μ m. Original magnification, $\times 100$ (**A-I** and insets). VUE, villitis of unknown etiology.

Figure 5 Representative high power images of placental sections after immunostaining for proinflammatory and anti-inflammatory cytokines. **A, C, and E:** VUE lesions were stained for IL-2 (**A**), IL-12 (**C**), and IL-4 (**E**). **B, D, and F:** Representative sections of placenta from pregnancies with a normal outcome were stained for IL-2 (**B**), IL-12 (**D**), and IL-4 (**F**). **Arrows** highlight staining of cells in the stromal core, **arrowheads** highlight staining associated with syncytiotrophoblast. Scale bar = 20 μ m. Original magnification, $\times 400$. VUE, villitis of unknown etiology.

significantly more pan-placental CD4 and CD8 cells than all other groups ($P < 0.001$) and were observed in every field of view, excluding lesions (Figure 3, C and D).

Cytokines

To fully characterize the immune response in VUE, immunostaining of characteristic Th1 (IL-2, IL-12) and Th2 (IL-4) cytokines was performed (Figure 4). Staining of IL-2 and IL-12 localized to cells within the stromal core and stillborn, as previously shown²² (Figure 5). Area of IL-12 staining was significantly increased in VUE lesions and live born FGR placentas compared with normal pregnancy ($P < 0.001$). A significant increase in area of IL-2 staining was observed in VUE lesions ($P < 0.05$) (Figure 6). IL-4 staining localized to both stromal and trophoblast cells²³ (Figure 5). A significant reduction was found in the area of IL-4 immunostaining in VUE lesions and areas devoid of lesions compared with controls ($P < 0.05$ and $P < 0.01$, respectively). A significant reduction in IL-4 staining area was also observed in the stillbirth with FGR group compared with live birth with FGR and controls ($P < 0.05$ and $P < 0.001$, respectively). IL-10 appeared significantly increased overall in areas of VUE placentas devoid of lesions ($P < 0.001$), but individual comparisons failed to

reach statistical significance (Figures 4 and 6, Supplemental Figure S1). Staining intensity for all cytokines was significantly different between samples (Supplemental Figure S2). Intensity of IL-2 was significantly reduced (stillbirth FGR, $P = 0.006$; live birth FGR, $P = 0.0004$) and IL-12 increased (stillbirth FGR, $P = 0.0002$; live birth FGR, $P < 0.001$) in FGR samples compared with control. IL-4 intensity was significantly reduced in VUE lesions compared with control ($P = 0.02$). IL-10 staining intensity was significantly increased in VUE placentas compared with stillbirth with FGR (VUE lesion, $P = 0.03$; VUE nonlesion, $P = 0.01$). Immunostaining for cytokines IL-6 and TGF- β was not detected in VUE lesions (Supplemental Figure S1).

Macrophage and T-Cell Phenotype

Dual immunofluorescence was performed to identify immune cell sources of cytokines of interest. IL-2 co-localized with CD4⁺ staining, CD163⁺ on the cell surface co-localized with cytoplasmic IL-12 staining, and IL-4 co-localized with CD4⁺ (Figure 7). Evidence of faint IL-4 fluorescence was found in the syncytiotrophoblast layer as observed in the images of chromogen staining (Figures 4 and 7).

Discussion

This study has fully characterized VUE in placentas from stillborn infants by quantifying the immune cells found in the lesions by using unbiased image analysis software, after IHC to detect specific immune cell types. We have also demonstrated specific pan-placental inflammatory changes in stillbirths (with and without VUE), indicating that a more widespread immune reaction was occurring in areas of placenta without VUE lesions. Furthermore, our cytokine analyses indicate that the immune response in the lesions is directed toward Th1-type response.

Previous studies of VUE in live birth have described lesions comprising mainly fetal macrophages²⁴ and maternal CD4⁺^{18,25} or CD8⁺^{12,14} T lymphocytes. Our results support these descriptions: in cases of stillbirth, macrophages and CD4⁺ T cells predominate, although elevated numbers of CD8 cells are also present. A key strength of the current study is the use of immunostaining to detect specific immune cell types. Pathologists examining hematoxylin and eosin-stained sections would not be able to identify individual leukocyte subsets, which are clearly visible after immunostaining for leukocyte, macrophage, CD4, and CD8 cell markers. VUE has been likened to graft rejection or graft-versus-host disease.^{11–13} The phenotype that we observed, with CD4⁺ lymphocytes outnumbering CD8⁺ lymphocytes, agrees with the 1989 study of Labarrere et al²⁵ and supports this hypothesis because the T-helper cells would assume responsibility for directing the immune response toward a Th1-type response.^{26,27} The presence of cytotoxic CD8 T cells in the lesions may contribute to both

Figure 6 Quantification of cytokine staining area using HistoQuest image analysis software. **A–D:** IL-2 (**A**), IL-12 (**B**), IL-4 (**C**), and IL-10 (**D**). Horizontal lines represent median. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, Kruskal-Wallis test with Dunn's post hoc test. DAB, diaminobenzidine; LB FGR, live birth with fetal growth restriction; NP, normal pregnancy; SB FGR, stillbirth with fetal growth restriction; VUE, villitis of unknown etiology.

the fetal placental vasculopathy often observed in VUE and the recently described elevated apoptosis of intravillous cells.^{9,17,28,29}

Since Medawar³⁰ first described the immunologic paradox of pregnancy, much has been published on the shift from Th1 to Th2 responses in pregnancy.^{31–35} Suppression of anti-fetal cell-mediated responses and a skew toward antibody-mediated immunity appears to be advantageous in the maintenance of a successful pregnancy.^{35,36} The cytokine profile differs between Th1 and Th2 responses; IL-2, IL-6, and IL-12 are associated with Th1 responses, whereas anti-inflammatory cytokines IL-4, IL-10, and TGF- β predominate in Th2-type responses. Our findings of increased levels of IL-2 and IL-12 in VUE lesions suggest that there may be an inappropriate shift toward cell-mediated immunity in VUE cases associated with stillbirth. Staining intensity of IL-2 and IL-12 was not significantly different between VUE samples and controls, implying that more cells are secreting the cytokine but the expression of it is not different. Co-localized immunofluorescence staining of CD163/IL-12 and CD4/IL-2 is indicative of proinflammatory macrophage and Th1 CD4 T-cell phenotypes. We have also detected a significant reduction in levels of anti-inflammatory IL-4 in VUE placentas from cases of stillbirth. The reduction in IL-4 levels was mirrored by a significant reduction in expression in VUE lesions. The placental anti-inflammatory cytokine environment characterized by IL-4, IL-10, and TGF- β , which induces maternal tolerance of the fetus, is

critical in maintaining a healthy pregnancy.^{32,36,37} Conversely, levels of proinflammatory IL-12 remain low throughout successful pregnancy.³⁶ Significant decreases in IL-4 in stillbirth placentas may be a contributory factor in permitting a shift from Th2-type to Th1-type immunity. These data support a reduction in Th2 and a Th1 bias in VUE lesions, which may lead to unresolved cell-mediated inflammation. Therefore, the critical balance appears to be disrupted in VUE lesions in cases ending in stillbirth.

It has been stated that macrophages and their cytokines are fundamental in establishing and maintaining the placenta at the start of pregnancy.^{38,39} It is therefore reasonable to suggest that an excess of immune cells and proinflammatory cytokines in late pregnancy may have a detrimental effect on placental function, which is then manifested as FGR or stillbirth. These effects would be in addition to the cytotoxic effects of any immune response mounted. Interestingly, systematic reviews suggest a general shift toward a proinflammatory cytokine profile in maternal serum and placentas from FGR and small for gestational age infants, confirming a role for a proinflammatory state in FGR.^{40,41} Conflicting reports of M2 placental macrophages potentially acting as either proinflammatory⁴² or immunosuppressive²⁹ cells only serve to compound the mystery underlying VUE. Our investigation has shown placental macrophages expressing the M2 marker CD163, yet producing the proinflammatory cytokine IL-12, further enhancing the evidence of the

Figure 7 Representative high power images of placental sections after dual immunofluorescence for proinflammatory and anti-inflammatory cytokines and cell markers. **A–C:** VUE lesions stained for CD4 (red), IL-2 (green), co-localization (yellow) (**A**), CD163 (red) and IL-12 (green) (**B**), and CD4 (red) and IL-4 (green), co-localization (yellow) (**C**). **D:** Mouse and rabbit IgG isotype control. **Red arrows** highlight dual staining; **yellow arrows** indicate co-localized immunofluorescence; and **white arrowheads** indicate trophoblast-localized IL-4. Scale bar = 20 μ m. Original magnification, $\times 630$ (**A–D** and inset). VUE, Villitis of unknown etiology.

plasticity of these cells and their potential role in pregnancy complications.

Pan-placental increases in leukocyte numbers in VUE are a novel finding. Significantly elevated numbers of leukocytes and macrophages were detected in all stillbirths compared with controls. The elevation in macrophages in live born FGR suggests that this is not a postmortem artifact. These data are consistent with our recent findings of elevated placental macrophage numbers in pregnancies at high risk of stillbirth.²¹ Staining intensity of cytokines in both live born and stillborn FGR were consistent throughout, further confirming that the structural characteristics observed is not related to postmortem tissue changes. The placentas from these pregnancies also exhibited a proinflammatory bias in relation to cytokine mRNA and protein expression (elevated IL-1 family members, reduced IL-10 and IL-4). Together, these data are highly suggestive of placental inflammation being an underlying factor in pregnancies that result in FGR and stillbirth.

Previous descriptions of VUE have stated the occurrence of focal villous lesions with adjacent unaffected tissue displaying normal structural characteristics.⁹ It has been suggested that the occurrence of VUE may have been underestimated because sampling of the placenta was not extensive enough to find evidence of lesions⁴³; Altamiani et al⁴³ reported that at least six blocks are needed to achieve an 85% chance of diagnosing 95% of cases. In our study, CD4 and CD8 T-cell numbers were increased in stillbirths with VUE compared with all other group samples, and these increases were not confined to VUE lesions. This finding is a strength of the specificity of the IHC in our study. A sensitive and unbiased image analysis tool that can accurately detect immunostained cells with the use of a relatively low magnification ($\times 100$) also contributes to the strength of the study. Our data suggest that a pan-placental increase in

CD4 and CD8 T cells should stimulate the pathologist to suspect an inflammatory condition, such as VUE, without relying on locating a specific lesion. With the use of IHC and sensitive image analysis software such as HistoQuest, the incidence of VUE could potentially be identified and accurately reported with fewer tissue samples. A general consensus on the sampling and definitions of placental lesions, as discussed by the Amsterdam Placental Workshop Group, attempts to address issues such as this (T.V. Khong et al, unpublished data). More sophisticated placental pathologic investigations may improve diagnosis of VUE (as was the case with estrogen receptor expression in breast cancer⁴⁴) and thus provide much sought after explanations for the cause of death.

By examining placental tissue after delivery, IHC can only provide a snapshot of VUE, rather than a temporal profile of the cell migration and its associated effects. This could explain why descriptions of the proportions of T cells in VUE differ between research studies because they sample different periods in the evolution of lesions.^{12,14,17,18} In addition, the pan-placental presence of CD4 and CD8 T cells may represent pre-lesions that, given time, would expand within the villous stroma, potentially explaining why VUE is considered a third-trimester phenomenon.⁹ The 1995 study by Khong,⁴⁵ describing expression of major histocompatibility complex type II in both nonvillitis and villitis-affected placentas, also suggests that VUE may be an evolving lesion. This dynamic process cannot be surmised from the current method of studying placental tissue. The results of this study would be further enhanced by including samples of placenta from live born infants with evidence of VUE. At the time the study was conducted, these were not available.

A greater understanding of the immune responses associated with VUE may lead to the development of screening

tests to enable antenatal detection of VUE. Our laboratory recently published data that indicate alterations in concentrations of maternal serum cytokines in pregnancies characterized by placental inflammation.²¹ This is supported by the observation of elevated maternal plasma chemokines (CXCL9, CXCL10, and CXCL11) in cases of VUE.¹³ It may therefore be possible to identify placental inflammation antenatally which would allow increased antenatal surveillance and potential anti-inflammatory therapies to prevent stillbirth.

Conclusion

We have fully characterized VUE in stillbirths with the use of IHC to identify specific immune cell types and have detected evidence of a Th1-type cytokine response. These data present novel insights into the pathogenesis of VUE and provide solid foundations for functional studies to determine the impact of inflammation on placental function.

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Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2015.12.010>.

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