SUPPLEMENTARY INFORMATION

Inflammasome-targeted therapy might prevent adverse perinatal outcomes of recurrent chronic intervillositis of unknown etiology

Short title: Inflammasome-targeted therapy in recurrent chronic intervillositis

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1. SUPPLEMENTARY METHODS

1.1. Selection of the placenta samples with CHI

Cases were identified as previously described (1). Briefly, cases were identified by searching the Department of Histopathology placenta database for the term ‘intervillositis.’ Serial 2-µm-thick sections of formalin-fixed paraffin-embedded potential CHI tissues, stained with hematoxylin–eosin–saffron (HES) or immunostained for CD68, were re-analyzed independently by three pathologists specialized in fetal pathology. Discordant cases were included or excluded according to consensus

among the three pathologists. Kappa values for interobserver variability of the diagnosis of CHI was 0.70. Diagnosis of CHI was confirmed according to the criteria of Bos et al (2): (1) presence of cellular infiltrate in the intervillous space; (2) ~ 80% of the mononuclear cells in the intervillous space positive for CD68; (3) infiltration occupying at least 5% of the intervillous space; and (4) no clinical or histopathological sign of infection.

1.2. Treatment protocol

The four following drugs were administrated as soon as the intra-uterine pregnancy was confirmed according to this protocol:

- Anakinra 100 mg once daily subcutaneously
- Hydroxychloroquine (HCQ) 400 mg orally per day
- Colchicine 1 mg orally per day,
- Low dose of aspirin (LDA) (100 mg) orally per day (in the evening).

The treatment protocol incorporates aspirin for mitigating the risk of intrauterine restriction, hydroxychloroquine for its immunomodulatory properties, anakinra, and colchicine to target inflammasome activation. While anakinra and colchicine are not explicitly mentioned in guidelines for intervillositis, their efficacy in managing other inflammasome-related conditions such as Still's disease and Mediterranean fever justifies their inclusion in this regimen. If possible, HCQ and colchicine were started preconceptionally for optimal tolerability. LDA was prescribed until 34 weeks of gestation in accordance to French guidelines in case of IUGR (3). The three other molecules were maintained until the delivery.

N.B: low-molecular-weight heparin was prescribed at preventive dose for patient 3 because of obstetrical antiphospholipid syndrome.

Rationale for using anakinra and colchicine as an inflammasome target therapy :

- Anakinra is an interleukin-1 (IL-1) receptor antagonist that competitively inhibits the binding of IL-1α and IL-1β to the IL-1 receptor. IL-1 is a pro-inflammatory cytokine that is produced through inflammasome activation. By blocking the IL-1 receptor, anakinra disrupts the downstream signaling cascade initiated by inflammasome activation. This interruption in the

IL-1 signaling pathway is a key mechanism through which anakinra exerts its anti-inflammatory effects.

- Colchicine: Colchicine, on the other hand, functions by disrupting microtubule polymerization. While its primary indication is for the treatment of gout and certain inflammatory conditions, colchicine has also been found to impact the inflammasome pathway. It interferes with the assembly of the NLRP3 (NOD-like receptor protein 3) inflammasome, which is a critical component of the innate immune system. Colchicine's ability to impede microtubule formation influences cellular processes and signaling pathways, ultimately preventing the activation of the NLRP3 inflammasome and subsequent release of pro-inflammatory cytokines.

In summary, colchicine disrupts microtubule formation, leading to interference with NLRP3 inflammasome assembly while anakinra inhibits the inflammasome pathway by blocking the IL-1 receptor. Both medications contribute to the downregulation of inflammatory responses associated with the inflammasome pathway.

1.3. Immunohistochemistry acquisition

The paraffin-embedded placental tissues were cut in serial 3-µm-thick sections. First, the tissue sections were dewaxed at 56°C for 2 hours and then antigen retrieval was performed using the PT Link pH 9 buffer (Agilent Dako, Santa Clara, CA) for 40 minutes at 95°C then cooled down to 65°C for 20 minutes. Next, sections were incubated with an endogenous peroxidase inhibitor (200 uL for each tissue section) for 10 minutes at room temperature and after being washed in TBS-tween buffer (tris-buffered saline and polysorbate 20, Dako) were incubated with the primary antibodies for one hour at room temperature (anti-CD68, Abcam, EPR20545, anti-NLRP3, Enzolife, ALX804819 ; anti-PYCARD, Sigma-Aldrich, HPA-049074). Each antibody was used at a 1/100 dilution. Sections were washed again, and immunoreactive signals were visualized using the Dako EnVision FLEX peroxidase detection system, as per the manufacturer’s instructions. Finally, the sections were counterstained with hematoxylin and mounted in Eukitt® mounting medium. Sections were visualized

with a Nikon DS-Fi2 microscope (Leica) and images were acquired using the NIS Elements imaging software.

Negative controls were performed by repeating the experiment in absence of the primary antibody.

1.4. RNA quantification and preparation for NanoString nCounter mRNA analysis

Total RNA from serial 20-µm-thick sections of formalin-fixed paraffin-embedded (FFPE) was extracted using a RNeasy FFPE kit (QIAGEN) according to the manufacturer's protocol. RNA quantification was performed using the Thermo Scientific™ NanoDrop 2000. For each sample, 185 ng of purified RNA was added to 8 µL of Master Mix Reporter CodeSet and 2 µL Capture ProbeSet using an nCounter master kit as recommended (NanoString Technologies)

1.5. Gene Set Enrichment and Modeling of Gene Interactions Networks

Upregulated genes were imported into the Ingenuity Pathways Analysis (IPA) software (Ingenuity Systems; Qiagen, Redwood City, CA, USA) www.ingenuity.com/). Mechanistic networks (MN) analysis was performed using IPA to generate signaling cascades. IPA uses precise algorithms to predict functional regulatory networks from gene expression data (4).

2. SUPPLEMENTARY FIGURES AND TABLES

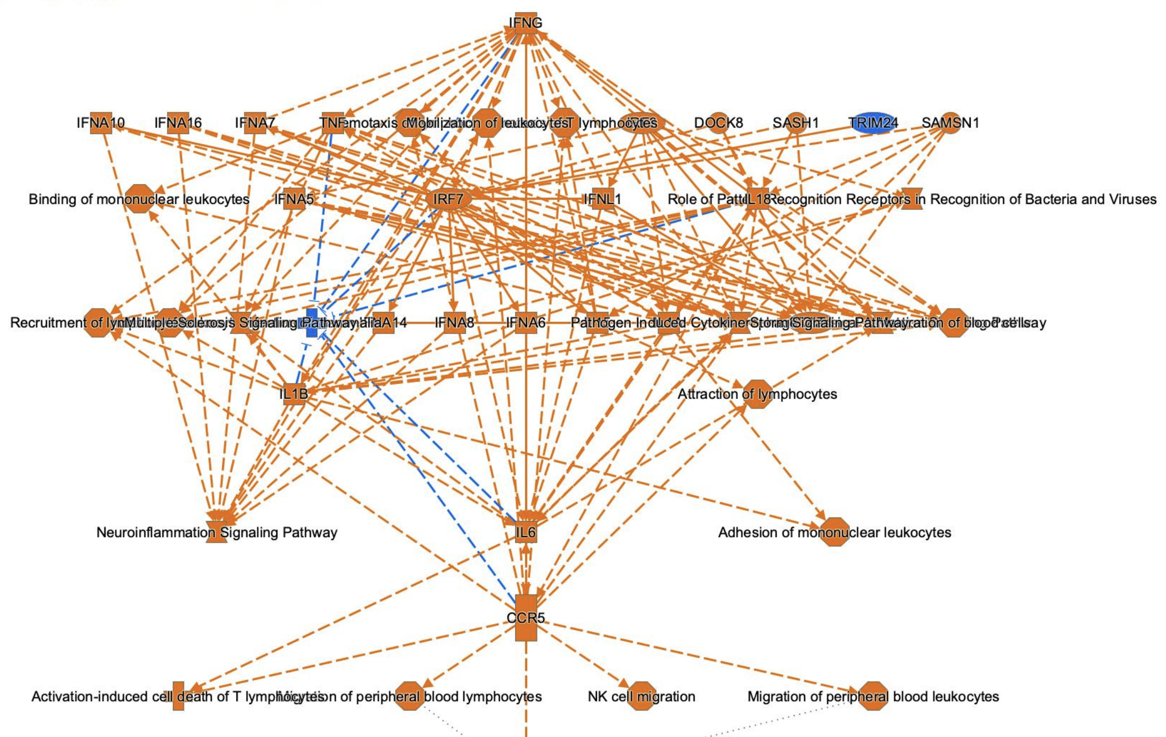
Supplementary Table 1: Patient characteristics for transcriptomic analysis of inflammasome pathway

	CHI	Healthy donors
Number of subjects	18	6
Age, years (median, range)	30.9 [22-43]	30.0 [21-39]
Race, n (%)		
Caucasian	6 (33)	5 (83)
Asian	0 (0)	0 (0)
Black	7 (39)	0 (0)
Missing data	5 (28)	1 (16)
BMI, kg/m^2 (median, range)	21.6 [17.1-34.0]	19.9 [17.2-28.0]
Active smoking, n (%)	8 (44)	2 (33)
Hypertension disorder history, n (%)	0 (0)	1 (17)
CHI history, n (%)	2 (11)	-
Ongoing Treatment (when analysis was performed), n (%)	None: 15 (83)	None: 5 (28)
	LDA: 2 (11)	LDA: 1 (6)
	LDA + LMWH + Ig IV + HCQ: 1 (6)	
Obstetrical outcomes, n		
Preeclampsia	1	0
SGA	16	0
Stillbirth	1	0
Cesarean section	7	3
Gestational age at birth (median, range)	37.7 [35.0-41.3]	37.8 [36.3-39]
Birth weight, g (median, range)	2247 [940-3160]	3018 [2590-3540]

Abbreviations: BMI, body mass index; CHI, chronic histiocytic intervillositis; SGA, small for gestational age; LDA, low dose aspirin; LMWH, low molecular weight heparin; IV Ig, intravenous immunoglobulins; HCQ, hydroxychloroquine

Supplementary Figure 1: TOP-Scoring regulatory cytokines

The Top-Scoring regulatory cytokines identified with the IPA software corresponded to interferon type I (IFN type I), tumor necrosis factor (TNF), interleukin-6 (IL-6), interleukin-1B (IL-1B), and interleukin-18 (IL-18). Figure obtained from Ingenuity Pathways Analysis (IPA) software (Ingenuity Systems; Qiagen, Redwood City, CA, USA, www.ingenuity.com/) and published with the permission of IPA Qiagen.



Supplementary Table 2: List of genes used to calculate zScores for each gene signature

	Signature Targets
IL-1 β	*CCL2, CCL20, CXCL2, IL1A, IL1B, IL6, LILRB1, NFKB1, NFKBIZ, POU2F2*
IL-18	*ARG1, B2M, BAX, BCL2, BID, CASP3, CASP8, CCL18, CCL19, CCL2, CCL20, CCL3, CCL4, CCL5, CD36, CD81, CD83, CEBPB, CHUK, CTNNB1, CXCL2, FADD, FAS, FN1, ICAM1, IFNG, IKBKB, IL10, IL12B, IL13, IL18, IL18R1, IL18RAP, IL1B, IL2RA, IL6, IL9, IRAK1, IRF1, ITGA2B, LCK, MAPK1, MYD88, NFKB1, NFKB2, NFKBIA, NFKBIZ, NOS2, PRKCD, PTGS2 RELA, SOCS3, SPP1, TBX21, TNF, TNFAIP3, TNFSF11, TP53, TRAF1, TRAF6*
Inflammasome	*IL1B, IL18, MEFV, casp1, NLRP3, PYCARD*

Supplementary Table 3: Baseline maternal characteristics of the three consecutive patients treated with inflammasome blockade therapies during pregnancy.

	Patient 1	Patient 2	Patient 3
Age range	30-35	40-45	25-30
Body Mass Index (kg/m^2)	22	27	24
Active smoking	No	Yes	Yes
Hypertension disorder history	No	No	No
Diabetes mellitus	No	No	No
History of pregnancy loss			
Preeclampsia	0	0	0
< 14 WG only—no	6	6	0
- Abortion	0	1	
- Miscarriage—no./total no.	6	5	0
- Ectopic or molar pregnancy—no./total no.	0	0	0
≥ 14 WG only—no	0	3	2
Number of live births	1/7 pregnancies	0/9 pregnancies	1/2
Confirmed CHI	3/6 fetal losses	5/8 fetal losses	2/2 fetal losses
Previous treatment received	LDA, LMWH, PRED, HCQ	LDA, LMWH, PRED, HCQ, IV Ig, AZA, ADA	LDA, LMWH

Abbreviations: CHI, chronic histiocytic intervillositis; IUGR, intra-uterine growth restriction; IV Ig, intravenous immunoglobulins; LDA, low dose aspirin; LMWH, low molecular weight heparin; PRED, prednisone; TOP, termination of pregnancy; AZA, azathioprine; ADA adalimumab

Supplementary Table 4: Maternal blood immune analysis of the three consecutive patients treated with inflammasome blockade therapies during pregnancy.

	Patient 1			Patient 2			Patient 3		
	Baseline	Pregnancy	Post partum	Baseline	Pregnancy	Post partum	Baseline	Pregnancy	Post partum
Leucocytes G/L	6,7	9,2	4,5	6,5	10	8	6,3	9	8,1
Neutrophiles G/L	4,3	6,9	2,6	4	6,3	5,2	3,7	6,4	5,6
Lymphocytes G/L	1,53	1,6	1,4	1,9	2,5	2	1,6	1,7	1,7
C Reactive Protein mg/dL	5,8	6,1	1,9	2,3	6,1	5	1,3	4,3	3,7
Immunoglobulines g/L	8,4	NA	9,3	9,2	8.9	7	10,5	6,9	NA
B Lymphocytes G/L (%)	0.20 (9)	0,18 (9,2)	0,13 (9,3)	0,2 (8,6)	0,22 (8)	0,24 (8,2)	0,2 (9)	0,21 (9,3)	0,19 (8,9)
CD4 T Lymphocytes G/L (%)	0,76 (43)	0,7 (45)	0,68 (48)	0,9 (56)	1 (54)	1,2 (55)	0,91 (53)	1 (50)	0,99 (52)
CD8 T Lymphocytes G/L (%)	0,42 (21)	0,33 (20)	0,33 (23,7)	0,36 (21,9)	0,41 (24)	0,4 (23)	0,54 (31)	0,55 (30)	0,5 (30)
HLA DR T Lymphocytes %	1,9	2	3	7,6	5	5,3	7,9	8	8,3
Antinuclear antibodies	0	0	0	0	NA	0	1/320	1/160	1/320
Anti-DNA antibodies	0	0	0	0	NA	0	0	0	0
Anti-ENA Antibodies	0	0	0	0	NA	0	0	0	0
C3 level g/l	0,9	NA	1,02	1,29	NA	1,26	1,5	1,65	1,18
C4 level g/l	0,18	NA	0,22	0,21	NA	0,22	0,27	0,42	0,3
Lupus anticoagulant	negative	negative	negative	negative	negative	negative	positive	positive	positive
Anti-cardiolipin antibody	negative	negative	negative	negative	negative	negative	negative	NA	negative
Anti-β2glycoprotein-I antibody	negative	negative	negative	negative	negative	negative	negative	NA	negative

Abbreviations : ENA : extractable nuclear antibodies, NA : not avail

3. REFERENCES

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