Bidirectional regulation between 1st trimester HTR8/SVneo trophoblast cells and in vitro differentiated Th17/Treg cells suggest a fetal-maternal regulatory loop in human pregnancy

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Problem: During normal pregnancy, delicate crosstalk is established between fetus-derived trophoblasts and maternal immune cells to ensure maternal-fetal tolerance and successful placentation. Dysfunction in these interactions has been highly linked to certain pregnancy complications.

Method of study: Naïve CD4⁺ T cells were cultivated with or without 1st trimester derived trophoblast cell line HTR8/SVneo cells in the absence or presence of T helper 17 (Th17) or regulatory (Treg) cell-inducing differentiation conditions. After 5 days of co-culture, HTR8/SVneo cells and CD4⁺ T cells were harvested and analyzed using flow cytometry.

Results: CD4⁺ T cells exposed to HTR8/SVneo cells showed enhanced induction of CD4⁺Foxp3⁺Treg cells with strong expression of TGF-β1 and inhibitory molecules (cytotoxic T lymphocyte-associated protein-4 [CTLA-4], T-cell immunoglobulin mucin-3 [Tim-3], and programmed cell death-1 [PD-1]). Though not effecting Th17 differentiation, exposure to HTR8/SVneo cells promoted increased expression of proliferative and apoptotic markers on Th17 cells. Co-culture with Th0 cells, or differentiated Th17 or Treg cells, down-regulated Caspase-3 and MMP-9 (but not MMP-2) expression in HTR8/SVneo cells, while promoting Ki67 expression.

Conclusions: HTR8/SVneo cells regulated maternal CD4⁺ T-cell differentiation, resulting in the expansion of immunosuppressive Treg cells, while CD4⁺ T cells might promote the growth, and control the invasiveness of HTR8/SVneo cells. Thus, a bidirectional regulatory loop might exist between trophoblasts and maternal immune cell subsets, thereby promoting harmonious maternal-fetal crosstalk.

Keywords
HTR8/SVneo cells, pregnancy, Th17 cells, Treg cells, trophoblasts
The establishment of maternal-fetal tolerance and successful placenta tion are key events during early pregnancy. Under normal conditions, the maternal immune system accepts the semi-allogene neic fetus, which protects both mother and fetus against infection. Disruption of this immune balance, however, causes the placenta and fetus to be attacked as a foreign organ transplant, resulting in pregnancy failure. Extravillous trophoblasts (EVT), the main cell type involved in the placenta tion process, invade the underlying decidua, dissolve the extracellular matrix (ECM), and migrate into the uterine spiral arteriolar walls, remodeling the uterine vasculature. Inadequate EVT invasion has been closely associated with several pregnancy-associated diseases, including recurrent pregnancy loss (RPL), pre-eclampsia (PE), and gestational trophoblastic diseases.

T-cell subsets, especially CD4+ helper T (Th) cells, play a pivotal role in successful pregnancy. Driven by a set of transcriptional regulators and cytokines, naive CD4+T cells are able to differentiate into distinct subsets, including Th1, Th2, Th17, and Treg cells. A polarization toward Th2 bias in the maternal immune response has long been considered the main mechanism of tolerance induction toward the fetus. According to recent studies, the balance between regulatory (Treg) and T helper 17 (Th17) cells is also important in the maintenance of normal pregnancy, whereas the shift in the Th17/Treg ratio toward Th17 cells has been proposed as a cause for several pregnancy-associated diseases, such as RPL, PE, and gestational diabetes mellitus.

Decidual immune cells (DICs) not only regulate the maternal immune response to promote fetal semi-allograft tolerance but also mediate the implantation and trophoblast invasion. At the same time, trophoblasts mediate interactions between the fetus and mother for the exchange of nutrients, gases, waste products, as well as for the regulation of immune tolerance. EVTIs potential candidates for educating maternal immune cells to generate a tolerant microenvironment at the maternal-fetal interface. At present, studies regarding the interaction between trophoblasts and DICs have shown that trophoblasts have the unique ability of instructing DICs to develop a regulatory phenotype for fetal tolerance. However, the regulatory effect of trophoblasts on CD4+T-cell differentiation, especially on Th17/Treg differentiation, remains poorly understood. Knowledge regarding the influence of Th17/Treg differentiation on the biological behaviors of trophoblasts is also limited.

Based on the aforementioned observations, we assumed that trophoblasts might affect Th17/Treg cell differentiation from naive CD4+T cells, leading to the induction of Treg cell expansion at the maternal-fetal interface. In turn, differentiated CD4+T cells might affect the biological functions of trophoblasts. Based on this hypothesis, we investigated the effect of trophoblasts on Th17/Treg cell differentiation from naive CD4+T cells and trophoblast phenotypic markers of function modulated by Th17/Treg cells generated in vitro using the immortalized human first-trimester EVT cell line HTR8/SVneo, which has been widely used as a substitute for human primary trophoblasts.
2.4 | Co-culture of CD4+T and HTR8/SVneo cells

HTR8/SVneo cells (2 × 10^5) were initially cultured in 6-well flat-bottom plates in RPMI 1640 medium supplemented with 10% FBS the day before co-culture with naïve CD4+T cells. When the adherent HTR8/SVneo cells reached 50%, 4 × 10^5 Th0, Th17, and Treg cells (according to the different differentiation agents mentioned previously) were seeded on the top of the HTR8/SVneo layer for 5 days in media comprising different differentiation agents. After induction, the number of CD4+T cells in each well was approximately 2 × 10^6. While in the co-culture system, the cell number was approximately double in this amount. To exclude the effect of HTR8/SVneo cell proliferation on CD4+T cells, HTR8/SVneo cells were pre-treated with mitomycin C for 30 min (10 mM) in some wells. For intracellular cytokine analysis of CD4+T cells, brefeldin A (10 μg/mL, BioLegend, USA), PM (50 ng/mL, BioLegend, USA), and ionomycin (1 μg/mL, BioLegend, USA) were added 4 hours before the end of the culture. HTR8/SVneo cells and CD4+T cells were then harvested, stained, and analyzed through flow cytometry. After culture, the proportion of live cells (assessed by the 7-AAD− cells) was around 90%.

2.5 | Flow cytometry

Cell surface molecular expression and intracellular cytokine production were evaluated using flow cytometry. AlexaFluor® 488-conjugated anti-human Foxp3 or MMP-9; FITC-conjugated anti-human CD4; PE-conjugated anti-human CD45RA, MMP-2, T-cell immunoglobulin mucin-3 (Tim-3), or TGF-β1; PE/CY7-conjugated anti-human IL-10, CD45 or IL-17A; PerCP-conjugated anti-human CD4; AlexaFluor® 647-conjugated anti-human Caspase-3, APC-conjugated anti-human CTLA-4; Brilliant Violet 421-conjugated anti-human Ki-67 or IL-17A; Brilliant Violet 510-conjugated anti-human CD45 or PD-1 antibodies (BioLegend, USA) were used. Details regarding Ab are presented in Table S1. For intracellular staining, cells were fixed and permeabilized using the Fix/Perm kit (BioLegend, USA). Flow cytometry was performed on a Beckman-Coulter CyAn ADP cytometer and analyzed with FlowJo software (Tree Star, USA).

2.6 | Statistical analysis

Normally distributed variables were presented as means and SD. ANOVA was used to evaluate differences in normally distributed variables with homogeneity of variance among groups. Variables with skewed distribution were described using median and interquartile range. The Kruskal-Wallis test was used to assess the differences in variables with skewed distribution among groups. The Bonferroni multiple comparisons test was performed for variables that showed a significant difference with Kruskal-Wallis test. A P value of <0.05 was considered significant. For variables with a P value less than 0.05 following ANOVA, the post hoc Dunnett t test was performed to determine differences between each group. All analyses were carried out using the GraphPad Prism 5 software (GraphPad, San Diego, CA).
been proposed as functional markers of specific T-cell subsets.\textsuperscript{19,20} Next, we analyzed CTLA-4, Tim-3, and PD-1 expression in in vitro-generated Th17/Treg cells cultivated with or without HTR8 cells. As shown in Figure 3, compared to Th0 cells, Th17 cells expressed higher levels of Tim-3 and PD-1, while Treg cells expressed more CTLA-4, Tim-3, and PD-1. HTR8/SVneo cells increased the expression of all inhibitory receptors in Treg cells, but had no influence on their expression in Th17 cells.

### 3.3 | CD4\(^+\)T cells modulate the biological behaviors of trophoblast

We then studied whether in vitro-generated Th17/Treg cells regulated the biological behaviors of HTR8/SVneo cells. After co-culture with Th0, Th17, or Treg cell, HTR8/SVneo cell apoptosis (assessed using Caspase-3\(^+\) cells) was inhibited, while HTR8/SVneo cell proliferation (assessed using the Ki-67\(^+\) cells) was promoted (Figure 4A,B). MMP-2 and MMP-9 are two important gelatinases involved in ECM remodeling during trophoblast invasion.\textsuperscript{21} The effects of CD4\(^+\)T cells on MMP-2 and MMP-9 expression were examined using flow cytometry. As shown in Figure 4C,D, CD4\(^+\)T cells decreased MMP-9 production in HTR8/SVneo cells, but had no effect on MMP-2 expression.

### 4 | DISCUSSION

A successful pregnancy relies on the maternal immune system's tolerance toward the semi-allogeneic fetus and sufficient placental...
As such, a complex and reciprocal interaction between the maternal immune system and fetal trophoblasts is required to maintain normal pregnancy. The present study utilized the well-characterized human first-trimester EVT cell line HTR8/SVneo to demonstrate the notable role of these cells in regulating CD4+ T-cell differentiation, resulting in the expansion of immunosuppressive Treg cells. Meanwhile, CD4+ T cells also modulated the biological functions of HTR8/SVneo cells. To our knowledge, this study presented novel findings suggesting a unique maternal-fetal co-culture system could be used to regulate the Th17/Treg ratio and that a positive regulatory loop might exist between trophoblasts and maternal immune cell subsets, promoting the harmonious maternal-fetal crosstalk.

Upon encountering antigens presented by antigen presenting cells or being driven by a set of cytokines, naïve CD4+ T cells are able to differentiate into distinct subsets, including Th1, Th2, Th17, and Treg cells. Th2 bias at the maternal-fetal interface has long been considered as the main mechanism of tolerance induction toward the fetus. Th17 cells play a critical role in inducing of inflammation, while abnormal Th17 cell levels have been associated with the
FIGURE 4 Differentiated CD4⁺T cells modulated the biological functions of HTR8/SVneo cells. HTR8/SVneo cells were co-cultured with Th0, Th17 and Treg cells (with the indicated agents) for 5 days. Caspase-3 (A), Ki-67 (B), MMP-2 (C) and MMP-9 (D) expression in HTR8/SVneo cells was analyzed through flow cytometry. ***P < 0.001, compared to the Ctrl group. Data are represented as the mean ± SD. Flow cytometry plots are representative of three independent experiments.
expression was promoted, suggesting that CD4+ T cells promoted the growth of trophoblasts and controlled the invasiveness of human trophoblasts. MMP-2 is the main gelatinase involved in the function of early first-trimester trophoblast (6-8 weeks). In the late first trimester (8-12 weeks), however, both MMP-2 and MMP-9 participate in trophoblast invasion. Accordingly, our findings show that CD4+ T cells down-regulated MMP-9 but not MMP-2 expression in HTR8/SVneo cells. As such, the interaction between CD4+ T cells and trophoblast might play a role in the prevention of excessive trophoblast invasion in the late first trimester of pregnancy. Though Th17 cells had been reported to participate in pregnancy-related pathologies, our study and others showed that Th17 cells played important roles in regulating trophoblast function. Recent findings have also indicated that not all Th17 cells are pro-inflammatory given that Th17 subpopulations with diverse functions may exist. More investigations are needed to explore whether non-pathogenic Th17 subsets and pro-inflammatory Th17 subpopulations co-exist at the maternal-fetal interface, which subsets are involved in the functional regulation of trophoblast, and which mechanisms are used to modulate Th17 subsets during pregnancy. Questions remain regarding the validity of using immortalized cell lines to represent the in vivo environment, though HTR8/SVneo cells have been proven effective for recapitulating key aspects of EVTs. Nonetheless, the interaction between primary trophoblasts and naïve CD4+ T cells and the related mechanisms still require further study. Moreover, further ELISA assay and flow cytometry sorting technology would be needed to determine cytokine production by different T-cell subsets. Despite these limitations, the results presented in the present study undoubtedly demonstrated that trophoblasts might influence the differentiation of naïve CD4+ T cells, while CD4+ T cells could in turn modulate the biological functions of trophoblasts, resulting in Treg expansion and adequate trophoblast invasion during pregnancy.

**CONFLICT OF INTEREST**

The authors disclose no conflict of interests.

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Additional supporting information may be found online in the Supporting Information section at the end of the article.