An imbalance of the IL-33/ST2-AXL-efferocytosis axis induces pregnancy loss through metabolic reprogramming of decidual macrophages

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Abstract
During embryo implantation, apoptosis is inevitable. These apoptotic cells (ACs) are removed by efferocytosis, in which macrophages are filled with a metabolite load nearly equal to the phagocyte itself. A timely question pertains to the relationship between efferocytosis-related metabolism and the immune behavior of decidual macrophages (dMΦs) and its effect on pregnancy outcome. Here, we report positive feedback of IL-33/ST2-AXL-efferocytosis leading to pregnancy failure through metabolic reprogramming of dMΦs. We compared the serum levels of IL-33 and sST2, along with IL-33 and ST2, efferocytosis and metabolism of dMΦs, from patients with normal pregnancies and unexplained recurrent pregnancy loss (RPL). We revealed disruption of the IL-33/ST2 axis, increased apoptotic cells and elevated efferocytosis of dMΦs from patients with RPL. The dMΦs that engulfed many apoptotic cells secreted more sST2 and less TGF-β, which polarized dMΦs toward the M1 phenotype. Moreover, the elevated sST2 biased the efferocytosis-related metabolism of RPL dMΦs toward oxidative phosphorylation and exacerbated the disruption of the IL-33/ST2 signaling pathway. Metabolic disorders also lead to dysfunction of efferocytosis, resulting in more uncleared apoptotic cells and secondary necrosis. We also screened the efferocytotic molecule AXL regulated by IL-33/ST2. This positive feedback axis of IL-33/ST2-AXL-efferocytosis led to pregnancy failure. IL-33 knockout mice demonstrated poor pregnancy outcomes, and exogenous supplementation with mouse IL-33 reduced the embryo losses. These findings highlight a new etiological mechanism whereby dMΦs leverage immunometabolism for homeostasis of the microenvironment at the maternal–fetal interface.

Keywords Efferocytosis · IL-33/ST2 axis · Metabolic immune reprogramming · Decidual macrophages · Recurrent pregnancy loss

Introduction
Efferocytosis is the clearance of dying and dead cells, which is performed by professional and nonprofessional phagocytes [1, 2]. This biological behavior has been artificially divided into recognition, engulfment and immunomodulation, three steps involving several signals (“find-me”, “eat-me” and “don’t-eat-me” signals) [3]. Many cells undergo apoptosis during embryonic development, and some studies have shown that a certain number of apoptotic cells (ACs) at the maternal–fetal interface modulate maternal immunological function [4]. Decidual macrophages (dMΦs) and epithelial cells at the interface can clear these dying or dead cells to ensure successful pregnancy [5]. Deficient or excessive efferocytosis can contribute to the origin and development of pathological...
conditions in pregnancy, such as unexplained recurrent pregnancy loss (RPL), preeclampsia, antiphospholipid syndrome, fetal growth restriction, and rupture of ectopic pregnancy, and research on its exact mechanisms is in the early stage [6].

Efferocytosis-related metabolism might be one of the mechanisms. One recent study found that efferocytosis could induce mitochondrial respiration to polarize macrophages to the anti-inflammatory phenotype to promote wound healing and organ repair following myocardial infarction in the heart. However, one study showed the uptake of glucose and the release of lactate during efferocytosis in the abdominal environment. During the first trimester of human pregnancy, low oxygen tension or hypoxia is essential for proper placentation and placental function. The maternal–fetal interface presents a unique microenvironment, while efferocytosis-related metabolism and its roles in the maternal–fetal interface are still poorly understood.

Interleukin-33 (IL-33) is a pleiotropic cytokine that is released from the nucleus to the outside of various cells in response to tissue injury, stress or infection, acting as an “alarmin” in the immune system. IL-33 exerts its biological function by binding to the IL-1RL1 receptor (also known as ST2) and the coreceptor IL-1 receptor accessory protein (IL-1RAcP). Additionally, the secreted isoform of ST2 (also known as sST2) is a decoy receptor for IL-33 and competes with membrane-bound ST2 to block the IL-33/ST2 signaling pathway [7]. IL-33 was shown to be closely associated with trophoblast cell proliferation and placental growth [8]. Our previous work revealed that IL-33 derived from decidual stromal cells (DSCs) promoted the proliferation and invasion of DSCs by upregulating the expression of the chemokine CCL2/CCR2, induced Th2 bias and suppressed the cytotoxicity of decidual natural killer cells (dNKs) [9, 10]. A clinical study found that women with a viable fetus who eventually miscarried had dysregulated levels of serum IL-33 and potentially sST2 at six weeks of gestation [11]. Of note, the IL-33/ST2 axis participates in regulating immune reprogramming of bone marrow-derived macrophages and FceR1α+ macrophages of squamous cell carcinoma by affecting macrophage metabolism [12, 13]. Therefore, a similar relationship may exist between IL-33/ST2 signaling and the efferocytosis of dMΦs, and the possible connections may play an important role in maintaining homeostasis at the maternal–fetal interface.

Here, we focused on the effect of the IL-33/ST2 axis on dMΦs efferocytosis and the underlying relationship between efferocytosis-related metabolism and the homeostasis of the maternal–fetal interface. In the current research, we identified a novel pathological mechanism of pregnancy failure resulting from disorder of the IL-33/ST2 axis followed by a pathological positive feedback loop involving the metabolism and polarization status of dMΦs at the maternal–fetal immune interface and clarified the molecule associated with efferocytosis downstream of the IL-33/ST2 axis.

Materials and methods

Tissue collection and primary isolation

Tissue samples used in this study were collected after obtaining informed consent approved by the Ethics Committee of Obstetrics and Gynecology Hospital, Fudan University. Decidual tissues were acquired from women with normal gestation (normal group; \( n = 145; \) age, 28.0 ± 2.93 years, mean ± SD; gestational age, 52.34 ± 3.25 days) who chose elective vaginal termination for nonmedical reasons and patients with RPL (RPL group; \( n = 72; \) age, 30.14 ± 3.07 years, mean ± SD; gestational age, 54.58 ± 3.57 days) who experienced the loss of two or more clinical pregnancies before 20 weeks of pregnancy that were not necessarily consecutive (documented by ultrasonography or histopathologic examination) [14]. Patients who had abnormal vaginal bleeding, severe abdominal pain, or other typical symptoms of inflammation were excluded. Fresh decidual tissues were rinsed in 1 × PBS twice to remove blood clots and finely minced within 60 min after the operation.

Then, decidual stromal cells (DSCs) and decidual immune cells (DICs) were isolated and cultured. The washed decidual tissues were cut into <1-mm-thick sections and digested with Dulbecco’s modified Eagle’s medium (DMEM)/F12 (Cat# SH30023.01, HyClone) containing 20% collagenase type IV (Cat# C5138, Sigma) and 5% trypsin-DNase I (Cat# A3778, PanReac AppliChem). After discontinuous Percoll gradient centrifugation (Cat# 17-0891-09, GE Healthcare), DSCs and DICs ranged in density from 1.042 to 1.062 g/mL, and 1.062 to 1.077 g/mL, respectively. Then, DSCs were isolated and cultured in DMEM/F12 complete medium with 10% fetal bovine serum (FBS, Cat# 10099-141, Gibco) in the presence of 100 U/mL penicillin and 100 mg/mL streptomycin, and DICs were cultured in complete RPMI 1640 medium (Cat# GNM-23471-S, Genom) and FBS. Both types of cells were placed in culture flasks at 37 °C under 5% CO₂ for subsequent processing.

Isolation and treatment of decidual macrophages (dMΦs)

Positive selection with anti-CD14 microbeads was used to isolate decidual macrophages from DICs according to the manufacturer’s instructions (Cat# 130-050-201, Miltenyi Biotec). The purity of the enriched dMΦs reached more than 90%, which was confirmed through flow cytometry [15]. The enriched dMΦs, as well as the THP-1 cell line,
were cultured in complete RPMI 1640 medium with 10% FBS with or without recombinant human ST2/IL-33R Fc Chimera (Cat# 523-ST-100, R&D Systems), recombinant human IL-33 (Cat# 3625-IL-010, R&D Systems), recombinant human GAS6 (Cat# 885-GSB-050, R&D Systems), the PI3K inhibitor LY294002 (Cat# S1777, Beyotime), the AKT inhibitor VIII (Cat# SF2784, Beyotime), the MEK1/2 inhibitor U0126 (Cat# S1901, Beyotime), the JNK inhibitor SP600125 (Cat# S1876, Beyotime), and the P38 MAPK inhibitor SB203580 (Cat# SC0380, Beyotime) and cultured in a 37 °C incubator under 5% CO₂ for further processing.

**Mice and the C57BL/6 J pregnant mouse model with IL-33 gene knockout (IL-33⁻/⁻)**

All experimental procedures that involved animals were performed in accordance with the Guide for the Care and Use of Laboratory Animals (China). The experimental methods in particular were carried out following the approved guidelines. IL-33⁻/⁻ mice were established by Shanghai Model Organisms Center, Inc. Eight-week-old wild-type (WT) C57BL/6 J ♂ mice and IL-33⁻/⁻ ♀ mice were mated in a 2:1 ratio as a control group. IL-33⁻/⁻ ♀ mice and WT ♂ mice were mated 2:1 as the experimental group and sacrificed at G7.5 and G14.5 days after the appearance of vaginal plugs (G0.5 day). In parallel experiments, IL-33⁻/⁻ ♀ mice were mated with WT ♂ mice, and IL-33⁻/⁻ ♀ mice were mated with vaginal plugs were randomly divided into two groups. One group of mice was injected intraperitoneally with 100 µl of normal saline on G3.5, 7.5 and 11.5 days and was used as the control group. The other group was intraperitoneally injected with 200 ng/100 µl mouse IL-33 protein on G3.5, 7.5 and 11.5 days. Both groups of mice were sacrificed at 14.5 days of gestation. The pregnancy rate, embryo number, embryo absorption rate, crown-rump length and weight of the embryos, and diameter and weight of the placentas of these groups were calculated. Moreover, minced uteri were digested in DMEM/F12 supplemented with 50% collagenase Type IV for 45 min at 37 °C with gentle agitation. The single-cell suspension of the mice was filtered through a 400-mesh sieve and used for subsequent FCM assays. Pregnancy rates were calculated and expressed as the percentage of pregnant mice among all mated female mice.

**Apoptotic assay**

To obtain apoptotic DSCs, we incubated primary DSCs with cobalt chloride (CoCl₂, 0–600 µM, Cat# 449776, Sigma) for 24 h. After three washes with PBS, the cells were digested with trypsin and resuspended in a PE Annexin V Apoptosis Detection Kit (Cat# 559763, BioLegend). The administration of CoCl₂ resulted in increases in the number of apoptotic DSCs compared with those only treated with trypsin (60–70% vs. 10–20%). The apoptosis rate was defined as follows: (Annexin V +7-AAD+ cells + Annexin V +7-AAD− cells)/total cells × 100%.

**In vitro efferocytosis assay**

In the in vitro efferocytosis assay, apoptotic DSCs were labeled with a CFSE Cell Division Tracker Kit (Cat# 423801, BioLegend) for 20 min at 37 °C. Following CFSE labeling, DSCs were incubated with dMΦs at a ratio of 2:1 (target: macrophage) for 120 min at 37 °C. The efficient efferocytosis rate was determined by flow cytometry, and each dataset was first gated on CD14-APC-positive macrophages. The efferocytosis rate was calculated as [CD14⁺CFSE⁺ cells/CD14⁺ cells × 100%]. In the assay with ST2-OE THP-1 cells, which could fluoresce spontaneously in green-FICT, apoptotic DSCs were labeled with Tag-it Violet Proliferation and Cell Tracking Dye (Cat# 425101, BioLegend). The efferocytosis rate was determined as follows: BV421⁺FITC⁺ cells/FITC⁺ cells × 100%.

**Seahorse analysis**

For measurement of the oxygen consumption rate (OCR) and glycolytic extracellular acidification rate (ECAR), normal dMΦs and RPL dMΦs were treated with apoptotic DSCs (ACs) for 1 h and added to XF96 cell culture microplates coated with CellTak (the number of dMΦs in each well was 1 × 10⁵, and the number of ACs was 5 × 10⁴). Oxidative phosphorylation (OXPHOS) was tested with a mitochondrial stress test kit (Cat# 103015–100, Seahorse Bioscience). The following were injected: the ATP synthesis inhibitor oligomycin (1.5 µM), carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP; 1.5 µM) to uncouple ATP synthesis, rotenone (100 nM) to block complex I, and antimycin A (1 µM) to block complex III. Glycolysis was measured using a glycolysis stress test kit (Cat# 103020–100, Seahorse Bioscience), and the following were injected successively: 20 mM glucose, 1 µM oligomycin (ATP synthesis inhibitor), and 80 mM 2-DG (glycolysis inhibitor). The changes in OCR and ECRA were measured by a Seahorse XF96 extracellular flux analyzer (Agilent Technologies). The basic OCR, ATP production, maximum respiration of OXPHOS, spare capacity, basic ECAR, glycolysis, maximum respiration of glycolysis and glycolytic reserve were generated by Wave Desktop software (Agilent Technologies).

**Mitochondrial membrane potential (mtΔΨ) assay**

The mitochondrial membrane potential (mtΔΨ) was detected with JC-1 (Cat# C2006, Beyotime). The dMΦs with or without AC were treated with 1 × JC-1 dye for 20 min followed by flow cytometry. JC-1 is a fluorescent probe
widely used to detect mitochondrial membrane potential. It has two forms: monomer and aggregates. Red fluorescence (Ex/Em = 585/590 nm) is a sign of active mitochondrial function and high mtΔΨ, while green fluorescence (Ex/Em = 514/529 nm) in the cells suggests poor mitochondrial function with low mtΔΨ. The red/green fluorescence ratio (590 nm/529 nm) indicated the value of mtΔΨ and mitochondrial function.

Flow cytometry (FCM)

Cells were collected and incubated with corresponding fluorochrome-conjugated antibodies for 35 min at room temperature. For endonuclear IL-33 and intracytoplasmic TGF-β IFN-γ, we first fixed and permeabilized the cells with Bio-Legend’s FOXP3 Fix/Perm Buffer Set (Cat# 421403). The fluorescein-labeled antibodies used in human cells were as follows: CD14-APC-CY7 (Cat# 301820), CD80-PE (Cat# 305412), CD86-APC (Cat# 305208), CD163-BV421 (Cat# 333612), and CD206-PE-CY7 (Cat# 321124), all obtained from BioLegend; and ST2-PE (Cat# FAB5231P), IL-33-APC (Cat# IC625A), and AXL-PE (Cat# FAB154P), all purchased from R&D Systems. In addition, the fluorescent-labeled antibodies applied in mouse cells are listed below: mCD45-BV421 (Cat# 103126), mCD11/b-PE-CY7 (Cat# 101216), mF4/80-FITC (Cat# 123108), mTGF-β-PE (Cat# 141305), mIFN-γ-BV421 (Cat# 505842), mCD206-APC (Cat# 147108), mCD86-BV421 (Cat# 105032), mCD209-PE (Cat# 833004), mCD80-APC (Cat# 104713), all obtained from BioLegend; and mIL-33-PE (Cat# UC2744529, Invitrogen), mAXL-PE (Cat# FAB8541P, R&D Systems), PI3K-AF488 (Cat# ab225371), p-PI3K-PE (Cat# ab278691), AKT-AF488 (Cat# 50845S, CST), p-AKT-PE (Cat# 558275, BD Pharmingen), ERK1/2-AF488 (Cat# 4780S, CST), and p-ERK1/2-PE (Cat# 612566, BD Pharmingen). The samples were assessed with a CytoFLEX analyzer (Beckman Coulter) and analyzed with FlowJo_V10 software for Windows (TreeStar, Inc.).

Enzyme-linked immunosorbent assay (ELISA)

Cell supernatants were collected and centrifuged at 1000g for 20 min to remove cell debris, and the remaining supernatants were collected for further assays. ELISAs were performed to detect the titers of human IL-33 (Cat# 42590, BioLegend), sST2 (Cat# DST200, R&D Systems), TGF-β (Cat# 436707, BioLegend), and IFN-γ (Cat# 430104, BioLegend) according to the manufacturer’s protocols.

Construction of a stable ST2-overexpressing THP-1 cell line with lentivirus

THP-1 cells were obtained from Shanghai Institute for Life Science and maintained under standard culture conditions with RPMI 1640 medium (Cat# GNM-23471-S, Genom) and fetal bovine serum (FBS) (Cat# 10099–141, Gibco) at 37 °C with 95% normal air and 5% CO2. The lentiviral expression systems were purchased from Shanghai GeneChem Co., Ltd. The modeling multiplicity of infection (MOI) was 50. The viral infection-enhancing reagent was HitransG A. After transfection, the virus media were harvested, and cells were treated for 72 h with negative control (NC) lentivirus and ST overexpression (ST2-OE) lentivirus. The negative control (NC) group and ST2-OE THP-1 cells treated with IL-33 (2 ng/ml, 48 h) were then detected by RNA-seq analysis (BerryGenomics Company, No: IBFC2018631).

RNA isolation and quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from cells with TRIzol (Cat# 15596026, TaKaRa) according to the manufacturer’s protocol. The RNA was reverse transcribed to cDNA with the PrimeScriptTM RT reagent Kit (Cat# RR047A, TaKaRa). Then, semiquantitative qRT-PCR was performed with TB Green Premix Ex Taq II (Cat# RR820A, TaKaRa). A comparative threshold cycle (CT) value was normalized to the values of GAPDH for each sample by using the 2−ΔΔCT method. Primer information is listed in Supplementary Table 1.

Western blot

Radioimmunoprecipitation assay (RIPA) buffer (Cat# P0013B, Beyotime), protease inhibitor cocktail (1:100, Cat# HY-K0010, MedChemExpress) and phosphatase inhibitor cocktail II (1:100, Cat# HY-K0022, MedChemExpress) were added to the cells to obtain the total proteins. A BCA Protein Assay Kit was used to measure the protein concentration (Cat# P0010, Beyotime). GoldBand 3-color regular range protein marker (Cat# 20351ES72, Yeasen) along with 10 µg protein was separated through electrophoresis on the 10% polyacrylamide gels (Cat# P0012AC, Beyotime), transblotted onto polyvinylidene difluoride membranes, and incubated with antibodies against GAPDH (1:2000, Cat# 4370, Cell Signaling Technology), actin (1:2000, Cat# 4695, Cell Signaling Technology), ST2 (1:1000, Cat# ab194113, Abcam), AXL (1:1000, Cat# ab227871, Abcam), p-AKT (1:2000, Cat# 4060, Cell Signaling Technology), AKT (1:1000, Cat# 4691, Cell Signaling Technology), p44/42 MAPK (Erk1/2) (1:2000, Cat# 4370, Cell Signaling Technology), p44/42 MAPK (Erk1/2) (1:1000, Cat# 4695,
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Dysfunction of the IL-33/ST2 axis promotes DSC apoptosis and dMΦs/THP-1 efferocytosis and results in an OXPHOS bias during efferocytosis

As previously mentioned, we observed that ST2 increases while IL-33 decreases in RPL dMΦs, and increased apoptotic cells in decidual tissue accompany these alterations. To further examine how the dysregulation of the IL-33/ST2 balance contributes to RPL, we treated DSCs or dMΦs/THP-1 cells with sST2 (200 ng/ml, 48 h) for the subsequent experiments.

As expected, exposure to sST2 led to increased apoptosis of DSCs (Fig. 2A). Additionally, treatment with sST2 promoted the efferocytosis of dMΦs from the women with normal pregnancies and THP-1 cells (Fig. 2B, D), suggesting that abnormally high levels of sST2 contribute to high apoptosis and efferocytosis in decidua from the patients with unexplained RPL by interfering with IL-33/ST2 signaling.

Mitochondrial membrane potential (mtΔΨ) can also reflect mitochondrial function. When fewer cells have low mtΔΨ, the intensity of OXPHOS is higher. The percentage of cells with low mtΔΨ was obviously decreased in dMΦs from the women with unexplained RPL (Fig. 2C), suggesting a higher level of OXPHOS in the RPL group during efferocytosis. Furthermore, this phenomenon was only observed in the presence of ACs. We treated THP-1 cells with IL-33 and sST2 with or without ACs. IL-33 increased the percentage of cells with low mtΔΨ, indicating a low level of OXPHOS. Treatment with sST2 had the opposite result (Fig. 2E). More importantly, IL-33 promoted glycolytic bias, while sST2 induced mitochondrial bias during efferocytosis of dMΦs (Fig. 2F, G) and THP-1 cells (Fig. 2H, I). These findings suggest that dysfunction of the IL-33/ST2 axis occurs in patients with RPL and increases apoptotic cells and efferocytosis with an OXPHOS bias. Of note, we found that the basic OCR and ECAR of dMΦs were lower than those of primary peritoneal macrophages and LR73 phagocytes in other studies [16, 17]. As shown in a comparison with the nondifferentiated THP1 cell line in Fig. 2H, I, this phenomenon might be a feature of this decidual population.
Efferocytosis is more dependent on glycolysis than OXPHOS

Owing to the disruption of energy metabolism of dMΦs from the patients with unexplained RPL, we further investigated the possible metabolic mechanism of the efferocytosis of macrophages using 2-deoxy-D-glucose (2-DG), a glucose analog that acts as a competitive inhibitor of glucose metabolism. Further analysis showed that the glycolytic level of THP-1 cells decreased immediately after 2-DG treatment, while 2-DG did not inhibit glycolytic metabolism after 24 h and 48 h of treatment (Fig. 3A). In subsequent experiments, therefore, 2-DG was added at the beginning of the efferocytosis assay, and it inhibited the efferocytosis of the THP-1 cell line and dMΦs (Fig. 3B, C). However, the OXPHOS inhibitor oligomycin had no such effect (Fig. 3D, E).

IL-33 deficiency increases the risk of pregnancy failure

Subsequently, TUNEL assay was carried out in pregnant WT and IL-33−/− mice to measure apoptotic cells at the maternal–fetal interface. To eliminate the interference of embryo genotypes, we observed apoptotic cells in the WT ♀ mouse and IL-33−/− ♂ mouse mating model and the IL-33−/− ♀ mouse and WT ♂ mouse mating model and found no significant difference in the number of apoptotic cells between the WT and IL-33−/− pregnant mice at G14.5 days (Fig. 4A). However, the number of apoptotic cells was markedly increased in the IL-33−/− pregnant mice at G14.5 days (Fig. 4B), suggesting that macrophages in mouse uterus (uMΦs) should compensate for the removal of increased apoptotic cells during early pregnancy, and the elevated efferocytosis might be a secondary reaction to the high apoptosis. Moreover, the uMΦs from pregnant IL-33−/− mice had a proinflammatory M1-biased phenotype (CD80highCD86highIFN-γhighCD163lowCD206low), whereas there was no M1 bias in dMΦs from the patients with unexplained RPL (Fig. 5A). Additionally, other proinflammatory markers, such as iNOS, IL1-β and TNF-α, were tested by RT-PCR after efferocytosis of dMΦs from the women with RPL (Fig. 5B). Similarly, these classical proinflammatory molecules (iNOS and TNF-α) did not decrease significantly in RPL dMΦs after efferocytosis.

Furthermore, we detected the secretion of IL-33, sST2, TGF-β and IFN-γ by dMΦs before and after efferocytosis. IL-33 secreted by dMΦs was relatively low, which was also consistent with our previous report. DSCs are one of the main sources of IL-33 at the maternal–fetal interface [10]. After efferocytosis, IL-33 secretion was increased but not significantly (Fig. 5C). After efferocytosis, RPL dMΦs secreted higher levels of sST2 (Fig. 5D) and thereby blocked IL-33/ST2 signal transduction. Normal dMΦs secreted higher levels of IFN-γ before and after efferocytosis, which should be helpful for trophoblast invasion and embryonic growth and development (Fig. 5E) [18]. In addition, RPL dMΦs secreted higher levels of IFN-γ before and after efferocytosis (Fig. 5F) [19], although its concentration was too low. The results suggest that dMΦs from the patients with RPL have a persistent M1 bias and high sST2 after efferocytosis.

IL-33 suppresses efferocytosis of dMΦs by downregulating the expression of the efferocytosis-related receptor AXL

To further investigate the regulatory mechanism of IL-33/ST2 on the efferocytosis of dMΦs, we generated ST2-overexpressing (ST2-OE) THP-1 cells (Figure S2A, B). The negative control (NC) cells and ST2-OE THP-1 cells treated with IL-33 were then detected by RNA-seq analysis. There
**A**

| TUNEL | WT | IL-33^- |
|-------|----|---------|
| DAPI |     |         |

**B**

| TUNEL | WT | IL-33^- |
|-------|----|---------|
| DAPI |     |         |

**C**

|       | G7.5 |       | G7.5 |       | G7.5 |       | G7.5 |       | G7.5 |       |
|-------|------|-------|------|-------|------|-------|------|-------|------|-------|
| CD80 (%) |      |       |      |       |      |       |      |       |      |       |
| of CD45/F480/CD11b+ |      |       |      |       |      |       |      |       |      |       |
| WT    |      |       |      |       |      |       |      |       |      |       |
| IL-33^- |      |       |      |       |      |       |      |       |      |       |

**D**

|       | G14.5 |       | G14.5 |       | G14.5 |       | G14.5 |       | G14.5 |       |
|-------|------|-------|------|-------|------|-------|------|-------|------|-------|
| CD80 (%) |      |       |      |       |      |       |      |       |      |       |
| of CD45/F480/CD11b+ |      |       |      |       |      |       |      |       |      |       |
| WT    |      |       |      |       |      |       |      |       |      |       |
| IL-33^- |      |       |      |       |      |       |      |       |      |       |

**E**

|       |     |       | Non-pregnant | Pregnant |
|-------|-----|-------|--------------|----------|
|       |     |       |              |          |
|       |     |       |              |          |

**F**

- WT
- IL-33^-  

**G**

|       |       |       |       |       |
|-------|-------|-------|-------|-------|
|       |       |       |       |       |

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The IL-33/ST2 axis inhibits AXL expression in dMΦs by activating the PI3K/AKT and ERK1/2 signaling pathways

To explore the signal transduction mechanism of the IL-33/ST2 axis and AXL, we analyzed the pathway enrichment of differentially expressed genes of the RNA-seq data. The PI3K/AKT and MAPK signaling pathways were mainly activated after IL-33 treatment of ST2-2OE THP-1 cells (Fig. 7A). Further analysis showed that stimulation of IL-33 inhibited AXL expression by upregulating the phosphorylation levels of AKT and ERK1/2 but not JNK or P38 (Figure S3A, B). Additionally, blocking the PI3K/AKT or ERK1/2 signaling pathway reversed the inhibitory effect of IL-33 on AXL expression in dMΦs and THP-1 cells (Fig. 7D–F and Figure S3C). However, treatment with the P38 inhibitor or JNK inhibitor did not have similar effects (Fig. 7B, C). We also observed that blocking the PI3K/AKT or ERK1/2 signaling pathway enhanced the efferocytosis of dMΦs and THP-1 cells (Fig. 7G and Figure S3D). Clinically, we tested the percentages of p-PI3K, p-AKT and p-ERK in dMΦs from the control subjects and the patients with RPL. We found that RPL dMΦs showed lower phosphorylation of PI3K, AKT and ERK than normal dMΦs (Fig. 7H). In an animal study, we also discovered lower phosphorylation of AKT and ERK in IL-33−/− mice (Figure S3E, F). Treatment of normal dMΦs with sST2 reduced the phosphorylation levels of PI3K, AKT and ERK. P-ERK was increased by IL-33, while p-PI3K and p-AKT levels were decreased (Fig. 7H). This finding is relevant as ERK inhibition had the greatest effect on AXL expression (with the lowest dose of 5 µM), while AKT and PI3K only had an effect with high doses of inhibitors. There were no significant differences in OXPHOS and glycolysis in the normal dMΦs treated with the PI3K inhibitor, AKT inhibitor or ERK inhibitor (Fig. S3G, H). We considered that there might be other pathways involved. Further protein–protein interaction network prediction analysis by the STRING database (https://www.string-db.org/) was performed (Figure S3I). Aryl hydrocarbon receptor (AHR), which plays an important regulatory role in a variety of biological functions of macrophages [23–25], may also be involved in the regulation of AXL by IL-33 and efferocytosis-related metabolism in macrophages. This issue requires further study. These data suggest that the suppression of IL-33 to efferocytosis of dMΦs is dependent on the downregulation of AXL expression mediated by the PI3K/AKT and ERK1/2 signaling pathways.

were 1131 genes with high expression and 16 genes with low expression in the ST2-OE group compared with the NC group (Figure S2C). Based on the differentially expressed genes, three signaling pathways (inflammatory pathway, phagocytosis pathway, and endocytosis pathway) were identified by Gene Ontology (GO) annotation (Fig. 6A). Among these genes, complement C3 and the efferocytosis-related receptor AXL should be emphasized, as they are involved in the three signaling pathways (Fig. 6B). Subsequently, the qRT-PCR results confirmed that the ST2-OE group of the implantation number of the pregnant WT and IL-33−/− mice at G7.5 days (G). The embryo absorption rate, weight and crown-rump length of embryos and the diameter and weight of placentas of pregnant WT and IL-33−/− mice at G14.5 days. Each experiment was performed at least three times. The data were analyzed by unpaired Student’s two-tailed t test for comparison. The data are shown as the mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, ns no statistically significant difference

Fig. 4 IL-33 deficiency increases the risk of pregnancy failure. The number of apoptotic cells at the maternal–fetal interface of pregnant WT and IL-33−/− mice at G7.5 days (A) and G14.5 days (B) tested by TUNEL staining. The expression of CD80, CD86, CD206, CD209, TGF-β and IFN-γ in CD45+ F4/80+ CD11b+ dMΦs from the pregnant WT and IL-33−/− mice at G7.5 days (C) and G14.5 days (D). (E) The pregnancy rate of the pregnant WT and IL-33−/− mice at G7.5 and G14.5 days. (F) The implantation number of the pregnant WT and IL-33−/− mice at G7.5 days. (G) The embryo absorption rate, weight and crown-rump length of embryos and the diameter and weight of placentas of pregnant WT and IL-33−/− mice at G14.5 days. Each experiment was performed at least three times. The data were analyzed by unpaired Student’s two-tailed t test for comparison. The data are shown as the mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, ns no statistically significant difference
Exogenous IL-33 prevents pregnancy failure in IL-33−/− pregnant mice

To further confirm the roles of IL-33 in the efferocytosis of dMΦs and normal pregnancy, we mated IL-33−/− females with WT males and then injected them intraperitoneally with or without recombinant mouse IL-33 protein (IL-33 group). As expected, supplementation with exogenous IL-33 significantly decreased the number of apoptotic cells at the maternal–fetal interface of the IL-33−/− pregnant mice (Fig. 8A). The CD45+F4/80+CD11b+ uMΦs of the IL-33 group expressed lower AXL (Fig. 8B). In addition, these uMΦs displayed the M2 phenotype (CD80lowCD86lowIFN-γlowCD209highTGF-βhigh) in the IL-33 group (Fig. 8C and Figure S1E). More importantly, the embryo absorption rate, crown-rump length and weight of the embryos, and diameter and weight of the placentas were improved in the IL-33−/− pregnant mice with a peritoneal injection of IL-33, although there was no significant difference in the number of embryos implanted (Fig. 8D). Moreover, we also tested the expression of AXL on CD45+F4/80+CD11b+ uMΦs from WT and IL-33−/− mice at G7.5 days (Fig. 8E) and G14.5 days (Fig. 8F) to reflect the efferocytosis of uMΦs. AXL expression increased...
An imbalance of the IL-33/ST2-AXL-efferocytosis axis induces pregnancy loss through metabolic...

Fig. 6 IL-33 suppresses efferocytosis of dMΦs by downregulating the expression of the efferocytosis-related receptor AXL. (A) The GO enrichment analysis diagram of the genes with downregulated expression (HIST1H4C, HIST1H1E, HIST1H2BE, SMIM11A, PLEC, KMT2D, SOGA1, SIGLEC1, C3, EVPL, AXL, GP1BB, PTGDS, HIST2H3A, HIST2H3C, HSPG2). (B) The genes involved in the inflammatory response, phagocytosis and endocytosis pathways were intersected in the form of a Venn diagram. (C) The expression level of human AXL protein in normal and RPL dMΦs. The impact of IL-33 (2 ng/ml, 48 h) and sST2 (200 ng/ml, 48 h) on the expression of AXL (D) and the efferocytosis efficiency (E) of normal dMΦs. (F) The effect of GAS6 (50 ng/ml, 100 ng/ml, 2 h) on the efferocytosis of normal dMΦs. All experiments were performed at least three times. The data were analyzed by unpaired Student’s two-tailed t test for comparison of two groups and by one-way ANOVA with Tukey’s post-hoc test for multiple comparisons for more than two groups. The data are shown as the mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, ns no statistically significant difference.
in IL-33−/− group at 7.5 days of gestation, but there was no significant difference at 14.5 days of gestation. These results demonstrate that exogenous IL-33 supplementation improves the pregnancy outcomes of the IL-33−/− pregnant mice, and this effect is dependent on inhibiting apoptosis and the expression of AXL and triggering the M2 bias of uMΦs. And considering the results of Fig. 4, the increased AXL and efferocytosis could remove the increased apoptotic cells at G7.5 days. The homeostatic microenvironment of the maternal–fetal immune interface was maintained (the apoptotic cells of IL-33−/− mice were equal to the WT mice, in Fig. 4A). While at G14.5, it is possible that other efferocytosis-related molecules besides AXL were involved in the regulation of efferocytosis and the homeostasis of maternal–fetal interface deteriorated (the apoptotic cells of IL-33−/− mice were increased, in Fig. 4B).

Discussion

Apoptosis in early embryogenesis is inevitable and necessary. Too many or too few apoptotic cells lead to pregnancy failure [4, 26]. Efferocytosis has a central role in scavenging apoptotic cells in differentiated tissues [5, 27]. Decidual macrophages, as the most important antigen-presenting cells at the maternal–fetal interface, are the main operators of efferocytosis. However, how efferocytosis and the subsequent metabolic and immunological changes of dMΦs function in this particular microenvironment remains unknown. Here, we found that the immunological phenotypes of dMΦs from patients with normal pregnancies and unexplained RPL were different. Although several researches have suggested that efferocytosis has anti-inflammatory effects, promoting the polarization of macrophages to M2. RPL dMΦs with the M1 phenotype did not convert to the M2 phenotype after efferocytosis. It has been reported that efferocytosis of necrotic debris promotes the occurrence of an inflammatory response [28, 29]. These findings led us to speculate that there might be different reactions of efferocytosis, that the death mode of the engulfed cells or the functional status of phagocytes themselves may determine the function of efferocytosis, and that the ultimate purpose of efferocytosis is to maintain the homeostasis of the local microenvironment.

DMΦs at the maternal–fetal interface account for 20% of the total number of decidual immune cells, which have strong plasticity and heterogeneity and play an important role in maintaining immune tolerance, protecting the fetus from infection, removing apoptotic cells [30, 31] and remodeling helical arterioles [32]. Therefore, rather than functioning as antigen-presenting cells, dMΦs should be considered cellular transducers that perceive sensory stimuli (such as invading trophoblast cells and apoptotic DSCs) and respond to maintain homeostasis of the maternal–fetal interface.

The adaptation of each type of macrophage to the metabolic environment is closely related to their main functions, and the metabolism of M1 and M2 macrophages is also different [33]. It was initially believed that proinflammatory M1-type macrophages mainly rely on aerobic glycolysis for energy supply, and this metabolic characteristic is conducive to the rapid production of ATP by macrophages to maintain their phagocytosis and scavenging function [34]. In these macrophages, HIF-1α is activated and plays a key role in the process of glycolysis [35]. In contrast, anti-inflammatory M2 macrophages have a complete TCA cycle, fatty acid oxidation (FAO) and OXPHOS [33]. However, some recent studies revealed that the differentiation of M2-type macrophages also requires glycolysis to support fatty acid synthesis and OXPHOS of M2 macrophages, and it was found that FAO exists in M1 macrophages to facilitate the activation of their inflammasomes [36]. The IL-33/ST2 axis has also been found to regulate the differentiation of macrophages through metabolism. Disorder of the IL-33/ST2 axis enhances the OXPHOS level of macrophages and decreases glucose uptake and the ECAR value. The number of mitochondria and DNA copies are increased, and the expression of mitochondrial fusion-related genes is increased [13]. Inconsistently, the IL-33/ST2 axis was also reported to promote glycolysis, inhibit OXPHOS and promote M2 polarization of macrophages through the mTOR pathway, promoting the occurrence and development of tumors [7]. These findings suggest a more complex metabolic network during macrophage activation, and macrophages of different tissues and organs have metabolic heterogeneity. In the current research, the activation of IL-33/ST2 was able to polarize dMΦs toward an M2 phenotype and restrict glycolysis-mediated efferocytosis. After efferocytosis, the phenotype of dMΦs...
remains tolerant, contributing to the maintenance of normal pregnancy. Moreover, the elevated sST2 biased the efferocytosis-related metabolism of RPL dMΦs toward OXPHOS and exacerbated the disruption of the IL-33/ST2 signaling pathway. Metabolic disorders, in turn, lead to dysfunction of efferocytosis, resulting in more uncleared apoptotic cells and excessive secondary necrosis. Our previous report showed that IL-33 also promoted the proliferation and invasion of DSCs [9]. As expected, we observed that knocking out IL-33 increased the risk of pregnancy loss with higher apoptotic cells and M1 polarization of dMΦs.

AXL, which, along with Tyro3 and the MerKT receptor, is a member of the TAM family of kinase receptors, mediates the “eat-me” signal during efferocytosis. This molecule inhibits the development of inflammation, which along with Tyro3 and MerKT receptor stands for the TAM family of kinase receptor, mediates tissue repair through efferocytosis and mediates immunosuppression in the tumor microenvironment, which is associated with poor prognosis of tumors [20]. Clinical studies found that in the plasma of patients with severe preeclampsia, free MerTK and AXL expression increases and is positively correlated with the severity of blood pressure and proteinuria. GAS6 was lower and negatively correlated with the level of proteinuria, suggesting that TAM-related signaling pathways (especially the AXL-GAS6 signaling pathway) might participate in the pathological process of preeclampsia [37]. In addition, clinical cohort studies found that AXL-mediated Zika virus infection leads to the occurrence of congenital microcephaly in human [38]. Although Zika virus infection in a mouse model did not require the TAM receptor family to mediate [39], knockout of AXL did not prevent Zika virus infection of human neural progenitor cells and the brain [40]. As the other molecule obtained by the Venn diagram with complement C3, AXL might also affect the microenvironment at the maternal–fetal interface. Therefore, AXL was selected as the follow-up molecule downstream of IL-33/ST2. Herein, we revealed the regulation of AXL by IL-33/ST2 and the effect on pregnancy outcomes for the first time.

Notably, we found that IL-33 downregulated the expression of AXL but not MerTK, and GAS6 was not involved in the regulation of efferocytosis in dMΦs. The downstream signaling pathways of IL-33/ST2 include ERK1/2, JNK, P38/MAPK, PI3K/AKT, and NF-κB [41], which are involved in the regulation of proinflammatory and anti-inflammatory responses in various microenvironments. In this study, we found that IL-33/ST2 decreased the expression of AXL in dMΦs by activating the PI3K/AKT and ERK1/2 signaling pathways. These two signaling pathways have also been widely reported in the etiology of RPL [42–44], suggesting that inactivation of the PI3K/AKT and ERK1/2 signaling pathways contributes to spontaneous abortion by an imbalance in efferocytosis. In vivo confirmation trials, we observed that administration of IL-33 led to a decrease in AXL, an increase in M2 differentiation, and homeostasis of efferocytosis at the maternal–fetal interface and improved the pregnancy outcome of IL-33−/− mice. IL-33 can be found in various cells, including mastocytes, dendritic cells and adipocytes. This molecule plays an active role in inducing immunotolerance and immunoregulation and can be used to develop treatments for autoimmune diseases. Similarly, we found that blocking the IL-33/ST2 axis shows promise for improving the outcome of miscarriage. Our hypothesis is based on clinical observations related to the IL-33/ST2 axis in some diseases, and we extend these discoveries to clinical patients with unexplained RPL. More importantly, sST2 is considered an important prognostic marker and indicator for monitoring therapy in patients with heart failure and has relatively mature clinical detection methods [45]. Interestingly, sST2 is expected to be a promising predictor of clinically abnormal pregnancies, such as preeclampsia and miscarriage. Circulating and placental sST2 levels were increased in preeclampsia, although plasma IL-33 levels showed no significant difference [46]. Additionally, the predictive value of IL-33/ST2 was reported for miscarriage at 6 weeks of gestation [11]. As expected, we observed increased ST2 levels in patients with missed abortion in this study. Notably, efferocytosis-targeted strategies are emerging for tumorigenesis and cancer management [3, 47]. In the current study, the “eat-me” signal AXL was identified as a downstream regulatory molecule of IL-33/ST2 in dMΦs. Therefore, the potential value of targeting the IL-33/ST2 axis and AXL in the prediction and intervention of unexplained RPL should be emphasized. However, due to the limited number of clinical samples in this study, the potential value of sST2 in the
prediction of unexplained RPL needs to be validated with a large clinical dataset in the future.

Conclusions

In conclusion, we described a complex regulatory network between efferocytosis and metabolism of dMΦs in the maternal–fetal microenvironment (Figure S4). Under normal conditions, the IL-33/ST2 axis activates the ERK/2 and PI3K/AKT signaling pathways to downregulate the expression of AXL, thereby restricting redundant efferocytosis to ensure a certain number of apoptotic cells at the maternal–fetal interface. Additionally, the activation of IL-33/ST2 was able to polarize dMΦs toward an M2 phenotype and control the efferocytosis dominated by glycolysis, which is beneficial to the maternal–fetal immunotolerance of normal pregnancy. Once the IL-33/ST2 axis was disturbed, the number of apoptotic cells increased, and the efferocytosis function of dMΦs was secondarily enhanced. DMΦs with higher efferocytosis secrete more sST2 and less TGF-β, which polarizes dMΦs toward an M1 phenotype. The elevated sST2 further disrupted the efferocytosis of dMΦs toward OXPHOS and exacerbated the disruption of the IL-33/ST2 signaling pathway. Metabolic disorders also lead to dysfunction of efferocytosis, resulting in more uncleared apoptotic cells and secondary necrosis and eventually leading to the occurrence of spontaneous abortion. This study provides a novel mechanism of the immune etiology of recurrent spontaneous abortion from the perspective of efferocytosis–related metabolism.

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Authors’ contributions Y-RS performed the experiments and data analysis, generated the figures and prepared the manuscript; W-TH designed the study, performed experiments and assisted with data interpretation; H-HS performed experiments, searched the literature and edited the manuscript; C-YW, Y-KL, X-QM searched the literature; M-QL designed the study, guided the experiments and edited the manuscript; X-YZ initiated and supervised the study and edited the manuscript.

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Availability of data and material Not applicable.

Declarations

Competing interests The authors declare no conflict of interest.

Ethics approval and consent to participate Tissue samples used in this study were collected after obtaining informed consent approved by the Ethics Committee of Obstetrics and Gynecology Hospital, Fudan University.

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