Trophoblast-derived hyaluronan promotes the regulatory phenotype of decidual macrophages

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Abstract

There is delicate crosstalk between fetus-derived trophoblasts (Tros) and maternal cells during normal pregnancy. Dysfunctions in interaction are highly linked to some pregnancy complications, such as recurrent spontaneous abortion (RSA), pre-eclampsia and fetal growth restriction. Hyaluronan (HA), the most abundant component of extracellular matrix, has been reported to act as both a pro- and an anti-inflammatory molecule. Previously, we reported that HA promotes the invasion and proliferation of Tros by activating PI3K/Akt and MAPK/ERK1/2 signaling pathways. While lower HA secretion by Tros was observed during miscarriages than that during normal pregnancies, in the present study, we further confirmed that higher secretion of HA by Tros could induce M2 polarization of macrophages at the maternal–fetal interface by interacting with CD44 and activating the downstream PI3K/Akt-STAT-3/STAT-6 signaling pathways. Furthermore, HA could restore the production of IL-10 and other normal pregnancy markers by decidual macrophages (dMφs) from RSA. These findings underline the important roles of HA in regulating the function of dMφs and maintaining a normal pregnancy.

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Introduction

In a successful pregnancy, the maternal immune system undergoes numerous changes in order to avoid the alienation of the semi-allogeneic fetus, while at the same time protecting the woman against pathogens. This balance is mainly established locally at the maternal–fetal interface, where the extravillous trophoblast cells invade the decidua and come into contact with the decidual immune cells (DICs). Failure to establish and maintain this proper crosstalk is associated with complications of pregnancy, such as pre-eclampsia and recurrent spontaneous abortion (RSA) (Arck & Hecher 2013).

Macrophages are the second most abundant leucocytes (10–20% of the DICs) and the most important specialized antigen-presenting cells at the maternal–fetal interface (Svensson-Arvelund & Ernerudh 2015). Decidual macrophages (dMφs) are associated with the success of pregnancy by promoting decidual vascular remodeling (Lash *et al.* 2016) and clearance of cell debris (Egashira *et al.* 2017). The depletion of macrophages

after conception has been reported to cause embryo implantation arrest (Care *et al.* 2013). Parallel to the T helper-1 (Th1)/T helper-2 (Th2) paradigm, environmental cues and molecular mediators polarize macrophages as type 1 macrophages (M1) and type 2 macrophages (M2). With the high expression of major histocompatibility complex class II (MHC II), CD80, CD86 and IL-12, M1 are more effective at antigen clearance and switching T-cell responses toward Th1 immune response (Mills *et al.* 2000). It has been reported that M1 play crucial roles in implantation (Jaiswal *et al.* 2012). While characterized by M2-type markers (CD163, CD206, CD209 and IL-10), M2 have more immunosuppressive capacities, contributing to tissue remodeling and promoting Th2 immune responses (Murray *et al.* 2014). It seems that dMφs maintain the balance between pro-inflammatory M1 and anti-inflammatory M2 during normal pregnancies. Imbalance of M1/M2 leads to a pathological pregnancy, such as RSA (Shimada *et al.* 2018, Tsao *et al.* 2018).

Hyaluronan (HA) is an unbranched polymer composed of repeating glucuronic acid and N-acetyl glucosamine

disaccharide units, and it is found ubiquitously in the extracellular matrix (ECM) of all mammalian tissues. HA is protective against infection-mediated preterm birth as it is crucial in maintaining an epithelial and mucosal barrier to limit pathogen infiltration of the lower reproductive tract during pregnancy (Akgul *et al.* 2014). In addition to its structural role in the ECM, HA has diverse functions in cell growth, migration and adhesion, dependent on the HA size and expression of HA-interacting molecules such as CD44 (Dicker *et al.* 2014). During early pregnancy, high-molecular-weight HA (HMW-HA) enhances the proliferation and invasive properties of trophoblasts (Tros) and inhibits apoptosis by activating PI3K/AKT and MAPK/ERK1/2 signaling pathways (Zhu *et al.* 2013a). Meanwhile low-molecular-weight HA (LMW-HA) has been reported to play a role in promoting angiogenesis and inflammation (Jiang *et al.* 2005).

Emerging evidence has suggested that HA creates a specific microenvironment that is favorable for tumor angiogenesis, invasion, metastasis and immune escape (Chanmee *et al.* 2016). Recently, it has been revealed in a study that the HA-CD44-ERK1/2-STAT3 pathway serves as an important regulator in the formation of M2-like tumor-associated macrophages (TAMs) (Zhang *et al.* 2016). Given the pseudo-malignant nature of the placenta, it is possible that HA plays a role in maternal-fetal tolerance by regulating the function of dMφs. Here we showed that, during normal pregnancies in humans, Tros-derived HA induces the M2 polarization of dMφs by interacting with CD44 and activating the PI3K/Akt-STAT3/STAT-6 pathway.

Materials and methods

Human samples

First-trimester (gestational age: 6–12 weeks) human villous and decidual tissues were obtained from normal pregnancies (terminated for non-medical reasons, $n=33$) and clinical miscarriages (two or more miscarriages diagnosed as RSA, excluding genetic, endocrine and anatomic abnormalities, infections, poor health habits and so forth; $n=28$). The miscarried decidual tissues were obtained very soon after they were identified. All participants provided written informed consent. The Human Research Ethics Committee of the Obstetrics and Gynecology Hospital of Fudan University approved the collection and use of the samples. All the methods were carried out in accordance with the approved guidelines.

Human cell isolation

Tros were isolated by trypsin-DNase-I digestion and discontinuous Percoll gradient centrifugation, as previously described (Wang *et al.* 2016).

DICs were isolated by collagenase IV/DNase-I digestion and discontinuous Percoll gradient centrifugation from the decidual tissue of normal pregnancies and RSA, as previously

described (Wang *et al.* 2015, 2016). dMφs were isolated from DICs by magnetic affinity cell sorting using CD14 microbeads (Miltenyi Biotec).

Treatment of dMφs

Freshly isolated dMφs were cultured with HMW-HA (50 or 100 µg/mL) or medium-molecular-weight HA (MMW-HA) (50 or 100 µg/mL) or LMW-HA (50 or 100 µg/mL) (all from R&D Systems) with or without the pretreatment of neutralizing anti-CD44 antibody (20 µg/mL, Clone 5F12, Scientific Inc, Fremont, CA, USA) at 37°C in 5% CO₂ for 48 h. In some experiments, the cells were pretreated with the PI3K signaling pathway inhibitor LY294002 (50 mM, Cell Signaling Technology) or MEK1/2 inhibitor U0126 (30 mM, Cell Signaling Technology) for 30 min before HA treatment.

Freshly isolated Tros were seeded at a density of 2×10^5 cells/mL per well in 24-well Matrigel-coated plates overnight. The cells were washed twice with phosphate-buffered saline and the supernatants were collected after culturing for 48 h. The supernatants were harvested and centrifuged at 2000g, and then moved to a new tube and stored at –80°C. dMφs were cultured with the supernatants of Tros (TCM). In some wells, an HA antagonist (pep-1) and control peptides (Cpep, 100 mg/mL, Invitrogen Life Technologies Corp) with or without the pretreatment of neutralizing anti-CD44 (20 µg/mL) antibody were added. The cells were then harvested for analysis by flow cytometry after 48 h of co-culture.

Western blot analysis

dMφs were stimulated by 100 µg/mL HMW-HA for 0, 15, 30, 60 or 120 min. The cells were harvested and homogenized in an ice-cold radioimmunoprecipitation assay (RIPA) lysis buffer. Total cell lysates were collected, and equal quantities of protein were separated by SDS-PAGE and blotted onto PVDF membranes. The PVDF membranes were blocked with Tris-buffered saline (TBS) containing 5% skimmed milk powder for 1 h at room temperature and incubated with anti-phospho-Akt (1:1000, Cell Signaling Technology), anti-Akt (1:1000, Cell Signaling Technology) or anti-GAPDH mAb (1:3000, Cell Signaling Technology) overnight at 4°C. Then, the membranes were washed with 1× TBS/Tween 20 buffer three times and incubated with horseradish peroxidase-conjugated polyclonal secondary antibodies for 1 h at room temperature. The membranes were developed with the enhanced plus chemiluminescence assay.

Flow cytometry

The expression of cell-surface molecules and intracellular cytokines was evaluated using flow cytometry. The following were used: fluorescein isothiocyanate (FITC)-conjugated anti-human CD80, CD209 and CD14, phycoerythrin (PE)-conjugated anti-human CXCL10, CCL22, CCL17, CXCL9, CD80, CD163 and CD86, PE/CY7-conjugated anti-human CD86, CD14, IL-10 and TNF-α, allophycocyanin (APC)-conjugated anti-human CD206, and ICOSL, Alexa Fluor 647-conjugated anti-human IL-12, Brilliant Violet 421-conjugated anti-human

PDL-1, PDL-2, HLA-DR, CD163 and IL-4 and Brilliant Violet 510-conjugated anti-human CD14, PDL-1 (BioLegend, San Diego, CA, USA). For intracellular staining, cells were fixed and permeabilized using the Fix/Perm Kit (BioLegend). A minimum of 10,000 events were acquired using a Beckman-Coulter CyAn ADP flow cytometer and analyzed with FlowJo software (Tree Star, Ashland, OR, USA).

Statistical analysis

The significance of differences between two groups was determined by Dunnett's *post hoc t*-test. Multiple groups were analyzed by one-way or two-way ANOVA with Bonferroni's posttests using Prism version 5 software (GraphPad). For all statistical tests, *P* values of <0.05 were considered statistically significant.

Results

Effects of HA on dMφs during early pregnancy

In order to investigate the potential role of HA in the function of dMφs during normal early pregnancies in humans, we first studied the effect of HA with different molecular weights on the expression of MHC II and co-stimulatory molecules of dMφs. As shown in Fig. 1A, 100 µg/mL HMW-HA (>950 kDa) downregulated the

expression of CD80 and CD86 but upregulated that of PDL-1 and PDL-2 on dMφs. PDL-1 and PDL-2 are related to the alternative activated phenotype in macrophages and are mechanisms of T-cell suppression by alternative activated macrophages (Smith *et al.* 2004, Terrazas *et al.* 2005). The expression of ICOSL and HLA-DR did not vary with the treatment with HA (Supplementary Fig. 1A, see section on supplementary data given at the end of this article).

Highly expressed pattern recognition receptors such as CD163 and CD206 on dMφs play important roles in clearing pathogens at the maternal–fetal interface (Tang *et al.* 2015). We found an increase of CD163 and CD206 on dMφs after treatment with HMW-HA (Fig. 1B). HMW-HA also increased the production of IL-10 by dMφs (Fig. 1C), but had no effect on the production of IL-12 (Supplementary Fig. 1B). Apart from these surface molecules and cytokines, chemokines have also been used as biomarkers of M1/M2 (Zhang *et al.* 2017). HMW-HA increased M2-associated CCL17 and CCL22 but decreased M1-associated CXCL10 expression on dMφs (Fig. 1D). Although LMW-HA was reported to induce a classically activated-like state of macrophages (Rayahin *et al.* 2015), as shown in Fig. 1, we did not observe the effects of MMW-HA (75–350 kDa) and LMW-HA (15–40 kDa) on the phenotype of dMφs.

Figure 1 Effects of HA on dMφs during early pregnancy. (A) Quantitation of flow cytometric analysis of the expression of MHC II and co-stimulatory molecules by dMφs stimulated with HMW-HA (HHA, 50 or 100 µg/mL) or MMW-HA (MHA, 50 or 100 µg/mL) or LMW-HA (LHA, 50 or 100 µg/mL). (B, C and D) Quantitation of flow cytometric analysis of the expression of CD206, CD163 cytokines and chemokines by dMφs stimulated with the indicated concentrations of HMW-HA, MMW-HA or LMW-HA. Data are represented as the mean ± standard error of the mean (s.e.m.) for three to five separate experiments. *n* = 12 dMφs in the first trimester of normal pregnancies. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

HMW-HA induces M2 polarization through CD44

CD44 is the principal receptor of HA, and HA-CD44 signaling has long been known to play a role in immune regulation (Heldin *et al.* 2008). In order to determine whether the functional regulation of HMW-HA on dMφs is dependent on CD44, we first examined the expression of CD44 on DICs and found that about 80% of dMφs expressed CD44 (Fig. 2A and B). Then, we used an anti-CD44 neutralizing antibody to directly analyze the interaction of HMW-HA with CD44 on dMφs. Pretreatment with anti-CD44 monoclonal antibodies (mAbs) notably reversed the induction of M2 polarization by HMW-HA, as confirmed by lower expression of PDL-1, PDL-2, CD206, CD209, CD163, IL-10, CCL17 and CCL22, and the higher expression of CD80, CD86 and CXCL10 on dMφs (Fig. 2C).

PI3K/Akt-STAT-3/STAT-6 pathways are involved in M2 polarization

We previously reported that HMW-HA could activate PI3K/Akt and MAPK/ERK1/2 signaling pathways in Tros and decidual stromal cells during early pregnancies (Zhu *et al.* 2013a,b). In order to further understand the intracellular mechanism after the binding of HMW-HA to CD44 on dMφs, the effect of the specific signal transduction inhibitors on dMφs was examined. The results showed that U0126 (an inhibitor of ERK1/2) had no effect on HMW-HA-mediated M2 polarization. Conversely, LY294002 (an inhibitor of PI3K) markedly reversed this effect (Fig. 3A). Additionally, treatment with U0126 or LY294002 alone did not affect the expression of M1/M2-associated markers on dMφs, suggesting that the reversal effect of LY294002 was

Figure 2 HMW-HA induces M2 polarization through CD44. (A) Quantitation of flow cytometric analysis of the expression of CD44 on DICs. (B) Flow cytometric analysis of the expression of CD44 on dMφs. The flow cytometric plot is from one representative experiment among three separate experiments. (C) Quantitation of flow cytometric analysis of M1/M2 associated markers on dMφs stimulated with HMW-HA (HHA, 100 μg/mL) in the presence or absence of anti-CD44 mAbs. Data are represented as the mean ± S.E.M. for three separate experiments. $n=9$ dMφs in the first trimester of normal pregnancies. * $P<0.05$, ** $P<0.01$, *** $P<0.001$, compared with the group Ctrl. # $P<0.05$, ## $P<0.01$, ### $P<0.001$, compared with the group HHA.

not due to its cytotoxic effect, but that it resulted in the inhibition of the HMW-HA-mediated activity. We further confirmed that HMW-HA could activate the Akt signaling pathway in a time-dependent manner by Western blotting (Fig. 3B).

The activation of the signal transducer and activator of transcription 3 (STAT-3) and STAT-6 is essential for the expression of M2-associated markers and TAMs bioactivities (Sica & Bronte 2007, Ishii *et al.* 2009). Our data showed that the phosphorylation levels of both STAT-3 and STAT-6 in dMφs were remarkably increased when treated with HMW-HA. Furthermore, the inhibition of PI3K suppressed the phosphorylation of STAT-3 and STAT-6 in dMφs stimulated by HMW-HA

(Fig. 3C). So, the PI3K/Akt-STAT-3/STAT-6 pathways were involved in the M2 polarization induced by HMW-HA.

Tros instruct dMφs to an M2 phenotype via HMW-HA

Previously, we have shown that Tros have the unique ability to instruct T cells, and primary Tros could secrete HA continuously (Zhu *et al.* 2013b, Wang *et al.* 2015, 2016). Furthermore, in normal pregnancies, the HA produced by Tros is mostly HMW (data not shown). So we questioned whether Tros could also instruct macrophages and whether this effect was dependent on HA secretion. As expected, treatment

Figure 3 PI3K/Akt-STAT-3/STAT-6 pathways are involved in the M2 polarization. (A) Quantitation of flow cytometric analysis of M1/M2 associated markers on dMφs stimulated with HMW-HA (HHA, 100 µg/mL) in the presence or absence of the indicated inhibitors of signal transduction. (B) dMφs were serum treated with 100 µg/mL HMW-HA at a different time. A Western blot was used to analyze the total levels and phosphorylation levels of Akt. HMW-HA induced Akt phosphorylation in dMφs in a time-dependent manner. The image is representative of four individual experiments. (C) Flow cytometric analysis (right) and quantitation (left) of STAT-3 and STAT-6 phosphorylation in dMφs stimulated with HMW-HA (HHA, 100 µg/mL) in the presence or absence of the PI3K signaling pathway inhibitor LY294002. The flow cytometric plot is from one representative experiment. Data are represented as the mean ± S.E.M. for three separate experiments, $n=9$. * $P<0.05$, ** $P<0.01$, *** $P<0.001$, compared with group Ctrl. ** $P<0.01$, *** $P<0.001$, compared with group HHA.

of the TCM increased the expression of M2-associated markers, while decreasing the M1-associated markers of dMφs. Treatment with an HA antagonist peptide and/or blocking HA-CD44 interaction by anti-CD44-neutralizing antibody significantly abrogated the Tros-induced M2 polarization, whereas CD80 and CD86 were upregulated (Fig. 4).

Dysregulation of dMφs correlates with human miscarriages

Our previous report has proven that lower HA secretion by Tros was observed in miscarriages than that of normal pregnancies (Zhu *et al.* 2013b). Because Tros could instruct dMφs into an M2 bias (Fig. 4), we analyzed the phenotype of dMφs from normal pregnant women and patients who experienced RSA to explore the possible clinical significance of dMφs instructed by Tros-secreted HA. Flow cytometric analysis revealed a much lower expression of PDL-1, PDL-2, IL-10, IL-4, CD206, CD209 and CD163 on dMφs from RSA compared with that from normal pregnancy (Fig. 5A, B and C). Conversely,

the expression of CD80, CD86 and IL-12 by dMφs was much higher in RSA (Fig. 5A and B), whereas the expression of HLA-DR and ICOSL on dMφs was not different between the two groups (Fig. 5A). Besides affecting the biological behaviors of Tros themselves (Zhu *et al.* 2013b), the reduced secretion of HA by Tros also affected the function of dMφs, further leading to the development of RSA.

Next, we treated dMφs isolated from patients with RSA with 100 µg/mL HMW-HA to explore whether HA could rebalance the M1/M2 polarization of dMφs from RSA. As shown in Fig. 5D, 100 µg/mL HMW-HA upregulated the expression of CD206, CD209, CD163, PDL-1 and PDL-2 but downregulated the expression of CD80 and CD86 on dMφs from RSA. Furthermore, HMW-HA restored the production of IL-10 by dMφs isolated from patients with RSA, although it had no effect on the production of IL-4 and IL-12. These data indicated that the reduced secretion of HA may act as an early warning sign of adverse pregnancy, and HA may represent novel therapeutic strategies to prevent pregnancy loss.

Figure 4 Tros instruct dMφs to an M2 phenotype via HMW-HA. Quantitation of flow cytometric analysis of M1/M2 associated markers on dMφs cultured with TCM in the presence or absence of pep-1 and Cpep with or without pretreatment of neutralizing anti-CD44 (20 µg/mL) antibody. Data are represented as the mean $\pm$ S.E.M. for three separate experiments, $n=12$. * $P<0.05$, ** $P<0.01$, *** $P<0.001$, compared with group Ctrl. # $P<0.05$, ## $P<0.01$, ### $P<0.001$, compared with group TCM+Cpep.

Figure 5 Dysregulation of dMφs correlates with human miscarriages. (A) Flow cytometric analysis (right) and quantitation (left) of MHC II and co-stimulatory molecules on dMφs from normal pregnancies ($n=12$) and RSA ($n=19$). (B) Flow cytometric analysis (right) and quantitation (left) of cytokine production by dMφs from normal pregnancies and RSA. (C) Flow cytometric analysis (right) and quantitation (left) of pattern recognition receptors on dMφs from normal pregnancy and RSA. The flow cytometric plot is from one representative experiment among six separate experiments. (D) Quantitation of flow cytometric analysis of the surface molecule expression and cytokine production by dMφs isolated from patients with RSA ($n=9$) stimulated with HMW-HA (HHA, 100μg/mL) from three separate experiments. Data are represented as the mean $\pm$ S.E.M. * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

Discussion

A successful pregnancy relies on adequate placental formation and the maternal immune system tolerating the semi-allogeneic fetus. Multiple mechanisms function to protect the fetus from attacks by the maternal immune system and promise the adequate invasion of extravillous Tros. In humans, dMφs display an M2 phenotype and may be involved in Tros invasion, vascular remodeling and the development of maternal-fetal tolerance (Petroff *et al.* 2002). Previously, we reported that a higher level and greater molecular mass of HA can promote Tros growth and invasion in an autocrine manner, which is beneficial to placentation, whereas the lower HA secretion by Tros was observed in miscarriages than in normal pregnancies (Zhu *et al.* 2013b). The current study shows that, during normal pregnancies in humans, Tros-derived HA induces M2 polarization by interacting with CD44 and activating the PI3K/Akt-STAT3/STAT-6 pathway. This is further evidence of the delicate crosstalk between the fetus-derived Tros and maternal cells during normal pregnancies. Additionally, a decrease in HA secretion results in an abnormal function of macrophages, leading to RSA. Due to the fact that HA could restore the production of IL-10 and

other normal pregnancy markers on dMφs isolated from patients with RSA, this indicates that HA may present novel therapeutic strategies to prevent pregnancy loss.

HA has received great attention as a versatile functional biopolymer since its discovery. The native form, HMW-HA, can be broken down into lower-molecular-weight fragments in response to environmental cues, such as reactive oxygen species and pH (Stern *et al.* 2007). The molecular weight variants of HA have been used in a variety of biomedical applications eliciting varying biologic responses (Highley *et al.* 2016). For example, LMW-HA was identified as inducing potent pro-inflammatory responses (Powell & Horton 2005), whereas HMW-HA was reported to regulate osteoclast formation (Ariyoshi *et al.* 2014) and create a specific microenvironment favorable for tumor development (Chanmee *et al.* 2016). Because of the lack of a standard size for HMM- and LMM-HA, HA may serve various roles in different studies. We found that HMW-HA could induce M2 polarization of dMφs, but MMW- and LMW-HA had no effects on the expression of biomarkers of M1/M2 (Fig. 1). However, it has been shown in a study that macrophages correspond to pro-inflammatory responses for LMW-HA (Rayahin *et al.* 2015). This may be due to the different molecular weights of HA used

Figure 6 A schematic diagram of Tros-derived HA induced M2 polarization at the maternal–fetal interface. Tros secrete HA, which in turn promotes the invasion and proliferation of human Tros. Tros-derived HMW-HA instructs dMφs to transform into immunosuppressive M2 through interacting with CD44 and activating the downstream PI3K/Akt-STAT-3/STAT-6 signaling pathways. While in RSA, the secretion of HA by Tros was decreased, and the M2 bias was disrupted at the maternal–fetal interface, which was harmful to the maintenance of normal pregnancy.

(≤ 5 kDa in the report) and the different characteristics between dMφs and murine macrophages.

Our results also showed that co-culture of Tros increased the expression of M2-associated markers while decreasing the M1-associated markers of dMφs. With the higher expression of IL-10 and CD163, CD206, CD209, PDL-1 and PDL-2, dMφs could better exert immunosuppressive effects and remove pathogens, thus promoting immune tolerance at the maternal–fetal interface (Smith *et al.* 2004, Terrazas *et al.* 2005, Tang *et al.* 2015). Treatment with an HA antagonist peptide significantly abrogated the Tros-induced M2 polarization. This is also supported by a recent study that displayed a link between HA accumulation and large numbers of M2-like macrophages (Tiainen *et al.* 2015). Our results provide further evidence that Tros could educate maternal immune cells to develop a unique phenotype and tolerate the fetus.

CD44 is the major cell-surface receptor for HA, and HA–CD44 signaling appears to be significant in regulating immunological responses, such as T cells recruitment, mature B cells activation and monocytes deactivation (Jiang *et al.* 2011). We found that antagonizing the HA–CD44 interaction on dMφs, or between Tros and dMφs, significantly inhibits the phenotype transformation, suggesting that the induction of M2 by HA is triggered by binding to CD44. However, besides CD44, HA could also bind to other receptors. For example, it has been reported in a study that CD44 and TLR4 were both engaged in the HA-mediated deactivation of monocytes (Del *et al.* 2005). Our study cannot exclude other HA receptors in M2 conversion at the maternal–fetal interface, which needs further investigation.

The signaling pathways in dMφs polarization are mostly unclear. It is well documented that ERK1/2 and

PI3K/Akt signal cascades are closely connected to the function of HA (Zhu *et al.* 2013a,b). Our data showed that the PI3K/Akt signal activated by HA–CD44 interaction induced the phenotype transformation of dMφs, implying a novel role of the PI3K/Akt pathway in macrophages function. Of note, it was found in one study that the PI3K/Akt pathway could also inhibit abnormal macrophage activation in the placenta (Zhou *et al.* 2017). STAT-3 and STAT-6 signals in macrophages are involved in the regulation of immune responses and are master regulator of M2 genes (Welch *et al.* 2002, Ishii *et al.* 2009). The activation of STAT-3 and STAT-6 is inhibited by suppressing the PI3K/AKT signal in dMφs. Our data suggest that the PI3K/Akt-STAT-3/STAT-6 pathway is the molecular cue responsible for HA-mediated M2 formation at the maternal–fetal interface.

Abnormally activated dMφs can disrupt the tolerance at the maternal–fetal interface, which is closely associated with RSA, pre-eclampsia and premature delivery (Xu *et al.* 2016). Our data, together with previous studies indicating M1 dominance in miscarriages (Shimada *et al.* 2018, Tsao *et al.* 2018), suggest that M1 bias is detrimental to pregnancy. HA was able to induce M2 polarization at the maternal–fetal interface, and the secretion of HA by Tros from RSA significantly decreased (Zhu *et al.* 2013b). Furthermore, HA could restore the M2 phenotype of dMφs isolated from patients with RSA. Based on these results, we conclude that failure to maintain M2 bias resulting from the low HA microenvironment at the maternal–fetal interface may be one of the major reasons leading to RSA. However, although Tros-derived HA polarizes M2 *in vitro*, its precise role *in vivo* remains to be established.

In summary, we have demonstrated that a high level and mature state of HA is beneficial to the maintenance of normal pregnancy. Additionally, Tros-derived HA instructs dMφs to transform into immunosuppressive M2 through interacting with CD44 and activating the downstream PI3K/Akt-STAT-3/STAT-6 signaling pathways (Fig. 6). Our study provides new insights into the mechanisms underlying the formation of M2 bias during normal pregnancies, which may be helpful in pregnancy loss immunotherapy.

Supplementary data

This is linked to the online version of the paper at <https://doi.org/10.1530/REP-18-0450>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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