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Correlations Between High-mobility Group Box 1 and Th1/Th2 Cytokines in Peripheral Blood of Patients with Unexplained Recurrent Spontaneous Abortion

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KEYWORDS High-mobility Group Box 1. Peripheral Blood. T Helper Cell. Unexplained Recurrent Spontaneous Abortion

ABSTRACT The researchers aimed to explore the correlations between high-mobility group box 1 (HMGB1) and T helper cell (Th1/Th2) cytokines in the peripheral blood of patients with unexplained recurrent spontaneous abortion (URSA). After treatment, IL-2, IFN-ã and HMGB1 expressions significantly declined, while IL-4 and IL-10 expressions rose in URSA group (P<0.05). HMGB1, IL-2 and IFN-ã expressions were significantly lower and IL-4 and IL-10 expressions were higher in the successful pregnancy group than those in the failed pregnancy group (P<0.05). HMGB1 was positively correlated with IL-2 and IFN-ã, while negatively correlated with IL-4 and IL-10 (P<0.01). Th1 cell percentage and Th1/Th2 ratio were significantly higher and Th2 cell percentage was lower at 12 and 24 hours, than those under stimulation with HMGB1 at 0 hours (P<0.05). URSA is associated with the shift of Th1/Th2 balance toward Th1. HMGB1 may regulate the balance of Th1/Th2 immune response, as a potential target for treating URSA.

INTRODUCTION

Recurrent abortion refers to a pathological state manifested as three times (or above) of pregnancy loss in the first 20 weeks of pregnancy, which is mainly affected by infection, heredity and endocrine factors (Yang et al. 2018). The cases with unknown reasons account for forty to fifty percent of all cases, which is referred to as unexplained recurrent spontaneous abortion (URSA) (Wang et al. 2021). The failure rate of re-pregnancy in URSA patients is up to eighty percent, indicating that URSA is refractory infertility that needs to be solved urgently (Roomandeh et al. 2018). In recent years, maternal-foetal immune tolerance has been reported as the leading cause for URSA (Dean et al. 2019; Li et al. 2019). Immune cells and secreted cytokines form a unique immune tolerance state at the maternal-foetal interface, thus benefiting pregnancy and protecting normal delivery of the foetus (Li et al. 2020). The T helper 2 (Th2)-involved humoral immunity is dominant in normal pregnancy, but Th1-dominated cell-mediated immune attack occurs in URSA patients, leading to the imbalance of maternal-foetal immune tolerance (Ou and Yu 2020). Notably, high-mobility group box 1 (HMGB1) has been reported as a pro-inflammatory mediator that induces pathological pregnancy, and facilitates the secretion of Th1 cytokines by cluster of differentiation (CD)4^+ T lymphocytes (Liu et al. 2019).

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Objectives

The aim of this study was to investigate the expressions of HMGB1 and Th1/Th2 cytokines in the peripheral blood of patients with URSA, and the correlations between HMGB1 and Th1/Th2 cytokines.

METHODOLOGY

Subjects

A total of 100 URSA patients treated in the hospital from June 2019 to August 2020 were enrolled as the URSA group. The patients (26-37 years old) were aged 28.74 (±3.41) years old on average, with a mean body mass index (BMI) of 20.54 (±2.30) kg/m^2. Meanwhile, 50 healthy women (normal childbirth once or more) receiving physical examinations were selected as the control group. They

were 25-40 years old, with an average of 29.12 (±3.29) years old, and a mean BMI of 20.69 (±1.93) kg/m^2. No significant difference was found in the age and BMI between the two groups. This study was approved by the medical ethics committee of the hospital, and the research methods were strictly in accordance with relevant regulations. All patients were informed of relevant rights and risks, and they signed the informed consent voluntarily.

Inclusion Criteria

The URSA group met the diagnostic criteria of URSA as follows (Quan and Yang 2017):

a) Patients who had 3 times (or more) of spontaneous abortion, and no history of live births, stillbirth and dead-birth.
b) Those who had no common abortion-related causes such as reproductive tract infections, reproductive tract malformations and morphological abnormalities, chromosomal abnormalities of both spouses and embryos, endocrine abnormalities and natural immune diseases after examination
c) Those with regular menstrual cycle and normal ovulation
d) Those with negative anti-human chorionic gonadotropin antibody, anti-endometrial antibody, anti-cardiolipin antibody and anti-nuclear antibody
e) Those who had no immunotherapy and vaccination in the first 3 months of pregnancy
f) Those whose spouses had normal semen in routine examination or returned to normal after treatment
g) Those who were in a non-pregnancy period at the time of consultation, and had no abortion in the past 3 months.

Inclusion criteria for the control group were:

a) Patients who had full-term pregnancy once or above, and whose children were alive
b) Those who had no medication recently, no history of related immunologic, genetic and infectious diseases, and no acute and chronic vaginal inflammations
c) Those who had no history of spontaneous abortion and no abnormal pregnancy history such as stillbirth and dead-birth
d) Those who were in a non-pregnancy period at the time of consultation, and had no abortion in the past 3 months.

Treatment Methods

All URSA patients were subjected to active immunotherapy. Firstly, 30 mL of fasting blood was drawn from the husband or healthy unrelated individuals of every patient (biochemical, hepatic function, hepatitis virus, HIV, Treponema pallidum and other tests indicated normal indicators), and mixed with the same amount of normal saline. Next, lymphocytes were collected using Ficoll density gradient centrifugation, and cell concentration was adjusted to 2×10^{10}-3×10^{10}/L. Subsequently, the lymphocytes were intradermally injected at the inner side of the left forearm at three points, once every 2 weeks. Two weeks after the last immunotherapy, conception was guided, followed by continued immunotherapy until 20 weeks of pregnancy. Successful pregnancy referred to pregnancy over 28 weeks, and no abnormalities in ultrasound or delivery of live births (Chen et al. 2016).

Sample Collection

About 10 mL of the forearm venous blood was drawn from every healthy person of the control group early morning on the day of the physical examination, and every patient of URSA group before treatment and 2 weeks after the last immunotherapy respectively, and anticoagulated with 0.2 mL of heparin. The peripheral blood mononuclear cells were collected using density gradient centrifugation, and washed with PBS 3 times. Cell concentration was adjusted to 2×10^6/mL, and the cells were detected by flow cytometry. After centrifugation, plasma was collected and stored in a refrigerator at -80°C for later enzyme-linked immunosorbent assay (ELISA).

Detection of Th1, Th2 Cells and Their Ratio in Peripheral Blood

A total of 200 ìL of peripheral blood mononuclear cell suspension prepared was equally assigned into two tubes A (Th1) and B (Th2). Then, A and B were stimulated with phorbol ester (1 ìg/mL), ionomycin (50 ìg/mL) and monensin (0.1 mg/mL) for 6 hours, and added with CD3, CD4 and CD8 antibodies, followed by incubation at room temperature for 15 minutes in the dark. After fixation and rupture of membrane, A and B tubes were added with interferon-gamma (IFN-â) and interleu-

kin (IL)-4 antibodies respectively, and incubated at room temperature for 20 minutes in the dark. Subsequently, flow cytometry was utilised to detect the percentages of Th1 ($CD3^+CD4^+CD8^-IFN\text{-}\gamma^+$) and Th2 ($CD3^+CD4^+CD8^-IL\text{-}4^+$) cells, and the Th1/Th2 ratio was calculated.

Detection of Plasma HMGB1 and Th1/Th2 Cytokines

The expression levels of plasma HMGB1, Th1 cytokines (IL-2 and IFN-γ) and Th2 cytokines (IL-4 and IL-10) were detected by ELISA. The kits were purchased from eBioscience, and all operations were conducted strictly according to the instructions.

Stimulation of $CD4^+$ T Lymphocytes with Recombinant Human HMGB1 *in Vitro*

The immunomagnetic bead method was adopted to isolate $CD4^+$ T lymphocytes in healthy controls. $CD4^+$ T lymphocytes of healthy women were stimulated with recombinant human HMGB1 (100 ng/mL, Sigma, USA) for 0, 6, 12 and 24 hours (Xiong et al. 2019). The culture plate was added with medium containing ten percent foetal bovine serum, 100 U/mL of penicillin and 100 U/mL of streptomycin, and incubated in an incubator at 37°C in five percent CO_2. Th1 and Th2 cells were treated using the methods mentioned above, and detected by flow cytometry.

Statistical Analysis

SPSS 19.0 software was employed for statistical analysis. The measurement data were expressed as mean ± standard deviation, and the *t*-test was used for comparison between groups. The correlations among indicators were explored by Spearman's and Pearson's analyses. Two-tailed $P<0.05$ indicated that a difference was statistically significant.

RESULTS

Th1 and Th2 Cells in Peripheral Blood and Th1/Th2 Ratio

The percentage of Th1 ($CD4^+IFN\text{-}\gamma^+$) cells in $CD4^+CD8^-$ T-cells [(6.94±0.62)%] and Th1/Th2 ratio [(4.63±0.83)%] were significantly higher, while the percentage of Th2 ($CD4^+IL\text{-}4^+$) cells in $CD4^+$ T-cells [(1.48±0.59)%] was significantly lower in URSA group than those in control group [(5.96±1.68)%, (2.84±1.05)%, (2.12±0.91)%] ($P<0.05$) (Fig.1 and Table 1).

Table 1: Th1 and Th2 cells in peripheral blood and Th1/Th2 ratio

Indicator	*URSA group (n=100)*	*Control group (n=50)*	*t*	*p*
Th1 (%)	6.94±0.62	5.96±1.68	5.183	<0.001
Th2 (%)	1.48±0.59	2.12±0.91	5.189	<0.001
Th1/Th2	4.63±0.83	2.84±1.05	11.372	<0.001

Expression Levels of HMGB1 and Th1 and Th2 Cytokines in Peripheral Blood

Before treatment, HMGB1, IL-2 and IFN-γ expression levels [(98.62±20.32) ng/mL, (15.63±1.93) ng/L, (587.62±143.93) ng/L] were significantly higher and IL-4 and IL-10 expression levels [(11.48±2.24)

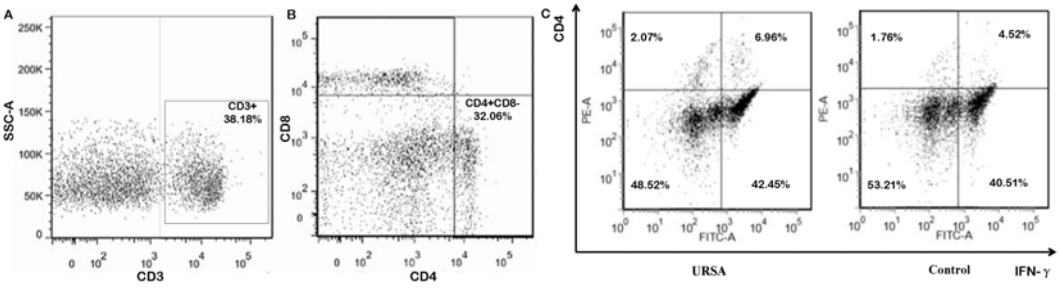

Fig. 1. Flow cytometry results of Th1 cells in peripheral blood of patients
A: CD3+ T lymphocyte gating
B: CD4+CD8- T lymphocyte gating
C: Percentage of Th1 ($CD4+IFN\text{-}\gamma^+$) cells in CD4+CD8- T-cells in peripheral blood

Table 2: Expression levels of HMGB1 and Th1 and Th2 cytokines in peripheral blood

Indicator	*URSA group (n=100)*		*Control group (n=50)*	*t*	*P*
	Before treatment	*After treatment*			
HMGB1 (ng/mL)	98.62± 20.32	49.84± 10.07*	48.82± 9.08	17.381	<0.001
IL-2 (ng/L)	15.63± 1.93	13.25± 2.11*	12.91± 2.04	7.983	<0.001
IFN-â (ng/L)	587.62± 143.93	489.25± 129.41*	463.21± 132.07	5.277	<0.001
IL-4 (ng/L)	11.482± 2.24	33.17± 3.82*	34.25± 4.45	6.712	<0.001
IL-10 (ng/L)	10.62± 1.70	44.91± 4.78*	43.56± 5.13	8.496	<0.001

t and P: URSA group *vs.* control group before treatment. *P<0.05 *vs.* before treatment.

ng/L, (10.62±1.70) ng/L] were significantly lower in URSA group than those in control group [(48.82±9.08) ng/mL, (12.91±2.04) ng/L, (463.21±132.07) ng/L, (34.25±4.45) ng/L, (43.56±5.13) ng/L] (P<0.05). After treatment, the difference was not statistically significant among the above indicators between URSA group and control group (P>0.05) (Table 2).

Expression Levels of HMGB1 and Th1 and Th2 Cytokines in Peripheral Blood between Patients with Different Pregnancy Outcomes

Active immunotherapy was administered in the 100 URSA patients, 89 cases with successful pregnancy and 11 cases with failed pregnancy (termination of embryonic development), indicating that the success rate of pregnancy was eighty-nine percent. The expression levels of HMGB1 and Th1 and Th2 cytokines in peripheral blood of patients with the two pregnancy outcomes were compared. There was no significant difference in the above indicators before treatment (P>0.05). After treatment, HMGB1, IL-2 and IFN-γ expression levels were significantly lower [(47.84±10.07) ng/mL, (12.12±2.30) ng/L, (467.15±133.01) ng/L] and IL-4 and IL-10 expression levels were significantly higher [(35.26±3.69) ng/L, (45.24±5.02) ng/L] in the successful pregnancy group than those in the failed pregnancy group [(94.46±10.13) ng/mL, (15.58±1.53) ng/L, (565.64±102.21) ng/L, (12.32±1.36) ng/L, (11.23±3.68) ng/L] (P<0.05) (Table 3).

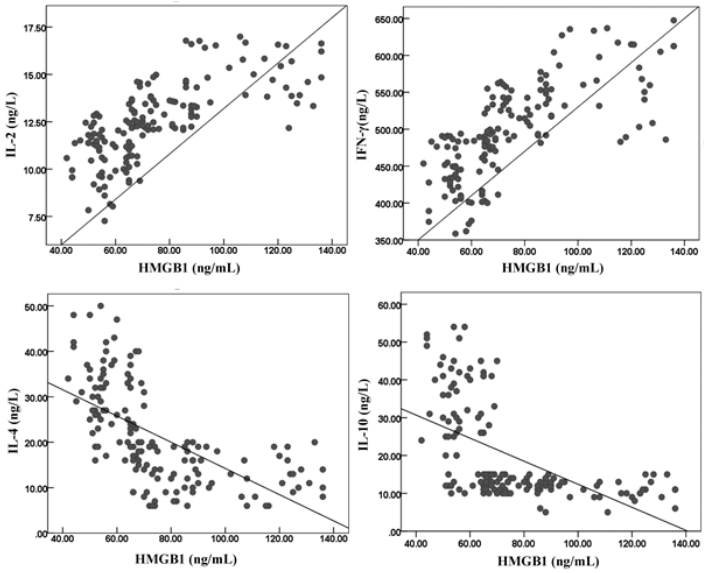

Fig. 2. Correlations of HMGB1 with IL-2, IFN-γ, IL-4 and IL-10

Table 3: Expression levels of HMGB1 and Th1 and Th2 cytokines in peripheral blood between patients with different pregnancy outcomes

Indicator	*Successful pregnancy group (n=89)*		*Failed pregnancy group (n=11)*	
	Before treatment	*After treatment*	*Before treatment*	*After treatment*
HMGB1 (ng/mL)	98.53± 18.69	47.84± 10.07*	98.84± 15.42	94.46± 10.13
IL-2 (ng/L)	15.46± 2.47	12.12± 2.30*	16.02± 1.25	15.58± 1.53
IFN-ã (ng/L)	583.48± 132.66	467.15± 133.01*	591.39± 98.27	565.64± 102.21
IL-4 (ng/L)	11.39± 2.18	35.26± 3.69*	11.98± 1.59	12.32± 1.36
IL-10 (ng/L)	10.68± 1.65	45.24± 5.02*	10.54± 1.23	11.23± 3.68

*P<0.05 *vs.* before treatment.

Correlations of HMGB1 with IL-2, IFN-γ, IL-4 and IL-10

In URSA group, HMGB1 had positive relations with IL-2 and IFN-γ in peripheral blood (r=0.766, r=0.779, P<0.01), while it was negatively correlated with IL-4 and IL-10 (r=–0.735, r=–0.641, P<0.01) (Fig. 2).

Percentages of Th1 and Th2 Cells and Th1/Th2 Ratio after HMGB1 Stimulating CD4+ T Lymphocytes

After CD4+ T lymphocytes were stimulated with HMGB1 for 0, 6, 12 and 24 hours, the percentage of Th1 cells and Th1/Th2 ratio gradually increased, while the percentage of Th2 cells gradually decreased. The percentage of Th1 cells and Th1/Th2 ratio were higher [(6.76±0.92)%, (7.48±1.03)%; 4.70±0.83, 5.96±0.95] and the percentage of Th2 cells was lower [(1.41±0.59)%; (1.23±0.41)%] at 12 and 24 hours than those under stimulation with HMGB1 at 0 hours [(5.89±0.73)%, 2.79±0.51, (2.12±0.48)%] (P<0.05) (Fig. 3 and Table 4).

Table 4: Percentages of Th1 and Th2 cells and Th1/Th2 ratio after HMGB1 stimulating CD4+ T lymphocytes for different times (n=10)

Indicator	*0 h*	*6 h*	*12 h*	*24 h*
Th1 (%)	5.89±0.73	6.06±1.08	6.76±0.92*	7.48±1.03*
Th2 (%)	2.12±0.48	1.90±0.51	1.41±0.59*	1.23±0.41*
Th1/Th2	2.79±0.51	3.14±0.85	4.70±0.83*	5.96±0.95*

*P<0.05 *vs.* 0 h.

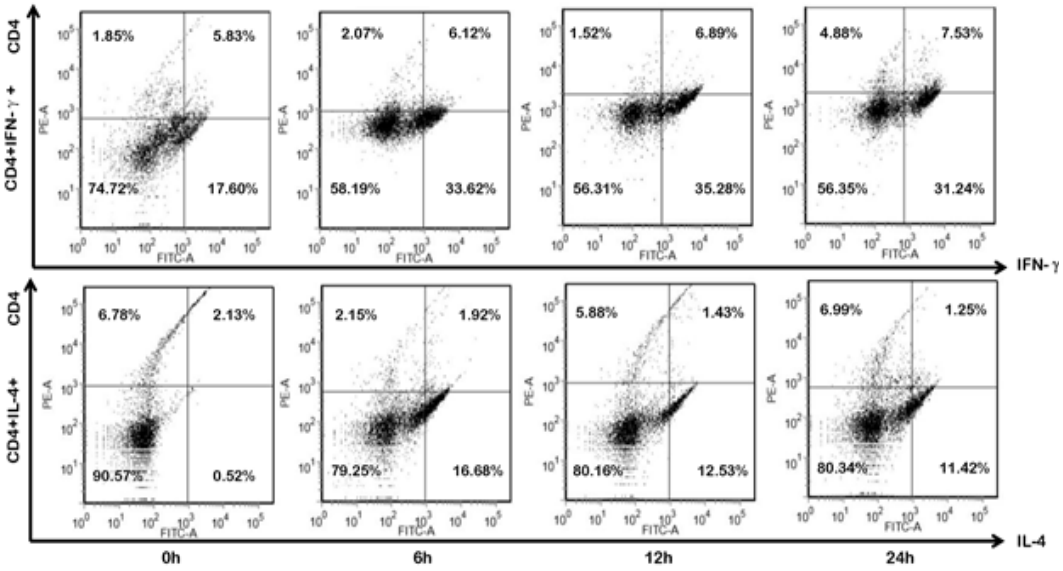

Fig. 3. Flow cytometry results of Th1 and Th2 cells after HMGB1 stimulating CD4+ T lymphocytes

DISCUSSION

Half of the foetal tissue is derived from the father, so embryo is equivalent to a semi-allotransplant for the mother (Würfel et al. 2019). During normal pregnancy, the maternal immune system forms a local foetal immune tolerance microenvironment through a series of complex reactions such as recognition, response and negative regulation (Zhao et al. 2018). The occurrence of URSA is closely related to the imbalance of immune microenvironment homeostasis. The activation and inhibition of T cells are essential for the regulation of immune homeostasis, while Th1/Th2 immunity plays an important role in pregnancy immunity (Azizi et al. 2019). The Th1/Th2 immune response toward Th2 during normal pregnancy can protect the foetus from rejection by the maternal immune system, and the occurrence of URSA is a Th1-dominated immune response, manifested as impairment of the Th1/Th2 balance, increased secretion of Th1 cytokines, and enhancement of cellular immunity (Verma et al. 2019). In this study, the occurrence of URSA was correlated with Th1/Th2 imbalance at the mother-foetal interface, which may be regulated by HMGB1.

Th1 mediates cellular immunity mainly through IFN-γ, tumour necrosis factor-alpha (TNF-γ) and IL-2, thus inducing inflammation and tissue damage, while Th2 mediates humoral immune response through secreting IL-4, IL-10 and IL-6, thereby mitigating inflammation and tissue damage (Kuroda et al. 2021). Generally, there is a dynamic negative feedback balance between Th1 and Th2 cells (Bora and Khan 2019). IL-2 and IFN-ã inhibit Th2 in the process of inducing Th1 differentiation and maturation, while IL-4 and IL-10 alleviate the immune damage and diseases mediated by IL-2 and IFN-γ (Khandhar et al. 2020). TNF-γ and IFN-γ are involved in vascular construction and transformation before and after embryo implantation, and the overexpression of Th1 cytokines has an adverse effect on pregnancy (Azizi et al. 2019). The excessive secretion of IFN-γ leads to the overexpression of foetal histocompatibility leukocyte antigen class I and class II molecules, thus inducing an inflammatory response in maternal T cells and inhibiting embryo implantation (Choi et al. 2018). Pregnancy is affected by this dynamic balance, which induces URSA in the case of imbalance. In this study, the percentage of Th1 cells, Th1/Th2 ratio and expression levels of IL-2 and IFN-γ significantly increased, while IL-4 and IL-10 decreased in URSA patients. The percentage of Th1 cells, Th1/Th2 ratio and Th1/Th2 cytokines returned to normal levels after active immunotherapy. Yang et al. (2018) reported that IFN-γ and TNF-α were highly expressed, while IL-4 and IL-10 were lowly expressed in the peripheral blood of URSA women, which were contrary to those in normal pregnant women. Sasaki et al. (2017) reported that the IL-6 and IL-10 levels were significantly higher and the IFN-γ level was lower in non-URSA women than those in URSA women. Likewise, the researchers herein found that the Th1/Th2 imbalance may be a crucial cause for URSA. Hence, lymphocyte immunotherapy to restore the balance of Th1/Th2 provides an important strategy for the successful pregnancy of URSA patients after treatment, which benefits the shift of Th1/Th2 cytokine levels toward Th2-dominated humoral immunity and promotes the occurrence of maternal-foetal immune tolerance.

As a DNA-binding protein in cells, HMGB1 can be actively secreted by multiple cells or passively released by dead cells outside, also participating in the occurrence of various inflammatory diseases (Aikawa et al. 2020). HMGB1 is highly expressed in placental tissues, which can be released into the maternal circulation when placental organs are under hypoxic stress upon preeclampsia (Liu 2019). Li et al. (2014) reported that HMGB1 was highly expressed in the peripheral blood of URSA women, and it up-regulated Th17-related cytokines and down-regulated Treg-related cytokines after stimulation, thus shifting the Th17/Treg balance toward Th17, leading to immune rejection of the foetus by the maternal immune system and finally inducing recurrent abortion. In the present study, the HMGB1 level was significantly higher in the peripheral blood of URSA patients than that of normal women, and declined significantly after treatment. Besides, HMGB1 was positively correlated with Th1 cytokines, whereas negatively correlated with Th2 cytokines. The $CD4^+$ T lymphocytes of healthy women were stimulated with HMGB1. With prolonged time, the percentage of Th1 cells and Th1/Th2 ratio gradually increased, while the percentage of Th2 cells decreased. Notably, after stimulation with HMGB1 for 12 hours, the Th1/Th2 ratio was close to the level before treatment, suggesting that HMGB1 may be involved in the Th1/Th2 immune response of URSA patients.

Lv et al. (2018) reported that after $CD4^+$ T lymphocytes were stimulated with HMGB1, Th1 cell subsets were predominant in the Th1/Th2 immunity response. By stimulating healthy human T lymphocytes with HMGB1 *in vitro*, Guo et al. (2018) reported that Th1 was dominant after stimulation for 12 hours, but it changed into Th2 dominance with increasing dosage and duration of stimulation. Taken together, HMGB1 can regulate the Th1/Th2 balance, but its regulatory effect on the Th1/Th2 balance may be bidirectional, which needs more in-depth research. Xiu et al. (2018) reported that the RAGE, NF-κB and TNF-α expression levels in the endometrium epithelial cells of pregnant rats treated with HMGB1 were elevated, indicating that HMGB1 may activate NF-κB by binding RAGE, thus inducing the expression of inflammatory factor TNF-α and participating in inflammatory response.

CONCLUSION

In conclusion, the pathogenesis of URSA is correlated with the shift of Th1/Th2 balance toward Th1. HMGB1 may play a regulatory role in balancing the Th1/Th2 immune response, as a new target for treating URSA.

RECOMMENDATIONS

It is of great significance to further study the specific characteristics and mechanisms of HMGB1 for URSA treatment.

ACKNOWLEDGMENTS

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ABBREVIATIONS

ELISA: Enzyme-linked immunosorbent assay
HMGB1: high-mobility group box 1
IFN-γ: interferon-gamma
IL: interleukin
Th: T helper
TNF-α: tumour necrosis factor-alpha
URSA: unexplained recurrent spontaneous abortion

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