Impaired Myeloid-derived Suppressor Cells Are Associated With Recurrent Implantation Failure: A Case-Control Study

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Research

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Abstract

Background: Studies have reported that myeloid-derived suppressor cells (MDSCs) contribute to maintain pregnancy. The aim of this case-control study was to test whether there is a dysregulation of peripheral MDSCs in recurrent implantation failure (RIF).

Methods: 26 RIF patients and 30 controls were recruited. Flow cytometry was applied to characterize polymorphonuclear (PMN)-MDSCs, monocytic-MDSCs (M-MDSCs), effector T cells (Teffs) and regulatory T cells (Tregs) in blood. ELISA was used to define MDSCs correlative cytokines and chemokines in serum from all patients.

Results: Compared with controls, RIF patients showed significant reductions of blood PMN-MDSCs, M-MDSCs, Tregs and NO production by PMN-MDSCs, whereas the expression of ζ chain on CD4⁺T cell receptor (TCR) and CD8⁺TCR displayed a remarkable upregulation in RIF patients. Moreover, RIF patients presented a lower concentration of serum chemokine (C-C motif) ligand (CCL) 5 and transforming growth factor (TGF)- β than those from controls. Furthermore, the level of TCR ζ chain on CD4⁺ and CD8⁺ Teffs was negatively correlated not only with the percentage of PMN-MDSCs, but also with the amount of NO produced by PMN-MDSCs. The frequency of PMN-MDSCs had positive correlations with the concentration of CCL5 and TGF- β .

Conclusions: This study indicated that the dysregulation of MDSCs might impair maternal-fetal immune balance thus resulting in RIF.

Introduction

Recurrent implantation failure (RIF) is diagnosed when women experienced 3 or more frozen or fresh cycles with being transferred high-quality embryos and failed to obtain a clinical pregnancy [1, 2]. Numerous studies have found that the identifiable causes include parental chromosomal abnormalities, defective embryonic development, uterine anatomic anomalies, and poor endometrium [3–6]. Nevertheless, more and more researches have focused on the immunological aspects, especially a failure to establish maternal-fetal immunotolerance for successful implantation.

Lédée et al. reports that, at the time of embryo implantation, the maternal immune system is featured by distinct immunological alterations with enrichment of various immune cells in both peripheral circulation and uterus microenvironment [7]. These changes make an immunological tolerance environment which protects embryo expressing paternal antigen from maternal antigen-specific T cells and contributes to successful implantation [7]. Breakdown of maternal-fetal tolerance was found to be associated with a poor clinical outcome in pregnant women [8, 9]. Furthermore, dysregulation of implicated immune cells such as uterine natural killer cells (NK), peripheral NK, regulatory T cells (Tregs), T-helper cells have been discovered in RIF [10–13].

Myeloid-derived suppressor cells (MDSCs) represent a heterogeneous cell group with attributes in myeloid origin, immature state, and immunosuppressive function [14]. Under pathogenic condition, MDSCs are accumulated by an inflammatory condition which contains mediators such as transforming growth factor (TGF)- β , tumor necrosis factor (TNF)- α , interleukin (IL)1 β , IL-6, IL-10 and chemokines such as chemokine (C-C motif) ligand (CCL) 2, CCL3, CCL4, CCL5 [15, 16]. They exert immunosuppressive function by different mechanisms: the activation of arginase-1 (Arg-1) and inducible nitric oxide synthase (iNOS) which generates nitric oxide (NO) [14]. These pathways caused a dysregulation of T cell functions by downregulating the ζ chain expression on T cell receptor (TCR), which has a key role in the TCR-mediated antigen recognition and signal transduction [14, 17]. In mice, MDSCs can be divided into CD11b⁺Ly6G⁻Ly6C^{high} monocytic MDSCs (M-MDSCs) and CD11b⁺Ly6G⁺Ly6C^{low} polymorphonuclear MDSCs (PMN-MDSCs). In human, they are identified as CD33⁺HLA-DR^{-/low}CD11b⁺CD14⁺CD15⁻ M-MDSCs and CD33⁺HLA-DR^{-/low}CD11b⁺CD14⁻CD15⁺ PMN-MDSCs [18]. MDSCs have been reported to expand in different pathological conditions such as cancer, traumas and infectious diseases [19–21]. Because of the immunosuppressive effect of MDSCs, studies focused on their roles in pregnancy and associated complications in recent years [22–26]. Studies demonstrated that MDSCs expanded not only in uterus but also in peripheral blood during gestation period [22, 24]. In human, PMN-MDSCs are accumulated in peripheral circulation of healthy pregnant women as compared to nonpregnant controls [27, 28]. Reduction of MDSCs was explored both in decidua and in peripheral blood from miscarriage patients [29]. In addition, depletion of MDSCs was found to result in a significant decrease of Tregs and severe embryo resorption in mice model [30].

These growing evidences strengthen that MDSCs have a strong ability to promote maternal immune tolerance. However, whether peripheral MDSCs are associated with RIF is still unknown. In this study, we have assessed the level and function of MDSCs along with its subtypes in RIF patients and pregnant women with first IVF. Our study is the initial research that discovers the reduction of PMN-MDSCs in RIF patients. These findings provide a strategy with which inducing MDSCs could be utilized as a therapeutic option in RIF women.

Materials And Methods

Patient selection

In our study, 26 RIF patients and 30 controls were recruited from the Reproductive Medicine Center of the First Affiliated Hospital of Anhui Medical University. All RIF patients were transferred high-quality embryos in more than 3 fresh or frozen cycles and failed to achieve clinical pregnancy. Ultrasonography and hysteroscopy were applied to confirm that every RIF patient had a normal morphology and thickness of endometrium and a normal uterine cavity. Women who underwent the first IVF-embryo transfer (ET) cycles because of oviduct infertility were recruited, and the ones who obtained clinical pregnancy were identified as control group. All patients whose ages ranged from 22 to 38 in this study had a normal ovarian reserve and accepted frozen ET. The exclusive criteria for all the volunteers were as follows: hydrosalpinx, genetic or anatomic abnormalities, polycystic ovary syndrome, endometriosis,

adenomyosis, recurrent spontaneous abortion, autoimmune disorder, infectious diseases, immunomodulator treatment which might affect immune system 1 year prior to the study. Characteristics of patients are listed in Table 1. The Ethics Review Board of the First Affiliated Hospital of Anhui Medical University has approved the study (PJ2018-07-20). All patients signed informed consent.

Sample collection

Blood samples were taken 1-2 hours before embryo transfer. Peripheral blood mononuclear cells (PBMCs) were obtained from heparinized venous blood by density gradient centrifugation using Ficoll-Paque1.077 g/ml (GE Healthcare, Sweden). The serum was stored at -80°C and the cell pellets were adjusted to 10^7 cells and cryopreserved at -80°C. The cryopreservation medium was Roswell Park Memorial Institute supplemented with 30% human serum and 10% Dimethyl Sulphoxide.

Flow cytometry analysis of human PBMCs

The following anti-human antibodies (mAbs) used are listed in Table 2. PBMCs (1×10^6 /mL) were preincubated with human True-Stain Monocyte Blocker™ (Biolegend, USA) to block nonspecific binding and cells were stained with different combinations of antibodies listed in Table 2. 4,5-Diaminofluorescein Diacetate (DAF-2DA; Abcam, USA) was stained for NO production according to the manufacturer's recommendation. During the intracellular staining of Foxp3, cells were preincubated with the Foxp3 fixation/permeabilization kit (eBioscience, USA) following the instruction. Acquisition was performed by FACSVerse™ with FACSuite software. The compensation control was performed. Data with at least 100,000 events was analyzed with FlowJo software (Tree Star). Pseudocolor were used in the study.

ELISA assay in human serum

The serum was thawed, and 11 cytokines were analyzed by ELISA kit (Multisciences Biotech, China) according to the instruction manual. They were: TGF-β, IL-1β, IL-6, IL-10, Interferon-γ, granulocyte macrophage colony-stimulating factor, TNF-α, CCL2, CCL3, CCL4 and CCL5.

Statistical analysis

GraphPad Prism software was applied for statistical analysis. For two groups, an unpaired two-tailed Student's *t* test was implemented. Results were tested with a Chi-squared analysis test for categorical variables. Correlations between MDSCs and other factors were detected by Spearman analysis. Correlation coefficient is showed as *r*. *P* < 0.05 was considered a statistically significant difference.

Results

Impairment of MDSCs in RIF patients

We examined MDSCs in the PBMCs from patients of each group. Using flow cytometry, we characterized M-MDSCs as CD33⁺HLA-DR^{-/low}CD11b⁺CD14⁺CD15⁻ cells and PMN-MDSCs as CD33⁺HLA-

DR^{-/low}CD11b⁺CD14⁻CD15⁺ cells (Fig. 1a). The frequencies of these two subpopulations were measured as a percentage within live cells. RIF patients showed a decreased frequency of PMN-MDSCs (Fig. 1b, $P < 0.001$) and M-MDSCs (Fig. 1c, $P < 0.01$) as compared with those in control group. Meanwhile, we investigated suppressive potential of MDSCs by assessing NO production in these cells. Intracellular NO production was detected by DAF-2DA utilizing mean fluorescence intensity (MFI). NO production by PMN-MDSCs of RIF women was significantly decreased as compared to controls (Fig. 1d, $P < 0.01$). Whereas the level of intracellular NO of M-MDSCs was similar between both groups.

Immunosuppressive effect of MDSCs in RIF patients

Subsequently, we examined the effect of MDSCs on effector T cells (Teffs) in each group. Teffs were identified as CD3⁺CD4⁺Foxp3⁻ and CD3⁺CD8⁺Foxp3⁻ (Fig. 2a). We found that the frequency of CD4⁺ and CD8⁺Teffs in two groups remained mostly at the same level, however, RIF patients showed an upregulation of TCR ζ chain expression on CD4⁺ (Fig. 2b, $P < 0.05$) and CD8⁺Teffs (Fig. 2c, $P < 0.05$) as compared to those in controls.

Reduction of Tregs in patients with RIF

Next, we identified Tregs as CD3⁺CD4⁺ CD25⁺CD127^{low}Foxp3⁺ cells (Fig. 3a), and the frequency of Tregs has been calculated among live cells. We discovered a decreased level of Tregs in RIF patients as compared to this value in control group (Fig. 3b, $P < 0.01$).

Cytokines in RIF women

We investigated the serum chemokines or cytokines in two groups and demonstrated that the concentrations of CCL5 and TGF- β in RIF women were significantly lower than those in controls (Fig. 4a, $P < 0.05$ and Fig. 4b, $P < 0.01$). No statistically significant differences in levels of other investigated cytokines and chemokines were found.

The correlation between MDSCs and other factors

There was a significant negative correlation between the percentage of PMN-MDSCs and the expressions of CD4⁺TCR ζ chain (Fig. 5a, $r = -0.412$, $P < 0.01$) and CD8⁺TCR ζ chain (Fig. 5b, $r = -0.3509$, $P < 0.01$). Moreover, the production of NO produced by PMN-MDSCs displayed negative correlations with the levels of CD4⁺TCR ζ chain (Fig. 5c, $r = -0.3706$, $P < 0.01$) and CD8⁺TCR ζ chain (Fig. 5d, $r = -0.4539$, $P < 0.001$). Whereas, there was a positive relationship between the level of PMN-MDSCs and the level of CCL5 (Fig. 5e, $r = 0.4317$, $P < 0.01$) and TGF- β (Fig. 5f, $r = 0.4383$, $P < 0.01$). We did not find any correlation between the level of PMN-MDSCs and other cells, nor was there any relevance between the percentage of M-MDSCs and other cells and cytokines.

Discussion

Embryo implantation is an exceedingly complex, convoluted process of reproductive biology. An immunity homeostasis induced by maternal fetal cross-talk protects semi-allogeneic fetus from attacking by powerful maternal immune system. Pregnancy-induced immunomodulatory effects present in local of maternal-fetal interface as well as circulation of maternal system.

In the last decades, the contribution of MDSCs to maternal-fetal immunotolerance has been recognized [22, 23]. Their roles have been described in different situations, early and mid-term pregnancy, early miscarriage, the neonatal period and preeclampsia [24, 27, 29, 31]. Little is known, however, about the effects of MDSCs on the window of implantation (WOI) in RIF patients. To clarify this question, we investigated the patterns of MDSCs in peripheral blood from RIF women on the day of ET to determine whether MDSCs are involved in immunotolerance during WOI.

In present study, we found impressive impairments in frequency of peripheral blood MDSCs from women with RIF as compared to those from control group. These data are comparable to previous studies which described that the ratio of MDSCs among PBMCs showed a positive correlation with pregnancy rate in IVF patients [26]. MDSCs have been demonstrated to be one of the powerful immunosuppressive cells and tied up with maternal immune tolerance [32]. Köstlin and his group observed that healthy pregnant women have a significant accumulation of PMN-MDSCs in the blood during all stages of pregnancy [27]. Studies in human and mice report that a decreased percentage and activity of MDSCs in peripheral blood is associated with early miscarriage [9, 29, 33, 34]. In microenvironment, MDSCs secrete iNOS to catabolize L-Arg to citrullin and produce NO which downregulates the expression of the TCR ζ chain on T cells hence restrain T cells activity [14]. As a pivotal antigen-recognition cells population in human, activated T cells may be a threat to the embryo expressing paternal antigen [35]. In agreement with these observations, when we considered measurement of intracellular NO as a biomarker of immunosuppressive potential for MDSCs [14] and compared those in each group, we found a significant reduction in intracellular NO produced by PMN-MDSCs from RIF patients. On the contrary, the levels of TCR ζ chain on CD4⁺ and CD8⁺Teffs are significantly upregulated in RIF patients. Moreover, the level of TCR ζ chain on CD4⁺ and CD8⁺Teffs was negatively correlated not only with the percentage of PMN-MDSCs, but also with the amount of NO produced by PMN-MDSCs.

MDSCs have been demonstrated to maintain maternal-fetal tolerance by inducing Foxp3 expression in CD4⁺CD25⁻T cells, hence expanding Tregs [30]. Tregs as a subpopulation of suppressor cells, express the transcription factor Foxp3 and play a critical role in preventing semi-allogeneic fetus from maternal immune system [36]. Anergia of conceptus-specific T cells during pregnancy relies on the persistent presence of Tregs [35]. Enhancement of Tregs in peripheral blood is concerned with a better IVF treatment outcome [37], whereas reduction of peripheral Tregs is associated with reproductive failure [38]. In line with these observations, we found an exhaustion of Tregs in RIF patients. However, the proportion of Tregs were not relevant to the proportion of MDSCs in this study. This may be due to the fact that Tregs can also be induced by hormones [39].

MDSCs require different signal molecules for their migration, proliferation and activation to suppress the immune response [15, 16]. CCL5 was reported to play a vital role in the recruitment and activation of MDSCs as well as the generation and mobilization of MDSCs [15]. Similar to these studies, we found a decrease of serum CCL5 in RIF patients as compared to controls. Moreover, our data showed that the level of serum CCL5 was positively correlated with PMN-DSCs. Using animal model, Bae et al. described that CCL5 may be involved in or promote the placental development [40]. Taken together, we would suggest that impaired CCL5 and MDSCs could be a cause for RIF. Furthermore, our finding revealed that RIF patients displayed a significantly lower concentration of serum TGF- β as compared to control women. As a major effective media, TGF- β was reported to be secreted by MDSCs and activate several signaling pathways in MDSCs, and consequently augment immunosuppressive capacity of MDSCs [14]. Meanwhile, as an important anti-inflammatory cytokine, TGF- β was significantly decreased in plasma from RIF patients [41]. Our study found that the concentration of serum TGF- β presents a positive correlation with the percentage of PMN-MDSCs. These discoveries provide further evidence that an appropriate amount of MDSCs may contribute to embryo implantation, whereas depletion of MDSCs is detrimental to embryo implantation.

Conclusion

Our study discovers the differential expression of MDSCs and their related mediators in RIF and control group. These outcomes revealed that the dysregulation of MDSCs might impair maternal-fetal immune balance thus resulting in RIF. Therefore, targeting these cells might provide new treatment methods in the future.

Abbreviations

RIF, Recurrent implantation failure; IVF, in vitro fertilization; ET, embryo transfer; NK, natural killer cells; Tregs, regulatory T cells; MDSCs, myeloid-derived suppressor cells; PMN-MDSCS, polymorphonuclear myeloid-derived suppressor cells; M-MDSCs, monocytic myeloid-derived suppressor cells; TGF- β , transforming growth factor- β ; TNF- α , tumor necrosis factor- α ; IL, interleukin; CCL, chemokine (C-C motif) ligand ; Arg-1, arginase-1; iNOS, inducible nitric oxide synthase; NO, nitric oxide; TCR, T cell receptor; PBMCs, peripheral blood mononuclear cells; Teffs, effector T cells; MFI, mean fluorescence intensity; WOI, window of implantation

Declarations

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Authors contributions

Author contributions: KW and YC participated in study design and manuscript drafting. HJ and MZ and PG contributed to study conduct and data analysis. KB and ZL helped to prepare samples. CL helped with the acquisition of clinical data. All authors approved the final version of this manuscript and are responsible for the aspects of the work.

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Availability of data and materials

The data supporting the conclusions of this article are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

This study was approved by the Ethics Review Board of the First Affiliated Hospital of Anhui Medical University, China (No. PJ2018-07-20). Written informed consent was signed by all patients.

Consent for publication

Not applicable.

Competing interests

The authors report no competing interests.

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Tables

Table 1
Basic information of RIF patients and control group.

	RIF patients n = 26	CG n = 30	P- value
Female age (years) ^a	30.58 ± 4.13	29.57 ± 3.57	0.330
Male age (years) ^a	30.58 ± 5.08	31.00 ± 3.90	0.726
BMI (kg/m ²) ^a	22.29 ± 2.87	22.35 ± 3.82	0.936
Baseline FSH (IU/L) ^a	6.38 ± 0.48	7.03 ± 2.06	0.479
Baseline LH (IU/L) ^a	4.68 ± 2.12	4.29 ± 1.91	0.7019
Primary infertility ^b	18/26(69.23%)	12/30(40%)	0.029*
Secondary infertility ^b	8/26(30.77%)	18/30(60%)	
No. of Retrieved oocytes ^a	15.08 ± 1.25	15.03 ± 1.35	0.9814
No. of MII ^a	14.31 ± 1.14	13.6 ± 1.20	0.6735
No. of fertilized eggs ^a	13.27 ± 1.11	12.6 ± 1.12	0.6748
No. of cleavage ^a	13.19 ± 1.11	12.37 ± 1.10	0.5964
No. of 2PN ^a	10.77 ± 1.06	9.77 ± 1.00	0.4937
No. of obtained embryo ^a	7.23 ± 0.71	6.73 ± 0.75	0.5068
No. of high quality embryo ^a	6.28 ± 0.76	6.10 ± 0.76	0.8534
No. of endometrium thickness ^a	10.34 ± 0.35	11.36 ± 0.32	0.9627
Data are presented as means ± standard deviation (SD) or n%. ^a unpaired Student's <i>t</i> -test, ^b Chi-square test.			
RIF-recurrent implantation failure; CG-control group; BMI-body mass index; FSH-follicle stimulating hormone; LH-luteinizing hormone.			
* <i>P</i> < 0.05			

Table 2
Antibodies used for MDSCs, Tregs, Teffs.

Antibodies	Company
CD33 Monoclonal Antibody, PE-Cy7	Biolegend, USA
CD11b Monoclonal Antibody, APC	BD Biosciences,USA
HLA-DR Monoclonal Antibody,APC-H7	Biolegend, USA
CD14 Monoclonal Antibody, Percp-Cy5.5	BD Biosciences,USA
CD15 Monoclonal Antibody, PE	BD Biosciences,USA
CD3 Monoclonal Antibody,FITC	BD Biosciences,USA
CD4 Monoclonal Antibody,PE-Cy7	BD Biosciences,USA
CD25 Monoclonal Antibody,PE	BD Biosciences,USA
C127 Monoclonal Antibody,Percp-Cy5.5	BD Biosciences,USA
Foxp3 Monoclonal Antibody,APC	BD Biosciences,USA
CD3 Monoclonal Antibody,AF647	BD Biosciences,USA
CD4 Monoclonal Antibody,Percp-Cy5.5	BD Biosciences,USA
CD8 Monoclonal Antibody,APC	BD Biosciences,USA
CD247 Monoclonal Antibody,FITC	BD Biosciences,USA
Foxp3 Monoclonal Antibody,PE	BD Biosciences,USA

Figures

Figure 1

Figure 1

Analysis of MDSCs in RIF patients. PBMCs obtained from the peripheral blood of 26 RIF patients and 30 control group (CG) women were analyzed by flow cytometry. (a) Representative pseudocolor with the gating strategy identifying M-MDSCs (CD33+HLA-DR-/lowCD11b+CD14+CD15-cells) and PMN-MDSCs (CD33+HLA-DR-/lowCD11b+CD14-CD15+cells). NO production was measured by DAF-2 DA. (b, c) The frequency of PMN-MDSCs (b) and M-MDSCs (c) in RIF patients and CG are presented as the percentage of these cells within live PBMCs. (d) Results of NO reproduction on PMN-MDSCs is shown as mean fluorescence intensity (MFI). ** $P < 0.01$, *** $P < 0.001$. RIF, recurrent implantation failure; CG, control group.

Figure 2

Figure 2

Evaluation of Teffs in RIF patients. PBMCs obtained from the peripheral blood of RIF patients and control group (CG) women were analyzed by flow cytometry. (a) Representative pseudocolor with Gating strategy of Teffs. Foxp3 negative population was gated for CD3+CD4+T cells and CD3+CD8+T cells. CD247 used to identify the expression of TCR ζ chain on Teffs. (b, c) The expressions of TCR ζ chain on CD4+Teffs (b) and CD8+Teffs (c) were shown as the percentage of these cells within live cells. *P < 0.05.

Figure 3

Figure 3

Assessment of Tregs in RIF patients. PBMCs obtained from the peripheral blood of RIF patients and control group (CG) were analyzed by flow cytometry. (a) Representative pseudocolor with the gating strategy to detect CD3+CD4+CD25+CD127-/lowFcxp3+Tregs. (b) The frequency of Tregs in each group is shown as the percentage of these cells within live cells. **P < 0.01

Figure 4

Figure 4

Production of cytokines in patients with RIF. Cytokines were measured in serum of RIF patients as well as in controls by ELISA. (a, b) Concentrations of CCL5 (a), TGFβ (b) detected in patients with each group are expressed as pg/ml. *P < 0.05, **P < 0.01.

Figure 5

Figure 5

Correlation between PMN-MDSCs and other factors. (a, b) show negative correlations between the level of PMN-MDSCs and the level of CD4+ ζ chain (a) and CD8+ ζ chain (b). (c, d) reveal the expressions of CD4+ ζ chain (c) and CD8+ ζ chain (d) are both negatively correlated with the production of NO produced by PMN-MDSCs. (e, f) display positive relationships between the frequency of PMN-MDSCs and the concentration of CCL5 (e) and TGF β (f).