Abnormal Expression of the LAG-3/FGL-1 Signaling Pathway in Patients with Early-Onset Preeclampsia

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Background: Preeclampsia (PE) is a serious pregnancy disorder associated with immune tolerance imbalance. The etiology of preeclampsia has not been fully elucidated. The aim of this study was to clarify the possible role of the lymphocyte activation gene 3 (LAG-3)/fibrinogen-like protein 1 (FGL-1) signaling pathway in the immune imbalance of early-onset PE.

Material/Methods: We enrolled 34 women with early-onset PE and 34 age-matched normal pregnancies (NPs). Flow cytometry was performed to determine the expression of LAG-3 on peripheral T cell subsets (CD3+, CD4+, and CD8+ T cells). We measured LAG-3 expression on decidual T cells to determine whether there was a difference in the expression of LAG-3 between decidual and peripheral T cells. Maternal plasma levels of FGL-1 were measured by ELISA.

Results: There was no significant difference in LAG-3 expression on peripheral CD3+ T cells between NP and early-onset PE. Compared to NP, the significant decrease expression of LAG-3 by peripheral CD4+ and CD8+ T cells was found in early-onset PE. The LAG-3 expression was higher on decidual T cells than peripheral counterparts in all pregnancies. The plasma level of FGL-1 was significantly elevated in early-onset PE compared with NP.

Conclusions: Abnormal expression of LAG-3/FGL-1 signaling pathway may be associated with immune activation of effector T cells and impaired immune tolerance in early-onset PE.

Keywords: CD223 Antigen • FGL1 Protein, Human • Hypertension, Pregnancy-Induced • Immune Checkpoint Proteins • T-Lymphocytes

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Background

Pregnancy poses an immunological challenge because a semi-allogeneic fetus must be supported within the pregnant female [1,2]. Therefore, immune tolerance is essential to the maintenance of successful pregnancy. Excessive activation of the immune system may result in abnormal pregnancies, such as pregnancy loss, preeclampsia (PE), and fetal growth restriction.

PE is a serious complication of pregnancy, combined with high blood pressure, renal insufficiency and other systemic diseases [3,4]. Early-onset preeclampsia (early-onset PE) refers to the onset of clinical symptoms and delivery before 34 weeks of gestation [5]. The pathophysiology of preeclampsia is considered to be due to insufficient trophoblastic invasion of the uterine spiral arteries, which leads to placental hypoperfusion and excessive inflammatory response [6]. However, the etiology of preeclampsia remains largely undefined and there is no effective treatment yet.

T cell is altered and is considered to play an essential part in immunological maladaptation and inadequate tolerance in preeclampsia, such as the well-known T-helper 1 (Th1)/Th2/Th17 and regulatory T (Treg) cells paradigm [7]. Effector T cells, CD8+ and CD4+T cells, have also been reported to be involved in the pathogenic mechanism of preeclampsia [8,9]. Lymphocyte activation gene 3 (LAG-3, CD223) is an immune checkpoint molecule with structural similarities to CD4 [10]. It is expressed on activated CD8+ and CD4+T cells and negatively regulates their function, activation, and proliferation [11-13]. Enhanced LAG-3 expression on T cells was observed in a number of autoimmune diseases and cancers, which finally led to T cell dysfunction [14]. Fibrinogen-like protein 1 (FGL-1), a main LAG-3 functional ligand, inhibits T cell activation by combining with LAG-3 [15]. FGL-1 is a hepatocyte-derived protein that is released into the plasma and may be involved in fine-tuning systemic inflammation [16]. Upregulation of FGL-1 is observed in the plasma of cancer patients and associated with poor prognosis [17].

LAG-3/FGL-1 have been extensively studied in the fields of oncology [18-20]. Recent data suggest that they also play an essential role in the maintenance of normal pregnancy via multiple inhibitory mechanisms [21,22]. Nevertheless, there is limited information on LAG-3/FGL-1 in the context of immune regulation in preeclampsia. In this study, we aimed to explore whether the aberrant expression of LAG-3/FGL-1 is associated with pathogenesis of early-onset preeclampsia.

Material and Methods

Study subjects

A total of 68 pregnant women delivered by cesarean section in Shandong Provincial Hospital from 2019 to 2021 were included in the study. Thirty-four patients with early-onset PE were selected as the experimental group, while 34 women with normal pregnancy (NP) who gave birth during the same period were selected as the control group (Table 1). Post hoc power analysis was performed (power=1.0), which indicated that the sample size was adequate. The inclusion criteria of the experimental group were: the occurrence of hypertension (systolic blood pressure ≥140 mmHg and/or diastolic blood pressure ≥90 mmHg) before 34 weeks of gestation in women who were normotensive before 20 weeks, accompanied by either proteinuria or multiple organ dysfunction. All women selected as the control group had normal blood pressure and no complications. Patients were excluded if they had multiple gestations, premature rupture of membranes, autoimmune problems, infectious diseases or metabolic diseases such as diabetes mellitus, hypertension, and kidney disease. The study was approved by the Ethics Committee of Shandong Provincial

Table 1. Clinical characteristics of women with NP (n=34) and early-onset PE (n=34).

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<th>NP</th>
<th>Early-onset PE</th>
<th>P-value</th>
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<tr>
<td>Age (years)</td>
<td>28.96±7.13</td>
<td>30.17±4.58</td>
<td>NS</td>
</tr>
<tr>
<td>Gestation age (weeks)</td>
<td>38.79±1.14</td>
<td>32.84±0.75</td>
<td>*</td>
</tr>
<tr>
<td>Gravidity</td>
<td>1.50±4.29</td>
<td>1.72±2.06</td>
<td>NS</td>
</tr>
<tr>
<td>Parity</td>
<td>0.69±0.20</td>
<td>0.71±4.30</td>
<td>NS</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>115.46±6.32</td>
<td>158.03±8.92</td>
<td>**</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>79.13±7.24</td>
<td>104.26±9.13</td>
<td>**</td>
</tr>
<tr>
<td>Proteinuria (g/24 h)</td>
<td>0.03±0.01</td>
<td>1.12±0.28</td>
<td>**</td>
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<tr>
<td>Neonatal birth weight (g)</td>
<td>3.471.16±128.64</td>
<td>2.982.27±155.35</td>
<td>*</td>
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Statistical comparisons were made by unpaired t test. Data are presented as the mean±SEM. * P<0.05. ** P<0.01. NS – nonsignificant; NP – normal pregnancy; Early-onset PE – early-onset preeclampsia.
Hospital, Jinan, China (grant number 2020-805). Informed consent was obtained from all participants.

**Isolation of Peripheral Blood Mononuclear Cells (PBMCs)**

Heparinized peripheral blood samples were used to separate PBMCs on Ficoll gradient (GE-Healthcare, Chicago, IL, USA). The PBMCs at the plasma/Ficoll interface were centrifuged, resuspended and washed twice in phosphate-buffered saline (PBS). Isolated cells were then resuspended in Roswell Park Memorial Institute (RPMI) 1640 medium (HyClone, Logan, UT, USA) containing 10% fetal bovine serum (FBS, Gibco, USA). Cell viability was measured by Trypan blue staining. Ultimately, the cell suspension was counted microscopically (Olympus, Tokyo, Japan) and the concentration was adjusted to 10^6 cells/ml.

**Isolation of Decidual Tissues Mononuclear Cells**

Decidual tissues were minced into 1-mm³ pieces and incubated in buffer containing 1 mg/ml IV collagenase and 0.1 mg/ml DNase for 1 h at 37°C. Then, the suspensions were filtered through a 70-µm strainer (BD Biosciences, Franklin Lakes, NJ, USA) and washed in RPMI 1640 (HyClone, Logan, UT, USA) at 1500 r/min for 10 min. The supernatant was aspirated, and the pellet was resuspended in PBS. The suspensions were laid onto lymphocyte separation medium (GE Healthcare, Chicago, IL, USA) and centrifuged at 2000 r/min at room temperature for 25 min. Isolated cells were then collected, washed, and resuspended in RPMI 1640 medium (HyClone, Logan, UT, USA) containing 10% FBS. Cell viability was measured by Trypan blue staining.

**Flow Cytometric Analysis**

The following monoclonal antibodies were used for staining surface antigens: V500-conjugated anti-human CD4 (BD Biosciences, Franklin Lakes, NJ, USA), Alexa Fluor (AF)488-conjugated anti-human CD3 (BD Biosciences, Franklin Lakes, NJ, USA), AF700-conjugated anti-human CD8 (BD Biosciences, Franklin Lakes, NJ, USA), and phycoerythrin (PE)-conjugated anti-human LAG-3 (BD Biosciences, Franklin Lakes, NJ, USA). We incubated 10^6 cells with fluorescein-labeled mAbs for 30 min in the dark. After the staining, cells were washed twice with PBS. Finally, the cells were resuspended in 300 µl of PBS containing 1% paraformaldehyde and stored at 4°C in the dark until FACs analysis. Data acquisition was performed using a FACS Aria III flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA), and the collected data were exported to FACSDIVA V6.5 software (BD Biosciences, Franklin Lakes, NJ, USA) for analysis.

**Measurement of FGL-1 Levels**

The plasma samples were extracted after centrifugation and kept in a -80°C mechanical freezer for cryopreservation prior to use. Plasma levels of FGL-1 were measured by using ELISA kits (R&D Systems, MN, USA) following the manufacturers’ instructions. All samples were tested in duplicate.

**Statistical Analysis**

Statistical analyses were performed using GraphPad Prism software, version 7 (GraphPad, CA, USA). Normality tests were used to check the normality of the data. The unpaired t test was applied to test the differences between NP and early-onset PE. Statistical comparison between peripheral blood and decidua was performed employing the paired t test. The results were reported as the mean±SEM. P value <0.05 indicated statistical significance.

**Results**

**Phenotypic Analysis of Different Peripheral T Cell Subsets in NP and Early-Onset PE**

We compared the percentages of different T cell subsets in the peripheral blood between NP and early-onset PE by flow cytometry. Cells were sorted using the indicated gates. Single cells and lymphocytes were gated based on forward and side scatters. Lymphocytes were then subdivided into CD3+, CD4+, and CD8+ T lymphocytes (Figure 1). No differences were observed in the percentages of different T cell subsets (Table 2).

**LAG-3 Expression by Different Peripheral T Cell Subsets in NP and Early-Onset PE**

Surface expression of LAG-3 by peripheral CD3+ T, CD4+ T, and CD8+ T cells was measured by flow cytometry. The results showed there were no significant differences in LAG-3 expression on CD3+ T cells between NP and early-onset PE (Figure 2A). The expression of LAG-3 on CD4+ T and CD8+ T cells was significantly decreased in early-onset PE compared with NP (Figure 2B, 2C).

**Comparison of LAG-3 Expression Between Peripheral and Decidual T Cells**

To understand the differences in LAG-3 expression between peripheral and decidual T cells, we compared NP and early-onset PE. We found that the expression of LAG-3 on decidual T cells was significantly higher than that on peripheral T cells in both NP and early-onset PE (Figure 3A, 3B).

**Serum Levels of FGL-1 in NP and Early-Onset PE**

Serum levels of FGL-1 were measured by ELISA. Compared to NP, the serum level of FGL-1 was significantly elevated in women with early-onset PE (Figure 4).
CD4+ T cells indirectly contributes to abnormal activation of T cells in early-onset PE. Our research found a significant decrease in LAG-3 expression of early-onset PE compared to NP. As mentioned above, LAG-3 has a similar structure to CD4 and a higher affinity for binding to major histocompatibility complex (MHC)-II molecules than CD4 [25]. LAG-3-MHC-II interaction negatively regulates T cell activation, cytotoxicity, and cytokine production [26]. In contrast, T cell activation was supported when MHC-II bound to CD4 [27]. It seems that LAG-3 acts as a negative competitor of CD4, suggesting that the decreased expression of LAG-3 by peripheral CD4+ T cells indirectly contributes to abnormal activation of T cells via reducing this negative competition in early-onset PE.

Discussion

Preeclampsia is an obstetric syndrome characterized by abnormal immune activation dependent on the imbalance of T cell subsets [23]. Our results showed that there were no significant differences in the proportion of CD3+, CD4+, and CD8+ T cells between early-onset PE and NP. Prior studies have shown increased levels of activated CD4+ and CD8+ T cells in preeclamptic patients [8]. These observations suggest that abnormal immune activation in preeclampsia was not due to alterations in the proportion of CD3+, CD4+, and CD8+ T cells, but rather was associated with their activity. The activation and exhaustion of immune cells requires multiple incoming signals [24]. In this study, we aimed to clarify the possible role of the LAG-3/FGL-1 signaling pathway in the immune imbalance of early-onset PE.

Our research found a significant decrease in LAG-3 expression on peripheral CD4+ T cells and CD8+ T cells of early-onset PE compared to NP. As mentioned above, LAG-3 has a similar structure to CD4 and a higher affinity for binding to major histocompatibility complex (MHC)-II molecules than CD4 [25]. LAG-3-MHC-II interaction negatively regulates T cell activation, cytotoxicity, and cytokine production [26]. In contrast, T cell activation was supported when MHC-II bound to CD4 [27]. It seems that LAG-3 acts as a negative competitor of CD4, suggesting that the decreased expression of LAG-3 by peripheral CD4+ T cells indirectly contributes to abnormal activation of T cells via reducing this negative competition in early-onset PE.

CD8+ T cells, which were also suppressed by LAG-3, do not interact with MHC-II [28]. LAG-3 has recently been shown to be highly expressed on decidual CD8+ T cells during NP [21,22]. LAG-3+ Treg cells were also observed both in decidua and periphery in human early pregnancy [29]. Several studies have suggested LAG-3 negatively regulates the proliferation, activation, effector function, and homeostasis of CD4+ and CD8+ T cells [11-13]. On the basis of these results, it could be assumed that T cells express the LAG-3 on the surface at a high level during pregnancy to reduce T cell effector responses. However, the reduced expression of LAG-3 directly contributes to impaired maternal immune tolerance of early-onset PE by inducing abnormal activation of T cells.

Immune tolerance microenvironment at the maternal-fetal interface is the basis for supporting a semi-allogenic fetus during pregnancy [30]. We evaluated the potential contribution of immune checkpoint molecule LAG-3 to immune tolerance at the maternal-fetal interface by comparing its expression between peripheral and decidual T cells. Recent data suggest increased LAG-3 expression in decidual CD8+ T cells compared to peripheral counterparts in the first trimester of pregnancy, along with CD8+ T cell dysfunction [22]. This finding was consistent with our results that the expression of LAG-3 was significantly elevated on decidual T cells compared to the peripheral T cells in NP. Furthermore, the same result was found in early-onset PE patients, although the total expression of the LAG-3 was low. These results indicate that the upregulated expression of LAG-3 may be one of the patterns in which the

Table 2. Phenotype analysis of peripheral T cell subsets in NP (n=34) and early-onset PE (n=34).

<table>
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<tr>
<th></th>
<th>NP</th>
<th>Early-onset PE</th>
<th>P-value</th>
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<tr>
<td>CD3+ T cells</td>
<td>65.51±3.05</td>
<td>60.63±2.96</td>
<td>NS</td>
</tr>
<tr>
<td>CD4+ T cells</td>
<td>47.57±1.88</td>
<td>47.39±2.02</td>
<td>NS</td>
</tr>
<tr>
<td>CD8+ T cells</td>
<td>28.27±1.81</td>
<td>31.40±1.88</td>
<td>NS</td>
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Statistical comparisons were made by unpaired t test. Data are presented as the mean±SEM. P value <0.05 was considered to be statistically significant. NS – nonsignificant; NP – normal pregnancy; Early-onset PE – early-onset preeclampsia.
Figure 2. LAG-3 expression by different peripheral T cell subsets in women with NP and early-onset PE. Flow cytometry was used to determine the expression of LAG-3 by peripheral CD3+ T (A), CD4+ T (B) and CD8+ T cells (C) in NP and early-onset PE. Statistical comparisons were made by unpaired t test. The solid bars represent the medians of 34 samples, the boxes indicate the interquartile ranges, and the lines show the most extreme observations. Differences were considered significant when the P value was equal to or less than 0.05. * P<0.05, ** P<0.01. NP – normal pregnancy; Early-onset PE – early-onset preeclampsia. FACSDIVA V6. software (BD Biosciences) and GraphPad Prism software, version 7 (GraphPad) were used to create the figures.

decidual microenvironment inhibits T cell activation to maintain immune tolerance to fetal antigens.

A previous study reported that upregulation of FGL-1 reduced the progression of PE [31]. As previously reported and shown in this study, significantly higher serum levels of FGL-1 were observed in early-onset PE. FGL-1 is a protein predominantly expressed in the liver [32]. It can induce proliferation of normal hepatocytes and increase the proliferation of trophoblasts [33]. In addition, FGL-1 is responsible for T cell inhibitory function and reduces the production of proinflammatory cytokine IL-2 when interacting with LAG-3 on the T cell surface [15]. Therefore, the upregulation of FGL-1 seems to be a self-protective mechanism acting against the immunological stress of early-onset PE. Because the suppressive effect of FGL-1 is dependent on LAG-3, another possible explanation is that the declining expression of LAG-3 makes more FGL-1 molecules available for increased binding to LAG-3, thereby inhibiting T cell activation.
Figure 3. (A, B) The comparison of LAG-3 expression between peripheral and decidual T cells in NP and early-onset PE. Statistical comparisons were made by paired t test. Data are presented as the mean±SEM. P value <0.05 was considered to be statistically significant. * P<0.05, ** P<0.01. NP – normal pregnancy; Early-onset PE – early-onset preeclampsia. GraphPad Prism software, version 7 (GraphPad) was used to create the figures.

Figure 4. Serum levels of FGL-1 in NP and early-onset PE. Statistical comparisons were made by unpaired t test. Data are presented as the mean±SEM. P value <0.05 was considered to be statistically significant. * P<0.05. NP – normal pregnancy; Early-onset PE – early-onset preeclampsia. GraphPad Prism software, version 7 (GraphPad) was used to create the figure.

There were several limitations to the present study. First, because of the difference in the optimal termination time between clinically normal pregnancy and preeclampsia, the delivery time in the early-onset PE group was significantly earlier than in the NP group in this study population. Second, only serum levels were detected for FGL-1, which limited exploration of the association between FGL-1 and early-onset PE. Third, functional assays should be performed in the future to confirm the definite role of the LAG-3/FGL-1 signaling pathway in impaired immune balance of early-onset PE.

Conclusions

In summary, abnormal expression of the LAG-3/FGL-1 signaling pathway may be involved in T cell activation and impaired immune tolerance in preeclampsia. However, how LAG-3/FGL-1 achieves its inhibitory effects is unknown. Further study is warranted to achieve a deeper mechanistic understanding of the LAG-3/FGL-1 modulation pathway in preeclampsia. Our research will be instrumental in further investigations of the immunological pathogenesis of early-onset PE.

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Declaration of Figures’ Authenticity

All figures submitted have been created by the authors, who confirm that the images are original with no duplication and have not been previously published in whole or in part.

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