Original Article

Innate Immunity

Innate Immunity
2018, Vol. 24(4) 203–209

DOI: 10.1177/1753425918767305
journals.sagepub.com/home/ini
SAGE

Hydrogen peroxide promotes the activation of preeclampsia peripheral T cells

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Abstract

Preeclampsia (PE) is a pregnancy disorder with a high mortality rate. Patients with PE exhibit systemic high oxidative stress status and inflammatory immune activation. This study aims to define the role of H_2O_2 in the activation of neutrophils and T lymphocytes in PE patients. $CD3^+$/HLA-DR^+ cells in blood from PE patients are remarkably increased compared with those of normal non-pregnancies or normal pregnancies, while the percentage of $CD3^+$/$CD62L^+$ cells is significantly reduced in PE patients compared to normal pregnancies. Furthermore, CD62L levels in granulocytes of periphery blood of PE patients are significantly higher than non-pregnancies, but significantly lower than normal pregnancies. To characterize the effects of intracellular reactive oxygen species (ROS) on T lymphocyte activation in PE patients, PBMCs from normal pregnancies were challenged with H_2O_2, and intracellular ROS levels in neutrophil granulocytes, as well as T cell surface marker levels, have been determined. We confirm that H_2O_2 exposure increases intracellular ROS levels in neutrophil granulocytes, and increases the proportion of $CD3^+$/HLA-DR^+ cells, but does not alter the percentage of $CD3^+$/$CD62L^+$ cells in PBMCs. Our study has confirmed dysregulated $CD3^+$/HLA-DR^+ and $CD3^+$/$CD62L^+$ T lymphocytes in PE patient peripheral blood, and the dysregulative effects of H_2O_2 on T lymphocyte activation, suggesting a novel mechanism of immune activation in PE.

Keywords

CD62L, HLA-DR, hydrogen peroxide, neutrophil, preeclampsia, T lymphocyte

Date received: 13 November 2017; revised: 2 March 2018; accepted: 6 March 2018

Introduction

Preeclampsia (PE) is a disorder of pregnancy characterized by high blood pressure and severe damage to the kidney.[1] Without known causes, PE leads to poor outcomes for both the mother and the baby, and contributes to over 30,000 deaths per year.[2] PE may cause severe complications including placental abruption, hemolysis, HELLP syndrome (elevated liver enzymes and low platelets), eclampsia, and some cardiovascular diseases.[1] The only current cure to PE is delivery,[1] and there is a high demand to understand the mechanistic pathobiology of PE, thereby identifying novel therapeutic strategies.

Numerous studies have suggested that there is a systemic high oxidative stress status and inflammatory immune activation in patients with PE,[3,4] with no known mechanistic linkage between the two pathological events. Previous studies by us and others suggested that the oxidative stress product H_2O_2 exists at a high level in patients' serum, which might be due to the oxidative stress in placental tissue and peripheral activated neutrophil granulocytes.[5,6] The damage and

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dysfunction of vascular endothelial cells by H_2O_2 is considered as the key link in the development of PE, thus oxidative stress and activation of immune cells may be the two main factors causing damage to the endothelial system.[7,8] However, the relationship between oxidative stress of PE and activation of immune cells is still unclear. Therefore, this current study further explores the effects of H_2O_2 on activation of neutrophil granulocytes and T lymphocytes in patients with PE.

Materials and methods

Reagents

Intracellular nonspecific reactive oxygen species (ROS) probe H_2DCFDA and 10% H_2O_2 were obtained from Sigma. RPMI 1640 culture medium and FBS were purchased from Gibco. Anti-human-CD3-FITC, anti-human-CD62L-PE, and anti-human-HLA-DR-PE Abs were obtained from BD Biosciences.

Sample resource and grouping

All the samples were collected after obtaining informed consents and according to related ethical codes. The peripheral blood from PE patients was procured from the Department of Obstetrics, The First Affiliated Hospital of Bengbu Medical College. Human immunodeficiency virus 1 tests of all samples showed negative results, and no other underlying disease history was reported. The diagnostic criteria for PE were as follows: hypertensive (blood pressure > 140/90 mmHg), urine protein (+), possibly accompanied by symptoms such as epigastric discomfort and headache, no systemic hypertension before pregnancy. A tube containing EDTA-Na anticoagulant (5 ml) was used to collect the peripheral venous blood, and the blood was stored adequately for standby application. All human related procedures have been approved by the Bengbu Medical College IRB committee.

Separation of PBMCs and obtaining neutrophil granulocytes

Ficoll-Paque density gradient centrifugation was used to separate PBMCs.[9] Trypan blue was used to detect the viability. The viability was more than 95%, and the concentration was adjusted to 1×10^6/ml. Neutrophil granulocytes were obtained by the same method, and the viability was also detected using trypan blue with viability more than 95%. Wright–Giemsa stain was used to identify the purity of neutrophil granulocytes. The purity was confirmed to be more than 98%, and the cell concentration was adjusted to 1×10^6/ml.

Detection of HLA-DR and CD62L expression levels in T lymphocytes and CD62L expression in granulocytes

Anticoagulant blood samples (60 µl) were taken from normal non-pregnant woman (control), normal pregnant woman (non-PE), and PE patients. CD3-FITC Ab (6 µl) was added to the samples, and then HLA-DR-PE Ab or CD62L-PE Ab was added (8 µl). The samples were further mixed and stained at room temperature, avoiding light for 30 min. RBC lysis buffer (3 ml) was added into the samples and mixed. After RBCs were lysed, PBS (1 ml) was added to dilute the samples. The samples were centrifuged at 500 *g* for 5 min. The supernatant was discarded, and 2 ml of PBS was added to suspend the cells. The centrifugation process was repeated once and supernatant was again discarded, then 200 µl of PBS was added finally, and the expression levels were detected using flow cytometry.

Influence of H_2O_2 on T cell activation and CD62L expression level in samples from normal pregnant woman

PBMCs from normal pregnant woman were loaded to a 96-well plate at a density of 2×10^5 in each well, in the following groups: Group A, blank control group; Group B, addition of H_2O_2 (40 µM, the average serum H_2O_2 concentration in patients with PE). After incubation (37°C and 5% CO_2) for 24 h, the cells were collected and washed in PBS once. Then, PBS (60 µl) was added to re-suspend the cells, followed by addition of anti-human-CD3-FITC Ab (6 µl) and then anti-human-HLA-DR-PE Ab or anti-CD62L-PE Ab (8 µl). The samples were further mixed and stained at 37°C, avoiding light for 30 min. PBS (2 ml) was added, and the samples were centrifuged at 500 *g* for 6 min. The supernatant was discarded, and 2 ml of PBS was added to suspend the cells. The centrifugation process was repeated once and supernatant was again discarded, then 200 µl of PBS was added finally, and the expression levels were detected using flow cytometry.

Influence of H_2O_2 on the ROS level and the CD62L expression level in neutrophil granulocytes from normal pregnant woman

Isolated neutrophils were added to a 96-well plate at a density of 2×10^5 in each well. H_2O_2 at a final concentration of 40 µM was added in the experimental group. After incubation (37°C and 5% CO_2) for 6 h, the cells were collected and washed in PBS once. PBS (100 µl) was added to re-suspend the cells, followed by addition of H_2DCFDA (4 µmol/ml) or anti-CD62L-PE Ab (8 µl) and incubation at 37°C, avoiding light for 30 min. The samples were centrifuged at 500 *g* for 6 min. The supernatant was discarded, and 2 ml PBS was added to

suspend the cells. The centrifugation process was repeated once and supernatant was again discarded, then 200 µl of PBS was added, and the ROS levels were detected using flow cytometry.

Influence of normal neutrophil granulocytes treated with H_2O_2 on the HLA-DR expression level in T lymphocytes from normal pregnant woman

Neutrophil granulocytes or PBMCs were added to a 96-well plate at a density of 2×10^5 in each well. H_2O_2 at a final concentration of 40 µM was added in the experiment group. After incubation (37°C and 5% CO_2) for 6 h, the cells were treated and grouped as follows: Group A, PBMCs with no stimulation; Group B, PBMCs combined with neutrophil granulocytes treated with H_2O_2; Group C, PBMCs combined with neutrophil granulocytes not treated with H_2O_2; Group D, PBMCs, whose adherent cells were removed, combined with neutrophil granulocytes treated with H_2O_2; Group E, PBMCs, whose adherent cells were removed by the adhesion method, combined with neutrophil granulocytes not treated with H_2O_2. The cells were further incubated for 24 h. After staining with fluorescent Ab, the cells were detected using flow cytometry.

Statistical analysis

Graphical results and written quantifications of flow cytometry data were expressed as mean ± standard deviation, and SPSS 11.0 was used to perform statistical analysis. Comparisons between independent samples from two groups were analyzed by the *t* test and reported as *P* value and 95% confidence interval (CI) of the fold changes between values. Comparison among multiple groups was tested by one-way analysis of variance. The Rank sum test or the nonparametric test was used to test heterogeneity of variance. A *P* value less than 0.05 was termed as statistically significant. Prism 4.0 was used to draw the curves.

Results

HLA-DR and CD62L expression level in lymphocytes from patients with PE

The peripheral blood $CD3^+/HLA\text{-}DR^+$ cell percentage in PE patients ($n = 16$) is ~6 fold significantly higher than that in normal non-pregnant woman ($n = 12$) ($P < 0.01$, 95% CI = 4.639–7.578), and ~2.5 fold significantly higher than in normal pregnant woman ($n = 19$) ($P < 0.01$, 95% CI = 2.109–2.749). Furthermore, as reported,[10] peripheral blood $CD3^+/HLA\text{-}DR^+$ cell percentage in normal pregnant woman is significantly higher than non-pregnant woman ($P < 0.01$, 95% CI = 1.919–3.155, Figure 1a). Similarly, the peripheral blood $CD3^+/CD62L^+$ cell percentage in PE patients ($n = 16$) is significantly higher than that in normal non-pregnant woman ($n = 12$) ($P = 0.0046$, 95% CI = 1.080–1.391), while significantly lower than that in normal pregnant woman ($n = 19$) ($P < 0.01$, 95% CI = 0.7126–0.8362). In addition, compared with those from non-pregnant woman, the peripheral blood $CD3^+/CD62L^+$ cell percentage in normal pregnant woman is significantly increased ($P < 0.01$, 95% CI = 1.457–1.716, Figure 1b).

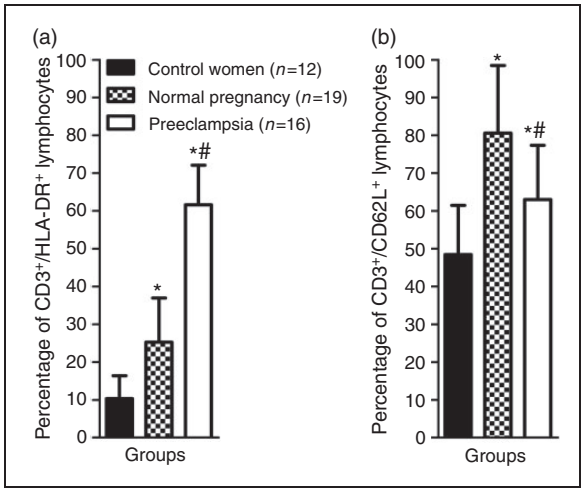

Figure 1. T lymphocyte profiles differ between control, normal pregnancy, and preeclampsia women. (a) $CD3^+/HLA\text{-}DR^+$ and (b) $CD3^+/CD62L^+$ T lymphocyte percentage in different peripheral blood samples ($^*P < 0.01$ compared with normal non-pregnancies; $^{\#}P < 0.01$ compared with normal pregnancies).

CD62L expression level in peripheral blood neutrophil granulocytes from patients with PE

When flow cytometry was used to analyze peripheral blood staining, granulocyte area R3 was chosen as gate in the scatter diagram.[11] CellQuest was used to analyze the average fluorescence strength of neutrophil granulocytes expressing CD62L at a high level (R4), moderate level (R5), and low level (R6) using a density map (see Supplementary Figure S1 online).[11] Compared with normal non-pregnant woman ($n = 19$), R4 values of normal pregnant woman ($n = 12$) ($P < 0.01$, 95% CI = 1.685–1.725) and PE patients ($n = 16$) ($P < 0.01$, 95% CI = 1.311–1.366) were significantly increased. Furthermore, R4 values of PE patients were significantly decreased compared with normal pregnant woman ($P < 0.01$, 95% CI = 0.7762–0.7923). The R5 values of normal non-pregnant woman, normal pregnant woman, and PE patients were 25.28 ± 6.32 ($n = 19$), 24.48 ± 7.21 ($n = 12$), and 23.70 ± 7.10 ($n = 16$), with no statistically significant difference among them ($P > 0.05$). The R6 values of the three groups were 6.69 ± 1.21 ($n = 19$), 6.87 ± 1.03 ($n = 12$), and 6.82 ± 1.37 ($n = 16$), with no statistically significant difference among them either ($P > 0.05$) (Figure 2).

Influence of H_2O_2 on HLA-DR and CD62L expression levels in T cells from normal pregnant woman

We next assessed the effects of H_2O_2 on HLA-DR and CD62L expression on T cell surfaces. PBMCs from normal pregnant woman were stimulated with 40 μM H_2O_2 for 24 h, and the cell percentage of $CD3^+$/HLA-DR^+ T lymphocytes was 13.98 ± 2.11, with no significant difference compared with the control group (17.19 ± 1.93) ($P = 0.2552$, 95% CI = 0.8821–1.038, Figure 3a). In parallel, the percentage of $CD3^+$/$CD62L^+$ T lymphocytes was 69.11 ± 6.72, compared with the control group (73.19 ± 5.77), with no significant difference either ($P = 0.0573$, 95% CI = 0.9450–1.001, Figure 3b). These results suggest that H_2O_2 (at 40 μM, 24 h) might not have a direct activating effect to alter HLA-DR and CD62L expression.

Influence of exogenous H_2O_2 on the ROS level and the CD62L expression level in neutrophil granulotytes from normal pregnant woman

We next examined the effects of exogenous H_2O_2 on neutrophil granulocyte ROS generation and CD62L expression. Incubation with exogenous H_2O_2 (40 μM, 6 h) significantly increased intracellular ROS inside the blood neutrophil granulocytes ($P < 0.01$, Figure 4a) collected from normal pregnant woman. In parallel, CD62L positive neutrophil granulocytes (with highly expressing CD62L, or R4) are significantly reduced by H_2O_2 incubation ($P < 0.01$, Supplementary Figure S1, Figure 4b). However, the neutrophil granulocytes moderately expressing CD62L (R5) or neutrophil granulocytes lowly expressing CD62L (R6) are not altered by H_2O_2 stimulation (Figure 4b).

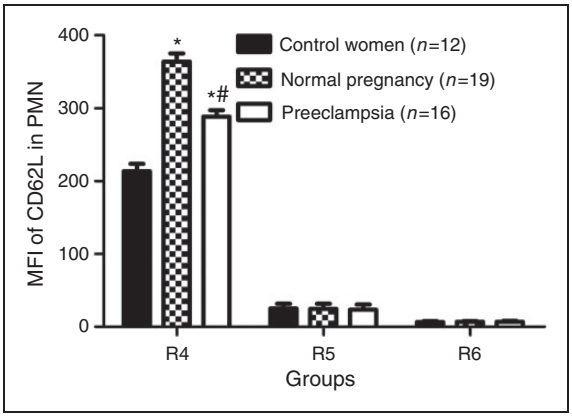

Figure 2. Neutrophil expression of CD62L is higher in preeclampsia than control women and higher in normal pregnancies than either control or preeclampsia women. CD62L fluorescence strength values expressed by neutrophil granulocytes in whole blood. MFI: mean fluorescence intensity; PMN: polymorphonuclear leukocytes (*$P < 0.01$ compared with normal non-pregnancies; #$P < 0.01$ compared with normal pregnancies).

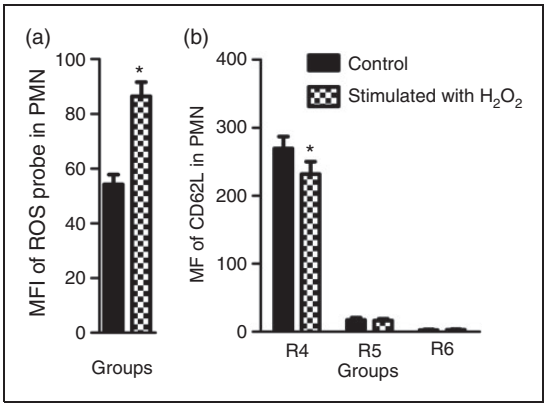

Figure 4. H_2O_2 treatment increases ROS production in neutrophils and reduces CD62L expression in R4 neutrophils of normal pregnancies. Influence of *in vitro* H_2O_2 (40 μM) stimulation on neutrophil granulocyte (a) ROS levels, and (b) CD62L surface expression in normal pregnancies. PMN: polymorphonuclear leukocytes (*$P < 0.01$ compared with the control of the same group, $n = 12$).

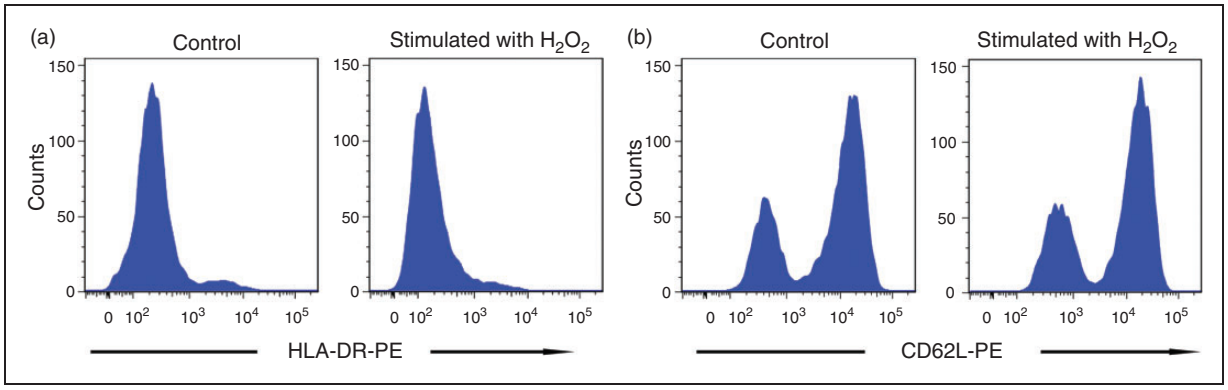

Figure 3. H_2O_2 treatment does not alter HLA-DR^+ or $CD62L^+$ populations in lymphocytes of normal pregnancies. Cell percentages of (a) $CD3^+$/HLA-DR^+ and (b) $CD3^+$/$CD62L^+$ in lymphocytes of normal pregnancies before and after treatment with 40 μM H_2O_2 ($n = 12$).

Influence of neutrophil granulocytes activated with H_2O_2 on the HLA-DR expression level in T lymphocytes from normal pregnant woman

After 24 h co-culture, the $CD3^+/HLA\text{-}DR^+$ cell percentage in Groups A, B, C, D, and E, were 4.67 ± 0.56, 12.91 ± 0.83, 6.73 ± 0.45, 32.51 ± 0.74, and 19.17 ± 0.69, respectively. Neutrophil co-culture increased HLA-DR expression lymphocytes (Groups B–E compared to Group A). Particularly, the cell percentage of $CD3^+/HLA\text{-}DR^+$ in Groups B and D were significantly higher than those in Groups C and E ($P < 0.01$, $n = 12$) respectively, suggesting that neutrophil granulocytes activated with H_2O_2 could promote activation of T cells (in both adherent cell intact or removal conditions). Also, significant difference between Groups B and D, or Groups C and E ($P < 0.01$, $n = 12$) was observed, indicating that adherent cells in PBMCs is influencing T cell activation by neutrophil granulocytes (Figure 5).

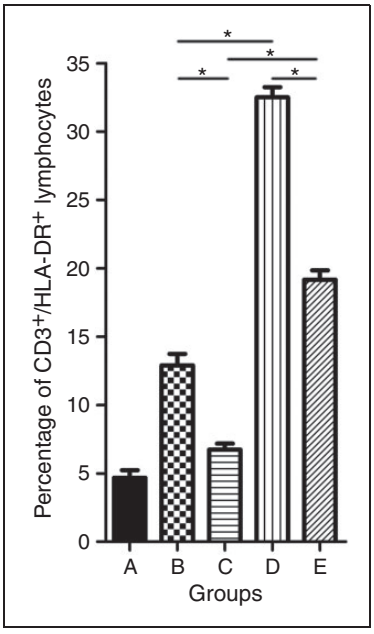

Figure 5. H_2O_2 treatment alters neutrophil interaction with PBMCs. Influence of neutrophil granulocytes in normal pregnancies pre-processed with 40 μM H_2O_2 on percentages of $CD3^+/HLA\text{-}DR^+$ expressed by PBMCs. The cells were treated and grouped as follows: Group A, PBMCs with no stimulation; Group B, PBMCs combined with neutrophil granulocytes treated with H_2O_2; Group C, PBMCs combined with neutrophil granulocytes not treated with H_2O_2; Group D, PBMCs, whose adherent cells were removed by the adhesion method, combined with neutrophil granulocytes treated with H_2O_2; Group E, PBMCs, whose adherent cells were removed by the adhesion method, combined with neutrophil granulocytes not treated with H_2O_2 (*$P < 0.01$ between groups).

Discussion

As a main intermediate of oxygen free radical metabolism, H_2O_2 is considered to be involved in biological behaviors of various cells.[12,13] In patients with PE, excessive ROS generation occurs due to hypoxia in the placenta, neutrophil granulocyte activation, and endothelial cell injury. After entering blood circulation, it also plays a very important role in promoting activation of immune cells and the inflammatory response.

However, how neutrophil granulocytes of PE patients are activated is still unclear. It has been reported that some unknown factors generated in the placenta from patients with PE may activate neutrophil granulocytes by promoting the generation of ROS and expression of adhesion molecules.[14] It was also found that exogenous H_2O_2 in the serum of patients with PE stimulating neutrophil granulocytes could significantly increase the generation of ROS and expression of adhesion molecules.[15] This indicates that a high level of H_2O_2 could play an important role in promoting the activation of neutrophil granulocytes in peripheral circulation. Thus one of the unknown neutrophil granulocyte activation factors above may be ROS itself, as preeclamptic placentae may result from hypoxic stress which leads to increased ROS.[16] A low level of ROS is often accompanied by mitochondrial respiration, and an increased ROS concentration increases ROS generation in turn, which is called ROS-induced ROS release (RIRR). The increased ROS in the mitochondrion can trigger the opening of mitochondrial membrane ion channels, leading to a rapid increase in ROS generation from the mitochondrial respiratory chain.[17] Whether the mechanism of exogenous H_2O_2 activating neutrophil granulocytes is related to RIRR still needs further exploration.

It has been reported that under hypoxia, ROS generated by endothelial cells can activate T cells. The activated T cells can further increase the injury exerted by neutrophil granulocytes on endothelial cells, and are involved in regulation of the complement system, endothelial cells, lymphocytes, and macrophages.[14] Activated neutrophil granulocytes are also one of the sources of two inflammatory cytokines (IL-1β and TNF-α). Therefore, the activated neutrophil granulocytes of PE may be the key cells during immune cell activation initiated by oxidative stress and the inflammatory response.

In line with the report by Saito et al.,[18] the present study found that compared with normal nonpregnancies and pregnancies, HLA-DR and CD62L expression increased in patients with PE, suggesting overactivation of lymphocytes in PE patients. It has also been shown that activated neutrophil granulocytes are responsible for the amplified inflammatory process in blood, and apoptotic neutrophils also initiate immune circuits via activation of monocytes/macrophages.[19] Compared with the study by Wang et al.,[14] the difference in the present study was that exogenous H_2O_2

could not directly increase the activation level of T lymphocytes in PE patients. However, it could mediate activation of T lymphocytes from normal pregnancies by activating neutrophil granulocytes; also, dendritic cells (DCs) or monocytes might play a key role in this process. It has been proved that the co-culture of DCs and neutrophil granulocytes could induce Th1-type immune responses.[20] This opinion not only supports the result of the present study, but also matches the condition of Th1 immune activation in patients with PE.

The mechanism of activated neutrophil granulocytes stimulating T lymphocytes could be as follows. The generated chemotaxis signal attracts monocytes and DCs. The generation of TNF-α and accumulation of DCs, as well as macrophage differentiation and activated cytokines, influences the Ag-presenting process. Thus, neutrophil granulocytes activated by PE not only recruit T cells and DCs to inflammatory sites, but also directly influence adaptive immunity by activating and inducing the adaptive immune response.[21] Furthermore, although the ability of neutrophil granulocytes to generate cytokines at a single-cell level is weaker than that of macrophages and lymphocytes, the number of neutrophils in inflammatory sites is higher than that of lymphocytes by one or two order of magnitudes. Thus, neutrophil granulocytes are an important resource for cytokines such as TNF-α, which decide the level of the inflammatory response.[21] Neutrophil granulocytes can also induce the generation of IL-12 in DCs, and improve the ability of activating T lymphocytes in primed DCs.[22]

CD62L, or L-selectin, is the early-stage adhesion molecule expressed on the surface of activated monocytes and neutrophil granulocytes. The decrease in the CD62L level on the surface of activated neutrophil granulocytes and T lymphocytes might be due to negative feedback regulation of the inflammatory response. Analysis of adhesion to activated endothelium under flow conditions revealed that CD16(bright)/CD62L(dim) neutrophils adhered less compared with CD16(bright)/CD62L(bright) and CD16(dim)/CD62L(bright) neutrophils.[23] Interestingly, the CD62L(bright) neutrophil subset is known to suppress lymphocyte proliferation *ex vivo*, and play a pivotal role in regulating lymphocyte-mediated inflammation and autoimmune diseases.[24] Despite a lot of suggestions, it remains unclear whether increases of CD62L expression have any functional consequences in PE. Compared to CD62L, or CD69, another early immune activation marker of TCR/CD3 receptor stimulation, HLA-DR antigen is expressed during the later stages (after 24 h of inflammatory stimulation) of T lymphocytes, and last for several wk.[25] By using two different markers (CD62L and HLA-DR), we were able to differentiate the dynamics in T cell activation (acute vs chronic, or transient vs persistent). Interestingly, the differences have confirmed a more persistent and chronic $CD3^+$ T cell activation in PE patients.

Taken together, our study has confirmed dysregulated $CD3^+/HLA\text{-}DR^+$ and $CD3^+/CD62L^+$ T lymphocytes in PE patient peripheral blood, and the effects of H_2O_2 on T lymphocyte activation. These findings confirm involvement of ROS/CD62L/HLA-DR in the immune activation in PE and might lead to novel anti-oxidant or cell-specific therapies for PE patients.

Acknowledgments

The authors thank Gabriel Kelly for his help in preparing the manuscript.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported by Anhui Provincial Natural Science Research Project of University (grant numbers KJ2013A188 and KJ2014A164), International Science and Technology Cooperation Project-Key Research and Development Program of Anhui Province (grant number1604b0602026), Science Foundation of Bengbu Medical College (grant number BYKY1404ZD), and National Natural Science Foundation of China (grant number 81570011).

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