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Circulating cell-free DNA indicates M1/M2 responses during septic peritonitis

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ABSTRACT

Circulating cell-free DNA (cfDNA) has been widely suggested as clinical indicator in diseases, including sepsis. It was thought that the cfDNA was coming from the cell lysis, necrosis and apoptosis caused by tissue damages during sepsis. M1 or M2 macrophage-type responses kill or repair in vivo, which is highly relevant with the tissue damages in sepsis. The correlation between cfDNA and M1/M2 responses during sepsis was never investigated. Here, we used bacteria injection induced septic peritonitis mouse model in both M1-dominant C57bl/6 and M2-dominant Balb/c mouse strains. We found that M2-dominant Balb/c mice showed better prognosis of septic peritonitis than C57bl/6 mice, which is corresponded with lower level of cfDNA in septic Balb/c mice compared to septic C57bl/6 mice. By assessing the M1 and M2 related cytokines in both septic Balb/c and C57bl/6 mice, we found out that Balb/c mice has lower tumor necrosis factor α (TNFα) and higher interleukin 10 (IL-10) productions than C57bl/6 mice during septic peritonitis. Especially, when monitoring the monocyte subtypes in peripheral blood of these septic mice, we found out that C57bl/6 showed higher inflammatory ($Ly6C^{high}$) monocyte (corresponding to M1 macrophage) proportion than Balb/c mice. Interestingly, we find out that cfDNA is highly correlated with the ratio of $Ly6C^{high}$ monocytes versus $Ly6C^{low}$ monocytes, which represents M1/M2 (killing/healing) responses. Our study suggested that the cfDNA is a good indicator for evaluating M1/M2 responses in septic peritonitis.

1. Introductions

Sepsis is a life-threatening condition that arises when the body's response to infection injures its own tissues and organs [1]. Sepsis is the leading cause of death in intensive care units (ICUs) [2–4]. The prevalence of sepsis internationally is estimated at 300 per 100,000 [5]. Sepsis occurs when infection fighting chemicals are released into the bloodstream and trigger inflammatory responses throughout the body. This inflammation can trigger a cascade of changes that can damage multiple organ systems [6], causing them to fail and shocks [3,4].

Circulating cell-free DNA (cfDNA) in human peripheral blood were identified in 1984 by Mandel and Metais [7,8]. cfDNA has been reported to be increased in various diseases, including sepsis [9,10], trauma [11], cancer [12–15], stroke [16], and myocardial infarction [17]. It was believed that cfDNA was from the dead cells of damaged tissue during inflammation of the diseases. The regulation of inflammation is modulated by macrophages, including responses of M1 and M2 macrophages [18]. M1 macrophages are the "killer" type macrophages, which can promote inflammation and rapidly eliminate the pathogens. M2 macrophages are the "heal" type macrophages, which routinely repair and maintain tissue integrity. The imbalance of M1/M2-type response causes diseases. In sepsis, inflammatory response (M1-type) is very important for eliminating pathogens at the earlier stages of infections [19]. However, continuously over-aggressive inflammatory response (M1-type) leads to the organ damage and shock [20], which are the main lethal causes [3]. Previous studies showed that enhancing M2-type response is helpful for the prognosis of sepsis [21–24]. The correlations between the M1/M2-type responses, which are the reasons of the inflammatory tissue damage, and the cfDNA concentration, which is the result of the inflammatory tissue damage, have not

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been investigated in sepsis previously.

Here, we monitored the cfDNA concentration in both M1-dominant C57bl/6 and M2-dominant Balb/c mouse in experimental induced septic peritonitis. We investigated the correlations of the cfDNA concentrations and the M1/M2-type response during the progress of septic peritonitis.

2. Materials and methods

2.1. Reagents

SYBR Gold Nucleic Acid Gel Staining, RNase A and DNase I were purchased from Thermo Fisher Scientific. Salmon sperm DNA was purchased from Sigma-Aldrich. Enzyme-linked immunosorbent assay (ELISA) kits for mouse TNFα and IL-10 were purchased from R&D Systems. UltraComp eBeads were purchased from eBioscience. APC-anti-CD115 and FITC-anti-Ly6C antibodies were purchased from Biolegend. Specific O18 antiserum was purchased from SSI Diagnostica.

2.2. Bacteria

Escherichia coli O18, a clinical isolate firstly from the cerebrospinal fluid of a newborn infant with meningitis [25], was isolated from a sepsis patient in the Qilu Hospital of Shandong University previously and typed by with the specific O18 antiserum. The typing protocol was developed according to the manufacturer's instructions. Bacteria were cultured in Luria-Bertani (LB) medium (10 g of tryptone, 5 g of NaCl, and 5 g of yeast extract perlite). Before each experiment, bacteria were incubated for 2 h in fresh medium to induce log phase.

2.3. Mice

Male C57BL/6J and BALB/c mice were bought from the Shandong University Laboratory Animal Center and were used at the age between eight to ten weeks. Mice were bred and housed in specific pathogen-free conditions in groups of 5 mice per cage with free access to food and water. Animal breeding and experiments were conducted according to Shandong University Animal Welfare guidelines and were approved by the Animal Ethics Committee of the Shandong University Medical School.

2.4. Septic peritonitis models

In the bacterial sepsis models, bacterial peritonitis was induced by an i.p. injection of 2×10^5 CFU *E. coli* O18 in 300 ml PBS. Clinical signs of sepsis were assessed using a murine sepsis score with a maximum of 28 points [26,27]. Survival was monitored in seven days after injections.

2.5. cfDNA measurements

cfDNA was measured directly in sera using SYBR Gold Nucleic Acid Gel Staining, according to the fluorometric method published before [10,28]. Briefly, samples were added to a black 96-well plate (Greiner Bio-One). SYBR Gold was applied (1:10,000) and fluorescence was measured with a microplate fluorometer (BioTek) at an emission wavelength of 530/25 nm and an excitation wavelength of 485/20 nm. Background fluorescence was subtracted by using sera treated with RNase A (100 U) and DNase I (500 U) for 5 h at 37 °C as negative control. Commercial Salmon sperm DNA were used as DNA standards.

2.6. Cytokine measurements

100 μL blood was collected through retro-orbital venules and was centrifuged down (500 × g, 5 min at 4 °C) to separate plasma and cells. TNFα and IL-10 in plasma were detected by using ELISA kits following manufacture's instruction. Quantification was performed by a microplate reader (Bio-Rad).

2.7. Flow cytometry

Erythrocytes in obtained blood cells (described above) were lysed using distilled water (room temperature, 10 s). Remaining leukocytes were washed with PBS (500 × g, 5 min at 4 °C) and were incubated with APC-anti-CD115 and FITC-anti-Ly6C at 4 °C for 30 min. After two washes, cells were resuspended in FACS buffer (0.5% BSA and 0.05% sodium azide in PBS) and were analyzed on a FACSCalibur (BD Biosciences). Compensation controls were performed using UltraComp eBeads incubated with relative antibodies at room temperature for 10 min. Flow cytometric data were analyzed using the FlowJo V9 software (FLOWJO, LLC). $CD115^{+}Ly6c^{high}$ cells were defined as classical/inflammatory monocytes, $CD115^{+}Ly6C^{low}$ cells were defined as patrolling/regulatory monocytes.

2.8. Statistics

Statistical analysis was performed with Prism 6 (GraphPad). Data are presented as mean ± SD. The means for the data sets were compared using unpaired student t-tests with equal variances. Linear regression or one phase association fits was applied for some data sets. The slope of the linear regression was tested against zero using an F-test. P values less than 0.05 were considered significant.

3. Results

3.1. cfDNA concentration is a marker of severity and prognosis in both Balb/c and C57bl/6 mouse sepsis models

We assessed the severity of septic peritonitis in both M1-dominant C57bl/6 and M2-domminant Balb/c mice. After injection of lethal dose bacteria (2×10^5 *Escherichia coli*, *E. coli*), C57bl/6 mice started to die at day 0.5 (12 h after injection, Fig. 1a, black line). Four mice (20%) were survived at day two, and only one mouse (5%) was survived at the end of the observation (day seven). In comparison, Balb/c mice showed a little bit better prognosis. They began to die at day 1 (24 h after injection, Fig. 1a, grey line). Five mice (25%) were left at day two, and no more death happened after that in our seven-day observation. When we evaluated the sepsis score during the monitoring (Fig. 1b), we found out that both the scores of C57bl/6 and Balb/c mice were increased within two days after pathogen injection. The score of C57bl/6 mice were kept in the high score (>20) during our observation. However, Balb/c mice showed improvement of the disease and the score dropped to ~15 at the end of our observation. These data showed that the M2-dominant Balb/c mice were more resistant to gram-negative *E. coli* induced septic peritonitis than the M1-dominant C57bl/6 mice.

When we assessed the cfDNA concentration during the progress of the septic peritonitis in C57bl/6 mice (Fig. 1c, black line), we found out that the cfDNA was increased to ~1300 ng/ml in the first three days after pathogen injection, then decreased and reached to ~600 ng/ml at the end of our observation. In comparison, the cfDNA in Balb/c mice (Fig. 1c, grey line) was increased to ~950 ng/ml in the first two days after the induction of the septic peritonitis, then decreased and dropped to less than 100 ng/ml at the end of our observations. Since the cfDNA concentration reflects the level of