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Decreased monocyte subsets and TLR4-mediated functions in patients with acute severe fever with thrombocytopenia syndrome (SFTS)

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SUMMARY

Objectives: The role of a newly discovered bunyavirus, the severe fever with thrombocytopenia syndrome virus (SFTSV), in the pathogenesis of severe fever with thrombocytopenia syndrome (SFTS) is poorly understood. In this study, it was hypothesized that peripheral monocytes, which are constantly exposed to viral infection in the blood, are likely targeted by the causative virus in SFTS patients.
Methods: Fifty-three patients and 25 healthy volunteers were enrolled in the study. Monocyte counts in the peripheral blood of all human subjects were monitored throughout the progress of the disease. SFTSV viral load and the expression of monocyte genes were investigated by real-time RT-PCR. Cytokine production of monocytes in SFTS patients upon lipopolysaccharide (LPS) stimulation was examined by ELISA.
Results: In comparison to SFTS patients in the convalescent stage and healthy controls, monocyte cell counts and percentages in patients at the acute stage were significantly lower. Decreased monocyte cell counts and subsets were positively correlated with SFTSV viral loads in the serum samples from SFTS patients. Despite their higher basal toll-like receptor 4 (TLR4) expression, monocytes from patients in the acute phase were shown to be compromised regarding the production of tumor necrosis factor alpha, but not interleukin 10, upon LPS stimulation.
Conclusions: These data strongly suggest that monocytes could be a major target during SFTSV infection. The decreased population and dysfunction of monocytes in acute SFTS patients may contribute to the disease severity.

1. Introduction

Severe fever with thrombocytopenia syndrome (SFTS) is an emerging infectious disease characterized by unexplained hemorrhagic fever-like illnesses. The causative agent, the severe fever with thrombocytopenia syndrome virus (SFTSV), also named Huaiyangshan mountain virus, is a novel tick-borne *Phlebovirus* classified within the *Bunyaviridae* family.[1] SFTSV is a single-stranded RNA virus, comprising three genes: L, M, and S.

Like other phleboviruses, SFTSV is transmitted mainly through vectors (ticks) to humans in most cases. However, some reports have indicated that SFTSV can be transmitted from human to human.[2] Since SFTS was first discovered in China in 2009,[3] SFTSV infection has spread to at least 16 Chinese provinces[4,5] and other East Asian countries, such as Japan[6] and Korea.[7] Patients with SFTS have shown a broad spectrum of clinical manifestations, ranging from an acute self-limited febrile illness to various grades of severe presentation, including a fatal outcome and multiple organ failure. Reported fatality rates vary between 12% and 30%.[4,8] Leukopenia and severe thrombocytopenia are the hallmarks of patients with SFTS in laboratory tests.[1,5] To date, neither effective antiviral strategies nor conventional vaccines have provided sustainable control of the spread of SFTSV.

A better understanding of the pathogenesis in SFTSV infection will facilitate the development of more effective prevention and control measures. Increasing evidence indicates that the immune

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status of the infected individual might play an important role in determining the severity and clinical outcome of this disease. Along these lines, the most severe SFTS patients are the elderly.[1] Studies by Deng et al.[9] and Sun et al.[10] have demonstrated that exaggerated immune activation and the overproduction of inflammatory cytokines such as interleukin (IL)-1β, IL-6, IL-10, tumor necrosis factor alpha (TNF-α), and monocyte chemoattractant protein-1 (MCP)-1 could create a 'cytokine storm', significantly contributing to the severity of SFTS. Various immune cell populations, including T lymphocytes and natural killer cells, have been found to be skewed in SFTSV-infected patients, especially those in the acute phase and the severe cases.[11] These results suggest that immunocompromised hematopoietic cells may contribute to the severity and clinical outcome in SFTS patients.[12,13]

Circulating monocytes, constituting about 10% of leukocytes in human blood, form a crucial part of the native immune system. While functional monocytes can fend off various infections, dysfunction of monocytes in immune-compromised individuals may lead to increased severity of viral infections. For example, monocytes, but not T- or B-cells, are the main targets of human peripheral blood mononuclear cells for dengue virus.[14] As for SFTSV, splenic macrophages have been shown to harbor infected viruses in a rodent model.[15] A similar result was obtained from an in vitro cell culture system using monocytic THP-1 cells.[16] These studies imply that monocytes/macrophages may support persistent SFTSV viral infection. However, the evidence that monocytes/macrophages are directly involved in the development of SFTS in patients, particularly those in the acute phase, is still missing.

Both the clinical and biological aspects of monocytes in SFTSV-infected patients were examined in this study. In addition, the functions of these monocytes were analyzed by treating them with a well-defined toll-like receptor 4 (TLR4) ligand, lipopolysaccharide (LPS). This study sheds some light on the pathological role of SFTSV-infected monocytes in the development of the disease.

2. Methods

2.1. Human subjects

All patients were admitted to Union Hospital in Wuhan City during the period April 20 to August 31, 2014. The diagnosis of SFTS was confirmed according to the clinical guidelines on severe fever with thrombocytopenia syndrome released by the Ministry of Health of the People's Republic of China. In all, 53 patients who developed SFTS (acute onset of high fever, leukopenia, and thrombocytopenia) were included in this study. Healthy volunteers recruited into the study were from the local area and there was no bias with respect to sex, age, or ethnic background.

All patients were admitted to the hospital on day 3 to day 6 following the onset of illness. Patients whose neutrophil granulocytes were lower than 1×10^9/l were given recombinant human granulocyte colony-stimulating factor (rhG-CSF; Qilu Pharmaceutical, Jinan, China) at 2 μg/kg daily until counts reached more than 2.5×10^9/l. Details of the patients' clinical history, physical examination, and routine hematology laboratory results were collected retrospectively by physicians. Blood samples were collected from patients in the acute and the convalescent phases from the date of admission until the 10th day without fever. Serum and peripheral blood mononuclear cells (PBMCs) obtained by Ficoll–Hypaque centrifugation of blood, were prepared from both patients and healthy subjects; these were used for further testing, as described below.

2.2. Monocyte preparation and cytokine detection

Human blood cells enriched in monocytes were isolated from PBMCs by an adherence method. Briefly, PBMCs from SFTS patients or healthy volunteers were incubated in microexudate-treated flasks at 1×10^7 cells/flask in RPMI 1640 containing 15% fetal bovine serum (FBS) at 37 °C for 2 h. Non-adherent cells were removed by repeat washing with phosphate buffered saline (PBS). The adherent cells were tested for purity by flow cytometry using a monocyte-specific marker anti-CD14 mAb FITC (BD Biosciences, San Jose, CA, USA) on a FACSCalibur instrument (BD Biosciences, San Jose, CA, USA). Purity of >80% could be reached routinely. The adherent cells were detached from the flasks with a cell scraper and stimulated with ultrapure LPS of an *Salmonella minnesota* strain (1 μg/ml; InvivoGen, San Diego, CA, USA) at 5×10^5 cells/ml in six-well plates for 18 h. The supernatant from these cultures was collected and assayed for the expression of TNF-α and IL-10 using a commercial ELISA kit (Elabscience, Wuhan, China). The detection limit for TNF-α was 8–1000 pg/ml and for IL-10 was 7.813–500 pg/ml.

2.3. RNA extraction, cDNA synthesis, and real-time RT-PCR

Total RNA was prepared from $0.5–2 \times 10^6$ purified monocytes using the TRIzol method (Life Technologies, Carlsbad, CA, USA). Single-stranded cDNA was synthesized using a PrimeScript Reagent Kit (TaKaRa/Clontech, Mountain View, USA). The quantitative real-time PCR was performed on a Bio-Rad Digital PCR system with SYBR Premix Ex Taq II (TaKaRa/Clontech) in 50 μl reaction using 1 μl cDNA. Data were collected and analyzed using QuantaSoft Software (TaKaRa/Clontech, Mountain View, USA). Primers for the detection of TLR4 (NM_0032663) and β-actin (ACTB) were purchased from GeneCopoeia (Rockville, USA). The fold change was calculated according to the formula $2^{\Delta CT(gene) - \Delta CT(ACTB)}$.

2.4. SFTS viral load assay

Serum viral RNA was extracted from patient samples (1 ml) using a viral RNA kit from DAAN Gene (Guangzhou, China); the manufacturer's procedures were followed. For intracellular viral RNA, total RNA was similarly extracted from 0.5×10^6 purified monocytes of SFTS patients and healthy controls, as described above; the SFTSV viral load (number of copies of SFTSV viral RNA) was determined by one-step real-time RT-PCR. A plasmid containing the full length of the SFTSV S segment was used as a standard for calculation of the SFTSV genome copies. All reagents were certified for clinical diagnostic use.

2.5. Statistical analysis

Continuous variables were recorded as the median (range) or mean ± standard error of the mean, unless indicated otherwise. Viral loads were subjected to correlation analysis after logarithmic transformation. The Mann–Whitney *t*-test was used to compare variables between two independent groups. Statistical analyses were performed using the Pearson Chi-square test or Fisher's exact test in tables to test relationships. Linear regression analysis was used to assess the correlation of two variables. The statistical graphs were constructed using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA) and SPSS version 17.0 (SPSS Inc., Chicago, IL, USA). A two-tailed *p*-value of less than 0.05 (95% confidence internal) was considered statistically significant.

2.6. Ethics statement

Written informed consent was obtained from all of the patients and volunteer participants. This study was approved by the Review Board of the Ethics Committee of Wuhan Union Hospital, Wuhan City, China.