

Detection and evaluation of immunofunction of patients with severe fever with thrombocytopenia syndrome

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Received: 12 April 2013 / Accepted: 2 September 2013 / Published online: 26 September 2013
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Abstract Severe fever with thrombocytopenia syndrome (SFTS) is an emerging infectious disease caused by SFTS virus (SFTSV) with a high fatality rate. But the immunofunction was still unclear. The objective of our study was to assess the immunofunction in SFTS patients. Immunofunction test with flow cytometry which contains CD3+, CD4+ and CD8+ T lymphocytes, B cells and NK cells would be used for detecting serum samples collected from 34 SFTS cases and 20 healthy donors. We found that CD3+ and CD4+ T lymphocytes were significantly diminished in SFTS compared to normal control. In contrast, the percentage of NK cells was elevated. Further analysis revealed that the number of CD3+ and CD4+ T lymphocytes showed that there was a more robust pattern of depression in acute phase and severe SFTS infection compared to the patients in recovery phase and mild SFTS infection. But NK cells were significantly increased in acute phase and severe SFTS. They reverted to the near normal levels in convalescent phase. Additionally, the levels of CD3+ and CD4+ T lymphocytes progressively decreased in death group when compared with the survival group, but the level of B cells was higher. The damages of immune system were obvious, and the immune dysfunction

might be partly responsible for disease progression of patients with SFTSV infection.

Keywords Severe fever with thrombocytopenia syndrome(SFTS) · Severe fever with thrombocytopenia syndrome virus (SFTSV) · Immunofunction

Introduction

Severe fever with thrombocytopenia syndrome (SFTS) is an emerging infectious disease caused by severe fever with thrombocytopenia bunyavirus (SFTSV), which is identified recently in multiple provinces in China. SFTSV is infected mainly by tick stings, and it also can be infected by contacting with the blood and secretion of SFTS patients [1, 2]. SFTSV is a novel phlebovirus within bunyavirus family [3], which can damage cells and tissue widely [4]. SFTS is an acute illness with clinical presentations of abrupt fever, thrombocytopenia, leukocytopenia, gastrointestinal symptoms, neural disorders, proteinuria, hematuria and bleeding tendency [5–8]. The case fatality rate for this severe disease was 30 % [3]. However, the pathogenic mechanism in patients with SFTSV infection is still unclear.

Viral interaction with the innate immune system played a core role in determining the outcomes of the infection [9]. Some authors had found that SFTS patients were at least partly immune-mediated, which might play an important role in determining the severity and clinical outcome [10]. Lymphocyte played an important role in the induction of cellular immunity in organism. Dysfunction of lymphocyte subsets caused immunofunction abnormality, which made disease deteriorate progressively and protractedly. Lymphocytes could stimulate the organism to produce immune

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response against viral antigens when organism was infected with SFTSV. It is clear that many viruses encode specific gene products that antagonize the immune response in recent years [11, 12]. Therefore, figuring out how SFTSV interacts with the host immune system is essential to understand the molecular mechanisms of SFTS. Until recently, immunofunction and lymphocyte subpopulation studies with respects to SFTSV infection are insufficient. We analyzed T-cell subgroups and their possible roles in SFTS disease.

Materials and methods

Patient demographics and clinical samples

The present study was a part of the study on pathogenetic mechanisms of SFTS disease involved 34 admitted SFTS patients (aged 30–78 years) from the Department of Infectious Diseases of Union Hospital, Tongji Medical College, Huazhong University of Science and Technology between May 2012 and September 2012. The viral RNA of all SFTS samples was detected to confirm positively for SFTSV infection by using a certificated real-time polymerase chain reaction (RT-PCR) kit [13]. Serum samples from 20 healthy volunteers (who neither suffer from any disease nor take medicine in recent weeks) were normal control, and we divided SFTS patients into different groups based on consistent clinical features. The serum samples of the acute phase of the patients were collected in an initial week, and after 2 weeks, they were in recovery phase. For mild and severe cases, the serum samples were collected around 7–12 days post the onset of illness according to the patients' condition. Human samples which were involved in the study were approved by the Research Ethics Committee of Tongji Medical College, Huazhong University of Science and Technology. The written informed consent was signed.

A series of blood samples from participators by peripheral venipuncture were drawn into ethylene diamine tetraacetic acid (EDTA) anticoagulation tube, and then they were immediately sent to the laboratory.

Flow cytometry

The peripheral blood samples were processed for an analysis of lymphocyte subsets by flow cytometry within 2 h. Lymphocyte subsets were identified by the following monoclonal antibodies: anti-CD45-PerCP, anti-CD3-FITC, anti-CD8-PE, anti-CD4-APC, anti-CD16/56-PE and anti-CD19-APC. Cell suspension was incubated in dark place at room temperature for 30 min. The red blood cells were removed by using 500 μ l lysis buffer in dark place at room temperature for 10 min. Finally, cells were washed and

resuspended in phosphate-buffered saline (PBS), and got the results on a flow cytometer (FACS CaliburTM, Becton Dickison Crop). Up to 5000–10000 total events were collected per sample. The lymphocyte population was selected by gating lymphocytes. The number of positive cells including the expression of CD3+, CD4+ and CD8+ T lymphocytes, B cells and NK cells was analyzed as a percentage of the cells in the lymphocyte gate.

Statistical analysis

The differences between the two groups were analyzed by the paired Student's *t* test. A *p* value <0.05 was considered statistically significant, using two-sided comparisons (confidence level 95 %). Data were stated by mean \pm S.D or median. Analyses were performed by using the Graph-Pad Prism 5 (Graph Pad Prism, La Jolla, CA, USA).

Results

Patients description and clinical laboratory parameters in SFTS patients

We recorded the clinical course and laboratory data of SFTS patients prospectively. Thirty-four SFTS patients were admitted from hilly or mountainous areas of Hubei and Henan province, and they described a history of field exposure within 2 weeks of the illness onset. The mean age was 55 years (range, 30–78 years), and 11(32.4 %) were male. All the patients suffered fever during the disease progression, and the mean days were 8 days (range, 5–14 days). The mean time of hospital stay was 9 days (range, 3–22 days). Three female patients who were 56–69 years old (8.82 %) died from severe SFTS owing to oral infection, lung infection with fungus and staphylococcus and so on in our research. However, some SFTS patients died of heart and respiratory failure during the initial several hours of hospitalization, so we did not get the samples. Among these patients, there were 12 patients (35.29 %) in acute phase, including 9 females and 3 males, with an average age of 57 years; 14 patients were severe, including 11 females and 3 males, with an average age of 58 years (Table 1). When taking into account the factor of age, we found that the patients in these two stages were elderly. We have confirmed that age is a significant predictor for multiple organ dysfunction syndrome (MODS) and death [14].

We also observed 12 sets of clinical laboratory parameters, including hematological and biochemical parameters (Table 2). All of the SFTS patients had thrombocytopenia and decreased WBC, NE %, LY % and ALB, but ALT, AST, LDH, PT, CK and CK-MB were elevated in acute phase and severe SFTS patients. Acute phase for SFTS

Table 1 Basic characteristics of samples involved in this study

Group	Number	Sex		Age		Duration of fever		Hospital stay		Death
		F	M	Median	Range	Median	Range	Median	Range	
Control	20	18	2	30	17–55	–	–	–	–	–
SFTS	34	23	11	55	30–78	8	5–14	9	3–22	3
Acute	12	9	3	57	32–78	5	3–7	–	–	3
Recovery	16	9	7	53	29–66	–	–	–	–	–
Mild	17	8	9	54	29–72	7	5–13	8	6–14	–
Severe	14	11	3	58	42–78	9	7–15	9	5–22	3

Table 2 Comparison of clinic parameters in different conditions of SFTS patients

Parameter	Acute phase	Recovery phase	Mild SFTS	Severe SFTS	Death	Survival
WBC × 109/L	1.94 (0.9–3.2) ^a	4.05 (1.2–10.8)	3.86 (1.2–9.9)	1.93 (0.9–15.6)	8.56 (2.0–15.6)	1.76 (0.9–13.3)
NE, %	68.9 (4.5–93)	68.3 (32.6–95.7)	61.2 (32.6–91)	70.4 (4.5–93)	75.7 (65.1–88.7)	57.9 (4.5–88.7)
LY, %	27.6 (5.5–79.9)	19.1 (3–63.1)	30.2 (6.6–63.)	25.9 (5.5–79.9)	17.3 (5.5–26.2)	32.3 (7.4–79.9)
PLT, × 109/L	51.5 (17–62) ^b	69 (31–246)	65 (31–246) ^c	44 (17–69)	83 (53–176)	67 (33–241)
ALT,U/L	108 (24–241)	70 (31–247)	77 (7–187)	81.5 (36–241)	176 (83–253)	67 (33–241)
AST,U/L	243.5 (32–862) ^b	88 (24–412)	117 (9–412) ^c	274 (64–862)	264 (258–579)	203 (27–862)
ALB,g/L	31.3 (26–38.8)	32.6 (23.5–37.8)	32.7 (23.5–37.8)	30.4 (21.4–37.3)	23.8 (21.4–29.7) ^d	31.5 (26–37.3)
CRP,μmol/L	80.8 (41.3–300.1)	59.55 (33.4–89.3)	49.8 (38.6–300.1)	72.3 (33.4–87.5)	46.8 (38.6–68.5)	67 (51.9–102.8)
BUN,mmol/L	5.1 (2.4–13.2)	3.9 (2.1–7.2)	4.0 (2.1–7.2)	4.4 (2.4–13.2)	3.4 (3.3–4.6)	3.8 (2.4–8.5)
CK,U/L	1,319 (149–4554) ^a	217 (28–1234)	230 (25–1234) ^c	1209 (115–4554)	1,598 (490–2001)	689 (29–4554)
LDH,U/L	1,107 (354–2100)	553.5 (221–2370)	540 (174–749) ^c	1016 (228–2370)	1,376 (828–1596)	1,016 (228–2370)
PT,s	12.8 (11.6–14.3)	13.1 (11.6–21.7)	13.7 (11.6–21.7)	12.7 (11.6–14.3)	13.2 (13.2–14.1)	12.7 (1.6–14.3)

Data are median (interquartile range); *WBC* white blood cell count, *NE* neutrophil, *LY* lymphocyte, *PLT* platelet count, *ALT* alanine aminotransferase, *AST* aspartate aminotransferase, *ALB* albumin, *CR* creatinine, *BUN* blood urea nitrogen, *CK* creatine kinase, *PT* prothrombin time
^a $p < 0.01$, ^b $p < 0.05$ compared with recovery phase; ^c $p < 0.05$ compared with severe SFTS; ^d $P < 0.05$ compared with survival

patients indicated that patients were in the first week of the onset, and clinical features and laboratory parameters were abnormal. In comparison with SFTS patients in recovery phase, serum levels of WBC, PLT, AST and CK were significantly lower in acute phase, but the levels of NE, LY, ALT, ALB, CRP, BUN, LDH and PT did not show statistical significance. Severe SFTS cases were categorized by having at least one of the following symptoms such as respiratory, renal, hepatic, cardiovascular and central nervous system (CNS) dysfunction, even with systemic inflammatory response syndrome (SIRS), disseminated intravascular coagulation (DIC) tendency and shock. There were significant differences in serum levels of PLT, AST, CK and LDH between severe and mild cases ($p < 0.05$). In death cases, only ALB was significant, but the difference might be due to the comparatively small study groups and big errors.

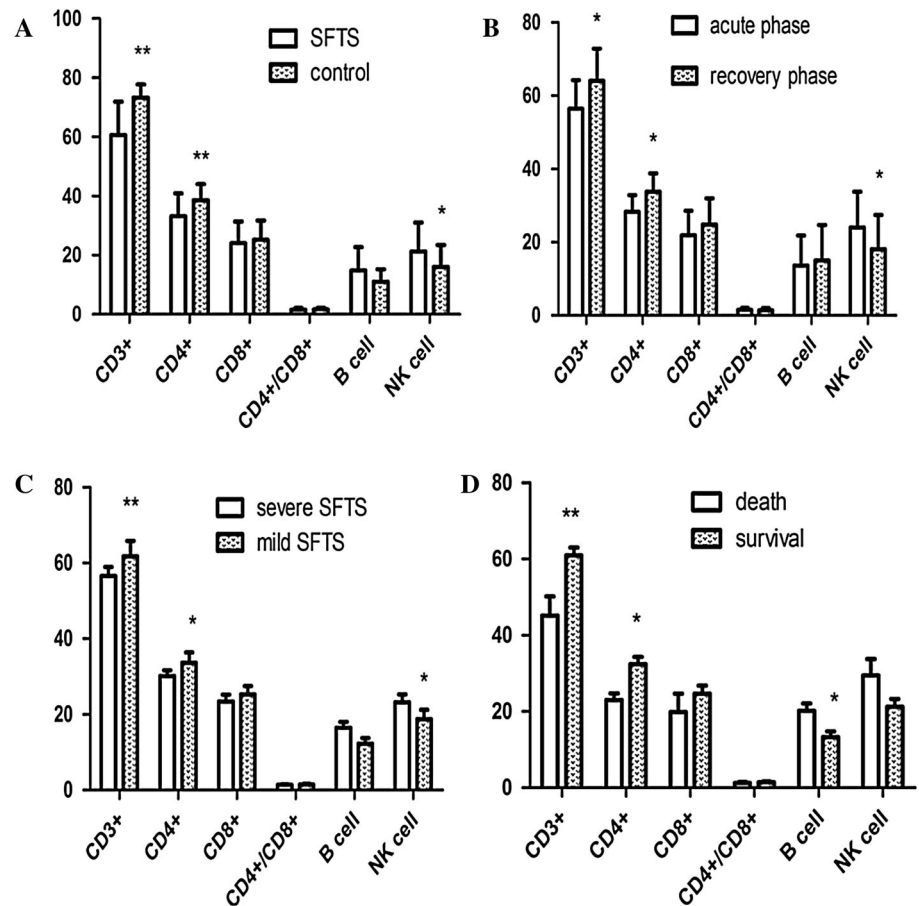
Lymphocyte subsets in peripheral blood

Thirty-four samples from SFTS patients and 20 healthy donors were subjected to flow cytometry for immunofunction

test. The values of T-lymphocyte subpopulation, B cells and NK cells are shown in Fig. 1. The CD3+ and CD4+ T lymphocytes were decreased significantly in SFTS with $60.66 \pm 11.19 \%$ ($p < 0.01$) and $33.15 \pm 7.73 \%$ ($p < 0.01$), respectively, but SFTS patients had a significantly higher percentage of NK cells in peripheral blood ($21.33 \pm 9.74 \%$, $p < 0.05$) compared to normal control. The proportions of CD8+ T cells, B cells and CD4 +/CD8+ T cells were also diminished or elevated in SFTS patients. However, there were no obvious statistical significances between SFTS and normal control regarding the values of CD8+ T cells, B cells and CD4 +/CD8+ T cells ($p > 0.05$).

According to the clinical manifestation and results of auxiliary examination, we divided the SFTS into different groups. Levels including acute and convalescent cases, mild and severe SFTS, death and survival SFTSV infection were compared. The levels of CD3+ T lymphocytes were $57.02 \pm 7.52 \%$ and $64.62 \pm 8.84 \%$ in patients with acute and convalescent cases, $56.3 \pm 8.72 \%$ and $61.9 \pm 15.4 \%$ in patients with mild and severe SFTSV infection, $45.17 \pm 8.61 \%$ and $60.97 \pm 6.86 \%$ in patients with death and survival SFTS, respectively, and the differences were

Fig. 1 Comparison of percentage of CD3+, CD4+ and CD8+ T lymphocyte, B cell and NK cell, as determined by flow cytometry. **a** The percentage of lymphocytes in 34 SFTS patients and 20 healthy donors. **b** The percentage of lymphocytes were compared between 12 acute cases and 16 convalescent cases. **c** The percentage of lymphocytes were compared between 14 severe cases and 17 mild cases. **d** The percentage of lymphocytes were in 3 death cases vs 11 survival cases. Data were expressed as means with SEM. * $p < 0.05$, ** $p < 0.01$



significant ($p < 0.05$). CD4+ T-lymphocytes' levels were $27.64 \pm 4.00\%$ and $34.21 \pm 4.91\%$ in patients with acute and convalescent cases, $29.88 \pm 5.51\%$ and $33.76 \pm 10.45\%$ in patients with mild and severe SFTSV infection, $23.04 \pm 2.96\%$ and $32.43 \pm 6.11\%$ in patients with death and survival SFTS, respectively, and the differences were significant ($p < 0.05$). The proportion of NK cells was significantly increased in patients with acute phase and severe SFTS compared to convalescent cases and mild SFTS ($24.03 \pm 9.77\%$ vs $16.76 \pm 8.11\%$ and $23.85 \pm 8.23\%$ vs $17.42 \pm 8.64\%$). However, within severe SFTS patients, the proportion of B cells was significantly diminished in survival cases compared to death cases ($13.3 \pm 4.81\%$ vs $20.16 \pm 3.39\%$). Although levels of CD8+ T cells and CD4+/CD8+ T cells were lower in SFTS group, acute phase and severe SFTSV infection than those in control group, convalescent phase and mild SFTSV infection, the differences were not significant ($p > 0.05$).

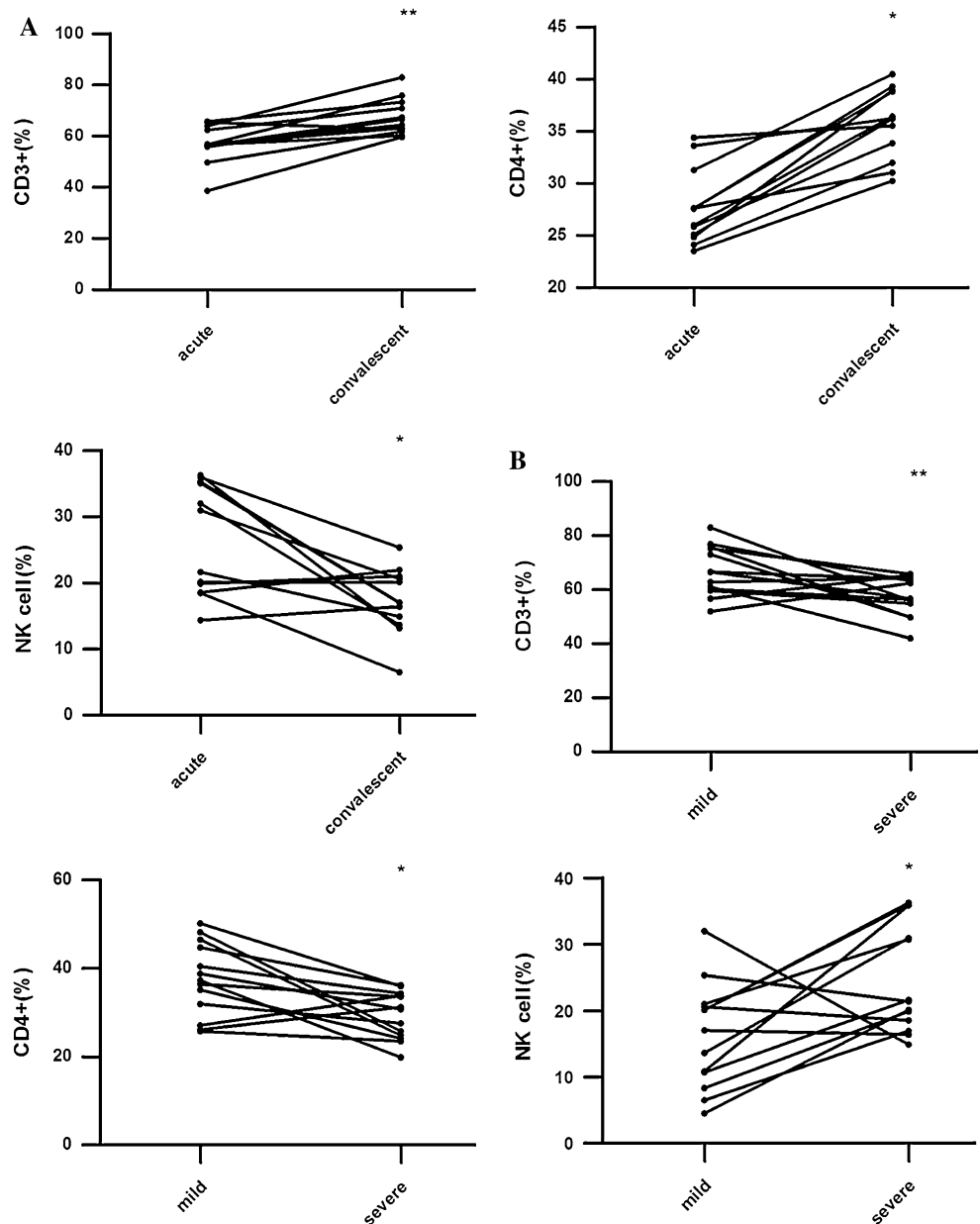
In order to acquire overall comprehension of immunofunction, we also statistically compared the variations of lymphocyte levels in the progression of the disease. The 12 paired samples were chosen from the acute to convalescent phase among 34 SFTS patients, as well as 14 paired samples from mild to severe SFTSV infection. The analysis

showed that the expression levels of CD3+ and CD4+ in the convalescent phase increased to the near normal levels (Fig. 2a). But the level of NK cells in the convalescent phase decreased compared to that in the acute phase. In contrast, when the condition of SFTS patients progressed from mildness to severity, the contents of CD3+ and CD4+ diminished gradually. And an impressive level elevation of NK cell was presented in severe SFTS patients (Fig. 2b). These dynamic analyses indicated that the immunofunction that back to physiological range was associated with the recovery and the mild state of SFTS disease.

Discussion

The pathogenesis of SFTS is not distinct at present. We described the clinical symptoms and laboratory parameters during the complete course of SFTS disease. The characteristics were more obvious for patients in acute phase and severe SFTS than that in recovery phase and mild SFTS. Those physiological balance of laboratory parameters was substantially altered in acute phase and returned to physiological ranges during the recovery phase. It had been

Fig. 2 Comparison of CD3+, CD4+ and NK cell in paired acute phase and convalescent phase, and in paired mild and severe SFTSV infection. **a** The percentage of CD3+, CD4+ and NK cell were compared in the paired samples from acute and convalescent phase. **b** The percentage of CD3+, CD4+ and NK cell were compared in the paired samples from mild and severe SFTS infection. Each *dot* shows the level in an individual. Each *line* connects a pair of samples chosen from the same case. * $p < 0.05$, ** $p < 0.01$



shown that innate immune responses determined the pathogenesis of SFTS, as well as high level of viral replication [10]. The amount of lymphocyte and its subsets was stable relatively in peripheral blood and tissue. And lymphocyte produced moderate immune response, which could eliminate foreign antigen without injuring intracorporal organism. But the balance was broken when SFTSV invaded and the amount of lymphocytic subpopulations changed. Our study showed that the expression level of lymphocyte subsets was abnormal in the peripheral blood of SFTS, especially in acute phase and severe SFTS patients.

In our prospective study, the most striking findings were the significant elevation of NK cell and the distinct

depression of CD3+ and CD4+ in peripheral blood of patients with SFTSV infection. The leukocyte count in SFTS was less than $4 \times 10^9/L$, and the average level was $2.86 \pm 1.56 \times 10^9/L$.

While the proportion of lymphocytes was decreased in acute and severe SFTS patients, together with these data, we found that activated T cells played a crucial role in the pathophysiology of SFTS since the main proportions of lymphocytes in peripheral blood were changed. The total number of T cells in SFTS patients decreased significantly and the decline in the number of CD3+ T lymphocytes prompted that the number of active cells was insufficient to participate in cellular immune response, and then, the cellular immune function was low in SFTS patients. The

expression of CD4+ on T cells was obviously reduced in SFTS, which made the function of immune globulin downgrade. At the same time, we also found that the percentage of NK cells was significantly high. It was well known that NK cells were classified as lymphocytes on the basis of their morphology, expression of lymphoid markers and common origin. NK cells had been shown in humans and mice to participate in the early control against virus infection [15]. NK cells could kill virally infected or transformed cells [16]. Therefore, NK cells were highly important for the pathogenesis of SFTS disease progression.

It was revealed in our study that the variation of lymphocyte subpopulation was associated with the phase of the disease and the patient's condition. In acute phase, the leukocyte count was less than $4 \times 10^9/L$, and it gradually returned to normal level in convalescence phase, while the leukocyte count was $2-4 \times 10^9/L$ in mild SFTS patients, and it was less than $2 \times 10^9/L$ in severe SFTSV infection. The percentages of CD3+ and CD4+ were significantly lower in the acute phase and severe SFTSV infection than those in recovery phase and mild SFTSV infection. The balance of immunocytes was broken and lymphocytopenia took place due to the effects of multiple stimuli. Li et al. [17] found that CD4+ T lymphocytes had a sharp decline in the research of severe acute respiratory syndromes (ARDS), which resulted in the damage of cellular immune function. CD4+ T lymphocytes could induce the virus specificity cytotoxic T cell forming specific polyclone immunity and assist B cell to produce neutralizing antibody, which made key roles in incubation period and acute phase when virus infected. We presumed that the reasons for the reduction of CD3+ and CD4+ T cells in acute and severe SFTS were as follows: firstly, T lymphocytes which struggled with SFTSV avoided fierce injuries to the organism; secondly, complicated immune made T lymphocytes damage and weakened the ability of immune response in the urgent condition of disease [18]. In contrast to T cells, we found that the count of NK cells was showed to increase, and the differences were statistically significant in acute and severe cases. NK cells, as components of innate immune defense, could directly induce the death of virus-infected cells in the absence of specific immunization, such as cellular immune. NK cells possessed immunoregulatory functions since they secreted cytokines such as IFN- γ , TNF- α , IL-10 and G-CSF. These inflammatory cytokines and chemokines played roles in the pathogenesis of virus infection, and the levels of them were related to the severity of the illness [19, 20]. Simultaneously, NK cells might exacerbate the disease by releasing multiple inflammatory cytokines and chemokines or damaging the affected tissues through direct cytotoxicity [21]. It had been found that the level of IFN- γ increased in fatal SFTS

cases[20], and IFN- γ could induce apoptosis of lymphocytes[22]. This might be one of the possible mechanisms of lymphocytopenia in SFTS patients due to direct and indirect attack of SFTSV. Therefore, we inferred that NK cells educed main roles against SFTSV infection when cellular immune function was weakened obviously. However, the indicators would rise again obviously during the recovery phase, which prompted that it was temporary for the cell immunosuppression resulting from SFTSV infection.

The expression of CD3+ and CD4+ T cells in three death cases of whom were older in severe SFTS diminished sharply. Moreover, as the SFTS disease developed, the level of T leukocyte count was progressively diminished, and there was a more significant decline in the expression of CD3+ and CD4+ T cells. Th1 was the positive cell in CD4+ T lymphocytes, and SFTSV triggered the immune system mechanisms mainly by stimulating the Th1 cells to produce immune responses against the viruses [21]. Deng et al. [21] had demonstrated that SFTSV activated mainly Th1 immune response against viral invasion. The decline of CD4+ T lymphocytes caused the suppression of immunofunction, which might aggravate the patient's condition. At the same time, we found that the percentage of B cells in death cases was significantly higher than that in the survival cases. It might indicate that humoral immunity could be activated when patient's condition was extremely serious, and then, humoral immunity produced antibody to develop the neutralization and enhance the phagocytosis. The immunofunction was extremely low in severe SFTS that increased the risk of secondary infection. However, the difference in the lymphocyte subgroups might not be helpful to judge the prognosis of death and survival owing to higher variations and lower case numbers.

In conclusion, the CD3+ and CD4+ T lymphocytes were significantly lower in SFTS than those in normal control, and the damages of cellular immune system were obvious in the disease progression of patients with SFTSV infection. NK cells were significantly higher and played important roles in killing virally infected cells. Furthermore, the immunofunction was damaged seriously in the severe SFTS patients and acute phase. Therefore, immunofunction detection might be an indicator to aid physicians in evaluating the disease severity of SFTS patients.

Acknowledgments This work was supported by the National Nature Science Foundation of China (Grant:81171638). We would like to thank the teachers at the Center for Stem Cell Research and Application for technical assistance and thank the professors in the Department of Infection Disease for their help in collecting samples.

Conflict of interest The authors who had taken part in this study declared that they did not have anything to disclose the conflict of interest with this manuscript.

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