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Research article

TLR2/NF-κB signaling may control expansion and function of regulatory T cells in patients with severe fever with thrombocytopenia syndrome (SFTS)

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ABSTRACT

Severe fever with thrombocytopenia syndrome (SFTS) is a recently identified infectious ailment triggered by a new strain of bunyavirus. It is distinguished by elevated fatality rates, ranging from 12 % to 30 %. The mechanism underlying the development of severe illness caused by SFTS bunyavirus (SFTSV) is not yet fully understood. To evaluate the role of the TLR2 receptor pathway in regulating Treg function in the progression of SFTS disease and possible mechanisms, sequential serum samples from 29 patients with SFTS (15 mild, 14 severe cases) were examined. Flow cytometry was employed to scrutinize the phenotypic and functional characteristics of TLR2 expression on circulating CD4 T cells, CD8 T cells, and Tregs. In all admitted patients, the evaluation of correlations between the frequencies of the aforementioned cells and SFTS index (SFTSI) was conducted. For SFTS, the levels of TLR2 on CD4 T cells and Tregs were significantly heightened when compared to those in healthy subjects. Additionally, the expression of TLR2 on Tregs exhibited a positive correlation with Ki-67 expression in Tregs and the severity of disease. Additionally, compared with those in uninfected controls, the expression levels of NF-κB in Tregs were significantly increased. Collectively, Tregs may be activated and proliferate through the stimulation of the TLR2/NF-κB pathway in reaction to SFTSV infection.

1. Introduction

Severe fever with thrombocytopenia syndrome (SFTS) manifests with an abrupt onset of extremely high fever, along with gastrointestinal or respiratory symptoms, a decrease in platelet count and white blood cell count, neurological manifestations, and the possibility of fatality between 7 and 14 days following the onset, with mortality rates between 12 % and 30 % [1–3]. At present, neither

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efficacious antiviral therapy nor traditional vaccines have been successful in attaining enduring management of the dissemination of SFTSV. Hence, there is an urgent requirement for a more comprehensive comprehension of the mechanisms governing the progression of SFTS. Additionally, establishing the human immune reactions to SFTSV infection, identifying protective factors, and elucidating disease pathogenesis are crucial for formulating efficient preventive and treatment approaches.

While the understanding of the mechanism behind SFTS remains lacking, there is a widely accepted consensus that the immune system could potentially play a pivotal role [4]. To establish an optimal defense against pathogens while simultaneously avoiding damage to healthy tissues, the immune system has a few regulatory systems. Regulatory T cells (Tregs) constitute a crucial component of the immune defense system, acting as a crucial regulator [5]. Our research team has recognized that deceased patients displayed elevated proportions of Tregs but reduced absolute Tregs counts compared to those who survived [6]. It appears that the immune system seems to be an uncontrolled force that requires guidance by regulators. Understanding the factors involved in Treg activation and expansion will provide novel therapeutic intervention strategies for SFTSV infection.

Toll-like receptors (TLRs) are a vital family of pattern recognition receptors, playing a significant role in discerning pathogen-associated molecular patterns (PAMPs) related to microorganisms and viruses [5]. Currently, accumulating profiling investigations have suggested that certain TLRs are expressed not solely in innate immune cells but also in Tregs [7]. Previous studies have shown that Tregs express multiple TLR mRNAs, encompassing TLR1, 2, 4, 5, 6, 7, and 8. Nevertheless, only a limited number of TLRs, specifically TLR2, TLR5, and TLR8, are activated, potentially influencing the proliferation and/or inhibitory capacity of Tregs [7,8]. Nevertheless, there has been a lack of studies examining the potential involvement of the TLR2 pathway in modulating Treg function and proliferation during the progression of SFTS disease.

In the ongoing investigation, we assessed the expression levels of TLR2 and NF-κB on CD4 T cells, CD8 T cells, and Tregs in 29 individuals diagnosed with SFTS, comprising 15 mild and 14 severe cases. This kind of information is valuable for facilitating more extensive and thorough investigations into the pathogenesis of SFTSV.

2. Materials and methods

2.1. Ethics statement

The research received approval from the Ethics Committee of Tongji Medical College and Tongji Medical College Affiliated Union Hospital, Huazhong University of Science and Technology (ethical approval number 2017S326 and 2017S031). Prior to blood collection, written informed consent was acquired from every participant.

2.2. Patients

Based on the clinical guidelines for SFTS set forth by the Ministry of Health of the People's Republic of China in 2010, our study included subjects infected with SFTSV from August 2017 to December 2017. Those who had concurrent hepatitis B virus (HBV), HCV, or human immunodeficiency virus (HIV)-1 infection; individuals meeting clinical and biological standards for fungal or/and bacterial infection; and those with Epstein–Barr virus positive were not included. SFTSV infected individuals are admitted to Wuhan Union Hospital. For retrospective analysis, patient information (including routine hematological laboratory results, clinical history and physical examination findings) was gathered from the medical documents. Table 1 displayed the fundamental clinical and laboratory features of patients with mild and severe conditions upon admission.

2.3. Procurement and handling of samples

Upon admission, all patients infected with SFTSV received conventional therapies as outlined in the SFTS treatment protocols

Table 1
Differences in clinical and laboratory characteristics between the mild and severe patients[a].

Characteritic	Healthy	ALL cases(N = 29)	*p*-Value	Mild(N = 15)	Severe(N = 14)	*p*-Value[b]
Age, years	54(28–71)	58(35–74)	0.07[b]	54(35–70)	61(40–74)	0.02[c e]
Male, sex,n(%)	8(47)	15(51.7)		9(60)	6(42.8)	0.36[d]
Duration of fever, days	N/A	6(3–10)	N/A	6(3–8)	6(3–10)	0.67[c]
PlasmaRNA, Log10	N/A	3.48(1.13–6.95)	N/A	2.22(1.13–5.61)	4.14(2.03–6.95)	0.02[c]
Platelet count, 10^9/l	186(127–287)	48(11–82)	<.0001[c e]	50(21–78)	49.5(11–82)	0.69[c]
Monocyte count, 10^9/l	0.46(0.34–0.60)	0.21(0.01–1.44)	0.59[c]	0.21(0.01–1.94)	0.19(0.01–1.44)	0.59[c]
Lymphocyte count, 10^9/l	1.61(1.33–2.4)	0.68(0.21–2.31)	0.007[c e]	0.74(0.31–2.31)	0.70(0.21–1.96)	0.59[c]
Leukocyte count, 10^9/l	6.57(4.51–9.1)	2.75(0.75–12.63)	<.0001[c e]	2.75(0.75–5.9)	5.8(0.95–12.63)	0.84[c]

Abbreviation: N/A, not applicable.
[a] Except for the proportion of males, all other data are presented as median (range).
[b] Statistic significance between the SFTS patients and the healthy controls, P < 0.05 was considered as significant.
[c] By means of the Mann–Whitney *U* test.
[d] By means of the Pearson Chi-square test.
[e] The parameter shows significant difference between the SFTS patients and the healthy controls.

authorized by the Chinese Ministry of Health. Throughout their entire hospitalization, blood specimens were methodically collected from the patients, starting within one day of hospital admission and subsequently every third day. Furthermore, we categorized the blood test results obtained from patients within the first day of hospitalization (within 7 days post disease inception) as the "acute period". The hematology findings derived from patients on the day prior to their release (when their clinical manifestations began to ameliorate and their laboratory parameters slowly normalized) signifying the "convalescent period". For this study, a total of eighteen blood donors who were free from any underlying diseases, not infected with SFTSV, and matched the infected patients in terms of gender, age, and racial background were included. Upon enrollment, blood samples were acquired from these participants at a solitary time point. To ensure timely processing, each sample was handled within the initial 4 h following collection. The separation of peripheral blood mononuclear cells (PBMCs) was performed following manufacturer's guidelines using Ficoll-Paque Plus (DAKEW Biotech, China) through density gradient centrifugation.

2.4. Flow cytometry

A sum of 2 × 105 PBMCs were gathered in containers and subsequently labeled with fluorescent conjugates of anti-human monoclonal antibodies (mAbs). Specifically, the APC-Cy7-labeled CD3 (300318, BioLegend, USA), PerCP-cy5.5-labeled CD4 (317428, BioLegend), APC-labeled CD25 (302610, BioLegend) and PE-Cy7-labeled CD127 (351320, BioLegend), Pacific Blue-labeled CD69 (310919, BioLegend) and PE-labeled Ki-67 (350504, BioLegend), PE-labeled NF-κB (565447, BD Bioscience, USA) and eFluor 506-labeled Fixable Viability Dye (65-0866-18, eBioscience, USA) antibodies were used. Furthermore, all experiments employed isotype-matched control monoclonal antibodies. As previous study [9] showed Tregs were labeled as CD4 + $CD25^{+}$ CD127 low.

For quantifying intracellular cytokine levels or intranuclear cytokines, cell permeabilization was carried out using Cytofix/Cytoperm (BD Bioscience, USA), subsequently proceeded with intracellular staining utilizing PE-labeled Ki-67 or intranuclear staining with PE-labeled NF-κB antibody. Following that, the samples were resuspended in 200 μl of phosphate-buffered saline (PBS) (HyClone, USA) prior to analysis using a FACSCalibur flow cytometer (BD Biosciences). To assess the frequencies of circulating lymphocytes, around 5 × 104–1 × 105 events per container were captured through flow cytometry. Subsequently, the data underwent analysis utilizing FlowJo software (TreeStar, Ashland, OR, USA).

2.5. Determination of SFTS viral load

As mentioned previously [10], total RNA was obtained from serum samples of each clinical patient using the DAAN Gene viral RNA kit (Guangzhou, China). The quantity of SFTSV RNA copies in SFTS patients was evaluated using an authorized real-time PCR kit (SFDA registration number 340166, China) following the manufacturer's guidelines.

2.6. Evaluating SFTS severity

In our earlier investigation [11], it was demonstrated that the SFTSI served as an evaluation instrument for evaluating the disease severity of SFTS upon admission. The SFTSI is calculated using the formula: 5 × level of neurological symptoms +4 × level of respiratory symptoms +3 × LG10 viral load - 2 × LN monocyte% - 7. The research highlighted that individuals with an SFTSI exceeding 16 faced a higher risk of mortality. For the current study, patients were categorized as "severe" if their SFTSI was above 16, and as "mild" if their SFTSI was below 16.

2.7. Statistical analysis

Data analysis was performed using SPSS version 17.0 (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 7.03 (GraphPad Software Inc., San Diego, CA, USA). The median ±95 % confidence interval (CI), median (range), or mean with the standard error of the mean (SEM) was employed to summarize continuous variables, whereas dichotomous variables were presented as absolute values and percentages. Comparisons of two independent groups were carried out using two-tailed Mann–Whitney U tests. The $\chi 2$ test or Fisher's exact test was used to evaluate dichotomous variables in the table. Additionally, a nonparametric Spearman correlation test was employed for conducting correlation analysis of two variables. In all tests, p values less than 0.05 (95 % CI) were considered statistically significant, and both two-tailed and one-tailed tests were utilized.

3. Results

3.1. Characterization of patients with mild and severe SFTS disease upon admission

This study included a cohort of 29 SFTS patients, with 15 classified as mild cases and 14 as severe cases. Table 1 presented the comprehensive clinical information and laboratory parameters for the patients involved in the study. In line with prior studies [4,12], no gender bias was observed among both mild and severe patients ($p = 0.36$). The median age of patients with severe illness surpassed that of individuals with mild conditions ($p = 0.02$). Furthermore, severe SFTS individuals exhibited notably elevated viral quantities compared to those with mild SFTS ($p = 0.02$). Furthermore, upon admission, patients diagnosed with SFTS demonstrated markedly decreased total lymphocyte, leukocyte and platelet counts in comparison to uninfected individuals. Nonetheless, there was no notable distinction among individuals with mild and severe conditions. Table 1 displayed the detailed characteristics of the hematology

laboratory tests for SFTS patients upon admission and individuals without infection.

3.2. SFTS severity exhibits a notable correlation with CD4+TLR2+ T cells rather than CD8+TLR2+ T cells

In order to assess the dynamic fluctuations of CD4+TLR2+ and CD8+TLR2+ T lymphocytes, we employed flow cytometry to analyze the proportions of CD4+TLR2+ and CD8+TLR2+ T cells at different stages in the cohort of 29 SFTS patients. Upon admission, it was identified from the data that the proportion of CD4+TLR2+ T cells, although not CD8+TLR2+ T cells, was markedly increased in both acute-phase and recovery-phase patients when compared with the healthy controls (millde panels of Fig. 1B and D). Contrarily, no significant distinction in the proportion of CD4+TLR2+ T cells was observed between mild patients and severe patients (left panel of Fig. 1B).

As shown in Fig. 1B and D, in the mild patients, the percentages of CD4+TLR2+ and CD8+TLR2+ T cells reverted back to normal levels after 17 days. In the severe patients, the percentages of CD4+TLR2+ and CD8+TLR2+ T cells did not return to normal levels until discharge. Additionally, the percentage of CD4+TLR2+ T cells in the severe patients was significantly increased on days 11–13 and after day 17 compared with that at admission. Moreover, the proportion of CD8+TLR2+ T cells in severe patients exhibited fluctuation above that observed in mild patients until their discharge. Moreover, linear regression analyses showed that the percentage of CD4+TLR2+ T cells, but not CD8+TLR2+ T cells, was positively correlated with SFTSI and viral loads (Figs. S2A and S2B).

We conducted additional assessments of Ki-67 and CD69 expression in CD4 and CD8 T cells using flow cytometry to evaluate T-cell proliferation and activation in all 29 SFTS patients during hospitalization. Upon admission, the Ki-67 expression levels in CD4 and CD8 T cells were noticeably elevated in SFTS patients when contrasted with uninfected controls (Fig. 2D and H), whereas the CD69 expression levels showed no significant variance (Fig. 2B and F). Nonetheless, in contrast to the severe cases, the proportion of CD4+/CD8+Ki-67+ T cells did not show a significant variance in the mild cases (left panels of Fig. 2D and H).

The Ki-67 expression dynamics in CD4 T cells mirrored those in CD8 T cells, oscillating above baseline levels, reaching a peak between days 11–13; however, the Ki-67 levels failed to normalize beyond day 17 (right panel of Fig. 2D and H). Moreover, CD69 expression in CD4 T cells peaked within days 11–13 across all SFTS patients. Conversely, CD69 expression in CD8 T cells reached its zenith during days 8–10 and days 14–16 among mild patients, and within days 11–13 among severe patients (right panel of Fig. 2F). Furthermore, our data indicated that there was no significant correlation between CD4/CD8+Ki-67+ T cells or CD4/CD8+CD69^{+} T cells and SFTSI or viral loads (Figs. S2C, S2D, S2E and S2F).

3.3. Significant correlation of the level of TLR2+ Tregs with the disease severity in SFTS patients

Due to the strong association between the proportion of CD4+TLR2+ T cells with the severity of SFTS and viral loads, we utilized flow cytometry to further examined the frequency of TLR2+ Tregs in the cohort of 29 SFTS patients during their hospital stay. Moreover, we further evaluated the frequency of TLR2+Tregs with the SFTSI and the viral loads upon admission through linear regression analyses. The findings indicated that the proportion of TLR2+Tregs was elevated in patients during both the acute-phase

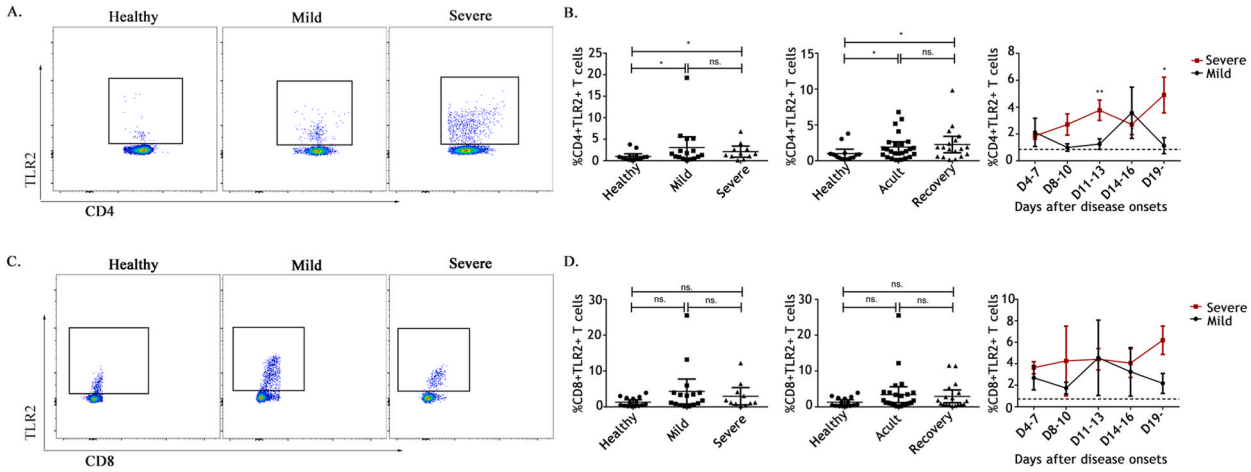

Fig. 1. Increases in the percentages of TLR2+ CD4/CD8 T-cells during the acute phase of SFTS.
(A and C): The expression levels of TLR2 on CD4/CD8 T-cells were analysed by flow cytometry in one representative healthy control, one representative patient with mild disease and one representative patient with severe disease. (Left panels of B and D): Percentages of TLR2+ CD4/CD8 T-cells in the healthy controls (n = 18) and the patients with mild (n = 15) and severe (n = 14) SFTS upon admission. (Middle panels of B and D): Percentages of TLR2+ CD4/CD8 T-cells in the healthy controls and the SFTS patients in the acute phase and SFTS in the recovery phase. (Right panels of B and D): Percentages of TLR2+ CD4/CD8 T-cells in the patients with mild (n = 15) and severe (n = 14) SFTS at different time intervals during their entire hospital stay. The dashed line represents the median of the healthy controls. Data are shown as the median ±95 % CI or mean with SEM. Statistical analysis was performed using the Mann–Whitney *U* test. The level of significance is indicated as follows: ns, not significant; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

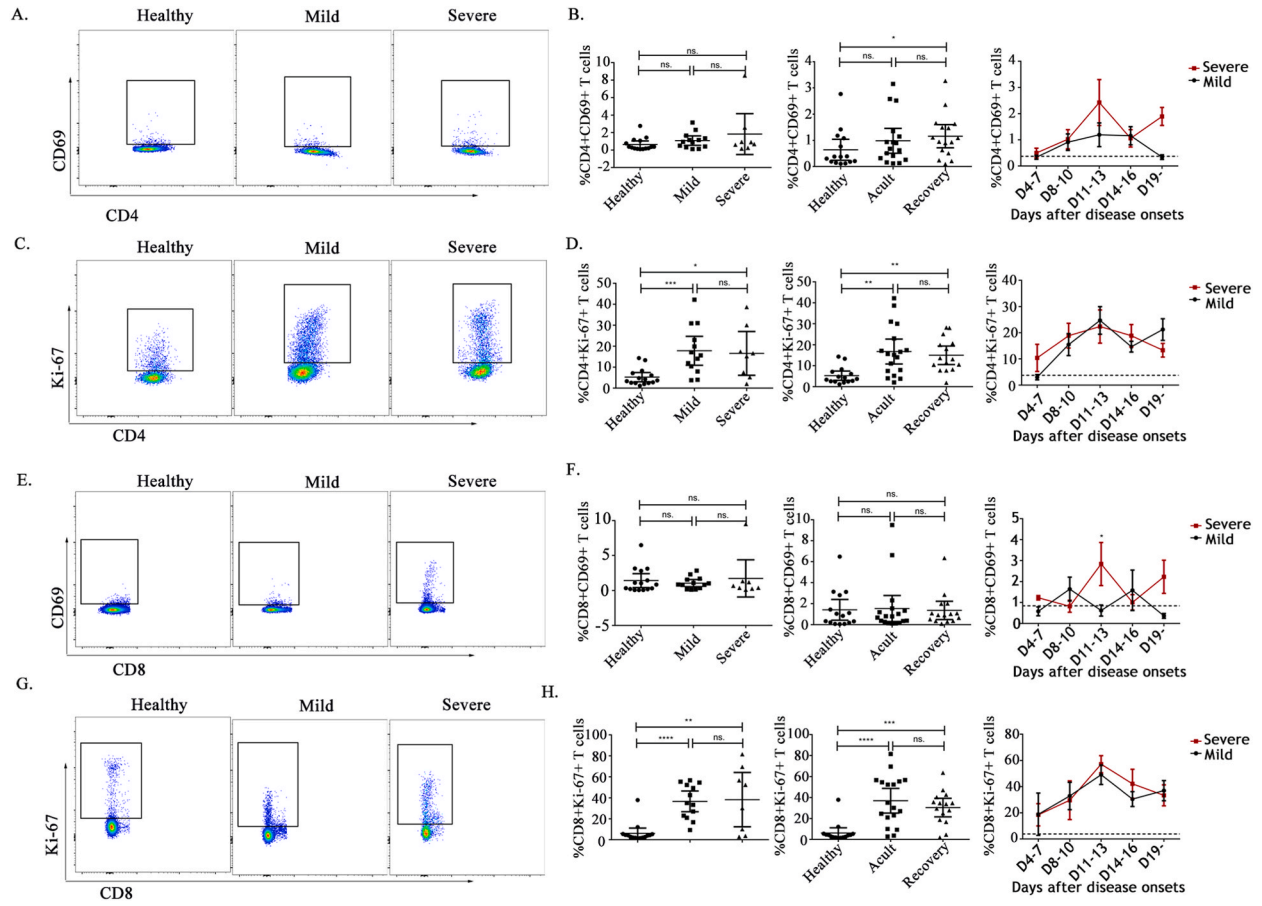

Fig. 2. Changes in the percentages of CD69 and Ki-67 of CD4/CD8 T-cells in SFTS patients.
(A, C, E and G): The expression levels of CD69 and Ki-67 on CD4/CD8 T-cells were analysed by flow cytometry in one representative healthy control, one representative patient with mild disease and one representative patient with severe disease. (Left panels of B and D): Percentages of CD69+CD4/CD8 T-cells in the healthy controls (n = 18) and the patients with mild (n = 15) and severe (n = 14) SFTS upon admission. (Middle panels of B and D): Percentages of $CD69^{+}$ CD4/CD8 T-cells in the healthy controls and the SFTS patients in the acute phase and SFTS in the recovery phase. (Right panels of B and D): Percentages of $CD69^{+}$ CD4/CD8 T-cells in the patients with mild (n = 15) and severe (n = 14) SFTS at different time intervals during their entire hospital stay. (Left panels of F and I): Percentages of Ki-67+CD4/CD8 T-cells in the healthy controls (n = 18) and the patients with mild (n = 15) and severe (n = 14) SFTS upon admission. (Middle panels of F and I): Percentages of Ki-67+ CD4/CD8 T-cells in the healthy controls and the SFTS patients in the acute phase and SFTS in the recovery phase. (Right panels of F and I): Percentages of Ki-67+ CD4/CD8 T-cells in the patients with mild (n = 15) and severe (n = 14) SFTS at different time intervals during their entire hospital stay. The dashed line represents the median of the healthy controls. Data are shown as the median ±95 % CI or mean with SEM. Statistical analysis was performed using the Mann–Whitney *U* test. The level of significance is indicated as follows: ns, not significant; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

and recovery-phase compared to that in the uninfected control group (middle panel of Fig. 3B). Nonetheless, the level of TLR2+Tregs upon admission did not exhibit a significant disparity compared to that in the severe patients (left panel of Fig. 3B). Throughout the hospitalization period, the TLR2 expression kinetics in Tregs of the severe patients fluctuated above those of the mild patients, reached a peak at days 11–13 and failed to normalize after day 17 (right panel of Fig. 3B). Additionally, linear regression analyses suggested a positive correlation between the proportion of TLR2+Tregs and SFTSI along with viral loads (Fig. 4A and B).

We further evaluated Ki-67 and CD69 expression on Tregs via flow cytometry to assess the proliferation and activation of Tregs in all 29 SFTS patients during hospitalization. The levels of Ki-67 and CD69 expression in Tregs were markedly elevated in SFTS patients at admission compared to the healthy cases (Fig. 3D and F). Nevertheless, there was no notable distinction in the proportion of Ki-67 or CD69 expression on Tregs between the mild patients and the severe patients (left panels of Fig. 3D and F).

The CD69 expression dynamics on Tregs reached a zenith between days 8–10 in individuals with mild illness and between days 11–13 in those with severe illness (right panel of Fig. 3D). Moreover, the Ki-67 expression dynamics in Tregs exhibited fluctuations exceeding the standard levels, reaching a peak between days 11–13 and failing to revert to normal levels post-day 17 (right panel of Fig. 3F). Moreover, based on our data, no significant correlation was observed between the percentage of CD69+Tregs or Ki-67+Tregs and the severity of SFTS or viral loads (Fig. 4C, D, 4E and 4F).

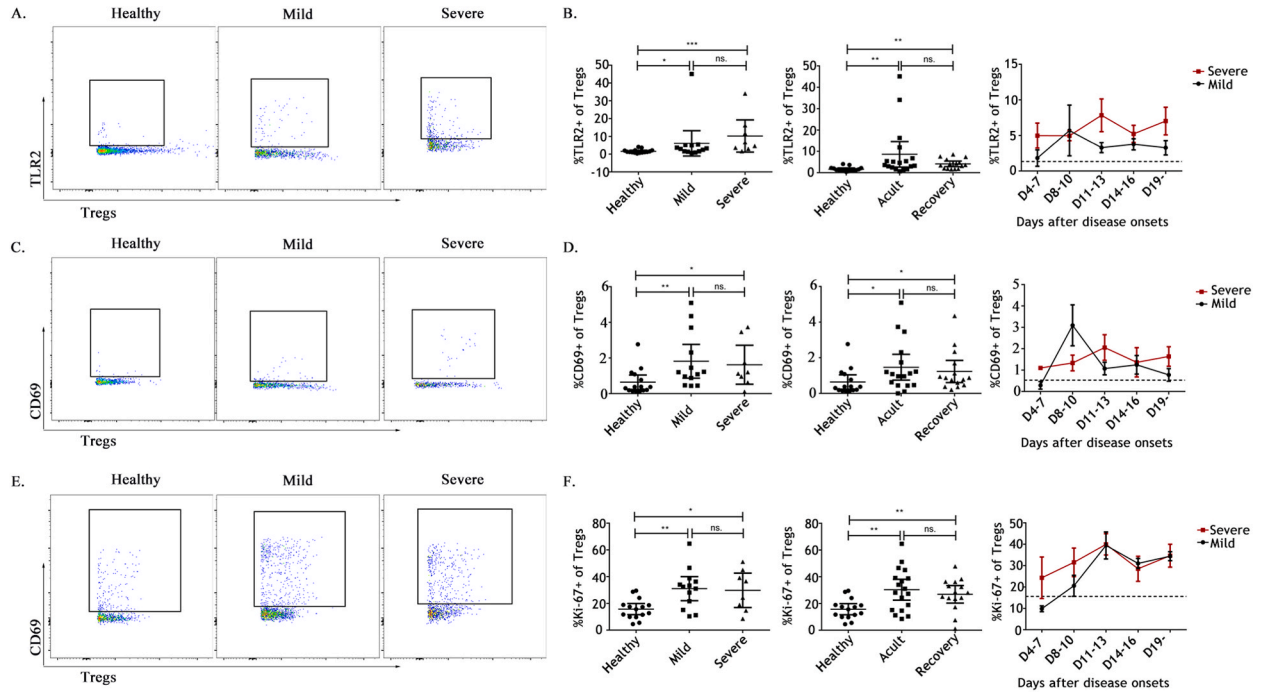

Fig. 3. Increases in the percentages of TLR2, CD69 and Ki-67 on Tregs during the acute phase of SFTS.
(A, C and E): The expression levels of TLR2, CD69 and Ki-67 on Tregs were analysed by flow cytometry in one representative healthy control, one representative patient with mild disease and one representative patient with severe disease. (Left panels of B, D and F): Percentages of TLR2, CD69 and Ki-67 on Tregs in the healthy controls (n = 18) and the patients with mild (n = 15) and severe (n = 14) SFTS upon admission. (Middle panels of B, D and F): Percentages of TLR2, CD69 and Ki-67 on Tregs in the healthy controls and the SFTS patients in the acute phase and SFTS in the recovery phase. (Right panels of B and D): Percentages of TLR2, CD69 and Ki-67 on Tregs in the patients with mild (n = 15) and severe (n = 14) SFTS at different time intervals during their entire hospital stay. The dashed line represents the median of the healthy controls. Data are shown as the median ±95 % CI or mean with SEM. Statistical analysis was performed using the Mann–Whitney *U* test. The level of significance is indicated as follows: ns, not significant; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

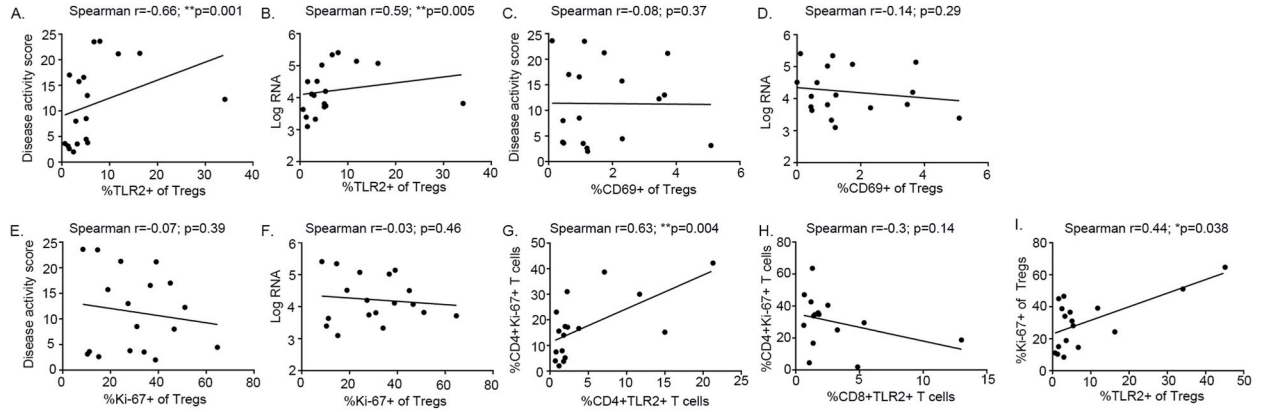

Fig. 4. Significant correlation of the level of TLR2+ Tregs with Ki-67+ Tregs in SFTS patients.
(A, C and E): Correlation of the percentages of TLR2, CD69 and Ki-67 on Tregs with SFTSI at admission in the 29 SFTS patient (including 15 mild and 14 severe patients). (B, D and F): Correlation of the percentages of TLR2, CD69 and Ki-67 on Tregs with the viral loads at admission in the 29 SFTS patient. (G): Correlation of the percentages of TLR2+CD4 T-cells with Ki-67+CD4 T-cells at admission in the 29 SFTS patient. (H): Correlation of the percentages of TLR2+CD8 T-cells with Ki-67+CD8 T-cells at admission in the 29 SFTS patient. (I): Correlation of the percentages of TLR2+Tregs with Ki-67+Tregs at admission in the 29 SFTS patient. Correlation analysis was performed via a non-parametric Spearman correlation test. In the graphs, r and p indicate the correlation coefficient and the p-value of significance, respectively. The level of significance is indicated as follows: ns, not significant; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

3.4. TLR2 may regulate the expansion of Tregs in patients with SFTS

Our current investigation revealed a substantial decrease in the quantities of CD4 T cells and Tregs among deceased patients when contrasted with their surviving counterparts [6,13]. A separate research investigation [14] demonstrated that dengue infection stimulated the expansion of Tregs through the TLR pathway, with surface TLR2 expression on Tregs being accountable for their amplification. In order to enhance comprehension regarding the engagement of the TLR2 pathway in the expansion of CD4 T cells, CD8 T cells, and Tregs in SFTSV-infected patients, we conducted an analysis to assess the relationship between the proportions of CD4+TLR2+ T cells, CD8+TLR2+ T cells, TLR2+ Tregs, and the intracellular Ki-67 expression levels associated with them. Our data showed that the percentages of TLR2+CD4 T cells and TLR2+Tregs, but not TLR2+ CD8 T cells, were positively correlated with the percentages of Ki-67 in the corresponding cells (Fig. 4G, H and 4I).

3.5. Variations in NK-κB expression levels in CD4 T cells and Tregs among SFTSV-infected patients

In our study, we examined the levels of NF-κB expression in CD4 T cells and Tregs from 29 SFTS patients. Fig. 5 depicted higher NF-κB expression percentages in CD4 T cells and Tregs among both acute-phase and recovery-phase patients in comparison to uninfected controls (middle panels of Fig. 5B and D). Nevertheless, we did not observe any noteworthy disparity in the percentages of NF-κB in CD4 T cells and Tregs between the mild and the severe cases (left panels of Fig. 5B and D).

Dynamic data indicated that the incidence of NF-κB expression in CD4 T cells of mild patients exhibited a continuous decrease, reaching a minimum value between days 11–13 before increasing again, without reverting to normal levels post day 17 following disease onset (right panel of Fig. 5B). Furthermore, the prevalence of NF-κB expression in Tregs among the mild patients oscillated above typical levels and failed to revert to normal beyond day 17. Additionally, in severe patients, the occurrence of NF-κB expression in both CD4 T-cells and Tregs steadily increased, peaking between days 11–13, with no return to usual levels post day 17 upon the onset of the disease (right panels of Fig. 5B and D). Moreover, the percentage of NF-κB in CD4 T cells in the severe patients was significantly increased on days 11–13 compared with that at admission (right panel of Fig. 5B).

4. Discussion

Establishing the human immune reaction to SFTSV invasion is a crucial aspect in identifying a defensive pattern essential for pathogenesis investigations and vaccine advancement. In addition to the aforementioned findings, our team made an intriguing discovery regarding the frequencies of immune cells in peripheral blood during the initial stage of SFTSV infection. Specifically, we observed a remarkable decrease in the counts of myeloid dendritic cells, monocytes, NK cells, and T lymphocytes. Furthermore, we found that these alterations in immune cell frequencies were closely linked to diverse clinical outcomes associated with SFTS [6,10,13,15]. In order to assess the potential impact of the TLR2 pathway on the control of Treg activity and proliferation during the course of SFTS, we closely observed and tracked a few of SFTS patients throughout the entirety of their hospitalization. Our data indicated that

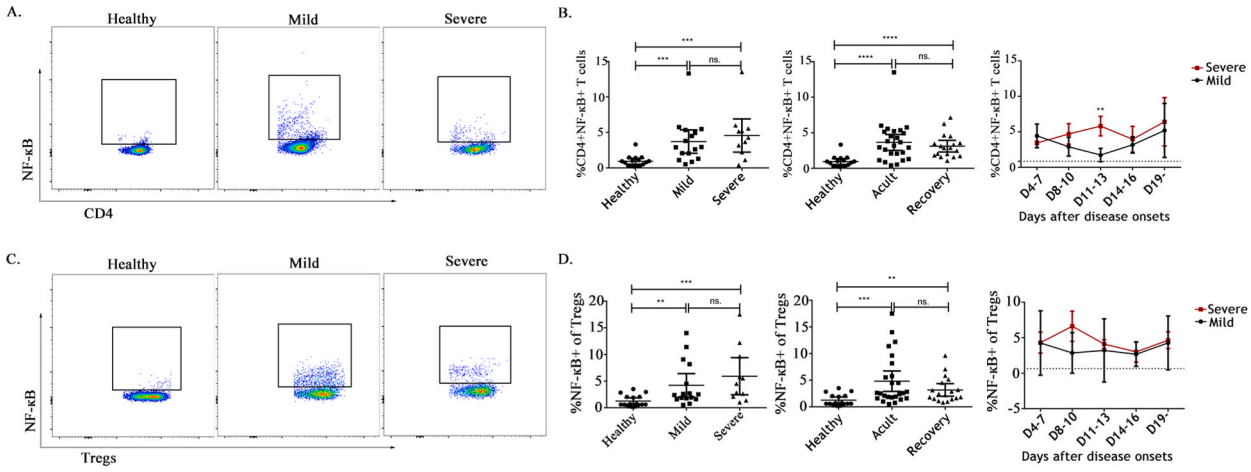

Fig. 5. Differential changes in NK-κB expression in CD4 T-cells and Tregs in SFTSV-infected patients.
(A and C): The expression levels of NK-κB in CD4 T-cells/Tregs were analysed by flow cytometry in one representative healthy control, one representative patient with mild disease and one representative patient with severe disease. (Left panels of B and D): Percentages of NK-κB in CD4 T-cells/Tregs in the healthy controls (n = 18) and the patients with mild (n = 15) and severe (n = 14) SFTS upon admission. (Middle panels of B and D): Percentages of NK-κB on CD4 T-cells/Tregs in the healthy controls and the SFTS patients in the acute phase and SFTS in the recovery phase. (Right panels of B and D): Percentages of NK-κB in CD4 T-cells/Tregs in the patients with mild (n = 15) and severe (n = 14) SFTS at different time intervals during their entire hospital stay. The dashed line represents the median of the healthy controls. Data are shown as the median ±95 % CI or mean with SEM. Statistical analysis was performed using the Mann–Whitney *U* test. The level of significance is indicated as follows: ns, not significant; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

acute SFTSV infection may induce the activation and expansion of Tregs via the TLR2/NF-κB pathway.

Tregs are pivotal in maintaining the equilibrium of peripheral T-cell tolerance, surveilling the host organs to prevent the activation of self-reactive cells under normal conditions, as well as during instances of infection [16]. The significance of Tregs in humans has been predominantly acknowledged in the context of chronic infectious conditions like hepatitis B virus (HBV) infection and tuberculosis [17]. Furthermore, it has been observed that Tregs are linked to a favorable outcome in Flavivirus infections like WNV and DenV [18–20]. However, there is currently insufficient evidence to suggest that host factors induce alterations and proliferation of Tregs during acute SFTSV infection.

In our prior investigation [13], we discovered that the proportion of Tregs was elevated, but the actual number of Tregs was diminished, which is a common observation during the initial phases of SFTSV infection, particularly among deceased patients. However, the precise host factors responsible for inducing the expansion of Tregs in SFTSV infection have yet to be determined. Previous studies [7,8] have reported the crucial role for TLR2 in regulating Treg amplification and inhibition by directly acting on Tregs themselves, and in the presence of TLR2 ligands, the inhibitory phenotype of Tregs was temporarily eliminated, thereby avoiding interference with the immune response both in laboratory settings (in vitro) and in experimental models of acute infection (in vivo). Our research illustrated a notable increase in TLR2 expression on Tregs in SFTS patients compared to healthy individuals, showing a positive correlation with the disease's severity. Moreover, the levels of Ki-67 expression in CD4 T cells and Tregs of SFTS patients surpassed those in healthy controls and exhibited a positive correlation with the expression of TLR2+Tregs. The heightened Ki-67 expression in CD4 T cells and Tregs among SFTS patients implied that the progressive rise in the quantity of CD4 T cells and Tregs were a consequence of stimulated proliferation. Similarly to a prior investigation [14], dengue infection triggered Tregs' proliferation via the TLR2/MyD88 pathway, where the surface TLR2 expression on Tregs facilitated their proliferation. Yet another research revealed that the proportion of Tregs within the complete CD4 T cell population in TLR2−/− mice and TLR-4−/− mice was inferior to that in wild-type mice, whereas the ratio in mixed lineage mice was superior to that in knockout mice, suggesting the mediation of TLR2/TLR4 in the number of Tregs [21]. Nonetheless, TLR-2−/− mice, unlike TLR-4−/− mice, harbored markedly decreased levels of Tregs compared to control mice [22,23]. In addition, administration of TLR-2 ligands to wild-type mice resulted in a noteworthy augmentation in Treg cell numbers [7,24].

Our data indicated that the NF-κB expression levels in Tregs were notably elevated in SFTS patients compared to healthy cases, which may indicate that the TLR2/NF-κB pathway may modulate Treg proliferation in SFTS patients. Hyunju Oh and colleagues demonstrated a dispensable yet pivotal function of canonical NF-κB activity in iTreg maturation and emphasized a distinctive role for individual NF-κB subunits in Treg advancement both in laboratory settings and within living organisms [25]. Moreover, research on genetic loss-of-function in mice suggested that the maturation and lineage constancy of Tregs heavily relied on stimuli triggering NF-κB activation and the distinct NF-κB proteins [26–28]. Despite extensive research focusing on the influence of TLR2 on the proliferation and suppressive functions of Tregs, few studies have examined these effects in the setting of a true acute viral infection. In light of this, the present study underscores the importance of the TLR2/NF-κB pathway in the activation and expansion of Tregs during a genuine acute SFTSV infection.

In conclusion, a potential pathway that possibly mediates Treg proliferation was uncovered in this study. The findings suggest that the TLR2/NF-κB pathway could play a role in Treg proliferation among individuals with SFTS, and that effector reactions are promptly and robustly triggered following the onset of symptoms. Furthermore, this investigation has provided insight into the progression of SFTS and the findings enhance our comprehension of the underlying mechanisms driving the development of this disease.

Availability of data

The data produced and analysed in the present investigation can be accessed from the Open Science Framework repository, identifier: https://osf.io/yvq5s.

CRediT authorship contribution statement

Meng-Meng Li: Project administration, Methodology, Funding acquisition, Formal analysis, Data curation. **Shan-Shan Hu:** Methodology, Investigation, Data curation. **Ling Xu:** Methodology. **Jing Gao:** Methodology, Investigation, Conceptualization. **Xin Zheng:** Writing – review & editing, Methodology, Funding acquisition. **Xiu-Ling Li:** Writing – review & editing, Investigation, Formal analysis. **Le-Le Liu:** Writing – review & editing, Visualization, Supervision, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e35950.

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