RESEARCH ARTICLE

Reduced phosphorylated Foxp3 levels in Crimean Congo haemorrhagic fever

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ABSTRACT

Crimean-Congo haemorrhagic fever (CCHF) is a severe human infection which can lead to fatal consequences. Acute CCHF patients were previously shown to exhibit frequencies of regulatory T-cell (T_{reg}) but lower T_{reg}-mediated suppressive activities than the healthy counterparts. This study aims is to investigate the phosphorylation levels of Foxp3 protein (master regulator of T_{reg} cells) in CCHF patients. Blood samples collected from 18 CCHF patients and nine healthy volunteers were used to isolate peripheral blood mononuclear cells (PBMCs). Total and phosphorylated Foxp3 expression levels in the isolated PBMC samples were monitored by western blot and quantified using ImageJ software. Total Foxp3 expression levels in CCHF patients displayed decreasing trend, but not significantly. In contrast, significantly lower expression levels of phosphorylated Foxp3 were reported in CCHF patients. Our results suggest a possible association between Foxp3 dephosphorylation and CCHF pathogenesis. Nevertheless, more studies are required to evaluate the effect of Foxp3 dephosphorylation on T_{reg} function, which would not only help to enlighten the CCHF pathogenesis but also contribute to the development of effective treatment strategies.

Keywords: Crimean-Congo haemorrhagic fever (CCHF); Treg cells; Foxp3; post-translational modifications; phosphorylation.

INTRODUCTION

Crimean-Congo haemorrhagic fever (CCHF) is a zoonotic tick-borne disease characterized by febrile disease with headache, myalgia, and petechial rash which can be fatal due to impaired haemostasis and multiorgan failure. (Akıncı *et al*., 2013). While it is endemic in many regions including Africa, Asia, Eastern and Southern Europe, as well as Central Asia; recent studies revealed its potential expansion into new areas (Spengler *et al*., 2019), with elevated fatality rates (Nasirian, 2020).

Disease pathogenesis is affected by both the virus and host anti-viral immune responses (Saksida *et al*., 2010; Papa *et al*., 2016). Analysis of cytokine levels in patient serum samples correlated disease severity and fatality with elevated levels of both inflammatory (Papa *et al*., 2006, 2016; Ergonul *et al*., 2006; Saksida *et al*., 2010; Kaya *et al*., 2014), and anti-inflammatory cytokines (Papa *et al*., 2006, 2016; Saksida *et al*., 2010; Yilmaz *et al*., 2017). Such associations led to hypothesize that anti-viral immune response is negatively regulated during the early onset of the infection, which is then followed by the induction of pro-inflammatory immune activities responsible for the organ failure and shock in severe cases (Saksida *et al*., 2010). Accordingly, in our previous study,

higher T_{reg} cell levels (CD4+CD25+CD127dim cells) were reported in subjects with ongoing CCHF infection than that in the healthy control counterparts (Gazi *et al*., 2018). However, when suppressive activities were compared, healthy control subject T_{reg} cells exhibited higher suppressive activities than the CCHF patient T_{reg} cells (Gazi *et al*., 2018).

One candidate mechanism responsible for the reduced T_{reg} cell-mediated inhibitory activity is the post-translational modification (PTM) of Foxp3. The Foxp3 protein is considered to be the master regulator of T_{reg} cells since its mutations lead to induction of severe autoimmune diseases by negatively influencing T_{reg} cell levels and function (Haiqi *et al*., 2011; Barbi *et al*., 2014). While Foxp3 expression and function levels were initially thought to be stable, Foxp3 PTMs, which can be induced by pro- and anti-inflammatory cytokines, are currently considered to have a vital role in Foxp3-mediated regulation of T_{reg} cell actions. Among the PTMs described for Foxp3 protein, phosphorylation influences nearly a third of cellular protein and is regarded as the most -studied PTM. Apart from stabilising Foxp3 protein expression, phosphorylation is also known to promote Foxp3 DNA binding and transcriptional activities (Barbi *et al*., 2014; van Loosdregt & Coffer, 2014). The aim of our study was to compare the total and phosphorylated Foxp3 expression

levels in the peripheral blood mononuclear cells (PBMCs) isolated from acute CCHF patients and healthy volunteers which would both contribute the current understanding of disease pathogenesis, and provide insights for development of effective treatment strategies.

MATERIALS AND METHODS

Ethical approval
All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/ or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This study was approved by Hitit University Clinical Research Ethics Committee in Corum/Turkey (08/09/2021-474).

Subjects
The study included 18 CCHF (10 mild and 8 moderate patients) and nine age- and sex-matched healthy subjects, administered to Hitit University Erol Olcok Training and Research Hospital (Corum / Turkey) between years of 2020 and 2022. Disease severity was evaluated according to the previously described parameters (Dokuzoguz *et al.*, 2013). All CCHF cases were confirmed with positive serology or real-time polymerase chain reaction (RT-PCR) test by the National Reference Laboratory of Turkey (Public Health Institute of Turkey) (Ergönül *et al.*, 2017). Subjects with known condition or medication that influence host immune response were not included in the study. After getting all patients' informed consents, the patients' database used in the study were obtained from the hospital information system.

T-cell isolation and proliferation
The peripheral blood samples (20 ml) were diluted 1:1 with 1xPBS (Gibco), and then added lymphocyte separation medium histopaque (Genaxxon). The mix was centrifuged at 540g for 30 minutes (mins), and the supernatant was removed. For washing steps, the cell pellet was re-suspended in 50 ml of RPMI-1640 (Gibco) media containing 2% fetal bovine serum (FBS, HyClone), 100IU/ml penicillin and streptomycin (Gibco) and L-glutamine (Sigma Aldrich) (R-2), and centrifuged at 540g for 10 mins. After the second washing step, the cells were suspended in FBS supplemented with 10% (v/v) DMSO (Sigma Aldrich). The cells were stored frozen in liquid nitrogen at temperature of -196°C.

Following thawing (incubation in the water bath for 10 mins), the PBMC samples were washed for two times. The MACS magnetic labelling system, and Human Pan T-cell Isolation kit (Miltenyi Biotec) were used to isolated T-cells. Following cell counting by NovoCyte flow cytometer (ACEA Biosciences), T-cells were diluted with R-2 to contain 1×10^6 cells/ml, and 1 ml of cell suspension was added to 6-welled plates. Cells were left for incubation at 37°C for seven days with proliferation solution (2 ml) (1 µg/ml anti-CD3 [Biolegend], 1 µg/ml anti-CD28 [Biolegend] and 300 U/ml recombinant IL-2 [Biolegend]). The proliferation media was renewed on the 4th day.

Western blot
The cell culture supernatant was separated after PBS wash at 2500 rpm for 3 mins. Cell pallets were lysed by incubation with ProtinEx Total Protein Extraction Solution (Geneall) plus 1x protease inhibitors (Gold Biotechnology) for 10 mins on ice. The cell lysate supernatant was collected after centrifugation at 16000 rpm at 4°C for 15 mins. Protein concentration was determined using Qubit Protein Assay kit (Thermo Fisher Scientific) and Qubit 3.0 Flourometer device (Thermo Fisher Scientific). The gel loading mixture (20 µl) was prepared by mixing 100 µg of cell pallet with 1× LDS NuPAGE Sample buffer containing 1× reducing agent (Invitrogen). The mix was heated at 70°C for 10 mins and then cooled on ice for 2 mins before electrophoresis on a 4%-12% NuPAGE gel (Invitrogen) with 1x MES buffer (Thermo Fisher) at 200V for 35 minutes. The proteins were then transferred overnight to Nitrocellulose membrane (Invitrogen). On the next day, the membranes were left for 1-h incubation in blocking buffer (5% bovine serum albumin in PBS and 0.1% Tween 20; 5% BSA-PBST), and were probed with rabbit anti-Foxp3 polyclonal (BT Lab; 1:50), rabbit anti-phospho-Foxp3 polyclonal (Affinity Biotech; 1:100), rabbit anti-β-actin polyclonal antibody (Affinity Biotech; 1:3000) for overnight. On the next day, after 3 washing steps with PBST on a shaker for 5 mins, the membranes were incubated with HRP-conjugated goat-anti-rabbit IgG antibodies (Advansta; 1:10 000) for 1 hour at room temperature. The protein expression levels were visualized by using NZY Supreme enhanced chemiluminescence reagent (Nzytech,) and UVP ChemiDoc-It^2 imaging system. The band intensities were quantified using ImageJ software (National Institutes of Health). Normalization was performed according to the housekeeping protein (β-actin) as loading control, and then to the control group samples.

Statistical analysis
Descriptive statistics including arithmetic mean and standard deviation was calculated. Levene's Test was applied for evaluating the homogeneity of the group variances. Independent Samples t Test with Welch's Correction was performed in accordance with the variance homogeneity test outcomes. Level of significance was accepted to be 0.05. All graphical representations and statistical hypothesis tests were performed with GraphPad Prism (Trial Version 9.4.1. for Mac) software.

RESULTS

Unaltered Foxp3 and reduced phospho-Foxp3 expression levels in PBMC samples isolated from CCHF patients
T_{reg} cells are important to maintain an effective immunoregulatory network to clear infection without any immunopathology by preventing excessive immune responses. Accordingly, acute-phase CCHF patients were previously demonstrated to display higher T_{reg} cell levels than their healthy counterparts (Gazi *et al.*, 2018). Since Foxp3 protein is regarded as the master regulator of T_{reg} cells, peripheral blood expression levels were monitored in PBMC samples collected from CCHF patient and control subjects. In contrast to our previous data (Gazi *et al.*, 2018), there was a trend of lower level of Foxp3 protein expression in the former group, but this did not reach statistical significance (Figure 1A and 1B).

The suppressive activities of T_{reg} cells were previously shown to be reduced by CCHF infection (Gazi *et al.*, 2018). This is thought to be associated with Foxp3 PTMs which can influence both Foxp3 protein transcription activity and T_{reg} function (Barbi *et al.*, 2014; van Loosdregt & Coffer, 2014). The western blot analysis revealed lower phosphorylated Foxp3 expression levels in PBMC samples isolated from CCHF patients when compared with those from healthy volunteers (Figure 1A and 1C).

DISCUSSION

CCHF is regarded as the most wide-spread rick-borne human disease with potential to expand to non-endemic areas as a result of factors including climate change, transportation of infested birds, and imported livestock (Kuehnert *et al.*, 2021). In humans, the infection is characterized by common symptoms such as high fever, malaise, myalgia and petechial rash which may be followed by various organ haemorrhages, shock, and/or multi-organ failure in severe cases (Akıncı *et al.*, 2013). Nevertheless, despite its high fatality rate that ranges between 10 and 40% (World Health Organisation (WHO, 2022), currently there is not yet any specific treatment option or vaccine available against CCHF (Kuehnert *et al.*, 2021). Today, while the CCHF disease pathogenesis is yet to be clarified, delayed anti-viral immune response is thought to facilitate the initial stages of the CCHFV infection (Saksida *et al.*, 2010).
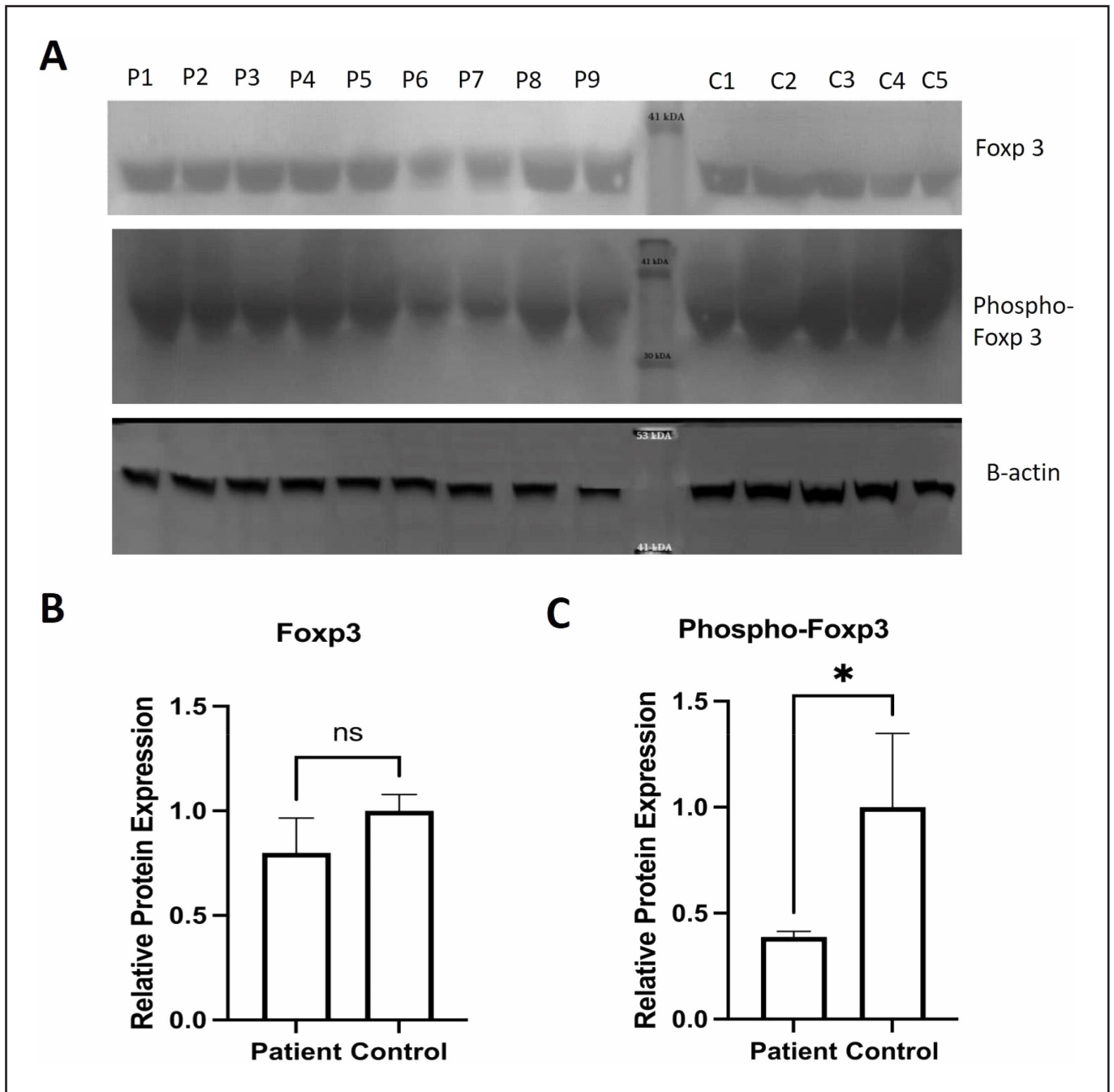
Figure 1. Comparison of Foxp3 and phospho-Foxp3 expression levels between control subjects and CCHF patients. Representative example of a Western blot analysis demonstrating the expression levels of Foxp3, phospho-Foxp3 and β-actin (housekeeping protein) in the PBMC samples isolated. The followings are shown on the blot: Patient No.1 (P1), P2, P3, P5, P6, P7, P8, P9 molecular weight marker, Control No.1 (C1), C2, C3, C4, C5 (A). Data from all volunteers (18 CCHF patients and nine healthy subjects) expressed as the means ± standard deviation of the relative levels of Foxp3 (B) and phosphor-Foxp3 (C) obtained after normalization to the housekeeping gene and control samples. Note: * and ns represents p < 0.05 and no significance, respectively.

In contrast to our previous data showing enhanced T_{reg} cells in CCHF blood samples (Gazi *et al.*, 2018), the results of the current study demonstrated a trend of reduced total Foxp3 expression levels but without any statistical significance. The conflictive data can at least be partially explained by the differences in the T_{reg} markers and methodologies used in both studies; CD4+CD25+CD127dim cell and Foxp3 protein expression levels in PBMC samples were monitored by flow cytometry (Gazi *et al.*, 2018) and western blot in our previous and current study, respectively. On the other hand, while CD4+CD25+CD127dim cells are known to be rich in immunosuppressive T-cell population (Liu *et al.*, 2006; Seddiki *et al.*, 2006; Hartigan-O'Connor *et al.*, 2007; Dunham *et al.*, 2008), Foxp3 protein expression alone was suggested not to be enough for generation of T_{reg} cells (Allan *et al.*, 2005), and was also detected in blood cells without any regulatory function (Gavin *et al.*, 2006; Allan *et al.*, 2007; Wang *et al.*, 2007; Miyara *et al.*, 2009). Therefore, the non-significant alteration in Foxp3 protein expression detected in our study may also be due to fluctuations in the frequencies of cells other than T_{reg} cells. One such cell population is Foxp3+ non-T_{reg} cells which represents a significant fraction of CD4+ T-cells in healthy subjects and was suggested to be an important CD4+ T-cell source of IL-17 production (Miyara *et al.*, 2009). The possible link

between Foxp3+ non-T$_{reg}$ cell levels and diminished IL-17 production in CCHF subjects (Ergυn l *et al.*, 2017) needs further investigation.

On contrary to the hypothesis that delayed anti-viral immune response is involved in the initial stages of the CCHFV infection (Saksida *et al.*, 2010), higher T$_{reg}$ cell levels but lower T$_{reg}$ suppressive activities were previously detected in acute-phase CCHF patients when compared with those that from the healthy control counterparts (Gazi *et al.*, 2018). Our results that demonstrated lower phosphorylated Foxp3 levels in CCHF blood samples, suggest Foxp3 dephosphorylation as the possible explanation for the inconsistency between T$_{reg}$ expression and functional levels.

Foxp3 protein possesses phosphorylation sites for kinases including Nemo-like kinase (NLK), cyclin-dependent kinase (CDK), Pim-2 kinase and lymphocyte-specific protein tyrosine kinase (LCK). Of those while the former promotes, others downregulate T$_{reg}$-mediated suppressive activities (Nakahira *et al.*, 2013; Deng *et al.*, 2019). Apart from phosphorylation, dephosphorylation of Foxp3 by protein phosphatase-1 (PP1) was also shown to negatively regulate T$_{reg}$-mediated suppressive activities (Nie *et al.*, 2013). Therefore, future studies are recommended to investigate the potential use of kinase-targeting therapies (Bhullar *et al.*, 2018) and dephosphorylation inhibitors such as TNF-β-specific inhibitor (Nie *et al.*, 2013) as promising therapy strategies against CCHF. The latter approach was also proposed in a recent study by Golden *et al.*, which demonstrated the protective effects of anti-TNF-β neutralizing antibodies in mice exposed to CCHF (Golden *et al.*, 2022).

Our study has some limitations. Firstly, small number of CCHF patients were included in our study that may have skewed our results. Because of the financial restrictions, polyclonal antibodies were used in the western blot analysis which can lead to cross-reaction. Apart from the antibody-based conventional analysis methods, approaches utilizing mass spectroscopy can be used alternatively for PTM analysis due to its high efficiency, sensitivity, and selectivity (Ke *et al.*, 2016). It was not also possible to investigate the possible association between Foxp3 dephosphorylation levels and fatality in CCHF, while neither total Foxp3 nor phosphorylated Foxp3 expression levels displayed difference between moderate and mild CCHF samples (data not shown). Therefore, while our results indicate a possible role for Foxp3 dephosphorylation in CCHF pathogenesis, future studies evaluating its effect on T$_{reg}$ activity in patients with different CCHF severity are required to confirm our suggestion. Apart from phosphorylation, future studies are also recommended to monitor the effects of other Foxp3 PTMs (e.g. acetylation, ubiquitination (Deng *et al.*, 2019) and identify the associated extracellular stimuli to further unravel the immunopathological mechanisms triggered by CCHFV.

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Conflict of interest
The authors declare that they have no conflict of interest.

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