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\textsubscript{reg}) but lower T\textsubscript{reg} mediated suppressive activities than the healthy counterparts. This study aims to investigate the phosphorylation levels of Foxp3 protein (master regulator of T\textsubscript{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 and CCHF pathogenesis.

Keywords: Crimean-Congo haemorrhagic fever (CCHF); T\textsubscript{reg} 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. (Akna 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\textsubscript{reg} cell levels (CD4\textsuperscript{+}CD25\textsuperscript{+}CD127\textsuperscript{dim} 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\textsubscript{reg} cells exhibited higher suppressive activities than the CCHF patient T\textsubscript{reg} cells (Gazi et al., 2018).

One candidate mechanism responsible for the reduced T\textsubscript{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\textsubscript{reg} cells since its mutations lead to induction of severe autoimmune diseases by negatively influencing T\textsubscript{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\textsubscript{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 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.

**Western blot**

The cell culture supernatant was separated after PBS wash at 2500 rpm for 3 mins. Cell pallets were lysed by incubation with ProteiEx 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 MDES 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-antibody IgG antibodies (Advansta; 1:10 000) for 1 hour at room temperature.

**RESULTS**

Unaltered Foxp3 and reduced phospho-Foxp3 expression levels in PBMC samples isolated from CCHF patients

While the expression of Foxp3 was not changed among the two groups, the expression levels of phospho-Foxp3 were lower in CCHF patients compared to controls. This finding is consistent with previous studies (Gazi et al., 2018). The expression levels of Foxp3 PTMs were not significantly different between the two groups.

**DISCUSSION**

CCHF is regarded as the most widespread tick-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 (Akna 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).
In contrast to our previous data showing enhanced T<sub>reg</sub> 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 conflicting data can at least be partially explained by the differences in the T<sub>reg</sub> markers and methodologies used in both studies; CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>dim</sup> 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<sup>+</sup>CD25<sup>+</sup>CD127<sup>dim</sup> 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<sub>reg</sub> 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<sub>reg</sub> cells. One such cell population is Foxp3<sup>+</sup> non-T<sub>reg</sub> cells which represents a significant fraction of CD4<sup>+</sup> T-cells in healthy subjects and was suggested to be an important CD4<sup>+</sup> T-cell source of IL-17 production (Miyara et al., 2009). The possible link...
between Foxp3+ non-Treg Cell levels and diminished IL-17 production in CCHF subjects (Ergün 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 Treg cell levels but lower Treg 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 Treg 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 Treg-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 Treg-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 Treg 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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