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The role of T_{reg} population in pathogenesis of Crimean Congo hemorrhagic fever

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ABSTRACT

Crimean-Congo hemorrhagic fever (CCHF) is a severe human infection caused by CCHF virus (CCHFV). Today, although the literature on CCHF pathogenesis is still limited, it is thought to be associated with immunosuppression in the early phase of infection followed by pro-inflammatory immune response that may lead to fatal outcomes. The aim of this study is to investigate the role of regulatory T-cells (T_{reg} cells) in the pathogenesis of CCHFV.

Peripheral blood mononuclear cell samples collected from 14 acute CCHF patients with mild disease course and 13 healthy subjects were included in this study. T_{reg} expression and functional levels were analyzed by flow cytometry. T_{reg} cells were identified as CD4 + CD25 + CD127dim cells, and their functional levels were compared by measuring their ability to suppress CD69 and CD154 expression by activated T-cells.

The flow cytometry analysis revealed that total T-cell and helper T-cell levels did not vary between the two groups. In contrast, CCHF patients displayed higher T_{reg} cell levels but lower T_{reg} suppressive activities when compared with control subjects.

This is the first study on the involvement of T_{reg} cells in CCHF pathogenesis. Our results indicate that even though T_{reg} cell levels are elevated during acute phase of CCHF infection, not all generated T_{reg} cells has immunosuppressive capacity, and therefore may not represent 'true' T_{reg} cell population. Future studies on the intrinsic mechanisms responsible for the reduced T_{reg} inhibitory activities are required for further enlightening the CCHF pathogenesis, especially in the acute phase of the disease.

1. Introduction

Crimean-Congo hemorrhagic fever (CCHF) is a severe fatal human infection caused by CCHF virus (CCHFV) which belongs to the Orthonairovirus genus in the Nairoviridae family of the order *Bunyavirales*. CCHF is characterized by febrile disease associated with headache, myalgia, and petechial rash, preceeding the signs of hemorrhagic diathesis, disseminated intravascular coagulation, and circulatory shock due to microvascular instability and impaired hemostasis (Akıncı et al., 2013).

There has not been a suitable animal model yet developed, and most of the literature regarding the CCHF pathogenesis is derived from human cases, in vitro studies as well as what is known from other viral hemorrhagic fever infections (Bente et al., 2010). Because of shared similarities with Ebola hemorrhagic fever, CCHF is thought to be associated with tissue damage caused by the virus directly, and indirectly via host anti-viral immune responses (Saksida et al., 2010; Papa et al., 2016). In support, CCHF patients with severe cases and fatal outcome were reported to have high levels of serum inflammatory chemokines and cytokines including TNF-α (Saksida et al., 2010; Papa et al., 2016; Ergonul et al., 2006; Papa et al., 2006; Kaya et al., 2014).

Nevertheless, immunosuppression was also suggested to be important for the severity of the disease course and fatal outcome. IL-10, which is an anti-inflammatory cytokine, was shown to be positively correlated with viral load and fatality of CCHF disease by different studies (Saksida et al., 2010; Papa et al., 2016, 2006). This supports the hypothesis that immune response is delayed during the initial stages of infection, providing the required time for virus to undergo increased replication and spread throughout the body. This is then followed by pro-inflammatory immune response leading to disseminated

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intravascular coagulation, organ failure and shock (Saksida et al., 2010).

Even though T-cells influence antiviral immunity at multiple stages of the immune response (Whitmire, 2011), there is still not much literature regarding human T-lymphocyte subpopulations affected by CCHFV. Among those studies, while Yilmaz et al. did not observe any difference between non-severe and severe CCHF cases regarding blood levels of lymphocytes, T-cells, inducer/helper T-cells, as well as cytotoxic T-cells, Akinci et al. reported a significant elavation of cytotoxic T-cell levels in fatal cases (Yilmaz et al., 2008; Akinci et al., 2009). However there is still not yet a study on human CD4 + T-lymphocyte subgroups, among which regulatory T-cells (T_{reg} cells) are involved in the regulation of immune responses, including CD8 + T-cell effector differentiation (McNally et al., 2011, 2014; Kastenmuller et al., 2011; Barbi et al., 2014).

Induction of T_{reg} cells is considered as a normal aspect of immunity and is important to maintain a balance between preventing immunopathology and allowing the immune response to clear infections. However, there are some exceptional cases in which T_{reg} cells appear to be responsible for the persistent state for pathogens including viruses such as hepatitis C virus, and human immunodeficiency virus (Robertson and Hasenkrug, 2006). T_{reg} cell level fluctuations were also reported during the acute stage of hemorrhagic fever with renal syndrome caused by hantaviruses which, like the CCHF virus, is a member of the *Bunyaviridae* family of viruses (Zhu et al., 2009). In addition, another study on hantaviruses concluded that the pathogenesis is associated with early suppression of the host innate immune response before the induction of host immune response that results in pulmonary damage and cardiac suppression (Safronetz et al., 2011).

The aim of this study is to investigate the role of T_{reg} cells in CCHF pathogenesis, an area which is yet to be explored. For this purpose, T_{reg} cell levels in CCHF patients and their suppressive activities were monitored and compared with those from healthy subjects. The study used patient samples obtained from Corum province of Turkey which is considered as an endemic region for CCHF (Gureser et al., 2015).

2. Materials and methods

2.1. Subjects

Fourteen CCHF patients and 13 healthy subjects, administered to Hitit University Erol Olcok Training and Research Hospital in Corum province of Turkey between years of 2015 and 2017, were included in this study. All patient and control subjects were white and Caucasian. The mean age of the control group was 30.5 ± 9.21 years (range 20–41 years), while it was 54 ± 10.02 years (range 20–66 years) for the CCHF group. Contol group consisted of 6 male and 7 female subjects while CCHF included 9 male and 5 female subjects. All CCHF cases were confirmed in the National Reference Laboratory of Turkey (Public Health Institute of Turkey), by positive serology or real-time polymerase chain reaction (RT-PCR) test of peripheral blood samples collected within the acute phase of infection as described before (Ergönül et al. (2017)). None of the subjects were under any treatment that may have an affect on host immune response. After getting all patients' informed consents, patients'database (clinical history, reason for splenectomy, vaccination status) were obtained from the hospital information system. This study was approved by the ethics committee of Numune Training and Research Hospital in Ankara/Turkey (E-15-625).

CCHF disease severity was evaluated according to the five previously described paremeters that included platelet count (0–3 points), activated partial thromboplastin time (0–3 points), fibrinogen (0–3 points), bleeding (0–3 points), and somnolence (0 or 1 points) (Dokuzoguz et al., 2013; Bakır et al., 2014). The scores given for each parameter were then added together, and the avarage taken from the two reseachers' scoring were used as the patient score.

2.2. Isolation of peripheral blood mononuclear cells (PBMCs)

Blood samples (20 ml) collected from patient and control subjects were diluted 1:1 with 1xPBS (Gibco, USA) which was then added an equal volume of lymphocyte separation medium histopaque (Genaxxon, Germany). Samples were centrifuged at 540*g* for 30 min (mins) after which the formed cloudy layer is removed. The cell pellet was suspended in 50 ml of RPMI-1640 (Gibco, USA) supplemented with 2% fetal bovine serum (FBS, HyClone, USA), 100IU/ml penicillin and streptomycin (Gibco, USA) and L-glutamine (Sigma Aldrich, Germany) (R-10). Cells were then centrifuged at the same speed for 10 min. After repeating this washing step one more time, cells were suspended in FBS supplemented with 10% (v/v) DMSO (Sigma Aldrich, Germany) to be stored frozen in liquid nitrogen at temperature of −196 °C.

2.3. Thawing cryopreserved cells

After 10 min of incubation in the water bath, thawed PBMC samples were transferred into 15 ml Falcon tubes, and 10 ml warm R-10 was added. Following centrifugation at 540*g* for 10 min, the cell pallet was suspended in 10 ml R-10. After repeating the same washing step, cells were counted by NovoCyte flow cytometer (ACEA Biosciences, USA).

2.4. Detection of T-cells, CD4 + T-cell and T_{reg} cell levels

From the thawed samples, 1×10^6 cells were used to study T_{reg} cells, and the rest was used for T-cell isolation. CD3-CFBlue (Immunostep, Spain), CD4-FITC (Biolegend, USA), CD25-APC (Biolegend, USA) and CD127-PE (Biolegend, USA) antibodies were used for the detection of T-cells, CD4 + T-cell and T_{reg} cell levels by NovoCyte flow cytometer.

2.5. T-cell isolation and proliferation

T-cells were isolated from PBMCs by using MACS magnetic labeling system, and Human Pan T-cell Isolation kit (Miltenyi Biotec, Germany) according to the manufacturer's guidelines. Cells were then counted and diluted with R-10 to contain 1×10^6 cells/ml. After adding 1 ml of cell suspension to 6-welled plates, cells were left for 7-day incubation with 2 ml of proliferation solution that contains 1 µg/ml anti-CD3 (Biolegend, USA), 1 µg/ml anti-CD28 (Biolegend, USA) and 300 U/ml recombinant IL-2 (Biolegend, USA). The proliferation media was renewed on the 4th day.

2.6. T_{reg} Cell isolation, and in vitro suppression assays

T_{reg} cells were isolated from T-cell population by using Human CD4 + CD25 + CD127dim/- Regulatory T Cell Isolation Kit (Miltenyi Biotec, Germany) according to the manufacturer's guidelines. For in vitro suppression assays, T_{reg} cells were co-incubated with 250×10^3 T-cells isolated from the same subject in 1:1 and 3:1 (T_{reg} cell:T-cell) ratio, and 2 µg/ml ConA (Invivogen, France) for 12 or 72 h. Incubation periods of 12 h and 72 h were used to monitor CD69 and CD154 expression levels by anti-human CD69-PE (Biolegend, USA), and CD154-PE (Biolegend, USA) respectively.

2.7. Statistical analysis

Descriptive statistics were calculated and provided throughout the text. Data were analyzed by Student's *t*-test and displayed as mean ± standard error of mean. The level of significance was accepted to be 0.05. All statistical analysis was performed, and graphs were drawn with GraphPad Prism (Version 7.00) software package.