

# Evaluation of the Immunomodulatory Effects of C-Vx on the Innate and Adaptive "Immune" System: Preliminary Results

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## ABSTRACT

**Objectives:** In this preliminary study, the *in vitro* effect of C-Vx in human PBMCs and the *in vivo* effect of C-Vx in rats were investigated.

**Methods:** The human part was analyzed in PBMCs isolated from healthy subjects. Apoptotic index, cytotoxic activity of CD8<sup>+</sup> T and NK cells, and cell proliferation of CD3<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>+</sup> T and NK cells in response to different doses of C-Vx were investigated. Also the hematological and biochemical parameters of the rats administered subcutaneously in three different doses of C-Vx were monitored for 14-days.

**Results:** Increased CD107a expression in response to C-Vx on NK cells but not on CD8<sup>+</sup> T cells support the increasing of NK cell cytotoxicity. C-Vx alone was capable of triggering proliferation of T and NK cells. The PHA-induced proliferation of CD3<sup>+</sup> and CD4<sup>+</sup> T cells was diminished in response to C-Vx, while PHA-induced CD8<sup>+</sup> T cell proliferation was up-regulated. PHA-triggered proliferation of total NK cells was enhanced with the existence of C-Vx.

C-Vx was well tolerated in rats with no serious adverse effects or mortality (death) after 14-days of follow-up. Biochemical-parameters (creatinine, blood urea nitrogen, etc.) were not significantly different among treated and control groups. The levels of white blood cells and lymphocytes were increased up to two-folds in the C-Vx group (especially 0.25 ml/day) as compared to the control group.

**Conclusion:** Taken together, these preliminary findings support the immunomodulatory effects of C-Vx. But these findings should cautiously be evaluated due to the low numbers of subjects in both human and experimental arms.

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## INTRODUCTION

SARS-CoV-2 cases continue to increase rapidly all over the world, including our country. With each passing day, the number of cases and deaths cause concern all over the world.

When SARS-CoV-2 first appeared, there was no vaccine or antiviral drug to prevent its spread (Wang et al., 2021). But now some drugs (favipiravir and remdesivir) have clinical utilization and many vaccines have been introduced to the market, such as the SARS-CoV-2 specific Coronavac, BNT162b2-BioNTech/Pfizer and mRNA-1273-Moderna vaccines (Scarabel, Guardascione, Dal Bo, & Toffoli, 2021).

Coronaviruses are a family of viruses that cause a variety of clinical outcomes from the common cold to severe acute respiratory syndrome (SARS) (D. Wang et al., 2020; Wu et al., 2020). Common symptoms of infection are respiratory symptoms, fever, cough, and dyspnoea. In more severe cases, pneumonia, acute respiratory distress syndrome (ARDS), multiple organ failure could be seen, which could end up with mortality (Cucinotta & Vanelli, 2020).

The cells and various molecules of the innate and adaptive immune systems become involved in the antiviral response (Hosseini et al., 2020). Several recent studies of SARS-CoV-2 have shown significant

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changes in both the innate and adaptive immune system. This immune disorder is characterized by lymphocytopenia and modulation of the total neutrophil count, and appears to be directly correlated with disease severity and mortality (Huang et al., 2020). In severe forms, there was a significant decrease in CD4<sup>+</sup> T, CD8<sup>+</sup> T, B cells, natural killer (NK) cells, monocytes, eosinophils, and basophils (Chen & John Wherry, 2020).

Since the last few decades, it is well known that live attenuated viruses simulate natural infection and are highly immunogenic and effective in induction of both humoral and cellular immune responses. Recent investigations have determined that the measles, mumps, and rubella (MMR) vaccine may protect against or reduce the severity of infection with the 2019 coronavirus (COVID-19). Interestingly, studies suggest that the MMR vaccine provides protection against other infections through trained immunity for up to 1 year and it may be a promising candidate for preclinical research and randomized clinical trials to combat (Pawlowski et al., 2021). A quasi-trial study reported the positive effect of the MMR vaccine on reducing the severity of COVID-19 symptoms among those who received it (Gold et al., 2020; Taheri Soodejani, Basti, Tabatabaei, & Rajabkhan, 2021).

C-Vx was developed by Hamida Pharma-USA together with Miracle Labs, Türkiye. Preliminary studies have started for a supportive treatment to cancer patients (unpublished data). However, with the outbreak of COVID-19 in the world recently, the scientific team made additional changes in the formula, and the formula is granted (patent number: 17/497,295). C-Vx is currently presented as an active vaccination and/or immunomodulatory product. A measles viral (MV) vector was part of the original C-Vx formula because of its strong oncolytic properties. The MV selectively replicates in and kills cancer cells and activates anti-tumor immune responses on use as a treatment. In addition to the MV vector in the formula, parts of Mumps and Rubella viruses were included to enhance the effect of C-Vx against SARS-CoV-2.

Regardless of the variant, the formulation of C-Vx is claimed to provide effective concomitant therapy against active COVID-19 infection while also being a vaccine against COVID-19. C-Vx might be used as an immunomodulatory agent and is believed to stimulate both innate and adaptive arms of immunity. It appears that the passive immune elements in C-Vx might destroy the virus and block the active cellular entry of SARS-CoV-2, both superficially and internally. The immunomodulatory effects of C-Vx on the human immune system, specifically on the innate and adaptive immunity against SARS-CoV-2, appear to be an interesting area of investigation. Its approach seems to be unique and would allow the immune system to react positively to external aggression, specifically on the innate and adaptive immune responses against SARS-CoV-2. This study aimed to investigate, *in vitro* effect of C-Vx in human peripheral blood mononuclear cells, and preliminary *in vivo* dose-response and evaluation of acute toxic effects of C-Vx in rats.

## MATERIAL AND METHODS

### Subjects

Healthy subjects (n=5) (3 female/2 male, age-mean: 44.6 - min. - max.: 32-56) were enrolled in the study, after signing the

“Informed Voluntary Consent” forms. Inclusion criteria were; to be over the age of 18, to accept the voluntary consent, not to have any systemic and neurological accompanying diseases, not to have a history of cancer, no administration of immunosuppressive drugs and not having an infectious disease in the last 3 months. The study protocol was approved by the Local Clinical Ethical Committee of Istanbul University (2020-1576).

### Cell Preparation

Peripheral blood mononuclear cells (PBMCs) were isolated with Ficoll-Hypaque (Sigma Chem. Co., St. Louis, MO, USA) density gradient centrifugation from heparinized blood samples. PBMCs were suspended in RPMI 1640 medium (Gibco, Life Technologies, UK) supplemented with 10% heat-inactivated fetal calf serum (FCS), penicillin (100 U/ml), streptomycin (100 mg/ml) and gentamicin (50 mg/ml) (all supplements purchased from Sigma Chem. Co., St. Louis, MO).

### Apoptotic Index of C-Vx and Dose Determination

To evaluate the non-toxic concentration of C-Vx, PBMC from one healthy subject is isolated and cultured at different concentrations for 24, 48 and 72 hours. Annexin V-Apoptosis Detection Kit I (Biolegend, San Jose, CA, USA) was used for the determination of apoptotic index by flow cytometry. All cultures were maintained at 37°C, 5% CO<sub>2</sub> with 100% humidity containing 1/50 C-Vx, 1/100 C-Vx, 1/250 C-Vx and 2 µg/ml phytohemagglutinin (PHA) and unstimulated (US) condition. Annexin V-FITC and propidium iodide (PI) (Biolegend, San Jose, CA, USA) were added then incubated, percentages of dead cells were analyzed in NovoCyte flow cytometry (Agilent Technologies, USA). Cells negative for PI and Annexin V were evaluated as viable, cells stained only with Annexin V were evaluated as early phase apoptosis, whereas cells stained with Annexin V and PI were considered to be late apoptotic PBMCs. Data analysis was performed using FlowJo™10.2 (Tree Star Inc., USA) analysis program.

### CD107a Degranulation Assay, Perforin and Granzyme B Expression

PBMCs were incubated with anti-human CD107a-APC with the absence and existence of K562 target cells at an effector/ target (E: T) ratio of 10:1 in the presence of C-Vx (1/250) for 5h at 37°C whereas medium alone served as unstimulated (US) control. After the culture, cells were stained with anti-human CD56-BV711, anti-human CD16-BV570, anti-human CD3-BV785 and anti-human CD8-FITC (Biolegend, San Jose, CA, USA) monoclonal antibodies. Samples were fixed and permeabilized according to manufacturer directions (Cytotfix & Cytoperm Kit, Biolegend, San Jose, CA, USA), and then stained with anti-human Perforin-PerCp-Cy5.5 and anti-human Granzyme B-Alexa Fluor700 (Biolegend, San Jose, CA, USA) monoclonal antibodies. Stained cells were fixed with 1% paraformaldehyde and analyzed by NovoCyte flow cytometry (Agilent Technologies, USA).

### CFSE-Based Lymphocyte Proliferation Analysis

Purified PBMCs were labelled with CFSE (Cell Trace, Invitrogen, USA) at 5 µM final concentration by incubation for 10 minutes, at +4°C and in the dark. CFSE-labelled PBMCs (1x10<sup>6</sup> cells/

ml) were cultured for 120 hours at 37°C in 5% CO<sub>2</sub> incubator with the absence or existence of 1/250 C-Vx and 2 µg/ml PHA (PHA, Thermo Fisher, USA), or their combinations. Following incubation, PBMCs were stained with anti-human-CD3-BV785, -CD4-PE-Cy7, -CD8-APC-Cy7, -CD56-BV711 and anti-human-CD16-BV570 monoclonal antibodies (Biolegend, San Jose, CA, USA). Proliferation capacities of total CD3<sup>+</sup> T, CD4<sup>+</sup> T, CD8<sup>+</sup> T and CD3<sup>+</sup>CD56<sup>+</sup> NK cells were analyzed with NovoCyte flow cytometry (Agilent Technologies, USA). Proliferation values were expressed as percentage of proliferating cells.

### Animals and Synchronisation

Adult female Wistar albino rats (weight 230-255 g; 12-14 weeks of age; purchased from Animal Facility of Laboratory Animals Science Department of Istanbul University Aziz Sancar DETAE. Adult female Wistar albino rats were included in the study to investigate the toxic effects of the C-Vx molecule. The *in vivo* studies in rats were approved by the Local Ethics Committee of Experimental Animals of Istanbul University (10/11/2020- 2020/28).

### Administration of C-Vx and Plasma/Tissue Sampling

C-Vx was performed in 3 doses four hours after the light onset (i.e., HALO 4). The product (i.e. active substances+vehicle+ adjuvant) was administered to 3 female Wistar rats in 0.1 (Dose-1), 0.25 (Dose-2) and 0.5 (Dose-3) ml each subcutaneously (s.c.) in a single dose. Isotonic saline was administered to control animals. The total animals were 12 and rats were followed up to day 14. Following the light anesthesia procedure with sevoflurane, 1 ml of blood was collected from the orbital venous plexus on day 5 (0.5 ml for hematological and 0.5 ml for biochemical analyses). Hematological and biochemical parameters were investigated (Bezmialem Vakıf University, Laboratory of Experimental Animals, Istanbul, Turkey). On the 14th day following the administration, the rats were sacrificed under anesthesia and 6 ml of blood was collected from the cardiac puncture; liver and kidney tissues were removed for histopathological examination and stored properly. The dose specified in such human immunomodulatory products or vaccines is given on a volume basis and administered as 0.5 ml for rabbits (Green MD, 2016). From this point of view, the highest volume of application was 0.5 ml (Dose-3) and smaller doses of C-Vx, i.e. 0.25 ml (Dose-2) and 0.1 ml volumes (Dose-1) were applied.

### Necropsy

Rats were sacrificed by cervical dislocation following anesthesia with sevoflurane inhalation. Organs were excised, fixed with 10% neutral buffered formalin for 48 hours at room temperature, dehydrated by passing from graded alcohol series and embedded in paraffine blocks. Sections were cut at 4 micrometers thick with microtome (Leica, Germany) and stained with hematoxylin & eosin, PAS and Masson trichrome stains, and examined with light microscope (Olympus BX41, Japan).

### Histological Evaluations

Tissue samples taken from kidneys and liver were fixed in 10% neutral formalin and processed for embedding in

paraffin blocks. Sections cut in 4 µm thick were stained with Hematoxylin & Eosin, Periodic Acid Schiff reactant and Masson trichrome stains for kidney and liver.

### Statistical Analyses

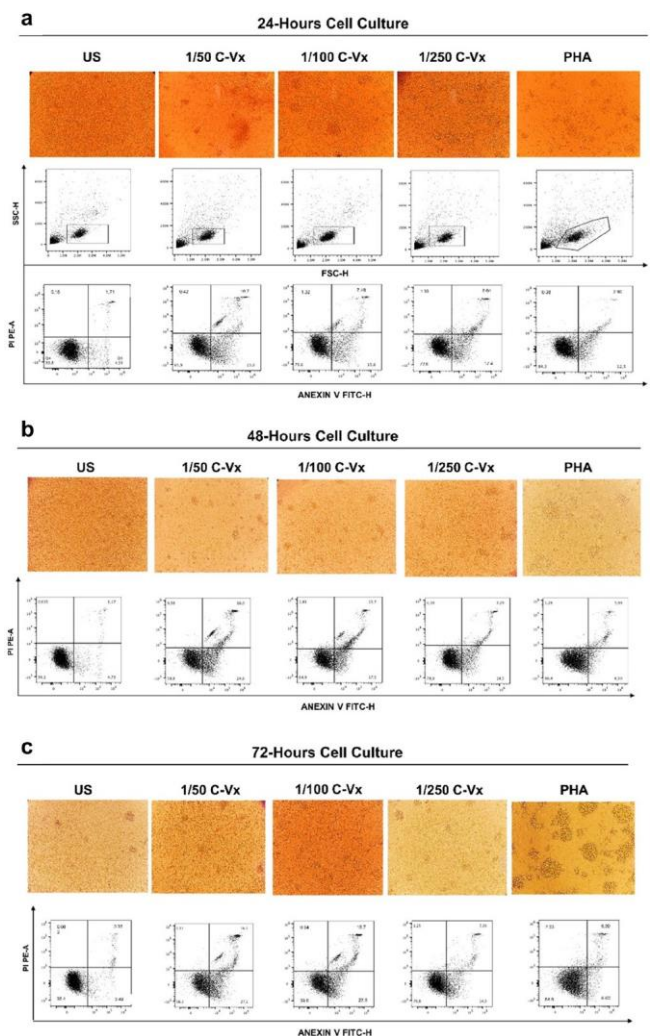
One-way ANOVA and Two-way ANOVA with post-hoc Tukey's test were used for comparison of multiple groups. Wilcoxon test was used for comparison of paired series. Biochemical and hematological parameters were evaluated with Two-way ANOVA, "Days" and "Treatment" factors were compared. A p-value less than 0.05 was accepted statistically significant. Graphpad Prism 8 Software (San Diego, CA) was used for statistical analyses as well as construction of graphics.

## RESULTS

### Determination of Apoptosis After C-Vx Stimulation

To obtain the optimal concentration of C-Vx substance, PBMC of one healthy subject were analysed at 24 (Figure 1a), 48 (Figure 1b) and 72 hours (Figure 1c).

Cell viabilities were found to be > 95% at 3 different culture time points without any stimulation. When the C-Vx dose



**Fig. 1:** Effect of C-Vx substance in PBMCs cultures. Images shown are healthy subject's microscope images and dot plots after C-Vx stimulation (1/50 C-Vx, 1/100 C-Vx, 1/250 C-Vx and PHA) in 24-hours (A), 48-hours (B), and 72-hours (C) cell cultures. FSC: Forward Scatter, PHA: phytohemagglutinin, SSC: Side Scatter, US: Unstimulated.

response was examined, the best viability response was obtained at 1/250 C-Vx concentration at 24, 48 and 72 hours of cell cultures (Figure 2a). C-Vx 1/250 concentration led to diminished apoptosis in both early and late stages when compared with other stimulation concentrations of C-Vx (Figure 2b). It was found that 1/50 and 1/100 concentrations reduced the cell viability < 80% and increased the late stage of apoptosis (Figure 2c). According to our findings, 1/250 C-Vx concentration and 72 hours culturing time were applied for following experiments.

### Cytotoxic Capacity of NK and CD8+ T Cells

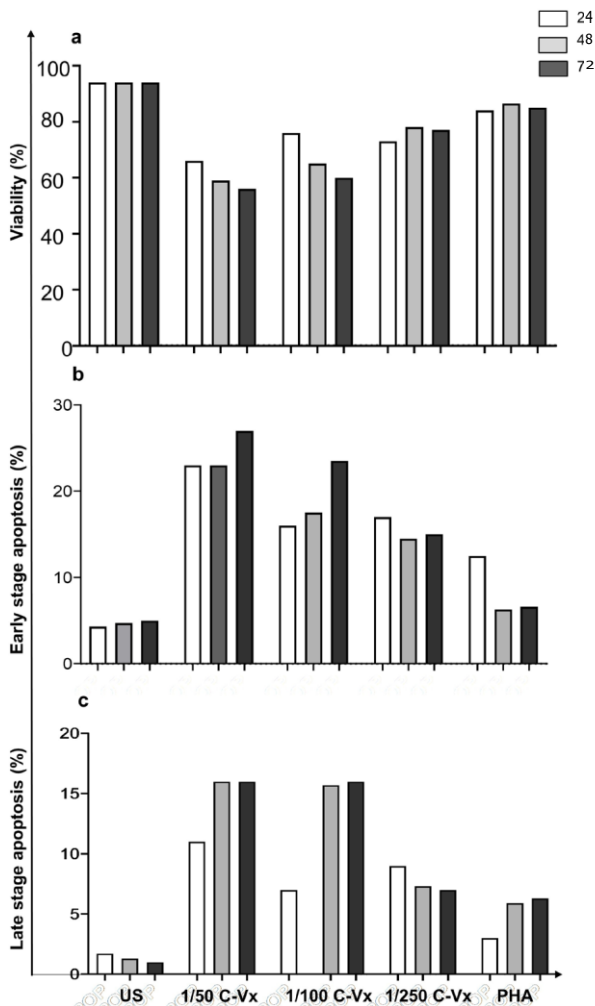
K562 erythromyeloid leukemia cell line was used for evaluation of cytotoxic capacities of CD3-CD16+CD56+ total NK and CD8+ T cells in healthy subjects (n=5). When the effect of C-Vx substance was evaluated on CD3-CD16+CD56+ NK cells, no significant differences were found for the perforin and granzyme expressions. However increased CD107a expression was observed both with the absence and existence of C-Vx stimulation, in comparison with the unstimulated control (US) (p=0.0079). This results supports the contribution of C-Vx in innate immunity by increasing NK cytotoxic capacity. Similar to

NK cells, CD8+ T cells also did not show any significant difference for perforin and granzyme expressions by C-Vx stimulation. In contrast to perforin and granzyme B, degranulation capacity of CD8+ T cells were significantly increased with K562 stimulation (p=0.0317). Even, there was an almost 2 fold increase by C-Vx stimulation, though with no significance (Figure 3). In general, perforin and granzyme release by NK and CD8+ T cells were diminished either with the absence or existence of C-Vx stimulation.

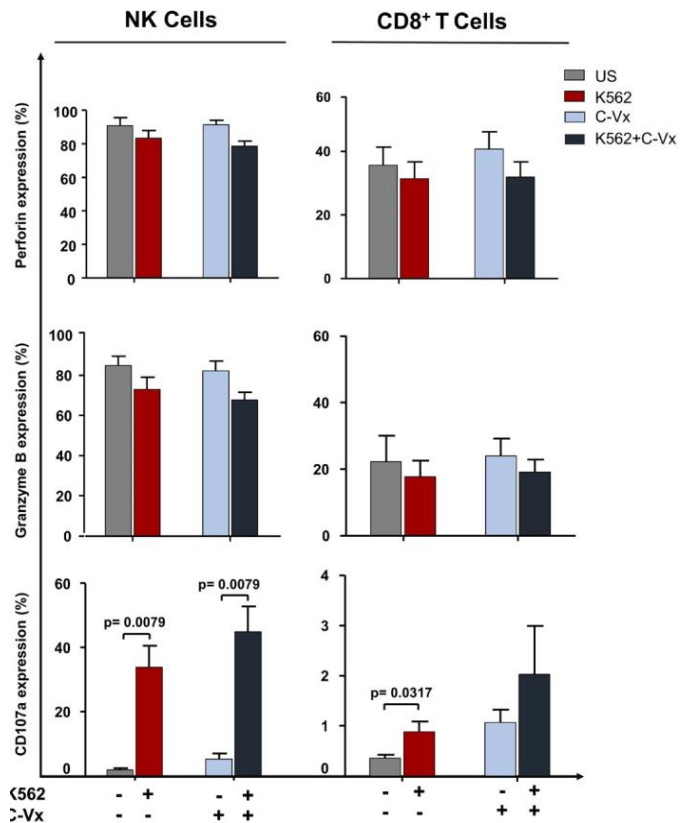
### C-Vx Increases the Proliferative Response of CD3+,CD4+, CD8+ T and CD3-CD16+CD56+ NK Cells

To evaluate the proliferative effects of C-Vx on lymphocytes and NK cells, PBMCs were labelled with CFSE and were cultured with the absence or existence of PHA, C-Vx and their combination. Following stimulation by C-Vx, proliferative responses of PBMCs and CD4+ T cells were increased significantly. (Figure 4a, c). In CD3+ T cells, proliferation ratio also was increased but with no statistical difference (p=0.06) (Figure 4b). Our results revealed that, C-Vx significantly reduced the PHA-induced proliferative responses in CD3+, and CD4+ T cells (p<0.05) (Figure 4a-c). However, the addition of C-Vx agent in CD8+ T cells significantly up-regulated PHA-induced proliferation (p<0.05) (Figure 4d).

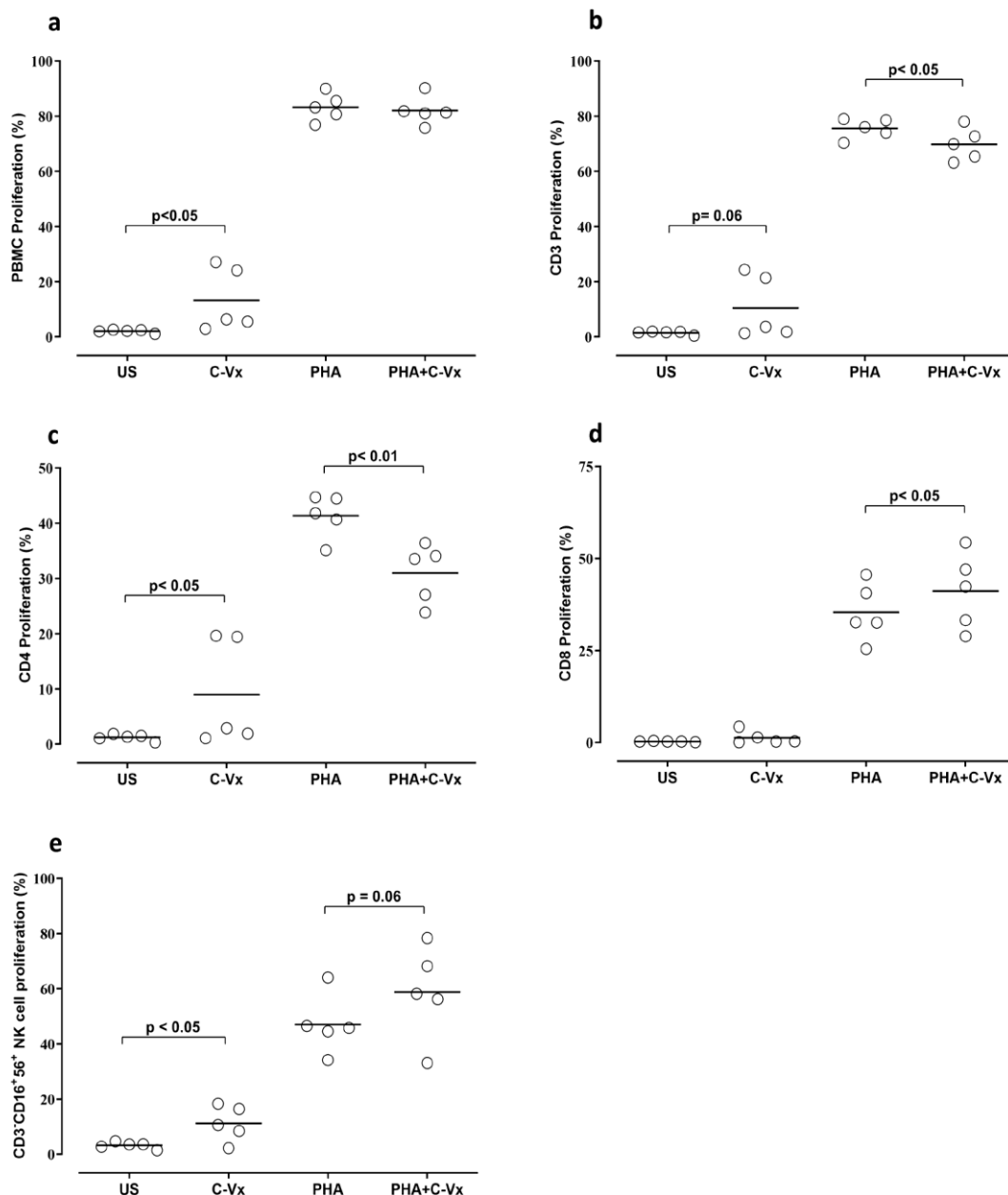
The proliferation of CD3-CD16+56+ NK cells with the absence and existence of C-Vx, PHA and their combination was investigated. It was found that CD3-16+56+ NK cell proliferation response was significantly upregulated by the C-Vx agent (p<0.05) (Figure 4e).



**Fig. 2:** Viability and apoptotic rate of PBMCs after C-Vx stimulation. Viable, early and late apoptotic PBMCs were evaluated by flow cytometry using Annexin V and propidium iodide after presence of 1/50 C-Vx, 1/100 C-Vx, 1/250 C-Vx dose and PHA stimulus for 24, 48 and 72 hours. US: Unstimulated, PBMC: Peripheral blood mononuclear cells isolated, PHA: phytohemagglutinin.



**Fig. 3:** Cytotoxic capacity of NK and CD8+ T cells. Perforin, granzyme B and CD107a expression levels of CD3-CD16+CD56+ NK and CD8+T cells were analysed with the absence or existence of C-Vx stimulation. US: unstimulated.



**Fig 4:** Proliferative responses of total PBMCs, CD3<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>+</sup> and NK cells in response to C-Vx stimulation. PBMC of healthy subjects were stained with CFSE and were cultured with the absence and existence of C-Vx, PHA and their combinations for 120 hours. Proliferative responses of cells were evaluated by flow cytometry, (e) Proliferative responses of CD3<sup>+</sup>CD16<sup>+</sup>56<sup>+</sup> NK cells in response to PHA and PHA+ adding of 1/250 concentration of C-Vx stimulation. Healthy controls PBMCs were stained with CFSE and were cultured with the absence and addition of C-Vx and PHA for 120 hours. Proliferative responses of cells were evaluated by flow cytometry. PBMC: Peripheral blood mononuclear cells isolated, PHA: phytohemagglutinin.

PHA-stimulated CD3<sup>+</sup>CD16<sup>+</sup>56<sup>+</sup> NK cell proliferation was further increased with C-Vx, but with no statistical significance (p=0.06) (Figure 4e).

As a result of *in vitro* studies, C-Vx seems to have an immunomodulatory effect. In order to further investigate *in vivo* effects of C-Vx and to research the possible toxic influences of C-Vx in animals, certain concentrations of C-Vx substance were given to animals and evaluated.

#### Effect of C-Vx on Body Temperature and Body Weight in Rats

After administration of C-Vx to healthy female Wistar albino rats, on day 1, 7 and 14, rats were weighed and body temperatures were measured with the rectal probe (Testo-108, Thermometer, Harvard-App.USA). There were no change in body

temperatures between days and between groups after C-Vx administration (Figure 5a). There was a minimal change in weight in the groups 0.1 ml (Dose-1) and 0.25 ml (Dose-2). In the Dose-1 group, body weight decreased by 4.4% on day 7 compared to day 1. On the other hand in the Dose-2 group, body weight increased slightly on day 14 compared to day 7. Despite these minor changes, there is no body weight difference between the C-Vx administered groups and the control group (Figure 5b).

#### Evaluation of Biochemical and Hematological Parameters After C-Vx Administration in Rats

On day 5th and 14th blood were drawn from the rats. Biochemical and hematological parameters were measured (Table 1). Although there were no statistical changes in BUN, CREA, ALT, AST, ALP biochemical parameters, serum GLU was slightly decreased in every group on day 14 compared to day 5, it was

Fatma B. OKTELIK, et al.: Evaluation of the Immunomodulatory Effects of C-Vx on the Innate and Adaptive System: Preliminary Results only statistically significant in Dose-2 group ( $p < 0.05$ ) (Table 1). the hematological parameters have slightly changed (Table 2). After administration of C-Vx to healthy female rats, some of Total WBC counts were increased in C-Vx groups compared

**Table 1:** Biochemical findings of control and C-Vx administered healthy female rats.

Biochemical parameters	Control		Dose-1		Dose-2		Dose-3	
	Day 5	Day 14	Day 5	Day 14	Day 5	Day 14	Day 5	Day 14
GLU (mg/dL)	132 ± 2	112 ± 8	140 ± 8	120 ± 7	147 ± 2*	121 ± 10	140 ± 4	125 ± 7
BUN (mg/dL)	20.0 ± 0.0	17.7 ± 2.4	17.7 ± 1.4	17.3 ± 1.8	17.7 ± 0.9	17.3 ± 0.9	19.0 ± 1.2	18.3 ± 1.8
CREA (mg/dL)	0.6 ± 0.1	0.8 ± 0.2	0.7 ± 0.0	0.67 ± 0.1	0.6 ± 0.1	0.7 ± 0.1	0.8 ± 0.1	0.67 ± 0.1
TBIL (mg/dL)	0.1 ± 0.0	0.13 ± 0.03	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.13 ± 0.03	0.2 ± 0.06
ALB (g/dL)	3.5 ± 0.2	3.8 ± 0.2	3.3 ± 0.1	3.7 ± 0.1	3.5 ± 0.1	3.4 ± 0.1	3.3 ± 0.2	3.4 ± 0.1
AST (U/L)	69 ± 7.7	164 ± 74	66 ± 10	63 ± 5	73 ± 8	72 ± 3	85 ± 14	61 ± 9
ALT (U/L)	56 ± 4	75 ± 20	54 ± 2	54 ± 8	64 ± 16	54 ± 7	54 ± 4	62 ± 8
ALP (U/L)	120 ± 22	108 ± 21	129 ± 10	137 ± 20	139 ± 34	124 ± 30	139 ± 38	125 ± 36

GLU: Glucose; BUN: Blood urea nitrogen; CREA: Creatinine; TBIL: Total bilirubin; ALB: Albumin AST: Aspartate aminotransferase; ALT: Alanine transaminase; ALP: Alkaline phosphatase. Biochemical parameters evaluated with Two-way ANOVA with Tukey post-hoc test. \* $p < 0.05$ ; day 5 vs day 14.

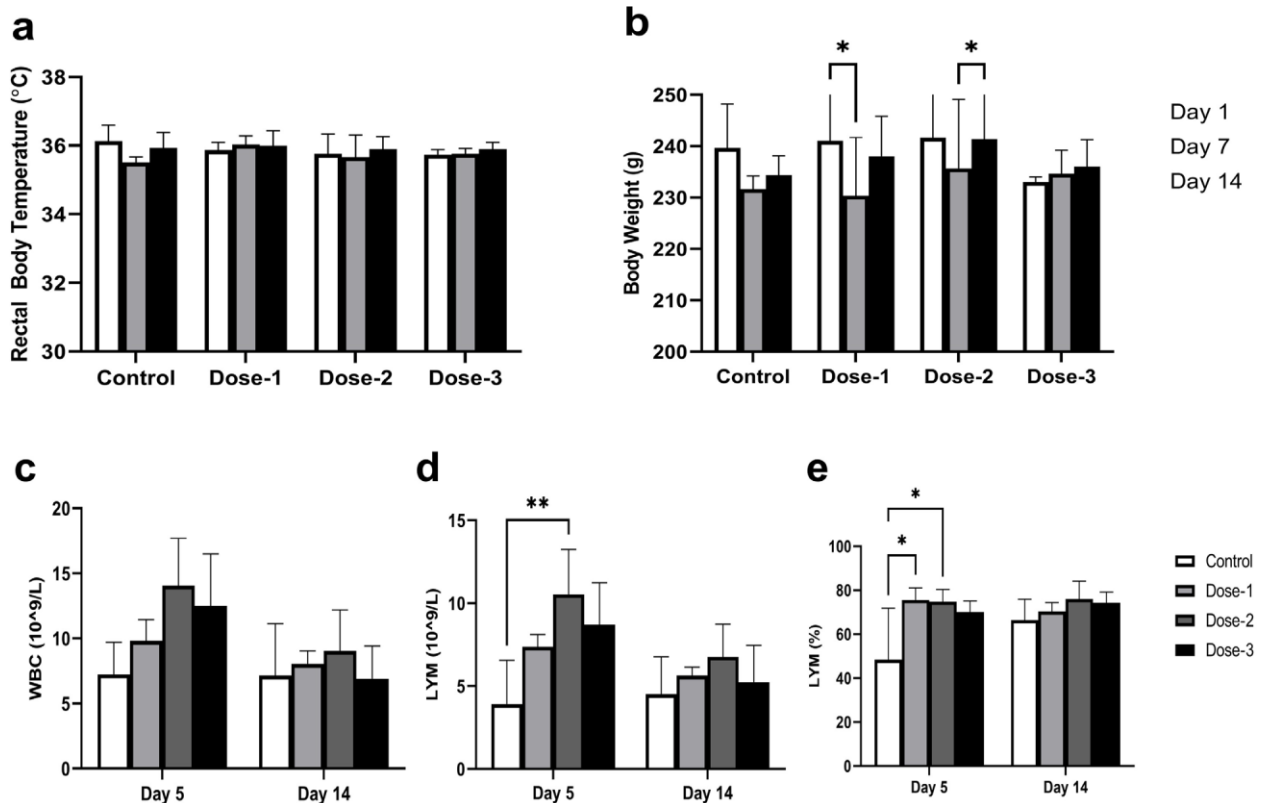
**Table 2:** Hematological findings of control and C -Vx administered healthy female rats.

Hematological parameters	Control		Dose-1		Dose-2		Dose-3	
	Day 5	Day 14	Day 5	Day 14	Day 5	Day 14	Day 5	Day 14
WBC ( $10^9/L$ )	7.2 ± 1.4	7.1 ± 2.3	9.8 ± 0.9	8.0 ± 0.6	14.1 ± 2.1*	9.0 ± 1.8	12.5 ± 2.3	6.9 ± 1.4
LYM ( $10^9/L$ )	3.9 ± 1.5	4.5 ± 1.3	7.4 ± 0.4	5.6 ± 0.3	10.5 ± 1.6	6.8 ± 1.1	8.7 ± 1.5	5.2 ± 1.3
MONO ( $10^9/L$ )	0.43 ± 0.18	0.50 ± 0.35	0.17 ± 0.07	0.73 ± 0.33	1.30 ± 0.67	0.63 ± 0.28	1.03 ± 0.5	0.23 ± 0.19
GRA ( $10^9/L$ )	2.9 ± 0.25	2.1 ± 0.9	2.3 ± 0.6	1.6 ± 0.12	2.3 ± 0.14	1.7 ± 0.5	2.8 ± 0.47	1.5 ± 0.26
LY (%)	48 ± 13	66 ± 5	76 ± 3*	70 ± 2	75 ± 3*	76 ± 5	70 ± 3	74 ± 3
MONO (%)	6.7 ± 3.1	6.9 ± 3.6	1.6 ± 1.0	9.1 ± 3.7	8.7 ± 4.1	6.3 ± 2.1	7.5 ± 3.5	3.8 ± 3.2
GRA (%)	45 ± 11	27 ± 6	23 ± 4*	21 ± 1	17 ± 1*	18 ± 3	23 ± 1*	22 ± 3
RBC ( $10^{12}/L$ )	8.2 ± 0.07	7.4 ± 1.1	7.9 ± 0.13	7.8 ± 0.24	7.8 ± 0.3	7.2 ± 0.3	9.0 ± 0.2	7.6 ± 0.06
HGB (g/dL)	14.9 ± 0.3	14.0 ± 2.0	14.2 ± 0.5	14.4 ± 0.3	14.6 ± 0.3	14.0 ± 0.5	13.4 ± 1.1	14.0 ± 0.1
HCT (%)	42.1 ± 0.8	39.7 ± 4.7	39.5 ± 1.5	40.1 ± 1.7	41.0 ± 0.1	39.1 ± 0.4	45.3 ± 1.0#	38.5 ± 1.1
MCV (fL)	51.3 ± 0.9	53.7 ± 1.8	50.0 ± 1.2	52.7 ± 0.7	52.3 ± 1.9	54.0 ± 1.5	50.3 ± 0.7	52.0 ± 0.6
MHC (pg)	18.1 ± 0.3	18.8 ± 0.2	18.0 ± 0.3	18.6 ± 0.3	18.7 ± 0.5	19.3 ± 0.3	14.8 ± 0.9*	18.5 ± 0.1
MHCH (g/dL)	35.4 ± 0.1	35.2 ± 0.7	36.0 ± 0.1	35.4 ± 0.9	35.6 ± 0.2	35.6 ± 0.9	29.5 ± 2.1*	35.6 ± 0.3
RDWc (%)	17.0 ± 0.2	17.3 ± 0.4	16.8 ± 0.2	17.4 ± 0.5	16.8 ± 0.1	17.2 ± 0.2	17.8 ± 0.3	17.3 ± 0.2
PLT (g/dL)	673 ± 80	860 ± 144	665 ± 122	814 ± 48	796 ± 44	1039 ± 206	763 ± 138	990 ± 35
PCT (%)	0.50 ± 0.06	0.63 ± 0.12	0.47 ± 0.09	0.53 ± 0.03	0.60 ± 0.0	0.73 ± 0.19	0.57 ± 0.09	0.67 ± 0.03

MPV (fL)	7.23 ± 0.18	7.00 ± 0.15	7.13 ± 0.27	6.77 ± 0.12	7.40 ± 0.36	7.07 ± 0.33	7.03 ± 0.13	6.77 ± 0.09
PDWc (%)	34.3 ± 1.4	34.4 ± 0.6	35.5 ± 0.4	32.8 ± 0.3	36.0 ± 1.0	34.3 ± 1.4	35.8 ± 0.8	32.8 ± 0.8

WBC: white blood cells; LYM: lymphocyte; MONO: monocyte; GRA: Granulocyte; LY%: Percentage of lymphocytes; MONO%: percentage of monocytes; GR%: Percentage of granulocytes. RBC: Red blood cells; HGB: Hemoglobin; HCT: Hematocrit; MCV: mean corpuscular volume; MCH: average corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration;

RDWc; red cell distribution width, PLT: Platelet; PCT: procalcitonin; MPV: mean platelet volume; PDWc Platelet distribution width. Hematological parameters evaluated with Two-way ANOVA with Tukey post-hoc test. \*p < 0.05; day 5 vs day 14, #p < 0.05; C-Vx treated vs control.



**Fig: 5:** Rectal body temperatures (a) and body weights (b) of control and C-Vx administered rats on day 1, 7 and 14. Data are presented as mean ± SD (n=3). Hematological parameters upon C-Vx administration in healthy female rats. WBC (c), LYM (d) and LYM% (e) of control and C-Vx administered rats on day 5 and 14. Data are presented as mean ± SD (n=3). Two-way ANOVA with Tukey post-hoc test, \*p<0.05, \*\* p<0.01.

to control group at day 5, however there was not any significant difference when Tukey post-hoc test was applied. Total LYM counts were also increased compared to control at day 5, it is statistically significant in the Dose-2 group (p<0.05). Total LYM counts were also decreased at day 14 in C-Vx groups. At day 5, LYM% was different between controls and C-Vx groups, it was increased significantly in the Dose-1 and Dose-2 groups (p<0.05) (Figure 5).

**Necroscopy Findings**

On the 14th day rats were sacrificed and the necropsy findings were evaluated macroscopically. Morphological changes were not detected in all groups. Abscess, exudate, lesion, granuloma or similar malignant formations were not observed in the cadavers of the rats constituting the control and experimental groups, and no bacterial, fungal or viral infection findings were noted.

**Histologic Evaluation**

The rats were sacrificed on 14th for evaluation of any histopathological change in liver and kidneys by light microscopy in this preliminary study. Any findings related with injury in tubular epithelium were not observed in C-Vx treated groups. Also, no serious impact of C-Vx were detected on glomeruli, except cellular proliferation and collapse in some extent in some glomeruli in one of the three rats in Dose-2 group (Figure 6a). On the other hand, local congestion and sparse thrombi in some of the hepatic lobules were observed in one of three rats in both Dose-1 (0.1 mL/day) and Dose-2 (0.25 mL/day) groups (Figure 6b).

**DISCUSSION**

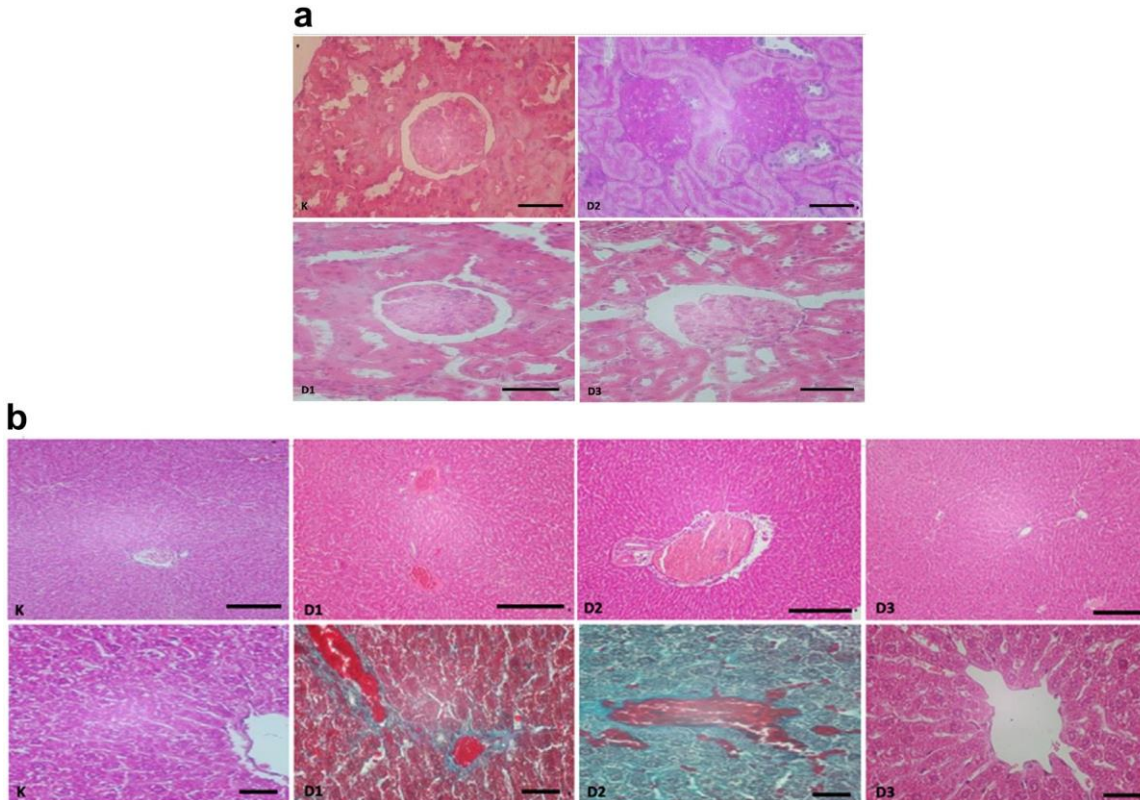
Genetic variants of SARS-CoV-2 have emerged and are

circulating all over the world, despite the different vaccine regimens available. An effective immune response to SARS-CoV-2 infection consists of two major components, rapid innate immunity and specific adaptive immunity and should be effective regardless of the variant.

The immunomodulatory effects of C-Vx on the human immune system, specifically on the innate and adaptive immune system against SARS-CoV-2, appear to be an interesting area of investigation. C-Vx is an agent developed for anti-tumor responses. However, considering the similarity of anti-viral defense mechanisms to anti-tumoral responses and the helplessness in drug development against SARS-CoV-2, various changes were made in the formulation of C-Vx and its use for COVID-19 disease was evaluated.

In our study, to observe the effects of C-Vx, PBMCs obtained from healthy individuals were cultured with C-Vx at certain time intervals. After stimulation with C-Vx, the viability and apoptotic rates of cultured cells were evaluated with AnnexinV and Propidium iodide. The best effect of C-Vx was observed at 1/250 dose at 72 hours and so that, the study was continued under these conditions.

NK cells are one of the initial responders against infections and they could kill target cells by cytotoxic degranulation and production of various cytokines (van Eeden, Khan, Osman, & Cohen Tervaert, 2020). Because of the cytotoxic functions of NK cells, their roles in the pathogenesis of COVID-19 have gained importance.



**Fig. 6:** (a) Light microscopic images of kidney glomeruli in control (K) and dose (D) groups. Collapsing appearance and mesangial increase in some glomeruli are seen in one of the three rats in 0.25 ml C-Vx administered (D2) group. (Stains: H&E for (K) and PAS for Dose (D) groups; Bars=150  $\mu$ m). (b) light microscopy of liver sections in control (K) and dose (D) groups. Congestion and thrombi in both hepatic and portal veins in some hepatic lobules are prominent in one of three rats in both Dose-1 (0.1 mL/day) and Dose-2 (0.25 mL/day) groups. (Stains: H&E for (K), PAS and Masson trichrome for Dose-1 (D1) and Dose-2, and PAS for Dose-3 (D-3) groups; Bars=200  $\mu$ m and 50  $\mu$ m for above and below pictures of low and high magnifications, respectively).

Studies have found that individuals infected with SARS-CoV-2 have reduced numbers of NK and CD8<sup>+</sup> T cells, and these cells exhibit a functionally depleted phenotype (Diao et al., 2020; F. Wang et al., 2020). The study revealed that the numbers and cytolytic activity of NK cells were important features of severe COVID-19 (Osman et al., 2020).

CD107a is a marker that correlates with the activity of NK cells and CD8<sup>+</sup> T cells. In our study, degranulation, and proliferation capacities of NK and CD8<sup>+</sup> T cells were evaluated after stimulation with C-Vx. Although an increase in CD107a is observed in C-Vx stimulated NK cells, but not in CD8<sup>+</sup> T cells, an increase in innate cytotoxic responses could be proposed.

When we evaluate the proliferation capacity of immune cells, C-Vx was observed to cause increased proliferative responses in

both CD4<sup>+</sup> T and NK cells. CD4<sup>+</sup> T cells regulate immune responses by their cytokine productions, and this regulation could result in potentiation of natural responses and targeting of B cell responses to the agent. Characterization of these responses require more detailed studies. PHA was used as a potent mitogen in our study, and the effect of C-Vx on PHA-stimulated proliferative responses was investigated. In CD4<sup>+</sup> T cells, PHA-stimulated proliferation was stabilized by C-Vx, while PHA-stimulated CD8<sup>+</sup> T and NK cell proliferation responses were enhanced by C-Vx effect. This agent is likely to cause an increase in immune responses.

Biochemical data obtained from Wistar albino female rats used in our preliminary study do not indicate any toxicity or any inflammation. On the 5th day after C-Vx application, while



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biochemical data in animals in the control and experimental groups were found to be at normal levels specific to strain (Harb et al., 2021), a significant increase in Aspartate aminotransferase (AST) enzyme activation was observed in the blood samples of the control group rats on the 14th day of the termination of the study. However, no similar findings were found in the C-Vx-administered groups.

Because of the absence of leukocytosis in the blood samples of the rats in the control group, either in the 5<sup>th</sup> day or 14<sup>th</sup> day as well as no body weight loss in rats, no increment in rectal temperature and hematological data shows the absence of general toxicity, infection or inflammation. Macroscopic evaluations in necropsy records also supported the above findings. It is claimed that AST levels in blood samples taken from the control group on the 14th day may have increased due to hemolysis.

In addition to biochemical and hematological data, histopathological results may also be determinant in the general evaluation. Since this product is administered to an experimental animal for the first time, possible serious adverse reactions or death after administration are major issues to be taken into consideration (Brennan & Dougan, 2005), so the number of animals has been limited in this preliminary study. Some of the morphological changes may be related with individual condition and response of animals or technical artifacts, depending on tissue sampling or processing. So, discrepancies in morphological findings in- and between groups should be interpreted cautiously, also by

correlating biochemical and functional data, due to the limited number of subjects in both control and experiment groups.

## CONCLUSIONS

In the light of these findings, it could be hypothesized that C-Vx regulates cytokine responses while supporting cytotoxic responses, but more detailed studies are needed for a clear view. C-Vx has immune modulatory effects, and it could be argued that an increase in non-specific cytotoxic responses possibly through NK cells could be the case.

**Ethics Committee Approvals:** This study was approved by the Ethics Committee of the Istanbul University (No: 2020-1576) and *in vivo* studies in rats were approved by the Local Ethics Committee of Experimental Animals of Istanbul University (IUHADYEK- No: 2020/28).

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Data Acquisition- F.B.O./ D.O.C./ G.D./ A.O./ E.C.A./ N.A./ and U.C.K

Data Analysis/ Interpretation- F.B.O./ D.O.C./ G.D./ A.O./ N.A./ U.C.K./ E.C.A./ S.S./ A.C.

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