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Effects of infliximab against carbon tetrachloride-induced spleen toxicity in rats

I. BAHCECI[1], L. TUMKAYA[2], T. MERCANTEPE[2], H. YILMAZ[3], Y.E. IBIK[1], O.F. DURAN[1], N. ARSLAN[1]

[1]Department of Medical Microbiology, [2]Department of Histology and Embryology, Faculty of Medicine, Recep Tayyip Erdogan University, Rize, Turkey
[3]Department of Medical Services and Techniques, Vocational School of Health Services, Recep Tayyip Erdogan University, Rize, Turkey

Abstract. – OBJECTIVE: Carbon tetrachloride (CCl_4) is a non-polar molecule used in industry in grain curing, insect-killing and especially in the production of chlorofluorocarbons. It is estimated that an average of 70,000 industry workers in Europe are exposed to this toxic compound.

MATERIALS AND METHODS: Twenty-four male Sprague-Dawley rats were randomly allocated into four groups: control group (saline only, Group I), infliximab (INF) group (Group II), CCl_4 group (Group III) and CCl_4+INF group (Group IV).

RESULTS: While there was an increase in the numerical density of CD3, CD68, and CD200R positive T lymphocytes and macrophages in the CCl_4 administration group (p=0.000), this was not the case in the CCl_4+INF administration group (p=0.000).

CONCLUSIONS: TNF-α inhibitors have a protective effect against CCl_4-induced spleen toxicity/inflammation as seen by the reduction in CD3, CD68, CD200R positive T lymphocytes and macrophages.

Key Words:
Carbon tetrachloride, CD3, CD68, CD200R, Spleen.

Introduction

Carbon tetrachloride (CCl_4) is a non-polar molecule and is used in industry (e.g., paint, dry cleaning, petroleum chemical production), grain curing, pest culling, and especially in the production of chlorofluorocarbons[1,2]. Although its use is limited due to toxicity, an average of 70,000 sector workers in Europe are exposed to CCl_4[3]. CCL_4 is a carcinogenic compound and easily penetrates the skin and mucosal surfaces due to its hydrophobicity. CCl_4 exerts multi-organ toxicity, especially on the liver, lung, and spleen[4].

The spleen has vital functions in filtering aged erythrocytes, regulating immune responses, and B cell differentiation. Oxidative damage significantly affects the structure and functions of the spleen[5,6]. Previous research established that CCl_4-induced splenic toxicity is associated with hypertension[7], low glycose and hypoxanthine levels and elevated uracil levels in rats[8]. Moderate congestion, decrease in antioxidants and increase in oxidative stress were reported in rat spleens treated with CCl_4[9,10]. It has been shown that CCl_4 is metabolized to trichloromethyl (CCl_3) and trichloromethyl peroxyl radical (CCl_3O_2) by the cytochrome P450 enzyme systems[11]. These free radicals cause damage to proteins, lipids, and DNA by peroxidation[12] as indicated by an increase in the amount of malondialdehyde (MDA), an important marker of lipid peroxidation[12]. Triggering the release of inflammatory cytokines like tumor necrosis factor-alpha (TNF-α) and interleukin-Iβ is responsible for the multi-organ toxicity of CCl_4[4,13,14]. TNF-α is a cytokine with important functions in inflammation and fibrosis in the spleen. Infliximab (INF), which binds and neutralizes free and cell-surface TNF-α, has a potent anti-inflammatory effect and is used in the treatment of severe inflammatory diseases and rheumatoid arthritis, INF suppresses ROS production[15,16].

This study investigated the effectiveness of INF, an anti-TNF-α antibody, against CCl_4 – induced splenic toxicity in rats.

Materials and Methods

Twenty-four male Sprague-Dawley rats with a mean weight of 243±27 g and aged 3-4 months

Corresponding Author: Ilkay Bahceci, MD; e-mail: bahceciie@hotmail.com

were used in the study. Rats were supplied by the Recep Tayyip Erdoğan University (RTEU) Animal Care and Research Unit (Rize, Turkey). All animals received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health. The study was approved by the RTEU Animal Ethical Committee (Approval Date: 31.03.2020, Approval No.: 2020/13).

Animal Study

All injections were performed intraperitoneally. Rats were kept under optimum laboratory conditions (22±2^{0}C room temperature, 40% humidity, and 12-hour light/dark cycle) throughout the experiments. Rats were fed standard pellet chow (Bayramoglu, Erzurum, Turkey) and had free access to tap water. Rats were randomly numbered using a computer-programmed numerator. Only 1 ml saline was administered to the control group. Infliximab (Remicade, Schering-Plough Medical Products Trade A.S., Istanbul, Turkey) was administered at 100 mg/kg/day for 5 days to the INF administration group (Group II). A single dose of 2 mL/kg of CCl_4 was administered to the CCl_4 treatment group (Group III). The CCl_4+INF group (Group IV) first received INF 100 mg/kg/day for five days before CCl_4 administration. At the end of the experiment, all groups were terminally anesthetized using 50 mg/kg ketamine hydrochloride (Ketalar®, Pfizer Ilacları Ltd. Sti, Istanbul, Turkey) and a 10 mg/kg Xylazine HCl (Rompun®, Bayer Turk, Istanbul, Turkey), and spleen tissues were quickly removed and trimmed.

Drugs and Chemicals

Saline (0.9%, Ibrahim Ethem Ulugay Ilac Sanayi Turk A.S, Istanbul, Turkey). CCl_4 (Saint Louis, MO 63103, USA). Remicade (Schering-Plough Medical Products Trade A.S., Istanbul, Turkey). Ketamine hydrochloride (Ketalar®, Pfizer Ilacları Ltd. Sti, Istanbul, Turkey). Xylazine HCl (Rompun®, Bayer Turk, Istanbul, Turkey).

Histopathological Analysis

Spleen tissues were trimmed and fixed in a neutral 10% formalin solution (Sigma-Aldrich, Germany) for 36 hours. After the fixation phase, routine histological tissue processing steps were performed using the tissue processor (Shendon Citedal 2000, Thermo Fisher Scientific, Waltham, MA, USA). Tissues were dehydrated by exposure to increasing ethanol series (50%, 70%, 80%, 90%, 96%, 100%) prior to xylol (Merck, Darmstadt, Germany) treatment. Following mordanting, the tissues were embedded in soft paraffin (Merck GmbH, Darmstadt, Germany) for one hour and then hard paraffin (Merck GmbH, Darmstadt, Germany) for 8 hours. Finally, the tissue samples were removed from the tissue processor and blocked with hard paraffin (Merck GmbH, Darmstadt, Germany) before embedding into cassettes (Isolab GmbH, Germany) using a paraffin dispenser (Leica 1150, Leica Biosystems, Wetzlar, Germany). Tissue blocks were kept overnight, and 2-3 µm thick sections were taken and placed on positively charged slides (PatoLab, Instabul,Turkey).

Immunohistochemistry (IHC) Analysis

To determine T lymphocytes and macrophages in splenic tissue; anti-CD3 (rabbit polyclonal anti-CD3, Abcam, UK), anti-CD68 (rabbit polyclonal anti-CD68, Abcam, UK), anti-CD200R (mouse monoclonal anti-CD200R, Abcam, UK) primary antibodies and secondary antibodies compatible with them were incubated for 60 minutes using IHC stainer (Lecia Bond Max, Leica Biosystems, Australia). After the incubations, the sections were counterstained with Harris' hematoxylin (Merck GmbH, Darmstadt, Germany).

Stereological Analysis

Numerical densities of anti-CD3+, anti-CD68+, and anti-CD200R+ cells were calculated using the fractional probe on the StereoInvestigator 9.0 (MicroBrightField, Colchester, VT, USA) software system and cells were enumerated as described previously[17]. Positive cells in 35 randomly assigned areas of each section by the Stereo Investigator Program were determined[18].

Statistical Analysis

The data obtained from the stereological analyzes were evaluated on SPSS 22.0 (IBM Corp., Armonk, NY, USA) statistical program. The Shapiro-Wilk test, Q-Q plot, Skewness-Kurtosis values and Levene's tests were used for normalization. Nonparametric data were expressed as medians with the 25%-75% interquartile range (IQR). These data were compared between the groups using the Kruskal Wallis test followed by the Tamhane T2 test. A p-value ≤ 0.05 was considered statistically significant.

Table I. Immunohistochemical Analysis Results (median-25%-75% interquartile range).

Group	CD3 positive cell numerical density (mm^3)	CD68 positive cell numerical density (mm^3)	CD200R positive cell numerical density (mm^3)
Control	6 (5-7)	9 (8-10)	4 (3-5)
Sham	5 (4-6)	8 (6-9)	5 (4-6)
CCl_4	16 (14-18)[a]	26 (24-28)[a]	15 (14-17)[a]
CCl_4+INF	9 (8-11)[a,b]	16 (15-18)[a,b]	8 (5-9)[a,b]

[a]$p = 0.000$ compared to the Control group, [b]$p = 0.000$ compared to the CCl_4 group, Kruskal Wallis/Tamhane T2 test.

Results

Immunohistochemical Analysis

Light microscopic examination showed the presence of $CD3^+$ T lymphocytes, especially in the white pulp of sections of the control group (Table I, Figure 1A). The structures of the white and red pulps were normal in this group. The INF-administered sham group (Group II) demonstrated a similar morphology (Table I, Figure 1B). On the other hand, there was a vast increase in the number of $CD3^+$ T lymphocytes in the

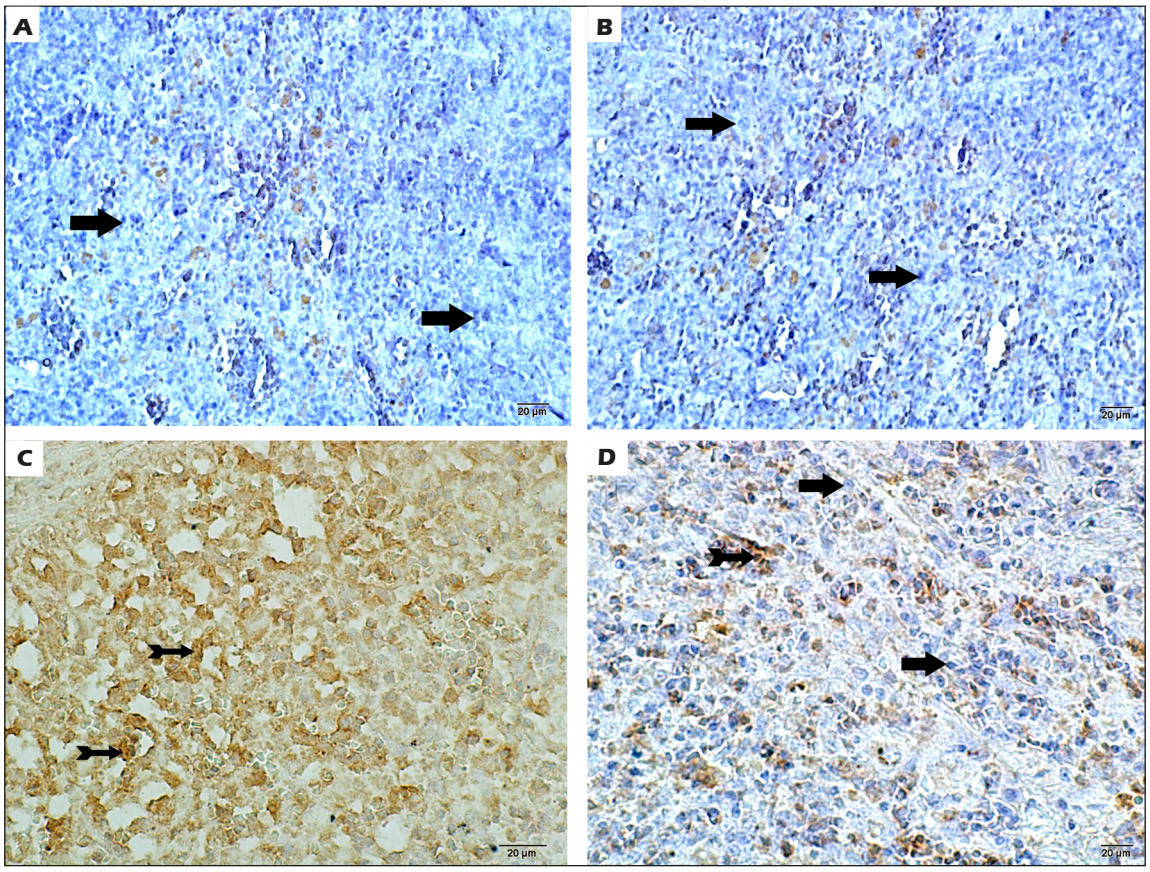

Figure 1. Representative light microscopic images of spleen tissue incubated with CD3 primary antibody. **A**, (×40) Control Group: CD3+ T lymphocytes are observed in the white pulp of normal spleen tissue (numerical density: 6(5-7) per mm^3). **B,** (×40) INF treatment Group: CD3+ T lymphocytes are observed in the white pulp of typical spleen tissue (numerical density: 5(4-6) per mm^3). **C,** (×40) CCl_4 treatment Group: An increase in CD3+ T lymphocytes is observed in atypical spleen tissue, especially in white pulp (numerical density:16(14-18) per mm^3). **D,** (×40) CCl_4+INF treatment Group: It is observed that there is a decrease in the number of CD3+ T lymphocytes, especially in the white pulp (numerical density: 9(8-11) per mm^3).

CCl_4-treated group (Table I, Figure 1C), which was contrary to the CCl_4+INF group (Table I, Figure 1D).

The presence and distribution of $CD68^+$ cells, which were mainly in the white pulp, were normal in the control and INF groups (Table I, Figures 2A and 2B, respectively). In contrast, the $CD68^+$ macrophages in the CCl_4-treated group (Table I, Figure 2C) were much higher in number than these two groups and the CCl_4+INF group (Table I, Figure 2D).

We observed occasional $CD200R^+$ macrophages, especially in the red pulp of the control and INF groups (Table I, Figure 3A and Figure 3B, respectively). However, the number of these cells increased in the CCl_4 group, especially in the red pulp (Table I, Figure 3C), which was the opposite of the CCl_4 + INF group (Table I, Figure 3D).

Stereological Analysis

There was no significant difference in the numerical density of $CD3^+$ T lymphocytes between the control and INF groups (Table I, Figure 1A-C). However, the number increased by two-fold, to 16 (14-18) mm^3, in the CCl_4 group (Table I, Figure 1A and 1C, p=0.000), which was much higher than the CCl_4+INF group, 9 (8-11) mm^3, (Table I, Figure 1C-D, p=0.000).

There was no difference in the numerical density of $CD68^+$ macrophages between the control group, 9 (8-10) mm^3 and the sham group, 8 (6-9) mm^3 (Table I, Figure 2A-B). However, the number increased to 26 (24-28) mm^3 in the CCl_4 group (Table I, Figure 2A and 2C, p=0.000). INF treatment reduced the density to 16 (15-18) mm^3 in the CCl_4+INF group (Table I, Figure 2C-D, p=0.000).

There was also no significant difference between the numerical density of CD200R+ mac-

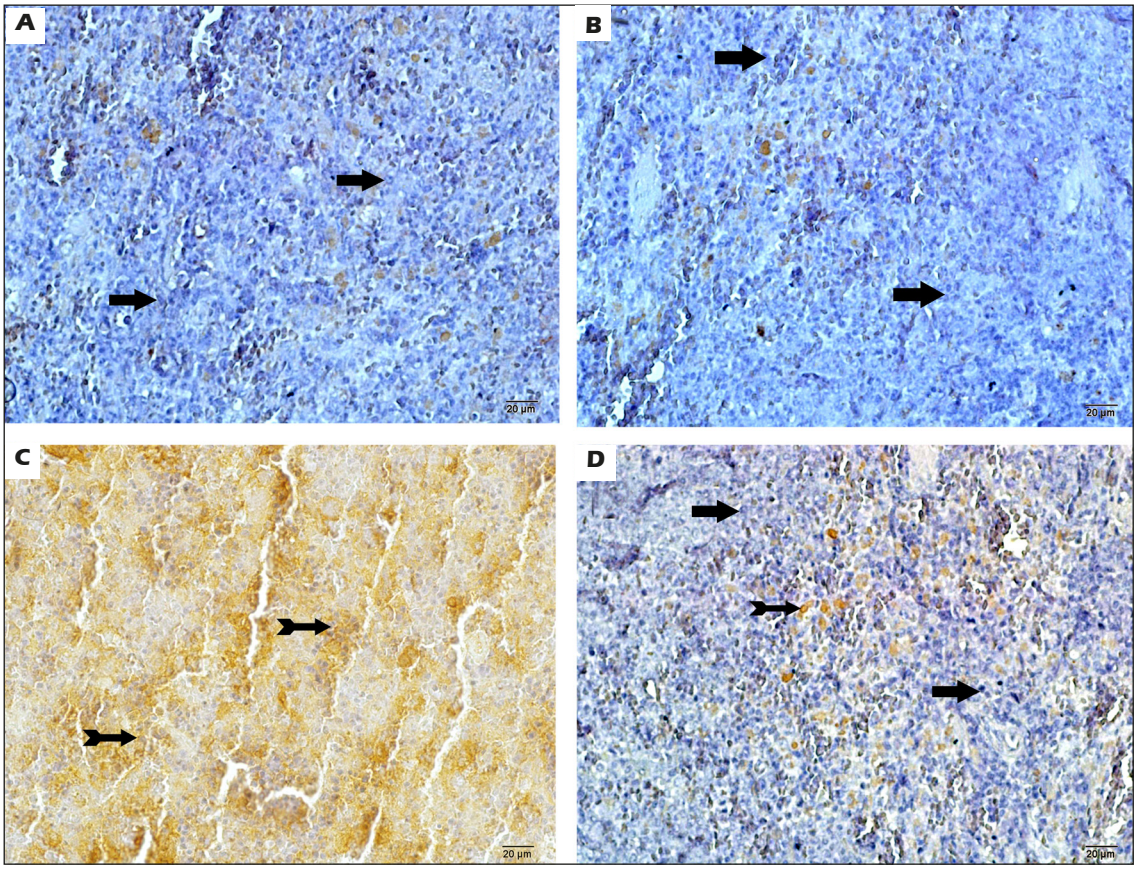

Figure 2. Representative light microscopic images of spleen tissue incubated with CD68 primary antibody. **A,** (×40) Control Group: CD68+ macrophages are observed in normal spleen tissue, especially in the red pulp (numerical density: 9(8-10) per mm^3). **B,** (×40) INF treatment Group: CD68+ macrophages are observed in the red pulp of typical spleen tissue (numerical density: 8(6-9) per mm^3). **C,** (×40) CCl_4 treatment Group: An increase in CD68+ macrophages is observed in the atypical spleen tissue, especially in the red pulp (numerical density: 26(24-28) per mm^3). **D,** (×40) CCl_4+INF treatment Group: It is observed that there is a decrease in the number of CD68+ macrophages, especially in the red pulp (numerical density: 16(15-18) per mm^3).

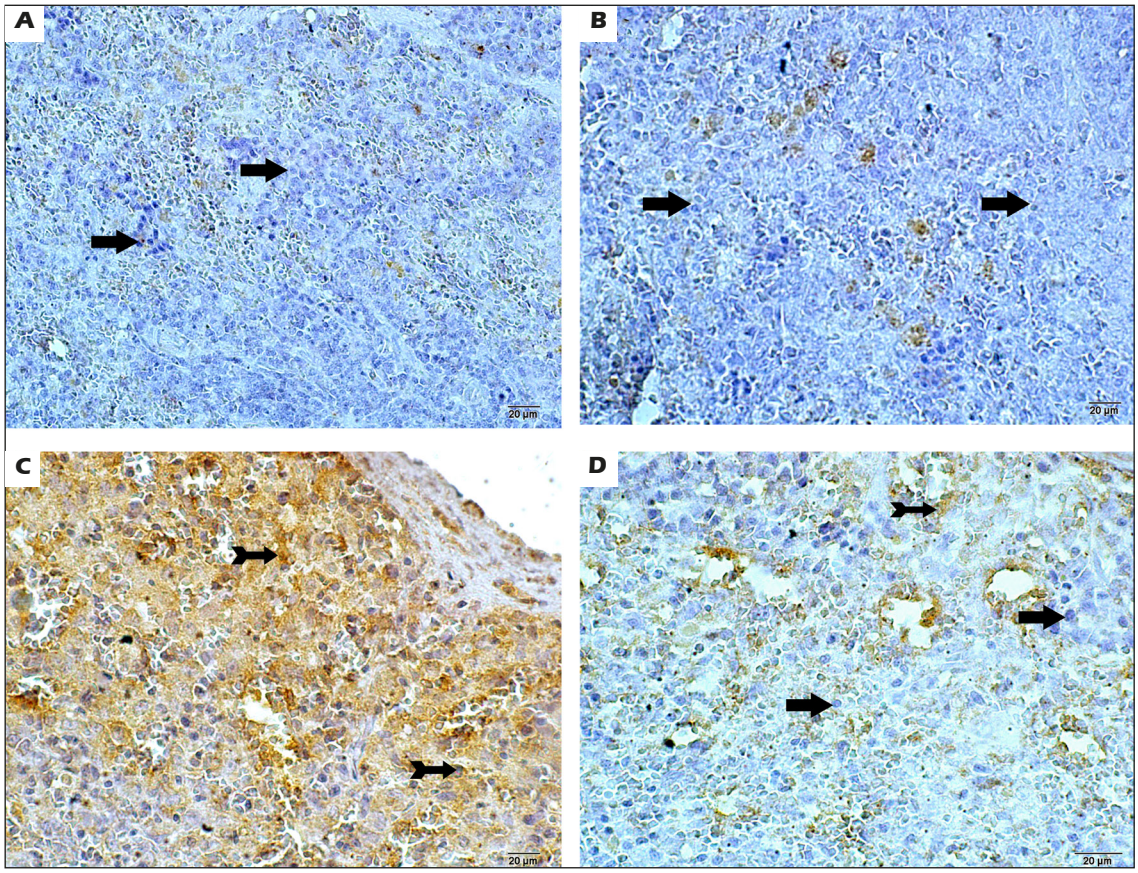

Figure 3. Representative light microscopic images of spleen tissue incubated with CD200R primary antibody. **A,** (×40) Control Group: CD200R+ macrophages are observed in normal spleen tissue, especially in the red pulp (numerical density: 4 (3-5) per mm^3). **B,** (×40) INF treatment Group: CD200R+ macrophages are observed in the red pulp of typical spleen tissue (numerical density: 5 (4-6) per mm^3). **C,** (×40) CCl_4 treatment Group: An increase in CD68+ macrophages is observed in the atypical spleen tissue, especially in the red pulp (numerical density:15 (14-17) per mm^3). **D,** (×40) CCl_4+INF treatment Group: It is observed that there is a decrease in the number of CD68+ macrophages, especially in the red pulp (numerical density: 8 (5-9) per mm^3).

rophages in the control group, 9 (8-10) mm^3, and the sham group, 4 (3-5) mm^3 (Table I, Figure 3A-C). On the contrary, the number increased to 15 (14-17) mm^3 in the CCl_4 group (Table I, Figure 3A and 3C, p=0.000). INF treatment reduced the cell density to 8 (5-9) mm^3 in the CCl_4+INF group (Table I, Figure 3C-D, p=0.000).

Discussion

CCl_4 is a colorless, clear, volatile and highly toxic substance. It can easily penetrate the body through inhalation and contact[19]. It is a carcinogen for many organs, especially the liver, lung, kidney and spleen[1]. Increased levels of TNF-α and IkB have been reported in studies of CCl_4-induced spleen toxicity[7]. Al-Tamimi et al[7] reported that CCl_4 increased hepatotoxicity and oxidative stress markers, including IkB and TNF-α expression in their study. In addition, CCl_4-induced oxidative stress increases NF-κB signaling. In this case, it causes the release of proinflammatory cytokines such as IL-1ß, TNF-α and IL-6[20]. Infliximab (INF) is an anti-TNF-α monoclonal antibody that is used in the treatment of many inflammatory diseases[21]. In several studies, it has been reported that INF ameliorates inflammation-induced tissue damage by inhibiting TNF-α and ROS production[22,23].

To the best of our knowledge, the effectiveness of INF in CCl_4-induced spleen toxicity as revealed by inflammation accompanied by enhanced CD3+, C68+, and CD200R+ cells in spleen tissue has not been investigated[23-25]. It has been shown that these cell populations increase in inflamma-

tory bowel diseases[26-29]. In addition, Mercantepe et al[18] reported a significant increase in these cells in methotrexate-induced splenic toxicity which was inhibited by INF treatment. Additional studies by Hove et al[30] and Petito et al[31] reported decreases in inflammatory cytokines and cell numbers in INF-treated inflammatory conditions.

The effect of INF on the expression of CD200R on activated macrophages T cells, and monocytes has been investigated. This marker is upregulated in inflammatory conditions which are suppressed by INF administration[32-35]. A recent study[18] showed that INF significantly reduced the amount of CD200R+ macrophages in methotrexate-induced splenic toxicity. Similarly, in this study, we observed that CCl_4 toxicity enhanced the number of $CD200R^+$ macrophages in the splenic red pulp, and the use of INF decreased the number of these cells.

This was a pilot study that examined the possible inflammation-reducing effect of TNF-α inhibitors by investigating the numerical alterations of CD3, CD68, and CD200R-positive T lymphocytes and macrophages in splenotoxicity. This study must be supported by investigating further inflammatory parameters.

Conclusions

In conclusion, it was found that CD3, CD68 and CD200R positive T lymphocytes and macrophages increased due to CCl_4-induced splenic toxicity, whereas TNF-α inhibitors showed protective effects against spleen toxicity by decreasing the expression of CD3, CD68 and CD200R.

Conflict of 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.

Availability of Data
The data that supports the findings of this study is available upon reasonable request.

Funding
None.

Ethical Approval
The study was approved by Recep Tayyip Erdogan University Animal Ethical Committee (Rize, Turkey). (Approval Date: 31.03.2020, Approval No.:2020/13).

Authors' Contribution
Ilkay Bahceci is the principal author and conducted most of the experimental work. Levent Tumkaya performed some of the experimental work and result in analysis. Tolga Mercantepe contributed to the conception and design of this study, data analysis and manuscript editing. Hamit Yilmaz and Yunus Emre Ibik contributed to data analysis and manuscript editing. Omer Faruk Duran and Nuray Arslan critically reviewed and revised the manuscript. All authors have read and approved the final manuscript.

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