Original Article

Acute *Brucella melitensis* M16 infection model in mice treated with tumor necrosis factor-alpha inhibitors

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Abstract
Introduction: There is limited data in the literature about brucellosis related to an intracellular pathogen and anti-tumor necrosis factor alpha (anti-TNFα) medication. The aim of this study was to evaluate acute *Brucella* infections in mice receiving anti-TNFα drug treatment.
Methodology: Anti-TNFα drugs were injected in mice on the first and fifth days of the study, after which the mice were infected with *B. melitensis* M16 strain. Mice were sacrificed on the fourteenth day after infection. Bacterial loads in the liver and spleen were defined, and histopathological changes were evaluated.
Results: Neither the liver nor the spleen showed an increased bacterial load in all anti-TNFα drug groups when compared to a non-treated, infected group. The most significant histopathological findings were neutrophil infiltrations in the red pulp of the spleen and apoptotic cells with hepatocellular pleomorphism in the liver. There was no significant difference among the groups in terms of previously reported histopathological findings, such as extramedullary hematopoiesis and granuloma formation.
Conclusions: There were no differences in hepatic and splenic bacterial load and granuloma formation, which indicate worsening of the acute *Brucella* infection in mice; in other words, anti-TNFα treatment did not exacerbate the acute *Brucella* spp. infection in mice.

Introduction

Anti-tumor necrosis factor alpha (TNFα) drugs are widely used for rheumatoid arthritis, ankylosing spondylitis, inflammatory bowel disease, psoriasis, psoriatic arthritis, and juvenile chronic arthritis. However, anti-TNFα drug therapy is associated with increased risk of infection, including non-typhoidal salmonellosis, listeriosis, pneumocystosis, and other fungal or viral infections, but *Mycobacterium tuberculosis* infection incidence is especially increased in patients who are treated with these drugs [1,2].

Brucellosis is still endemic in some parts of the world, and approximately 500,000 new cases are reported annually [3]. *Brucella* spp. are intracellular bacteria that release cytokines such as IFN-gamma, IL-2, and TNFα. Activated macrophages and dendritic cells are required for the immune response against *Brucella* infection [4-6]. TNFα has a critical role in macrophage activation, apoptosis, proinflammatory cytokine release, and granuloma formation. It is required for bactericidal effects and clearance of *Brucella* infection [7-9]. Although it was reported that TNFα was not secreted by mouse macrophages infected with *B. melitensis* [10], TNFα gene expression and induction was demonstrated in *in vitro* and *in vivo* studies conducted with mice macrophage, splenocyte, and dendritic cells [11-13]. Additionally, the increased TNFα serum level was shown with *B. abortus* infection, but not with *B. melitensis* [14]. Although brucellosis is an endemic disease and there is a widespread use of anti-TNFα drugs for different indications, reactivation of brucellosis can be expected more frequently. However, there are only three case reports about brucellosis reactivation due to anti-TNFα

drug therapy [15-17]. In this study, we aimed to investigate the responses to acute *Brucella* infection in mice receiving adalimumab (ADA), etanercept (ETN), and infliximab (IFX) by evaluating the splenic and hepatic bacterial loads as well as histopathological findings.

Methodology

Bacteria

This study used a standard smooth strain of *B. melitensis* provided by the Pendik Veterinary Control and Research Institute in Istanbul, Turkey. Lyophilized bacteria were suspended in phosphate buffered saline (PBS) and cultured on horse blood agar (HBA) plates for three days at 37°C with 5% CO_2.

Animals, infections, and anti-TNFα drugs

The study was carried out according to the ethical guidelines (no. 2011.035) of the Medical Ethics Committee of Pamukkale University, Denizli, Turkey. A total of 56 BALB/c mice between eight and ten weeks of age, weighing between 30 and 40 grams, were included in the study. During the experiment period, six mice were excluded from the analysis due to preterm death. The control groups (CG) (non-infected-CG, n = 6; infected-CG, n = 7) and following test groups were examined: ADA (adalimumab, n = 6); ADA + *B. melitensis* (adalimumab with acute *B. melitensis* infection, n = 6); ETN (etanercept, n = 6); ETN + *B.melitensis* (etanercept with acute *B. melitensis* infection, n = 7); IFX (infliximab, n = 6); and IFX + *B. melitensis* (infliximab with acute *B. melitensis* infection, n = 6).

On days 1 and 5 of the experiment period, drugs were administered subcutaneously to the ADA (2 mg/kg), ETN (0.4 mg/kg), and IFX (10 mg/kg) groups, while 100 µL PBS injections were given to the control group. The aim of this study was to evaluate acute *Brucella* infection in mice receiving anti-TNFα drug treatment, so a pre-infection regime was preferred, and the treatment was repeatedly performed as in previous studies [18-20]. Test groups were intraperitoneally inoculated with 4×10^4 colony-forming units (cfu) of *B. melitensis* M16 strain on day 5. Optimal doses were described by previous studies for virulent *Brucella*, and in this study, the infective dose was administered as in some other studies on a *B. melitensis* model [11,13,21].

Measurement of bacterial load in liver and spleen

On day 19 (the 14th day of infection), the spleen and liver were aseptically removed, weighed, and placed in sterile Petri dishes with 10 mL of saline. The organs were homogenized for quantitative bacterial load by mincing them with two no. 15 scalpels and homogenizing by pushing them through the mesh with a sterile syringe plunger. Aliquots of 0.1 mL spleen and liver homogenate samples were diluted tenfold serially in PBS. Next, 100 µL homogenates of each dilution were cultured on HBA plates at 37°C with 5% CO_2 for five days. The quantitative bacterial load of each sample was determined by log conversion.

Histopathology

Tissue samples of the spleen and liver taken from each mouse were fixed in 10% buffered formalin placed in paraffin blocks, sectioned at 5 µm, and stained with haematoxylin and eosin for histological evaluation. Two pathologists performed a blinded evaluation of histopathological changes in the spleen and liver, using the following histopathological evaluation criteria:

Spleen

Granuloma – 0: absent, 1: micro granuloma, 2: macro granuloma; extramedullary hematopoiesis – 0: absent, 1: mild, 2: moderate, 3: severe; lymphoid tissue depletion – 0: absent, 1: mild, 2: moderate, 3: severe; neutrophil infiltration in the red pulp – 0: absent, 1: present.

Liver

Granuloma – 0: absent, 1: micro granuloma, 2: macro granuloma; extramedullary hematopoiesis – 0: absent, 1: mild, 2: moderate, 3: severe; apoptotic cells – 0: absent/minimal, 1: present; portal inflammation – 0: absent, 1: mild, 2: moderate, 3: severe; perivascular mononuclear infiltration – 0: absent, 1: present; Kuppfer cell hyperplasia – 0: absent, 1: mild, 2: moderate, 3: severe; and hepatocellular pleomorphism – 0: absent, 1: present.

Statistical analysis

The statistical analysis was carried out using SPSS software version 17. The bacterial load in the spleen and liver were analyzed using the Mann-Whitney *U* test (two group comparison) and the Kruskal-Wallis test (multi group comparison). The histopathological findings of the spleen and liver were analyzed by the Chi-square and Kruskal-Wallis tests. Statistical significance was set at $p < 0.05$.

Results

Quantitative spleen bacterial load

Bacterial growth was observed in spleen tissue cultures of all infected mice. The lowest bacteria number was in the ETN + *B.melitensis* group, while the highest was in the IFX + *B. melitensis* group (Figure 1). There was no difference in quantitative splenic bacterial load between the groups receiving infected-CG and anti-TNFα. The IFX + *B. melitensis* group had a higher bacterial load when compared to the ETN + *B. melitensis* and ADA + *B. melitensis* groups (p = 0.010 and p = 0.025, respectively).

Quantitative liver bacterial load

There was bacterial growth in the majority of hepatic tissue cultures except in two mice in the ETN + *B. melitensis* group. These two subjects were not excluded from the analysis, because both demonstrated bacterial growth in the spleen and histopathological changes in the liver and spleen. Bacterial load was lower in the ETN + *B. melitensis* group than in the infected-CG, ADA + *B. melitensis*, and IFX + *B. melitensis* groups, and there was a significant difference between the ETN + *B. melitensis* and infected-CG groups (p = 0.025) (Figure 2).

Spleen histopathology

Extramedullary hematopoiesis was determined in all infected samples, but there was no significant difference between the scores of the groups. Granuloma formation was detected in 19 samples (73%), and there was no significant difference in granuloma formation and lymphoid tissue changes between the groups (Table 1). Neutrophil infiltration

in the red pulp was more prominent in the groups that were administered anti-TNFα, and there was a statistically significant difference between the ADA + *B. melitensis* and infected-CG groups (p = 0.026) (Figure 3).

Liver histopathology

Although granuloma was determined in all samples, extramedullary hematopoiesis was detected in only 13 samples (50%). There was no significant difference in terms of granuloma formation, extramedullary hematopoiesis, portal inflammation, perivascular mononuclear infiltration, and Kuppfer cell hyperplasia between the groups (Table 1). Hepatocellular pleomorphism was more prominent in the IFX + *B. melitensis* group than in the infected-CG group (p = 0.026) (Figure 4). Additionally, apoptotic cells in the IFX + *B. melitensis* group were more distinctive than in the infected-CG, ADA + *B. melitensis*, and ETN + *B. melitensis* groups (p = 0.026, p = 0.046, and p = 0.067, respectively) (Figure 5).

Discussion

The results of this study revealed that there was no significant increase in splenic and hepatic bacterial load during acute *B. melitensis* infection in mice resulting from anti-TNFα drug treatment. Previously, it was shown that TNFα was required for bacterial clearance in a mouse model of brucellosis [7]. Furthermore, splenic bacterial load was increased during acute *Brucella* infection in both TNFα gene deficient mice and mice receiving anti-TNFα monoclonal antibody [22,23].

Figure 1. Comparisons of the groups are shown in terms of the spleen tissue bacterial load. Values are mean ± standard error of mean (SEM)
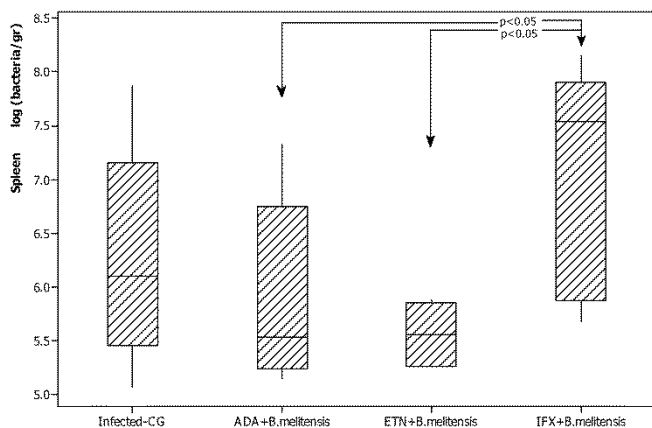
*p < 0.05 vs. ADA + *B. melitensis*, ETN + *B. melitensis* by Mann-Whitney *U* test

Figure 2. Comparisons of the groups are shown in terms of the liver tissue bacterial load. Values are mean ± standard error of mean (SEM)
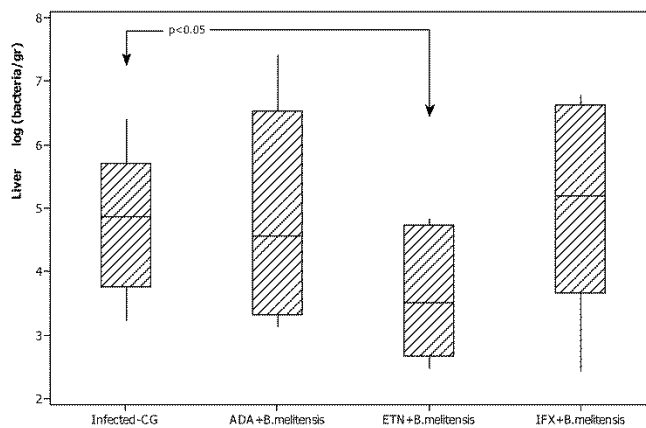

*p < 0.05 vs. ETN + *B. melitensis* by the Mann-Whitney *U* test

Table 1. Histopathological findings of acute *Brucella melitensis* M16 infection in mice treated with anti tumor necrosis factor-alpha drugs

| Organ, pathological findings	Infected-CG (n = 7)	ADA + *B. melitensis* (n = 6)	ETN + *B. melitensis* (n = 7)	IFX + *B. melitensis* (n = 6)	P
Spleen					
Granuloma mean grade (SEM)	1.14 (0.34)	1.67 (0.61)	1.14 (0.40)	1.17 (0.30)	.257
Extramedullary hematopoiesis, mean grade (SEM)	0.57 (0.37)	1.17 (0.40)	1.29 (0.47)	2.0 (0.4)	.242
Lymphoid tissue depletion, mean grade (SEM)	1.71 (0.5)	2.17 (0.4)	1.43 (0.2)	2.5 (0.34)	.209
Neutrophil infiltration in the red pulp (present/absent)	3/4	6/0*	5/2	5/1	.122
Liver					
Granuloma, mean grade (SEM)	2.71 (0.28)	2.33 (0.33)	1.71 (0.36)	2.67 (0.33)	.122
Extramedullary hematopoiesis, mean grade (SEM)	0.57 (0.37)	1.17 (0.40)	1.29 (0.47)	1.17 (0.54)	.651
Apoptotic cells (present/absent)	3/4	3/3	3/4	6/0**	.159
Portal inflammation, mean grade (SEM)	1.43 (0.36)	0.67 (0.33)	1.57 (0.42)	1 (0.36)	.369
Perivascular mononuclear infiltration (present/absent)	4/3	1/5	5/2	2/4	.201
Kuppfer cell hyperplasia, mean grade (SEM)	1.86 (0.34)	2.0 (0.51)	1.71 (0.36)	2.67 (0.21)	.256
Hepatocellular pleomorphism (present/absent)	3/4	5/1	6/1	6/0***	.078

SEM: standard error of mean
*p < 0.05 *vs*. infected-CG; **p < 0.05 *vs*. infected-CG and ADA + *B. melitensis* group; ***p < 0.05 *vs*. infected-CG

Figure 3. Neutrophil infiltration in the spleen red pulp: **a)** infected-CG group; **b)** ADA + *B. melitensis* group; **c)** ETN + *B. melitensis* group; **d)** IFX + *B. melitensis* group (H&E, x400 magnification)
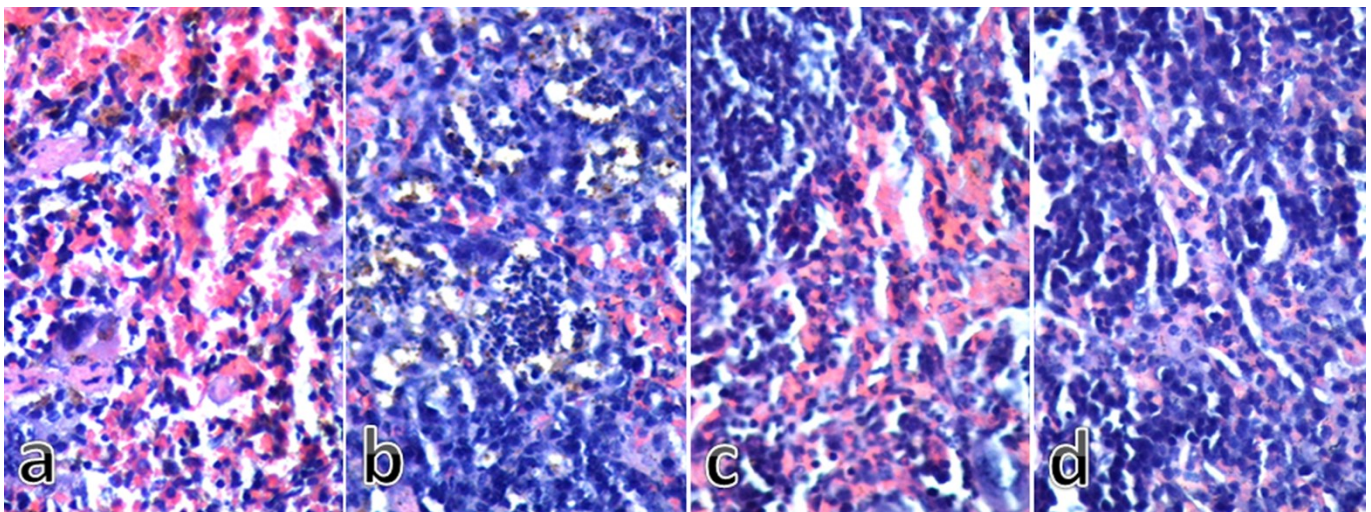
Figure 4. Hepatocellular pleomorphism in the liver: **a)** infected-CG group; **b)** ADA + *B. melitensis* group; **c)** ETN + *B. melitensis* group; **d)** IFX + *B. melitensis* group (H&E, x400 magnification)
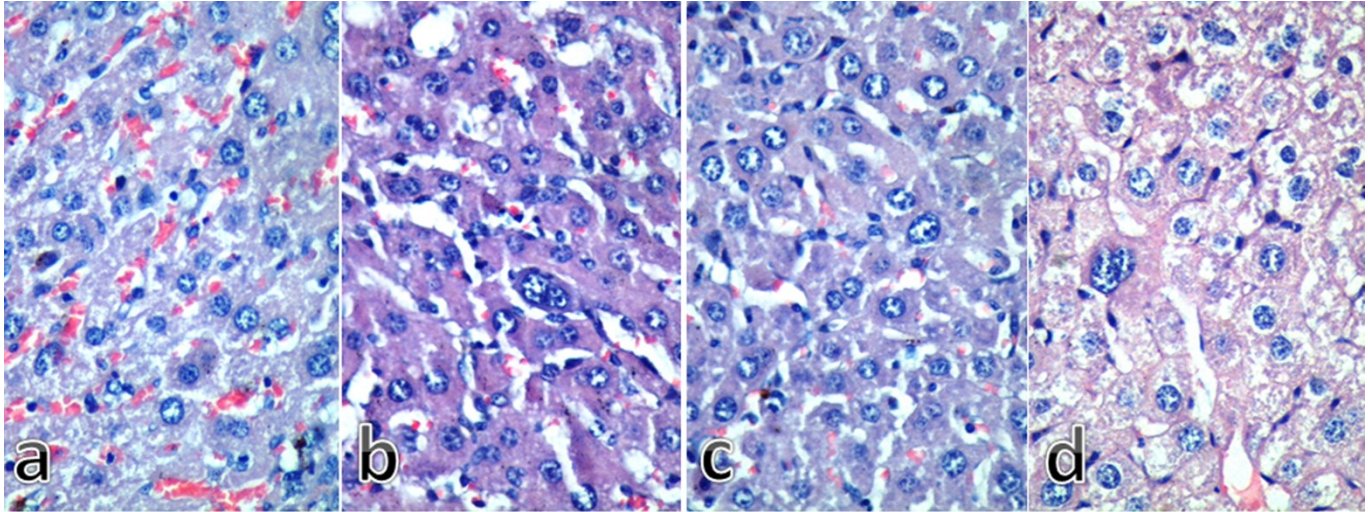
Figure 5. Apoptotic cells in the liver: **a)** infected-CG group; **b)** ADA + *B. melitensis* group; **c)** ETN + *B. melitensis* group; **d)** IFX + *B. melitensis* group (H&E, x400 magnification)
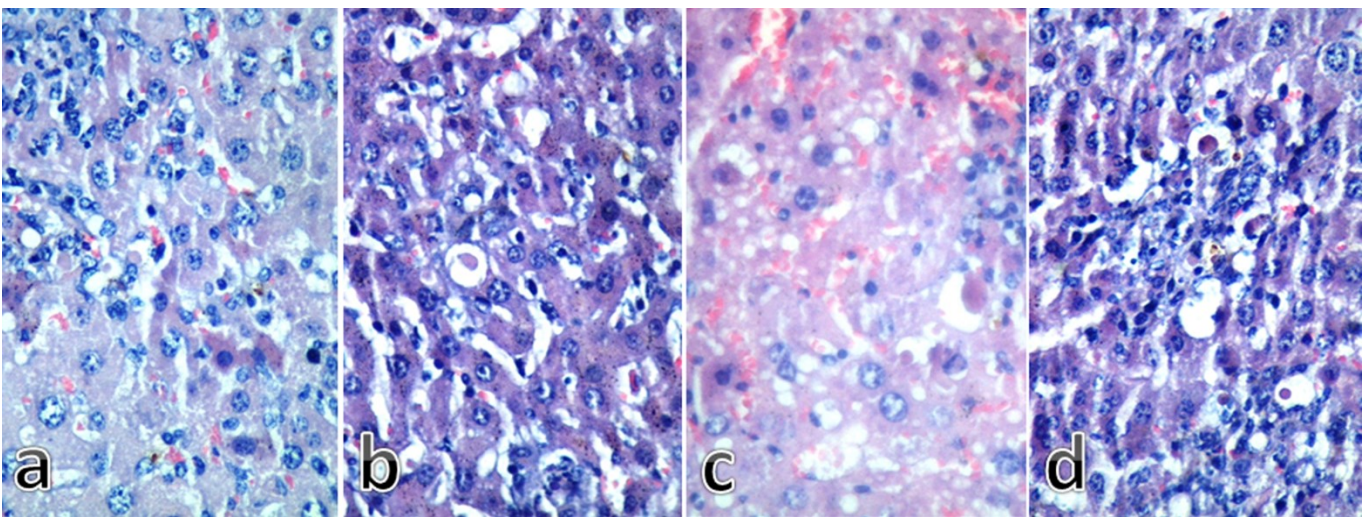
The reason our findings differed from those of previous reports may be the administration of anti-TNFα drugs used in daily medical practice.

In the present study, there was no higher bacterial load in groups receiving anti-TNFα drugs when compared to the control group, but some findings are still worthy of note.

First, hepatic bacterial load was lower in the ETN + *B. melitensis* group than in the infected-CG group (p = 0.025). Previous studies showed that TNFα stimulation of the hepatocytes of mice leads to TLR 2 and TLR 4 up-regulation, whereas TNFα blockage leads to their down-regulation [24]. In a recent study, it was reported that TLR 2 and 4 did not have a role in controlling hepatic infection in a mouse model of *B. melitensis*, and hepatic bacterial clearance started in TLR 2 and TLR 4 of knock-out mice after the second week of infection [25]. However, in the spleen, it was shown that TLR 4, but not TLR 2, was required to control *Brucella* infection [13,25,26]. These data may explain the lower hepatic bacterial load in the ETN + *B. melitensis* group as well as the absence of bacterial growth in two liver samples. However, TLR 4 is required for bacterial clearance in the spleen, and therefore splenic bacterial load was unchanged. Another factor influencing this result may be related to ineffectiveness of etanercept on the events related to the p55 receptor, unlike other drugs [27].

Second, splenic bacterial loads of the experiment groups are shown in Figure 1. In the IFX + *B. melitensis* group, the splenic bacterial load was significantly higher than the other two drug groups; however, none of the drug groups demonstrated a splenic bacterial load different from the control group. IFX is a human-mouse chimeric TNFα inhibitor, and it exerts its potential effect by binding both to soluble TNFα and transmembranal TNFα. Structural and pharmacological differences of the drugs may play a role in detecting higher bacterial load in the IFX + *B. melitensis* group than the other two drug groups [27]. As mentioned earlier, TLR 4 is essential for splenic bacterial clearance, and anti-TNFα drugs inhibit the expression of TLR 4 and TLR 2. It has been demonstrated that IFX and ETN have similar inhibitory effects on TLR 4 or TLR 2 expression in human synovial tissue [28]. However, there is no clear evidence about the altering effects of these drugs on TLR 2 and TLR 4 activities in mice. The drugs used in this study were previously administered for TNFα blockage in mouse models; it has been demonstrated that they lead to a decrease in TNFα levels in serum [29]. However, there is no clear information about the

dosing of these drugs in mice; therefore, we administered the doses applied for humans. In addition to this, drug doses may influence the differences between the groups.

Third, the histopathological evidence is critical. In the present study, extramedullary hematopoiesis was detected in spleen samples of mice infected with *B. melitensis* M16, and granuloma formation was detected in the majority (19/26; 73%) of them. It was previously reported that those two changes were the main signs in the spleen of the *B. melitensis* M16 mouse model [30]. Our results revealed that there was granuloma formation in all liver samples, and extramedullary hematopoiesis was encountered in 50% of the subjects. To date, we are not aware of a study investigating effects of TNFα neutralization on granuloma formation during *Brucella* infection. In one study, there was no significant difference in terms of granuloma formation between IL-10 knock-out mice in a *B. abortus* infection model and wild-type mice, even though higher TNFα levels were detected in the second week of infection [4]. The results of our study revealed that anti-TNFα drug use did not have any effect on granuloma formation either in the spleen or liver. This was also demonstrated in a *M. tuberculosis* mouse model [31].

In all anti-TNFα drug groups, there was neutrophil infiltration in the red pulp, but this difference reached statistical significance only in the ADA + *B. melitensis* group. In liver samples, apoptotic cells and hepatocyte pleomorphism were more prominent in the IFX + *B. melitensis* group. There are conflicting data about the hepatic effects of anti-TNFα drugs. In some patients, hepatotoxicity can develop due to IFX administration [32]. When the treatment was changed with ETN or ADA, hepatotoxicity disappeared in some patients who received IFX previously [33,34]. On the other hand, TNFα blockage was associated with the hepatoprotective effect in some conditions in mice models [35,36]. Another study reported that IFX treatment can control chronic hepatitis C infections by promoting apoptotic cells and preventing liver regeneration in a transgenic mouse model [37]. In our study, the detection of apoptotic cells and hepatocyte pleomorphism in the IFX + *B. melitensis* group, but not in the sham group, may indicate an altered response against *Brucella* infection.

Using a mouse model to investigate the effects of anti-TNFα treatment in human brucellosis is the major limitation of our study; ethical restrictions make it impossible to conduct such a study on humans. Still, the critical role of TNFα on disease control is well

known for mouse brucellosis, allowing an argument from analogy with human brucellosis. It was shown *in vitro* that there was no TNFα production in human macrophages and dendritic cells after some *Brucella* spp. infections [38-40]. Some studies also reported that TNFα was absent or was not increased in human brucellosis [41,42]. In spite of these findings, Demirdag *et al*. reported that TNFα increased in human brucellosis and it was correlated with other inflammatory parameters such as C-reactive protein during the disease follow-up [5]. That study also showed that high TNFα levels were decreased by brucellosis treatment. A recently published study reported results similar to those of Demirdag *et al*. [6].

Conclusions

Our results showed that anti-TNFα treatment was not associated with any difference in signs indicating disease severity, such as increased hepatic and splenic bacterial loads, granuloma formation, and extramedullary hematopoiesis in acute *Brucella* infection in mice. In order to safely use anti-TNFα drugs, we need to know whether there is a change in the prognosis or a reactivation of *Brucella* infection in the long term. Therefore, further studies are required to reveal the effects of anti-TNFα drugs on chronic *Brucella* infections and to follow the potential complications of anti-TNFα treatment, such as brucellosis reactivation, especially in endemic areas.

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