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Bacterial translocation is downregulated by anti-TNF-α monoclonal antibody administration in rats with cirrhosis and ascites☆

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***Background/Aims*: TNF-α is involved in the development of bacterial translocation in rats with cirrhosis. The aim of the current study was to evaluate the effect of anti-TNF-α mAb treatment on the incidence of bacterial translocation and systemic infections in rats with cirrhosis and ascites.**

***Methods*: Thirty rats with cirrhosis and ascites were randomly assigned to receive two intraperitoneal doses of anti-TNF-α mAb, distilled water or immunoglobulin on days 0 and 4. On day 10, a laparotomy was performed.**

***Results*: One out of 11 animals receiving anti-TNF-α mAb treatment, 7 out of 10 of the placebo group ($p < 0.01$), and 5 out of 9 of the IgG group developed bacterial translocation ($p < 0.05$). A significantly reduced number of systemic infections were observed in animals receiving anti TNF-α mAb treatment vs animals receiving placebo ($p < 0.01$). TNF-α in serum at laparotomy in animals receiving anti-TNF-α mAb was higher than that in the rest of groups and was also higher in the overall series of animals showing bacterial translocation.**

***Conclusions*: In the experimental model of CCl_4-induced rat with cirrhosis and ascitic fluid, anti-TNF-α mAb administration decreases the incidence of bacterial translocation, in a TNF-α/sTNF-α receptor-independent manner, without increasing the risk of systemic infections.**

***Keywords*: Bacterial translocation; Tumour necrosis factor alpha; Anti-TNF-α; Cirrhosis; Ascites; Bacterial infections; Cytokines**

1. Introduction

Bacterial translocation (BT) from the intestinal lumen to mesenteric lymph nodes (MLN) is considered one of the main events in the pathogenesis of spontaneous bacterial peritonitis (SBP) and other infections in cirrhosis [1,2]. The intimate mechanisms by which bacteria can exit the intestinal lumen, cross the epithelial wall and reach MLN may be different, and are not completely well understood in humans. Some of the factors involved are bacteria-dependent, such as virulence or overgrowth [3], while others may be related to intestinal permeability [4], mucosal edema or epithelial

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Abbreviations: TNF-α, tumour necrosis factor alpha; p55, soluble TNF-α receptor type I; p75, soluble TNF-α receptor type II; BT, bacterial translocation; MLNs, mesenteric lymph nodes.

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ultrastructural changes [5], mucosal peroxidation [6], and probably many others.

The presence of increased levels of endotoxin is a frequently described phenomenon in patients with advanced cirrhosis [7], which may increase BT by itself, or by inducing the secretion of proinflammatory cytokines such as tumour necrosis factor alpha (TNF-α). The question of whether increased levels of TNF-α are cause or effect of bacterial product trafficking remains uncertain. BT is significantly induced in rats subjected to hemorrhagic shock [8] or to injection of lipopolysaccharide [9], and these events may be abrogated when TNF-α is blocked [10], suggesting a role for TNF-α in the development of BT. However, TNF-α is a key cytokine in the inflammatory cascade that protects the host from infections, and thus its blockade might increase the risk of infections.

The subset of patients with advanced cirrhosis and ascites that develop nosocomial episodes of SBP shows increased levels of TNF-α in blood [11], which has been associated so far with a bacterial-induced secretion of proinflammatory cytokines, prior to SBP development. However, it is likely that increased levels of TNF-α observed in a subset of patients with advanced cirrhosis and presence of ascitic fluid may, in turn, increase the likelihood of new episodes of BT, closing the loop that might lead to the eventual development of SBP. This scenario suggests a complex and incompletely understood role of TNF-α in patients and animal models of cirrhosis.

The aim of this study is to assess first if TNF-α blockade may inhibit BT, and subsequently if this experimental approach may increase the risk of systemic infections in an animal model of cirrhosis and ascites.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats were included in this study. Animals with an initial weight of 100–120 g were individually caged at a constant room temperature of 21 °C and exposed to a 12:12 light/dark cycle. This study was approved by the Animal Research Committee of the Institut de Recerca of Hospital de la Santa Creu i Sant Pau (Barcelona) and by the Departament de Agricultura, Ramaderia i Pesca de la Generalitat de Catalunya (DARP). Animals received care according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals.

2.2. Induction of cirrhosis

Cirrhosis was induced as previously described by Runyon [12]. Rats weighing 100–120 g were fed standard rodent chow (B/K) and were treated with 1.5 mmol/L phenobarbital in tap water. When rats reached a weight of >200 g, weekly doses of CCl_4 (J.T. Baker Inc., Phillipsburg, NJ) were given intragastrically using a sterile pyrogen free syringe (Artsana p.p.a., Greenclate) with an attached stainless steel animal feeding tube (Popper and Sons, New Hyde Park, NY) without anesthesia. The first dose of CCl_4 was 20 μL and subsequent doses were adjusted based on changes in weight 48 hours after the last dose as previously reported [13]. After a minimum of 12 weeks of induction of cirrhosis, ascites was developed as shown by paracentesis.

2.3. Experimental design

Once ascites was evident, it was confirmed by paracentesis under anesthesia with 10 mg/kg xylazine (Rompun, Bayer) and 50 mg/kg ketamine (Ketolar, Parke–Davis). Animals were then randomly allocated into three groups: Group I received an intraperitoneal dose of anti-TNF-α monoclonal antibody (mAb, Centocor R&D, Inc., 15 mg/kg) on days 0 and 4, and animals included in Group II received an intraperitoneal dose of distilled water (1.5 ml) the same days of the study. Due to the immunomodulatory effects that may cause immunoglobulin [14–17], a third group of animals (Group III) was treated with an IgG isotype control (IgG2a), with the same dosis and conditions as Group I. Animals with infected ascites at paracentesis on day 0 were excluded from the study. A laparotomy was performed 10 days after the first dose of drug or placebo administration. Anti-TNF-α used in this study is composed of the variable region of a mouse anti-rat TNF monoclonal fused to a rat immunoglobulin (Ig) G Fc.

A sample of blood was obtained from the tail vein at day 0, centrifuged and stored at −40 °C until cytokines and TNF-α receptors were assessed.

2.4. Laparotomy

All rats were sacrificed at the end of the study period. Laparotomy was performed under anesthesia with the same drugs and doses used for paracentesis in strictly sterile conditions. Abdominal fur was removed with a depilatory and the skin was sterilized with iodine. A short incision in the abdominal wall was performed and a sample of ascitic fluid was obtained for bacterial culture. The abdomen was then opened widely and the remaining ascitic fluid was evacuated. If no free ascitic fluid was present, sterile swabs were passed over the parietal peritoneal surface and then plated. Samples of pleural fluid were also collected for microbiological study. The mesenteric lymph nodes from the ileo-cecal area were aseptically dissected, removed, weighed, and then liquefied in sterile saline for bacterial culture. Blood was collected from the cava vein in a non-additive sterile interior vacutainer (Becton–Dickinson Vacutainer Systems Eur., Meylan Cedex, France), centrifuged and stored for cytokine and TNF-α measurement.

Rats were then euthanized with intravenous sodium thiopentate (Penthotal, Abbott Laboratories).

2.5. Bacterial translocation and systemic infections

Samples of mesenteric lymph nodes, liver, spleen, ascites and pleural fluid when available were collected under sterile conditions before death of the rat, and cultured in MacConkey agar (Oxoid), Columbia sheep blood (Oxoid) and Esculin-Bile-Azide agar (MERCK), and incubated at 37 °C for 48 h. Bacterial translocation was defined as the positivity of culture of mesenteric lymph nodes. Systemic infections were considered as the positivity of culture of any of the remaining biological samples.

2.6. Serum quantitative enzyme immunoassays

Immunoassays for quantitative measurement of rat TNF-α, soluble TNF-α receptor type I (p55) and soluble TNF-α receptor type II (p75) in blood samples were performed using TNF-α, p55 and p75 Quantikine rat Immunoassays (R&D Systems, Abingdon, UK), according to the manufacturer's instructions. All samples were tested in duplicate and read at 450 nm and 490 nm in a ThermoMax microplate reader (Molecular Devices, Sunnyvale, California, USA). Standard curves were generated for each plate needed and the average zero standard optical densities were subtracted from the rest of standards, controls and samples to obtain a corrected concentration for all molecules.

2.7. Statistical analysis of experimental data

All observations are reported as means ± SD. Differences between groups were analyzed using the non-parametric Mann–Whitney *U*-test. The Wilcoxon signed-rank test was used for paired data. Qualitative variables are expressed as frequency or percentage and differences between groups are evaluated using χ^2 test. Correlations between variables are evaluated using the Spearman bivariate correlation test. A two-tailed p value below 0.05 is considered statistically significant. Analyses were performed with the SPSS statistical package (SPSS Inc. version 12.0, Chicago, Illinois, USA).

3. Results

3.1. Groups and characteristics of rats

Cirrhosis was induced in 67 animals, and 20 died during the induction period (29%). Surviving rats with ascites were then randomly allocated into Group I ($n = 16$), Group II ($n = 19$) and Group III ($n = 12$). Two rats from Group I, five rats from Group II and none from Group III presented a positive culture at paracentesis and were excluded from the study. In addition, 3 rats from Group I, 4 rats from Group II and 3 rats from Group III died during the study and were also excluded from the analysis. Group I finally comprised 11 animals, Group II 10 animals and Group III 9 animals.

According to the study protocol, animals included in Group I received anti-TNF-α mAb, animals in Group II received placebo and animals in Group III received an IgG isotype control (see Section 2).

Age of rats at the beginning of the different protocol treatments was 17.4 ± 2.7 weeks in Group I, 16.3 ± 3.6 weeks in Group II and 13.3 ± 3.0 weeks in Group III. Weight of rats with cirrhosis prior to treatment was: 413 ± 5 g in Group I, 418 ± 5 g in Group II and 420 ± 58 g in Group III. Differences were not statistically significant for these parameters among groups.

3.2. Anti-TNF-α mAb treatment downregulates BT in rats with cirrhosis and ascites

At day 10, BT was evaluated by microbiological culture of MLNs and systemic infections were assessed by microbiological culture of ascitic and pleural fluid, liver and spleen following the usual protocol of laparotomy. BT was observed in one out of 11 rats included in the anti-TNF-α administration group (9%), in 7 out of 10 rats in the placebo group (70%) ($p < 0.01$) and in 5 out of 9 rats in the IgG isotype control group (55.5%) ($p < 0.05$) (Fig. 1). Bacteria isolated in the different cultures are shown in Table 1. As can be observed *E. coli* was the most commonly isolated bacteria. Anti-TNF-α mAb treatment did not increase the risk of systemic infections.

A logistic regression test was performed using BT as dependent variable and treatment (anti-TNF-α, placebo or IgG isotype control), serum TNF-α, p55 and p75 at laparotomy as factors. Only treatment was significantly correlated with BT ($p < 0.05$).

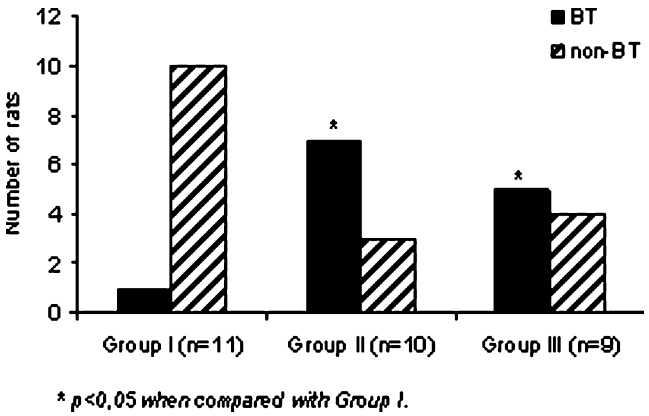

Fig. 1. Incidence of bacterial translocation in animals receiving anti-TNF-α mAb, placebo or an IgG isotype control. *$p < 0.05$ compared with animals treated with anti-TNF-α mAb.

3.3. Serum TNF-α levels are increased in animals receiving anti-TNF-α mAb

Serum TNF-α levels were measured in blood at the moment of paracentesis to evaluate possible differences between groups prior to treatment. No statistically significant differences were observed at baseline between groups.

TNF-α levels at laparotomy in serum of rats with cirrhosis in Group I (47.66 ± 71.14 pg/mL) were higher than serum TNF-α levels from rats receiving placebo (20.66 ± 19.70 pg/mL) and rats receiving IgG (22.66 ± 11.76 pg/mL), although these differences were not statistically significant. However, a surprising and

Table 1
Isolated bacteria in different cultures at laparotomy from animals included in the study

MLNs	Ascitic fluid	Pleural fluid	Liver	Spleen
Group I ($n = 1$)				
E. coli				
Group II ($n = 7$)				
E. coli		*E. coli*		
Enterococcus				
E. coli				
E. coli				*E. coli*
E. coli	*E. coli*			*E. coli*
E. coli	*E. coli*			
Enterococcus	*E. coli*	*E. coli*	*E. coli*	*E. coli*
Group III ($n = 5$)				
E. coli				
		Enterococcus		
E. coli				
E. coli				
		E. coli		

statistically significant increment of TNF-α values at laparotomy, compared with the corresponding levels at the moment of paracentesis, was observed in animals receiving anti-TNF-α mAb (9.93 ± 12.02 vs 47.66 ± 71.14 pg/mL, $p < 0.05$), but neither in animals receiving placebo (20.07 ± 17.48 vs 20.66 ± 19.70 pg/mL) nor in those treated with an IgG isotype control (17.76 ± 11.46 vs 22.66 ± 11.76 pg/mL).

When considering the group of animals receiving placebo (Group II), serum TNF-α levels at laparotomy were slightly higher in animals showing BT ($n = 7$) compared with those without BT ($n = 3$) (24.41 ± 27.22 vs 18.79 ± 17.67 pg/mL). Among animals from Group III, the same tendency was observed when comparing BT ($n = 5$) versus non-BT ($n = 4$) (25.02 ± 14.57 vs 19.71 ± 8.06 pg/mL) (Fig. 2a). Differences in both groups, though, did not reach significance probably due to the reduced number of animals. This comparison could not be undertaken in group I, since just one single animal showed BT.

3.4. Bacterial translocation and serum soluble TNF-α receptor levels are not related

Serum levels of soluble TNF-α receptors I (p55) and II (p75) at the moment of paracentesis were similar among groups.

Table 2
Serum soluble TNF-α receptors (p55 and p75) levels at the moment of paracentesis and laparotomy in all study groups

	Paracentesis	Laparotomy	*p*
Group 1($n = 11$)			
p55 (pg/mL)	134.23 ± 35.73	791.52 ± 508.80	<0.01
p75 (pg/mL)	1457.35 ± 423.05	1863.40 ± 485.43	<0.05
Group II ($n = 10$)			
p55 (pg/mL)	169.36 ± 90.71	813.08 ± 435.62	<0.01
p75 (pg/mL)	1614.29 ± 466.71	2028.75 ± 477.85	ns
Group III ($n = 9$)			
p55 (pg/mL)	133.50 ± 36.42	621.27 ± 63.45	<0.01
p75 (pg/mL)	1310.38 ± 500.16	1870.63 ± 480.22	ns

Values are shown as means ± standard deviation; ns, not significant. *p* values indicate differences between laparotomy and paracentesis for a given parameter.

Table 2 shows serum levels of p55 and p75 at paracentesis and at laparotomy in all studied animals. A statistically significant upregulation during the treatment period was observed for both receptor levels in animals receiving anti-TNF-α mAb (Group I). However, in Groups II and III, only p55 showed a statistically significant increment at laparotomy. A comparison of p55 and p75 serum levels at laparotomy revealed no statistically significant differences between groups.

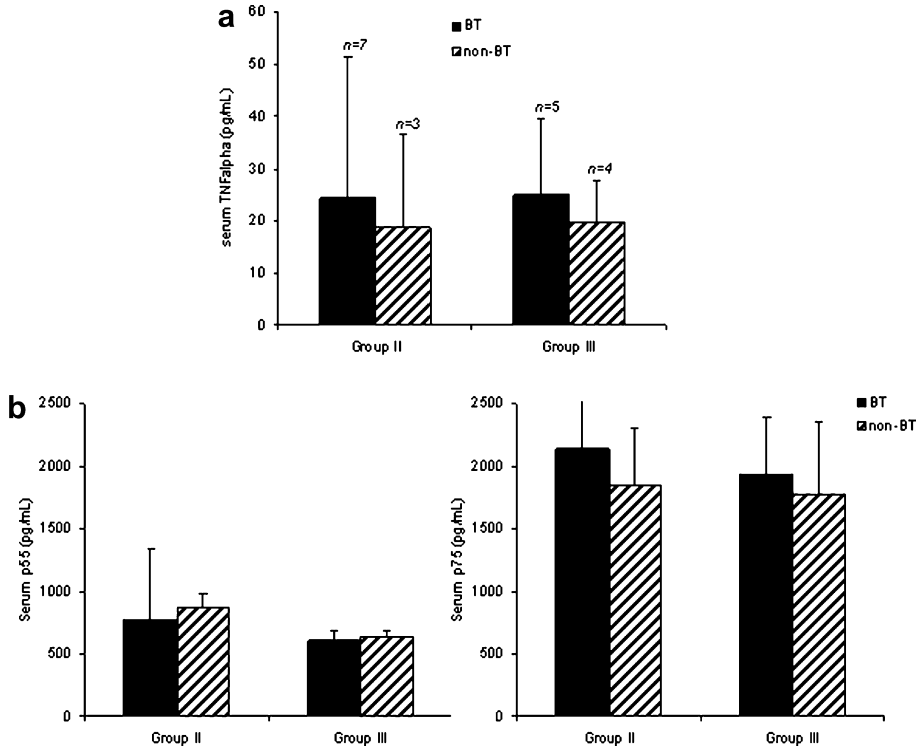

Fig. 2. (a) Serum TNF-α levels in animals from Group II (placebo) and Group III (IgG) at laparotomy, according to presence of bacterial translocation. (b) Serum soluble TNF-α receptor (p55 and p75) levels in animals from Group II and Group III at laparotomy, according to presence of bacterial translocation.

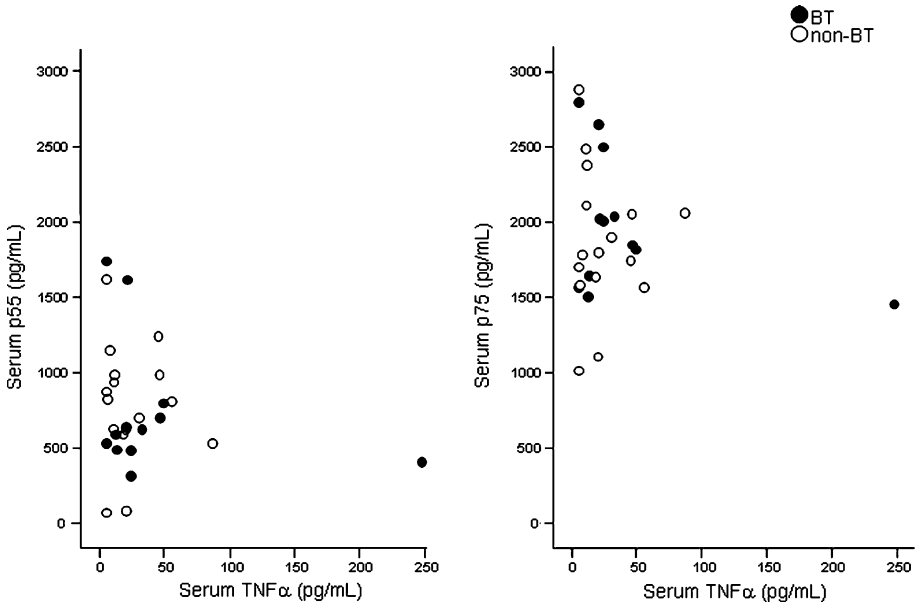

Fig. 3. Lack of correlation between serum levels of TNF-α and soluble TNF-α receptors (p55 and p75), according to presence of bacterial translocation.

Differences in serum levels of both soluble receptors were evaluated at laparotomy according to BT development. Both serum p55 and p75 levels were similar between animals with or without BT in groups II and III (Fig. 2b). As previously mentioned, Group I was excluded from this comparison since just one animal showed BT.

Lack of statistically significant correlation was evidenced between TNF-α and its soluble receptors when animals were studied according to the development of BT (Fig. 3), suggesting that BT is a TNF-α/sTNF-α receptors-independent event in the experimental model of CCl_4-induced rat with cirrhosis and ascites.

4. Discussion

BT is an important event that provides a mechanism for the pathogenesis of bacterial infections in cirrhosis [9,18,19]. Repeated access of bacteria from the intestinal lumen reaching MLNs and, from there, to other territories is the basis of this complex process [9,20] and the start point for the host's immune response activation.

The presence of increased levels of endotoxin has been reported in patients with advanced cirrhosis [7], and its presence induces the synthesis of multiple proinflammatory cytokines such as TNF-α. It has been shown that the injection of endotoxin to experimental animals significantly induces BT [9], and TNF-α blockade in animals subjected to hemorrhagic shock significantly decreases BT [10], suggesting a role for TNF-α in the development of BT.

TNF-α levels observed at admission in patients with sterile ascites that subsequently develop an episode of SBP are significantly higher than those in patients maintaining an sterile ascites during the admission period, as previously described [21–23], suggesting that covert seeding of bacteria or their products in blood and ascitic fluid and subsequent cytokine activation in a subset of patients with sterile ascites might indicate the likelihood of development of an SBP episode. Similarly, a marked activation of the cytokine cascade occurs during the development of spontaneous bacterial peritonitis in cirrhosis and TNF-α levels at diagnosis of infection has been shown to directly relate to the development of significant complications and death [21–23]. It might be then hypothesized that TNF-α blockade could be of interest in this setting, to compete for and to downregulate the amount of this molecule to prevent it from binding to surface receptors present mainly in macrophages and subsequently control the negative effects of an excessive amount of TNF-α. However, TNF-α is part of the normal immune response against bacterial infections and the administration of anti-TNF-α mAb might result in an increased risk of bacterial infection.

This investigation was designed to assess first, if TNF-α blockade might decrease BT in an animal model of cirrhosis and ascites, and secondly, if this therapeutic approach resulted in an increased risk of bacterial infections.

BT was significantly reduced in rats with cirrhosis receiving anti-TNF-α mAb treatment *vs* animals receiving placebo (Fig. 1) and this was not associated with a downregulation of TNF-α levels in animals from Group I. On the contrary, total TNF-α levels were increased during the time-course of the study in this group, between paracentesis and laparotomy. These counterintuitive findings can be explained by the commercially

available TNF-α immunoassay design, that detects both the amount of free TNF-α plus the amount of TNF-α bound to anti-TNF-α mAb. In addition, the slower shedding kinetics of immune complexes formed by TNF-α and anti-TNF-α compared with free TNF-α (US FDA clinical pharmacology review BLA 99-0128, www.fda.gov) could also account for the increased level of TNF-α, which in most part would be inactive, as it would be complexed with the anti-TNF-α mAb administered. The lower amount of TNF-α present in the group treated with placebo, on the other hand, in which the physiologic clearance rate is taking place, must be mostly active, which explains the higher incidence of BT in animals included in this group.

Soluble forms of TNF (sTNF) receptors I and II (p55 and p75) represent truncated forms of the two types of TNF receptors, and arise as a result of shedding of their extracellular domains. Elevated levels of sTNF receptors have been found previously in plasma and ascites of patients in association with infections and malignancies [24]. Although mechanisms are not well understood, there are reports in which increased TNF-α levels also trigger the shedding of TNF receptors [25–27]. This process seems to be independently regulated for each sTNF receptor [25], though, and could explain the differences observed in p55 and p75 in the placebo group at laparotomy in this study. p55 was upregulated at laparotomy in animals with cirrhosis receiving placebo. p75, however, was not significantly increased at laparotomy in this group. These results have been also observed in other studies regarding secretion of TNF-α after spinal cord injury [28]. p55 upregulation, that is, the shedding of TNF RI, could serve as a binding and inhibiting mechanism for competing with cell surface receptors for TNF-α, protecting other cells from the proapoptotic effect of this cytokine and localizing the inflammatory response.

In this study, the administration of anti-TNF-α mAb to animals with cirrhosis has not increased the incidence of systemic infections (Table 1). These findings, however, must be taken with caution, since this investigation has been designed to detect the development of bacterial infections in a short-term fashion. It is not known if different results might be obtained in a long-term setting.

Finally, results and conclusions on effects of anti-TNF-α mAb treatment in animals with cirrhosis and ascites cannot be completely considered without ruling out the possibility of an unspecific response caused by the immunoglobulin Fc portion of the anti-TNF-α monoclonal antibody. The immunomodulatory effect of intravenous immunoglobulin (IVIg) and its mechanisms has been widely reported in the past, including (i) T-cell proliferation and T-cell cytokine production through the inhibition of antigen-presenting cell activity, as it has been shown to inhibit the differentiation and maturation of dendritic cells in vitro [14], (ii) downregulation of costimulatory molecules associated with cytokine secretion, (iii) suppression of NFkB activation and IkB degradation, (iv) effects on the Fas apoptotic pathway (16) and likely others. For these reasons, a third group of animals was treated with an IgG isotype control, in the same doses and conditions as the anti-TNF-α mAb group was, and included in the study. Results show that the IgG control treatment did not decrease the BT rate among these CCl_4-induced rats with cirrhosis and ascites, at least as drastically as the anti-TNF-α mAb treatment did.

In conclusion, an increased production of TNF-α may play a role in the process of BT in rats with cirrhosis and ascites because anti-TNF-α mAb is able to downregulate BT without increasing the incidence of systemic infections in a short-term manner.

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