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The Therapeutic Efficacy of MS473, a Fully Human Single-Chain Variable Fragment Targeting *Staphylococcus aureus* Toxic Shock Syndrome Toxin-1, in a D-Galactosamine-Sensitized Mouse Model of Lethal Shock

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Abstract

Toxic shock syndrome toxin-1 (TSST-1), produced by *Staphylococcus aureus*, is one of the most potent superantigens involved in causing life-threatening TSS and contributes to the onset of some autoimmune diseases. To this end, we have previously identified a fully human single-chain variable fragment antibody (scFv), MS473, exhibiting high binding affinity and specificity for TSST-1 and demonstrating in vitro neutralization activity. In the present study, the therapeutic activity of MS473 was assessed in a D-galactosamine-sensitized mouse model of lethal shock. D-galactosamine-sensitized mice were injected with TSST-1 and then received a single dose of MS473 intraperitoneally (15 mg per kg of mouse body weight) after five minutes or intravenously (3 mg per kg of mouse body weight) after 10 min. The survival rate was examined for seven days. Furthermore, blood samples from different groups of mice were subjected to biochemical assessment, and their kidneys and livers were analyzed histopathologically 24 h after the toxin injection. The findings demonstrated a 100% survival rate with no significant damage to kidney and liver function in the treated groups, receiving MS473 through two different administration routes compared to the control groups, including the toxin-injected mice receiving normal saline or an unrelated scFv. Targeting disseminated TSST-1 with the scFv, which has appropriate permeability and distribution throughout the body, may be an effective way to alleviate the malfunctioning of the immune system caused by TSST-1.

Keywords *Staphylococcus aureus*, Toxic shock syndrome, Superantigens, Toxic shock syndrome toxin-1, Single-chain variable fragment

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Introduction

Staphylococcus aureus, a member of the ESKAPE pathogens, is a significant concern due to its potential to cause life-threatening diseases such as bacteremia, endocarditis, pneumonia, and toxic shock syndrome (TSS) [1–3]. Equally alarming is its potential contribution to the development or exacerbation of immune system-related disorders with high morbidity, such as atopic dermatitis, Kawasaki syndrome, and rheumatoid arthritis [3–6]. This pathogen is armed with various virulence factors, some of which have functional redundancy, giving it an exceptional ability to survive, evade the host immune system, and withstand antibiotics [3, 5, 7]. Operating within the complex system of the host immune system, *S. aureus* employs a sophisticated strategy to suppress its responses, such as inducing apoptosis or anergy in T cells and hindering an appropriate humoral response. The bacterium accomplishes these effects through the production of superantigens (e.g. staphylococcal enterotoxins serotypes A to E and toxic shock syndrome toxin [TSST]−1) that bypass antigen processing in antigen-presenting cells (APCs) via cross-bridging between the major histocompatibility complex (MHC) class II on APCs and the T cell receptors (TCRs) on T cells [6, 8]. This interaction recruits various immune cells, including T cells, monocytes, B cells, and macrophages, leading to the excessive secretion of cytokines and compromising the immune response's effectiveness [6, 9].

The success of monoclonal antibodies (mAbs) in treating malignancies and autoimmune disorders over the past few decades has drawn worldwide attention to efforts focusing on the development of mAbs to be utilized urgently in pandemic infectious diseases or bioterrorism (e.g. *Bacillus anthracis* toxins) [10, 11]. In the fight against bioterrorism, mAbs have long been regarded as one of the most effective biotherapeutics for targeting toxins [6, 12]. With this in mind, the FDA has approved three mAbs for the protective antigen component of the anthrax toxin of *Bacillus anthracis* (Obiltoxaximab and Raxibacumab) and toxin B of *Clostridium difficile* (Bezlotoxumab) [6, 10, 13]. Developing mAbs targeting TSST-1 holds immense potential. Anti-TSST-1 mAbs can prevent the antigen-independent activation of a large subset of T cells and APCs and the subsequent cytokine storm, leading to rare but fatal syndromes (e.g. TSS and sudden infant death syndrome) [6, 12]. Several studies have attempted to identify mAbs against TSST-1, with some showing promising results in vivo models [6, 12, 14–16]. A major point to note is that the Fc domain of an antibody is not involved in neutralizing toxins; therefore, antibody fragments such as single-chain variable fragments (scFvs) may be promising alternatives [6, 12]. An scFv consists only of two variable domains of light and heavy chains of an antibody connected by a peptide linker; however, it can bind to an antigen with appropriate affinity and specificity, has acceptable pharmacokinetics, and does not exhibit significant immunogenicity [11, 17]. In this regard, a list of anti-toxin scFvs has been developed [6, 12, 18, 19], some of which have demonstrated protective activity in vivo [18].

Our previous study found a novel fully human scFv, MS473, with high affinity and specificity for TSST-1, which decreased TSST-1-induced mitogenesis and cytokine release in vitro [6]. In the present study, we initially assessed the administration of the MS473 scFv in healthy mice to determine whether it has a toxic potential. Next, D-galactosamine-sensitized mice injected with TSST-1 were administrated with the MS473 scFv in two different routes and durations to assess its neutralization activity and therapeutic efficacy in a D-galactosamine-sensitized mouse model of lethal shock.

Results

No toxicity potential of the MS473 scFv

The MS473 scFv was administered to healthy mice to evaluate its potential side effects on the kidneys and liver. As shown in Fig. 1A, the administration of MS473—intraperitoneally at a dosage of 15 mg per kg of mouse body weight or intravenously at a dosage of 3 mg per kg of mouse body weight—did not increase blood urea nitrogen (BUN) and creatinine serum levels, comparable to the results observed with normal saline. Histopathological analysis of the kidneys conducted 24 h after the administration of MS473 revealed no signs of damage

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Fig. 1 No toxic effects of the MS473 scFv on the kidneys of healthy mice. Female BALB/c mice (eight mice per group) were administered a single dose of the MS473 scFv through intraperitoneal injection (IP) at 15 mg per kg of mouse body weight or intravenous injection (IV) at 3 mg per kg of mouse body weight. After 24 h, (A) the administration of MS473 did not increase BUN and creatinine serum levels, similar to the results observed with normal saline (NS). (B) Normal architecture was observed in the kidney tissue samples of healthy mice receiving NS or D-galactosamine (D-Gal). However, the mice sensitized with D-galactosamine and injected with TSST-1 (D-Gal + TSST-1) exhibited vacuolation and degeneration of the renal tubular epithelium (asterisks), infiltration of inflammatory cells between the renal tubules (yellow arrows), disorganized appearance of renal architecture, and interstitial hemorrhage (white arrows). No histopathological manifestations were observed in healthy mice receiving the MS473 scFv. Yellow arrowheads indicate the renal corpuscles

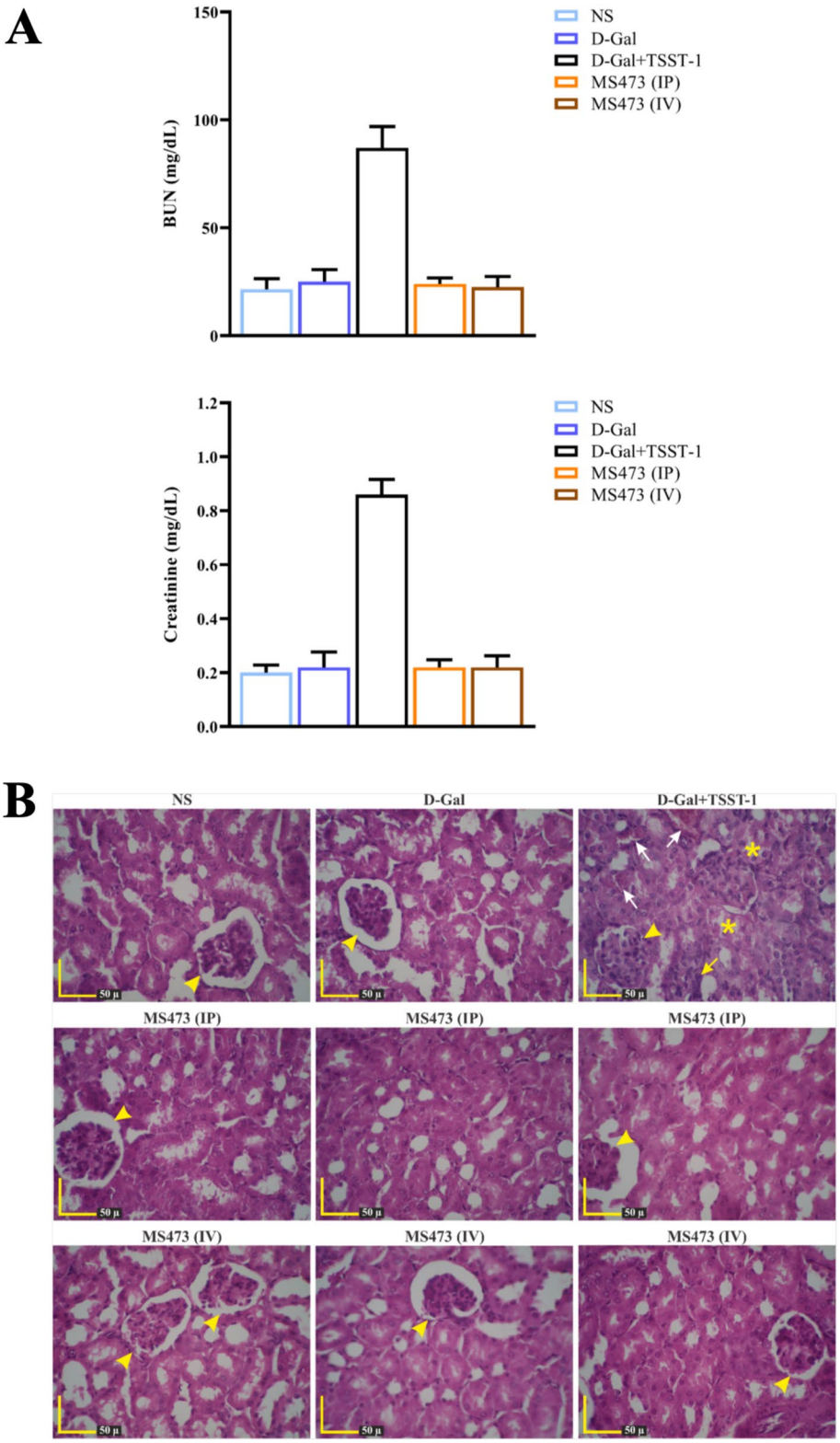

Fig. 1 (See legend on previous page.)

(Fig. 1B). Additionally, there was no increase in serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) (Fig. 2A), and no pathological damage was observed in the liver of the healthy mice that received a single dose of MS473 (Fig. 2B).

The treatment effectiveness of the MS473 scFv in the mouse model of TSST-1-induced lethality

The mouse model of TSST-1-induced lethality was created by injecting TSST-1 at the LD_{50} into D-galactosamine-sensitized mice. Administering the MS473 scFv intraperitoneally at a dosage of 15 mg per kg of mouse body weight after five minutes or intravenously at a dosage of 3 mg per kg of mouse body weight after 10 min resulted in a 100% survival rate after seven days (Fig. 3). The surviving mice showed no weight loss or distress signs during this time. In contrast, D-galactosamine-sensitized mice injected with the LD_{50} of TSST-1 and then received normal saline intraperitoneally showed ruffled fur, obvious weight loss, hunched posture, and lethargy, leading to death (survival rate: 50%). Furthermore, administering 15 mg per kg of EB211 intraperitoneally to mice after five minutes or delivering an intravenous injection of 3 mg per kg of EB211 after 10 min produced clinical signs similar to those observed in TSST-1-injected mice that received normal saline, resulting in a survival rate of 50% (Fig. 3).

To evaluate kidney function, we assessed the BUN and creatinine levels in the sera of mice from different groups 24 h after the toxin injection. As demonstrated in Fig. 4A, the MS473 scFv prevented the increase in serum levels of BUN and creatinine, thereby effectively mitigating the detrimental effects of TSST-1 on kidney function in D-galactosamine-sensitized mice injected with TSST-1. In stark contrast, BUN and creatinine serum levels in TSST-1-injected mice receiving normal saline or EB211 were markedly elevated, indicating significant damage to kidney function (Fig. 4A). Moreover, the kidneys of mice in MS473-treated and control groups were removed 24 h after the toxin injection, and tissue samples were examined histopathologically. The kidneys were predominately unaffected in MS473-treated groups, while vacuolation and degeneration of renal tubular epithelium, infiltration of the inflammatory cells between the renal tubules, disorganized appearance of renal architecture, and interstitial hemorrhage were observed in TSST-1-injected mice receiving normal saline or EB211 (Fig. 4B).

To assess liver function, we measured ALT and AST in the serum of mice 24 h after the toxin injection. As illustrated in Fig. 5A, the group of mice treated with the MS473 scFv showed no significant increase in ALT and AST serum levels compared to TSST-1-injected mice that received normal saline or EB211. This suggests that liver function remained normal, and no notable side effects were observed from administering MS473. Furthermore, histopathologic analysis did not indicate any significant evidence of necrosis or damage to the hepatocyte architecture in the liver of MS473-treated mice. In contrast, necrosis of hepatocytes, extensive disruption of the hepatocyte plates, outright rupture of the plasma membrane, and hemorrhage were remarkably seen in TSST-1-injected mice receiving normal saline or EB211 (Fig. 5B).

The therapeutic effect of the MS473 scFv was evaluated by administering it through two different routes and durations. The scFv was administered intraperitoneally five minutes after the TSST-1 injection and intravenously 10 min after the TSST-1 injection. Despite the higher dose (15 mg per kg of mouse body weight) when administered intraperitoneally, the results were similar to the lower intravenous dose (3 mg per kg of mouse body weight) administered 10 min later. This outcome may be attributed to the improved distribution and bioavailability of the scFv when administered intravenously compared to intraperitoneal administration.

Discussion

The presence of antibodies against TSST-1 in patients is crucial for preventing TSS; however, certain key issues must be considered. The absence of anti-TSST-1 antibodies during acute illness and their development during convalescence does not provide immediate protection for those exposed to TSST-1 for the first time [20]. It should be noted that some patients fail to develop the necessary antibodies, even during convalescence [9]. Last but not least, vulnerable populations,

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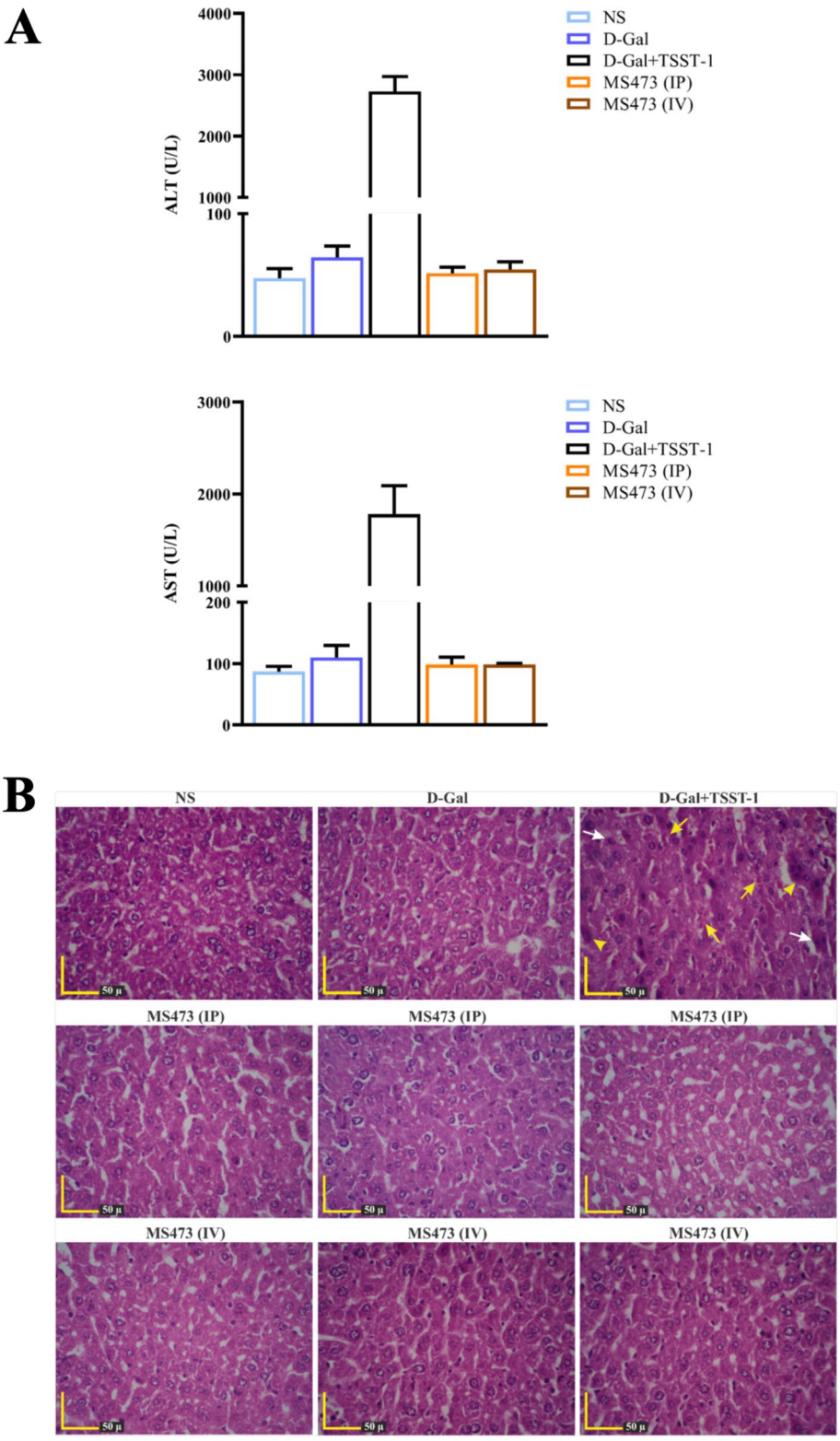
Fig. 2 No toxic effects of the MS473 scFv on the liver of healthy mice. Female BALB/c mice (eight mice per group) were administered a single dose of the MS473 scFv through intraperitoneal injection (IP) at 15 mg per kg of mouse body weight or intravenous injection (IV) at 3 mg per kg of mouse body weight. After 24 h, (A) No increase in ALT and AST serum levels was observed in the mice receiving MS473, similar to the control group receiving normal saline (NS). (B) In healthy mice receiving NS, D-galactosamine (D-Gal), or MS473, the liver tissue structure was normal, and no histopathological changes were observed. However, the liver tissue of D-galactosamine-sensitized mice injected with TSST-1 (D-Gal + TSST-1) showed necrosis of the hepatocytes (white arrows), extensive disruption of the hepatocyte plates, rupture of the plasma membrane (yellow arrowheads), and hemorrhage (yellow arrows)



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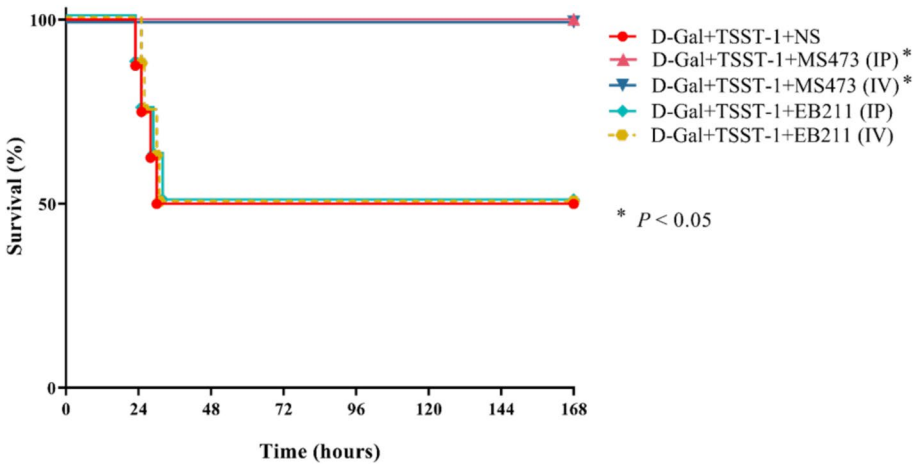

Fig. 3 Passive protection by the MS473 scFv against lethal shock from TSST-1 in a D-galactosamine-sensitized mouse model. A 100% survival rate was achieved after seven days in D-galactosamine-sensitized mice injected with the LD_{50} of TSST-1 and administered a single dose of MS473 (D-Gal + TSST-1 + MS473) intraperitoneally (IP) five minutes after the toxin injection or intravenously (IV) 10 min after the toxin injection. In comparison, D-galactosamine-sensitized mice injected with the LD_{50} of TSST-1 and then received normal saline (D-Gal + TSST-1 + NS) showed a 50% survival rate after seven days. Administering an unrelated scFv intraperitoneally five minutes after the toxin injection or intravenously 10 min after the toxin injection (D-Gal + TSST-1 + EB211) did not improve the survival rate. The asterisks denote statistical significance versus TSST-1-injected mice receiving NS using the log-rank test

such as immunosuppressed patients, premature infants, and the elderly, may not be able to generate a sufficient immune response [5, 21]. Therefore, passive immunization with anti-toxin antibodies holds promise for these patients [5]. Monoclonal antibodies have always been considered one of the most effective biotherapeutics for preventing the binding of TSST-1 to T cells and APCs, thus avoiding antigen-independent interactions [6, 12]. To this end, several studies have been conducted to develop mAbs and antibody fragments against TSST-1 [6, 12, 14, 15]. In our previous study, we identified a fully human scFv with high specificity and affinity binding to TSST-1 ($K_{aff} = 0.4 \times 10^9$ M^{-1}), diminishing TSST-1-induced mitogenicity and multiple cytokines secretion (IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, IL-17A, IFN-γ, TNF-α, G-CSF, and TGF-β) from human PBMCs.

Monoclonal antibodies have significantly advanced the treatment of prevalent global disorders and have proven highly effective in therapy, especially when used with other chemotherapeutics [11]. Nevertheless, their administration to patients may lead to various undesired effects [3]. Even though scFv lacking the Fc domain is not comparable in terms of causing side effects to a whole antibody, we examined the possibility of MS473 causing toxic effects on healthy mice. As expected, no pathological effect was observed on two vital organs, including the kidneys and liver.

The efficacy and protective potential of anti-TSST-1 antibodies are frequently assessed in animal models of

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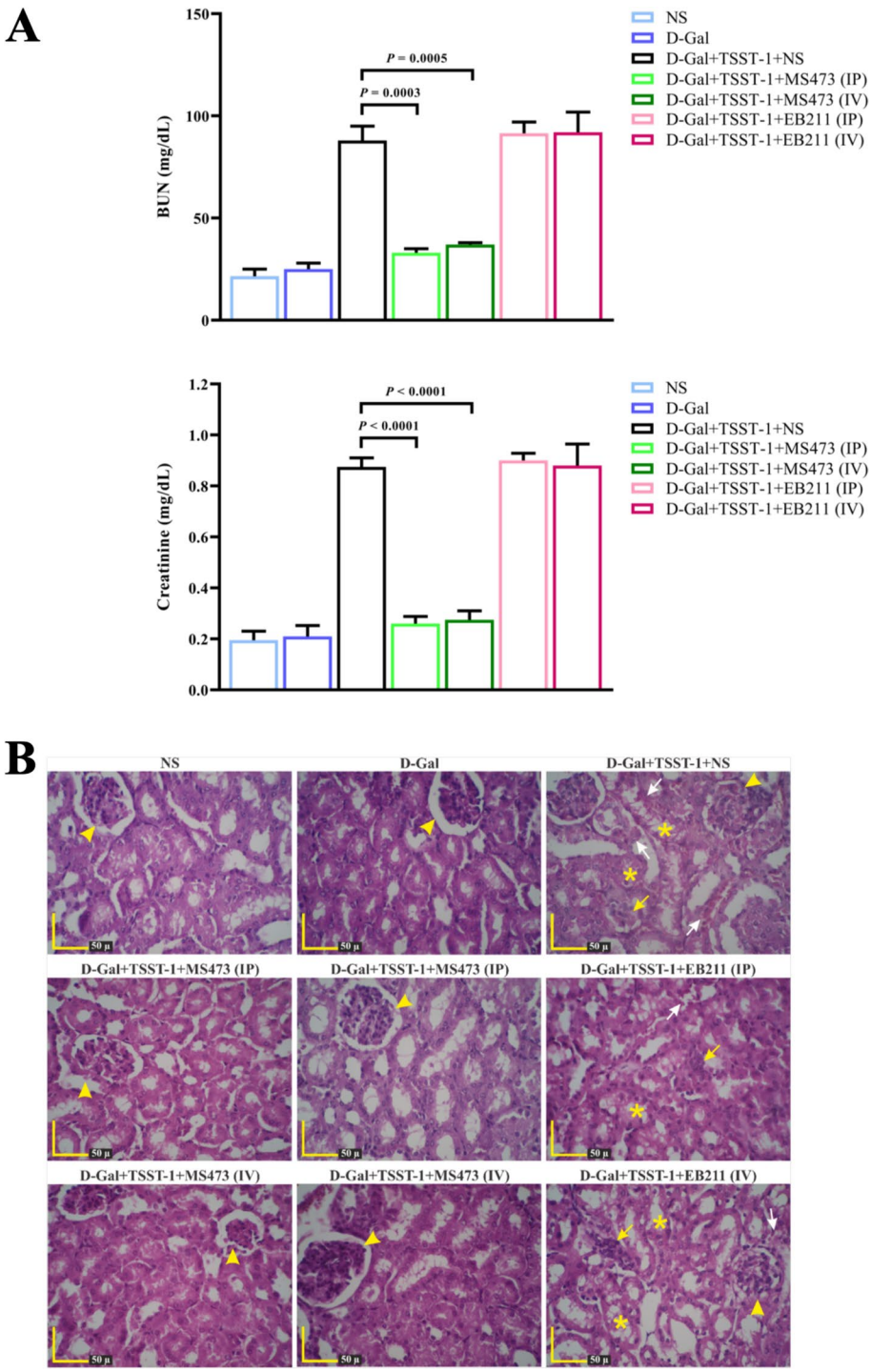
Fig. 4 The protective effect of the MS473 scFv on the kidneys of D-galactosamine-sensitized mice injected with TSST-1. (A) The MS473 scFv effectively prevented increases in serum levels of BUN and creatinine in D-galactosamine-sensitized mice injected with TSST-1 and administered a single dose of MS473 (D-Gal + TSST-1 + MS473) intraperitoneally (IP) five minutes after the toxin injection or intravenously (IV) 10 min after the toxin injection. In contrast, elevated serum levels were observed in D-galactosamine-sensitized mice injected with TSST-1 and then received normal saline (D-Gal + TSST-1 + NS). Furthermore, similar results were found in TSST-1-injected mice that received an unrelated scFv (D-Gal + TSST-1 + EB211) intraperitoneally five minutes after the toxin injection or intravenously 10 min after the toxin injection. Statistical significance was determined by one-way ANOVA, followed by Dunnett's multiple-comparison test against the control group, D-galactosamine-sensitized mice injected with the LD_{50} of TSST-1 and then received NS. (B) Normal architecture was observed in the kidney tissue samples of healthy mice that received NS or D-Gal. In contrast, D-galactosamine-sensitized mice injected with TSST-1 and then received NS or EB211 showed vacuolation and degeneration of the renal tubular epithelium (asterisks), infiltration of inflammatory cells between the renal tubules (yellow arrows), disorganized appearance of renal architecture, and interstitial hemorrhage (white arrows). However, no marked pathological changes were observed in D-galactosamine-sensitized mice injected with TSST-1 and administered a single dose of MS473. Yellow arrowheads indicate the renal corpuscles



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TSST-1-induced lethality [14, 15, 22]. For instance, Bonventre et al. established a rabbit model of toxic shock syndrome to assess the protective effects of a murine anti-TSST-1 mAb designated MAb 8–5–7 [14]. In rabbits receiving a constant subcutaneous infusion of TSST-1, MAb 8–5–7 was administered intravenously just before the toxin pump implant and after 48 h. In addition, the intraperitoneal administration of MAb 8–5–7 was evaluated by inserting an osmotic pump containing the mAb into the peritoneal cavity one day before subcutaneous

placement of the pump, releasing TSST-1. Their study indicated that intravenous bolus and constant intraperitoneal infusion of MAb 8–5–7 inhibited significant changes in serum chemistry and rabbit mortality. Of note, they reported that MAb 8–5–7, administered intraperitoneally, did not provide the same level of protection as the intravenous bolus, possibly due to a reduced insertion of MAb 8–5–7 into the systemic circulation [14]. Similarly, in a study by Kum et al., the protection activity of different mouse anti-TSST-1 mAbs was assessed in two animal models of TSST-1-induced lethality. In the mouse model, each D-galactosamine-sensitized mouse concurrently received an intraperitoneal injection of TSST-1 (0.2 mg per kg mouse body weight) plus different mAbs at five-fold higher molar concentrations than TSST-1. After three days, the mice treated with an anti-TSST-1 mAb designated MAb5 showed significantly higher survival rates than D-galactosamine-sensitized mice treated with a combination of TSST-1 and a control mAb or normal saline [15]. These two studies meticulously evaluated the protective activity of murine mAbs against TSST-1 [14, 15]. This attention to detail led us to investigate the effectiveness of MS473, a fully human scFv against TSST-1, in the mouse model of TSST-1-induced lethality. The MS473 scFv was administered through two different routes (intraperitoneally and intravenously) and at different times (five and 10 min) after the toxin injection instead of being administered before (prophylactic protection) or simultaneously with the toxin challenge. The results showed a 100% survival rate, with no significant alterations in kidney and liver function and minimal histopathological damage, emphasizing the effectiveness of the MS473 scFv.

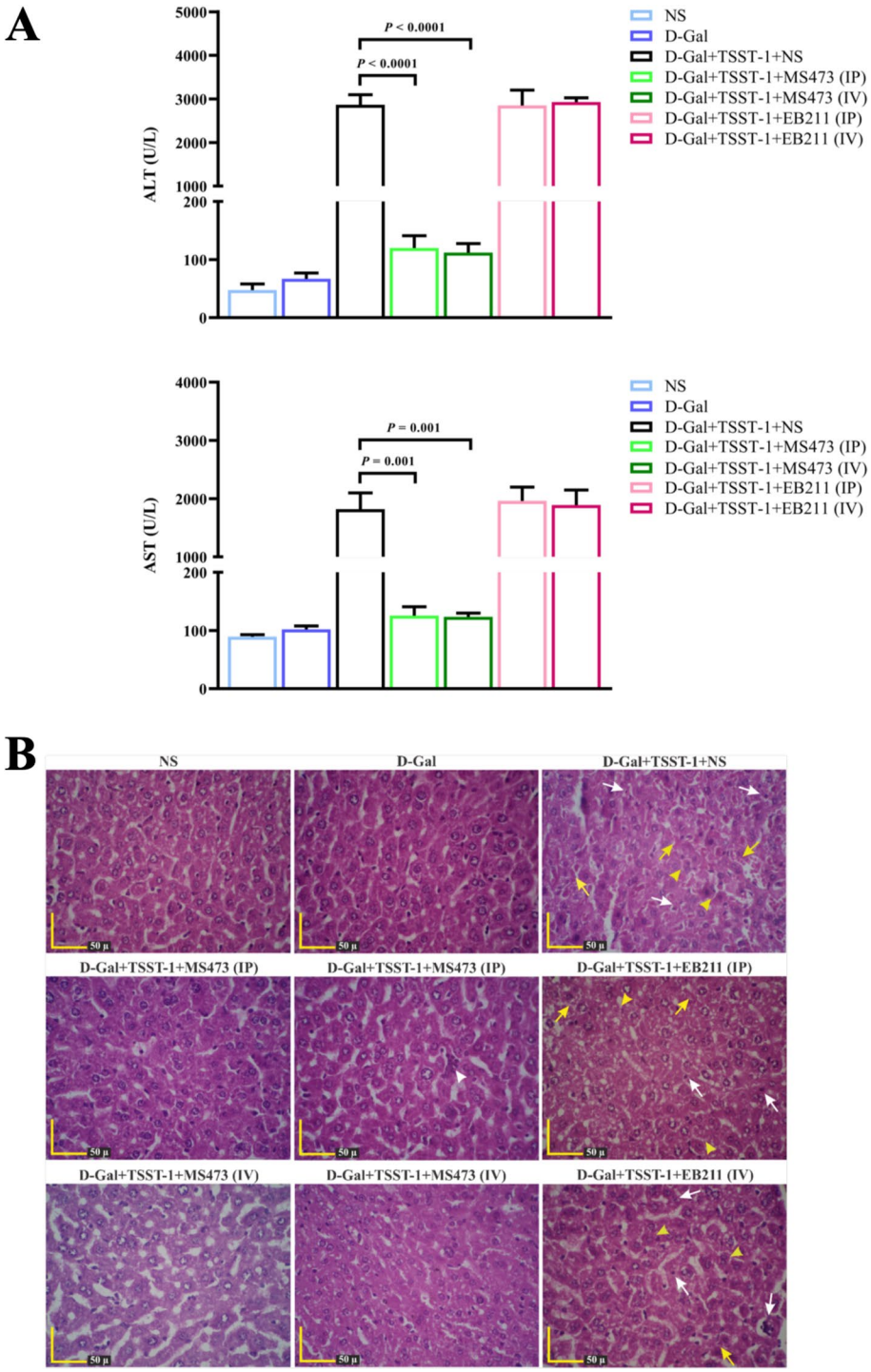
In preclinical and in vivo studies on rodents, the intraperitoneal route of administration is preferred for assessing pharmacodynamics [23]. This route is safe and less stressful for the animal, making it a more ethical choice. Despite the first-pass metabolism (passing through the liver) as a disadvantage of intraperitoneal administration, macromolecules (over 5 kDa) administered intraperitoneally enter the bloodstream directly through lymphatic vessels, bypassing the liver [23]. It is important to note that the absorption rate following intraperitoneal administration is half to a fourth as rapid as intravenous administration. Additionally, the higher volumes used in an intraperitoneal injection induce diuresis and increase the clearance of the drug, leading to a lower area under the curve (AUC) [23]. In line with the points above, it is noteworthy that administering a lower dose of MS473 via intravenous injection 10 min after the TSST-1 injection yielded comparable results to the intraperitoneal administration of the scFv at a higher dose (five-fold) five minutes after the toxin injection. These findings underscore the potential effectiveness of the intravenous route for delivering the scFv.

Conclusions

Our previous study identified an anti-TSST-1 scFv, MS473, which remarkably decreased mitogenesis and cytokine secretion from human PBMCs induced by TSST-1 in vitro. Our current study aimed to evaluate the potentially toxic effects of the MS473 scFv on healthy mice and its protective efficacy against TSST-1-induced lethal shock. The results demonstrated that the MS473 scFv had no toxic effects on healthy mice and effectively mitigated the detrimental effects of TSST-1, leading to a promising survival rate with minimal damage to vital organs. These findings underscore the potential of MS473 as a safe and effective treatment, instilling confidence in its effectiveness and fostering optimism for its future applications. Considering the detrimental effects of TSST-1 on the immune system and the resulting disorders due to immune system dysfunction, deploying this scFv with a suitable safety profile to target disseminated toxins holds promise in preventing the toxic shock syndrome caused by TSST-1.

(See figure on next page.)

Fig. 5 The protective effect of the MS473 scFv on the liver of D-galactosamine-sensitized mice injected with TSST-1. (A) D-galactosamine-sensitized mice injected with TSST-1 did not show increases in serum ALT and AST levels when treated with MS473 (D-Gal + TSST-1 + MS473) intraperitoneally (IP) five minutes after the toxin injection or intravenously (IV) ten minutes after the toxin injection. In contrast, these levels were elevated in D-galactosamine-sensitized mice injected with TSST-1 and then received normal saline (D-Gal + TSST-1 + NS). Furthermore, similar results were found in TSST-1-injected mice receiving an unrelated scFv (D-Gal + TSST-1 + EB211) intraperitoneally five minutes after the toxin injection or intravenously 10 min after the toxin injection. Statistical significance was determined by one-way ANOVA, followed by Dunnett's multiple-comparison test against the control group, D-galactosamine-sensitized mice injected with the LD_{50} of TSST-1 and then received NS. (B) The liver tissue samples from healthy mice receiving NS or D-Gal showed normal architecture. However, necrosis of the hepatocytes (white arrows), extensive disruption of the hepatocyte plates, rupture of the plasma membrane (yellow arrowheads), and hemorrhage (yellow arrows) were observed in D-galactosamine-sensitized mice injected with TSST-1 and then received NS or EB211. On the other hand, when D-galactosamine-sensitized mice were injected with TSST-1 and administered a single dose of MS473, the liver tissue structure was normal. However, slight disorganization of the hepatocyte plates and foci of lobular inflammation (white arrowhead) were observed in some fields



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Methods

Determination of the toxicity potential of the MS473 scFv on healthy mice

Female BALB/c mice (eight to ten weeks; 18–20 g; eight mice per group) were purchased from the Animal Laboratory of the Pasteur Institute of Iran. The mice were administered a single dose of the MS473 scFv through intraperitoneal injection at 15 mg per kg of mouse body weight or intravenous injection at 3 mg per kg of mouse body weight. The control groups were mice receiving

normal saline or D-galactosamine (3.8 mmol per kg of mouse body weight [24]) (Sigma, Steinheim Germany) via intraperitoneal injection. The third control group received D-galactosamine (3.8 mmol per kg of mouse body weight) intraperitoneally, followed by an intraperitoneal injection of TSST-1 (Sigma) at the LD_{50} (0.25 mg per kg of mouse body weight) two hours later [25, 26]. To determine the 50% lethal dose (LD_{50}), TSST-1 was administered to D-galactosamine-sensitized mice at varying doses, ranging from 0.1 to 0.5 mg per kg of mouse body weight [15]. The doses of 0.25 mg and 0.5 mg per kg of mouse body weight resulted in 50% and 100% lethality, respectively (unpublished data).

All the mice were anesthetized with an intraperitoneal injection of ketamine and xylazine (in a 2:1 ratio) at 24 h. They were then bled via cardiac puncture and euthanized through cervical dislocation [27, 28]. Afterward, their kidneys and liver were collected and weighed.

Assessment of the neutralizing ability of the MS473 scFv in the mouse model of TSST-1-induced lethality

Female BALB/c mice (eight to ten weeks; 18–20 g; eight mice per group) received an intraperitoneal injection of D-galactosamine at 3.8 mmol per kg of mouse body weight. After two hours, the mice were injected intraperitoneally with TSST-1 at the LD_{50} of 0.25 mg per kg of mouse body weight to establish a D-galactosamine-sensitized mouse model of lethal shock [25, 26]. Several treatment and control groups were considered to evaluate the therapeutic effectiveness of the MS473 scFv accurately. Two treatment groups were included: D-galactosamine-sensitized mice were administered a single dose of MS473 intraperitoneally five minutes after the TSST-1 injection or intravenously 10 min after—the timing after toxin injection was specifically chosen based on the route of administration. The control groups consisted of mice that received either normal saline or D-galactosamine. Moreover, D-galactosamine-sensitized mice were injected with TSST-1 and then administered normal saline. Another set of control groups included D-galactosamine-sensitized mice that were injected with TSST-1 and then received EB211 (an unrelated scFv antibody targeting *Acinetobacter baumannii)* either intraperitoneally five minutes after the toxin injection or intravenously 10 min after the toxin injection. All the mice were observed for clinical signs, including ruffled fur, weight loss, hunched posture, and lethargy, and the survival rate was recorded over seven days [29].

To evaluate kidney and liver function in TSST-1-injected mice treated with MS473, separate treatment and control groups (eight mice per group) were considered. All mice in the MS473 treatment groups, the mice receiving normal saline or D-galactosamine, and D-galactosamine-sensitized mice injected with TSST-1 and then received normal saline or EB211 (exhibiting clinical signs), were anesthetized using an intraperitoneal injection of ketamine and xylazine 24 h after the toxin injection. They were then bled via cardiac puncture and euthanized by cervical dislocation [27, 28]. Their kidneys and liver were promptly harvested and weighed. Furthermore, the mice that succumbed around 24 h after the toxin injection, due to the severity of their clinical signs, were immediately bled via cardiac puncture, and their kidneys and liver were collected.

Evaluation of kidney and liver function

The blood collected via cardiac puncture was allowed to clot at room temperature for 30 min, followed by centrifugation at 2,000 × g for 10 min. The separated serum was analyzed for several biochemical parameters, focusing on the two main kidney markers—BUN and creatinine—and the key liver enzymes—ALT and AST. All four parameters were measured using the cobas® 6,000 analyzer (c 501 module; Roche Diagnostics, Germany).

Histopathology

Following euthanasia, the kidneys and liver, aseptically removed, were fixed in 10% formalin for 24 h and then embedded in paraffin. After that, thin tissue sections were dyed with hematoxylin-eosin and examined using a Dino-Lite digital lens, Dino Capture 2 Software, and a light microscope [3, 17].

Statistical analyses

All results are presented as the mean ± standard deviation (SD) of data from at least three independent experiments. Statistical significance was determined using a one-way analysis of variance (ANOVA), followed by Dunnett's multiple-comparison test. The survival curve was generated using the Kaplan–Meier method, and significance was assessed using the log-rank (Mantel-Cox) test. The differences were considered statistically significant at $P < 0.05$. GraphPad Prism version 8 software (https://www.graphpad.com/) was used for all analyses.

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Authors' contributions

FRJ supervised and directed the study and wrote the manuscript. HRM performed histopathological analysis. AF, MA, and SDS helped interpret the data. FN helped perform experiments and was involved in the manuscript preparation. MS helped perform experiments. All authors read and approved the final manuscript.

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Data availability
All data generated or analyzed during this study are included in the manuscript.

Declarations

Ethics approval and consent to participate
Animal experiments were conducted in accordance with ARRIVE guidelines (https://arriveguidelines.org) and approved by the Animal Care and Use Committees of the Pasteur Institute of Iran (IR.PII.REC.1398.028). All methods were performed in accordance with relevant guidelines and regulations.

Consent for publication
Not applicable.

Clinical trial number
Not applicable.

Competing interests
The authors declare no competing interests.

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