Vaccination with Plasmid DNA Encoding a Mutant Toxic Shock Syndrome Toxin-1 Ameliorates Toxin-induced Lethal Shock in Mice

Mao-Hui Feng,1,3* Jing-Chun Cui,2,3* Akio Nakane3 and Dong-Liang Hu3,4

1Department of Oncology Surgery, Zhongnan Hospital, Wuhan University, Wuhan, Hubei, P.R. China
2Department of Bio-Engineering, Dalian Nationalities University, Dalian, Liaoning, P.R. China
3Department of Microbiology and Immunology, Hirosaki University Graduate School of Medicine, Hirosaki, Aomori, Japan
4Department of Zoonoses, Kitasato University School of Veterinary Medicine, Towada, Aomori, Japan

Staphylococcal toxic shock syndrome toxin-1 (TSST-1), a superantigenic toxin produced by Staphylococcus (S.) aureus, is a major cause of septic shock and toxic shock syndrome. To investigate whether vaccination with a plasmid DNA encoding a non-toxic mutant TSST-1 (mTSST-1) can protect mice against wild-type TSST-1-induced lethal shock, the mice were intranasally immunized with the plasmid DNA (named pcDNA-mTSST-1) plus a mucosal adjuvant, a non-toxic mutant labile toxin (mLT). After the immunization, the mice were challenged with TSST-1 and lipopolysaccharide (LPS). The survival rate of mice immunized with pcDNA-mTSST-1 plus mLT was higher than that of the control mice immunized with PBS alone, mLT alone, pcDNA-mTSST-1 alone, or a parent plasmid plus mLT. The titers of interferon-γ (IFN-γ) in the sera of mice immunized with pcDNA-mTSST-1 plus mLT were significantly lower than those of the mLT control mice. Immunization with pcDNA-mTSST-1 plus mLT increased the serum levels of TSST-1-specific antibodies, especially immunoglobulin G1 (IgG1) and IgG2a subclasses. Furthermore, the sera obtained from mice immunized with pcDNA-mTSST-1 plus mLT significantly inhibited the TSST-1-induced secretion of IFN-γ and tumor necrosis factor-α (TNF-α) in murine spleen cells in vitro. These results indicate that immunization with pcDNA-mTSST-1 plus mLT provides protection against the lethal toxic shock of mice induced by wild-type TSST-1. The protective effect could be due to TSST-1-specific neutralizing antibodies as well as the inhibition of IFN-γ and TNF-α secretions. Since TSST-1 is commonly released by invasive S. aureus, the pcDNA-mTSST-1 should be useful in preventing toxin-induced shock resulting from S. aureus infection.

Keywords: infection; mucosal immunization; Staphylococcus aureus; superantigen; toxic shock syndrome toxin

Introduction

Staphylococcus (S.) aureus is an important bacterial pathogen in human infections, ranging from superficial skin infections to invasive and potentially life-threatening infections, such as bacteremia, endocarditis, pneumonia and toxic shock syndrome (Hu et al. 2003; Maree et al. 2007). S. aureus is also an important pathogen in animals that causes diverse infectious diseases such as mastitis, dermatitis and arthritis (Fitzgerald et al. 2001; Foster 2012; Allard et al. 2013). In addition, emergence of antibiotic resistance among clinical isolates has made treatment of staphylococcal infections difficult. The growing prevalence of antibiotic resistant S. aureus strains threatens the effectiveness of current strategies for managing S. aureus infection and demonstrates the need for other means of controlling and preventing staphylococcal infections (Etz et al. 2002; Sheen et al. 2011).

S. aureus produces several superantigenic toxins that play important roles in establishing and maintaining infections (Dinges et al. 2000). Staphylococcal toxic shock syndrome toxin-1 (TSST-1) and staphylococcal enterotoxins (SEs) are pyrogenic toxin superantigens produced by pathogenic strains of S. aureus. These toxins are single-chain protein toxins of 19 to 29 kDa and have very similar three-dimensional structures that include both A and B domains (Dinges et al. 2000). TSST-1 and SEs are the causes of toxic shock syndrome (TSS) that manifests as fever, rash,
desquamation and hypotension (Musser et al. 1990; Jarraud et al. 2002). Previous studies suggest that atopic dermatitis and mastitis might be associated with TSST-1 (Fitzgerald et al. 2001; Foster 2012; Allard et al. 2013). TSST-1 can directly bind both major histocompatibility complex (MHC) class II molecules on antigen-presenting cells and the receptor of T-cells (TCR), thereby leading to massive proliferation of T cells and uncontrolled release of proinflammatory cytokines, including interleukin-1 (IL-1), IL-2, IL-6, IFN-γ, and TNF-α. In contrast, TSST-1 does not induce the secretion of anti-inflammatory cytokines such as IL-4 and IL-10 (Jupin et al. 1988; Marrack and Kappler 1990; Kotzin et al. 1993).

TSST-1 is commonly secreted by invasive S. aureus isolates, and especially methicillin-resistant S. aureus (MRSA) strains, and can cause severe pathologies. It is necessary to develop vaccines and therapeutic approaches that are capable of eliminating their toxicity (Schlievert 2001; Narita et al. 2008). Several reports have described toxicity and biological activity of wild-type and/or mutant SEs and TSST-1, and have shown that genetically altered SEs were highly immunogenic in mice and rhesus monkeys (Bavari et al. 1996; Ulrich et al. 1998). These recombinant SEs elicited neutralizing antibodies against wild-type SEs. The non-toxic mutant TSST-1 (mTSST-1) carries the alanine residue at position 135 instead of histidine and does not bind to TCR (Cullen et al. 1995). The mTSST-1 was shown to be less toxic because of the decrease in mitogenic and cytokine-releasing activities as well as the reduced mortality of laboratory animals (Bonventre et al. 1995; Hu et al. 2003).

DNA vaccines are easier for preparation, handling and long-term storage. In addition, they are suitable for intranasal immunization which is a non-invasive and safe method for administration. Therefore, in the present study, we constructed a plasmid DNA which encodes mTSST-1 (named pcDNA-mTSST-1), and investigated whether vaccination with pcDNA-mTSST-1 could protect mice against toxin-induced lethal shock. Our results show that intranasal vaccination with pcDNA-mTSST-1 gives rise to protection against the toxin-induced lethal shock and suggest that the protective effect could be due to the TSST-1-specific neutralizing antibody and the inhibitory effect on the production of inflammatory cytokine.

**Materials and Methods**

**Bacterial strains and growth condition**

*Staphylococcus aureus* strain 834, a clinical septic isolate that expresses TSST-1 and staphylococcal enterotoxin C2 (SEC2) was used (Nakane et al. 1995). *Escherichia coli* DH5α (Toyobo Co., Ltd., Tokyo, Japan) was grown in Luria broth (LB) at 37°C with shaking (110 rpm). Ampicillin (100 µg/ml) was used to maintain plasmids in *E. coli*. The *E. coli* DH5α containing pGXTSST plasmid, which expresses nontoxic mTSST-1, was grown in 2 × YTA medium (BD Bacto, Sparks, MD, USA) containing 100 µg/ml ampicillin at 37°C with shaking (Hu et al. 2003).

**Construction of plasmid DNA encoding non-toxic mTSST-1**

To construct a plasmid DNA, which can express in eukaryotic cells as a vaccine candidate, plasmid pGXmTSST (Hu et al. 2003) and pcDNA6B (In Vitrogen Co., Carlsbad, CA, USA), an eukaryotic expression plasmid, were purified by QIAPrep® Spin Miniprep kit (QIAGEN K.K., Tokyo, Japan) and were digested with EcoRI and SauI. The fragments of mTSST-1 gene from pGXMSTST were cloned into the pcDNA6B, and the resulting plasmid, named pcDNA-mTSST-1, was used for transformation of *E. coli* DH5α cells. The pcDNA-mTSST-1 plasmid was purified from the isolated clone culture by QIAprep Mega kit (QIAGEN) according to the manufacturer’s instruction.

**Expression of pcDNA-mTSST-1 in eukaryotic cells**

Fifteen µg of pcDNA-mTSST-1 DNA was added and mixed in HEK 293 cells (1 × 10⁷ cells), a human embryonic kidney cells. The mixture was placed aseptically into 0.4 cm wide electroporation cuvette, and electrical pulse was carried out. The cells were promptly returned to the culture flask with Dulbecco’s Modified Eagle Medium (DMEM) and cultured in CO₂ incubator at 37°C for 48 h. The cells were harvested and lysed with CelLyte-M Cell Lysis Reagent (Sigma-Aldrich, Corp., St. Louis, MO, USA). Expression of mTSST-1 was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting analysis.

**Purification of TSST-1**

Overnight cultures of *E. coli* DH5α harboring the pGXTSST plasmid were diluted 1:50 in 1 liter of 2 × YTA medium containing 100 µg/ml ampicillin. TSST-1 expression was induced by isopropyl-β-thiogalactopyranoside. After 3-h induction, the bacteria were collected, and lysed. Purification of TSST-1 was performed as in the previous study (Hu et al. 2003).

**Immunization and challenges of mice**

BALB/c mice, 6-8 week-old, were purchased from Animal Experiment Center, Dalian Medical University (Production license: SCXK (Liao) 2008-0002). The mice were kept on a cycle consisting of 12 h of light and 12 h of darkness. Food and water were available at all time. One hundred µg of purified pcDNA-mTSST-1, or pcDNA6B, was emulsified with 10 µg of mutant labile toxin (mLT) of *E. coli*, a mucosal immune adjuvant, in a total volume of 25 µl of dilution buffer (Hu et al. 2006). There were five groups of treated mice: 1) PBS alone, a solution control; 2) mLT alone, an adjuvant control; 3) pcDNA-mTSST-1 alone, a non-adjuvant control; 4) pcDNA6B plus mLT combination, a plasmid vector control; 5) pcDNA-mTSST-1 plus mLT combination, a DNA vaccine group. All mice were immunized intranasally and booster immunizations were performed 1 and 2 weeks after the initial vaccination. After the anti-TSST-1 antibodies in the sera of mice were confirmed (showing in the following section), the mice were challenged with a lethal dose of wild-type TSST-1 (15 µg/animal) and lipopolysaccharide (LPS, *E. coli*-derived; 80 µg/animal, Sigma-Aldrich) intraperitoneally on day 42 after the last boosting. The deaths of mice were recorded over 5 days. Animal challenged with TSST-1 plus LPS is a well-recognized lethal toxic shock animal model, because TSST-1 alone or LPS alone do not induce lethal shock (Hu et al. 2003).
mTSST-1 DNA Vaccine Protects against Lethal Shock

Determinations of cytokine titers in sera of the immunized and challenged mice

IFN-γ and IL-10 in sera of mice were determined by double-sandwich enzyme-linked immunosorbent assays (ELISAs). Briefly, microplates were coated with rat anti-mouse IL-10 monoclonal antibody (MAB) or rat anti-mouse IFN-γ MAB in 0.05 M carbonate buffer (pH 9.6) at 4°C overnight. After blocking with 5% non-fat milk in PBS (PBSM), the wells were incubated with 100 µl of each sample (you means each sample of the above five group mice) in triplicate overnight at 4°C and then exposed to biotinylated rabbit anti-mouse IL-10 MAB or rabbit anti-mouse IFN-γ antibody (Hu et al. 2003; Cui et al. 2005). Avidin-biotinylated peroxidase complex and o-phenylenediamine substrate were added to each well for color development. The reaction was measured at 490 nm in an ELISA plate reader.

Assay of specific anti-TSST-1 antibody

The serum samples from vaccinated mice were obtained on day 35 (5 weeks) after the last boosting, since our preliminary experiments began to show a high antibody production in the sera of mice at this time point. mTSST-1 still has the same antigenic epitope with wild-type TSST-1. As a result, vaccination with pcDNA-mTSST-1 can induce anti-TSST-1 antibody production in the serum of the mice. TSST-1-specific antibody in the serum of mice was measured by ELISA. Briefly, microplates were coated with wild-type TSST-1 (10 µg/ml) in 0.05 M carbonate buffer (pH 9.5) overnight at 4°C. The plates were blocked with PBSM. Serum samples were added to wells for 2 h at 37°C. Goat anti-mouse immunoglobulin G1 (IgG1), IgG2a, IgG2b, IgG3, IgA or IgM (Southern Biotechnology Associates, Inc., Birmingham, AL, USA) were added for 2 h at 37°C. Wells were washed and a substrate solution containing o-phenylenediamine and H2O2 were added for color development. The reaction was measured at 490 nm in an ELISA plate reader.

Assay of neutralization

To determine whether anti-TSST-1 serum can neutralize wild-type TSST-1 that induces cytokine production in spleen cells in vitro, we prepared spleen cells (1 × 106 cells/ml) from non-treated naïve mice. Anti-TSST-1 serum or control serum was pre-incubated with TSST-1 at 37°C for 1 h and then was added to the spleen cell cultures. For the control of cytokine production, wild-type TSST-1 alone without serum was also added to the spleen cell cultures. After 72 h of incubation at 37°C in a 5% CO2 incubator, each culture supernatant was collected. IFN-γ and TNF-α titers in the supernatants were determined by double-sandwich ELISAs as described previously (Nakane et al. 1995). The neutralizing activity of the sera was expressed as the ratio of cytokine production of TSST-1 incubated with the sera to TSST-1 alone without sera.

Statistical analysis

Data were expressed as means ± standard deviations (S.D.), and the Mann-Whitney U-test was used to determine the significance of the differences in antibody production and cytokine titers between control and experimental group. For survival experiments, Kaplan-Meier method was applied to obtain the survival fractions, and significance was determined by log rank test.

Results

Expression and characteristics of pcDNA-mTSST-1

We constructed a plasmid DNA, pcDNA-mTSST-1, which encodes non-toxic mutant TSST-1. The plasmid DNA was confirmed by DNA sequence and restriction enzyme analysis (Fig. 1A). The expression of mTSST-1 was characterized in HEK293 cells by Western blot analysis with specific anti-TSST-1 antibody. The results showed that mTSST-1 protein was expressed in the eukaryotic cells (Fig. 1B), and indicated that mTSST-1 protein encoded by pcDNA-mTSST-1 retains the same antibody-binding epitopes as wild-type TSST-1.

To confirm whether mTSST-1 has any superantigenic toxicity, we treated mice with the mTSST-1 (10 µg) plus LPS (80 µg), or wild-type TSST-1 (10 µg) plus LPS (80 µg), and determined the mortality rate. In striking contrast to 78.6% (11/14 mice) mortality rate of wild-type TSST-1 plus LPS, none of the mice (0/12 mice) died when they were given an equivalent dose of mTSST-1 plus LPS (data not shown). Thus, the lack of toxicity of mTSST-1 was confirmed in LPS-treated mice, consistent with previous results (Hu et al. 2003).
Effect of vaccination with pcDNA-mTSST-1 on host resistance against lethal toxic shock

Five groups of mice were respectively immunized intranasally 3 times with PBS alone, mLT alone, pcDNA-mTSST-1 alone, pcDNA-mTSST-1 plus mLT, or pcDNA6B plus mLT, and then were challenged intraperitoneally with a lethal dose of wild-type TSST-1 plus LPS on day 42 after the last boosting. At 72 h after challenge, 66.7% (8/12) of the mice vaccinated with pcDNA-mTSST-1 plus mLT survived (Table 1). In contrast, the survival rates for the mice injected with PBS alone, the mice injected with mLT alone, the mice injected with pcDNA-mTSST-1 alone, and the mice injected with pcDNA6B plus mLT were 0%, 18.2%, 27.3% and 12.5%, respectively (Table 1). These results indicate that vaccination with pcDNA-mTSST-1 plus mLT gives rise to significant protection against the lethal toxic shock. Because of these results, we mainly analyzed the group vaccinated with pcDNA-mTSST-1 plus mLT and the group administrated with mLT alone, the adjuvant control in the remaining experiments of our current study.

Effect of vaccination with pcDNA-mTSST-1 on cytokine responses after toxin challenge

To further investigate the effect of vaccination with pcDNA-mTSST-1 on cytokine production in sera of mice after challenge with wild-type TSST-1 plus LPS, we immunized mice with pcDNA-mTSST-1 plus mLT or mLT alone. The mice were then challenged with a lethal dose of wild-type TSST-1 plus LPS intraperitoneally. At 4, 8, 12, 24 h after toxin challenge, a representative Th1 type inflammatory cytokine (IFN-γ) and a Th2 type anti-inflammatory cytokine (IL-10) in the sera were determined by sandwich ELISA (Fig. 2). IFN-γ in the sera of mLT control mice increased from 4 h, showed higher titers and peaked at 8 h after toxin challenge. In contrast, the titers of IFN-γ in pcDNA-mTSST-1-immunized mice showed a small peak at 12 h, and were significantly lower than those of the control mice at 8, 12 and 24 h after toxin challenge (Fig. 2A). Interestingly, the titers of IL-10 in the sera of pcDNA-mTSST-1-vaccinated mice increased from 4 h to 12 h, and were significantly higher than those of control mice (Fig. 2B).

Antibody production in pcDNA-mTSST-1-vaccinated mice

Antibody responses were evaluated to analyze the protective mechanism resulting from pcDNA-mTSST-1 immunizations. ELISA plates, coated with wild-type TSST-1, were used to determine the antibody production. A strong IgG antibody response to TSST-1 was shown in the sera obtained from mice immunized with pcDNA-mTSST-1 plus...
mLT (Fig. 3). The levels of IgG1 and IgG2a were higher than other antibody subclasses. In contrast, the sera from mice administrated with mLT alone failed to react to TSST-1.

Neutralizing effect of immunized sera on cytokine production induced by TSST-1 in vitro

We further analyzed the effect of sera from pcDNA-mTSST-1 plus mLT-vaccinated mice on IFN-γ and TNF-α production induced by wild-type TSST-1 in naïve murine spleen cell cultures. Serum samples from the mice immunized with pcDNA-mTSST-1 plus mLT effectively inhibited IFN-γ and TNF-α production induced by TSST-1, compared with serum samples from the mice administrated with mLT alone (P < 0.05; Fig. 4). These results suggest that the inhibition of inflammatory cytokine production may be involved in the protective effect obtained from immunization with pcDNA-mTSST-1 plus mLT.

Discussion

*S. aureus* infection has a significant economic impact on health care (Maree et al. 2007; Allard et al. 2013). A variety of *S. aureus* preparations have been investigated as vaccines to prevent staphylococcal infections in human and veterinary clinical trials. None of them showed convincing benefit in patients, animal models or farm animals (Michie...
Several studies have demonstrated that toxoid vaccines, including non-superantigenic SEs, TSST-1 and streptococcal pyrogenic exotoxins, may protect against *S. aureus* infection and sepsis (Stiles et al. 2001; Gampfer et al. 2002; Hu et al. 2003). Nilsson et al. (1999) demonstrated that immunization with non-superantigenic SEA protected *S. aureus*-induced lethal septic shock. Gampfer et al. (2002) demonstrated that vaccination with non-superantigenic TSST-1 developed antibody responses against these toxins and protected against challenge with lethal doses of superantigen potentiated with LPS. Our previous studies have described the biological activity and characteristics of mTSST-1 (Hu et al. 2003; Cui et al. 2005). Since the DNA vaccine is easier for preparation, transport and suitable for long-term storage, we investigated whether vaccination with a DNA vaccine, pcDNA-mTSST-1, could protect mice against superantigenic toxin-induced lethal shock in this study. Our results showed that pcDNA-mTSST-1 vaccine is highly effective in inducing toxin-specific antibodies that are capable of neutralizing superantigenicity, decreasing proinflammatory cytokine production and protecting mice from lethal toxic shock.

In the present study, mice immunized with pcDNA-mTSST-1 plus mLT produced high titers of TSST-1-specific antibodies, especially higher levels of IgG1 and IgG2a. The sera from pcDNA-mTSST-1-immunized mice also significantly inhibited the IFN-γ and TNF-α production in murine spleen cells stimulated by wild-type TSST-1. Furthermore, the immunization also decreased serum IFN-γ level and increased IL-10 level after challenged with wild-type TSST-1 in mice. These results indicated that the vaccination with pcDNA-mTSST-1 plus mLT could induce Th2-type immune responses and production of the specific anti-TSST-1 antibodies. IL-10 and TSST-1-specific antibodies might play an important role in host resistance against superantigenic toxin induced lethal shock (Hu et al. 2003; Narita et al. 2008). Immunization with pcDNA-mTSST-1 alone without mLT did not induce significant immune response, suggesting that mLT, a mucosal adjuvant, is important for enhancing the cellular uptake of the plasmid and the immunity-inducing activities (Hu et al. 2006; Narita et al. 2008).

Previous studies indicated that the neutralizing activities of antibodies to staphylococcal superantigens could play an important role in the protective effects against systemic *S. aureus* infection and septic shock (Nilsson et al. 1999; Hu et al. 2003, Narita et al. 2008). The mechanism of TSST-1-immunization that protects against toxic shock and infection induced by *S. aureus* remains elusive. The possible explanations include the anti-inflammatory activity of the specific antibodies (Schlievert 2001; LeClaire et al. 2002), and the neutralization of the toxicity of *S. aureus*-surface molecules and/or secreted products (Nilsson et al. 1999; Schlievert 2001; Spaulding et al. 2012). Previous studies reported that there are cross-reactive antibodies among TSST-1, SEs and Streptococcal pyrogenic exotoxins A (Bohach et al. 1988; Ulrich et al. 1998; Bavari et al. 1999). Mice vaccinated with TSST-1 survived when challenged with staphylococcal enterotoxin A (SEA), SEB, or SEC. Anti-TSST-1 MAb also cross-inhibits SEA-induced

---

**Fig. 4.** Inhibitory effects of anti-mTSST-1 sera on cytokine secretion in spleen cells induced by wild-type TSST-1. Spleen cells were obtained from naïve mice. Wild-type TSST-1 was incubated with the sera (diluted 1:5 and 1:10) from mice vaccinated with pcDNA-mTSST plus mLT, or with mLT alone at 37°C for 1 h and then was added to the spleen cell cultures. The titers of IFN-γ (A) and TNF-α (B) were determined by ELISAs. The inhibitory effects were expressed as the ratio of cytokine production of TSST-1 incubated with the serum to TSST-1 alone without serum. The results are representative of two experiments, and the data are means ± s.d. based on five or six mice for each group. An asterisk indicates that the value is significantly different from the value obtained for the mice immunized with mLT alone (*P* < 0.05).
mitogenic activity and TNF-α secretion in vitro and protects against SEA-induced lethality in mouse model (Kum and Chow 2001). Our present study demonstrated that intranasal immunization with pcDNA-mTSST-1 vaccine profoundly altered the course of disease by neutralizing the pyrogenic superantigenic toxins, decreasing the production of proinflammatory cytokines. Since expression of TSST-1 and SEs is common among invasive S. aureus isolates, this non-toxic pcDNA-mTSST-1 and the specific antibodies could be useful in the treatment of toxic shock caused by S. aureus infection.

Acknowledgements

This study was supported in part by The Fund for the Promotion of International Scientific Research (The Karouji Medical Foundation B2), Japan, and The National Natural Science Foundation of China (30671554).

Conflict of Interest

The authors declare no conflict of interest.

References


