Vaccination with Nontoxic Mutant Toxic Shock Syndrome Toxin 1 Protects against *Staphylococcus aureus* Infection

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To investigate whether vaccination with nontoxic mutant toxic shock syndrome toxin 1 (mTSST-1) can protect against *Staphylococcus aureus* infection, mice were vaccinated with mTSST-1 and challenged with viable *S. aureus*. Survival in the mTSST-1–vaccinated group was higher, and bacterial counts in organs were significantly lower than those of control mice. Passive transfer of mTSST-1–specific antibodies also provided protection against *S. aureus*–induced septic death. Interferon (IFN)–γ production in the serum samples and spleens from vaccinated mice was significantly decreased compared with that in controls, whereas interleukin-10 titers were significantly higher in vaccinated mice. IFN-γ and tumor necrosis factor–α production in vitro were significantly inhibited by serum samples from mTSST-1–immunized mice but not from control mice. These results suggest that vaccination with mTSST-1 devoid of superantigenic properties provides protection against *S. aureus* infection and that the protection might be mediated by TSST-1–neutralizing antibodies as well as by the down-regulation of IFN-γ production.

*Staphylococcus aureus* is a frequently isolated bacterial pathogen in both hospital- and community-acquired infections; it causes diverse infections, ranging from superficial skin infections to invasive and potentially life-threatening infections, such as endocarditis, osteomyelitis, septic arthritis, pneumonia, and abscesses [1]. *S. aureus* is also a significant pathogen in economically important animals [2–4]. In addition, the 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. This scenario has sparked renewed interest in the development of immunotherapeutics and vaccines [5–7].

*S. aureus* produces several exotoxins that play important roles in establishing and maintaining infections [8]. Toxic shock syndrome toxin 1 (TSST-1), a pyrogenic toxin superantigen produced by pathogenic strains of *S. aureus*, is best known for its involvement in toxic shock syndrome (TSS), which manifests as fever, rash, desquamation, and hypotension [9–12]. It has been responsible for 90% of menstrual and 50% of nonmenstrual TSS cases [13]. The results of recent studies have suggested that at least a portion of atopic dermatitis [14], Kawasaki syndrome [15, 16], some forms of psoriasis, neonatal TSS-like exanthematous disease [17], and other illnesses might be associated with TSST-1. TSST-1 can directly bind to the major histocompatibility complex class II molecules on antigen-presenting cells and to T cell receptors bearing specific Vβ elements [18, 19]. This subsequently leads to a massive proliferation of T cells and the uncontrolled release of proinflammatory cytokines, including interleukin (IL)–1, IL-2, IL-6, interferon (IFN)–γ, and...
tumor necrosis factor (TNF)–α [18–20], which causes life-threatening TSS [16, 21–24].

Because TSST-1 is common among invasive *S. aureus* isolates, especially methicillin-resistant *S. aureus* strains, and it can cause severe pathologies, there is considerable need to develop vaccines and therapeutic approaches capable of eliminating its toxicity [14]. The mutagenesis of potential T cell receptors and major histocompatibility complex class II binding sites is a central effort of modern vaccine strategies and has been extensively used during the past 10 years. Several reports have described toxicity and biological activity of wild type and mutant staphylococcal enterotoxin A (SEA), staphylococcal enterotoxin B (SEB), and TSST-1 and showed that genetically altered SEA and SEB inactivated by site-directed mutagenesis and lacking superantigenic effects were highly immunogenic in mice and rhesus monkeys [25–28]. These recombinant vaccines elicited neutralizing antibodies against wild-type SEA and SEB. The mutation of histidine at position 135 to alanine negatively affects the binding of TSST-1 to the T cell receptors [29–32]. Mutant TSST-1 molecules were shown to be less toxic, as has been documented in vitro by a decrease in mitogenic and cytokine-releasing capacity as well as by the reduced mortality rate of laboratory animals [30, 32–35]. The results of previous studies have shown that immunization with recombinant and/or mutant SEA, SEB, and TSST-1 could protect mice or rabbits against lethal shock induced by the wild-type superantigenic toxin [25, 26, 36–38].

For the present study, we were interested in whether vaccination with mutant (m) TSST-1 could protect against systemic *S. aureus* infection in a mouse model and the mediators of that protection.

**MATERIALS AND METHODS**

**Animals.** BALB/c mice, 6–8 weeks old, were purchased from Clear Japan. The mice were housed in plastic cages under specific pathogen–free conditions at the Institute for Experiments, Hiroasaki University School of Medicine. They were kept on a 12 h:12 h light:dark cycle, and food and water were available at all times. All animal experiments were carried out in accordance with the Guidelines for Animal Experimentation of Hiroasaki University.

**Bacterial strains.** *S. aureus* 834 is a clinical septic isolate that expresses TSST-1 and staphylococcal enterotoxin C2 (SEC2) [39]. For the preparation of genomic DNA, *S. aureus* was inoculated into 5 mL of soybean-casein-digest broth (Nissui) and grown overnight at 37°C with shaking (at 110 rpm). For infection, *S. aureus* 834 was cultured at 37°C in tryptic soy broth (Difco) for 15 h and then collected by centrifugation and washed with sterile PBS. The washed bacteria were diluted with PBS and adjusted spectrophotometrically at 550 nm to the appropriate concentration. *Escherichia coli* DH5α (Toyobo Biochem) and *E. coli* NM522 mutS (Amersham Pharmacia Biotech) strains were routinely grown in Luria broth (Difco) at 37°C with shaking (at 110 rpm). Ampicillin at 100 µg/mL was used to maintain plasmids in *E. coli*. *E. coli* DH5α derivatives were grown in 2× yeast extract, tryptone, and ampicillin (YTA) medium that contained 100 µg/mL ampicillin at 37°C with shaking.

**Construction of plasmid expressing recombinant (r) TSST-1.** Genomic DNA containing the *gst* gene was isolated from *S. aureus* 834, as described elsewhere [40]. The DNA was used as a template in polymerase chain reaction (25 amplification cycles of 1 min of denaturing at 95°C, 1 min of annealing at 55°C, and an extension step of 1 min at 72°C), by using oligonucleotide primers corresponding to the N-terminal and C-terminal amino acid residues of TSST-1 that flanked the portion of the gene encoding the mature TSST-1: forward, 5′-CCCTACACAAAAGATATAATACACG-3′; reverse, 5′-CCACACCG-GGTGCATATTAATTTCTGCTTCTATAG-3′. To construct the rTSST-1 expression plasmid, the DNA fragments were digested with EcoRI and SalI. The fragments were cloned into pGEX-6p-1 (Amersham Pharmacia Biotech), a glutathione-S-transferase (GST) fusion expression vector, and then transformed into *E. coli* DH5α cells. The resultant plasmid was named pGXTSST. The nucleotide sequences of both strands were determined with the ABI 373 automatic DNA sequencer (PerkinElmer Applied Biosystems). Recombinant proteins expressed from this vector contain a N-terminal tail of GPLGSPEF residues.

**Construction of plasmid expressing mTSST-1.** The histidine at position 135 of the TSST-1 molecule has been reported to be an important site for superantigenicity [30]. To change this histidine to alanine, a selection primer (5′-GGTGAACGCA-ACGATGCCGGCGAATGGCAACAACG-3′) and a mutagenic primer (5′-AGACTTTGAAATTCCTGCTAG-3′) were designed to change the oligonucleotides CAT to GCT in bp 403 and 404 of the *gst* gene. The plasmid DNA was denatured at 100°C for 5 min in the presence of both the selection and mutagenic primers. The mutant DNA strand was synthesized with T4 DNA polymerase and T4 DNA ligase (TaKaRa). The enzymes were inactivated, and the reaction mixture was digested with PstI. After digestion, an aliquot of the sample was introduced into *E. coli* NM522 mutS. The bacteria were cultured at 37°C overnight in SOC broth (Nissui) that contained 100 µg/mL ampicillin. The DNA from the mixed plasmid pool was extracted and digested with PstI again. For the final transformation, an aliquot of the reaction mixture was introduced into *E. coli* DH5α. Several colonies were grown overnight for plasmid DNA extraction, and the plasmid DNA was digested in 2 separate reactions with PstI and SalI and analyzed on an agarose gel. Clones that had lost the unique PstI site and acquired an additional SalI site were selected.
and the mutant DNA sequences were conformed as described above. The histidine-to-alanine mutant plasmid was identified as pGXmTSST and transformed into E. coli DH5α.

**Expression and purification of rTSST-1 and mTSST-1.** Overnight cultures of E. coli DH5α cells harboring the recombinant or mutant plasmids were diluted 1:50 in 1 L of 2× YTA medium that contained 100 µg/mL ampicillin. E. coli cells were grown until the culture reached an optical density at 600 nm of 0.5–0.8. Expression of the recombinant and mutant proteins was then induced by adding isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 0.5 mmol/L. After a 3-h induction period, the bacteria were collected by centrifugation, and the bacterial pellets were resuspended in bacterial protein extraction reagent (B-PER; Pierce). Purification of GST–rTSST-1 and GST–mTSST-1, cleavage, and removal of the GST tag from rTSST-1 or mTSST-1 were done by using the bulk GST purification modules (Amersham Pharmacia Biotech) according to the manufacturer’s instructions. The purified proteins were determined by Bradford assay (Bio-Rad) and analyzed by SDS-PAGE.

**Immunodiffusion assay.** Gel double-immunodiffusion assays of rTSST-1 and mTSST-1 with anti–TSST-1 antibody were done basically according to the method described by Robbins et al. [41], with minor modifications. Noble agar (Difco), 1.2% in 0.01 mol/L PBS, which contained 10 ppm thimerosal, was poured into plastic petri dishes. Wells of equal size (8.0 mm diameter) were cut, and the agar plugs were removed by suction. Rabbit anti–rTSST-1 serum (1:8 dilution) and samples that contained recombinant or mutant toxins were added to wells (75 µL/well) and incubated in a humidified box at 37°C for 24 h. Nonprecipitated proteins were removed by soaking the gel in 0.01 mol/L PBS for 48 h with frequent changes. The gel was stained with 0.1% Coomassie brilliant blue R-250 in 10% acetic acid and 40% methanol in distilled water, followed by destaining in the solvent.

**Toxicity assay of rTSST-1 and mTSST-1.** The toxic effect of rTSST-1 and mTSST-1 for BALB/c mice was tested in a lipopolysaccharide (LPS)–potentiated mouse lethality model [42]. Mice were first injected intraperitoneally with rTSST-1 or mTSST-1 diluted in PBS (0.1–10 µg/mouse); 4 h later, the mice were injected again with 75 µg of LPS from E. coli O55:B5 (Sigma). The controls included animals given either TSST-1 or LPS alone, and lethality was recorded over 72 h.

**Immunization of mice and S. aureus challenge.** Purified rTSST-1 or mTSST-1 was dissolved in PBS and emulsified 1:1 in alum adjuvant (Pierce). Mice were injected with 200 µL of the emulsion that contained 10 µg of rTSST-1 or mTSST-1 protein, or adjuvant alone, at 2 subcutaneous sites on their backs. Booster immunizations were done 2 and 4 weeks after the initial vaccination. Mice were challenged at day 7 after the last booster with a lethal dose of S. aureus 834 by intravenous injection. The deaths of mice were recorded over 15 days. Blood samples were obtained before and after bacterial challenge. Bacteria in the spleen, liver, and kidneys were enumerated at day 3 after infection by preparing homogenates of these organs in PBS and by plating 10-fold serial dilutions on tryptic soy agar (Difco). Colonies were counted after 24 h of incubation at 37°C.

**Assays of specific antibodies.** Serum samples from vaccinated and challenged mice were obtained 7 days after the last immunization and after 3 days of challenge. Specific antibodies against TSST-1 and antibodies against the bacterial cell protein were measured by ELISA. Microplates (96-well; Nunc) were coated overnight at 4°C with rTSST-1 (10 µg/mL) or cell protein of sonicated S. aureus in carbonate buffer (pH 9.5). The plates were washed with PBS that contained 0.05% Tween 20 (PBST) and blocked for 2 h at 37°C with 10% Blockace (Dainippon Pharmaceutical) in PBS (PB SB). Serum samples were diluted in PB SB and added to washed wells for 2 h at 37°C. Wells were again washed with PBST, and goat anti-mouse IgG1, IgG2a, IgG2b, IgG3, IgA, or IgM (Southern Biotechnology Associates) was diluted in PBSB and added for 2 h at 37°C. Wells were washed with PBST and a substrate solution that contained o-phenylenediamine, and H₂O₂ was added to each well for color development. The reaction was terminated by the addition of 25 µL of 8 N HSO₄. The reaction was measured at 490 nm in an ELISA plate reader.

**Passive immunization.** To generate anti–mTSST-1 antibodies, rabbits were hyperimmunized with mTSST-1 as described elsewhere [40]. The serum samples were pooled and the immunoglobulin fraction was obtained by precipitation with a saturated ammonium sulfate solution, followed by extensive dialysis against PBS. The concentration of immunoglobulin in the precipitate was determined by using Bradford assay (Bio-Rad). Naïve mice were passively immunized intraperitoneally on day 1 with 10 mg of the immunoglobulin fraction that contained antibodies specific for TSST-1 or normal rabbit IgG (Sigma). On day 0, the mice were challenged intravenously with S. aureus 834 at 5 × 10⁷ cfu/mouse, mortality was monitored, and bacteria in the organs of the mice were enumerated at day 3 after infection as described above.

**Spleen cell cultures.** Spleens were removed aseptically from naïve or immunized mice, and spleen cells were obtained by squeezing the organs in RPMI 1640 medium (Nissui). Each cell suspension was filtered through stainless steel mesh (size 100). After lysis of erythrocytes with 0.85% NH₄Cl, the cells were washed 3 times and resuspended in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 U/mL penicillin G, and 100 µg/mL streptomycin and then placed in a 24-well tissue culture plate (Greiner) at a density of 10⁶ cells/well in the presence of rTSST-1, mTSST-1, or heat-killed S. aureus. After 72 h of incubation at 37°C in a 5% CO₂ incubator, the supernatants were collected and stored at −80°C until the cy-
tokine assays were done. For the determination of the neutralizing activity of anti–mTSST-1 serum samples against cytokines induced by rTSST-1 in vitro, anti–mTSST-1 serum or control serum was preincubated with rTSST-1 at 37°C for 1 h before the rTSST-1 was added to the spleen cell cultures.

In vitro proliferation assays. To investigate mTSST-1 and rTSST-1 responses in mice, spleen cells were suspended in RPMI 1640 medium supplemented with 10% fetal calf serum, 10 μmol/L sodium pyruvate (Wako Pure Chemical), and 50 μmol/L 2-mercaptoethanol (Wako). Spleen cells at 10^6/mL were incubated with varying amounts of mTSST-1 or rTSST-1 in round-bottomed microplates at 37°C for 2 days. The cultures were pulsed for 1 day with 20 kBq/well [3H]thymidine (ICN Biomedicals) and then harvested on glass-fiber filters. The amount of incorporated [3H]thymidine was measured by liquid scintillation counting. To investigate the response to mTSST-1 and rTSST-1 in human peripheral blood lymphocytes (PBLs), human PBLs from healthy volunteers were prepared by Lymphoprep-Tube (Nycomed) centrifugation, and 10^5 cells were added to microplate wells that contained various dilutions of rTSST-1 or mTSST-1 in 0.1 mL of culture medium as described above. Cellular proliferation was measured as described above.

Assays of cytokine production. Titers of IFN-γ, TNF-α, and IL-10 in the serum samples and organ extracts of mice were determined by double-sandwich ELISAs as described elsewhere [39, 43]. Organ extracts were prepared by centrifuging 10% (wt/vol) spleen and homogenates in RPMI 1640 medium that contained 1% (wt/vol) CHAPS (Wako) at 2000 g for 20 min. IL-2 was measured by use of the mouse IL-2 ELISA kit (BioSource International). To assay cytokine production in human PBLs, these were placed in a 24-well tissue culture plate (Greiner) at a density of 10^6 cells/well in the presence of rTSST-1 or mTSST-1. Supernatants of cell culture were collected after 72 h of incubation at 37°C in a 5% CO₂ incubator. Cytokine titers were determined by the human IFN-γ and TNF-α ELISA kits (BioSource International) and the IL-2 EASIA kit (BioSource Europe).

Statistical analysis. Data were expressed as means ± SDs, and the Mann-Whitney U test was used to determine the significance of the differences in bacterial counts in the organs and in cytokine titers between control and experimental groups.

RESULTS

Biological characteristics and immunological reactivity of mTSST-1. Wild-type rTSST-1 and the mutant gene product (mTSST-1) were identified and assayed for biological function and immunological reactivity. mTSST-1 from the mutant pGXmTSST strain was compared on Coomassie blue–stained SDS-PAGE with wild-type rTSST-1 from the pGXTSST strain and showed the presence of a readily detectable purified protein band that comigrated with purified rTSST-1 (data not shown). The immunological reactivity of mTSST-1 and rTSST-1 with polyclonal rabbit anti–rTSST-1 antibody was assayed using gel immunodiffusion. The antibody reacted readily with purified mTSST-1 and rTSST-1, and the precipitation lines between mTSST-1 and rTSST-1 coincided (data not shown). These results indicate that mTSST-1 retains the same antibody-binding epitopes as wild-type rTSST-1.

To confirm whether the superantigenic activity of mTSST-1 was deleted, proliferation (figure 1A) and cytokine production (figure 1B–D) induced by mTSST-1 and rTSST-1 in mouse spleen cells were determined. Substantial amounts of IFN-γ, TNF-α, and IL-2 were induced in mouse spleen cell cultures at all concentrations of rTSST-1 used and showed higher proliferation activity. In contrast, mTSST-1 had no detectable or lower cytokine production and proliferation activity (figure 1). In addition, the superantigenic activity of mTSST-1 in human PBLs was determined. mTSST-1 was devoid of human PBL stimulatory activity (figure 2A) and was completely devoid of the production of IFN-γ (figure 2B) and IL-2 (figure 2D), even at a concentration of 1000 ng/mL. TNF-α production induced by mTSST-1 was 10²-fold less than that induced by wild-type rTSST-1 (figure 2C). These results confirm that mTSST-1 is devoid of superantigenic activity.

Figure 1. Comparison of superantigenic activities of recombinant (r) toxic shock syndrome toxin 1 (TSST-1) and mutant (m) TSST-1 in mouse spleen cells. A, Proliferation of mouse splenic cells induced by rTSST-1 and mTSST-1. Cultures were incubated with rTSST-1 or mTSST-1 for 48 h and pulse-labeled with [3H]thymidine for 24 h. Results are mean cpm of triplicate wells ± SD. B–D, Cytokine production (interferon [IFN]-γ, B; tumor necrosis factor [TNF]-α, C, and interleukin [IL]-2, D) in supernatants of cultures of mouse splenic cells stimulated with rTSST-1 and mTSST-1, as measured by ELISA. Data are mean ± SD of results from 3 wells.
Comparison of superantigenic activities of recombinant (r) toxic shock syndrome toxin 1 (TSST-1) and mutant (m) TSST-1 in human peripheral blood lymphocytes (PBLs). A, Proliferation of human PBLs induced by rTSST-1 and mTSST-1. Cell cultures were incubated with rTSST-1 or mTSST-1 for 48 h and pulse-labeled with [3H]thymidine for 24 h. Data are mean cpm of triplicate of samples obtained from wells of 5 donors.

B–D, Cytokine production (interferon [IFN]–γ, B; tumor necrosis factor [TNF]–α, C; and interleukin [IL]-2, D) in supernatants of cultures of human PBLs stimulated with rTSST-1 and mTSST-1, as measured by ELISA. Data are mean ± SD of samples from 5 donors.

To further examine whether the toxicity of mTSST-1 protein is also detected in vivo, mice were injected with LPS plus mTSST-1 or rTSST-1, and the lethal effect was determined in mice. In striking contrast to the 80% mortality rate of rTSST-1 (at 10 μg/mouse) plus LPS, none of the mice died when they were given an equivalent dose of mTSST-1 plus LPS (data not shown). The lack of toxicity by mTSST-1 in our LPS-potentiated mouse model corresponded well to previous results of an in vivo assay [42].

Effect of vaccination with mTSST-1 and rTSST-1 on host resistance against S. aureus infection. Mice were vaccinated subcutaneously 3 times with mTSST-1, rTSST-1 plus alum, or alum only and then intravenously challenged with S. aureus strain 834 at 5 × 10^7 cfu/mouse. On day 8 after challenge, 83% of mice vaccinated with mTSST-1 survived. Conversely, only 22% of mice injected with alum alone survived. All mice injected with alum alone died 11 days after bacterial challenge, whereas 60% of the mTSST-1–immunized group still survived (P < .05). The experiment was terminated at day 15, with no further deaths occurring among mTSST-1–immunized mice (figure 3A). In addition, we also immunized with rTSST-1 in the same way. At days 8 and 11 after challenge, the survival of mice was similar to that of mTSST-1 vaccination, but, at day 13, only 22% of the mice survived. These results indicate that vaccination with mTSST-1 gives rise to significant protection compared with the control group.

To further confirm the effect of vaccination with mTSST-1 and rTSST-1 on bacterial growth in the organs, mice were immunized subcutaneously with mTSST-1 plus alum, rTSST-1 plus alum, or alum only; mice were then challenged intravenously with S. aureus strain 834 at 5 × 10^7 cfu/mouse. Three days after inoculation, the numbers of bacterial cells in the spleens, livers, and kidneys were determined. There were significantly fewer bacterial cells in the spleens, livers, and kidneys of mTSST-1–immunized mice than in the organs of control mice (P < .05; figure 3B), whereas no significant difference in bacterial counts in the kidneys was observed between the r-
Figure 4. Serum antibody responses of mutant (m) toxic shock syndrome toxin 1 (TSST-1)–vaccinated mice. Animals were immunized with recombinant (r) TSST-1 or mTSST-1 with alum adjuvant or adjuvant alone and then were infected with $5 \times 10^7$ cfu of Staphylococcus aureus on day 7 after the last boost. Serum samples were obtained on day 3 after infection. A, Anti–TSST-1–specific antibody titers were determined by ELISA. Plates were coated with rTSST-1 (5 µg/mL), and serum samples pooled from each group of 5 mice were diluted to 1:500. B, Anti–S. aureus antibody titers of serum samples pooled from each group of 5 mice were determined by ELISA, as in panel A. S. aureus cell protein–coated wells (5 µg/mL) were incubated with pooled serum samples (diluted 1:100). Data are mean optical density (OD) at 490 nm (± SD) of samples from 5 mice.

Humoral responses induced in mTSST-1–and rTSST-1–vaccinated mice. Humoral responses were evaluated, to analyze the protective mechanism provided by antibodies that resulted from mTSST-1 and rTSST-1 immunizations. ELISA plates, coated with either wild-type rTSST-1 or S. aureus cell proteins, were used to determine the humoral responses. A strong IgG antibody response to TSST-1 (figure 4A), but not to S. aureus cell proteins (figure 4B), was seen in the serum samples obtained from mice immunized with mTSST-1 or rTSST-1. In contrast, serum samples from mice injected with alum only did not react significantly to either TSST-1 or S. aureus cell proteins. The levels of IgG1 and IgG2b responses were similar between mTSST-1– and rTSST-1–immunized mice. Of interest, levels of IgG2a and IgG3 were higher in serum samples from rTSST-1–immunized mice ($P < .01$), whereas mTSST-1–immunized mice did not develop IgG2a and IgG3 responses (figure 4A). The lack of production of these antibodies might have been due to the deletion of the superantigenicity of mTSST-1. There was no difference in IgM production between immunized and control mice ($P > .05$).

Passive immunization. To verify that the protection of mice against S. aureus challenge was mediated by TSST-1–specific antibodies, a passive immunization study was conducted. Rabbits were actively immunized with mTSST-1; subsequently, blood was obtained, and serum samples were collected and pooled. The immunoglobulin fraction from the pooled hyperimmune serum samples or normal rabbit serum was administered to naive mice intraperitoneally, and immunized mice were challenged intravenously with S. aureus 834 at $5 \times 10^7$ cfu/mouse 24 h later. On day 6 after bacterial challenge, 90% of animals in the anti–mTSST-1 immunoglobulin–treated group survived, compared with 30% of mice in the control group ($P < .05$; figure 5A).

To assess whether the protection in mice passively immunized with anti–mTSST-1 was related to the inhibition of bacterial growth, bacterial counts in the organs of mice were determined. Mice were administered rabbit anti–TSST-1 immunoglobulin or normal rabbit IgG intraperitoneally and

Figure 5. Effect of the passive transfer of antibodies to mutant (m) toxic shock syndrome toxin 1 (TSST-1) on host resistance against Staphylococcus aureus infection. A, Mice were passively given 10 mg of anti–TSST-1 immunoglobulin or normal rabbit IgG and then were infected with $5 \times 10^7$ cfu of S. aureus 24 h after transfer. Survival was observed for each group of 10 mice. B, The no. of bacteria in spleens, livers, and kidneys of passively immunized mice and nonimmunized mice was determined. Mice were administered rabbit anti–TSST-1 immunoglobulin or normal rabbit IgG intraperitoneally and
Figure 6. Kinetics of endogenous cytokine production within 20 h after infection with *Staphylococcus aureus*. Mice were immunized with mutant (m) toxic shock syndrome toxin 1 (TSST-1) plus alum adjuvant or adjuvant alone and then were infected intravenously with 5 × 10^7 cfu of *S. aureus* on day 7 after the last boost. Titers of interferon (IFN)–γ (A) and interleukin (IL)–10 (B) in serum samples and spleens were determined by ELISA. Each point represents the mean ± SD for 3–5 mice. *Significantly different from control mice ( ).

Figure 7. Inhibitory effect of serum samples from mice immunized with mutant (m) toxic shock syndrome toxin 1 (mTSST-1) on cytokine production induced by TSST-1 in vitro. Because inflammatory cytokines play a central role in the lethal toxicity triggered by the TSST-1 produced by *S. aureus*, we examined the effect of serum samples from mTSST-1–vaccinated mice on TSST-1–induced IFN-γ and TNF-α production, which are commonly associated with superantigenic activity. Serum samples from mice immunized with mTSST-1 plus alum effectively inhibited IFN-γ and TNF-α production from murine spleen cells by TSST-1, relative to serum samples from alum-injected controls (figure 7; P < .01). These results suggest that the protective effect obtained from immunization with mTSST-1 may be linked to an inhibition of the production of inflammatory cytokines.

**DISCUSSION**

*S. aureus* is a well-adapted mammalian bacterium. It has a significant economic impact on health care and the dairy industry [7, 44]. The emergence of antibiotic resistance among *S. aureus* isolates has made the treatment of staphylococcal infections more challenging. The immunization of mice with mutant TSST-1 (mTSST-1) has been shown to protect against *S. aureus* infection, possibly through the induction of anti–TSST-1 antibodies, which may play an important role in resistance.

**Induction of IFN-γ and IL-10 in vaccinated mice during *S. aureus* infection.** To determine the role of endogenous IFN-γ and IL-10 in mTSST-1–immunized mice against *S. aureus* infection, mice were vaccinated with mTSST-1 plus alum or alum only and then were challenged with *S. aureus* 834 at 5 × 10^7 cfu/mouse. IFN-γ and IL-10 production in serum samples and spleen homogenates was determined by sandwich ELISA at 2, 4, 6, 10, and 20 h after infection (figure 6). Endogenous IFN-γ production in the serum samples of mTSST-1–immunized mice peaked at 10 h and disappeared at 20 h of infection (figure 6A). Although IFN-γ production in serum samples from control mice also peaked at 10 h and decreased at 20 h, titers were significantly higher than those of mTSST-1–immunized mice (P < .05). IFN-γ titers in the spleens of mTSST-1–vaccinated mice peaked 6–10 h of infection and then decreased at 20 h. In contrast, in control mice, IFN-γ levels increased from 4 to 20 h, and the titers were significantly higher than those of the vaccinated group (figure 6A). The titers of IL-10 in the serum samples of mTSST-1–vaccinated mice increased from 6 to 20 h and were significantly higher than those of control mice at 20 h (figure 6B). In the spleens of vaccinated mice, IL-10 levels increased at 2 h, peaked at 4 h, and then decreased slowly after 10 h of infection, whereas, in spleens of control mice, IL-10 showed a small peak at 4 and 6 h and then significantly decreased at 20 h of infection.

The data suggest that anti–TSST-1 antibodies, either transferred or elicited by active immunization, can protect mice against *S. aureus* infection and that the anti–TSST-1 antibodies might play an important role in resistance.

**Inhibitory effect of anti–TSST-1 antibodies on cytokine production induced by TSST-1 in vitro.** The protective effect obtained from immunization with mTSST-1 may be linked to an inhibition of the production of inflammatory cytokines.
immunoglobulin subclasses in mTSST-1–immunized mice did not affect the protection against S. aureus infection. Furthermore, passive transfer of the antibodies from mTSST-1–immunized animals also effectively protected against bacterial infection in naive mice. These results indicate that the Th2-induced immunoglobulin subclasses, but not Th1-induced antibodies, may play an important role in host resistance against S. aureus infection.

The results of our previous study showed that IFN-γ, a Th1-type cytokine, plays a detrimental role in S. aureus infection in vivo [39]. The administration of anti–IFN-γ monoclonal antibody resulted in the suppression of bacterial growth in the kidneys and protected mice from the lethal effects of S. aureus infection [39]. Zhao and Tarkowski [52] also demonstrated that IFN-γ receptor–deficient mice developed severe sepsis with high mortality rates after S. aureus infection. They also reported that anti–IFN-γ monoclonal antibody treatment increased the bacterial numbers in the kidneys and that the administration of recombinant IFN-γ ameliorated S. aureus sepsis [53]. Recently, we showed that IFN-γ plays an important role in the pathogenesis of S. aureus infection, because there was an increase in survival rates, a decrease in bacterial numbers in the kidneys, and an amelioration of histological changes observed in the kidneys in IFN-γ–/– mice compared with those in IFN-γ +/+ mice [54]. On the other hand, IL-10, a Th2-type cytokine, plays a beneficial role in protecting the host from shock due to endotoxin [55], septic shock [56], and shock due to staphylococcal enterotoxin [57, 58]. The results of our previous study showed that the administration of anti–IL-10 monoclonal antibody to mice inhibited the elimination of S. aureus from the kidneys, in which infectious foci were easily formed, which suggested that IL-10 might play a beneficial role in host resistance to S. aureus infection [54]. IL-10 is known to have anti-inflammatory actions in various inflammatory diseases [55–57]. It is possible that this cytokine may regulate excess inflammatory responses in S. aureus infection. In the present study, serum samples and spleens obtained from mTSST-1–immunized mice produced lower titers of IFN-γ and higher titers of IL-10 in response to S. aureus infection than did those obtained from the control mice (figure 6), which suggests that mTSST-1 vaccination may polarize Th0 toward Th2 in vivo and that the regulation of cytokine production might be involved in the acquisition of protection in mTSST-1–immunized mice. Moreover, the production of IFN-γ in spleen cells stimulated with mTSST-1 in vitro was significantly inhibited by incubation with TSST-1–specific antibodies obtained from mTSST-1–vaccinated mice (figure 7). These results, together with antibody transfer data, indicate that protection might be mediated by TSST-1–specific antibodies neutralizing the S. aureus–produced TSST-1 as well as by down-regulating IFN-γ production induced by S. aureus and toxins.

The mechanism of action of serum immunoglobulin in S.
Staphylococcus aureus infections remains elusive. One of the demonstrated effects of antibodies is anti-inflammatory activity [14, 59], and the other is neutralization of the toxicity of S. aureus surface contents and secreted products [14, 47]. Previous studies identified cross-reactive antibodies between staphylococcal enterotoxins and streptococcal pyrogenic exotoxin A [60, 61]. Ulrich et al. [25] and Bavari et al. [62] demonstrated cross-reactivity among TSST-1, SEA, SEB, and SEC. Mice vaccinated with TSST-1 survived when challenged with SEA, SEB, or SEC. More recently, Kum and Chow [63] reported that anti-TSST-1 monoclonal antibody also cross-inhibited SEA-induced mitogenic activity and TNF-α secretion in vitro and protects against SEA-induced lethality in a mouse model. It is important to consider that the majority of methicillin-resistant S. aureus strains in the United States produce SEB or SEC in very high concentrations. In Japan, the majority of methicillin-resistant S. aureus strains produce large amounts of TSST-1: up to 50–100 μg/mL of pyrogenic toxin superantigens in vitro. Considering that 2 physicians injected themselves with 3–5 μg of pyrogenic toxin superantigens and subsequently developed TSS, it is probable that a very slight infection could induce TSS [14]. Our results demonstrate that immunization with mTSST-1 profoundly alters the course of disease by neutralizing pyrogenic toxin superantigens, inhibiting IFN-γ production, and decreasing the mortality and bacterial growth rate, compared with control mice. Because the expression of TSST-1 and staphylococcal enterotoxins is common among invasive S. aureus isolates [1, 14], this nontoxicity of mTSST-1 and its specific antibodies should be useful in the control of S. aureus infection and treatment of TSS.

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References