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Adipose Tissue-Derived Mesenchymal Stem Cells Attenuate Staphylococcal Enterotoxin A-Induced Toxic Shock

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Adipose tissue-derived stem cells (ASCs), which are mesenchymal stromal cells isolated from adipose tissues, exhibit immunomodulatory effects that are promising for several applications, including the therapeutics of inflammatory diseases. In the present study, the effect of ASCs on bacterial toxin-induced inflammation was investigated. Intraperitoneal administration of ASCs rescued mice from lethal shock induced by staphylococcal enterotoxin A (SEA) potentiated with lipopolysaccharide. In the sera and/or spleens of mice administered ASCs, the production of proinflammatory cytokines, including interferon gamma, tumor necrosis factor alpha, interleukin-6 (IL-6), and IL-2 was reduced. By quantitative real-time PCR, the expression of Foxp3 in the mice administered ASCs was not altered. On the other hand, the expression of IL-12 receptor and STAT4 was decreased with ASC administration. These results imply that the effect of ASCs is not involved in the lineage of regulatory T cells but that these cells may modulate T_H1 differentiation. This information provides evidence that ASCs have properties that are effective to attenuate SEA-induced toxic shock and should prompt further exploration on other inflammatory diseases caused by bacterial toxins or bacterial infections.

Staphylococcus aureus, an important human pathogen, causes a variety of infections ranging from superficial infections to life-threatening diseases (1). The most common isolated strains from patients with sepsis caused by *S. aureus* infection produce a wide array of enterotoxins, including staphylococcal enterotoxin A (SEA) (2). In addition to having enterotoxic activity, SEA serves as a superantigen causing toxic shock syndrome in humans and animals. SEA cross-links between major histocompatibility complex (MHC) class II molecules on antigen-presenting cells and T-cell receptor (TCR) via specific Vβ regions. The specific interaction of this bacterial toxin with multiple cell types in the host results in an activation of both monocytes/macrophages and T lymphocytes and leads to an excessive release of proinflammatory cytokines, including interferon gamma (IFN-γ), tumor necrotic factor alpha (TNF-α), interleukin-6 (IL-6), and IL-2. These cytokines enhance immune reactions and tissue injuries, causing clinical features that include fever, hypotension, multiorgan dysfunction, shock, and death (3–5).

Mesenchymal stem cells (MSCs) are somatic stem cells residing in bone marrow and other various tissues. They possess the ability to differentiate into adipocytes, osteocytes, or chondrocytes (6, 7). In addition to having a role in tissue repair, MSCs can particularly affect various immune cells by suppressing the activation and proliferation of these cells (8). These characteristics make MSCs attractive for applications in inflammatory diseases. Adipose tissue is one of the richest sources of MSCs (9). MSCs from adipose tissue, referred to adipose tissue-derived mesenchymal stem cells (ASCs), have differentiation and immunoregulation abilities similar to those of the bone marrow-derived MSCs (10–12). A previous study has shown that ASCs attenuate liver injury and mortality in a mouse model of concanavalin A-induced hepatitis. In this hepatitis model, the production of TNF-α and IFN-γ, which play a crucial role in activation of T cells and NKT cells, is suppressed by administration of ASCs (13). These data prompted us to examine the effect of ASCs on inflammation caused by bacterial infections or bacterial toxins.

In the present study, the immunomodulatory effect of ASCs on toxic shock caused by SEA was investigated. In the mouse model, the biological effects of SEA are potentiated by lipopolysaccharide (LPS), the bacterial endotoxin that binds to Toll-like receptor 4 on the surfaces of cells. The SEA and LPS synergistically amplify the proinflammatory cytokines that lead to severe toxicity (14, 15). Reduction in lethality and proinflammatory cytokine production in this mouse model suggest that ASCs are effective to attenuate SEA-induced toxic shock.

MATERIALS AND METHODS

Mice. BALB/c mice (Clea Japan, Tokyo, Japan) (6- to 10-week-old females) were housed in plastic cages with a controlled light and dark cycle and fed a standard diet with water *ad libitum*. All the animal experiments were performed in accordance with Guidelines for Animal Experimentation of Hirosaki University. The present study was approved by the Committee on the Ethics of Animal Experimentation of Hirosaki University (permission number M10034).

Isolation and expansion of ASCs. ASCs were isolated and expanded as described previously with minor modifications (13). Briefly, subcutaneous and visceral adipose tissues were collected from BALB/c mice and placed in Iscove's modified Dulbecco's medium (IMDM) (Invitrogen, NY). Tissues were minced finely and incubated at 37°C for 40 min in 3 ml IMDM containing 0.2% collagenase (type II; Sigma, St. Louis, MO) and 1% bovine serum albumin (Sigma). Cells were collected from the digested tissues through 70-μm mesh. After centrifuging at 800 × *g* for 10 min, the

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TABLE 1 Primers used for quantitative real-time PCR

Gene product	Primer sequence (5′→3′)	
	Forward	Reverse
GAPDH	TGAAGGTCGGTGTGAACGGATTTGG	ACGACATACTCAGCACCGGCCTCAC
IFN-γ	AGCGGCTGACTGAACTCAGATTGTAG	GTCACAGTTTTCAGCTGTATAGGG
TNF-α	GGCAGGTCTACTTTGGAGTCATTGC	ACATTCGAGGCTCCAGTGAATTCGG
IL-6	TGGAGTCACAGAAGGAGTGGCTAAG	TCTGACCACAGTGAGGAATGTCCAC
IL-2	AGCAGCTGTTGATGGACCTA	CGCAGAGGTCCAAGTTCAT
Foxp3	GGCCCTTCTCCAGGACAGA	GCTGATCATGGCTGGGTTGT
T-bet	TCCTGCAGTCTCTCCACAAGT	CAGCTGAGTGATCTCTGCGT
STAT4	GAATGTGGAACACAAAGTGTCTGCC	CATTTTAGTAGATTGCTCCTGTAG
IL-12p35	ACCTGCTGAAGACCACAGATGACA	TAGCCAGGCAACTCTCGTTCTTGT
IL-12Rβ2	ACATAGTGGACCTATGTGGC	GCTTATTGGATGTGAGTTTTG

cells were suspended in 3 ml of 0.83% NH_4Cl for hemolysis and subsequently washed three times with IMDM. The cells were seeded in IMDM containing 9% fetal bovine serum (FBS) (Nichirei Bioscience, Tokyo, Japan), 9% horse serum (HyClone Laboratories, Logan, UT), 100 U/ml penicillin (Wako, Osaka, Japan), and 100 μg/ml streptomycin (Wako). The medium was changed every 3 or 4 days. The cells were subcultured for 3 passages and then prepared at a concentration of 2.0×10^6 cells/ml in phosphate-buffered saline containing 2% FBS (2% FBS–PBS).

Isolation of SPCs. Spleens were removed aseptically from BALB/c mice, and splenocytes (SPCs) were obtained by squeezing the spleen in RPMI 1640 medium (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan). The cell suspension was then filtered through 70-μm mesh. After lysis of erythrocytes with 0.83% NH_4Cl, the cells were washed 3 times and resuspended in 2% FBS–PBS. The cells were then prepared at a concentration of 2.0×10^6 cells/ml.

SEA-induced lethal shock and administration of ASCs. Recombinant SEA was prepared from a glutathione *S*-transferase (GST) fusion system as described previously (16). BALB/c mice were intraperitoneally administered 10 μg SEA, followed by 80 μg *Escherichia coli* O111:B4-derived LPS (Sigma) 4 h later. To examine the effect of ASCs, mice were intraperitoneally administered 1×10^6 ASCs or SPCs (as a control) at 30 min after SEA administration. Mice were then monitored, and mortality was recorded for 72 h. At 2 h and 18 h after LPS administration, sera and spleens were collected to further examine the cytokine response and the expression of T-cell-related genes.

Measurement of cytokine production. The titers of IFN-γ, TNF-α, IL-6, and IL-2 in serum and spleen homogenates were measured by an enzyme-linked immunosorbent assay (ELISA). Determination of IFN-γ production was carried out as described previously (17). The production of TNF-α, IL-6, and IL-10 was measured using CytoSet ELISA kits (Invitrogen) according to the manufacturer's recommendations. The production of IL-2 was measured with the mouse IL-2 DuoSet (R&D Systems, Minneapolis, MN) according to the manufacturer's instruction.

Quantitative real-time PCR. Total RNAs were prepared from the spleen using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. First-strand cDNAs were synthesized by reverse transcription of 1 μg RNAs using random primers (TaKaRa Shuzo Co., Ltd., Kyoto, Japan) and Moloney murine leukemia virus reverse transcriptase (Invitrogen). The primers used in this study are shown in Table 1. Noncontamination of genomic DNA and specificity of primers were confirmed by PCR using AmpliTaq Gold (Applied Biosystems, NJ). SYBR green Supermix (Bio-Rad Laboratories, Hercules, CA) was used for quantification of RNA targets by real-time PCR. PCRs were run with the following protocols. For IFN-γ, TNF-α, IL-6, IL-12(p35), and the signal transducer and activator of transcription 4 (STAT4), *Taq* DNA polymerase was initially activated at 95°C for 5 min, and 40 PCR cycles were subsequently performed with 30 s at 95°C for denaturing, 30 s at 55°C for annealing, and 30 s at 72°C for elongation. For IL-2, forkhead box P3 (Foxp3), IL-2 receptor (IL-12R), and T-cell-specific T-box transcription factor (T-bet), *Taq* DNA polymerase was initially activated at 95°C for 5 min, and 40 PCR cycles was subsequently performed with 30 s at 95°C for denaturing, 30 s at 50°C for annealing, and 30 s at 72°C for elongation. All experiments were performed in duplicate. Nontemplate controls and dissociation curves were used to detect primer-dimer conformation and nonspecific amplification. The threshold was set to the log linear range of the amplification curve and kept constant (0.05) for all data analysis. The threshold cycle (C_T) of each target product was determined and set in relation to the amplification plot of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The difference in C_T values (ΔC_T) between the target and GAPDH genes was used to calculate the relative expression [relative expression $= 2^{-(C_T \text{ of target genes} - C_T \text{ of GADPH})} = 2^{-\Delta C_T}$].

Statistical analysis. Statistical analysis was performed using Student's *t* test. For survival experiments, the Kaplan-Meier method was used to obtain survival fractions. A significant difference between the experimental and control groups was indicated by a *P* value of <0.05.

RESULTS

Lethal toxic shock caused by LPS-potentiated SEA is attenuated by ASCs. To establish a lethal toxic shock model, mice were intraperitoneally administered 10 μg SEA for 4 h before 80 μg LPS. To observe the effect of ASCs, 1×10^6 ASCs prepared in 2% FBS–PBS were intraperitoneally administered at 30 min after SEA injection. For controls, mice were administered an equivalent amount of SPCs or an equivalent volume of 2% FBS–PBS instead of the ASCs. The survival of each group of mice was monitored for 72 h. The results in Fig. 1 demonstrate that in the group administered buffer, 73% of mice died within 40 h. The survival of mice in the group administered SPCs was comparable to that of mice in the group administered buffer. Up to 75% and 87.5% of mice administered SPCs died within 40 h and 67 h after LPS administration, respectively. In contrast, up to 78% and 64% of mice administered ASCs survived at 40 h and 72 h, respectively. These results indicate that intraperitoneal administration of ASCs rescues mice from LPS potentiated SEA-induced lethal toxic shock. It should be noted that this effect is specific to SEA, because ASCs could not rescue mice from toxic shock induced by LPS alone (see Fig. S1 in the supplemental material).

ASCs suppress the cytokine response in sera of mice with LPS-potentiated SEA-induced toxic shock. To observe the effect of ASCs on cytokine response, sera from mice with LPS-potentiated SEA-induced toxic shock were collected at 18 h after LPS administration. The titers of IFN-γ, TNF-α, IL-6, and IL-2 in the sera were then determined by ELISA. The results in Fig. 2A to C demonstrate that ASC administration significantly suppressed the production of IFN-γ, TNF-α, and IL-6 in the sera of mice in

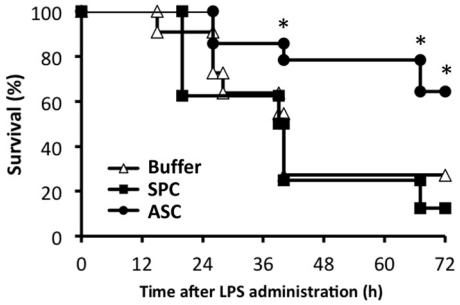

FIG 1 ASCs attenuate lethal toxic shock caused by LPS-potentiated SEA. Mice were injected intraperitoneally with 10 μg SEA, followed by 80 μg LPS, as described in Materials and Methods. At 30 min after SEA injection, mice were intraperitoneally administered 0.5 ml of 2% FBS–PBS (buffer), 0.5 ml of 1×10^6 SPCs in 2% FBS–PBS (SPC), or 0.5 ml of 1×10^6 ASCs in 2% FBS–PBS (ASC). The survival of mice was monitored for 72 h. The results are pooled from 4 independent experiments ($n = 3$ to 5). Values significantly different between mice administered SPCs and those administered ASCs are indicated (*, $P < 0.05$).

comparison with the control group administered SPCs. Although IL-2 has been shown to regulate staphylococcal enterotoxin-induced toxic shock (5), the production of IL-2 in the serum was too low to be detected in all groups of mice (Fig. 2D).

ASCs suppress the cytokine response in spleens of mice with LPS-potentiated SEA-induced toxic shock. The effect of ASCs on the cytokine response in spleen was further examined. Spleens from mice with SEA-induced toxic shock were collected at 18 h after LPS administration. The production of IFN-γ, TNF-α, IL-6, and IL-2 in the spleen homogenates was determined by ELISA. In addition, the gene expression of these cytokines was determined by quantitative real-time PCR. The results in Fig. 3 demonstrate that the production of IFN-γ, IL-6, and IL-2 in the spleens of mice with LPS-potentiated SEA-induced toxic shock was significantly reduced by ASC administration (Fig. 3A, C, and D). This cytokine production correlated to the mRNA expression determined by quantitative real-time PCR (Fig. 3D to F). At this time point, the production of TNF-α and its mRNA expression could not be detected in the spleen, even in those form from the control mice administered SPCs (Fig. 3B and F). During LPS-potentiated toxic shock, TNF-α production in the spleen would appear at an early time point. Thus, the production of TNF-α was assessed at 2 h after LPS administration. Although TNF-α production in the spleen was found at this time point, a significant effect of ASCs on this cytokine response was not found (see Fig. S2 in the supplemental material). This result suggests that ASCs had no effect on early production of TNF-α.

ASCs do not suppress the expression of Foxp3. Regulatory T cells (Treg cells) have been shown to regulate the differentiation of T helper cells (18). Thus, we observed the effect of ASCs on the expansion of Treg cells by determining the expression of Foxp3. The results in Fig. 4A and B demonstrate that expression of Foxp3 was not enhanced in mice administered ASCs whether at an early (2 h) or late (18 h) phase after LPS administration. Immunomodulation of Treg cells is mediated by the secretion of an anti-inflammatory cytokine, IL-10 (19). Thus, the production of IL-10 was also assessed by ELISA and quantitative real-time PCR. The results in Fig. S3 in the supplemental material demonstrate that the production and mRNA expression of IL-10 in the spleen were not altered by ASC administration (see Fig. S3A and B in the supplemental material). These results suggest that the immunomodulatory effect of ASCs in the LPS-potentiated SEA-induced toxic shock model is not involved in the enhancement of Treg cell lineage.

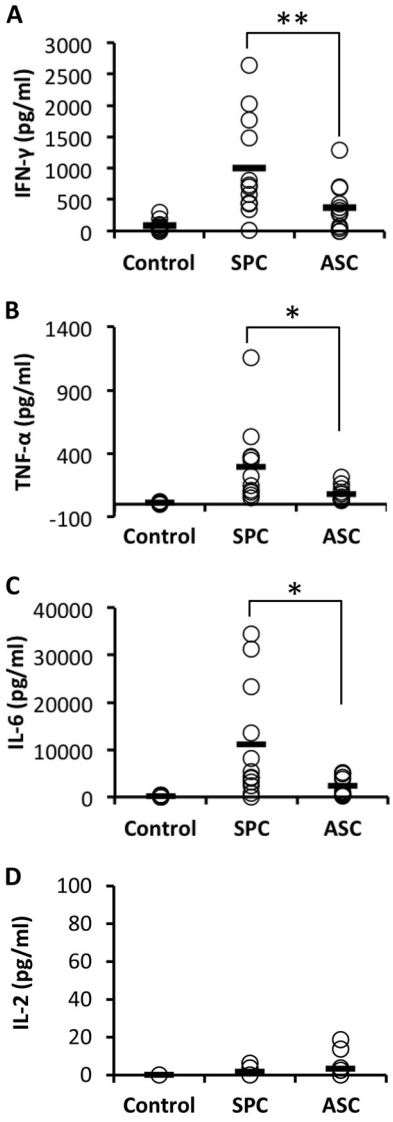

FIG 2 The cytokine response in sera of mice with LPS-potentiated SEA-induced toxic shock is attenuated by ASC administration. Mice were injected intraperitoneally with 10 μg SEA, followed by 80 μg LPS, as described in Materials and Methods. At 30 min after SEA injection, mice were intraperitoneally administered 1×10^6 SPCs or 1×10^6 ASCs in 2% FBS–PBS. At 18 h after LPS injection, serum was collected and the titers of IFN-γ (A), TNF-α (B), IL-6 (C), and IL-2 (D) in the sera were determined by ELISA. Control indicates the cytokine production from naive mice without LPS-potentiated SEA-induced toxic shock. The data are pooled from 3 independent experiments ($n = 3$ or 4). Each dot on the graph represents an individual mouse. The horizontal bar indicates the mean value for each group. The values that are significantly different between mice administered SPCs and those administered ASCs are indicated (*, $P < 0.05$; **, $P < 0.01$).

ASCs suppress the expression of IL-12R and STAT4 in mice with LPS-potentiated SEA-induced toxic shock. Due to activated T_H1 cells playing a crucial role in superantigen-induced toxic shock (20, 21), we further assessed the effect of ASCs on T_H1-related genes. At 18 h after LPS injection, the mRNA expression of

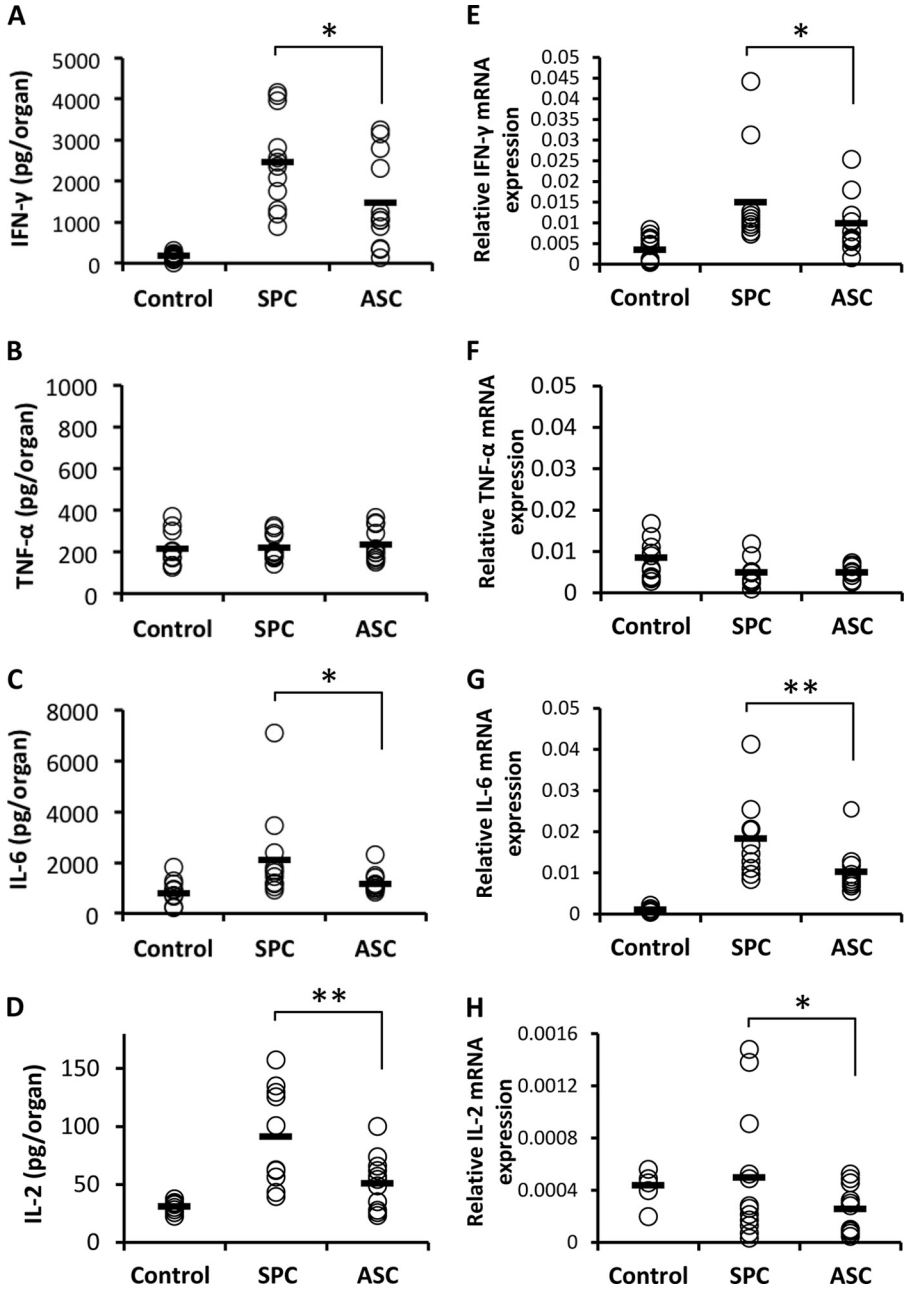

FIG 3 The cytokine response in spleens of mice with LPS-potentiated SEA-induced toxic shock is attenuated by ASC administration. Mice were injected intraperitoneally with 10 μg SEA, followed by 80 μg LPS, as described in Materials and Methods. At 30 min after SEA injection, mice were intraperitoneally administered 1×10^6 SPCs or 1×10^6 ASCs in 2% FBS–PBS. At 18 h after LPS injection, spleens were collected. The titers of IFN-γ (A), TNF-α (B), IL-6 (C), and IL-2 (D) in the spleen homogenates were determined by ELISA. The mRNA expression of IFN-γ (E), TNF-α (F), IL-6 (G), and IL-2 (H) in the spleen was examined by quantitative real-time PCR. Control indicates the cytokine production from naive mice without LPS-potentiated SEA-induced toxic shock. The data are pooled from 3 independent experiments ($n = 3$ or 4). Each dot on the graph represents an individual mouse. The horizontal bar indicates the mean value for each group. The values that are significantly different between mice administered SPCs and those administered ASCs are indicated (*, $P < 0.05$; **, $P < 0.01$).

T_H1 transcription factors T-bet and STAT4 was determined. The results in Fig. 5A and B demonstrate that the expression of STAT4 but not T-bet was suppressed by ASC administration. Thus, we further examined the expression of IL-12 and IL-12R, which are the important signaling factors for STAT4 expression and T_H1 differentiation (22). The results in Fig. 5C and D demonstrate that ASC administration significantly suppressed the expression of IL-12Rβ2 at 2 h after LPS injection. These results suggest that attenuation of LPS-potentiated SEA-induced toxic shock by ASCs may modulate the expression of IL-12R, which then affects the expression of STAT4 and T_H1 differentiation.

DISCUSSION

Staphylococcal enterotoxins (SEs) are potent activators of the immune system and cause a myriad of maladies, ranging from food poisoning to potentially life-threatening toxic shock. Re-

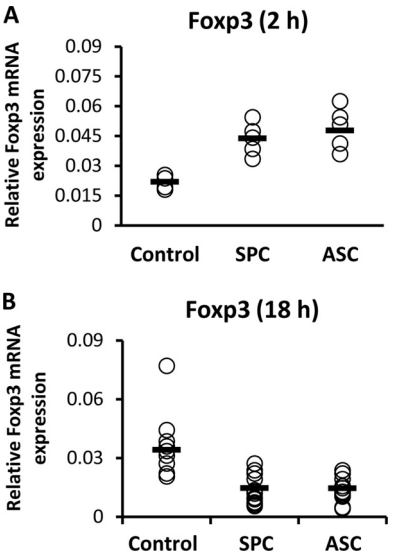

FIG 4 Expression of Foxp3 in the spleens of mice with LPS-potentiated SEA-induced toxic shock is not altered by ASC administration. Mice were injected intraperitoneally with 10 μg SEA, followed by 80 μg LPS, as described in Materials and Methods. At 30 min after SEA injection, mice were intraperitoneally administered 1×10^6 SPCs or 1×10^6 ASCs. At 2 h or 18 h after LPS injection, spleens were collected. The mRNA expression of Foxp3 in the spleen at 2 h (A) or 18 h (B) after LPS injection was examined by quantitative real-time PCR. Control indicates the cytokine production from naive mice without LPS-potentiated SEA-induced toxic shock. The data are pooled from 3 independent experiments ($n = 3$ or 4). Each dot on the graph represents an individual mouse. The horizontal bar indicates the mean value for each group.

garding their superantigenic activity, SEs stimulate immune-cell expansion and uncontrolled release of proinflammatory cytokines (2–5). Thus, suppression of inflammation and immune-cell proliferation is a main target to ameliorate clinical symptoms of toxic shock caused by SEs. ASCs have become attractive for clinical applications (23). Adipose tissues in which abundant ASCs exist are easily attainable. Particularly, lipoaspiration, a procedure for collecting adipose tissues, represents a minimally invasive option comparing to the commonly used bone marrow procedure (24, 25). Regarding the profound immunomodulatory impact of ASCs, application of these cells has already been demonstrated in a variety of experimental models of diseases, including autoimmune diseases and graft-versus-host diseases (13, 26, 27). However, the suppressing effect of ASCs in the toxic shock model caused by bacterial toxins has not yet been reported. In the present study, the effect of ASCs on toxic shock caused by SEA was examined. To establish SEA-induced toxic shock in mouse model, LPS is required, because mice are more resistant to the toxic effect of SEA than humans (28). Although the mechanism underlying the synergistic induction of LPS remains unclear, LPS enhances lethality caused by SEA *in vivo* (14, 15).

Previous studies have demonstrated that ASCs localize at inflamed sites and modulate the immune response (13, 29, 30). In addition, the inflammatory cytokines produced by activated T cells play a crucial role in increasing the immunosuppressive effect of ASCs (11). In the concanavalin A-induced hepatitis model, efficient immunosuppression of ASCs was found after administration of 1×10^6 ASCs at 30 min after induction with concanavalin A (13). Therefore, in our study, 1×10^6 ASCs were administered

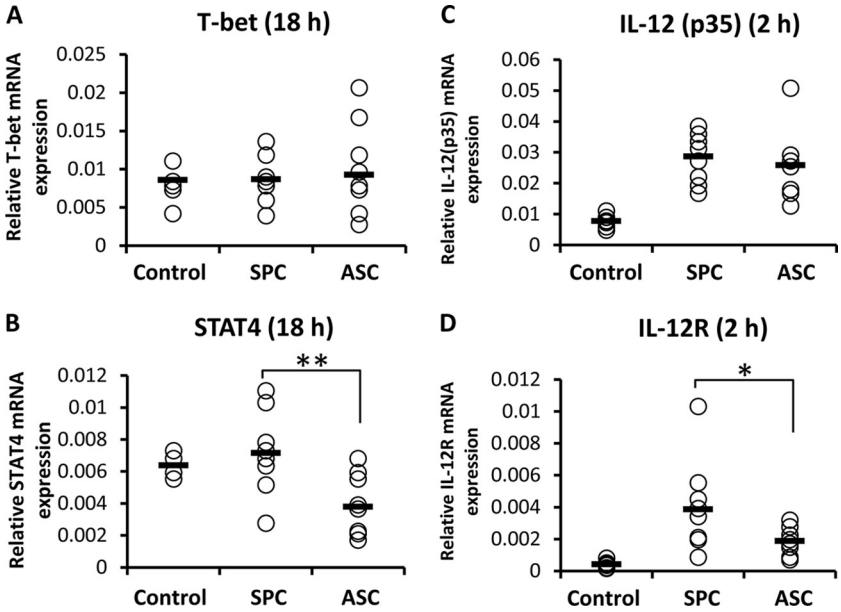

FIG 5 Expression of IL-12R and STAT4 in the spleens of mice with LPS-potentiated SEA-induced toxic shock is suppressed by ASC administration. Mice were injected intraperitoneally with 10 μg SEA, followed by 80 μg LPS, as described in Materials and Methods. At 30 min after SEA injection, mice were intraperitoneally administered 1×10^6 SPCs or 1×10^6 ASCs. At 2 or 18 h after LPS injection, spleens was collected and the mRNA expression of T_H1-related genes was determined by quantitative real-time PCR. The expression of T-bet (A) and STAT4 (B) was examined at 18 h, whereas the expression of IL-12(p35) (C) and IL-12Rβ2 (D) was examined at 2 h after LPS injection. Control indicates the cytokine production from naive mice without LPS-potentiated SEA-induced toxic shock. The data are pooled from 3 independent experiments ($n = 3$ or 4). Each dot on the graph represents an individual mouse. The horizontal bar indicates the mean value for each group. The values that are significantly different between mice administered SPCs and those administered ASCs are indicated (*, $P < 0.05$; **, $P < 0.01$).

intraperitoneally at 30 min after SEA injection. The results demonstrate that intraperitoneal administration of ASCs improved the survival of mice from LPS-potentiated SEA-induced toxic shock. Importantly, ASCs could not attenuate lethal toxic shock caused by LPS alone. This result suggests an immunomodulatory effect of ASCs on the SEA response. Indeed, SEA and LPS have different mechanisms to activate T cells. SEA, as well as other superantigens, activates T cells via cross-linking between major histocompatibility complex class II and the TCR (4). On the other hand, LPS acts as neither a mitogen, a superantigen, or an antigen. The activation of T cells by LPS requires the help of accessory functions by primed monocytes and is MHC unrestricted (31). A study demonstrated that MSCs modulate the immune response by inhibiting mitogen- and antigen-stimulated T-cell proliferation (32). In addition, MSCs have been shown to reduce the expression of major histocompatibility complex class II molecules on mature dendritic cells (33). These known mechanisms of MSCs would explain the noneffectiveness of ASCs in the LPS-induced toxic shock model. In our study, not only was the survival of mice with LPS-potentiated SEA-induced toxic shock improved, but the inflammatory response also was attenuated by ASC administration. Nonalteration of Foxp3 gene expression in both early and late phases of toxin-induced toxic shock, as well as IL-10 production, suggests that ASCs do not have an influence on Treg cells. On the other hand, ASCs seem to affect T_H1 cell expansion. Even though the expression of T-bet, a key transcription regulator of T_H1 via the IFN-γ receptor and TCR, was not altered, the expression of IL-12R and STAT4 was significantly reduced by ASC administration. Thus, the ASCs may modulate T_H1 cell expansion through IL-12R and STAT4 signaling. In order to apply ASCs for prevention of toxic shock, the detailed mechanism underlying this suppression effect of ASCs remains to be clarified. It is important to address whether this suppressing effect results from the secreted soluble factors, cell-cell contact, or absorption of SEA by ASCs.

In the present study, we clearly demonstrated that ASCs attenuate toxic shock caused by LPS-potentiated SEA. This information addresses an emerging and important role of ASCs in attenuating inflammatory responses caused by infections with *S. aureus*. In addition, this information significantly advances the understanding of *S. aureus* infection biology and will be very useful for persons who have to deal with serious *S. aureus* infections.

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We declare that we have no conflict of interest.

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