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Distinct cytokine-inducing ability of *Streptococcus pyogenes* strains isolated from toxic shock-like syndrome cases

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Summary. Streptococcal toxic shock-like syndrome (TSLS) is one of the fulminant types of toxic infectious diseases caused by *Streptococcus pyogenes*. Although there are several candidates as the virulence factors including M protein, little is known why TSLS is caused by only some of *S. pyogenes* strains but not by others. In this study, we compared the cytokine inducing activity in mice between *S. pyogenes* M-3 type strains derived from TSLS and non-TSLS cases. After infection into mice, TSLS-derived strains persisted longer in the blood than non-TSLS-derived strains. Moreover, TSLS-derived strains were highly capable of inducing proinflammatory cytokines both *in vivo* and *in vitro*. TSLS-derived strains also showed a higher ability to produce streptolysin O (SLO). These findings suggest that SLO plays a role as a possible virulence factor accounting for the shock induction in the infected host.

Key words — *Streptococcus pyogenes*, TSLS, cytokine.

INTRODUCTION

Streptococcus pyogenes is a gram-positive coccus and is known as the representative of group A *streptococci* (GAS). GAS causes a variety of acute infectious diseases including not only local tonsillitis, cellulitis, lymphangitis or erysipelas, but also systemic diseases like scarlet fever, rheumatic fever and acute glomerulonephritis due to immune response of the infected host. In addition to these long-known streptococcal diseases, invasive streptococcal infections appear to be increasing. Based on a recent population-based data, the global burden of disease caused by GAS is estimated to be at least 517,000 deaths each year, and the burden of invasive GAS diseases is unexpectedly high, with at least 663,000 new cases and 163,000 deaths each year [(1)].

Streptococcal toxic shock syndrome, which is referred to as toxic shock-like syndrome (TSLS) is one of the invasive GAS diseases and is known to be potentially fatal. TSLS typically manifests with high fever and severe pain at the site of infection, accompanied by low blood pressure, malaise and confusion, which can rapidly progress to coma, multi-organ failure and necrotizing fasciitis. In Japan, since the report of the first case of TSLS in 1992, more than 300 cases have been observed with a mortality as high as 60%[(2)]. Though streptococci belonging to serogroups other than group A are also known to cause an invasive infection, GAS comprises more than 94% of the reported cases. Among various virulence-associated factors of GAS, the M type has been implicated widely as involved in the ability to cause the invasive infections[(3)]. In contrast to the carbohydrate group antigen, M antigen is a bacterial surface protein and has been employed for serotyping of GAS. As serotyping based on M-agglutination test does not always give convincing results, the genotyping of M protein gene (*emm*) has been introduced recently. The *emm* genotyping of the causative isolates in Japan during 2000 to 2004 revealed that *emm1* type strains were the most frequently isolated strains followed by *emm3*, then

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Abbreviations — TSLS, toxic shock-like syndrome; SLO, streptolysin O; GAS, group A *streptococci*; CFU, colony forming unit; TNF-α, tumor necrosis factor-α; IL, interleukin; IFN-γ, interferon-γ.

emm89, *emm28*, and so on[2]. This tendency toward the domination of *emm1* strains also applies to the strains isolated in countries outside Japan. Although there are some differences in the incidence of particular *emm* types according to the place of survey conducted, M-1 and M-3 types accounted for more than 50% of the cases with M-3 type being always less frequent compared with M-1 type[3].

Thus epidemiological study clearly shows that GAS strains of particular M serotype or *emm* genotype predominantly cause streptococcal TSLS. However, it may be controversial whether M protein itself is a virulence factor contributing to the direct invasiveness or it is just a marker of causative strains. M-1 or M-3 strains can be isolated also from non-invasive streptococcal infection like acute pharyngitis or tonsillitis without development into invasive, life-threatening disease.

The clinical symptoms of streptococcal TSLS are characterized by a suddenly appearing toxic shock resembling to the typical toxic shock syndrome (TSS) in which the robust production of a massive amount of host cytokines is implicated. In this regard, it is of interest to test whether there is any difference in the cytokine-inducing ability between the GAS strains isolated from TSLS or from non-invasive local infections. In this study, we have compared the levels of various inflammatory cytokines produced by mouse macrophages after stimulation with the whole cell of streptococcal strains. To rule out the contribution of various M proteins, strains of GAS with the same M type have been employed for the present study.

MATERIALS AND METHODS

Bacterial strains and growth

The group A *S. pyogenes* strains were obtained from the Department of Bacteriology, National Institute of Infectious Diseases, Tokyo, Japan and Kanagawa Research Laboratory of Public Health, Kanagawa, Japan. All strains were identified as GAS according to conventional bacteriological profile including Gram stain, β-hemolysis on blood agar plate, absence of catalase activity and Lancefield serotyping. Typing for M antigens of *S. pyogenes* strains was also carried out at those institutes. In order to compare the cytokine-inducing activity between the isolates from TSLS cases and those from non-TSLS cases without participation of different M-type, a possible virulence factor, we have selected only M-3 type strains. Four M-3 type strains, 920522, 930040, 940130 and 950323 were isolated mainly from the throat of non-TSLS cases, and other four M-3 type strains, NIH#12, NIH#16, NIH#20 and NIH#34 were all derived from TSLS cases. Each bacterial strain was cultured in a brain heart infusion broth (Eiken Chemicals, Tokyo, Japan) at 37°C overnight and then stored at -80°C in aliquots. The viable number of bacterial stock was determined by counting the colonies grown on blood agar after cultivating the serially diluted sample.

Hemolytic activity of each bacterial strain

Each bacterial strain was suspended at 1×10^6 CFU (colony-forming unit) /ml in brain heart infusion broth (Beckton Dickinson Microbiology Systems, MD, USA) and cultured with gentle shaking at 37°C for 6 h. By centrifugation, bacterial culture supernatants were obtained and subjected for a quantitative assay of hemolytic activity. Serially two-fold diluted samples were added to the equal volume of 1% suspension of sheep erythrocytes and incubated for 60 min in a round-bottomed 96-well plate. The plate was centrifuged at 800 x g for 10 min and the hemolytic activity was expressed as a hemolytic unit (HU) that was defined as the reciprocal of the highest dilution that showed complete hemolysis (absence of erythrocytes sediment). The hemolytic activity in the culture supernatants was completely inhibited by treatment with cholesterol, indicating that the hemolytic activity was largely responsible for streptolysin O (SLO), a cholesterol-dependent hemolysin of *S. pyogenes* (data not shown).

Mouse and macrophages

Male mice of BALB/c strain raised and maintained under specific pathogen-free condition (Japan SLC, Shizuoka, Japan) were used at the age of 6 to 8 weeks. Three days after an intraperitoneal injection of 2 ml of thioglycollate medium (Beckton Dickinson), peritoneal exudate cells were harvested. Cells suspended at 2×10^6 /ml were allowed to adhere to 96-well flat-bottomed culture plate for 2 h and then non-adherent cells were removed by washing with warm Hanks' balanced salt solution (HBSS). Resulting adherent cells were used as peritoneal macrophages.

In vivo infection

Mice were injected intraperitoneally with 5×10^7 CFU of bacterial strain and the venous blood sample was obtained in 24 h. Blood samples were diluted in Heparin-PBS and the colony count was done by using blood agar plate. To check the cytokine induction *in vivo*, sera were obtained 6 h and 24 h after intraperitoneal infection and subjected for cytokine assay using ELSIA kit specific for each cytokine (Pharmingen, CA, USA).

Cytokine induction assay

Mouse peritoneal macrophages were stimulated with *S. pyogenes* suspended in HBSS at MOI of 10 and cultured at 37°C in a humidified incubator with 5% CO_2. After 6 h of culture, the supernatants were saved and examined for the level of several inflammatory cytokines including interleukin 1α (IL-1α), IL-6 and tumor necrosis factor-α (TNF-α). Cytokine levels in the culture supernatants were determined by ELSIA.

Statistical analysis

The statistical significance of data was determined by Student's *t*-test and a *p* value of <0.05 was considered to be statistically significant.

RESULTS

Comparison of bacterial virulence between TSLS-derived strains and non-TSLS-related strains

In order to compare the virulence between TSLS-derived strains and non-TSLS-derived strains, three mice were infected intraperitoneally with 5×10^7 CFU of each strain. Blood sample was obtained 24 h after the infection and the number of viable bacteria in the blood was determined by plating the serially diluted sample on blood agar plates. The mean CFU value calculated per ml of blood for each strain was plotted. As shown in Fig. 1, the mean of 4 TSLS-derived strains was significantly higher than that of 4 non-TSLS-derived strains. It was clear that TSLS-derived strains persist for a longer period than non-TSLS-derived strains *in vivo*, suggesting the higher virulence of TSLS-derived strains.

Comparison of the cytokine levels in the sera of mice infected with each group of strains

Mice were infected intraperitoneally with 5×10^7 CFU of each strain. As the response of cytokine production in vivo occurs at an early stage, blood samples were obtained 6 h after the infection and sera were collected. The levels of proinflammatory cytokines, TNF-α, IL-1α and IL-6 were determined by EIA. The average level of each cytokine was always higher in the sera taken from mice infected with TSLS-derived strains than those from mice infected with non-TSLS-derived strains (Fig. 2). A difference of *p* value less than 0.05 could be observed for both TNF-α and IL-1α, but the difference of IL-6 was not statistically significant ($p> 0.1$).

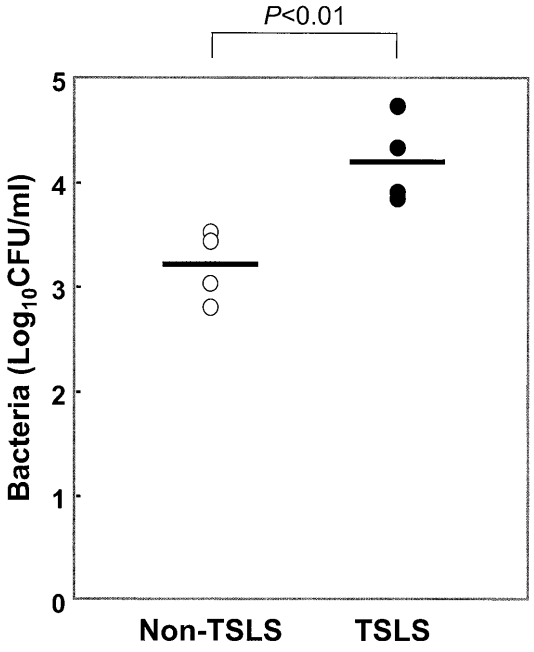

Fig. 1. Comparison of the virulence between TSLS-derived and non-TSLS-derived *S.pyogenes* strains. Balb/c mice were infected intraperitoneally with 5 x 10^7 CFU of *S.pyogenes* strains that were isolated from TSLS or non-TSLS cases. Blood samples were obtained 24 h after infection and diluted with heparin-PBS. The number of bacteria was enumerated by inoculation of the samples on blood agar plates.

Since IFN-γ is an essential cytokine usually produced after initial production of proinflammatory cytokines, sera were also obtained 24 h after the infection for determination of serum IFN-γ level (Fig. 3). As the mean value, IFN-γ as high as 330 pg/ml was detected in the sera of mice infected with TSLS-derived strains, while the level in the sera of mice infected with non-TSLS-derived strains was significantly low ($p < 0.01$).

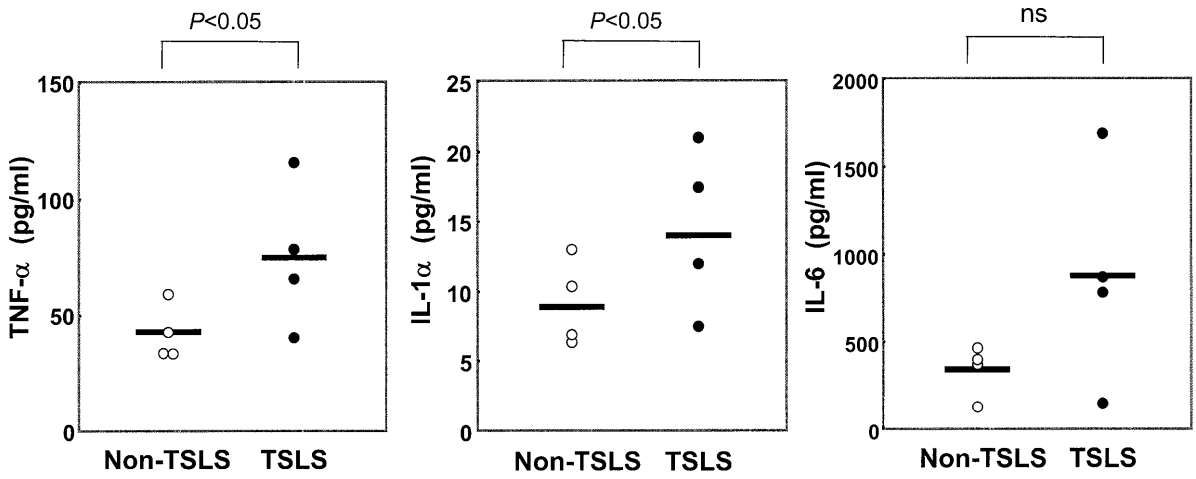

Fig. 2. Comparison of the cytokine levels in the sera of mice infected with TSLS-derived and non-TSLS-derived *S. pyogenes* strains. Mice were infected intraperitoneally with 5 x 10^7 CFU of *S. pyogenes* strains. Sera were prepared 6 h after infection. The levels of TNF-α, IL-1α and IL-6 in the sera were measured by ELISA.
ns, not significant.

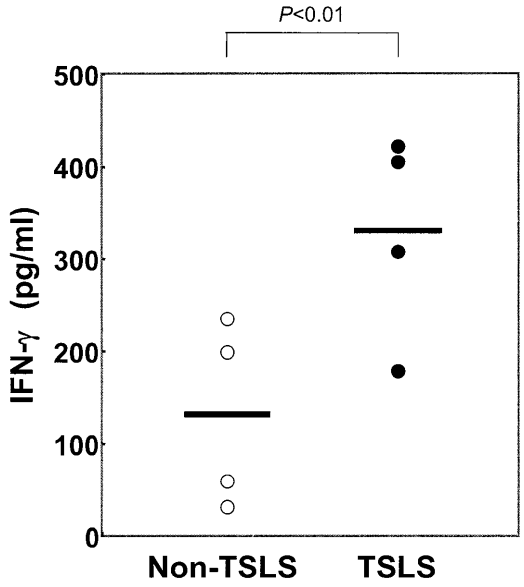

Fig. 3. The level of IFN-γ in the sera of mice infected with TSLS-derived and non-TSLS-derived *S.pyogenes* strains. Mice were infected intraperitoneally with 5 x 10^7 CFU of *S. pyogenes* strains. Sera were prepared 24 h after infection. The levels of IFN-γ in the sera were measured by ELISA.

Comparison of cytokine-inducing activity *in vitro*

As there was significant difference in the *in vivo* level of cytokines between the groups infected with TSLS-derived and non-TSLS-derived strains, next we examined the *in vitro* cytokine-inducing activity. Peritoneal exudates macrophages were stimulated with the suspension of each strain at a MOI of 10, then the culture supernatants were examined for cytokine production 6 h after culturing (Fig. 4). In general, there was a tendency for a higher cytokine inducing activity in TSLS-derived strains as compared with non-TSLS-derived strains, though a statistical significance ($p < 0.05$) was obtained only in TNF-α level and not in IL-1α and IL-6.

Comparison of hemolysin production

The TSLS-derived and non-TSLS-derived strains were inoculated into fresh brain heart infusion broth at 1×10^6 /ml and then cultured at 37°C for 8h. A densitometric measurement revealed that all the strains grew well to almost the same turbidity. By centrifugation, supernatants were collected and examined for the hemolysin production. The TSLS-derived strains produced a high level of hemolysin while the levels of hemolysin produced by non-TSLS-derived strains were significantly low (Fig. 5).

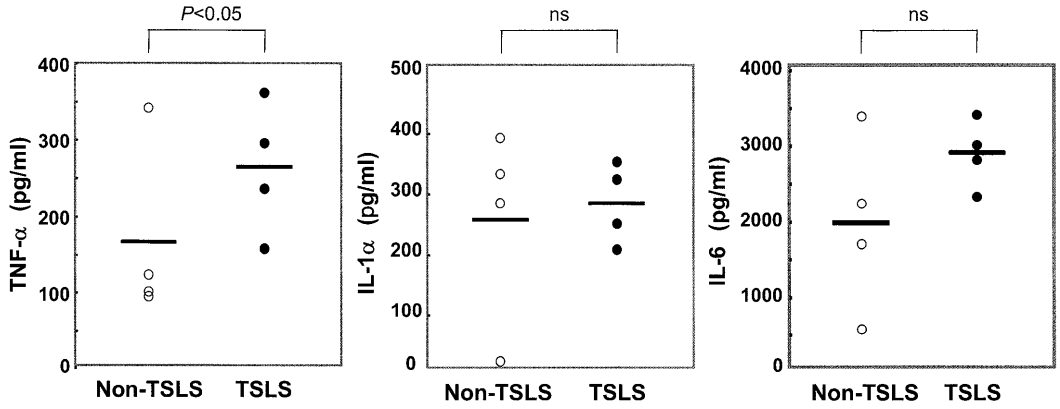

Fig. 4. Cytokine production from macrophages after infection with TSLS-derived and non-TSLS-derived *S. pyogenes* strains. Peritoneal macrophages were infected with *S. pyogenes* strains at MOI of 10 for 6h. The culture supernatants were collected and the levels of cytokines were measured by ELISA.
ns, not significant.

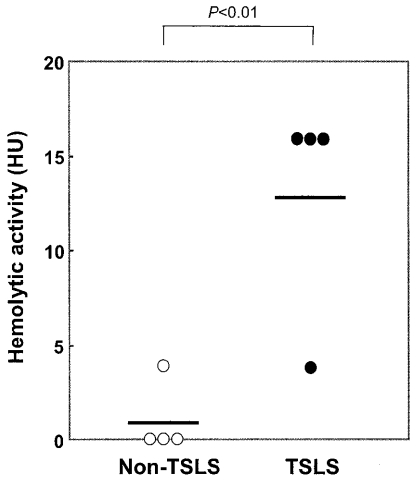

Fig. 5. Comparison of the hemolysin-producing ability between TSLS-derived and non-TSLS-derived *S. pyogenes* strains. Various strains of *S. pyogenes* were cultured for 8 h in brain heart infusion broth and the supernatants were collected. The amount of hemolysin secreted in the supernatant was estimated by measuring the hemolytic activity to 1% suspension of sheep erythrocytes.

DISCUSSION

Among various forms of infectious diseases caused by *S. pyogenes*, TSLS is the most recently recognized, fulminant type of infection. TSLS appears to have emerged in the mid-1980s and was first described on two fatal cases with a typical clinical manifestation in 1987[4], 10 years after the first description of original toxic shock syndrome due to *Staphylococcus aureus* by Todd et al[5]. In 1993, TSLS was established as an independent clinical entity and its diagnostic criteria were defined by a working group at Center for Disease Control and Prevention (CDC)[6]. Since then, this severe type of *S. pyogenes* infection has been accumulating and the attack rate estimated in 2000 by CDC was one case in 100,000 population[7]. According to the brief review by Y. Shimizu who described the first case of TSLS in Japan, 97 cases matching the diagnostic criteria were detected by 1997[8]. Though most of the cases are following infection with GAS, there are several reports indicating that group B[9] or even group C or G *streptococci*[10] can cause TSLS as well. The rather rare incidence and the occurrence of TSLS mainly in otherwise healthy adults suggest a contribution of some host factors but so far no definitive evidence has been obtained.

All the *S. pyogenes* strains are of group A carbohydrate antigen but can be divided further according to surface protein antigens. M typing is based on the antigenicity of M proteins comprising more than 50 types, and M protein has been implicated in both colonization and resistance to phagocytosis of GAS[11]. The idea that M protein may be an important virulence determinant is based on the predominance of *S. pyogenes* having particular M type among clinical isolates from TSLS cases. It is true that the vast majority of strains isolated from TSLS cases have been of the M-1 and M-3 types, however, some other M-types including those that could not be typed have also been isolated from such cases. Moreover, M-1 and M-3 types are also commonly isolated from asymptomatic carriers and from patients with transient pharyngitis or mild scarlet fever[12]. Besides, even if the M protein plays the major role in the colonization or antiphagocytic action, it is unlikely that such kind of escape mechanism results in a rapid induction of clinical shock by itself. Thus, the contribution of M protein to TSLS is still controversial, and in this study we have compared the TSLS isolates and non-TSLS isolates of the same M-3 type.

The bacterial clearance or persistence in the blood after systemic infection is a measure for an overall virulence. In mice, TSLS-derived strains showed about 1.0 log higher level in the blood 24 h after infection and this result was indicative of the higher virulence of TSLS strains. Upon systemic infection, macrophages or dendritic cells show a quick response to the invading bacteria presumably via Toll-like receptors (TLRs) and produce various cytokines as an innate immune response[13]. TLR ligands of Gram-positive bacteria are believed to stimulate the innate immune response mainly via TLR2[14], but some recent reports indicate TLR4 is also involved in the response to streptococcal cell wall[15]. Though TLR involvement was not examined in this study, there was *in vivo* response of cytokines that could be detected by cytokine ELISA in the sera of mice infected with *S. pyogenes*. An interesting observation was that a higher response was detected in TNF-α and IL-1α production when infected with TSLS-derived strains than after infection with non-TSLS strains. Though statistical significance was not obtained in IL-6 production, 3 out of 4 mice showed IL-6 levels above 780 pg/ml which were far higher than the highest level (465 pg/ml) observed among mice infected with 4 non-TSLS-derived strains. This result indicated that TSLS-derived strains have higher activity in inducing the host proinflammatory cytokines. IFN-γ is not a proinflammatory cytokine but an important TH1 cytokine contributing host defense[16]. Again, the level of IFN-γ in the sera was higher in mice infected with TSLS-derived strains. As IFN-γ response at the early stage of infection is believed to be dependent on both macrophage-mediated IL-12 and IL-18, we have examined the level of these IFN-γ-inducing cytokines in the sera but could not detect a significant amount in both groups of sera (data not shown).

A CFU assay in the blood after infection demonstrated that TSLS-derived strains persisted at a higher level than non-TSLS-derived strains. Therefore, the *in vivo* response could be a reflection of a higher number of viable bacteria *in vivo*. To rule out this possibility, we have done the *in vitro* study by using peritoneal exudates macrophages. After 6 h of stimulation with the same number of TSLS-derived and non-TSLS-derived strains, proinflammatory cytokines were induced in both groups. A higher level of macrophage response was observed after stimulation with TSLS-derived strains regarding two cytokines, IL-6 and TNF-α. In this *in vitro* infection model, no intracellular bacterial growth was observed, and the bacterial number associating with macrophages was almost the same for both groups, so the difference in the cytokine response appeared to be due to some difference in the cytokine-inducing factor.

Some host cytokines are known to play a major role in the development of lethal shock in bacterial infection[17]. Among various cytokines, macrophage-derived TNF-α is the most implicated in the induction of septic shock[18]. TNF-α is a multifunctional cytokine mediating the fever, cachexia, inflammation, bone

resorption, tissue remodeling, cellular apoptosis and septic shock[18, 19]. This cytokine causes shock and tissue injury identical to the sepsis, and anti-TNF antibodies given early protect animals from septic shock[20]. In our present study, macrophages showed a significant level of TNF-α production particularly to the stimulation with TSLS-derived strains. TNF-α can be produced by not only macrophages but also a variety of host cells, but the finding that adherent macrophages produced a high level of TNF-α *in vitro* suggest the presence of TNF-α-inducing property especially in TSLS-derived strains.

Peptidoglycan, lipoteichoic acid, and killed organisms are reported to be capable of inducing TNF-α production by mononuclear cells *in vitro*[21]. These bacterial surface components that are representative TLR ligands may not account for the difference we have observed between TSLS-derived strains and non-TSLS-derived strains. Cell-free bacterial supernatant has been shown to induce proinflammatory cytokines including TNF-α in human mononuclear cells[22], suggesting the contribution of secreted protein from bacteria to the induction of key cytokines. *S. pyogenes* is known to produce a variety of toxins or exoenzymes contributing to the destruction of connecting tissues or skin manifestation. Among protein toxins produced by *S. pyogenes*, streptococcal pyrogenic exotoxin (Spe) is a unique protein toxin now recognized as one of the bacterial superantigens. Following the discovery of TSST-1 as the causative bacterial superantigen for staphylococcal toxic shock syndrome in 1981[23], many superantigens have been identified from not only *S. aureus* (such as Staphylococcal enterotoxin (SE) A, SEB, SEC1-3 and SED) but also *S. pyogenes* (such as SpeA, SpeC, SpeG and SpeH). SpeA is the designation as superantigen for streptococcal pyrogenic (erythrogenic) exotoxin A[24]. This superantigen is believed to stimulate T cell populations expressing particular TcR Vβ repertoire in the context of class II MHC on antigen presenting cells[25]. Murine cells are also known to produce several cytokines after stimulation with this superantigen[26]. Although superantigen seems to participate in the pathophysiology of TSLS, the T cell-dependent induction of cytokines by SpeA and other streptococcal superantigen(s) cannot explain the induction of macrophage-derived proinflammatory cytokines. On the other hand, IFN-γ production was highly induced after infection with TSLS-derived strains. Since IFN-γ has been shown to be produced mainly from T cells and NK cells, but not from macrophages, a further study is necessary to clarify whether the difference in IFN-γ-inducing activity between TSLS-derived and non-TSLS-derived strains is responsible for the difference in their superantigen activity for T cell activation.

The data have suggested a distinct ability of TSLS-derived strains to induce host proinflammatory cytokines which are implicated in the pathophysiology of *S. pyogenes* infection. In order to get some insight into the possible virulence factor that may account the cytokine-inducing activity, the hemolysin-producing ability was determined. Recently, it is reported that listeriolysin O (LLO) produced by *Listeria monocytogenes* is highly capable of inducing proinflammatory cytokines followed by induction of IFN-γ without participation of T cells or superantigen activity[27]. Similar activity was observed in other allied protein toxins produced by several Gram-positive bacterial species[28, 29]. LLO and allied protein toxins are the family protein belonging to cholesterol-dependent cytolysin and its cytokine-inducing activity has been shown to be dependent on the N-terminus portion of the protein[30]. Streptolysin O (SLO) is a hemolysin produced by *S. pyogenes* and is one of the members of cholesterol-dependent cytolysin family proteins. Taking these finding into consideration, we have examined the strains employed in this study for the ability to produce SLO by semi-quantitative assay. There was a significant difference in the SLO-producing activity between TSLS-derived strains and non-TSLS-derived strains. Although a further study in detail is to be done to clarify whether SLO is responsible for the induction of proinflammatory cytokines in TSLS, there is a possibility that the SLO-producing ability in the different *S. pyogenes* strains is related to the aetiology of TSLS that cannot be explained by particular M-type or other known virulent determinants. SLO is reported to be capable of inducing chemokines[31] and one report suggests the cytokine inducing ability of SLO[32], therefore, direct study in the future using recombinant SLO may reveal the direct evidence of SLO contribution to cytokine induction in TSLS.

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REFERENCES

1. Carapetis JR, Steer AC, Mulholland EK, Weber M: The global burden of group A streptococcal diseases. *Lancet Infect Dis* **5:** 685-694, 2005.
2. The topics of this month. Streptococcal infections 2000-2004. *Infect Agent Surv Rep* **25:** 252-253, 2005. (http://idsc.nih.go.jp/iasr/25/296/tpc296-j.html)
3. Stevens DL: Invasive streptococcal infections. *J Infect Chemother* **7:** 69-80, 2001.
4. Cone LA, Woodard DR, Schlievert PM, Tomory

GS: Clinical and bacteriologic observations of a toxic shock-like syndrome due to *Streptococcus pyogenes*. *N Engl J Med* **317:**146-149, 1987.
5. Todd J, Fishaut F, Welch T: Toxic-shock syndrome associated with phage-group-I *staphylococci*. *Lancet* **2:** 1116-1118, 1978.
6. The working group on severe streptococcal infections: Defining the group A streptococcal toxic shock-like syndrome. *JAMA* **269:** 390-391, 1993.
7. Stevens DL: Streptococcal toxic shock syndrome associated with necrotizing fasciitis. *Ann Rev Med* **51:** 271-288, 2000.
8. Shimizu Y: Streptococcal toxic shock-like syndrome. *Intern Med* **39:** 195-196, 2000.
9. Crum NF, Wallace MR: Group B streptococcal necrotizing fasciitis and toxic shock-like syndrome: a case report and review of the literature. *Scand J Infect Dis* **35:** 878-881, 2003.
10. Williams GS: Group C and G *streptococci* infections: emerging challenges. *Clin Lab Sci* **16:** 209-213, 2003.
11. Cleary PP, LaPenta D, Heath D, Haanes EJ, Chen C: A virulence regulon in *Streptococcus pyogenes*. In: Dunnyu GM, Cleary PP and Makay LL (eds). Genetics and molecular biology of *Streptococci*, *Lactococci* and *Enterococci*. ASM Press, Washington DC 1991, p 147-151.
12. Johnson DR, Stevens DL, Kaplan EL: Epidemiologic analysis of group A streptococcal serotypes associated with severe systemic infections, rheumatic fever, or uncomplicated pharyngitis. *J Infect Dis* **166:** 374-382, 1992.
13. Takeda K, Akira S: Toll-like receptors in innate immunity. *Int Immunol* **17:**1-14, 2005.
14. Yoshimura A, Lien E, Ingalls RR, Tuomanen E, Dziarski R, Golenbock D: Recognition of Gram-positive bacterial cell wall components by the innate immune system occurs via Toll-like receptor 2. *J Immunol* **163:** 1-5, 1999.
15. Okamoto M, Oshikawa T, Tano T, Ahmed SU, Kan S, Sasai A, Akashi S, Miyake K, Moriya Y, Ryoma Y, Saito M, Sato M: Mechanism of anticancer host response induced by OK-432, a streptococcal preparation, mediated by phagocytosis and Toll-like receptor 4 signaling. *J Immunother* **29:** 78-86, 2006.
16. Salgame P: Host innate and Th1 responses and the bacterial factors that control infection. *Curr Opin Immunol* **17:** 374-380, 2005.
17. Stevens DL, Bryant AE, Hackett SP: Gram-positive shock. *Curr Opin Infect Dis* **5;** 355-363, 1992.
18. Lin WJ, Yeh WC: Implication of Toll-like receptor and tumor necrosis factor alpha signaling in septic shock. *Shock* **24:** 206-209, 2005.
19. Liu SF, Malik AB: NF-kappa B activation as a pathological mechanism of septic shock and inflammation. *Am J Physiol Lung Cell Mol Physiol* **290:** 622-645, 2006.
20. Aggarwal BB, Samanta A, Feldmann M: A compendium of cytokines and other mediators of host defense. TNFα. In: Oppenheim JJ, Feldmann J (eds). Cytokine reference. Academic press London 2001, p 413-434.
21. Muller-Alouf H, Alouf JE, Gerlach D, Ozegowski JH, Fitting C, Cavaillon JM: Comparative study of cytokine release by human peripheral blood mononuclear cells stimulated with *Streptococcus pyogenes* superantigenic erythrogenic toxins, heat-killed *streptococci*, and lipopolysaccharide. *Infect Immun* **62:** 4915-4921, 1994.
22. Soto A, Evans TJ, Cohen J: Proinflammatory cytokine production by human peripheral blood mononuclear cells stimulated with cell-free supernatants of viridans *streptococci*. *Cytokine* **8:** 300-304, 1996.
23. Schlievert PM, Shands KN, Dan BB, Schmid GP, Nishimura RD: Identification and characterization of an exotoxin from *Staphylococcus aureus* associated with toxic-shock syndrome. *J Infect Dis* **143:** 509-516, 1981.
24. Abe J, Forrester J, Nakahara T, Lafferty JA: Selective stimulation of human T cells with streptococcal erythrogenic toxins A and B. *J Immunol* **146:** 3747-3750, 1991.
25. Leonard BA, Lee PK, Jenkins MK, Schlievert PM: Cell and receptor requirements for streptococcal pyrogenic exotoxin T-cell mitogenicity. *Infect Immun* **59:** 1210-1214, 1991.
26. Muller-alouf H, Alouf JE, Gerlach D, Fitting C, Cavaillon JM: Cytokine production by murine cells activated by erythrogenic toxin type A superantigen of *Streptococcus pyogenes*. *Immunobiology* **186:** 435-448, 1992.
27. Nomura T, Kawamura I, Tsuchiya K, Kohda C, Baba H, Ito Y, Kimoto T, Watanabe I, Mitsuyama M: Essential role of interleukin 12 (IL-12) and Il-18 for gamma interferon production induced by listeriolysin O in the spleen cells of mice. *Infect Immun* **70:** 1049-1055, 2002.
28. Baba H, Kawamura I, Kohda C, Nomura T, Ito Y, Kimoto T, Watanabe I, Ichiyama S, Mitsuyama M: Induction of gamma interferon and nitric oxide by truncated pneumolysin that lacks pore-forming activity. *Infect Immun* **70:** 107-113, 2002.
29. Ito Y, Kawamura I, Kohda C, Baba H, Nomura T, Kimoto T, Watanabe I, Masao Mitsuyama: Seeligeriolysin O, a cholesterol-dependent cytolysin of *Listeria seeligeri*, induces gamma interferon from spleen cells of mice. *Infect Immun* **71:** 234-241, 2003.

30. Kohda C, Kawamura I, Baba H, Nomura T, Ito Y, Kimoto T, Watanabe I, Mitsuyama, M: Dissociated linkage of cytokine-inducing activity and cytotoxicity to different domains of listeriolysin O from *Listeria monocytogenes*. *Infect Immun* **70:** 1334-1341, 2002.
31. Mitsui K, Takano K, Nakatani S, Nambu H, Shibata F, Nakagawa H: Chemokine production by rat macrophages stimulated with streptolysin O from *Streptococcus pyogenes*. *Microbiol Immunol* **46:** 37-45, 2002.
32. Kappur V, Majesky MW, Li LL, Black RA, Musser JM: Cleavage of interleukin 1β (IL-1β) precursor to produce active IL-1β by a conserved extracellular cysteine protease from *Streptococcus pyogenes*. *Proc Natl Acad Sci USA* **90:** 7676-7680, 1993.