Regular Article

Selective Cytotoxicity of Staphylococcal α -Hemolysin (α -Toxin) against Human Leukocyte Populations

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Staphylococcus aureus produces a variety of exoproteins that interfere with host immune systems. We attempted to purify cytotoxins against human leukocytic cells from the culture supernatant of *S. aureus* by a combination of ammonium sulfate precipitation, ion-exchange chromatography on a CM-cellulose column and HPLC on a Mono S 5/50 column. A major protein possessing cytotoxicity to HL60 human promyelocytic leukemia cells was purified, and the protein was identified as α -hemolysin (Hla, α -toxin) based on its molecular weight (34 kDa) and N-terminal amino acid sequence. Flow cytometric analysis suggested differential cytotoxicity of Hla against different human peripheral blood leukocyte populations. After cell fractionation with density-gradient centrifugation, we found that peripheral blood mononuclear cells (PBMCs) were more susceptible to Hla than polymorphonuclear leukocytes. Moreover, cell surface marker analysis suggested that Hla exhibited slightly higher cytotoxicity against CD14-positive PBMCs (mainly monocytes) than CD3- or CD19-positive cells (T or B lymphocytes). From these results, we conclude that human leukocytes have different susceptibility to Hla depending on their cell lineages, and thereby the toxin may modulate the host immune response.

Key words α -hemolysin; leukocyte; cytotoxin; immune evasion; flow cytometry

INTRODUCTION

The Gram-positive bacterium *Staphylococcus aureus* is a common human pathogen that causes infectious diseases in various tissues, with the potential for severe morbidity and mortality, especially in compromised patients. The emergence of multidrug-resistant strains of *S. aureus*, including methicillin-resistant *S. aureus* (MRSA), has become a serious threat to global public health. It is known that *S. aureus* produces numerous virulence factors that play crucial roles in the pathogenesis of the infection. These factors include hemolysins, leukocidins, enterotoxins, and toxic shock syndrome toxin-1.^{1–4)} Several studies including ours have shown that *S. aureus* also secretes various exoproteins that cause perturbations of the host immune system, such as complement inhibitors, immunoglobulin-binding proteins, chemotaxis inhibitory proteins, and superantigen-like proteins (SSL).^{5–8)} The SSL family of exoproteins have been reported to influence a wide range of host immune responses (e.g., Toll-like receptor antagonist, immunoglobulin receptor antagonist, and leukocyte trafficking inhibitors).^{9–12)} Thus, exoproteins from *S. aureus* are thought to interact with host immune cells and thereby facilitate the development of infections. One of the most direct actions disturbing the host immunity seems to be the secretion of cytotoxins causing serious damage to immune cells.

In this study, we attempted to purify cytotoxins from a culture supernatant of *S. aureus* using HL60 human pro-

myelocytic leukemia cells as a target, and a major cytotoxic protein with a molecular weight of ca. 34 kDa was isolated. Based on its N-terminal amino acid sequence analysis, the cytotoxin was identified as α -hemolysin (Hla; also known as α -toxin). We then examined the cytotoxicity of Hla against human peripheral blood leukocytes, and found that each leukocyte population exhibited differential susceptibility to Hla. We further analyzed the selective cytotoxicity of Hla by cell fractionation and flow cytometry.

MATERIALS AND METHODS

Reagents and Antibodies Heparin (porcine) was purchased from Mochida Pharmaceutical Co., Ltd. (Tokyo, Japan). Dextran 200000, sodium dodecyl sulfate (SDS), acrylamide, *N,N'*-methylenebis(acrylamide), *N,N,N',N'*-tetramethylethylenediamine (TEMED), and 2-mercaptoethanol were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Casamino acids, yeast extract, and 7-aminoactinomycin D (7-AAD) were purchased from BD Biosciences (San Jose, CA, U.S.A.). RPMI 1640 medium and fetal bovine serum (FBS) were supplied by Sigma-Aldrich (St. Louis, MO, U.S.A.) and Biosera (Boussens, France), respectively. Lymphocyte Separation Solutions were purchased from Nacalai Tesque (Kyoto, Japan). CM-cellulofine C-200m (CM-cellulose) was a product of Seikagaku Corp. (Tokyo, Japan). Mono S 5/50 GL and Superose 12 10/300 GL columns were purchased from GE Healthcare (Piscataway, NJ, U.S.A.). A mixture of molecular weight markers (thyroglobulin,

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γ -globulin, ovalbumin, myoglobin, and vitamin B₁₂) (gel filtration standard) was purchased from Bio-Rad (Hercules, CA, U.S.A.). Coomassie brilliant blue (CBB) R250 was purchased from Merck (Darmstadt, Germany).

Anti- α -hemolysin antibody and horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (IgG) antibody were purchased from Sigma-Aldrich and Kirkegaard & Perry Laboratories (Gaithersburg, MD, U.S.A.), respectively. Phycoerythrin (PE)-labeled anti-CD3 antibody, Alexa Fluor 488-labeled anti-CD14 antibody and PE-Cy7-labeled anti-CD19 antibody were purchased from BD Biosciences.

Cells HL60 (a human promyelocytic leukemia cell line), THP-1 (a human monocytic leukemia cell line), U937 (a human monoblastic leukemia cell line), NALL-1 (a human acute lymphoblastic leukemia cell line) and K562 (a human chronic myelogenous leukemia cell line) were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) at 37°C under humidified 5% CO₂.

Human peripheral blood leukocytes were prepared essentially as described previously.¹³⁾ Briefly, human blood collected from healthy volunteers using heparin as an anticoagulant was mixed with an equal volume of 3% dextran 200000/saline. After the mixture was left to stand for 30 min to sediment most of the erythrocytes, the supernatant was centrifuged at 900rpm for 5 min. Leukocytes were purified from the pelleted cells by hypotonic lysis of the remaining erythrocytes. The preparation of mononuclear leukocytes (lymphocytes and monocytes) and polymorphonuclear leukocytes (granulocytes) was conducted by density gradient centrifugation using Lymphocyte Separation Solutions ($d = 1.077$ and 1.119) according to the manufacturer's instructions. These experimental protocols were approved by the Hoshi University Institutional Review Board (#29-002).

Purification of Cytotoxins from the Bacterial Culture Supernatant The *S. aureus* strain (ATCC 27733) was grown in CCY medium (30.0 g/L yeast extract, 20.0 g/L casamino acids, 23.2 g/L sodium pyruvate, 6.25 g/L Na₂HPO₄·12H₂O, 0.41 g/L KH₂PO₄, 20.0 mg/L MgSO₄·7H₂O, 7.5 mg/L MnSO₄·H₂O, 6.4 mg/L FeSO₄·7H₂O, 6.4 mg/L citric acid) under aerobic conditions at 37°C for 16 h.¹⁴⁾ The culture supernatant was centrifuged at 5000rpm for 20 min, then fractionated by ammonium sulfate precipitation (70% saturation) and centrifuged at 8000rpm for 20 min. The precipitate was dissolved in a small volume of 20 mM sodium phosphate buffer (pH 5.8) and dialyzed against the same buffer. After the centrifugation to remove insoluble materials, the supernatant was applied to a column (1.5 × 12 cm) of CM-cellulose (CM-cellulofine C-200 m) that had been equilibrated with 20 mM sodium phosphate buffer (pH 5.8). The column was washed with the same buffer, and the proteins bound to the column were eluted successively with the same buffer containing 0.12, 0.3 and 0.75 M NaCl. The eluate with 0.12 M NaCl was pooled and subjected to HPLC on a Mono S 5/50 GL column (GE Healthcare). The elution was conducted at a flow rate of 0.5 mL/min with a concentration gradient of NaCl (0–0.25 M) in 20 mM sodium phosphate buffer (pH 5.8).

Cytotoxic Assay Cytotoxicity was measured by the water-soluble tetrazolium method (WST-8; Seikagaku Corp.). Briefly, a serial dilution of cytotoxins was prepared in a 96-well culture plate, and a cell suspension (1 × 10⁶ cells/mL, 0.05 mL) in RPMI 1640 medium/10% FBS was added to

each well. After the plate was incubated at 37°C for 5–9 h, WST-reagent (10 μ L) was added to the well, and the plate was further incubated at 37°C for 90 min. The number of surviving cells was estimated by measuring A₄₅₀ (reference: A₆₂₀) with a microplate reader (Model MTP-450; Corona Electric, Ibaraki, Japan).

Immunoblotting SDS-polyacrylamide gel electrophoresis and immunoblotting were conducted as described previously.¹⁵⁾ A specimen purified from *S. aureus* culture supernatant was electrophoresed on 12.5% polyacrylamide gel in the presence of SDS under reducing conditions and immunoblotted using anti-Hla antibody and HRP-conjugated anti-rabbit IgG antibody. Chemiluminescence was detected with an imaging analyzer (ImageQuant LAS 500; GE Healthcare).

Amino Acid Sequence Analysis N-terminal amino acid sequence determination of purified proteins based on the Edman degradation method was conducted using an Applied Biosystems model 492 ProciseTM cLC Protein Sequencer (Thermo Fisher Scientific, Waltham, MA, U.S.A.).

Flow Cytometry Flow cytometric analysis was performed with a FACSariaTM III cell sorter (BD Biosciences) essentially as described previously.¹⁶⁾ The profile of human leukocyte populations (granulocytes, lymphocytes and monocytes) was analyzed by the forward scatter *versus* side scatter (FSC *vs.* SSC) plots. Cells positive for 7-AAD were considered dead cells. For the analysis of leukocyte cell surface markers, PE-labeled anti-CD3 antibody, Alexa Fluor 488-labeled anti-CD14 antibody and PE-Cy7-labeled anti-CD19 antibody were used. We also analyzed the expression of a disintegrin and metalloproteinase domain-containing protein 10 (ADAM 10) on human peripheral leukocytes by flow cytometry using anti-ADAM 10 monoclonal antibody (Santa Cruz Biotechnology, Dallas, TX, U.S.A.) and PE/DazzleTM 594-labeled anti-mouse IgG antibody (BioLegend, San Diego, CA, U.S.A.).

RESULTS

Purification of Cytotoxin from *S. aureus* Culture Supernatant The crude sample after ammonium sulfate precipitation was separated by ion-exchange chromatography on a CM-cellulose column. The most active fraction for the cytotoxicity against HL60 human leukemic cells was found to be an eluate with 0.12 M NaCl (Fig. 1). This fraction was estimated to account for approximately 58% of the total cytotoxic activity. We next applied the active fraction to HPLC on a Mono S column eluted with a concentration gradient of NaCl. As shown in Fig. 2A, we detected three consecutive protein peaks at an approximate elution time of 20 min. The cytotoxicity assay revealed that the first peak eluted from the column exhibited strong activity but that no significant activity was associated with the other two protein peaks. After the active fraction was rechromatographed under the same conditions, the proteins in the corresponding fraction were analyzed by SDS-polyacrylamide gel electrophoresis. When the polyacrylamide gel was stained with CBB, a single band at *ca.* 34 kDa was observed (Fig. 2B). We then analyzed the N-terminal amino acid sequence of the purified protein by a protein sequencer, and the sequence of the first 17 amino acid residues was deduced to be ADSDINIKTGTTDGSN. The NCBI-BLAST search indicated that this sequence was identical with that of *S. aureus* α -hemolysin (Hla, α -toxin) (Sequence ID:

Fig. 1. Ion-Exchange Chromatography of the Culture Supernatant of *Staphylococcus aureus*

The crude sample after ammonium sulfate precipitation was applied to a column (1.5×12 cm) of CM-cellulose that had been equilibrated with 20 mM sodium phosphate buffer (pH 5.8). After the column was washed with the same buffer, the proteins bound to the column were eluted in a stepwise fashion with the same buffer containing 0.12, 0.3 and 0.75 M NaCl. Each fraction (220 drops) of the eluate was collected and monitored with A_{280} (closed circle) and assayed for cytotoxicity against HL60 leukemic cells using a water-soluble tetrazolium (WST)-based cell viability test (grey bar). The toxin concentration that gave 50% survival (IC_{50}) under the condition described in MATERIALS AND METHODS was defined as 1 unit/mL. The fractions eluted with 0.12 M NaCl (indicated by a bar) were pooled and further subjected to purification with HPLC.

Fig. 2. Purification of Cytotoxin from the Culture Supernatant of *Staphylococcus aureus* by HPLC

(A) The active fraction after CM-cellulose column chromatography was applied to HPLC on a Mono S 5/50 GL column and eluted with a concentration gradient of NaCl (0–0.25 M) in 20 mM sodium phosphate buffer (pH 5.8) at a flow rate of 0.5 mL/min. The elution was monitored with A_{280} , and each fraction (0.5 mL) was collected and assayed for cytotoxic activity using a WST-based cell viability test. (B) The proteins in the active fraction from HPLC were analyzed by SDS-polyacrylamide gel electrophoresis/Coomassie brilliant blue staining (left) and Western blotting using anti- α -hemolysin antibody (right).

WP_084927791.1). When the protein sample was subjected to immunoblotting analysis using anti-Hla antibody, it gave a single band with the same electrophoretic mobility as the band detected by CBB staining (Fig. 2B). To estimate the approximate molecular weight without denaturation, the sample was applied to size exclusion chromatography on a Superose 12 10/300 GL column. Compared with the elution positions of

Fig. 3. Cytotoxicity of Purified Hla against Various Human Leukemic Cell Lines

(A) The cell suspension (1×10^6 cells/mL, 0.05 mL) was mixed with a serial dilution of Hla (0.05 mL) and incubated at 37°C for 6 h. The cytotoxicity was measured by WST-based cell viability assay. (B) The HL60 cell suspension (1×10^6 cells/mL, 0.05 mL) was mixed with Hla ($6 \mu\text{g/mL}$, 0.05 mL) and incubated at 37°C for 0–9 h. The cytotoxicity was measured as described above.

standard proteins, the molecular weight of the purified protein was estimated to be 30–35 kDa (data not shown), suggesting that the protein was composed of a monomer with *ca.* 34 kDa. Based on these results, we concluded that the cytotoxin purified from the *S. aureus* culture supernatant was Hla.^{17,18)}

Susceptibility of Human Leukemia Cell Lines to Hla We then examined several human leukemic cell lines for their susceptibility to Hla. Among the cell lines tested, NALL-1 lymphoblastic leukemia cells were most susceptible ($IC_{50} = 0.008 \mu\text{g/mL}$), followed by HL60 promyelocytic leukemia cells ($IC_{50} = 0.025 \mu\text{g/mL}$), U937 monocytic leukemia cells ($IC_{50} = 0.072 \mu\text{g/mL}$), and THP-1 monocytic leukemia cells ($IC_{50} = 0.082 \mu\text{g/mL}$) (Fig. 3A). By contrast, K562 myelogenous leukemia cells appeared to be resistant ($IC_{50} > 3.0 \mu\text{g/mL}$). Next, we examined the time period required for exhibiting cytotoxicity by Hla. As shown in Fig. 3B, incubation of HL60 cells for 6 h or longer in the presence of Hla was required for efficient cytotoxicity, but no substantial cytotoxicity was observed after the incubation for 3 h or shorter periods.

Differential Susceptibility of Human Leukocyte Populations to Hla We next examined the cytotoxicity of Hla against human peripheral blood leukocytes. The dose response of cytotoxicity to THP-1 cells by Hla showed a typical sigmoid curve (Fig. 4A). By contrast, the cytotoxicity of Hla to human blood leukocytes was rather complicated; *i.e.*, the cytotoxicity was dose-dependently increased up to $0.3 \mu\text{g/mL}$ of Hla but was almost unchanged at concentrations between 0.3 – $9 \mu\text{g/mL}$ (the approximate cytotoxicity was 40%). This result suggests that human leukocytes consist of multiple cell populations with different susceptibility to Hla. To further ex-

Fig. 4. Viability of Human Leukocytes after the Treatment with Hla

(A) THP-1 leukemic cells and leukocytes isolated from human peripheral blood (2×10^6 cells/mL, 0.05 mL) were incubated with various concentrations of Hla (0.05 mL) at 37°C for 5 h. The cytotoxicity was measured by WST-based cell viability assay. (B) Human peripheral blood leukocytes (2×10^6 cells/mL) were incubated with Hla (0, 0.3 or 27 μg/mL) at 37°C for 5 h. After the treatment with 7-AAD, the cells were analyzed by flow cytometry. The results are shown in the forward versus side scatter (FSC vs. SSC) plots. The dots in the area surrounded by a broken line (left and middle panels) represent 7-AAD-positive dead cells. G, granulocyte; L, lymphocyte; M, monocyte.

Table 1. Viability of Human Leukocytes after the Treatment with Hla

Hla (μg/mL)	Cell viability (%)			
	Leukocyte (whole)	Monocyte	Lymphocyte	Granulocyte
0	100.0	100.0	100.0	100.0
0.3	87.7	1.8	4.4	103.5
3.0	86.4	2.7	3.3	102.1
27.0	37.5	0.0	0.0	44.5

amine this possibility, we utilized flow cytometry to analyze the cell profile after treatment with various concentrations of Hla. Figure 4B (right panel) shows the profile of human leukocytes without Hla treatment. After the treatment with Hla (0.3 μg/mL), the lymphocyte and monocyte populations had almost completely disappeared (Fig. 4B, middle panel). When the concentration of Hla was increased to 27 μg/mL, a significant population of granulocytes was injured in addition to the lymphocytes and monocytes (Fig. 4B, left panel). The cell viability of each leukocyte population after the treatment with various concentrations of Hla is summarized in Table 1. The viability of monocytes or lymphocytes was markedly decreased after the treatment with Hla at 0.3 μg/mL, and more than 95% of these leukocyte populations were injured by Hla. By contrast, the viability of granulocytes was not substantially influenced by concentrations up to 3.0 μg/mL and was decreased to 45% when the Hla concentration was increased to 27 μg/mL.

We next isolated mononuclear cells (PBMCs) and polymorphonuclear leukocytes (granulocytes) from human peripheral

Fig. 5. Susceptibility of Human Leukocyte Populations to Hla

(A) Granulocyte (left) and PBMC (right) fractions separated from human peripheral blood by density-gradient centrifugation were analyzed by flow cytometry. The results for viable cells are shown in the forward versus side scatter (FSC vs. SSC) plots. (B) Granulocytes or PBMCs (2×10^6 cells/mL, 0.05 mL) were incubated with various concentrations of α -HL (0.05 mL) at 37°C for 5 h. The cytotoxicity was measured by WST-based cell viability assay.

blood and separately examined their susceptibility to Hla. The flow cytometric analysis indicated that the granulocyte fraction consisted mainly of granulocytes with a small percentage (approximately 3%) of lymphocytes, and that the PBMC fraction was composed mainly of lymphocytes and monocytes (Fig. 5A). The results of the cytotoxicity assay shown in Fig. 5B clearly indicated that these two cell populations were quite different in their susceptibility to Hla; *i.e.*, PBMCs were susceptible ($IC_{50} = 0.19 \mu\text{g/mL}$) but granulocytes were almost resistant at 9 μg/mL or lower concentrations. We next confirmed the susceptibility of lymphocytes and monocytes to Hla by analyzing their cell surface markers. The viable leukocytes were analyzed by flow cytometry after the treatment with Hla for CD19 (a marker for B lymphocytes), CD3 (a marker for T lymphocytes), and CD14 (a marker for monocytes). The cell profiles with or without Hla treatment (0 or 1.0 μg/mL) are shown in Fig. 6. We observed that all three populations (CD19-, CD3-, and CD14-positive cells) were considerably decreased after the Hla treatment. The results summarized in Table 2 indicated that viable CD3- or CD19-positive lymphocytes and CD14-positive monocytes were decreased after the Hla treatment in a dose-dependent manner. In addition, CD14-positive monocytes appeared to be more susceptible to Hla as compared to CD3- or CD19-positive lymphocytes; the percentages of viable cells (Hla: 0.3 μg/mL) were 7.3% (monocytes) and 73.9% (lymphocytes).

Expression of ADAM10 on Human Leukocytes Previous studies have demonstrated that a disintegrin and metalloproteinase domain-containing protein 10 (ADAM10) serves as a high affinity receptor for Hla and that its surface expression and the binding of Hla to the cells were well correlated.^{19–21)} We therefore analyzed ADAM10 expression of the leukocyte populations by flow cytometry. The results shown in Fig. 7 indicated that all populations (monocytes, lymphocytes, and

Fig. 6. Flow Cytometric Analysis of Human Leukocyte Populations after Hla Treatment

Human leukocytes after treatment with or without Hla (1 $\mu\text{g/mL}$) were analyzed by a flow cytometer using PE-labeled anti-CD3 antibody, Alexa Fluor 488-labeled anti-CD14 antibody, and PE-Cy7-labeled anti-CD19 antibody. The results for viable cells are shown in the plots of side scatter (SSC) versus fluorescence intensity.

Table 2. Surface Marker Analysis of Viable Cells after the Treatment with Hla

Hla ($\mu\text{g/mL}$)	CD3 (+) or CD19 (+)		CD14 (+)	
	Cell number	%	Cell number	%
0	2086	100.0	259	100.0
0.3	1542	73.9	19	7.3
1.0	448	21.5	0	0.0
3.0	358	17.2	0	0.0

Fig. 7. Flow Cytometric Analysis of the Expression of ADAM 10 on Human Leukocyte Populations

Human leukocytes were treated with anti-ADAM 10 antibody (2 $\mu\text{g/mL}$ at 0°C for 1 h). After washing with PBS/2% FCS, the cells were treated with PE/Dazzle™ 594-labeled anti-mouse IgG antibody at 0°C for 30 min and subjected to flow cytometry. In the FSC/SSC plot, monocytes, lymphocytes and granulocytes were gated as shown in Fig. 4, and the fluorescence profile of each cell population is shown in a histogram (dark grey). Each population was also analyzed without first antibody treatment as a control (light grey). Values of mean fluorescence intensity (MFI) for both specimens are shown.

granulocytes) expressed ADAM10 but that monocytes exhibited slightly higher expression as compared to lymphocytes and granulocytes. The higher ADAM10 expression on monocytes seems to parallel their higher susceptibility to Hla, but the ADAM 10 expression on lymphocytes was slightly lower than that on granulocytes.

DISCUSSION

In the present study, we purified a major cytotoxin against HL60 leukocytic cells from an *S. aureus* culture supernatant and identified the cytotoxin as Hla, based on its molecular weight (ca. 34 kDa) (SDS-polyacrylamide gel electrophoresis and size exclusion chromatography), N-terminal amino acid

sequence, and immunoblotting using anti-Hla antibody. The purified protein had a strong hemolytic activity against rabbit erythrocytes but not against human erythrocytes, which was consistent with the previous studies.^{1,17)} Hla is known as a pore-forming exotoxin that assembles cylindrical oligomers on target cell membranes, causing cytolysis of various mammalian cells, including platelets, leukocytes, respiratory epithelial cells, keratinocytes, and endothelial cells.^{18,22,23)} The present study demonstrated selective cytotoxicity toward human leukocyte populations; *i.e.*, monocytes and lymphocytes were much more susceptible to Hla than granulocytes. The importance of Hla in the leukocidal activity of *S. aureus* was suggested by a previous study showing correlation between the levels of Hla production and cytotoxicity against human PBMCs among community-associated *S. aureus* strains.²⁴⁾ Nygaard *et al.* also reported that the culture supernatant of a Hla-deletion mutant of a community-associated MRSA strain caused a much lower level of cell membrane damage to CD3-positive lymphocytes than the culture supernatant of the wild-type strain, suggesting that Hla plays a critical role in the membrane damage of T lymphocytes caused by *S. aureus* infection.²⁵⁾

In addition to leukocidal activity, Hla was reported to modulate various types of immune responses—*e.g.*, induction of neutrophil adhesion to endothelial cells,²⁶⁾ production of pro-inflammatory cytokines by macrophages and monocytes,²³⁾ interleukin (IL)-1 β - and IL-18-mediated inflammasome activation in microglia,²⁷⁾ and upregulation of Toll-like receptor-2 expression on monocytes.²⁸⁾ Thus, Hla may cause serious immune perturbation in association with *S. aureus* infection.

The cause of the selective susceptibility of leukocyte populations to Hla is not clear at present, but the higher susceptibility of monocytes to Hla may result from higher expression of ADAM 10 on their cell surfaces. The observation that CD14-positive monocytes expressed ADAM 10 at a moderate level is in good agreement with a previous study.²⁹⁾ By contrast, the marked difference in the susceptibility of lymphocytes and granulocytes cannot be explained by ADAM 10 expression levels on these leukocyte populations, because both cell types expressed comparable levels of ADAM 10. It was also reported that phosphocholine groups of sphingomyelin clustered in the cell membranes were crucial for stable attachment of the toxin to target cells.³⁰⁾ In addition to the expression levels of these receptors for Hla on the cell surface,

differences in oligomerization and conformational changes of Hla in cell membranes and/or intracellular processes including signaling pathways leading to cell death may be involved in the differential cytotoxicity by Hla. Characterization of the molecular mechanism underlying the toxin activity would thus appear to be important, since it would provide valuable information on the prevention of Hla-dependent cytotoxicity in infectious diseases by *S. aureus*.

It has also been established that so-called two-component toxins such as γ -hemolysin/PV-leukocidin possess potent leukotoxic activity.^{1,31)} Because these toxins are secreted from *S. aureus* as two non-associated subunits, we did not detect their cytotoxicity during the purification process in this study. Diep *et al.* reported that PV-leukocidin showed strong cytotoxicity against human polymorphonuclear leukocytes (IC₅₀ values between 0.1 and 1 nM),³²⁾ suggesting that Hla and PV-leukocidin have different target specificities for cytotoxicity. Therefore, it appears that Hla and leukocidins cooperatively injure leukocytes to suppress the host immune responses. They also demonstrated in animal models that a monoclonal antibody cross-reacting with Hla and PV-leukocidin effectively protected the animals from pneumonia induced by a community-associated MRSA.³²⁾ However, the contribution of these cytotoxins to the pathogenesis by MRSA infection may depend on the strain of *S. aureus* and the animal model. In a mouse model of *S. aureus* pneumonia, the expression levels of Hla in *S. aureus* strains were shown to directly correlate with their virulence, and the vaccination of mice with Hla afforded protection against staphylococcal pneumonia.³³⁾ We are hopeful that Hla vaccination or immunotherapy may also prevent *S. aureus* pneumonia in humans.

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Conflict of Interest The authors declare no conflict of interest.

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