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SHIP1 DELETION ENHANCES ROS PRODUCTION AND DECREASES SURVIVAL OF THE *S. AUREUS* INFECTED MICE

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Abstract
Reactive oxygen species (ROS) production by neutrophils is essential for host innate immune defense. However, although ROS production is advantageous for killing various infectious organisms, excessive ROS can cause tissue damage. Thus, ROS production by neutrophils must be tightly controlled. In this study, we investigated a role for SH2 domain–containing inositol–5–phosphatase 1 (SHIP1), a PtdIns(3,4,5)P_3 phosphatase, in ROS production by neutrophils using *ship1*$^{-/-}$ mice. SHIP1 deletion enhanced ROS production as well as the bacterial killing capability of neutrophils. However, the *ship1*$^{-/-}$ mice showed decreases survival of the mice infected with *Staphylococcus aureus* (*S. aureus*). Our results suggest that SHIP1 controls appropriate host defenses against *S. aureus* infections in mice.

Key words : Reactive oxygen species, NADPH oxidase, neutrophil, phospholipid

Introduction

Generation of reactive oxygen species (ROS) by NADPH oxidase plays a critical role in antimicrobial mechanisms[1,2]. Phagocytes are essential players in host defense and contribute to the innate immune system by ingesting pathogens and producing ROS for microbial killing. NADPH oxidase generates superoxide anions (O_2^-) that are metabolized to other toxic molecules, such as hydrogen peroxide, hypochlorous acid, and hydroxyl radicals[3,4]. Genetic defects in the components of NADPH oxidase result in chronic granulomatous disease (CGD). Because of their reduced ROS production, patients with CGD are susceptible to serious infections due to various microbes including *Staphylococcus*, *Aspergillus*, and *Candida* species[5].

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Phagocyte NADPH oxidase is composed of gp91^{phox}, p22^{phox}, p47^{phox}, p40^{phox}, p67^{phox}, and small GTPase Rac2. In resting cells, Rac2 and a tertiary complex comprising p47^{phox} and p40^{phox} along with p67^{phox} are found in the cytosol, whereas gp91^{phox} and p22^{phox} (cytochrome b_{558}) are localized in the membranes of secretory vesicles and specific granules[1,2]. When neutrophils become activated, these components assemble in endosomal and/or plasma membranes to form a functional NADPH complex in order to stimulate ROS production.

Phosphoinositides, phosphorylated derivatives of the membrane phospholipid phosphatidylinositol, are involved in NADPH oxidase formation. For example, PtdIns(3,4)P_2 and PtdIns3P bind to the Phox homology domains of p47^{phox} and p40^{phox}, respectively, leading to recruitment of these two components to membranes where they interact with the core catalytic components of NADPH oxidase[6]. PtdIns(3,4,5)P_3 activates Rac2 through Rac–GEFs (P–Rex, DOCK2, and Vav)[7], which in turn directly binds to and activates NADPH oxidase[8]. Therefore, kinases and phosphatases that regulate phosphoinositide metabolism must play key roles in the ap-

propriate generation of ROS in phagocytes.

SH2 domain-containing inositol-5-phosphatase 1 (SHIP1) is a hematopoietic phosphatase that dephosphorylates PtdIns(3,4,5)P_3 to form PtdIns(3,4)P_2[9]. Several lines of evidence have shown regulatory roles for SHIP1 in the functions of phagocytes[10,11]. Our group demonstrated that SHIP1 was required for the chemoattractant-induced polarization of and chemotaxis by macrophages and neutrophils[12]. However, a role for SHIP1 in NADPH oxidase activation remains controversial. SHIP1 has been implicated in both positive and negative regulation of FcγR-induced ROS production in macrophages[13,14]. In this study, we investigated the effect of SHIP1 deficiency on ROS production in neutrophils and bacterial killing capability and survival of the mice infected with *S. aureus*.

Materials and methods

Mice

Ship$^{-/-}$ mice were generated as described previously[15]. In all experiments, *ship*$^{+/+}$ littermates were used as wild-type (WT) controls. Mice aged 4-6 weeks were used in this study. All experimental protocols were reviewed and approved by the Akita University Institutional Committee for Animal Studies.

Isolation of bone marrow neutrophils

Bone marrow was harvested from mice by flushing their femurs and suspended in hypotonic buffer to lyse red blood cells. The cells were resuspended in Hanks' Balanced Salts Solution (HBSS), without Ca^{2+} and Mg^{2+}, and were centrifuged (1,000 *g*, 30 min, room temperature) over a discontinuous Percoll (GE Healthcare) gradient consisting of 62% Percoll in HBSS. After washing, mature neutrophils were resuspended in Krebs-Ringer-HEPES (KRH) buffer (10 mM HEPES/NaOH at pH 7.4, 130 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 5 mM glucose, 1.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, and 0.1% BSA). Neutrophil purity was typically >85% as determined by FACS analysis.

Preparation of bacterial strain

Staphylococcus aureus (ATCC strain 25923) cells were incubated at 37°C to logarithmic growth from overnight culture in Luria-Bertani medium. The cells were opsonized by incubation with 10% mouse serum at 37°C for 30 min and washed with PBS. The washed *S. aureus* cells were resuspended in KRH.

Measurement of ROS production

ROS production was determined using a luminol-dependent chemiluminescence assay with 96-well plates (no. 655075 ; Greiner Bio-One). Neutrophils were primed with 145 pM tumor necrosis factor (TNF-α) or KRH for 30 min at 37°C. A total of 2×10^5 neutrophils in KRH were incubated with 56 μM luminol (Tokyo Kasei Kogyo Co.) for 10 min at 37°C. Then, 10 μM *N*-formyl-l-methionyl-l-leucyl-l-phenylalanine (fMLP, Sigma), 40 nM phorbol 2-myristate 13-acetate (PMA), or opsonized *S. aureus* cells (final bacteria/BMN ratio of 20 : 1) were added to the reaction mixture via the injection port of the SpectraMax L microplate reader (Molecular Devices), and light emission was recorded at fixed time intervals.

Phagocytosis assay

TNF-α-primed (145 pM, 30 min) neutrophils were incubated with rhodamine isothiocyanate (RITC)-labeled serum-opsonized *S. aureus* cells for 20 min at 37°C (final bacteria/BMN ratio of 20 : 1). Non-phagocytosed bacteria were lysed with 56 nM lysostaphin (Sigma) for 10 min. Neutrophils were cytospun onto glass microslides and fixed in 10% formaldehyde for 10 min at room temperature, and coverslips were mounted onto the slides. Phagocytosed bacteria were visualized using a DMIRE2 microscope (Leica) and quantitated.

Nitroblue tetrazolium assay

TNF-α-primed neutrophils were pre-incubated with 0.6 mM nitroblue tetrazolium (NBT, Sigma) for 30 min at 37°C, and then the cells were incubated with serum-opsonized *S. aureus* cells for 20 min at 37°C (final bacteria/BMN ratio of 20 : 1). Non-phagocytosed bacteria were lysed with 56 nM lysostaphin for 10 min. Neutrophils were cytospun onto glass microslides and fixed in 10% formaldehyde for 10 min at room temperature, and coverslips were mounted on the slides. Dark formazan deposits were detected by light microscopy.

Determination of bacterial number in organs

Mice were administered 0.2 ml of a bacterial solution containing 1×10^8 colony-forming units (CFU) of viable *S. aureus* cells by i.p. injections. A mouse was humanely sacrificed 24 h after infection, its peritoneal cavity was thoroughly flushed with 10 ml of PBS, and its liver and spleen were aseptically dissected and homogenized. 10-fold serial dilutions of PBS or homogenized liver and spleen were plated on Luria-Bertani agar, and the numbers of CFU were determined after overnight incubation at 37°C.

S. aureus infection

S. aureus cells were incubated at 37°C to logarithmic growth from overnight culture, and bacterial cells were harvested and resuspended in PBS. Mice were administered 0.2 ml of a bacterial solution containing 2×10^8 CFU of viable *S. aureus* cells by i.p. injections, and their survival was monitored for 10 days.

Statistical analysis

Statistical comparisons were made by Student's t-test. Statistical significance was defined at $P<0.05$.

Results and discussion

To study a role for SHIP1 in ROS production by neutrophils, we investigated whether SHIP1 deficiency had any effect on ROS production in neutrophils. Opsonized antigen-mediated cross-linking of immunoglobulin G Fc-gamma receptors (FcγRs) on neutrophils leads to PI3K activation, ROS production, and phagocytosis[16]. We first examined ROS production in response to serum-opsonized *S. aureus* cells[17] using a luminol-dependent luminescence assay[18]. Compared with the WT neutrophils, ROS production was significantly enhanced in the *ship1*$^{-/-}$ neutrophils (Fig. 1A).

Biphasic ROS production was observed when neutrophils were stimulated with the G protein-coupled receptor agonist fMLP. In the WT neutrophils, fMLP-induced ROS production was terminated within 8 min, whereas it was sustained for >20 min in the *ship1*$^{-/-}$ neutrophils (Fig. 1B). When neutrophils were treated with PMA, a protein kinase C activator, no difference was observed in ROS production between the WT and *ship1*$^{-/-}$ neutrophils (Fig. 1C). Taking into consideration that class I PI3Ks are activated after stimulation by fMLP or cross-linking FcγRs but not by PMA, our results suggest that SHIP1 negatively regulated the oxidative burst of neutrophils that involves PI3K activity.

It is well documented that neutrophils become "primed" by prior exposure to TNF-α, which by itself does not stimulate ROS production[19]. Because potentiating ROS production by the loss of SHIP1 was reminiscent of TNF-α priming, the underlying mechanism of which remains poorly understood, we examined the effect of TNF-α on the oxidative burst in *ship1*$^{-/-}$ neutrophils. As shown in Fig. 1D, both *ship1*$^{-/-}$ and WT neutrophils incubated with TNF-α responded more rapidly and to a greater extent to opsonized *S. aureus* cells. Similarly, SHIP1 deficiency further enhanced fMLP-induced ROS generation in TNF-α-primed neutrophils (Fig. 1E). Thus, it appeared that SHIP1 was not the site where TNF-α signaling interacted with FcγRs or fMLP receptor signaling to induce its priming effect.

PI3K signaling has been also implicated in the phagocytosis[20]. We next examined whether SHIP1 deficiency also affected phagocytosis by neutrophils. To test this, we quantified *S. aureus* cells engulfed by neutrophils using the nitroblue tetrazolium (NBT) assay and the RITC-labeled *S. aureus* assay. No difference was observed between the WT and *ship1*$^{-/-}$ neutrophils in their capability to phagocytose opsonized *S. aureus* cells in either assay (Fig. 2). These results were in contrast to those of studies of macrophages that lacked SHIP1, which indicated that SHIP1 was a negative regulator of phagocytosis by dephosphorylating PtdIns(3,4,5)P_3 at the phagocytic cup[10,21]. These results suggested that different phagocytosis mechanisms might operate in neutrophils compared with those in macrophages.

S. aureus is one of the leading causes of life-threatening infections[22]. To examine a role for SHIP1 in host defense against *S. aureus* infection, the WT and *ship1*$^{-/-}$ mice were inoculated intraperitoneally with 1×10^8 CFU of *S. aureus* cells. The bacterial counts in the peritoneal cavity, liver, and spleen were all significantly lower in the *ship1*$^{-/-}$ mice 24 h after infection (Fig. 3). This en-

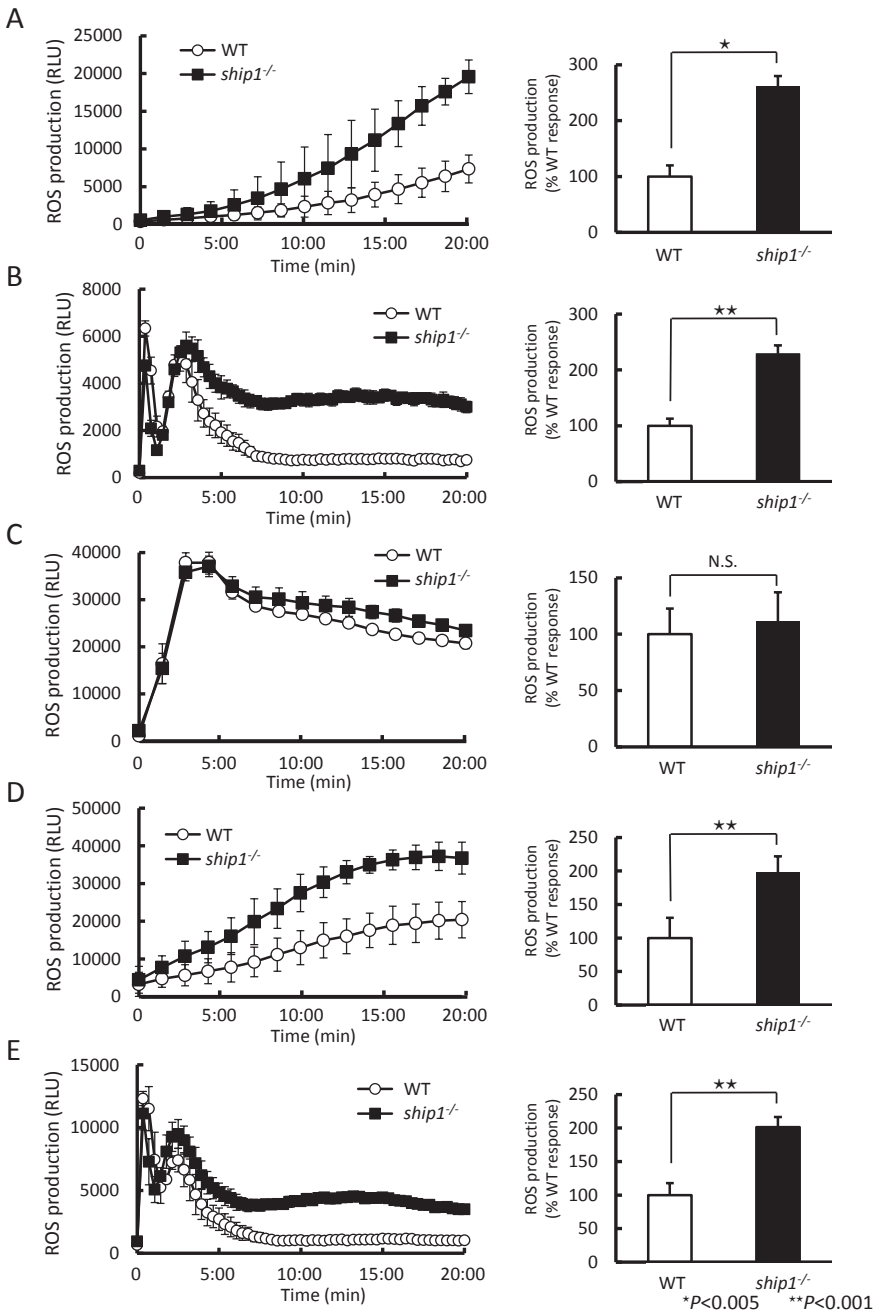

Fig. 1. ROS production by bone marrow neutrophils in *ship1*$^{-/-}$ and WT mice. (A–E) WT and *ship1*$^{-/-}$ neutrophils (2×10^6/well) were stimulated with opsonized *S. aureus* cells (A), 10 μM fMLP (B), or 40 nM PMA (C), and light emission was recorded (relative light units ; RLU). (D and E) Neutrophils were primed with 145 pM TNF-α and stimulated with opsonized *S. aureus* cells (D) or 10 μM fMLP (E). Response kinetics (line graph) and total integrated responses over 20 min as percentage of WT (bar graph) are shown. Results are means ± SEs of 3 independent experiments, with duplicate measurements for each.

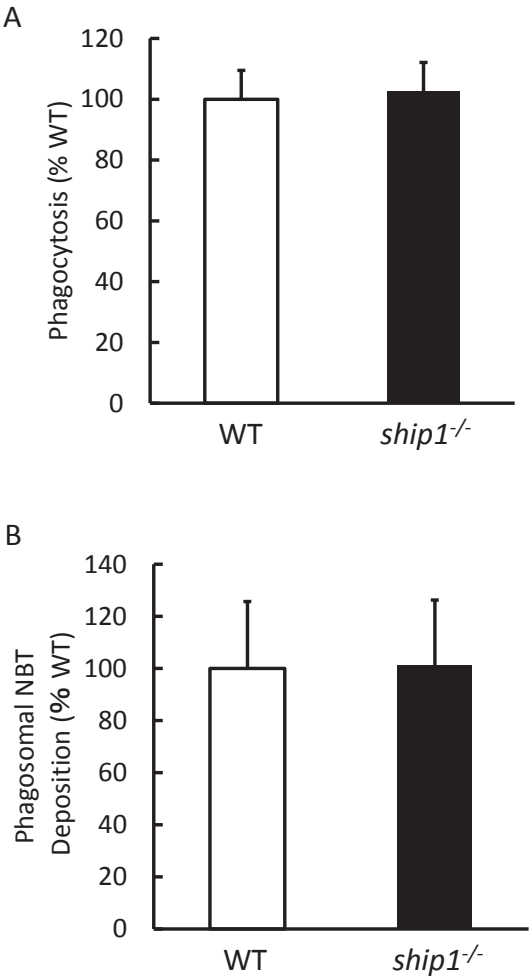

Fig. 2. Effect of SHIP1 deficiency on neutrophil phagocytosis of *S. aureus* cells. (A) Primed bone marrow neutrophils were incubated with RITC-labeled, serum-opsonized *S. aureus* cells. *S. aureus* cells in phagosomes were detected by confocal microscopy. (B) Primed bone marrow neutrophils were pre-incubated with NBT and incubated with serum-opsonized *S. aureus* cells. Dark formazan deposits were detected by light microscopy. Quantitative results for dark formazan deposits or RITC-labeled *S. aureus* cells are expressed as percentages of WT values (mean±SE ; $n>200$).

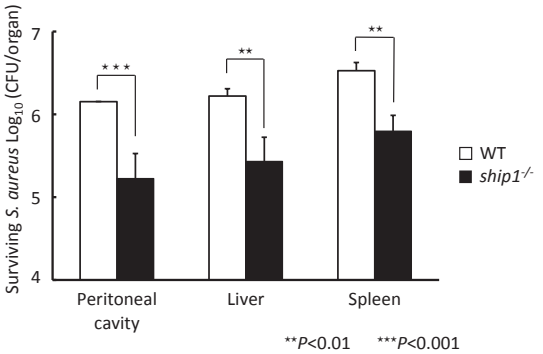

Fig. 3. Numbers of colonies in *ship1*$^{-/-}$ and WT mice after *S. aureus* injection. Mice ($n=3$ or 4) were infected i.p. with 1×10^8 CFU of *S. aureus*. The peritoneal cavity was thoroughly flushed with PBS, and organs were harvested 24 h after injection. Bacterial counts were determined by culture on agar. Results are means ± SEs of 3 experiments.

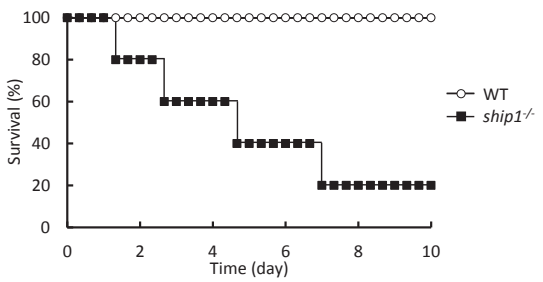

Fig. 4. Decreased survival of *ship1*$^{-/-}$ mice against *S. aureus* infection. Survival of *ship1*$^{-/-}$ and WT mice ($n=5$/group) after i.p. infection with 2×10^8 CFU of *S. aureus* cells. Mouse survival was monitored for 10 days.

hanced clearance of bacteria *in vivo* was consistent with the greater ROS production concomitant with normal phagocytosis by *ship1*$^{-/-}$ neutrophils. However, interestingly and surprisingly, while all WT mice survived after *S. aureus* inoculation, 80% *ship1*$^{-/-}$ mice died within 7 days of infection (Fig. 4).

In this study, we showed that SHIP1 negatively controlled ROS production in neutrophils. After *S. aureus* infection, a loss of SHIP1 improved bacterial clearance but was not beneficial for host survival. SHIP1 was also essential for an appropriate antimicrobial defense. An excessive, sustained increase in ROS production has been implicated in the pathogenesis of numerous diseases including systemic inflammatory response syndrome[23]. It has been reported that *ship1*$^{-/-}$ macrophages exhibited enhanced IL-1β and IL-6 production in response to FcγR

clustering[11]. We also observed that serum IL-6 levels were higher in the *ship1*$^{-/-}$ mice than in the WT mice (data not shown).

Thus, we postulate that prolonged ROS production by neutrophils may result in systemic inflammation and culminate in the death of *ship1*$^{-/-}$ mice. In future studies, it will be important to investigate the interaction between p47^{phox} and SHIP1 product, PI(3,4)P_2. Additional knowledge on the function of SHIP1 in ROS production, though being beneficial for killing bacteria, may provide a new therapeutic approach for inflammatory diseases and severe sepsis.

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