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Adenosine derived from *Staphylococcus aureus*-engulfed macrophages functions as a potent stimulant for the induction of inflammatory cytokines in mast cells

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In this study, we attempted to isolate novel mast cell-stimulating molecules from *Staphylococcus aureus*. Water-soluble extract of *S. aureus* cell lysate strongly induced human interleukin-8 in human mast cell line-1 and mouse interleukin-6 in mouse bone marrow-derived mast cells. The active molecule was purified to homogeneity through a C_{18} reverse phase HPLC column. By determination of its structure by MALDI-TOF and ^{1}H- and ^{13}C-NMR, adenosine was revealed to be responsible for the observed cytokine induction activities. Further studies using 8-sulfophenyl theophylline, a selective adenosine receptor blocker, verified that purified adenosine can induce interleukin-8 production via adenosine receptors on mast cells. Moreover, adenosine was purified from *S. aureus*-engulfed RAW264.7 cells, a murine macrophage cell line, used to induce phagocytosis of *S. aureus*. These results show a novel view of the source of exogenous adenosine *in vivo* and provide a mechanistic link between inflammatory disease and bacterial infection. [BMB reports 2011; 44(5): 335-340]

INTRODUCTION

Innate immunity serves as a crucial host defense mechanism against microbial infection in all animals. The ability of a host to distinguish between self and non-self remains a central hallmark of innate immunity (1). Pathogenic microbes possess distinct pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS) and flagellin from Gram-negative bacteria and lipoproteins from Gram-positive bacteria (2). Recognition of these PAMPs is achieved by a group of germ-line encoded pattern-recognition receptors (PRRs) (3). The Toll-like receptors (TLRs), a family of mammalian PRRs, are type-1 integral transmembrane glycoproteins with extracellular domains containing leucine-rich repeats, which recognize microbial products, and then transfer recognition signals via a cytoplasmic Toll/interleukin (IL)-receptor homology domain, leading to the production of pro-inflammatory cytokines (4). Some TLRs (e.g. TLR1, TLR2, TLR4, TLR5, and TLR6) are expressed on the immune cell surface and are known to recognize bacterial lipoproteins, LPS, and flagellins, whereas TLR7, TLR8, and TLR9 are exclusively expressed within intracellular endosomes and mainly sense microbial nucleic acids.

Adenosine is a ubiquitous purine nucleoside that has long been recognized as a potent biological signaling molecule. It plays important roles in cardiovascular, neurological, and renal diseases (5). In addition, adenosine functions as a potent pro-inflammatory mediator and contributes to the pathogenesis of inflammatory lung diseases, such as asthma and chronic obstructive pulmonary disease (6). This molecule is also known to be able to influence mast cell functions through the activation of adenosine receptors expressed on the cell surface (7). Four adenosine receptors mediating biological responses, A_1, A_{2a}, A_{2b}, and A_3, have been identified. Each receptor has its own unique tissue distribution, ligand affinity, and signal transduction pathways (8). Mast cells express the A_{2a}, A_{2b}, and A_3 adenosine receptors. Among them, the A_{2b} and A_3 adenosine receptors are known to be associated with adenosine-mediated mast cell activation (9). Endogenous adenosine is either released through metabolically active cells or generated via degradation of extracellular adenosine monophosphate (AMP) (10). However, the source and full effects of exogenous adenosine derived from bacteria such as *S. aureus* are largely unknown.

S. aureus is a pathogenic Gram-positive bacterium as well as a major cause of mortality in medical facilities. This bacterium causes various infectious diseases, including sepsis, endocarditis, and pneumonia. However, detailed information on the molecular structures of *S. aureus* PAMPs is not available, except

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for lipoproteins (11). In the current study, upon isolating novel mast cell-stimulating molecules from *S. aureus*, we found that water-soluble extract (WSE) derived from *S. aureus* strongly induced human interleukin-8 (hIL-8) in human mast cell-1 (HMC-1). Eventually, the WSE was purified to homogeneity through several columns and the responsible compound finally identified as adenosine. Furthermore, we also confirmed that adenosine could potently induce hIL-8 expression via adenosine receptor in HMC-1 by examination using 8-sulfophenyl theophylline (8-SPT), a specific adenosine receptor blocker. In addition, we showed that adenosine was secreted from *S. aureus*-engulfed macrophages. These results reveal that one source of exogenous adenosine is derived from engulfed *S. aureus*, which can be used to increase our understanding of the biological functions of adenosine in association with mast cell-mediated inflammatory diseases.

RESULTS

Biochemical characteristics of crude WSE of *S. aureus*

To identify the novel biologically active molecule functioning as a stimulant toward the mast cells, we prepared water-soluble extract (WSE) from *S. aureus* lysate. When the cytokine-induction ability of WSE was examined in mouse bone marrow-derived mast cells (mBMMCs) and human mast cell line-1 (HMC-1), mouse IL-6 (mIL-6) was induced in a dose-dependent manner after 6 h of stimulation in mBMMCs (Fig. 1A). This cytokine induction was not attenuated in the presence of polymyxin B sulfate (PMB), which functions as a potent blocker of LPS, suggesting that there was no LPS contamination in the crude WSE of *S. aureus*. As controls, tripalmitoyl (Pam_3)-Cys-Ser-$(Lys)_4$ (Pam3CSK4, lipopeptide), soluble peptidoglycan (PG) and lipoteichoic acid (LTA) purified from *S. aureus*, β-1,3-glucan, and CpG oligonucleotide (ODN), which are suggested to be potent stimulators of immune cells, did not produce any mIL-6 production in mBMMCs. The hIL-8 production by WSE was also induced in a dose-dependent manner in HMC-1 cells (Fig. 1B). Taken together, these results suggest that crude WSE of *S. aureus* contained a novel molecule that can produce inflammatory cytokines in mast cells.

Purification of mast cell-stimulating molecule from *S. aureus*

In order to further purify active molecule from WSE, we added methanol to *S. aureus* WSE. When the supernatant and precipitate were used for the examination of inflammatory cytokine production, only the supernatant induced inflammatory cytokines in mast cells. When the supernatant was further fractionated through a Sep-Pak cartridge column, the fractions eluted with 30% acetonitrile showed the strongest cytokine-inducing activity (Fig. 2A) (see the purification procedure in Supplemental Fig. S1). Subsequently, when we performed C_{18} reverse-phase

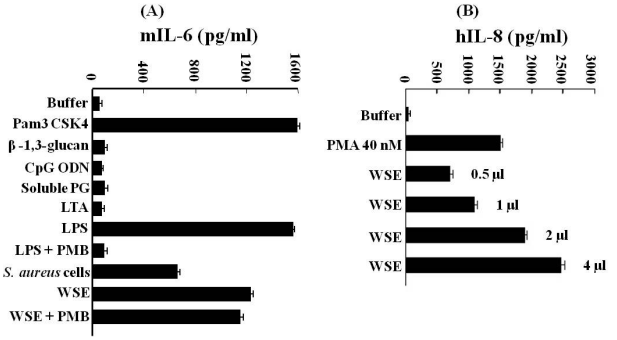

Fig. 1. Characteristics of crude WSE. (A) mBMMC was stimulated with PAMPs, such as tripalmitoyl cysteinyl lipopeptide (Pam3CSK4, 2.5 μg/ml), β-1,3-glucan (10 μg/ml), CpG ODN (20 μg/ml), soluble PG (10 μg/ml), LTA (2 μg/ml), and LPS (300 ng/ml), and mIL-6 was determined in the culture supernatant after stimulation for 6 h. Moreover, WSE was treated with polymyxin B sulfate (PMB, 25 μg/ml) prior to stimulation of mBMMCs. Pam3CSK4 was used for the positive control of mBMMCs. (B) Induction of hIL-8 production by WSE in HMC-1. Added volumes of WSE are indicated above each histogram. One representative result is shown from at least three experiments. Phorbol 12-myristate 13-acetate (PMA, 40 nm) were used as positive control HMC-1. Data was presented as average ± standard error from triplet experiments.

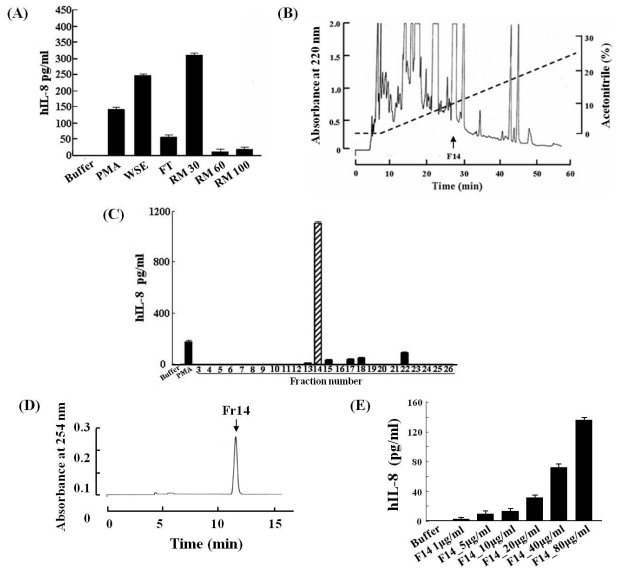

Fig. 2. Purification of active molecule from WSE. (A) hIL-8 stimulatory activity by prepared WSE. The hIL-8 levels were determined in culture supernatant after stimulation with prepared WSE, flow-through (FT), 30% methanol elute (RM-30), 60% methanol elute (RM-60), and 100% methanol elute (RM-100). PMA (40 nM) was used as a positive control in HMC-1 cells. (B) The elution pattern of the first HPLC chromatography. RM-30 was separated by C_{18} reverse phase HPLC chromatography with an acetonitrile gradient of 0-30%, and fractionation was monitored at A_{220nm}. (C) hIL-8 stimulatory activities on HMC-1 cells by each fraction of (B) are shown. (D) Final elution profile of purified molecule from WSE. (E) The effect of the purified molecule induced hIL-8 production in a dose-dependent manner in HMC-1 cells.

column chromatography, the active fraction (fraction number 14) showed cytokine production activity (Fig. 2B, 2C). Following this, the active fraction was purified through a Phenyl-column and C_{18} reverse-phase column, which showed a symmetrical peak. Finally, the purified molecule also induced hIL-8 in a dose-dependent manner (Fig. 2D, 2E). When the structure of the purified molecule was determined by MALDI-TOF and NMR analyses (supplemental Fig. S2), the purified compound was finally determined to be adenosine, which perfectly matched that of commercially available adenosine. These results demonstrate that adenosine derived from WSE was purified to homogeneity and induced mIL-6 and hIL-8 production in mast cells.

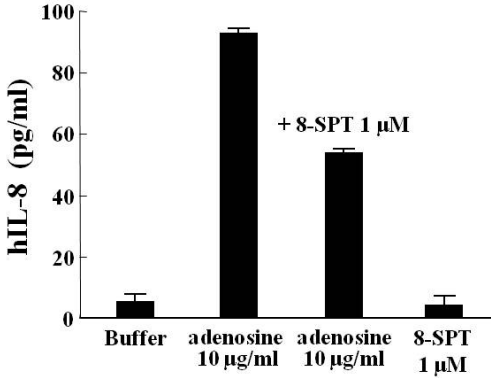

Fig. 3. Adenosine receptor blocker inhibits adenosine-mediated cytokine production on mast cells. Adenosine (10 μg/ml, Fluka) was preincubated with 8-SPT (1 μM) for 80 min, and its mixture was allowed to stimulate HMC-1 cells. The hIL-8 level in the culture supernatant was determined after stimulation for 6 h. Data are presented as average ± standard error of triplet experiments.

Specific adenosine receptor blocker inhibits adenosine-mediated inflammatory cytokine production

To further verify whether or not the adenosine purified from *S. aureus* really produces inflammatory cytokine production via adenosine receptors expressed on mast cells, we performed competitive inhibitory experiments using 8-sulfophenyl theophylline (8-SPT), a specific adenosine receptor blocker (12). As expected, *S. aureus* adenosine-mediated hIL-8 production was specifically inhibited by 8-SPT, suggesting that adenosine induced hIL-8 expression via adenosine receptors in HMC-1 (Fig. 3).

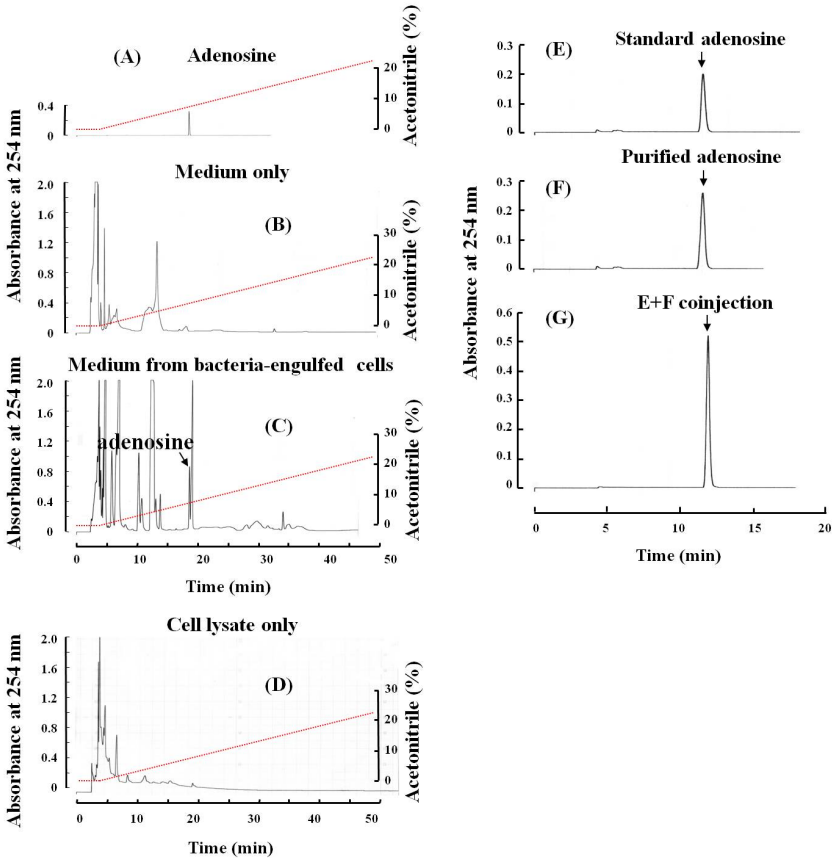

Fig. 4. Adenosine is secreted from *S. aureus*-engulfed macrophages. (A) and (E) indicate C_{18} reverse phase HPLC analyses patterns of standard adenosine; (B), HPLC analysis of pattern of only culture medium; (C), *S. aureus* was co-incubated with RAW264.7 cells to induce phagocytosis. After phagocytosis induction, the collected culture medium was analyzed on C_{18} reverse phase HPLC; (D), analysis pattern of RAW264.7 cell lysate only; (F), the purified adenosine from (C) is injected into the C_{18} reverse phase HPLC column. (G) The purified adenosine, which was secreted from *S. aureus*-engulfed macrophages, was mixed with standard adenosine and then co-injected into a C_{18} reverse phase HPLC column.

Adenosine is secreted from *S. aureus*-engulfed macrophages
To confirm whether or not adenosine is really released from *S. aureus*-engulfed phagocytic cells, we utilized RAW264.7, a kind of murine macrophage cell line, in order to induce phagocytosis of *S. aureus*. After induction of phagocytosis, the culture medium was analyzed to examine whether or not adenosine was secreted from *S. aureus*-engulfed macrophages. As shown in Fig. 4, when the medium recovered from the *S. aureus* engulfed-macrophage cells was injected into the C_{18} reverse HPLC column, one peak showing the same retention time as that of authentic adenosine was eluted (Fig. 4A, 4C). This peak was not observed when only the medium or only the lysate of RAW264.7 cells was injected (Fig. 4B, 4D). Further, when we co-injected the purified adenosine from the medium along with authentic adenosine into the C_{18} reverse column, two molecules were co-eluted as a one peak, suggesting that the purified molecule was adenosine (Fig. 4E - 4G). These results suggest that adenosine was released from *S. aureus*-engulfed macrophages, after which the released adenosine induced inflammatory cytokine production via adenosine receptors.

DISCUSSION

S. aureus bacteria infect humans and often cause superficial skin lesions as well as food poisoning by releasing enterotoxins into food. Further, this bacterium induces toxic shock syndrome by releasing superantigens into the blood stream, as well as sepsis and multiorgan failure (13). In these serious situations, it is important to determine the infection-inducing molecules of *S. aureus* at the molecular level. Recently, we screened mutant strains of *S. aureus* that lack various cell wall components in order to determine which molecules of the cell wall are recognized by host innate immune receptors. Among them, we demonstrated that wall teichoic acid (WTA) of *S. aureus* is a native ligand of human mannose binding lectin (MBL), a typical recognition molecule involved in the activation of the human lectin complement pathway (14), and that triacylated lipoproteins but not LTA derived from *S. aureus* are major ligands of mammalian Toll-like receptor-2 (15). Here, we provide additional evidence that adenosine released from *S. aureus*-engulfed macrophages can function as an inducing molecule of inflammatory cytokines in mast cells.

Many studies have reported that adenosine has potent effects on both the cardiovascular and immune systems (5). Exposure of host tissues to adenosine results in increased vascular permeability and extravasation of serum proteins. The mechanism by which adenosine regulates these physiological changes is through the activation of its four receptors, A_1, A_{2a}, A_{2b}, and A_3 (16), and is poorly defined. In many cell types, adenosine secretion is up-regulated in response to injury. Enhanced production of adenosine has been detected in numerous pathological conditions, such as inflammation, hypoxia, and ischemia (17). Although several studies have provided evidence that endogenous adenosine is derived from many cells during normal metabolic function *in vivo*, we here clearly demonstrate that exogenous adenosine derived from *S. aureus*-engulfed macrophages may be an additional source as well as a cause of pathological conditions.

Based on these results, we propose a putative mechanism regarding the generation of exogenous adenosine from pathogenic bacteria. Briefly, when bacteria invade the host body, some bacteria will be lysed by the membrane attack complex (MAC), which is generated by complement activation. On the other hand, some bacteria will be opsonized by IgG and C3b complement component, which can bind to Fc receptor and complement receptor 3, respectively. Accordingly, upon evoking phagocytosis after recognition of opsonized bacteria, these bacteria will be engulfed by phagocytic cells. In this case, adenosine would be excreted from the phagocytic cells, followed by subsequent mast cell activation via adenosine receptors. Ultimately, this will enhance inflammatory responses in hosts. If adenosine excreted from bacteria stimulates adenosine receptors on the surfaces of ubiquitous tissue cells, then cellular signaling involved in the pathogenesis of *S. aureus* will be promoted, causing disorders such as sepsis, asthma, glaucoma, and diabetes.

In summary, this study demonstrates that adenosine derived from *S. aureus*-engulfed phagocytic cells functions as a potent inflammatory cytokine stimulant. Moreover, these findings provide insights into the deleterious influence of *Staphylococcal* components during bacterial infection and also how mast cells respond to bacterial infection.

MATERIALS AND METHODS

Mice
C57BL/6 mice were purchased from Hyochang Science (Daegu, Korea). Mice aged 6-8 weeks were bred under specific pathogen-free conditions in the animal facilities of Pusan National University.

Cell culture
Mouse bone marrow-derived mast cells (mBMMCs) were prepared and cultured as previously described (18). Briefly, mBMMCs were differentiated from bone marrow stem cells, which were isolated from the femur and tibia of 6-8 week old C57BL/6 mice. The isolated bone barrow stem cells were incubated to density of 1×10^6 cells/ml in Iscove's modified Dulbecco's medium (IMDM) (Gibco) supplemented with L-glutamine (2 mM, Sigma), HI-FCS (10%, Gibco), penicillin (100 IU/ml, Sigma), streptomycin (100 μg/ml, Sigma), murine stem cell factor (SCF) (30 ng/ml, Peprotech), and IL-3 (30 ng/ml, Peprotech) at 37°C, 5% (v/v) CO_2 in air and 95% humidity. Cultural medium was changed every 5-7 days. Non-adherent cells were collected and transferred to fresh medium, which were maintained for approximately 2 months. The homogeneity of mast cells was determined by staining

with toluidine blue and FACS analysis. Cells cultured for 7-9 weeks were used in the experiments. HMC-1 was provided by Dr J. H. Butterfield (Mayo Clinic, Rochester, MN, USA). HMC-1 was cultured in Iscove's modified Dulbecco's medium (IMDM) (Gibco) supplemented with L-glutamine (2 mM, Sigma), monothioglycerol (10 μM, Sigma), HI-FCS (10%, Gibco), penicillin (100 IU/ml, Sigma), and streptomycin (100 μg/ml, Sigma) at 37°C in atmosphere containing 5% (v/v) CO_2. Culture medium was changed every 4-6 days at a final density of 0.1×10^6 cells/ml. RAW264.7, a murine macrophage cell line, was cultured in Dulbecco's Modified Eagle's medium (DMEM) (Gibco) (containing 4,500 mg/L of glucose, 2 mM L-glutamine, 110 mg/l of sodium pyruvate, and sodium bicarbonate) supplemented with HI-FCS (10%, Gibco), L-glutamine (2 mM, Sigma), penicillin (100 IU/ml, Sigma), and streptomycin (100 μg/ml, Sigma) at 37°C in atmosphere containing 5% (v/v) CO_2.

Cytokine assay

To determine the levels of hIL-8 and mIL-6 present in mast cells, an hIL-8 and mIL-6 ELISA kit (BD Bioscience) was used (15). Mast cells were harvested by centrifugation at 1,000 rpm for 5 min and cultivated to starvation overnight with basal medium in the absence of SCF and IL-3 before stimulation. For induction of cytokines, cells were suspended with fresh basal medium, seeded in 96-well plates (SPL, Korea) (0.25×10^6 cells/150 μl of basal medium/well), and then stimulated in triplicate with desired ligand for 6-12 h at 37°C. After stimulation, supernatant from the wells was harvested by centrifugation at 1,000 rpm for 7 min and stored at -20°C.

Murine macrophage-mediated phagocytosis

RAW264.7 cells (8.0×10^6 cells) were suspended in DMEM containing 10% HI-FCS and transferred to culture flask. After adherence of RAW264.7 cells for 4 h, non-adherent cells were removed by aspiration. *S. aureus* suspended in 30 ml of DMEM were co-incubated with the prepared cells at a ratio of 10 to 1 and incubated for 3 h at 37°C. Then, the supernatant was collected, followed by treatment with absolute methanol, and was used as a further purification source. Culture medium alone or cell lysate of RAW264.7 cells was also subjected to HPLC analyses as negative controls. The commercially available adenosine was used as a positive control.

Isolation and purification of mast cell-stimulating molecule from *S. aureus*

S. aureus cowan 1 strain was grown in Todd-Hewitt broth medium (Bacto) at 37°C with vigorous shaking for 18 h. After cooling, bacterial cells were sedimented from the culture medium by centrifugation (6,000 × g, 4°C, 30 min), washed three times with PBS (pH 7.2), again two times with distilled water (DW). The bacterial cells were suspended with 25 ml of DW, boiled at 95°C for 30 min, and then disrupted by merging suspensions of bacterial cells with the same volume of 0.1 mm glass beads in DYNO mill, followed by boiling at 95°C for 30 min again. After the glass beads were settled down to bottom of flask, materials except for glass beads were centrifuged (6,000 × g, 4°C, 30 min) and the supernatant was obtained. This solution was lyophilized and designated as water-soluble extract (WSE). The WSE dissolved with sterile DW was treated with absolute methanol at a ratio of 1 : 3 at 4°C for 4 h, After centrifugation (6,000 × g, 4°C, 30 min), the supernatant was loaded onto Sep-pak C_{18} cartridge column (Waters) and then fractionated into flow-through (FT), RM30 (elute of 30% methanol), RM60 (elute of 60% methanol), RM100 (elute of 100% methanol), respectively (supplemental Fig. S1).

HPLC analysis

The RM30 from Sep-pak C_{18} cartridge column was isolated by high pressure liquid chromatography (HPLC) on a C_{18} reverse-phase column (9.2 × 25 mm, Gilson) and eluted at a flow rate of 1 ml/min with a linear gradient between water and 30% acetonitrile in water. The eluted compounds were detected by measurement of absorbance at 220 nm. The active fractions, which were determined by hIL-8 assay as described above, were eluted by 7% acetonitrile in water. The active fractions from the 1st C_{18} reverse-phase HPLC column were applied to the Phenyl-HPLC column (3.9 × 150 mm) (Waters), and the active fraction was eluted isocratically at a flow rate of 1 ml/ml by 2% acetonitrile in water. The eluted compounds were detected by measurement of absorbance at 254 nm, as we observed optimal maximal absorbance of the purified compounds at 254 nm, suggesting that the compounds contained aromatic components. Then, the active fractions obtained from the 2nd Phenyl-HPLC column were applied to the C_{18} reversephage HPLC column (4.6 × 250 mm, Gilson) again. Finally, a pure compound was eluted isocratically at a flow rate of 1 ml/min by 7% acetonitrile in water, which were also monitored by $A_{254\ nm}$ (supplemental Fig. S1).

Structure analysis by MALDI-TOF and NMR

The determination of the structure of the purified molecule from WSE was carried out by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry in the Mass Spectrometry Facility at the Ewha Womans University in Seoul, Korea. Briefly, 1 μl of sample and 1 μl of matrix solution were applied to a sample plate. After drying, the sample was analyzed with a mass spectrometer. Further, the structure was determined on the basis of ^1H and ^{13}C nuclear magnetic resonance (NMR) analysis. The ^1H and ^{13}C NMR spectra were recorded on a Varian Unity Inova 500 MHz spectrometer. Chemical shifts were reported with reference to the respective residual solvent or deuterated solvent peaks.

Statistical analysis

The mean and SD or SE were calculated by Excel software (Microsoft). Student's t test was used to calculate significance, and $P < 0.05$ was considered to represent a statistically significant difference between two sample means.

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REFERENCES

1. Medzhitov, R. and Janeway, C. A. Jr. (1997) Innate immunity: the virtues of a nonclonal system of recognition. *Cell* **91**, 295-298.
2. Hoffmann, J. A., Kafatos, F. C., Janeway, C. A. and Ezekowitz, R. A. (1999) Phylogenetic perspectives in innate immunity. *Science* **284**, 1313-1318.
3. Janeway, C. A. and Medzhitov, R. Jr. (2002) Innate immune recognition. *Annu. Rev. Immunol.* **20**, 197-216.
4. Akira, S., Uematsu, S. and Takeuchi, O. (2006) Pathogen recognition and innate immunity. *Cell* **124**, 783-801.
5. Trincavelli, M. L., Daniele, S. and Martini, C. (2010) Adenosine receptors: what we know and what we are learning. *Curr. Top. Med. Chem.* **10**, 860-877.
6. Spicuzza, L., Bonfiglio, C. and Polosa, R. (2003) Research applications and implications of adenosine in diseased airways. *Trends. Pharmacol. Sci.* **24**, 409-413.
7. Kumar, V. and Sharma, A. (2009) Adenosine: an endogenous modulator of innate immune system with therapeutic potential. *Eur. J. Pharmacol.* **616**, 7-15.
8. Olah, M. E. and Stiles, G. L. (1995) Adenosine receptor subtypes: characterization and therapeutic regulation. *Annu. Rev. Pharmacol. Toxicol.* **35**, 581-606.
9. Zhong, H., Shlykov, S. G., Molina, J. G., Sanborn, B. M., Jacobson, M. A., Tilley, S. L. and Blackburn, M. R. (2003) Activation of murine lung mast cells by the adenosine A3 receptor. *J. Immunol.* **171**, 338-345.
10. Fredholm, B. B. and Dunwiddie, T. V. (1988) How does adenosine inhibit transmitter release? *Trends. Pharmacol. Sci.* **9**, 130-134.
11. Fournier, B. and Philpott, D. J. (2005) Recognition of Staphylococcus aureus by the innate immune system. *Clin. Microbiol. Rev.* **18**, 521-540.
12. Sommerschild, H. T., Lunde, P. K., Deindl, E., Jynge, P., Ilebekk, A. and Kirkeboen, K. A. (1999) Elevated levels of endogenous adenosine alter metabolism and enhance reduction in contractile function during low-flow ischemia: associated changes in expression of Ca(2+)-ATPase and phospholamban. *J. Mol. Cell Cardiol.* **31**, 1897-1911.
13. Peschel, A., Jack, R. W., Otto, M., Collins, L. V., Staubitz, P., Nicholson, G., Kalbacher, H., Nieuwenhuizen, W. F., Jung, G., Tarkowski, A., van Kessel, K. P. and van Strijp, J. A. (2001) *Staphylococcus aureus* resistance to human defensins and evasion of neutrophil killing via the novel virulence factor MprF is based on modification of membrane lipids with L-lysine. *J. Exp. Med.* **193**, 1067-1076.
14. Park, K. H., Kurokawa, K., Zheng, L., Jung, D. J., Tateishi, K., Jin, J. O., Ha, N. C., Kang, H. J., Matsushita, M., Kwak, J. Y., Takahashi, K. and Lee, B. L. (2010) Human Serum Mannose-binding Lectin Senses Wall Teichoic Acid Glycopolymer of Staphylococcus aureus, Which Is Restricted in Infancy. *J. Biol. Chem.* **285**, 27167-27175.
15. Kurokawa, K., Lee, H., Roh, K. B., Asanuma, M., Kim, Y. S., Nakayama, H., Shiratsuchi, A., Choi, Y., Takeuchi, O., Kang, H. J., Dohmae, N., Nakanishi, Y., Akira, S., Sekimizu, K. and Lee, B. L. (2009) The Triacylated ATP Binding Cluster Transporter Substrate-binding Lipoprotein of Staphylococcus aureus Functions as a Native Ligand for Toll-like Receptor 2. *J. Biol. Chem.* **284**, 8406-8411.
16. Ohta, A. and Sitkovsky, M. (2001) Role of G-protein-coupled adenosine receptors in downregulation of inflammation and protection from tissue damage. *Nature* **414**, 916-920.
17. Peralta, C., Hotter, G., Closa, D., Prats, N., Xaus, C., Gelpi, E. and Rosello-Catafau, J. (1999) The protective role of adenosine in inducing nitric oxide synthesis in rat liver ischemia preconditioning is mediated by activation of adenosine A2 receptors. *Hepatology* **29**, 126-132.
18. Zhu, F. G., Gomi, K. and Marshall, J. S. (1998) Short-term and long-term cytokine release by mouse bone marrow mast cells and the differentiated KU-812 cell line are inhibited by brefeldin A. *J. Immunol.* **161**, 2541-2551.