Serum proteins potentiate therapeutic efficacy of lysocin E against *S. aureus*

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Abstract

The development of antimicrobials that exert therapeutic efficacy by novel mechanisms is crucial toward combatting multidrug-resistant bacteria. We previously reported a novel antibiotic, lysocin E, that is effective against methicillin-resistant *Staphylococcus aureus* (MRSA)¹. Lysocin E exhibits more prominent therapeutic effects against *S. aureus* in a mouse systemic infection model compared with its basal antibacterial activity *in vitro*, but the detailed mechanism underlying this discrepancy was unclear. Here we show that serum or plasma from various species, including humans, increases the antimicrobial activity of lysocin E, and identified apolipoprotein A-I (ApoA-I) as an enhancing factor in the serum. Using gene knockout mice, we further revealed that ApoA-I contributes to the therapeutic effects of lysocin E. The binding capacity of lysocin E to ApoA-I was enhanced by lipid II, an intermediate component of *S. aureus* cell wall synthesis in the membrane, and ApoA-I enhanced the membrane-damaging activity of lysocin E at a sub-minimum inhibitory concentration (sub-MIC). Our results are the first to demonstrate that antimicrobial activity can be potentiated through interactions of host serum proteins with microbial components to enhance the therapeutic effect, which broadens the strategies for developing antimicrobials by taking advantage of host-microbe interactions.

Main text

Since the discovery of penicillin, antimicrobial agents have been screened on the basis of their *in vitro* antimicrobial activity, which does not accurately reflect the host environment. In general, the binding of host proteins to antimicrobials has long been considered a major obstacle for the *in vivo* therapeutic activities of antimicrobials, drastically reducing the antimicrobial efficacies expected from *in vitro* analyses in the absence of host factors. Our unique strategy to search for antimicrobial agents, utilising the silkworm as a host for evaluating the *in vivo* effects of antimicrobials at early stages of screening^{2,3}, which could exclude compounds with reduced antimicrobial activity due to host protein binding^{4,5}, led to the discovery of a novel antibiotic, lysocin E¹ (Extended Data Fig.1a). Lysocin E is therapeutically effective against a clinical isolate of an MRSA strain at doses lower than that expected on the basis of its *in vitro* antimicrobial activity (Table 1). The ratio of the median effective dose to the minimal inhibitory concentration (ED₅₀/MIC) is

more than 16-fold smaller for lysocin E compared with other anti-MRSA drugs, indicating high therapeutic efficacy of lysocin E compared with the other anti-MRSA drugs. We therefore hypothesised that the antimicrobial activity of lysocin E is promoted by factors in the host environment.

a

b

Biotinylated menaquinone (5)

c

Delipidated lipid II (6)

Extended Data Fig.1| Structure of compounds used in this study

a, Structure of lysocin E and lysocin E derivative used in this study. Lysocin E natural type is **1**, Bu-type lysocin E is **2**, and biotinylated lysocin E natural-type and Bu-type used in bio-layer interferometry (BLI) analysis or pull-down assay are **3** and **4**, respectively. **b**, Structure of biotinylated menaquinone used in BLI analysis. **c**, Structure of delipidated lipid II.

67 **Table 1| Therapeutic effect in a mouse systemic MRSA infection model and**
68 **antimicrobial activity of anti-MRSA agent against MRSA.**

	Lysocin E	Vancomycin	Linezolid	Daptomycin
Antimicrobial activity (MIC : $\mu\text{g ml}^{-1}$)	4	1	2	0.5
Therapeutic effect (ED ₅₀ : mg kg^{-1})	0.36	4.2	3.8	0.73
ED ₅₀ /MIC	0.098	4.2	1.9	1.6

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Serum ApoA-I enhances lysocin E activity

In contrast to vancomycin or daptomycin, we found that the antimicrobial activity of lysocin E against a methicillin-sensitive *S. aureus* (MSSA) strain was increased by the addition of various animal sera, human plasma, or silkworm haemolymph (Fig. 1a). The increased antimicrobial activity of lysocin E induced by the addition of bovine serum was also observed against other *S. aureus* strains, and Gram-positive bacteria such as *Staphylococcus* spp. and *Bacillus subtilis* (Extended DATA Table 1). To understand the mechanism by which serum enhanced the activity of lysocin E, we purified the responsible factors in the bovine serum by measuring the increase in antimicrobial activity of lysocin E due to exposure to serum components (Extended Data Fig.1a). Ethanol extraction, ODS column chromatography, and gel filtration chromatography were performed. Analysis by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) revealed that the elution patterns of 32-kDa and 10-kDa proteins from gel filtration column chromatography were consistent with the antimicrobial enhancing activity of lysocin E (Extended Data Fig. 1b,c). Peptide mass fingerprinting (PMF) analysis revealed that the proteins were ApoA-I and II (Extended Data Fig. 1d). Both human recombinant ApoA-I and purified human ApoA-II reduced the MIC of lysocin E (Fig. 1b), confirming that ApoA-I and II are the serum factors responsible for enhancing the antimicrobial activity of lysocin E. Although ApoA-I exhibits antimicrobial activity against Gram-negative bacteria such as *Escherichia coli* and *Klebsiella pneumoniae*^{6,7}, the addition of purified bovine and human ApoA-I or II did not inhibit the growth of *S. aureus* at up to 300 $\mu\text{g ml}^{-1}$. Thus, the enhanced antimicrobial activity of lysocin E induced by ApoA-I, II is not due to direct antimicrobial activity of ApoA-I and II against *S. aureus*.

To obtain genetic evidence for the involvement of ApoA in the therapeutic effects of lysocin E, we examined the therapeutic effect of lysocin E using gene knockout mice⁸. The concentration of ApoA-I is approximately 7-fold higher than that of ApoA-II in mouse plasma⁹; therefore, we performed further analysis using *Apoa1* gene knockout mice. The enhancement of the antimicrobial activity of lysocin E in the serum of *Apoa1* homozygous deficient mice was 6-fold lower than that in wild-type mice (Fig. 1c). ApoA-II is present in the serum of *Apoa1* homozygous deficient mice at the same level as in wild-type mice¹⁰ and ApoA-I and ApoA-II showed similar enhancing activity (Extended Data Fig. 1a); thus, the residual activity in the serum of *Apoa1* homozygous deficient

mice can be explained by the activity of ApoA-II. Next, we examined the therapeutic efficacy of lysocin E in an *S. aureus* systemic infection model using *ApoA1* homozygous deficient mice. Lysocin E treatment did not reduce the number of surviving bacteria in the kidney of *ApoA1* homozygous deficient mice, but did reduce the number of bacteria number in the kidney of wild-type mice (Fig. 1d). These results suggest that ApoA-I contributes to the therapeutic effect of lysocin E in mice.

Figure 1 | Apolipoprotein A enhanced antimicrobial and therapeutic activity of lysocin E

a, Effect of serum and plasma from various animals on the antimicrobial activities of lysocin E, vancomycin, and daptomycin against *S. aureus* MSSA1 strain. The same results were obtained in 3 independent experiments. **b**, Reduction of the MIC of lysocin E against *S. aureus* by the addition of human recombinant ApoA-I protein and human ApoA-II. The data are representative of duplicate experiments with similar results. **c**, The lysocin E enhancing activity of serum prepared from *ApoA1* deficient mice. The data represent the mean $\pm$ S.E.M from three independent experiments. **d**, Effect of lysocin E in a systemic infection model of *S. aureus* using *ApoA1* deficient mice. Wild-type and *ApoA1* homozygous-deficient mice were infected with the Newman strain and the number of viable bacteria in the kidney 1 day after infection was compared between the lysocin E and vehicle administered groups (n=9/group). The data represent the mean $\pm$ S.E.M. and statistical analysis was performed by 1-way ANOVA with Tukey's multiple comparisons test. The data are representative of duplicate experiments with similar results.

Extended Data Table 1| Effect of bovine serum on the antimicrobial action of lysocin E against different microorganisms

Microorganisms / strains	MIC ($\mu\text{g ml}^{-1}$)		Fold (A/B)
	Serum 0% (A)	Serum 10% (B)	
<i>Staphylococcus aureus</i> Smith ATCC13709	2.0	0.063	1/32
RN4220	4.0	0.13	1/32
MSSA1	4.0	0.13	1/32
MRSA4	4.0	0.25	1/16
<i>Staphylococcus haemolyticus</i> JCM2416	2.0	0.0078	1/256
<i>Staphylococcus pseudintermedius</i> JCM17571	4.0	0.13	1/32
<i>Bacillus subtilis</i> JCM2499	2.0	0.25	1/8
<i>Bacillus cereus</i> JCM20037	2.0	0.50	1/4
RN4220 wild type	4.0	0.13	1/32
ΔmenA	64	64	1
ΔmenB	64	64	1

a

Fraction	Protein (mg)	Total activity (unit)	Yield (%)	Specific activity (unit/mg protein)	Purification (fold)
I. Calf serum	1100	13000	100	11	1
II. Ethanol extract	17	20000	160	1200	110
III. ODS column	4.3	15000	120	3600	330
IV. Superose12 column (peak 1)	3.4	20000	160	5900	540
IV. Superose12 column (peak 2)	0.2	1400	11	7000	640

b

c

d

Peak 1; Apolipoprotein A-I [*Bos taurus*]

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1  RHFQQDDPQSSWDRVKDFATVYVEAIKDSGRDYVAQFEA 40
41  SALGKQLNLKLLDNWDTLASTLSKVREQLGPVTQEFWDNL 80
81  EKETASLRQEMHKDL EEVKQKVQPYLDEFQKKWHEEVEIY 120
121 RQKVAPLGEFREGARQKVQELQDKLSPLAQELRDRAAH 160
161 VETLRQQLAPYSDDLRLRLTARLEALKEGGSLAEYHAKA 200
201 SEQLKALGEKAKPVLEDLRQGLLPVLESLLKVSILAAIDEA 240
241 SKKLNAQ 247
```

Peak 2; Apolipoprotein A-II [*Bos taurus*]

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1  ALVRRQAEESNLQSLVSQYFQTVADYGKDLVEKAKGSELQ 40
41  TQAKAYFEKTQEELTPFFK KAGTDLLNFLSSFIDPKKQPA 80
81  TR
```

Extended Data Fig 2| Biochemical identification of apolipoprotein A-I as an enhancing factor of lysocin E activity

a, Purification table of lysocin E antimicrobial enhancing factors from bovine serum. **b**, Elution patterns of the Superose12 column chromatography. The black line shows the amount of protein (right axis) and the red line shows the lysocin E antimicrobial promoting activity (left axis) in each fraction. **c**, Applied sample. **c**, Coomassie Brilliant Blue-stained images after separation by 12% SDS-PAGE for fractions 13 to 31 obtained from the Superose12 column chromatography. **d**, Amino acid sequence identified by PMF analysis for samples eluted from SDS-PAGE gels. Red letters indicate fragments obtained by PMF analysis.

ApoA-I activity is mediated via lipid II

ApoA-I and high-density lipoprotein-containing ApoA-I bind to the surface of bacteria^{11,12}. We found that ApoA-I binds the surface of *S. aureus* in a concentration-dependent manner (Fig. 2a). Because the addition of ApoA-I did not increase the amount of lysocin E that binds to the surface of *S. aureus* (Extended Data Fig. 3), the enhancing effect of ApoA-I cannot be explained by increased accumulation of lysocin E to the cell surface. To elucidate the lysocin E-enhancing functions of ApoA-I, we searched for lysocin E derivatives that did not respond to the addition of ApoA-I, although the antimicrobial activity of the lysocin E derivatives in our synthetic library does not differ from that of the natural type in the absence of ApoA-I¹³. We found that the activity of Bu-type lysocin E (Extended Data Fig. 1a), which has a modified fatty acid chain moiety, was not enhanced by ApoA-I (Extended Data Table 2). We assumed that the interaction of lysocin E Bu-type with ApoA-I differs from that of the natural-type lysocin E. We compared the affinities of the natural- and Bu-type lysocin E for ApoA-I, and found that their binding constants were similar, 3.6 and 3.4 μM , respectively (Extended Data Fig. 4a). The results suggested that lysocin E interacts with ApoA-I, although this interaction does not explain the difference in the ApoA-I response between natural- and Bu-type lysocin E. We then tested menaquinone, a cofactor of the electron transport chain of *S. aureus* that is a target of lysocin E¹. The binding constant of natural- and Bu-type lysocin E with a menaquinone was also similar, 4.0 and 5.0 μM , respectively (Extended Data Fig. 4b). Further, the binding capacity of lysocin E to ApoA-I in an ApoA-I pull-down assay did not significantly differ between the natural- and Bu-type lysocin E in the presence of menaquinone (Fig. 2b), suggesting that the presence of menaquinone does not affect the affinities of natural- and Bu-type lysocin E for ApoA-I.

Lipid II was recently reported to interact with lysocin E, and treatment of *S. aureus* with lysocin E at a sub-MIC doses leads to the accumulation of lipid II in the *S. aureus* membrane¹⁴. We hypothesised that lipid II is involved in the lysocin E antimicrobial-enhancing effect of ApoA-I, and tested this hypothesis in the following experiments. We first compared the binding constant of lipid II to natural-type and Bu-type lysocin E, and found that the binding constant of natural-type lysocin E with lipid II was 3-times lower than that of Bu-type lysocin E (Extended Data Fig. 4c). In addition, the binding capacity of lipid II to the natural-type lysocin E was significantly higher than that of the Bu-type (Extended Data Fig. 4d). We further demonstrated that lipid II

increased the binding of the natural-type lysocin E to ApoA-I (Fig. 2b) in a pull-down assay in the presence of menaquinone, and this phenomenon was not observed in the absence of menaquinone (Fig. 2c). To further confirm the importance of the lipid II–ApoA-I interaction, we constructed ApoA-I with deletion of the lipid-binding domain (ApoA-I Δ NM), which is less capable of enhancing the antimicrobial activity of lysocin E (Fig. 2d). Compared with wild-type ApoA-I, ApoA-I Δ NM had a lower lipid II-dependent binding capacity to lysocin E (Fig. 2e). These findings suggested that lipid II enhanced the recruitment of the lysocin E and menaquinone complex to ApoA-I.

Nisin is another antimicrobial that binds lipid II with membrane-damaging and bactericidal activities against *S. aureus*¹⁵. We found that the antimicrobial activity of nisin was enhanced by adding ApoA-I as in case of lysocin E (Extended Data Table 2). We then demonstrated that the binding of nisin to ApoA-I was increased by lipid II in a pull-down assay (Fig. 3a). These findings suggest that binding of nisin to ApoA-I is mediated by lipid II, resulting in enhancement of the antimicrobial activity. We further tested whether the membrane damage and bactericidal antimicrobial activities of lysocin E and nisin are enhanced by ApoA-I, and found that ApoA-I enhanced the bactericidal and membrane damaging activities of both antimicrobials in sub-MIC conditions (Fig. 3b,c). The membrane potential loss induced by the antimicrobials was observed at concentrations substantially lower than the MIC, even in the absence of ApoA-I (Fig. 3d). These findings implied that a small amount of membrane damage was induced by lysocin E or nisin at sub-MIC, and then ApoA-I greatly increased the membrane damage, resulting in the bactericidal actions of both antimicrobials (Fig. 3e).

Figure 2| Lipid II enhances the interaction of lysocin E with ApoA-I

a, Binding of ApoA-I to the cell surface of *S. aureus* RN4220. Different concentrations of ApoA-I were added to *S. aureus*. The cells were then treated with SDS-PAGE sample buffer, and centrifuged. Next, the supernatant was boiled and applied to SDS-PAGE, and band intensities were quantified using ImageJ. Data represent mean $\pm$ S.E.M. from 3 independent experiments. **b**, Lipid II-mediated binding of lysocin E to ApoA-I in an ApoA-I pull-down assay in the presence of menaquinone. Data represent mean $\pm$ S.E.M. of triplicate results. Statistical analysis was performed by 1-way ANOVA with Tukey's multiple comparisons test. **c**, Lipid II effect for lysocin E binding to ApoA-I in the absence of menaquinone. Data represent mean $\pm$ S.E.M. from 3 independent experiments, and statistical analysis was performed the Student 2-tailed *t*-test. **d**, ApoA-I Δ NM decreased the lysocin E antimicrobial activity enhancing effect against *S. aureus* RN4220 compared with wild-type ApoA-I. MIC values were determined by the micro-dilution assay described in the methods and representative data are shown from triplicate independent experiments with similar results. **e**, Requirement of lipid-binding domain of ApoA-I for lipid II-mediated lysocin E binding to ApoA-I. Data represent mean $\pm$ S.E.M. of triplicate results. Statistical analysis was performed by 1-way ANOVA Tukey's multiple

comparisons test.

Figure 3| Nisin and lysocin E showed common characteristics under sub-MIC

a, Increased lipid II-mediated nisin binding to ApoA-I. Data represent mean $\pm$ S.E.M. of triplicate results. Statistical analysis was performed by 1-way ANOVA with Tukey's multiple comparisons test. **b, c**, ApoA-I enhanced the membrane disruption activity (**b**) and bactericidal activity (**c**) of lysocin E and nisin at sub-MIC. Data represent mean $\pm$ S.E.M of triplicates, and statistical analyses were performed by the Student *t*-test. **d**, Loss of membrane potential of *S. aureus* after treatment with lysocin E and nisin. Data from triplicate experiments (mean $\pm$ S.E.M) are shown and represent an arbitrary fluorescent unit at 60 s after addition of the compounds. **e**, Presumed model for ApoA-I enhancing activity of lysocin E.

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226 **Extended Data Fig. 3| Effect of ApoA-I on the amount of lysocin E that binds to the cell surface**
 227 **of *S. aureus***

228 RN4220 (2 ml, OD₆₀₀ = 0.5) was incubated for 5 min with the indicated concentrations of ApoA-I in
 229 MHB, and further incubated with 2 µg ml⁻¹ lysocin E. The cells were precipitated by centrifugation at
 230 6000 x g for 5 min and washed with the same volume of PBS (-). Lysocin E was extracted from
 231 precipitated cells by 50% acetone and analysed by RP-HPLC. HPLC was performed as previously
 232 described¹. Data represent mean ± S.D. of triplicate results.

233

234 **Extended Data Table 2| Antimicrobial activity of derivatives of lysocin E and nisin**
 235 **against *S. aureus* RN4220 strain in the presence of ApoA-I**

Compounds	MIC ($\mu\text{g ml}^{-1}$)	
	Control	+ApoA-I 25 $\mu\text{g ml}^{-1}$ (fold)
Lysocin E (natural, 1)	4	0.5 (1/8)
Bu type lysocin E (2)	4	4 (1/1)
Nisin	8	2 (1/4)
Vancomycin	1	1 (1/1)

236

Extended Data Fig. 4| A comparison of natural- and Bu-type lysocin E for interactions to ApoA-I, menaquinone and lipid II

a, BLI analysis of the ApoA-I and natural- or Bu-type lysocin E interaction. Biotinylated natural or Bu type lysocin E was immobilised on a streptavidin biosensor and kinetic analysis was performed using 2.5 μ M of ApoA-I. **b**, BLI analysis for menaquinone and natural or Bu-type lysocin E interaction. Biotinylated menaquinone was immobilised on a streptavidin biosensor and kinetic analysis was performed using 12.5 μ M lysocin E derivatives. **c**, BLI analysis for the lipid II and natural or Bu-type lysocin E interaction. Biotinylated natural- or Bu-type lysocin E was immobilised on a streptavidin biosensor and kinetic analysis was performed using 24 μ M of lipid II. Representative data showing similar results from 3 independent experiments are shown (**a-c**). **d**, Lipid II binding to biotinylated natural- or Bu-type lysocin E in a pull-down assay. Data represent mean $\pm$ S.E.M. from 3 independent experiments, and statistical analysis was performed using the unpaired Student 2-tailed *t*-test.

Discussion

The findings of the present study demonstrated that the therapeutic efficacy of lysocin E is promoted by the host factor ApoA-I (Fig. 1). To our knowledge, this is the first study providing genetic evidence that specific host factors enhance the activity of an antimicrobial, thereby contributing to its therapeutic effects. Lysocin E was identified by screening based on therapeutic efficacy using a silkworm *S. aureus* infection model¹. The antimicrobial activity of lysocin E is also enhanced by silkworm haemolymph (Fig. 1a), and the silkworm contains apolipoprotein, which has similar functions as ApoA-I and binds the surface of *S. aureus*¹⁶. We consider that the use of the silkworm infection model to screen for compounds with therapeutic effectiveness led to the discovery of the antibiotic lysocin E, which has unique features.

Here, we present a presumed model that small amounts of membrane damage induced by lysocin E and nisin trigger a coordinated action between ApoA-I and lipid II that finally leads to massive membrane disruption, resulting in bactericidal action (Fig. 3e). ApoA-I constitutes a major protein of high-density lipoproteins in mammals and high-density lipoprotein carries lipids such as cholesterol. In addition, phosphatidylglycerol and cardiolipin, which are acidic phospholipids constituting the cell membrane of *S. aureus*¹⁷, bind ApoA-I^{18,19}. It is possible that interactions between these phospholipids and ApoA-I also contribute to expanding the disruption of cell membranes caused by lysocin E or nisin. The detailed process underlying the formation of these complexes requires further clarification.

It is important to elucidate the essentiality of lipid II for the ApoA-I enhancing effect on the antimicrobial activity of lysocin E, but it is difficult to verify using gene disruption strains because lipid II is an essential component for bacterial growth. By performing a biochemical analysis using lipid II-containing artificial liposomes, we demonstrated that lipid II was not involved in the membrane disruptive activity of lysocin E (Extended Data Fig. 5a). The necessity of lipid II for the antimicrobial activity of lysocin E in live bacteria should be clarified by further analysis. Regarding menaquinone, it appears to be an essential component for the antimicrobial activity of lysocin E, because the antimicrobial activity of lysocin E is greatly decreased in a mutant with disrupted *menA* and *menB* genes, which are essential genes for menaquinone synthesis, even in the presence of serum (Extended Data Table 1). Nisin disrupted liposomes containing lipid II as previously reported²⁰, but not those containing menaquinone (Extended Data Fig. 5b).

Therefore, although the roles of lipid II in the antimicrobial activity of lysocin E and nisin seem to differ, they share a common mechanism by which the antimicrobial activity modulated by ApoA-I via lipid II is enhanced, implying the existence of a series of similar antimicrobials. Other than ApoA-I, proteins in a host such as albumin are known to interact with bacteria^{21,22}. Proteins in serum are thought to act principally as inhibitory molecules against the antimicrobial activity of antimicrobial agents. On the basis of our results, we propose that the screening for antimicrobials whose activity is enhanced by host proteins that interact with the bacterial surface is an effective way to discover novel and therapeutically effective antimicrobials with different mechanisms from those of previously identified antimicrobials.

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a

b

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298 **Extended Data Fig. 5| Lipid II requirement for membrane disruption activity of lysocin E and**
 299 **nisin**

300 Membrane disruption activity of lysocin E (a) or nisin (b) on liposomes containing menaquinone and
 301 lipid II fractions. The data are representative of duplicate experiments with similar results.

302

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Methods

Bacteria, animals, and reagents

S. aureus RN4220²³, Newman, and MSSA1²⁴ strains were cultured in tryptic soy broth (TSB; Becton Dickinson and Co., Franklin, NJ, USA) overnight at 37°C with shaking. In the case of the *menA^{ts}* mutant, all cultures and assays were performed at 30°C. Other bacterial species were cultured as described previously¹. Various kinds of serum and human plasma were purchased from MilliporeSigma (St. Louis, MO, USA), heat-inactivated by treatment of 56°C for 30 min, and stored at -20°C until use. Recombinant human ApoA-I was purified as described in the Supplementary Information and human ApoA-II was purchased from Calbiochem (Merck Millipore, Billerica, MA, USA). Mice (ICR and C57BL/6J) were obtained from and CLEA Japan, Inc. *Apoa1* gene knockout mice were obtained from Jackson Laboratory (Bar Harbor, ME, USA) and bred until 8 to 12 weeks old. Lysocin E was prepared as previously described¹, and lysocin E derivatives were synthesised as previously reported¹³. The other reagents were purchased from Fujifilm Wako Pure Chemicals (Tokyo Japan) or MilliporeSigma except where noted.

Measurement of antimicrobial and bactericidal activity of lysocin E

The antimicrobial activities of the compounds were measured by the microdilution method²⁵. Measurement of the bactericidal activity of lysocin E (0.25 µg ml⁻¹) and nisin (2 µg ml⁻¹) with or without 25 µg ml⁻¹ ApoA-I was performed according to the National Committee for Clinical Laboratory Standards method²⁶. Sera and ApoA-I, II were added to the bacterial culture before distributing into 96-well plates. ApoA-I and II antimicrobial activity was evaluated using the RN4220 strain.

Measurement of membrane damaging activity

Membrane damaging activity was measured as follows²⁷. *S. aureus* MSSA1 strain was incubated with 25 µg ml⁻¹ ApoA-I, together with 0.25 µg ml⁻¹ lysocin E or 2 µg ml⁻¹ nisin at 37 °C, for 30 min, filtered through a 0.45-µm filter, and then the absorbance at 260 nm of the eluents was measured. Membrane potential was measured according to a fluorescence-based assay¹ with slight modification. Briefly, *S. aureus* MSSA1 or Newman strain was grown in TSB at 37°C with shaking at 200 rpm overnight. The overnight culture was diluted 100-fold in cation-adjusted Mueller Hinton Broth (CA-

MHB) and grown until the A_{600} reached 1.0. The cells were then collected by centrifugation at 8000 rpm for 10 min at 4°C, washed with HEPES buffer (pH 7.0) containing 5 mM HEPES, 50 mM glucose, and 5 mM EDTA, and resuspended in the same buffer to an A_{600} of 0.05. A membrane potential-sensitive dye, 3,3'-dipropylthiadicarbocyanine iodide, was added to the cells at a final concentration of 250 nM, and the cells were incubated in the dark at 37°C for 30 min. The fluorescence intensities (excitation 622 nm; emission 670 nm) were measured by a spectrofluorometer (Jasco FP-6200) equipped with a heated chamber at 37°C after adding the respective antimicrobials. All experiments were performed under minimal light conditions.

Purification of lysocin E antimicrobial activity-enhancing factors from calf serum

Calf serum (10 ml) was added to 15 ml EtOH (60% v/v), and the mixture was centrifuged for 15 min. The collected supernatant was evaporated and suspended in 8 ml Milli-Q water (fraction [fr.] II). Fr. II with formic acid (0.1%) was applied to 10 ml of ODS resin (Waters Corporation, Milford, MA, USA). After washing with 25% EtOH and then 50% EtOH, the resin was eluted with 75% EtOH containing 0.1% formic acid. The eluted sample was evaporated and dissolved in 0.8 ml buffer A (10 mM phosphate buffer [pH 7.1], 6 M urea; Fr. III). Fr. III was applied to the Superose 12 10/300 GL gel filtration column (GE Healthcare) and eluted with the AKTApurifier system (GE Healthcare). The elution was performed in buffer A at a flow rate of 0.5 ml min⁻¹, and 60 fractions (0.5 ml per fr.) were collected in 2-ml tubes. All fractions except for Fr. I were dialysed in 10 mM phosphate buffer (pH 7.1) before assessment of their activities. Protein concentrations were measured using Coomassie PlusTM protein assay reagent (ThermoFisher, Waltham, MA, USA) with bovine serum albumin as the standard. Amino acid sequence analysis was performed essentially as described previously²⁸. One unit was defined as the activity that decreases the MIC of lysocin E to 1 µg ml⁻¹.

Plasmid construction, expression and purification of ApoA-I

The oligonucleotide sequence of the human apolipoprotein A-I open reading frame (ORF), modified for optimized codon usage in *E. coli* according to a previous report²⁹, was synthesized at Eurofins Genomics and cloned in the pET20b vector (Novagen). The plasmid, designated pET-ApoWT, was introduced into *E. coli* BL21(DE3)/pLysS, and cells were selected with 100 µg ml⁻¹ ampicillin and 12.5 µg ml⁻¹ chloramphenicol. To

construct lipid domain-deleted ApoA-I, we first amplified the 2 regions within the ORF (nucleotide Nos. 1-435 and 481-729) using degenerated primers with linker sequences (Extended Data Table 3) and the pET-ApoWT plasmid as a template. The 2 products were then connected by another round of PCR and ligated to the EcoR V-digested pET20b vector, resulting in pET-ApoΔM. Next, the internal ORF region (nucleotide No. 196-729) was amplified by PCR using degenerated primers (Extended Data Table 3) and the pET-ApoΔM plasmid as a template. The PCR product was digested with Nde I and Hind III, and then ligated with the pET20b vector linearized with the same restriction enzymes, resulting in pET-ApoΔNM. Colonies were inoculated with NZCYM broth (10 g of pancreatic digest of casein, 1 g of casamino acids, 5 g of yeast extract, 5 g of NaCl, 1 g of magnesium sulfate anhydrous, dissolved in 1 L of water and autoclaved at 121°C for 15 min) in the presence of the above-mentioned antibiotics. After overnight incubation, 0.5 ml of the full-growth cultures were added to 50 ml of the fresh NZCYM broth containing the above-mentioned antibiotics, followed by aerobic incubation at 37°C to OD₆₀₀ = 0.6. Then, 50 μl of 1 M isopropyl β-D-1-thiogalactopyranoside (final 1 mM) was added, and the cultures were further incubated for 3 h. Cells were collected by centrifugation, resuspended in 1xGnPB (50 mM phosphate buffer pH 8, 0.5 M NaCl, 3 M guanidine hydrochloride), sonicated and centrifuged at maximum speed for 10 min. The supernatants were collected and applied to Probond, a nickel-chelating resin (Life Technologies). The column was washed successively with 1xGnPB and 20 mM imidazole in 1xGnPB, and then eluted with 200 mM imidazole in 1xGnPB. The eluted fractions were dialyzed against 50 mM phosphate buffer (pH 8) and 100 mM NaCl. Approximately 5 to 6 mg of the recombinant protein was obtained from 50 ml of the initial *E. coli* culture.

Extended Data Table 3| Nucleotide sequences of primers used in this study. Letters in lower case indicate linker sequences and an underlines indicate restriction enzyme recognition sites.

Target	Forward	Reverse
rApoA-I ΔN, nucleotide No. 196-729	5'- GGTACCC <u>CATATG</u> CATCACCATCACCATCA CCCGGTGACCCAGGAATTCT-3'	5'- GGTACCA <u>AAGCTT</u> AGATCTACTAGTTCTA GATCACTGGG-3'

rApoA-I ΔM, nucleotide No.	5'-GGTACCCATATGCATCACCATC-3'	5'-gcccagtgggctcagcttctctt-3'
1-435		
rApoA-I ΔM, nucleotide No.	5'-	5'-
481-729	agaagctgagccactgggcACGCATCTGGCGCC	AAGCTTAGATCTACTAGTTCTAGATCACT
	GTACAG-3'	GGG-3'

Mouse infection experiments

Mouse protocols followed the Regulations for Animal Care and Use of the University of Tokyo under approval by the Graduate School of Pharmaceutical Science at the University of Tokyo (approval No. 24-55). For Table 1, clinically isolated MRSA MR6 strain³⁰ was cultured overnight, suspended in 7% type II porcine stomach mucin supplemented with 0.2 mM FeNH₄-citrate and intraperitoneally administered to mice (ICR, female, 4 weeks of age) at 5.7x10⁷ colony-forming units. Lysocin E (4 to 0.25 mg kg⁻¹), vancomycin (8 to 0.5 mg kg⁻¹), linezolid (8 to 0.5 mg kg⁻¹), or daptomycin (4 to 0.25 mg kg⁻¹) were administered subcutaneously to 5 animals/group at 1 h and 6 h after infection. Survival of mice was assessed after 5 days and ED₅₀ values were calculated by multiple logistic regression analysis. For Fig. 1d, the Newman strain was cultured in TSB overnight and cells (4.0x10⁷ colony-forming units) washed in phosphate-buffered saline (PBS) were injected into the tail vein of 8 to 12-week-old female C57BL/6J wild-type mice or *ApoA1* homozygous deficient mice. After 2 h, lysocin E (0.5 mg kg⁻¹) was administered subcutaneously. Twenty-four hours later, the kidneys were harvested and crushed with a BioMasher II (Nippi Incorporated, Tokyo, Japan). The suspensions were diluted with PBS(-), plated on TSB agar medium, and the number of colonies was counted.

ApoA-I pull-down assay

MK-4 (100 μl of 1.25 mM) was mixed with N-dodecyl β-D-maltoside (250 μl of 100 mM) in the presence or absence of lipid II fraction (300 μl of 70 μM). Lipid II fraction was prepared from *S. aureus* RN4220 treated with moenomycin and followed by park nucleotide removal³¹. The mixture was dried *in-vacuo* and dissolved in 2 ml of water. The micelles were then filtered through a 0.45-μm polyvinylidene difluoride membrane filter, and a 200-μl aliquot of the filtered micelles were used for the binding of antimicrobials in the presence of ApoA-I. Briefly, 5 μg of antimicrobials was added to the aliquots and

the mixture was incubated for 15 min on ice in the presence of 4 µg ApoA-I. The mixture was then transferred to His-Tag magnetic beads (Dynabeads, ThermoFisher). The bound protein was then eluted with 250 mM imidazole and the eluted antimicrobials were quantified using an ACQUITY UPLC H-Class PLUS attached to Xevo G2-XS QToF system (Waters Corporation). After injecting the sample into a 2.1 × 100 mm Acquity UPLC HSS T3 1.8 µm column (Waters Corporation) equilibrated with 0.3 mL min⁻¹ of 30% acetonitrile and 0.1% formic acid in water, the column was developed with a linear gradient to 60% acetonitrile and 0.1% formic acid in water over 10 min. The eluate was continuously applied to a Waters Xevo G2-XS QToF mass spectrometer and the spectra obtained using MassLynx 4.1 (Waters Corporation) in electrospray ionisation (ESI)-positive mode were analysed by UNIFI Scientific Information System ver. 1.9 (Waters Corporation).

Bio-Layer Interferometry analysis.

Bio-layer interferometry (BLI) measurements were performed on a BLItz system (ForteBio, Fremont, CA, USA) maintained at room temperature. The biotinylated ligand, diluted to 0.1 mg/ml in BLItz buffer (0.05% [v/v] Tween-20 in PBS), was immobilised on a streptavidin biosensor and the kinetic analysis was performed by running BLItz buffer for 300 s each, in the presence and absence of analyte for association and dissociation, respectively. Biotinylated menaquinone and biotinylated lysocin E derivatives (natural and Bu-type) were synthesised as described in the Supplementary Information.

Amount of ApoA-I binding on the surface of *S. aureus*

S. aureus RN4220 strain was grown overnight in TSB at 37°C with shaking at 150 rpm. The A₆₀₀ was adjusted to 0.5 with CA-MHB, 1 ml of which was treated with 0, 10, 20, or 40 µg ml⁻¹ of ApoA-I and further incubated at 37°C with shaking at 150 rpm for 5 min. The cells were then centrifuged at 3000 rpm at 4°C for 30 min, washed twice with PBS, and then the pellets were suspended in SDS-PAGE sample buffer. Under these conditions, the *S. aureus* cells were not lysed. The supernatants were collected, boiled, and applied to 12.5% SDS-PAGE. The gel was stained with Coomassie brilliant blue (CBB), and the band intensities were quantified using ImageJ software (1.47v, NIH, USA)³².

Liposome disruption assay

Liposome preparation was performed as previously described with modifications¹. Briefly, a lipid film prepared from 1,2-dioleoyl-sn-glycero-3-phosphocholine containing 1.0 mol % menaquinone-4 or/and 0.1 mol % lipid II was rehydrated with 5 mM sodium HEPES (pH 7.5) containing 100 mM calcein. The liposome suspension was freeze-thawed 5 times and filtered through a Sephadex G-50 column with 20 mM sodium HEPES (pH 7.5) containing 1 mM EDTA to remove free calcein. The liposome fraction was diluted 5-fold with buffer, and the increase in fluorescence intensity (excitation: 310 nm, emission: 530 nm) was measured by a spectrofluorometer after adding the antimicrobials.

Quantification of lipid II

The lipid II fraction was prepared from 5-ml cultures as previously described¹⁴ and hydrolysed by incubating at 99°C for 30 min in 100 µl of 10 mM ammonium acetate pH 4.2. The samples were freeze-dried and dissolved in 30 µl water. The supernatant, after centrifugation at 16,000g for 10 min, was analysed by a quantitative liquid chromatography-mass spectrometry (LC-MS). The LC-MS system comprised an ACQUITY UPLC H-Class PLUS and Xevo G2-XS QToF system, and CORTECS UPLC T3 1.6 µm 2.1 x 150 mm column (Waters Corporation) maintained at 40°C with a flow rate of 0.3 ml min⁻¹. Solvent A was 0.1% aqueous formic acid and solvent B was 0.1% formic acid in acetonitrile. After 8 µl of the sample was applied to the column equilibrated with solvent A, the column was developed with the same solvent for 2 min followed by a linear gradient of 100 to 80% of A over 15 min. Mass spectra were collected in ESI-negative mode using MassLynx 4.1 (Waters Corporation), and analysed by UNIFI Scientific Information System ver. 1.9). The peak area of delipidated lipid II (Extended Data Fig. 1c) to assess the exact mass m/z 664.255 [M-2H]²⁻ (calculated mass m/z 664.256 [M-2H]²⁻) was quantified and the structure was confirmed by the fragment pattern generated from MS^E analysis using the UNIFI Scientific Information System. The concentration of lipid II in the extracted fraction was calculated using standard compound purified from delipidated lipid II by HPLC (Alliance HPLC system, Waters Corporation) using PEGASIL ODS SP100AQ, 4.6 x 250 mm column (Senshu Scientific, Tokyo, Japan) with the same mobile phase system as described for the UPLC system above except the flow rate was 1 ml min⁻¹.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 8.4.3 (GraphPad Software, San Diego, CA, USA). *P* values over 0.05 were considered non-significant.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request. Source data are provided with this paper.

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Author contributions

H.H. conceived the idea. H.H. and S.P. designed the experiments. S.P. analysed the interactions of ApoA-I, lipid II, and lysocin E. A.P., S. J., K. I., and J. Y. purified ApoA-I and assayed the antimicrobial activity. H.H., S.P., and A.P. performed the membrane disruption assay. H.I. and K.T. synthesised the lysocin E derivatives. M.I. and K.S. provided critical discussion for writing the manuscript. H.H., S.P., and A.P. wrote the manuscript. All authors contributed critically to the interpretation of the results and gave final approval for publication.

Competing interest declaration

The authors declare competing financial interests as follows: Dr. Sekimizu is a consultant for Genome Pharmaceutical Institute Co, Ltd.

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Figures

Figure 1

Apolipoprotein A enhanced antimicrobial and therapeutic activity of lysocin E a, Effect of serum and plasma from various animals on the antimicrobial activities of lysocin E, vancomycin, and daptomycin against *S. aureus* MSSA1 strain. The same results were obtained in 3 independent experiments. b, Reduction of the MIC of lysocin E against *S. aureus* by the addition of human recombinant ApoA-I protein and human ApoA-II. The data are representative of duplicate experiments with similar results. c, The lysocin E enhancing activity of serum prepared from ApoA1 deficient mice. The data represent the mean $\pm$ S.E.M from three independent experiments. d, Effect of lysocin E in a systemic infection model of *S. aureus* using ApoA1 deficient mice. Wild-type and ApoA1 homozygous-deficient mice were infected with the Newman strain and the number of viable bacteria in the kidney 1 day after infection was compared between the lysocin E and vehicle administered groups ($n=9/\text{group}$). The data represent the mean $\pm$ S.E.M. and statistical analysis was performed by 1-way ANOVA with Tukey's multiple comparisons test. The data are representative of duplicate experiments with similar results.

Figure 2

Lipid II enhances the interaction of lysocin E with ApoA-I a, Binding of ApoA-I to the cell surface of *S. aureus* RN4220. Different concentrations of ApoA-I were added to *S. aureus*. The cells were then treated with SDS-PAGE sample buffer, and centrifuged. Next, the supernatant was boiled and applied to SDS-PAGE, and band intensities were quantified using ImageJ. Data represent mean $\pm$ S.E.M. from 3 independent experiments. b, Lipid II-mediated binding of lysocin E to ApoA-I in an ApoA-I pull-down assay in the presence of menaquinone. Data represent mean $\pm$ S.E.M. of triplicate results. Statistical analysis was performed by 1-way ANOVA with Tukey's multiple comparisons test. c, Lipid II effect for lysocin E binding to ApoA-I in the absence of menaquinone. Data represent mean $\pm$ S.E.M. from 3 independent experiments, and statistical analysis was performed the Student 2-tailed t-test. d, ApoA-I ΔNM decreased the lysocin E antimicrobial activity enhancing effect against *S. aureus* RN4220 compared with wild-type ApoA-I. MIC values were determined by the micro-dilution assay described in the methods and

representative data are shown from triplicate independent experiments with similar results. e, Requirement of lipid-binding domain of ApoA-I for lipid II-mediated lysocin E binding to ApoA-I. Data represent mean $\pm$ S.E.M. of triplicate results. Statistical analysis was performed by 1-way ANOVA Tukey's multiple comparisons test.

Figure 3

Nisin and lysocin E showed common characteristics under sub-MIC a, Increased lipid II-mediated nisin binding to ApoA-I. Data represent mean $\pm$ S.E.M. of triplicate results. Statistical analysis was performed by 1-way ANOVA with Tukey's multiple comparisons test. b, c, ApoA-I enhanced the membrane disruption activity (b) and bactericidal activity (c) of lysocin E and nisin at sub-MIC. Data represent mean $\pm$ S.E.M of triplicates, and statistical analyses were performed by the Student t-test. d, Loss of membrane potential of *S. aureus* after treatment with lysocin E and nisin. Data from triplicate experiments (mean $\pm$ S.E.M) are

shown and represent an arbitrary fluorescent unit at 60 s after addition of the compounds. e, Presumed model for ApoA-I enhancing activity of lysocin E.

Supplementary Files

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