

Outer Membrane Permeabilization Is an Essential Step in the Killing of Gram-Negative Bacteria by the Lectin RegIII β

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Abstract

The C-type lectin RegIII β can kill certain Gram-positive and Gram-negative bacteria. The susceptibility of *S. Typhimurium* depends on the bacterial growth phase, i.e., bacteria from the logarithmic growth phase do bind RegIII β and are subsequently killed. Lipid A is one of the bacterial targets for RegIII β . However, at the molecular level, it is not understood how RegIII β interacts with and kills Gram-negative bacteria. Here, we show that RegIII β interacts with Gram-negative bacteria in two distinct steps. Initially, it binds to surface-exposed lipid A. The lipid A can be shielded by the O-antigen of lipopolysaccharide (LPS), as indicated by the exquisite susceptibility of *wbaP* mutants to RegIII β -mediated killing. Increased cell viability after incubation with an anti-lipid A antibody also supports this conclusion. This RegIII β -binding permeabilizes the outer membrane to hydrophobic dyes like Ethidium bromide or to bulky bacteriolytic enzymes like lysozyme. Conversely, compromising the outer membrane integrity by the mild detergent Triton X-100 enhances the antibacterial effect of RegIII β . Based on our observations, we conclude that RegIII β interacts with Gram-negative bacteria in two subsequent steps. Initially, it binds to the outer membrane thus leading to outer membrane permeabilization. This initial step is necessary for RegIII β to reach a second, still not well understood target site (presumably localized in the periplasm or the cytoplasmic membrane), thereby triggering bacterial death. This provides novel insights into the outer membrane-step of the bactericidal mechanism of RegIII β .

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Introduction

The surface of the mammalian intestine interacts directly with the external environment. Hence, these tissues are continuously exposed to bacteria, viruses, fungi and parasites that could act as pathogens. Epithelial antimicrobial proteins such as α -defensins are of critical importance for keeping the large numbers of microorganisms in check [1–3]. In addition to limiting microbial challenges, antimicrobial proteins also affect the composition of gut microbiota and may prevent opportunistic invasion by symbiotic bacteria [2,4].

The RegIII (regenerating gene family protein III) lectins belong to the epithelial antimicrobial proteins. RegIII β and RegIII γ are expressed in the murine intestine, and have bactericidal activity [5–8]. The production of RegIII β and RegIII γ is dramatically increased in response to bacterial colonization and pathogenic infection leading to inflammation [5,6,8]. The matured RegIII lectins (molecular mass of ~16 kDa) are secreted from Paneth cells and epithelial cells of the intestine into the gut lumen [5,6], and RegIII β and RegIII γ have been shown to protect against pathogens and contribute to the maintenance of microbiota homeostasis [5,8–13]. However, their antimicrobial mechanism is still not completely understood.

RegIII β and RegIII γ have distinct spectra of antimicrobial activity. RegIII γ can kill Gram-positive bacteria but not Gram-negative bacteria whereas RegIII β displays bactericidal activity against both certain Gram-positive and Gram-negative bacteria [5,8]. For example, RegIII β was found to kill various bacteria taken from stationary phase cultures, i.e. *Clostridium butyricum*, *Lactobacillus reuteri*, and different *Escherichia coli* strains but not *Enterococcus faecalis*, *Lactobacillus murinus*, and *Salmonella Typhimurium* [8]. The bactericidal effect of RegIII lectins depends on their ability to bind to target bacterial structures [14,15]. RegIII γ binds to Gram-positive bacteria by recognizing peptidoglycan, and then seems to kill by damaging the cell wall and provoking cytoplasmic leakage [5,14]. Similarly, it is conceivable that RegIII β kills Gram-positive bacteria by the same mechanism as RegIII γ because RegIII β can also bind to peptidoglycan [14,15]. In contrast to RegIII γ , RegIII β can also bind to Gram-negative bacteria. This has been studied best using the Gram-negative enteropathogen *Salmonella Typhimurium* (*S. Typhimurium*). Here, RegIII β was shown to bind to the bacterial surface and inhibition studies indicated that it can bind directly to the lipid A moiety of lipopolysaccharide [15]. However, the killing mechanism of RegIII β is not well understood and it had remained unclear, how it is linked to lipid A binding.

Interestingly, RegIII β was able to kill *S. Typhimurium* taken from a logarithmic growth phase culture. In fact, the bactericidal activity of RegIII β against *S. Typhimurium* was found to be strictly dependent on the pathogen's growth phase and kills *S. Typhimurium* grown at the logarithmic growth phase, but not bacteria taken from stationary cultures [15]. A tripeptide ERN (E134, R135 and N136) and D142 in the Loop 2 of RegIII β are involved in bacterial recognition and bactericidal activity [15]. However, the mechanism determining this growth phase dependency had remained enigmatic.

Here, we have analyzed the bactericidal mechanism of RegIII β against *S. Typhimurium*. In particular, we focused on the initial interaction with the outer membrane. This demonstrated that binding of surface-exposed lipid A and subsequent outer membrane permeabilization represents an important step of the bactericidal mechanism. Our study provides novel insights into the bactericidal mechanism of RegIII family lectins against Gram-negative bacteria.

Materials and Methods

Bacterial Strains

S. Typhimurium wild-type strain SL1344 was used in this study [16]. SKI12 harboring a mutation of *wbaP* gene is a SL1344 derivative lacking an LPS O-antigen [17]. Bacteria were diluted (1:200) from an overnight culture in 20 ml of LB broth (100-ml Erlenmeyer flask) and grown to the indicated OD₆₀₀ under mild aeration (160 rpm) at 37°C. Streptomycin (50 μ g/ml) and kanamycin (50 μ g/ml) were used when required.

Antibiotics and Antibodies

Polymyxin B was purchased from Novo Nordisk (Copenhagen, Denmark). Gentamicin and ampicillin were purchased from Axonlab (Baden, Switzerland). Ciprofloxacin was purchased from Bayer (Leverkusen, Germany). Anti-lipid A antibody [26-5] and anti-*Salmonella* Typhimurium LPS antibody [1E6] were purchased from abcam (Cambridge, UK). IgG from mouse serum was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Purification of Recombinant Proteins

Recombinant untagged-RegIII β and its point-mutated variants were prepared as described previously [8,15]. Briefly, *E. coli* expressing RegIII β were lysed by sonication, and then the resulting inclusion bodies including RegIII β were purified. The purified RegIII β inclusion bodies were solubilized in denaturing buffer containing guanidine-HCl and then subjected to refolding buffer containing arginine-HCl to refold the RegIII β protein. Finally, the refolded RegIII β was purified by dialysis in binding buffer (25 mM 4-morpholineethanesulfonic acid (MES) pH 6.0, 25 mM NaCl).

In vitro Killing Assay

The *in vitro* killing assay was performed as described previously [15]. In brief, bacteria grown at the indicated growth phase were washed and resuspended in binding buffer (25 mM MES pH 6.0, 25 mM NaCl) at a density of $1-3 \times 10^6$ cfu/ml. The diluted bacterial suspension was exposed to RegIII β (2.5 or 10 μ M) or polymyxin B (1 μ g/ml) at 37°C for 30 min. Bacteria were then plated on selective LB media. The recovered cfu were normalized for the original cfu of the inoculum, thus yielding the bacterial "survival" (in %). Preincubation steps using anti-lipid A or mouse IgG were performed for 10 min at 37°C, as indicated.

Bacterial Binding Assay

The bacterial binding assay was performed as described previously [15]. In brief, 50 μ l aliquots (1.0×10^7 cfu) of *S. Typhimurium* cells grown up to the logarithmic growth phase were pelleted by centrifugation, washed with binding buffer (25 mM MES pH 6.0, 25 mM NaCl), suspended in binding buffer and incubated with various concentrations of anti-lipid A antibody or mouse IgG, as indicated. Ten micrograms of RegIII β or BSA (in 50 μ l total volume) was added and incubated for 15 min at 37°C. The reaction mixtures were centrifuged at 6000 *g* for 5 min, and the resulting supernatant was isolated, mixed with SDS-PAGE sample buffer and boiled for 5 min. The pellet was washed once with binding buffer and boiled for 5 min in SDS-PAGE sample buffer (50 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 0.1% (w/v) bromophenol blue, 10% (v/v) glycerol, 100 mM 2-mercaptoethanol). The samples were subjected to SDS-PAGE and analyzed by Coomassie brilliant blue (CBB) staining. The corresponding band of RegIII β was identified by comparing with a reaction mixture control containing *S. Typhimurium*, but not RegIII β , or purified RegIII β proteins.

Bacterial Whole-cell ELISA

A 96-well microtiter plate (Nunc MaxiSorp® flat-bottom 96 well plate) was coated with 100 μ l of *S. Typhimurium* (grown to mid-logarithmic growth phase or stationary phase) in PBS (5×10^7 cfu/well) for 1 h at 37°C, and incubated at 4°C overnight. After washing with wash buffer (0.05% Tween-20 in dH₂O), 100 μ l of anti-lipid A antibody (0.02 mg/ml) or anti-*Salmonella* Typhimurium LPS antibody (1 μ g/ml) in blocking buffer (4% BSA, 0.05% Tween-20 in PBS) were incubated for 1 h at 37°C. After incubation, the wells were washed with washing buffer extensively, 100 μ l of anti-mouse IgG conjugated to horseradish peroxidase (HRP, Sigma; 1:100 for lipid A or 1:1000 for LPS, diluted in blocking buffer) was incubated for 1 h at 37°C. After extensively washing with wash buffer, ABTS (2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid); Calbiochem, San Diego, CA, USA) was added, and then incubated for 15 min at room temperature. The bound HRP was detected by measuring OD₄₀₅ of each well using a Spectra max plus microplate spectrophotometer (Molecular devices, Sunnyvale, CA, USA).

Outer Membrane Permeability Assay (EtBr Influx Assay)

S. Typhimurium grown to the mid-logarithmic growth phase was washed with binding buffer (25 mM MES pH 6.0, 25 mM NaCl), and diluted to OD₆₀₀ = 0.4/ml in binding buffer. The bacterial suspension was incubated with RegIII β (2.5 or 5 or 10 μ M final concentration) or point-mutant RegIII β variants (10 μ M), polymyxin B (1 μ g/ml), gentamicin (100 μ g/ml), ciprofloxacin (1 μ g/ml) or BSA (160 μ g/ml) for 20 min at 37°C. If necessary, anti-lipid A antibody (16 μ g/ml) or mouse IgG (16 μ g/ml) were preincubated for 10 min at 37°C. After addition of ethidium bromide (EtBr) (6 μ M), the fluorescence of the EtBr-nucleic acid complex was immediately measured by using Cary Eclipse fluorescence spectrometer (Varian Inc., Walnut Creek, CA, USA) with excitation and emission wavelengths of 545 and 600 nm, respectively. The widths of the slits are 5 and 10 nm, respectively.

Statistical Analysis

Statistical analysis was performed using the Mann Whitney *U*-test or unpaired Student's *t*-test. Correlation analysis was performed using Pearson's correlation coefficient. *P* < 0.05 was considered to be statistically significant.

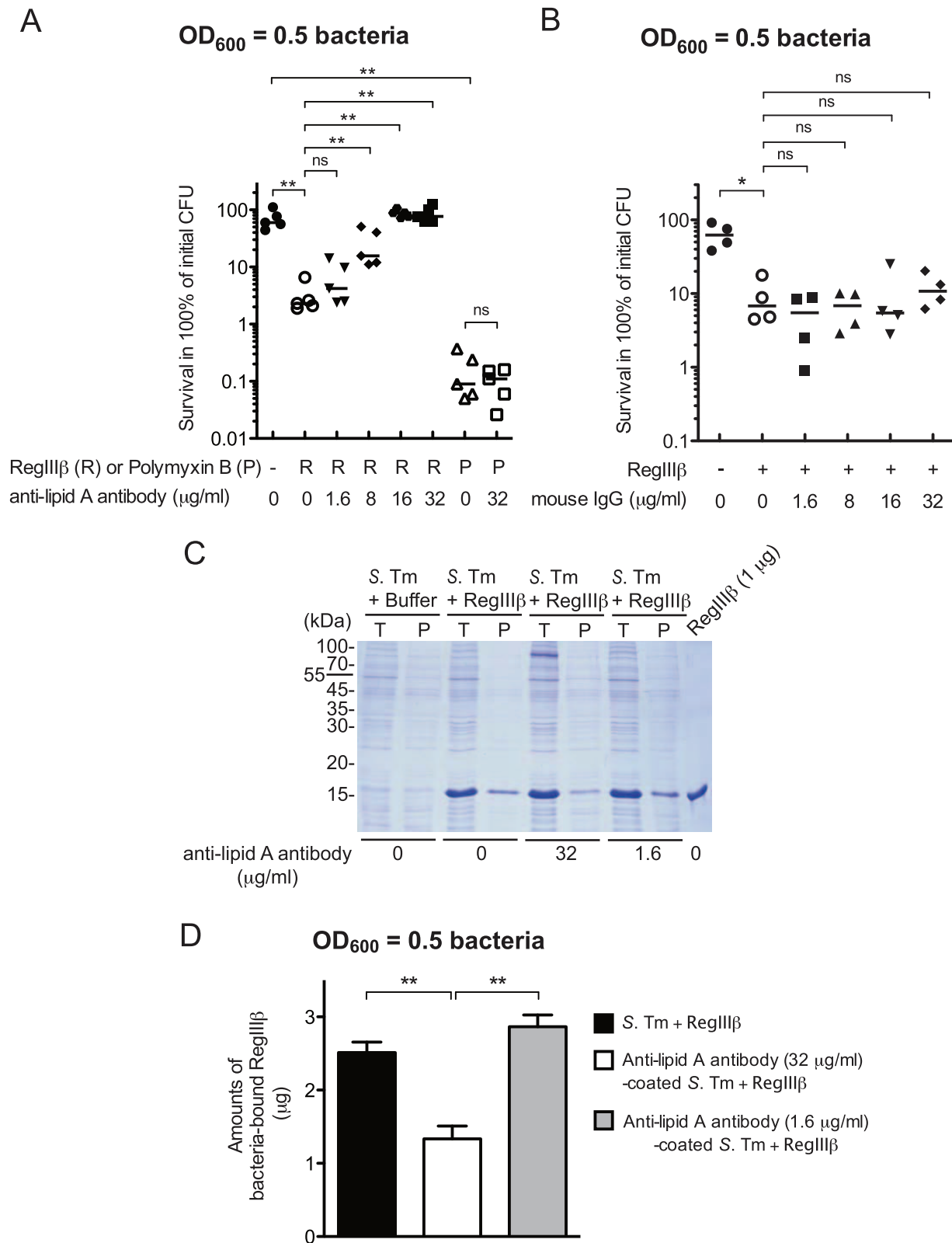


Figure 1. Preincubation with anti-lipid A antibody inhibits the bactericidal effect of RegIII β . A) *S. Typhimurium* wild-type (SL1344) from the mid-logarithmic growth phase was incubated with the indicated concentrations of anti-lipid A antibody for 10 min at 37°C. Afterwards, purified RegIII β (10 μM) or polymyxin B (1 $\mu\text{g/ml}$) was added and the mixture was incubated for 30 min at 37°C. Bacterial survival was quantified by dilution-plating. Assays were done in five independent experiments. The bar shows the median. ** $P < 0.01$; ns, not significant (Mann-Whitney U test). B) *S. Typhimurium* wild-type (SL1344) from the mid-logarithmic growth phase was incubated with the indicated concentrations of mouse IgG for 10 min at 37°C. Purified RegIII β (10 μM) was incubated with the preincubated mixture for 30 min at 37°C and bacterial survival was quantified by dilution-plating. Assays were done in four independent experiments. The bar shows the median. * $P < 0.05$; ns, not significant (Mann-Whitney U test). C)

Bacteria (1×10^7 cfu) were grown up to the mid-logarithmic growth phase, preincubated with anti-lipid A antibody (1.6 or 32 $\mu\text{g/ml}$) at 37°C for 10 min, and then incubated with RegIII β (10 μg) at 37°C for 15 min (T , total fraction). The RegIII β bound or unbound to bacteria was separated by centrifugation (P , pellet), and then analyzed by SDS-PAGE and CBB staining. D) Quantitative analysis. Amounts of bacteria-bound RegIII β (μg) were determined by comparing the band intensity of 1 μg of RegIII β as a control. The error bars represent the S.D. of the mean from at least three independent experiments. $^{***}P < 0.01$; ns, not significant (unpaired Student's t -test). doi:10.1371/journal.pone.0069901.g001

Results

Binding of RegIII β to Surface-exposed Lipid A is Essential for its Bactericidal Activity

Earlier work has established that RegIII β binds to the lipid A moiety of LPS and that this bacterial binding is critical for its bactericidal activity against Gram-negative bacteria [15]. In the unperturbed outer membrane, the lipid A moiety of LPS is mostly shielded by the LPS O-antigen. This raised the question if the accessibility of the lipid A might represent a limiting step in RegIII β -mediated killing. Therefore, we investigated whether masking of surface-exposed lipid A by using anti-lipid A antibody can inhibit the bactericidal activity of RegIII β . We first evaluated binding-specificity of the anti-lipid A antibody. The anti-lipid A antibody used in this study bound to lipid A moiety of purified LPS and extracted lipid A, but not to any other moieties of the purified LPS, especially the O-antigen (Figure S1A and C). In contrast, the anti-LPS antibody bound to the O-antigen of LPS, but not to the lipid A moiety (Figure S1B and D). Furthermore, lipid A in the fractionated outer membrane, but not any other proteins, was detected by the anti-lipid A antibody (Figure S1E–G). Altogether, these results verify the binding specificity of the anti-lipid A antibody. Therefore, this reagent should be suitable for masking the surface-exposed lipid A when incubated with Gram-negative bacteria.

To this end, we preincubated *S. Typhimurium* grown to $\text{OD}_{600} = 0.5$ (note: at $\text{OD}_{600} = 0.5$ the bacteria are sensitive to RegIII β -mediated killing; [15]) with an anti-lipid A antibody, before the bacteria were exposed to RegIII β . Preincubation with low concentrations (1.6 $\mu\text{g/ml}$) of anti-lipid A antibody had no detectable effect on the bactericidal activity of RegIII β (Fig. 1A). However, at concentrations of ≥ 8 $\mu\text{g/ml}$ the anti-lipid A antibody inhibited RegIII β -mediated killing. Complete inhibition was observed at anti-lipid A antibody concentrations of 16 or 32 $\mu\text{g/ml}$ (Fig. 1A).

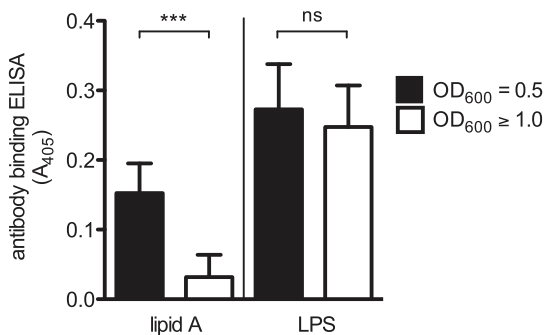


Figure 2. Difference in the amount of surface-exposed lipid A in distinct bacterial growth phases of *S. Typhimurium*. Amounts of exposed lipid A and LPS in *S. Typhimurium* wild-type strain (SL1344) grown at mid-logarithmic growth phase ($\text{OD}_{600} = 0.5$) or at stationary phase ($\text{OD}_{600} \geq 1.0$) were compared by whole-cell ELISA using anti-lipid A or anti-LPS antibodies (Experimental Procedures). The error bars represent the SD of the mean from three independent experiments. $^{***}P < 0.001$; ns, not significant (unpaired Student's t -test). doi:10.1371/journal.pone.0069901.g002

It remained unclear if the anti-lipid A antibody specifically inhibited RegIII β . Alternatively, it might provide a non-specific shield against LPS-binding agents. To test this, we analyzed the effect of the anti-lipid A antibody on polymyxin B-mediated killing. Polymyxin B is a small cationic antimicrobial antibiotic targeting the lipid A and 3-deoxy-D-manno-oct-2-ulosonic acid (Kdo) moieties of LPS [18]. However, unlike RegIII β , the bactericidal activity of polymyxin B was not inhibited by the anti-lipid A antibody, even at concentration of 32 $\mu\text{g/ml}$ (Fig. 1A), indicating that the anti-lipid A antibody-mediated masking may not block the PMB-(lipid A)-Kdo interaction. Furthermore, we performed a control with non-specific mouse IgG. Preincubation with the control mouse IgG did not affect the bactericidal activity of RegIII β at any antibody concentration (Fig. 1B). This verified that the anti-lipid A antibody specifically inhibited the bactericidal activity of RegIII β .

Binding of RegIII β to the bacterial surface is necessary for its bactericidal effect [15]. Therefore, we investigated whether the masking surface-exposed lipid A by the anti-lipid A antibody can prevent the binding of RegIII β to the bacterial surface. Indeed, preincubation with high concentrations of anti-lipid A antibody (32 $\mu\text{g/ml}$), but not low concentrations of the same antibody (1.6 $\mu\text{g/ml}$), inhibited the binding of RegIII β to the bacteria (Fig. 1C and 1D). Collectively, these results suggest that the masking surface-exposed lipid A by preincubation with anti-lipid A antibody protects *S. Typhimurium* from the bactericidal effect of RegIII β by inhibiting the access of RegIII β to lipid A. Moreover, these data indicated that the growing bacteria display “exposed” lipid A moieties on their surface which can be bound by either the anti-lipid A antibody, or by RegIII β .

The Bactericidal Activity of RegIII β Correlates with the Amount of Surface-exposed Lipid A

The bactericidal activity of RegIII β is dependent on bacterial growth phase, i.e., RegIII β kills *S. Typhimurium* cells grown at logarithmic growth phase, but not cells taken from the stationary phase [15]. Together with the data presented in Fig. 1, this suggested that *S. Typhimurium* cells from the logarithmic growth phase display more surface exposed lipid A than cells from the stationary phase. To investigate this, we performed an ELISA assay on whole bacterial cells. To this end, ELISA plates were coated with *S. Typhimurium* grown at mid-log phase ($A_{600} = 0.5$) or the stationary phase ($A_{600} \geq 1.0$) and used to analyze the binding of the anti-lipid A antibody. An antibody directed against the O-antigen served as a positive control, as this epitope should be exposed on logarithmic growth phase and stationary phase bacteria, alike.

S. Typhimurium from the mid-log phase ($A_{600} = 0.5$) displayed more surface-exposed lipid A than cells from the stationary phase ($A_{600} \geq 1.0$) (Fig. 2, left side of the figure). In contrast, the anti-O-antigen antibody bound equally well to mid-log and stationary phase bacteria (Fig. 2, right side of the figure). This indicated that the differential binding detected with the anti-lipid A antibody was not attributable to loss or reduced expression of O-antigen. These data provided additional evidence that the high susceptibility of logarithmic growth phase bacteria to RegIII β -mediated killing was

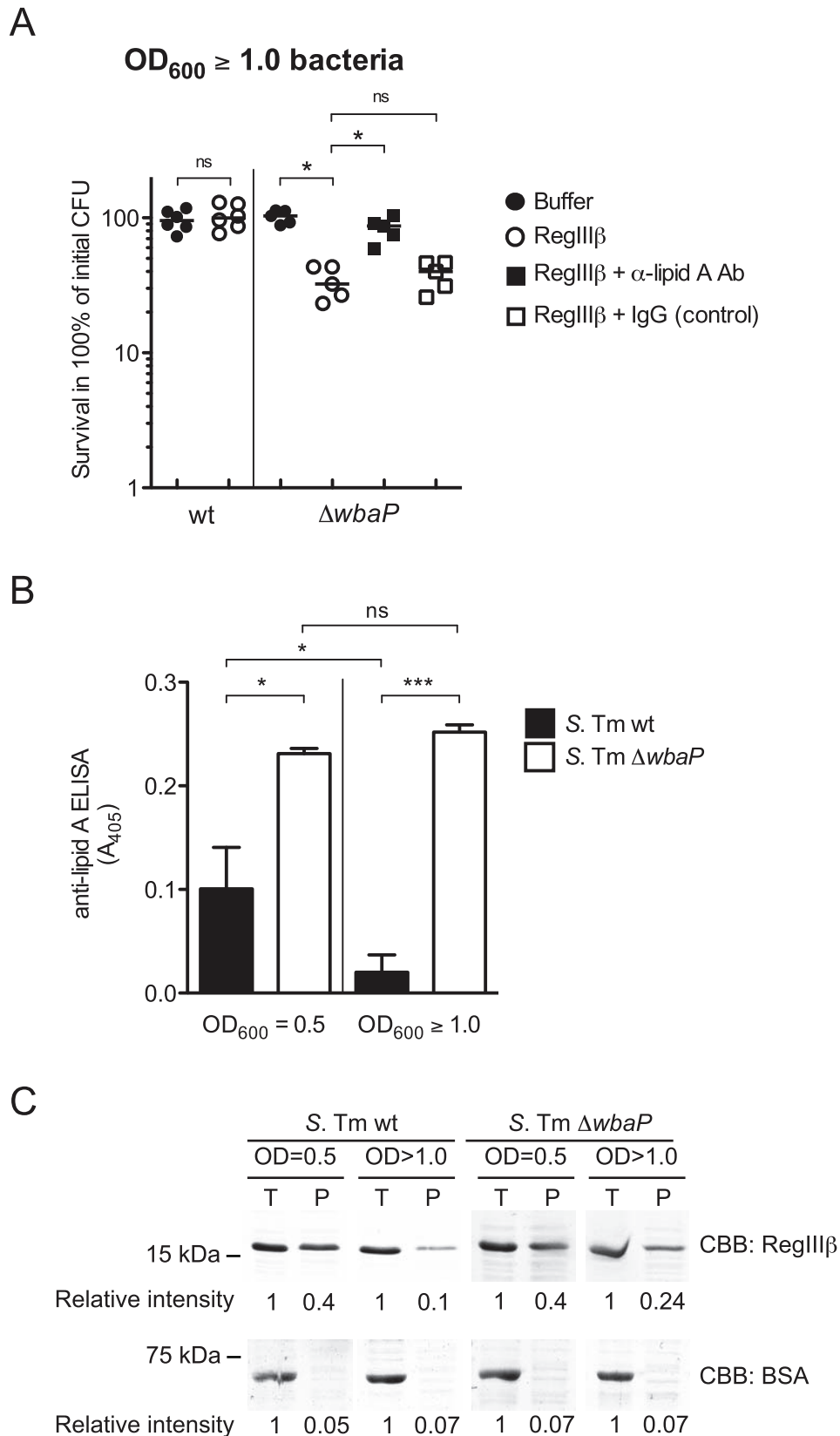


Figure 3. O-antigen deficient *S. Typhimurium* $\Delta wbaP$ is highly RegIII β -susceptible at stationary phase, and displays high amounts of surface-exposed lipid A. A) Inhibition of RegIII β killing. *S. Typhimurium* $\Delta wbaP$ (SK112) was grown to stationary phase and incubated for 10 min at 37°C with 32 μ g per ml of anti-lipid A antibody or mouse IgG. Purified RegIII β (10 μ M) was incubated with the antibody- or IgG-preincubated SK112 strain or untreated control bacteria for 30 min at 37°C. Bacterial survival was quantified by dilution-plating. Assays were performed in at least five independent experiments. The bar shows the median. * $P < 0.05$; ns, not significant (Mann-Whitney U test). B) Amounts of surface-exposed lipid A in *S.*

Typhimurium wild-type strain (*S. Tm wt*) or the O-antigen deficient strain (*S. Tm $\Delta wbaP$*) grown at mid-logarithmic growth phase ($OD_{600} = 0.5$) or at stationary phase ($OD_{600} \geq 1.0$). Surface binding was analyzed by whole-cell ELISA using the anti-lipid A antibody. The error bars represent the SD of the mean from three independent experiments. * $P < 0.05$; *** $P < 0.001$; ns, not significant (unpaired Student's *t*-test). C) Bacterial binding assay. Wild type *S. Typhimurium* or the O-antigen deficient strain (*S. Tm $\Delta wbaP$* ; 1×10^7 cfu) were grown up to the mid-logarithmic growth phase or the stationary phase, and then incubated with RegIII β (10 μ g) or BSA (10 μ g) at 37°C for 15 min (*T*, total fraction: 50 μ l). The RegIII β bound or unbound to bacteria was separated by centrifugation (*P*, pellet), and then analyzed by SDS-PAGE and CBB staining. The band intensity was calculated using ImageJ software by defining the relative intensity of the total fraction (*T*) as "1".
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indeed attributable to increased amounts of surface-exposed lipid A.

The O-specific Polysaccharide Inhibits the Access of RegIII β to Surface-exposed Lipid A

Our previous data have established that the O-specific polysaccharide (O-antigen)-deficient *S. Typhimurium* ($\Delta wbaP$ mutant) is susceptible to RegIII β -mediated killing even when bacteria are taken from the stationary phase [8]. Considering the data presented above, this suggested that the susceptibility of $\Delta wbaP$ mutant might be explained by the lack of O-antigen mediated shielding of the lipid A. First, this was tested in the antibody shielding assay presented in Fig. 1. Indeed, the bactericidal effect of RegIII β against the $\Delta wbaP$ mutant was inhibited significantly upon preincubation with the anti-lipid A antibody, but not with the control IgG (Fig. 3A). These results confirmed that binding to surface-exposed lipid A is an essential step of RegIII β -mediated killing of *S. Typhimurium* $\Delta wbaP$ taken from the stationary.

In order to substantiate this, we compared the amount of surface-exposed lipid A between wt *S. Typhimurium* and the $\Delta wbaP$ mutant using the whole-bacterial cell ELISA presented in Fig. 2. Indeed, the $\Delta wbaP$ mutant displayed significantly higher levels of anti-lipid A antibody-binding than the wt strain. This was true for bacteria from the logarithmic growth phase and from the stationary phase (Fig. 3B). Furthermore, we observed that the $wbaP$ mutant bound significantly higher amounts of RegIII β , but not BSA as a control, i.e. in the stationary phase, as shown in the

bacterial pull-down assay (Fig. 3C). This was in line with our hypothesis that the levels of surface exposed lipid A determine the susceptibility of *S. Typhimurium* to RegIII β -mediated killing.

Compromised Outer Membrane Integrity Increases the Susceptibility to RegIII β

To further assess the critical role of outer membrane structure in RegIII β -mediated killing, we investigated whether compromising the outer membrane barrier integrity may increase the susceptibility to RegIII β . Control experiments confirmed that *S. Typhimurium* was completely resistant to the killing by RegIII β when taken from the stationary phase (Fig. 4). However, treatment with sublethal concentrations of Triton X-100 (a mild detergent) or EDTA (a Mg²⁺ chelator known to disrupt barrier of outer membrane) rendered the stationary phase bacteria highly susceptible to RegIII β (Fig. 4). Furthermore, Mg²⁺ does not seem to be essential for RegIII β activity, as RegIII β displayed a pronounced bactericidal activity in the presence of EDTA.

Collectively, these results suggest that the structure of the LPS defines the degree of susceptibility of *S. Typhimurium* against RegIII β -mediated killing. RegIII β binding to the lipid A of the outer membrane seems to be an essential step of the killing mechanism.

RegIII β Reduces the Outer Membrane Barrier Function of *S. Typhimurium*

First, we were testing if RegIII β does indeed affect outer membrane barrier function. To assess outer membrane strength, we performed an ethidium bromide (EtBr) influx assay [19]. EtBr, a DNA-intercalating fluorescent dye, cannot effectively enter *S. Typhimurium*, as long as the outer membrane barrier is intact. However, upon passing the outer membrane, EtBr rapidly traverses the cytoplasmic membrane, enters the bacterial cytosol and binds to intracellular nucleic acids. The latter can be detected by fluorescence spectrometry, as EtBr displays strongly enhanced fluorescence when intercalating with nucleic acids. We also employed polymyxin B (disrupts the outer membrane [20,21]), gentamicin and ciprofloxacin two antibiotics known to kill *S. Typhimurium* but would (most likely) leave the outer membrane intact. BSA served as a negative control. Enhanced fluorescence signal intensities were found with RegIII β and with polymyxin B, whereas gentamicin-, ciprofloxacin-, or BSA-treated cells did not display enhanced signal intensity (Fig. 5A). Plating assays determining the number of colony forming units verified that RegIII β , polymyxin B and the antibiotics displayed the expected antimicrobial activities whereas BSA did not affect bacterial viability (Fig. 5B). Please note that this assay employed 10-fold higher bacterial densities than the other assays employed in our study. This explains why just 50% of the bacteria are indeed killed by RegIII β in the control experiment (Fig. 5B). In conclusion, these data established that RegIII β does indeed permeabilize the outer membrane of *S. Typhimurium*.

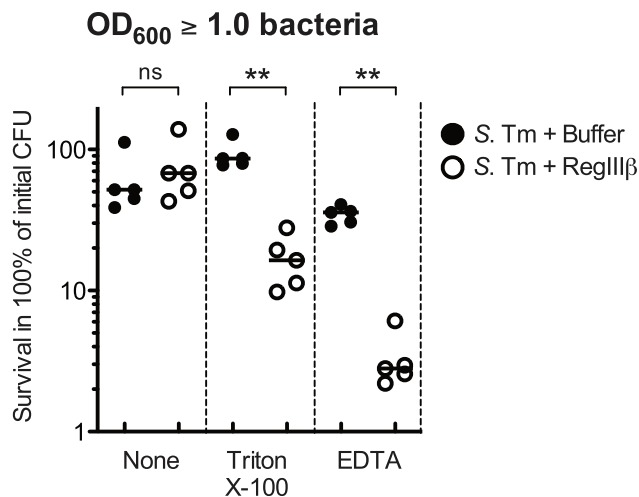


Figure 4. Triton X-100 and EDTA increase the susceptibility to RegIII β -killing of *S. Typhimurium* from the stationary phase. The *S. Typhimurium* wild-type strain (*S. Tm*) was grown to stationary phase ($OD_{600} \geq 1.0$), and coincubated with purified RegIII β (10 μ M) and Triton X-100 (1%) or EDTA (1 mM) for 30 min at 37°C. Bacterial survival was quantified by dilution-plating. Assays were repeated five times. The bars show the median. ** $P < 0.01$; ns, not significant (Mann-Whitney *U* test).
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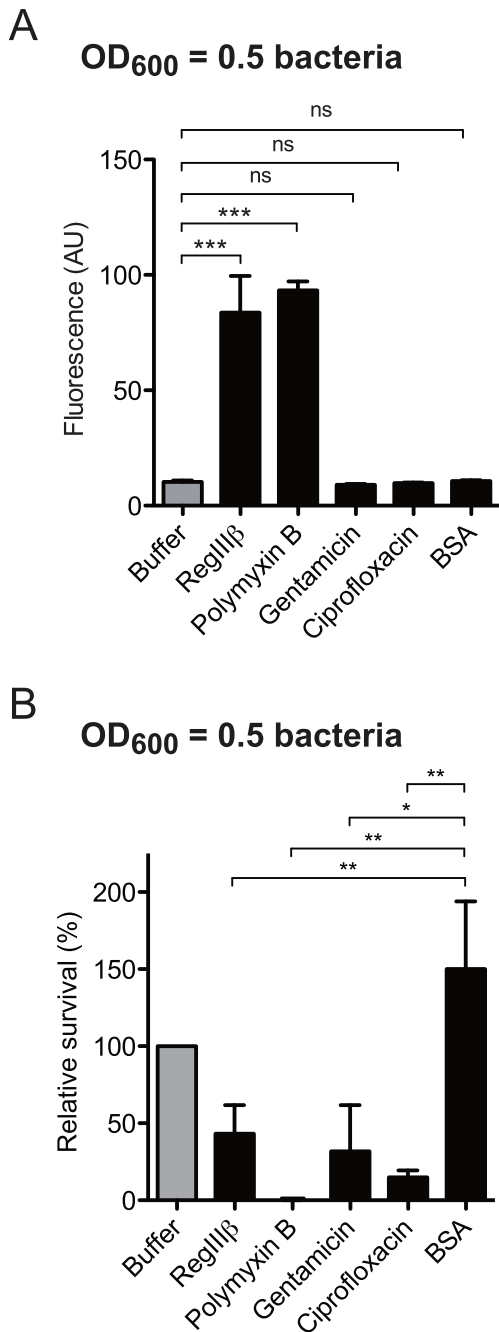


Figure 5. RegIII β permeabilizes the outer membrane. A) The outer membrane permeability of wild-type *S. Typhimurium* from the mid-logarithmic growth phase was measured in the presence of RegIII β (10 μ M), polymyxin B (1 μ g/ml), gentamicin (10 μ g/ml), ciprofloxacin (1 μ g/ml) or BSA (160 μ g/ml) using the ethidium bromide influx assay. The error bars represent the SD of the mean from at least three independent experiments. *** $P < 0.001$; ns, not significant (unpaired Student's *t*-test). B) Activity control of the antimicrobial agents. Bacterial survival (%) of bacteria from the assay shown in (A) was assessed by plating. The data was plotted as 100% of the buffer-treated samples. The error bars represent the SD of the mean from at least three independent experiments. ** $P < 0.01$; *** $P < 0.001$; ns, not significant (unpaired Student's *t*-test).
doi:10.1371/journal.pone.0069901.g005

RegIII β -mediated Membrane Permeabilization Requires the Bacterial Binding of RegIII β

Our previous data have shown that high concentrations of NaCl (more than 75 mM) completely inhibits binding of and killing by RegIII β [15]. Therefore, to investigate whether the bacterial binding is required for membrane permeabilization by RegIII β , we performed the EtBr influx assay in the presence of 100 mM or 25 mM NaCl. High concentrations of NaCl inhibited the RegIII β -mediated membrane permeabilization (Fig. 6A).

Furthermore, we investigated whether RegIII β -mediated membrane permeabilization requires the binding to surface-exposed lipid A. Preincubation with the anti-lipid A antibody, but not control IgG, significantly reduced the fluorescence signal intensity (Fig. 6B). Control assays monitoring bacterial viability verified that the anti-lipid A antibody indeed inhibited RegIII β -mediated killing under the conditions of this assay (Fig. 6C). In conclusion, the binding of RegIII β to surface-exposed lipid A in the outer membrane is essential for outer membrane permeabilization.

The Ability of RegIII β to Permeabilize the Outer Membrane Correlates with the Bactericidal Activity

Next, we examined whether the ability of RegIII β to permeabilize outer membrane correlates with its bactericidal activity. A dose-dependent enhancement of the fluorescence signal intensity was observed upon incubating logarithmic growth phase bacteria with 2.5 μ M, 5 μ M or 10 μ M RegIII β (Fig. 7). Interestingly, sublethal concentrations of RegIII β (2.5 and 5 μ M) were still capable of eliciting detectable outer membrane permeabilization (Fig. 7, green and orange symbols).

The Loop 2 mutants E134Q and N136A of RegIII β , which display attenuated bactericidal activity [15], also displayed reduced permeabilization (Fig. 7). The mutant D142A showed a slightly different effect: it had a detectable bactericidal effect already at a concentration yielding just slightly enhanced outer membrane permeability. The slightly different phenotypes of these two RegIII β mutants might be explained by different capacities to bind to lipid A [15]. I.e. the more pronounced outer membrane permeability of D142A could be attributable to a reduced capacity to bind lipid A [15]. Additional work would have to confirm the significance of this observation. Altogether, these results indicate that the ability of RegIII β to permeabilize the outer membrane correlates with its bactericidal activity (Pearson's correlation coefficient, $P = 0.0001$). Wild type RegIII β seems to permeabilize outer membrane before killing can be detected.

RegIII β -mediated Membrane Permeabilization Sensitizes *S. Typhimurium* to Sublethal Concentrations of Lysozyme or Triton X-100

Effective increase in outer membrane permeability sensitizes Gram-negative bacteria to hydrophobic antibiotics such as erythromycin or bacteriolytic reagents including lysozyme or Triton X-100 that cannot traverse the outer membrane effectively [22,23]. Therefore, we next investigated whether RegIII β sensitizes *S. Typhimurium* to sublethal concentrations of lysozyme or Triton X-100. In logarithmic growth phase *S. Typhimurium*, sublethal concentrations of RegIII β (2.5 μ M) increase the outer membrane permeability slightly (Fig. 7), but did not (yet) affect bacterial viability (Fig. 7 and 8). One hundred microgram per ml of lysozyme or 1% of Triton X-100 alone also had no effect on bacterial viability (Fig. 8). In contrast, co-incubation with RegIII β and lysozyme or Triton X-100 significantly reduced the number of viable bacteria (Fig. 8). These results confirm that sublethal doses of RegIII β can permeabilize the outer membrane and that this

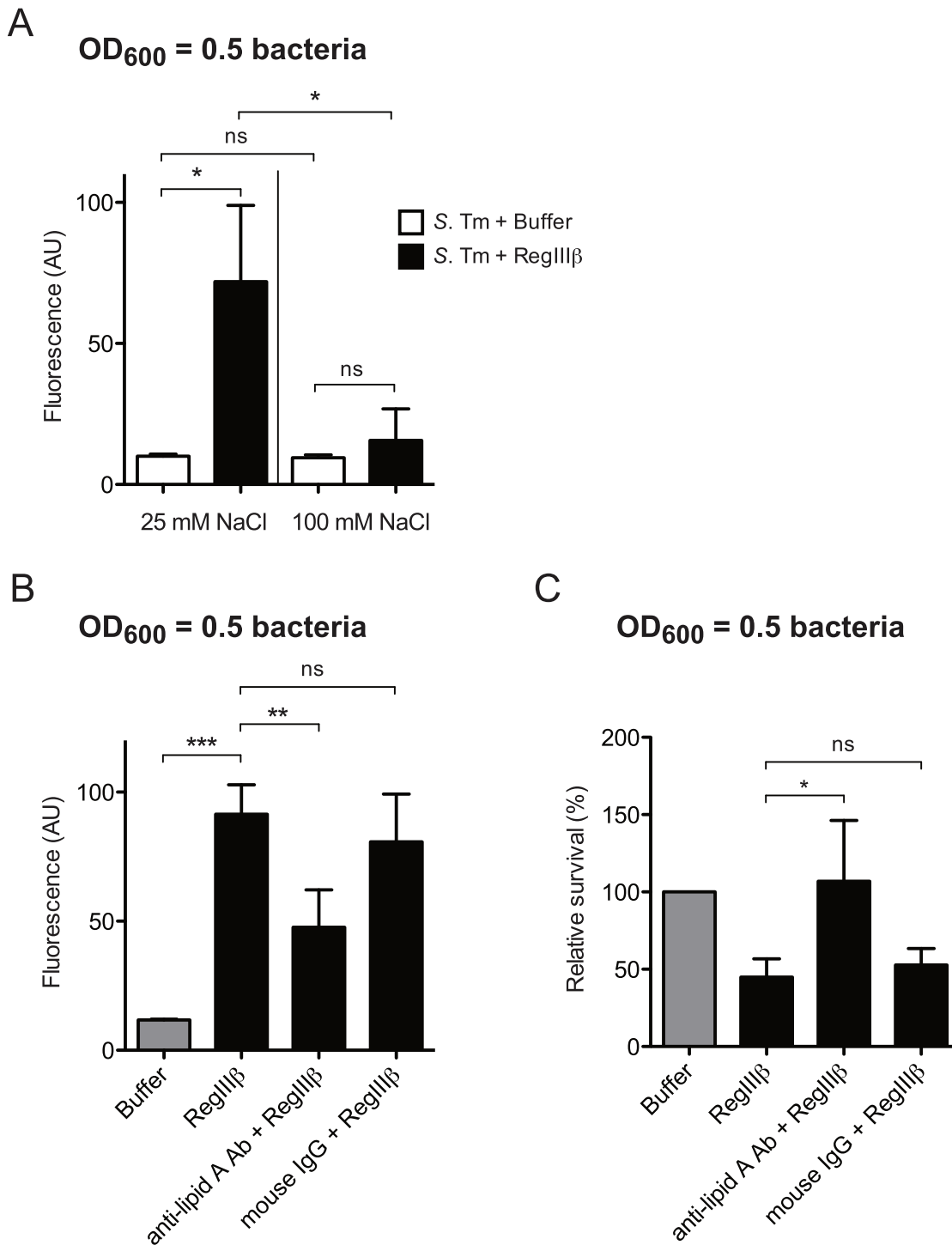


Figure 6. Surface binding is required for RegIII β -mediated outer membrane permeabilization. A) Salt-concentration dependency of RegIII β -mediated membrane permeabilization. Outer membrane permeabilization in wild-type *S. Typhimurium* (*S. Tm*) from the mid-logarithmic growth phase by RegIII β (10 μ M) in the presence of 25 mM or 100 mM sodium chloride. The outer membrane permeability was measured using the ethidium bromide influx assay. The error bars represent the SD of the mean from at least three independent experiments. * $P < 0.05$; ns, not significant (unpaired Student's *t*-test). B) Preincubation with the anti-lipid A antibody inhibits the RegIII β -mediated outer membrane permeabilization. RegIII β (10 μ M)-mediated outer membrane permeability of wild-type *S. Typhimurium* from the mid-logarithmic growth phase preincubated with anti-lipid A antibody (16 μ g/ml) or mouse IgG (16 μ g/ml) was analyzed using the ethidium bromide influx assay. The error bars represent the SD of the mean from at least three independent experiments. ** $P < 0.01$; *** $P < 0.001$; ns, not significant (unpaired Student's *t*-test). C) Control experiment assessing bacterial survival in the ethidium bromide influx assay (B). Relative survival (%) was evaluated as 100% of the buffer-treated samples. The error bars represent the SD of the mean from at least three independent experiments. ** $P < 0.01$; *** $P < 0.001$; ns, not significant (unpaired Student's *t*-test). doi:10.1371/journal.pone.0069901.g006

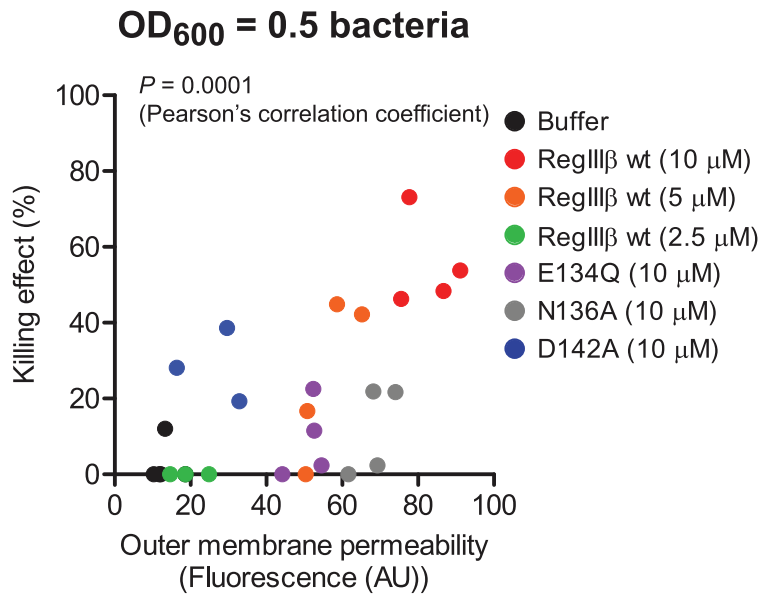


Figure 7. The ability of RegIIIβ to permeabilize outer membrane is correlated with its bactericidal activity. Outer membrane permeability was analyzed using the ethidium bromide influx assay. We analyzed logarithmic growth phase wild-type *S. Typhimurium* incubated with RegIIIβ wt (2.5, or 5, or 10 μM) and the RegIIIβ point mutants E134Q (10 μM), N136A (10 μM) and D142A (10 μM). Killing (%) was analyzed by dilution-plating. The correlation analysis was performed using Pearson's correlation coefficient ($P = 0.0001$). doi:10.1371/journal.pone.0069901.g007

OD₆₀₀ = 0.5 bacteria

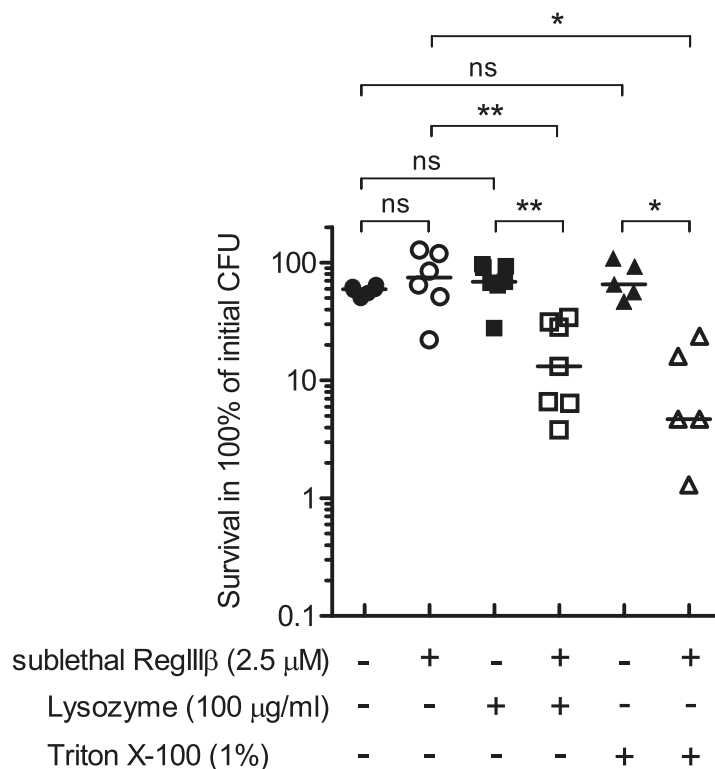


Figure 8. The outer membrane permeabilization by RegIIIβ sensitizes *S. Typhimurium* to lysozyme or Triton X-100. The *in vitro* killing assay was performed using wild-type *S. Typhimurium* from the mid-logarithmic growth phase. Sublethal concentrations of RegIIIβ (2.5 μM), lysozyme (100 μg/ml), Triton X-100 (1%), or the indicated mixtures were incubated with the bacteria for 30 min at 37°C. Bacterial survival was quantified by dilution-plating. Assays were done in at least five independent experiments. The bar shows the median. * $P < 0.05$; ns, not significant (Mann-Whitney *U* test). doi:10.1371/journal.pone.0069901.g008

sensitizes *S. Typhimurium* to detergents like Triton X-100 or bacteriolytic compounds such as lysozyme which interact with periplasmic target sites.

Discussion

We have previously shown that the bactericidal effect of RegIII β depends on the bacterial growth phase. *S. Typhimurium* from the log (not the stationary) phase, was efficiently bound and killed by RegIII β [15]. Competitive binding data established that the lipid A moiety of LPS was one target for RegIII β . Here, we have extended this work by demonstrating that the binding of RegIII β to surface-accessible lipid A is absolutely essential for its bactericidal effect. This surface binding seems to facilitate permeabilization of the outer membrane and represents the initial step of the interaction of RegIII β with Gram-negative bacteria.

Surface accessibility of lipid A may also explain the growth phase dependence of the antimicrobial activity of RegIII β . In the logarithmic growth phase (but not the stationary phase), *S. Typhimurium* displays high amounts of lipid A on its surface as indicated by its increased binding capacity for anti-lipid A antibody (this work) and for RegIII β [15]. Furthermore, a $\Delta wbaP$ mutant which lacks O-specific polysaccharide, is susceptible to RegIII β not only in the logarithmic growth phase but also in the stationary phase. Again, this went along with increased levels of surface-exposed lipid A in both growth phases. Together, these data suggest that the O-side chain provides a steric shield for the lipid A moiety. In wild type *S. Typhimurium*, this shield seems to be less effective in the logarithmic growth phase than in the stationary phase (Figure S2A–B). The $\Delta wbaP$ mutant lacks this shield and is consequently susceptible to RegIII β binding (and killing) in either growth phase (Figure S2C).

Different scenarios are conceivable for explaining why wild type *S. Typhimurium* remains susceptible to RegIII β surface binding during the log phase. First of all, the outer leaflet of the outer membrane is composed not only of LPS, but also harbors significant quantities of membrane proteins, including porins. In logarithmic growth phase bacteria, LPS is thought to account for 73.1% of the outer surface of *S. Typhimurium* [24]. Therefore, it is conceivable that RegIII β could get access to lipid A by passing through the remaining area (26.9%) (Figure S2A). Alternatively, the surface-exposed lipid A might be located at specific sites like the growth zones of the cell wall or the septum at division sites (Figure S2A). These sites are clearly more prominent in the logarithmic growth phase than in the stationary phase. It will be an interesting topic for future work to assess these hypotheses.

In this study, we showed that when stationary phase bacteria (which are normally resistant to RegIII β) were treated with agents compromising outer membrane integrity, i.e. Triton X-100, *S. Typhimurium* becomes highly susceptible to RegIII β -mediated killing. Moreover, Triton X-100 was able to reduce the bactericidal doses of RegIII β which are required to kill *S. Typhimurium* from the log growth phase. Furthermore, this allowed outer membrane passage of lysozyme (14.3 kDa), a small protein of similar size as RegIII β (16 kDa), which can kill Gram-

negative bacteria by enzymatic digestion of the murein sacculus, a structure located within the periplasm. These observations indicated that permeabilizing the outer membrane by RegIII β is an important first step of the bactericidal mechanism of this antimicrobial lectin against wild type *S. Typhimurium*.

It is still unclear how RegIII β triggers bacterial death upon traversal of the outer membrane. The substantial (though reduced) bactericidal dose of RegIII β required to kill Triton X-100 treated bacteria, suggests that this second step requires significant amounts of RegIII β . Our data suggest that this RegIII β must traverse the outer membrane, presumably to reach the bactericidal target site somewhere in the periplasm or the cytoplasmic membrane. In the case of RegIII β , these targets are still unknown. Recently, it has been shown that peptidoglycan recognition proteins (PGRPs) kill Gram-positive and Gram-negative bacteria by activating the stress-control two-component regulatory system (C_{ss}R-C_{ss}S for *Bacillus subtilis* or C_{px}R-C_{px}A for *Escherichia coli*), the accompanying hydroxyl radical production and by membrane depolarization, but not membrane permeabilization [25]. It is tempting to speculate that RegIII γ , which can bind peptidoglycan of Gram-positive bacteria as well as PGRPs, has the same killing mechanism as PGRPs. However, it remains to be elucidated whether this holds true for RegIII β -mediated killing of *S. Typhimurium*.

In conclusion, the bactericidal lectin RegIII β seems to kill Gram-negative bacteria by a mechanism involving (at least) two distinct steps. In the first step, RegIII β binds to surface-exposed lipid A and permeabilizes the outer membrane. The O-antigen confers some protection against this binding-step. The second, bactericidal step seems to be distinct from the initial outer membrane-interaction and awaits further analysis. Our findings advance the mechanistic understanding of the antimicrobial activity of RegIII-family lectins and prepare the ground for analysis of the second, bactericidal step.

Supporting Information

Figure S1
(EPS)

Figure S2
(EPS)

File S1
(DOCX)

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Author Contributions

Conceived and designed the experiments: TM WDH. Performed the experiments: TM. Analyzed the data: TM WDH. Contributed reagents/materials/analysis tools: WDH. Wrote the paper: TM WDH.

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