The Staphylococcus aureus Peptidoglycan Protects Mice against the Pathogen and Eradicates Experimentally Induced Infection

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Abstract

Staphylococcus aureus, in spite of antibiotics, is still a major human pathogen causing a wide range of infections. The present study describes the new vaccine A170PG, a peptidoglycan-based vaccine. In a mouse model of infection, A170PG protects mice against a lethal dose of S. aureus. Protection lasts at least 40 weeks and correlates with increased survival and reduced colonization. Protection extends into drug-resistant (MRSA or VISA) and genetically diverse clinical strains. The vaccine is effective when administered - in a single dose and without adjuvant - by the intramuscular, intravenous or the aerosol routes and induces active as well as passive immunization. Of note, A170PG also displays therapeutic activity, eradicating staphylococci, even when infection is systemic. Sustained antibacterial activity and induction of a strong and rapid anti-inflammatory response are the mechanisms conferring therapeutic efficacy to A170PG.

Introduction

S. aureus has developed a successful strategy of evading the human immune system, entailing the accumulation of an impressive array of virulence factors. It can colonize several niches of the human body and cause life-threatening diseases, such as pneumonia, osteomyelitis, septicemia and endocarditis. S. aureus infects immunocompromised patients as well as patients without apparent risk factors [1]. About 30% of the human population carries S. aureus [2], a circumstance testifying the ability of this pathogen to modulate its virulence and to colonize the human host.

Strain differences in the resistance to S. aureus infection have been demonstrated in mice [3]. There is suggestive evidence that individual differences in the defence against S. aureus might also exist in humans. A polymorphism in the TIR domain of the human TLR2 gene has been reported to be associated with S. aureus susceptibility [4], although this association was not confirmed in a later study [5]. In addition to the capacity to cause diverse and serious diseases, S. aureus also displays an extraordinary potential to develop antimicrobial resistance [6]. The last decade has witnessed the emergence and rapid spread of community-associated and antibiotic-resistant strains [1]. In this context, the development of an effective vaccine appears particularly urgent.

The present article exploits the use of S. aureus peptidoglycan (PG) as a potential vaccine. PG, a linear polymer of repeating -1-4-linked N-acetylmuramic acid and N-acetylgalactosamine, accounts for approximately 50% in weight of the cell wall of Gram-positive bacteria, enabling them to resist osmotic pressure [7]. The S. aureus PG is recognized by the host nucleotide-binding oligomerization domain (Nod) 1 and Nod 2 [7] intracellular receptors and is involved in the activation of the complement [8], cell-mediated immunity [9] and opsonization [8]. PG is present on all the S. aureus bacterial strains, is exposed on the cell wall and can thus sense the external environment [8]. These features make it attractive as a vaccine.

This paper presents evidence that the PG-based vaccine A170PG, administered by the intramuscular, intravenous or aerosol routes in a single dose and without adjuvant, protects mice against an otherwise lethal dose of S. aureus. Protection lasts at least 40 weeks and extends to S. epidermidis and L. monocytogenes. The serum from vaccinated mice protects naive mice against a lethal dose of S. aureus. Remarkably, the vaccine, administered to already infected mice, eradicates infection in one month.

Results

Peptidoglycan protects mice against S. aureus

The cell wall (CW) from the S. aureus strain A170 was extracted with trichloroacetic acid [10] and preliminarily purified with concanavalin A (ConA) -Agarose. Two groups of mice were then immunized with the ConA negative (ConA-CW) or the ConA positive (ConA+CW) fraction of the CW, respectively. Two weeks later the mice were challenged with an otherwise lethal dose of S. aureus A170. The mice immunized with the ConA+CW fraction survived all (10/10), while those immunized with the ConA+CW fraction died all (10/10) (Table 1; experiment 1; P: <0.0001).
that PG is the component affording protection.

In experiment 2; P:

The serum from mice immunized with the ConA CW fraction was shown to react with the homologous antigen ConA CW (as expected), with PG, but not with LTA (Figure 1, panel A). The test provided preliminary evidence that PG, but not LTA, is present in the ConA CW preparation. The absence of LTA is not surprising since presumably removed by the trichloroacetic acid used to extract the CW [11].

The serum from mice immunized with the ConA CW preparation protects naive mice from a lethal dose of A170. The same serum lost this property if first absorbed with PG (Table 1; experiment 1; P: <0.00001).

Mindful of drawbacks often associated with S. aureus CW purification by chemical methods [11,12], the identification of the ConA CW component protecting mice against S. aureus was pursued by serological and enzymatic methods. Using as antigens the ConA CW fraction, peptidoglycan (PG) and lipoteichoic acid (LTA) from S. aureus, the serum from mice immunized with the ConA CW preparation was shown to react with the homologous antigen ConA CW [11,13]. The test provided preliminary evidence that PG, but not LTA, is present in the ConA CW preparation. The absence of LTA is not surprising since presumably removed by the trichloroacetic acid used to extract the CW [11].

The serum from mice immunized with the ConA CW preparation protects naive mice from a lethal dose of A170. The same serum lost this property if first absorbed with PG (Table 1; experiment 2; P: <0.00001). This result is the first line of evidence that PG is the component affording protection.

The ConA CW preparation was digested with lysozyme or lysostaphin. The enzyme-digested preparations were then tested to see whether they conserved the capacity to protect mice challenged with a lethal dose of the A170 strain. Digestion with lysozyme or lysostaphin destroyed the ability of ConA CW to immunize mice (Table 1; experiment 3; P: 1). Given that these enzymes both hydrolyze specifically the PG [11,13], the result provides further evidence that PG is indeed the ConA CW component conferring protection against S. aureus.

The S. aureus strain A170 was grown in the presence of penicillin G (5 μg/ml). When the culture reached the stationary phase, the soluble PG (sPG) was isolated from the medium by ConA-Sepharose affinity chromatography, tested for reactivity with ConA CW antibodies (Figure 1, panel B) and used to immunize mice. Challenge was performed two weeks later with a lethal dose of A170, the immunized mice survived all (10/10) (Table 1; experiment 4; P: <0.0001). Since sPG is deprived of any CW component [11], the experiment provides compelling evidence that the protection observed in this experiment is afforded by PG.

Pentaglycine (Gl5) is an epitope unique to staphylococcal PG [14,15] while muramic acid (MA) and ribitol (RBT) are monosaccharides unique to PG and teichoic acid (TA), respectively [11,15]. Gly5, MA and RBT were therefore ideal reagents to confirm the presence of PG and the absence of TA in the ConA CW preparation. In a direct ELISA assay, Gly5 and MA bound to the ConA CW antibodies, while RBT did not (Figure 1, panel B); similarly, in a competition test only Gly5 and MA, along with PG, inhibited the binding of ConA CW antibodies to the homologous antigen (Figure 1, panel C).

Taken together, the above experiments offer stringent and independent lines of evidence that the component of ConA CW conferring protection against S. aureus infection corresponds to the PG; this component will be referred to as A170PG.

### Table 1. PG is the protective component of the ConA CW fraction.

| Exp | Dose/mouse | Treatment | Interval Treatment-challenge: weeks (w)/ hours (h) | Challenge* | Pathogen | Survival | P-valueb |
|-----|------------|-----------|-----------------------------------------------|------------|----------|---------|----------|
| 1   | 15 μg      | ConA CW   | 2 w                                           | A170       | 10/10    | <0.0001 |          |
|     | 15 μg      | Heat-treated ConA CW | 2 w | A170       | 10/10    | <0.0001 |          |
| 2   | 10 μl      | Non-absorbed ConA CW Antibodies | 24 h | A170       | 10/10    | <0.0001 |          |
|     | 15 μg      | PG-Absorbed ConA CW Antibodies | 24 h | A170       | 10/10    | <0.0001 |          |
| 3   | 15 μg      | Lysozyme-Digested ConA CW | 2 w | A170       | 10/10    | 1       |          |
| 4   | 15 μg      | Lysostaphin-Digested ConA CW | 2 w | A170       | 10/10    | <0.0001 |          |
| 5   | 15 μg      | sPG       | 2 w                                           | A170       | 10/10    | <0.0001 |          |

*10⁶ CFU/mouse.

bKaplan-Meier test.

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A170PG prevents S. aureus infection

Protection experiments with A170PG were extended to a larger number of animals. To present the results more effectively, the details of the experiments described in this and the following sections are shown in the relevant tables or figure legends. Mice were immunized with A170PG and two weeks later infected with a lethal dose of S. aureus A170. At the time of infection, the antibody titre against A170PG was 10⁻¹⁻⁵. The vaccinated mice survived (20/20), while control (non-vaccinated) mice died (20/20) within 4–5 days (Table 2; experiment 1; P: <0.0001). Four days after challenge, the kidneys of the A170PG-treated animals displayed significantly fewer CFU (log₁₀ of CFU number: 4.7 ± 3.6; P: <0.0001) compared to the kidneys of the control mice. Inspected four weeks after challenge, the kidneys of the vaccinated mice were sterile (Table 2; experiment 1–2). When the interval between vaccination and challenge was extended to 40 weeks, A170PG was still protective (Table 2; experiment 2; P: <0.0001).

Next experiments were aimed at optimizing the dose of the vaccine. The smallest dose of A170PG tested – 3 μg/mouse – was fully protective and safe, even when administered intravenously (Table 3; experiments 1 and 2; P: <0.0001; Figure S2). The experiments to be described were all carried out using the dose of 5 μg/mouse.
A170PG is broadly protective

The efficacy of the vaccine was also tested against 28 heterologous strains of *S. aureus*, 26 of which derived from patients with staphylococcal infections. Strains represent different genotypes, as assessed by several approaches (Table S1). Mice were vaccinated intramuscularly with 3 μg of A170PG and two weeks later were infected by the same route with a lethal dose of one of the bacterial strains. The difference in survival between vaccinated and control mice at four weeks from challenge was statistically significant in each case (Table S2). At time of death (4–5 days from infection) the kidney colonization levels among control mice ranged from 7.1 ± 6.6 to 7.8 ± 6.1 (log10 of CFU number). At four weeks from challenge the kidneys of vaccinated mice were sterile. Remarkably, the vaccine was effective against the methicillin-resistant (MRSA) strains A174, A175, RIMD31092 and the vancomycin-intermediate (VISA) strain A176 (Table S2). A170PG also protected mice against the challenge with a lethal dose of the Gram-positive bacteria *S. epidermidis* or *L. monocytogenes* (surviving mice: 10/10) (Table S2), a result concurring with the broad cross-reaction displayed by the PG preparations from *S. aureus, S. epidermidis* and *L. monocytogenes* (Figure S1) and the evidence that PG is highly conserved among Gram positive bacteria [16].

The A170PG antibodies protect against *S. aureus* infection in vivo

Two groups of mice were injected intramuscularly with protein A-purified Ig from normal mouse serum or from the serum of mice immunized with A170PG (10 μl at 50 μg/ml diluted 1:10 with sterile saline/mouse). The next day, the mice were challenged with a lethal dose of *S. aureus* A170. Survival was monitored for seven days. In two independent experiments, the serum from mice immunized with A170PG antibodies protected mice from death (20/20) (Table 4; experiments 1–2; P: <0.0001). The same serum—given at the dose of 20 μl/mouse - killed 20 out of 20 mice (Table 4; experiments 3–4; P: 1). Absorption of A170PG antibodies (10 μl at 50 μg/ml diluted 1:10 with saline) with rat anti-total mouse immunoglobulin (5 μl at 100 μg/ml) or with A170PG (5 μl at 100 μg/ml) abrogated the protective effect of the

Table 2. A170PG, administered by the intramuscular route at 15 μg/mouse, protects mice against *S. aureus* infection.

| Exp | Vaccination | Interval vaccination/challenge (weeks) | Challenge dose (CFU/mouse) | Pathogen | Survival | P- value a | CFU/g kidneys | (mean ± SD) log10 |
|-----|-------------|---------------------------------------|---------------------------|----------|----------|------------|--------------|----------------|
| 1   | A170PG      | 15                                    | 2                         | 10⁸      | A170     | 20/20      | <0.0001      | 0 ± 2         |
|     |             |                                       |                           | 10⁸      | A170     | 0/20       | 7.4 ± 0.7    |               |
| 2   | A170PG      | 15                                    | 40                        | 10⁸      | A170     | 20/20      | <0.0001      | 0 ± 2         |
|     |             |                                       |                           | 10⁸      | A170     | 0/20       | 7.3 ± 6.7    |               |

aKaplan-Meier test.
bCFU counted at 4 weeks from challenge.
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serum (Table 4; experiments 5–6; P: 1). The above experiments demonstrate that protection afforded by the A170PG antibodies is specific (is removed by rat anti total mouse immunoglobulin or the homologous antigen); second, A170PG antibodies can be protective or detrimental, depending upon the dose being given.

The A170PG vaccine eradicates lung infections

*S. aureus* is one of the most common causes of pneumonia [17] and one of the bacterial pathogens most frequently detected in the respiratory secretions of patients with cystic fibrosis [18]. To explore the therapeutic potential of A170PG against lung infection, groups of mice were infected via aerosol with the A170 bacteria. Further, to ascertain whether the bacterial load influenced the result, three different infection regimens were used (10^6, 10^7 or 10^8 CFU/mouse). When visibly sick (3–14 days from infection, depending upon the infection regimen used), the mice were treated with A170PG via aerosol (3 µg/mouse). The difference in survival between treated and untreated groups was statistically significant in each case (Table 5; P: 0.0001 or <0.0001). Remarkably, the lung colonization level was greatly reduced at one week after treatment and infection eradicated in 30 days (Table 5); by this time, in addition to the lungs, also the liver, kidneys and spleen of the treated mice were free from *S. aureus* (Table S3).

Protective mechanisms elicited by the A170PG vaccine

Vaccines generally work by inducing antibodies with bactericidal activity, triggering a secondary response and preventing the inflammation that follows infection. The A170 bacteria were incubated (2 h at 37°C) with (a) anti-A170WTA purified antibodies alone, (b) complement alone or (c) with both antibodies and complement. Bacteria were then plated and the CFU counted. Antibodies alone exerted a bactericidal activity significantly greater than that of the complement; when antibodies and complement were used in combination, complement did not enhance the bactericidal activity of the antibodies (Figure 2). Also,
mice which received a boost dose of vaccine at 40-week distance from the first, displayed the main features of the vaccine-induced B cell memory: switch from the IgM to the IgG or IgA isotypes and rapid antibody production (Figure 3). Mice, vaccinated and 2 weeks later infected, displayed a cytokine profile very similar to that of naive mice; instead, the mice infected without being first vaccinated displayed significantly higher levels of the pro-inflammatory cytokines TNF-α (P: 0.0001) and IFN-γ (P: 0.01) compared to the levels of the vaccinated mice (Figure 4). Finally, *S. aureus* colonized lungs or kidneys from non-vaccinated mice, while was unable to colonize the same organs from vaccinated mice (Figure 5).

The therapeutic activity of A170PG was also characterized by a rapid reduction of the bacterial load and inflammation level of treated mice. Mice, infected and 72 h later treated with A170PG, returned free of *S. aureus* within 30 days from treatment (Table 5; Table S3) and displayed significantly lower transcription levels of the cytokines TNF-α (P: <0.0001) and IFN-γ (P: 0.03) compared to the mice which were infected, but not treated (Figure 4).

### Table 5. Aerosol administration of A170PG vaccine (3 μg/mouse) eradicates the *S. aureus* lung infection.

| Mice | Infection regimen | Interval infection treatment (days) | Colonization level (CFU±SD; log_{10}) at time of: | Colonization level (CFU±SD; log_{10}) at indicated days from treatment | Survival | P-value *
|------|------------------|----------------------------------|-----------------------------------------------|------------------------------------------------|----------|---------|
|      |                  |                                  | Infection b | Treatment b | Death b | 7 | 14 | 30 |
| Treated | 10⁸             | 3                               | 5.3± 5.0 | 6.8± 6.4 | 4.7± 3.3 | 2.6± 1.6 | 0 | 9/10 | 0.0001 |
| Untreated | 10⁸             |                                  | 5.4± 5.1 | 7.2± 5.8 | 0 | 0/10 |
| Treated | 10⁷             | 9                               | 4.5± 4.3 | 6.7± 6.3 | 4.7± 3.6 | 2.3± 1.5 | 0 | 10/10 | <0.0001 |
| Untreated | 10⁷             |                                  | 4.4± 4.2 | 7.3± 6.1 | 0 | 0/10 |
| Treated | 10⁶             | 14                              | 3.7± 3.3 | 6.8± 5.6 | 4.6± 3.5 | 2.3± 1.7 | 0 | 10/10 | <0.0001 |
| Untreated | 10⁶             |                                  | 3.8± 3.2 | 7.2± 5.3 | 0 | 0/10 |

*Values calculated on 3 mice.

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**Figure 2.** A170PG antibodies display strong bactericidal activity also in the absence of complement. A170 bacteria (10⁷ CFU/100 μl TSB medium) were incubated (1 h, 37°C) alone, with complement (5 μl), with purified A170PG antibodies (5 μl), or with complement (5 μl) and antibodies (5 μl). Bacteria were cultured on agar plates and CFU counted. Each histogram represents the results (mean±SD) of 3 independent experiments. doi:10.1371/journal.pone.0028377.g002
Discussion

In a mouse model of infection, the A170PG vaccine protects mice against a lethal dose of *S. aureus*. Protection correlates with increased survival and reduced colonization. It lasts at least 40 weeks (Table 2) and extends into genetically diverse (Tables S1) and drug-resistant (MRSA or VISA) (Table S2) clinical strains. The vaccine is effective when administered - in a single dose and without adjuvant - by the intramuscular, intravenous (Table 3) or aerosol (Table 5) routes. Significantly, A170PG eradicates the pathogen by the time infection has become systemic (Table S3).

The vaccine induces active as well as passive immunization: adoptively transferred A170PG antibodies protect mice against a lethal *S. aureus* challenge (Table 4).

Several and independent experiments critically demonstrate that the biologically active component of the vaccine is the PG. First, the serum from vaccinated mice protects naïve mice from a lethal dose of A170; however, this property is lost if the serum is absorbed with PG (Table 1, experiment 2). Second, the vaccine loses the capacity to protect mice if first digested with lysozyme or lysostaphin, enzymes which hydrolyze specifically the PG (Table 1, experiment 3). Third, grown in the presence of penicillin G (which inhibits the PG incorporation into the CW), the PG accumulates in the culture medium in a soluble form (sPG) deprived of any CW component [11]; the sPG from the A170 strain effectively protects mice against *S. aureus* (Table 1, experiment 4). Fourth, the serum from mice vaccinated with A170PG recognizes two components unique of the PG: Gl5 and MA (Figure 1, panel B).

The results described in this study, while promising, require a critical analysis in the light of the current literature. The mouse strain used in this study (Balb/c) is highly susceptible to *S. aureus* [3]. Still, large bacterial doses are needed for infection. The model therefore does not mimic closely human staphylococcal infections, which are generally caused by a small initial inoculum. Second, unlike humans, the mouse does not have pre-existing antibodies to *S. aureus* [19]. Therefore, one must contemplate the possibility that A170PG - which works well in mice - might not work equally well in humans since it could be neutralized by pre-existing antibodies. More important, there is evidence that staphylococcal escape strategies from immune defence mechanisms are by far more efficient in humans than in mice [20].

PG shows limited serological variability [16] (Figure S1), confirmed by the capacity of A170PG to protect against *S. aureus*, *S. epidermidis* and *L. monocytogenes* (Table S2). PG thus represents an attractive choice as vaccine. However, previous single component vaccines have given disappointing results in humans [19]. StaphVAX (a capsular polysaccharide vaccine) in the confirmatory phase III clinical trial offered no significant protection against *S. aureus*, compared to the control group [19]. Thus, the efficacy of
A170PG as single component vaccine in humans remains to be confirmed by clinical testing.

Some categories of patients (such as premature neonates and haemodialysis patients), at high risk of staphylococcal infections, do not respond adequately to immunization. These patients benefit particularly from passive immunoprophylaxis. A170PG antibodies provide passive protection, at least in mice (Table 4). In vivo, A170PG antibodies display prozone effect (see below), a property that could not be anticipated on the basis of their functions in vitro and potentially relevant to passive immunoprophylaxis against *S. aureus*. Purified A170PG antibodies were protective only when administered within a restricted range: the dose of 10 µl/mouse rescued all mice (20/20) from a lethal dose of A170 bacteria (Table 4; experiments 1 and 2); the dose of 20 µl/mouse of the same preparation instead killed all (20/20) the mice (Table 4, experiments 3 and 4). Incidentally, the observation that antibodies are not protective if used in excess is not new and in the literature the phenomenon is described with the term of “prozone effect” [21].

In a trial with AltaStaph (a pool of sera from volunteers immunized with StaphVAX), 24% of the patients treated with AltaStaph and standard therapy died, compared to 11% of those treated with the standard therapy alone [19]. The result was interpreted as evidence of the limited use of the antibodies against capsular polysaccharides [19]. The data reported above make it plausible also that failure of AltaStaph might have been caused by the use of an excess of antibodies (and concurrent prozone effect). More in general, the prozone effect perhaps deserves careful consideration when interpreting passive protection experiments.

A170PG exerts its prophylactic activity by inducing antibodies able to kill bacteria (Figure 2), inhibit organ colonization (Figure 5), evoke B cell memory (Figure 3) and an anti-inflammatory response (Figure 4). A170PG has also therapeutic capacity. The vaccine eradicates infection (even when systemic) with surprising efficacy and rapidity (Table S3; Table 5). One single dose of vaccine administered via aerosol to mice previously infected with a lethal dose of A170 rescued all the mice, while control (infected but not treated) mice all died (Table 5). In these experiments, mice were treated with A170PG when severely sick. Still, treated mice all eradicated the staphylococci within one month from treatment (Table S3; Table 5). In addition, A170PG induced also a strong anti-inflammatory response (Figure 4). While these results are

**Figure 5. A170PG vaccination inhibits mouse organ colonization by *S. aureus*.** Bacteria colonized lungs and kidneys from non-vaccinated mice, but not the same organs from vaccinated mice. Sections from vaccinated and non-vaccinated mice were incubated (2 h) with Dapi (10 µl at 0.5 µg/ml), fluorescein isothiocyanate (10 µl at 0.5 µg/ml) or neither.

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robust evidence of the vaccine’s therapeutic potential, the mechanisms responsible for the therapeutic efficacy remain conjectural. Monocytes and macrophages are the main source of pro-inflammatory cytokines [16]. At the same time they play a major role also in the antibacterial response [22,23]. This study shows that macrophage exposure to A170PG suppresses the production of pro-inflammatory cytokines (Figure 4), but leaves the antibacterial activity intact, which culminates in the eradication of the pathogen (Table S3; Table 3). Though seemingly contradictory, these results become consistent if one assumes that PG can induce macrophage tolerance, much as LPS does [22]. Exposure to LPS inactivates the macrophage pro-inflammatory genes, while leaving inducible the antibacterial response genes; this permits the avoidance of excessive inflammation and septic shock while fighting pathogens [22]. Here it is proposed that the same might occur in the case of macrophages exposure to PG. Phrased another way, PG would dampen inflammation in the case of Gram-positive infections, just as LPS does in the case of Gram-negative infections. This hypothesis – though to be confirmed by future studies - is attractive also because it would explain why PG - a known bacterial virulence factor [8,9] - is not harmful when used as vaccine: an excess of PG, as in this study (3–15 μg/mouse), it would induce desensitization of the downstream inflammation signalling pathways.

In conclusion, A170PG potentially can find application in the prophylaxis as well as the therapy of staphylococcal infections, a rare property for a vaccine. At the same time, one must prudently consider that A170PG has been tested in a mouse model and the mouse is not a natural host for S. aureus. Whether this seemingly promising vaccine will protect humans can only emerge from clinical trial.

Materials and Methods

Ethics Statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the European Community. The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of Naples (Permit Number: 86/609/EEC). All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

Bacteria

The sources of the Staphylococcus aureus strains used in the study are shown in Table S1. The Staphylococcus epidermidis and Listeria monocytogenes strains were collected from patients hospitalized at the Medical School of the University of Naples. Molecular characterization of the S. aureus strains included analysis of the rDNA V3 spacer region [24], capsular polysaccharide [25], enterotoxin genes [26,27], ScmA 1 restriction endonuclease analysis [28] and protein A gene (spa) typing [29]. Clinical samples were streaked on trypticase soy agar (TSA) (Oxoid, Milan, Italy) and single colonies expanded in trypticase soy broth (TSB) (Oxoid). For in vivo and in vitro experiments, bacteria were grown in TSB at 37°C, harvested while in the exponential growth phase (ODs600 1.5 to 1.8), centrifuged (8 x 10^5 x g for 10 min) and washed with saline (0.15 M NaCl). Serial 10-fold dilutions in saline were then plated on TSA. To isolate the soluble form of the peptidoglycan (sPG), the S. aureus strain A170 bacteria was grown in TSB in the presence of penicillin G (Biopharma, Italy; 5 μg/ml). When the culture was in the exponential growth phase, the sPG was isolated from the medium by ConA-Agarose affinity chromatography.

ConA-Agarose affinity chromatography

CW was extracted from S. aureus, S. epidermidis or L. monocytogenes with trichloroacetic acid [10] and further purified with concanavalin A conjugated to agarose (ConA-Agarose; Sigma, Milan, Italy). The lectin suspension (500 μl) was centrifuged, washed with Tris-HCl buffer and the pellet suspended in 500 μl of the same buffer. CW (2.8 mg in 5 ml Tris-HCl buffer pH 7.5) was mixed with the ConA-Agarose, incubated for 1 h, centrifuged and the supernatant (ConA fraction) recovered. The pellet was washed three times with Tris-HCl buffer and the CW eluted from the matrix with 5 ml of 0.2 M α-methyl-diglycoside (Sigma) (ConA-CW fraction). The ConA-CW and ConA^+CW fractions were desalted (Sephadex G-25), lyophilized, weighed and tested separately for the capacity to protect mice.

Mice

Experiments were carried out on female Balb/c mice (aged 8 to 10 weeks) at the animal facility of the University of Naples. Mice infection and vaccination were carried out via the intramuscular, intravenous or aerosol routes. For intramuscular or intravenous infection, individual mice were injected with 10⁷ colony forming units (CFU)/100 μl saline. For aerosol infection, five mice were placed in a nebulizing chamber and exposed to the aerosol bacterial suspension (10⁶ to 10⁸ CFU/100 μl saline). The ConA-CW fraction from the S. aureus strains A170 was administered intramuscularly (3–15 μg in 50 μl/mouse) or by aerosol (3–15 μg/50 μl saline/mouse). Organs were dissected and weighed. Samples were homogenized in 1 ml saline and serially diluted in the same medium. CFU were evaluated by the plate count assay and expressed as CFU/g.

Detection of ConA^±CW antibodies

The wells of a 96-well plate (Falcon, Milan) were coated with the antigen being tested: muramic acid (MA), pentaglycine (Gly5), ribitol (RBT), lipoteichoic acid (LTA) (all from Sigma); ConA^+ or ConA fractions of the CW from S. aureus, S. epidermidis or L. monocytogenes; soluble peptidoglycan (sPG). Antigens were used at 0.1–15 μg in 50 μl/well. The plate was quenched with 3% bovine serum albumin (50 μl/well; 2 h), washed with PBS, incubated overnight with purified antibodies from mice immunized with ConA-CW (diluted 10⁻² to 10⁻⁴; 50 μl/well). Antibodies were purified by protein A affinity chromatography [30] and brought to 50 mg/ml. Plates were washed with PBS and incubated, in succession, with peroxidase-labelled secondary antibodies (rat anti total mouse immunoglobulin, rat anti mouse-IgG, -IgM or -IgA diluted 10⁻² to 10⁻⁵; 50 μl/well; 2 h, Sigma) and peroxidase substrate (100 μl/well; 1 h; Bio-Rad, Milan). Optical density (OD) was measured at 600 nm. Samples were run in triplicate.

Other methods

To test the biological activity of the A170PG, S. aureus A170 bacteria (10⁷ CFU/100 μl saline) were incubated (1 h at 37°C) with 5 μl of A170PG purified antibodies or with 5 μl of the same antibodies plus 5 μl of complement (BioMerieux, France). CFU were evaluated by the plate count assay. ConA-CW (0.28 mg in 500 μl Tris-HCl pH7.5) was digested with lysozyme (Sigma; 220 U/ml; 2 h) or lysostaphin (Sigma; 220 U/ml; 2 h) and then used to immunize mice (50 μl/mouse). The transcription level of mouse cytokine genes was analyzed by real-time reverse transcription PCR (RT-PCR) as described [31]. Survival rates of mice were analyzed using the Kaplan-Meier test. Bacterial counts and gene expression levels were analyzed using the paired t test (P values are two-tailed values).
Supporting Information

Figure S1 Cross-reaction of PG preparations from *L. monocytogenes* (A), *S. epidermidis* (B) and *S. aureus* (C). Conditions of the ELISA assay 1.5 μg PG preparation in 50 μl/well; serum from mice immunized with A170PG diluted 10^-3: 50 μl/well; rat anti mouse IgG diluted 10^-3: 50 μl/well.

(TIF)

Figure S2 Kaplan-Meier survival curves of mice vaccinated with A170PG (3 μg/mouse) by the aerosol, intramuscular or intravenous routes. Vaccinated and control mice were challenged with 10^6 CFU/mouse.

(TIF)

References

1. Liu G (2009) Molecular pathogenesis of *Staphylococcus aureus* infection. Pediatric Res 65: 71R–77R.
2. Graham P, Lin S, Larson E (2006) A U.S. population based survey of *Staphylococcus aureus* colonization. Annals of Internal Medicine 144: 318–325.
3. Koek-Noreswede M, Rodhe M, Oechsecke S, Miller L, Cheung A, et al. (2008) Immunological mechanisms underlying the genetic predisposition to severe *Staphylococcus aureus* infection in the mouse model. Am J Pathol 173: 1657–1668.
4. Lorenz F, Mira J, Cornish K, Arbour N, Cheung A, et al. (2004) Lack of association between *Staphylococcus aureus* lipoteichoic acid in sera from blood donors and patients with staphylococcal skin infections. J Clin Microbiol 27: 1286–1291.
5. Moore C, Segal S, Berendt A, Hill A, Day N (2004) Lack of association between *Staphylococcus aureus* peptidoglycans in human and rabbit sera. J Clin Microbiol 25: 540–545.
6. Wergeland H, Haaland L, Natsø H, Werenberg F, Oreleng P (1989) Antibodies to staphylococcal peptidoglycan and its peptide epitopes, teichoic acid and lipoteichoic acid in sera from blood donors and patients with staphylococcal infections. J Clin Microbiol 27: 1286–1291.
7. Fournier B, Philipp (2005) Recognition of *Staphylococcus aureus* by the innate immune system. Clin Microbiol Rev 18: 521–540.
8. Boneca I (2005) The role of peptidoglycan in pathogenesis. Curr Op Microbiol 8: 46–53.
9. Peterson P, Wilkinson B, Kiem Y, Schmelz D, Douglas S, et al. (1978) The key role of peptidoglycan in the opsonization of *Staphylococcus aureus*. J Clin Invest 61: 597–609.
10. Lowy FD (1987) *Staphylococcus aureus* - The anti-S aureus antibody response. Rev Infect Dis 20: 52–61.
11. Goodner K, Horsfall F (1935) The protective action of type 1 antipneumococcus serum in mice. J Exp Med 62: 359–374.
12. Gantner B, Singh M (2007) Short-term memory. Nature 447: 916–917.
13. Nau G, Richard F, Schlesinger A, Jennings E, Landre P, et al. (2002) Human macrophage activation programs induced by bacterial pathogens. Proc Nat Acad Sci 99: 1503–1508.
14. Blaiotta G, Pennacchia C, Ercolini D, Moschetti G, Villani F (2003) Combining denaturing gradient gel electrophoresis of 16S-23S rDNA intergenic spacer region polymorphism analyses for the identification of *Staphylococcus* from Italian fermented sausages. Systematic Appl Microbiol 26: 423–433.
15. Verdier J, Durand G, Bes M, Kimberley L, Lina G, et al. (2007) Identification of the capsular polysaccharides in *Staphylococcus aureus* clinical isolates by PCR and agglutination tests. J Clin Microbiol 45: 725–729.
16. Blaiotta G, Ercolini D, Pennacchia C, Fusco V, Casaburi A, et al. (2004) PCR detection of staphylococcal enterotoxin genes in *Staphylococcus* spp. strains isolated from meat and dairy products. Evidence for new variants of *se* and of *se* in *S. aureus* AB-8802. J Appl Microbiol 97: 719–730.
17. Blaiotta G, Fusco V, von Eiff G, Villani F, Becker K (2006) Restriction nucleases analysis of the enterotoxin gene cluster (eg) polymorphism and spa typing analyses. Appl Environ Microbiol 72: 6117–6123.
18. Blaiotta G, Moschetti G, Simeoli E, Andolfi R, Villani F, et al. (2001) Monitoring lactic acid bacteria strains during “cacioricotta” cheese production by restriction endonucleases analysis and pulsed-field gel electrophoresis. J Dairy Res 68: 139–144.
19. Meffmann A, Friedrich AW, Rosenkotter N, Rothganger J, Karch H, et al. (2006) Automated DNA sequence-based early warning system for the detection of methicillin-resistant *Staphylococcus aureus* outbreaks. PLoS Med 3: e33.
20. Goding J (1978) Use of the staphylococcal protein A as an immunological reagent. J Immun Meth 20: 241–253.
21. Capparelli N, Nocerino N, Innamone M, Ercolini D, Parlaro M, et al. (2009) Phage therapy of *Salmonella enterica*: a fresh appraisal of phage therapy. J Infect Dis 201: 52–61.

Table S1 Molecular characterization of *S. aureus* strains.

| (DOC) |

Table S2 Broad protective activity of the A170PG vaccine.

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Table S3 Aerosol administration of A170PG eradicates *S. aureus* systemic infection.

| (DOC) |

Author Contributions

Conceived and designed the experiments: RC DI. Performed the experiments: NN CM GB PB. Analyzed the data: RC DI NN CM GB PB. Wrote the paper: DI.