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Engineered liposomes sequester bacterial exotoxins and protect from severe invasive infections in mice

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Gram-positive bacterial pathogens that secrete cytotoxic pore-forming toxins, such as *Staphylococcus aureus* and *Streptococcus pneumoniae*, cause a substantial burden of disease. Inspired by the principles that govern natural toxin-host interactions, we have engineered artificial liposomes that are tailored to effectively compete with host cells for toxin binding. Liposome-bound toxins are unable to lyse mammalian cells *in vitro.* We use these artificial liposomes as decoy targets to sequester bacterial toxins that are produced during active infection *in vivo.* Administration of artificial liposomes within 10 h after infection rescues mice from septicemia caused by *S. aureus* and *S. pneumoniae,* whereas untreated mice die within 24–33 h. Furthermore, liposomes protect mice against invasive pneumococcal pneumonia. Composed exclusively of naturally occurring lipids, tailored liposomes are not bactericidal and could be used therapeutically either alone or in conjunction with antibiotics to combat bacterial infections and to minimize toxin-induced tissue damage that occurs during bacterial clearance.

The global burden of bacterial disease remains high and is set against a backdrop of increasing antimicrobial resistance[1,2]. There is a pressing need for new therapeutic strategies that are less likely to lead to drug resistance[3].

Many bacterial pathogens secrete protein toxins that kill or damage host cells by forming pores in the host cell membrane (pore-forming toxins) or by degrading plasmalemmal lipids (lipases). Membrane-damaging toxins such as cholesterol-dependent cytolysins (CDCs: pneumolysin, streptolysin O, tetanolysin), α-hemolysin or bacterial phospholipase C have a crucial role in the establishment and progression of infectious diseases[4–7].

The CDC family comprises more than 20 members from different Gram-positive bacterial genera including *Clostridium, Streptococcus, Listeria* and *Bacillus*[8]. Pneumolysin, secreted by *S. pneumoniae*, and streptolysin O, secreted by *Streptococcus pyogenes*, insert directly in the plasmalemmal lipid bilayer of target cells and form oligomeric pores that are large enough to allow the leakage of intracellular proteins[8–10].

α-Hemolysin, which is secreted by Gram-positive *S. aureus*, forms small pores that primarily affect the intracellular ion balance. Its oligomers insert directly into the lipid bilayer: a basic and aromatic amino-acid-rich crevice between the rim domain and the stem domain forms a binding site for phospholipid headgroups[9,11]. Gram-negative pathogens also secrete a variety of repeats-in-toxins that possess pore-forming or lipase activity[12].

The lipid bilayer of the plasmalemma is mainly composed of glycerophospholipids, sphingolipids and cholesterol. Cholesterol and sphingomyelin are logical choices as target molecules for bacterial toxins as they confer specificity for animal cells as opposed to bacterial cell membranes[8,9,13]. It appears that increased levels of cholesterol within distinct membrane microdomains, but not the mere presence of individual cholesterol molecules within the plasma membrane, are important for pore-forming toxin activity[8–10]. Such lipid microdomains assemble within the plasmalemma of eukaryotic cells as a result of the interactions of sphingolipids with one another and with cholesterol, followed by the accumulation of these lipids within membrane rafts[14–17]. Rafts are characterized by a tight packing of saturated acyl chains within the liquid ordered phase, whereas glycerophospholipids, with preferentially unsaturated acyl chains, reside in a more fluid liquid-disordered phase[14–16]. Raft microdomains *in vivo* are unstable structures less than 40 nm in diameter[14,15].

Unlike unstable *in vivo* rafts, stable, liquid-ordered, lipid microdomains can be artificially created in liposomes consisting of cholesterol and sphingomyelin[18]. Here, we explore whether liposomes comprising higher than *in vivo* relative concentrations of selected lipids can be used as decoy targets to sequester bacterial membrane-damaging toxins. Using individual, purified bacterial toxins we establish that liposomes composed of sphingomyelin in combination with artificially high concentration of cholesterol (cholesterol: sphingomyelin liposomes; 66 mol/% cholesterol) efficiently bind

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CDCs, phospholipase C and α-hemolysin. Furthermore, a mixture of cholesterol:sphingomyelin liposomes (66 mol/% cholesterol) with sphingomyelin liposomes (sphingomyelin-only liposomes) efficiently sequester the whole palette of toxins released by a variety of staphylococcal and streptococcal pathogens. The same mixture of liposomes is successfully used to treat fatal invasive staphylococcal and streptococcal diseases in mouse models, either alone or in combination with antibiotic therapy.

RESULTS

Sequestration of purified bacterial toxins

We initially assessed whether an artificially high concentration of a particular target lipid increases the affinity of liposomes for membrane-damaging toxins and thereby allows a preferential binding of the toxin to liposomes compared to mammalian cells. As CDCs bind directly to plasmalemmal cholesterol, and this interaction is augmented within cholesterol- and sphingomyelin-rich lipid microdomains, we made liposomes that were saturated with cholesterol (the maximal concentration of cholesterol that can be accommodated within a lipid bilayer is 66 mol/% (ref. 19)) and contained sphingomyelin as a bilayer-forming lipid (cholesterol:sphingomyelin; 66 mol/% cholesterol). When added to the cell-liposome mixture, pneumolysin exclusively targeted the cholesterol:sphingomyelin liposomes, whereas the cells remained toxin-free (**Fig. 1a**; full-sized blot is shown in **Supplementary Fig. 1**).

We next investigated whether cholesterol:sphingomyelin liposomes were able to protect human epithelial cells, endothelial cells and cells of the innate immune system, which are the major effector cells responsible for fending off a pathogen attack, from a number of purified bacterial toxins. Additionally, cholesterol:sphingomyelin liposomes (66 mol/% cholesterol) were compared with liposomes of other lipid compositions in order to gain insights into basic principles that govern liposome-toxin interaction. The amounts of purified toxins required for complete inhibition of proliferation of human monocyte THP-1 cells were established (**Supplementary Fig. 2**) and used in all subsequent experiments.

Cholesterol:sphingomyelin liposomes (cholesterol = 66 mol/%) completely protected THP-1 cells from CDCs (streptolysin O, tetanolysin, pneumolysin), α-hemolysin and phospholipase C (**Fig. 1b** and **Supplementary Fig. 3a**). Liposomes that contained no cholesterol were ineffective against pneumolysin and α-hemolysin; limited protection against phospholipase C was observed for sphingomyelin-only but not for phosphatidylcholine-only liposomes (**Fig. 1c**). The amounts of the cholesterol:sphingomyelin liposomes that were required for the neutralization of the cytotoxic activity of pneumolysin (**Supplementary Fig. 3b**) were comparable to those that were

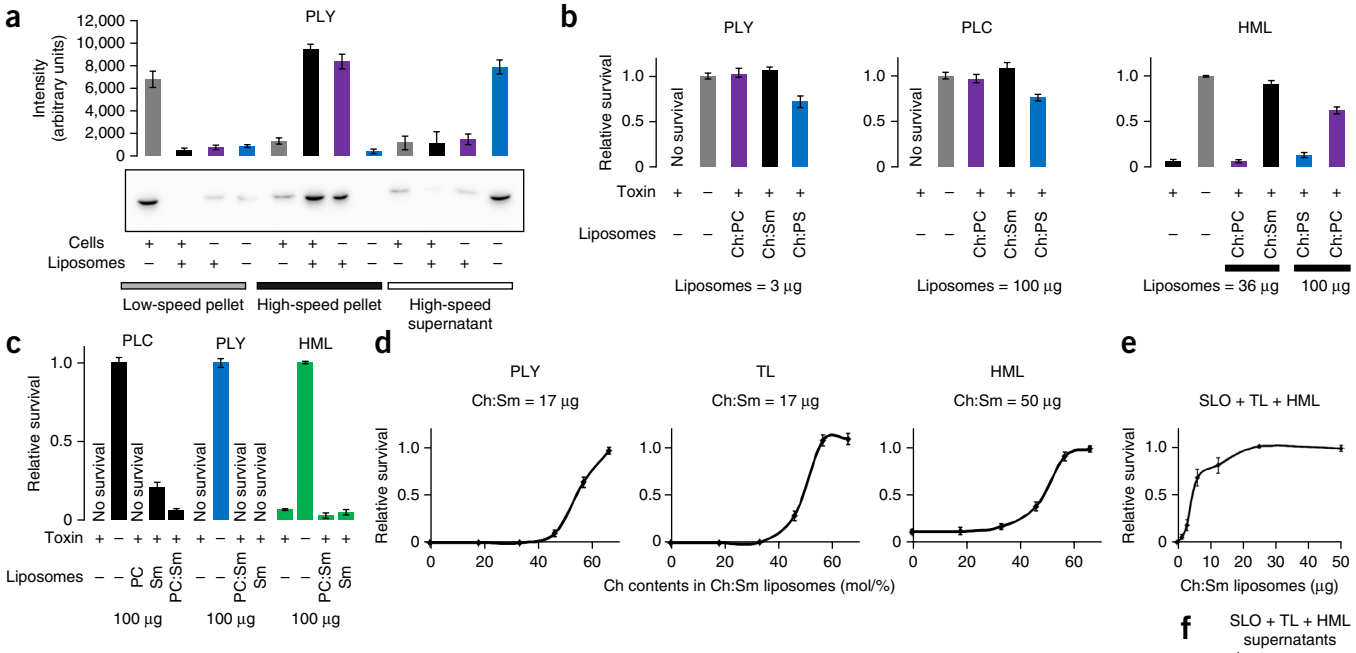

Figure 1 Liposomes composed of cholesterol and sphingomyelin protect monocytes from cholesterol-dependent cytolysins, α-hemolysin (HML) and phospholipase C (PLC). (**a**) Pneumolysin (PLY) was added to THP-1 cells without liposomes (+,–); to a mixture of THP-1 cells with cholesterol:sphingomyelin (Ch:Sm) liposomes (+,+); to Ch:Sm liposomes without cells (–,+) or to buffer-only (–,–). The samples were centrifuged at low-speed (cell pellet), then at high-speed (liposome pellet). The resulting pellets and the high-speed supernatant were analyzed by western blotting for PLY content. In the absence of either cells or liposomes, PLY was recovered in the high-speed supernatant; in the absence of liposomes, PLY was bound by the cells and was recovered preferentially in the low-speed pellet; in the absence of cells, PLY was bound by the liposomes and was recovered in the high-speed pellet. In the cell-liposome mixture, PLY exclusively targeted the liposomes. ($n = 6$). Full-sized blot in **Supplementary Figure 1**. (**b**) Liposomes composed of Ch (66 mol/%) in combination with phosphatidylcholine (PC), Sm or phosphatidylserine (PS) protect THP-1 cells from PLY or PLC. Ch:Sm liposomes are most effective against HML ($n = 9$). (**c**) Liposomes without cholesterol do not protect THP-1 cells from PLY or HML. Sm-only, but not PC-only liposomes, give limited protection against PLC ($n = 6$). (**d**) Artificially high concentrations of cholesterol within Ch:Sm liposomes are required for protection against PLY, tetanolysin (TL) or HML. ($n = 9$). (**e**) Ch:Sm liposomes protect THP-1 cells from the combination of streptolysin O (SLO) (400 ng), TL (200 ng) and HML (1.2 μg) ($n = 7$). (**f**) A mixture of SLO + TL + HML was incubated with or without Ch:Sm liposomes, followed by centrifugation. The liposomal pellet was discarded; the supernatants were added to the cells. The liposome-free supernatants of the toxin-liposome mixture do not damage exposed cells. This is in contrast to toxin preparations that were centrifuged in the absence of liposomes. ($n = 4$). PC:Sm = (1:1 w/w). Error bars, mean ± s.e.m.

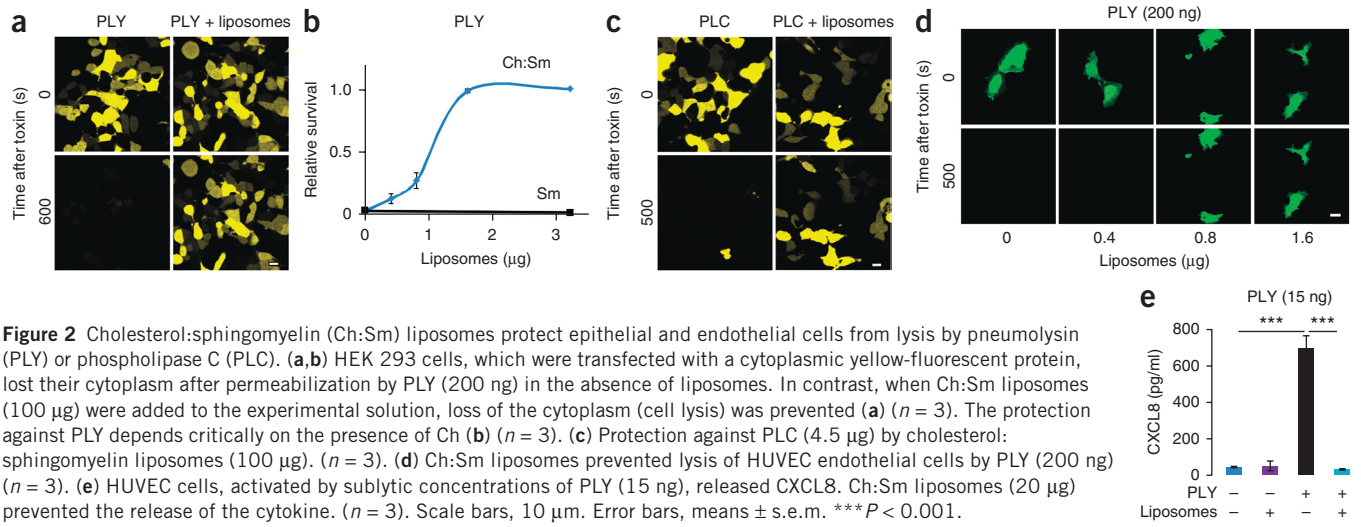

Figure 2 Cholesterol:sphingomyelin (Ch:Sm) liposomes protect epithelial and endothelial cells from lysis by pneumolysin (PLY) or phospholipase C (PLC). (**a**,**b**) HEK 293 cells, which were transfected with a cytoplasmic yellow-fluorescent protein, lost their cytoplasm after permeabilization by PLY (200 ng) in the absence of liposomes. In contrast, when Ch:Sm liposomes (100 μg) were added to the experimental solution, loss of the cytoplasm (cell lysis) was prevented (**a**) ($n = 3$). The protection against PLY depends critically on the presence of Ch (**b**) ($n = 3$). (**c**) Protection against PLC (4.5 μg) by cholesterol: sphingomyelin liposomes (100 μg). ($n = 3$). (**d**) Ch:Sm liposomes prevented lysis of HUVEC endothelial cells by PLY (200 ng) ($n = 3$). (**e**) HUVEC cells, activated by sublytic concentrations of PLY (15 ng), released CXCL8. Ch:Sm liposomes (20 μg) prevented the release of the cytokine. ($n = 3$). Scale bars, 10 μm. Error bars, means ± s.e.m. $^{***}P < 0.001$.

required for the sequestration of the toxin, established in a direct binding experiment (**Supplementary Fig. 3c**).

Liposomes containing cholesterol at 20–30 mol/% concentration, which is similar to total cholesterol concentration occurring *in vivo*[20–22], were unable to compete with cells for toxin binding; artificially high cholesterol concentrations above 50 mol/% were required for cholesterol: sphingomyelin liposomes to fully protect monocytes from lysis by pneumolysin, tetanolysin or α-hemolysin (**Fig. 1d**).

The relative ratio of cholesterol to phospholipid within lipid bilayers rather than its total concentration defined the protective potential of cholesterol-containing liposomes: 3 μg (total cholesterol = 1.5 μg) of cholesterol:sphingomyelin liposomes with high contents of cholesterol (66 mol/%) provided complete protection against 200 ng of tetanolysin (**Supplementary Fig. 3b**), whereas 17 μg (total cholesterol = 3.4 μg) of cholesterol:sphingomyelin containing 33 mol/% of cholesterol provided no protection (**Fig. 1d**).

Liposomal cholesterol was >1,000 times more effective in neutralizing streptolysin O than cholesterol that was present *in vivo* in the form of high-density lipoprotein (HDL); 0.2 μg of liposomal cholesterol was required for half-maximal neutralization of 400 ng of streptolysin O (**Supplementary Fig. 3b**), whereas >300 μg of HDL cholesterol was needed for the same degree of protection (**Supplementary Fig. 3d**).

Cholesterol:sphingomyelin liposomes exerted fully protective effects against the combined action of streptolysin O, tetanolysin and α-hemolysin (**Fig. 1e**). Sequestration of all three toxins by cholesterol: sphingomyelin liposomes was confirmed in a direct binding experiment (**Fig. 1f**).

Collectively, our results demonstrate that cholesterol:sphingomyelin liposomes (cholesterol = 66 mol/%) efficiently sequestered a variety of purified toxins belonging to different toxin classes with diverse modes of membrane-damaging action.

Epithelial and endothelial cells constitute physical barriers to pathogens. Human embryonic kidney epithelial cells (HEK 293) that were treated with pneumolysin or with phospholipase C lost their entire cytosol within 10 min of the toxin exposure (**Fig. 2a–c**). The loss of cytosol, that is, immediate cell lysis, was prevented when cholesterol: sphingomyelin liposomes were added to the cells before the toxin exposure (**Fig. 2a–c**), whereas sphingomyelin-only liposomes were not protective (**Fig. 2b**).

Cholesterol:sphingomyelin liposomes (cholesterol = 66 mol/%) also protected human endothelial HUVEC cells from pneumolysin-induced lysis (**Fig. 2d**) and prevented pneumolysin-induced release of CXCL8 (also known as IL-8) by these cells at sublytic pneumolysin concentrations (**Fig. 2e**). Thus, liposomal toxin sequestration not only prevented endothelial cell damage but also precluded their toxin-induced inflammatory activation. The latter might be of utmost importance *in vivo*, because uncontrolled, overwhelming inflammatory responses during bacterial infection can lead to extensive tissue damage in the host[23]. Pneumolysin concentrations used in our study (60–800 ng/ml) were comparable to those occurring during invasive disease *in vivo*[24–26].

Sequestration of toxins secreted by human pathogens

We next checked whether artificial liposomes could sequester the full panoply of toxins secreted by clinically relevant human pathogens.

Cholesterol:sphingomyelin liposomes completely protected THP-1 cells from the effect of toxins secreted by all of the *S. pyogenes* strains tested (**Fig. 3a**,**b**). The amount of cholesterol:sphingomyelin liposomes that was required to achieve complete protection against *S. pyogenes* DSM 11728 (**Fig. 3b**) was comparable to the amounts of liposomes that were fully protective against purified streptolysin O (**Supplementary Fig. 3b**), suggesting that this toxin is the single cytotoxic agent that is secreted by this pathogen.

In contrast, cholesterol-containing liposomes (either cholesterol: sphingomyelin or cholesterol:phosphatidylcholine) provided only limited protection against toxins secreted by a methicillin-resistant strain of *S. aureus* (MRSA 2040) (**Fig. 3c**). However, a combination of cholesterol-containing liposomes (cholesterol = 66 mol/%) with cholesterol-free, sphingomyelin-only liposomes yielded full protection against this pathogen (**Fig. 3d**,**e**). **Figure 3d** shows the results of an experiment in which the survival of THP-1 cells was assessed in the presence of an invariable amount (600 μg) of cholesterol: phosphatidylcholine liposomes and increasing amounts of either phosphatidylcholine-only or sphingomyelin-only liposomes. At these experimental conditions, the relative survival at the "0 μg" point (**Fig. 3d**; black arrow) corresponds to that of the "600 μg" point (**Fig. 3c**, black arrow). Used alone, 600 μg of cholesterol-containing liposomes provided only limited protection against toxins secreted by MRSA 2040 (**Fig. 3c**,**d**; black arrows). Combining 600 μg of cholesterol-containing liposomes with up to 225 μg of phosphatidylcholine-only liposomes provided no additional protection (**Fig. 3d**). However, the addition of as little as 75 μg of sphingomyelin-only liposomes rendered the liposomal mixture fully protective against toxins secreted by MRSA 2040 (**Fig. 3d**). In a reciprocal experiment, the survival of THP-1 cells was assessed

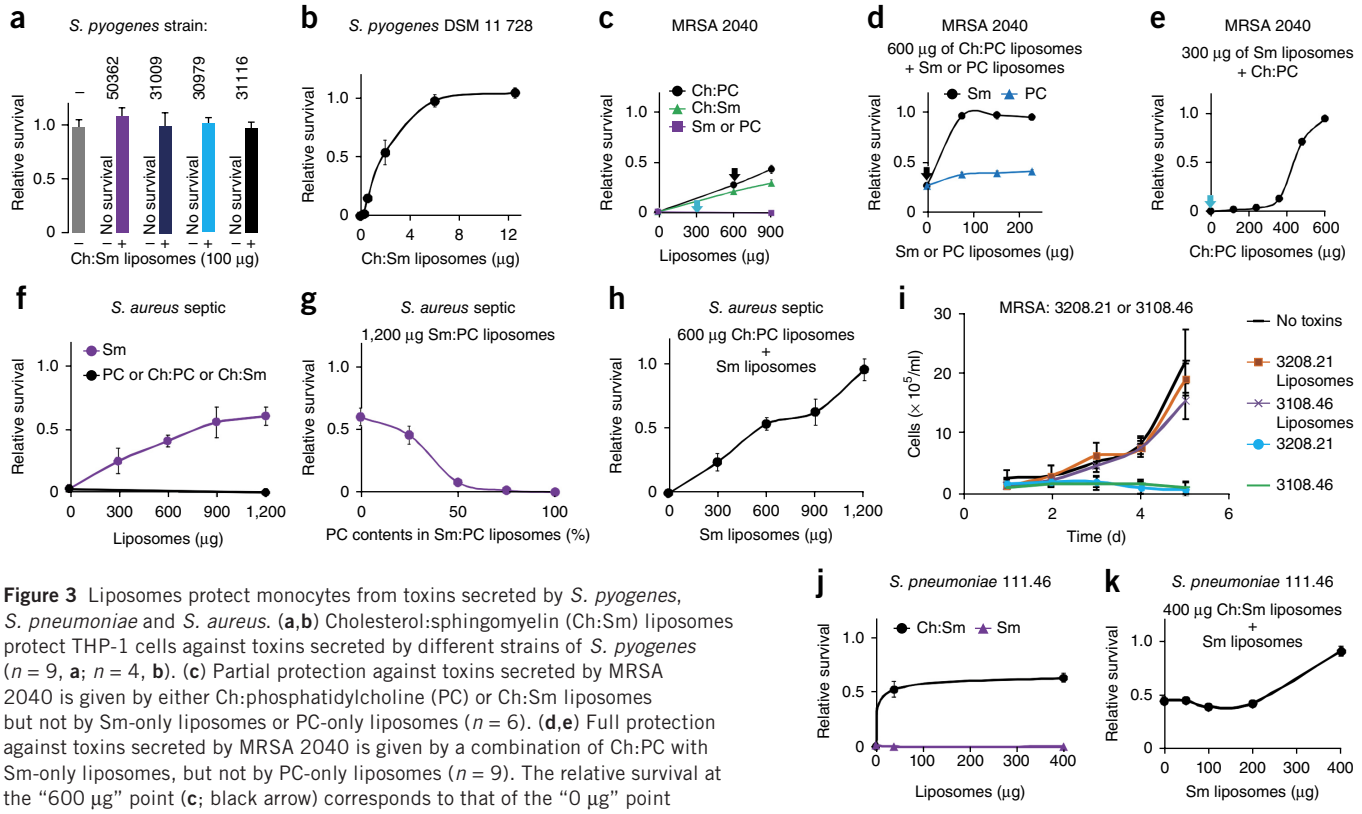

Figure 3 Liposomes protect monocytes from toxins secreted by *S. pyogenes*, *S. pneumoniae* and *S. aureus*. (**a**,**b**) Cholesterol:sphingomyelin (Ch:Sm) liposomes protect THP-1 cells against toxins secreted by different strains of *S. pyogenes* ($n = 9$, **a**; $n = 4$, **b**). (**c**) Partial protection against toxins secreted by MRSA 2040 is given by either Ch:phosphatidylcholine (PC) or Ch:Sm liposomes but not by Sm-only liposomes or PC-only liposomes ($n = 6$). (**d**,**e**) Full protection against toxins secreted by MRSA 2040 is given by a combination of Ch:PC with Sm-only liposomes, but not by PC-only liposomes ($n = 9$). The relative survival at the "600 µg" point (**c**; black arrow) corresponds to that of the "0 µg" point (**d**; black arrow); the relative survival at the "300 µg" point (**c**; blue arrow) corresponds to that of the "0 µg" point (**e**; blue arrow). (**f**) Partial protection against toxins secreted by the septic *S. aureus* strain is given by Sm-only but not by PC-only, Ch:Sm or Ch:PC liposomes ($n = 6$). (**g**) Concentrations of Sm above 75 mol/% are required for the maximal possible protection against the septic *S. aureus* strain by the Ch-free, Sm-containing liposomes ($n = 6$). (**h**) Full protection against toxins secreted by the septic *S. aureus* strain by a mixture of sphingomyelin-only and cholesterol-containing liposomes ($n = 9$). (**i**) A mixture of Ch:Sm liposomes (66 mol/% of cholesterol; 600 µg) and sphingomyelin-only liposomes (300 µg) protect THP-1 cells from culture supernatants obtained from clinically isolated strains of MRSA ($n = 3$). (**j**) Partial protection against toxins secreted by clinically isolated *S. pneumoniae* strain 111.46 is given by Ch:Sm but not by Sm-only liposomes ($n = 9$). (**k**) Full protection against toxins secreted by *S. pneumoniae* strain 111.46 by a mixture of Ch:Sm and Sm-only liposomes ($n = 9$). Error bars, mean ± s.e.m.

in the presence of invariable, saturating amounts of sphingomyelin-only liposomes (300 µg) and increasing amounts of cholesterol:phosphatidylcholine liposomes (**Fig. 3e**). At such experimental conditions the relative survival at the "0 µg" point (**Fig. 3e**; blue arrow) corresponds to that of the "300 µg" point (**Fig. 3c**, blue arrow). Used alone, 300 µg of sphingomyelin-only liposomes provided no protection against toxins secreted by MRSA 2040 (**Fig. 3c**,**e**, blue arrows). Combining 300 µg of sphingomyelin-only liposomes with up to 400 µg of cholesterol-containing liposomes provided no additional protection (**Fig. 3e**), but increasing the amount of cholesterol-containing liposomes to 600 µg rendered the liposomal mixture fully protective against toxins secreted by MRSA 2040 (**Fig. 3e**).

Taken together, these results suggest that MRSA 2040 secretes at least two toxins. One toxin is neutralized by sphingomyelin-only liposomes (complete neutralization at ≤75 µg), whereas the second toxin is neutralized by 400–600 µg of cholesterol-containing liposomes.

Liposomal treatment was also effective against a strain of *S. aureus* that was isolated from a septic patient (septic *S. aureus* strain). In contrast to the MRSA 2040 strain, the cytolytic toxins secreted by the septic *S. aureus* strain were partially sequestered by sphingomyelin-only liposomes but not by the cholesterol-containing liposomes (**Fig. 3f**), suggesting that the relative contribution of sphingomyelin-binding toxin(s) to the total cytotoxic activity of this pathogen was higher than that of the cholesterol-binding toxin(s). The toxin-sequestering activity of sphingomyelin-containing liposomes was drastically decreased when sphingomyelin was replaced by the same-headgroup glycerolipid, phosphatidylcholine (**Fig. 3g**). As in the MRSA 2040 strain, the combination of sphingomyelin-only liposomes with cholesterol-containing liposomes (66 mol/%) was required for full protection against the septic *S. aureus* strain (**Fig. 3h**).

The mixture of cholesterol:sphingomyelin liposomes (cholesterol = 66 mol/%) and sphingomyelin-only liposomes was also protective against clinically isolated MRSA strains (**Fig. 3i**).

For *S. pneumoniae*, limited protection achieved with low amounts of cholesterol:sphingomyelin liposomes alone (**Fig. 3j**) can be attributed to sequestration of pneumolysin (**Supplementary Fig. 3b**). The mixture of cholesterol-containing and sphingomyelin-only liposomes was fully protective against this pathogen (**Fig. 3k**).

Sequestration of bacterial toxins *in vivo*

We next investigated whether the mixture of cholesterol-containing (cholesterol = 66 mol/%) and cholesterol-free, sphingomyelin-containing liposomes protects mice from fatal staphylococcal and pneumococcal disease.

Initially, we evaluated biotoxicity and biodistribution of the liposomes in healthy mice. The doses of liposomes that were routinely used in our study (100–150 mg/kg) are known to be nontoxic when liposomes are used as carriers for the intravenous delivery of antibiotics in rats (400 mg/kg)[27]. This is consistent with our findings that the hearts, kidneys, livers, lungs and spleens of mice that were

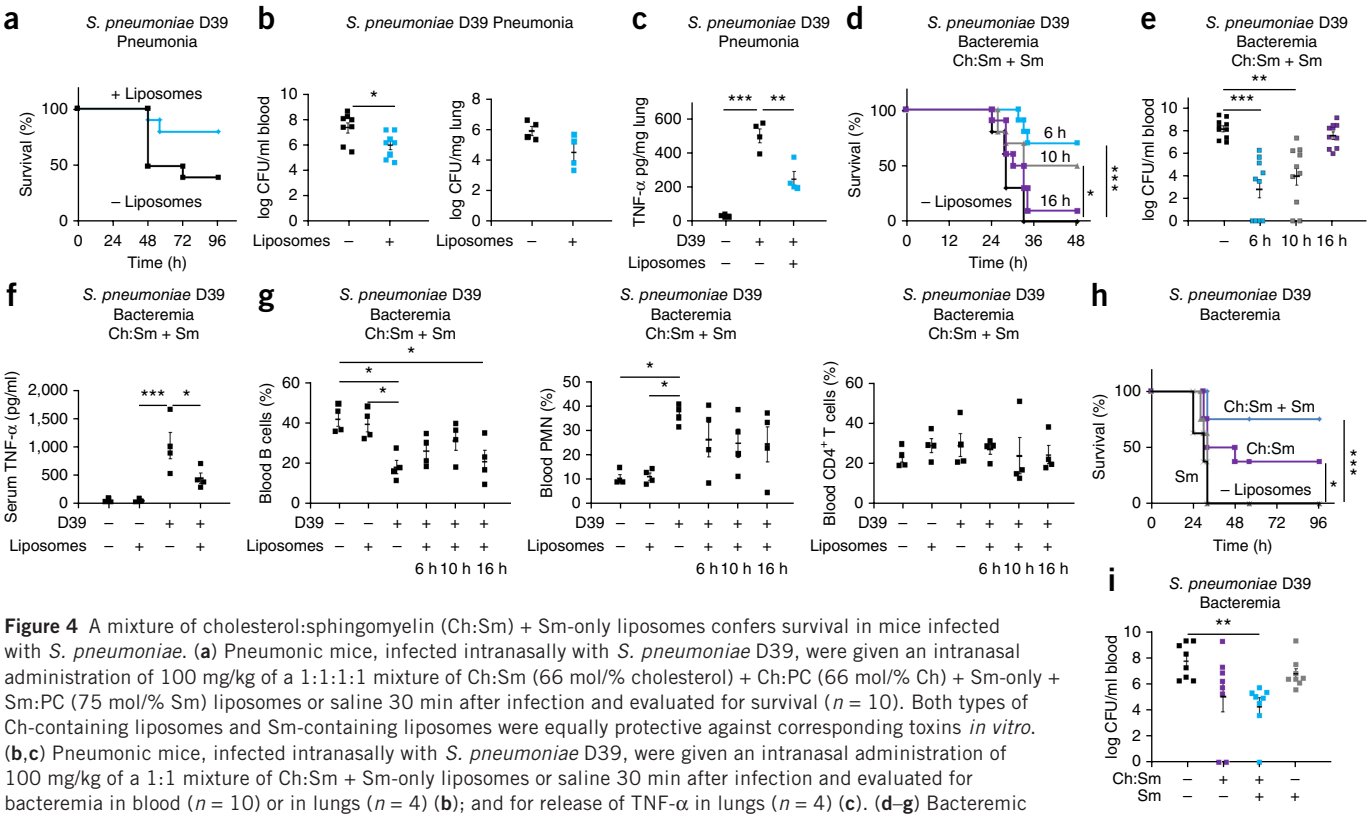

Figure 4 A mixture of cholesterol:sphingomyelin (Ch:Sm) + Sm-only liposomes confers survival in mice infected with *S. pneumoniae*. (**a**) Pneumonic mice, infected intranasally with *S. pneumoniae* D39, were given an intranasal administration of 100 mg/kg of a 1:1:1:1 mixture of Ch:Sm (66 mol/% cholesterol) + Ch:PC (66 mol/% Ch) + Sm-only + Sm:PC (75 mol/% Sm) liposomes or saline 30 min after infection and evaluated for survival ($n = 10$). Both types of Ch-containing liposomes and Sm-containing liposomes were equally protective against corresponding toxins *in vitro*. (**b**,**c**) Pneumonic mice, infected intranasally with *S. pneumoniae* D39, were given an intranasal administration of 100 mg/kg of a 1:1 mixture of Ch:Sm + Sm-only liposomes or saline 30 min after infection and evaluated for bacteremia in blood ($n = 10$) or in lungs ($n = 4$) (**b**); and for release of TNF-α in lungs ($n = 4$) (**c**). (**d**–**g**) Bacteremic mice, infected intravenously with *S. pneumoniae* D39, were given an intravenous administration of 100 mg/kg liposomes 6 h or 10 h or 16 h after infection and evaluated for survival ($n = 10$) (**d**); for bacteremia in blood ($n = 10$) (**e**); for release of TNF-α in blood ($n = 4$) (**f**); and for changes in blood leukocytes ($n = 4$) (**g**). (**h**,**i**) Bacteremic mice, infected intravenously with *S. pneumoniae* D39, were given intravenous administrations of 150 mg/kg/injection of Ch:Sm + Sm-only or Ch:Sm or Sm-only liposomes or saline 8 and 12 h after infection and evaluated for survival (**h**; $n = 8$) and bacteremia in blood (**i**; $n = 8$). Error bars, mean ± s.e.m. $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$.

treated with 100 mg/kg of the liposomal mixture showed no obvious macroscopic or microscopic changes (**Supplementary Fig. 4**). Biodistribution was assessed by mass spectrometry in mice that were injected with liposomes that contained 6% of deuterated sphingomyelin. The half-maximal decline in the level of circulating liposomes occurred at 4 h, whereas ~20% of the liposomes were still circulating at 24 h after injection (**Supplementary Fig. 5**). The highest per organ accumulation of deuterated sphingomyelin was observed in the liver, followed by the spleen and the intestine. The highest specific (per mg of protein) retention of liposomes was detected in spleens 6–24 h after injection (**Supplementary Fig. 5**), which is indicative of their elimination by splenic macrophages.

We next tested the efficacy of liposomes as treatment for severe bacterial disease in mice. In an invasive pneumococcal pneumonia model, mice were infected intranasally with *S. pneumoniae* serotype 2 (strain D39). Liposomes provided protection against invasive pneumonia (**Fig. 4a**). In accord, reductions of bacterial load were observed in the blood and lungs of mice treated with the liposomal mixture (**Fig. 4b**). Importantly, the levels of TNF-α, a signature pro-inflammatory cytokine associated with pneumococcal infection, were considerably reduced in the liposome-treated mice (**Fig. 4c**). Histological analysis of lungs obtained 24 h after infection from mice infected intranasally with *S. pneumoniae* showed prominent peribronchiolar and perivascular inflammatory cuffs (**Supplementary Fig. 6**). In lungs of the liposome-treated mice edematous changes in the peribronchiolar and perivascular connective tissue were marked, but infiltration by leukocytes was reduced (**Supplementary Fig. 6**). No obvious microscopic changes were observed in the lungs of healthy mice that were treated intranasally with the liposomal mixture (**Supplementary Fig. 6**).

In a fatal pneumococcal sepsis model, mice were injected intravenously with a lethal dose of *S. pneumoniae* D39. Mice that were not treated with liposomes died within 33 h of bacterial challenge (**Fig. 4d**). A single injection of the liposomal mixture was protective when given at 6 h or 10 h after infection, whereas injection at 16 h was ineffective (**Fig. 4d**). A considerable reduction of the bacterial load was observed in the blood of mice that had been injected with a single dose of the liposomes at 6 h or at 10 h after infection (**Fig. 4e**). As we observed in the lungs of mice in the invasive pneumococcal pneumonia model, blood levels of TNF-α were also substantially reduced in the pneumococcal sepsis model in mice that were injected with the liposomal mixture 6 h after intravenous infection (**Fig. 4f**). Furthermore, a single injection of liposomes attenuated the bacteremia-induced reduction of blood B-cells and reduced the pro-inflammatory recruitment of blood polymorphonuclear leukocytes, a major contributing factor to uncontrolled inflammation, whereas the levels of T-cells were not affected either by bacteremia or by the liposomal treatment (**Fig. 4g**). Injection of the liposomal mixture in healthy mice did not influence levels of either TNF-α or that of blood leukocytes (**Fig. 4f**,**g**).

Because the levels of liposomes in blood decreased by 50% 4 h after injection (**Supplementary Fig. 5a**), we injected mice with liposomes twice with a 4 h interval to maintain a high level of circulating liposomes. No control or sphingomyelin-only mice survived beyond 32 h (**Fig. 4h**). However, 6 out of 8 mice that received the cholesterol:sphingomyelin plus sphingomyelin-only liposomal mixture and 3 out of 8 mice that received cholesterol:sphingomyelin liposomes survived. The cholesterol:sphingomyelin

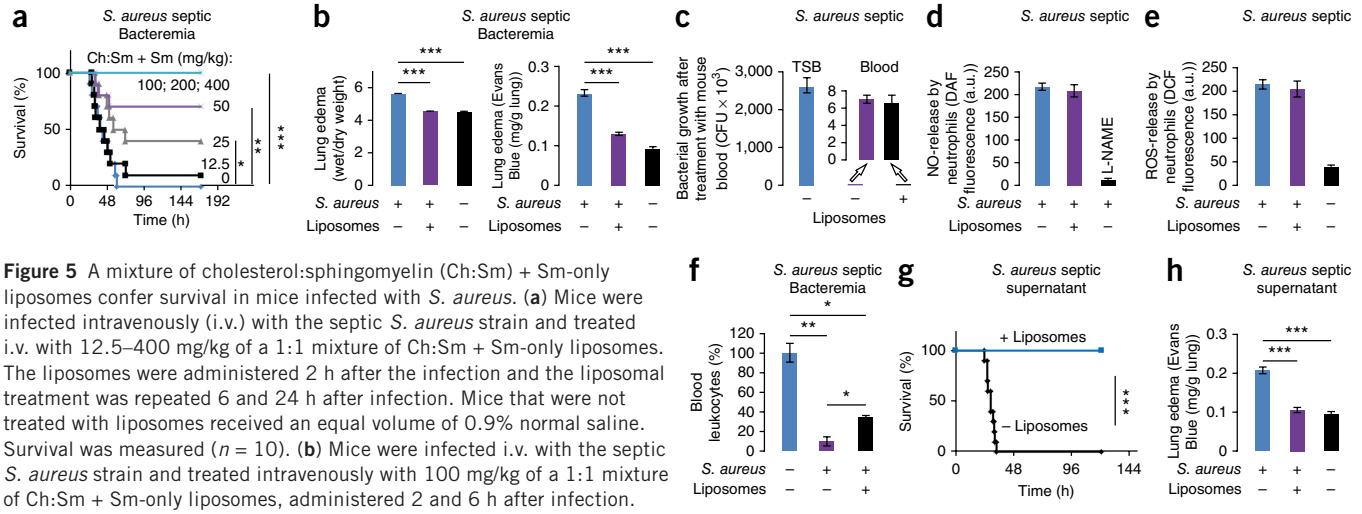

Figure 5 A mixture of cholesterol:sphingomyelin (Ch:Sm) + Sm-only liposomes confer survival in mice infected with *S. aureus*. (**a**) Mice were infected intravenously (i.v.) with the septic *S. aureus* strain and treated i.v. with 12.5–400 mg/kg of a 1:1 mixture of Ch:Sm + Sm-only liposomes. The liposomes were administered 2 h after the infection and the liposomal treatment was repeated 6 and 24 h after infection. Mice that were not treated with liposomes received an equal volume of 0.9% normal saline. Survival was measured ($n = 10$). (**b**) Mice were infected i.v. with the septic *S. aureus* strain and treated intravenously with 100 mg/kg of a 1:1 mixture of Ch:Sm + Sm-only liposomes, administered 2 and 6 h after infection. Lung edema was evaluated by comparing wet/dry weight of the lungs ($n = 4$) and by monitoring the intrapulmonal leakage of Evans Blue ($n = 4$). (**c**) Blood samples obtained from healthy mice were incubated with *S. aureus* in the presence or absence of Ch:Sm + Sm-only liposomes. After the incubation, aliquots were plated on LB plates, grown overnight and the CFU were counted ($n = 4$). Corresponding data (indicated by arrows) are shown on a magnified scale to highlight that liposomes had no effect on bacterial killing by mouse blood. TSB, tryptic soy broth. (**d**,**e**) Ch:Sm + Sm-only liposomes had no effect on the capability of purified mouse neutrophils to release nitric oxide (NO) ($n = 4$) (**d**) or reactive oxygen species (ROS) ($n = 4$) (**e**) upon infection with *S. aureus*. NO was determined in the presence or absence of L-NAME (i-NOS inhibitor). DAF, 4-amino-5-methylamino-2′,7′-difluorofluorescein; DCF, 2′,7′dichlorodihydrofluorescein diacetate acetyl ester. (**f**) Mice were infected i.v. with the septic *S. aureus* strain and were treated 2 and 6 h after infection i.v. with Ch:Sm + Sm-only liposomes or PBS. Control mice received PBS only. The number of leukocytes was measured by FACS ($n = 4$). (**g**,**h**) *S. aureus* culture supernatant was incubated with Ch:Sm + Sm-only liposomes or with an equal volume of PBS. Liposomes were removed by centrifugation and supernatants were injected i.v. in mice. Survival ($n = 10$) (**g**) and lung edema ($n = 4$) (**h**) were evaluated. Error bars, mean ± s.e.m. $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$.

plus sphingomyelin-only group had the lowest average colony-forming units (CFU) counts after 24 h of bacteremia (**Fig. 4i**).

In a staphylococcal sepsis model, mice were injected intravenously with a lethal dose of the septic *S. aureus* strain. None of the control mice survived beyond day 3 (**Fig. 5a**). Treatment with ≥100 mg/kg per injection of the liposomal mixture resulted in complete recovery of the infected mice (**Fig. 5a**), whereas lower doses provided partial protection. The liposomal treatment (100 mg/kg per injection) prevented development of pulmonary edema in the infected mice, a typical feature of severe *S. aureus* sepsis (**Fig. 5b**). A single injection of the liposomes (100 mg/kg) was equally protective at 2 h, 6 h or 10 h after infection (4 mice for each time point; all survived), whereas an injection 16 h after infection was ineffective (4 mice; death after 36, 40, 48 and 52 h).

Mechanism of protection by liposomes

Liposomal treatment considerably reduced TNF-α levels both in the invasive pneumococcal pneumonia model and in the pneumococcal sepsis model (**Fig. 4c**,**f**). Although TNF-α aids in the bacterial clearance process, excessive production of this cytokine during infection leads to uncontrolled inflammation (cytokine storm), and increased mortality. Liposomes that contain negatively charged phosphatidylserine have been shown to inhibit TNF-α-release from lipopolysaccharide (LPS)-treated cells[28]. The liposomes used in this study did not contain phosphatidylserine, and their protective action was not due to an inhibition of LPS-induced TNF-α overproduction. Mice that were injected intravenously with 150 mg/kg of the cholesterol:sphingomyelin plus sphingomyelin-only liposomal mixture 45 min after administration of LPS from *Escherichia coli* or *Pseudomonas aeruginosa* ($n = 2$ for each group) and control mice that received an injection of normal saline 45 min after LPS administration ($n = 3$) died within 36 h of LPS administration.

The reduced TNF-α levels seen in liposome-treated mice may be the result of decreased bacterial numbers and/or reduced toxin-mediated damage and cellular activation. Liposomal treatment considerably decreased bacterial loads in infected animals (**Fig. 4b**,**e**). Yet, the liposomes themselves are not bactericidal: the minimal bactericidal concentration was >16 mg/ml for *S. aureus* and *S. pneumoniae*. In addition, we observed no evidence that the liposomes either modified bacterial killing by mouse blood (**Fig. 5c**) or activated neutrophils to release nitric oxide (**Fig. 5d**) and reactive oxygen species (**Fig. 5e**).

Our *in vitro* data suggest that the major decrease in bacterial load in infected animals after liposomal treatment is due to the protection of host immune cells from lysis by the bacterial toxins. Accordingly, in the staphylococcal sepsis model, injection of the cholesterol:sphingomyelin plus sphingomyelin-only liposomal mixture protected mouse leukocytes from killing by *S. aureus in vivo* (**Fig. 5f**).

To provide further evidence that the liposomes prevented fatal infection by sequestering bacterial toxins, mice were injected with bacteria-free culture supernatants obtained from the septic *S. aureus* strain (**Fig. 5g**). All mice that received bacterial supernatants, incubated with PBS, died within 34 h after the injection, whereas all mice that received liposome-treated supernatants survived. Liposomal pre-treatment prevented the development of lung edema in mice that were injected with bacterial supernatants (**Fig. 5h**).

Collectively these experiments demonstrate that liposomal protection *in vivo* was due neither to direct bactericidal activity nor to a direct activation of host defenses by the liposomes. Nor did it require direct liposome-host, bacteria-host or bacteria-liposome interactions; the sequestration of secreted bacterial toxins by the mixture of cholesterol:sphingomyelin plus sphingomyelin-only liposomes was sufficient to protect host cells from lysis and uncontrolled inflammatory hyperactivation and to prevent death of infected animals.

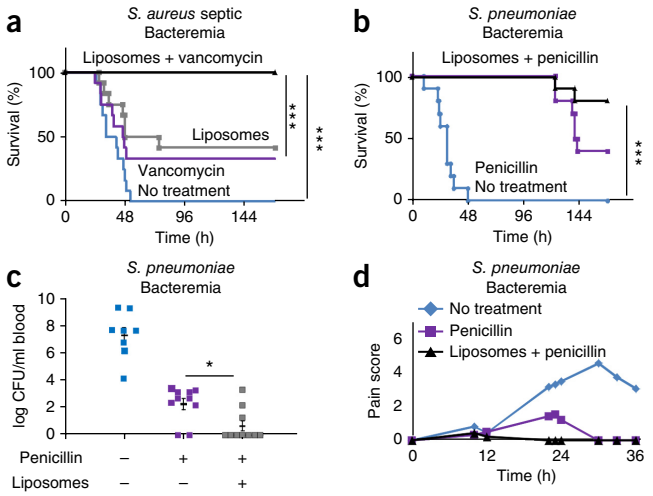

Figure 6 A combination of toxin-sequestration with antibiotic treatment to treat fatal *S. aureus and S. pneumoniae* infections. (**a**) Mice were infected i.v. with the septic *S. aureus* strain and randomly separated into four groups. Mice of the first group received injections of PBS as control. All 12 mice of this group died within 52 h of infection. Mice of the second group were treated i.v. with low doses of Ch:Sm + Sm-only liposomes, which were administered 2, 6 and 24 h after infection (total dose = 37.5 mg/kg). As the liposome dose is too low to provide complete protection, only 5 out of 12 mice survived the infection. Mice of the third group were treated i.v. with 100 mg/kg of vancomycin at 1 and 9 h after infection. Only 4 out of 12 mice survived the infection if treated with vancomycin alone. Mice of the fourth group were treated i.v. with Ch:Sm + Sm-only liposomes and vancomycin administered as above. All 12 mice survived the infection. (**b**–**d**) Mice were infected i.v. with *S. pneumoniae* D39 and randomly separated into three groups. Mice of the first group received injections of PBS as a control. All ten mice of this group died within 48 h of infection (**b**). Mice of the second group were treated intravenously with 25 mg/kg of penicillin at 10 and 24 h after infection. Only 4 out of 10 mice survived in this group (**b**). Mice of the third group were treated intravenously with 100 mg/kg Ch:Sm + Sm-only liposomes and with 25 mg/kg of penicillin at 10 and 24 h post-infection. 8 out of 10 mice survived the infection (**b**). The mice were further evaluated for bacteremia 24 h after infection ($n = 10$) (**c**) and for visible disease signs ($n = 10$) (**d**). Error bars, mean ± s.e.m. $^{***}P < 0.001$; $^{*}P < 0.05$.

Adjunct therapy with liposomes

Antibiotics are the mainstay of treatment against bacterial infection but massive release of bacterial toxins owing to bacterial lysis during antibiotic therapy might result in more severe pathology than the infection itself[23,29]. Therefore, we investigated whether the combination of liposomes with antibiotic treatment could be beneficial in mouse *S. aureus* and *S. pneumoniae* bacteremia models.

Neither the very low doses of 12.5 mg/kg/injection of the liposomal mixture nor 100 mg/kg/injection vancomycin were sufficient to protect the mice from the lethal effects of a systemic *S. aureus* infection, whereas the combination of both provided complete protection (**Fig. 6a**). A combination of liposomal toxin-sequestration and antibiotic treatment was also most effective against fatal sepsis caused by *S. pneumoniae* (**Fig. 6b**). In the course of antibiotic-liposome combination therapy, pneumococcal loads in blood 24 h after infection were considerably reduced as compared to antibiotic alone and the pain score (i.e., signs of disease) of mice that were infected with *S. pneumoniae* were completely eradicated (**Fig. 6c**,**d**).

DISCUSSION

Survival of patients with bacteremia and sepsis is dependent on timely antimicrobial treatment. However, with an increasing number of antibiotic-resistant pathogens, the selection of empiric antimicrobial treatment has become difficult[30].

We have engineered artificial liposomal decoys that bind bacterial toxins and used them to treat fatal infections in mice. We show that the binding of toxins depends on the presence of certain lipids and/or liquid-ordered lipid microdomains within the liposomes, consistent with the makeup of host cell plasmalemma. Artificial liposomes efficiently sequestered multiple purified toxins belonging to different toxin classes with diverse modes of membrane-damaging action and protected human epithelial and endothelial cells as well as cells of the innate immune system from the toxin-induced lysis. Protection against CDCs by cholesterol-containing liposomes is consistent with the notion that these toxins bind directly to cholesterol[9]. However, as the relative ratio of cholesterol to phospholipid within lipid bilayers rather than its total concentration defined the protective potential of cholesterol-containing liposomes, it is conceivable that cholesterol-rich raft microdomains are preferentially targeted by these toxins *in vivo*. In addition, the preferential protection against α-hemolysin by cholesterol:sphingomyelin liposomes is consistent with the notion that α-hemolysin binds to sphingomyelin, which is clustered within raft microdomains[11].

Liposomes sequestered the full panoply of toxins secreted by individual strains of staphylococcal and streptococcal pathogens. Whereas a single toxin was responsible for the cytolytic activity of *S. pyogenes*, multiple toxins with different modes of plasma membrane binding were secreted by *S. aureus*. At least one staphylococcal toxin was sequestered by cholesterol-containing liposomes, whereas another toxin(s) bound to cholesterol-free, sphingomyelin-containing liposomes. The similarities between liposomal neutralization of bacterial culture supernatants and the neutralization of purified bacterial toxins allow, with a high degree of confidence, matching of the cytotoxic activity of *S. pneumoniae* and *S. pyogenes* to that of pneumolysin and streptolysin O, respectively. Phospholipase C or α-hemolysin might be responsible for the cytotoxic activity of staphylococci, although yet unidentified toxin(s) that bind to cholesterol-containing liposomes might also contribute to the cytotoxic activity of this pathogen. The nature of sphingomyelin-binding toxin(s) also requires definition. Although establishing the exact nature of cytotoxic agents that are targeted by cholesterol:sphingomyelin and sphingomyelin-only liposomes requires further investigation, this caveat will not compromise the applicability of toxin-sequestration as a therapeutic approach, because, at least during the onset of a therapeutic intervention, the exact nature of the bacterial pathogens and their individual toxins will be unknown. Most importantly, the mixture of cholesterol: sphingomyelin and sphingomyelin-only liposomes sequestered toxins that were secreted by all of the staphylococcal and streptococcal strains tested so far, including clinical isolates and antibiotic-resistant pathogens.

We show that upon binding of exotoxins by artificial liposomes, bacteria were efficiently cleared by the host's immune system in the mouse bacteremia models. Likewise, the mucosal, intranasal application of liposomes protected mice from invasive pneumococcal pneumonia, significantly reducing pneumococcal loads. Although local protection of the host epithelia may prevent bacterial invasion, binding of membrane-damaging toxins in the bloodstream may facilitate the resolution of severe infection and bacteremia by preventing the death of immune cells. Liposome-mediated prevention of uncontrolled release of pro-inflammatory mediators might also be crucial for host survival and may contribute to preventing the development of life-threatening cytokine storm and septic shock. Furthermore, removal of toxins at the time of antibiotic therapy is of paramount

importance, as the bacteriolytic nature of antibiotics can lead to acute release of bacterial toxins causing organ-specific defects.

We show that liposomal therapy is effective against Gram-positive pathogens. Two Gram-negative pathogens, *E. coli* and *P. aeruginosa*, were tested in our pilot study. However, these pathogens were not cytotoxic for THP-1 cells in our *in vitro* screening experiments. Therefore, tailoring of liposomal formulations, which is critical for *in vivo* testing, could not be carried out. We are currently testing alternative approaches for establishing liposomal formulations that can be used to sequester toxins secreted by Gram-negative pathogens.

Liposomes are currently used as carriers for drug delivery and are nontoxic for humans[31]. All components of our liposomal formulations are naturally occurring lipids that are present in the outer leaflet of the plasmalemmal lipid bilayer, that is, they are exposed to the systemic circulation *in vivo* and thus are nonimmunogenic and biologically neutral. Because the liposomes are not bactericidal, it is unlikely that they will exert evolutionary pressure that would select for the emergence of drug-resistant bacteria[32].

Our data provide a compelling argument for pursuing liposome-based, toxin-sequestrating therapy for use as a treatment for life-threatening bacterial infections. Liposomes could be used for the treatment of antibiotic-resistant bacterial pathogens, because they neutralize the bacterial toxins and allow time for the host immune system to eliminate the bacteria. The neutralization of toxins, however, is also important for the treatment of antibiotic-sensitive bacteria, as antibiotic-mediated killing of the bacteria may result in a massive release of toxins that results in deterioration of the clinical condition of the patient.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

B.D.H., D.R.N. and K.A.B. contributed equally to this study. B.D.H., K.A.B., R.Z., M.J.E. and E.G. performed *in vivo* experiments with *S. aureus*. D.R.N., S.G. and L.B.-M. performed *in vivo* experiments with *S. pneumoniae*. K.M. and J.S. provided bacterial culture supernatants for the *in vitro* experiments; J.S. performed MBC experiments; B.K. and L.J. performed bio-distribution experiments. M.L., H.W., A.D. and E.B.B. performed *in vitro* experiments; E.G. and A.K. designed the *in vivo* experiments with *S. aureus* and *S. pneumoniae*, respectively, and provided bacterial culture supernatants for the *in vitro* experiments. A.K. provided purified pneumolysin for *in vitro* experiments. E.B.B. designed the *in vitro* experiments. A.S. provided statistical advice for the paper. A.D. designed the study. E.B.B. designed and coordinated the study and wrote the paper. D.R.N., E.G., A.S., A.K. and A.D. edited and contributed to the writing of the paper. All authors analyzed and discussed the results and commented on the manuscript. E.G., A.K., A.D. and E.B.B. are co-senior authors.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the online version of the paper.

1. Stefani, S. & Goglio, A. Methicillin-resistant Staphylococcus aureus: related infections and antibiotic resistance. *Int. J. Infect. Dis.* **14**. (suppl. 4), S19–S22 (2010).
2. Bush, K. *et al.* Tackling antibiotic resistance. *Nat. Rev. Microbiol.* **9**, 894–896 (2011).
3. Alksne, L.E. & Projan, S.J. Bacterial virulence as a target for antimicrobial chemotherapy. *Curr. Opin. Biotechnol.* **11**, 625–636 (2000).
4. Titball, R.W. Bacterial phospholipases C. *Microbiol. Rev.* **57**, 347–366 (1993).
5. Kadioglu, A., Weiser, J.N., Paton, J.C. & Andrew, P.W. The role of Streptococcus pneumoniae virulence factors in host respiratory colonization and disease. *Nat. Rev. Microbiol.* **6**, 288–301 (2008).
6. Bubeck Wardenburg, J., Bae, T., Otto, M., DeLeo, F.R. & Schneewind, O. Poring over pores: α-hemolysin and Panton-Valentine leukocidin in *Staphylococcus aureus* pneumonia. *Nat. Med.* **13**, 1405–1406 (2007).
7. Timmer, A.M. *et al.* Streptolysin O promotes group A Streptococcus immune evasion by accelerated macrophage apoptosis. *J. Biol. Chem.* **284**, 862–871 (2009).
8. Gonzalez, M.R., Bischofberger, M., Pernot, L., van der Goot, F.G. & Freche, B. Bacterial pore-forming toxins: The (w)hole story? *Cell. Mol. Life Sci.* **65**, 493–507 (2008).
9. Parker, M.W. & Feil, S.C. Pore-forming protein toxins: from structure to function. *Prog. Biophys. Mol. Biol.* **88**, 91–142 (2005).
10. Tweten, R.K. Cholesterol-dependent cytolysins, a family of versatile pore-forming toxins. *Infect. Immun.* **73**, 6199–6209 (2005).
11. Valeva, A. *et al.* Evidence that clustered phosphocholine head groups serve as sites for binding and assembly of an oligomeric protein pore. *J. Biol. cholesterolem.* **281**, 26014–26021 (2006).
12. Linhartová, I. *et al.* RTX proteins: a highly diverse family secreted by a common mechanism. *FEMS Microbiol. Rev.* **34**, 1076–1112 (2010).
13. Olsen, I. & Jantzen, E. Sphingolipids in bacteria and fungi. *Anaerobe* **7**, 103–112 (2001).
14. Simons, K. & Ikonen, E. Functional rafts in cell membranes. *Nature* **387**, 569–572 (1997).
15. Brown, D.A. & Rose, J.K. Sorting of GPI-anchored proteins to glycolipid-enriched membrane subdomains during transport to the apical cell surface. *Cell* **68**, 533–544 (1992).
16. Harder, T. & Simons, K. Caveolae, DIGs, and the dynamics of sphingolipid-cholesterol microdomains. *Curr. Opin. Cell Biol.* **9**, 534–542 (1997).
17. Simons, K. & Gerl, M.J. Revitalizing membrane rafts: new tools and insights. *Nat. Rev. Mol. Cell Biol.* **11**, 688–699 (2010).
18. Klose, C. *et al.* Yeast lipids can phase-separate into micrometer-scale membrane domains. *J. Biol. Chem.* **285**, 30224–30232 (2010).
19. Huang, J., Buboltz, J.T. & Feigenson, G.W. Maximum solubility of cholesterol in phosphatidylcholine and phosphatidylethanolamine bilayers. *Biochim. Biophys. Acta* **1417**, 89–100 (1999).
20. Dietschy, J.M. & Turley, S.D. Cholesterol metabolism in the central nervous system during early development and in the mature animal. *J. Lipid Res.* **45**, 1375–1397 (2004).
21. Ikonen, E. Cellular cholesterol trafficking and compartmentalization. *Nat. Rev. Mol. Cell Biol.* **9**, 125–138 (2008).
22. Mesmin, B. & Maxfield, F.R. Intracellular sterol dynamics. *Biochim. Biophys. Acta* **1791**, 636–645 (2009).
23. Rittirsch, D., Flierl, M.A. & Ward, P.A. Harmful molecular mechanisms in sepsis. *Nat. Rev. Immunol.* **8**, 776–787 (2008).
24. Stringaris, A.K. *et al.* Neurotoxicity of pneumolysin, a major pneumococcal virulence factor, involves calcium influx and depends on activation of p38 mitogen-activated protein kinase. *Neurobiol. Dis.* **11**, 355–368 (2002).
25. Spreer, A. *et al.* Reduced release of pneumolysin by *Streptococcus pneumoniae* in vitro and in vivo after treatment with nonbacteriolytic antibiotics in comparison to ceftriaxone. *Antimicrob. Agents Chemother.* **47**, 2649–2654 (2003).
26. Wall, E.C. *et al.* Persistence of pneumolysin in the cerebrospinal fluid of patients with pneumococcal meningitis is associated with mortality. *Clin. Infect. Dis.* **54**, 701–705 (2012).
27. Bakker-Woudenberg, I.A., ten Kate, M.T., Guo, L., Working, P. & Mouton, J.W. Improved efficacy of ciprofloxacin administered in polyethylene glycol-coated liposomes for treatment of *Klebsiella pneumoniae* pneumonia in rats. *Antimicrob. Agents Chemother.* **45**, 1487–1492 (2001).
28. Brisseau, G.F. *et al.* Unilamellar liposomes modulate secretion of tumor necrosis factor by lipopolysaccharide-stimulated macrophages. *Antimicrob. Agents cholesterolemother.* **38**, 2671–2675 (1994).
29. Moore, C.L. *et al.* Prediction of failure in vancomycin-treated methicillin-resistant *Staphylococcus aureus* bloodstream infection: a clinically useful risk stratification tool. *Antimicrob. Agents Chemother.* **55**, 4581–4588 (2011).
30. Huttunen, R. & Aittoniemi, J. New concepts in the pathogenesis, diagnosis and treatment of bacteremia and sepsis. *J. Infect.* **63**, 407–419 (2011).
31. Drulis-Kawa, Z. & Dorotkiewicz-Jach, A. Liposomes as delivery systems for antibiotics. *Int. J. Pharm.* **387**, 187–198 (2010).
32. Allen, R.C., Popat, R., Diggle, S.P. & Brown, S.P. Targeting virulence: can we make evolution-proof drugs? *Nat. Rev. Microbiol.* **12**, 300–308 (2014).

ONLINE METHODS

This study was performed with the approval of and in strict accordance with the UK Home Office and the University of Liverpool ethics committee (Home Office Project License Number 40/3602) as well as the Bezirksregierung Düsseldorf and the University of Duisburg-Essen ethics committee (protocol number Az. 84-02.04.2013.A282) or the University of Cincinnati ethics committee (IACUC# 10-05-10-01). Every effort was made to minimize suffering, and in bacterial infection experiments, mice were humanely euthanized if they became lethargic. All animal experiments were carried out at the Universities of Liverpool, Duisburg-Essen and Cincinnati.

Reagents. Streptolysin O from *S. pyogenes*, α-hemolysin from *S. aureus*, tetanolysin from *Clostridium tetani*, phospholipase C from *Clostridium perfringens* were from Sigma. Pneumolysin was prepared as previously described[33]. HDL (3.2 mg/ml cholesterol) was from Calbiochem. LPS from *E. coli* or *P. aeruginosa* was from Sigma. Deuterated sphingomyelin (16:0-d31) was from Avanti Polar Lipids.

Cells. The human embryonic kidney cell line (HEK 293; ATCC CRL-1573) was maintained as previously described[34]. Human umbilical vein endothelial cells (HUVEC) were obtained from Promocell and maintained in Promocell's Endothelial Cell Growth Medium. The human acute monocytic leukemia cell line (THP-1; ATCC TIB-202) was maintained in RPMI 1640 medium containing 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin.

Bacterial cultures and supernatants. Bacterial culture supernatants were prepared from *S. pyogenes* strains 50362, 31009 (clinical isolates from blood culture), 30979 (clinical isolate from tissue biopsy), 31116 (clinical isolate from nasopharynx) and DSM 11728; *S. aureus* strains MRSA 2040, MRSA 3208.21 (clinical isolate from nasopharynx), MRSA 3108.46 (clinical isolate from a wound) and a methicillin-sensitive *S. aureus* strain isolated from a septic patient (septic *S. aureus* strain) as well as from *S. pneumoniae* (clinical isolate, 111.46). The septic *S. aureus* strain, also used for *in vivo* experiments, produced enterotoxin D and was tested negative for Panton-Valentin-leukocidin (PVL) and toxic shock syndrome toxin (TSST).

The bacteria were grown overnight on blood agar plates, resuspended at an OD of 0.225 in 40 ml tryptic soy broth (TSB) and allowed to grow at 37 °C with shaking at 125 r.p.m. to an OD_{600} = 0.6–0.8. Bacterial cultures were centrifuged at 2,800 r.p.m. for 10 min at room temperature. Culture supernatants were harvested and stored at −70 °C.

Liposomes. Cholesterol, sphingomyelin from egg yolk, phosphatidylcholine from egg yolk and phospatidylserine (sodium salt) from bovine brain were from Sigma. The lipids were individually dissolved in chloroform at 1 mg/ml concentrations. For the preparation of liposomes the chloroform solutions of the individual lipids were mixed in the composition and the proportions, which are given in the text. Chloroform was completely evaporated for 30 min at 60 °C, followed by 2 h under vacuum. Tyrode's buffer (140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM glucose, 10 mM HEPES; pH = 7.4) containing 2.5 mM $CaCl_2$ was added to the films of dried lipids. Following incubation for 30 min at 45 °C in an Eppendorf thermomixer with vigorous shaking, the liposomes were sonicated three times for 5 s at 6 °C in a Bandelin Sonopuls sonicator at 80% power. The liposomal preparations were left for at least 1 h at 6 °C before they were used in experiments.

Alternatively, unilamellar cholesterol:sphingomyelin (66 mol/% cholesterol; mean diameter = 130 nm) and sphingomyelin (100%; mean diameter = 90 nm) liposomes, produced according to a good manufacturing practice (GMP)-compliant process protocol, were provided by Lascco (Geneva, Switzerland – product name CAL02). The amounts of liposomes are given as the amount of total lipids used for their preparation.

Toxin-induced cell lysis and protective effects of liposomes *in vitro*. The effects of purified toxins or bacterial culture supernatants on the proliferation of a human monocyte cell line (THP-1) were assessed in the presence or absence of liposomes of various lipid compositions. Routinely, 100–200 µl of a purified toxin solution or a bacterial culture supernatant (Ca^{2+}-Tyrode's buffer or bacterial culture broth were used as controls) were added to 100 µl (5×10^4 cells/ml or 5×10^5 cells/ml) of cells pre-mixed with 50 µl of liposomes. After incubation for 3 h, 2 ml of cell culture medium was added to the tubes. The cells were counted for 5–14 d. The toxins and the liposomes were present for the whole duration of an experiment.

In human embryonic kidney epithelial cells (HEK 293) and in human umbilical vein endothelial cells (HUVEC), toxin-induced lysis was monitored as a decline of cytoplasmic fluorescence due to an efflux of intracellular GFP (green-fluorescent protein) or YFP (yellow-fluorescent protein). GFP or YFP were transiently expressed as described[34]. GFP- or YFP-expressing cells were used for laser scanning module (LSM) imaging experiments 2 d after transfection. Cells, seeded on 15 mm glass coverslips (5×10^5 cells per coverslip), were mounted in a perfusion chamber at 25 °C in 200 µl of Tyrode's buffer containing 2.5 mM $CaCl_2$, and their fluorescence was recorded in an Axiovert 200 M microscope with a laser scanning module LSM 510 META (Zeiss) using a × 63 oil immersion lens. At time-point 0, the buffer was replaced by 100 µl of solution containing liposomes, followed (with 20–30 s of handling delay) by 100 µl of the toxin-containing solution. The images were analyzed using the "Physiology evaluation" software package (Zeiss).

Pneumolysin binding to Ch:Sm liposomes. Pneumolysin was diluted in Ca^{2+}-Tyrode's buffer and centrifuged at 100,000*g* for 1 h to remove aggregated protein.

In a direct binding assay, 200 ng of pneumolysin was incubated with 0–9 µg of cholesterol:sphingomyelin liposomes (66 mol/% cholesterol) for 20 min at 20 °C. Liposomes were pelleted by centrifugation at 100,000*g* for 2 h. pneumolysin content in the pellets and in the supernatants was assessed by western blot analysis.

In a competition binding assay, 100 ng of pneumolysin was incubated with or without THP-1 cells (5×10^5 cells/ml) and with or without 100 µg of cholesterol:sphingomyelin liposomes (66 mol/% cholesterol) for 20 min at 20 °C. The samples were centrifuged at 134 *g*; supernatants were collected and further centrifuged at 100,000*g* for 2 h. Low-speed and high-speed pellets were dissolved in 50 µl of SDS-PAGE sample buffer. The protein of the high-speed supernatants was precipitated by 10% trichloroacetic acid in the presence of 1 mg/ml bovine serum albumin, pelleted and dissolved in 50 µl of SDS-PAGE sample buffer. Pneumolysin content in the samples was assessed by western blot analysis. Monoclonal antibody against pneumolysin (1F11; Santa Cruz Biotechnology) was used at 1:500 dilution.

Release of CXCL8 was measured in culture supernatants of HUVEC cells 24 h after treatment with 15 ng (30 ng/ml) of pneumolysin in the presence or absence of 40 µg/ml cholesterol:sphingomyelin (66 mol/% cholesterol) liposomes by Bioplex assay according to the manufacturer's instructions.

Protective effects of liposomes *in vivo*. *6- to 8-week-old, age matched, C57BL6/J male mice were used for S. aureus in vivo infection experiments.* All experiments undertaken were approved by local authorities. *S. aureus* were grown overnight on blood agar plates, the bacteria were resuspended at an OD of 0.225 in 40 ml TSB and allowed to grow at 37 °C with shaking at 125 r.p.m. to the early logarithmic phase for 1 h. Bacterial cultures were then pelleted by centrifugation at 2,800*g* r.p.m. and washed in phosphate buffered saline. 3×10^6 CFU bacteria were resuspended in 100 µl of 0.9% saline and injected intravenously into mice. A 1:1 mixture of cholesterol:sphingomyelin (66 mol/% cholesterol) and sphingomyelin-only liposomes was injected at the indicated times and doses in 0.9% saline. Controls received saline only. Survival was determined over 7 d.

6- to 8-week-old, age matched, MF1 (Charles River, UK) female mice were used for S. pneumoniae infection experiments. Bacterial inoculum for infection was prepared from stocks frozen in mid-log phase. Aliquots were thawed, centrifuged (13,000*g*, 2 min) and washed with endotoxin-free PBS before infection. For induction of pneumococcal pneumonia, mice were anesthetized with a mixture of O_2 and isofluorane and infected intranasally with 1×10^6 CFU *S. pneumoniae* serotype 2 (strain D39) in PBS. Liposome preparations were administered intranasally 30 min after infection. For induction of bacteremia, 1×10^6 CFU D39 were injected into the tail vein. Liposome preparations were

administered intravenously at the indicated times and doses. Tail bleeds were done 24 h after infection to assess bacteremia.

In all experiments mice were monitored for signs of disease using the scheme of Morton[35] and were euthanized once disease had reached the "lethargic" state.

Biodistribution of liposomes. Cholesterol:sphingomyelin (66 mol/% cholesterol) and sphingomyelin-only liposomes, each containing 6% of deuterated sphingomyelin were prepared as described above. Healthy mice and mice that were intravenously infected with *S. aureus* were injected with 100 mg/kg of the deuterated liposomes. Mice were euthanized immediately, 6.5 h or 24 h after liposomal injection ($n = 4$ for each time point). Blood, lungs, kidneys, aortas, livers, intestines and spleens were removed and analyzed by mass spectrometry. 16:0-d31 sphingomyelin was extracted and quantified as recently described[36]. Sample analysis was carried out by rapid resolution liquid chromatography-MS/MS using a Q-TOF 6530 mass spectrometer (Agilent Technologies) operating in the positive electrospray ionization mode. Chromatographic separation on the Zobrax Eclipse column (C8, 2.1 × 150 mm, 3.5 µm particle size; Agilent Technologies) was achieved by gradient elution using mobile-phase A (water/formic acid 100:0.1 v/v) and mobile-phase B (acetonitrile/methanol/formic acid 50:50:0.1 v/v). The precursor ions of 12:0 sphingomyelin (m/z 647.5123) as internal standard and 16:0-d31 sphingomyelin (m/z 734.7619) were cleaved into the fragment ion of m/z 184.3. Quantification was performed with Mass Hunter Software (Agilent Technologies).

Antibiotic treatment. In the pneumococcal sepsis model, penicillin (25 mg/kg) was given intraperitoneally (i.p.) at 10 and 24 h after infection. In the staphylococcal sepsis model, vancomycin (100 mg/kg) was given i.p. at 1 and 9 h after infection.

Injection of bacterial supernatants. *S. aureus* was grown overnight on blood agar plates. Bacteria were resuspended at an OD of 0.225 in 40 ml TSB and allowed to grow at 37 °C with shaking at 125 r.p.m. to the early logarithmic phase for 1 h. Bacteria were centrifuged at 3,200 r.p.m., the supernatant collected, centrifuged again, a 150 µl aliquot of the supernatant collected and incubated for 4 h with 100 mg/kg liposomes consisting of a 1:1 mixture of cholesterol:sphingomyelin (66 mol/% cholesterol) and sphingomyelin-only liposomes at 37 °C. Controls were supplemented with the same volume PBS. After the 4 h incubation, samples were centrifuged at 20,000*g* and 150 µl of supernatant was injected into mice i.v.

Liposomal treatment of LPS-septic mice. LPS from *E. coli* or *P. aeruginosa* was injected i.p. 45 min before intravenous application of liposomes.

TNF-alpha release. TNF-α was measured in lung homogenates or serum (diluted 1:10 in PBS) obtained from healthy mice; healthy liposome-treated mice; mice infected with *S. pneumoniae* D39; and D39-infected mice that were treated with the liposomes by enzyme-linked immunosorbent assay (ELISA) (BioLegend), according to manufacturer's instructions.

Blood leukocytes. Blood leukocytes were counted in lung homogenates or serum (diluted 1:10 in PBS) by flow cytometry. Mouse tissue cell suspensions, at 2×10^8 cells/ml were incubated with purified anti-Fc receptor blocking antibody (anti-CD16/CD32) before addition of antibodies against cell surface markers. A combination of fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, PE-Cy7- and allophycocyanin (APC)-conjugated monoclonal antibodies (eBioscience) were used. CD19 was used to identify B cells, CD3, CD4 and CD8 to identify T cells and Gr-1 and CD11b to identify neutrophils. In each experiment the appropriate monoclonal control antibodies and single conjugate controls were included. Alternatively, blood leukocytes were quantified by their typical forward versus sideward scatter using flow cytometry. Samples were analyzed using a Becton Dickinson FACScalibur flow cytometer running CellQuest acquisition and analyzed using FlowJo software (Tree Star).

Lung edema *in vivo* after *S. aureus* infection. *S. aureus* was grown overnight on blood agar plates, the bacteria were resuspended at an OD of 0.225 in 40 ml TSB and allowed to grow at 37 °C with shaking at 125 r.p.m. to the early logarithmic phase for 1 h. Bacteria were centrifuged at 2,800 r.p.m., washed once in PBS and 5×10^6 CFU were i.v. injected in a volume of 100 µl. Controls were injected with PBS. We then determined survival and lung edema by measuring wet and dry weight as well as Evans Blue leakage 12 h after the infection. Liposomes (100 mg/kg; 1:1 mixture of cholesterol:sphingomyelin (66 mol/% cholesterol) and sphingomyelin-only liposomes) were injected 2 and 6 h after the infection. Evans Blue (20 mg/kg) was i.v. injected, mice were euthanized after 10 min, the lung was flushed with PBS via the right heart, removed and dried for 2 d at 56 °C. The dried tissue was suspended in 0.8 ml formamide per 100 mg dry tissue and incubated for 24 h at 60 °C. The OD of the samples was then analyzed by 620 nm and the concentration determined using a standard curve of Evans Blue.

Killing capacity of blood samples. 40 µl of mouse blood were incubated with 10,000 CFU *S. aureus* at 37 °C for 4 h with shaking at 125 r.p.m. in the presence of either liposomes at 1 mg/ml (1:1 mixture of cholesterol:sphingomyelin (66 mol/% cholesterol) and sphingomyelin-only liposomes) or the same volume PBS. After the incubation, aliquots were plated on LB plates, grown o/n and the CFU were counted.

NO release. Blood samples were taken from healthy mice and we performed a Percoll density gradient centrifugation to purify neutrophils. Neutrophils were incubated *ex vivo* with 5 µM 4-amino-5-methylamino-2′,7′-difluorofluorescein (DAFFM) (Molecular Probes) at 37 °C for 30 min. DAFFM is nonfluorescent and reacts with NO to a fluorescent substrate. Samples were washed with PBS and then further incubated with 0.5 mg/ml of each cholesterol:sphingomyelin (66 mol/% cholesterol) and sphingomyelin-only liposomes (1 mg/ml total) at 37 °C for 30 min or with the same volume of PBS. They were then challenged with *S. aureus* at an multiplicity of infection (MOI) of 1 cell per 10 bacteria and NO was determined over a period of 90 min in the presence or absence of L-NAME, an i-NOS inhibitor. Samples were then analyzed by fluorescence-activated cell sorting (FACS). The relative NO production induced by *S. aureus*, as is indicated by the change of the fluorescence of the DAFFM, was calculated by using the formula ΔDAFFM mean fluorescence after *S. aureus* = mean fluorescence after *S. aureus* – mean fluorescence without infection. Fluorescence intensity of the L-NAME group was calculated as following: ΔDAFFM mean fluorescence after *S. aureus* + L-NAME = mean fluorescence after *S. aureus* + L-NAME – mean fluorescence L-NAME only.

ROS release. Blood samples were taken from healthy mice, and we performed a Percoll density gradient centrifugation to obtain neutrophils. The cells were incubated *ex vivo* with 10 µM 2′,7′ dichlorodihydrofluorescein diacetate acetyl ester (DCF) (H2DCFDA, Molecular Probes) at 37 °C for 30 min, incubated with 0.5 mg/ml of each liposome (1 mg/ml total as above) at 37 °C for 30 min or with the same volume of PBS, challenged with *S. aureus* at an MOI of 1 cell per 10 bacteria and ROS was determined over a period of 60 min. Controls were incubated with 100 U/ml superoxide dismutase (SOD) and 10 U/ml catalase. Samples were analyzed by FACS. The relative ROS production induced by *S. aureus*, as is indicated by the change of the fluorescence of the DCF, was calculated by using the formula ΔDCF mean fluorescence = mean fluorescence after *S. aureus* - mean fluorescence after [*S. aureus* + SOD + catalase].

Bactericidal activity of liposomes. To define the minimum bactericidal concentration (MBC), we followed the guidelines proposed by the Clinical and Laboratory Standards Institute[37]. For the broth microtiter dilution tests, 96-well plates supplemented with 50 µl of 0.5–16 mg/ml liposomes were inoculated with 50 µl of Mueller Hinton broth containing a bacterial cell suspension of $1–5 \times 10^5$ colony-forming units (CFU)/ml. The plates were incubated for 24 h at 36 °C. MBC was tested by transferring 10 µl aliquots from the wells of the broth microtiter dilution plates onto Columbia blood agar (Oxoid). The inoculated plates were further incubated for 24 h at 36 °C and then colonies were counted.

Statistical analysis, sample size planning and measures against bias. For the comparison of continuous variables from independent groups we used Student's *t*-test for two groups and one-way ANOVA for more than two groups followed by post-hoc Student's *t*-tests for all pairwise comparisons applying

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Bonferroni correction for multiple testing. Between-group comparisons of survival variables was performed by log-rank test.

As sensitivity analyses, which generally supported the claim of the prespecified analyses, we applied Welch's *t*-tests and exact Wilcoxon-Mann-Whitney tests for continuous variables and the latter also for survival variables given that no censoring events happened during the follow-up. Furthermore, to follow our sample size plan, we also applied Fisher's exact test to compare the survival rates at the last observation between groups. All reported *P* values are two-sided and corrected for comparison-wise multiplicity.

The sample size planning of the mouse experiments with outcome death was based on two-sided Fisher's exact tests (i.e., a comparison of event rates as suggested by Shah[38]) even though the pre-planned analyses was for survival times. When aiming to detect a between-group difference in survival rates between 15% and 90% (at the last follow-up, assuming no censoring events before), ten animals per group (1:1 allocation) are required for a comparison-wise power of 90% at a significance level $\alpha = 5\%$ (two-sided); for $n = 8/n = 12$ per group the power will be about 87%/98%. The sample size planning for the continuous variables in, for example, the *S. aureus in vivo* infection experiments, was based on two-sided Wilcoxon-Mann-Whitney tests (software: G*Power Version 3.1.7 of the University of Duesseldorf, Germany). When aiming at detecting a large standardized effect of 2.5 (in units of a s.d. of a standard normal distribution) between means of two groups, four animals per group (1:1 allocations) are required for a comparison-wise power of 80% at a significance level $\alpha = 5\%$ (two-sided). Thus, the animal experiments were powered to detect large effects.

Mice were divided into cages of equal size (usually three to five mice) on arrival by animal unit technical staff with no involvement in study design. Thus, for a single experiment, all mice used were of the same age and sex and were within 2 g weight of each other. Cages were numbered and the numbers randomly assigned to an experimental treatment group. The investigators were not blinded to the group allocation during the experiment and/or when assessing the outcome.

For the pain score, mice were scored separately by two independent researchers and one animal unit technical staff member with no involvement in study design and recorded.

Investigators were blinded for histology and Evans Blue experiments.

33. McNeela, E.A. *et al.* Pneumolysin activates the NLRP3 inflammasome and promotes proinflammatory cytokines independently of TLR4. *PLoS Pathog.* **6**, e1001191 (2010).
34. Babiychuk, E.B., Monastyrskaya, K., Potez, S. & Draeger, A. Intracellular Ca(2+) operates a switch between repair and lysis of streptolysin O-perforated cells. *Cell Death Differ.* **16**, 1126–1134 (2009).
35. Morton, D.B. Pain and laboratory animals. *Nature* **317**, 106 (1985).
36. Merrill, A.H. Jr., Sullards, M.C., Allegood, J.C., Kelly, S. & Wang, E. Sphingolipidomics: high-throughput, structure-specific, and quantitative analysis of sphingolipids by liquid chromatography tandem mass spectrometry. *Methods* **36**, 207–224 (2005).
37. Clinical and Laboratory Standards Institute. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically – Twenty second information supplement: Approved standard M07–A8. (CLSI, Wayne, PA, USA, 2009).
38. Shah, H. How to calculate sample size in animal studies. *Natl. J. Physiol. Pharm. Pharmacol.* **1**, 35–39 (2011).