

The Ashwell receptor mitigates the lethal coagulopathy of sepsis

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The Ashwell receptor, the major lectin of hepatocytes, rapidly clears from blood circulation glycoproteins bearing glycan ligands that include galactose and *N*-acetylgalactosamine. This asialoglycoprotein receptor activity remains a key factor in the development and administration of glycoprotein pharmaceuticals, yet a biological purpose of the Ashwell receptor has remained elusive. We have identified endogenous ligands of the Ashwell receptor as glycoproteins and regulatory components in blood coagulation and thrombosis that include von Willebrand factor (vWF) and platelets. The Ashwell receptor normally modulates vWF homeostasis and is responsible for thrombocytopenia during systemic *Streptococcus pneumoniae* infection by eliminating platelets desialylated by the bacterium's neuraminidase. Hemostatic adaptation by the Ashwell receptor moderates the onset and severity of disseminated intravascular coagulation during sepsis and improves the probability of host survival.

The liver controls the removal of exogenously administered glycoproteins from circulation, as discovered over 35 years ago^{1–4}. These classical investigations identified the first vertebrate lectin as a hepatic receptor for glycoproteins bearing glycan chains that lack sialic acid, termed asialoglycoproteins^{5,6}. Today, the Ashwell receptor is known to be one of multiple lectins of the C-type lectin family that bind asialoglycoproteins, and its activity remains a fundamental consideration in the design of clinical treatments to provide therapeutic levels of glycoproteins in circulation^{7,8}. Mammalian asialoglycoprotein receptors (ASGPRs) mediate the capture and endocytosis of a wide range of exogenously administered glycoproteins that have galactose or *N*-acetylgalactosamine residues at the termini of their glycan chains. More recent findings have indicated that some sialylated glycans are also ligands for the Ashwell receptor⁹. Localized to the vascular face of the hepatocyte cell surface, the Ashwell receptor is positioned to remove and degrade potentially deleterious circulating glycoproteins^{6,10,11}. Nonetheless, the biological purpose of this lectin has remained an enigma. Endogenous ligands have not been identified, and the conservation of the Ashwell receptor throughout vertebrate evolution remains unexplained.

The Ashwell receptor is composed of type 2 transmembrane glycoproteins termed asialoglycoprotein receptor-1 (Asgr-1) and asialoglycoprotein receptor-2 (Asgr-2) that are encoded by distinct but closely linked genes, with variation in Asgr-2 structure due to RNA splicing^{12–14}. Both *Asgr1* and *Asgr2* are highly conserved among mammalian species and may have originated from a single ancestral gene^{15–17}. Although *Asgr1* and *Asgr2* are detectable in some extrahepatic tissues, they are predominantly expressed in the liver and are often used as markers of hepatocytes. Oligomerization of the Asgr-1

and Asgr-2 glycoproteins has been observed in various cellular contexts, with these findings supporting the possibility that Ashwell receptors may exist as Asgr-1–Asgr-2 hetero-oligomers, Asgr-1 homotrimers and homotetramers, and Asgr-2 homodimers and homotetramers, perhaps thereby altering substrate selectivity, binding affinities and rates of endocytosis^{18–24}. Notably, although mice deficient in either *Asgr-1* or *Asgr-2* show decreased clearance of exogenous desialylated glycoproteins, they do not accumulate endogenous asialoglycoproteins in the circulation, and they lack phenotypic abnormalities^{25–27}. Because α 2,3-linked sialic acid can mask underlying ASGPR ligands on glycoproteins, we suspected that reducing the expression of these sialic acid linkages *in vivo* among endogenous glycoproteins might unmask ligand identity and thereby facilitate investigations of Ashwell receptor function in biology and disease.

The ST3Gal family of sialyltransferases includes enzymes that add sialic acid in α 2,3 linkage to galactose residues at the termini of *N*- and *O*-glycan chains and that can thereby mask endogenous ASGPR ligands. Thus, a genetic approach to disrupt sialyltransferase function in the mammalian germline might expose endogenous ligands for one or more lectins with asialoglycoprotein-binding activity, including the Ashwell receptor, the Kupffer cell receptor, the macrophage galactose receptors and the galectins. Indeed, when the *St3gal4*-encoded sialyltransferase (ST3Gal-IV) is limiting or absent, such ligands appear on a subset of regulatory and prothrombotic components of the mammalian blood coagulation system, including vWF and platelets²⁸. ST3Gal-IV deficiency causes prolonged bleeding and coagulation times attributable to ASGPR-mediated clearance of these clotting factors from circulation²⁸. These studies have revealed that endogenous ASGPR activity is poised to cause rapid hemostatic modulation in

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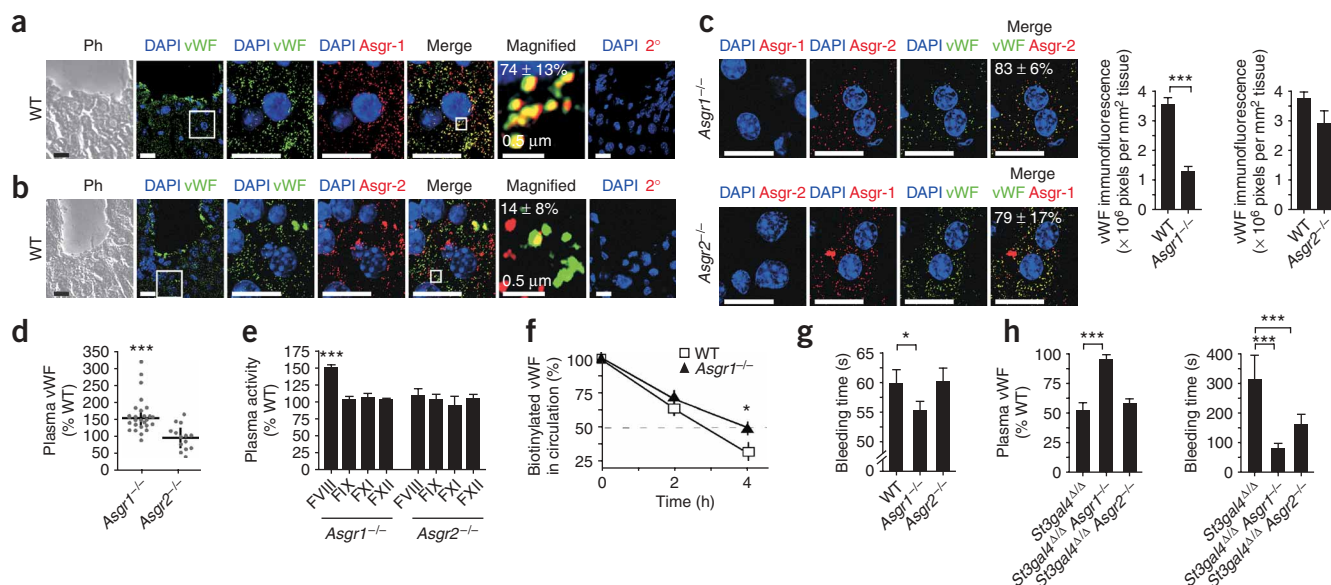


Figure 1 Ashwell receptors of hepatocytes modulate vWF homeostasis and blood coagulation. **(a–c)** Liver sections from 8-week-old WT C57BL/6NHsd and Asgr-deficient mice imaged by phase-contrast (Ph) and fluorescent deconvolution microscopy using antibodies to vWF (green) and Asgr-1 or Asgr-2 (red); DNA is stained by DAPI (blue). The percentage of vWF colocalized (yellow) with Asgr-1 **(a)** or Asgr-2 **(b)** in WT hepatocytes is indicated. Magnified views of the boxed regions are shown. Staining with Texas Red-conjugated secondary antibody to goat IgG (2°) alone is also shown. vWF abundance in Asgr-1-deficient hepatocytes compared to WT or Asgr-2-deficient hepatocytes is quantified **(c)**. The percentage of vWF colocalized in Asgr-1-deficient or Asgr-2-deficient hepatocytes is indicated. The micrographs shown are representative of ten fields of view obtained from three mice of each genotype. All scale bars denote 5 μm unless otherwise indicated. **(d)** Plasma vWF abundance in mice lacking either Asgr-1 or Asgr-2. Horizontal bars indicate median vWF abundance, and vertical bars denote the interquartile range. **(e)** Coagulation factor measurements in mice lacking either Asgr-1 or Asgr-2 compared with WT littermates, $***P < 0.001$. **(f)** Half-life of biotinylated plasma vWF from WT mice transfused into either WT or Asgr-1-deficient recipients. Plots represent data from eight WT and nine Asgr-1-deficient recipients. **(g,h)** Bleeding times **(g)** and bleeding times and plasma vWF abundance **(h)** in mice of the indicated genotypes. Mice homozygous for a null (deletion) mutation in the *St3gal4* gene are denoted as *St3gal4* ^{Δ/Δ} . Histograms include data from 20–25 mice of each genotype. All values are means \pm s.e.m.; $***P < 0.001$; $*P < 0.05$.

response to a reduced sialylation state of platelets, vWF and possibly other blood components. Here we investigate whether vWF and platelets may be endogenous ligands of the Ashwell receptor and whether they are subject to clearance during pathologic conditions of rapid desialylation, such as bloodstream infection with a microbe expressing sialidase (neuraminidase) activity.

RESULTS

Ashwell receptors govern vWF homeostasis

We observed localization of the vWF glycoprotein *in situ* primarily among liver endothelial cells, but also among hepatocytes; in which the majority of vWF colocalized with the Asgr-1 chain of the Ashwell receptor (**Fig. 1a**). In contrast, there was markedly less colocalization of hepatocyte vWF with Asgr-2 (**Fig. 1b**). In mice homozygous for null mutations in either *Asgr1* or *Asgr2* (refs. 25,29), the other closely linked gene retained function, and the corresponding Asgr glycoprotein remained expressed (**Fig. 1c**). Furthermore, the absence of Asgr-1, but not of Asgr-2, reduced the total amount of vWF associated with hepatocytes, resulting in an increased percentage of the remaining vWF that colocalized with Asgr-2 (**Fig. 1c**).

Circulating plasma vWF abundance was increased 1.5-fold in mice lacking Asgr-1 in comparison either to mice lacking Asgr-2 or wild-type (WT) littermates (**Fig. 1d**). The elevated vWF in Asgr-1 deficiency was paralleled by an increased abundance of plasma factor VIII, a procoagulation factor that binds to and is stabilized by vWF in the circulation (**Fig. 1e**). No changes in vWF or factor VIII were observed in Asgr-2-deficient mice, and factor IX, factor XI and factor XII were unaffected in mice with null mutations in either *Asgr1* or *Asgr2*

(**Fig. 1e**). Elevated abundance of plasma vWF correlated with increased vWF half-life in the circulation (**Fig. 1f**). Nonetheless, the elevation of vWF levels did not result in an increase in the frequency of asialo-vWF (data not shown). These findings reveal that the Ashwell receptor is normally engaged in the control of endogenous vWF clearance and homeostasis, implying a receptor-ligand relationship and suggesting a role in modulating blood coagulation and thrombosis.

Functional intersection of the Ashwell receptor and ST3Gal-IV

Mice lacking Asgr-1 had a reduced bleeding time compared to WT littermates, whereas their counterparts lacking Asgr-2 were unaffected (**Fig. 1g**). Because ASGPR-mediated clearance of vWF occurs in ST3Gal-IV sialyltransferase deficiency, we hypothesized that the Ashwell receptor and ST3Gal-IV may jointly control endogenous vWF homeostasis. We studied mice bred to lack ST3Gal-IV function as well either Asgr-1 or Asgr-2. Asialo-vWF was detected by the absence of sialic acid linkages at the termini of various glycan chains using plant-derived galactose binding lectins as previously described²⁸ and was the dominant vWF glycoform in circulation in all strains of mice lacking ST3Gal-IV function (data not shown). Notably, the diminished abundance and shortened half-life of plasma vWF due to ST3Gal-IV deficiency were restored to normal by the additional loss of Asgr-1, whereas, in contrast, loss of Asgr-2 did not result in such a rescue (**Fig. 1h** and data not shown). Moreover, Asgr-1 deficiency also corrected the prolonged bleeding and activated partial thromboplastin times in the absence of ST3Gal-IV, which is consistent with normalization

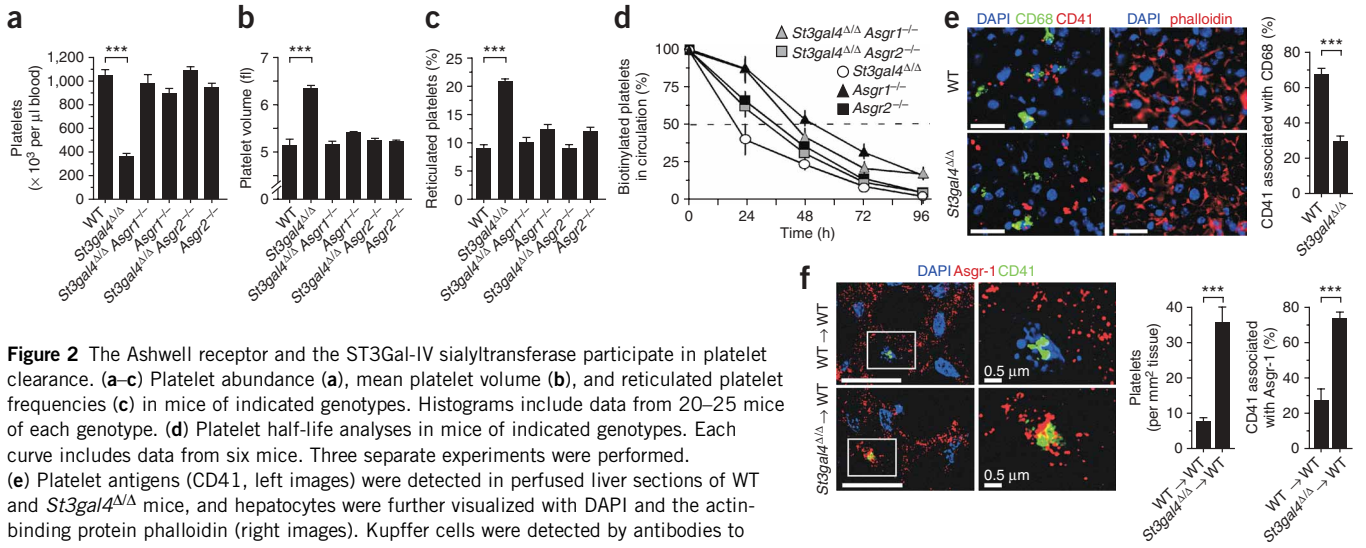


Figure 2 The Ashwell receptor and the ST3Gal-IV sialyltransferase participate in platelet clearance. **(a–c)** Platelet abundance **(a)**, mean platelet volume **(b)**, and reticulated platelet frequencies **(c)** in mice of indicated genotypes. Histograms include data from 20–25 mice of each genotype. **(d)** Platelet half-life analyses in mice of indicated genotypes. Each curve includes data from six mice. Three separate experiments were performed. **(e)** Platelet antigens (CD41, left images) were detected in perfused liver sections of WT and *St3gal4 Δ/Δ* mice, and hepatocytes were further visualized with DAPI and the actin-binding protein phalloidin (right images). Kupffer cells were detected by antibodies to CD68 (green). The images shown are representative of multiple fields of view from three mice of each genotype, comprising a total of 200 separate CD41 signals. **(f)** Sections of perfused liver were analyzed from WT recipients of biotinylated platelet-rich plasma from donors of the indicated genotypes to detect platelets (CD41, green) and Asgr-1 (red). Images are representative of multiple fields of view from three mice of each genotype, comprising a total of 46 separate CD41 signals. Scale bars denote 5 μ m unless otherwise indicated. ****P* < 0.001. All values are means \pm s.e.m.

of circulating vWF and consequently factor VIII abundance (**Fig. 1h** and data not shown).

Unexpectedly, mice lacking *Asgr-2* and ST3Gal-IV, despite their vWF deficit, had a marked reduction in bleeding time compared to

mice lacking only ST3Gal-IV (**Fig. 1h**). Because platelets that are deficient in sialic acid linkages (asialo-platelets) are also found in the circulation of mice lacking ST3Gal-IV function, with resultant thrombocytopenia (low platelet count) due to ASGPR-mediated

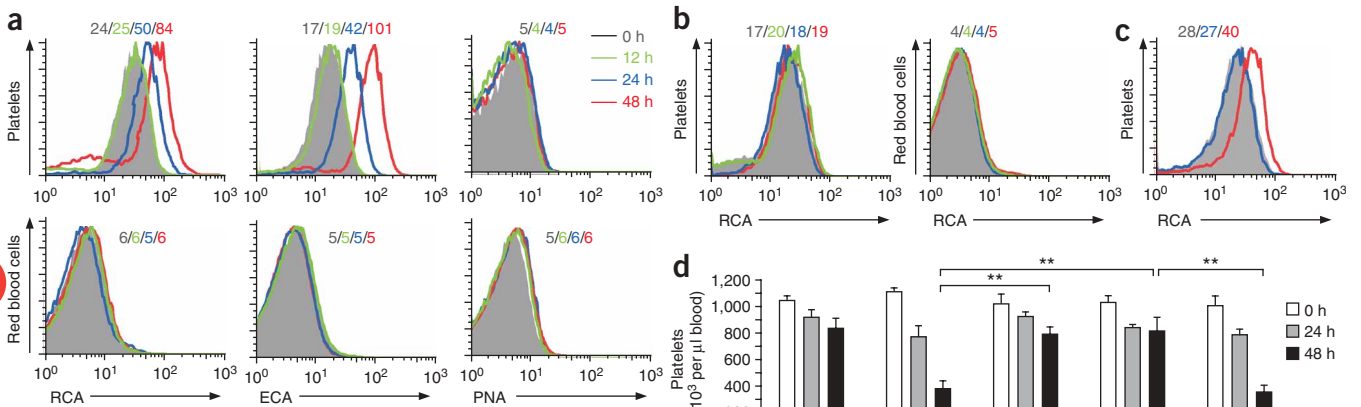


Figure 3 Thrombocytopenia caused by NanA-dependent platelet desialylation and Ashwell receptor function in *S. pneumoniae* infection. **(a)** Asialoglycoproteins were detected by *Ricinus communis* agglutinin (RCA) and *Erythrina cristagalli* agglutinin (ECA) lectin binding at the indicated times after infection with *S. pneumoniae* strain D39 (*S. pneumoniae* WT). PNA lectin binding was used to detect the Thomsen Friedenrich antigen. Mean fluorescence is enumerated at the indicated times after infection. **(b)** Detection of asialoglycoproteins on platelets and red blood cells after infection with the *S. pneumoniae* *NanA $^-$* mutant. Data in **a** and **b** are representative of three to five independent experiments comparing at least six mice per group. **(c)** Detection of asialoglycoproteins on platelets after infection with the *S. pneumoniae* *NanA $^-$* mutant complemented by a functional *nanA* gene in *trans* (*NanA T*). Data are representative of four mice analyzed. **(d)** Platelet abundance in four to eight mice of the indicated genotypes after infection with *S. pneumoniae* WT, *S. pneumoniae* *NanA $^-$* or *S. pneumoniae* *NanA $^-$* complemented with *NanA T* (*NanA $^-$* + *NanA T*). Similar results were obtained after *S. pneumoniae* WT infection of *Asgr-2*-deficient mice (data not shown). SPN, *S. pneumoniae*. **(e)** Platelet turnover in uninfected WT mice and in WT or *Asgr1 $^{-/-}$* mice after *S. pneumoniae* WT infection. Each plot includes data from six mice. **(f)** Detection of asialo-vWF by RCA lectin binding and total vWF levels at 0 and 48 h after infection with *S. pneumoniae* WT when bacteremia was $> 1 \times 10^9$ CFU/ml. Each histogram presents data from four to six mice. All comparative analyses were performed on mice with equivalent levels of bacteremia at each timepoint. ****P* < 0.001 and ***P* < 0.01. All values are means \pm s.e.m.

clearance²⁸, we suspected that the Ashwell receptor might also control platelet homeostasis.

The Ashwell receptor in platelet homeostasis

Circulating platelet counts were unaffected in mice homozygous for either *Asgr1* or *Asgr2* mutation; however, the low platelet counts of mice with ST3Gal-IV deficiency were restored to normal in littermates that further lacked either *Asgr-1* or *Asgr-2* (Fig. 2a). Platelet volume, reticulated (newly formed) platelet abundance, and platelet half-life were also normalized (Fig. 2b–d). No alterations in red blood cell half-life occurred in parallel measurements (data not shown).

The liver is a major anatomic site of normal platelet clearance, and Kupffer cells, the resident macrophages of the liver, contribute to this clearance activity^{30,31}. Hepatocytes are not generally considered to have a significant role. However, platelets have been observed within hepatocytes in some contexts, and the ability of hepatocytes to clear desialylated platelets has been recently reported in studies of platelet binding and internalization^{32,33}. We observed that the majority of platelets in the livers of WT mice are normally associated with Kupffer cells (Fig. 2e). In ST3Gal-IV-deficient mice, despite an overall increase in platelet sequestration in the liver, there was a decrease in the percentage of platelets associated with Kupffer cells (Fig. 2e). In the acute setting of plasma transfusion, biotinylated asialo-platelets derived from ST3Gal-IV-deficient donors were more abundant in the livers of post-transfused wild-type recipients, and the majority were associated with hepatocytes, as compared to the abundance and localization of post-transfused biotinylated WT platelets bearing normal abundance of sialic acid linkages (Fig. 2f). We conclude that ST3Gal-IV deficiency exposes endogenous glycan ligands on platelets that are recognized by the Ashwell receptor in an interaction that promotes asialo-platelet clearance by hepatocytes and leads to thrombocytopenia.

Bacterial sialidase activity and the Ashwell receptor

The location, high capacity and rapid kinetics of ligand clearance by the Ashwell receptor is consistent with its anticipation of a sudden and widespread alteration of endogenous glycoprotein structure within the circulatory system. Such conditions might arise after infection by pathogens that encode sialidase (neuraminidase) activity, such as *Streptococcus pneumoniae*, a leading cause of sepsis and the life-threatening thrombohemorrhagic disorder known as disseminated intravascular coagulation, manifested by rapid consumption of platelets and coagulation factors^{34–36}.

We administered a lethal dose of *S. pneumoniae* (isolate D39) to WT mice by intraperitoneal (i.p.) injection and monitored platelet glycosylation in mice with comparable bacterial counts in the blood before death. Circulating platelets were progressively desialylated during *S. pneumoniae* sepsis, as evidenced by an increase in galactose exposure (Fig. 3a). In contrast, loss of sialic acid from the surface of red blood cells was not detected. Infection of WT mice with an isogenic *nanA* mutant strain of *S. pneumoniae* lacking the sialidase enzyme NanA³⁷ (NanA⁻) did not generate asialo-platelets in circulation (Fig. 3b). Bacteremia during the progression of sepsis after infection of WT mice with the *S. pneumoniae* NanA⁻ isogenic mutant was similar to that seen with infection by the parental *S. pneumoniae* D39 strain (*S. pneumoniae* WT), in contrast to a previous finding in which an *S. pneumoniae* NanA⁻ isolate failed to propagate in circulation³⁸. Complementation of NanA activity in *trans* (NanA^T) restored platelet desialylation similar to that seen with *S. pneumoniae* WT (Fig. 3c). These studies identify the bacterial NanA neuraminidase as the sialidase responsible in sepsis for platelet desialylation.

The thrombocytopenia that occurs in sepsis is believed to reflect accelerated platelet consumption due to increased blood coagulation. Upon infection of WT mice with *S. pneumoniae* WT, thrombocytopenia developed before death, as expected (Fig. 3d). In contrast, when the same challenge was applied to *Asgr1*-deficient mice, platelet levels

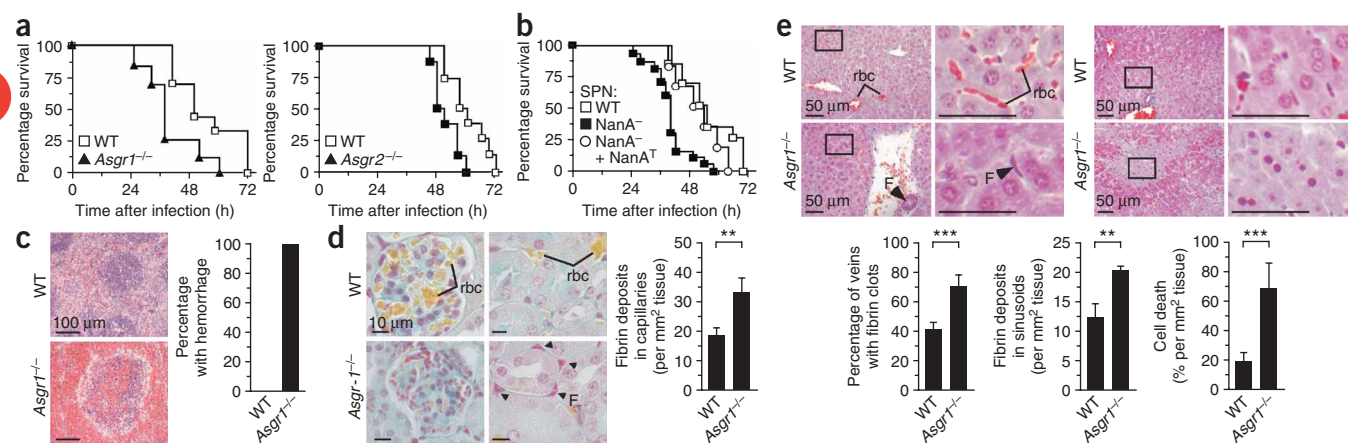


Figure 4 Extension of lifespan and reduction in coagulopathy by the Ashwell receptor in lethal *S. pneumoniae* infection. **(a)** Survival times of WT and *Asgr1*-deficient mice after infection with a lethal dose of *S. pneumoniae* WT. **(b)** Survival times of WT mice infected with *S. pneumoniae* variants that retain or lack NanA sialidase activity. **(c–e)** Tissue histopathology in mice of the indicated genotypes 24 h after infection with *S. pneumoniae* WT. **(c)** Hemorrhage of the spleen with involvement of the lymphoid follicles occurred in all mice lacking *Asgr-1*. Data are representative of results from eight littermate pairs. **(d)** In the kidney, reduced vascular flow and increased deposition of fibrin (F) were evident in capillaries of the glomeruli and between the tubules. Rbc, red blood cell. **(e)** In the left four images, fibrin deposition was also markedly increased in the veins and sinusoids of the liver in *Asgr-1* deficiency (left and center graphs). In the right four images, hepatocyte death was detected by the presence of pyknotic nuclei (right graph). Boxed regions are shown enlarged to the right. Scale bars denote 100 μ m (c), 10 μ m (d) and 50 μ m (e). Images in c–e are representative of 20–30 randomly selected fields of view used for quantitation. These studies included six or more littermate comparisons of the indicated genotypes infected with equivalent doses of *S. pneumoniae* variants (2×10^5 CFU administered i.p.). All comparisons were performed on mice with equivalent levels of bacteremia. *** $P < 0.001$ and ** $P < 0.01$. All values are means \pm s.e.m.

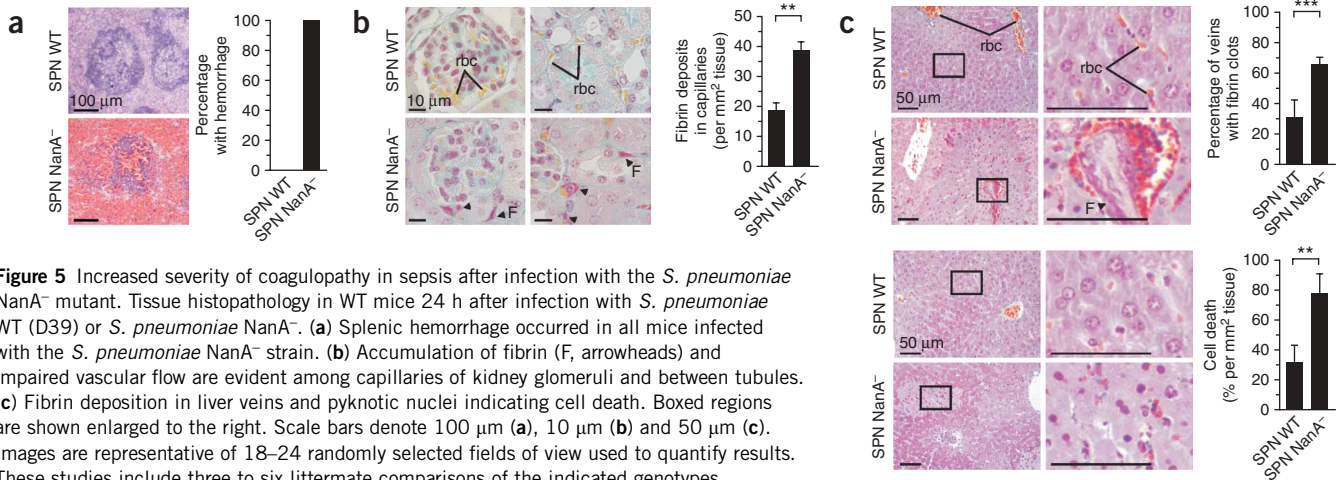


Figure 5 Increased severity of coagulopathy in sepsis after infection with the *S. pneumoniae* NanA⁻ mutant. Tissue histopathology in WT mice 24 h after infection with *S. pneumoniae* WT (D39) or *S. pneumoniae* NanA⁻. (a) Splenic hemorrhage occurred in all mice infected with the *S. pneumoniae* NanA⁻ strain. (b) Accumulation of fibrin (F, arrowheads) and impaired vascular flow are evident among capillaries of kidney glomeruli and between tubules. (c) Fibrin deposition in liver veins and pyknotic nuclei indicating cell death. Boxed regions are shown enlarged to the right. Scale bars denote 100 μ m (a), 10 μ m (b) and 50 μ m (c). Images are representative of 18–24 randomly selected fields of view used to quantify results. These studies include three to six littermate comparisons of the indicated genotypes infected with equivalent doses of *S. pneumoniae* variants (2×10^5 CFU administered i.p.). All comparisons were performed on mice with equivalent levels of bacteremia. *** $P < 0.001$ and ** $P < 0.01$. All values plotted are means \pm s.e.m.

were comparable to those in uninfected mice (Fig. 3d). Normal platelet counts were also present in WT mice infected with the *S. pneumoniae* NanA⁻ mutant, whereas thrombocytopenia was again triggered when the *S. pneumoniae* NanA⁻ mutant was complemented with the intact *nanA* gene (Fig. 3d). Consistent with these findings, platelet half-life decreased during *S. pneumoniae* WT infection of WT mice but was unaltered in mice lacking Asgr-1 after an identical challenge (Fig. 3e). The Ashwell receptor is not itself involved in controlling sialidase function, as the amount of platelet desialylation produced by *S. pneumoniae* NanA was identical in WT and Asgr-deficient hosts (data not shown). In addition, vWF was desialylated during *S. pneumoniae* WT infection at higher bacterial loads, and under these conditions there was evidence of increased clearance of asialo-vWF (Fig. 3f). Thus, during *S. pneumoniae* sepsis, the hepatic Ashwell receptor is responsible for the induction of thrombocytopenia in response to platelet desialylation by the bacterial NanA sialidase.

The Ashwell receptor reduces coagulopathy and increases survival

We suspected that the reduction of prothrombotic factors during *S. pneumoniae* sepsis might moderate the pathologic outcome in the host. Thus, we determined the time to death after *S. pneumoniae* WT infection. Mice retaining Ashwell receptor function survived considerably longer than littermates that were deficient in either Asgr-1 or Asgr-2; whereas those lacking Asgr-1 succumbed most rapidly (Fig. 4a). Of note, WT mice rendered septic by the *S. pneumoniae*

NanA⁻ mutant also died sooner than those infected with *S. pneumoniae* WT, mirroring the decreased survival time associated with Asgr-1 deficiency, whereas complementation of the NanA⁻ mutant restored survival times to similar values as observed with *S. pneumoniae* WT infection (Fig. 4b). We suspected that Ashwell receptor function may moderate the severity of disseminated intravascular coagulation during sepsis. When mice had comparable bacteremia, and on average 12 h before their expected time of death, we undertook histopathological analysis of organs and tissues. Hemorrhage of the spleen was observed in all mice lacking Asgr-1 but was absent from WT littermates (Fig. 4c). In addition, vascular occlusion and fibrin deposition were markedly elevated in the kidney in the absence of Asgr-1 (Fig. 4d). Further signs of severe disseminated intravascular coagulation were observed in the liver of Asgr-1-deficient mice, with increased fibrin deposition in sinusoids and more severe venous thromboembolic occlusion (Fig. 4e). These pathological findings in Asgr-1-deficient mice were coincident with an increase in hepatocyte cell death (Fig. 4e). A similar increase in the severity of disseminated intravascular coagulation, including fibrin deposition, thrombosis and hemorrhage portending multi-organ failure, was also observed in WT mice rendered septic by the *S. pneumoniae* NanA⁻ mutant (Fig. 5). Notably, in a lower-dose challenge model using *S. pneumoniae* WT, all Asgr-1-deficient mice succumbed to infection, compared to a 37% survival rate of WT hosts (Fig. 6). The Ashwell receptor thus impedes the pathogenesis of disseminated intravascular coagulation and improves the probability of host survival in *S. pneumoniae* sepsis.

DISCUSSION

S. pneumoniae is a preeminent human pathogen and one of the leading causes of death worldwide, especially in infants and young children^{39,40}. Sepsis caused by *S. pneumoniae* infection can trigger disseminated intravascular coagulation (DIC), a life-threatening complication involving the consumption of procoagulants and platelets with the deposition of intravascular fibrin throughout the body, resulting in multi-organ failure^{35,36,40}. This thrombohemorrhagic pathology is lethal among a significant fraction of patients with severe septicemia and in intensive care. Our findings indicate that the marked thrombocytopenia closely associated with *S. pneumoniae* sepsis is neither mediated by the pathogen *per se* nor due to platelet consumption in DIC. Instead, thrombocytopenia is the result of

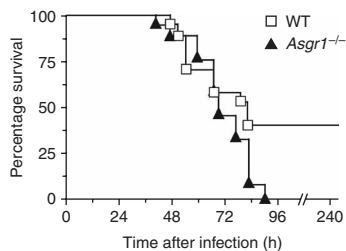


Figure 6 The Ashwell receptor decreases mortality in *S. pneumoniae* sepsis. None of the 15 Asgr-1-deficient mice survived infection with a low dose of *S. pneumoniae* WT (D39) (1×10^3 CFU administered i.p.). In contrast, 6 of 16 WT littermates survived. This finding was reproduced in three independent experiments with additional cohorts of mice.

Ashwell receptor–dependent clearance of platelets that are first desialylated by the NanA sialidase of the pathogen. Host glycoprotein remodeling by *S. pneumoniae* NanA retards the onset of severe hematologic changes that are indicative of acute DIC. Consequently, a subset of normal mice can survive challenge with limiting doses of *S. pneumoniae* that are lethal to littermates deficient in Ashwell receptor function.

Both the Asgr-1 and Asgr-2 chains of the Ashwell receptor are required for asialo-platelet clearance, and a deficiency of either leads to a shortened survival time in *S. pneumoniae* sepsis. The time to death upon challenge with a lethal dose of *S. pneumoniae* WT was prolonged to a lesser extent by Asgr-2 deficiency as compared to Asgr-1 deficiency. This suggests that asialo-vWF clearance, mediated by Asgr-1 but not Asgr-2, may be important for the relative efficacy of Asgr-1 deficiency in prolonging survival. However, an increased protective effect of Asgr-1 deficiency as compared to Asgr-2 deficiency against severe DIC and morbidity is not yet clearly established, and additional studies are needed to determine the precise lethal dose range of *S. pneumoniae* in mice deficient in either Asgr-1 or Asgr-2.

The Asgr-1 chain of the Ashwell receptor normally participates in plasma vWF homeostasis independently of sialylation and sepsis. Although we found that asialo-vWF is rapidly removed from circulation by Asgr-1, asialo-vWF accumulation did not occur in Asgr-1–deficient mice. This may be explained by the previously documented ability of the Ashwell receptor to bind sialo- as well as asialo-glycoproteins as potential endogenous ligands⁹. We observed colocalization of vWF with Asgr-2 in Asgr-1–deficient mice, even after adjustment for reduced hepatocyte vWF abundance in these mice, consistent with the possibility that there may be some compensation by Asgr-2. Nevertheless, plasma vWF abundance is not altered by Asgr-2 deficiency. There seems to be less interdependence and overlap between Asgr-1 and Asgr-2 function than might be expected, and we observed that either chain of the Ashwell receptor can be expressed in the absence of the other *in vivo*. Our findings reveal that vWF and platelets are endogenous ligands of the Ashwell receptor, implying that hepatocytes are involved in the clearance of these coagulation factors from the circulation, which is an unexpected biological activity for this cell type.

Respiratory pathogens other than *S. pneumoniae*, including *Haemophilus influenzae*, *Pseudomonas aeruginosa* and influenza virus, also express one or more sialidases that target host glycoconjugates to reveal cellular receptors for mucosal adherence or cell penetration^{41–44}. Not all pathogens that evoke DIC express sialidase activity, however; thus, for these pathogens, the Ashwell receptor would not be expected to modulate pathogenicity by asialoglycoprotein clearance. All *S. pneumoniae* strains isolated in nature express the NanA sialidase as a virulence factor that promotes mucosal colonization^{45,46}. However, we find that NanA also reduces the severity of DIC, revealing an antivirulence activity of NanA that may serve the *S. pneumoniae* pathogen by lessening the likelihood of host mortality. NanA expressed by *S. pneumoniae* during sepsis removes a subset of α 2,3-linked sialic acids but does not appear to remove this sialic acid linkage from the core 1 O-glycan structure and thus does not expose the Thomsen-Friedenreich antigen, as indicated by unaltered peanut agglutinin lectin binding on either platelets or red blood cells. Although different sialic acid linkages often have unique functions⁴⁷, loss of sialic acids in general can reduce the negative charge at the platelet surface and may in some cases alter their aggregation properties and contribute to disease⁴⁸. We hypothesize that the effect of such changes may be exaggerated by aspects of the acute cellular pathophysiology of sepsis, including endothelial dysfunction and leukocyte

activation. Of note, the asialo-platelets that accumulate in uninfected ST3Gal-IV deficient mice did not show altered activation profiles or responses (P.K.G., S.U., D.D., N.V., D.T.L. *et al.*, unpublished data), and the substantial elevation of circulating asialo-platelet concentration in mice lacking ST3Gal-IV and either Asgr-1 or Asgr-2 was not associated with obvious pathologic changes.

Ashwell receptor expression in hepatocytes is induced rapidly upon birth; the fetus lacks this mechanism of removing circulating glycoproteins⁴⁹. As a post-partum glycoprotein and platelet clearance system, the Ashwell receptor may have had a major evolutionary role in the outcome of infection by *S. pneumoniae* and perhaps other systemic pathogens. Moreover, the changing ligand specificity of Ashwell receptors in phylogeny^{50,51} reflects increasing glycan complexity in the host and perhaps mirrors the evolving infectious nature of various pathogens. It is unclear whether the Ashwell receptor provides a selective advantage among vertebrates in the absence of infection. ST3Gal-IV and perhaps other sialyltransferases may be regulated in some circumstances to desialylate endogenous glycoproteins and thereby engage Ashwell receptor clearance activity. Such regulation has yet to be documented, however, and the normal turnover of endogenous circulating glycoproteins has not been linked to ASGPR activity¹⁰. Nevertheless, endogenous sialidases in vertebrates may be involved in some clinical and pathogenic contexts. For example, loss of both sialic acid and galactose from the surface of chilled platelets prepared for transfusion may reflect extracellular glycosidase activities that thereby provoke rapid platelet clearance via binding of exposed *N*-acetylglucosamine to $\alpha_M\beta_2$ integrin on liver-resident Kupffer cells³¹.

The Ashwell receptor provides an adaptive response to *S. pneumoniae* infection by detecting the byproducts of NanA sialidase activity on host blood factors and removing these prothrombotic components from circulation, preventing them from promoting the lethal complications of DIC. These results reveal a critical hemostatic role for hepatocytes in the acute pathology of sepsis and show that the Ashwell receptor is a key factor in determining host survival. Similar hemostatic modulation may be induced by treatments that likewise deplete platelet and prothrombotic coagulation factor abundance and function. Exogenously administered sialidase is, for example, capable of desialylating platelets and inducing thrombocytopenia⁵². Methods to engage and enhance the Ashwell receptor system may provide a new therapeutic opportunity for impeding the development of DIC in life-threatening systemic infections.

METHODS

Mice. *Asgr1* mutant mice (ref. 29) were kindly provided by B. Sauer (Stowers Institute for Medical Research, Kansas City, Missouri). We purchased *Asgr2* mutant mice from The Jackson Laboratory, stock 002387 (ref. 25). Mice bearing a mutation in the gene encoding ST3Gal-IV (*St3gal4*) have been previously described²⁸. We generated mice homozygous for multiple mutant alleles from parental mice with *St3gal4* Δ/Δ *Asgr1*^{+/+} or *St3gal4* Δ/Δ *Asgr2*^{+/+} genotypes. We observed no phenotypic differences between males and females. Prior to experimentation, we backcrossed all strains of mice to C57BL/6NHsd mates for at least eight generations and provided sterile pellet food and water *ad libitum*. We used littermates aged 8–13 weeks in all studies presented. All animal use and procedures were approved by the Institutional Animal Care and Use Committee of the University of California, San Diego.

Histology. For histological staining, we used tissues fixed in 10% buffered formalin, trimmed, processed, embedded in paraffin and sectioned at a width of 5 μ m. For immunofluorescence studies, we stained glass slides bearing 5- μ m sections of frozen embedded liver tissue (in OCT medium; Sakura Finetek). Antibodies included FITC-conjugated sheep antibody to human vWF (Abcam), goat antibody to mouse ASGPR1 and goat antibody to human ASGPR2 (Santa Cruz Biotech), phycoerythrin (PE)-conjugated rat antibody to mouse CD41

(BD Pharmingen), Alexa Fluor 647–phalloidin to detect actin (Invitrogen), FITC-conjugated rat antibody to mouse CD68 (Serotec) and Texas Red-conjugated antibody to goat IgG secondary antibodies (Jackson Immuno-Research Labs). We performed all primary antibody incubations (1:200) at 4 °C overnight and secondary antibody incubations (1:1,000) at 22 °C for 1 h and then mounted the slides with aqueous gel mount (Biomed). We obtained images by fluorescence deconvolution microscopy with a DeltaVision Restoration microscope (Applied Precision) and analyzed them with DeltaVision SoftWorx (Version 2.50). We acquired and processed images identically within all comparative studies. Images were quantified for immunofluorescence signal, colocalization or both with MetaMorph software (Universal Imaging).

At 24–48 h after infection with *S. pneumoniae*, we collected organs for histopathological and morphometric examination. Organs from mice determined to have equivalent amounts of bacteria in the blood were immersion-fixed, embedded in paraffin, sectioned and stained with H&E (Surgipath) using standard histological techniques. We prepared the Fraser-Lendrum stain (Sigma) and used it to identify fibrin deposition (red) and red blood cells (yellow or orange). Two investigators blind to the experimental group microscopically examined the stained tissue sections for semiquantitative analysis. We scored 20 randomly selected fields of view for fibrin deposits in capillaries. We counted 100 central and portal veins from random liver sections at three tissue depths and scored them as unclotted owing to the presence of red blood cells, clotted owing to visible fibrin thrombi or empty owing to upstream thrombi. We scored 20 randomly selected fields of view for fibrin deposits in sinusoids and used 30 randomly selected fields of view to assess pyknotic bodies and hepatocyte necrosis.

Hematology. We anesthetized mice by a mixture of 3% isoflurane with oxygen in an induction chamber maintained with a nose cone outside of the induction chamber. We transected tails of mice with a sterile razor blade 2 mm from the tip, collected whole blood (100 µl) in EDTA-containing polypropylene microtubes (Becton Dickinson) to ensure proper anticoagulation and kept the tubes at 22 °C until we could analyze them (within 4 h). We acquired blood cell counts with leukocyte differentials and platelet counts in duplicate on a Hemavet 850FS Multi Species Hematology System (Drew Scientific) programmed with mouse hematology settings. We prepared a whole-blood smear from each sample and Wright stained it for manual viewing. We performed coagulation factor analyses and clotting time assays as previously described²⁸.

von Willebrand factor analysis. We used vWF antibody and lectin binding assays to determine vWF glycosylation and glycoprotein abundance as previously described²⁸. We determined vWF half-life after *ex vivo* biotinylation also as previously described²⁸.

Platelet measurements. We collected whole blood from retro-orbital or tail bleeds into EDTA-Vacutainer tubes (Becton Dickinson) and diluted it 1:30 in Tyrode's buffer (150 mM NaCl, 5 mM KCl, 1 mM MgSO₄, 10 mM HEPES, pH 7.4) before staining for flow cytometric analyses to determine glycosylation status²⁸, circulating platelet half-life or percentage of reticulated platelets. We performed all platelet analyses at 22 °C. We determined platelet half-lives after *in vivo* biotinylation by infusion of *N*-hydroxysuccinimide-biotin (Pierce). We injected 10 mg of *N*-hydroxysuccinimide-biotin (dissolved in DMSO, diluted to 500 µl volume in PBS) per kg of mouse body weight into the lateral tail vein. We collected 30 µl of whole blood by tail bleed from anesthetized mice at 24-h intervals as indicated in **Figures 2** and **3** for analysis by flow cytometry; time 0 measurements were determined 1 h after biotin injection. We determined the percentage of biotinylated cells by flow cytometric measurements of light scatter and detection by binding of PE-conjugated streptavidin and FITC-conjugated antibody to CD41 (platelets) and APC-conjugated antibody to ter199 (red blood cells) (BD Pharmingen). Biotinylation efficiency varied between 80–95%. Levels of biotinylated platelets and red blood cells in circulation were based on measurements at time 0, denoted as 100%.

We calculated the percentage of reticulated platelets using RNA staining methods with Thiazole Orange (TO; Sigma). We diluted 2 µl of whole blood 1:30 in Tyrode's buffer and incubated it with 0.1 µg of PE-conjugated CD41-specific antibody at a final concentration of 1 µg/ml TO in PBS (stock of 1 mg/ml dissolved in methanol) for 10 min in the dark at 22 °C. We collected

and analyzed flow cytometric data on 10,000 platelets. We confirmed identification of TO-positive platelets with RNase-pretreated whole blood.

***Streptococcus pneumoniae* infection.** Wild-type *S. pneumoniae* serotype 2 strain D39 and its isogenic NanA⁻ mutant³⁵ were kindly provided by T. Mitchell (University of Glasgow) and were grown in Todd-Hewitt broth (Acumedia) with 2% yeast extract. After overnight incubation at 37 °C and 5% CO₂, we re-inoculated bacteria into fresh media, cultured them to mid-logarithmic growth phase ($A_{600} = 0.4$), centrifuged them at 1,500g for 5 min, washed them once and resuspended them in PBS. We performed infections as follows unless otherwise stated. We infected mice weighing 18–20 g with a dose of 2×10^5 colony-forming units (CFU) of bacteria in 100 µl by i.p. injection. At 24-h intervals after injection, we collected blood to enumerate bacterial CFU in circulation and to measure hematologic parameters as we monitored the time to death. Unless otherwise indicated, those mice with equivalent bacteremia (between 1×10^4 CFU/ml and 1×10^6 CFU/ml at 24 h after infection followed by an increase to between 1×10^6 CFU/ml and 1×10^8 CFU/ml at 48 h after infection) were studied further. For platelet half-life determination, we injected mice with biotin 1 h before *S. pneumoniae* infection.

We generated the *S. pneumoniae* NanA^T strain (expressing neuraminidase *in trans*) from the isogenic *S. pneumoniae* D39 NanA mutant strain transformed with a PDC123 plasmid containing the *S. pneumoniae nanA* gene. An overnight culture of *S. pneumoniae* grown in brain heart infusion medium (Fluka) was re-inoculated into fresh media with 1 mM CaCl₂. When the cultures had reached $A_{600} = 0.1$, we added competent stimulate peptide-1 (CSP-1) to a final concentration of 100 ng/ml before further incubation at 37 °C for 15 min. We added 100 ng plasmid PDC123 containing the *nanA* gene to this culture before plating it on a chloramphenicol-selective Todd-Hewitt broth plus 2% yeast extract (Becton Dickinson) plate for growth at 37 °C with 5% CO₂ overnight for positive colony selection.

Statistical analyses. All data are presented as the means \pm s.e.m. unless otherwise indicated. We analyzed numeric data for statistical significance using Student's unpaired *t*-test with Prism software (GraphPad). We considered *P* values of less than 0.05 as statistically significant. Degrees of statistical significance are presented as ****P* < 0.001, ***P* < 0.01 or **P* < 0.05.

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