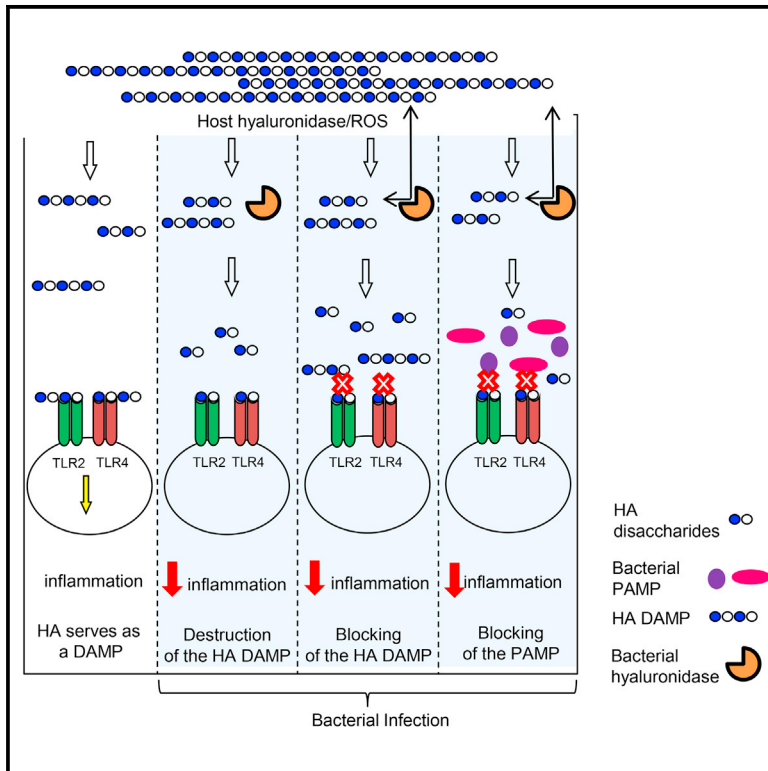


Cell Host & Microbe

Group B *Streptococcus* Evades Host Immunity by Degrading Hyaluronan

Graphical Abstract



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In Brief

During tissue injury, host hyaluronidases cleave high molecular-weight hyaluronan (HA) into pro-inflammatory fragments that activate TLR2 or TLR4. Kolar et al. demonstrate that bacterial hyaluronidases, secreted by certain Gram-positive pathogens, cleave intact or fragmented HA into disaccharides that are themselves non-stimulatory and block binding of stimulatory HA fragments.

Highlights

- GBS hyaluronidase degrades pro-inflammatory hyaluronan (HA) fragments to disaccharides
- HA disaccharides block TLR2/4 signaling by both HA fragments and TLR2/4 agonists
- Hyaluronidases secreted by Gram-positive pathogens promote immune evasion
- HA disaccharides and GBS hyaluronidase inhibit inflammation in a lung injury model



Group B *Streptococcus* Evades Host Immunity by Degrading Hyaluronan

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SUMMARY

In response to tissue injury, hyaluronan (HA) polymers are cleaved by host hyaluronidases, generating small fragments that ligate Toll-like receptors (TLRs) to elicit inflammatory responses. Pathogenic bacteria such as group B *Streptococcus* (GBS) express and secrete hyaluronidases as a mechanism for tissue invasion, but it is not known how this activity relates to immune detection of HA. We found that bacterial hyaluronidases secreted by GBS and other Gram-positive pathogens degrade pro-inflammatory HA fragments to their component disaccharides. In addition, HA disaccharides block TLR2/4 signaling elicited by both host-derived HA fragments and other TLR2/4 ligands, including lipopolysaccharide. Application of GBS hyaluronidase or HA disaccharides reduced pulmonary pathology and pro-inflammatory cytokine levels in an acute lung injury model. We conclude that breakdown of host-generated pro-inflammatory HA fragments to disaccharides allows bacterial pathogens to evade immune detection and could be exploited as a strategy to treat inflammatory diseases.

INTRODUCTION

Hyaluronan (HA) is a linear glycosaminoglycan polymer with a molecular weight greater than 5,000 Da. The polymer, composed of repeating units of the disaccharide D-glucuronic acid β 1-3-N-acetyl-D-glucosamine β -1,4, is found in the extracellular matrix of nearly all tissues and is synthesized by many cell types, including fibroblasts, endothelial cells, and keratinocytes (Jiang et al., 2007). Its primary and most obvious function is to contribute toward the stability and structure of the extracellular matrix, but a broad literature also suggests its involvement in a number of other physiologic and pathologic conditions, including cancer, atherosclerosis, pulmonary fibrosis, pulmo-

nary emphysema, nephritis, arthritis, cerebral infarct, and diabetes (Back et al., 2005; Cuff et al., 2001; Hall and Turley, 1995; Jiang et al., 2007; Li et al., 2011; Mikecz et al., 1995; Weiss et al., 2000).

A major function of HA appears to be immune surveillance. In response to infectious or non-infectious tissue injury, HA is rapidly degraded by reactive oxygen species and host hyaluronidases into low-molecular-weight HA fragments that possess immunostimulatory activity (Jiang et al., 2007). These HA fragments are coined damage-associated molecular patterns (DAMPs) (Matzinger, 2002) and have been shown to interact with macrophages, dendritic cells, and endothelial cells via Toll-like receptor 2 (TLR2) and/or TLR4 to initiate a pro-inflammatory response (Scheibner et al., 2006; Taylor et al., 2004; Termeer et al., 2002). This “endogenous” defense mechanism is believed to complement the more direct pathogen detection mechanisms, whereby TLRs and other pattern recognition receptors recognize molecules or molecular motifs produced only by microbes.

Hyaluronidases are produced by mammals, as well as by other eukaryotes, parasites, fungi, and bacteria. Unlike hyaluronidases from Gram-negative bacteria, which are trapped within the periplasmic space, hyaluronidases from Gram-positive bacteria are secreted (Hynes et al., 2000). The long-standing belief is that these enzymes serve two purposes. Bacterial hyaluronidases degrade HA to small subunits that could serve as nutrients. During host invasion, bacterial hyaluronidases could facilitate bacterial dissemination through tissues by acting as “spreading factors” (Hynes et al., 2000). However, spreading presents a conundrum: if invasion of the host by hyaluronidase-expressing bacteria is inherently linked to HA breakdown, how do pathogens circumvent HA-mediated immune activation? It was demonstrated that hyaluronidase is expressed by most clinical strains of GBS (Benchetrit et al., 1987; Kjems et al., 1980), and serotype III GBS isolated from infected neonates expresses higher levels of hyaluronidase compared to commensal serotype III GBS isolated from asymptomatic infants (Milligan et al., 1978). Furthermore, a recent study suggested that GBS hyaluronidase deficiency is associated with increased inflammation in fish and mice, but how this might relate to hyaluronidase activity was not clear (Wang et al., 2014). The most common bacterial hyaluronidase tested for immunostimulatory activity is the commercially

available enzyme purified from *S. hyalurolyticus*. This hyaluronidase degrades HA to 4- to 16-mer fragments and induces pro-inflammatory signaling, similar to the function of host hyaluronidases noted earlier (Shimada and Matsumura, 1980). In comparison to the *S. hyalurolyticus* enzyme, hyaluronidases from major pathogens, including group B *Streptococcus* (GBS) and *S. pneumoniae*, degrade HA to component disaccharides (Ponnuraj and Jedrzejewski, 2000; Pritchard et al., 1994). Kinetic studies of GBS hyaluronidase suggest that the enzyme makes an initial cut within the HA polymer then moves along the HA chain, cleaving off disaccharide subunits; this leads to the accumulation of HA disaccharides almost exclusively, irrespective of whether the enzymatic reaction is partial or complete (Pritchard et al., 1994). We therefore hypothesize that a pathogen-associated hyaluronidase (e.g., from GBS) would degrade host-derived HA fragments to non-stimulatory disaccharides to facilitate immune evasion.

RESULTS

GBS Hyaluronidase Degrades Pro-inflammatory HA Fragments

To address our hypothesis, we stimulated bone marrow-derived macrophages (BMDMs) with commercial HA disaccharides and confirmed that HA disaccharides are non-immunostimulatory (Figure 1A). Next, we investigated the enzymatic activity of purified GBS hyaluronidase. As reported previously (Taylor et al., 2004), exhaustive digestion of commercial HA, containing fragments of different sizes, by GBS hyaluronidase reduced these fragments to the component disaccharides (data not shown). When added to BMDM, the HA disaccharides induced less tumor necrosis factor alpha (TNF- α) release than did commercial HA fragments or a digest of HA by mammalian hyaluronidase (Figure 1A). These findings demonstrate that GBS and mammalian hyaluronidases have opposite functions. Next, we asked whether GBS hyaluronidase could degrade stimulatory HA fragments (DAMPs) generated by mammalian hyaluronidases. Exhaustive treatment with mammalian hyaluronidase degraded HA into 2- to 8-mers, and subsequent treatment with GBS hyaluronidase converted these 2- to 8-mers into a single disaccharide peak (Figure 1B) that had reduced ability to stimulate macrophages (Figure 1C). These data indicate that GBS hyaluronidase abrogates the immunostimulatory activity of HA fragments.

To investigate the impact of hyaluronidase expression on GBS immune evasion, we generated an isogenic GBS hyaluronidase mutant of a human clinical isolate by allelic replacement of *hylB* deletion and complemented the mutant with the *hylB* gene on a plasmid vector (*phylB*) (Figure S1A; Table S1). HA incubated with the Δ *hylB* GBS supernatant induced higher levels of TNF- α and interleukin-6 (IL-6) from macrophages than did HA incubated with supernatants from either the wild-type (WT) or the complemented mutant Δ *hylB*+*phylB* strain (Figure 1D). In vivo, mice infected with Δ *hylB* GBS had elevated pro-inflammatory cytokines (at 24 hr) and reduced bacterial burden (at 48 hr but not at 24 hr) compared to mice infected with WT GBS (Figures 1E, 1F, and S1B–S1D). Consistent with the immunomodulatory properties of GBS hyaluronidase, mice infected with WT GBS had significantly reduced immunopathology (lower high-mobility

group box 1[HMGB1] and KC and higher fibrinogen) compared to mice infected with the isogenic Δ *hylB* mutant (Figures 1G, S2A, and S2B). To investigate whether heterologous expression of GBS hyaluronidase confers protection to other bacteria, we transferred the GBS *hylB* expression vector into a strain of group A *Streptococcus* (GAS) that lacks endogenous hyaluronidase expression (Table S1). GBS hyaluronidase expression in the GAS background reduced pro-inflammatory cytokine release and promoted survival of the GAS in a murine sepsis model (Figures S3A and S3B). These in vivo findings suggest that GBS hyaluronidase promotes pathogen survival by reducing immune recognition and pro-inflammatory cytokine production.

Although GBS is increasingly recognized as an important cause of infection in the elderly population (Farley, 2001), GBS infection is most devastating in neonates, who acquire the pathogen from colonized mothers during birth (Baker et al., 2011). Therefore, we tested the role of GBS hyaluronidase in models of neonatal sepsis and vaginal colonization. In 4-day-old neonates infected intraperitoneally (i.p.) with GBS, hyaluronidase reduced TNF- α release and promoted survival of the pathogen. Likewise, in female adult mice colonized vaginally with GBS, the hyaluronidase-expressing GBS induced lower MIP-2 release and exhibited enhanced colonization of the host compared to Δ *hylB* GBS (Figures 1H, 1I, and S3C–S3E).

GBS Hyaluronidase Inhibits the TLR2 and TLR4 Stimulatory Activity of HA DAMPs

To verify that the anti-inflammatory activity of GBS hyaluronidase involved interruption of HA signaling through TLR2 or TLR4, undigested HA or HA predigested with mammalian or GBS hyaluronidase was used to stimulate normal, TLR2-deficient, or TLR4-deficient macrophages. Whereas HA digested with mammalian hyaluronidase stimulated greater TNF- α release from WT macrophages compared to undigested HA, these differences were clearly diminished in *Tlr2*^{-/-} or *Tlr4*^{-/-} macrophages (Figure 2A). In comparison, HA degradation by GBS hyaluronidase reduced its ability to stimulate TNF- α release from WT macrophages to the level observed in *Tlr2*^{-/-} or *Tlr4*^{-/-} macrophages (Figure 2A). The significance of HA signaling via TLRs was confirmed in vivo. Differences in the levels of pro-inflammatory cytokines and colony-forming units (CFUs) induced by WT versus Δ *hylB* GBS in WT mice were not observed in *Tlr2*^{-/-} mice (Figures 2B–2D and S4). In addition, the survival difference between mice infected with WT and those with Δ *hylB* GBS was not observed in *Tlr2*^{-/-} mice (Figures 2E and 2F). These findings confirm the importance of these TLR pathways in the immunomodulatory activity of GBS hyaluronidase. WT GBS did not induce mortality in Figure 2E, and it is possible that the difference in immunopathology induced by WT and Δ *hylB* GBS could be more fully appreciated using a higher bacterial inoculum.

HA Disaccharides Inhibit Immunostimulatory Activity of Bacteria, Lipopolysaccharide, and Pam3CSK4

The preceding data are consistent with a mechanism whereby destruction of stimulatory HA fragments allows GBS to invade the host without triggering a DAMP-mediated immune response. However, macrophage stimulation experiments suggested that this mechanism alone could not explain the full effect of the bacterial hyaluronidase. When HA stimulatory

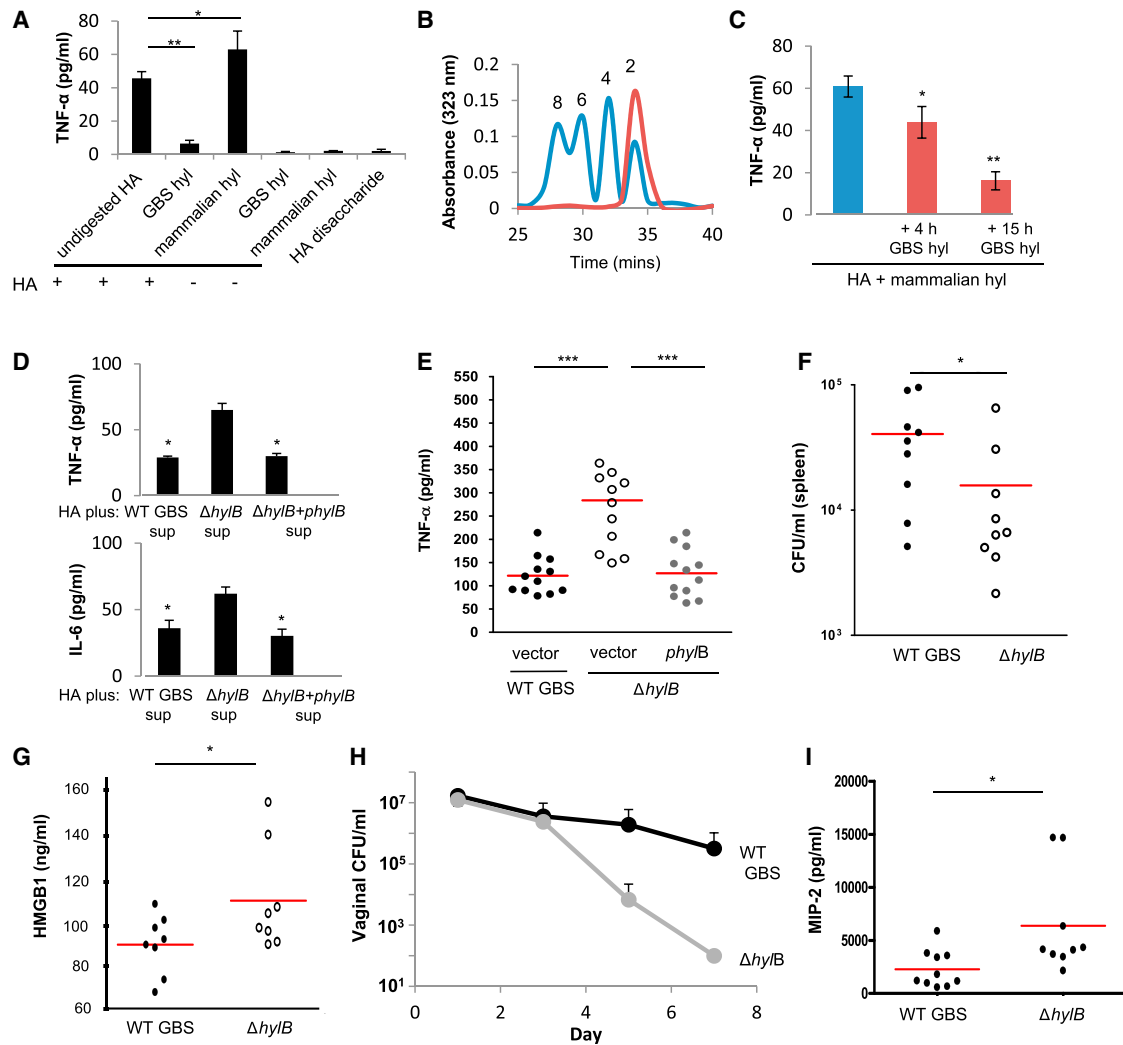


Figure 1. GBS Hyaluronidase Promotes Immune Evasion by Degrading Immunostimulatory HA Fragments

(A) TNF- α production by BMDM after stimulation with HA or digested HA.

(B and C) HA digested with mammalian hyaluronidase (blue) or with mammalian hyaluronidase and subsequently with GBS hyaluronidase (red). (B) HPLC profiles of the digests. (C) Stimulatory activity of the digests measured by macrophage TNF- α production.

(D) TNF- α and IL-6 production by macrophages stimulated with HA and digested with supernatant from WT, $\Delta hylB$, or $\Delta hylB+phyIB$ GBS.

(E–G) CD1 mice infected i.p. with 2×10^7 CFUs of GBS. (E) Splenic TNF- α at 24 hr. (F) Bacterial burden at 48 hr. (G) HMGB1 at 48 hr.

(H and I) Vaginal colonization. CD1 mice were injected with 17β -estradiol i.p. to synchronize estrus. After 24 hr, the mice were administered WT or $\Delta hylB$ GBS into the vaginal lumen. The vaginal cavities were swabbed every other day for a week, and (H) CFU counts and (I) MIP-2 concentrations were determined.

For (A)–(D), data are shown as mean \pm SD, and results are representative of at least three experiments. For (H), data are shown as mean \pm SEM. For (E)–(G) and (I), each data point represents an individual mouse. Data analysis was performed using ANOVA for (A) and (C)–(E), unpaired two-tailed t test for (G) and (I), and Mann-Whitney U-test for (F). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

fragments were digested by WT GBS supernatant, the levels of cytokines stimulated did not simply return to that of the supernatant alone but instead decreased by an additional 60% (Figure 3A). These findings suggest that HA disaccharides may have anti-inflammatory properties. We found that stimulation of TNF- α release by macrophages exposed to heat-killed GBS, *S. aureus*, *E. coli*, or *P. aeruginosa* was reduced in the presence of HA disaccharides (Figure 3B). HA disaccharides were not cytotoxic; hence, suppressed release of pro-inflammatory cytokines was not an indirect consequence of reduced BMDM viability (Figure S5A).

Because HA stimulates pro-inflammatory signaling through TLR2 and TLR4, we evaluated the effect of HA disaccharides on macrophage activation with the TLR2 agonist Pam3CSK4 and the TLR4 ligand lipopolysaccharide (LPS). HA disaccharides suppressed TNF- α release induced by both Pam3CSK4 and LPS but not pro-inflammatory cytokines induced by CpG (TLR9 agonist), poly(I:C) (TLR3 agonist), flagellin (TLR5 agonist), or imiquimod (TLR7 agonist) (Figures 3C, 3D, and S5B), suggesting that the inhibition was specific to TLR2 and TLR4. In addition, the inhibition was dependent on the dose of HA disaccharides or HA predigested with GBS hyaluronidase (Figure 3E),

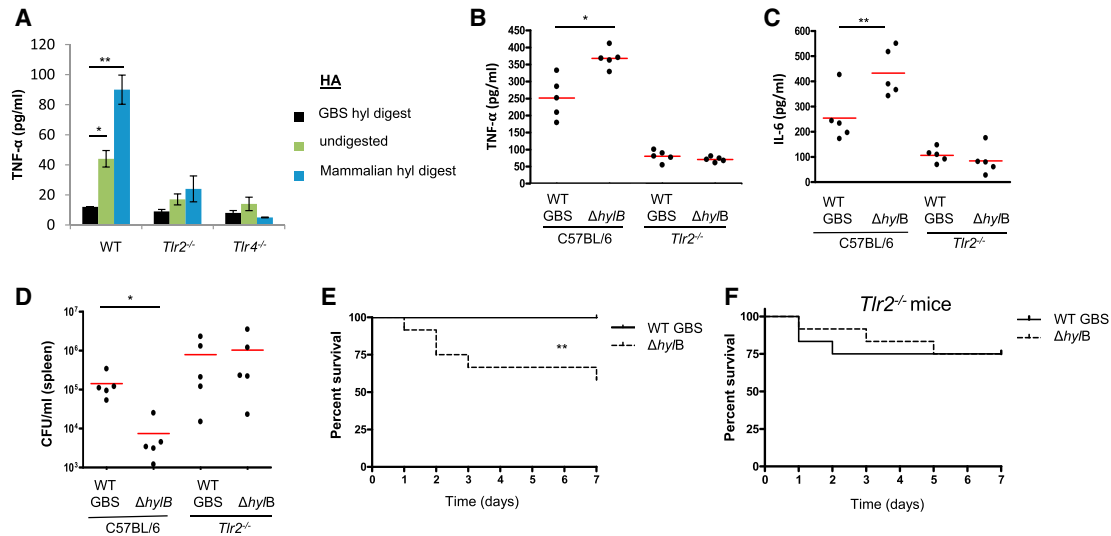


Figure 2. GBS Hyaluronidase Reduces TLR2-Induced Inflammation

(A) Stimulatory effect of HA or HA digests on WT, *Tlr2*^{-/-}, or *Tlr4*^{-/-} macrophages.

(B–D) WT and *Tlr2*^{-/-} mice infected with WT or Δ *hyiB* GBS. (B) Splenic TNF- α and (C) IL-6 at 24 hr (n = 5). (D) Splenic bacterial burden at 48 hr.

(E and F) Survival curve of WT and *Tlr2*^{-/-} mice injected i.p. with 10^8 (WT mice) or 10^7 (*Tlr2*^{-/-} mice) of WT or Δ *hyiB* GBS.

For (A), data are shown as mean \pm SD, and results are representative of at least three experiments. For (B)–(D), each data point represents an individual mouse. Data analysis was performed using ANOVA for (A), Mann-Whitney U-test for (B)–(D), and chi-square test for (E) and (F). *p < 0.05, **p < 0.01.

and the inhibitory effect of HA disaccharides was observed with the human monocytic cell line THP1 (Figure 3F). In the absence of MyD88, there was minimal stimulation of IL-6 release by LPS, Pam3CSK4, or heat-killed GBS, and hyaluronan disaccharide had no discernible effect on cytokine release (Figure S5C).

HA Disaccharides Block TLR2 and TLR4 Pathways

To verify that HA disaccharides impede pathogen-associated molecular pattern (PAMP) signaling specifically through TLR2 and TLR4 and to minimize interference from signaling through other receptors, we assessed the effect of HA disaccharides on HEK293 cells transfected with a necrosis factor κ B (NF- κ B) luciferase reporter plasmid and TLR2, TLR4/MD2, or TLR9 expression constructs. Consistent with findings in BMDM, HA disaccharides reduced NF- κ B reporter (luciferase) activity induced via TLR2 or TLR4 but not TLR9 (Figures 4A–4C). In addition, HA disaccharides block the direct binding of biotinylated stimulatory HA fragments to TLR2 or TLR4 immobilized on a plate. In the same assay, HA disaccharides block PAMP Pam3CSK4 (Figures 4D and 4E). Control chondroitin sulfate disaccharides have no effect on binding. Together, these data indicated that GBS hyaluronidase interferes with TLR2 and TLR4 signaling by PAMPs and DAMP via generation of HA disaccharides.

Major Gram-Positive Pathogens Secrete Hyaluronidases that Inhibit Inflammation

Aside from GBS, many other Gram-positive commensal bacteria and pathogens secrete hyaluronidases that are believed to degrade HA into disaccharides (Hynes et al., 2000). Hyaluronidases from a different group of Gram-positive bacteria, made up mostly of environmental *Streptomyces* species, share signif-

icant amino acid sequence similarity with the pathogens but form a separate cluster upon phylogenetic analysis (Hynes et al., 2000). The primary function of hyaluronidases from soil *Streptomyces* bacteria is likely to degrade HA in decaying plants for nutrition. Based on published data on the *S. hyalurolyticus* enzyme (Shimada and Matsumura, 1980) and our findings given earlier, we hypothesized that Gram-positive pathogens secrete anti-inflammatory hyaluronidases to evade the immune system. By contrast, *Streptomyces* species are not exposed to the same selective pressure and therefore produce hyaluronidases that do not necessarily reduce mammalian inflammation. We addressed this hypothesis by investigating hyaluronidases from two pathogens (*S. aureus* and *S. pneumoniae*) and two *Streptomyces* species (*S. hyalurolyticus* and *S. coelicolor*). To directly compare the immunomodulatory function of the bacterial hyaluronidases during infection, we expressed some hyaluronidases in the Δ *hyiB* GBS background (Table S1). Analyzed by high-performance liquid chromatography (HPLC), hyaluronidases from *S. pneumoniae* and *S. aureus* reduced HA to a single peak of disaccharides, whereas hyaluronidase from *S. hyalurolyticus* degraded HA to 4- to 8-mers (Figure 5A). Consistent with the HPLC findings, HA digested with purified hyaluronidases from the Gram-positive pathogens (Figure 5B) or with *S. aureus* hyaluronidase expressed in the Δ *hyiB* background (Figures 5C and 5D) reduced the immunostimulatory activity of commercial HA. In comparison, digest of HA either with purified *S. hyalurolyticus* hyaluronidase or with *S. coelicolor* hyaluronidase expressed in the Δ *hyiB* GBS background stimulated increased TNF- α release compared to commercial HA (Figures 5B and 5D). The significance of these findings were confirmed in vivo by demonstration that Δ *hyiB* GBS expressing the *S. aureus* hyaluronidase on a plasmid vector induced lower

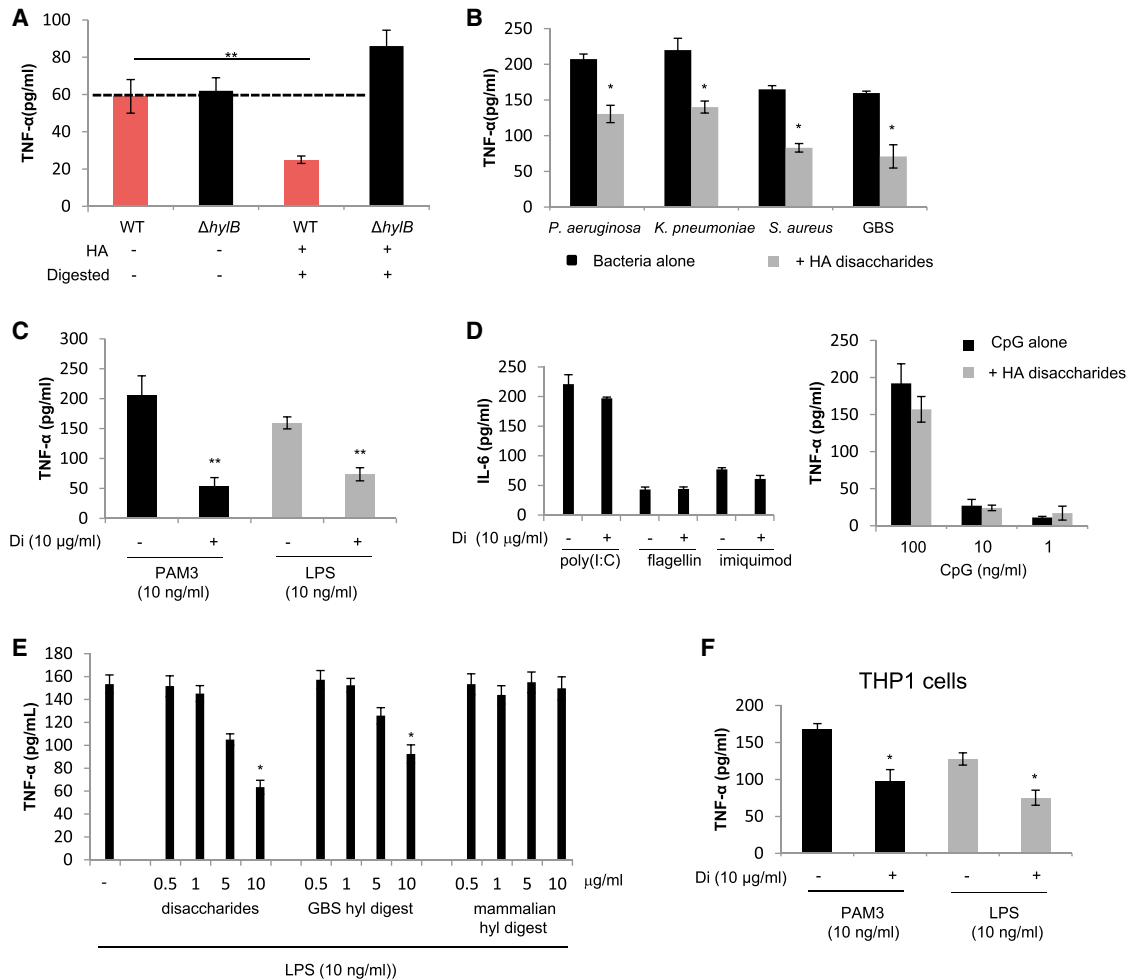


Figure 3. HA Disaccharides Dampen the Immune Response to Bacterial PAMPs

(A) Inhibitory effect of GBS hyaluronidase extending beyond abrogation of HA fragment stimulatory activity. TNF- α production by BMDM stimulated with supernatant from WT or Δ hyIB GBS \pm digested HA.

(B–D) Effect of HA disaccharides on TNF- α production by BMDM, induced by bacteria or TLR ligands. Effect of HA disaccharides on macrophage stimulation by (B) heat-killed bacteria, (C) Pam3CSK4 or LPS, or (D) poly(I:C) (100 ng/ml), flagellin (100 ng/ml), imiquimod (100 ng/ml), or CpG.

(E) Effect of titrating commercial HA disaccharides or HA disaccharides generated by GBS hyaluronidase on LPS (10 ng/ml) stimulation of macrophages.

(F) THP1 cells stimulated with Pam3CSK4 or LPS in the presence or absence of HA disaccharides (10 μ g/ml).

Data are shown as mean \pm SD, and results from (A)–(F) are each representative of three experiments. Data analysis was performed using ANOVA for (A) and unpaired two-tailed t test for (B)–(D) and (F). * $p < 0.05$, ** $p < 0.01$.

TNF- α release (Figure 5E), whereas Δ hyIB GBS expressing the *S. coelicolor* enzyme induced higher TNF- α release compared to Δ hyIB GBS (Figure 5F), which is not because of a difference in CFUs (data not shown). Overall, these data support the idea that the selective pressure of the inflammatory immune response drove the functional divergence of the hyaluronidases from major Gram-positive pathogens and from the soil microbial genus *Streptomyces*.

GBS Hyaluronidase and HA Disaccharides Ameliorate Inflammation in an Acute Lung Injury Model

The anti-inflammatory properties of GBS hyaluronidase and HA disaccharides prompted us to ask whether these bacterial tools or strategies could be used to treat inflammatory diseases, in particular HA-related diseases. The LPS acute

lung injury model is a commonly used experimental model of generalized lung inflammation. Because HA breakdown is an important pathophysiologic feature of the model (Liang et al., 2007), we used the model to evaluate the therapeutic efficacy of disaccharides and GBS hyaluronidase. We administered LPS to mice intra-tracheally with or without HA disaccharides or purified GBS hyaluronidase. Lung histology after 24 hr was scored in a blinded fashion, and cytokines from lung homogenates were measured. Treatment with either HA disaccharides or GBS enzyme effectively blocked the manifestation of LPS-mediated pathology and significantly reduced the levels of pro-inflammatory cytokines (Figures 6A and 6B). This provides proof of principle of the potential of GBS hyaluronidase and HA disaccharides as anti-inflammatory agents.

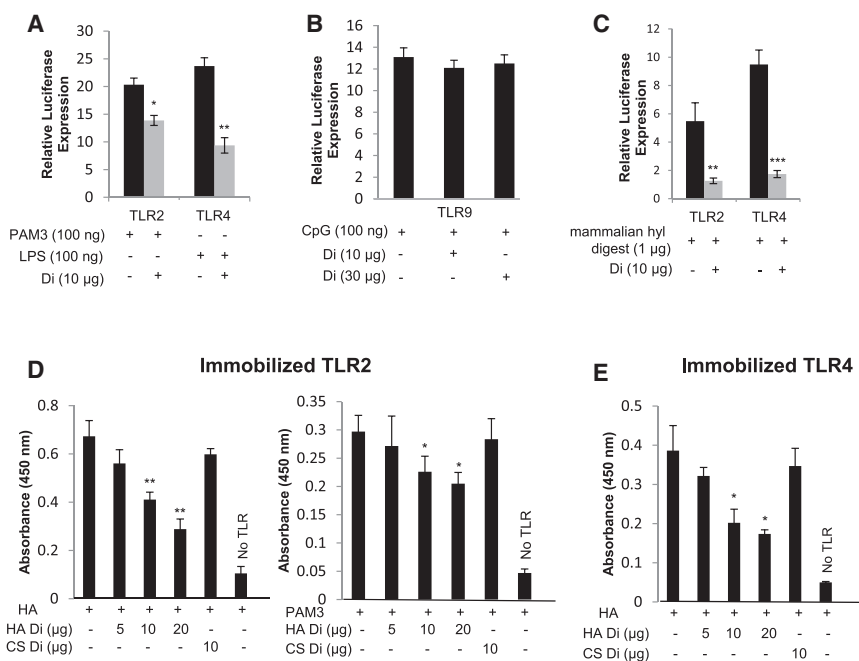


Figure 4. HA Disaccharides Interfere with Signaling through TLR2 and TLR4

(A–C) Effect of HA disaccharides on NF- κ B luciferase activity in HEK293 cells transfected with various constructs. (A) HEK cells transfected with TLR2 or TLR4/MD2 and stimulated with either Pam3CSK4 or LPS. (B) HEK cells transfected with a TLR9 construct and stimulated with CpG. (C) HEK cells transfected with TLR2 or TLR4/MD2 and stimulated with HA fragments generated by mammalian hyaluronidase.

(D and E) Disaccharide blocking of Pam3CSK4 to TLR2 and HA binding to TLR2 or TLR4/MD2 immobilized on a plate. CS, chondroitin sulfate.

Data are shown as mean \pm SD. Concentrations are expressed per milliliter. Results are representative of at least three experiments. Data analysis was performed using an unpaired two-tailed t test for (A)–(C) and ANOVA for (D) and (E). * $p < 0.05$, ** $p < 0.01$.

DISCUSSION

Coexistence of hosts and microbes has fostered the development of various immune strategies and counter-strategies by the warring hosts and pathogens. The linkage of HA breakdown with TLR activation is one seemingly brilliant host surveillance strategy that should be able to detect tissue damage due to sterile or microbial injury. However, its specific role in limiting bacterial infection has not been previously demonstrated. Here, we provided evidence for the importance of this immune surveillance mechanism by showing TLR2-dependent increased survival of hyaluronidase-expressing GBS compared to Δ hylB GBS.

From a bacterial perspective, we showed that major Gram-positive pathogens take advantage of the unique properties associated with HA fragment lengths to undermine the host surveillance mechanisms. Hyaluronidases from Gram-positive pathogens act as endo-*N*-acetyl-hexosamines by cleavage of the β -1,4 linkage and produce primarily HA disaccharides (Kreil, 1995). Shorter incubation of the enzyme with HA does not lead to release of intermediate-sized HA products, e.g., 4- to 16-mers. In comparison, eukaryotic hyaluronidases are endoglycosidases that hydrolyze the β -1,4 linkages between *N*-acetyl-hexosamines and glucuronic acid (Aronson and Davidson, 1967; Gushulak et al., 2012), and the end products of the enzymatic reaction are tetrameric HA or larger fragments. This difference in enzymatic activities allows the Gram-positive pathogens to achieve three tasks during infection. First, because non-immunostimulatory HA fragments are produced from their enzymatic activity, hyaluronidases from pathogens facilitate “spread” of the bacteria without sacrificing stealth. Second, the bacterial enzyme destroys pro-inflammatory HA fragments generated in the infectious process. Third, the HA disaccharide by-product arising from DAMP degradation could further block PAMP stim-

ulation of TLR2/4 pathways. Overall, bacterial hyaluronidases retool the immune surveillance system into an immune evasion device.

Fragment length is a well-recognized determinant of HA polymer functions. High-molecular-weight HA provides structural support to tissues, but also exhibits anti-inflammatory properties by preventing TLR signaling and by blocking phagocytosis by macrophages (Forrester and Balazs, 1980; Scheibner et al., 2006). High-molecular-weight HA has been applied successfully for treatment of various experimental inflammatory conditions (Asari et al., 2010; Liu et al., 2008; Nakamura et al., 2004). Shorter HA fragments have specific function not only in cellular physiology but also in pathology. For example HA fragments of 10–15 disaccharide subunits produced by highly invasive bladder cancer stimulate endothelial cell proliferation and capillary formation (Lokeshwar and Selzer, 2000). In the context of inflammation, shorter HA fragments have been generally associated with pro-inflammatory responses. Shorter HA fragments activate macrophages, as well as dendritic cells, and stimulate transcription of pro-inflammatory cytokines, antimicrobial peptides, and metalloproteinases (Scheibner et al., 2006; Taylor et al., 2004; Termeer et al., 2002). Our finding that HA disaccharides (at 5 μ g/ml) block TLR2 and TLR4 adds to the complexity of existing HA biology. Because an estimated 5 g of HA are turned over daily within the human host (Fraser et al., 1997) and HA plays an important role in various diseases, the presence of these anti-inflammatory disaccharides bears investigation in their involvement in the pathophysiology of diseases.

In our study, we provided proof of principle that the strategies used by pathogens to evade HA or TLR2/4-induced inflammation could be applied to ameliorate inflammatory diseases. The LPS acute lung model shows that purchased HA disaccharides or HA disaccharides generated by GBS hyaluronidase *in vivo* are capable of blocking the TLR4 agonist. HA disaccharides

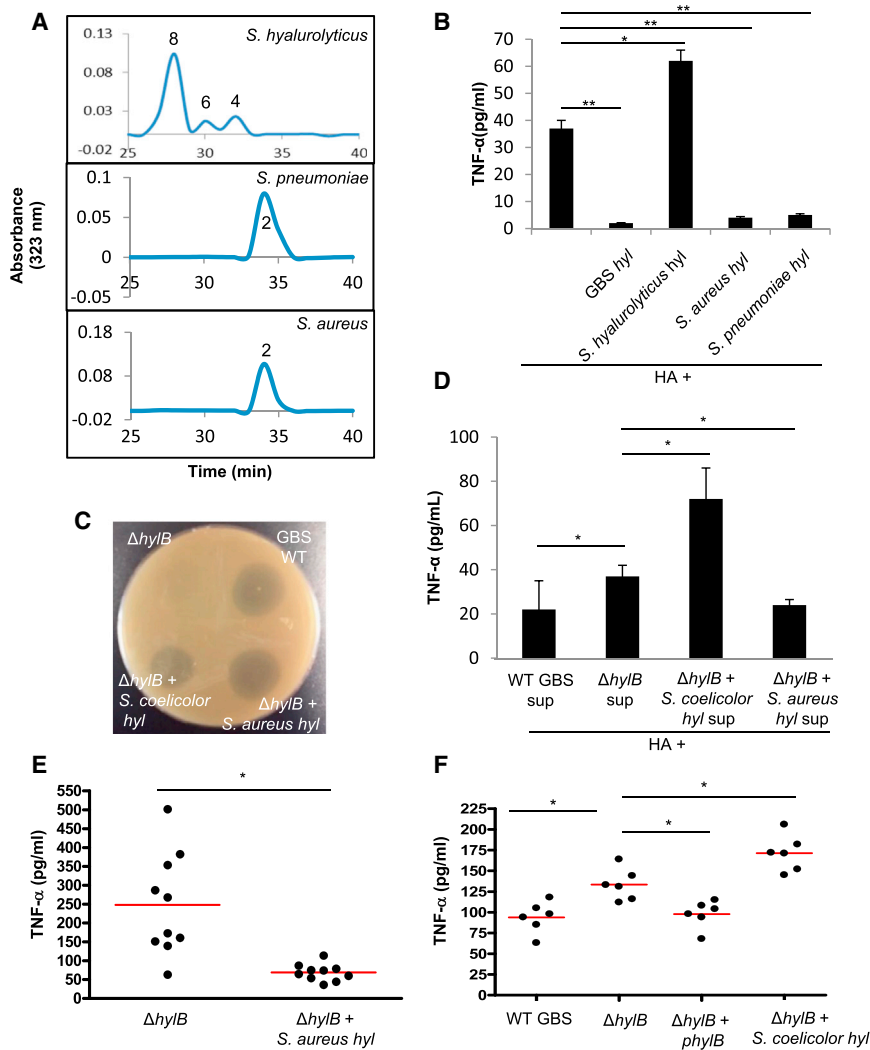


Figure 5. Hyaluronidases from Gram-Positive Pathogens and Soil *Streptomyces* Species Have Different Immunomodulatory Functions

(A and B) Hyaluronidase purified from *S. hyalurolyticus*, *S. pneumoniae*, or *S. aureus* used to degrade HA. (A) Digestion products were analyzed on HPLC or (B) used to stimulate BMDM. (C) Hyaluronidase gene from *S. coelicolor* or *S. aureus* introduced into the Δ *hylB* GBS background. Hyaluronidase activity of the Δ *hylB* GBS constructs was visualized on a HA plate.

(D) Supernatants of the various GBS constructs incubated with HA. The digestion products were used to stimulate BMDM.

(E and F) CD1 mice infected i.p. with WT GBS, Δ *hylB* GBS, or Δ *hylB* expressing hyaluronidases from various bacteria. TNF- α production in the spleen was measured after 24 hr.

For (B) and (D), data are shown as mean \pm SD, and results are representative of three experiments. Data analysis was performed using ANOVA for (B), (D), and (F) and unpaired two-tailed t test for (E). **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

influence against bacterial infection (de la Motte and Kessler, 2015). In the dextran sulfate sodium (DSS) colitis model, DSS administration induced production of HA fragments, which promoted TLR4-dependent production of PGE2 by macrophages with an overall cytoprotective effect on epithelial cells against inflammation (Zheng et al., 2009). Many gut commensal bacteria, including *C. perfringens*, *C. difficile*, and *E. faecalis*, produce extracellular hyaluronidases (Canard et al., 1994; Hafiz and Oakley, 1976; Rosan and Williams,

1966). How hyaluronidase expression by these bacteria affects gut immunity and inflammation is a potentially interesting area of research.

could inhibit TLR2- and TLR4-mediated inflammatory conditions, though many TLR2/4 blockers already exist and have shown good efficacy in experimental disease models (Hennessy et al., 2010). In comparison, GBS hyaluronidase and hyaluronidases from other Gram-positive pathogens represent unique tools that could address needs unmet by current therapeutics. These enzymes produce TLR2/4 blocking HA disaccharides but also degrade potentially pathogenic HA fragments. HA fragments play prominent roles in various human diseases as described earlier (Back et al., 2005; Cuff et al., 2001; Hall and Turley, 1995; Jiang et al., 2007; Li et al., 2011; Mikecz et al., 1995; Weiss et al., 2000). Therefore, bacterial hyaluronidases could be exploited as novel therapeutic agents.

Beyond infections, hyaluronidase expression by commensal bacteria also has the potential to influence host inflammation and immunity. For example, the host gastrointestinal tract is an important site of constant host-commensal interactions and exposure to HA, and many studies have documented a role of HA in both homeostasis and colonic inflammation (de la Motte and Kessler, 2015). HA is found in abundance, especially in the colon, and is believed to have an overall protective

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EXPERIMENTAL PROCEDURES

Reagents

HA disaccharide sodium salt, HA (from rooster comb), *S. hyalurolyticus* hyaluronidase, bovine testis hyaluronidase, and LPS (from *E. coli* 055:B5) were acquired from Sigma-Aldrich. Pam3CSK4, poly(I:C), imiquimod, and CpG were purchased from InvivoGen. *Salmonella* flagellin was a gift from Dr. Edward Miao.

Mice

Tlr2^{-/-} and *Tlr4*^{-/-} mice have been backcrossed for 16 generations with C57BL/6 mice (Bulut et al., 2009). *MyD88*^{-/-} mice were also maintained at Cedars-Sinai Medical Center. The mice were housed in specific-pathogen-free facilities, and

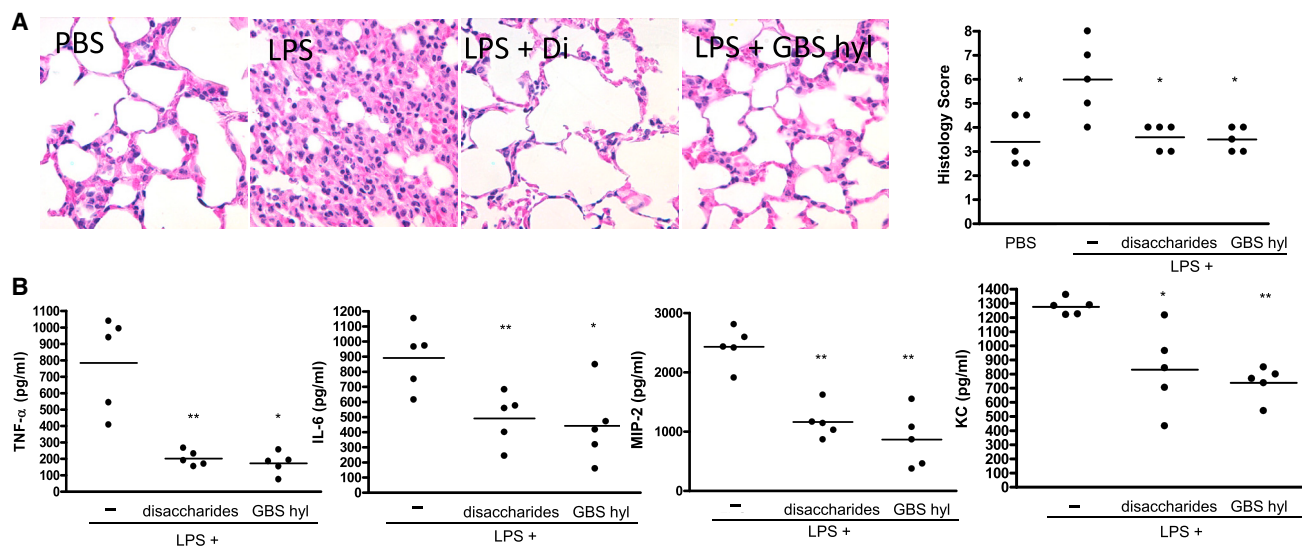


Figure 6. GBS Hyaluronidase and HA Disaccharides Ameliorate Inflammation in an Experimental Lung Injury Model

Mice were injected intra-tracheally with LPS (5 μ g) \pm HA disaccharides (100 μ g) or GBS hyaluronidase (0.5 mg). The mice were sacrificed after 24 hr.

(A) H&E staining and pathology scores of the lungs.

(B) Cytokines and chemokines in lung homogenates. Each data point represents an individual mouse.

Data analysis was performed using ANOVA. * $p < 0.05$, ** $p < 0.01$ versus LPS group.

10- to 12-week-old sex-matched mice were used for in vitro and in vivo experiments.

Bacterial Strains, Growth Conditions, and Heat-Killed Bacteria

Unless otherwise stated, GBS (A909), *S. pneumoniae* (TIGR), and GAS (M49) were cultured overnight at 37°C without shaking, and *K. pneumoniae* (American Type Culture Collection [ATCC] 10031), *P. aeruginosa* (ATCC 35032), and *S. aureus* (LAC) were grown overnight at 37°C with shaking. The culture media used were Todd-Hewitt broth supplemented with 0.5% yeast extract (GBS and GAS), brain-heart infusion medium (*S. pneumoniae*), Luria broth (*K. pneumoniae* and *P. aeruginosa*), and tryptic soy broth (*S. aureus*). Heat-killed bacteria were generated by heating bacteria to 65°C for 1 hr.

Construction of the Δ hyIB Mutant

Δ hyIB mutant was generated by precise in-frame allelic replacement of *hyIB* with chloramphenicol acetyltransferase (*cat*) in the WT GBS strain A909, as previously described (Locke et al., 2007). Briefly, PCR was used to amplify approximately 500 bp upstream and 500 bp downstream of the targeted GBS chromosomal gene region. Primers adjacent to the upstream and downstream regions of *hyIB* were constructed with 25 bp 5' extensions corresponding to the 5' and 3' ends of the *cat* gene from pACYC (see Table S1 for list of primers) (Nakano et al., 1995). Fusion PCR was then performed to combine upstream and downstream products with a 660 bp amplicon of the *cat* gene (Buchanan et al., 2006). The fusion product resulting from this PCR contained an in-frame substitution of *hyIB* with *cat* and was subcloned into the Gateway entry vector pCR8/GW/TOPO. This vector was then used to transform chemically competent Mach 1 *E. coli* cells (Invitrogen). Plasmid DNA was extracted, and the fusion PCR amplicon was transferred into the temperature-sensitive knockout vector pKODestErm (Locke et al., 2007) via an attL-attR recombination reaction to create the knockout plasmid pKOhyIB. After its propagation in MC1061 *E. coli*, the pKOhyIB construct was introduced into WT GBS through electroporation. Transformants were identified at 30°C by Erm selection and shifted to 37°C (a non-permissive temperature for plasmid replication). Differential antibiotic selection of Cm^r and Erm^s allowed identification of candidate colonies as potential allelic exchange mutants. Targeted in-frame replacement was confirmed unambiguously through PCR, documenting the desired insertion of *cat* and the absence of the *hyIB* sequence in chromosomal DNA isolated from the Δ hyIB mutant.

Complementation and Heterologous Expression Studies

Flanking primers were used to amplify *hyIB* and contiguous regions 1 kb upstream and downstream of the gene (from the chromosome of WT GBS A909 strain) (Table S1). The PCR product was directionally cloned into the shuttle expression vector pDCerm (Jeng et al., 2003), and the recombinant plasmid was used to transform by electroporation competent GBS A909 Δ hyIB strain or M49 GAS strain.

For heterologous expression of hyaluronidases from *S. aureus*, primers were tailored from flanking regions of the *S. aureus* (Colorado strain) *hysA* gene (Table S1). The gene was amplified by PCR, directionally cloned into pDCerm, and introduced into the Δ hyIB GBS background as described earlier. For cloning of the *S. coelicolor* hyaluronidase (SC1C2.15) gene, amplification by PCR proved to be difficult. Therefore, the *S. coelicolor* cosmid St1C2 was acquired (Redenbach et al., 1996) and digested with BamHI. A 6 kb fragment containing the hyaluronidase gene was ligated to shuttle vector pUC19 and subsequently subcloned into pDCerm and electroporated into the GBS Δ hyIB strain.

Purification of GBS Hyaluronidase

GBS, *S. aureus*, and *S. pneumoniae* were cultured overnight and then centrifuged at 4,000 rpm for 10 min. The supernatants were removed and filter sterilized. Solid ammonium sulfate was added slowly to each supernatant until 75% saturation was reached, at 4°C, with gentle stirring for at least 2 hr. Proteins were precipitated by centrifuging for 30 min at 10,000 rpm at 4°C. The precipitate was resuspended in 20 mM phosphate buffer (pH 6.0), and dialyzed against 4 l of 20 mM phosphate buffer (pH 6.0) for 2–3 hr at 4°C with stirring. The phosphate buffer was exchanged for new buffer, and dialysis was continued overnight. The dialyzed protein solution was centrifuged at 10,000 rpm for 10 min, and any salt was removed. The protein sample was loaded onto a HiTrap SP XL (GE Healthcare) column (5 ml) that has been pre-equilibrated with 20 mM phosphate buffer (pH 6.0). Proteins were eluted using a sodium chloride gradient (0–300 mM), and 5 ml fractions were collected. The fractions were analyzed for purity using SDS-PAGE silver staining.

HA Plate Assay

HA plates were prepared by adding sterilized bovine serum albumin (0.8%) and hyaluronic acid (0.3 mg/ml) to warm (46°C) autoclaved media containing 1% Noble agar, 1% yeast extract, and 3% Todd-Hewitt broth. After the agar

solidified, hyaluronidase activity was assessed by adding bacterial culture supernatant or inoculating bacteria on the plate. A zone of clearing indicated the presence of hyaluronidase activity.

HPLC Analysis

Hyaluronidase enzymes, specified in the text, were mixed with 5 mg/ml of HA from rooster comb (Sigma-Aldrich) and, unless indicated, were incubated at 37°C for 18 hr in 0.1 M sodium acetate buffer (pH 5.3). The enzymes were heat inactivated at 85°C for 10–20 min. The liquid was removed by Speedvac, and the digest was resuspended in 0.1 M ammonium bicarbonate buffer. Chromatography was carried out using an Ultrahydrogel 120 column (7.8 mm × 30 cm, Waters) at room temperature, equilibrated with 0.1 M ammonium bicarbonate buffer. The flow rate was 0.5 ml/min, and absorbance was read at 232 nm.

Digestion Reactions Used in Macrophage and HEK Cell Assays

HA (2 mg) was digested using 0.5 mg of purified hyaluronidase from GBS, *S. aureus*, or *S. pneumoniae* or using 100 μ l supernatant from overnight bacterial cultures. The exhaustive digestion was performed in 50 mM ammonium acetate with 10 mM CaCl₂ (pH 6.5) at 37°C for 18 hr. Exhaustive digest of HA (2.5 mg) was also carried out using bovine testis hyaluronidase (275 turbidity units) or *S. hyalurolyticus* hyaluronidase (100 turbidity units) in 50 mM sodium acetate buffer (pH 5.0) at 37°C for 18 hr. Digestions were stopped by heat inactivating the enzymes at 85°C for 10–20 min. For macrophage and HEK cell assays, an equivalent of 10 μ g/ml of HA digest was added to ~80,000 cells unless otherwise specified. Unless otherwise specified, macrophages were stimulated with the HA products for 4 hr, and culture supernatants were stored at –80°C for cytokine analyses.

Enzyme-Linked Immunosorbent Assay and LDH Assay

Mouse TNF- α , IL-6, MIP-2, KC (BioLegend), fibrinogen (Innovative Research), and HMGB1 (MyBiosource) specific ELISAs were performed according to the manufacturer's instructions. Lactate dehydrogenase (LDH) cytotoxicity assay was performed using an LDH kit (Clontech) according to the manufacturer's instructions.

Cell Cultures

For BMDMs, bone marrow cells were isolated from the femurs and tibiae of 12-week-old C57BL/6 mice and suspended in RPMI 1640 medium with 10% heat-inactivated fetal bovine serum (FBS). Ten percent supernatant from L929 cells, containing macrophage colony stimulating factor, was added to induce differentiation of bone marrow cells into macrophages. The cells were cultured in 5% CO₂ at 37°C for 7 days before use.

THP1, a human monocytic cell line derived from an acute monocytic leukemia patient (ATCC TIB-202), was maintained in RPMI 1640 media with the addition of 10% heat-inactivated FBS, 2.0 mM L-glutamine, and 10 mM HEPES. THP1 cells were seeded at an appropriate density in tissue culture plates and induced to differentiate by the addition of 10 ng/ml of phorbol 12-myristate 13-acetate (Sigma-Aldrich) for 18 hr.

HEK293 cells (ATCC CRL-1573) were maintained in DMEM (Invitrogen) with the addition of 10% heat-inactivated FBS and 2 mM L-glutamine (Gibco-BRL).

Luciferase Assays

HEK293 cell reporter assays were performed as described previously (Underhill et al., 1999) using the indicated plasmids. Briefly, HEK293 cells were transfected using lipofectamine 2000 (Invitrogen) with 2 μ g of the NF- κ B reporter construct endothelial cell-leukocyte adhesion molecule (ELAM)-luciferase, together with 1 μ g of murine TLR2, TLR9, or TLR4 and MD2 expression construct, as indicated in the text. The cells were stimulated with 100 ng of LPS, Pam3CSK4, or CpG for 4 hr, and luciferase activity was measured by using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Background ELAM-luciferase activity was subtracted.

Murine Model of Sepsis

The 10-week-old female mice were injected i.p. with 1×10^7 – 3×10^7 CFUs of GBS or GAS. After 24 or 48 hr, the mice were euthanized, spleen and blood were aseptically harvested, and bacterial CFUs were determined on agar

plates. In addition, homogenized spleens were centrifuged at 10,000 rpm for 10 min, and the supernatants were stored at –80°C for cytokine analysis by ELISA.

Murine Model of Vaginal Colonization

An established murine model of the vaginal colonization murine model was used (Sheen et al., 2011). Briefly, 10-week-old female CD1 mice were injected i.p. with 0.5 mg 17 β -estradiol to synchronize estrus. After 24 hr, the mice were injected with 1×10^7 CFUs of GBS into the vaginal lumen. On days 1, 3, 5, and 7, colonizing GBS were recovered by swabbing the vaginal cavity with calcium alginate tips. Samples were vortexed for 10 min, serially diluted, and plated on CHROMagar StrepB plates.

Murine Model of Neonatal Sepsis

The 4-day-old C57BL/6 mice were injected i.p. with 1×10^4 CFUs of GBS in 50 μ l PBS. After 48 hr, the pups were euthanized, kidneys were aseptically harvested, and bacterial CFUs were determined on agar plates. Homogenized kidneys were centrifuged at 10,000 rpm for 10 min, and the supernatants were stored at –80°C for cytokine analysis.

TLR2 and TLR4 Binding Assays

Recombinant TLR2 (20 μ g/ml, R&D) or TLR4 and MD2 (20 μ g/ml, R&D) was reconstituted in coating buffer and added to a 96-well plate at 4°C overnight. The plate was washed three times with wash buffer (PBS + 0.05% Tween 20) and blocked for an hour with PBS + 10% FBS. The plate was washed three times in washing buffer. Biotin-labeled HA (1 μ g/ml, mw 10K Creative PEG works) or Pam3CSK4 (100 ng/ml, InvivoGen) was added with or without HA disaccharide or chondroitin sulfate disaccharide and incubated at room temperature for 2 hr. The plate was washed three times, and 100 μ l of Avidin horseradish peroxidase was added to each well and incubated at room temperature for 30 min. The plate was washed five times, and 100 μ l of 3,3',5,5'-tetramethylbenzidine solution added to each well and incubated in the dark at room temperature until desired coloration was observed. The reaction was stopped with 2 N H₂SO₄, and the absorbance was read at 450 nm.

Murine Acute Lung Injury Model

The 10-week-old female C57BL/6 mice were injected intra-tracheally with LPS (5 μ g), LPS plus HA disaccharides (100 μ g), or LPS plus GBS hyaluronidase (0.5 mg). After 24 hr, the mice were euthanized, the lungs were homogenized in 200 μ l of PBS and centrifuged at 10,000 rpm for 5 min, and the supernatants were analyzed for chemokine and cytokine composition by ELISA. For histology, lung tissues were fixed in 10% formalin (Medical Chemical), embedded in paraffin, and submitted to the Department of Pathology at Cedars-Sinai Medical Center for H&E staining. Lung histology slides were analyzed and scored by an investigator (J.L.) in a blinded manner using a modified scoring system, according to a previously published protocol (Matute-Bello et al., 2001).

Statistics

Data are expressed as mean \pm SD. Two-group analysis used either unpaired two-tailed t test or non-parametric Mann-Whitney U-test in the case of missing normality. Comparisons of multiple groups were performed using one-way ANOVA and subsequent Bonferroni multiple comparisons. If normality or equal variance tests failed, then a Kruskal-Wallis test and subsequent Dunn's multiple comparisons were used. Fisher exact test was used for analysis of percentage colonization, and chi-square test was used for analysis of mouse survival. All in vitro studies were done with at least three sets of independent experiments. GraphPad Prism and Excel were used for all analyses.

Ethics Statement

This study was performed under strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals. Cedars-Sinai Medical Center is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International and in compliance with NIH guideline of laboratory animal care and use. The protocol was approved by the institutional animal use and care committee of the Cedars-Sinai Medical Center (Institutional Animal Care and Use Committee 3402).

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and one table and can be found with this article online at <http://dx.doi.org/10.1016/j.chom.2015.11.001>.

AUTHOR CONTRIBUTIONS

S.L.K., P.K., and G.Y.L. conceived and directed the project, designed experiments, and prepared the manuscript. S.L.K. and P.K. conducted most of the experiments and analyzed the data. C.W.T., A.S., and A.K. performed some of the in vitro molecular and cellular experiments. J.L. and D.J. helped with scoring of the histology and design of the in vivo LPS experiment. R.M. facilitated the HPLC studies. V.N., M.A., and D.M.U. helped with the design of in vitro assays.

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