Gram-positive bacterial cell wall components inhibit herpes simplex virus infection

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Abstract

The role of the mucosal microbiome in viral infections remains unclear. Genital herpes, caused by herpes simplex virus 1 and 2 (HSV-1 and HSV-2), is among the most prevalent sexually transmitted infections. Despite evidence linking vaginal *Lactobacillus* to protection against viral infection, the specific mechanisms mediating this defense are not well understood. Here, we show that multiple cell wall components from gram-positive bacteria, including lactobacilli, inhibit HSV-1 and HSV-2 infection in cells and in a mouse model of genital herpes infection. Peptidoglycan and lipoteichoic acid, significantly reduced HSV infectivity in cells and improved disease outcomes in mice. Antiviral effects of cell wall components relied on intact peptidoglycan structure and were independent of TLR2-mediated host signaling. Our findings identify a species-independent antiviral function for multiple gram-positive bacterial cell wall components against HSV-1 and HSV-2 infection and suggest that the composition of the mucosal microbiome may play an underappreciated role in suppressing mucosal herpes infection in humans.

The vaginal microbiome as a primary defense against pathogens

Question: What makes some vaginal microbiomes better at protecting against herpes infection than others?

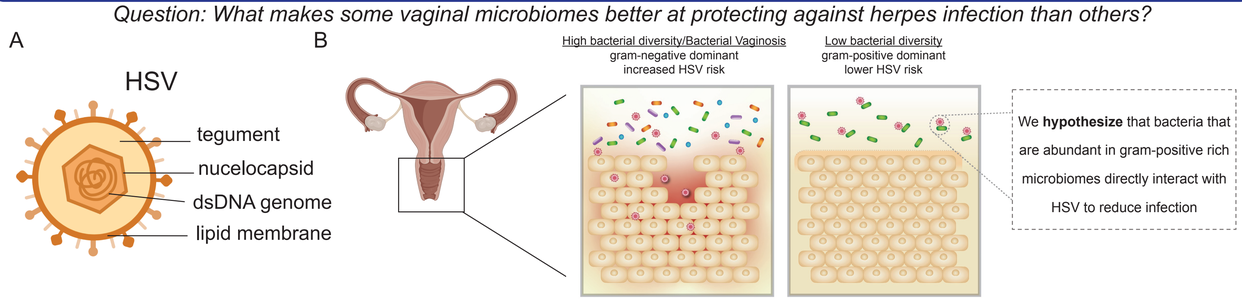
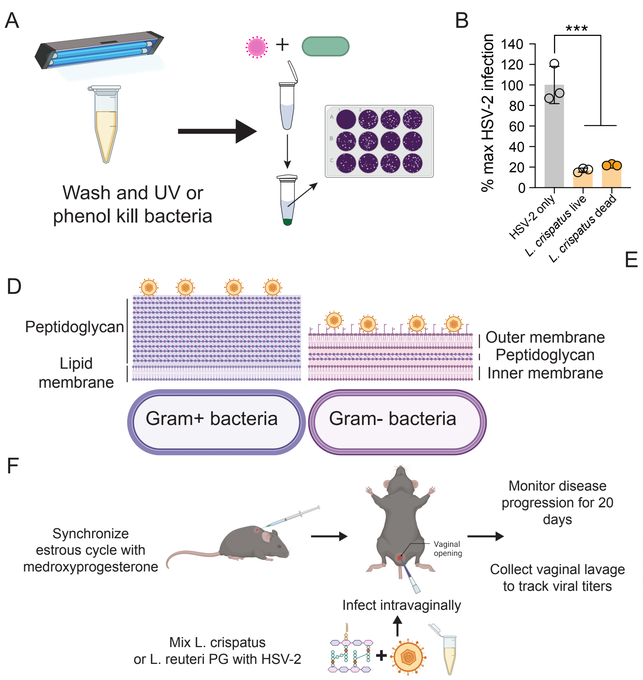


Figure 1. (A) HSV is an enveloped neurotropic dsDNA virus that infects the mucosal epithelia and establishes a lifelong latent infection in the dorsal root ganglia. (B) The vaginal mucosa is colonized by microbes that protect the host from invading pathogens, including viruses. Healthy vaginal communities are generally characterized by low bacterial diversity, with gram-positive *Lactobacillus* species dominant, and are associated with lower HSV infection risk. Bacterial Vaginosis (BV) is a common vaginal disorder associated with malodor, itching, abnormal discharge, and burning. BV is associated with high-diversity, *Lactobacillus*-low, gram-negative-dominant communities and is a known risk multiplier for viral infection, including HSV.

Lactobacillus cells and isolated peptidoglycan inhibit HSV-2 infection



Disease score
1. genital erythema
2. genital hair loss
3. ruffled fur, hunched posture
4. hind limb paralysis
5. death

Figure 2 summary: *Lactobacillus* species dominate healthy vaginal communities and are associated with reduced HSV risk. To determine if the bacterial cell body, rather than secreted factors, drives this protection, we exposed HSV-2 to live and killed washed vaginal *Lactobacillus crispatus (Lc)*. Both live and dead *Lc* reduced HSV-2 infection. Given that peptidoglycan (PG) is enriched in healthy vaginal communities with *Lactobacillus* vs. BV communities, we isolated PG from *Lc* and gut-associated *Limosilactobacillus reuteri (Lr)* (for comparison) and found that both PGs reduced HSV-2 infection in cells and improved survival and disease outcomes in a mouse model of vaginal herpes.

Figure 2 details: (A) Schematic for the assays shown in (B-C). (B-C) Bacterial cultures of *L. crispatus* MV-1A-US were grown in NYC III, washed, OD-normalized and killed by either UV (B) or phenol (C). (B) A representative experiment is shown n=3, and (C) n=4 across two experiments. Bacteria were then mixed with HSV-2 and incubated before pelleting the bacteria by centrifugation. Infectious HSV-2 in the supernatant was titered by plaque assay on Vero cells as shown in (A). (B-C, E) Datapoints are normalized to the average of HSV-2. (D) Lactobacilli are gram-positive bacteria with surface exposed PG. In gram-negtive bacteria, PG is not surface exposed and lies between the inner and outer membrane. Thus, herpes virions could potentially interact with the PG of gram-positive bacteria and the outer membrane and its surface structures in gram-negative bacteria. (E) We isolated *Lc* and *L. reuteri CF48-3A* PG and mixed them with HSV-2, incubated, and then added the mix to Vero cells to quantify infectious virus using a plaque assay (n=3-6, a representative experiment is shown). (F) Schematic and (G) timeline of mouse treatments for experiments shown in (H-J). Mice received depot medroxyprogesterone acetate (DMPA) to synchronize estrous cycles and then were infected intravaginally with 10,000 PFU HSV-2 and either 200 µg *Lc* or *Lr* PG at the time of infection and an additional 200 µg of PG 24 hours later (n=8-9, a representative experiment is shown). Survival was tracked over 12 days (H), and disease severity tracked for 12 days (I). (J) Vaginal lavage viral titers were quantified using plaque assays (J) The dashed line represents the Limit Of Detection (LOD). Error bars represent mean and the standard deviation (SD) (B-C, E) or mean and standard error of

Gram-positive PG reduces HSV-2 infection *in vitro* and *in vivo*

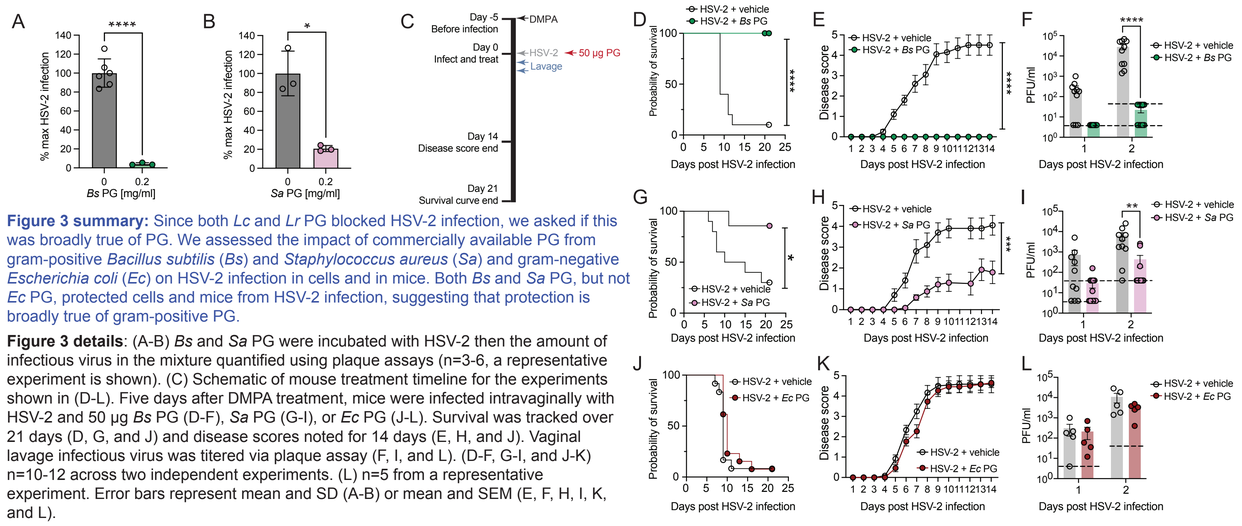


Figure 3 summary: Since both *Lc* and *Lr* PG blocked HSV-2 infection, we asked if this was broadly true of PG. We assessed the impact of commercially available PG from gram-positive *Bacillus subtilis* (*Bs*) and *Staphylococcus aureus* (*Sa*) and gram-negative *Escherichia coli* (*Ec*) on HSV-2 infection in cells and in mice. Both *Bs* and *Sa* PG, but not *Ec* PG, protected cells and mice from HSV-2 infection, suggesting that protection is broadly true of gram-positive PG.

Figure 3 details: (A-B) *Bs* and *Sa* PG were incubated with HSV-2 then the amount of infectious virus in the mixture quantified using plaque assays (n=3-6, a representative experiment is shown). (C) Schematic of mouse treatment timeline for the experiments shown in (D-L). Five days after DMPA treatment, mice were infected intravaginally with HSV-2 and 50 µg *Bs* PG (D-F), *Sa* PG (G-I), or *Ec* PG (J-L). Survival was tracked over 21 days (D, G, and J) and disease scores noted for 14 days (E, H, and J). Vaginal lavage infectious virus was titered via plaque assay (F, I, and L). (D-F, G-I, and J-K) n=10-12 across two independent experiments. (L) n=5 from a representative experiment. Error bars represent mean and SD (A-B) or mean and SEM (E, F, H, I, K, and L).

PG protection of HSV-2 infection is lysozyme sensitive

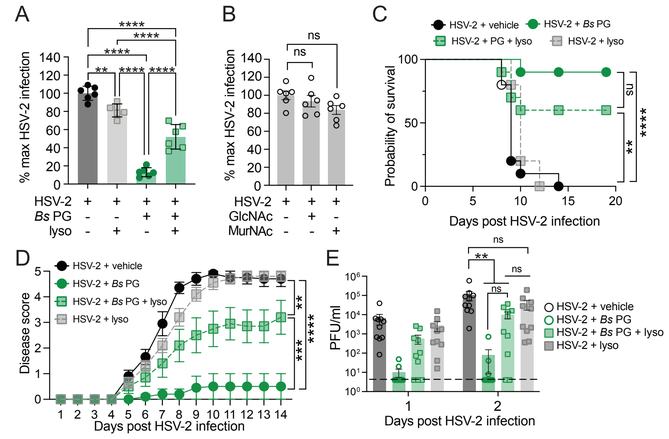


Figure 4 summary: To determine whether antiviral activity was attributable to PG itself rather than co-purifying compounds, we treated *Bs* PG with lysozyme, which degrades the PG backbone. Lysozyme treatment reduced antiviral protection in cells and in mice. The individual PG sugar subunits GlcNAc and MurNAc did not protect cells from HSV-2 infection, suggesting that long-chain PG is required for antiviral activity.

Figure 4 details: (A) *Bs* PG with or without lysozyme (lyso) was mixed with HSV-2, incubated, and infectious virus quantified by plaque assay on Vero cells (n=6, across two independent experiments). (B) GlcNAc or MurNAc [0.2 mg/ml] was mixed with HSV-2, incubated, and infectious virus quantified as in (A) (n=6, across two independent experiments). (C-E) Five days post DMPA-treatment, mice (n=10, across two independent experiments) were infected intravaginally with HSV-2 and the following treatments at the time of infection: 50 µg of *Bs* PG with or without lysozyme or lysozyme alone. Survival was tracked over 20 days (C), and disease severity tracked for 14 days (D). (E) Vaginal lavage infectious virus was quantified using plaque assays. Error bars represent mean and SD (A) or mean and SEM (B, D, and E).

LTA inhibits HSV-1 and HSV-2 infection *in vitro* and *in vivo*

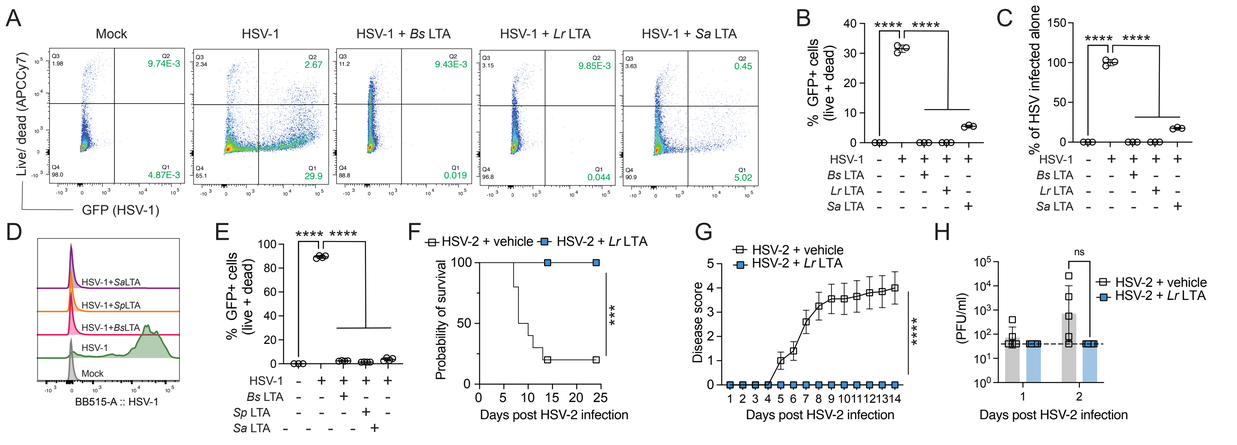


Figure 5 summary: Having established that PG inhibits HSV infection in Vero cells and mice, we asked whether lipoteichoic acid (LTA), another hallmark of gram-positive cell walls, also had antiviral activity. We found that LTA from multiple gram-positive species reduced HSV-1 infection in human endocervical cells (End1) and human foreskin fibroblasts (HFF). LTA also improved survival and disease outcomes when mice were infected with HSV-2, demonstrating that multiple cell wall components protect against HSV.

Figure 5 details: 50 µg LTA from *Bs*, *Lr* and *Sa* was incubated with HSV-1 K26GFP (MOI 1) and added to human End1 cells and frequency of live and dead GFP+ cells quantified (A-B) and normalized against HSV-1 infection condition alone (C) 22 hours post-infection (n=3 a representative experiment shown). 50 µg LTA from *Bs*, *Streptococcus pyogenes* (*Sp*), and *Sa* was incubated with HSV-1 K26GFP (MOI 1) and added to HFF cells and GFP expression from representative conditions shown (D) and quantified (E) 22 hours post-infection (n=4, a representative experiment is shown). (F-H) Five days after DMPA treatment, mice (n=10, across two independent experiments) were infected with HSV-2 alone or with 50 µg *Lr* LTA. Survival was tracked over 20 days (F), and disease severity tracked for 14 days (G). Vaginal lavage was collected and infectious virus quantified using plaque assays (n=5, a representative experiment is shown) (H). Error bars represent mean and SD (B, C, and E) or mean and SEM (G-H).

Cell wall inhibition of HSV-1 infection is independent of TLR2 and NOD1/2 sigaling

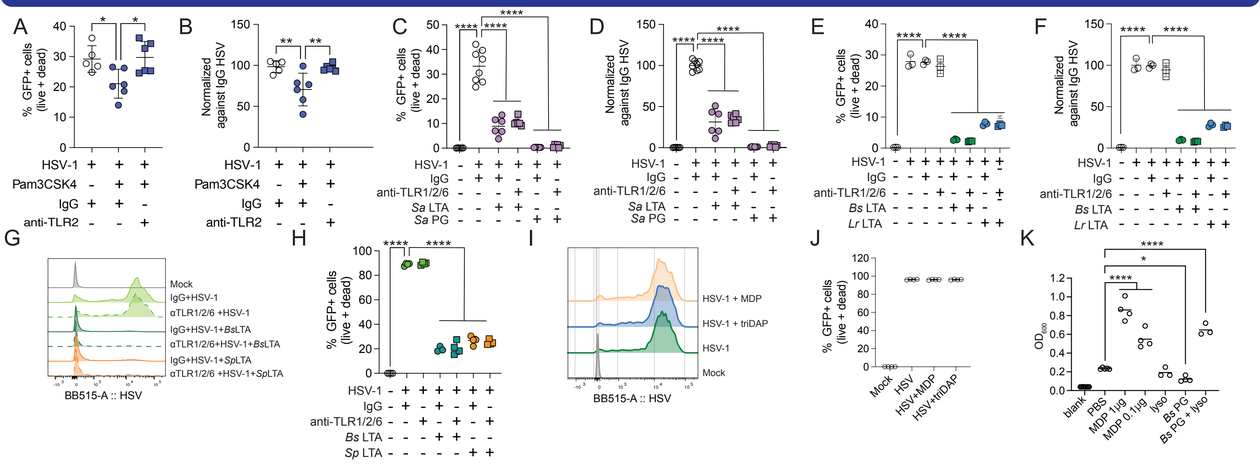
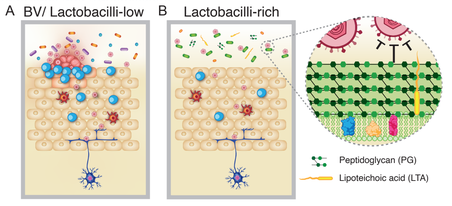


Figure 6 summary: LTA and PG are known to activate innate immune pattern recognition receptors TLR2, NOD1, and NOD2 in some systems. We asked whether cell wall antiviral activity in End1 cells and HFFs was mediated through these receptors. Blocking TLR1, 2, and 6 did not abrogate LTA or PG antiviral activity in End1 (PG and LTA) or HFF (LTA) cells. *Bs* PG fragments were able to activate NOD2, but not intact *Bs* PG in HEK-Blue reporter cells. However, NOD1 agonist triDAP and NOD2 agonist MDP did not protect against HSV-1 infection in HFFs, suggesting that NOD1/2 are dispensable for HSV-1 infection in HFFs. These data suggest that gram-positive cell wall antiviral activity is independent of canonical innate immune signaling, pointing to a role for direct physical interaction between intact cell wall components and the virion in blocking infection.

Figure 6 details: (A-E) End1 cells were pre-treated with anti-TLR1, 2, and 6 antibodies or control IgG and then infected with HSV-1 K26GFP at an MOI of 1 in the presence of TLR-2 agonist Pam3CSK4 (A-B), *Sa* LTA or PG (C-D), *Bs* LTA, or *Lr* LTA (E-F). GFP+ live and dead cells were quantified 22 hours post-infection (A, C, and E) and data normalized against HSV-1 infected cells alone (B, D, and F). (G-H) HFFs were treated with anti-TLR1, 2, and 6 or control IgG antibodies and then infected with HSV-1 K26GFP at an MOI of 1 in the presence of LTA from *Bs* or *Sp*, (n=4, a representative experiment is shown). GFP fluorescence profiles from representative samples are shown (E) and quantified (F) 22-hour post-infection. (I-J) HFFs were infected with an MOI of 1 HSV-1 K26GFP along with MDP or triDAP and GFP+ live and dead cells quantified 22 hours post-infection (J) (n=4, a representative experiment shown). (K) HEK293-NOD2 reporter cells were treated with *Bs* PG, *Bs* PG + lysozyme, 1 mg or 0.1 mg MDP and NOD2 activity measured by NF-κB-inducible SEAP (n=3-4, a representative experiment shown). Error bars represent mean and SD.

Model of PG and LTA inhibition of HSV infection

Our data show that gram-positive bacterial cell wall components PG and LTA inhibit HSV infection independently of canonical host innate immune signaling. We hypothesize that in *Lactobacillus*-dominant vaginal communities, PG and LTA interact with HSV virions to reduce infection at the mucosa, and that loss of this protection in BV may contribute to increased HSV susceptibility. These findings suggest that gram-positive bacterial cell wall components could be harnessed in a topical transmission-blocking gel to reduce HSV outbreaks and transmission.



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An earlier version of this work is on BioRxiv where further details on methods and statistics can be found: https://doi.org/10.1101/2025.11.02.686169

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