Lymphatic dissemination is a common route of systemic invasion by diverse extracellular bacteria in soft tissue infection .

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Abstract

Extracellular bacterial pathogens cause an estimated 6 million deaths annually and drive antimicrobial resistance. Systemic bacterial infection is typically attributed to direct blood vessel invasion or intracellular transit, while draining lymph nodes are generally assumed to trap and eliminate extracellular bacteria to prevent onward lymphatic spread. However, we previously demonstrated that *Streptococcus pyogenes* disseminates extracellularly via the lymphatic system. Here, we show that lymphatic dissemination extends across diverse clinically important extracellular bacterial pathogens, including *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus,* and *Streptococcus agalactiae*. In a murine soft tissue infection model, clinical isolates reached local draining lymph nodes, sequential distant draining lymph nodes, and systemic organs. In contrast, non-draining lymph nodes contained few or no bacteria, even during bacteraemia, consistent with lymphatic spread rather than haematogenous seeding. Dissemination efficiency varied between species and among isolates, with *S. aureus* largely restricted to local draining lymph nodes. Notably, capsular polysaccharide enhanced lymphatic dissemination independently of bacterial burden at the infection site. These findings identify lymphatic dissemination as a common route of invasion by extracellular bacterial pathogens, exposing sequential draining lymph nodes to viable bacteria.

Introduction

Lymphatic vessels permeate tissues throughout the body, returning fluid from interstitial spaces to the blood via filtering lymph nodes that are critical for generating adaptive immune responses to pathogens[1-3]. Despite providing a direct anatomical route from peripheral tissues to the circulation, the role of the lymphatic system in extracellular bacterial invasion remains poorly defined. Lymph nodes are commonly assumed to efficiently contain and eliminate extracellular bacteria, preventing onward spread to sequential draining lymph nodes and the bloodstream[4-7], reinforcing emphasis on direct vascular invasion or intracellular transport as dominant mechanisms of systemic dissemination[4,8-11]. However, these assumptions are largely inferred from studies using viral antigens, weakly virulent bacteria, or inactivated particles, and data from virulent clinical isolates is lacking[4,6,12-14].

We previously demonstrated that lymphatic dissemination of virulent *Streptococcus pyogenes* can drive systemic bloodstream infection from an initial soft tissue site[15]. In contrast to previously reported mechanisms of intracellular transport by phagocytes[9,16,17], *S. pyogenes* remained extracellular as it transited from the infection site into afferent lymphatics, traversed draining lymph nodes, and exited via efferent lymphatics. This process was augmented by streptococcal virulence mechanisms, including hyaluronan capsule, which can bind the lymphatic endothelial receptor LYVE-1[15,18]. Whether this extracellular lymphatic dissemination is unique to *S. pyogenes* or represents a broader feature of extracellular bacterial infection remains unknown[4].

Extracellular bacterial pathogens account for an estimated 6 million deaths annually and dominate WHO antimicrobial resistance priority lists, but incomplete understanding of systemic invasion constrains efforts to prevent, predict, and treat serious disease[19,20]. Over a quarter of bacterial bloodstream infections lack a defined primary focus[21-23], challenging the assumption that bacteraemia necessarily arises from direct vascular invasion at an overt local site[8,11]. Although most bacteria (~0.5-2 µm) exceed the size range considered optimal for passive lymphatic transport[24,25] , both bacterial[15] and host cells can enter afferent lymphatics[26,27].

Here, we investigated whether lymphatic dissemination is a conserved feature of infection across clinically important extracellular bacterial pathogens. We show that, following intramuscular infection, diverse bacterial species traverse and accumulate within sequential lymph nodes and disseminate to systemic organs. These findings identify lymphatic transit as a major route of systemic invasion across extracellular bacterial pathogens and have implications for host immunity.

Results

Diverse clinically important extracellular bacteria disseminate through sequential draining lymph nodes and to systemic organs.

Building on our prior demonstration that invasive *Streptococcus pyogenes* infection is driven by extracellular lymphatic dissemination[15], we examined whether this route is shared by other major extracellular bacterial pathogens. We analysed twelve soft-tissue clinical isolates from six clinically important pathogens (*Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus agalactiae*, and *S. pyogenes*) (Table 1) using a standardised murine hindlimb infection model.

Six hours after intramuscular infection, we quantified bacterial burden in blood, liver, spleen, and lymph nodes. We assessed lymph nodes draining the infected hindlimb as well as non-draining nodes to distinguish lymphatic from haematogenous dissemination. Although species- and strain-dependent variability was observed, all isolates of *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *S. agalactiae*, and *S. pyogenes* were recovered from local (inguinal and iliac) and sequential distant draining (axillary) lymph nodes and systemic organs (Figure 1A-E). In contrast, non-draining (brachial) lymph nodes consistently contained few or no detectable bacteria and were significantly lower than levels in draining lymph nodes, even during high-grade bacteraemia. Clinical isolates of *S. aureus* accumulated predominantly in local draining lymph nodes and showed limited dissemination to sequential distant draining nodes, blood, or systemic organs (Figure 1F). *S. aureus* strain USA 300, which is commonly used for infection studies, showed particularly weak

dissemination. Together, these findings demonstrate that lymphatic transit is a common invasive route across diverse clinically important extracellular bacterial pathogens.

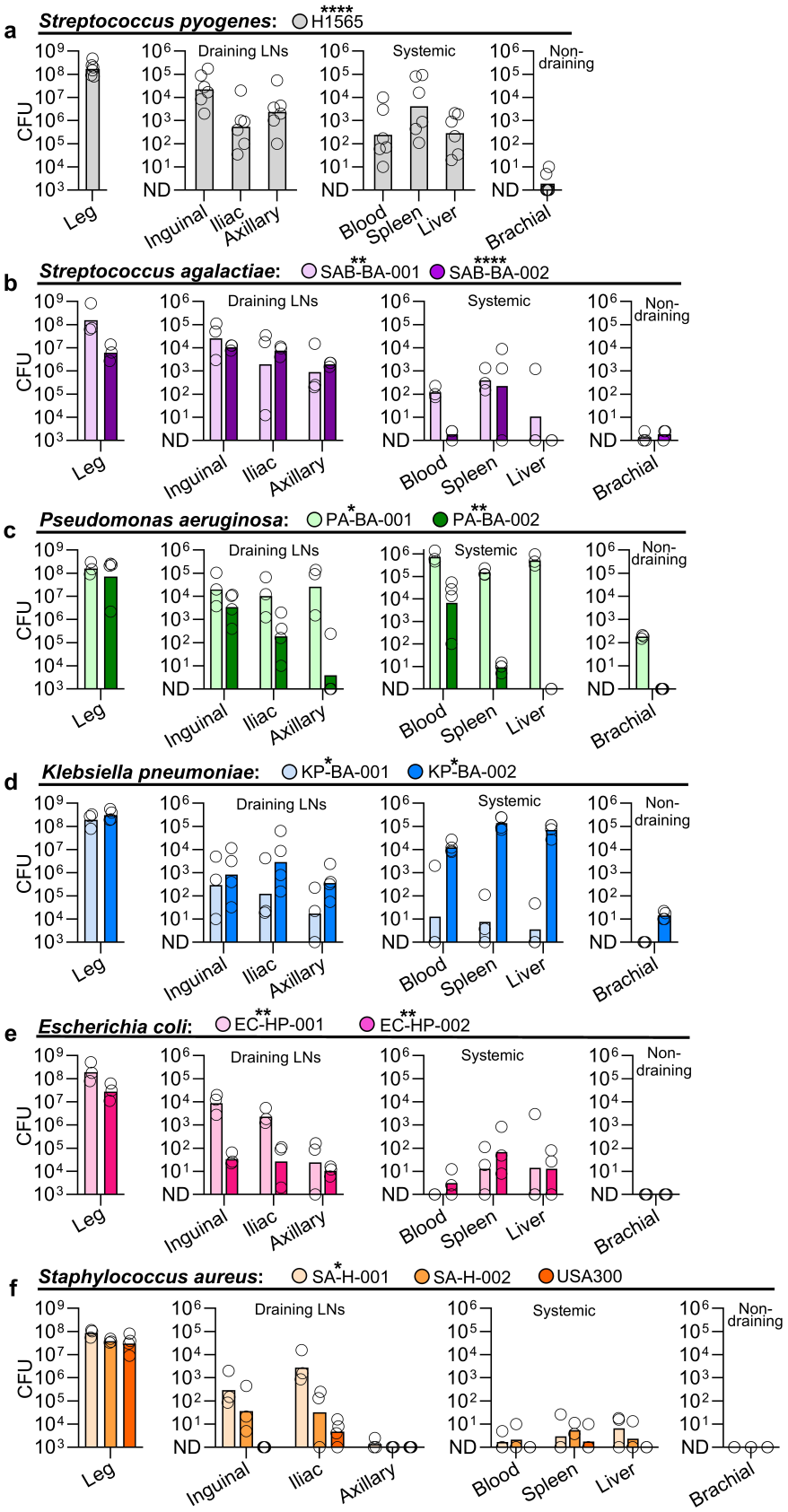

Figure 1 | Diverse clinically important extracellular bacteria disseminate through sequential draining lymph nodes and to systemic organs

Clinical isolates of *S. pyogenes* (H1565) (a), *S. agalactiae* (SAB-BA-001 and SAB-BA-002) (b), *P. aeruginosa* (PA-BA-001 and PA-BA-002) (c), *K. pneumoniae* (KP-BA-001 and KP-BA-002) (d), *E. coli* (EC-HP-001 and EC-HP-002) (e), and *S. aureus* (SA-H-001, SA-H-002, and USA300) (f) recovered from the hindlimb infection site, draining and non-draining lymph nodes, blood, and systemic organs of FVB/n mice 6 h after intramuscular infection with 10^8 CFU. Symbols represent individual mice, n = 3-6 per group, and bars indicate geometric means. CFU are reported per ml of blood, per g of liver, per spleen, per lymph node, and per infected hindlimb as indicated. Welch's t-tests comparing CFU between draining and non-draining lymph nodes were performed on $\log_{10}$-transformed data, with p-values adjusted for multiple comparisons using the Benjamini-Hochberg false discovery rate method across all strains. ****$p \leq 0.0001$; **$p \leq 0.01$; *$p \leq 0.05$; ns, $p > 0.05$.

Genetic deletion of capsule abrogates lymphatic spread

Our previous work showed that the hyaluronan capsule of *S. pyogenes* significantly enhances lymphatic dissemination[15,18]. To determine whether this effect extends beyond *S. pyogenes* and reflects a broader role for bacterial capsules, we examined *S. agalactiae* strain A909, which produces a structurally distinct polysaccharide capsule[28], alongside an otherwise isogenic acapsular mutant (A909 Δ*cpsD*).

Using the same intramuscular murine infection model, we quantified bacterial burden in blood, liver, spleen, ipsilateral lymph nodes draining the hindlimb infection site, and contralateral non-draining lymph nodes. Encapsulated *S. agalactiae* disseminated to local and sequential distant draining lymph nodes as well as systemic organs, with minimal recovery from non-draining lymph nodes. In contrast, deletion of the capsule reduced bacterial counts in local draining lymph nodes and abrogated dissemination to distant draining lymph nodes, blood and systemic organs (Figure 2). Bacterial numbers at the infection site did not differ between encapsulated and acapsular strains, indicating that the reduced dissemination was not attributable to impaired local survival.

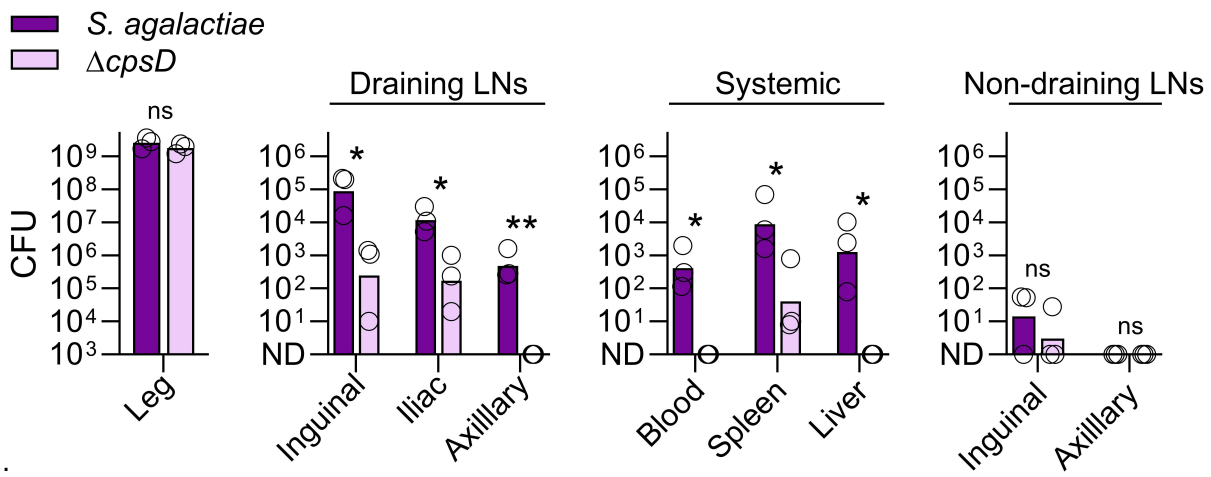

Figure 2 | Genetic deletion of capsule abrogates lymphatic spread of *S. agalactiae*

Recovery of *S. agalactiae* A909 or an otherwise isogenic capsule-deficient Δ*cpsD* mutant from the hindlimb infection site, ipsilateral draining and contralateral non-draining lymph nodes, blood, liver and spleen of mice 3 h after intramuscular infection with 10^8 CFU. Symbols represent individual mice, n = 3 per group, and bars indicate geometric means. CFU are reported per ml of blood, per g of liver, per spleen, per lymph node, and per infected hindlimb, as indicated. Welch's t-tests were performed on $\log_{10}$-transformed data, with p-values adjusted for multiple comparisons using the Benjamini-Hochberg false discovery rate method. **$p \leq 0.01$; *$p \leq 0.05$; ns, $p > 0.05$.

Enzymatic digestion of alginate exopolysaccharide does not influence *P. aeruginosa* dissemination .

Whereas two distinct surface-bound streptococcal bacterial capsules promoted significant lymphatic dissemination, *P. aeruginosa* strain PA-BA-001 exhibited substantial lymphatic dissemination despite lacking a classical capsule (Figure 1C). However, strain PA-BA-001 does produce abundant secreted exopolysaccharide, primarily alginate. To determine whether alginate contributed to dissemination, we infected mice intramuscularly with PA-BA-001 that had been pre-treated with alginate lyase (200 U, 30 min at 37°C) or left untreated. Bacterial burden was quantified 3 h after infection in blood, liver, spleen, ipsilateral draining lymph nodes and contralateral non-draining lymph nodes. Alginate lyase treatment did not alter bacterial counts in any compartment. High bacterial numbers were recovered from ipsilateral draining lymph nodes and systemic organs, whereas contralateral non-draining lymph nodes contained significantly fewer bacteria irrespective of alginate lyase treatment ($p \leq 0.01$; Welch's t-tests).

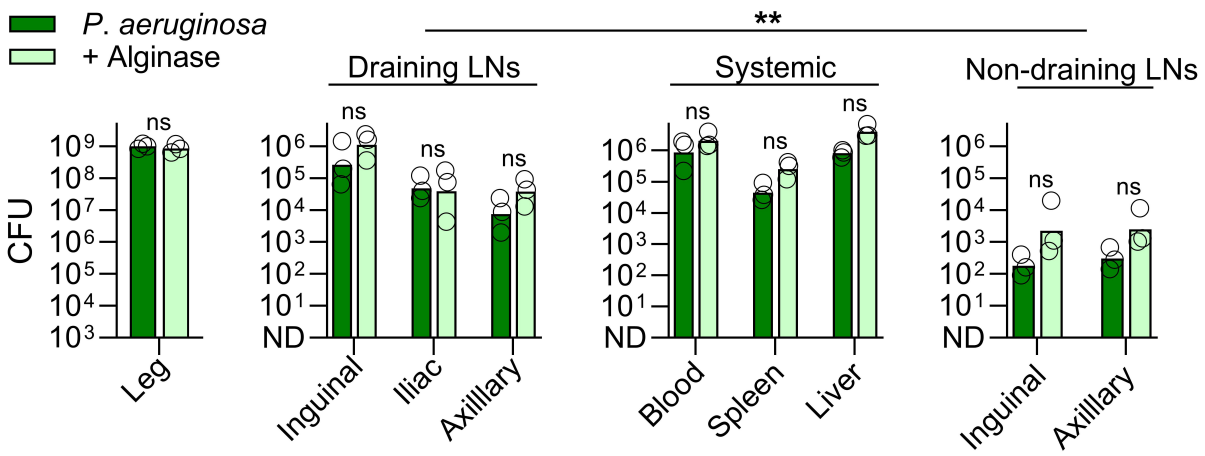

Figure 3 | Enzymatic digestion of alginate exopolysaccharide does not influence *P. aeruginosa* dissemination

Recovery of *P. aeruginosa* (PA-BA-001) with or without pre-treatment with alginate lyase (200 U), from the hindlimb infection site, ipsilateral draining and contralateral non-draining lymph nodes, blood, liver and spleen of mice 3 h after intramuscular infection with 10^8 CFU. Symbols represent individual mice, n = 3 per group, and bars indicate geometric means. CFU are reported per ml of blood, per g of liver, per spleen, per lymph node, and per infected hindlimb as indicated. Welch's t-tests were performed on log10-transformed data, with p-values adjusted for multiple comparisons using the Benjamini-Hochberg false discovery rate method. **$p \leq 0.01$; ns, $p > 0.05$.

Discussion

We previously demonstrated that extracellular lymphatic dissemination through sequential lymph nodes is the predominant route of invasive spread for *S. pyogenes*[15]. Here, we show that lymphatic transit through sequential lymph nodes occurs across diverse extracellular bacterial pathogens, including *E. coli*, *K. pneumoniae*, *P. aeruginosa*, and *S. agalactiae*. These findings support lymphatic dissemination as a common pathway of systemic invasion that also delivers extracellular bacteria to lymph nodes central to adaptive immune generation.

Across all pathogens examined, bacteria were consistently recovered at high burdens in local draining lymph nodes and were frequently present in the sequential distant draining axillary lymph node. In contrast, the brachial lymph node, which is adjacent to the axillary node and shares blood supply but does not receive lymphatic drainage from the infected hindlimb[15,29], contained no bacteria or significantly lower bacterial burdens than draining lymph nodes. Greater bacterial burdens in axillary than brachial lymph nodes persisted during bacteraemia, indicating lymphatic dissemination rather than haematogenous seeding.

These findings challenge the prevailing view that lymph nodes function as terminal bacterial traps [6,7,30,31]. This perspective has been shaped disproportionately by studies of *Staphylococcus aureus* USA300 LAC, which is poorly virulent in murine soft-tissue infection models[32,33]. Extending this literature, we found that modern clinical isolates of MRSA and MSSA likewise accumulated predominantly within local draining lymph nodes and rarely seeded distant lymph nodes or systemic organs. This identifies *S. aureus* as an important exception compared with the other extracellular pathogens examined, which showed efficient lymphatic dissemination accompanied by systemic infection. This concordance between limited lymphatic dissemination and limited systemic infection further supports lymphatic transit as the dominant route of invasive spread.

Particles of roughly 10-100 nm are considered optimal for passive lymphatic uptake[24,25], whereas bacteria (0.25-2 µm width) are substantially larger. Nonetheless, our

results show that diverse extracellular bacteria can access the lymphatic system, and that dissemination varies across species and strains, indicating pathogen-specific determinants. Capsule deletion in *S. agalactiae* markedly reduced lymphatic and systemic dissemination without reducing bacterial burdens at the infection site, suggesting that this effect was not explained simply by loss of capsule-mediated resistance to opsonophagocytic killing. Consistent with this, our previous work with *S. pyogenes* showed that clodronate-mediated depletion of subcapsular and medullary sinus lymph node macrophages had no impact on either lymphatic or systemic bacterial counts[15], demonstrating that inhibition of phagocytosis alone does not explain lymphatic spread. Bacterial capsules are polyanionic and hydrophilic, properties that can enhance lymphatic uptake of particles by masking adhesins[34,35] and increasing electrostatic repulsion within the extracellular matrix[36,37]. While the surface-associated distinct capsules of *S. pyogenes*[15,18,38] and *S. agalactiae* both promoted lymphatic dissemination, enzymatic digestion of the secreted alginate exopolysaccharide of *P. aeruginosa* did not alter dissemination, although incomplete enzymatic digestion in vivo cannot be excluded. These findings indicate that surface-associated capsular polysaccharide promotes lymphatic dissemination beyond immune evasion.

Clinically, these findings provide a potential anatomical explanation for cryptic bloodstream infections, which account for more than 25% of bacteraemia episodes and arise despite limited superficial peripheral tissue involvement[21,22]. Lymphatic dissemination from peripheral infection offers a plausible route to systemic invasion without overt vascular breach and helps to explain discrepancies between superficial infection severity and serious systemic outcomes. Although *S. pyogenes* is particularly associated with bacterial lymphangitis and lymphadenitis in humans, other bacterial species have also been recovered from lymph nodes during surgery[39-41], with evidence that lymphatic dissemination drives systemic spread[42].

The implications of bacterial lymphatic dissemination extend beyond systemic spread. Prior work has shown that bacterial infection can disrupt subcapsular sinus macrophage organisation, impair germinal centre maintenance, and alter adaptive priming dynamics in lymphoid organs[31,43-46]. In *S. pyogenes* and *S. aureus*, toxin production within

lymphoid tissue provides a direct mechanism for altered T-cell activation and inflammatory amplification[47,48]. Our data demonstrate that multiple extracellular bacterial species access and persist within sequential draining lymph nodes during acute infection, extending opportunities for immune dysregulation. Bacterial interactions with collecting lymphatic vessels may also affect lymphatic function and immune responses[49].

Several limitations warrant consideration. Although the dissemination patterns observed are strongly consistent with lymphatic spread, direct visualisation of efferent lymphatic transit and surgical interruption of lymphatic flow via lymphadenectomy, previously demonstrated for *S. pyogenes*, was not repeated here across all bacterial species[15]. Experiments were conducted in a single mouse strain; however, we have previously shown that lymphatic dissemination of a given bacterial strain is reproducible across multiple strains of mice[15]. We also assessed a single bacterial dose typical of intramuscular infection models; though our prior work with *S. pyogenes* demonstrates that lymphatic dissemination occurs across a range of bacterial concentrations, including burdens comparable to those observed in human infection[15,50]. While these data support lymphatic dissemination as a major route of spread, they do not exclude contributions from haematogenous pathways, the relative importance of which may vary across bacterial species, strains, and infection contexts.

In summary, extracellular lymphatic dissemination through sequential lymph nodes is a common route of invasion across clinically important bacterial pathogens following peripheral tissue infection. This pathway links local infection to systemic spread while exposing immune-inductive tissues to bacteria. Accounting for lymphatic dissemination will be important for interpreting invasive infection and the models used to study it.

Table 1 | Bacterial strains used in this study

Species	Strain ID	Typing	Description	Reference or Bioresource and identifier
E. coli	EC-HP-001	ST131	Bacteraemia isolate	Microbial Products
E. coli	EC-HP-002	ST88	Bacteraemia isolate	Microbial Products
K. pneumoniae	KP-BA-001	Undefined	Hypermucoviscous (string test+)	BioAID (H1733)
K. pneumoniae	KP-BA-002	Undefined	Skin infection, Hypermucoviscous (string test+)	BioAID (H1521)
P. aeruginosa	PA-BA-001	Undefined	Bacteraemia	BioAID (H1730)
P. aeruginosa	PA-BA-002	Undefined	Breast abscess	BioAID (H1522)
S. agalactiae	A909	Serotype Ia	Neonatal bacteraemia	Reference strain (1934)
S. agalactiae	A909 Δ*cps*	Acapsular	Acapsular Δ*cpsD* isogenic A909 mutant, constructed with pJRS233[51]	Areschoug, T., and Lindahl, G. (Unpublished)
S. agalactiae	SAB-H-001	Serotype Ia	Neonatal bacteraemia	BioAID (H1146)
S. agalactiae	SAB-H-002	Serotype V	Neonatal bacteraemia	BioAID (H1148)
S. aureus	SA-H-001	CC15	MSSA bacteraemia	“HHS-8”[52]
S. aureus	SA-H-002	CC22	MRSA bacteraemia	“HHS-9”[52]
S. aureus	USA300	CC8	MRSA (acapsular)	[53]
S. pyogenes	H1565	*emm*1	Encapsulated *covR* mutant in vivo-passaged derivative of H584 (maternal sepsis)	[38]

Bacterial isolates taken from BioAID[54] and Microbial Products[55] patient bioresources.

Methods

Bacterial strains and growth conditions

Streptococcus pyogenes and *Streptococcus agalactiae* were cultured on Columbia horse blood agar (CBA) (EO Labs) or in Todd Hewitt broth with yeast extract (both Oxoid) at 37°C, in 5% CO2. *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Staphylococcus aureus* were grown on CBA at 37°C in air overnight. All strains and origins described in Table 1. Bioresource in Adult Infectious Diseases (BioAID) and Microbial Products are REC-approved bioresources of patient isolates. BioAID is a multicentre Bioresource, collecting biological samples from patients with suspected infection at presentation to the Emergency Department.

Mice

In vivo experiments were performed in accordance with the Animal (Scientific Procedures) Act 1986, with appropriate UK Home Office licenses according to established institutional guidelines using 6-8-week-old FVB/n female mice (Charles River UK).

Infections

Mice were challenged intramuscularly with 10^8 CFU of *S. pyogenes*, *S. agalactiae*, *E. coli*, *L. lactis*, *K. pneumoniae*, *P. aeruginosa*, or *S. aureus* in 50 µl PBS and quantitative endpoints compared at 3- or 6-hours post-infection, as indicated. For alginate exopolysaccharide digestion, 200 U of alginate lyase was added to a *P. aeruginosa* inoculum and incubated for 30 minutes at 37°C prior to infection.

Enumeration of bacteria from organs and blood

At specified endpoints, mice were anaesthetised with isoflurane (4% induction, 2% maintenance), their blood taken by cardiac puncture into tubes containing heparin, then euthanised. Thigh muscle, liver, spleen, and ipsilateral axillary, brachial, iliac, and inguinal lymph nodes were dissected for further analyses. Dissected lymph nodes were first disrupted with a motorised pellet pestle, and all organs and tissues were homogenised with scissors in PBS. Bacterial CFU counts were determined by plating of homogenised tissue and blood samples onto the specified agar, with or without dilution in PBS, as appropriate. CFU are displayed as per ml of blood, per g of liver, per leg, or per organ for all figures.

Statistics

All statistical analyses were performed with GraphPad Prism 11. Comparison of two datasets was carried out using a two-tailed Welch's t-tests on $\log_{10}$-transformed data, with p-values adjusted for multiple comparisons using the Benjamini-Hochberg false discovery rate method. Comparisons of groups of draining lymph nodes and groups of non-draining lymph nodes normalised CFU counts by the number of lymph nodes per group.

Ethics

Use of clinical isolates were approved by South Central - Oxford C Research Ethics Committee: 14/SC/0008 and 19/SC/0116 (BioAID) and West London Research Ethics Committee: 06/Q0406/20 (HPRU).

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Curation, H.K.L., S.S.; Writing - Original Draft, M.K.S.; Visualization, M.K.S.; Funding Acquisition, M.K.S., D.G.J., and S.S.

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