Lymphatic dissemination drives systemic invasion by diverse clinically relevant extracellular bacterial pathogens

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Abstract

Systemic bacterial infection is typically attributed to direct blood vessel invasion or intracellular transit, while the role of the lymphatic system remains largely unexplored. We previously showed that *Streptococcus pyogenes* disseminates extracellularly via the lymphatics. Here, we extend this observation to a diverse group of important extracellular bacterial pathogens, including *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Streptococcus agalactiae*. In a murine soft tissue infection model, nearly all clinical isolates reached both local and distant draining lymph nodes and systemic organs, with minimal bacterial presence in non-draining nodes. This pattern demonstrates that diverse bacteria can traverse filtering lymph nodes and supports lymphatic rather than haematogenous spread as the primary driver of systemic invasion. Bacterial dissemination varied between species and among isolates of the same species, with capsular polysaccharides emerging as one key determinant. These findings establish lymphatic metastasis as a common but under-recognised route of bacterial invasion, with implications for pathogenesis, immunity, and treatment.

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[1,2]. Despite forming a direct anatomical route for pathogen invasion, the role of the lymphatic system in bacterial infection is poorly defined, with intracellular routes of systemic dissemination dominating perceptions of bacterial invasion[3-6]. Early studies provided evidence for bacterial lymphatic spread, but the lymphatic system is commonly assumed to efficiently contain and kill bacteria[5,7-9]. However, current perspectives on bacterial lymphatic dissemination are largely inferred from studies using viral antigens, weakly virulent bacteria, or inactivated particles, with data from live virulent bacteria lacking[5,8,10-12].

We recently demonstrated previously unappreciated extracellular bacterial lymphatic metastasis of virulent strains of *Streptococcus pyogenes* drives systemic infection[13]. Strikingly, unlike previously reported mechanisms of intracellular transport by phagocytes[4,14,15], we showed *S. pyogenes* remained extracellular as it moved from the infection site into afferent lymphatics, through draining lymph nodes and onward via efferent lymphatics. This lymphatic dissemination was augmented by streptococcal virulence mechanisms, particularly hyaluronan capsule. Inside infected lymph nodes, we showed *S. pyogenes* subverted phagocytic clearance to replicate and seed intense bloodstream infection. Collectively, these findings highlighted the lymphatic system as key in *S. pyogenes* pathogenesis, contrary to previous expectations[5,13,16].

Over a quarter of all bacterial bloodstream infections lack a defined primary focus of infection[17-19], conflicting with the assumption that bacteraemia arises from direct invasion of blood vessels at a significant local site of infection[3]. This discrepancy suggests that lymphatic dissemination may be an under-recognised and potentially dominant route for systemic invasion for more bacterial species. Lymphatic uptake of particles is influenced by size, shape, surface charge, and hydrophobicity[20,21]. Most bacteria are much larger than the optimal size for

lymphatic clearance of particles (20–100 nm), however both bacterial (~1–2 μm) and human (5–15 μm) cells can enter initial afferent lymphatics[13,22-24]. Like *S. pyogenes*, most species of clinically important extracellular bacterial pathogens produce capsular polysaccharides which alter their physiochemical properties[25]. Bacterial capsular polysaccharides can increase electrostatic repulsion to the extracellular matrix[26], mask underlying adhesion molecules on the bacterial surface[27,28], and inhibit phagocytic clearance[29,30]; properties that enhance uptake of particles into lymphatic vessels[20,21].

Here, we broaden our investigation of bacterial invasion to a diverse set of clinically important extracellular pathogens and show that lymphatic dissemination is a common and under-recognised feature of infection. We demonstrate diverse bacteria traverse filtering lymph nodes and present evidence consistent with lymphatic, rather than haematogenous, spread as a dominant driver of systemic invasion across species. These findings prompt reconsideration of how bacterial dissemination is understood, with important implications for pathogenesis, immunity, and treatment.

Results

A wide range of clinically important extracellular bacterial pathogens reach distant lymph nodes and systemic organs via the lymphatics.

Building on our recent demonstration of the previously unappreciated importance of extracellular bacterial lymphatic dissemination in *Streptococcus pyogenes* infection[13], we hypothesised that lymphatic metastasis might also be pertinent to the pathogenesis of other bacterial species. To investigate this, we systematically analysed lymphatic involvement in bacterial dissemination using an established murine model of bacterial tissue invasion[13]. We assessed within-host dissemination of isolates from a broad panel of clinically important bacterial pathogens, including Gram-positive and -negative species: *Escherichia coli*, *Klebsiella*

pneumoniae, *Pseudomonas aeruginosa*, *Staphylococcus aureus, Streptococcus pyogenes,* and *Streptococcus agalactiae* (group B streptococcus) (Table 1). Most isolates were obtained from bacterial bloodstream infections that had emanated from a soft tissue focus, and we tested an average of two strains of each bacterial species.

Six hours post intramuscular infection, we quantified bacterial burden in blood, liver, spleen, and lymph nodes that do or do not drain the hindlimb injection site[31]. Despite notable 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 and distant draining lymph nodes and systemic organs, but absent or present in significantly lower numbers in non-draining lymph nodes (Fig. 1a–e), indicating prominent lymphatic spread rather than haematogenous seeding of lymph nodes. Draining lymph enters the bloodstream via the lymphatic ducts and the spleen and liver can remove bacteria from blood. Bacterial counts in non-draining lymph nodes were low, even in infections that caused high-grade bacteraemia and large systemic bacterial burden, such as *P. aeruginosa* I-P-0087 and *K. pneumoniae* I-P-0811 highlighting the inefficiency of haematogenous seeding. For *S. aureus*, the greatest bacterial counts outside of the infection site were present in the local draining lymph nodes, though there low bacterial numbers in distant draining lymph nodes and minimal systemic bacterial burden (Fig. 1f). Together, these data demonstrate that diverse clinically important extracellular bacteria, including both Gram-positive and Gram-negative species, are capable of spreading through the murine lymphatic system during infection.

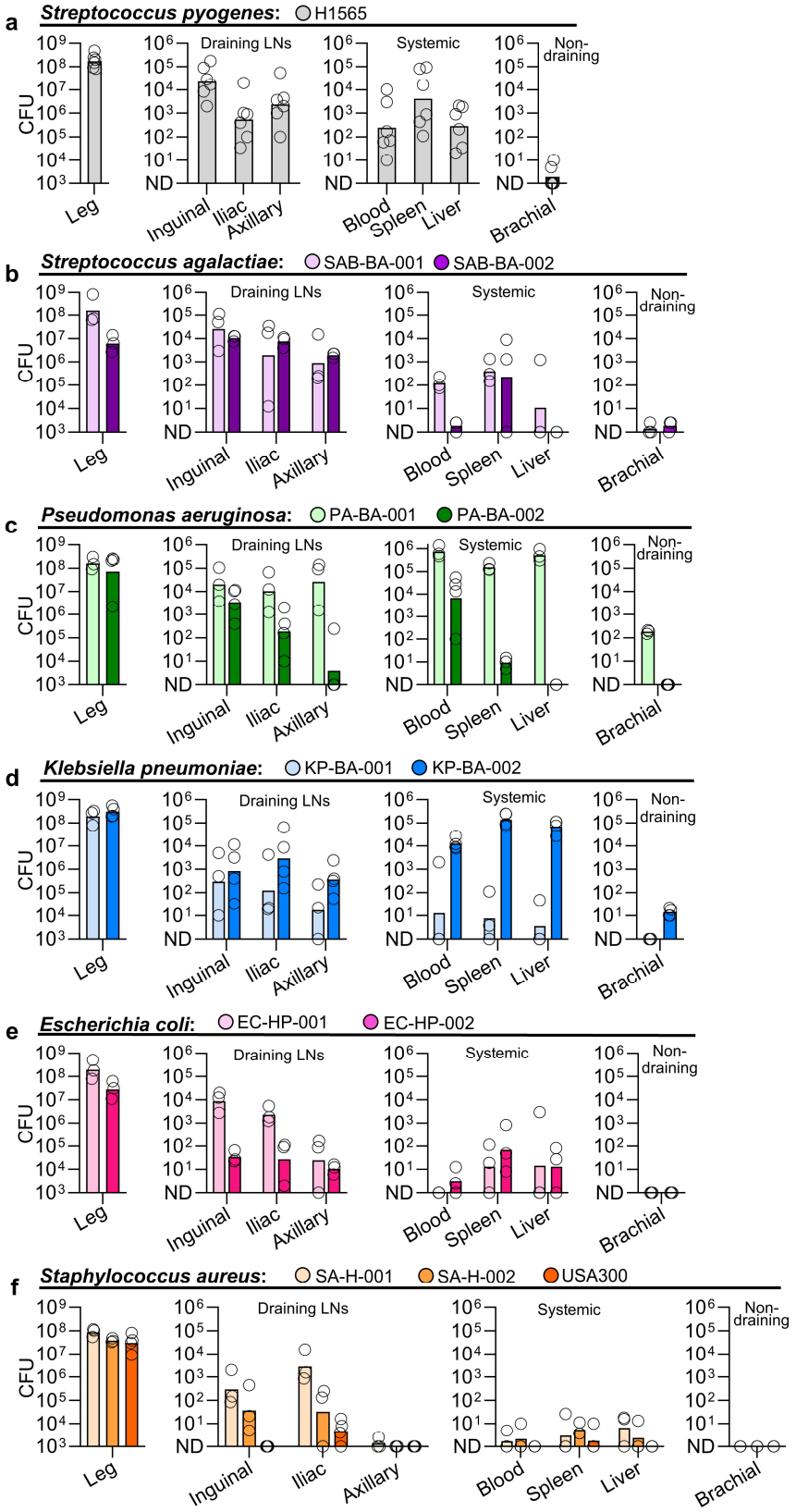

Figure 1 | A wide range o clinically important extracellular bacterial pathogens reach distant lymph nodes and systemic organs via the lymphatics.

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 USA 300) (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 leg, or per organ.

Genetic deletion of capsule abrogates lymphatic spread of *S. agalactiae*.

Our previous work showed that the hyaluronan capsule of *S. pyogenes* significantly enhances lymphatic dissemination[13,32]. Because bacterial capsules confer physiochemical properties that could enhance lymphatic uptake, we investigated whether this effect was unique to *S. pyogenes* or reflected a broader feature of capsular polysaccharides. To directly test this, we evaluated the bacterial spread of *S. agalactiae* strain A909, which produces a sialylated polysaccharide serotype Ia capsule, that is distinct in structure to the hyaluronan capsule of *S. pyogenes*[33], and an otherwise isogenic acapsular mutant (A909 Δ*cpsD*).

Following intramuscular infection, we quantified bacterial burden in blood, liver, spleen, ipsilateral lymph nodes that drain the hindlimb infection site, and non-draining contralateral lymph nodes. Like other *S. agalactiae* isolates, A909 showed extensive lymphatic dissemination to local and distant draining lymph nodes and systemic organs, with little to no bacteria recovered from non-draining lymph nodes. Deletion of the capsule dramatically reduced bacterial numbers in the local draining lymph nodes and abrogated spread to distant sequentially draining lymph nodes and systemic sites (Figure 2). Importantly, bacterial numbers at the infection site did not differ between strains, indicating that dissemination defects were not due to impaired local survival. As in other infections studied, colony counts in draining lymph nodes were significantly higher than in non-draining nodes. The highest burden outside the infection site was consistently found in local draining lymph nodes, reinforcing the

conclusion that lymphatic transit is the dominant route of systemic invasion.

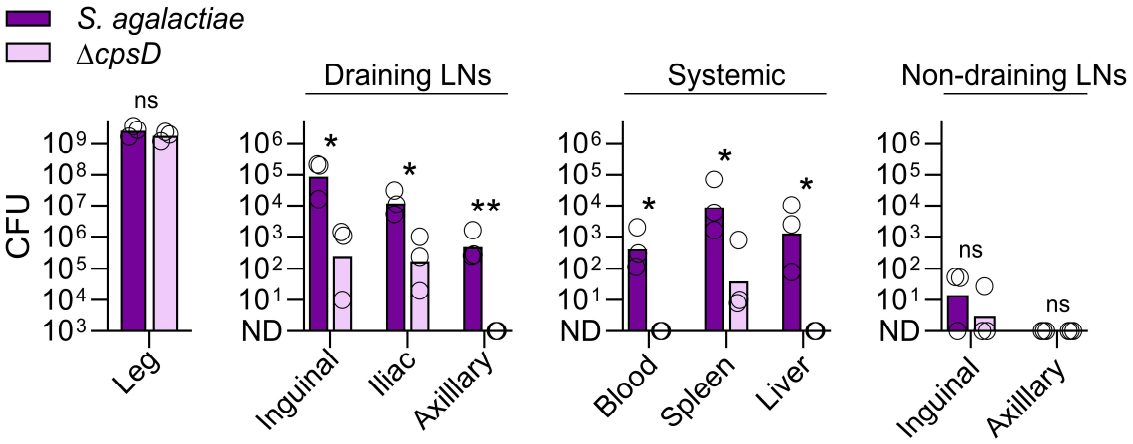

Figure 2. Genetic deletion o capsule abrogates lymphatic spread o *S. agalactiae*.

Recovery of *S. agalactiae* (A909, serotype Ia) or an otherwise isogenic capsule-deficient Δ*cpsD* mutant from the hindlimb infection site, draining and non-draining lymph nodes, blood, and systemic organs of mice 3 h after intramuscular infection with 10^8 CFU. Symbols represent individual mice, n = 3 per group, and bar heights indicate geometric means. **p ≤ 0.001; *p ≤ 0.05; ns, p > 0.05: Welch's t tests were performed on $\log_{10}$-transformed data. CFU are reported per ml of blood, per g of liver, per leg, and per organ.

Enzymatic digestion o alginate exopolysaccharide does not influence *P. aeruginosa* invasion.

Having demonstrated that two distinct streptococcal capsules promote lymphatic dissemination, we next investigated whether *P. aeruginosa* spread involves a similar mechanism. Although it lacks a capsule, *P. aeruginosa* PA-BA-001 exhibited substantial lymphatic dissemination (Fig. 1c) and produces large amounts of hydrophilic, anionic exopolysaccharide, primarily alginate, which is not surface-bound. To test whether alginate contributes to spread, we compared bacterial dissemination following intramuscular infection with *P. aeruginosa* strain PA-BA-001, with or without pre-treatment using 200 U alginate lyase for 30 minutes at 37 °C. Bacterial burden was quantified 3 hours post-infection in blood, liver, spleen, ipsilateral draining lymph nodes, and contralateral non-draining lymph nodes. Alginate

lyase treatment had no measurable effect on bacterial counts in any tissue (Fig. 3). High levels of *P. aeruginosa* were consistently recovered from draining lymph nodes and systemic organs, with minimal presence in non-draining nodes. This pattern again indicates that lymphatic spread is the primary route of dissemination, rather than seeding of lymph nodes from blood.

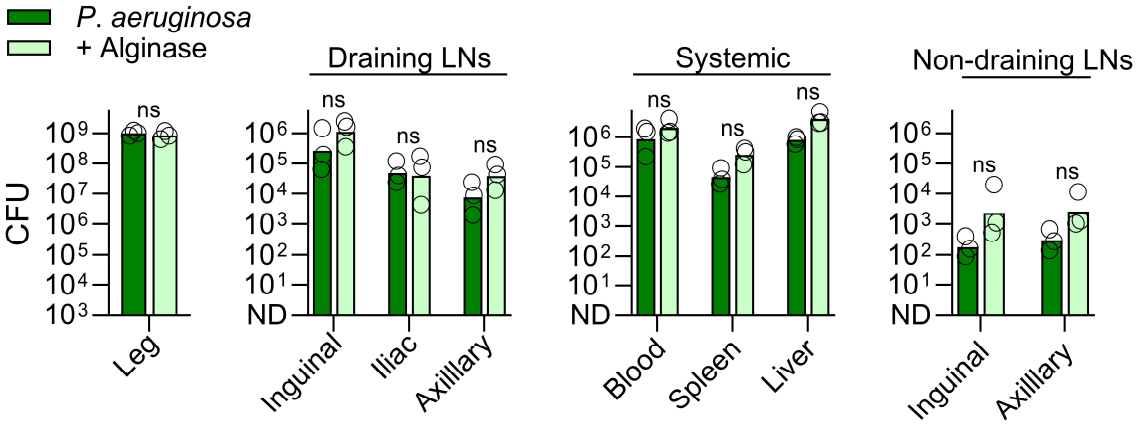

Figure 3. Enzymatic digestion o alginate exopolysaccharide does not influence *P. aeruginosa* invasion.

Recovery of *P. aeruginosa* (PA-BA-001) with or without pre-treatment in 200U alginate lyase, from the hindlimb infection site, draining and non-draining lymph nodes, blood, and systemic organs of mice 3 h after intramuscular infection with 10^8 CFU. Symbols represent individual mice, n = 3 per group, and bar heights indicate geometric means. *$p \leq 0.05$; ns, $p > 0.05$: Welch's t tests were performed on $\log_{10}$-transformed data. CFU are reported per ml of blood, per g of liver, per leg, and per organ.

Discussion

The mechanisms by which extracellular bacteria disseminate from peripheral tissue to the bloodstream in infection remain poorly defined[5]. Systemic invasion is typically attributed to blood vascular endothelial translocation at the primary infection site or intracellular transport within phagocytes[3,4,14,34]. Our recent findings revealed that extracellular dissemination through sequential lymph nodes is the predominant route of invasive spread for clinical isolates of the important human pathogen *S. pyogenes*[13]. Here, we present evidence that redefines current

models of systemic bacterial infection, demonstrating that lymphatic transit is a major invasive route for a broad range of extracellular bacterial, including *E. coli*, *K. pneumoniae*, *P. aeruginosa*, and *S. agalactiae*, that are among the top 10 bacterial causes of global mortality[35]

In all bacterial species tested, CFU counts in draining lymph nodes, particularly in distal sequential draining nodes, were strongly associated with systemic spread. In contrast, non-draining lymph nodes contained very few bacteria, even during high-grade bacteraemia, indicating negligible blood-borne seeding. Lymph from the hindlimb infection site reaches the distant-draining ipsilateral axillary lymph node only after passing through the local-draining ipsilateral inguinal lymph node[13,36]. Blood flowing from the same hindlimb, by contrast, returns to the heart and lungs before being distributed in parallel to all arterial beds. Since the non-draining brachial node and the distant-draining axillary node receive the same arterial blood, they share the same baseline risk of blood-borne seeding. This contrast demonstrates that lymph, not blood, is the primary source of bacteria in draining lymph nodes and shows that most species can traverse nodal filtration. Our results establish the lymphatic system as the dominant pathway by which bacteria reach both local-draining and distant-draining lymph nodes and subsequently enter the circulation and seed systemic organs such as the liver and spleen. This pattern of spread reflects normal lymphatic physiology, in which lymph passes through a series of lymph nodes before entering the bloodstream.

In addition to their role in generating adaptive immunity, lymph nodes are regarded to capture bacteria and coordinate innate immune responses that eliminate pathogens[5,8,9,37]. Consequently, the lymphatic system is commonly considered a dead-end for bacterial spread[8,38-41]. However, this paradigm is primarily supported by a limited number of studies using *S. aureus*[41-43] which, though a robust human pathogen, generally exhibits poor systemic spread in murine models[9,44]. This view has also been shaped by inferred relevance from viral infection models, where strategically positioned CD169^{+} subcapsular and medullary sinus macrophages in lymph nodes were shown to prevent systemic spread[10,11]. Our data update these perceptions

by confirming that a wide range of bacterial species can traverse lymph nodes. We also build on data with reference and GFP-expressing strains of *S. aureus,* that even highly virulent *S. aureus* human isolates, including MRSA and MSSA, show limited lymphatic dissemination and subsequently fail to establish systemic infection efficiently in mice. Notably, despite host differences between mice and humans in response to infection[45,46], *S. aureus* was the only bacterial species for which none of the tested human isolates robustly disseminated beyond local-draining lymphatics or reached systemic organs.

Experimental depletion of neutrophils and lymph node macrophages has been associated with increased systemic burden of *S. aureus* and *P. aeruginosa,* suggesting these phagocytes may be important for restricting lymphatic spread[8,9]. However, our previous work with human- and mouse-virulent *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[13]. Furthermore, we observed that neither neutrophils nor macrophages could effectively engulf virulent *S. pyogenes* within lymph nodes. Bacterial virulence is likely a critical factor, while neutrophils can eliminate avirulent bacteria within minutes, they can be ineffective against hypervirulent strains[47,48]. The impaired phagocytosis of *S. pyogenes* we observed was driven by streptococcal virulence factors, including the hyaluronan capsule, C5a peptidase, and the chemokine-cleaving protease SpyCEP, which interfere with opsonisation, phagocytosis, and neutrophil recruitment[13,49]. These findings suggest that particularly virulent bacteria can overcome lymph node defences, much like they can overwhelm systemic clearance by the spleen and liver that ordinarily control mild and transient bacteraemia in healthy individuals[50-54].

Early lymphatic cannulation studies in dogs and rabbits reported incomplete filtration of haemolytic streptococci (likely *S. pyogenes* and *S. canis*), as well as *Bacillus subtilis* and *Bacillus anthracis* spores and vegetative cells[38,40]. More recent work using killed *S. aureus* bioparticles has reached similar conclusions, demonstrating that lymph node filtration is

incomplete[33]. In addition to active cellular clearance, filtration is also driven by lymph node architecture; the meandering sinuses of the lymph node medulla slow lymph flow resulting in passive settling of particles[38,55]. For hypervirulent bacterial strains that resist phagocytosis and killing, this environment can become a niche for bacterial survival and replication[13]. Here we extend this evidence by showing diverse clinical isolates of extracellular bacteria can escape lymph node defences and disseminate systemically.

The hyaluronan capsule of *S. pyogenes* facilitates lymphatic dissemination, in part through binding to LYVE-1, a hyaluronan receptor that mediates leukocyte entry into initial lymphatic vessels. While LYVE-1 appears to promote retention of encapsulated streptococci within lymph nodes, it is not required for lymphatic entry[13,32]. Here, we found that deleting the sialylated polysaccharide capsule of *S. agalactiae* markedly reduced lymphatic and systemic dissemination, despite unchanged bacterial load at the infection site. The terminal sialic acids of this capsule can bind Siglec receptors expressed by lymph node macrophages[56,57], and this interaction may also influence retention. However, these capsules are also polyanionic and hydrophilic, which can enhance lymphatic uptake by masking adhesins[27,28], inhibiting phagocytosis[29,58,59], and increasing electrostatic repulsion in the extracellular matrix[26,29]. These general properties likely contribute broadly to efficient lymphatic uptake and spread[21,60]. By contrast, the exopolysaccharide alginate from *P. aeruginosa*, though also polyanionic and hydrophilic, did not impact dissemination following alginate lyase treatment, possibly due to its lack of bacteria surface association or alternatively incomplete digestion in vivo. Collectively, our results support a functional role for diverse bacterial capsules in enhancing lymphatic uptake and immune evasion.

We observed substantial heterogeneity in lymphatic spread and systemic invasion across bacterial species and isolates. This variation likely reflects differences in the presence and expression of species- and strain-specific virulence factors, including bacterial capsules. However, interpreting bacterial counts in blood and organs is challenging, as they reflect the

combined effects of dissemination, replication, immune clearance, and tissue-specific retention. Disentangling these factors will require comprehensive and quantitative assessment of bacterial distribution and immune activity across multiple organs in parallel. Advances in explainable machine learning, particularly when combined with Bayesian approaches, offer the potential to integrate complex datasets and identify the pathogen and host factors that govern dissemination routes. These approaches may be critical for integrating fragmented findings on bacterial invasion, where lymphatic spread is increasingly recognised as a fundamental route to systemic infection.

There are limitations to the data presented. For *S. pyogenes* we visualised extracellular bacteria in efferent lymphatics, established the requirement for lymphatic spread for systemic invasion with lymphadenectomy, and evidenced prominent lymphatic dissemination occurred over a range of mouse strains, infection titres and injection routes. These specific aspects were not confirmed for all bacterial species assessed in this study. However, there is no clear biological rationale to suggest that *S. pyogenes* is uniquely capable of lymphatic dissemination, and the broader patterns we observed are consistent with generalisable mechanisms.

Our data have substantial implications for understanding pathogenesis and immunity against major human bacterial pathogens. While our investigation focused on a wide range of clinically important extracellular bacteria, there is also compelling evidence that extracellular lymphatic transit contributes to the pathogenesis of traditionally intracellular pathogens such as nontyphoidal *Salmonella* and *Yersinia pestis*[15,61-67]. 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[68-70], and there is evidence that lymphatic dissemination may play a broader role in systemic spread[71]. Our findings provide a plausible mechanism for cryptic bloodstream infections, which account for over a quarter of all bacteraemia episodes and frequently lack a clear tissue focus, helping to explain discrepancies between superficial infection severity and systemic outcomes[17,18].

As lymph nodes are central to generating effective adaptive responses, our findings raise important considerations for immunity. Even temporary extracellular bacterial presence within these tissues has the potential to disrupt generation of immunity through inflammation or production of virulence factors[44]. The presence of *S. aureus* in lymph nodes has been shown to impair immunity, while increased *Salmonella* burden has been associated with delayed germinal centre formation[43,72-76].

Conclusion

In conclusion, our work reveals that lymphatic dissemination is a widespread and under-recognised route of bacterial invasion, with major implications for pathogenesis, immunity, and clinical understanding. Defining the consequences of lymphatic interactions and integrating lymphatic data into infection models will be essential for a comprehensive understanding of bacterial disease and the development of targeted interventions.

Table 1 | Bacterial strains used in this study

Species	Strain ID	Typing	Description	Reference
E. coli	EC-HP-001	ST131	Bacteraemia isolate	HPRU
E. coli	EC-HP-002	ST88	Bacteraemia isolate	HPRU
K. pneumoniae	KP-BA-001	Undefined	Hypermucoviscous (string test+)	BioAID[77] (H1733)
K. pneumoniae	KP-BA-002	Undefined	Hypermucoviscous (string test+) isolated from foot ulcer	BioAID[77] (H1521)
P. aeruginosa	PA-BA-001	Undefined	Bacteraemia isolate	BioAID[77] (H1730)
P. aeruginosa	PA-BA-002	Undefined	Breast abscess aspirate isolate	BioAID[77] (H1522)
S. agalactiae	A909	Serotype Ia	Neonatal bacteraemia isolate	Reference strain (isolated in 1934)
S. agalactiae	A909 Δ*cps*	Acapsular	Acapsular Δ*cpsD* isogenic A909 mutant constructed with pJRS233	Areschoug, T., and Lindahl, G. (Unpublished) and [78]
S. agalactiae	SAB-H-001	Serotype Ia	Neonatal bacteraemia isolate	BioAID[77] (H1146)
S. agalactiae	SAB-H-002	Serotype V	Neonatal bacteraemia isolate	BioAID[77] (H1148)
S. aureus	SA-H-001	CC15	MSSA bacteraemia isolate	"HHS-8"[79]
S. aureus	SA-H-002	CC22	MRSA bacteraemia isolate	"HHS-9"[79]
S. aureus	USA300	CC8	MRSA isolate (acapsular)	[80]
S. pyogenes	H1565	*emm*1	Hyper-virulent, encapsulated *covR* mutant of H584 M1T1 invasive puerperal sepsis blood isolate, (selected from mouse spleen in vivo)	[81]

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 described in Table 1.

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 10.4. Comparison of two datasets was carried out using a two-tailed Welch's t-test on $\log_{10}$-transformed data. For comparison of three or more data sets, Brown-Forsythe and Welch One-Way ANOVA followed by Dunnett T3 multiple comparisons post-tests were performed on $\log_{10}$-transformed data. A p-value of ≤ 0.05 was considered significant.

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