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REVIEW

By dawn or dusk—how circadian timing rewrites bacterial infection outcomes

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The immune system exists in perpetual co-evolution with pathogens, and microbial pathogenesis is inexorably linked to the cyclical interactions between the pathogen and the host. Because pathogens exploit the immune system in unique ways, the antimicrobial efficacy of any given immune process varies between pathogens, and the consequences of activation or inhibition of antimicrobial programs must be interpreted in the context of the given pathogen. An increasing body of literature shows that numerous facets of the immune system are tightly regulated by the circadian clock, with multiple immune processes demonstrating increased activity during certain times of the day. However, the field of circadian immunology has generally given its attention to unraveling the mechanism of circadian regulation and comparatively little attention to how these circadian oscillations may influence the ultimate outcome of diseases. Therefore, this review aims to interpret these findings in the context of a select number of clinically relevant pathogens: *Salmonella enterica*, *Listeria monocytogenes*, and *Streptococcus pneumoniae*. In this way, we hope to discuss the complex factors that determine how the circadian clock regulates disease progression.

Keywords: bacteria; circadian; clock; immunity; infection; pathogenesis; microbe; *Streptococcus pneumoniae*; *Salmonella enterica*; *Listeria monocytogenes*

In mammals, a large set of genes oscillate in expression in a nearly 24-h cycle. These rhythmic expressions are known as circadian rhythms (from the Latin *circa diem*, 'around a day') and are utilized by the organism to adapt to changing environmental conditions throughout the day, such as temperature and ambient light. To synchronize these oscillations to environmental conditions, mammals receive temporal information from a variety of sources. Chief among these is light exposure, which is sensed in the retina, and the light information is then provided to the suprachiasmatic nucleus in the brain via the optic nerves [1]. The suprachiasmatic nucleus subsequently relays this temporal information to other cells in the body by regulating body temperature and hormone production [1]. The result of these processes is daily oscillations in a

Abbreviations
BMDM, bone marrow-derived macrophages; CRY, CRYPTOCHROME; CT, circadian time; DC, dendritic cell; i.n., intranasal; i.p., intraperitoneal; i.t., intratracheal; i.v., intravenous; InlA, internalin A; $InlA^m$, internalin A, mouse adapted; iNOS, inducible nitric oxide synthase; LLO, Listeriolysin O; *Lm*, *Listeria monocytogenes*; *Lm*-OVA, *Listeria monocytogenes* [which expresses] ovalbumin; LPS, lipopolysaccharide; LTA, lipoteichoic acid; MMM, marginal zone metallophilic macrophages; MZM, marginal zone macrophages; NET, neutrophil extracellular trap; NOS, nitric oxide synthase; OT-I, MHC class I-restricted, ovalbumin-specific, CD8+ T cells; OVA, ovalbumin; PER, PERIOD; PRR, pattern recognition receptor; ROS, reactive oxygen species; SCV, *Salmonella*-containing vacuole; SFB, segmented filamentous bacteria; SNP, single nucleotide polymorphism; *Spn*, *Streptococcus pneumoniae*; T3SS, type 3 secretion system; TipDC, TNF-α/iNOS-producing dendritic cells; TLR, Toll-like receptor; ZT, zeitgeber time.

variety of cellular pathways, including many immune pathways. In appreciation of this observation, a growing body of literature reports that many immune functions are circadian, with immune pathways such as cytokine production and immune cell migration being reported to have differential expression throughout the course of a day [2,3]. We posit that this time-dependent difference produces a phenomenon in which all infections experience times of day at which the risk of severe disease outcome is worse. Although only a few pathogens have been examined thus far, these studies show clear evidence that susceptibility to pathogenic diseases is partially dependent on the time of the host's circadian rhythms. This is corroborated by clinical data, which show that disruption in the circadian clock (e.g., in a night shift worker) is a predictor of more severe disease outcomes [4,5]. Given the diverse strategies by which pathogens cause disease, our goal in this review was to place existing circadian studies into the broader framework of pathogenesis.

Circadian rhythms are maintained by a self-perpetuating transcriptional–translational feedback loop known as the circadian clock. In mammals, this loop consists of the circadian transcription factors CLOCK and BMAL1, which together bind to E-box regions in gene promoters to induce the transcription of a plethora of genes [6]. Among these are circadian repressors: the PERIOD proteins (PER1, PER2, and PER3) and CRYPTOCHROME proteins (CRY1 and CRY2), which upon expression dimerize and translocate into the nucleus, ultimately binding to CLOCK: BMAL1 complexes [6]. In this heterotetrameric complex, CLOCK and BMAL1 are unable to efficiently bind to E-box sites, preventing further activation of these genes [6]. Without further production of PER and CRY, the concentration of these repressors decrease, eventually releasing CLOCK and BMAL1 from repression and allowing the feedback loop to enter a new cycle [6]. This cycle requires roughly 24 h to complete and occurs in cells from numerous organs throughout the host [7,8].

A second feedback loop interlinks with the circadian clock, allowing for the fine-tuning of the output and oscillation of the circadian clock. In this feedback loop, CLOCK:BMAL1 enhances the expression of the transcriptional repressor REV-ERBα [9]. REV-ERBα competes with the transcription factors RORα and RORγ for promoter binding at ROR/REV-ERB response elements; without ROR binding, the expression of an additional set of genes is downregulated [10,11]. Among the genes repressed by REV-ERBα binding are *Clock* and *Bmal1* [9–11]. This series of events therefore produces a feedback loop which causes *Clock* and *Bmal1* transcriptional expression to oscillate [9,10].

The circadian regulation of immune processes and their impact on infection has been thoroughly reviewed elsewhere [2,3,12–14]. Given that there are relatively few circadian studies available for any given pathogen, most reviews necessarily generalize across organisms to provide a broad and holistic overview of the field. However, it is well known that there is substantial variation in the virulence strategies employed by each pathogen and the immune responses necessary to defend against each infection. The host response to a pathogenic invasion is dependent on the site of infection and host cell type; a pathogen therefore faces vastly distinct immunological landscapes in different organs [15,16]. The nature of the immune response also distinctly differs between bacterial pathogens [17,18]. Thus, while many studies use lipopolysaccharide (LPS) to mimic bacterial-triggered inflammation, it is important to acknowledge that LPS is only made by Gram-negative bacteria, and these findings might not be relevant to the pathogenesis of Gram-positive bacteria, which lack LPS. Additionally, through extensive co-evolution between the bacterial pathogen and the host, successful pathogens have developed distinct adaptations to evade, resist, or subvert the immune responses of their host. It is therefore not surprising that immune responses that are most beneficial for the clearance of one pathogen are rarely similar to the responses most beneficial for the clearance of other pathogens. In many cases, an immune response that is protective against one pathogen is ineffective or even detrimental to the clearance of another pathogen.

The wide variation in pathogenesis, combined with the small number of circadian studies on bacterial infections, is difficult to capture in a single review. Here, we instead focus on the pathogenesis of three clinically relevant bacterial pathogens—*Salmonella enterica*, *Listeria monocytogenes*, and *Streptococcus pneumoniae*—and examine what is known about circadian regulation of host physiology and immune processes that are most relevant to each infection. For reference, we have generated a simplified figure that denotes the time of best host outcome for all pathogenesis studies discussed here (Fig. 1). A cursory review of the presented information suggests disagreements in the published literature regarding optimal times of infection; however, the figure leaves out many important variables necessary for interpretation—such as what phenotype was measured (bacterial burden? In which tissue? At what time point?) and what infection times were tested (did the authors only infect animals during the day?). For this reason, we have produced additional figures (Figs 3–5) that include these relevant variables, which will be discussed in greater detail in

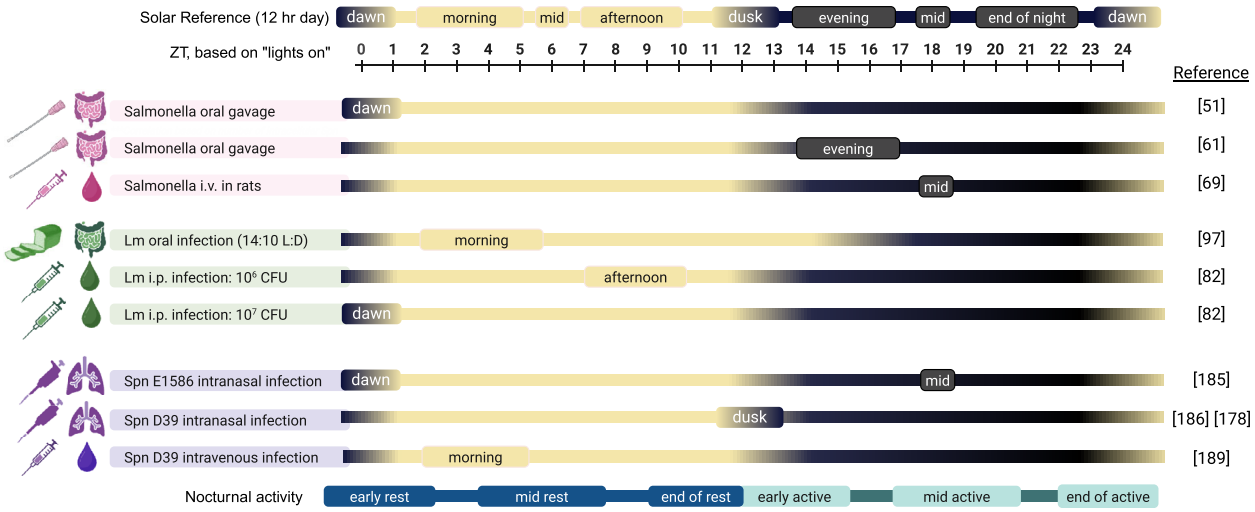

Fig. 1. Summary of optimal time-of-day control of bacterial infections. To ease comparisons, the times listed here denote the 'best' outcome for the host from each study reviewed here that analyzed direct measures of bacterial pathogenesis (increased survival or decreased bacterial burden). Icons to the left depict the route of infection and target organ for bacterial inoculation to signify differences between experimental approaches: gavage needle + intestines (oral infection delivered by oral gavage), bread + intestines (oral infection delivered by contaminated food), needle + blood drop (intravenous), pipette + lung (respiratory infection delivered by intranasal inoculation). *Streptococcus pneumoniae* (Spn) is in purple, *Listeria monocytogenes* (Lm) is in green, and *Salmonella enterica* Typhimurium (Salmonella) is in pink. Note that this summary does not take into account what specific outcome was measured (survival or bacterial burden at different timepoints) or what infection times were analyzed (some compared two daytime exposures). Expanded summaries of this data based on each pathogen are provided in Figs 3–5. Created in BioRender. Kimmey, J. (2025).

the following sections. We will attempt to contextualize immune processes in a pathogen-specific manner and provide potential explanations for how the circadian clock contributes to differential disease outcomes.

How to tell time

Researchers use multiple timekeeping systems to quantify circadian phase, each with distinct advantages and interpretation challenges. In this review, we report times in as many formats as possible in an attempt to improve accessibility while maintaining scientific precision.

Social time (e.g., 14:00 or 2:00 PM) is rarely used in circadian biology because it does not properly account for experimental light schedules, which may vary from the external light cycle depending on experimental design. Instead, times are often given as zeitgeber time (ZT), defined as the number of hours after a synchronizing environmental cue. In animal studies, ZT0 almost always refers to lights on (analogous to dawn), while ZT12 is 12 h later, typically coinciding with lights off (analogous to dusk). However, for animals maintained on a 14:10 light schedule (14 h light, 10 h dark), which mimics summer lighting patterns, lights off or 'dusk' occurs at ZT14.

In vitro, where mammalian cells do not directly respond to light, ZT0 typically marks a synchronizing event such as a medium change or addition of a chemical cue. Because these cues do not directly align with solar time, translating this to an *in vivo* 'time' is not straightforward. Further, many *in vitro* studies are performed in the absence of timekeeping cues to prevent confounding effects from such stimuli. In such cases, the intrinsic circadian period often deviates from 24 h; thus, a time provided in hours will not consistently correspond to any given circadian phase. To standardize comparisons in these cases, time can be expressed in **circadian time (CT)**, which normalizes the cycle to 24 equal circadian hours, defined as 1/24 of the measured period length for a given sample.

While this nomenclature is critical for experimental precision and reproducibility, it can be a barrier, particularly for nonspecialists. Furthermore, many studies use nocturnal mice, whose active phase occurs at night, which is opposite to the human active phase. Consequently, many processes are inverted between mice and humans, and it is common to contextualize reported times as active phase (wake) or rest phase (sleep). However, not all rhythms are inverted between diurnal and nocturnal species [19] and oversimplification can cause unintended bias. For example, one

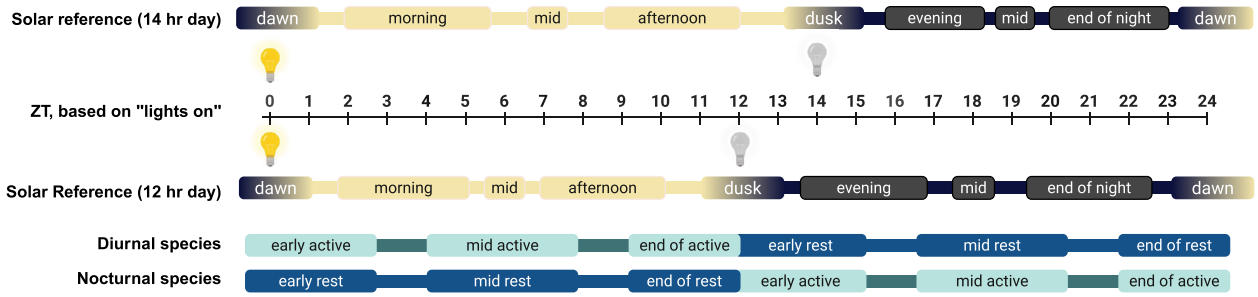

Fig. 2. Reference of different time-keeping mechanisms used in circadian literature. Zeitgeber time (ZT) is counted in hours after a 'time-giving' cue, or *zeitgeber*. Here, we provide ZT with a reference based on lights on (ZT0, depicted by a yellow light bulb), which is the most common zeitgeber used in animal studies. Lights off is depicted by a gray light bulb. Phrases used to denote solar time (dawn, morning, etc.) are provided above the ZT axis based on a 14 h day/10 h night (i.e., summer day) or below the ZT axis based on a 12 h day/12 h night (i.e., fall/spring day—most common in animal facilities). Due to space, mid-day and midnight have been shortened to 'mid'. Note that 'dusk' and the 'mid' day or night will correlate with different ZT, depending on the lighting cycle used for animal experiments. At the bottom, references based on activity patterns of diurnal and nocturnal animals are provided which roughly divide the activity and rest periods into 'early', 'mid', and 'late'. A gap is left between named time periods to denote that these terms are not universally agreed upon nor consistent in the literature (i.e., between early and mid active). Created in BioRender. Kimmey, J. (2025).

might compare a phenotype that peaks 'at the start of the rest phase' to one that peaks 'at the end of the active phase' without realizing that both occur at ZT0 in mice.

For ease of interpretation by noncircadian readers, we have attempted to translate ZT or CT into familiar solar references whenever possible (dawn, morning, mid-day, afternoon, dusk, evening, midnight), followed by active/rest phase annotation appropriate for the species used in the study. In this review, mid-day and midnight refer to halfway between lights on and lights off (ZT6 and ZT18 if on a 12:12 light cycle), not necessarily 12:00 AM/PM local time (Fig. 2).

Salmonella enterica

Salmonella enterica is a Gram-negative gastrointestinal pathogen that primarily infects intestinal epithelial cells and macrophages following consumption of contaminated food or water. Although thousands of serovars exist [20], pathogenic *Salmonella* are broadly classified as either typhoidal or nontyphoidal, based on their capacity for systemic dissemination and disease presentation. Typhoidal serovars, such as *S.* Typhi and *S.* Paratyphi, are human-restricted and cause typhoid fever, whereas nontyphoidal serovars (e.g., *S.* Typhimurium) cause self-limiting gastroenteritis in immunocompetent hosts but can become invasive in immunocompromised individuals [21]. In both cases, infection induces an inflammatory response that is critical for pathogen clearance. Typhoidal strains have evolved mechanisms to suppress early gut inflammation, thereby facilitating immune evasion. This decrease in initial inflammation enables the pathogen to breach the intestinal barrier and disseminate systemically to cause typhoid fever [21].

The *Salmonella enterica* serovar Typhimurium is nontyphoidal in humans, with infections typically self-limiting within 1 week postexposure and exhibiting low mortality [22]. However, mice exposed to this serovar are unable to successfully clear the infection, leading to the development of systemic infection exhibiting symptoms reminiscent of typhoid fever [23,24]. As most research into *Salmonella* utilizes the Typhimurium serovar, we will use the term *Salmonella* to refer to *S.* Typhimurium for the remainder of this section.

The pathogenesis of *Salmonella* depends heavily on type III secretion systems (T3SS), a needle-like apparatus that attaches to the host cell membrane and injects bacterial effector proteins directly into the host cytoplasm. This system allows *Salmonella* to manipulate host cell signaling, cytoskeletal dynamics, and immune responses. *Salmonella* contains two known T3SSs, encoded on the *Salmonella* pathogenicity islands SPI-1 and SPI-2 [25]. The SPI-1-encoded T3SS facilitates invasion of intestinal epithelial cells by inducing cytoskeletal rearrangements to promote bacterial uptake [25]. Once internalized, *Salmonella* switches to the SPI-2-encoded T3SS to remodel the nascent vacuole into a replication-permissive compartment known as the *Salmonella*-containing vacuole (SCV) [25,26].

Within this niche, the bacteria hijack host trafficking pathways and access nutrients to support intracellular replication. *Salmonella* also replicate within macrophages by inducing phagocytosis, then modifying the resulting phagosome into an SCV via SPI-2-encoded

effectors [26]. Regulation of secretion systems and production of virulence factors are central to bacterial success and critical for pathogenesis. As invasion involves extensive hijacking of host cellular processes, it remains unclear how or whether the SCV manipulates the circadian clock and circadian regulation of cellular processes. Hence, the relevance of intrinsic circadian regulation becomes uncertain once *Salmonella* have successfully established its replicative niche within host cells and will not be discussed further here.

Colonization resistance

The first barrier against *Salmonella* is the acidic environment of the stomach, which significantly limits the number of bacteria reaching the intestine [27–30]. The bacteria that survive encounter the intestinal microbiota, which provides a secondary layer of defense through competitive exclusion and metabolic interference. The presence of the microbiome provides hosts with 'colonization resistance' by occupying ecological niches in the intestinal lumen, which excludes *Salmonella* from efficiently colonizing the intestines. Supporting this idea, germ-free mice, which lack a microbiome, or mice treated with antibiotics to ablate the intestinal microbiome exhibit increased susceptibility to *Salmonella* infection [31,32].

In addition to providing colonization resistance, the gut microbiome interacts extensively with the host immune system to modulate and maintain immune readiness. For instance, attachment of commensal bacteria to the intestinal epithelium can stimulate production of antimicrobial peptides, which provide nonspecific defenses against many bacteria [33]. Commensals also contribute to barrier immunity by promoting the development of gut-associated lymphoid tissues [34–36] and producing short-chain fatty acids that can affect the circadian oscillation of peripheral clocks [37]. Importantly, these various functions are associated with specific microbial taxa, making the composition of the microbiome, rather than its mere presence, critical for effective colonization resistance. Many factors influence the composition of the gut microbiome, including host genetics, age, and diet [38,39]. Highlighting the importance of gut microbiome composition on infection resistance, laboratory mice sourced from different vendors are known to have different abilities to resist *Salmonella* infection, a trait that was traced to differences in the gut microbiome composition across vendors [40].

Increasing studies have now established that the diversity and composition of gut microbial populations exhibit diel oscillations and are influenced by host circadian rhythms [41–48], suggesting that the colonization resistance provided by these commensal microbes may similarly change throughout the day. A study by Brooks *et al.* showed that the adherence of segmented filamentous bacteria (SFB) to intestinal epithelial cells experienced daily oscillations and contributed to the rhythmic expression of the antimicrobial peptide REG3γ [49] (Fig. 3). In C57BL/6 mice sourced from Taconic Biosciences, a facility known to have mice colonized with SFB, the intestinal REG3γ abundance in these mice increased throughout the day (peaking at the start of the active phase in mice) and declined throughout the night. This rhythmicity could be reversed by daytime restricted feeding and was lost in mice expressing a dominant negative *Clock* allele, suggesting that clock-controlled feeding rhythms drive SFB attachment. No significant expression of REG3γ was observed in mice lacking SFB due to being raised germ-free mice (lacking microbiome), or being sourced from Jackson Labs (not colonized with SFB), or in mice lacking MyD88 (defective in microbial detection). Thus, expression and rhythmicity of this antibacterial defense mechanism are intricately controlled by complex dynamics between the clock, feeding, and the microbiome.

Salmonella is resistant to certain antimicrobial peptides such as LCN2 and REG3γ; thus, the induction of such antimicrobial peptides preferentially kills the microbiome and therefore facilitates *Salmonella* colonization [50,51]. Counterintuitively, then, the expression of REG3γ promotes rather than prevents *Salmonella* infection. In agreement with this observation, *Salmonella* infection of mice colonized with SFB had higher *Salmonella* bacterial titers and more rapidly succumbed to infection when exposed at dusk (ZT12, end of rest phase) compared to dawn (ZT0, end of active phase) [49]. As expected, no time-of-day difference in bacterial titers was observed during *Salmonella* infection of mice lacking SFB, either due to vendor source or pretreatment with antibiotics. Given that numerous members of the intestinal microbiome are known to modulate the host environment, it is probable that other bacterial species may influence the circadian host susceptibility to *Salmonella* or to other gastrointestinal pathogens, but further research is required to fully understand these effects.

Sensing and cytokine production

During invasion, detection of *Salmonella* virulence factors, such as components of the T3SS, cell wall, or flagellin, leads to the assembly of various inflammasomes including NLRP3, NAIP/NLRC4, and NLRP6 in

macrophages and/or epithelial cells [52–54]. Inflammasomes are large, multiprotein complexes that initiate pro-inflammatory cascades by activating intracellular proteases known as caspases. Once activated, caspases cleave and therefore induce the maturation of the pro-inflammatory cytokines IL-1β and IL-18 [55,56]. The

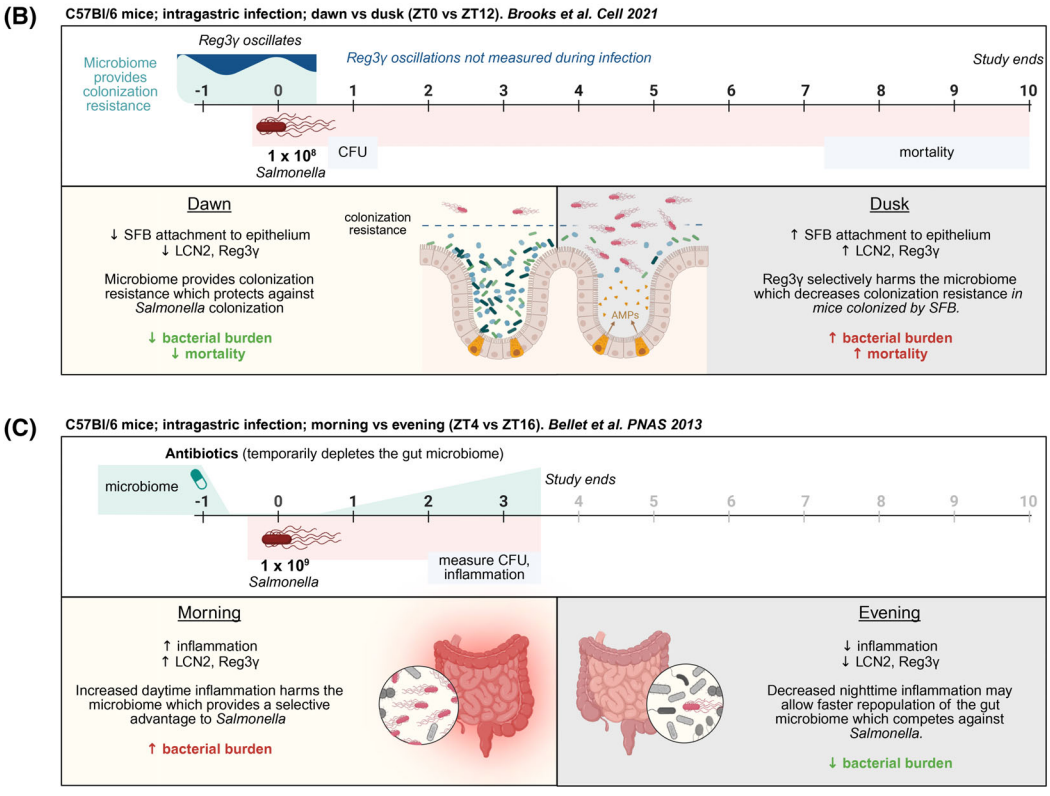

Fig. 3. Summary of host outcomes following time-of-day studies with *Salmonella*. (A) Summary of studies reviewed here. For reference, timelines denoting activity, solar time and ZT (relative to lights on at ZT0) are provided at the top. Each study is denoted by a pink header with title followed by the citation number used in this review in brackets [#]. Icons are placed based on the ZT at which the infection occurred (not necessarily the time at which the parameter was measured). Symbols indicate increased (↑), highly increased (↑↑), or decreased (↓) levels of a parameter measured in the study that is thought to drive differences in bacterial outcome. Ultimate outcomes of pathogenesis are summarized as better for the host (✓, green fill) or worse for the host (✕, red fill). n.s. in gray means there was no significant differences in that parameter based on the time of infection. Note that studies often vary in the timepoints tested. In study [51], improved host outcome occurred at dawn and is associated with decreased segmented filamentous bacteria (SFB) attachment and REG3γ levels. Based on REG3γ levels, one would expect an improved outcome at ZT18 (and worse at ZT6), but these times were not tested. (B, C) Summary of the two oral infection models used to investigate *Salmonella* infection. The study in (B) [51] found oscillations in REG3γ which are thought to increase susceptibility to *Salmonella* during the night by reducing colonization resistance. The study in (C) [61] found increased daytime inflammation during *Salmonella* infection that worsens outcome by limiting competition from the microbiome. While similar mechanisms of pathogenesis are uncovered in both studies, differences in diel-susceptibility were identified, which may be due to differences in timepoints measured or the use of antibiotics prior to infection. Created in BioRender. Kimmey, J. (2025).

activation of inflammasomes also induces pyroptosis, a form of pro-inflammatory programmed cell death, which prevents *Salmonella* from accessing its intracellular replicative niche [55–58]. *Salmonella* additionally activates Toll-like receptors (TLRs), which are important microbe-detecting proteins, and in this way induce the production of additional pro-inflammatory cytokines such as TNF-α, CXCL1, and CXCL2 [59–62]. The collective production of these pro-inflammatory cytokines is critical to induce neutrophil migration into the infected tissues, an essential step toward resolution of the infection.

The circadian clock is known to regulate inflammasome activation, as the circadian-associated transcription factor REV-ERBα is known to suppress the expression of the prototypical inflammasome NLRP3. The circadian control of the NLRP3 inflammasome in different disease contexts is discussed in more detail in another review [13]; briefly, REV-ERBα prevents *Nlrp3* transcription, leading to the oscillatory expression of *Nlrp3* transcript and protein abundance [63]. Accordingly, NLRP3-mediated cytokine production is similarly circadian-controlled, with the greatest amount of IL-1β produced when peritoneal macrophages are stimulated at dusk (ZT12, end of rest phase) [63]. Additionally, circadian disruption has been found to increase the abundance of mature caspase-1 in the brain, though no difference was observed in the abundances of the inflammasome proteins NLRP1, NLRP3, and NLRC4 nor of the inflammasome adaptor proteins ASC and AIM2 [64]. Because abundances of inflammasome-associated proteins appear unchanged, the authors conclude that the increased caspase maturation is due to a circadian regulation of inflammasome assembly rather than a circadian-driven regulation of transcription or translation, suggesting that the circadian clock is able to regulate multiple steps of inflammasome activation. Caspase-1 is the downstream effector of both the NLRP3 and NAIP/NLRC4 inflammasomes [55,56]; thus, it is possible that the observed maturation of caspase-1 is driven in part by a circadian-regulated activation of both NLRP3 and NAIP/NLRC4 inflammasomes.

The production of pro-inflammatory cytokines and chemokines relevant to *Salmonella* infection is also under circadian control. For instance, it has been shown that leukocytes from human blood samples respond to stimulation with the Gram-negative bacterial component, LPS, by producing pro-inflammatory cytokines such as TNF-α and IL-8 (human homolog of murine CXCL1 and 2) in a diel manner, with greater amounts being produced with blood extracted at around 9–10 AM, during the human early-mid active phase [65]. A separate study with blood extracted from human volunteers found that stimulation with LPS extracted from the *Salmonella* serovar Abortusequi induced IFN-γ and IL-8 in a diel manner, with greater amounts being produced from blood extracted at around dusk to dawn (human rest phase) [66]. Notably, stimulation with the Gram-positive bacterial component, LTA (which is not made by *Salmonella*), did not induce a similar diel effect, highlighting the differences between the immune responses to different bacterial pathogens [66]. It remains as yet unclear the precise mechanism by which the production of these cytokines exhibits circadian oscillations, but it is possible that oscillation of microbial detection capabilities (i.e., Toll-like receptors) may be at least partially responsible for this diel phenotype. Taken together with the known circadian control of the inflammasome, the ability to detect *Salmonella* colonization and the subsequent signaling for a pro-inflammatory immune response is therefore likely to be circadian, with greater sensitivity of detection occurring during the host active phase.

One of the earliest investigations into the time-of-day susceptibility of *Salmonella* was published in 1983, in

which rats injected with *Salmonella* at night (active phase for Sprague–Dawley rats) showed greater survival compared to rats injected in the day [67] (Fig. 3). While the mechanism for this effect was not determined, the authors hypothesized this may be due to increases in circulatory pyrogens at night, which could allow for increased immune responses and therefore clearance of *Salmonella*. A separate study in mice found that mice exposed intragastrically to *S*. Typhimurium had greater bacterial clearance and lower inflammation of the intestinal lining when exposed at night (ZT16, mid-active phase) compared to mice exposed in the morning (ZT4, mid-rest phase) [59] (Fig. 3). This report identified differences in the production of pro-inflammatory cytokines as a driving factor for this difference in outcomes. Night-infected mice were associated with higher abundance of *Tnfα* transcript at 60 h postinfection. However, at 72 h postinfection, this phenotype was reversed, with day-infected mice now displaying higher transcript levels of *Tnfα* and *Cxcl1*. This is consistent with the idea that an early, robust immune response is important for effective *Salmonella* clearance, as failure to clear invading pathogens rapidly will prompt a greater immune response later as the pathogen replicates and worsens the infection.

The report additionally found that the expression of certain antimicrobial peptides—LCN2 and REG3γ—following *Salmonella* infection differed depending on the time of infection. The expression of these antimicrobial peptides was greater upon *Salmonella* exposure in the daytime, correlating with the window of susceptibility to *Salmonella* infection. Similar to the study by Brooks *et al*., the expression of LCN2 and REG3γ preferentially disrupted the intestinal microbiome and provided an open niche for the invading *Salmonella*, leading to increased susceptibility of mice during the day. Agreeing with this observation, wild-type *Salmonella* experienced a competitive advantage over a *Salmonella* mutant susceptible to LCN2, but this advantage was present only when mice were exposed in the day (which correlates with infection-induced timing of LCN2 expression). In total, this study reveals that immune signaling and colonization resistance together are important drivers of the circadian susceptibility to *Salmonella*.

Nutritional immunity

As mentioned previously, Salmonella can engage with and activate host cell inflammasomes such as NLRP3, NAIP/NLRC4, and NLRP6. While activation of the NLRP6 inflammasome by *Salmonella* can activate key pro-inflammatory cytokines and induce pyroptosis, it also activates a noncanonical pathway, which induces an iron sequestration response in host cells [53]. All organisms, including pathogens, require access to essential metals such as iron to carry out basic biological processes. During infection, host cells capture and sequester essential metals to prevent and hamper the spread of the pathogen in a strategy known as nutritional immunity [68]. Paradoxically, this response is detrimental during *Salmonella* infection, as accumulation of iron in macrophages and epithelial cells provides a direct source of iron to intracellular *Salmonella* [53]. Hence, despite losing a pathway for producing beneficial cytokine and pyroptotic responses, mice lacking NLRP6 have improved bacterial clearance of *Salmonella* compared to wild-type controls [53].

Similar to NLRP3, NLRP6 was observed to be suppressed by REV-ERBα [69]. Intriguingly, this report finds that *Nlrp6* mRNA expression is lowest at dusk (ZT12, end of rest phase) and highest at dawn (ZT0, end of active phase), suggesting a difference in transcriptional kinetics between *Nlrp6* and *Nlrp3* despite both being in part regulated by REV-ERBα. As NLRP6-mediated iron aggregation is deleterious for *Salmonella* infection, lower amounts of REV-ERBα activity are expected to improve *Salmonella* clearance. Indeed, applying a pharmacological inhibitor of REV-ERBα was found to improve bacterial clearance to a similar degree as deletion of *Nlrp6*, and pharmacological inhibition similarly improved inflammation-mediated injuries of colonic tissues during *Salmonella* infection. Taken together, REV-ERBα-mediated regulation of *Nlrp6* suggests that the greatest degree of nutritional immunity-related *Salmonella* resistance occurs at the peak of REV-ERBα oscillations, which in mice occurs at dusk (ZT12, end of rest phase).

Nutritional immunity may therefore provide an alternative interpretation for the results found in the early investigations into *Salmonella* susceptibility discussed above. The report documented that serum availability of iron and zinc, two biologically essential metals, was higher during the day (when the rats were more susceptible) as compared to the night (when the rats were less susceptible) [67]. Therefore, in addition to the hypothesis that changes in circulatory pyrogens drive a time-of-day difference in *Salmonella* susceptibility, *Salmonella* bacterial burden may be greater when infected in the day due to better accessibility of essential metals and therefore greater bacterial replication.

Neutrophil recruitment and function

In response to the pro-inflammatory signals produced by macrophages and epithelial cells, neutrophils

translocate to the infection site. At the infection site, neutrophils clear *Salmonella* through degranulation and generation of reactive oxygen species (ROS), thus making neutrophils valuable for the resolution of infection [70,71]. The circadian control of immune cell recruitment to target organs is well-established in non-infected hosts, with many individual aspects of recruitment known to be regulated by the circadian clock. For instance, multiple adhesion molecules on endothelial and infiltrating myeloid cells oscillate over circadian time, leading to time-of-day differences in the ability of circulating myeloid cells to adhere to the endothelial lining and extravasate into infection sites [72]. Notably, cells in different tissues exhibit different circadian regulation of these adhesion molecules; thus, the ideal time of myeloid cell recruitment is organ-specific [72].

Additionally, the process of neutrophil migration has itself been found to be circadian, with murine neutrophils extracted at ZT13 (early active phase) exhibiting greater migratory ability than neutrophils extracted at ZT5 (mid-rest phase) [73]. The report attributed this difference in migratory efficacy to differences in the speed of neutrophil rolling and strength of neutrophil adhesion to endothelial cells. In turn, this difference is associated with a circadian clock-dependent pattern of neutrophil aging, in which mouse neutrophils are maintained during the night (active phase) and aged during the day (rest phase). A neutrophil-specific deletion of the core circadian clock gene *Bmal1* revealed a loss in this circadian aging phenotype and subsequently an increase in neutrophil rolling, adhesion, and extravasation. The same study found a similar phenotype in human neutrophils, with a higher proportion of aged neutrophils in the circulation at 4:00 PM (mid-late active phase) and a lower proportion at 4:00 AM (mid-late rest phase), suggesting that the circadian-dependent neutrophil aging phenotype may be more generally conserved across various species. However, human and mouse neutrophils exhibit aging at different circadian times (rest phase in mice, active phase in humans), suggesting that additional or altogether different pathways regulate the circadian neutrophil aging phenotype in humans. The degree to which this difference affects human susceptibility to *Salmonella* is unclear and warrants further investigation.

At the infection site, neutrophils help to clear invading bacteria by enacting a variety of antimicrobial processes such as NETosis and degranulation. A study investigating the circadian control of neutrophils found clear oscillations in a variety of such processes in neutrophils from both mice and humans [74]. In a sterile inflammation model of tissue injury through ischemia, neutrophil extracellular trap (NET) formation in the cremaster muscle of mice was increased during the night (ZT13, early active phase) compared to the day (ZT5, mid rest phase) [74]. The authors ascribe this observation to a neutrophil deprogramming that occurs over the course of a day, which causes neutrophils to be less capable of undergoing NETosis. To measure circadian degranulation potential, the authors assessed the granule content of neutrophils extracted from mice at different times of the day and found that the time of peak granule content in neutrophils was the middle of the night (ZT17, mid active phase), with the trough occurring near dawn (ZT0, end of active phase). Additionally, the authors associate this peak with the release of neutrophils from the bone marrow and hypothesize that, over time in circulation, neutrophils lose their granules. All these data suggest that the most potent neutrophil responses would be during the early-mid active phase (at night in mice). A similar circadian phenomenon was observed in human neutrophils (though the times are flipped as humans are diurnal). Human neutrophils exhibited peak granule content and NET-forming capacity early in the morning (8:00 AM, early active phase) and decreased by the afternoon (2:00 PM).

Additionally, some evidence exists that suggests that neutrophil ROS production is circadian. While circadian ROS production has not yet been observed in murine neutrophils, it has been documented in zebrafish models of *Salmonella* infection. In zebrafish, injection of *Salmonella* into the hindbrain led to greater survival when exposure occurred in the light phase (active phase) compared to the dark phase (rest phase) [75]. This difference in survival was in part attributable to neutrophil activation, as a neutrophil-specific deletion of the circadian clock gene *per2* led to impaired *Salmonella* killing and ROS production.

In total, studies show that *Salmonella* clearance is dependent on the time of infection, with murine and zebrafish models of infections showing better bacterial clearance when exposed during the host active phase. This difference is attributable to circadian regulation of microbial detection mechanisms, immune signaling, and neutrophil activity. Evidence shows that the advantageous times for each of these immune aspects occur during the host's active phase, corroborating the *in vivo* observation of host susceptibility to *Salmonella*. Circadian regulation of NLRP6 is noteworthy, as the most beneficial time for NLRP6 activation occurs at the end of the murine rest phase, suggesting that the time of greatest *Salmonella* resistance *in vivo* may trend toward the earlier portion of the host active phase.

One major caveat to these estimates is that the protective effects of the microbiome are highly variable—in perhaps the most extreme scenario, mice colonized with SFB showed circadian susceptibility to *Salmonella* that was nearly inverted to these estimates. Other as-yet unidentified members of the host microbiome are likely to similarly modify circadian susceptibility to *Salmonella* via unique modulatory pathways, suggesting that the time of greatest susceptibility may be at least somewhat specific to each individual.

Additionally, consideration must be given to the fact that studies on *Salmonella* infections are typically performed with *S.* Typhimurium, only one of a multitude of *Salmonella* serovars capable of infecting humans. *S.* Typhi, for instance, features adaptations for suppressing the immune response, thereby allowing *S.* Typhi to replicate unimpeded and develop into a systemic infection [21]. Given these immune-suppressing adaptations, it is unclear whether susceptibility to *S.* Typhi exhibits similar circadian patterns to *S.* Typhimurium. Specifically, suppression of many of the immune pathways discussed above may result in a phenomenon in which the circadian expression of many of these immune pathways is irrelevant in the context of *S.* Typhi infections. Further research is therefore required to determine the circadian nature of infections with other *Salmonella* serovars.

Listeria monocytogenes

Listeria monocytogenes (*Lm*) is a Gram-positive bacterial pathogen that infects humans through contaminated food. While it can cause severe gastrointestinal illness in healthy adults, *Lm* is typically restricted to the gut [76], as anatomical and immunological barriers such as the acidic gastric environment, the presence of antimicrobial peptides, and colonization resistance prevent bacterial access to deeper tissues [77,78]. However, newborns, older adults, and immunocompromised individuals are at risk of developing systemic listeriosis [76]. In such cases, *Lm* can initiate invasion by binding to E-cadherin on intestinal epithelial cells via its virulence factor InlA, which triggers bacterial endocytosis via a clathrin-mediated mechanism, allowing *Lm* to gain access to the host cell cytosol [76]. Here, *Lm* replicates efficiently, utilizing a suite of virulence factors to manipulate host cellular processes and ensure bacterial survival. *Lm* is also able to spread directly between cells by using actin-based motility, which enables *Lm* to evade detection by most extracellular immune components while disseminating to new host cells [79,80].

Eventually, *Lm* spreads to lymphatic and blood vessels and disseminates to the liver and spleen. In some cases, *Lm* can cross the blood–brain barrier, resulting in meningoencephalitis or cross the placental barrier and cause fetal death [76]. Early detection of *Lm* and the subsequent production of pro-inflammatory cytokines are necessary to prevent these severe invasive diseases [81]. Cytokines prime innate and adaptive immune effectors to combat *Lm* and resolve infection. Innate immune cells, including macrophages, neutrophils, monocytes, and dendritic cells, are crucial for early control of infection, but are insufficient for clearance in most cases of *Lm* infection. Because *Lm* can largely evade immune detection by remaining within infected host cells, $CD8^+$ cytotoxic T cells, which can detect and destroy infected host cells, are required for the ultimate resolution of infection [81]. Out of the many cytokines that contribute to efficient control of *Lm*, IFN-γ plays a particularly important role due to its ability to increase antibacterial efficacy in macrophages [82,83] and enhance $CD8^+$ T cell activity [84].

The majority of our understanding of *Lm* pathogenesis and immunity derives from studies performed on mouse models. Similar to most infections, *Lm* pathogenesis depends on the dose [85], mouse strain [86], sex [87], age [88], and route of infection [85]. For example, following foodborne transmission, *Lm* bacteria are not detectable in the spleen and liver until 48 h after exposure [89], and it is thought that initial interactions with *Lm* at mucosal surfaces during this time could preactivate cells in the liver and spleen [85]. In contrast, intravenous (i.v.) infection bypasses gut barriers and mucosal surfaces. This infection model leads to detectable *Lm* in the spleen and liver within 15 min of infection [90,91], where *Lm* encounters naïve immune cells, potentially leading to immune responses and disease outcomes that differ markedly from those observed in foodborne infection models.

Establishing infection

As discussed previously, a key step in the pathogenesis of *Lm* in the gut relies on the binding of the bacterial surface protein Internalin A (InlA) to E-cadherin on host epithelial cells [76,92]. InlA binds efficiently to human E-cadherin but not the murine homolog, which limits *L. monocytogenes*' ability to establish infection via oral exposure in wild-type mice [93]. To model oral infection, models have been developed to allow *Lm* to bind to the intestinal epithelial cells, either by genetically modifying mice to express human E-cadherin [94] or *Lm* to express a modified version of InlA engineered to bind to mouse E-cadherin ($InlA^m$) [95]. The development and utilization of these models have helped elucidate the kinetics of foodborne *Lm*

infection, complementing the existing knowledge of *Lm* pathogenesis, which is typically derived from systemic infections or *in vitro* models.

Bou Ghanem and colleagues demonstrated clear time-of-day differences in a natural route of infection by feeding mice bread saturated with *Lm* $InlA^m$-contaminated butter [96] (Fig. 4). Mice infected at mid-day (ZT5, mid rest phase) were better able to restrict *Lm* burden in the gut (primary infection) and systemic tissues (disseminated infection) as compared to mice infected at night (ZT14.5, early active phase) [96]. Despite well-established differences in susceptibility between BALB/c and C57BL/6 mouse strains [86], both strains showed increased resistance during the day (rest phase), suggesting the possibility of a shared clock-dependent driver of *Lm* resistance in the gut. Importantly, this difference was not attributable to reduced daytime feeding, as C57BL/6 mice infected at mid-day (ZT5, mid rest phase) exhibited a slightly higher initial gut burden than those infected at night (ZT14.5, early active phase), yet subsequently achieved greater bacterial clearance. Resistance correlated with decreased numbers of cell-associated *Lm*, suggesting the possibility of rhythmic modulation of bacterial adhesion to E-cadherin. While there is some evidence that E-cadherin can oscillate throughout the day [97], the authors found only a modest role for InlA and therefore bacterial adhesion in early intestinal infection. Hence, it is likely that this effect is driven by other, unidentified features of the gastrointestinal environment that are under circadian control. In the complexity of host–pathogen interactions, time-of-day analyses may offer a powerful filter to identify factors whose temporal dynamics align with susceptibility windows and allow prioritization of factors most likely to shape disease outcomes.

Sensing and cytokine production

While the determinants of early gut colonization remain incompletely defined, *in vitro* and systemic infection models have demonstrated a key role for

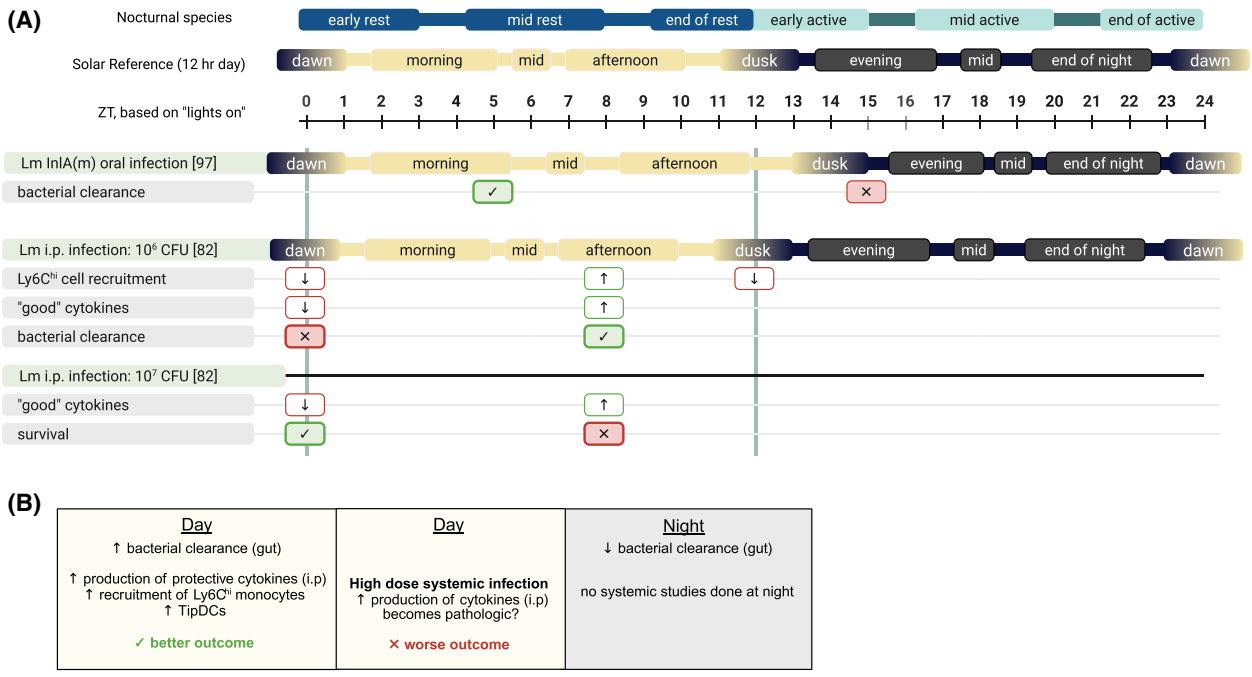

Fig. 4. Summary of host outcomes following time-of-day studies with *Listeria monocytogenes* (*Lm*). (A) Summary of studies reviewed here. For reference, timelines denoting activity, solar time and ZT (relative to lights on at ZT0) are provided at the top. Each study is denoted by a pink header with title followed by the citation number used in this review in brackets [#]. Icons are placed based on the ZT at which the infection occurred (not necessarily the time at which the parameter was measured). Symbols indicate increased (↑) or decreased (↓) levels of a parameter measured in the study that is thought to drive differences in bacterial outcome. Ultimate outcomes of pathogenesis are summarized as better for the host (✓, green fill) or worse for the host (X, red fill). Note that studies vary in the route (oral [97] vs systemic [82]), dose (10^6 vs 10^7 CFU) and lighting parameters (14 h day [97] vs 12 h day [82]) which limits cross-study interpretation. (B) Based on the limited data available, mice may be more resistant to *Lm* during the day (rest phase), but protective immune responses may contribute to immunopathology and mortality at high doses. Created in BioRender. Kimmey, J. (2025).

innate immune cells in the early response to *Lm*. Tissue-resident phagocytes are among the first cell types to respond to *Lm*. *In vitro* studies have demonstrated that after phagocytosis by naïve macrophages, *Lm* secretes virulence factors such as listeriolysin O (LLO) to escape from the phagosome [76]. Once in the cytosol, *Lm* replicates and expresses its virulence factor ActA, which allows the bacteria to hijack host Arp2/3 machinery to induce actin-based motility and propel itself into neighboring cells [98]. By providing a protected intracellular niche, these macrophages permit *Lm* replication and expansion, exacerbating infection. However, recognition of *Lm* components by host pattern recognition receptors (PRRs) such as TLR2 and CLEC5A on these cells triggers the production of pro-inflammatory cytokines, including IL-6, TNF-α, IL-12 and IFN-γ [99–101], which are critical for control of Lm.

Differences in TLR expression may affect the sensitivity of microbial detection and could thereby drive significant changes in resistance to *Lm*, as loss of MyD88, which is required for signaling through most TLR receptors, renders mice highly susceptible to *Lm* [102,103]. The expression of several TLRs exhibits circadian oscillation in the intestinal epithelium of mice [104], with the peak at dawn (ZT0, end of active phase) and trough at dusk (ZT12, end of rest phase), potentially contributing to the increased daytime resistance observed in oral *Lm* infection. Indeed, splenic macrophages showed greater production of *Il6*, *Il1b*, and *Tnf* transcripts following stimulation with heat-inactivated *Lm* at dawn (ZT1, early rest phase) than at dusk (ZT13, early active phase), agreeing with the idea that sensitivity to *Lm* detection can fluctuate over the course of the day [105]. Hence, the ability to detect the presence of *Lm* is an important prerequisite for bacterial clearance.

Following *Lm* detection, the production of IFN-γ is also essential for bacterial clearance; the importance of this response is underscored by the extreme susceptibility of *Ifngr*$^{-/-}$ mice, which lack the receptor for IFN-γ and fail to control infection [84]. IFN-γ enhances the antimicrobial capacity of macrophages by increasing the production of reactive oxygen and nitrogen intermediates [83] and autophagy-mediated clearance [82], thereby allowing the macrophage to restrict *Lm* growth. Daily oscillations in IFN-γ transcript and protein have been reported [106,107], which may contribute to differential activation during infection. When combined with signals produced by the activation of TLRs, IFN-γ also drives polarization of macrophages into the pro-inflammatory M1 state, which is associated with control of *Lm* [100,108]. Many aspects of macrophage biology, including metabolism, polarization, phagocytosis, trafficking, cytokine production, and activation, either oscillate or become disrupted when the circadian clock is perturbed and has been reviewed in detail separately [12].

Myeloid cell recruitment and function

The production of these cytokines and chemokines ultimately promotes the recruitment of innate immune cells such as neutrophils and monocytes. Genetic approaches disrupting the factors necessary for neutrophil [109] or monocyte recruitment [110–115] render mice more susceptible to *Lm* infection, demonstrating a critical role in the recruitment of these infiltrating myeloid cells. Furthermore, antibody-mediated depletion of myeloid cells using anti-Gr-1 mAb leads to increased susceptibility to *Lm* [90,116–120]. The anti-Gr-1 antibody recognizes two distinct cell surface receptors: Ly6G, which is on neutrophils, and Ly6C, which is on monocytes, and accordingly, anti-Gr-1 depletes both populations [119]. Subsequent depletion studies using a neutrophil-specific antibody (anti-Ly6G) found that while neutrophils are not required to control low-dose infections [121], they play important roles in controlling bacterial burden in the liver during high-dose infections [122,123]. In contrast, neutrophil depletion has minimal impact on bacterial control in the spleen, even in high-dose infections [122,123]. This heightened importance for neutrophils in the liver may reflect differential antimicrobial capacity between Kupffer cells (resident macrophages in the liver) and marginal zone macrophages (resident macrophages in the spleen).

As discussed previously in the *Salmonella* section, adhesion molecules for myeloid cell recruitment are expressed in a circadian manner, although the specific timing of circadian regulation differs according to tissue [72]. Several studies have reported the circadian recruitment of myeloid cells to the liver: following adoptive transfer, Gr-1^{hi} cells (neutrophils and inflammatory monocytes) showed enhanced homing to the liver in the evening (ZT13, early active phase) as compared to morning (ZT1, early rest phase), while no differences were seen in noninflammatory monocytes [72]. Similar kinetics have been reported for recruitment of neutrophils to the liver in a model of endotoxic shock (higher in the evening (ZT13) as compared to midday (ZT5)) [124]. However, recruitment of Ly6C^{hi} monocytes to the peritoneum after a sterile inflammatory stimulus (thioglycollate administration) shows slightly different kinetics, with a peak in late afternoon (ZT8, mid-late rest phase) as compared to dawn and dusk

(ZT0 and ZT12, end of active and rest phase) [89]. Because chemotaxis of each inflammatory cell type depends on the specific chemoattractants and the adhesion molecules expressed in each tissue, it is not surprising that these studies in disparate tissues yielded different results.

Recruitment of inflammatory monocytes is critical to bacterial control of *Lm*, in part because these cells differentiate into specialized antimicrobial effectors at the site of infection. There are two subsets of circulating monocytes: Ly6C^{hi} monocytes, which are inflammatory, and Ly6C^{low} monocytes, which are typically involved in the resolution of infection [81]. In response to IFN-γ, Ly6C^{hi} monocytes differentiate into TNF-α/iNOS-producing dendritic cells (TipDCs), which play a central role in bacterial clearance [112,125]. TNF-α promotes further recruitment and activation of myeloid cells [81] while iNOS (inducible Nitric Oxide Synthase) catalyzes the production of reactive nitrogen species, which can directly kill *Lm* [112].

Nitric oxide synthases (NOS) have been shown to exhibit circadian behavior in host cells. Basal NOS activity oscillates in a 24-h pattern in various mouse tissues, including lung, blood, and kidneys, peaking shortly before dawn (ZT21, late active phase) [126]. In the liver, *iNOS* expression is oscillatory and depends on the rhythmic presence of Nocturnin, a deadenylase that stabilizes *iNOS* mRNA shortly before dawn (ZT20, late active phase) [127]. These data suggest that both monocyte recruitment and the antimicrobial effector functions of TipDCs, such as iNOS-mediated killing, may be under circadian control, with the greatest degree of recruitment and microbial killing occurring in the murine early active and late active phases, respectively.

These mechanistic links between circadian control of monocyte recruitment and TipDC effector function are supported by *in vivo* evidence demonstrating time-of-day-dependent susceptibility to *Lm* infection (Fig. 4). In a study using intraperitoneal infection of *Lm*, mice challenged with 10^6 colony-forming units of *Lm* in the afternoon (ZT8, mid-late rest phase) exhibited increased bacterial clearance compared to mice infected at dawn (ZT0, end of active phase) [89]. This protection correlated with increased numbers of TipDC in the peritoneum, spleen, and liver, as well as increased levels of CCL2, IL-1β, IL-6, and IFN-γ following infection in the afternoon (ZT8, mid-late rest phase) compared to dawn (ZT0, end of active phase). Based on our current understanding of host defense against *Lm*, this immune profile is expected to enhance monocyte recruitment, macrophage activation, and antibacterial effector function, providing a mechanistic explanation for the improved survival observed in the afternoon (ZT8).

When mice were challenged with a higher, lethal dose of *Lm* (10^7 colony forming units), mice infected in the afternoon (ZT8, mid-late rest phase) still exhibited elevated production of the same 'protective' cytokines (CCL2, IL-6, IL-1β, and IFN-γ), consistent with a circadian influence on the early detection and immune sensing of *Lm* [81]. Despite this, mice given the high-dose infection of *Lm* in the afternoon (ZT8) succumbed to infection faster than mice infected at dawn (ZT0, end of active phase). This suggests that either cytokine amplitude alone is not sufficient to account for bacterial clearance or that circadian augmentation of cytokine responses may become pathologic under increased infectious burden. A similar outcome is seen in mice lacking γδ T cells, which exhibit elevated IFN-γ, IL-6, and IL-12 in response to *Lm* and succumb despite high cytokine levels, reinforcing the idea that excessive inflammation can drive mortality when immune regulation is compromised [128]. Thus, greater cytokine induction is not inherently protective; in contexts of high pathogen burden or disrupted regulation, times of day associated with amplified inflammatory responses may instead contribute to immune-mediated pathology.

CD8$^+$ T cell activation and responses

Although innate immunity is necessary for the detection and early clearance of *Lm*, the development of a strong cytotoxic T-cell (CD8$^+$ T cell) response is essential to eliminate most infections. As an intracellular pathogen, *Lm* can evade extracellular sensing and persist within host cells, necessitating CD8$^+$ T-cell-mediated surveillance. CD8$^+$ T cells identify *Lm*-infected cells and induce their elimination via Fas–FasL interactions [129], TNF-α-dependent apoptosis [130], or granule exocytosis [130,131]. The spleen is the primary site of T cell priming during *Lm* infection [132]. Here, dendritic cells (DCs) serve as the principal antigen-presenting cells, presenting *Lm*-derived peptides with appropriate costimulation to naïve CD8$^+$ T cells to activate and mature the T cell [81]. Once activated, CD8$^+$ T cells undergo coordinated phases of expansion and trafficking to infected tissues to eliminate *Lm* infection.

Due to its intracellular lifestyle and capacity to elicit strong cytotoxic T-cell responses, *Lm* has long served as a model system for studying CD8$^+$ T-cell biology. In the intraperitoneal infection model described above, mice infected in the afternoon (ZT8, mid-late rest phase) exhibited greater numbers of IFN-γ-producing

$CD8^+$ T cells – which correlate with protective immunity – as compared to those infected at dawn (ZT0, end of active phase) [89]. Several studies have leveraged *Lm* expressing ovalbumin (OVA) to investigate $CD8^+$ T-cell responses. Because bacteria produce many antigenic epitopes and therefore activate a diverse population of $CD8^+$ T cells, heterologous expression of OVA simplifies tracking of antigen-specific responses. In one study, mice infected at night (ZT16; early-mid active phase) with OVA-expressing *Lm* (*Lm*-OVA) generated a greater number of OVA-specific $CD8^+$ T cells than mice infected in the morning (ZT4, early-mid rest phase) [133]. This time-of-day effect was absent in mice lacking glucocorticoid receptors [133], implying that hormonal circadian signals, at least in part, contribute to $CD8^+$ T-cell responses.

A second study used the *Lm*-OVA infection model to investigate whether the T-cell intrinsic circadian clock is necessary for time-of-day dependent differences in T-cell function. Here, the authors performed adoptive transfers of OVA-specific $CD8^+$ T cells (OT-I cells) that were either wild-type or *Bmal1*$^{-/-}$ into mice, and then infected with *Lm*-OVA [134]. The use of OT-I cells increases the number of antigen-specific T cells present at the start of infection, making it easier to track their responses over time. This study found that T cells from mice infected in the morning (ZT2, early rest phase) produced more IL-2 (a cytokine which supports T cell proliferation), as compared to T cells from mice infected at night (ZT14, early active phase) [134]. Interestingly, this time-of-day effect did not depend on the T cell clock, as similar effects were observed in *Bmal1*$^{-/-}$ T cells, suggesting that extrinsic factors or cues such as the cytokine milieu or oscillations in antigen-presenting cells are responsible for this effect. Notably, there was no difference in the frequency of IFN-γ^+ or TNF-α^+ T cells, two effector cytokines that are important for control of *Lm*, leaving open the question of how the observed circadian oscillation of $CD8^+$ T cell response would impact *Lm* infection.

While the times of day that yielded the strongest T-cell responses differed between the two studies, this discrepancy may reflect differences in what was measured. The first study found greater numbers of antigen-specific $CD8^+$ T cells after night infection [133], while the other observed increased IL-2 production following morning infection [134]. The generation of T-cell responses involves sequential steps, such as antigen uptake, presentation and migration of antigen-presenting cells, T cell priming, and clonal expansion of effector T cells, each of which has its own kinetics of activation [135,136]. Combined with the complex dynamics of bacterial pathogenesis, therefore, it is unlikely that there exists a singular optimal time for T-cell generation.

The kinetics of antigen presentation can be bypassed by preloading dendritic cells (DCs) with OVA antigen *in vitro* then adoptively transferring these OVA-DCs into mice. Using this model, $CD8^+$ T-cell responses were greatest when OVA-DCs were transferred in the middle of the expected day (CT6, expected mid rest phase) compared to the middle of the expected night (CT18, expected mid active phase) [137]. [Note: in this study, mice were maintained in constant darkness, so times were reported using circadian time (CT) instead of zeitgeber time (ZT) due to the absence of external time cues (a.k.a. zeitgebers).] Mice that received OVA-DCs in the expected day (CT6) generated higher percentages of activated, antigen-specific $CD8^+$ T cells, and a greater percentage of this T-cell population produced IFN-γ upon restimulation.

While a small role for the DC-intrinsic clock was found in the control of DC trafficking to the spleen (a site of T cell priming), this time-of-day effect was found to be largely dependent on the T-cell intrinsic clock, as the circadian effect in the $CD8^+$ T-cell response was lost when wild-type OVA-DCs were transferred into mice with *Bmal1*-deficient T cells. In contrast, the circadian effect was preserved when *Bmal1*-deficient DCs were transferred into mice with wild-type $CD8^+$ T cells, confirming that the DC-intrinsic clock plays only a minor role in driving this phenomenon. Further analysis showed enhanced activation of TCR signaling pathways during the expected day (CT6), suggesting that naïve T cells are primed for more efficient activation during the day. Importantly, the observed differences in the $CD8^+$ T-cell response had functional outcomes—mice vaccinated with OVA-loaded DCs during the expected day (CT6) showed significantly greater control of *Lm* burden in the liver and spleen as compared to mice vaccinated during the expected night (CT18).

Vaccination studies can offer insight into circadian regulation of T-cell responses, but translating these findings to an infection context requires careful consideration. In all infections, the kinetics of antigen presentation are affected by the location where antigen-presenting cells encounter the antigen, which in turn affects how quickly these cells traffic to lymphoid tissues to prime $CD8^+$ cells. The study that used OVA-loaded DCs detected the adoptively transferred DCs in the spleen within 4 h of i.v. transfer [137]. This suggests that the optimal time of $CD8^+$ T cell priming may occur somewhere between CT6 (when the DCs were transferred) and CT10 (when the DCs were measured in the spleen), roughly corresponding to ‘early to late

afternoon'. While no circadian pathogenesis studies have been conducted using the i.v. model of *Lm* infection, the timing is consistent with an i.p. infection model that reported augmented IFN-γ^+ $CD8^+$ T cells and improved clearance of *Lm* following infection during the afternoon (ZT8, mid-late rest phase) vs dawn (ZT0, end of active phase) [89]. That being said, the study which infected mice with *Lm*-OVA bacteria found greater antigen-specific T cells at night (ZT16, early-mid active phase) as compared to morning (ZT2, early rest phase), which may reflect differences in antigen presentation or cytokine milieu during an *in vivo* infection with naïve immune cells. Finally, as the kinetics of dissemination from the gut are not well defined, further studies are needed to predict how these T-cell dynamics would translate to natural infection.

In total, these studies show circadian regulation over the severity of *Lm* infections, but the optimal time for resolution of the infection greatly depends on the dose and route of exposure. This variability reflects the differences in the kinetics of bacterial invasion and the degree of immune response following different models of *Lm* infection, which together inform disease severity. For instance, while pro-inflammatory signals can augment immune cell recruitment and activation to protect against low infectious doses, these same signals can also induce harmful levels of inflammation in cases of high infectious dose. Therefore, the time of greatest inflammatory response is not necessarily the time of the most optimal outcome for the host.

The route of infection can similarly influence disease outcome. Oral infections of *Lm* showed increased bacterial clearance during the middle of the murine rest phase, seemingly agreeing with the results obtained during a low-dose systemic infection. However, as *Lm* must overcome antimicrobial barriers in the gastrointestinal tract in order to establish infection following oral inoculation, it is unclear whether the time of greatest resistance reflects changes in gastrointestinal dynamics or systemic immune responses. Further, caution must also be taken to interpret these results, as no studies published thus far have investigated time points in the middle of the night (murine active phase). More research is therefore required for comparisons across infection models and to determine the precise cycle of infection susceptibility.

The complex pathogenesis of *Lm* infection also underlies an important facet of circadian regulation of immune responses—there is unlikely to be a time at which every aspect of the immune response is completely optimal. In the case of $CD8^+$ T-cell activation, many sequential steps are required from the initial infection to the ultimate killing of infected host cells by activated T cells. Each of these steps studied in isolation has its own kinetics and circadian regulation. As the generation of adaptive immune responses *in vivo* typically spans several days, optimal activation of immunity may therefore necessitate coordination across different circadian times.

Streptococcus pneumoniae

Streptococcus pneumoniae (*Spn*, also known as the pneumococcus) is a Gram-positive bacterium that can asymptomatically colonize the upper respiratory tract of humans [138,139]. However, *Spn* also causes a wide range of infections including of the sinuses (sinusitis), middle ear (otitis media), bloodstream (invasive pneumococcal disease), and remains the leading cause of community-acquired bacterial pneumonia [140,141]. While these infections are generally uncommon among immunocompetent adults, people such as newborns, older adults, and the immunocompromised exhibit a greater risk of pneumococcal infection [142]. The establishment of infection involves a migration out of the upper respiratory tract, which in turn involves a complex interplay of the host immune system and bacterial anti-immune defenses that have not been fully elucidated, highlighting an as-yet unappreciated layer of complexity to host–pathogen interactions in the context of *Spn* infections [143,144].

As of 2025, more than 100 serotypes have been documented [145], with an average of only 74% of a strain's genome being shared with all other *Spn* strains [146]. As more than one-quarter of the genome varies between strains, *Spn* experiences significant genetic and therefore phenotypic diversity, contributing to differences in virulence factors, metabolic capacity, and host interactions [147–151]. As a result, the pathogenesis of different *Spn* strains varies considerably, with many strains exhibiting specific tissue tropisms, such as the well-studied D39 (serotype 2) entering the bloodstream and causing systemic disease, TIGR4 (serotype 4) accessing the brain and causing meningitis, and A66.1 (serotype 3) remaining in the lung to cause sustained pneumonia [152]. Additionally, a specific human isolate was found to prefer colonizing the ear over systemic infection based on a single nucleotide polymorphism (SNP) [153].

To study pneumococcal lung infection in a mouse model, the bacteria are typically administered intranasally (i.n.) or intratracheally (i.t.), with these exposure routes leading to similar outcomes in mouse models [154]. In this infection model, many, but not all strains of *Spn* can disseminate from the lung to the

bloodstream, thereby leading to systemic infection [152]. As the virulence factors necessary for systemic infections can differ from those of lung infection, systemic infections can also be directly modeled with intraperitoneal (i.p.) or intravenous (i.v.) infection models [155]. Disseminated infection occurs in about 5% of community-acquired pneumonia cases [156] with such infections having approximately 17–20% mortality, though this is highly dependent on host risk factors and *Spn* serotype [157,158]. Thus, while uncommon, systemic infection models with *Spn* nevertheless still represent a clinically relevant model of pneumococcal infection.

The heterogeneity of *Spn* strains and the varied immune landscapes of their target tissues present a challenge to summarize pneumococcal infections as a singular form of bacterial infection. Additionally, unlike *Salmonella* and *Lm*, *Spn* is an extracellular pathogen and generally replicates outside of a host cell [144,159]. During an infection, therefore, *Spn* is exposed to the cumulative antimicrobial efforts of multiple immune cells, making infection and immune dynamics more difficult to model for pneumococcal infections. Hence, although *Spn* was the first pathogen shown to elicit circadian variation in host susceptibility, with studies from the late 1960s and early 1970s reporting time-of-day-dependent survival in mice [160–162], the underlying mechanisms of this circadian susceptibility remain poorly understood. Here, we aim to discuss the nuances of these pathogeneses in the context of circadian immunology.

Mucociliary clearance

For *Spn* to gain access to the lower respiratory tract, the bacteria must resist mucociliary clearance, a process by which microbes are trapped by the mucin secreted by airway epithelial cells and are moved out of the lower airways by the coordinated beating of epithelial cilia. *Spn* produces a polysaccharide capsule that reduces bacterial attachment to mucin [163–165] and can cleave mucin through expression of its neuraminidases (NanA and NanB) [143]. Additionally, some evidence exists that the major *Spn* virulence factor pneumolysin reduces ciliary beating, suggesting that resisting mucociliary clearance is essential for *Spn* to maintain lower airway infections [166,167]. Mucin production in murine lungs has been reported to be under circadian control, as mice were observed to have greater amounts of mucin in their airways during the expected night (active phase) than during the expected day (rest phase) [168]. This oscillation in mucin was attributed to mucin transport out of airway cells and was found to be regulated by circadian signals from the suprachiasmatic nucleus of the brain, as this circadian oscillation was lost upon disruption of the vagal nerve innervating the airway passages [168].

Likewise, in a lung inflammation model, mice stimulated with ovalbumin to induce inflammation produced differing amounts of MUC1 in a time-of-day manner, with greater amounts of MUC1 produced when stimulated at dusk (ZT12, end of rest phase) than when stimulated at dawn (ZT0, end of active phase) [169]. [Note: ZT0 was defined as the time of light off (dusk) in this study. For consistency, we have converted these times such that ZT0 corresponds to lights on (dawn) instead.] The authors found that *Muc1* expression was suppressed by *Bmal1* in a circadian manner, as silencing of *Bmal1* induced MUC1 protein abundance while simultaneously ablating the circadian phenotype. Notably, the authors also found that *Muc5ac* transcript expression in response to ovalbumin stimulation was similarly circadian, although *Muc5ac* transcript oscillation was different than observed with *Muc1*. *Muc5ac* transcript was found to be higher between the night and the following afternoon (ZT20–8, mid-late active phase to late rest phase). Although the authors did not investigate whether this oscillatory mRNA translates into oscillatory MUC5AC protein abundance, this result suggests that different mucins may exhibit different circadian properties. Nevertheless, mucin abundance has been shown to be circadian in both a homeostatic and an inflammatory model, suggesting that *Spn* resistance may similarly be circadian both during exposure and during infection, with better resistance during the host's active phase.

Macrophage phagocytosis

During infection, *Spn* encounters tissue-resident macrophages, which phagocytose the bacteria to mitigate further spread of the bacteria. Studies investigating the circadian regulation of murine macrophage phagocytosis have generally found greater phagocytic ability during the mouse active phase, although the precise timing of greatest phagocytosis depends on the stimulation model and the consequences of these circadian oscillations can differ according to the site of infection. Macrophage populations are highly variable and tissue-dependent, with tissue-resident macrophages within one organ having different phagocytic and antimicrobial capabilities than macrophages within another organ [170–172]. Hence, phagocytosis of *Spn* presents a nuanced and often unintuitive aspect of *Spn* pathology. For instance, a study into alveolar macrophages, the resident macrophages in the lung,

found that alveolar macrophages are important for resistance to *Spn* infection only in low-dose infections [173], possibly because higher doses overwhelm their ability to control *Spn* infection. However, alveolar macrophages from humans that are nasally colonized with *Spn* show increased capacity to phagocytose *Spn* and other bacterial pathogens for up to 3 months postcolonization, suggesting this defense plays an important role in protection against natural infections [174]. Additionally, as different strains of *Spn* localize to different organs during infections, *Spn* encounters different populations of macrophages, contributing to variations in the host–pathogen interactions between *Spn* strains.

Most studies into the circadian nature of macrophage phagocytic ability utilize either peritoneal macrophages or bone marrow-derived macrophages (BMDMs), which do not cleanly reflect the macrophage population of any given organ and hence cannot be used to estimate general antimicrobial efficacy during *Spn* infection [175,176]. Nevertheless, peritoneal macrophages and BMDMs provide valuable insights into the mechanisms of circadian regulation on macrophage phagocytosis. One study observed that peritoneal macrophages uptake more *E. coli* bioparticles when exposed at the end of the day (increased at ZT8, peaking at ZT12) as compared to the morning or night (ZT0, 4, 16 or 20) [177]. Similarly, peritoneal macrophages isolated at different times and incubated with *S. aureus* bioparticles showed increased phagocytosis at dusk (ZT12) compared to dawn (ZT0) [178], suggesting peritoneal macrophage phagocytosis is improved shortly before dusk (late rest phase of the mouse). In contrast, the circadian phagocytic activity of BMDMs is less clear, as BMDMs are best suited to *in vitro* assays and circadian phases of *in vitro* assays do not readily translate to *in vivo* host circadian times. A study investigating phagocytosis of zymosan, which mimics fungal particles, by BMDMs found the greatest phagocytosis occurring at 8 h after the peak of *Per2* mRNA expression [179], whereas another study in BMDM found that phagocytosis of amyloid particles (nonmicrobial particles associated with Alzheimer's) was greatest at the peak of PER2 protein expression [180]. These times roughly correspond to dawn (end of active phase) and dusk (end of rest phase) in the whole animal, respectively [8,181]. The discrepancies in the time at which maximal phagocytosis is observed may reflect differences in macrophage subtype or the particle being phagocytosed, as both can influence the efficiency and kinetics of internalization [182–184].

Several studies have reported time-of-day resistance to *Spn* infection *in vivo* driven at least in part due to differences in circadian-driven oscillations in macrophage phagocytosis. In a recent study with an invasive infection model of *Spn* strain E1586 (Serotype 1), mice infected intranasally at midnight and dawn (ZT18 and ZT0, mid to end of active phase) showed greater bacterial clearance after 24 h in the lung and spleen compared to infection at mid-day and dusk (ZT6 and ZT12, mid- and end of rest phase) [185] (Fig. 5). This difference in phagocytic activity was linked to the circadian clock protein REV-ERBα, which was found to negatively regulate expression of *Apln* (apelin), a hormone responsible for maintaining a wide variety of cellular functions in multiple tissues [186]. In alveolar macrophages and BMDMs, apelin signaling was found to improve phagocytic ability, with *Apln* expression greater at dawn (ZT0, antiphase of REV-ERB activity) as compared to dusk (ZT12). In turn, this was correlated with greater clearance of Spn at ZT0, compared to ZT12. Exogenous supplementation of apelin was sufficient to improve macrophage uptake of *Spn in vitro* and decrease bacterial burden *in vivo*, confirming its role in regulating this process. Similarly, pharmacological inhibition of REV-ERBα improved phagocytosis across all tested cell types *in vitro* and *ex vivo*, decreased bacterial burdens, and improved survival *in vivo* following *Spn* infection at dusk (ZT12).

Relatedly, it is known that mice lacking *Bmal1* in myeloid-derived cells showed increased control of *Spn* following intranasal infection with an invasive strain of *Spn*, D39 (Serotype 2) due to increased phagocytosis [178]. While this study did not directly investigate alveolar macrophages, it demonstrated that peritoneal macrophages lacking *Bmal1* exhibited higher expression of genes involved in cytoskeletal remodeling and actin polymerization, which facilitated cell migration and phagocytosis, suggesting that *Bmal1* expression suppresses phagocytic capability [178]. Indeed, loss of *Bmal1* led to increased phagocytosis of Spn *ex vivo* by peritoneal macrophages and increased uptake of *S. aureus* bioparticles in peritoneal exudate cells *in vivo* [178].

It is important to recognize that not all *Spn* infections are equivalent, as serotype-specific traits can drive markedly different outcomes in infection. In contrast to the findings by Angulo *et al.*, which used strain E1586 (serotype 1) [185] and observed increased resistance at dawn (ZT0), a study by Gibbs *et al.* used strain D39 (serotype 2) [187] and reported increased resistance at dusk (ZT12) (Fig. 5). Both studies used C57Bl/6-derived male mice, an intranasal route of infection, observed bacterial dissemination from the lung within the first 24 h of infection, and measured both pulmonary and disseminated (spleen or blood)

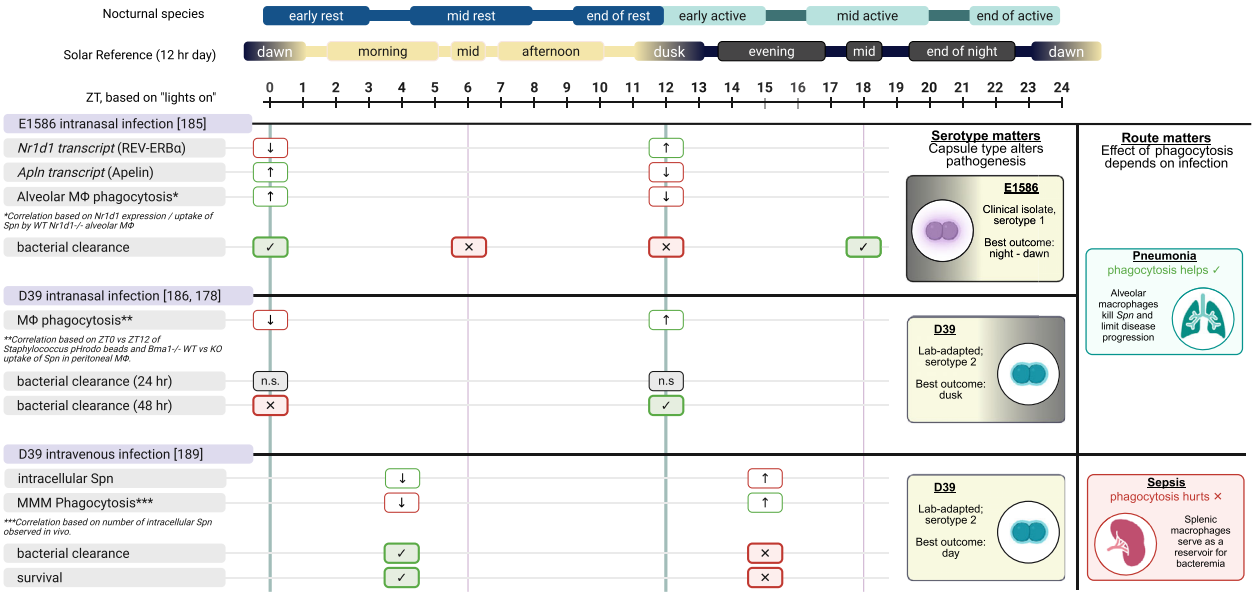

Fig. 5. Summary of host outcomes following time-of-day studies with *Streptococcus pneumoniae* (*Spn*). Summary of studies reviewed here. For reference, timelines denoting activity, solar time and ZT (relative to lights on at ZT0) are provided at the top. Each study is denoted by a pink header with title followed by the citation number used in this review in brackets [#]. Icons are placed based on the ZT at which the infection occurred (not necessarily the time at which the parameter was measured). Symbols indicate increased (↑) or decreased (↓) levels of a parameter measured in the study that is thought to drive differences in bacterial outcome. Ultimate outcomes of pathogenesis are summarized as better for the host (✓, green fill) or worse for the host (×, red fill). n.s. in gray means there was no significant differences in that parameter based on the time of infection. Note that studies vary in the route (oral [97] vs systemic [82]), dose (10^6 vs 10^7 CFU) and lighting parameters (14 h day [97] vs 12 h day [82]) which limits cross-study interpretation. (Right-hand panels) Several studies identified differences in phagocytosis as driving time-of-day dependent outcomes during infection. However, these studies highlight the diversity of *Spn* pathogenesis; where serotype and infection route can drastically alter relative contribution of immune defenses and the time of best host outcome. Created in BioRender. Kimmey, J. (2025).

burden. However, in addition to the different bacterial strains used, Angulo *et al.* used a higher dose (1×10^6 CFU of E1586) and measured bacterial burden at 24 h postinfection, while Gibbs *et al.* used a lower dose (2×10^4 CFU of D39) and only observed statistically significant differences at 48 h postinfection.

Dissemination of *Spn* from the lung requires utilization of several pneumococcal virulence factors that allow the bacteria to evade antimicrobial pathways such as the expression of soluble antimicrobial factors and phagocytosis by alveolar macrophages [188]; once in the bloodstream, *Spn* faces a different milieu of immune defenses [189]. The observed difference in *Spn* resistance between the two studies may therefore reflect inherent serotype differences and differences in stage-specific immune dynamics. These factors highlight the challenges of lung infection models using naturally invasive *Spn* strains, where rapid dissemination obscures the timing and localization of specific immune responses due to complex, tissue-specific immune interactions.

A separate study injected mice with *Spn* strain D39 intravenously, which bypasses these challenges by avoiding the kinetics of crossing the lung barrier [190]. In this model, microbes in the bloodstream rapidly reach the spleen, where they are filtered through the marginal zone of the spleen, a specialized anatomical interface containing marginal zone macrophages (MZMs) and marginal zone metallophilic macrophages (MMMs) [191]. However, it is known that *Spn* can replicate intracellularly in these cells, ultimately leading to host cell lysis, which releases viable bacteria into the bloodstream and drives fatal septicemia [192,193]. Thus, phagocytosis of *Spn* by splenic MMM is detrimental for the host [193]. In line with this, Hames *et al.* found that mice infected during the day (ZT3, associated with greater resistance) had lower percentages of *Spn*-infected MZM and MMM (Fig. 5). Infection at night (ZT15, associated with greater susceptibility) led to a higher percentage of infected MZM and MMM and increased signs of bacterial replication [190].

It is noteworthy that during *Spn* infection, circadian control of phagocytosis by alveolar macrophages has been shown to be protective [185], while circadian control of phagocytosis by splenic MMM was detrimental [190]. Interestingly, however, due to differences in the circadian phagocytosis of alveolar macrophages and splenic MMM, the most protective time for phagocytosis nevertheless occurs in the day (rest phase in mice) for both types of macrophages.

Because capsular serotype strongly influences the susceptibility of *Spn* to macrophage uptake [194], the overall impact of phagocytosis on disease outcome likely depends on the infecting strain. Moreover, in systemic infection, increased MMM phagocytosis impairs clearance of *Spn* [193] but is essential for protection against *Lm* [195], providing an example of an immune response that may be beneficial for one pathogen yet harmful for another, highlighting the pathogen- and tissue-specific nature of immune responses and their potential interactions with the circadian clock.

Neutrophil recruitment and function

Clearance of *Spn* is also dependent on the early detection of the bacteria and the subsequent recruitment of nonresident immune cells. As with *S.* Typhimurium and *Lm*, *Spn* engages various Toll-like receptors and NOD-like receptors. The binding of these receptors activates NF-κB and induces production of the pro-inflammatory cytokines IL-1α, IL-1β, IL-6, and TNF-α. These pro-inflammatory signals lead primarily to the production of the neutrophil-attracting chemokines CXCL1, CXCL2, CXCL5, and IL-17. Recruitment of neutrophils is critical for the resolution of *Spn* infection, as depletion of neutrophils in mice before infection with either the anti-Gr-1 antibody [196,197] or the anti-Ly6G antibody [198,199] increases susceptibility to *Spn* and leads to higher mortality. In the previous sections discussing *Salmonella* and *Lm*, we have highlighted the connection between neutrophil recruitment and the circadian clock. In the lung, neutrophil influx in response to LPS (a molecule found on Gram-negative bacteria) is circadian, with the greatest recruitment seen at dawn (ZT0, end of active phase) [187]. However, the contribution of this phenotype to time-of-day dependent control of *Spn* is unclear, as *Spn* lacks LPS and infection with strain D39 (serotype 2) did not elicit differential amounts of neutrophils following infection at dawn (ZT0) or dusk (ZT12) [187]. Neutrophils are also known to contribute to the control of *Spn* in the spleen [200], but it is not known if these effects vary over circadian time.

Though the infiltration of neutrophils is known to be essential for early infection, the role of neutrophils across all models of *Spn* infection is nuanced, as sustained neutrophil influx and inflammation can be damaging to host tissue and exacerbate host pathologies [201–203]. During *Spn* infection, neutrophils help to eliminate invading bacteria via phagocytosis [204], neutrophil extracellular trap (NET) formation [205,206], and degranulation [207]. Neutrophil elastase and cathepsin G are serine proteases and key mediators of bacterial killing [208], and mice lacking these enzymes have increased susceptibility to *Spn* infection [199]. As discussed previously in the *Salmonella* section, many neutrophil antimicrobial processes are under circadian regulation, with the greatest neutrophil responses generally occurring at night in mice (active phase in mice) or morning in humans (active phase in humans). Surprisingly, this pattern of maximal neutrophil activity coincides with worse clinical outcomes. A total of 5000 pneumonia patients showed that both pneumonia severity (assessed by pneumonia severity index) and mortality peaked around 9:00 AM and reached their lowest point in the evening (6:00–11:00 PM) [74]. This temporal pattern suggests that heightened neutrophil inflammatory capacity does not necessarily translate to improved disease control, but may instead contribute to immunopathology and worse patient outcomes during peak activation periods.

In total, these pieces of evidence suggest that immune responses to *Spn* infection are generally most efficient when activated during the active phase compared to the rest phase. However, care must be taken to interpret these observations and to predict the circadian nature of *Spn* infection. As demonstrated by the investigations into the circadian nature of macrophage phagocytosis, an immune pathway may be beneficial or detrimental, depending on the bacterial strain, exposure route, and tissue being investigated. One important consideration for comparison across tissues is that, even within a single organism, different tissues are known to oscillate on different phases: a circadian peak in one tissue may be a circadian trough in another. This presents an additional challenge for interpretation when utilizing pathogens such as *Spn*, which are capable of infecting multiple organs simultaneously. Combined with the understanding that immune responses and their efficacies may differ across organs, the time-of-day influence on *Spn* susceptibility can often be circuitous and unintuitive. For instance, MMM phagocytosis is more efficacious during the murine active phase, but is detrimental to *Spn* clearance; meanwhile, alveolar macrophage phagocytosis is more efficacious during the murine rest phase

and is essential for *Spn* clearance. Therefore, although much is known about the circadian regulation of immune pathways, more research is required to interpret their relevance in the context of pneumococcal infections.

Conclusion

The circadian clock has become recognized as a core mediator of immune activity that demonstrably impacts the outcome of bacterial infection, but the specific mechanisms and optimal timing windows depend heavily on the pathogen. Here, we showed that many of the immune pathways that are engaged during infections caused by *S.* Typhimurium, *Lm*, and *Spn* are circadian-controlled and that the development of the diseases caused by these pathogens therefore changes depending on the time of infection. From a broader perspective, a large number of immune pathways have now been identified as being regulated by the circadian clock; thus, it is not surprising that the susceptibilities to these pathogens were found to be circadian as well. Indeed, given the role of the circadian clock as a central regulator of multiple immune processes, it is likely that many other pathogens, if not most pathogens, share a similar phenotype of circadian susceptibility.

The specific nature of circadian susceptibility, however, is more difficult to predict, as it is often unclear whether an improved immune response truly correlates with improved disease outcome. In the discussion of just these select few pathogens, we have examined multiple examples of these unintuitive interactions. In the case of *Salmonella*, the production of antimicrobial peptides worsens disease outcome by inhibiting colonization resistance, and activation of the microbe-detecting inflammasome NLRP6 induces iron sequestration, which improves *Salmonella* growth. In the case of *Lm*, increased cytokine production during the day was correlated with improved bacterial clearance at a low dose but correlated with faster mortality at a higher dose, suggesting even protective cytokines can drive pathologic outcomes. Finally, in the case of *Spn*, several studies identify phagocytosis as a driver of time-of-day dependent differences in disease outcome. However, the effect depends heavily on the model used—pulmonary studies point toward a beneficial role of phagocytosis by alveolar macrophages, but a systemic study identifies a detrimental role for phagocytosis by MMM. Because the consequences of a given immune response are dependent on the invading pathogen, much of this aspect of pathogenesis is unknown; contextualizing specific immune processes to the overall susceptibility of a pathogen is therefore likely to be a fruitful area of future research.

Additionally, variation in circadian timing across human populations is well-documented in circadian literature. An individual's chronotype, or preferred sleep timing, is known to be regulated at least in part by their genetic makeup [209], suggesting that the same drivers of chronotype variability may be an additional regulatory factor in circadian immunity. Research into human chronotypes has revealed that both age and sex influence chronotype [210], offering a possible explanation for observed age- and sex-dependent differences in disease susceptibility. Additionally, aging is known to reduce the robustness of circadian oscillations in a variety of biological measures, including blood cortisol [211] and lipid levels [212], which may contribute to differences in disease susceptibility across age groups. For instance, the circadian oscillation of *Apln*, the hormone that regulates alveolar macrophage phagocytosis, diminishes with age, leading to an overall greater susceptibility to *Spn* infection in aged mice compared to young mice [185]. Similarly, sex-related differences in circadian rhythmicity may drive differences in disease outcome, but more research is required to determine the precise relationships between sex, the circadian clock, and infectious disease outcome. Beyond investigations into circadian immunology, greater awareness of circadian influences on infection characteristics may improve reproducibility in noncircadian studies, as circadian differences in immune responses may prove to be an uncontrolled source of experimental variability. We therefore recommend reporting the times of infection in all infection models, even when circadian influences are not the focus of investigation.

Finally, recent studies have described circadian rhythms in bacterial species beyond cyanobacteria including *Bacillus subtilis* (found in soil) [213], *Klebsiella aerogenes* (formerly *Enterobacter aerogenes*, found in the gut microbiome) [214,215], and *Acinetobacter baumannii* (an opportunistic human pathogen, preprint) [216], suggesting bacterial-intrinsic clocks might exist more broadly and could, at least in principle, influence infection outcome. At present, however, no evidence exists to demonstrate that bacterial-intrinsic rhythms occur *in vivo* or affect host–pathogen interactions, and there are no reports of circadian oscillations in *Salmonella, Listeria*, or *Streptococcus* species. Importantly, however, the absence of existing reports for these questions may simply reflect a topic that has not yet been systematically examined, and the presence or absence of such rhythms remains an open question.

The circadian nature of disease susceptibility additionally presents a unique opportunity for uncovering deeper insights into the field of immunology. Because immune activity changes throughout the day, circadian studies may reveal which processes most strongly influence disease outcomes and to what extent they shape pathogenesis. The field of circadian pathogenesis remains in its infancy, with significant gaps and limited pathogen coverage constraining our understanding of temporal host–pathogen dynamics. Current studies often employ different infection routes, doses, timing protocols, and outcome measures, making cross-pathogen comparisons challenging and highlighting the need for more standardized approaches. Nevertheless, the consistent observation that infection timing influences disease outcomes across diverse bacterial systems suggests that circadian regulation represents a fundamental but underexplored dimension of host defense. An increased understanding of this field may lead to improvements in therapeutic technologies, as drug treatments or vaccinations may be timed with a patient's circadian clock to optimize for maximal efficacy while minimizing side effects. Future research that systematically examines circadian–pathogen interactions within the context of specific virulence mechanisms may therefore not only reveal previously unrecognized principles governing immune function and bacterial pathogenesis but also have practical applications for disease treatment and prevention.

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Author contributions

DM, CSP, and JMK contributed to the conceptualization, writing, and visualization of this manuscript.

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