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Contrasting persistence strategies in *Salmonella* and *Mycobacterium*

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Summary

Long-term survival of persistent bacterial pathogens in mammalian hosts critically depends on their ability to avoid elimination by innate and adaptive immune responses. The persistent human pathogens that cause typhoid fever and tuberculosis exemplify alternative strategies for survival in the host: immune evasion and immune adaptation, respectively. *Salmonella enterica* serotype Typhi evades host innate immune responses and inflammation by expressing factors that interfere with its detection as a Gram-negative bacterium, enabling persistent colonization of an immunologically privileged niche, the gallbladder. In contrast, *Mycobacterium tuberculosis* has adapted to survive within phagocytic cells, which typically eliminate invading microbes, by deploying stress resistance mechanisms that counteract the harsh environment of the phagolysosome.

Introduction

Many pathogenic bacteria cause acute infections characterized by an adaptive immune response that clears the invading microbe and generates immunological memory. Some bacterial pathogens, however, maintain infections for the lifetime of their mammalian hosts without causing overt disease signs or symptoms, despite triggering a robust adaptive immune response [1,2]. These persistent bacterial pathogens depend on infected carriers for survival in the host population and transmission to naïve individuals.

Salmonella enterica serotype Typhi (*S*. Typhi), the bacterium that causes systemic typhoid fever, establishes persistent infection of the gallbladder in 1–4% of typhoid patients [3]. Typhoid carriers are asymptomatic but periodically shed large numbers of *S*. Typhi in their stools. Periodic transmission from asymptomatic carriers is essential for long-term maintenance of *S*. Typhi in human populations. Mary Mallone, a notorious typhoid carrier better known as “Typhoid Mary”, infected at least 57 people in New York City before she was confined to lifelong quarantine in 1907. Similarly, *Mycobacterium tuberculosis* typically persists as an asymptomatic latent infection of immune competent humans before reactivating to cause full-blown tuberculosis (TB) disease that can be transmitted to new hosts. Compelling

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molecular evidence for decades-long persistence of latent *M. tuberculosis* came from molecular subtyping experiments conducted on more than 2,000 clinical *M. tuberculosis* specimens [4]. Remarkably, isolates from a father and son whose TB diagnoses were separated by 33 years had identical DNA restriction fragment patterns indicating an epidemiological relationship. These were the only samples with a specific molecular signature, strongly suggesting direct transmission of *M. tuberculosis* from father to son, followed by a 33 year period of latency [5].

Although both *S.* Typhi and *M. tuberculosis* avoid elimination by the immune response, these pathogens use different strategies to persist in their hosts. *S.* Typhi *evades* host immunity by expressing factors that reduce the host inflammatory response, enabling systemic invasion and colonization of the gallbladder, a privileged anatomical site that receives little immune surveillance. In contrast, *M. tuberculosis* employs stress resistance mechanisms to *counteract* the harsh environment of the activated macrophage phagolysosome, a compartment that typically eliminates microbial invaders.

S. Typhi immune evasion - molecular masking

S. Typhi is a human-specific pathogen that is related to the non-typhoidal serotype *Salmonella enterica* serotype Typhimurium (*S.* Typhimurium), which causes gastroenteritis. Because *S.* Typhi is host-specific, it does not persist in animals that are used as infection models. *S.* Typhimurium has a broader host range that includes mice as a model to study *Salmonella* persistence (Box 1). Both *Salmonella* species initiate infection by invading the intestinal mucosa, a process that requires two type 3 secretion systems (T3SS-1 and T3SS-2), which promote uptake by intestinal epithelial cells and survival in host macrophages, respectively (Figure 1) [6,7]. Mucosal invasion by *S.* Typhimurium is detected by pattern recognition receptors including Toll-like receptor 4 (TLR4), which recognizes lipopolysaccharide (LPS), and TLR5, which binds flagellin. Activation of TLR signaling induces expression of inflammatory cytokines such as IL-8 and TNF-α, which recruit neutrophils to contain the infection [6]. In contrast, *S.* Typhi invasion of the intestinal mucosa does not trigger neutrophil influx, allowing the bacteria to disseminate to the liver, spleen, bone marrow, and gall bladder (Figure 1) [8]. *S.* Typhi evasion of innate immunity suggests that it can disguise its identity as a Gram-negative bacterial pathogen.

Whole-genome sequencing revealed striking genetic differences between *S.* Typhi and other *Salmonellae*, including the presence of a unique 134 kbp region designated *Salmonella* pathogenicity island 7 (SPI-7) [9]. SPI-7 is genetically unstable and is readily lost during passage of *S.* Typhi in the laboratory [6]. SPI-7 deficient *S.* Typhi strains fail to inhibit inflammatory cytokine production in cultured human colonic epithelial cells, suggesting that this locus is important for immune evasion [10].

SPI-7 encodes functions for production and export of the Vi capsular polysaccharide antigen. The Vi capsule is expressed during human *S.* Typhi infection and contributes significantly to *S.* Typhi pathogenesis in human volunteers [6]. Ectopic expression of the Vi capsule in *S.* Typhimurium reduces TLR4-dependent production of the pro-inflammatory cytokine TNF-α in cultured macrophages and in mice [11•]. Although the mechanism by which Vi capsule prevents TLR4 signaling is unclear, it is possible that the capsule layer shields LPS from detection by TLR4, since strains expressing the Vi capsule are not agglutinated by antibody against the LPS O-antigen [11•].

SPI-7 also encodes TviA, a regulatory protein that controls expression of the Vi capsule, flagellar motility, and the invasion-associated T3SS-1 in response to osmolarity, in cooperation with the RcsC/RcsD/RcsB signal transduction system [12••]. Under conditions of low osmolarity, Vi capsule production is induced and genes encoding T3SS-1 and the flagellar

apparatus are repressed in a TviA- and RcsB-dependent manner [12••]. Inverse osmoregulation of flagellar motility and T3SS-1 (required for mucosal invasion) and Vi capsule (required for systemic immune evasion) has important consequences for *S*. Typhi pathogenesis. During invasion of the intestinal mucosa, *S*. Typhi encounters relatively high osmolarity in the intestinal lumen followed by low osmolarity inside host tissue. This environmental shift should promote expression of Vi capsule and repression of motility and T3SS-1 post-invasion. Analysis of Vi capsule expression in a bovine ligated ileal loop model revealed increased expression of Vi capsule by *S*. Typhi associated with host tissue compared to bacteria in the intestinal lumen [14]. In addition, TviA-mediated repression of flagellin expression avoids detection of *S*. Typhi by host TLR5. Compared to wild-type *S*. Typhi, a *tviA* mutant produced more flagellin and induced more TLR5-dependent pro-inflammatory IL-8 production by human colonic epithelial cells [13].

S. Typhi gallbladder persistence - bile resistance

While induction of Vi capsule and repression of flagellin contribute to *S*. Typhi immune evasion and systemic infection, persistent colonization of the gallbladder depends on additional factors including bile resistance. Bile - a lipid-rich, detergent-like digestive secretion with antimicrobial properties - is produced by the liver and concentrated in the gallbladder for delivery to the small intestine [15]. A genome-wide screen identified more than 150 *S*. Typhi genes required for bile tolerance [16]. Among the putative bile resistance genes are *acrAB* and *tolC*, encoding a bile acids efflux system, and LPS biosynthesis genes [16].

S. Typhi may also resist bile by forming biofilms on gallstones. *S*. Typhi biofilms, comprising microcolonies encased in an exopolysaccharide (EPS) matrix, are resistant to environmental insults and host immune mechanisms [17]. Bile induces production of an EPS O-antigen that facilitates *S*. Typhi biofilm formation on human gallstones [18]. Gallstone biofilms may promote *S*. Typhi carriage in the gallbladder by increasing bile resistance. Indeed, conversion to the chronic typhoid carrier state is strongly correlated with the presence of gallstones [3].

S. Typhi may also persist within gallbladder epithelial cells, a unique niche of *S*. Typhimurium replication identified in a mouse model of acute typhoid fever [19•]. Invasion of the gallbladder epithelium requires a functional T3SS-1, but induces strong inflammatory responses and neutrophil influx [19•]. It is conceivable that *S*. Typhi could limit this inflammatory response to persist in the gallbladder epithelium using the same immune evasion tactics that enable it to disseminate systemically.

Mycobacterium tuberculosis persistence in macrophages - stress management

Throughout infection *M. tuberculosis* persists within host phagocytic cells (Figure 2), which normally serve as a first line of host defense by internalizing and destroying microorganisms within phagolysosomes. The acidic pH of phagolysosome compartments suppresses microbial metabolism and activates intralumenal hydrolytic enzymes that degrade bacterial components such as proteins and lipids [20,21]. Reactive oxygen and nitrogen species (ROS and RNS) are generated in the maturing phagolysosome by the NADPH phagocyte oxidase (NOX2) and inducible nitric oxide synthase (iNOS), respectively [22]. ROS and RNS kill bacteria by damaging protein tyrosine residues, DNA bases, lipids, thiols, and metal centers [22]. Phagolysosomes also contain cationic antimicrobial peptides (CAMPs) that permeabilize bacterial cell membranes [21,23].

M. tuberculosis produces lipid and protein factors that block phagosome maturation and phagolysosome fusion in non-activated macrophages [24]. Although IFN-γ-activated macrophages

deliver *M. tuberculosis* to a mature phagolysosome, the bacteria are nonetheless capable of surviving in this harsh environment [25]. Consistent with these observations, *M. tuberculosis* mutants that fail to block phagosome-lysosome fusion in resting macrophages are not impaired for intracellular survival [26,27]. A growing body of evidence (see below) indicates that persistence of *M. tuberculosis* in mature phagolysosomes is due to specific mechanisms that counteract the stresses inflicted by activated macrophages. Thus, in contrast to *S*. Typhi, which persists by **evading** the host immune response and colonizing a niche **outside** immune surveillance, *M. tuberculosis* persists by **counteracting** host immune mechanisms and colonizing a niche **within** the immune system. This "counter-immune" strategy is shared by some but not all mycobacterial pathogens. For example, the related pathogen *Mycobacterium ulcerans* evades host immunity by secreting mycolactone, a cytotoxic compound with immunomodulatory properties (Box 2).

M. tuberculosis counteracts the toxic ROS and RNS encountered in the phagolysosome by three general strategies: enzymatic detoxification, scavenging, and damage repair (reviewed in [28]). *M. tuberculosis* also requires a protein degradation complex, the proteasome, to counteract damage caused by RNS. Mutations in genes encoding two putative accessory factors of the proteasome, *mpa* and *pafA*, confer sensitivity to RNS [29]. Attenuation of Mpa-deficient bacteria in wild-type mice is partially reversed in $iNOS^{-/-}$ mice, suggesting that proteasome-mediated protein degradation is required to counteract RNS and other stresses that *M. tuberculosis* encounters in the host [29]. Depletion of the proteasomal core subunits PrcBA, which have proteolytic activity against a broad range of peptide substrates, also renders *M. tuberculosis* more sensitive to RNS and impairs persistence in the lungs of mice [30•].

Mpa is the proteasomal ATPase that interacts with the core proteasome in the presence of ATP and probably unfolds and translocates proteins into the protease core for degradation [31]. Mpa specifically recognizes proteins that are targeted for degradation by a short peptide tag, the prokaryotic ubiquitin-like protein Pup [32••,33]. The Pup tag is coupled to target proteins in two steps: deamidation of the Pup C-terminal glutamine by Dop and subsequent conjugation to target proteins by PafA [34]. Proteasomal degradation of Pup-tagged proteins, mediated by Mpa and PafA, might be required for survival of nitrosative stress because it removes proteins that are irreversibly damaged by RNS, removes specific RNS-damaged proteins that are toxic, or upregulates transcription of genes encoding anti-oxidants by removing a transcriptional repressor [35].

M. tuberculosis factors that contribute to acid resistance and pH homeostasis were identified by screening for acid-sensitive mutants. Most of the identified mutations affect genes required for biogenesis of the mycobacterial cell wall, a complex lipid-rich structure that functions as a permeability barrier [36]. These mutations also confer hypersensitivity to other stressors, including lipophilic antibiotics and detergents, suggesting increased permeability of the cell wall to these compounds as well as to protons [36]. Apparently the cell wall permeability barrier also contributes to survival of *M. tuberculosis* in acidified phagolysosomes, since cell wall deficient mutants are attenuated in IFN-γ-activated macrophages and in mice [36].

One of the factors required for acid resistance is Rv3671c, a membrane-localized serine protease that is critical for maintenance of *M. tuberculosis* pH homeostasis. Unlike wild-type *M. tuberculosis*, the Rv3671c mutant failed to maintain neutral cytoplasmic pH either *in vitro* in acidified medium or *in vivo* in the phagolysosomes of IFN-γ-activated macrophages [37••]. Although its precise function is unknown, the Rv3671c protease might modify the mycobacterial cell wall or activate stress-response signaling pathways to maintain intrabacterial pH and promote survival in acidified phagolysosomes [38].

Within mature phagolysosomes *M. tuberculosis* also encounters CAMPs including cathelicidin, hepcidin, and ubiquitin-derived peptides that kill by disrupting the bacterial cell wall [39–41]. Other bacteria avoid CAMP-mediated cytolysis by reducing their surface negative charge, thus reducing their affinity for the positively charged CAMPs [42]. *M. tuberculosis* also resists CAMPs by cell surface modification. The LysX lysine transferase is required for linkage of positively charged lysine moieties to phosphatidyl glycerol (PG), a lipid component of the *M. tuberculosis* membrane [43•]. LysX-deficient bacteria are more sensitive to HNP-1, a CAMP produced by neutrophils, as well as the cationic antibiotics vancomycin and polymyxin-B [43•]. Replication of *lysX* mutant bacteria is impaired in animal infection models, indicating that PG lysinylation contributes to *M. tuberculosis* survival in the host [43•].

An unusually impermeable cell wall also protects *M. tuberculosis* from certain CAMPs. *Mycobacterium smegmatis*, a non-pathogenic relative of *M. tuberculosis*, is sensitive to ubiquitin-derived peptides within the mature phagolysosome [41]. Mutations in *mspA*, encoding a porin protein that forms aqueous channels in the *M. smegmatis* cell wall, confer resistance to ubiquitin-derived peptides [44]. Although the *M. tuberculosis* genome does not encode an MspA ortholog, expression of *M. smegmatis mspA* in *M. tuberculosis* increases membrane permeability and sensitivity to ubiquitin-derived peptides, and impairs survival in autophagic macrophages that deliver ubiquitin-derived peptides to the maturing phagolysosome [44]. Thus, the relative impermeability of the *M. tuberculosis* cell wall may have evolved as a mechanism to increase resistance to acidic pH and CAMPs encountered within mature phagolysosomes.

Conclusions

Although *S.* Typhi and *M. tuberculosis* use contrasting strategies to persist in their human hosts, both effectively thwart the immune system. Whereas *S.* Typhi **evades** host immunity in order to invade and colonize an immunologically privileged niche (the gallbladder), *M. tuberculosis* **counteracts** host immunity in order to establish a niche within the cell-mediated immune system (the macrophage). Recent work has begun to identify the gene products that allow *M. tuberculosis* to counteract the stresses encountered in macrophage phagolysosomes, but the underlying mechanisms of stress resistance remain ill defined. For example, although the proteasome is clearly important for resistance to RNS and persistence in mice, it is unclear whether the proteasome's critical role is general turnover of proteins that have incurred irreparable damage, or degradation of specific proteins that control the nitrosative stress response. Similarly, it is still largely unknown which mycobacterial cell wall components confer impermeability to protons and CAMPs. In the case of *S.* Typhi, although examination of genetic elements unique to this species has revealed novel mechanisms of immune evasion, the mechanisms that contribute to *S.* Typhi persistence in chronic carriers remain largely a black box. Despite the impressive recent advances reviewed here, for persistent investigators in both fields the most exciting discoveries undoubtedly still lie ahead.

Box 1. Persistent Infection by *Salmonella enterica* serotype Typhimurium

A mouse model of persistent *S.* Typhimurium infection that recapitulates important aspects of typhoid fever has begun to shed light on *Salmonella* immune evasion mechanisms. Persistent infection with *S.* Typhimurium can be achieved in mice that express wild type Nramp1, an ion transport protein that restricts availability of divalent cations to intracellular pathogens [45]. *S.* Typhimurium colonizes the mesenteric lymph nodes of these mice within infected macrophages, and is occasionally found in the gallbladder, liver, and spleen [45]. Some mice (~25%) become "super-shedders" that continuously excrete *S.* Typhimurium in their stools and spread the infection to naïve mice [46]. Factors that contribute to *S.*

Typhimurium persistence include components of both type 3 secretion systems (T3SS-1 and T3SS-2) [47]. The T3SS-2 effector protein SseI is required for long-term systemic infection of mice [48]. SseI interferes with the migration of infected cells by specifically binding the cell migration regulator IQGAP, thereby preventing normal dendritic cell migration to lymphoid tissues. These activities may constitute a mechanism for limiting presentation of *Salmonella* antigens and naïve T cell priming to inhibit adaptive immunity [48].

Box 2. Immune Suppression by *Mycobacterium ulcerans*

Mycobacterium ulcerans is closely related phylogenetically to *M. tuberculosis*, but causes a markedly different disease, Buruli ulcer (BU), which is characterized by painless necrotic skin lesions [49•]. Replication and persistence of *M. ulcerans* in BU lesions is profoundly influenced by production of mycolactone, a macrolide cytotoxin that has immunosuppressive properties. Although the precise cellular targets of mycolactone remain mysterious, it induces apoptosis of infected host cells [49•], inhibits production of the pro-inflammatory cytokine TNF-α by macrophages [50], and suppresses dendritic cell priming of T cells [51]. Mycolactone synthesized by *M. ulcerans* can diffuse from infected skin tissue to lymphoid organs within mononuclear cells, where it may exert some of these immune suppressive functions [52]. Mycolactone also damages nerve cells, which contributes to the painlessness of BU [53]. The genes required for mycolactone synthesis are carried on a virulence plasmid, pMUM001, that was apparently acquired by horizontal gene transfer from an unknown source [49•]. Perhaps because mycolactone came to dominate the interaction of *M. ulcerans* with its human host, following acquisition of pMUM001 other mycobacterial virulence factors were apparently lost by reductive evolution [49•].

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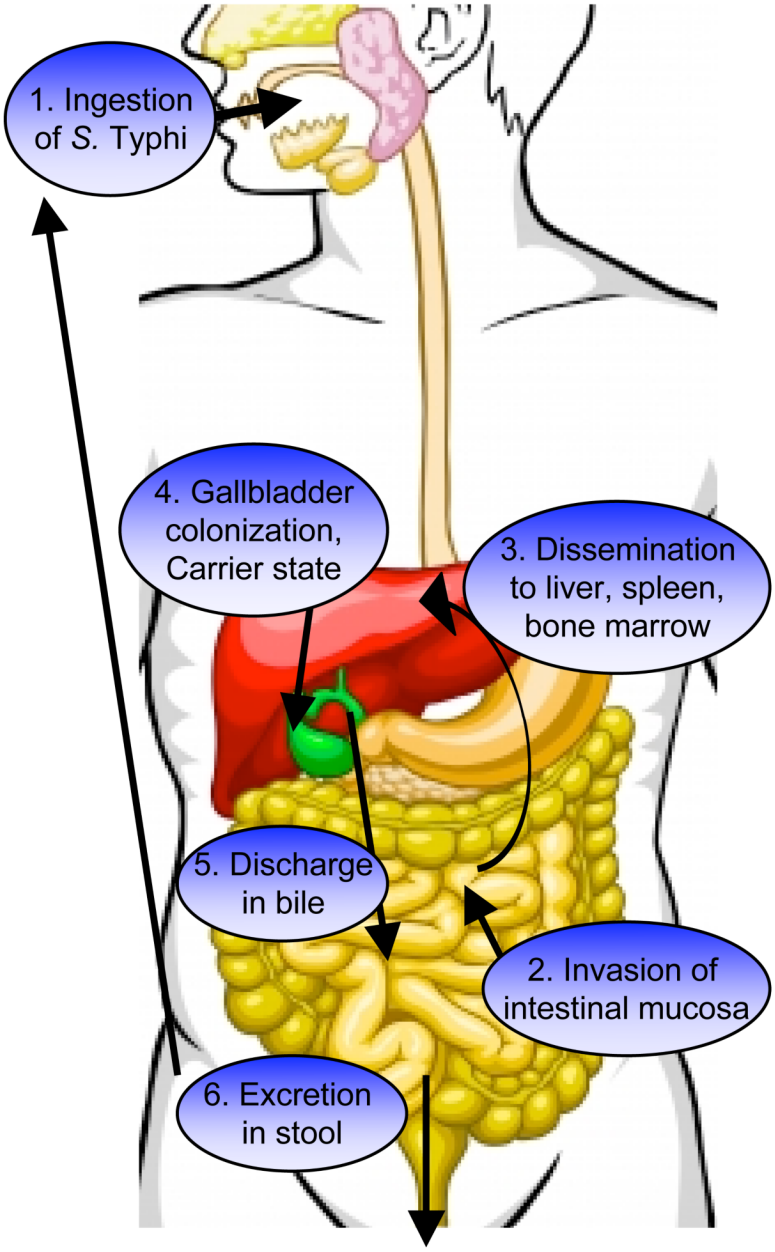

Figure 1. Pathogenesis of *Salmonella* Typhi infection

Typhoid fever is acquired by ingestion of food or water contaminated with *S*. Typhi (1). Bacteria that survive passage through the gastric acid barrier of the stomach invade intestinal epithelial cells and migrate through them to reach the lamina propria (2). In the intestinal mucosa, *S*. Typhi is phagocytosed by macrophages and survives within these phagocytic cells by T3SS-2 mediated secretion of effectors that interfere with host cell function. Following invasion, *S*. Typhi expresses factors that inhibit detection by the host innate immune system. This “masking” enables the bacteria to disseminate systemically to colonize macrophages in the liver (shown in red), spleen, and bone marrow (3). From the liver, *S*. Typhi can reach the gallbladder (shown in green) in bile. Infection of the gallbladder can lead to conversion to an

asymptomatic carrier state (4). *S*. Typhi carriers continuously discharge the typhoid bacillus from the gallbladder to the small intestine in bile (5) and excrete viable bacteria in their stools (6) that can infect naïve hosts.

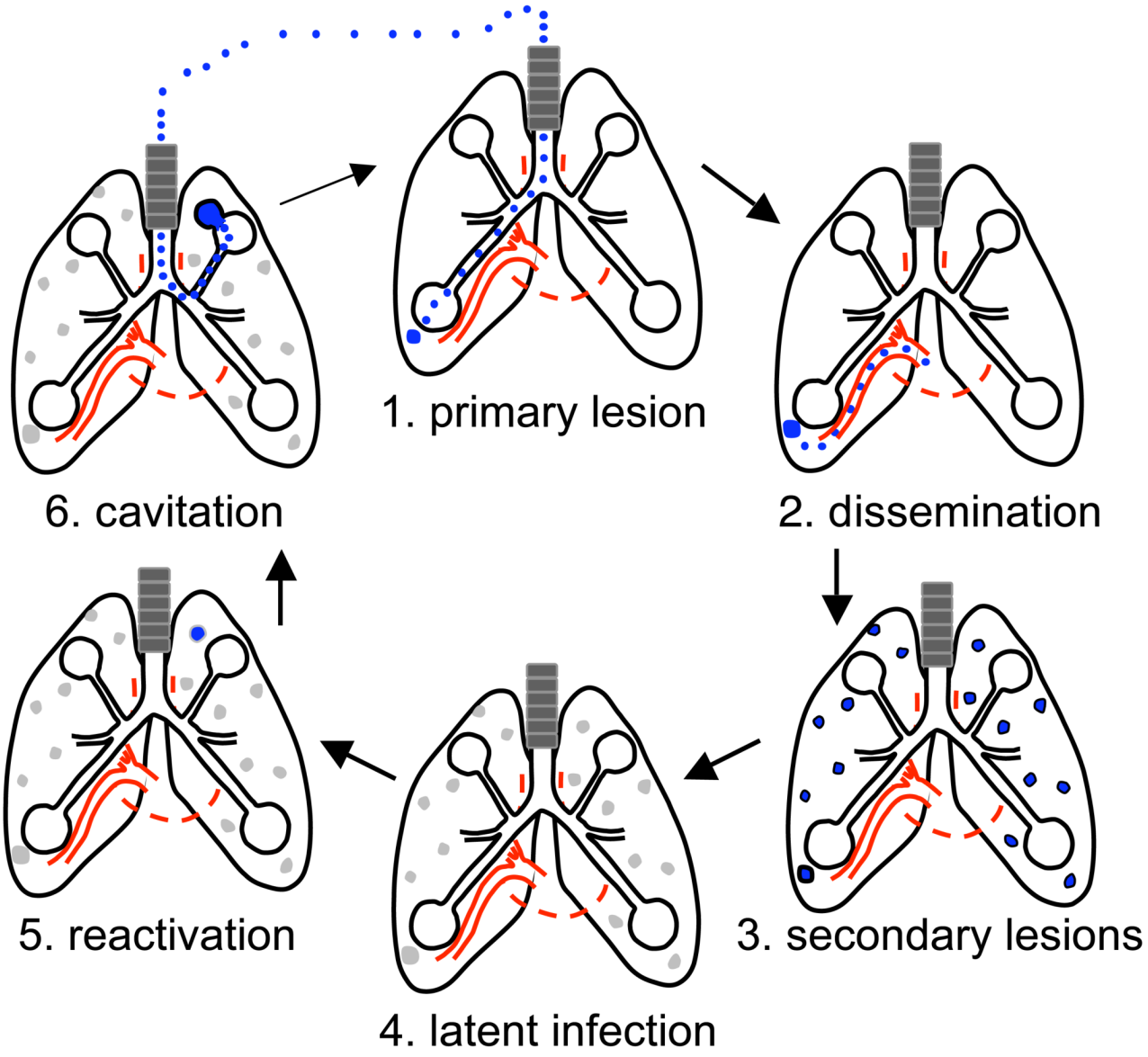

Figure 2. Pathogenesis of *Mycobacterium tuberculosis infection*

Infection with *M. tuberculosis* is initiated by inhalation of aerosols containing the bacterium (1). In the lung, *M. tuberculosis* is rapidly phagocytosed by resident alveolar macrophages. Actively replicating bacilli (shown in blue) induce inflammatory responses that recruit blood monocyte-derived macrophages. During growth of the primary lesion, some infected cells disseminate systemically to seed secondary lesions elsewhere in the lung (2, 3). In the majority of individuals, *M. tuberculosis* enters a latent state characterized by bacteria that are relatively quiescent (shown in grey) (4). When the immune system is compromised, for example by old age or HIV infection, *M. tuberculosis* resumes active growth (usually in secondary lesions) and elicits overt signs and symptoms of disease (5). Replication of *M. tuberculosis* leads to growth of the lesion and tissue destruction, which releases infectious bacteria into the lung airways (6). The characteristic cough of tuberculosis generates aerosols containing bacteria that can be inhaled by a naïve host to initiate a new round of infection.