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Do chance encounters between heterogeneous cells shape the outcome of tuberculosis infections?

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The sum of all of the interactions between single bacteria and host cells determines if an infection is cleared, controlled, or progresses at the whole host-organism level. These individual interactions have independent trajectories defined by diverse and dynamic host-cell and bacterial responses. Focusing on *Mycobacterium tuberculosis* infection, we discuss how advances in single-cell technologies allow investigation of heterogeneity in host-pathogen interactions and how different layers of heterogeneity in the host affect disease outcome. At late stages of infection, many single interactions co-exist and different outcomes depend on inter-granuloma and intra-granuloma heterogeneity. However, during bottleneck events involving small numbers of bacteria, random events, such as chance interactions with more or less permissive host cells, play a decisive role and may explain why some exposed individuals never develop the disease.

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Introduction

A *Mycobacterium tuberculosis* (*Mtb*) infection starts when airborne droplets containing single bacteria enter an individual's lungs and reach the alveoli. There, alveolar macrophages act as the first line of defense against lung pathogens by phagocytosing and killing the invading bacteria. However, *Mtb* has evolved mechanisms to survive and even replicate inside these immune cells, thus avoiding clearance and propagating the infection. As the disease progresses, other cell types are recruited to the site of infection, inducing the formation of granulomas, multi-cellular structures that can limit *Mtb* propagation (Figure 1). Over time, diverse infection outcomes can occur, ranging from sterilization of the bacteria, persistence of bacteria in a latent stage, to active infectious tuberculosis [1]. These heterogeneous outcomes can depend on diversity in granuloma maturation at later stages of infection [2]. For example, secondary granulomas originating in the upper lungs from bacteria re-seeded from the vascular or lymphatic systems are more likely to undergo caseation and disseminate bacteria than lesions originating from the primary infection in the lower lungs [3].

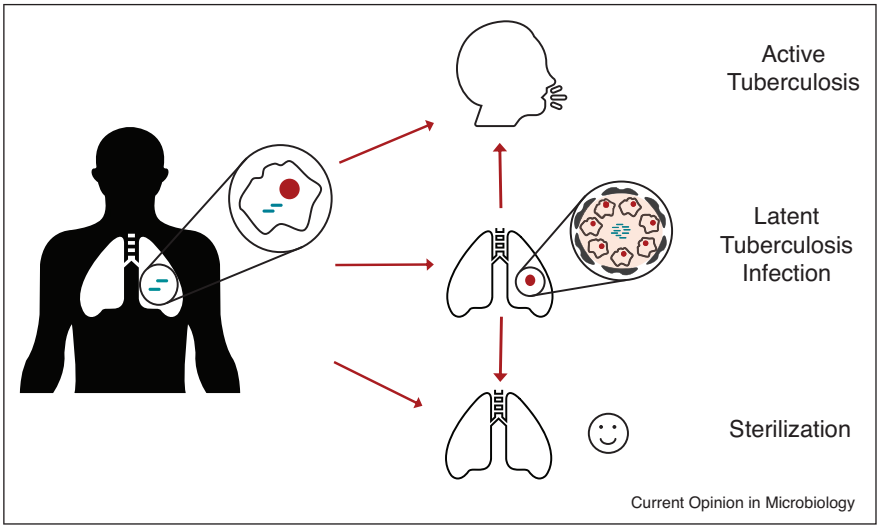
Interestingly, some highly exposed individuals never develop any lesion or adaptive immune response to *Mtb*, suggesting that sterilization is possible even before granuloma formation [4]. At earlier stages, diverse disease outcomes may thus be explained by differences in the host cells first encountered by the bacteria. Elucidating diversity in these 'first contacts' requires advanced technologies, such as single-cell RNAseq and time-lapse microscopy, which have become widely available only recently. In this review, we will discuss emerging evidence suggesting that exposure of *Mtb* to random or programmed heterogeneous host environments during different stages of the infection could contribute to diversity in disease progression (Figure 2).

Heterogeneous granulomas

Each granuloma is formed from a single initiating bacterium and progresses independently from others in the lungs, with outcomes ranging from sterilization to cavitation and dissemination of bacteria [2,5•]. Inter-granuloma heterogeneity not only plays a role in determining the outcome of a lesion but can also affect the efficacy of anti-tuberculosis drug treatment. MALDI mass spectrometry imaging of lung slices and PET-scans of human lungs indeed showed that drug penetration varies from granuloma to granuloma and that some drugs preferentially accumulate in specific areas within a granuloma. For example, fluoroquinolones penetrate more easily in regions with a high density of macrophages, while pyrazynamide and isoniazid accumulate in the central caseum of granulomas [6,7,8•]. Thus, in some local environments, subpopulations of bacteria could be suboptimally exposed to drugs and have a better chance to survive.

Heterogeneous environments are not only observed between different granulomas, but also within the same lesion. By using mass spectrometry or *in-situ* hybridization on human, mouse, and rabbit lungs, it was observed that protein expression varies significantly in different regions of the granulomas, and that pro-inflammatory and anti-inflammatory proteins are preferentially

Figure 1

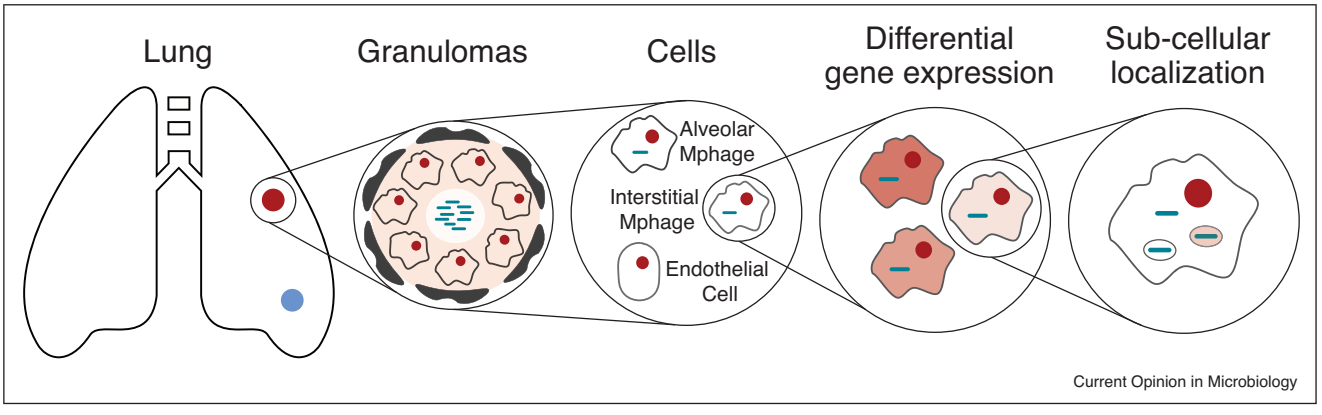


Tuberculosis disease progression.
An infection with *Mtb* starts when droplets containing bacteria enter the host's lungs. *Mtb* has a minimal infective dose of one bacterium; thus, even a single bacterium internalized by a single host cell is enough to initiate an infection. The outcomes of an *Mtb* infection are highly variable, with some individuals sterilizing the infection, others containing the bacteria inside granulomas (latent tuberculosis), and yet others falling sick and developing symptoms (active tuberculosis). Individuals with latent tuberculosis may eventually sterilize the infection, continue to harbor bacteria without developing active disease, or develop symptoms years after having their first contact with *Mtb* (reactivation tuberculosis).

expressed in the center or periphery of certain lesions, respectively (Figure 3) [9–11].

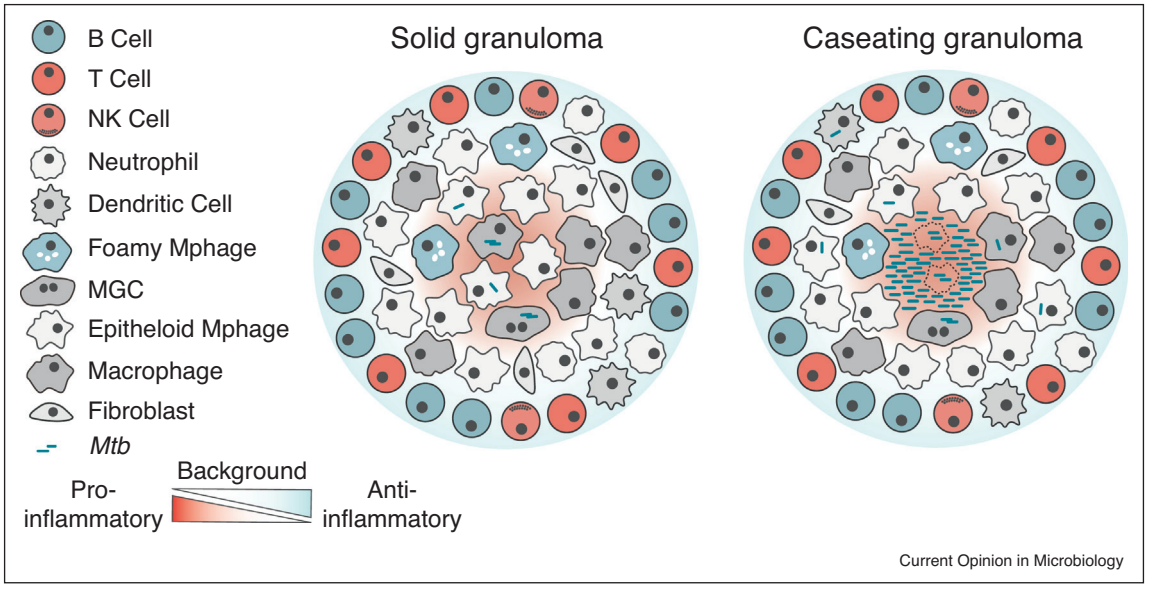
These studies provide valuable information on spatial heterogeneity *in vivo*, but the techniques used lack the ability to study the dynamics of the infection with a high spatiotemporal resolution. To address this caveat, *in vitro* models of granulomas have been developed recently [12,13,14•,15]. These models do not recapitulate complex lung physiology, but can be used to address basic

Figure 2



Mtb is exposed to diverse host environments.
An *Mtb* infection triggers an immune reaction, which results in the formation of primary granulomas that may occasionally sterilize the bacteria (blue). As the disease progresses, bacteria can escape these lesions and enter the vasculature. From there, they can be reseeded in the lungs and contained in secondary granulomas (red). Both primary and secondary granulomas can display a solid or caseating center, with the latter type being more prone to disease progression and bacterial dissemination. Heterogeneity is not only observed between granulomas, but also at the cellular level within a single granuloma. Indeed, *Mtb* can infect many different cell types, some of which (e.g. epithelial cells) are more permissive to its growth than others (e.g. interstitial macrophages). Differential gene expression in a host-cell population can also contribute to the diversity of environments to which *Mtb* is exposed and can influence bacterial dissemination. Finally, even within a single cell, bacteria can be exposed to diverse stressors in different subcellular compartments.

Figure 3



Structures of TB granulomas.
TB granulomas are solid organized aggregates of immune cells recruited to the site of infection to circumscribe bacteria and limit their spreading. The core of granulomas consists of macrophages that can fuse into multinucleated giant cells (MGCs) or differentiate into other specialized cell types, such as foamy or epithelioid macrophages. The macrophage-rich center is surrounded by a lymphocytic cuff composed of B and T cells. Many other cell types populate granulomas, including neutrophils, dendritic cells, natural killer (NK) cells, and fibroblasts. All of these cell types can be potentially infected by *Mtb*; however, bacteria are most commonly observed in the macrophage core. Spatial distribution of pro-inflammatory and anti-inflammatory signatures in different granuloma compartments can define a local balance ensuring bacterial control. However, during disease progression, host cell death events can lead to the formation of a caseating center comprising necrotizing cellular material and extracellular bacteria. Caseum liquefaction can result in granuloma cavitation and disruption, thereby facilitating bacterial dissemination or release into the airways.

questions about formation and maturation of granulomas, and offer great promise for studying heterogeneity.

Heterogeneous host cells

Granulomas are composed of different types of cells, such as macrophages, lymphocytes, and fibroblasts, which could all potentially be infected by *Mtb* [1] (Figure 3). Since all of these cells display different inflammatory signatures and exert different control on intracellular bacteria, random heterogeneity in their distribution and infection could influence the progression of a lesion [5•,16]. Furthermore, recent studies showed that during the course of infection, *Mtb* can also infect other non-immune cells, such as endothelial and epithelial cells, and that these cell types are more permissive to bacterial growth than macrophages [17,18].

Diversity can also be observed within a single class of immune cells. This is for instance the case for macrophages, which are probably the most common cell type infected by *Mtb*. Over the course of the disease, bacteria come in contact with different types of macrophages, including alveolar macrophages at the beginning of the infection and interstitial macrophages at later time points [19]. Interestingly, interstitial macrophages control bacterial growth better than alveolar macrophages and express higher levels of proteins related to hypoxia and inflammation [20••,21].

Finally, differences can exist even within populations of alveolar or interstitial macrophages, further increasing the diversity of environments to which *Mtb* is exposed. At least two distinct subpopulations of alveolar macrophages are simultaneously present in the lungs of mice, and their distribution may affect disease progression [22]. Even amongst interstitial macrophages, different subpopulations displaying either pro-inflammatory or anti-inflammatory characteristics co-exist [23]. It will be interesting to determine whether these subpopulations of macrophages interact with *Mtb* differently, thereby contributing to heterogeneity in disease progression.

Heterogeneous intracellular localization

Single-cell approaches have revealed a fascinating diversity of host-pathogen interactions in different tissues and cell types. However, bacteria can be exposed to heterogeneous environments even within a single host cell. By using single-cell fluorescence and electron microscopy of infected macrophages, *Mtb* was observed within different membrane-bound compartments, including permissive

phagosomes and less permissive phagolysosomes and autophagosomes [16]. *Mtb*-containing phagosomes are dynamic structures able to fuse with other compartments, mature, and acquire different markers [24•]. Moreover, mycobacteria can use the ESX-1 type VII secretion system to damage the membranes of these compartments and escape to the cytosol, which may be a more permissive environment for *Mtb* growth [25,26]. These observations call attention to two interesting points. First, the subcellular localization of *Mtb* can be heterogeneous not only in space but also over time. Second, dynamic subcellular localization of *Mtb* may depend on a fine balance between cellular defence mechanisms and bacterial virulence factors.

Mtb fluorescent reporter strains for stress responses, bacterial growth, or metabolic activity have revealed that subpopulations of bacteria sensing different stresses and adopting heterogeneous growth dynamics can co-exist within the same host cells [27]. Moreover, combination of a fluorescent biosensor for mycothiol redox potential in *Mtb* with markers for host-cell intracellular compartments highlighted that autophagosomes and endosomes are enriched for bacteria with an oxidized or reduced signature, respectively [28]. These studies suggest that heterogeneous intracellular localization of *Mtb* could induce or select phenotypically divergent subpopulations of bacteria, which could be differently controlled by the host.

Mtb subpopulations with an oxidized or reduced signature are differently killed by antibiotics [28], suggesting that bacterial subpopulations within heterogeneous subcellular microenvironments could be relevant in the context of persistence to drugs. Moreover, differential killing of bacteria in selected cellular microenvironments may also depend on different local drug accumulation. For example, correlative electron and ion microscopy showed that bedaquiline preferentially accumulates in host cell lipid droplets and selectively depletes a subpopulation of droplet-associated *Mtb* [29•].

Phenotypic heterogeneity of host cells and bacteria

As discussed, differences between host cells may be programmed and depend on external environments and signals or different cell ontologies [30]. However, even cell populations cultivated under homogeneous conditions *in vitro* can display heterogeneous phenotypes, likely due to differential gene expression [31]. Based on results of single-cell RNAseq, unstimulated human monocyte-derived macrophages can be clustered into distinct subpopulations, confirming the existence of a basal phenotypic heterogeneity in this cell population [31]. Infection with *Mtb* induces different shifts in gene expression within each cluster, suggesting that basal cellular heterogeneity may influence responses to infection [31]. It will be interesting to determine whether random encounters of bacteria with host cells from these different clusters may lead to diverse infection outcomes.

Subpopulations of human monocyte-derived macrophages that are restrictive or permissive for intracellular *Mtb* growth can be discriminated by GM-CSF signalling. Heterogeneous GM-CSF signalling in the cells is not pre-existent and is differently stimulated by growing and non-growing intracellular bacteria upon infection [32••]. Similarly, heterogeneous growth of intracellular *Salmonella* correlates with M1 or M2 polarization in macrophages [33]. From these examples, it is not possible to unambiguously assess whether the observed heterogeneous host-cell response to the infection is the cause or the consequence of fast and slow bacterial growth. However, in other studies, stochastic heterogeneous expression of *Salmonella* virulence factors has been shown to be sufficient to drive different responses in individual infected host cells [34,35]. We are not aware of similar studies on *Mtb*, but results obtained with mutant strains suggest that heterogeneous expression of virulence factors may influence not only the host response to the infection, but also the intracellular localization of the bacteria [36,37].

Discussion

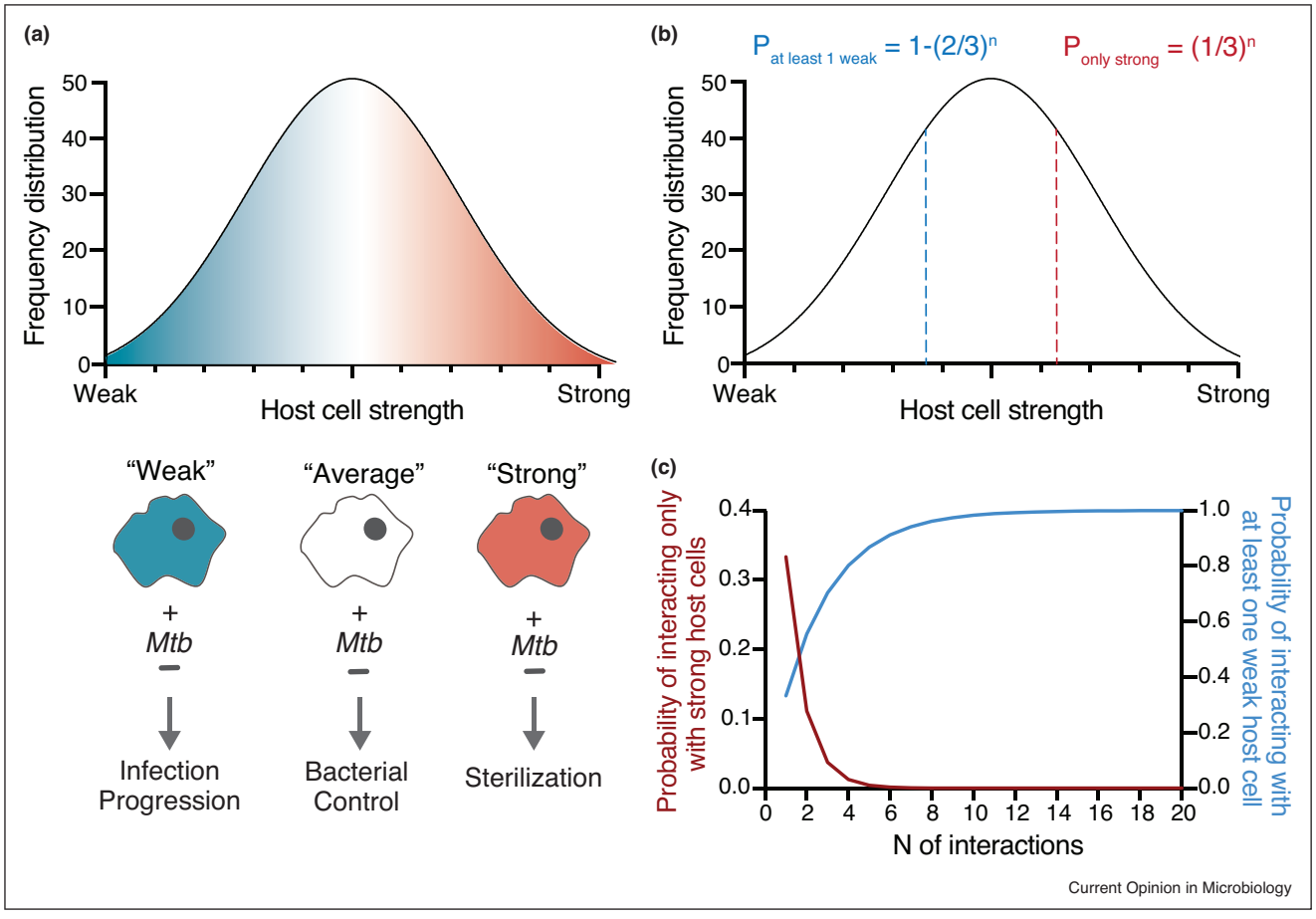
Intracellular pathogens such as *Mtb* are exposed to several layers of heterogeneity in the host, ranging from different intracellular microenvironments to inter-lesion diversity. At the level of single cells, this phenotypic heterogeneity can either predate the interaction with the bacteria or be triggered by it [21,32••]. Similar phenotypic diversity is also observed in bacteria and can be further amplified by exposure to host stresses [38]. These observations highlight that control of bacteria by the host is a dynamic and spatially heterogeneous process that continuously evolves as a balance between host responses to the infection and bacterial virulence mechanisms. According to this view, global progression of an infection is thus the sum of all of the outcomes of the single heterogeneous host-pathogen interactions that co-exist in the same host organism.

After having been obscured by decades of studies based on bulk average readouts, diversity in host-pathogen interactions is now increasingly appreciated thanks to recent advances in single-cell techniques, such as RNA-seq and proteomics of single cells. Dual single-cell RNA-seq can quantify the expression of thousands of host and pathogen genes in parallel in single infected host cells. However, current protocols allow the analysis only of highly expressed bacterial genes due to the low abundance of bacterial RNA within a single infected host cell [39,40]. Moreover, single-cell 'omics techniques provide only a static snapshot of phenotypic heterogeneity in host and pathogen cells at any given time, whereas most of these phenotypes are highly dynamic. Conversely, time-lapse microscopy with fluorescent reporters can reveal dynamic changes in host and pathogen gene expression

over time, but with a limited throughput. Technical improvements and the combination of time-lapse microscopy with live cytoplasmic pico-sampling [41] or endpoint dual single-cell RNAseq would push the boundaries of each technique and provide the temporal resolution needed to understand how heterogeneity originates, evolves, and shapes the outcome of host-pathogen interactions.

Dynamic studies would be useful not only to define the causality of the observed heterogeneity, but also to investigate the role of chance in host-pathogen interactions. Chance is an understudied phenomenon in infection biology; however, some of the studies discussed in this review suggest that individual bacteria could encounter, by chance, a more or less permissive host-cell environment, resulting in different infection outcomes (Figure 4a). For example, a bacterium encountering an aggressive macrophage or a more permissive epithelial cell may be killed or may replicate, respectively [18]. At late stages of infection, when large numbers of bacteria and host cells interact, the net outcome presumably corresponds to the average of all of the individual interactions. In this case, heterogeneity and chance may have a less decisive role in defining the outcome of the infection (Figure 4b and c). However, whenever small numbers of bacteria interact with small numbers of host cells, heterogeneity in the host could play a major role in defining

Figure 4



Encounters with heterogeneous host cells can determine infection outcome.
(a) Single bacteria can interact with more-permissive or less-permissive host cells, which may result in different fates for the intracellular bacteria (growth, death, or non-replicating persistence). We hypothesize that phenotypic heterogeneity in host cell gene expression can define a spectrum of 'strength' ranging from 'weak' (host cells unable to control bacterial replication) to 'strong' (host cells kill the intracellular bacteria). **(b)** If we assume that host-cell 'strength' has a Gaussian distribution in the host cell population, the probability to meet only 'strong' host cells (defined here as the strongest one-third of the population) is $P = (1/3)^x$ with x equal to the number of interactions between a single bacterium and a host cell. Conversely, the probability to meet at least one 'weak' host cell (the weakest one-third) is $P = 1 - (2/3)^x$. **(c)** The probability to meet only 'strong' host cells decreases quickly as the number of interactions increases. This observation suggests that when the number of interactions is very low, heterogeneity in host cells is likely to define the outcome of the infection. As the number of interactions increases, this scenario is less likely to happen and the alterative scenario, where at least one bacterium interacts with a 'weak' host cell, become highly probable, possibly leading to progression of the infection.

the outcome of the interaction because, due to the 'finite-number effect', extreme events are not averaged out (Figure 4b and c). This condition occurs during bottleneck events, such as transmission of an infectious agent from one host to another. We speculate that when numbers are sufficiently low, even small differences, such as basal phenotypic heterogeneity in gene expression or diverse intracellular localization of bacteria in host cells, could be significant in determining the course of disease progression. The potential role of programmed or stochastic phenotypic heterogeneity in the outcome of host-pathogen interactions has received relatively little attention so far. However, especially in the context of infections initiated by very small numbers of bacteria, such as tuberculosis, chance encounters between individual bacteria and host cells with a more-permissive or less-permissive phenotype could potentially play a decisive role in determining whether the bacteria will grow, persist in a non-replicating state, or be eliminated by the host.

Conflict of interest statement

Nothing declared.

CRediT authorship contribution statement

Chiara Toniolo: Conceptualization, Writing - original draft, Writing - review & editing, Visualization. **Ophélie Rutschmann:** Conceptualization, Writing - original draft, Writing - review & editing, Visualization. **John D McKinney:** Conceptualization, Writing - review & editing, Supervision, Funding acquisition.

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