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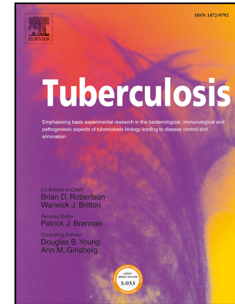

Defining Dormancy in mycobacterial disease

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Defining Dormancy in mycobacterial disease

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Abstract

Tuberculosis remains a threat to global health and recent attempts to shorten therapy have not succeeded mainly due to cases of clinical relapse. This has focussed attention on the importance of "dormancy" in tuberculosis. There are a number of different definitions of the term and a similar multiplicity of different *in vitro* and *in vivo* models. The danger with this is the implicit assumption of equivalence between the terms and models, which will make even more difficult to unravel this complex conundrum. In this review we summarise the main models and definitions and their impact on susceptibility of *Mycobacterium tuberculosis*. We also suggest a potential nomenclature for debate. Dormancy researchers agree that factors underpinning this phenomenon are complex and nuanced. If we are to make progress we must agree the terms to be used and be consistent in using them.

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Keywords;

Mycobacterium tuberculosis, dormancy, latency, persistence, mycobacteria.

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Introduction

Improvement in tuberculosis treatment is seen widely as an important goal of research to reduce preventable deaths. One of the major barriers to progress is the prolonged duration of treatment, which has put an emphasis on the unusual nature of tuberculosis where the organism forms a life-long relationship with the host persisting in a low metabolic state in the tissues. Recent phase III clinical trials of shortened regimens have failed to show non-inferiority mainly due to higher relapse rates in the experimental arms despite more rapid clearance of bacteria[1-3]. This implies that hidden populations of bacteria may be important in defining outcome as has been implied by recent publications [4,5]. Unravelling the biology of the relationship between dormancy and response to antibiotics are critical for the development of better treatments for tuberculosis.

The diversity of terms used to describe the physiology of *M .tuberculosis* and other mycobacterial cells in tissues creates a communication challenge. This has the effect that bacteriologists, immunologists, clinical trialists, clinicians and diagnosticians, are surrounded by a cloud of uncertain meaning. That the same words have different meanings to scientists blocks progress in this already difficult area. To address this issue we have reviewed and summarised the literature surrounding the term dormancy and its companion terms persistence and latency, whilst identifying some of the key biological questions that need to be addressed. We believe that it is only by creating a common language to underpin the scientific conversation that efficient progress in scientific knowledge is possible and will allow a future consensus to emerge and this is what we attempt to do in this manuscript.

Dormant

It is important to distinguish the terms dormant and dormancy from others that are often erroneously used as synonyms: latent/latency and persistent and persistence (see below). A state of dormancy has been hypothesised for *M.tuberculosis* cells in human infection and it is a feature that has been recognised in a wide range of bacteria since 1944 [6]. At its simplest, the term dormant might be defined as “having normal physical functions suspended or slowed down for a period of time; in or as if in a deep sleep”, but how can this anthropomorphic concept be applied to bacteria in humans? The working

definition of mycobacterial dormancy is complicated by the fact that it is usually described negatively: inability to grow on solid medium, the presence of low metabolic activity, alteration of gene regulation with the accumulation of tri-acylglycerides in intracellular lipid bodies, loss of acid fastness, tolerance to antibiotics, and the imputation that growth rate is slow. To explore this state a number of dormancy models have been developed and these are described in more detail in Table 1 and in the text below. The critical, implicit and as yet untested assumption is that they all result in a similar phenotype and that these phenotypes are predictive of a state found in the human host. Although there have been many descriptions of the histopathological changes found in human and animal models of disease none of these unequivocally identify mycobacterial cells in a dormant state[7,8].

Experimental data are starting to reveal the physiological state of organisms that might be candidates to be the elusive dormant cell, for example some organisms in human and animal specimens cannot be recovered on solid medium and can only be detected by growth in liquid culture broth or by the addition of resuscitation promotion factor (Rpf)[9-11]. Studies have shown that, after a course of chemotherapy, liquid culture and the tissues of mice contain un-culturable cells that transcribe messenger RNA[12]. Using a fluorescent marked reporter strain the lungs of chronically infected mice have been shown to harbor a subpopulation of non-growing but metabolically active bacteria. Such bacterial cells are notable in mice treated with the isoniazid, suggesting that they may have a role in post-chemotherapeutic relapses.[13] Reports also indicate that, as mycobacteria become increasingly difficult to resuscitate, they have less capacity to cause infection in mice[14].

The physiological conditions within a patient with tuberculosis are starting to be understood and research has been directed towards extending this knowledge and finding models that recreate these conditions. The caseous granuloma is thought to be an anaerobic environment providing circumstantial support for the importance of some *in vitro* models described in more detail below. A “dormancy” phenotype can be created in the laboratory by applying different forms of stress: oxygen or nutrient deficiency, hyper-acidity, antibiotics and multiple combinations of these. The published models include bacterial starvation[15], successive shift-down or anaerobiasis (the Wayne model)[16], external acidification[17], antibiotic survival[12,18], multiple stress [19-21]

and these are reviewed in turn. For the moment we will call all of these "dormancy" models, recognising that, at present, we only have an atlas of phenomenology and there is a need to identify the characteristics of each model comprehensively.

Lipid body positive

Lipid bodies were first described in a range of bacteria among which was *M.tuberculosis* and other mycobacteria including *M. leprae* where it was classified as "fatty material usually present but sometimes absent in common media[22,23]. These enigmatic bodies were little studied until the work of Garton and colleagues who showed that lipid bodies could be formed rapidly from uptake of fatty acids or from simple carbon sources in low-nitrogen conditions[24]. The lipid bodies contain poly-β-hydroxyalkanoates, tri-acylglycerides and wax esters[24]. Such lipid bodies are found more commonly as bacteria progress to stationary phase as illustrated in Figure 1. Lipid bodies are found more commonly in late stationary phase cultures of mycobacteria, which are known to be more resistant to drugs and the concentration of drug required to clear all bacteria (the MBC- minimum bactericidal concentration) rises significantly[25]. Older cells express lipid bodies [24,26,27] and it was assumed they are responsible for the phenotypic resistance found.

Lipid body positive bacteria have been found to predominate in human sputum and this was associated with transcriptional signatures similar to those found in non-replicating mycobacteria[9] The authors found that the lipid body count was highly correlated with time to positivity in diagnostic liquid cultures, which they concluded established a direct link between this cytological feature and the size of a potential non-replicating population. More recent work indicates that a range of growth-limiting stresses trigger a common signal transduction pathway slowing growth by redirecting carbon flux towards storage[7]. These data suggest that dormant bacteria survive chemotherapy both in vitro and in mice, and are metabolically active, although at a low level. Other studies show that there is a difference between number of organisms cultivated on solid medium and detected by use of MGIT [28]. In a study of sequential sputum samples

from patients receiving standard chemotherapy between day 21 and 28 the change in the odds ratio of an unfavorable outcome for each percentage rise in percentage lipid body positive and the acid fast bacilli count was 1.21 (95% CI, .97–1.50; P = .088). Thus it appears that patients with a greater percentage of lipid body positive mycobacterial cells detected in their sputum smear at later time points are more likely to have an unfavourable outcome[29]. The importance of lipid body positive bacteria has been emphasised by an in vitro study that demonstrates lipid body positive cells can be purified from all mycobacterial cultures tested and are phenotypically resistant to antibiotics irrespective of whether they are derived from "old" or "young" cultures indicating that the presence of a lipid body is associated with phenotypic antibiotic resistance[30].

Hypoxia

To create an *in vitro* hypoxic model Wayne and colleagues uses a long term culture without agitation and oxygen excluded[16]. They demonstrated a fourfold increase in the isoctrate lyase activity and ten-fold increase in glycine dehydogenase. In a later variant of the model where anaerobiasis was achieved sequentially, relative resistance to isoniazid rifampicin and ciprofloxacin was demonstrated[16] accompanied by a loss of glycine dehydrogenase activity. The Wayne model also includes an element of starvation (see below) through nutrient depletion[16]. The chaperonin alpha crystallin was found to be up-regulated in this model [31]and knockout of its gene led to an increased rate of growth in mice, suggesting that its function may be to slow growth in dormancy[32]. Many genes, including alpha crystallin alter their expression in response to hypoxia and are regulated by the DosR regulon. This two component regulator alters the expression of a series of genes in response to hypoxia and nitric oxide[33] and redox stress[34]. A total of 50 genes are up-regulated including tri-acylglyceride synthase[19,21]. Comparing cells in a Wayne model with SWATH mass spectrometry it was shown that the DosR regulon induced proteins make up 20% of hypoxic cells compared with 1% in exponentially growing cells. In comparison there was little change in ribosomal proteins. Many components of the electron transport chain and the energy generating machinery changed in abundance. Differential regulation of enzymes

in the sub-networks of acetyl-CoA alanine/ glutamate, and trehalose metabolism were found. Competing incorporation of acetyl-CoA either into TCA cycle or triacylglycerol biosynthesis was found consistently. Lipid pathways were significantly upregulated when absolute protein concentration and Vmax enzyme data were compared[35].

DosR is not an essential gene for survival in *M.tuberculosis* as knock out strains have only a modest decrease in viability in a hypoxic environment[36,37]. In a macaque model of infection, despite being able to replicate initially, animals infected with DosR regulon mutants failed to develop persistent infection or develop disease. This was associated with the appearance of a Th1 response and organization of hypoxic lesions where *M.tuberculosis* expressed *dosR*[38]. Interestingly, some Beijing lineage strains have been shown to express DosR constitutively as well as accumulating large quantities of lipid in lipid bodies[19]. A recent study where the DosR regulon was induced to wild type levels did not change *M.tuberculosis* replication kinetics and the authors concluded that DosR expression alone is insufficient to explain latency[39]. A recent hypothesis published by Orme suggests that the triggering of the DosR is an adaptation to these inimical conditions created by necrosis of tissue by host generated radicals. The necrotic centre created is hypoxic, but the dead neutrophils and other cells mean that the bacteria are not starved. His argument is that the DosR response readies the organism for transmission: survival as an extracellular state[40].

The importance of a hypoxia is supported by the accumulating evidence that anaerobic conditions are found in granulomas. A hypoxia marker EF5 demonstrated granulomas with necrotic centres in human tissue, which contrasts with mouse granulomatous lesions where hypoxia does not appear to occur[41]. Significantly it has been shown that metronidazole has no effect on the outcome from the Cornell model of tuberculosis treatment (see below)[42] whereas this drug has been shown to be bactericidal with dormant mycobacteria[43]. The probe pimonidazole (PIMO), which binds to thiol-containing peptides only at low partial pressures of oxygen, colocalised with areas of caseous necrosis in guinea pigs, rabbits, and cynomologous macaques [44], but not with cellular granulomas in the standard mouse model[46]. However, metronidazole lacked activity in a murine hypoxic granuloma model and in guinea pigs [47,48].

In human lung tissue endothelial cells have been detected with an anti-CD31 immunohistochemistry probe in granulomas with necrotic centres; thought to represent mature lesions. These cells are not found in lesions without necrotic centres. This observation may support the idea that hypoxia is associated with granuloma maturity [41]. The importance of the nature of the animal model is confirmed by a study study that compared the virulence of a strain of *M. tuberculosis* with a *dosR-dosS* deletion mutant (*dosR-dosS* [*dosR-S*]), comparing it to complemented strain and wild-type H37Rv in rabbits, guinea pigs, and mice infected by the aerosol route as well as using a mouse hollow-fiber system. In the mouse and the guinea pig models, the *dosR-S* mutant exhibited a growth defect but did not replicate more than the wild type[49]. In another guinea pig model, bedaquiline failed to eradicate bacteria in residual primary lesion necrosis with incomplete dystrophic calcification that is hypoxic and morphologically similar to that described in human lung lesions. This suggests that this acellular rim may be a primary hypoxic location populated by bacilli phenotypically resistant to this drug[50].

These results contrast with those obtained when mycobacteria are exposed to a higher than atmospheric oxygen concentrations where there is increased multiplication by H37Ra and BCG strains[51].

Starvation

Loebel first demonstrated that starved cells had minimal evidence of respiration[9,15,52]. Gegenbacher also used the Loebel model and showed that depletion of oxygen killed starved bacilli but death was prevented by nitrate indicating that survival and respiration was possible in the presence of an alternative exogenous electron acceptor. Nutrient-starved bacilli lacking isocitrate lyase failed to reduce their intracellular ATP level and died, thus establishing a link between ATP control and intermediary metabolism. These data demonstrate a critical role for the glyoxylate shunt in survival in a nutrient limited environment[53].

Betts et al developed this model further and demonstrated phenotypic resistance to rifampicin and isoniazid in starved cells that contrasted with full activity against exponentially growing cells. Importantly, metronidazole had no effect on starved bacteria[54] as would be expected as its mode of action depends on reducing conditions adding to the importance of recognising that not all dormant phenotypes are identical.

Using this combined model they demonstrated differential up-regulation of proteins by micro-array[54,55]. They found evidence for slow- down of the transcription apparatus, energy metabolism, lipid biosynthesis and cell division in addition to induction of the stringent response and several other genes that may play a role in maintaining long-term survival within the host. Energy metabolism genes (15%), 16% of lipid metabolism genes 59% of the translation apparatus were down-regulated. Of the 12 *M.tuberculosis* sigma factors (*sigB*, *sigE, sigF* and *sigD)* four were up-regulated, but *sigA* expression did not change significantly. The majority of differentially expressed cell process genes belonged to the transport/binding protein subclass[54].

Another fermenter nutrient starvation model with controlled oxygen concentration, monitored nutrient depletion found up-regulation of genes in the glyoxylate shunt including isocitrate lyase, along with components of the TCA cycle. *Sig B sig E* and *sigH* were up-regulated and ATP synthase genes down-regulated. In contrast to the results obtained by Betts et al., *rpoA, B* and *rpoC* was up-regulated. All genes associated with the *mas* gene cluster were up-regulated throughout the period of stationary phase adaptation (*mas*, *fad*D26, *fad*D28, *mmp*L7, and *pps*A-E) and at day 75 there was a sharp rise in expression of *fad*D26. The enzyme Relmtb, which is required for survival of mycobacteria under long term in vitro starvation conditions was up-regulated by day 18 and then declined slowly[56].

Xie varied the PBS starvation model and added 16 different agents to cultures suspended only in PBS and incubated these for 6 weeks. Of these 16, only four (chlorpromazine, isoniazid, rifampin, trifluoperizine) where able to kill 99% of bacteria at concentrations less than 160mg/L[57]. There was one log difference in rifampicin susceptibility between log phase and starved cells and this increased to 2 orders for isoniazid.

Nitrogen limitation

Anuchin and colleagues developed a nitrogen limitation model in which *M.tuberculosis* cells adopted an ovoid morphology that were more resistant to heat, hygromycin and doxycycline stresses compared to a 48 hour culture[58]. In further studies, the authors found intra-species morphological heterogeneity of dormancy. They defined cyst-like, coccoid and poorly differentiated cells that all showed dormant-like properties and yet

varied in their response to exogenous stress. Cyst-like cells were shown to contain electron transparent lipid bodies and were most resistant to heat stress and displayed the greatest difference (two orders of magnitude) between viable count measured by colony forming units (cfu) and most probably number methods (MPN).

Phosphate limitation

It has been hypothesised that *M. tuberculosis* encounter a phosphate limited environment in the macrophage and that its response mediated through pkk1 and RelA are important for establishing tolerance to antibiotics[59,60].

Acidification

M.tuberculosis is sensitive to low pH and survives in the phagolysosome by arresting acidification[61]. Pyrazinamide, a drug only active at low pH, can reduce treatment duration of tuberculosis chemotherapy from nine to six months, and it was postulated that this was due to its effect on cells in an acid environment [62]. When the pH was reduced, ovoid cells with thickened cell wall, a low metabolic activity and elevated resistance to antibiotics and heating developed and failed to form colonies on solid medium[17]. This phenomenon has been described in *M.tuberculosis* from sputum samples.[9]. Acidification was also used as part of a multiple stress model in *M.tuberculosis* that included low oxygen (5%), high CO_2 (10%), low nutrient (10% Dubos medium) [20,63]. The cells stopped replicating, lost their acid-fastness, and had reduced susceptibility to isoniazid and rifampicin whilst accumulating tri-acylglycerols and wax esters (WE) and up-regulating putative neutral lipid biosynthetic genes[18,20,64].f

Multiple Stress

Since *M.tuberculosis* faces a range of stresses it is logical that a more pathophysiological model would be one with multiple stresses. Most models apply a single stress even though most impart additional unmeasured stresses on the culture (eg. accumulation of metabolites, gradual acidification, nutrient depletion). Deb and colleagues created a 'multiple stress' model: low-nutrient (10% Dubos medium), acidic (pH 5.0), high CO_2

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(10%) and low O_2 (5%)[20]. In these conditions the cells accumulated non-polar lipids as measured by TLC and [^{14}C]oelic acid incorporation, were resistant to isoniazid and rifampicin, lost acid fastness, increased Nile Red flouresence and decreased in buoyant density. Genes involved in the glyoxylate cycle (*icl, gltA1*), carbon storage (*tgs*), stress response and hypoxia (*hspX, Rv2031c, dosR*) and anaerobic respiration (*frdA, narG/H/X, nirA*) were up-regulated. Genes coding for expression of ATP and NAD energy regeneration systems and the transcription/translation apparatus were down-regulated. The authors believe that this provides a model where cells display all of the proposed hallmarks of dormancy.

Stringent response

The stringent response, which utilizes hyperphosphorylated guanine [(p)ppGpp] as a signalling molecule, controls gene expression during starvation and is under the control of the RelA and SpoT proteins in other species and by Rel(Mtb) in *M. tuberculosis*. This response has been shown to be critical to establish persistent infection in mouse and guinea pig models of tuberculosis and is associated with shut down of key metabolic pathways[65,66]. There is a link to differential antibiotic response as polyphosphate accumulation has been associated with tolerance to isoniazid in *M. tuberculosis* [67].

Resuscitation dependency

Bacterial cells with phenotypic similarities with dormant bacilli as measured by the presence of lipid bodies have been detected in human sputum specimens[9,27] and can be the dominant cell type making up to 99.9% of cells[12]. These cells may require the addition of a bacterial cytokine, resuscitation promotion factor (Rpf) to be detected by culture[9,68]. A study of smear-positive sputum samples from the UK and The Gambia revealed that up to 86% of those staining positively with Zhiel-Neelsen were also positive with Nile Red, a stain that preferentially binds lipid and showed these organisms had lipid bodies. The transcriptome of these cells is similar to slowly replicating or non-replicating mycobacteria. Since the lipid body count was correlated with time to positivity in diagnostic liquid cultures the authors concluded that the presence of lipid bodies was linked to the size of the non-replicating population[27]. It

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was notable that the characteristic of Rpf dependence was retained in long-term storage at 4°C. There was a more rapid decline in the cfu count of non-Rpf dependent cells compared to the Rpf dependent count indicating that these latter cells are relatively resistant to rifampicin. This resuscitation index continued to increase among a small number of subjects who were monitored over an extended period. Direct treatment of sputum samples with rifampicin had less effect on Rpf cells supporting the idea that these cells were tolerant of this antibiotic at the low dose applied (1mg/L)[9].

Antibiotic survival or persistence

The term "persistence" is often used to describe bacteria surviving after a period of treatment. The first description of rifampicin tolerant cultures in which no bacteria could be cultured but messenger RNA of specific *M.tuberculosis* genes could be detected and radioactive uracil was incorporated, was performed by Hu and colleagues[11]. These data suggest that dormant unculturable bacterial cells are metabolically active[69]. Further work led to the development of three separate models of antibiotic stress, namely 100 day microaerophilic cultures, pretreatment with rifampicin and the pulsed introduction of air[69-71]. These 100 day cultures contain 10-100 fold more unculturable cells which can be resuscitated with Rpf than culturable bacteria [11].

Clinical relapse was noted in the relapse of patients infected with resistant organisms in the first streptomycin trial[72] and it was McDermott who first suggested that the inability of drugs to kill all of the organisms was responsible for this phenomenon. It was recognised early that the organisms were not eliminated by treatment in the mouse model but could not be identified by any method that required their multiplication although small numbers of acid fast bacilli could be seen[73]. Following cortisol therapy or spontaneously the infection relapsed and mycobacteria became detectable again. It was in a subsequent paper that this group showed the importance of pyrazinamide in killing these "persisting" cells. A characteristic that the authors described as "sterilisation"[74]. These experiments were fundamental in developing what became known as the Cornell Model[75]. Further work with this model demonstrates that after the completion of chemotherapy, the tissues of the mice became "sterile", no bacteria could be cultured from the tissues even in liquid cultures, yet

messenger RNA of specific *M tuberculosis* genes could be detected, suggesting that the bacteria, although dormant, were metabolically active[12]. The"sterile" phase of the Cornell model contains a high proportion of Rpf-dependent *M.tuberculosis* cells which can be killed by high but not low dose rifampicin[11]. A second "persistence" model is described where a relatively resistant mouse strain C57/BL6 is inoculated by the aerosol or parenteral route. The initial infection is controlled but the organism persists without causing overt disease[63].

Keren and colleagues have selected for antibiotic persister cells by treating an exponentially growing culture with cycloserine and performing an Afymetrix array. They found that 1408 genes were down regulated and 282 up-regulated with growth and energy related genes extensively down regulated. Of 65 Transcriptional activators listed in the *M.tuberculosis* genome 10 were over-expressed in persisters. One of these, Rv2866, is a *relE* homologue and was recently shown to increase drug tolerance when over-expressed in an *E. coli* system. Key genes up-regulated included *sigF, dnaE2, acr2, lat* and *sig G*. They created a four-dimensional Venn diagram summarizing the differences and commonalities of four models: persisters, starvation, enduring hypoxic response (her), and non-replicating persistence. There are five genes that are common to all models and 55 genes that are common to persisters and at least 2 other models. Of special interest are the five genes that are overexpressed in all models. These genes are Rv0251c (acr2), encoding an alpha-crystallin, Rv1152, a transcriptional regulator of the gntR family, Rv2497c (pdhA), encoding a probable pyruvate dehydrogenase component, Rv2517c, encoding a hypothetical protein, and Rv3290c (lat), encoding an L-lysine-epsilon-aminotransferase[18,64].

Biofilm dormancy models

Ackart and colleagues showed that *M tuberculosis* failed to form biofilms when cultured planktonically in the presence of Tween-80 and did not reach logarithmic phase growth and remained highly susceptible to antimicrobial drugs. In contrast when Tween is absent bacilli formed complex microbial communities attached to untreated well surfaces or to the extracellular matrix derived from lysed human leukocytes and tolerated drug treatment. This could be reversed by treatment of microbial communities with DNase I or Tween[76].

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Implications of dormancy

Susceptibility

The importance of dormancy in *M.tuberculosis* is the repeated observation of phenotypic resistance to anti-tuberculosis agents although the degree is not fully delineated due to the different models of dormancy employed, the incomplete spectrum of agents tested and the diversity of susceptibility methods employed. Cell wall inhibitors are most affected but the action of the RNA polymerase inhibitor rifampicin and fluoroquinolones acting on DNA gyrase is also reduced[16]. This is not to say that testing susceptibility of "dormant" cells is easy since, *a priori,* they may not grow well on standard solid media making methodology problematic. In Table 2 we summarise the effect of different models on the susceptibility to antibiotics with the methods that were used.

Through *in vitro* dormancy models a range of susceptibility test are used (MIC, MBC and percentage of cell surviving after different exposure time using CFU or resazurin reduction). This results in different drug dose for the same species through different models. For example if we look at rifampicin in one species *M.tuberculosis*, in the Wayne model 0.1 μg/mL correspond to 4.2 % of bacilli surviving after 192 h of drug exposure and for the in vitro granuloma model a MIC was found between 0.47 and 0.94 μg/mL, illustrating the difficulties in comparing susceptibility results from different models even if they are generating "dormancy" cells types. To some extent these observations have been clarified by the report that indicates that it is the presence of a lipid body irrespective of cell age that is associated with a significant increase in the minimal bactericidal concentration of any mycobacterial species suggesting that this is a phenomenon common to the genus[30].

Relationship to disease/pathology/treatment

A number of narratives have been created to link the concept of dormancy with clinical disease and chemotherapy.

Special populations hypothesis

The first "model" that attempted to this was the special populations hypothesis[62] based on clinical trial results with *in vitro* and mouse experiments. It has four cell "types": continuously growing cells, which isoniazid and rifampicin are said to act on, those in acid conditions, the target of pyrazinamide, intermittently metabolising cells against which rifampicin had its major activity and non-growing cells that are resistant to antibiotics[62]. This hypothesis has been influential in the design of clinical trials but there is limited direct bacterial or pathological evidence to support it .

Yin-yang hypothesis

Zhang and colleagues describe mixed growing and non-growing subpopulations that exist in a continuum of metabolic states. Among the growing sub-population (yang), are a small non- or slow -growing population (yin). The evidence comes from batch culture growth curves and the authors describe that as bacteria enter stationary phase the yin increase and the yang decrease. This model, they postulate, explains why, after two months of treatment the persister cells revert to activity and can be killed by isoniazid and rifampicin and why isoniazid is effective in the treatment of latent disease[77]. This idea is supported by the observation that both lipid rich and lipid poor cells are found in cultures of all ages.

The Scout hypothesis

Shleeva and colleagues created a three stage model that described the change in status from active growth, through a transition stage to "deep dormancy"[78] n which the cells were viable but not culturable. The authors postulate that cells can reverse the journey and become active again and contrast the results from the Wayne model where cells retain the ability to be cultured with their own model where cells do not grow on solid medium without the addition of Rpf or culture supernatant[16]. This apparent discrepancy is resolved by the description of a "Wayne zone" that arises because of the shorter time to achieve the state. They also describe a "Cornell zone" in which cells enter a non-culturable state that precedes cell death. These cells require external intervention before they can transition to the Wayne zone where resuscitation can occur if the correct conditions are encountered. Epstein built on this idea to postulate a dormant

state from which microbial cells awake at random. The stochastic nature of this event could be precipitated by a change in a master regulatory gene. These newly activated cells become scouts that, if they survive in the environment go on to form new colonies and through quorum sensing link to the larger bacterial population[79]. Disease recurrence would occur when scouts encounter a favourable environment before the bacterial population is eradicated by the immune system or chemotherapy. Although this appears to be an anthropomorphic explanation of events, it remains a stochastic process because "scouts" are continually produced and reactivation only occurs when one survives to multiply and cause relapse. How dormancy might be linked with clinical models of latency is illustrated in the Figure 2.

Disambiguation of dormancy

Latency

This term is an important concept in tuberculosis and the majority of those with primary infection are able to control the infection and may not show any clinical illness and some authors have suggested that latency and active disease are the extremes of a spectrum[80]. Epidemiological studies suggest that there is a 5-10% chance that the controlled, or latent infection will become patent to produce post primary disease. This is thought to be the most frequent route to pulmonary tuberculosis[81]. Our ability to study and manage the latent form of disease has been transformed by enhanced diagnostic methods depending on tuberculosis specific T cell response assays or Interferon gamma response assays (IGRAs) that are superior to tuberculosis skin tests[82].

The importance of latent infection in the natural history of tuberculosis has often led to a potentially false link with bacteriological considerations of dormancy. The assumption is that the mycobacterial cells in latent infection are dormant. Yet, there is little experimental evidence to support this hypothesis. Patients with latent infection can be treated successfully with isoniazid, a drug that inhibits cell wall synthesis. The observations of Rpf dependent cells that show reduced metabolic activity go some way to creating a bridge between the concepts of dormancy and latency[9,27]. PCR has detected *M.tuberculosis* in sections of adipose tissue samples from 39 subjects from Mexico and France who had died from causes other than TB. *M.tuberculosis* DNA could

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be detected fat tissue surrounding the kidneys, the stomach, the lymph nodes, the heart and the skin in 9/57 Mexican samples (6/19 individuals), and in 8/26 French samples (6/20 individuals)[83]. Other post mortem studies reported similar results[84]. A subsequent study identified viable bacilli in extrapulmonary sites, as evidenced by the detection of mycobacterial mRNA.[85]. Further work is required using RNA based methods to confirm that activity level of bacterial cells in tissue. To avoid misunderstanding the authors believe the term latency should only be used in relation to a clinical disease state and dormancy to the bacterial cell state.

A new nomenclature

Using the data set out in this review we propose that the term dormancy should only apply to bacterial cells and specifically those that are capable of regrowth: it should be a reversible phenomenon. Bacteria in a viable non-culturable state, for example, would have to be capable of resuscitation to qualify as "dormant" and to distinguish them from "dead".

Given the multiplicity and complexity of models described we propose here that a dormancy model should be sub-defined by additional terms. Thus, the Loebel model would be described as dormancy-starvation. Examples of how this approach could be notated are found in Text box 2 and applying this could do much to create clarity in this important area of mycobacterial research

Future challenges and research

In this review we have described the range of models of "dormancy", yet, there are only a few reports of such cells from patient samples[9,27]. Further study of patient material is needed to understand the role of different dormancy phenotypes in human disease. This would test the relationship between in vitro and animal models and clinical disease. Descriptions of the cell state of *M.tuberculosis* in the tissues of patients with disease are handicapped by the absence of comprehensive specimen collections and methodologies to differentiate cells states reliably *in situ*. If we are to understand the effect of these dormancy phenotypes on chemotherapy it is necessary to chart the number, occasion and cell state of *M. tuberculosis* in patients with disease, and to understand the rate that

"dormant" cells transition to "active" states. The challenge for the future is to integrate these disparate models and data into a cohesive theory of pathology and its impact on the natural history of tuberculosis and the therapy of the disease. Dormancy researchers agree that the factors underpinning the phenomenon of dormancy are important, complex and nuanced. If we are to make progress we need to agree and use a consistent nomenclature. To support this approach we have proposed a candiate terminology for dormant TB, which should be updated regularly as new data become available. If we do not, progress will continue to stumble through mists of uncertain meaning.

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Table1. Summary of mycobacterial dormancy models with a description of methods of selecting cellular sub-types, and the phenotypic and metabolic characteristics

Principle of model and organism	**Morphology of cells**	**Buoyant density**	**Metabolism**	**Microscopic observations**	**Viability challenges**	**Lipids in resultant cells**
Gradual acidification, *M. smegmatis* [17,86]	Three types of cells formed: ovoid, transitional, rod shaped – 60% ovoid after 30 days	Ovoid cells higher buoyant density 1.8M zone in 1.4-2.2 M sucrose gradient	Post-stationary cultures exhibit low but non zero uptake of radioactive DNA/RNA precursors no detectable respiration	Thickened, multi-layered cell wall, electron dense cytoplasm	Ovoid forms more resistant to antibiotics and heat	Not reported
Growth in modified potassium free medium, *M. smegmatis* [87]	Spherical cells, condensation of cytoplasm		Decreased total metabolic activity and total respiratory activity	Increased number vacuoles, decreased ribosomes. Cells with “rarefied” cytoplasm non-viable		Total amount of lipid 2-fold lower than metabolically active cells.

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Multiple-stress model on *M.tuberculosis*[16,20]		Buoyant density decreased with increasing time under multiple stress	-Enzymes of glyoxylate cycle are up regulated. Shutdown of ATP and NAD energy regeneration systems. -Overall slowdown of transcription/ translation apparatus	Loss of acid fatness – 30% after 30 days of multiple stress	Resistance to RIF and INH – at 18 days c. 12% survive 5µg/mL RIF and c. 35% survive 0.8%µg/mL INH	Increased lipid bodies (70% lipid bodies positive at 18 days). Accumulation of storage lipids (WE and TG) in anaerobic but not aerobic samples
Nutrient starvation *M.tuberculosis* [54]			Significantly decreased respiration rate		No loss of viability after 6 weeks. Starved cultures resistant to RIF and INH at 1 $\mu g mL^{-1}$ Sensitive to metronidazole	Down-regulation of genes involved in lipid biosynthesis (micro-array)

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Low oxygen tension *M. bovis* and *M.tuberculosis* [88]	Cell diameter of anaerobically cultured cells significantly thinner than aerobic: *M. bovis* 255 vs 284 nm. *M.tb* 278 vs 338 nm			*M.tuberculosis*, *M. bovis*- Markedly thickened (21.2 vs 16.1 nm) cell wall with rupturing and pealing after 6 months in anaerobic or micro-aerobic but not aerobic conditions. -No thickening in *M. smegmatis* – less than 1 % of cells intact in 35 culture days.		Not reported

Long term anaerobic culture, *M.tuberculosis* [88]	No difference in cell length between anaerobic and aerobic cultures (1.14 vs 1.06 µm)			Thickened cell wall, dependent on anaerobiosis and not cell density. APP1 pigment produced, only significantly in anaerobic cultures		No qualitative difference in cell wall lipids
Plate based anaerobiosis *M. smegmatis* [89]					Sensitivity to MTZ in anaerobically grown cultures	Not reported
Oxygen depletion *M. smegmatis*[90]	Percentage of bacilli survival *M. smegmatis* CFU counting after 24h exposure to the drug		Low level DNA and RNA synthesis		Anaerobic culture sensitive to MTZ but not OF	

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Oxygen shift down *M.tuberculosis* [16]			DNA synthesis terminates with cell division but some low level RNA synthesis continues. Slight fall in ATP as cells enter stationary phase		Non-replicating persistent cells resistant to INH and CIP but not to MTZ	Not reported
Wayne like oxygen depletion *M.tuberculosis* [91]			Specific glycine dehydrogenase activity increases sharply in NRP1 but declines in NRP2. Isocitrate lyase down-regulated in anaerobic cultures		Anaerobic cultures develop dose dependent Metronidazole susceptibility	Nor reported

Culture in mHdeB[a] medium *M. smegmatis* [92]	Bacteria are Clumping and 2 to 3 times smaller		Low metabolism (rhodamine-123 staining) and negligible endogenous respiration			Not reported
Human granuloma *in vitro* model *M.tuberculosis* [93]					Significantly more cells resistant to rifampin after 8 days exposure to model	Loss of acid fastness and increase in Nile Red positive staining after 8 days of exposure to model

[a] Hartman's–de Bont medium

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Text box1. Assumptions around dormancy

The critical, implicit and as yet untested assumption is that

all dormancy phenotypes are equivalent

that the phenotypes are predictive of a state found in the human host.

bacteria in specimens and in tissue samples and animal models of disease share some phenotypic characteristics associated with dormancy

there is no unequivocal marker that identify mycobacterial cells in a dormant state

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Text box 2 Examples of nomenclature for different dormancy states and the application of the scheme to describe a cell or cells

A = Actively dividing cells

M = Multiplication rate high, medium, low , undetectable (h,m,l, u) qualified by medium used:

Growth medium S=solid, L=Liquid, A=animals(Mi=mice, R=rats, G=guinea-pigs, S=simian) e.g., $M^{u}S$

$Met^{h,m,l}$ =Metabolic rate high, medium, low. Addition of method used to detect growth could be: S35=Methionine S35 uptake. U=Uridine Tritiated uptake . ATP=ATP production

Tol = Tolerant to antbiotics R=Rif, H=Isoniazid, E=ethambutol , Z=pyrazinamide

S =Sensitive to antibiotics R, H, E, Z

Rpf = Resuscitation Promoting Factor dependent

λ +/- =Lipid body present (+) or absent(-)

Thus, a cell or group of cells that was/were unculturable in solid and liquid but grow in mice, are tolerant of rifampicin, and resuscitated by the addition of Rpf would be represented by M^{u} $SLM^{h}MiAm$, TolR, Rpf.

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Table 2 Relative susceptibility of mycobacterial species to anti-tuberculosis agents in differing models of "dormancy".

Model	**Methodologies** How was susceptibility measured?	**Susceptibilities** Isoniazid	Rifampicin	Pyrazinamide	Ciprofloxacin	Metronidazole
Gradual acidification TB	MIC [92,94]	0.05µg/mL	0.5µg/mL	20µg/mL		
Growth in modified potassium free medium	MIC [95]Plate counting methodology lowest viable number- TB	1.0µg/mL	5.0 µg/mL			

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Multiple-stress model TB	Cell survival after ≥96h exposure to indicated levels of drug- CFU count [20]	0.8µg/mL	5.0µg/mL			12µg/mL
Nutrient starvation TB	Cell survival after 168h exposure to indicated levels of drug- CFU count [54]	10µg/mL	10µg/mL			

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Low oxygen tension TB	MBC_{90} Wayne cidal concentration- the concentration of drug that reduced the CFU of the initial inoculum by 90% after 5 days exposure [96]	4.0 µg/mL				
Long term anaerobic culture TB	7, 14, 21 day exposures to drugs – CFU / MGIT (non-growth determined after negative for 100 days) [97]		8µg/mL	100µg/mL		8µg/mL

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Plate based anaerobiosis	*M.tuberculosis* number of viable cells determined by CFU on 7h11 [98] *M. smegmatis* Solid media plating for CFU counting.	25 uM viable cell count is reduced by 50% compared to the control [98].				Wild type viable counts are reduced a 100 fold at 100 μg/mL. [89]
Oxygen depletion	BCG Solid media plating for CFU counting. [89,91]					BCG viable count are reduced a 1000 fold at 100 μg/mL and 3 fold at 10 μg/mL. There is a dose dependent effect using MTZ.

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Oxygen shift down (Wayne model) TB	*M.tuberculosis* cells survival. Solid media plating for CFU counting.	0.4µg/mL [16]	0.1µg/mL[16]		1µg/mL[16]	12 µg/mL in "non replicating stage 2"(Wayne & Hayes, 1996) 8 µg/mL (Wayne & Sramek, 1994)
Oxygen depletion	Percentage of bacilli survival *M. smegmatis* CFU counting after 24h exposure to the drug[90]					120 µg/mL 20 ± 10 percent survival for persistent culture (240 hours old sealed culture). 95 ± 9 percent survival for active culture (logarithmic aerobic culture).

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Culture in mHdeB[a] medium	*M. smegmatis* percentage of resazurin (0 percent resazurin reduction)[99] Mtb h37rv MIC90 using resazurin microplate assay (REMA) [99]	10 µM	1µM less than 0.19µM MBC90: >100µM		1µM	
Human granuloma *in vitro* model[93]	M.tb MIC defined using Alamar blue reduction [76]	MIC: 1.25-2.5 µg/mL[76]	MIC: 0.47-0.94 µg/mL [76]			

[a] Hartman's–de Bont medium

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Table 3 Summary of publications and their associated definitions of "dormancy".

Authors and reference	Definition
(Shleeva, Salina, & Kaprelyants, 2010)[100]	The dormant state is understood here as a *reversible* state of a bacterial cell, when the level of metabolic activity is decreased and the cell is able to survive for a long time without division
(Gengenbacher & Kaufmann, 2012)[53]	Non- replicating bacilli maintaining full viability at a very low metabolic rate; organisms show minor susceptibility or phenotypic drug resistance to antibiotics targeting functions required for growth.
(Voskuil, Visconti, & Schoolnik, 2004)[33]	Without replication or significant metabolism.
(Mariotti et al., 2013)[101]	Dormancy is defined as a stable but reversible non-replicating state of *Mycobacterium tuberculosis*.
(Kaprelyants, Gottschal, & Kell, 1993)[102]	A reversible state of low metabolic activity, in which cells can persist for extended periods without division.
Karakousis et al., 2004) [103]	Altered physiologic state characterized by stationary-state colony-forming unit counts and decreased metabolic activity and greater susceptibility to rifampin than isoniazid.

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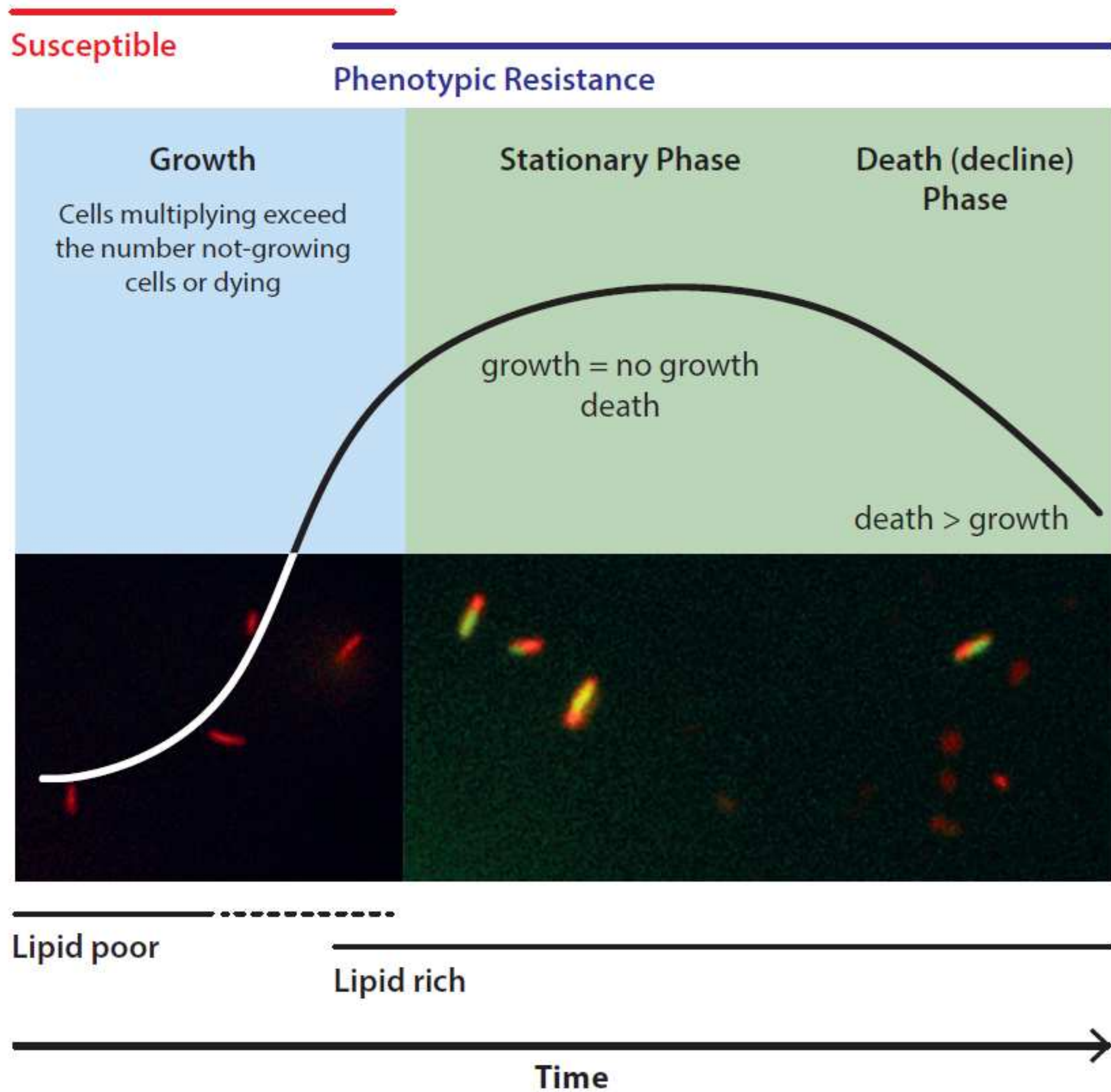

Figure 1. Mycobacterial cells stained with Nile Red and viewed by fluorescence microscopy at 100X (Leica DM5500) (excitation; 480/40, 540/40. Emission 527/30, 645/75) showing lipid poor cells (red) and lipid rich cells (red with central green lipid body. This is set alongside a hypothetical mycobacterial growth curve and the change in the phenotypic susceptibility over time in a batch culture.

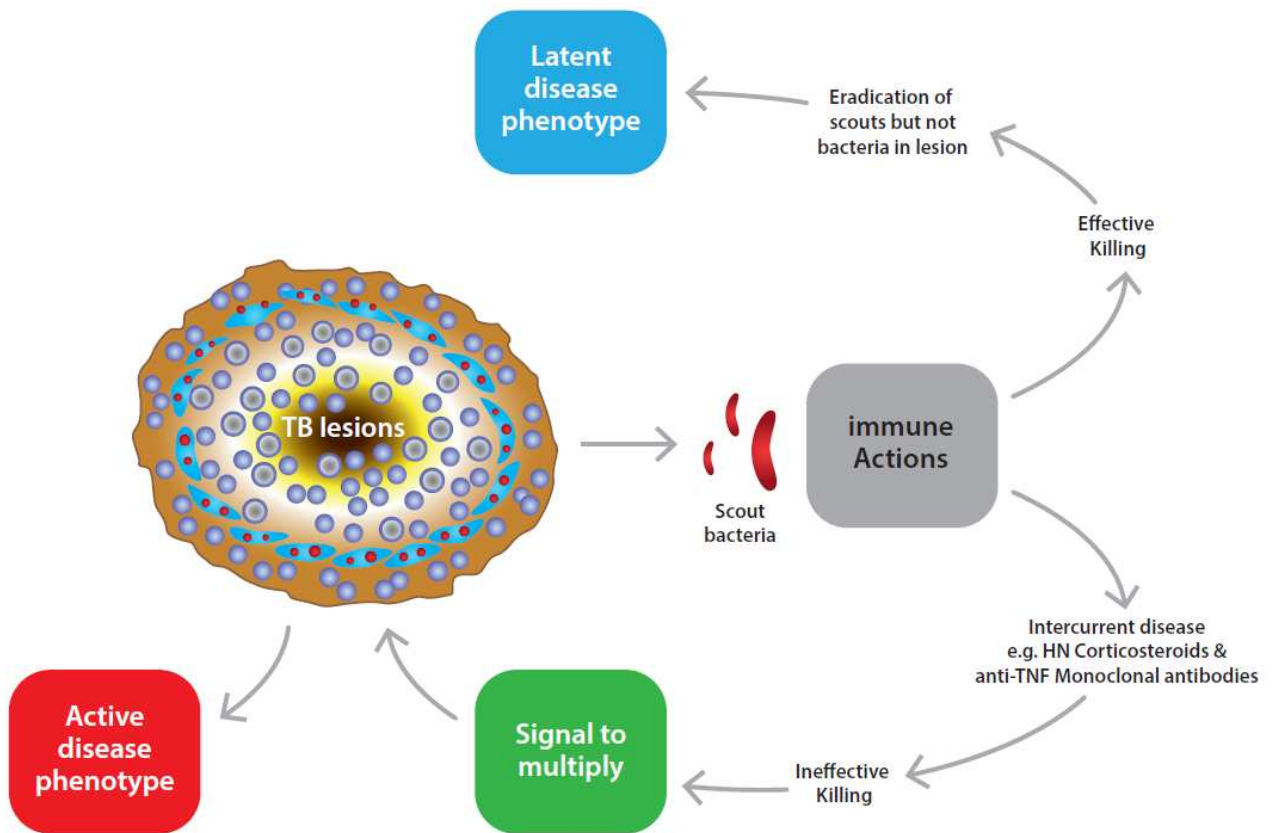

Figure 2. Illustration of how the "Scout" hypothesis might link to concepts of dormancy with dormant mycobacterial cells in the tuberculosis lesion "reactivating" and emerging. The fate of the lesion would depend on whether the re-activated or resuscitated scout to survive and give a signal.

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