ESAT-6 Independent survival and virulence: need for paradigm shift?

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Tuberculosis is a major health concern that has received lot of attention from researchers across the world. The virulent form of tuberculosis is caused by *Mycobacterium tuberculosis* (*M. tb*), a highly evolved, slow-growing, facultative pathogen. In the literature, the major suspected pathway responsible for its virulence is the Esx-1 secretion system owing to the properties attributed by ESAT-6 secretion. By arguing through literature instances that support ESAT-6 secretion independent virulence, this review not only questions ESAT-6 secretion as a sole readout to virulence, but also, suggests survivability as a likely separable property from virulence in mycobacterium, ultimately trying to create a tiny breach to the existing paradigm of thinking in mycobacterial pathogenesis in relation to ESAT-6 secretion.

Keywords: ESAT-6 secretion, *Mycobacterium tuberculosis*, virulence, survivability.

TUBERCULOSIS is one of the oldest diseases and continues to be among the deadliest worldwide. The success rate of treatment through chemotherapy and vaccination is much lower, thereby necessitating newer ways of TB treatment[1]. Comparative analysis of genomes of virulent *Mycobacterium tuberculosis* (*M. tb*) H37Rv and *Mycobacterium bovis* attenuated BCG strain revealed major differences in genomic regions, region of differences or RDs, among mycobacterial species. One such genomic region, called RD-1, encompassing the Rv3871–Rv3879c, is absent in all BCG strains of *M. bovis*, but present in all virulent, laboratory and clinical strains of *M. bovis* and *M. tb*, suggesting that deletion of RD-1 locus was perhaps the primary deletion event, responsible for attenuation of virulent *M. bovis*[2–4]. The initial experimental evidence to support this premise was obtained when introduction of differently sized RD-1 from *M. tb* to *M. bovis* BCG and attenuated *M. microti* restored a functional ESX-1 secretion system with 'an apparent virulent-like phenotype' assessed through increased immunogenicity and virulence of these strains. On the other hand, targeted deletion of the RD-1 locus from *M. tb* (resultant strain, also referred as ΔRD1 strain) has attenuated the bacteria[2,3,5,6]. In addition to these evidences, numerous studies have widely linked the role of RD-1 locus to survival and pathogenicity of *M. tb*[7–14]. Interestingly enough, in a one-off observation involving long-term infection, i.e. >1 year, the ΔRD1 strain, not only managed to survive *in vivo* but also showed that elevated bacterial loads and histopathological features of the infected mice lungs highly resembled that of the infection by the virulent TB strain[15]. This suggests that *M. tb*, upon removal of certain phenotype(s) associated with existing 'survival' or 'virulence' mechanism(s), can evolve with alternative approach(es) to sustain itself inside the host system that can, in turn, only be witnessed in a long-term infection.

The RD-1 region and its flanking genes, a gene cluster referred to as extRD-1 region (Rv3866–Rv3881c), encodes a specialized secretion system with two best studied substrates ESAT-6 (also called EsxA or Rv3875) and CFP-10 (also known as EsxB or Rv3874). This secretion system has been named after the secretory component ESAT-6 as ESAT-6 system-1 (ESX-1), also referred to as type VII secretion system, specific to mycobacterial species[8,11,16]. Various components coded through extRD-1 genes have been found to be essential for the functional ESX-1 apparatus, but their specific roles associated in ESX-1 function have not been well characterized[8,16–20]. Most ESX-1 encoding core regions come under extRD-1 region, but neighbouring genes of extRD-1 region also corresponds to ESX-1 system. The ESX-1 genetic locus thus extends from *espE* (*rv3864*) through *mycP1* (*rv3883c*) that code for the ESX-1 core components necessary for ESX-1 mediated secretion. Some other additional loci including *whiB6* (*rv3862c*), a gene upstream of the ESX-1 locus[21], an unlinked *espACD* (*rv3616c–rv3615c–rv3614c*) operon and a trans acting element *espR* (*rv3849*) are also found to be involved in ESX-1 secretion regulation through an interplay of PhoPR and MprAB two-component systems. In fact, in addition to the two major substrates ESAT-6 and CFP-10, the proteins EspA, EspC, EspR along with EspB (Rv3881c) are also substrates of the ESX-1 secretion system[22–31]. However, the secretory nature of EspR appears to bear a strain-specific difference as investigated by Cox group with *M. tb* Erdman strain and Cole's group with *M. tb* H37Rv using the same growth condition (Sautons medium) and similar technique (immunoblotting)[2,23].

The ESX-1 secretion system is considered to play a critical role in survival, growth and virulence of *M. tb*

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Box 1. Key terms

Attenuation: Attenuation refers to the loss of virulence or decreased virulence shown by a given bacterial strain/population relative to wild type, if not mentioned otherwise in relation to a particular function/activity.

'Avirulent' strains: Avirulent strains are the ones which are incapable of bringing any of the disease symptoms that otherwise exhibit upon infection with the virulent strains of the same bacteria. Thus, avirulent strain is devoid of virulence potential. The difference between avirulent and attenuated strain lies in the feature that attenuated strains are more prone to turning back to virulent form compared with the avirulent strain that has a rare possibility for the same.

Complementation: To supply back a functional copy of the gene(s) that is being lost or disrupted in the genome of the bacteria either under its native promoter in the genome or ectopically under some inducible promoter.

Cost: 'Cost' in this review defined in relation to a given phenotype is exhibited through expression of specific protein or set of proteins. The cost in this context refers to the energy investment at metabolic level associated with expression of that phenotype or physiological consequences the functional state of the phenotype brings to the bacterium under given set of environmental condition.

Fitness: It refers to the ability of one bacterial strain/population to differentially survive or differentially reproduce over other bacterial strain/population in a given environment.

Phenotype: This term used to indicate the specific feature/function the bacterium exhibits upon expression of gene or set of genes associated with that function in a given environment.

'Switching on' of phenotype: This term in this review is used to refer to the resultant change produced in the interaction of a bacterium with its environment, upon the induction of expression of some gene(s) that otherwise remains in an unexpressed/silent form.

inside the host. The ESX-1 mediated secretion has been attributed to important activities including phagosomal maturation inhibition, macrophage response modulation, intracellular replication, cytosolic translocation, necrosis induction, intercellular spread, down regulation of T-cell and dendritic cell functions and granuloma formation[7–12,14,20,32,33]. The various ESX-1 secretion-mediated functions have been ascribed to the secretion of ESAT-6 (EsxA) and CFP-10 (EsxB), the two key ESX-1 substrates. Although there are long standing evidences that associate ESAT-6 secretion with different ESX-1 secretion mediated survivability and virulence functions of *M. tb*, literature provides insufficient evidences for these activities as a direct consequence of ESAT-6 secretion. The exact mechanism(s) through which ESAT-6 secretion could mediate these activities at molecular and cellular level are also not very well-defined. The association of multiple phenotypes with ESX-1 secretion apparatus also suggests that ESAT-6 secretion may not be solely involved in ESX-1-mediated virulence, but there may be other substrates or components associated with ESX-1 apparatus that are yet to be explored. Moreover, all the ESX-1 substrates and the possible ways they could affect mycobacterial survival or virulence have not been well characterized.

Mutual dependency of Esx-1 substrates

An unusual feature associated with ESX-1 is that various ESX-1 substrates require each other for secretion. Secretion of EspA and EspC is mutually dependent on ESAT-6 and CFP-10 secretion, since blocking of EspA or EspC secretion abolishes the secretion of ESAT-6 and CFP-10 (refs 25, 26, 29, 34). EspR, a transcription factor, is also known to regulate ESAT-6 secretion through transcriptional control over *espA* and *espC* genes[22–24,31]. The secretion requirements of EspB are distinct from those of EsxA and EsxB and proposed to overlap with EsxA and EsxB secretion mechanism at the binding of Rv3871[28–30]. This could be a probable reason for the unobstructed secretion of EspB in EsxA or EsxB secretion deficient mutants in *M. tb*. EspB cleavage by MycP1 protease (Rv3883c) upon secretion was found to limit the translocation of other ESX-1 secretion[28]. This apparent co-dependency of the known ESX-1 substrates for secretion makes it difficult to identify the relative contribution of individual ESX1 substrates to these ESX-1 associated activities.

M. tb survival and virulence is independent of EsxA (ESAT-6) secretion

Voluminous studies published till date indicate the critical role of ESX-1 locus in the intracellular survival and virulence of *M. tb*. The ESX-1 locus associated virulence has been hypothesized to be mediated primarily by one of the ESX-1 substrates, ESAT-6. The ESX-1 substrate proteins other than ESAT-6/CFP-10, i.e. EspA, EspB and others, also possess distinct roles in *M. tb* virulence separate from their facilitation to the secretion of

ESAT-6, although their underlying virulence functions/mechanisms remain undefined[25,29,30]. These studies suggest that different substrates make independent and possibly, additive contributions to the ESX-1 mediated virulence in *M. tb*. In the light of these findings and the apparent co-dependency of different ESX-1 substrates for secretion, it is difficult to define or attribute one unique function and the relative contribution of individual substrate protein(s) to the ESX-1 mediated virulence. The absence of *espACD* operon in non-pathogenic *M. smegmatis*, perhaps, suggests the virulence role of ESX-1 substrates coded through this operon[35].

An array of reports correlate the presence of ESAT-6 and CFP-10 secretion with virulence phenotype, while secretion blockage correlated to attenuation of the mycobacterial strains. Also, the ESX-1 locus, including ESAT-6, is highly conserved in both pathogenic and nonpathogenic bacteria, wherein the latter, interestingly, has been shown to be involved in conjugation[32,36]. This highly contrasting observation suggests that the ESX-1 locus could be essential for virulence but that might not directly mediate the same. Several lines of evidences also support this view point, as the virulence of pathogenic mycobacterium could be independent of ESAT-6 secretion (Table 1). These reports reveal the contribution of proteins, associated with functional ESX-1 system, to the ESX-1 mediated virulence, in an ESAT-6 secretion independent manner. Thereby, it indicates that ESAT-6/CFP-10 secretion cannot be the sole read-out for a functional ESX-1 system and its associated 'virulence definition'. This, in turn, should question the way investigators relate the role of a functional ESX-1 system in mycobacterial pathogenesis, to the ESAT-6/CFP-10 secretion assuming it as the only appropriate read-out for a functional ESX-1 system.

In vivo versus *in vitro* secretion

Based on the literature, the ESAT-6 secretion of mycobacterium can be said to be modulated by the change in environment or growth conditions: for instance, the growth of nonpathogenic mycobacterial species *M. smegmatis* in sauton's medium induces ESAT-6 secretion, but not when it is grown in 7H9 medium, the most commonly used mycobacterial growth medium[28,37]. Most reports that separate the ESAT-6 secretion phenotype and the mycobacterial virulence have analysed the secretion *in vitro* through immuno-blotting technique (Table 1). But, the *in vitro* secretion levels of ESAT-6 may not be similar to that of the levels in cellular and animal models, as it involves the influence of host factors, which could affect the ESAT-6 secretion levels. Therefore, it necessitates a thorough investigation of ESAT-6 secretion status *in vivo* to precisely relate the *in vivo* virulence potential of the strain with its ESAT-6 secretion ability *in vitro*, to rule out any possible modulation of ESAT-6 secretion under *in vivo* condition. ESAT-6 can find access to host cell cytosol by breaching the phagosomal membrane that can be easily sampled and presented through MHC class I molecule. The evaluation of EsxA/EsxB-specific CD8+ T-cell response priming, thus, can be used to assess the *in vivo* secretion of EsxA/EsxB[25,38–40]. Among different reports that separate the ESAT-6 secretion ability from the virulence of the strain (Table 1), only few have analysed the *in vivo* ESAT-6 secretion by testing the strains for their ability to induce specific T-cell responses against EsxA/EsxB antigens[25,40,41]. These reports find the ESAT-6 secretion status of the strains under *in vivo* conditions, similar to that detected through *in vitro* secretion assays, thereby, ruling out the possibility of ESAT-6 secretion status modulation between *in vivo* and *in vitro* conditions. Hence, these observations validate the notion that mycobacterial virulence can be independent of ESAT-6 secretion under *in vivo* conditions through *in vivo* ESAT-6 secretion assays. In other words, the virulence of ESAT-6 secretion deficient *M. tb* strains (Table 1) suggest that other mycobacterial factors or host factors could compensate the loss of ESAT-6 secretion in these strains and thereby, retain virulence of the strains. Similarly, the attenuation of strains showing unimpeded ESAT-6 secretion (Table 1) proposes that ESAT-6 secretion cannot be the sole factor to determine the infectivity or virulence in *M. tb*.

Gene inactivation approaches to study ESAT-6 secretion role in mycobacterial virulence

The secretion of ESAT-6 is not only under the regulation of extRD-1 region but also regulated by some unlinked, distant gene loci as well[8,21,24,25,28,30,31,33,40]. This, in turn, indicates the existence of complex networking at proteome levels in mycobacterium. In other words, the function associated with a protein not only gets compromised upon disruption of its coding gene directly, but could also be compromised by disturbances that occur at other gene loci as well, if the product of the disrupted gene is in someway needed for the proper functioning of the protein as a matter of direct or indirect consequence. To screen out all the genes/proteins from the genome, that could be needed for the proper functioning of a given protein, is a difficult task. To accomplish this, one needs to compare various aspects of the functions of a given protein in the wild type bacteria versus mutant strain carrying the disrupted copy of the gene, whose effect over protein functioning is under investigation. Thus, upon knock out of any gene, the effect comes on to the bacterial virulence and may or may not directly relate to the function of the cognate protein, but could be a result of the downstream effect(s) that took place in the absence of the protein being knocked out. This could be in the form of a compromised function of some other protein(s), whose

Table 1. List of strains, ESAT6 secretion and virulence status described in literature

Strain	ESAT-6 secretion detection assay	ESAT-6 secretion	Virulence status	Report
M. tb H37rvΔespACD:: pAC138ACD	IVTSA	Yes	Attenuated for survival and growth in mice and in macrophages, strain also showed compromised functional integrity of mycobacterial cell wall.	Garces *et al.*[25]
	IVIVSA	Yes		
M. tb Erdman espA:: Tn + pMDesp AF5RCD and *M. tb* Erdman espA::Tn + pMDesp AK41ACD	IVITSA	No	Cytotoxic to THP-1 cells and virulent during acute phase of infection in mice; cell surface integrity remains unaffected.	Chen *et al.*[26]
M. tb Erdman espA::Tn and *M. tb* Erdman 5′Tn::pe35	IVITSA	No	espA::Tn mutant induced more IL-1β secretion and greater cytotoxicity to THP-1 and MRC-5 cells, owing to the unimpeded secretion of EspB by espA::Tn mutant; shows EspB role in EsxA independent host cell killing.	Chen *et al.*[29]
*M. tb*ΔespF and MtbΔespG1	IVIVSA IVITSA	Yes Yes	Strains found to be attenuated for their growth in BMDMs as well as mice. Attenuation of strains also confirmed by histopathological analysis.	Bottai *et al.*[41]
*M. microti*Δrv3860-66	IVITSA IVIVSA	Yes Yes	Attenuated for its growth *in vivo* in SCID mice.	Brodin *et al.*[40]
M. marinum M1663::Tn:: 1664 strain and the complemented strain 1663::Tn::1664/ *p* = 1663–1668	IVITSA	No	The complemented strain was able to lyse amoeba monolayer and RAW cells partially.	Kennedy *et al.*[33]

Examples of the strains showed ESAT-6 secretion independent survival or virulence are quoted in the table. *IVITSA – *In vitro* secretion assay (immunoblotting). **IVIVSA – *in vivo* secretion assay (Spleenocyte T cell response assays).

functionality is in some way dependent on the protein being knocked out.

Different gene inactivation approaches (such as transposon insertion, point mutations or gene knockout) have been used to associate the normal ESAT-6 secretion to the virulence of mycobacterium where different ESAT-6 secretion regulatory genes upon knock out, results in compromised ESAT-6 secretion and attenuation of resultant strains, and complementation of wild type copy of that gene restores back the virulence and normal ESAT-6 secretion. It is possible that some other 'virulent phenotype(s)' also gets co-regulated with ESAT-6 secretion and levels of which, would also be fluctuating with ESAT-6, upon disturbances in some common regulatory centres of the two, if any. The complex interactive networks that exist at proteome level could make the involvement of single protein in regulation of more than one phenotype happen. Specifically with the ESAT-6 secretion regulation, the occurrence of multiple and wide spread regulatory centres for ESAT-6 expression regulation[8,21,24,25,28,30,31,33,40] in the genome adds to the probable occurrence of this possibility. Thus, when experimenting through inactivation of regulatory loci, any virulent phenotype(s) that could have their possible co-regulation with ESAT-6 secretion would go unnoticed or unexplored for their role in virulence, while focusing only on the ESAT-6 secretion. The role of other virulent phenotype(s) that could have their possible co-regulation with ESAT-6 secretion would also become the subject matter of investigation, when questioning the role of ESAT-6 secretion as a sole read-out for virulence, based on the available instances that support ESAT-6 secretion-independent virulence (Table 1). If the levels of other virulent phenotype(s) also show fluctuations, the complementation back with normal levels of that particular phenotype(s) would clarify the contribution of that phenotype in the virulence or attenuation of mutants for ESAT-6 regulatory gene(s). Thus, upon looking at the gene inactivation approaches to define ESAT-6 secretion role in mycobacterial virulence, one possibility that could emerge out is the role of some ESAT-6 co-regulated virulent phenotype(s) that might have remained unnoticed, while focusing on to the normal ESAT-6 secretion ability as the only readout for virulence of the strain.

Compensation for loss of ESAT-6 secretion

It is important to accept that a variety of phenotypes can work in harmony for the successful pathogenesis of *M. tb*. It is possible that, upon the loss of ESAT-6 secretion,

other mycobacterial factors might have compensated the role of ESAT-6 secretion. The recognition of loss of ESAT-6 secretion by the bacteria could perhaps occur by the generation of feedback signalling and possibly operate through other regulatory locus/loci (described above) in ESAT-6 secretion deficient strains. The sensing of loss of an active phenotype in the form of ESAT-6 secretion could be followed by the modulation of the other active phenotype(s) that might have compensated the virulence role associated with ESAT-6 secretion in the virulent strains devoid of ESAT-6 secretion (Table 1). The changes to the levels of active phenotypes could readily be achieved through the variation in the level of protein(s) associated with that phenotype(s). Thus, the compensation that happens through this mode provides fitness advantage to the bacteria, if the environment demands the compensatory mechanism to come up readily. Another possible way the compensation to ESAT-6 secretion loss could happen is through the 'switching on' of some other needful phenotype(s), which otherwise (in the presence of ESAT-6 secretion) remain silent. The 'switching on' or 'induction' of such silent phenotype(s) would possibly need the expression of some new set of genes, which otherwise remain dormant. But, for discovering any such new phenotype, the bacteria may have to bear a 'cost', whose magnitude depends upon the time stretch taken to induce the needed phenotype for that environment. Once discovered, this time stretch can be brought down by the bacteria, if it keeps the 'environment experience' as a memory imprint in the genome and uses that for any needful re-networking of signalling for the induction of that silent phenotype(s) in any future need. The compensation through such silent phenotype(s), not only provides an opportunity to study novel phenotypes that could contribute to the virulence, but also helps to understand the evolutionary mechanism involved in the emergence of new phenotypes. Another possible way to keep the ready availability of any phenotype after being discovered is through maintaining a variable sub-population (expressing that phenotype) that would be better adapted for past encountered environment, as a bet hedging strategy (for detailed discussion see ref. 40). But, for any of the above compensatory events to happen, there must be some strong sensing mechanism(s) associated with ESAT-6 secretion, which should get activated in the strains lacking ESAT-6 secretion, upon sensing suitable environment, possibly formed by the presence of specific host factors. Thus, one of the possibilities, through which *M. tb* strain devoid of ESAT-6 secretion can retain the virulence, is via compensation by other virulent phenotypes.

Survivability: a distinct and separable feature from the virulence potential in *M. tb*

'Virulence' is the ability of pathogenic bacteria to establish the 'disease' or 'pathogenesis in the host. Pathogenic bacteria can, in principle, carry multiple 'phenotypes' that can help in its survival, reproduction and 'setting up of disease condition' inside the host. Several phenotypes, can thus contribute their part at different phases of disease progression for bacterial pathogenesis. The host environment, in turn, poses barriers including immune barriers to prevent the infection by the pathogen. The degree of virulence is therefore, determined through the complex balance between pathogen and host factors. Thus, the bacterial virulence is dictated by multiple variables that include host immune status, infectivity of pathogenic bacteria, number of infecting bacteria, infection route in the host, to name a few, besides other factors.

'Survivability' is the property of a pathogen which enables it to survive and reproduce inside the host against host immune responses. The survivability is also, most of the time, determined by the activity of 'multiple phenotypes acting in concert', like virulence. Thus, survivability must come prior to any observation that defines the virulence of the bacteria. In summary, pathogenic bacteria must be able to survive and reproduce inside the host to raise its number sufficiently high to produce disease symptoms or observable manifestations.

In case of *M. tb*, several published instances show that 'avirulent' or 'attenuated' strains retain their survivability comparable to that of the wild type (H37Rv). A few instances from *M. tb* literature can be described as:

Example 1: Ohol *et al.*[28] worked to elucidate MycP1 regulation over ESAT-6 secretion. They have shown ΔmycP1 strain unable to secrete ESAT-6 and is shown to be attenuated *in vivo* through histopathological examination of infected lung sections (figure 5 *h*; ref. 28). However, a close glance at the growth kinetics of ΔmycP1 in *in vivo* infection (figure 5 *d*; ref. 28), makes it quite clear that ΔmycP1 is able to replicate inside mice, despite an initial dip in its growth compared to wild type *M. tb* on the 7th day post infection, and by 56 DPI, it was able to achieve the CFU very close to the wild type.

Example 2: Chen *et al.*[29] while describing the role of EspB in mycobacterial virulence, independent of ESAT-6 secretion, worked with two mutants viz. *espA*::Tn and 5′Tn::*pe35*; both of them were found to be deficient for ESAT-6 secretion and were significantly attenuated in comparison to the wild type based on *in vitro* virulence assays (figure 2; ref. 29). These mutants were also analysed for their *in vivo* survivability and it is clear from their work that the CFU values by 56 DPI were similar to that of wild type in infected mice lungs despite marginally slower growth rate during the acute phase of infection (figure 3 *a*; ref. 29).

Example 3: Strain H37Ra is the most studied avirulent *M. tb* strain which is deficient for ESAT-6 secretion[39,42]. The *in vivo* survivability of *M. tb* H37Ra has been

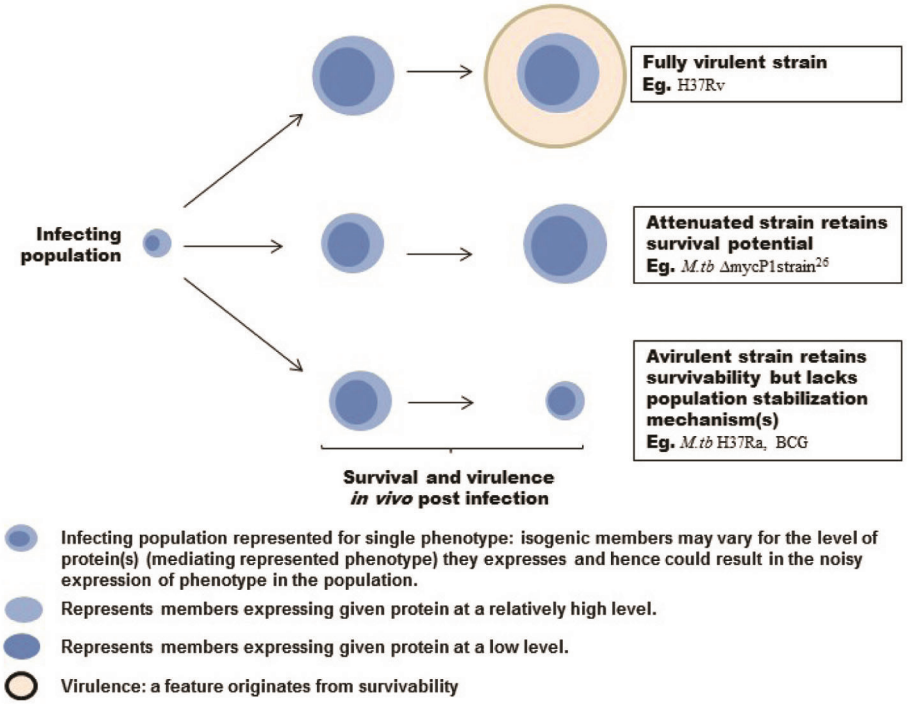

Figure 1. From the 'infecting population' (on the left), the top-group represents the mycobacterial strain(s) that replicates, stabilizes its population and produces pathological fingerprints, e.g. *Caseaous granuloma*, inside host. The middle group represents the strain that replicates and stabilizes its population but is unable to produce any characteristic 'finger print pathology' in the host tissue. The bottom group represents the strain that replicates, but not able to stabilizes its population inside the host (which may eventually lead to its clearance) and is unable to produce any finger print pathology in the host tissue. Although not drawn to scale, the size of the filled circle implies the growth of population from the initial infection stage.

investigated in various studies. For example: Kim *et al.*[43] and Rapasy *et al.*[44] have compared H37Ra and H37Rv strains survivability in figures 1 *b* and *a* respectively. The CFU values at 4 WPI clearly show that H37Ra growth kinetics reflected almost parallel to that of the H37Rv, although H37Ra multiplies at marginally slower growth rate compared to H37Rv. However, one month post-infection, CFU values for H37Ra begin to decline while H37Rv stabilizes its population. But, H37Ra maintains a two-fold high CFU (log scale) compared to the initial infecting CFU and is not cleared in mice even after 25 weeks post-infection.

The above is a meagre representation out of large evidences, demonstrating the survivability of 'attenuated' or 'avirulent' *M. tb* strains under *in vivo* condition. All these observations (including not cited) suggest that:

(1) It is important to delink the survivability of *M. tb* with ESAT-6 secretion. A bulk of literature associates ESAT-6 secretion role in mycobacterial survival and virulence inside the host as described in the previous sections. However, the strains deficient for ESAT-6 secretion, showed only a marginal decline in replication rate during the acute phase of infection, which was eventually overcome by these strains and the observed CFU were very similar to that of wild type by the time of chronic phase of infection (examples 1 and 2 above). The initial 'slow-down' of replication rate could be a result of the time frame taken by bacteria to adapt to the loss of an active phenotype in the form of ESAT-6 secretion, that, in turn would be reflected into the population growth kinetics of these strains. Moreover, the avirulent H37Ra strain, that lacks ESAT-6 secretion[39,42], also possess survival potential, and is able to replicate and maintain its CFU two-fold higher than the initial infecting CFU even after 25 weeks post infection[43,44].

(2) Although survivability is an inextricable feature of virulent strain, it (the features associated with survivability) can be retained even after losing virulence in the *M. tb* strains as seen with the avirulent and attenuated *M. tb* strains that retains their survival potential (examples 1–3 above). This also suggests that survivability could be a distinct feature of *M. tb* that can be maintained independent of the virulence potential. In our opinion, this survival potential is a key facet to virulence of any pathogen, including *M. tb*. Thus, attacking the survivability is anticipated to render the bacterium incapable of replicating inside the host and thereby, not letting the bacterial numbers to raise, which in turn, would not allow the infection to progress. Therefore, attenuated *M. tb* strains that retain the survival potential could be used to study the survivability and potential ways to interfere the same, to limit the *M. tb* infection.

(3) Most pathogens, including *M. tb*, essentially have to maintain a balanced relationship between survival and virulence at various stages of their life cycle, to optimize the fitness. For example, under host immune pressure, a pathogen may down-express some virulence factors to limit the immune response elicited against it, to survive through the immune surveillance. On the contrary, at appropriate times, *M. tb* may be needed to deploy specific virulence factors to retain the viablility under host stresses, like that encountered in the macrophage. Thus, the context of prevailing environment and the inherent abilities of a pathogen to interact with that environment, might evoke a decision-making in the pathogen, to decide upon the preferential deployment of available strategies toward survival and/or virulence.

Summary

An immense array of studies link the mycobacterial survival and virulence to its normal ESAT-6 secretion ability. However, several instances can also be noted, where mycobacterial virulence was found to be independent of ESAT-6 secretion. These reports likely suggest contributions of other phenotypes to the survival and virulence of mycobacterium. These phenotypes could be in the form of other ESX-1 linked substrates/proteins or, the compensatory changes arise in response to disturbed ESAT-6 secretion or, might be some unexplored phenotype sharing common regulatory loci with ESAT-6 secretion regulation. A dissection of observations on some of these studies suggests survivability as a likely separable property of mycobacterium from its virulence potential (Figure 1). Concisely, this review calls the role of ESAT-6 secretion as a sole determinant to mycobacterial survival and virulence into question and tries to create a tiny breach to this existing paradigm of thinking. Viewing through this breach, would probably open doors to new and unexplored possibilities in the *M. tb* research.

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