R E V I E W

Targeting of immune signalling networks by bacterial pathogens

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Host defence against microbial pathogens requires appropriate coordination of multiple signalling pathways. These pathways are triggered by innate immune recognition of conserved microbial molecules, and initiate an inflammatory cascade that involves recruitment of leukocytes to the site of infection, activation of antimicrobial effector mechanisms and induction of an adaptive immune response that promotes clearance of infection and long-term immune memory. Microbial pathogens possess specialized proteins termed virulence factors, which interfere with host defence at several levels. Many virulence factors from diverse pathogens have been identified in recent years and their functions linked to disruption of essential processes of immune defence, from signalling to phagocytosis. Although the diversity of pathogens and virulence factors is immense, common themes have emerged with regard to how microbial pathogens interfere with immune responses. Here we discuss recent advances in our understanding of how virulence factors target innate and adaptive immune responses, focusing on bacterial pathogens. We also propose that pathogens responsible for causing acute infection tend to target central components (hubs) of cellular signalling pathways, causing global disruption of the host response. By contrast, pathogens that cause chronic or persistent infections tend to target more peripheral signalling network components (nodes) to promote pathogen persistence.

Multicellular eukaryotic organisms possess a complex set of antimicrobial recognition and defence systems that enables their survival despite continuous interactions with both pathogenic and non-pathogenic microbes. These defence systems include innate mechanisms, (for example, mucosal epithelial surface barriers, pattern recognition receptors, such as the Toll-like (TLR) and Nod-like (NLR) receptor families, and antimicrobial effector molecules) and adaptive immune responses mediated by T and B lymphocytes expressing antigen receptors. These immune defence systems control host–microbe interactions, the outcome of which can range from symbiotic coexistence with commensal microflora, to mild asymptomatic infections, to highly virulent infectious diseases, depending on the particular host and microbial organisms involved.

Successful pathogens depend on the coordinated activities of virulence factors to overcome host barriers to colonization and to evade or subvert the subsequent immune response. Most pathogens that are highly co-evolved with their hosts have reached a state of relative equilibrium in which the degree of overt pathology is minor, allowing long-term association with a particular host or host population. This is true for pathogens such as *Mycobacterium tuberculosis* and *Helicobacter pylori* that cause persistent infections[1]. The degree of pathogenesis tends to be more severe when pathogen and host have not co-evolved; the latter often serves as a dead-end host for that pathogen[2], as exemplified by *Bacillus anthracis* infection of humans. However, exceptions to this general trend exist in situations where increased transmission depends on greater virulence[3]. For example, the host-restricted pathogen *Salmonella enterica* serovar Typhi shows greater invasiveness and increased lethality in its preferred higher primate hosts; high invasiveness in this case correlates with the establishment of a persistent state of infection in a minority of individuals, enabling long-term shedding of the bacteria into the environment[4].

All these interactions are mediated by the interplay between bacterial virulence factors and host immune responses. Rapidly increasing knowledge about immune detection of and responses to microbes has led to a better understanding of how pathogens target the host immune response. Given their central role in immune signalling, it is not surprising that NF-κB and mitogen-activated protein kinase (MAPK) pathways are targeted by numerous virulence factors, including the YopJ/P proteins of pathogenic *Yersinia* species[5–7], AvrA and SpvC of *Salmonella*[8–10], OspF of *Shigella* species[11,12] and lethal factor of *B. anthracis*[13].

A greater appreciation for the central role of protein ubiquitylation in immune signalling has also led to the discovery of virulence proteins that target this modification, as shown by the identification of both ubiquitin ligases and deubiquitinases among bacterial virulence factors. In a number of instances, a single pathogen expresses members of both classes of enzymes, such as the OspG and IpaH proteins of *Shigella*[14,15] or SopA and SseL proteins of *Salmonella*[16–18]. Inhibition of NF-κB and MAPK signalling, and modulation of protein ubiquitylation by microbial pathogens have recently been extensively reviewed[19,20]. The goal of this review is to suggest a context for the diversity of virulence factors that target host immune responses, and to propose a rationale for the interaction of bacterial virulence factors with their targets, using several key examples.

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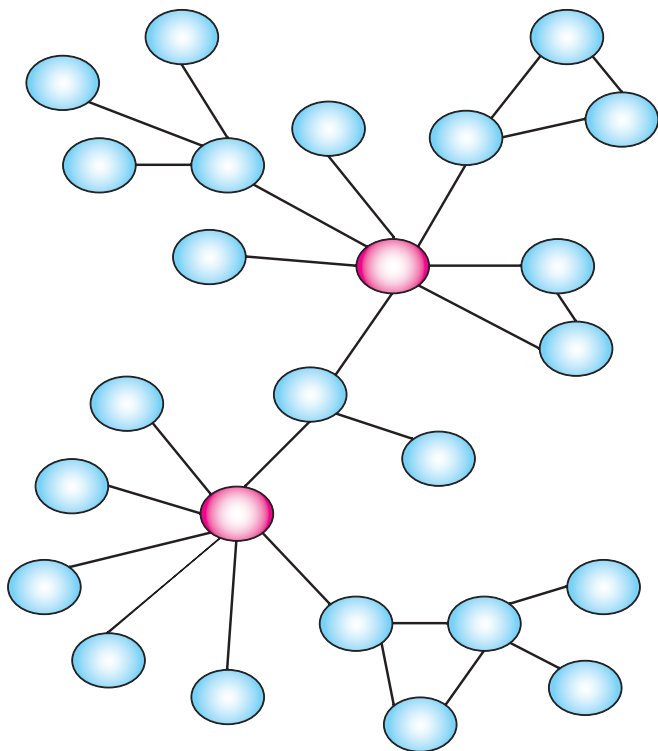
Figure 1 Model scale-free network. This model network contains elements termed nodes (coloured ovals) that interact with one another (connecting lines). Most of the nodes in the network interact with only one or two other nodes (blue ovals) but a small number have many connections and are termed hubs (pink ovals). In this idealized network, the hubs can be seen to interact with approximately 30% of the elements in the network, whereas the nodes interact with, at most, 8% of other nodes. Random elimination of one of these nodes is unlikely to disrupt the network, whereas disruption of either of the hubs, which account for only 8% of total network, would disrupt about 30% of network functions. This disproportionate dependence on a limited number of highly connected hubs is a key feature of scale-free networks.

Immune signalling pathways as scale-free networks

Global analyses of protein–protein interactions in several experimental systems have revealed that biological systems show properties of scale-free networks[21]. Components of the networks are defined as 'nodes', with each node being connected to a particular number of other nodes. In cellular protein interaction networks, the nodes are individual proteins connected through protein–protein interactions that can be either stable, as in multiprotein complexes, or transient, as between a kinase and its target. In a scale-free network, the likelihood that any given node has a specified number of connections is inversely related to that number of connections. This type of relationship is known as a power law and confers unique properties to these networks[22]. In particular, scale-free biological networks contain a small number of highly connected proteins, termed 'hubs', and a large number of proteins with low connectivity termed 'nodes'[21] (Fig. 1). Scale-free networks are thought to arise naturally through evolution by preferential 'attachment' of new proteins to pre-existing hubs rather than to other nodes[23]. One advantage of such networks is that they are highly robust in the face of random disruption, as highlighted by the observation that approximately 80% of genes in *Saccharomyces cerevisiae* are non-essential[24]. Conversely, scale-free network architecture is highly susceptible to disruption of the central hubs[25].

We suggest that these properties of signalling networks may explain, in part, the choice of cellular targets of pathogens' virulence factors. Thus, we propose that pathogens that cause acute infections tend to target central hubs of cellular signalling networks. However, the goal of certain pathogens, particularly those that show long-term survival within host cells or have a chronic phase during their infection life cycle, is not necessarily complete disruption of the host immune response. Such pathogens are more likely to target nodes rather than hubs to modulate specific aspects of the response.

Interactions of acute and chronic pathogens with the host

The outcome of acute infection is generally very dramatic for the host or for the pathogen, as it leads to either complete resolution of infection and pathogen clearance (following a brief period of intensive replication), or to death of the host. The goal of acute pathogens is to replicate rapidly, to as great an extent as possible, and to facilitate transmission to new hosts. This strategy often requires extreme effects on the host, involving large-scale disruption of host signalling pathways and cellular physiology. On the other hand, pathogens that cause chronic infections excel in adapting to the host in a manner that ensures long-term residence either within a given host or within a host population. This strategy benefits from subtle and highly specialized manipulations of the host, while preserving key components of normal host physiology. These distinctions are not always clear-cut and, in some instances, an initial acute infection can lead to long-term persistence of the same pathogen.

Targeting hubs of innate host defence networks

Disruption of pro-inflammatory gene expression and innate immune responses by pathogen virulence factors occurs at several stages of immune signalling. Among the best studied is the disruption of NF-κB and MAPK pathways by *B. anthracis* lethal factor and the *Yersinia pestis* YopJ protein[7,13] (Fig. 2). Disruption of these signalling hubs blocks production of pro-inflammatory cytokines and causes macrophage death[26,27]. More recently, a class of bacterial proteins that shares homology with the Toll/IL-1 receptor (TIR) domain was identified. The TIR protein–protein interaction module is found in TLR and IL-1 receptor proteins and in their signalling adaptors[28,29]. Interactions between TIR-domain-containing receptors and adaptors are required to initiate signalling cascades in response to IL-1 family cytokines and to TLR ligands. The bacterial TIR-domain containing proteins TcpC of uropathogenic *Escherichia coli,* TcpB of *Brucella miletensis*[30] and TlpA of *Salmonella enterica* serovar Enteritidis[31] interfere with TLR signalling, as demonstrated by a decrease in the production of pro-inflammatory cytokines by cells infected with bacteria expressing these proteins. Compared with isogenic mutants, these bacteria also show enhanced intracellular replication and virulence in animal infection models, indicating that disruption of TLR signalling not only inhibits production of pro-inflammatory cytokines, but also promotes intracellular bacterial replication. This may be due to to the role of TLR signalling in enhanced trafficking of phagosomal content to lysosomes[32] and disruption of this aspect of TLR signalling by TcpC/TlpA.

A search in public databases for TIR-domain-containing proteins in the bacterial kingdom yields several hundred proteins, many of which have no known function, that are found in environmental bacterial species, as well as in those that interact with eukaryotic organisms. Therefore, it remains to be seen whether other bacterial TIR domain proteins also
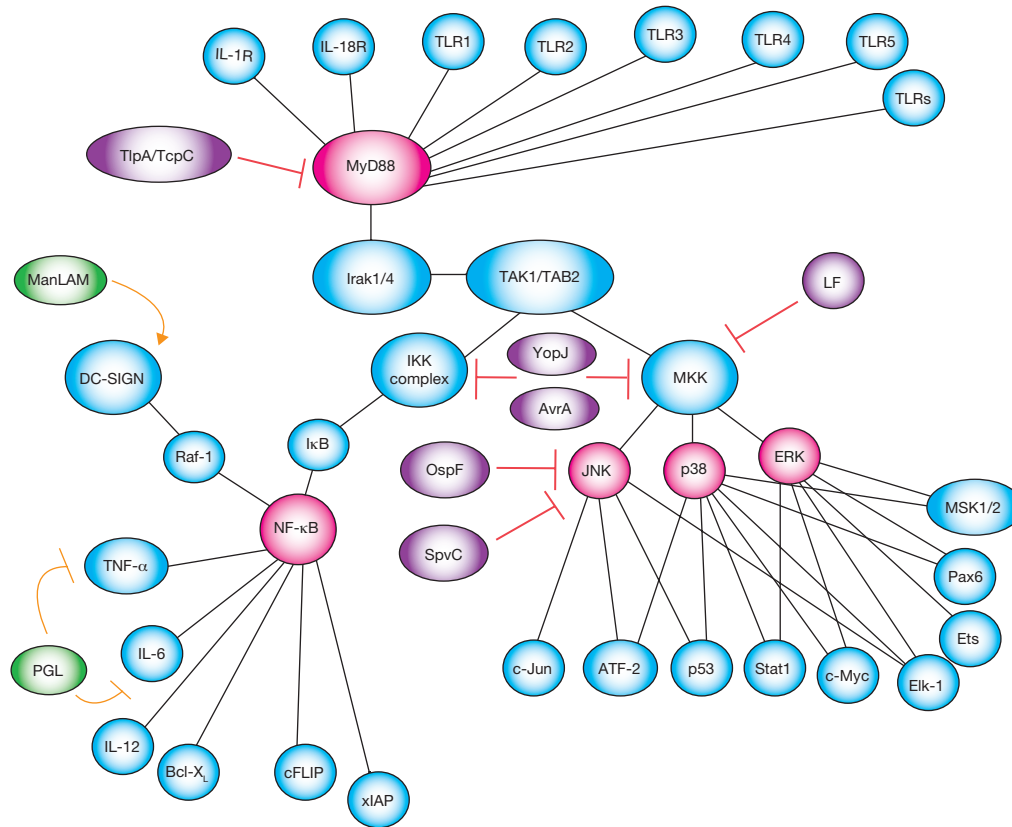
Figure 2 Innate immune network components (blue and pink ovals) and their targeting by bacterial virulence factors. Virulence factors of acute pathogens (purple ovals) preferentially target central hubs (pink ovals) or essential regulators of hub proteins in the signalling network. A large body of work from several laboratories has contributed to this understanding of how acute pathogens disrupt immune defence networks. Lethal factor (LF) of *B. anthracis* targets proteins of the MAPK kinase (MKK) family by cleaving and inactivating them[13], whereas the YopJ/AvrA family of virulence factors targets both MKK and IKK proteins[7–9]. SpvC and OspF are members of a newly identified class of bacterial enzymes, phospho-threonine lyases, which inhibit MAPK signalling by preventing their phosphorylation[10–12]. TlpA and TcpC, on the other hand,

are bacterial TIR-domain-containing proteins that interfere with TLR signalling by targeting MyD88 (refs 30, 31). Inhibition of these targets leads to major disruption of transcriptional programs required for robust immune responses. By contrast, how persistent pathogens selectively modulate specific aspects of the immune response is only beginning to be understood. A number of mycobacterial glycolipids, such as ManLAM and PGL (green ovals), modulate the innate immune response either by interacting with the surface protein DC-SIGN on dendritic cells (ManLAM)[55], or inhibiting production of pro-inflammatory cytokines by macrophages (PGL)[57]. However, PGL expression seems to be selected against in *Mycobacteria*, suggesting selective pressure against virulence factors that might act to globally disrupt the immune response.

function in pathogenesis. However, in addition to the role of eukaryotic TIR-domain-containing proteins in host defence, the TirA protein seems to also have a broader role in the social amoeba *Dictyostelium*, allowing the organism to feed on normally non-pathogenic bacteria[33]. Bacterial acquisition of TIR-domain-containing proteins may therefore have served an ancient function enabling bacteria to overcome predation by unicellular eukaryotes, as has been proposed for bacterial virulence factors that target other innate immune functions such as phagocytosis[34].

Targeting hubs of adaptive host defence networks

The presence of an adaptive immune system in vertebrates has led to selection pressure on microbial pathogens to produce virulence factors that also interfere with this aspect of host defence. Indeed, a number of pathogens show a counterintuitive tropism for lymphatic tissues, the sites of initiation of adaptive immunity to pathogens. Therefore, acute pathogens with tropism for lymphoid organs, such as pathogenic *Yersinia* species, produce virulence factors that target signalling hubs of both innate and adaptive immune responses. The tyrosine phosphatase YopH, long known to be involved in blocking macrophage and neutrophil

phagocytosis[35–37], also interferes with the ability of B cells to upregulate MHC class II and co-stimulatory molecules[38]. More recently, YopH was shown to specifically bind and dephosphorylate two central regulators of T cell receptor signalling, the proteins LAT and SLP-76 (ref. 39). This was observed even at extremely low multiplicities of infection, suggesting that YopH may interfere with T cell activation during early stages of infection *in vivo*. Although preferential delivery of *Yersinia* effector proteins into monocyte-derived cells has been reported[40], it is highly likely that *Yersinia* encounters lymphocytes while replicating in lymphoid tissues, and that the ability of *Yersinia* to interfere with T cell activation subverts the adaptive host response. The extremely potent tyrosine phosphatase activity of YopH has marked effects on both macrophages and T cells, thus making it difficult to separate the effects of YopH on these cell types during *Yersinia* infection. However, the substrate binding region of YopH has been mapped[41], potentially allowing identification of bacterial mutants unable to inhibit T cell activation while retaining their ability to block phagocytosis. Analysis of such mutants will provide a definitive understanding of how YopH-mediated inhibition of T cell activation contributes to virulence *in vivo*.

Targeting the immune response during persistent infection

Chronic or persistent infection is characterized by long-term association between hosts and microbial pathogens, and is often clinically asymptomatic, despite a functional immune response, as many chronic infections are not accompanied by global immunosuppression. How persistent pathogens maintain this state is not entirely clear, but the nature of the host-microbial interactions is fundamentally different during acute and persistent infections. Persistent infection, in which active replication of the bacteria is limited and the infectious organisms are thought to be 'latent', requires a finely tuned balance between virulence factors of the pathogen and an active host immune response. Disrupting this balance can rapidly reactivate the latent organisms and lead to an acute infection state, accompanied by high replicating pathogen loads. In experimental models of persistent *Salmonella* infection, treatment of infected mice with neutralizing antibodies against interferon-γ (IFN-γ) resulted in bacterial reactivation, symptoms of acute infection and increased host mortality[42]. Virulence factors that function during persistent infection must therefore accomplish a different goal from those that function during acute infection: they must allow for bacterial survival and replication while preventing global disruption of the immune response and preserving other vital host processes. These virulence factors are therefore more likely to act by fine-tuning the host immune response and target specific node proteins rather than global signalling hubs.

Targeting nodes of innate host defence networks

M. tuberculosis is a prototypical persistent pathogen, and can be considered as one of the most successful human pathogens; in 1997 the WHO estimated that 32% of the world's population (1.86 billion people) was infected, with approximately 8 million new cases occurring that year[43]. In the absence of treatment, infection with *M. tuberculosis* is life-long, yet most infected individuals never develop active disease, carrying the organism within immune foci termed granulomas[44].

Maintenance of the granuloma is thought to depend on a long-term but labile balance between the immune system and the bacteria, involving low-level replication of metabolically active bacilli, which are normally prevented from escape and replication throughout systemic sites by an active and ongoing immune response[45]. Similarly to persistent infections with *Haemophilus* or *S. pneumoniae*, circulating antibodies against the organism do not prevent superinfections with *M. tuberculosis*, indicating that the immune response is somehow incomplete[46]. Indeed, a zebrafish model of *Mycobacterium marinum* infection demonstrated that superinfecting *Mycobacteria* home to pre-existing granulomas, within which they are able to survive and replicate[47]. The signals that drive this homing remain uncharacterized, but they depend on trafficking of monocyte-derived cells to the granuloma. Bacteria that arrive at the pre-existing granulomas rapidly induce expression of granuloma-specific genes, which presumably are necessary for bacterial survival and persistence within the granuloma[47].

The interplay that must occur between bacteria and host cells to direct superinfecting bacteria to pre-existing granulomas suggests that bacteria must fine-tune localized immune responses, rather than trigger their global shut-down. A recent study demonstrated that *Mycobacteria* in granulomas recruit new macrophages that phagocytose dead or dying bacteria, providing the bacteria with a constantly self-replenishing replicative niche[48]. The mycobacterial RD1 secretion system is required for both granuloma formation and recruitment of new macrophages, leading

to the proposal that the development of granulomas is orchestrated by the bacterium to take advantage of the host response[48]. However, several studies have demonstrated that the host immune system can directly detect and respond to either secretion systems or secretion system activity of diverse pathogens[49–52]. Granuloma formation may therefore be the result of a highly co-evolved interplay between host immune responses and bacterial virulence mechanisms.

In addition to their ability to redirect trafficking of infected macrophages, *Mycobacteria* produce a complex array of unique surface lipids that have immunomodulatory properties and are important for mycobacterial virulence[53]. Such molecules include phenolic glycolipid (PGL), which has been reported to inhibit production of the pro-inflammatory cytokines TNF-α, IL-6, and MCP-1 (ref. 54), and lipoarabinomannan (ManLAM), which prevents dendritic cell maturation by binding to a lectin specific for dendritic cells designated DC-specific ICAM-3 grabbing non-integrin (DC-SIGN)[53,55]. How these bioactive lipids modulate the host immune response is not entirely clear. However, recent data suggest that interaction of a number of persistent pathogens, including *M. tuberculosis*, with DC-SIGN modulates TLR-dependent NF-κB activation[56]. It also seems that, at least in some cases where immunomodulatory lipids cause broad immunosuppression, their expression is selected against within the mycobacterial population or downregulated during the chronic phase of infection[54,57].

These data support the notion that long-term persistence of pathogens in a host population selects for virulence factors that act to modulate or fine-tune immune responses, and may select against those that globally inhibit the host response. In many cases, the exact targets for these virulence factors remain to be identified. However, they are likely to be different from the ones targeted by virulence factors of acute pathogens. Alternatively, the targets may be the same but the mechanism by which they are affected may be different, resulting in modulation of target protein function rather than complete inhibition.

Intracellular pathogens such as *M. tuberculosis* and *Salmonella* have fundamentally different metabolic requirements during persistent and acute infections, as demonstrated by the observation that for both organisms, enzymes of the glyoxylate shunt pathway of fatty acid utilization are essential for persistence but dispensable for acute infection[58–60]. Recently, genome-wide screens identified gene products specifically required for persistence of *Salmonella* and *M. tuberculosis*[61,62]. Many of these genes have no previously defined function, indicating that further studies are necessary to delineate the nature of the host–pathogen interactions that occur during chronic infection. In the case of *Salmonella*, several genes, such as those encoding the *Salmonella* secretion system involved in invasion of mammalian cells, now seem to be necessary for both acute and persistent infection[61]. The precise role of these gene products during persistent infection remains to be identified, but it is possible that they function analogously to the *M. tuberculosis* secretion system in promoting re-infection of new cells at sites of bacterial persistence.

Targeting nodes of adaptive host defence networks

Long-term association of pathogens with their hosts requires that virulence factors also target adaptive immune responses in a manner that does not produce complete immunosuppression yet enables pathogen persistence. *Haemophilus influenzae*, *Streptococcus pneumoniae*, and *Neisseria* species are routinely found as part of the normal flora in mucosal epithelia of the nasopharynx of most healthy individuals, but can also cause acute,

sometimes life-threatening, disease[63]. How these bacteria switch from a persistent subclinical to an acute inflammatory state remains an area of active investigation, but in addition to a capsule that enables these bacteria to avoid phagocytosis, they secrete a virulence factor, IgA protease, that cleaves IgA at the hinge region of the heavy chain[64]. IgA is the major immunoglobulin isotype found at mucosal sites, and is secreted in large quantities into the intestinal and respiratory lumen through uptake at the basolateral side of the mucosal epithelium by specialized polymeric Ig receptors[65]. Secretory IgA (sIgA) is primarily found as a dimer of IgA molecules and controls the homeostasis of mucosal tissues by forming immune complexes with particulate antigens and preventing binding of bacteria to epithelial surfaces. sIgA is greatly reduced in the intestinal lumen of animals that are raised under germ-free conditions; sIgA production is therefore thought to be driven by the commensal flora and to shape the commensal microflora found at mucosal sites[66,67]. Interestingly, IgA protease is associated with members of the microbial flora that cause invasive disease, suggesting that it has an important role in pathogenesis[68–70]. However, because these organisms are also found as components of the normal flora in the absence of disease, IgA protease may enable these bacteria to establish a persistent subclinical infection, with the shift to acute infection depending on other virulence factors or a change in host immune status. IgA protease is a prime example of fine-tuned modulation of the adaptive immune response by bacteria that establish persistent infections of mucosal surfaces.

Conclusions and future perspectives

Microbial pathogens use a diverse arsenal of virulence factors that enable them to colonize and replicate within a broad spectrum of both intracellular and extracellular host environments. As our understanding of host defence mechanisms and microbial recognition has advanced, we have gained further insight into the host targets and mechanisms of microbial virulence factor activity, and how these factors modulate the host response. The recently identified TIR-domain-containing proteins that interfere with TLR signalling[30,31], as well as the recent finding of a previously uncharacterized enzymatic activity in a class of virulence factors that irreversibly dephosphorylate MAPK proteins[10–12], provides additional evidence of the diversity of strategies taken by bacterial pathogens to manipulate the host response. Descriptions of newly identified cellular processes are often rapidly followed by identification of a virulence factor that interferes with that process. This suggests that our knowledge of bacterial virulence factors is heavily biased by the preferential analysis of their effects on well-characterized host processes. In addition, a great deal of work remains to be done to understand both the biochemical function and the biological role of the vast majority of known virulence factors.

We have suggested here that acute infections are caused by pathogens that target classes of proteins different from those targeted by pathogens during chronic infection. Specifically, acute pathogens, given their 'objectives', tend to target central hubs in immune signalling networks. As networks are vulnerable to deliberate attacks on central hubs[71], acute pathogens efficiently disable host defences and other physiological functions. In contrast, persistent pathogens have a different agenda: they tend to maintain a long-term, often subclinical association with their host and therefore must avoid targeting the hubs in host defence networks, given that their disruption results in strong immunosuppression that disrupts the balance necessary to maintain the persistent state. This is, of course,

a simplified view, and examples can doubtless be found that do not fit neatly into this framework. Host–pathogen interactions are not static and are subject to changes in immune status and niche availability, as occurs with cystic fibrosis patients colonized by *Pseudomonas aeruginosa*, or burn patients colonized by the same organism, with very different outcomes[72]. The few examples provided here are not intended to be all-encompassing, but rather, illustrative of a general framework that may provide a useful starting point to better understand the diversity of host–pathogen interactions. As the nature of persistent infections is often subclinical, the actual host targets, and indeed many of the virulence factors involved in maintaining persistence, remain unknown or poorly characterized, providing an important area for future investigation.

Furthermore, the combinatorial activities of virulence factors of host-adapted pathogens can result in modulated or fine-tuned responses even for those pathogens that cause acute infection. This has been proposed to occur in the case of *Shigella*, which initially triggers a robust pro-inflammatory response in the intestine to facilitate its dissemination, but then modulates this response by means of a virulence factor that downregulates a subset of NF-κB dependent genes[11]. *Salmonella* invasion of host cells requires virulence factor-induced disruption and rearrangement of cellular actin, which is then rapidly restored by another virulence factor[73]. These observations demonstrate the requirement for host-adapted pathogens to modulate their interactions with the host, even during acute infections. It is therefore likely that the expression or secretion of factors with seemingly opposing activities is tailored to particular times during infection. Future advances in the field of host–pathogen interactions will therefore require a higher-resolution understanding of the kinetics and location of virulence factor expression within the host over the course of infection, as well as whether the targets of virulence factors are nodes or hubs in signalling networks. Such an understanding is likely to reveal new general principles of host-pathogen interactions and to provide new targets and strategies for antimicrobial therapies.

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