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Review

Bacterial pore-forming toxins: The (w)hole story?

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Abstract. Pore-forming toxins (PFTs) are the most common class of bacterial protein toxins and constitute important bacterial virulence factors. The mode of action of PFT is starting to be better understood. In contrast, little is known about the cellular response to this threat. Recent studies reveal that cells do not just swell and lyse, but are able to sense and react to pore formation, mount a defense, even repair the damaged membrane and thus survive. These responses involve a variety of signal-transduction pathways and sophisticated cellular mechanisms such as the pathway regulating lipid metabolism. In this review we discuss the different classes of bacterial PFTs and their modes of action, and provide examples of how the different bacteria use PFTs. Finally, we address the more recent field dealing with the eukaryotic cell response to PFT-induced damage.

Keywords. Pore-forming toxins, aerolysin, cholesterol-dependent cytolysin, cellular response, caspase-1.

Introduction

Pore-forming toxins (PFTs) represent the largest class of bacterial protein toxins and are often important virulence factors of a pathogen [1]. Forming pores in the membrane of target cells, which leads to cellular ion imbalance, is a widely used form of attack. Beside bacteria, many organisms as different as cnidarians, mushrooms and plants also produce PFTs [2–4]. Strikingly, even mammals utilize PFT-like proteins such as perforins as a part of their innate immune defense [5]. The fact that the use of pore formation is so widely spread suggests that PFTs evolved early in time and, therefore, represent an ancient form of attack. Until now, bacterial PFTs are the best described (for a review see [1, 6]). They are secreted by the pathogens in a water-soluble form that binds to the target cell and generally multimerizes into an amphipathic structure that finally inserts in the target cell membrane and forms a pore (Fig. 1). This ability to convert from a soluble to a transmembrane form is common to all PFTs and constitutes one of their most remarkable features. In this review, we discuss the different classes of bacterial PFTs and their modes of action. We also provide a few examples of how the different bacteria use PFTs and then address the more recent field of eukaryotic cell responses to the damage induced by the PFTs. However, it should be noted already here that the knowledge about toxin-induced cellular phenomena lags behind our understanding of the structural mechanisms. This is probably because cell lysis was previously assumed to be the only cell fate after pore formation and, as such, the (w)hole story. Most probably it is just the beginning.

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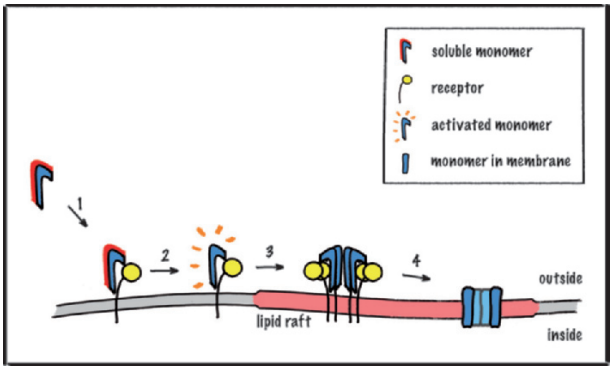

Figure 1. General mechanism of pore formation by β-pore-forming toxins (PFTs). Most PFTs are produced by the bacteria in form of soluble precursor toxins that diffuse towards the target cell and bind with high affinity through the interaction with a specific receptor (1). Some PFTs may then require activation by proteolytic cleavage (2). Common to all β-PFTs is the absolute requirement for oligomerization (3), a process that can be promoted by lipid rafts, into ring like structures that insert into the membrane and form pores (4).

Classifications of PFTs

PFTs are best classified according to the type of structures they use to insert into the lipid bilayer upon pore formation, *i.e.*, α-PFT cross the membrane as α-helices and β-PFTs as β-sheets (Fig. 2). Pore-forming colicins secreted by *Escherichia coli* are representative members of the α-PFTs family [7]. This family also includes the translocation domain of Diphtheria toxin [8], as well as the mammalian anti-apoptotic protein Bcl2 [9]. The pore-forming domains of multiple α-PFTs such as colicins and Cry toxins from *Bacillus thuringiensis* have been crystallized in their water-soluble forms [6, 10–13]. They all form a three-layered structure of up to ten α-helices, which sandwich a hydrophobic helical hairpin. Partial unfolding of the protein, triggered by locally low pH and possibly other mechanisms, is thought to expose this central hydrophobic helical hairpin that then spontaneously inserts into the lipid bilayer [6]. Whereas partial unfolding might be sufficient to allow membrane insertion, channel formation most likely requires oligomerization of the protein. No definitive proof for multimerization is available for this class of toxins and thus the exact mechanism of pore formation remains unclear (Fig. 2a).

Formation of the transmembrane channel is much better understood for the β-PFT family. In contrast to α-PFTs, sequence analysis predicts entirely soluble β-PFTs with no detectable hydrophobic stretches [1, 6]. β-PFTs contain a high percentage of β-structure, and, in the early 90 s, it was suggested that these toxins might cross lipid bilayers as β-barrels as observed for bacterial porins [14]. Studies on different toxins from this family have confirmed this hypothesis and led to the elucidation of an overall common mode of action. As all toxins, β-PFTs are synthesized as soluble proteins. At high concentration, they have the ability to multimerize into circular polymers, a step that for certain toxins, such as aerolysin, requires proteolytic activation [15, 16]. Upon multimerization, each monomer contributes one (aerolysin from *Aeromonas hydrophila* and α-toxin from *Staphylococcus aureus*) or 2 (cholesterol-dependent cytolysins [17]) amphipatic β-hairpins, which together generate an amphipathic β-barrel. This β-barrel exhibits a hydrophilic cavity and a hydrophobic outer surface thus allowing membrane insertion [17–20]. The number of monomers varies from 7 in the case of aerolysin and staphylococcal α-toxin [21–23] to up to 50 in the case of the cholesterol-dependent cytolysins (CDC), which include streptolysin O (SLO) and perfringolysin O [24, 25]. The pores formed by β-PFTs vary greatly in size, from 2 nm in diameter for aerolysin and α-toxin, to 50 nm in diameter for CDC [6]. CDC pores can allow the passage of fully folded proteins and have, therefore, widely been used in cell biology to generate semi-permeabilized cell systems [26]. The difference in size between aerolysin- and CDC-type PFTs has a number of cellular consequences that are discussed below. It is presently not clear why different bacteria have evolved to produce toxins that generate pores of different sizes. The required cellular response during infection might provide the key to this interesting question.

A number of proteins that do not fully qualify as PFTs should also be mentioned briefly. Most prominent amongst these are the translocation domains of certain non-pore-forming toxins. These are the so-called AB toxins, where the B subunit is responsible for binding to the target cell and translocation of the A subunit into the cytoplasm. The A subunit bears the enzymatic activity. Examples include the already mentioned Diphtheria toxin, the translocation domain of which resembles colicins [27], and anthrax toxin, the B subunit of which forms a heptameric transmembrane channel reminiscent of the aerolysin and Staphylococcal α-toxin channel [19, 28, 29]. In contrast to *bona fide* PFTs, B subunits of AB toxins do not insert into the plasma membrane of target cells because they require an acidic environment for membrane insertion to occur. Pore formation by B subunits only occurs in the endocytic pathway upon internalization [30–32].

Also related to PFTs are the tips of bacterial type III or type IV secretion systems (for review see [33]). Many pathogenic Gram-negative bacteria inject bacterial proteins into the host cell cytoplasm [34]. They do so by injecting proteins directly from their cyto-

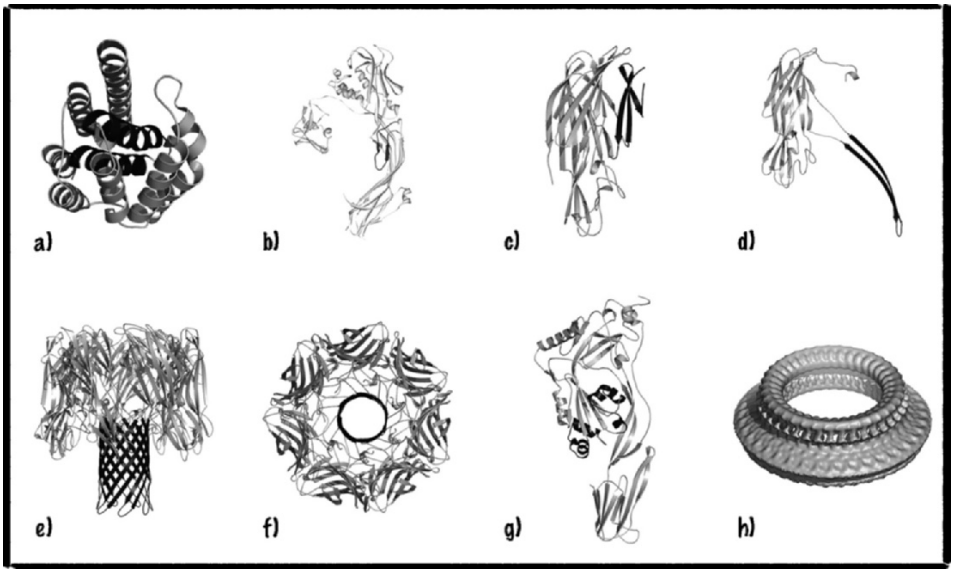

Figure 2. α- and β-PFTs. Ribbon representations of α-and β-PFT structures. For each toxin, the region involved in forming the lipid-spanning region of the pore is highlighted by dark shading. (*a*) α-PFT family member colicin B secreted by *Escherichia coli.* (*b*) The structure of the water-soluble aerolysin by *Aeromonas hydrophila.* (*c*) The water-soluble LukF monomer. LukF is a PFT produced by *Staphylococcus aureus* and is thought to have a structure similar to that of the α-toxin from the same organism. (*d*) β-PFT family member α-toxin protomer, produced by *S. aureus,* as observed in the pore structure shown in (*e*, *f*). (*e*, *f*) *S. aureus* α-toxin heptameric pore viewed (*e*) perpendicular to the pore axis, and (*f*) down the axis [45]. (*g*) *Clostridium perfringens* perfringolysin O as an example of a water-soluble CDC monomer. (*h*) Cryo-EM reconstruction of the *Streptococcus pneumoniae* pneumolysin pore [90]. Note the difference in monomer number and size between (*f*) and (*h*) (not same scale). All figures were produced by the computer program PyMol, except (*h*) which was taken from [90].

plasm into that of the host cell through syringe-like structures called type III and IV secretion system. The type III secretion system (T3SS) is evolutionarily related to the flagellum and crosses both inner and outer bacterial membranes [35, 36]. It must also cross the host cell membrane and does so by producing proteins that have pore-forming ability. Examples include IpaB from *Shigella flexneri*, SipB from *Salmonella* sp. or YopB and YopD from *Yersinia* sp. [33]. Little is known about the structure by which these proteins cross the lipid bilayer. The high helical content and the presence of hydrophobic stretches indeed suggest an α-helical transmembrane domain [37].

Here we focus on PFTs and proteins that affect mammalian cells, *i.e.*, β-PFTs (since *bona fide* α-PFTs such as colicins affect bacteria) and effectors of T3SSs.

PFTs and their general mode of action

Once secreted by the bacteria, PFTs diffuse towards their target cell, to which they bind *via* a specific receptor, often with high affinity [1]. The toxin thus hijacks cell surface structures, which are normally destined to other functions. Binding of the toxin to the cell surface is the first step in the intoxication process. If the receptor is not ubiquitous, this leads to cell-type-specific intoxication. In the absence of toxin-specific receptors, cells are indeed resistant to the action of the PFTs [38]. Receptors for a variety of PFTs have been identified (see Table 1 for a selection). These include transmembrane proteins, acting as receptors for the B subunit of AB toxins [39], lipid-anchored proteins in the case of aerolysin [40], lipids for cholesterol-dependent cytolysins [41], and even specific lipid clusters proposed for Staphylococcal α-toxin binding [42]. These receptors provide a number of advantages to the toxins. First, they provide binding sites to the cell surface. Lowering the diffusion space from three dimensions in the extracellular medium to two dimensions on the cell surface leads to a 1000-fold increase in concentration [43], which in turn increases the probability of monomer collision, a step essential in the oligomerization process, and thereby the efficiency of oligomerization. Further increase in local toxin concentration is obtained either because the receptor is pre-clustered or has a tendency to associate with specific membrane domains such as lipid rafts that are enriched both in glycosyl-phosphatidylinositol (GPI)-anchored proteins (the receptors for aerolysin) and cholesterol (the receptors for CDCs) [44].

As mentioned, oligomerization occurs in a circular fashion, leading to ring-like structures [45]. Certain toxins such as Staphylococcal α-toxin initially form a

Table 1. Bacterial pore-forming toxin (PFT) and related proteins.

Toxin	Bacterium	Receptor	Toxin type	Reference
Aerolysin	*Aeromonas hydrophila*	GPI-APs	Small β-PFT	[60]
α-toxin	*Staphylococcus aureus*	Lipid clusters	Small β-PFT	[42]
α-toxin	*Clostridium septicum*	GPI-APs	Small β-PFT	[59]
Cry5B	*Bacillus thurgiensis*	GPI-APs, glycolipids, lipids	Small α-PFT	[168]
Streptolysin O (SLO)	*Streptococcus pyogenes*	Lipid/cholesterol	CDCs (β-PFT)	[169]
Listeriolysin O (LLO)	*Listeria monocytogenes*	Lipid/cholesterol	CDCs (β-PFT)	[41]
Pneumolysin O (PLY)	*Streptococcus pneumoniae*	Lipid/cholesterol	CDCs (β-PFT)	[41]
Perfringolysin O (PFO)	*Clostridium perfringens*	Lipid/cholesterol	CDCs (β-PFT)	[41]
Anthrax toxin	*Bacillus anthracis*	CMG2, TEM8	AB/Small β-PFT	[96, 97]
Diphtheria toxin	*Corynebacterium diphtheriae*	HBEGF	AB	[170]
Vcc	*Vibrio cholerae*	Carbohydrate receptors	Small β-PFT	[171]

pre-pore that undergoes a second series of conformational changes that lead to membrane insertion [46]. For others, such as aerolysin, oligomerization, folding of the transmembrane β-barrel and membrane insertion appear to be coupled events [18]. Exposure of the hydrophobic surface on the outside of the formed β-barrel is thought to provide the energy for membrane insertion, although no evidence is currently available to support this hypothesis.

Toxins forming small pores: Aerolysin and related PFTs

Aerolysin is one of the best-studied toxins from the category of small channel-forming toxins (reviewed in [47]). The toxin is produced by various species of the genus *Aeromonas* and is synthesized by the bacteria as a pre-protoxin. Pre-proaerolysin has a N-terminal signal sequence that directs it to translocate across the inner membrane of the bacteria [48]. In the periplasm of the bacteria, the toxin folds [49] and the precursor, proaerolysin, is then translocated into the extracellular medium through a type II secretion apparatus [50, 51]. After binding to GPI-anchored proteins at the target cell surface [40, 52–54], proaerolysin monomers are activated by proteolytic cleavage. However, activated aerolysin also readily binds to GPI-anchored proteins. Efficient proteolysis can be performed by proteases produced by the bacterium itself [16], enzymes of the digestive tract such as trypsin [16] or α-chymotrypsin, or by cell surface-associated proteases, such as furin [15]. Activation, consisting of cleavage of the 40-amino acid C-terminal precursor peptide, is accompanied by a conformational change required for heptamerization and pore formation. Although the toxin may exist as a dimer in solution [55, 56], surface binding occurs in the monomeric state [57].

Aerolysin is L-shaped (Fig. 2b) with a small N-terminal lobe (domain 1) and a large lobe organized in three additional domains [58]. Domains 1 and 2 are involved in binding to the GPI-anchored receptors. Domain 2 binds to the glycan core of the GPI anchor [59] and domain 1 binds to N-linked sugar on the protein moiety [60, 61]. This double binding leads to a high-affinity interaction of aerolysin with its receptor. Virtually all GPI-anchored proteins, which are generally highly glycosylated, can be used by aerolysin as a receptor, including Thy-1 [40], contactin [54], CD14, carboxypeptidase M or NCAM (neural cell-adhesion molecule) [62]. Notable exceptions are the prion protein (unpublished) and CD59 [60].

Domain 2 is also involved in initiating the oligomerization process [63], while domains 3 and 4 keep the heptamer assembled. The stability of this complex is reminiscent of that of prion aggregates, being resistant to boiling, detergents and high levels of chaotropic agents [64]. The membrane-spanning region corresponds to a ~20-amino acid loop that localizes to domain 3 (Fig. 2b), which folds into an amphipathic β-hairpin upon oligomerization and membrane insertion [18].

A homologue of aerolysin, α-toxin, is produced by *Clostridium septicum*. It shares 27 % sequence identity and 72 % sequence similarity with aerolysin and is the main virulence factor of the bacterium [65]. As aerolysin, α-toxin is secreted as an inactive protoxin, which is proteolytically activated by removal of a C-terminal peptide and binds to GPI-anchored membrane proteins [59]. The affinity for GPI-anchored proteins is, however, lower than aerolysin due to the fact that α-toxin lacks the amino acid sequence corresponding to domain 1 of aerolysin, so that binding occurs only to the glycan core of the GPI anchor [61].

Another PFT forming small pores is α-toxin from *S. aureus*. Studies on α-toxin insertion showed that α-toxin precursors bind to specific lipid clusters at the membrane of target cells [42]. Pore formation differs from that of aerolysin in that α-toxin first forms a prepore [22, 66–69]. A conformational change then leads to the detachment of 3 small β-strands from the core of the protein (Fig. 2c) that unfold and refold to form a 14-stranded β-barrel with equivalent segments in the other protomers (Fig. 2e, f) [70–73].

Although the crystal structures of the water-soluble monomers of several small-pore-forming β-PFTs have been solved, only a single high resolution structure of the oligomer in a transmembrane state is available due to the difficulty of growing crystals of membrane proteins. The crystal structure of the *S. aureus* α-toxin in its oligomeric form (Fig. 2e, f) [45] shows that the complex has a mushroom-like shape and measures approximately 10 nm in height and 10 nm in diameter. The structure revealed that seven β-hairpins associate together to form a 14-stranded antiparallel β-barrel that constitutes the transmembrane channel. Alternating polar and non-polar residues compose the transmembrane β-hairpin region. This structural element is also present in aerolysin [18], the B subunit of anthrax toxin, called protective antigen, [19] and *C. septicum* α-toxin [20].

Large-pore formers: The CDCs

CDCs are around 60 kDa in size and form the largest family of PFTs. As mentioned above, CDC pores can be formed by up to 50 monomers, each contributing 2 β-hairpins to the transmembrane structure, thus leading to a 200-stranded β-barrel, hence the classification of large-pore former CDCs (for a review see [6, 74]). To date, the CDC family comprises over 20 members from 24 different Gram-positive bacterial species such as *Clostridium*, *Streptococcus*, *Listeria* or *Bacillus* [6, 74]. Interestingly, almost all CDC-producing bacteria are pathogenic for humans and/or animals. Most CDCs are produced extracellularly and insert into the plasma membrane of the target cell. This plasma membrane permeabilization may serve to affect the host cell (see below), or to transfer proteins secreted by the bacterium into the host cell as proposed for SLO from *Streptococcus pyogenes* [75]. Certain pathogens, such as *Listeria monocytogenes*, can produce their CDC both extracellularly [76] as well as after entry into the target cell by phagocytosis [77]. Insertion of this CDC, termed listeriolysin O (LLO), into the phagosomal membrane allows the bacterium to escape into the cytoplasm of the target cell. Interestingly, of all CDCs, LLO is the only one that shows pH sensitivity [76], with a greatly increased pore-forming activity at low pH corresponding to the lumen of the phagosome. In contrast, activity is lower at the neutral pH corresponding to that of the extracellular space, as well as that of the host cell cytoplasm after bacterial release [76]. It can, however, not be excluded that LLO has additional roles when produced in the cytoplasm of the host cell.

At the level of the primary structure, CDCs display a high degree of sequence similarity ranging from 40% to 80% [6]. This is mainly reflected in the conserved core of about 471 amino acids shared by all CDCs, which essentially corresponds to the sequence of pneumolysin (PLY), the shortest member of the family [78]. CDCs with longer sequences usually display variations in the N terminus, the functions of which are unknown for many members. Furthermore, all CDCs contain a highly conserved undecapeptide, which is thought to be critical for cholesterol-mediated membrane recognition [79, 80]. CDCs indeed all share a strict cholesterol dependency for oligomerization, which gave them their name. Most CDCs seem to use cholesterol directly as a receptor [81, 82]. Intermedilysin (ILY) from *Streptococcus intermedius,* however, was shown to have a proteinaceous receptor, *i.e.*, the GPI-anchored protein CD59 [83]. Interestingly, ILY shows a lower degree of conservation in the conserved undecapeptide important for cholesterol binding [84]. As for all CDCs, pore formation by ILY requires the presence of cholesterol for the membrane insertion step [85]. The concentration and distribution of cholesterol in the membrane therefore probably plays a major role in the oligomerization process of CDCs and binding to cholesterol alone does not seem to be the full story [85]. Since microdomains (or lipid rafts) are also modulated by membrane cholesterol levels, it has been proposed that these lipid rafts further regulate CDC oligomerization [86, 87]. *Vice versa*, binding of CDCs to the membrane could stabilize or enlarge lipid rafts [88, 89] and trigger signaling events.

A recent electron microscopy reconstruction of PLY from *Streptococcus pneumoniae* nicely illustrates the hydrophobic pore complex (Fig. 2h), while Figure 2g shows the x-ray structure of the closely related perfringolysin O (PFO) monomer from *Clostridium perfringens* [90]. After membrane binding, oligomerization leads to a ring like structure termed the prepore. Membrane insertion occurs through a subsequent conformational change, the trigger for which might be the completion of the oligomerization process. During this transition, two α-helical bundles in each monomer convert to a pair of amphiphatic β-hairpins that will ultimately line the pore [17, 41, 90]. There is still debate concerning the size of the

channels that CDCs can make. Pores of up to 40 nm [90] diameters are generally admitted and *in vitro* experiments excluded the possibility of intermediate size pores by showing that PFO can form only large channels [91, 92]. However, intermediate arc-shaped oligomers, lined by protein on one side and by lipid on the other have been observed by cryo-electron microscopy and are thought to be functional [93, 94]. Such structures, however, seem highly unfavorable in terms of energy and raise the possibility that the arc-like structures are a consequence of the preparation for electron microscopy. CDCs could also make smaller size pores, formed by fewer monomers.

Channel formation by the anthrax toxin B subunit, or protective antigen

Among the bacterial protein toxins, some members of the so-called AB toxins can be regarded as PFTs, such as diphtheria and anthrax toxins. The anthrax toxin from *Bacillus anthracis* is an AB toxin (for review see [95]), the B subunit of which shares many characteristics with β-PFTs. Anthrax toxin is composed of three independent polypeptide chains: the protective antigen (PA), the lethal factor (LF) and the edema factor (EF). Anthrax PA is secreted in a soluble form that has high affinity for two mammalian transmembrane proteins: tumor endothelial marker 8 (TEM8) and capillary morphogenesis gene 2 (CMG2) that act as its receptors [96, 97]. Like proaerolysin, PA undergoes proteolytic activation at the cell surface *via* members of the furin family [98] and subsequent oligomerization into heptameric structures [30]. In contrast to aerolysin, pore formation does not occur at the cell surface, but only at the acidic pH of endosomes [31, 99]. Interestingly, the pH sensitivity is not dictated solely by the PA molecules but is provided by the interaction with the receptor [100, 101]. Upon acidification, PA is thought to be partially released from the receptor [102], allowing the membrane-spanning region to unwind in each monomer and assemble together into a 14-stranded β-barrel [19].

The two enzymatic subunits have no surface receptors but interact with the PA heptamer and are co-endocytosed with the complex, *via* clathrin-coated pits [103]. At acidic pH, the PA heptamer inserts into the membrane [99, 104]. Low pH also triggers partial unfolding of EF and LF, which can then cross the membrane through the PA channel [105, 106]. Interestingly, the PA channel was found to form not in the limiting membrane of endosomes, but rather in the intraluminal vesicles of this multivesicular organelle [31]. Release into the cytoplasm of the enzymatic subunits, therefore, requires back fusion of these intraluminal vesicles with the limiting membrane of the organelle. Little is known about this back fusion process, which could be promoted by the PA channel. The anthrax toxin is, therefore, an interesting example of a combination between a PFT structure and enzymatically active proteins.

Type III secretion systems

Another bacterial virulence factor that represents some similarities with PFTs is the T3SS, which is produced by many Gram-negative bacteria. This secretion machinery allows bacteria to modulate eukaryotic cells by injecting proteins directly into their cytosol (for review see [33]). The T3SS has been well characterized in bacteria like *Shigella*, *Yersinia* or *Salmonella* and is thought to be derived from the bacterial flagellum due to structural similarities [34–36]. The T3SS injection system is found in both pathogenic and symbiotic bacteria. It is composed of an injectisome, which represents a needle-like structure and a translocator, which forms a pore in the membrane of the target cell allowing the transport of proteins directly into the eukaryotic cell. These proteins, called effectors, are able to interfere with the normal control and signaling of the target cell. IcsB, for example, is secreted by the invasive bacteria *Shigella flexneri,* and allows the bacteria to escape from the cellular autophagic pathway by preventing bacterial recognition by the cell [107]. Other effectors have been shown to act on the actin cytoskeleton by modulating phagocytosis through induction [76] or inhibition [34, 108] of bacterial uptake. Additionally, some effectors have also been shown to modulate vesicular trafficking once the bacterium has been internalized [109]. It has also been reported that some of them modulate the innate and adaptive immune response [110, 111].

Role of PFTs in pathogenesis

Although it has been clearly established for a variety of bacterial pathogens that their PFT(s) contribute and are sometimes essential for pathogenesis, the exact role of PFTs is not always clear. It is also noteworthy that the events triggered by pore formation in the plasma membrane of a target cell vary depending on toxin concentration, exposure time to the toxin, as well as target cell type (Fig. 3). Toxin concentrations during infection have, however, not been established and are likely to vary depending of the site of infection and the distance between the target cell and the producing bacterium.

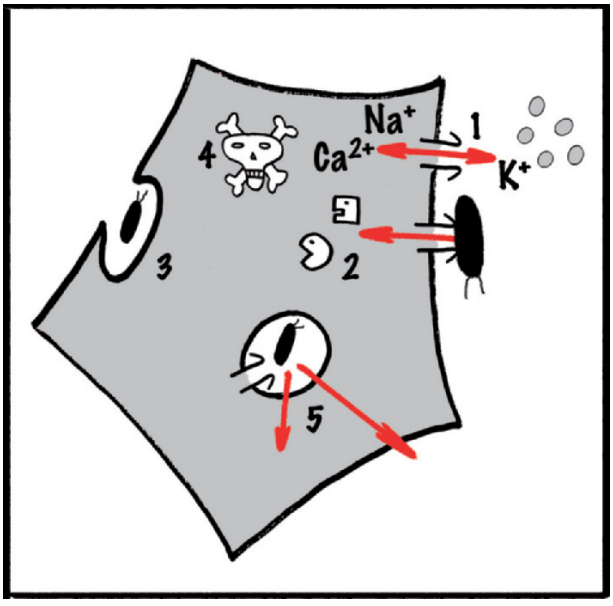

Figure 3. Induced cellular effects of PFTs. One effect of pore formation at the plasma membrane is the permeabilization of the plasma membrane to ions leading to changes in cytoplasmic ion composition and the release of nutrients (1). The large pores formed by members of the CDC also allow the efflux of intracellular proteins (1). Bacteria can also directly inject proteins, termed effectors, into the target cell cytoplasm *via* type III or type IV secretion machineries (2). These bacterial proteins are able to modify specific cellular mechanisms for the benefit of the bacterium, *e.g.*, inducing phagocytosis of the bacterium (3). All these effects of bacterial toxins and effectors may eventually lead to the death of the host cell (4). Pore formation can also occur after the bacterium has been internalized. This allows, for example, escape of the bacterium from the phagosome into the cytosol, where it can replicate, or egress of the bacterium outside the cell once replication in the target cell has occurred (5).

One of the clearest absolute requirement for a PFT during infection is that of *Listeria* for LLO. This toxin mediates escape of the bacterium into the cytoplasm, thus preventing phagosome maturation and killing of the bacterium in phagolysosomes [112]. LLO, however, appears to contribute to pathogenesis in a variety of other fashions. Pore formation of LLO at the cell surface generates calcium-dependent and lipid-mediated signaling, leading to a variety of events such as tyrosine phosphorylation events [89], actin rearrangement [113], NF-κB activation [114] and regulation of gene expression through histone modification [115]. LLO was also found to induce calcium influx or, in certain cases, even oscillations [116]. These oscillations were also observed in the case of *E. coli* α-hemolysin (HlyA) [117, 118]. Calcium puffs and spikes are known to be complex mechanisms that modulate transcriptional regulation in an intensity- and frequency-dependent manner [119, 120]. The LLO-induced calcium waves have been shown to regulate different cellular functions such as bacterial internalization, endocytic vesicle fusion, degranulation, cytokine synthesis and apoptosis [121, 122]. Activation of similar intracellular events has been reported for other PFTs, such as actin rearrangement or NF-κB activation by PLY [123].

The importance of PFTs during infection has also been clearly established for PLY [124], produced by all serotypes of *Streptococcus pneumoniae* [125–127] and is essential for virulence. PLY contributes to the pathogenesis of various pneumococcal disease syndromes in a number of ways (for review see [128]). PLY has several distinct functions, especially in the early pathogenesis of pneumococcal infection. The enzyme is cytotoxic to ciliated bronchial epithelial cells and disrupts tight junctions and the integrity of the bronchial epithelial monolayer [129, 130]. In addition, PLY action disrupts the alveolar-capillary boundary favoring the spread of the bacteria into the pulmonary interstitium and ultimately into the bloodstream [128]. The cytotoxic effects of PLY can directly inhibit phagocyte and immune cell function, which leads to suppression of the host inflammatory and immune responses [128].

Another situation where PFT requirement has been put forward is that of the delivery by *Streptococcus* of bacterial NAD-glycohydrolase into the eukaryotic target cell cytoplasm. Translocation was shown to occur *via* the SLO pore by a polarized process [75]. The authors describe that SLO, the CDC of the Gram-positive pathogen *S. pyogenes,* directs the translocation of a streptococcal effector molecule into the host cell cytosol. Furthermore, the effector, which is capable of producing the potent eukaryotic second messenger molecule cyclic ADP-ribose, contributes to a cytotoxic response in keratinocytes during infection by *S. pyogenes* [45]. These data provide a novel function for the large family of CDCs and suggest that toxin-mediated translocation may be a general mechanism for injection of effectors and, thus, serve as the functional equivalent of T3SSs for Gram-positive pathogens.

Sensing bacterial toxins: The Nod-like receptors

Considering that pore-formation constitutes a very ancient form of attack that resembles mechanical membrane rupture, it is likely that cells have evolved mechanisms to resist and/or repair this kind of damage. This implies that cells have means to sense impairment of their plasma membrane, one of the first consequences of which is changes in ionic composition (Fig. 4).

The innate immune system provides a first line of host cell defense against many microorganisms including bacteria. The cells of the innate immune system,

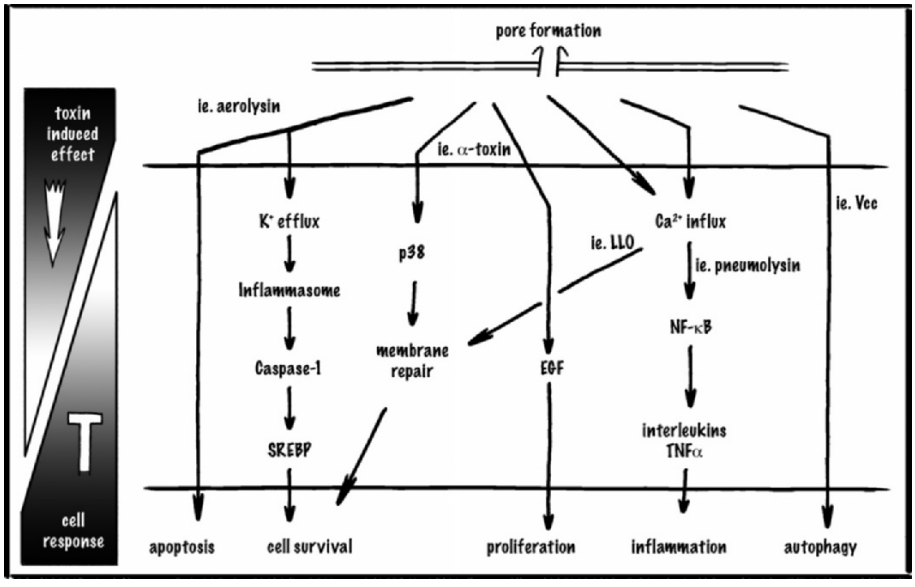

Figure 4. Cellular responses induced by PFTs. Several pathways activated in the host cell by PFTs are depicted. EGF, epidermal growth factor; SREBP, sterol responsive element binding protein; LLO, listeriolysin O; SLO, streptolysin O.

usually include macrophages and neutrophils but also epithelial cells, which constitute the first physical barrier to pathogens. Unlike adaptive immunity, the innate immune system is a nonspecific immune response based on a much smaller number of receptors called pattern recognition receptors (PRRs). These receptors are microbial sensing proteins that recognize conserved structural components of the microorganisms, called pathogen-associated molecular patterns (PAMPs) [131]. Bacterial cell wall components such as peptidoglycan, bacterial flagellin, and nucleic acid structures, unique to bacteria and viruses, are examples of PAMPs.

The first family of PRRs studied in detail was the Toll-like receptor (TLR) family [132]. TLRs are transmembrane proteins with a ligand-binding domain composed of leucine-rich repeats (LRRs). They are involved in the detection of microbes in the extracellular compartment and in endosomes. More recently, another family of PAMP detectors has been discovered, which in analogy to the TLRs have been termed NLRs or Nod-like receptors [133]. In contrast to the TLRs, they reside in the cytoplasm and have been shown to be activated upon treatment with several PFTs [134, 135]. The NLRs comprise two large sub-classes, the NODs (NOD1–5) and the NALPs (NALP1–14), that have ligand-binding domains composed of LRRs. Upon ligand binding they form large multiprotein complexes allowing interactions with downstream effectors and/or adaptor proteins. The two major documented downstream effects of NLR activation are NF-κB and caspase-1 activation, events that involve different NLR members [136].

NF-κB, a heterodimeric transcription factor, is a key regulator of the pro-inflammatory response, activating genes that encode cytokines and co-stimulatory factors [137]. Interleukin (IL)-8 production, as a consequence of the activation of the NF-κB pathway, was observed upon treatment with Staphylococcal α-toxin [138], aerolysin [139], PLY [140] and LLO [141]. The multiprotein complexes involved in caspase-1 activation have been termed, by Tschopp and coworkers, the inflammasome [142]. Caspase-1 is responsible for the processing and activation of the pro-inflammatory cytokines IL-1β and IL-18 [143]. Among the toxins forming small pores, α-toxin from *S. aureus* and aerolysin from *A. hydrophila* have been shown to activate the inflammasome [134, 135]. In this case, the trigger of inflammasome activation is potassium efflux through the toxin pore. LLO produced by *L. monocytogenes* also results in caspase-1 activation and subsequent IL-1β secretion [144, 145]. It is, however, not clear at what step LLO pore formation is involved in caspase-1 activation: pore formation in the plasma membrane or in the phagosome? Is cytoplasmic release of the bacterium the required event? In other words, is inflammasome assembly triggered by changes in ion composition or by the presence of the bacteria inside the cytoplasm?

Another toxin to which cells seem to respond in an inflammasome-dependent manner is the anthrax toxin. Macrophages treated with anthrax lethal toxin were found to secrete IL-1β and IL-18 after caspase-1 activation [146, 147]. The mechanisms leading to inflammasome activation in response to lethal toxin are still unclear. However, it is unlikely that the LF itself is recognized by intracellular PRRs, since a catalytically inactive LF was unable to trigger caspase-1 activation, despite its delivery to the cytoplasm (Reig, Mellman and van der Goot, submitted). It has

recently been proposed that caspase-1 activation by anthrax toxin depends on the release of intracellular potassium and on the proteasome [148].

Inflammasome activation has also been described upon host cell invasion by type III or type IV secretion harboring bacteria. In these instances, the responsible PAMP was, however, found to be flagellin [136, 149–151]. At present, recognition of bacterial effectors has not been reported. A recent work, however, highlights the importance of membrane impairment induced by the injection needle, suggesting that as for PFTs making small pores, potassium efflux could contribute to inflammasome activation [152].

A consequence of inflammasome activation by PFTs is the processing of proinflammatory cytokines. More recently described is the activation of the central regulators of lipid metabolism, the sterol responsive element binding proteins (SREBPs) [134]. These are membrane-bound transcription factors that control the synthesis of enzymes involved in lipid metabolism [153]. Caspase-1-mediated SREBP activation was, furthermore, found to promote cell survival after toxin attack revealing a mechanism by which the cells seem to react to the damage induced by PFTs.

Other pathways induced by PFTs: P38 MAPK and autophagy

Among the signaling pathways induced by PFTs, some promote cell survival (Fig. 4). The p38 mitogen-activated protein kinase (MAPK) pathway has been shown to be essential to respond to toxin injury in both nematodes and mammalians and was the first one to be described in promoting cell survival. Huffman et al. [154] showed that transcription of p38 was up-regulated in *Caenorhabditis elegans* treated with the Crystal toxin Cry5B produced by *B. thuringiensis*, and that blocking downstream targets of p38 using siRNA produced animals hypersensitive to toxin injury. These results are in agreement with a preview study that showed enhanced *C. elegans* susceptibility to bacterial pathogens when the p38 homolog *pmk-1* was knocked-down [155]. The p38 pathway was also found to be involved in the cellular response of Baby Hamster Kidney cells to aerolysin, since p38 inhibitors led to an increase in toxin-induced cell death [154]. Activation of p38 was also observed upon exposure of epithelial cells to other PFTs like PLY, SLO, *S. aureus* α-toxin and anthrolysin from *B. anthracis* [156–159]. Interestingly, activation of p38 was dependent on osmotic stress induced by pore formation, as it could be prevented by the addition of high molecular weight dextran to the extracellular medium [157]. Furthermore, p38 was shown to contribute to membrane resealing after exposure to Staphylococcal α-toxin, and the p38 pathway was protective to the cells [156]. These results did not extend to SLO treatment [156]. In contrast, upon exposure to the large PFT PLY, p38 was found to mediate neuronal cell death [159]. Thus, p38 activation seems to be a conserved feature of cells responding to a pore; however, the effects of p38 activation might differ from one toxin to the other, possibly related to the size and stability of the formed pore and/or the type of target cell.

In addition to the p38 pathways, PFTs might lead to the activation of other MAPK pathways such as the observed activation of c-Jun N-terminal kinase (JNK) by SLO, leading to TNF-α production [158].

Interestingly, pore formation by Staphylococcal α-toxin was found to trigger cell proliferation through the activation of epidermal growth factor receptor (EGFR) and its crucial adaptor protein Shc [160]. This activation leads to an increase in cell proliferation, to an extend similar to that of treatment with the epidermal growth factor HB-EGF. The mitogenic effect was also induced in cells treated with SLO and seems to be dependent on the hole-forming activity of the toxin, since an inactive Staphylococcal α-toxin mutant did not induce proliferation.

Autophagy has also been reported to be a cellular defense induced upon pore formation by a cytolysin from *Vibrio cholerae* (Vcc) [161]. This PFT has been shown to induce vacuolation and autophagy in target cells. Strikingly, inhibition of autophagosome formation by RNAi or drugs resulted in decreased cell survival upon Vcc intoxication. The invasive bacteria *S. flexneri* as a whole is also known to be targeted by the cell's autophagic machinery, but, as mentioned, escapes it by injecting a specific effector called IcsB into the cytoplasm *via* a T3SS [107]. The innate defense role of autophagy against other bacterial pathogens has also been demonstrated with *S. pyogenes* and *M. tuberculosis* [162, 163]. Recently, autophagy has also been suggested to protect *Shigella*-invaded macrophages, since inhibition of autophagy promoted cell death [164]. Interestingly, caspase-1 seems to inhibit autophagy in this setup, while the adaptor protein ASC positively regulates autophagosome maturation. Autophagy, usually known for degradation of organelles, can therefore act as a specific cellular response during infection with certain pathogens and even upon intoxication with purified PFTs and, thereby, promote cell survival.

Membrane repair

After membrane disruption by a PFT, some cell types have been shown to undergo a recovery process in terms of potassium and ATP levels leading to a prolonged survival. This recovery mechanism has been described for *S. aureus* α-toxin injuries of fibroblasts and also involves p38 [165]. Cells treated with LLO and SLO are also able to repair their lesions, but with distinctly different time scales. While membrane damage induced by large CDC pores is repaired in less than 1 h, cells treated with small-pore formers need many hours to regain normal intracellular potassium levels [26]. Furthermore, it seems that the resealing mechanism after SLO membrane permeabilization does not depend on p38, but on calcium [156]. Calcium influx is generally accepted to be important for membrane repair in physically injured cells, where fusion of intracellular organelles with the plasma membrane constitutes part of the repair mechanism [166]. Plasma membrane repair, therefore, seems to be the most obvious candidate process of a cell's defense, since the main problem is indeed a hole in the membrane. The difference in time scale between the repair of CDC-induced damage (<1 h) and that of Staphylococcal α-toxin induced damage (hours) suggest that different repair mechanisms are involved, the former being calcium dependent and the later p38 dependent. One important differentiating factor could be the stability of the pores, although there is currently no evidence for this. It indeed appears that Staphylococcal α-toxin and even more so aerolysin oligomers are far more stable [167] than those formed by CDCs.

The recently reported link between the activation of the SREBP pathway [134] upon treatment with aerolysin also suggests a role for lipogenic genes in cell survival. An alternative possibility has been suggested for cell recovery following exposure to Staphylococcal α-toxin, which is closure of the pore *via* constriction of the transmembrane β-barrel [165]. Clearly further studies are required to understand the mechanisms leading to cell survival following attack by small-pore-forming PFTs.

Concluding remarks

Majors advance have been made over the last decade in the understanding of the structure and pore-formation mechanisms of PFTs. In contrast, little is known still about the consequences of pore formation at the cellular level. Ion imbalance such as increase in cytosolic calcium and decrease in intracellular potassium appear to be sensed by cells, leading to a variety of signaling cascades, some of which are starting to be elucidated. Pore formation is, however, a major threat for cells and it appears that this stress leads to a great variety of signaling pathways that need to be identified and elucidated.

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