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# Pore-forming toxins and cellular non-immune defenses (CNIDs)

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Pore-forming toxins (PFTs) are the most common class of bacterial protein toxin and are important for bacterial pathogenesis. Recent studies have shown that the previous model stating that epithelial cells lyse in response to these toxins and have no defenses against these pores is oversimplified. Rather, it appears that cells have sophisticated mechanisms and signal-transduction pathways with which to respond to such an attack. There is a growing body of knowledge about how cells respond to and protect themselves against PFTs; this protection against PFTs is likely to be important in host survival to attack by bacterial pathogens, but does not neatly fit into current concepts of adaptive or innate immunity. Therefore, it is proposed that the terminology cellular non-immune defenses (CNIDs) be used to describe defenses that are employed by non-immune cells to protect against bacterial attack.

## Addresses

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## Introduction

Many bacterial pathogens, both Gram-positive and Gram-negative, produce pore-forming toxins (PFTs) that contribute to and are sometimes essential for virulence [1]. PFTs thus comprise about 25% of all known bacterial protein toxins (Table 1). PFTs are also produced by amoeba [2], hydra [3] sea anemones [4], worms [5] and plants [6]. Pore formation thus appears to be a very ancient form of attack. The targets of PFTs are equally diverse, including bacteria, as is the case for colicins [7], insects as is the case for *Bacillus thuringiensis* crystal toxin [8], fish and mammals. Most PFTs share the following mode of action: they are released by the organism in a soluble, generally monomeric, form that freely diffuses towards target cells. With the recently published exception of *Escherichia coli*

hemolysin [9], binding to targets cells occurs by specific interactions with cell surface molecules that can be either lipids, such as for *Listeria monocytogenes* listeriolysin O (LLO) or *B. thuringiensis* crystal toxin [10], proteins, such as for intermedilysin [11], or carbohydrates bound to proteins, such as for *Aeromonas hydrophila* aerolysin [12,13]. Interestingly, specific organization or clusters of lipids can also act as receptors, as recently proposed for earthworm lysenin [14] or  $\alpha$  toxin from *Staphylococcus aureus* [15]. Once bound to its receptor, the toxin undergoes circular polymerization into ring-like structures, the stoichiometry of which varies amongst toxins, for example hexamers, such as for staphylococcal leukotoxins [16], heptamers, such as for aerolysin [17], and 50-mers for members of the cholesterol dependent toxins, such as LLO or streptolysin O (SLO) [18].

Pore formation can occur at the cell surface. This is generally the case for toxins produced by extracellular bacteria such *Aeromonas* species and *S. aureus*. The benefit for the bacterium of host cell plasma membrane permeabilization is not clear. It is unlikely that cell death is the desired effect. Pore formation at the plasma membrane has also been proposed to enable the translocation of bacterial proteins from Gram-positive bacteria, which lack type III or IV secretion systems, to the host cell cytoplasm. Indeed it was found that Streptococci secrete proteins in a very polarized manner and that these secreted proteins gain access to the host cell cytoplasm through pores form by SLO [19]. Pore formation can also occur after the bacterium has been internalized by the host cell. Pore formation then mediates release of the microorganism into the cytoplasm. Examples include LLO mediated release of *Listeria* [20] and SLO mediated release of Group A Streptococci [21]. Finally it has been proposed that egress of *Legionella*, once replication in the target cell has occurred, is mediated by a pore-forming bacterial protein [22].

## Reported cellular effects

Formation of transmembrane pores by PFTs is clearly a major threat to cells and can provoke a spectrum of events that depend on the type of target cell, the type of PFT, the toxin concentration and the exposure time. We first mention cellular effects that have been observed upon pore formation mostly in mammalian cells and then focus on two pathways that have been characterized in more detail and for which contribution to cell survival was shown.

The earliest documented effect of PFTs is their ability to rapidly kill erythrocytes through osmotic lysis.

However, this cell type is not necessarily encountered during infection, as least at an early stage. Nucleated mammalian cells are far more resistant to PFTs, and cell death is dependent on cell type and cell dose. Although not strictly known, it is likely that physiological concentrations of toxins during infection are sublytic, especially for toxins that are produced in the gut or blood stream where there is a significant amount of fluid flow.

The earliest effect of pore formation is clearly the permeabilization of the plasma membrane to ions, and in particular calcium, along their respective electrochemical gradients, leading to changes in cytoplasmic ion composition [1]. The large pores formed by members of the cholesterol dependent toxins (e.g. LLO, SLO and pneumolysin) in addition allow for the efflux of intracellular proteins [18,23].

As a consequence of pore formation by Staphylococcal  $\alpha$  toxin, aerolysin and LLO, cells of the immune system, such as lymphocytes [24,25] or dendritic cells [26], can undergo apoptosis. Interestingly, aerolysin-treated macrophages were also found to upregulate the anti-apoptotic protein Bcl-2 [27], suggesting that multiple, and sometimes opposing pathways can be simultaneously stimulated. The final outcome of these opposing pathways will depend, probably in a cell type dependent manner, on the balance (or the cross-talk) between these pathways.

Cells of the immune system were also found to produce numerous inflammatory molecules. Interleukin (IL)-8 production, as a consequence of the activation of the nuclear factor  $\kappa$ B (NF- $\kappa$ B) pathway, was observed upon treatment with Staphylococcal  $\alpha$  toxin [28], aerolysin [27], pneumolysin [29] and LLO [30]. In the case of pneumolysin, activation of NF- $\kappa$ B was shown to require toxin mediated calcium influx into the cell. Production of IL-1 $\beta$  was observed for cells treated with aerolysin [27] and Staphylococcal  $\alpha$  toxin [31]. Tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) release was found for cells treated with aerolysin [27] and SLO [32]. And finally, prostaglandin E2 was produced upon aerolysin treatment through the cyclooxygenase-2 pathway [27].

Non-immune cells were found to vacuolate in response to treatment with aerolysin or *Serratia marcescens* hemolysin [33,34]; the vacuoles originated from the endoplasmic reticulum (ER) [33]. Pore formation was also found to trigger release of calcium from the ER, through mechanisms that involve activation of G proteins, phospholipase C and IP3 production [35]. In a recent study, transient plasma membrane pore formation by Staphylococcal  $\alpha$  toxin in keratinocytes was shown to trigger activation of the epidermal growth factor (EGF)-receptor and cell proliferation [36\*].

## Cellular immunity pathways to PFTs

### Activation of the p38 stress pathway

The first characterized cell survival pathway activated in response to pore-formation was obtained by microarray analysis of the nematode *Caenorhabditis elegans* treated with the *B. thuringiensis* Crystal toxin Cry5B, against those not treated [37\*\*]. It was found that transcription of the p38 mitogen-activated protein kinase (MAPK) was upregulated in toxin-treated worms and that p38 pathway knock out worms were hypersensitive to both low chronic doses and high brief doses of toxin, revealing the activation of a p38-dependent survival pathway. One of the downstream targets of p38 was found to be *ttm-1*, the human orthologue of which is a divalent cation transporter [37\*\*]. The knock-down of *ttm-1* rendered worms hypersensitive to Cry5B, but the underlying mechanism remains to be elucidated.

The involvement of p38 was extended to mammalian cells. Inhibition of p38-rendered Baby Hamster Kidney cells more sensitive to aerolysin [37\*\*]. These findings were further supported by the observation that p38 becomes phosphorylated in response to various PFTs such as Staphylococcal  $\alpha$  toxin, SLO [38\*,39\*], pneumolysin [40] and anthrolysin [39\*]. Interestingly, phosphorylation of p38 could be prevented by the addition of high molecular weight dextran to the extracellular medium, suggesting that osmotic stress following pore formation, rather than signaling through toxin receptors, is involved in p38 activation [39\*]. It was indeed observed that p38 contributes to membrane resealing after exposure to Staphylococcal  $\alpha$  toxin, and that the p38 pathway was protective to the cells [38\*]. These results did not extend to SLO treatment [38\*]. In another system, p38 was found to mediate neuronal cell death, instead of survival, upon exposure to the large pore-forming toxin pneumolysin [40]. Thus, 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 cell type involved. Nonetheless, activation of the p38 pathway appears to be a conserved feature of cells responding to a pore and that, in several instances involving small pores, this activation is protective.

### Activation of lipid metabolic pathways

Toxins forming small pores, such as aerolysin and Staphylococcal  $\alpha$  toxin were recently found to trigger the activation of the central regulators of lipid metabolism, the sterol responsive element binding proteins (SREBPs) in epithelial cells [41\*\*]. These are membrane-bound transcription factors that control the synthesis of enzymes involved in lipid metabolism [42]. The pathway found to lead to the activation of SREBPs was the following: pore-formation in the plasma membrane led to the permeabilization to small ions and in particular to an efflux of cytosolic potassium. This drop in cellular potassium led to the assembly of the so-called inflammasome, a

multi-protein complex involved in the activation of caspase-1. This caspase is well known to process pro-IL-1 $\beta$  as well as IL-18 and IL-33 into their active forms, however in this pathway that is triggered by pore-forming toxin, the caspase was found to mediate SREBP activation through a yet to be determined intermediate. Finally, activation of lipogenic genes by SREBPs promoted cell survival after toxin attack.

### Membrane repair mechanisms

The p38 and SREBP pathways both contribute to cell survival, but it is unclear at present by which mechanisms. One possibility is clearly lesion repair. That repair can occur has been shown for small pores such as Staphylococcal  $\alpha$  toxin pores [43], as well as large SLO type pores [23]. The repair mechanisms might however differ for the two types of toxins since for SLO resealing is rapid (<1hr) and is p38-independent, whereas recovery after  $\alpha$  toxin treatment is slower and p38-dependent [38\*].

Rapid plasma membrane repair mechanisms have been studied upon cell injury induced by laser treatment or cell scratching. These involve calcium-mediated fusion of intracellular organelles (it is debated exactly which organelles are involved) with the plasma membrane [44]. Because resealing after SLO permeabilization is calcium/calmodulin-dependent, it is possible that repair after cholesterol dependent toxin action involves membrane donation by intracellular organelles [44]. This mechanism is unlikely to occur during recovery of cells after exposure to Staphylococcal  $\alpha$  toxin or aerolysin, considering the time frame of recovery. It is interesting to note that the more stable the oligomeric toxin assembly (aerolysin, Staphylococcal  $\alpha$  toxin) it appears, the more difficult for the cell to repair the lesions. The aerolysin heptamer is heat- and detergent-resistant, whereas the Staphylococcal  $\alpha$  toxin heptamer is heat-sensitive and the cholesterol-dependent toxins (e.g. SLO, and LLO) multimers are sensitive to both. When combining the observations in the literature, it thus appears that the ability of cells to repair their membrane inversely correlates with the oligomer stability. We propose the following speculation: lesions formed by multimeric PFTs are lined by proteins, and not lipid, in contrast to mechanical lesions of cell membranes. The protein lining prevents radial expansion of the pore, which would occur if the lesions were lined by lipids, thus preventing rapid cell lysis. On the other hand however, the protein ring would prevent rapid resealing of membrane such as might occur upon fusion with intracellular organelles. One could imagine that for less stable pores, such as those formed by SLO or LLO, disassembly of the protein ring with time would expose lipid edges that would then enable either resealing or, under specific circumstances, membrane rupture. Resealing would be important upon pore formation at the plasma membrane leading to cell recovery, whereas

radial expansion would be important upon pore formation in phagosomes, enabling organelle rupture, and release of the PFT-producing bacterium into the cytoplasm. In addition to pore formation, this membrane rupture event might require cytosolic host components, such as the cytoskeleton.

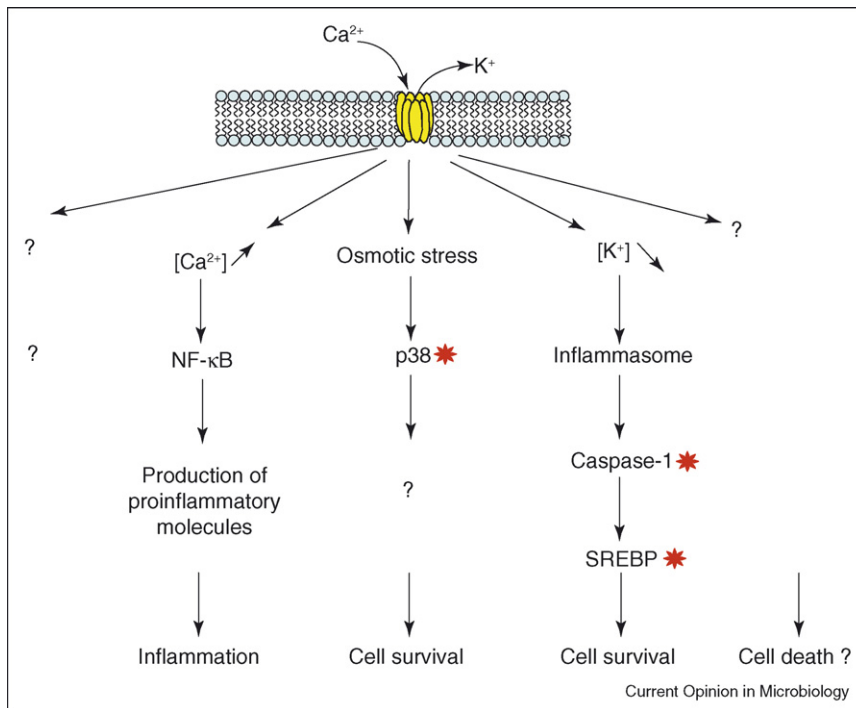
### Conclusions: cellular non-immune defenses

Many bacterial pathogens produce PFTs, which appear to represent an ancient bacterial weapon. These toxins have been found to be important *in vivo* for the infections by numerous and diverse bacteria (e.g. *Staphylococcus aureus* [45], Group A and Group B Streptococci [46,47], and *Clostridium septicum* [48\*]). Thus, inhibiting the action of PFTs or conversely promoting general cellular defenses against PFTs might be an alternative therapeutic approach towards simultaneously improving outcomes of diseases caused by several important bacterial pathogens. We hypothesize that cells in general have evolved conserved mechanisms (e.g. the p38 pathway) to deal with impairment of their membrane permeability (e.g. caused by stresses such as cell stretching or unregulated channel openings) and that they have adapted these mechanisms to deal with PFTs.

The recent studies on cellular responses to PFTs indicate that cells in general are equipped with various devices that enable them to detect plasma membrane permeabilization (Figure 1). These include changes in osmotic pressure, in ion composition and in intracellular calcium concentration, however additional sensing mechanisms are likely to operate. Thus, defense against PFTs and against the bacteria that use them is not strictly limited to cells of the immune system, but is more broadly present in most if not all cell types. The work on PFTs suggests that we should broaden our notion of innate immunity to include more generalized cellular defenses that are not limited to cells of the immune system, leading to the term we prefer, cellular non-immune defenses (CNIDs). The numerous signaling cascades that are triggered upon pore formation have different consequences: some promote cell survival, such as the p38 and SREBP pathways, whereas others are important for initiation and propagation of inflammatory lesions such as the NF- $\kappa$ B pathway [28], whereas still others might have little importance.

Gene profiling studies and RNAi screens using model organisms as well as different types of mammalian cells should provide a more complete view of the various alterations that are sensed by PFT-treated cells, which pathways are subsequently activated and how the balance and cross talk between these pathways determines the final outcomes. It will also be interesting to evaluate the benefits that each pathway provides both to the host and the pathogen, keeping in mind that cell survival is likely to be beneficial to both in numerous cases.

Figure 1



Schematic representation of the various pathways that are activated upon pore-formation by bacterial toxins.

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