REVISTA CUBANA DE CIENCIAS BIOLÓGICAS
http://www.rccb.uh.cu

ARTÍCULO DE REVISIÓN

# Cell death mechanisms induced by pore forming toxins with special focus on actinoporins

*Mecanismos de muerte celular inducidos por toxinas formadoras de poros con especial enfoque en actinoporinas*

**Carmen Soto Febles[1]*, Lena de León Esperón[1], Javier Alvarado-Mesén[2], Flavia Llorente Álvarez[1], Arianna Yglesias[3], Hermis Rodríguez[3], Rancés Blanco Santana[4], María E. Lanio[1], Isabel Fabiola Pazos Santos[1], Ana M. Hernández [4], Carlos Álvarez[1]***

[1] Centro de Estudio de Proteínas, Facultad de Biología, Universidad de La Habana, Cuba
[2] Universidad Nacional, Costa Rica.
[3] Instituto de Medicina Tropical "Pedro Kourí", La Habana, Cuba
[4]Centro de Inmunología Molecular, La Habana, Cuba

*Autores para correspondencia:
*carmensoto@fbio.uh.cu*
*calvarez@fbio.uh.cu*

**Recibido:** 2020-09-19

**Aceptado:** 2020-12-30

**ABSTRACT**

Pore-forming toxins (PFTs) are one of the weapons used by living beings to attack or self-defend. These proteins are secreted in soluble form but they undergo conformational changes, creating a pore in the host membrane. PFTs are distributed from bacteria to mammals. Their binding to membrane leads to cell swelling, imbalance of ionic gradients and cell death. The cell death mechanisms induced by bacterial PFTs are the best characterized; by contrast, eukaryotic PFTs have been less studied than their bacterial counterparts, perhaps due to their lower impact on human health. Among eukaryotic PFTs, actinoporins (APs) constitute a particular family produced by sea anemones. The interest in actinoporins has risen due to their high cytotoxicity that can be used to design immunotoxins against tumor cells and molecule delivery systems to cell cytosol. In this review we describe the main cell death mechanism induced by PFTs with particular focus on sticholysins I and II (Sts, StI/StII) two APs thoroughly studied in our laboratory. Understanding the death cell mechanisms triggered by Sts and other APs is of the utmost importance due to their potential applications to treat several pathological conditions in humans. Work is in progress in our Center with these toxins in this direction.

**Keywords:** pore-forming toxins, cell death mechanism, actinoporin

***RESUMEN***

Las toxinas formadoras de poro (PFTs) son una de las armas usadas por los seres vivos para atacar o autodefenderse. Estas proteínas se secretan en forma soluble, pero sufren un cambio conformacional al crear un poro en la membrana diana. Las PFTs se distribuyen desde las bacterias hasta los mamíferos. Su unión a la membrana provoca un incremento del volumen celular, el desequilibrio de los gradientes iónicos y la muerte de la célula. Los mecanismos de muerte celular inducidos por las PFTs de origen bacteriano son los mejor caracterizados; en contraste, los desencadenados por las PFTs eucarióticas han sido menos estudiados, quizás debido a su menor

impacto en la salud humana. Entre las toxinas formadoras de poro eucarióticas, las actinoporins (APs), producidas por las anémonas marinas, constituyen una familia particular. El creciente interés en las APs se debe a su elevada citotoxicidad que puede usarse para diseñar inmunotoxinas contra células tumorales y sistemas de liberación de moléculas al citosol celular. En esta revisión describimos los principales mecanismos de muerte celular inducidos por las PFTs con un enfoque particular en las sticholisinas I y II (Sts, StI/StII), dos APs ampliamente estudiadas en nuestro laboratorio. El conocimiento de los mecanismos celulares involucrados en la muerte inducida por las Sts y otras APs es de suma importancia debido a sus aplicaciones potenciales en el tratamiento de varias condiciones patológicas en humanos. En esta dirección, se encamina parte del trabajo con estas toxinas en nuestro Centro.

***Palabras claves:*** *toxinas formadoras de poro, mecanismo de muerte celular, actinoporina*

## INTRODUCTION

Pore forming toxins (PFTs) are one of the tools used by living organisms to attack or self-defend (Bischofberger *et al.*, 2012). These proteins are synthesized in soluble form and in response to various stimuli undergo conformational changes that lead to pore formation on the plasma membrane (PM) of target cells. Pore formation impairs cell membrane permeability inducing the uncontrolled passage of solutes and ions across the membrane (Cosentino *et al.*, 2016). This non-regulated movement leads to an ionic and osmotic imbalance and finally to cell death (Maček *et al.*, 1994). Cell death is characterized by an irreversible loss of cell vital functions and may occur in response to different stressors including PFTs action (Galluzi *et al.*, 2015; Tang *et al.*, 2019).

Most of the studies on cell death mechanisms and intracellular pathways activation induced by PFTs have been carried out with bacterial PFTs (Cancino-Rodezno *et al.*, 2009). However, there is limited information on cell death mechanisms or signaling pathways triggered by eukaryotic PFTs.

PFTs from sea anemones are called actinoporins (APs) and represent a quite interesting family of PFTs due to their significant membranotropic action and high cytolytic activity. For these reasons, APs constitute promising therapeutic agents against tumor cells and attractive molecules for other biomedical and biotechnological applications (reviewed in Alvarez *et al.*, 2020). Cell death mechanisms induced by APs have been poorly explored; indeed, only a few studies have been documented with non-consistent results (Fedorov *et al.*, 2010; Soletti *et al.*, 2010; Soto *et al.*, 2018). A common aspect in the mechanism involved in cell death by PFTs is its dependence on cellular type, cell metabolic condition and toxin concentration (Katayama *et al.* 2007, Cancino-Rodezno *et al.*, 2009; Soto *et al.*, 2018). Due to their importance for developing treatments or biotechnological tools, this review aims at summarizing the main cell death mechanism induced by PFTs with particular focus on APs sticholysins I and II (Sts, StI/StII) two APs thoroughly studied by CEP.

### Pore forming toxins (PFTs)

#### *General characteristics of pore formation*

PFTs have a broad taxonomy distribution from bacteria to mammals (Bischofberger *et al.*, 2012). Secreted in a monomeric soluble form, they can assemble into oligomeric structures with the ability to insert in the membrane via specific receptors leading to pore formation and as a consequence occurs the membrane permeabilization (Bischofberger *et al.*, 2012). Membrane perforation by PFTs leads to changes in ion concentration in the cytosol of the target cell. These pores can be either lytic to the target cell, for example by creating an osmotic imbalance, or can mediate the translocation of proteins, namely toxins, into the cytoplasm (Lesieur *et al.*, 1997).

The multiple outcomes of pore formation by a PFT depend on its concentration, the pore diameter, the number of pores/cell and the stability of the pore (Hotze and Tweten, 2012). Consequently, the cellular response to PFTs will also depend on the abovementioned factors. Calcium and potassium have been identified as the main mediators in the cellular response to cell membrane damage by these proteins (Bischofberger *et al.*, 2012).

PFTs are classified in two broad groups depending on the elements of secondary structure used to span the cell membrane (Lesieur *et al.*, 1997). α-PFTs utilize amphipathic α-helices to pierce the membrane whereas β-PFTs form amphipathic β-barrel pores (Dal Peraro and van der Goot, 2016). Three families of α-PFT (colicins, actinoporins, and cytolysin A) and three

of β-PFT (hemolysins β, aerolysin β and Cholesterol Dependent Cytolysins (CDC)) have been identified (Dal Peraro and van der Goot, 2016).

Their common functional feature is the ability to penetrate cell membranes eventually leading to cell death. This is carried out through a variety of molecular strategies for assembly into the lipid bilayer that are not very clear yet.

Since PFTs kill cells, they are potential candidates for targeted toxin therapy for the treatment of cancer and immune diseases, as well as other biotechnological applications (Li *et al.*, 2017).

### *Bacterial PFTs*

Many pathogenic bacteria produce PFTs being important components of their virulence arsenal. In spite of sequence and structural diversity, all bacterial PFTs follow the same mode of action (Gonzalez *et al.*, 2008).

They are secreted as monomeric and soluble proteins that can diffuse toward target cells to which they bind via specific receptors (Gonzalez *et al.*, 2008). The receptors include transmembrane or glycosylphosphatidyl inositol–anchored proteins (GPI-anchored proteins), lipids or cluster of lipids (Coskun and Simons, 2011; Skocaj *et al.*, 2014), generally associated with lipid rafts (Coskun and Simons, 2011). Indeed, cholesterol is the receptor of CDCs such as perfringolysin O (PLO) or streptolysin O (SLO) whereas GPI-anchored proteins are the receptors for *Aeromonas aerolysin* and *Clostridium septicum* α-toxin (Abrami *et al.*, 2002). In addition, other receptors such as claudins, components of tight junctions (Van Itallie and Anderson, 2013), are receptors for *Clostridium perfringens* enterotoxin (CPE) (Shinoda *et al.*, 2016), and the metalloproteinase domain-containing protein 10 (ADAM10) was proposed to be a receptor for *Staphylococcus aureus* α-hemolysin on epithelial cells (Berube and Bubeck, 2013) (Table 1).

**Table 1.** Some representative examples of bacterial PFTs
***Table 1.*** *Algunos ejemplos representativos de PFTs bacterianas*

| Type of PFT | Family | Organism | Examples | Receptor | References |
|---|---|---|---|---|---|
| α-PFT | Colicins | *Escherichia coli* | Colicin A<br>Colicin Ia<br>Colicin E1<br>Colicin N | At IM/OM | Lackey *et al.,* 1994 |
| | Cytolysin A | *Escherichia coli* | Cytolysyin A (ClyA) | Chol | Eifler *et al*., 2006 |
| | | *Bacillus cereus* | Non-haemolytic tripartite enterotoxin (Nhe) | Chol | Ganash *et al*., 2013 |
| | | | Haemolysin BL (Hbl) | Chol | Madegowda *et al*., 2008 |
| | Repeats-in-toxin (RTX) | *Escherichia coli* | HlyA | N.D | Welch, 2001 |
| | Cholesterol-dependent cytolysins (CDC) | *Listeria monocytogenes* | Listeriolysin (LLO) | Chol | Koster et al., 2014 |
| | | *Clostridium perfringens* | Perfringolysin O (PFO) | | Feil *et al*., 2012 |
| | | *Streptococcus pyogenes* | Streptolysin O (SLO) | | Sierig *et al*., 2003 |
| | | *Bacillus anthracis* | Anthrolysin (ALO) | | Bourdeau *et al*., 2009 |
| | | *Staphylococcus pneumoniae* | Pneumolysin (PLY) | | Braun *et al.,* 2007 |
| β-PFT | Haemolysins β | *Stapylococcus aureus* | Panton–Valentine leukocidin | C5a receptors (C5aRs) | Spaan *et al*., 2013 |
| | | | α-haemolysin (HlA) | PC/ADAM10/ disintegrin | Wilke and Bubeck Wanderburg, 2010 |
| | Aerolysin | *Aeromonas hydrophila* | Aerolysin | GPI-anchored proteins | Abrami *et al.,* 2000 |
| | Aerolysin-like β-PFT | *Clostridium perfringens* | Enterotoxin (CPE) | Claudin | Shinoda *et al*., 2016 |

At IM/OM: inner membrane/outer membrane, Chol: cholesterol, N.D: Non-determined, PC:phosphatidylcholine, ADAM10: metalloproteinase domain-containing protein 10, GPI-anchored proteins: glycosylphosphatidyl inositol–anchored proteins

On the other hand, the stoichiometry, and the pore diameter, depends on the toxin and its concentration in the vicinity of the target cell (Hotze and Tweten, 2012). These differences lead to a large diversity in the cellular responses such as cell death, membrane repair or cellular survival (Hotze and Tweten, 2012).

Bacterial PFTs have been used with different biomedical and biotechnology purposes. These toxins can destroy tumors and can also be used for PFT-based cancer vaccines (Patyar *et al.,* 2010). In spite of their potential exhibited for the construction of immunotoxins, only one from *Pseudomonas* has been approved by the FDA for application in B-cell cancer (Kreitman *et al.,* 2018). Currently it is necessary to develop novel immunotoxins from other sources due to the immunogenicity and limited penetration of some bacterial PFT-based immunotoxins (Tejuca *et al.,* 2009). In addition, PFTs have been also used for the design of biosensors based on protein nanopores for sequencing of polypeptides and nucleic acids (Zhao *et al.*, 2009; Robertson and Reiner, 2018).

***APs***

Among PFTs, APs, produced by sea anemones, are extremely interesting due to their biomedical or biotechnological potential to build immunotoxins (Tejuca *et al,* 2009; Pentón *et al*, 2011; Mutter *et al,* 2018), vaccine platforms (Lanio *et al,* 2014; Laborde *et al,* 2017), biosensors (Watanabe *et al,* 2017; Huang et al, 2019) or even as a component of their defense or attack molecular arsenal. APs are monomeric, soluble, α-helical PFTs with Mr of around 20 kDa and most of them exhibit highly basic pI. They oligomerize and integrate into natural and model sphingomyelin (SM)-containing membranes where they form pores of ~2 nm diameter (Maček et al., 1994; Tejuca et al., 1996; Tejuca et al., 2001; Anderluh and Macek, 2002; Alvarez et al., 2009). They are extremely cytotoxic and lytic to a variety of cells in the nM range (Fedorov et al., 2010; Soletti et al., 2010; Cabezas et al., 2017; Soto et al., 2018; Alvarado-Mesén et al., 2019).

Fragaceatoxin C (FraC) from *Actinia fragacea* (Tanaka *et al.*, 2015), equinatoxin II (EqtII) from *Actinia equina* (Athanasiadis et al, 2001) and Sts produced by *Stichodactyla helianthus* (Lanio *et al.*, 2001; Mancheño *et al.*, 2003; Alvarez *et al.* 2009; García-Linares *et al.*, 2013) are the best characterized members of this protein family.

Particularly, StI and StII exhibit high sequence similarity (99%) and identity (93%) (Huerta *et al.*, 2001; Lanio *et al.*, 2001). These toxins bind to membranes, leading to the formation of pores both in cell and model membranes. The transmembrane α-helical barrel pores disturb cellular ionic gradients, cause osmotic swelling and finally lead to cell death (Tejuca *et al.*, 1996; 2001).

The three-dimensional (3D) solution structures of four APs have been solved: StI (García-Linares *et al.*, 2013), StII (Mancheño *et al.*, 2003), EqtII (Athanasiadis *et al.*, 2001; Hinds *et al.*, 2002) and FraC (Tanaka *et al.*, 2015). The comparison of these structures in solution reveals a similar 3D fold formed by a central rigid and compact core consisting of two β sheets. On the opposite sides of this β-core, two α-helices are oriented perpendicularly to each other (Figure 1). The first α -helix located close to the N-terminus is amphipathic in nature, mobile and flexible and is involved in pore-formation (Hong *et al.*, 2002; Mancheño *et al.*, 2003; García-Linares *et al.*, 2013). Soluble StII complexed with phosphocholine (POC) structure revealed a phospholipid headgroup binding site (Figure 1), which is located in a region with an unusually high abundance of aromatic amino acid residues. The residues involved in the POC binding site are strictly conserved in APs, suggesting that the same mechanism of lipid headgroup recognition is followed by other members of AP family (Bakrac *et al.*, 2008). In the case of the nigrelysin (Ng), the most recently described AP from the Costa Rica´s Pacific coast anemone *Anthopleura nigrescens,* only two amino acid residues in the POC binding site (N78 and Y108) involved in protein-lipid interaction are not conserved respect to StII (D76 and F106). However, these changes do not compromise the order of permeabilizing activity of Ng (Alvarado-Mesén *et al.,* 2019).

This binding site together with the aromatic amino acid cluster forms an essential structural assembly for binding of these toxins to membranes known as the interfacial binding site (Bakrac *et al*., 2008). Recently, the crystallographic structure of FraC has been solved. This study revealed the existence of multiple sites for lipid binding (Tanaka *et al.*, 2015); in fact, two of these sites (L2 and L3) were considered similar to the POC binding site initially described for StII, while L4 and L5 were hypothesized to be sites of low affinity for POC or perhaps high affinity binding sites for lipids with headgroups other than POC (Tanaka *et al.*, 2015).

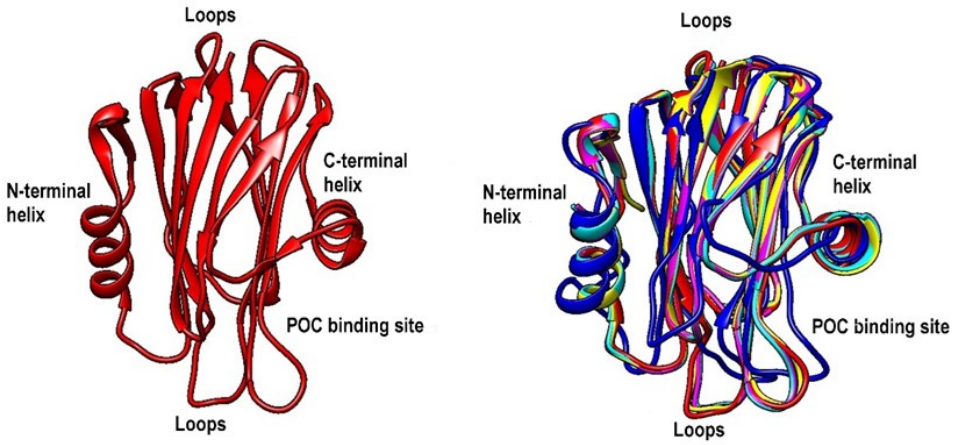


**Figure 1.** Superposition of the tridimensional structures of StII, StI, EqtII, FraC and Ng-model. A) Ribbon diagram of StII structure (PDB: 1O72) which shows the common structural elements of APs. Also, the POC binding site is showed. B) The 3D structure of StII (red color; PDB: 1O72) was compared with StI (blue; PDB: 2ks4), FraC (magenta; PDB: 3vwi), EqtII (yellow; PDB: 1iaz) and Ng model (cyan). The resulting RMSD values were 1.185 Å (between118 atoms pairs) for the pair StII/StI; 0.532 Å (between 173 atoms pairs) for StII/FraC; 0.579 Å (between 171 atoms pairs) for StII/EqtII and 0.646 Å (between 173 atoms pairs) for StII/Ng model. For the construction of the Ng model, the SWISS-MODEL platform (https://swissmodel.expasy.org/) was used. Images were produced with the UCSF Chimera program for Windows (Pettersen et al., 2004).

***Figure 1.*** *Superposición de las estructuras tridimensionales de StII, StI, EqtII, FraC y el modelo de Ng A) Diagrama en cintas de la estructura de StII (PDB: 1O72) que muestra los elementos estructurales comunes de las APs. Además, se muestra el sitio de unión a POC. B) La estructura 3D de StII (rojo; PDB: 1O72) se comparó con StI (azul; PDB: 2ks4), FraC (la magenta; PDB: 3vwi), EqtII (amarillo; PDB: 1iaz) y el modelo de Ng (azul claro). Los valores de RMSD resultantes fueron 1.185 Å (entre los 118 pares de átomos) para el par StII/StI; 0.532 Å (entre 173 pares de los átomos) para StII/FraC; 0.579 Å (entre los 171 pares de los átomos) para StII/EqtII y 0.646 Å (entre 173 pares de los átomos) para modelo de StII/Ng. Para la construcción del modelo de Ng, se utilizó la plataforma SWISS-MODEL (https://swissmodel.expasy.org /). Las imágenes se obtuvieron con el programa UCSF Quimera para Windows (Pettersen et al., 2004).*

The pores generated by APs in membranes result from a several stages-mechanism: attachment to membrane, oligomerization, detachment and insertion of the toxin N-terminal region into the hydrophobic core of the membrane, and eventually pore structuring (Mancheño *et al.*, 2003; García-Linares *et al.*, 2013; Rojko *et al.*, 2016). The first step in this sequence is binding of soluble monomers to membrane via the aromatic cluster and lipid binding sites. As this step does not involve significant protein conformational changes, the global structure of the membrane-bound monomer is similar to that in solution (Alvarez *et al.*, 2003; Alegre-Cebollada *et al.*, 2007). Once the toxin binds to the membrane, the association of several monomers and the transfer of the aminoterminal region from the body of the protein to the hydrophobic core of the bilayer must occur (Rojko *et al.*, 2013).

**Main cell death mechanisms**

Cell death can be classified into accidental or regulated. Several factors are considered as inducers of cell death; among them, insults of physical- (high pressures, osmotic forces), chemical- (extreme pH changes) or mechanical- (shear forces) nature have been described (Galluzzi *et al.,* 2015). They can elicit the immediate and catastrophic demise of cells known as accidental cell death. In contrast, regulated cell death (RCD) is characterized by the involvement of intracellular pathways that modulate cell death (Galluzzi *et al.*, 2018) and is sensitive to pharmacologic agents and genetic manipulations (Galluzzi *et al.*, 2015). In this sense, when RCD takes place in the absence of external disruptions, it is named programed cell death which has relevant functions in tissue development or turnover (Galluzzi *et al.*, 2018).

In addition to accidental or regulated, cell death mechanisms can be classified into different subclasses based on their molecular aspects. The main recognized types are apoptosis, necroptosis and pyroptosis (Tang *et al.*, 2019) (Table 2).

Below we will describe the main features that characterize apoptosis, necrosis, and necroptosis.

**Table 2.** Main types of regulated cell death
***Table 2.*** *Principales tipos de muerte celular regulada*

| Type of cell death | Morphological characteristics | Biochemical characteristics | Some inhibitors |
|---|---|---|---|
| Apoptosis | Cell volume reduction.<br>Nuclear condensation.<br>Apoptotic bodies formation. | Caspase activation.<br>DNA fragmentation.<br>ΔΨm dissipation.<br>PS exposure. | Pan caspase inhibitors (Z-DEVD-FMK, Z-VDVAD-FMK). |
| Necroptosis | Cell swelling.<br>Rupture of PM.<br>Moderate chromatin condensation. | Activation of RIP1, RIP3, and MLKL.<br>Necrosome formation. MLKL translocation to PM and pore formation. | Necrostatin-1, GSK872, HS-1371, Necrosulfonamide. |
| Pyroptosis | Cell swelling.<br>Rupture of PM.<br>Moderate chromatin condensation. | Activation of caspase 1 or 11 and gasdermin.<br>Pore formation induced by gasdermin. | Caspase 1 and 11 inhibitors. |

PM: Plasma membrane; ΔΨm: changes in mitochondrial membrane potential; PS: phosphatidylserine. Taken from Tang *et al.*, 2019.

**Apoptosis**

Apoptosis is a RCD mechanism (Galluzzi *et al.*, 2015) characterized by caspase activation (Mariño *et al.*, 2014), phosphatidylserine (PS) translocation from the inner PM monolayer toward the outer monolayer (Suzuki *et al.*, 2016), DNA fragmentation (Mariño *et al.*, 2014) and permeabilization of outer mitochondrial membrane (OMM) (Mariño *et al.*, 2014). These biochemical changes are responsible for morphological alterations such as cell volume reduction (Orrenius *et al.*, 2011), chromatin condensation (Fink *et al.*, 2005), nucleolus disintegration (Orrenius *et al.*, 2011), nucleus fragmentation (Kerr *et al.*, 1972; Burgess, 2013) and apoptotic bodies formation (Mariño *et al.*, 2014) (Table 2).

Caspases are a family of cysteine proteases, synthetized as zymogens and activated by proteolysis (Shalini *et al.*, 2015). These enzymes provoke the rupture of peptide bonds when the carboxylic group of their substrate is an aspartate residue (McIlwain *et al.*, 2013). Two types of caspases participate in apoptosis. Initiator caspases (i.e. caspases 2, 8, 9 and 10), that are activated in response to stress signals, cellular damage or a signal of cell death, and effector caspases (i.e. caspases 3, 6 and 7) (Julien and Wells, 2017) activated by initiator caspases (Kumar, 2007).

Apoptosis can occur by extrinsic or intrinsic pathways (Nagata and Tanaka, 2017). The extrinsic pathway is mediated by a complex formed between death receptors of the PM and ligands such as Fas ligand (FasL), tumor necrosis factor (TNF) and TNF-related apoptosis inducing ligand (TRAIL) (Von Karstedt *et al.*, 2017). The complex induces trimerization and activation of the death receptor. Signaling from cell surface toward intracellular medium is mediated by Death Domain (DD) of the receptor´s cytoplasmic region. In addition, adaptor molecules that contain DD such as Fas Associated protein with Death Domain (FADD) (Fu *et al.*, 2016), are sequestered to receptor DD. All of them constitute the molecular complex named Death Inducing Signaling Complex (DISC) (Koff *et al.*, 2015). FADD protein contains a Death Effector Domain (DED), that participates in procaspase 8 sequester to DISC complex where it is activated (Koff *et al.*, 2015) (Figure 2). On the other hand, the activation of the intrinsic pathway is caused by cell stress generated by hypoxia, DNA irreparable damage, Reactive Oxygen Species (ROS), microtubules alterations, Endoplasmic Reticulum (ER) stress and elevated concentrations of cytosolic calcium (Vitale *et al.*, 2017). The outer mitochondrial membrane (OMM) permeabilization determines the irreversibility of the intrinsic apoptosis (Galluzzi, *et al.*, 2016), which is favored by the proapoptotic members of the Bcl-2 protein family (Delbridge *et al.*, 2016). Bax and Bak are members of the proapoptotic protein family (Luna-Vargas and Chipuk, 2016) that predominate in the cytosol of healthy cells but during apoptosis undergo conformational changes, translocate to the OMM where oligomerize and form a pore (Groose *et al.*, 2016).

This pore provokes an increase of OMM permeability that leads to proapoptotic factors release such as Apoptosis-Inducing Factor (IAP) and cytochrome c and ATP from mitochondria to cytosol (Delbridge *et al.*, 2016). Cytochrome c and Apoptosis protease-activating factor-1 (Apaf-1) are components of the apoptosome, complex responsible of procaspase 9 activation; moreover, ATP participates in the formation of this complex, as well (Li, *et al.*, 1997) (Figure 2). Caspases 8 and 9 activation allows the effector caspases activation, enzymes responsible of the morphological and biochemical changes observed during the apoptosis mechanism (Figure 2).

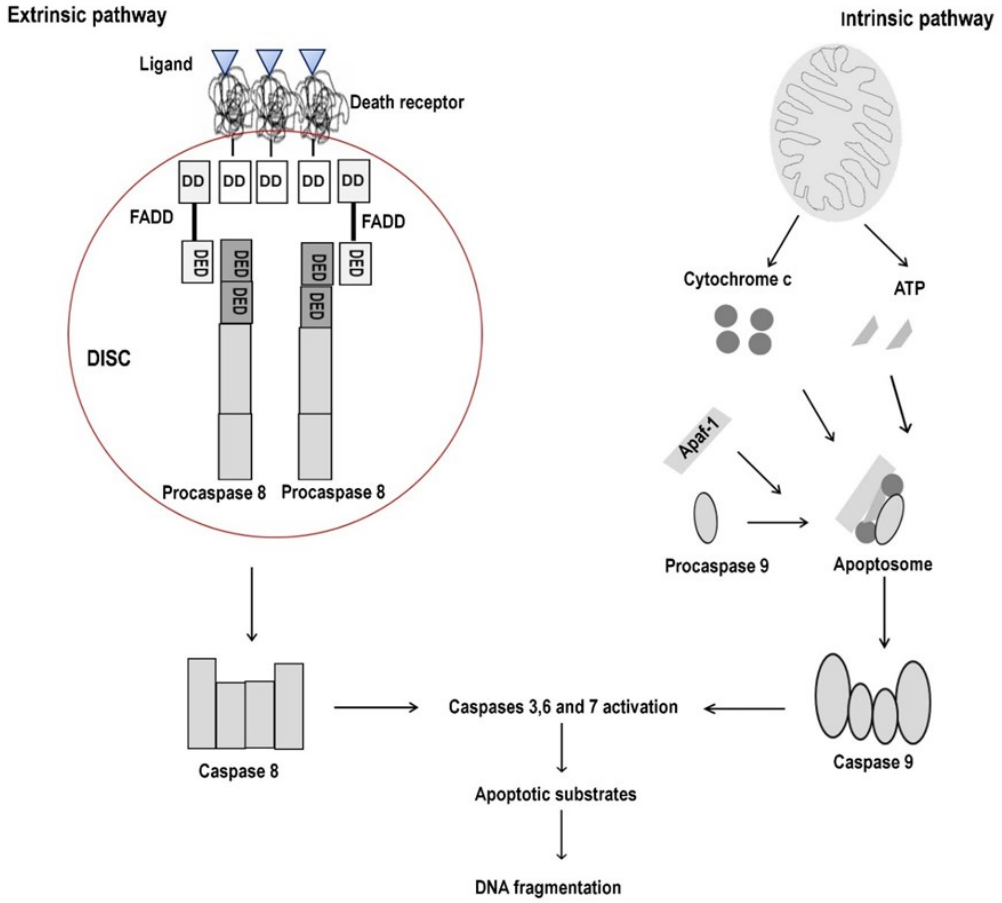


**Figure 2.** Schematic representation of apoptosis activation. *Extrinsic pathway*: After ligand binding to its receptor, the death receptor recruits adaptor proteins through its cytosolic death domains (DD). Besides DD, the adaptors contain death effector domains (DED) which recruit procaspase 8 to the complex resulting in Death Inducing Signalling Complex (DISC) formation. Thus procaspase 8 is activated which in turn activates effector caspases. *Intrinsic pathway*: the apoptotic stimulus produces the release of apoptogenic factors from the intermembranous space of mitochondria to the cytosol, such as cytochrome c and ATP, which induce the formation of the apoptosome and the activation of procaspase-9. Apaf-1 protein activates procaspase-9 by proteolytic degradation. In turn, caspase-9 mediates the activation of effector caspases. Effector caspase activation leads to morphological and biochemical changes that occur during apoptosis.

***Figure 2.*** *Representación esquemática de la activación de la apoptosis. Vía extrínseca: Después de la unión del ligando a su receptor, el receptor de muerte recluta a las proteínas adaptadoras a través de sus dominios citosólicos de muerte (DD). Además de DD, los adaptadores contienen los dominios efectores de muerte (DED) que reclutan a la procaspasa 8, lo que resulta en la formación del complejo de señalización que induce la muerte (DISC). De esta forma, se activa la procaspase 8, la cual s su vez activa a las caspasas efectoras. Vía intrínseca: el estímulo apoptótico produce la liberación de factores apoptogénicos tales como el citocromo c y el ATP, desde el espacio intermembranoso de la mitocondria hacia el citosol, los cuales inducen la formación del apoptosoma y la activación de la procaspasae-9. La proteína Apaf-1 activa a la procaspasa-9 por degradación proteolítica. A su vez, la caspasa-9 media la activación de caspasas efectoras. La activación de caspasas efectoras conduce a los cambios morfológicos y bioquímicos que ocurren durante la apoptosis.*

**Necrosis**

The term necrosis refers to the accidental cell death occurring in a non-regulated way (Belizário *et al.*, 2015). The main identified causes of necrosis are: hypoxia, the ischemic insult, ROS, extreme temperature changes, nutrient deficiency and toxin action (Orrenius *et al.*, 2011). Necrosis is characterized by an ionic imbalance, increased water entry to the cell and consequently increase in mitochondria and cell size (Savva et al., 2013), ATP depletion, loss of membrane integrity (Nagahama et al., 2011) and the release of intracellular content to extracellular medium (Savva et al., 2013). The early phenomenon of cell swelling is named oncosis, a prelethal phase of this cell death mechanism (Orrenius *et al.*, 2011). As results, the rupture of cellular membrane and organelles, as well as the release of intracellular content take place (Kroemer *et al.*, 2009). At present, necrosis is classified in accidental or regulated depending on the stressor and cell response (Galluzzi *et al*., 2018).

***Regulated necrosis***

The main regulated necrosis mechanisms induced by PFTs are necroptosis and pyroptosis (Table 2).

***Necroptosis.***

Necroptosis is a form of regulated cell death with morphological features of necrosis (Zhang *et al.*, 2020) (Table 2). Necroptosis can be initiated by the TNF superfamily receptors, Toll-like receptors (TLR3 and TLR4), and interferon receptors. This type of cell death is classified into three categories: extrinsic, intrinsic and intrinsic mediated by ischemia. Extrinsic necroptosis is induced by TNFα and constitutes the most studied mechanism (Berghe *et al.*, 2016) while the intrinsic necroptosis is stimulated by ROS (Dhuriya and Sharma, 2018). Other stimuli related to necroptosis are viral diseases, interferons, antitumor drugs, cytoplasmic calcium increase and ceramide nanoliposomes (Huang *et al.*, 2015; Galluzzi *et al.*, 2018; Zhang *et al.*, 2018; Choi *et al.*, 2019).

At molecular level, this type of cell death is dependent on receptor-interacting proteins (RIP), RIP1 and RIP3 (Petrie *et al.*, 2019) and the pseudokinase MLKL (Mixed Lineage Kinase Domain-like protein) (Table 2) (Petrie *et al.*, 2019) not involved in accidental necrosis (Galluzzi *et al.*, 2018; Zhang *et al.*, 2018).

The interaction of executioner MLKL with membrane lipids is essential for necroptosis (Weinlich *et al.*, 2017). All RIPs are Thr/Ser kinases with a conserved kinase domain located at the polypeptide amino end; however, they differ in those domains without functional kinase activity (Zhang *et al.*, 2010). RIP1 has a DD in its carboxyl end that interacts with some receptors and adaptor proteins, a polyubiquitinated intermediate domain and a RIP homotypic interaction motif (RHIM) (Newton, 2015). This later domain interacts with RIP3 (Ofengeim and Yuan, 2013) which has a kinase and a RHIM domain (Mompean *et al.*, 2018).

TNFα binding to its receptor (TNFR1) elicits its oligomerization. Subsequently, proteins such as Tumor Necrosis Factor 1 Receptor associated Death Domain Protein (TRADD), RIP1, apoptosis inhibited cellular proteins (cIAP) and Tumor Receptor Associated Factor 2 (TRAF2) are recruited by the DD receptor. Upon stimulation of the TNFR 1, a signaling complex termed Complex I is formed containing TRADD, TRAF2, RIP1, and cIAP (Vandenabeele *et al.*, 2010) (Figure 3). In this complex, RIP1 is modified by cIAP polyubiquitination (Goncharov *et al.*, 2013) promoting MAPK activation and the NF-kB signaling pathway. In consequence, survival gene expression is increased (Newton *et al.*, 2014). If TNFR1 activation matches up with RIP1 deubiquitination or lack of cIAP, RIP1 translocates to complex II formation. In turn, complex IIa is formed by RIP1, TRADD, FADD and procaspase 8 (Zhang *et al.*, 2020) (Figure 3).

Caspase 8 activation promotes effector caspases activation leading to RIP1 and RIP3 proteolysis and therefore apoptosis is favored (Salvesen and Walsh, 2014). However, if caspases are inactive, RIP3 interacts with RIP1 by RHIM domain, resulting in RIP3 phosphorylation, and necrosome formation also called complex IIb, the major molecular complex of necroptosis (Grootjans *et al.*, 2017). Phosphorylated RIP3 phosphorylates MLKL (Chen *et al.*, 2014) a hallmark of necroptotic death (Petrie *et al.*, 2018) which in turn oligomerizes in the cytosol and translocates to the plasma membrane (Rodriguez *et al.*, 2016) where interacts with phosphotidyl inositol phosphate and promotes pore formation (Figure 3). Pore formation leads to ion imbalance, water influx, release of DAMPs, increase in cellular size, plasma membrane rupture, release of intracellular content and cell death (Petrie *et al.*, 2018). In addition, MLKL acts as platform for recruitment of $Na^+$ or $Ca^{2+}$ channels in PM (Cai *et al.*, 2014).

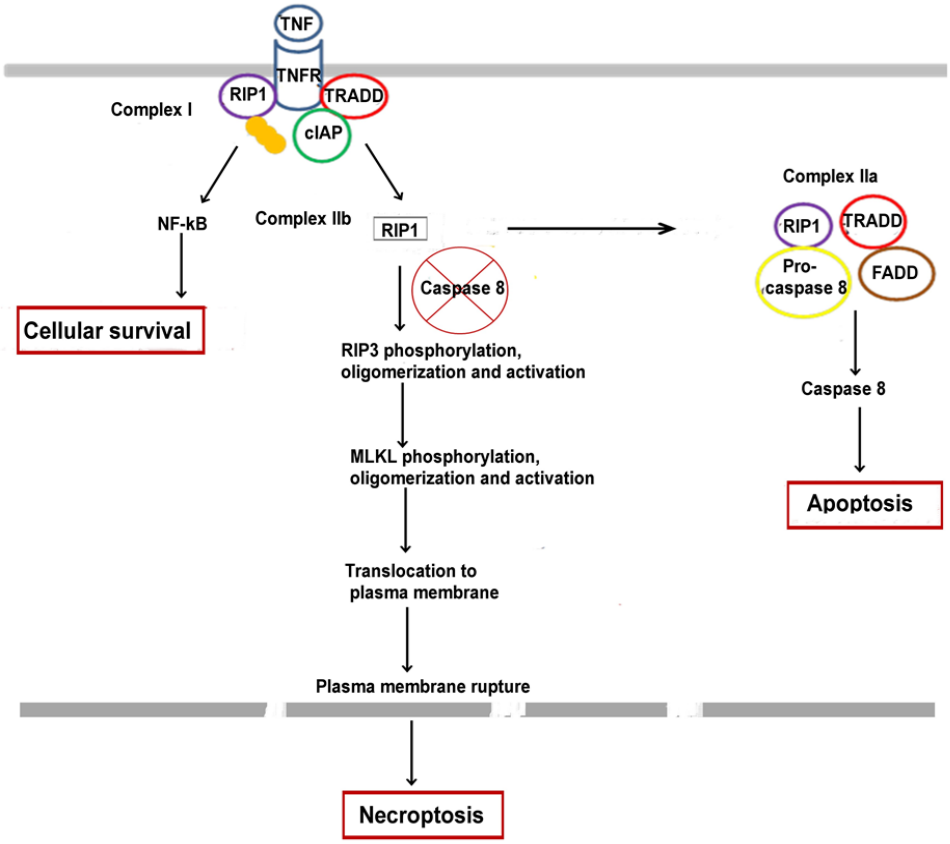


**Figure 3.** Schematic representation of the classical pathway of necroptosis via TNFα. After formation of the TNFα-TNFR1 complex, a sequence of steps takes place and complex I, IIa or IIb are formed. Complex I containing RIP1 polyubiquitinated induces the NF-kB intracellular pathway activation resulting in cellular survival. In contrast, complex IIa or IIb leads to cell death. Complex IIb induces apoptosis when caspases are activated. However, in the absence of activated caspases, RIP3 and MLKL activation are essential to induce necroptosis. MLKL phosphorylation leads to its oligomerization and translocation to the plasma membrane generating a pore, ion imbalance and eventually cell death.

***Figure 3.*** *Representación esquemática de la vía clásica de necroptosis a través del TNFα. Después de la formación del complejo de TNFα-TNFR1, ocurre una serie de pasos y se forman en complejo I, IIa o IIb. El Complejo I que contiene a RIP1 poliubicuitinada induce la activación de la vía intracelular del NF-kB que conduce a la supervivencia celular. En el contraste, en complejo IIa o IIb conduce a la muerte celular. Cuando se activan las caspasas el complejo IIb induce apoptosis. Sin embargo, en la ausencia de caspasas activas, la activación de RIP3 y de MLKL son esenciales en la inducción de necroptosis. La fosforilación de MLKL conduce a su oligomerización y translocación a la membrana plasmática generando un poro, desbalance iónico y finalmente la muerte celular*

Recently, differences have been observed between mouse and human MLKL oligomerization (Petrie *et al.*, 2018). In mouse cells, MLKL phosphorylation by RIP3 is sufficient to induce oligomerization and translocation to membranes (Tanzer *et al.*, 2015). In contrast, the role of RIP3-mediated phosphorylation of MLKL in human cells is unclear, but promotes MLKL oligomer formation, reorganization of these oligomers that promote cell death and/or the release of activated MLKL from the necrosome to allow membrane translocation and permeabilization (Petrie *et al.*, 2018).

On the other hand, some studies have revealed that mitochondrial ROS production, activation of the mitochondrial phosphatase PGAM family member 5 (PGAM5), or the presence of the mitochondrial permeability transition pore can trigger a necroptotic mechanism of cell death (Wang *et al.*, 2012). The mechanism by which the mitochondrial ROS production contributes to necroptosis induction is not clear, but it might include a redox sensing upstream of RIP1 activation and RIP3 recruitment (Zhang *et al.*, 2017). In addition, some studies suggest a connection between aerobic metabolism and necroptosis because glutaminolysis and pyruvate dehydrogenase activity are regulated by RIP3 (Yang *et al.*, 2018). In other studies, NADPH oxidase derived ROS have been implicated in necroptosis in neutrophils (Wang *et al.*, 2018). The significance of different ROS sources in necroptosis and how they impact the signal transduction remain to be further investigated.

***Pyroptosis.***

Pyroptosis is a RCD mechanism activated in response to homeostasis perturbations (Jorgensen and Miao, 2015) and mediated by the activation of the inflammasome, a cytosolic multiprotein complex which recruits and activates caspase 1 (Fink and Cookson, 2005; Lu *et al.*, 2014) (Table 2). This caspase mediates the proteolytic processing of pro -IL1β and pro-IL18 into mature IL1β and IL18, respectively. In mice, this type of inflammatory cell death can be triggered by the activation of caspase 1 or 11 while in humans is related to caspase1, 4 or 5 according to the cell type (Broz and Dixit, 2016).

Caspase 1 also activates the protein Gasdermin D (GSDMD) (Wang *et al.*, 2017), which is the key effector of pyroptosis (He *et al.*, 2015; Ding *et al.*, 2016; Liu and Lieberman, 2020) and is cleaved by caspase 1 or caspase 11 to produce Gasdermin C and a N-terminal fragment named Gasdermin N (He *et al.*, 2015). Once formed, Gasdermin N translocates to the inner layer of the PM where it binds phosphatidylinositol phosphates, phosphatidic acid, and PS.

Ensuing, the protein undergoes conformational changes facilitating its oligomerization and membrane insertion to generate a pore with inner diameter of 10-15 nm (Lieberman *et al.*, 2019). Pore formation elicits cellular ionic gradient dissipation, water influx (Fink and Cookson, 2006), cellular swelling, osmotic lysis (Van Opdenbosch and Lamkanfi, 2019) as well as proinflammatory cytokine (e.g. IL1 β) (Liu *et al.*, 2016) and damage-associated molecular pattern (DAMPs) release (de Vasconcelos *et a*l., 2018).

In this type of RCD, the presence of nuclear condensation coupled to cell swelling and the formation of large bubbles at the PM that eventually break down are observed (Rathkey *et al*., 2018). In summary, these events lead to the release of cytosolic content to the extracellular medium favoring the inflammatory response (Degterev *et al.*, 2008) (Figure 4).

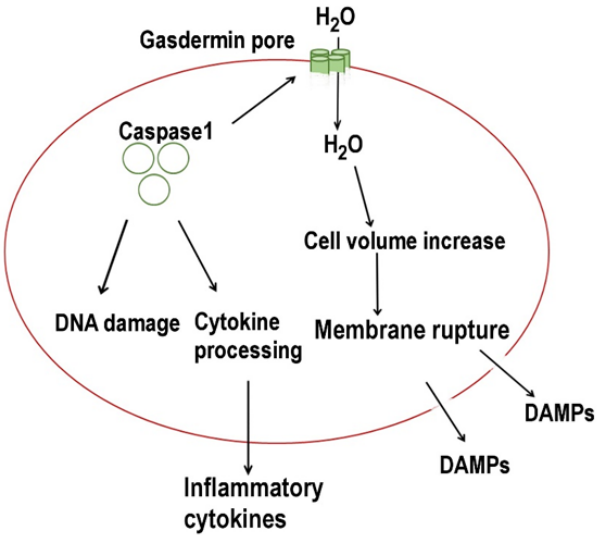


**Figure 4.** *Schematic representation of pyroptosis.* During pyroptosis caspase 1 is activated which induces DNA damage and cytokines processing. On the other hand, gasdermin activation occurs with the participation of caspase 1, generating pores in the plasma membrane. Pore formation leads to cellular ionic gradient dissipation, water influx, cellular swelling, osmotic lysis with proinflammatory cytokines and other cytosolic components release to the extracellular medium, eventually favoring an inflammatory response and cell death.

***Figure 4.*** *Representación esquemática de la piroptosis. Durante la piroptosis se activa la caspasa 1 la cual induce daño al ADN y el procesamiento de las citosinas. Por otra parte, la activación de la gasdermina tiene lugar con la a participación de la caspasa 1, generando poros en la membrana plasmática. La formación del poro conduce a la dispersión del gradiente iónico celular, el influjo de agua, el hinchamiento celular, la lisis osmótica con la liberación de citosinas proinflamatorias y otros componentes citosólicos hacia el medio extracelular, lo que favorece una respuesta inflamatoria y la muerte celular.*

## Intracellular pathways and cell death mechanisms induced by bacterial PFTs and APs

### *Selected examples from bacterial PFTs*

Pore formation caused by PFTs leads to a decrease in intracellular potassium concentration and can provoke a spectrum of events depending on the target cell, the type and concentration of PFT and the time of cell exposure to the toxin.

At lytic concentrations, bacterial PFTs induce accidental necrosis (Menzies and Kourteva, 2000; McClane and Chakrabarti, 2004; Cancino-Rodezno *et al.*, 2009; Hernández-Flores and Vivanco-Cid, 2015).

However, at sublyttic concentrations (those that do not cause cell death under certain conditions), cells respond to the aggression of PFTs by activating complex regulatory mechanisms, involving intracellular signaling pathways, which can lead to cell survival or death (González et al., 2011). Throughout this process, many PFTs induce an increase in intracellular calcium which, in turn, triggers the activation of several signaling cascades (Bischofberger et al., 2012)..

In this sense, bacterial PFTs induce a regulated cell death mechanism that involves the activation of intracellular signaling pathways such as Mitogen-Activated Protein Kinases (MAPKs) (Gonzalez *et al*., 2011; (Chakravorty *et al*., 2015). These enzymes are serine/threonine kinases that largely respond to stress stimuli. Conventional MAPKs include the extracellular signal -regulated kinases 1 and 2 (ERK1/2), p38 isoforms and c-Jun amino (N)-terminal kinases 1, 2, and 3 (JNK1/2/3) (Brito *et al, 2019*). The ERK pathway (Ras-Raf-MEK1/2-ERK1/2) is involved in cellular proliferation, differentiation, survival, migration and development (McCubrey *et al*., 2006) as well as in cell death (Lu and Xu, 2006; Mebratu and Tesfaigzi, 2009). JNK and p38 pathways can be activated by cell stressors such as osmotic stress, cytokines, heat shock, potassium efflux and are involved in apoptosis, inflammation and cellular differentiation (Wagner and Nebreda, 2009). MAPKs carry out the regulation of a great variety of cellular process. Once activated, they phosphorylate a variety of intracellular proteins including transcription factors, membrane transporters, cytoskeleton elements and other kinases (Zhang and Liu, 2002).

In particular, bacterial PFTs, at sublytic concentrations, activate the three families of MAPKs (Chakravorty *et al.,* 2015). For instance, the PFT produced by *C. septicum* is toxic to many types of cells (Kennedy *et al.,* 2009; Chakravorty *et al.,* 2015). Experimental evidence showed that this toxin induces MAPK activation in a dose dependent manner both *in vitro* and *in vivo* (Chakravorty *et al.,* 2015). Preincubation of cells with MAPK inhibitors provoked a decrease in the cytotoxicity induced by the toxin (Chakravorty *et al.,* 2015), suggesting the involvement of these pathways in the cell death mechanism prompted by the toxin. Moreover, in the presence of the calcium chelator EGTA, MAPK activation and cell death induced by the toxin decreased, indicating the important role of this ion in toxin cytotoxicity (Chakravorty *et al.,* 2015).

Several members of the CDC PFT family, such as SLO, vanginolysin (VLY) produced by *Gardnerella vaginalis*, anthrolysin O (AnlO) produced by *B. anthracis*, PLY, α-hemolysin, aerolysin, β hemolysin from Group *B Streptococcus*, β- toxin from C. perfringens, LLO at sublytic concentrations also induced p38 activation (Essmann *et al.,* 2003; Huffman *et al.,* 2004; Fick *et al*., 2005; Saenz *et al.,* 2010, Nagahama *et al.,* 2013). In Baby Hamster Kidney (BHK) cells the aerolysin at sublytic nanomolar concentrations induced p38 activation without cellular lysis (Huffman *et al.,* 2004; Bischof *et al.,* 2008). In the presence of SB 203580, a specific inhibitor of p38, cells were not capable to recover plasma membrane integrity (Husmann *et al.,* 2004). Moreover, p38 activation in HeLa cells by aerolysin induced an unfolding protein response (UPR), a type of stress response of endoplasmic reticulum (RE). This UPR is a complex reaction to different situations aiming at protecting the cell of the deleterious effect of unfolded proteins and it can be activated as a defense mechanism against the toxin effects (Bischof *et al.,* 2008).

The activation of p38 is dependent of pore formation, is triggered by potassium efflux (Kloft *et al.,* 2009), takes place rapidly and transiently (Porta *et al.,* 2011), might differ from one toxin to other (Aroian and van der Goot, 2007), and constitutes a defense mechanism against bacterial PFT-mediated damage in different mammalian cell lines (Hussmann *et al.,* 2006, Gonzalez *et al.,* 2011, Porta *et al.,* 2011, Bischofberger *et al.*, 2012, Nagahama *et al., 2013*).

Besides the recognized involvement of p38 in the defense response against PFTs, lethal consequences of its activation by PFTs have also been documented. Thus, activation of p38 mediated the neuronal cell death upon exposition to pneumolysin (Stringaris *et al.,* 2002). Pneumolysin also induced p38 activation which was related to neuroblastoma cell death (Guessan *et al.,* 2005).

JNK is the third type family of MAPKs. Both p38 and JNK regulate multiple downstream signals, which have been shown to be protective upon PFT intoxication *in vivo*. JNK is considered a regulator of PFT-induced transcriptional responses (Kao *et al.,* 2011). For example, *C. perfringens* β-toxin, β-hemolysin from GBS, SLO, PLY, LLO induce the JNK phosphorylation (Stassen *et al.,* 2003; Aguilar *et al.,* 2009; Gonzalez *et al*., 2011; Bebien *et al.,* 2012; Nagahama *et al.,* 2013).

Beyond accidental necrosis, at sublytic concentrations bacterial PFTs also induce regulated cell death such as apoptosis, necroptosis and pyroptosis (Bischofberger *et al,* 2012). Thus, the bacterial PFTs: α -toxin, LLO, PLY, PVL, HlyA, parasporin-1 from *B. thuringiensis,* SLO, α-hemolysin from *E. coli*, α-toxin from *C. septicum*, VacA, PFT from *H. pylori,* among others, induce apoptosis in different cell types (Nelson *et al.,* 1999; Genestier *et al.,* 2005; Katayama *et al.,* 2007; Wiles *et al.,* 2008; Cancino-Rodezno *et al.*, 2009; Timmer *et al.,* 2009; Imre *et al.,* 2012; Akazawa *et al.,* 2013; Bielaszewska *et al*., 2013).

Apoptosis induced by bacterial toxins is characterized by cell ATP depletion (Katayama *et al.*, 2007), cytochrome c release (Labandeira-Rey *et al.*, 2007), PS exposition in the outer monolayer of the PM (Carrero *et al.*, 2004), activation of caspases 9, 3 and 7 (Genestier *et al.*, 2005; Bielaszewska *et al.*, 2013), increase in mitochondrial permeability (Bielaszewska *et al*., 2013; Nerlich *et al.*, 2018), loss of mitochondrial membrane potential (Nerlich *et al.*, 2018) and DNA fragmentation (Wiles *et al.*, 2008). In particular, PLY toxin induces apoptosis in human endothelial and neuroblastoma cells through p38 and JNK MAPK activation. Inhibition of p38 and JNK phosphorylation reduced caspases activation and apoptosis (Guessan *et al*., 2005). The activation of p38 was related to calcium influx through toxin pore, suggesting a role of this ion in the cell death induced by PLY (Stringaris *et al.,* 2002).

Necroptosis is induced by sublytic concentrations of β-toxin from *C. perfringens* in porcine endothelial cells (Autheman *et al.*, 2013). Consistently, Lactate Dehydrogenase release, propidium iodide uptake, ATP depletion, potassium efflux, and cytosolic calcium increase from the external medium were observed. Interestingly, the release of the High Mobility Group Box 1 protein (HMGB1), a DAMPs associated with the immunogenic response induced by necroptosis, was also reported. In the presence of a calcium chelating agent and necrostatin-1 (Nec-1), an inhibitor of RIP1 phosphorylation, the cytotoxic effect of the toxin was inhibited, suggesting that calcium and RIP1 are involved in the cell death. Additionaly, in the presence of cyclosporin A, an inhibitor of the mitochondrial permeability transition pore formation, the cell death was also inhibited showing a role of mitochondria in the cytotoxicity induced by the toxin (Autheman *et al.*, 2013).

Recently, it was documented that α-toxin from *S. aureus,* hemolysin from *E. coli* and VaC also induce cell death by necroptosis (Radin *et al.,* 2011; Kitur *et al.,* 2015). Toxin from *Serratia marcescens* induced necroptosis on A549 cells by a mechanism independent of death receptors and TLR but related to the intracellular calcium and potassium imbalance (González-Juarbe *et al.*, 2017).

Pyroptosis is another cell death mechanism induced by sublytic concentrations of bacterial PFTs (Soong *et al.,* 2012). PVL, LLO, SLO and the lethal toxin from *B. anthracis* activate caspase 1 (Fink *et al.*, 2008; Shoma *et al.*, 2008; Timmer *et al.*, 2009) which promote the proinflammatory cytokines activation and the lysis mediated by gasdermin pore formation (Fink *et al.*, 2008).

***Studies on APs***

In contrast to bacterial PFTs, there are only a few studies on the cell death mechanisms induced by APs.

The cytotoxic effects of StII and Nigrelysin (Ng) from the sea anemone *Anthopleura nigrescens* (Alvarado-Mesén *et al.,* 2019), take place at nanomolar concentrations (Table 3) in mammalian cells (Cabezas *et al.*, 2017; Soto *et al.*, 2018; Alvarado-Mesén *et al.*, 2019).

Pore formation by lytic concentrations of StII leads to cellular swelling and membrane blebbing on Baby Hamster Kidney (BHK) cells a few minutes after toxin addition (Cabezas *et al.,* 2017). Blebs have been observed in other animal cells by the action of Eqt II (García-Sáez *et al.,* 2011), and these structures are considered protrusions formed on the cell surface after intense membrane injury (García-Sáez *et al.,* 2011). Nevertheless, this mechanism against cell lysis do not seem to be effective at high toxin concentration at which the cell cannot recover and finally die by necrosis (Cabezas *et al.,* 2017).

In this sense, we demonstrated that StII (Soto *et al.*, 2018) and Ng (Alvarado-Masén *et al*., 2019) similarly to other actinoporins (Soletti *et al.*, 2010) induce an increase in cellular size and the release of intracellular content in different cell types. Under StII treatment, cells such as A549 and L929 with fibroblast morphology, adopt an oval shape most likely due to water entry into the cell (Figure 5).

**Table 3.** Cytotoxic activity of StII and Ng on cell lines
***Table 3.* Actividad citotóxica de StII y Ng sobre líneas celulares**

| Cell line | Origin | Toxicity ($C_{50}$, nM) |
|---|---|---|
| L1210 cmah-kd or SH44 | Modified murine B lymphoma | 1.2 ± 0.1[a *] |
| L1210 | Murine B lymphoma | 2.3 ± 0.1[a*]<br>2.2 ± 0.1[b**] |
| J774 | Murine macrophages | 0.9 ± 0.1[a]* |
| MB16F10 | Murine melanoma | 20[a]**** |
| L929 | Murine fibrosarcoma | 15[a****] |
| BHK | Baby Hamster Kidney | <10[a ***] |
| Raji | Human Burkitt B Lymphoma | ± 0.2[a*] |
| A459 | Human non-small lung cancer cells | 10[a****] |
| Lymphocyte from peripherical blood | Human | 2.5 ± 0.2[a*] |

$C_{50}$: cytotoxic activity (mean concentration required for 50% reduction of cell viability. Values represent the mean ± SD (n = 3). [a] StII, [b] Ng, * Soto et al., 2018, ** Alvarado-Mesén et al., 2019, *** Cabezas *et al.,* 2017, ****non published results

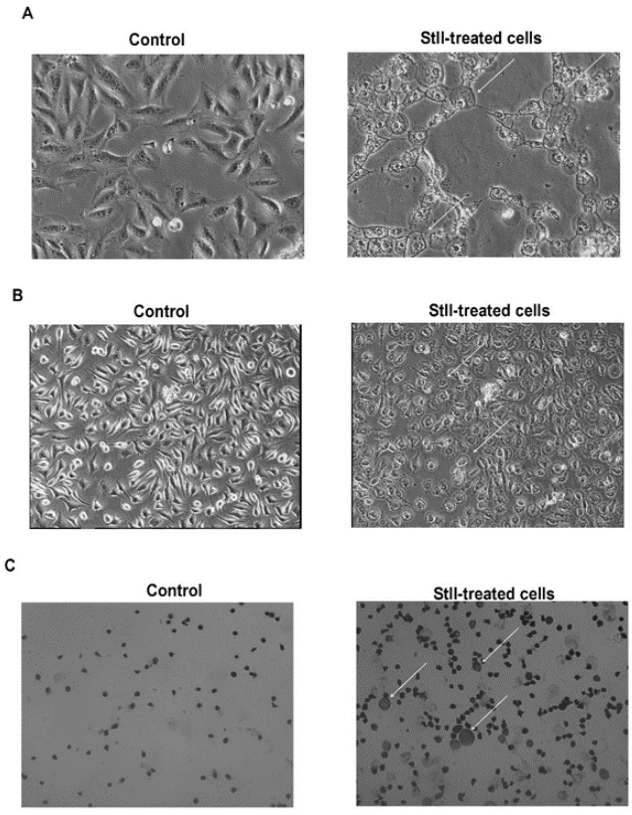


**Figure 5**. Morphological changes of cells upon treatment with StII. A) Human lung cancer cells (A549) treated with 40 nM StII. B) Murine fibrosarcoma cells (L929) treated with 20 nM toxin, C) Murine macrophage J774 treated with 1 nM toxin. Cells were cultured without (control) or with StII for 30 min at room temperature. The cells were observed by phase contrast microscopy. In the case of J774, cells were stained with hematoxylin and eosin and were observed by optical microscopy. Examples of swollen cells are identified by white arrows. Non published results.

***Figure 5**. Cambios morfológicos de células tratadas con StII. A) Las células de cáncer de pulmón humano (A549) se trataron con StII 40 nM. B) Células de fibrosarcoma murino (L929) se trataron con 20 nM de toxina, C) Macrófagos murinos (J774) se trataron con 1 nM de la toxina. Las células se cultivaron sin (control) o con StII durante 30 min a temperatura ambiente. Las células se observaron al microscopio de contraste de fase. En el caso de las células J774, las células se tiñeron con hematoxilina y eosina y se observaron al microscopio óptico. Los ejemplos de células hinchadas se señalan con flechas blancas. Estos resultados no han sido publicados.*

At sublytic concentrations in BHK cells, StII causes a decrease in intracellular potassium which is related to p38 and ERK1/2 activation, indicating that their activation upon membrane injury by StII is crucial to restore the intracellular concentration of this ion (Cabezas *et al.,* 2017). This result suggests that p38 and ERK1/2 are the main kinases participating in the mechanism of cell defense against AP action. In addition, ERK1/2 phosphorylation was inhibited when cells were treated with U0126, a specific MEK1/2 inhibitor (Favata et al., 1998), before StII addition (Cabezas *et al.,* 2017) indicating that their activation occurred via MEK1/2. The pre-incubation of cells with U0126 and with the p38 inhibitor (SB203580) (Husmann *et al.,* 2006), significantly impaired the intracellular potassium recovery (Cabezas *et al.,* 2017).

On the other hand, StII induced the ERK1/2 activation on B-cell lymphoma (Raji) cells, but in contrast to BHK cells, in the presence of PD98059, another inhibitor of MEK1/2, the cytotoxic effect of the toxin was reduced, suggesting an involvement of this pathway in the cell death mechanism induced by the toxin (Soto *et al.,* 2018). In addition, KN62, an inhibitor of $Ca^{2+}$/CaMKII reduced cell death in Raji cells demonstrating its contribution to the cytotoxicity induced by StII (Soto *et al*., 2018). In correspondence with these results, the cell death induced by EqtII and Bc2 (from the sea anemone *Bunodosoma caissarum)* on human glioblastoma U87, involves the activation of $Ca^{2+}$/ CaMKII and ERK1/2 MAPK (Soletti *et al.,* 2010) Interestingly, members of CAMKII cascade are implicated in ERK1/2 activation (Zhong and Su, 2002).

In Raji cells, at sublytic concentrations, StII induced calcium release mainly from the Endoplasmic Reticulum (ER), the primary storage site for intracellular $Ca^{2+}$. To examine the involvement of ER on cell death mechanism induced by StII, we analyzed the effect of salubrinal, an inhibitor of eukaryotic initiation factor 2α (eIF2α) dephosphorylation (Methippar *et al.,* 2009). In the presence of salubrinal, cytosolic calcium concentration was reduced suggesting the involvement of ER in this process (Soto *et al.,* 2018).

Taking into account, the relation between ER-mitochondria and the ability of mitochondria to uptake calcium (Bravo et al., 2011), it is tempting to speculate that the change in mitochondrial membrane potential in Raji cells upon StII treatment (Soto et al., 2018) might be related to the increase of cytosolic calcium level.

It has been described that eIF2α phosphorylation contributes to the immunogenicity of cell death caused at least by clinically chemotherapeutics. Indeed, eIF2α phosphorylation within cancer tissues is related to DAMPs exposure which is favorable to anticancer immune responses (Bezu *et al.,* 2018). StII induces eIF2α phosphorylation in tumoral cells, which contributes to their cytotoxic effect (Soto *et al.,* 2018).

Moreover, at sublytic concentrations, StII on tumoral cells, cause the activation of intracellular pathways related to cell death by regulated necrosis. StII cytotoxicity on Raji cells occurs without caspases activation in comparison with doxorubicin-treated cells, a classical apoptosis inducer (Figure 6A). Consistently, the cytotoxicity is not affected by the broad-spectrum caspase inhibitor QVD-OPh indicating that caspases are not involved in StII-induced cell death in this and other cell lines such as MB16 F10 (murine melanoma) and A549 (human lung cancer cells) (Figure 6B). Other apoptotic markers such as DNA fragmentation or apoptotic bodies formation were not observed as well (Soto *et al.*, 2018).

In contrast, it was claimed that actinoporin RTX-A produced by the anemone *Radianthus macrodactylus*, at nM concentrations, elicited cell death by apoptosis in mouse epidermic cells JB6P+Cl41. This conclusion was drawn from the demonstration that StII triggered the translocation of PS from PM inner to outer monolayer (Fedorov et al., 2010). This is the only record of apoptosis induced by an AP. In the same year, Soletti *et al.* (2010) concluded that Eqt II from *Actinia equina* induced, at sublytic concentrations, a mechanism similar to necrosis that involved the activation of some intracellular pathways in the light of our current knowledge of the complexity and diversity of cell death mechanisms, it is possible to catalogue the cell death caused by RTXA as *regulated necrosis* given the participation of intracellular pathways.

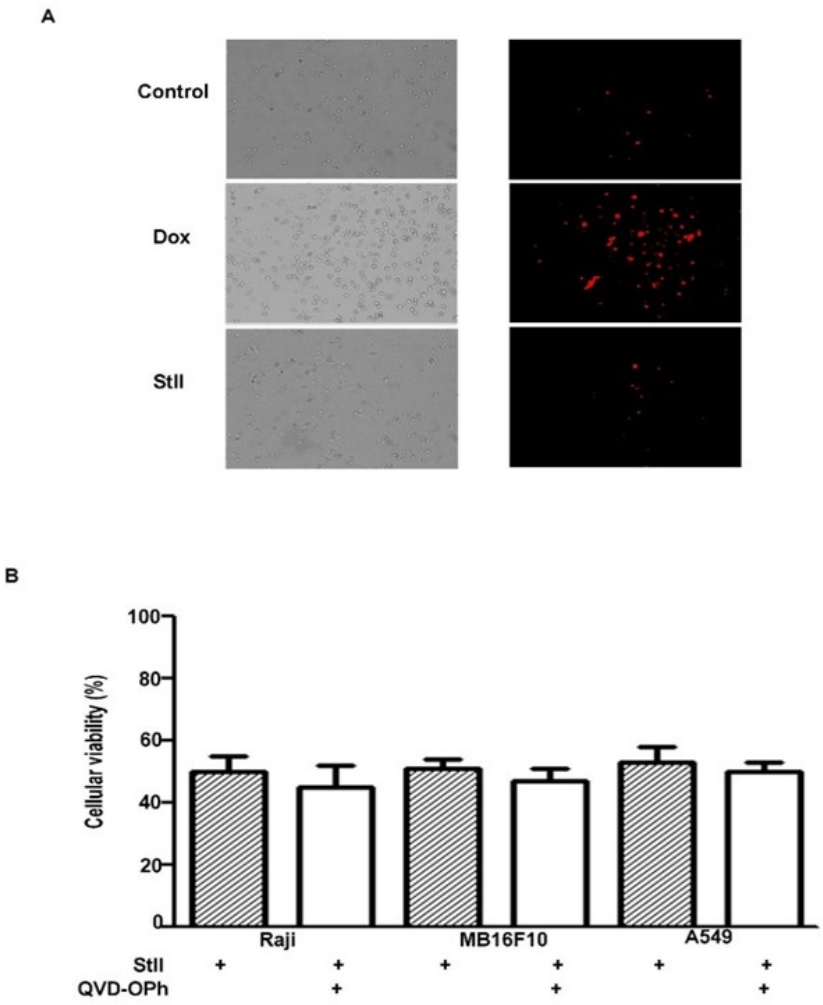


**Figure 6.** *StII does not induce apoptosis markers.* A) Caspases- 3 and 7 activation in Raji cells treated with sublytic concentrations of StII for 24 h as determined by fluorescence microscopy. StII: 0.1 nM, doxorubicin (Dox): 0.5 µM. Left panels: Morphology of cells under optical microscopy. Non treated cells are considered control. Cells treated with Dox are considered apoptosis positive control. Cells treated with StII show swelling and death cells are observed as ghost cells. Right panels: caspases 3 and 7 activation estimated by fluorescence microscopy. Positive cells to caspase-3 and 7 appear as red cells as a consequence of binding of activated caspases to the caspase inhibitor DEVD-FMK (L-Asp-Glu-Val-Asp-fluoromethylketone) coupled to fluorophore sulforodamin. In all cases the magnification was 400X. B) Cellular viability estimated by MTT assay after treatment with QVD-POh and StII for 24 h in different cell types: Raji cells, murine melanoma (MB16F10) and human lung cancer cells (A549). Data represents media and standard deviation. No differences were observed among treatments. Non published results.

***Figure 6.*** *StII no induce marcadores de apoptosis. A) Activación de caspasas 3 y 7 en células Raji tratadas con concentraciones sublíticas de StII durante 24 h determinadas por microscopía de fluorescencia. StII: 0.1 nM, doxorrubicina (Dox): 0.5 µM. Paneles izquierdos: Morfología de las células al microscopio óptico. Las células no tratadas se consideran control. Las células tratadas con doxorrubicina se consideraron el control positivo. En las células tratadas con StII se observa hinchazón celular y células muertas aparecen como fantasmas celulares. Paneles derechos: Activación de caspasas 3 y 7 estimada mediante microscopía de fluorescencia. Las células positivas a las caspasas-3 y 7 aparecen como células rojas debido a la unión del inhibidor de caspasas DEVD-FMK (L-Asp-Glu-Val-Asp-fluorometilcetona) acoplado al fluoróforo rodamina a la caspasa activa. En todos los casos la amplificación fue de 400X. B) Viabilidad Celular estimada por el ensayo de MTT después del tratamiento con QVD-POh y StII para 24 h en los distintos tipos celulares: células Raji, melanoma murino (MB16F10) y células de cáncer de pulmón humano (A549). Los datos representan la media y la desviación estándar. No se observaron diferencias entre los tratamientos. Estos resultados no han sido publicados*

To the best of our knowledge, there is only one study trying to explore whether AP activity can result in necroptosis. In fact, we recently reported that pretreating Raji cells with Nec-1, inhibited the cytotoxic effect of StII, suggesting a contribution of RIP1 to the cell death mechanism induced by the toxin. However, in the presence of necrosulfonamide (NSA), a necroptosis inhibitor that specifically inhibits human MLKL (Sun *et al*., 2012), the cell death induced by StII was not affected. Also, StII induced significant activation of RIP1 but no RIP3 activation could be detected. Since RIP3 activates MLKL, this result is in agreement with the lack of effect found for NSA in StII cytotoxicity (Soto *et al.*, 2018). In fact, StII did not induce MLKL phosphorylation estimated by western blot assays (unpublished results). Therefore, the cell death promoted by StII does not seem to occur through RIP3-MLKL pathway a characteristic of necroptotic mechanisms. Taken together, these findings reinforce the notion that APs can induce a regulated necrosis at sublytic concentrations.

So far, pyroptosis has not been described to be induced by any AP. In fact, our studies showed that StII´s lethal action is not abrogated by the presence of QVD-OPh, a broad-spectrum caspase inhibitor. Therefore, this AP does not seem to induce pyroptosis on Raji cells (Soto *et al.*, 2018) or in other cellular types (Figure 6).

**Conclusions**

In general, cell death pathways show a high degree of diversity and plasticity. The type and the intensity of the damage signal, the cellular type, as well as the metabolic state of the cells among other factors determine how would happen the cellular death. Due to the existence of redundant response mechanisms, the blockade of a particular cellular death pathway does not necessarily avoid the destruction of the cell, but rather it can redirect it toward an alternative pathway. PFTs are able to kill different cellular types efficiently becoming potential tools for biomedical applications to eliminate undesirable cells. Bacterial PFTs at sublytic concentrations cause RCD such as apoptosis, necroptosis and pyroptosis. However, only apoptosis or regulated necrosis have been claimed to be cell death mechanisms induced by APs at sublytic concentrations. Differences between cell death mechanisms induced by these two broad groups of PFTs might have their origin in several factors.

On the one side, bacterial PFTs, besides a lipidic receptor like cholesterol can require a protein receptor that has not been described for any AP. Bacterial toxins have suffered a higher selection pressure due to their co-evolution with humans and the interplay between the human immune system and the bacterial infective and survival arsenal. This has probably led to more specialized infective mechanisms to survive involving protein receptors and escape mechanisms. In contrast, sea anemone toxins whose encounters with humans are quite rare and, in terms of space and evolutionary time, probably insignificant, have rely their toxin action on an ancient and global mechanism of interaction with the lipidic bilayer. On the other side, experimentally, the cellular type studied in one or other cases could explain at least partially different responses. Moreover, we should keep in mind that in contrast to bacterial PFTs, there are much fewer studies on the cell death mechanisms induced by AP, particularly at sublytic concentrations where the problem is more interesting. Therefore, we still have time to discover novel death mechanisms induced by AP PFTs and their control.

Due to their highly cytotoxic activity APs constitute attractive tools for the construction of membrane-acting immunotoxins or molecule delivery systems to cell cytosol, alternatively to their per se killing activity. In addition, these molecules after a rational modification could be used to strengthen the effects of chemotherapeutic agents and common anti-cancer drugs by facilitating their access into the cell cytosol. A profound understanding of the molecular mechanisms involved in toxin-cell interaction and the implications for cell functioning as described in this contribution would facilitate the rational design of their applications.

## CITED LITERATURE

Abrami, L., M. Fivaz, and F.G. van Der Goot (2000). Adventures of a pore-forming toxin at the target cell surface. Trends Microbiol. 8: 168–172.

Abrami, L., M.C. Velluz, Y. Hong, K. Ohishi, *et al.* (2002). The glycan core of GPI-anchored proteins modulates aerolysim binding but is not sufficient: The polypeptide moiety is required for the toxin-interaction. FEBS Lett 512: 249-254.

Aguilar, J.L., R. Kulkarni, T.M. Randis, S. Soman, *et al.* (2009). Phosphatase-dependent regulation of epithelial mitogen-activated protein kinase responses to toxin induced membrane pores. PLoS One 4(11): e8076.

Akazawa, Y., H. Isomoto, K. Matsushima, T. Kanda, *et al*. (2013). Endoplasmic reticulum stress contributes to Helicobacter pylori VacA-induced apoptosis. PLoS One 8(12): e82322

Alegre-Cebollada, J., M. Oñaderra, J.G. Gavilanes and A. Martínez del Pozo (2007). Sea Anemone Actinoporins: The Transition from a Folded Soluble State to a Functionally Active Membrane-Bound Oligomeric Pore. Curr. Prot. Pept. Sci. 8: 558-572.

Álvarado-Mesén, J., Solano-Campos, F., Canet, L., Pedrera, *et al.* (2019). Cloning, purification and characterization of nigrelysin, a novel actinoporin from the sea anemone *Anthopleura nigrescens*. Biochimie 156: 206–223.

Álvarez, C., F. Casallanovo, C.S. Shida, L.V. Nogueira, *et al.* (2003). Binding of sea anemone pore-forming toxins sticholysins I and II to interfaces. Modulation of conformation and activity, and lipid-protein interaction. Chem. Phys. Lipids. 122: 97-105.

Alvarez, C., F. Pazos, C. Soto, R. Laborde, *et al.* (2020). Pore-forming toxins from sea anemones: from protein-membrane interaction to its implications for developing biomedical applications. In: Advances in biomembranes and lipid self-assembly. Academic Press.pp:129-184.

Álvarez, C., J.M. Mancheño, D. Martínez, M. Tejuca, *et al.* (2009). Sticholysins, two pore-forming toxins produced by the Caribbean Sea anemone Stichodactyla helianthus: Their interaction with membranes. Toxicon. 54: 1135–1147.

Anderluh, G and P Maček (2002). Cytolytic peptide and protein toxins from sea anemones (Anthozoa: Actiniaria). Toxicon. 40: 111-124.

Aroian, R., and F.G. van der Goot (2007). Pore-forming toxins and cellular non-immune defenses (CNIDs). Curr Opin Microbiol 10: 57–61.

Athanasiadis, A., G. Anderluh, P. Maček and D. Turk (2001). Crystal structure of the soluble form of equinatoxin II, a pore-forming toxin from the sea anemone *Actinia equina*. Structure. 9: 341-346.

Autheman, D., M. Wyder, M. Popoff, K. D'herde, *et al.* (2013). Clostridium perfringens b-toxin induces necrostatin-inhibitable, calpain dependent necrosis in primary porcine endothelial cells. PLoS One. 8: e64644.

Bakrač, B., I. Gutiérrez-Aguirre, Z. Podlesek, A. Sonnen, *et al.* (2008). Molecular determinants of sphingomyelin specificity of a eukaryotic pore-forming toxin. J. Biol. Chem. 283: 18665-18677.

Bebien, M., M.E. Hensler, S. Davanture, L.C Hsu, *et al.* (2012). The pore-forming toxin β hemolysin/cytolysin triggers p38 MAPK-dependent IL-10 production in macrophages and inhibits innate immunity. PLoS Pathog.8: e1002812.

Belizário, J. E., L. Vieira-Cordeiro and S. Enns (2015). Necroptotic cell death signalling and Execution Pathways: Lessons from Knockout mice. Mediators Inflamm.1-15.

Berghe, T. V., W. Kaiser, J.M. Mathieu and P. Vandenabeele (2016). Molecular crosstalk between apoptosis, Necroptosis, and survival signaling. Molecular & Cellular Oncology. 2: e975093.

Berube, B and W. Bubeck (2013). Staphylococcus aureus α-toxin: Nearly a century of intrigue. Toxins 5:1140-1166.

Bezu, L., A. Sauvat, J. Humeau, M. Leduc, *et al*. (2018). eIF2a phosphorylation: A hallmark of immunogenic cell death. OncoImmunology e 1431089

Bielaszewska, M., C. Ruter, L. Kunsmann, L. Greune, *et al.* (2013). Enterohemorrhagic *Escherichia coli* hemolysin employs outer membrane vesicles to target mitochondria and cause endothelial and epithelial apoptosis. PLoS Pathog 9(12): e1003797.

Bischof, L. J., C. Kao, F.C. Los, M.R. Gonzalez, *et al*. (2008). Activation of the unfolded protein response is required for defenses against bacterial pore forming toxin *in vivo*. *PLoS Pathog.* 4: e1000176.

Bischofberger, M., I. Iacovache and & F.G. van der Goot (2012). Pathogenic pore-forming proteins: function and host response. Cell Host Microbe. 12: 266-275.

Bourdeau, R.W., E. Malito, A. Chenal, B.L. Bishop, et al. (2009). Cellular functions and X-ray structure of anthrolysin O, a cholesterol-dependent cytolysin secreted by Bacillus anthracis. J. Biol. Chem. 284: 14645–14656.

Braun, J.S., O. Hoffmann, M. Schickhaus, D. Freyer, *et al.* (2007). Pneumolysin causes neuronal cell death through mitochondrial damage. Infect. Immun. 75, 4245–4254.

Bravo, R., J.M. Vicencio, V. Parra, R. Troncoso, et al. (2011). Increased ER-mitochondrial coupling promotes mitochondrial respiration and bioenergetics during early phases of ER stress., J. Cell Sci. 124: 2143–2152.

Brito, C., D. Cabanes, F. Sarmento, S. Souza (2019). Mechanisms protecting host cells against bacterial pore-forming toxins. Cellular and Molecular Life Sciences 76:1319–1339.

Broz, P., and V.M. Dixit (2016). Inflammasomes: mechanism of assembly, regulation and signalling. Nat. Rev. Immunol. 16: 407 –420.

Burgess, D. J. (2013). Apoptosis: Refined and lethal. Nat. Rev. Cancer. 13:79.

Cabezas, S., S. Ho, U. Ros, M.E. Lanio, *et al.* (2017). Damage of eukaryotic cells by the pore-forming toxin sticholysin II: consequences of the potassium efflux. Biochim. Biophys. Acta. 1859: 982-992.

Cai, Z., S. Jitkaew, J. Zhao, H.C. Chiang, et al. (2014). Plasma membrane translocation of trimerized MLKL protein is required for TNF-induced necroptosis. Nat Cell Biol 16:55–65.

Cancino-Rodezno, A., H. Porta, M. Soberón and A. Bravo (2009). Defense and death responses to pore forming toxins. Biotechnol. Genet. Eng. Rev. 26: 65-82.

Carrero, J. A., B. Calderon and E.R. Unanue (2004). Type I interferon sensitizes lymphocytes to apoptosis and reduces resistance to Listeria infection. J. Exp. Med. 200: 535–540.

Chakravorty, A., Awad, M. M., Cheung, J. K., Hiscox, T. J., Lyras, D., & Rood, J. I. (2015). The pore-forming α-toxin from *Clostridium septicum* activates the MAPK pathway in a Ras-c-Raf-dependent and independent manner. *Toxins* 7: 516-534.

Chen, X., W. Li, J. Ren, D. Huang, *et al.* (2014). Translocation of mixed lineage kinase domain-like protein to plasma membrane leads to necrotic cell death. Cell Res. 24: 105–121.

Choi, M.E., D.R. Price, S.W. Ryter and A.M.K Choi (2019). Necroptosis: A crucial pathogenic mediator of human disease. JCI Insight 4.

Cosentino, K., U. Ros and A.J. Garcia-Saez (2016). Assembling the puzzle: Oligomerization of alpha-pore forming proteins in membranes, Biochim. Biophys. Acta 1858: 457–466.

Coskun, U., and K. Simons (2011). Cell membranes: the lipid perspective.Structure 19, 1543–1548.

Dal Peraro, M and G. van der Goot (2016). Pore-forming toxins: ancient, but never really out of fashion. Nat. Rev. Microbiol. 14: 77-92.

de Vasconcelos, N. M., N. Van Opdenbosch, H. Van Gorp, E. Parthoens, *et al*. (2018). Single-cell analysis of pyroptosis dynamics reveals conserved GSDMD-mediated subcellular events that precede plasma membrane rupture. Cell Death Differ. https://doi.org/10.1038/s41418-018-0106.

Degterev, A., J. Hitomi, M. Germscheid, I.L. Chen, *et al.* (2008). Identification of RIP1 kinase as a specific cellular target of necrostatins. Nat. Chem. Biol. 4, 313–321.

Delbridge, A. R., S. Grabow, A. Strasser and D.L. Vaux (2016). Thirty years of BCL-2: translating cell death discoveries into novel cancer therapies. Nat. Rev. Cancer 16**:** 99-109.

Dhuriya, Y. and D. Sharma (2018). Necroptosis: a regulated inflammatory mode of cell death. Journal of Neuroinflammation 15:199.

Ding, J., K. Wang, W. Liu, Y. She, *et al.* (2016). Pore-forming activity and structural autoinhibition of the gasdermin family. Nature 535:111–116.

Eifler, N., M. Vetsch, M. Gregorini, P. Ringler, *et al.* (2006). Cytotoxin ClyA from Escherichia coli assembles to a 13-meric pore independent of its redox-state. The EMBO Journal 25:2652-2661.

Essmann, F., H. Bantel, G. Totzke, I.H. Engels, *et al.* (2003). Staphylococcus aureus alpha-toxin-induced cell death: predominant necrosis despite apoptotic caspase activation. Cell Death Differ. 10: 1260–1272.

Favata, M. F., K. Y. Horiuchi, E. J. Manos, A. J. Daulerio, *et al.* (1998). Identification of a novel inhibitor of mitogen-activated protein kinase kinase. J. Biol. Chem. 273:18623.

Fedorov, S., S. Dyshlovoy, M. Monastyrnaya, L. Shubina, *et al.* (2010). The anticancer effects of actinoporin RTX-A from the sea anemone Heteractis crispa (=Radianthus macrodactylus), Toxicon. 55 : 811–817.

Feil, S. C., S. Lawrence, T.D. Mulhern, J.K. Holien, *et al.* (2012). Structure of the lectin regulatory domain of the cholesterol-dependent cytolysin lectinolysin reveals the basis for its lewis antigen specificity. *Structure* **20:** 248–258.

Fick, H., R. Cockeran, H.C. Steel, C. Feldman, *et al* (2005). Pneumolysin-mediated activation of NFkappaB in human neutrophils is antagonized by docosahexaenoic acid. Clin Exp Immunol. 140:274-281.

Fink, S. L., T. Bergsbaken, and B.T. Cookson (2008). Anthrax lethal toxin and Salmonella elicit the common cell death pathway of caspase-1-dependent pyroptosis via distinct mechanisms. Proc. Natl.Acad. Sci. 105: 4312–4317.

Fink, S.L., and B.T. Cookson (2005) Apoptosis, pyroptosis and necrosis: mechanistic description of dead and dying eukaryotic cells. Infection and Immunity. 73:1907-1916.

Fink, S.L., and B.T. Cookson (2006). Caspase-1-dependent pore formation during pyroptosis leads to osmotic lysis of infected host macrophages. Cell. Microbiol. 8: 1812–1825.

Fu, Q., T.M. Fu, A.C. Cruz, P. Sengupta, *et al.* (2016). Structural Basis and Functional Role of Intramembrane Trimerization of the Fas/CD95 Death Receptor. Mol Cell. 61: 602-613.

Galluzzi, L., A. Lopez-Soto, S. Kumar and G. Kroemer (2016). Caspases connect cell-death signaling to organismal homeostasis. Immunity 44: 221–231.

Galluzzi, L., I. Vitale, S.A. Aaronson, J.M. Abrams, *et al.* (2018). Molecular mechanisms of cell death: recommendations of the Nomenclature Committee on Cell Death. Cell Death Differ. 25 (3):486-541.

Galluzzi, L., J.M. Bravo-San Pedro, I. Vitale, S.A. Aaronson, *et al.* (2015). Essential versus accessory aspects of cell death: recommendations of the NCCD. Cell Death Differ. 22 (1): 58– 73.

Ganash, M., D. Phung, S. Sedelnikova, T. Linback, *et al. (2013).* Structure of the NheA component of the Nhe toxin from *Bacillus cereus*: implications for function. *PLoS ONE* 8, e74748 (2013).

García-Linares, S., I. Castrillo, M. Bruix, M. Menéndez, *et al.* (2013). Three-dimensional structure of the actinoporin sticholysin I. Influence of long-distance effects on protein function. Arch. Biochem. Biophys. 532(1): 39-45.

García-Sáez, A.J., S.B. Buschhorn, H. Keller, G. Anderluh, *et al*. (2011). Oligomerization and Pore Formation by Equinatoxin II Inhibit Endocytosis and Lead to Plasma Membrane Reorganization, J. Biol. Chem. 286: 37768–37777.

Genestier, A. L., M.C. Michallet, G. Prevost, G. Bellot, *et al.* (2005). Staphylococcus aureus Panton-Valentine leukocidin directly targets mitochondria and induces Bax-independent apoptosis of human neutrophils. J. Clin. Invest. 115: 3117–3127.

Goncharov, T., K. Niessen, M.C. de Almagro, A. Izrael-Tomasevic, *et al.* (2013). OTUB1 modulates c-IAP1 stability to regulate signaling pathways. EMBO J. 32:1103–1114.

Gonzalez, M. R., Bischofberger, M., Frêche, B., Ho, S., Parton, R. G., & van der Goot, F. G. (2011). Pore-forming toxins induce multiple cellular responses promoting survival. *Cell. Microbiol*. 13(7):1026-1043.

Gonzalez, M.R., M. Bischofberger, L. Pernot, G. van Der Goot, *et al.* (2008).Bacterial pore-forming toxins : The (w)hole story ?, Cell. Mol .Life. Sci. 65: 493–507.

González-Juarbe, N., K.M.N. Bradley, A.T. Shenoy, R.P.Gilley, *et al.* (2017). Pore forming toxin-mediated ion dysregulation leads to death receptor independent necroptosis of lung epithelial cells during bacterial pneumonia. Cell Death Differ. 24: 917-928.

Groose, L., C.A. Wurm, C. Bruser, D. Neumann, *et al.* (2016). Bax assembles into large ring-like structures remodeling the mitochondrial outer membrane in apoptosis. EMBO J. 35: 402-413.

Grootjans, S., T. VandenBerghe, and P. Vandenabeele (2017) Initiation and execution mechanisms of necroptosis: an overview. Cell Death Differ. 1–12.

Guessan P.D.N., B. Schmeck, A. Ayim, *et al*. (2005). Streptococcus pneumoniae R6x induced p38 and JNK-mediated caspase-ependent apoptosis in human endothelial cells. Thrombosis and Haemosttasis 94: 295-303.

He, W., H. Wan, L. Hu, P. Chen, *et al.* (2015). Gasdermin D is an executor of pyroptosis and required for interleukin-1β secretion. Cell Res 25:1285–1298.

Hernández-Flores, K.G. and H. Vivanco-Cid (2015). Biological Effects of Listeriolysin O: Implications for Vaccination. BioMed Research International. ID 360741.

Hinds, M. G., W. Zhang, G. Anderluh, P.E. Hansen, *et al.* (2002). Solution structure of the eukariotic pore-forming cytolisin equinatoxin II: implication for pore formation. J. Mol. Biol. 315: 1219-1229.

Hong, Q., I. Gutiérrez-Aguirre, A. Barlič, P. Marlovrh, *et al.* (2002). Two step membrane binding by Equinatoxin II, a pore-forming toxin from the sea anemone, involves an exposed aromatic cluster and a flexible helix. J. Biol. Chem. 277: 41916-41924.

Hotze, E.M. and R.K. Tweten (2012). Membrane assembly of the cholesterol-dependent cytolysin pore complex, Biochim. Biophys. Acta - Biomembr. 1818: 1028–1038.

Huang, G., A. Voet and G. Maglia (2019). FraC nanopores with adjustable diameter identify the mass of opposite-charge peptides with 44 dalton resolution. Nat. Commun.10, 835.

Huang, Z., S. Wu and Y. Liang (2015). RIP1/RIP3 binding to HSV-1 ICP6 initiates necroptosis to restrict virus propagation in mice. Cell Host Microbe 17:229–242.

Huerta, V., V. Morera, Y. Guanche, G. Chinea, *et al.* (2001). Primary structure of two cytolysin isoforms from Stichodactyla helianthus differing in their hemolytic activity, Toxicon. 39: 1253–1256.

Huffman, D. L., L. Abrami, R. Sasik, J. Corbeil, et al. (2004). Mitogen-activated protein kinase pathways defend against bacterial poreforming toxins. *Proc Natl Acad Sci* 101: 10995–11000.

Husmann, M., K. Dersch, W. Bobkiewicz, E. Beckmann, et al. (2006) Differential role of p38 mitogen activated protein kinase for cellular recovery from attack by pore-forming S. aureus alpha-toxin or streptolysin O. Biochem Biophys Res Commun 344(4):1128–1134.

Imre, G., J. Heering, A.N. Takeda, M. Husmann, *et al.* (2012). Caspase-2 is an initiator caspase responsible for pore-forming toxin-mediated apoptosis. EMBO J. 31, 2615–2628.

Jorgensen, I., and E.A. Miao (2015). Pyroptotic celldeath defends against intracellular pathogens. Immunol Rev. 265(1):130-142.

Julien, O and J.A. Wells (2017). Caspases and their substrates. Cell Death Differ.24: 1380–1389.

Kao, C.Y., F.C. Los, D.L. Huffman, S. Wachi, *et al* (2011). Global functional analyses of cellular responses to pore-forming toxins. PLoS Pathog 7(3):e1001314.

Katayama, H., Y. Kusaka, H. Yokota,T. Akao, *et al.* (2007). Parasporin-1 a novel cytotoxic protein from Bacillus thuringiensis induces $Ca^{2+}$ influx and sustained elevation of the cytoplasmic $Ca^{2+}$ concentration in toxin sensitive cells. J. Biol. Chem. 282: 7742-7752.

Kennedy, C.L., D.J. Smith, D. Lyras, A. Chakravorty, J.I. Rood (2009). Programmed cellular necrosis mediated by the poreforming alpha-toxin from Clostridium septicum. PLoS Pathog 5(7): e1000516

Kerr, J. F., A.H. Wyllie and A.R. Currie (1972). Apoptosis: a basic biological phenomenon with wide ranging implications in tissue kinetics. Br. J. Canc. 26:239-257.

Kitur, K., D. Parker, P. Nieto, D.S. Ahn, *et al*. (2015). Toxin-induced necroptosis is a major mechanism of Staphylococcus aureus lung damage. PLoS Pathog.11(4): e1004820.

Kloft, N., T. Busch, C. Neukirch, S. Weis, et al. (2009). Pore-forming toxins activate MAPK p38 by causing loss of cellular potassium. Biochem. Biophys. Res. Commun. 385, 503–506.

Koff, L., S. Ramachandiran, and L. Bernal-Mizrachi (2015). A time to kill: targeting apoptosis in cancer. Int. J. Mol. Sci. 16: 2942-2955.

Koster, S. K. Van Pee, M. Hudel et al. (2014). Crystal structure of listeriolysin O reveals molecular details of oligomerization and pore formation. Nature Communications. 5: 3690.

Kreitman, R.J., C. Dearden, P.L. Zinzani, J. Delgado, *et al.* (2018). Moxetumomab pasudotox in relapsed/refractory hairy cell leukemia. Leukemia 32: 1768–1777.

Kroemer, G., L. Galluzzi, P. Vandenabeele, J. Abrams, *et al.* (2009). Classification of cell death: recommendations of the Nomenclature Committee on Cell Death. Cell Death Differ 16(1):3-11.

Kumar, S. (2007). Caspase function in programmed cell death. Cell Death Differ. 14(1):32-43.

Labandeira-Rey, M., F. Couzon, S. Boisset, E.L. Brown, *et al.* (2007). Staphylococcus aureus Panton-Valentine leukocidin causes necrotizing pneumonia. Science 315: 1130–1133.

Laborde, R. J., O. Sanchez-Ferras, M.C. Luzardo, Y. Cruz-Leal, *et al.* (2017). Novel adjuvant based on the pore-forming protein sticholysin II encapsulated into liposomes effectively enhances the antigen specific CTLmediated immune response. J. Immunol. 198: 2772-2784.

Lakey, J.H., F.G. van der Goot., F. Pattus (1994). All in the family: the toxic activity of pore-forming colicins. Toxicology 87(1–3):85–108

Lanio, M. E., L. Fernández, R. Laborde, Y. Cruz, *et al.* (2014). Vaccine composition based on sticholysin encapsulated into liposomes. Patente US 8,697,093 B2.

Lanio, M. E., V. Morera, C. Álvarez, M. Tejuca, *et al.* (2001). Purification and characterization of two hemolysins from *Stichodactyla helianthus*. Toxicon. 39: 187-194.

Lesieur, C., R. Mo and G. van Der Goot (1997). Conformational Changes Due to Membrane Binding and Channel Formation by Staphylococcal Alpha-Toxin, J. Biol. Chem. 272: 5709–5717.

Li, M., Z-S. Liu, X-L. Liu, Q. Hui, *et al.* (2017). Clinically targeting recombinant immunotoxins for cancer therapy. Onco Targets Ther. 10:3645–3665.

Li, Y., P. Maher and D. Schubert (1997). A role for 12-lipoxygenase in nerve cell death caused by glutathione depletion. Neuron. 19:453–463.

Lieberman, J., H. Wu, and J.C. Kagan (2019) Gasdermin D activity in inflammation and host defense. Sci Immunol 4: eaav1447.

Linkermann, A., T. VandenBerghe, N. Takahashi, U. Kunzendorf, *et al.* (2014) The Potential Role of Necroptosis in Diseases. In Necrotic Cell Death. eds. Shen HM, Vandenabeele P. 1–22, New York: Humana Press, Springer.

Liu, X and J. Lieberman (2020). Knocking 'em Dead: Pore-Forming Proteins in Immune Defense. Annu.Rev. Immunol.38:455–485.

Liu, X., Z. Zhang, J. Ruan, Y. Pan, *et al.* (2016) Inflammasome-activated gasdermin D causes pyroptosis by forming membrane pores. Nature 535:153–158.

Lu, A., V.G. Magupalli, J. Ruan, Q. Yin, *et al.* (2014) Unified Polymerization Mechanism for the Assembly of ASC-Dependent Inflammasomes. Cell. 156: 1193–1206

Luna-Vargas, M. P. and J.E. Chipuk (2016). Physiological and Pharmacological Control of BAK, BAX, and Beyond. Trends Cell Biol. 26: 906-917.

Maček, P., G. Belmonte, C. Pederzolli, C and G. Menestrina (1994). Mechanism of action of equinatoxin II, a cytolysin from the sea anemone Actinia equina L. belonging to the family of actinoporins. Toxicology. 87: 205-227.

Madegowda, M., S. Eswaramoorthy, S.K. Burley, & S. Swaminathan (2008). X-ray crystal structure of the B component of hemolysin BL from *Bacillus cereus*. Proteins 71**:** 534–540.

Mancheño, J. M., J. Martín-Benito, M. Martínez-Ripoll, J.G. Gavilanes, *et al.* (2003). Crystal and electron microscopy structures of sticholysin II actinoporin reveal insight into the mechanism of membrane pore formation. *Structure.* 11: 1319-1328.

Mariño, G., M. Nino-Santanao, E.H. Baeherecke and G. Kroemer (2014). Self-consumption: the interplay of autophagy and apoptosis. Nat. Rev. Mol. Cell. Biol. 15:81-94.

McClane, B. A., & G. Chakrabarti (2004). New insights into the cytotoxic mechanisms of *Clostridium perfringens* enterotoxin. *Anaerobe* 10: 107-114.

McCubrey, J., M.M. LaHair & R.A. Franklin (2006). OSU-03012 in the treatment of Glioblastoma. *Molecular Pharmacology*. 70 (2):437–439.

McIlwain, D. R., T. Berger and T.E. Mak (2013). Caspase functions in cell death and disease. Cold Spring Harb Perspect Biol. 5(4): a008656.

Mebratu, Y., & Y. Tesfaigzi. (2009). How ERK1/2 activation controls cell proliferation and cell death: Is subcellular localization the answer? *Cell Cycle*. 8(8):1168-1175.

Menzies, B. E., I Kourteva. (2000). Staphylococcus aureus alpha-toxin induces apoptosis in endothelial cells. FEMS Immunol. Med. Microbiol. 29:3945.

Methippar, M.M., T. Bashir, S. Kumar, N, Alam, et al. (2009). Salubrinal, an inhibitor of protein synthesis, promotes deep slow wave sleep, Am J Physiol Regul Integr Comp Physiol 296: R178–R184

Mompean, M., W. Li, J. Li, *et al* (2018). The structure of the necrosome RIPK1-RIPK3 core, a human hetero-amyloid signaling complex. Cell 173: 1244–1253.

Mutter, N.L., M. Soskine, G. Huang, I.S. Albuquerque, *et al.* (2018) Modular Pore-Forming Immunotoxins with Caged CytotoxicityTailored by Directed Evolution. ACSChem. Biol.13:3153–3160.

Nagahama, M., M. Shibutani, S. Seike, M. Yonezaki, *et al.* (2013). The p38 MAPK and JNK pathways protect host cells against *Clostridium perfringens* beta-toxin. Infect Immun. 81(10):3703-3708.

Nagahama, M., M. Umezaki, M. Oda, K. Kobayashi, *et al.* (2011). Clostridium perfringens iota-toxin b induces rapid cell necrosis. Infect Immun. 79(11):4353-4360.

Nagata S and M. Tanaka (2017). Programmed cell death and the immune system. Nat Rev Immunol 17:333–340.

Nelson, K.L., R.A. Brodsky, and J.T. Buckley (1999). Channels formed by subnanomolar concentrations of the toxin aerolysin trigger apoptosis of T lymphomas. Cell Microbiol 1: 69–74.

Nerlich, A., M. Mieth, E. Letsiou, D. Fatykhova, *et al.* (2018). Pneumolysin induced mitochondrial dysfunction leads to release of mitochondrial DNA. Nat. Sci. Rep. 8:182.

Newton, K. (2015). RIPK1 and RIPK3: critical regulators of inflammation and cell death Trends Cell Biol. 25(6):347-353.

Newton, K., D.L. Dugger, K.E. Wickliffe, N. Kapoor, *et al.* (2014). Activity of protein kinase RIPK3 determines whether cells die by necroptosis or apoptosis. Science. 343:1357–1360.

Ofengeim, D., and J. Yuan (2013). Regulation of RIP1 kinase signalling at the crossroads of inflammation and cell death. Nat. Rev. Mol. Cell. Biol. 14(11):727–736.

Orrenius, S., P. Nicotera and B. Zhivotovsky (2011). Cell death mechanisms and their implications in toxicology. Toxicological Sciences. 119(1): 3–19.

Patyar S., R. Joshi., D.P. Byrav, A. Prakash, et al. (2010). Bacteria in cancer therapy: a novel experimental strategy. J Biomed Sci.17:21.

Pentón, D., V. Pérez-Barzaga, I.Díaz, M.L. Reytor, *et al.* (2011). Validation of a mutant of the pore-forming toxin sticholysin-I for the construction of proteinase-activated immunotoxins. Protein Eng. Des. Sel. 24: 485–493.

Pettersen, E. F., T.D. Goddard, C.C. Huang, G.S. Couch, *et al.* (2004). UCSF chimera a visualization system for exploratory research and analysis. J. Comput. Chem. 25:1605–1612.

Petrie, E.J., J.J. Sandow, A.V. Jacobsen, B.J. Smith, *et al.* (2018). Conformational switching of the pseudokinase domain promotes human MLKL tetramerization and cell death by necroptosis. Nat. Commun. 9, 2422.

Petrie, E.J., P.E. Czabotar and J.M. Murphy (2019). The Structural Basis of Necroptotic Cell Death Signaling. Trends in Biochemical Sciences (In press).

Porta, H., A. Cancino-Rodezno, M. Soberon, A. Bravo. (2011) Role of MAPK p38 in the cellular responses to pore-forming toxins. Peptides 32(3):601–606.

Radin, J.N., C. Gonzalez-Rivera, S.E. Ivie, M.S. McClain, *et al.* (2011). Helicobacter pylori VacA induces programmed necrosis in gastric epithelial cells. Infect. Immun. 79, 2535–2543.

Rathkey, J. K., J. Zhao, Z. Liu, Y. Chen, *et al.* (2018). Chemical disruption of the pyroptotic pore-forming protein gasdermin D inhibits inflammatory cell death and sepsis. Sci.Immunol.3: eaat2738.

Robertson, J.W.F and J.E. Reiner (2018). The Utility of Nanopore Technology for Protein and Peptide Sensing. Proteomics 18, e1800026.

Rodriguez, D. A., R. Weinlich, S. Brown, C. Guy, *et al.* (2016). Characterization of RIPK3-mediated phosphorylation of the activation loop of MLKL during necroptosis. Cell Death Differ. 23: 76–88.

Rojko N, Kristan KC, Viero G et al (2013) Membrane damage by an alpha helical pore-forming protein, equinatoxin II, proceeds through a succession of ordered steps. J Biol Chem 288:23704 –23715.

Rojko, N., M. Dalla Serra, P. Maček and G. Anderluh (2016). Pore formation by actinoporins, cytolysins from sea anemones. Biochim. Biophys. Acta 1858: 446–456.

Saenz, J. B., J. Li, and D.B. Hasiam. (2010). The MAP kinase-activated protein kinase 2(MK2) contributes to the shiga toxin-induces inflammatory response. Cell Microbiol 12:516–29

Salvesen, G.S., and C.M. Walsh (2014) Functions of caspase 8: the identified and the mysterious. Semin Immunol. 26:246–252.

Savva, C. G., S.P. Fernandes da Costa, M. Bokori-Brown, C.E. Naylor, *et al.* (2013). Molecular architecture and functional analysis of NetB, a pore-forming toxin from Clostridium perfringens. J. Biol. Chem. 288: 3512–3522.

Shalini, S., L. Dorstyn, S. Dawar and S. Kumar (2015). Old, new and emerging functions of caspases. Cell Death Differ 22:526–539.

Shinoda, T., N. Shinya, K. Ito, N. Ohsawa, *et al.* (2016). Structural basis for disruption of claudin assembly in tight junctions by an enterotoxin. Scientific Repots 6:33632. doi: 10.1038/srep33632.

Shoma, S., K. Tsuchiya, I. Kawamura, T. Nomura, *et al.* (2008). Critical involvement of pneumolysin in production of interleukin-1alpha and caspase-1-dependent cytokines in infection with Streptococcus pneumoniae in vitro: a novel function of pneumolysin in caspase-1 activation. Infect. Immun. 76:1547-1557.

Sierig, G., C. Cywes, M.R. Wessels, C.D Ashbaugh (2003). Cytotoxic effects of streptolysin O and streptolysin S enhance the virulence of poorly encapsulated group a streptococci. Infect Immun 71: 446–55

Skocaj, M., N. Resnik, M. Grundner, K. Ota, *et al*. (2014). Tracking cholesterol/sphingomyelin-rich membrane domains with the ostreolysinA-mCherry protein. PLoS ONE 9: e92783.

Soletti, R.C., T. Alves, J. Vernal, H. Terenzi, *et al.* (2010). Inhibition of MAPK / ERK , PKC and CaMKII Signaling Blocks Cytolysin-induced Human Glioma Cell Death, Anticancer Res. 30: 1209–1215.

Soong, G., J. Chun, D. Parker and A. Prince (2012). S. aureus activation of caspase-1/calpain signaling mediates invasion through human keratinocytes. J. Infect. Dis. 205: 1571–1579.

Soto, C., G. Bergado, R. Blanco, T. Griñán, *et al.* (2018). Sticholysin II-mediated cytotoxicity involves the activation of regulated intracellular responses that anticipates cell death Biochimie 148:18-35.

Spaan, A. N., A. Schieperset, C.J.C de Plaas, D. van Hoogdonk, *et al.* (2013). The staphylococcal toxin PantonValentine leukocidin targets human C5a receptors. Cell Host Microbe 13: 584–594.

Stassen, M., C. Muller, C. Richter, et al. (2003). The streptococcal exotoxin streptolysin O activates mast cells to produce tumor necrosis factor alpha by p38 mitogen-activated protein kinase- and protein kinase C-dependent pathways. Infection and Immunity 71: 6171-6177.

Stringaris, A.K., J. Geisenhainer, F. Bergmann, C. Balshusemann, *et al.* (2002) Neurotoxicity of pneumolysin, a major pneumococcal virulence factor, involves calcium influx and depends on activation of p38 mitogen-activated protein kinase. Neurobiol Dis 11(3):355–368.

Sun, L., H. Wang, Z. Wang, S. He, *et al.* (2012). Mixed lineage kinase domain-like protein mediates necrosis signaling downstream of RIP3 kinase. Cell. 148: 213-227.

Suzuki, J., E. Imanishi and S. Nagata, S. (2016). Xkr8 phospholipid scrambling complex in apoptotic phosphatidylserine exposure. Proc Natl Acad Sci 113: 9509-9514.

Tanaka, K., J.M. Caaveiro, K. Morante, J.M. González-Mañas, *et al.* (2015). Structural basis for self-assembly of a cytolytic pore lined by protein and lipid. Nat. Commun. 6: 6337.

Tang, D., R. Kang, T. Vanden Berghe, P. Vandenabeele, *et al.* (2019). The molecular machinery of regulated cell death. Cell Research 29:347–364.

Tanzer, M., A. Tripaydonis, A.I Webb, S.N. Young, *et al.* (2015). Necroptosis signaling is tuned by phosphorylation of MLKL residues outside the pseudokinase domain activation loop. Biochem J. 471:255-265.

Tejuca, M., M. Dalla Serra, C. Potrich, C. Álvarez, *et al.* (2001). Sizing the radius of the pore formed in erythrocytes and lipid vesicles by the toxin sticholysin I from the sea anemone Stichodactyla helianthus. J. Membr.Biol. 183: 125-1351135–1147.

Tejuca, M., M. Dalla Serra, M., Ferreras, M.E. Lanio, *et al.* (1996). Mechanism of membrane permeabilization by sticholysin I, a cytolysin isolated from the venom of the sea anemone *Stichodactyla helianthus*. Biochemistry 35: 14947-14957.

Tejuca. M., G. Anderluh, M. Dalla-Serra (2009) Sea anemone cytolysins as toxic components of immunotoxins. Toxicon 54:1206–1214.

Timmer, A.M., J.C. Timmer, M.A. Pence, L.C. Hsu, *et al.* (2009). Streptopysin O promotes group a Streptococcus immune evasion by accelerated macrophage apoptosis. J. Biol. Chem. 284: 862-871.

Van Itallie, C. M. and J.M. Anderson (2013) Claudin interactions in and out of the tight junction. Tissue Barriers 1: e25247.

Van Opdenbosch, N., and M. Lamkanfi (2019). Caspases in Cell Death, Inflammation, and Disease. Immunity 50:1352–1364.

Vandenabeele, P., L. Galluzzi, T.V. Berghe, and G. Kroemer (2010) Molecular mechanisms of necroptosis: an ordered cellular explosion. Nat. Rev. Mol. Cell Biol. 11(10):700–714.

Vitale, I., G. Manic, R. De Maria, G. Kroemer et al. (2017). DNA Damage in Stem Cells. Mol. Cell. 66**:** 306-319.

von Karstedt S, A. Montinaro, H. Walczak, *et al.* (2017). Exploring the TRAILs less travelled: TRAIL in cancer biology and therapy. Nat Rev Cancer. 7:352–366.

Wagner, E. F. and A.R. Nebreda (2009). Signal integration by JNK and p38 MAPK pathways in cancer development. Nat. Rev. Cancer. 9:537-549.

Wang, X., S. Yousefi, and H.U. Simon (2018) Necroptosis and neutrophil-associated disorders. Cell Death Dis. 9, 111.

Wang, Y., W. Gao, X. Shi, J. Ding, W. Liu, H. He, K. Wang and F. Shao (2017) Chemotherapy drugs induce pyroptosis through caspase-3 cleavage of a gasdermin. Nature 547:99–103.

Wang, Z., H. Jiang, S. Chen, F. Du, *et al.* (2012). The mitochondrial phosphatase PGAM5 functions at the convergence point of multiple necrotic death pathways. Cell. 148:228–243.

Watanabe, H., A. Gubbiotti, M. Chinappi, N. Takai, *et al.* (2017). Analysis of Pore Formation and Protein Translocation Using Large Biological Nanopores. Anal. Chem. 89: 11269–11277.

Weinlich, R., A. Oberst, H.M. Beere and D.R.Green (2017). Necroptosis in development, inflammation and disease. Nat. Rev. Mol. Cell Biol. 18: 127–136.

Welch, R.A. (2001). RTX toxin structure and function: a story of numerous anomalies and few analogies in toxin biology. Curr Top Microbiol Immunol. 25:85-111.

Wiles, T. J., B.K. Dhakal, D.S. Eto and M.A. Mulvey (2008). Inactivation of host akt/protein kinase B signaling by bacterial pore-forming toxins. Mol. Biol. Cell. 19: 1427-1438.

Wilke, G.A., and J. Bubeck Wardenburg (2010). Role of a disintegrin and metalloprotease 10 in *Staphylococcus aureus* alpha-hemolysin-mediated cellular injury. Proc. Natl. Acad. Sci. USA 107: 13473–13478.

Yang, M., M. Lee, Z. Huang Z. (2018). TFAM is a novel mediator of immunogenic cancer cell death. Oncoimmunology 7, e1431086.

Zhang, D., J. Lin and J. Han (2010). Receptor -interacting protein (RIP) kinase family. Cell. Mol Immunoly. 7: 243–249.

Zhang, W., and H. T. Liu (2002). MAPK signal pathways in the regulation of cell proliferation in mammalian cells. Cell. Res. 12 (1): 9-18.

Zhang, X., M. Matsuda, N. Yaegashi, T. Nabe, *et al.* (2020). Regulation of Necroptosis by Phospholipids and Sphingolipids. Cells 9: 627; doi:10.3390/cells9030627.

Zhang, Y., SS. Su, S. Zhao, Z. Yang, *et al.* (2017). RIP1 autophosphorylation is promoted by mitochondrial ROS and is essential for RIP3recruitment into necrosome. Nat Commun. 8:14329.

Zhang, Y., X. Chen, C. Gueydan and J. Han (2018). Plasma membrane changes during programmed cell deaths. Cell Res. 28: 9–21.

Zhao, Q., R.S.S de Zoysa, D. Wang, D.A. Jayawardhana (2009) Real-time monitoring of peptide cleavage using a nanopore probe. J. Am. Chem. Soc. 131: 6324–6325.

Zhong, L., and J.Y Su (2002). Isoflurane activates PKC and $Ca^{2+}$/ Calmodulin dependent protein kinase II via MAP kinase signaling in cultured vascular smooth muscle cells. Anesthesiology. 96(1): 148-154.

• • •