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IMMUNO-MODULATORY FUNCTIONS OF THE TYPE -3 SECRETION SYSTEM AND IMPACTS ON THE PULMONARY HOST DEFENSE: A ROLE FOR EXOS OF PSEUDOMONAS AERUGINOSA IN CYSTIC FIBROSIS.

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INTRODUCTION

1. PAMPs and innate immune response

One of the main functions of innate immunity/inflammation is to allow the host to recognize invading pathogens. Charles Janeway (1) suggested that pathogens express structures that allow their recognition by the host defense systems. He named these structures 'pathogen-associated molecular patterns' or PAMPs that are conserved motifs expressed by families of microorganisms and are essential for their survival. PAMPs include numerous virulence factors such as lipopolysaccharides (LPS) of Gram-negative bacteria, lipopeptides of Gram-positive bacteria and double-stranded RNA (dsRNA) of certain viruses. PAMPs recognition is a key step in anti-infectious immunity that involves interactions with cellular receptors called 'pattern recognition receptors', or PRRs. In addition to PAMPs, a number of toxins are produced by bacteria in response to various environmental circumstances. Exotoxins are actively secreted during the infection process, in contrast to endotoxins which are part of the bacterial outer membranes, and are secreted in extracellular media only after bacterial death. Both exo- and endotoxins are virulence factors that stimulate and/or manipulate host cell functions to favor infections. In contrast to PAMPs, exotoxins do not exhibit specific receptors like PRRs but are injected in the in host cells and directly target various enzymatic and signaling pathways.

In this review we summarize some relevant data of literatures on ExoS, an exotoxin from the type-3 secretion system of *Pseudomonas aeruginosa*, with a particular focus on the role of this toxin in the elimination of *Staphylococcus aureus* from airways of patients with cystic fibrosis.

2. The type 3 secretion system of *P. aeruginosa*

Opportunistic bacteria such as *Pseudomonas aeruginosa* can colonize virtually all human mucosal surfaces and invade tissues and blood of patients leading to various injuries or diseases. The seriousness of *P. aeruginosa* infections and its natural resistance to antimicrobial agents, led to a considerable interest in elucidating the pathogenesis of *P. aeruginosa* infections. The WTA critical priority list of antibiotic-resistant bacteria, that most urgently needs the development of new antibacterial drugs, includes *P. aeruginosa* (<http://www.who.int/mediacentre/news/releases/2017/bacteria-antibioticsneeded/en/>).

Like other Gram-negative bacteria, *P. aeruginosa* exhibits various secretion systems including the type-II and the type-III secretion systems, T2SS and T3SS, respectively). The T2SS is a membrane bound complex generally found in Gram-negative bacteria and used to secrete proteins, also called exotoxins, into the extracellular milieu and/or host organisms. The recent genome sequencing indicated that T2SS is largely restricted to the *Proteobacteria* and is present in many genera in the *Alphaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, and *Deltaproteobacteria* classes. The T2SS system produces secreted proteins encompassing degradative enzymes, such as proteases, lipases, phosphatases and several enzymes that process complex carbohydrates, involved in various pathogenic processes. The latter include host cells death, tissue degradation, innate immunity suppression, adherence to host surfaces, biofilm formation, invasion into and growth within host cells, etc. (2).

The T3SS is a needle-like structure which functions as a sensory probe to detect the presence of host cell systems and to inject toxins within these cells that help the bacteria to infect them (3). This needle-like structure is called type three-secretion system (T3SS, or Injectisome). These effectors molecules are injected directly from the bacterial cells into the eukaryotic (host) cell, where they exert various biological effects that allow the bacteria to proliferate and to escape the immune response of

the host. The term T3SS was coined in 1993 and distinguishes this system from other secretion systems found in Gram-negative bacteria. The T3SS, found in many animal and plant associated bacteria, are similar in their sequences and structures as a result of divergent evolution. Phylogenetic analysis suggested a model of horizontal transfers between Gram-negative bacteria of the T3SS gene cassette. The T3SS is composed of approximately 30 different proteins, making it one of the most complex secretion systems. Its structure shows many similarities with bacterial flagella (long, rigid, extracellular structures used for motility).

Four T3SS effector molecules (also named exotoxins) – ExoS, ExoT, ExoU and ExoY – have been identified in *P. aeruginosa* isolates, but only some of these isolates secrete simultaneously all these toxins. It should be stressed that although almost all *P. aeruginosa* strains examined so far harbor the genes that encode the T3SS machinery, the majority of these strains do not carry a complete set of the genes that encode the effectors (4,5). For example, in isolates from acute infections, the *exoS* gene is found in 58–72% of isolates, the *exoU* gene in 28–42%, the *exoY* gene in 89% and the *exoT* gene in 92–100%. Of a great interest it has been shown that nearly all strains have either the *exoS* or the *exoU* gene but not both. The reasons for this mutually exclusive expression are not clear.

3. ExoS, structure and particularities

ExoS is considered as a bifunctional toxin that exhibits both a GTPase-activating protein (GAP) and an ADP ribosyl transferase (ADPRT) activity. This effector protein has a modular domain structure that underscores the complexity of its interactions with host cells. The extreme amino terminus of ExoS carries the information for its targeting to the T3SS apparatus. Although no consensus sequence has been identified, the first 15 amino acids of ExoS presumably encode the information that is necessary for secretion through this apparatus (6) Residues 15–51 are believed to constitute a binding site for the ExoS chaperone, SpsC (7). On the other hand, the residues 51–72 exhibit a symmetrical leucine-rich sequence that forms the membrane localization domain (MLD) (8), which plays a key role in the initial transient localization of ExoS to the plasma membrane of the host cell (9). The residues 96–233 of ExoS form a GAP domain that targets Rho, Rac and cell division cycle 42 (CDC42) (10,11), that are small GTPases that control the organization of the host cell actin cytoskeleton. It has been shown that the irreversible disruption of the

cytoskeletal structure contribute to a reduction in cell–cell adherence, which in turn facilitate *P. aeruginosa* penetration through epithelial barriers (12–14). This leads to a cell death which has features of apoptosis, associated to a loss of cell membrane integrity and subsequent necrosis (13,15). Studies suggested that ExoS induces killing of host immune cells that allows *P. aeruginosa* to persist in spite of a vigorous host immune response (16). However the cell types targeted by ExoS *in vivo* have not been identified.

4. Roles of T3SS in host immune response

A number of studies have investigated the implications of *P. aeruginosa* T3SS toxins in inducing inflammatory and/or deleterious (toxic) effects on host cells such as epithelial and endothelial cells with various pathophysiological consequences for the host. As an example, our previous studies (17) demonstrated that both the T2SS and T3SS of *P. aeruginosa* are capable of causing death in lung infections and that the onset of action differs between the T2SS and the T3SS, with the T3SS acting rapidly within 24h, and the T2SS causing death at a slower rate. It is likely that this difference in the timing of lung injury is due to the fact that T2SS activation requires bacteria to reach high concentrations to achieve quorum sensing (18). Alternatively, it is possible that the pathologic lesions in this model are late in appearing. Due the fact that bacterial counts in the lungs had increased significantly by 24h, the observations are consistent with a role of quorum sensing in mediating lung injury and death. The ExoU toxin injected by *P. aeruginosa* within host cells exhibits a potent cytosolic phospholipase A2 (cPLA2)-like activity. The latter has been shown to account for membrane lysis and acute death of infected cells. We have addressed the question whether ExoU would exhibit a pro-inflammatory activity by enhancing the synthesis of eicosanoids by host organisms (19). Indeed, the host cPLA2 α belongs to a family of lipolytic enzymes which hydrolyse membrane phospholipids at the sn-2 position, thereby releasing fatty acids, particularly arachidonic acid (AA) (20,21). The latter is the precursor of a variety of pro-inflammatory lipid mediators such as prostaglandins and leukotrienes, which are involved in inflammatory processes. We showed that endothelial cells from the HMEC-1 line infected with PA103, a laboratory strain of *P. aeruginosa* expressing the toxin ExoU, exhibited potent release of AA, as well as significant amounts of the cyclooxygenase (COX)-derived prostaglandins PGE(2) and PGI(2). Both AA, PGE2 and PGI2 releases could be significantly inhibited by

methyl arachidonyl fluorophosphonate (MAFP), a specific cPLA2 α inhibitor (20,21). However, an isogenic mutant defective in ExoU synthesis failed to induce the releases of these mediators. Most importantly, using an experimental respiratory infection model, we showed that bronchoalveolar lavage (BAL) from mice instilled with PA103 exhibited a marked influx of inflammatory cells and PGE(2) release. Both processes were significantly reduced by indomethacin, a non-selective COX inhibitor. Thus, these results suggest that ExoU may contribute to *P. aeruginosa* pathogenesis by inducing an eicosanoid-mediated inflammatory response of host organisms. Since non-enzymatic AA oxidation produces reactive radicals, we next examined the ability of PA103 to stimulate oxidative stress in infected cells. Additional FACS analysis of cell labeled with the C-11 Fluor probe and anti-4-hydroxynonol antibody revealed a significant peroxidation of cell membrane lipids (22,23). Taken together these results and our finding that PA103-infected epithelial cells death was significantly attenuated by pre-treating these cells with alpha-tocopherol, a known potent antioxidant, before infection. This finding suggested that AA-induced oxidative stress is a parallel mechanism of cell damage in the course of infection by ExoU-producing *P. aeruginosa* (22,23).

DO T3SS PLAY A ROLE IN THE EVOLUTION OF AIRWAYS INFECTION IN CYSTIC FIBROSIS?

1. Induction of the host sPLA2-IIA production by T3SS and role in S. aureus killing

Cystic fibrosis (CF) is a recessive lethal autosomal genetic disorder that commonly affects Caucasians (24,25). It is due to mutations present in the CF trans-membrane conductance regulator (CFTR) gene (26,27) with F508del being the most frequent mutation (28). This mutation corresponds to a deletion of 3 nucleotides at the positions 507 and 508 of the CFTR gene located on the chromosome 7, resulting in the loss of a single codon for the amino acid phenylalanine (F). This gene encodes a protein channel in epithelial cells where it regulates the luminal secretion of chloride and water transport. Airways disease in CF is the major problem that determines the life span of patient (24) and contributes to 80–95% death in CF (29). In the lungs, mutations of CFTR cause depletion of airway surface liquid and mucus dehydration, thus providing a niche favorable for chronic infections by opportunistic pathogens.

Bacterial airways infection varies significantly with the age of patients and *Staphylococcus aureus* is the most commonly isolated bacterium from young CF patients (30,31). With the increase of age of CF patients, *Pseudomonas aeruginosa* becomes progressively predominant representing over 80% of bacteria in adult patients airways (32). However, the mechanisms responsible for this age-related and progressive infection switch from *S. aureus* to *P. aeruginosa* have not been clearly established.

Based on preliminary data from our laboratory (Wu et al. unpublished data) and findings in literature (for more details see ref (33)), we have hypothesized that a selective elimination of *S. aureus* in CF airways by a host molecule endowed with potent antibacterial activity might be involved in the progressive replacement of *S. aureus* by *P. aeruginosa*. Using various pharmacological approaches we identified group IIA secreted phospholipase A2 (sPLA2-IIA) (34,35) as a major bactericidal factor involved in *S. aureus* killing by CF expectorations. This enzyme is known to kill selectively the Gram-positive bacteria such as *S. aureus* and this was confirmed by our studies (33). Indeed, we observed that expectorations from adult CF patients contain sufficient levels of sPLA2-IIA to kill *S. aureus* with no or only minor effects on *P. aeruginosa* (33). The mechanisms leading to selective killing of *S. aureus* by sPLA2-IIA are detailed in the figure 1. This selective killing is mainly due to the presence at the cell wall of *S. aureus* of lipo-Teichoic acid (LTA) with high negative charges. Given that sPLA2-IIA is a very cationic enzyme, this results in a tight binding of sPLA2-IIA to LTA, thus promoting the cell wall penetration by sPLA2-IIA and ultimate hydrolysis of membrane phospholipids of *S. aureus*. However, whether the charges of these membrane phospholipids play a role in sPLA2-IIA interaction with *S. aureus* is still unknown (for more details see refs (32,33)).

This role was confirmed by using transgenic mice over-expressing sPLA2-IIA. Indeed, in a pulmonary model of bacterial infection these mice were able to clear more efficiently *S. aureus* and to survive compared to corresponding Wt mice. In addition, no significant differences were observed between sPLA2-IIA Tg compared to wt mice when *S. aureus* was replaced by *P. aeruginosa* in this model (33).

2. Cell sources of sPLA2-IIA and the mechanism of its induction by ExoS

The presence of sPLA2-IIA in the CF expectorations prompted us to search the source of this enzyme in the lungs by using immunohistochemistry (IHC) on lung

explants from 10 non-CF and nine CF patients. Non-CF patients are non-smoking individuals undergoing lung resection for peripheral lung cancer. IHC analyses showed a marked immunostaining of sPLA2-IIA in all sections of CF bronchial explants as compared with non-CF patients (33). Most of positive staining was observed in PMNs and in the cell surface of bronchial epithelial cells (BECs) suggesting that these cells are a main source of sPLA2-IIA in bronchial explants of CF patients (33). This prompted us to investigate the effects of *P. aeruginosa* to those of *S. aureus* on sPLA2-IIA expression by the BEC line IB3-1 bearing the F508del-CFTR mutation. All tested laboratory and clinical *P. aeruginosa* strains induced sPLA2-IIA expression in IB3-1 cells in contrast to *S. aureus* strains which failed to stimulate this sPLA2-IIA.

Next, we performed experiments to identify the virulence factors of *P. aeruginosa* and the signaling pathways involved in sPLA2-IIA expression by BECs. We first showed that the major known PAMPs of *P. aeruginosa* (LPS, HSL, CpG, pili and flagellin) failed to induce sPLA2-IIA expression by IB3 cells. In this study, cells were infected with PAK, a laboratory strain of *P. aeruginosa*, and its mutants. Remarkably, we showed that the PAK mutant Δ pscF, lacking T3SS, induced sPLA2-IIA expression at much lower levels compared with the parent strain PAK in these cells. Deletion of T3SS but not T2SS increased IL8 secretion compared with the parent strain, thus indicating that failure of this mutant to induce sPLA2-IIA expression was not due to an inability to simulate host cells. Induction of sPLA2-IIA seems to be a specific property of T3SS as the Δ xcpQ strain lacking T2SS induced both sPLA2-IIA and IL-8 expressions at similar levels compared to the wt parent PAK strain (33). Similar findings were observed with primary BEC obtained from CF airways. Indeed, T3SS-deficient strain induced in these cells a sPLA2-IIA production at lower levels compared with the parent wt PAK strain, but induced similar IL8 secretion (33).

3. KLF2 is a key factor in ExoS-induced sPLA2-IIA expression

In a subsequent step we searched which exotoxins from the T3SS are involved in the stimulation of sPLA2-IIA expression by *P. aeruginosa*. Our results showed that among all the T3SS toxins produced by this bacterium, only ExoS is involved in sPLA2-IIA expression. Indeed, we observed that the ExoS-deficient mutant of PAK

(Δ ExoS) induced sPLA2-IIA expression in IB3-1 cells at much lower extent compared with the parent strain and the mutants' deficient for the other toxins ExoT (Δ ExoT) and ExoY (Δ ExoY). These findings prompted us to search the mechanisms by which ExoS induces sPLA2-IIA expression by BEC of CF patients. Using pharmacological approaches we showed that the classical pathways (NF- κ B, AP-1 MAPK known to modulate sPLA2 expression in various cell systems) were not involved in ExoS-induced sPLA2-IIA expression in BECs. This expression is under the control of Krüppel-Like Factor 2 (KLF2), a transcription factor known to be induced by bacterial toxins (36). We also showed that PAK ExoS, but not ExoT or ExoY is able to stimulate KLF2 expression in BECs. KLF2 is also called lung KLF, belongs to the SP-1 zinc-finger transcription factor (37). KLF2 is known to exert anti-inflammatory activities in endothelial cells, monocytes and epithelial cells (38–40). Thus, our studies report for the first time the implication of a transcription factor of the KLF family in sPLA2-IIA transcription. Although the mechanism by which ExoS induces KLF2 expression is still unclear, our studies suggest that this induction is independent of RhoA, a potential target suggested by others (41). Indeed, ExoS is a bifunctional toxin with two enzymatic domains, GAP and ADPRT (42), and only the GAP domain is responsible of RhoA inactivation (11). We demonstrated that ADPRT, but not GAP domain, is involved in ExoS-induced sPLA2-IIA expression.

It remains to be investigated whether the inability to stimulate sPLA2-IIA expression is a general property of Gram-positive bacteria such as *S. aureus*. Given that Gram-positive bacteria are in general highly sensitive to sPLA2-IIA bactericidal effects (compared with Gram-negative bacteria); the inability to induce the production of sPLA2-IIA by Gram-positive bacteria may represent an evolutionary adaptation to escape sPLA2-IIA bactericidal action and to colonize the host.

CONCLUSIONS

Our findings suggest that sPLA2-IIA participates to the progressive elimination of *S. aureus* from CF airways. We propose that *P. aeruginosa* colonization improves this elimination by increasing sPLA2-IIA expression of airways cells by a mechanism involving the injection in BECs of CF patients of ExoS through the T3SS. In

agreement, previous studies showed that sPLA2-IIA is present in human and animal biological fluids or cell supernatants at sufficient levels to kill (34,35,43,44). Antimicrobial peptides (AMPs) such as LL-37 have also been shown to play a role in pulmonary host defense toward *S. aureus* and *P. aeruginosa* in CF lungs (45), although the antimicrobial activity of these AMPs is impaired in CF airways. In addition, to our knowledge among all AMPs and sPLA2s reported in the literature, sPLA2-IIA displays the most potent and selective bactericidal action toward Gram-positive bacteria, such as *S. aureus*. However, it should be kept in mind that, in addition to sPLA2-IIA, other processes might also potentially contribute to the age-dependent decrease of *S. aureus* loads in CF airways (46,47).

The present work indicates that one bacterium can kill another one by manipulating the innate immunity of their common host. This opens a new area of research to investigate the role of host antimicrobial molecules in the dynamic of microbiota (see figure 2).

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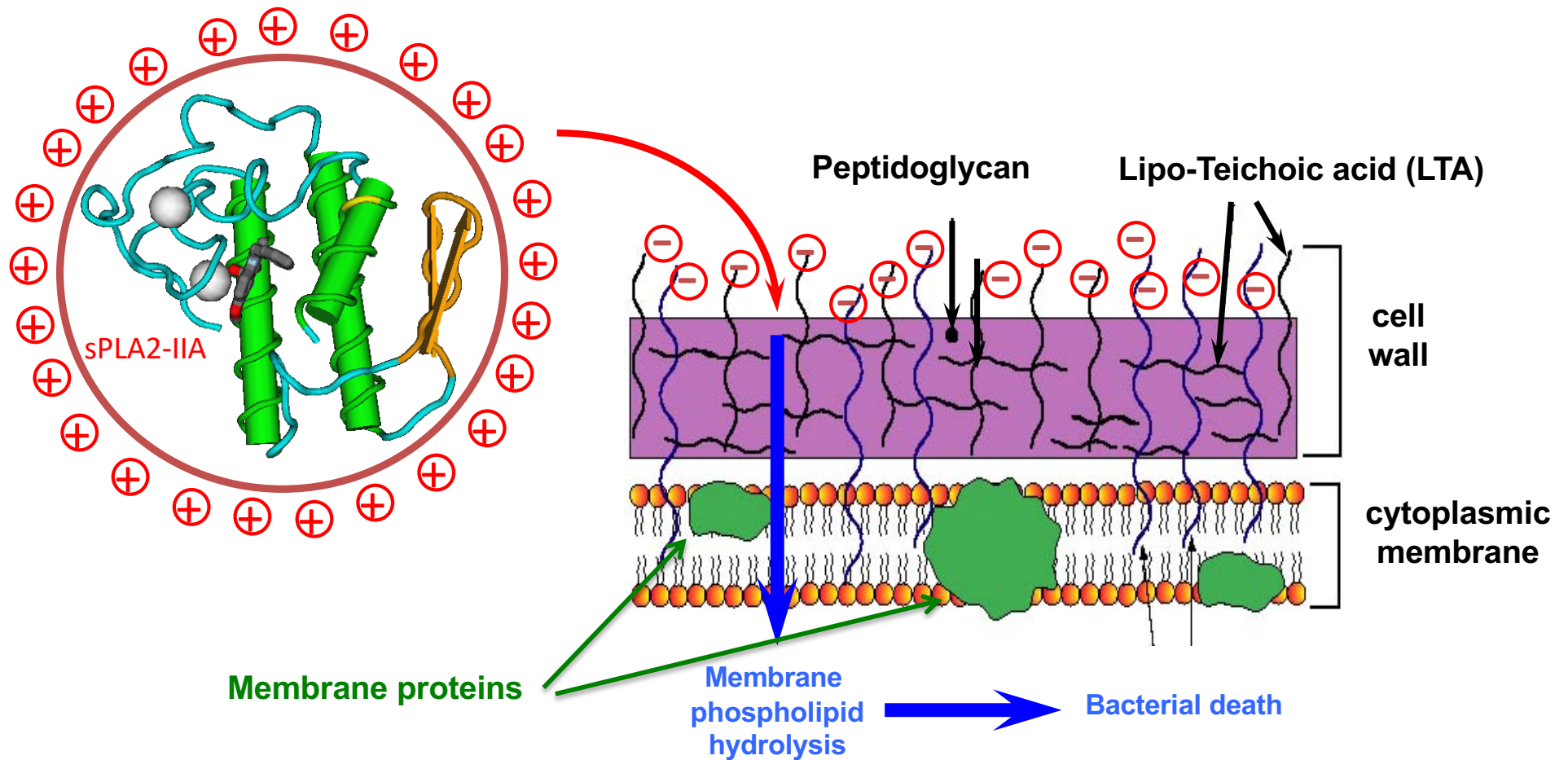


Figure 1 : Why does sPLA2-IIA efficiently kill *S. aureus* ?: Lipo-Teichoic acid (LTA) is a main component of the cell wall of the Gram-positive bacterium *S. aureus*. LTA is highly negatively charged in contrast to sPLA2-IIA that is a very cationic enzyme. This leads to a tight binding of sPLA2-IIA to LTA by electrostatic interaction, promoting the cell wall penetration by this enzyme and hydrolysis of membrane phospholipids and ultimate bacterial death (for more details see refs 34, 35)

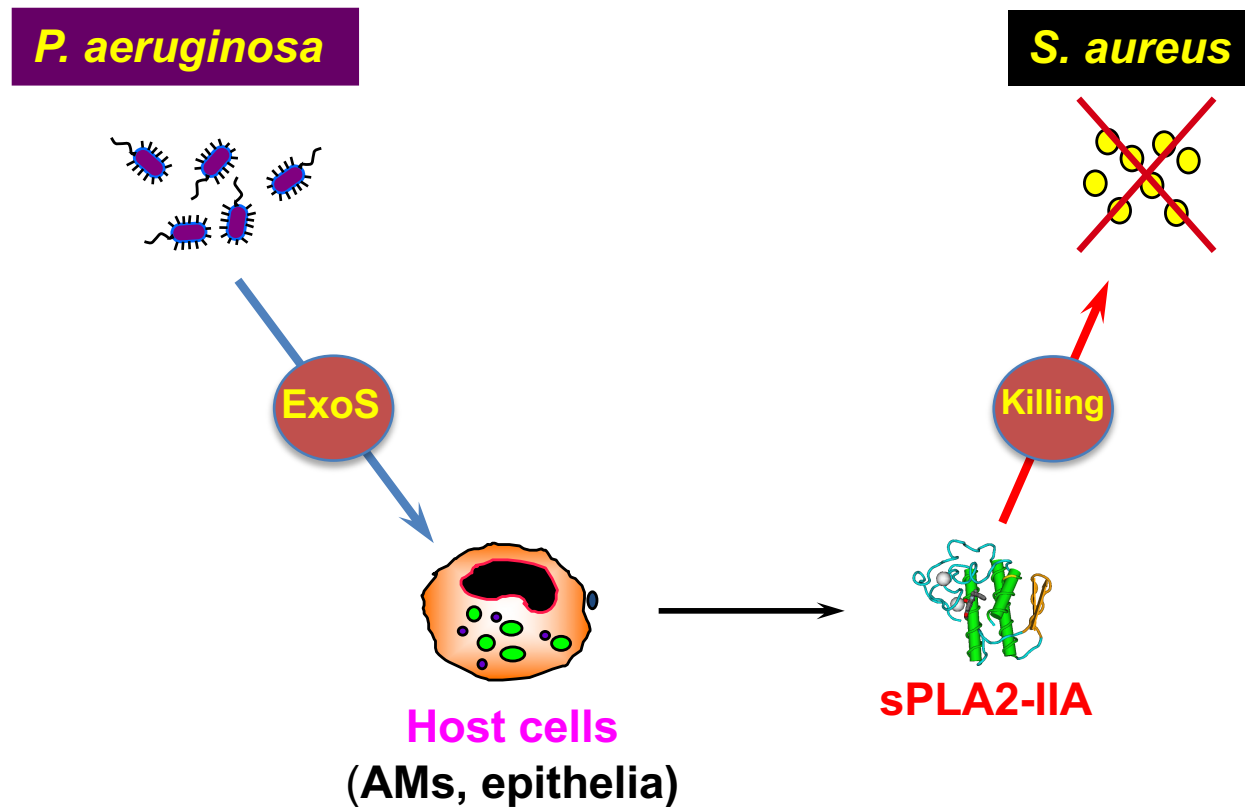


Figure 2 : one bacterium can kill another bacterium by using a molecule of the host : a role for the ExoS of *P. aeruginosa*. Bacterial airways infection varies significantly with the age of CF patients and *S. aureus* is the most commonly isolated bacterium from young CF patients (30, 31). With the increase of age of CF patients, *P. aeruginosa* becomes progressively predominant representing over 80% of bacteria in adult patients airways (32). We propose that *P. aeruginosa* stimulates host cells (mainly epithelial cells but may be also alveolar macrophages (AMs)) that subsequently produces sPLA2-IIA. This enzyme that selectively and efficiently kills *S. aureus* leading to its progressive elimination from CF airways. The toxin ExoS plays a key role in the induction of sPLA2-IIA production by epithelial cells.