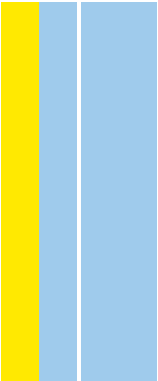


DOUTORAMENTO
BIOLOGIA MOLECULAR E CELULAR

Mechanisms of host resistance to pneumolysin attack

Joana Maria de Abreu Carvalho Pereira

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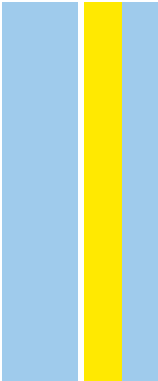
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JOANA MARIA DE ABREU CARVALHO PEREIRA

MECHANISMS OF HOST RESISTANCE TO PNEUMOLYSIN ATTACK

Tese de Candidatura ao grau de Doutor em Biologia Celular e Molecular;
Programa Doutoral da Universidade do Porto (Instituto de Ciências Biomédicas
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De acordo com o disposto no ponto n.º 2 do Art.º 31º do Decreto-Lei n.º 74/2006, de 24 de Março, aditado pelo Decreto-Lei n.º 230/2009, de 14 de Setembro, o autor declara que na elaboração desta tese foram incluídos dados das publicações abaixo indicadas. O autor participou ativamente na conceção e execução dos trabalhos que estiveram na origem dos mesmos, assim como na sua interpretação, discussão e redação.

According to the relevant national legislation, the author declares that this thesis includes data from the publications indicated below. The author participated actively in the conception and execution of the work that originated that data, as well as in their interpretation, discussion, and writing.

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2. Alves, S.*; **Pereira, J.M.***; Mayer, R.L.; Gonçalves, A.D.A.; Impens, F.; Cabanes, D.; Sousa, S. Cells Responding to Closely Related Cholesterol-Dependent Cytolysins Release Extracellular Vesicles with a Common Proteomic Content Including Membrane Repair Proteins. *Toxins*. **2023**, *15*, 4. *These authors contributed equally to this work. doi.org/10.3390/toxins15010004
3. Brito, C.; **Pereira, J.M.**; Mesquita, F.S.; Cabanes, D.; Sousa, S. Src-Dependent NM2A Tyrosine Phosphorylation Regulates Actomyosin Remodeling. *Cells*. **2023**, *12*, 1871. doi.org/10.3390/cells12141871
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Abstract

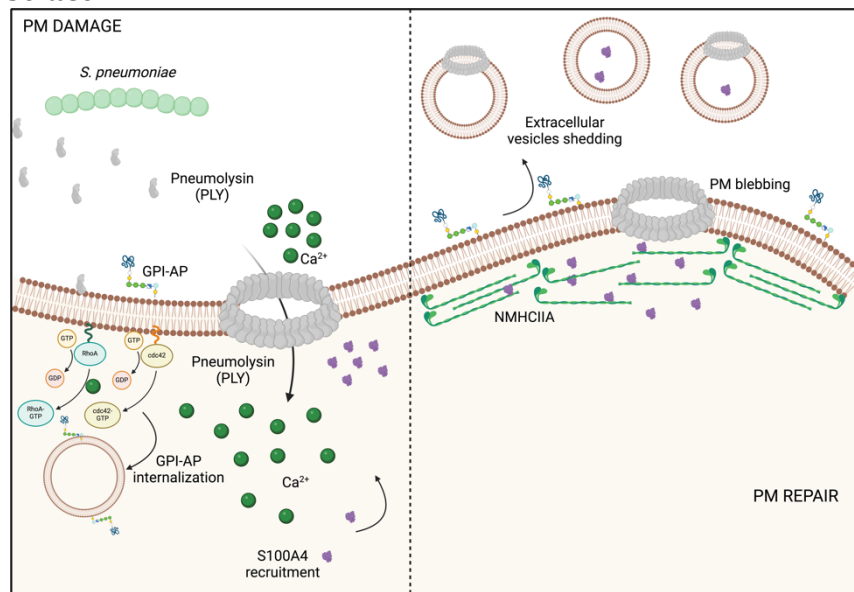
The plasma membrane (PM) acts as a shield protecting cells from external cues, thus maintaining cellular homeostasis. Both healthy and pathologic conditions induce PM lesions, which may lead to cell death and tissue inflammation. Cells cope with PM damage by mobilizing effective repair mechanisms that, if dysregulated, threaten cell survival and induce tissue pathology. Pore-forming toxins (PFTs) are major bacterial virulence factors that generate pores on the PM of host cells thereby disrupting epithelial barriers and fostering bacterial dissemination.

Pneumolysin (PLY) is a PFT considered a major virulence factor released by the highly pathogenic bacteria *Streptococcus pneumoniae*. PLY circulates at low levels early in infection and cells activate repair mechanisms to ensure cell survival and limit bacterial dissemination. Despite the significant progress made in the last years to uncover such repair pathways our current knowledge of those mechanisms is still limited. Therefore, here, we aim to elucidate the host cell protective mechanisms that allow PM repair after damage caused by PLY in the context of *Sp* infection. We uncover that PM damage triggered by sub-lytic PLY doses and infection with *Sp* wild-type (WT) but not its counterpart PLY-deficient mutant, was accompanied by a fast and transient reorganization of the host cell cytoskeleton. Non-muscle heavy chain myosin IIA (NMHCIIA) rapidly and transiently concentrates in cortical clusters at sites of PM remodeling. Super-resolution microscopy analysis reveals that NMHCIIA accumulates as soluble protein disassembling from filament stacks and this occurs concomitantly with the dephosphorylation of the Myosin Regulatory Light Chain (MRLC). As PM integrity is restored, NMHCIIA clusters dissipate, filaments reassemble and its phosphorylation levels are reestablished, suggesting that PM repair requires tight regulation of the dynamics of the actomyosin network. This remodeling is key for PM repair as it coordinates PM remodeling events such as PM blebbing and shedding of extracellular vesicles (EVs). We isolated and characterized the proteomic repertoire of EVs released by PLY-intoxicated cells. Those EVs contain PLY itself, proteins already described in PM repair and new proteins potentially involved in repair, namely Ca^{2+} -binding proteins and cytoskeletal components. S100A4 is a Ca^{2+} -responding protein present in PLY EVs that was previously described to interact with NMHCIIA and directly destabilize NMHCIIA filament stacks. We thus investigated the role of this protein in cytoskeletal remodeling triggered by PLY. We showed that upon PLY intoxication,

S100A4 redistributes to the cell periphery, co-localizes, and interacts with NMHCIIA cortical clusters. Besides, we showed that S100A4-depleted cells display increased sensitivity to PLY damage during *Sp* infection. Also, the absence of S100A4 reduced the number of cells displaying NMHCIIA accumulations, increased the release of large-size EVs and enhanced blebbing upon PLY challenge. Ultimately, we showed that besides activating intracellular pathways required for proper PM repair and survival, PLY also directly modulates cell surface composition through the removal by internalization of glycosylphosphatidylinositol-anchored proteins (GPI-APs) which also supported cell survival to *Sp* infection through a mechanism that remains understudied.

Our findings elucidate the mechanisms supporting actomyosin cytoskeleton remodeling required for the orchestration of PM repair mechanisms upon PLY damage during infection with *Sp*.

Graphical abstract



- PLY released during infection with *S. pneumoniae* induces PM damage
- PLY induces internalization of GPI-APs through the activation of cdc42 and RhoA GTPases
- PLY pore-formation triggers a massive influx of Ca^{2+} and recruitment of S100A4
- S100A4 interacts with NMHCIIA and supports actomyosin remodeling
- Shedding of extracellular vesicles allows full PM repair and supports PLY elimination from the cell surface

Resumo

A membrana plasmática (MP) atua como um escudo protegendo as células de estímulos externos, mantendo assim a homeostase celular. Tanto em condições saudáveis como patológicas a MP está exposta a constantes lesões, que podem levar à morte celular e inflamação dos tecidos. As células lidam com o dano na MP activando mecanismos de reparação eficazes que, se desregulados, ameaçam a sobrevivência celular e induzem patologia tecidual. As toxinas formadoras de poros (TFPs) são importantes fatores de virulência bacterianos que geram poros na MP das células hospedeiras, perturbando assim as barreiras epiteliais e promovendo a disseminação de bactérias.

A pneumolisina (PLY), uma TFP, é um importante fator de virulência libertado pela bactéria altamente patogénica *Streptococcus pneumoniae* (*Sp*). No início da infeção, a PLY circula em níveis reduzidos, e as células ativam a sua maquinaria de reparação para garantir a sobrevivência celular e limitar a disseminação bacteriana. Apesar do significativo progresso feito nos últimos anos para desvendar tais vias de reparação, o nosso conhecimento atual desses mecanismos é ainda limitado. Assim sendo, pretendemos elucidar os mecanismos de proteção celular que permitem a reparação da MP após dano com PLY no contexto da infeção por *Sp*. Demonstramos que o dano na MP desencadeado por doses sub-letais de PLY e durante a infeção com *Sp* selvagem mas não com o seu mutante deficiente em PLY, foi acompanhado por uma reorganização rápida e transitória do citoesqueleto da célula hospedeira. A cadeia pesada da miosina não muscular IIA (NMHCIIA) concentra-se rapidamente e de forma transitória em estruturas corticais nos locais de remodelação da MP. A microscopia de super-resolução revelou que a NMHCIIA se acumula como proteína solúvel, disassociando-se dos filamentos de miosina, e isso ocorre concomitantemente com a desfosforilação da cadeia leve reguladora de miosina (CLRM). À medida que a integridade da MP é restaurada, os aglomerados corticais de NMHCIIA dissipam-se, os filamentos reconstituem-se e os níveis de fosforilação da CLRM são restabelecidos, sugerindo que a reparação da MP requer uma regulação rigorosa da dinâmica do citoesqueleto de actomiosina. Esta remodelação é fundamental para a reparação da MP, pois coordena eventos de remodelação membranares, como a formação de blebs e a libertação de vesículas extracelulares (VEs). Adicionalmente, isolámos e caracterizámos o repertório proteómico das VEs libertadas por células intoxicadas

com PLY. Essas VEs contêm PLY, proteínas previamente descritas na reparação da MP e novas proteínas potencialmente envolvidas na reparação, nomeadamente proteínas responsivas a cálcio e componentes do citoesqueleto. A S100A4 é uma proteína que responde ao cálcio que se encontra presente nas VEs libertadas pelas células intoxicadas com PLY. Esta foi previamente descrita como sendo capaz de interagir com a NMIIA e destabilizar diretamente os seus filamentos. Assim, investigámos o papel desta proteína na remodelação do citoesqueleto desencadeada pela PLY e mostramos que, em resposta à intoxicação por PLY e infeção com *Sp*, a S100A4 redistribui-se para a periferia da célula, co-localiza e interage com as estruturas corticais de NMIIA. Além disso, verificamos que as células depletadas para a S100A4 apresentam uma sensibilidade aumentada ao dano por PLY durante a infeção por *Sp*. Adicionalmente, a ausência de S100A4 reduziu o número de acumulações corticais de NMHCIIA, aumentou a libertação de vesículas extracelulares de grande tamanho e aumentou a formação blebs da MP em resposta ao dano por PLY. Finalmente, revelamos que, para além de ativar vias intracelulares necessárias para uma reparação adequada da MP e garantir a sobrevivência celular, a PLY também modula diretamente a composição da MP através da remoção por internalização de proteínas ancoradas ao glicosilfosfatidilinositol, fenómeno que também suporta a sobrevivência celular em resposta à infeção com *Sp* através de um mecanismo ainda por explorar.

Concluindo, os nossos resultados elucidam os mecanismos que suportam a remodelação do citoesqueleto de actomiosina necessária para a orquestração adequada dos mecanismos de reparação da MP após o dano por PLY durante a infeção por *Sp*.

List of abbreviations

ACD - Assembly competence domain
AHNAK - Neuroblast differentiation-associated protein
AIF - Apoptosis-inducing factor
ALG-2 - Apoptosis-linked gene 2
ALIX - ALG-2-interacting protein X
ASM - Acid sphingomyelinase
ATP - Adenosine tri-phosphate
CDC - Cholesterol-dependent cytolysin
CHMP - Charged multivesicular body protein
CFP - Cyan Fluorescence Protein
CFU - Colony-forming unit
ClyA - Cytolysin A
CPNE - Copine
CTR - Control
DAG - Diacylglycerol
DAPI - 4',6'-diamino-2-phenyl-indol
DCs - Dendritic cells
DIC - Differential interference contrast
Did2 - Vacuolar protein-sorting-associated protein 46
DMSO: Dimethyl Sulfoxide
EEA-1 - Early Endosome Antigen 1
EIF2 - Eukaryotic initiation factor 2
ER - Endoplasmic reticulum
ESCRT - Endosomal sorting complex required for transport
FBS - Fetal bovine serum
FDA - Food and Drug Administration
FLAER - Fluorescein-labeled pro-aerolysin
FRET - Fluorescence resonance energy transfer
GPI - Glycosylphosphatidylinositol
GSMD - Gasdermin D
HBSS - Hank's buffer salt solution
IF - Immunofluorescence

IFN- β - Interferon beta
 IL-1 β - Interleukin 1 β
 ILV - Intraluminal Vesicles
 IPD - Invasive pulmonary disease
 K⁺ - Potassium
 LAMP1- Lysosomal associated glycoprotein 1
 LC3 - Microtubule-associated *protein* 1A/1B-light chain 3
 LLO – Listeriolysin-O
 MAC - Membrane attack complex
 MAPK – Mitogen-activated protein kinase
 MFI - Mean Fluorescence Intensity
 MG53 - Tripartite motif-containing protein 72
 MMPs - Metalloproteinases
 MRC-1 - Mannose receptor C-type 1
 MRLC – Myosin regulatory light chain
 MS – Mass spectrometry
 mtDNA – mitochondrial DNA
 MV - Microvesicles
 MVB - Multivesicular body
 NE - Neutrophil elastase
 NETs - Neutrophil extracellular traps
 NI – Non-infected
 NI - Non-intoxicated
 NLRP3 - NLR Family Pyrin Domain Containing 3
 NMHCIIA – Non-muscle myosin heavy chain IIA or heavy chain of myosin IIA
 NMII - Non-muscle myosin II
 NMIIA - Non-muscle myosin II A
 NMIIB – Non-muscle myosin II B
 NT - Non-treated
 PAMPs - Pathogen Associated Molecular Patterns
 PBS – Phosphate buffer solution
 PFO - Perfringolysin O
 PFT – Pore-forming toxin

PI - Propidium iodide
PKC - Protein kinase C
PLY - Pneumolysin
PM - Plasma membrane
PMN - Polymorphonuclear neutrophils
pMRLC - Ser19-phosphorylated MRLC
PS - Phosphatidylserine
RLC - Regulatory light chain
ROI - Region of interest
ROS - Reactive oxygen species
Sp - *Streptococcus pneumoniae*
SLO - Streptolysin O
Slp4-a - Synaptotagmin-like protein 4a
SNARES - N-ethylmaleimide sensitive factor attachment protein receptors
T3SS - Type-3 secretion system
TEM - Transmission electron microscopy
TMEM16 - Transmembrane protein 16
TNF- α - Tumor necrosis factor
VAMP-7 - Vesicle-associated membrane protein 7
Vps4 - Vacuolar protein sorting-associated protein 4
Vta1 - Vesicle Trafficking 1
UPR- Unfolded protein response
WCL - Whole cell lysate
WGA - Wheat germ agglutinin
YFP - Yellow fluorescence protein

Keywords

Pore-forming toxins; Plasma membrane; Repair; Pneumolysin; Extracellular vesicles; Blebbing; Shedding; Calcium signaling; *Streptococcus pneumoniae*; S100A4; Non-muscle myosin IIA; Glycosylphosphatidylinositol-anchored proteins.

Chapter I – Introduction

“When, at last, the work is done
Don't sit down, it's time to dig another one.”
“Breathe” by Pink Floyd

The introduction chapter includes the literature review from two different relevant topics in the scope of this PhD thesis:

A) A story about resilience: Reviewing Plasma Membrane Damage and Repair.

Here, I will delve into plasma membrane damage and intrinsic repair strategies. I will further discuss different sources of PM injury and explore which mechanisms and protein machinery are activated by eukaryotic cells to overcome damage and promote the repair of PM.

B) The Yin and Yang of Pneumolysin During Pneumococcal Infection. In this sub-section, I will discuss the dual and controversial roles of pneumolysin during infection with *S. pneumoniae*. This review was published in *Frontiers in Immunology* (Pereira J.M., Xu S., Leong J.M. and Sousa S. (2022). The Yin and Yang of Pneumolysin During Pneumococcal Infection. *Front. Immunol.* 13:878244. doi: 10.3389/fimmu.2022.878244).

A story about resilience: Reviewing Plasma Membrane Damage and Repair

1. Plasma membrane

1.1. Importance in homeostasis

The plasma membrane (PM) is a vital and selective semi-permeable barrier [1] that plays a pivotal role in preserving cellular homeostasis by protecting cellular organization and facilitating optimal cellular function [1, 2]. Besides its inherent function to isolate the intracellular space from the extracellular milieu, the PM also participates in the activation of intracellular cell signaling and signal transduction pathways [3]. Furthermore, it is actively involved in intercellular communication, facilitates cell adhesion, and enables the selective uptake of ions and nutrients.

1.2. Plasma membrane composition

Regarding its structural organization, the PM is characterized by a dynamic asymmetric fluidic lipid bilayer mainly composed of phospholipids, which are molecules constituted by a hydrophobic core and a polar head region. PM organization is affected by how membrane lipids are asymmetrically distributed in the inner and outer leaflets. Its exact composition is still an issue under debate and even though there is no consensus, several models have been proposed over the years to explain the complexity of this cellular component [4]. Currently, the most accepted model to explain the organization of cell membranes is the “Fluid Mosaic model”, published in 1972 by Singer N. J. and Nicolson G. L. In this model, PM is dynamic and heterogeneous. It possesses a large number of phospholipids organized as a fluid discontinuous bilayer alternated with proteins containing globular amphipathic molecules interspersed within [5]. Nevertheless, it is well-established that PM is a complex cellular component mainly constituted by a diverse mixture of hundreds of phospholipid species, including, sphingolipids, glycerophospholipids, and sterols, cholesterol being the most abundant among them [6]. While lipids are fundamental to structurally support PM, it also contains a substantial number of associated proteins that execute vital functions. These proteins are classified as integral if anchored to the lipid bilayer through transmembrane domains; peripheral membrane proteins if

anchored via lipid moieties; and membrane-associated when associated with electrostatic, protein-protein, or protein-glycan interactions (**Figure 1**) [6, 7].

Glycosylphosphatidylinositol-anchored proteins (GPI-APs) are integral membrane proteins that are attached to the outer leaflet of the cell PM through a highly conserved glycosylphosphatidylinositol anchor. The GPI anchor results from post-translational modifications and comprises a phospholipid portion and a glycan chain [8-10]. GPI-APs present a higher affinity for lipid rafts, enriched in cholesterol, in the PM and its insertion relies on sorting and membrane fusion machinery [8, 11]. At the cell surface, GPI-APs are organized in clusters that are actively formed. Interestingly, their assembly is dependent on a fine regulation of cholesterol levels at the cell surface [12]. Regarding their function in cells, GPI-APs can act as receptors for numerous viruses and bacterial toxins [13], adhesion molecules, protease inhibitors, hydrolytic enzymes, and even interact with complement components [10].

The interaction between lipids and proteins across the membrane allows the formation of dynamic domains [14]. Another interesting aspect of the PM is its different composition between the inner and outer leaflets, which exhibit notable disparities between each other. The inner leaflet is characterized by the presence of a higher amount of charged phospholipids like phosphatidylethanolamines, phosphatidylserines, and phosphatidylinositol. In contrast, the outer leaflet is mainly composed of sphingomyelin, phosphatidylcholine, and glycosphingolipids (**Figure 1**). The presence of cholesterol is common in both leaflets. This heterogeneity is found not only between leaflets but also laterally within each leaflet, resulting in the formation of lipid rafts, which are specialized microdomains enriched in cholesterol and sphingolipids that accumulate PM-associated proteins and serve as signaling scaffolds. All these leaflet-specific features allow the PM to sustain a localized response independently of the fact that both leaflets are interconnected [7].

Underlying the PM resides a thin actin cytoskeleton meshwork that interacts with membrane lipids and constitutes an essential factor in the regulation of membrane organization serving as a framework for diverse membrane components [15]. Cortical actin is found associated with a plethora of actin-binding proteins that together generate and control contractile forces needed to modulate membrane dynamics and tension. All these features need to be tightly regulated to allow cells to change their shape and exert a multitude of cellular functions ranging from cell migration, division,

and tissue morphogenesis [16, 17]. Additionally, the actin cytoskeleton was shown to interact with ionic transport systems at the PM which consequently, directly or indirectly, modulates the transport across the PM [18, 19].

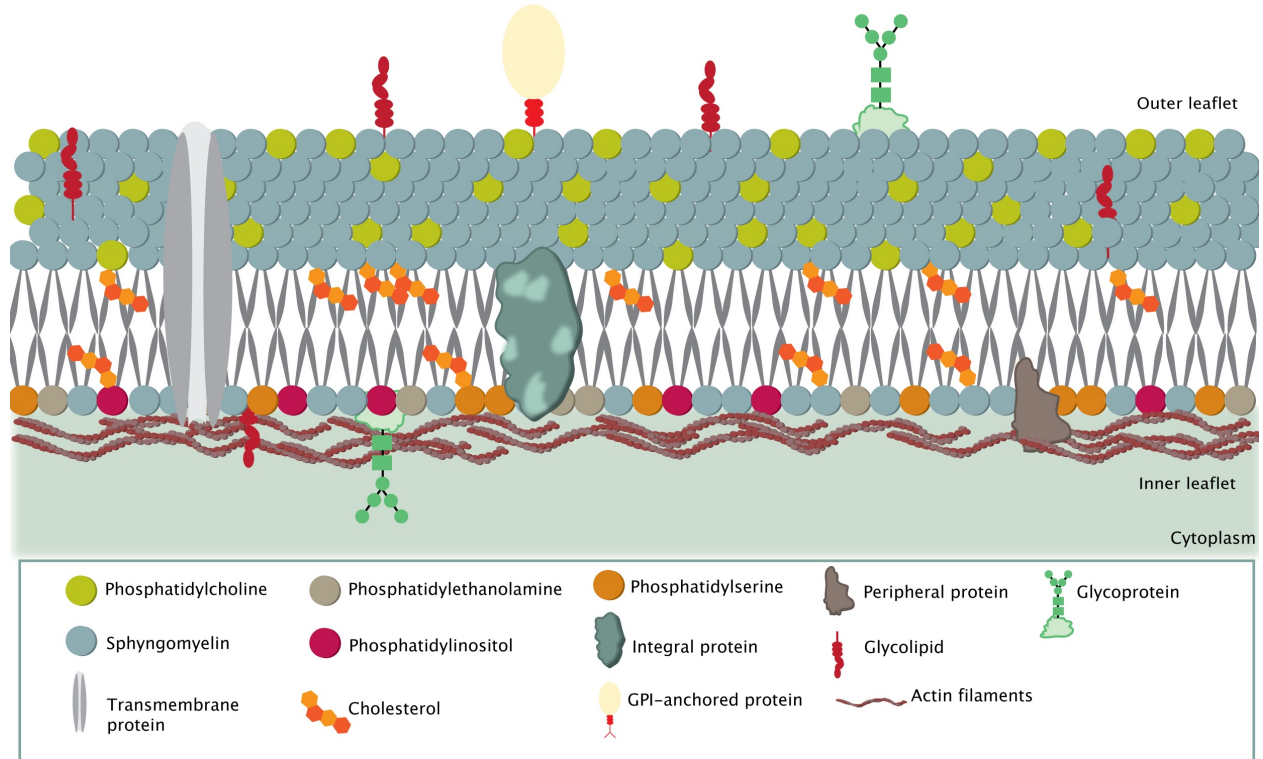


Figure 1- Plasma membrane composition. PM is a complex asymmetric lipid bilayer structure constituted by a diverse mixture of different phospholipid species: sphingolipids, glycerophospholipids, and sterols. Besides phospholipids, different classes of proteins can be found: integral if anchored to the lipid bilayer through transmembrane domains; peripheral membrane proteins if anchored via lipid moiety; and membrane-associated when associated by electrostatic, protein-protein, or protein-glycan interactions. Cholesterol is common in both leaflets. While the inner leaflet is characterized by the presence of charged phospholipids like phosphatidylethanolamines, phosphatidylserines, and phosphatidylinositol, the outer leaflet is mainly composed of sphingomyelin, phosphatidylcholine, and glycosphingolipids.

2. Plasma membrane damage

2.1. Sources of PM damage

Unlike procaryotic, eucaryotic cells lack an external cell wall to protect PM. Consequently, the PM of eucaryotic cells is more susceptible to external and internal cues [20]. Given its protective role, the maintenance of PM integrity is fundamental for

any organism to support its survival. Under physiological conditions, PM is continuously under stress and suffers a diversity of insults that can arise from the extracellular environment (mechanical, chemical, microbiological, and immune damage) or the intracellular milieu, including, oxidative stress, activation of cell death pathways or lysosomal leakage [21].

2.1.1. Mechanical damage

Under physiological conditions, mechanical stress constitutes one of the major sources of PM damage as our cells are constantly exposed to mechanical forces. Muscle cells are particularly susceptible to this type of damage, since, during body movement or intensive physical exercise, these cells stretch inducing physical membrane breaching and causing nanometer lesions, however, usually, cells can rapidly seal (**Figure 2**) [22, 23]. A similar injury process occurs in cardiac myocytes during heartbeat, where PM is essential to mediate cardiac contraction (**Figure 2**). Therefore, if cells fail to rapidly restore PM integrity, this can severely compromise heart function [1, 24, 25]. It was also demonstrated that endothelial cells are subjected to mechanical stress derived by forces of shear and stretch and by physical distress caused by external ultrasounds, related wounding has been associated with the initiation of atherosclerosis (**Figure 2**) [26-28]. Other described physical distress includes the exposure of cells to noise and heat that can induce microlesions and protein denaturation, respectively, at the cell PM therefore disrupting its integrity (**Figure 2**) [29]. The formation of protein aggregates resulting from misfolding of soluble monomers can similarly induce mechanical damage through the formation of pore-like structures (**Figure 2**) [30]. Even epithelial cells' PM integrity in the gut is constantly compromised by potential chemicals present in ingested foods. In addition, peristaltic movement in the gut can press undigested food and cause mechanical injuries [31]. An *in vivo* study showed that cells residing at the margins of the discontinuous epithelial gut are more prone to PM damage, when repair occurs their migration capacity is enhanced and those cells actively contribute to tissue wound healing. Thereby, injury and repair of gut cells may contribute to the fast and continuous cell turnover of the epithelium thereby maintaining its layer integrity [31].

2.1.2. Chemical disruption

As noted in section 1.2, PM has a complex lipid composition that contributes to sustain its biophysical properties. PM is frequently exposed to chemicals that can alter its unique biophysical properties and compromise its integrity. Reactive oxygen species (ROS) arise from a partial reduction of oxygen molecules and are produced endogenously in a variety of pathophysiological conditions. It is most frequent in metabolically active cells (such as liver cells) or during hypoxia, constituting a major source of chemical damage [32]. Once formed, they are highly reactive and PM lipids are preferential targets. Thus, frequently this process results in lipid peroxidation and membrane disruption (**Figure 2**) [33-35]. Moreover, ROS constitutes an end product of phospholipase A2 (PLA₂) reaction, a cellular enzyme that cleaves ester bonds within the phospholipids [36], changing PM fluidity and permeability [37]. Additionally, some compounds like methyl- β -cyclodextrin, an FDA-approved excipient for drug development, induce membrane cholesterol depletion, which alters membrane dynamics and tension in turn rendering cells susceptible to subsequent damage (**Figure 2**) [38, 39]. It has also been demonstrated that phenothiazine derivatives, which are widely used in clinics, strongly sensitize cancer cells to a variety of injury sources by modifying the biophysical properties of their PM and inhibiting PM repair [40]. Besides, alcohol, a widely used recreational substance, has been shown to disrupt cell membrane's physical structures by different means, it can induce lipid membrane disordering and directly interact with membrane proteins amending their normal functions (**Figure 2**) [41, 42]. Interestingly, during ferroptosis, a form of cell death, cells lose an important lipid repair enzyme, the glutathione peroxidase 4, which favors the accumulation of oxidized phospholipids that leaner the PM favoring PM damage (**Figure 2**) [43].

2.1.3. Immune-mediated damage

Immune cells often cause harm to the PM of host cells while mounting an effective immune response against pathogens. Cytotoxic T-cells release cytolytic granules (granzyme B (GzmB)) along with perforin (PFN), a small pore-forming protein that inserts into the membrane of cells to generate holes of approximately 20 nm, thereby compromising PM integrity and often inducing cell lysis (**Figure 2**) [44, 45]. During the innate immune response, the activation of the complement pathway

through different mechanisms results in the synchronized assembly of a variety of complement proteins to form the membrane attack complex (MAC), whose primary function is to be inserted into pathogens' membranes and lyse them [46]. However, when uncontrolled, MAC can also target host cells by distorting the PM lipid bilayer and compromising their integrity (**Figure 2**) [47, 48]. Gasdermins (GSDM) are pore-forming proteins produced during pyroptosis, a mechanism of inflammatory programmed cell death employed by cells during the immune response to release pro-inflammatory cytokines [49]. Cells express up to six GSMD proteins that reside in the cytosol in its inactive conformation. To exert its pore-forming activity, they need to be activated by proteolytic cleavage mediated by different proteases (caspases, cathepsin G, elastase) that release GSMDs N-terminal domain that translocate to the PM and assemble to form a transmembrane pore that destabilizes PM (**Figure 2**) [50].

2.1.4. Microbial-mediated injury

The PM is a fundamental cellular barrier that protects cells against a diversity of microbial pathogens. As such, they devise virulence strategies to bypass this protection and become experts in breaching the PM to injure host cells, escape the immune system, invade and spread to distant organs, and get access to intracellular rich-nutrients environment [21].

Many fungi species employ this strategy to invade host cells. Recently it has been identified in *C. albicans* a pore-forming toxin, candidalysin, that assembles into polymers to insert into the host cell membranes leading to membrane permeabilization (**Figure 2**) [51, 52]. Besides, *C. albicans*, *A. fumigatus*, and *C. neoformans* release phospholipases, which are enzymes responsible for hydrolyzing important lipids present at the host cell's PM thereby contributing to its remodeling and disturbance (**Figure 2**) [53, 54]. In addition, some fungi like *A. fumigatus* and *C. albicans* have the ability to grow in different forms and evolve from yeasts to hyphae [55]. Noteworthy, during hyphae expansion, an increased physical force is applied against the PM of target epithelial cells which may perturb PM integrity and facilitate fungal invasion (**Figure 2**) [53, 56, 57]. *Plasmodium* sporozoites also compromise epithelial host cell membrane integrity through a specific type of migration called “cell transversal” which they employ to cross cells and invade the host [58, 59].

Viruses also evolved tactics to overcome PM hindrance, in fact, influenza A, coronaviruses, hepatitis C virus, among others, produce small pore-forming proteins called viroporins that oligomerize into the host cell PM to create small pores and facilitate the delivery of viral machinery needed for replication into the host intracellular space (**Figure 2**) [60-62]. Interestingly, the hemolytic paramyxovirus envelope can fuse with PM to also generate small pores of about 1 nm (**Figure 2**) [63]. Adenovirus has the fascinating capacity to hijack host PM repair machinery to facilitate its entry into the host cells. Through the expression of protein-VI, a membrane lytic protein that wounds PM, it activates PM repair mechanisms such as lysosome exocytosis that favors membrane remodeling through the release of acid sphingomyelinase that consequently favors endocytosis and facilitates viral internalization [64].

Bacteria are complex microorganisms that employ sophisticated strategies to invade and surpass host cell protective barriers. They evolved specialized virulence factors to compromise host PM and get access to intracellular space [65]. An example of this is the complex type III secretion system (T3SS), present mostly in Gram-negative bacteria. T3SS generates transient pores at the PM through the assembly and insertion of translocon components into the host PM that function as channels to facilitate the direct delivery of bacterial effectors into the host cells and promote its intracellular replication and survival (**Figure 2**) [66-69]. The release of bacterial enzymes, such as phospholipases and sphingomyelinases, that catalyze the hydrolysis of glycerophospholipids and sphingomyelin respectively, which are the major components of eukaryotic cell's PM (referred to in section 1.2), also constitutes a central virulence strategy evolved by both Gram-positive and Gram-negative bacteria to perturb host cell membrane composition and biophysical properties [21, 70]. Pore-forming toxins (PFTs) are major virulence factors produced by many bacteria. They are produced as water-soluble monomers and released into the extracellular milieu. From the extracellular milieu, they interact and bind to PM lipids and/or protein receptors by adopting a compatible structure with the amphipathic environment of the PM lipid bilayer (**Figure 2**) [71, 72]. Upon PM insertion, PFTs form stable transmembrane pores dramatically altering cell permeability. Pores allow the uncontrolled exchange of ions, small molecules, or proteins between the intracellular and extracellular environment, perturbing cellular homeostasis. The outcome of PFT insertion depends on the PFT

identity, activity, and concentration [72, 73]. Bacterial PFTs are categorized into two major groups, the α -toxins and β -toxins, defined based on the secondary structure of the transmembrane pore [72]. While the α -toxins (colicin, cytolysin A (ClyA) and actinoporin families) insertion and oligomerization occur simultaneously to create a transmembrane α -helical pore; in β -toxins (the hemolysin, the aerolysin and the cholesterol-dependent cytolysin (CDC) families) toxin monomers bind cellular receptors, oligomerizes to create a pre-pore that subsequently structurally reorganize into antiparallel β -hairpins to create a transmembrane β -barrel structure that perforates PM [74, 75]. In the scope of this thesis, we will study a β -toxin that belongs to the CDC family of PFTs.

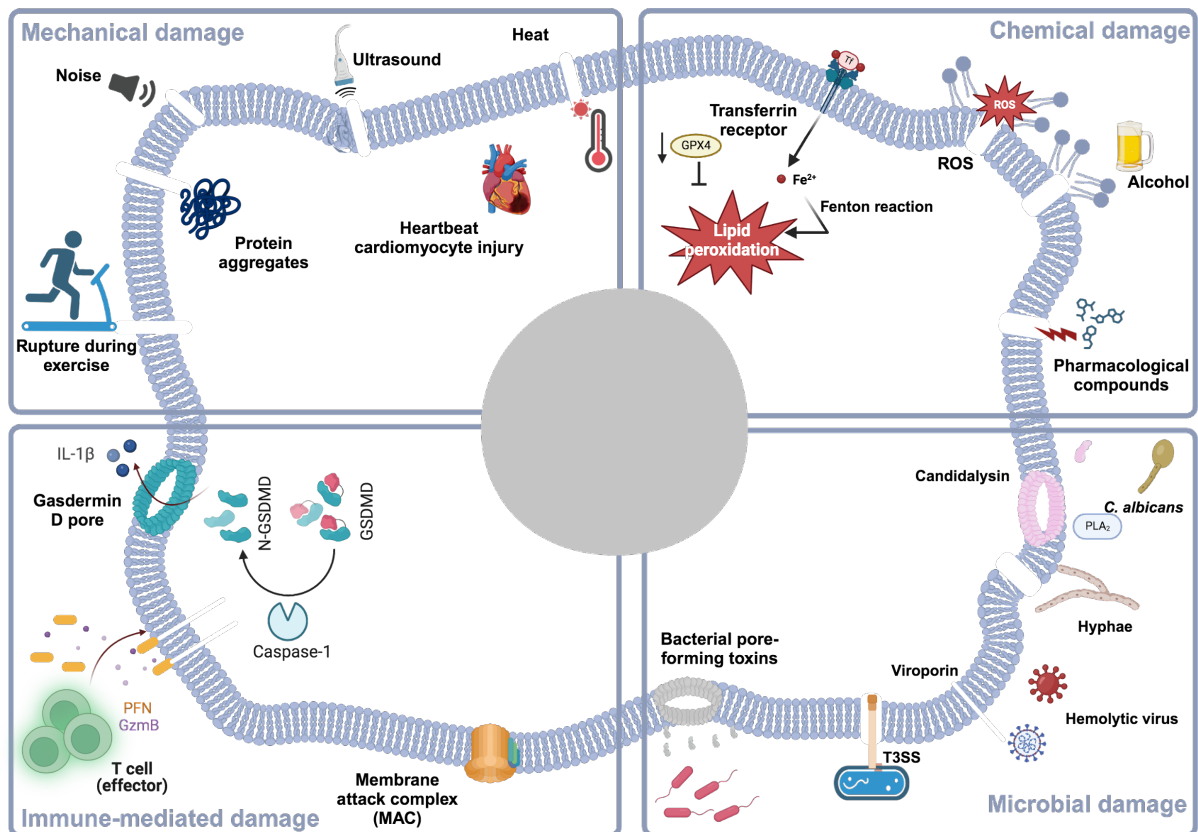


Figure 2- Sources of PM damage. Under physiological conditions, the PM suffers stresses of different natures that threaten cellular homeostasis. Mechanical-induced injury is frequently observed in muscle cells during intense exercise or in cardiomyocytes during a heartbeat, but may also arise from external stressors like noise, ultrasounds, or heat that induce PM microlesions and protein denaturation. It may also originate from chemical damage mainly mediated by ROS which favors membrane lipid peroxidation, some pharmacological

compounds may alter PM biophysical properties which renders cells susceptible to damage. Immune-mediated damage is often triggered when mounting an effective immune response against pathogens. Cytotoxic T-cells release perforin and granzyme B and during an uncontrolled innate immune response, membrane attack complex (MAC) may insert into the PM of epithelial cells disrupting its integrity. During pyroptosis, an inflammatory programmed cell death pathway, caspases cleave gasdermin D and expose its N-terminal which then binds to PM and creates a pore that disrupts homeostasis and allows the release of inflammatory cytokines. Microbial-mediated damage can arise from different pathogens ranging from yeasts which release pore-forming proteins and phospholipases or even when growing hyphae; viruses can also release small proteins called viroporins that inserts into the PM; the T3SS from bacteria inserts a needle structure into the host cells' PM to deliver their content and some other bacteria may produce pore-forming toxins that oligomerize and insert into the PM creating pores and disrupting homeostasis. Created with BioRender.com.

3. Plasma membrane repair

Cells need to tightly maintain homeostasis to survive and exert their physiological functions. PM serves as a critical barrier to protect cells, yet its integrity is constantly being challenged under physiological conditions by multiple sources of damage, already discussed above, which undermine its protective role. Independently of the source of damage and the cell type, cells can effectively sense the breaches and mount a response that depending on the extent of damage, includes either engagement in cell death pathways or activation of specific PM repair mechanisms that cooperate to reestablish membrane integrity and ensure cell survival [76].

3.1. Sensing the breaches

Perceiving the damage is pivotal for cells to rapidly engage PM repair mechanisms. Under physiological conditions, cells maintain a 10 000-fold gradient of Ca^{2+} between the extracellular (concentrations in the range of mM) and intracellular milieu (nM concentration range) [65, 77]. It is thus intuitive that upon membrane disruption, independently of the source, there is a substantial influx of Ca^{2+} that will be proportional to the magnitude of the damage. It is well established that a rise in intracellular Ca^{2+} concentration is key and constitutes the primordial step for cells to activate PM repair mechanisms [76, 78]. However, this increase needs to be tightly regulated by cells. To activate repair mechanisms, an abrupt and rapid overload of Ca^{2+} needs to be sensed by cells, however, these levels cannot reach elevated toxic

concentrations [76, 78]. Otherwise, it will have deleterious consequences for cells such as dysregulation of cellular signaling and activation of cell death pathways [76, 79]. It is accepted that the levels of intracellular calcium supported by cells to efficiently repair the damage is around 20 μM , which corresponds to the concentration that allows both the translocation of repair proteins (e.g. annexins that will be further discussed in the next sections) to the damaged PM limiting Ca^{2+} influx and the effective clearance of excess Ca^{2+} ions by cellular organelles that have limited capacity to execute these function due to the high ATP demanding [80].

Interestingly, small pores, of about 2 nm, as the ones formed by α -toxins or aerolysin, produced by *S. aureus* and *A. hydrophila*, respectively, were shown to take longer times to seal when compared to large pores, of about 30 nm, created by CDCs. This feature was associated with the lower or complete lack of Ca^{2+} influx that crosses small pores, which results in later detection of the damage by host cells and consequent increase in cytotoxicity [81].

Membrane damage leads to an ion imbalance, not only due to the massive influx of Ca^{2+} but also the loss of intracellular potassium (K^+), which has been described to play a critical role in controlling PM repair. This control is exerted through the regulation of several pathways, including the activation of mitogen-activated protein kinase (MAPK) and p38 pathways and downstream Endoplasmic Reticulum Unfolded Protein Response (UPR) which might be important to eliminate unfolded proteins that might accumulate upon Ca^{2+} overload (**Figure 3, panel 1**) [82, 83]; the phosphorylation of eukaryotic initiation factor 2 alpha (eIF2), leading to inhibition of protein translation (**Figure 3, panel 1**); and the initiation of the autophagy-related pathway mediated by LC3 conversion. These mechanisms collectively contribute to conserving energy, which is crucial for membrane recovery and restoration of cellular homeostasis [76, 84-87]. Despite its described role in PM repair, the depletion of intracellular K^+ can also contribute to aggravate PM damage through the activation of innate immune responses. Studies indicated that the decrease in intracellular K^+ levels is sufficient to activate NLRP3 inflammasome, thereby exacerbating inflammation and promoting tissue damage, suggesting that K^+ efflux might have a dual role in repair, which is likely dependent on the extent of the drop in intracellular K^+ levels (**Figure 3, panel 1**) [88].

The loss of intracellular adenosine-5' triphosphate (ATP) into the extracellular space is a phenomenon occurring upon loss of membrane integrity due to the ATP diffusion either through voltage-gated channels opened by membrane depolarization or through hemichannels (**Figure 3, panel 1**) [89]. The decrease in intracellular ATP was associated with efficient PM repair. Opening of voltage-gated channels also favors Ca^{2+} influx and promotes exocytosis. Curiously, using a model of mechanical injury in alveolar epithelial cells, the removal of ATP from the extracellular space reduced PM repair efficiency and decreased the levels of LAMP-1 exposed at the PM suggesting the impaired fusion of lysosomes with the PM, a mechanism described to facilitate repair. Contrarily, increased levels of extracellular ATP facilitates repair [89-91]. Extracellular ATP partially activates the purinergic receptor P_2Y_2 , which is frequently upregulated in response to stress, and depletion of this receptor impaired repair supporting an active role for ATP in PM repair possibly through the activation of purinergic receptors that control membrane trafficking events [90]. Additionally, α -toxin from *S. aureus* enhances cellular damage by activating the purinergic receptor P_2X_7 , which acts as a cation channel (**Figure 3, panel 1**) [92] that aids Ca^{2+} influx not only in injured and but also in neighboring cells that were not injured yet. Initiation of downstream pathways activated by increased Ca^{2+} influx recruits repair machinery beforehand thereby averting subsequent damage [93].

3.2. Repair the holes

The ability to self-repair, under physiological or pathological conditions in response to different insults, is intrinsic to cells, tissues, and organisms. Deregulated PM repair jeopardizes cellular survival and underlies widespread debilitating disorders, such as cancer, muscular and neurodegenerative diseases [94]. Several mechanisms have been proposed to participate in the seal of membrane breaches and they rely on the size and source of damage. If small lesions are created by mechanical or chemical damage, they commonly seal spontaneously due to the increased tension along the pore edge where forces are propelled to bring together the lesioned ends and seal the membrane opening [95]. Conversely, if damage is inflicted by the insertion of a protein at the membrane (e.g. PFTs), membranes cannot self-reseal as they need to not only close the hole but also remove the inserted protein from the PM which requires the activation of more complex mechanisms [65]. Several mechanisms have been

proposed over the years, however, they might be interconnected and possibly complement each other. By this means, an efficient and coordinated activation of repair mechanisms is essential to guarantee membrane integrity restoration and organism survival.

3.2.1. Damage confinement: PM blebbing

Blebbing is a cellular process frequently occurring in mammalian cells during cell division, migration, and apoptotic cell death [96, 97]. Blebs are spherical membrane protrusions that rapidly expand after detachment of the PM from the underlying actomyosin cytoskeleton decreasing membrane tension and increasing intracellular pressure favoring membrane outgrowing and cytosol leakage into the bleb area [98, 99]. Blebbing is a universal response to PM damage, regardless of its source, and is directly associated with increased cellular recovery capacity [100, 101]. Resulting blebs allow the isolation of the damaged membrane thereby restricting Ca^{2+} rising to the bleb area and avoiding that intracellular concentration reaches deleterious levels for cells while coping with PM disruption (**Figure 3, panel 2**) [101], thus although it is not an active mechanism of repair, it is fundamental to restrict the damage [101]. Interestingly, the assembly of cortical endoplasmic reticulum (ER)-actomyosin bundles mediated by the ER chaperone Gp96 through the control of the cytoskeletal motor protein heavy chain of the non-muscle myosin IIA (NMHCIIA) activity is indispensable for the proper coordination of bleb expansion and retraction (**Figure 3, panel 2**) [102, 103]. Depletion of Gp96 is detrimental to cell survival during *L. monocytogenes* infection as cells cannot properly control membrane blebbing and repair of damage induced by the PFT LLO is compromised [102, 103]. Blebbing was also associated with the action of the lipid scramblase TMEM16F. This transmembrane protein was shown to be activated in a calcium-dependent manner upon challenging with pore-forming agents [104]. TMEM16F-mediated exposure of phosphatidylserine at the PM outer leaflet leads to increased fluidity and plasticity of the PM and favors the formation of membrane blebs (**Figure 3, panel 2**) [104]. Another lipid scramblase (TMEM16E) was associated with successful PM repair in muscle cells after laser injury, by modulating Ca^{2+} homeostasis through its ion channel activity. Specifically, this scramblase seems to be important to keep proximity between PM and ER and sustain ER integrity in damaged cells, supporting the role of the lipid scramblases in PM repair [105].

Although blebbing mostly supports cell repair and survival, it can be associated with cell death and in some cases it might be detrimental to cells in the context of bacterial infection [98]. *P. aeruginosa* utilizes blebs generated upon PM damage triggered by the insertion of the translocon of the T3SS to allow its intracellular motility and replication inside epithelial cells [106]. Also, *L. monocytogenes* exploits large PM blebs instigated upon damage induced by LLO to promote cell-to-cell spread and facilitate its dissemination across host cells [107]. As cells recover their PM integrity, the majority of the blebs retract, however, some are released in a process called “ectocytosis” [108].

3.2.2. Get rid of the damage: Shedding

Directly linked to PM blebbing, PM shedding is a well-characterized mechanism crucial for damage resolution and lesion removal [109]. This process, also known as “ectocytosis”, involves the passive extrusion of PM portions as microvesicles (MV) [1] and constitutes the preferential repair mechanism triggered by cells in response to pore-forming proteins. Although pore formation and consequently Ca^{2+} influx potentiates membrane shedding, it has been shown that in the case of PFTs-induced damage (LLO, SLO, ILY), toxin oligomerization at the PM is sufficient to trigger this event even in the absence of a complete pore [110]. Despite being a transversal process across cell types, cells may exploit this mechanism differently and it has a direct impact on cellular recovery after damage. In a study using PLY, a pore-forming toxin produced by *S. pneumoniae*, distinct immune cell types demonstrated distinct damage resolution capacities, with reduced MVs release correlating with increased susceptibility to damage [111]. Regarding the protein machinery involved in MV formation and release, the pore-formation by the *S. pyogenes* PFT streptolysin O (SLO) was shown to trigger the sequential recruitment of specific annexins (A1 and A6), which are calcium-dependent proteins that bind charged phospholipids with different calcium sensitivities (**Figure 3, panel 2**) [112, 113] to the PM vicinity to fuse with the membrane and quarantine the area before the elimination of damaged membrane areas thereby facilitating their removal and minimizing cellular consequences, allowing cells to return to normal functions [79, 113]. Shedding of vesicles may also rely on an active mechanism dependent on the Ca^{2+} mediated translocation of the endosomal sorting complex required for transport (ESCRT) to the PM in response to

different damage sources (mechanical, chemical, and mediated by pore-forming proteins) [114]. Particularly, in small lesions (<100 nm), the assembly of ESCRT-III components and accessory proteins nearby PM, assists repair [114, 115] in a mechanism that requires the initial recruitment of apoptosis-linked gene (ALG)-2 that complexes with annexin A7 and facilitate the recruitment of ALG-2-interacting proteins X (ALIX) to the damaged membrane to assemble ESCRT-III complex and the sequential recruitment of the Vps4 that directly contributes to the cleavage and removal of the damaged portion of the cell membrane (**Figure 3, panel 2**) [115, 116]. Comparative proteomic analysis of MVs released upon CDC pore formation revealed that annexins and ESCRT components are specifically packed inside EVs released from intoxicated cells, supporting the notion that these protein groups are recruited to damage sites and actively participate in the outward budding and pinching off the injured membrane [100]. Vesicle shedding primarily serves to eliminate damage from the cell surface, however, research in the field established that MVs also interact and control neighboring cells by delivering their content cargo and exerting signaling functions [117]. Studies carried out using MVs released from PLY-injured cells have demonstrated that MVs containing the PFT can be engulfed by macrophages and modulate the immune response by altering their polarization phenotype towards an enhanced response to Gram-positive bacteria [118]. Another recent investigation focusing on MVs released from alveolar epithelial cells upon PLY damage in the context of *S. pneumoniae* infection revealed another functional role of these MVs to alter neutrophil immune functions. Specifically, MVs compromised the formation of ROS, a vital component of the host-defense mechanism against bacterial pathogens in the lungs [119]. Exosomes are smaller EVs whose biogenesis differs from MVs [120]. Exosome release was recently associated with the effective repair of PM damage caused by mechanical and SLO-induced damage [121]. Upon PM disruption, annexin A6 is recruited to MVB in a Ca^{2+} -dependent fashion where it serves as a membrane tether linking MVBs to the PM and facilitating exosome delivery to the extracellular space (**Figure 3, panel 2**) [121].

Overall, while initially exerting a role in damage elimination from the cell surface, MVs arising from injured cells have an intricate role in intracellular communication and immune regulation, possibly contributing to improving damage resolution.

3.2.3. Get rid of the damage: Endocytosis

Solving damage at the PM is a complex process that entails the coordination of different mechanisms that cooperate to fulfill diverse cellular requirements [79]. Blebbing and shedding are interconnected events that operate to quickly isolate and remove the initial damage made by pore-forming proteins. However, recent evidence suggests that while cells are coping with pore removal, additional mechanical damage usually occurs, and complementary mechanisms are activated to promote cell survival. Lysosomal fusion has been described as an interlocking mechanism to repair secondary injuries happening after pore formation [79]. In fact, wound patching through the delivery of intracellular membranes to the injured cell surface was one of the first mechanisms described to seal lipid-based damage by decreasing PM tension and providing membrane constituents [122, 123]. Lysosomal fusion with the PM during repair is a complex mechanism governed by the action of multiple proteins, such as synaptotagmin VII (Syt VII), that senses intracellular Ca^{2+} overload, dysferlin that tether the organelles to the PM and SNARE protein VAMP7 that facilitates their fusion (**Figure 3, panel 3**) [124-129]. Lysosomal position during membrane repair is controlled by the small GTPases Arl8b that mediates kinesin-1 dependent microtubules trafficking towards PM [130, 131]; and the secretory Rab3a and its effector synaptotagmin-like protein 4a (Slp4-a) [131] that recruits NMHCIIA which consequently attaches lysosomes to cortical actin keeping them in the vicinity of PM [132]. Fusion of lysosomes with PM actively contributes to patching as they deliver membrane components. In addition to that, these organelles have a complex lumen filled with hydrolytic enzymes that after fusion with PM are discharged to the extracellular milieu, where they may interact with and modulate membrane properties [133]. Acid sphingomyelinase (ASM) is a lipase released from the lumen of lysosomes upon membrane fusion that converts PM sphingomyelin into ceramide domains favoring endocytosis (**Figure 3, panel 3**). Interestingly, ASM activity is dependent on the action of other lysosomal proteases, such as cysteine proteases cathepsin B, L and D. The model proposed by Castro-Gomes et al. suggests that the extracellular delivery of cathepsin B and L immediately after lysosomal fusion increases proteolysis of cell surface proteins and potentiates the activity of ASM. ASM-mediated ceramide domain

formation putatively activates cathepsin D which down-regulates ASM activity. Altogether contributing to the reestablishment of cellular homeostasis [134].

As mentioned, the resolution of PM lesions from the cell surface, especially the ones induced by pore-forming proteins, requires not only the seal of the membrane hole but also the physical removal of the protein from the cell surface. Morgan B. P. and colleagues first demonstrated that endocytosis of membrane attack complexes (MAC) actively contributed to neutrophil evasion to lysis [91]. Later, PM lesions triggered by the immune pore-forming glycoprotein perforin were shown to also trigger endocytosis of granzymes into large EEA-1 vesicles in a process mediated by clathrin and dynamin [135, 136]. The pore-forming toxin PLY was shown to induce endocytosis during infection of *S. pneumoniae* in neuronal cells to boost neuroinflammation and increase tissue damage. This mechanism relies on the presence of cholesterol and requires its pore formation activity. By testing different inhibitors, the authors show that the endocytic process that enhances inflammation (by the increased release of proinflammatory cytokines such as TNF- α and IL-6) is dependent on dynamin, phosphatidylinositol-3-kinase, and potassium efflux. Nevertheless, PLY was localized inside cells associated with flotillin-1 positive endosomal vesicles and was engulfed in a mechanism independent of dynamin, suggesting that PLY internalization might be regulated by a distinct endocytic pathway [137].

Additional studies using the pore-forming toxin SLO have shown that endocytosis is facilitated by negative PM curvature stimulated by ceramide-rich membrane platforms triggered by the activity of ASM released upon lysosomal fusion [138, 139]. Contrary to what was described for perforin-induced lesions, removal of damage induced by pore-forming toxins or mechanical injuries relies on caveolar endocytosis [140]. The mechanism proposed involves the internalization of the toxin, which is promptly ubiquitinated and sorted by the ESCRT-III complex into multivesicular bodies (MVB) for further degradation intracellularly (**Figure 3, panel 3**) [141]. Interestingly, cells get rid of the *S. aureus* α -toxin by endocytosis but instead of being degraded, the toxin is recycled back to the PM and expelled by exocytosis (**Figure 3, panel 3**) [142]. Although endocytosis participates in damage removal, this is not transversal to all sources of PM damage, a recent study using LLO toxin suggested endocytosis is important to remodel host cell PM after injury favoring repair, but does not actively

participate in toxin removal from the cell surface [143]. Intriguingly, endocytosis was proposed as a compensatory mechanism succeeding membrane repair events such as blebbing, shedding, and patching to fully restore cellular homeostasis through the removal of inactivated or monomeric toxins from the cell boundary, clearance of blebs that failed to shed or retract, and disposing of intracellular machinery that was recruited to damaged sites [110]. Importantly, endocytosis of injured membranes was validated *in vivo* using *Caenorhabditis elegans* as a model organism. Damage to the intestinal epithelium triggered by the PFT Cry5B induces endocytosis in a process dependent on the activity of the GTPase Rab-5 [144].

In conclusion, there is no unified mechanism of repair, to fully restore homeostasis and PM integrity cells undergo different mechanisms depending on the source and extent of damage, that might be concomitantly or sequentially activated to accomplish full recovery. Using laser damage and Ca^{2+} imaging to determine the spatiotemporal Ca^{2+} distribution which correlated with PM damage, a developed mathematical model predicts that PM holes are closed very quickly in a kinetic magnitude of seconds [145]. Although this immediate response to PM damage only takes a few seconds and is required to constrain the damage and rapidly close the PM holes to avoid cell death, a sequential restructuration is needed afterward to reestablish membrane integrity and remove potentially damaging agents which are estimated to take about 30 minutes [146]. The restructuration of the PM involves a process denominated macropinocytosis which involves actin remodeling and formation of protrusions around the wounded PM. Then several proteins such as Rab 35, Rab 5, EEA1 and LC-3 are recruited to facilitate endocytosis of the membrane that is then targeted by autophagic proteins (LC3-II and Rubicon) which target membrane portions for lysosomal fusion for consequent degradation [146].

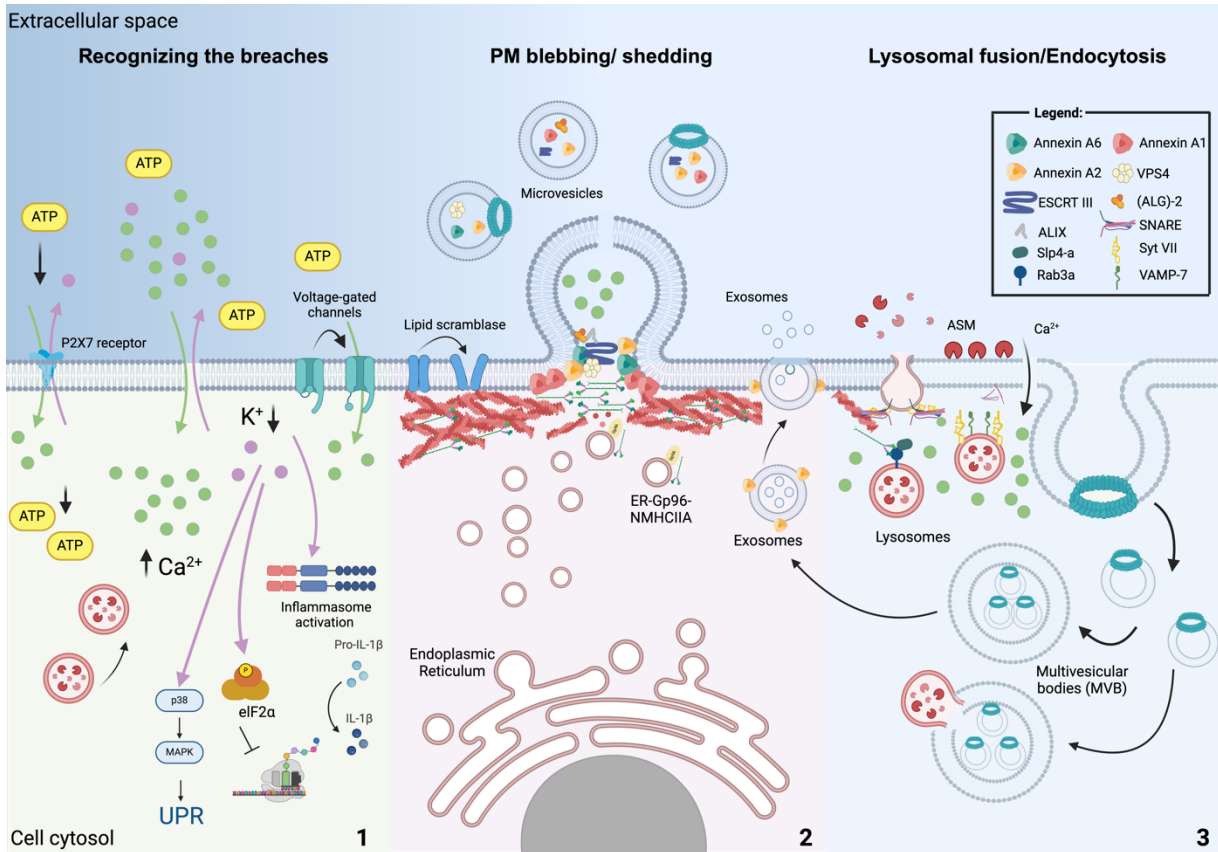


Figure 3- Schematic representation of PM repair mechanisms. 1) PM disruption can be sensed by cells by different mechanisms that will further activate different PM repair pathways. Given the 10,000-fold gradient of Ca^{2+} between extracellular and intracellular space, PM breaches allow the massive influx of Ca^{2+} through the pores which is sensed by cells and activates downstream pathways and cell signaling required for survival. Besides, K^+ depletion inside cells can modulate inflammasome activation, protein translation repression and UPR responses. ATP efflux can occur through voltage-gated channels and increased extracellular ATP partially activates the purinergic receptor P_2Y_7 , which is a cationic channel that aids Ca^{2+} influx and thereby recruits lysosomes to the PM. 2) Blebbing is a universal response to PM damage, it is triggered by a local detachment of the PM from the underlying cortical cytoskeleton and acts as a damage confinement avoiding the deleterious influx of Ca^{2+} . This is regulated by the local recruitment of annexins and Endoplasmic Reticulum (ER)-NMHCIIA remodeling occurring immediately after damage. Extracellular vesicles (EVs) shedding may arise from the direct extrusion of the blebs and damaged PM which allows the elimination of the damaged PM and associated core repair machinery. Furthermore, EVs may arise from the direct fusion of MVB which also contributes to PM reseal. 3) PM disruption and Ca^{2+} influx trigger the recruitment and docking with the PM through SNARE complex. Subsequently, lysosomes fuse with the PM and release intraluminal enzymes to the extracellular space. Acid

sphingomyelinase (ASM) hydrolyses sphingomyelin residues at the PM which generates ceramide domains, that facilitate membrane internalization and endocytosis of damaged PM. Upon internalization, the damaged PM portions are sorted to MVB which can be degraded upon lysosomal fusion or recycled back to the PM and released in exosomes (2). Created with BioRender.com.

3.3. Major effectors in PM repair

Upon disruption of membrane integrity, cells need to quickly seal the breaches to contain intracellular content loss, keep homeostasis, and survive. I already discussed which mechanisms can be employed by cells during PM repair. These organized processes are highly dependent on key proteins that exert important roles at different time points. Ca^{2+} is the major intracellular signaling molecule acting after PM disruption, therefore cells have several internal systems to fine-tune intracellular Ca^{2+} levels. Several intracellular proteins contain EF-hand domains, thereby they can sense Ca^{2+} influx intracellularly and activate different functions that contribute to PM repair [147].

3.3.1. Annexins

Annexins are by far the most extensively studied family of proteins known to assist PM repair. Annexins constitute a large family of proteins that has the interesting feature of including in its core domain, Ca^{2+} and phospholipid binding sites. Thus, in the presence of Ca^{2+} , annexins undergo conformational changes and bind to negatively charged phospholipids at the PM [148]. Other than that, its N-terminal domain is capable of establishing interactions with other cytosolic proteins. Intriguingly, annexins have the unique characteristic of presenting different Ca^{2+} sensitivities, there are annexins with low Ca^{2+} sensitivity (e.g., Annexin A4 and A5), intermediate Ca^{2+} sensitivity (e.g., Annexin A1), and excessive Ca^{2+} sensitivity (e.g., Annexin A2 and A6) [149]. Considering its inherent functionality in cells and being Ca^{2+} one of the major switches to activate PM repair mechanisms, annexins have long been associated with effective repair processes.

Annexin A1 and A2 were the first described to play an active role during PM repair. As shown by using muscle cells from a muscular dystrophy mouse model, upon mechanical injury, both annexins A1 and A2 associate with dysferlin, a protein previously linked to PM repair, in a Ca^{2+} -dependent manner and accumulate at PM

damage sites where they facilitate aggregation and fusion of intracellular vesicles that repair the injured membranes (**Figure 4**) [150]. Equivalent recruitment was observed in epithelial cells. Annexin A1 was shown to be rapidly recruited from the cytosol to the disrupted membranes after laser damage. Inhibition of annexin A1 function using specific inhibitors or truncated mutants impairs PM repair, thus reinforcing its importance in the emergent response to PM damage [151]. During PM blebbing (discussed in the previous section) the local increase in Ca^{2+} levels triggered by pore-formation favors the binding of annexin A1 to the PM where it concentrates and cross-links contiguous membranes thereby creating a macromolecular plug that isolates blebs from the cell core, thereby limiting the loss of cytosolic material and restricting the rise of extracellular components like Ca^{2+} inside the blebs [101]. Annexin A1 seems to play a role *in vivo* by significantly enhancing the healing of gastric mucosal injuries [152]. Not only annexin A1 is important to regulate membrane fusion events, but also annexin A2 mediated coating of enlargeosomes is necessary for efficient recruitment and fusion of these structures with the PM [153]. Interestingly, annexin A2 mediates F-actin polymerization and patches formation at early endosomes which are required early-to-late endosome transport and endosome biogenesis [154]. In the context of PM wounding occurring during cancer cell migration and invasion, annexin A2 complexes with S100A11 and accumulates at sites of damage where together they interact with actin filaments to decrease their depolymerization rate triggered by Ca^{2+} influx and allow the polymerization of actin filaments around the wound site thereby contributing to the restitution of membrane tension required for complete wound closure or excision of damaged areas (**Figure 4**) [155]. Through its C-terminal domain, annexin A1 can also interact with S100A11 [156]. PFT damage also recruits annexin A2 to specific PM extensions denominated nanotubes that eventually break down and are released from the cells as microvesicles allowing concomitantly the elimination of the toxin. However, the exact contribution of annexin A2 to the formation of nanotubes was not fully addressed [100]. Annexin A5 assembles with annexin A1 and A2 upon intracellular Ca^{2+} rise, and cluster together near PM (**Figure 4**) [157]. Interestingly, all these annexins were found overexpressed after traumatic spinal cord injury *in vivo* suggesting their protective role in the restoration of membrane integrity [158]. Upon Ca^{2+} influx triggered by PM disruption, Annexin A5 may also self-assemble into two-dimensional arrays which bind to phosphatidylserine residues at the PM and

stabilizes and strengthens membranes to prevent wound expansion and facilitate repair [159].

The importance of annexins was also validated *in vivo* using a zebrafish model of myopathy. Immediately after laser-damage of muscle cells, annexin A6 rapidly translocates to the PM, and it is succeeded by the relocation of annexin A2 and ultimately A1. The late recruitment of annexin A2 and A1 is dependent on annexin A6 suggesting that this is a highly organized event [160]. Altogether they form a dense patch that clogs the membrane opening. Whole genome and RNA sequencing data from a mouse model of muscular dystrophies revealed a truncated version of annexin A6 associated with disruption of the annexin cap allowing cell leakage and abnormal muscle repair capacity [161]. Annexin A6 is key to produce constriction forces during epithelial PM repair. Assisting annexin 6-driven events, annexin A4 is similarly recruited to the injured sites, self-assemble into trimers near PM, and forces membrane curvature directing the strength to wound closure. Both annexins work together to exert forces that bring together the wound edges, facilitating the patching of internal vesicles and reducing the hole size [162]. Recently, using different sources of PM disruption (microbial-mediated or mechanical stress), annexin A6 was also proposed to play a role in PM repair as a mediator of exosome secretion. In particular, Annexin 6 is recruited to multivesicular bodies (MVBs), which are recycled back to the PM lesions, to function as a membrane anchor facilitating the fusion of MVBs with the damaged PM and coordinating the release of exosomes [121]. Annexins may also participate in the shedding of the PM by regulating the assembly of the ESCRT-III. Following injury, annexin A7 complexes with ALG-2. This interaction enhances the binding of ALG-2 to ALG-2-interacting protein X (ALIX) necessary for the proper assembly of ESCRT-III complex and removal of damaged membranes during repair [116].

Annexins have multiple functions in repair acting as major sensors of injury and initiators of repair. Given their putative capacity to bind PM phospholipids they stabilize membranes and coordinate important membrane remodeling events such as intracellular vesicle patching, pore-clogging, blebbing and shedding. Their different Ca^{2+} sensitivities allow the fine regulation of their function at different timescales to properly coordinate membrane remodeling events.

3.3.2. Synaptotagmins and SNARES

Synaptotagmins are a family of multifunctional proteins that have long been associated with Ca^{2+} -mediated exocytosis, especially at the synapses in the brain [163]. They share structural features that include a transmembrane domain at the N-terminal, a linker, and two C-terminal domains that bind up to three Ca^{2+} ions [164]. Contrarily to annexins, the binding of Ca^{2+} to synaptotagmins does not induce any conformational changes but instead alters the electrostatic potential which facilitates interactions with phospholipids at the PM. Although they are structurally similar, synaptotagmins may appear at different locations within secretory vesicles or at the PM which is correlated with different Ca^{2+} binding capacities and distinct functions at the cellular level [164]. Synaptotagmin I (Syt I) is the most extensively studied member of the family. It is mainly found in the brain where it operates as a Ca^{2+} sensor together with the SNARE complex to regulate neurotransmitter release at the synapses [165]. To our knowledge, synaptotagmins have been first associated with the repair of axolemma damage in invertebrate axons and vertebrate neurites damage in a mechanism that requires the Ca^{2+} -dependent binding of Syt I to syntaxin that allows the fusion of vesicles with the PM [166, 167].

Syt VII is ubiquitously expressed in mammalian cells and has long been associated with Ca^{2+} -dependent lysosomal exocytosis during PM repair [124, 125]. This effect was later shown to be relevant in PM repair *in vivo*, as Syt VII-deficient mice presented defective resealing capacity due to impaired lysosomal exocytosis [126]. Analysis of macrophages infected with attenuated and virulent *Mycobacterium tuberculosis* (*Mtb*) revealed that bacteria generate microlesions in the PM of infected cells that are rapidly sealed by the fusion of lysosomes and Golgi-derived vesicles with the PM in attenuated *Mtb*-infected macrophages but not in cells infected with virulent *Mtb*. This difference was associated with the increased capacity of attenuated *Mtb* to induce the production of prostaglandin E_2 (PGE_2) that consequently regulates Syt VII expression and lysosomal fusion [168]. The function of Syt VII in the control of lysosomal exocytosis seems to be dependent on its Ca^{2+} -mediated interaction with PM SNARE proteins SNAP-23 and VAMP7 (**Figure 4**) [169]. Interestingly, in astrocytes, whose lysosomes lack Syt VII, the isoform that is associated with efficient repair through lysosomal fusion is Syt XI [170].

Extended-synaptotagmins (E-Syt) are ER-resident proteins that share not only the name but also some structural features with synaptotagmins. This family of proteins also has an N-terminal membrane anchor and cytosolic C-terminal domain, and they play an active role in PM repair. However, they have different localization and functionalities [171]. Regarding their primary role, E-Syt mediates the contact between the endoplasmic reticulum (ER) and the PM in a process regulated by intracellular Ca^{2+} rise. These ER-PM contact sites are crucial to regulate Ca^{2+} and lipid homeostasis [172, 173]. In the context of PM repair, E-Syt1 has recently been described to mediate the formation of ER-PM contact sites enriched in Ca^{2+} -bound E-Syt. Additionally, E-Syt interacts and mediates the recruitment of S100A11 to the PM where it interacts with annexin A2 to remodel the actin cytoskeleton that assists PM repair [156].

3.3.3. Other Ca^{2+} -responding proteins

Dysferlin is a type-II transmembrane phospholipid-binding protein that is structurally similar to Syt family proteins [174]. In the presence of Ca^{2+} dysferlin binds negatively charged phospholipids and [175] anchors to the PM by a transmembrane sequence close to its c-terminal domain. It has seven C2 domains (C2A-C2G) [174] which somehow contribute to PM repair, although at different extends as assessed by deletion mutant functions of different C2 domains. All the C2 domains except C2B modulate Ca^{2+} signaling [176]. The first evidence for the involvement of dysferlin in PM repair arose from the fact that mutations in the dysferlin coding gene were responsible for the development of muscle-related diseases limb-girdle muscle dystrophy type 2B and Miyoshi myopathy associated with impaired repair of sarcolemma damage [177]. Using a dysferlin-deficient mouse model and laser-induced wounding of muscle fibers, it was demonstrated that upon damage dysferlin-carrying vesicles are recruited to the injured membrane and dysferlin accumulates in patches. Contrarily, in the absence of dysferlin, muscle fibers were unable to repair the damage therefore supporting the role of dysferlin in proper muscle membrane repair [178, 179]. Dysferlin was also associated with cardiomyocyte repair [180] possibly by participating in the docking and fusion of intracellular patches with PM [181]. Co-immunoprecipitation and proteomics assays performed in muscle cells showed that upon membrane rupture, dysferlin interacts with more than 100 proteins suggesting that its function is possibly dependent on its interaction with other ligands [150, 182,

183]. In agreement, dysferlin-deficient muscle cells still translocate vesicles to the sarcolemma, thereby suggesting that dysferlin is crucial for fusion events but other proteins may participate in the trafficking and anchoring of intracellular vesicles to the PM [184]. Mitsugumin 53 (MG53) is a tripartite motif-containing (TRIM) family protein associated with repair in skeletal cells [185]. It interacts with dysferlin and mediates the assembly and trafficking of the protein together with intracellular vesicles towards PM injured sites [184, 186, 187]. Annexin A1 and A2 also interact with dysferlin and this interaction is required for the efficient fusion of dysferlin-rich vesicles with the membrane [150, 188]. AHNK is another key interactor, it binds to the C2A domain of dysferlin and regulates vesicle recruitment and stabilization at the site of injury [182]. Possibly this is due to its inherent role in the control of cortical actin cytoskeleton reorganization upon binding to annexin A2/S100A10 complex (**Figure 4**) [189]. Indeed, pharmacological disruption of the actin cytoskeleton in adult mice muscle fibers impaired the recruitment of dysferlin-containing vesicles to the injured membranes, thereby supporting a role for AHNK in vesicle positioning [190]. Patients bearing mutations in the caveolin-3 gene also presented mislocalization of dysferlin and impaired sarcolemma repair [191]. Muscle and non-muscle cells expressing mutant isoforms of caveolin-1 and -3 presented an accumulation of dysferlin in the Golgi complex [192], suggesting a role for caveolins in dysferlin trafficking. However, additional work showed that the exit of dysferlin from the Golgi is not solely dependent on caveolins, dysferlin may use alternative pathways to exit this organelle [193]. A novel role for caveolins in the regulation of dysferlin endocytosis was reported, in the absence of caveolin-1 and -3, dysferlin reaches the PM but is quickly removed by endocytosis. Altogether this suggests that caveolins may regulate not only pos-Golgi trafficking of dysferlin but also its retention in the PM by negatively regulating endocytosis via a clathrin-independent pathway [193]. Besides the coordination of intracellular vesicle patching, dysferlin may also control lysosomal fusion with the PM through its C2A domain in coronary endothelial cells [194]. The role of dysferlin in lysosomal tethering and fusion with the PM was further validated in mouse and human myoblast dysferlin-deficient cells. These models show a reduced amount of lysosomes at the PM and deficient ASM secretion [129]. Overall dysferlin together with their interaction partners plays an active role in the repair process,

however, how these complexes assemble during repair and the spatiotemporal sequence of the events is still undefined.

Calpains are a group of Ca^{2+} -dependent intracellular cysteine proteases [195]. The best-characterized calpain -1 and -2, are ubiquitously expressed and display different Ca^{2+} sensitivities. While calpain-1 activation requires a Ca^{2+} concentration within the μM range, calpain-2 demands an even higher concentration around the mM range [196]. Calpains were first thought to contribute to repair in a study performed using neurons where the authors observed that the addition of exogenous proteases favored neurite membrane resealing after laser-wounding in a Ca^{2+} -dependent manner [197]. Using crayfish giant axons and myelinated rat dorsal root axons, it was effectively demonstrated that endogenous calpain contributes to the closure of injured axons. Also, calpain-depleted cells accumulated vesicles at the cell surface, suggesting that calpains are implicated in vesicle fusion rather than vesicle accumulation at the cell PM [198, 199]. Mouse fibroblasts knocked-out for calpain -1 showed impaired PM resealing capacity, which was rescued by the addition of purified calpain -1 and -2 in the presence of Ca^{2+} . Furthermore, two calpain substrates involved in the dynamics of actin cytoskeleton, talin and vimentin, are recruited and cleaved at the PM suggesting that calpains might be acting as remodelers of the cortical cytoskeleton [196]. Mouse embryonic fibroblast expressing reduced levels of both calpain -1 and -2 have impaired PM resealing capacity [200], however decreased expression of each calpain individually had barely no effect on the repair capacity of cells suggesting that they may play redundant roles in PM repair [200]. Interestingly, loss of calpain-3, which is frequently associated with a specific limb-girdle muscular dystrophy: LGMD-2A, had no effect on repair efficiency after injury [200], suggesting that calpain -1 and -2 are the major players in repair. Cells treated with PFTs showed an accumulation of calpain-2 at the cell surface in sites of cytoskeletal-ER remodeling [102]. Another evidence of the role of calpain during PM repair was observed when cells pre-treated with the inhibitor of calpain activity, calpeptin, showed reduced vesicle shedding after damage with a PFT [79]. Contrary to what was suggested before, a recent study demonstrated that epithelial cells that do not express both calpain -1 and -2 were impaired in their capacity to repair damage induced by mechanical injury, but were able to recover from injury caused by PFTs and the detergent saponin, suggesting that the active role of calpain -1 and -2 in membrane repair is linked to the size and type of the injury [201].

Moreover, calpain acts as an upstream regulator of dysferlin-mediated repair since it can cleave, in a Ca^{2+} -dependent manner, dysferlin to mini-dysferlin_{C72}, which loses its c-terminal domain and keeps only the transmembrane domain together with C2F, C2G. Vesicles enriched in mini-dysferlin_{C72} were found to be rapidly recruited to injury sites where they fuse with PM. Being dysferlin associated with muscle damage and large wound repair, this might also explain the active role of calpains in mechanical wound repair [202-204].

ESCRT machinery is key in cellular membrane remodeling processes such as cytokinesis, neuronal development, intraluminal vesicle formation and budding, and PM repair [205]. The ESCRT system is composed of four highly conserved multimeric protein complexes denominated ESCRT-0, ESCRT-I, ESCRT-II and ESCRT-III and additional ESCRT-associated proteins (Vps4, Vta1, Did2) [206]. The participation of ESCRT complexes in PM repair is a common feature of different mechanisms. One of them entails the removal of PFTs from the PM by endocytosis [141]. In PM assaults by PFTs, upon cellular internalization, the toxin is rapidly ubiquitinated and consequently recognized by ESCRT proteins that sort the toxin into the lumen of MVB for internal degradation [141]. ESCRT proteins also contribute to the shedding of damaged membranes. The recruitment of ESCRT-III components to injured membranes occurs within seconds after damage and ESCRT-positive membrane budding and shedding was also observed. Additionally, the depletion of important ESCRT-III and ESCRT-associated proteins (CHMP2A, CHMP4B and Vps4) compromised cell survival and repair, which revealed the crucial role of this specific complex for membrane repair [114]. The mechanism by which ESCRT machinery regulates the scission of the PM is highly dependent on Ca^{2+} influx. The accumulation of ESCRT-III and Vps4 complex near lesioned membranes depends on the membrane recruitment of the Ca^{2+} -binding protein apoptosis-linked gene (ALG-2) and its binding to Ca^{2+} and ALG-2-interacting protein X (ALIX) (**Figure 4**) [115, 207]. This was validated in response to several sources of PM damage, bacterial and fungal PFTs, mechanical injury and recently immune-mediated damage by gasdermin D [208, 209]. The presence of protein members of ESCRT machinery assessed by proteomics in vesicles isolated from injured cells also supports its role in vesicle shedding [100]. Importantly, the requirement of ESCRT components for proper membrane resealing was further validated *in vivo* using *C. elegans* as a model organism, where ESCRT III mediates the sequential recruitment

of syntaxin-2 (SYX-2) and fusexin (EFF-1) to accomplish epidermal membrane repair [210].

As mentioned, ESCRT proteins mediate the repair of gasdermin D pores during pyroptotic cell death in a Ca^{2+} -dependent manner, which restricts the release of pro-inflammatory cytokines allowing cell survival [209]. Interestingly, this knowledge led to the research of new strategies to prevent ESCRT-mediated repair using Ca^{2+} chelator agents in tumor cells, thereby preventing membrane repair and increasing cellular sensitivity to gasdermin D pores [211].

3.3.4. S100 proteins and repair

S100 proteins consist of a group of 21 low-molecular-weight cytosolic proteins [147]. They exhibit Ca^{2+} -binding capacity and share structural features. They all contain two different EF-hand Ca^{2+} binding loops. One of them is located in the C-terminal domain and resembles the ones from other Ca^{2+} sensor proteins, and a second one is located in the N-terminal domain which is exclusive from this protein family [147]. These proteins are usually found in cells as symmetric homodimers [147, 212], but exceptionally they can also form heterodimers. The most extensively study heterodimer of the family is S100A8:S100A9 which have functional pro-inflammatory role in cells [213-215]. Recently, biochemical studies on different S100 heterodimers provided new insights on heterodimer formation and proposed that other non-characterized heterodimers can assemble in cells (S100A1:S100B, S100A1:S100P and S100A11:S100B) [216].

Besides its Ca^{2+} -binding capacity, these proteins usually interact with other proteins. To properly recognize their protein target, S100 proteins need to undergo structural reorientation to unveil their hydrophobic pocket imposed for target recognition. Ca^{2+} binding to S100 proteins favors these conformational rearrangements [217]. Annexins, which are described above as major PM repair proteins, are among the proteins known to interact and form complexes with S100s [217, 218]. Thereby, some S100 proteins were found to actively participate in PM repair [218]. S100A10 and S100A11 were the most extensively studied proteins in the context of PM resealing. S100A10 interacts with annexin A1 and A2 to form heterotetrameric complexes that function as a bridge bringing together two adjacent membrane phospholipids during vesicle fusion with PM [219, 220]. The participation

of S100A10 in PM repair comprises the formation and recruitment of a multiprotein complex composed not only by annexins but also AHNAK and dysferlin, altogether they sustain proper cortical actin cytoskeleton organization and cell membrane structure and facilitate membrane repair and fusion events (**Figure 4**) [189, 218, 221, 222]. S100A11 also specifically interacts with Annexin A1 and A2 [155]. After epithelial cell membrane damage, Ca^{2+} influx favors the interaction of annexin A2 and S100A11 which co-localize and accumulate at the cell surface. Deletion mutant analysis shows that the recruitment of S100A11 to the PM is strictly dependent on its interaction with annexin A2, suggesting that its role in PM repair is dependent on the binding to a target protein (**Figure 4**) [155]. As described in previous sections, S100A10-Annexin A2 complex regulates actin cytoskeleton organization by increasing cortical F-actin polymerization state and regulating damaged membrane expulsion by vesicle shedding [155].

S100A4 concomitantly accumulates with S100A11 at sites of PM damage. Interestingly, this accumulation anteceded the accumulation of F-actin at sites of membrane remodeling [218]. S100A4 can also be designated as metastasin or calvasculin, the name arises from the first described function of the protein as a tumor metastasis promotor [223]. It accumulates at the leading edge of migrating cells and has been associated with cell motility and invasion [224, 225]. This characteristic was early on associated with the high-affinity binding ($K_D \sim 1 \text{ nM}$) of S100A4 to the motor enzyme non-muscle myosin IIA heavy chain (NMHCIIA). This interaction is favored by Ca^{2+} binding to S100A4 which induces protein dimerization and conformational rearrangements that expose its target binding region [226-230]. S100A4 binds the assembly competence domain (ACD) in the coiled-coil tail located in the C-terminal region of the NMHCIIA molecule. This binding destabilizes non-muscle Myosin IIA (NMIIA) filaments by unwrapping the coiled-coil tail, which consequently destabilizes the intermolecular interactions between contiguous NMIIA molecules and promotes filament disassembly [229, 231]. Besides inducing filament disassembly, two-dimensional phosphopeptide studies demonstrated that S100A4 also modulates NMIIA filament assembly/disassembly through the inhibition of protein kinase C phosphorylation [232, 233]. Although S100A4 preferentially binds NMHCIIA and this interaction is the most extensively described in the literature, S100A4 can bind other cytoskeletal regulators such as tropomyosin [234]; F-actin where it potentially acts as

a crosslinker [224, 235]; liprin $\beta 1$, whose interaction diminishes the phosphorylation status of the protein and may interfere with cell-cell adhesion processes [227, 236]; Rho-binding domain (RBD) of Rhotekin and even forms a complex with GTP-bound RhoA altering Rho signaling function [237]. Thus, S100A4 acts as a main regulator of the cell cytoskeleton architecture and its Ca^{2+} -dependent function points to an active role of cytoskeletal remodeling required during PM repair. Curiously, recently S100A4 was described as an interactor partner of Annexin A2, its asymmetric binding to the N-terminal domain of Annexin A2 exposes its concave side and reestablishes the membrane-bridging activity of the protein, however, this interaction requires Ca^{2+} concentrations above the physiological intracellular threshold, suggesting that Ca^{2+} influx mediated by PM disruption may favor this interaction and functionalities [238]. The relevance of S100A4 in PM repair will be further addressed in this dissertation.

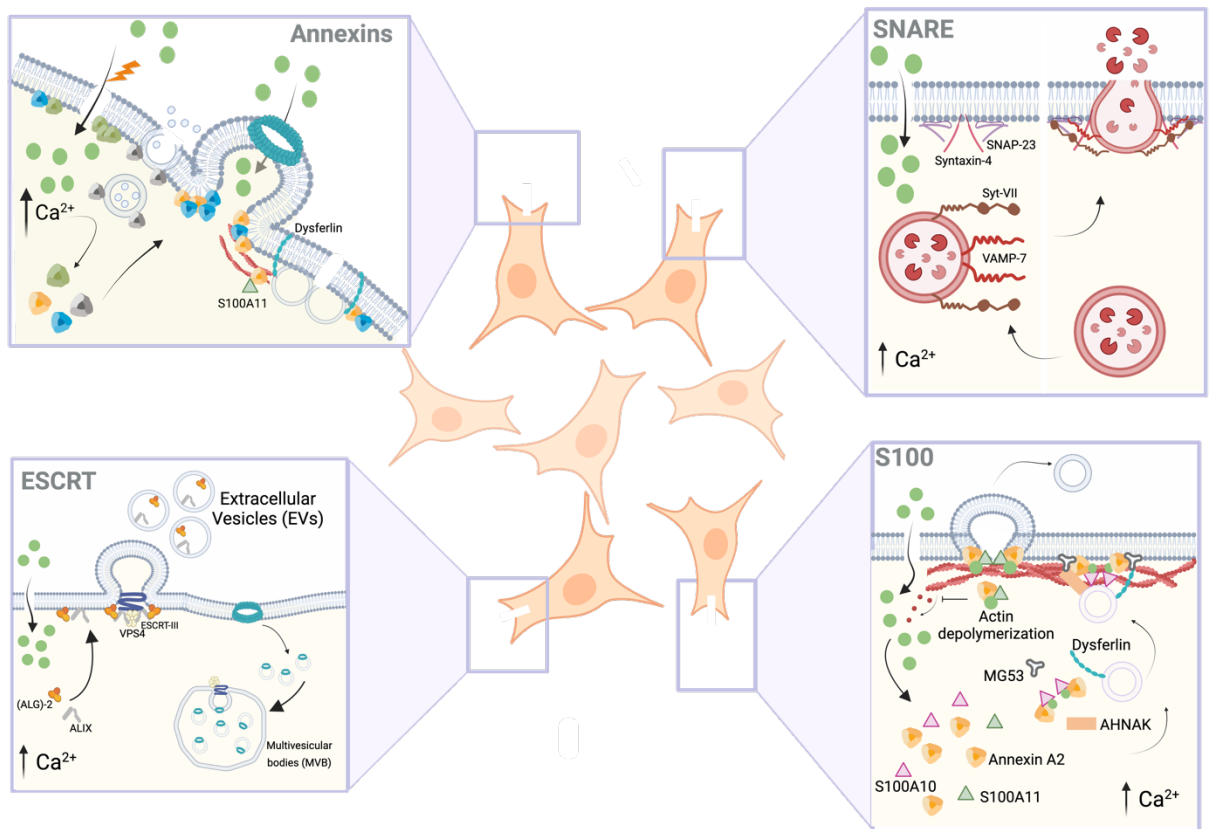


Figure 4- Calcium-binding proteins are major players in the repair of the PM. PM disruption leads to a massive influx of extracellular Ca^{2+} which can bind and activate multiple intracellular proteins that individually or in complexes cooperate to reseal PM. Annexins are the most extensively described family of proteins involved in PM repair, they present different Ca^{2+} sensitivities, therefore they are recruited to the PM at different time rates. They all have the

interesting feature of binding negatively charged phospholipids at the PM, they can assemble and form protein arrays that contribute to membrane deformation or even act as a clog at the base of blebbing sites avoiding deleterious cytosolic loss. Besides, annexin A2 interacts with S100A11 and dysferlin to coordinate vesicular trafficking and patching of intracellular vesicles and actin remodeling required for PM repair. Annexin A6 was also associated with MVB tethering and exosome release in the context of PM repair. SNARES and synaptotagmins act together to mediate lysosomal recruitment and fusion to the PM. Ca^{2+} influx favors the interaction between Syt VII and VAMP-7 with SNAP-23 at the PM which is facilitated by syntaxin 4, altogether creating a complex that facilitates the fusion and release of lysosomal content to the extracellular milieu. ESCRT is a large family of proteins that are recruited upon Ca^{2+} signaling and can mediate both the exocytosis of injured membranes by accumulating underneath damaged areas and contributing to membrane remodel and pinching off, or regulating endocytosis by assisting the sorting endocytosed membranes into the lumen of multivesicular bodies (MVB). S100 proteins have lately been associated with PM repair through their association with annexins. Annexin A2-S100A11 complex assists cortical actin polymerization which contributes to the excision of the damaged portions of the plasma membrane. Besides, intracellular Ca^{2+} rising activates multiple proteins such as dysferlin and Annexin A2-S100A10 complex which recruits additional protein machinery such as AHNK and MG53 to the vesicle surface. These complex interactions trigger the migration of vesicles to the cell periphery where they modulate the underlying cytoskeleton and fuse with damaged membranes. Created with BioRender.com.

3.4. Cytoskeletal remodeling associated with PM repair

The cytoskeleton is a vital cellular framework that sustains proper cell shape and organization. It is mainly composed of filamentous proteins that together provide the tension and mechanical support that cells need to effectively exert their functions. Cytoskeleton is intimately linked to the PM as described in section 1.2. Therefore, the disruption of PM will consequently affect the underlying cytoskeletal organization. PM repair mechanisms involve active PM remodeling events, that depend on the proper coordination of the cortical cytoskeleton required to modulate membrane tension and dynamics to facilitate vesicle recruitment and membrane deformation events [239]. An intact cortical cytoskeleton impairs such remodeling events. However, upon PM disruption, the influx of Ca^{2+} triggers actin depolymerization at damaged surrounding areas which results in decreased membrane tether forces and tension thereby contributing to membrane patching and deformation [240, 241]. This is supported by

the fact that pre-treatment of laser-injured fibroblasts with actin depolymerization agent cytochalasin D rescues their repair capacity even in the absence of Ca^{2+} [241]. The actin remodeling occurring upon membrane damage is extensively described in cell wound repair models of *Drosophila* and *Xenopus* oocytes. Briefly, actin is mobilized in association with microtubules to the damaged cell surface, where together with the activity of myosin and RhoGTPases form a contractile ring around the wound edge that actively contributes to efficient wound closure [242-246]. F-actin was found enriched at injured PM surrounding areas after laser-damage of muscle cells together with other important repair proteins such as annexins and dysferlin [187, 247], its pharmacological destabilization by lantruculin significantly delayed annexin A6 cap formation at the PM, suggesting that although not essential, fast actin remodeling regulates a faster response of cells to PM damage [187]. In mammalian epithelial cells, intracellular Ca^{2+} rising upon PM damage induces F-actin depolymerization around the damaged sites, but the recruitment of the protein complex Annexin A2-S100A11 allows the rebuilding of F-actin by regulating the recruitment and polymerization of actin, event crucial for PM repair [155]. The association of this complex with ANHAK was also shown to be critical as it mediates the cortical actin reorganization [189]. Moreover, the recruitment of dysferlin to the PM also relies on proper actin cytoskeleton dynamin since pharmacological ablation of actin polymerization significantly impaired dysferlin recruitment to sites of PM damage and membrane integrity restoration [190]. The formation of an actomyosin ring is essential to close wounds in distinct organisms as previously described. In mammalian cells, non-muscle myosin II (NMII) was also reported to assist PM repair. Interestingly, distinct roles in the PM repair process were identified for distinct NMII isoforms through studies involving human fibroblasts and rat corneal epithelial cells. While non-muscle NMII B is located at the cell surface and is essential for the first response of cells to damage and its downregulation impaired vesicle exocytosis, the downregulation of NMII A only had an impact in repeated injuries where it facilitates the rate of membrane repair by regulating vesicle formation at the trans-Golgi network [248, 249]. Besides, downregulation of NMII A expression hinders MG53 redistribution to the PM and efficient PM repair, suggesting that NMII A controls MG53-mediated membrane repair by governing the recruitment of MG53 to sites of PM damage [250]. NMII A is also fundamental to control lysosomal positioning and exocytosis, which are

critical events for PM repair [132]. As referenced in previous sections, PM blebbing is a mechanism that protects cells against lysis during PM damage, and its formation depends on cortical actomyosin contraction which is driven by NMII. Inhibition of NMII ATPase activity by blebbistatin impairs PM blebbing occurring after damage with distinct PFTs and renders cells more susceptible to injury [101, 102]. PM disruption induced by different PFTs (LLO, PFO) induces NMIIA remodeling, transient cortical accumulation and interaction with the ER chaperone Gp96 at venues of PM blebbing [102, 251]. This ER-NMIIA interaction is critical for PM repair as it controls PM remodeling events such as blebbing and membrane shedding [102]. Supporting the notion that cytoskeletal reorganization is required and occurs upon membrane damage, besides NMIIA, other cytoskeletal proteins and additional regulators were found in these cortical accumulations, such as NMIIIB, tubulin, the actin cross-linker filamin-A and calpain-2 [102]. Calpains are also essential cytoskeletal components of PM repair, they are rapidly recruited after membrane disruption and their main function is to remodel cortical cytoskeleton near injured sites, possibly by orchestrating the proteolytic degradation of cortical F-actin and focal adhesion molecules (talin, vimentin) that will further facilitate PM resealing [196]. The PFT PLY was proposed to directly interact with actin beneath PM and activating the RhoGTPases RhoA and Rac1, which directly regulates actin cytoskeleton dynamics and organization [252-254]. Thus, the insertion of PLY into the host PM may initiate the reorganization of the actin cytoskeleton, however, how this interaction favors PM repair was not further addressed [254].

Altogether this supports the critical role of a tight regulation of the cytoskeleton reorganization and remodeling during membrane repair. Nevertheless, complementary studies, especially in mammalian cells, are required to further understand the dynamics and spatiotemporal distribution of the cytoskeleton remodeling, since cells might require different mechanisms at different times. For instance, immediately after damage, the disassembly of the cortical cytoskeleton might be required to release tension and allow membrane remodeling events to occur. But later on, to reestablish membrane properties other mechanisms might be activated.

3.5. Implications in Disease

The maintenance of PM integrity is crucial to sustain cellular homeostasis. Under physiological conditions, and as detailed in section 2.1, cells are continuously subjected to external insults. Fortunately, under physiological and pathological conditions, cells have an inherent capacity to self-repair. If the activation of those mechanisms is partially impaired and restoration of membrane integrity repeatedly fails, it eventually leads to the development of debilitating acute and/or chronic disorders.

Muscle cells are responsible for multiple skeletal and cardiac physiological functions. In these cells, the sarcolemma is constantly under forces of contraction and is highly susceptible to mechanical stress. In fact, muscle cells are the cell type that most frequently suffer from PM lesions [23]. Eccentric exercise further increases the rate of skeletal muscle PM damage. However, a large proportion of the cells rapidly reseal and survive after this stress [22, 255]. If those mechanisms are impaired PM vulnerability and permeability are increased and may eventually be associated to the development of severe pathologies such as muscular dystrophies [24, 94]. Interestingly, several mutations in genes encoding for proteins involved in effective PM repair are associated with the development of muscular dystrophies. A mutation affecting dysferlin function, one of the major proteins involved in muscle repair, leads to the development of either Limb-Girdle muscular dystrophy type 2B or Miyoshi Myopathy diseases [178, 256]. Additionally, mutations in calpain-3, an important regulator of annexins, also result in Limb-Girdle muscular dystrophy type 2A [257]. Rippling Muscle Disease is a consequence of the loss of caveolin-3, which somehow controls the translocation of dysferlin-associated repair machinery to cell PM [191, 193] (**Figure 5**). Thereby, therapeutical strategies aiming to potentiate PM repair have been proposed to mitigate muscular dystrophy clinical disease outcomes. Although it is still very preliminary, the injection of recombinant Annexin A6 protects mice against acute muscle injury. Furthermore, mdx mice, which are the most widely used model to study Duchenne Muscular Dystrophy, have demonstrated an enhanced ability to repair the damage on skeletal muscle fibers and show significant improvements in disease pathology characteristics when injected with recombinant MG53 [258]. Besides, MG53 was also proposed to possess therapeutic potential to treat cardiomyocytes following myocardial infarction episodes. This is because ischemia-

reperfusion events that occur after infarction episodes trigger an overwhelming increase in oxygen levels that favors the generation of reactive oxygen species (ROS) and initiate membrane lipid peroxidation that leads to PM damage. Therefore, enhancing the ability of cardiomyocytes to repair these lesions may play a protective role in preventing cardiac injury [259]. Indeed, mice treated with recombinant MG53 display improved cardiac structure and function after myocardial infarction [259] **(Figure 5)**.

Compromised PM integrity in neurons has been associated with neuronal disorders. Diseases such as Alzheimer's or Parkinson's are characterized by the accumulation of misfolded proteins, amyloid beta protein (A β) and α -synuclein, respectively. Both proteins interact with PM components, especially lipids, including cholesterol [260, 261]. These interactions with lipids facilitate the formation of PM pore-like structures which alter the biophysical properties and permeability of membranes and compromise PM integrity of neurons which has deleterious effects and contributes to the pathogenesis of these diseases [260, 262-265]. Thus, understanding the mechanisms of neuronal repair after PM injury might contribute to the development of novel strategies to delay the onset of these pathologies [266] **(Figure 5)**.

Lung cells are particularly susceptible to PM damage triggered by microbial pathogens that release specific pore-forming toxins (e.g. *S. pneumoniae* and its PFT PLY) that compromise lung epithelial cells' integrity [267, 268] and can also induce indirect PM damage through the exacerbated immune response and release of pro-inflammatory cytokines [267]. Environmental cues such as inhalation of particles or cigarette smoke may lead to oxidative stress which enhance chemical PM damage in the lung cells [21, 269, 270]. Direct physical stress associated with surfactant dysfunction may also occur and may lead to increased surface tension and mechanical damage [271, 272] thereby disrupting lung epithelium. There are also common diseases associated with PM damage and impaired PM repair phenotype, the most well-described diseases are acute respiratory distress syndrome (ARDS) which is characterized by increased alveolar damage; and idiopathic pulmonary fibrosis (IPF) which has been associated with increased cell injury and impaired cell repair capacity [273] **(Figure 5)**.

Although engagement of PM repair mechanisms is generally beneficial for cells to cope with several sources of physiological stresses they undertake and compromised repair capacity leads to the development of severe diseases, in the context of cancer cells, the hyperactivation of these repair mechanisms may result in enhanced cell invasion capacity and metastasis. In fact, to spread throughout the body, cancer cells need to reach the blood or lymphatic system which implies the migration through difficult barriers and increased physical stress. Thus, these cells frequently have their PM integrity compromised but they also have overactivation of the repair machinery which ensures a fast and efficient PM repair supporting tumor cell survival even under high mechanical stress [94] **(Figure 5)**. Interestingly, investigations using human clinical samples from breast, pancreatic, laryngeal and colorectal cancer showed that these cells overexpressed several annexins, which are intimately related to effective repair [274, 275]. In addition to that, a study using metastatic cell lines showed that these cells expressed high levels of S100A11 which together with Annexin A2 improved PM repair capacity [155]. Supporting this, Bouvet, F. and colleagues recently showed that the impaired membrane repair capacity of cancer cells abolished their survival and migration capacity [276].

As mentioned previously, one of the major sources of PM damage is microbial infection. By releasing pore-forming proteins or by directly interacting with cells they can compromise PM integrity and invade organisms causing severe pathologies. Inhibiting PM repair capacity of cells through the knockdown of proteins actively involved in PM repair compromised the host survival capacity *in vitro* and *in vivo* [102] **(Figure 5)**. Thereby, modulation PM repair mechanisms can protect cells from this attack and promote survival.

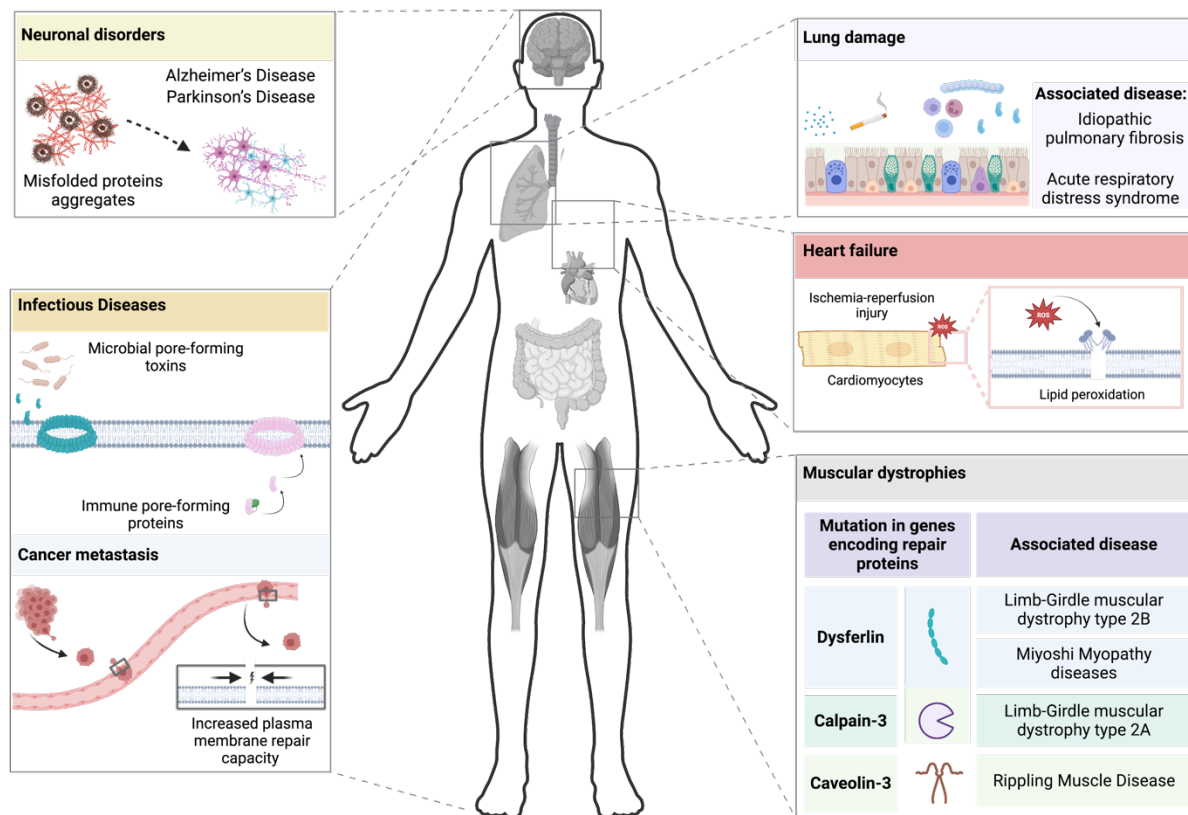


Figure 5- PM damage-associated diseases. Misfolded proteins such as amyloid beta protein ($A\beta$) and α -synuclein in the brain are characteristic of neurodegenerative disorders such as Alzheimer's or Parkinson's. These misfolded proteins can induce “pore-like” structures in neuronal cells disrupting PM integrity and contributing to neuronal injury and degeneration. Lung cells are frequently exposed to several sources of PM damage, ranging from inhaled aerosols, and bacterial pathogens that can release pore-forming toxins that cause direct damage in epithelial cells or indirect damage by exacerbating immune response (immune-mediated damage), also cigarette smoke might induce chemical damage due to increased ROS production, and even mechanical damage when lung epithelium present surfactant dysfunction. After infarction, heart ischemia-reperfusion results in high levels of ROS which triggers PM lipid peroxidation that compromises cardiomyocyte PM integrity. Muscular dystrophies are usually associated with defects in PM repair, indeed, the development of some specific diseases arise as a result of specific mutations in genes that encode proteins extensively described to play a role in PM repair. Infectious agents usually produce pore-forming toxins that compromise the PM of host cells thereby facilitating infection; additionally, pathogens usually induce an immune response from the host that can also produce pore-forming proteins that again disrupt PM integrity. Counterintuitively, cancer cells have enhanced PM repair capacity, this feature allows cells to quickly repair PM damage occurring during cell

migration to distant tissues. Overall, compromised PM integrity and repair capacity may lead to severe complications and complex human disorders. Created with BioRender.com.

Although PM repair mechanisms are implicated in a variety of human diseases, in my dissertation I will delve into the intricate role of those mechanisms in response to damage caused by bacterial pore-forming toxins and in the context of infectious diseases. Specifically, I dug into the mechanisms of PM repair activated in response to pneumolysin (PLY), a PFT from *S. pneumoniae*, to further elucidate their impact on the outcome of infection caused by this pathogen. Considering this, the subsequent chapter will comprehensively discuss the current knowledge regarding the significance of PLY in pneumococcal infection.

The Yin and Yang of pneumolysin during pneumococcal infection

Pneumolysin (PLY) is a pore-forming toxin produced by the human pathobiont *Streptococcus pneumoniae*, the major cause of pneumonia worldwide. PLY, a key pneumococcal virulence factor, can form transmembrane pores in host cells, disrupting plasma membrane integrity and deregulating cellular homeostasis. At lytic concentrations, PLY causes cell death. At sub-lytic concentrations, PLY triggers host cell survival pathways that cooperate to reseal the damaged plasma membrane and restore cell homeostasis. While PLY is generally considered a pivotal factor promoting *S. pneumoniae* colonization and survival, it is also a powerful trigger of the innate and adaptive host immune response against bacterial infection. The dichotomy of PLY as both a key bacterial virulence factor and a trigger for host immune modulation allows the toxin to display both “Yin” and “Yang” properties during infection, promoting disease by membrane perforation and activating inflammatory pathways, while also mitigating damage by triggering host cell repair and initiating anti-inflammatory responses. Due to its cytolytic activity and diverse immunomodulatory properties, PLY is integral to every stage of *S. pneumoniae* pathogenesis and may tip the balance towards either the pathogen or the host depending on the context of infection.

1. Introduction

Streptococcus pneumoniae (*Sp* or pneumococcus) is an extracellular Gram-positive bacterium that asymptomatically colonizes the upper respiratory tract of 5–10% healthy adults and 20–40% of children [277]. *Sp* was considered the major cause of lower respiratory infections (LRI) with a global incidence of 2.7% worldwide, resulting in more than a million deaths per year and thus ranking among the deadliest bacteria [278]. In the United States alone, pneumococcal pneumonia leads to over 150,000 annual hospitalizations. Other forms of *Sp* infectious complications such as otitis media, bacteremia, and meningitis are also significant healthcare burdens, leading to an average of 5 000 000, 4 000, and 2 000 cases/year respectively (source CDC: <https://www.cdc.gov/pneumococcal/clinicians/clinical-features.html>). Elderly and children under 5 are particularly susceptible to severe disease, with an invasive pulmonary disease (IPD) incidence of 7 %. A major health concern and economic burden, *Sp* infection results in over \$17 billion in direct medical costs annually in the US [279, 280].

Sp is naturally transformable and displays high genome plasticity, contributing to the rapid emergence of antibiotic resistance and evasion of vaccine-mediated immunity [277, 281]. Effective *Sp* prevention and treatment is complicated by the appearance of multidrug-resistant infections, non-vaccine serotypes and aging of the world's population [280, 282]. Approximately 30% of *Sp* cases involved isolates resistant to one or more antibiotics (<https://www.cdc.gov/drugresistance/pdf/threats-report/strep-pneumoniae-508.pdf>). Worldwide, pneumococci resistant to penicillin, erythromycin, and trimethoprim-sulfamethoxazole are on the rise [283]. To a lower extent, resistance to Tetracycline, chloramphenicol and fluoroquinolone have also been identified [284]. While in the United States, multi-drug resistant pneumococci prevalence has been reduced since introduction of pneumococcal vaccines, the risk remains high in susceptible populations, especially individuals aged 65 and over [285]. Vaccination has been the cornerstone of pneumococcal disease prevention. However, currently, the two classes of pneumococcal vaccines, the 23-valent pneumococcal polysaccharide vaccine (PPSV23) and the pneumococcal vaccines based on protein-conjugated polysaccharides (PCV13/PCV23), only protect against a subset of over 90 different pneumococcal capsular variants (capsular serotypes) [286]. Through both the expansion of pre-existing non-vaccine pneumococcal serotypes and serotype 'switching', an exchange of capsular polysaccharide genes through transformation, infectious strains not covered by standard vaccination are on the rise [287]. As the efficacy of traditional antibiotics and vaccines become compromised, understanding the mechanisms of action of pneumococcal virulence determinants is of critical importance for the development of new therapeutics.

Sp transmits predominantly via aerosol as the bacteria is harbored in the nasopharynx. Asymptomatic *Sp* carriage can lead to localized infection of tissues contiguous with the nasopharynx, causing sinusitis, otitis media, and pneumonia [277]. The bacterium is also capable of subsequent systemic spread to the heart and brain upon access to the bloodstream, causing serious diseases such as cardiac dysfunction and meningitis [277] (**Figure 1**). The versatility of *Sp* is reflected by the diversity of interactions with its host depending on the site of infection and degree of disease. To colonize the host nasopharynx, *Sp* forms biofilms on the mucosa of the upper respiratory tract [288]. This causes mucosal inflammation which promotes

bacterial shedding in secretions leading to transmission [289]. Long-term asymptomatic nasopharyngeal carriage supports inflammation-induced transmission and predisposes the host to develop disease (**Figure 1A**). *Sp* can then spread from the nasopharynx to neighboring tissues, such as the sinusoidal cavities to cause sinusitis, the middle ear to cause otitis media, or the eye to cause keratitis (**Figure 1B-C**), all of which can be recurrent infections especially in immunocompromised patients [290]. *Sp* aspiration into the lower respiratory tract causes pneumonia, where infection damages alveolar epithelial and endothelial cells promoting tissue permeability and induces thrombotic events (**Figure 1D**). If the immune response mounted in the lungs is not sufficient to eliminate the bacteria, *Sp* may gain access to the bloodstream and disseminate to cause IPD (**Figure 1 E-G**). During IPD, *Sp* may invade the spleen [291], the heart [292-294], and/or may cross the blood-brain barrier [295]. In the heart, *Sp* forms biofilms and damages both cardiomyocytes and immune cells, leading to myocardial dysfunction and long-term pneumonia-associated adverse cardiac events (**PACE; Figure 1F**). *Sp*-induced remodeling of brain tissue during meningitis likely accounts for permanent neurological damage reported in about 50% of the survivors (**Figure 1G**).

Integral to all stages of *Sp* pathogenesis is the host-pathogen interaction mediated by pneumolysin (PLY), a key virulence factor produced by almost all clinical isolates and currently known *Sp* serotypes [296]. PLY can promote asymptomatic carriage, colonization, transmission, immune evasion and dissemination, highlighting the multifaceted functions of this bacterial toxin. As a member of the cholesterol-dependent cytolysin (CDC) family, PLY is a pore-forming toxin (PFT) that at lytic concentrations disrupts host cell plasma membrane (PM) and promotes the uncontrolled influx and efflux of ions, small molecules, and proteins [297]. PLY-induced pore formation interferes with a plethora of cellular signal transduction pathways. Depending on PLY concentration, which dictates the extent of PM damage and intracellular Ca^{2+} overload, host cells respond by activation of either cell death or cell survival and damage repair pathways. At lytic levels, PLY-induced pores can overwhelm cellular homeostatic mechanisms, triggering both cellular proinflammatory signaling pathways and irreversible cellular injury that results in the release of molecules heightening inflammation. The ensuing tissue damage is integral to pneumococcal disease [298]. For example, in the lung, exacerbated inflammation

promotes tissue invasion and systemic spread [297, 299]. In contrast, at the sub-lytic concentrations likely present during the early stages of infection, PLY stimulates cell-autonomous repair mechanisms that overcome membrane damage, restore cell integrity, and promote cell survival [300, 301], curtailing inflammation. In addition, depending on infection dose, site, disease stage, target cell, and the immune state of the host, PLY may display either “Yin” or “Yang” components, each with potentially opposing effects on *Sp* pathogenesis. The Yin of PLY promotes disease by promoting cell damage either directly or by excessive inflammation. The Yang of PLY mitigates the damage by triggering host repair mechanisms and modulating inflammation, allowing the carriage of *Sp* without symptoms. The remarkable ability of PLY to trigger both “Yin” and “Yang” responses depending on infection context is showcased in several *in vitro* and *in vivo* infection models [302, 303]. Interestingly, heterogeneous expression of PLY within individual bacteria of an isogenic *Sp* population favors the appearance of subpopulations expressing different levels of PLY. Recent studies showed that genetically modified PLY expressing *Sp* strains producing different levels of PLY triggered different outcomes during infection. Specifically, high-PLY producing bacteria induced extensive autophagosome damage allowing efficient clearance of *Sp* from the host cells, yet, low-PLY producing bacteria facilitated evasion of host defense mechanism and promoted the cross of the blood-brain barrier [304, 305]. Furthermore, rapid and high levels of PLY release drives hypervirulence in infection by serotype 1 pneumococci [306, 307], showcasing that the plasticity of PLY expression during *Sp* pathogenesis is fundamental and can influence the outcome of the infection.

Here we provide a comprehensive review of PLY functions and interactions with host cells by describing the mechanism of pore formation, its multiple targets and effects on host cells, and its antipodal interactions with the host immune system. We summarize the available data and recent discoveries that highlight the “Yin” and “Yang” perspectives of PLY activity contributing to *Sp* disease progression, unveiling the myriad and sometimes conflicting properties of this fascinating bacterial toxin.

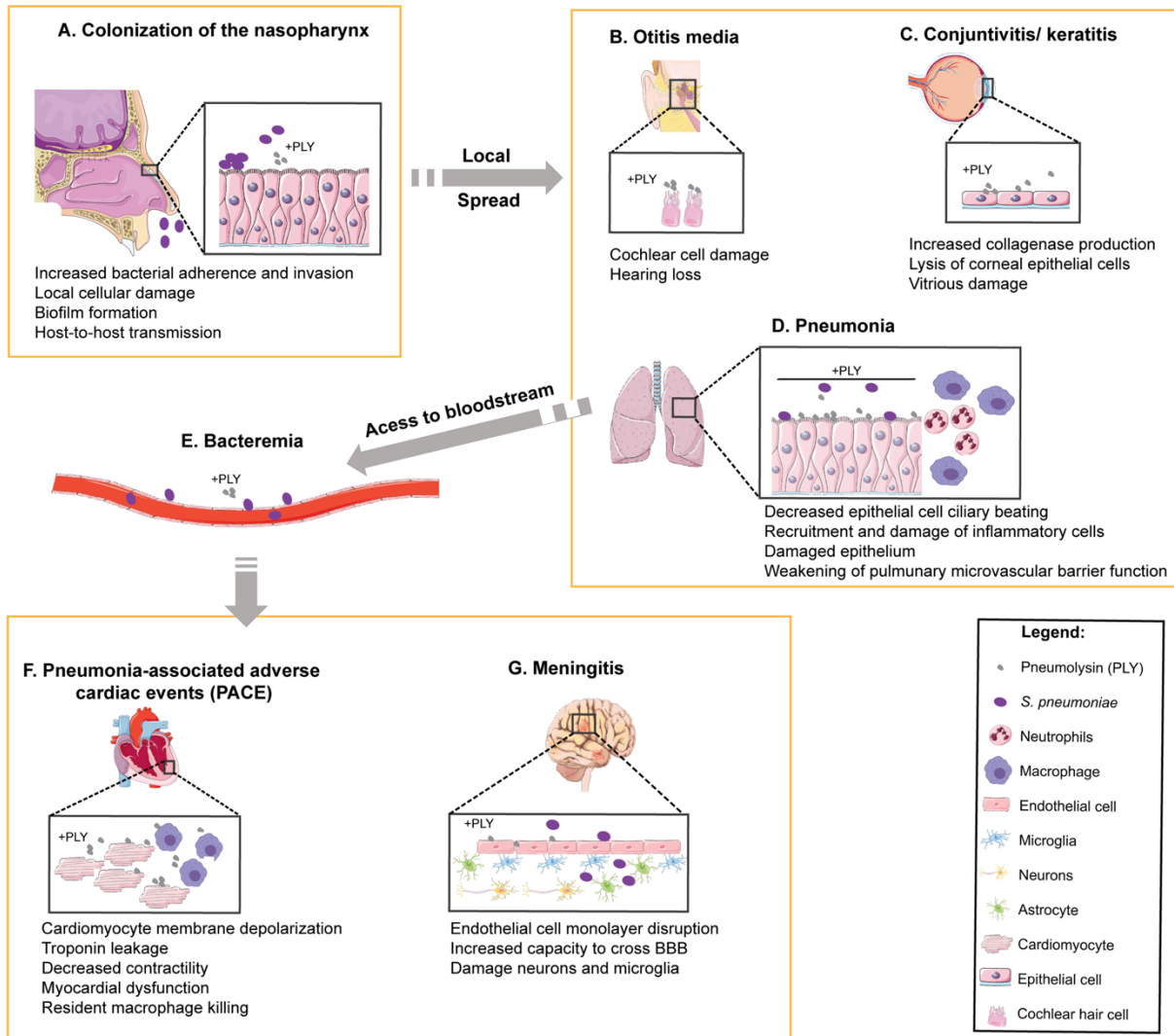


Figure 1- Role of Ply in human pneumococcal pathogenesis and in the targeting of multiple organs. In the multiple steps of pathogenesis during human infection by *S. pneumoniae*, the target organs and the cell types involved are indicated and the reported functions of Ply are listed below each step. (A) *S. pneumoniae* colonizes the human upper respiratory tract. Prolonged colonization of the nasopharynx may favor bacterial spread to neighboring tissues such as (B) the middle ear, causing otitis media and massive damage of cochlear hair cells, and (C) the eyes, leading to the development of keratitis and endophthalmitis. Upon aspiration, *S. pneumoniae* can reach (D) the lower respiratory tract and cause pneumonia. In the lungs, Ply causes the dysfunction of epithelial barrier, facilitating bacterial access to (E) the bloodstream, where Ply diminishes phagocytosis and disrupts endothelial/epithelial cells to promote tissue invasion. The bacteria are then able to infect (F) the heart, causing pneumonia-associated adverse cardiac events (PACE) by targeting the cardiomyocytes and resident macrophages, and (G) the brain, causing damage of both neurons and microglia.

2. Structural and functional insights of pneumolysin

2.1. PLY export

Secreted PLY is key for infection; however, the localization and export mechanism of PLY is still debated [308]. PLY can be detected in the bacterial cytosol [308, 309], non-covalently attached to the cell wall [308], in culture supernatants [308], and in circulation during *Sp* infection [310]. Given the lack of a canonical N-terminal signal peptide that promotes protein secretion, PLY was thought to be released solely by autolysis, an idea supported by experimental data showing reduced PLY release in the presence of non-bacteriolytic antibiotics [311]. Nevertheless, autolysis may not be the only mechanism for PLY secretion [308, 312]. In fact, although attenuated for virulence, *Sp* strains lacking autolysin release wild-type levels of PLY when cultured *in vitro* [313, 314]. In addition, a functional SecA2 system is required for the non-covalent association of PLY to cell wall peptidoglycan [315], a structure that can hinder PLY delivery to the bacterial surface [316]. Indeed, enzymatic digestion of *Sp* peptidoglycan promotes PLY release and is detrimental for virulence [316]. Thus, PLY secretion is likely determined by several mechanisms that may cooperate to foster disease [307].

2.2. Mechanism of PLY-mediated membrane permeabilization

PLY shares structural and functional properties with other bacterial PFTs belonging to the CDC family, such as listeriolysin (LLO), perfringolysin (PFO), and streptolysin (SLO) [317]. It binds to cholesterol residues at the PM of host cells, oligomerizes, and undergoes conformational changes to form stable pores with well-defined sizes (**Figure 2**).

To generate pores, soluble PLY interacts with the PM, multimerizes into two successive “prepore” complexes, and then inserts into the PM to generate a large pore (**Figure 2A**). Soluble PLY, in its monomeric state, has been shown by crystallographic methods to be an asymmetric molecule composed of 4 major domains (D1 to D4), including α -helices and β -sheets (**Figure 2B**). The N-terminal D1 domain, although not essential for cell binding, stabilizes overall protein structure and enables hemolytic activity [318, 319]. The non-contiguous D1 and D3 are adjacent in the 3D crystal structure and linked to the C-terminal of D4 via D2 (**Figure 2B**) [320]. Blocking monoclonal antibodies and the analysis of binding-defective point mutants indicate

that D4 promotes membrane binding [297, 321-325]. In particular, two residues (T459 and L460) in a conserved D4 undecapeptide that comprises a Trp-rich loop are essential for cholesterol recognition (**Figure 2B, arrow**) [297, 317, 326, 327]. In addition, the recombinant PLY toxoid B “PdB” harboring a mutation in Trp 433 in D4 is unable to undergo the conformational changes required for membrane insertion and strains expressing this mutant show reduced virulence, further reinforcing the role of D4 in membrane binding and pore formation [328, 329].

PLY monomers multimerize, forming transient pre-pore asymmetric structures with variable diameters and composed of 40 to 44 monomers (**Figure 2A**) [320, 322, 330-334]. D2 undergoes a 90° rotation, bringing D1 and D3 towards the PM, compacting the overall structure and establishing the late pre-pore (**Figure 2A, C**) [297, 331]. PM insertion occurs when the D3 helix bundles (HB1 and 2) are refolded into 85Å β-hairpins (HP1 and HP2) that perforate the hydrophobic membrane and assemble into an approximately 260Å diameter transmembrane channel (**Figure 2D**) [330, 331]. Mutations in D3 residues Y150 and T172 are found in a naturally occurring non-hemolytic variant of PLY [321, 335], pointing to their critical role in the conformational alterations required for transmembrane hairpin formation, PM insertion and subsequent pore-formation [321]. Although it is unclear what advantage *Sp* derives from loss of PLY hemolytic activity, serotypes harboring naturally occurring non-hemolytic PLY are commonly associated with non-lethal respiratory tract infections [336] pointing to roles played by non-pore formation dependent mechanisms of PLY during *Sp* pathogenesis.

Additionally, it has been reported that PLY monomers may be trapped in arcs or slit-shaped oligomers [297] that have the ability to generate pores [337, 338], possibly with variable sizes, different permeabilities and distinct functional roles [333, 339]. At low PLY concentrations, likely occurring at early stages of infection, complete prepore ring structures are expected to form less efficiently than arcs, and so pore formation may rely on the efficiency of the conversion of arc oligomers to pores. At high PLY concentrations, potentially reached later in infection, binding of soluble PLY to PM is efficiently followed by assembly of full rings, so cell lysis mainly depends on the PLY affinity for cholesterol [339]. PM disruption following arc or full ring assembly possibly dictates different host responses according to the phase of infection and could influence the balance of Yin and Yang outcomes.

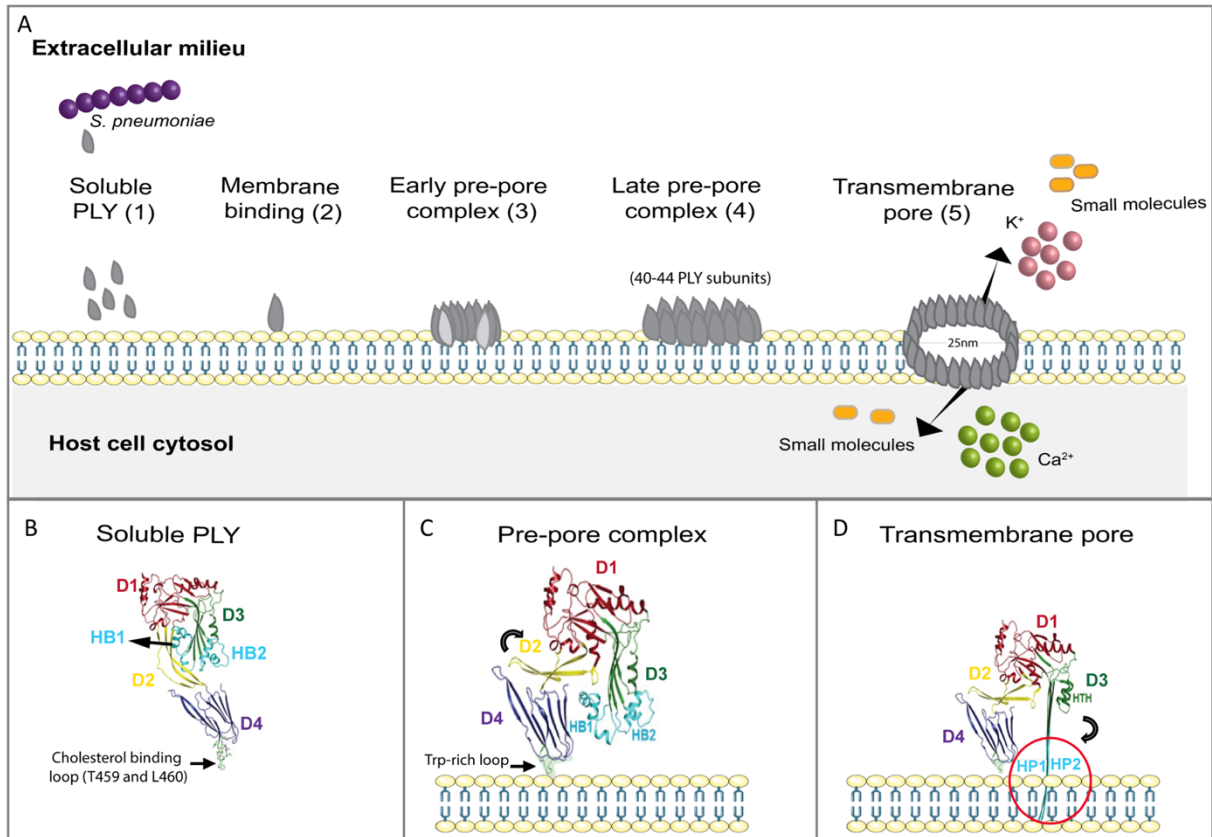


Figure 2- Mechanisms of PLY-mediated host plasma membrane permeabilization and conformational changes associated to pore formation. (A) PLY pore-formation is a multi-step process. PLY is released by *S. pneumoniae* as a water-soluble monomer (1) which specifically bind to cholesterol residues on the host cell plasma membrane (2). PLY monomers oligomerize by interacting with each other to form the early pre pore complex (3), which protrudes into the membrane surface establishing the late pre-pore (4). Finally, PLY inserts hairpins HP1 and HP2 across the membrane forming an open transmembrane channel, which allows the uncontrolled influx and efflux of ions and small molecules (5). (B) Soluble PLY. The 3D crystal structure of PLY monomer as it is released from *S. pneumoniae* is shown. The 4 major domains, from D1 to D4, as well as Helix Bundles (HB) 1 and 2 and cholesterol binding loop are indicated. The arrow indicates residues T459 and L460 in the D4 Trp-rich loop which are essential for cholesterol recognition. (C) PLY in pre-pore complex. Structure of PLY upon cholesterol binding via the conserved D4 Trp-rich loop. Interaction with cholesterol induces a 90° rotation of D2 (in yellow, indicated by the curved arrow) bringing D1 and D3 towards the host plasma membrane. HB1 and HB2 are positioned just above the host membrane. This structural organization is maintained in the pre-pore stage. (D) PLY in the transmembrane pore. The 3D structure of PLY when inserted in the host cell plasma membrane is depicted. HB1 and HB2 refold into 85 Å β -hairpins HP1 and HP2 (shown by the curved arrow), which insert (red

circle) and cross the hydrophobic membrane to form an open transmembrane pore. Adapted from [319].

2.3. **PLY as a ligand for host molecules**

In addition to binding cholesterol, PLY interacts with several other host molecules, interactions that contribute its function as a both pro- and anti-inflammatory agent, further discussed in Section 4 below. First, through its D4 domain, PLY binds the mannose receptor C type 1 (MRC-1), expressed at the cell surface of multiple immune cell types in the airways [340]. MRC-1 acts as an internalization receptor, allowing *Sp* to invade MRC-1-expressing dendritic cells (DCs) and alveolar macrophages. At low infectious doses when immune stimulation by bacterial PAMPs is limited, MRC-1-PLY interaction and the consequent *Sp* phagocytosis modulate inflammation (discussed in section 4.2.2) and promote intracellular bacterial survival in the lungs in a murine pneumococcal pneumonia model [340]. Second, PLY also has the capacity to modulate the interaction of *Sp* with complement by functioning as a molecular decoy, binding the Fc portion of human IgG and triggering C1q recruitment and complement cascade activation [341, 342] (reviewed in section 4.2.4). This interaction is dependent on specific residues within short non-contiguous PLY sequences that are homologous to the human C-reactive protein (CRP), which also activates the complement [342]. *In vivo*, diversion of complement proteins by PLY promotes both pulmonary and systemic infection, as PLY-deficient *Sp* become more virulent in complement-deficient mice [343]. Lastly, PLY was suggested to bind host cell glycans such as sialylated fucosylated glycan divalent-LewisX (sLeX) [344], and computational docking studies suggested the D4 and the interface between D3 and D4 as putative binding sites for LeX and sLeX, respectively [322]. Nevertheless, interactions detected *in vitro* utilized high sLeX : PLY ratios and thus appear to be of low affinity, and PLY : sLeX co-crystals have never been observed [322], indicating the need for further studies are needed to explore potentially biological interactions between PLY and sLeX.

2.4. **PLY-mediated pathogenesis and targeted cells**

Due to its ability to bind to cholesterol commonly found in mammalian PMs, PLY is able to target and modulate the function of virtually all cell types and thus plays key

roles in many modes of infection, such as asymptomatic carriage, local disease, or life-threatening systemic disease (**Figure 1**) [345].

Early in infection, PLY has vital roles in colonization of the nasopharynx and in host-to-host transmission (**Figure 1A**) [289, 346, 347]. *In vitro*, PLY-deficient mutants are impaired in adherence [348] and show lower bacterial burden in the nasopharynx of intranasally infected mice [346, 347]. *Sp* grown *in vitro* as a biofilm show increased *ply* transcription and produced high levels of PLY [349, 350], suggesting that *Sp* biofilms developed in the respiratory mucosa might also produce high levels of PLY. The PLY-induced mucosal inflammation promotes increased bacterial shedding in secretions and promotes transmission in an infant mouse model of *Sp* infection [289]. PLY is also a key molecule in the progression of *Sp* infection from the nasopharynx to neighboring tissues. A PLY-deficient mutant causes mild histopathological changes and lower middle ear bacterial loads in chinchilla OM model [351, 352]. In guinea pig infection models, PLY directly damages cochlear hair cells [353, 354]. In addition, PLY intracochlear perfusion induces a strong cytotoxic effect possibly related to pore formation at the PM of inner hair cells (**Figure 1B**) [355, 356]. PLY also plays a role in pneumococcal ocular infections causing endophthalmitis and keratitis [357, 358]. Intravitreally injection of PLY causes inflammation and tissue damage [359, 360], likely related to PLY-induced corneal epithelial cell lysis (**Figure 1C**) [361].

In the lungs, PLY targets pivotal cell types during acute injury at the early phases of pneumonia and becomes a potent inducer of inflammation, facilitating damage of the respiratory epithelial barrier and host tissue penetration (**Figure 1D**). Purified PLY damages bronchial and alveolar epithelium and slows human ciliary beating *in vitro* [362]; it damages alveolar epithelial and endothelial cells impairing barrier function [363, 364]; and causes platelet destruction thus inhibiting platelet thrombus formation [365, 366]. Anti-PLY polyvalent antibodies were reported to inhibit PLY-mediated platelet annihilation and were proposed to be of pharmacological interest to treat *Sp* community-acquired pneumonia [365, 366]. In addition, PLY can compromise effective immune response in the lungs through several mechanisms: it induces the necroptosis of alveolar macrophages [367-369], triggers caspase-dependent cell death of dendritic cells [370, 371], promotes neutrophil transepithelial cell migration and elastase release, thus impairing macrophage phagocytosis, and damages epithelial cells [372-374]. The pro-inflammatory role of PLY is discussed in detail in section 4.

If not controlled in the lungs, the bacteria can reach the bloodstream and cause IPD (**Figure 1E**). In the heart, sub-lytic doses of PLY disrupt the PM of cardiomyocytes leading to the influx of Ca^{2+} , membrane depolarization and induction of ER stress, which together cause alterations in cardiac rhythm and depression in contractile force. At higher concentrations, PLY causes cardiomyocyte cell death, inhibiting cell contractility and promoting extensive cardiac damage which may lead to microlesion formation and cardiac remodeling likely associated with long-term adverse cardiac events described in IPD patients (**Figure 1F**) [310, 375, 376]. *Sp* biofilms, established in the heart of infected mice, release PLY causing rapid macrophage killing and impairing cytokine production [377].

During brain infection, PLY induces neuronal cell death [378, 379] and targets astrocytes, rearranging cytoskeleton and altering astrocyte cell shape, which leads to remodeling of brain tissue, astrocytic retraction, and cortical astroglial reorganization (**Figure 1G**) [380-382]. PLY-dependent astrocyte cell death is mediated by connexin 43 (Cx43), a gap junction protein forming hemichannels, that amplifies ATP release and cytosolic Ca^{2+} influx, ultimately leading to astrocyte depletion and blood-brain barrier destabilization [379]. PLY also targets other cells in the brain promoting endothelial cell disruption [383], limiting microglia motility [384], and decreasing cilia beating frequency in ependymal cells [385-387], which correlates with loss of cilia and damage of ependymal ultrastructure described in rat meningitis model [388].

3. **PLY triggers multiple cellular responses: irreversible damage or repair**

Upon interaction with cells, PLY interferes with a plethora of signal transduction pathways to induce multiple and antipodal cellular responses that define the pathogenesis in a whole organism (**Figure 3**). Such responses are cell-type specific and are dependent on PLY concentration, which correlates with the extent of the PM damage and of the intracellular Ca^{2+} overload. Activation of different cellular responses can tip the balance towards activation of cell death or cell survival pathways, thus promoting tissue damage or repair, respectively. At lytic concentrations, PLY triggers irreversible damage and cell death by causing massive mitochondrial damage, excessive inflammation, and tissue injury. In contrast, at sub-lytic amounts, PLY activates repair pathways triggering PM remodeling, cytoskeleton reorganization, and, by transiently activating MAPK, cell survival.

3.1. Mitochondrial damage

The role of PLY in mitochondrial damage was first described in primary rat neurons infected with *Sp* or incubated with culture supernatants, which both induce the Ca^{2+} -dependent mitochondrial release of apoptosis-inducing factor (AIF), a likely manifestation of massive mitochondrial damage (**Figure 3, left**) [389]. In addition, PLY was shown to trigger mitochondrial swelling, loss of mitochondrial membrane potential, and impairment in mitochondrial metabolism [390, 391]. Electron microscopy analysis of PLY-intoxicated neurons show direct binding of PLY to the mitochondrial membrane [390]. In human alveolar epithelial cells, PLY also induces dramatic morphological alterations of the mitochondria, which are accompanied by cytosolic Ca^{2+} overload, reduction in ATP levels, membrane depolarization, increased mitochondria permeability and release of mitochondrial DNA (mtDNA) into the cytosol (**Figure 3, left**) [391]. mtDNA can then be released extracellularly through microvesicle shedding and act as a danger signal, thus contributing to inflammation (**Figure 3, right**) [391]. In macrophages, cytosolic mtDNA released by PLY-damaged mitochondria is recognized by STING and upregulates the expression and secretion of IFN- β , demonstrated both *in vitro* and *in vivo* in lungs of *Sp* infected mice [392].

3.2. Interactions with the host cytoskeleton

PLY was reported to bind and promote actin polymerization *in vitro* [254] and was shown to interact with actin filaments underneath the PM of astrocytes [393] or exposed at the surface of damaged neuronal cells (**Figure 3, left**) [378]. In fact, PLY D4 domain interacting with cholesterol in the PM of neurons enables β -actin exposure to the outer surface of the cell, facilitating *Sp* adhesion and invasion and increasing cell death [378]. In addition, in those cells, PLY induces cytoskeleton instability depolymerizing intracellular actin filaments likely due to increased Ca^{2+} levels [378]. How these data relate to the PLY intrinsic ability to polymerize actin *in vitro* needs further investigation, and an understanding of the consequence of PLY-mediated *Sp* interactions with the actin cytoskeleton may require kinetic analysis of the infection in relevant models.

PLY also bundles and stabilizes host cell microtubules during intoxication of neuronal cells and during pneumococcal meningitis in rabbit infection models (**Figure**

3, left) [381]. Consistent with increased microtubule stabilization, high levels of acetylated tubulin were found in both the cell culture and animal model [381]. PLY-triggered microtubule stabilization is comparable to pharmacological microtubule-stabilizing agents (e.g., taxol) and has been suggested to perturb axonal transport, likely contributing to neuronal damage during pneumococcal meningitis [381]. PLY-induced microtubule perturbations are dependent on cholesterol binding and require Src kinase activity but are independent of Ca^{2+} influx and actin remodeling [381].

Several reports, primarily in neuronal cells, suggest that at low concentrations, PLY interaction with host PM causes rapid cytoskeleton remodeling which translates into cell shape changes (**Figure 3, right**). Alterations in the brain structure detected in infected rats were associated with PLY-mediated astrocytes retraction and reshaping of focal adhesions, which are underlined by cytoskeleton reorganization [380, 382]. In primary mouse astrocytes and neuronal cells, sub-lytic concentrations of PLY binding to cholesterol promotes the formation of actin stress fibers, filopodia, and lamellipodia through the activation of RhoA and Rac1 GTPases (**Figure 3, right**) [254, 393].

3.3. Activation of cell survival pathways

PLY binding to the PM triggers activation of cell survival pathways such as the mitogen-activated protein kinase p38 (p38/MAPK) [394, 395], which is a conserved response to sub-lytic doses of PFTs dependent on K^+ efflux [396], that can stimulate cell survival pathways [395]. In epithelial cells, activation of p38/MAPK by sub-lytic concentrations of PLY [397, 398] triggers the production of pro-inflammatory cytokines (e.g., IL-8) to attract neutrophils, thus promoting an effective immune response early in infection when bacterial numbers are low (**Figure 3, right**) [397]. In macrophages, PLY-induced p38/MAPK activation and cytokine production take place at the PM upon pore formation [397, 398] and has been suggested to promote *Sp* clearance [399]. Later in infection, following *Sp* uptake by macrophages, PLY induces the trafficking of pneumococcal cell wall components to the host cell cytosol, presumably through phagosomal damage [400]. If p38/MAPK activation is blocked, leakage of *Sp* cell wall components to the host cell cytosol is exacerbated and results in macrophage cell death. Thus, PLY-induced p38/MAPK activation protects phagosomal integrity and limits the release of bacterial components, likely modulating

the recognition of pathogen-associated molecular patterns by the host surveillance systems (**Figure 3, right**) [400, 401].

The timing of the MAPK response to PFTs can influence tissue injury. Although MAPK activation can promote cell survival responses upon intoxication by PFTs, its subsequent modulation by protein phosphatases PP1 and PP2A, observed in epithelial cells [398], can prevent excessive inflammatory responses that could lead to irreversible tissue damage. Conversely, the transient nature of p38/MAPK activation might not be sufficient to block cell death triggered by the recognition of *Sp* components in the macrophage cytosol [400].

Finally, in some cell types, activation of p38/MAPK is detrimental. In SH-SY5Y neuronal cells, p38/MAPK is associated with increased neuronal cell death and neurotoxic effects [402], and in microglial, PLY-mediated MAPK activation increases ROS production and promotes senescence (**Figure 3, left**) [403].

3.4. Activation of plasma membrane repair mechanisms

Supernatants from stationary phase cultures of *Sp* produce sufficient amounts of PLY to permeabilize cells [100]; however, during infection, the majority of these perforated cells are able to recover from damage and survive. PLY pore assembly renders host cell PM permeable to ions and small molecules [394, 404, 405]. An increase of Ca^{2+} concentrations to above $20\mu\text{M}$ impairs host-cell signaling and engages cell death pathways [394, 406]. However, an initial increase in intracellular Ca^{2+} levels act as a danger signal and activates PM repair mechanisms to prevent cell lysis [397, 402, 407-409]. Thus, at otherwise sub-lytic PLY concentrations, reduced extracellular Ca^{2+} enhances PLY toxicity and result in lysis [410, 411]. In the presence of extracellular Ca^{2+} , PLY-induced Ca^{2+} influx triggers the recruitment of annexins, cytoplasmic Ca^{2+} responsive proteins that bind to negatively charged phospholipids at the sites of PM injury [113]. Annexin A2, which displays the highest Ca^{2+} sensitivity, is the first to translocate to the site of damage, followed by annexin A6 and A1 (**Figure 3, right**). Annexin translocation fosters the formation of plasmalemmal nanotubes at the pore site that culminates in the release of microvesicles enriched in PLY, annexins, actin-binding and Ca^{2+} regulated proteins, and ESCRT components (**Figure 3, right**) [100, 411], thus shedding the PLY pore and modulating the rise in intracellular Ca^{2+} [406]. This repair process has potential immunological implications for *Sp* infection, as PLY-

containing microvesicles induce macrophage polarization that enhances the immune response towards molecular patterns of Gram-positive bacteria [118]. By contrast, in alveolar epithelial cells, human lung explants and infected mice, *PLY* mediates the release of microvesicles containing mitochondrial cargo that, when uptake by neutrophils suppress their ability to release ROS and thus impair efficient immune response against *Sp* [119]. The differential *PLY* sensitivity of immune cells is thought to be due to differential efficiency in PM repair [111]. Myeloid cells, the first-line defenders, show enhanced shedding of *PLY*-containing microvesicles and increased resistance to *PLY*. In contrast, lymphoid cells are enriched in cholesterol-containing lipid rafts, which facilitate *PLY* binding and pore formation, and are impaired in microvesicles formation and PM resealing activity, leading to high *PLY* susceptibility [111].

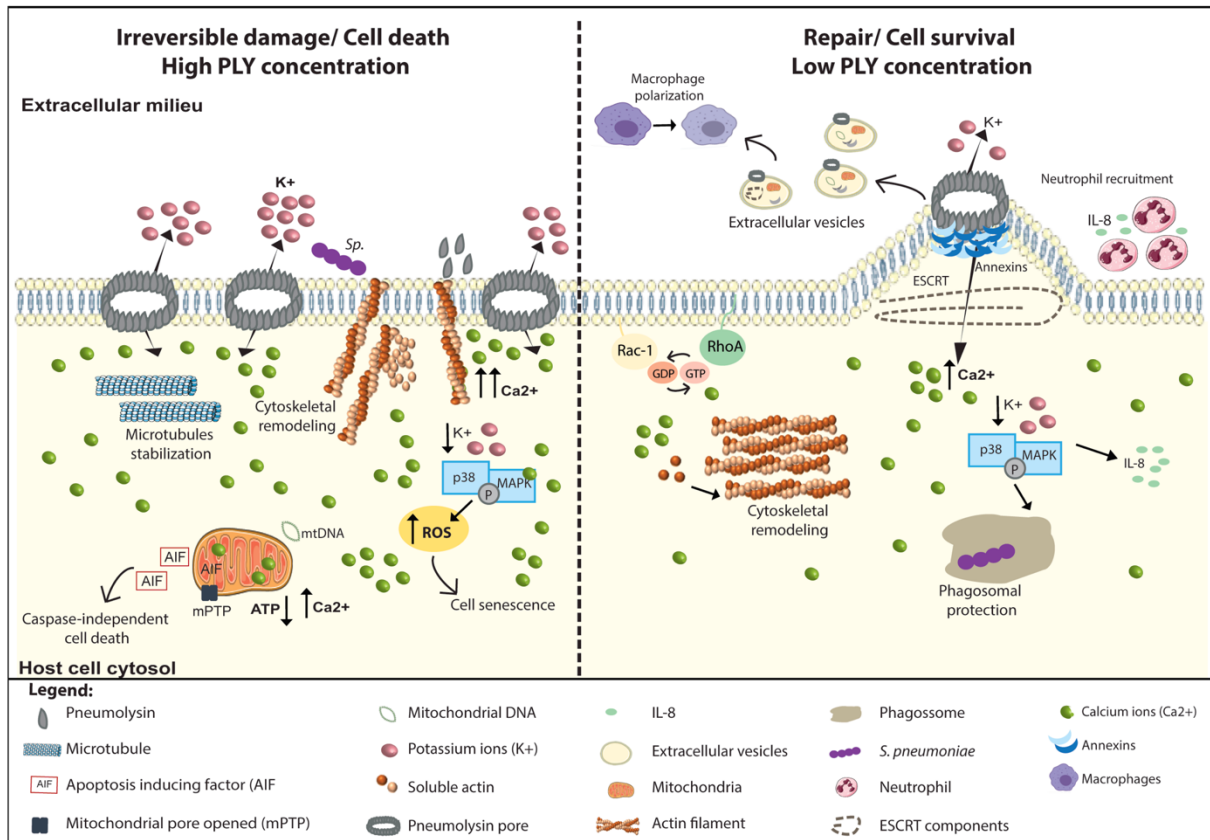


Figure 3- *PLY* is a trigger for multiple cellular responses. *PLY* interacts with cells and, depending on *PLY* concentration and the intracellular Ca²⁺ levels, induces a variety of antipodal cellular responses that can lead to irreversible damage or the induction of cellular repair mechanisms. Left Panel: At lytic amounts, the overwhelming increase in intracellular Ca²⁺ levels induces the surface exposure of actin which facilitate *Sp* adhesion and invasion,

increasing cell death. In addition, PLY-mediated microtubule stabilization may perturb axonal transport, likely contributing neuronal damage. Also, in neuronal cells, p38/MAPK activation is detrimental for the host cell as it increases ROS production and induces senescence. High PLY concentrations cause irreversible mitochondrial damage by inducing swelling, loss of mitochondrial membrane potential, and morphologic and metabolic alterations. Concomitantly with Ca^{2+} overload, mitochondrial permeability increases, the ATP levels decrease, and mitochondrial DNA is released into the cytosol. Following these events, the mitochondrial apoptosis-induced factor (AIF) reaches the cytoplasm and activates caspase-independent cell death. Right Panel: At sub-lytic amounts, the influx of limited amounts of extracellular Ca^{2+} triggers the sequential recruitment of annexins to the damaged sites where they assemble in 3D arrays to clog the PM pore. Increased intracellular Ca^{2+} also induces cytoskeleton remodeling through the activation of small GTPases Rac1 and RhoA and triggers PM rearrangements culminating in PM blebbing and ESCRT-mediated release of microvesicles containing PLY, annexins, actin-binding and Ca^{2+} regulated proteins, ESCRT components and mitochondrial DNA among others. Released microvesicles promote survival by eliminating the pore, transporting danger signals and enhancing immune responses. In response to K^{+} efflux cell survival pathways such p38/MAPK are activated and stimulate the production of pro-inflammatory cytokines such as IL-8, promoting neutrophil recruitment, and enhancing phagosomal integrity, thus limiting the release of toxic bacterial components into the cytosol.

4. PLY interactions with the immune system: pro- or anti-inflammatory responses

PLY can either exacerbate or mitigate damage during infection depending on the state of the immune system as well as the infection site, dose, timing, and interacting host cell type [118, 302, 307]. It can trigger inflammation-mediated tissue damage and promote bacterial dissemination [294, 412, 413]. However, PLY can elicit host-protective responses in the innate and adaptive branches of the immune system, including activation and stimulation of cytokine production by macrophages, neutrophils, endothelial, epithelial, and dendritic cells [298, 414-416]. The multifaceted nature of PLY-induced immune responses may account for some of the disparate outcomes of infection by PLY-deficient strains in different infection models (**Figure 4 and Table 1**). This also reflects the dual relationship of *Sp* with host cells, sometimes invasive and inducing severe disease, and other times remaining localized as an asymptomatic colonizer (as discussed in sections 1 and 2.4.).

4.1. Pro-inflammatory properties

4.1.1. Epithelial cell activation

Epithelial cells are a first line of defense against *Sp*, in part by functioning as a physical barrier against invasion and, in the lower respiratory tract, maintaining the mucociliary elevator that removes microbes from the lung. PLY both disrupts epithelial cell junctions, compromising this barrier, and interfering with the number and organization of cilia in human and mouse airway epithelium-derived air-liquid interface organoid cultures [417, 418], crippling the mucociliary elevator. In addition, as the sentinels for infection, the mucosal epithelium produces a range of antimicrobial peptides and pro-inflammatory signaling molecules to eliminate invading pathogens and alert immune cells to respond to invasion. PLY induces β -defensin 2 secretion in A549 human lung epithelial cells and in human middle-ear cells [419]. PLY, is also a potent activator of cytokine and chemoattractant secretion through pore-forming insult to airway epithelial cells (**Figure 4, left**) [373, 397, 420]. PLY induces epithelial cell release of IL-1 β , TNF- α [420], and the neutrophil chemoattractants IL-8 [397] and heparin A₃ (HXA₃) [373, 421, 422]. Accumulation of inflammatory cytokines and chemokines facilitate the recruitment of leukocytes responding to and clearing *Sp*. Nevertheless, in both a lung epithelial tissue culture and a mouse pulmonary infection model, HXA₃-mediated basolateral-to-apical neutrophil transmigration exacerbates damage to epithelial barrier and promotes lethal *Sp* systemic spread [423, 424].

4.1.2. Neutrophil activation

As described above, neutrophil infiltration and the associated epithelial damage promote bacterial dissemination into the bloodstream, resulting in lethal systemic disease [423]. While neutrophil recruitment to the lungs during early stages of infection aids *Sp* clearance, overtime, reduction in adenosine production by lowered CD73 expression correlated with loss of neutrophil antimicrobial efficacy [425]. Extracellular ATP is shown to neutralize PLY-mediated neutrophil activation and may explain this shift in neutrophil response profile [426]. Excessive neutrophil activities can be harmful to the host [412]. Indeed, the prolonged accumulation of PMNs in a murine model of pneumococcal pneumonia results in increased burden and high tissue damage [425]. Upon extravasation and encountering invading bacteria,

exposure of PMNs to PLY can trigger the release of additional neutrophil-recruiting signals, including Ca^{2+} -dependent increases in IL-8, f-MLP, phospholipase A₂, prostaglandin E₂, and leukotriene B₄ [427, 428], reinforcing neutrophil-direction inflammation. Neutrophils at the site of infection can be further activated by *Sp* and PLY to produce reactive oxygen species (ROS) [372, 414, 429], degranulate, and form neutrophil extracellular traps (NETs) (Figure 4, left).

ROS have multiple immune-modulatory functions during bacterial infection: in addition to direct antimicrobial activity and signaling to modulate immune cell function, ROS can also cause damage to host cells and tissues [430]. Interestingly, pretreating neutrophils with PLY promotes sensitivity to f-MLP [429], which conditions neutrophils to release higher and more sustained levels of ROS, and become more prone to degranulation (Figure 4, left) [414].

During degranulation, neutrophils secrete proteolytic enzymes capable of propagating tissue damage. These include serine proteases neutrophil elastase (NE), cathepsin G and proteinase-3, and neutrophil metalloproteinases (MMPs). PLY can trigger NE release either by degranulation of primary granules or by direct cell lysis [372, 414]. Although NE degrades pneumococcal cell wall-localized aminopeptidase N and can facilitate opsonophagocytic killing in the presence of an antiphagocytic capsule [372, 431-433], excessive NE activity is detrimental to the host [434]. NE has potent catalytic activity against a range of extracellular matrix proteins, including elastin, proteoglycan, fibronectin, and several collagen types [435], surfactant proteins [434], and alveolar epithelial cell junction protein such as E-cadherin [436]. In addition, NE stimulates the lung epithelium to release proinflammatory cytokines and induces epithelial apoptosis [437-439]. Severe pneumonia in human patients and experimental animals is associated with increased NE levels in lung bronchoalveolar lavage fluid [440, 441] and plasma [442]. NE may thus be a critical inducer of epithelial permeability during pneumococcal pneumonia, compromising lung epithelial barrier and promoting bacterial dissemination [443]. Cathepsin G and proteinase-3, which like NE are stored in azurophilic granules, also contribute to infection-related lung injury, albeit to a much lesser degree than NE [444]. Metalloproteinases (MMPs) are neutrophil granule components that are also released following the increase in cytosolic Ca^{2+} levels resulting from PLY-mediated pore formation and can be augmented by concurrent stimulation by f-MLP [445]. MMPs, especially MMP-8 and -9, have been

correlated with tissue injury in pneumococcal pneumonia and meningitis [446-448]. Thus, the PLY-mediated release of neutrophil granule components contributes to inflammation and pathogenesis, especially during severe forms of *Sp* disease.

Finally, pneumococcal capsule and PLY work in synergy to promote neutrophil extracellular trap (NET) production [434], a vital neutrophil effector function for trapping and killing extracellular microbes (**Figure 4, left**). While NETs exhibit significant antibacterial activity against *Sp* [449], their efficacy is counteracted by *Sp*-secreted endonucleases EndA and TatD that digest NETs and facilitate escape [434]. NETs are intercalated with NE and other proteases that can damage host cells just as they can a pathogen, and excessive NETosis against *Sp* is implicated in promoting lung injury [450], sepsis [451], and increasing mortality [452]. PLY is thus a potent activator of multiple neutrophil effector functions, most of which can propagate inflammatory tissue damage in the lung and are associated with severe pathology.

4.1.3. Inflammasome activation

PLY induces inflammasome-dependent IL-1 β secretion and initiates the pro-inflammatory cascade associated with *Sp* infection [453-455]. PLY-mediated K⁺ efflux, which is sufficient to trigger NLRP3 activation [456], results in caspase-1 cleavage and the consequent production and secretion of IL-1 β [453, 457]. An alternative inflammasome activation pathway aided by PLY is the AIM2 inflammasome pathway. AIM2 senses double-stranded DNA from lysed bacteria, which can localize to host cell cytosol after PLY-mediated phagosomal membrane disruption [458, 459]. PLY-deficient *Sp* induces less inflammasome-dependent cytokines production (including IL-1 α , IL-1 β , and IL-18), which influence downstream cytokine and chemokine responses to *Sp*. Indeed, inflammasome-dependent cytokines promote secretion of IL-17A, IFN- γ , and neutrophil chemoattractants, each of which aids bacterial clearance [401, 454, 460]. Inflammasome activation is host protective in mouse and human macrophages *ex vivo* and in mouse pneumococcal infection models [454, 460, 461]. *Sp* strains impaired in inflammasome activation, including some serotype 1, serotype 8, serotype 7F, and strains expressing the nonhemolytic allele 5 of PLY, tend to cause more invasive disease [461-463] and chronic infection [293]. In contrast, in pneumococcal meningitis models, PLY-dependent inflammasome activation increasing neurotoxicity and pathology [464], possibly because PLY-triggered inflammasome

activation results in rapid pyroptotic death in microglial cells [465]. Thus, while inflammasome activation is in general a protective response against *Sp* infection, tissue types such as the brain can be susceptible to pathologies due to pyroptotic death (**Figure 4, left**).

4.1.4. Macrophage activation

PLY activates macrophages to release of IL-1 β , IL-6, TNF- α , IFN- β , IL-23, granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage migration inhibitory factor (MIF), and nitric oxide (NO) [466-469]. Many of these pro-inflammatory and chemotactic responses rely on p38 or inflammasome activation. Some PLY-induced macrophage responses require additional *Sp* factors as co-stimulants [470]. For instance, phagosomal membrane disruption by PLY allows for pneumococcal DNA to enter the cell cytoplasm, triggering cytosolic DNA sensors for IFN- β production [392]. PLY can also modulate macrophage activation when sequestered into vesicles shed during membrane repair; interaction with PLY-containing vesicles increases macrophage IL-1 β , TNF α , CCL5, CCL8, and CCL1 [118].

In the monocyte-derived THP-1 cell line, PLY is responsible for the majority of gene modulations upon exposure to *Sp* [466]. Upregulated genes include those encoding proinflammatory molecules such as IL-8 and monocyte chemotactic protein 3 (MCP-3), and cell surface receptors that impact inflammation, such as macrophage inflammatory protein 1 β (MIP-1 β), IL-2 receptor β (IL-2R β), IL-15 receptor α (IL-15R α), and interferon receptor 2, promoting pro-inflammatory cytokine propagation (**Figure 4, left**).

4.1.5. Necroptosis of multiple cell types

PLY induces necroptosis, an inflammatory pathway of programmed cell death, in the lungs [369] and heart [471]. In the lungs, PLY-mediated necroptosis of alveolar macrophages [369] and epithelial cells [369] is triggered in response to loss of ion homeostasis, ATP depletion, and ROS generation [369], and depends on the phosphorylation of MLKL [268], a master effector of necroptosis. The rapid depletion of alveolar macrophages by necroptosis may greatly contribute to extensive lung damage (**Figure 4, left**). In the heart, PLY-mediated necroptosis occurs in macrophages that infiltrate the infected myocardium and in cardiomyocytes, thereby suppressing

the anti-*Sp* immune response at that site [471] and contributing to cardiac injury [472]. The use of pharmacological inhibitors of necroptosis reduces acute injury in the lungs and heart during *Sp* infection, providing a novel therapeutic target to overcome infection [472]. Interestingly, PLY-induced necroptosis was also reported in nasopharyngeal epithelial cells during *Sp* asymptomatic colonization and was associated with the increased production of anti-pneumococcal antibodies [473], suggesting a role in the development of protective immunity against *Sp* and highlighting the infection context dependent nature of PLY-host interactions.

4.2. Anti-inflammatory properties

4.2.1. Epithelial cell tolerization

As a prerequisite to becoming a deadly pathogen, *Sp* asymptotically colonizes the human nasopharynx. This immune-silent colonization and long-term *Sp* carriage are thought to be sustained by anti-inflammatory responses, which prevent the disruption of the epithelial barrier and the infiltration of neutrophils [474]. After nasopharyngeal inoculation of 10^5 *Sp* in mice, a dose that results in asymptomatic carriage, the production of PLY is associated with higher levels of the immunosuppressive cytokine TGF- β 1 and immunomodulatory T regulatory cells (Tregs) and lower levels of neutrophil recruitment (**Figure 4, right**). *Sp* infection of human nasopharyngeal epithelial cells and fibroblasts resulted in PLY-dependent secretion of TGF- β 1. Treatment of these cells with purified PLY likewise results in TGF- β 1 secretion [474]. In contrast, compared to the 10^5 dose, nasopharyngeal inoculation of 10^7 *Sp*, which leads to bacterial clearance, results in the production of lower levels of TGF- β 1 and Tregs, and higher levels of IFN- γ and neutrophil infiltration [474]. These findings indicate that PLY is capable of fostering nasopharyngeal carriage by limiting proinflammatory responses.

Interestingly, compared to other bacterial respiratory pathogens, such as *Haemophilus influenzae*, *Sp* induced cytokine production by epithelial cells *in vitro* appears delayed [420], consistent with the low numbers of infiltrated neutrophils detected in early stages of lobular pneumonia in human patients, despite bacterial load [475]. At these early infection stages, PLY promotes the expression of MAPK phosphatase 1, which dephosphorylates p38 to suppress TNF- α production [476] (**Figure 4, right**). Later during infection, secretion of proinflammatory cytokines by

epithelial cells increase [420]. This delay in the initiation of epithelial cell-mediated inflammation may delay immune cell infiltration and provide *Sp* with a window of unchecked growth to establish infection. In addition, *PLY* downregulated many binding receptors that aid *Sp* detection by macrophages, including complement component receptor 2/CD21, platelet-activating factor acetylhydrolase, and oxidized low-density lipoprotein receptor 1 (OLR1). Thus, on top of directly targeting epithelial cells for tolerization, *PLY* evades surveilling immune cells by downregulating critical receptors for *Sp* recognition.

4.2.2. MRC-1 as a mediator of anti-inflammatory response

As mentioned, *PLY* was recently described to directly interact with MRC-1, which is expressed by DCs and alveolar macrophages, two cell types that represent a first line of defense mounted against *Sp* in the lungs [340]. The *PLY*-MRC1 interaction impairs inflammatory response, limiting inflammatory cytokine secretion through the cytokine suppressor SOCS1, as well as neutrophil infiltration (**Figure 4, right**) [340]. Consistent with its role as a phagocytic receptor, MRC-1 expressed on DCs binds to sub-lytic concentrations of *PLY* and promotes internalization of *PLY*-producing *Sp*. In mouse alveolar macrophages, internalized *PLY*-producing *Sp* colocalizes with MRC-1, whereas *PLY*-deficient *Sp* colocalizes with lysosomes (**Figure 4, right**). Upon pulmonary challenge of mice, *PLY* production is associated with lower levels of TNF- α and greater numbers of bacteria in lavage fluid. Similarly, genetic ablation or antibody inhibition of MRC-1 results in higher levels of TNF- α and lower bacterial load [340].

These findings opened new therapeutic strategies to fight pneumococcus infection. Indeed, molecular docking approaches identified MRC-1-derived peptides that neutralize *PLY*-MRC-1 interaction that impair *Sp* internalization and promote pathogen killing by autophagy [477]. Furthermore, MRC-1-derived peptides were shown to inhibit *PLY*-driven cell lysis, inflammation, and lung epithelium damage by limiting *PLY* interaction with cells and decreasing IL-8 and TNF- α secretion [477]. In zebrafish and mouse models of pneumococcal infection, MRC-1-derived peptides reduce disease development, promote host survival, and decrease bacterial burden [477].

4.2.3. Triggering of autophagic processes

Autophagy is a natural self-degradative mechanism that is activated in epithelial and immune cells to degrade cytoplasmic content. Autophagy also plays major roles in pathogen elimination controlling both inflammation and adaptive immune response [478]. *Sp* activates autophagy, which functions as a host protective mechanism by promoting *Sp* clearance [479]. As anticipated by a membrane-damaging agent, PLY was shown to initiate autophagy in a variety of cells such as human alveolar epithelial cells [479], murine microglia [465], and osteoblast cells [480]. ROS generation triggered by PLY leads to the inhibition of the PI3K/AKT/mTOR pathway and the consequent activation of canonical autophagy [465, 479], which degrades intracellular *Sp* and limits infection in host cells [479]. In murine microglia, autophagy activated by *Sp* transiently blocks caspase-1 activation and IL-1 β secretion, delaying pyroptosis, the caspase-1-dependent inflammatory cell death pathway [465]. In these cells, *Sp* infection increases the expression of autophagy-related genes in the early phase of infection as a host protective mechanism before pyroptosis and concomitant extensive tissue damage can occur (**Figure 4, right**). However, at later stages of infection, sustained PLY-mediated high-level ROS generation activates caspase-1 and microglia pyroptosis [465]. In osteoblasts, PLY-mediated activation of autophagy impairs differentiation by regulating the expression of differentiation-related genes, which require mTOR signaling [480], a finding with potential relevance to *Sp* osteomyelitis. Finally, PLY induces selective autophagy promoting the delivery of *Sp* entrapped in autophagosomes to lysosomes for further degradation [481]. In fibroblasts, PLY triggers (LC3)-associated phagocytosis (LAP), followed by canonical autophagy activation, suggesting a hierarchical autophagy activation process leading to *Sp* clearance [482]. In fibroblasts, the activation of canonical autophagy is required for *Sp* degradation, but in bone-marrow-derived macrophages (BMDMs), PLY-induced LAP is sufficient for bacterial clearance, allowing degradation to occur much more rapidly [483]. In aged BMDMs, LAP is compromised, and cells display reduced *Sp* killing capacity and increased expression of proinflammatory cytokines (**Figure 4, right**) [483].

4.2.4. Complement subversion

Complement activation has a crucial role in host protection against pathogens, but *Sp* utilizes PLY-mediated complement activation to sequester complement

components away from *Sp* surface, thus protecting *Sp* from host defenses and facilitating bacterial spread and survival. Purified PLY activates the human complement cascade *via* the classical pathway independently of its lytic activity [341, 484]. Homology domains shared with CRP, mediate the PLY binding to the Fc portion of immunoglobulins, which in turn recruit and activate C1q, and are required for PLY-triggered complement activation [341, 342]. While in serum from C1q KO mice PLY fails to trigger complement activation, in human C1q-depleted serum, PLY still activates the complement and C3b deposition is observed [485], suggesting that in humans, PLY may stimulate complement through C1q-independent pathways that directly target C3. PLY was further shown to trigger lectin pathway by binding to L-ficolin with high affinity [485]. However, PLY-triggered complement activation decreased the serum opsonic activity for *Sp* both *in vitro* and *in vivo* in mouse models, reducing bacterial uptake by neutrophils and impairing the recruitment of T-cells to the sites of infection in the lung [341, 343, 484].

4.2.5. Induction of apoptosis

Pathogen-induced apoptosis, a non-inflammatory programmed cell death pathway, plays an important role in tissue damage caused by infectious diseases and constitutes an important mechanism of protection from invasive disease. PLY cytolytic activity can prompt cells to engage in apoptosis by different mechanisms depending on PLY concentration and the cell type involved (**Figure 4, right**). One of the first studies connecting PLY to apoptosis showed that in neurons, PLY pore-forming activity triggers Ca^{2+} influx and mitochondrial damage which results in the release of pro-apoptotic factor (AIF) and induces apoptosis in a caspase-independent manner [389, 390]. The same mechanism was described in brain microvascular endothelial cells, inner cochlear hair cells, and DCs [356, 370, 486]. In addition, intracellular PLY induces caspase-dependent apoptosis in *Sp*-infected human dendritic cells, whereby blocking their maturation and the production of inflammatory cytokines, thus inhibiting dendritic cell-mediated inflammatory responses [371]. Caspase-dependent apoptosis, triggered by PLY-induced phagolysosomal membrane permeabilization, also occurs in human monocyte-derived macrophages [487, 488]. In endothelial cells, PLY-triggered caspase activation and apoptosis depend on the activation of p38/MAPK and suppression of extracellular signaling regulation kinase (ERK)1/2 [489]. Further

supporting *PLY*'s ability to trigger apoptosis, in human epithelial lung alveolar cells *PLY* causes double-stranded DNA breaks, which leads to cell cycle arrest followed by non-homologous end-joining DNA repair or, if DNA damage persists, by engagement in apoptosis [490]. Clinical isolates deficient for *PLY* are unable to induce DCs apoptosis and trigger a strong proinflammatory response leading to excessive lung inflammation [371]. Thus, the deleterious effect of *PLY* on immune cells, blocking their maturation, inhibiting the secretion of inflammatory cytokines, and inducing apoptosis, promotes bacterial evasion from immune detection and allows for immune-silent colonization.

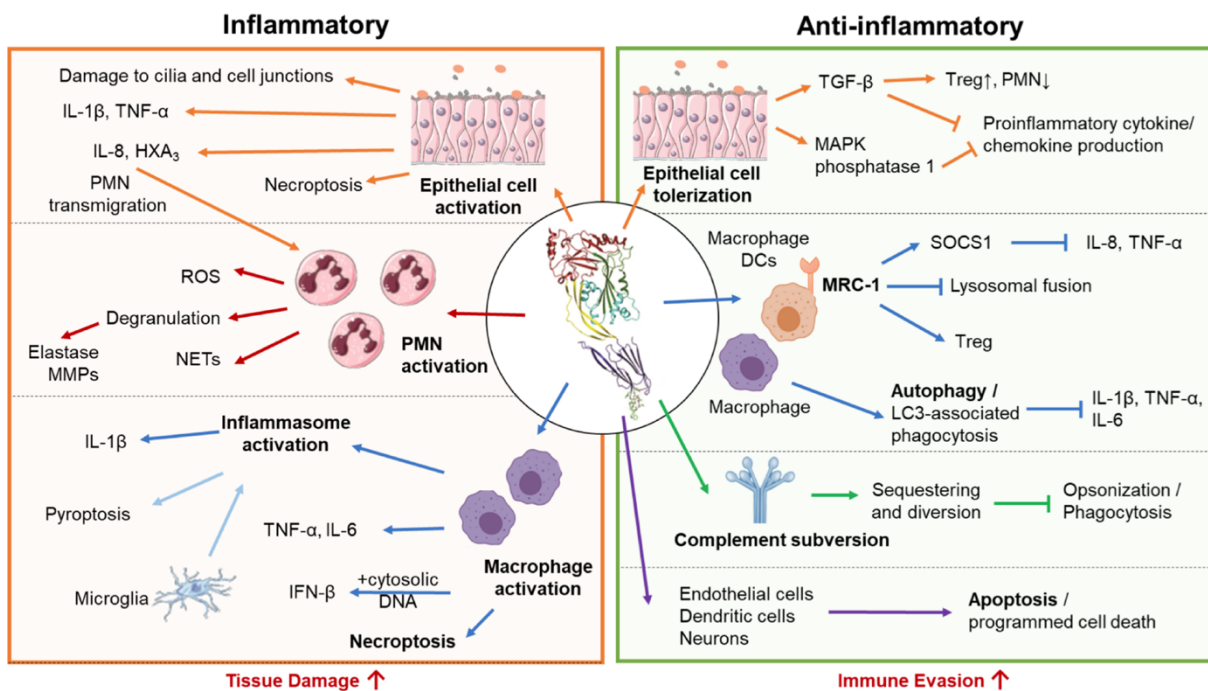


Figure 4– Pro-inflammatory and anti-inflammatory host responses to *PLY*. *S. pneumoniae* triggers both pro-inflammatory and anti-inflammatory responses depending on interacting host cell type and infection context. Left Panel: In excess, pro-inflammatory actions of *PLY* enhance tissue damage and promote bacterial spread. In epithelial cells, *PLY* induces production of pro-inflammatory cytokines and chemokines, promoting neutrophil transmigration and compromising epithelial barrier function. *PLY*-stimulated neutrophils engage in a wide range of effector functions, including degranulation, reactive oxygen species (ROS) production, and neutrophil extracellular trap (NET) release, many of which propagate inflammatory tissue damage and are associated with severe pathology in the lung. *PLY* can activate macrophages independently or in conjunction with other co-stimulants to cause the release of pro-inflammatory cytokines and chemokines. Macrophages can also be activated by

PLY-dependent inflammasome activation, which in bone marrow-derived macrophages leads to IL-1 β -mediated inflammation, and in microglia, pyroptotic cell death. PLY is also a potent inducer of macrophage necroptosis, often leading to acute tissue injury. Right Panel: Anti-inflammatory activities of PLY downregulate immune responses and may aid bacterial evasion. During pneumococcal colonization and early stages of lung infection, PLY suppresses inflammatory cytokine production by airway epithelial cells and enhances recruitment of T regulatory cells, promoting unchecked bacterial colonization. Internalization of PLY by alveolar macrophages and dendritic cells via the mannose receptor MRC-1, or, in bone marrow derived macrophages, PLY triggered LC3- associated phagocytosis, suppress the production of inflammatory cytokines. To avoid complement mediated detection and opsonization, PLY acts as a decoy molecule to sequester complement proteins. Finally, PLY triggers apoptosis in a wide range of cell types, including endothelial cells, neurons, and dendritic cells, allowing for non-inflammatory removal of these cell types.

Table1. PLY-triggered pro- and anti-inflammatory immune modulations.

Nature of PLY-triggered immune response	Immune processes activated	Target host cell or factor type	Specific actions	Effect on infection outcome
Pro-inflammatory	Epithelial cell activation	Epithelial cells	Disruption to cilia organization and movement Disruption to cell junction complexes	Compromised airway epithelial barrier function
			Release of pro-inflammatory cytokines IL-1 β , TNF- α Release of chemokines IL-8, and HXA ₃	Increased tissue damage
	Neutrophil activation	Neutrophils	Release of chemokines IL-8, f-MLP, PLA2s, PGE2, LTB ₄ Increased neutrophil transmigration	Increased tissue damage and systemic spread

			Prolonged neutrophil accumulation	
			Enhanced pro-inflammatory secretory profile: ROS, serine proteases, MMPs Increased NETosis	Disrupted ECM, surfactants and cell junction proteins increasing lung permeability Increased systemic spread
	Inflammasome activation	Macrophage and dendritic cells	NLRP3 or AIM2 triggered inflammasome-dependent cytokine production: IL-1 α , IL-1 β , and IL-18	Enhanced bacterial clearance
		Microglia	Pyroptotic death	Increased neurotoxicity
	Macrophage activation	Macrophage	Enhanced pro-inflammatory secretory profile: IL-1 β , IL-6, TNF- α , IFN- β , IL-23, GM-CSF, MIF, NO, and pro-inflammatory exosomes	Enhanced bacterial clearance and immune cell recruitment
	Necroptosis	Tissue resident macrophages and cardiomyocytes	Alveolar macrophage depletion Cardiac macrophage depletion and cardiomyocyte death	Increased tissue injury
		Epithelial cells	Epithelial cell death	Anti- <i>Sp.</i> antibody production
Anti-inflammatory	Tolerization to colonization	Epithelial cell	TGF- β secretion Suppressed TNF- α production	Increased Treg activity and reduced neutrophil infiltration

				Increased nasopharyngeal colonization
			Downregulation of <i>Sp.</i> -detecting receptors: CD21, PAFR, OLR1	Nasopharyngeal colonization evading immune detection
	Anti-inflammatory polarization through MRC-1	Alveolar macrophage and dendritic cells	SOCS1-mediated suppression to TNF- α	Decreased cytokine production
			Decreased lysosomal fusion	Increased bacterial burden
	Autophagy	Epithelial cells, fibroblasts, and macrophages	Canonical autophagy and/or LC3-associated phagocytosis	Decreased cytokine production
				Increased intracellular bacterial clearance
		Microglia	Delay of caspase-1 activation and pyroptotic death	Decreased neurotoxicity
	Complement subversion	Complement proteins	Sequestration of complement components by binding to immunoglobulins (classical pathway) and L-ficolin (MBL pathway)	Decreased serum opsonic activity
	Apoptosis	Neurons, endothelial cells, cochlear hair cells, and dendritic cells	Mitochondrial damage-associated release of AIF	Decreased inflammatory cytokine production
		Epithelial cells	DNA damage-induced cell cycle arrest	Depletion of responding immune cells

		Macrophages and dendritic cells	Phagolysosome membrane disruption- induced caspase activation	
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5. Future perspectives

A better understanding of the complex “Yin” and “Yang” properties of PLY will facilitate the translation of critical *Sp* pathogenesis mechanisms into clinical intervention strategies. PLY stands as an attractive therapeutic target to complement classical antibiotic therapy, which, besides fostering the development of resistance, can trigger bacterial lysis and consequent release of PLY that alone can have deleterious effects on cells and the immune system [301]. Several repurposed drugs and PLY-neutralizing compounds have been investigated. Statins, which inhibit cholesterol production, administered prior to infection both *in vitro* or *in vivo* confer significant resistance to PLY cytotoxicity by impairing binding [491, 492]. Also, some natural compounds were shown to target the oligomerization process by binding to specific D3 and 4 residues, resulting in reduced cytolytic activity [493-497]. The use of artificial liposomes was also suggested as a way to sequester PLY. The administration of liposomes prior to pneumococcal infection reduced septicemia and invasive disease in a mouse model [443]. Recent efforts have been made to find novel therapeutic tools to inhibit PLY release, and recently, a study demonstrated that clarithromycin downregulates *ply* transcription *in vitro* and *in vivo* and consequently reduces PLY production by bacteria [498], proposing a new strategy to treat pneumococcal disease. The clinical potential of current Ply-neutralizing therapeutic strategies and their limitations have been extensively reviewed elsewhere [298, 301]. Since PLY is also a potent trigger for anti-*Sp* immune responses, harnessing the immunogenic potentials of PLY has long been of therapeutic interest. PLY-immunized mice displayed significantly increased survival upon infection, and thus suggested that PLY should be considered for inclusion in a human vaccine [499]. Further studies using different animal models aimed to obtain an active immunization using a genetic toxoid derivative of PLY, alone or in combination with other proteins [500-505]. In fact, PLY toxoid demonstrated potential effectiveness as an immunogenic component and in controlling bacteremia and bacterial colonization [500-506]. Phase I clinical trials

concerning the use of PLY toxoid vaccine formulations were performed in adults and children, and results demonstrated that it is well-tolerated and immunogenic when administered as individual protein vaccines or combined with capsule polysaccharide conjugates [501, 507, 508]. Experimental evolution studies revealed the emergence of variants that produce low levels of PLY showing decreased virulence but increased persistence [509]. Such lineages have been proposed to serve as starting point to the development of live-attenuated pneumococcal vaccines.

To use PLY-targeting therapies and treatment tailored to the stage and severity of pneumococcal infection, requires a better elucidation of the role of PLY in pathogenesis. As highlighted in this review, PLY induces multiple contradicting responses. The parameters that dictate the exact mechanisms triggered in the host cells during pore-formation are still elusive. It is known that PLY is present in circulation during early pneumococcus infection and sub-lytic doses induced toxicity and modulated host immune response; furthermore, host cells can trigger PM repair mechanisms to recover from damage and get rid of the toxin [394, 404, 407, 510]. However, it is still not clear which molecular mechanisms and host proteins are involved during the process of cell survival and the importance of these events in the context of infection. Cells can expel the toxin through vesicle shedding, but whether they can function as a danger signal to neighboring cells or as a modulator of the immune response remains unclear [100, 111, 118, 406]. PLY can also have several effects in intracellular signaling and modulate host cytoskeleton but remains uncertain how these events can help cells to survive and repair the damage [254, 380, 382, 393, 511]. Furthermore, the use of complex models like 3D cell culture or *in vivo* models should now be considered to clarify the crosstalk between different host cell types and their microenvironment. Insights into the mechanistic control for cellular response to PLY pores may also help resolve the apparent contradictory inflammatory and anti-inflammatory implications under different infection contexts. The discovery of new mechanisms of host PM repair and survival may bring new insights to the development of therapies against the injurious actions of PLY during pneumococcus infection.

Thesis aims and scope

The plasma membrane (PM) is a highly selective permeable barrier that protects cells against extracellular threats by physically separating the intracellular space from the outside cellular environment, thereby preserving cellular homeostasis. Pore-forming toxins (PFTs) represent a major class of bacterial virulence factors. Given their intrinsic capacity to create pores on the PM of host cells, PFTs disrupt cellular integrity, compromise epithelial barriers, disturb cellular homeostasis and cause organism threats by actively contributing to bacterial dissemination. *Streptococcus pneumoniae* (pneumococcus) is a severe human pathogen that besides causing upper respiratory tract infections, represents the most frequent cause of deadly pneumonia worldwide, accounting for 1 million under-five deaths in developing countries. Through the release of its major virulence factor, the PFT pneumolysin (PLY), pneumococcus induces an overwhelming immune response and extensive damage in the lung cell PM, leading to extensive tissue damage and fostering infection dissemination. Whether at low concentrations of PLY, presumably occurring early during pneumococcal infection, cells have the ability to efficiently activate PM repair mechanisms that cooperate to limit bacterial dissemination and sustain cell survival was poorly explored.

My PhD work thesis, aimed to investigate host cell responses to PM damage triggered by sub-lytic PLY concentrations and uncover the molecular mechanisms underlying host resistance to PLY-induced damage, likely controlling the early infection. This work was based on previous findings from the host laboratory showing that sub-lytic concentrations of other PFTs (LLO, PFO) induce a massive but transient actomyosin and endoplasmic reticulum reorganization that supports PM remodeling events such as blebbing crucial to fully repair the damaged PM [102].

In line with these observations, my PhD project aimed to:

I. Identify novel host factors implicated in cellular resistance to PLY attack and characterize their role in PM repair

II. Evaluate the role of actomyosin remodeling and uncover its underlying molecular mechanisms in the response to PLY intoxication

III. Assess the role of proteins involved in PM repair in host survival to pneumococcus infection

Chapter II – Results

“A soul in tension that's learning to fly,
condition grounded but determined to try”

"Learning to Fly" by Pink Floyd

The results obtained during my PhD are presented and will be discussed in three different sections. Each of these sections is formatted as research manuscripts already published, submitted or in preparation.

- 1- Cells Responding to Closely Related Cholesterol-Dependent Cytolysins Release Extracellular Vesicles with a Common Proteomic Content Including Membrane Repair Proteins (Published in Toxins, DOI: <https://doi.org/10.3390/toxins15010004>)
- 2- S100A4 is a central orchestrator of cytoskeletal remodeling triggered by pneumolysin during pneumococcal infection (In preparation)
- 3- *Streptococcus pneumoniae* infection of lung epithelial cells induces internalization of surface GPI-anchored proteins through PLY-mediated activation of host Rho GTPases (Submitted to PloS Pathogens, DOI: doi.org/10.1101/2024.04.17.590015)

Section 1

Cells Responding to Closely Related Cholesterol-Dependent Cytolysins Release Extracellular Vesicles with a Common Proteomic Content Including Membrane Repair Proteins

We aimed to assess whether different cholesterol-dependent pore-forming toxins trigger the same cellular responses and share the same repair machinery. In this section, we demonstrate that host cells can effectively cope with damage induced by different cholesterol-dependent cytolysins (LLO and PLY). This repair capacity relies on a prompt influx of Ca^{2+} . PM shedding of extracellular vesicles (EVs) by host cells partially contributes to the removal of two different cholesterol-dependent cytolysins from the cell PM. Furthermore, we uncover new proteins potentially involved in PM repair and give new insights into common mechanisms and machinery engaged by cells in response to PM damage.

I contributed to the conceptualization and design of the experiments, collected and analyzed all the data regarding PLY toxin. I also prepared the majority of the Figures, actively participated in the drafting of the manuscript and helped in the response to the referees.

This article was published in the peer-reviewed journal *Toxins*.

Toxins 2023, 15, 4.
<https://doi.org/10.3390/toxins15010004>

Cells Responding to Closely Related Cholesterol-Dependent Cytolysins Release Extracellular Vesicles with a Common Proteomic Content Including Membrane Repair Proteins

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Abstract

The plasma membrane (PM) protects cells from extracellular threats and supports cellular homeostasis. Some pathogens produce pore-forming toxins (PFTs) that disrupt PM integrity by forming transmembrane pores. High PFT concentrations cause massive damage leading to cell death and facilitating infection. Sub-lytic PFT doses activate repair mechanisms to restore PM integrity, support cell survival and limit disease. Shedding of extracellular vesicles (EVs) has been proposed as a key mechanism to eliminate PFT pores and restore PM integrity. We show here that cholesterol-dependent cytolysins (CDCs), a specific family of PFTs, are at least partially eliminated through EVs release, and we hypothesize that proteins important for PM repair might be included in EVs shed by cells during repair. To identify new PM repair proteins, we collected EVs released by cells challenged with sub-lytic doses of two different bacterial CDCs, listeriolysin O and pneumolysin, and determined the EV proteomic repertoire by LC-MS/MS. Intoxicated cells release similar EVs irrespectively of the CDC used. Also, they release more and larger EVs than non-intoxicated cells. A cluster of 70 proteins including calcium-binding proteins, molecular chaperones, cytoskeletal, scaffold and membrane trafficking proteins, was detected enriched in EVs collected from intoxicated cells. While some of these proteins have well-characterized roles in repair, the involvement of others requires further study. As proof of concept, we show here that Copine-1 and Copine-3, proteins abundantly detected in EVs released by intoxicated cells, are required for efficient repair of CDC-induced PM damage. Additionally, we reveal here new proteins potentially involved in PM repair and give new insights into common mechanisms and machinery engaged by cells in response to PM damage.

1. Introduction

The integrity of the plasma membrane (PM) is crucial for the survival of single cells and for the maintenance of tissue organization in a full organism. PM lesions cause massive Ca^{2+} influx, loss of cytoplasmic content and disruption of cellular homeostasis, and lead to cell death if rapid reseal does not occur [512]. Therefore, to survive PM damage, cells promptly engage repair mechanisms that aim to restore PM integrity and cellular homeostasis. In physiological conditions, PM lesions and repair often occur in tissues under mechanical stress such as skeletal and cardiac muscle,

intestinal and respiratory epithelia, and vascular endothelium [94]. Deregulation of the PM repair pathways was largely found to be associated with the development of different human disorders [94, 276]. Infection by microbial pathogens and exacerbated immune responses also cause PM damage that ultimately determines cell fate and disease progression [76, 513].

Bacterial pore-forming toxins (PFTs) are major virulence factors produced by a variety of severe human pathogens [514]. They are secreted by the bacteria as soluble monomers, interact with the PM of host cells and assemble into stable transmembrane pores [72] to disrupt epithelial barriers, support immune evasion, and promote infection dissemination [76, 515]. Cholesterol-dependent cytolysins (CDCs) are well conserved reaching at least 45% of structural homology and share similar mechanisms of pore formation being the most studied sub-family of PFTs. Monomers of CDCs directly bind PM cholesterol residues, oligomerize and undergo conformational changes that allow their stable insertion across the PM, forming a large well-defined pore including around 40 monomers [510, 516]. The toxin concentration critically determines the level of the induced PM damage, which in turn dictates cell fate [517]. While cells are unable to cope with high toxin concentrations engaging in cell death pathways, at sub-lytic concentrations of PFTs the cells orchestrate a multifactorial repair response that supports cell survival and limit the progression of the infection [76, 102, 517].

Distinct mechanisms, including protein clogging, wound patching, membrane replacement by endocytosis or shedding of damaged sites, were proposed to participate in the re-establishment of PM integrity [76, 94]. However, their hierarchy in effective repair and their level of interdependency and/or cooperation remains unknown. The proper activation of repair mechanisms relies on the controlled influx of extracellular Ca^{2+} [145]. Fluctuations in intracellular Ca^{2+} concentrations are efficiently sensed by Ca^{2+} -binding proteins, such as annexins that are activated and recruited to the sites of damage to participate in the PM reseal [518]. In addition, Ca^{2+} influx causes the disruption and reorganization of the cortical cytoskeleton and fosters PM remodeling events promoting blebbing and shedding of extracellular vesicles, which were both previously correlated with PM damage resolution [102, 519, 520]. PM blebs were suggested to confine the pore away from the cytosol avoiding further Ca^{2+} increase and thus protecting the cell cytosol from irreversible damage

[101]. Such blebs can either retract or be released as extracellular vesicles allowing pore elimination and/or playing immunomodulatory functions [101, 118, 119, 521, 522].

Previous studies demonstrated that proteins involved in PM repair regulate the dynamics of PM blebbing and the shedding of extracellular vesicles upon intoxication [102][13]. These data lead us to hypothesize that, upon pore formation, increased intracellular Ca^{2+} levels induce the recruitment of the cellular machinery for repair to the cortex of the cells next to the sites of pore insertion. There, such machinery might control PM remodeling and shedding of vesicles to assist pore elimination. We thus propose that shed vesicles might carry at least part of the cellular machinery required for PM repair. Here, we seek to determine the proteomic repertoire of vesicles released by intoxicated cells during the repair of PM damage, with the aim to identify novel proteins involved in repair. To further assess whether repair mechanisms were conserved among damage induced by different CDCs, we used two different toxins and performed a comparative analysis. Purified Listeriolysin O (LLO) and Pneumolysin (PLY), which are produced by the pathogenic gram-positive bacteria *Listeria monocytogenes* and *Streptococcus pneumoniae*, respectively, were used [517, 523]. A previous study determined the proteomic content of vesicles released by cells intoxicated with PLY [521]. Here, we seek to advance on this research and perform a comparative analysis revealing the proteins that are common in the response to different CDCs and specific to the release of extracellular vesicles triggered by such toxins.

We reveal here new protein candidates potentially involved in the repair of PM damage induced by CDCs. Additionally, our data open new perspectives and provide new insights into common mechanisms and machinery engaged by cells to respond to a broad range of PM damage.

2. Results and Discussion

2.1. The concentration of CDCs determines the extent of PM damage and the efficacy of repair

We first set up the experimental conditions in which we cause the maximum PM damage that triggers the maximum capacity to repair and recover PM integrity, assuming that this correlates with maximal release of extracellular vesicles. We started

by determining the highest concentration of CDCs that allow the full recovery of PM integrity following toxin washout. HeLa cells were incubated with increasing concentrations of purified LLO or PLY for 15 min and allowed to recover from induced PM damage for 20 h. PM permeability was monitored immediately after toxin washout (0 h) and after 20 h of recovery by propidium iodide (PI) permeability assays coupled with flow cytometry analysis. We observed that the percentage of PI-positive cells increased in response to growing concentrations of LLO and PLY (gray bars, Figure 1A, B), thus confirming that both LLO and PLY permeabilize the PM in a dose-dependent manner [102, 521, 524]. Specifically, 20 h after toxin washout, to allow recovery from PM damage, the percentage of PI-positive cells dropped to the levels detected in non-intoxicated (NI) cells (blue bars, Figure 1A, B) in samples challenged with lower doses of toxins, thus indicating that these cells are able to fully recover PM integrity. At the highest CDC concentrations (2 nM LLO and 0.5 nM PLY), at least 80% of the cells displayed PI permeability after toxin washout (0 h, gray bars, Figure 1A,B); and after 20 h of recovery, the percentage of PI-positive cells remained significantly higher than in the NI samples (blue bars, Figure 1A, B), suggesting that at such CDC concentrations cells do not recover from inflicted damage.

To ensure that the decreased percentage of PI-positive cells after 20 h of recovery was indeed due to PM repair and not related to the loss of damaged cells, we quantified by microscopy the number of adherent cells for each sample. In NI control conditions, the number of adherent cells increased after 20 h, which corresponds to normal cell growth (Figure 1C, D). In samples intoxicated with low CDCs doses, the number of adherent cells remained roughly the same immediately after toxin washout (0 h) and recovery (20 h), suggesting that cells intoxicated with lower doses of CDCs recover PM integrity, do not die but do not proliferate (Figure 1C, D). At the highest CDC concentrations (2 nM LLO and 0.5 nM PLY), the number of adherent cells (at 0 and 20 h) was significantly lower than in the NI condition (Figure 1C, D), suggesting that at such concentrations the cells undergo immediate overwhelming damage, are unable to repair the damage and recover PM integrity, and detach from the support (Figure 1C, D).

Together, these data indicate that at sub-lytic CDC concentrations cells become permeabilized but do not massively detach and thus are not lost during the experiment. While at high CDC doses, the cells detach and are lost; and thus, the

percentage of PI-positive cells is likely underestimated in our experimental setup. Expectedly, our results also indicate that the level of PM permeabilization depends on the concentration and activity of CDCs and ability of cells to repair the injury decreases as damage increases. Recovery of PM damage is achieved in a cell population in which about 60% of the cells are permeabilized. The concentration and activity of CDCs are thus key determinants for the effectiveness of repair.

Given that we aim to cause the maximum damage that cells are able to cope with, in the next experiments we will use 1 nM for LLO and 0.2 nM for PLY, concentrations that permeabilize roughly 60% of the cells and allow complete recovery as compared to the NI condition (Figure 1A, B).

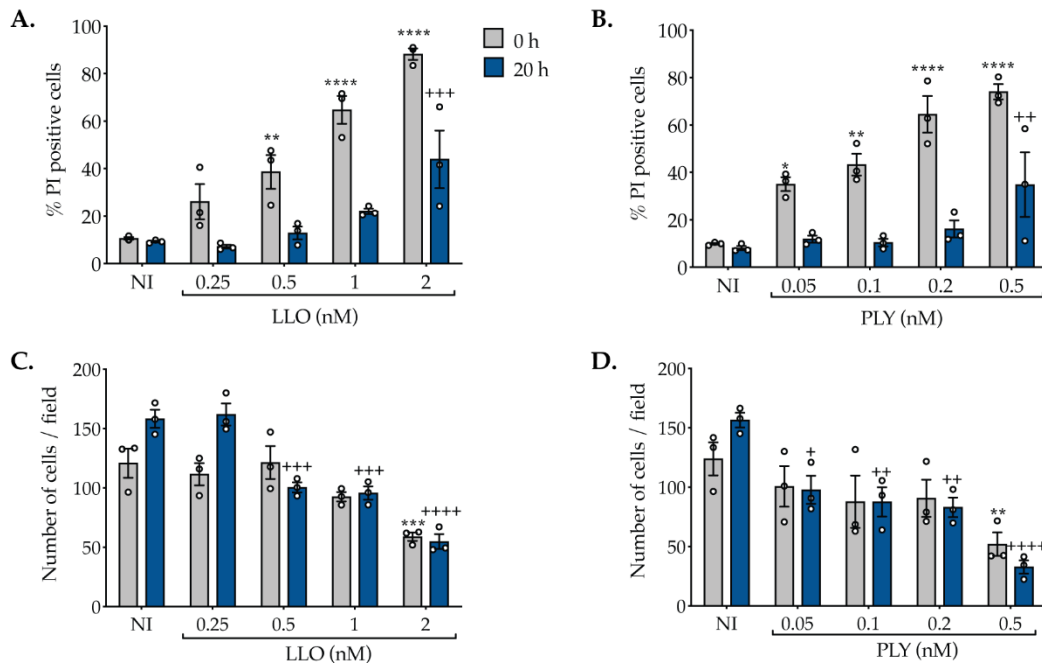


Figure 1. CDC concentration determines the extent of PM damage and the efficacy of repair. HeLa cells were left non-intoxicated (NI) or were intoxicated with the indicated concentrations of (A) LLO or (B) PLY for 15 min. PM permeability was measured by flow cytometry following incorporation of the membrane impermeable dye propidium iodide (PI). The graphs show the percentage of PI-positive cells immediately after PFT washout (0 h, gray bars) or following PFT washout and recovery (20 h, blue bars). (C and D) Quantification of the total number of cells per field (5 fields corresponding to at least 250 cells were quantified per experiment in each condition) after DAPI nuclear staining. HeLa cells were left NI or were intoxicated with the indicated concentrations of (C) LLO or (D) PLY for 15 min. Quantifications were performed immediately after PFT washout (0 h, gray bars) or following PFT washout and recovery (20 h, blue bars). Values represent the mean $\pm$ SEM of three independent experiments.

Open circles represent values for each independent experiment. P values were calculated using two-way ANOVA followed by Dunnett's test. **P<0.01; ***P<0.001; ****P<0.0001. * refers to NI at 0 h vs intoxicated at 0 h; + refers to NI at 20 h vs intoxicated at 20 h.

2.2. Repair of CDC-induced PM damage relies on an abrupt shot of Ca^{2+} early in intoxication

In addition to the concentration and associated activity of the toxin, the concentration of extracellular Ca^{2+} is another key factor determining the repair and recovery of PM integrity [4]. Of note, under physiological conditions, Ca^{2+} exists at ~100 nM in the cell's cytosol and may reach ~2 mM in the extracellular milieu [525]. To determine the extracellular Ca^{2+} concentration that would allow maximum repair of CDC-induced damage, HeLa cells were challenged with 1 nM LLO for 15 min in the presence of physiological concentration of extracellular Ca^{2+} (1.2 mM) and left to recover from damage, after toxin washout, for 20 h in Hanks' Balanced Salt Solution (HBSS) supplemented with growing Ca^{2+} concentrations. PM integrity was assessed by PI permeability assays immediately after toxin washout (0 h) and after 20 h of recovery in Ca^{2+} concentrations ranging from 0 to 1.2 mM. We found that cells do not recover PM integrity in the absence of extracellular Ca^{2+} ; notably, in such conditions, the level of damage even increased during the 20 h, as indicated by the increased percentage of cells permeable to PI (Figure 2A). However, in the presence of extracellular Ca^{2+} , cells recovered their PM integrity at similar levels irrespective of the Ca^{2+} concentration that we tested (Figure 2A). These results indicate that a low concentration of extracellular Ca^{2+} (75 μM) is necessary and sufficient to enable PM repair, and that higher concentrations of extracellular Ca^{2+} have no significant effect on PM repair.

We next investigated whether Ca^{2+} is only important during the repair stage or also during the intoxication. In particular, we aimed to assess if the availability of extracellular Ca^{2+} during the repair process would overcome the damage caused in the absence of Ca^{2+} . For that, HeLa cells were intoxicated with 1 nM LLO for 15 min in the absence of extracellular Ca^{2+} and allowed to recover from damage for 20 h in HBSS supplemented with growing concentrations of Ca^{2+} . As above, PM integrity was assessed by PI permeability assays after toxin washout (0 h) and 20 h of recovery. As expected, samples intoxicated in the absence of Ca^{2+} showed higher percentages of PI-positive cells as compared with cells intoxicated in the presence of Ca^{2+} (Figure 2A,

B). This indicates that in the absence of Ca^{2+} , cells undergo increased PM damage. Additionally, we found that cells intoxicated in the absence of extracellular Ca^{2+} do not recover PM integrity, even if physiological Ca^{2+} concentrations are provided during the recovery period (Figure 2B). Indeed, the percentages of PI permeable cells remained unchanged after 20 h of recovery (Figure 2B). Altogether these results suggest that during exposure to CDCs, extracellular Ca^{2+} and its consequent rapid influx at early stages of intoxication determine the ability of damaged cells to reseal their PM. If intoxication occurs in the absence of extracellular Ca^{2+} , the later addition of Ca^{2+} cannot promote repair and recovery of PM integrity. The early entry of Ca^{2+} upon pore formation likely activates mechanisms of repair that cannot be triggered at later stages. Our data are in agreement with previous studies reporting that in vitro chelation of extracellular Ca^{2+} during PLY intoxication leads to a twofold reduction in the production and shedding of Evs [119, 521] and also results in inefficient Ca^{2+} -dependent repair [406].

In this context, we investigated whether different extracellular Ca^{2+} concentrations impact CDC-induced PM damage in the initial intoxication stages. For that, HeLa cells were incubated with 1 nM LLO for 15 min, in the presence of Ca^{2+} concentrations ranging from 0 to 1.2 mM, and PM permeability was immediately assessed. Intoxications performed in concentrations of Ca^{2+} below 1.2 mM resulted in equivalent percentages of PI-positive cells (Figure 2C), which were significantly higher than those obtained for intoxications under physiological Ca^{2+} concentrations (1.2 mM). Surprisingly, at 0.1 mM of Ca^{2+} , which corresponds to 10-fold less than the physiological extracellular Ca^{2+} concentration, but that is still 1000-fold higher than the intracellular concentration; the damage caused by CDCs is already too high and likely irreversible (Figure 2C). These data suggest that repair of PM damage induced by CDCs relies on an at least 12,000-fold gradient maintained by cells between intracellular and extracellular Ca^{2+} concentrations, which causes an abrupt entry of Ca^{2+} at early stages upon pore formation. Our results thus indicate that levels of Ca^{2+} under the physiological concentrations during the intoxication have a profound impact on the ability of cells to recover PM integrity.

We concluded that to obtain the maximum recovery of PM integrity upon CDC pore formation, both the intoxication and recovery time periods should be performed in physiological Ca^{2+} concentrations (1.2 mM); otherwise, PM damage increases and reaches levels that are incompatible with full PM recovery. Also, given the similarities between LLO and PLV [317, 510], we decided to use the same Ca^{2+} concentrations in intoxications with either LLO or PLV.

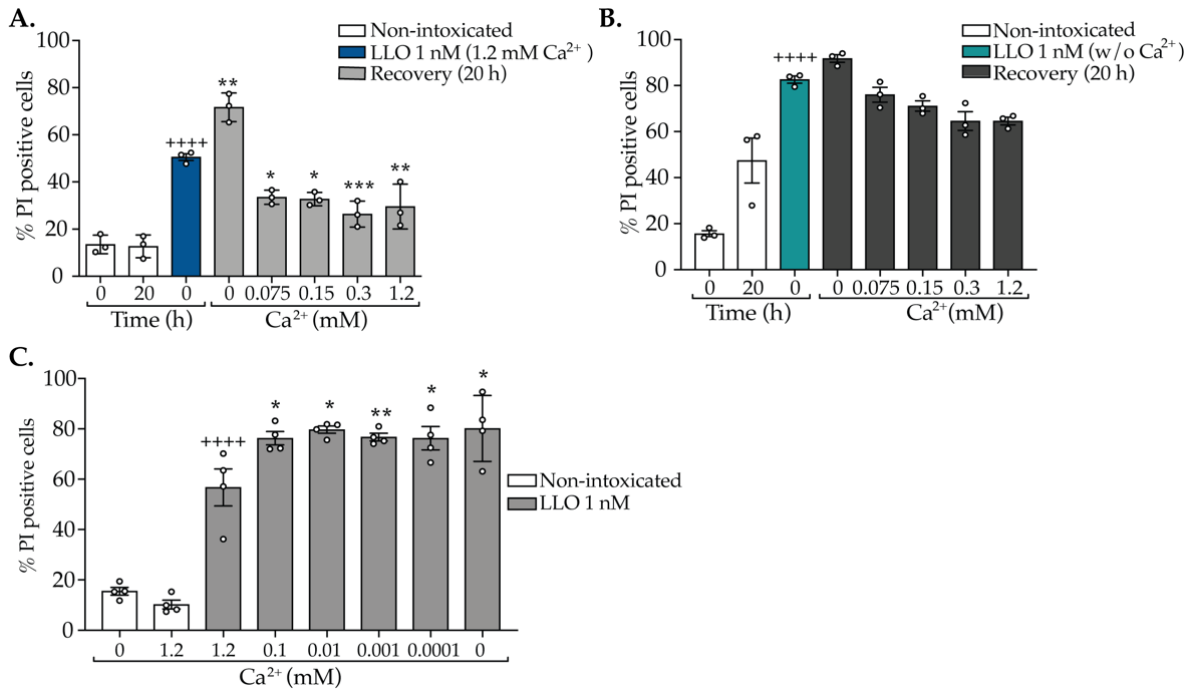


Figure 2. Physiologic concentrations of extracellular Ca^{2+} are required for the resealing of damaged PM. HeLa cells were left non-intoxicated (NI) or were intoxicated for 15 min with 1 nM of LLO in (A) HBSS containing 1.2 mM Ca^{2+} or in (B) HBSS without Ca^{2+} . (A, B) After toxin washout, cells were allowed to recover PM integrity for 20 h in HBSS supplemented with increasing Ca^{2+} concentrations. Graphs (A, B) show the percentage of PI-positive cells determined by flow cytometry at the end of intoxication after LLO washout (0 h) and after 20 h of recovery in HBSS with the indicated Ca^{2+} concentrations. (C) HeLa cells were left NI or were intoxicated with 1 nM LLO in HBSS with the indicated Ca^{2+} concentrations. The percentage of PI-positive cells was assessed immediately after washout. Values are the mean $\pm$ SEM of four independent experiments. P values were calculated using one-way ANOVA followed by Tukey's test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$. * refers to intoxicated cells in the presence of 1.2 mM Ca^{2+} at 0 h vs recovery in growing Ca^{2+} concentrations (in A) and vs cells intoxicated in the presence of Ca^{2+} concentrations ranging from 0 - 0.1 mM (in C); + refers to NI at 0 h vs intoxicated cells at 0 h (in A-C).

2.3. Cells intoxicated with CDCs release large extracellular vesicles during PM damage repair

As mentioned above, vesicle shedding is a major mechanism deployed by cells to repair PM damage [76]. To characterize shed vesicles during repair of LLO- and PLY-induced PM damage, HeLa cells were intoxicated in the conditions determined above (1 nM LLO or 0.2 nM PLY for 15 min in the presence of 1.2 mM Ca^{2+}) and allowed to recover from damage in the presence of 1.2 mM Ca^{2+} . After 3 h of repair, which we found sufficient for full recovery (Figure S1), shed vesicles were isolated from cell supernatants by ultracentrifugation. As the control, we collected vesicles shed by the same number of NI cells during 3 h. The concentration and size distribution of purified extracellular vesicles (EVs) were determined by Nanoparticle Tracking Analysis (NTA). We found that, during recovery, LLO- and PLY-intoxicated cells released similar amounts of vesicles (Figure 3A-C). Additionally, samples from supernatants of intoxicated cells contained a two-fold higher EV concentration than samples from control NI cells (Figure 3B). This confirms that increased EV shedding is a common mechanism responding to CDC-induced damage, activated during the recovery of PM integrity. Size distribution analysis revealed that while the concentration of small-size EVs (<120 nm) was equivalent in the supernatants of intoxicated and NI cells (Figure 3C), larger EVs (120 nm to 500 nm) were shed in significantly increased amounts by intoxicated cells as compared with NI control cells (Figure 3C). Transmission electron microscopy (TEM) analysis of purified EVs confirmed that those recovered from supernatants of LLO- and PLY-intoxicated cells displayed a heterogeneous profile with sizes ranging from 120 to 500 nm, while EVs from NI cells showed a homogeneous profile with sizes up to 120 nm (Figure 3D).

These data indicate that vesicle shedding is increased during repair of CDC-induced damage and that intoxicated cells mainly release large EVs, which are rarely released by NI cells. Furthermore, LLO- and PLY-intoxicated cells release similar amounts of vesicles, which share the same size distribution profile, reinforcing the

idea that vesicle shedding is a common response triggered by different CDCs to promote PM repair.

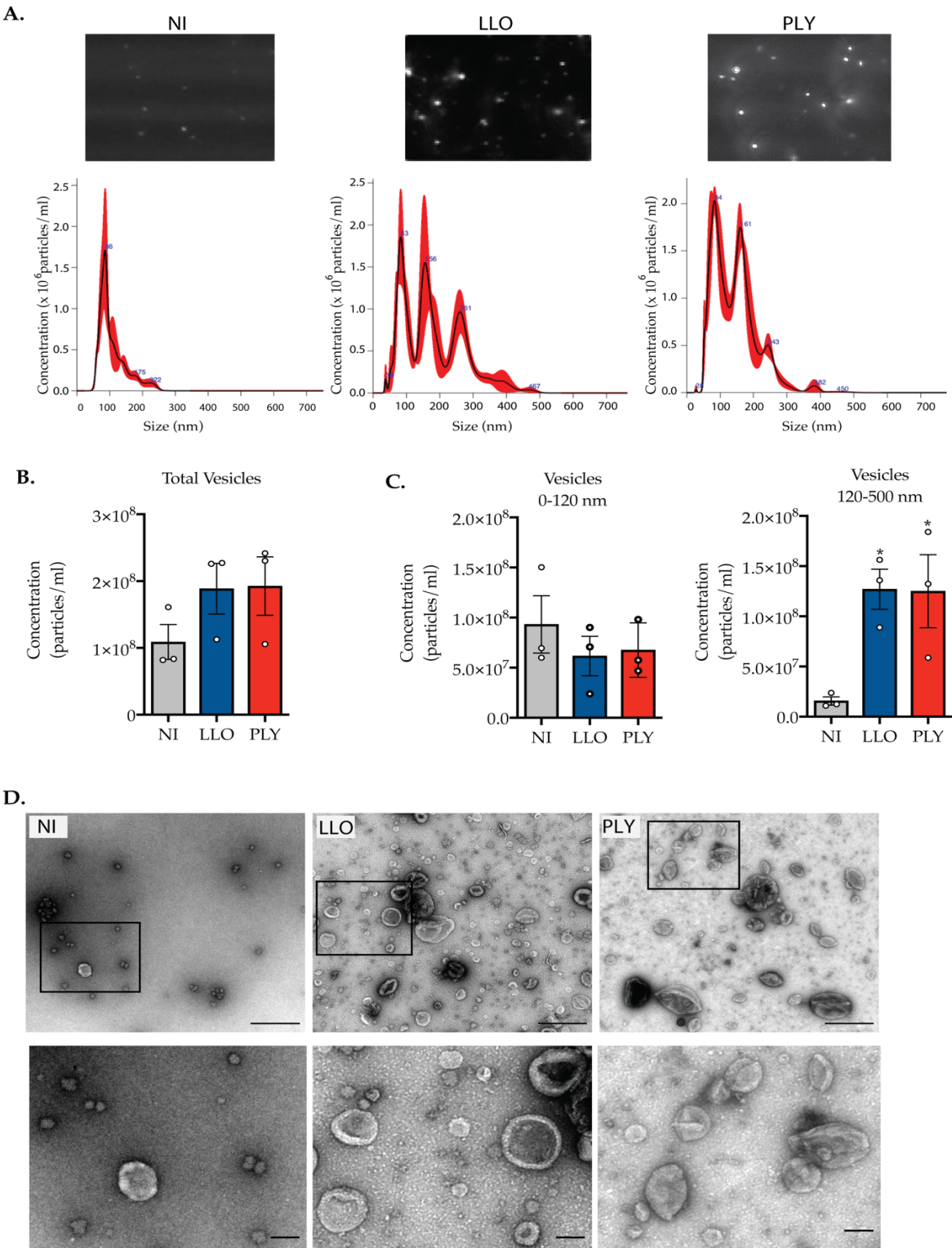


Figure 3. Release of large extracellular vesicles is a specific response to intoxication by CDCs. (A-C) Nanoparticle tracking analysis (NTA) of purified EVs isolated from the cell supernatants of HeLa cells non-intoxicated (NI) or intoxicated with 1 nM of LLO or 0.2 nM of PLY, for 15 min. (A) Light scattering snapshots from videos recorded from NanoSight analysis (upper panel). Representative histograms obtained from NTA analysis showing, for each sample, the concentration of vesicles according to their size (bottom panel). (B) The graph shows the total concentration of EVs recovered in each sample. Values are the mean $\pm$ SEM of three independent experiments. (C) Size distribution of quantified EVs. Graphs show the concentration of vesicles with sizes between 0 to 120 nm and between 120 to 500 nm. Values are the mean $\pm$ SEM of three independent experiments. One-way ANOVA test was performed (* $P < 0.05$). (D) Transmission electron microscopy (TEM) of purified EVs. Scale bar = 500 nm. Insets show EVs at higher magnification (Scale bar = 100 nm).

2.4. The release of large extracellular vesicles contributes to the removal of CDCs from the PM

We further confirm the concept that shedding of EVs constitutes a mechanism for the physical removal of the pore from the PM, thus allowing CDC elimination and recovery of PM integrity [79, 108, 110, 521]. To ascertain if EVs collected from supernatants of CDC-intoxicated cells contain the CDCs, we performed immunogold labeling on purified EVs using specific antibodies raised against LLO or recognizing GFP (to detect GFP-PLY). Electron microscopy images displayed in Figures 4A, B, respectively, show the presence of LLO or PLY associated with EVs released during recovery of damage. The percentage of EVs associated with gold-labeled particles was determined per field in TEM images. EVs associated with at least one gold particle were considered positive for LLO or PLY. We found that the majority of vesicles released are positive for either LLO or PLY (Figure 4C). However, LLO and PLY were undetectable in about 40 % of the EVs, suggesting that vesicle shedding may serve different purposes and that cells may also release vesicles in areas where the PM is intact. LLO and PLY were also detected in purified EVs by immunoblotting (Figure 4D) and by mass spectrometry (MS)-based proteome analysis (Figure 4E) on vesicles shed by LLO- and PLY-intoxicated cells, respectively. As expected, CDCs were not detected in EVs released by NI cells.

Altogether these data confirm that shedding of EVs allows the cells to get rid of PM bound CDCs, as previously proposed [108, 110, 411]. Additionally, these results

strongly suggest that shedding has an active role in pore elimination and protects the cell from damage supporting cell survival. Nevertheless, the absence of CDCs in 40 % of the EVs also raises the possibility that released EVs might have additional functions such as transport of the repair machinery to the cell cortex, PM reorganization and/or delivery of signals to neighboring cells. In line with this, a previous study had proposed that PLY triggers the release of distinct vesicle populations: PLY-positive and PLY-negative EVs which would supposedly be derived from different regions of the PM [521]. One may therefore speculate that shed EVs originate from different routes and have distinct functions during PM repair, which may also correlate with the different size sub-populations.

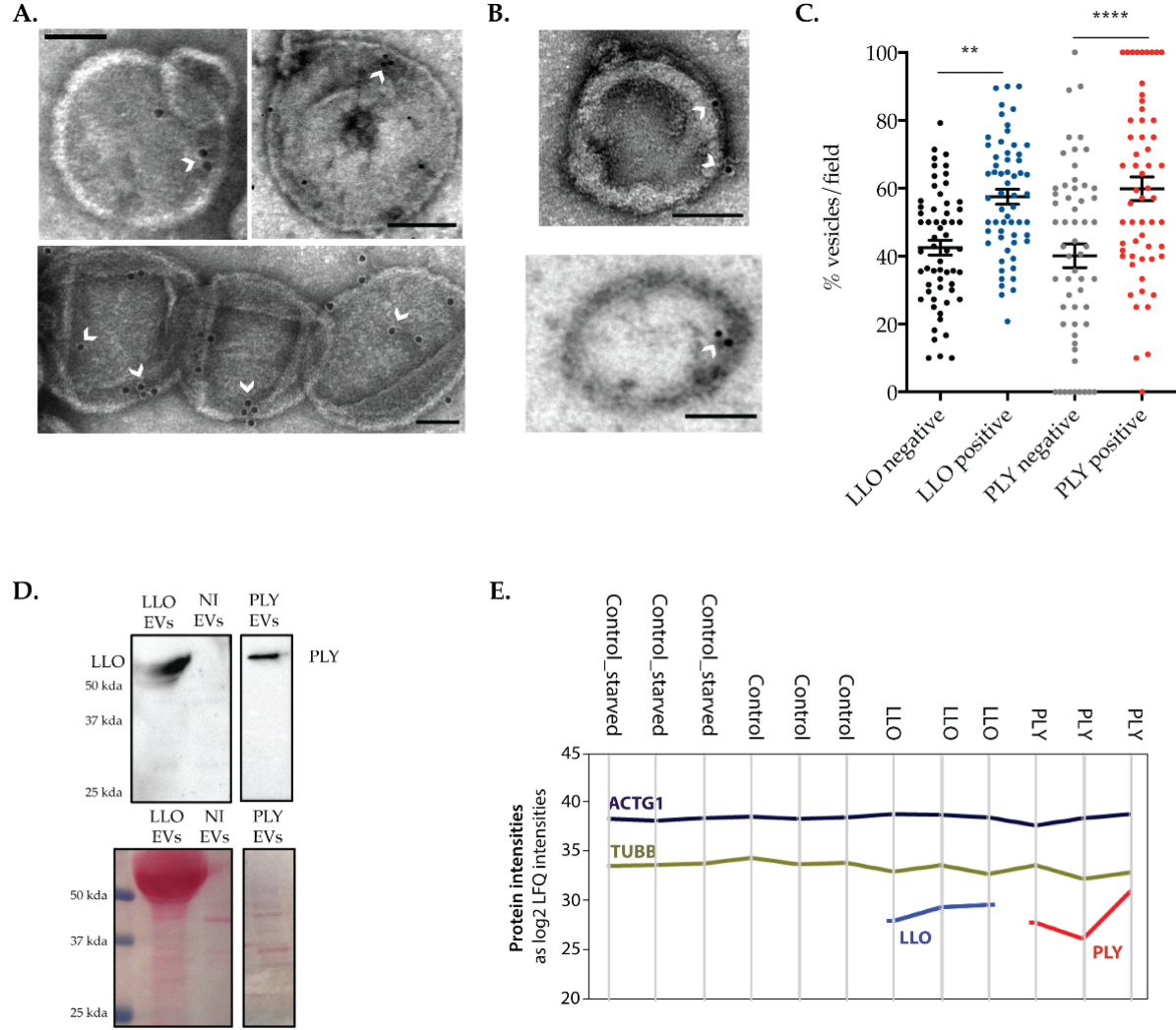


Figure 4. The release of EVs contributes to the removal of CDCs from the PM of host cells.

(A,B) Transmission electron microscopy (TEM) images showing immunogold labeling of (A) LLO in EVs collected from supernatants of HeLa cells intoxicated with 1 nM of LLO for 15 min and (B) GFP-PLY in EVs collected from supernatants of HeLa cells intoxicated with 0.2 nM of GFP-PLY for 15 min. Arrowheads indicate gold particles in the EVs collected from LLO- and PLY-intoxicated cells. Scale bar, 100 nm. (C) Quantification of LLO- and PLY-positive EVs in TEM images. Evs associated at least with one gold particle were considered positive. Data from three independent experiments are shown as percentage of CDC-positive or -negative vesicles per field. A one-way ANOVA test was performed (** $p < 0.01$, **** $p < 0.0001$). (D) Immunoblot showing LLO and PLY protein levels in EVs released from HeLa upon intoxication. EVs released by non-intoxicated (NI) cells were used as the control. Ponceau staining (lower panel) was used as loading control to detect proteins in membranes before immunoblotting. (E) Profile plots showing protein levels (as log₂ LFQ intensities) of PLY, LLO, as well as TUBB and ACTG1 as loading controls. All LFQ intensities based on match-between-runs with missing MS/MS scans were removed prior to visualization. Detection of LLO and PLY in EVs of the respective CDC-treated cells confirms release into EVs.

2.5. Proteins present in EVs released by intoxicated cells are potential actors in PM repair

To gain further information on the function of EVs during the repair of PM damage induced by CDCs, we determined their protein content by MS-based proteomics. As a control, EVs were collected from cells cultured in standard conditions (10% EV-depleted FBS, control) for 72 h and cells cultured under serum starvation (1% EV-depleted FBS, control-starved) for 48 h. Serum starvation was expected to act as a stress stimulus for EVs release [526]. As NI cells shed a lesser amount of EVs than intoxicated cells, the time before collection of control EVs was extended to ensure that we had enough material for analysis. Considering that donor cells can specifically pack into EVs the cellular components required to respond to a specific condition, we postulated that proteins overrepresented in EVs released by intoxicated cells might potentially be involved in repair or intercellular signaling responses.

EVs collected from three independent experiments were lysed, proteins were digested by trypsin and peptide mixtures were separated and analyzed by liquid chromatography- tandem mass spectrometry (LC-MS/MS). A total of 2110 proteins were reliably quantified (Table S1). Principal component analysis (PCA), a statistical method used to reduce the number of variables from a dataset to produce strong

patterns, clearly demonstrated that EVs from NI HeLa cells cluster differently from EVs released by intoxicated cells (Figure 5A). This suggests that proteins are selectively packed in EVs released by intoxicated cells, further supporting the idea that the identification of the proteins enriched in EVs is an invaluable step toward the holistic understanding of PM repair mechanisms. Additionally, together with the differences in EV concentration and size distribution (Figure 3), our results suggest that the release of EVs triggered by CDCs is a specific response to damage different from canonical EV secretion. Moreover, EVs from LLO- or PLY-intoxicated cells cluster together indicating that they share similar proteins (Figure 5A), corroborating the idea that different CDCs trigger equivalent cell protective responses.

To identify the proteins overrepresented in EVs released by intoxicated cells, which we postulate might be involved in PM repair, several comparative analyses of the proteomic data were performed. Volcano plots were generated for pairwise comparison of protein intensities in the different conditions: control vs. control-starved, LLO vs. PLY, LLO vs. control; PLY vs. control and all CDCs (LLO and PLY, mentioned as PFTs) vs. all controls. The control vs. control-starved comparison revealed 50 significantly regulated proteins (though 41 of these are potential contaminants, Volcano plot in Figure S2 and Table S2), while comparing LLO vs. PLY did not show any significant differences (Volcano plot in Figure S2). Together, these comparisons confirm that EVs from control cells have similar proteomic profiles and EVs released by LLO- or PLY-intoxicated cells share the same proteomic signature, corroborating the PCA analysis (Figure 5A). Notably, the LLO vs. control comparison revealed 360 significantly regulated (including 57 potential contaminants), of which 46 were overrepresented in LLO samples, including LLO itself (Figure 4E, Table S3). Similarly, the PLY vs. control comparison resulted in 102 regulated proteins (including 48 potential contaminants), of which 13 were overrepresented in PLY samples, including PLY itself (Figure 4E, Table S4). Of note, the potential contaminants are defined by the software tool that we have used, which includes a list of common contaminants in proteomic analysis.

Given the reported similarities between the modes of action of LLO and PLY and associated cellular responses [72, 76, 527], as well as the closely related proteomic signature of the EVs released by LLO- or PLY-intoxicated cells (Figure 5A), we also compared protein intensities in all EVs samples from intoxicated cells (by either LLO

or PLY) with the intensities in all control samples. This comparison revealed 615 proteins with significantly different abundances, though 45 of these are potential contaminants (Figure 5B, Table S5). The intensity of these proteins was also visualized in a heatmap, clustering in three distinct groups (Figure 5C). Cluster 1 comprises 70 proteins that are overrepresented in the EVs collected from supernatants of intoxicated cells (Figure 5C). Proteins included in this cluster, which according to our assumption may be key for the repair of PM damage, were classified following Gene Ontology analysis (Figure 5D and Table 1) using PANTHER [528-530] [34-36]. Calcium-binding proteins, molecular chaperones, cell adhesion molecules, cytoskeletal, scaffold and membrane trafficking proteins were found among the most enriched categories in the EVs released by intoxicated cells (Figure 5D and Table 1). In particular, we detected several annexins (Table 1), which have been extensively described as key players in Ca^{2+} -driven repair responses [149, 221, 406, 521, 531]. Chaperones and cytoskeletal proteins were also previously reported to be essential for effective repair of damage caused by CDCs [102, 519].

The evidence that proteins with well-established roles in PM repair were detected enriched in the EVs released by intoxicated cells validates our approach. Our data further reveal other EV-enriched proteins that might be important for the repair of damage induced by CDCs. Finally, results also suggest that repair relies on complex cellular responses that require the cooperation of a variety of protein families. The specific function of newly detected candidates in the response to CDCs and infection needs to be further assessed in proper cellular and animal models.

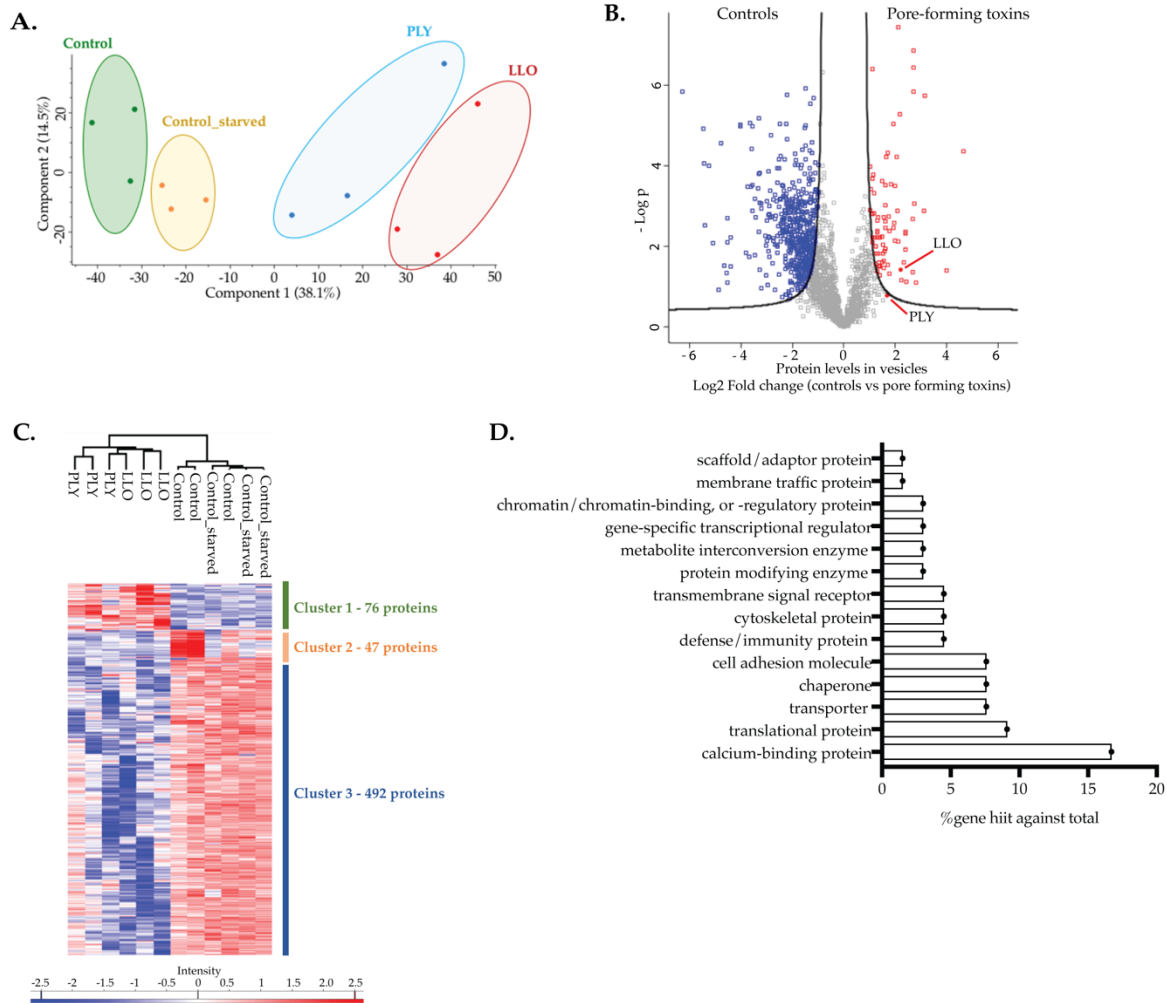


Figure 5. CDC-damaged cells release vesicles with specific proteomic profiles enriched in proteins involved in PM repair. (A) Principal component analysis (PCA) based on all proteins detected in EVs released from HeLa cells non-intoxicated (NI; control and control_starved) and intoxicated with LLO or PLY. Scatter plot shows sample mappings along the two principal components. X and Y axis show principal component 1 and principal component 2 which explain 38.1 % and 14.5 % of the total variance, respectively. While both principal components represent a dimensionality reduction of all protein abundances, they consider different protein abundances to a different extend. (B) Volcano plot showing the protein level represented in fold change (log2) of EVs released from NI HeLa cells (controls) compared to EVs collected from HeLa cells intoxicated with LLO and PLY (x axis). Replicate samples from three independent experiments were analyzed and Student t-tests were applied to calculate $-\log p$ -values for each protein (y axis). The curved black lines represent the threshold for statistical significance determined by t-testing (FDR=0.05 and S0=1). Blue squares correspond to underrepresented proteins and red squares are overrepresented proteins. (C) Heatmap visualizing the protein

intensity of significant hits from (B) after non-supervised hierarchical clustering. (D) Gene ontology analysis of proteins from cluster 1 in (C), performed on PANTHER.

Table 1. Full list of proteins detected in cluster 1, classified following Gene Ontology analysis. All these proteins were found statistically enriched in the EVs released by CDC-damaged cells.

Cluster 1	
Calcium binding proteins Annexin A1 Annexin A2 Annexin A3 Annexin A4 Annexin A5 Annexin A6 Annexin A7 Annexin A11 Calmodulin-like protein 5 Copine-1 Copine-3 Hippocalcin-like protein 1 Translational proteins 40S ribosomal protein S20 40S ribosomal protein S18 60S ribosomal protein L8 60S ribosomal protein L31 60S ribosomal protein L23a Eukaryotic translation initiation factor 4B Transporters ATP synthase subunit G2, mitochondrial Cationic amino acid transporter 2	Cytoskeletal proteins Keratin, type I cytoskeletal 18 Vimentin PDZ and LIM domain protein 5 Defense/immunity proteins HLA class I histocompatibility antigen, B alpha chain HLA class I histocompatibility antigen, C alpha chain UL16-binding protein 3 Transmembrane signal receptors CD44 antigen Ephrin type-A receptor 2 Tissue factor Protein modifying enzymes Protein kinase C alpha type Multifunctional procollagen lysine hydroxylase and glycosyltransferase LH3 Membrane trafficking proteins Alpha-taxilin Gene-specific transcriptional regulators Glucocorticoid receptor

Monocarboxylate transporter 4 Sodium- and chloride-dependent taurine transporter Solute carrier family 12 member 2 Chaperones DnaJ homolog subfamily A member 1 Peptidyl-prolyl cis-trans isomerase B Proteasome assembly chaperone 1 Protein disulfide-isomerase A3 Cell adhesion molecules Cadherin-2 Cadherin-13 C-X-C chemokine receptor type 4 Desmocollin-1 Podocalyxin Chromatin-binding, or -regulatory proteins Histone H2A.V Histone H2B type 1-L Histone H3.3 Histone H4 Scaffold/adaptor proteins A-kinase anchor protein 12	Leucine-rich repeat flightless-interacting protein 1 Metabolite interconversion enzymes 5-nucleotidase Carnitine O-palmitoyltransferase 1, liver isoform Others Proteasomal ubiquitin receptor ADRM1 Glycogen synthase, muscle CD59 glycoprotein Neuroblast differentiation-associated protein AHNAK Neuroplastin Brain acid soluble protein 1 CD99 antigen Nuclease-sensitive element-binding protein 1 Y-box-binding protein 3 Myristoylated alanine-rich-C-kinase substrate Sorcin Ubiquitin-40S ribosomal protein S27a Dermcidin RNA-binding protein 14 78 kDa glucose-regulated protein RNA-binding protein 4 Uncharacterized protein C7orf50
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2.6. Copine-1 and Copine-3 are required for efficient PM repair

To validate the proteomic data, we investigated whether Copine-1 and Copine-3 (CPNE1 and CPNE3, respectively) were important for the repair of PM damage induced by PLY. Some observations supported a role for CPNE1 and CPNE3 in the response to CDCs: although copine's exact function in cells remains unknown, CPNE1 and CPNE3 are ubiquitously expressed in several tissues [532] and were described as calcium-

dependent phospholipid-binding proteins [533, 534]. Also, they were both found enriched in EVs released from intoxicated cells (Table 1), along with other proteins with established functions in PM repair. We generated HeLa cell lines depleted for CPNE1 or CPNE3 by CRISPR-Cas9 genome editing and investigated their ability to repair PLY-induced PM damage. The expression of CPNE1 and CPNE3 in the generated cell lines was verified by Western blot using specific antibodies recognizing as either CPNE1 or CPNE3 (Figure S3). We found that the cell line deficient in CPNE1 (KO-CPNE1) was notably not expressing CPNE1 but expressing CPNE3 as their wild-type (WT) parental cells. However, the cell line knockout for CPNE3 (KO-CPNE3) fails to express either CPNE3 or CPNE1, which suggests that the selected guide RNAs might be targeting both proteins or that in the absence of CPNE3, CPNE1 is less expressed or degraded. Both cell lines, together with HeLa WT cells, were challenged with 0.2 nM of PLY, and their PM integrity was assessed by PI permeability assays immediately after toxin washout (0 h) and at different time points (30, 60, and 120 min) after toxin washout and recovery in complete medium. At 0 h, immediately after toxin washout, the three cell lines were permeabilized at comparable levels, about 70% of the cells were detected as PI-positive (Figure 6A). In HeLa WT cells, the PM permeability rapidly decreased to the levels of NI cells. Specifically, 30 min after toxin washout only 20% of the cells were detected as PI-positive, indicating that these cells efficiently recover from PM damage induced by sub-lytic doses of PLY (Figure 6A). Contrarily, intoxicated KO-CPNE1 and KO-CPNE3 HeLa cells displayed percentages of PI-positive cells significantly higher than NI cells throughout the recovery period (Figure 6A), indicating that in the absence of CPNE1 and/or CPNE3 cells do not efficiently repair the injury inflicted by PLY. Of note, the repair defect appears to be amplified in KO-CPNE3 as compared with CPNE1 cells (Figure 6A), which may be related to the fact that KO-CPNE3 cells lack both CPNE1 and CPNE3. These data point out that CPNE1 and CPNE3 are required for efficient repair of damage induced by PLY and reinforce our hypothesis suggesting that the EVs released from intoxicated cells carry into their cargo intracellular machinery that is specifically recruited to damaged areas and plays a pivotal role in the re-establishment of the PM integrity.

Considering that CPNE1 and CPNE3 translocate to the PM in response to increased intracellular Ca^{2+} concentrations, we investigated the intracellular localization of both CPNE1 and CPNE3 upon incubation of cells with PLY. For that, HeLa

cells were transfected to express GFP-tagged CPNE1 or CPNE3 and the cellular localization of the GFP protein fusions was assessed by confocal microscopy in non-intoxicated cells or upon the 0.2 nM PLY challenge. In non-intoxicated cells, the GFP-associated fluorescence was homogeneously detected in the cytoplasm, indicating that both GFP-CPNE1 and GFP-CPNE3 are uniformly distributed in the cytoplasm (Figure 6B). However, upon PLY intoxication, GFP-CPNE1 and GFP-CPNE3 were found enriched at the cell cortex, suggesting that the Ca^{2+} influx generated by PLY pore formation induces the translocation of CPNE1 and CPNE3 to PM-damaged sites (Figure 6B). Additionally, in intoxicated cells, CPNE1 and CPNE3 were found to accumulate together with NMHCIIA (Figure 6C) at cortical sites where the actomyosin cytoskeleton is remodeled to support effective repair [102, 519]. Considering that accumulations of NMHCIIA in the cortex of intoxicated cells are established as hallmarks of PM damage repair, the detection of CPNE1 and CPNE3 at these specific sites strongly suggests their role in assisting repair.

Together, these data point to the important role of CPNE1 and CPNE3 in the recovery of PM integrity possibly through the interaction with the repair machinery at specific cortical sites. Importantly, these results serve as proof-of-concept supporting our original hypothesis proposing that proteins present in the EVs released by intoxicated cells are potentially required for damage repair.

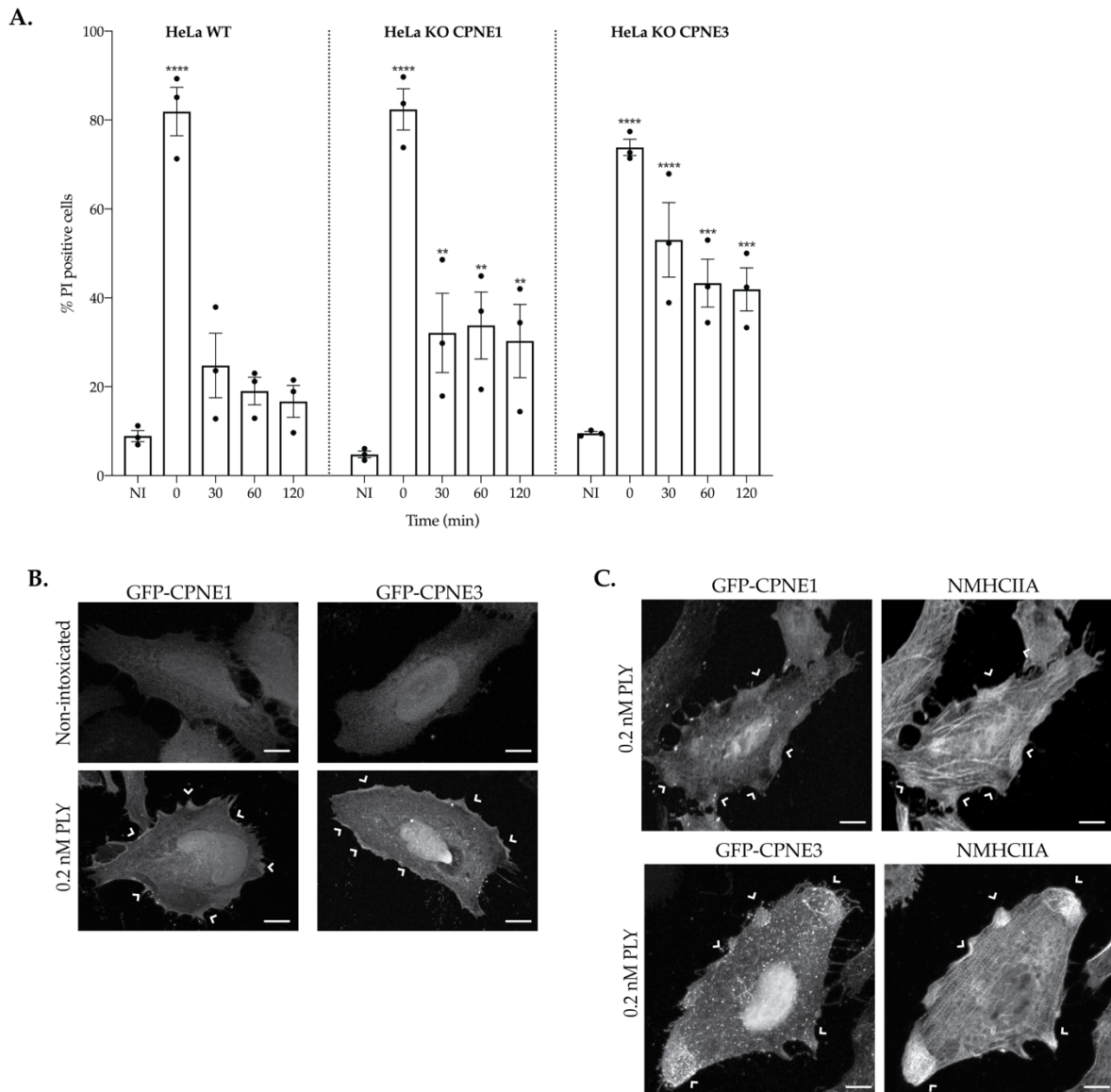


Figure 6. CPNE1 and CPNE3 are required for efficient PM repair of damage induced by PLY.

(A) WT, KO-CPNE1, and KO-CPNE3 HeLa cells were left non-intoxicated (NI) or were intoxicated with 0.2 nM of PLY (10 min) followed by toxin washout and recovery allowed for 30, 60 or 120 min. Permeability of the PM was determined by flow cytometry following PI incorporation at the indicated time points. The graph shows quantifications of PI-positive cells for all cell lines in non-intoxicated (NI) or intoxicated conditions. Values are the mean $\pm$ SEM ($n = 3$) and p values were calculated using two-way ANOVA followed by Dunnett's test. ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; statistics refer to NI vs intoxicated with different times of recovery. (B) Representative confocal microscopy images of HeLa cells overexpressing GFP-CPNE1 or GFP-CPNE3 left non-intoxicated or challenged with 0.2 nM PLY for 10 min. GFP signal is shown. Arrows indicate GFP-CPNE1 and GFP-CPNE3 enrichments at the cortex of PLY intoxicated cells. (C) Confocal microscopy images of HeLa cells overexpressing GFP-CPNE1 or GFP-CPNE3 intoxicated with 0.2 nM PLY (10 min) and stained for NMHCIIA. GFP-CPNEs and NMHCIIA signals

are shown. Arrows show the co-accumulations of GFP-CPNEs and NMHCIIA at sites of actomyosin remodeling for repair. Scale bar, 10 μ m.

3. Conclusions

PM shedding is considered a major repair mechanism in response to several sources of PM injury, including laser, mechanical, detergent, and PFT-induced damage [78, 79, 102, 108, 110, 114, 521]. Our data demonstrate that shedding of EVs is more than a stochastic event that only serves to eliminate the damaged PM. Notably, in addition to allowing the physical removal of CDCs from the cell surface [110, 411, 521], the release of EVs may serve as a communication vehicle and a transporter of repair proteins. Importantly, both CDC-positive and CDC-negative EVs were detected, raising the hypothesis that these two populations may have different functions, and accordingly the CDC might not be the only difference in their proteomic profile. Also, given that the proteomic profile of EVs released by NI and intoxicated cells is substantially different, it is worth speculating that EVs released upon PM damage follow secretion routes that differ from canonical EVs, with a cargo that might be stimulus-specific.

Here, we provide the full protein repertoire of EVs released in the context of intoxication and supply a high value list of proteins that are potentially new actors in PM repair of CDC-induced damage, as well as in the response to other types of PM damage. In particular, EVs released by intoxicated cells appear to be enriched in calcium-binding proteins, molecular chaperones, cell adhesion molecules; and cytoskeletal, scaffold and membrane trafficking proteins. Of note, a previous study analyzed the proteins enriched in microvesicles released by PLY intoxicated cells using as a control the total membrane isolates from intoxicated cells [521]. Despite the identification of some overlapping proteins (e.g., annexins), we also found differences in the identified proteins, which may be related to the different controls used.

Among the proteins of interest identified here are CPNE1 and CPNE3, AHNAK and sorcin. As mentioned, CPNE1 and CPNE3 are phospholipid-binding proteins that respond to intracellular Ca^{2+} increases, thereby translocating from the cytoplasm to the cell cortex [533, 534]. Additionally, there is increasing evidence suggesting that copines may mediate signal transduction [535, 536] and membrane transport, namely by interacting with annexins [537, 538]. Our data here reveal CPNE1 and CPNE3 as critical components for an efficient PM repair in response to PLY-induced damage;

however, their specific molecular role in this process needs to be further studied. In particular, CPNE1 and CPNE3 protein partners during repair need to be identified. Additionally, whether CPNEs are required for the repair of other types of PM damage is still unknown. The neuroblast differentiation- associated protein AHNK was previously associated with actin cytoskeleton remodeling and membrane repair through interactions with annexins [189, 539]. Notably, AHNK was found in vesicles that are rapidly exocytosed and recycled back in response to Ca^{2+} increase [540, 541]. Finally, sorcin, which is also a Ca^{2+} -binding protein, was reported to be involved in vesicular trafficking and co-localizes with Rab11 [542], a vesicular trafficking protein required for PM repair and pore removal of a non-CDC PFT [144].

Additionally, some chromatin-associated proteins (essentially histones) and translational proteins (ribosomal proteins) were unexpectedly detected enriched in EVs. However, their presence in EVs does not necessarily indicate that they are involved in repair and may have signaling functions that need to be investigated. Several histones were modified in response to different CDCs to regulate gene expression [543, 544]. Noteworthy, histones were detected in EVs released in other contexts, and it was speculated that they may act as damage-associated molecular pattern signals controlling cell signaling and innate immunity [545]. Concerning the translational proteins, it is possible that such proteins are packed together with specific RNA molecules, which together with proteins are commonly included in EVs. Ribosome-associated proteins were often detected in the EVs released in other contexts [546, 547]. The presence of RNA in EVs released by intoxicated cells and their association with ribosomal proteins needs to be further investigated.

Our work reveals the proteomic content of EVs released by cells upon bacterial CDCs attack, confirms the role of these EVs in the elimination of the CDC pore and identifies new proteins involved in PM repair, giving new insights into the cell machinery engaged in response to PM damage. Further studies are now required to assess the exact molecular role of these proteins in PM repair.

4. Materials and Methods

4.1. Cell lines and reagents culture

HeLa (ATCC CCL-2) and HEK293T (ATCC CRL-3216) cells (ATCC, Manassas, VA, USA) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with glucose and

L-glutamine (Lonza, Basel, Switzerland) and supplemented with 10 % fetal bovine serum (FBS; Biowest, Nuaille, France). Cells were maintained at 37 °C in a humidified 5 % CO₂ atmosphere.

4.2. Cell intoxications

LLO and PLY toxins were purified as described [548]. Intoxications were performed in Hanks' balanced salt solution (HBSS) (Lonza, Basel, Switzerland) for 15 min, at the indicated toxin concentrations. For recovery assays, the toxins were washed out twice with PBS Ca²⁺-free and allowed to recover from damage in DMEM supplemented with 10 % FBS for 20 h. For experiments aiming to assess the importance of extracellular Ca²⁺ concentration, intoxications and recovery assays were carried out in HBSS Ca²⁺-free medium and supplemented with the indicated Ca²⁺ concentrations.

4.3. Flow cytometry analysis

Plasma membrane integrity was assessed by flow cytometry, following propidium iodide (PI) permeability assays. HeLa cells (2.5×10^5) were seeded in six-well plates 20 h before intoxicated as indicated and allowed to recover from damage. Immediately after toxin washout (T 0 h) and after 20 h of recovery, cells were trypsinized and resuspended in 0.5 ml of PBS containing 2 % of FBS. Samples were then incubated with PI at 2 µg/ml for 1 min and fluorescence intensity was measured at the FL3 channel on a BD Accuri™ C6 flow cytometer (BD Biosciences, San Jose, CA, USA). At least 10 000 cells were collected after doublet discrimination and data was analyzed using FlowJo Software (Version 10.8 TreeStar Inc., Ashland, OR, EUA).

For the analysis of HeLa WT, KO CPNE1 and KO CPNE3 cell lines, 3×10^5 cells were seeded in 6-well plates 24 h before intoxication with 0.2 nM of PLY. PI incorporation was measured as described above immediately after toxin washout (T 0 h) and after recovery during the indicated time points. At least 16 000 cells were collected and analyzed.

4.4. Immunofluorescence microscopy quantifications

Cells intoxicated as described in section 4.2, were fixed with 4 % paraformaldehyde (PFA; 15713, EMS, Hatfield, PA, USA) for at least 10 min at room

temperature, quenched with 0.1 M NH_4Cl for 40 min, permeabilized with 0.1 % Triton-X100 in PBS for 5 min and washed 3x in PBS. Coverslips were incubated for 30 min with Phalloidin Alexa Fluor 488 (Invitrogen, Waltham, MA, USA) and DNA was stained with DAPI (Sigma, St Louis, MO, USA). Coverslips were mounted onto microscope slides with Aqua-Poly/Mount (Polysciences, Warrington, PA, USA). Images were collected with an epifluorescence Olympus BX63 (Olympus, Tokyo, Japan) microscope equipped with a 20 x 0.75 NA objective lens. DAPI nuclear staining was used to quantify the number of cells after washout (0 h) and after 20 h of recovery after intoxication. The number of cells/field was quantified in blind in 5 random fields for each condition from three independent experiments.

4.5. Isolation of extracellular vesicles (EVs)

HeLa cells (3×10^6) were seeded in 100 mm tissue culture dishes and incubated in DMEM supplemented with 10% EVs-depleted FBS. Then, 20 h later, cells were left non- intoxicated (NI) in HBSS or intoxicated with 1 nM of LLO, 0.2 nM of PLY or GFP-PLY (for immunogold labeling assays) during 15 min. NI and intoxicated cells were then washed twice in PBS Ca^{2+} -free and allowed to recover from damage in serum-free DMEM for 3 h. Cell culture supernatant was collected from 20 tissue culture dishes and EVs were isolated by differential centrifugation as described in [549] with slight modifications. Briefly, cell culture supernatants were collected and centrifuged at $2000 \times g$ for 20 min at 4 °C to remove cell debris, transferred to polypropylene tubes and centrifuged at $30,000 \times g$ for 40 min at 4 °C. The resulting EV pellet was washed in PBS, centrifuged again at $30,000 \times g$ (40 min at 4 °C) and resuspended in 70 μL of PBS. Samples were immediately processed for transmission electron microscopy (TEM) experiments or stored at 4 °C for 24 h for immunogold labeling and LC-MS/MS analysis. For longtime storage, EVs were kept at -80 °C until further processing.

4.6. Nanoparticle tracking assay

Size distribution profiles of isolated EVs were obtained using the NanoSight NS300 system (Malvern Panalytical, Worcestershire, UK). Diluted vesicle suspensions were loaded into the instrument and 30 s videos were recorded in triplicates for each sample, with camera level set at 15 and detection threshold set at 5. The videos were

analyzed with the NTA 3.2 software (Malvern Panalytical, Worcestershire, UK) to assess size distribution.

4.7. Electron Microscopy and immunogold labeling

The morphology of EVs was assessed using negative staining TEM. Briefly, formvar- carbon coated EM-grids were glow discharged for 20 s at 10 mA. 5 μ L of EV sample was added to the grid and allowed to adhere for 5 min. EVs were then stained with 7 μ L of 1% uranyl acetate, excess of liquid was dropped onto a piece of filter paper and grids were allowed to dry prior to imaging. For immunogold labeling, EVs samples were fixed in 2% PFA for at least 10 min at 4 °C. Glow discharged formvar-carbon coated EM-grids were floated on top of 10 μ L drops of fixed EVs for 20 min, washed in PBS and incubated with PBS 0.02% glycine for 10 min, to quench free aldehyde groups. Grids were washed in PBS, incubated in PBS 2% BSA containing 0.1% saponin for 30 min, washed in PBS 0.5% BSA and incubated with PBS 1% BSA containing primary antibody mouse anti-LLO (1:5, antibody B8B20-3-2 [550]) or rabbit anti-GFP (1:10, antibody Ia1 produced in our lab) for 2 h 30 min at room temperature. The grids were washed three times in PBS 0.5% BSA then incubated with a goat anti-mouse IgG conjugated to 15 nm gold (1:20, EM GMHL15/2, BBI-Solutions OEM Limited, Cardiff, UK) or anti-rabbit IgG conjugated to 10 nm gold (1:20; Sigma, St Louis, MO, USA) for 1 h at room temperature. The grids were washed three times in PBS 0.5% BSA, incubated with 1% glutaraldehyde for 5 min and washed three times in deionized water prior to negative staining. Images were acquired on a JEOL JEM 1400 TEM (JEOL, Tokyo, Japan) 80 kV equipped with a CCD digital camera Orious 1100 W. Digital images of EVs from each sample were taken from 3 independent experiments.

4.8. Proteomics sample preparation and LC-MS/MS analysis

LLO- and PLY-induced EVs were isolated from the supernatant of intoxicated HeLa cells allowed to recover for 3 h in serum-free medium. Control EVs were isolated from the supernatant of NI HeLa cells cultured in DMEM supplemented with 10% EV-depleted FBS (control) or 1% EV-depleted FBS (control-starved), for 72 h or 48 h, respectively. Proteins were solubilized with 100 mM Tris pH 8.5, 1% sodium deoxycholate, 10 mM tris(2- carboxyethyl) phosphine (TCEP) and 40 mM chloroacetamide for 10 min at 95 °C under 1000 rpm (Thermomixer, Eppendorf, Hamburg, Germany). Each sample was

processed for proteomics analysis following the solid-phase-enhanced sample-preparation (SP3) protocol as described in [551]. Enzymatic digestion was performed with 2 µg of Trypsin/LysC overnight at 37 °C under 1000 rpm. Tryptic sample peptides were dissolved in 0.1% formic acid and 500 ng per sample injected for LC-MS/MS analysis into an Ultimate 3000 RSLC- nano system. Trapping was performed at 10 µL/min for 3 min in loading solvent (0.1% formic acid) on a 0.5 cm trapping cartridge (Acclaim PepMap C18 100 Å, 5 mm × 0.3 mm, 160454, Thermo Scientific, Waltham, MA, USA). Peptide separation after trapping was performed on a 50 cm EASY-Spray column (ES803, PepMap RSLC, C18, 2 µm, Thermo Scientific, Waltham, MA, USA) at 35 °C.

Peptides were eluted by a non-linear gradient from 2.5 to 50% MS solvent B (0.1% FA 80% ACN) over 148 min, at a constant flow rate of 250 nL/min, followed by a 15 min washing at 99% MS solvent B. Re-equilibration with 97.5% MS solvent A (0.1% FA in water) was performed at 250 nL/min for 17 min adding up to a total run length of 180 min. Peptides were analyzed by a data-dependent top10 acquisition method in positive ion mode on a Q Exactive Orbitrap instrument (Thermo Scientific, Waltham, MA, USA). MS1 scans were acquired at m/z 380–1580 with an MS1 AGC target of 3E6 ions with a max. ion injection time of 60 ms at a resolution of 70,000. MS2 scans were acquired at resolution 35,000 with an AGC target of 2E5 with 110 ms max. injection time, 2Da isolation window, intensity threshold 7.3E4, exclusion of unassigned, 1, 8, >8 positively charged precursors, peptide match preferred, exclude isotopes on, dynamic exclusion time 45 s. The scan range for MS2 spectra was set from 200 to 2000 m/z. HCD energy was set to 27% NCE, and the background ion at 445.12003 Da was used as lock mass. The spray voltage was set to 1.9 kV.

4.9. Proteomics data analysis

LC-MS/MS runs of all 12 biological samples were searched together combining technical replicates for statistical reasons using the MaxQuant algorithm (created by Jürgen Cox, version 1.6.9.0, <https://www.maxquant.org/>, accessed on 9 November 2022, Max-Planck Institute of Biochemistry, Martinsried, Germany). Default search settings were used, including a false discovery rate set at 1% on PSM and protein level. Spectra were searched against the human protein sequences in the Swiss-Prot database (database release version of 2018_11), containing 20,412 sequences [552],

supplemented with the LLO and PLY sequences (P13128 and Q04IN8, respectively) available at UniProt database. The mass tolerance for precursor and fragment ions was set to 4.5 and 20 ppm, respectively, during the main search. Enzyme specificity was set to the C-terminal of arginine and lysine, also allowing cleavage next to prolines with a maximum of two missed cleavages. Variable modifications were set to oxidation of methionine residues and acetylation of protein N- termini. Matching between runs was enabled with a matching time window of 0.7 min and an alignment time window of 20 min. Only proteins with at least one unique or razor peptide were retained, leading to the identification of 4585 protein groups. Proteins were quantified by the MaxLFQ algorithm integrated into the MaxQuant software. A minimum ratio count of two unique or razor peptides was required for quantification. Further data analysis was performed with the Perseus software (version 1.6.7.0) after uploading the protein groups file from MaxQuant [553]. Reverse database hits were removed, and replicate samples were grouped. Proteins with less than three valid values in at least one group were removed, and missing values were imputed from a normal distribution around the detection limit resulting in 2110 quantified proteins, which were subsequently used for further data analysis. These quantified proteins were subjected to two-sided, unpaired t-tests using permutation-based multiparameter correction with 1000 randomizations and an FDR of 1% plotting eVs from LLO-treated cells against control eVs, eVs from PLY-treated cells against control eVs, and eVs from all toxin-treated cells (PLY and LLO, mentioned as PFTs) against all control eVs (control and control-starved). The result of the latter t-test is shown in the volcano plots in Figure 5B. For each protein, the $\log_2$ (PFT/controls) fold change value is indicated on the X-axis, whereas the statistical significance ($-\log$ p-value) is indicated on the Y-axis. Proteins outside the curved lines, set by an FDR value of 0.05 and an S0 value of 1 in the Perseus software, represent specific significant upregulated or downregulated proteins. The 615 proteins identified with statistically different abundances between toxin-treated eVs were taken further in Perseus for z-scoring of the LFQ intensities and hierarchical clustering, resulting in the heat maps presented in Figure 5C.

4.10. Establishment of CPNE1 and CPNE3 knockout HeLa cells by CRISPR/Cas9

Oligonucleotides targeting the gene encoding for CPNE1 and CPNE3 were designed using Benchling [554]. Two independent sequences were designed for each gene, targeting exons 4 and 10 of CPNE1 and exons 4 and 13 of CPNE3 (pre-gRNA

listed in Table S6). Forward and reverse sequences were annealed and cloned in pLentiCRISPR V2 plasmid gift from Feng Zhang; Addgene plasmid #52961) [555] using the *BsmBI* site. HEK293T cells transfected to assemble and release lentivirus expressing the gRNAs targeting CPNE1 and CPNE3 encoding genes. Briefly, transfections were performed using LipofectamineTM 2000 (Invitrogen, Waltham, MA, USA) diluted in OptiMEM (Thermo Scientific, Waltham, MA, USA) and mixed with 0.5 µg of plasmid p8.91 (gift from Simon Davis; Addgene plasmid #187441); 0.5 µg of plasmid pMDG (gift from Simon Davis; Addgene plasmid #187440) [556] and 0.25 µg of pLentiCRISPR V2 harboring the selected gRNAs. Lentivirus was recovered 72 h later from the supernatants of transfected HEK293T cells. HeLa cells were seeded at 1×10^6 cells per well in 6-well plates and incubated at 37 °C in a humidified atmosphere with 5% CO₂. When 50% confluency was reached, they were incubated with the recovered lentivirus and selected with 2.5 µg/mL of puromycin.

4.11. Generation of GFP-tagged CPNE1 and CPNE3

DNA fragments encoding CPNE1 and CPNE3 were amplified by PCR from the cDNA of HeLa cells, using the high fidelity ACCUZYMETM DNA polymerase (Bioline, London, UK) and the primers listed in Table S6. The generated PCR fragments were cloned into the plasmid pEGFP-C1 (Clontech, Mountain View, CA, USA). The CPNE1 encoding fragment was inserted using the restriction sites XhoI and Sall (New England BioLabs, Ipswich, MA, USA), and the CPNE3 fragment was cloned into XhoI and BamHI (New England BioLabs, Ipswich, MA, USA) restriction sites. The generated plasmids allowed the expression of GFP-tagged CPNE1 or CPNE3 variants, in which the GFP is placed at the N-terminus of the fusion protein (GFP-CPNE1 or GFP-CPNE3). The constructs were verified by sequencing.

4.12. Transfection and immunofluorescence microscopy

HeLa cells (3×10^5) were seeded on glass coverslips in 6-well plates and transfected 20 h after with 3 µg of plasmid encoding GFP-CPNE1 or GFP-CPNE3 using jetPRIME[®] transfection reagent (Polyplus, Illkirch, France) following manufacturer's instructions. Cells were left non-intoxicated or incubated with 0.2 nM PLY for 10 min, fixed with 4% PFA for at least 10 min at room temperature, quenched with 0.1 M NH₄Cl for 40 min at 4 °C, permeabilized with 0.1% Triton-X100 in PBS for 5 min, and washed

3× in PBS. Afterward, coverslips were incubated for 1 h with rabbit anti-NMHCIIA (Sigma, St Louis, MO, USA) followed by 45 min with a goat anti-rabbit coupled to CY3 (Jackson ImmunoResearch, West Grove, PA, USA). DNA was stained with DAPI (Sigma, St Louis, MO, USA). Coverslips were mounted onto microscope slides with Aqua-Poly/Mount (Polysciences, Warrington, PA, USA). Images were acquired with a Leica Scanning Confocal SP5 equipped with a 63 × 1.30 NA Glycerol objective lens and processed using Image J [557].

4.13. Immunoblotting assay

HeLa cells or EVs were directly lysed in Laemmli buffer (0.25 mM Tris-HCl pH 6.8; 10% SDS; 50% glycerol; and 5% β-mercaptoethanol). Total protein extracts were resolved by SDS-PAGE and transferred onto nitrocellulose membranes (Hybond ECL; GE Healthcare, Chicago, IL, USA). Primary antibodies (mouse anti-LLO (1:100, antibody B8B20-3-2 [62]), rabbit anti-GFP (1:100, antibody Ia1 produced in our lab), mouse anti-Copine 1 (1:250, sc-101269 Santa Cruz Biotechnology, Dallas, TX, USA), mouse anti-Copine 3 (1:500, sc-390143, Santa Cruz Biotechnology, Dallas, TX, USA) and anti-actin (1:5000, AC15 Sigma Aldrich, St Louis, MO, USA) and secondary antibodies (anti-mouse HRP or anti-rabbit HRP 1:1000, Abliance, Compiègne, France) were diluted in TBS-Tween (150 mM NaCl; 50 mM Tris-HCl, pH 7.4; and 0.1% Tween). A signal was detected using ECL (Thermo Scientific, Waltham, MA, USA).

4.14. Statistical analysis

Statistical analyses were performed using Prism GraphPad 6 software (GraphPad Software, La Jolla, CA, USA, www.graphpad.com, accessed on 2 January 2019). Two-way ANOVA with Dunnett's post-hoc analyses was used to compare the means of samples in relation to a control sample, within each condition. One-way ANOVA with Tukey's post-hoc analyses were used for pairwise comparisons of more than two different means. Two-tailed unpaired Student's *t*-test was used for the comparison of means between two samples. Details regarding the statistical analysis of proteomic data are provided in the corresponding figure legends and/or methods sections above.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/toxins15010004/s1>. Figure S1: Cells

recover full PM integrity 3 h after CDCs washout. Figure S2: Volcano plots showing the protein levels. Figure S3: Immunoblot showing the levels of CPNE1 and CPNE3. Supplementary Tables. Tables providing proteomics data. Table S1: List of proteins detected and reliably quantified in EVs released from control HeLa cells, LLO- and PLY-intoxicated cells. Table S2: List of proteins showing significantly different abundances in EVs released by control and control-starved HeLa cells. Table S3: List of proteins overrepresented in EVs released by LLO-intoxicated HeLa as compared to control EVs. Table S4: List of proteins overrepresented in EVs released by PLY-intoxicated HeLa cells as compared to control EVs. Table S5: List of proteins showing significantly different abundances in EVs released from both LLO- and PLY-intoxicated HeLa cells as compared to EVs from control cells. Table S6: Sequence of the oligonucleotides designed as gRNA for CRISPR/Cas-9 gene editing and used for the cloning of CPNE1 and CPNE3 into pEGFP-C1. Video S1: Representative video recorded in a NanoSight NS300 system using EVs from non-intoxicated cells. Video S2: Representative video recorded in a NanoSight NS300 system using EVs from LLO-intoxicated cells. Video S3: Representative video recorded in a NanoSight NS300 system using EVs from PLY-intoxicated cells.

Supplementary Materials

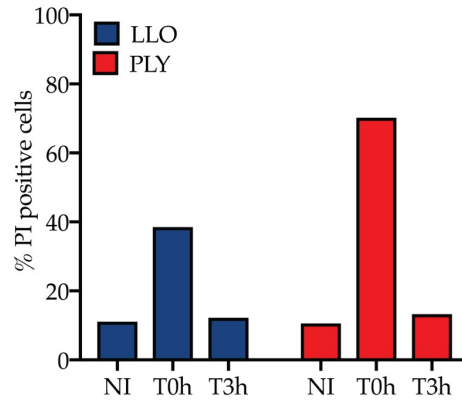


Figure S1. Cells recover full PM integrity 3 h after PFTs washout. Cells were left non-intoxicated (NI) or were intoxicated with LLO (1 nM, blue bars) or PLY (0.2 nM, red bars) for 15 min. PM permeability was measured immediately after 15 min of intoxication (T 0 h) and after 3 h of PFTs washout (T 3 h), by flow cytometry following incorporation of the membrane impermeable dye propidium iodide (PI). Graph shows representative data from a single experiment.

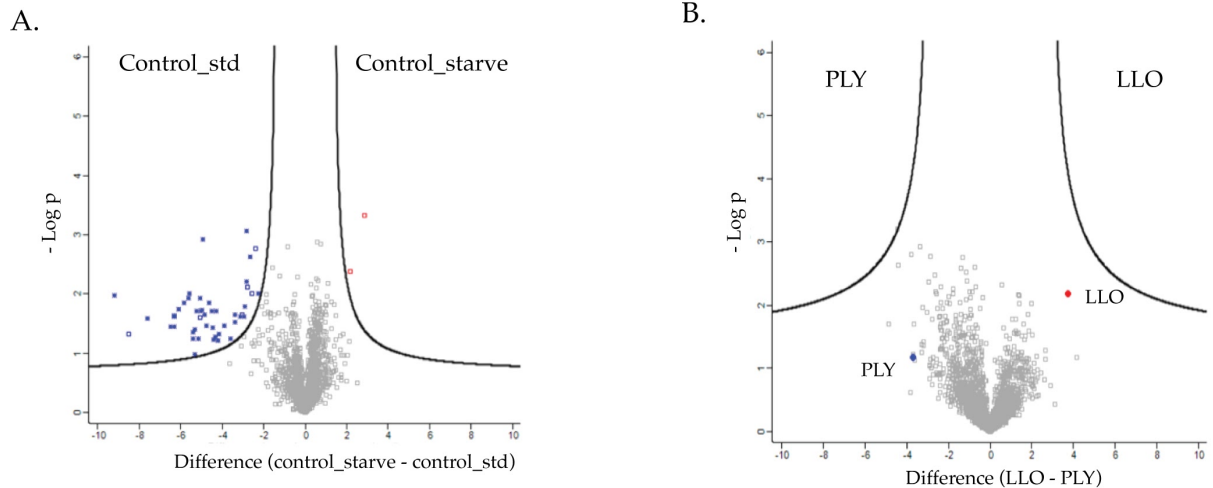


Figure S2. Volcano plots showing the protein levels (x axis), represented in fold change (in $\log_2$), detected on A) EVs released from control HeLa cells compared to EVs collected from control starved HeLa cells and B) EVs released from PLY-intoxicated HeLa cells compared to EVs collected from LLO-intoxicated HeLa cells. Three independent experiments were analysed and Student t-tests were applied to calculate $-\log p$ values for each protein (y axis). The curved black lines represent the threshold for statistical significance determined by t-test (FDR=0.05).

and $S0=1$). Blue and red squares correspond to overrepresented proteins in the specific condition.

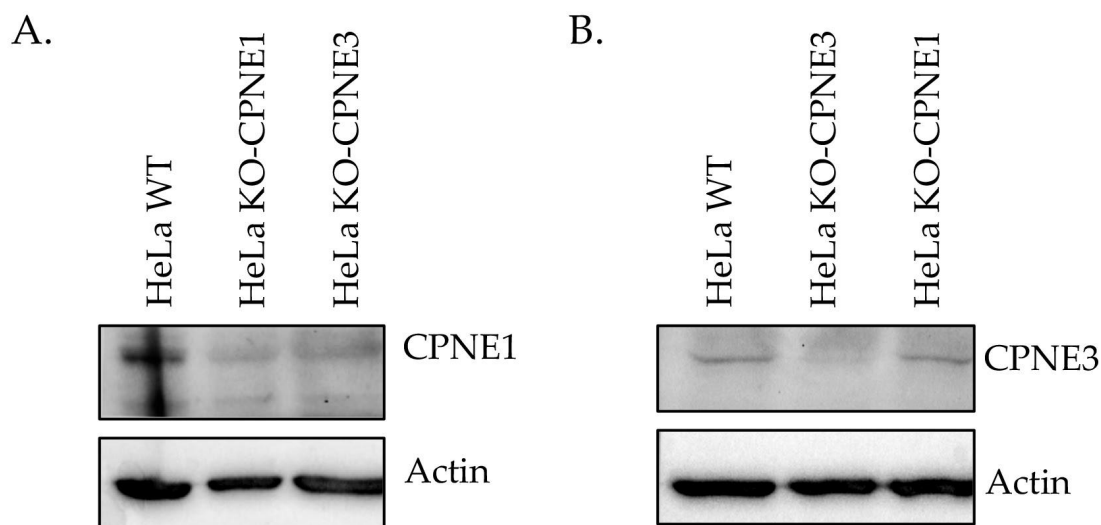


Figure S3. Immunoblot showing the levels of (A) CPNE1 and (B) CPNE3 in WT, KO-CPNE1 and KO-CPNE3 HeLa cells. Total protein extracts were collected from WT, KO-CPNE1 and KO-CPNE3 HeLa cells, western blot analysis was performed, and membranes were stained using (A) CPNE1 or (B) CPNE3 antibody. Actin was used as loading control.

Supplementary Tables. Tables provide proteomics data. **Table S1.** List of proteins detected and reliably quantified in EVs released from control HeLa cells, LLO and PLY-intoxicated cells. **Table S2.** List of proteins showing significantly different abundances in EVs released by control and control starved HeLa cells. **Table S3.** List of proteins showing significantly different abundances in EVs released from PLY-intoxicated and LLO-intoxicated HeLa cells. **Table S4.** List of proteins overrepresented in EVs released by LLO-intoxicated HeLa as compared to control EVs. **Table S5.** List of proteins overrepresented in EVs released by PLY-intoxicated HeLa cells as compared to control EVs. **Table S6.** List of proteins showing significantly different abundances in EVs released from both LLO and PLY-intoxicated HeLa cells as compared to EVs from control cells

Table S7. Sequence of the oligonucleotides designed as gRNA for CRISPR/Cas-9 gene editing and used for the cloning of CPNE1 and CPNE3 into pEGFP-C1.

CPNE1 exon 4 (gRNA)	Forward	5´ CAC CGT TGG CCG GAC TGA ACG GGT G 3´
	Reverse	5´ AAA CCA CCC GTT CAG TCC GGC CAA 3´
CPNE1 exon 10 (gRNA)	Forward	5´ CAC CGT TTT CTG CTG CTT CTC AGG G 3´
	Reverse	5´ AAA CCC CTG AGA AGC AGC AGA AAA C 3´
CPNE3 exon 4 (gRNA)	Forward	5´ CAC CGA GTG ATG ATG ACT TCT TAG 3´
	Reverse	5´ AAA CCT AAG AAG TCA TCA TCA CTC 3´
CPNE3 exon 13 (gRNA)	Forward	5´ CAC CGC GCT CAG ATA CCT CCT CAG 3´
	Reverse	5´ AAA CCT GAG GAG GTA TCT GAG CGC 3´
GFP-CPNE1 (cloning in pEGFP-C1)	Forward	5´ ATT CTC GAG ACG CCC ACT GCG TGA CCT TGG 3´
	Reverse	5´ ATT GTC GAC CTA GGC CTG GGG GGC CTG TG 3´
GFP-CPNE3 (cloning in pEGFP-C1)	Forward	5´ ATA CTC GAG CAG CTG CCC AGT GTG TCA C 3´
	Reverse	5´ TCC GGA TCC TCA CTG CTT CTG TTG TTT CGT GGC 3´

Section 2

S100A4 is a central orchestrator of cytoskeletal remodeling triggered by pneumolysin during pneumococcal infection

In this section, we explored and characterized the cytoskeletal remodeling triggered by PLY during intoxication and infection by *S. pneumoniae*. We discovered that PLY triggers a transient remodeling of the actomyosin cytoskeleton that correlates with PM blebbing, depicted by the cortical accumulation of soluble non-muscle heavy chain IIA (NMHCIIA). We characterized these accumulations and identified S100A4 as a major regulator of the cytoskeletal remodeling triggered upon PLY challenge.

I contributed to the conceptualization and design of the experiments, collected and analyzed all the data. I also prepared all the figures and drafted the manuscript.

This article is still in preparation for submission.

(Manuscript in preparation)

S100A4 is a central orchestrator of cytoskeletal remodeling triggered by pneumolysin during pneumococcal infection

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Abstract

The plasma membrane (PM) serves as a critical barrier, shielding cells from external cues and contributing to the maintenance of cellular homeostasis. Pore-forming toxins (PFTs) are major bacterial virulence factors that generate pores on the PM of host cells thereby breaching epithelial barriers and facilitating bacterial dissemination. Among PFTs, Pneumolysin (PLY) stands out as a major virulence factor released by the highly pathogenic *Streptococcus pneumoniae*. In the early stages of infection, PLY is released at low levels prompting cells to engage repair mechanisms to ensure survival and limit bacterial spread. Here, we show that S100A4 protects cells against damage inflicted by PLY during infection with *S. pneumoniae*. S100A4 stands out as a novel regulator of the transient remodeling of the host cell cytoskeleton required for the proper activation of key PM repair processes such as blebbing and shedding. Collectively, our findings point to an active involvement of S100A4 in the host protection upon damage with PLY during infection with *pneumococcus*.

1. Introduction

Streptococcus pneumoniae (pneumococcus) is a gram-positive extracellular bacteria that often asymptotically colonizes the human upper respiratory tract [277]. However, pneumococci have high capacity to environment adaptation, and can invade aseptic regions of the lower respiratory tract and spread throughout the organism leading to severe diseases such as pneumonia and meningitis [558]. Despite the globally implemented vaccination strategies, pneumococcal infections are still a leading cause of morbidity and mortality worldwide [559], being a major public health concern aggravated by the recently pointed out high rates of antibiotic resistance in *S. pneumoniae* [560-562]. Thus, comprehending how pneumococcal virulence determinants act and how host cells can effectively block them is critical to developing new therapeutic tools.

Pneumolysin (PLY) is a pivotal virulence factor required during all stages of *S. pneumoniae* pathogenesis, from the colonization of the nasal cavity to the invasion of deep tissues [517]. It is a cholesterol-dependent pore-forming toxin (PFT) that creates stable pores in the plasma membrane (PM) of host cells altering its permeability properties, disrupting cellular homeostasis and eventually causing cell death, concomitantly allowing bacterial dissemination [76, 517]. We had previously shown that *in vitro* in cultured epithelial cell lines the extent of damage and the cell fate rely on PLY concentration and that the PM resealing depends on calcium-mediated activation of PM repair mechanisms [563].

In addition, we demonstrated that distinct PFTs induce PM remodeling events such as blebbing and shedding, which correlate with effective membrane repair [102, 519, 520, 563]. Coordinated PM blebbing is supported by the reorganization of the cortical actomyosin cytoskeleton, characterized by the calcium-dependent accumulation of the heavy chain of non-muscle myosin-IIA (NMHCIIA) and its intricate interplay with endoplasmic reticulum (ER) components such as Gp96 [102, 519, 520]. These events are required for the recovery of PM integrity, indeed the disruption of cytoskeleton-ER interconnection leads to uncontrolled blebbing, impaired PM repair, and heightened host susceptibility to bacterial infection [102]. Challenge of human epithelial cell lines with purified PLY or Listeriolysin O (LLO, a cholesterol-dependent cytolysin produced by *Listeria monocytogenes*) also promote increased vesicular shedding, which has been proposed as a major PM repair mechanism [563]. Indeed,

extracellular vesicles released by intoxicated cells contain the toxin itself and are packed with hallmarks of PM repair (several annexins, ESCRT-III components) and with new proteins that despite not being previously associated to PM damage might possibly function in PM repair such as calcium-responding and cytoskeleton-associated proteins [563].

Our aim here is to investigate the molecular pathways underlying PM events and repair mechanisms triggered by PLY pore formation, with particular emphasis on the reorganization of the actomyosin cytoskeleton. We unveil that cells challenged with sub-lytic doses of purified PLY or infected with PLY-producing pneumococci undergo transient remodeling of their actomyosin cytoskeleton. First, cortical NMHCIIA filaments are disrupted and NMHCIIA accumulates in its soluble form, to later reorganize into filaments, these transient changes correlate with damage resolution and restoration of PM integrity. We show that PLY triggers the calcium-dependent interaction of NMHCIIA with S100A4, a calcium-responsive protein present in the EVs released by cells responding to PLY [563]. In addition, we demonstrate that S100A4 is actively recruited to the PM upon PLY challenge and shields cells against PM damage triggered by PLY alone and during infection with pneumococcus. Furthermore, our investigation suggests that S100A4 potentially orchestrates cytoskeletal remodeling required for efficient PM blebbing and shedding, which are crucial for proficient PM repair following PLY-induced damage in the context of lung infection by *S. pneumoniae*.

2. Results

2.1. PLY triggers a transient remodeling of the host cytoskeleton that correlates with host PM damage

We previously reported the cortical accumulation of non-muscle heavy chain myosin IIA (NMHCIIA) as a hallmark of PM repair of damage inflicted by the bacterial cholesterol-dependent cytolysins LLO and perfringolysin (PFO) [102, 519]. Given that PLY shares functional and structural features with LLO and PFO (all belonging to the CDC family) we assess here whether PLY also triggers similar cytoskeleton remodeling and if this process is co-related to damage resolution.

HeLa cells were challenged with two different concentrations of PLY for 10 minutes. We then assessed PM damage and cellular capacity to restore membrane

integrity after PLY washout by using propidium iodide (PI) incorporation assays. Actomyosin remodeling was evaluated at different time points by confocal microscopy imaging after NMHCIIA immunostaining. We found that PLY, as we described before for LLO and PFO [102, 519], triggers a massive cytoskeleton remodeling with specific accumulations of NMHCIIA being observed in the cortex of challenged cells, in a dose-dependent manner (Figure 1 A, B). These cytoskeletal alterations correlate with the loss of PM membrane integrity determined by the increasing percentage of PI-positive cells 10 min after PLY intoxication (timepoint 0 h, Figure 1C). Of note, cells incubated with the highest PLY concentration (0.5 nM) were not able to fully recover from PLY-induced damage, suggesting that such concentration is already too high, causing at some extent irreversible damage (Figure 1C). Concomitantly with damage and actomyosin remodeling, cells undergo massive blebbing immediately after the toxin challenge (Movie S1). Following toxin washout, NMHCIIA accumulations dissipate, as shown in the representative images and by the reduced percentage of cells displaying such accumulations (Figure 1 A, B). The integrity of the PM is rapidly restored as indicated by the decreased levels of PI-positive cells at timepoints 1, 4 and 20 h (Figure 1 C). Given that different cell types may have different susceptibilities to PLY [522] and that *S. pneumoniae* is a respiratory pathogen, we aimed to confirm the data obtained in HeLa cells challenged with purified PLY, in more relevant experimental models such as epithelial lung cells. For that, we used H292, a lung epithelial cell line widely used to study host-pathogen interactions in the respiratory tract [564, 565]. H292 cells were intoxicated with growing concentrations of purified PLY for 10 min and analyzed as described above for HeLa cells. We found that PLY also triggers the transient cytoskeleton remodeling in H292 cells, with specific cortical NMHCIIA accumulations (Figure 1 D). The percentage of cells displaying these accumulations increased with growing PLY concentrations, showing a dose-dependent response (Figure 1E), however, the percentage of PI-positive cells remains roughly the same irrespectively to PLY concentration, suggesting that the lower PLY concentration is already sufficient to cause damage in a high percentage of cells (Figure 1 F, 0 h). Similarly, to what we described for HeLa cells, NMHCIIA accumulations and the permeability to PI strongly decrease upon PLY washout (Figure 1 D, E, F). Nevertheless, cells intoxicated with the highest PLY concentration (1 nM) still display NMHCIIA accumulations 4 h after toxin washout and do not recover PM integrity as efficiently as cells treated with lower doses

of PLY, suggesting that cells do not cope with 1 nM PLY induced damage. Altogether, our findings indicated that the NMHCIIA cortical accumulations are intimately related to PLY-induced damage and that these accumulations occur in different cell lines, underscoring their overarching significance.

PLY is produced and released by almost all clinical isolates and *S. pneumoniae* serotypes [566] being a crucial virulence factor for the development of pathogenesis in the host [517]. To investigate whether the remodeling of the cytoskeleton also takes place during pneumococcal infection or is instead a specific response to purified PLY, HeLa cells were infected with *S. pneumoniae* TIGR4 (serotype 4) wild-type (*Sp.* WT) or with its PLY-deficient mutant (*Sp. Δply*) strains for 1 h. Infected cells were fixed and processed for confocal microscopy analysis. While the infection with *Sp.* WT strain led to actomyosin remodeling and NMHCIIA accumulations in the cell cortex, the infection with *Sp. Δply* strain had no detectable effect as compared to non-infected (NI) cells (Figure 1G). Quantification on confocal microscopy images shows that 20 % of the *Sp.* WT-infected cells display NMHCIIA accumulations while those were undetectable in NI and *Sp. Δply*-infected cells (Figure 1H). These data show that the remodeling of the host cytoskeleton is a global response that also occurs during pneumococcal infection and confirms that it relies on PLY. We further assessed PLY-induced PM damage and cellular capacity to repair in the context of infection with *Sp.* WT or *Sp. Δply*. Consistent with our previous results, cells infected with *Sp.* WT, but not those infected with *Sp. Δply*, showed increased permeability to PI, indicating that *S. pneumoniae* causes PLY-mediated PM damage during cellular infection. Additionally, cells exhibit the capacity to partially restore PM integrity after bacterial washout, confirming their ability to repair the damage inflicted by PLY in the context of *S. pneumoniae* infection. Our data points to the intricate interplay between PLY-induced damage and the cytoskeletal remodeling crucial for cells to restore PM integrity and survive.

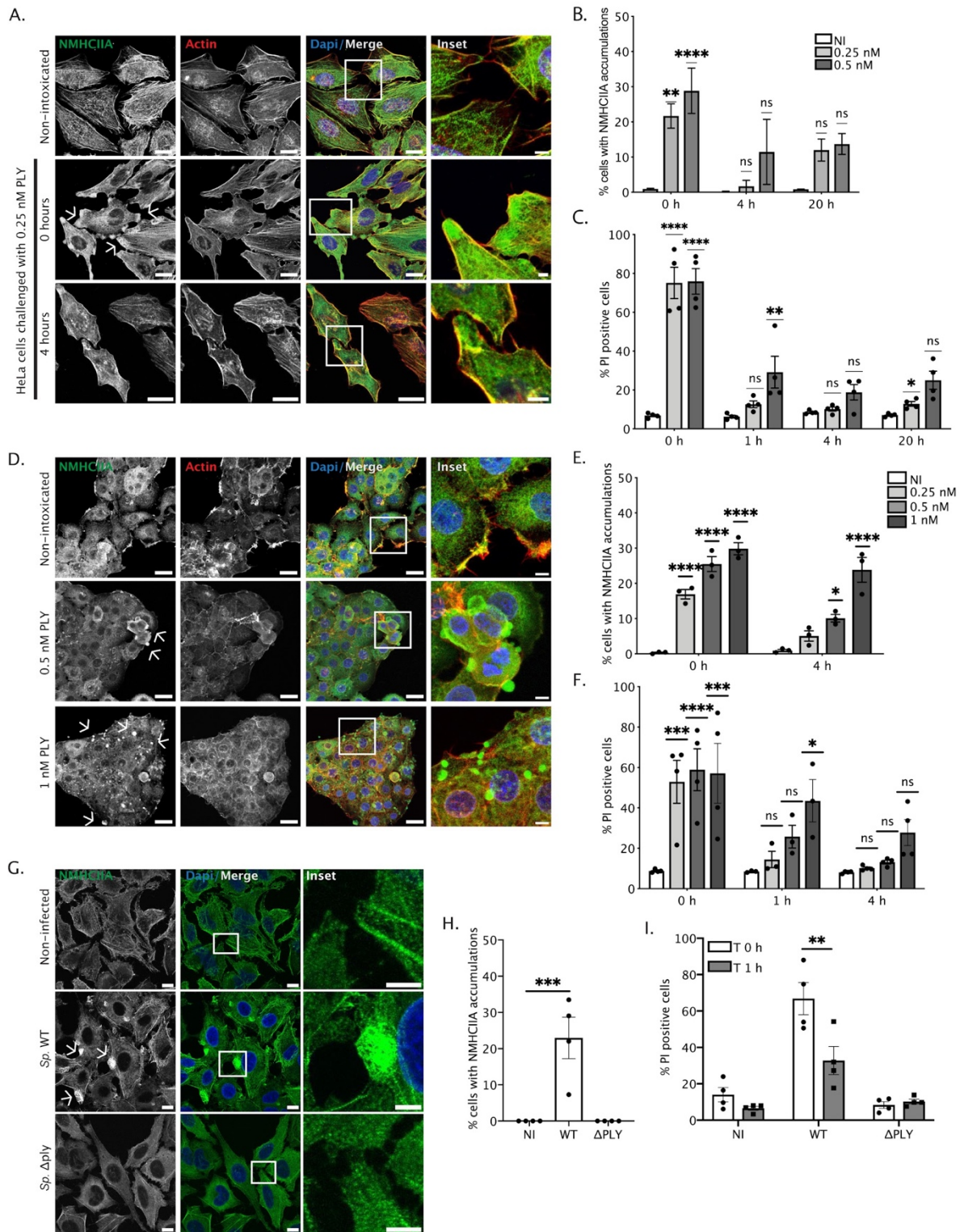


Figure 1. PLY triggers a transient remodeling of the host cytoskeleton that correlates with PM damage. A) Confocal microscopy images of HeLa cells non-intoxicated, immediately after incubation with 0.25 nM of PLY for 10 min (0 h) and after 4 h of PLY washout. Cells were

immunolabeled for non-muscle myosin IIA heavy chain (NMHCIIA) (green) and stained for actin using phalloidin (red) or nucleus using DAPI (blue) (scale bar = 20 μm). The arrows and the inset shows NMHCIIA accumulation at the cell cortex (scale bar = 5 μm) B) Quantification of the percentage of cells displaying NMHCIIA accumulations after incubation with increasing PLY concentrations for 10 min (labeled as 0 h) and following 4 h or 20 h of PLY washout. Values are the mean $\pm$ SEM ($n \geq 3$), p-values were calculated using two-way ANOVA with Dunnett's multiple comparison test of NI vs intoxicated for each timepoint. C) Percentage of PI-positive HeLa cells determined by flow cytometry analysis. Cells were challenged with increasing PLY concentrations for 10 min (initial damage, 0 h) and allowed to recover after PLY washout for 1, 4 or 20 h. Cells were incubated with PI to determine the percentage of cells with compromised PM integrity (PI-positive cells). Values are the mean $\pm$ SEM ($n \geq 3$), p-values were calculated using two-way ANOVA with Dunnett's multiple comparison test of NI vs intoxicated for each timepoint. D) Representative confocal microscopy images of H292 cells left non-intoxicated or immediately after treatment (0 h) with 0.5 nM and 1 nM of PLY for 10 min. Cells were immunolabeled for non-muscle myosin IIA heavy chain (NMHCIIA) (green) and stained for actin using phalloidin (red) or nucleus using DAPI (blue) (scale bar = 20 μm). The arrows and the inset shows NMHCIIA accumulation at the cortex (scale bar = 5 μm) E) Percentage of H292 cells with NMHCIIA accumulations upon incubation with increasing PLY doses for 10 min and following 4 h of PLY washout. Values are the mean $\pm$ SEM ($n \geq 3$), and p-values were calculated using two-way ANOVA with Dunnett's multiple comparison test of NI vs intoxicated for each time point. F) Quantification of PM damaged H292 cells (PI-positive) determined by flow cytometry. Cells were challenged with increasing PLY concentrations for 10 min (0 h) and allowed to recover after toxin washout for 1 and 4 h. Values are the mean $\pm$ SEM ($n \geq 3$), and p-values were calculated using two-way ANOVA with Dunnett's multiple comparison test of NI vs intoxicated for each timepoint. G) Confocal microscopy images of HeLa cells left non-infected or infected with *S. pneumoniae* wild-type TIGR4 (*Sp* WT) or its corresponding PLY-deficient mutant (*Sp* Δply) for 1 h (scale bar = 10 μm). The arrows and the inset shows NMHCIIA organization at the cortex (scale bar = 5 μm). H) Percentage of cells displaying NMHCIIA accumulations in HeLa cells left uninfected or after infection for 1 h with *Sp*. WT or with *Sp* Δply . Values are the mean $\pm$ SEM ($n = 4$), and p-values were calculated using one-way ANOVA with Dunnett's multiple comparison test of NI vs infected cells. I) Quantification of the percentage of PI-positive HeLa cells. Cells were infected with *Sp*. WT or *Sp*. Δply for 1 h and allowed to recover after bacterial washout for 1 h. Values are the mean $\pm$ SEM ($n \geq 3$), and p-values were calculated using one-way ANOVA with Dunnett's multiple comparison test of T 0h vs T 1h. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$; $p \leq 0.0001$.

2.2. **PLY modulates the assembly and disassembly of myosin-IIA filaments presumably at the sites of PM damage**

The cortical accumulation of NMHCIIA in cells was proposed as a hallmark event occurring during cellular response to PM damage inflicted by different CDCs [102, 519]. It is well-established that those NMHCIIA rearrangements are intimately interrelated to the control of PM remodeling events such as blebbing and shedding [102]. However, the structural organization of these accumulations has never been addressed. Confocal microscopy of HeLa cells challenged with sub-lytic PLY concentrations, show accumulation of NMHCIIA at specific cell periphery sites, but also reveal NMHCIIA filament stacks (as described in [567]) organized alongside the actin fibers in the cell cytosol (Figure 2 A, inset 1 and 2). In the NMHCIIA cortical accumulations no discernible pattern or organization was detected (Figure 2 A, inset 1). To further investigate the structural organization of those accumulations, we performed stimulated-emission-depletion (STED) fluorescence microscopy to overcome the diffraction limit encountered using confocal microscopy and improve the resolution [568]. In non-intoxicated cells, we detected NMHCIIA filaments associated with actin bundles and aligned in “stacks” (Figure 2 B, upper panel). In cells challenged with PLY, NMHCIIA stacks are mainly detected in the cytosol. At the cortical regions NMHCIIA stacks were barely detected, instead NMHCIIA appeared to accumulate in its soluble form (Figure 2 B, middle and bottom panels). Our observations suggest that NMHCIIA filament stacks disassemble at specific cortical sites which leads to the accumulation of soluble NMHCIIA. To support these observations and assess the dynamics of the cytoskeletal remodeling, we performed confocal live-cell microscopy in HeLa cells overexpressing NMHCIIA fused to GFP (GFP-NMHCIIA) (Movie S2). Before the PLY challenge, NMHCIIA filaments were detected at the cell periphery (Movie S2, Figure 2 C, time frame 0). Upon PLY, NMHCIIA filaments rapidly disassembled and soluble NMHCIIA started to accumulate and spread outwards (Movie S2 Figure 2 C, time frame 5-9). Subsequently, NMHCIIA re-organizes back into filaments and NMHCIIA GFP signal retracts towards the cell body, a process illustrated in Movie S2 and Figure 2 C (time frame 11-24). NMHCIIA gradually reverted to its initial organization, as evidenced by Figure 2 C (time frame 54). As actomyosin remodeling was shown to be required to control membrane blebbing and shedding [102], we investigated whether NMHCIIA disassembly and accumulation occur in sites of

membrane remodeling. To do so, HeLa cells expressing GFP-NMHCIIA were stained with fluorescently conjugated wheat germ agglutinin (WGA) which labels the PM, intoxicated with PLY and followed by live-cell imaging (Movie S3). NMHCIIA rapidly disassembled and accumulated at the base of a bleb undergoing outward expansion (Movie S3, Figure 2 D, red arrows). As NMHCIIA molecules re-organize the bleb expands and later retracts back towards cell cytoplasm suggesting that the reorganization of NMHCIIA filaments may play a role in PLY-induced blebbing (Movie S3, Figure 2 D, green arrows).

Myosin-IIA activity and filament stability are regulated by the phosphorylation of myosin II regulatory light chain (MRLC) in serine 19 and threonine 18 residues [569, 570]. Given our data showing the remodeling of NMHCIIA, we aimed to evaluate myosin IIA activity upon PLY challenge and during recovery after washout. For that we assessed the levels of pMRLC by immunoblot using a specific antibody that recognizes Thr18/Ser19 MRLC phosphorylation. We found that MRLC is rapidly dephosphorylated upon PLY challenge. The phosphorylation levels of MRLC are reestablished and return to basal levels upon toxin washout (Figure 2 E). Altogether our data support a role for myosin IIA activity during membrane repair and indicates that a tight regulation of the actomyosin network is required for efficient recovery of the PM integrity.

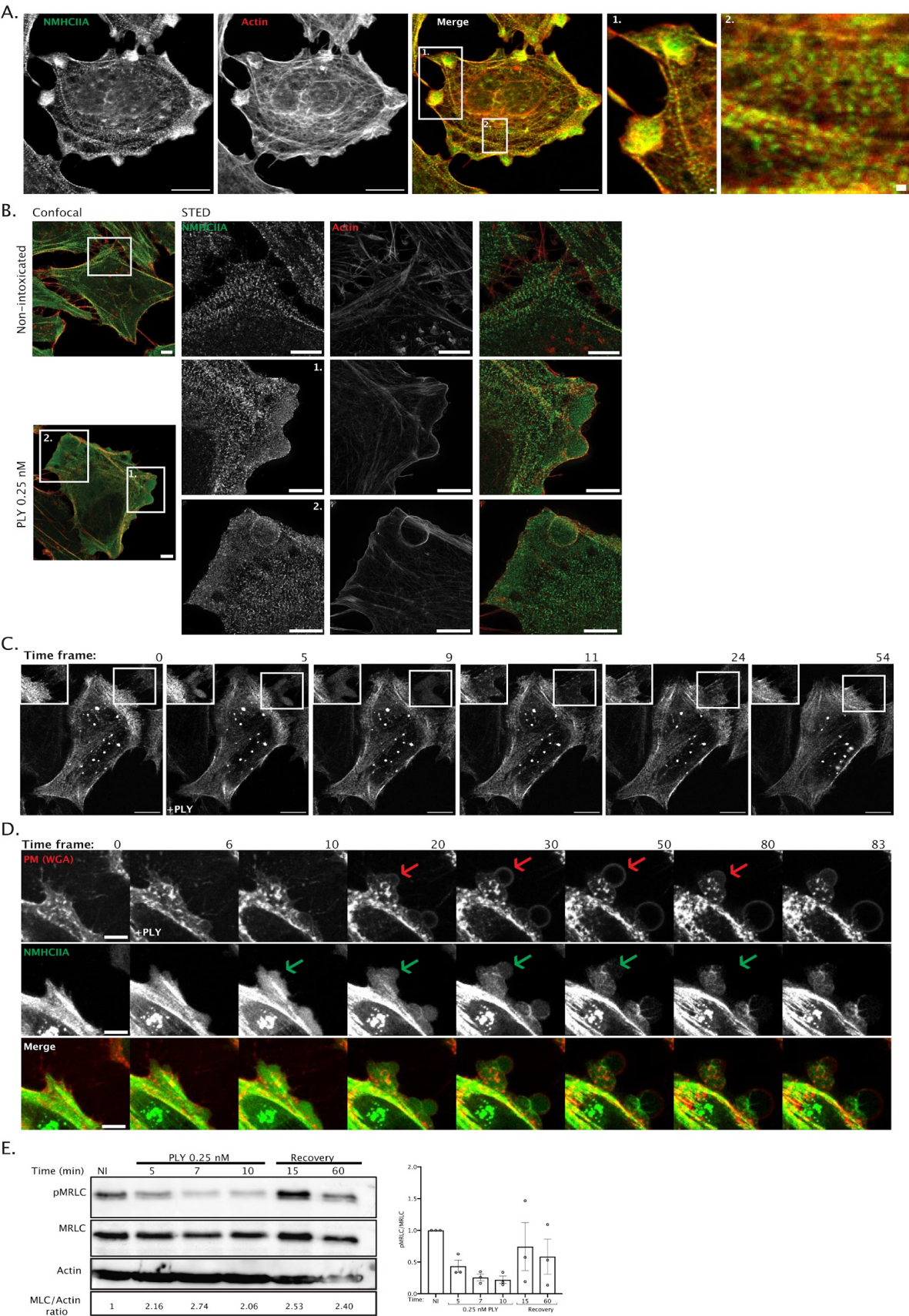


Figure 2. PLY modulates myosin-IIA filament assembly and activity. A, B) Structural characterization of the NMHCIIA accumulations displayed upon intoxication with PLY. Cells were intoxicated and fixed. NMHCIIA was immunolabeled (green) and actin was stained with phalloidin (red). Cells were imaged using a Leica Scanning Confocal SP8 (A) and a Stellaris 8 STED Falcon (B). Scale bar = 10 μm and 0.5 μm in the insets (A) and Scale bar = 5 μm (B) C) Sequential frames of time-lapse confocal microscopy analysis of PLY intoxicated HeLa cells expressing GFPNMHCIIA. PLY was added to the culture medium 10 s before t_5 . Frame interval before PLY addition is 1 minute and after PLY challenge 17.4 seconds. Scale bar = 10 μm D) Sequential frames of time-lapse confocal microscopy analysis of PLY intoxicated HeLa cells expressing GFPNMHCIIA and labeled with tetramethylrhodamine-WGA. PLY was added to the culture medium 10 s before t_6 . Frame interval before PLY addition is 1 minute and after PLY challenge 32.5 seconds. Scale bar = 5 μm (E) Immunoblots for Ser19/Threo18-phosphorylated MRLC (pMRLC), MRLC and actin on lysates from non-intoxicated (NI) or PLY-intoxicated (0.25 nM) cells. Graph shows the quantification of pMRLC levels normalized to non-intoxicated cells. Values are the mean $\pm$ SEM (n=4), each dot corresponds to an independent experiment.

2.3. PLY triggers the interaction between S100A4 and NMHCIIA

We previously unveiled the full proteome of vesicles released by intoxicated cells during the recovery from PLY-induced damage. Several calcium-responding proteins, including S100A4 were found enriched in those vesicles [563]. S100A4 was reported to bind the tail assembly competence domain (ACD) of non-muscle myosin IIA (NMIIA) through direct interaction with NMHCIIA, which occurs in a Ca^{2+} -dependent manner [230, 571, 572]. This interaction promotes both NMIIA filament disassembly and impairs filament assembly of monomeric NMHCIIA (Figure 3 A). Considering our data showing the accumulation of soluble NMHCIIA at the cell periphery, we hypothesize that S100A4 might play a role in the cytoskeleton remodeling occurring upon PLY-mediated damage. To address this issue, we determined the cellular localization of the endogenous S100A4 in non-intoxicated or PLY-intoxicated HeLa cells, by immunofluorescence confocal microscopy. We found that, while in non-intoxicated cells S100A4 is homogeneously distributed in the cell cytoplasm, following PLY challenge, S100A4 specifically translocates from the cytoplasm to the cortex, where it accumulates alongside with NMHCIIA (Figure 3 B). Given that NMHCIIA accumulations highly depend on Ca^{2+} influx and since Ca^{2+} binding to S100A4 induces conformational changes that favor its interaction with NMIIA molecules, we wonder whether S100A4 cortical recruitment would be driven by Ca^{2+} [519, 573]. As expected, S100A4

translocation to the cell cortex was abolished when cells were intoxicated in the absence of extracellular calcium (Figure 3 B). In agreement with the representative images shown in Figure 3 B, the quantification of the S100A4 mean fluorescence intensity (MFI) at the cell periphery (approach depicted in figure 3 C) reveals higher levels of S100A4 associated to the cortex when cells are intoxicated in the presence of extracellular Ca^{2+} . Thus, supporting the idea that S100A4 recruitment to the cell cortex depends on Ca^{2+} influx triggered by PLY-pore formation (Figure 3 B, C). To further understand the dynamics of the recruitment of S100A4 and NMHCIIA filament disassembly and further accumulation, we conducted time-lapse microscopy of HeLa cells overexpressing both GFPNMHCIIA and SNAP-S100A4 (Movie S4). SNAP-S100A4 was recruited to the cell cortex immediately observed after PLY challenge and simultaneously accumulated with GFPNMHCIIA. Both proteins strongly accumulated at the same sites and co-localized in areas where PM later initiates blebbing (Movie S4, Figure 3 D, white square). These data suggest that S100A4 and NMHCIIA work together in the early response to PLY-induced damage, possibly having a role in cytoskeletal remodeling required for blebbing initiation. However, our data did not allow us to establish whether S100A4 recruitment precedes NMHCIIA soluble accumulation. Consistent with previous results, we confirmed by co-immunoprecipitation assay (IP) that upon PLY challenge S100A4 interacts with NMHCIIA (Figure 3 E). Interestingly, this interaction is diminished after toxin washout during recovery of PM integrity, thus corroborating our hypothesis that PLY intoxication favors the interaction between S100A4 and NMHCIIA and consequent filament disassembly and accumulation of soluble NMHCIIA.

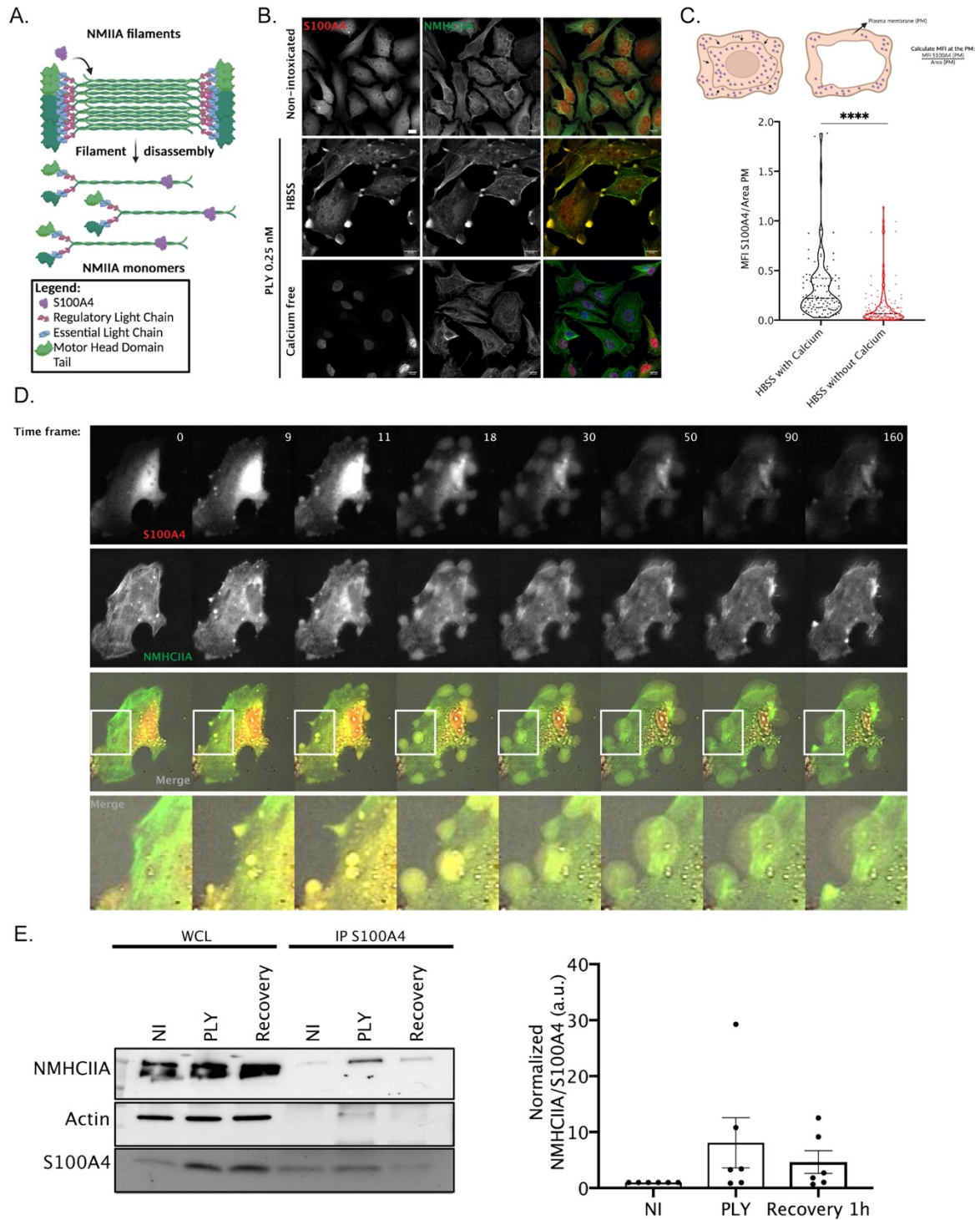


Figure 3: S100A4 interacts with NMHCIIA upon PLY pore-formation. A) Schematic representation of S100A4 binding to the c-terminal of NMHCIIA filaments that leads to NMHCIIA filament disassembly. B) Confocal microscopy images of HeLa cells left non-intoxicated or

intoxicated with PLY (0.25 nM) in the presence (HBSS) or absence (Calcium free) of extracellular calcium. NMHCIIA and S100A4 were immunolabeled (green and red, respectively). C) Quantification of S100A4 mean fluorescence intensity (MFI) calculated at the PM. Values are normalized to the area of the PM of each cell. D) Sequential frames of time-lapse widefield microscopy of PLY intoxicated HeLa cells expressing GFPNMHCIIA and SNAP-S100A4 simultaneously. PLY was added to the culture medium 10 s before time-frame 0. DIC: differential interference contrast. White squares indicate NMHCIIA accumulations at PM blebbing locations. Arrows point towards a retracting bleb. Highlighted inset shows the recruitment of S100A4 and NMHCIIA accumulation at a PM blebbing site. Frame interval before PLY addition to the cells (between -6 to 0) is 1 minute and after PLY challenge (between 0-246) is 10 seconds. E) Immunoblots showing NMHCIIA, S100A4 and actin levels in whole-cell lysates (WCL) and S100A4 IP fractions of HeLa cells non-intoxicated, challenged with PLY (0.25 nM) for 10 min and allowed to recover for 1 h after toxin washout. Graph shows the quantification of NMHCIIA levels in the S100A4 IP fractions. Values are the mean $\pm$ SEM (n = 6) (a.u., arbitrary units).

2.4. S100A4 modulates PM events and cytoskeleton remodeling in response to PLY-induced damage

Considering our data showing that S100A4 and NMHCIIA co-localize and interact upon PLY challenge and the role of S100A4 in modulating NMHCIIA filament organization, we investigated whether this interplay is associated with the accumulation of NMHCIIA at the cortex of intoxicated cells. To tackle this, we assessed if S100A4-depleted cells retain their ability to respond to PLY by accumulating cortical NMHCIIA. We used HeLa cells wild-type non-transfected (WT), or transfected with a control siRNA (HeLa siCtr) or with a mix of siRNAs specifically targeting the expression of S100A4 (HeLa siS100A). Those cells were intoxicated with PLY (0.25 nM) and by immunofluorescence microscopy we determined the percentage of cells displaying NMHCIIA accumulations. About 30 % of the HeLa WT and siCtr cells showed comparable levels of NMHCIIA accumulations (Figure 4 A, B). However, in siS100A cells, which express reduced levels of S100A4 as verified by immunoblot, only 20 % of the cells had detectable NMHCIIA cortical accumulations. Our data supports a role for S100A4 in the NMHCIIA reorganization induced by PLY intoxication.

As already mentioned, NMHCIIA accumulations serve as a hallmark of PFT-induced PM blebbing and are important for effective PM repair [101, 520]. Given the presumed role of S100A4 in promoting NMHCIIA filament disassembly and the

accumulation of NMHCIIA at the cortex, we hypothesize that it may contribute to the control of PM remodeling events such as blebbing and shedding. To explore this, we used a human lung epithelial cell line A549 cells wild-type (A549 WT) or expressing a control shRNA (A549 shCtr) or a specific shRNA targeting S100A4 (A549 shS100A4) [574]. Those cells were intoxicated with PLY and we examined their capacity to undergo PM blebbing. The blebs were detected by scanning electron microscopy (SEM) and the percentage of cells with blebs was blindly scored. A549 WT and shCtr samples displayed a comparable percentage of cells with blebs; approximately 20 % of the cell population showed at least one bleb (Figure 4 C, D). Conversely, the percentage of cells with blebs significantly increases to 30 % in A549 shS100A4 samples (Figure 4 C, D). Together, our data suggest that in the absence of S100A4 cells are impaired in their capacity to accumulate NMHCIIA at the regions of PM blebbing which may lead to increased and uncontrolled PM blebbing.

Given that the release of vesicles, PM shedding, is another mechanism of PM repair engaged in response to different PFTs [76, 563], and that S100A4 was detected in vesicles released by PLY-intoxicated cells, we wonder if besides PM blebbing, shedding could also be altered in cells depleted for the expression of S100A4. A549 shCtr and shS100A4 cells were challenged with sub-lytic amounts of PLY and allowed to recover for 3 h after PLY washout. The vesicles released during repair were then isolated by differential centrifugation and characterized according to their morphology (Transmission Electron Microscopy, TEM) and size (Nanoparticle Tracking Analysis, NTA). We found that A549 shCtr and A549 shS100A4 cells released comparable amounts of vesicles (Figure 4 E, F). However, A549 shS100A4 cells released higher amounts of vesicles of larger sizes (between 120-500 nm) compared to control cells (Figure 4 E, G). Altogether our data underscore the role of S100A4 to tightly control cytoskeletal rearrangements necessary for the activation of imperative PM repair processes such as blebbing and shedding.

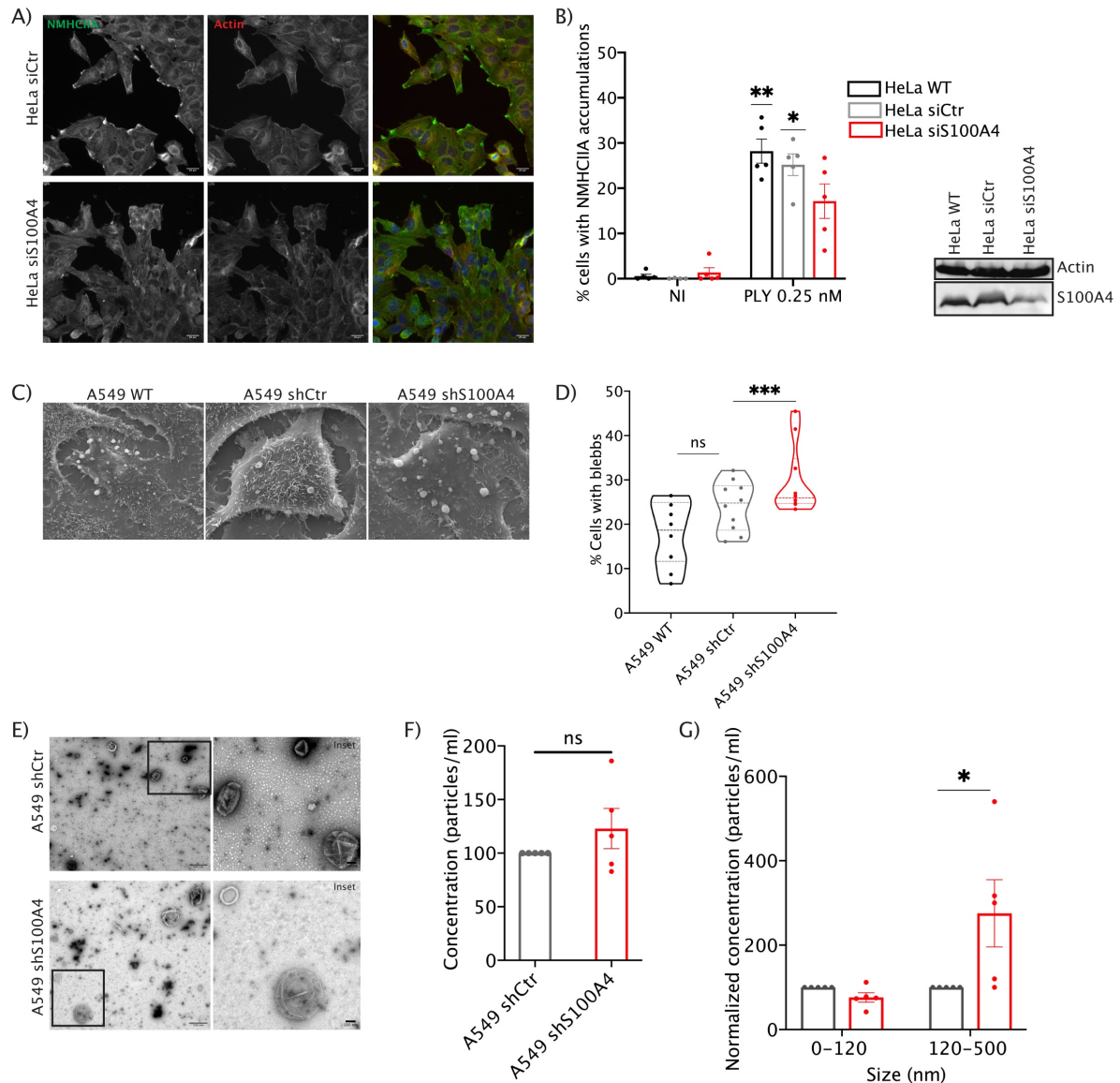


Figure 4: S100A4 coordinates cytoskeleton remodeling and PM repair events. A) Representative confocal immunofluorescence images of Control (siCtr) and S100A4-depleted (siS100A4) HeLa cells intoxicated with PLY (0.25 nM, 10 min). NMHCIIA was immunolabeled (green) and actin (red) and nucleus (blue) were stained with phalloidin and DAPI, respectively. Scale bar = 20 μ m B) (Left) Percentage of HeLa cells (WT or transfected with control non-targeting (siCtr) siRNA, or siRNA targeting S100A4 (siS100A4)) displaying NMHCIIA cortical accumulations non-intoxicated and after challenge with PLY 0.25 nM. (Right) Representative immunoblot showing S100A4 levels from HeLa WT, siCtr and siS100A4 cells. Actin was used as a loading control. C) Representative Scanning Electron Microscopy (SEM) images of A549 WT, A549 shCtr or A549 shS100A4 treated with 0.25 nM PLY for 10 min. D) Graph represents the percentage of cells with blebs quantified in 10 fields from SEM images of A549 WT, shCtr

or shS100A4 intoxicated with 0.25 nM PLY for 10 min. E) Representative transmission electron microscopy (TEM) of vesicles released by A549 shCtr and A549 shS100A4 after challenge with 0.1 nM of PLY and recovery for 3h. Scale bar = 500 nm. Insets show vesicles at higher magnification (Scale bar = 100 nm). F) The graph shows the total concentration of vesicles recovered from A549 shCtr and A549 shS100A4 cells. Values were normalized to the A549 shCtr cells for each experiment and represent the mean $\pm$ SEM of four independent experiments. G) Size distribution of quantified vesicles. Graphs show the concentration of vesicles normalized to A549 shCtr with sizes between 0 to 120 nm and between 120 to 500 nm recovered from A549 shCtr and A549 shS100A4 cells. Values are the mean $\pm$ SEM of four independent experiments. A two-way ANOVA test with Sidak's multiple comparisons was performed (* $p < 0.05$).

2.5. S100A4 promotes PM integrity during intoxication and PLY-driven *S. pneumoniae* infection

Bearing in mind the protective role of cytoskeletal remodeling and NMHCIIA accumulation in response to PFTs intoxication [102, 519], we thus assessed the involvement of S100A4 in the cellular response to PLY-induced damage. We took advantage of the availability of A549 WT, shCtr and shS100A4 to evaluate the levels of PM damage following PLY challenge and infection with *S. pneumoniae*. The levels of PM permeability were assessed through flow cytometry using PI incorporation assay. We first intoxicated A549 WT and shS100A4 cells with increasing PLY concentrations. Both cell lines exhibit a dose-dependent increase of PI incorporation, indicating that growing PLY concentrations triggered increased damage, as expected. However, A549 shS100A4 cells displayed higher levels of PI incorporation for the same PLY concentration when compared to the A549 WT (Figure 5 A), suggesting that the absence of S100A4 renders cells more susceptible to PLY-induced damage. This difference was more pronounced at lower PLY concentrations, likely because at higher PLY doses cells undergo overwhelming damage and engage in cell death pathways. To investigate if the capacity of cells to recover from the damage caused by PLY was also compromised in the absence of S100A4, we performed recovery assays. A549 WT, shCtr and shS100A4 cells were intoxicated with PLY for 10 min, then PLY was washed out and the PM permeability was evaluated by PI incorporation assays at different time points. We found that although A549 shS100A4 are consistently more sensitive than control cells to PLY damage, they retain comparable ability to recover from damage

(Figure 5 B), suggesting a role for S100A4 only in the initial response to intoxication possibly through damage restraint.

We aimed to validate our data in a more relevant model and assess if similar responses to damage were triggered by PLY directly released by *S. pneumoniae* (*Sp*) during cellular infection. For that, we infected the mentioned A549 cell lines with either *Sp*. TIGR4 wild-type (WT) or the corresponding PLY deficient mutant (Δply), for different time points (30 min and 1 h) and assessed PM integrity. At 30 min of infection, in A549 shCtr the percentage of PI positive cells remain the same in non-infected or infected conditions, indicating that this infection time is not sufficient to detect damage even in cells infected with the *Sp* WT strain (Figure 5C). However, cells depleted for S100A4 (shS100A4) already showed significant levels of damage at 30 min of infection, with 40 % of the cells being detected as PI-positive (Figure 5 C). These data confirm that in the absence of S100A4 the cells are more susceptible to PLY-induced damage. After 1 h infection, A549 shCtr cell population showed 40 % of PI-positive cells when infected with the *Sp* WT strain (Figure 5C). The infection with *Sp* Δply did not induce significant damage in control cells. S100A4-depleted cell population showed increased damage when infected by *Sp* WT for 1 h, reaching 60 % of PI-positive cells (Figure 5 C). In addition, at this time point these cells also showed significant damage when infected by *Sp* Δply (Figure 5C), which suggests that other bacterial virulence factors than PLY, such as SpxB and GlpO, increase H₂O₂ production which can induce membrane lipid peroxidation and alter membrane properties and fluidity compromising PM integrity as well [21, 277, 575]. Overall, these data show that *Sp* infection disrupted PM integrity in a PLY-dependent manner and that cells depleted for S100A4 are more susceptible to PLY-induced PM damage during infection by *Sp*. Also, our results point to the protective role of S100A4 not only against PLY-induced PM damage but also counteracting damage caused by other bacterial factors.

Lastly, we assessed the capacity of the cells to effectively reseal the damage inflicted by PLY during infection with *Sp*. We found that for all infection times tested, although A549 shS100A4 consistently presented higher levels of PI incorporation, meaning increased levels of PM damage, these cells were equally capable of repairing membrane integrity as compared to A549 shCtr cells (Figure 5 D). These results are in line with the ones we obtained using the purified protein, supporting the role of S100A4 only in the initial response of cells to damage by PLY.

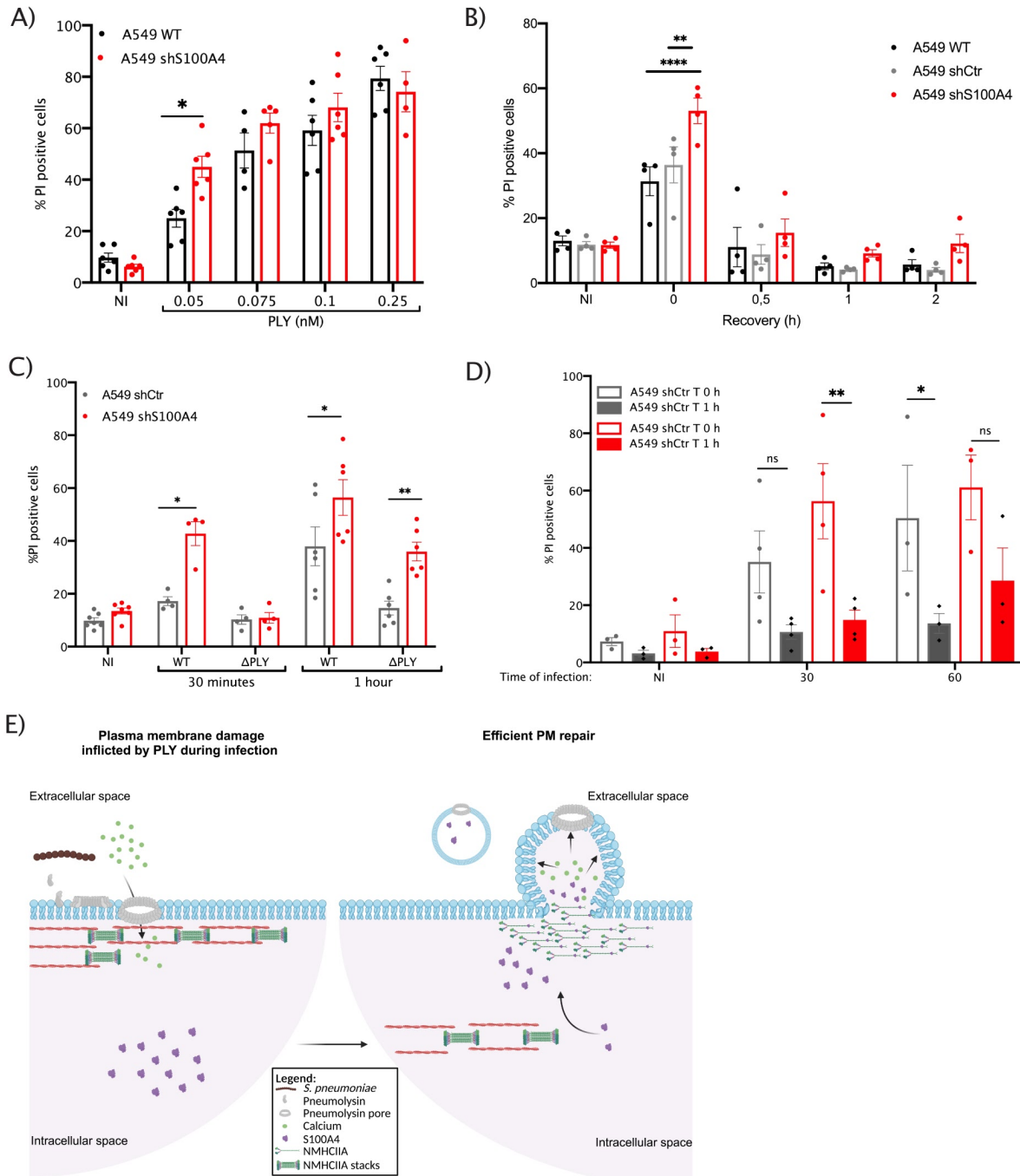


Figure 5: S100A4 protects cells from damage during *S. pneumoniae* infection. A) Plasma membrane permeability represented by the percentage of PI-positive cells, assessed by flow cytometry after increasing doses of Ply in A549 WT and shS100A4 cells. Values are the mean $\pm$ SEM ($n \geq 4$), p-values were calculated using two-way ANOVA Sidak's multiple comparison test of A549 WT vs A549 shS100A4 cells for each Ply concentration. B) Graph showing the percentage of PI-positive cells after intoxication with 0.05 nM Ply and allowed to recover for

the indicated time points in A549, A549 shCtr and A549 shS100A4 cells. Values are the mean $\pm$ SEM ($n = 4$), p-values were calculated using two-way ANOVA Tukey's multiple comparison test of A549 WT vs A549 shCtr vs A549 shS100A4 cells for each timepoint of recovery. C) Plasma membrane permeability assessed by flow cytometry in A549 shCtr and shS100A4 cells infected for 30 minutes and 1 hour with *Sp* TIGR4 WT (WT) or PLY-mutant strains (Δ PLY) (1h, MOI=100). Values are the mean $\pm$ SEM ($n \geq 4$), p-values were calculated using two-way ANOVA Sidak's multiple comparison test of A549 shCtr vs A549 shS100A4 cells. D) Quantification of the percentage of PI-positive cells determined by flow cytometry using A549 shCtr and shS100A4 cells after 30 and 60 minutes following infection with *Sp* TIGR4 WT (WT) or PLY-mutant strains (Δ PLY) (1h, MOI=100) and allowed to recover for 1 h after bacterial washout. Values are the mean $\pm$ SEM ($n = 4$), p-values were calculated using two-way ANOVA Turkey's multiple comparison test of T 0h vs T 1h. E) Schematic model. During infection, *S. pneumoniae* releases PLY that binds cholesterol residues at the PM and creates transmembrane pores that allow the massive influx of Ca^{2+} . The intracellular Ca^{2+} rising triggers the recruitment of S100A4 from the cytosol to the cell periphery where it interacts with the myosin IIA tail leading to filament stack disassembly and the accumulation of soluble NMHCIIA at the cell cortex. This cytoskeletal remodeling is important to tightly control important PM repair mechanisms such as blebbing and shedding.

3. Discussion

Loss of PM integrity triggered by the insertion of different cholesterol-dependent cytolysins into the PM of host cells triggers a massive remodeling of the actomyosin cytoskeleton and induces ER disruption into vacuoles that are recruited to the cell periphery where the actomyosin undergoes remodeling and NMHCIIA strongly accumulates [102]. These events were directly linked to successful PM repair, which is highly dependent on the massive influx of extracellular Ca^{2+} . Here we reported that actomyosin remodeling is critical for efficient and timely host response to PLY damage during *S. pneumoniae* infection and further characterize the structural features of the NMHCIIA accumulations, aiming to better understand their function. As the purified toxin, PLY released during cellular infection with *Sp*. induces a fast and transient accumulation of NMHCIIA at the cell periphery presumably at sites of damage. The dissipation of these NMHCIIA accumulations directly correlates with the reestablishment of PM integrity thus supporting their active role in PM repair.

Non-muscle myosin-II (NMII) is a hexamer molecular motor enzyme that associates with actin filaments in non-muscle cells to convert chemical energy into

mechanical forces. It is composed by two NMII heavy chains (NMHCII) that forms parallel homodimers which binds two pairs of essential and regulatory light chains. Cells possess three different NMII paralogs, NMIIA, NMIIB and NMIIC that differ from each other in its heavy chain. Due to its inherent properties, NMIIA plays a pivotal role in the reorganization of cell cytoskeleton and is imperative for key cellular processes such as cell migration and cytokinesis [570, 576]. To further understand the functional role of the cytoskeletal alterations observed upon PLY challenge, we deeply studied the structural features of NMHCIIA accumulations, the most abundantly expressed NMII paralog [577], by super-resolution microscopy and live-cell imaging. Interestingly, we found that at the cell periphery, NMIIA filament stacks rapidly dissipate as PLY is added and NMHCIIA rapidly accumulates in its soluble form. When dephosphorylated, NMIIA can adopt a folded conformation (10s-fold monomer) in which the motor and polymerization activities are impaired. Upon phosphorylation of the myosin regulatory light chain (MRLC), NMII acquires an extended conformation (6s monomer) which has motor activity and is available to assemble into new bipolar filaments [570]. Although it lacks motor activity, the 10s folded monomer diffuses faster, which is critical to keep the equilibrium between the assembly and disassembly of NMII in cells. As such, the activity of NMII is highly regulated and mediated by the phosphorylation of its RLC. Our data shows that MRLC is rapidly dephosphorylated upon PLY challenge but after 15 minutes of recovery, the levels of phosphorylated MRLC increased, suggesting that a rapid regulation of NMIIA assembly state is required during damage and repair. This is supported by our live-cell experiments showing a fast disassembly of NMIIA stacks at the cell periphery and accumulation of soluble NMHCIIA that quickly reorganizes in stacks in a retrograde movement towards the cell body, typical of the formation of new NMIIA filaments at the leading edge of protruding cells [578]. Possibly, the disruption of pre-existing NMIIA filaments allows the generation of an additional pool of NMIIA monomers that becomes available to assemble new filaments, a process that is critical to support such a dynamic process as blebbing. Our data showing the disassembly and reorganization of NMHCIIA at the base of newly formed bleb supports this hypothesis. In fact, the recruitment and activation of NMII is critical to drive bleb retraction, being NMIIA paralog the major contributor due to its faster turnover [579]. Together with previous observations that impaired NMHCIIA accumulations lead to uncontrolled PM blebbing, we postulate that

fine-tuned regulation of the NMIIA assembly state is critical to control PM blebbing triggered by PLY-induced damage. Interestingly, PLY was reported to interact with actin cytoskeleton and modulate the activity of Rho GTPases Rac1 and RhoA [393]. Bleb dynamics rely on the actin cytoskeleton reorganization, with RhoA being recruited and activated at the PM to drive actin reassembly and NMII contraction propelling bleb retraction [580-583]. Altogether suggesting that pore-formation by PLY favors cytoskeletal remodeling required to support bleb formation and retraction, a mechanism that protects cells against lysis [101].

Supported by our previous findings showing that S100A4 is present in vesicles released by PLY-intoxicated cells and by its putative role as a critical regulator of NMIIA filament organization [572], we further investigated its contribution to the cytoskeletal remodeling triggered by PLY and its relevance in cell resistance to PLY attack. S100A4 is an homodimer that possesses two Ca^{2+} binding sites. In the presence of Ca^{2+} the molecule undergoes conformational changes that reveal a hydrophobic binding pocket which provides a binding site for other proteins. Accordingly, S100A4 interaction with NMIIA, through the C-terminal of NMHCIIA, relies on Ca^{2+} binding [230]. We demonstrated that cellular intoxication with PLY triggers the recruitment of S100A4 to the cell periphery, where it accumulates at the same sites as NMHCIIA, in a process strongly dependent on the presence of Ca^{2+} . This is a very dynamic process and usually occurs in sites of PM blebbing, where Ca^{2+} is usually concentrated. Our live-cell studies revealed that the recruitment of S100A4 to the cell periphery is immediate to the addition of PLY, NMHCIIA quickly accumulates and as soon as blebs starts to expand S100A4 seems to fade away, possibly to allow NMIIA re-assembly into stacks. S100A4 is extensively studied for its role in cancer metastasis which has been associated with its interaction with NMIIA that promoted directional cell migration. At the leading edge of migrating cells, a tight regulation of NMII filament assembly is required and mediated by calcium-regulated proteins, among which S100A4 seems to play a role [225]. Interestingly, we previously demonstrated that the typical cytoskeletal dynamics occurring during PM blebbing after PFT-damage, also occurs during cell migration and assembly of uropod-like WRAMP structures, suggesting that similar mechanisms might be activated in both processes and thereby supporting our hypothesis that fine-tune modulation of NMIIA activity is required to control PM blebbing. S100A4 is possibly the molecular regulator responsible to maintain this

equilibrium by keeping a pool of soluble NMIIA. This is also supported by our findings showing that in the absence of S100A4 cells have less NMIIA accumulations, bleb more and shed larger PM portions, which indicate that cells are not properly controlling these events. Although the absence of S100A4 did not completely abolish NMHCIIA accumulations, we need to take into consideration that our approach using siRNA only guarantees the partial depletion of S100A4.

In this study, we reveal the protective role of S100A4 in orchestrating host cell cytoskeleton remodeling required for the activation of PM repair mechanisms, upon PLY intoxication as well as in the context of infection with *S. pneumoniae*. Upon PLY damage, S100A4 is specifically recruited to the cell periphery and interacts with NMHCIIA in sites of PM blebbing. There, soluble NMHCIIA rapidly accumulates and re-organizes to drive bleb expansion and retraction (Figure 5 E). The absence of S100A4 impairs proper PM remodeling events (blebbing and shedding) and renders cells more susceptible to damage by PLY. Altogether sustaining that S100A4 provides cellular protection against damage induced by PLY by tightly controlling cytoskeletal remodeling critical to achieve full PM repair.

4. Materials and Methods

4.1. Cell lines

Human cervix epithelial cell line (HeLa, ATCC CCL-2) and human alveolar basal epithelial cell line (A549, ATCC CCL-185) were kept in DMEM with glucose and L-glutamine (Biowest), supplemented with 10 % fetal bovine serum (FBS) (Biowest). Human pulmonary mucoepidermoid carcinoma-derived cell line (H292, ATCC CRL-1848) was grown in RPMI 1640 medium (Gibco) supplemented with 10 % FBS (Biowest). A549 cells depleted for the expression of S100A4, previously generated as described [574], were a kind gift from Dr. Min Chen (University of Kentucky, USA) and were maintained in culture using DMEM with glucose and L-glutamine (Biowest), complemented with 10 % FBS (Biowest) and 2 µg/mL of puromycin. All cell lines were kept at 37 °C in a 5 % CO₂ atmosphere.

4.2. Plasmids, dyes, and antibodies

The plasmid encoding for GFP-NMHCIIA (#11347) was acquired from Addgene [584]. To generate SNAP-S100A4 plasmid, the S100A4 coding sequence was amplified

from HeLa cells cDNA using ACCUZYME™ DNA polymerase (Bioline) and the following primers: S100A4_Fw 5'-ATAGGATCCGCGTGGCCTCTGGAGAAG-3' and S100A4_Rv 5'-CTTACTCGAGTCATTTCTTCCTGGGCTG-3'. The amplified fragment was cloned at the BamHI and XhoI restriction sites into the pSNAP-tag (m) vector, purchased from Addgene (a gift from New England Biolabs & Ana Egana (#101135; [585])). The SNAP-S100A4 construct was verified by DNA sequencing analysis.

For SNAP-S100A4 labeling cells were incubated with 0.6 μ M SNAP-Cell TMR-Star (New England Biolabs) for 30 minutes following manufacturer's instructions. F-actin was stained, using 1:200 Alexa Fluor 647- or 555-conjugated phalloidin (Invitrogen) for confocal microscopy or with 1:50 phalloidin 635P (Abberior GmbH) for STED microscopy. Plasma membrane was labeled with WGA-Tetramethylrhodamine (Thermo Fisher Scientific), a lectin selectively binding N-acetylglucosamine and N-acetylneuraminic acid, following manufacturer's indications.

Primary antibodies were used at 1:200 for immunofluorescence, 1:50 for STED microscopy and Transwell's membrane staining, and 1:1000 for immunoblot. The following antibodies were used: rabbit anti-NMHCIIA (Sigma); mouse anti-actin AC-15 (Sigma); rabbit anti-MRLC and anti-phospho-MRLCSer19 (Cell Signaling); mouse anti-S100A4 (DSHB); rat anti-E-cadherin antibody (ectodomain, DCMA-1) (Abcam). The secondary antibodies were used at 1:500 for immunofluorescence or at 1:50 for STED: goat anti-rabbit Alexa Fluor 488 (Invitrogen); goat anti-mouse Cy3 (Jackson ImmunoResearch); goat anti-rat Alexa Fluor 568 or 647 (Invitrogen); goat anti-rabbit or anti-mouse Cy5 (Jackson ImmunoResearch); goat polyclonal anti-rabbit STAR 580 (Abberior GmbH). For immunoblot goat anti-rabbit or anti-mouse HRP (PARIS) were used at 1:2000.

4.3. Transfections

For live-cell imaging experiments, 7×10^4 or 1.5×10^5 HeLa cells were seeded into 2- or 4-well Ibittreat μ -dishes (Ibidi), respectively. Cells were transfected 24 h later with 0.5 μ g or 1 μ g of DNA, respectively, using jetPRIME™ Polyplus-transfection reagent as indicated by the manufacturer.

For S100A4 knockdown, HeLa cells (5×10^5) were seeded in 6-well plates with or without glass coverslips. Cells were transfected 24 h later with 1 μ g of control siRNA-D (sc-44232, Santa Cruz Biotechnology) or specific siRNAs targeting S100A4 (sc-

106781, Santa Cruz Biotechnology) using Lipofectamine™ 2000 following manufacturer's instructions. Cells were used in subsequent experiments 48 h post-transfection.

4.4. **PLY purification and cell intoxication**

PLY expression and purification was carried out as described [519, 548]. Toxin challenge at the indicated concentrations was performed in Hanks' balanced salt solution (HBSS) or, in HBSS Ca²⁺-free medium, when specified, for 10 minutes in a humidified incubator at 37 °C with 5 % CO₂.

4.5. **Bacterial strains and growth conditions**

Frozen aliquots of *S. pneumoniae* TIGR4 (serotype 4) were prepared by growing bacteria in Todd-Hewitt broth (BD Biosciences) enriched with 0.5 % yeast extract and Oxyrase at 37 °C with 5 % CO₂ until mid-exponential growth phase. Then, bacterial cultures were centrifuged (4000 rpm for 5 min) and bacterial pellets resuspended in growth media containing 20 % (v/v) glycerol and immediately frozen. Bacterial concentrations were determined by serial dilutions and plating on tryptic soy agar supplemented with 5 % sheep blood agar (Northeast Laboratory Services). The TIGR4 PLY-deficient mutant strain (Δply) was kindly given by Dr. Andrew Camilli (Tufts University, Boston, MA).

For infection experiments, *S. pneumoniae* strains (WT and Δply) from frozen aliquots were cultured in Todd-Hewitt (BD Biosciences), supplemented with 0.5 % yeast extract and Oxyrase at 37 °C with 5 % CO₂ and handled at log-phase. Before infection, *S. pneumoniae* strains were washed and resuspended in HBSS supplemented with Ca²⁺ and Mg²⁺, bacterial suspension was added to cells at the indicated MOIs and incubated at 37 °C with 5 % CO₂ for the indicated times.

4.6. **Immunofluorescence assays**

For immunofluorescence assays, cells were grown in 13 mm diameter coverslips for 24 h and intoxicated as described above with the indicated PLY concentrations. Cells were washed three times with PBS 1x, fixed with 4 % paraformaldehyde (PFA) for 15 minutes at room temperature, and quenched with 0.1 M NH₄Cl for 40 minutes at 4 °C. Prior to immunostaining, cells were permeabilized with 0.1 % Triton X-100 for 5

minutes at room temperature, washed with PBS 1x, blocked with 1 % BSA in PBS for 1 h and incubated with primary antibody for 1 h at room temperature, washed three times in PBS 1x and incubated with secondary antibody for 45 minutes. Antibodies were diluted in 1 % BSA in PBS. Coverslips were mounted with Aqua-Poly/Mount medium onto microscope slides. For the quantification of NMHCIIA cortical accumulations and S100A4 mean fluorescence intensity (MFI), images were acquired using the epifluorescence Olympus BX63 microscope equipped with a 60x 1.35 NA objective or Leica DMI6000 equipped with a 40x 0.60 NA objective lens. For representative image acquisition, a Leica Scanning Confocal SP5 II or a Leica Scanning Confocal SP8 microscope equipped with a 63x 1.30 NA objective lens were used. For super-resolution acquisition, we took advantage of the Leica Stellaris 8 STED Falcon microscope equipped with a 93x 1.30 NA objective lens.

4.7. Immunofluorescence quantifications

NMHCIIA accumulations were blind scored. Briefly, cells were deemed positive if they exhibited at least one dense NMHCIIA accumulation at the cell cortex. Nuclear staining (DAPI) was used to quantify the number of cells per field. A minimum of 10 fields per condition and 200 cells were quantified for each condition across three independent experiments.

The recruitment of S100A4 to the cell cortex was quantified using ImageJ and the following steps: 1) total cell area was determined using NMHCIIA staining by manually drawing cell borders; 2) the ImageJ “enlarge” tool was used to proportionally reduce cell size; 3) the PM associated region of each cell was selected with the tool “XOR” by subtracting the reduced size cell to the total area; 4) S100A4 MFI in both total cell area and PM area was calculated; 5) the ratio between the MFI of S100A4 at the PM and total cell area was calculated for at least 10 fields per condition and 40 cells in three independent experiments.

4.8. Flow cytometry

Cells (HeLa, H292, A549 WT, A549 shCtr or A549 shS100A4) were seeded in a 6-well plate at a density of 5×10^5 cells/well. 20 h later, cells were left non-intoxicated (NI) or were intoxicated with the indicated PLY concentrations for 10 minutes at 37 °C in 5 % CO₂ atmosphere. For recovery assays, PLY was washed out with PBS 1x (3

washes) and complete medium was added for the indicated times. For infection assays, complete medium (DMEM + 10 % FBS) supplemented with 200 µg/mL gentamicin was added to cells to kill extracellular bacteria and were incubated for 1 h at 37 °C with 5 % CO₂. Cells were then processed for flow cytometry: washed in PBS 1 x and resuspended in FACS buffer (2 % FBS in PBS 1 x). Immediately before analysis, propidium iodide (PI, 2 µg/mL) was added and samples incubated for 1 min at room temperature. Fluorescence intensity was assessed using a BD Accuri™ C6 flow cytometer (BD Biosciences) or FACS Calibur flow cytometer (BD Biosciences) at the FL3 channel. At least 10,000 cells per sample were collected after doublet discrimination. Data was analyzed using FlowJo Software (Version 10.8 TreeStar Inc.).

4.9. Immunoprecipitation and immunoblotting assays

HeLa cells (6×10^6) were seeded in 100 mm dishes and treated as specified 20 h later. Subsequently, cells were washed in cold PBS 1x three times and lysed using RIPA lysis buffer supplemented with PMSF (Santa Cruz). Cell lysates were collected by centrifugation (14 000 g, 10 minutes, 4 °C). The total protein content was determined using Protein Assay Dye Reagent (Biorad) following manufacturer's instructions. 1000 µg of total protein extract was pre-cleared using protein G-immobilized PureProteome magnetic beads (Milipore) and incubated overnight with 1 µg of anti-S100A4 antibody (DHSB). 30 µL of protein G-immobilized magnetic beads (Milipore) were utilized to capture the immune complexes, followed by immunoblotting. For immunoblotting, total protein lysates and IP fractions were resuspended in sample buffer (0.25 mM Tris-Cl, pH 6.8; 10% SDS; 50% glycerol; and 5% β-mercaptoethanol), separated by SDS-PAGE and transferred into nitrocellulose membranes (Biorad). Membranes were blocked in 5 % fat milk or 5 % BSA (Sigma) in TBS-tween (150 mM NaCl; 50 mM Tris-HCl, pH 7.4; 0.1% Tween) for at least 1 h primary and secondary antibodies were diluted as specified above in blocking solution. Immunoblot signals were detected using ECL (Thermo Scientific). Densitometry was executed in Chemidoc (Biorad).

4.10. Extracellular vesicles isolation and characterization

A549 shCtr (control) and A549 shS100A4 cells (3×10^6) were seeded on 100 mm tissue culture dishes and 20 h later they were intoxicated with 0.1 nM of PLY for 10 min, washed twice in PBS Ca²⁺-free buffer and allowed to recover from damage in

serum-free DMEM for 3 h. Cell culture supernatants from 10 tissue culture dishes were collected and extracellular vesicles (EVs) were isolated by differential centrifugation following the protocol described in [563]. The resulting EV pellet from both cell lines was resuspended in 100 μ L of Ca^{2+} -free PBS. Samples were immediately processed for transmission electron microscopy (TEM) experiments. For extended period storage, EVs were preserved at -80°C .

EVs were characterized according to their morphology by negative staining TEM. Formvar-carbon coated EM-grids were glow discharged for 30 s at 10 mA, then 5 μ L of each EV sample was adsorbed to the grid for 10 min. EVs were stained using 5 μ L of 1% uranyl acetate, excess liquid was dropped onto a piece of filter paper and grids were allowed to dry before imaging. Images were obtained using a JEOL JEM 1400 TEM (JEOL, Tokyo, Japan) 80 kV equipped with a CCD digital camera Orious 1100 W.

The size distribution profile of the isolated EVs was assessed by nanotracking particle analysis (NTA). Briefly, EVs were diluted 1000 x and injected into a NanoSight NS3000 system (Malvern Panalytical). Prior to analysis, camera settings were adjusted to a camera level of 16 and a detection threshold of 5, and 25 frame-per-second videos were recorded in triplicates for each sample. To assess size distribution profiles, the videos were processed and analyzed using the NTA 3.2 software (Malvern Panalytical, Worcestershire, UK).

4.11. Scanning Electron Microscopy

Cells (A549 WT, A549 shCtr and A549 shS100A4) were seeded on coverslips at a density of 1.5×10^5 . 24 h later, cells were left non-intoxicated or challenged with PLV 0.1 nM for 10 minutes and fixed with 2 % glutaraldehyde (GA) in 0.1 M Na-Cacodylate buffer for 4 h at room temperature. Samples were rinsed 3 times for 15-minute periods with 0.1 M Na-Cacodylate buffer and fixed again using 1 % osmium tetroxide (OsO_4) in H_2O for 1 h at 4°C in the dark. Samples were washed 6 times with dH_2O during 15-minute periods. For dehydration of the samples, increasing concentrations of ethanol aqueous solutions (30 %: 60 %: 90 %: 96 %) were sequentially added to the coverslips. Each solution was incubated for 5 minutes at room temperature, followed by two final incubations of 5 minutes each with 100 % ethanol. Samples were submitted to the critical point dryer (CPD 7501, Polaron Range) to conclude the dehydration process. Before imaging the samples were coated with a thin

conductive film (AuPd metal alloy) deposited by Sputtering using the SPI Module Sputter Coater equipment. The coating thickness is in the range of 10 nm to 15 nm. Images were acquired using FEI Quanta 400 FEG ESEM.

4.12. Statistic

Statistical analyses were executed using GraphPad Prism Version 8.1.1 (224) software (GraphPad Software, La Jolla, CA, USA, www.graphpad.com). The statistical test applied to each experiment is indicated in the figures legends.

Section 3

***Streptococcus pneumoniae* infection of lung epithelial cells induces internalization of surface GPI-anchored proteins through pneumolysin-mediated activation of host Rho GTPases**

In this manuscript we explored the impact of *S. pneumoniae* infection on the surface expression of GPI-anchored proteins (GPI-APs) and in particular CD73, which was previously shown crucial for host resistance to *S. pneumoniae* infection. This work was performed in the framework of a collaboration established with Prof John Leong (Tufts University School of Medicine) and Dr Elsa Bou Ghanem (University at Buffalo School of Medicine), during the course of my PhD. We discovered that *S. pneumoniae* infection reduces the expression of GPI-APs at the cell surface in a way that depends onPLY pore-formation capacity. Additionally, we characterize the mechanism by which PLY mediates GPI-APs cell surface expression. In response to PLY pore-formation, GPI-APs are internalized in a process that depends on the activation of RhoA and Cdc42 GTPases. My contribution to this work included the acquisition and analysis of microscopy data (live-cell imaging, FRET and confocal microscopy) and flow cytometry to determine PM integrity after PLY challenge. In addition, I participated in Figure design and actively contributed to the drafting of the manuscript.

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***Streptococcus pneumoniae* infection of lung epithelial cells induces internalization of surface GPI-anchored proteins through PLY-mediated activation of host Rho GTPases**

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Abstract

A return to homeostasis after infection-associated cellular injury can be accelerated by a rapid damage response. *S. pneumoniae*, a typically asymptomatic colonizer of the host upper respiratory tract, can cause serious and life-threatening infections when it gains access to the lungs and other organs. The cholesterol binding *S. pneumoniae* pore-forming toxin, pneumolysin (PLY), is central to the induction of host cell damage. Here, we first found that mouse lung infection by *S. pneumoniae* diminished pulmonary expression of CD73, a glycosylphosphatidylinositol anchored protein (GPI-AP) that modulates inflammation. Infection of the human pulmonary epithelial cell line H292 resulted in a PLY-dependent reduction of not only cell surface CD73, but also the population of surface expressed GPI-APs. The decrease in cell surface GPI-APs was rapid, required pore-forming activity, and could be recapitulated by purified PLY and other cholesterol binding cytolysins. In response to PLY-mediated insult, GPI-APs were not released from the surface of epithelial cells in extracellular vesicles but rather internalized by a mechanism dependent on the Rho GTPases RhoA and Cdc42. Internalization of GPI-APs was associated with lower levels of PLY-induced apoptosis and membrane permeabilization. These findings suggest that internalization of GPI-APs from epithelial cell membranes may constitute a rapid innate repair response to cell damage induced by PLY and other pore forming toxins that could help bacteria evade host defenses as many GPI-APs have roles in immunity.

Author summary

Streptococcus pneumoniae causes serious infections that can result in mortality. The pore-forming toxin, pneumolysin (PLY) produced by these bacteria is important for their ability to cause disease. Understanding how the host responds to damage by this toxin can result in better treatment against infection. In this study, we found that PLY-mediated injury results in decreased expression of glycosylphosphatidylinositol anchored proteins (GPI-AP) from the cell surface by internalization. GPI-AP co-localize in cholesterol-rich areas of the membrane where PLY inserts to form pores and cells with decreased surface GPI-APs were associated with less of PLY-induced cell death and membrane permeabilization. These results suggest that GPI-AP are internalized as part of repair mechanisms activated in response to infection-induced cell injury. As many GPI-APs have important roles in the immune response, their removal from the cell may inadvertently help the bacteria establish better infection.

1. Introduction

Streptococcus pneumoniae (pneumococcus), although an asymptomatic colonizer of the upper respiratory tract of healthy hosts, can, in susceptible populations, cause severe lower respiratory tract infections with high mortality and morbidity rates [278, 586]. *S. pneumoniae* predominantly causes pneumonia but can reach the bloodstream and cause serious extrapulmonary diseases, such as septicemia, meningitis, and endocarditis [587, 588]. More than 100 different *S. pneumoniae* serotypes have been identified based upon capsular polysaccharide content [277, 589], which plays a role in the severity of pneumococcal disease [590]. *S. pneumoniae* harbors many other virulence factors that promote its transition from the colonizer state to an infectious stage. These range from surface proteins involved in interaction with host pulmonary epithelium to the expression of factors that mediate metabolic adaptation or immune evasion [591].

Among these bacterial factors is a 53-kDa heat-labile cholesterol-dependent cytotoxin (CDC), pneumolysin (PLY), which is a highly conserved and important virulence factor that promotes the establishment of infection and evasion of host immune responses [342, 592]. PLY creates pores in the cell membrane [298, 373, 517] through a multistep process [593] in which 30-50 toxin monomers insert in the cholesterol-rich domains of host cells, oligomerize to form a large pre-pore complex [594, 595] that then undergoes conformational changes to produce a large 25-nm β -barrel pore in the membrane [331, 373]. Numerous studies have highlighted the role of PLY in damaging the pulmonary epithelium, activating the classical complement pathway, inducing inflammation, and inhibiting neutrophil function [298, 591]. Strains lacking PLY show reduced virulence [596].

Glycosylphosphatidylinositol-anchored proteins (GPI-APs) are expressed in highly divergent organisms, e.g., from protozoa to humans, and mediate diverse functions, such as environmental sensing, cell adhesion and migration, and immunomodulation [8, 597, 598]. GPI-APs are attached to the outer leaflet of the cell membrane through a highly conserved glycosylphosphatidylinositol (GPI) anchor, which is composed of a phospholipid portion and a glycan chain [8]. The GPI anchor is covalently linked to the C-terminus of the protein in a multi-step post-translational modification process that occurs in the endoplasmic reticulum (ER) [9, 10]. The GPI-APs then migrate to the cell

surface via the secretory pathway, which involves trafficking through the ER, Golgi apparatus, and vesicular transport mechanisms, steps that are at least partially dictated by the GPI anchor [599]. Machinery that controls membrane fusion and sorting drives GPI-AP insertion in the plasma membrane, and in polarized epithelial cells, GPI-APs are selectively sorted to the apical domain [600] where they are organized into lipid rafts, specialized microdomains enriched in cholesterol and sphingolipids [8, 11]. In polarized cells the clusters are assembled in the Golgi apparatus and traffic to the apical domain, while in non-polarized cells the clusters organize from GPI-APs monomers already expressed at the cell membrane [12]. The expression of GPI-APs on the cell surface is highly regulated. GPI-APs can be released from the cell surface by the action of specific phospholipases, such as phospholipase C (PLC) [598], which cleave the GPI anchor resulting in the release of the protein into the extracellular environment. They can also be internalized through endocytosis, sorted and recycled back to the cell surface following vesicular trafficking pathways [601].

Rho GTPases are small GTP-binding proteins that act as molecular switches by cycling between an inactive GDP-bound state and an active GTP-bound state [602]. When activated, Rho GTPases interact with downstream effector proteins to initiate specific signaling cascades, which ultimately regulate key cellular functions [602]. In particular, Rho GTPases are implicated in the regulation of intracellular membrane trafficking processes such as endocytosis, exocytosis, and vesicle transport, and they control the cytoskeleton remodeling required for vesicle movement and fusion with target membranes [603]. The Rho GTPase Cdc42 has been proposed as a regulator of GPI-APs uptake [601]. During GPI-APs endocytosis, Cdc42 activation and the level of cholesterol at the cell membrane are coupled, with concomitant recruitment of actin polymerization machinery [604]. Following their internalization through a pathway that is cholesterol-sensitive and dependent on Cdc42-activation, GPI-APs traffic to the recycling endosomes and are recycled back to the cell surface, thus replenishing the pool of proteins that may have been removed or degraded.

Many GPI-APs have roles in immune modulation [10, 598]. Among them is CD73, the enzyme required for production of extracellular adenosine (EAD). EAD is produced in response to cellular damage whereby the ATP released in the extracellular milieu is sequentially converted to adenosine monophosphate (AMP) and then to adenosine by

two surface ecto-nucleotidases, CD39, a transmembrane protein and the GPI-AP CD73, respectively [605]. CD73 is crucial for host resistance to infection by *S. pneumoniae* [425] as mice lacking this enzyme are highly susceptible to infection and cannot control bacterial numbers [425]. We previously reported that the absence of CD73 on neutrophils diminished their ability to kill pneumococcus [425], thus favoring bacterial growth. Interestingly, the expression of CD73 is significantly reduced on neutrophils migrating into the lungs in response to pneumococcal infection [606], indicating that pneumococcal infection may result in the modulation of CD73 on host cell surfaces.

In this study, we report that infection with *S. pneumoniae* reduces CD73 surface expression on host pulmonary epithelial cells. This reduction reflects a general effect of PLY on the surface GPI-AP expression and depends on its pore forming capacity. The reduction in CD73 expression was not due to its release from the cell surface but rather due to internalization of GPI-APs in a RhoA- and Cdc42-dependent manner. Internalization of GPI-APs was associated with reduced host cell damage in response to PLY. This study identifies a possible mechanism through which repair of damage caused by *S. pneumoniae* helps the bacteria potentially escape the host immune response.

2. Results

2.1. Infection with *S. pneumoniae* reduces the expression of GPI-APs on the surface of lung epithelial cells.

We previously found that the EAD-producing enzymes CD73 and CD39 were crucial for host resistance against pneumococcal pneumonia [425, 607, 608]. To study the effect of *S. pneumoniae* infection on the expression of CD73 and CD39, we intra-tracheally (i.t.) infected C57BL/6 mice with *S. pneumoniae* TIGR4 strain or mock-treated with PBS. Forty-eight hours post infection (hpi), the lungs were harvested and the expression of CD73 and CD39 on total lung cells quantified using flow cytometry. *S. pneumoniae* significantly reduced the percentage of lung cells that express CD73 compared to uninfected controls, while the expression of CD39 remained unchanged (Fig 1A). In addition, a reduction in the amount of surface CD73 was detected on lung epithelial (EPCAM⁺) cells (Fig 1B).

To further explore the mechanisms involved, we modeled the decreased expression of CD73 on lung epithelial cells *ex vivo* using H292 cells, a human

pulmonary epithelial cell line extensively used to study the interaction between the host factors and respiratory pathogens [423, 564, 565, 609]. Infection of H292 cells with increasing numbers of *S. pneumoniae* TIGR4 resulted in a dose-dependent decrease in the expression levels of CD73 (Fig 1C), mimicking the *in vivo* finding.

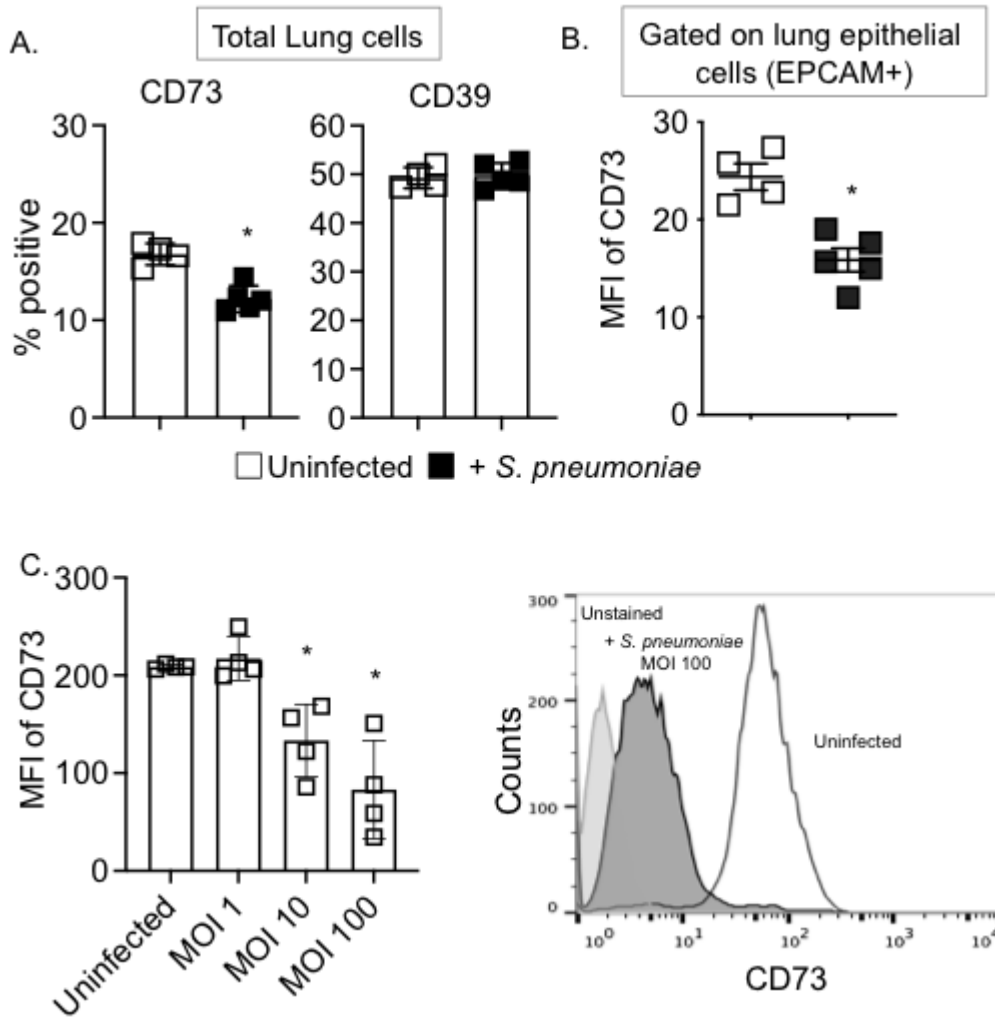


Figure 1: Pulmonary infection by *S. pneumoniae* significantly reduces CD73 expression on the surface of lung epithelial cells. C57BL/6 (8-weeks old) male mice were infected intra-tracheally with 2×10^6 CFU of *S. pneumoniae* TIGR4 strain or mock infected with PBS. Mice were euthanized 48 hpi, the lungs harvested and digested into a single cell suspension, and surface stained for CD73 and CD39. Flow cytometry was used to determine (A) the percentage of CD73⁺ and CD39⁺ total lung cells and (B) the expression (mean fluorescent intensity or MFI) of CD73 on lung epithelial cells (gated on EPCAM⁺ cell population). The data shown are pooled from 4-5 mice per group with each box representing one mouse. *, $p < 0.05$ by Student's t-test indicates significant differences from uninfected controls. (C) H292 cells were challenged with

S. pneumoniae TIGR4 strain at indicated MOIs for 1 h and MFI of surface exposed CD73 was measured by flow cytometry. The panel on the right shows a representative histogram showing the intensity of CD73 labeling comparing uninfected and infected conditions. Data are pooled from four independent experiments. *, $p < 0.05$ by One-way ANOVA followed by Dunnet's test.

These data indicate that *S. pneumoniae* infection specifically regulates the expression of CD73, a GPI-AP, but does not affect the levels of CD39, a transmembrane protein [610]. We thus tested if infection with *S. pneumoniae* selectively targets the surface expression of GPI-APs in general. H292 cells infected with *S. pneumoniae* TIGR4 were labelled with antibodies recognizing the GPI-APs CD55 and CD59 or stained with fluorescently labeled inactive variant of the protein aerolysin (FLAER), which selectively binds GPI anchors at the cell membrane [43]. Flow cytometric analysis showed that *S. pneumoniae* infection significantly reduced the expression of both CD55 and CD59, as well as FLAER staining intensity (Fig 2A). This reduction was not common to all surface proteins, as *S. pneumoniae* infection increased the expression of transmembrane proteins such as CD47 and CD54 (Fig 2B). These findings indicate that pneumococcus specifically reduces the surface expression of GPI-APs.

In vivo, epithelial cells are polarized, and GPI-APs mainly localize at the apical domain [44]. To determine if the pneumococcus-induced reduction of GPI-APs surface exposure also occurs on polarized epithelial cells, H292 cells were grown as polarized monolayers on Transwell filters [39, 45]. The cells were then apically infected with *S. pneumoniae* TIGR4 for an hour and the expression of GPI-APs was analyzed through flow cytometry. The expression levels of the GPI-APs CD73, CD55 and CD59 were significantly reduced in infected cells as compared to the uninfected controls (Fig S1). Overall, these data show that infection with *S. pneumoniae* reduces the surface expression of GPI-APs on lung epithelial cells.

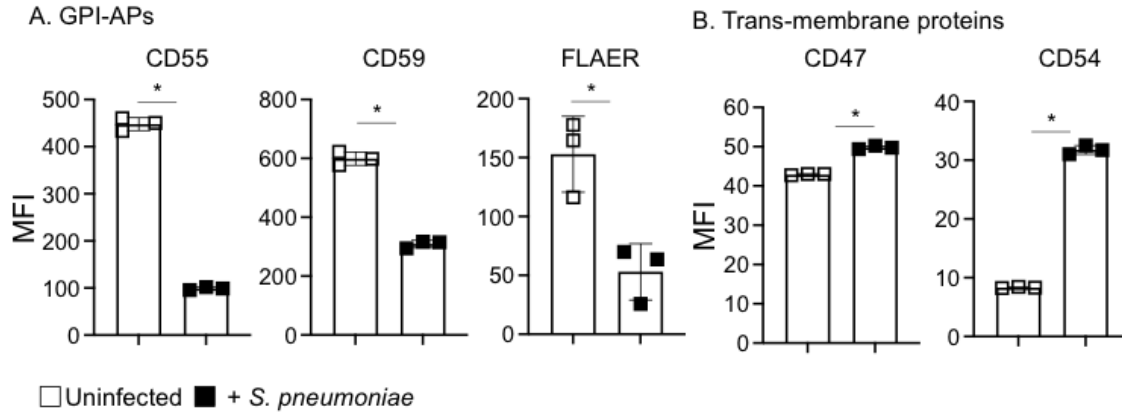


Figure 2: *S. pneumoniae* infection of lung epithelial cell lines decreases surface associated Glycosylphosphatidylinositol anchored proteins (GPI-APs). H292 cells were infected with *S. pneumoniae* TIGR4 strain at an MOI of 10 for 1 h. Flow cytometry was used to measure the expression (MFI) of (A) CD55 and CD59, GPI-APs detected using specific antibodies, and of bulk GPI-APs detected by using the fluorescent-labeled mutant of aerolysin (FLAER) or (B) CD47 and CD54, both transmembrane proteins detected by using specific antibodies. Data are pooled from three independent experiments. *, $p < 0.05$ by Student's t-test indicates significant differences from uninfected controls.

2.2. *S. pneumoniae*-induced reduction of GPI-APs is dependent on the pore-forming ability of PLY.

To determine if the reduced surface exposure of CD73 was strain dependent, H292 were infected with D39, a serotype 2 strain of *S. pneumoniae*. Like TIGR4 infection, infection with D39 significantly reduced the expression of CD73 on H292 cells (Fig S2A), suggesting that the effect of *S. pneumoniae* on CD73 surface expression is not strain specific. Infection of H292 cells with Δcps TIGR4, lacking the polysaccharide capsule, also reduced surface CD73 similarly to wild-type TIGR4 (Fig S2A), thus indicating that the reduction of CD73 expression is independent from the capsule. However, we found that heat-killed *S. pneumoniae* TIGR4 not only failed to reduce CD73 expression on H292 cells but rather increased it (Fig S2B). This indicates that a heat-labile bacterial factor is involved in reducing the expression of CD73 on lung epithelial cells.

S. pneumoniae pneumolysin (PLY) is heat-labile [298, 517], so we tested if the reduction in CD73 is PLY-dependent. H292 cells infected with Δply TIGR4 did not show diminished CD73 expression on the cell surface compared to the uninfected controls

(Fig 3A). Moreover, infection of H292 cells with *Bacillus subtilis* expressing *ply* (*B. subtilis* PLY) significantly reduced levels of CD73 as compared to cells challenged with wild-type *B. subtilis* (Fig 3A). To test if PLY alone is sufficient for reducing the expression of CD73, H292 cells were treated with increasing concentrations of purified recombinant PLY (rPLY). The expression of CD73 was reduced upon incubation with rPLY in a dose-dependent manner (Fig 3A). Altogether, these findings demonstrate that PLY is required and sufficient for decreasing CD73 from the cell surface of pulmonary epithelial cells.

To determine if pore formation is required to decrease CD73 expression on host cell surface, we tested PLY mutants defective at different stages of pore formation. 2.5 µg/ml of wild-type PLY elicited more than 3-fold reduction in CD73 expression (Fig 3A and B) but the same concentration of PLY^{T456G}, which is incapable of binding cholesterol [516], PLY^{M-1}, which is locked at an early stage of pore formation [373, 594, 611], or PLY^{P-1}, which is locked at late stage [373, 594, 611], failed to induce any change in CD73 expression on lung epithelial cells (Fig 3B). These results indicate that the phenotype observed requires the pore-forming ability of PLY.

As PLY pores may result in the death of lung epithelial cells during infection [302, 612], we tested if the reduced expression of GPI-APs could be a consequence of cell death. We tracked cell death following infection with *S. pneumoniae* TIGR4 by measuring apoptosis and found that infection resulted in rapid apoptosis of H292 cells (Annexin⁺) as early as 10 minutes post-infection (Fig S3A). This depended on PLY, as infection with Δply *S. pneumoniae* TIGR4 was not associated with a significant effect on cell viability (Fig S3A). However, flow cytometric analysis showed that infection with *S. pneumoniae* induced a similar significant 2-fold reduction in the intensity of FLAER on both apoptotic (Annexin⁺) and non-apoptotic (Annexin⁻) cell populations compared to the uninfected control groups (Fig S3B), demonstrating that decrease in GPI-APs is not a result of cell death. We further confirmed that PLY-dependent reduction in GPI-APs could be observed at this early 10-minute timepoint, as 10-minutes post-infection of H292 cells with wild-type but not Δply TIGR4 resulted in significant reduction of FLAER binding (Fig 3C). Altogether, these data indicate that *S. pneumoniae*-induced reduction in GPI-APs on host lung epithelial cells is PLY-mediated.

2.3. Cholesterol-dependent-cytolysins reduce the expression of GPI-APs at the surface of lung epithelial cells.

PLY belongs to a large family of cholesterol-dependent-cytolysins (CDC) that share structural and functional properties [298]. To determine if the PLY-mediated reduction of GPI-APs expression is a common feature shared among different members of CDCs, H292 cells were challenged with two other CDCs: Streptolysin O (SLO) and Intermedilysin (ILY), produced by Group A *Streptococci* and *Streptococcus intermedius*, respectively [613]. As pore formation is required for reduction of GPI-AP expression, we treated H292s at concentrations sufficient to induce equal levels of pore-formation, but that did not result in H292 cell death [373]. We found that incubation of cells with SLO and ILY with 5 units of pore forming activity caused a reduction in the FLAER intensity like that induced by PLY (Fig 3D), indicating that the decrease of GPI-AP expression is a feature common to different CDCs.

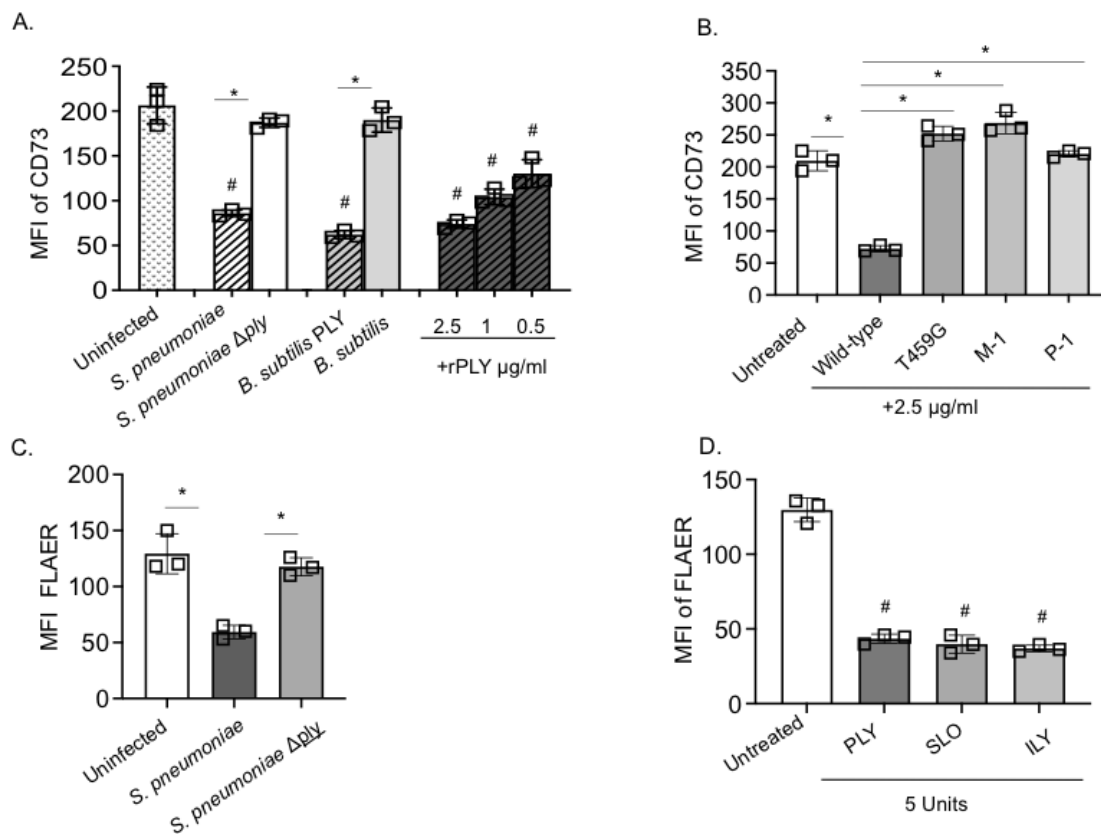


Figure 3: The decrease in cell surface expression of GPI-APs is dependent on PLY and its pore formation capacity. H292 cells were infected with the indicated bacterial strains at an MOI of 10 or treated with the indicated concentrations of recombinant toxins for 1 h (A-B) or

10 min (C-D). The cells were then stained and the MFI of CD73 or FLAER was measured by flow cytometry. Data pooled from three independent experiments are shown. #, $p < 0.05$ indicates significant differences from uninfected controls and *, $p < 0.05$ indicates significant differences between the indicated groups by One-way ANOVA followed by Tukey's test (A-C) or Dunnet's test (D). Units for the indicated toxins were determined by their ability to induce pores in the cells. 1U of toxin is equivalent to the concentration of toxin that causes pores in 50% of the cells. rPLY (recombinant PLY), PLY (Pneumolysin), SLO (Streptolysin O) and ILY (intermedilysin). T459G, M-1 and P-1 refer to *S. pneumoniae* strains expressing mutant forms of PLY that cannot form fully functional pores (see Materials & Methods).

2.4. GPI-APs are not released from the cell surface upon infection with *S. pneumoniae*

GPI-APs can be removed from the host cell surface through cleavage by phosphatidylinositol phospholipase C (PI-PLC) [614]. In fact, *S. pneumoniae* that produces the pneumococcal surface protein C (PspC) can activate PI-PLC [615]. However, we found that PspC-deficient *S. pneumoniae*, which are incapable of activating host PI-PLC, showed no defect in triggering a reduction of surface CD73 (Fig S4A). In addition, treatment of H292 cells with the phospholipase C specific inhibitor U-73122 (or its inactive analogue U-73343 [616]) did not abrogate the *S. pneumoniae*-mediated reduction in CD73 surface expression (Fig S4B). These findings indicate that reduction in cell surface-associated CD73 upon *S. pneumoniae* infection does not depend on the activity of host PI-PLC.

As part of a membrane repair mechanism, host cells infected with *S. pneumoniae* can release extracellular membrane vesicles through which they expel membrane-bound bacterial products such as PLY [119, 563]. To determine if CD73 was shed in host-derived vesicles, H292 cells were treated with CellTracker Green dye CMFDA, which stains the cell cytoplasm and can be used to track host-derived vesicles. Following a challenge with *S. pneumoniae* TIGR4, cell free supernatants were collected, stained with FLAER and the presence of vesicles simultaneously positive for FLAER and CMFDA was analyzed through flow cytometry. Surprisingly, we observed a significant reduction in the percentage of vesicles FLAER⁺ CMFDA⁺ released by H292 cells infected by *S. pneumoniae* compared to the uninfected controls (Fig S5), suggesting that the decrease of GPI-APs induced by *S. pneumoniae* infection is not due to the release of extracellular vesicles by the infected cells. As injured cells can also release parts of

injured membranes via the formation of protrusions named blebs [101, 521], we tested the effect of treating H292 cells with blebbistatin, a drug reported to inhibit blebbing [617]. We found that treatment with blebbistatin did not affect the levels of GPI-APs (Fig S6). Collectively, these data indicate that the reduction in GPI-APs following pneumococcal infection is not due to their release from the cell surface by the activity of phospholipases, shedding in vesicles or blebs.

2.5. Infection with *S. pneumoniae* triggers the internalization of GPI-APs

As we found that the decreased levels of GPI-APs at the cell surface are not related to their extracellular release, we tested if infection of lung epithelial cells by *S. pneumoniae* triggers their internalization. To do so, H292 cells were infected with *S. pneumoniae*, stained with FLAER to identify GPI-APs, and imaged by confocal microscopy. In conditions where H292 cells were treated with HBSS (uninfected controls) or were challenged with Δply *S. pneumoniae*, GPI-APs were uniformly detected on the surface of the cells (Fig 4A), following the outline of the cell membrane. However, in *S. pneumoniae* infected cells, GPI-APs lose their uniform association to the cell cortex and appear in clusters inside cells (Fig 4A, white arrows). These data suggest that infection with *S. pneumoniae* triggers the internalization of GPI-APs.

If GPI-APs are removed from the cell surface by internalization (vs. extracellular shedding), the total amount of GPI-AP associated with cells is predicted to be unchanged. To test that, H292 cells were stained with FLAER before infection by *S. pneumoniae* ("PRE"). After 1 h, cell-associated GPI-APs was quantitated by flow cytometry. Indeed, we found that FLAER staining remained unchanged, i.e., was indistinguishable from FLAER staining of uninfected cells (Fig. 4B, "PRE"). To confirm that under these experimental conditions, *S. pneumoniae* resulted in the removal of GPI-APs from the cell surface, in parallel we stained H292 only after the 1 h infection ("POST"). As predicted, surface staining by FLAER after infection revealed decreased surface GPI-APs (Fig. 4B, "POST"). These findings confirm that infection with *S. pneumoniae* induces the internalization of GPI-APs.

To test whether PLY was sufficient to trigger this process, as well as to follow GPI-AP internalization in living cells, HeLa cells (chosen due to their ease of imaging by microscopy) were pre-labelled with FLAER and then challenged with purified PLY or mock-treated. FLAER-stained GPI-APs were visualized by fluorescent microscopy for 45

minutes (Supplementary video 1). FLAER intensity was rapidly reduced on the cell surface upon challenged with PLY (Fig 4C and Supplementary video 1), in addition FLAER signal was detected intracellularly within 3 minutes of incubation with PLY (Fig 4C and Supplementary video 1) in structures resembling intracellular vesicles (Fig 4C and Fig S7). At 45 min after PLY incubation FLAER signal at the surface of the cells appeared to increase (Figure 4C and S7), suggesting that internalized GPI-APs are recycled back to the cell surface. Overall, these data show that the infection with *S. pneumoniae* triggers the internalization of surface GPI-APs which are then found intracellularly in vesicle-like structures.

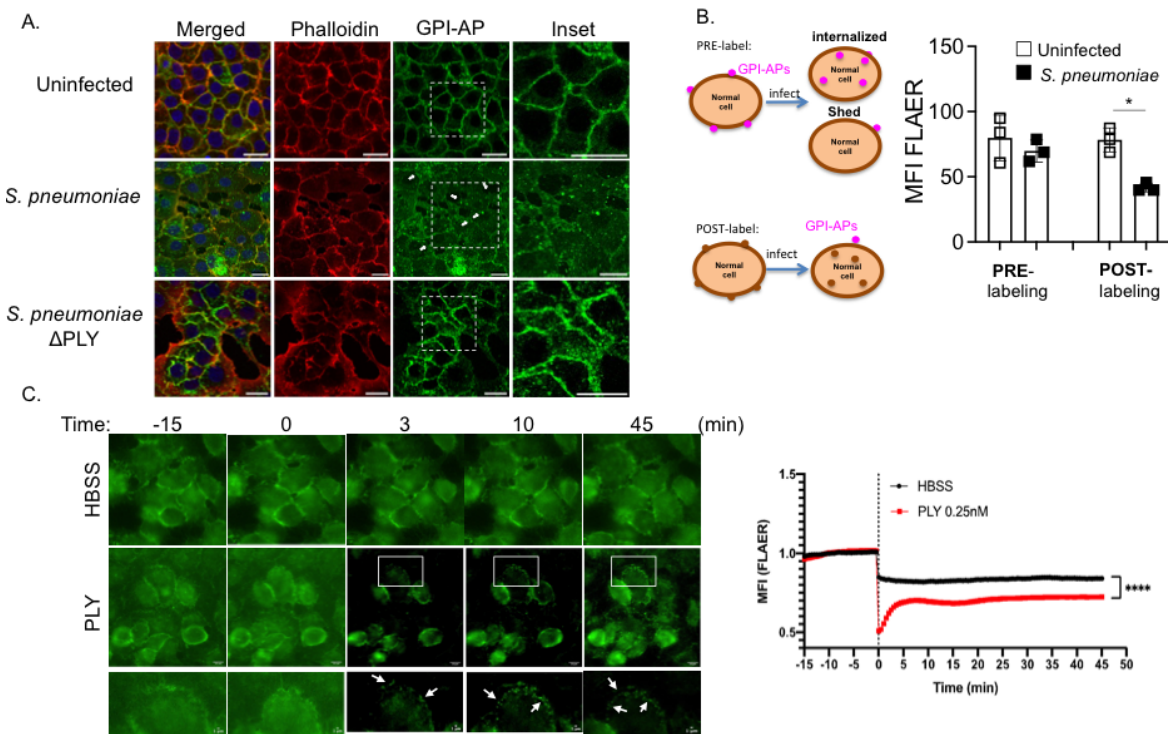


Figure 4: Infection with *S. pneumoniae* triggers the internalization of GPI-APs from the host cell surface. (A) H292 cells were infected with wildtype or *S. pneumoniae* Δ ply at an MOI of 20 for 1 h, fixed with PFA for 20 min, permeabilized with 0.1% Tx-100 and stained with phalloidin (to detect actin filaments, red) and FLAER (to detect GPI-APs, green). White arrows point GPI-APs concentrating intracellularly into vesicle-like structures. Insets highlight FLAER staining in the different conditions. Bars indicate a 20 μ m scale. Representative images from four independent experiments are shown. (B) Left panel shows the schematic illustration of FLAER labeling of H292 cells either pre- or post-infection with *S. pneumoniae* TIGR4 strain (MOI of 10 for 1 h). The right panel shows the MFI of FLAER measured by flow cytometry. Data pooled

from three independent experiments are shown. *, $p < 0.05$ indicates significant differences between the indicated groups by One-way ANOVA followed by Tukey's test. (C) The left panel shows sequential frames of time-lapse microscopy video of HeLa cells pre-labeled with FLAER and challenged with PLY (0.25 nM) or HBSS (control). PLY was added at time point 0 min. The inset shows detailed distribution of GPI-APs up to 45 min post PLY challenge. The right panel shows quantified MFI for the FLAER signal overtime. Image analysis was performed using ImageJ by randomly selecting a region of interest (ROI) in the membrane of at least 53 cells per condition and calculating MFI overtime. The values were then normalized to the mean MFI calculated before adding PLY or HBSS. Scale bar for the middle lane is 10 μm and for the bottom lane is 1 μm . White arrows in the bottom lane indicate where GPI-APs appear in "vesicle-like" structures likely inside the cells. *, $p < 0.05$ by Student's paired t-test.

2.6. Internalization of surface GPI-APs is mediated by PLY-induced activation of host Rho GTPases RhoA and Cdc42.

To explore the mechanism behind *S. pneumoniae*-mediated internalization of GPI-APs, we first assessed a possible role of the actin cytoskeleton, given that dynamic changes in the actin machinery are often required for endocytosis [618]. H292 cells were pre-treated with Cytochalasin D, an inhibitor of actin polymerization, at 2 mM, a concentration that prevented the actin-dependent cell-cell spread of *Listeria monocytogenes* ([619] and data not shown). Treatment with Cytochalasin D did not affect the reduction of FLAER intensity following *S. pneumoniae* infection (Fig S8A), suggesting an actin-independent mechanism for the internalization of GPI-APs. Similar results were observed when Cytochalasin D pre-treated H292 cells were challenged with PLY or SLO (Fig S8B), indicating that other CDCs also induce the internalization of GPI-APs through a mechanism independent of actin polymerization.

The host Rho family of guanosine 5'-triphosphate (GTP)-binding proteins (GTPases) are known to play important roles in protein trafficking, receptor-mediated endocytosis, and internalization of GPI-APs [620]. To examine the role of Rho GTPases in the internalization of GPI-APs induced by *S. pneumoniae* infection, H292 cells were treated either with *Clostridium difficile* toxin B (ToxB), which irreversibly inactivates Rho GTPases by glucosylation [621], or with vehicle control, before infection with *S. pneumoniae*. While infection with *S. pneumoniae* in vehicle-treated cells caused the expected reduction in FLAER intensity indicating the decreased surface expression of GPI-APs (Fig 5A), cell surface expression of GPI-APs was not affected in cells pre-treated with ToxB (Fig 5A), suggesting a role for Rho GTPases in PLY-mediated GPI-APs

internalization. To confirm the role of Rho GTPases in the internalization of GPI-APs, we knocked down their expression using RNAi. H292 cells were transfected with siRNAs targeting Cdc42, RhoA and Rac-1 or with scrambled non-targeting siRNA as control. At 48 h post transfection, cells were challenged with *S. pneumoniae* at a MOI of 10 for 1 h and FLAER intensity was quantified through flow cytometry. Depletion of Cdc42 and RhoA but not Rac-1 abrogated the expected reduction in FLAER intensity usually observed in H292 cells infected by *S. pneumoniae* (Fig 5B). This indicates that the internalization of pneumococcal-mediated GPI-APs depends on the Rho GTPases Cdc42 and RhoA.

Rho GTPases were previously reported to be activated by PLY in neuronal cells [393, 517]. We assessed if Cdc42 and RhoA are activated by PLY in epithelial cells in the time frame of PLY-mediated internalization of GPI-APs. We transfected HeLa cells with plasmids encoding the fluorescence resonance energy transfer (FRET) probes (CFP/YFP FRET pairs) that reflect the activation state of RhoA or Cdc42. Each probe comprises a truncated form of the GTPase and the Rho-binding domain (RBD) of effectors, bound to the CFP or YFP, respectively. The binding of GTP to the GTPase (resulting in activation) brings the YFP-bound GTPase domain and the CFP-bound RBD into close proximity, allowing FRET from CFP to YFP [101, 563]. Transfected HeLa cells were challenged with 0.25 nM of PLY and live-cell microscopy was performed to visualize the activation status of RhoA and Cdc42. FRET ratio (FRET/CFP) was calculated specifically at the cell cortex using Image J. Cells were imaged for 20 min before PLY was added and for additional 10 min after PLY challenge. For both probes, the ratio of FRET/CFP remained stable during the 20 min before PLY addition, showing that the levels of GTPase activation do not vary in these conditions (Fig 5C). Shortly after the addition of PLY, we observed a modest but consistent increase in the FRET/CFP ratio levels. The increase detected for the Cdc42 probe was very rapid and of short duration (about 40 s), while the increase for the RhoA probe appeared more sustained in time (140s) (Fig 5C). These data suggest that PLY triggers a fast and transient activation of both RhoA (Supplementary video 2) and Cdc42 (Supplementary video 3) at the cell cortex. The ratio of FRET/CFP, for both probes, rapidly decreased to values that tended to be lower than those obtained before PLY incubation (Fig 5C), indicating that although the activity of RhoA and Cdc42 is triggered during the very early response to PLY induced damage, it is rapidly shutdown. Overall, these data

indicate that PLY induces internalization of GPI-APs through the rapid, spatially, and temporally regulated activation of the Rho GTPases RhoA and Cdc42.

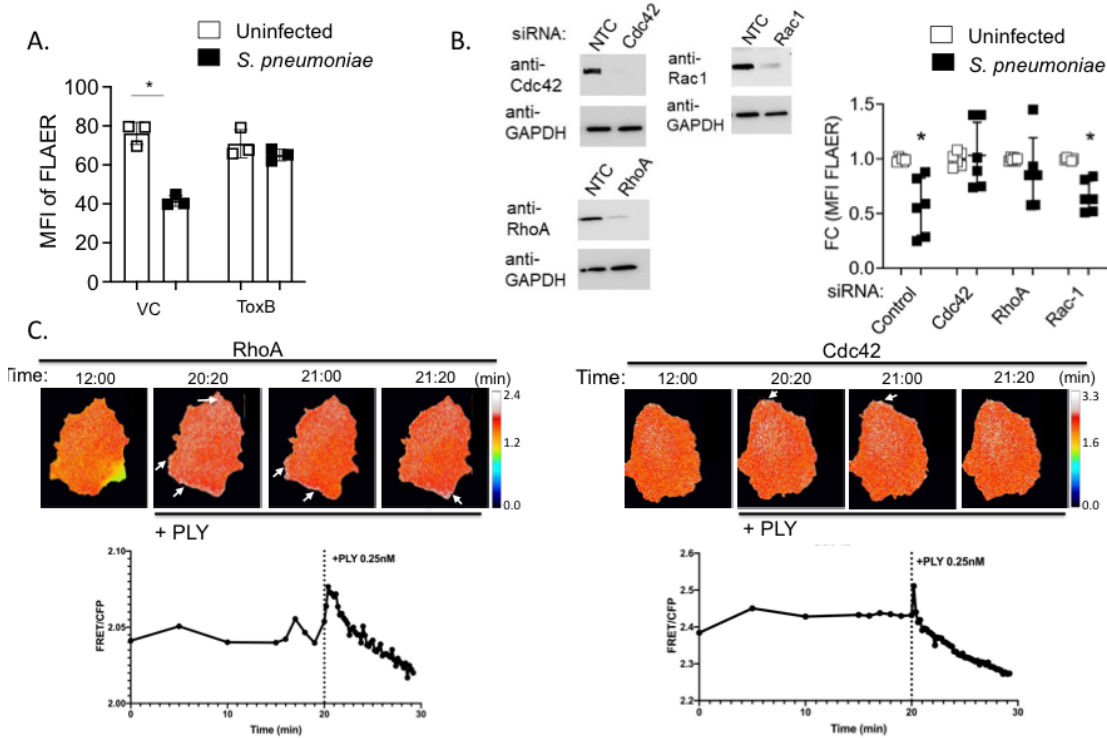


Figure 5: Removal of GPI-APs from the cell surface largely depends on host GTPases RhoA and Cdc42 activity. (A) H292 cells were pre-treated with 100 ng/ml *C. difficile* toxin B (Tox B) or vehicle control (VC) overnight and infected with *S. pneumoniae* TIGR4 strain at an MOI of 10 for 30 min, labeled with FLAER and the MFI was assessed by flow cytometry. Data are pooled from three independent experiments. *, $p < 0.05$ by One-way ANOVA followed by Tukey's test. (B) H292 cells were transfected with a control non-targeting (NTC) siRNA, or siRNA targeting GTPases Cdc42, RhoA and Rac1. At 48 h post-transfection, whole cell lysates were used to confirm the depletion of the target proteins through Western blotting. Detection of GAPDH was used as a loading control (left panel). Depleted and control cells were infected with *S. pneumoniae* TIGR4 at an MOI of 10 for 30 min, labeled with FLAER and the MFI assessed by flow cytometry (right panel). The MFI values of FLAER are displayed as fold changes with respect to the uninfected baseline for the respective siRNA condition. The data shown are the mean of three independent experiments done in duplicates. *, $p < 0.05$ indicates significantly different than 1 by one-sample t-test. (C) HeLa cells over-expressing a FRET probe for RhoA or Cdc42 were imaged for 20 min before and 10 min after incubation with 0.25 nM of PLY. The upper panel shows representative sequential frames of time-lapse images of HeLa cells before and

after incubation with PLY. FRET/CFP ratio images are shown in blue-to-white pseudocolor scale with white being high activation. Arrows show the regions where RhoA (left) or Cdc42 (right) are activated (FRET/CFP signal is high). The lower panel shows the quantitative analysis for the ratio between the FRET and CFP signal (FRET/CFP), which measures the RhoA and Cdc42 activation levels. The quantification was performed using a custom-made macro to calculate FRET/CFP ratio specifically at the cell membrane. 79 cells (for RhoA) and 74 cells (for Cdc42) were analyzed from two independent experiments.

2.7. GPI-APs are not required for PLY-mediated pore formation

The PLY-related CDC ILY utilizes the human GPI-AP CD59 as a receptor to induce oligomerization and pore formation [622] and the cytolytic pore forming toxin aerolysin binds the GPI moiety of the GPI-anchored T-lymphocyte protein Thy-1 [597]. To test whether PLY requires the expression of GPI-APs on the host cell surface to form pores, H292 cells were either treated with recombinant PI-PLC to cleave GPI-APs from the cell surface [614], or mock-treated with PBS. As expected, treatment of cells with PI-PLC caused a significant reduction in the percentage of GPI⁺ cells (FLAER⁺) compared to the untreated control group (Fig S9A). Upon addition of PLY to H292 cells, we detected the expected decrease in GPI⁺ cells compared to the untreated control group (Fig S9A). We did not see a further reduction in the number of cells expressing GPI-APs when PLY was added to PI-PLC pre-treated cells (Fig S9A).

We then assessed whether reduced expression of GPI-APs impact PLY-mediated pore formation using PI permeability assays. We observed that PLY challenge induced a 4-fold increase in the number of permeabilized cells (PI⁺) in both the untreated and PI-PLC pre-treated conditions in comparison to the respective baseline controls (Fig S9B). Finally, the average intensity (MFI) of PI staining was unaltered by PI-PLC treatment. These data indicate that PLY does not require surface GPI-APs to bind and form pores on host cell membranes.

2.8. Internalization of GPI-APs is associated with lower levels of PLY-induced cellular damage

PLY forms pores in cholesterol rich domains, where GPI-APs are also colocalized [298]. Depending on the extent of damage, host cells can repair the damaged membrane and revert to homeostasis [76]. To couple the kinetics of the PLY-mediated removal of GPI-AP from the cell surface with an analysis of the population of cells that

have undergone this reduction, H292 cells were challenged with *S. pneumoniae* or mock-treated with PBS for different time periods and the kinetics of reduction in FLAER intensity was determined throughout infection. According to our previous live-cell imaging experiments of cells challenged with rPLY (Fig 4C), we expected the reduction to occur rapidly. Indeed, *S. pneumoniae* significantly reduced FLAER intensity within 10 minutes of infection compared to the PBS-treated cells and retained this reduced level for the remainder of the 90-minute observation period (Fig 6A). To analyze the specific population of cells that reduced GPI-AP, we utilized flow cytometry and found that by 10 minutes after infection approximately 60% of the infected cells showed reduced levels of GPI-AP (termed “GPI^{low}” cells) (Fig 6B), a percentage that also remained stable through the rest of the experiment.

To test if the reduction of GPI-APs from the cell surface affected cellular viability, we measured the staining of the population of GPI^{low} or GPI^{high} H292 cells by Annexin V, which stains apoptotic cells, and PI, which stains cells with permeable membranes. Interestingly, we found that, throughout infection, the population of infected GPI^{high} H292 cells had a significantly higher percentage of membrane damaged (PI⁺) (Fig 6C) and apoptotic (Annexin V⁺) cells than the population of GPI^{low} cells (Fig 6D). These findings suggest that reduced GPI-APs surface expression is associated with reduced levels of PLY-induced damage. Altogether, these findings suggest that cells with reduced expression of GPI-APs are more resistant to PLY-induced toxicity and that the internalization of GPI-anchored proteins seems to be part of membrane repair that limits the extent of PLY-induced cellular damage.

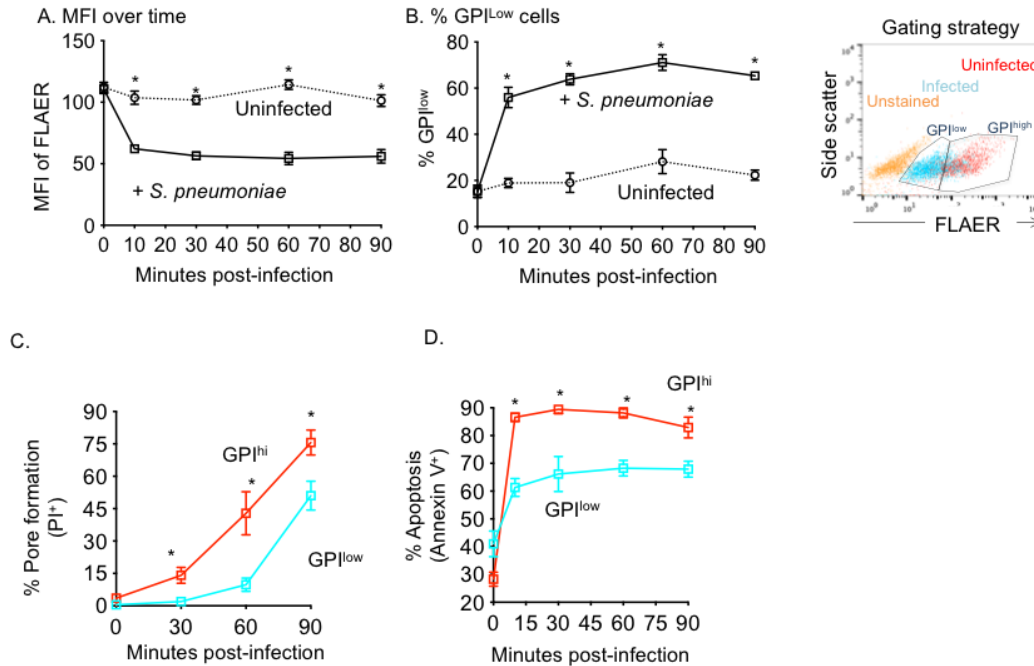


Figure 6: Internalization of GPI-APs is associated with lower levels of PLY-induced cellular damage. H292 cells were infected with *S. pneumoniae* TIGR4 strain at an MOI of 10 or left uninfected. The cells were then labeled with FLAER and flow cytometry was used to measure (A) the MFI of FLAER at indicated time points of infection and (B) calculate the percentage of H292 cells (left panel) with down-regulated FLAER expression (termed as GPI^{low}) based upon the gating strategy shown in the right panel. (C) H292 cells suspended in media containing propidium iodide (PI) were infected with *S. pneumoniae* TIGR4 strain at an MOI of 10 and labeled with FLAER. GPI^{low} vs GPI^{hi} cell populations were then gated on, as indicated in the left panel of (B) and the percentage of H292 cells within each population that incorporated the PI dye was assessed by flow cytometry. (D) H292 cells were infected with *S. pneumoniae* for the indicated times and stained with Annexin V and FLAER. GPI^{low} vs GPI^{hi} populations were gated (right panel in B) and the percentage of apoptotic (Annexin V +) cells within each population was determined through flow cytometry. Data pooled from three independent experiments are shown. * $p < 0.05$ indicate differences by one-way ANOVA comparison between the different groups at indicated time points.

3. Discussion

The outcome of host-pathogen interaction is proposed to be mediated by the damage response [623, 624]. This damage can be caused by the invading organism and/ or the host's immune response to infection. *S. pneumoniae*, a typically

asymptomatic colonizer of the host upper respiratory tract, can cause serious and life-threatening infections when it gains access to the lungs and other organs and this is directly linked to its ability to cause organ damage and impair organ function [373, 625-627]. One key component of this is the bacterial toxin PLY, found in all the clinical isolates of *S. pneumoniae* [308]. PLY induces host cell damage by making pores in cells [627]. However, host survival can be contingent on repairing/controlling infection-induced damage [628]. In this study, we found that PLY-induced pores are associated with the rapid removal of GPI-APs from the host cell surface, which can have profound effects on host response to infection. PLY activated host RhoGTPases RhoA and Cdc42, resulting in the internalization of GPI-APs (Figure 7). This internalization of GPI-APs was associated with reduced cellular damage thus, suggesting the induction of host repair mechanisms.

GPI-APs are a heterogeneous group of proteins which lack transmembrane domains and are dependent on the GPI moiety for their expression on the cell surface [10]. Through their interactions with the sphingolipids and cholesterol in the lipid bilayer, the GPI moieties anchor the proteins in lipid-rich microdomains or membrane rafts [10, 629]. We found here that removal of GPI-APs from the cell surface was not only mediated by PLY, but also occurred upon treatment of host cells with other cholesterol dependent cytolysins (CDCs) including SLO and ILY. CDCs bind to the cholesterol moieties in the host cell surface through the undecapeptide domain of the peptide to mediate their cytolytic activity [593, 622]. Binding of the soluble monomeric form of the CDCs to the cholesterol-rich membrane results in a sequence of structural changes causing oligomerization of the monomers with subsequent formation of transmembrane β -barrel that results in pore formation in the host cell membrane [622, 630]. Therefore, CDCs and GPI-APs will likely localize together in cholesterol-rich microdomains at the cell surface where the damage occurs. Consequently, internalization of GPI-APs following CDC-induced damage may be part of membrane repair. Prior work with SLO showed that pores formed by the toxin are endocytosed as part of the membrane repair process [141]. Here, we found that removal of GPI-APs by PLY was dependent on the pore forming ability of the toxin and that internalization of GPI-APs was associated with reduced cellular damage, suggesting that the host can repair the damage at the membrane.

The type and extent of host cell damage as well as the host response to CDCs is highly concentration dependent [76]. At high concentration levels, PLY induces irreversible loss of cell membrane integrity and cellular homeostasis, while at lower concentrations, it activates host cell repair mechanisms to restore their membrane integrity and to survive [76]. Initiation of host cell repair of the plasma membrane depends on a limited influx of Ca^{2+} that triggers cytoskeletal rearrangements to plug the holes in the plasma membrane [408, 517]. This has been described to be mediated by the activation of host GTPases, re-localization of host intracellular organelles and recruitment of cytoplasmic Ca^{2+} responsive proteins including annexins that interact with the plasma membrane phospholipids at the injury site [408, 517, 631]. Host Rho GTPases, including Rho, Rac and Cdc42, play a major role as molecular switches that regulate key cellular signaling pathways and link surface receptors to the downstream processes culminating in endocytosis, exocytosis and actin polymerization that affect cell polarity, migration, cytokinesis and vesicle trafficking [632]. While exploring the cellular mechanisms that drive GPI-APs reduction at the cell surface, we found that removal of GPI-APs from the cell surface upon *S. pneumoniae* infection was dependent on Cdc42 and RhoA while Rac1 was not involved in this process. These data fit with what has been reported in the literature regarding the role of Rho GTPases in the turnover of GPI-APs under homeostatic conditions. GPI-APs are normally recycled from the cell surface through an endocytic process called potocytosis involving caveolae, which is independent of both dynamin and clathrin-mediated pathways [601, 633]. Once internalized, GPI-APs within the cell can be seen in tubular structures called GPI-enriched endosomal compartments [633, 634]. Internalization of GPI-APs via endocytosis is under the regulation of host GTPases [633, 634]. This includes CD59, one of the GPI-APs for which we observed reduction in surface expression upon infection, whose internalization via endosomes is reported to be regulated by Cdc42 [635]. Additionally, perturbation in cholesterol moieties in the lipid bilayer also leads to internalization of GPI-APs via clathrin- and dynamin-independent endocytic pathway [633]. Therefore, CDC binding to the cell surface and pore formation results in the perturbation of the cholesterol rich microdomains where GPI-APs are localized resulting in initiation of the endocytic pathway which may lead to the repair of the injured membrane.

One of the interesting findings of this study was that actin polymerization had no apparent role in the internalization of GPI-APs despite the activation of Cdc42 and RhoA. Cdc42 is a key regulator of actin polymerization through the regulation of N-WASP and Arp2/3 complex and similarly, RhoA also regulates actin polymerization [632]. Several recent reports have described actin-independent endocytic events in various cell types. Work on non-adherent K562 human erythroleukemic cells or adherent Cos-7 cells showed a lack of actin polymerization role in the endocytosis process in these cells [636]. In another study, treatment of A431 cells (adherent and in suspension) with cytochalasin D did not affect the process of endocytosis indicating an actin-independent mechanism of endocytosis in these cells [636]. Additionally, the rationale that the polymerization of actin is important to generate the mechanical force required for vesicle fission during clathrin-mediated endocytosis was recently challenged. In mouse chromaffin cells, inhibition of dynamin GTPase but not of actin polymerization resulted in changes in the fission-pore conductance in the endocytic membranes indicating that the pinching off of the endocytic vesicle from the plasma membrane is actin-independent [637]. The requirement of actin in endocytosis could be spatially regulated as in polarized cells inhibition of actin polymerization blocked clathrin-mediated endocytosis from the apical but not the basolateral surface of polarized Madin-Darby Canine Kidney Cells (MDCK) [638]. This study observed PLV-mediated reduction in GPI-AP expression in pulmonary epithelial cells from infected murine models as well as in non-polarized and polarized H292 cells. Overall, our findings point to a signaling pathway whereby PLV activated Cdc42 and RhoA initiate internalization of GPI-APs independent of actin polymerization machinery. Other bacterial pathogens have evolved to use actin polymerization-independent mechanisms to trigger host responses. For example, *Salmonella enterica* serovar Typhimurium was reported to activate Rho kinases to mediate entry into the cell by activating myosin II independent of actin polymerization [639]. Similarly, *Listeria monocytogenes* was shown to manipulate host exocytosis independent of actin polymerization to enter the host cell [640].

Apart from stimulating membrane repair through annexins, injury to plasma membrane by PLV, LLO or SLO also results in a phenomenon called shedding whereby the host cell releases extracellular vesicles to not only remove the injured part of the membrane but also to remove the membrane bound toxin [119, 563]. Blebbing is one

of the ways to mediate the shedding of larger vesicular structures whereby the cell undergoes actomyosin-dependent localized contraction in the plasma membrane to create confined spaces or a localized bulge with elevated Ca^{2+} levels to prevent leakage to cytoplasmic contents and proteins in the extracellular milieu [76, 517]. These localized bulges in the plasma membrane are eventually released by the cells either to remove the areas of pores in case of a low degree of plasma membrane injury or to initiate the processes of regulated apoptosis and inflammation in case of extensive cell damage [76, 101]. Since shedding and blebbing result in the loss of part of the plasma membrane, it could also result in the loss of expression of GPI-APs. However, data presented here using CMFDA dye to track the vesicles and using chemical inhibition of blebbing showed that this was not the case. Surprisingly, we observed a significant reduction in the percentage of vesicles FLAER+ CMFDA+ released by H292 cells infected by *S. pneumoniae* compared to the uninfected controls. We also found no evidence for the enzymatic release of GPI-APs from the cell surface to the extracellular milieu. Certain bacterial species including *S. pneumoniae* have evolved to induce the expression of host lipolytic enzyme phospholipase [641]. Given that the anchors of GPIs interact with the lipid moieties of the plasma membrane, the GPI-APs are therefore susceptible to cleavage by phospholipases [598, 642]. The findings here with phospholipase C specific inhibitor U-73122 and using a ΔpspC *S. pneumoniae* strain showed that the reduction in CD73 expression following PLY treatment was not a consequence of cleavage of GPI-APs by the host phospholipase.

The removal of GPI-APs from the host cell surface can seriously affect the host's ability to control infection. These GPI-bound proteins are essential for many host physiological functions including but not limited to enzymatic activities, nutrient uptake, complement regulators, immune antigens and adhesion molecules [12, 425, 598, 643, 644]. This work shows that treatment with PLY reduces CD55, CD59 and CD73 levels on pulmonary epithelial cells, all of which are GPI-APs and important regulators of host inflammatory response. CD73 is an ectonucleosidase that is required for initiating host responses to damage [610]. The host can sense infection-induced damage by recognizing several damage-associated molecular patterns (DAMPS), including direct recognition of extracellular ATP and its breakdown product adenosine in the extracellular environment [645]. CD73 is required to dephosphorylate ATP, which has leaked from damaged cells, into adenosine [646]. We

previously found that extracellular adenosine production by CD73 was required for host resistance against *S. pneumoniae* in part by modulating epithelial responses to these bacteria [425, 606, 607]. Other groups have also found that CD73 expression on epithelial cells is important in other infections where CD73 expression on intestinal epithelial cells was needed to control *Salmonella enterica* serovar Typhimurium infection [647]. Apart from CD73, both CD55 and CD59 act as inhibitors of complement membrane attack complex (MAC) [648, 649] and have a role in neutrophil trans-epithelial migration [650]. Therefore, PLV's downregulation of CD55 and CD59 could also have important implications on host immune responses. In summary, our findings suggest that repair mechanisms activated in response to infection-induced cellular damage may inadvertently help *S. pneumoniae* and other bacterial pathogens evade host defenses, reflecting the dual function of PLV during infection.

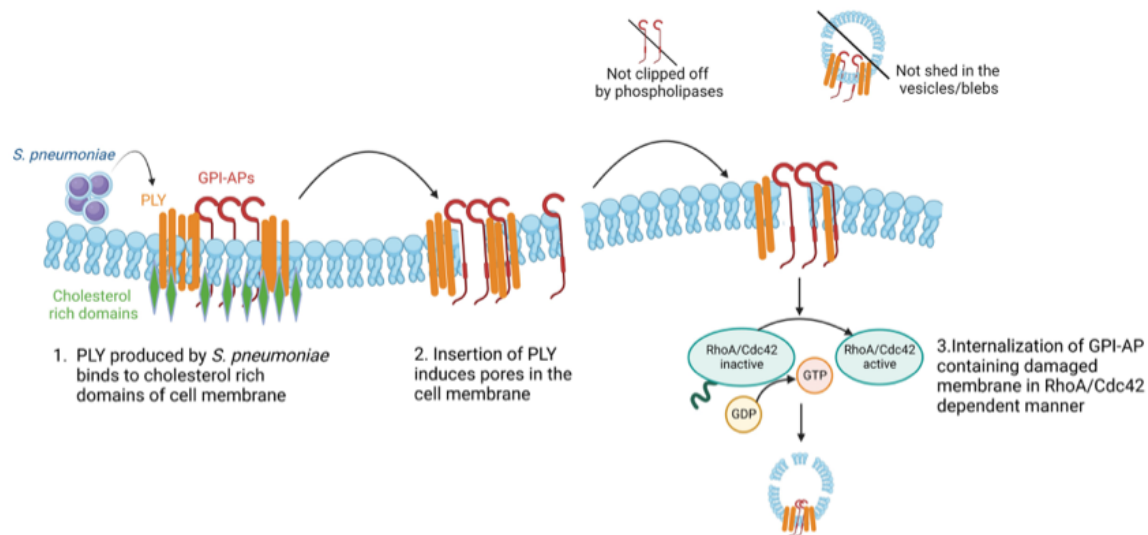


Figure 7: Working Model. PLV inserts in cholesterol-rich microdomains where GPI-APs are colocalized. PLV-induced pores trigger membrane repair resulting in internalization of the GPI-AP containing damaged membrane in a RhoA/Cdc42-dependent manner.

4. Materials and Methods

Ethics Statement: Mice were handled in accordance with the recommended Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. All procedures were done according to the protocol approved by the University at

Buffalo Institutional Animal Care and Use Committee (IACUC), approval number MIC33018Y.

Mice: All the animal work was performed in 8-12 weeks C57BL/6J male mice purchased from Jackson Laboratories (Bar Harbor, ME) and housed at a specific-pathogen free facility at the University at Buffalo.

Mammalian cell lines and media: Human epithelial cervical cancer cell line HeLa (ATTC) and pulmonary mucoepidermoid carcinoma cell line NCL-H292 (ATCC) were grown in Dulbecco's modified Eagle's medium (ThermoFisher Scientific) and RPMI 1640 media (ThermoFisher Scientific), respectively, supplemented with 10% fetal bovine serum (FBS) (ThermoFisher Scientific) and 100 U penicillin/streptomycin (ThermoFisher Scientific). For all the experiments involving lifting and/or seeding, the cells were washed twice with dPBS (Gibco) and treated with Trypsin/EDTA (Gibco).

Bacterial strains and growth conditions: The following bacterial strains were used: *S. pneumoniae* TIGR4 and D39 [316], *PLY*-deletion (Δply) [316], capsule-deletion (Δcps) [651], and pneumococcal surface protein C-deletion ($\Delta pspC$) mutant strains of *S. pneumoniae* TIGR4 [652] *B. subtilis* and its mutant expressing *PLY* (*B. subtilis* *PLY*) [312]. The pneumococcal strains were grown to mid-log phase in Todd-Hewitt broth (BD Biosciences) supplemented with Oxyrase (Fisher) and 0.5 % yeast extract at 37 °C/5 % carbon dioxide. Bacterial aliquots were then frozen at -80 °C in growth media with 20 % glycerol. Tryptic Soy Agar plates with 5 % sheep blood (Northeast Laboratory Services, P1100) were used to confirm the titers of *S. pneumoniae* in the aliquots. Before infecting animals or H292 cells, bacterial aliquots were thawed and diluted in PBS to the desired number. *B. subtilis* strains were grown overnight at 37 °C in Luria-Bertani (LB) Broth.

Toxin purification: Purification of SLO (Streptolysin O), ILY (intermedilysin) and *PLY* (*PLY*) along with its mutant forms was carried out as previously described [373, 611]. Briefly, the cultures of *E. coli* BL-XL-1 Blue expressing polyhistidine-tagged toxins were grown in sterile TB broth (with 1:100 of overnight culture) grown at 37 °C with shaking at 200 rpm supplemented with 100 µg/mL ampicillin. Expression of toxins was induced by the addition of isopropyl β-D-thiogalactopyranoside (IPTG, Fisher Scientific) to a final concentration of 1 mM when the OD₆₀₀ of the culture reached 0.5-0.6 after which the cultures were allowed to grow overnight. This was followed by

centrifugation, resuspending and lysing bacterial pellets in PBS supplemented with halt protease inhibitor cocktail (Thermo Fisher Scientific) using microfluidizer. The cell lysates were centrifuged at 10000 g for 30 min at 4 °C and the clear supernatant was collected and passed through Ni-NTA agarose column (Qiagen). Contaminating proteins were washed away by passing a 20–120 mM imidazole gradient through the column while the bound toxins were eluted using PBS containing 500 mM imidazole. The purity of the toxin was confirmed by running the collected fraction on SDS-PAGE. The toxin-containing fractions were mixed with 10% (v/v) glycerol and 5 μ M Tris(2-carboxyethyl) phosphine hydrochloride (TCEP) and flash-frozen on dry ice and then shifted to –80 °C until use. *C. difficile* toxin B (Tox B) was purchased from Sigma. Unless indicated, we used 0.25 nM of toxin for experiments with purified PLY as this concentration corresponded to 1 pore forming Unit which causes PI uptake in only 50% of the cell population and was sufficient to induce low-grade cellular damage.

Toxin activity assay: The activity of purified toxins was measured as previously described [373]. Briefly, aliquots of different toxins were thawed on ice and centrifuged at 14,000 rpm at 4 °C for 10 min to remove protein precipitates. Bradford assay (Bio-Rad) was used to measure the concentrations of toxins per the manufacturer's instructions. Toxins at different concentrations were added in wells of non-adherent 96-well plates containing approximately 1×10^5 H292 cells and incubated for 10 min at 37 °C. Following incubation, cells were stained with propidium iodide (PI) (1 mg/mL), incubated in dark at RT for 15 min and analyzed through flow cytometry. The toxin concentration that caused permeabilization of 50 % of the H292 cells (PI+) was referred as “1 unit” (1U) of toxin activity. Cells treated with 5 % Triton X-100 acted as a positive control while HBSS was used as negative control.

Murine infection studies: Mice anesthetized under isoflurane treatment were intratracheally (it) instilled with *S. pneumoniae* TIGR4 at a dose of 2×10^6 CFU, as described previously [425, 653]. At 48 h post-infection, the mice were euthanized, lungs were harvested and single-cell suspension was obtained by digesting the tissue in RPMI 1640 containing 10 % FBS and 2 mg/mL of type 2 collagenase (Worthington) and 30 μ L/mL of 10 mg/mL DNase (Worthington) stock prepared in dH₂O as previously described [653].

Preparing heat killed *S. pneumoniae* TIGR4: Frozen aliquots of *S. pneumoniae* TIGR4 were thawed on ice, centrifuged at 1700 g for 5 min and resuspended in PBS in the original volume. The bacterial suspension was then incubated at 65 °C for 2.5 h. Bacterial killing was confirmed by plating the suspension on blood agar plate.

Preparation of polarized H292 monolayers and infection with *S. pneumoniae*: Polarized H292 monolayers were prepared as previously described [654]. Briefly, the inverted side of the filter membrane of 24-well Transwells with a pore size of 3 µm were coated with 70µL of 30µg/mL of sterile collagen solution (3 mg/mL of collagen stock diluted in 60% ethanol) and allowed to dry for 3 to 4 h at RT under sterile conditions. Approximately 1×10^6 H292 cells resuspended in RPMI 1640 media (with FBS and Pen/Strep) were added in the volume of 70µL on the coated filter membrane and allowed to grow overnight at 37 °C with 5 % CO₂. The following day, the Transwells with cell were inverted into a sterile 24-well plate containing 1mL of H292 complete media and cells were allowed to grow and polarize for a week at 37 °C with 5 % CO₂. Cells were then washed, infected apically with *S. pneumoniae*, treated with trypsin and type II collagenase, stained for GPI-APs, and analyzed by flow cytometry.

Antibodies, inhibitors, and other reagents: The antibodies used in flow cytometry studies to measure the expression of specific GPI-APs included the following antibodies: anti-CD73 (AD2, eBioscience), anti-CD39 (eBioA1, eBioscience), anti-CD55 (JS11, Biolegend), anti-CD47 (CC2C6, Biolegend) and anti-CD54 (HA58, Biolegend). The primary antibodies used for Western blotting included anti-Rac1 (Proteintech) (1:1000), anti-Cdc42 (Cell Signaling) (1:1000), anti-RhoA (Cell Signaling) (1:1000) and anti-GAPDH (MA5-15738) (1:5000). Horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Jackson Immunolabs. The following anti-mouse antibodies were also used: Anti-CD326 (EpCAM) (G8.8, eBioscience), anti-CD39 (24DMS1, eBioscience) and anti-CD73 (eBioTY/11.8, eBioscience). The inhibitors Cytochalasin D, PI-PLC, PI-PLC inhibitor U-73122 and its control U-73343 (Invitrogen, ThermoFisher Scientific) and Blebbistatin were purchased from Sigma and dissolved in DMSO (unless indicated). As appropriate, control groups were treated with diluted DMSO, PBS or HBSS.

Detection of plasma membrane integrity: *S. pneumoniae* or PLY-induced cellular damage was determined by using ANNEXIN/PI staining (BioLegend), per the

manufacturer's protocol. Briefly, infected H292 cells were centrifuged at 1200 rpm at 4 °C, the supernatants were discarded, and pellets were re-suspended in 100 µL of Ca²⁺ free staining buffer (supplied with the kit). 2.5 µL of FITC Annexin V and 5 µL of Propidium Iodide were used per sample. The samples were then incubated for 15 min at room temperature (RT) in the dark and immediately analyzed by flow cytometry.

FLAER labeling: Following challenge with different bacterial strains or toxins, H292 cells were centrifuged at 1200 rpm at 4 °C, the supernatants were discarded, and pellets were re-suspended in 50 µL of FLAER (FL2S, Cederlane) diluted in FACS buffer (HBSS Ca/Mg⁺ containing 1% FBS) at the final dilution of 1:100. The cells were then incubated for 30 min on ice in the dark. Following incubation, the cells were washed twice with 100 µL of FACS buffer, resuspended in 300 µL of FACS buffer and analyzed by flow cytometry.

Flow cytometry: Fluorescence intensities for different GPI-APs, Annexin V and Propidium Iodide were measured on a BD Fortessa cytometer. At least 50,000 events for lung tissue and 10,000 events for *ex vivo* assays with H292 cells were captured for analysis. For the analysis of FLAER-positive cell vesicles (Figure S5), the events were captured by legacy MoFlo cytometer. For the data in Figure S9, the BD Accuri C6 flow cytometer was used to capture the events. All the analysis was done using FlowJo software.

RNA-interference: Approximately, 1.5×10^4 H292 cells were seeded in 24-well plates (Falcon) and cells were transfected the following day in serum free media using 100 nM of short interfering RNA (siRNA), Opti-mem (Invitrogen) and the lipid reagent LF2000 (Invitrogen) as described previously [278]. Serum was added on day 3 at a final concentration of 10% in the transfected wells. On day 4, the cells were challenged with *S. pneumoniae* TIGR4 strain or lysed for Western blotting to confirm protein depletion. SMARTpool siRNAs from Dharmacon were used to deplete Cdc42 (M-005057-01-0005), Rac1 (M-003560-06-0005) and RhoA (M-003860-03-0005). A non-targeting NTC siRNA (D-001210-02-05) was used as a control.

Western blotting: To confirm protein depletion, approximately 48 h post-transfection H292 cells were lysed on ice for 30 min in ice-cold RIPA buffer (1% Triton X-100, 0.25% sodium deoxycholate, 0.05% SDS, 50 mM Tris-HCl [pH 7.5], 2 mM EDTA, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 10 mg/liter each

of aprotinin and leupeptin), as previously described [655]. The cell lysates were centrifuged at 12,000 rpm at 4 °C and bicinchoninic acid kit (Pierce) was used to determine the protein concentration in the samples. An equal amount of protein (20 µg) was run on Mini-PROTEAN TGX Stain-Free Precast Gels (BioRad) SDS-polyacrylamide gel and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked in 3% BSA for 1 h at RT and incubated overnight at 4 °C with primary antibodies diluted in TBS/T with 3 % BSA. The membranes were washed thrice with TBST (5 min per wash) followed by incubation with HRP-tagged secondary antibodies for 1 h at RT. The bands were detected using Clarity™ Western ECL substrate (BioRad) on the ChemiDoc XRS+ system (BioRad).

Measuring phosphatidylinositol-specific phospholipase C (PI-PLC) and PLY-induced cell damage: H292 cells were seeded in a 6-well plate (5×10^5 cells/well) and 20 h later were pre-treated with PI-PLC (0.1U) prepared in (Opti-MEM supplemented with 10mM Hepes, 1mM EDTA and 0.1%BSA) [656]. Cells were then challenged for 10 min with PLY at 0.25 nM diluted in HBSS (or HBSS only in the untreated condition). Cells were then harvested, centrifuged at 1200 rpm for 5 min at 4 °C and incubated for 30 min on ice in 50 µL of FACS buffer containing 0.5 µL of FLAER (1:100). Following incubation, cells were washed and centrifuged at 1200 rpm for 5 min at 4 °C and resuspended in FACS buffer. Just before flow cytometry analysis, propidium iodide (PI) was added at 2 µg/mL and incubated for 1 minute. Then, cells were analyzed for FLAER and PI staining in BD Accuri C6 flow cytometer. At least 20,000 events per sample were collected. Data obtained were analyzed using FlowJo software (Version 10.8).

Immunofluorescence microscopy: Approximately, 2×10^5 H292 cells were grown in a complete medium on 13 mm diameter coverslips in 6-well plates. The day after, the cells were washed and challenged with *S. pneumoniae* in serum-free RPMI 1640 medium. The plate was centrifuged at 1000 rpm for 2 min to enhance contact between bacteria and H292 cells and then incubated for 1 h at 37 °C in 5 % CO₂. Cells were washed in PBS, fixed in PBS with 4 % paraformaldehyde, quenched with 20 mM NH₄Cl (20 min), permeabilized with 0.1 % Triton X-100 (5 min) and blocked with 3 % BSA in PBS (1 h). The antibodies were then diluted in PBS containing 1% BSA and cells were labeled for GPI-AP nucleus and F-actin. GPI-AP using FLAER-Alexa 488 (Cedarlane) (1:50), DAPI (Sigma) (1:1000) and Alexa fluor 680 phalloidin (Life technologies) (1:50), respectively as described previously [657]. Cells were then washed and mounted with

Vectashield (Vector laboratories) and imaged using a 63 x 1.4 NA objective lens on a confocal laser-scanning microscope (Leica SP8) and processed using ImageJ software.

RhoA and Cdc42 activation and FRET: HeLa cells seeded in Ibidi 8-well dishes (4×10^4 cells/well) (Ibidi) were transfected approximately 20 h later with 300 ng of plasmid encoding FRET probes (CFP/YFP FRET pairs) for RhoA or Cdc42 using jetPrime transfection reagent (Polyplus) and incubated for 16–20 h to allow the expression of the recombinant proteins. FRET probes used were described in [658, 659]. Cells were then kept in Opti-MEM (Gibco, without phenol red) at 37 °C in 5 % CO₂ and imaged using Nikon TI microscope coupled with IRIS9 camera and using 60 x 1.4 NA objective. Cell imaging was started 20 min before PLY (0.25 nM) addition and continued for 10 min after challenge with the toxin. CFP/FRET/YFP fluorescence data were acquired after every 20 s. Image sequences were analyzed using ImageJ and FRET/CFP signal ratio were calculated only at the cell cortex using a custom-made macro. A total number of 79 cells from 2 independent experiments were analyzed for RhoA activation and 74 cells were analyzed from 2 independent experiments for Cdc42 activation.

Live cell imaging and signal quantification: HeLa cells were seeded in 8-well Ibidi plates (1×10^5 cells/well) and 20 h later stained with FLAER (1:100) for 30 min. Cells were then imaged using a Nikon Ti microscope coupled with IRIS9 camera with 40x 0.95 NA objective, for 15 min with a time interval of 30 s before the addition of PLY (0.25 nM diluted in HBSS) or HBSS (used as untreated control), and then for additional 45 min post PLY addition. Quantification of the FLAER signal was obtained by image analysis using ImageJ. A total of 53 cells were analyzed. The mean fluorescence intensity (MFI) was determined overtime in randomly selected regions of interest (ROIs) at the cell membranes. These values were then normalized to the mean MFI obtained from the same cells before these were challenged with PLY or HBSS.

Statistical analysis: Graph Pad Prism9 was used to perform all the statistical analysis. All the data graphs represent the mean values +/- standard deviation (SD). Significant differences were determined using One-way ANOVA followed by Tukey's or Dunnett's multiple comparison test or Student's t-test or one sample t and Wilcoxon test, as indicated. *p* values of 0.05 or lower were considered significant.

Supplementary Data

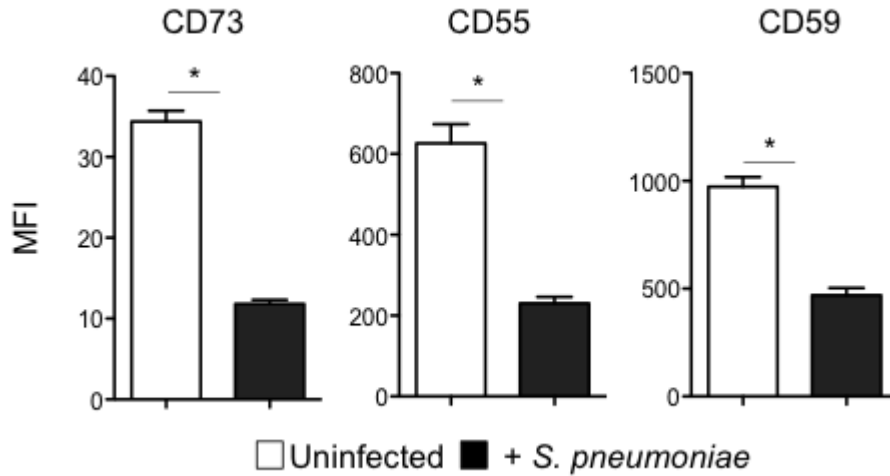


Figure S1: *S. pneumoniae* reduces the expression of GPI-APs on polarized cells. H292 cells seeded on transwells were allowed to polarize for 1-week. The cells were then infected apically with *S. pneumoniae* TIGR4 strain at an MOI of 10 for 1 h, stained for the indicated proteins and MFI was measured by flow cytometry. Representative data from one of three independent biological replicates are shown where each condition was tested in triplicate (n = 3 technical replicates per experiment). *, p < 0.05 by Student's t-test indicates significant differences from uninfected controls.

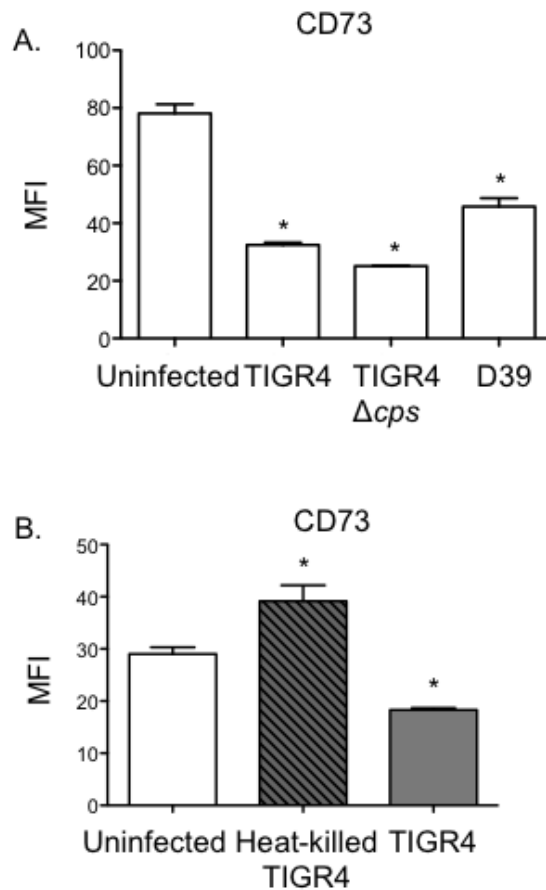


Figure S2: *S. pneumoniae*-induced reduction in CD73 expression is not strain- or capsule-dependent but mediated by a heat-labile bacterial factor. (A-B) H292 cells were infected with the indicated strains of *S. pneumoniae* at an MOI of 10 for 1 h and the expression of CD73 (MFI) measured by flow cytometry. Representative data from one of three independent experiments are shown where each condition was tested in triplicate (n = 3 technical replicates per experiment). *, p < 0.05 by one-way ANOVA comparison with uninfected controls.

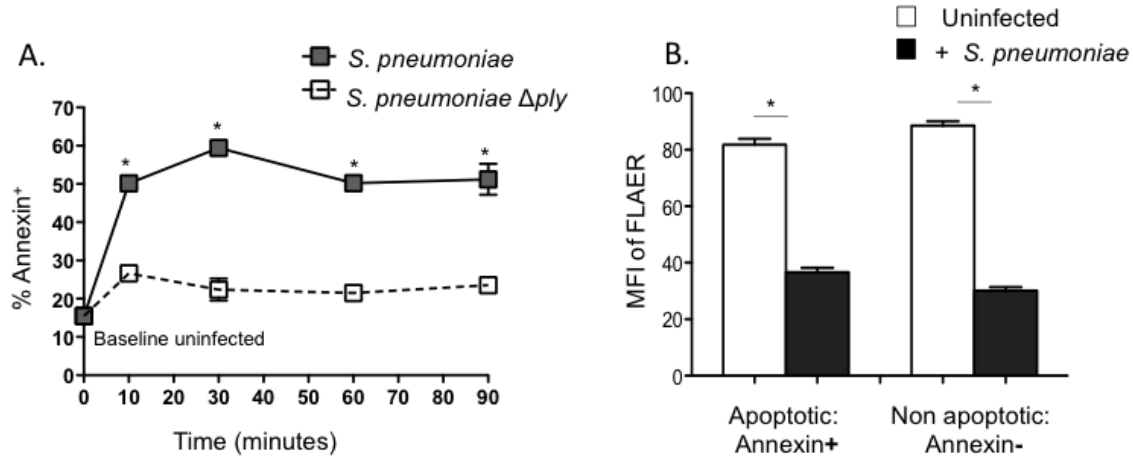


Figure S3: PLY-induced decrease in GPI-APs expression does not result from cell death. H292 cells were infected with wildtype or *S. pneumoniae* Δply at an MOI of 10. The cells were then stained with Annexin V and FLAER and analyzed by flow cytometry to determine (A) the percentage of apoptotic (Annexin V⁺) cells throughout infection and (B) the MFI of FLAER of both apoptotic (gated on Annexin V⁺ cells) and non-apoptotic (gated on Annexin V⁻ cells) cell populations at the 10 min timepoint. Representative data from one of three independent experiments where each condition was tested in triplicate (n = 3 technical replicates per experiment) are shown. *, $p < 0.05$ by one-way ANOVA comparison with corresponding time points (A) and (B) uninfected controls.

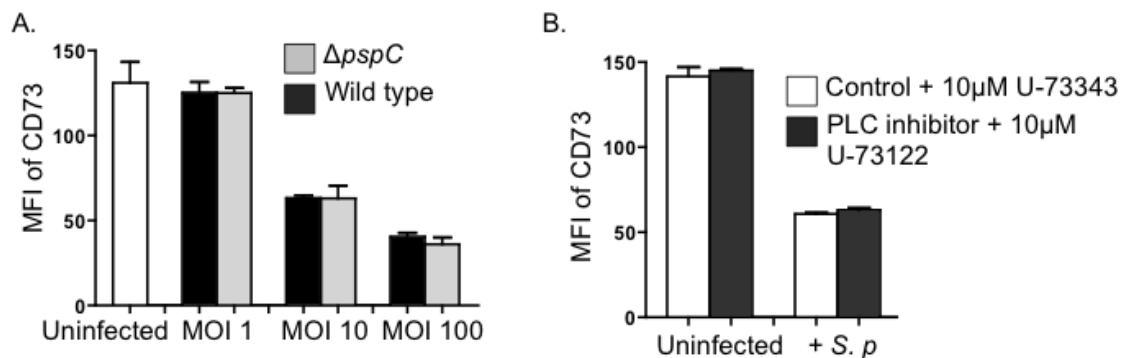


Figure S4: PLY-induced decrease of surface CD73 expression is not mediated by host phospholipases (PLC). (A) H292 cells were infected with wildtype or *S. pneumoniae* $\Delta pspC$ at indicated MOIs for 1 h and analyzed for CD73 expression through flow cytometry. (B) H292 cells were treated with 10 μM PLC inhibitor U-73122 or its inactive form U-73343 for 30 min followed by infection (in the presence of inhibitors) with *S. pneumoniae* TIGR4 strain at an MOI of 10 for 1 h. Cells were then stained and the expression (MFI) of CD73 was measured by flow

cytometry. Representative data from one of three independent experiments where each condition was tested in triplicate ($n = 3$ technical replicates per experiment) are shown.

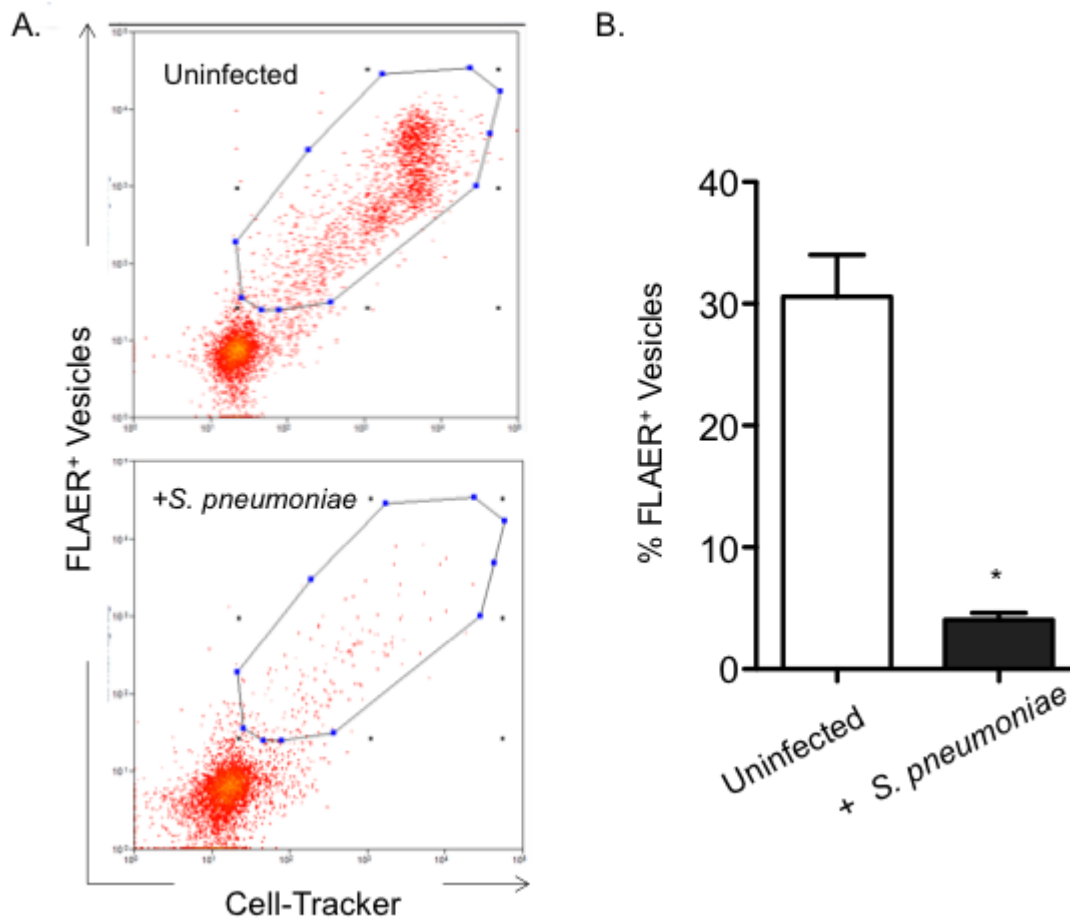


Figure S5: *S. pneumoniae* infection decreases the release of vesicles containing GPI-APs from lung epithelial cells. H292 cells were loaded with 1 μ M of CellTracker Green dye CMFDA and challenged with *S. pneumoniae* TIGR4 strain at an MOI of 10 or left uninfected for 1 h. The cell culture supernatants were collected, centrifuged and stained with FLAER. The presence of FLAER-positive vesicles (under CMFDA+ gate) was analyzed by Legacy MoFlo cell sorter. (A) shows a representative dot plot of vesicles analyzed and (B) shows the quantification of samples under each condition. Data shown are representative from one of three independent experiments where each condition was tested in triplicate ($n = 3$ technical replicates per experiment). *, $p < 0.05$ by Student's t-test, indicate significant difference from the uninfected controls. CMFDA (5-chloromethylfluorescein diacetate).

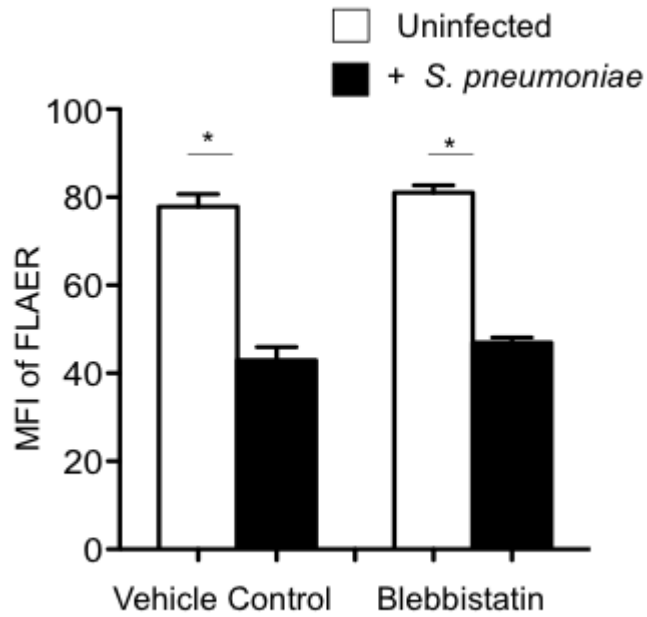


Figure S6: *S. pneumoniae*-mediated decrease in GPI-APs expression is independent of blebbing. H292 cells were pre-treated with 50 μ M Blebbistatin or vehicle control for 20 min followed by infection with *S. pneumoniae* TIGR4 strain at an MOI of 10 for 1 h. The cells were then stained with FLAER and MFI analyzed by flow cytometry. Data shown are representative from one of two independent experiments where each condition was tested in triplicate (n=3 technical replicates per experiment). *, $p < 0.05$ by one-way ANOVA comparison with uninfected controls.

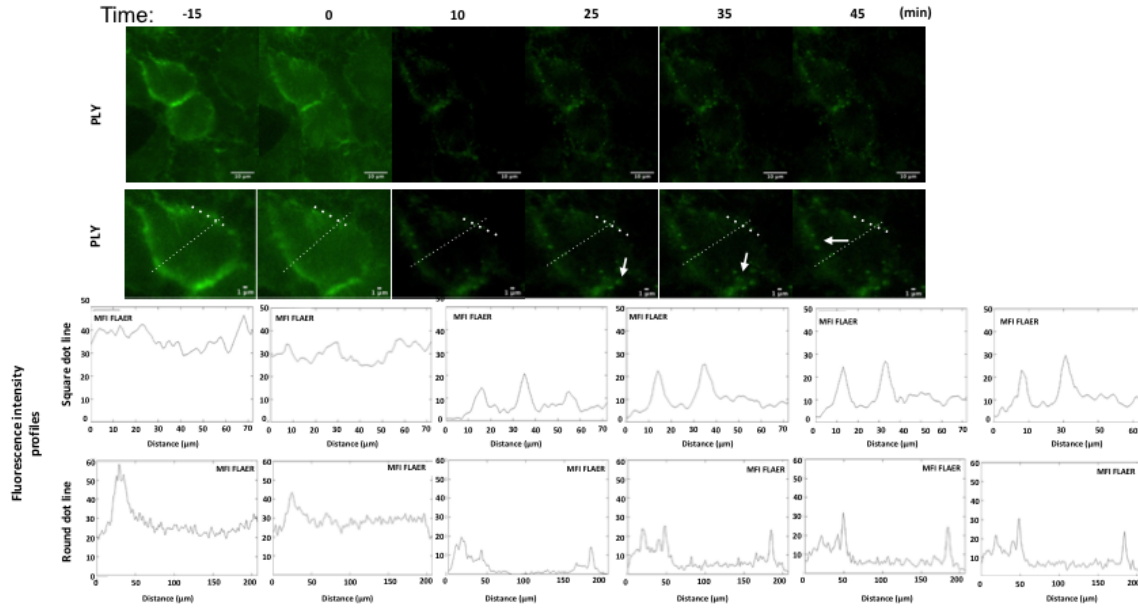


Figure S7: GPI-APs are detected inside the cells in structures resembling intracellular vesicles upon PLY challenge. Sequential frames of time-lapse microscopy video of HeLa cells pre-labeled with FLAER. PLY (0.25 nM) was added at time point 0 min. The inset (bottom row) shows details of GPI-APs distribution. FLAER intensity in line scans (lower panels) was determined along the dotted lines drawn in the inset images. Fluorescence intensity profiles (indicating FLAER distribution) at the bottom were obtained in a cortical region (thick line, upper panel profiles) and across the entire cell (thin line, lower panel profiles). Scale bar for the top lane is 10 μm and for the bottom lane is 1 μm .

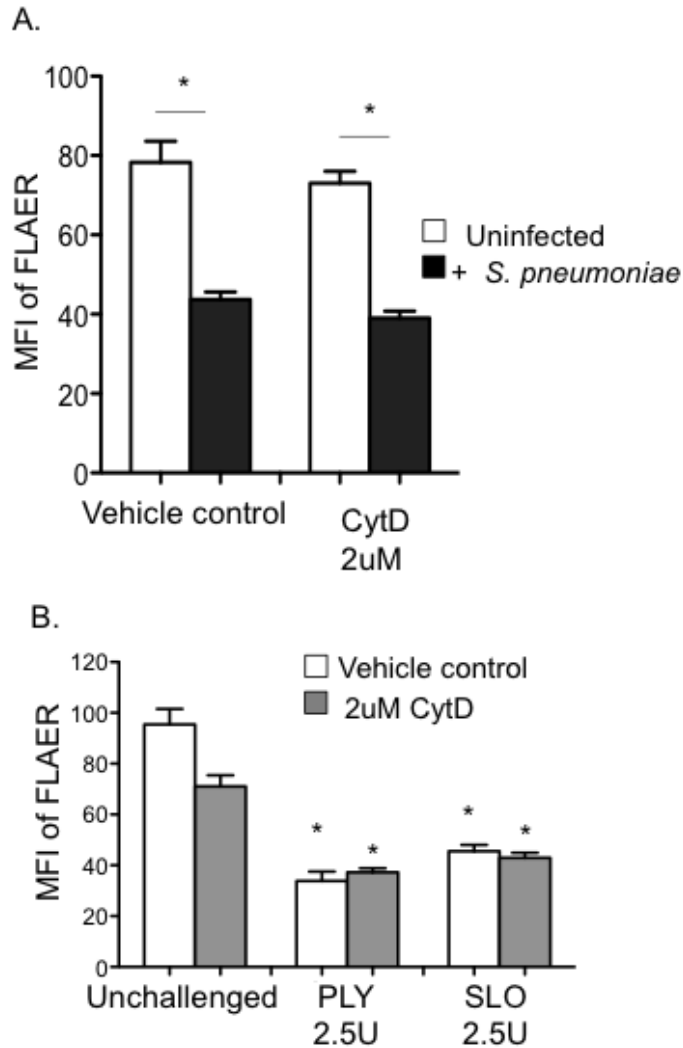


Figure S8: PLY-mediated decrease in GPI-APs surface expression is not dependent on actin polymerization. H292 cells were pre-treated with 2 μ M cytochalasin D (CytD) or vehicle control for 30 min followed by challenge with (A) *S. pneumoniae* TIGR4 strain at an MOI of 10 for 30 min or (B) 2.5 U of recombinant PLY or SLO for 15 min. Cells were then labeled with FLAER and the MFI was assessed by flow cytometry. The graphs show representative data from one of three independent experiments where each condition was tested in triplicate ($n = 3$ technical replicates per experiment). *, $p < 0.05$ indicates significant differences by one-way ANOVA comparison with corresponding uninfected controls under the same treatment conditions.

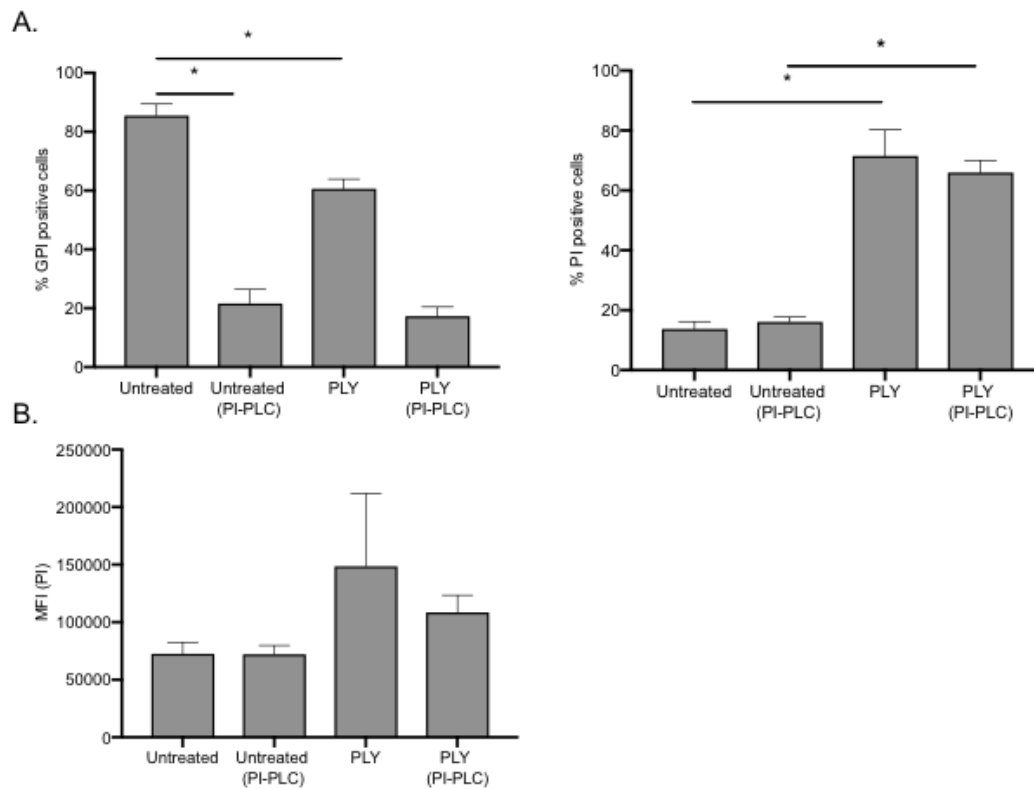


Figure S9: Surface-exposed GPI-APs are not required for PLY-induced plasma membrane permeabilization. (A) H292 cells incubated with or without PI-PLC (0.1 U, 2 h at 37 °C) were challenged with purified PLY (0.25 nM) for 10 min or left untreated. Cells were then collected and processed for flow cytometry analysis to determine the percentage of GPI⁺ and PI⁺ cells. Data shown are pooled from at least three independent experiments. *, $p < 0.05$ by one-way ANOVA comparison with untreated cells. (B) The mean fluorescence intensity (MFI) of the PI is shown in (B).

Chapter III – General discussion

“Encumbered forever by desire and ambition
There's a hunger still unsatisfied
Our weary eyes still stray to the horizon
Though down this road we've been so many times”
"High Hopes" by Pink Floyd

Cells are protected by a complex lipidic bilayer, the plasma membrane (PM), which constitutes an important physical barrier that guarantees efficient cellular fitness and homeostasis. Given its main function, PM is constantly targeted by external insults that compromise barrier integrity, cellular functions and tissue physiology. As such, to counteract these frequent assaults, different cell autonomous repair mechanisms have been described to respond to PM damage thereby sustaining the cell's physiological functions. Microbial pathogens naturally evolved sophisticated strategies during evolution to overcome host cells' natural barriers by inducing PM damage either through the direct action of their virulence factors (enzymes, pore-forming toxins) or by triggering strong pro-inflammatory responses resulting in immune-mediated damage.

S. pneumoniae (*Sp*) is a common colonizer of the human upper respiratory tract [288], nevertheless, *Sp* can migrate and penetrate distant tissues to cause severe infections, especially in immunocompromised individuals. *Sp* constitutes the major cause of bacterial pneumonia worldwide with a high mortality rate associated, posing a serious health threat, especially to children and the elderly [660]. Colonization and infection by *Sp* rely on the release of its pore-forming toxin (PFT), pneumolysin (PLY). As other bacterial PFTs, PLY forms transmembrane pores in the host cell PM disrupting host cell PM integrity, compromising epithelial barriers, facilitating immune evasion, and fostering infection. In addition, PLY elicits strong pro-inflammatory responses ultimately leading to the loss of lung function [517]. Nevertheless, at low doses of PLY, presumably occurring early in infection, cells activate calcium-dependent PM repair mechanisms that sustain cell survival by removing the lesioned PM and the pore [517].

During my PhD, I deeply investigated the mechanisms underlying host resistance to sub-lytic PLY-induced damage strictly using purified PLY or in the context of infection with *Sp*.

Together with my colleagues, I found that an abrupt shot of calcium (Ca^{2+}) is required to guarantee the proper engagement of the PM repair machinery. Furthermore, I identified extracellular vesicles (EVs) shedding and PM blebbing as key mechanisms employed by host cells to fully restore PM integrity and remove the toxin from the cell surface. By characterizing the proteomic content of EVs shed by PLY-intoxicated cells, during the repair process, I identified novel proteins potentially

involved in PM repair and contributed to further expanding our knowledge on the molecular machinery participating in PM resealing (Chapter II - Section 1).

I also characterized the cytoskeletal alterations endured by epithelial cells in response to PLY-induced damage. Concretely, a transient and dynamic accumulation of soluble non-muscle heavy chain myosin IIA (NMHCIIA) at the cell cortex is required to support PM remodeling events. My data also shows that such events are likely coordinated by S100A4, a pivotal Ca^{2+} -responding protein found in the EVs released by PLY-intoxicated cells (Chapter II - Section 2).

Finally, I significantly collaborated in a study showing that infection with *Sp* diminished the surface expression of the glycosylphosphatidylinositol-anchored proteins (GPI-APs) in a PLY-dependent manner. The reduction of GPI-APs was associated with their internalization by host cells, in a process mediated by the Rho GTPases RhoA and cdc42 activated by PLY itself. We propose that the transient decrease of the surface expression of GPI-APs favors cellular innate repair response to PM damage induced by PLY (Chapter II - Section 3).

I. Intracellular responses to PLY-induced damage

Sensing the PM disruption is a critical step for damage resolution, as cells can timely and efficiently mend PM and restore barrier integrity to ensure survival. Ca^{2+} is highly abundant in the extracellular milieu and cells keep a 10 000 fold gradient between extracellular and intracellular space. In this work, I reported that to achieve maximum recovery of PM integrity, extracellular physiological Ca^{2+} concentrations are mandatory during damage and recovery of PM integrity [563]. Ca^{2+} ions are promiscuous signaling molecules and act as intracellular messengers that regulate many cellular functions [661]. Indeed, Ca^{2+} ions control gene expression, induce post-translational modifications, and interact and modulate the functions of a variety of proteins, including cytoskeletal proteins [662]. The maintenance of Ca^{2+} homeostasis is thus of utmost importance and is usually achieved by the activity of Ca^{2+} pumps located in the Endoplasmic Reticulum (ER) (SERCA pump) and mitochondria (mitochondria uniporter pump) [663, 664]. The substantial Ca^{2+} influx triggered by PLY pore formation triggers a swift intracellular response and can potentially recruit these organelles to mitigate intracellular Ca^{2+} overload [663, 664]. It has been previously shown that PM damage induced by different cholesterol-dependent cytolysins

listeriolysin O (LLO, produced by *Listeria monocytogenes*) or perfringolysin O (PFO, produced by *Clostridium perfringens*) induces ER fragmentation, vacuolization and accumulation near PM damage sites [102, 519, 520]. Gp96 an ER-resident chaperone becomes cell surface exposed upon LLO and PFO intoxication and plays a crucial role in PM repair and host survival to *L. monocytogenes* infection [102]. Accordingly, I found that PLY also induces ER vacuolization and Gp96 recruitment to the cell periphery (data not shown). However, after toxin washout and PM resealing, ER organization returns to normal, suggesting that this is a transient event and possibly an emergency response to locally control Ca^{2+} levels. Therefore, I postulate that upon membrane disruption, ER is recruited to the cell periphery where it might act as a Ca^{2+} buffering system avoiding deleterious intracellular Ca^{2+} overload. In support of this hypothesis, the ER chaperone Gp96 was previously described as the major Ca^{2+} binding protein of the ER and might regulate Ca^{2+} -mediated signals including mitochondrial activation [665]. As mentioned, mitochondria is an important organelle that also regulates Ca^{2+} homeostasis. Supporting mitochondria importance during PM damage, PLY was described to induce mitophagy during infection by *Sp*. Intracellular Ca^{2+} rise induced by PLY pore formation triggered mitochondrial fragmentation and led to an increase in mitochondrial Ca^{2+} influx, supporting the notion that mitochondrial pumps might also contribute to buffering intracellular Ca^{2+} rise [391]. Intriguingly, I conducted transmission electron microscopy (TEM) analysis on epithelial cells challenged with sub-lytic concentrations of PLY (data not shown) and detected significant mitochondrial and ER fragmentation in close proximity to the PM. This observation lends support to the hypothesis that these organelles may serve as buffering systems, preventing cells from reaching harmful cytosolic Ca^{2+} levels while coping with damage. It is worth noting that this organelle remodeling might also have additional functions in PM repair. As an example, Mesquita et al showed that NMHCIIA strongly accumulates with ER vacuoles at sites of PM remodeling and that Gp96 controls cytoskeletal remodeling by interacting with NMHCIIA and the actin-binding protein Filamin A. Thus suggesting that ER remodeling serves not only to control Ca^{2+} levels but also to support cytoskeletal rearrangements required for efficient PM repair [102].

As pointed out in the introduction, Ca^{2+} ions can bind and activate multiple intracellular proteins that are recruited to the damaged PM sites and cooperate to fully restore PM integrity. In agreement, our proteomic analysis revealed that extracellular

vesicles (EVs) isolated from PLY-intoxicated cells are enriched in Ca^{2+} -binding proteins. Taking all these into account, we postulated that proteins significantly enriched in EVs derived from PLY-intoxicated cells may directly arise from PM-damaged sites and thus should play important roles in PM repair. To support our hypothesis, some of these proteins have already described functions in PM resealing (annexins and AHNK), however, others were never associated with PM repair, opening new perspectives in the field. Copine-1 and -3 are calcium-dependent phospholipid-binding proteins [666] identified in EVs from PLY-intoxicated cells and, to our knowledge, their functional role in PM repair was never been addressed. This work demonstrates the involvement of both copine-1 and -3 in the repair of damage induced by PLY. Additionally, recent work suggested that copine-3 might be involved in the anchoring of lysosomes and MVBs at the plasma membrane during PM repair [121]. Altogether these findings open new perspectives on the functional role of copines in the control vesicle patching and shedding in response to different sources of damage.

Given the differences between the proteomic profiles of EVs released by intoxicated and non-intoxicated cells, we postulated that the EVs shed during PM repair would primarily arise from the direct outward budding of the PM as a way to expel the toxin from the cell surface. However, considering the heterogeneous size distribution and that about 40 % of the EVs do not contain PLY, we cannot exclude the possibility that EVs may originate from alternative pathways, potentially including exosomes, which have been previously associated with PM repair. Indeed, I generated preliminary data suggesting that during PM repair cells accumulate high numbers of multivesicular bodies (MVB), reinforcing the possibility that exosomes could play a role in PM repair. Whether copines are actively involved in vesicular trafficking is still unknown and additional studies are required to address the relevance of such proteins in EV shedding.

PM is intimately linked to the cell cytoskeleton, therefore it is expected that the disruption of PM integrity also destabilizes the underlying cytoskeleton. Cortical actomyosin meshwork has long been implicated with anchoring and fusion of vesicles with the PM and the weakening of the cortical actomyosin barrier is critical to support successful remodeling events such as vesicle patching or PM blebbing [667]. In the context of PM repair, it was previously shown that actin depolymerization occurring after PM disruption facilitates repair by decreasing PM tension and favoring PM

deformation events [241]. Here, I showed that PLY induces a rapid accumulation of soluble NMHCIIA at the cell cortex and that this remodeling is controlled by S100A4, a Ca^{2+} -binding protein shown to directly interact with the c-terminal of NMHCIIA promoting NMIIA filament disassembly [572]. S100A4 co-localizes with NMHCIIA immediately after PLY challenge and in the absence of S100A4, cells are more susceptible to PLY-induced PM damage. It is conceivable that S100A4-NMHCIIA interaction leads to the disassembly of NMIIA filaments, thereby releasing a new pool of soluble NMHCIIA. These monomers subsequently accumulate prominently at sites of PM blebbing. Besides S100A4 recruitment and NMHCIIA disassembly, I also reported the rapid dephosphorylation of myosin regulatory light chain (MRLC) that returned to basal levels quickly after recovery. Dephosphorylation of MRLC has long been associated with the loss of both non-muscle myosin II (NMII) MgATPase activity and bipolar filament integrity, which consequently destabilizes the F-actin network due to the dissociation of NMII from actin which results in the disruption of actin-myosin cross-bridges [668, 669]. Thus, our observations support that immediately after PLY challenge, the recruitment of S100A4 contributes to the rapid disassembly of NMHCIIA filament stacks that possibly leads to the local destabilization of the F-actin meshwork and increased actin depolymerization rate. These events are transient since NMIIA filaments are rapidly reorganized. In agreement with the profound PLY-induced actomyosin remodeling, my preliminary data show that PLY-intoxicated cells transiently display reduced numbers of filopodia-like structures. During repair both filopodia length and number are reestablished returning to pre-intoxication levels, thus confirming that response to intoxication relies on actomyosin remodeling.

Whether PLY-pores localize at sites of highest PM and actomyosin remodeling is still unknown. To assess that, correlative light and electron microscopy (CLEM) together with electron tomography (ET) techniques need to be implemented in intoxicated cells to detect the PLY pore and deeply characterize the cellular areas in the vicinity of the pore. In particular, PM blebbing, shedding of vesicles, cytoskeletal remodeling and recruited organelles could be analyzed in great structural detail.

Taking into account all the generated data, I propose a model in which upon PLY challenge, the rapid disassembly of NMIIA filaments mediated by S100A4 and NMIIA dephosphorylation destabilizes actomyosin meshwork and contributes to the decrease of PM tension which facilitates bleb expansion and vesicle trafficking toward

PM. Altogether these events should favor the rapid resealing and clogging of pores. This is also supported by our findings showing ER vacuoles localized in these specific NMIIA accumulations.

II. Plasma membrane lipidic and proteomic alterations triggered by PLY-damage

PLY directly binds cholesterol residues at the PM and pore-formation requires the extraction of lipids from the hydrophilic inner edge of the inserted pore [332]. Therefore, PLY-induced PM damage inevitably alters local lipid abundance and distribution, and it also affects protein composition. Hence, successful repair entails the remodeling and replacement of those lipids and proteins that structurally support PM barrier function. PM injury prompts the compartmentalization and reorganization of lipids around the injury edge [670] which becomes evident as lipids start moving due to the increased fluidity of individual lipids. This increased fluidity enables lipids to shift laterally and even flip, driven by the activation of lipid scramblases during membrane repair [104, 105]. This lipid dynamics aids the fluidic membrane to adapt to the changes in PM tension required for efficient repair and was associated with the establishment of lipid microdomains close to the damaged sites which could facilitate PM repair. This occurs because lipids may serve as signaling scaffolds, either by direct signaling or by attaching to specific proteins directly involved in PM repair [7]. Phosphatidylserine (PS) is one of the lipids that was found to accumulate around the damaged PM after mechanical damage [670]. This accumulation near the damage sites favors the recruitment and binding of proteins already known to be involved in PM repair such as annexins, MG53 and dysferlin. Thus, local lipid remodeling directly contributes to the functional role of those proteins in the stabilization of the damaged sites. Another important lipid that accumulates near damaged sites is diacylglycerol (DAG) which results from the ceramide/phosphatidylcholine (PC) hydrolysis process via sphingomyelin synthase (SMS) [670]. DAG has as downstream effectors PKC which consequently regulates the recruitment and activity of Rho GTPases RhoA and Cdc42, which in turn modulate the underlying cytoskeletal remodeling required for proper PM repair [670]. Here I show that the remodeling of PM surface through the removal of glycosylphosphatidylinositol-anchored proteins (GPI-APs) also depends on RhoA and Cdc42 activation, one could imagine that lipid remodeling around the wound site

facilitates shaving of GPI-APs from the cell surface. Local modification of lipids might also be a result of new membrane delivery through the fusion of lysosomes, which are major lipid suppliers in the context of repair, with the PM. Furthermore, lysosomal fusion allows the release of acid sphingomyelinase (ASM) that locally modifies PM lipid content by mediating the hydrolysis of sphingomyelin to ceramide which has membrane-bending properties that support inward budding of the PM contributing to damage removal by endocytosis [139].

Together with my colleagues, we reported here that PLY, as other cholesterol-dependent cytolysins, induces the internalization of GPI-APs thus decreasing their surface expression. One of those GPI-APs, CD73 has been previously described as an important regulator of immune response against *Sp*, indeed CD73 KO mice show similar bacterial burden in the lungs but increased bacteremia after 3 days of infection, suggesting that CD73 is crucial to control bacterial spread [425]. The role of CD73 in infection was mainly associated with its role in the conversion of ATP released by damaged cells into extracellular adenosine, which in turn recruits neutrophils to infection sites to control bacterial burden [425]. GPI-APs typically form clusters that are mainly located in cholesterol-enriched PM domains (lipid rafts), which are also preferential binding sites for PLY. In addition, PM cholesterol depletion has been associated with intracellular retention of GPI-APs [671]. Given that endocytosis is a described mechanism for PM repair and that our data show that the internalization of GPI-APs reduced cellular damage, we may speculate that endocytosis has multiple roles in repair first by eliminating the damage, but also by decreasing the levels of GPI-APs at the cell surface as thus limiting immune-mediated damage and finally by decreasing the levels of cholesterol at the surface thus limiting further damage. Nevertheless, all these possibilities need further investigation. Overall, a combination of multiple factors orchestrates PM repair mechanisms in cells, ranging from lipid alterations and signaling to intracellular ion imbalance. Altogether cooperating to recruit intracellular protein machinery that will quickly reseal the damage and later restore PM organization and function.

III. Relevance of PM repair mechanisms in the outcome of infection

Recent work suggests that to fully restore PM integrity and functions, cells need to undergo two different stages: the first is considered an acute phase where the

damage is crudely repaired to avoid cell death and relies on the rapid reorganization of the actin network, and the second is a restructuration phase that guarantees full re-establishment of PM integrity [146]. In this work, we were able to demonstrate that both PM blebbing and shedding contribute to PM repair upon PLY challenge. We believe that both events are interconnected and may occur at different stages of PM repair. Indeed, PM blebbing has been recognized as a mechanism by which cells immediately constrain the damage [101] and we show here that it represents an immediate response to PLY damage, emerging within seconds after PLY challenging. The formation of blebs is supported by a massive remodeling of the associated actomyosin cytoskeleton characterized by the cortical accumulation of soluble NMHCIIA. Regarding the fate of those blebs during PM repair, our live-cell imaging data indicates that some of those blebs retract, but others keep growing or stabilize and are eventually released from the cell surface. PLY thus also induces the release of extracellular vesicles (EVs) from the cell surface during PM repair. It is noteworthy that those vesicles were isolated after toxin washout and 3 h of repair, therefore they do not represent an immediate response to PLY challenge but instead, a process of PM restructuration and physical removal of the toxin from the PM. Nevertheless, 40 % of the EVs do not contain PLY protein suggesting additional roles for released EVs. Cells are capable of internalizing EVs arising from neighboring cells either by direct fusion with the PM or via endocytosis, and those EVs can effectively deliver their cargo (proteins, lipids, DNA) into recipient cells and alter intracellular signaling pathways [672]. Indeed, others have shown that EVs arising from PLY-intoxicated cells can effectively modulate the host innate immune response, either by suppressing the capacity of neutrophils to produce and release reactive oxygen species (ROS) or by altering macrophage polarization towards a phenotype that exhibits enhanced response towards Gram-positive bacteria [118, 119], suggesting that vesicles arising from PLY-intoxicated cells might have an impact on the outcome of the infection with *Sp.*

We found that besides PLY, EVs released from intoxicated cells contain multiple proteins involved in PM repair. One question that remains unanswered is whether the content of those EVs can be effectively delivered to neighboring epithelial cells through EVs internalization and activate intracellular signaling pathways that support PM repair therefore functioning as a paracrine signaling that favors their subsequent

response to PLY damage. Indeed, EVs containing Annexin A1 activate wound repair pathways in the intestinal mucosa, therefore, this might also happen in our model [673]. This would not only impact the response of cells that are near damaged areas but modulate the response from cells that are far away from the infection foci therefore protecting them from later PLY-mediated damage. It would be interesting to further understand these mechanisms because, in the context of infection PLY is released freely in the extracellular milieu and cells are constantly targeted by the toxin, if by delivering repair machinery these EVs enhance the capacity of cells to resist PLY intoxication that would pave the way for new therapeutic strategies to enhance cellular fitness and repair capacity.

In the context of lung infection with *Sp* it is important to consider how single-cell repair might contribute to the outcome of the infection. For that, we should take into account that the lung epithelium is a complex system composed of many epithelial cell types, endothelial cells, and immune cells [674]. Growing evidence suggests that PM repair may control inflammation associated with tissue injury. However, how single-cell PM repair exactly contributes to tissue inflammation remains elusive. Upon PM disruption cells can release “danger” signaling molecules to the extracellular milieu which can be recognized by immune cells to initiate a cascade of immunological responses. Indeed, PLY has been shown to have a potent proinflammatory signature [517]. Pore-formation induces the release of cytokines and chemoattractants which elicits neutrophil transmigration across both endothelial and epithelial barriers which enhances tissue damage by the production of ROS (one of the major sources of chemical PM damage) and neutrophil extracellular trap (NET) release [373, 517, 675]. Although the recruitment of immune cells to damaged sites positively contributes to *Sp* elimination, it is important to remember that exacerbated tissue damage triggered by these immune cells may also facilitate bacterial spread to deeper tissues where it can cause severe disease. A fast activation of PM repair mechanisms would not only protect single-cell integrity and homeostasis but also reduce overall inflammatory responses associated with early infection stages to limit bacterial spread. We indeed have unpublished data indicating that Gp96, a protein crucial for PM repair in response to LLO damage, sustained tail regeneration capacity in a zebrafish tail amputation model reinforcing the idea that enhancing single-cell repair might actively contribute to tissue repair and regeneration. Supporting our hypothesis an interesting study

using recombinant MG53, a protein described to facilitate PM repair, showed that besides enhancing PM resealing at a single-cell level in a mouse model of dermal injuries, the protein also reduced the release of inflammatory cytokines in the wound area which positively affected early wound closure and avoided excessive immunogenic remodeling which can be detrimental for the tissues [676]. We then speculate that a fast and proficient PM repair early during *Sp* infection will boost damage resolution and control inflammation.

Concluding remarks

The severity of *Sp*-associated infections is often not a result of microbial burden but instead a consequence of lung damage mediated by uncontrolled immune responses. In this context, targeting a cellular response instigated during infection instead of directly focusing on the bacteria may reduce the risk of global resistance emergence, which is a concern regarding the few available drugs for *Sp* infection treatment that currently exclusively inhibit bacterial functions. In particular, understanding the molecular mechanisms supporting PM repair would help to identify specific targets for pharmacological intervention. Enhancing PM repair can directly improve infection outcomes. This is because repairing individual cells may also aid in overall tissue repair, as previously discussed, and a deficient repair might exacerbate chronic inflammation. Moreover, the intracellular biological processes instigated by PLV damage that contributes to repair are usually common to other sources of PM damage in other pathological contexts, thus our findings have a broader impact and may set the stage for the development of therapies in the context of acute respiratory distress syndrome, pulmonary fibrosis and even lung unrelated diseases such as muscular dystrophies and neurological disorders

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APPENDIX

Article

Src-Dependent NM2A Tyrosine Phosphorylation Regulates Actomyosin Remodeling

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Abstract: Non-muscle myosin 2A (NM2A) is a key cytoskeletal enzyme that, along with actin, assembles into actomyosin filaments inside cells. NM2A is fundamental for cell adhesion and motility, playing important functions in different stages of development and during the progression of viral and bacterial infections. Phosphorylation events regulate the activity and the cellular localization of NM2A. We previously identified the tyrosine phosphorylation of residue 158 (pTyr¹⁵⁸) in the motor domain of the NM2A heavy chain. This phosphorylation can be promoted by *Listeria monocytogenes* infection of epithelial cells and is dependent on Src kinase; however, its molecular role is unknown. Here, we show that the status of pTyr¹⁵⁸ defines cytoskeletal organization, affects the assembly/disassembly of focal adhesions, and interferes with cell migration. Cells overexpressing a non-phosphorylatable NM2A variant or expressing reduced levels of Src kinase display increased stress fibers and larger focal adhesions, suggesting an altered contraction status consistent with the increased NM2A activity that we also observed. We propose NM2A pTyr¹⁵⁸ as a novel layer of regulation of actomyosin cytoskeleton organization.

Keywords: NM2A; Src kinase; actomyosin cytoskeleton; cytoskeletal remodeling; cell migration



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1. Introduction

The non-muscle myosin 2A (NM2A) is a major cytoskeletal motor protein that plays a central role in the contractility of the actin cytoskeleton [1,2]. Each NM2A molecule is a hexameric complex composed of two non-muscle myosin heavy chains (NMHC2A) and two pairs of light chains: the regulatory (RLCs) and essential (ELCs) light chains [1,2]. NMHC2A folds into three different domains: the conserved head/motor domain comprising the sites for ATP hydrolysis and actin binding; the neck domain that interacts with the light chains, stabilizing the protein and regulating NM2A activity; and the tail, containing a coiled coil region responsible for heavy chain homodimerization and non-muscle myosin II (NMII) filament formation [2,3]. Phosphorylation of the RLCs at threonine 18 and serine 19 regulates the NM2A enzymatic activity and filament formation [4–7]. The inactive form of NM2A, associated with unphosphorylated RLCs, folds into a compact structure in which motor heads and tails directly interact [2,8,9]. This autoinhibited molecule does not form filaments, interacts only weakly with actin, and freely diffuses in cells [10–13]. Upon phosphorylation of the RLC, the active NM2A molecules undergo antiparallel self-association to form bipolar filaments [10,14], which interact with actin filaments and power

actomyosin network contraction by converting chemical energy into mechanical force via ATP hydrolysis [15]. NM2A subcellular distribution and filament assembly are additionally regulated by the specific phosphorylation on RLC tyrosine 155 in migrating cells [16] and on serine and threonine residues at the NMHC2A tail domain [17,18].

NM2A is broadly required for processes dependent on force generation and remodeling of the actomyosin cytoskeleton [19,20]. NM2A self-organizes into ordered superstructures, such as super clusters and stress fibers, through a combination of mechanical interactions and biochemical signaling [21–24]. Its activity and self-organization properties modulate the architecture of actomyosin networks, thus determining its function. In addition to regulating NM2A activity, phosphorylation plays critical roles in its organization within actomyosin networks [25,26]. The remodeling and contractility of such actomyosin networks shape cells and tissues during physiological processes such as development and wound repair.

Defects in NM2A functions are associated with a multitude of human diseases [22,27,28], including various viral and bacterial infections [29–31]. Upon cellular infection by the human pathogen *Listeria monocytogenes*, Src kinase is activated [32] and NMHC2A is phosphorylated in the conserved tyrosine 158 (Tyr¹⁵⁸) residue, located in its motor domain [30]. Phosphorylation of Tyr¹⁵⁸ (pTyr¹⁵⁸) occurs in a Src-dependent manner and restricts bacterial cellular invasion [30]. Whether this phosphorylation controls the cellular processes NM2A are involved in, thus regulating the active cellular remodeling of actomyosin networks in space and time, has not been addressed yet.

Herein, we investigated the impact of NMHC2A pTyr¹⁵⁸ on actomyosin cytoskeleton organization, cell adhesion, and migration. We found that the Src-dependent regulation of pTyr¹⁵⁸ is essential to control the organization of the actomyosin cytoskeleton, assembly/disassembly of focal adhesions, and cell motility. Our results provide new insights into NM2A regulation through a novel phosphorylation event, which modulates subcellular events where NM2A's function is crucial.

2. Materials and Methods

2.1. Cell Lines

HeLa cells (ATCC CCL-2) were grown in DMEM with glucose and L-glutamine (Lonza), supplemented with 10% fetal bovine serum (FBS; Biowest, Nuaillé, France), and maintained at 37 °C in a 5% CO₂ atmosphere.

2.2. Reagents, Toxins, Antibodies and Dyes

Dasatinib (Santa Cruz Biotechnology, Dallas, TX, USA) was used at 300 nM in complete medium for 1 h before the experiment. The following antibodies were used at 1/200 for immunofluorescence microscopy (IF) or 1/1000 for immunoblotting (IB): mouse anti-β-actin (#A1978, Sigma, St Louis, MO, USA); rabbit anti-NMHC2A (#M8064, Sigma, St Louis, MO, USA); rabbit anti-calnexin (#AB2301, Merck Millipore, Burlington, MA, USA), rabbit anti-vinculin (#700062, Fisher Scientific, Waltham, MA, USA); rabbit anti-pMLC (Thr18/Ser19) (#3674, Cell Signaling, Danvers, MA, USA); and monoclonal anti-Src (GD11, abcam, Cambridge, UK). For IF analysis, DNA was stained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; Sigma, St Louis, MO, USA) and actin with Rhodamine Phalloidin (Thermo Fisher Scientific, Waltham, MA, USA) at 1/200. Secondary antibodies were used at 1/500: goat anti-rabbit Alexa Fluor 488 (Invitrogen, Waltham, MA, USA), goat anti-rabbit Alexa Fluor 594 (Invitrogen, Waltham, MA, USA), and goat anti-mouse Cy3 (Jackson ImmunoResearch, West Grove, PA, USA). For IB, goat anti-rabbit or anti-mouse HRP (Abliance, Compiègne, France) was used at 1/10,000.

2.3. Plasmids

CMV-GFP-NMHCII-A plasmid was a gift from Robert Adelstein (Addgene # 11347) [33]. Insertion of point mutations Y158F and -Y158E was achieved by site-directed mutagenesis

using a QuickChange II Site-directed mutagenesis kit (Agilent, Santa Clara, CA, USA) as described in [30]. Hsp90 HA was a gift from William Sessa (Addgene # 22487) [34].

2.4. Transfections and shRNA Lentiviral Transductions

HeLa cells seeded on top of glass coverslips in Nunc™ 24-well plates (5×10^4 cells/well), into Ibitreat μ -dishes (Ibidi, Martinsried, Bayern, Germany) (1×10^5 cells/well), or Nunc™ 6-well plates (5×10^5 cells/well) were, respectively, transfected with 0.5 μ g, 1 μ g, or 2.5 μ g of DNA using jetPRIME® Polyplus transfection reagent or Lipofectamine 2000® (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturers' instructions. Protein expression was allowed for 18 to 24 h before cells were processed. shRNA lentiviral transductions were performed as described in [30] and the sequences used are available in the Supplementary Materials (Table S1).

2.5. Immunoblot Assays

HeLa cells total protein extracts were recovered in sample buffer (0.25 mM Tris-Cl, pH 6.8; 10% SDS; 50% glycerol; and 5% β -mercaptoethanol), resolved by SDS-PAGE (10% acrylamide), and transferred onto nitrocellulose membranes using a TransBlot Turbo™ system (BioRad, Hercules, CA, USA) at 0.3 A for 1 h. Primary and secondary HRP-conjugated antibodies were diluted in TBS-Tween 0.1% (150 mM NaCl; 50 mM Tris-HCl, pH 7.4; and 0.1% Tween) with 5% (*m/v*) milk. For the immunoblot assessing the expression levels of Src, 20 μ g of total protein extracts from WT and shSrc HeLa cells were loaded. Washes were performed with TBS-Tween 0.2%. The signal was detected using Western Blotting Substrate (Thermo Fisher Scientific, Waltham, MA, USA) and collected in a ChemiDoc™ XRS+ System with Image Lab™ Software (version 3.0.1.14, BioRad, Hercules, CA, USA).

2.6. Immunofluorescence Microscopy

Cells were fixed in 3% paraformaldehyde (15 min at room temperature), quenched with 20 mM NH₄Cl (1 h), and permeabilized with 0.1% Triton X-100 (5 min). Coverslips were incubated for 1 h with primary antibodies, washed three times in PBS 1 $\times$, and incubated for 45 min with secondary antibodies and, when indicated, Rhodamine Phalloidin and DAPI. Antibodies and dyes were diluted in PBS containing 1% BSA. Coverslips were mounted onto microscope slides with Aqua-Poly/Mount (Polysciences, Warrington, PA, USA). Images were collected with a confocal laser scanning microscope (Leica SP5 II, Wetzlar, Germany) and processed using Fiji™ (version 2.9.0/1.53t) or Adobe Photoshop (CS6 version 13.0) softwares.

2.7. g-Actin and f-Actin Separation

HeLa cells (5×10^5) were seeded in six-well plates and either transfected to express GFP-NMHC2A-WT or -Y158F variants or treated with dasatinib for 1 h. Cells were washed twice with PBS 1 $\times$ and homogenized for 10 min at 37 °C in actin stabilization buffer (0.1 M Hepes, pH 6.9, 30% glycerol (*v/v*), 5% DMSO (*v/v*), 1 mM MgSO₄, 1 mM EGTA, 1% Triton X-100 (*v/v*), 1 mM ATP, complete protease, and phosphatase inhibitors). Total protein fractions were ultracentrifuged at 100,000 $\times g$ for 75 min at 37 °C. Supernatants containing g-actin were collected, and pellets containing f-actin were resuspended in an equivalent volume of RIPA buffer. Total, supernatant, and pellet fractions were processed for immunoblotting.

2.8. Quantification of Immunofluorescence Images: NMHC2A Aggregates, Stress Fibers, Focal Adhesions, Phospho-Regulatory Light Chain (pMLC), Cell Area, and Aspect Ratio

The percentage of cells displaying stress fibers or NMHC2A aggregates was quantified in at least 200 cells per sample. Cells showing approximately 50% (arbitrary) of their total area covered by dense actomyosin fibers were considered positive for stress fibers. Cells displaying at least two distinct aggregates larger than 2 μ m or one larger than 5 μ m were

considered positive for aggregation. Anisotropy was quantified using the Fiji™ plug-in FibrilTool as described in [35] and focal adhesions were quantified as described in [36] using the CLAHE and Log3D Fiji™ plug-ins. The levels of pMLC were quantified using Fiji™ by measuring the raw integrated density of pMLC in each cell normalized by the total cell area in at least 100 cells per sample. The area of the cells and respective aspect ratio were measured using the analysis tool in Fiji™.

2.9. Cell Motility Analysis

Cells were seeded into Ibitreat μ -dishes (Ibidi, Martinsried, Bayern, Germany), transfected to express the different GFP-NMHC2A variants, maintained in OptiMEM (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) at 37 °C and 5% CO₂, and imaged using a widefield microscope (Leica DMI6000 Time-lapse, Wetzlar, Germany) equipped with a 20 $\times$ 0.4 NA objective. Data sets of transmitted light and the fluorescence of GFP-NMHC2A were acquired every 10 min between 16 and 20 h. Fiji™ was used for image sequence analyses and video assembly. The velocity and accumulated distance were analyzed in at least 200 cells/condition using the Manual tracking plug-in from Fiji™.

2.10. Fluorescence Recovery after Photobleaching (FRAP)

HeLa cells were transfected to express either GFP-NMHC2A-WT or -Y158F. Images were acquired 20 h after transfection using a laser scanning confocal microscope SP8 (Leica) equipped with a 63 $\times$ 1.3 NA objective at 37 °C and 5% CO₂. Five pre-bleach images were acquired every 138 ms, followed by five bleaching scans with 100% intensity on the 488 nm laser line over the region of interest (ROI). The recovery of fluorescence was monitored for 56 s every 138 ms (400 frames), followed by 60 s every 300 ms (200 frames). Mean fluorescence intensities (MFI) were measured in Fiji™ and a fluorescence recovery analysis was performed as described in [37] using the easyFRAP-web on-line platform (<https://easyfrap.vimnet.upatras.gr/> (accessed on 3 June 2019)). Briefly, the MFI of the bleached ROI was normalized to non-bleached ROIs. We followed a full-scale normalization which subtracts background values at each time point and corrects for laser fluctuations, fluorescence losses during photobleaching, differences in starting intensities, and losses in total fluorescence. Additionally, the MFI was corrected for differences in the bleaching efficiency following easyFRAP-web on-line platform recommendations [37]. The mobile fraction and the half-maximal recovery time (T-half) values were obtained by curve fitting. Curve fitting was performed using a single- and double-term exponential equation and obtained R² values served as a goodness-of-fit measure. Only cells for which fitted curves showed an R² $\geq$ 0.8 were considered to quantify the mobile fraction and T-half.

2.11. Statistical Analysis

Statistical analyses were carried out with Prism 8 (version 8.1.1, GraphPad Software, La Jolla, CA, USA), using a two-tailed unpaired Student's *t*-test for comparison of means between two samples.

3. Results and Discussion

3.1. Actomyosin Cytoskeleton Organization Is Modulated by NMHC2A pTyr¹⁵⁸

Previous data demonstrated that *Listeria monocytogenes* infection activates Src kinase [32], which in turn phosphorylates NMHC2A on the conserved tyrosine 158 (Tyr¹⁵⁸) residue [30]. Considering the central role of NM2A in key cellular processes [2], we hypothesized that NMHC2A pTyr¹⁵⁸ may control physiological events that rely on the remodeling of the actomyosin cytoskeleton. To address this, we tested the impact of NMHC2A pTyr¹⁵⁸ in the overall organization of the actomyosin cytoskeleton. HeLa cells were transfected to express either a GFP-tagged wild-type NMHC2A (GFP-NMHC2A-WT), a mutant mimicking a permanent phosphorylation in which Tyr¹⁵⁸ was replaced by a glutamate (GFP-NMHC2A-Y158E), or a non-phosphorylatable NMHC2A mutant in which Tyr¹⁵⁸ was substituted by a phenylalanine (GFP-NMHC2A-Y158F). The percentage

of transfected cells (Figure 1A) and the expression levels, measured as the mean GFP signal intensity (Figure 1B), were similar for the three NMHC2A variants.

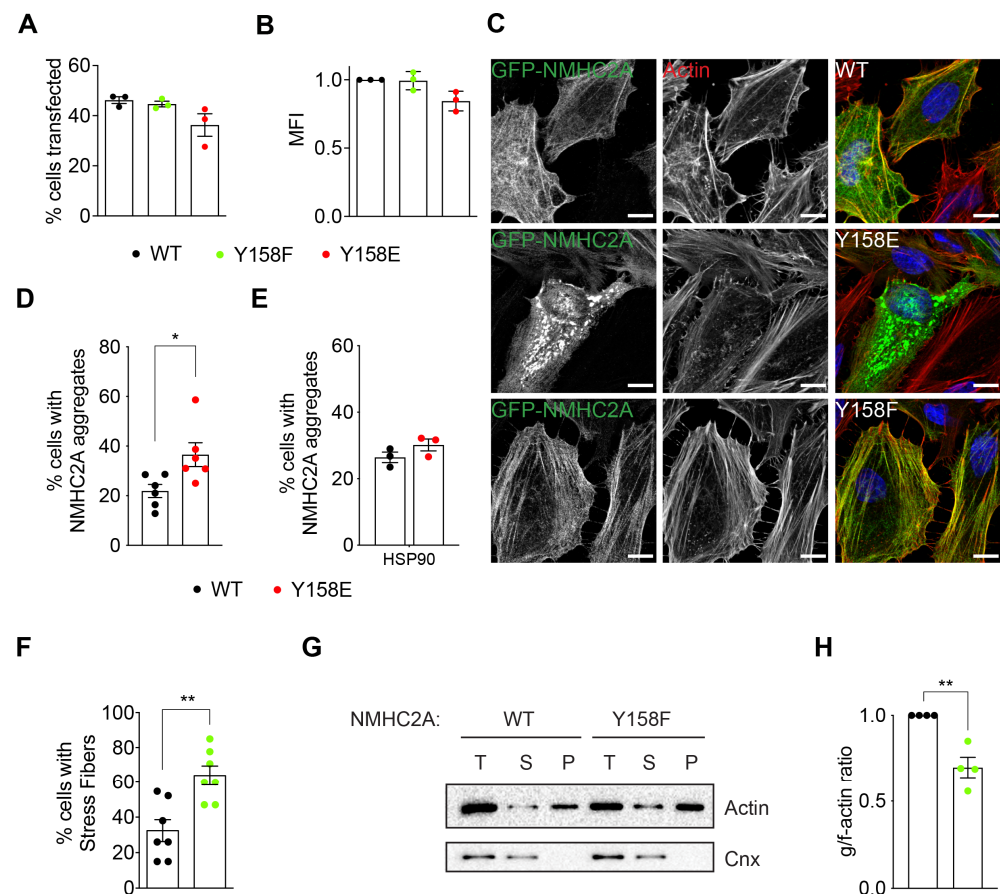


Figure 1. Actomyosin organization is regulated by the NMHC2A pTyr¹⁵⁸ status. (A) Percentage of transfected cells determined by the quantification of GFP positive cells by flow cytometry. Each dot corresponds to a single independent experiment. Values are the means $\pm$ SEM ($n = 3$). (B) Expression levels of the different variants shown as the MFI (mean fluorescence intensity) of the GFP positive cells determined by flow cytometry. Each dot corresponds to a single independent experiment. Values are the means $\pm$ SEM ($n = 3$). (C) Representative confocal microscopy images of HeLa cells ectopically expressing either GFP-NMHC2A-WT, -Y158E, or -Y158F (green) and stained with phalloidin for f-actin (red) and DAPI (blue). Scale bar, 10 μ m. (D–F) Scoring of the percentage of cells displaying (D,E) NMHC2A aggregation or (F) stress fibers in HeLa cells ectopically expressing the indicated NMHC2A variants or co-expressing HSP90 in (E). Each dot corresponds to an independent experiment. Values are the means $\pm$ SEM; p -values were calculated using a two-tailed unpaired Student's t -test; * $p < 0.05$, ** $p < 0.01$. (G) Immunoblots showing the levels of globular (g)- and filamentous (f)-actin in HeLa cells ectopically expressing NMHC2A WT or Y158F variants. g- and f-actin from the total cell lysates (T) were separated by ultracentrifugation. g-actin was recovered from supernatant fractions (S) while f-actin remained associated with pellet fractions (P). Calnexin was used as a loading control (Cnx). (H) The g-/f-actin ratio quantified from immunoblot images. Each dot corresponds to an independent experiment. Values are the means $\pm$ SEM ($n = 4$); the p -value was calculated using a two-tailed unpaired Student's t -test; ** $p < 0.01$.

A confocal microscopy analysis of transfected cells revealed that GFP-NMHC2A-Y158E aggregated in 40% of the cells, while GFP-NMHC2A-WT was detected in aggregates in 20% of the cells (Figure 1C,D). This aggregation phenotype, of which an acute example is depicted in Figure 1C, was reverted when GFP-NMHC2A-Y158E was co-expressed with the chaperone HSP90 (Figure 1E), reported to facilitate NMHC2A folding and actomyosin

filament assembly [38–40]. This observation suggests that the aggregation phenotype might be related to GFP-NMHC2A-Y158E folding and/or assembly defects, which are overcome by the overexpression of HSP90. NM2A aggregation was previously reported for mutants affecting ATPase's activity and ability to translocate actin filaments [41]. Thus, considering that GFP-NMHC2A-Y158E aggregation may trigger toxic effects and/or be associated with a decreased NM2A activity and that phospho-mimetic mutations do not always mimic the presumed phosphorylations [42,43], we pursued our study with only the non-phosphorylatable mutant (GFP-NMHC2A-Y158F).

About 60% of the cells expressing GFP-NMHC2A-Y158F were found enriched in actin filaments, displaying large areas covered by stress fibers (Figure 1C,F) organized in an anisotropic orientation (Figure S1). Only 20% of the GFP-NMHC2A-WT-expressing cells were enriched in stress fibers, which also displayed anisotropic orientation (Figures 1C,F and S1). To support these findings, we performed actin fractionation assays to assess the levels of globular (g-) and filamentous (f-) actin in cells overexpressing NMHC2A-WT or -Y158F variants. The amount of f-actin (pellet fraction, P) was higher in GFP-NMHC2A-Y158F- than in GFP-NMHC2A-WT-expressing cells (Figure 1G) and thus, the g-/f-actin ratio was significantly decreased in cells expressing the GFP-NMHC2A-Y158F variant (Figure 1H). Our data thus show that cells expressing the non-phosphorylatable NMHC2A variant accumulate stress fibers and f-actin filaments. Since the total levels of actin remained comparable (Figure 1G), the accumulation of stress fibers and f-actin detected in cells expressing the non-phosphorylatable NMHC2A variant (GFP-NMHC2A-Y158F) may be due to increased actin polymerization and/or decreased depolymerization, which would alter the filament turnover and dynamics. We speculate that GFP-NMHC2A-Y158F may have a higher f-actin crosslinking activity than its WT counterpart, inducing filaments to coalesce and thus increasing the overall density of the network and contraction [44]. Besides motor activity, the NMHC2A-mediated crosslinking of actin filaments was proposed to increase tension and to contribute to cortical integrity [44–46]. Additionally, cells with stress fibers have been demonstrated to produce high contractile forces which are not locally restricted but propagate to the surrounding actin network, impacting the overall contractile energy of the cell [47]. The accumulation of f-actin or stress fibers is associated with an increased cell stiffness and rigidity, adhesion to the substratum, and decreased cell migration capabilities [48,49]. Our data thus suggest that by accumulating stress fibers and reorganizing their actomyosin cytoskeleton, cells expressing the non-phosphorylatable variant GFP-NMHC2A-Y158F may display strong adhesion to the substratum and an impaired ability to migrate. In addition, it raises the hypothesis that through the phosphorylation of NMHC2A Tyr¹⁵⁸, the cell may reorganize its actomyosin cytoskeleton and adapt its contractile state to respond to external cues.

3.2. Src Kinase Coordinates Actomyosin Cytoskeleton Remodeling

Given that the phosphorylation status of Tyr¹⁵⁸ affects the organization of the actomyosin cytoskeleton and that during *Listeria* infection, NMHC2A pTyr¹⁵⁸ depends on Src kinase [30], we further investigated the role of Src activity in the overall organization of the actomyosin cytoskeleton. For that, we interfered with the Src activity by using the pharmacological inhibitor Dasatinib (Dasa) or downregulating the Src expression through specific shRNAs (shSrc). The reduced expression of Src kinase in shSrc cells was confirmed by immunoblotting (Figure S2). A confocal microscopy analysis consistently showed that cells with a reduced Src activity or Src expression display more stress fibers than control cells (Figure 2A). Indeed, the percentage of cells displaying stress fibers significantly increased under impaired Src conditions (Figure 2B). In addition, actin fractionation assays on total lysates of Src-impaired cells showed decreased levels of g-actin (supernatant fraction, S) and, concomitantly, increased levels of f-actin in the pellet fraction (Figure 2C). In agreement with the increased stress fibers detected by fluorescence microscopy, the g-/f-actin ratio was significantly reduced in Src-impaired cells (Figure 2D). These data show that cells with impaired Src activity display an accumulation of stress fibers, as observed in

those expressing the non-phosphorylatable GFP-NMHC2A-Y158F. Of note, our previous data demonstrated that NMHC2A pTyr¹⁵⁸ triggered by bacterial infection does not occur in cells expressing reduced levels of Src kinase [30]. Supporting this crosstalk between Src and NMHC2A, previous studies showed that Src activity controls cortical tension and/or membrane dynamics in NMII-dependent processes such as non-apoptotic plasma membrane blebbing [50], cell volume regulation upon osmotic stress [51,52], and neuronal growth cone [53].

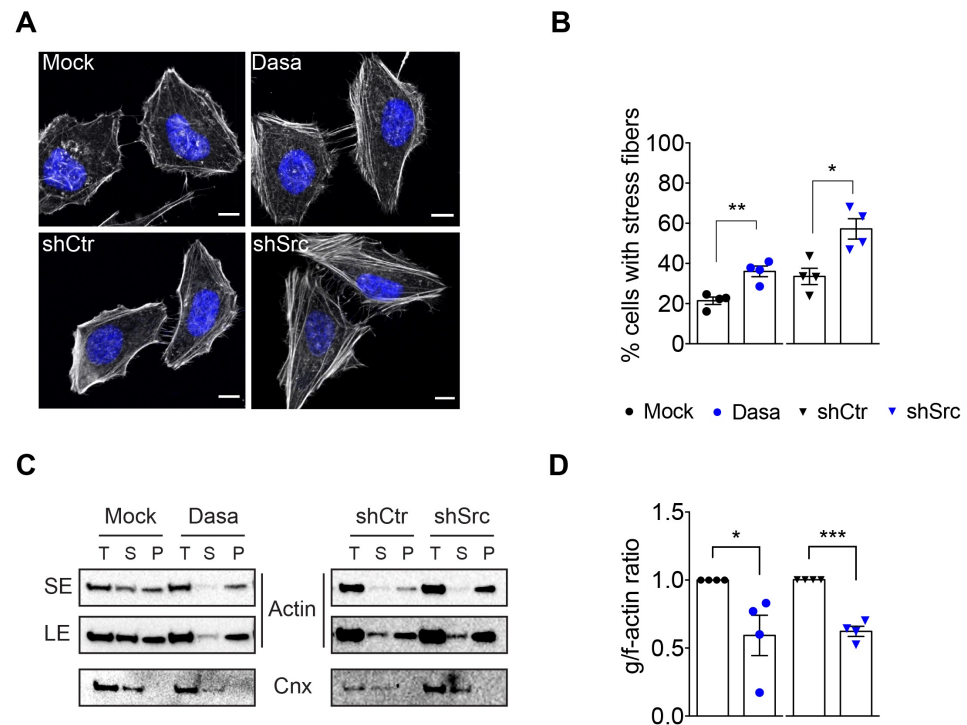


Figure 2. Src kinase controls actomyosin cytoskeleton organization in HeLa cells. (A) Confocal microscopy images of control (mock and shCtr) and Src-impaired (Dasatinib-treated and shSrc) HeLa cells stained with phalloidin for f-actin (greyscale) and with DAPI (blue). Scale bar, 10 μ m. (B) Percentage of cells with stress fibers quantified from images similar to those shown in (A). Values are the means $\pm$ SEM (n = 4); *p*-values were calculated using a two-tailed unpaired Student's *t*-test, * *p* < 0.05, ** *p* < 0.01. (C) Immunoblots showing the levels of globular (g)- and filamentous (f)-actin in HeLa cells treated as in (A). g- and f-actin from the total cell lysates (T) were separated by ultracentrifugation. g-actin was recovered from supernatant fractions (S) while f-actin was associated with pellet fractions (P). Calnexin was used as a loading control (Cnx). Actin levels are shown with both short exposure (SE) and long exposure (LE). (D) Quantification of the g-/f-actin ratio was conducted using immunoblot signals. Values are the means $\pm$ SEM (n = 4); *p*-values were calculated using a two-tailed unpaired Student's *t*-test, * *p* < 0.05, *** *p* < 0.001. In (B,D), each dot or triangle corresponds to an independent experiment.

A high Src activity reduces cell adhesion by triggering stress fiber disassembly [54–56], which enhances cell migration, invasion, and tumor metastasis [48,57]. Contrarily, and in agreement with our observations, a low Src activity was reported to promote stress fiber assembly, which is associated with cell stiffening [48] and a reduced cell migration [58]. In line with this, Src-deficient fibroblasts show reinforced integrin and actomyosin cytoskeleton interactions [59], also limiting migration. Our data strikingly show that the overexpression of a NMHC2A molecule non-phosphorylatable on Tyr¹⁵⁸ phenocopies the behavior of cells with impaired Src activity, which suggests that Src-dependent phosphorylation of NMHC2A Tyr¹⁵⁸ might contribute to Src-mediated cell processes. Thus, this raises the hypothesis that, besides coordinating the actomyosin cytoskeleton by targeting ROCK and

FAK [60], Src kinase might also regulate the actomyosin cytoskeleton through the direct control of NMHC2A pTyr.

3.3. The Focal Adhesion Morphology Is Regulated by NMHC2A pTyr¹⁵⁸

Stress fibers and focal adhesions are structurally and functionally interdependent [49,61]. Despite controversy in the field, much evidence pinpoints that the assembly of stress fibers and focal adhesions depends on tension generated by non-muscle myosin II (NMII) [49]. Indeed, the activity of NMII was shown to drive the assembly of stress fibers and focal adhesions [62] and NMII expression is required for the maturation of nascent adhesions [63,64]. Considering the described interconnections between Src, NMII activity, stress fibers, and focal adhesions and our observations regarding the stress fiber accumulation in cells expressing the non-phosphorylatable GFP-NMHC2A-Y158F variant, we investigated whether focal adhesions would also be affected by the status of NMHC2A pTyr¹⁵⁸. Cells over-expressing GFP-NMHC2A-WT or -Y158F were fixed and immunolabeled for vinculin, a marker for focal adhesions [65]. The number, size, and shape of the focal adhesions were analyzed by confocal microscopy. Although in GFP-NMHC2A-Y158F-expressing cells the number of focal adhesions per cell was significantly decreased (Figure 3A,B), they were larger and more elongated, displaying increased areas and aspect ratios (Figure 3A,C,D).

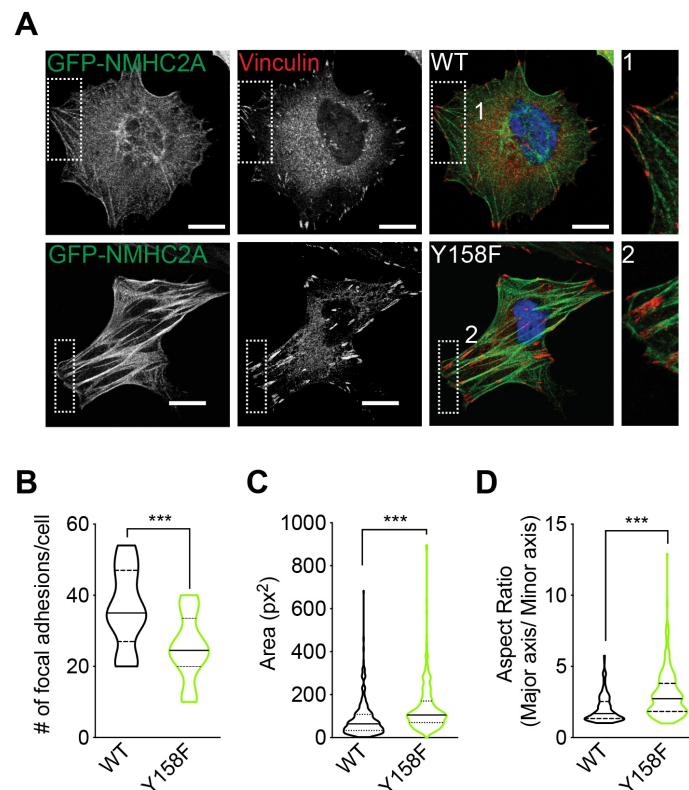


Figure 3. The number and the morphology of focal adhesions in HeLa cells are determined by the phosphorylation status of NMHC2A in Tyr¹⁵⁸. (A) Representative confocal microscopy images of HeLa cells ectopically expressing GFP-NMHC2A-WT or GFP-NMHC2A-Y158F (green), immunolabeled for vinculin (red) and stained with DAPI (blue). The insets (1 and 2) show the focal adhesion morphology. Scale bar, 10 μ m. (B) Quantification of the number of focal adhesions per cell from images similar to those shown in (A). Each dot corresponds to the mean number of focal adhesions per cell obtained from three independent experiments. Values are the means $\pm$ SEM; p -value was calculated using a two-tailed unpaired Student's t -test, *** $p < 0.001$. (C,D) Quantification of the focal adhesion (C) area and (D) aspect ratio from images similar to those shown in A. Each dot corresponds to a single focal adhesion. Values are the means $\pm$ SEM ($n > 140$); p -values were calculated using a two-tailed unpaired Student's t -test, *** $p < 0.001$.

The traction force that focal adhesions exert on the cell substrate was shown to be directly related to their size [66,67]. Possibly, under increasing tension, new proteins are recruited to focal adhesions whose composition may thus be regulated by mechanical stress [68,69]. In agreement with this, several proteins such as vinculin were found to be associated with focal adhesions in an NMII-dependent manner [70–72].

While the assembly of ventral stress fibers and focal adhesions depends on the tension powered by NM2A contractility [62–64,70], other studies reported that the tension and NMII activity had little effect on both stress fiber and focal adhesion assembly [73–75]. Our data favor the involvement of NM2A-mediated mechanical tension in the assembly of stress fibers and their associated focal adhesions.

Together with the increased stress fibers detected in cells overexpressing GFP-NMHC2A-Y158F, our results also suggest that focal adhesions are under increased tension and that the NMHC2A pTyr¹⁵⁸ status not only regulates stress fiber assembly but also interferes with focal adhesion morphology, thus indicating a role in their formation and/or maturation. Overall, we show here the critical importance of NMHC2A pTyr¹⁵⁸ in cytoskeletal organization and cell adhesion, also suggesting its involvement in cell migration.

3.4. The Lack of NMHC2A pTyr¹⁵⁸ Affects Cell Size and Impairs Cell Motility

Considering the well-established roles of Src kinase, NMHC2A, and actin cytoskeleton in the regulation of cell size/shape and migration [76–78], and our data showing that the Src-mediated phosphorylation of NMHC2A Tyr¹⁵⁸ impacts actomyosin cytoskeleton organization, we investigated if the status of NMHC2A pTyr¹⁵⁸ may affect the cell shape parameters and cell migration. We compared the area and aspect ratio of cells expressing GFP-NMHC2A-WT or -Y158F. Expression of GFP-NMHC2A-Y158F led to a slightly increased cell area (Figure 4A). However, aspect ratio values were similar in both conditions (Figure 4B). To evaluate the consequences of the cytoskeletal alterations induced by the expression of NMHC2A-Y158F, we monitored the motility of HeLa cells using fluorescence timelapse microscopy (Figure 4C and Movie S1). A cell tracking analysis of the timelapse videos revealed that cells expressing GFP-NMHC2A-Y158F migrated with a reduced velocity (Figure 4D) and spanned shorter distances (Figure 4E) when compared to cells expressing GFP-NMHC2A-WT. These results are in line with the elongated focal adhesions and increased stress fibers we observed, indicating that cells are possibly under increased tension if the non-phosphorylatable NMHC2A-Y158F is overexpressed. The NMHC2A pTyr¹⁵⁸ status thus modulates cell motility, most likely through the control of actomyosin cytoskeleton organization.

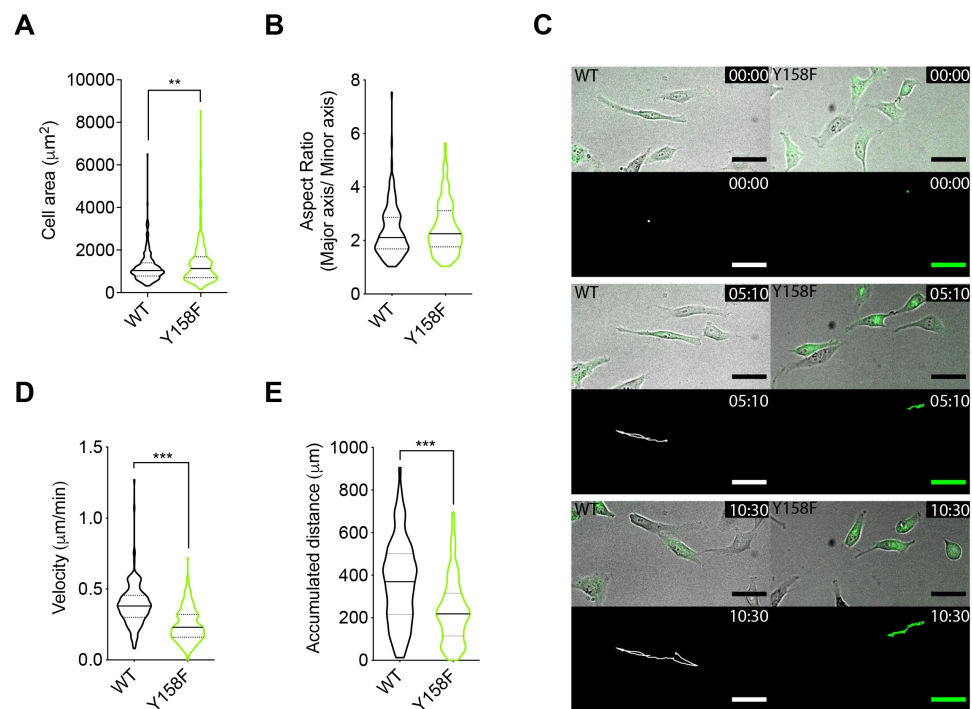


Figure 4. The lack of NMHC2A pTyr¹⁵⁸ impairs the motility of HeLa cells. (A,B) Quantification of cell shape parameters of HeLa cells ectopically expressing GFP-NMHC2A-WT or -Y158F: (A) area and (B) aspect ratio. Cells were stained with phalloidin and quantifications were performed using FijiTM. Each dot represents a single cell. Values are the means $\pm$ SEM ($n > 160$); p -values were calculated using a two-tailed unpaired Student's t -test, ** $p < 0.01$. (C) Sequential frames of the timelapse microscopy Movie S1, from 0 to 10 h 30 min. HeLa cells ectopically expressing GFP-NMHC2A-WT or GFP-NMHC2A-Y158F are shown. The movement of individual cells over time was analyzed using the FijiTM Manual Tracking plugin and is represented by colored lines. Scale bar, 50 μ m. (D,E) Quantification of cell motility parameters using the Manual Tracking plugin of FijiTM: (D) velocity and (E) accumulated distance in HeLa cells as in A. The data were obtained from movies similar to Movie S1. Each dot represents a single cell. Values are the means $\pm$ SEM ($n > 200$); p -value was calculated using a two-tailed unpaired Student's t -test, *** $p < 0.001$.

3.5. NMHC2A pTyr Regulates NM2A Activation and Dynamics

Given our observations, we next examined whether pTyr¹⁵⁸ could affect the NM2A turnover on actin filaments. To investigate this, we performed Fluorescence Recovery After Photobleaching (FRAP) in HeLa cells overexpressing either GFP-NMHC2A-WT or -Y158F. The GFP signal of NMHC2A was photobleached in a single region per cell where NMHC2A filaments were detected, the recovery of the signal was followed over time and the data were analyzed using the easyFRAP-web on-line platform [37]. The GFP signal within the photobleached region in cells overexpressing GFP-NMHC2A-Y158F recovered faster than that in cells overexpressing GFP-NMHC2A-WT (Figure 5A,B). The mobile fraction, corresponding to the fraction of molecules able to turnover, and the half time required for signal recovery (T-half) were obtained from the easyFRAP-web mean curve fitting tool [37]. The calculated mobile fractions were similar under both conditions (Figure 5C). However, the half time for signal recovery was significantly shorter in cells expressing GFP-NMHC2A-Y158F as compared to those expressing GFP-NMHC2A-WT (Figure 5D), indicating that the expression of non-phosphorylatable NMHC2A-Y158F allows for a faster NM2A turnover.

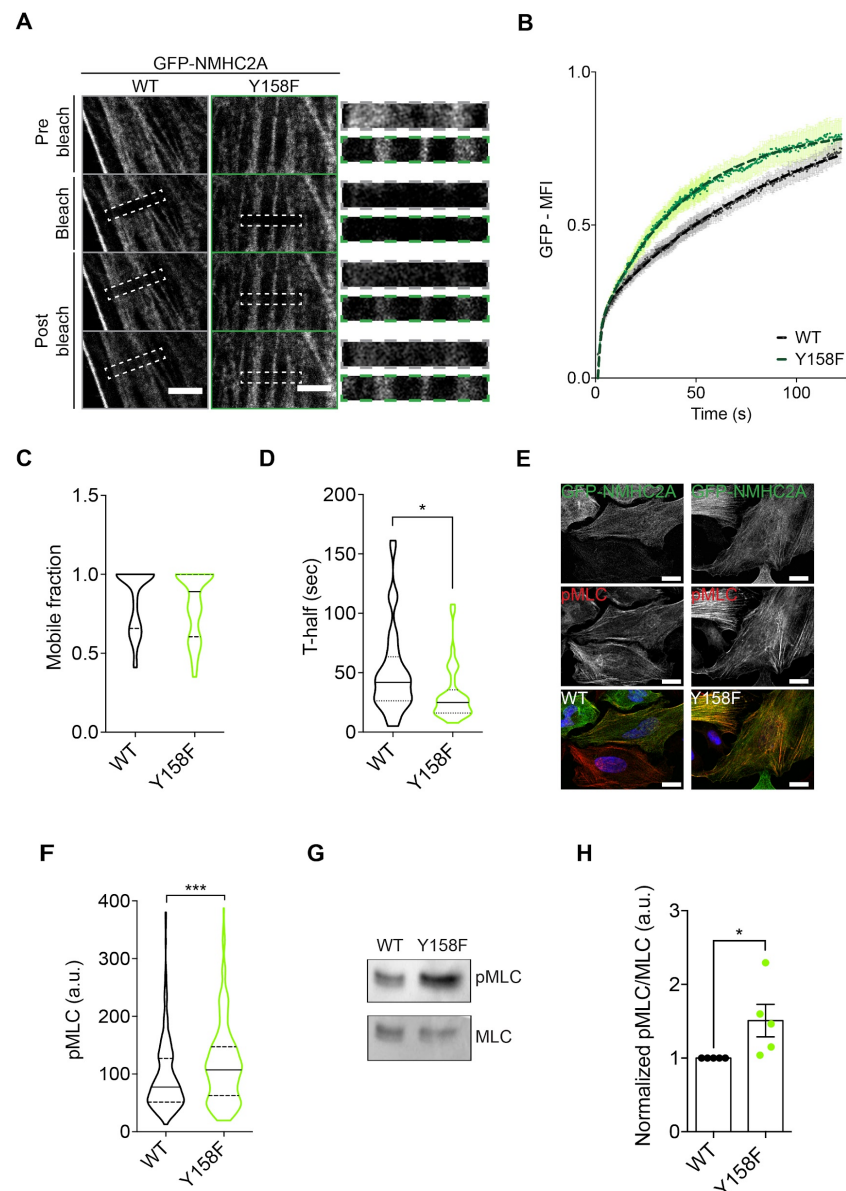


Figure 5. NMHC2A pTyr¹⁵⁸ controls the dynamic assembly of bipolar filaments in HeLa cells. (A) Representative confocal microscopy timelapse images of Fluorescence Recovery After Photo-bleaching (FRAP) experiments in HeLa cells ectopically overexpressing either GFP-NMHC2A-WT or -Y158F. Insets of the bleached region in dashed lines. Scale bar, 5 μ m. (B) Plot of the normalized mean fluorescence intensity recovery curves obtained for each condition tested (black, WT; green, Y158F). Values are the means $\pm$ SEM ($n \geq 40$ cells). (C,D) Quantification of the (C) mobile fraction and the (D) half-maximal recovery time (T-half) obtained from the curve fitting of each individual recovery curve. Each dot represents a single cell. Values are the means $\pm$ SEM ($n \geq 40$ cells); p -values were calculated using a two-tailed unpaired Student's t -test, * $p < 0.05$. (E) Representative confocal microscopy images of HeLa cells ectopically expressing either GFP-NMHC2A-WT or -Y158F (green), immunolabeled to detect pMLC (red) and stained with DAPI (blue). Scale bar, 15 μ m. (F) Levels of pMLC in cells expressing the different NMHC2A variants, quantified using FijiTM. Each dot corresponds to a single cell. Values are the means $\pm$ SEM ($n > 300$); p -value was calculated using a two-tailed unpaired Student's t -test, *** $p < 0.001$. (G) Immunoblot showing the levels of phosphorylated MLC (pMLC) and the total levels of MLC in whole cell lysates of HeLa cells expressing either GFP-NMHC2A-WT or -Y158F. (H) Quantification of pMLC immunoblot signals normalized to that of MLC. Each dot corresponds to an independent experiment. Data correspond to means $\pm$ SEM ($n = 5$); p -value was calculated using a two-tailed unpaired Student's t -test, * $p < 0.05$.

NM2A filament dynamics and self-organization require NM2A contractility, which is in turn regulated by RLC phosphorylation [2,21]. Thus, we quantified phosphorylated RLC in residues threonine 18 and serine 19 (pMLC) to assess the levels of NM2A activation. In agreement with a faster turnover, we found that cells overexpressing the non-phosphorylatable GFP-NMHC2A-Y158F displayed higher levels of pMLC than those expressing GFP-NMHC2A-WT (Figure 5E–H). In addition, pMLC co-localized with stress fibers, induced by the expression of GFP-NMHC2A-Y158F (Figure 5E). Overall, NM2A-Y158F-expressing cells displayed increased levels of Thr18/Ser19 phosphorylated myosin regulatory light chain and showed a faster turnover of NM2A, which might be associated with an increased contractility [49]. Our results suggest that non-phosphorylated Tyr¹⁵⁸ triggers NM2A activation and increases its dynamics within assembled filaments and not at the single molecule level.

4. Conclusions

Phosphorylations in tyrosine residues control cell signaling and regulate a variety of biological processes, including the reorganization of the cytoskeleton [79,80]. We previously reported an uncharacterized tyrosine phosphorylation event in NMHC2A triggered by bacterial infections [30]. Infection with *L. monocytogenes* induced Src-dependent NMHC2A phosphorylation on Tyr¹⁵⁸, a residue that is located close to the ATP-binding pocket of NMHC2A and was predicted to be accessible for phosphorylation [81]. Here, we showed that the regulation of NMHC2A pTyr¹⁵⁸ contributes to the cellular organization of the actomyosin cytoskeleton.

Larger and more elongated focal adhesions, together with increased stress fibers and higher pMLC levels detected in NMHC2A-Y158F-expressing cells, suggest an altered contraction status that may be associated with an increased tension, a stronger adhesion to the substratum, and increased forces exerted on the extracellular matrix [48,82,83]. The phosphorylation status of NMHC2A Tyr¹⁵⁸ could therefore regulate the assembly/disassembly of focal adhesions and stress fibers, thus providing a novel mechanism to modulate cytoskeletal organization. Phosphorylation events in the regulatory light chain and the heavy chain tail domain, respectively, regulate NM2A activity (reviewed in [2]), the spatial localization [16], and the dynamics of NM2A filaments [84,85]. Whether the phosphorylation of Y158 interferes with other phosphorylation events reported to regulate the NM2A activity or filament assembly is unknown, but worth investigating in the future. To our knowledge, pTyr¹⁵⁸ is the only phosphorylation occurring in the motor domain of NMHC2A that contributes to regulating the dynamics of the actomyosin cytoskeleton. Previous studies proposed that the formation of heterotypic filaments [86,87] and the assembly of NMII supramolecular structures, named NMII stacks [21,88], may modulate actomyosin dynamics likely to accomplish specific functions in particular spatiotemporal contexts [42,89]. It is possible that pTyr¹⁵⁸ regulates the assembly of such heterotypic filaments and/or NMII stacks. By interfering with actomyosin remodeling, one can speculate that the phosphorylation status of NMHC2A Tyr¹⁵⁸ may affect NM2A-driven cellular processes such as cytokinesis, membrane trafficking, and organelle positioning. As a proof of concept, data from our laboratory indicate that permanent phosphorylation of Tyr¹⁵⁸ impairs cytokinesis in *C. elegans* embryos, while worms expressing a non-phosphorylatable NMHC2A are more susceptible to stress cues such as heat and bacterial infections (Brito et al., unpublished data).

We thus propose a new conserved mechanism for the regulation of the actomyosin cytoskeleton through the control of NM2A pTyr¹⁵⁸. This adds a new layer of complexity in the control of actomyosin dynamics with a major impact on cell adhesion to a substrate and cell migration.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/cells12141871/s1>, Figure S1: Quantification of the anisotropic distribution of stress fibers; Figure S2: Immunoblots showing Src and actin levels in extracts from WT and shSrc HeLa cells. Actin was used as a loading control. Table S1: shRNA sequences for Src knockdown in HeLa cells; Movie S1: Ectopic expression of the different GFP-NMHC2A variants affects cell motility in HeLa cells.

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Supplementary Materials: “Src-dependent NM2A tyrosine-phosphorylation regulates actomyosin dynamics”

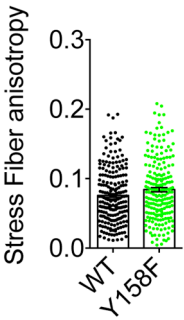


Figure S1. Quantification of the anisotropic distribution of stress fibers in HeLa cells ectopically expressing GFP-NMHC2A-WT or Y158F. Each dot corresponds to a single cell. Values are the mean $\pm$ SEM ($n > 200$)

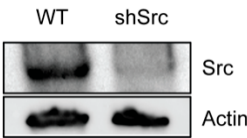


Figure S2. Immunoblots showing Src and actin levels in extracts from WT and shSrc HeLa cells. Actin was used as loading control.

Table S1. shRNA sequences for Src knockdown in HeLa cells.

shRNA Sequences (5'-3')		Source
<i>shControl-SHC016</i>	CCGGGCGCGATAGCGCTAATAATTTCTCGAGAAA TTATTAGCGCTATCGCGCTTTTT	Sigma
<i>shSrc-TRCN0000023597</i>	CCGGGTGGCTTACTACTCAAACATCTCGAGATGT TTGGAGTAGTAAGCCACTTTTT	

Movie S1. Ectopic expression of the different GFP-NMHC2A variants affects cell motility in HeLa cells. HeLa cells ectopically expressing GFP-NMHC2A-WT or -Y158F were followed by time-lapse microscopy for more than 15 h. (Upper panel) Sequential frames acquired every 10 min (8 frames per second display rate). Overlay of the transmitted light and the fluorescence of GFP-NMHC2A variants is shown. Scale bar, 50 μm . (Lower panel) Representative tracking of the movement of cells expressing WT or Y158 versions of NMHC2A, produced by the Manual Tracking plug-in on Fiji™. Scale bar, 50 μm .