Control of cytoskeletal dynamics during cellular responses to pore forming toxins

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\textbf{ABSTRACT}

Following damage by pore forming toxins (PFTs) host cells engage repair processes and display profound cytoskeletal remodeling and concomitant plasma membrane (PM) blebbing. We have recently demonstrated that host cells utilize similar mechanisms to control cytoskeletal dynamics in response to PFTs and during cell migration. This involves assembly of cortical actomyosin bundles, reorganisation of the endoplasmic reticulum (ER) network, and the interaction between the ER chaperone Gp96 and the molecular motor Non-muscle Myosin Heavy Chain IIA (NMHCIIA). Consequently, Gp96 regulates actomyosin activity, PM blebbing and cell migration, and protects PM integrity against PFTs. In addition, we observed that PFTs increase association of Gp96 and ER vacuoles with the cell surface or within PM blebs loosely attached to the cell body. Similarly, gut epithelial cells damaged by PFTs \textit{in vivo} were shown to release microvilli structures or directly purge cytoplasmic content. Cytoplasmic purging involves profound cytoskeletal remodeling and ER vacuolation, suggesting that our observations recapitulate recovery processes \textit{in vivo}. Here, we discuss our findings in light of the current understanding of PM repair mechanisms and \textit{in vivo} recovery responses to PFTs.

\textbf{INTRODUCTION}

Evolutionary conserved mechanisms allow eukaryotic cells to sustain mechanical and chemical stress that injure the PM.\textsuperscript{1-3} The changes in the intracellular concentration of calcium and potassium caused by PM rupture initiate recovery processes which depend on the size of the damage, the cell types involved and the nature of the inflicted stress (e.g. mechanical injuries or insertion of stable protein pores such as those created by bacterial PFTs).\textsuperscript{1-3} In general, cells engage PM repair pathways, rearrange the cytoskeleton, control their metabolic state and activate stress-associated signaling.\textsuperscript{2,3}

PM damage promotes calcium influx, which enhances exocytosis, predominantly of lysosomes. These vesicles patch large mechanical wounds (> 100 nm),\textsuperscript{4} and promote acid-sphingomyelinase (ASM) release, which generates PM-ceramide domains that engulf PM damage in caveolae-derived endosomes.\textsuperscript{2,3} Stable protein pores cannot be patched and are removed by endocytosis or shedding within small PM vesicles (nm size).\textsuperscript{6} PM shedding may actually constitute an intrinsic repair mechanism that senses PFT oligomerisation and is potentiated upon damage and calcium influx.\textsuperscript{7} Shedding depends on endosomal sorting complexes required for transport (ESCRT) and is similar to the budding of viral particles.\textsuperscript{6-8} \textit{In vivo}, recovery from PFT-mediated damage appears to involve the cooperation between different mechanisms. Host survival requires regulators of both endocytic and exocytic trafficking and epithelial cells display increased rates of endocytosis, shedding of PM material\textsuperscript{9} and/or direct purging of cytoplasmic content.\textsuperscript{10} In addition, epithelia compact its cytoskeletal network and display alterations of cellular organelles while preserving coherence and functionality.\textsuperscript{10}

The fine control of the cytoskeletal dynamics is therefore necessary to promote PM recovery.\textsuperscript{11} Indeed, following mechanically-induced PM damage, microtubules allow recruitment of distal vesicles while local actin rearrangements and myosin activity relief tension facilitate vesicle delivery and provide force to re-establish PM integrity.\textsuperscript{12-16} The importance of cytoskeletal dynamics in cells targeted by PFTs remains poorly defined.
**Novel regulators of cytoskeletal dynamics protect against PFTs**

We recently identified the ER chaperone Gp96 and NMHCIIA as regulators of cytoskeletal dynamics following PFT-mediated PM damage. Gp96 and NMHCIIA interact upon PFT intoxication and accumulate into distinct bundles at sites of PM blebbing (Fig. 1 and Supp Mov 1). These processes require calcium influx generated by PM damage and occur during *Listeria monocytogenes* (*Lm*) infection, which depends on the PFT listeriolysin O (LLO). The reorganisation of the actomyosin network is mediated by Gp96, which modulates myosin II activity and coordinates PM blebbing during PFT intoxication. Both Gp96 and NMHCIIA promote cell survival upon LLO intoxication.

We characterized further the formation of NMHCIIA bundles during PFT intoxication and found that host cells utilise similar mechanisms to regulate cytoskeletal dynamics during recovery of PM integrity and cell migration. (i) PFT-induced actomyosin bundles accumulate proteins found at the trailing edge of migrating cells; (ii) upon PFT intoxication, Gp96 interacts with Filamin-A, an actin cross-linker that regulates cell migration; and (iii) stimulation of cell migration with Wnt5a, which promotes assembly of rear-end ER-actomyosin structures, also enhances NMHCIIA-Gp96 interaction. In line with these observations, we showed that Gp96 regulates general cytoskeletal organization and therefore modulates cell shape and cell motility.

Recent independent studies have also proposed a role for Gp96 in cytoskeletal organization, cell polarity and cell migration. This may occur through the control of vesicular trafficking and/or interaction with different cytoskeletal proteins such as F-actin-capping protein 1, Actin, Radixin and ROCK2. Of note, Gp96 is predominantly expressed at early stages of development and contributes to the establishment of epithelial gut morphology and apical specification. Polarized lysosome secretion and establishment of cell polarity are regulated by NMHCIIA. Therefore, it is possible that Gp96 and NMHCIIA interact to coordinate vesicular trafficking and cytoskeletal dynamics necessary for the definition of cell polarity and for efficient PM repair. Whether NMHCIIA and Gp96 directly interact remains unknown. Yet, Gp96 is the ER paralogue of the cytosolic chaperone HSP90, which binds myosin head domains and is necessary to coordinate assembly and folding of myosin thick filaments.

Few additional molecules were associated with the cytoskeletal reorganisation following PFT-mediated PM damage. RhoA and Rac1 GTPases promote actin remodelling and Src-family kinases mediate microtubule bundling and stabilization. The importance of such processes for cell recovery from PFT-mediated wounding is uncertain. Nevertheless, GTPases (RhoA, Rac and Cdc42) coordinate the assembly and dynamics of actomyosin rings, which promote closure of laser-induced wounds in *Xenopus* oocytes, and Src, together with myosin light chain kinase (MLCK), regulate PM expansion during osmotic stress.

Besides actomyosin reorganisation and simultaneous PM blebbing, cells modify the entire ER network following PFT intoxication, as depicted by the alteration of the characteristic ER reticular pattern and formation of vacuoles containing mCherry-Sec61β (a subunit of the ER membrane translocon complex Sec61) (Fig. 1 and Supp Mov 1). Vacuolation of the ER and other cellular structures is an important mechanism for cell recovery and survival.

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**Figure 1.** Redistribution of NMHCIIA and ER network upon LLO treatment. Sequential frames of time-lapse confocal microscopy sequence of LLO-treated HeLa cells expressing simultaneously GFPNMHCIIA and mCherrySec61. LLO was added to culture medium 10 seconds before t0. DIC – differential interference contrast. Highlighted inset depicts ER structures within NMHCIIA bundles and PM blebs.
Figure 2. Exposure of ER and Gp96 at blebs from LLO treated cells. (A) Confocal microscopy Z-stack projections of HeLa cells treated with LLO (0.5 nM, 15 min) and immunolabelled for the C-terminal sequence present in ER resident proteins, ER-KDEL (red), NMHCIIA (green) and stained with DAPI (blue). Orthogonal views and 3D projections illustrate exposure of ER vacuoles at the cell surface (arrow). (B-C) Confocal microscopy images of HeLa cells left untreated or treated with LLO and immunolabelled for (B) ER-Gp96 (blue), NMHCIIA (green) and stained with FITC-WGA (Plasma membrane, PM-red) and DAPI (white), or (C) Sec61 (red), NMHCIIA (blue) and stained with FITC-WGA (green) and DAPI (white). Insets and arrows indicate NMHCIIA-positive PM blebs containing Gp96 or Sec61, loosely attached to the cell body. Arrow-heads show cortical NMHCIIA-Sec61 within the cell body. All scale bars are 10 μm. (D) Longitudinal TEM images of HeLa cells left untreated or treated with 0.5 nM LLO for 15 min. ER - ER cisternae in untreated cells and ER vacuoles in LLO-treated cells; N - nucleus. Arrows show vesicles and bleb-like structures at the proximity of the PM containing ER vacuoles and apparently detached from the cell body.
organelles has been reported in response to different PFTs in various cell types and in vivo.1,10 The relevance of such morphological alteration is not understood and has been mainly associated with organelle damage and cell death.1 However, following toxin wash-out, cells recover normal actomyosin and ER distribution with equivalent kinetics.17

Lysosomes and the ER are major intracellular calcium stores and their dynamics are crucial for functioning. In particular, the transient distribution of ER and lysosomes to the trailing edge of migrating cells directs calcium signaling and assembly of cytoskeletal complexes that mediate tail retraction.18 Of note, stimulation of such process enhances Gp96-NMHCIIA interaction.17 However, the role of the ER during recovery from PFT-induced PM damage remains unclear. ER proteins have been detected at PM wounds of mechanically injured cells30 and inhibition of ER stress pathways or calcium sequestration compromises survival after PFT intoxication.31,32 Whether lysosomes or the ER control calcium signaling and actomyosin dynamics during PM repair is still speculation.30 Nevertheless, Gp96 regulates calcium homeostasis at the ER.33 We observed that certain LLO-intoxicated cells appear to expose ER compartments containing ER-retention sequence KDEL, Gp96 and Sec61α at the cell surface or within large PM blebs loosely attached to the cell body (Fig. 2A-C).17 Transmission electron microscopy (TEM) of intoxicated HeLa cells confirmed that such vacuoles are detected within large bleb-like structures at the proximity of the PM and apparently detached from the cell body (Fig. 2D). Thus, upon damage, cells can release ER-derived compartments to the extracellular environment. Whether the release of ER vacuoles only occurs in dying cells or upon organelle damage is still unclear. Yet these processes may constitute a common feature of cellular material, which protect host tissues from PFT attack. Moreover, it will be important to further analyze the physiologic relevance of recovery mechanisms in the context of bacterial infections: What are the consequences of PM blebbing and cytosolic purging in the context of different infections? Are these processes related to the shedding of apoptotic bodies and damaged cells from infected epithelia?

As PM recovery processes display important evolutionary conserved features,2,3,11 the ground-breaking use of amenable models such as zebrafish (Danio rerio) and drosophila (Drosophila melanogaster) to the direct visualization of infectious processes in vivo will continue to be of critical importance.10,17

## Conclusion and future perspectives

We have highlighted the importance of NMHCIIA and uncovered an unexpected role for the ER chaperone Gp96 in host cell recovery against PFTs. Future studies are now necessary to understand how cytoskeletal dynamics interfere with polarized secretion and shedding of cellular material, which protect host tissues from PFT attack. Moreover, it will be important to further analyze the physiologic relevance of recovery mechanisms in the context of bacterial infections: What are the consequences of PM blebbing and cytosolic purging in the context of different infections? Are these processes related to the shedding of apoptotic bodies and damaged cells from infected epithelia?

Materials and methods

### Plasmids and antibodies

Plasmid GFPNMHCIIA (#11347) was obtained from Addgene and mCherry-Sec61-N-18 was a gift from M. Davidson through Addgene (# 55130). Rabbit anti-NMHCIIA (Sigma); mouse anti-NMHCIIA (Abcam); rat anti-Gp96 (Enzo); mouse anti-Sec61α G-2 (Santa Cruz) were used at 1/200 for immunofluorescence microscopy (IF). PM was labeled with FITC-conjugated WGA (Sigma) DNA with 4',6-Diamidino-2-phenylindole dihydrochloride, DAPI (Sigma) and IF fluorescently-conjugated secondary antibodies (Invitrogen) were used at 1/500.

### Cell lines and toxin

HeLa (ATCC CCL-2) cells were cultivated in DMEM with glucose and L-glutamine, supplemented with 10% FBS. Cells were maintained at 37°C in a 5% CO2 atmosphere. Cell culture media and supplements were from Lonza. LLO was purified as previously17 and treatments and washes were carried in Hank’s Balanced Salt Solution (HBSS) as indicated.

### Immunofluorescence microscopy

Cells were fixed in 4% paraformaldehyde (15 min), quenched with 20 mM NH4Cl (1 h), permeabilized with 0.1% Triton X-100 (5 min), and blocked with 10% BSA in PBS (30 min). Antibodies were diluted in PBS...
containing 1% BSA. Coverslips were incubated for 1 h with primary antibodies, washed 3 times in PBS and incubated 45 min with secondary antibodies. DNA was counterstained with DAPI (Sigma). Coverslips were mounted onto microscope slides with Aqua-Poly/Mount (Polysciences). Images were collected with a confocal laser-scanning microscope (Leica SP5II) and processed using ImageJ64 or Adobe Photoshop software.

**Live imaging and quantification of PM blebbing of LLO-treated cells**

Cells seeded into Ibidi dishes (Ibidi), simultaneously transfected with GFPNMHCIIA and mcherrySEC61, maintained in HBSS at 37°C with 5% CO₂ were imaged using an Andor Revolution XD Spinning-disc confocal system with an EMCCD iXonEM+ camera, 488 nm laser lines, and a Yokogawa CSU-22 unit on an inverted microscope (IX81; Olympus), driven by Andor IQ live-cell imaging software. LLO (0.5 nM) was added 10 min after initial image acquisition. Differential interference contrast (DIC) images and GFP fluorescent data sets with 0.5 μm Z-steps were acquired using a UPLSAPO 100×/1.40 objective lens every 15 sec. ImageJ64 was used for image sequence analysis and video assembly.

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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