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A *Staphylococcus aureus* Pore-Forming Toxin Subverts the Activity of ADAM10 to Cause Lethal Infection

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Abstract

Staphylococcus aureus is a major cause of human disease, responsible for half a million infections and approximately 20,000 deaths per year in the United States alone [1,2]. This pathogen secretes α-hemolysin, a pore-forming cytotoxin that contributes to the pathogenesis of pneumonia [3–5]. α-hemolysin injures epithelial cells by interacting with its receptor, the zinc-dependent metalloprotease ADAM10 [6]. We show that mice harboring a conditional disruption of the *Adam10* gene in lung epithelium are resistant to lethal pneumonia. Investigation of the molecular mechanism of toxin-receptor function revealed that α-hemolysin upregulates ADAM10 metalloprotease activity in alveolar epithelial cells, resulting in cleavage of the adherens junction protein E-cadherin. Cleavage is associated with disruption of epithelial barrier function, contributing to the pathogenesis of lethal acute lung injury. A metalloprotease inhibitor of ADAM10 prevents E-cadherin cleavage; similarly, E-cadherin proteolysis and barrier disruption is attenuated in ADAM10 knockout mice. Together, these data attest to the function of ADAM10 as the cellular receptor for α-hemolysin. The observation that Hla can usurp the metalloprotease activity of its receptor reveals a novel mechanism of pore-forming cytotoxin action in which pathologic insults are not solely the result of irreversible membrane injury, and defines ADAM10 inhibition as a strategy for disease modification.

Pore-forming cytotoxins (PFTs) are a family of bacterial virulence factors that cause eukaryotic cell injury and death [7]. *Staphylococcus aureus* encodes multiple PFTs, the most prominent being α-hemolysin (Hla) [8]. Hla is essential for diseases involving epithelial cell-

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AUTHOR CONTRIBUTIONS I.I. performed murine infection modeling, in vivo E-cadherin cleavage studies and ECIS studies. N.I. performed mouse breeding and genetic analysis and assisted with infection modeling. G.W. performed siRNA transfections, analyzed ADAM10 expression on 16HBE14o- cells and performed cellular assays of metalloprotease activity. M.P. examined the effects of GI254023X on toxin binding and performed ECIS experiments. K. F. generated the Hla^{PPL} mutant. Y.W. performed ECIS experiments. J.B.W. performed cellular assays of metalloprotease activity, E-cadherin cleavage, immunofluorescence microscopy, and wrote the paper. All authors discussed the results and commented on the manuscript.

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lined interfaces, including pneumonia, dermonecrotic skin infection, corneal infection, and toxic shock syndrome [3,4,9–11]. Hla induces injury by forming a lipid-bilayer penetrating heptameric pore [12]. The identification of ADAM10 as the Hla receptor provides an opportunity to elucidate receptor function in disease and define mechanisms of toxin-induced injury.

ADAM10 knockout mice exhibit early embryonic lethality [13]. To examine the requirement for ADAM10 in staphylococcal pneumonia, we utilized surfactant protein C (SP-C) promoter-driven expression of Cre-recombinase to generate mice harboring *Adam10* deletion in respiratory epithelium (Supplementary Fig. 1a) [14,15]. This system achieves recombination in SP-C-expressing progenitor type II pneumocytes, yielding ADAM10-deficient type I pneumocytes that constitute the alveolar epithelium; genomic analysis and protein expression confirmed recombination (Supplementary Fig. 1b, c). Knockout mice ($ADAM10^{-/-}$) were resistant to lethal pneumonia caused by methicillin-sensitive (Fig. 1a) and methicillin-resistant (Supplementary Fig. 1d) *S. aureus*. Histopathologic examination of $ADAM10^{-/-}$ lungs following sublethal infection revealed limited inflammatory cell influx and preservation of alveolar structure (Fig. 1b). Lethal infection in controls caused widespread consolidation and cellular infiltration, while $ADAM10^{-/-}$ mice exhibited more localized disease (Fig. 1c). ADAM10 knockout did not alter lung bacterial load (Supplementary Fig. 1e), in contrast to the reduction observed in Hla-deficient *S. aureus* infection or upon toxin neutralization [5,16]. Toxin-mediated injury therefore augments bacterial recovery independent of epithelial ADAM10 expression, whereas ADAM10 expression is essential for progressive, lethal disease.

Zinc-dependent catalysis by ADAM10 results in ectodomain cleavage of proteins that modulate immunity in addition to E-cadherin that facilitates inter-epithelial cell adhesion [17,18]. As epithelial barrier integrity is disrupted in pneumonia, we hypothesized that ADAM10 enzymatic activity may contribute to toxin-mediated injury. To assess whether Hla alters ADAM10 metalloprotease activity, we performed a fluorogenic peptide cleavage assay on A549 alveolar epithelial cells treated with 10 μg ml^{-1} active Hla (300 nM), a subcytolytic concentration (Supplementary Fig. 2a). Intoxication induced metalloprotease activity in irrelevant siRNA-treated cells (Fig. 2a), a response that was not elicited by a non-oligomerizing Hla_{H35L} mutant [19]. ADAM10 siRNA-treated cells only exhibited activity comparable to irrelevant siRNA-transfected cells at 30 minutes, likely owing to residual ADAM10 expression (Fig. 2a, Supplementary Fig. 2b and ref.[6]); activity in ADAM10 siRNA-treated cells thereafter was markedly reduced.

To evaluate the effects of increased ADAM10 activity on barrier function, electrical cell substrate impedance sensing (ECIS) was utilized, recording toxin-induced changes in A549 monolayer resistance. Intoxication caused rapid loss of resistance in irrelevant transfectants (Fig. 2b, blue) whereas ADAM10 knockdown preserved barrier function (green) similar to controls (PBS, black, and Hla_{H35L}, red). The requirement for ADAM10 in barrier disruption was evident even at 50 μg ml^{-1} Hla wherein cytolytic injury occurs shortly post-intoxication and can be receptor-independent (Supplementary Fig. 2a and ref. [6]).

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Bacteria circumvent host barrier defenses through virulence factors that dismantle the structural framework of the epithelium [20]. Proteolysis of the E-cadherin extracellular domain by ADAM10 severs the homotypic, adherens junction-based linkage between adjacent cells [21]. To examine whether Hla-induced ADAM10 activation caused E-cadherin cleavage, lysates from A549 cells were analyzed by immunoprecipitation and immunoblotting. ADAM10-mediated cleavage releases the E-cadherin N-terminal extracellular domain, leading to a reduction in full-length (FL) protein and appearance of an intracellular C-terminal fragment (CTF) [21]. Ionomycin induces ADAM10 activity [22], enhancing cleavage compared to DMSO or Hla_{H35L} treatment, while Hla (20 μg ml^{-1}) triggered rapid cleavage (Fig. 2c). Concentrations of Hla as low as 1 μg ml^{-1} induced cleavage within 1 hour (Fig. 2d). Immunofluorescence microscopy confirmed loss of E-cadherin (Fig. 2e). Cells transfected with ADAM10 siRNA did not reveal toxin-induced CTF generation (Supplementary Fig. 2c), maintaining E-cadherin expression (Supplementary Fig. 2d).

To define the structural form of Hla that activates ADAM10, we examined toxin assembly intermediates. These include monomeric Hla_{H35L} that binds to ADAM10 but cannot form stable heptamers, and a pre-pore locked mutant (Hla_{PPL}) containing an engineered disulfide bond that allows heptamer formation but prevents toxin insertion into the membrane until dithiothreitol is present (Hla_{PPL}+DTT). Only Hla_{PPL}+DTT activated ADAM10 and led to E-cadherin cleavage (Fig. 2f), demonstrating a requirement for the pore. Methyl-β-cyclodextrin (MβCD)-mediated blocade of the pore abrogated metalloprotease activation and E-cadherin cleavage. These findings suggested that the pore may serve as an ion conduit, triggering downstream signaling. Indeed, calcium in the media as observed in F12K, Dulbecco's PBS (DPBS, containing Ca^{2+}, Mg^{2+} and K^{+}) and PBS+Ca^{2+} was required (Fig. 2g).

To further examine pore formation in ADAM10 activation, we treated A549 cells with *S. pneumoniae* pneumolysin (PLY), a cholesterol-dependent PFT implicated in pneumococcal pneumonia [23]. PLY is highly cytotoxic (Supplementary Fig. 2e, upper), inducing cell-associated metalloprotease activity (middle), notably at lower toxin concentrations wherein E-cadherin cleavage was observed (lower). These data suggest that ADAM10 may be utilized by multiple PFTs for pathologic E-cadherin cleavage. Cleavage was most prominent at overtly cytotoxic PLY concentrations, likely reflecting metalloprotease-dependent E-cadherin cleavage during apoptosis as triggered by PLY [24,25]. The receptor function of ADAM10 may specifically target Hla to the epithelium, potentiating the ability of the small pore to activate ADAM10 and cause E-cadherin cleavage at subcytolytic concentrations.

Acute lung injury is associated with loss of epithelial barrier function, leading to accumulation of cellular, proteinaceous edema [26]. To examine the role of Hla in E-cadherin cleavage and barrier disruption *in vivo*, we utilized a mouse *S. aureus* pneumonia model. Hla-deficient strains demonstrate limited virulence in this model, and toxin neutralization protects against disease [5,16]. C57BL/6J mice were infected with wild-type (WT) *S. aureus* USA300/LAC or an isogenic Hla-deficient mutant (Hla-) [3]. Bronchoalveolar lavage (BAL) was performed to evaluate E-cadherin cleavage, measuring N-terminal fragment (NTF) release. Infection with non-toxigenic *S. aureus* led to minimal NTF detection in contrast to WT infection (Fig. 3a). Alveolar barrier disruption was observed as increased BAL cell

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counts following WT infection, a response that was blunted in Hla- infection (Fig. 3b). BAL protein analysis revealed exudation by 6 hours in WT-infected animals, rising through 24 hours (Fig. 3c). Minimal influx was seen in Hla- infected mice at 6 and 24 hours, with increased protein at 12 hours likely reflective of the host inflammatory state [3,5]. Toxin-independent inflammation in this setting may result from other virulence factors, notably Protein A-mediated TNF receptor activation [27], or the effects of CXCR3 chemokines on host immune cells [28].

To examine Hla sufficiency in eliciting E-cadherin cleavage, we delivered 0.4 μg Hla or Hla_{H35L} intranasally to mice. Only Hla treatment led to NTF accumulation (Fig. 3d) with increasing cell counts and alveolar protein (Supplementary Fig. 3a). Within 4 hours, when signs of injury were not yet apparent (Fig. 3e, upper), E-cadherin cleavage was already evident (lower). Hla induced minimal E-cadherin cleavage in $ADAM10^{-/-}$ mice (Fig. 3f). BAL cell recovery in $ADAM10^{-/-}$ mice was blunted, with markedly reduced protein accumulation (Fig. 3g). Infection of C57BL/6J mice with a ~2.5-fold greater inoculum of Hla- *S. aureus* did not result in increased E-cadherin cleavage (Fig. 3h), protein leakage (Fig. 3i, right), or death (data not shown), in spite of a trend toward increased cell recovery (Fig. 3i, left). Delivery of this increased Hla- *S. aureus* inoculum to $ADAM10^{-/-}$ mice and controls resulted in similar disease severity (Supplementary Fig. 3b).

These observations raised the possibility that metalloprotease inhibition may mitigate toxin action. Treatment of A549 cells with the ADAM10-specific inhibitor GI254023X [29] abrogated E-cadherin cleavage (Fig. 4a and Supplementary Fig. 4a online), correlating with preservation of monolayer resistance (Fig. 4b). A549 cells do not form a polarized epithelium, thus observations were confirmed in the 16HBE14o- bronchial epithelial line (Supplementary Fig. 4b–d). ADAM10 expression in 16HBE14o- did not display polarity (Supplementary Fig. 4e and ref. [30]). Unexpectedly, GI254023X impaired toxin binding and oligomerization on A549 cells (Fig. 4c), perhaps owing to conformational changes that alter toxin association. GI254023X treatment of mice prevented E-cadherin cleavage (Fig. 4d), reduced alveolar exudates (Supplementary Fig. 5a) and protected against lethal pneumonia (Fig. 4e). GI254023X pretreatment of rabbit red cells, but not toxin, blunted hemolysis, confirming that this strategy targets the host (Supplementary Fig. 5b). GI254023X prevented PLY-induced E-cadherin cleavage (Supplementary Fig. 6), thus may represent a broadly-applicable strategy against PFT-mediated injury.

These studies enhance knowledge of PFT biology, defining the role of a proteinaceous receptor in *S. aureus* pathogenesis. The use of ADAM10 as the Hla receptor confers functionality to the toxin, coupling target binding with epithelial barrier disruption. This mechanism of action permits a clearer understanding of the toxin's tropism for barrier-forming cells, and barrier disruption emerges as a critical early pathologic disturbance that is directly linked to lethal disease outcome. Additional studies are necessary to appreciate the independent contributions of Hla-induced cytolysis and ADAM10-mediated E-cadherin cleavage to barrier disruption; these may be quantitatively and temporally distinct during infection. Investigation of ADAM10 as a mediator of barrier injury induced by PFTs may reveal a more generalized role for the enzyme in bacterial pathogenesis, highlighting avenues for disease modification.

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METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemedicine/.

Supplementary Material

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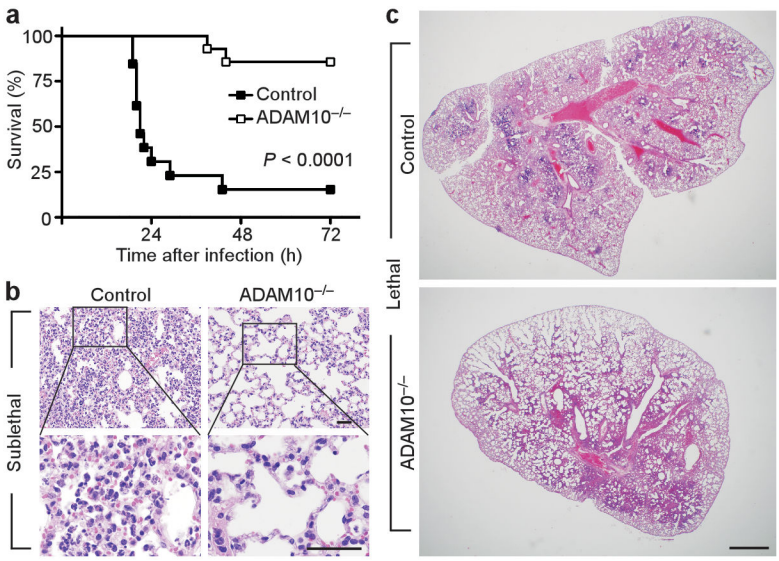

Figure 1.

ADAM10 contributes to lethal *S. aureus* pneumonia. **(a)** Survival curves for mice harboring conditional deletion of *Adam10* in the respiratory epithelium (ADAM10$^{-/-}$) relative to non-deleted littermate controls following infection with *S. aureus* strain Newman. n = 14 mice, control and 15 mice, ADAM10$^{-/-}$. Hematoxylin and eosin stained lung tissues derived from control and ADAM10$^{-/-}$ mice 18–24 hours post-infection with a sublethal **(b)** or lethal **(c)** inoculum of *S. aureus*. Scale bars = 40 μm **(b)** and 1 mm **(c)**.

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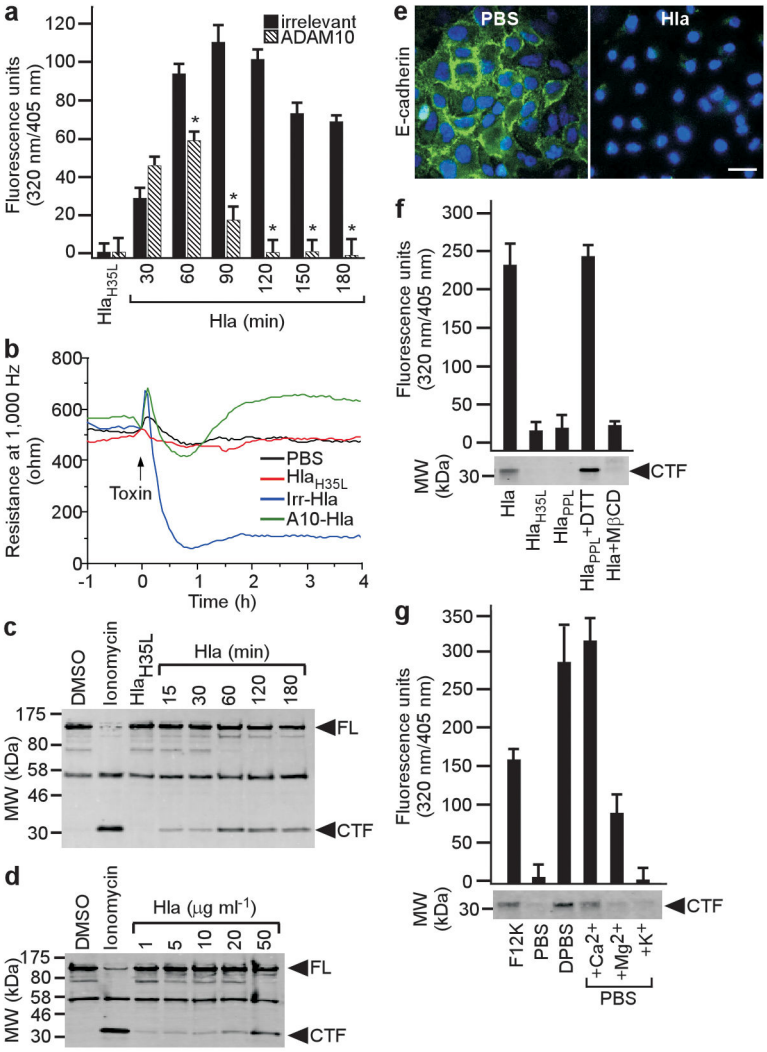

Figure 2.

Hla induces ADAM10-dependent epithelial barrier disruption and E-cadherin cleavage. **(a)** Cell-associated metalloprotease activity measured in A549 cells transfected with irrelevant (Irr) or ADAM10 siRNA following treatment with 10 μg ml^{-1} (300 nM) active Hla or the non-toxigenic mutant Hla_{H35L}. Activity was quantified by detection of a fluorescent substrate product. **(b)** Electrical cell substrate impedance sensing (ECIS) recordings of A549 monolayers treated with PBS (black), the Hla_{H35L} mutant (50 μg ml^{-1}, red), or irrelevant (Irr, blue) and ADAM10 (A10, green) siRNA transfectants treated with 50 μg ml^{-1} Hla. **(c)** Immunoblot analysis of full-length E-cadherin (FL) and accumulation of the C-terminal cleavage fragment (CTF) following treatment of A549 cells with controls DMSO and ionomycin compared to 20 μg ml^{-1} Hla_{H35L} or Hla over the time course indicated. **(d)** Concentration dependence of E-cadherin cleavage in A549 cells exposed to 1–50 μg ml^{-1} (30 nM – 1.5 μM) Hla for 1 hour. **(e)** Immunofluorescence microscopy images demonstrating surface expression of E-cadherin (green) following treatment with Hla. Nuclei (blue) are stained with the fluorescent DNA stain DAPI. Mean pixel intensity scored for ≥75 cells, 20.5 ± 0.87 (PBS) and 16.5 ± 1.72 (Hla), $P = 0.02$. Scale bar = 20 μm. **(f and**

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g) Cellular metalloprotease activity and E-cadherin cleavage induced by treatment of A549 cells with 10 μg ml^{-1} Hla, the monomeric Hla_{H35L} mutant, a pre-pore locked mutant (Hla_{PPL}) that is reverted to the wild-type toxin in the presence of dithiothreitol (Hla_{PPL}+DTT), or Hla in the presence of the pore-blocking methyl-β-cyclodextrin (MβCD) **(f)**, or by Hla in the presence of media (F12K), PBS, Dulbecco's PBS (DPBS) or PBS supplemented with 0.9 mM Ca^{2+}, 0.493 mM Mg^{2+} or 2.67 mM K^{+} **(g)**. Error bars represent SEM.

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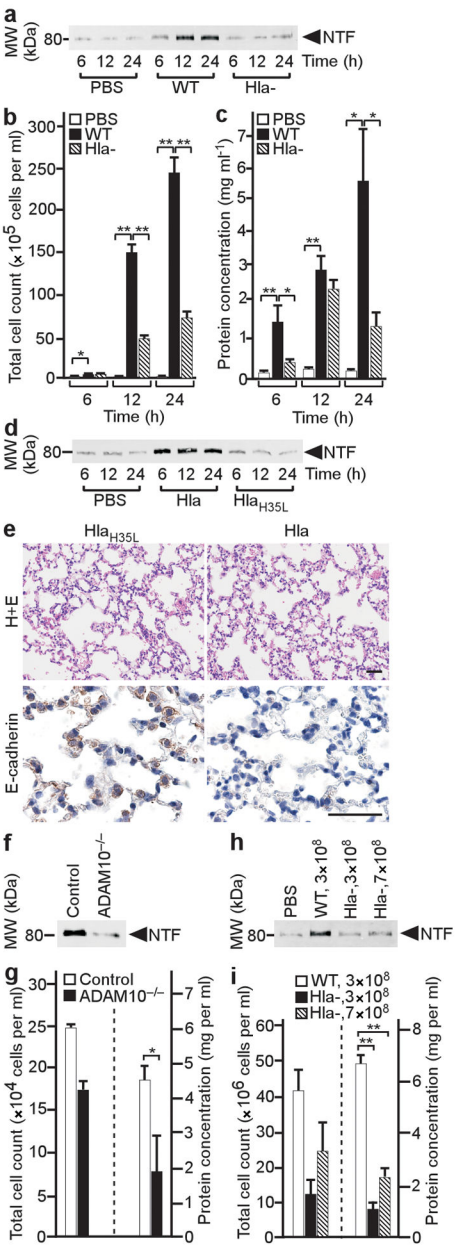

Figure 3.

Hla is required for E-cadherin cleavage and disruption of epithelial barrier function in *S. aureus* pneumonia. Bronchoalveolar lavage (BAL) fluid analysis 6, 12, and 24 hours post-infection of C57BL/6J mice infected with *S. aureus* USA300 or its isogenic mutant harboring a disruption of the *hla* locus (Hla-) to assess E-cadherin cleavage, measured by immunoblotting for the released N-terminal extracellular fragment (NTF) **(a).** Simultaneous evaluations of barrier disruption were made assessing BAL for cell count **(b)** and protein concentration **(c)** in groups of 7 animals. **(d)** BAL fluid analysis from C57BL/6J mice that received an intranasal dose of 0.4 μg purified Hla or Hla_{H35L}, or control PBS, assessed as described in **(a). (e)** Histopathology of murine lung tissues 4 hours post-treatment with PBS or Hla. Tissues were analyzed by hematoxylin and eosin staining (upper panels) or E-

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cadherin immunohistochemistry (lower panels). Scale bars = 40 μm. Immunoblot analysis of cleaved E-cadherin NTF **(f)** and quantification of cell and protein content **(g)** present in BAL fluid from ADAM10$^{-/-}$ mice relative to control littermates following treatment with 0.4 μg purified Hla delivered by intranasal route. E-cadherin cleavage **(h)** and cell/protein recovery **(i)** from BAL samples of C57BL/6J mice infected with either 3×10^8 WT or Hla- *S. aureus* as compared to infection with 7×10^8 Hla- *S. aureus*. Statistical analysis for panels **b**, **c**, **g** and **i** was performed using a two-tailed Student's t-test, where * denotes $P < 0.05$ and ** denotes $P < 0.02$.

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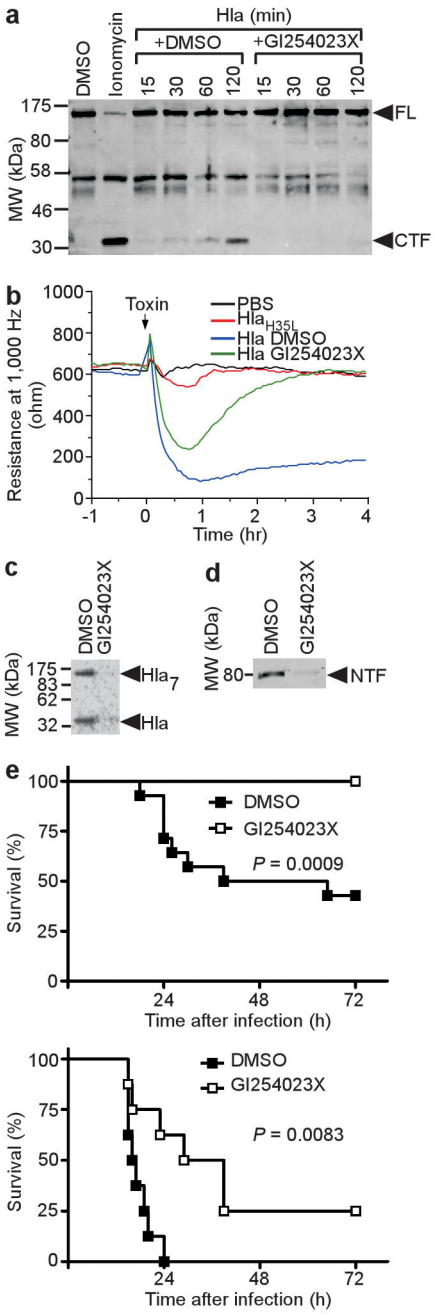

Figure 4.

An ADAM10-specific metalloprotease inhibitor prevents Hla-mediated injury. **(a)** Toxin-induced (20 μg ml^{-1}) E-cadherin cleavage detected by immunoblot analysis of Hla treated A549 cells that were pretreated with the metalloprotease inhibitor GI254023X (20 μM) or DMSO vehicle (FL, full length; CTF, C-terminal fragment). **(b)** ECIS-based monitoring of A549 monolayer resistance following toxin treatment (20 μg ml^{-1}) of cells exposed to GI254023X (20 μM) or DMSO vehicle control. **(c)** Binding and oligomerization of radiolabelled, active Hla to A549 cells following treatment with GI254023X (Hla_7, oligomeric Hla; Hla, monomeric Hla). **(d)** Immunoblot analysis of cleaved E-cadherin NTF

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present in BAL fluid from GI254023X-treated mice relative to DMSO treated animals following intranasal instillation of purified Hla. **(e)** Mortality curves in mice treated with DMSO vehicle or GI254023X upon challenge with lethal inocula of strain Newman (upper panel, n = 14 mice, 5×10^8 *S. aureus* per mouse, lower panel, n = 8 mice, 6.3×10^8 *S. aureus* per mouse).