ORIGINAL ARTICLE

# Syndecan-1 Attenuates Lung Injury during Influenza Infection by Potentiating c-Met Signaling to Suppress Epithelial Apoptosis

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## Abstract

**Rationale:** Syndecan-1 is a cell surface heparan sulfate proteoglycan primarily expressed in the lung epithelium. Because the influenza virus is tropic to the airway epithelium, we investigated the role of syndecan-1 in influenza infection.

**Objectives:** To determine the mechanism by which syndecan-1 regulates the lung mucosal response to influenza infection.

**Methods:** Wild-type (WT) and *Sdc1*$^{-/-}$ mice were infected with a H1N1 virus (PR8) as an experimental model of influenza infection. Human and murine airway epithelial cell cultures were also infected with PR8 to study the mechanism by which syndecan-1 regulates the inflammatory response.

**Measurement and Main Results:** We found worsened outcomes and lung injury in *Sdc1*$^{-/-}$ mice compared with WT mice after influenza infection. Our data demonstrated that syndecan-1 suppresses bronchial epithelial apoptosis during influenza infection to limit widespread lung inflammation. Furthermore, we determined that syndecan-1 attenuated apoptosis by crosstalking with c-Met to potentiate its cytoprotective signals in airway epithelial cells during influenza infection.

**Conclusions:** Our work shows that cell-associated syndecan-1 has an important role in regulating lung injury. Our findings demonstrate a novel mechanism in which cell membrane–associated syndecan-1 regulates the innate immune response to influenza infection by facilitating cytoprotective signals through c-Met signaling to limit bronchial epithelial apoptosis, thereby attenuating lung injury and inflammation.

**Keywords:** influenza; lung injury; syndecan-1; proteoglycan; c-Met

Influenza is a devastating respiratory disease that causes up to 500,000 deaths annually (1). The virus is tropic for the airway epithelium, where it replicates, causing epithelial death and tracheobronchitis (2). Lethality from influenza typically occurs with the development of acute respiratory distress syndrome (ARDS) secondary to viral bronchopneumonia, and may commonly occur with a concomitant bacterial superinfection (1, 3). The high mutability of the viral genome (i.e., antigenic drift) prevents long-term immunity and necessitates yearly vaccinations (4). More concerning is the appearance of novel influenza strains from viral genetic rearrangement (i.e., antigenic shift), which leads to pandemic strains (5).

Syndecan-1 is a proteoglycan that is primarily expressed by epithelia (6). All syndecans (1 through 4) are type I transmembrane proteins that contain similar structural domains. The syndecan-1 ectodomain contains attachment sites for glycosaminoglycan side chains, which can bind other proteins (e.g., chemokines or growth factors) primarily

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## At a Glance Commentary

**Scientific Knowledge on the Subject:** The influenza virus initially infects the airway epithelium, which induces the robust innate immune response necessary to contain the pathogen. However, exuberant inflammation can be harmful and result in excessive lung injury that significantly contributes to mortality. The airway epithelium has an important role in regulating the inflammatory response after influenza infection.

**What This Study Adds to the Field:** This study further defines mechanisms by which the airway epithelium controls lung injury after influenza infection. We show that syndecan-1 regulates the lung epithelial innate immune response to influenza by limiting cellular apoptosis through crosstalk with the c-Met receptor. Our data demonstrate a novel mechanism of action by which the transmembrane syndecan-1, and not the shed ectodomain, regulates the lung inflammatory response. Furthermore, we provide evidence that limiting programmed cell death of the lung epithelium has dramatic effects on attenuating lung inflammation after influenza infection. Thus, our work defines a unique mechanism by which membrane-bound syndecan-1 regulates cytoprotective signals within the lung epithelium and can potentially be therapeutically targeted to limit lung injury.

through its heparan sulfate chains. The transmembrane domain appears to regulate dimerization, whereas the cytoplasmic domain interacts with scaffolding and signaling molecules.

The biological functions of syndecan-1 in the lungs are elicited during various injuries. Our previous work demonstrated that cell-surface syndecan-1 regulates re-epithelialization in the lungs (7–9). In this situation, shedding of syndecan-1 is a mechanism of turning off the cell surface receptor, and excessive shedding may have adverse effects because of loss of receptor function (10, 11). Syndecan-1 has also been found to regulate lung inflammation, but the mechanisms have all involved the function of the shed ectodomain (12–16). An unexplored area of how syndecan-1 regulates lung inflammation is the ability of the transmembrane protein to modulate cellular function while it is intact on the cell surface. Syndecan-1 can associate with a variety of co-receptors on the cell membrane to modulate cellular signaling and function (17).

Because the lung epithelium is the primary location of influenza infection and is where syndecan-1 is expressed to regulate lung immunity, we postulated that syndecan-1 affects the host response to viral infection through regulation of co-receptors while associated on the cell membrane. We found that syndecan-1 has a cytoprotective role that limits viral-induced apoptosis, which in turn attenuates lung injury after influenza infection. Moreover, syndecan-1 confers an anti-apoptotic signal by acting on the cell surface to potentiate signaling through c-Met, the hepatocyte growth factor (HGF) receptor.

Some of the results of these studies have been previously reported in the form of abstracts (18, 19).

## Methods

### Influenza Model

A/PR/8/34 (PR8) stocks, a mouse-adapted H1N1 influenza, were expanded by incubating them in embryonated chicken eggs as previously described (20). Wild-type (WT) and *Sdc1*$^{-/-}$ mice (C57BL/6) were intranasally instilled with an H1N1 virus (A/PR/8/34; PR8; 250–500 plaque-forming units in 50 μl of sterile phosphate-buffered saline).

### Cell Culture

The creation of BEAS-2b cells stably expressing human syndecan-1 shRNA (Sdc1 KD) and Sdc1 KD cells that express WT and mutant syndecan-1 were previously validated (7, 9). Primary cultures of airway epithelial cells grown at an air–liquid interface (ALI) were created from WT and *Sdc1*$^{-/-}$ mice as previously described (7).

### Statistics

Statistical significance was determined using the Student's *t* test or the two-way analysis of variance. $P < 0.05$ was considered statistically significant. Survival analysis was calculated by using the log-rank test. All data points are means ± SEM unless stated otherwise.

## Results

### Syndecan-1 Regulates the Host Response by Attenuating Disease after Influenza Infection

Syndecan-1 is primarily expressed by the lung epithelium with airway epithelial cells having the highest expression (Figure 1A). After PR8 infection, syndecan-1 was shed from the lung epithelium (Figures 1A–1C). WT mice had inflammatory cell infiltration largely confined to the peri-bronchiolar region as expected with influenza (Figure 1D). However, *Sdc1*$^{-/-}$ mice had extensive alveolitis in addition to the peri-bronchiolar inflammation. Furthermore, body weight loss, a good indicator of disease severity, was greater in *Sdc1*$^{-/-}$ mice than WT mice (Figure 1E). More importantly, syndecan-1 conferred protection from lethality because WT mice had better survival after influenza compared with *Sdc1*$^{-/-}$ mice (Day 12 postinfection: 46% vs. 0%, respectively; $P < 0.05$; Figure 1F).

Influenza-infected *Sdc1*$^{-/-}$ mice also had more inflammatory cells (Figure 1G), neutrophils (Figure 1H), total protein (Figure 1I), and IgM (Figure 1J) in the bronchoalveolar lavage (BAL) than WT mice. Furthermore, there were significantly higher expression of the pro-inflammatory chemokines and viral response genes in *Sdc1*$^{-/-}$ compared with WT animals (*see* Figures E1A–1E in the online supplement). However, influenza viral load (see Figures E1F–E1H) and secretion of IL-1β and IL-18 (*see* Figures E1I and E1J) were similar between genotypes. Together, these findings indicate that syndecan-1 dampens the inflammatory response to influenza infection, thereby limiting widespread lung injury and protecting the host from death.

### Lung Epithelial Syndecan-1 Regulates the Host Response to Influenza Infection

Syndecan-1 expression is primarily confined to the lung epithelium (Figure 1A). To confirm that influenza-mediated inflammation was controlled by lung epithelial expression of syndecan-1, we used an adeno-associated virus (AAV) vector to transduce syndecan-1 within the lung epithelium (*see* Figure E2 in the online supplement) (21). Restoration of lung epithelial expression of syndecan-1 in *Sdc1*$^{-/-}$ mice improved lung injury, weight

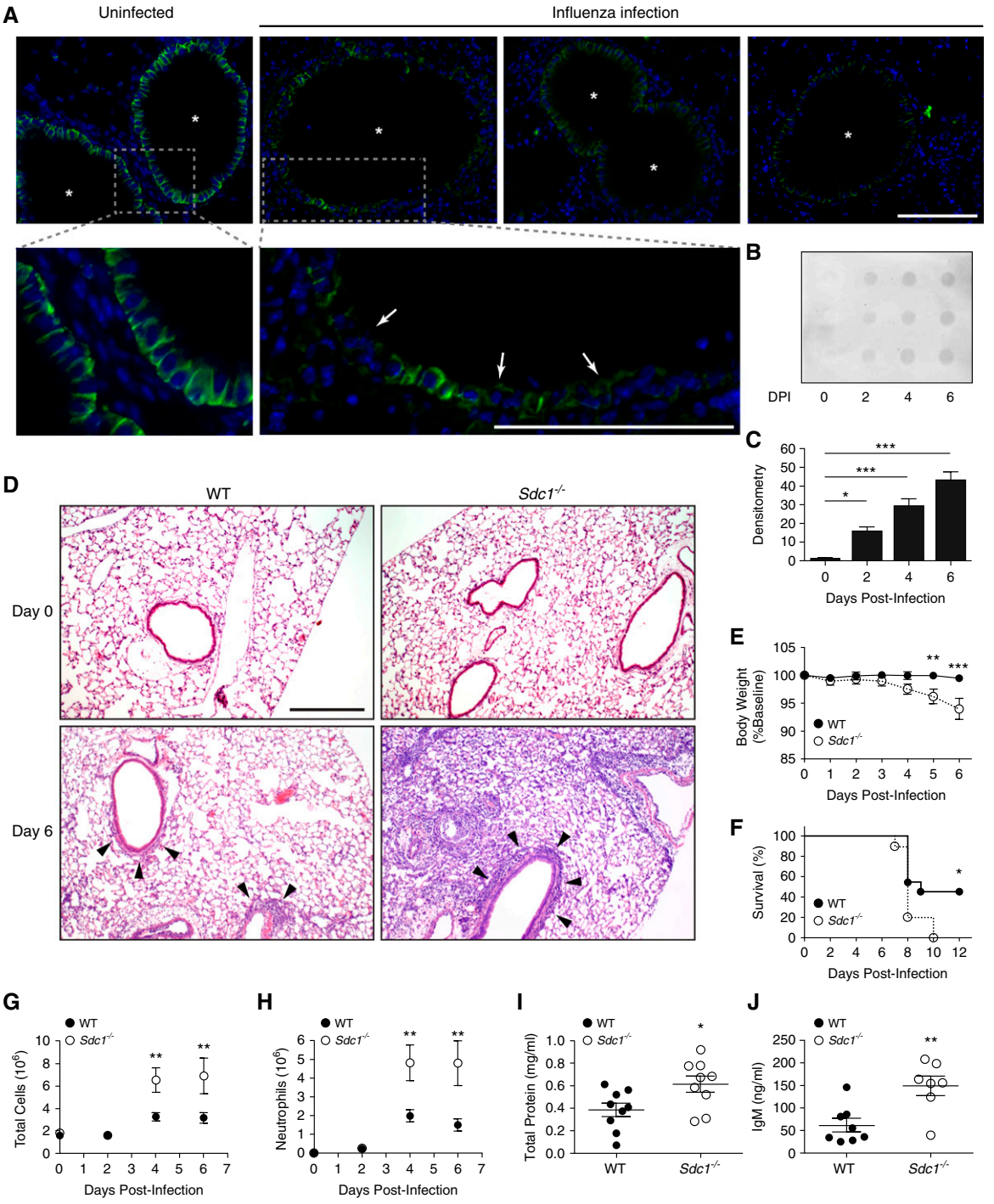


**Figure 1.** Syndecan-1 limits lung inflammation and injury during influenza infection. Wild-type (WT) and *Sdc1*$^{-/-}$ mice were infected intranasally with 250 plaque-forming units of PR8 virus. (*A*) Syndecan-1 expression (*green*) lines the basolateral surface of the airway epithelium at baseline (*airway lumen). After influenza infection (Day 6), syndecan-1 expression is diminished throughout the lung epithelium (*arrows*). *Scale bar*, 100 μm. (*B*) Syndecan-1 dot blot of bronchoalveolar lavage (BAL) from influenza-infected mice shows shedding into the airspaces after infection. (*C*) Densitometry of dot blot showing a significant increase in syndecan-1 shedding after influenza infection. Day 1 (0.901 ± 0.76), Day 2 (15.56 ± 2.61), Day 3 (29.28 ± 3.99), Day 4 (42.76 ± 4.80); n = 3 per group. (*D*) Representative hematoxylin and eosin images of control (Day 0) and virus-infected lungs from WT and *Sdc1*$^{-/-}$ mice. Peribronchiolar infiltrates are evident in both genotypes (*arrowhead*). However, *Sdc1*$^{-/-}$ mice have more widespread lung injury during influenza infection. *Scale bar*, 200 μm. (*E*) Body weight loss (WT: n = 24 for Days 0–4; n = 10 for Days 5–6; WT: n = 22 for Days 0–4; n = 9 for Days 5–6) and (*F*) survival in WT and *Sdc1*$^{-/-}$ mice after influenza infection (n = 11 and 10, respectively). (*G–J*) Analysis of BAL fluid from mice during influenza infection for (*G*) total cell count (n = 3–15), (*H*) neutrophils (n = 3 – 14), (*I*) total protein (n = 9), and (*J*) IgM levels (n = 7–8). Data shown as mean ± SEM. DPI = days postinfection. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

loss, and survival compared with control conditions that expressed green fluorescent protein in *Sdc1*$^{-/-}$ mice (Figures 2A–2C). Lung permeability and inflammation were also reduced in *Sdc1*$^{-/-}$ mice with epithelial expression of syndecan-1 compared with controls (Figures 2D–2G). These data establish that syndecan-1 expression in the lung epithelial compartment regulates lung inflammation during influenza infection.

### Syndecan-1 Regulates Bronchial Epithelial Apoptosis during Influenza Infection

Influenza infection mediates much of its early damage by inducing apoptosis within the lung epithelium (22–26). We found that airway epithelial cells were apoptotic after influenza infection (Figure 3A) and more abundant in *Sdc1*$^{-/-}$ mice than WT mice (*see* Figure E3 in the online supplement). Caspase-3 activity was also higher in *Sdc1*$^{-/-}$ (365.7 ± 106.4) compared with WT conditions (96.04 ± 30.42) after infection (Figure 3B). When syndecan-1 expression was replenished within the lung epithelium of *Sdc1*$^{-/-}$ mice, caspase-3 activity was reduced in comparison to control *Sdc1*$^{-/-}$ mice (Figures 3C and 3D).

The airway epithelium is the target of influenza, where apoptosis is localized (Figures 3A–3D) (27, 28). We specifically evaluated airway epithelial apoptosis using murine ALI cultures, and we found *Sdc1*$^{-/-}$ ALI cultures had 1.82-fold more apoptotic activity compared with WT airway epithelium after influenza infection (Figure 3E). The augmented apoptosis in *Sdc1*$^{-/-}$ conditions could be attenuated by replenishing syndecan-1 expression in *Sdc1*$^{-/-}$ ALI cultures (Figure 3F). In addition, using the BEAS-2b human bronchial epithelial cell line, cells with suppressed syndecan-1 expression had 4.5-fold augmented apoptosis after influenza infection compared with control cells replete with syndecan-1 (Figure 3G). Moreover, syndecan-1 must be cell-associated with a "shed" ectodomain that has no effect in attenuating apoptosis after influenza infection (Figure 3H). These results demonstrate syndecan-1 restrains apoptosis in both human and murine airway epithelium after influenza infection. Furthermore, our data indicate that syndecan-1 must be membrane bound to modulate cellular apoptosis during influenza infection.

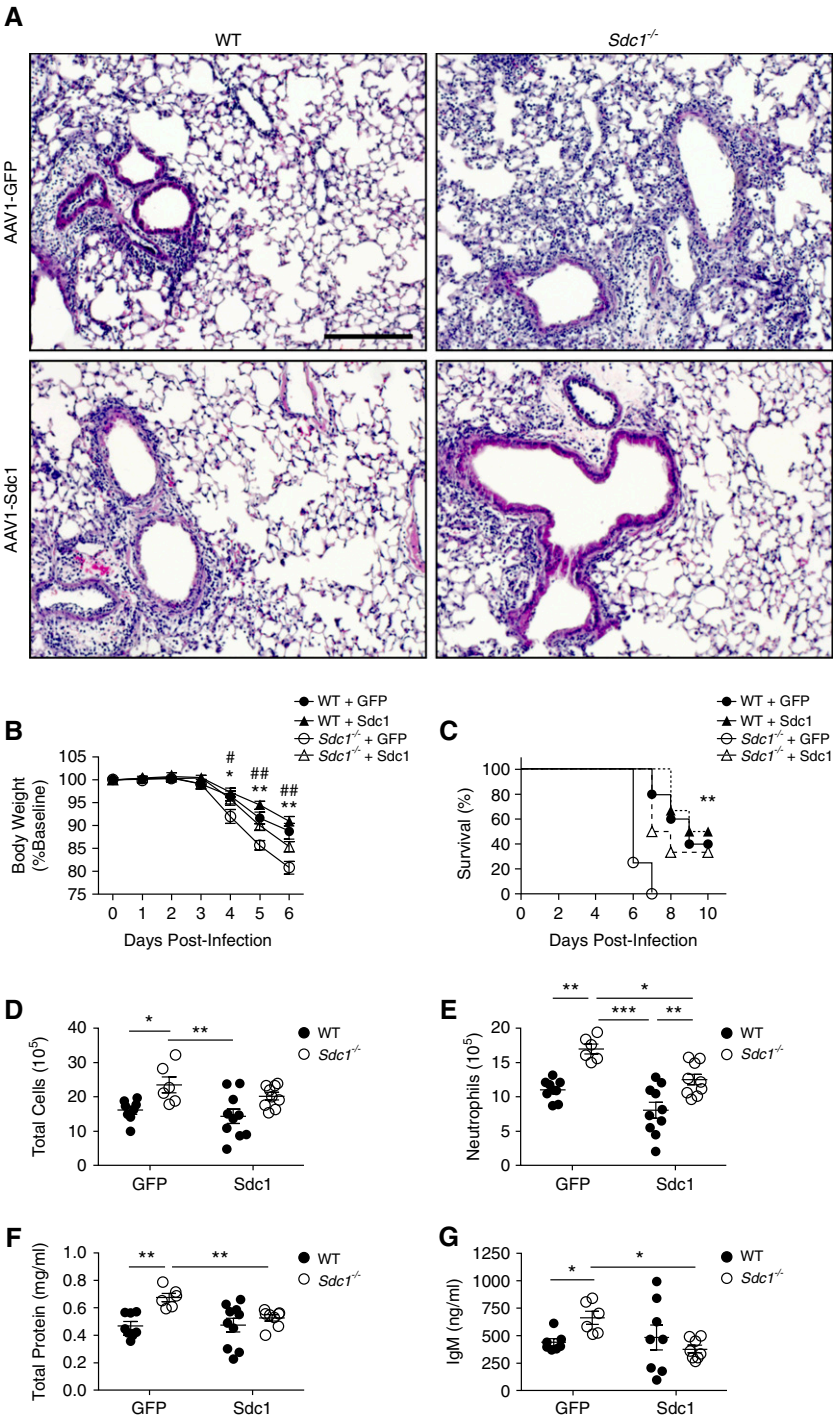


**Figure 2.** Lung epithelial expression of syndecan-1 reduces lung inflammation and injury during influenza infection. Adeno-associated virus serotype 1 (AAV1) vectors were intranasally instilled into wild-type (WT) and *Sdc1*$^{-/-}$ mice to transduce either green fluorescent protein (GFP) or syndecan-1 in the lung epithelium, and mice were subsequently infected intranasally with 500 plaque-forming units of PR8 virus. (*A*) Representative hematoxylin and eosin images of virus-infected lungs from WT and *Sdc1*$^{-/-}$ mice transduced with either GFP or syndecan-1 show diminished alveolitis in *Sdc1*$^{-/-}$ mice transduced with AAV-Sdc1 compared with AAV-GFP. *Scale bar*, 200 μm. (*B*) Body weight loss (n = 6–10; $^{*}P < 0.01$; $^{**}P < 0.001$ WT + GFP vs. *Sdc1*$^{-/-}$ + GFP; $^{\#}P < 0.05$; $^{\#\#}P < 0.01$ *Sdc1*$^{-/-}$ + GFP vs. *Sdc1*$^{-/-}$ + Sdc1) and (*C*) survival (n = 4–6; $^{*}P < 0.001$) after influenza infection. (*D–G*) Analysis of bronchoalveolar lavage fluid from mice (n = 6–10) during influenza infection for (*D*) total cell count, (*E*) neutrophils, (*F*) total protein, and (*G*) IgM levels. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. All data are shown as mean ± SEM.

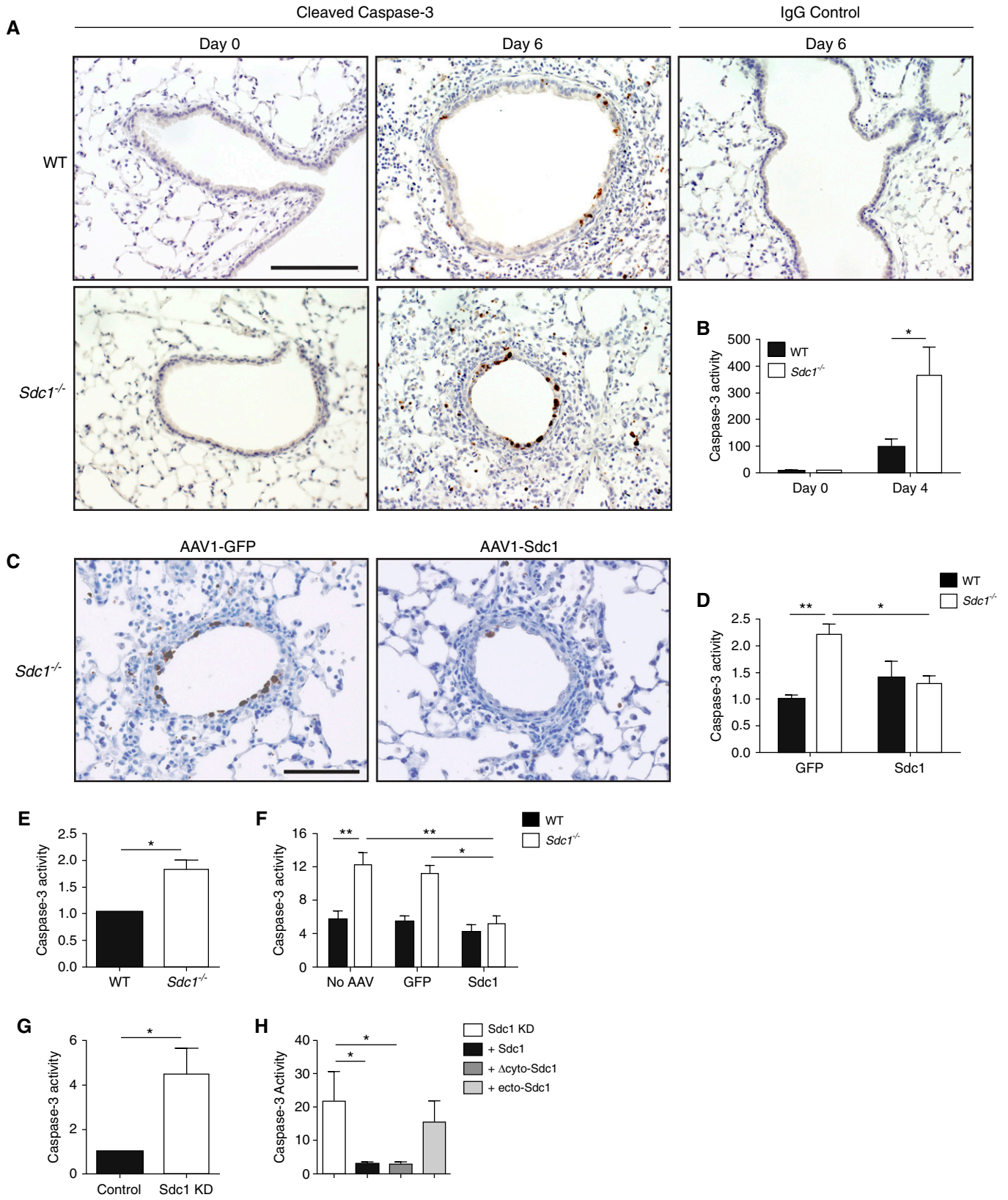


**Figure 3.** Syndecan-1 expression by the lung epithelium attenuates apoptosis during influenza infection. (*A*) Cleaved caspase-3 immunohistochemistry (*brown*; *scale bar*, 200 μm) and (*B*) lung homogenate caspase-3 activity (normalized to Day 0; n = 5, *$P < 0.05$) in influenza-infected lungs from wild-type

### Syndecan-1 Controls Airway Epithelial Apoptosis to Moderate Lung Inflammation after Influenza Infection

To determine if augmented apoptotic activity in the lungs of infected *Sdc1*$^{-/-}$ mice caused more lung injury, we treated mice with zVAD, a pan-caspase inhibitor. This treatment attenuated the exuberant apoptosis and significantly improved lung injury, weight loss, and survival in infected *Sdc1*$^{-/-}$ mice, bringing the levels closer to those seen in infected WT mice (*see* Figure E4 in the online supplement; Figures 4A–4C). Moreover, zVAD reduced lung inflammation in *Sdc1*$^{-/-}$ mice with relatively little impact on WT mice (Figures 4D and 4E; *see* Figure E5 in the online supplement). Altogether, our data demonstrate reduction of the excessive lung epithelial apoptosis in *Sdc1*$^{-/-}$ mice after influenza infection attenuates lung injury and inflammation.

### Syndecan-1 Potentiates c-Met Signaling in the Bronchial Epithelium during Influenza Infection

c-Met has been found to attenuate lung injury and apoptosis in bronchial epithelial cells (29, 30), so we evaluated if syndecan-1 facilitated c-Met signaling during influenza infection. Influenza-infected cells had more c-Met activation in murine and human lung bronchial epithelial cells that expressed syndecan-1 (Figure 5A). c-Met mediates its pro-survival and anti-apoptotic effects via AKT (31), and AKT activation was also dampened in influenza-infected murine and human bronchial epithelial cultures lacking syndecan-1 expression (Figure 5B). Immunoblotting lung homogenates from influenza-infected mice also revealed differential activation of c-Met (Figure 5C) and AKT (Figure 5D) with more activation in the lungs of WT mice compared with *Sdc1*$^{-/-}$ mice after influenza infection. Furthermore, immunohistochemistry for activated AKT revealed more intense staining within the airway epithelium of WT mice compared with *Sdc1*$^{-/-}$ mice (Figure 5E). These data demonstrate that syndecan-1 facilitates activation of c-Met and its downstream effector, AKT, in the lung epithelium after infection.

To determine if manipulation of this signaling pathway could affect outcomes after influenza infection, we delivered an activated (myristolated) AKT via AAV vectors to the lung epithelium (32). We found that the augmented apoptosis in *Sdc1*$^{-/-}$ cultures during influenza infection could be suppressed to comparable levels with WT conditions by the expression of activated AKT (*see* Figure E6A in the online supplement). Moreover, activated AKT expressed in the lung epithelium *in vivo* improved outcomes after influenza infection (*see* Figures E6B and E6C). These data provide direct evidence that suppression of apoptosis specifically within the epithelial compartment attenuates the severity of disease in *Sdc1*$^{-/-}$ mice during influenza infection and highlight the importance of syndecan-1 in regulating lung bronchial epithelial apoptosis via the c-Met/AKT axis during the onset of influenza infection.

### HGF-stimulated Signaling Is Dampened In Syndecan-1–Deficient Bronchial Epithelial Cells

HGF (scatter factor) is the only known ligand for c-Met (29). Therefore, HGF stimulation of cells specifically activates c-Met, which allowed us to interrogate syndecan-1 regulation of this pathway. Murine and human bronchial epithelial cells treated with HGF revealed greater activation of c-Met in syndecan-1–expressing cells compared with cells deficient in syndecan-1 expression (Figure 6A; *see* Figure E7 in the online supplement). Furthermore, differential AKT activation was also demonstrated (Figure 6B; *see* Figure E7) and mirrored the c-Met results. Together, these data further demonstrate that c-Met signaling is enhanced by syndecan-1 when stimulated by its cognate ligand, HGF.

We predicted other cellular functions of c-Met activation would be diminished in conditions that lack syndecan-1 expression. The mitogenic effects of c-Met signaling were blunted in the absence of syndecan-1 (Figures 6C and 6D). Furthermore, the syndecan-1 ectodomain must be tethered to the cell membrane to augment HGF-mediated cell proliferation (Figure 6D). In contrast, a shed ectodomain had no effect on c-Met function. These findings are congruous with our previous data that demonstrated that syndecan-1 regulates c-Met function only when associated with the cell membrane.

Our findings suggest beneficial effects from c-Met-AKT activation during influenza infection. When BEAS-2b cells (that express syndecan-1) were infected with PR8, apoptosis was attenuated with HGF and enhanced with c-Met inhibition (Figure 6E). In contrast, apoptosis was minimally affected by HGF or c-Met inhibitor in BEAS-2b cells that lacked syndecan-1 expression. A similar trend was found when WT and *Sdc1*$^{-/-}$ ALI cultures were treated with HGF and a c-MET inhibitor (*see* Figure E8 in the online supplement). These data indicate that c-Met requires the presence of syndecan-1 to be fully functional in mediating its pro-survival effects.

### Cytoprotective Effects from c-Met Signaling Is Blunted in *Sdc1*$^{-/-}$ Conditions *In Vivo* during Influenza Infection

Next, we modulated c-Met activity *in vivo* to test the ability of syndecan-1 to regulate its protective effects during influenza infection.

**Figure 3.** (Continued). (WT) and *Sdc1*$^{-/-}$ mice (250 plaque-forming units). (*C*) Cleaved caspase-3 immunohistochemistry (*brown*; *scale bar*, 200 μm) and (*D*) lung homogenate caspase-3 activity (normalized to the WT GFP condition) in influenza-infected lungs (500 plaque-forming units) from WT and *Sdc1*$^{-/-}$ mice transduced with either AAV1-GFP (1.0 ± 0.07 vs. 2.21 ± 0.19, respectively) or AAV1-Sdc1 (1.39 ± 0.31 vs. 1.29 ± 0.14, respectively; n = 6–10, $^{*}P < 0.05$; $^{**}P < 0.01$). (*E–H*) Caspase-3 activity was measured in lysates of (*E*) WT and *Sdc1*$^{-/-}$ air–liquid interface (ALI) cultures infected with PR8 (n = 4, $^{*}P < 0.05$); (*F*) WT and *Sdc1*$^{-/-}$ ALI cultures transduced with either AAV carrying GFP (control) or murine syndecan-1 followed by PR8 infection. No AAV: 5.66 ± 1.0 versus 12.23 ± 1.46; GFP: 5.39 ± 0.72 versus 11.2 ± 0.93; Sdc1: 4.16 ± 0.89 versus 5.21 ± 0.91 (n = 3, $^{*}P < 0.005$; $^{**}P < 0.001$); (*G*) Infected BEAS-2b cells that had syndecan-1 knocked down by small hairpin RNA expression (Sdc1 KD) versus its control (expressing a scrambled shRNA; n = 8, $^{*}P < 0.05$). (*H*) Infected Sdc1 KD cells stably express various mutants of mouse syndecan-1. (n = 3, $^{*}P < 0.05$). Sdc1 KD (21.78 ± 8.77); +Sdc1 (2.86 ± 0.61, full-length syndecan-1); +Δctyo-Sdc1 (2.83 ± 0.63, truncated syndecan-1 lacking the cytoplasmic domain but still tethered to the cell membrane); +ecto-Sdc1 (15.50 ± 6.35, syndecan-1 ectodomain that is secreted from cell simulating shed syndecan-1). All data are shown as mean ± SEM. AAV = adeno-associated virus; GFP = green fluorescent protein; KD = knockdown.

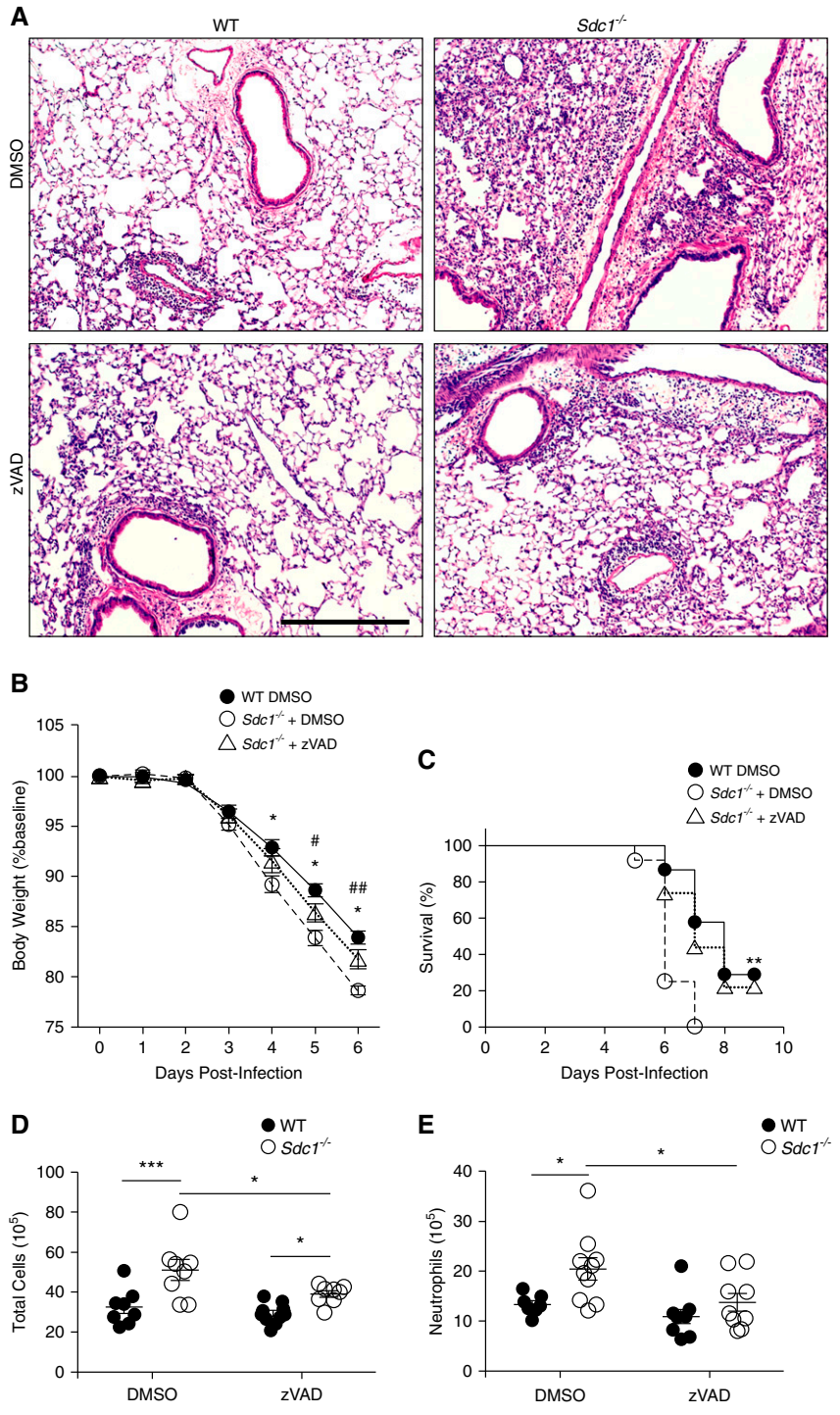


**Figure 4.** Suppressing apoptosis reduces lung injury and increases survival in *Sdc1*$^{-/-}$ mice during influenza infection. Wild-type (WT) and *Sdc1*$^{-/-}$ mice treated with zVAD during influenza infection (500 plaque-forming units). (*A*) Representative hematoxylin and eosin images of influenza infected lung tissue 6 days postinfection. *Scale bar*, 200 μm. (*B*) Weight loss in WT and *Sdc1*$^{-/-}$ mice after influenza infection (n = 8–10). $^{*}P < 0.005$ for WT + dimethyl sulfoxide (DMSO) versus *Sdc1*$^{-/-}$ + DMSO; $^{\#}P < 0.05$; $^{\#\#}P < 0.01$ for *Sdc1*$^{-/-}$ + DMSO versus *Sdc1*$^{-/-}$ + zVAD. (*C*) Survival in WT and *Sdc1*$^{-/-}$ mice after influenza infection (n = 15). $^{**}P < 0.01$. WT + zVAD conditions had no significant difference compared with WT + DMSO conditions and was removed in *B* and *C* for improved visualization of the graphs. (*D*) Total cell count and (*E*) neutrophils in the bronchoalveolar lavage 6 days postinfection (n = 8–10). $^{*}P < 0.05$; $^{***}P < 0.001$. All data are shown as mean ± SEM.

c-Met inhibition in mice during influenza infection augmented lung injury and inflammation in WT conditions, but this had minimal effect on *Sdc1*$^{-/-}$ conditions (Figures 7A–7C). Conversely, when HGF was given to influenza-infected mice, WT mice treated with HGF had improvement in lung inflammation, whereas *Sdc1*$^{-/-}$ mice had no effect on the total BAL cell count and only a modest decline in neutrophils (Figures 7D–7F). Moreover, HGF improved survival and attenuated weight loss in WT mice after influenza infection (*see* Figure E9 in the online supplement). Furthermore, we found c-Met inhibition augmented and HGF treatment attenuated lung apoptosis during influenza infection *in vivo* (see Figure E10 in the online supplement). Because c-Met is primarily expressed by the epithelium, these results provide additional proof that modulating lung epithelial apoptosis can regulate the inflammatory response during influenza infection. These data demonstrate that syndecan-1 modulates c-Met signaling to provide cytoprotective signals to the lung epithelium and limits inflammation after influenza infection.

## Discussion

Although the mechanisms that lead to lung injury and inflammation after influenza infection are not fully understood, much of the mortality that occurs during the yearly flu epidemic, especially among otherwise healthy people, is because of an excessive host response. Our study indicates that syndecan-1 is an important host survival factor that moderates inflammation during influenza infection. Previous studies have found that lung inflammation is regulated by the shed ectodomain (12–16). Our results show that syndecan-1, as a transmembrane receptor on the cell surface, also has an important role in regulating lung injury and inflammation. Moreover, our work unravels a novel mechanism whereby syndecan-1 crosstalks with c-Met to potentiate its cytoprotective effects and moderates inflammation during influenza infection by controlling epithelial apoptosis.

Inflammation is clearly necessary to fight influenza infection, but an exaggerated response characterized by exuberant and dysregulated cytokines results in widespread

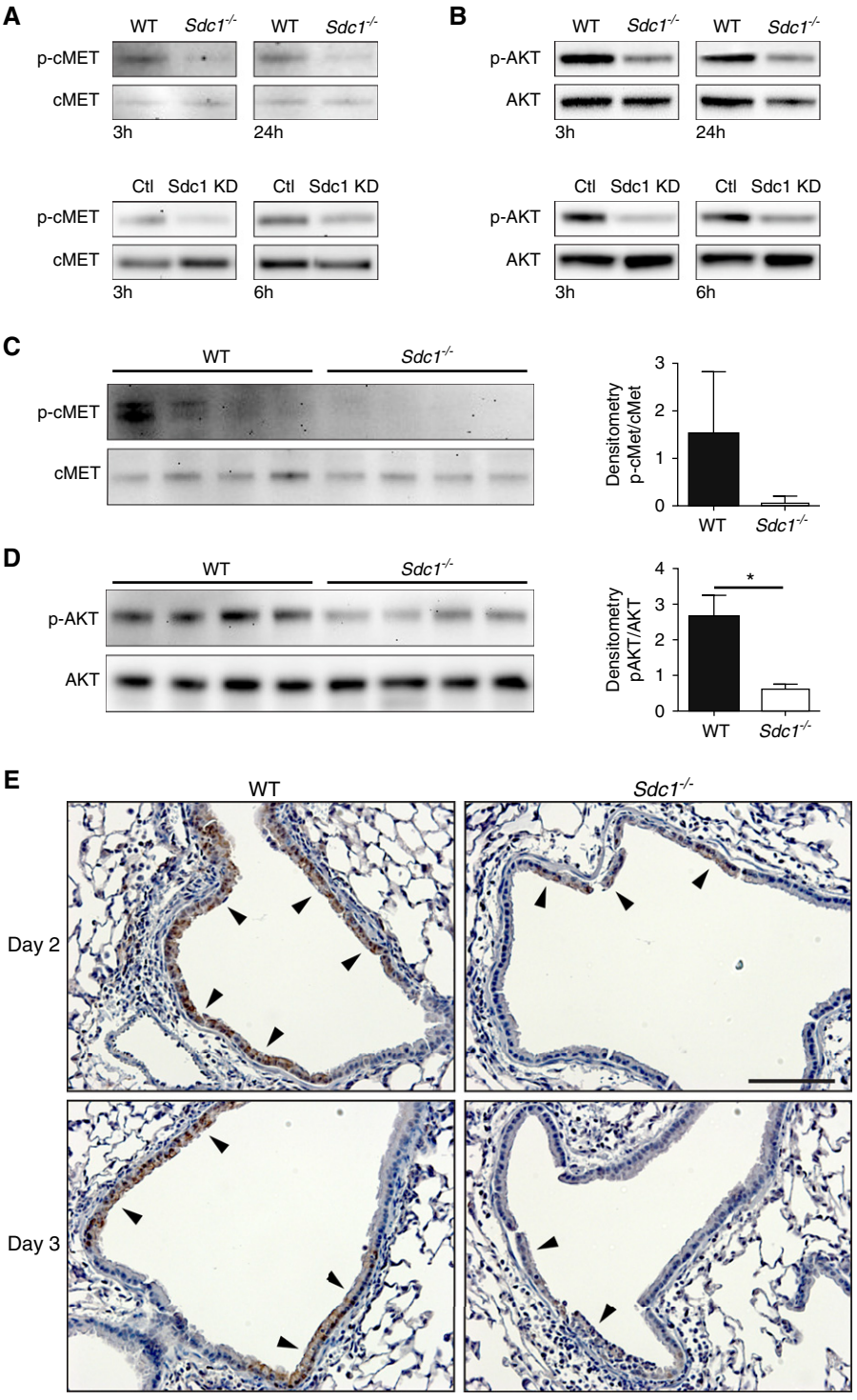


**Figure 5.** Syndecan-1 facilitates activation of c-Met and its downstream effector, AKT, after influenza infection. Western blot analysis for total and activated (*A*) c-Met and (*B*) AKT in PR8 infected wild-type (WT) and *Sdc1*$^{-/-}$ air–liquid interface cultures and BEAS-2b cells that had syndecan-1 knocked down (Sdc1 KD) and its control. Representative results of three independent experiments are shown. Immunoblot for total and activated (*C*) c-Met and (*D*) AKT in whole lung homogenates of influenza-infected WT and *Sdc1*$^{-/-}$ mice (500 plaque-forming units; Day 3 post-infection; n = 4). Densitometry results: c-Met (1.52 ± 1.31 vs. 0.06 ± 0.15); AKT (2.64 ± 0.62 vs. 0.60 ± 0.15). *$P < 0.05$. (*E*) Immunohistochemistry for $^{pS473}$AKT (*brown*; *arrowheads*) in influenza-infected mice reveal less intense expression in the airway epithelium of *Sdc1*$^{-/-}$ mice. *Scale bar*, 100 μm. Ctl = control.

lung injury that can cause the host to succumb. Our data indicate syndecan-1 functions as an important cytoprotective factor in the lung epithelium during influenza infection to limit inflammation and minimize lung injury. Because our studies evaluated early time points, these findings implicate syndecan-1 in governing innate immunity during influenza infection, which is consistent with the fact that clinical outcomes are determined by mucosal innate immune responses (33). Previous studies have found that alveolar cell apoptosis is an important process for the adaptive immune response to this viral pathogen (26, 34). Our data suggest that syndecan-1 also controls the alveolar apoptotic response during influenza infection, but that the role of syndecan-1 in regulating the adaptive immune response has yet to be delineated.

The lung epithelium is not solely responsible for regulating the inflammatory response, but because of its location, it functions as a first responder to quickly sense pathogenic insults and coordinate inflammatory cell influx into the airspaces (14). An interesting aspect of these data is that controlling the initial response in the airway epithelial compartment can limit the overall lung injury. Influenza is primarily tropic to the airway epithelium, and an ensuing alveolitis can occur even in the absence of viral dissemination (28, 35). ARDS can happen with extra-pulmonary diseases (36), so the fact that airway pathology causes alveolitis is not overly surprising. Airway specific inflammation has been experimentally shown to cause broader lung injury (37, 38). The proximity of the terminal bronchioles to the alveolar spaces could allow for paracrine signals between these compartments that mediate the lung injury response after influenza. Recent evidence has also implicated the endothelium in propagating the cytokine storm during influenza (39), and the bronchial epithelium could potentially crosstalk with the endothelial compartment to mediate the recruitment of inflammatory cells.

IL-1β and IL-18 levels were identical between WT and *Sdc1*$^{-/-}$ mice, indicating that syndecan-1 regulates lung injury independent of inflammasome activation. Congruous to this finding, our data demonstrated that apoptosis of the bronchial epithelial cells regulates lung

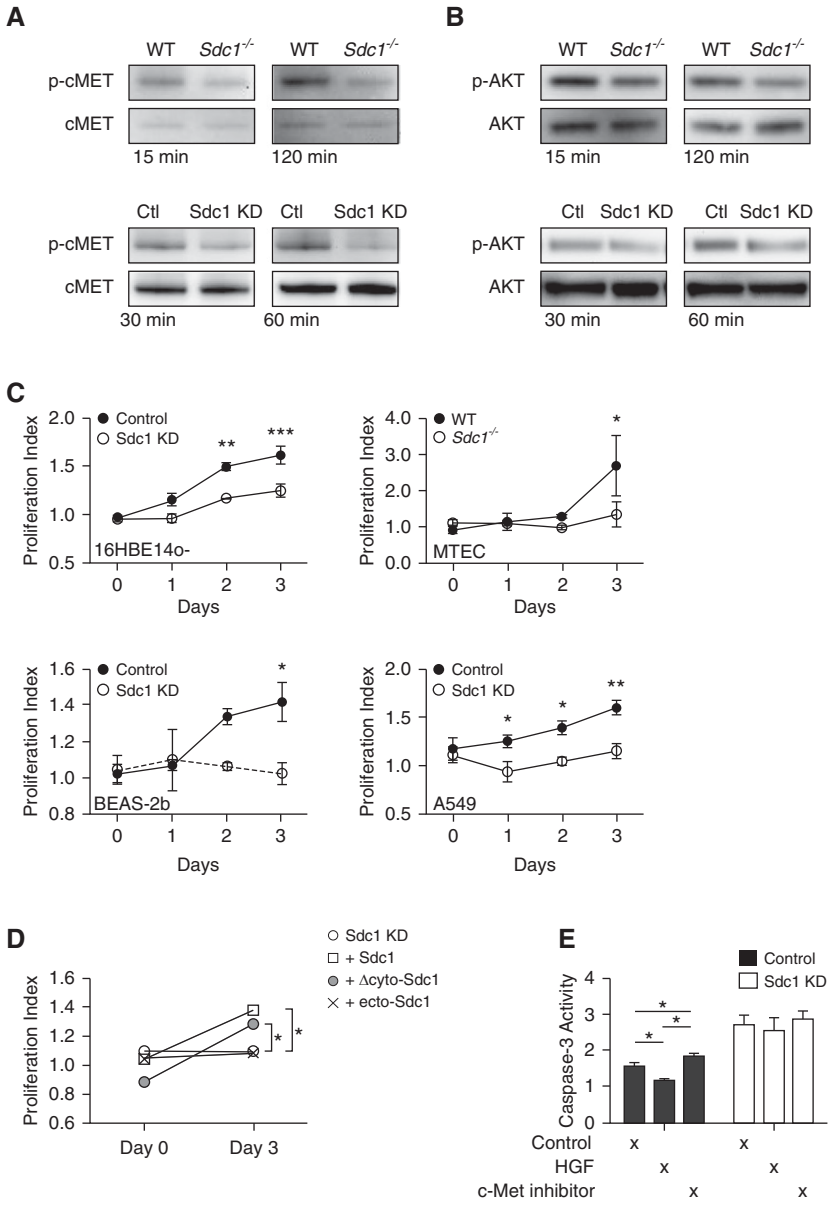


**Figure 6.** Syndecan-1 promotes c-Met signaling and downstream functions when stimulated with hepatocyte growth factor (HGF). Western blot analysis for total and activated (*A*) c-Met and (*B*) AKT in HGF stimulated wild-type (WT) and *Sdc1*$^{-/-}$ air–liquid interface cultures and BEAS-2b cells that had syndecan-1 knocked down (Sdc1 KD) and its control. Representative results of three independent experiments are shown. (*C*) Proliferation of control and Sdc1 KD cells were measured after HGF stimulation using CyQuant (Life Technologies, Carlsbad, CA). Various cell lines are respectively labeled. The proliferation index is the number of cells with HGF stimulation compared with cells without HGF stimulation per cell line; n = 3. *$P < 0.05$; **$P < 0.005$; ***$P < 0.001$. (*D*) Sdc1 KD cells that stably express various mutants of mouse syndecan-1 were stimulated with HGF, and the proliferation index was determined. n = 3, *$P < 0.05$. (*E*) Caspase-3 activity was measured in control and Sdc1 KD BEAS-2b cells infected with PR8 virus with or without treatment with HGF or the c-Met inhibitor, SGX-523 (n = 3). Control (PR8 only: 1.55 ± 0.071, HGF: 1.16 ± 0.03, c-Met inhibitor: 1.82 ± 0.05). Sdc1 KD (PR8 only: 2.69 ± 0.16, HGF: 2.53 ± 0.21, c-Met inhibitor: 2.86 ± 0.12). All data are shown as mean ± SEM. Ctl = control; ecto-Sdc1 = syndecan-1 ectodomain that is secreted from cell simulating shed syndecan-1; MTEC = primary mouse tracheal epithelial cells; Sdc1 = full-length syndecan-1; Δctyo-Sdc1 = truncated syndecan-1 lacking the cytoplasmic domain but still tethered to the cell membrane.

inflammation, whereas the inflammasome response is moderated by hematopoietic cells during influenza infection (40). Epithelial apoptosis is predominantly a proviral event that augments lung injury (22–26), and programmed cell death has a prominent role in regulating lung inflammation and injury (41–43). Influenza mediates many of its cytotoxic effects through induction of apoptosis, and our data and studies by others demonstrate that blocking apoptosis during influenza infection significantly reduces lung injury and mortality (26, 44, 45). Simplistically, apoptotic death of the epithelium breaks down the nature barriers that prevent lung edema. However, apoptotic cells can also act as a stimulus to promote lung injury (41–43). Furthermore, impaired clearance of apoptotic cells can lead to autoimmune disease, augment septic shock and lung injury, and result in secondary necrosis, which is an additional pro-inflammatory signal (46–51). Factors released from the apoptotic cell can also modulate phagocytic cell migration and tissue repair (52–58). Thus, intrinsic mechanisms that function to mitigate bronchial epithelial apoptosis in response to infection would predictably reduce tissue injury and inflammation, thereby promoting recovery and survival.

Apoptosis is also necessary for viral proliferation (24, 25, 59). However, the augmented apoptosis in *Sdc1*$^{-/-}$ animals had no effect on viral titer compared with WT conditions. One possible explanation is that the caspase activity in WT conditions exceeds a maximal threshold necessary for viral proliferation; therefore, augmented apoptosis in *Sdc1*$^{-/-}$ conditions had no further effect. In addition, because of the complexities of the entire animal, other factors not modeled in culture may affect viral proliferation *in vivo*. Our results are not unique because others have also shown that augmented apoptosis has no effect on viral titers *in vivo* in the acute inflammatory phase of infection (26).

Unlike previous studies that found a significant role for the shed syndecan-1 ectodomain in mediating lung inflammation (12–16), our data delineated a new mechanism by which syndecan-1 functions on the cell surface as a cytoprotective signaling modulator to control lung inflammation after injury. We found that

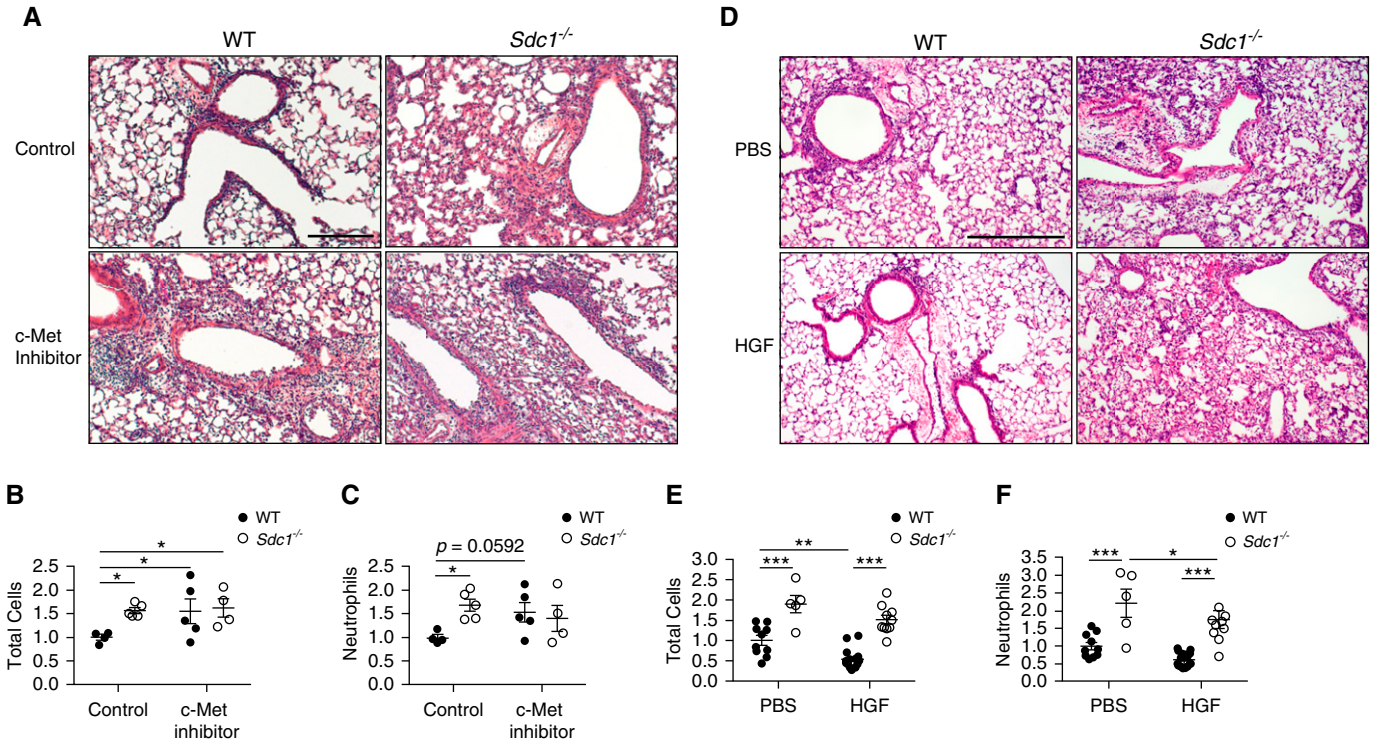


**Figure 7.** c-Met stimulation is protective during influenza infection *in vivo* and requires syndecan-1 for optimal signaling. (*A–C*) Wild-type (WT) and *Sdc1*$^{-/-}$ mice were infected with PR8 and concurrently treated with c-Met inhibitor (SGX-523) or control. (*A*) Representative hematoxylin and eosin–stained lung sections from Day 6 postinfection demonstrate worsened lung injury in WT conditions treated with c-Met inhibitor compared with phosphate-buffered saline (PBS). c-Met inhibition had minimal effect in *Sdc1*$^{-/-}$ mice. *Scale bar*, 200 μm. (*B*) Total cellular and (*C*) neutrophil infiltrates into the airspaces 6 days postinfection (n = 4–5; normalized to WT control conditions). *$P < 0.05$. All data are shown as mean ± SEM. (*D–F*) WT and *Sdc1*$^{-/-}$ mice were infected with PR8 and concurrently treated with hepatocyte growth factor (HGF) or PBS control. (*D*) Representative hematoxylin and eosin–stained lung sections from Day 6 postinfection demonstrate improvement in lung injury in WT conditions treated with HGF compared with PBS. HGF had minimal effect on *Sdc1*$^{-/-}$ mice. *Scale bar,* 200 μm. (*E*) Total cellular and (*F*) neutrophil infiltrates into the airspaces 6 days postinfection (n = 5–16; normalized to WT PBS conditions). All data are shown as mean ± SEM. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

syndecan-1 regulates the apoptotic response in cells by potentiating c-Met signaling. As the receptor for HGF, c-Met activates an important pathway that limits lung injury (29). The bronchial epithelium is the predominant location of c-Met expression in the lungs and also primarily produces HGF in the early phase after bacterial injury (30, 60, 61). Furthermore, the HGF/c-Met signaling axis is a pro-survival signal in the bronchial epithelium that promotes proliferation and suppresses apoptosis after injury (30, 62).

Mice treated with a c-Met inhibitor had worsening lung injury after influenza infection. c-Met inhibitors are currently being studied in clinical trials as treatments for cancer (63), and our data would suggest that patients might become susceptible to more severe lung injury after influenza infection. HGF treatment significantly improved total cell counts in WT mice, but neutrophils, although trending to improvement, were not statistically significant. Several possibilities exist that limit the efficacy of intranasally delivered HGF. The dose of HGF given may not sufficiently saturate c-Met receptors. Moreover, receptor–ligand interactions require proper compartmentalization that may not be effectively replicated with exogenously administered HGF (31). HGF could also non-specifically bind cell debris, and expose the extracellular matrix or inflammatory cells, thus reducing the effective dose. As we continue to further our understanding of how syndecan-1 regulates c-Met activation, this research may lead to alternative methods (e.g., pharmacologic) that can circumvent these therapeutic limitations while manipulating this pathway to dampen lung injury after influenza infection.

*Sdc1*$^{-/-}$ mice are fertile and develop without any abnormalities in contrast to *Met*$^{-/-}$ mice (64, 65). This divergence in phenotypes most likely is because syndecan-1 has the capability to tune c-Met activity and is not absolutely necessary for its function. Moreover, the functional role of syndecan-1 is only elicited in perturbed states, which may suggest syndecan-1 modulation of c-Met is only necessary in non-homeostatic, non-developmental situations. Analogously, syndecan-1 interacts with and modulates the insulin growth factor-1 receptor, which when deficient, causes severe pulmonary developmental issues and neonatal lethality (66–68). In contrast, hypomorphic expression of insulin growth factor-1 receptor had no developmental abnormalities (66). In a similar fashion, lower levels of c-Met activation in the absences of syndecan-1 may be sufficient for normal

development. What is the evolutionary advantage for syndecan-1 potentiation of c-Met signaling? One possibility is that the lung epithelium gains a migratory phenotype as part of the reparative programming when syndecan-1 is shed from the cell surface (7–9). After the epithelial surface is reestablished, cells must re-differentiate and regain the expression of syndecan-1. Those that do not will be more susceptible to death, which has a teleological advantage to prevent pathological outcomes that may benefit from less differentiated, pro-migratory cells (e.g., cancer).

We have identified syndecan-1 as an important pro-survival factor in the lung epithelium that limits acute lung injury after influenza infection. We provide evidence for a novel mechanism in which transmembrane syndecan-1 potentiates c-Met activity, thereby providing anti-apoptotic signals that limit epithelial cell death after influenza infection. Because apoptosis is no longer considered a quiescent process, syndecan-1 may be a factor that controls lung inflammation by regulating the apoptotic response in the lung epithelium. Identifying methods to control this pathway or limit the shedding of syndecan-1 after infection may lead to new therapeutics to limit lung injury. ■

**Author disclosures** are available with the text of this article at www.atsjournals.org.

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