A Novel Role for PDZ-Binding Motif of Influenza A Virus Nonstructural Protein 1 in Regulation of Host Susceptibility to Postinfluenza Bacterial Superinfections

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Abstract

Influenza A viruses (IAVs) have multiple mechanisms for altering the host immune response to aid in virus survival and propagation. While both type I and II interferons (IFNs) have been associated with increased bacterial superinfection (BSI) susceptibility, we found that in some cases type I IFNs can be beneficial for BSI outcome. Specifically, we have shown that antagonism of the type I IFN response during infection by some IAVs can lead to the development of deadly BSI. The nonstructural protein 1 (NS1) from IAV is well known for manipulating host type I IFN responses, but the viral proteins mediating BSI severity remain unknown. In this study, we demonstrate that the PDZ-binding motif (PDZ-bm) of the NS1 C-terminal region from mouse-adapted A/Puerto Rico/8/34-H1N1 (PR8) IAV dictates BSI susceptibility through regulation of IFN- α/β production. Deletion of the NS1 PDZ-bm from PR8 IAV (PR8-TRUNC) resulted in 100% survival and decreased bacterial burden in superinfected mice compared with 0% survival in mice superinfected after PR8 infection. This reduction in BSI susceptibility after infection with PR8-TRUNC was due to the presence of IFN- β , as protection from BSI was lost in *Ifn- β ^{-/-}* mice, resembling BSI during PR8 infection. PDZ-bm in PR8-infected mice inhibited the production of IFN- β posttranscriptionally, and both delayed and reduced expression of the tunable interferon-stimulated genes. Finally, a similar lack of BSI susceptibility, due to the presence of IFN- β on day 7 post-IAV infection, was also observed after infection of mice with A/TX98-H3N2 virus that naturally lacks a PDZ-bm in NS1, indicating that this mechanism of BSI regulation by NS1 PDZ-bm may not be restricted to PR8 IAV. These results demonstrate that the NS1 C-terminal PDZ-bm, like the one present in PR8 IAV, is involved in controlling susceptibility to BSI through the regulation of IFN- β , providing new mechanisms for NS1-mediated manipulation of host immunity and BSI severity.

Keywords: influenza, bacterial superinfection, interferons, NS1, antiviral immunity

Introduction

INFLUENZA VIRUS INFECTIONS are a common cause of illness, and they can lead to the development of deadly secondary bacterial infections. The historical record of these bacterial superinfections (BSIs) dates back to the 1700s (1) with significant impacts noted during influenza epidemics and pandemics (39,40,60). However, laboratory studies into mechanisms of BSI severity have been largely conducted using the mouse-adapted A/Puerto Rico/8/34-H1N1 (PR8) in-

fluenza virus model (52–54,57,59,61,64). It is well accepted in the field of postinfluenza BSIs that dynamic host:virus interactions contribute to an aberrant inflammatory response around day 7 postinfluenza infection (30,42,54). This host response is associated with impaired bacterial clearance, allowing for bacterial dissemination and tissue damage.

While both innate and adaptive immune mechanisms can contribute to increased BSI susceptibility, central to these aberrant inflammatory responses seems to be the activity of interferons (IFNs) (37,54,57,59). In this regard, both type I

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and type II IFNs have been implicated in increased susceptibility of mice to BSI, through mechanisms leading to reduced production of antimicrobial peptides (52), inadequate bacterial clearance by macrophages (5,42) and neutrophils (59,62), and inhibition of T and NK cell responses (30,37).

More recently, utilizing the PR8 influenza virus, *Staphylococcus aureus* BSI model, our group reported that type I IFN signaling can aid in bacterial clearance at earlier time points after PR8 influenza virus infection (59), adding to the role and the divergence of specific type I IFN cytokines in shaping host susceptibility to BSI. Specifically, we found that the duration of IFN- β production by the host, which in the case of PR8 influenza virus infection is limited to the first 3 days postinfection (pi), and contributes to BSI resolution. At the time of increased BSI susceptibility (~day 7 post-PR8 influenza infection), IFN- β production was minimal and largely replaced by IFN- α . Because of the presence of IFN- β in lungs, mice that were challenged with *S. aureus* on days 2–3 post-PR8 were able to clear a BSI and recover from infection. However, mice challenged at day 7 post-PR8 exhibited impaired bacterial clearance and survival after BSI, which was due to the absence of IFN- β .

While it is well accepted that BSIs exacerbate severity of disease during influenza epidemics and pandemics, it is also known that not all strains of influenza virus uniformly increase host susceptibility to BSIs. Previously, we found that infection of mice with the A/swine/Texas/4199-2/98-H3N2 (TX98) influenza virus did not result in increased severity of BSI (64). This lack of increased BSI susceptibility in mice infected with TX98 virus occurred regardless of whether the BSI was caused by *S. aureus*, *Streptococcus pneumoniae*, or *Streptococcus pyogenes*. Based on this observation, we sought to determine the viral genes and host responses that contribute to these observed differences in BSI susceptibility. Toward this goal, we have used reverse genetics to create reassortant viruses that express combinations of PR8 and TX98 genes and we recently identified the hemagglutinin (HA; 27) and the nonstructural (NS) gene expressed by influenza A virus (IAV) as individual regulators of lethal BSI severity (27). In this current study, we focused on understanding how the NS gene contributes to BSI severity.

The NS gene is alternatively spliced to yield different gene products, including NS protein 1 (NS1) and NS2. NS2 is a nuclear export protein that aids in the production of new IAV particles. On the other hand, NS1 is pivotal for virus replication and it has been shown to antagonize type I IFN signaling (12,16,19). Type I IFNs appear to play an important role in the regulation of host susceptibility to post-influenza BSIs (37,54,57–59,66). The majority of studies consider type I IFN signaling around day 7 postinfluenza as detrimental to the outcome of BSI. However, little is known about the contribution of individual type I IFNs to this susceptibility. In this regard, we found that duration of IFN- β production by the host in response to IAV dictates the severity of BSI susceptibility (59). Despite this clear connection between aberrant type I IFN signaling and increased BSI susceptibility, the contribution of IAV NS1 protein toward BSI severity has not been examined.

To address this obvious gap in our understanding of influenza contributions toward BSI severity, in this study, we

set out to test our hypothesis that the NS1 protein of PR8 virus contributes to BSI severity by interfering with IFN- β production and type I IFN receptor signaling. Building upon our previous observation that the PR8 NS gene increased BSI severity of TX98[PR8NS]-infected mice (27), we began by investigating whether obvious structural differences existed between the NS gene products of PR8 and TX98 viruses. Through alignment of the NS1 proteins expressed by PR8 and TX98 we identified that the PDZ-binding motif (PDZ-bm) present at the C terminus of the PR8 NS1 protein was absent from TX98. Truncation of the PR8 NS1 protein from 230 to 219 amino acids, eliminating the PDZ-bm, allowed for IFN- β expression to persist until day 7 after infection, similar to that found during TX98 infection. This persistence of IFN- β expression increased survival after BSI.

Our evaluation of host immune responses after influenza virus infection show that IFN- β expression establishes an immune environment that helps with clearance of bacteria from the lungs of infected animals. Moreover, our results demonstrate that regulation of IFN- β during PR8 influenza infection can be virally controlled based on the presence of the PDZ-bm in the NS1 protein. Thus, we provide a new mechanism for NS1 antagonism of IFNs and the corresponding effects on host immunity and BSI outcome.

Materials and Methods

Reverse genetics, chimeric viruses

We used site-directed mutagenesis (QuickChange Kit; Stratagene, La Jolla, CA), to insert a stop codon at position 220 of the PR8-WT NS gene product, replacing the arginine residue that was naturally present. A PR8 virus expressing truncated PR8 NS1 protein, referred to as PR8-TRUNC was created using the eight-plasmid reverse genetics system as described (22,23,27). Influenza virus NS1 domain mutant (NS1dm) variants were created by reverse genetics, using synthetic DNA generated by GeneWiz (South Plainfield, NJ), with *BsmBI* sites inserted to move the genetic material into pHW2000, as described (21).

Mice

Breeding pairs for C57BL/6J mice were obtained from Jackson Laboratories (Bar Harbor, ME) and were bred in-house and housed at the Animal Resources Center at Montana State University (MSU). MSU is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC; number 713). Adult (6–8-week-old), female Balb/c mice (Harlan Laboratories, Indianapolis, IN) were housed at the University of South Dakota. IFN- β null mice on a C57BL/6 background were a kind gift from Dr. Stefanie Vogel (University of Maryland in Baltimore). All mice used in this study were between 6 and 8 weeks of age. Mice were handled in accordance with the guidelines established by the Animal Care and Use Committee at the University of either MSU or South Dakota. All care and procedures were in accordance with the recommendations of the National Institutes of Health, the U.S. Department of Agriculture, and the Guide for the Care and Use of Laboratory Animals (8th ed.).

Infections and determination of bacterial and viral burdens

For nonsurgical intratracheal inoculations, mice were lightly anesthetized as described elsewhere (51,59) and instilled with 0.1 LD₅₀ of mouse-adapted IAV strain A/PR8/8/34 (PR8-WT; H1N1) or PR8-based NS1dm viruses in 100 μ L of normal saline.

The D39 strain of *S. pneumoniae* type 4 (ATCC 6304; American Type Culture Collection, Manassas, VA) and LAC strain of *S. aureus* (pulsed-field USA 300 MRSA) were grown in Todd Hewitt broth (Becton Dickinson and Co., Sparks, MD) supplemented with 0.5% yeast extract (THY; Fisher Scientific, Fair Lawn, NJ) at 37°C with 5% CO₂ until mid-log phase of growth. Stock cultures in THY with 10% glycerol (Sigma, St. Louis, MO) were snap frozen in liquid nitrogen and stored at -80°C until needed. Before use, stock cultures were washed twice at 16,000 $\times$ g with Dulbecco's Phosphate Buffered Saline (PBS) (D-PBS; Gibco, Grand Island, NY) and resuspended to the appropriate concentration. Bacterial colony-forming units (CFUs) were approximated using the optical density at 450 nm. CFUs were confirmed by plating serial dilutions on blood agar plates (Becton Dickinson Microbiology Systems, Cockeysville, MD) using the drop plate method, as previously described (41).

The *S. pyogenes* strain used in this study (MGAS315, serotype M3) was obtained from the American Type Culture Collection (Manassas, VA), and was prepared and inoculated based on an LD₅₀ of 10⁷ CFU in mice, as described previously (4,27,64).

To determine lung viral titers, lung homogenate samples were serially diluted and incubated with Madin-Darby canine kidney cells. Viral titers were either determined by plaque assay method (53,59,65) or were reported as a TCID₅₀, as previously described (3). CFU and PFU were determined from lung homogenate samples.

Preparation of bronchoalveolar lavage fluid samples and cytokine analyses

Mice were sacrificed by intraperitoneal (ip) administration of 90 mg/kg of body weight sodium pentobarbital followed by blood collection. Bronchoalveolar lavage was obtained by lavaging the lungs with 2 mL of 3 mM EDTA in PBS (20), and cellular composition was determined by hemocytometer cell counts and differential counts of cytopspins after staining with Quick-Diff solution (Siemens; Medical Solutions Diagnostics, Tarrytown, NY). Levels of IFN- α (31.3–2,000 pg/mL), IFN- β (1.9–500 pg/mL), IL-27 (16–2,000 pg/mL), IL-28 (31–2,000 pg/mL), and IFN γ (15.6–1,000 pg/mL) in cell-free bronchoalveolar lavage fluid (BALF) were determined using a sandwich Enzyme-Linked Immunosorbent Assay Kit with the BALF samples (Ready-SET-Go; eBioscience, San Diego, CA, or BioLegend). Results from analysis of BALF samples are reported as mean with % standard error of the mean from five mice (tested in triplicate).

PCR array

Mice were inoculated as described above with 0.1 LD₅₀ of either PR8-WT or PR8-TRUNC virus. At the depicted time points, mice were sacrificed by ip administration of 90 mg/kg of body weight sodium pentobarbital. Lungs were

immediately cut into pieces and placed into 3 mL of RNeasy lysis buffer. RNA was extracted following the RNeasy Kit protocol (Qiagen) and reverse transcribed into cDNA using the RT² First-Strand Kit (Qiagen). The cDNA was analyzed with a custom-designed RT² profiler PCR Array (Qiagen) with RT² SYBR Green ROX qPCR Mastermix (Qiagen) and run using an ABI 7900HT (384-well block) (Dr. Bridget Barker; Tgen, Flagstaff, AZ). Data (average result of two biological replicates run in triplicate) are represented as a heat map of fold change over control (naive mice).

Statistical analyses

Unless otherwise specified in the figure legends, reported results are mean $\pm$ standard deviations of five mice/group from a single experiment. Each experiment for which results are presented in the article was independently performed at least twice with similar results. The differences between treatment groups were analyzed using Student's *t* test or analysis of variance. For the differences in survival rates, Kaplan–Meier curves were plotted and analyzed using Prism software (version 4.0; GraphPad, La Jolla, CA). Statistical differences with *p*-values of <0.05 were considered significant.

Results

The NS gene expressed by IAV PR8 contributes to BSI severity

To evaluate the contribution of the NS gene expressed by IAV to BSI severity, we utilized reverse genetics to create reassortant viruses expressing either the PR8-derived NS gene on a TX98 virus backbone, or the TX98-derived NS gene on the PR8 virus backbone (27). Within each reassortant virus of the parental strains (PR8 or TX98), we only altered the NS gene segment to create the PR8[TX98_NS] and TX98[PR8_NS] reassortant viruses, with the remaining seven of eight gene segments in each strain being unaltered. Our results demonstrate that inclusion of the NS gene segment from PR8 virus in TX98 (TX98[PR8_NS]) was sufficient to reduce the survival of wild type (WT) mice after BSI from the 100% found in mice infected with TX98-WT virus to only 43% survival found with mice infected with TX98[PR8_NS] reassortant virus (Fig. 1A). Moreover, when lung bacterial load was evaluated at 24 h post-BSI, we observed a striking 8 log increase in bacterial load of mice infected with TX98[PR8_NS] compared with mice infected with parental TX98-WT virus (Fig. 1B), which correlated with the observed reduction in survival (Fig. 1A).

While infection of wild type (WT) mice with parental PR8-WT virus resulted in 0% survival after BSI, the reassortant PR8 virus expressing the TX98 NS gene PR8[TX98_NS] resulted in only a moderate improvement, to 20% survival (Fig. 1A). Although the TX98[PR8_NS] virus significantly impaired bacterial clearance after BSI (8 log increase over TX98-WT-infected mice), the PR8[TX98_NS] did not significantly improve bacterial clearance over PR8-WT-infected mice (Fig. 1B). This suggests that the NS genes of PR8 and TX98 influenza viruses have opposite effects on BSI susceptibility. Specifically, while the NS gene of PR8 influenza appears to worsen the outcome of subsequent BSI (Fig. 1; TX98[PR8_NS] vs. TX98-WT), the NS gene of TX98 virus slightly improves

FIG. 1. PR8 Influenza NS gene influences host susceptibility to superinfections. Balb/c mice were i.n. infected with 0.1 LD₅₀ of IAV virus on day 0 and challenged with 0.1 LD₅₀ of MGAS315 strain of *Streptococcus pyogenes* on day 7 (indicated by arrow in A). (A) Survival, (B) lung *S. pyogenes* titers at 24 h postchallenge, and (C) lung virus titers at day 7 after infection (at the time of BSI) are depicted as TCID₅₀/mL. (A) $^{**}p < 0.01$ by log-rank test. (B, C) $^{***}p < 0.001$; $^{*}p < 0.05$ by one-way ANOVA followed by Mann-Whitney test. Data for PR8-WT and TX98 viruses used in this figure have been previously published (27) and are reported here as controls. ANOVA, analysis of variance; IAV, influenza A virus; BSIs, bacterial superinfections; NS, nonstructural.

the BSI susceptibility (PR8[TX98_NS] vs. PR8-WT). Overall, our results suggest that the PR8 NS gene is a stronger modulator of BSI susceptibility than the NS gene of the TX98 virus.

Interestingly, lung virus titers at day 7 pi were similar in mice infected with PR8-WT, PR8[TX98_NS], and TX98[PR8_NS] viruses, whereas the TX98-WT lung titer was ~2 log lower, indicating that differences in the kinetics of virus clearance alone could not account for the differences in BSI severity (Fig. 1C). This suggests that the PR8 NS gene contributes to the death versus survival phenotype after BSI by regulation of the host's ability to clear bacteria. Additionally, because we observed 43% survival after BSI in mice infected with TX98(PR8_NS), but only 20% survival in mice infected with PR8(TX98_NS) (Fig. 1A), and because we found no significant differences in either viral and bacterial lung burden in mice infected with either of these two reassortant strains (Fig. 1B, C), this suggests that the changes in mortality track with the NS origin rather than level of either pathogen burden.

Presence of the PDZ-bm of PR8-IAV at the C terminus of its NS1 protein contributes to increased BSI severity

To determine which gene product of PR8 NS enhances BSI susceptibility, we compared the PR8 and TX98 NS gene segments based on defined protein domains (17,19). We found the majority of differences in the NS gene occurred in the NS1 protein (Supplementary Fig. S1). The NS1 protein of IAV is comprised of three domains, including the effector domain (ED), RNA-binding domain (RBD), and the C-terminal domain (CTD) (Fig. 2A and Supplementary Fig. S1). Alignment of the NS1 protein sequences of PR8-WT and TX98-WT viruses allowed us to identify an 11-amino-acid truncation in the C-terminal portion of the TX98 NS1 protein

that was not present in the PR8 NS1 protein (Fig. 2A and Supplementary Fig. S1). This 11-amino-acid sequence at the C-terminus of PR8's NS1 contains a PDZ-bm. Conversely, the 11-amino-acid truncation of TX98 resulted in a shorter TX98 NS1 protein that, at 219 amino acids long, lacked the PDZ-bm (Supplementary Fig. S1). In addition to this truncation at the C-terminus, the TX98 NS1 also contains several amino acid substitutions within all three NS1 protein domains when compared with PR8 NS1 (Supplementary Fig. S1). All three of the NS1 domains are known to antagonize type I IFN responses, and thus affect host immune responses against an influenza virus infection (17,19).

While our results with the TX98/PR8 reassortants allowed us to establish that the NS genes of PR8-WT and TX98-WT viruses vary in their contribution to BSI susceptibility, we found that other PR8 gene segments may also affect BSI susceptibility (27,64). Thus, to overcome the inherent bias of other viral genes confounding the effect NS1 may have on BSI susceptibility, we analyzed the contribution of each PR8 NS1 domain in isolation. To determine which protein domain of PR8's NS1 contributes to increased host BSI susceptibility mediated by PR8 virus, we created NS1dm viruses on the PR8 influenza backbone.

Specifically, in each NS1dm virus we replaced only one of the three PR8 NS1 protein domains (RBD, ED, or CTD) with the corresponding domain from the TX98 NS1 protein. As such, we created three PR8 NS1dm viruses: PR8[TX98_NS1-RBD], PR8[TX98_NS1-ED], and PR8[TX98_NS1-CTD] (Fig. 2A). Importantly, because NS1 CTD of TX98-WT was naturally truncated (no PDZ-bm) (Supplementary Fig. S1), upon creating the PR8[TX98_NS1-CTD] NS1dm reassortant virus, we added PR8's PDZ-bm to the C-terminus of the TX98 NS1 CTD (Fig. 2A). In parallel, we created an additional virus (PR8-TRUNC), where we introduced a stop codon at position 220 of the PR8 NS1 (no TX98 NS1

FIG. 2. Truncation of PR8's NS1 PDZ-bm reduces mice survival after BSI. **(A)** Schematic showing the domains present/absent within the PR8 virus, including PR8-TRUNC and the reassortant domain mutant viruses; PR8(TX98_NS1-RBD), PR8(TX98_NS1-ED), and PR8(TX98_NS1-CTD) as defined by Hale (17). **(B–D)** C57BL/6 (WT) mice were infected with 0.1LD₅₀ of a designated virus. **(B, C)** Mice were challenged with 0.1 LD₅₀ of *Streptococcus pneumoniae*. **(B)** Survival and **(C)** body weight loss as a measure of morbidity were determined daily. Arrow indicates day of BSI challenge (d7). **(D)** Lung virus titers were determined on day 7 (at the time of BSI) by plaque assay method. ** $p < 0.01$ by log-rank test. NS1, NS protein 1; PDZ-bm, PDZ-binding motif; RBD, RNA-binding domain; CTD, C-terminal domain; i.n., intranasally.

domains). This allowed us to directly evaluate the role of the PR8 NS1 PDZ-bm in host susceptibility to BSI.

Using the *S. pneumoniae* BSI model, we found that truncation of the PR8 NS1 gene yields 100% survival (PR8-TRUNC; gray square), compared with the 0% survival seen with PR8-WT (Fig. 2B; black circle). Replacing the PR8 CTD with the TX98 CTD, a mutant that still contains the PR8 PDZ-bm (open diamonds) yielded 0% survival. Expression of either the TX98 RBD or ED in the PR8 virus containing PR8 PDZ-bm (open triangle and reversed open triangle, respectively), yielded a severe BSI that was similar to PR8-WT, with 0% survival (Fig. 2B). Additionally, we found no difference in lung pathology (measured by Hematoxylin and Eosin-stained lung sections) or cellular damage (measured by lactate dehydrogenase in the BALF) between any of the NS1dm viruses, PR8-TRUNC, or PR8-WT virus (data not shown). These results demonstrate that for PR8, the PDZ-bm of the NS1 protein is driving the BSI susceptibility phenotype and that the other domains of PR8-NS1 are not required for BSI susceptibility at day 7.

Similarly, when weight loss was used as an indicator of morbidity, only the PR8-TRUNC virus-infected mice lost less body weight than the PR8-WT-infected mice (Fig. 2C). This was despite the observation that mice in-

fecting with the PR8-TRUNC virus had similar levels of virus in their lungs at day 8 as mice infected with either PR8-WT virus or the other NS1dm viruses (Fig. 2D). We also found that mice infected with the PR8-TRUNC virus exhibited a significant advantage over mice infected with PR8-WT after either *S. pyogenes* or *S. aureus* BSI, indicating that the presence of the PR8 PDZ-bm in influenza NS1 protein increases host susceptibility to infection caused by any of the three most prevalent agents of post-influenza BSIs (Fig. 3). This suggests that the PDZ-bm expressed by the PR8 NS1 protein can reduce both survival (Fig. 3B) and bacterial clearance (Fig. 3C) after BSI. It also indicates that these detrimental effects of the PR8 PDZ-bm on BSI outcome occur regardless of the bacterial species involved.

Absence of the PR8 PDZ-bm allows for sustained production of IFN- β during PR8-TRUNC IAV infection, which is required for reduced host susceptibility to BSI

While previous reports show that type I IFN signaling at day 7 post-PR8-WT infection increased susceptibility to BSI (37,57), we recently reported that type I IFN signaling at day 3 pi with the same virus significantly improved bacterial

clearance from the lung (59). The improved bacterial clearance at day 3 pi with PR8-WT was due to the simultaneous presence of IFN- α and IFN- β in the lung, whereas increases in BSI severity at day 7 pi with PR8-WT occurred in the absence of IFN- β and presence of IFN- α (59). While extensive studies of the IAV NS1 protein identified multiple mechanisms by which NS1 inhibits type I IFN signaling (12,16,19), the idea that NS1 can selectively suppress only IFN- β but not IFN- α expression has not been entertained. Because we found that PR8-TRUNC-infected mice were less susceptible to BSI than PR8-WT-infected mice, we next

sought to determine whether IFN- β was present in the lungs of these mice at day 7 pi.

Consistent with our previous report (59), we found that WT mice infected with PR8-WT produced primarily IFN- α at day 7 pi (>98% of total type I IFNs; Fig. 4A, B). In contrast to these mice, when we evaluated type I IFN production at day 7 pi in lung homogenates of PR8-TRUNC-infected WT mice, we found that both IFN- α (60% of total type I IFNs) and IFN- β (40% of total type I IFNs) were present. We also found that mice infected with TX98-WT virus, which was not associated with increased BSI susceptibility (Fig. 1A) that expresses a

naturally truncated NS1 protein (Fig. 2A and Supplementary Fig. S1) also had sustained IFN- β production up to day 7 pi (Fig. 4A).

Since other IFNs, such as type II IFN (IFN γ) and type III IFNs (IL-27 and IL-28 in mice), have been found to play a role in BSI susceptibility (1,2,6,15,34), we next examined whether infection of mice with PR8-TRUNC virus affected production of these cytokines when compared with mice infected with PR8-WT virus at the time when PR8-WT-infected mice become susceptible to BSI (day 7 post-PR8). We found no differences in the levels of IFN γ , IL-27, or IL-28 in BALF taken from WT mice that were infected with either PR8-WT or PR8-TRUNC viruses (Supplementary Fig. S2), suggesting that these cytokines are not involved in the BSI susceptibility mediated by PR8 PDZ-bm. Therefore, our results imply that the absence of the PDZ-bm in the NS1 protein of IAV correlates with the extended production of IFN- β observed through day 7 pi. Further highlighting the differences between PR8-WT and TX98-WT viruses, we found that infection with TX98-WT resulted in decreased production of IL-27, IL-28, and IFN γ at day 7 post-BSI (Supplementary Fig. S2), most likely due to the decreased viral burden of TX98-WT-infected mice at this time after infection (Fig. 1C).

To investigate whether the extended production of IFN- β in WT mice infected with PR8-TRUNC virus corresponded with reduced BSI severity compared with PR8-WT-infected mice, we analyzed lung bacterial burden of WT mice at 24 h post-*S. pneumoniae* BSI. Indeed, we found that mice infected with PR8-TRUNC virus at day 7 pi exhibited 10 times less *S. pneumoniae* in their lungs, compared with PR8-WT-infected mice (Fig. 4C). This enhancement of bacterial clearance after BSI in mice infected with PR8-TRUNC virus complemented our previous results (59), where we found that the presence of IFN- β during IAV infection results in decreased lung bacterial burden after BSI. It is important to note that a 1 log decrease in lung bacterial burden at 24 h post-BSI is accepted in the influenza superinfection field as biologically relevant, as it extends survival and allows for bacterial clearance. As we found that mice infected with either PR8-WT or PR8-TRUNC viruses have similar viral burden at day 7 post-IAV (Fig. 2D), our results suggest that at least for PR8, the NS1 PDZ-bm does not alter virus clearance, but it does lead to an increase in BSI susceptibility (Fig. 4C).

We found that the absence of NS1's PDZ-bm correlated with increased IFN- β levels at day 7 pi with PR8-TRUNC virus compared with PR8-WT virus. To determine whether persistence of IFN- β could directly account for improved bacterial clearance in mice infected with PR8-TRUNC virus, we utilized *Ifn- β ^{-/-}* mice (kindly gifted by Dr. S. Vogel, University of Maryland, Baltimore). Using these mice, we found that at day 7 pi with either virus (PR8-WT or PR8-TRUNC), *Ifn- β ^{-/-}* mice did not produce detectable levels of IFN- β (Fig. 5A, B). Importantly, regardless of viral strain, we found that the *Ifn- β ^{-/-}* mice did produce high levels of IFN- α , which has been shown to be involved in worsening BSI outcome at day 7 (59). When tested in our BSI model, we found that *Ifn- β ^{-/-}* mice infected with PR8-TRUNC virus did not show a reduction in lung bacterial burden after BSI (Fig. 5C) in contrast to what was found with the WT mice (Fig. 4C). In fact, *Ifn- β ^{-/-}* mice infected with the PR8-TRUNC virus had similar lung bacterial burdens as either WT or *Ifn- β ^{-/-}* mice infected with PR8-WT virus (Figs. 4C and 5C).

We also found that *Ifn- β ^{-/-}* mice infected with influenza that naturally lacks the PDZ-bm on NS1, the TX98-WT strain, resulted in increased lung bacterial burden after BSI compared with PBS-inoculated mice and no difference in bacterial burden compared with WT-infected mice (Supplementary Fig. S3A, B). Interestingly, WT mice infected with PR8-TRUNC virus had reduced BSI-induced neutrophil recruitment when compared with PR8-WT-infected WT mice (Fig. 5D). Additionally, *Ifn- β ^{-/-}* mice infected with PR8-TRUNC showed significantly increased levels of neutrophils recruited to the lung after BSI than was observed in WT mice (Fig. 5D).

Furthermore, while differences in IFN- β production had a profound effect on the ability of influenza-infected mice to clear BSI, IFN- β production had no effect on lung virus clearance by either PR8-WT or PR8-TRUNC viruses (Fig. 5E). We also found that morbidity (determined by weight loss; Fig. 5F) and survival (Fig. 5G) of *Ifn- β ^{-/-}* mice to BSI was not affected by the absence of PR8's PDZ-bm (PR8-WT vs. PR8-TRUNC). Thus, deleting the PDZ-bm from the influenza NS1 reduces bacterial burden in mice in an IFN- β -dependent manner, providing insight into how these host (IFN- β) and viral (NS1) factors interact to regulate BSI susceptibility.

Expression of PDZ-bm both delays and reduces robustness of expression of tunable IFN-stimulated genes

While all type I IFNs signal through the same type I IFN heterodimeric receptor (IFNAR1/2), IFN- β is known to have a substantially higher binding affinity for either IFNAR subunit than any IFN- α (8,32). This unique interaction allows IFN- β to induce the same antiviral "robust" genes that are induced by IFN- α , while also inducing antiproliferative and proapoptotic "tunable" genes that are not known to be induced by IFN- α (36,56). Since these tunable genes have been implicated in regulation of the intensity of IFN responses, we wanted to determine whether they may contribute to BSI severity.

As mentioned above, we demonstrated that presence of the PDZ-bm correlated with a reduction in the duration of IFN- β production after IAV infection, we next sought to determine whether expression of the PDZ-bm affected the activation of other IFN-related genes. Using the Custom RT2 Profiler PCR Array (Qiagen) designed to quantify transcription of 24 IFN-related genes most commonly associated with induction of tunable versus robust IFN activity (56), we evaluated whole lung homogenates of WT mice infected with either PR8-WT or PR8-TRUNC virus (Fig. 6). At 6 h pi with PR8-WT virus, most of the IFN-related genes tested were expressed at the baseline level observed in PBS-inoculated mice (Fig. 6A—left panel). Consistent with induction of "robust" antiviral responses, expression of *Mx1* was upregulated 2-fold above the baseline expression level. While not statistically significant, expression of *Cxcl10* and *Oas1a* was also upregulated 6 h after PR8 inoculation. In contrast to PR8-WT infection, at 6 h pi with PR8-TRUNC virus, nearly all 24 IFN-related genes, with the exception of IFN- β , IFN- $\alpha 4$, and IFN- $\alpha 2$ (*Ifn- α*) were significantly downregulated (over 2-fold) in PR8-TRUNC-infected mice.

FIG. 5. Suppression of IFN- β production by IAV NS1 PDZ-bm contributes to increased BSI susceptibility. (**A**, **B**) Levels of IFN- α and IFN- β levels were evaluated in lungs of *Ifn-β^{-/-}* mice infected with either PR8-WT or PR8-TRUNC IAV for 7 days. Depicted are (**A**) concentration levels and (**B**) relative amounts of IFN- α versus IFN- β from total lung concentrations. (**C**) *Ifn-β^{-/-}* mice were infected with 0.1 LD₅₀ of either PR8-WT or PR8-TRUNC IAV on day 0 and were challenged with *S. pneumoniae* on day 7. Lung bacterial burden was evaluated 24 h after challenge. (**D**, **E**) WT and *Ifn-β^{-/-}* mice were infected as described above. At 24 h post-BSI (**D**) cells from bronchoalveolar lavages were isolated and cytopspins were prepared and stained using DiffQuick, and viral burden (**E**) was evaluated by plaque assay. (**F**, **G**) WT and *Ifn-β^{-/-}* mice were infected as described above. (**F**) Weights (depicted as percent starting weight) and (**G**) survival were monitored daily. Arrow indicated day of BSI challenge (d7). * $p < 0.05$; ** $p < 0.01$ by one-way ANOVA.

Consistent with the protection from BSI observed at day 3 pi with PR8-WT virus (53), murine lungs showed ~5-fold increase in the expression of *IFN-β* and *IFN-α4* (Fig. 6A—right panel). By day 3 pi, lungs of mice infected with PR8-TRUNC virus significantly (>100-fold increase) upregulated eight genes that marked activation of both antiviral (*Mx1*, *IFN-α2*, *Oas1a*) and tunable (*Cxcl10*, *Cxcl11*, *IFN-β*, *Usp18*) patterns (Fig. 6A). By day 7 pi, the expression pattern of the IFN-related genes was similar in mice infected with either PR8-WT or PR8-TRUNC viruses, although mice infected with PR8-TRUNC virus expressed almost 2-fold more *IFN-β* and *Irf7* (Fig. 6B). Additionally, despite detection of *IFN-β* transcript, little to no IFN- β protein was detected at day 7 pi in lung homogenates of mice infected with PR8-WT virus (Fig. 4A, B). Thus, both the timing and the diversity of IFNAR signaling are directly affected by the expression of a PDZ-bm by a viral NS1, and the absence of the PDZ-bm allows for both accelerated and enhanced expression of IFN- β -induced (*Cxcl10*, *Cxcl11*) and IFN- β -supporting (*Usp18*) genes as early as day 3pi.

Discussion

Viral manipulation of host immunity, specifically through inhibition of the type I IFN response (28), and its effects on viral infection are well established. How virus-mediated antagonism of the immune response affects susceptibility to BSI and which viral proteins are mediating this response remains unknown. In this study, we demonstrate that the class I PDZ-bm sequence expressed at the C-terminal end of the H1N1 PR8 NS1 protein is involved in controlling susceptibility to BSI through the regulation of IFN- β responses.

Many of the well-characterized functions of NS1 during viral infection are mediated through different portions of the NS1 protein, including the N-terminal dsRNA-binding domain and the C-terminal ED (17,19). These regions of NS1, either together or on their own, are important for mediating pre- and posttranscriptional regulation of the antiviral IFN response (7,18,33). As differences in these key regions exist, it suggests that NS1 proteins from different viruses may have distinct effects on BSI susceptibility. Specifically, the

A	6 hours pi		3 days pi		B	7 days pi	
	PR8 WT	TRUNC	PR8 WT	TRUNC		PR8 WT	TRUNC
Usp18	0.3	-3.5	0.1	10.7	Usp18	3.4	4.2
Mavs	-2.1	-4.8	-2.0	-3.7	Mavs	-2.7	-2.9
STING	0.6	-4.0	-2.2	0.9	STING	1.7	3.6
Irf1	0.1	-2.3	-2.2	1.4	Irf1	2.7	-4.2
Irf3	0.1	-2.3	-2.1	-2.1	Irf3	-2.3	-2.5
Irf5	0.1	-4.2	-2.2	0.6	Irf5	1.0	3.1
Irf7	0.5	-3.4	0.3	41.4	Irf7	16.5	25.4
Stat6	-2.2	-5.2	-2.9	-3.0	Stat6	-2.7	-8.5
Stat3	0.1	-3.7	-2.2	0.1	Stat3	0.3	0.6
Ifn-β1	0.3	4.1	5.3	606.3	Ifn-β1	147.5	278.9
Ifn- α 4	0.1	3.4	3.4	3.3	Ifn- α 4	3.0	0.7
Ifn- α 2	-2.1	2.5	1.2	13.7	Ifn- α 2	2.2	-2.0
Ifnar1	0.1	-3.2	-2.1	-2.1	Ifnar1	-2.0	0.2
Ifnar2	0.1	-2.3	-2.3	0.4	Ifnar2	0.1	0.2
Jak1	0.1	-2.0	-2.5	-2.3	Jak1	-2.3	-2.5
Tyk2	-2.1	-2.5	-2.5	-2.1	Tyk2	-2.1	0.4
Mx1	2.1	-6.1	-2.9	37.3	Mx1	21.0	44.0
Cxcl10	0.8	-2.6	2.0	211.8	Cxcl10	132.6	167.4
Cxcl11	-5.4	-2.1	1.3	177.3	Cxcl11	82.3	49.8
Oas1a	0.5	-3.3	0.3	13.1	Oas1a	7.4	12.8
Trail	0.2	-2.8	-2.1	-2.1	Trail	0.3	-2.2
Syk	-2.1	-7.7	-3.2	-2.9	Syk	-2.1	-2.1

FIG. 6. Expression of PDZ-bm delays and reduces robustness of tunable interferon-stimulated genes. WT mice were infected with 0.1 LD₅₀ of either PR8-WT or PR8-TRUNC IAV on day 0. RNA isolated from whole-lung preparations at (A) 6 h and 3 days or (B) at 7 days after infections were evaluated for expression of interferon-related genes by Custom RT2 Profiler PCR Array, Qiagen. Data (average result of two biological replicated run in triplicates) are presented as fold change over control (naive mice). IFN- β -induced tunable genes are *bolded*. Color images are available online.

PDZ-bm sequences located in the C-terminal region of NS1 can vary greatly within IAV (17,19), such as with recent human H3N2 isolates that express either RSEV (A/New York/1128/2008-H3N2) or GPEV (A/Pavia/07/2014-H3N2) PDZ-bm sequences, or recent human H1N1 isolates that were truncated to 219 amino acids (A/California/4/09-H1N1 and A/Gainesville/05/2014-H1N1). This variation in the PDZ-bm has been found to affect IAV morbidity (17) or, as presented in this study, BSI susceptibility.

Intracellular protein–protein recognition domains, like PDZ, are involved in the assembly and organization of complexes that mediate diverse cellular functions (45). Initial studies using PDZ domain arrays identified both an increased number and intensity of PDZ proteins that bind to NS1 expressing an avian PDZ-bm (ESEV) compared with NS1 expressing a human PDZ-bm (RSKV) (14,25,31,38,46,67). However, we only found a single published report that considered interactions with the PR8 RSEV PDZ-bm, which focused exclusively on interactions with the PDZ protein PDLim2 (67). Using a PDZ:PDZ-bm web-based database that considers interactions with both human and mouse PDZ domain proteins (24,63), we identified Dlg1 and MAGI-1, but not Scribble, as predicted interactors with RSEV PDZ-bm (data not shown). To our knowledge, neither the specific interactions between an NS1 RSEV PDZ-bm and Dlg1 or MAGI-1, nor the consequences of these potential interactions, have been confirmed experimentally.

Previous work with avian influenza viruses expressing ESEV PDZ-bm, different from the RSEV PDZ-bm studied here, indicated an interaction between ESEV PDZ-bm and MAGI-1 that impaired NS1 inhibition of IFN- β expression (31), as well as with Dlg1 and Scribble, which disrupts cellular junctions and prevents apoptosis (14,38). While the RSEV PDZ-bm may induce similar responses as those previously described, understanding how different PDZ-bm sequences impact IAV infection, specifically alterations of host immune response, and progression to a BSI need to be determined. Direct effects on IFN- β , cellular junctions, and apoptosis are all likely to influence susceptibility to BSIs and are of significant interest in our future research plans.

In this study, we demonstrate that the PDZ-bm of the NS1 protein from PR8 IAV increases host susceptibility to BSI on day 7 post-IAV. The NS1 protein from IAV has been shown to have a major role in viral infection pathology, and early expression of NS1 during infection is known to act primarily by antagonizing the host-generated IFN response (12,13). Our recent work demonstrated that susceptibility to BSI is regulated by the time-dependent production of type I IFNs during IAV (59), where we reported that the lack of IFN- β and presence of IFN- α at day 7 was associated with negative BSI outcome. Importantly, we also recently demonstrated that the timing of IFN- β signaling during IAV infection contributes to regulation of both morbidity and

survival, with early signaling enhancing recovery (58). In this study, we expand upon this mechanism by demonstrating that the PDZ-bm of the NS1 protein from PR8 IAV regulates host susceptibility to BSI on day 7 post-IAV through downregulation of IFN- β . Therefore, our results suggest that the PDZ-bm of NS1 is involved in the regulation of the time-dependent type I IFN response during IAV infection, which in turn determines BSI susceptibility.

Not surprisingly, recent reports suggest a nonredundant role for IFN- β during both viral and bacterial infections. As such, IFN- β -deficient mice can be more susceptible to viral infections, including those caused by influenza (29) and vaccinia viruses (10), depending on the strain and infectious dose used. In this study, using a low inoculum of 0.1LD₅₀ of PR8 IAV, we did not find increased IAV susceptibility of *Ifn- β ^{-/-}* mice. This allowed us to consider the contribution of the host factor, IFN- β , to BSI susceptibility in the absence of the increased morbidity resulting from high-dose IAV infection.

While type I IFNs, including IFN- β , are largely considered to be beneficial in the resolution of virus infections, it should be noted that blockage of IFN- β during Lymphocytic choriomeningitis virus (LCMV) infection, while not affecting early virus dissemination, resulted in accelerated virus clearance (44), indicating that the contribution through induction of IFN- β may be virus specific. Similarly, IFN- β can either promote or interfere with the immune response during infection with bacterial pathogens (9). Although type I IFN signaling has been associated with disease progression during *Mycobacterium tuberculosis* infection (3), recent evidence suggests that IFN- β signaling may dampen virulence of *M. tuberculosis* (3). In a murine model of bacterial pneumonia, the virulence level of *S. aureus* strains correlated with its ability to stimulate the type I IFN signaling pathway (48).

We found that production of IFN- β in response to IAV infection was required for improved *S. aureus* clearance during BSI (59). Consistent with our finding, others reported that failure to induce IFN- β contributed to increased pathogenicity of cutaneous *S. aureus* infection (26). While type I IFNs have been implicated in increased host susceptibility to post-IAV *S. pneumoniae* pneumonia (57), IFN- β was also reported to reduce invasive pneumococcal bacteremia (35) and contribute to protection from Group B Streptococcus (66). Thus, it appears that both the timing of IFN- β production and the type of pathogen eliciting type I IFN signaling can independently dictate the outcome of this signaling to pulmonary bacterial infections.

Our results demonstrate that the IFN- β antagonism by NS1 of PR8 that leads to increased BSI susceptibility requires the PDZ-bm RSEV, which is absent in the non-BSI-inducing TX98 strain of IAV. While type I IFN signaling has been characterized as detrimental for BSI outcomes (57), both our previous work (59) and the results reported in this study support our position that the lack of IFN- β signaling accompanied by signaling with only IFN- α at day 7, rather than overall type I IFN signaling, increases BSI susceptibility. These results further expand the IAV-induced mechanisms regulating the type I IFN response involved in BSI susceptibility, specifically through the PDZ-bm, demonstrating that susceptibility to BSI during PR8 infection is a virus-dependent mechanism. Since BSIs typically occur in the weeks following a primary influenza virus infection,

communication between the virus and the host likely defines downstream effects that modulate susceptibility to BSI, and our work suggests that this decision may be made during the initial hours after virus infection (58,59).

Importantly, our results imply that the inhibition of IFN- β by the PDZ-bm of NS1 was at the posttranscriptional level, as both PR8-WT and PR8-TRUNC induced high levels of IFN- β mRNA, but only PR8-TRUNC-infected mice produced IFN- β protein on day 7 post-IAV. These results suggest a new NS1-mediated mechanism, where the PDZ-bm of NS1 acts to regulate the posttranscriptional IFN response. The pretranscriptional regulation of IFN- β is thought to occur by inhibition of retinoic acid-inducible gene I (RIG-I) activation by direct interaction of PR8 NS1 with RIG-I, preventing induction of IFN- β mRNA transcription (16,43,47,49). This inhibition of RIG-I is thought to require the N-terminal portion of NS1, which is still present in PR8-TRUNC and PR8-WT. This suggests that at day 7 post-IAV NS1 is not involved in inhibition of IFN- β transcription. Potentially, the PDZ-bm of NS1 may interact with proteins that are directly involved in mRNA maturation, nucleocytoplasmic transport, or by forming an inhibitory complex with the mRNA nuclear export machinery (11,50,55).

While our results describe a novel function for IAV's NS1 in controlling IFN- β production during IAV infection, we also found that the NS1 proteins of PR8-WT and TX98-WT viruses differ in their capacity to regulate host BSI susceptibility. Whether the modest effect of TX98 NS gene segment on BSI susceptibility of PR8[TX98_NS]-infected mice could be explained by a compensatory effect of other PR8 gene segments remains to be determined. In this regard we recently described the contribution of PR8's HA in modulating BSI susceptibility (27). There we found that replacement of either HA or NS gene segment in TX98 with the HA or NS gene segment from PR8 allowed the TX98 virus expressing either a PR8's HA or a PR8's NS to increase BSI susceptibility at day 7 pi.

In this study, we demonstrate further that while PR8's NS gene worsens outcome to BSI when added to TX98, the NS gene from TX98 is not able to overcome PR8-induced BSI susceptibility. This suggests that PR8 NS is a stronger modulator of the host BSI susceptibility than TX98's NS gene. Altogether, these and our previous results suggest that other genes in the PR8 virus, such as HA (27), may be able to overcome the effect of the NS gene of TX98 in PR8[TX98_NS] reassortant virus. Nonetheless, our reverse genetics-based approach allowed us to discover that the RSEV PDZ-bm facilitates part of PR8's NS1 antagonism of the host-BSI response through IFN- β regulation.

In summary, while our previous work focused on host type I IFN regulation of post-IAV susceptibility to BSI (59), in this study, we expand upon this mechanism by providing evidence for the ability of the IAV PR8 NS1 protein to direct host type I IFN responses that determine BSI severity. Importantly, we determined that the PDZ-bm of NS1 acts posttranscriptionally to inhibit the production of IFN- β protein, and this inhibition of IFN- β production negatively impacts BSI susceptibility. Future work will provide insight into which proteins are interacting with the PDZ-bm of NS1 to inhibit IFN- β production in an effort to better understand IAV-mediated modulation of the antiviral immune response.

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Author Disclosure Statement

No competing financial interests exist.

Supplementary Material

Supplementary Figure S1
Supplementary Figure S2
Supplementary Figure S3

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