Nonstructural Protein 1 (NS1)-Mediated Inhibition of c-Abl Results in Acute Lung Injury and Priming for Bacterial Co-infections: Insights Into 1918 H1N1 Pandemic?

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Background. Nonstructural protein 1 (NS1) proteins from avian influenza viruses like the 1918 pandemic NS1 are capable of inhibiting the key signaling integrator c-Abl (Abl1), resulting in massive cytopathic cell alterations.

Methods. In the current study, we addressed the consequences of NS1-mediated alteration of c-Abl on acute lung injury and pathogenicity in an in vivo mouse model.

Results. Comparing isogenic strains that differ only in their ability to inhibit c-Abl, we observed elevated pathogenicity for the c-Abl-inhibiting virus. NS1-mediated blockade of c-Abl resulted in severe lung pathology and massive edema formation and facilitated secondary bacterial pneumonia. This phenotype was independent of differences in replication and immune responses, defining it as an NS1 virulence mechanism distinct from its canonical functions. Microarray analysis revealed extensive downregulation of genes involved in cell integrity and vascular endothelial regulation.

Conclusions. NS1 protein-mediated blockade of c-Abl signaling drives acute lung injury and primes for bacterial coinfections revealing potential insights into the pathogenicity of the 1918 pandemic virus.

Keywords. Influenza A virus; NS1 protein; c-Abl; acute lung injury; bacterial coinfections.

Infections of the respiratory tract are a major burden to human health, with influenza A viruses (IAVs) ranking as one of the major pathogens. In addition to seasonal epidemics, IAVs are able to cause pandemic outbreaks with millions of victims, such as the Spanish flu outbreak from 1918 [1]. A better understanding of the fundamentals of the interplay between IAV virulence factors and host immune responses is needed to elucidate differences in severity and outcomes of infections with different strains. The Nonstructural protein 1 (NS1) has been described as a crucial virulence factor for IAVs and linked to suppression of antiviral type I interferon (IFN) responses [2], as have been the IAV polymerase proteins [3]. The NS1 protein lacks intrinsic enzymatic activity and thereby fulfills these functions almost exclusively via interaction with and manipulation of host cell proteins [4]. In recent years, much of the research in the field has been directed toward binding motifs within NS1 to clarify interactions with cellular proteins and explicate resulting functions for NS1.

The NS1 protein harbors src homology (SH) binding motifs (SHbms) that are capable of interacting with cellular SH domain-bearing proteins. Human and avian IAVs differ in expression of SHbms within the NS1 protein. Whereas NS1 of human IAVs carry one SH2bm and one SH3bm, prototype avian IAVs express NS1 proteins with an additional SH3bm (SH3(II)bm), in the C-terminal part of NS1 (Figure 1A) [5, 6]. The

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SH2bm is highly conserved among IAV and was shown to be crucial for IAV pathogenicity [7, 8]. Interestingly, the pandemic 1918 H1N1 strain carried the SH3(II)bm when it crossed over into humans but rapidly lost it during adaptation to the human host so that the recently circulating seasonal human IAVs do not express the SH3(II)bm. We recently demonstrated that prototype avian IAVs inhibit the cellular signaling integrator c-Abl (Abil) via this SH3(II)bm [9]. Alteration of c-Abl kinase activity affected highly pathogenic avian IAV virus replication and pathogenicity [9]. The cellular kinase c-Abl is connected to multiple host cell functions and has been shown to be crucial for cytoskeleton organization [10–12]. NS1-mediated inhibition of c-Abl kinase activity resulted in severe cytopathic effects of infected cells resulting in sudden and robust disruption of epithelial cell layers (Figure 1B) [9].

In the current study, we examined the direct contribution of SH3(II)bm-mediated inhibition of c-Abl by the NS1 protein to the pathogenicity of human IAVs. Our data clarify that NS1-mediated blockade of c-Abl kinase activity drives acute lung injury, resulting in fatal outcome of IAV infections and bacterial superinfections in a replication state and immune response independent fashion. This introduces an entirely new concept of NS1-mediated pathology independent of its canonical type I interferon suppression activity.

MATERIALS AND METHODS

Viruses and Bacteria
The mouse-adapted human recombinant influenza virus A/Puerto-Rico/8/34 (H1N1) (wt) and a virus carrying the mutation T215P within the NS1 (T215P) as well as reassortant viruses (7:1) containing the PR8 backbone and the A/chicken/California/139/01 (H6N2) nonstructural (NS) gene segment (PR8 NS H6N2 wt or PR8 NS H6N2 NS1 P215T) were generated using the pHW2000 based reverse genetic system [13], as described elsewhere [5]. The Streptococcus pneumoniae strain A66.1 was grown as described elsewhere [14]. Cultivation and infection procedures for A549 and Madin-Darby canine kidney (MDCK) cells were conducted as described elsewhere [5, 15].

Mouse Experiments
All mouse studies were performed in compliance with animal welfare regulations under an animal protocol approved by the Animal Care and Use Committee of St Jude Children’s Research Hospital. Female BALB/c mice 7–8 weeks old (Jackson Laboratory) were infected under anesthesia with 2.5% inhaled isoflurane (Baxter). Intranasal infection was done in a final volume of 50 μL for virus or 100 μL for bacteria. Mice were monitored daily for morbidity and weight loss. In accordance with animal welfare restrictions, mice were euthanized after weight loss of 30% and were considered to have died that day.

Western Blotting and Antibodies
Cell lysis and performance of Western blot was conducted as described elsewhere [5]. To monitor c-Abl inhibition, phosphorylation of CT10 Regulator of Kinase (CRKI) (rabbit anti-CRKL [pY221]) polyclonal antibody [pAb]; Cell Signaling) and CRKL (rabbit anti-CRKL [pY207] pAb; Cell Signaling) was analyzed. CRKI/II (mouse anti-CRKI/II monoclonal antibody [mAb]; BD) and CRKL (rabbit anti-CRKL [C-20] pAb; Santa Cruz) analyses were used as controls. ERK2 (anti-ERK2 [C-14] rabbit pAb; Santa Cruz) and Tubulin (anti-β Tubulin [9F3] rabbit mAb; Cell Signaling) detection served as loading control. Infection control was done by visualization of IAV NS1 protein expression (mouse anti-NS1 [clone NS1-23-1] mAb; Institute of Molecular Virology, Muenster, Germany).

Analysis of Virus Titers and Cytokine and Chemokine Levels
The analysis of infectious virus particles in infected mouse lungs and bronchoalveolar wash fluid (BALF) was performed as described elsewhere [7]. In brief, samples were harvested (lungs were homogenized). Afterward, samples were clarified from debris by centrifugation and supernatants were used for assessment of plaque-forming units (PFU) per milliliter in standard plaque titrations on MDCK cells. For determination of cytokine/chemokine, natriuretic peptide A (NPPa), natriuretic peptide B (NPPb), and angiotensin-converting enzyme 2 (ACE2) messenger RNA levels, lungs were collected in TRIzol. RNA isolation was done using the TRIZol method [16] and a PureLink RNA Mini kit (Ambion). Reverse transcription was done using M-MLV Reverse Transcriptase (Promega) according to the manufacturers protocol and real-time quantitative polymerase chain reaction (PCR) reaction was conducted with QuantiTect SYBR Green PCR Kit (Qiagen) and QuantiTect primer (Qiagen), according to the manufacturers recommendation. Relative messenger RNA concentrations were determined (Applied Biosystems 7300 Real Time PCR System) by using the 2−ΔΔCT method [17]. The housekeeping gene Gapdh served as an internal control.

Immune Cell Infiltration and Lung Pathology
For analysis of lung pathology markers and inflammatory cell infiltration, BALF was collected as described elsewhere [7]. In brief, BALF cells were purified and counted, and fluorescence-activated cell sorter analysis was performed as described in detail elsewhere [7]. BALF cells were analyzed for resident macrophages (F4/80+, Gr1low, CD11c−high, and MHC-II−low), exudate macrophages (F4/80+, Gr1high, CD11cintermediate and MHC-II−low), neutrophils (Gr1+, F4/80−, and CD11c−), CD4+ T cells (CD3+ and CD4+), and cytotoxic T cells (CD3+ and CD8+). The remaining cell free BALF was used for lung pathology markers analysis (total protein concentration via the Bradford assay [Bio-Rad] and lactate dehydrogenase (LDH) measurement via an LDH assay [Sigma]). Detailed pathology investigations and
immunohistochemistry were conducted as described elsewhere [7]. Blinded pathology scoring of denuded bronchioles was obtained and immunohistochemical studies for viral antigen distribution (anti-influenza A, USSR [H1N1]; US Biological) and type II pneumocytes (anti–surfactant protein C) were conducted; slides were scanned and quantified as described elsewhere [7].

For lung edema analysis, total lungs were collected, wet lung weights were determined immediately, and dry lung weights were determined after drying at 70°C for 48 hours. Afterward, the wet-dry lung weight ratio was calculated.

**Microarray Assay**

For investigation of gene expression in mouse lungs, total lung RNA was isolated. RNA quality was analyzed (Agilent 2100 Bioanalyzer) and total RNA (100 ng) was processed according to the Affymetrix GeneChip Whole Transcript Labeling protocol. Biotinylated complementary DNA (5.5 µg) was fragmented and hybridized to Affymetrix GeneChip Mouse Gene 2.0 ST arrays and then stained and scanned according to the manufacturer’s instructions. Data were robust multi-array average summarized [18] and visualized by principle component analysis. Next, each transcript was statistically tested using analysis of variance, and the resultant P values were corrected for multiple testing [19] using Partek Genomics Suite software (version 6.6) (Partek). Hierarchical clustering and heat map visualization of z score–normalized data for complete data or select genes were performed using Spotfire Decision Site software (version 9.1) (TIBCO). Gene Ontology enrichment analysis for genes either upregulated or downregulated in T215P virus–infected lungs was performed by the DAVID Bioinformatics Resources software (version 6.7) developed and provided by the National Institute of Allergy and Infectious Diseases [20]. Gene Ontology bar charts were scored and visualized with STATA/MP software (version 11.2) (StataCorp), and heat maps for candidate genes were generated with Spotfire Decision Site software (version 9.1) (TIBCO).

**Serum Markers**

For analysis of serum markers, total blood was collected and serum was produced using serum gel tubes (Sarstedt). Serum carbon...
dioxide and sodium were analyzed in the veterinary pathology core using the Trilogy Multi-purpose Analyzer (Drew Scientific), and serum atrial natriuretic peptide (ANP) was analyzed using mouse ANP enzyme-linked immunosorbent assay (Elabscience).

RESULTS

Impact of NS1-Mediated c-Abl Inhibition on IAV Pathogenicity

The NS1 protein of IAVs has been described as a crucial pathogenicity determinant with several different functions. Our earlier studies revealed NS1-mediated c-Abl inhibition as a mechanism inducing severe cytopathic effects in infected epithelial cells [9]. Most avian viruses, including the avianlike 1918 pandemic virus, harbor a proline (P) at amino acid 215, whereas human H1N1 strains carry a threonine (T) in this amino acid position (Figure 1A). Here, we created 2 isogenic human IAVs differing only in the expression of the SH3(II) bm (wt and T215P).

To confirm that the introduction of the avian SH3(II) bm into the NS1 protein of a human IAV (T215P mutation) resulted in c-Abl inhibitory capacity of human IAVs in our model, we infected A549 lung epithelial cells (Figure 1C) and mice (Figure 1D). Inhibition of c-Abl after introduction of the SH3(II) bm was confirmed on the level of CRK protein phosphorylation in A549 cells and in mouse lungs. Using this model, we examined the impact of c-Abl inhibition on the pathogenicity of human IAVs. Using 2 different virus doses (30 PFU in Figure 2A and 2B and 100 PFU in Figure 2C and 2D) we found clearly elevated pathogenicity manifested as weight loss and death with the c-Abl-inhibiting virus. Accumulation of viral particles in total lung (Figure 2E) and BALF (Figure 2F) was comparable, confirming the earlier found similar replication capacity of

Figure 2. Nonstructural protein 1 (NS1)-mediated c-Abl inhibition increases human influenza A virus pathogenicity. Ten (A–E) and 5 (F) BALB/c mice per group were infected intranasally with 30 plaque-forming units (PFU) per mouse (A, B) or 100 PFU per mouse of PR8 wild type (wt) or PR8 T215P (C–F). A–D, Mice were monitored for weight loss (A, C) and survival (B, D) for 15 days. B, D, Kaplan–Meier survival curve analysis was performed. A, C, Data are represented as means ± standard deviations. E, F, Virus titers in mouse lungs (E) and bronchoalveolar lavage fluid (BALF) (F) were determined 3, 5, and 8 days after infection by standard plaque titration assay. Each dot represents result from 1 mouse. *P < .05 for comparison between PR8 wt– and PR8 T215P–infected animals. A, C, Student t test analysis with correction for multiple tests (Holm-Sidak method); B, D, log rank (χ2) test for statistical analysis of Kaplan–Meier survival data; (E, F), Student t test analysis with Welch’s correction for samples with unequal variance.
these 2 virus variants in in vitro assays [9] and ruling out gross differences in growth as a mechanism for pathogenicity. Taken together, these data demonstrate that the introduction of the c-Abl inhibitory motif into the NS1 of a human IAV mediates pathogenicity through a mechanism not involving increased virus production.

Influence of Manipulation of c-Abl Kinase Activity on Immune Responses After IAV Infection

One hallmark of canonical NS1 functions is interference with host immune responses. Because c-Abl is a central signaling integrator and was also described as potentially involved in manifestation of antiviral immune responses [21], we analyzed a possible effect of the NS1-mediated inhibition of c-Abl kinase activity on the immune response to IAVs. Monitoring immune relevant cytokine/chemokine expression (IFN-β, IFN-γ, tumor necrosis factor (TNF) α, and CCL2) (Figure 3A) as well total BALF cell counts and counts for specific immune cells, such as resident macrophages, exudate macrophages, and neutrophils (Figure 3B), we found sustained inflammatory responses after infection with both viruses, compared to phosphate buffered saline infected animals. However, there were no strong or broad differences between the 2 viruses in cytokine/chemokine expression and overall composition of BALF. Only slightly elevated counts for exudate macrophages and neutrophils were present at certain points in time. In addition, the adaptive T-cell immune response did not differ substantially between the viruses (Supplementary Figure 1). Taken together, although c-Abl is involved in a multitude of signaling networks, our data suggest that NS1-mediated inhibition of c-Abl kinase activity does not substantially alter immune responses after IAV infections, making it unlikely that an alteration of lung inflammation is responsible for the observed differences in IAV pathogenicity.

Effect of NS1-Mediated Inhibition of c-Abl Kinase Activity on Lung Transcriptome After IAV Infections

Because we did not observe substantial alterations in overall immune responses after infection with the wt and T215P viruses, we queried whether a global impact of NS1-mediated c-Abl inhibition was present, using microarray analysis to investigate the whole lung transcriptome. Principle component analysis revealed separate gene expression profiles for the wt and T215P viruses as well as for naive mice (Figure 4A). Heat map analysis revealed patterns of interest in gene expression profiles, showing groups of upregulated or downregulated genes in T215P infections versus wt infections (Figure 4B).

To determine the biological features of these different groups of genes, we analyzed the composition using gene annotation approaches. Interestingly, many genes that are downregulated in T215P virus infections show involvement in cytoskeleton regulation and thereby cell integrity, smooth muscle and extracellular matrix organization (Figure 4C) with particular genes...
such as actin or actin-binding proteins (\textit{Actc1}, \textit{Actn2}) or proteins involved in endothelium regulation, such as \textit{Nppa} (Figure 4D). Genes upregulated in T215P infections compared with wt virus infections can be grouped primarily as genes

**Figure 4.** Nonstructural protein 1 (NS1)-mediated inhibition of c-Abl has global consequences on lung transcriptome after influenza A virus (IAV) infection. Three BALB/c mice per group were infected with 100 plaque-forming units (PFU) per mouse of PR8 wild type (wt), PR8 T215P, or phosphate-buffered saline (PBS) as naive control. Five days after infection, total lung RNA was isolated and analysis for global lung transcriptome was conducted. \(A\), Principle component analysis (PCA) visualization of IAV-infected and PBS control mice is shown. This visualization captures 45.3% of the variability across samples in the top 2 components PC1 and PC2, principal component 1 and 2. \(B\), The \(z\)-transformed complete data set shown as heat map analysis (color-coded expression) clustered by gene and displayed by experimental class. \(C\)–\(F\), Selected groups of genes downregulated \((C, D)\) and upregulated \((E, F)\) in T215P compared with wt/mock infections were tested for Gene Ontology enrichment analysis using DAVID software \((C, E)\). Redundant categories were removed to simplify visualization and maximize category diversity among the statistically significant categories. The \(P\) value of enrichment was \(-\log_{10}\) transformed to create a score for visualization in the bar chart. These enrichment \(P\) values passed false discovery rate at 5% by the Benjamini–Hochberg method. \(D, F\), Heat map visualization for genes specified as downregulated \((\geq 0.5 \log_2 \text{ratio})\) \((D)\) and upregulated \((\geq 0.3 \log_2 \text{ratio})\) \((F)\) in T215P compared with wt/mock. Abbreviation: SDs, standard deviations.
Blockade of c-Abl signaling capacity via the nonstructural protein 1 results in excessive lung pathology after influenza A virus infection. Five (A–D) or 4 (E–H) BALB/c mice per group were infected intranasally with 100 plaque-forming units per mouse of PR8 wild type (wt), PR8 T215P or phosphate-buffered saline (PBS) as mock control. A, B, At the indicated time points, bronchoalveolar lavage fluid (BALF) was collected and total protein (A) and lactatedehydrogenase (LDH) (B) in BALF were analyzed as pathology markers. C, D, Eight days after infection, lungs were harvested, weighed, then dried and weighed again; wet lung weight (C) and wet-dry lung weight ratio (D) are depicted. E–G, Lungs obtained on day 5 after infection were fixed, embedded, sectioned, and stained with hematoxylin-eosin for histological analysis (E, F) or immunohistochemistry (IHC) for influenza antigen (G). E, Representative images for each group are shown (×40 magnification); arrowheads indicate sites of denudation F, Pathology scores for denuded bronchioles. G, Overall distribution of influenza antigen in the entire lung (percentage of lung field positive for flu antigen). H, Lungs were collected on days 3, 5, and 8 after infection, fixed, embedded, sectioned, and IHC stained for type II pneumocytes (surfactant protein C [SPC] staining). Afterward, lung slides were scanned, and whole lung field SPC staining was quantified. Then the percentage of total lung field positive for SPC staining (type II pneumocytes) was calculated and depicted. Data in A–D and F–H are represented as means ± standard deviations. *P<.05 for comparison between PR8 T215P and all other groups analyzed by using analysis of variance with Tukey test for multiple comparisons.

Figure 5. Blockade of c-Abl signaling capacity via the nonstructural protein 1 results in excessive lung pathology after influenza A virus infection. Five (A–D) or 4 (E–H) BALB/c mice per group were infected intranasally with 100 plaque-forming units per mouse of PR8 wild type (wt), PR8 T215P or phosphate-buffered saline (PBS) as mock control. A, B, At the indicated time points, bronchoalveolar lavage fluid (BALF) was collected and total protein (A) and lactatedehydrogenase (LDH) (B) in BALF were analyzed as pathology markers. C, D, Eight days after infection, lungs were harvested, weighed, then dried and weighed again; wet lung weight (C) and wet-dry lung weight ratio (D) are depicted. E–G, Lungs obtained on day 5 after infection were fixed, embedded, sectioned, and stained with hematoxylin-eosin for histological analysis (E, F) or immunohistochemistry (IHC) for influenza antigen (G). E, Representative images for each group are shown (×40 magnification); arrowheads indicate sites of denudation F, Pathology scores for denuded bronchioles. G, Overall distribution of influenza antigen in the entire lung (percentage of lung field positive for flu antigen). H, Lungs were collected on days 3, 5, and 8 after infection, fixed, embedded, sectioned, and IHC stained for type II pneumocytes (surfactant protein C [SPC] staining). Afterward, lung slides were scanned, and whole lung field SPC staining was quantified. Then the percentage of total lung field positive for SPC staining (type II pneumocytes) was calculated and depicted. Data in A–D and F–H are represented as means ± standard deviations. *P<.05 for comparison between PR8 T215P and all other groups analyzed by using analysis of variance with Tukey test for multiple comparisons.
involved in wound repair and defense mechanisms (Figure 4E). Focusing on specific genes, we obtained several micro-RNAs as well as immunoglobulins upregulated in T215P infected lungs compared with wt infection (Figure 4F). Taken together, these data reflect dysregulation of processes that might be expected to be involved in the observed phenotype during infection, the cellular dysmorphology and loss of epithelial barrier integrity seen in our previous in vitro studies [9].

Lung Damage and Dysfunction Due to Inhibition of c-Abl Activity by IAV NS1 Protein

After characterizing the global impact of NS1-mediated c-Abl inhibition on expression of genes involved in lung structure and homeostasis, we examined the impact on acute lung injury as a mechanism of increased pathogenicity after T215P infection. Indeed, measurement of lung damage markers such as total protein (Figure 5A) and LDH (Figure 5B) in BALF and investigation of lung edema (Figure 5C and 5D) revealed excessive lung injury after infection with the c-Abl–inhibiting virus. Furthermore, histology analysis showed increased direct denudation of bronchioles (Figure 5E and 5F), a finding not affected by the extent of virus spread at this particular point in time (Figure 5G). Finally, we found a strong depletion of type II pneumocytes after T215P virus infections, a complication affecting lung tissue regeneration and wound repair as well as alveolar fluid clearance and lung fluid balance. As consequence of these findings, we observed elevated levels for serum carbon dioxide as a marker for impaired alveolar-capillary gas exchange (respiratory acidosis) (Figure 6A). We could also detect strongly elevated serum sodium levels (Figure 6B) along with decreased serum ANP (Figure 6C), both markers for systemic dehydra- tion in T215P virus–infected animals. Taken together, our data suggest that direct lung pathology leads to excessive edema formation with potential lack of capillary integrity and alveolar flooding affecting lung functionality. This results in respiratory failure and systemic dehydra- tion as a mechanism for the increased morbidity and mortality rates observed after infection with the c-Abl–inhibiting virus.

Relevance of NS1 Mediated c-Abl Inhibition in IAV Infections to Human Health

A crucial complication of influenza infections can be the priming for bacterial coinfections. Opening of epithelial barriers and deregulation of lung functions and homeostasis, as seen after infection with our c-Abl–inhibiting IAV, are discussed as a mechanism for increased susceptibility to bacteria. To analyze a potential impact of the SH3(II)bm on the priming for bacterial coinfections, we used a well-established bacterial coinfection model after primary flu infection [22]. Indeed, strong and rapid development of bacteria-induced weight loss (Figure 7A) and death (Figure 7B) was observed after primary infection with the SH3(II)bm-bearing T215P variant followed by bacterial challenge on day 7, compared with a weaker priming for bacterial coinfections after wt IAV infection.

The here depicted pathogenicity mediating SH3(II)bm within the NS1 is still highly conserved in circulating avian IAVs. Therefore, a reassortant pandemic IAV carrying this pathogenicity-boosing motif by introduction via an entire avian NS segment as seen in the 1918 H1N1 IAV is feasible. To study this issue, we generated a human IAV carrying an NS segment from a low-pathogenic avian H6N2 IAV expressing the SH3(II)bm, together with an isogenic mutant lacking the key motif. Interestingly, the reassortant virus carrying the motif and thereby being able to inhibit c-Abl showed increased weight loss (Figure 7C) and mortality rates (Figure 7D) compared with the virus carrying an NS segment with a disrupted SH3(II)bm. This finding implicates the transferability of the pathogenicity-determining motif to other virus systems. Taken together, our data establish NS1-mediated blockade of c-Abl signaling as a new mechanism for IAV virulence by establishing respiratory failure and priming for bacterial coinfections in IAV infections as seen in the 1918 H1N1 pandemic.
DISCUSSION

An understanding of the complex details of respiratory pathogen-induced lung pathology is of extreme importance to guide more specific future treatment interventions. To understand the versatile mechanisms of microbe-induced acute lung injury and loss of lung function, the integration of pathogen virulence factors, host immune responses, and host susceptibility has to be considered. The NS1 protein is widely understood to be an important pathogenicity determinant [4, 7]. To this point, NS1-mediated virulence has been described almost exclusively as relating to NS1-mediated facilitation of virus replication through inhibition of host immune responses [23–26]. Here, we could demonstrate that the NS1 protein can directly affect lung pathology in a virus replication-and immune response-independent fashion, introducing a new concept of NS1-mediated IAV pathology. Lung homeostasis with intact and tight epithelial and endothelial barriers and proper ion and water transport avoiding alveolar flooding is crucial for lung function [27]. Loss of lung functionality through reduced surfactant due to disturbed fluid homeostasis is a common consequence of diminished epithelial integrity, resulting in acute lung injury [28]. Molecularly, the regulation of cell-cell interactions, such as tight junctions, is extremely important for maintaining epithelial cell barrier functions, and therefore the accurate regulation of the cellular cytoskeletal network is crucial [29]. Any agent disturbing this lung homeostasis may induce acute lung injury with further complications, including acute respiratory distress syndrome [30]. Interestingly, our data show that NS1-mediated blockade of c-Abl directly interferes with this lung homeostasis in IAV infection, resulting in loss of epithelial-endothelial barriers and massive lung edema.

Mechanistically, several candidate genes involved in cellular integrity and epithelial and vascular barrier function are downregulated by the NS1-mediated c-Abl inhibition. The downregulation of genes involved in cytoskeleton organization, and therefore cell shape and cell-cell contact organization, can contribute to the loss of epithelial-endothelial barrier, as shown in our recent findings on NS1 blockade of c-Abl and cytopathic host cell alterations [9]. Such potential candidate genes are the natriuretic peptide expressing genes Nppa and Nppb. Interestingly, the NPPa peptide (expressed from the Nppa gene) was recently linked to regulation of endothelial barriers and thereby vascular permeability [31] and protection from bacteria-induced acute lung injury [32–34]. Therefore, the downregulation of NPPa peptide expression represents one potential mechanism of direct NS1 protein–induced lung pathology, a concept strengthened by recent work using Nppa knockout mice.
[35]. Recently, Zou et al [36] demonstrated a critical role of downregulated angiotensin-converting enzyme 2 (Ace2) expression in IAV-induced acute lung injury, a gene that was also decreased in expression by NS1 blockade of c-Abl kinase activity in our study (Supplementary Figure 2).

In addition to direct pathological changes after IAV infection, we also showed that NS1 blockade of c-Abl and the resulting alterations in the lung primes for bacterial coinfections, confirming the broad importance of c-Abl regulation in lung infections. Mechanistically, the opening of epithelial barriers may enhance bacterial access to the lung, whereas pathological alterations, such as alveolar flooding, may both provide enhanced carbon sources for bacterial growth and contribute to decreased physical and immune barriers to bacterial proliferation. Thus, our data clearly emphasize the need to investigate more integrated concepts of microbe-induced lung pathology in addition to the well-described interplay of pathogens and host on the level of antimicrobial immune responses.

Sequencing analysis of the SH3(II)bm revealed interesting distinct expression patterns of the motif with highest conservation in almost all avian subtypes (Supplementary Table 1). Focusing on zoonotic introductions of avian IAVs into humans in the past (Supplementary Table 1) and evolution after introduction as seen after the 1918 pandemic reveals dynamics of SH3(II)bm expression. These analyses highlight the importance of this motif and suggest further evaluation in different animal model systems, including ferrets.

In conclusion, we have identified the SH3(II)bm within NS1 as a new crucial molecular pathogenicity signature. This finding has important epidemiological relevance since the pandemic 1918 H1N1, a highly pathogenic IAV that took its NS1 from an avian strain, carrying the SH3(II)bm in the NS1. Indeed, there is an overall high conservation of this motif in recent avian IAVs, suggesting that a repeat of this scenario is possible and, therefore, that the potential consequences shown here have to be considered for pandemic planning and that excessive surveillance efforts are demanded.

**Supplementary Data**

Supplementary materials are available at The Journal of Infectious Diseases online (http://jid.oxfordjournals.org). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyrighted. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

**Notes**

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