Overcoming NS1-Mediated Immune Antagonism Involves Both Interferon-Dependent and Independent Mechanisms

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To ensure survival, our immune system must overcome the action of pathogen-encoded immune antagonists, such as influenza A nonstructural protein-1 (NS1). NS1 subverts the host interferon (IFN) response at multiple levels and blocks the induction of IFN- β , a critical antiviral cytokine. This immune antagonism can be overcome in some cases. It has been shown that IFN- β is upregulated by 48 h in the lungs of wild-type C57BL/6 mice infected with influenza A. In contrast, it is shown here that IFNB1 continues to be repressed in IFNAR1^{-/-} IL28R α ^{-/-} mice, which are deficient in Type-I and III IFN signaling, implying induction of IFNB1 depends on effective IFN signaling. Despite the complete lack of IFN signaling in this system, some IFN stimulated genes (ISGs) were induced following infection with a Flu strain lacking NS1. While the expression of these viral stress-inducible genes (VSIGs) was initially repressed following infection with wild-type Flu, many of these genes became upregulated by 48 h postinfection. These results demonstrate the existence of IFN-independent mechanisms that can overcome NS1-mediated immune antagonism of VSIGs.

Background

CIRCULATING STRAINS of influenza vary year to year in their genetic composition and potential pathogenicity. Between 1976 and 2007 influenza-associated deaths ranged from 3,000 to 49,000 per year in the United States (Simonsen and others 1997; Thompson and others 2010). The emergence of highly pathogenic strains of influenza A, such as the one in 1918, resulted in the deaths of 20–40 million people worldwide (Webster 1999; Taubenberger and others 2000). Although some aspects of increased pathogenicity have been traced back to individual influenza genes (Presanis and others 2011), the mechanisms underlying the balance between viral immune antagonism and the resulting host response remain largely unknown.

Influenza A virus has a negative-strand RNA genome that encodes 11 or 12 proteins, depending on the strain, on 8 RNA segments (Wise and others 2009). Segment 8 encodes an mRNA that is alternatively spliced to express the nonstructural protein-1 (NS1) and the nuclear export protein (NEP). NS1 is a nonessential protein, but it performs several important accessory functions for the optimal replication of the virus in its host. The role of NS1 has been studied through comparison between a variety of wild-type influenza A strains, such as H1N1, H7N7, and their recombinant analogs where NS1 has been deleted or mutated (Grimm and others

2007; Kochs and others 2007). Variation in the NS1 sequence across influenza strains produces strain-specific antagonist activity (Geiss and others 2002). NS1 binds and sequesters double-stranded RNA (dsRNA) to prevent activation of pathogen-recognition receptors (PRRs) and the downstream transcription factors interferon regulatory factor 3 (IRF3), IRF7, and NF κ B, which are essential for the production of interferon- β (IFN- β) (Talon and others 2000; Wang and others 2000b; Hayman and others 2007; Hale and others 2008). However, this inhibition is released by 48 hours postinfection (hpi), resulting in the upregulation of *IFNB1* gene expression and the production of IFN- β (Horisberger and others 1983; Kochs and others 2007; Mordstein and others 2008; Moltedo and others 2009). NS1 also inhibits IFN-inducible dsRNA-activated kinase PKR (protein kinase RNA-regulated) (Hale and others 2008; Randall and Goodbourn 2008). Despite several studies identifying targets of NS1-mediated antagonism, we know very little about how this antagonism is overcome to yield IFN- β production and an ultimately successful immune response.

Type I IFNs are pleiotropic cytokines that are essential for antiviral immune responses (Honda and others 2006). Type I IFNs are comprised of multiple IFN- α isoforms, IFN- β , IFN- γ , IFN- ϵ , and IFN- κ ; of these, IFN- α/β are produced by virus-infected cells. Signaling by type I IFNs through the JAK-STAT pathway leads to the expression of a multitude of

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genes, termed interferon-stimulated genes (ISGs) through activation of the ISGF3 complex (composed of *IRF9*, *STAT1*, and *STAT2*). ISGF3 is also activated by type III IFNs (IFN-III), a family containing 2 (mice) or 3 (humans) IFN- λ members (Dumoutier and others 2004). Expression of ISGs is an important component of the host response, as these gene products have important antiviral and immunostimulatory functions.

The role of type-I and III IFN signaling in the response to influenza infection has been studied using mice with genetic disruptions in both the IFN- α/β receptor I (*IFNAR1*^{-/-}) and the interleukin 28 receptor α (*IL28RA*^{-/-}) (Mordstein and others 2008), thus preventing IFN signaling through the JAK-STAT pathway. Following infection with a recombinant influenza strain (SC35M) deficient in NS1 (Flu- Δ NS1), *IFNAR1*^{-/-} *IL28RA*^{-/-} mice induce a significant number of ISGs, referred to as viral stress-inducible genes (VSIG), despite the complete absence of ISGF3 activation (Schmid and others 2011). Schmid and others (2011) proposed that Flu- Δ NS1-induced IRF3/7 activation was responsible for the upregulation of these VSIGs, and that this redundancy with the IFN response was an important defense against viral infection. However, this study did not consider the action of NS1 on the induction of VSIGs in wild-type influenza (Flu-wt) infections.

To investigate whether NS1 blocks IFN-independent induction of VSIGs, we compared the temporal responses to infection with Flu-wt and Flu- Δ NS1 in *IFNAR1*^{-/-} *IL28RA*^{-/-} mice. Using lung tissue of uninfected and infected mice, we performed differential gene expression analysis to identify genes modulated in an NS1-dependent manner at 12, 24, and 48 hpi. Our analysis revealed that NS1 inhibits VSIGs for 24 hpi, but that a restoration of IRF3/7 activity partially released VSIG inhibition by 48 hpi. Moreover, we find that NS1 globally represses the expression and activity of many other transcription factors, and our analysis identifies several novel effects, including modulation of ETS-domain proteins.

Methods

Microarray data

Experimental procedures are fully described in Schmid and others (2011). Briefly, *IFNAR1*^{-/-} *IL28RA*^{-/-} B6.A2G-Mx1 mice (Mordstein and others 2008), carrying intact Mx1 alleles and defective alleles for *IFNAR1* and *IL28RA*, were infected through the intranasal route with 10⁵ plaque-forming units of either influenza A virus strains SC35M wild-type (Flu-wt) or SC35M deficient in the NS1 (Flu- Δ NS1) (Kochs and others 2007). Animals treated with phosphate-buffered saline/0.3% bovine serum albumin served as negative controls. Lungs of infected and uninfected mice were harvested at 12, 24, and 48 hpi and total RNA was isolated. RNA from 3 lungs per cohort was pooled to perform standard Affymetrix analysis. Affymetrix experiments were performed at the biopolymers facility at Harvard Medical School (<http://genome.med.harvard.edu/>). Raw Affymetrix microarray data from these experiments were downloaded from GEO (GSE24695). While these data were previously deposited in GEO (Schmid and others 2011), the Flu-wt data have never been analyzed or compared with the Flu- Δ NS1 response.

Microarray analysis

Raw Affymetrix microarray data were normalized using gcRMA. Differential expression was defined for each probe set using 2 criteria. For at least one time point, we required: (1) a minimum log₂ intensity of 5, which was indicative of nonexpressed genes by visual inspection of the expression intensity histogram at time zero, and (2) an absolute fold-change of at least 3. Affymetrix Mouse Genome 430 2.0 Array annotation data (mouse4302.db) were used for mapping between probe set identifiers, official gene symbols, and Entrez Gene identifiers. Sets of probes that mapped to the same gene symbol were filtered to retain probes with the highest average fold change.

At each time point genes that were differentially expressed only during Flu-wt infections or Flu- Δ NS1 infections were classified into Flu-wt and Flu- Δ NS1-specific gene sets, respectively. The shared gene set was defined at each time point as the set of genes differentially expressed in the same direction during both infections. In addition, the set of Flu- Δ NS1-specific genes was expanded to include genes that were upregulated in Flu- Δ NS1 relative to Flu-wt infections. Similarly, genes that were upregulated in Flu-wt relative to Flu- Δ NS1 were classified as Flu-wt-specific. For time-series analysis each gene was associated with the first time point at which that gene was differentially expressed. All analysis was performed using the BioConductor software package in R (Gentleman and others 2004). Gene ontology analysis was performed using the conditional test in the GO stats package 2.24.0 (Falcon and Gentleman 2007).

Network construction

The network of gene–gene interactions for Flu-wt and Flu- Δ NS1-specific gene sets was constructed using STRING version 9.05 (www.string-db.org/). STRING was run using the default settings and interactions were stored in text format. The final network was visualized using Cytoscape 2.7, which was also used to calculate network statistics including node-degree. Disconnected nodes were removed from the network.

Transcription factor target identification

Using the UCSC Genome Bioinformatics site, we downloaded the transcription start sites (TSSs) for all mouse RefSeq genes, defined by the July 2009 refGene table (Pruitt and others 2005). For all mouse genes, the regions 2kb around the TSS were identified from the mouse (mm9) genome. These regions were then aligned to human (hg18) and chimpanzee (panTro2) genomes using multiz30way (Blanchette and others 2004). All genome sequences were obtained from the UCSC Genome Bioinformatics Site. Transcription factor binding sites were obtained from either the TRANSFAC database (Matys and others 2003) (all high-quality vertebrate transcription factor matrices for version 2008.3) or independent publications (Schmid and others 2011). The MATCH algorithm (Kel and others 2003) was used to scan all mouse sequences and aligned sequences for putative binding sites using TRANSFAC defined cutoff scores to minimize the sum of false positive and false negative matches. Putative binding sites were considered to be evolutionarily conserved if a match occurred in the same position for all aligned species and no gap was present in the alignment of any of those

species. Each TRANSFAC matrix was linked to a set of gene symbols describing the potential binding factors using annotations present in the "binding factor" field of the matrix. Only vertebrate TRANSFAC matrices that could be linked to an HGNC gene symbol (Dec. 2008) (Bruford and others 2008), either directly or through an alias listed in NCBI gene, were included. A total of 722 binding sites were tested and the p-values were corrected for multiple hypothesis testing by the Benjamini-Hochberg method. These binding sites mapped to 654 transcription factors.

Results

NS1 blocks *IFNB1* expression in the absence of IFN signaling

NS1 inhibits *IFNB1* production by antagonizing IRF3/7 and NF κ B (Talon and others 2000; Wang and others 2000a, 2000b). In wild-type mice, NS1-mediated inhibition of the IFN response is overcome by 48 hpi, leading to induction of IFN- β (Kochs and others 2007; Moltedo and others 2009). To determine whether the induction of *IFNB1* expression was dependent on IFN signaling, we analyzed time-series gene expression data from *IFNAR1*^{-/-} *IL28R α* ^{-/-} B6.A2G-Mx1 mice following infection with Flu-wt. (Hale and others 2008; Mordstein and others 2008; Wolff and Ludwig 2009). Strikingly, we found that *IFNB1* remained at baseline levels at all time points (Fig. 1A). This subversion of the IFN response was mediated by NS1, since *IFNB1* became highly expressed following infection with Flu- Δ NS1 (Fig. 1A). Thus, in the absence of Type-I and Type-III IFN signaling NS1- continues to inhibit *IFNB1* expression for at least 48 hpi.

To investigate the mechanism(s) underlying this NS1-mediated subversion of the IFN response, we studied the temporal patterns of IRF3/7, c-JUN, and NF κ B activity (Panne and others 2004). Together, these 3 factors form the enhancosome, which regulates *IFNB1* expression. We hypothesized that NS1 would inhibit the activity of at least one of these factors at all time points up to 48 hpi with Flu-wt. The temporal activity pattern for each of these factors was estimated using a bioinformatics approach. First, genes that

were differentially-expressed at each time point were identified by comparing infected mice with uninfected controls (Supplementary Table S1 and Methods section; Supplementary Data are available online at www.liebertpub.com/jir). Second, by comparing the differential expression patterns following infection with each virus strain, we identified genes whose expression was changed only in Flu-wt infection (Flu-wt-specific), genes whose expression was modulated only in Flu- Δ NS1 infection (Flu- Δ NS1-specific), and genes that were differentially expressed in the same direction upon either infection (shared) (Fig. 1B, C and Supplementary Table S1). Analysis of these gene sets showed that IRF3/7, c-JUN, and NF κ B targets were all significantly over-represented among Flu- Δ NS1-specific genes at 24 hpi (Fig. 2A, B, definitions of the TF binding sites are shown in Supplementary Table S2), which is consistent with the observed upregulation of *IFNB1* following infection with this strain. In contrast, activity of IRF3/7 was absent at 24 hpi among Flu-wt-specific and shared genes, while c-JUN and NF κ B activity were both absent at 48 hpi (Fig. 2A). This inferred activity pattern is further supported by the observation that only target genes in the Flu- Δ NS1 response exhibit a significant shift in transcription factor binding site location closer to the TSS at 48 hpi (Fig. 3A–D). It has been observed that true cis-regulatory binding sites tend to be located close to the TSS (Beer and Tavazoie 2004; Koudritsky and Domany 2008). Thus, NS1-mediated inhibition of IRF3/7 activity during the first 24 hpi, combined with suppression of c-JUN and NF κ B activity later in the response, prevents the upregulation of *IFNB1* over the first 48 h of infection *in vivo*.

NS1-mediated antagonism is partially released in the absence of IFN signaling

Despite the lack of Type-I and -III IFN signaling, 30% of the ISGs defined by Schoggins and others (2011) were included among all upregulated genes. These genes were previously identified by Schmid and others (2011) as VSIGs, and their activity was linked to IRF3/7. To investigate the role of NS1 in modulating VSIG expression, we compared Flu-wt and Flu- Δ NS1 infection, under the assumption that

FIG. 1. The impact of NS1 on the host immune response. (A) Expression levels of *IFNB1* mRNA in uninfected mice (diamonds), Flu-wt-infected mice (squares) and Flu- Δ NS1-infected mice (triangles). (B) Number of up (upper y-axis) or down (lower y-axis) regulated genes classified as Flu-wt-specific (differentially expressed only in Flu-wt-infected mice), Flu- Δ NS1-specific (differentially expressed only in Flu- Δ NS1-infected mice) or shared (differentially expressed in both infections at the same time point). (C) Number of up (upper y-axis) or down (lower y-axis) regulated interferon-stimulated genes. NS1, nonstructural protein-1; IFN, interferon beta.

FIG. 2. NS1-dependent suppression of genes regulated by c-Jun, NFκB, and IRF3/7. **(A)** Enrichment of predicted c-JUN and NFκB targets among Flu-wt-specific, Flu-ΔNS1-specific, and shared genes. c-JUN targets were identified by the presence of V\$AP1_Q4 (empty circle), V\$AP1_Q6_01 (cross), V\$AP1_Q4_01 (gray circle), V\$AP1_Q2 (empty square), V\$AP1_Q1 (diamond), V\$AP1_C (triangle pointing up), V\$AP1_Q2_01 (triangle pointing down), or V\$AP1_Q6 (grey triangle) binding sites in the promoter region. NFκB targets were identified by the presence of: V\$NFkB_Q6_01 (filled square) or V\$NFKAPAB65_01 (filled diamond) binding sites in the promoter region. Horizontal black line indicates the threshold for statistical significance after correction for multiple testing. **(B)** Enrichment of predicted IRF3/7 targets defined by the presence of V\$ISRE_01 (from TRANSFAC, square) or IRF3/7 (defined in Schmid and others 2011, empty triangle) binding sites in the promoter region. Horizontal black line indicates the threshold for statistical significance after correction for multiple testing. **(C)** Fold changes of *IRF7* (diamonds), *IFIT1* (squares), and *IFIT3* (triangles) mRNA following Flu-wt (solid lines) and Flu-ΔNS1 (dashed lines) infections.

the differences in expression between these strains are driven by NS1-mediated processes. Consequently, genes upregulated in a Flu-ΔNS1-specific manner are referred to as being inhibited by NS1, while genes upregulated in a Flu-wt-specific manner are referred to as being induced by NS1. Similarly, NS1 is assumed to block the repression of genes downregulated in a Flu-ΔNS1-specific manner, and induce the repression of genes downregulated in Flu-wt-specific manner. Shared genes are considered to be regulated in an NS1-independent manner, and this pattern was observed for 14% of the ISGs (Fig. 1C). The expression of 59 ISGs (16%) was inhibited by NS1. This included important viral resistance genes such as *IRF7*, *IFIT1*, and *IFIT3* (Fig. 2C). An additional 33 genes (9%) were induced by NS1, including *TNFSF13B*, *IL6ST*, and *IL1R1*. Unlike the case for NS1-mediated antagonism of the IFN response, which continued over the entire time-course, 10 out of the 23 VSIGs that were inhibited within the first 24 hpi were observed to be induced by 48 hpi following Flu-wt infection. This included key antiviral response genes such as *IRF7* and *IFIT3*. The late gene induction was not due to viral clearance, since high levels of NS1 were still observed in the lungs of Flu-wt-infected mice at 48 hpi (Supplementary Fig. S1). These results show that NS1-mediated suppression of the VSIG response is eventually overcome in an IFN-independent manner.

Schmid and others (2011) suggested that the differential expression of VSIGs in the absence of IFN signaling was driven by *IRF3/IRF7* activity, which can also regulate genes through ISREs. To investigate whether this mechanism was also involved in the late upregulation of VSIGs following Flu-wt infection, we carried out a promoter analysis of differentially expressed genes. An initial screen of all IRF and ISRE binding motifs defined by TRANSFAC indicated that NS1 significantly inhibited induction of genes containing ISREs (defined by the V\$ISRE_01 matrix) ($P < 0.01$) at 24 hpi (Fig. 2B), definitions of binding sites are shown in Supple-

mentary Table S2). However, these genes showed a trend toward enrichment in Flu-wt-specific genes at 48 hpi, although this was not statistically significant after correcting for multiple hypothesis testing. Since the presence of ISREs does not specifically define IRF3/7 targets, we conducted a more focused analysis using the IRF3/7 motif WNNNGAAANN GAAA, where W=A/T and N=A/T/G/C (C\$IRF7_long) (Lin and others 2000; Schmid and others 2011) (Fig. 2B). In this case, significant IRF3/7 activity was detected in Flu-wt-infected mice at 48 hpi. Further, the IRF3/7-regulated genes that were enriched at 48 hpi in Flu-wt were a subset of those upregulated at 24 hpi following Flu-ΔNS1 infection. Interestingly, IRF3/7-targets were not significantly enriched following infection with Flu-ΔNS1. Although the number of upregulated IRF3/7 targets increased over time, the large number of other genes upregulated in Flu-ΔNS1 infection led to higher P values compared with Flu-wt infection, where only ~100 genes are upregulated. Thus, we conclude that IRF3/7 activity is likely to play a role in the eventual upregulation of VSIGs that are initially antagonized by NS1.

Further support for the involvement of IRF3/7 in overcoming NS1-mediated antagonism of VSIGs comes from the analysis of binding site locations. While ISRE and IRF3/7 binding sites were located significantly closer to the TSS among Flu-ΔNS1-specific genes at 24 hpi, this shift in binding site locations was not observed until 48 hpi among Flu-wt-specific genes (Fig. 3C, D). Overall, these results support a model where IRF3/7 activity is initially inhibited by NS1, preventing the induction of many VSIGs, but that this inhibition is overcome by 48 hpi.

The global gene expression signature of NS1-mediated immune antagonism

To identify additional NS1-mediated gene expression changes that were not dependent on IFN signaling, we

FIG. 3. Binding site location shifts are consistent with predicted transcription factor activity. The absolute location of binding sites relative to the TSS for **(A)** NFκB, **(B)** c-JUN, and **(C, D)** IRF3/7 (**C**: V\$ISRE_01 and **D**: IRF7_long) in promoter regions of genes first upregulated at each time point. The number of genes in each group is indicated above the box. The lower and upper edges of the boxes represent the lower and upper quartile, respectively. The medians are indicated by horizontal bars within the box and upper and lower horizontal bars out of the boxes represent 1.5 times interquartile range. 'O' indicate outliers. Group "C" is a control set including all RefSeq genes that contain binding sites for the same TRANSFAC matrix, but that are not differentially expressed. Filled boxes indicate that the median location is significantly closer to the TSS compared with the control group ($p \leq 0.05$, Wilcoxon rank sum test). Binding site definitions are provided in the Fig. 2 caption, and the closest site to the TSS is used when multiple binding sites are present for a single gene. TSS, transcription start site.

compared genome-wide expression patterns following Flu-wt and Flu-ΔNS1 infections (Fig. 1B). NS1 inhibited 971 genes and induced 376 genes. NS1 blocked the repression of 337 genes and induced the repression of 398 genes. Following Flu-wt infection, the maximum number of differentially expressed genes was observed at 24 hpi, while the number of genes continued to increase over time following infection with Flu-ΔNS1. In total, NS1 inhibited 1,308 genes and induced 774 genes either through direct or downstream effects, such as modulating cytokine secretion from infected cells (Fig. 1B).

NS1 suppressed the induction of many genes associated with PRRs and their downstream adaptor proteins, including melanoma differentiation-associated gene 5 (*MDA5*) (fold-change=8 and 2.5 in Flu-ΔNS1 and Flu-wt, respectively) and *TLR2* (fold-change=8 and 2 in Flu-ΔNS1 and Flu-wt, respectively). Notably, *DDX58* (RIG-I) was not differentially expressed in any condition. NS1 also inhibited induction of *TRAF1* (fold-change=3 and -3 in Flu-ΔNS1 and Flu-wt, respectively), an adaptor protein TNF-superfamily signaling, and *MyD88* (fold-change=4 and 2 in Flu-ΔNS1 and Flu-wt, respectively) and *TRAF6* (fold-change=4

and 1 in Flu- Δ NS1 and Flu-wt, respectively), which are adaptor proteins for many TLRs (Akira and others 2001; Schnare and others 2001). NS1 modulated the host response by inhibiting the expression of several chemokines and cytokines, including *TNF*, *CCL5*, *CXCL16*, *CXCL12*, *IL13*, and *IL23*. Gene ontology analysis was used to define the biological function(s) modulated by NS1 (Supplementary Table S3). Many core immune response functions were NS1-independent. Neutrophil chemotaxis, leukocyte migration, and some of the inflammatory responses were all enriched among the shared genes. However, NS1 inhibited several important pathways including the positive regulation of IL-6 production, the JNK cascade, along with antigen processing and presentation. Interestingly, B cell activation appeared to be induced in the presence of NS1. Overall, NS1 was found to modulate the activity of several important immune-related pathways.

Network analysis was applied to identify global regulators of early gene expression changes (up to 24 hpi) that were specific to Flu-wt and Flu- Δ NS1 infections. The network of Flu- Δ NS1-specific genes (Supplementary Fig. S1A) identified *TNF* as the most important driver of the early response when NS1 is absent (based on the number of connections). Other important genes included *TLR2*, *CCL5*, *IRF7*, *MyD88*, and *IL12B*. We find 5% of Flu- Δ NS1-specific upregulated genes are defined as TNF-induced genes by (Waddell and others 2010). In contrast, the Flu-wt-specific network (Supplementary Fig. S2) suggested an *IFNG*-driven response, with *CD44* also playing a potential role. We find 3% of Flu-wt specific upregulated genes are defined as *IFNG*-induced genes by (Waddell and others 2010). These results suggest a qualitative divergence in the early

immune response to influenza depending on whether or not NS1 is present.

NS1-mediated modulation of transcription factors

Consistent with observations made in cell lines, NS1 suppressed induction of many transcription factors with known immune involvement (Roach and others 2007). In particular, NS1 inhibited induction of 19 transcription factors within the first 24 hpi (Supplementary Table S2), including *IRF7* and *NF κ B2*. With the exception of *IRF7*, all of these transcription factors continued to be repressed over the entire time-course, similar to the pattern observed with *IFNB1*. Interestingly, NS1 also induced 8 transcription factors during this same period, including *RUNX3* and *NFIC* (Supplementary Table S2). Twenty-two TFs were upregulated at equivalent times in Flu-wt and Flu- Δ NS1 infections (eg, *ATF3*, *MYC*, and *MXD1*) (Fig. 4A). Nevertheless, most of these transcription factors had higher maximum expression levels in the absence of NS1 indicating at least a partial repression (Fig. 4B). Thus, NS1 has a global inhibitory effect on transcription factor mRNA levels, although a small number of factors are induced.

The activity of many transcription factors can be modulated by post-transcriptional modifications or small changes in mRNA expression. Analyzing the set of transcription factor target genes for coordinated changes can thus provide a broader view of transcription factor activity (Zaslavsky and others 2010). Eleven transcription factors were identified as NS1 dependent through over-representation of target genes at each time point among the Flu-wt-specific, Flu- Δ NS1-specific, and shared gene sets (Supplementary Tables S4 and 5

FIG. 4. The impact of NS1 on transcription factors expression. **(A)** Consistency in the differential expression time for transcription factors following Flu-wt (y-axis) and Flu- Δ NS1 (x-axis) infections. A gradient from white to black depicts lower to higher overlap, as quantified by $-\log_{10}(P \text{ values})$ from Fisher's exact test. Overlapping transcription factors at the indicated times for (Flu-wt and Flu- Δ NS1) are *ATF3* at (12, 12 hpi); *Junb*, *Bcl3*, *Maff*, *Cebpd*, *Myc*, *Egr2*, *Cebpb*, *Nr4a3*, *Zbtb16*, *Hif1a*, *Arid5a*, *Mafk*, *Mbd1*, and *Mxd1* at (24, 24 hpi); *Per2*, *Tcf23*, *Gatad2b*, *Hif3a*, *Nsd1*, *Rbbp4*, *Preb*, and *Nfe2l2* at (48, 48 hpi); *IRF7* at (48, 24 hpi); *Klf9* and *Runx3* at (24, 48 hpi). **(B)** Comparison of the maximum fold-changes across all time points for individual transcription factors mRNAs upregulated in Flu- Δ NS1 and Flu-wt infections. The inset is a density plot showing the distribution of $\log_2$ expression intensities across all microarrays. hpi, hours postinfection.

and Supplementary Fig. S3A–C). Among these, the activity of 4 transcription factors (*ETS1/2*, *TEL*, *MYOG*, and *SRF*) was observed even though their mRNA was not differentially expressed. Moreover, though upregulation of *NFE2* and *JUNB* mRNA were not dependent on NS1, their activity was only observed in Flu-wt and Flu-ΔNS1, respectively (Supplementary Fig. S3C). Interestingly, some of the ETS-domain transcription factors were induced (*PUI1*), while others were repressed (*ETS1/2* and *TEL*) by NS1. This may explain the variable effect of NS1 on ISGs, since ETS-domain proteins have been shown to coregulate ISRE-mediated expression (Wang and others 2000a; Honda and others 2006). The activity of several transcription factors was associated with the shared response (*STAT1*, *STAT3*, *STAT5A*, *NFκB*, and *C/EBP*), suggesting NS1-independent regulation (Supplementary Tables S4 and S5). Thus, NS1 modulates the activity of many TFs, through both transcriptional and post-transcriptional mechanisms.

Discussion

IFNAR1^{-/-} *IL28Rα*^{-/-} mice are deficient in Type-I and Type-III IFN signaling, but retain an ability to induce VSIGs through activation of IRF3/7, resulting in an IFN-like transcriptome, after infection with Flu-ΔNS1 (Schmid and others 2011). Extending previous observations that NS1 antagonizes the host IFN response (Talon and others 2000; Wang and others 2000b; Hayman and others 2007; Hale and others 2008), we report that the IFN-independent VSIG response is also antagonized by NS1, blocking the expression of many genes involved in antigen presentation and immune pathways. By comparing the temporal gene expression response to Flu-wt and Flu-ΔNS1 infection, we found that the host response utilizes both IFN-dependent and independent mechanisms to overcome this viral immune antagonism.

We find that while *IFNB1* expression in the presence of NS1 is dependent on IFN signaling, IRF3/7-mediated activation of VSIGs is not. In wild-type C57BL/6 mice, NS1 inhibits IFN-β for 36 hpi (Molledo and others 2009). However, full induction of IFN-β is observed by 48 hpi. This release of NS1-mediated inhibition may occur through a positive feedback loop induced through initial secretion of IFN-β by a small number of infected cells (Hu and others 2007; Molledo and others 2009). Our data in B6.A2G.Mx1 mice lacking IFN signaling support the hypothesis that IFN signaling is required to overcome this NS1-mediated inhibition of *IFNB1*, since *IFNB1* gene expression remains at baseline levels 48 hpi with Flu-wt. Note that type I and III signaling is believed to be more effective in these B6.A2G.Mx1 mice compared with C57BL/6 mice due to the presence of *MX1* (Grimm and others 2007). Our results suggest that the continued inhibition of *IFNB1* expression 48 hpi with Flu-wt is due to the suppression of c-JUN and NFκB activity, which are required along with IRF3/7 for upregulation of *IFNB1* (Talon and others 2000; Wang and others 2000b). A role for NS1-mediated inhibition of NFκB is also supported by previous studies (Wang and others 2000b; Tisoncik and others 2011). These results demonstrate that overcoming NS1-mediated antagonism of IFN-β is dependent on IFN signaling.

Interestingly, IRF3/7 activity was apparent by 48 hpi with Flu-wt indicating that at least a partial release of NS1-mediated suppression does not require IFN-signaling. Since NS1 levels were still high at this time, it is likely that host-mediated mechanism drives this release (Chua and others

2013). Several mechanisms are possible. High multiplicity of infection leading to higher levels of virus-derived molecular patterns or massive host-cell death could be sensed by cells, such as DCs and macrophages, in the lungs thus initiating an abrupt inflammatory cascade (Martinon and others 2006; Idzko and others 2007; Kumagai and others 2007). Our results also identify several transcription factors whose activity is independent of NS1, and which are involved in antiviral response. These factors, including STAT and CEBP family members, may also play a role in initiating the inflammatory cascade.

In addition to the specific effects of NS1 on IRF3/7, NFκB, and c-JUN activity, we identified several other transcription factors inhibited by NS1. These include MyoD, an inhibitor of cell proliferation (Potthoff and Olson 2007), raising the possibility that NS1 could induce epithelial cell proliferation. This function might facilitate viral establishment by increasing the pool of nearby target cells. Additionally, MyoD is known to interact with c-Jun (Bengal and others 1992) suggesting a possible mechanism of NS1-mediated regulation of c-JUN. *Mef2*, which is involved in stress responses initiation (Potthoff and Olson 2007), was also inhibited by NS1. This inhibition may be indirect, since activation of *Mef2* may have resulted from the strong inflammatory response in Flu-ΔNS1 mice (Haye and others 2009; Molledo and others 2009). Finally, NS1 also inhibited 2 transcription factors from the family of ETS-domain proteins, which have been implicated as cofactors in the regulation of IRFs 1, 4, and 8 (Wang and others 2000a; Honda and others 2006). Overall, the presence of NS1 significantly antagonized the activity of several transcription factors, and led to a global decrease in the expression levels of most transcription factors. However, at this point it is unclear whether these effects are a direct consequence of NS1 interaction, or secondary to differential cytokine secretion across cells infected with Flu-wt and Flu-ΔNS1.

The study of the *in vivo* response is critical for the understanding of immune regulation by viral antagonists. However, the *in vivo* response is complex and subject to the effects of infiltrating immune agents and the pathology of resident cells. In particular, the IRF3/7-mediated response and transcriptional regulation might exist in cell type-specific manner. *IRF3* is constitutively expressed in every cell type tested to date. In contrast, *IRF7* is a lymphoid-specific factor, which is constitutively expressed in the cytoplasm of B cells, plasmacytoid dendritic cells, and monocytes (Ning and others 2011). Additionally, the observed expression is also affected by the number of infected and uninfected cells. For example, *IRF7* stability varies between infected (stable for 0.5–1 h) and uninfected (stable for 5 h) cells (Ning and others 2011).

In conclusion, NS1 antagonizes both the IFN response, and IFN-independent expression of VSIGs. Our studies performed in the absence of type I and III signaling reveal that *IFNB1* expression is dependent on IFN signaling. In contrast, the release of NS1-mediated inhibition of IRF3/7 activity occurs in an IFN-independent manner, although IFN-signaling may still be required for full activity. Our results provide a kinetic signature of immune antagonism by NS1 that should aid future studies of influenza infection.

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