HHS Public Access

Author manuscript

Antiviral Res. Author manuscript; available in PMC 2024 January 08.

Published in final edited form as:

Antiviral Res. 2020 April ; 176: 104747. doi:10.1016/j.antiviral.2020.104747.

Influenza virus NS1- C/EBPβ gene regulatory complex inhibits RIG-I transcription

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Abstract

Influenza virus non-structural protein 1 (NS1) counteracts host antiviral innate immune responses by inhibiting Retinoic acid inducible gene-I (RIG-I) activation. However, whether NS1 also specifically regulates RIG-I transcription is unknown. Here, we identify a CCAAT/Enhancer Binding Protein beta(C/EBPβ) binding site in the RIG-I promoter as a repressor element, and show that NS1 promotes C/EBPβ phosphorylation and its recruitment to the RIG-I promoter as a C/EBPβ/NS1 complex. C/EBPβ overexpression and siRNA knockdown in human lung epithelial cells resulted in suppression and activation of RIG-I expression respectively, implying a negative regulatory role of C/EBPβ. Further, C/EBPβ phosphorylation, its interaction with NS1 and occupancy at the RIG-I promoter was associated with RIG-I transcriptional inhibition. These

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Conflict of interest: The authors declare that they have no conflicts of interest with the contents of this article.

The findings and conclusions in this report are those of the authors and do not necessarily represent the views of Centers for Disease Control and Prevention.

findings provide an important insight into the molecular mechanism by which influenza NS1 commandeers RIG-I transcriptional regulation and suppresses host antiviral responses.

Keywords

Influenza; RIG-I; NS1; C/EBPβ; transcriptional regulation

1. Introduction

The host innate immune system is critical for protection against invading microbes. To achieve this objective it relies on evolutionarily conserved pattern recognition receptors (PRRs) that sense structural motifs of invading microbes and initiate a rapid protective response (Ranjan et al., 2009). However, pathogens have developed strategies to overcome the host defence mechanisms and target host proteins involved in innate immune pathways and inhibit their function by expressing one or more viral antagonistic proteins. In the case of influenza virus, NS1, a 219-237 amino acid long viral protein, is critical for suppression of host antiviral response (Conenello et al., 2011; Guo et al., 2007; Mibayashi et al., 2007; Tisoncik et al., 2011; Yoshizumi et al., 2014). Pathogen sensor, RIG-I has been shown to be indispensable for protection against many viruses including influenza (Barral et al., 2009; Breiman et al., 2005; Cheng et al., 2006; Pichlmair et al., 2006; Ranjan et al., 2010; Yoneyama et al., 2015). Following influenza virus infection, RIG-I detects incoming viral genome and/or replication intermediates and undergoes an activation process that involves its structural rearrangement (Cui et al., 2008; Yoneyama and Fujita, 2008). Previously we (Guo et al., 2007; Ranjan et al., 2010) and others (Mibayashi et al., 2007; Pichlmair et al., 2006) have reported that influenza virus NS1 inhibits RIG-I-mediated type I interferon induction. Furthermore, we have recently shown that the NS1 expression in virus infected cells leads to inhibition of RIG-I expression (Ranjan et al., 2014). Influenza A virus NS1 protein is a multifunctional virulence factor that inhibits various cellular processes to facilitate viral gene expression, has been shown to intronic sequences with a subset of pre-mRNAs, including RIG-I pre-mRNA which can impair RIG-I expression (Zhang et al., 2018). Further, influenza virus NS1 protein binds cellular DNA to block transcription of antiviral genes (Anastasina et al., 2016). However, the molecular mechanism for the inhibition of RIG-I expression has not been characterized. Gene expression is under tight control of transcription factors that bind to unique DNA enhancer/repressor elements. We have shown earlier that Interferon Regulatory Factor-1 (IRF-1) positively regulates interferon- or dsRNA-induced RIG-I transcription (Su et al., 2007). In the present investigation, analysis of potential binding sites of transcription factors within the promoter region of RIG-I using TFSEARCH revealed NF-κB and C/EBPβ binding sites in addition to IRF-1. NF-κB and C/EBPβ belong to distinct families of transcription that target unique DNA enhancer elements. The transcription factor NF-kB consists of two subunits: a 50- and a 65-kDa proteins, and bind to κB enhancer motifs, found in promoters of a variety of genes in response to various stimuli (Baeuerle, 1991; Nolan et al., 1991; Stein et al., 1993). The C/EBPβ family of transcription factors belong to bZIP proteins (Vinson et al., 1989) characterized two motifs in the C-terminal half of the proteins, a basic region involved in DNA binding and a leucine zipper motif involved in dimerization. The C/EBP

family consists of several members including C/EBPα, C/EBPβ, C/EBPγ and C/EBPδ that form homo- and hetero-dimers (Akira et al., 1990; Cao et al., 1991; Poli et al., 1990). Transcriptional control by bzip family proteins is also determined by post-translational modifications. For example, C/EBPβ phosphorylation is involved in its translocation and formation of homo- or hetero-dimers that promote its binding to other factors and to regulatory elements (Chinery et al., 1997; Stein et al., 1993). Transcriptional regulation by C/EBPβ is rather complex as both transcriptional activation and repressor roles have been reported (Prosch et al., 2001; Zwergal et al., 2006).

The presence of a C/EBPβ binding site upstream of the transcription initiation site of RIG-I prompted us to investigate the role of C/EBPβ in RIG-I transcriptional activation during influenza virus infection.

2. Material and methods

2.1 Cell cultures, antibodies and reagents

Human lung epithelial cell line A549, and normal human bronchial epithelial (NHBE) cells (Lonza, Switzerland) were maintained as described (Ranjan et al., 2010). Transfection studies were carried out with expression vectors (1 μg) in cells (10^6/well) plated in 6-well plates. Virus infection was carried out with A/PR8/1934 (PR8), A/California/7/2009 (H1N1) (Influenza Research Repository, CDC, Atlanta, USA), A/Singapore/16-0019/2016 (H3N2) (https://www.seqirus.com) or PR8ΔNS1 (provided by Adolfo Garcia-Sastre, School of Medicine at Mount Sinai, NY, USA) viruses at a multiplicity of infection (MOI) of 1.0 or 1.0 focal forming units (f.f.u.) with trypsin supplementation as described previously (Ranjan et al., 2010). Antibodies used in this studies are Anti-RIG-I, anti-C/EBPβ, anti-phospho-C/EBPβ, anti-ERK, anti-phospho-ERK (Cell Signaling Technology, USA). Upstate Biotechnology), (Cell Signaling Technology, USA), (Cell Signaling Technology); Anti-NS1 antibodies were provided by Adolfo García-Sastre (Icahn, School of Medicine at Mount Sinai, NY, USA) Anti-myc, anti-β-actin antibodies and MEK inhibitor U0126 (Sigma Aldrich, USA); Rabbit IgG, polyclonal - Isotype Control (ChIP Grade) was purchased from Abcam (MA, USA).

pCAGGS myc-NS1 expression vector and pGL3-RIG-I promoter reporter were described previously (Guo et al., 2007; Su et al., 2007). NFκB and IFNβ luciferase reporter plasmid was obtained from Dr. Rongtuan Lin (McGill University, Canada). pCDNA hC/EBPβ expression vector and mutants T235A, Y274F and L320R were kindly provided by Dr. Mary B Goldring (Tsuchimochi et al., 2010).

2.2 Construction of RIG-I promoter reporter mutants

hC/EBPβ-deleted or P65-deleted RIG-I promoter luciferase reporters were constructed by using the QuickChange Lightning Site-directed Mutagenesis kit (Agilent Technologies, USA) and following manufacturer's protocol. The following primers were used: 5'tgggtacacaggtgtactcgaattgcacactcag3' and 5'ctgagtgtgcaattcgagtacacctgtgtaccca3' for hC/EBPβ deletion; 5'atgtaatcagtcattttggacaacaggtcctataaagctaaacatagac3' and 5' gtctatgtttagctttataggacctgttgtccaaaatgactgattacat3' for p65 deletion.

2.3 Nuclear extract preparation and Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extracts for EMSA were prepared using Nuclear Extract Kit (Active Motif, CA, USA). EMSA was performed using LightShift Chemiluminescent EMSA kit (Thermo Fisher Scientific, USA) as per manufacturer's instructions with some modifications. Instead of Biotinylated probe, we used Cy5-tagged probes for double stranded spanning the potential binding sites for transcription factor hC/EBPβ, non-specific and consensus hC/EBPβ EMSA cold probes (synthesized at CDC Biotechnology Core Facility, Atlanta, USA) that are shown in Fig.1. Protein-DNA complex was resolved on a % non-denaturing polyacrylamide gel. Cy5 labelled DNA was detected using Fluorescent Imaging Odyssey (Li-cor, Nebraska, USA). In template competition experiments, a 100-fold molar excess of cold non-specific or consensus hC/EBPβ probes were added 10 min prior to the addition of labelled probe.

2.4 Luciferase assay

A549 or NHBE cells were co-transfected with hC/EBPβ expression vector (2 μg/well) and pGL3-RIG-I, IFNβ or NFκB promoter luciferase-reporter plasmids (0.5 μg/well) and 10ng/well of pRL (Renilla luciferase) (for normalizing transfection efficiency) for indicated treatment and harvested at indicated time-points for luciferase activity using dual luciferase assay kit (Promega, USA) as per manufacturer's instructions. Results are normalized against Renilla luciferase activity.

2.5 siRNA knockdown studies

Custom designed gene-specific siRNAs (2 sets) to silence hC/EBPβ in A549 or NHBE were purchased from Thermo Fisher Scientific, USA, and were used as per the manufacturer's protocol.

2.6 Real Time RT-PCR

Total RNA was isolated from cells using the RNAeasy kit (Qiagen, USA) and real time RT-PCR was conducted using a Stratagene Mx3005P Q-PCR machine for mRNA expression of RIG-I, IFNβ, NPvRNA, RANTES and β-actin as described previously (Ranjan et al., 2014)

2.7 Gene profiler RNA expression analysis

RNA isolated from cells were also analyzed for a panel of antiviral genes using Human Antiviral Response PCR Array (QIAGEN, USA) as per manufacturer's protocol.

2.8 Co-immunoprecipitation and Immunoblotting

Cells plated in 6-well plates or 100-mm tissue culture plates were harvested in RIPA buffer (Sigma Aldrich, St Louis, USA), and cell lysates were incubated with primary antibody overnight at 4°C followed by incubation with protein A Dynabeads (Invitrogen, USA) for 2 h. The beads were washed three times with PBS, suspended in Laemmli buffer (62.5 mM Tris-HCl pH 6.8, 25% glycerol, 2% SDS, 0.01% Bromophenol blue), boiled for 10 min and centrifuged at 12 000 x g for 10 min at 4°C. Supernatants were collected and analyzed by western blotting as described previously (Ranjan et al., 2014).

2.9 Chromatin Immunoprecipitation (ChIP) Assay

ChIP assays were performed using formaldehyde-cross-linked nuclei lysates prepared from 4 x 10^6 of the indicated cells as described previously (Ranjan and Boss, 2006). Anti-NS1 and anti-C/EBPβ antibodies used in ChIP assays were purchased from Cell Signaling Technology, Danvers, MA, USA. Real-time PCR was performed in a Stratagene PCR machine. The following primer sets were used for the RIG-I and human CXCL10 promoters:

RIG-IFwd, GCGGGTGGGAATGTAAACTG

RIG-IRev, TCTGAGTGTGCAATTCGATGAG,

hCXCL10Fwd, TTT GGA AAG TGA AAC CTA ATT CA

hCXCL10Rev, CAG GAA CAG CCA GCA GGT TTT

These primer sets were used in a PCR with denaturation at 94 °C for 15 s, annealing at 57 °C for 30 s, and extension at 72 °C for 30 s. The amount of immunoprecipitated DNA was determined by comparison of the threshold cycle value with a standard curve generated using a known amount of genomic DNA as template. To correct for differences in chromatin preparations among samples, the values of immunoprecipitated DNA were normalized to the amount of chromatin DNA added to each ChIP reaction. All experiments were performed at least three times from independent chromatin preparations. The average values of the samples were plotted as fold over the TCR antibody control.

2.10 Cell Viability Assay

Cell viability assay was performed using MTT assay kit (Thermo Fisher Scientific, USA) as per manufacturer's protocol.

2.11 Statistical Analysis

To determine the statistical significance, we used analysis of variance (ANOVA) using GraphPad PRISM 5 (GraphPad Software) and a value of $p \leq 0.05$ was considered significant when compared with respective controls.

2. Results

2.1 RIG-I promoter contains C/EBPβ binding site

To identify transcription factors that are involved in regulation of RIG-I expression, potential binding sites of transcription factors within the ~400-bp promoter region of RIG-I were analysed with TFSEARCH software (http://www.cbrc.jp/papia/howtouse/howtouse_tfsearch.html). Putative consensus binding sites for NFκB, C/EBPβ and IRF-1 were identified with locations of 298, 215, and 4-bp upstream of the transcription initiation site, respectively (Fig. 1A). Transcription factors bind to their cognate DNA motifs in order to exert their function. To this end, we tested the binding ability of C/EBPβ to its putative site in the RIG-I promoter by electrophoretic mobility shift assay (EMSA). Sequences of the probes used in EMSA are shown (Fig. 1B). The probe spans the sequence from −201/−236 of the RIG-I promoter and contains the predicted C/EBPβ binding site. Nuclear extracts

from influenza virus A/PR8-infected A549 cells showed a shift in the band suggesting binding activity to cy5 labelled DNA containing the potential C/EBPβ binding element (Fig. 1C; lane 3). Nuclear extracts pre-incubated with cold competitor probes (predicted or consensus) in 100 molar excess failed to bind to the Cy5 probe (Fig. 1C; lanes 4 and 5). However, nuclear extracts pre-incubated with mutant non-specific cold probe did not prevent C/EBPβ binding to the Cy5 probe (Fig. 1C; lane 6 and 7). These findings suggest that C/EBPβ binds to its predicted binding site in RIG-I promoter.

2.2 C/EBPβ negatively regulates RIG-I transcriptional activation

Cytosolic pathogen sensor, RIG-I is critical for host protection against influenza infection (Hornung et al., 2006; Pichlmair et al., 2006; Ranjan et al., 2010). On the other hand, influenza virus NS1 supresses RIG-I-mediated antiviral response by interacting with RIG-I and TRIM25 (Gack et al., 2009; Mibayashi et al., 2007). Previously, we reported that influenza NS1 expression coincides with the inhibition of RIG-I expression in A549 cells (Ranjan et al., 2010; Ranjan et al., 2014). RIG-I transcription has been reported to be impaired in human lungs by pandemic H1N1 virus (Wu et al., 2012). However, the precise mechanism by which NS1 expression results in inhibition of RIG-I expression is unknown. During early stages of influenza infection NS1 may not be present, though a recent report suggests the presence of NS1 in virions (Hutchinson et al., 2014; Osterlund et al., 2012). However, the copy number of NS1 protein per virion is very low and may not be sufficient for inhibition of early type I interferon. Influenza virus NS1 accumulates in host cells as infection progresses. This also correlates with the infection dose of influenza virus. At lower MOI (0.5 MOI), we observed weak inhibitory action of NS1 on RIG-I expression and function (not shown). Time-kinetic studies of RIG-I protein expression by immunoblot revealed that RIG-I was detectable at 6 h post-PR8 influenza A virus (IAV) infection in A549 cells. However, RIG-I was undetectable at 24 h post-infection (Fig. 2A). This suggests that PR8-induced RIG-I expression was repressed during influenza virus infection. Notably, NS1 expression was detectable at as early as 6 h. It was further supported by the analysis of NP vRNA (Fig. 2B), RIG-I mRNA (Fig. 2C) and IFNβ (Fig. 2D) mRNA expression that are consistent with RIG-I and NS1 protein expression patterns following PR8 infection (Fig. 2A).

To investigate the functional role of C/EBPβ in RIG-I expression, we overexpressed human C/EBPβ in A549 cells followed by PR8-infection for 6 h. At this time-point RIG-I was detectable in PR8-infected lung epithelial cells. Overexpression of hC/EBPβ resulted in reduced RIG-I and increased NS1 protein expression when compared to vector-transfected cells (Fig. 2E). We also observed a slight but not significant increase in NP vRNA expression in C/EBPβ transfected A549 cells (Fig. 2F). Consistent with protein expression data, PR8 infection resulted in reduced RIG-I (Fig. 2G) and IFNβ (Fig. 2H) mRNA expression in hC/EBPβ transfected A549 cells.

To further confirm the role of C/EBPβ, we used gene-specific siRNA to knockdown C/EBPβ expression in A549 and NHBE cells. C/EBPβ siRNA transfection significantly decreased C/EBPβ protein expression in A549 (Fig. 2I) and in NHBE cells (Fig. S1A) as compared to scrambled siRNA controls. NP vRNA analysis showed slight decrease in

C/EBPβ knockdown A549 (Fig. 2J) and NHBE cells (Fig. S1B) which is consistent with NS1 protein expression (Fig. 2I and Fig. S1A). C/EBPβ knockdown resulted in increased RIG-I expression in A549 (Fig. 2I) and in NHBE cells (Fig. S1A) infected with PR8 virus at 6 h. Further, Knockdown of C/EBPβ by siRNA significantly ($p<0.05$) increased RIG-I and IFNβ mRNA expression following PR8 infection in A549 (Fig. 2K and 2L) and NHBE cells (Fig. S1C and S1D) at 6 h. Mock infection did not induce RIG-I protein expression or RIG-I and IFNβ mRNA expression in scrambled or C/EBPβ siRNA treated A549 or NHBE (Fig. 2K, 2L, S1C and S1D).

C/EBPβ knockdown studies were further confirmed by using another set of siRNA (pool) which significantly reduced C/EBPβ mRNA expression in NHBE cells (Fig. S1E). We did observe slight, but not significant increase in NPvRNA (Fig. S1F) at 6 h post-infection. However, C/EBPβ knockdown significantly increased RIG-I (Fig. S1G) and IFNβ (Fig. S1H) as observed previously.

To study RIG-I transcriptional regulation during influenza virus infection, a 1902-bp fragment containing the RIG-I promoter region cloned in a luciferase reporter plasmid was used as described previously (Su et al., 2007). The time-kinetics studies of RIG-I promoter following PR8 infection showed a time-dependent increase in promoter activation that peaked at 6 h and subsequently declined at 24 h (Fig. 2M). This is consistent with data shown in Fig. 2A and Fig. 2C. To determine the role of the C/EBPβ binding site within the RIG-I promoter, a mutant RIG-I promoter luciferase reporter construct with a deleted (−215/−230) C/EBPβ binding site was used. PR8 infection resulted in increase in the wild-type RIG-I promoter activity which peaked at 6 hr. (Fig. 2M). hC/EBPβ overexpression resulted in decrease in the wild-type RIG-I promoter activity (Fig. 2N).Notably, hC/EBPβ overexpression did not reduce C/EBPβ-deleted RIG-I promoter activation during influenza virus infection as observed in with the wild-type RIG-I promoter (Fig. 2N and 2O). As a control, another mutant RIG-I promoter luciferase reporter construct with a deleted NF-κB (p65) binding site (−298/−306) was used in this study. Deletion of the p65 binding site in the RIG-I promoter resulted in complete loss of promoter activation following PR8 infection as compared to wild type control (Fig. 2P and 2N). These findings confirm the repressor and the enhancer functions of C/EBPβ and NF-kB, respectively, on the RIG-I promoter.

Results of the above studies suggest that the C/EBPβ expression negatively regulates RIG-I and IFNβ expression. However, we observed only slight increase in NP vRNA expression which also indicates viral replication. These studies were carried out only at 6 h post infection, and viruses may not have undergone enough replication cycle. Therefore, we infected vector- or hC/EBPβ-transfected A549 cells with PR8 or its NS1-deficient counterpart, PR8ΔNS1 (1.0 MOI) and analysed NP vRNA, RIG-I and IFNβ mRNA expression as well as cell viability at 24 h post-infection. Data obtained showed a significant increase in NP vRNA (Fig. S2A) in C/EBPβ transfected when compared to vector control in PR8 infected A549 cells. RIG-I and IFNβ expression was not detectable and remained unchanged in vector or in hC/EBPβ transfected A549 cells infected with PR8. PR8ΔNS1 infection did not show significant difference in NP vRNA expression level in vector- or hC/EBPβ-transfected A549 cells. In contrast to PR8 infection, infection with PR8ΔNS1 increased RIG-I and IFNβ mRNA expression in both vector- or hC/EBPβ-transfected A549

cells (Fig. S2B and S2C). Analysis of cell viability by MTT assay revealed a significant decline in hC/EBPβ-transfected A549 cells, but not in vector-transfected A549 cells infected with PR8. PR8ΔNS1 infection did not cause cell death (Fig. S2D). Our results differ from reports (Ehrhardt et al., 2007; Jackson et al., 2010) that suggest that absence or loss in NS1 protein function promotes apoptosis in infected cells. One of the reasons for this discrepancy could be high m.o.i. of influenza A virus they used in their studies. Increased cell death in hC/EBPβ-transfected A549 cells following PR8 infection could be due to enhanced PR8 replication as a result of supressed RIG-I and IFNβ induction.

Negative regulation of RIG-I by hC/EBPβ at 24 h, was further confirmed by gene-specific siRNA knockdown experiments, where we knocked-down C/EBPβ and studied the expression of NP vRNA, RIG-I mRNA, IFNβ mRNA and cell viability at 24 h post-infection with PR8 or PR8ΔNS1. In hC/EBPβ-knockdown of A549 cells infected with PR8, NP vRNA mRNA expression level declined significantly when compared to control siRNA (Fig. S2E). We also observed increased RIG-I and IFNβ mRNA expression (Fig. S2F and S2G). In contrast to hC/EBPβ overexpression experiments, we did not observe significant decline in cell viability (Fig. S2H). On the other hand, infection with PR8ΔNS1, that lacks NS1, did not alter NP vRNA expression, and we did observe increased RIG-I and IFNβ mRNA expression. These data not only suggest a role for hC/EBPβ in the regulation of influenza virus replication and innate immune activation, but also implicates NS1 as critical regulator in these processes.

Different influenza virus strains can alter host response differently. Therefore, we infected A549 cells with pandemic H1N1 (pH1N1) and seasonal H3N2 viruses to study role of C/EBPβ. Overexpression of hC/EBPβ and siRNA knockdown of C/EBPβ in A549 cells resulted in decrease and increase in RIG-I expression respectively by both pH1N1 and H3N2 viruses similar to that of PR8 infection. This increase or decrease in RIG-I expression was well correlated with decrease or increase in NS1 expression. (Fig. S3A-F).

2.3 NS1-induced C/EBPβ phosphorylation inhibits RIG-I transcription

C/EBPβ transcriptional regulatory activity is controlled by several mechanisms including protein-protein interaction and post-translational modifications which also determines its activation (Kowenz-Leutz et al., 1994) and binding to DNA (Mahoney et al., 1992). In an un-induced state, C/EBPβ remains in a repressed inactive form and is activated by phosphorylation of its repressor domain located between the N-terminal transactivation domain and the C-terminal bzip region (Park et al., 2004; Piwien-Pilipuk et al., 2002; Piwien-Pilipuk et al., 2001). This domain contains several serine and threonine residues that are constitutively phosphorylated to some extent. C/EBPβ phosphorylation is carried out by several protein kinases (Park et al., 2004; Prusty et al., 2002). Human C/EBPβ contains known phosphorylation sites, including Thr^{235}, Thr^{266} and Thr^{273} in the DNA binding domain of C/EBPβ, and that have been shown to be critical for transcriptional functions (Park et al., 2004). ERK-dependent phosphorylation of C/EBPβ on Thr235 by ERK has been reported to promote its transcriptional activity. To assess the role of C/EBPβ phosphorylation, we used U0126, a selective inhibitor of both MEK1 and MEK2(a type of MAPK/ERK kinase) which inhibits ERK activation (Zhao et al., 2013). In mock infected

A549 cells, DMSO or U0126 alone did not induce RIG-I expression. Furthermore, DMSO treatment did not inhibit PR8-induced RIG-I expression. However, in the presence of U0126, PR8 infection resulted in increased RIG-I expression. U0126 inhibited C/EBPβ and ERK phosphorylation as compared to DMSO treatment in A549 cells (Fig. 3A). In addition, we tested U0126 on RIG-I promoter activity induced by PR8 or NS1ΔPR8 (PR8 that lacks functional NS1) infection. In the presence of DMSO, both PR8- and PR8ΔNS1, induced RIG-I promoter activity. Notably, PR8ΔNS1-induced RIG-I promoter activation was significantly ($p<0.05$) higher as compared to that of PR8, consistent with a role for NS1 in inhibiting RIG-I transcription. U0126 treatment enhanced RIG-I promoter activity in both PR8- and PR8ΔNS1-infected A549 cells when compared to their respective DMSO controls at 6h (Fig. 3B). It has been suggested that U0126 inhibits IAV replication (Droebner et al., 2011). Our data indicate that MEK1/2 U0126 inhibitor promoted RIG-I and IFNβ induction which can impact IAV replication. Longer incubation in U0126 resulted in increased cell death (not shown).

These findings indicate that ERK-mediated phosphorylation of C/EBPβ may inhibit transcriptional activity on the RIG-I promoter. To investigate further, we used several human C/EBPβ mutant constructs: T235A (affects phosphorylation and the consensus ERK phosphorylation site at Thr-235); Y274F (affects DNA binding) and L320R (affects protein dimerization) (Tsuchimochi et al., 2010). A549 cells were co-transfected with vector, wild-type C/EBPβ or mutant hC/EBPβ constructs and RIG-I promoter reporter constructs followed by PR8 infection. Similar to previous findings, wild-type C/EBPβ significantly ($p<0.05$) inhibited PR8-induced RIG-I promoter activation as compared to vector control. Remarkably, T235A mutant expression vector failed to inhibit PR8-induced RIG-I promoter activation, and RIG-I promoter activation was comparable to that of vector-transfected A549 cells. Similar results were observed for Y274A and L320R mutants (Fig. 3C). These findings not only strongly support a role of C/EBPβ phosphorylation in RIG-I transcriptional regulation but also indicate Thr^{235} phosphorylation site as critical determinant. Additionally, these results also indicate the significance of phosphorylation dependant DNA binding and dimerization of C/EBPβ in context to RIG-I promoter regulation.

2.4 NS1 forms complex with C/EBPβ

C/EBPβ phosphorylation following NS1 overexpression or PR8 infection in A549 cells, prompted us to investigate if influenza NS1 interacts with C/EBPβ. To this end, we carried out co-immunoprecipitation studies. A549 cells were infected with PR8 (1.0 MOI) and were subjected to co-immunoprecipitation using anti-NS1 or anti-C/EBPβ antibodies. Immunoprecipitation of NS1 or C/EBPβ co-purified C/EBPβ and NS1 in PR8-infected A549 cells (Fig. 3D).

NS1 is crucial for counteracting host antiviral response and is required for virus fitness during its replication cycle in the host (Garcia-Sastre et al., 1998; Hale et al., 2008). In addition, NS1 has been shown to interfere with antiviral gene expression (Marazzi et al., 2012). Our data from the PR8ΔNS1 infection experiments were consistent with a potential role for NS1 in decreasing RIG-I transcription. To investigate whether influenza

NS1 could alter RIG-I transcriptional activation, A549 cells were transfected with vector alone or myc-NS1 expression vector and were analysed for C/EBPβ phosphorylation. myc-NS1 transfection alone induced C/EBPβ (1.6-fold) and ERK (1.8-fold) phosphorylation as determined by densitometric analysis (Fig. 3E). This also suggests that NS1 was sufficient to induce C/EBPβ phosphorylation even in the absence of virus infection or replication. To evaluate its functional impact, we studied RIG-I and IFNβ promoter activation induced by PR8ΔNS1. As expected NS1 overexpression significantly ($p<0.05$) reduced PR8ΔNS1-induced promoter activity of RIG-I and IFNβ when compared to vector control (Fig. 3F and 3G).

We also investigated if myc-NS1 transfection in A549 cells results in its interaction with C/EBPβ. To this end, we carried out co-immunoprecipitation studies in myc-NS1 transfected A549 cells using anti-myc or anti-C/EBPβ antibodies. Immunoprecipitation of NS1 or C/EBPβ co-purified NS1 and C/EBPβ (Fig. 3H). These data also suggest that NS1 interaction with C/EBPβ can take place even in the absence of active virus replication.

2.5 NS1 promotes phospho-C/EBPβ recruitment to the RIG-I promoter

As a transcriptional regulator, C/EBPβ is recruited to promoters of genes it regulates. Presence of potential C/EBPβ binding site at RIG-I promoter, and the physical and functional interaction between NS1 and C/EBPβ prompted us to investigate if NS1 and/or C/EBPβ gets recruited to the RIG-I promoter and whether C/EBPβ phosphorylation is required for this recruitment. We carried out chromatin immunoprecipitation (ChIP) assays coupled with qRT-PCR studies in A549 cells infected with PR8 or PR8ΔNS1 in the presence or absence of MEK1/2 inhibitor U0126 (Fig. 4). Notably, only PR8 infection induced a time-dependent increase in phospho C/EBPβ occupancy at the RIG-I promoter that peaked at 12 h post-infection (Fig. 4A). PR8ΔNS1-induced C/EBPβ recruitment to the RIG-I promoter was comparable with controls at any time point of this study. U0126 completely inhibited PR8-induced C/EBPβ recruitment to the RIG-I promoter. These results suggest NS1-dependent recruitment of phosphorylated C/EBPβ on the RIG-I promoter. We also investigated NS1 recruitment to RIG-I promoter. NS1 recruitment to RIG-I promoter was evident at 6 h post-infection which reached maximum at 12 h. Intriguingly, U0126 also suppressed NS1 recruitment at the RIG-I promoter (Fig. 4B).

We also carried out ChIP analysis in A549 cells, transfected with wild-type hC/EBPβ-expression vector or its mutants T235A, Y274F or L320R and infected with PR8. p-hC/EBPβ immunoprecipitation did not show the presence of RIG-I promoter in vector or mutant hC/EBPβ-transfected A549 cells. However, we did observe an increased p-C/EBPβ binding to RIG-I promoter in wild-type hC/EBPβ-transfected A549 cells following PR8 infection (Fig. 4C). To determine the specificity of hC/EBPβ binding to RIG-I promoter, we analysed CXCL10 promoter which is known to have a C/EBPβ-binding site, and C/EBPβ binding negatively regulates its expression (Brownell et al., 2013). We did not detect presence of CXCL10 promoter in p-hC/EBPβ immunoprecipitates (Fig. 4D) or in NS1 immunoprecipitation in response to PR8 or PR8ΔNS1 infection in the presence or absence of U1026 (Fig. 4E). These data clearly demonstrate that C/EBPβ binding to its RIG-I promoter is specific. Interestingly hC/EBPβ pulldown revealed the presence of CXCL10

in wild-type hC/EBPβ overexpressing A549 cells (Fig. 4F). Chip with Isotype control antibodies did not show the presence of RIG-I promoter (Fig. S4).

Results of above studies indicate that the transcription factor, C/EBPβ negatively regulates RIG-I transcription. Given that C/EBPβ can bind to several other genes in response to various immune stimuli, it is not clear whether overexpression or siRNA knockdown of C/EBPβ could alter the expression of other genes as well. To address this, we analysed a panel of antiviral genes in A549 cells transfected with hC/EBPβ or hC/EBPβ-siRNA and infected with PR8. PR8 infection resulted in upregulation of several genes including RIG-I (DDX58), IFNβ and CXCL10 (Fig. 5). However, this also includes upregulation of type I interferon-responsive genes as a result of RIG-I activation. IFNβ is known to induce a number of PRRs and other immune genes in an autocrine and paracrine manner (Ranjan et al., 2014) and could have impacted other gene expression in these experiments. C/EBPβ knockdown upregulated many other genes including RIG-I and CXCL10. In contrast, overexpression of hC/EBPβ downregulated the expression of these genes. However, several genes did not show any change in their expression pattern following PR8 infection or hC/EBPβ transfection or its knockdown. These data provide evidence that negative role of C/EBPβ was specific to certain genes including RIG-I.

4. Discussion

Results of the present study clearly indicate a repressor role for C/EBPβ in RIG-I transcriptional activation by influenza A virus infection. The NS1 protein of influenza A viruses is a known antagonist of host interferon, and has been shown to target multiple innate immune receptors including RIG-I (Guo et al., 2007; Ranjan et al., 2010) and others (Mibayashi et al., 2007; Pichlmair et al., 2006). However, the precise mechanism(s) that leads to RIG-I transcriptional inhibition is not known. In this investigation, we show that influenza NS1 suppresses RIG-I transcription via transcription factor C/EBPβ and thus contributes to NS1 interferon antagonizing functions as one of the mechanisms. Negative regulation of genes by C/EBP family proteins has been reported previously. C/EBPβ has been shown to block p65 phosphorylation and inhibit NF-κB-mediated transcriptional activation in TNF-tolerant cells (Zwergal et al., 2006). C/EBPβ also interacts with p65 and inhibits human CMG IE1/2 enhancer promoter (Prosch et al., 2001). Presence of adjacent or overlapping binding sites for C/EBP and NF-κB is not uncommon and promoters of many immune and acute phase response genes possess this feature (Lopez-Rodriguez et al., 1997; Stein and Baldwin, 1993). C/EBPβ has been shown to synergistically activate promoter with C/EBPβ binding sites, while they inhibit promoter with functional κB motifs (Prosch et al., 2001; Stein and Baldwin, 1993). One of the explanations for this inhibition is cross-coupling between NF-κB/Rel and C/EBP family proteins (Stein et al., 1993). Interestingly, RIG-I promoter contains binding sites for both NF-κB and C/EBPβ that are 60bp apart from each other. In the present study, we observed NS1 and C/EBPβ complex formation and its recruitment to RIG-I promoter. It is likely that NS1 stabilizes the C/EBPβ-DNA complex by forming complex with C/EBPβ. Another possibility is that NS1 transiently interacts with C/EBPβ and triggers a conformational change in C/EBPβ that favours increased C/EBPβ binding to its DNA binding sites. The molecular nature of NS1 and C/EBPβ interaction is not known and needs further investigation. However, our MEK1/2 kinase inhibition studies

together with C/EBPβ mutants experiments (Fig. 3) suggest that ERK-induced-C/EBPβ phosphorylation, dimerization and DNA binding ability are critical for its inhibitory action on RIG-I promoter. While the precise mechanism of this inhibition is unknown, it is likely that NS1-C/EBPβ complex formation at C/EBPβ binding site on RIG-I promoter may prevent p65 binding to RIG-I promoter, thereby, abolishing p65 enhancer function. In the present study p65 enhancer function was confirmed by using p65 promoter mutant (Fig. 2). Another likelihood is that NS1 interaction with p65, makes p65 unavailable to bind to RIG-I promoter and perform its enhancer function. These data also indicate that C/EBPβ phosphorylation is associated with the level of transcriptional control by which C/EBPβ DNA-binding activity is regulated. Apart from DNA binding, C/EBPβ phosphorylation of a MAPK site adjacent to bZIP region coincides with a conformation change of C/EBPβ that facilitates direct interaction with distinct mediator complexes (Mo et al., 2004). It is likely that NS1 triggers conformational changes in C/EBPβ via phosphorylation, thereby, enabling complex formation with NS1 and other mediators. Although further studies are needed to understand the functional signification of NS1 and C/EBPβ interaction at RIG-I promoter, these ideas support independent models for NS1-mediated RIG-I transcriptional inhibition.

In the present study, we identified C/EBPβ as an important transcription factor for RIG-I transcriptional regulation in influenza virus infection. Our study revealed a novel mechanism by which NS1 supresses RIG-I transcription via C/EBPβ phosphorylation, NS1-C/EBPβ complex formation and its recruitment to the RIG-I promoter.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements:

PBF holds the Thelma Newmeyer Corman Chair in Cancer Research in the VCU Massey Cancer Center (MCC) and DS is a Harrison Scholar in the VCU MCC.

AGS: This work was partly supported by CRIP (Center for Research on Influenza Pathogenesis), an NIAID funded Center of Excellence for Influenza Research and Surveillance (CEIRS, contract # HHSN272201400008C) to AG-S

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Highlights

- We identified C/EBPβ binding site in the RIG-I promoter as a repressor element.
- C/EBPβ overexpression and siRNA knockdown resulted in suppression and activation of RIG-I expression respectively.
- Influenza virus NS1 binds with C/EBPβ and promotes its occupancy at RIG-I promoter and prevents RIG-I expression.

A

AAGTTAAAATTAAAAAAAAAAAAGAAAGGCTGACCAATGTGTCTATCTACTGGAGCTCTC
CTACTGCGGGTGGGAATGTAAACTGGATGTAATCAGTCATTTTGGACAACAGGTGGCGGT p65 (-298/-306)
TTCCTATAAAGCTAAACATAGACTTACCCTGGCACCCAGCCGACGTGGGAGAACTGGGTA
CACAGGTGTACACTTTACAAAACTCATCGAATTGCACACTCAGATGTGTGTCATTTTACT C/EBPβ (-215/-230)
GTGTCTAAACGTTAGCGCTATAAAAGCAAAACAAAATATAAGATCATAAAAATAAAAGCA
ATTCCTAGGGGTCCTCTCCGTTCCCGGGCTTCCTCGGTGCGGAGGGAAACGAAACTAGCC
CGAGGCAAAACAGCCTCCGCGAACCCCGCCCGCCGCTAGTTGCACTTTCGATTTTCCCTT IRF-1 (-4/-18)

B

EMSA probes	Sequence (5'-3')
predicted C/EBPβ binding	GTGTACACTTTACAAAACTCATCGAATTGCACACT
consensus C/EBPb binding site	CCTTACCTT TTA GTCTTTCAACAAACT
mutant 1 C/EBPb binding site	GTGTACGGAGTGGAGGGAGGGTCGAATTGCACACT
mutant 2 C/EBPb binding site	GTGTACGGGGGGGGGGGGGGTCGAATTGCACACT

C

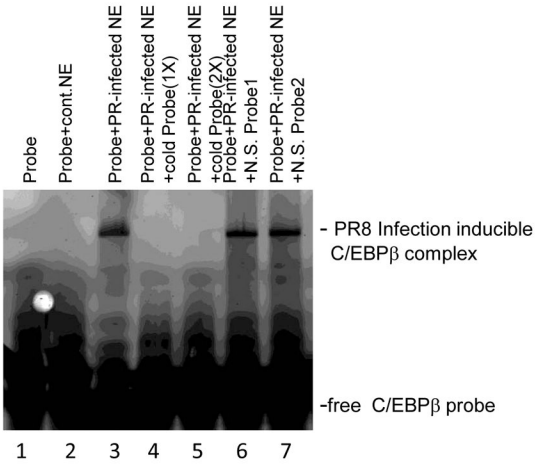

Fig. 1.

hC/EBPβ binds to predicted binding site on RIG-I promoter. (A) RIG-I promoter region sequence with potential regulatory elements (underlined) identified by TFSEARCH. (B) Sequence of probes used in EMSA. (C) EMSA showing hC/EBPβ binding to hC/EBPβ sequence in RIG-I promoter. Lanes 1, free Cy5 hC/EBPβ probe without incubation with A549 nuclear extracts (NE); lane 2, Cy5 hC/EBPβ probe incubated with uninfected A549 NE; Lane 3, Cy5 hC/EBPβ probe incubated with PR8-infected A549 NE; lane 4, PR8-infected A549 NE pre-incubated with hC/EBPβ cold competitor (100 molar excess) followed by Cy5 probe incubation; lane 5, PR8-infected A549 NE pre-incubated with consensus cold competitor (100 molar excess) followed by Cy5 probe incubation; lane 6, PR8-infected A549 NE pre-incubated with nonspecific (N.S.) cold competitor 1 (100 molar excess) followed by Cy5 probe incubation; lane 7, PR8-infected A549 NE pre-incubated with nonspecific (N.S.) cold competitor 2 (100 molar excess) followed by Cy5 probe incubation. Probes were separated on a 4-12% gradient non-denaturing polyacrylamide gel and detected using Fluorescent Imaging Odyssey as described.

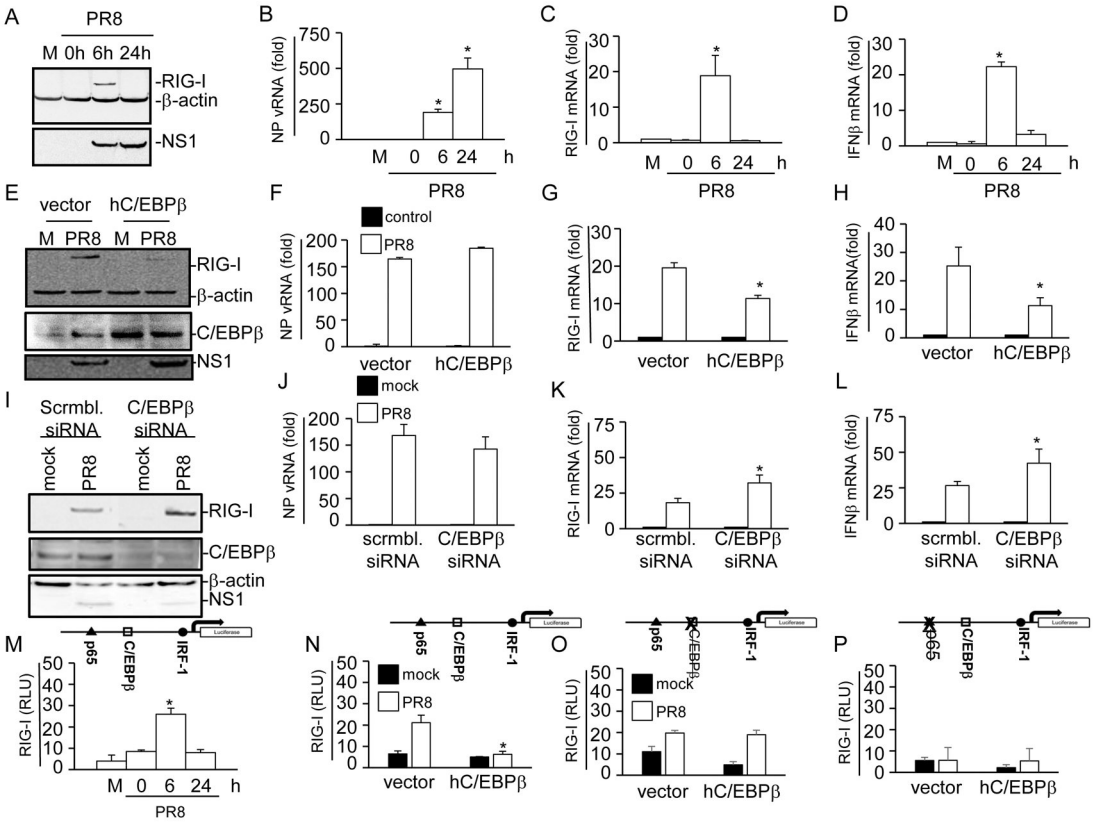

Fig. 2.

hC/EBPβ negatively regulates RIG-I and IFNβ activation. A549 cells were untransfected or co-transfected with vector or hC/EBPβ expression vector/ control siRNA or hC/EBPβ siRNA and RIG-I or mutant RIG-I promoter luciferase reporter plasmids for 24 h. Cells were then infected with PR8 (1.0 MOI) for indicated time periods and harvested for various analysis. A549 cells were mock infected or infected with PR8 (1.0 MOI) infection at indicated time period and analysed for (A) RIG-I and NS1 protein expression by western blot and (B) NPvRNA, (C) RIG-I mRNA and (D) IFNβ mRNA expression by qRT-PCR. A549 cells transfected with vector or hC/EBPβ expression vector were mock infected or infected with PR8 (1.0 MOI) infection for 6 h and analysed for (E) RIG-I, C/EBPβ and NS1 protein expression by western blot and (F) NPvRNA, (G) RIG-I mRNA and (H) IFNβ mRNA expression by qRT-PCR. A549 cells transfected with control siRNA or hC/EBPβ siRNA were mock infected or infected with PR8 (1.0 MOI) infection for 6 h and analysed for (I) RIG-I, C/EBPβ and NS1 protein expression by western blot and (J) NPvRNA, (K) RIG-I mRNA and (L) IFNβ mRNA expression by qRT-PCR. (M, N, O and P) Luciferase reporter activity (RLU) in A549 cells untransfected or co-transfected with vector or hC/EBPβ expression vector and RIG-I promoter luciferase reporter plasmids for 24 h, followed by PR8 (1.0 MOI) infection for 6 h. Data shown are means ± S.D. from three independent experiments. *p<0.05 compared to respective controls.

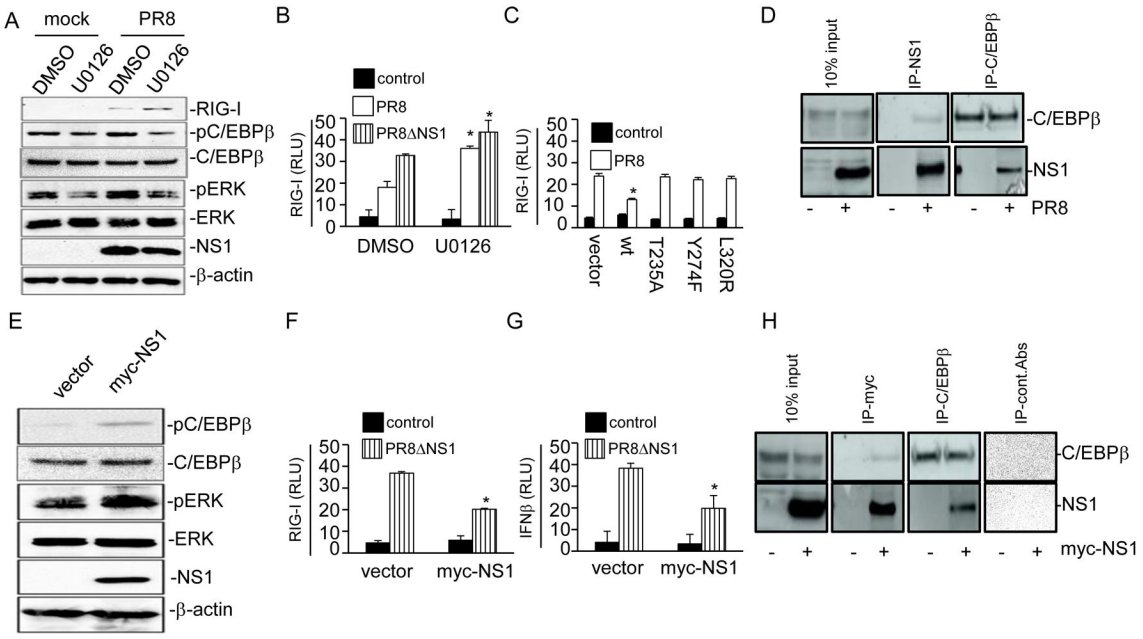

Fig. 3.

NS1 promotes hC/EBPβ phosphorylation and interacts with hC/EBPβ. A549 cells, untransfected or co-transfected with vector, wild type hC/EBPβ or mutants threonine 235 to alanine (T235A), tyrosine 274 to phenylalanine (Y274F) or leucine 320 to arginine (L320R) or myc-NS1 expression vector and/or RIG-I promoter reporter plasmids for 24 h. Cells were then infected or mock infected with PR8 or PR8ΔNS1 (1.0 MOI) for 6 h in the presence or absence of U0126 (10 μM). (A) Immunoblot analysis of RIG-I, phC/EBPβ, hC/EBPβ, pERK, ERK and NS1 in A549 cells mock infected or infected with PR8 (1.0 MOI) for 6 h in the presence or absence of U0126 (10 μM). (B) RIG-I promoter activity (RLU) in A549 cells transfected with RIG-I promoter reporter plasmids for 24 h followed by mock or PR8 or PR8ΔNS1 (1.0 MOI) infection for 6 h in the presence or absence of U0126 (10 μM). (C) RIG-I promoter activity (RLU) in A549 cells, transfected with vector or wild type hC/EBPβ or hC/EBPβ mutants threonine 235 to alanine (T235A), tyrosine 274 to phenylalanine (Y274F) or leucine 320 to arginine (L320R) and/or RIG-I promoter reporter plasmids for 24 h followed by mock or PR8 or PR8ΔNS1 (1.0 MOI) infection for 6 h. (D) Total cell lysate from PR8-infected A549 cells were subjected to immunoprecipitation using anti-C/EBPβ or anti-NS1 antibodies. Immunoprecipitates were analysed for presence of NS1 and hC/EBPβ by immunoblotting. (E) Immunoblot analysis of phospho hC/EBPβ, hC/EBPβ, pERK, ERK and NS1 in A549 cells transfected with vector or myc-NS1 expression vector. (F) RIG-I promoter activity (RLU) in A549 cells co-transfected with RIG-I promoter reporter plasmids and vector or myc-NS1 expression vector, followed by mock or PR8ΔNS1 (1.0 MOI) infection. (G) IFNβ promoter activity (RLU) in A549 cells co-transfected with RIG-I promoter reporter plasmids and vector or myc-NS1 expression vector, followed by mock or PR8ΔNS1 (1.0 MOI) infection. (H) Total cell lysate from vector or myc-NS1 expression vector transfected A549 cells were subjected to immunoprecipitation using anti-myc, anti-C/EBPβ or control antibodies. Immunoprecipitates were analysed for presence of NS1

and hC/EBPβ by immunoblotting. Data shown are means ± S.D. from three independent experiments. *p<0.05 compared to respective controls.

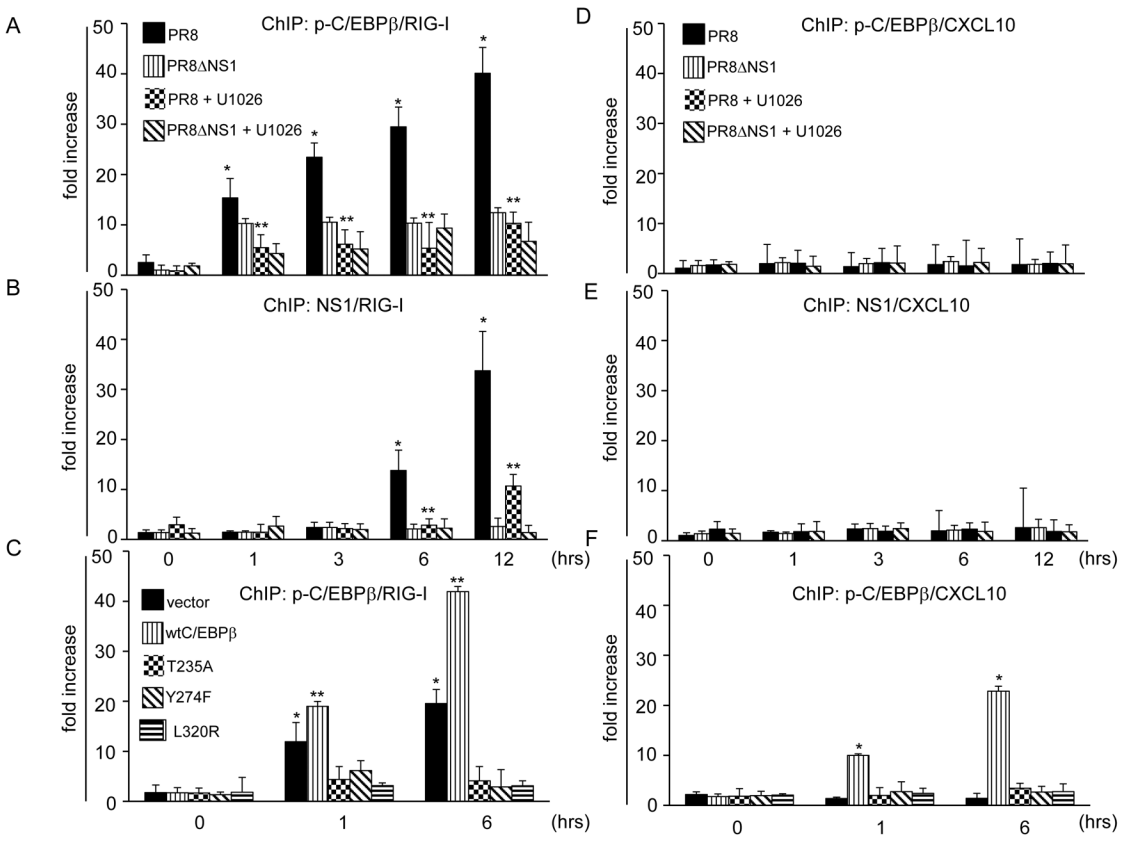

Fig. 4.

NS1 co-operatively enhances hC/EBPβ recruitment at the RIG-I promoter region. ChIP assay using antibodies against phospho-hC/EBPβ and NS1 coupled with real-time PCR were performed on A549 cells infected with PR8 (1.0 MOI) or NS1ΔPR8 (1.0 MOI) in the presence or absence of U0126 at the indicated times. The precipitated DNA was analysed for the presence of RIG-I or CXCL10 promoter region by quantitative real-time PCR. (A) p-hC/EBPβ occupancy at RIG-I promoter in A549 cells infected with infected with PR8 or NS1ΔPR8 in the presence or absence of U0126. (B) NS1 occupancy at RIG-I promoter in A549 cells infected with PR8 or NS1ΔPR8 in the presence or absence of U0126. (C) p-hC/EBPβ occupancy at RIG-I promoter in A549 cells transfected with vector, wt hC/EBPβ or mutants T235A, Y274F or L320R followed by PR8 or NS1ΔPR8 infection. (D) p-hC/EBPβ occupancy at CXCL10 promoter in A549 cells infected with infected with PR8 or NS1ΔPR8 in the presence or absence of U0126. (E) NS1 occupancy at CXCL10 promoter in A549 cells infected with infected with PR8 or NS1ΔPR8 in the presence or absence of U0126. (F) p-hC/EBPβ occupancy at CXCL10 promoter in A549 cells transfected with vector, wt hC/EBPβ or mutants T235A, Y274F or L320R followed by PR8 or NS1ΔPR8 infection. All data were normalized to the amount of chromatin added to each precipitation reaction and expressed as fold-increase over irrelevant (TCR) antibody controls. Data shown are means ± S.D. from three independent experiments. *p<0.05 compared to respective controls.

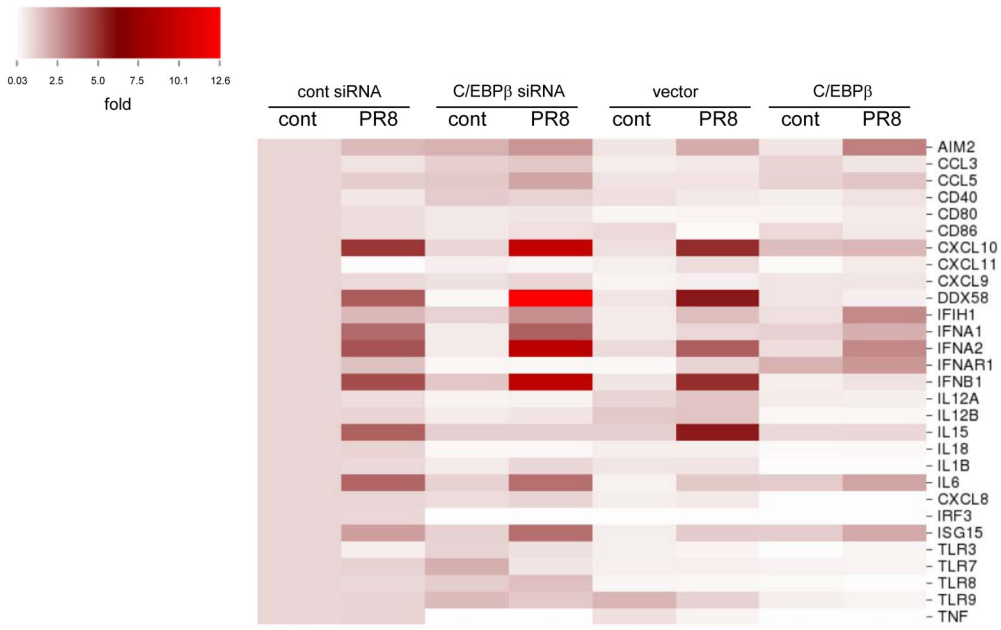

Fig. 5.

Heat map of differentially expressed genes based on PCR Array analysis. NHBE cells were transfected with vector, hC/EBPβ, control siRNA or hC/EBPβ siRNA for 24h. Cells were then mock infected or infected with PR8 (1.0 MOI) for 6 h. Gene expression were analysed by real-time RT-PCR using PCR array kit as per manufacturer protocol. Data shown are average from two independent experiments. The scale shows the level of gene expression where red corresponds to upregulation (log2 fold).