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Title: NFκB2 p52 has a role in antiviral immunity through IKKα-dependent induction of Sp1 and IL-15

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Running Title: A role for NFκB2 in anti-*viral immunity*

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Background: IKKα can promote the ability of p52 to transactivate gene expression in a manner requiring p65.
Results: p52 is induced by TLR3 activation and regulates Sp1 transcription. Sp1 promotes the transcription of IL-15. Both events require the presence of IKKα and p52.
Conclusion: p52 is a target for IKKα in anti-viral immunity.
Significance: This study reports a role for NFκB2 in the induction of anti-viral gene expression.

SUMMARY:
In this study we describe a previously unreported function for NFκB2, an NFκB family transcription factor, in antiviral immunity. NFκB2 is induced in response to Poly(I:C), a mimic of viral dsRNA. Poly(I:C), acting via TLR3, induces p52-dependent transactivation of a reporter gene in a manner that requires the kinase activity of IKKα and the transactivating potential of RelA/p65. We identify a novel NFκB2 binding site in the promoter of the transcription factor Sp1 which is required for *Sp1* gene transcription activated by Poly(I:C). We show that Sp1 is required for IL-15 induction by both Poly(I:C) and Respiratory Syncitial Virus, a response that also requires NFκB2 and IKKα. Our study identifies NFκB2 as a target for IKKα in anti-viral immunity and describes, for the first time, a role for NFκB2 in the regulation of gene expression in response to viral infection.

INTRODUCTION:

NF□B2 (p100/p52), is a member of the NF□B family of transcription factors, that comprises five mammalian members: Rel/c-Rel; RelA/p65; RelB; NF□B1 (p50 and its precursor p105); and NF□B2 (p52 and its precursor p100). These proteins exist in various homo- and heterodimeric complexes and control many biological processes, particularly in inflammation and immunity (1). There are two distinct NF□B activation pathways termed the canonical and the alternative pathways. The canonical pathway is the better characterised NF□B pathway. It is activated by innate immune receptors, inflammatory cytokines and stress pathways, and leads to the phosphorylation of the inhibitory subunit I□B□ by IKK□ and IKK□, leading to its degradation. The subsequent release and nuclear translocation of the p50/p65 dimer leads to the induction of a wide range of immune and inflammatory genes (1). The alternative pathway involves NF□B2. Known activators of this pathway are CD40, BAFF receptor and LT-□ receptor. Activation of this pathway involves NF□B-inducing kinase (NIK). NIK activates IKK□ which phosphorylates p100, causing p100 to be partially processed to produce the active p52 subunit. Typically p52 is described as part of a heterodimer with RelB. This complex is essential for NF□B2's role in humoral immunity and secondary lymphoid organogenesis.

A third IKK, termed IKK□ has been described. IKK□ is activated downstream of the dsRNA receptors TLR3, RIG-I and MDA5 and by TLR4 and IFN-□ (2, 3) and, in turn, activates the transcription factors IRF3, IRF7, STAT1 and p65. IKK□ has also been shown to be activated by Respiratory Syncitial Virus (RSV) and Influenza B virus (3, 4).

We identified p52 as a binding partner for IKK□ in a yeast-two-hybrid screen and subsequently determined that over expression of IKK□ could promote the transactivating potential of p52 (5), however the functional importance of this interaction remains elusive. Here we report the uncovering of a signalling pathway activated by TLR3, or RSV, that involves IKK□, NF□B2 and p65. We identify a conserved binding site for p52 on the Sp1 promoter and confirm IL-15, an anti-viral cytokine, as a target for Sp1 on this pathway. Our study provides a previously undescribed function for both NF□B2 and Sp1 in anti-viral immunity.

EXPERIMENTAL PROCEDURES:

Reagents and plasmids - LPS, (Alexis Corporation), Poly(I:C) and Poly(A:U) (Invivogen). Antibodies; p100/p52 cell signalling #4882, □-actin Sigma-Aldrich #A1978, Sp1 Milllipore #07-645. Oligonucleotides (Eurofins). Taqman probes (Applied Biosystems). FLAG-IKK□ and IKK□(K38A) were provided by Shizuo Akira (Osaka University, Japan). The TBK1-encoding plasmid was a gift from Dr. Makato Nakanishi (National Institute for Longevity Sciences, Japan). HA-p52 was a gift from Neil Perkins (University of Dundee, Scotland). Gal-luciferase reporter gene (Stratagene). The construction of the p52-Gal4 has been described (5). TRAF1, TRAF3, TRAF6, RIP1 and Nap1 plasmids were gifts from Andrew Bowie (Trinity College Dublin, Ireland). The HA-p65(S536A) plasmid was generated from the HA-p65 plasmid using the QuikChange XL site-directed mutagenesis kit (Stratagene).

Cell culture and Isolation – WT and IKK□+/- MEFs obtained from Shizuo Akira (Osaka University, Japan) and HEK293 cells, HEK293 cells stably expressing either TLR3 (HEK293-TLR3) (Invivogen), were cultured in DMEM. WT, NF□B2-/- and IKK□+/- (Kate Fitzgerald, University of Massachusetts, USA) bone marrow, were isolated from the tibias and femurs of C57/Bl6 mice, resulting cells were grown in MCSF conditioned DMEM. Human peripheral blood mononuclear cells (hPBMC) were isolated from whole blood using a Ficoll gradient and cultured in RPMI. In all cases, DMEM and RPMI medium were supplemented with 10% fetal calf serum, 2 mm l-glutamine, 1% penicillin/streptomycin solution (v/v). Cells were plated at 1 x 10^5 cells/ml, treated as described, before isolation of RNA or lysate for qPCR or Western blot respectively.

Sp1-promoter luciferase construct- For construction of the Sp1-promoter- luciferase reporter gene, we cloned its 5' regulatory region of Sp1 from -1303 nt from the translational start site

(ATG) between the *Nhe*I and *Xho*I sites of the reporter luciferase vector pGL3-enhancer (Promega). Progressive deletion constructs were generated using a common reverse primer and five different forward primers. The *Sp1*-specific sequences for these primers were taken from the EMBL-EBI AF261690 source (in uppercase, see below). For the forward primers these specific sequences were preceded by an arbitrary sequence (in lowercase, see below) including a *Nhe*I restriction site (italics). The reverse primer followed a similar structure but contained a *Xho*I restriction site (italics) in the arbitrary sequence. The numbers indicated after the primer sequences correspond to the distance in nt from the 5′-end of the sequence in uppercase to the translational start site.

For 5'
tcaagtcag*ggctagc*TTGCTTTATGCATAGGCGGT-3'
(-1303)
For 5'
tcaagtcag*ggctagc*CGGATTCTGGTTGGCCGTTGT-3'
(-477)
For 5'
tcaagtcag*ggctagc*CTATCAAAGCTTTGCCTATCC-3'
(-443)
For 5'
tcaagtcag*ggctagc*GGGAGCCCGCCTGCCGGTTG -3'
(-415)
For 5'
tcaagtcag*ggctagc*TCCTTCCAAGCCAATCATCTCC-3'
(-388)
for 5'
tcaagtcag*ggctagc*GCTCCCGCCCATCTTCACTTC -3'
(-365)
Rev 5'
cagtgctgc*ctcgag*GCTCAAGGGGGTCCTGTCCGG
3' (-20)

Transfection-based Reporter Gene Assays – Cells were transfected with GeneJuice transfection reagent (Novagen, Madison, WI) with a total amount of 350–400 g/well containing 150ng of p-55UAS$_G$Luc and 50 ng of p52-Gal4 fusion construct (MEFs) or with a total of 250ng of DNA containing 100ng of p-55UAS$_G$Luc or 30ng of p52-Gal4 (HEK293s). Assays also contained the plasmid DNA of interest, an empty vector and 30ng of *Renilla reniformis* luciferase construct. For Sp1-promoter-luciferase assays, HEK293 cells were transfected with a total amount of 220ng of DNA/well comprising 80ng of reporter construct, the plasmid DNA of interest, 40ng of *R. reniformis* luciferase construct, and empty vector. Cell extracts were monitored 24–36h post-transfection for firefly luciferase activity following standard protocols with values were normalized for transfection efficiency with *R. reniformis* luciferase.

RNA extraction and PCR - MEFs and BMDM or hPBMC were set up at 5×10^5 or 1×10^6 cells/ml, respectively. Cells were stimulated with Poly(I:C). Total RNA was extracted using the RNeasy kit (Qiagen). For mRNA expression analysis, cDNA was prepared from 20 to 100 ng/ml total RNA using the High-Capacity cDNA archive kit (Applied Biosystems). Individual mRNAs were monitored with the following inventoried The AB7900FAST platform (Applied Biosystems) was used for all PCR, done in triplicate. Changes in expression were calculated by the change in threshold (C_T) method with *Gapdh* as an endogenous control for gene-expression analysis and were normalized to results obtained with untreated cells. TaqMan assays were from Applied Biosystems: mouse *Sp1* assay (Mm00489039_m1), mouse *IL-15* assay (Mm00434210_m1), mouse *Gapdh* (glyceraldehyde phosphate dehydrogenase) assay, human *Sp1* assay (Hs00916521_m1), human *IL-15* assay (Hs01003713), human *Gapdh* assay.

Small interfering RNA (siRNA) – The following RNA interference duplex was purchased from Qiagen, Hs_NF B2_1 FlexiTube siRNA SI00300965 and Allstars negative control siRNA (#1027281) or Dharmacon ON-TARGET plus siRNA Sp1 (L-026959). Following optimization, 50 nM of siRNA was used. Human PBMC were transfected with siRNA using an Amaxa electroportator and a Cell Line Nucleofector Kit V, program V-01 (PBMC). 1×10^6cells/ml PBMC were used per point for nucleofection. Cells were harvested after 72 h and used for further analysis.

Immunoblotting- MEFs and BMDM were seeded at 5×10^5 cells/ml, HEK293TLR3 cells were seeded at 1 x 10^5 cells/ml or hPBMC were set up 1

× 10^6 cells/ml, one day prior to stimulation with 2 % FCS. Cells were stimulated with Poly(I:C) and lysed in 1 ml of low stringency lysis buffer (50 mm HEPES, 100 mm NaCl, 1 mm EDTA, 10% glycerol, 0.5% Nonidet P-40, and protease inhibitors). Protein concentration was measured by Bradford and equal amounts of protein were separated by SDS-gel electrophoresis, transferred to PVDF membrane and incubated with antibody and visualised by autoradiography.

Chromatin Immunoprecipitation– Genpathway, Inc. (CA, USA), carried out an analysis of gene promoters that bound to p52 using samples prepared from WT and IKK□KO MEFs according to their instructions. Briefly, MEFs were set up at 5 x 10^5 cell/ml. A final volume of 1% formaldehyde was added directly to existing media, incubated for 15 min. 1/20 volume of 2.5 M glycine was then added to each flask and allowed to set at room temperature for 5 min. Cells were scraped, washed in PBS and sent on dry ice to Genpathway. BMDMs were set up at 5 x 10^5 cell/ml, media was removed, replaced with PBS and fixed by adding Final concentration of 1% Formaldehyde to each culture dish. Flasks were incubated for 10 min at room temperature. 1/20 volume of 2.5 M glycine was then added to each flask and allowed to set at room temperature for 5 min. Primary antibody. Anti-p52 (Abcam #7972), anti-p50 (Millipore #06-886) and anti-p65 (Santa Cruz (F-6) sc-8008) were determined to give the best ChIP results. Quantitative RT-PCR was carried out using primers for either Sp1-promoter or □-actin promoter as indicated. Data are presented as % of input.

Affinity Purification with Biotinylated oligonucleotides - HEK293 cells were seeded at 1 x 10^5 cell/ml and incubated overnight. Cells were then transfected with either 2 □g of HA-p52 (five plates) or an empty vector control (five plates). 24 h later cells were lysed in 100□l of Oligonucleotide Buffer (ONB) (25mM TRIS, 50mM EDTA, 5% glycerol, 5 mM NaF, NP40 1%, 1mM DTT, 150 mM NaCl and protease- and phosphatise- inhibitors) pooled and snap frozen. Samples were then thawed on ice and diluted with a further 4.5 ml of ONB without NaCl, a 50 □l sample of lysate was kept and the remainder was divided into 5 tubes and incubated for 2 h with streptavidin-agarose beads conjugated to biotinylated promoter regions, termed Seq 1-5, as depicted in Fig. 4f. Lysates were then centrifuged to pellet the beads which were washed 3 times before 50 □l of 5 x SDS sample buffer was added to the beads. Samples were then immunoblotted as indicated.

Viral infection of BMDM- RSV long strain (group A) was obtained from American Type Culture Collection (Manassas, VA), and propagated in HEp-2 cells with serial plaque purifications to reduce defective-interfering particles (6). WT and NFkB2 deficient BMDM were plated in 6-well ($3x10^6$ cells per well) tissue culture plates. Macrophages were infected with RSV (multiplicity of infection = 2) or treated with medium alone and incubated at 37°C for the indicated times.

RESULTS:

Poly(I:C) promotes p52-driven transactivation in an IKK□ and p65-dependent manner. Having previously identified p52 as a protein that interacts with IKK□, and furthermore, showing that IKK□ promotes transactivation by p52 (5), we wished to probe the functional relevance of this interaction. Given the anti-viral role of IKK□, we chose to test whether dsRNA analogue P(I:C) could promote p52-mediated transactivation. We co-transfected HEK293-TLR3 cells with a plasmid encoding full-length p52 fused to the DNA-binding-domain (DBD) of Gal4 (p52-Gal4) and a Gal4-driven luciferase construct. Fig.1A shows that increasing concentrations of dsRNA analogues P(I:C) and P(A:U) promote p52-driven transactivation. Since P(A:U) is a dsRNA analogue only recognised by TLR3 (7) and P(I:C) and P(A:U) both promoted p52-driven transactivation to almost identical levels, the implication is that TLR3, and not the cytosolic RIG-1-like receptors, promote p52-driven transactivation. Having previously identified IKK□ and p65 in a complex with p52 (5), we next examined whether IKK□ and/or p65 mediates P(I:C)-inducible p52- driven transactivation. P(I:C) failed to promote p52-driven transactivation in IKK□$^{-/-}$ MEFs or p65$^{-/-}$ MEFs compared with WT MEFs (Fig.1B). These MEF strains were responsive to TLR3 ligation as

demonstrated by comparable levels of P(I:C) inducible phosphorylation of p38 (not shown). We next determined whether the kinase activity of IKK□ was required for P(I:C)-inducible transactivation by p52. As shown in Fig. 1C, P(I:C) promoted p52-driven transactivation to ~5-fold over control, whereas a kinase dead form of IKK□ (IKK□K38A) inhibited this induction, presumably acting as a dominant negative inhibitor.

Of the five NF□B family members, only c-Rel, p65 and RelB have transactivating potential (9). p50 and p52 are DNA binding subunits, and are unable to transactivate gene expression on their own. Since we and others have shown that p52 interacts with p65 (5, 10), we next tested whether p65 was the transactivation partner for p52 downstream of P(I:C). Even small amounts of p65 could strongly drive p52-dependent transactivation, and this ability of p65 to induce p52 transactivation was substantially impaired when the serine residue at position 536 in p65 was mutated to an alanine (p65S536A) (Fig.1D). Phosphorylation of p65 at position S536 is known to be very important for the efficient transactivating potential of p65 in response to many ligands, and IKK□ is known to phosphorylate S536 in response to P(I:C) (8-11). We next investigated whether p65 could induce p52-dependent transactivation in IKK□$^{-/-}$ MEFs. As shown in Fig.1E, p65 induced p52-transactivation in WT MEFs but this induction was substantially impaired in IKK□$^{-/-}$ MEFs, indicating that p65 requires IKK□ to confer its transactivation potential to p52. IRF3, another transcription factor with transactivating potential, is activated downstream of P(I:C) and phosphorylated by IKK□ (10, 12). However, IRF3 is unable to mediate transactivation by p52 in HEK293-TLR3 cells (Fig.1F). Together, these results imply that P(I:C) activates IKK□ that, in turn, mediates p65 transactivation of the p52-dependent reporter gene, likely by phosphorylating p65 at position S536 (11-14).

We further investigated whether components of the TLR3 signalling pathway could promote p52-driven transactivation. Fig.1G-I demonstrates that TRIF, when over-expressed, can strongly induce p52-driven transactivation; TRAF3, TRAF6 and RIP1, IKK□ and TBK1, all of which are known to be downstream of TRIF (13, 14), can promote p52-dependent transactivation in a dose-dependent manner, whereas TRAF1 and the IKK□/TBK1 adaptor NAP1 cannot. To determine whether TRIF-induced p52 transactivation is mediated by IKK□, we compared the ability of TRIF to drive p52 transactivation in WT and IKK□$^{-/-}$ MEFs (Fig 1J). IKK□ is required for TRIF-induced p52 transactivation, as TRIF was unable to induce p52 transactivation in IKK□$^{-/-}$ MEFs (Fig.1J).

All of these data pointed to p52 as an important target for TLR3 signaling and we further confirmed this by demonstrating that expression levels of both p100 and p52 were induced by P(I:C) in a dose-dependent manner (Fig. 1K).

p52 binds to the promoter of the Sp1 gene to activate its transcription - Having shown that p52 is induced and activated by P(I:C), we next interrogated the gene promoters that were bound by p52 in response to P(I:C). We carried out ChIP analysis to determine genes that p52 might bind to. This was carried out commercially by Genpathway Inc. who revealed that p52 binds to an enhancer sequence in the *Sp1* gene promoter in the region shown in Fig.2A. We next carried out ChIP analysis comparing WT and IKK□$^{-/-}$ BMDMs treated with P(I:C) for 3 h. As shown in Fig.2B, there was no difference between WT and IKK□$^{-/-}$ cells in the basal binding of p52 to the *Sp1* promoter. However P(I:C) induced a five-fold increase in binding of p52 to the *Sp1* promoter in WT BMDMs and this was abrogated in IKK□$^{-/-}$ BMDMs. We also investigated the binding of the NF□B subunit p65, to the *Sp1* promoter. p65 binding to the promoter was significantly increased 2 fold in response to P(I:C). Binding of p65 to the *TNF* promoter was measured as a positive control for p65 binding (not shown). p52 binding was again evident in the P(I:C)-treated cells (Fig. 2C).

To examine this region further and locate the DNA element important for the transcriptional regulation of the *Sp1* gene by p52, a series of 5′-deletion promoter constructs were generated by PCR and cloned into the promoterless pGL3-enhancer luciferase reporter vector. The resulting constructs, contain 5′-flanking regions from −20 to

−474, -443, -412, -385 and -362 relative to the translational start codon.. HEK293 cells were transfected with the deletion constructs in conjunction with increasing amounts of plasmid expressing HA-p52. A 4-fold increase in activity was induced by co-transfection of pGL3-474 with HA-p52, compared with that of pGL3-474 alone (Fig.2D). Deletion to -443 abolished this activity, which was also abolished in all the other constructs (not shown). These results demonstrate that the *Sp1* promoter is activated by p52 in the region of sequence between -443 and -474 nts relative to the start site. We next employed an oligo-pulldown assay to assess if P(I:C) could induce the binding of p52 and p65 to this region of the promoter (-443 to -474). This is clearly the case, since P(I:C) specifically induced the binding of both p52 (second panel) and p65 (third panel) but not p100 (top panel) or p50 (bottom panel) to the oligonucleotide sequence indentified from the Sp1-luciferase assay (Fig. 2E). Interestingly the bound form of p65 appears to be in a phosphorylated state, since we were also able to weakly detect P-p65S536 in the induced complex (fourth panel).

We further defined the p52 binding site using this assay. Overexpressed HA-p52 binds to the oligonucleotide consisting of the sequence from -434 to -472 (Fig 2F, Seq 1, lane 2), and also to the sequences from -443 to -472 (Seq 2, lane 4), from -452 to -472 (Seq 3, lane 6) and from -434 to -462 (Seq 4, lane 8). However, HA-p52 does not bind to the oligonucleotide consisting of the sequence from -434 to -452 (Seq 5, lane 10) (Fig. 2F). Sequence 5 differs from the sequences 1-4 in that it lacks the sequence GGCCGTTGTT. Interestingly, this area in the promoter of Sp1 is conserved among species (Fig.2G). This identifies, for the first time, GGCCGTTGTT as a binding site for p52.

Having demonstrated that p52 binds to the Sp1 promoter we next tested the functional consequences of this response. As shown in Fig. 2H. P(I:C) induced the expression of *Sp1* mRNA in WT BMDMs whereas this effect was not observed in either IKK$\alpha^{-/-}$ or NFκB2$^{-/-}$ BMDMs. To determine whether this effect could be seen in human cells, siRNA directed against NFκB2, or a non-targeting control, were transfected into PBMC and Sp1 induction was measured. P(I:C) induced Sp1 expression in control cells, this induction was lost in NFκB2-knockdown cells (Fig.2I).

Sp1, IKKα and NFκB2 regulate IL-15 gene transcription - We next determined target genes for Sp1 that might be relevant to the anti-viral response. The promoter of IL-15, a pro-inflammatory, anti-viral cytokine, was strongly predicted to be regulated by Sp1. IL-15 is known to be induced by P(I:C) and by viral infection (16, 17). We hypothesized that a P(I:C)-inducible gene regulated by Sp1 should not be induced in either IKKα or NFκB2 knockout cells. To validate IL-15 as a Sp1 target gene, human PBMC were transfected with siRNA directed against Sp1 or a non-targeting control and IL-15 induction was measured. P(I:C) caused an increase in the level of IL-15 mRNA over time that was lost in cells deficient in Sp1 (Fig. 3A). Importantly Sp1 knock down did not reduce the ability of the PBMCs to induce IL-6 (not shown) or RANTES, which was in fact increased upon Sp1 knock down (Fig. 3B). These are Sp1-independent, P(I:C)-inducible genes, indicating specificity in the IL-15 observation. This effect was confirmed pharmacologically in PBMC pretreated with 1 μM Mithramycin A (MMA), an Sp1 inhibitor. P(I:C) caused a ~5 fold increase in the level of IL-15 mRNA over basal levels after 8 h. This effect was inhibited by pretreatment with MMA (Fig.3C).

Following this we measured the IL-15 transcript in response to P(I:C) in WT, NFκB2$^{-/-}$ and IKK$\alpha^{-/-}$ BMDMs. IL-15 transcript levels increased 60-fold in WT BMDMs after 8 h Poly(I:C) treatment, whereas a marked inhibition of this response was observed in both NFκB2$^{-/-}$ and IKK$\alpha^{-/-}$ BMDMs (Fig.3D). This dependency of P(I:C)-induced IL-15 levels on NFκB2 was confirmed in human PBMC, where no increase was observed in PBMC transfected with siRNA targetted against NFκB2 (Fig. 3E). These data therefore implicate IKKα, NFκB2 and Sp1 in the induction of IL-15 by P(I:C).

RSV infection fails to upregulate Sp1 and IL-15 transcripts in both IKKα and NFκB2 knockout BMDMs - Finally we examined whether this

pathway was important for induction of IL-15 by a virus. RSV is recognised by TLR3 during infection (18, 19) and is a powerful inducer of IL-15 (16). We infected WT, IKK$\beta^{-/-}$ and NFκB2$^{-/-}$ BMDMs with RSV (moi =2) for 4, 8 or 24 h and measured Sp1 mRNA levels. As shown in Fig.4A left hand panel, Sp1 transcript was induced 10 fold after 4 h RSV infection in WT BMDM. Conversely no induction of Sp1 was observed in IKK$\beta^{-/-}$ BMDMs after RSV infection (Fig.4A, right hand panel). Similarly no Sp1 was inducible by RSV infection in NFκB2$^{-/-}$ BMDMs (Fig.4B). As shown in Fig.5C left hand panel, IL-15 is induced by RSV in WT BMDMs. However this induction of IL-15 is completely abrogated in RSV-infected IKK$\beta^{-/-}$ BMDMs (Fig.4C, right hand panel). Similarly, RSV induced IL-15 is abrogated in NFκB2$^{-/-}$ BMDMs (Fig.4D, right hand panel). In order to determine the specificity of the effect of NFκB2 and IKKβ on RSV-inducible IL-15, we measured levels of IFNβ and IL-12p40, two further RSV inducible genes, and found that neither NFκB2 or IKKβ deficiency reduced the levels of these cytokines in response to RSV infection (Fig.4E-H). These results, therefore, indicate that similar to P(I:C), RSV infection will trigger a pathway involving NFκB2, activated by IKKβ, leading to upregulation of Sp1 and induction of IL-15, which could be critical for anti-viral immunity

DISCUSSION:

In the NFκB field, the majority of studies concerned with infection and inflammation have centred on the canonical NFκB pathway, whereas NFκB2 is better known for its functions in lymphoid organogenesis and humoral immunity (20).

In this study, we present a novel inducer and activator of NFκB2 in the form of Poly(I:C), that acts via TLR3. A few studies have identified an indirect role for NFκB2 in host defense. With respect to viral immunity, RSV infection has been shown to induce the release of p65 from p100/p65 complexes (21). However, the elucidation of genes potentially regulated by NFκB2 in the host response to infection has been unexplored.

Sp1 was identified as a target gene for p52 in response to Poly(I:C) through ChIP analysis. Sp1 is a transcription factor, first identified based on its ability to interact with the GC-rich motif of simian virus 40 regulatory sequences (22). Sp1 plays a critical role in many diverse cellular events such as cell growth (23), differentiation (24), apoptosis (23), angiogenesis (25), and viral latency (26), by regulating the expression of other genes. Sp1 was once thought to serve mainly as a constitutive activator of housekeeping genes. However, growing evidence indicates that various post-translational modifications can influence the transcriptional activity and stability of Sp1, making it a transcription factor responsive to extracellular signals.

Sp1 associates physically and cooperates functionally with several cellular transcriptional activators and also with several viral regulatory proteins including the HIV-1 regulatory protein, Tat (27) and HCMV's IE gene products (28). These associations determine the level of Sp1-mediated, viral or host gene transcription (29, 30). Furthermore, Sp1 regulated elements are found in the promoters of various viruses such as HIV-1 (26), SV40 (22), HSV-1 (31), HCMV (32) and EBV (33). The fact that recognition elements of Sp1 are frequently found in the promoters of various viral genes and, furthermore, that viral regulatory proteins associate with Sp1 to affect its transactivating potential, implies that viruses have hijacked the host response to infection, in the form of increased Sp1 availability, to their own advantage. This indicates that Sp1 induction upon viral infection is probably a common event during host defence. However, although there is an abundance of circumstantial evidence indicating a role for Sp1 in host defence against viral infection, only one study has demonstrated a functional role for Sp1 in the antiviral response in the skin to Vaccinia virus through its regulation of OAS2 expression (34). Therefore, we considered Sp1 a valid target for further investigation.

The identification of sequences to which NFκB dimers bind and effect gene expression in a dimer-specific manner is under intense investigation. Active NFκB dimers bind to specific DNA sites in the promoters of target genes that are collectively known as κB-sites (35). The classical κB-sites follow the 5'-GGGRNWYYCC-3' consensus (R=purine, Y=pyrimidine, W=A or T and N= any

nucleotide), however it has been reported that the p52/RelB heterodimer binds to and activates a unique class of κB-site with the consensus 5'-RGGAGAYTTR-3' (R=A or G and Y= C or T). This consensus sequence is present in the promoters of chemokines involved in lymphoid development and the maintenance of the splenic architecture (36). With increasing numbers of NFκB-regulated DNA target sites being discovered, the diversity of these κB-sequences is becoming more apparent, with even the stringency of the GGG and CC core sequences called into question (37). Our study has identified a previously undescribed κB-site 5'-GGCCGTTGTT -3' targeted by p52/p65 in the promoter of the Sp1 gene in the region between -474 and -443 nts from the translational start site.

Functionally, we have demonstrated that Sp1 mRNA and protein levels increase upon P(I:C) treatment and that this increase is not observed in cells lacking either IKKα, p65 or NFκB2. We chose IL-15 as a possible candidate when considering what target genes might be regulated by Sp1. The role of IL-15 in host defense against viral infections is well documented, and it is known to be induced in response to numerous viruses including RSV (16, 38). IL-15 is a potently pro-inflammatory cytokine with a diverse range of immune-regulatory functions (39). The IL-15 gene promoter is also predicted to have two Sp1 sites (40). We confirmed that IL-15 gene expression required the presence of active Sp1 in PBMC in response to P(I:C) and that its induction required both IKKα and NFκB2.

Finally, we examined the role of IKKα and NFκB2 in a viral infection model. RSV is a major human respiratory pathogen and the leading cause of lower respiratory tract infection in infants worldwide (19). RSV is a ssRNA virus, however it makes dsRNA during its replication cycle and it has been reported that TLR3 mediates inflammatory cytokine and chemokine production in response to RSV infection (19, 41).

Knocking out IKKα alone completely abrogates any Sp1 or IL-15 gene expression in response to RSV. It is known that IKKα phosphorylates both IRF3 and p65 in response to RSV to increase the transactivation potential of these transcription factors (4, 42). As the IL-15 promoter has also been shown to have a virus-inducible region, encompassing an IRF-element (IRF-E) and a consensus NFκB motif (43), the lack of IL-15 gene expression in response to RSV in IKKα- deficient BMDMs could conceivably be due to the insufficient transactivation of p65 and IRF3. However, we believe this is unlikely as other kinases can act in place of IKKα in this role, most notably TBK1 and IKKε (8, 12). In addition, we observed that BMDMs that lack NFκB2 also fail to upregulate IL-15 gene expression in response to either P(I:C) or RSV, suggesting that IKKα acts upstream of NFκB2 in our system. The mechanism of P(I:C)- and RSV-induced IL-15 expression is likely due to the ligation of TLR3, which both activates IKKα and induces p52. p52 then binds the promoter of Sp1 with p65, inducing its expression, Sp1 then binds the promoter of IL-15, upregulating its expression. We were unable to test RSV *in vivo* in NFκB2$^{-/-}$ mice since they are severely immunocompromised due to defective lymphoid organogenesis (20, 44).

The number of genes regulated by IKKα in a non-redundant manner are very few (3), so it is of interest that we report two new genes to add to this list, *i.e.*, Sp1 and IL-15. Similarly, the number of genes known to be regulated by NFκB2 is small in number and relate only to lymphoid organogenesis, humoral immunity, and DNA damage (36, 45, 46). Considering the abundance of functions of IL-15 (41), we therefore present a role for NFκB2 as a key regulator of anti-viral immunity. Furthermore, TLR3 signalling is activated by viral, bacterial and parasite-derived dsRNA or by host-derived mRNA (47). Therefore, it is conceivable that NFκB2 alone or in conjunction with Sp1 will be found to play a role in host defence against a broader range of infectious agents and also in autoimmunity.

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FIGURE LEGENDS:

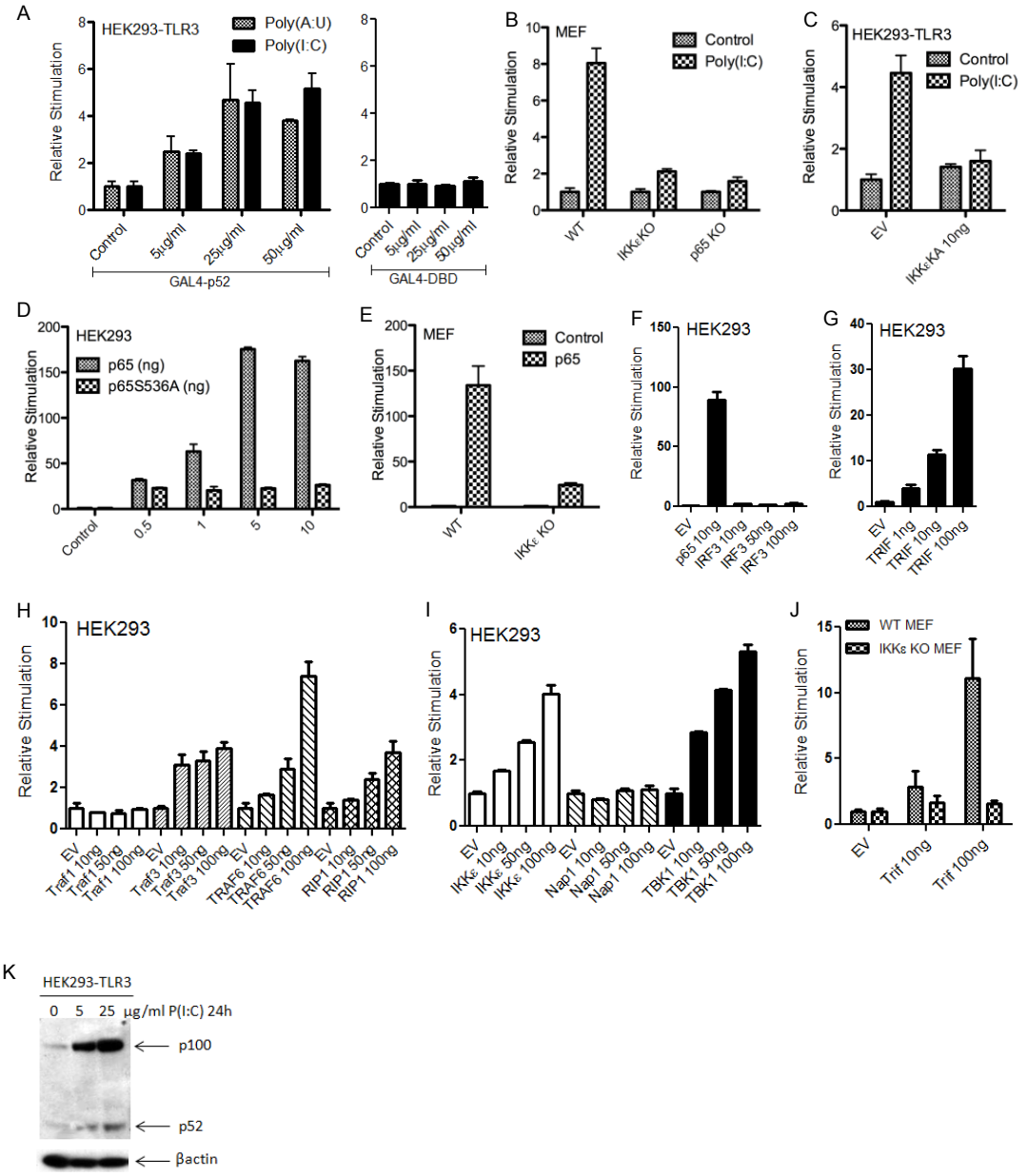
Figure 1 *Poly(I:C) induces p100 and p52 expression and p52-dependent transactivation in an IKK-and p65-dependent manner*: (A-J) p52-dependent transactivation was assayed in all cells using 150ng of p-55UAS$_G$Luc and 50ng of p52-Gal4 or Gal4-DBD. HEK293-TLR3 cells were (A) stimulated with Poly(A:U) or Poly(I:C) 24h post-transfection or left untreated (contol) and incubated for 6h, or co-transfected with plasmid encoding (C) IKKKA (D) p65, p65S536A or EV. (B) WT, IKKKO or p65 KO MEFs were stimulated with P(I:C) 24h post-transfection or left untreated (control) and incubated for 6h. (E) WT and IKKKO MEFs were co-transfected with p65 or EV as indicated. (F-I) HEK293 cells or (J) wildtype and IKK$^{-/-}$ MEFs, were co-transfected with plasmid encoding (F) IRF3, p65 (G, J) Trif, (H) TRAF1, TRAF3, TRAF6, RIP1, or (I) IKK, Nap1, TBK1 or with empty vector only (EV) as indicated. Luciferase activity was determined 24-36 h after transfection. Data are the means of three measurements, with *error bars* representing S.D. (K) HEK293-TLR3 cellswere treated with increasing doses of P(I:C) for 24h lysed and probed for p100/p52. Data are representative of 3 separate experiments.

Figure 2 *Poly(I:C) induces p52 and p65 to bind a previously undescribed site in the Sp1 promoter to drive transcription in* an IKK-, *p65-* and NFB2*-dependent manner* (A) A partial sequence of the Sp1 promoter region -474 nts to -362 nts 5' from the start codon. The primers used for the ChIP assay are underlined. (B) WT and IKKKO BMDMs were treated with P(I:C) for 3h or left untreated, after which a ChIP assay was performed using an anti-p52 antibody. Primers specific for promoters of Sp1 or -actin were designed, and binding events of p52 were measured as % of input. (C) WT BMDMs were treated with P(I:C) for 0, 3 and 6h, after which a ChIP assay was performed using antibodies against HA (control), p65 or p52, binding events were measured as % of input. Data are the means of three measurements with error bars representing S.D. Statistical analysis was carried out using Student T-test, (*) = P<0.05 (**) = P<0.001 (***) = P<0.0001. Values are representative of three separate experiments. (D) Sp1 promoter truncations were cloned into a pGL3 luciferase reporter vector, Sp1 promoter activity was assayed in HEK293 cells transfected with 80ng of pGL3 vector containing Sp1 promoter truncations -474 and -443 nts 5' from the start site respectively, or with pGL3 vector alone. Cells were co-transfected with plasmid encoding HA-p52. Luciferase activity was determined 24h after transfection and is represented as fold increase in luciferase over each individual pGL3-Sp1-promoter construct EV control. Data are the means of three measurements, with *error bars* representing S.D. Values are representative of three independent experiments. (E) HEK293-TLR3 cells were treated with P(I:C) for 0 and 3h, lysed and an Oligopulldown (OPD) assay was carried out with the -472 to -434 oligo sequence, samples were probed for p100, p52, p65, P-p65S536 and p50. (F) An OPD assay was carried out in HEK293 cells using the oligonucleotide sequence from -472 to -434 5' from the Sp1 translational start codon (seq 1) and truncations of this nucleotide sequence (sequences 2-5) as shown in (F, bottom panel). Cells were transfected with plasmid encoding either HA-p52 (+) or empty vector (-). 24h later cells were lysed, incubated with oligos as indicated and probed for HA (F, top panel). OPD assays are representative of two separate experiments. (G) Species sequence alignment of the site in the Sp1-promoter. Upstream regions were obtained via biomart taking the flanked regions 2500 base pairs upstream. An alignment was created using MUSCLE. The alignment was viewed and an image exported via Jalview. The ten central base pairs in the alignment are the binding site, the binding site starts -364 upstream from the gene start site in the human sequence. (H) WT, IKKKO and NFB2 KO BMDMs or (I) PBMCs transfected with either siRNA targeting NFB2 or a non-targeting control siRNA for 48h, were stimulated with P(I:C) for 0, 4 and 8h as indicated. (H-I) Quantitative RT-PCR analysis of RNA from these cells was carried out with primers specific for Sp1; expression is normalised to that of GAPDH and is presented relative to that of untreated controls. Data are the mean of at least three separate experiments with each point assayed in triplicate, with *error bars* representing S.D.

Figure 3 *IL-15 induction in response to Poly(I:C) re*quires Sp1, IKK□and NF□B2: (A,B,E) PBMCs were transfected with siRNA targeting (A, B) Sp1 (E) NF□B2 or a non-targeting control siRNA for 48h, cells were then stimulated with P(I:C) for 0, 4 and 8h as indicated. (C) PBMC were pre-treated for 1h with 1□M Mithramycin A (MMA) or left untreated before stimulation with P(I:C). (D) WT, IKK□$^{-/-}$ and NF□B2$^{-/-}$ BMDMs were stimulated with P(I:C) for 4, 8 and 24h or left untreated as indicated. In each case, RT-PCR analysis of RNA was carried out with primers specific for IL-15, or RANTES as indicated; expression is normalised to that of GAPDH and is presented relative to that of untreated controls. Data are the mean of at least three separate experiments with each point assayed in triplicate, with *error bars* representing S.D.

Figure 4 *RSV infection induces both Sp1 and IL*-15 in an IKK□- and NF□B2-*dependent manner*: WT, IKK□$^{-/-}$ and NF□B2$^{-/-}$ BMDMs were infected with RSV long strain (group A) (moi=2) or treated with medium for 2, 4, 8 or 24h. Cells were lysed and RNA was extracted for RT-PCR analysis, with primers specific for (A,B) Sp1, or (C,D) IL-15, or (E,F) IFN□, or (G,H) IL-12p40; expression is normalised to that of GAPDH and is presented relative to that of untreated controls. Data are representative of three separate experiments, with each point assayed in triplicate, with error bars representing S.D.

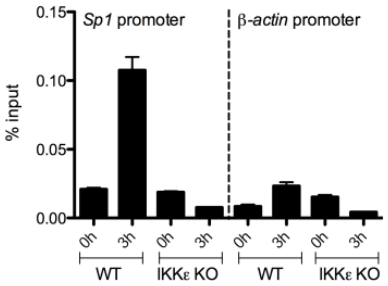
Figure 1


A
HEK293-TLR3
Poly(A:U)
Poly(I:C)
Relative Stimulation
Control
5μg/ml
25μg/ml
50μg/ml
GAL4-p52
GAL4-DBD
B
MEF
Control
Poly(I:C)
WT
IKKε KO
p65 KO
C
HEK293-TLR3
EV
IKKεKA 10ng
D
HEK293
p65 (ng)
p65S536A (ng)
E
MEF
p65
F
HEK293
p65 10ng
IRF3 10ng
IRF3 50ng
IRF3 100ng
G
HEK293
TRIF 1ng
TRIF 10ng
TRIF 100ng
H
HEK293
Traf1 10ng
Traf1 50ng
Traf1 100ng
Traf3 10ng
Traf3 50ng
Traf3 100ng
TRAF6 10ng
TRAF6 50ng
TRAF6 100ng
RIP1 10ng
RIP1 50ng
RIP1 100ng
I
HEK293
IKKε 10ng
IKKε 50ng
IKKε 100ng
Nap1 10ng
Nap1 50ng
Nap1 100ng
TBK1 10ng
TBK1 50ng
TBK1 100ng
J
WT MEF
IKKε KO MEF
Trif 10ng
Trif 100ng

K
HEK293-TLR3
0 5 25 μg/ml P(I:C) 24h
p100
p52
βactin

Figure 2

B



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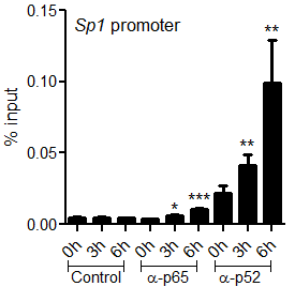

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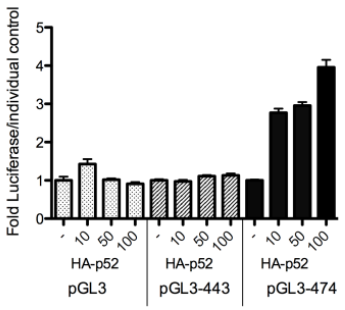

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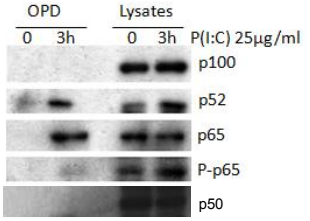

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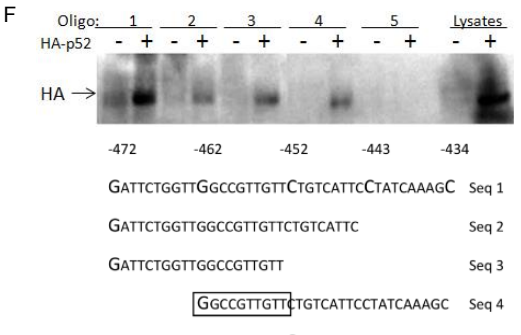

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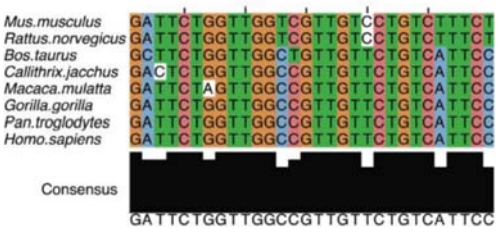

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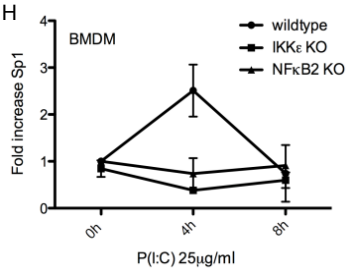


I

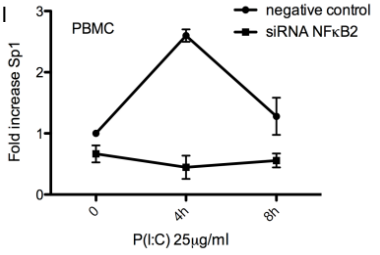
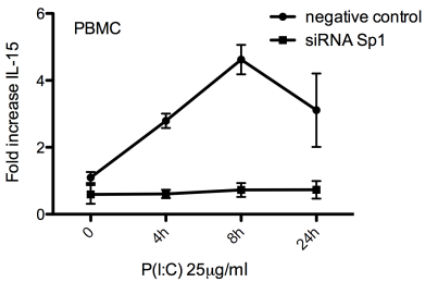


Figure 3

A



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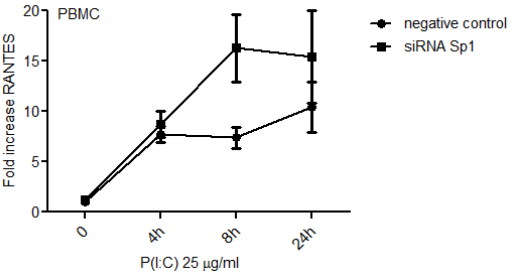

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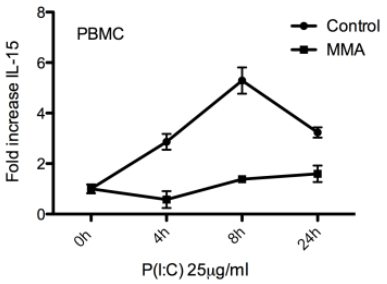

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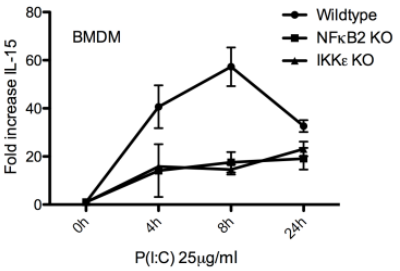

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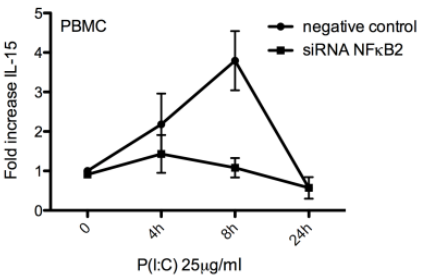
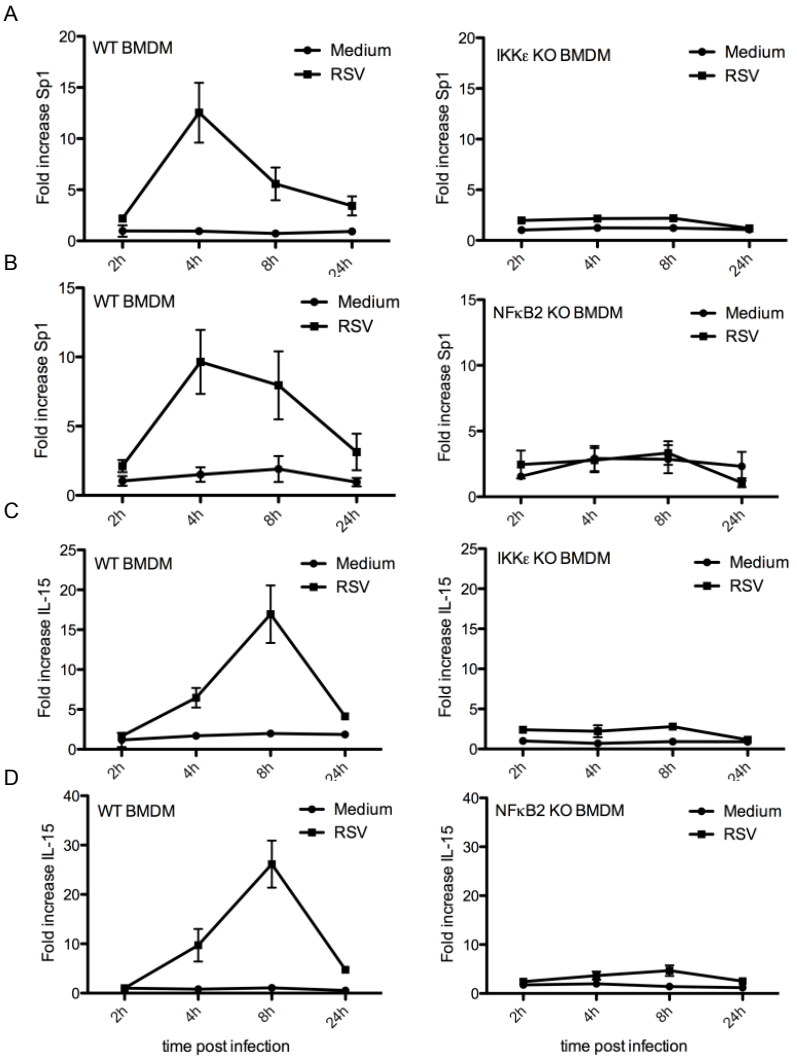
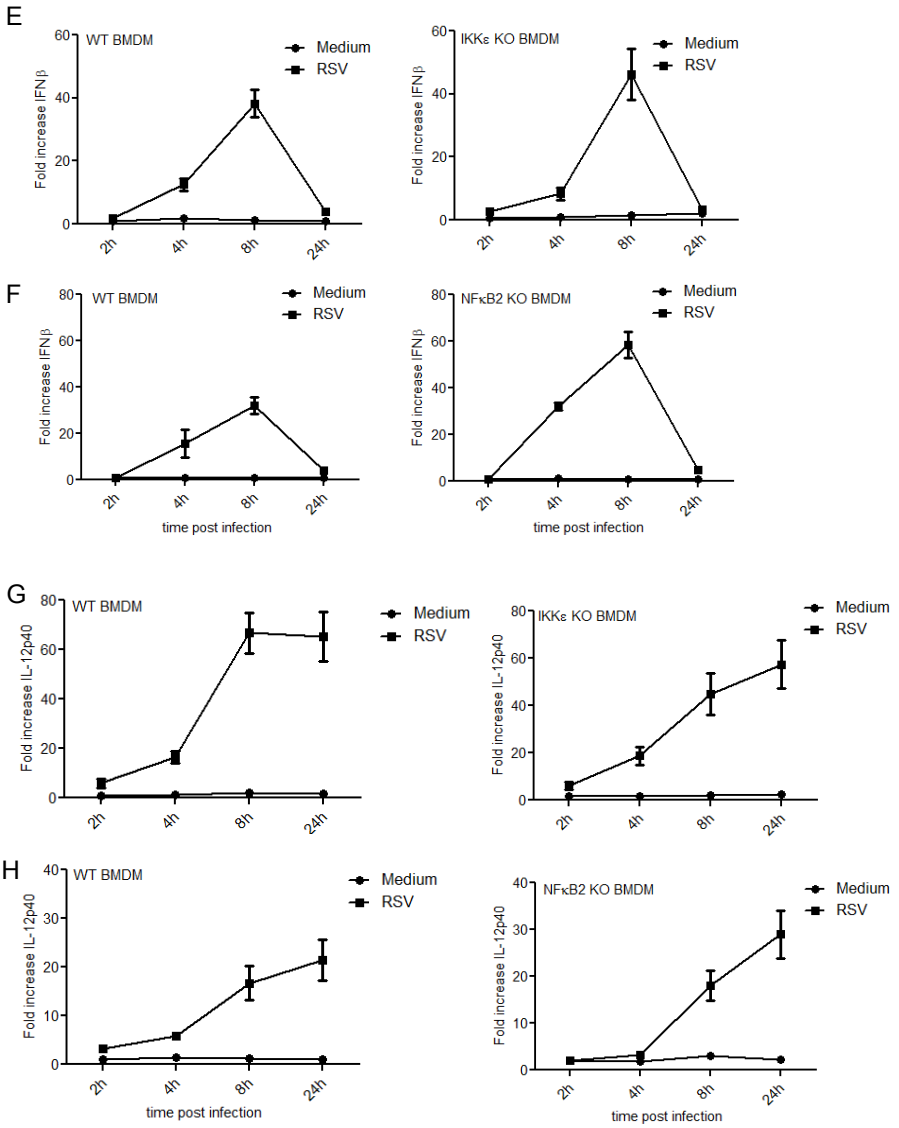


Figure 4


A
WT BMDM
Medium
RSV
Fold increase Sp1
IKKε KO BMDM
B
NFκB2 KO BMDM
C
Fold increase IL-15
D
2h
4h
8h
24h
time post infection


E
WT BMDM
IKKε KO BMDM
Medium
RSV
Fold increase IFNβ
F
WT BMDM
NFκB2 KO BMDM
Medium
RSV
Fold increase IFNβ
time post infection
G
WT BMDM
IKKε KO BMDM
Medium
RSV
Fold increase IL-12p40
H
WT BMDM
NFκB2 KO BMDM
Medium
RSV
Fold increase IL-12p40
time post infection
2h
4h
8h
24h