SUPPLEMENTAL MATERIAL

Activation of innate immune antiviral response by NOD2

Ahmed Sabbah, Te Hung Chang, Rosalinda Harnack, Victoria Frohlich, Kaoru Tominaga, Peter H. Dube, Yan Xiang & Santanu Bose

SUPPLEMENTARY TABLE 1 – Primers and siRNAs

RT-PCR primers

Gene	forward	reverse
GAPDH	*5'*GTCAGTGGTGGACCTGACCT	*5'*AGGGGTCTACATGGCAACTG
Human NOD2	*5'*GAAGTACATCCGCACCGAG	*5'*GACACCATCCATGAGAAGACAG
Human NOD1	*5'*TCCAAAGCCAAACAGAAACTC	*5'*CAGCATCCAGATGAACGTG
RSV N	*5'*AAGGGATTTTTGCAGGATTGTTT	*5'*TCCCCACCGTAACATCACTTG
MAVS	*5'*ATGCCGTTTGCTGAAGAC	*5'*CTAGTGCAGACGCCGCCG
IFN-β	*5'*GATTCATCTAGCACTGGCTGG	*5'*CTTCAGGTAATGCAGAATCC
RIGI	*5'*GCATATTGACTGGACGTGGCA	*5'*CAGTCATGGCTGCAGTTCTGTC
Mouse NOD2	*5'*GCCCTACAGCTGGATTACAAC	*5'*CGCCTGTGATGTGATTGTTC
RICK	*5'*GTTGCTTGGCCATTGAGATT	*5'*ATGCGCCACTTTGATAAACC
TNF-α	*5'*GAGTGACAAGCCTGTAGCCCATGTTG TAGCA	*5'*GCAATGATCCCAAAGTAGACCT GCCCAGACT

siRNAs

Gene	siRNA (sense)	siRNA (antisense)
NOD2	CGGUGAAAGCGAAUGGAUU	AAUCCAUUCGCUUUCACCG
MAVS	CCGUUUGCUGAAGACAAGA	UCUUGUCUUCAGCAAACGG
RIGI	AAUUCAUCAGAGAUAGUCA	UGACUAUCUCUGAUGAAUU

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SUPPLEMENTAL FIGURES

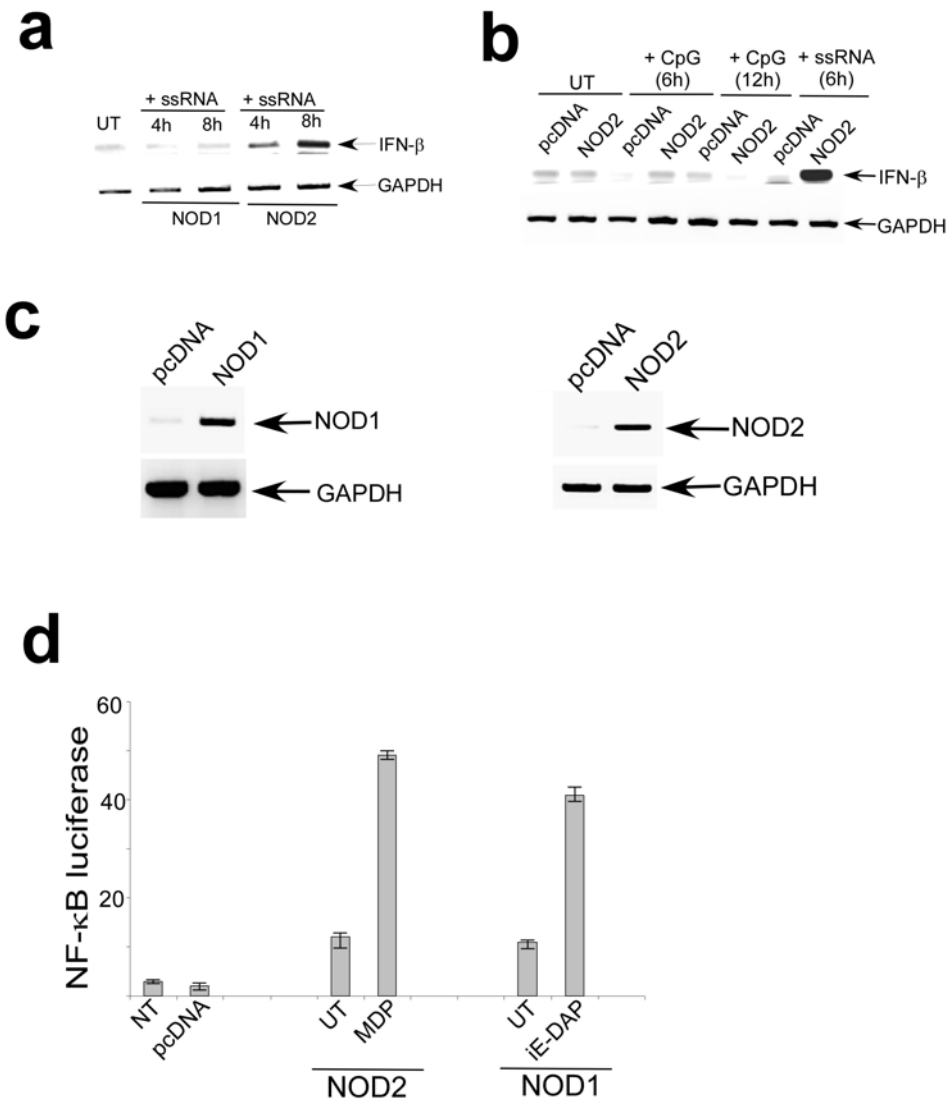

Figure 1 Induction of IFN-β expression by ssRNA. **(a)** RT-PCR analysis of IFN-β expression in pcDNA, human HA-NOD1 and human HA-NOD2 transfected 293 cells treated with ssRNA. UT; untreated. **(b)** RT-PCR analysis of interferon-β (IFN-β) expression in UT, ssRNA and CpG treated 293 cells transfected with either pcDNA or HA-NOD2. **(c)** RT-PCR analysis of NOD1 and NOD2 expression in 293 cells transfected with either HA-NOD1 or HA-NOD2. **(d)** Activation of NF-κB-luciferase in non-transfected (NT), pcDNA, HA-NOD1 and HA-NOD2 transfected 293 cells that were either untreated (UT) or treated with MDP (10 μg/ml for 12h) or iE-DAP (10 μg/ml for 24h). The luciferase assay results are presented as mean ± s.d. from three independent experiments.

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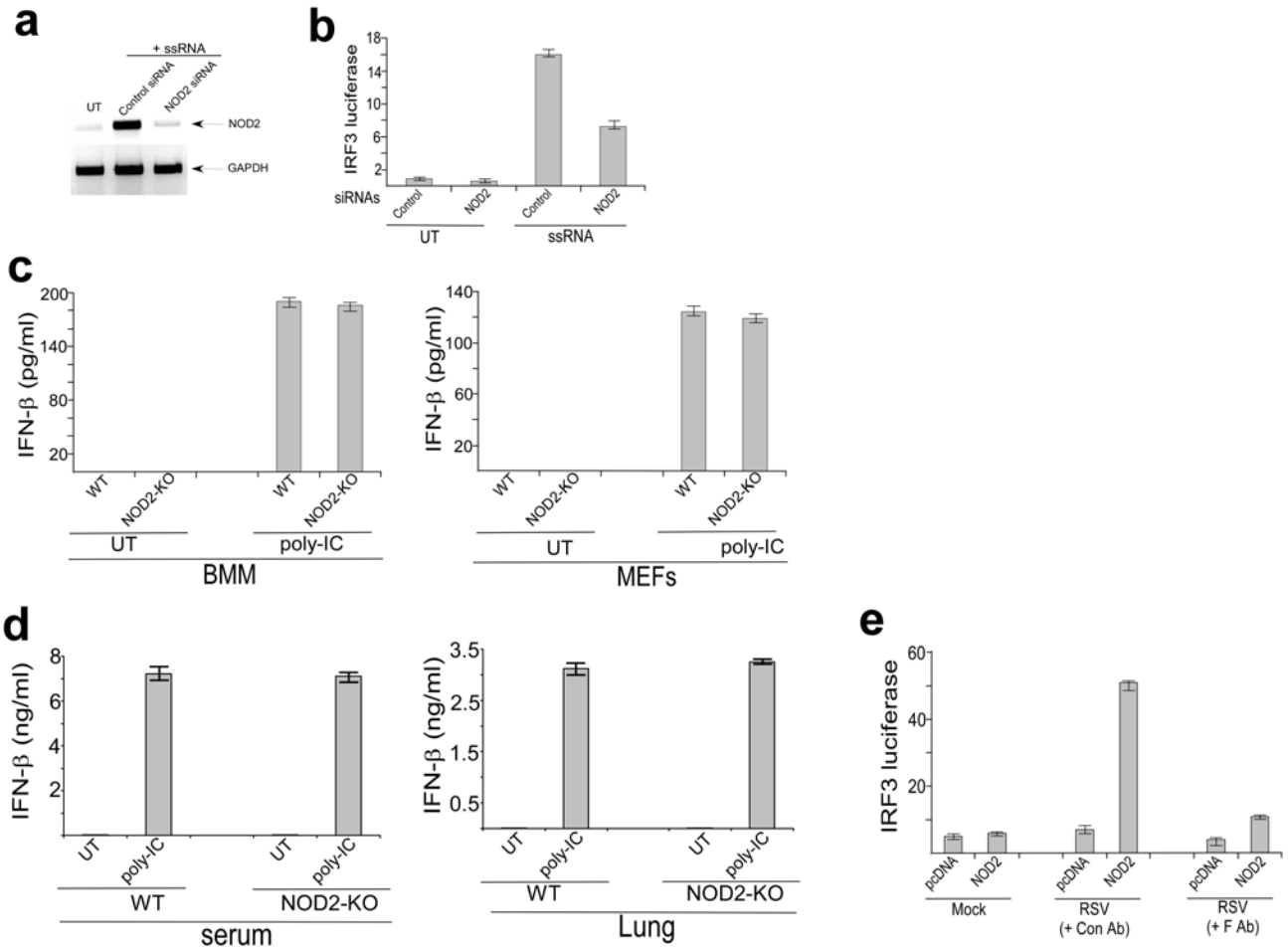

Figure 2 NOD2 is required for antiviral response. **(a)** Efficiency of NOD2 specific siRNA was examined by analyzing NOD2 expression (by RT-PCR) in control or NOD2 siRNA transfected A549 cells +/- ssRNA treatment (6h). **(b)** Activation of IRF3-luciferase in untreated (UT) and ssRNA treated (6h) A549 cells transfected with either control siRNA or NOD2 siRNA. **(c)** IFN-β production from UT and poly-IC treated (10 μg/ml for 18h) bone marrow derived macrophages (BMM) and mouse embryo fibroblasts (MEFs) isolated from wild type (WT) or NOD2 knock-out (KO) mice. IFN-β was measured by ELISA assay and each value represents the mean ± s.d. from three independent experiments. **(d)** IFN-β concentrations in the serum and lung homogenate of WT and NOD2-KO mice following poly-IC administration by intravenous or intranasal route, respectively. IFN-β values (n=8 mice/group) are presented as mean ± s.e.m. $p < .05$, by t-test when data were normally distributed, or by Mann-Whitney Rank sum test when data were not normally distributed. **(e)** Activation of IRF3-luciferase in 293 cells (expressing either pcDNA or HA-NOD2) infected (6h) with RSV in the presence of either control antibody (Con Ab) or RSV fusion (F protein) protein specific neutralizing antibody (F Ab). The luciferase assay results are presented as mean ± s.d. from three independent experiments.

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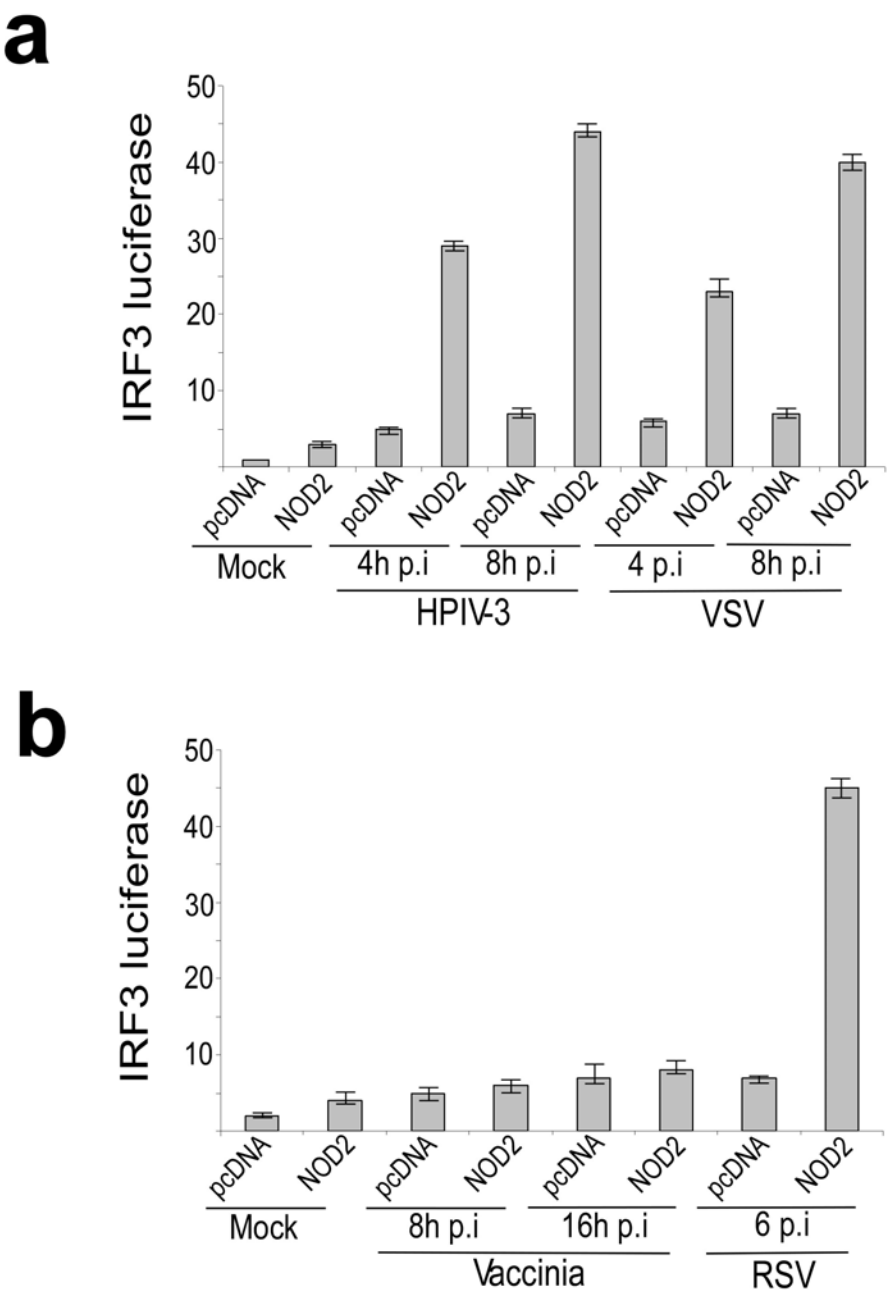

Figure 3 NOD2 mediated antiviral response following infection with ssRNA viruses. **(a)** Activation of IRF3-luciferase in human parainfluenza virus type-3 (HPIV-3) (0.5 MOI) or vesicular stomatitis virus (VSV) (0.5 MOI) infected (at indicated post-infection or p.i time-periods) 293 cells transfected with either pcDNA or HA-NOD2. **(b)** Activation of IRF3-luciferase in vaccinia virus or RSV infected 293 cells transfected with either pcDNA or HA-NOD2. The luciferase assay results are presented as mean ± s.d. from three independent experiments.

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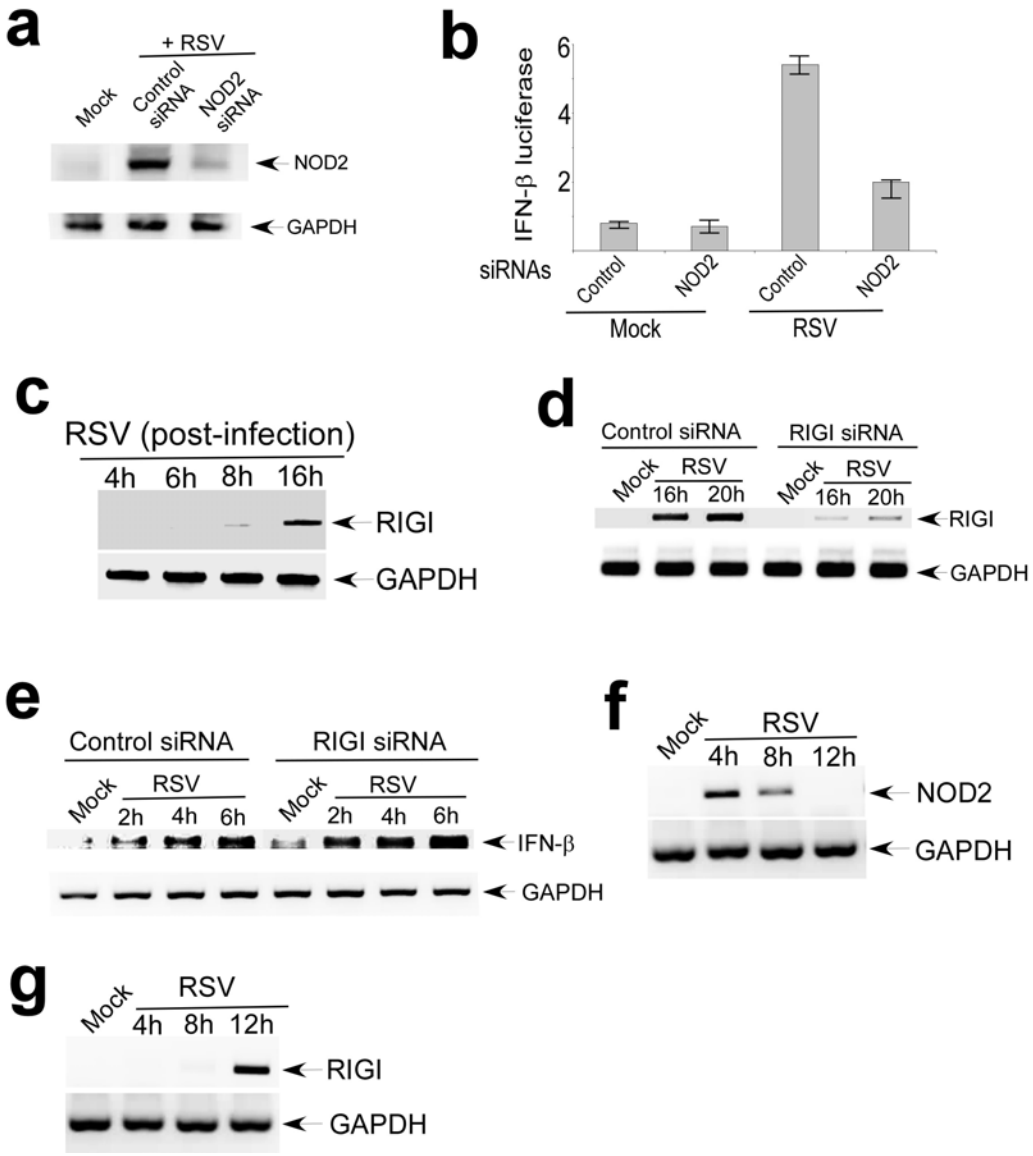

Figure 4 NOD2 but not RIGI is required for antiviral response during early RSV infection. **(a)** Efficiency of NOD2 specific siRNA was examined by analyzing NOD2 expression (by RT-PCR) in control or NOD2 siRNA transfected A549 cells infected with RSV for 4h. **(b)** Activation of IFN-β-luciferase reporter gene in control siRNA or NOD2 siRNA trasfected A549 cells infected with RSV for 6h. The luciferase assay results are presented as mean ± s.d. from three independent experiments. **(c)** RT-PCR analysis of RIGI expression in RSV infected (4h – 16h post-infection) A549 cells. **(d)** Efficiency of RIGI specific siRNA was examined by analyzing RIGI expression in control siRNA or RIGI siRNA transfected A549 cells infected with RSV for 16h or 20h. **(e)** RT-PCR analysis of IFN-β expression in control siRNA or RIGI specific siRNA trasfected A549 cells infected with RSV for 2h-6h. **(f)** RT-PCR analysis of NOD2 expression in MEFs infected with RSV for 0h-12h. **(g)** RT-PCR analysis of RIGI expression in MEFs infected with RSV for 0h-12h.

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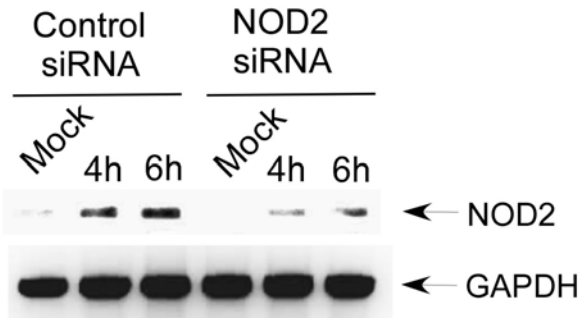

Figure 5 NOD2 expression in NHBE cells and silencing of NOD2 in NHBE cells. Efficiency of NOD2 specific siRNA was examined by analyzing NOD2 expression in control siRNA or NOD2 siRNA transfected NHBE cells infected with RSV.

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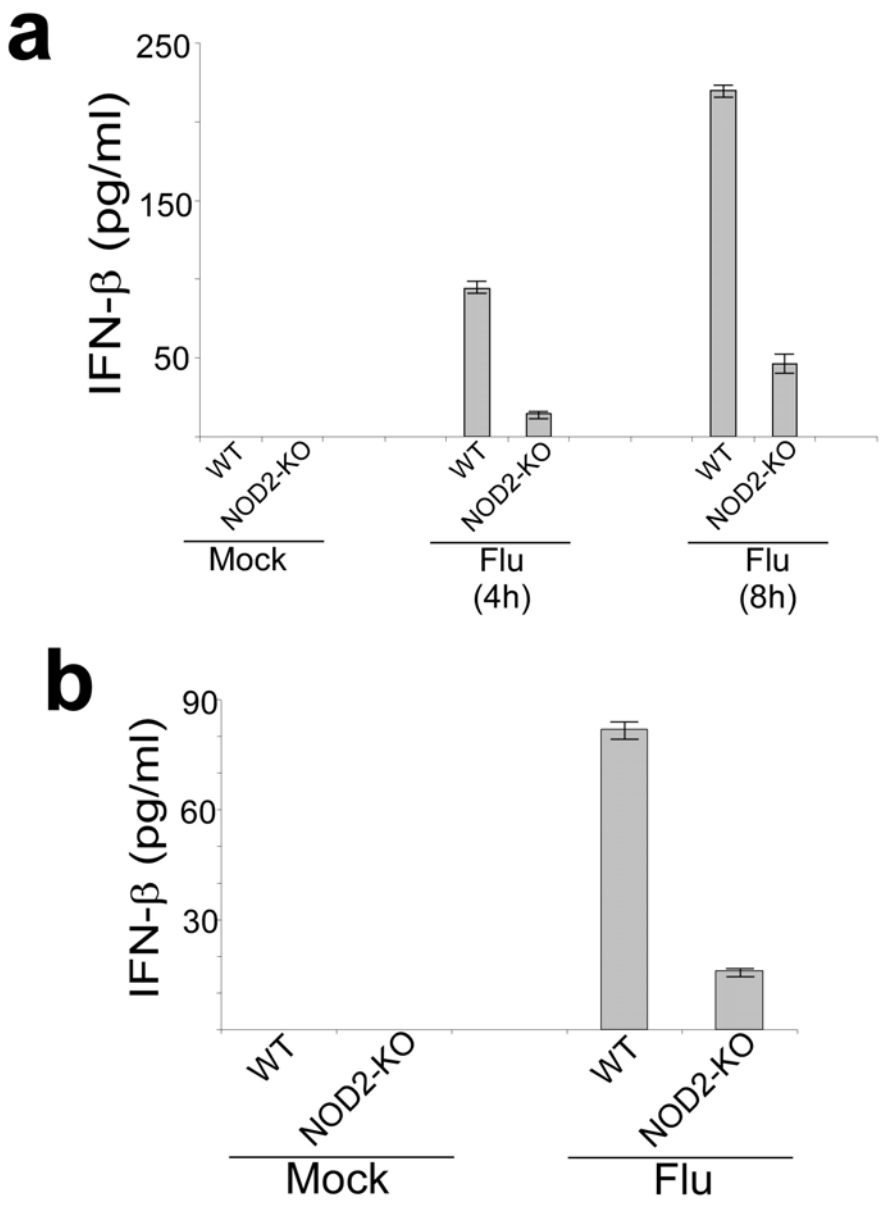

Figure 6 NOD2 is required for IFN production from influenza A [mouse-adapted influenza A/PR/8/34 (H1N1) virus] virus infected cells. **(a)** IFN-β production from mock and influenza A virus (Flu) infected (infected at 1 MOI for 4h and 8h) mouse embryo fibroblasts (MEFs) isolated from wild type (WT) and NOD2 knock-out (KO) mice. **(b)** IFN-β production from mock and Flu infected (infected at 1 MOI for 4h) bone marrow derived macrophages (BMM) isolated from WT and NOD2-KO mice. Amount of IFN-β was measured by ELISA assay and each value represents the mean ± s.d. from three independent experiments.

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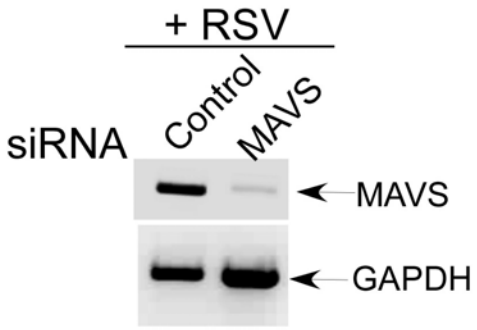

Figure 7 MAVS silencing efficiency in NHBE cells. Efficiency of MAVS specific siRNA was examined by analyzing MAVS expression (by RT-PCR) in control siRNA or MAVS siRNA transfected NHBE cells infected with RSV for 4h.

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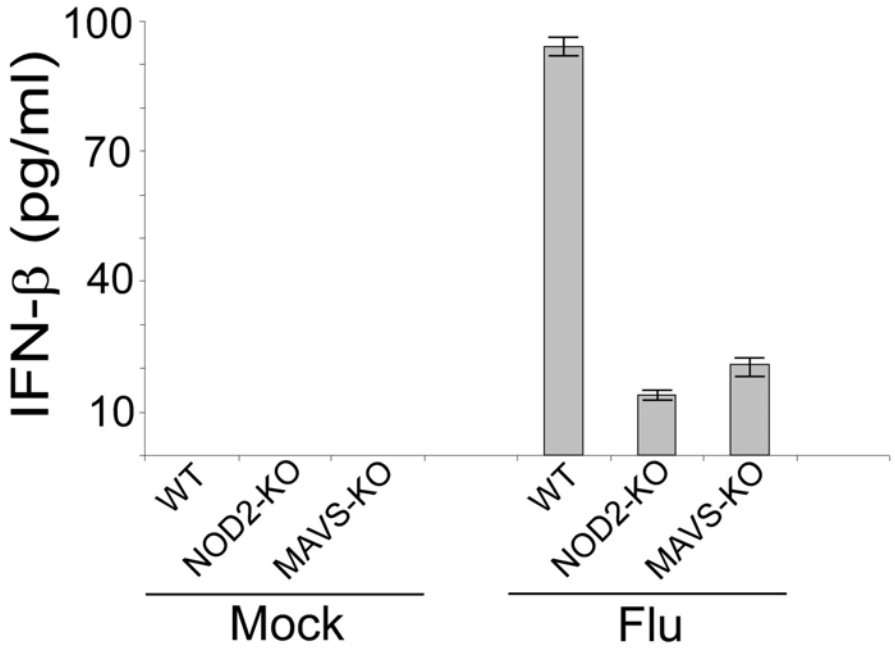

Figure 8 MAVS is required for NOD2 mediated IFN production from influenza A [mouse-adapted influenza A/PR/8/34 (H1N1) virus] virus infected cells. IFN-β production from mock and influenza A virus (Flu) infected (infected at 1 MOI for 4h) mouse embryo fibroblasts (MEFs) isolated from WT, NOD2-KO and MAVS-KO mice. Amount of IFN-β was measured by ELISA assay and each value represents the mean ± s.d. from three independent experiments.

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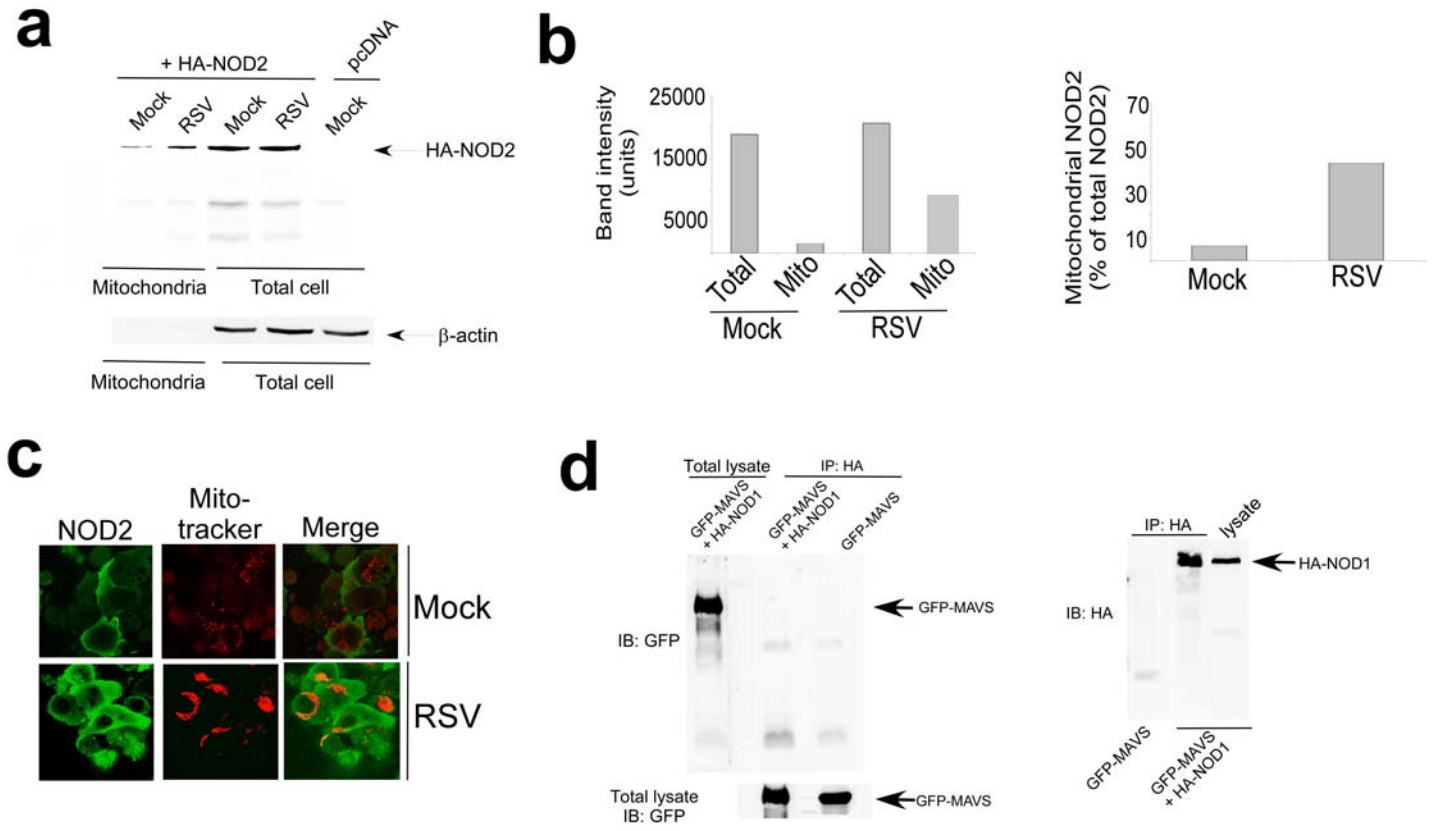

Figure 9 Interaction of NOD2 with MAVS. **(a)** Mitochondrial and total cellular extract prepared from RSV infected (4h post-infection) 293 cells expressing HA-NOD2 was subjected to immunoblotting with anti-HA (to detect NOD2) and β-actin (to ascertain the purity of mitochondrial extract) antibody. **(b)** The band corresponding to the HA-NOD2 protein [in mitochondrial (Mito) and total cell (Total) extracts] from the immunoblot analysis in Supplemental Fig. 9a was quantified by GeneTools program (Syngene) and the values (corresponding to the band intensities) are presented as a bar graph. The values are also presented to demonstrate the percentage of total NOD2 protein that is localized in mitochondria in mock (uninfected) cells vs. infected cells. **(c)** Confocal immuno-fluorescent analysis of RSV infected (at 3h post-infection) A549 cells following labeling with NOD2 (green) antibody (NOD2 antibody was purchased from Cayman Chemical company) and Mito-tracker (red, to detect mitochondria) (Mito-tracker Red was purchased from Invitrogen). The merged yellow image shows co-localization of NOD2 with mitochondria in infected cells. **(d)** Left panel: Co-immunoprecipitation analysis was performed by immuno-precipitating (IP) RSV infected 293 cell (co-expressing HA-NOD1 and GFP-MAVS) lysate with anti-HA-agarose and immunoblotting with anti-GFP antibody. Expression of GFP-MAVS in the cell lysate (by immunoblotting 25 μg of total cellular lysate with anti-GFP antibody) is also shown in the lower panel. Right panel: Expression of HA-NOD1 in the cell lysate (25 μg total lysate) and amount of HA-NOD1 bound to anti-HA-agarose beads was examined by immunoblotting with anti-HA antibody.

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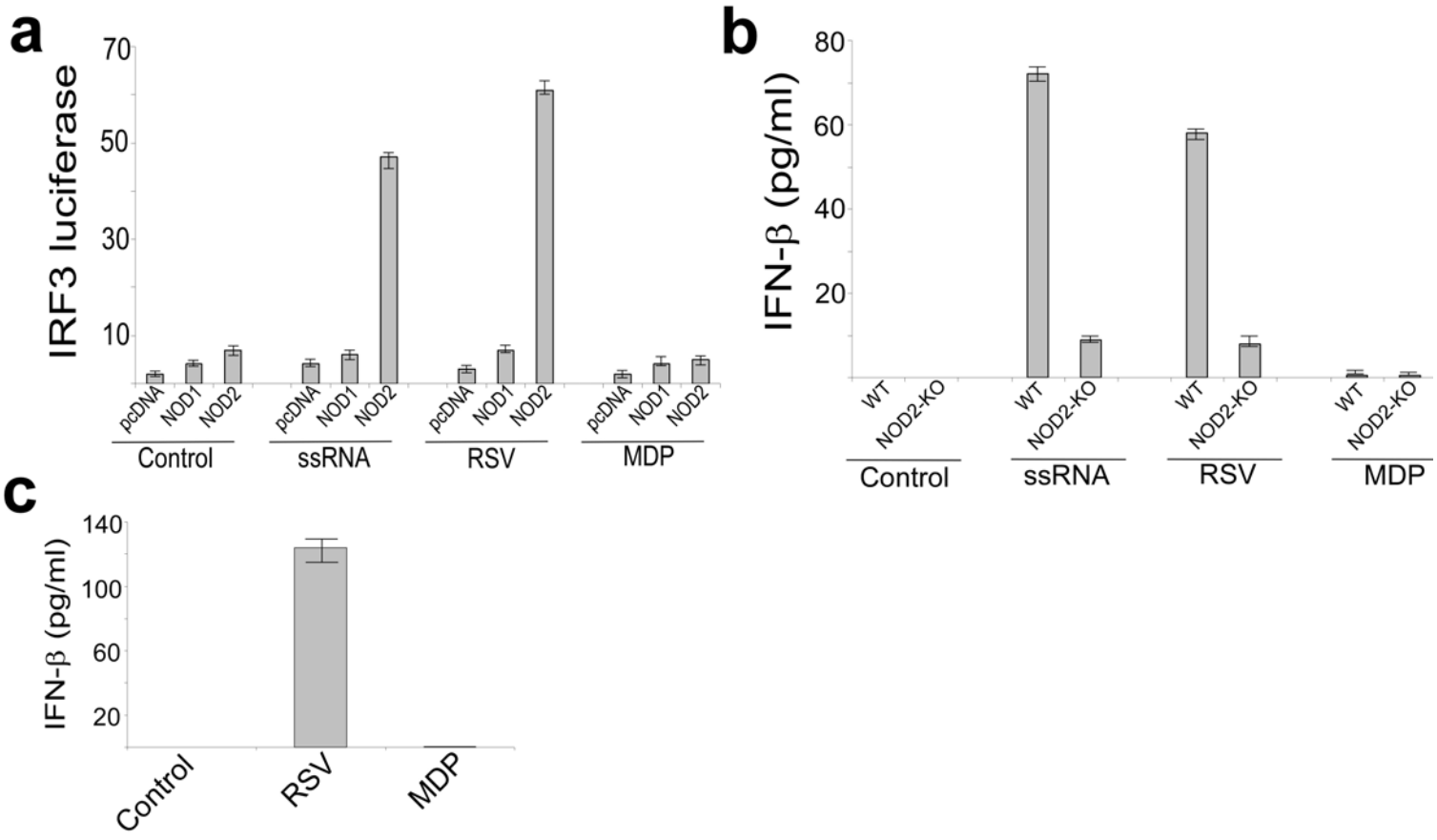

Figure 10 IFN induction by MDP. **(a)** Activation of IRF3-luciferase reporter gene in ssRNA (1 μg/ml, 10h), MDP (10 μg/ml, 24h) treated and RSV infected (6h) 293 cells expressing either pcDNA, HA-NOD2 or HA-NOD1. Control; untreated or mock infected cells. The luciferase assay results are presented as mean ± s.d. from three independent experiments. **(b)** IFN-β production from control, ssRNA (1 μg/ml, 24h), MDP (15 μg/ml, 24h) treated and RSV infected (6h) bone marrow derived macrophages (BMM) isolated from wild type (WT) and NOD2-knock-out (KO) mice. **(c)** IFN-β production from control, MDP treated (15 μg/ml, 24h) and RSV infected (6h) NHBE cells. Amount of IFN-β was measured by ELISA and each value represents the mean ± s.d. from three independent experiments.

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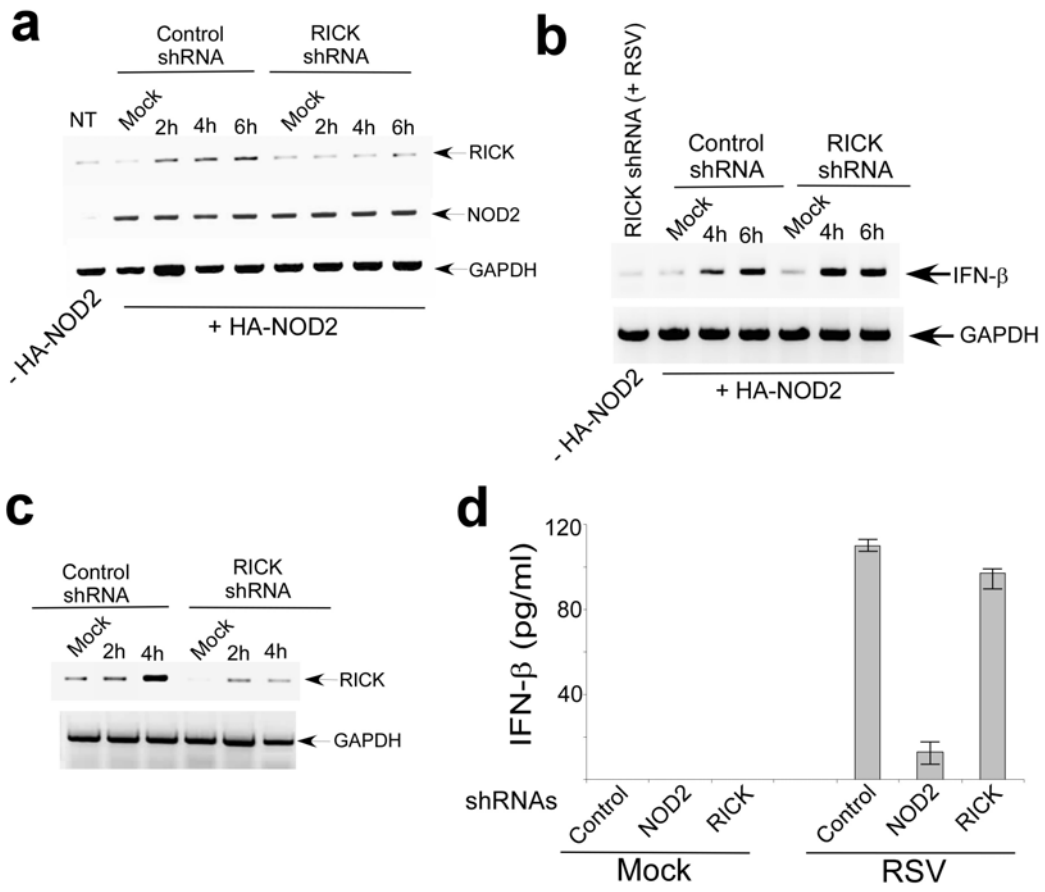

Figure 11 Role of RICK during activation of antiviral response by NOD2. **(a)** Endogenous RICK and HA-NOD2 expression in RSV infected (4h post-infection) HA-NOD2 expressing 293 cells transfected with either control shRNA or RICK shRNA. NT; non-transfected cells that is not expressing HA-NOD2. Please note that shRNA was utilized to silence expression of endogenous RICK in 293 cells. **(b)** RT-PCR analysis of IFN-β expression in mock and RSV infected 293 cells (expressing HA-NOD2) transfected with control shRNA or RICK shRNA. The first lane of the gel shows that 293 cells transfected with RICK shRNA in the absence of HA-NOD2 expression failed to induce IFN-β following RSV infection. **(c)** Efficiency of RICK specific shRNA was examined by analyzing endogenous RICK expression (by RT-PCR) in control or RICK shRNA transfected NHBE cells infected with RSV. **(d)** IFN-β production from mock and RSV infected NHBE cells transfected with either control shRNA, NOD2 shRNA or RICK shRNA. Amount of IFN-β was measured by ELISA and each value represents the mean ± s.d. from three independent experiments.

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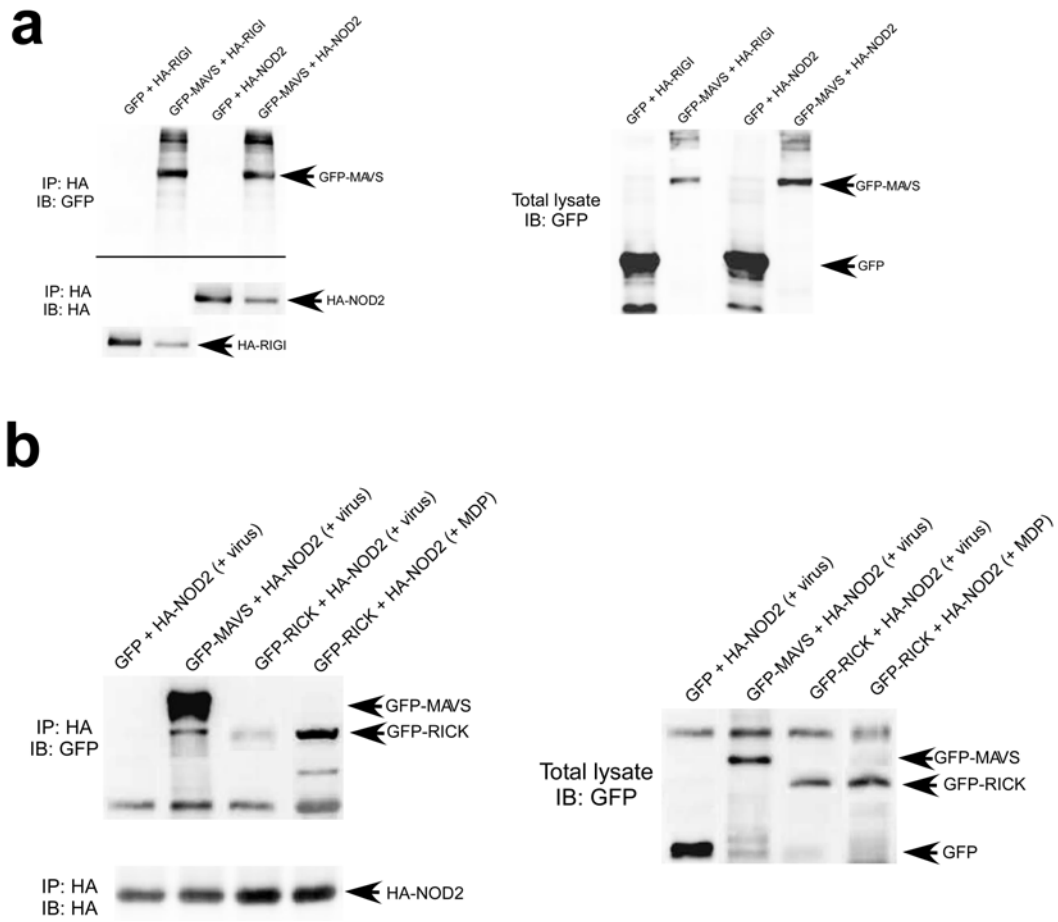

Figure 12 Interactions of RICK and RIGI with MAVS and NOD2. **(a)** Left panel: Co-immunoprecipitation analysis was performed by immuno-precipitating (IP) 293 cell [co-expressing HA-NOD2/GFP-MAVS, HA-RIGI/GFP-MAVS, HA-NOD2/GFP (empty GFP expressing vector), HA-RIGI/GFP)] lysate with anti-HA antibody (covalently conjugated to agarose beads) and immunoblotting with anti-GFP antibody. In the lower panel, amount of HA-NOD2 and HA-RIGI bound to anti-HA-agarose beads is also shown by immunoblotting the HA-agarose bound proteins with anti-HA antibody. Right panel: Expression of GFP- MAVS and GFP in the cell lysate was analyzed by immunoblotting 12 μg of total cellular lysate with anti-GFP antibody. **(b)** Left panel: Co-immunoprecipitation analysis was performed by immuno-precipitating (IP) 293 cell [co-expressing HA-NOD2/GFP-MAVS, HA-NOD2/GFP-RICK, HA-NOD2/GFP (empty GFP expressing vector) +/- RSV infection or MDP treatment (20 μg/ml, 3h)] lysate with anti-HA antibody (covalently conjugated to agarose beads) and immunoblotting with anti-GFP antibody. In the lower panel, amount of HA-NOD2 bound to anti-HA-agarose beads is also shown by immunoblotting the HA-agarose bound proteins with anti-HA antibody. Right panel: Expression of GFP-MAVS, GFP-RICK and GFP in the cell lysate was analyzed by immunoblotting 12 μg of total cellular lysate with anti-GFP antibody.

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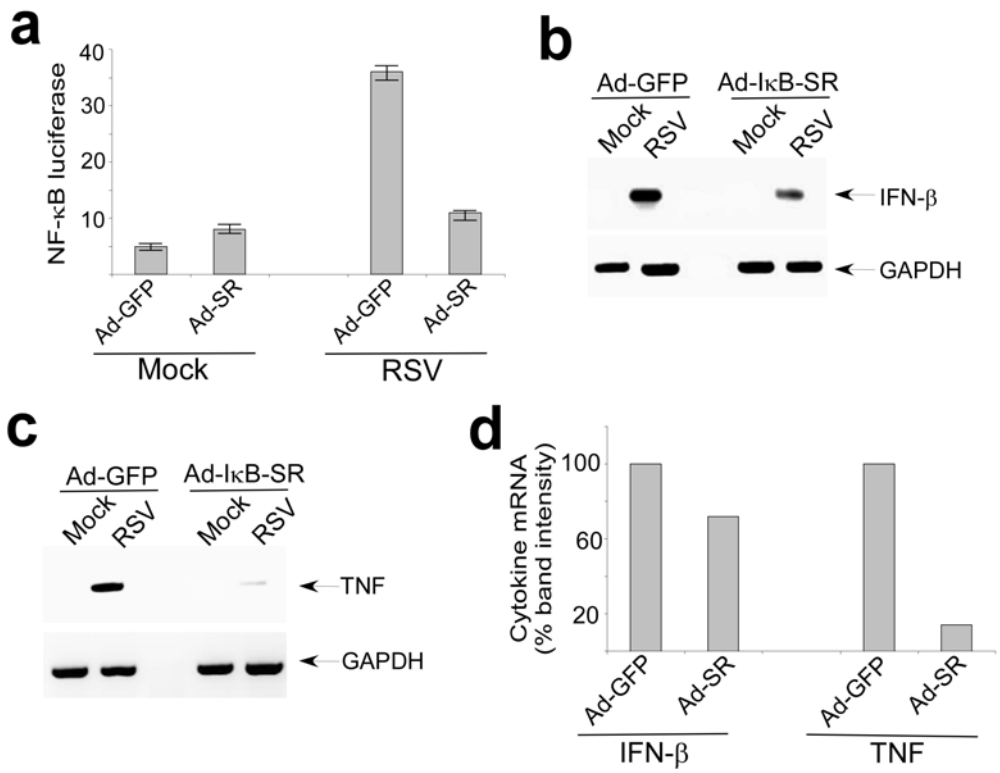

Figure 13 Role of NF-κB in NOD2 mediated IFN gene expression. **(a)** NHBE cells transfected with NF-κB luciferase plasmid were infected with non-infectious recombinant adenoviruses expressing GFP (Ad-GFP) or IκB-super-repressor (Ad-SR). The GFP and IκB-SR expressing cells were infected with RSV and luciferase activity was measured at 16h post-infection. The luciferase assay results are presented as mean ± s.d. from three independent experiments. Please note that while RSV activated NF-κB in control GFP expressing cells, expression of IκB-SR led to drastic reduction in NF-κB activity. **(b)** RT-PCR analysis of IFN-β expression in GFP or IκB-SR expressing NHBE cells infected with RSV for 4h. **(c)** RT-PCR analysis of TNF-α expression in GFP or IκB-SR expressing NHBE cells infected with RSV for 16h. **(d)** The band corresponding to IFN and TNF from the RT-PCR analysis in Supplemental Fig. 16b was quantified by GeneTools program (Syngene) and the values (corresponding to the band intensities) are presented as a bar graph. 100% represents band intensity from RSV infected cells expressing control GFP.

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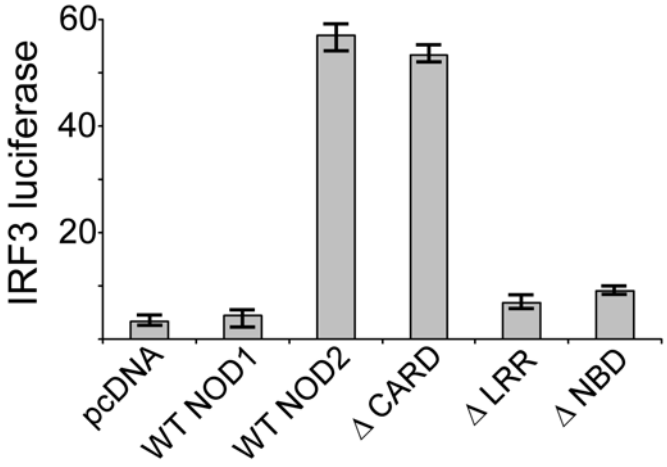

Figure 14 LRR and NBD domains of NOD2 are required for induction of IFN response. Activation of IRF3-luciferase reporter gene in mock infected and RSV infected (6h post-infection) 293 cells expressing pcDNA, WT NOD2 and various NOD2 deletion mutants [ΔCARD (NOD2 mutant lacking both CARD domains), ΔNBD (NOD2 mutant lacking the NBD domain) and ΔLRR (NOD2 mutant lacking the LRR domain)]. The luciferase assay results are presented as mean ± s.d. from three independent experiments.

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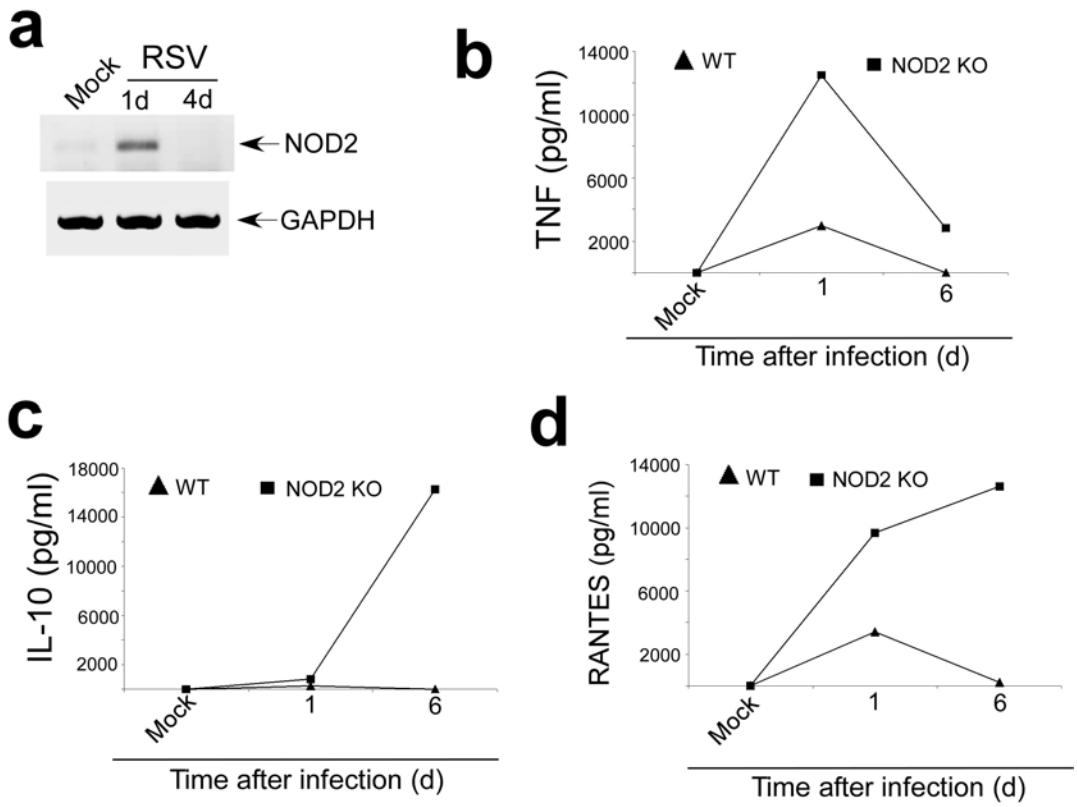

Figure 15 NOD2 expression in RSV infected mice lungs and pro-inflammatory cytokine and chemokine production from RSV infected wild-type (WT) and NOD2-KO mice. **(a)** RT-PCR analysis of murine NOD2 expression in lungs following infection of wild type mice with RSV ($5X10^6$ pfu/animal) for 1d and 4d. Concentrations of TNF-α **(b)**, IL-10 **(c)** and RANTES **(d)** were measured in bronchoalveolar (BAL) specimens at 1d and 6d post-infection by FACS analysis using the BD Cytometric mouse cytokine/chemokine Bead Array kit (BD BioSciences). The cytokine values (n=4 mice/group) are presented as mean ± s.e.m. $p < .05$, by t-test when data were normally distributed, or by Mann-Whitney Rank sum test when data were not normally distributed.

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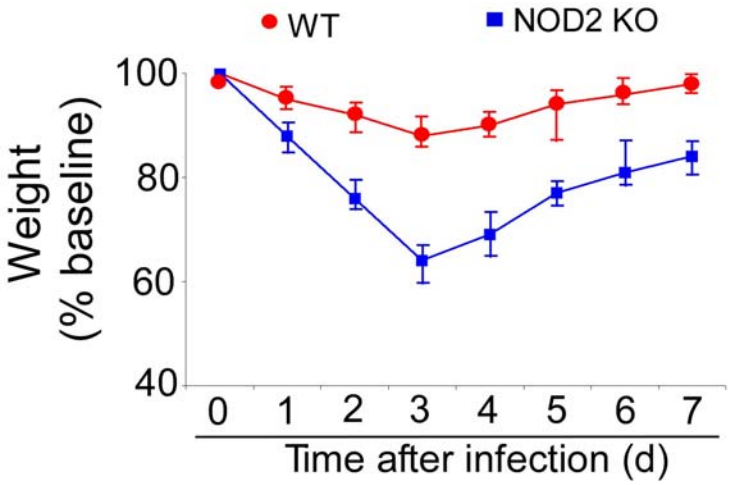

Figure 16 Loss of body weight of RSV infected mice. Body weight of RSV infected ($5X10^6$ pfu/animal) wild-type (WT) (n=6 mice/day) and NOD2-KO (n=6 mice/day) mice during the course of infection. Results are expressed as the % change in body weight from the baseline (uninfected animals) and the results represent mean ± s.e.m. (by t-test $p < 0.05$, WT vs. NOD2-KO mice).

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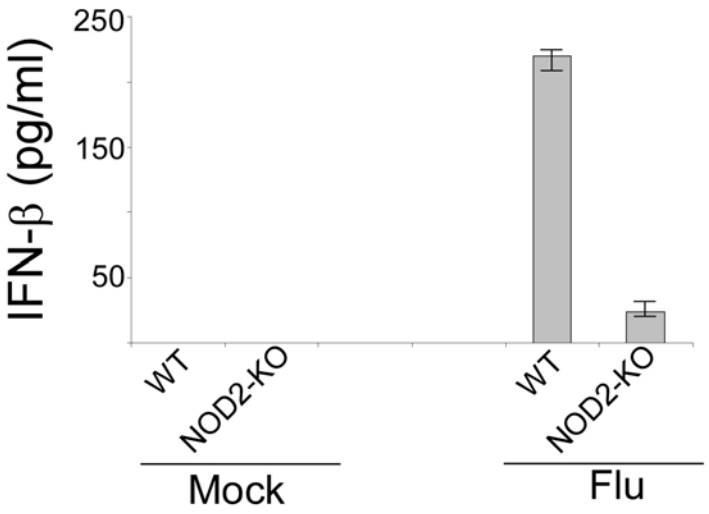

Figure 17 NOD2 is required for IFN production during early influenza A virus infection of mice. IFN-β concentrations in the bronchoalveolar lavage (BAL) of influenza A [A/PR/8/34 (H1N1) virus] infected (1 X 10^5 pfu/mouse) WT and NOD2 KO mice were measured at 2d post-infection. IFN-β was measured by ELISA and each value (n=5 mice/group) represents mean ± s.e.m. $p < .05$, by t-test when data were normally distributed, or by Mann-Whitney Rank sum test when data were not normally distributed.

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