Negative control of TLR3 signaling by TICAM1 downregulation

Shasha Tao, Lingxiang Zhu, Pakkei Lee, Wai-ming Lee, Kenneth Knox, Jie Chen, Yuanpu Peter Di and Yin Chen

ONLINE DATA SUPPLEMENT

Supplemental Materials and Methods:

1. Viruses, chemicals, inhibitors, antibodies

RV16 stock was amplified and purified based on the previous published protocol (18). Briefly, HeLa cell suspension was infected at room temperature for 1 h with a multiplicity of infection (MOI) of 10–15 PFU per cell. Infected cell suspensions were diluted 10-fold in prewarmed medium B and incubated at 35°C for 7–8 h. The cells were then pelleted and resuspended in phosphate-buffered saline (PBS). Virus was released from cells by three cycles of freezing and thawing and then harvested as the supernatant after centrifugation of pellet cell debris. Sucrose gradient was used to purify the virus particles released from cells. Synthetic dsRNA was purchased from InvivoGen (San Diego, CA). Chemical inhibitors (zVAD, MG132) were purchased from Calbiochem (EMD Biosciences, Inc., San Diego, CA). Anti-RV16 antibody was used to determine the expression of viral coat protein (VP0 and VP2) as described before (1). For western blot, anti-TLR3 antibody was purchased from Abcam (Cambridge, MA). Antibodies against IRF3, TBK1, Caspase 3, TICAM1, phospho-STAT1 were purchased from Cell signaling technology (Danvers, MA). Anti-Nrf2 and anti-actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). TICAM1-CFP (TICAM1 was in-frame fused with CFP at its C-terminus) was purchased from Addgene (Cambridge, MA) under a MTA.

2. Cell culture conditions

Normally, primary cells were plated on a 25-mm Transwell (Costar, Corning, NY) chamber at 1–2 x 10^4 cells/cm^2 in Ham's F-12-DMEM (1:1) supplemented with insulin (5

μg/ml), transferrin (5 μg/ml), epidermal growth factor (EGF, 10 ng/ml), dexamethasone (0.1 μM), cholera toxin (10 ng/ml), bovine hypothalamus extract (15 μg/ml), BSA (0.5 mg/ml), and all-*trans*-retinoic acid (30 nM). The cells were subjected to an immersion culture condition for 1 wk and then shifted to an air-liquid interface culture condition. Under this biphasic culture condition, high transepithelial resistance (>500 $\Omega \cdot cm^2$), multiple cell layers, beating cilia, and the formation of mucus-secreting granules were observed. Normally, experiments were performed 21 days or 2 wk after the culture condition was switched from immersion to air-liquid interface. The medium was routinely changed once every other day. BEAS-2B was cultivated in serum-free medium as described before (20). Human primary lung fibroblast and macrophage were cultivated in RPMI media plus 10% fetal bovine serum (FBS). To count for the donor variation, every experiment involving primary cells was repeated on at least three independent donors. NCIH292 and HeLa were obtained from ATCC. NCIH292 was cultivated in RPMI media plus 10% FBS. HeLa was cultivated in DMEM plus 10% FBS. All RV infection and dsRNA treatment were performed at growth factor (or serum)-starved conditions.

3. Real-time PCR

cDNA was prepared from 3 μg of total RNA with Moloney murine leukemia virus (MoMLV)–reverse transcriptase (Promega, Inc.) by oligo-dT primers for 90 min at 42°C in a 20-μl reaction solution, and was then further diluted to 100 μl with water for the following procedures. Two microliters of diluted cDNA was analyzed using 2x SYBR Green PCR Master Mix by an ABI 5700 or ABI Prism 7900HT Sequence Detection

System (Applied Biosystems Inc., Foster City, CA), following the manufacturer protocol. Primers (Table.1) were used at 0.2 μM. The PCR reaction was performed in 96-well optical reaction plates, and each well contained a 50-μl reaction mixture. The SYBR green dye was measured at 530 nm during the extension phase. The relative mRNA amount in each sample was calculated based on the $\Delta\Delta C_t$ method using housekeeping gene *GAPDH*. The purity of amplified product was determined from a single peak of a dissociation curve. Efficiency curves were performed for each gene of interest relative to the housekeeping gene, based on the manufacturers instructions.

4. Western blot

Total cellular protein was collected and the western blot analyses were based on the methods described previously (12). Equal protein load for both total and nuclear proteins was confirmed using the staining of anti–actin antibody.

5. ELISA assay of IFNβ and IFNλ1

Cell culture media was collected for ELISA assay as described before (19). IFNβ kit was purchased from Invitrogen (Carlsbad, CA). And IFNλ1 kit was purchased from R&D systems (Minneapolis, MN). The concentration of either IFNβ or IFNλ1 was determined by titrating against the standard made with purified IFNβ protein or IFNλ1 protein, respectively. IFNβ standard was labeled as international units (IU)/ml, and IFNλ1 standard was labeled as pg/ml.

Supplemental Figures:

Fig. E1: TICAM1 knockdown by different siRNA. A) NCI-H292 cells were mock-transfected (lipo), or transfected with control siRNA (siC), scrambled TICAM1 siRNA control (siTS), two different siRNA against TICAM1 (the sequence of siT was described in Materials and Methods, siT1: CCATAGACCACTCAGCTTT(2)), respectively. 24hrs later, total cellular protein was collected for western blot analysis of TICAM1. Actin was used as the loading control. B) TICAM1-dependent IFNβ gene expression by dsRNA. Cells were transfected with different siRNA as described in A). 24hrs later, the cells were treated with dsRNA (25ug/ml) for 3hrs and RNA was extracted. IFNβ expression was determined by real-time PCR. The data are presented as the fold inductions by compared with mock-transfected (lipo) and mock (saline)-treated control. nd: no difference. #,$: $p < 0.05$ when comparing siRNA (siT or siT1) transfected samples with mock transfected (lipo) samples, n= 5.

Fig. E2: Specificity of TBK1 and IRF3 antibodies. NCI-H292 cells were transfected with control siRNA (siC), siRNA against TBK1 (siTBK1: GACAGAAGTTGTGATCACATT(3)), and siRNA against IRF3(siIRF3: GGAGGATTTCGGAATCTTC(4)), respectively. 24hrs later, total cellular protein was collected for western blot analysis of TBK1 or IRF3, respectively. Actin was used as the loading control.

Fig.E3: TICAM downregulation was dependent on RV replication. To make replication-deficient RV16 (UV-RV), stocks of RV16 were ultraviolet irradiated, as described previously (1). RV16 (MOI=10) or UV-irradiated RV16 was used to infect HeLa cells for

24hrs. Cellular protein was collected for western blot analysis of TICAM1. Actin was used as the loading control.

References:

E1. Chen Y, Hamati E, Lee PK, Lee WM, Wachi S, Schnurr D, Yagi S, Dolganov G, Boushey H, Avila P, et al. Rhinovirus induces airway epithelial gene expression through double-stranded rna and ifn-dependent pathways. *Am J Respir Cell Mol Biol* 2006;34(2):192-203.

E2. Wang Q, Nagarkar DR, Bowman ER, Schneider D, Gosangi B, Lei J, Zhao Y, McHenry CL, Burgens RV, Miller DJ, et al. Role of double-stranded rna pattern recognition receptors in rhinovirus-induced airway epithelial cell responses. *J Immunol* 2009;183(11):6989-6997.

E3. Thurston TL, Ryzhakov G, Bloor S, von Muhlinen N, Randow F. The tbk1 adaptor and autophagy receptor ndp52 restricts the proliferation of ubiquitin-coated bacteria. *Nat Immunol* 2009;10(11):1215-1221.

E4. Opitz B, Vinzing M, van Laak V, Schmeck B, Heine G, Gunther S, Preissner R, Slevogt H, N'Guessan PD, Eitel J, et al. Legionella pneumophila induces ifnbeta in lung epithelial cells via ips-1 and irf3, which also control bacterial replication. *J Biol Chem* 2006;281(47):36173-36179.

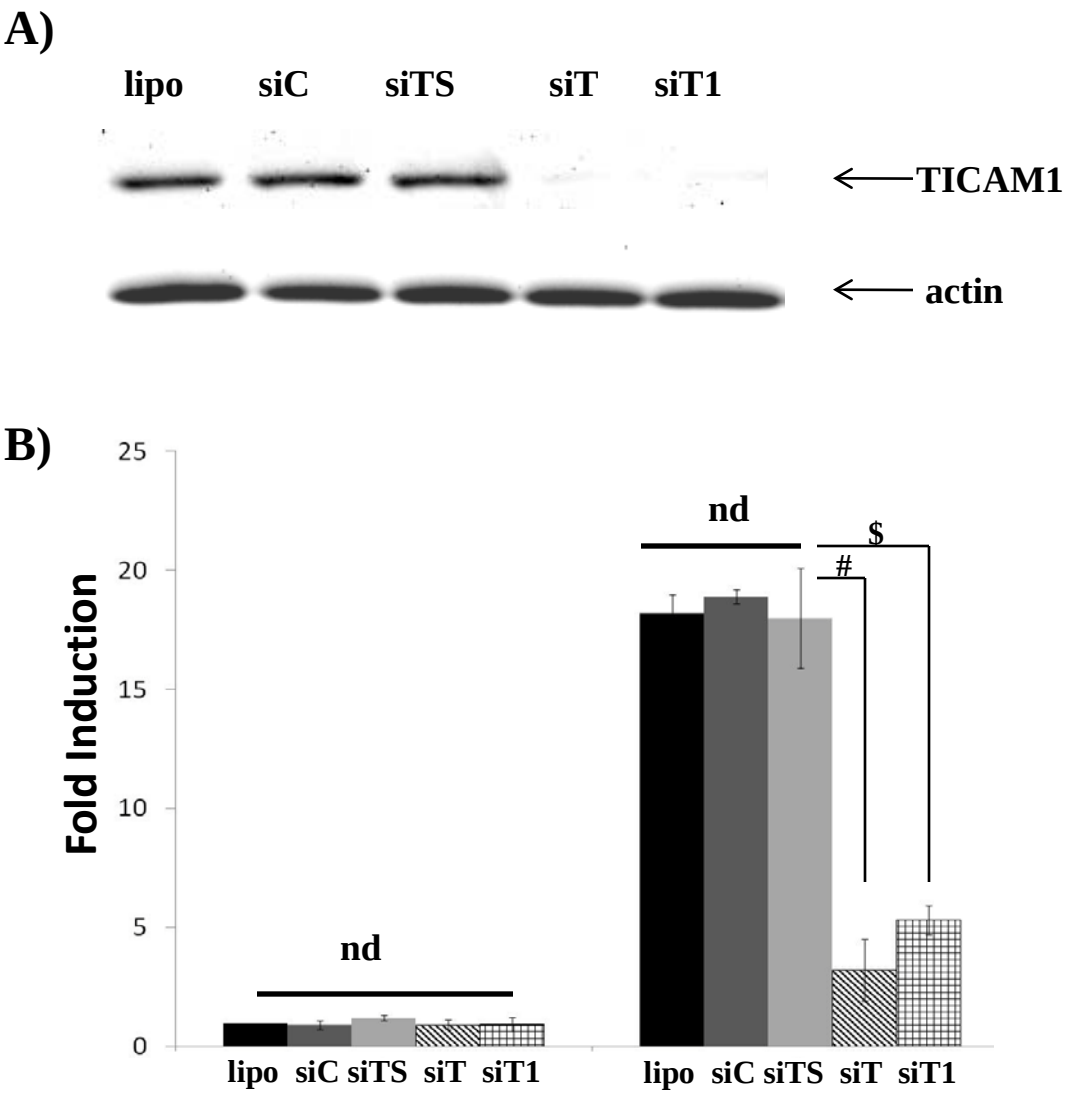
A)
lipo
siC
siTS
siT
siT1
TICAM1
actin
B)
Fold Induction
25
20
15
10
5
0
nd
nd
$
#
lipo siC siTS siT siT1
lipo siC siTS siT siT1

Fig. E1

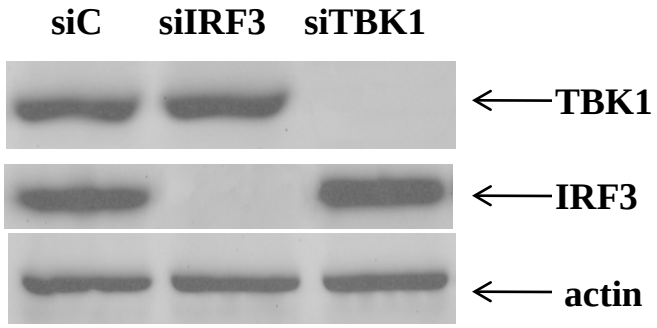

Fig. E2

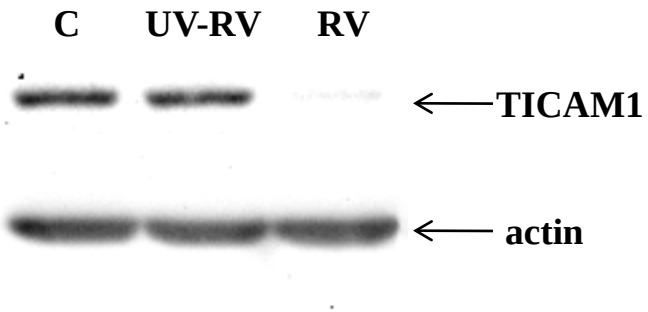

Fig. E3