Virus Research 253 (2018) 28–37

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

TNF-α exerts potent anti-rotavirus effects via the activation of classical NF-κB pathway

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ARTICLE INFO

Keywords:
Antiviral
Cytokines
NF-κB
Signaling
Rotavirus
TNF-α

ABSTRACT

Active virus-host interactions determine the outcome of pathogen invasions. It has been shown that in isolated dendritic cells (DCs), rotavirus can induce the expression of tumor necrosis factor α (TNF-α), a vital cytokine mediating host immune responses. However, the role of TNF-α in rotavirus infection is unknown. In this study, we demonstrated that TNF-α has potent anti-rotavirus effects, independent of type I interferon production. Blocking of TNF-α by infliximab, a clinically available TNFα antibody, totally abrogated this effect. Mechanistic studies revealed that the anti-rotavirus effect of TNF-α was achieved by NFκB-regulated genes via the activation of classical nuclear factor κB (NF-κB) signaling. Our study reveals the pivotal role and the mechanism-of-actions of TNF-α in the host defense against rotavirus. Thus, this knowledge may contribute to the better understanding of the complexity of rotavirus-host interactions.

1. Introduction

Rotavirus is a viral pathogen that causes severe gastroenteritis worldwide, especially in children under five years of age (Tate et al., 2012). It is also prevalent in immunocompromised patients, such as pediatric and adult organ transplant recipients (Yin et al., 2015b). Notably, gastrointestinal infections including rotavirus have been recognized as potential cause of exacerbation and induction of flares in inflammatory bowel disease (IBD) patients (Masclee et al., 2013). Rotavirus primarily infects mature enterocytes at the tips of the intestinal villus, leading to acute diarrhea (Greenberg and Estes, 2009). However, systemic infections can occur which involve multiple extra-intestinal organs, including liver, kidney and the central nervous system (Chiappini et al., 2005).

The innate and adaptive immune systems play an essential role in the limitation of rotavirus infection in infected hosts (Angel et al., 2012; Holloway and Coulson, 2013). Rotavirus can induce the production of interferons (IFNs) and cytokines, including interferon α (IFN-α), IFN-β, tumor necrosis factor α (TNF-α), interleukin 6 (IL-6) and also IL-8 in dendritic cells (DCs) (Deal et al., 2010; Mesa et al., 2007; Rosales-Martinez et al., 2016). Previous studies have demonstrated a role for the different types of IFNs in constraining rotavirus infection (Hernandez et al., 2015; Lin et al., 2016; Saxena et al., 2017). In addition, we have previously shown that rotavirus modulates the expression of interferon-stimulated genes (ISGs) that cooperatively mediate an anti-viral state in the infected cells (Yin et al., 2015a). On the other hand, rotavirus efficiently develops strategies to counteract these anti-viral responses (Arnold et al., 2013; Ding et al., 2016), indicating an active and dynamic virus-host interplay following infection.

TNF-α was first described as a serum factor that mediates killing of tumors in vitro, from which it derives its name (Carswell et al., 1975). Further studies discovered that TNF-α is a potent and essential mediator of inflammatory responses. Aberrant regulations of TNF-α have been associated with many immune-mediated inflammatory diseases, such as rheumatoid arthritis (RA) and IBD. This has led to the development of therapeutic agents targeting TNF-α that are now successfully used in the clinic (Kalliolias and Ivashkiv, 2016; Sedger and McDermott, 2014). However, these TNF-α antagonists are well known to increase the risk of severe viral (including rotavirus) and bacterial infections, thus limiting their use in groups of patients (Kim and Solomon, 2010; Sedger and McDermott, 2014). This phenomenon highlights the fact that TNF-α has either direct or indirect effects against bacterial and viral

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https://doi.org/10.1016/j.virusres.2018.05.022
Received 17 November 2017; Received in revised form 11 May 2018; Accepted 29 May 2018
Available online 31 May 2018

infections. Indeed, several studies have demonstrated the anti-viral and anti-bacterial properties of TNF-α against influenza virus (Seo and Webster, 2002), hepatitis C (HCV) and E (HEV) viruses (Wang et al., 2016), poxviruses (Bartee et al., 2009), and *Mycobacterium tuberculosis* (Flynn et al., 1995), either alone or in combination with IFNs.

However, the potential activity of TNF-α on rotavirus has not yet been described. Here we report that TNF-α has potent anti-viral effects against rotavirus. However, its anti-rotavirus effect is totally independent of interferon production and the IFN signaling pathway. Importantly, these effects are achieved by the induction of NFκB-regulated genes through the activation of classical nuclear factor κB (NF-κB) signaling. This study therefore strengthens the role of TNF-α as a host immune response in defending against viral infections.

2. Materials and methods

2.1. Reagents

Recombinant human TNF-α (Peprotech, USA) was dissolved in phosphate-buffered saline (PBS) to a final concentration of 100 µg/mL. Human recombinant IL32 A (GeneTex), IL8 (Abnova), CXCL10 (BioLegend), CXCL11 (BioLegend), CCL20 (BioLegend) and CCL2 (BioLegend) was dissolved to a final concentration of 10 µg/mL. Stock of JAK I inhibitor (Santa Cruz Biotech, CA) was dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St Louis, MO, USA) with a final concentration of 5 mM. ReIA (p65) antibody (C22B4, #4764) was purchased from Cell Signaling Technology (Leiden, The Netherlands). β-actin and TNFR1 antibody (sc-8436) were purchased from Santa Cruz Biotechnology. β-tubulin antibody (ab6046) was purchased from Abcam. Anti-VP4 mouse monoclonal antibody (HS-2) was generously provided by Professor Harry Greenberg (Standford University School of Medicine, USA). Anti-rabbit or anti-mouse IRDye-conjugated antibodies were used as secondary antibodies for western blotting (Stressgen, Victoria, BC, Canada).

2.2. Rotavirus SA11 and human-derived strain

A well-characterized and broadly used laboratory strain, simian rotavirus SA11, was employed. SA11 rotavirus strain used in this study was prepared as previously described (Knipping et al., 2012). Rotavirus genome copy numbers were determined by quantitative real-time polymerase chain reaction (qRT-PCR) referring to a plasmid template using a standard curve calculation method as described previously (Yin et al., 2015a). A standard curve was generated by plotting the log copy number versus the cycle threshold (C_T) value (Supplementary Fig. S1). Human rotavirus (huRV) strains were isolated form rotavirus diarrhea patients as described previously (Yin et al., 2015a).

2.3. Cell culture

Caco2 cell line (human caucasian colon adenocarcinoma ECACC) was cultured in Dulbecco's modified Eagles's medium (DMEM; Lonza, Verviers, Belgium) supplemented with 20% (vol/vol) heat-inactivated fetal calf serum (FCS; Sigma-Aldrich, St. Louis USA), 100 U/mL penicillin and 100 U/mL streptomycin (Gibco, Grand Island, USA). The cells were maintained in 5% CO_2 at 37 °C in a humidified incubator.

2.4. Inoculation of SA11 and huRV rotavirus and treatment

Caco2 cell lines (not differentiated) maintained in T75 flasks were suspended and subsequently seeded into 48-well plates (5 * 10^4 cells/well) in DMEM containing 20% (vol/vol) FCS and 100 U/mL penicillin-streptomycin. When the cell confluence was approximately 80% after 2–3 days of culture, culture medium was discarded. The cell monolayer was then washed twice with PBS. 100 µL of serum-free DMEM medium containing 5 µg/mL of trypsin (Gibco, Paisley, UK) and SA11 rotavirus (MOI 0.7) were added and incubated at 37 °C with 5% CO_2 for 60 min for infection, followed by three times washing with PBS to remove free, uninfecting virus particles. Subsequently, the cells were added with serum-free culture medium containing 5 µg/mL of trypsin (and indicated treatments) and incubated at 37 °C with 5% CO_2 for 48 h. Inoculation protocol of huRV strains is similar to inoculation of SA11 rotavirus.

2.5. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated with a Machery-NucleoSpin RNA II kit (Bioke, Leiden, The Netherlands) and quantified using a Nanodrop ND-1000 (Wilmington, DE, USA). cDNA was prepared from total RNA using a cDNA Synthesis Kit (Takara Bio Inc) with random hexamer primers. qRT-PCR was performed with a SYBRGreen-based real-time PCR (MJ Research Opticon, Hercules, CA, USA) according to the manufacturer's instructions. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was considered as a reference gene to normalize gene expression. Relative gene expressions were normalized to GAPDH using the formula $2^{-\Delta\Delta CT}$ ($\Delta\Delta CT_{sample} - \Delta\Delta CT_{control}$). The SA11 rotavirus sense and antisense primers target 564–585 and 719-699 of the VP6 segment of SA11 genome, respectively. All primers used in this study are listed in Supplementary Table S1.

2.6. IFN production bioassay

The IFN production bioassay was performed to detect secreted IFN proteins in the culture medium as described previously (Xu et al., 2016). Caco2 cells were seeded into 48-well plates and stimulated with TNF-α 10 ng/mL. After 24 h, medium was removed and the cell monolayer was washed three times with PBS. Subsequently, the medium was refreshed and cultured for another 24 h to let the produced IFNs secreted into the medium. The culture (conditioned) medium were then collected and filtered through 0.45 µm pore size membrane. To detect the presence of IFNs, Huh7-ISRE-luc reporter cell lines was used. Huh7-ISRE-luc is a luciferase reporter model in which the firefly luciferase gene was driven by multiple IFN-stimulated response elements (ISRE) promoter. In this model, the firefly luciferase activity can be potently stimulated by a low concentration of IFN-α treatment. Huh7-ISRE-luc cells were cultured in DMEM supplemented with 10% FCS (vol/vol), 100 U/mL penicillin and streptomycin.

2.7. Gene knockdown using lentiviral vectors

Lentiviral pLKO knockdown vectors (Sigma-Aldrich) targeting TNFR1 and ReIA (p65) or scrambled control, were obtained from the Erasmus Center of Biomics and produced in human embryonic kidney epithelial cell line HEK 293 T cells as described previously (Pan et al., 2009). After a pilot study, the shRNA vectors exerting optimal gene knockdown were selected. All shRNA sequences are listed in Supplementary Table S2. As the lentiviral vectors also encode a puromycin resistance gene, transduced cells were subsequently selected by adding puromycin (8 µg/mL; Sigma) to the cell culture medium. Knockdown and control Caco2 cells were infected with rotavirus SA11 as previously described.

2.8. Caco2-based NF-κB and AP-1 luciferase reporter cell lines and measurement of luciferase activity

NF-κB and AP-1 luciferase reporter cells were generated by transducing Caco2 cells with lentiviral vectors expressing the firefly luciferase gene under the control of the promoters containing the NF-κB and AP-1 motifs, respectively (System Biosciences).

To measure the luciferase activity, luciferin potassium salt (100 mM; Sigma) was added to the cells and incubated for 20 min at 37 °C. The luciferase activity was quantified with a LumiStar Optima

luminescence counter (BMG Lab Tech, Offenburg, Germany).

2.9. Western blot assay

After discarding the culture medium (supernatants), cultured cells were lysed in Laemmli sample buffer containing 0.1 M DTT and heated for 5 min at 95 °C, followed by loading onto a 10% sodium dodecyl sulfate polyacrylamide gel and separation by electrophoresis (SDS-PAGE). After 90 min. running at 120 V, proteins were electrophoretically transferred onto a polyvinylidene difluoride (PVDF) membrane (Immobilon-FL) for 1.5 h with an electric current of 250 mA. Subsequently, the membrane was blocked with a mixture of 2.5 ml blocking buffer (Odyssey) and 2.5 ml PBS containing 0.05% Tween 20, followed by overnight incubation with primary antibodies (1:1000) at 4 °C. The membrane was then washed 3 times, followed by incubation for 1 h with IRDye-conjugated secondary antibody (1:5000). After washing 3 times, protein bands were detected with the Odyssey 3.0 Infrared Imaging System.

2.10. Enzyme-linked immunosorbent assay (ELISA)

Serum samples were collected from Crohn's disease patients and stored at −80 °C. Serum TNF-α levels were measured by an ELISA kit (eBioscience, USA) according to the manufacturer's instructions. The absorbance value was measured at 450 nm in an automatic microplate reader. The results were calculated based on a standard curve.

2.11. MTT assay

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma) (10 mM) was added to Caco2 cells cultured in 96-well plates at indicated time points (24 and 48 h) following TNF-α and cytokine treatments. The cells were then incubated at 37 °C with 5% CO_2 for 3 h. Subsequently, the culture medium was removed and 100 µl of dimethyl sulfoxide (DMSO) was added to each well. The absorbance of each well was measured on the microplate absorbance readers (BIO-RAD) at the wavelength of 490 nm.

2.12. Immunofluorescence and confocal microscope assay

Caco2 cells were seeded on glass coverslips in 6-well plates. Then, Caco2 cells were infected with SA11 for 48 h. Subsequently, cells were washed with PBS, fixed in 4% PBS-buffered formalin for 10 min. and blocked with tween-milk-glycine medium (PBS, 0.05% tween, 5 g/L skim milk and 1.5 g/L glycine). Samples were incubated with 1:500 dilution of anti-rotavirus (ab181695) antibody (Abcam) overnight at 4 °C. Subsequently, samples were incubated with 1:1000 dilution of Alexa Fluor™ 594 goat anti-mouse secondary antibodies (Invitrogen). Nuclei were stained with DAPI (4,6-diamidino-2-phenylindole; Invitrogen). Finally, the images were detected using immunofluorescence microscope.

For analysis of ReIA protein, Caco2 cells on glass coverslips in 6-well plates were infected with either mock, SA11, huRV 1 and huRV 2 rotavirus for 8 h. Subsequently, cells were washed twice with PBS. The mock- or rotavirus-infected cells were then stimulated with TNF-α (100 ng/mL) for 1 h. Fixed and permeabilized cells were stained with antibodies to ReIA (C22B4, #4764, Cell Signaling Technology, Leiden, The Netherlands), followed by anti-rabbit IgG(H + L),F(ab') 2 Fragment (Alexa Fluor 488 conjugate) secondary antibodies. The ReIA proteins were visualized with confocal microscopy. Nuclei were stained by DAPI (blue).

2.13. Statistical analysis

Statistical analysis was performed using the nonpaired, nonparametric test (Mann-Whitney test; GraphPad Prism software, GraphPad Software Inc., La Jolla, CA). P values < 0.05 were considered statistically significant.

2.14. Ethical statement

The use of serum samples from IBD patients was approved by the Medical Ethical Committee of the Erasmus Medical Center (Medisch Ethische Toetsings Commissie Erasmus MC). Informed consents were signed by the volunteers or patients who agreed to participate.

3. Results

3.1. TNF-α has potent anti-viral activity against rotavirus

TNF-α serves as a host immune response against a variety of pathogen invasions. It has been previously reported that virus infection, such as influenza virus, induces TNF-α expression in lung epithelial cells, which subsequently inhibits influenza virus replication (Seo and Webster, 2002). To investigate whether rotavirus infection can also induce *tnfa* gene transcription, we employed the widely used Caco2 intestinal epithelium cell line. Infection of these cells with SA11 rotavirus did not trigger transcription of *tnfa* gene at 6, 24, 36 and 48 h post-infection (Supplementary Fig. S2A). In addition, analysis of *IL1B* and *IL8* gene expression levels showed that there were no changes of these genes upon rotavirus infection in Caco2 cells (Supplementary Fig. S2B and C). This finding, which is in agreement with previous studies (Cuadras et al., 2002), suggests that in our experimental system, Caco2 cells cells do not produce TNF-α upon rotavirus infection.

Since rotavirus does not stimulate *tnfa* gene transcription in Caco2 cells, we next investigated the effects of exogenous TNF-α treatment on rotavirus replication. Treatment of SA11 rotavirus-infected Caco2 cells with human recombinant TNF-α for 24 and 48 h significantly inhibited viral RNA levels. At concentrations of 10 and 100 ng/mL for 24 h, TNF-α decreased total viral RNA levels by 41 ± 8% ($P <$ 0.05) and 61 ± 4% ($P <$ 0.01), respectively (Fig. 1A). The inhibitions were more pronounced at 48 h after infections, where TNF-α decreased total viral RNA levels by 67 ± 5% ($P <$ 0.01) and 71 ± 4% ($P <$ 0.01) at concentrations of 10 and 100 ng/mL, respectively (Fig. 1A). In addition, intracellular viral RNA levels quantified at 48 h post-infections were significantly reduced by 92 ± 0.3% ($P <$ 0.001) and 92 ± 2% ($P <$ 0.001) at 10 and 100 ng/mL, respectively (Fig. 1B). These inhibitions were also more potent than those at 24 h, where TNF-α inhibited 55 ± 7% ($P <$ 0.01) and 70 ± 4% ($P <$ 0.001) of intracellular viral RNA levels at concentrations of 10 and 100 ng/mL, respectively (Fig. 1B). Consistently, human TNF-α significantly reduced rotavirus secretion by the infected Caco2 cells as shown in viral titer quantification (Fig. 1C). A notable reduction was also shown in the western blot analysis of VP4 protein (Fig. 1D) and immunofluorescence analysis of VP6 protein of SA11-infected Caco2 cells (Fig. 1E). Importantly, MTT assay demonstrated that TNF-α did not exert cytotoxicity to Caco2 cells (Supplementary Fig. S3A).

It is well-known that TNF-α is involved in IBD pathogenesis and is associated with the disease's activity. TNF-α may influence the expression of pro-inflammatory genes in IBD patients (Slebioda and Kmiec, 2014). To get further insight about the role of TNF-α in rotavirus infection, we collected serum samples from anti-TNF-α naïve Crohn's disease patients. The serum TNF-α levels were measured by ELISA and 4 serum samples with relatively high TNF-α levels were selected (Supplementary Fig. S4A). Notably, all selected serum samples exerted anti-rotavirus effect (as determined by total viral RNA levels) as compared to the control serum (Supplementary Fig. S4B). These results suggest that TNF-α may indirectly influence rotavirus infection.

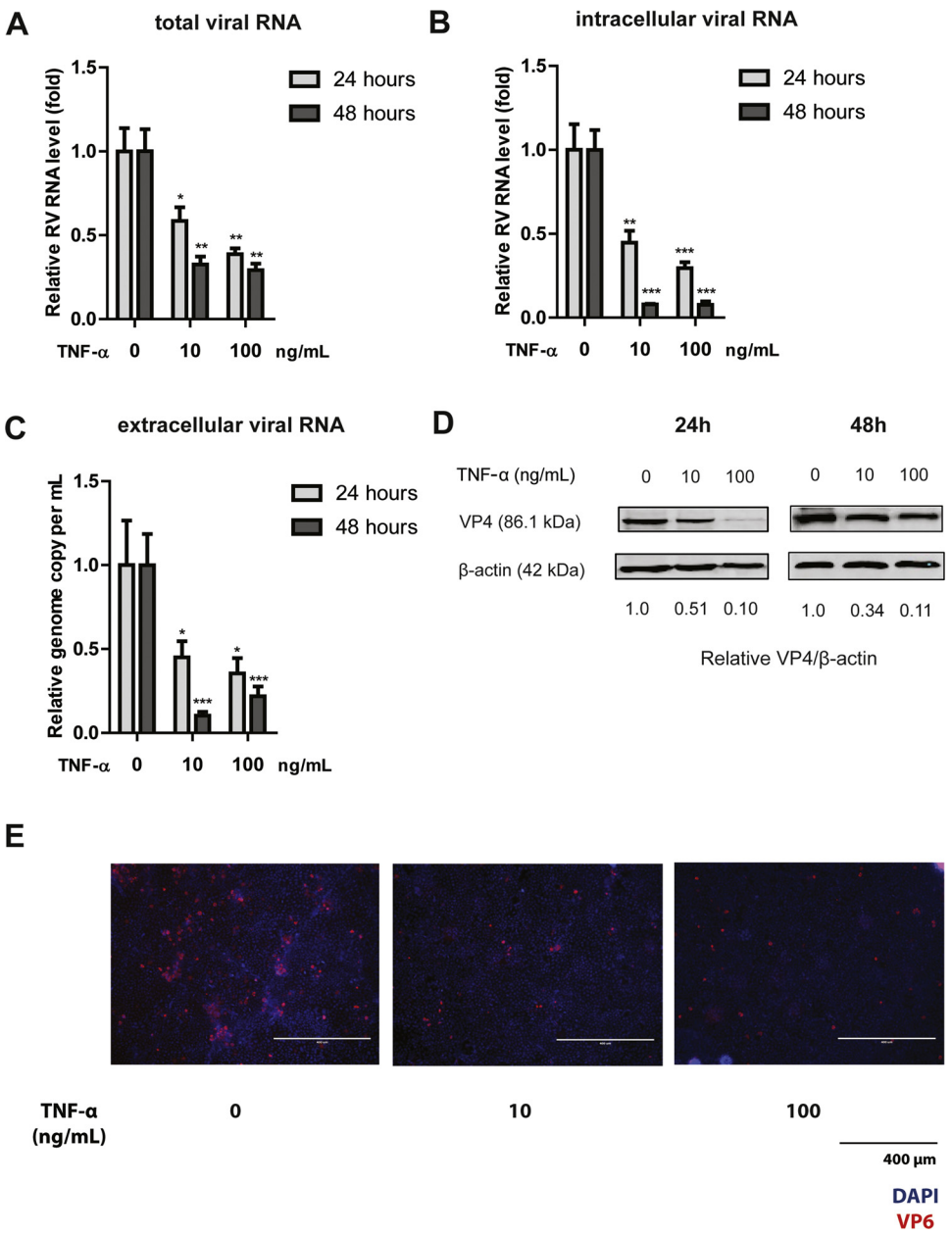


Fig. 1. Anti-rotavirus effects of TNF-α. TNF-α treatment (10 and 100 ng/mL) significantly inhibits rotavirus replication as measured by total **(A)**, intracellular **(B)** and extracellular (secreted) **(C)** rotavirus RNA levels in Caco2 cells both at 24 and 48 h post-infections (n = 6–8, means ± SEM; (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; Mann-Whitney test). **(D)** Western blot analysis of VP4 protein indicates inhibitory effect of 10 and 100 ng/mL TNF-α against rotavirus at 24 and 48 h post-infections. **(E)** Immunofluorescence staining of VP6 rotavirus protein (red) after 48 h infection of Caco2 cells with rotavirus SA11. Nuclei were visualized by DAPI (blue) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

3.2. Anti-rotavirus effect of TNF-α is independent of interferon production and JAK-STAT pathway activity

Since TNF-α can induce type I IFNs in several cell lines, we first investigated the straightforward possibility that TNF-α merely induces IFN-α and IFN-β expression to mediate its anti-rotavirus effect in Caco2 cells. As demonstrated by qRT-PCR, basal constitutive expression of *IFN-α* and *IFN-β* is low, compared to the reference genes *GAPDH* (Fig. 2A). In addition, TNF-α treatment for 48 h did not significantly increase *IFN-α* and *IFN-β1* (Fig. 2B) gene expression. To further confirm the absence of IFN production in our experimental systems, the conditioned medium from the TNFα-stimulated cells were collected and were used to perform an IFN production bioassay. We used Huh7-ISRE-luciferase reporter cell lines, in which the firefly luciferase gene expression is driven by multiple IFN-stimulated response elements (ISRE) promoter. As shown in Fig. 2C, the conditioned medium was not able to stimulate IFN response and confirmed the absence of IFN proteins secreted in the culture medium.

Type I IFNs signal through activation of the Janus Kinases (JAKs) protein which leads to phosphorylation of Signal Transducers and Activators of Transcription (STAT) family of proteins. This JAK-STAT signaling ultimately promotes expression of IFN-stimulated genes (ISGs) as the ultimate anti-viral effector molecules (Wang et al., 2017). To further rule out the possibility of JAK-STAT pathway involvement, we combined TNF-α with *pan*-JAK I inhibitors which potently block JAK-STAT signaling (Wang et al., 2016). Consistently, addition of JAK I inhibitor (5 μM) did not abolish the anti-rotavirus effects of TNF-α (Fig. 2D).

3.3. TNF receptor 1 is essential for TNF-α induced anti-rotavirus effect

TNF-α exerts its biological effect through interaction with two different receptors expressed in the target cells, TNF receptor 1 (TNFR1) and TNFR2. TNFR1 is ubiquitously expressed by almost all human tissues and can be activated by both soluble and transmembrane TNF-α. In contrast, TNFR2 expression is limited to certain cell types, such as immune cells, mesenchymal stem cells and endothelial cells (Kalliolias and Ivashkiv, 2016). In addition, it is suggested that TNFR2 can only be

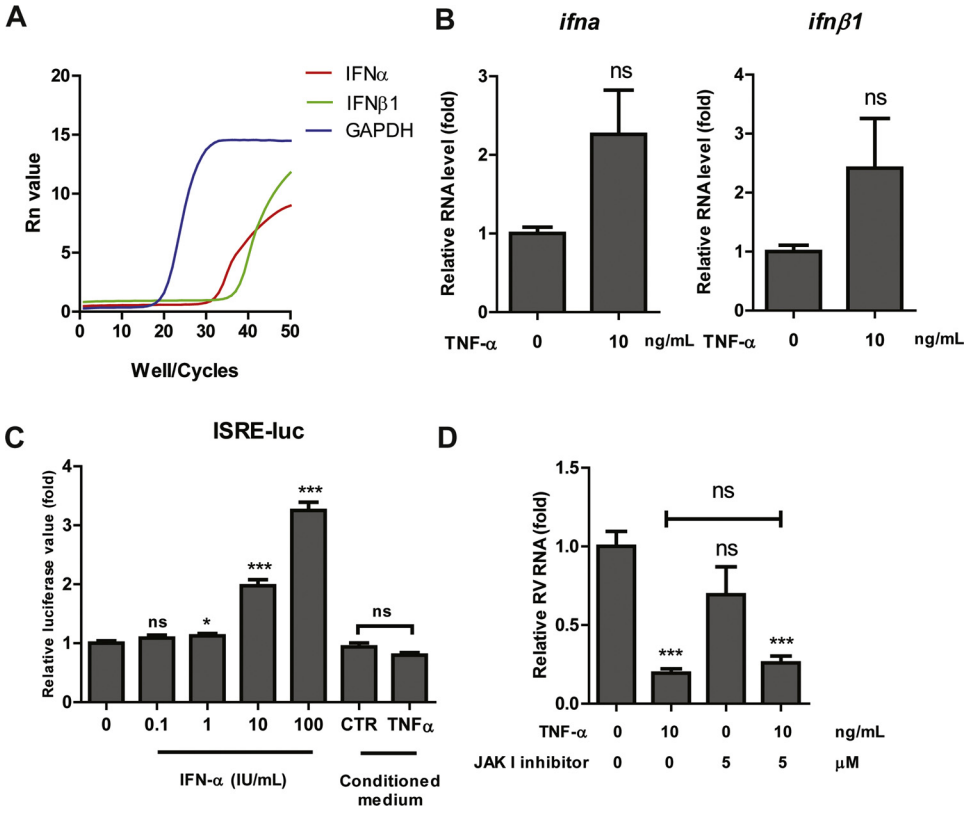


Fig. 2. Anti-rotavirus effect of TNF-α is independent of interferon and the JAK-STAT signaling. (A) The relative basal IFN-α and IFN-β1 expression levels of Caco2 cell lines were determined by qRT-PCR. GAPDH served as a reference gene. **(B)** *IFN-α* and *IFN-β1* gene expression levels in Caco2 cells were not induced following 10 ng/mL TNF-α stimulation for 48 h as quantified by qRT-PCR (n = 12). **(C)** ISRE *firefly* luciferase activity in Huh7-ISRE-luc model treated with the conditioned medium for 48 h (n = 11). **(D)** JAK I inhibitor (5 μM) did not abolish the anti-rotavirus effects of TNF-α at 48 h post-infections (n = 6). Data are presented as means ± SEM (*$P < 0.05$; ***$P < 0.001$; ns, not significant; Mann-Whitney test).

activated by transmembrane TNF-α. Thus, TNFR1 serves as the major signaling component for soluble TNF-α in vivo (Kalliolias and Ivashkiv, 2016). Therefore, this encouraged us to investigate whether anti-rotavirus effects of TNF-α was mediated via TNFR1.

First, Caco2 cells were transduced with integrating lentiviral vectors expressing shRNA that specifically target TNFR1. Among three tested shRNA vectors, sh-96 vector showed the most potent gene silencing capacity, as determined by qRT-PCR (Fig. 3A) and western blot analysis (Fig. 3B). As expected, TNFα-induced *CXCL10* expression was abolished in TNFR1 knockdown cells which was examined at 6 h following TNF-α stimulation (Fig. 3C). Caco2 cells with sh-96 TNFR1 knockdown and scrambled sh-RNA (sh-CTR) as control were subsequently infected with SA11 rotavirus. In sh-CTR transfected cells, treatment with 10 ng/mL TNF-α significantly reduced viral RNA levels (80 ± 2.4% inhibition; $P < 0.001$). However, the anti-rotavirus effect of TNF-α was abolished upon TNFR1 silencing (Fig. 3D). To validate these results, the clinically used anti-TNFα antibody, infliximab (Remicade®), was used. Infliximab binds specifically to TNF-α and blocks its interaction with TNF receptors (Sedger and McDermott, 2014). As expected, infliximab efficiently abrogated TNFα-induced *CXCL10* expression at 6 h after stimulation (Fig. 3E). Consistently, combining TNF-α with infliximab completely blocked the anti-rotavirus effects of TNF-α at 48 h after infections (Fig. 3F).

3.4. TNF-α mediates anti-rotavirus activity through NF-κB signaling

At the cellular level, binding of TNF-α to TNFR1 activates downstream c-JUN N-terminal kinase (JNK), leading to the transcription of AP-1 target genes. Additionally, TNFR1 activates NF-κB, resulting in induced expression of NF-κB target genes (Cabal-Hierro and Lazo, 2012). To dissect whether the anti-rotavirus effects of TNF-α are mediated via the AP-1 or NF-κB pathway, we first constructed Caco2-based stable NF-κB and AP-1 driven luciferase reporter cell lines. As shown in Fig. 4A, TNF-α stimulation resulted in profound induction NF-κB driven luciferase activity in a dose-dependent manner at 12, 24 and 48 h after stimulation, yet no effect on AP-1 driven luciferase activity was observed. Noteworthy, the maximum induction of NF-κB driven luciferase activity was observed at 12 h after stimulation (Fig. 4A). Based on these results, we hypothesized that the anti-rotavirus effects of TNF-α on Caco2 cells are mediated via the classical NF-κB signaling pathway, rather than the AP-1 pathway.

The endpoint of TNF-α signaling is activation of the heterodimeric NF-κB complex, consisting of the ReIA (p65) and p50 (Cabal-Hierro and Lazo, 2012). To examine the role of this ReIA (p65)-p50 complex in mediating anti-rotavirus effects of TNF-α, Caco2 cells were transduced with integrating lentiviral shRNA vectors to silence ReIA. Two of the three tested shRNA vectors targeting ReIA profoundly downregulate *ReIA* both at mRNA and protein level (Fig. 4B and C). Importantly, ReIA knockdown promoted SA11 rotavirus replication by 2.2 ± 0.3 fold ($P < 0.01$) (Fig. 4D), supporting its importance in the control of rotavirus infection. In ReIA knockdown cells, induction of *CXCL10* expression was efficiently blocked following TNF-α (10 ng/mL) stimulation for 6 h (Fig. 4E). Next, Caco2 cells with sh-10 ReIA knockdown and scrambled sh-RNA as control (sh-CTR) were infected with SA11 rotavirus with and without treatment of 10 ng/mL of TNF-α. As before, treatment of sh-CTR transfected cells with 10 ng/mL TNF-α significantly decreased viral RNA levels (60 ± 6.5% inhibition; $P < 0.001$). However, this anti-rotavirus effect was abrogated in ReIA knockdown cells (Fig. 4F), demonstrating that NF-κB signaling pathway is essential to mediate anti-rotavirus effects of TNF-α.

3.5. TNFα-induced cytokines are the downstream effectors to exert anti-rotavirus activity

Following TNF-α stimulation, the activated p65:p50 NF-κB complex translocates to the nucleus where it binds to specific DNA motifs located in the promoter region of its target genes. This event will regulate, either induce or suppress, the expression of TNFα-modulated genes in a cell-type-specific manner (Kalliolias and Ivashkiv, 2016). These NFκB-regulated genes play an essential role in the host immune response such

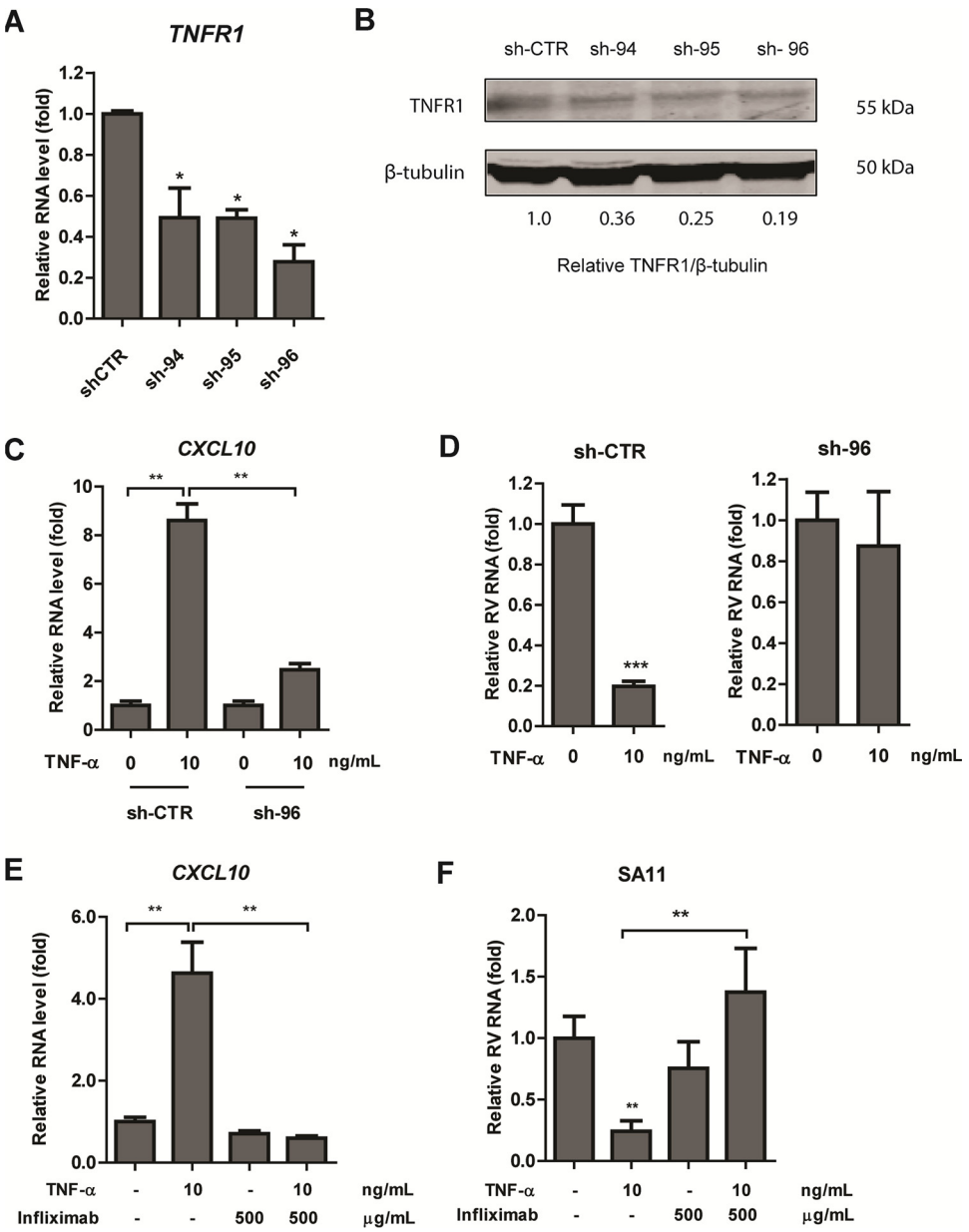


Fig. 3. Anti-rotavirus effect of TNF-α is mediated via TNF receptor 1 (TNFR1). (A) qRT-PCR (n = 4) and **(B)** western blot analysis of TNFR1 knockdown by lentiviral sh-RNA vectors in Caco2 cells. **(C)** TNFR1 knockdown (sh-96) abolished TNFα-induced *CXCL10* expression at 6 h following stimulation (n = 6). **(D)** TNFR1 knockdown (sh-96) led to the blockade of TNFα-induced anti-rotavirus activity at 48 h post-infections (n = 6). **(E)** The TNF-α inhibitor (Infliximab) (500 μg/mL) abrogated the induction of *CXCL10* gene following TNF-α stimulation (10 ng/mL) for 6 h (n = 5). **(F)** Infliximab (500 μg/mL) blocked anti-rotavirus effects of 10 ng/mL TNF-α as measured by qRT-PCR at 48 h post infection (n = 6). Data are presented as means ± SEM (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; Mann-Whitney test).

as pro-inflammatory cytokine and chemokine regulation. Therefore, we examined a panel of cytokines and chemokines which may be induced by human TNF-α in Caco2 cell lines.

As shown in Fig. 5**A**, 10 ng/mL TNF-α stimulation for 6 and 24 h led to increased transcription of the genes encoding IL-8, IL-32, CCL2, CCL20, CXCL10 and CXCL11 genes in Caco2 cells. To dissect whether these TNFα-induced cytokines could inhibit rotavirus infection, we treated SA11-infected Caco2 cells with those cytokines at a concentration of 10 ng/mL. As shown in Fig. 5B, treatment with IL32, IL8, CXCL11 and CCL20 potently inhibited rotavirus replication as measured by qRT-PCR. Western blot analysis of VP4 protein confirmed these results (Fig. 5C). Importantly, MTT assay revealed that these cytokines did not affect cell growth and cytotoxicity to Caco2 cells (Supplementary Fig. S3B). These results collectively show that TNFα-induced cytokines exert a powerful anti-rotavirus activity.

3.6. Sensitivity of human rotavirus (huRV) derived from clinical strains to TNF-α treatment

Previous studies have indicated that several rotavirus strains, including Wa (human), RRV (simian), and OSU (porcine), have different mechanisms of antagonizing NF-κB activation. Those mechanisms include degradation of β-TrCP and inhibition the nuclear translocation of NF-κB (Graff et al., 2009; Holloway et al., 2009). To extend our results, we employed human rotavirus (huRV)-derived clinical strains isolated from two diarrhea patients (Yin et al., 2015a). Caco2 cells infected with two huRV-derived clinical strains were treated with TNF-α 100 ng/mL for 48 h. As shown in Fig. 6, huRV strain 1 replication was notably inhibited by TNF-α treatment (70 ± 6% inhibition; $P < 0.01$), in contrast to huRV strain 2 which was resistant to TNF-α treatment. These results suggest that huRV strains have different sensitivity to TNF-α treatment.

To further investigate the different sensitivity of rotavirus strains against TNF-α treatment observed in our study, we analyzed the ability of different rotavirus strains to block translocation of ReIA to the nucleus (Supplementary Fig. S5). In mock-infected Caco2 cells, the ReIA protein level in the nucleus was increased following TNF-α stimulation (100 ng/mL) for 1 h, compared with mock-infected unstimulated cells. Unexpectedly, in SA11-, huRV 1- and huRV2-infected Caco2 cells, the nuclear accumulation of ReIA was also elevated following TNF-α

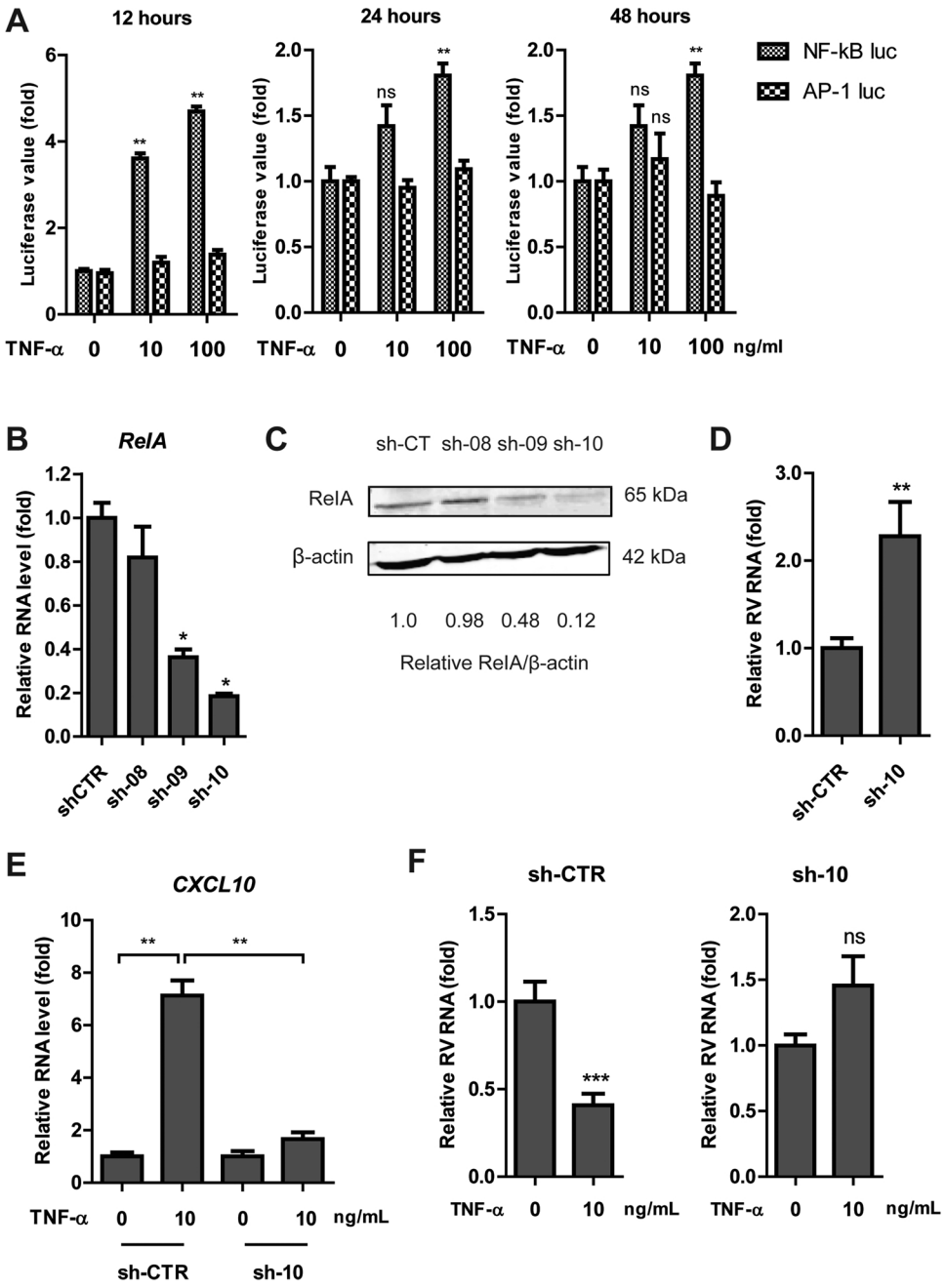


Fig. 4. Anti-rotavirus effect of TNF-α is mediated via NF-κB signaling. (A) In Caco2-based NF-κB reporter cell lines, TNF-α induced activation of NF-κB-related luciferase signals in a dose-dependent manner as measured at 12, 24 and 48 h, while AP1-related luciferase signals were not affected (n = 4–6). qRT-PCR (n = 4) **(B)** and western blot **(C)** analysis of ReIA (p65) knockdown by lentiviral sh-RNA vectors in Caco2 cells. **(D)** ReIA (p65) knockdown (sh-10) promoted rotavirus replication as quantified by total RNA levels in Caco2 cells at 48 h post-infections (n = 8). **(E)** ReIA (p65) knockdown (sh-10) led to the blockade of TNFα-induced *CXCL10* expression at 6 h post-stimulations (n = 6). **(F)** ReIA knockdown (sh-10) resulted in the blockade of TNFα-induced anti-rotavirus activity at 48 h post-infections (n = 6–8). Data are presented as means ± SEM ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; ns, not significant; Mann-Whitney test).

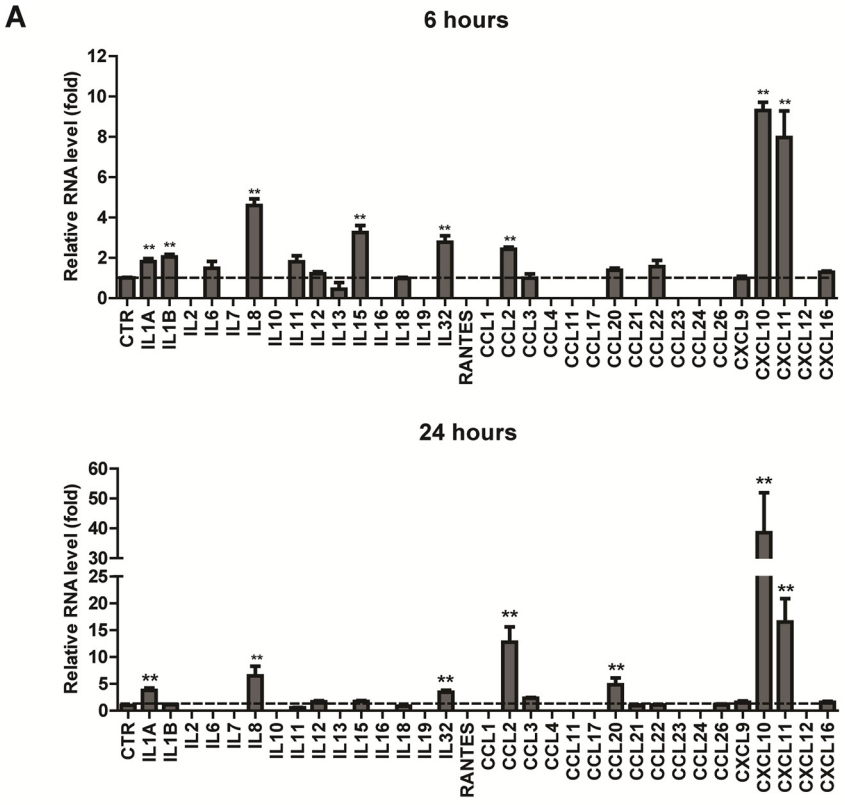
stimulation (Supplementary Fig. S5).

4. Discussion

TNF-α was first known for its anti-cancer activity in vitro (Carswell et al., 1975). The subsequent development of recombinant technology allowed the dissection of the multiple roles of TNF-α in the human body, which includes inflammatory responses, immune regulatory functions, and anti-microbial immunity (Sedger and McDermott, 2014). Inappropriate regulation of TNF-α has been implicated in various human diseases, leading to the use of TNF-α inhibitors in the clinic to treat these TNFα-related diseases. The subsequent increased of viral and bacterial infections in these patients suggests that TNF-α plays an essential role in anti-viral and anti-microbial immunity (Kim and Solomon, 2010).

Timely and rapid immune responses against invading viral pathogens are critical for the host to clear such infections. These early responses mainly depend on anti-viral immunity mediated by innate immune cells that produce various cytokines, such as IFNs, interleukins and TNF-α that act directly to eliminate infections and indirectly by promoting subsequent development of a more specific adaptive immunity (Holloway and Coulson, 2013). In infected children, increased levels of IFN-α were detected in the blood, and related to the clinical outcome (De Boissieu et al., 1993). Thus, investigating innate responses is crucial for understanding virus-host pathogenesis during rotavirus infections (Arnold et al., 2013).

Previous studies have shown the increased production of TNF-α by DCs (Deal et al., 2010) and macrophages (Mohanty et al., 2010) upon rotavirus stimulation. Elevated levels of TNF-α were found in children with rotavirus diarrhea as compared to healthy controls (Azim et al., 1999). Furthermore, in children with rotavirus diarrhea, a significantly higher level of TNF-α was found in serum of those who developed fever and had more frequent episodes of diarrhea (Jiang et al., 2003). It is probably associated with the effect of TNF-α on ion secretion in human



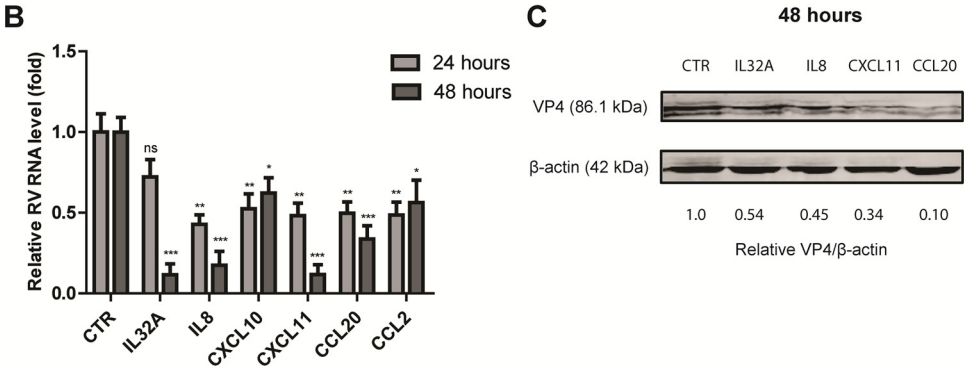


Fig. 5. TNFα-induced cytokines exert anti-rotavirus activity. (A) Expression profile of 33 cytokines in Caco2 cells as quantified by qRT-PCR analysis. Some cytokines were up-regulated with 10 ng/mL TNF-α stimulation of Caco2 cells for 6 and 24 h (n = 6). **(B)** IL32 A, IL8, CXCL10, CXCL11, CCL20 and CCL2 significantly inhibited rotavirus replication as quantified by qRT-PCR of total RNA levels, both at 24 and 48 h (n = 6–8). **(C)** Western blot analysis of VP4 protein indicates the inhibitory effect of 10 ng/mL of IL32, IL8, CXCL11 and CCL20 against rotavirus at 48 h post-infections. Data are presented as means ± SEM (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ns, not significant; Mann-Whitney test).

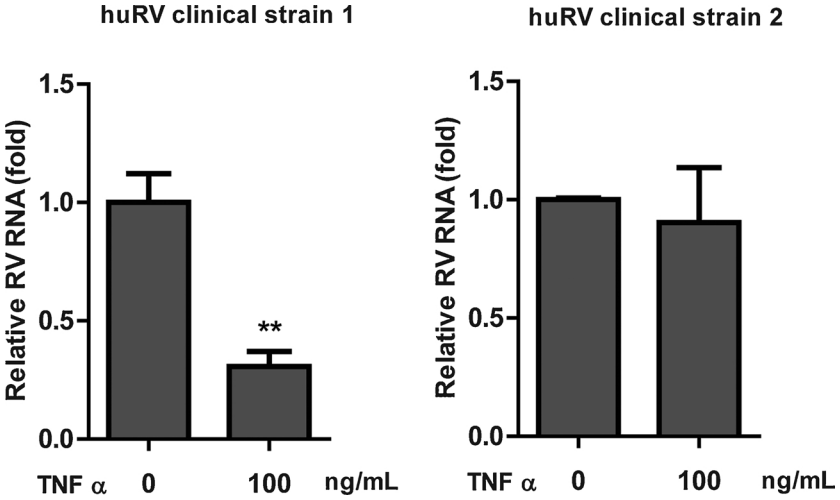


Fig. 6. Sensitivity of human rotavirus (huRV) clinical strains to TNF-α treatment. TNF-α treatment for 48 h (100 ng/mL) significantly inhibits replication of huRV clinical strain 1 (huRV 1) as measured by total rotavirus RNA levels in Caco2 cells; while replication of huRV clinical strain 2 (huRV 2) was not affected. (n = 3, means ± SEM, **$P < 0.01$, Mann-Whitney test).

intestinal epithelial cells (Oprins et al., 2000). This suggests that TNF-α may contribute to the immunity and the disease pathogenesis. However, the exact role of TNF-α in rotavirus infection has not yet been extensively studied. Here we demonstrated that TNF-α has a strong anti-rotavirus effect both at intracellular and extracellular (excreted) levels. We also showed that the clinically used TNF-α inhibitors (infliximab) can completely block this effect, supporting caution of the use of these treatments in patients with latent or active viral infections.

In several cell lines, TNF-α induces the production of type I IFNs (IFN-β) (Ahrens et al., 1990; Hughes et al., 1988; Jacobsen et al., 1989; Yarilina et al., 2008). Thus, we first hypothesized a straightforward explanation that anti-rotavirus effect of TNF-α is merely mediated via type I IFN and consequently, classical IFN-signaling (JAK-STAT pathway activation). However, treatment of Caco2 cells with TNF-α did not significantly increase *ifna* and *ifnb1* gene expression levels, and consequently did not result in IFN production (Fig. 2B and C). In addition, combination treatment with TNF-α and the *pan*-JAK I inhibitor did not abolish the anti-rotavirus effect of TNF-α. Thus, we conclude that the anti-rotavirus effect of TNF-α is independent of IFN production and the JAK-STAT pathway.

TNF-α signals mainly through TNFR1 receptor and stimulates downstream signaling pathways that ultimately activates two major transcription factors, NF-κB (classical NF-κB pathway) and c-Jun (AP-1 pathway) (Cabal-Hierro and Lazo, 2012; Kalliolias and Ivashkiv, 2016). Our experiments using Caco2-based NF-κB and AP-1 reporter cell lines showed that TNF-α signals via NF-κB and not via AP-1. NF-κB is activated following rotavirus infection and is involved in the regulation of cytokine productions (Rollo et al., 1999), indicating its importance in the host defense against rotavirus. Indeed, the following loss-of-function assays by knocking down ReIA (p65), one of the main components of the NF-κB protein complex, led to an increase of rotavirus replication. Furthermore, knockdown of ReIA abrogates anti-rotavirus effect of TNF-α. This indicates the importance of NF-κB in restricting rotavirus infection and also in mediating anti-rotavirus effects of TNF-α.

It is important to note that several studies have reported that other rotavirus strains can inhibit NF-κB activation (Graff et al., 2009) or its TNFα-induced translocation to the nucleus (Holloway et al., 2009). Noteworthy, the NSP1 protein of rotavirus contains a conserved C-terminal phosphodegron-like (PDL) motif which disrupts NF-κB activation by interfering with IκB degradation (Morelli et al., 2015). However, our data suggested that translocation of ReIA to the nucleus was not blocked by SA11 as well as both human rotavirus strains 1 and 2, as demonstrated by confocal microscopy analysis (Fig. S5). Other mechanisms, including blocking of ReIA binding to its promoter, may be involved. Collectively, our results showed that anti-rotavirus effect of TNF-α is mediated via TNFR1 receptor and classical NF-κB signaling.

Several cytokines are induced following exposure of dendritic cells to rotavirus, such as IL-6, IL-8, CXCL-10 and CCL5 (Deal et al., 2010; Rosales-Martinez et al., 2016). Several cytokines have been associated with clinical symptoms in rotavirus diarrheal children (Jiang et al., 2003). Surprisingly, we found that TNFα-induced cytokines, such as IL-8, CXCL10, CXCL11, have potent anti-rotavirus effects. Further experiments are therefore required to clarify their mechanism of action in inhibiting rotavirus infection. Noteworthy, anti-TNFα treatment in Crohn's disease patients is associated with modulation of pro-inflammatory genes, including IL1B and CXCL11 (Leal et al., 2015). Accordingly, our results from patients' samples suggest that a high level of TNF-α in serum may modulate rotavirus infection indirectly by affecting cytokine levels in these patients' sera.

It is important to note that under inflammatory conditions, infected cells are continuously exposed to various cytokines. The effect of these cytokines could be influenced by the presence of other cytokines (Bartee et al., 2008). Several studies have shown a synergistic anti-viral effect of TNF-α and type I IFNs (IFN-α and IFN-β) in the setting of HCV, HEV (Wang et al., 2016), respiratory virus (Fink et al., 2013) and poxvirus infections (Bartee et al., 2009). TNF-α can also synergize with type II IFNs (IFN-γ) in the setting of cytomegalovirus (CMV) infection (Lucin et al., 1994). Therefore, it is interesting to further investigate whether these combined effects can be observed in rotavirus infections.

5. Conclusion

We demonstrate a novel role of TNF-α in inhibiting rotavirus replication. This anti-rotavirus effect is mediated via the classical NF-κB signaling pathway, independent of IFN production and JAK-STAT pathway activity. Furthermore, this finding elucidates the increased risk of viral infection upon TNF-α inhibitor treatments used in the clinic. Thus, this knowledge may contribute to a better clinical management of these patients.

Potential conflicts of interest

The authors declare no conflict of interest.

Author contributions

M. S. H. contributed to study concept and design, acquisition of data, analysis and interpretation of data, drafting of the manuscript and statistical analysis; S. D., S. C., Y. Y., and J. S. contributed to acquisition of data and critical revision of the manuscript; G. M. F. and C. J. W. contributed to IBD patient serum sample collection and data analysis; M. P. P. contributed to study concept, study supervision and critical revision of the manuscript; Q.P. contributed to study concept, obtained funding, study supervision and critical revision of the manuscript; W.W. contributed to study concept and design, study supervision and critical revision of the manuscript.

Acknowledgements

The authors thank to Professor Harry Greenberg (Standford University School of Medicine, USA) for generously providing the mouse monoclonal antibody against rotavirus VP4 protein. This work is supported by the Dutch Digestive Foundation for a career development grant (No. CDG 1304 to Q. P.). This research is also supported by the Indonesia Endowment Fund for Education (LPDP) for funding PhD fellowship to Mohamad S. Hakim and the China Scholarship Council for funding PhD fellowship to W. Wang (201303250056), S. Chen (201606760056) and Y. Yin (201307720045).

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.virusres.2018.05.022.

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