The Effects of Lithium on Inflammation Profiles and Nf-κB Nuclear Translocation in Raw 264.7 Macrophages Exposed to Rift Valley Fever Virus

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Research

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Abstract

Introduction

Rift Valley fever virus is a mosquito-transmitted zoonotic viral infection that results in Rift Valley fever disease. RVFV and other viruses have developed mechanism to circumvent the immune recognition in an attempt to advance their viral progeny. The RVFV S segment encodes a non-structural protein (NSs) known to be the main virulence factor that aid in immune suppression and viral replication. Thus, this study is aimed at investigating the inflammatory effects of lithium using macrophages as innate immune model.

Methods

The supernatant from RVFV-infected Raw 264.7 cells and treated with lithium, was examined with Elisa assay kit to measure production levels of the cytokines and chemokines. The H2DCF-DA and DAF-2 DA oxidant florigenic assays were used to determine the levels of ROS and RNS by measuring the fluorescence intensity shown by the cells post RVFV-infection and lithium treatment. While on the other hand Western blotting assay was used to measure expression levels of the inflammatory proteins and immunocytochemistry measured the cellular location of the NF-κB.

Results

Lithium have shown to stimulate production of IFN-γ 3 hrs pi and reached its peak 12 hrs later. Moreover, the secondary pro-inflammatory cytokine and chemokine, IL-6, and RANTES were elevated at 12 hrs pi. Lithium was shown to stimulate expression of the primary pro-inflammatory molecule, TNF-α as early as 3 hrs pi. In addition to TNF-α expression, the regulatory cytokine, IL-10, was stimulated by lithium and reached its peak 12 hrs pi. The RVFV-infected cells treated with lithium have shown lowered ROS and RNS production as opposed to lithium-free RVFV-infected control cells. The regulatory properties of lithium were further supported by the protein expression assay that showed low expression of the iNOS while stimulating heme oxygenase (HO) and IκB. Lithium was shown to reverse NF-κB nuclear translocation in RVFV-infected Raw 264.7 cells as shown by the Immunocytochemistry assay. Moreover, lithium lowered the presence of nuclear NF-κB in RVFV-infected cells as opposed to untreated RVFV-infected cells and 5 mg/ml LPS controls as shown by the protein expression assay.

Conclusion

This study demonstrates that lithium inhibits NF-kB nuclear translocation and modulate inflammation profiles in RVFV-infected Raw 264.7 cells.

Introduction

Immune response is the vertebrate-dependent phylogenetic defence mechanism against infectious agents such as toxins and microbes. It is classified into innate immune response and humoral immune response (Akira *et al*., 2006; Xiao, 2017). Macrophages play a central role in the innate immune system and inflammation. These cells are antigen presenter cells (APC) located in various locations as tissue residential cells and as well as circulating cells in plasma (Plowden *et al*., 2004). The innate immune response is not entirely non-specific since myeloid lineage cells such as dendritic and macrophages express the pathogen recognition receptors (PRR) that recognise the conserved microbial components termed pathogen associated molecular patterns (PAMP) (Wang *et al*., 2004).

The toll like receptors (TLRs), NOD-1 & 2 and retinoic acid–inducible gene I (RIG-I)–like helicase receptors (RLRs) are PRR known to recognise conserved microbial components and activate onset of various signalling pathways (Akira *et al*., 2006; Wang *et al*., 2013). Viral components are recognised by a number of receptors that include TLR-7 & 8 recognising viral ssRNA while RIG-I and TLR-3 detect viral dsRNA. These molecules are expressed intracellularly on the endosome membrane. Viral glycol proteins are shown to be recognised by TLR 2 & 4, thereby stimulating other inflammatory mediators and not the anti-viral specific molecules such as type I IFN. Binding of viral ligands to the receptors recruit adaptor molecules such as MyD88, TIRAP and TRIF to the cytoplasmic domain of the receptors (Akira *et al*., 2006).

Toll like receptor-3 (TLR3) induce production of the IFN via a MyD88-independent signalling pathway. TRIF is then recruited and dimerise with TLR-3 on the cytoplasmic domain. The pathway culminates with activation of IRF-3 and IRF-7 which translocate into the nucleus and bind to the IFN-stimulated response elements (ISREs), resulting in the expression of IFN-inducible genes. Conversely, TLR 7 & 9 induce production of IFN via the MyD88 signalling pathway (Akira *et al*., 2006). RIG-I is a cytoplasmic dsRNA detecting receptor which is not accessible to TLR-3. This molecule interacts with IPS-1 to RIG-I using the CARD domain as its adaptor molecule. The resulting signalling cascade activates IRF-3 and IRF-7 in a similar manner used by TLR-3. The RIG-I was shown to detect cytoplasmic replicating dsRNA while TLR-3 detect dsRNA in the apoptotic bodies of virally infected cells undergoing apoptosis (Akira *et al*., 2006).

RVFV is a mosquito-born zoonotic viral infection that result in various symptoms from flue like symptoms to a sever encephalitis and haemorrhagic fever. It contains a negative sense single-stranded RNA genome made up of 3 segments namely the L segments that encode viral RNA dependent RNA polymerase, the M segments that encode envelop glycoproteins (Gn and Gc) a 78 kDa protein and a 14 kDa non-structural protein (NSm). (Nfon *et al*., 2012). Moreover, The S segments encode the nucleoprotein (N) in the negative-sense and a non-structural protein (NSs) in the genomic direction (Nfon *et al*., 2012). The non-structural protein NSs was shown to be the main virulence molecule, this protein has innate immune suppressive properties that aid in the viral replication and viremia (Nfon *et al*., 2012). RVFV NSs protein circumvent the innate immune response system through inhibition of type I IFN (α & β) (Nfon *et al*., 2012), other studies (Le May *et al*., 2004; Wood *et al*., 2004) have shown RVFV NSs protein to induce suppression of mRNA synthesis machinery. This virulent molecules have shown to interact with p44 and XPD subunit of the TFIIH basal transcription factor.

The NSs disturb the assembly of this basal transcription factor suggesting the suppression of several mechanism of gene expression (Le May *et al*., 2004; Wood *et al*., 2004). NSs aggregate with p44 and XPD and form nuclear filament-like structures that are thought to impair the transcription machinery (Le May *et al*., 2004; Wood *et al*., 2004). NSs is involved in the RVFV pathogenesis through transcriptional shut down that lead to a weakened anti-viral response and IFNs production system. IFNs are important antiviral factors that stimulate antiviral molecules and recruit other immune cells to the inflamed site in order to limit viral spread. The type I IFNs have been shown to enhance the constitutively expressed protein kinase RNA-activation (PKR). In addition to the IFNs system the PKR expression is enhanced by dsRNA and ssRNA. The role of this serine threonine kinase is to phosphorylate eukaryotic translational inhibition factor 2 (eIF2), leading to translational arrest of both cellular and viral mRNAs (Habjan *et al*., 2009).

The PKR has shown some activity in the absence of NSs protein since NSs was shown to directly degrade PKR (Habjan *et al*., 2009). Monkeys that have shown high expression of IFNs cytokines have not developed RVF disease after exposure to RVFV. As a result these cytokines where suggest to possess protective properties against RVFV. Animal model studies show selective inhibition of the IFN-α since production of IFN-γ, TNF-α, IL-6, IL-12, and IL-1 β was observed without detectable levels of IFN-α. Interestingly, IFN-γ and IL-12 have been suggested to lower viremia by stimulating NK cells and cytotoxic role (Nfon *et al*., 2012). The evidence that NSs antagonise the IFNs cytokines makes RVFV infection difficult to clear. An *in vitro* vivo study conducted by Jansen van Vuren and colleagues showed contradictory findings with those that postulate that NSs result in transcriptional shut down and weakened inflammatory response (van Vuren *et al*., 2015, Le May *et al*., 2004).

The work by Jansen van Vuren *et al* showed that immune response was mounted to a similar extend in both the fatal cases and none fatal cases. This study shows that early infection samples have high levels of IL-8 and CCL-2/MCP-1 in serum as compared to sera from uninfected controls. Interestingly, fatal case serum has shown 10-fold increased levels of IL-6 (pro-inflammatory cytokine) than non-fatal cases. The serum level of IL-10 in both early and late samples of the fatal and non-fatal cases were statistically not different. This study demonstrated that the fatality and survival of patients might not rely on inflammatory response but rather the extent of the regulation of inflammatory response (Jansen van Vuren *et al*., 2015). This immune regulatory evidence reported is in agreement with other similar publications that have shown deleterious outcomes of under-regulated or persistent inflammation (Reuter *et al*., 2010).

It was shown that during inflammation the innate immune cell produces an excess of toxic reactive oxygen/ nitrogen species primarily used in digesting invading pathogens. Thus, persistent inflammation exposes cellular biomolecules such as proteins nucleic acids and lipids to these toxic free radical molecules. Damage to these biomolecules molecules attenuate cellular function, triggering cell death (Reuter *et al*., 2010). The uncontrolled production of these inflammatory molecules has been linked to pathogenesis of most of the chronic ailments such as neurodegeneration and cancer (Jope *et al*., 2007). Wang and colleagues have linked West Nile virus (WNV) infection outcomes to elevated inflammation

and damage to the endothelial integrity. This lead to leukocyte extravasation, encephalitis and death (Wang *et al*., 2004). Induced autoimmune encephalomyelitis studies has further shown that under controlled inflammation weakens endothelial barrier and uncontrolled leukocyte extravasation (De Sarno *et al*., 2008). It is therefore the aim of the study to determine the effects of lithium on inflammation profiles and NF-κB nuclear translocation in Raw 264.7 macrophages exposed to Rift valley fever virus.

Materials And Methods

Cell Culture and Viral Propagation

The RVFV AR 20368 strain was isolated during the 1974 RVF outbreak in South Africa. The virus was propagated on Vero C1008 cells at an MOI of 0.2, followed by harvesting the monolayer after extensive cytopathic effect (cpe). The supernatant fluid was store at -70°C after centrifugation at 3,000 xg for 30 minutes (Martín-Folgar *et al* ., 2010). The RVFV Raw 264.7 macrophage cells were obtained from Prof Lyndy McGraw (University of Pretoria, photochemistry division, Onderstepoort campus), MNA cells from Dr Kgaladi (NICD, Arbo). The cells were maintained in cell culture flasks at 37^{o}C, in a humidified 95% air and 5% CO_2 atmosphere. Raw 264.7 and MNA cells were propagated in Dulbecco Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 2 mM L-glutamine and 1x penicillin-streptomycin. Vero C1008 cells where purchased from ATCC and propagated in Minimum Essential Medium (MEM), supplemented with 10 % FBS, 2 mM L-glutamine and 1x penicillin-streptomycin (Lonza). Trypan blue dye and haemocytometer where used to determine cell density (Matsebatlela *et al*., 2012).

Cell treatment

Lithium Chloride was purchased from Fluka (Chemika, Switzerland) and the stock was prepared at 500 mM LiCl stored at 4 °C. Sodium Chloride was purchased from Sigma-aldrich (USA) and the stock was prepared at 500 mM NaCl stored at 4 °C. The experiments were executed by seeding the Raw 264.7 at various cell densities depending on the experimental setting and then treated with LiCl (2.5, 1.25 and 0.625 mM) as well as NaCl (2.5 mM).

Determination of the cytokines expression pattern using ELISA

In an attempt to measure the production of the cytokines and chemokines Raw 264.7 cell as a model of macrophages where seeded at 2.4 X10^6 Cells for 3 hrs in T25 flasks and inoculation with $10^{4.8}$ $TCID_{50}$/mL for an hour and then the supernatant was removed. Thereafter the cells were treated with lithium (as outlined in Cell treatment) and 5 mg/ml LPS for 24 hrs while the supernatant was collected in 3, 6, 12 24 hrs time intervals. The collected supernatant was frozen at -20 °C for later use. Enzyme linked immunosorbent assay (ELISA) was executed according to manufacturer's protocol (Preprotech, USA). The capture antibody (for IL-10, IL-6, TNF-α, IFN-γ and RANTES) was diluted with PBS to 0.5 µg/ml and l00 µL was added to each well in a 96 well plate and incubated at RT overnight. Liquid was aspirated followed by 4 times washing with 300µL wash buffer (0.05 % tween-20 in 1x PBS) per well using ELx 405 auto plate washer (Bio-TEK instruments-Inc).

The standard was serially diluted 2 fold and 100µL was added in triplicates, the samples were added in triplicates as well for 2 hrs. The added samples were aspirated and washed 4 times with wash buffer. The detection antibody was diluted in diluent to 0.5µg/mL and 100 µL was added in each well for 2 hrs at RT. This was then followed aspiration of the excess detection antibody and plates were washed 4 times with wash buffer. The 5.5µL avian peroxidase was then diluted 1: 2000 in 11 ml diluent, thereafter 100 µL of this diluted avian peroxidase was added to each well and incubated for 30 min at RT. This solution was aspirated followed by 4 times wash with wash buffer, and then 100 µL of ABTS substrates solution was added to each well and the plates were incubated for colour development. Thereafter the colour was measured by reading the OD at 405 nm with ELx 802 universal microplate reader (Bio-TEK instruments-Inc)

Determination of the production of the reactive oxygen species (ROS)

2',7'-Dichlorofluorescin diacetate (H_2DCF-DA) is a cell-permeable reactive oxygen species (ROS) non-fluorescent probe. This molecule is deacetylate by cellular esterase and then react with ROS turning in to a highly fluorescent 2',7'-dichlorofluorescein. The Raw 264.7 cells were seeded at $4x10^5$ cells/well in a 6 well plate for 3 hrs and treated with lithium (as outlined in Cell treatment) and 5 mg/ml LPS, this was then followed by inoculation with RVFV at $1x10^{4.8}$ $TCID_{50}$/ 100µL for 24 hrs. After 24 hrs of inoculation, cells were staining with permeant H_2DCF-DA at RT for 30 min in the dark then cell were fixed with 3.7% paraformaldehyde for an hour. The pictures where captured at 480 and 535 nm (Ex/Em) with EVOS FL Colour imaging system (Life technologies, USA). On the other hand the Raw 264.7 cells where seeded in at $5x10^5$ cells/well in a 96 well plate for 3 hrs and treatment as well as inoculation was done as above for 12 and 24 hrs. After the incubation time H_2DCF-DA was added for 30 min and then fluorescence intensity was measured at ex/em 480/535 nm using a Fluoroskan Ascent FL (Thermo Fisher Scientific, USA).

Determination of the production of the reactive nitrogen species (RNS)

5,6-Diaminofluorescein diacetate (DAF-2 Da) is a cell permeant NO indicator that is deacetylated by intracellular esterases to DAF-2 that react with NO to yield a highly fluorescent triazolofluorescein (DAF-2T). The Raw 264.7 cells were seeded at $4x10^5$ cells/well in a 6 well plate for 3 hrs and treated with lithium (as outlined in Cell treatment) and 5 mg/ml LPS, this was then followed by inoculation with RVFV at $1x10^{4.8}$ $TCID_{50}$ /100 µL for 24 hrs. After 24 hrs of inoculation, cells were staining with permeant DAF-2 DA at RT for 30 min in the dark then cell were fixed with 3.7% paraformaldehyde for an hour. The pictures where captured at 480 and 535 nm (Ex/Em) with EVOS FL Colour imaging system (Life technologies, USA). For Fluorescence intensity, Raw 264.7 cells where seeded in at $5x10^5$ cells/well in a 96 well plate for 3 hrs and treatment as well as inoculation was done as above for 12 and 24 hrs. After the incubation time DAF-2 Da was added for 30 min and then fluorescence intensity was measured at ex/em 480/535 nm using a Fluoroskan Ascent FL (Thermo Fisher Scientific, USA).

NF-κB translocation immunofluorescence assay

Raw 264.7 macrophage cells were cultured in 6 well plates on the slides at 4 x10^5 cells/well for 3hrs before treatment with lithium (as outlined in Cell treatment) and 5 mg/ml LPS, this was then followed by inoculation with RVFV at 1x$10^{4.8}$ $TCID_{50}$ /100 µL for 24 hrs. After fixation with 4% paraformaldehyde cells were permeablised with 0.1% Triton X-100, 1%BSA for 60 min. The nonspecific binding sites were blocked by adding 1% BSA for 1 hr, followed by 2 x wash with wash buffer. Cells were incubated for 60 min with rabbit anti-p65 antibody (1:500) (Santa Cruiz) followed by 3x wash with wash buffer, and then FITC-labelled goat anti-rabbit secondary Ab incubation for 60 min. After 5 min nuclear staining with DAPI cells were mounted on slides using 50% glycerol and analysed using the fluorescent inverted Nikon Ti-E microscope at 20x magnification.

Examination of inflammatory protein expression using Western blotting assay

In order to examine apoptosis/NF-κB related protein expression on Raw 264.7 cells seeded in T25 flasks for 3 hrs at a density of 1x10^6 cell/ml. The cells where treated with lithium (as outlined in Cell treatment) and infected for 3 days with RVFV $10^{4.8}$ $TCID_{50}$ /ml. Cells were washed once with 1x PBS, thereafter cells lysis was accomplished with 500µL lysis buffer [10 mM Tris-HCl, pH 6.8, 1 % SDS, 100 mM sodium chloride, 1 mM EDTA, 1% NP 40, protease inhibitor], the cells where vortexed for 10 sec and incubated on ice for 30 minutes. The supernatant was collected by centrifugation at 15 000 xg for 20 minutes at 4°C and then protein concentration was determined using BCA protein assay at 562 nm. For SDS PAGE 50µg proteins was mixed with the sample buffer [1 mM Tris buffer pH 6.8, 20 % SDS, 20 % glycerol, 0.05 % β-mercaptoethanol, 0.002 % bromophenol blue] and then separated by 12 % SDS-PAGE then transferred to a polyvinyilidene fluoride (PVDF) membrane using a semi-dry blotting system (Bio-Rad).

The membranes was blocked with Tris buffered saline (TBS) [150 mM NaCl, 50 mM Tris, 0.1 % Tween, pH 7.5] containing 3 % fat free dried milk. The membranes was washed with wash buffer [0.05 % TBS-Tween] and then incubated each time with one of the 1:500 dilutions of anti- NF-κB-p65, IκB, HO-1, iNOS and β-actin primary antibodies for 1 hr at RT. After incubation, the membranes was washed 3 X with wash buffer and the primary will be bound by corresponding peroxidase-conjugated secondary antibodies at 1:10 000 dilutions for 1hr at RT. The membranes was washed with wash buffer and the immune-reactive proteins was detected using the supersignal west pico chemiluminescent substrate (Thermo Scientific, Rockford, USA) thereafter visualised and photographed using the ChemiDoc XRS+ (Bio-RAD, USA).

Extraction of the cytoplasmic and nuclear proteins

In order to extract bot the cytosolic and nuclear NF-kB protein the on Raw 264.7 cells where seeded in T25 flasks for 3 hrs at a density of 1x10^6 cell/ml. The cells where treated with lithium (as outlined in Cell treatment) and infected for 3 days with RVFV $10^{4.8}$ viral titer/ml, cells where then washed once with 1x PBS and then harvested. For cytoplasmic proteins buffer A composed of (10 mM HEPES, 10 mM KCl, 1 mM MgCl2, 5% glycerol, 0.5 mM EDTA, 0.1 mM EGTA, 1X protease inhibitor solution, 2 mM PMSF and 0.5 mM DTT) was added and the cell pallet where incubated on ice for 15 min with an addition of 0.5 % NP-40. This was flowered by vortexing for 10 sec then centrifugation at 12,000 x g for 1 min. For nuclear NF-

kB protein extraction the centrifugation pellet was exposed to 500ml high salt buffer B (20 mM HEPES, 1% NP-40, 400 mM NaCl, 10 mM KCl, 1 mM MgCl2, 20% glycerol, 0.5 mM EDTA, 0.1 mM EGTA, 1X protease inhibitor solution, 2 mM PMSF and 0.5 mM DTT) for 1 hr on ice. The mixture was followed by vortexed for 15 sec and then 12,000 x g for 1 min.

Statistical analyses

All assays were performed 3 times in duplicates and the error bars represent the degree of variance. Graph-Pad prism 6 software was used to plot the graphs and statistical analysis was executed with Instat 3 software. Duncan's multiple comparison t-test was used to determine significant differences between the means of treated and untreated groups. Differences were considered significant at * $p \leq 0.05$; ** $p \leq 0.01$, *** $p \leq 0.001$.

Results

Influence of lithium on production of the inflammatory pro and anti-inflammatory Cytokines

Most viruses have developed mechanisms to invade the immune surveillance system which favours viral replication and increased viral progeny. RVFV is not an exception to this type of virus-induced immune invasion mechanism. RVFV is known to inhibit the innate immune system, particularly the type I IFN cytokine production as its mechanism of host invasion (Nfon *et al.*, 2012); while other studies (Caroline *et al.,* 2016; van Vuren *et al.*, 2015) outlined prolonged immune response as the primary detrimental factor in patients who are suffering from this type of viral infection. This study examined the impact of lithium on cytokine and chemokines production after RVFV inoculation. This study has demonstrated that lithium stimulates production of INF-γ in the Raw 264.7 macrophage model system.

Increase in lithium concentrations in RVFV-infected Raw 264.7 cells resulted in elevated INF-γ production in comparison to the RVFV-infected control cells not exposed to lithium (Fig 1A). The increase in INF-γ production also increased proportionally with increase in incubation time. INF-γ production in RVFV-infected Raw 264.7 cells treated with lithium was even higher than treatments with LPS *albeit* reaching comparable levels at 24 hrs post inoculation (pi). Lithium at 1.25 mM seemed to induce the highest amount of INF-γ production compared to other concentrations in all the time points. Another prominent inflammatory cytokine, IL-6, was shown to be produced after 12 hrs pi (Fig 1B). As depicted in figure 1B, lithium 1.25 mM stimulate elevated levels of IL-6 as from 6 hrs (pi) compared to untreated control RVFV-infected cells. After 12 hrs (pi), 0.625mM lithium dramatically increased IL-6 production in RVFV-infected cells compared to untreated RVFV-infected cells and those treated with LPS. The chemokine (RANTES/CCL-5) showed similar increment profiles to those observed in IL-6. Lithium induced CCL-5 production after 12 hrs pi the three concentrations of lithium induced production levels CCL-5 (Fig 1D). Although lithium generally increased CCL-5 production compared to control cells, CCL-5 production seems to be inversely proportional to LiCl concentrations in RVFV-infected cells at 12 and 24 hrs pi.

Furthermore, increases in lithium concentrations was shown to increase production of the TNF-α compared to that of RVFV-infected control cells as from 6 hrs pi (Fig 1E). The increase in TNF-α production was also shown to be time-dependent. The level of TNF-α after treatment with lithium was shown tobe above that of positive control 5 mg/ml LPS at all three concentrations. The effect of lithium on production of pro-inflammatory cytokines such as IL-10 was also studied. Lithium was shown to stimulate IL-10 production in RVFV-infected cells (Fig 1C). Lithium was shown to stimulate IL-10 production as from 3 hrs pi, increasing with increase in incubation time. IL-10 production was maximal at 1.25 mM LiCl compared to other concentrations. IL-10 production in control RVFV showed improvement as from 6 hrs pi and reached the same levels in all lithium concentrations after 24 hrs pi (Fig 1C).

Effects of lithium on production of reactive oxygen and nitrogen species after RVFV inoculation.

Reactive oxygen species are generated as metabolic by-products during mitochondrial respiratory chain and excessively produced during inflammation as a result of encounter with inflammatory stimulus (Reuter *et al*., 2010). In this study fluorescent probes such as 2',7'–dichlorofluorescein diacetate (H_2DCF-DA) and 4,5-diamino-fluorescein diacetate (DAF-2 DA) are used to determine the level of ROS and NOS after a viral infection. Figure 2 A and 3 A represent qualitative levels of ROS and RNS production in Raw 264.7 cells after treatment with lithium and infection with RVFV. Lithium concentrations are shown to downregulate production of this reactive molecules as the green fluorescence intensity is reduced with lithium treatment. Quantitative measurement of fluorescence intensity (Fig 2 B and 3 B) show that lithium lowered production of ROS in a concentration dependent manner. Maximum inhibition of RNS production was observed in cells treated with lithium at 2.5 mM. These findings also show that production of this ROS/RNS molecules only reach their peak levels at 24 hpi. Moreover, lithium concentrations are shown to upregulate antioxidant enzyme Heme oxygenase-1 (HO-1) (fig 2 C). Lithium is shown to induce HO-1 expression in a concentration dependent manner.

Determination of RNS production using DAF-2 show that lithium inhibited RNS production in RVFV-infected Raw 264.7 cells. Treatment of cells with lithium notably reduced DAF-2 fluorescence intensity. These observations were accompanied by elevated NOS-2 expression as represented in figure 3 C. Higher expression levels of the inducible nitric oxide synthase were elevated in control RVFV and LPS. Expression of NOS-2 was shown to be lowered by lithium in a concentration-dependent manner. Inducible nitric oxide synthase is an enzyme that stimulate production of the nitric oxide. These findings are consistent with the findings in figure 3 A and B.

Effects of lithium on nuclear translocation and expression of the NF-κB signalling pathway molecules

The molecular location of a transcription factor NF-κB is used as a measure of inflammatory responsive. NF-κB binds to the kappa responsive element and induce inflammatory mediators. Thus, cellular location parameters of the NF-κB molecule is an essential phenomenon studying inflammatory patterns. The molecular location of the NF-κB was examined using immunocytochemistry depicted in figure 4 A. Figure 4 A shows that NF-κB is located in both the nucleus and cytoplasm in lithium-treated cells, since green

fluorescence is on either side of the nucleus. However, control untreated cells show less of the green fluorescence in the nucleus and the image overlays showed an intense blue nuclear fluorescence, displaying that the two stains are not in the same location.

The positive control 5 mg/ml LPS shows light blue nuclear fluorescence since all the green (NF-kB staining) and the blue (nucleus staining) are all in the same location (nucleus). The control RVFV show similar patterns as control LPS, with more NF-κB nuclear staining observed, indicative of nuclear translocation. On the other hand, figure 4 B and C shows that lithium inhibit translocation of NF-κB with LiCl 2.5 mM showing the most inhibition. The IκB-α expression 12 hpi (Fig 4 D) shows to be elevated in lithium-treated cells in a dose dependent manner. IκB-α is the inhibitory molecule that keeps NF-κB in the cytoplasm and is known to regulate activity of this transcription factor (Garcia *et al*., 2009).

Discussion

Innate immune response plays a central role in the immune system as the first line of defence against foreign and infectious agents. Innate immunity, although non-specific, has ability to orchestrate humoral immune system through antigen presentation to the $CD^{+}4$ T-cells (Xiao, 2017). It facilitates the first line of defence systems through inflammation, an ontogenetically old defence mechanism regulated by cytokines, products of the plasma enzyme systems, lipid mediators released from different cells, and vasoactive mediators released from mast cells, basophils, platelets and macrophages (Ross *et al.,* 2002). Macrophages are antigen presenter cells (APC) that produce cytokines as inflammatory mediators, which recruitment other immune cells to the inflamed site and link the innate and adaptive immune response (McElroy and Nichol, 2012).

Inflammation pays a pivotal role in eliminating infections. However, deregulated inflammation can lead to tissue damage and various adverse conditions that include neurodegeneration disorders, diabetes, cancer and endothelial leakage (Koriyama *et al*., 2013; Jope *et al*., 2007; Wang *et al*., 2004). Viruses such as RVFV target macrophages to invade the innate immune system and use them as a vehicle to target tissues such as brain and liver (McElroy and Nichol, 2012). This virus is known to inhibit production of the IFNs as targeted by the NSs virulence factor. This is thought to be the mechanism in which RVFV use to circumvent the immune system in favour of viral replication (Nfon *et al*., 2012). In addition to inhibited IFNs, NSs is shown to induce direct degradation and inhibition of the PKR involved in translational arrest of both cellular and viral mRNA (Habjan *et al*., 2009).

Weakened inflammatory responses from RVFV infection has been suggested to contribute to RVFV pathogenesis and fatality (Nfon *et al*., 2012). Contrary to these suggestions, a recent body of evidence (Caroline *et al.,* 2016; van Vuren *et al*., 2015; Gray *et al*., 2012) suggests that deregulation and prolonged inflammation correlate with viral pathogenesis and fatality. The combination of this contradicting inflammatory evidence and the IFNs inhibitory role of the NSs led to the hypothesis that unbalanced and deregulated inflammation could be central to the RVFV pathogenesis and lethality. This work examined lithium as a potential drug to restore regulatory patterns of inflammation and innate immune system. In

this study lithium has shown to stimulate production of the primary pro-inflammatory cytokine, TNF-α, in Raw 264.7 cells as early as 3 hrs post infected with RVFV. All three concentration of lithium were shown to significantly stimulate production of this primary pro-inflammatory cytokine.

Kleinerman *et al*, have observed TNF-α increment in LPS-stimulated macrophages treated with lithium as early as 10 min post stimulation with the production plateau reached within 12 hrs post stimulation. Kleinerman and colleagues hypothesised that lithium induced production of TNF-α by macrophages and the produced TNF-α subsequently stimulate production of the granulocyte-macrophage CSF (GM-CSF) from the endothelial cells. These observation can be associated with the observed lithium-induced leukocytosis and granulocytosis as a result of GM-CSF (Merendino *et al*., 1994; Kleinerman *et al*., 1989). The secondary pro-inflammatory cytokine, IL-6, and a chemokine, RANTES, were shown to be produced during the late hours of infection 12 and 24 hrs with 1.25 mM LiCl being the most effective (fig 1 B & D). These findings coincide with observations by Maes *et al* which showed that lithium did not induce significant production of the IL-6 in both stimulated and unstimulated cells (Maes *at al*., 1999).

Moreover, Makola *et al* showed inhibitory properties of 10 mM lithium on RANTES production 24 hrs post stimulation with lipopolysaccharide (LPS) in a GSK-independent manner (Makola *et al*., 2020). This could suggest that lithium may not possess sufficient ability to stimulate IL-6 and RANTES production. *In vitro* studies by van Vuren showed a 10 times elevated production of IL-6 in fatal cases as compared to non-fatal patients. This observation suggests that elevated production of this cytokine could be favouring virus survival as opposed to host defence (van Vuren *et al*., 2015). Interestingly, lithium was shown to reverse the elevated production of this pro-inflammatory cytokine in RVFV-infected cells. In another study, Maes *et al* has shown that lithium enhance production of another secondary pro-inflammatory cytokine IL-8 in both LPS and phytohemagglutinin (PHA) stimulated and unstimulated cells (Maes *at al*., 1999).

This current work demonstrated significant up-regulation of IFN-γ by lithium from 3 hrs post infection in a concentration-dependent manner (Fig 1, A). This work shows that lithium stimulates production of some pro-inflammation cytokines which is the most important phenomenon since Nfon *et al* link weakened inflammation with pathogenesis and lethality. A review by Nassar and Azab, support findings from this current study as they are in agreement with previous reports. However, most of the reports show the inhibitory role of lithium on this cytokine rather than stimulation (Nassar and Azab, 2014). Nfon *et al* have linked elevated levels of IFN-γ to survival of infected goats in animal experimental models. IFN-γ is suggested to inhibit viral replication and stimulate cytotoxic activity of the NK cells since lowered viremia has been observed in surviving infected goats (Nfon *et al.,* 2012). In the current *in vitro* model system, lithium has been suggested to inhibit viral replication through induction of early apoptosis in virus-infected cells, leading to abortion of replicating viral progeny.

Elevated IFN-γ levels could be another mechanism used by lithium to lower viral replication. In addition to pro-inflammatory cytokines, lithium stimulated production of the anti-inflammatory cytokine, IL-10, and the cytokine production reached its peak as early as 12 hpi (fig 1 C). Similar findings have been reported in other studies (Maes *at al* 1999; Rapaport and Manji, 2001). These studies showed that lithium

stimulate expression of IL-10 and IL-1R anti-inflammatory molecules. This is suggested to be a regulatory mechanism as a result of overwhelming production of inflammatory mediators known to have deleterious outcomes. Lithium is shown to stimulate both the pro and anti-inflammatory cytokines in the current and previous studies (Nassar and Azab, 2014; Maes *at al*., 1999).

It is suggested that lithium could be restoring the balance in production of inflammatory mediators, as pro-inflammatory molecules are later balanced by regulatory cytokines to limit over production of pro-inflammatory molecules. Lithium remains an effective and preferred treatment option for bipolar disorders despite the sparse and limited understanding of its mechanism of action (Nassar and Azab, 2014). Under-regulated Inflammation has been linked to pathological processes behind manic depression and bipolar disorders. Hence, studies suggest that lithium could be restoring inflammatory deregulation as the mechanism underlying its anti-depressant property (Nassar and Azab, 2014; Maes *at al*., 1999). An *in vitro* study by Gray *et al* observed strong and prolonged expression of IL-6, GCSF and MCP-1 three days pi, this has led to suggestion that these molecules could be involved in endothelial leakage that lead to haemorrhagic fever (Gray *et al*., 2012).

The RVFV-infected lithium treated cells have shown to lower production of the reactive oxygen and nitrogen species. The lowered production of this reactive molecules has been depicted in figure 2 A and 3 A in a qualitative assay. Quantitative findings (fig 2 B and 3 B) show the same trend as in the pictures. As represented in Figure 1 B and 2B, there is a significant difference in production of these reactive species 24 hrs post infection. Previous work has shown lithium at 10 mM to reduce ROS while 5 mM being effective in reduction of NO production in LPS-stimulated Raw 264.7 cells (Makola *et al*., 2020). More interestingly, in the current study, lithium down-regulated expression of iNOS enzyme (fig 3 C), which correlate with lowered NO production. In addition to the inhibited iNOS, lithium stimulate HO-1 expression, an antioxidant enzyme (fig 2 C). This work aligns inflammation regulatory properties of lithium with activation of the NF-κB transcription factor.

Lithium-treated cells show the presence of the NF-κB in both the cytoplasm and the nucleus, suggesting the reversal/ inhibition of the transcription factor from translocating to the nucleus (fig 4 A). Molecular translocation of NF-κB into the nucleus is observed to be lowered by lithium treatments in a concentration dependent manner (fig 4 B & C). Previous studies (Narayanan *et al*., 2014) have shown that RVFV stimulate NF-κB nuclear translocation, culminating in production of inflammatory mediators and resulting in oxidative stress. Oxidative stress is a condition emanating from excessive production of oxidants and free radicals, leading to imbalance between oxidants and antioxidants. Studies (Christen, 2000; Reuter *et al*., 2010; Narayanan *et al*., 2014) have shown that oxidative stress conditions elicit biomolecules deformation that lead to altered cell function and then cell demise.

Narayanan *et al*, hypothesise that RVFV prevalent liver disease emanate from oxidative stress that lead to hepatic cell demise (Narayanan *et al*., 2014). Thus, inflammatory deregulation and oxidative stress has been linked with a number of pathogenic outcomes. This study suggest that lithium could ameliorate detrimental outcomes emanating from this viral infection. Figure 4 D, show that lithium concentrations

upregulate the inhibitory molecules, IκB-α. IkB-α inhibit the translocation of the NF-κB by masking its nuclear translocation domain (Garcia *et al*., 2009). Our previous work at high lithium concentration (10 mM) showed expression of the NF-κB inhibitors IκB-α, TRAF3, Tollip and NF-κB1/p50 to be lithium inhibition biomarkers (Makola *et al*., 2020). What remains profound about lithium is that in as much as it was shown to promote expression of some pro-inflammatory cytokines it stimulates anti-inflammatory cytokines in an attempt to avoid oxidative stress and nonspecific damage to host cell biomolecules.

Previous studies show that NSs selectively tempers with the type I IFN signalling while sparing the other inflammation mediators such as ROS, NOS and Pro/ anti-inflammation cytokines/chemokines. On a signalling level this could mean that NSs inhibit nuclear translocation of IRF3 and 7 as the transcription factors involved in production of type I IFNs or perhaps targeting the transcription of ISGs, leading to silencing of antiviral molecules (Ghaemi-Bafghi and Haghparast, 2013) (fig 5). Since NSs selectively inhibit IFNs which are linked to IRFs transcription factors, it then implies that other inflammatory mediators expressed by other transcription factors such as AP-1 and NF-κB will continually be produced leading elevated inflammatory mediators and oxidative stress. Thus, this work link regulatory mechanism of lithium with inhibition of the NF-κB signalling pathway as the inflammatory transcription factor that is not targeted by NSs.

The NF-κB signalling pathway is suggested to be stimulated by the glycol proteins detected by TLR-4 or ssRNA detected by TLR-7 or dsRNA detected by the RIG-I. All these PRRs are linked to the NF-κB signalling pathway in as much as others stimulate IRF signalling as well (fig 5). The hypothesis that lithium restore balance in the inflammatory system emanates from its regulatory mechanism on the NF-κB pathway as NSs inhibit translocation of IRFs and not other transcription factors. The *in vitro* and *ex vivo* studies have shown cytokine and chemokines production excluding type I IFN during RVFV infection (Nfon *et al*., 2012; van Vuren *et al*., 2015). The activated NF-κB pathway continue producing these inflammatory mediators that are suggested to participate in the RVFV pathogenesis. Therefore, lithium restores production of excessive inflammatory mediators as it has been observed to limit NF-κB translocation through upregulation of the IκB molecule.

Conclusion

The NF-κB transcription factor is shown to be the targeted molecule behind the anti-inflammation properties displayed by lithium. Results from this work show that lithium inhibit NF-κB-activity and that is linked to the observed inhibition of production of the inflammatory mediators at various concentrations. Although additional work is required, the current findings suggest that lithium could be used as an anti-haemorrhagic fever agent since RVFV-induced oxidative stress, which is suggested to be the critical factor that lead to haemorrhagic fever, which is shown to be reversed by lithium treatment. This study predicts that the RVFV virulence factor NSs inhibits production of the type I IFN at a transcription factor level (IRF) since production of other inflammatory mediators such as RNS, ROS, cytokines and chemokines are observed with the exception of the type I IFN.

Abbreviations

MyD88:	Myeloid differentiation factor 88	TLR:	Toll Like Receptors
TRIF:	TIR-domain-containing adaptor protein-inducing IFN-β	Tirap:	TIR Domain containing adaptor protein
γ-GCS:	γ- glutamylcysteine synthetase	Traf:	TNF receptor-associated factor
FDA:	US Food and Drug Administration	NaCl	Sodium Chloride
RNS	Reactive Nitrogen species	RIG-1:	Retinoic acid-indicible gene 1
Tollip:	Toll interacting protein	NSs:	78-kDa non-structural protein
TRAF3:	TNF receptor-associated factor 3	LPS:	Lipopolysaccharide
HO:	Haem Oxygenase	RTCA:	real time cell analyser system (rtca)
iNOS:	Inducible nitric Oxide Synthase	TNF-α:	Tumor necrosis alpha
IκB-α:	Inhibitor of kappaB kinase alpha	IFNs:	Interferons
MTT:	3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide	RANTES:	Regulated on activation, normal T cell expressed and secreted
DAF-2DA:	5,6-Diaminofluorescein diacetate	IL-6:	Interleukine-6
H_2DCF-DA:	2',7'-dichlorodihydrofluorescein diacetate	IRF:	Interferon Regulatory Factor
GSK-3:	Glycogen Synthase Kinase-3	NOS:	Reactive nitrogen species
FBS:	Fetal Bovine Serum	ROS:	Reactive oxygen species

Declarations

All authors have declared that there is no conflict of interest regarding publication of this work.

Ethics approval and consent to participate

Not applicable

Consent to publication

Not applicable

Data Availability

The raw data are available upon reasonable request.

Competing interests

There is no financial and non-financial competing interests.

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Authors' contribution

RT Makola, TM Matsebatlela, JT Paweska and PJ van Vuren were involved in experimental design and project conceptualisation. RT Makola, PJ van Vuren, GK More and J Kgaladi were involved in running the experiments. RT Makola, TM Matsebatlela, JT Paweska and PJ van Vuren were involved in data analysis. This manuscript was written by RT Makola. While RT Makola and all the co-authors reviewed the manuscript.

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Figures

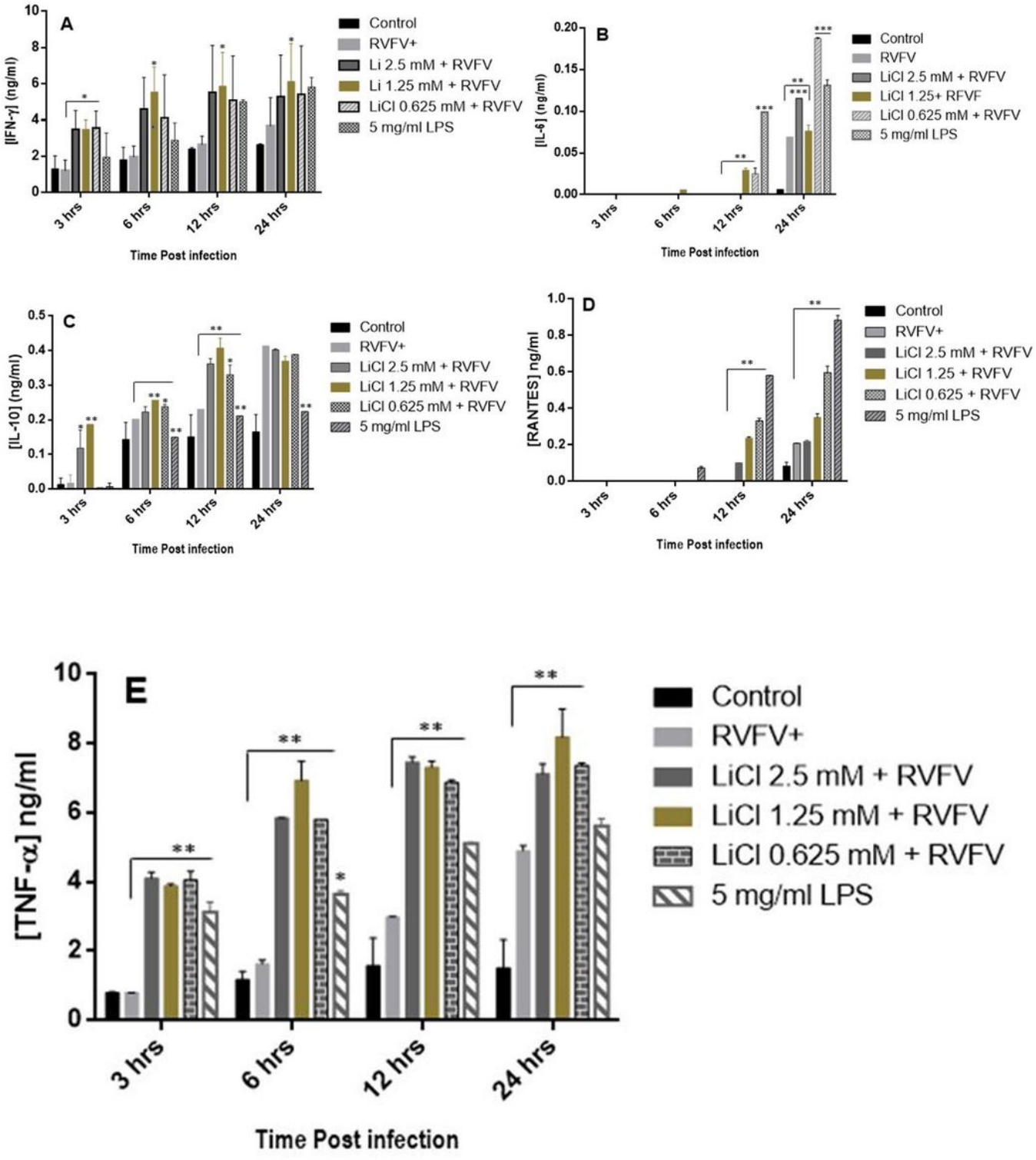

Figure 1

Determination of the effects lithium on TNF-α production after inoculation with the RVFV. In order to measure effects of lithium in TNF-α, IL-6, IL-10, IFN-γ and RANTES production, Raw 264.7 cells where seeded at the 2.4x106 cells per T 25 flask for 3 hrs inoculated with 1x104.8 viral tite for an hour. The excess virus was washed and the cells were treated with various concentration of lithium and then supernatant was collected in 3, 6, 12 and 24 hrs time intervals. The Sandwich Elisa (PeproTech, USA) was

executed to measure the amount of these cytokines from various samples. The data points represent mean + standard deviation (error bar). The plot was developed with Graph pad prism-6 software and instat-3 was used to establish the statistical analysis.

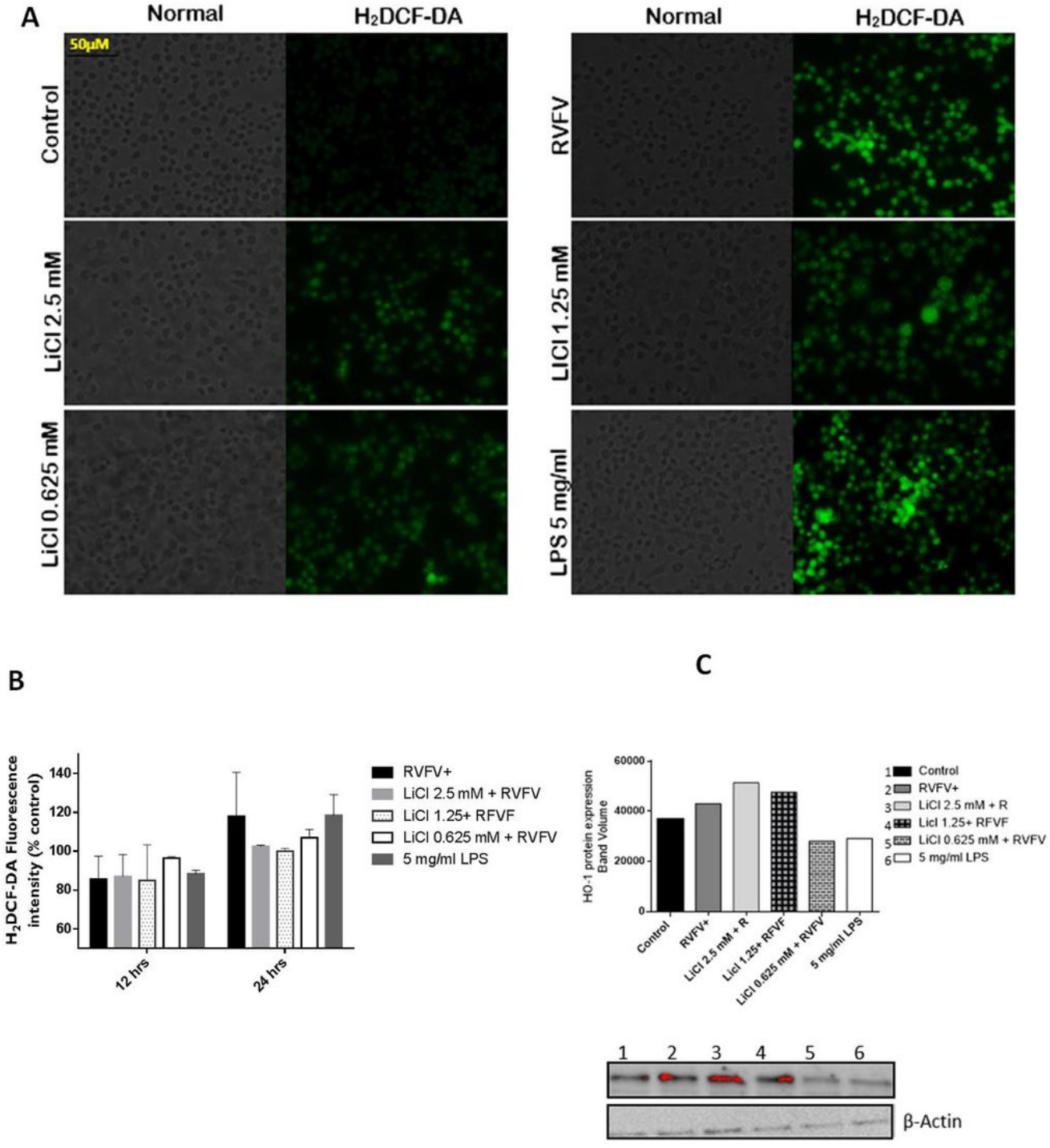

Figure 2

Determination of the effects of lithium on oxidative burst after Raw 264.7 cell are challenged with RVFV and expression of levels of antioxidant enzyme. (A) Cells were seeded at 4x105 cells/well in a 6 well plate

for 3 hrs then pre-treated with various concentration of lithium and 5 mg/ml LPS. Later the some wells were inoculated with RVFV at 1x104.8 viral titer for 24 hrs. After 24 hrs of inoculation, cells were staining with permeant H2DCF-DA at RT for 30 min in the dark then cell were fixed with 3.7% paraformaldehyde for an hour. The pictures where captured with EVOS FL Colour imaging system (Life technologies, USA) Ex: 495nm; Em: 515nm. (B) Cells were seeded at 1x106 cells/well for 3 hrs then inoculated with RVFV at 103.8 viral titter/100uL and then treated with lithium concentrations and stimulated with 5 mg/ml LPS for 12 and 24 hrs. After the incubation hours the cells were stained with H2DCF-DA for 30 min in the dark, and then the fluorescence intensity was measured with Fluoroskan Ascent FL (Thermo Fisher Scientific, USA) at ex (485 nm)-em (538 nm). The graphs were developed with Graph pad prism-6 software and instat-3 was used to establish the statistical analysis. (C) In order to determine the expression of HO-1 protein, Raw 264.7 cell where seeded at 1x106 cell/ml for 3 hrs then treated as above, and then cells were inoculated with 104.8 viral titer/mL RVFV for 12 hrs. This was then followed by isolation of proteins and then western blotting assay. The pictures were captured with ChemiDoc XRS+ (Bio-RAD, USA).

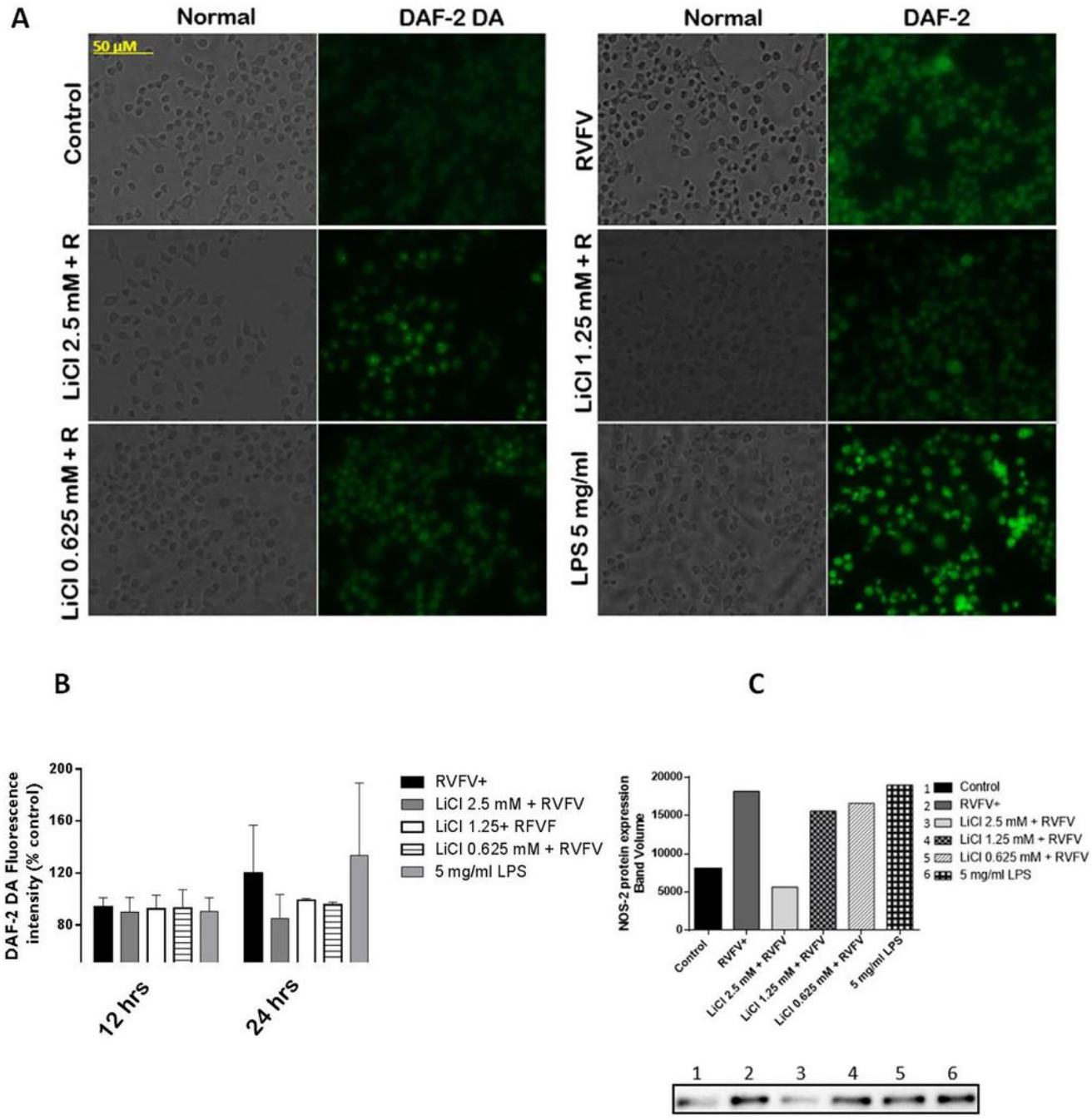

Figure 3

Determination of the effects of lithium on production of inflammatory reactive nitrogen species 24 hrs post RVFV inoculation and expression levels of the NOS enzyme. (A) Cells were seeded at 4x105 cells/well in a 6 well plate for 3 hrs then pre-treated with various concentration of lithium and 5 mg/ml LPS. Later the some wells were inoculated with RVFV at 1x104.8 viral titer for 24 hrs. After 24 hrs of inoculation, cells were staining with permeant DAF-2 DA at RT for 30 min in the dark then cell were fixed

with 3.7% paraformaldehyde for an hour. The pictures where captured with EVOS FL Colour imaging system (Life technologies, USA). (B) Cells were seeded at 1x106 cells/well for 3 hrs then inoculated with RVFV at 103.8 viral titter/100uL and then treated with lithium concentrations and stimulated with 5 mg/ml LPS for 12 and 24 hrs. After the incubation hours the cells were stained with DAF-2 DA for 30 min in the dark, and then the fluorescence intensity was measured with Fluoroskan Ascent FL (Thermo Fisher Scientific, USA) at ex (485 nm)-em (538 nm). The plot were developed with Graph pad prism-6 software and instat-3 was used to establish the statistical analysis. (C) In order to determine the expression of NOS-2 protein, Raw 264.7 cell where seeded at 1x106 cell/ml for 3 hrs then treated with 2.5, 1.25, and 0.625 mM LiCl, and then cells were inoculated with 104.8 viral titer/mL RVFV for 12 hrs. This was then followed by isolation of proteins and then western blotting assay. The pictures were captured with ChemiDoc XRS+ (Bio-RAD, USA).

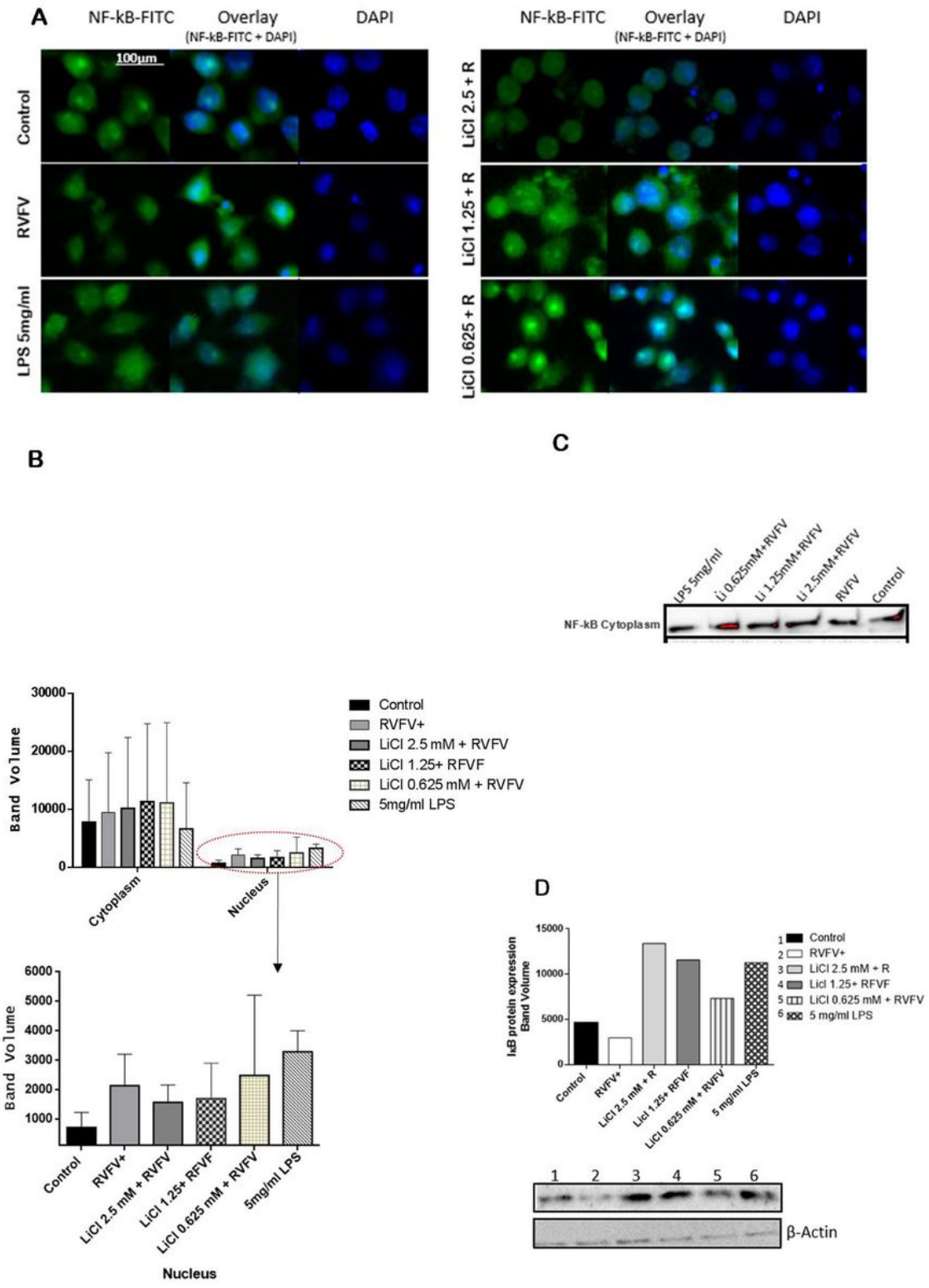

Figure 4

Effects of lithium on translocation of NF-kB between the cytoplasm and nucleus, and expression levels of IkB. (A) The cells where seeded Cells were seeded at 4x105 cells/well in a 6 well plate for 3 hrs then treated with 2.5, 1.25, and 0.625 mM LiCl concentration of lithium, 5 mg/ml LPS and inoculated with RVFV at 1x104.8 viral titer for 24 hrs. After 24 hrs of inoculation, cells were fixed with 4% paraformaldehyde for an hour, and then permeabilized with (0.1% Triton X-100, 1%BSA) for 30 min.

Permeabilised cells were incubated for 60 min with rabbit anti-p65 antibody (1:500). The primary antibody was followed by FITC-labelled goat anti-rabbit secondary Ab incubation for 60 min. After 5 min nuclear staining with DAPI cells were mounted on slides using 50 % glycerol and analysed usingusing fluorescent inverted Nikon Ti-E microscope at 20x magnification. (B&C) In order to determine the translocation quantity of the NF-κB, Raw 264.7 cell where seeded at 1x106 cell/ml for 3 hrs in the T25 cell culture flasks and then treated with various concentration of lithium and other cells were inoculated with 104.8 viral titer/mL RVFV for 24 hrs. This was then followed by isolation of cytoplasmic proteins as well the nucleus proteins and then western blotting assay followed. The pictures were captured with ChemiDoc XRS+ (Bio-RAD, USA). The ChemiDoc XRS+ image lab 5.2.1software was used to measure band volume (Bio-RAD, USA). (C) The plot was developed with Graph pad prism-6 software and instat-3 was used to establish the statistical analysis.(D) In order to determine the expression of the above depicted of Ikb-α, protein, Raw 264.7 cell where seeded and inoculated as above for 12 hrs. This was then followed by isolation of proteins and then western blotting assay. The pictures were captured with ChemiDoc XRS+ (Bio-RAD, USA)

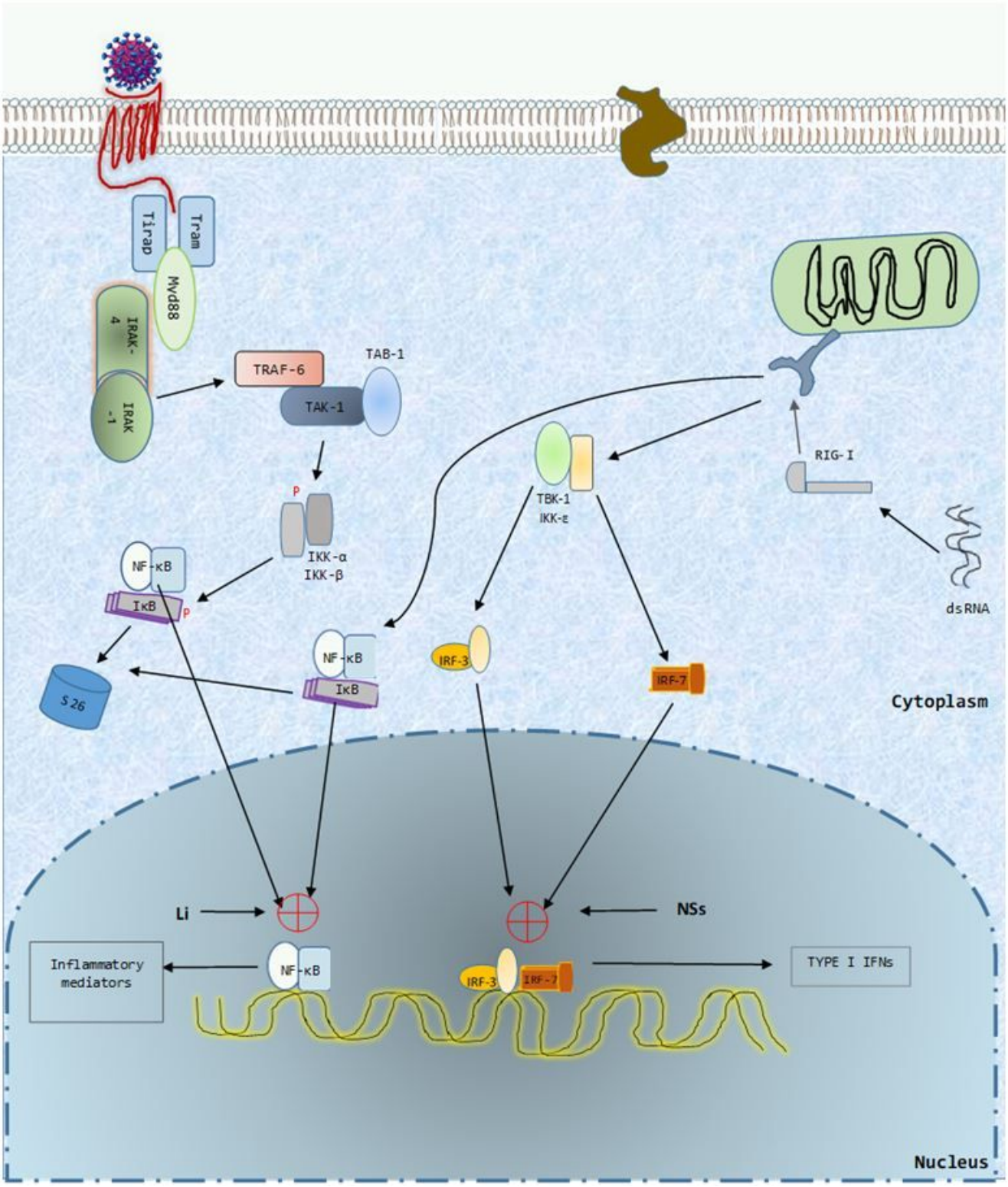

Figure 5

Determination of the canonical NF-kB and IRF3/7 signalling pathways and the effects of lithium post RVFV infection. TLR 2 and 4 are stimulated by the viral glycoproteins that in turn recruit adapter molecule Myd88 via tirap. The adapter molecules recruit Irak4 which phosphorylate recruit irak-1 which then associate with Traf-6. Traf-6 recruit Tak1 and Tab2. Tak1 phosphorylate IKK-β, which then phosphorylate IkB which is then tagged for ubiquitination and then degradation by cytoplasmic proteases. This then

allows translocation of NF-kB to the nucleus and inflammatory genes expression. The RIG-1 is known to be stimulated by dsRNA from replicating viral genome, which is said to be hidden from the TLR-3. This cytoplasmic receptor is shown to be essential for viral IFN expression. The RIG-1 is shown to associate with IPS-1 with its N-terminal card domain. The IPS-1 and RIG-1 association activate TBK1 and IKK-ε which phosphorylate IRF-3 and 7. The NSs is suggested to interfere with the IFN signalling at the transcription factor level since there is an expression of other inflammatory mediators except for IFNs. Since, the NSs inhibit the interferon production via IRF inhibition other transcription factors such NF-kB continue to produce inflammatory mediators, hence, elevated production of other inflammatory mediators except the IFN. This diagram suggests that NF-kB inhibition as a result of upregulated IkB could be the, mechanism in which lithium restore dysregulated inflammation after RVFV infection leading to haemorrhagic fever pathogeneses observed during this viral infection.