

Calcium dependent host innate immune resistance to
influenza A virus infection

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Declaration

I hereby declare this thesis has been composed by myself and has not been submitted for a degree elsewhere. The work presented is my own and the work of other authors and material from other sources is duly acknowledged. Collaboration with other persons during this work has been fully acknowledged.

Leah Goulding

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Abstract

Influenza A virus infection is a major threat to public health, claiming over half a million lives globally each year. Strategies to combat influenza virus infection include vaccination and antiviral therapy. Influenza virus vaccine escape mutants, arising from mutations resulting in antigenic alterations of their surface glycoproteins so they are no longer recognised by the host immune system, can limit the effectiveness of vaccines. Secondly, antivirals that directly target the virus inevitably apply selection pressure on the virus and lead to drug resistance. The onset of resistance has been documented for the neuraminidase inhibitors, currently the only class of anti-influenza drugs recommended by the World Health Organisation.

Targeting host factors that are required by the pathogen or enhancing host immune responses has been identified as an alternative to direct antiviral therapy. Host targeted treatment could be a viable alternative to overcome the serious problem of antiviral resistance. The evidence presented here indicates that activation of a specific host Ca^{2+} mobilisation pathway, store-operated calcium entry (SOCE), mediates a potent antiviral innate immune response that dramatically inhibits influenza virus production in a variety of cell types. Non-cytotoxic doses of an SOCE agonist, a sesquiterpene lactone thapsigargin (TG), induced a sustained and broad spectrum innate immune state which was effective against the virus pre- and post-infection. TG priming induced the endoplasmic reticulum stress unfolded protein response and enhanced type I/III IFN associated response in the face of infection. In addition, TG priming reduced

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the expression of a range proviral host factors required for post-translational viral protein transport and modification.

Antiviral activity was also identified in related sesquiterpenes, (+)-ledene and dehydroleucodine (DHL). Their antiviral mechanism of action was distinct from TG and did not elevate type I IFN signalling or induce host stress signalling pathways.

In summary, the strategic pharmacological or genetic activation of SOCE could underpin a new host-centric therapeutic approach capable of effectively inhibiting influenza A virus replication. TG was identified as a potential broad spectrum inhibitor of RNA virus replication capable of activating multiple host innate immune responses.

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Abbreviations

2'-5'-Oligoadenylate Synthetase = OAS

2-Aminoethoxydiphenyl borate = 2-APB

Adenosine triphosphate = ATP

Biological function = BF

Calcium ion = Ca^{2+}

Calcium release-activated channels = CRAC

Calmodulin = CaM

Calnexin = CNX

Calreticulin = CRT

Cyclopiazonic acid = CPA

Degree celcius = $^{\circ}\text{C}$

Dehydroleucodine = DHL

Dendritic cells = DCs

Deoxyribonucleic acid = DNA

Deoxynucleotide triphosphates = dNTPs

Differentially expressed genes = DEGs

Differentiation medium = DM

Dimethyl sulfoxide = DMSO

Dulbecco's modified eagle medium = DMEM

Endoplasmic reticulum = ER

Fast Ca^{2+} dependent inactivation = FCID

Fetal calf serum = FCS

Green fluorescent protein = GFP

lx

Hemagglutinin = HA

Hour(s) = h

IFN-stimulated genes = ISGs

Ingenuity pathway analysis = IPA

Interferon = IFN

Interferon regulatory factor = IRF

Interleukin = IL

Madin-Darby Canine Kidney cells = MDCK cells

Matrix = M

Messenger RNA = mRNA

Molecular function = MF

Microliter = μ l

Micromolar = μ M

Minute(s) = min

Multidimensional scaling = MDS

Nanomolar = nM

Neuraminidase inhibitor = NAI

Newborn pig tracheal cells = NPTr cells

Next generation sequencing = NGS

NOD like receptor = NLR

Non structural = NS

Normal human bronchial epithelial = NHBE cells

Nuclear localisation signal = NLS

Nucleoprotein = NP

Pattern recognition receptor = PRR

Phosphate buffered saline = PBS

Plasma membrane = PM

Porcine tracheal epithelial cells = PTECs

Principle component analysis = PCA

Retinoic acid-inducible gene-I = RIG-I

Revolutions per minute = RPM

Ribonucleic acid = RNA

RNA dependent RNA polymerase = RdRp

Room temperature = RT

Sesquiterpene lactone = SL

Slow Ca^{2+} dependent inactivation = SCID

Stromal interaction molecule = STIM

Store operated calcium entry = SOCE

Thapsigargin = TG

Toll like receptor = TLR

Transcripts per million = TPM

Transient receptor potential cation = TRPC

Tris (hydroxymethyl) aminomethane = Tris

Tris buffered saline = TBS

Ultrosor G = UG

Unfolded protein response = UPR

Universal tube = UT

Virus like particle = VLP

Yellow fluorescent protein = YFP

1. Chapter 1: Literature Review

1.1 Influenza A viruses

Within the *Orthomyxoviridae* family there are four genera of influenza viruses: *Influenza virus A*, *Influenza virus B*, *Influenza virus C* and *Influenza virus D* (Hause et al., 2014). Three genera of influenza virus types (A, B and C) are capable of infecting humans (Taubenberger & Morens, 2010). Influenza A viruses are categorised according to the antigenic specificity of the membrane glycoproteins hemagglutinin (HA) and neuraminidase (NA) (W.H.O, 1980). Presently, 18 HA subtypes and 11 NA subtypes have been identified (Wohlbald & Krammer, 2014).

Influenza A virus is a continual and persistent threat to human health. Seasonal influenza claims an estimated 291 243 – 645 832 lives annually, based on data from 1999-2015 obtained from 37 countries (Iuliano *et al.*, 2018). Clinical symptoms of influenza virus infection include high fever, cough, myalgia headache and malaise. Individuals generally recover in 3-7 days but children, the elderly, pregnant women and immune compromised individuals or with chronic conditions are more susceptible to complications (Ghebrehewet *et al.*, 2016). The severity of infection depends on the contribution of host and virus factors (Byrd-Leotis *et al.*, 2017). The pathogenesis of influenza virus infection varies across influenza virus subtypes, with particular interest regarding the highly pathogenic H5N1 subtype with a mortality rate of ~60% (Neumann, 2015, Kuiken *et al.*, 2012). Clinical symptoms following infection with highly pathogenic influenza viruses has spanned asymptomatic infection to pneumonia and multi-organ failure (Hui, 2008). The epithelial cells lining with respiratory tract of the host are the initial targets of the influenza A virus (Wu *et al.*, 2016). Lower respiratory tract infections are associated with greater morbidity and

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mortality while non-fatal influenza virus infections are usually associated with upper respiratory tract infections (Taubenberger & Morens, 2008). This may be due to influenza virus glycoprotein hemagglutinin (HA) which mediates virus attachment to the host cells by binding to sialylated glycans. Seasonal human influenza prefers to bind to the sialic acid α 2, 6 galactose linkage, a linkage located predominantly in the upper respiratory tract. On the other hand, avian viruses such as the highly pathogenic avian H5N1 virus, preferentially bind to the sialic acid α 2, 3 galactose linkage located in the lower respiratory tract and thereby increasing the risk of the patient in developing pneumonia (Shinya *et al.*, 2006b, Korteweg & Gu, 2008). Pneumonia, arising from primary viral pneumonia or bacterial secondary pneumonia, is a major contributor to seasonal and pandemic influenza virus morbidity and mortality (Rothberg *et al.*, 2008).

Host immune factors contribute to the pathogenesis of influenza virus infection. A severe inflammatory response, due to overproduction of cytokines and chemokines commonly referred to as a 'cytokine storm', can result in a fatal degree of tissue damage and can lead to organ dysfunction (Tisoncik *et al.*, 2012, Liu *et al.*, 2016a, To *et al.*, 2010). Mortality from influenza virus infections directly correlates with cytokine storm development (Oldstone *et al.*, 2013). Influenza virus induced cytokine storm can induce acute lung injury (ALI) or a greater degree of lung damage resulting in acute respiratory distress syndrome (ARDS), the latter resulting in a mortality rate up to 60% (Short *et al.*, 2014, Riscili *et al.*, 2011). An influenza induced cytokine storm is initially triggered by epithelial cell infection, resulting in cytokine and chemokine production from neighbouring endothelial cells (Oldstone *et al.*, 2013). The cytokines damage the epithelial-endothelial barrier and direct infection of the epithelial and endothelial cells by influenza A virus also contributes to lung damage,

with infection inducing cell death and contributing to pulmonary oedema (Short et al., 2014). Innate immune cells are recruited to the site of infection by the pro-inflammatory signals and contribute to the production of the cytokines and chemokines (Oldstone et al., 2013). Migration of immune cells, such neutrophils and macrophages, may cause temporary damage to the epithelial-endothelial barrier. However, the damage caused by neutrophils is attributed to their production of reactive oxygen species, pro-inflammatory cytokines and neutrophil extracellular traps while expression of cytokines and TRAIL in macrophages is thought to underpin innate immune cell mediated lung damage (Short et al., 2014). In the later stages of cytokine storm development, influenza virus specific T cells exacerbate the pro-inflammatory state by further production of pro-inflammatory molecules. In addition, cytolytic T cells lyse virus infected cells, thereby contributing to tissue damage (Oldstone et al., 2013). The unregulated cytokine response includes a wide range of cytokines from the interferons (IFNs), the interleukins and tumour necrosis factor (TNF) (Tisoncik et al., 2012). However, the type I IFNs are substantial contributors to cytokine storm development (Oldstone et al., 2013). The role of the cytokine storm in influenza virus mortality was reflected in its contribution to the pathogenesis of the avian H5N1 influenza A virus (Peiris *et al.*, 2004). Preventing the onset of hyperctokinemia may be essential for reducing disease severity and improving the outcome of the patient (de Jong *et al.*, 2006).

Within the last century five influenza A virus pandemics have arisen in addition to regional epidemics (Taubenberger & Morens, 2010). An H1N1 influenza virus, the 1918 ‘Spanish flu’, infected approximately one third of the world population and claimed an estimated 50 million deaths globally (Taubenberger & Morens, 2006,

Johnson & Mueller, 2002). The mortality curve of this pandemic was particularly striking, with a peak in young adults between approximately 20-40 years of age, contrasting with the traditionally observed U shaped mortality curve (Taubenberger & Morens, 2006). This has been attributed to prior exposure to a previous influenza virus pandemic, a H3N8 virus which circulated from 1889-1900. The immunological memory to the earlier strain may have resulted in a dysregulated immune response following infection to the antigenically novel H1N1 virus later in life (Gagnon *et al.*, 2013, Worobey *et al.*, 2014). Alternatively, previous exposure of individuals >65 years of age to the 1847 epidemic virus may have conferred a protective effect, resulting in the lower mortality rate for the older proportion of the population (Taubenberger & Morens, 2009). Secondary bacterial pneumonia contributed to the high mortality rate of the 1918 pandemic, suggesting treatment of secondary infections may be essential when considering the response to future pandemics (Morens *et al.*, 2008). In 1957 an H2N2 virus, arising from the human 1918 H1N1 virus and containing novel gene segments encoding avian-like H2, N2 and PB1, replaced the circulating H1N1 virus (Furuse & Oshitani, 2016a, Lindstrom *et al.*, 2004, Smith *et al.*, 2009). The 1957-58 pandemic of the H2N2 virus, also known as ‘Asian flu’ due to its first reported emergence, claimed over one million lives (Viboud *et al.*, 2016). As previously observed, this influenza A virus strain was subsequently expelled by the emergence of ‘Hong Kong flu,’ a H3N2 virus, in 1968. This new virus consisted of the human H2N2 virus gene segments but with novel H3 HA and polymerase basic protein 1 (PB1) gene segments of avian origin (Kawaoka *et al.*, 1989, Gething *et al.*, 1980, Smith *et al.*, 2009). Unlike previous viruses, the H3N2 virus was not displaced by the re-emergence of the H1N1 virus in 1977, instead both viruses co-circulated (Furuse & Oshitani, 2016b, Nguyen-Van-Tam & Hampson, 2003). The re-emergence of an H1N1 virus

was attributed to an accidental release of a H1N1 virus strain preserved from the 1950s from a laboratory (Kendal *et al.*, 1978, Zimmer & Burke, 2009, Nakajima *et al.*, 1978).

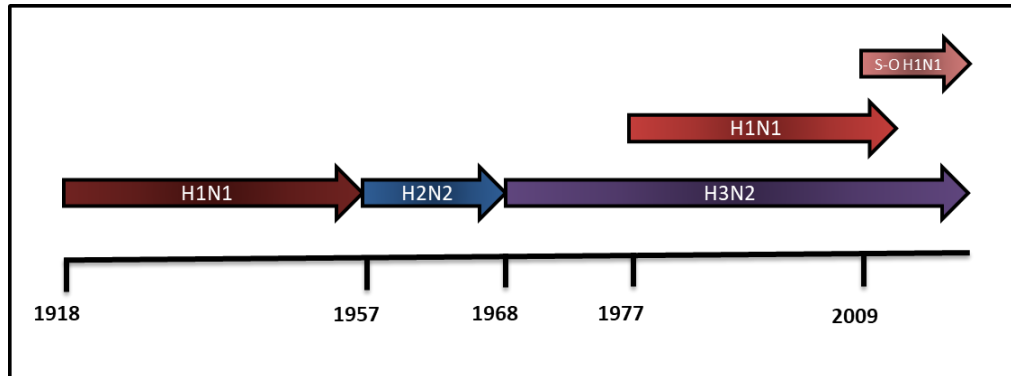


Figure 1-1. Emergence of influenza virus pandemics. The 1918-1919 ‘Spanish flu’ pandemic remained in circulation for approximately 40 years until it was replaced in 1957 by a H2N2 virus variant containing three novel gene segments. In 1968 a H3N2 pandemic emerged and replaced the H2N2 virus. However, the re-emergence of the 1918 H1N1 virus in 1977 did not replace the H3N2 virus, instead co-circulating with both viruses continuing to persist. Most recently, in 2009 a novel H1N1 virus of swine origin appeared in humans. Figure adapted from (Taubenberger & Morens, 2010, Furuse & Oshitani, 2016b)

The recurrent challenge posed by influenza A virus across the globe was highlighted by the most recent pandemic; the 2009 outbreak of a novel influenza A H1N1 virus. A global mortality figure ranging between 151 700 to 575 400 respiratory and cardiovascular deaths were attributed to the 2009 pandemic influenza A H1N1 virus (Dawood *et al.*, 2009). The risk of mortality for those infected with the 2009 H1N1 virus may have been increased for those exposed to the 1957 H2N2 virus pandemic

(Gagnon *et al.*, 2018). Those within the population with impaired immunity, such as young children and the elderly, were particularly susceptible to infection, associated complications resulting in hospital admission and increased mortality (Thompson *et al.*, 2004, Iuliano *et al.*, 2018).

1.1.1 Influenza A virus replication cycle

Influenza A virus contains eight segments of negative single stranded RNA (Eisfeld *et al.*, 2015). The eight segments encode up to 13 proteins through alternative splicing; hemagglutinin (HA), neuraminidase (NA), nucleoprotein (NP), matrix 1 (M1), matrix 2 (M2), polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2) and polymerase acidic protein (PA), non-structural protein 1 (NS1), non-structural protein 2 (NS2) which is also referred to as nuclear export protein (NEP), polymerase basic protein 1 – F2 (PB1-F2), PA-X and PB1-N40 (de Chasse *et al.*, 2013). The viral particle is formed of a core of viral ribonucleoproteins (vRNPs) formed of viral negative stranded RNA associated with a double helix of NP. Three polymerase proteins (PB1, PB2 and PA) make the viral RNA polymerase complex located at the end of the vRNPs. The vRNPs are held by the matrix 1 (M1) shell underneath the viral envelope formed of host cell plasma membrane (PM) (Eisfeld *et al.*, 2015). Three viral transmembrane proteins, hemagglutinin (HA), neuraminidase (NM) and matrix 2 (M2) protein are located within the lipid bilayer (Zhao *et al.*, 2017)

HA2 subunits linked by a disulphide bond (Huang *et al.*, 1980, Toyozo & Shun-ichi, 1980, Chen *et al.*, 1998). Cleavage is believed to occur after virion release or in the exocytic pathway (Böttcher-Friebertshäuser *et al.*, 2010, Walker *et al.*, 1992). Two main HA0 cleavage sites have been described. Low pathogenicity influenza viruses contain HA with a monobasic cleavage site of arginine or lysine that is recognised by trypsin like proteases (Sun *et al.*, 2010, Günther *et al.*, 1993). High pathogenic avian viruses contain H5 and H7 subtypes with a polybasic cleave motif recognised by subtilisin-like proteases (Suguitan *et al.*, 2012, Stieneke-Gröber *et al.*, 1992). The hemagglutinin cleavage site contributes to virus pathogenicity due to the localisation of host proteases. The monobasic cleavage site of low pathogenic viruses is recognised by proteases localised to the respiratory tract and other internal organs in mammals, while the polybasic sequence of highly pathogenic viruses is recognised by ubiquitously expressed proteases (Sun *et al.*, 2010, Steinhauer, 1999, Horimoto *et al.*, 1994, Peitsch *et al.*, 2014). Approximately 7 trypsin-type endoproteases recognise the carboxyl moiety of a single basic R residue within the cleavage motif Q(E)-T/X-R of seasonal influenza A virus, such as TMPRSS2 and plasmin (Kido *et al.*, 2009, Laporte & Naesens, 2017). However, for highly pathogenic avian influenza A viruses, the multi-basic cleavage site motifs (e.g. R-X-K/R-R) are cleaved by ubiquitous proteases such as furin, PC6 and mosaic serine protease large form (MSPL) (Suguitan *et al.*, 2012, Kido, 2015).

Each HA monomer consists of a globular head domain and a stem domain. The globular head consists of the HA1 region only while the stem domain is composed of HA1 and HA2. A short hydrophobic region within the stem of coiled coil α helices attaches HA to the virion envelope (Skehel & Wiley, 2000, Mair *et al.*, 2014) The sialic acid binding domain, essential for the first stage of infection, is located on the

highly conserved globular head of antiparallel β sheets (Gamblin *et al.*, 2004, Weis *et al.*, 1988). The binding site is formed of conserved secondary structures (the 130 loop, 190 helix and 220 loop) and a base of four conserved residues (Chen *et al.*, 2012b, Zhang *et al.*, 2013). Binding specificity of HA for sialic acid determines the host range of the virus (Neumann & Kawaoka, 2006). The linkage between the galactose and the terminal sialic acid residue confers host specificity. The avian-like HAs prefer the α 2-3 linkage and human and swine viruses display preference for the α 2-6 linked sialic acid (Rogers & Paulson, 1983, Ito *et al.*, 1998). Due to the prevalence of both linkages on the respiratory epithelium of pigs, they are considered to act as a ‘mixing vessel’ which facilitates reassortment between avian and human influenza viruses (Ito *et al.*, 1998). The α 2-6 linked sialic acid is the predominant linkage in the upper airway tracheal epithelium of humans while the less common α 2-3 linkage is predominantly located in the lower respiratory tract (Baum & Paulson, 1990, Shinya *et al.*, 2006a). In cultured primary human epithelial cells, the α 2-6 linkage was predominant in non-ciliated cells while the α 2-3 linkage preferred by avian viruses was prevalent in ciliated cells (Matrosovich *et al.*, 2004). There have been reports describing α 2-3 linkage on numerous human respiratory cells, including goblet cells, ciliated epithelial cells and alveoli pneumocytes (Nicholls *et al.*, 2007, Kumlin *et al.*, 2008). Children appear to display a greater abundance of the α 2-3 linkage and this was proposed to account for differences in susceptibility between children and adults for avian influenza (Nicholls *et al.*, 2007).

1.1.1.2 Endocytosis and fusion

Following binding to SA, the influenza A virus is internalised via receptor mediated endocytosis. Clathrin mediated endocytosis underlies the primary internalisation mechanism employed by influenza viruses (Rust *et al.*, 2004). However, influenza A virus enters via clathrin mediated endocytosis, caveolae endocytosis and a clathrin-independent non caveolae mediated endocytosis (Sieczkarski & Whittaker, 2002). The internalised viruses are trafficked along the endocytic pathway and undergo two acidification steps in the early and late endosomes. Viral uncoating initiates in the early endosome and is completed in the late endosomes (Li *et al.*, 2014). The low pH facilitates fusion of virus and endosomal membranes and vRNP release (Yoshimura & Ohnishi, 1984, Bui *et al.*, 1996). The M2 ion channel is a single pass membrane composed of four transmembrane helices with the H37 residue functioning as the pH sensor and W41 as the gating residue (Schnell & Chou, 2008, Pielak & Chou, 2011). Acidification of the viral lumen is mediated by M2 ion channel which facilitates proton movement into the viral lumen (Schnell & Chou, 2008, Pielak & Chou, 2011). The low pH induces dissociation of the vRNPs from the matrix 1 (M1) protein (Bui *et al.*, 1996). In addition, the pH triggers fusion of the viral and endosomal membrane by inducing a conformational change in HA. While the HA1 globular domain is necessary for binding to the host membrane, the HA2 fusion peptide is required for membrane insertion (Wang *et al.*, 2015). The second acidification step is essential for activating HA mediated membrane fusion (Lakadamyali *et al.*, 2003). At low pH (~5) HA undergoes an irreversible conformational change which projects the N terminal fusion peptide of HA2 to facilitate fusion with the endosomal membrane (Wang *et al.*, 2015). Interaction between the transmembrane domain and the amphipathic helix HA2 fusion

peptide facilitates fusion pore formation (Han *et al.*, 2001, Bonnafeous & Stegmann, 2000, Armstrong *et al.*, 2000).

1.1.1.3 Virus replication and transcription

The free cytosolic vRNPs are transported into the nucleus. Two nuclear localisation signals (NLS) of nucleoprotein (NP) mediate import of the vRNPs via the importin- α/β pathway (Wu *et al.*, 2007, Nakada *et al.*, 2015). Viral RNA (vRNA) is essential for transcription and replication of the viral genome, acting as a template for viral mRNA or complementary RNA (cRNA) synthesis. Primary transcription of the vRNA generates viral mRNA via a primer dependent mechanism. Primers are obtained via a cap snatching mechanism dependent on cleaving the 5' methylated cap structure (m⁷GpppXm) from host mRNA (Pflug *et al.*, 2017). The viral RNA dependent RNA polymerase (RdRp) consists of PB1, PB2 and PA subunits. The PB2 domain binds to the cap of host mRNAs and the endonuclease activity of the PA subunit cleaves the cap preferentially at purines located 8-14 residues from the cap (de Velthuis & Fodor, 2016, Plotch *et al.*, 1981). The 5' capped primers are obtained from nascent RNA polymerase II (RNA pol II) transcripts through direct interaction between the viral RdRp and the C terminal domain of host RNA Pol II (Lukarska *et al.*, 2016). Capped primers 10-13 nucleotides long facilitate transcription initiation (Pflug *et al.*, 2018). The PB1 subunit contains the polymerase activity of the RdRp (Kobayashi *et al.*, 1996). Transcription terminates at the uridine tract of 5-7 residues located 17 to 22 residues from the 5' end of the segment. The poly(A) tail is formed by the viral polymerase reiteratively copying the uridine tract (Robertson *et al.*, 1981). Due to the stuttering activity of the viral polymerase at the uridine tract, mRNAs are shorter than

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viral RNA and cRNA as they lack the noncoding region at the 3' end (Wang *et al.*, 2014).

Replication of the viral genome requires the replication of (-) stranded vRNA through (+) cRNA intermediates (Scull & Rice, 2010). A priming loop in the viral polymerase is required for primer independent cRNA synthesis. This priming loop, formed of a β -hairpin loop in the PB1 subunit, facilitates terminal *de novo* initiation at the first and second residues at the 3' end of the vRNA template (Reich *et al.*, 2014, te Velthuis *et al.*, 2016). cRNA subsequently facilitates the production of full length progeny vRNA by RdRp through a *de novo* initiation mechanism referred to as internal initiation. Truncation of the priming loop did not impair vRNA synthesis from cRNA synthesis (te Velthuis *et al.*, 2016). Instead, the dinucleotide AG primer was synthesised from the fourth (U) and fifth residue (C) from the 3' end. Realignment to the cRNA template so the AG primer aligns to the first two residues of the 3' end facilitates vRNA synthesis (Zhang *et al.*, 2010). The mechanism behind a switch between transcription and replication by the viral polymerase is unclear (Vreede *et al.*, 2004, Scull & Rice, 2010).

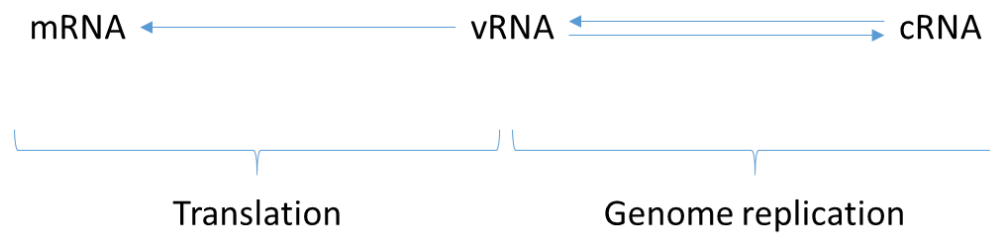


Figure 1-3. Viral RNA is converted into mRNA or cRNA to facilitate translation or genome replication.

1.1.1.4 Translation

Viral mRNA is preferentially translated by the host cell translation machinery over host mRNA in the cytosol (Garfinkel & Katze, 1993, Park & Katze, 1995). Viral mRNA translation is dependent on association with multiple host factors, including the eukaryotic initiation factor (eIF) 4F complex (formed of eIF4A, eIF4E, and eIF4G subunits) responsible for initiating eukaryotic mRNA translation (Merrick, 2015). Viral mRNA associates with cap protein eIF4E via its snatched 5' methylated cap structure, which recruits other components of the translation initiation complex (Bier *et al.*, 2011). However, others have reported influenza virus replication was not affected by impaired eIF4E. Instead, viral mRNA may depend on a modified translation initiation complex where the eIF4E subunit may be replaced by the viral polymerase (Burgui *et al.*, 2007). In addition, the NS1 protein associates with the eIF4GI subunit and poly(A)-binding protein (PABP1) to promote viral mRNA translation (Burgui *et al.*, 2003). NS1 also mediates negative regulation of host mRNA

translation, such as inhibition of cellular pre-mRNA 3' end processing (Nemeroff *et al.*, 1998).

The protein subunits of the vRNP (NP, PB1, PB2 and PA) are imported into the nucleus for vRNP assembly. The β -importin Ran-binding protein 5 (RanBP5) mediates PB1 and PA nuclear import while PB2 has been proposed to associate with importins $\alpha 3$ and $\alpha 7$ (Hutchinson *et al.*, 2011, Pumroy *et al.*, 2015). NP nuclear import is mediated by the importin- α/β pathway. The synergistic activity of the NLSs confers high preference for importin- $\alpha 7$ (Wu *et al.*, 2017). M1, NEP (NS2) and NS1 are synthesised in the cytosol and subsequently imported into the nucleus (Ye *et al.*, 1995, Greenspan *et al.*, 1988, Greenspan *et al.*, 1985). In contrast, HA and NA are co-translationally inserted into the ER while M2 is inserted into the ER membrane (Copeland *et al.*, 1986, Wang *et al.*, 2008a, Hull *et al.*, 1988) .

1.1.1.5 vRNP assembly and nuclear export

Following progeny viral vRNA and protein synthesis, the vRNPs are assembled in the nucleus (Wu *et al.*, 2011). The vRNP consists of a helical structure of NP which associates with the viral polymerase via the PB1 and PB2 subunits (Coloma *et al.*, 2009, Martín-Benito *et al.*, 2001). Positively charged residues of NP are proposed to associate with the progeny vRNA phosphate backbone (Elton *et al.*, 1999). The NP double helix forms the positively charged RNA binding groove that associates with the negatively charged vRNA (Arranz *et al.*, 2012b). In addition, the vRNA genomic ends associate with the viral polymerase (Hsu *et al.*, 1987, Arranz *et al.*, 2012a).

The newly assembled vRNPs are exported from the host nucleus, mediated by M1 and nuclear export protein (NEP or non-structural protein 2) (Huang *et al.*, 2013, Martin & Helenius, 1991). Chromosome region maintenance 1 (CRM1) mediates nuclear export of proteins containing leucine rich nuclear export signals (NES). NEP contains two nuclear export signals (NES1 and NES2) required for CRM1 protein mediated nuclear export of the vRNP-M1-NEP complex (Huang *et al.*, 2013). The NES of M1 does not associate with CRM1 but its association with the vRNPs is required for nuclear export. It has been proposed to function as a bridge between the vRNPs and NEP (Cao *et al.*, 2012, Sakaguchi *et al.*, 2003). M1 may promote efficient export by mediating vRNP export complex by binding to host cell chromatin, thereby bringing the complex into close proximity to components of CRM1 mediated nuclear export (Chase *et al.*, 2011). Finally, M1 association may prevent subsequent import of the cytosolic progeny vRNPs (Whittaker *et al.*, 1996). In addition, HA accumulation triggers activation of the host Raf/MEK/ERK signalling pathway which is essential for vRNP nuclear export, thereby coordinating the different components of virus replication (Marjuki *et al.*, 2006).

1.1.1.6 Assembly at the plasma membrane

Multiple mechanisms have been proposed for vRNP transport from the nucleus to the PM and other virus proteins, such as NEP, may depend on vRNP trafficking to the PM (Yasuda *et al.*, 1993b). Following vRNP nuclear export, the vRNPs accumulate near the microtubule organising centre (MTOC) where they co-localise with Rab11, potentially via the PB2 subunit, and are transported to the PM (Amorim *et al.*, 2011).

A Rab11 and microtubule dependent vesicular transport mechanism underlying vRNP

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movement to the PM has been widely reported (Bruce *et al.*, 2010, Eisfeld *et al.*, 2011a, Momose *et al.*, 2011). Briefly, human immunodeficiency virus Rev binding protein (HRB) has been proposed to facilitate vRNP release from the CRM1 export complex following nuclear export (Eisfeld *et al.*, 2011b). Host factor Y-box-binding protein 1 (YB-1) may mediate vRNP binding to microtubules around the MTOC (Kawaguchi *et al.*, 2012). Finally, the vRNPs may be transported from the MTOC and to the apical PM along microtubules by riding on Rab11 positive recycling endosomes (Momose *et al.*, 2011, Eisfeld *et al.*, 2011a). An alternative mechanism proposed Rab11 dependent formation of irregularly coated vesicles (ICVs) and ER remodelling around the MTOC were essential for vRNP transport (de Castro Martin *et al.*, 2017). The eight vRNPs are asymmetrically assembled in the influenza virion as a supramolecular complex (Noda *et al.*, 2012, Fournier *et al.*, 2012). In particular, a pattern of assembly for the longer RNPs was observed, suggesting specific interactions between the RNPs underlie their assembly in the progeny virion (Noda *et al.*, 2012). M1 associates with the RNA and NP in the vRNP complex. M1 binding to the vRNP requires a basic amino acid residue domain and N terminal hydrophobic domains (Ye *et al.*, 1999, Noton *et al.*, 2007). NEP associates with the M1 matrix in the progeny virus particle (Yasuda *et al.*, 1993a).

The influenza glycoproteins are post-translationally modified and trafficked to the PM through the host secretory pathway (Copeland *et al.*, 1986, Wang *et al.*, 2008b). N-linked glycosylation is essential for correct glycoprotein folding and oligomerisation (Wang *et al.*, 2008b, Daniels *et al.*, 2003). HA is synthesised as its HA0 precursor and forms trimers in the ER before it is transported to the Golgi apparatus (Copeland *et al.*, 1986). HA contains seven N-linked glycosylation sites (Daniels *et al.*, 2003). NA

is co-translationally inserted into the ER and forms tetramers in the ER (Wang et al., 2008a, Hogue & Nayak, 1992). NA displays multiple glycosylation states in its head domain and four conserved sites in its stem domain (Wang et al., 2008a, Chen *et al.*, 2012a). The influenza glycoproteins associate with the calnexin/calreticulin (CNX/CRT) ER resident lectins (Wang et al., 2008a, Daniels et al., 2003). Quality control and maturation of N-linked glycosylated proteins is mediated by association with the CNX/CRT lectins (Caramelo & Parodi, 2008b). For example, mutation of the head domain glycosylation sites inhibited NA maturation, interfering with NA co-translational association with ER chaperones CNX and CRT (Wang et al., 2008a).

Processed HA and NA are trafficked from the trans-Golgi to the apical PM. The transmembrane domains of the influenza glycoproteins are essential for apical PM targeting (Barman & Nayak, 2000, Lin *et al.*, 1998). However, the host factors required for trafficking the virus proteins to the PM have not been fully described (Wang *et al.*, 2012, Yadav *et al.*, 2016). GTPase Cdc42 and its GTPase-activating protein, Rho GTPase Activating Protein 21 protein (ARHGAP21) are host factors that have been identified as facilitators of virus glycoprotein trafficking to the PM through actin cytoskeleton remodelling (Wang et al., 2012).

At the host PM, HA and NA accumulate in cholesterol/sphingolipid-rich rafts (Ohkura *et al.*, 2014). Progeny virus assembly at the host PM is initiated by HA and NA mediated recruitment of M1 at the PM (Rossman & Lamb, 2011, Ali *et al.*, 2000). HA and NA cytoplasmic tail mutants failed to recruit M1 (Chen *et al.*, 2007). In addition to the cytoplasmic tail, the transmembrane domain of HA may mediate its association with M1 (Rossman & Lamb, 2011, Ali et al., 2000). The cytoplasmic tail of the M2 ion channel has been proposed to contribute to M1 recruitment to the PM. Deletion of M2 residues 71 to 97 impaired direct association with M1 and its incorporation into

budding virions (Chen *et al.*, 2008). The M2 protein is synthesised in the cytosol and subsequently inserted into the ER membrane, mediated by a signal recognition particle in the transmembrane domain (Hull *et al.*, 1988). M2 is trafficked through the secretory system and its activity interferes with vesicular transport in the trans-Golgi (Henkel & Weisz, 1998). It is trafficked to the PM in association with host factors including transport protein particle complex 6A delta (TRAPPC6Δ), a subunit of the TRAPP complex, and ubiquitin protein ligase E3 component N-recognin 4 (UBR4) (Zhu *et al.*, 2017a, Tripathi *et al.*, 2015). The TRAPP complexes (TRAPP1 and TRAPP2) are involved in ER to Golgi and intra-Golgi transport. TRAPPC6Δ may regulate M2 trafficking to the PM, interacting with the M2 C terminal L96 residue (Zhu *et al.*, 2017a). M2 association with UBR4, via its transmembrane and cytosolic domain, also appears to be required for efficient M2 intracellular transport. In the absence of UBR4, M2 localisation to the PM is impaired and the M2 protein is directed to the autophagosomes (Tripathi *et al.*, 2015).

1.1.1.7 Virus budding and release

Virus infections may be broadly split into two categories; persistent or cytopathic infections. The latter, also referred to as lytic infection, induces changes in the host cell due to viral infection and results in lysis of the majority of host cells soon after infection (Heaton, 2017). Influenza A virus has long been established as a cytolytic virus (Scholtissek *et al.*, 1967).

Accumulation of HA and NA at the PM in lipid raft microdomains was proposed to trigger influenza virus budding (Scheiffele *et al.*, 1999, Zhang *et al.*, 2000). The contribution of the influenza membrane proteins, particularly HA, was highlighted by

the formation of virus like particles (VLPs) following glycoprotein overexpression (Zhang *et al.*, 2000, Chen *et al.*, 2007). A later study found M1 and M2 were necessary for glycoprotein mediated VLP formation (Chlanda *et al.*, 2015). Furthermore, M2 facilitates the final stage of virion budding by mediating ESCRT independent membrane scission (Rossman *et al.*, 2010). Residues 54 to 62 of M2 form an amphipathic helix essential for binding to host PM cholesterol to facilitate membrane curvature and scission (Roberts *et al.*, 2013).

NA is a neuraminidase, of which there are nine subtypes, which is essential for the release of progeny virus particles. Eight conserved residues (R118, D151, R152, R224, Q276, R292, R371 and Y406) form the active site of the first binding site while the N9 neuraminidase contains a second binding site dependent on six conserved residues (S367, S370, S372, R400, W403 and K432) (Varghese *et al.*, 1997, Shtyrya *et al.*, 2009). NA cleaves the host cell sialic acid residues and prevents accumulation of progeny viruses at the PM of the 'parent' host cell (Liu *et al.*, 1995, Shtyrya *et al.*, 2009). In addition, NA facilitates infection of host respiratory epithelial cells by cleaving the sialic acid residues of the mucins within the defensive mucus layer, preventing HA binding to the sialylated mucin decoys (Cohen *et al.*, 2013).

1.1.1.8 Influenza virus suppression of the host antiviral response

Influenza virus inhibits the host innate immune response. NS1 impairs activation of interferon regulatory factor (IRF) 3, NF- κ B and c-Jun/ATF-2 transcription factors and subsequent type I interferon (IFN) production (Talon *et al.*, 2000, Wang *et al.*, 2000, Ludwig *et al.*, 2002). IRF3 transcription may be impaired by NS1 mediated inhibition of mRNA nuclear export and host 3' end mRNA processing (Krug, 2015, Qian *et al.*, 20

1994). NS1 inhibits host antiviral proteins: retinoic acid-inducible gene-I (RIG-I), 2'-5'-oligoadenylate synthetase (OAS) and protein kinase R (PKR) (Pichlmair *et al.*, 2006, Min & Krug, 2006, Min *et al.*, 2007). However, only certain strains of influenza virus contain NS1 proteins capable of inhibiting IRF3 activation (Kuo *et al.*, 2010). The PB1 gene (segment 2 of the IAV genome) encodes a second protein, PB1-F2, through the +1 open reading frame. PB1-F2 interacts with the mitochondrial antiviral signalling protein (MAVS) and inhibits activation of the type I IFN pathway (Varga *et al.*, 2012). PB1-F2 also facilitates apoptosis by forming pores through self-oligomerisation in the mitochondrial membrane and promoting formation of the permeability-transition pore complex (PTPC) (Bruns *et al.*, 2007, Zamarin *et al.*, 2005). In addition, the PB1 mRNA gives rise to a truncated PB1 protein, PB1-N40, arising from an alternative AUG codon. The function of this protein is unclear and may not contribute to virus replication (Wise *et al.*, 2009). A second open reading frame in segment 3 encodes an alternative product, PA-X (Jagger *et al.*, 2012). PA-X contributes to virus dependent shutoff of host protein synthesis by mediating host mRNA degradation, suppression of the type I IFN response and inhibition of the humoral immune response (Hayashi *et al.*, 2015). NS1, PA-X and PB1-F are not incorporated into the virus particles (Marc, 2014)

1.1.2 Prevention and treatment of influenza A virus infection

1.1.2.1 Non-pharmaceutical Interventions

Influenza virus is transmitted through large respiratory droplets ($>5\mu\text{m}$) and smaller droplet nuclei particles ($<5\mu\text{m}$) arising from coughing and sneezing of infected individuals, also referred to as aerosol transmission (Cowling *et al.*, 2013, Brankston *et al.*, 2007). The virus may also be spread through exposure to infectious fluids via contact with infected individuals or contaminated surfaces or objects (fomites) (Brankston *et al.*, 2007, Edenborough *et al.*, 2012, Mubareka *et al.*, 2009). Encouraging good hand hygiene practices and respiratory etiquette are among the most basic non-pharmaceutical interventions (NPIs) for limiting the spread of influenza virus (Aledort *et al.*, 2007, Wong *et al.*, 2014). Guidelines for non-pharmaceutical dependent strategies for limiting virus spread also include isolating patients from the public but this strategy has limited success due to the cooperation of the public and limited success in accurate diagnosis (World Health Organization Writing, 2006). Social distancing measures, such as school closures for children and working from home for adults, may provide limited effectiveness in managing virus transmission within the population (Perlroth *et al.*, 2010).

1.1.2.2 Vaccination

Current strategies regarding influenza A virus outbreaks are predominantly dependent on preventing infection, relying on the production and delivery of an effective vaccine for the predicted predominant strains of influenza A virus (W.H.O. Writing Group *et al.*, 2012, Steinbruck *et al.*, 2014, Kostova *et al.*, 2013). Vaccination exploits the humoral immune response, eliciting the production of neutralising antibodies primarily against HA and NA (Grebe *et al.*, 2008). Vaccination is currently the most effective strategy for preventing infection and reducing the spread of influenza virus

infection across the population, despite challenges in vaccination rates (Restivo *et al.*, 2018, Nichol & Treanor, 2006, de Lusignan *et al.*, 2016). Vaccination is particularly beneficial for those in the population considered to be high risk groups, such as children, pregnant women and those with underlying medical conditions (Restivo *et al.*, 2018). However, due to antigenic drift and antigenic shift facilitating the introduction of novel influenza A virus strains, the recommended vaccination strategy must be continually amended and updated (Grohskopf *et al.*, 2017, Chen & Deng, 2009, Steinbrück *et al.*, 2014). While the impact of the anticipated annual epidemics of influenza A viruses may be reduced through the current vaccination strategy, the threat of an unanticipated epidemic or pandemic from the exposure of an immunologically naïve host population to novel antigenic variants remains. This is due to the delay between the onset of an epidemic and the widespread availability of an effective vaccine, a process potentially taking up to 6-9 months (Laskowski *et al.*, 2015, Carrat & Flahault, 2007).

1.1.2.3 Antiviral strategies

Under conditions where vaccination is not an available strategy, antiviral compounds provide the next line of defence. Currently, three classes of antiviral compounds have been developed that target conserved elements of the influenza virus; the neuraminidase inhibitors (NAIs), M2 channel inhibitors and viral polymerase inhibitors.

1.1.2.3.1 Adamantanes

The M2 ion channel regulates acidification of the virion interior and is essential for virus uncoating (reviewed by Manzoor *et al.*, 2017). The adamantane drugs, amantadine and rimantadine, inhibit the M2 channel and interfere with virus entry to the host cell (Davies *et al.*, 1964). This family of antivirals target two sites on the M2 channel: a high affinity binding site located on the ion channel pore and a low affinity binding site at a lipid facing pocket (Rosenberg & Casarotto, 2010). However, they are no longer recommended for the treatment or prophylaxis of influenza A virus due to the prevalence of adamantane resistance. Approximately 45% of influenza viruses from HA subtypes H1-H17 display adamantane resistance. Over 90% of adamantane resistant influenza viruses display mutations in the M2 S31 residue (Bright *et al.*, 2006, Dong *et al.*, 2015). Due to the prevalence of adamantane drug resistance, only the NAIs are recommended for influenza treatment (von Itzstein *et al.*, 1993, Kim *et al.*, 1997, Bright *et al.*, 2005).

1.1.2.3.2 Neuraminidase inhibitors

Neuraminidase is required for cleavage of sialic acid from cell surface receptors (Wagner *et al.*, 2002). NA facilitates progeny virus release from the host cells by preventing virus accumulation at the cell surface (Wagner *et al.*, 2002). The release of influenza virus from infected cells is inhibited by the NAIs (Liao *et al.*, 2017). Four NAIs (oseltamivir, zanamivir, peramivir and laninamivir) have been licenced for use, although their availability varies across the world with only zanamivir and oseltamivir

licensed globally for influenza virus treatment (Ikematsu & Kawai, 2011, Shetty & Peek, 2012, Dreitlein *et al.*, 2001).

NAI	Common brand name	Licensed for use	Administered
Oseltamivir	Tamiflu	Global	Capsule or oral suspension /Intravenous
Peramivir	Rapivab	United States, Canada, Australia, Japan, Taiwan, Korea and EU	Intravenous
Zanamivir	Relenza	Global	Inhalation
Laninamivir	Inavir	Japan	Inhalation

Table 1-1. Licensed NAIs for treatment of influenza A virus infection

The NAIs are effective against multiple strains of influenza A and B viruses (Mendel *et al.*, 1998, Burnham *et al.*, 2013). This group of inhibitors is based on the identification of 2,3-dehydro-2-deoxy-N-acetylneuraminic acid (DANA) as a weak inhibitor of NA (Burmeister *et al.*, 1993). Modification of the C4 on the sugar ring,

replacing C4 hydroxyl group with a guanidino group dramatically increased the binding affinity of the compound and resulted in the production of 2,3-didehydro-2,4-dideoxy-4-guanidinyl-N-acetylneuraminic acid, more commonly referred to as zanamivir (Varghese *et al.*, 1992, von Itzstein *et al.*, 1994, von Itzstein *et al.*, 1993). Zanamivir is inhaled to alleviate influenza symptoms (Hayden *et al.*, 1997, Group, 1998). The 4-guanidino group forms hydrogen bonds with charged amino acids such as D151, E227 W178 and E227, conserved residues which are important for supporting the catalytic activity of NA (Colman *et al.*, 1983, Varghese *et al.*, 1995, Wu *et al.*, 2013). Similar to zanamivir, the structure of oseltamivir was based on the modification of DANA. It contains a C4 amino group and a substitution of the glycerol side chain with a hydrophobic pentyl ether side chain (Mendel *et al.*, 1998). Oseltamivir is administered orally as a prodrug (oseltamivir phosphate) which is converted to the active form, oseltamivir carboxylate, by hepatic esterases (Davies, 2010). Hydrogen bonds between the inhibitor carboxylate moiety with residues R292 and R371 and its amino group with residues E119, D151 and W178 are responsible for the majority of interactions between the drug and the binding pocket (Le *et al.*, 2010, Maturos *et al.*, 2008). Additionally, the pentyl ether group binds to residues I222, R224, A246 and E276 of a hydrophobic pocket (Yen *et al.*, 2006, Le *et al.*, 2010). Structure based design methods gave rise to the development of peramivir, an intravenously delivered NAI effective against influenza A and B viruses (Alame *et al.*, 2016). The cyclopentane derivative contains a carboxyl group which forms eight hydrogen bonds with residues R118, R292 and R371, in comparison to the six hydrogen bonds formed by oseltamivir and zanamivir (Malaisree *et al.*, 2008). The acetamido group associates with residues I222 and W178 while the guanidino group interacts with the acidic groups of residues E119, D151 and E227 (Alame *et al.*, 2016).

Finally, laninamivir is administered as an octanoate pro-drug which is converted to its active form in the lung. It contains a 4-guanidino group and an additional 7-methoxy group (Kubo *et al.*, 2010, Vavricka *et al.*, 2011). The 4-guanidino group of laninamivir binds beneath the 150 cavity of neuraminidase (Vavricka *et al.*, 2011). Laninamivir was determined to directly interact with seven catalytic residues (R118, D151, R152, R224, E276, R292 and R371) and four residues forming the NA framework (E119, W178, E227 and E277) (Meeprasert *et al.*, 2012). Laninamivir is effective against highly pathogenic H5N1 avian viruses and oseltamivir resistant viruses (Yamashita *et al.*, 2009, Kiso *et al.*, 2010a). Furthermore, the protective effect of a single intranasal dose of laninamivir was sustained up to seven days while oseltamivir and zanamivir require twice daily administration (Yamashita *et al.*, 2009, Kiso *et al.*, 2010a, Kubo *et al.*, 2010).

However, the effectiveness of the NAIs is a continued source of debate, with particular focus on the modest alleviation of symptoms (Nguyen-Van-Tam *et al.*, 2015). Clinical studies suggest zanamivir and oseltamivir reduce the duration of influenza virus mediated symptoms by approximately one day, provided the patient receives the antiviral medication within 24-48 h of the emergence of symptoms (Dreitlein *et al.*, 2001, Doll *et al.*, 2017, Dobson *et al.*, 2015, Nicholson *et al.*, 2000). The protective effect of oseltamivir against mortality has been demonstrated in mice (Dreitlein *et al.*, 2001, Baranovich *et al.*, 2014). Its protective effect was dependent on swift NAI administration. This was demonstrated by a reduction in survival rate in mice infected with H7N9 virus when treatment was delayed to 48 h after infection in comparison to 24 h post-infection (Baranovich *et al.*, 2014). Twice daily oral administration of oseltamivir was able to protect ferrets from mortality following infection with the

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highly pathogenic avian H5N1 virus (Boltz *et al.*, 2008). In humans, an association between NAI administration and a significant reduction in mortality was determined in a cohort study in Ontario, Canada (McGeer *et al.*, 2007). A review of systemic reviews and meta-analyses support a reduction in mortality in humans following administration of oseltamivir or zanamivir (Doll *et al.*, 2017). NAI treatment significantly reduced the risk of developing pneumonia and other lower respiratory tract complications (Smith & Dutkowski, 2005, Kaiser *et al.*, 2003, Dobson *et al.*, 2015). However, while NAI treatment of the 2009 H1N1 virus reduced the likelihood of a severe outcome, the reduction in mortality was dependent on early treatment (Muthuri *et al.*, 2013, Heneghan *et al.*, 2016). Furthermore, meta analysis of the 2009 pandemic suggested there was not a significant reduction in mortality in children (Muthuri *et al.*, 2014). The prophylactic capabilities of oseltamivir and zanamivir have also been assessed, with a relative reduction of 70-90% in the likelihood of infection depending on the prevention strategy (Cooper *et al.*, 2003).

Regardless of the efficacy of the NAIs, the encroaching onset of NAI resistance provides a further complication in treating influenza infections, particularly regarding the potential for further pandemics. Antiviral resistance has been identified in numerous influenza A virus subtypes, including the highly pathogenic avian influenza virus H5N1 subtype (de Jong *et al.*, 2005, Earhart *et al.*, 2009). The susceptibility of the NAIs to resistance when treating avian influenza viruses is of particular importance when their role in the development of pandemic influenza A outbreaks is considered (Smith, 2010). The persistence of the active form of oseltamivir, oseltamivir carboxylate, in the environment following use in treating seasonal influenza virus outbreaks may contribute to the development of antiviral resistance and highlights the

need for careful usage. Oseltamivir carboxylate has been detected in surface water and sewage plants where it may come in to contact with influenza virus carried by waterfowl. Exposure to low concentrations of oseltamivir carboxylate fostered the development of antiviral resistance in influenza A H1N1 virus in mallards (Järhult, 2012).

NAI resistance is dependent on substitutions of the eight residues in the catalytic active site or the 11 essential framework residues. Mutation of these residues impairs association of the NAI and the NA sialic binding pocket (Eshaghi *et al.*, 2014). Mutations which confer antiviral resistance may be NA subtype specific while others are shared across NA subtypes (Song *et al.*, 2015, Choi *et al.*, 2017). Substitution at residues E119 and R292 conferred resistance to avian N3, N7 and N9 influenza viruses (Song *et al.*, 2015). E119V, R292K and Q136K substitutions and deletion of residues 245-248 reduced the sensitivity of H3N2 influenza A virus to NAIs oseltamivir, zanamivir, and peramivir (Eshaghi *et al.*, 2014). Substitutions may not confer resistance to all of the NAIs due to differences in NAI binding mechanisms. This is reflected by the H5N1 virus H274Y mutation conferring resistance to oseltamivir but not zanamivir (de Jong *et al.*, 2005, Moscona, 2005).

The novel Q136K substitution also reduced zanamivir and peramivir sensitivity of seasonal H1N1 influenza virus but did not affect oseltamivir susceptibility (Hurt *et al.*, 2009). Most recently, oseltamivir resistance of the 2009 pandemic of swine H1N1 influenza A virus has been described (Calatayud *et al.*, 2011, Kiso *et al.*, 2010b). Resistance to H1N1 viruses was most commonly associated with a H275Y substitution (Hurt *et al.*, 2012). Viruses with the H275Y substitution were found to display reduced fitness in animal models (Duan *et al.*, 2010, Ives *et al.*, 2002). Although others suggested the NA H274Y substitution conferred oseltamivir resistance without

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impairing pathogenicity or transmissibility in animal models (Kiso *et al.*, 2010b). In addition, a novel pandemic 2009 H1N1 virus was identified with an NA S247N mutations. The S247N/ H275Y double mutant was highly resistant to oseltamivir treatment. The oseltamivir concentration required for 50% inhibition increased nearly 6000 fold compared to oseltamivir susceptible viruses (Hurt *et al.*, 2011). The onset of resistance is a key limitation of NAIs which directly target the influenza virus.

1.1.2.3.3 Viral RNA dependent RNA polymerase inhibitors

The viral RNA dependent RNA polymerase (RdRp) has been proposed as an alternative target for virus directed antiviral therapy (Zhou *et al.*, 2018). The viral RNA polymerase is composed of three subunits: PA, PB1 and PB2 (Boivin *et al.*, 2010).

The PA subunit of the RdRp facilitates the cap snatching mechanism of viral mRNA synthesis through its endonuclease activity (Dias *et al.*, 2009). The active site of the PA endonuclease is highly conserved (Crepin *et al.*, 2010, DuBois *et al.*, 2012). The 4-substituted 2,4-dioxobutanoic acids and their derivatives were identified as inhibitors of the PA endonuclease (Hastings *et al.*, 1996, Tomassini *et al.*, 1994). Other PA inhibitors such as RO-7 were later identified. RO-7 significantly reduced virus titre through inhibition of influenza polymerase activity with minimal toxicity to the host cell (Jones *et al.*, 2016). RO-7 was an effective prophylactic and therapeutic treatment against multiple strains of influenza A and B viruses (Jones *et al.*, 2016, Jones *et al.*, 2017). S-033188 (also known as Baloxavir marboxil or Xofluza) is a potent inhibitor of the cap-dependent endonuclease. S-033188 significantly alleviated symptoms and

reduced viral titres in a phase 3 clinical trial of patients between 12 and 64 years of age who received a single oral administration of S-033188 within 48 hours of symptom onset (Portsmouth *et al.*, 2017). It has been approved for treatment of uncomplicated influenza A or B virus infections in Japan and is under review by the U.S. Food and Drug Administration (Heo, 2018). Potential mutations at the endonuclease active site which may confer antiviral resistance to S-033188 have been identified (Omoto *et al.*, 2018). Substitution at I38T in the PA protein reduced influenza susceptibility to inhibitors S-033188 and RO-7 (Omoto *et al.*, 2018, Jones *et al.*, 2018). However, the viruses displaying the I38T substitution also exhibited impaired fitness. The identification of substitutions following treatment with the inhibitor may aid surveillance of emerging virus strains and provide valuable insight into future challenges presented by antiviral treatment (Omoto *et al.*, 2018).

In addition to the PA subunit, the PB2 subunit of the influenza virus RNA polymerase may be targeted. The PB2 subunit binds to the 7 methylguanosine (m⁷G) cap of the host pre-mRNA (Boivin *et al.*, 2010). m⁷G derivatives occupy the PB2 m⁷G cap binding site and are effective inhibitors of PB2 binding activity (Pautus *et al.*, 2013). VX-787 (also known as pimodivir) has been identified as an effective inhibitor of the PB2 subunit of multiple influenza A virus strains. In mice, VX-787 inhibited influenza A virus replication in prophylaxis and treatment regimens, including treatment regimens delayed up to 120 hours after initial infection (Byrn *et al.*, 2015). In a phase IIa clinical study VX-787 significantly reduced viral load and clinical symptoms following once daily administration 24 hours after infection (Trevejo *et al.*, 2017).

T-705 (favipiravir) is an inhibitor of influenza virus RNA polymerase activity. The mechanism of action proposed for T-705 suggested T-705 was converted to T-705RTP

by cellular kinases and T-705RTP may be misidentified as a purine nucleotide by the virus RNA polymerase (Furuta *et al.*, 2005). Incorporation of T-705RTP into the viral RNA strand may inhibit extension of the viral strand (Furuta *et al.*, 2017). T-705 has been proposed to be an effective treatment for the 2009 H1N1 pandemic viruses and overcome the challenge of oseltamivir resistance (Kiso *et al.*, 2010b).

1.1.2.4 Host directed antiviral therapy

Host based therapy has been proposed as an alternative to antivirals that target the virus directly, potentially overcoming the onset of resistance observed for the current antivirals available (Marois *et al.*, 2014, Shaw, 2011). Multiple host components are essential for influenza virus replication (Marois *et al.*, 2014, Mehle & Doudna, 2010). Host directed therapy may depend on inhibition of pro-viral host signalling pathways or activation of host antiviral responses (Planz, 2013, Loo *et al.*, 2016). DAS181 (Fludase) is a recombinant fusion protein consisting of a cell surface anchoring sequence and sialidase catalytic domain. The protein prevents virus attachment to the airway epithelium by pre-emptively removing the sialic acids used by human and avian influenza viruses (Malakhov *et al.*, 2006). Influenza viruses are internalised via receptor mediated endocytosis and fusion of the virus and endosome membranes, allowing vRNP release into the cytosol, is triggered by low pH (Li *et al.*, 2014). Lysosomotropic alkalinizing agents, such as amodiaquine, chloroquine, primaquine or quinacrine, significantly inhibited influenza virus replication through interfering with endosomal acidification (Marois *et al.*, 2014). An alternative strategy also proposed was targeting the pro-influenza Ca^{2+} dependent proteins. The calcium/calmodulin-dependent protein kinase (CaM kinase) II beta regulated viral RNA transcription,

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demonstrating pro-viral activity for calcium dependent host proteins (König *et al.*, 2010). Modulation of calcium sensitive proteins by interfering with intracellular Ca^{2+} concentration through exposure to calcimycin, TMB-8, and verapamil inhibited virus replication (Marois *et al.*, 2014). Activation of the host immune signalling pathways is an alternative host directed therapy. Activation of IFN-stimulated genes (ISGs) has been proposed as a target for novel therapeutics (Crosse *et al.*, 2018). Activation of host ISGs through stimulating IRF3 activation through small molecular RIG-I agonists has been proposed (Loo *et al.*, 2016). Targeting the host factors was suitable for prophylaxis and treatment post infection (Marois *et al.*, 2014).

1.1.3 Innate immune response

The innate immune response provides a non-specific defence to potential pathogens and is the first line of defence to an infection. It is comprised of physical barriers to infection (such as epithelial surfaces), humoral components (such as the complement system) and cellular components (such as macrophages, dendritic cells and neutrophils) (Shishido *et al.*, 2012, Gasteiger *et al.*, 2017). Pathogens are recognised by the innate immune system via germline encoded pattern recognition receptors (PRRs). The germline encoded PRRs allow the organism to mount an immune response to an immunological challenge without the requirement of prior exposure through recognition of pathogen associated molecular patterns (PAMPs) (Medzhitov & Janeway, 2000, Janeway, 2013). There are four broad families of these receptors: Toll like receptors (TLRs), C-type lectin receptors (CLRs), NOD like receptors (NLRs) and Retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) (Basset *et al.*, 2003, Iwasaki & Medzhitov, 2004, Takeuchi & Akira, 2010, Palm & Medzhitov, 2009). Viral nucleic acid is recognised by the PRRs. For example, TLR9 recognises viral DNA, TLR3 recognises double stranded RNA while TLR7 and TLR8 recognised single stranded RNA. In addition, TLR2 and TLR4 may also recognise viral glycoproteins (Akira *et al.*, 2006). PRRs capable of recognising influenza virus infection include CLR macrophage mannose receptor, TLRs 3, 4 and 7, RLR RIG-I and NLRs NLRP3, NOD2 and NLRX1 (Pulendran & Maddur, 2015). Activation of the PRRs triggers signalling cascades, including pro-inflammatory cytokine production mediated by NF- κ B or the type I IFN response (Newton & Dixit). NF- κ B regulates the expression of a multitude of pro-inflammatory genes such as chemokines for immune cell recruitment, pro-inflammatory cytokines and growth factors

promoting immune cell differentiation and activation (Newton & Dixit, Hayden *et al.*, 2006). The IFNs regulate the expression of interferon stimulated genes (ISGs) such as PKR, OAS and RNaseL (Samuel, 2001). The ISGs inhibit influenza virus replication through multiple mechanisms. For example, RNaseL is an endoribonuclease, activated by binding 2-5A oligonucleotides produced by OAS, that catalyses viral RNA degradation, including influenza A virus RNA (Samuel, 2001, Cooper *et al.*, 2015). IFN inducible viperin interferes with influenza virus release through modulating plasma membrane fluidity (Wang *et al.*, 2007).

The expression of pro-inflammatory cytokines and chemokines stimulates the cellular innate immune antiviral response, activating innate immune cells such as macrophages, neutrophils and natural killer (NK) cells. Infected epithelial cells produce CCL2 which recruits migration of macrophages and monocytes to the site of infection through their CCR2 receptor (Lin *et al.*, 2008, Herold *et al.*, 2006). Recruited innate immune cells, such as macrophages and neutrophils, contribute to pathogen clearance through phagocytosis of influenza virus infected cells (Hashimoto *et al.*, 2007). Macrophages contribute to clearance of influenza A virus via opsonophagocytosis (Huber *et al.*, 2001). NK cells are activated by influenza A virus infection and respond to viral infections through cytolysis of infected cells and cytokine production (Hwang *et al.*, 2013).

Innate immune cells are also integral to the function of the adaptive immune response. Antibody titres and the percentage of CD8⁺ T lymphocytes was reduced in alveolar macrophage depleted pigs infected with influenza virus (Kim *et al.*, 2008). However, dendritic cells (DCs) underpin the bridge between the innate and adaptive immune

response, functioning as the professional antigen presenting cells. Through direct infection or phagocytosis of virus particles or apoptotic epithelial cells, DCs obtain and present the viral antigens to naïve and memory B and T cells (Banchereau & Steinman, 1998, Bhardwaj *et al.*, 1994).

1.1.4 Adaptive immune response

The adaptive immune response is essential for protection against influenza virus infection, demonstrated by high susceptibility of immunodeficient mice lacking B or T cells to virus infection (Bot *et al.*, 1996). In contrast to innate immunity, adaptive immune responses are specific to the pathogen and dependent on B cell and T cell activity. However, upon first exposure to the pathogen this response is slow to develop. Following pathogen recognition, innate immune cells such as dendritic cells present antigens and co-stimulatory molecules to adaptive immune cells, triggering activation of naïve T lymphocytes (Medzhitov & Janeway, 1998). Cytotoxic CD8⁺ effector T cells control influenza virus infection through elimination of virus infected respiratory epithelial cells facilitating replication, as well as cytokine and chemokine production (reviewed by (Hufford *et al.*, 2015). CD4⁺ T cells may be split into two subtypes, Th1 or Th2, according to their cytokine profile. The former promotes antibody production in B cells while the latter regulates CD8⁺ T cell activity (reviewed by (van de Sandt *et al.*, 2012). Memory CD4⁺ and CD8⁺ T cells provide long lasting protection against influenza virus and facilitate a rapid immune response for subsequent infections (reviewed by (Grant *et al.*, 2016, Brown *et al.*, 2004)).

Humoral immunity is key to overcoming influenza virus infection. Influenza viruses stimulate production of virus specific homo- and hetero-subtypic antibodies by B cells (Wrammert *et al.*, 2008, Nguyen *et al.*, 2007). The B cells are activated by the follicular helper T cell, if they present the corresponding antigen acquired through their B cells receptor (Medzhitov & Janeway, 1998). Protective IgM antibodies are constitutively produced by the B-1 subset of B cells (Choi & Baumgarth, 2008). IgG and IgA antibodies, the former produced systemically and the latter mostly located in mucosal tissues, are both integral for protection against influenza virus infection (Muramatsu *et al.*, 2014, Renegar *et al.*, 2004).

1.1.5 The type I IFN response

An essential component of the immune response following influenza virus infection is the IFN response (Killip *et al.*, 2015). Regulation of the type I IFN response has been proposed for host directed therapy (Loo *et al.*, 2016). The IFNs, discovered in 1957, were first identified as an ‘interfering agent’ from influenza virus infected chick embryo cells, capable of inhibiting virus production (Isaacs & Lindenmann, 1957). The capacity of the IFNs to impede infection was further explored and its ability to impede the growth of intracellular bacteria, such as *Chlamydia psittaci* and *C. trachomatis*, recognised (Sueltenfuss & Pollard, 1963, de la Maza *et al.*, 1985). The IFNs can be split into three classes according to their corresponding receptor; type I, type II and type III (Pestka *et al.*, 2004). The type I IFNs consists of IFN- β , δ , ϵ , κ , τ , ω , ζ and 13 types of IFN- α , which bind to a receptor formed of IFNs- α/β receptor (IFNAR) 1 and IFNAR2 subunits (Pestka *et al.*, 2004). The family of type I IFNs are

cytokines responsible for orchestrating an immune response to provide defence against viral infection (Killip *et al.*, 2015).

The type I IFN response is activated following recognition of a viral challenge by the pattern recognition receptors, such as the Toll-like receptors (TLRs), nucleotide oligomerization domain-like receptors (NLRs) and retinoic acid-inducible gene I-like receptors (RLRs) (Thompson *et al.*, 2011, Ranjan *et al.*, 2015). Within the TLRs, the dsRNA sensor TLR3 and the ssRNA sensor TLR7 are essential for recognition and induction of the IFN response in response to influenza virus infection (Wu *et al.*, 2015, Diebold *et al.*, 2004). NOD2 deficient bone marrow-derived macrophages and mouse embryonic fibroblasts and NOD2 knock out mice displayed impaired type I IFN production in response to H1N1 virus infection (Sabbah *et al.*, 2009). In addition, the NLRP3 inflammasome has been proposed as a key regulator of immunity in response to influenza (Pang & Iwasaki, 2011). RNA helicases retinoic acid inducible gene-I (RIG-I) and melanoma differentiation antigen 5 (MDA5) are essential cytosolic double stranded RNA sensors which activate the type I IFN response in response to influenza virus infection (Loo *et al.*, 2008, Benitez *et al.*, 2015). The cytosolic receptors activate the adaptor molecule interferon-beta promoter stimulator 1 (IPS-1) which in turn activates the IRFs 3 and 7 (Kawai *et al.*, 2005). IRF3 and IRF7 have been identified as key regulators of innate immunity induced by viruses (Hatesuer *et al.*, 2017, Wong & Chen, 2014, Hiscott, 2007). IFN- α and IFN- β responses following influenza A virus infection were absent in IRF3 and IRF7 deficient mice (Hatesuer *et al.*, 2017). IPS-1 associates with the N-terminal tandem caspase recruitment domain (CARD) of RIG-I/MDA5 through its own CARD like domain. Knockdown of IPS-1 impaired RIG-I/MDA5 induction of type I IFN signalling while IPS-1 overexpression

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induced IRF3 and IRF7 phosphorylation and activation (Kawai et al., 2005). IRF-3 phosphorylation at serine and threonine residues in the C terminus by kinases TANK-binding kinase-1 (TBK1) and IKK ϵ facilitates its dimerisation and nuclear localisation. The IRF3 dimer associates with CBP/p300 in order to regulate expression of the type I IFNs such as IFN- β (Fitzgerald *et al.*, 2003, Takahasi *et al.*, 2010). IRF3 and IRF7 form homodimers or heterodimers to regulate type I IFN production (Honda *et al.*, 2006, Andrienas *et al.*, 2018). Preferential induction of *IFNA4*, *IFNA7*, and *IFNA14* expression by IRF7 and induction of *IFNB* and *IFNA1* by IRF3 was reported in humans (Lin *et al.*, 2000). The TLRs differentially regulate the IRFs. TLR3 and TLR4 activate IRF3 to induce IFN- β production while TLR9 and TLR7 activates IFN- β and IFN- α through IRF7 (reviewed by (Colonna, 2007, Uematsu & Akira, 2007). TLR3 and TLR4 induce IFN- β and expression of the interferon stimulated genes (ISGs) through activation of the adaptor protein TRIF. TRIF induces IRF3 phosphorylation through IKK ϵ and TBK1 (Fitzgerald et al., 2003, Doyle *et al.*, 2002). The serine 473 residue of Akt is also a target of TBK1 and contributes to maximal IRF3 activation in response to TLR4 and TLR3 agonists (Joung *et al.*, 2011). IRF7 is activated by myeloid differentiation primary response 88 (MyD88) dependent TLR 7 and TLR9 signalling pathway. The TLR adaptor molecule MyD88 recruits IRAK-4 and IRAK-1, stimulating IRAK-1 phosphorylation by IRAK-4 (Kim *et al.*, 2007). IRAK-1 recruits TRAF6 and the former mediates IRF7 activation via its kinase activity while the latter activates IRF7 via ubiquitination (Uematsu *et al.*, 2005, Kawai *et al.*, 2004).

Following production of the type I IFNs, the IFNs bind to their receptors and induce ISG expression via the IFN-stimulated gene factor 3 (ISGF3) transcriptional complex.

The canonical type I IFN signalling pathway signals via the Janus family kinases (JAKs) Tyk2 and Jak2. The JAKs activate signal transducer and activator of transcription 1 (STAT1) and STAT2 (McNab *et al.*, 2015). The phosphorylated STAT molecules form a heterodimer which translocate to the nucleus. The STAT heterodimer forms a complex with IRF9, forming the transcription factor ISGF3 which facilitates transcription of the ISGs by binding to the IFN-stimulated response element in ISG promoters (Au-Yeung *et al.*, 2013). Activation of the IFNs signalling pathway results in the upregulation of a multitude of ISGs to limit virus replication, including influenza specific genes such as the Mx GTPase family, viperin and IFN-induced transmembrane (IFITM) family members (Killip *et al.*, 2015). Within the adaptive immune response, IFN signalling regulates expansion and differentiation of virus specific CD8 T cells (Kolumam *et al.*, 2005)

1.2 Store operated calcium entry

Calcium ions are essential for numerous functions, affecting nearly every cellular process from cell survival to cell death. Various immune challenges, such as viruses and bacteria, alter calcium ion homeostasis in order to facilitate their replication (Zhou *et al.*, 2009, Asmat *et al.*, 2011). Ca^{2+} plays an essential role in influenza virus replication. Influenza A virus infection induces oscillations in cytosolic Ca^{2+} (Hartshorn *et al.*, 1988, Hartshorn *et al.*, 1990, Ueda *et al.*, 2010, Fujioka *et al.*, 2013). An increase in cytosolic Ca^{2+} regulates influenza virus internalisation via clathrin mediated and independent endocytosis (CME and CIE respectively). The former is dependent on Ca^{2+} regulation of RhoA, Rho-kinase (ROCK), phosphatidylinositol 4-phosphate 5-kinase (PIP5K) and phospholipase C (PLC) pathway and the latter on Ca^{2+} mediated Ras activation. The increase in cytosolic Ca^{2+} was dependent on intracellular Ca^{2+} store mobilisation and an influx of extracellular Ca^{2+} . Influenza A virus-mediated increase in cytosolic Ca^{2+} and Ras activation were inhibited in the presence of the extracellular Ca^{2+} chelator EGTA (Fujioka *et al.*, 2013). Extracellular Ca^{2+} influx was responsible for H5N1 virus induced increases in cytosolic Ca^{2+} , with minimal contribution from intracellular Ca^{2+} sources. The extracellular Ca^{2+} influx was responsible for mediating influenza A virus induced apoptosis (Ueda *et al.*, 2010). Influenza virus induced dysregulation of Ca^{2+} homeostasis impaired store dependent increases in Ca^{2+} in neutrophils. This was proposed to underlie a Ca^{2+} mediated mechanism of influenza virus immune cell deactivation (Hartshorn *et al.*, 1988). Activation of Ca^{2+} dependent host proteins such as the calcium/calmodulin-dependent protein kinase (CaM kinase) II beta may facilitate influenza virus replication, through regulating viral RNA transcription (König *et al.*, 2010). Activation of sialidase activity

of progeny virus is dependent on Ca^{2+} , demonstrating Ca^{2+} is essential for facilitating internalisation and release of influenza virus (Chong *et al.*, 1991, Fujioka *et al.*, 2013).

The cytosolic concentration of Ca^{2+} is tightly regulated. The majority of intracellular Ca^{2+} is located within organelles such as the endoplasmic reticulum (ER), sarcoplasmic reticulum (SR) and the mitochondria (Pozzan *et al.*, 1994, Contreras *et al.*, 2010, Carafoli, 2002). Similar to the extracellular space, the ER contains a considerably higher concentration of calcium ions than the cytosol. Ca^{2+} concentrations range between 1-2mM in the extracellular space in contrast to 100nM Ca^{2+} in the cytosol of resting cells (Meldolesi & Pozzan, 1998, Rizzuto & Pozzan, 2006, Samtleben *et al.*, 2013b). Calcium ions within the ER exist within two pools, those bound to protein chaperones and those considered 'free.' The latter ranges between 60-800 μM when the store is full and below 50 μM when empty (Miyawaki *et al.*, 1997, Michalak *et al.*, 2002, Samtleben *et al.*, 2013a). Ca^{2+} efflux from the ER is predominantly mediated by inositol 1,4,5-trisphosphate receptor (IP_3R) and the ryanodine-receptor (RyR) (Gillespie & Fill, 2008).

Store operated calcium entry (SOCE) mediates Ca^{2+} entry through channels present in the PM in response to reduced Ca^{2+} concentrations within the intracellular calcium stores (Smyth *et al.*, 2010). The Ca^{2+} Release Activated Ca^{2+} (CRAC) current, a Ca^{2+} selective current initiated by depletion of intracellular Ca^{2+} stores labelled I_{CRAC} , was first identified in mast cells (Hoth & Penner, 1992). A highly calcium selective channel in the PM was initially identified as responsible, the calcium release activated calcium (CRAC) channel, which is now known to be a prototypic store operated calcium (SOC) channel (Prakriya & Lewis, 2003). Depletion of Ca^{2+} in the ER lumen is sensed

by stromal interaction molecule (STIM) which subsequently activates the SOC channels to facilitate extracellular Ca^{2+} influx (Liou *et al.*, 2005a, Roos *et al.*, 2005a)

1.2.1 Store operated Ca^{2+} channel

The SOC channels are a tetrameric protein complex of ORAI subunits spanning the PM. They are responsible for transporting Ca^{2+} ions from the extracellular space into the cytosol upon ER Ca^{2+} store depletion (Penna *et al.*, 2008, Feske *et al.*, 2006). The SOC channels are highly selective for Ca^{2+} ions under physiological conditions where extracellular calcium ion concentration is in the range of 1-2mM (Hou *et al.*, 2012). In the presence of extracellular Ca^{2+} , the SOC channels display a 1000 fold preference for Ca^{2+} over monovalent cations. In the absence of extracellular Ca^{2+} they are permeable to monovalent cations such as Na^+ , Li^+ , K^+ , Rb^+ and Cs^+ (Lepple-Wienhues & Cahalan, 1996, Bakowski & Parekh, 2002, Hoth & Penner, 1993).

The importance of the ORAI proteins in mediating SOCE has been well established. A homozygous missense mutation in *ORAI1* was identified in SOCE deficient patients displaying severe combined immune deficiency (SCID) syndrome. Expression of wild type *ORAI1* in SCID T cells restored SOC channel function (Feske *et al.*, 2006). In the same year, two other studies identified ORAI1 (also referred to as CRACM1) as an essential component for SOCE. Knockdown or over-expression of *ORAI1* suppressed and enhanced extracellular Ca^{2+} influx respectively (Vig *et al.*, 2006c, Zhang *et al.*, 2006). Two other homologues of ORAI1 have been identified, ORAI2 and ORAI3. The latter is considered the ‘newest’ of the ORAI subunits from an evolutionary vantage point and is only present in mammals (Shuttleworth, 2012). All of the

homologues are capable of activating store operated calcium entry but each display differing characteristics (DeHaven *et al.*, 2007, Mercer *et al.*, 2006, Feske *et al.*, 2006). The homologues share approximately 80-90% pairwise sequence identity in their transmembrane domains but the N- and C termini varies greatly (Hogan & Rao, 2015, Shuttleworth, 2012). All of the ORAI homologues facilitate SOCE, demonstrated by enhanced SOCE in HEK293 cells overexpressing *ORAI1*, *ORAI2* or *ORAI3*. However, of the three isoforms, *ORAI1* over-expression induced the greatest influx of Ca^{2+} in HEK293 cells and *ORAI3* the least (DeHaven *et al.*, 2007, Lis *et al.*, 2007a). The increased magnitude of SOCE following *ORAI1* expression relative to the other homologues may be attributable to an N terminal P/R rich region in the SOC channel subunit. Over-expression of an *ORAI2* chimera containing the *ORAI1* N terminus strongly induced SOCE. In contrast, only a marginal increase in SOCE was observed following over-expression of an *ORAI1* chimera containing the *ORAI2* N terminus (Takahashi *et al.*, 2007). Of note, the presence of heterotetrameric ORAI SOC channels may account for some discrepancies between over-expression and knock down studies (Takahashi *et al.*, 2007, Lis *et al.*, 2007b). For example, *ORAI1* and *ORAI2* form heteromeric channels in mouse T cells which are as capable as homomeric SOC channels in conducting Ca^{2+} (Vaeth *et al.*, 2017b).

Each of the ORAI subunits is formed of four transmembrane α helices (TM1-TM4), a C terminal M4 extension helix and N terminal M1 extension sequence (Rothberg *et al.*, 2013). Cross linking between the cysteine side chains of amino acids within the M1 helices (at position 88, 95, 102 or 106 in human *ORAI1*) is thought to be responsible for the formation of the tetrameric SOC channel structure (Zhou *et al.*, 2010). The pore of the channel itself is lined by the M1 transmembrane helices

(Rothberg et al., 2013). The extracellular entrance to the pore is negatively charged and facilitates cation accumulation (Dong *et al.*, 2013). A ring of acidic glutamate (E) side chains is responsible for mediating ion selectivity (Yamashita *et al.*, 2007, Yamashita & Prakriya, 2014). The E106 carboxyl groups of ORAI1 TM1 (E81 in ORAI3) have been implicated in the formation of the glutamate ring binding site for Ca^{2+} (Hou et al., 2012, Schindl *et al.*, 2008). In addition, E190 in ORAI1 has also been implicated in ion selectivity (Zhou et al., 2010). Substitution of residues E106 and E190 in ORAI1, located in TM helices 1 and 3 respectively, reduced the specificity of the channel for Ca^{2+} (Prakriya *et al.*, 2006, Yamashita et al., 2007, McNally *et al.*, 2009, Vig *et al.*, 2006a). Residues within the first extracellular loop between the TM regions of ORAI1 (D110/112/114) are involved in facilitating binding of a second Ca^{2+} at the pore mouth. A D110/112 double mutant allowed increased permeation of monovalent Na^+ and K^+ ions, suggesting these residues are also necessary for selecting Ca^{2+} over monovalent cations (Vig *et al.*, 2006b). In divalent free conditions, ORAI2 and ORAI3 displayed increased permeability for Na^+ (Vig et al., 2006b, Lis et al., 2007b). Differences in the extracellular loop may account for the differences in ion selectivity across the homologues. The glutamate selectivity ring is conserved across the ORAI homologues but the aspartate residues located in the extracellular loop of ORAI1 are not conserved. Instead the loop contains residues E110/Q112/Q114 in ORAI2 and E110/D112/E114 in ORAI3 (Lis et al., 2007b). Below the glutamate ring, conserved hydrophobic side chains line the central region of the pore and maintain the channel pore structure (Hou et al., 2012). A hydrophobic gate consisting of residues V102 and F99 has been identified in ORAI1 channels (Yamashita *et al.*, 2017). As the Ca^{2+} ions travel through the pore with its central hydrophobic domain flanked by hydrophilic regions, the ions may undergo a process of dehydration and subsequent

rehydration (Rothberg et al., 2013, Hou et al., 2012). Pore hydration has been proposed to be a driving force behind ion movement through the pore (Hou et al., 2012, Dong et al., 2013). The base of the ORAI1 pore spanning the PM was proposed to consist of a flexible basic region formed of three residues (R83, K87 and R91) from each subunit (Zhang *et al.*, 2011, Rothberg et al., 2013). R91 may form a physical gate at the cytosolic side of the pore to prevent Ca^{2+} entry. Residue G98 may serve as the gating hinge, providing flexibility to the channel to allow channel opening, widening the pore diameter and allowing Ca^{2+} to pass the R91 gate (Zhang et al., 2011). Electrostatic repulsion between the lower basic region and the cation was proposed to facilitate Ca^{2+} exit from the channel (Dong et al., 2013).

1.2.2 Stromal interaction molecule

In response to depleted calcium stores, a sensor of intracellular Ca^{2+} levels within these stores is required. The stromal interaction molecule (STIM) is a conserved modular type 1 transmembrane protein essential for SOCE. STIM monitors ER/SR Ca^{2+} concentration and activates the SOC channels in the PM upon Ca^{2+} store depletion (Liou *et al.*, 2005b, Roos *et al.*, 2005b, Zhang *et al.*, 2005a). There are two mammalian homologues, STIM1 and STIM2, responsible for detecting calcium levels within the endoplasmic reticulum (ER) (Liou et al., 2005b). STIM1 was first identified as a ubiquitously expressed tumour suppressor gene and led to the discovery of a second gene, labelled STIM2 (Williams *et al.*, 2001). Although the STIM homologues are highly similar, displaying >65% sequence similarity, they play different roles in the cell (Stathopoulos *et al.*, 2009, Brandman *et al.*, 2007). STIM2 functions to maintain basal cytosolic and ER Ca^{2+} concentrations within a tight range, whilst STIM1 is

predominantly responsible for SOCE activation (Brandman *et al.*, 2007, Stathopoulos *et al.*, 2009, Gruszczynska-Biegala *et al.*, 2011). STIM2 displays greater sensitivity to ER calcium concentrations than STIM1. This greater sensitivity allows STIM2 activation following smaller alterations in the level of intracellular calcium, permitting tight regulation of calcium influx (Brandman *et al.*, 2007). A splice variant of STIM2, STIM2.1 or STIM2 β , displays an inhibitory role in SOCE activation, opposing STIM1 and STIM2 function. This splice variant contains an additional exon 9, located in the region encoding the domain responsible for interacting with the SOC channel (Miederer *et al.*, 2015, Rana *et al.*, 2015). STIM1 isoforms have also been identified. An alternative STIM1 isoform, STIM1L, is an 115kD protein splice variant derived from alternative splicing at exon 11. STIM1 was ubiquitously expressed while the longer variant, STIM1L, was found only in the skeletal muscle of humans (Horinouchi *et al.*, 2012). Disruption of the STIM1L and SOC channel clusters delayed SOCE activation in myoblasts, suggesting STIM1L plays a vital role in mediating rapid SOCE activation (Darbellay *et al.*, 2011). In murine embryonic fibroblasts (MEFs), STIM1L was not located in preformed clusters and was less efficient at associating with ORAI1 channels, suggesting STIM1L alone was not sufficient for enhancing SOCE. PM lipid composition may account for cell type specific variation in STIM1L function, dictating the ability of the STIM1 isoforms to interact with the PM and ORAI1 channels. Alternatively, additional proteins which enhance STIM1 association with the SOC channel may account for differences observed in STIM1L function in different cell types (Saüc *et al.*, 2015).

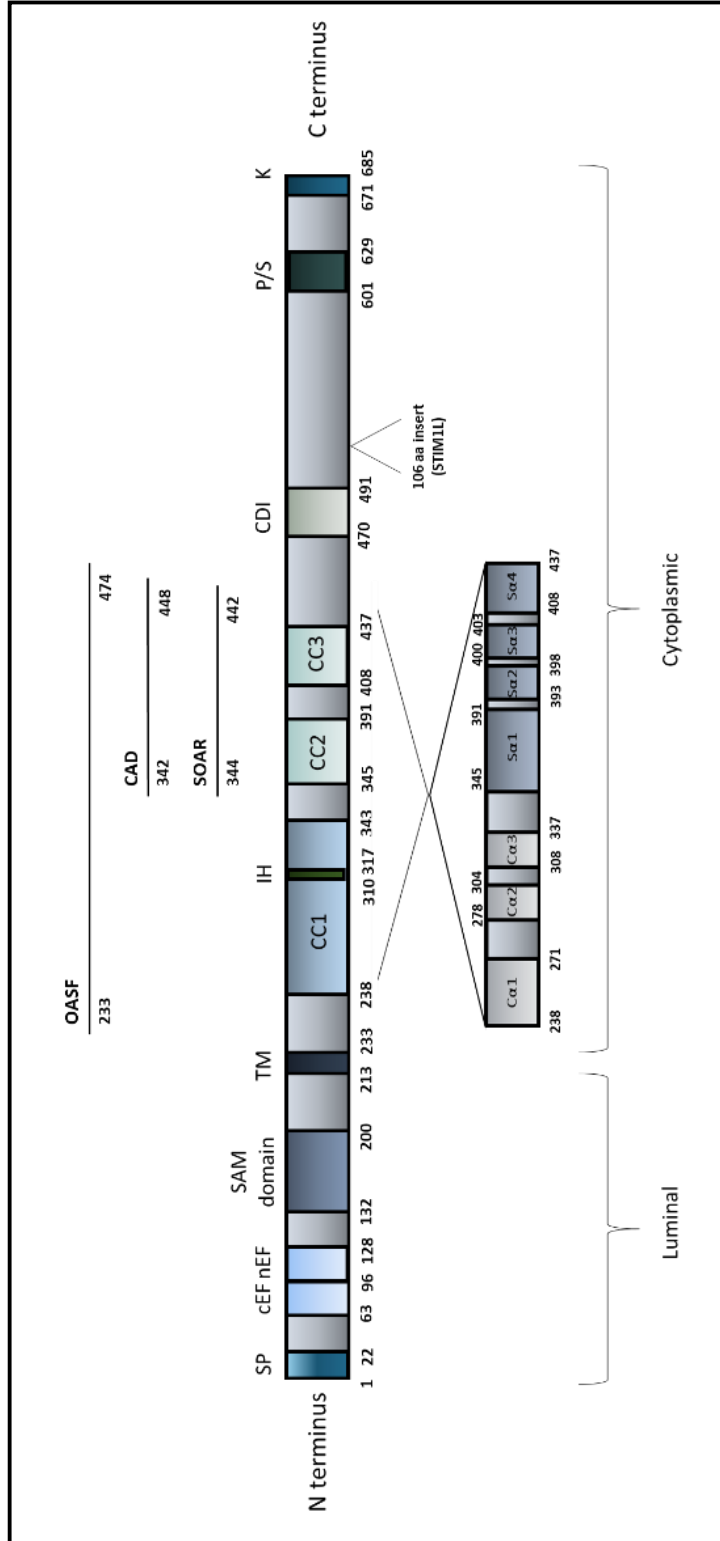


Figure 1-4. STIM1 domain structure. Functional domains indicated by shaded boxes. Amino acid residue labelling for human STIM1.

SP= signal peptide; cEF= canonical EF hand; nEF = non-canonical EF hand; SAM = sterile α motif; TM = transmembrane domain; CC = coiled coil; IH = inhibitory helix; CDI = Ca^{2+} dependent inactivation (CDI) region; P/S = proline/serine rich domain; K = lysine rich poly basic domain. Figure reproduced from (Prakriya & Lewis, 2015, Yu et al., 2013b, Muik et al., 2011, Mullins et al., 2009).

1.2.3 STIM-SOC channel activation

At the resting state when Ca^{2+} stores are full, STIM1 is evenly distributed throughout the ER membrane as a dimer (Zhou *et al.*, 2015, Covington *et al.*, 2010, Penna *et al.*, 2008). The canonical EF hand domain, located adjacent to a sterile alpha motif (SAM) in the luminal N terminal domain of STIM1, is responsible for sensing Ca^{2+} concentration in the ER lumen (Zhang *et al.*, 2005b, Stathopulos *et al.*, 2008). The EF hand motif, first identified in parvalbumin, displays a characteristic helix-loop-helix fold and undergoes a conformational change upon alterations in calcium ion concentration (Moews & Kretsinger, 1975, Huang *et al.*, 2009). STIM1 is held in a stable, inactive conformation mediated by hydrophobic interactions between the canonical EF (cEF) hand and SAM. This interaction requires the contribution of a second ‘hidden’ non canonical EF (nEF) hand motif which, unlike the cEF hand, does not bind to Ca^{2+} . It forms a hydrophobic cleft with the canonical EF hand that interacts with residues in the C terminus of SAM (Stathopulos *et al.*, 2008). The EF hand-SAM interaction binds to a single Ca^{2+} with low affinity and upon Ca^{2+} depletion the EF-SAM interaction is destabilised (Stathopulos *et al.*, 2006). The conformational changes in STIM1 exposes EF-SAM domain hydrophobic surfaces and this facilitates STIM1 oligomerisation (Stathopulos *et al.*, 2006, Covington *et al.*, 2010). The formation of stable, higher order oligomers also requires interactions between the cytoplasmic coiled coils (CCs) of STIM1, in addition to EF-SAM oligomerisation (Fahrner *et al.*, 2014, Zhou *et al.*, 2013). The STIM1 ORAI1 activating region (SOAR), also known as the CRAC activating domain (CAD), is the minimum region of STIM1 required for activating the SOC channel (Park *et al.*, 2009, Yuan *et al.*, 2009). CC1 has been proposed to function as a SOAR/CAD tether to hold STIM1 in

a quiescent state (Zhou et al., 2013). Interactions between CC1 and the CC3 of SOAR have been proposed to mediate STIM1 inhibition. CC1 and CC3 mutations induced the extended, active conformation of the ORAI1 activating small fragment (OASF) of STIM1 (Yu et al., 2013a, Muik et al., 2011). CC1 may hold STIM1 in its quiescent state through association of an inhibitory acidic segment located in the CC1 α helix 3 and a basic region within SOAR (Korzeniowski et al., 2010). Others disagreed with this model, suggesting the CC1 acidic helix was not ideally located to interact with the basic region of SOAR (Yang et al., 2012). Although, their model supports a CC1 inhibitory helix (IH) (spanning residues 310-337 in human STIM1) mediated inhibition of the SOAR dimer (Yang et al., 2012). A later study suggested residues 310-317 (in human STIM1) formed the inhibitory domain and residue Y316 played a key role in maintaining the quiescent STIM1 conformation (Yu et al., 2013b). One model suggested CC1 may interact with CC3 via its α 1 helix. This was supported by constitutive activation of SOC channels by STIM1 mutants lacking CC1 α 1 or STIM1 mutants containing point mutations abolishing CC1 α 1 interaction with CC3 (Fahrner et al., 2014). Ca^{2+} dependent alterations in the luminal domain were proposed to induce conformational changes that disrupt the inhibitory association with SOAR/CAD and allow STIM1 to form its active extended conformation (Zhou et al., 2013). Furthermore, STIM1 may be held in an inactive conformation with the aid of additional proteins (Jha et al., 2013, Srikanth et al., 2010).

STIM1 oligomerisation is a critical step in regulating STIM1 activation. multiple lines of evidence support a sequential process in which STIM1 must first oligomerise in order to form puncta at the ER-PM junctions (Luik *et al.*, 2008, Liou *et al.*, 2007).

Stimulation of STIM1 oligomerisation in the presence of Ca^{2+} was sufficient to induce

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its translocation and activation of SOC channels (Luik et al., 2008). STIM1 must redistribute to the ER- PM junctions to associate with the SOC channels in the PM (Wu et al., 2006). The STIM1 puncta are located 10-25nm below the PM and their formation occurs seconds prior to SOC channel activation (Wu et al., 2006). Amino acid residues 425-671 contain a P/S rich region that functions as a targeting region and mediates STIM1 migration to the ER/PM junctions (Li et al., 2007). The P/S rich regions of STIM1 may allow STIM1 translocation to the ER/PM junction through association with the C terminus of EB1, a microtubule plus end tracking protein, via a SxIP motif located within the STIM1 S/P rich region (Grigoriev et al., 2008, Honnappa et al., 2009). The I/P dipeptide was essential within this motif, as mutation of the dipeptide prevented EB1 binding and microtubule plus end tracking (Honnappa et al., 2009). STIM1/EB1 association is regulated by phosphorylation of STIM1 by ERK1/2. Phosphorylation of residues S575, S608 and S621 induced dissociation of EB1 and allowed SOCE activation (Pozo-Guisado et al., 2013). Additionally, STIM1 translocation may be mediated by interaction between a polybasic motif in the STIM1 C terminus and polyphosphoinositides present in the PM (Liou et al., 2007, Li et al., 2007). Once at the PM, STIM1 associates with the SOC channels in order to facilitate an influx of Ca^{2+} from the extracellular space. The STIM1/SOC channel complex consists of eight STIM1 proteins and the tetrameric ORAI channel. Each STIM1 SOAR dimer associates with one ORAI molecule (Penna et al., 2008, Li et al., 2011, Soboloff et al., 2012).

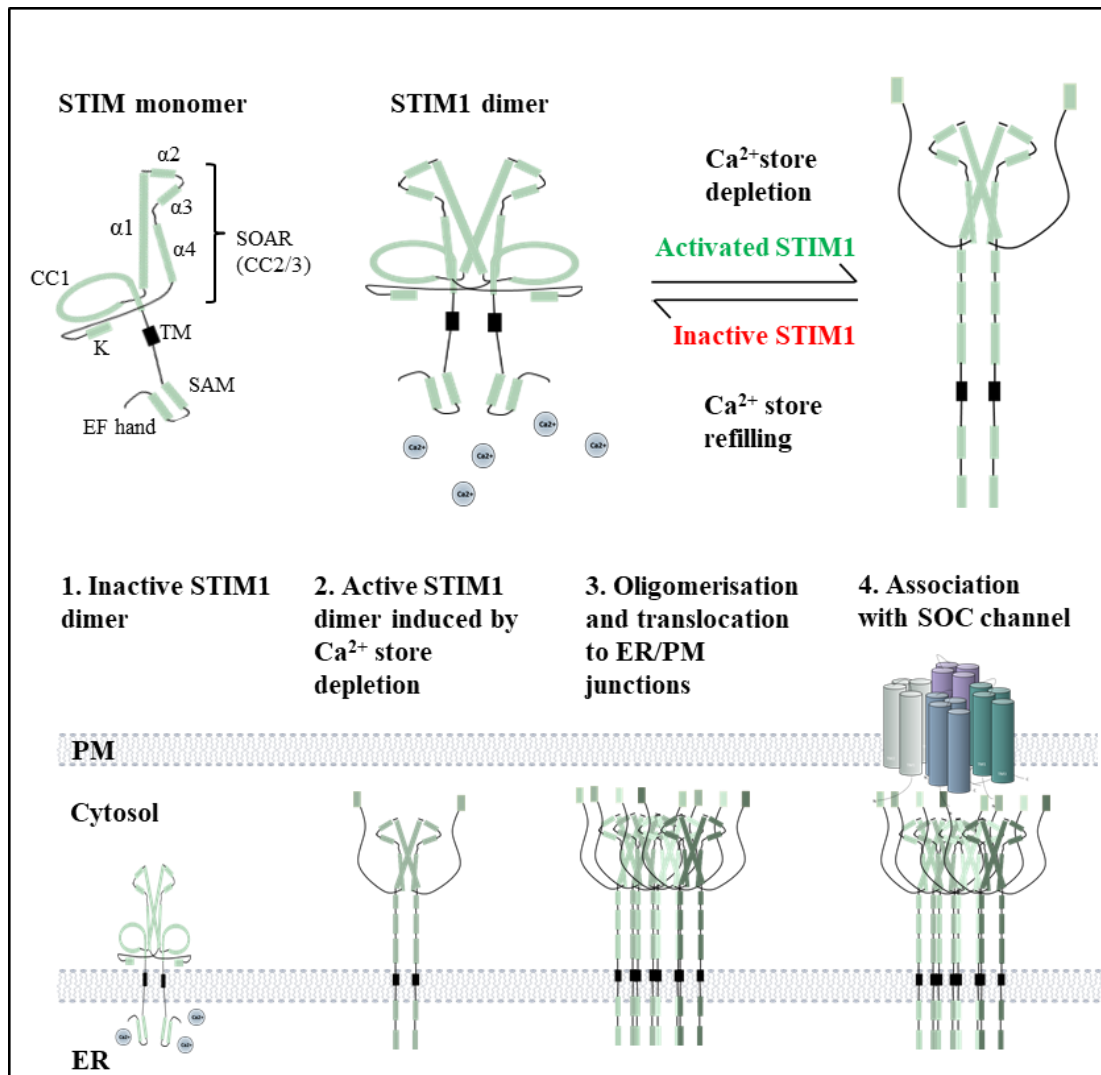


Figure 1-5. STIM1 adopts an extended active conformation following Ca^{2+} store depletion to form puncta with the tetrameric SOC channels in the PM. ER = endoplasmic reticulum; CC = coiled coil; K = lysine rich domain; SAM = sterile α motif; SOAR = STIM1 Orai1 activating region; TM = transmembrane domain. Figure adapted from (Yang et al., 2012, Feske & Prakriya, 2013).

STIM1 binds to the C terminal region in the M4 extension helix of Orai1 via its SOAR/CAD (Park et al., 2009, Yuan et al., 2009, Hou et al., 2012). Within the C

terminus of the ORAI proteins, a putative coiled coil domain was proposed to mediate association with STIM1 (Muik *et al.*, 2008, Hou *et al.*, 2012). Deletion of the ORAI1 C terminus impaired STIM1 mediated activation of the SOC channel (Muik *et al.*, 2008, Li *et al.*, 2007, Zheng *et al.*, 2013). Disruption of the coiled coil, through introduction of point mutations L273S and L276D in human ORAI1, impaired SOCE activation (Muik *et al.*, 2008, Zheng *et al.*, 2013, Navarro-Borelly *et al.*, 2008). Hydrophobic interactions with the C terminal residues L273 and L276 were proposed to underlie STIM1 binding to the ORAI1 subunits. STIM1 binding subsequently induced a conformational change in ORAI1 TM4 at the proline bend (P245 in human ORAI1) which repositioned residues of the hydrophobic gate lining the channel pore (Palty *et al.*, 2015). The C terminal binding domain is shared by all the ORAI homologues, suggesting a shared STIM mediated mechanism of activation (Muik *et al.*, 2008). Acidic residues within the C terminal binding domain were proposed to interact with a region of exposed basic residues within the CC2 of the SOAR/CAD of STIM1 through electrostatic interactions (Calloway *et al.*, 2010, Korzeniowski *et al.*, 2010, Soboloff *et al.*, 2012). However, the electrostatic interactions may be dispensable for channel gating and instead function to stabilise STIM1/ORAI1 binding (Palty *et al.*, 2015). In addition, STIM1 binds to an N terminal binding site of ORAI1. This interaction is not necessary for STIM1 binding to the SOC channel but is required for channel activation. Deletion of residues 1-73 in the ORAI1 N terminus did not impair SOCE. However, the highly conserved N terminal residues 74–90 were essential for SOC channel activation (Li *et al.*, 2007). STIM1 binding to the ORAI1 N terminus was proposed to induce a conformational change in TM1, the transmembrane helix lining the pore, to induce channel opening (Zheng *et al.*, 2013). In summary, STIM1 binding to the N and C terminus was proposed to induce conformational

changes in the ORAI1 TM1 and TM4, disrupting the hydrophobic gate and increasing channel hydration to facilitate Ca^{2+} movement through the channel (Yamashita et al., 2017, Palty et al., 2015).

1.2.4 SOCE regulation

1.2.4.1 Ca^{2+} dependent inactivation

Ca^{2+} dependent inactivation of the SOC channels is essential for preventing excessive influx of extracellular Ca^{2+} (Lee *et al.*, 2009). Two mechanisms exist, fast Ca^{2+} dependent inactivation (FCDI) which acts within milliseconds and a comparatively slower mechanism taking tens of seconds, labelled slow Ca^{2+} dependent inactivation (SCDI) (Fierro & Parekh, 1999). SCID is thought to be the dominant Ca^{2+} dependent inhibitory mechanism (Parekh, 1998). SCID is dependent on a global increase in Ca^{2+} rather than local increases. This was indicated by SCID suppression by high concentrations of EGTA (>10mM) which suppresses a bulk increase in Ca^{2+} but does not reduce the concentration of Ca^{2+} in microdomains (Zweifach & Lewis, 1995b).

In contrast, FCDI was not dependent on Ca^{2+} changes across the whole cell but local, microdomain Ca^{2+} concentrations in close proximity to the Ca^{2+} channel (Zweifach & Lewis, 1995a, Fierro & Parekh, 1999). The dependency of FCDI on local Ca^{2+} was demonstrated by increased suppression of FCDI by exposure to a faster Ca^{2+} chelating agent (BAPTA) than a slower buffer (EGTA) (Fierro & Parekh, 1999, Zweifach & Lewis, 1995a).

The sensitivity of the ORAI proteins to CDI varies. For example, ORAI1 α displayed greater sensitivity to CDI than ORAI1 β (Desai *et al.*, 2015). Furthermore, ORAI1 displayed sensitivity to SCDI while ORAI2 and ORAI3 were sensitive to FCDI (Lis *et al.*, 2007b, Lee *et al.*, 2009). ORAI3 displayed a greater sensitivity to FCDI than ORAI2 (Lis *et al.*, 2007b). The difference between the homologues regarding FCDI may be attributable to three conserved glutamates found in the C termini of ORAI2 and ORAI3 but not ORAI1 (Lee *et al.*, 2009). Transferring the C terminal tail of ORAI2 to ORAI1 conferred FCDI to ORAI1 chimeras. In contrast, transferring the ORAI1 tail to ORAI2 or ORAI3 produced channels lacking FCDI (Lee *et al.*, 2009). The differences between the homologues may allow for regulation by different signalling pathways, thereby enabling tight regulation of calcium influx by multiple pathways (DeHaven *et al.*, 2007).

1.2.4.1.1 Calmodulin

Calmodulin (CaM) is a key regulator of Ca²⁺ dependent inactivation and has been proposed to function as a mediator of SCID and FCID (Singh *et al.*, 2002, Li *et al.*, 2017). CaM functions as a regulator of multiple other channels, from ion channels and glutamate receptors to growth factor receptors (Levitan, 1999, Ehlers *et al.*, 1996, Sanchez-Gonzalez *et al.*, 2010).

In a rat liver cell line, CaM was proposed to contribute to FCDI, supported by a 30% reduction in FCDI following over-expression of a Ca²⁺ insensitive CaM mutant (Litjens *et al.*, 2004b). CaM facilitates rapid inactivation through association with an N terminal calmodulin binding domain (CMBD) of ORAI1 to mediate CDI (Mullins *et al.*, 2009). Hydrophobic interactions are responsible for association of CaM and

ORAI1. The W76 residue within the CMBD may interact with a hydrophobic cleft in C terminal lobe of CaM. Although a later study proposed W76 did not mediate CaM dependent FCDI (Liu *et al.*, 2012b, Mullins *et al.*, 2016). Further analysis suggested the N terminal and C terminal lobes bind to different ORAI1 molecules in order to facilitate CDI (Liu *et al.*, 2012b).. N terminal residues 1-63 of ORAI1 α have also been implicated in mediating FCDI as stronger CDI was observed for the longer ORAI1 α form (Desai *et al.*, 2015, Fukushima *et al.*, 2012).

However, CaM has also been implicated as a facilitator of SCID. The conflict in reports is highlighted by the differences in over-expression of Ca²⁺ insensitive CaM mutants. While FCID was impaired in H4IIE cells overexpressing a Ca²⁺ insensitive CaM mutant, in salivary gland cells SCID was impaired by over-expression of a Ca²⁺ insensitive CaM mutant (Singh *et al.*, 2002, Litjens *et al.*, 2004a). CaM may mediate SCID through interacting with residues adjacent to the ORAI1 binding site of STIM1 in the presence of Ca²⁺, disrupting the interaction between SOAR and ORAI1 and disassembling STIM1 oligomers within the puncta (Li *et al.*, 2017, Bauer *et al.*, 2008).

1.2.4.1.2 SARAF

An additional mediator of SCID has been identified. SARAF, a protein located in the ER and PM, was proposed to facilitate SCID through association with STIM1 (Palty *et al.*, 2012, Albarran *et al.*, 2016b). The inhibitory association of SARAF with the SOAR of STIM1 was regulated by a C terminal inhibitory domain (CTID) within STIM1 (Jha *et al.*, 2013). Deletion of the CTID severely reduced SARAF/SOAR association and diminished SCID (Jha *et al.*, 2013). However, reduced SCID was still possible in the presence of STIM1 mutants lacking domains necessary for SARAF

mediated SCID, resulting in the proposal of an additional SARAF independent SCDI mechanism (Jha et al., 2013).

An alternative model proposed SARAF associated with ORAI1 and STIM1. In agreement with the previous mode of action, SARAF associates with STIM1 under resting conditions and this inhibitory interaction is relieved following store depletion. Over-expression of *SARAF* eradicated TG stimulated STIM1/ORAI1 interaction and silencing *SARAF* enhanced this association. Upon store depletion SARAF was proposed to interact with the C terminus of ORAI1 to facilitate Ca^{2+} entry before associating once more with STIM1 to induce SCDI (Albarran *et al.*, 2016a).

1.2.4.2 CRACR2A

CRAC regulator 2A (CRACR2A) has been identified as a key regulator of SOCE, acting to stabilise the STIM1-ORAI1 complex at the ER-PM junction. Depletion of CRACR2A reduced TG stimulated Ca^{2+} influx and impaired STIM1 and TG mediated ORAI1 cluster formation. In the absence of Ca^{2+} , CRACR2A associated with positive residues within the N terminal domain of ORAI1 and the coiled coil and proline/lysine rich domains of STIM (Srikanth et al., 2010). ORAI K85 has been implicated in channel gating and puncta formation (Srikanth et al., 2010, McNally *et al.*, 2013, Lis *et al.*, 2010). Secondly, K87 has been identified as one of the positively charged amino acids lining the SOC channel pore and contributes to STIM1 binding (Rothberg et al., 2013). Interestingly, K85 and K87 within the N terminus of ORAI1 were essential for CRACR2A association. A double ORAI1 mutant (K85A/K87A) displayed reduced residual binding to CRACR2A and impaired clustering following store depletion (Srikanth et al., 2010).

A long variant of CRACR2A (CRACR2A-L) approximately twice the size of CRACR2A has been identified in endothelial cells, alternative splicing giving rise to a variant containing a C terminal extension which places the protein within the Rab GTPase family (Wilson *et al.*, 2015). Despite sharing the N terminus with CRACR2A, CRACR2A-L is unable to regulate CRAC channels but its sensitivity to Ca^{2+} and GTPase function allows bridging between signalling pathways, such as the Ca^{2+} -NFAT and JNK signalling pathways within T cells (Wilson *et al.*, 2015, Srikanth *et al.*, 2016).

A homologue of CRACR2A, CRACR2B shared the conserved predicted EF hand and coiled coil domains. However, CRACR2A depletion impaired SOCE to a greater extent in Jurkat T cells than CRACR2B. Secondly, co expression of *CRACR2A* and *ORAI1* in murine embryonic fibroblasts enhanced intracellular Ca^{2+} concentration while co expression of *ORAI1* and *CRACR2B* minimally enhanced SOCE. This suggests there is limited functional redundancy between the two homologues (Srikanth *et al.*, 2010).

1.2.4.3 STIMATE

The STIM-activating-enhancer (STIMATE), encoded by *TMEM110*, is an ER membrane protein which functions as a positive regulator of SOCE. STIMATE co-localises with STIM1 at the ER and translocates with STIM1 to the ER-PM junctions upon store depletion (Jing *et al.*, 2015, Quintana *et al.*, 2015). Over-expression of *STIMATE* stimulated puncta formation in the absence of store depletion and enhanced puncta formation and size in the presence of store depletion. STIMATE dependent enhancement of puncta formation was supported by the reduction in the number, size

and rate of formation of STIM1 puncta at the ER-PM junctions in *STIMATE* knockout HEK293 cells (Jing et al., 2015). Knockdown of *STIMATE* impaired SOCE in HeLa and Jurkat T cells, reflected by a reduction in Ca^{2+} dependent NFAT signalling and impaired influx of Ca^{2+} following store depletion (Quintana et al., 2015). Interaction between SOAR and CC1 of STIM1 underpins an intramolecular autoinhibitory mechanism of STIM1, preventing interaction between the ORAI1 and SOAR in the absence of store depletion (Korzeniowski et al., 2010, Muik et al., 2011, Ma *et al.*, 2015). *STIMATE* may enhance puncta formation by relieving this auto-inhibition, interfering with the association between CC1 and SOAR and promoting the formation of an active STIM1 dimer (Jing et al., 2015).

1.2.5 SOCE function

1.2.5.1 Cell proliferation

SOCE mediated Ca^{2+} influx is inhibited during mitosis, indicated by the loss of TG and histamine mediated extracellular Ca^{2+} influx into the cytosol during mitosis (Preston *et al.*, 1991, Volpi & Berlin, 1988). During mitosis, STIM1 is phosphorylated at multiple residues, such as S668 and S486, to suppress its activity (Smyth *et al.*, 2009). SOCE regulates the G1/S transition, supported by inhibition of the cell cycle G1/S transition by a fast Ca^{2+} chelator or SOCE inhibitor, SKF-96365 (Chen *et al.*, 2016).

The ORAI SOC channels and STIM1 have been implicated in a variety of cancers, regulating aberrant cell proliferation and metastasis (Zhang *et al.*, 2014, Ay *et al.*, 2013). Elevated expression of *STIM1* and *ORAI1* was associated with disease advancement and increased mortality rates in gastric cancer patients. Knock down of the SOCE components in gastric cancer cell lines reduced their proliferation and altered cell cycle marker expression (Xia *et al.*, 2016). Over-expression of *ORAI1* was found to mediate proliferation in non-small cell lung carcinoma via the PI3k/AKT/ERK pathway (Zhan *et al.*, 2015). *ORAI3* has also been implicated in non-small cell lung cancer cell proliferation. *ORAI3* knockdown inhibited cell proliferation; arresting the cells in the G0/1 phase and downregulating the expression of several key cyclins and cyclin dependent kinases essential for cell cycle progression (Ay *et al.*, 2013).

1.2.5.2 Differentiation

In addition to regulating cell proliferation, STIM1 and ORAI dependent regulation of differentiation has also been reported. Human myoblast differentiation was dependent on STIM1, ORAI1 and ORAI3. Silencing these components impaired myoblast differentiation while over-expression of *STIM1* and *ORAI1* enhanced myoblast differentiation (Darbellay *et al.*, 2009). STIM1 and ORAI1 are necessary for myoblast hyperpolarisation, a process which triggers myoblast fusion, indicating SOCE is essential for the early stages of differentiation (Entwistle *et al.*, 1988, Liu *et al.*, 1998, Darbellay *et al.*, 2009, Park *et al.*, 2002). SOCE may regulate myoblast differentiation via STIM1 dependent activation of the nuclear factor of activated T cells (NFAT) c3 pathway. Netrin/cdo dependent activation of ERK2 regulates STIM1 activity through phosphorylating residue S575, enhancing STIM1/Cdo association (Lee *et al.*, 2012b). The STIM1/Cdo complex was proposed to subsequently activate the calcineurin/NFATc3 pathway required for initiation of differentiation (Delling *et al.*, 2000, Lee *et al.*, 2012b). SOCE was required for proliferation of undifferentiated epidermal keratinocyte cells and mediated the Ca^{2+} dependent onset of keratinocyte differentiation (Numaga-Tomita & Putney, 2013).

1.2.5.3 Skeletal muscle contraction

Repetitive skeletal muscle contraction depletes Ca^{2+} within the sarcoplasmic reticulum (Kurebayashi & Ogawa, 2001, Gutierrez-Martin *et al.*, 2005). In healthy muscle, SOCE is critical for strenuous exercise, replenishing intracellular Ca^{2+} stores to enable successive contractions and to limit fatigue (Wei-LaPierre *et al.*, 2013, Thornton *et al.*, 61

2011). Extracellular Ca^{2+} influx through the SOC channels is required for maintaining contractile force following repetitive myotube stimulation. Inhibitors of SOCE reduced the high frequency contractile force in skeletal muscle (Thornton et al., 2011). Mice expressing a muscle specific dominant negative *ORAI1* survived into adulthood but displayed increased muscle fatigability. The performance of dnORAI mice following continual exercise decreased significantly. Isolated extensor digitorum longus muscles were unable to maintain contractile force following recurrent stimulation (Wei-LaPierre et al., 2013). In humans, SOCE deficient patients with *STIM1* or *ORAI1* mutations displayed non-progressive myopathy (Feske et al., 2006, Fuchs et al., 2012). Defective SOCE has been implicated in the development of reduced contractile strength of aged skeletal muscle (Thornton et al., 2011, Zhao et al., 2008).

1.2.5.4 Apoptosis

Conflicting reports on ORAI1 and STIM1 dependent regulation of apoptosis have been described. In pancreatic adenocarcinoma cell lines, ORAI1 and STIM1, perform an anti-apoptotic role and knockdown of *ORAI1* and *STIM1* increased cell sensitivity to chemotherapy drugs (Kondratska et al., 2014). Stress induced apoptosis was also mediated by ORAI1 in the mouse hippocampal cell line HT22 in response to oxidative stress. Inhibition of SOCE with pharmacological inhibitor 2-APB or knockdown of *ORAI1* protected HT22 cells from oxidative stress (Henke et al., 2013). In a human prostate cancer cell line, knockdown of *ORAI1* or expression of non-functional ORAI1 mutants conferred resistance to TG induced apoptosis, suggesting a pro-apoptotic role for ORAI1 (Flourakis et al., 2010).

In contrast, *ORAI1* knockdown conferred sensitivity to type I IFN (IFN- α/β) induced apoptosis in T cells (Yue *et al.*, 2012). Secondly, knockdown of *ORAI1* or treatment with SOCE inhibitors (SKF-96365 and DES) in glioma cells enhanced apoptosis, providing evidence of an anti-apoptotic function for SOCE (Liu *et al.*, 2011a).

1.2.6 Studying SOCE: Thapsigargin

Thapsigargin (TG), a sesquiterpene lactone originating from the roots of *Thapsia garganica* is an essential tool for studying the effect of altering Ca^{2+} homeostasis (Patkar *et al.*, 1979, Santarius *et al.*, 1987). Plants within the *Thapsia* genus have been utilised for centuries. A relatively recent example from 1937 revealed resin from the root of *Thapsia* was used as a remedy for rheumatic pains (Patkar *et al.*, 1979, Brøgger Christensen *et al.*, 1984). One of the first properties attributed to this molecule was its ability to release histamine from a variety of cell types, both rat and human, in a calcium dependent manner (Rasmussen *et al.*, 1978, Ali *et al.*, 1985). TG induces an increase in cytosolic Ca^{2+} concentration through preventing ER store refilling and inducing extracellular Ca^{2+} influx via SOCE (Yamamoto *et al.*, 1995, Gericke *et al.*, 1993). TG mediates its effect on cytosolic Ca^{2+} through inhibition of the sarcoplasmic or endoplasmic reticulum Ca^{2+} ATPase (SERCA) family of calcium pumps and preventing Ca^{2+} uptake into a major intracellular Ca^{2+} store (Thastrup, 1990, Thastrup *et al.*, 1990, Lytton *et al.*, 1991). Transmembrane helix 3 (M3) was first identified as a target for TG mediated inhibition of SERCA. Mutation of amino acid Phe 256 in M3 of SERCA conferred resistance to TG mediated inhibition (Yu *et al.*, 1998, Hua & Inesi, 1997). A cavity flanked by the M3, M5 and M7 helices was subsequently proposed to form the TG binding site. Hydrophobic residues and a hydrogen bond between O8 of TG and the NH group of isoleucine 829 facilitates its association with the ATPase (Toyoshima & Nomura, 2002). Within these helices, belonging to M3, M5 and M7 respectively, Phe 256, Ile 765 and Tyr 837 were particularly important for TG binding (Xu *et al.*, 2004). The ability of TG to deplete intracellular calcium ion

stores has made the SERCA inhibitor an invaluable tool for studying store operated calcium entry.

1.3 Hypothesis

Influenza virus replication is inhibited when Ca^{2+} homeostasis in the host cell is disrupted.

1.4 Aims and objectives

The aim of this project was to identify and characterise the antiviral activity of Ca^{2+} mobilisation on host innate immunity in response to influenza A virus infection. In order to achieve this aim, the specific objectives are:

1. To establish the role of Ca^{2+} mobilisation on influenza virus replication, and associated changes in host innate immune processes

For this objective, the effect of intracellular Ca^{2+} mobilisation and extracellular Ca^{2+} influx through activation of store operated calcium entry (SOCE) will be considered. The effect of different extracellular Ca^{2+} concentrations on influenza virus replication will be assessed. The contribution of SOCE to the antiviral response will be explored through chemical activation with non-toxic concentrations of TG, chemical inhibition with BTP-2 and Synta-66, overexpression and knockdown of SOCE components. Antiviral activity will be determined by characterising progeny virus output through focus forming assay (FFA), M gene expression through real time quantitative PCR (RT-qPCR) and viral protein production through Western blotting. Host cellular and innate immune responses will be assessed through RT-qPCR, specifically focussing on the host type I IFNs response and induction of endoplasmic reticulum stress.

2. To characterise the effect of TG priming on the host transcriptome and identify host signalling pathways regulated by TG which could impact on influenza A virus replication

To this end, next generation sequencing of the host transcriptome will be performed. Human bronchial epithelial cells will be primed with non-cytotoxic concentrations of TG and infected with USSR H1N1 influenza virus. Total RNA-sequencing will be used to identify differentially expressed transcripts regulated by TG priming and infection. This will be used to identify key host molecular targets and signalling networks regulated by TG. This will also be used to identify any off target effects associated with possible cytotoxicity.

3. To assess the antiviral activity of additional sesquiterpenes and assess the underlying antiviral mechanisms

In this objective, the antiviral activity of selected sesquiterpenes and sesquiterpene lactones will be assessed. Tracheal epithelial cells will be briefly primed with the compounds and viral output quantified through FFAs. The stage at which virus replication is inhibited will be evaluated by M gene expression through real time quantitative PCR (RT-qPCR), viral protein production through Western blotting and viral nucleoprotein localisation by immunofluorescence confocal microscopy.

2. Chapter 2: General Materials and Methods

2.1 Cell culture media

2.1.1 Proliferation media for porcine myoblasts

Proliferation media (PM) was composed of skeletal muscle cell growth basal medium (Ready-to-use) (PromoCell) supplemented with 10% FCS (Gibco) and 0.2% PlasmocinTM Prophylactic (Invivogen).

2.1.2 Differentiation media for pig myoblasts

UG differentiation media was composed of DMEM-Glutamax (high glucose) (Gibco), 0.4% UltrosorTM G (UG) (Pall Corporation), 0.1% Gentamicin 50mg/ml (Lonza) and 0.2% PlasmocinTM Prophylactic.

2.1.3 NPTr and MDCK culture media

Immortalised newborn pig tracheal (NPTr) cells and Madin-Darby Canine Kidney (MDCK) cells were cultured in DMEM-Glutamax (high glucose), 10% FCS and 100U/ml penicillin and streptomycin (P/S) (Gibco).

2.1.4 PTEC and NHBE cell culture media

Porcine tracheal epithelial cells (PTECs) and normal human bronchial epithelial (NHBE) cells (Lonza) were cultured in airway epithelial cell media (Ready to use) (PromoCell).

2.1.5 Cryopreservation media

Cryopreservation media for NPTr cell, MDCK cell and porcine myoblast storage in liquid N₂ consisted of 40% growth media, 50% FCS and 10% Dimethyl sulfoxide Hybri-MaxTM (DMSO, Sigma-Aldrich). Cryopreservation media for primary epithelial cells was composed of a 1:1 ratio of growth media and freeze media (ProFreeze-CDM Medium, Lonza) supplemented with 15% DMSO.

2.1.6 Infection media

Serum free media for influenza viral infection of NPTr cells, MDCK cells and porcine myoblasts was composed of UltraCULTURE media (Lonza), 100U/ml P/S, 2mM L-glutamine (Gibco) and 250ng/ml L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK) trypsin (Sigma-Aldrich). Infection media (IM) of NHBE cells and PTECs consisted of airway epithelial cell media supplemented with 250ng/ml TPCK trypsin (Sigma-Aldrich).

2.2 Primary Cell Culture

2.2.1 Culture vessel preparation for porcine myoblasts and myotubes

The culture vessels for myoblast and extended myotube culture were coated with a Maxgel:collagen (M:C) solution. Rat tail collagen stock solution (2% rat type 1 collagen (Sigma-Aldrich) in PBS (Gibco)) and Maxgel ECM solution (1% Maxgel ECM (Sigma-Aldrich) in DMEM-Glutamax (high glucose)) were prepared and combined in a 1:1 ratio. The appropriate volume (Table 2-1) was added to the vessel

and left in a sterile environment until completely dry. Once dry, the vessels were washed with PBS and dried again.

Culture Vessel	Volume of M:C Solution
T75	4ml
T25	2ml
Well of 6 well plate	800ul
Well of 12 well plate	400ul
Well of 24 well plate	200ul

Table 2-1. Volume of M:C coating solution required for different tissue culture vessels

2.2.2 Porcine Primary Myoblast Isolation

The isolation protocol was based on previous protocols described (Baquero-Perez *et al.*, 2012, Sebastian *et al.*, 2015). Skeletal muscle samples (*longissimus dorsi* and *psoas*) from 4-6 weeks old commercial Large White cross pigs (~5g) were aseptically removed and washed in 30ml of 1X PBS followed by 30ml of collection medium (DMEM-Glutamax (high glucose), 100U/ml P/S, 2.5µg/mL Fungizone (Gibco)) at room temperature (RT). The muscle samples were washed with PBS and excess connective tissue removed using a sterile scalpel. The sample was thoroughly minced, washed with collection medium and the tissue pelleted by centrifugation at low speed. The tissue pellet was suspended in dissociation medium (collection media

supplemented with 0.06 U/ml pronase (Sigma-Aldrich)) and incubated at 37°C for 1 h, using approximately 10ml for every 1g of sample. The sample was agitated every 10 min. After incubation the sample was triturated using a 25ml pipette. The muscle fragments were allowed to settle before the supernatant was collected, filtered through a 40µm cell strainer (fisher scientific) into a fresh collection tube and centrifuged at 300g for 5 min. The supernatant was removed and the cell pellet re-suspended in 15ml of an equal proportion of collection medium and DMEM-Glutamax (high glucose) containing 10% FCS and 100U/ml P/S. The cells were centrifuged at 300g for 5 min, the supernatant disposed of and the pellet re-suspended in 15ml of PBS. The cells were pelleted a final time, the supernatant disposed of and the pellet re-suspended in 10-12ml of myoblast PM and seeded in a coated T75 flask.

2.2.3 Porcine Tracheal Epithelial Cell Isolation

PTECs were isolated from the tracheal epithelium of 3-4 months old pigs. The trachea was collected and washed in PBS supplemented with 100U/ml P/S and 2.5µg/mL Fungizone. The tracheobronchial mucosae was stripped, minced and incubated overnight at 4°C in DMEM-F12 medium (Lonza) containing 0.06 U/ml pronase. The supernatant was collected, filtered through a cell strainer (40µm) and centrifuged at 150g for 5 min. The supernatant was removed and the cells washed twice in DMEM-Glutamax (high glucose) supplemented with 100U/ml P/S and 2.5µg/mL Fungizone. The collected cells were cultured in airway epithelial cell media.

2.2.4 Cryopreservation

Cells were proliferated to reach over 60% confluence on prepared T75 flasks. If not intended for immediate use the cells were stored in liquid N₂. The culture media was removed from the T75 and the cells were washed with PBS before incubated for 2-3 min at 37⁰C with 1X 0.25% trypsin (Gibco) for MDCK cells, NPTr cells and porcine myoblasts. Once the cells had detached from the T75, 4ml of growth media containing FCS was added to inactivate the trypsin and the suspended cells were transferred to a falcon tube. For the primary epithelial cells, the DetachKit (PromoCell) was used according to the manufacturers recommended subcultivation protocol. The suspended cells were transferred to a bench top centrifuge and centrifuged at 150*g* for 2 min. The supernatant was removed and the cells resuspended in cryopreservation media. For one T75, the cells were resuspended in 3ml of cryopreservation media and 1ml of the suspended cells transferred to a sterile cryovial and stored at -80⁰C before transfer to liquid N₂.

2.2.5 Frozen cell revival

Cells preserved in liquid N₂ were thawed at 37⁰C in a water bath. The suspended cells were transferred to 10ml of the appropriate media and transferred to a T75. The media was replaced 24 h post seeding and subsequent media changes performed every 48 h. Once ~70% confluent, the cells were passaged and seeded into the required culture vessels, using the volumes described in (Table 2-2).

Flask/Plate	Volume
T75	10-12ml
T25	4ml
Well of 6 well plate	2ml
Well of 12 well plate	1ml
Well of 24 well plate	0.5ml
Well of 48 well plate	0.25ml
Well of 96 well plate	0.1ml

Table 2-2 Volume of media required for different tissue culture vessels

The NPTr cells, MDCK cells, PTECs and NHBE cells were incubated at 37°C in 5% CO₂. The myoblasts were incubated at 37°C in 5% CO₂ and 5% O₂.

2.2.6 Cell passage

At 60-70% confluence the cells were passaged. After removal of the PM, the cells were washed with PBS. The cells were incubated at 37°C until detached from the culture surface in 5ml of 1X 0.25% trypsin for MDCK cells, NPTr cells and myoblasts. Addition of 4ml of PM inactivated the trypsin. For the primary epithelial cells, the DetachKit (PromoCell) was used according to the manufacturers recommended subcultivation protocol. The entire volume was transferred to a Falcon tube and centrifuged at 150g for 2 min. The supernatant was removed and the pellet re-suspended in 1ml of PM before transferred to the required volume of media (Table 2-2).

2.2.7 Differentiation of porcine myoblasts

UG differentiation media was used to induce differentiation once the porcine myoblasts reached 90-95% confluence in TC treated 24 well plates with Cell+ growth surface (Sarstedt) and coated with M:C solution. The myoblasts were washed with PBS before the addition of 0.5ml UG DM per well. The plates were incubated in 5% O₂ and 5% CO₂ at 37°C. Myotubes were visible by 5 days. After 5-7 days, 25% of the media was replaced with fresh differentiation media every 2 days.

2.3 Plasmid Preparation

Plasmids (Abcam) sent as bacteria in agar stabs were stored at 5-8°C until processing. Agar (Oxoid) plates were prepared according to the manufacturer's protocol with either 100µg/ml ampicillin (Sigma-Aldrich) or 50µg/ml kanamycin (Sigma-Aldrich), as required. In the presence of a flame, the wire loop was sterilised and the bacteria spread across the plates to allow single, defined colonies to form. The agar plates were incubated at 37°C overnight in a dry incubator. Alternatively, 1µl of plasmid (0.5µg/ml) was transferred to 40µl of competent bacteria and left on ice for 30 min. The bacteria were subjected to heat shock by placing the Eppendorf of bacteria on a heat block at 42°C for 30 seconds. 500µl of SOC outgrowth medium (NEB) was added to the Eppendorf and shaken horizontally at 37°C for 1 h at 225rpm. The bacteria were plated on prepared agar plates at serial dilutions to allow for defined colony formation and incubated at 37°C overnight in a dry incubator.

2.4 Maxiprep

Bacterial broth was prepared, suspending the required volume of nutrient broth powder (Oxoid) in distilled water according to the manufacturer's protocol, and autoclaved. Under sterile conditions, the appropriate antibiotic was added, giving a final concentration of 100µg/ml ampicillin or 50µg/ml kanamycin. Approximately 5ml of broth was aliquoted into each universal tube (UT). Using a sterile toothpick, a single colony was picked and the sterile toothpick placed in the UT. The UTs were shaken at 150rpm overnight at 37°C. After overnight incubation, the 5ml of broth was transferred to an autoclaved conical flask containing 200ml of bacterial broth containing the appropriate antibiotic. The conical flask was sealed and shaken at 225rpm at 37°C overnight. The solution was transferred to a fresh vessel and all vessels equalised in weight using distilled water. The bacterial cells were pelleted through centrifugation at 6000x *g* for 15 min at 4°C. The supernatant was removed and the pellet frozen at -20°C until processing. The plasmid DNA was obtained from the pellet using the EndoFree Plasmid Maxi Kit (Qiagen), following the manufacturer's protocol.

2.5 Cell transfection

At ~60% confluence the cells were transfected using transfection reagent Lipofectamine 2000 (Invitrogen). The plasmids were diluted in Opti-MEM (Gibco) to give the recommended final concentration of DNA per well recommended by the manufacturer's protocol. Lipofectamine2000 was diluted in Opti-MEM, volumes for a 24 well plate described in Table 2-3. The diluted DNA and Lipofectamine reagent

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were thoroughly mixed together, incubated at RT for 10 min and added to the cells. The duration of exposure to the DNA-lipid complex for each cell type described in Table 2-3.

Cell type	LPF2000 (μl)	Duration
NHBE cells	1	6 h
NPTr cells	1.5	‘Overnight’ (>12 h)
Porcine myoblasts	2	‘Overnight’
PTECs	1	6 h

Table 2-3 Volume of Lipofectamine transfection reagent recommended per well of a 24 well plate and duration of exposure to the DNA lipid complex for the corresponding cell type

2.6 Cell viability assay

The cells were primed with the indicated chemicals at the indicated concentrations for 1 h or 30 min as stated, rinsed 3 times with PBS and replaced with fresh media. Cell viability was measured 24 h later using the CellTiter-Glo® Luminescent Cell Viability Assay (Promega) and the Caspase-Glo® 3/7 Assay (Promega). Both assays were performed according to the manufacturer’s protocol.

2.7 Fluo- 8 assay

The cells were cultured until >90% confluent in black Nunc MicroWell 96-well optical-bottom plates with cell culture treated polymer base (Thermo Scientific). The cells were treated as stated and cytosolic calcium concentration measured using the Fluo-8 Calcium Flux Assay Kit (Abcam) according to the manufacturer's instructions. Fluorescence, indicative of cytosolic calcium concentration, was measured using FLUOstar OPTIMA (BMG Labtech).

2.8 Influenza virus infection of cells

2.8.1 Influenza virus subtype

Two subtypes of human influenza A were utilised in this study; USSR H1N1 (A/USSR/77) and pandemic (pdm) H1N1 2009 (A/California/07/2009). Viruses were supplied by the Animal and Plant Health Agency and a master stock virus grown in 10 day old embryonated chicken eggs and stored in liquid N₂ until use. An aliquot of master stock virus was thawed once and used to generate experimental stock virus (stored at -80°C until use) to reduce the likelihood of egg adaptation. Experimental stock virus was freeze thawed a maximum of three times and was not used to amplify virus stock. For each replicate a new aliquot of experimental virus stock was used.

2.8.2 Influenza virus propagation

Embryonated Dekalb white chicken eggs (Henry Stewart & Co) were incubated at 37.5°C with humidity set between 40-50%. At 10 days old, the eggs were inoculated with the respective virus subtype. The seed virus was diluted to the appropriate concentration in PBS supplemented with 2% tryptose phosphate broth (Sigma-Aldrich) and 5% P/S, which had been filtered through a 0.22µm filter (Fisher scientific). Holes were pierced in the shell at the site of inoculation within the allantoic cavity and at the air sac. Using a sterile 25G needle and syringe, 0.1ml of the virus dilution was injected into the allantoic cavity and the holes sealed with hot wax. The eggs were returned to the incubator and 72 h later placed at 4°C for 12- 24 h. The allantoic fluid was harvested, the viruses were aliquoted and stored until use at -80°C. The viral suspensions were prepared at a dilution of 1.0 multiplicity of infection (MOI). The dilution at which approximately 100% of confluent MDCK cells in a 96 well plate (Fisher Scientific) were positively stained for viral NP protein was classified as 1.0 MOI. The diluted virus stock was stored at -80°C until use and were freeze thawed a maximum of three times. All experiments were performed using the virus stock suspended in allantoic fluid to avoid cell adaptation of the influenza A viruses.

2.8.3 Influenza virus infection of cells

At ~80-90% confluence, the cells were infected with USSR H1N1 virus (A/USSR/77) or pdm H1N1 2009 (A/California/07/2009) at the appropriate MOI for each cell type (Table 2-4).

Cell type	MOI
A549	1.0
NHBE cells	1.0
NPTr cells	0.5
Porcine myoblasts	2.0

Table 2-4 Multiplicity of infection used for each cell type

The appropriate volume of stock virus was suspended in IM and the cells exposed for 2 h to the suspended virus. After 2 h the IM was removed and the cells washed twice with PBS. The media was replaced with fresh IM and the cells incubated at 37°C in 5% CO₂ for 24 h. Following the 24 h incubation the IM was removed and centrifuged at maximum speed for 2 min. The supernatant was removed and stored at -80°C until required. The cells were treated with the appropriate lysis buffer for RNA extraction and the lysed cells collected with their respective pellets. RNA extraction was performed immediately or the cell lysate stored at -80°C until required.

2.8.4 Quantification of influenza virus released into the supernatant from transfected cells

The focus forming assay (FFA) was used to quantify progeny virus through immunostaining techniques and was not dependent on cell lysis. MDCK cells were seeded in 96 well plates. Once 100% confluent, the cells were exposed to the appropriate volume of infected supernatant for 2 h and incubated at 37°C in 5% CO₂.

The volume of infected supernatant used varied according to progeny viral output and the minimum representative volume, determined through serial dilutions, was used to quantify virus titer. After the 2 h incubation, the media was removed and the cells washed twice with PBS. 100µl of infection media was added to each well and the cells incubated for 4 h. After 4 h, the infection media was removed and the wells washed with 1X Tris buffered saline (TBS) (Tris base (Fisher Scientific) in distilled water), using 100µl per well of a 96 well plate. Acetone: methanol (Fisher scientific) (1:1) was added to the wells for 10 min at RT, using 40µl per well of a 96 well plate. The fixed cells were washed thrice with 1X TBS to remove the acetone: methanol and left at 5-8°C in 1X TBS until staining.

2.8.5 Immunostaining

Immunostaining of the infected MDCK cells was performed using the ImmPRESS HRP Anti-Mouse IgG (Peroxidase) Polymer Detection Kit (Vector Laboratories) and the Liquid DAB+ Substrate Chromogen System (Dako). The 1X TBS was removed and 40µl of ImmPRESS 2.5% normal goat serum block buffer added to each well and left for 20 min at RT. This was removed and the cells washed thrice with 1X TBS. The cells were incubated for 1 h at RT in 40µl of primary antibody, Anti- Influenza-A NP ab20343 produced in mouse (abcam), diluted 1/8000 in 1X TBS. Following the incubation period, the antibody was removed and the cells washed thrice with TBS. 40µl of undiluted ImmPRESS™ (Peroxidase) Polymer Anti-Mouse IgG Reagent was added to each well and left at RT for 45 min. One drop of DAB+chromogen (Dako) was diluted in every 1ml of the DAB substrate buffer until appropriate final volume was obtained. After washing thoroughly 4 times with 1X TBS to reduce background

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staining, 40µl of the prepared chromogen mix was added to the wells and left to develop for approximately 5 min until colour developed. The cells were washed a final 3 times in TBS before imaged with an inverted light microscope at 100X magnification. Focal forming units per ml (ffu/ml) of supernatant was calculated using the following formula

$$\frac{30 \times (\text{average infected cells per field})}{\mu\text{l of infectious supernatant}}$$

2.9 Quantification of gene expression by RT-PCR

2.9.1 RNA extraction

RNA extraction was performed using the RNeasy Plus Mini Kit (Qiagen) for the NPTr cells, porcine myoblasts and NHBE cells, following the recommended protocol. RNA extraction from porcine myotubes and post-natal tissue samples was performed using the RNeasy fibrous tissue minikit (Qiagen) following the recommended protocol with an additional sonication step. Following cell lysis by Qiagen's RLT buffer containing the recommended volume of β- Mercaptoethanol (Sigma-Aldrich), the reaction mix was sonicated for 30 seconds with a Bioruptor UCD 200 Sonicator (Diagenode).

For each reaction, two wells of a 24 well plate were lysed and treated as one sample or one well of a 12 well plate. RNA concentration was measured using nanodrop8000

2.2.0.

2.9.2 cDNA reverse transcription

For cDNA conversion, 0.5µg of RNA was suspended in DPEC treated H₂O to make a final volume of 11.5µl for each reaction. Using the SuperScript™ III First-Strand Synthesis System (Invitrogen) the isolated RNA was converted cDNA. For each reaction 1µl of 10mM dNTP, 1µl of random hexamers (50ng/µl), 4µl of first strand buffer, 1µl 0.1M DTT, 1µl RnaseOUT (40U/µl) and 0.5µl of SuperScript III reverse transcriptase (200U/µl) was used. The mix was incubated at 50°C for 50 min and subsequently at 85°C for 5 min. The cDNA was stored at -20°C until use.

2.9.3 Quantitative real time PCR (qRT-PCR)

The expression levels of numerous genes were quantified using SYBR green based quantitative real time polymerase chain reaction (qRT-PCR). A LightCycler® 96 Real-Time PCR System (Roche) was used for qPCR using the QuantiNova SYBR Green PCR Kit (Qiagen). For each well of a white LightCycler 480 multiwell 96 well plate (Roche) the PCR reaction was set up (Table 2-5), with three technical replicates for each sample.

SYBR green	
qPCR master mix components	Volume
1X master mix	10µl
Forward primer (1:10 dilution of 100pmol/µl stock)	1µl
Reverse primer (1:10 dilution of 100pmol/µl stock)	1µl
DPEC treated water	3µl
Template cDNA (1:100 dilution)	5µl

Table 2-5 Components and corresponding volumes for a single reaction of SYBR Green dependent qPCR.

The cycle parameters for qPCR using SYBR green are described in Table 2-6.

Step		Temperature (°C)	Time (s)	Cycle
Initial denature		95	300	1
3 step amplification	Denature	95	10	45
	Annealing	60	10	
	Extention	72	10	
Melting	Denature	95	5	1
	Annealing	60	60	
	Extension	97	1	
Cooling		4	Hold	1

Table 2-6 Cycle parameters for qPCR using SYBR green.

2.9.4 Normalisation of qRT-PCR

18S rRNA was used as the house keeping gene for the normalisation of target gene expression. The $\Delta\Delta C_q$ method was utilised, where the 18s C_q was subtracted from the C_q -value of the target gene in the corresponding sample. The control C_q -value of the samples compared was subtracted from the C_q -values of the other samples. This value (x) was used to calculate the relative expression level, using the formula; $2^{-(x)}$.

2.10 Western Blotting

Protein lysate was collected using the radioimmunoprecipitation (RIPA) lysis buffer (Santa Cruz), prepared according to the manufacturer's instructions. Each sample, consisting of one or two wells of a 12 well plate according to cell density, was treated with 112µl of RIPA buffer and incubated for 15 min at 2-5°C, tapping the plate to redistribute the buffer within the wells every 5 min. A cell scraper was used to detach any cells remaining within the well. The protein lysate was transferred to an Eppendorf and centrifuged at 12 000 rpm for 15 min at 4°C.

For protein quantification, a standard curve using serial dilutions of 50, 25, 12.5, 6.25 and 3.125ng/µl of bovine serum albumin standard (Thermo Scientific) was generated, diluting 2mg/ml BSA stock in distilled water containing 1% RIPA buffer. 5µl of protein sample was diluted in 500µl of distilled water. Protein Assay Dye Reagent Concentrate (bio-rad) was diluted ¼ and 100µl added to each well of a 96 well plate. An equal volume of the standards, samples and blank was added to the protein assay dye reagent and mixed thoroughly. Absorbance at 595nm was measured on a plate reader and protein concentration quantified relative to the standard curve.

For each well 5, 10 or 20µg of protein, determined according to relative abundance of proteins of interest, was diluted in 3.75µl pre-warmed NuPAGE LDS sample buffer 4X (Invitrogen) and 1.5µl NuPAGE reducing agent 10X (Invitrogen) and made up to 15µl with distilled water. The samples were incubated for 10 min at 70°C and briefly spun down. 40ml of NuPAGE™ MOPS SDS Running Buffer 20X (Invitrogen) was diluted in 800ml distilled water. 500µl of NuPage antioxidant (Invitrogen) was added

to 200ml of the prepared running buffer. The 1.0 mm NuPAGE™ 4-12% Bis-Tris Protein Gel (Invitrogen) was prepared by flushing the 12 wells with 10ml of 1X running buffer once the comb was removed and placed in the running tank. The 200ml of running buffer and antioxidant was poured into the inner chamber and the remaining, unaltered 1X running buffer poured into the outer chamber. 15µl of the prepared sample was added per well of the protein gel, while 7µl of SeeBlue™ Pre-stained Protein Standard (Invitrogen) was run in one lane. Gels were run at 200V and 115mA for 50 min.

The Trans-Blot SD semi-dry transfer cell (bio-rad) was used for protein transfer onto a PVDF membrane. 1 sheet of Whatman 3MM Chr chromatography paper (GE Healthcare Whatman), cut to the size of the gel, was soaked in anode buffer I (0.3M Tris, 10% methanol and adjusted to pH 10.4), 2 sheets soaked in anode buffer II (25mM Tris, 10% methanol and adjusted to pH 10.4) and 3 sheets soaked in cathode buffer I (25mM Tris, 40mM glycine, 10% methanol and adjusted to pH 9.4). The PVDF membrane (Thermo Scientific) was first rehydrated in methanol for 15 seconds, washed in water for 5 min and subsequently immersed in anode buffer II. After 50 min, the protein gel was removed from its plastic frame and immersed in cathode buffer I for 5 min. The Trans-Blot SD semi-dry transfer cell was cleaned with distilled water and the filter paper stacked, first the sheet immersed in anode I buffer, the 2 sheets immersed in anode II buffer and then the PVDF membrane. The gel was placed onto the PVDF membrane and the final 3 sheets of cathode buffer I soaked blotting paper placed atop the gel. Gently, air bubbles were removed and the transfer unit assembled. The unit was run at 10V and 70mA for 1 h. The unit was disassembled and the corner of the PVDF membrane marked to record the order of the proteins. The PVDF membrane was twice washed in 1X TBST (0.5M Tris, 1.5M NaCl, 1% Tween

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20 and adjusted to pH 7.4) for 5 min and blocked at RT by incubation in blocking buffer (5% milk (Marvel) in 1X TBST) for 1 h on a bench top rocker at approximately 30rpm. After washing for 5 min in 1X TBST, the membrane was incubated with the appropriate concentration of primary antibody (Table 2-7), diluted in blocking buffer at 4⁰C overnight. The membrane was incubated for 5 min 3 times in 1X TBST before incubation with the appropriate HRP-linked secondary antibody (Table 2-7) for 1 h. The membrane was again washed for 5 min 3 times in 1X TBST. Excess TBST was blotted off. 500µl of each solution of the Amersham™ ECL™ Prime (GE healthcare) kit was thoroughly mixed together and pipetted onto the membrane. After a 2-3 min incubation, the membrane was transferred to a layer of saran wrap and sealed within. Protein bands were detected using the ChemiDoc MP Imaging System (bio-rad) according to the manufacturer's instructions. ImageJ was used to quantify the protein bands and β-actin used as the house keeping gene for normalisation.

Primary antibodies				
	Predicted size	Origin	Dilution	Supplier
Beta-actin	42 kDa	Mouse	1:10000	Sigma
NP	56 kDa	Rabbit	1:3000	Pierce
M1	27 kDa	Goat	1:2000	Abcam
Secondary antibodies				
		Origin	Dilution	Supplier
anti-goat IgG-HRP		Mouse	1:2000	Santa Cruz
Anti-mouse IgG, HRP-linked Antibody		Horse	1:2000	Cell signalling technology
Anti-rabbit IgG, HRP-linked Antibody		Goat	1:2000	Cell signalling technology

Table 2-7 Primary and secondary antibodies used for western blot

2.11 Immunofluorescence confocal microscopy

The samples were washed twice with PBS and fixed in fresh 4% paraformaldehyde (VWR) in PBS (Sigma Aldrich), adjusted to pH 7.4, for 20 min at RT. The cells were permeabilised with 0.1% TRITON X-100 (Sigma-Aldrich) for 5 min at 4⁰C. After washing with PBS, the samples were incubated in PBS supplemented with 3% BSA (Sigma-Aldrich) and 1% glycine (Sigma-Aldrich) for 30 min in a humidity chamber at RT, washed twice with PBS (5 min each time) and incubated under the same

conditions in 10% serum from the secondary antibody donor, diluted in PBS. The samples were incubated with the primary antibody, diluted according to the manufacturer's recommendation in 10% serum in PBS, overnight at 4°C. The samples were washed with PBS and the secondary antibody, diluted according to the manufacturer's recommendation in 10% serum in PBS, was added for 1 h at RT in a humidity chamber. The secondary antibody was removed and the cells thoroughly washed with PBS. After immunostaining for all of the proteins of interest, the cells were washed thoroughly with PBS. The PBS was removed and ProLong™ Gold Antifade Mountant with DAPI (Invitrogen) was added to each well at a sufficient volume to cover the base of the well and incubated at RT for 24 h. The NHBE cells were subsequently stored at 4°C until imaging. The cells were imaged with a Zeiss 710 confocal microscope in oil immersion with a 40x/1.3 objective.

2.12 Statistical analysis

GraphPad Prism (GraphPad Software) was used for statistical analysis. Experiments were performed with a minimum of three replicates and the standard deviation calculated and displayed on the graphs. The distribution of the data was determined with the Shapiro-Wilk normality test and the appropriate statistical test chosen according to distribution of the data. The corresponding statistical test utilised was described in the figure legends.

An unpaired t-test, which uses the mean to determine the likelihood the groups belonged to the same population, was used to compare a single treatment to the control. Where indicated, a one way ANOVA was used to compare multiple treatments to the control and a two-way ANOVA was used to determine the effect of

two factors on the treatment/response being analysed. The control group was indicated in each figure legend.

P-values <0.05 considered significant. * indicated significance, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

Statistical analysis and analysis of data quality of next generation sequencing data described in Section 4.2.5.2.

3. Chapter 3: Store operated Ca^{2+} entry is a potent host innate immune pathway against influenza A virus

Influenza A virus infection presents a major threat to public health. The emergence of drug resistance to the available antivirals is a problem which may be overcome by the development of host directed therapy. Here activation of a host Ca^{2+} signalling pathway, store operated calcium entry (SOCE), facilitates a potent innate immune response is reported. Activation of SOCE, through brief exposure to non-toxic levels of thapsigargin (TG), dramatically elevated activation of innate immune signalling pathways and reduced influenza A virus production from tracheal epithelial cells and muscle cells. SOCE is a key regulator of innate immunity in response to influenza A virus infection and we have demonstrated here that it is amenable to the development of novel therapeutics which target the host response to inhibit influenza A virus replication.

3.1 Introduction

The function of SOCE in host cells during influenza A virus infection was unclear as conflicting roles for SOCE have been reported. Pro-viral functions have been attributed to SOCE. Epstein–Barr virus (EBV) altered Ca^{2+} homeostasis through its latent membrane protein-1 (LMP-1), which increased expression of *ORAI1* and promoted Ca^{2+} entry through the SOC channels in B cells (Dellis *et al.*, 2011). Hepatitis B virus increased cytosolic Ca^{2+} to facilitate virus replication through HBx protein dependent activation of SOC channels in primary rat and human hepatocytes (Casciano *et al.*, 2017, Casciano & Bouchard, 2018). Inhibition of the SERCA pumps by TG stimulated HIV production in latently infected ACH-2 T cell line (Papp & Byrn, 1995). Finally, rotavirus nonstructural protein 4 (NSP4) mediated depletion of the intracellular Ca^{2+} stores and STIM1 activation was essential for rotavirus replication (Hyser *et al.*, 2013).

However, the alteration in Ca^{2+} homeostasis may be utilised by the host cell to orchestrate an immune response. Despite its pro-viral functions, extracellular Ca^{2+} influx through SOCE mediates the immune response at multiple levels. The role of SOCE in cells of the adaptive immune response has been well described. SOCE deficient patients, displaying mutations in *ORAI1* or *STIM1*, suffered from severe recurrent infections of viral, bacterial and fungal pathogens, highlighting the importance of this pathway in mediating an immune response to a variety of challenges (Feske *et al.*, 2006, Picard *et al.*, 2009). SOCE regulates the development and activity of T cell populations. Regulatory T cell development and function was dependent on SOCE, indicated by a dramatic reduction of regulatory T cells in mice with T cells lacking STIM1 and STIM2 (Oh-hora *et al.*, 2008). Effector CD8^+ T cell development

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and function in response to lymphocytic choriomeningitis virus infection was also dependent on STIM1 and STIM2. Furthermore, SOCE dependent regulation of CD40L expression in CD4⁺ T cells was essential for memory CD8⁺ T cell responses (Shaw *et al.*, 2014). CD8⁺ T cells and natural killer cell degranulation and subsequent target cell lysis was dependent on ORAI1 and STIM1 (Maul-Pavicic *et al.*, 2011). Impaired cytokine production and proliferation was observed in ORAI1 and ORAI1/ORAI2 deficient mouse T cells (Vaeth *et al.*, 2017b, McCarl *et al.*, 2009). Natural killer cell cytokine (TNF- α and IFN- γ) and chemokine (macrophage inflammatory protein-1 β) production was impaired in the absence of ORAI1 (Maul-Pavicic *et al.*, 2011).

SOCE regulates immune cell activity through activation of Ca²⁺ sensitive transcription factors, such as nuclear factor of activated T cells (NFAT) (Vaeth & Feske, 2018). T cells lacking STIM1 displayed reduced production of cytokines IL-2, IFN- γ and IL-4, due to impaired nuclear localisation of NFAT (Oh-hora *et al.*, 2008). Ca²⁺ dependent activation of the transcription factor myocyte enhancer factor 2 (MEF2) in T cells has been described (Youn *et al.*, 1999), while SOCE dependent activation of MEF2 was observed in human myoblasts (Darbellay *et al.*, 2009). T cell receptor antigen binding induced Ca²⁺ mobilisation and activation of NF- κ B are dependent on STIM1/ORAI1 mediated extracellular Ca²⁺ entry (Liu *et al.*, 2016b). SOCE protected T cells from type I IFN induced apoptosis via Ca²⁺ dependent activation of NF- κ B. Inhibition of SOCE via overexpression of a dominant negative form of *ORAI1*, significantly enhanced IFN- α induced apoptosis of Jurkat T cells and CD4⁺ T cell line CEM391 (Yue *et al.*, 2012). STIM1 and STIM2 were also implicated in the production of virus neutralising antibodies (Shaw *et al.*, 2014, Vaeth *et al.*, 2016). ER Ca²⁺ store depletion and SOCE have been implicated in B cell receptor (BCR) mediated increase in

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cytosolic Ca^{2+} (Baba *et al.*, 2014). BCR mediated B cell proliferation was inhibited in mice lacking STIM1 and STIM2, while B cell development was unaffected (Matsumoto *et al.*, 2011). Anti-inflammatory IL-10 production was impaired in B cells lacking the STIM homologues due to defective NFAT activation (Matsumoto *et al.*, 2011).

Components of the innate immune system are also regulated by SOCE. STIM1 mediated Ca^{2+} influx regulated high affinity IgE receptor FcεRI-mediated mast cell activation. Mast cells lacking STIM1 displayed reduced NF-κB and NFAT activation in response to FcεRI stimulation (Baba *et al.*, 2007). STIM2 was essential for TNF-α, IL-10 and IFN-γ production in mouse neutrophils (Clemens *et al.*, 2017). STIM2 and ORAI1 were necessary for IL-6 and TNF-α production in mouse spinal astrocytes in response to lipopolysaccharide (LPS) (Gao *et al.*, 2016). Activation of the NLR Family Pyrin Domain Containing 3 (NLRP3) inflammasome is dependent on Ca^{2+} mobilisation from the ER (Murakami *et al.*, 2012, Lee *et al.*, 2012a). Extracellular Ca^{2+} alone can also activate the NLRP3 inflammasome via activation of G protein-coupled receptor family C group 6 member A, subsequently resulting in an IP_3 mediated increase of cytosolic Ca^{2+} (Rossol *et al.*, 2012). Furthermore, 2-APB, at a concentration sufficient to inhibit SOCE, inhibited NLRP3 inflammasome dependent IL-1β and caspase-1 processing (Murakami *et al.*, 2012). Extracellular Ca^{2+} influx, induced by agonists for TLR4, TLR3 and TLR9, regulated macrophage cytokine and chemokine production (Tang *et al.*, 2015). An extracellular Ca^{2+} chelator (EGTA) and knockdown of *STIM1* impaired activation of TLR signalling pathway in macrophages in response to TLR agonists LPS, oly (I:C) and CpG ODN (Tang *et al.*, 2015). In RAW 264.7 macrophages, LPS mediated IRF3 activation was dependent on phospholipase C γ2 induced intracellular Ca^{2+} mobilisation (Chiang *et al.*, 2012). Increased

extracellular Ca^{2+} induced IL-1 β , IL-1 α and IL-6 and TNF production in monocytes, supporting extracellular Ca^{2+} dependent activation of the pro-inflammatory response (Rossol et al., 2012).

Ca^{2+} clearly has a dual role in orchestrating pro-viral and antiviral host signalling responses. This is reflected in the conflicting evidence presented for SOCE as a target for virus replication and its essential role in regulating the immune response. The potential of calcium modulators in inhibiting viral replication through targeting host cell pathways has already been identified (Marois et al., 2014). Therefore, the aims of this chapter are to (i) establish the pro- or antiviral activity of SOCE activation via pharmacological agonists and overexpression of SOCE components and (ii) establish the mechanisms behind SOCE dependent regulation of influenza A virus replication.

3.2 Materials and Methods

3.2.1 Transfection

3.2.1.1 Overexpression studies

Transfection of NPTr cells, myoblasts and NHBE cells at 70% confluence was performed as described in Section 2.5, with the indicated plasmids (Table 4-1). Overexpression of YFP (Elden *et al.*, 2010) served as the negative control for ORAI1 YFP (Prakriya *et al.*, 2006) and pEX-SP-YFP-STIM1(23-685) (Liou *et al.*, 2005a) to account for the effect of transfection and the YFP tag on the host immune response. The pBK-CMV plasmid functioned as the negative control plasmid for the ORAI2 and ORAI3 plasmids (Gwack *et al.*, 2007). mCherry2-N1 functioned as the negative control for p-mCherry-C1-hCRACR2A-isoform-a (Srikanth *et al.*, 2016) and STIMATE-mCherry (Jing *et al.*, 2015). NPTr cells were stably transfected with mCherry2-N1, STIMATE-mCherry or p-mCherry-C1-hCRACR2A-isoform-a; cells expressing the plasmids were selected in proliferation media supplemented with 2mg/ml G418 over several passages.

Plasmid	Plasmid #	Supplier
pcDNA3.2 YFP	84910	pcDNA3.2 YFP was a gift from Aaron Gitler (Addgene plasmid # 84910)
pEX-SP-YFP-STIM1(23-685)	18857	pEX-SP-YFP-STIM1(23-685) was a gift from Tobias Meyer (Addgene plasmid # 18857)
ORAI1 YFP	19756	ORAI1 YFP was a gift from Anjana Rao (Addgene plasmid # 19756)
pBK-CMV		Agilent
pcDNA3.1 ORAI2	16369	pcDNA3.1 ORAI2 was a gift from Anjana Rao (Addgene plasmid # 16369)
pcDNA3.1 ORAI3	16370	pcDNA3.1 ORAI3 was a gift from Anjana Rao (Addgene plasmid # 16370)
mCherry2-N1	54517	mCherry2-N1 was a gift from Michael Davidson (Addgene plasmid # 54517)
p-mCherry-C1-hCRACR2A-isoform-a	79593	p-mCherry-C1-hCRACR2A-isoform-a was a gift from Yousang Gwack (Addgene plasmid # 79593)
STIMATE-mCherry	70676	STIMATE-mCherry was a gift from Yubin Zhou (Addgene plasmid # 70676)

Table 3-1. Plasmids used for cell transfection

3.2.1.2 siRNA mediated knock down

NHBE cells at 70% confluence in 12-well plates were transfected with the indicated siRNA. NHBE cells were transfected with predesigned Silencer® Select siRNA (Ambion) against *ORAI1* (siRNA ID s228396) or *STIM1* (siRNA ID s531229) and the Silencer™ Select Negative Control No. 1 siRNA (Invitrogen) was used as a negative control. For each well, 2µl Lipofectamine RNAiMAX transfection reagent (Invitrogen) was diluted in 50µl Opti-MEM (Gibco) and the 10µM siRNA work stock was diluted in 50µl Opti-MEM (Gibco) to give 10pmol/ml of siRNA per well. The diluted siRNA was added to the diluted Lipofectamine RNAiMAX (Invitrogen) at a 1:1 ratio and incubated for 5 min at RT. The cells in each well were incubated with 100µl of siRNA-lipid complex for 6 h before washing with PBS and placed in fresh proliferation media. Subsequent infection of transfected cells with influenza A virus took place 48 h post transfection.

3.2.2 Cell viability

3.2.2.1 Extracellular calcium

NPTr cells and 12 day porcine myotubes were cultured in 96-well plates until near confluence in culture conditions described in Section 2.2. The media were replaced with equivalent media containing 100mg/L, 200mg/L or 300mg/L CaCl₂ for 24 h. DMEM-Glutamax (high glucose) contained 200mg/L CaCl₂. Media containing 100mg/L CaCl₂ was composed of a 1:1 ratio of DMEM-Glutamax (high glucose) and

DMEM (high glucose, no glutamine, no calcium) (Gibco). DMEM-Glutamax (high glucose) was supplemented with CaCl₂ (Sigma-Aldrich) to give a final concentration of 300mg/L CaCl₂. Cell viability was measured after 24 h of incubation using the CellTiter-Glo® Luminescent Cell Viability Assay according to the manufacturer's instructions.

3.2.2.2 Compounds priming

NPTr cells were primed with 0.25µM, 0.5µM, 1µM or 2µM TG; 0.0005µM, 0.005µM, 0.05µM, 0.5µM or 5µM CPA; 0.5µg/ml, 1µg/ml or 5µg/ml tunicamycin; 2.5nM, 10nM, 50nM, 150nM BTP2 or 0.05µM, 0.2µM, 1µM, 3µM or 30µM Synta66. Porcine myoblasts were primed with 0.25µM, 0.5µM or 1µM TG. NHBE cells were primed with 0.001µM, 0.005µM or 0.01µM TG; 10nM, 50nM or 150nM BTP2; 1µM, 3µM or 30µM Synta66. The cells were primed with the indicated compounds for 30 min. Post-priming, the cells were washed with PBS and placed in the appropriate culture media for 24 h. Cell viability was determined using the CellTiter-Glo® Luminescent Cell Viability Assay (Progenia) or the Caspase-Glo® 3/7 Assay (Promega) according to the manufacturer's instructions.

3.2.3 Fluo-8 assay

NPTr cells, myoblasts, PTECs and NHBE cells were cultured until near confluence in 96-well black Nunc MicroWell 96-well optical-bottom plates with cell culture-treated polymer base (Thermo Fisher Scientific). Culture media were replaced with DMEM-

Glutamax (high glucose) supplemented with 1% FCS and the cells were loaded with Fluo-8 from the Fluo-8 Calcium Flux Assay Kit (Abcam) and incubated for 1 h at RT. The media and loading dye was removed and the cells washed with PBS. (i) Cells were treated with the indicated concentrations of TG in high glucose DMEM without calcium (Gibco) or high glucose DMEM containing 200mg/L CaCl_2 . The cells were then incubated for 10 min before fluorescence was measured. (ii) Fluo-8 loaded NPTr cells were incubated in 1 μM , 10 μM or 20 μM CPA for 30 mins before fluorescence was recorded. (iii) NPTr cells stably transfected with mCherry2-N1, STIMATE-mCherry or p-mCherry-C1-hCRACR2A-isoform-a were treated with 0.5 μM TG for 10 min before fluorescence was measured. (iv) NPTr cells, PTECs, porcine myoblasts and NHBE cells were cultured until near confluence, loaded with Fluo-8 dye as previously described and infected with 1.0 MOI USSR H1N1 (A/USSR/77) for 10 mins in the appropriate infection media (IM) before fluorescence was measured. Fluorescence measured using a FLUOstar OPTIMA (BMG Labtech).

3.2.4 Influenza A virus infection

3.2.4.1 Influenza virus infection in differing extracellular CaCl_2 concentrations

Serum free infection media with CaCl_2 concentrations of 100mg/L, 200mg/L or 300mg/L were prepared. DMEM-Glutamax (high glucose) contained 200mg/L CaCl_2 . IM containing 100mg/L CaCl_2 was composed of a 1:1 ratio of DMEM-Glutamax (high glucose) and DMEM, high glucose, no glutamine, no calcium (Gibco). DMEM-Glutamax (high glucose) was supplemented with CaCl_2 (Sigma-Aldrich) to give an

IM with a final concentration of 300mg/L CaCl₂. Each IM was supplemented with P/S, L-glutamine and TPCK trypsin to give a final concentration of 100U/ml, 2mM and 250ng/ml, respectively. NPTr cells and 12 day differentiated myotubes, cultured as described in Section 2.2, were infected with USSR H1N1 virus (A/USSR/77) at 0.5 MOI and 2.0 MOI, respectively. The cells remained in IM of differing CaCl₂ concentrations for the duration of the 24 h infection. Virus titration based on 6 h focus forming assay was performed on MDCK cells.

3.2.4.2 Influenza virus infection of transfected cells

Transfected cells were infected with USSR H1N1 (A/USSR/77) or pandemic H1N1 (A/California/07/2009) virus 48 h post transfection. NPTr cells, porcine myoblasts and NHBE cells were infected for 24 h with the indicated H1N1 influenza A virus strain at 0.5 MOI, 2.0 MOI and 1.0 MOI, respectively.

3.2.4.3 Influenza virus infection of primed cells

NPTr cells were primed with 0.5μM and 1μM TG; 0.005μM, 0.05μM and 0.5μM CPA; 0.5μg/ml and 1μg/ml tunicamycin; 150nM BTP2 or 0.05μM; 5μM Synta66. Porcine myoblasts were primed with 0.25μM and 0.5μM TG. NHBE cells were primed with 0.001μM and 0.005μM TG, 150nM BTP2 and 5μM Synta66. Cells were incubated with the specified compounds for 30 min or 1 h as indicated. The cells were then washed with PBS and infected with USSR H1N1 (A/USSR/77) or pandemic H1N1 (A/California/07/2009) at the indicated MOI for 24 h.

Spun supernatant was collected for quantification of viral titre as described in Section 2.8 and total RNA was extracted and processed as described in Section 2.9 for quantification of gene expression by RT-PCR.

3.2.5 RT-qPCR

Porcine post-natal tissues (lung, spleen, tracheal epithelium, *longissimus dorsi* and *psoas*) were isolated from 3 commercial Large White-cross 3 year old sows. The tissue samples were collected and washed in PBS before processing. Approximately 0.2g of sample was minced and total RNA was isolated using the RNeasy fibrous tissue Mini Kit (Qiagen) according to the manufacturer's instructions. Total RNA collected was collected from cultured cells using the RNeasy Plus Mini Kit. Gene expression quantified as described in Section 2.9, cycling conditions and volumes are described in Section 2.9.3. USSR H1N1 virus *M* gene expression analysed using primers (F) 5' AGATGAGCCTTCTAACCGAGGTCG 3' and (R) 5' TGCAAAAACATCTTCAAG TCTCTG (Eurofins). Pandemic H1N1 *M* gene expression analysed using primers (F) 5' AGATGAGTCTTCTAACCGAGGTCG 3' and (R) 5' TGCAAAGACACTTTCCAGTCTCTG 3' (Eurofins). Expression of human *RIG-I*, *OAS1*, *IFN-β1*, *DDIT3*, *HSPA5*, *HSP90B1*, *ATP2C1*, *ATP2C2*, *GCP60*, *WIPI49*, *ORAI1*, *ORAI2*, *ORAI3*, *STIM1* and *STIM2* was determined using predesigned KSPQ12012 KiCqStart primer pairs (Sigma-Aldrich). Pig *DDIT3*, *HSPA5* and *HSP90B1* expression was determined using predesigned KSPQ12012 KiCqStart primer pairs (Sigma-Aldrich). Additional primers for porcine genes in Table 4-2 were designed using PrimerExpress 3.0.1 based on publicly available sequence data. RT-qPCR relative fold expression was normalised to 18s rRNA, using universal primers

(F) 5' ACGGCTACCACATCCAAGGA 3', (R) 5' CCAATTACAGGGCCTCGAAA 3') (Sigma-Aldrich).

Gene	Primer	Sequence (5'-3')
RIG-I	F	CCCTGGTTTAGGGACGATGAG
	R	AACAGGAACTGGAGAAAAAGTGATG
OAS1	F	CAAACCCAACCCGGAAATCT
	R	TTTCCCTCGGGACTTGCA
ORAI1	F	CAGAGCTACTCCGAGGTGATGA
	R	AGTAGAGCTTGCGCCAAGACA
ORAI2	F	GGACCTCTGTCTGACTTGCTT
	R	GCAGTGACGCTGACATTCTTCT
ORAI3	F	AGGGCTTGTATTTGTGGTCTTTG
	R	TGTGGCGGTCTGTCTTGTGT
STIM1	F	GGATCTCAGAGGGATTGAC
	R	TTGGAAGGCATGGCACTGA
STIM2	F	GCCTCACCTCTTCCCTCTATTCT
	R	CTAAATCATCAACTCCTCCAGCAA

Table 3-2. Primer sequences for SOCE and type I IFN dependent genes.

Total RNA was extracted from the supernatant of NPTr cells primed with 0.5μM TG, and NHBE cells primed with 0.01μM TG for 30 min. The primed cells were subsequently infected with USSR H1N1 (A/USSR/77) virus at 0.5 MOI and 1.0 MOI,

respectively. Supernatants were collected 24 h later and viral RNA isolated using the QIAamp Viral RNA Mini Kit (QIAGEN) according to the manufacturer's instructions. The extracted RNA was converted to cDNA, described in Section 2.9, with the volume of random primers reduced from 1µl to 0.25µl per reaction. Viral M gene expression was assessed through RT-qPCR, using primers (F) 5' AGATGAGCCTTCTAACCGAGGTCG 3' and (R) 5' TGCAAAAACATCTTCAAG TCTCTG (Eurofins). M gene expression detected in supernatants was not normalised and was dependent on progeny virus release. The normalisation step was removed from the comparative Ct method used to calculate relative fold gene expression.

3.2.6 Western Blotting

NPTr cells were infected with USSR H1N1 (A/USSR/77) at 0.5 MOI for 24 h in IM with CaCl₂ concentrations of 100mg/L, 200mg/L and 300mg/L. NPTr cells were primed with 0.5µM TG for 30 mins before infection with USSR H1N1 or pandemic H1N1 (A/California/07/2009) virus at 0.5 MOI for 24 h. Porcine myoblasts were primed with 0.5µM TG for 30 mins before infection with USSR H1N1 or pandemic H1N1 virus at 2.0 MOI for 24 h. NHBE cells were primed with 0.005µM for 30 mins and subsequently infected with USSR H1N1 or pandemic H1N1 virus at 1.0 MOI for 24 h. NPTr cells, porcine myoblasts and NHBE cells were transiently transfected with the indicated plasmid, and two days post-transfection were infected with USSR H1N1 or pandemic H1N1 virus at the indicated MOI for 24 h. After the 24 h infection, the protein lysates were collected and processed as described in Section 2.11. β-actin, NP and M1 protein were detected and the protein bands quantified by densitometry using ImageJ. Viral proteins were normalised to β-actin.

3.2.7 Transmission electron microscopy

NPTr cells or A549 cells were seeded in 24 well plates (Starstedt) on tissue culture treated, 13mm diameter Thermo Scientific™ Nunc™ Thermanox™ coverslips (Fisher scientific). At near confluence, the cells were treated with TG for 30 min at the appropriate concentration, washed with PBS and subsequently infected with USSR H1N1 virus (A/USSR/77) at 0.5 and 1.0 MOI respectively. Twelve h post infection, the samples were fixed. The cells were initially fixed within the wells in 3% glutaraldehyde in 0.1M sodium cacodylate buffer (pH 7.4) for 1 h, in a large enough volume to cover the cell monolayer. The cells were washed with 0.1M cacodylate buffer and stored at 4⁰C in fresh buffer until subsequent processing. The samples were post fixed in 1% aqueous osmium tetroxide for 30 min and washed for 5 min in distilled water. The samples were then dehydrated in a series of ethanol solutions; twice immersed for 5 min in 50% ethanol, followed by 70% ethanol, 90% ethanol and finally thrice immersed in 100% ethanol for 10 min. The coverslips were transferred to a fresh container and twice immersed in 100% propylene oxide for 5 min. The samples were then infiltrated in epoxy resin in a series of resin: propylene oxide solutions. The samples were submerged for 30 min in a 1:3 mix of resin: propylene oxide, 1 h in a 1:1 mix of resin: propylene oxide and immersed in pure resin for 3 h, the pure resin replaced each h. The coverslips were transferred to a BEEM capsule (TAAB laboratories) and covered with fresh pure resin, cells facing upward and in contact with the resin. The resin was polymerised in an embedding oven at 60⁰C for 48 h. The polymerised resin was removed from the capsule and the Thermanox coverslip removed. The resin block was sectioned and ultrathin sections collected using a diamond knife on a Leica UC6 ultramicrotome, at 80nm from each block. The

ultrathin sections were mounted on 200 mesh copper grids (EM Resolutions Ltd) and stained in Uranylless (TAAB laboratories) and lead citrate (TAAB laboratories), according to the manufacturer's protocol. The copper grids were visualised on a Tecnai T12 Biotwin TEM, which runs at an accelerating voltage of 110kV and images captured using a Mega View SIS camera for analysis.

3.2.8 Immunofluorescence confocal microscopy

NHBE cells were cultured in an eight well μ -slide (ibidi) and at 70% confluence primed with 0.01 μ M TG or DMSO for 30 min. The cells were washed with PBS and infected with USSR H1N1 (A/USSR/77) virus at 1.0 MOI for 6 h. After 6 h, the samples were processed according to Section 2.11, using the indicated antibodies (Table 4-3). The cells were imaged with a Zeiss 710 confocal microscope in oil immersion with a 40x/1.3 objective.

1^o Antibody	Host species	Working concentration	Supplier
Anti-beta actin antibody	Rabbit	1µg/ml	Abcam
Anti-Influenza A Virus nucleoprotein antibody [AA5H]	Mouse	6.48µg/ml	Abcam
Anti-Influenza A H1N1 hemagglutinin antibody [C102 (IV.C102)]	Mouse	1µg/ml	Abcam
2^o Antibody	Working concentration		Supplier
Anti-Rabbit IgG H&L (Alexa Fluor® 488)	4µg/ml		Abcam
Anti-Mouse IgG H&L (Alexa Fluor® 647)	4µg/ml		Abcam

Table 3-3. Primary and secondary antibodies and their corresponding working concentrations used for confocal microscopy in human NHBE cells infected with USSR H1N1 virus.

3.3 Results

3.3.1 Reduced extracellular calcium corresponded with increased progeny virus output

A simplistic approach to determine the effect of extracellular Ca^{2+} on the innate immune response was to alter the availability of extracellular Ca^{2+} upon influenza A virus infection. Ca^{2+} concentration ($[\text{Ca}^{2+}]$) in the infection media (IM) was adjusted to give the indicated concentrations. Due to the adverse effect of altering extracellular calcium ions on cell viability, only NPTr cells and 12 day differentiated porcine myotubes were suitable for study. Altering extracellular Ca^{2+} had no effect on NPTr cell and 12 day differentiated myotubes viability (Figure 3-1A and 3-2A). Porcine myoblasts and NHBE cells were unable to survive for 24 h in IM with altered $[\text{Ca}^{2+}]$ without significant reductions in cell viability (data not shown). A non-toxic increase in extracellular Ca^{2+} corresponded with decreased progeny virus output from both NPTr cells and 12 day differentiated myotubes (Figure 3-1B and 3-2B). Viral M-gene expression was only significantly reduced at 300mg/L CaCl_2 in NPTr cells (Figure 3-1C) and was unaffected in porcine myotubes (Figure 3-2C). This suggests extracellular Ca^{2+} regulates the immune response and may mediate a post-transcriptional dependent antiviral mechanism.

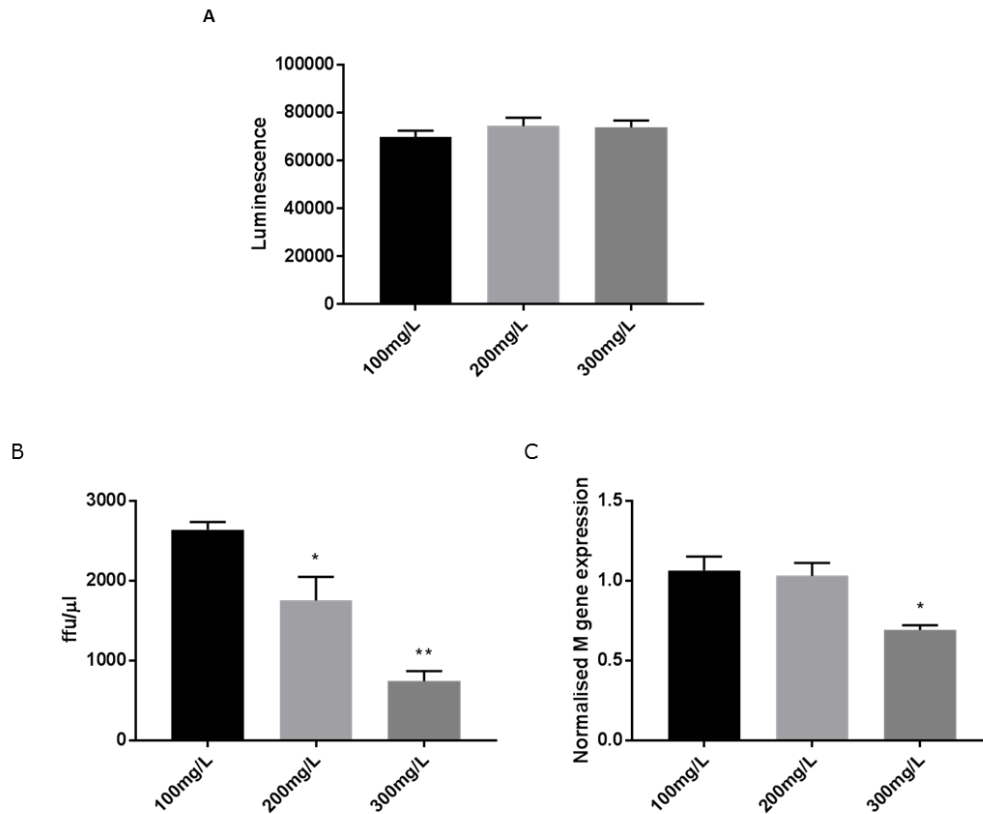


Figure 3-1. Increased extracellular calcium corresponds with reduced viral output from NPTr cells. NPTr cells were infected with USSR H1N1 at 0.5MOI for 24 h in indicated extracellular concentrations. NPTr cells were cultured in 200mg/L CaCl₂ until the 24 h infection. (A) Cell viability assessed with CellTiter-Glo® Luminescent Cell Viability Assay of uninfected NPTr cells cultured for 24 h in indicated CaCl₂ concentrations. (B) Spun supernatants were used to infect MDCK cells for 6 h in focus forming assays (FFAs). (C) Total RNA was extracted from each sample for viral M gene expression normalised to 18S rRNA. Significance determined by one way ANOVA, relative to 100mg/L CaCl₂.

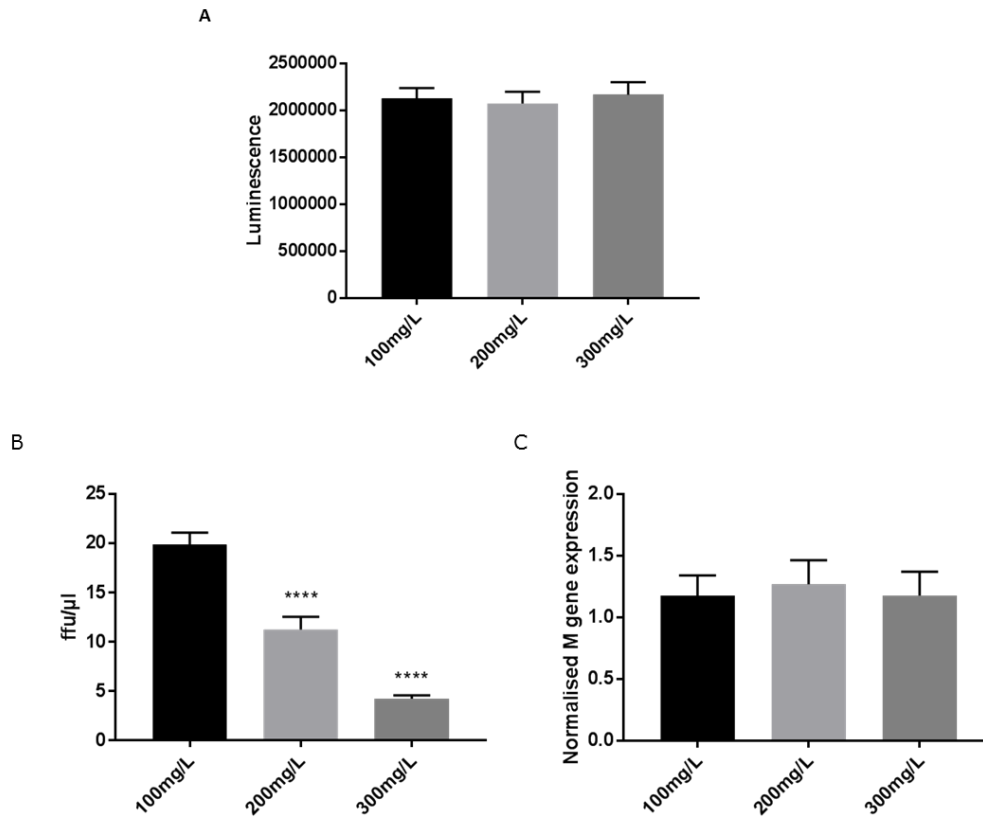


Figure 3-2. Increased extracellular calcium corresponded with reduced viral output from porcine myotubes. 12 day differentiated porcine myotubes cultured in UG DM, 25% media change performed every 2 days. (A) Cell viability of uninfected myotubes cultured in indicated CaCl₂ concentrations for 24 h assessed with CellTiter-Glo® Luminescent Cell Viability Assay. Porcine myotubes were infected with USSR H1N1 virus at 2.0 MOI for 24 h at the indicated calcium concentrations. (B) Spun supernatants were used to infect MDCK cells for 6 h in focus forming assays (FFAs). (C) Total RNA was extracted from each sample for viral M gene expression, normalised to 18S rRNA. Significance determined by one way ANOVA, relative to 100mg/L CaCl₂.

In NPTr cells infected with USSR H1N1, a significant reduction in M1 protein at higher $[Ca^{2+}]$ was observed, although this was not mirrored by viral NP protein levels (Figure 3-3).

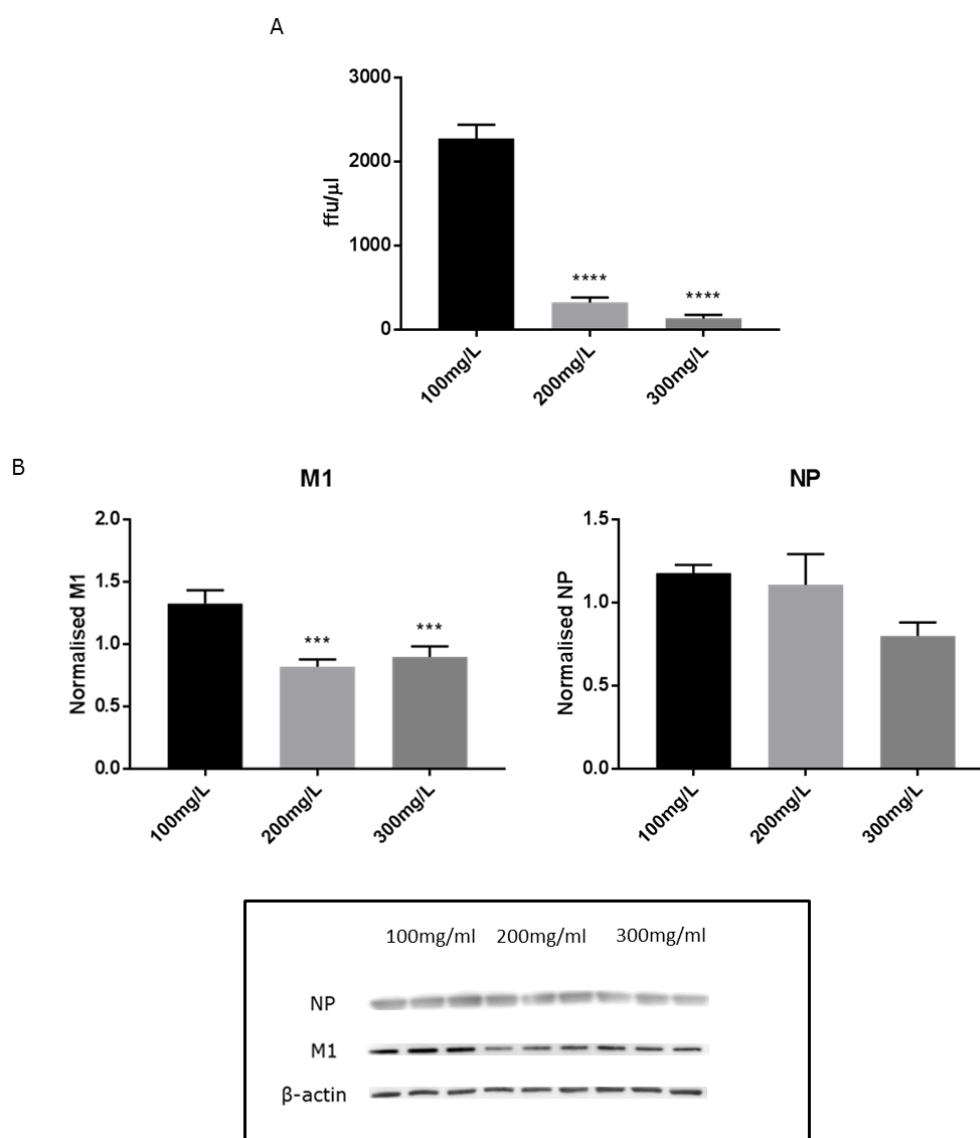


Figure 3-3. Virus protein concentrations were differentially affected in the presence of increased extracellular Ca^{2+} . NPTr cells were infected with USSR H1N1 at 0.5MOI for 24 h in the indicated extracellular $CaCl_2$ concentrations. (A) Spun supernatants were used to infect MDCK cells for 6 h in focus forming assays (FFAs). (B,) Viral

nucleoprotein and M1 protein detected via western blot and normalised to β -actin. Significance determined by one way ANOVA. Significance relative to 100mg/L CaCl_2 unless otherwise indicated.

3.3.2 Influenza A virus infection did not induce an increase in cytosolic Ca^{2+}

Elevation of cytosolic Ca^{2+} following influenza A virus infection has been reported (Fujioka et al., 2013, Ueda et al., 2010). Infection with USSR H1N1 virus did not induce a significant increase in cytosolic Ca^{2+} in any of the cell types described (Figure 3-4). This may be attributed to pathogenicity of the influenza virus, with highly pathogenic influenza virus inducing excessive extracellular Ca^{2+} influx and subsequently inducing cell death (Ueda et al., 2010). Infection with low pathogenic influenza A viruses may not induce a similar increase in cytosolic $[\text{Ca}^{2+}]$.

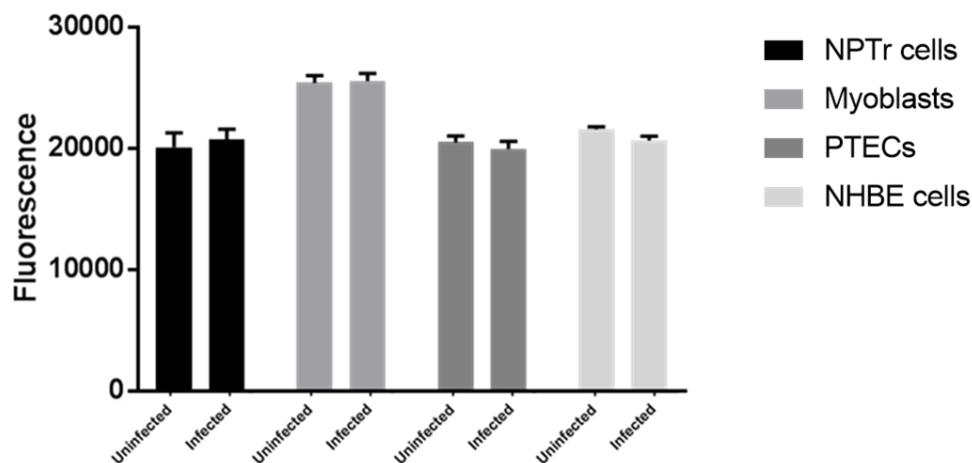


Figure 3-4. H1N1 influenza A virus infection did not induce an influx of extracellular Ca^{2+} into the cytosol. Fluo-8 fluorescence intensity measured 10 min post infection with USSR H1N1 at 1.0 MOI. Significance determined by unpaired t-test within indicated cell types.

3.3.3 Brief TG priming reduces progeny virus output in tracheal epithelial and muscle cells of pig and human origin

The well established role of TG as a small molecule inhibitor of SERCA and agonist of SOCE, made it an ideal candidate for assessing the antiviral activity of SOCE. Brief exposure of non-toxic doses of TG immediately prior to infection with USSR H1N1 virus or pandemic H1N1 virus, was sufficient to significantly reduce progeny virus production from NPTTr cells (Figure 3-5), porcine myoblasts (Figure 3-6) and NHBE cells (Figure 3-7). The effect of TG was consistent across cell lines (NPTTr cells) and primary cells (primary myoblasts and NHBE cells), across different cell types (tracheal epithelial cells and primary myoblasts) and across species (pig and human).

Although TG priming consistently resulted in a greater reduction in virus output in epithelial cells than muscle cells, it potently inhibited influenza virus replication in all cell types. At 0.5 μ M, TG priming consistently reduced viral titre by ~90% in epithelial cells and an average reduction in viral titre of ~60% in porcine myoblasts. In addition, as non-toxic doses of TG were used, indicated by ATP production (CellTiter-Glo assay) and caspase3/7 activity (Caspase-Glo 3/7 assay), reductions in progeny virus were not attributable to TG cytotoxicity.

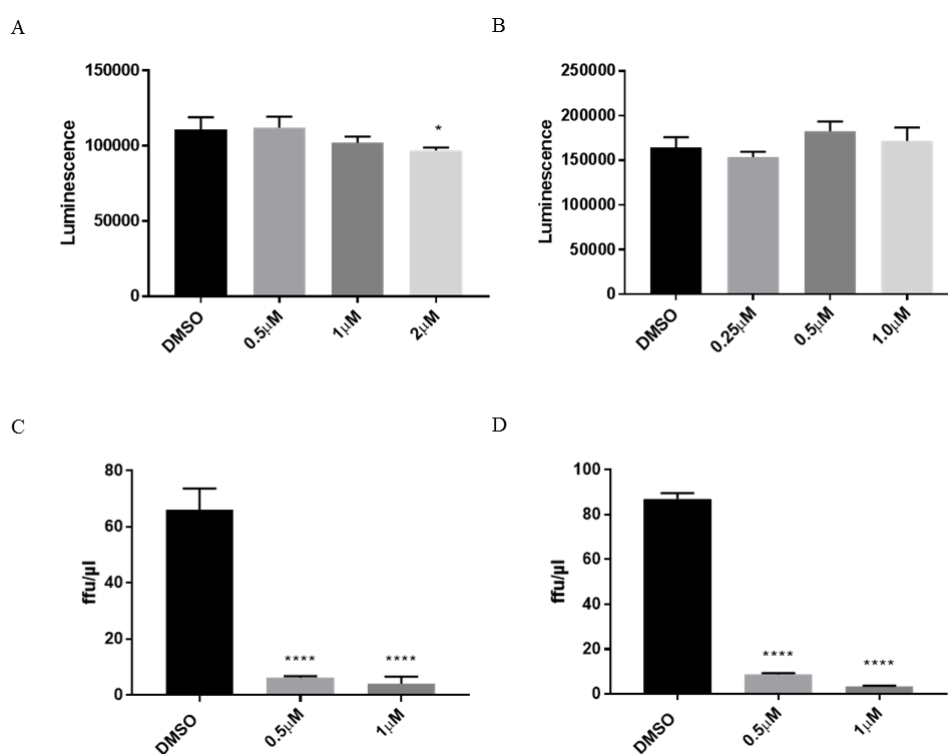


Figure 3-5. Transient priming of NPTr cells with non-toxic concentrations of TG reduced progeny virus production. (A, B) Incubation with 1 μ M TG for 1 h did not alter NPTr cell viability. Cell viability of NPTr cells transiently exposed to indicated concentrations of TG measured using (A) CellTiter-Glo® Luminescent Cell Viability Assay and (B) Caspase 3/7 Glo assay. NPTr cells exposed to the inhibitor for 1 h

before washing and media replacement. Cell viability measured 24 h later. (C, D) TG priming of NPTr cells reduced progeny virus output. NPTr cells, incubated with 0.5 μ M or 1 μ M TG for 1 h, were subsequently infected with (C) USSR H1N1 or (D) pandemic H1N1 virus at 0.5 MOI for 24 h. Spun supernatants were used to infect MDCK cells for 6 h in focus forming assays (FFAs). Significance determined by one way ANOVA, comparing to the DMSO control

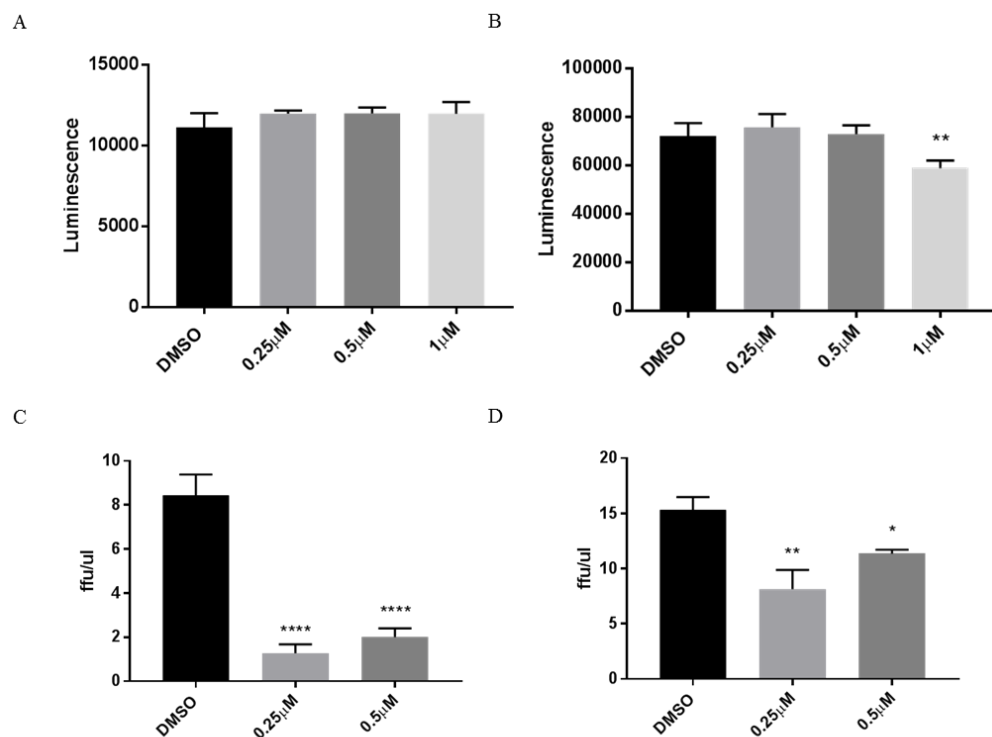


Figure 3-6. Transient priming of primary porcine myoblasts with non-toxic concentrations of TG reduced progeny virus production. (A, B) Incubation with 0.5 μ M TG for 1 h did not reduce myoblast viability. Cell viability of porcine myoblasts transiently exposed to indicated concentrations of TG measured using (A) CellTiter-Glo® Luminescent Cell Viability Assay and (B) Caspase3/7 Glo assay. Porcine myoblasts exposed to the inhibitor for 1 h before washing and media

replacement. Cell viability measured 24 h later. (C, D) TG priming of porcine myoblasts reduced progeny virus output. Porcine myoblasts, incubated with 0.25 μ M or 0.5 μ M TG for 30 min, were subsequently infected with (C) USSR H1N1 or (D) pandemic H1N1 virus at 2.0 MOI for 24 h. Spun supernatants were used to infect MDCK cells for 6 h in focus forming assays (FFAs). Significance determined by one way ANOVA, comparing to the DMSO control.

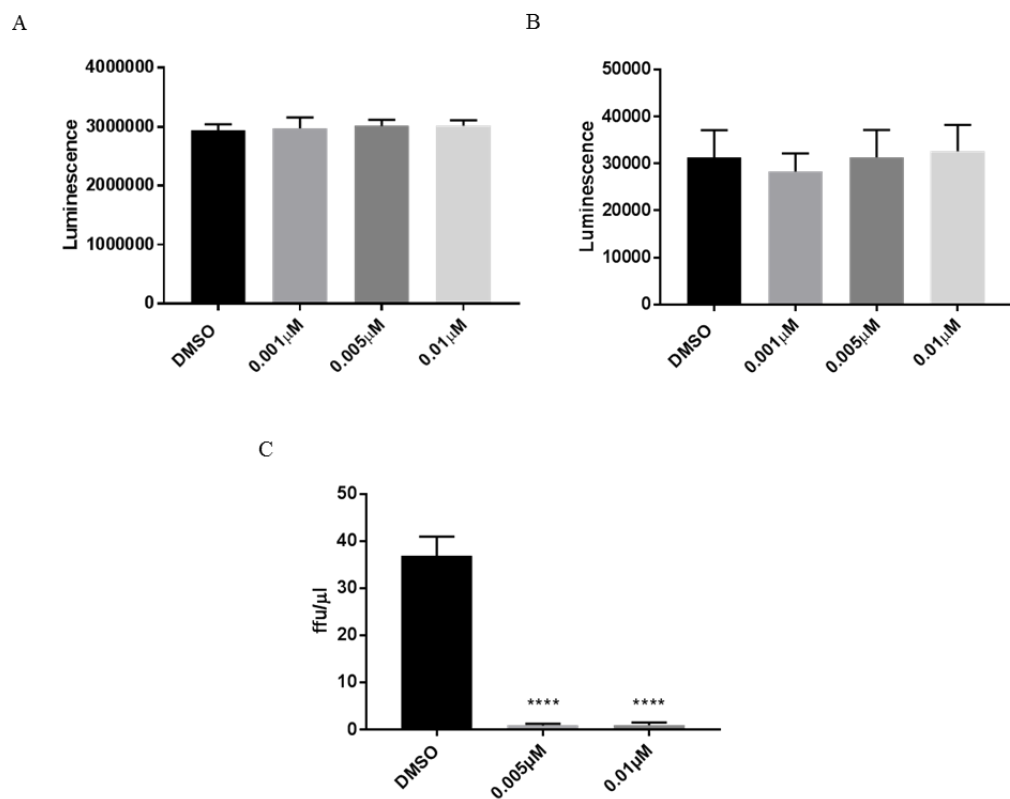


Figure 3-7. Transient priming of NHBE cells with non-toxic concentrations of TG reduced progeny virus production. (A, B) Incubation with 0.01 μ M TG for 30 min did not alter NHBE cell viability. Cell viability of NHBE cells transiently exposed to indicated concentrations of TG measured using (A) CellTiter-Glo® Luminescent Cell Viability Assay and (B) Caspase3/7 Glo assay. NHBE cells exposed to the inhibitor

for 30 min before washing and media replacement. Cell viability measured 24 h later.

(C) TG priming of NHBE cells reduced progeny virus output. NHBE cells, incubated with 0.005 μ M or 0.01 μ M TG for 30 min, were subsequently infected with USSR H1N1 at 0.5 MOI for 24 h. Spun supernatants were used to infect MDCK cells for 6 h in focus forming assays (FFAs). Significance determined by one way ANOVA, comparing to the DMSO control.

DMSO was used as a solvent for TG and its impact on progeny virus output was assessed in NPTr cells and porcine myoblasts. DMSO priming had no effect on virus production (Figure 3-8). Therefore, the significant reduction in progeny virus output cannot be attributed to the solvent.

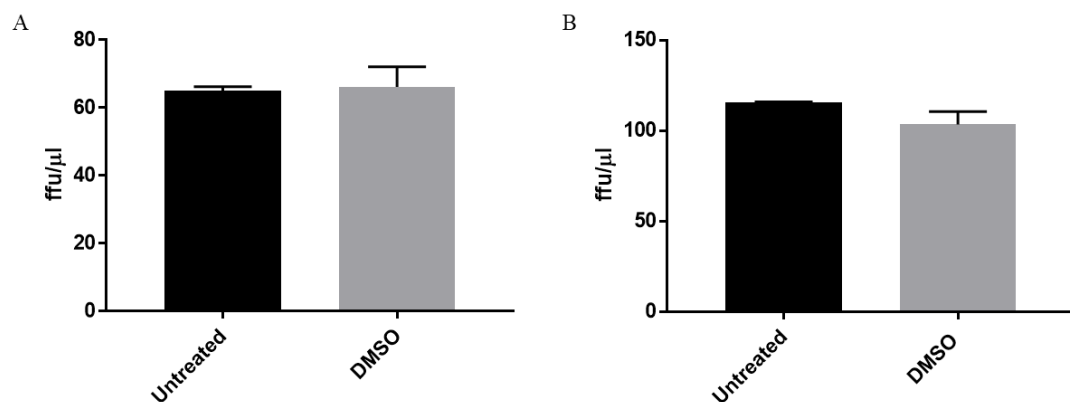


Figure 3-8. DMSO priming does not reduce progeny virus output. (A) NPTr cells and (B) porcine myoblasts were incubated in DMSO for 30 min and subsequently infected with USSR H1N1 at 0.5 MOI for 24 h. Spun supernatants were used to infect MDCK cells for 6 h in focus forming assays (FFAs). Significance determined by unpaired t-test.

3.3.4 TG induced increase in cytosolic calcium was dependent on extracellular Ca^{2+} influx

As described in Section 3.3.1, the availability of extracellular Ca^{2+} was associated with progeny virus output and an increase in extracellular Ca^{2+} accompanied a decrease in influenza virus replication. To analyse the dependence of the TG mediated increase in cytosolic Ca^{2+} on extracellular Ca^{2+} influx, the effect of TG on cytosolic $[\text{Ca}^{2+}]$ was measured in the presence and absence of extracellular calcium. In the absence of extracellular Ca^{2+} , TG did not significantly increase cytosolic $[\text{Ca}^{2+}]$ for tracheal epithelial cells or muscle cells (Figure 3-9). However, in the presence of extracellular Ca^{2+} , TG induced a significant increase in cytosolic Ca^{2+} at the concentrations associated with a reduction in viral titre (Figure 3-9).

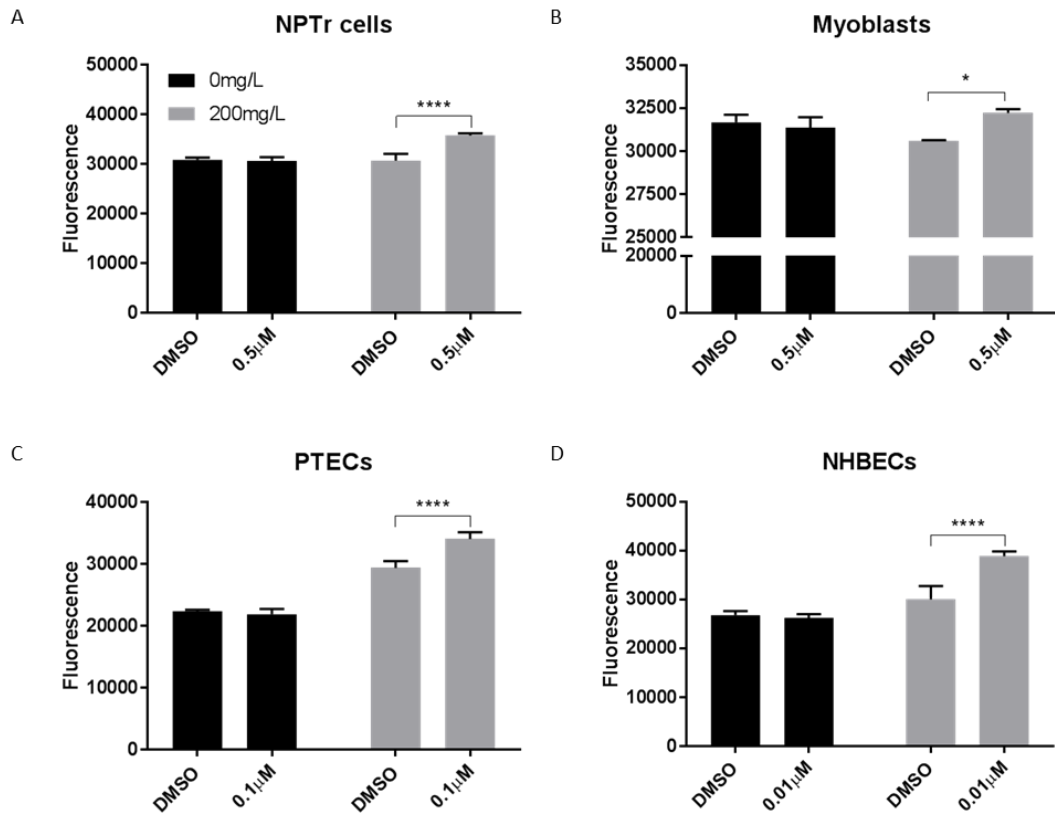


Figure 3-9. TG induced Ca^{2+} mobilisation was dependent on the availability of extracellular calcium. Fluo-8 fluorescence intensity measured in (A) NPTr cells incubated in 0.5 μM TG, (B) porcine myoblasts incubated in 0.5 μM TG, (C) PTECs in 0.1 μM TG and (D) NHBE cells in 0.01 μM TG for 10 min in 0mg/L extracellular CaCl_2 (black) or 200mg/L extracellular CaCl_2 (grey). Significance determined by two way ANOVA. Significance relative to corresponding DMSO treatment.

In addition, the greater reduction in viral titre from epithelial cells (Figure 3-5 and 3-7) corresponded to greater sensitivity to TG mediated extracellular Ca^{2+} influx (Figure 3-9A and 3-9D). This provides further support for a TG mediated antiviral response dependent on facilitating extracellular Ca^{2+} influx.

3.3.5 TG inhibited the influenza A virus replication post-transcriptionally

The current antivirals available inhibit virus replication at different stages within the life cycle. The NAIs interfere with release of progeny virus through inhibition of NA, while an inhibitor of the cap-dependent endonuclease within the influenza virus RNA polymerase has recently been identified as a viable treatment of influenza A virus infection (Liao et al., 2017, Omoto et al., 2018). The stage at which TG priming inhibited virus replication was determined to be post-transcriptional, as there was no reduction in virus M gene expression observed in porcine or human tracheal epithelial cells (Figure 3-10). Although, a significant reduction in pandemic H1N1 virus M gene expression in porcine myoblasts was consistently observed (Figure 3-10D). This may be attributed to a virus specific response in primary myoblasts or may have no biological significance. However, overall, TG priming did not inhibit influenza virus transcription and inhibited virus replication at a later point.

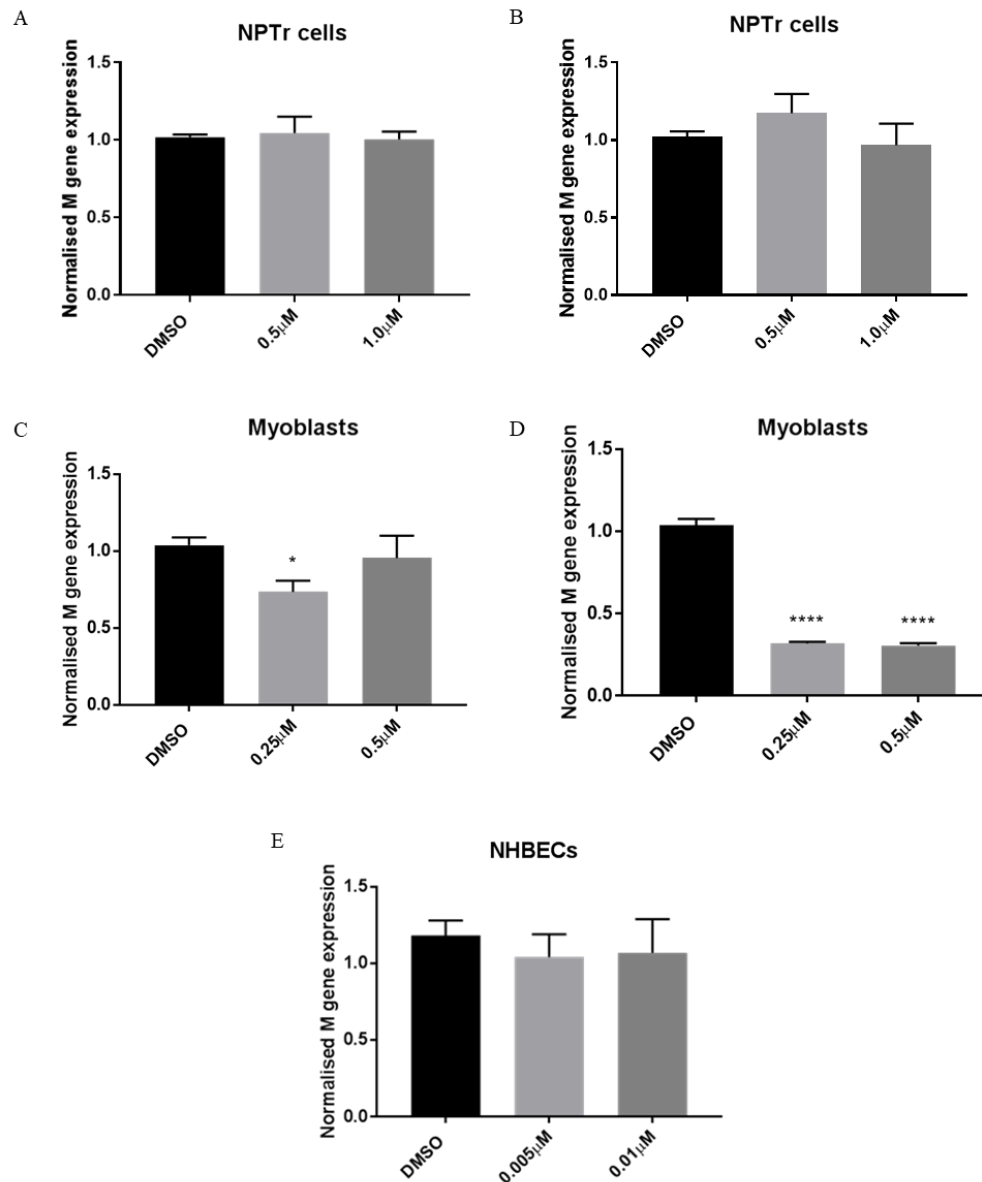


Figure 3-10. Post-transcriptional inhibition of virus replication by TG. (A, B) NPTTr cells, incubated with 0.5 μ M or 1 μ M TG for 1 h, were subsequently infected with (A) USSR H1N1 or (B) pandemic H1N1 virus at 0.5 MOI for 24 h. (C, D) Porcine myoblasts, incubated with 0.25 μ M or 0.5 μ M TG for 30 min, were subsequently infected with (C) USSR H1N1 or (D) pandemic H1N1 virus at 2.0 MOI for 24 h. (E) NHBE cells, incubated with 0.005 μ M or 0.01 μ M TG for 30 min, were subsequently infected with USSR H1N1 at 0.5 MOI for 24 h. Total RNA was extracted from each

sample for fold viral M gene expression, normalised to 18s rRNA, calculated using the comparative Ct method. Gene expression compared to the DMSO control, awarded a relative expression of 1. Significance determined by one way ANOVA, comparing to the DMSO control.

3.3.6 TG did not inhibit viral protein synthesis

The host cell translation machinery is utilised by influenza virus for viral mRNA translation while host protein synthesis is repressed (Yángüez & Nieto, 2011). The dynamic relationship between calcium and protein synthesis has long been established and depletion of ER Ca^{2+} stores found to impair protein synthesis (Chin *et al.*, 1987, Kimball & Jefferson, 1992). Therefore, the rapid activity of TG may be dependent on inhibition of viral protein synthesis via Ca^{2+} mobilisation. Western blots were performed to quantify viral protein (NP and M1) in TG primed cells. In MDCK cells infected with influenza A virus (influenza A /Puerto Rico/8/1934 H1N1), all viral proteins were detected 6 hours post infection and virus protein synthesis plateaued at 10 hours post infection. Viral proteins HA, M1, NA and NP displayed the highest synthesis rates between 4 and 12 hours post infection (Kummer *et al.*, 2014).

TG priming did not impair translation of virus proteins in NPTr cells (Figure 3-11), and had minimal impact on virus protein production in myoblasts, with only a significant reduction observed in M1 protein in TG primed myoblasts infected with USSR H1N1 virus (Figure 3-12). No significant alteration in M1 protein was observed in NHBE cells but NP protein levels were significantly decreased in TG primed cells infected with USSR H1N1 virus but was significantly increased in TG primed NHBE cells infected with pandemic H1N1 virus (Figure 3-13). A corresponding significant

reduction in virus output was consistently recorded across all cell types. The significant increases in virus proteins in TG primed cells may reflect an accumulation of virus proteins within the cells, suggesting virus release may be impaired (Figure 3-11 and Figure 3-13B).

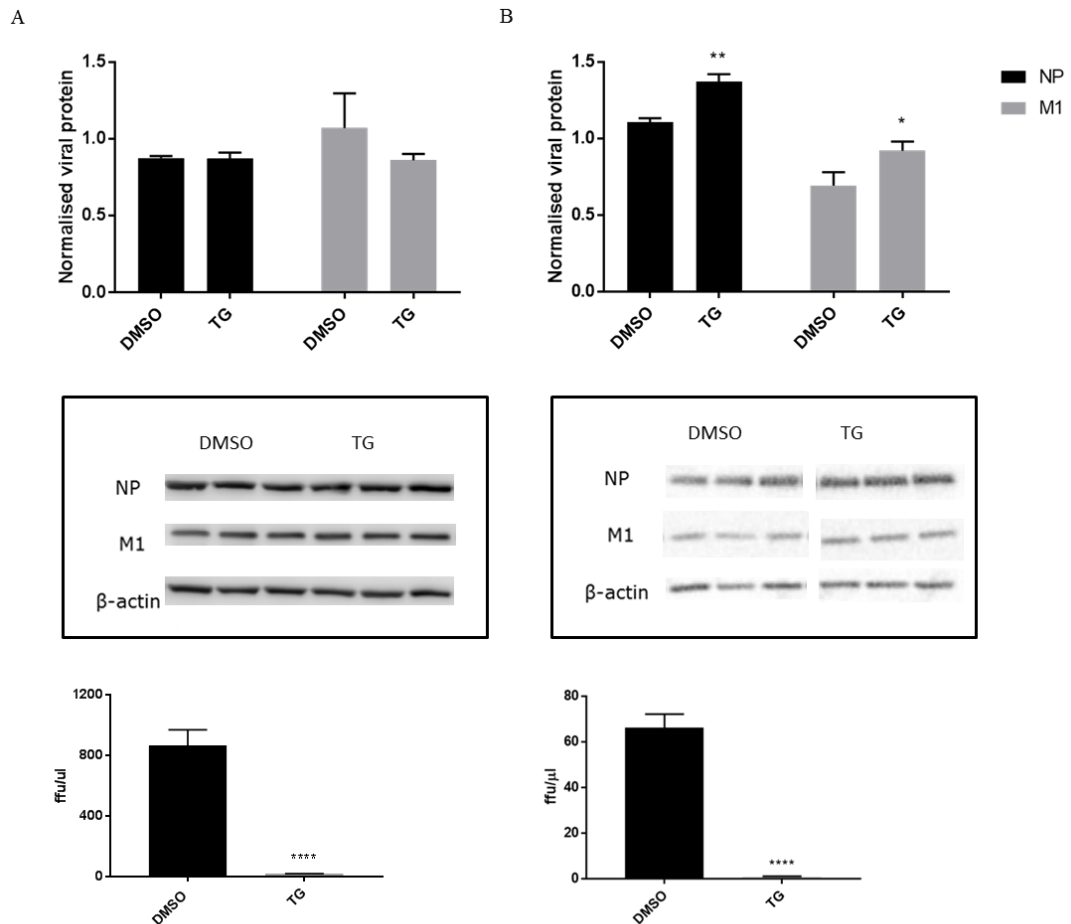


Figure 3-11. No reduction in viral proteins in infected NPTr cells pre-primed with TG. NPTr cells were pretreated with 0.5 μ M TG for 30 min and subsequently infected with (A) USSR H1N1 or (B) pandemic H1N1 at 0.5 MOI for 24 h. Viral NP and M1 was detected via western blot and normalised to β -actin. Unrelated intervening wells from pandemic H1N1 virus infection removed. Significance determined by two way ANOVA or paired t-test. Significance relative to corresponding DMSO treatment.

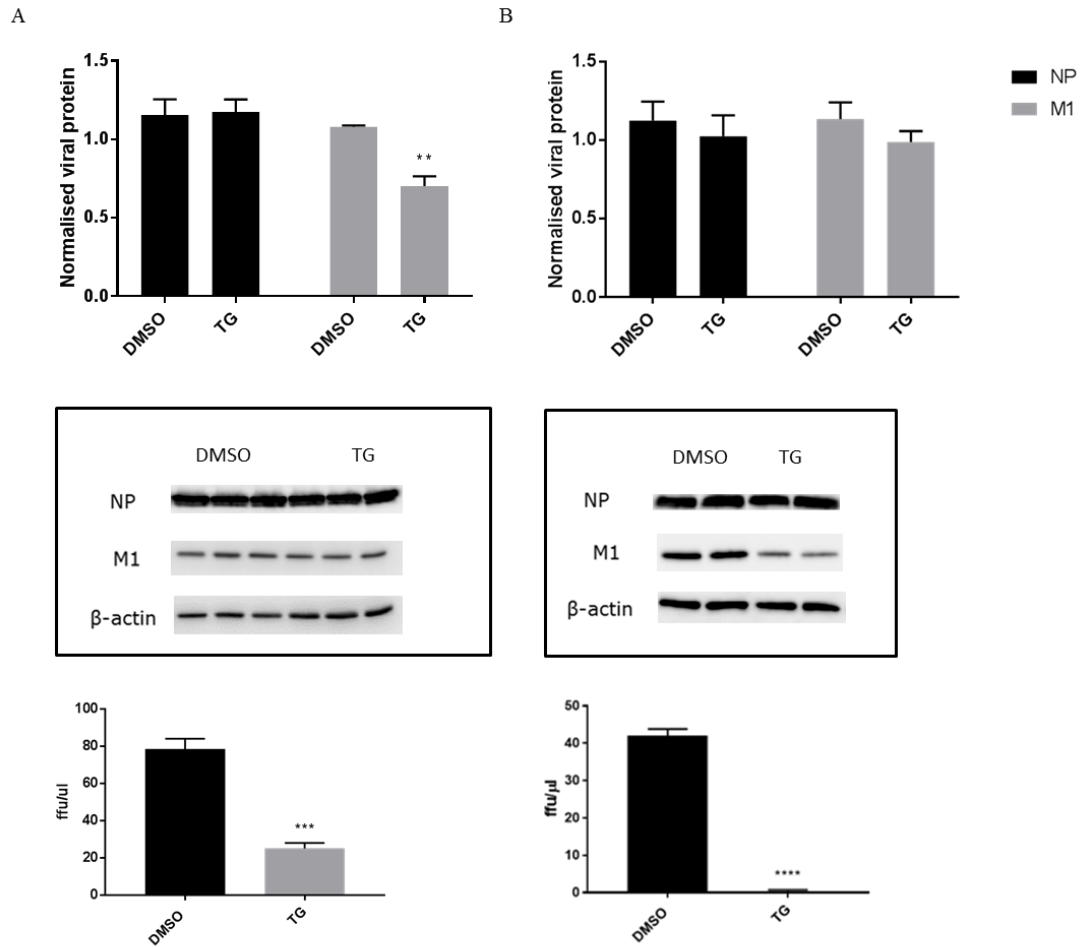


Figure 3-12. Minimal reduction in viral proteins in infected porcine myoblasts pre-primed with TG. Porcine myoblasts were pretreated with 0.5μM TG for 30 min and subsequently infected with (A) USSR H1N1 or (B) pandemic H1N1 at 2.0 MOI for 24 h. Viral NP and M1 was detected via western blot and normalised to β-actin. Significance determined by two way ANOVA or paired t-test. Significance relative to corresponding DMSO treatment.

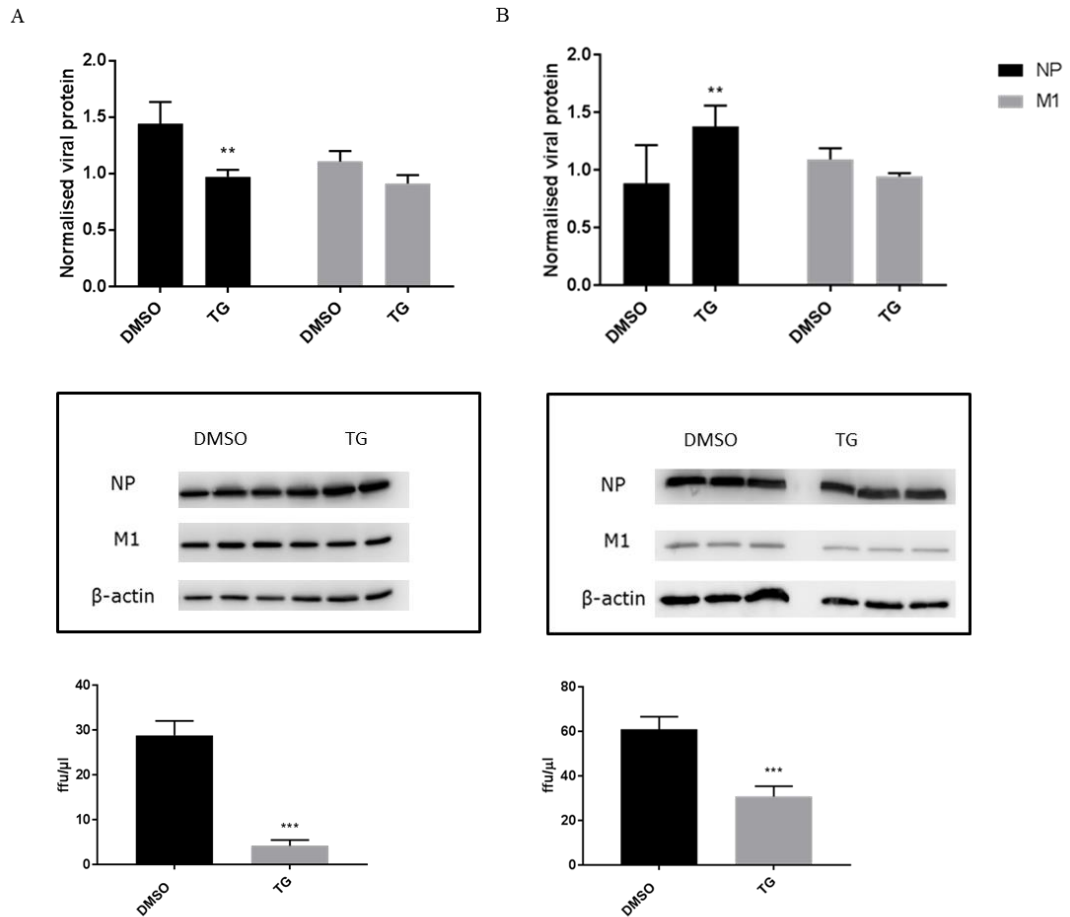


Figure 3-13. Inconsistent alteration in viral proteins in infected NHBE cells pre-primed with TG. NHBE cells were pretreated with 0.005μM TG for 30 min and subsequently infected with pandemic H1N1 at 1.0 MOI for 24 h. Viral NP and M1 was detected via western blot and normalised to β-actin. Spun supernatants were used to infect MDCK cells for 6 h in focus forming assays (FFAs). Significance determined by unpaired t-test. Significance determined by two way ANOVA or paired t-test. Significance relative to corresponding DMSO treatment.

3.3.7 SOCE activator CPA did not induce extracellular Ca^{2+} influx at non-toxic concentrations

To assess if the effect of TG on viral progeny output was mediated by SOCE or by TG specific mechanisms, NPTr cells were primed with cyclopiazonic acid (CPA) prior to infection. CPA was identified as a selective inhibitor of ER and SR Ca^{2+} ATPase, inhibiting Ca^{2+} store refilling and enhancing Ca^{2+} entry into the cytosol via SOCE (Seidler *et al.*, 1989, Uyama *et al.*, 1993). Knock down of *ORAI1* or *STIM1* in airway smooth muscle cells and human airway myocytes respectively, impaired CPA mediated Ca^{2+} influx (Peel *et al.*, 2006, Peel *et al.*, 2008).

NPTr cells were exposed to non-toxic doses of CPA (0.005 μM , 0.05 μM or 0.5 μM) for 30 min immediately prior to infection with USSR or pandemic H1N1 virus for 24 h. In contrast to the dramatic reduction in progeny output following TG pre-treatment, there was no significant reduction in viral output from NPTr cells pre-treated with any of the indicated non-toxic CPA concentrations (Figure 3-14). The lack of reduction in progeny viral output may be attributed to the dose of CPA used. In other studies, CPA was used at doses ranging between 5 μM - 20 μM (Noble *et al.*, 2014, Cui *et al.*, 2016, Peel *et al.*, 2008, Ethier *et al.*, 2001). However, NPTr cell viability was significantly reduced following exposure to CPA at 5 μM , thereby limiting use of the compound to concentrations below this threshold to prevent a reduction in progeny virus output attributable to cytotoxicity (Figure 3-14A). The lack of antiviral activity of CPA at the lower doses may be attributed to the lack of Ca^{2+} influx. At a non-cytotoxic concentration of 10 μM , CPA inhibited human respiratory syncytial virus (RSV) replication (Cui *et al.*, 2016). This inhibition was thought to be dependent on an increase in intracellular Ca^{2+} , indicated by the ability of other SOCE activators, such

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as TG and ionomycin, to reduce the viral titre of RSV (Cui et al., 2016). In NPTr cells, CPA was only able to induce an increase in cytosolic Ca^{2+} when used at doses higher than $10\mu\text{M}$ CPA (Figure 3-14B). At $1\mu\text{M}$, no significant increase in cytosolic Ca^{2+} was measured, suggesting SOCE was not activated at the lower, non-toxic doses of CPA used prior to infection. This indirectly further supports the proposed antiviral role for SOCE, as a lack of extracellular Ca^{2+} influx was not associated with a reduction in viral titre.

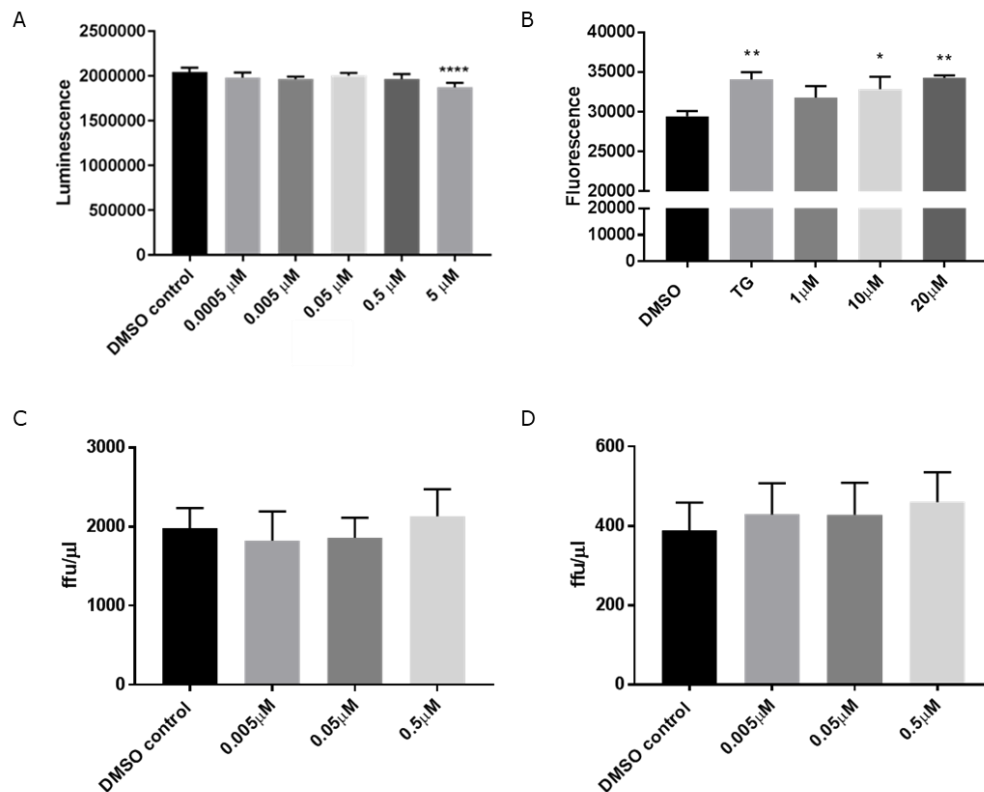


Figure 3-14. Pre-treatment of NPTr cells with non-toxic doses of CPA did not affect virus production. (A) Cells were transiently exposed to indicated concentrations of CPA for 30 min before rinsing with PBS and media replacement. Cell viability measured 24 h later with CellTiter-Glo® Luminescent Cell Viability Assay. (B)

Priming 10 μ M CPA elicited extracellular Ca^{2+} influx based on Fluo-8 Ca^{2+} assays. (C, D) NPTr cells, primed with indicated concentrations of CPA for 30 min, were subsequently infected with (C) USSR H1N1 or (D) pandemic H1N1 virus at 0.5MOI for 24 h. Spun supernatants were used to infect MDCK cells for 6 h in focus forming assays (FFAs) and immunostained for viral NP positive cells. Significance determined by one way ANOVA, comparing to the DMSO control.

3.3.8 Transient exposure to SOC channel inhibitors increased progeny virus production in epithelial cells

BTP2 and Synta66 are small molecule inhibitors of the ORAI proteins (Zitt *et al.*, 2004, Jairaman & Prakriya, 2013). They were utilised to analyse the effect of pharmacological SOCE inhibition on virus replication. BTP2 and Synta66 priming did not significantly reduce cell viability in NPTr cells or NHBE cells 24 hours after priming (Figure 3-15 and 3-16). Although, an increase in ATP synthesis was observed in Synta66 primed NPTr cells (Figure 3-15B) but not NHBE cells (Figure 3-16B).

Brief pre-treatment of NPTr cells (Figure 3-15) and NHBE cells (Figure 3-16) with non-toxic doses of BTP2 or Synta66 resulted in a significant increase in progeny virus output. This indicated inhibition of the SOC channels impaired the host immune response to influenza virus infection.

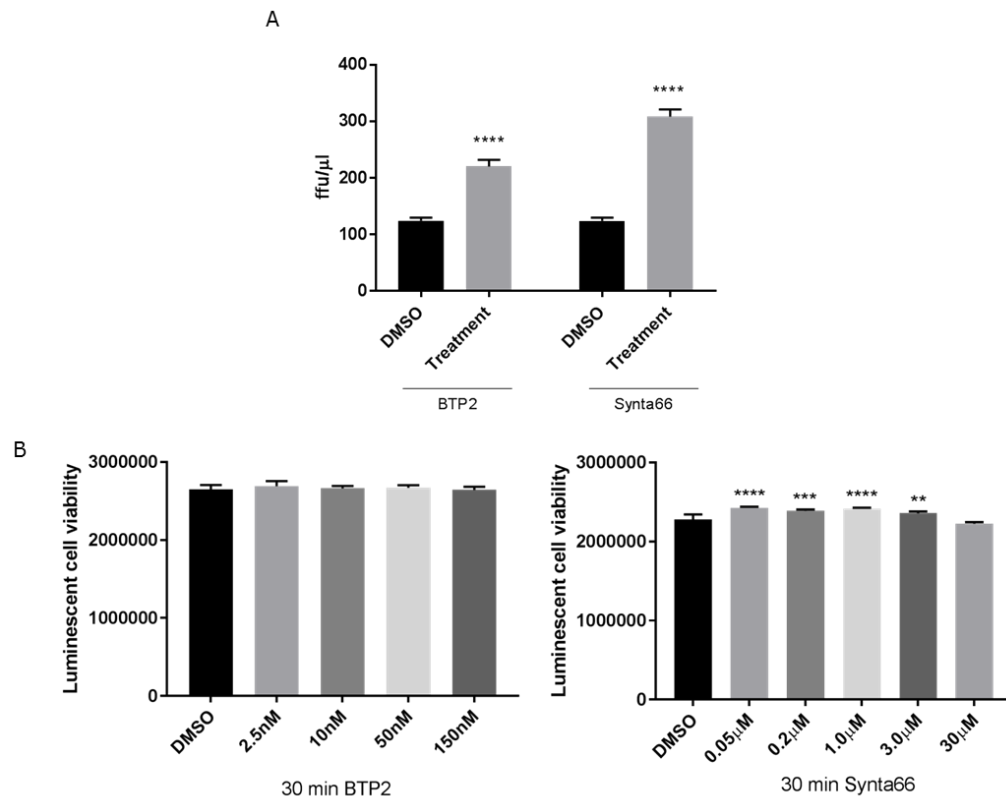


Figure 3-15. Pre-treatment with ORAI1 inhibitors increased progeny virus output.

(A) NPTr cells incubated with 150nM BTP2 or 5 μM Synta66 for 30 min were subsequently infected with USSR H1N1 virus at 0.5 MOI for 24 h. Spun supernatants were used to infect MDCK cells for 6 h in focus forming assays (FFAs). Significance determined by paired t-test, comparing to the DMSO control. (B) Non-toxic doses of BTP2 and Synta66 were used in the priming of NPTr cells. NPTr cells incubated with BTP2 or Synta66 for 30 min at the indicated concentrations, the cells washed with PBS and replaced with fresh media. Cell viability measured 24 h later using CellTiter-Glo® Luminescent Cell Viability Assay. Significance determined by (A) unpaired t-test or (B, C) one-way ANOVA in relation to corresponding DMSO control.

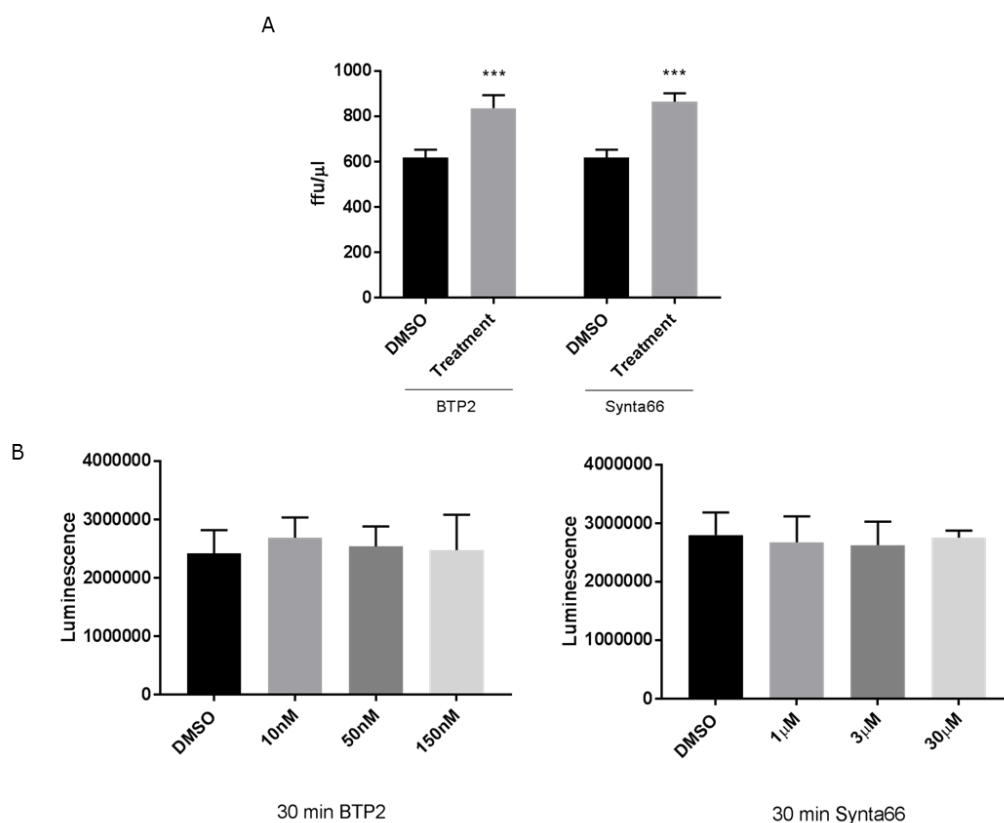


Figure 3-16. Pre-treatment with ORAI1 inhibitors increased progeny virus output.

(A) NHBE cells incubated with 150nM BTP2 or 5 μ M Synta66 for 30 min were subsequently infected with USSR H1N1 virus at 1.0 MOI for 24 h. Spun supernatants were used to infect MDCK cells for 6 h in focus forming assays (FFAs). Significance determined by paired t-test, comparing to the DMSO control. (B) Non-toxic doses of BTP2 and Synta66 were used in the priming of NHBE cells. NHBE cells incubated with BTP2 or Synta66 for 30 min at the indicated concentrations, the cells washed with PBS and replaced with fresh media. Cell viability measured 24 h later using CellTiter-Glo® Luminescent Cell Viability Assay. Significance determined by (A) unpaired t-test or (B, C) one-way ANOVA in relation to corresponding DMSO control.

3.3.9 ORAI1 and STIM1 mediate an antiviral response against influenza A virus replication

Unintended side effects from SERCA inhibition may account for the antiviral activity of TG, other than activation of SOCE. Therefore, over-expression of components of the SOCE machinery provided a valuable insight into the contribution of SOCE to the antiviral response. *ORAI1* was the dominant homologue expressed in post-natal tissue (Figure 3-17) while *ORAI3* was the highest expressed SOC channel subunit expressed in cultured cells (Figure 3-18). Greater abundance of STIM1 mRNA was identified in post-natal skeletal muscle and cultured cells (Figure 3-17 and 3-18).

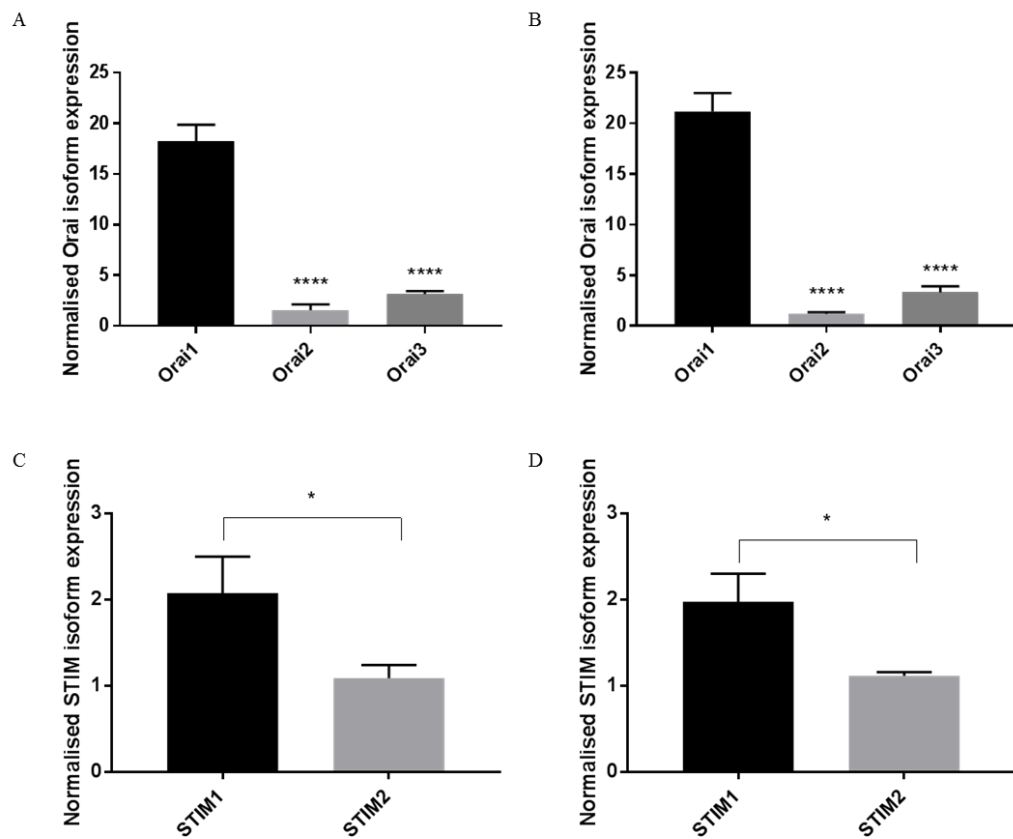


Figure 3-17. ORAI1 and STIM1 were the dominant SOC channel subunit expressed in porcine postnatal skeletal muscle. Fold gene expression of SOC components, normalised to 18s rRNA, in porcine postnatal skeletal muscle quantified by real time PCR. Expression relative to (A, C) ORAI2 and (C, D) STIM2, awarded a relative value of 1. (A, C) Longissimus dorsi and (B, D) psoas obtained from three-year-old sows. Significance determined by (A) one way ANOVA relative to ORAI1 unless otherwise indicated or (C) unpaired t-test.

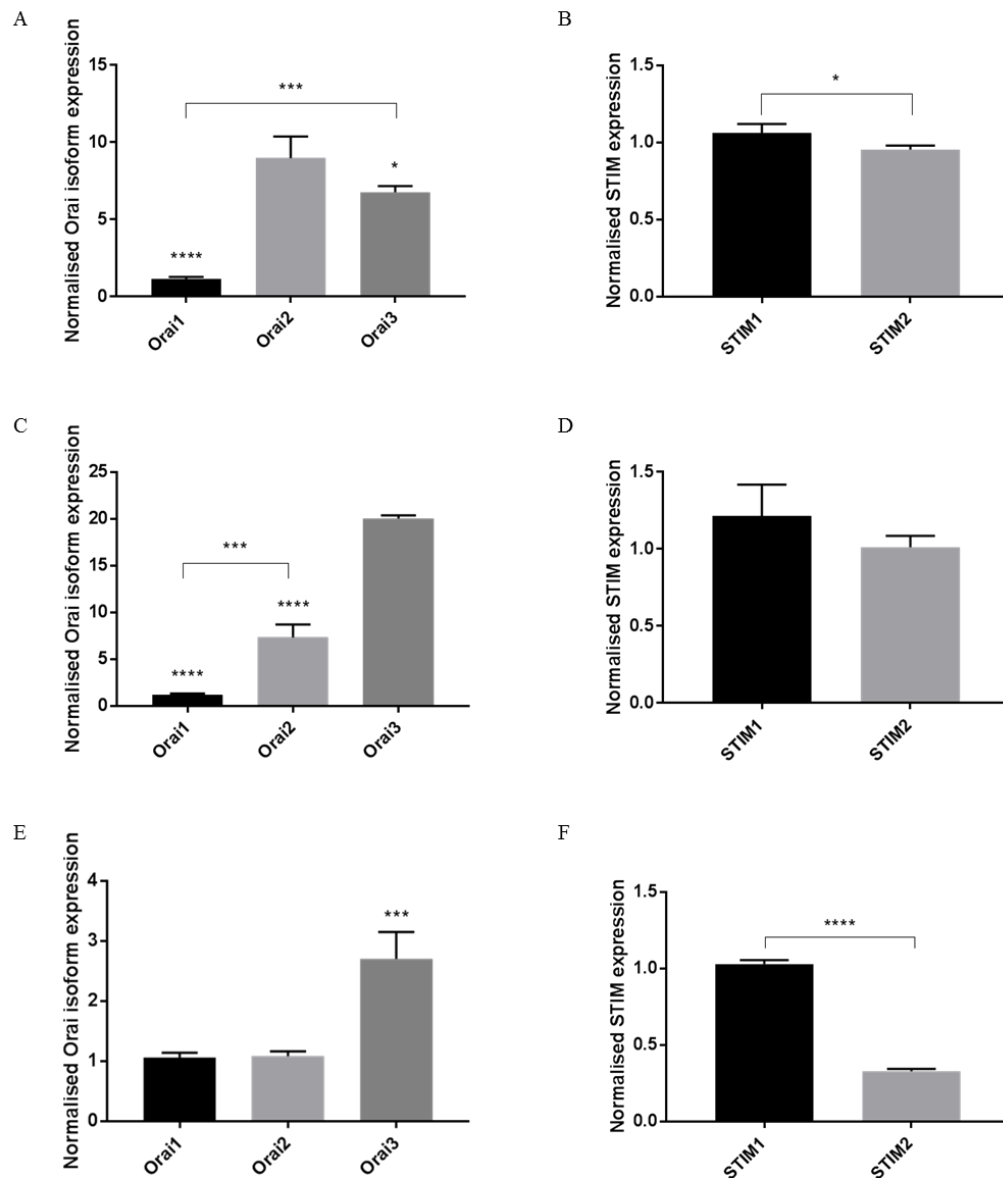


Figure 3-18. SOCE component expression in vitro. Expression of SOC channel subunits, ORAI1-3, in (A) NPTr cells, (C) myoblasts and (E) NHBE cells. Fold gene expression, normalised to 18s rRNA, comparing gene expression to ORAI1, awarded a relative expression of 1. Significance determined by one way ANOVA, comparing expression to ORAI3. Expression of endoplasmic reticulum luminal calcium sensors, STIM1 and STIM2, in (B) NPTr cells, (D) myoblasts and (F) NHBE cells. Fold gene

expression, normalised to 18s rRNA, comparing gene expression to STIM1, awarded a relative expression of 1. Significance determined by unpaired t-test.

Over-expression of the each of the indicated ORAI isoforms (*ORAI1*, *ORAI2* and *ORAI3*) and *STIM1* all significantly reduced progeny virus output in NPTr cells (Figure 3-19) and porcine myoblasts (Figure 3-20). Having established the relative abundance of *ORAI1* and *ORAI3* *in vitro* and *in vivo*, co-over-expression of *STIM1* with *ORAI1* and *ORAI3* on virus replication was analysed. Co-over-expression of *ORAI1* or *ORAI3* with *STIM1* significantly impaired virus replication (Figure 3-19 and 3-20). In NPTr cells, over-expression of *ORAI3* alone induced a ~80% reduction in USSR H1N1 and pandemic progeny virus output while co-over-expression of *STIM1* and *ORAI3* induced a 75-80% reduction in progeny virus output. This is comparable to the ~90% reduction following TG priming of NPTr cells (Figure 3-5).

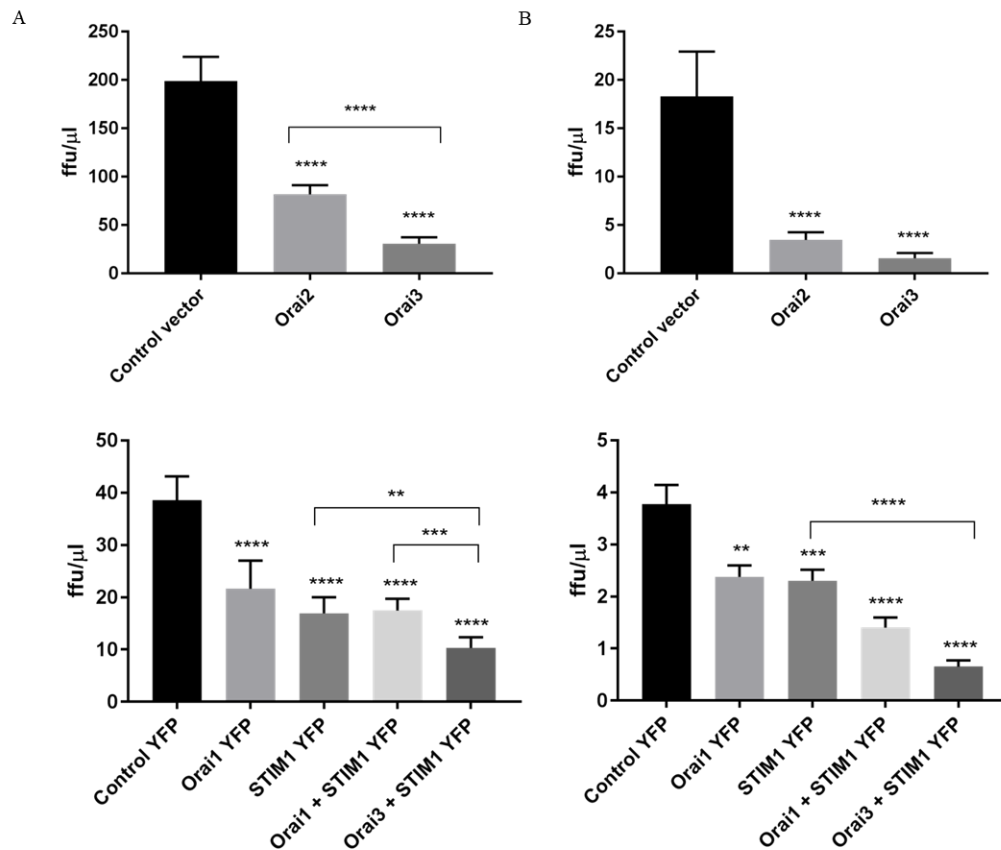


Figure 3-19. Over-expression of STIM1, ORAI1 and ORAI3 in NPTr cells significantly reduced progeny influenza virus release. NPTr cells were transfected with indicated plasmid for 2 days. Cells were subsequently infected with (A) pandemic H1N1 or (B) USSR H1N1 virus at 1.0 and 0.5 MOI respectively. Spun supernatants were used to infect MDCK cells for 6 h in focus forming assays (FFAs). Significance determined by one way ANOVA, comparing transfected cells to the corresponding controls unless otherwise indicated

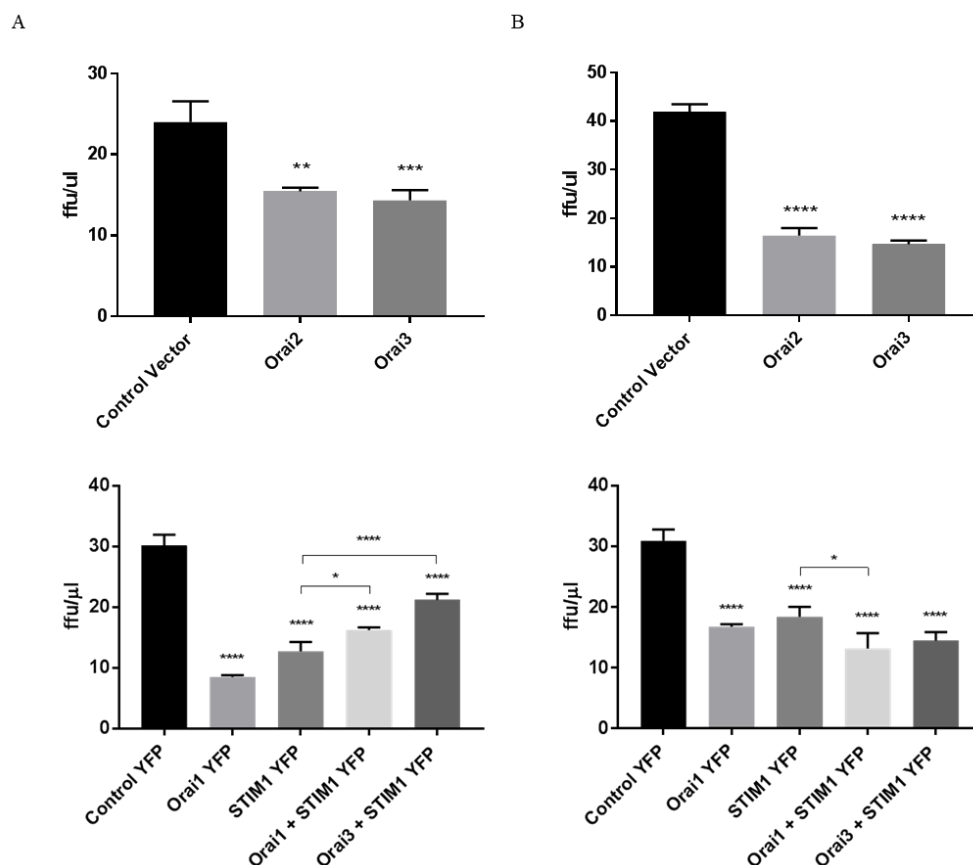


Figure 3-20. Over-expression of ORAI1-YFP, ORAI2-YFP and ORAI3-YFP with STIM1-YFP in myoblasts reduced viral progeny output. Porcine myoblasts were transiently transfected with indicated plasmids for 2 days and infected with (A) USSR H1N1 or (B) pandemic virus at 2.0 MOI for 24 h. Spun supernatants were used to infect MDCK cells for 6 h in focus forming assays (FFAs). Viral output analysed via one way ANOVA, comparing viral output to the control vector or YFP. Significance determined by one way ANOVA comparing viral output to the control vector.

Over-expression and co-over-expression of *STIM1* and *ORAI1* in NHBE cells significantly reduced USSR H1N1 progeny virus output (Figure 3-21), demonstrating over-expression of *STIM1* and *ORAI1* impaired influenza virus replication in porcine

and human tracheal epithelial cells alike. However, over-expression of *STIM1* or *ORAI1* in NHBE cells induced a comparatively modest reduction in progeny virus output of 44% and 49%, respectively, and a significant reduction of 31% in NHBE cells co-overexpressing *STIM1* and *ORAI1*. The significant but modest reduction in progeny virus output may be a reflection of variability in transfection efficiency. In comparison, TG priming induced a significant reduction in progeny virus output of ~95% in NHBE cells (Figure 3-7).

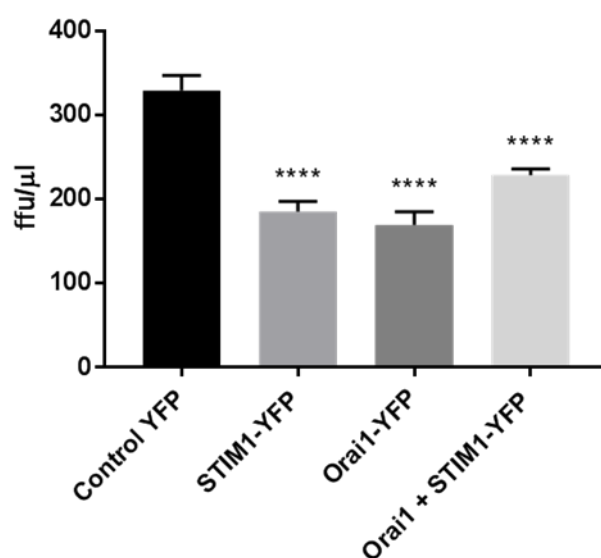


Figure 3-21. Over-expression of ORAI1-YFP and STIM1-YFP in NHBE cells reduced viral progeny output. NHBE cells, transiently transfected with the indicated plasmids for 2 days, were infected with USSR H1N1 at 1.0 MOI for 24 h. Spun supernatants were used to infect MDCK cells for 6 h in focus forming assays (FFAs). Viral output analysed via one way ANOVA, comparing viral output to the control vector or YFP. Significance determined by one way ANOVA comparing viral output to the control vector.

Knock down of *ORAI1* and *STIM1* provided further evidence for the novel antiviral activity of SOCE. Efficient knock down of *STIM1* (siRNA mediated knockdown resulting a ~75% reduction in *STIM1* mRNA) significantly increased progeny virus production in NHBE cells infected with USSR H1N1 virus (Figure 3-22). Knock down of *ORAI1* did not have a similar impact on virus replication, most likely due to the presence of *ORAI2* and *ORAI3*, compensating for the reduction in *ORAI1* expression.

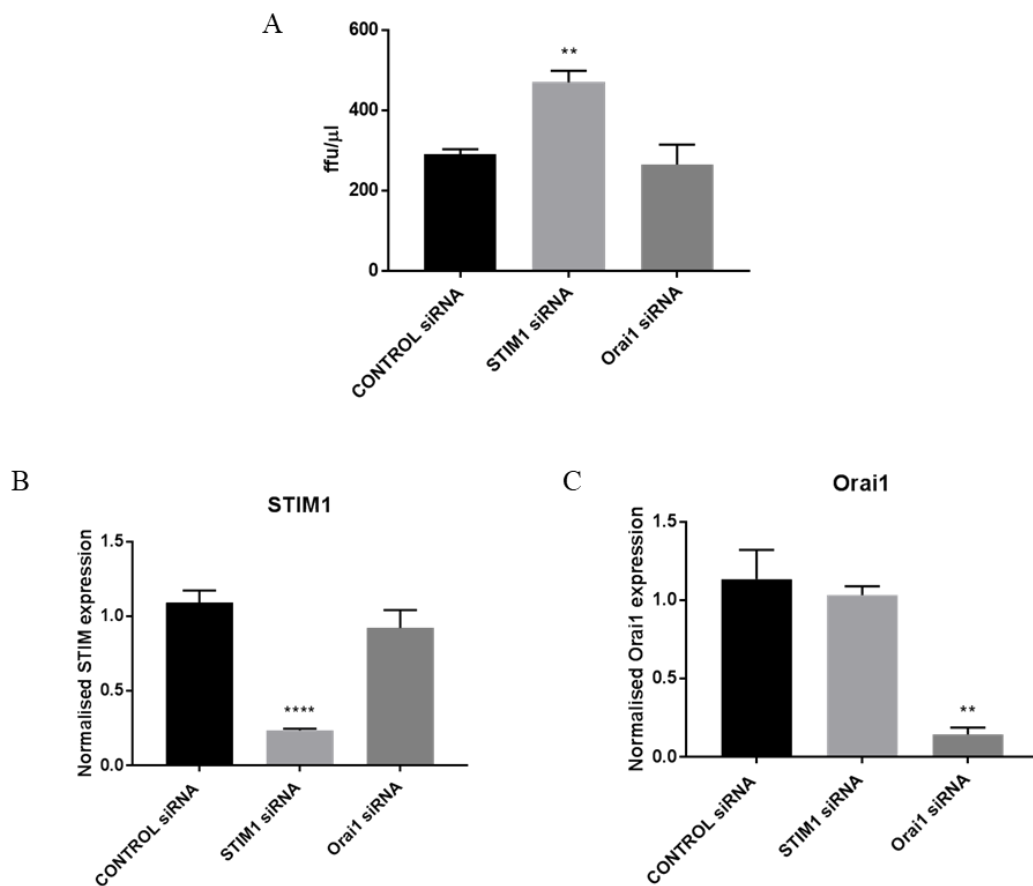


Figure 3-22. Knockdown of *STIM1* in NHBE cells increased progeny virus output.

NHBE cells, transiently transfected with the indicated siRNAs for 48 h were subsequently infected with USSR H1N1 at 1.0 MOI for 24 h. (A) Spun supernatants were used to infect MDCK cells for 6 h in focus forming assays (FFAs). (B, C, D)

Total RNA was extracted from each sample for indicated gene expression, normalised to 18S rRNA, calculated using the comparative Ct method. Fold gene expression relative to the uninfected control siRNA. Significance determined by a one way ANOVA, comparing to the corresponding control vector.

3.3.10 Over-expression of the SOCE components impaired influenza virus transcription

Over-expression of *STIM1* and the *ORAI* homologues reduced progeny virus production, as also observed following TG priming. However, while TG priming did not reduce viral M gene expression in the cells, over-expression of *STIM1*, *ORAI1*, *ORAI2* and *ORAI3* significantly inhibited viral M gene expression in NPTr cells and porcine myoblasts (Figure 3-23). This suggested SOCE facilitates an antiviral response that inhibits influenza A virus replication at the transcriptional stage, conflicting with the post-transcriptional antiviral response induced by TG priming.

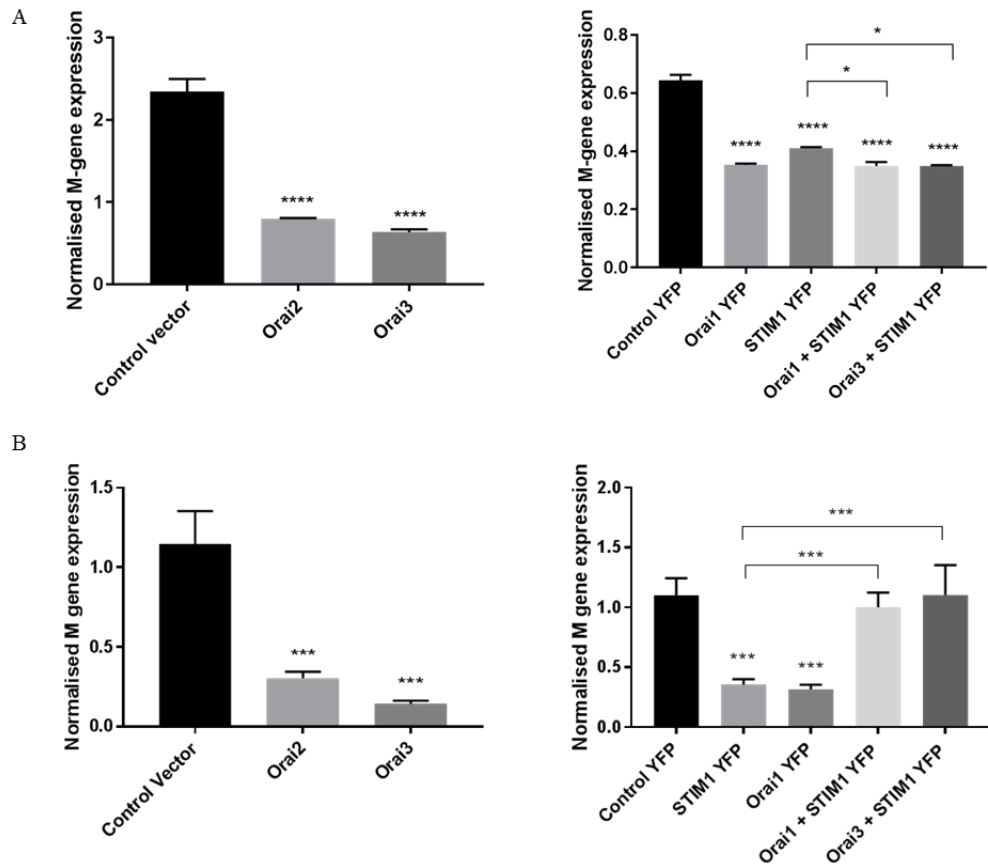


Figure 3-23. Over-expression of *STIM1* or *ORAI* significantly reduced viral *M* gene expression. (A) NPTr cells and (B) porcine myoblasts were transiently transfected with the indicated plasmids for 2 days and infected with USSR H1N1 virus at 0.5 MOI and 2.0 MOI, respectively, for 24 h. Total RNA was extracted from each sample for viral *M* gene expression normalised to 18S rRNA. Significance determined by one way ANOVA, comparing transfected cells to the corresponding controls unless otherwise indicated. YFP = yellow fluorescent protein.

However, NHBE cells overexpressing *STIM1* or *ORAI1* did not display a significant reduction in viral *M* gene expression (Figure 3-24). The reasons for this are unknown

but may reflect a difference between porcine and human cells following over-expression of the SOCE machinery.

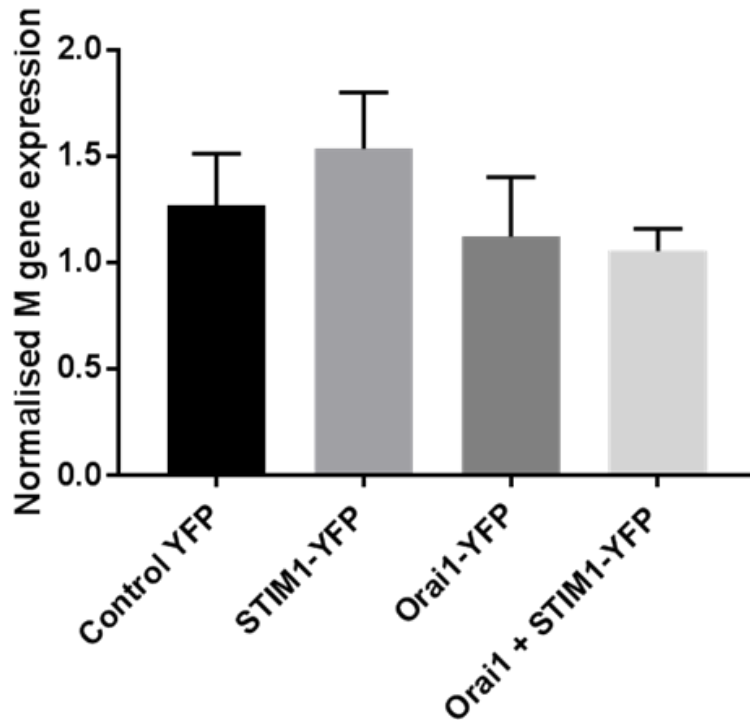


Figure 3-24. Over-expression of *STIM1* or *ORAI1* did not reduce viral *M* gene expression. NHBE cells, transiently transfected with the indicated plasmids for 2 days, were infected with USSR H1N1 virus at 1.0 MOI for 24 h. Total RNA was extracted from each sample for viral *M* gene expression normalised to 18S rRNA. Significance determined by one way ANOVA, comparing transfected cells to the corresponding controls unless otherwise indicated. YFP = yellow fluorescent protein.

Furthermore, knockdown of *STIM1* or *ORAI1* in NHBE cells had no significant effect on viral *M* gene expression, supporting a post-transcriptional inhibition of influenza virus replication in human tracheal epithelial cells (Figure 3-25).

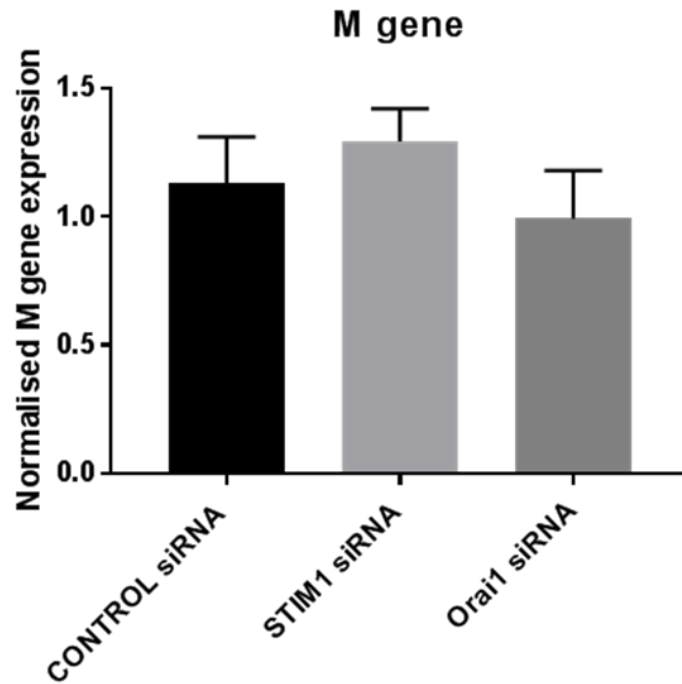


Figure 3-25. Knockdown of *STIM1* or *ORAI1* in NHBE cells did not alter *M* gene expression. NHBE cells, transiently transfected with the indicated siRNAs for 48 h were subsequently infected with USSR H1N1 at 1.0 MOI for 24 h. Total RNA was extracted from each sample for *M* gene expression, normalised to 18S rRNA, calculated using the comparative ct method. Fold gene expression relative to the uninfected control siRNA. Significance determined by a one way ANOVA, comparing to the corresponding control vector.

3.3.11 Over-expression of *STIM1* and *ORAI1* did not impair viral protein synthesis

Viral *M* gene expression was significantly reduced in NPTr cells and porcine myoblasts overexpressing *STIM1* or the SOC channel subunits, therefore viral protein levels were expected to be correspondingly reduced. However, viral M1 or NP protein

levels were not significantly reduced in NPTr cells (Figure 3-26) and primary myoblasts (Figure 3-27) overexpressing *ORAI1* or *STIM1*. Discrepancies between mRNA and corresponding protein levels are not uncommon (Greenbaum *et al.*, 2003, Koussounadis *et al.*, 2015). Therefore, it is possible the M gene mRNA levels do not reflect M1 protein and vice versa. The evidence presented here indicates *ORAI1* and *STIM1* dependent SOCE did not inhibit virus mRNA translation. SOCE may mediate a post-translational mechanism for inhibiting influenza virus replication.

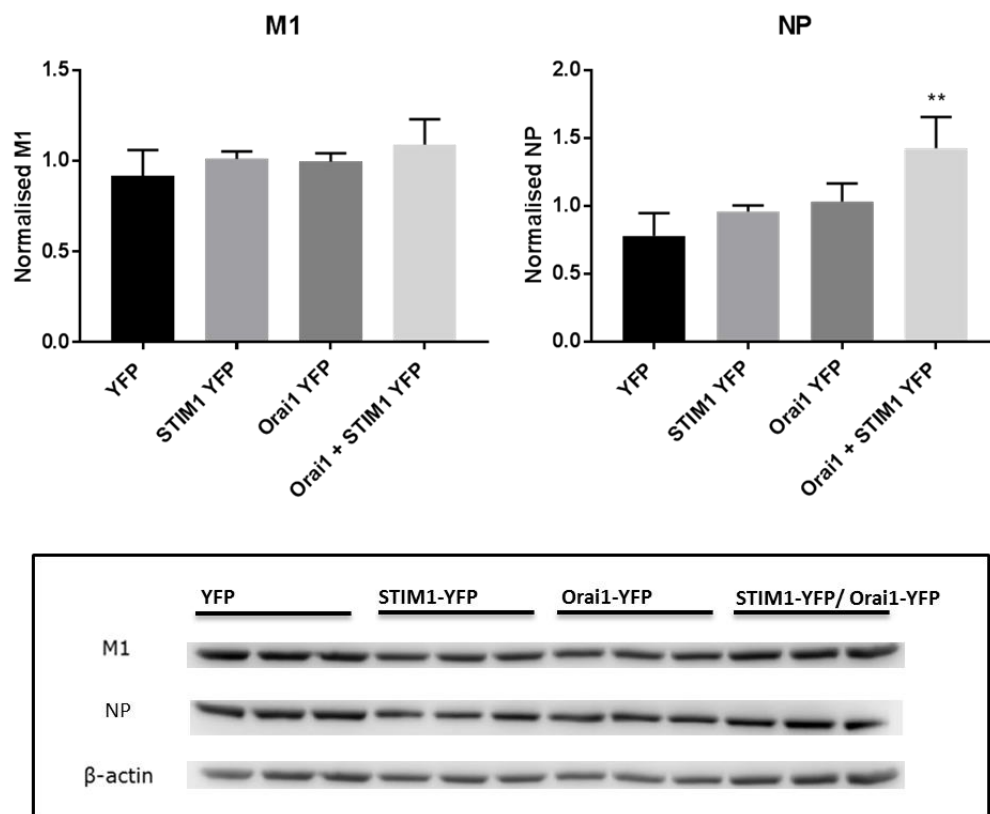


Figure 3-26. Over-expression of *STIM1* or *ORAI1* in infected NPTr cells did not reduce viral protein. NPTr cells, transiently transfected with *STIM1*-YFP and *ORAI1*-YFP for 2 days, were infected with pandemic H1N1 at 0.5 MOI for 24 h.

Western detection of viral M1 and NP was normalised to β -actin. Significance determined by one way ANOVA relative to the control vector.

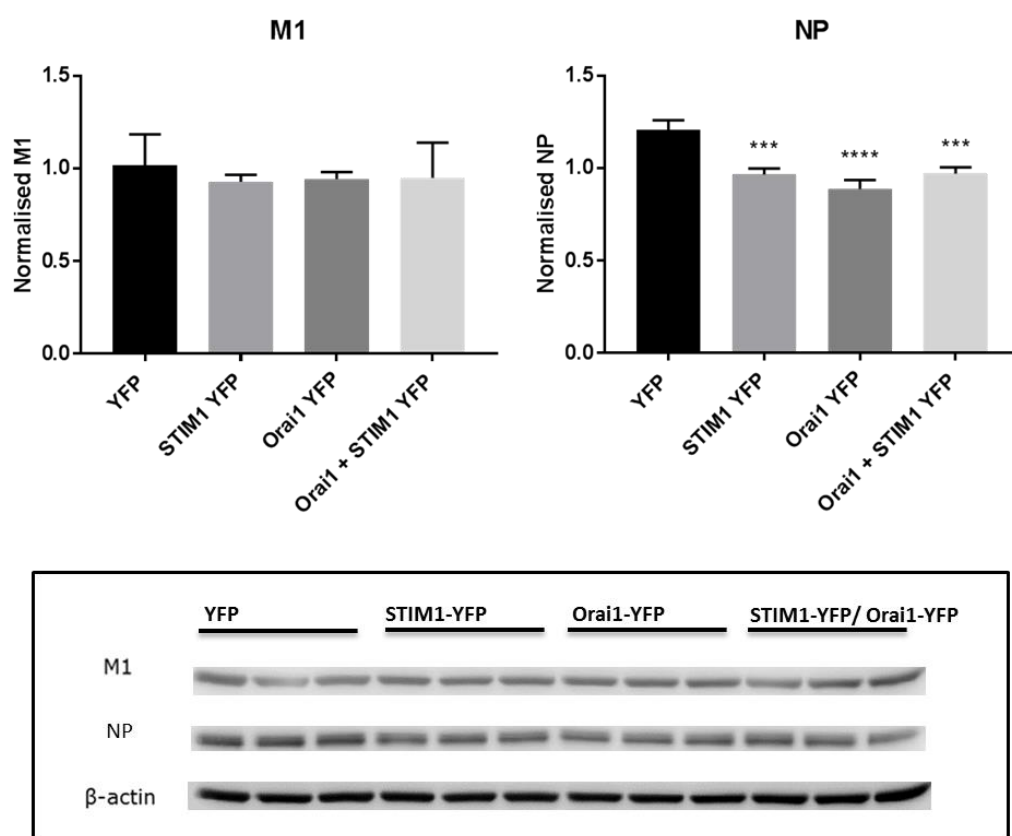


Figure 3-27. Over-expression of STIM1 or ORAI1 in porcine myoblasts did not reduce viral M1 protein. Porcine myoblasts, transiently transfected with STIM1-YFP and ORAI1-YFP for 2 days, were infected with pandemic H1N1 at 2.0 MOI for 24 h. Western detection of viral M1 and NP was normalised to β -actin. Significance determined by one way ANOVA relative to the control vector.

3.3.12 Over-expression of positive regulators of SOCE, STIMATE and CRACR2A, inhibited influenza A virus replication

The SOCE components, STIM1 and the ORAI proteins, require other proteins to function correctly and regulate their activity. STIMATE associates with STIM1 and positively regulates puncta formation, associating with STIM1 to facilitate the release of the STIM1-Orai-activating-region (SOAR) upon Ca^{2+} depletion of the ER store (Hooper & Soboloff, 2015, Jing et al., 2015). CRACR2A regulates STIM1 and ORAI1 association and is essential for STIM1/ORAI1 clustering at the PM-ER junctions (Srikanth et al., 2010). Over-expression of the SOCE facilitators, *CRACR2A* and *STIMATE*, significantly reduced progeny virus output from tracheal epithelial cells and muscle cells (Figure 3-28). Facilitating SOCE activation had the greatest effect on tracheal epithelial cells. *CRACR2A* over-expression induced the greatest impairment of virus replication, reducing USSR H1N1 progeny virus output by 66.21% in NPTr cells, 28.38% in porcine myoblasts and 80.16% in NHBE cells.

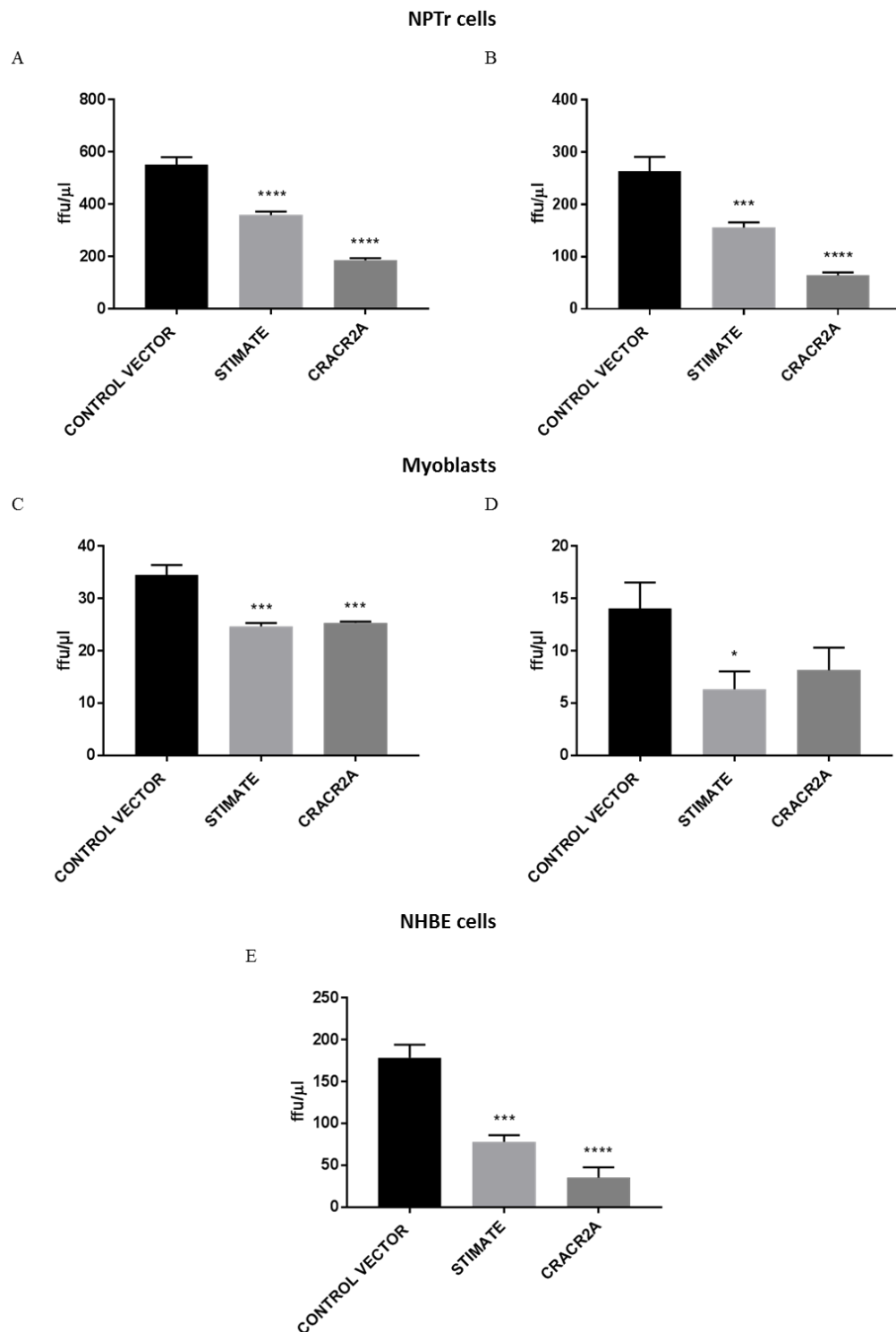


Figure 3-28. Over-expression of SOCE regulators, CRAC2RA and STIMATE, significantly reduced progeny influenza virus release. NPTTr cells, stably transfected with indicated plasmids, were infected with (A) USSR H1N1 or (B) pandemic H1N1 at 0.5 MOI for 24 h. Porcine myoblasts, transiently transfected with indicated

plasmids, were infected with (C) USSR H1N1 or (D) pandemic H1N1 at 2.0 MOI for 24 h. (E) NHBE cells, transiently transfected with indicated plasmids for 48 h, were infected with USSR H1N1 at 1.0 MOI for 24 h. Spun supernatants were used to infect MDCK cells for 6 h in focus forming assays (FFAs). Significance determined by a one way ANOVA, comparing to the control vector.

3.3.13 Over-expression of SOCE facilitators, CRACR2A and STIMATE, inhibited influenza A virus replication post-transcriptionally

Over-expressing the ORAI homologues or *STIM1* significantly reduced viral M gene expression (Section 3.3.10). In contrast, overexpressing *STIMATE* or *CRACR2A* did not reduce viral M gene expression (Figure 3-29). Again, porcine myoblasts differed from the overall pattern, as transfected myoblasts infected with USSR H1N1 virus displayed a significant downregulation in viral M gene expression (Figure 3-29C). This was not consistent with pandemic H1N1 virus infection or the tracheal epithelial cells and may suggest cell type specific differences to SOCE activation. However, the evidence presented here continues to provide support for a SOCE mediated antiviral mechanism.

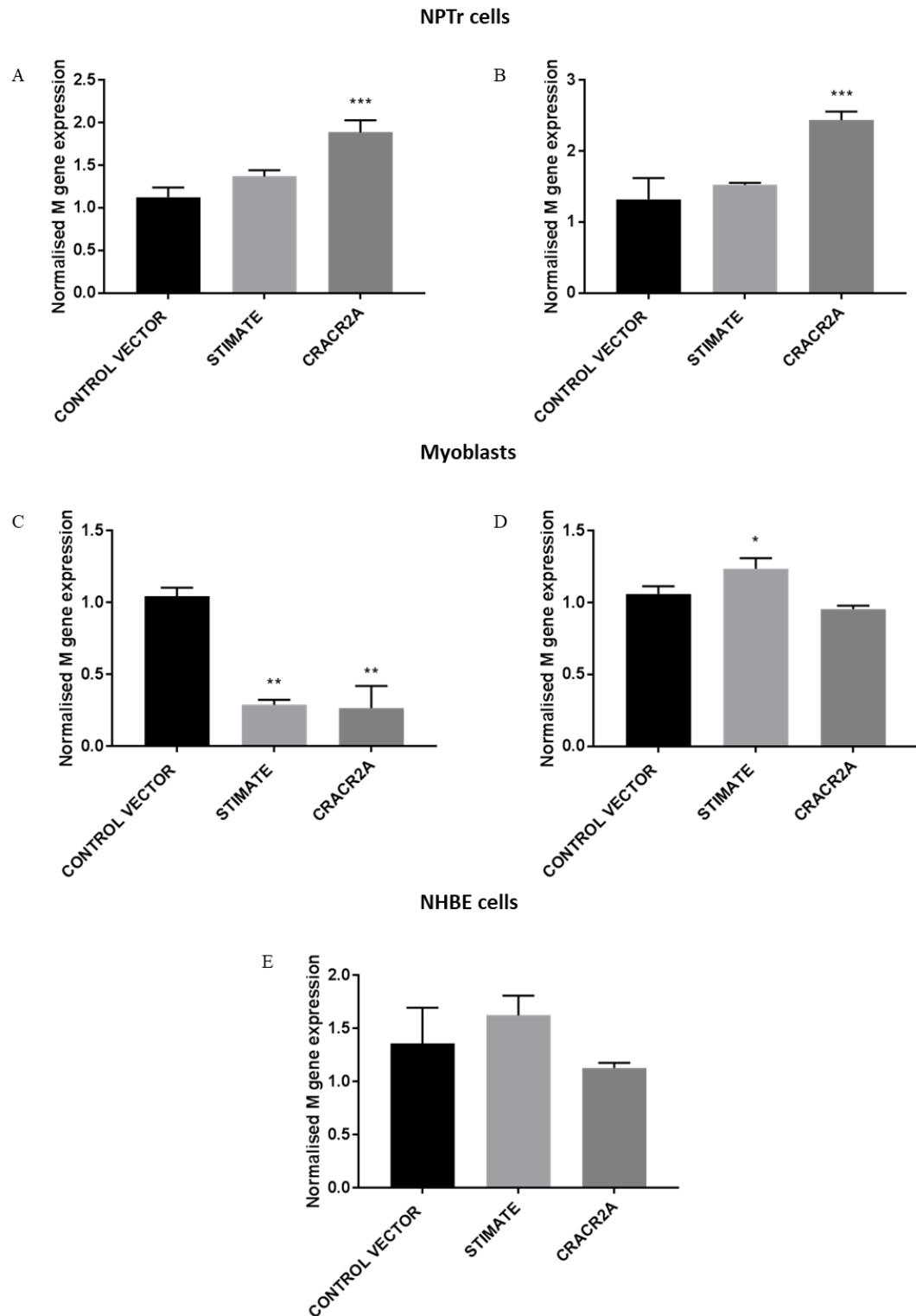


Figure 3-29. Over-expression of SOCE regulators, CRAC2RA and STIMATE, did not inhibit H1N1 virus transcription. NPTr cells, stably transfected with indicated plasmids, were infected with (A) USSR H1N1 or (B) pandemic H1N1 at 0.5MOI for 150

24 h. Porcine myoblasts, transiently transfected with indicated plasmids, were infected with (C) USSR H1N1 or (D) pandemic H1N1 at 2.0 MOI for 24 h. NHBE cells, transiently transfected with indicated plasmids for 48 h, were infected with (E) USSR H1N1 at 1.0 MOI for 24 h. Total RNA was extracted from each sample for fold viral M gene expression, normalised to 18s rRNA, calculated using the comparative *ct* method. Gene expression compared to the control vector (mCherry) awarded a relative expression of 1. Significance determined by a one way ANOVA, comparing to the control vector.

3.3.14 Increased cytosolic localisation of virus nucleoprotein in TG primed NHBE cells

Influenza virus nucleoprotein (NP) is transported into the nucleus for packing into the vRNP complex following translation in the cytosol, mediated by the two nuclear localisation signals (NLS) of NP (Davey *et al.*, 1985, Wu & Panté, 2009, Cros *et al.*, 2005, Wu *et al.*, 2017). The nuclear localisation signals of NP associate with importin- α of the importin- α/β nuclear import pathway (Nakada *et al.*, 2015, Labaronne *et al.*, 2017, Wu *et al.*, 2017). Intracellular Ca^{2+} dependent regulation nuclear import has been reported (Kaur *et al.*, 2014, Sweitzer & Hanover, 1996, Greber & Gerace, 1995). Transient fluctuations in cytosolic Ca^{2+} , induced by intracellular store depletion by TG treatment, inhibited passive and signal mediated nuclear translocation of proteins from the cytosol (Greber & Gerace, 1995). Elevated intracellular Ca^{2+} levels, induced by TG, were found to inhibit importin- α/β 1 and importin- β 1 mediated nuclear import (Kaur *et al.*, 2014). Therefore, as viral M gene expression and protein levels were unaffected by TG priming, a post-translational mechanism for TG mediated antiviral

activity dependent on impaired viral protein processing or intracellular transport was proposed. The subcellular localisation of NP in NHBE cells infected with USSR H1N1 virus for 6 h was assessed. Increased cytosolic localisation of NP was observed in NHBE cells primed with TG for 30 min prior to infection (Figure 3-30).

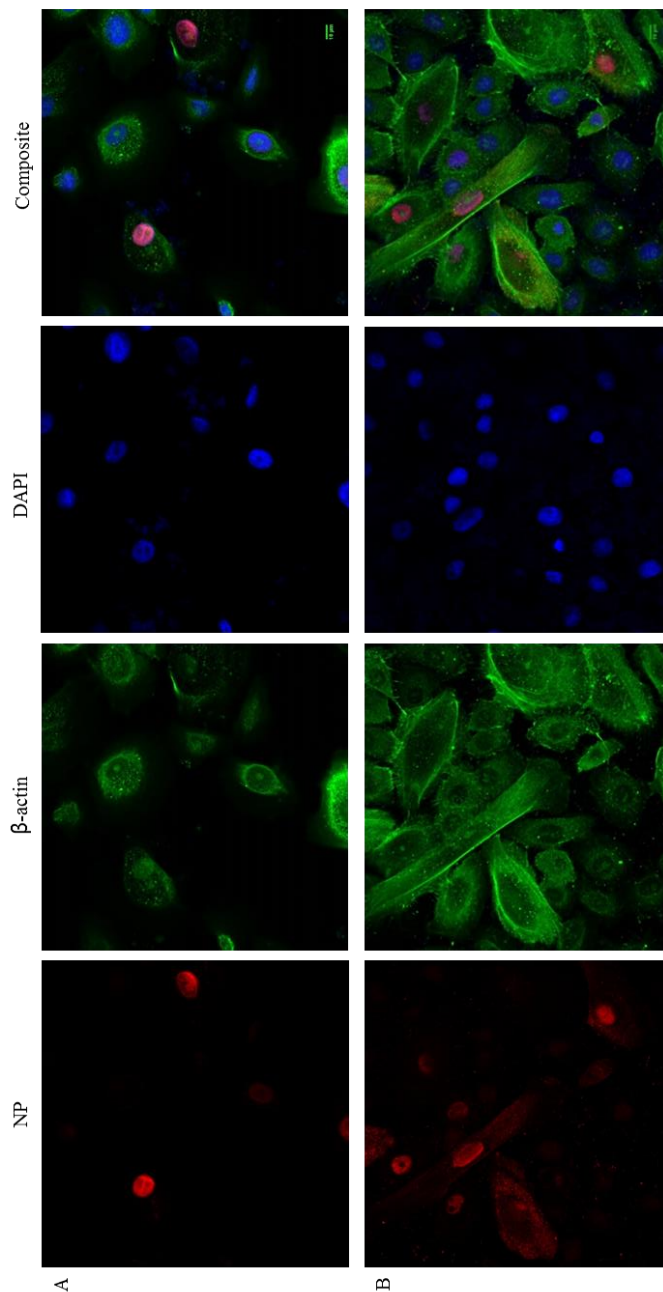


Figure 3-30. Increased cytosolic accumulation of viral nucleoprotein in TG treated NHBE cells. NHBE cells were primed with (A) DMSO or (B) 0.01 μ M TG for 30 mins and subsequently infected with USSR H1N1 at 1.0 MOI for 6 h. Cells were fixed in 4% paraformaldehyde and immunostained for beta actin (rabbit anti-beta actin primary antibody detected by goat anti-rabbit IgG H&L conjugated to Alexa Fluor 488 secondary antibody, green fluorescence) and viral nucleoprotein (mouse anti-influenza A virus nucleoprotein primary antibody detected by donkey anti-mouse IgG 153

H&L conjugated to Alexa Fluor 647 secondary antibody, red fluorescence). DNA was stained with DAPI (blue fluorescence). NHBE cells imaged on Zeiss 710 confocal microscope.

Quantitative analysis, comparing the ratio of nuclear NP fluorescent signal to cytosolic fluorescent signal, indicated a significant increase in cytoplasmic NP in TG primed cells (Figure 3-31). There was a >2.5 fold decrease in mean nuclear to cytosolic localisation ratio in TG treated cells. This proposes transport of NP into the nucleus was impaired by the TG mediated increase in cytosolic Ca^{2+} .

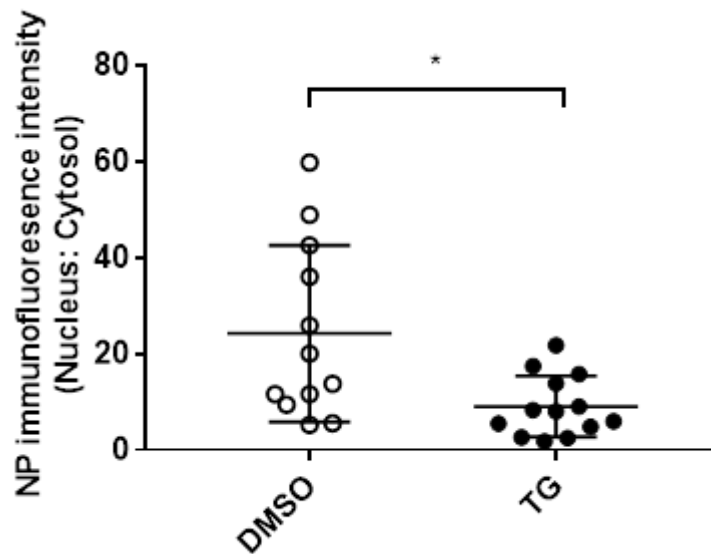


Figure 3-31. Reduced nuclear localisation of viral nucleoprotein in TG treated NHBE cells. *NHBE cells were primed with 0.005 μM TG for 30 min and subsequently infected with USSR H1N1 at 1.0 MOI for 6 h. The ratio of nuclear to cytoplasmic NP fluorescence signal in DMSO and TG primed NHBE cells was calculated using image J for each individual cell. Significance determined by paired t-test.*

Further work is required to explore any morphological changes observed in TG primed NHBE cells when compared to DMSO primed NHBE cells (Figure 3-32).

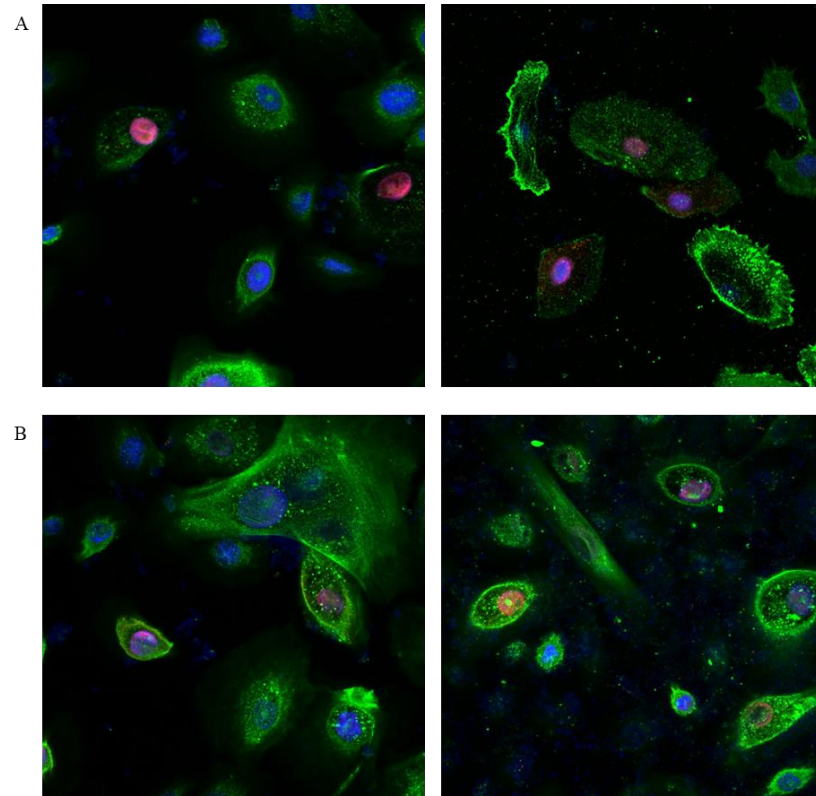


Figure 3-32. The impact of TG priming on NHBE cells morphology is unclear. NHBE cells were primed with (A) DMSO or (B) 0.01μM TG for 30 mins and subsequently infected with USSR H1N1 at 1.0 MOI for 6 h. Cells were fixed in 4% paraformaldehyde and immunostained for beta actin (rabbit anti-beta actin primary antibody detected by goat anti-rabbit IgG H&L conjugated to Alexa Fluor 488 secondary antibody, green fluorescence) and viral nucleoprotein (mouse anti-influenza A virus nucleoprotein primary antibody detected by donkey anti-mouse IgG H&L conjugated to Alexa Fluor 647 secondary antibody, red fluorescence). DNA was stained with DAPI (blue fluorescence). NHBE cells imaged on Zeiss 710 confocal microscope.

3.3.15 TG priming prior to influenza A virus infection reduced total progeny virus production

TG priming may reduce the number of viruses produced by the cells, perhaps interfering with virus budding. Alternatively, the total number of progeny virus may not be reduced and instead display reduced infectivity due to impaired virus protein folding, virus protein packaging or other mechanisms. The FFA measures progeny virus production through subsequent infection of MDCK cells by progeny virus present in the supernatant of cells infected with influenza H1N1 virus for 24 h. However, this only reflects the production of infective viruses. It does not differentiate between the possibility of defective virions with reduced infectivity or a reduced number of progeny virus. Viral M gene expression is proportional to the quantity of progeny virus, as viral RNA extraction was limited to progeny virus in the supernatant. Extraction of viral RNA from the progeny virus of tracheal epithelial cells (NPTr cells and NHBE cells) infected with USSR H1N1 virus for 24 h, revealed a significant reduction in viral M gene expression (Figure 3-33A). The reduction in viral M gene expression corresponded to the reduction of NP positive MDCK cells in the corresponding FFAs (Figure 3-33B and 3-33C). While this does not eliminate the possibility of the production of virions with reduced infectivity, it verifies TG priming induced an overall reduction in progeny virus output.

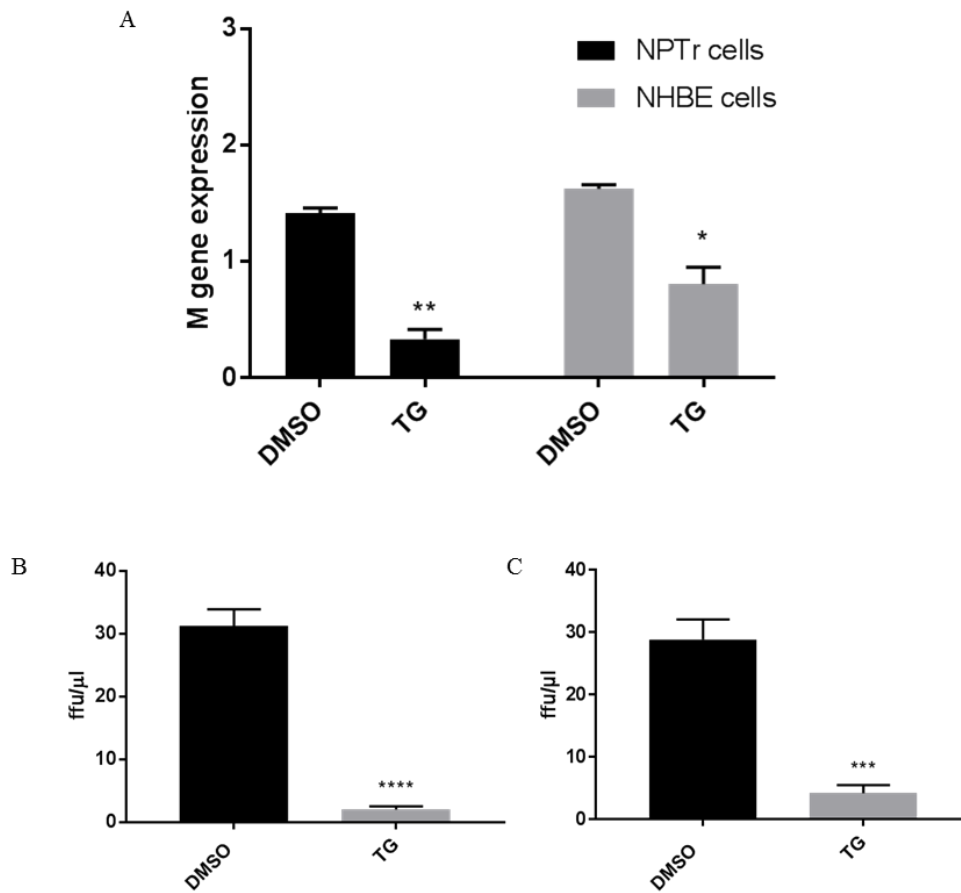


Figure 3-33. TG priming reduced total progeny virus production in tracheal epithelial cells. NPTTr cells primed with 0.5 μ M TG and NHBE cells primed with 0.01 μ M TG for 30 min prior to infection with USSR H1N1 virus at 0.5 and 1.0 MOI, respectively. (A) Viral RNA was extracted from the supernatant 24 h post infection and viral M gene expression relative to the corresponding DMSO control calculated. Spun supernatants were used to infect MDCK cells for 6 h in focus forming assays (FFAs) for (B) NPTTr cells and (C) NHBE cells. Significance determined by paired t-test.

3.3.16 TG pre-treatment did not impair virion morphology from infected NPTr cells

As TG treatment did not appear to impair the early stages of influenza virus replication (indicated by M gene expression and viral protein quantification), the effect of TG treatment in the later stages of virus replication was analysed. Influenza virus structure and budding through transmission electron microscopy (TEM) were analysed. A549 cells and NPTr cells were selected for TEM as they were suitable for TEM sample processing.

There was no significant difference in the morphology of the influenza A virus particles in TG primed cells relative to DMSO treatment. TG primed A549 cells and NPTr cells displayed the characteristic circular morphology with the darkened band of stained viral coat proteins, as observed following DMSO priming (Figure 3-34 and 3-35). Influenza virus structure was consistent with TEM images within the literature (Bruce et al., 2010, Ma *et al.*, 2012).

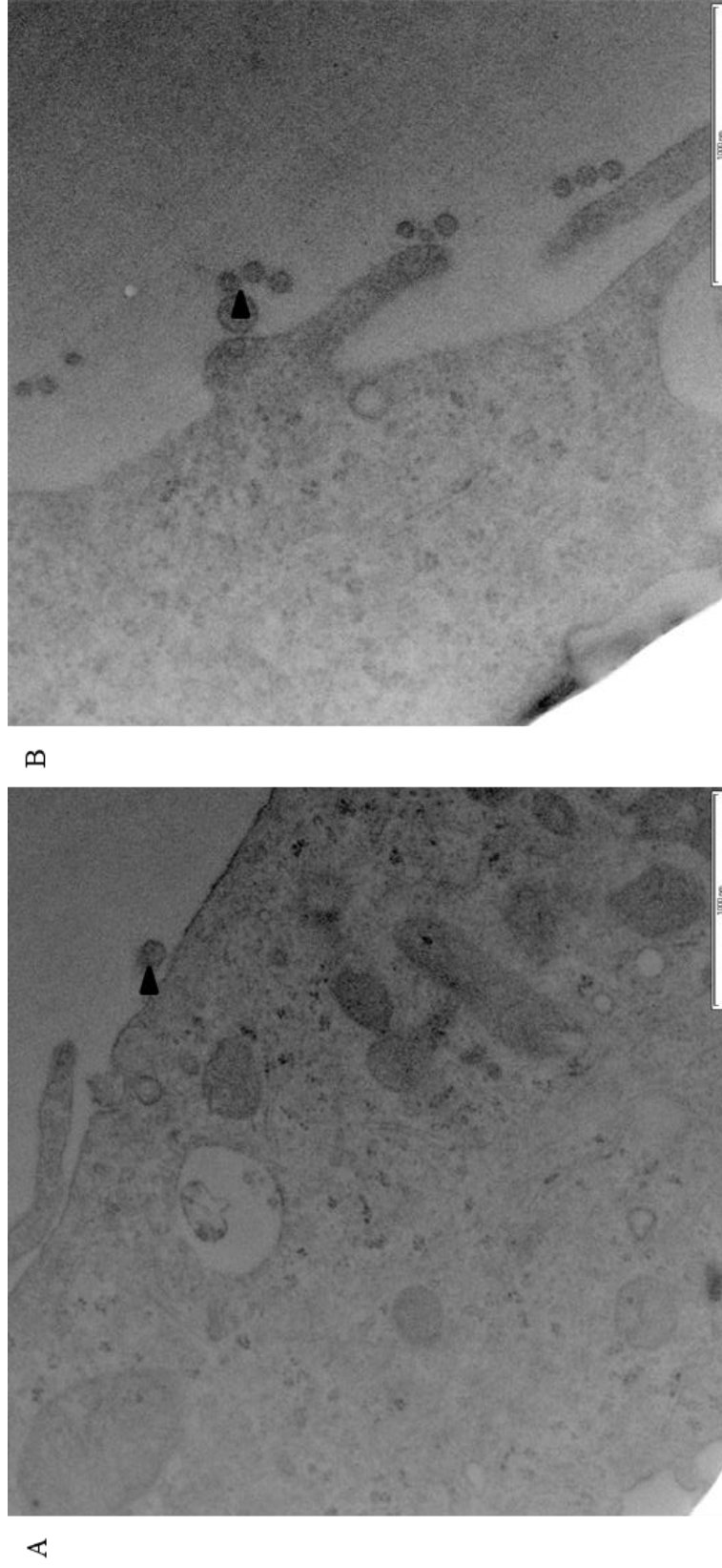


Figure 3-34. Pre-treatment with TG did not impair morphology of influenza virions from infected A549 cells. A549 cells were grown on Thermanox coverslips and pretreated with (A) DMSO or (B) 0.5μM TG for 30 min and subsequently washed with PBS and infected with USSR H1N1 at 1.0MOI for 12 h. The NPTr cells were processed for TEM. Examples of influenza virions marked with arrowheads. Scale bars: 1000nm.

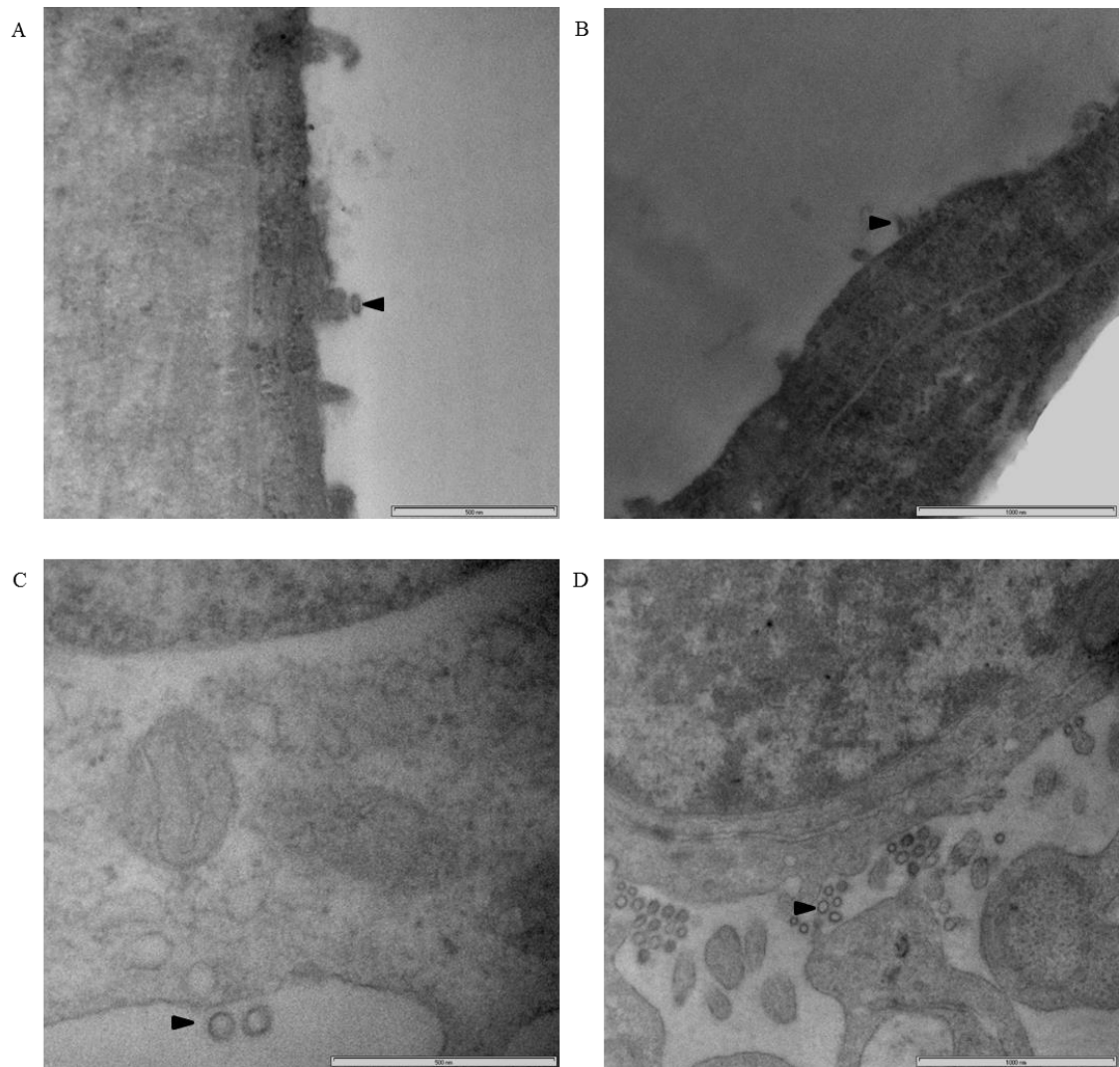


Figure 3-35. Pre-treatment with TG did not impair morphology of influenza virions from H1N1 infected NPTr cells. NPTr cells were grown on Thermanox coverslips and pretreated with (A,B) DMSO or (C,D) 0.5μM TG for 30 min, washed with PBS and infected with USSR H1N1 at 0.5MOI for 12 h. The NPTr cells were processed for TEM. Examples of influenza virions marked with arrowheads. Scale bars: (A, C) 500nm, (B,D) 1000nm.

3.3.17 TG elevated select innate immune signalling pathways in influenza A virus infected cells

In addition to evaluating the effect of TG directly on virus replication, the effect of TG priming on host innate immunity was considered. Modulation of the host signalling pathways has been proposed as a potential therapy following virus infection (Pattabhi *et al.*, 2015, Crosse *et al.*, 2018). The type I IFN signalling pathway is an essential component of the innate immune response following influenza virus infection. *RIG-I* and *OAS1* are well established as type I IFN dependent genes, the former a cytosolic dsRNA receptor and the latter generating 2'-5'-oligoadenylates which activates ribonuclease L in order to degrade viral RNA (Loo & Gale, 2011, Kwon *et al.*, 2013, Chakrabarti *et al.*, 2011b). TG priming significantly elevated expression of these type I IFN dependent genes in NPTr cells (Figure 3-36), primary porcine myoblasts (Figure 3-37) and NHBE cells (Figure 3-38) infected with USSR H1N1 virus or pandemic H1N1 virus. In addition, expression of *IFN β 1* was significantly enhanced in NHBE cells infected with USSR H1N1 virus but no expression was detected in uninfected NHBE cells (Figure 3-38). Elevated *OAS1* and *RIG-I* gene expression was limited to TG primed influenza virus infected cells, indicating the effect of TG on the type I IFN signalling pathway is specific to virus infected cells.

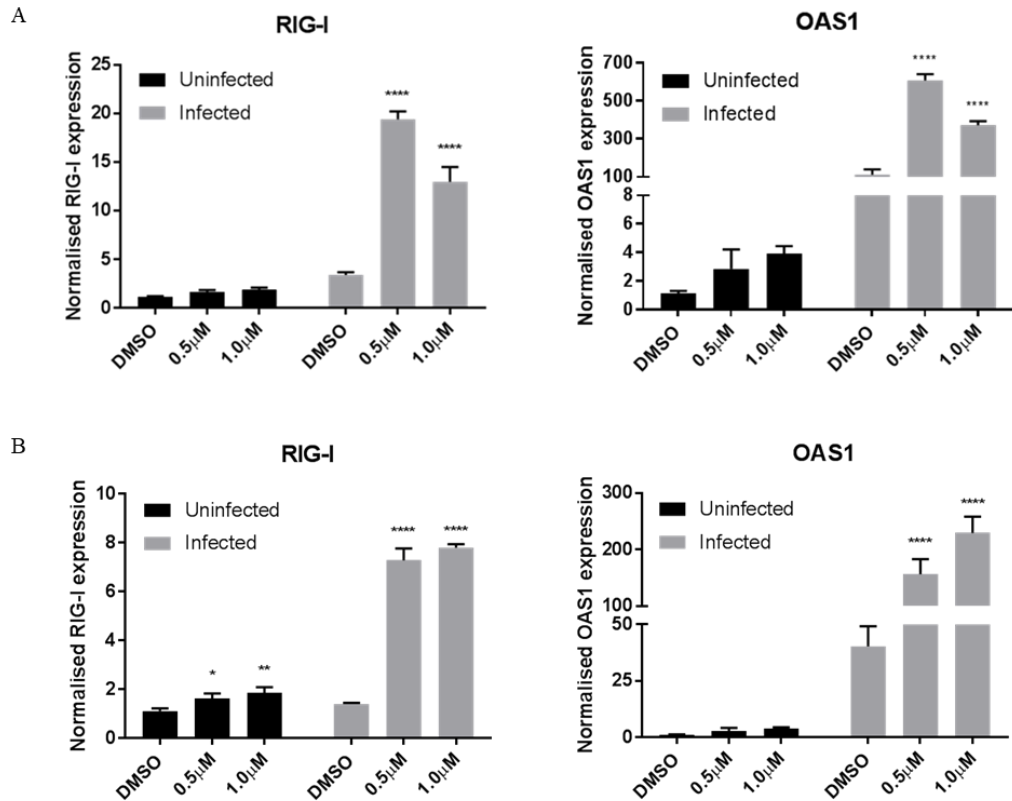


Figure 3-36. TG priming of NPTr cells elevated type I IFN dependent gene expression in response to infection. NPTr cells, pre-treated with 0.5 μ M or 1 μ M TG for 1 h, were subsequently infected with (A) USSR or (B) pandemic H1N1 virus at 0.5 MOI for 24 h. Total RNA extracted 24 h later. Fold type I IFN dependent gene expression, normalised to 18s rRNA, calculated using the comparative Ct method. Gene expression compared to the uninfected DMSO control awarded a relative expression of 1. Significance determined by two way ANOVA, relative to the corresponding DMSO control.

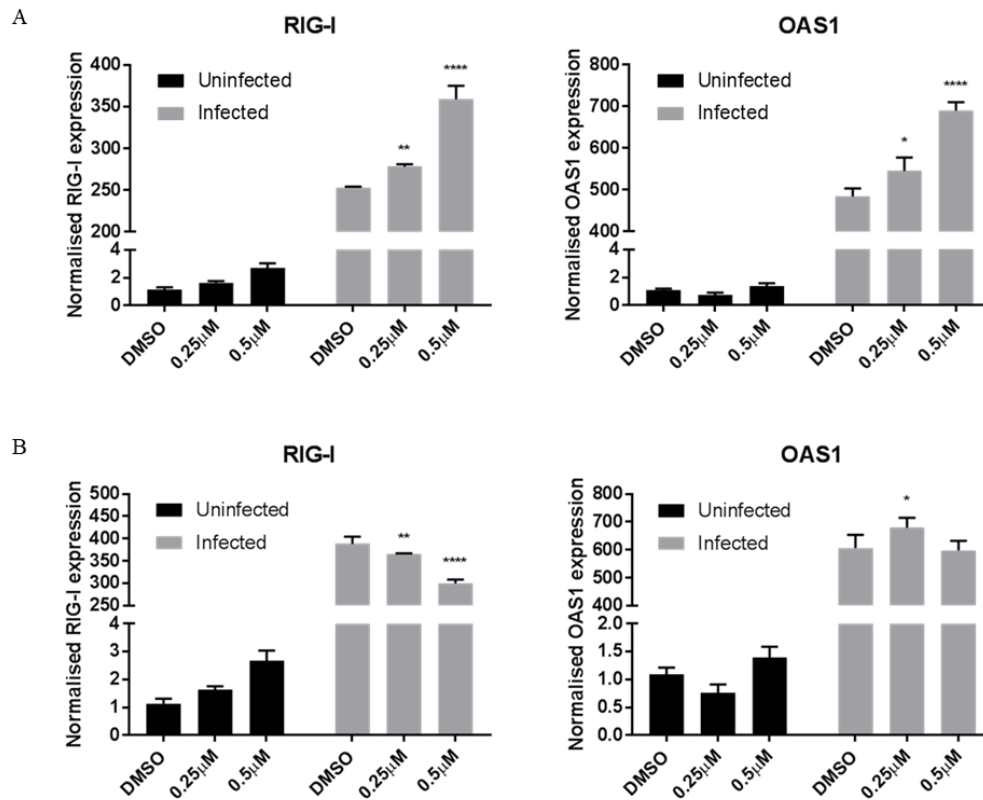


Figure 3-37. TG priming of porcine myoblasts elevated type I IFN dependent gene expression in response to infection. Porcine myoblasts, pre-treated with 0.25 μ M or 0.5 μ M TG for 30 min, were subsequently infected with (A) USSR or (B) pandemic H1N1 virus at 2.0 MOI for 24 h. Total RNA extracted 24 h later. Fold type I IFN dependent gene expression, normalised to 18s rRNA, calculated using the comparative Ct method. Gene expression compared to the uninfected DMSO control awarded a relative expression of 1. Significance determined by two way ANOVA, relative to the corresponding DMSO control.

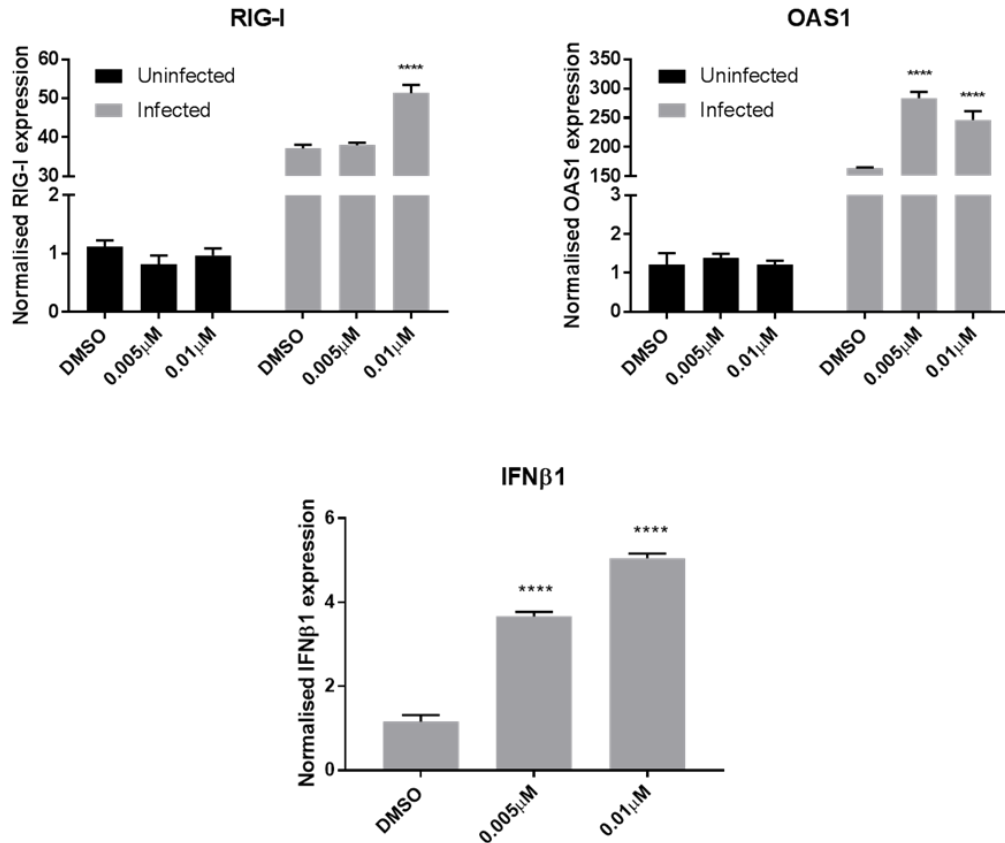


Figure 3-38. TG priming of NHBE cells elevated type I IFN dependent gene expression in response to infection. NHBE cells, pre-treated with 0.005μM or 0.01μM TG for 30 min, were subsequently infected with USSR H1N1 at 0.5 MOI for 24 h. Total RNA extracted 24 h later. Fold type I IFN dependent gene expression, normalised to 18s rRNA, calculated using the comparative Ct method. IFNβ RNA was below the threshold of detection in uninfected NHBE cells. Gene expression compared to the uninfected DMSO control awarded a relative expression of 1. Significance determined by two way ANOVA, relative to the corresponding DMSO control.

Primary porcine myoblasts infected with pandemic H1N1 virus displayed down regulation of type I dependent gene expression in TG primed cells (Figure 3-37).

However, if the duration of TG priming was increased to an hour, *RIG-I* and *OAS1* expression was elevated in pandemic H1N1 virus infection (Figure 3-39).

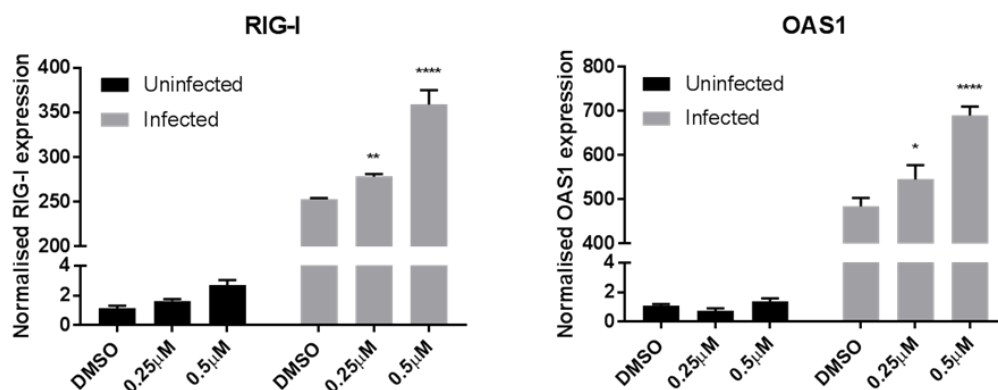


Figure 3-39. Extended TG priming of porcine myoblasts elevated type I IFN dependent gene expression in response to pandemic H1N1 virus infection. Porcine myoblasts, pre-treated with 0.25 μ M or 0.5 μ M TG for 1 h, were subsequently infected with pandemic H1N1 virus at 2.0 MOI for 24 h. Total RNA extracted 24 h later. Fold type I IFN dependent gene expression, normalised to 18s rRNA, calculated using the comparative Ct method. Gene expression compared to the uninfected DMSO control awarded a relative expression of 1. Significance determined by two way ANOVA, relative to the corresponding DMSO control.

In contrast, the NF- κ B signalling pathway was inhibited by TG priming, reflected by the downregulated expression of target genes *interleukin-6* (*IL-6*), and *TNF α induced protein 3* (*TNFAIP3*) in NHBE cells (Figure 3-40). The inhibitory activity of sesquiterpene lactones on the NF- κ B signalling pathway has already been described (Rüngeler *et al.*, 1999). However, TG mediated inducement of ER stress has been implicated in NF- κ B signalling activation (Kaneko *et al.*, 2003, Zhu *et al.*, 2017b).

The discrepancy may be attributed to the severity of the ER stress response. Priming with non-cytotoxic concentrations of TG for a short period of time may inhibit NF- κ B dependent gene expression while priming with detrimental TG concentrations may induce NF- κ B signalling.

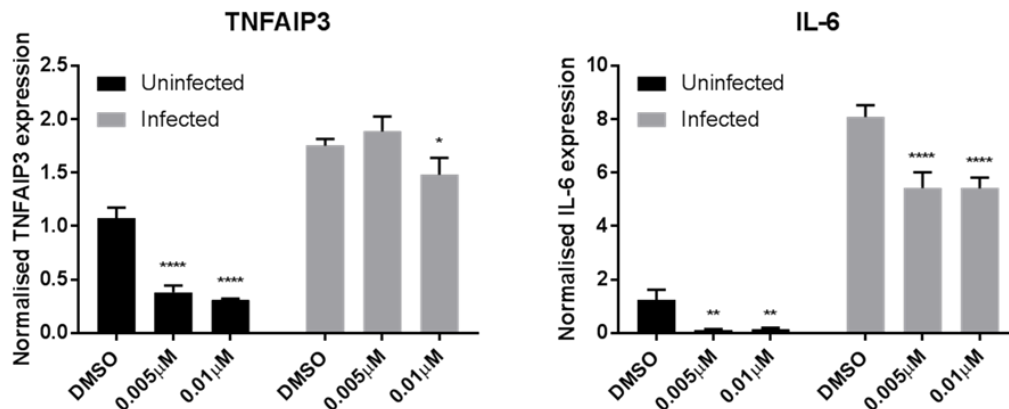


Figure 3-40. TG priming inhibited NF- κ B dependent gene expression in NHBE cells. NHBE cells, pre-treated with 0.005 μ M or 0.01 μ M TG for 30 mins, were subsequently infected with USSR H1N1 at 1.0 MOI for 24 h. Total RNA extracted 24 h later. Fold NF- κ B dependent gene expression, normalised to 18s rRNA, calculated using the comparative ct method. Gene expression compared to the uninfected DMSO control awarded a relative expression of 1. Significance determined by two way ANOVA, relative to the corresponding DMSO control.

3.3.18 Facilitating SOCE through over-expression studies did not elevate expression of type I IFN dependent genes

Over-expression of SOCE components significantly impaired influenza virus replication, an effect shared by TG. However, while TG has been established as a

potent inducer of type I IFN dependent gene expression in tracheal epithelial cells and muscle cells, over-expression of *STIM1* or *ORAI1-3* revealed a distinction between the TG mediated antiviral response and STIM1/ORAI mediated immunity. Over-expression of *STIM* and the ORAI homologues did not elevate expression of the type I IFN responsive genes, *RIG-I* and *OAS1* in NPTr cells (Figure 3-41), primary porcine myoblasts (Figure 3-42) and NHBE cells (Figure 3-43), regardless of infection with USSR H1N1 virus.

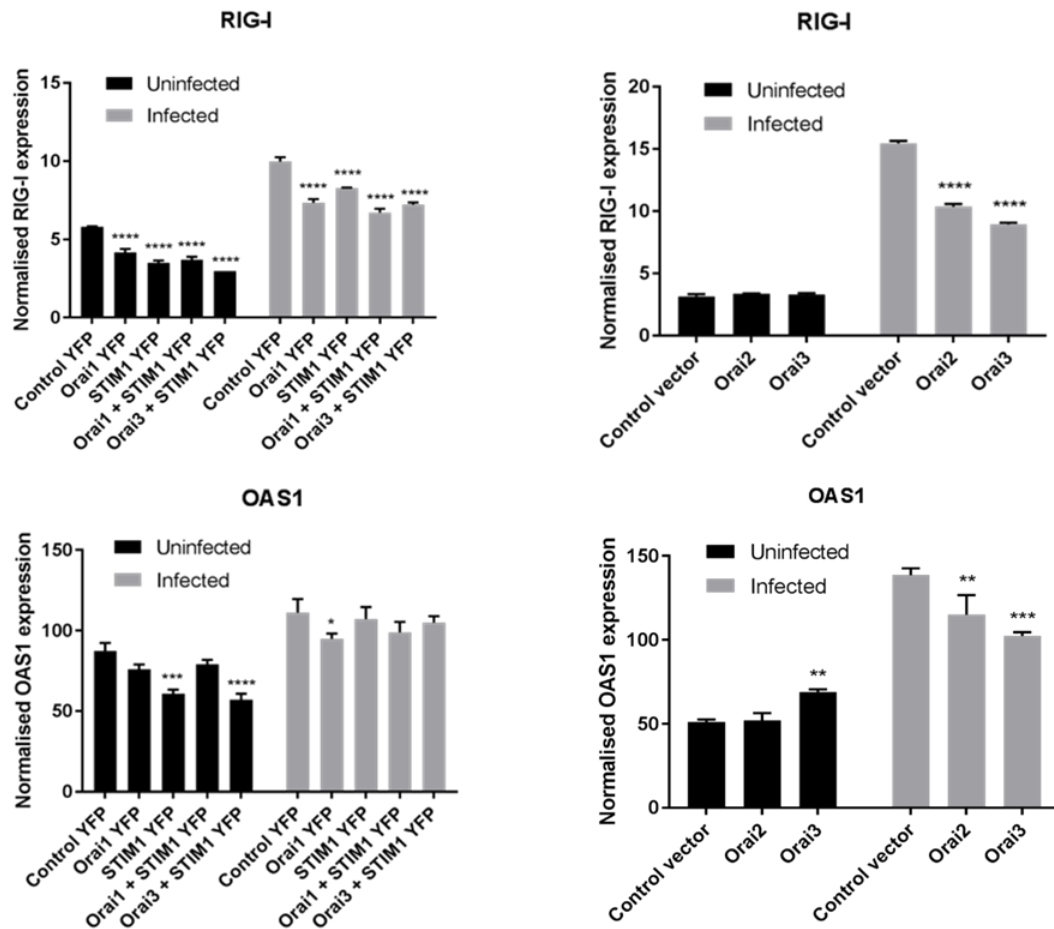


Figure 3-41. Over-expression of *STIM1* and *ORAI* did not increase *RIG-I* or *OAS1* expression in NPTr cells. NPTr cells, briefly transfected with the indicated plasmids for 2 days, were infected with USSR H1N1 virus at 0.5 MOI for 24 h. Total RNA was extracted from each sample for type I dependent IFN gene expression, normalised to 18S rRNA. Significance determined by one way ANOVA, calculated using the comparative ct method. Gene expression compared to the uninfected DMSO control awarded a relative expression of 1. YFP = yellow fluorescent protein.

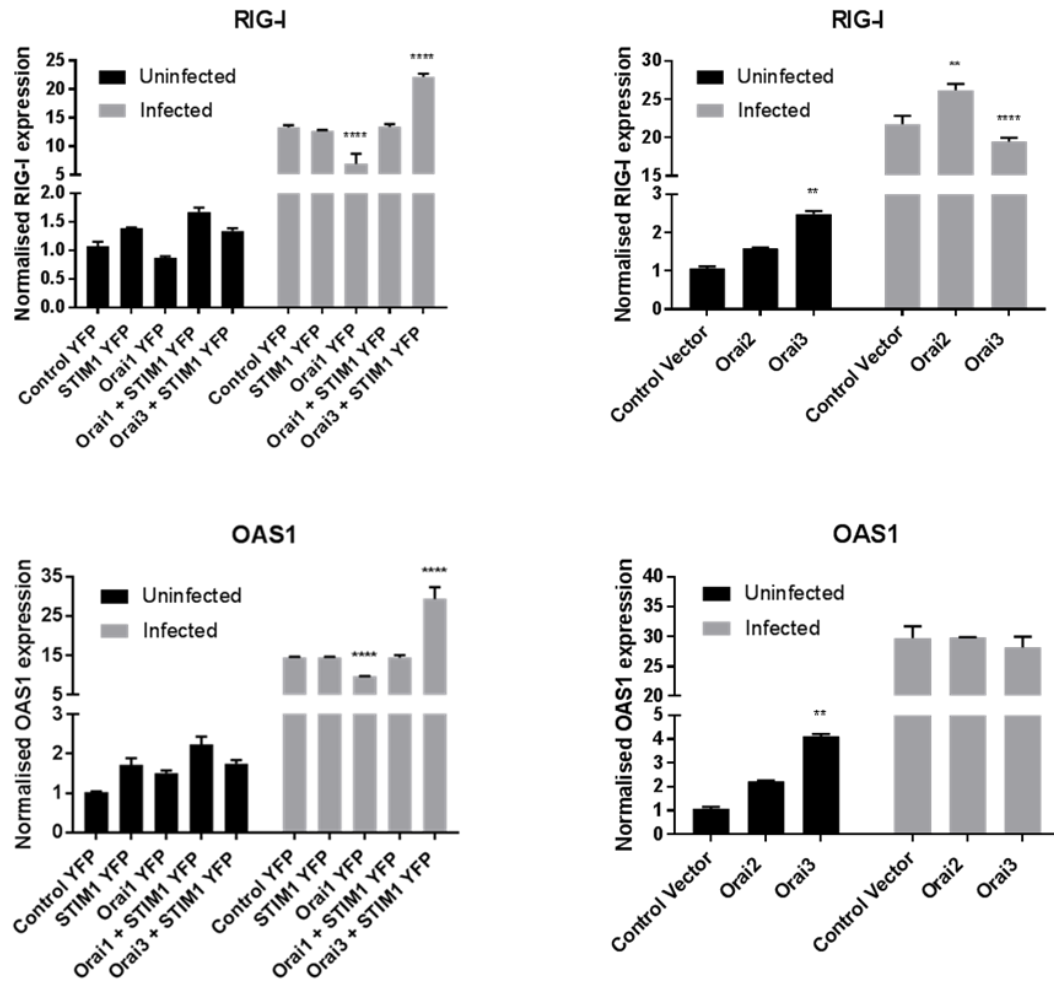


Figure 3-42. Over-expression of STIM1 and ORAI did not increase RIG-I or OAS1 expression. Porcine myoblasts, briefly transfected with the indicated plasmids for 2 days, were infected with USSR H1N1 virus at 2.0 MOI for 24 h. Total RNA was extracted from each sample for type I dependent IFN gene expression, normalised to 18S rRNA. Significance determined by one way ANOVA, calculated using the comparative ct method. Gene expression compared to the uninfected DMSO control awarded a relative expression of 1. YFP = yellow fluorescent protein

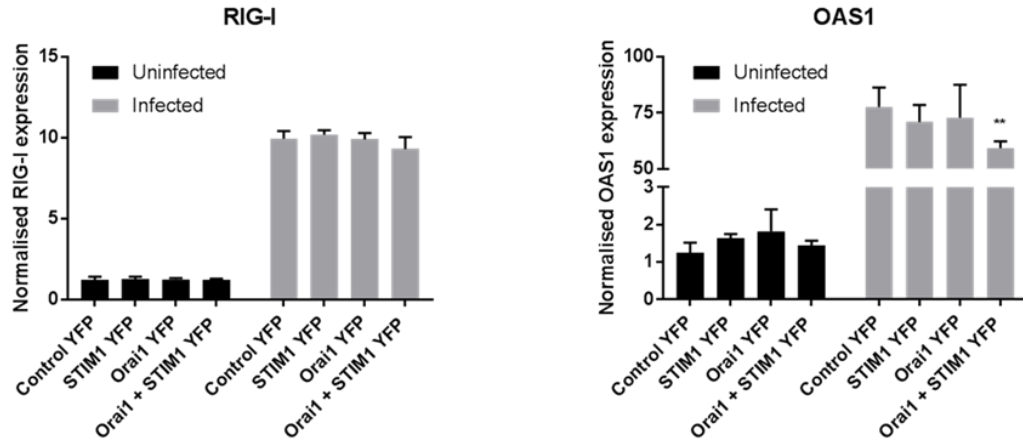


Figure 3-43. Over-expression of *STIM1* and *ORAI 1* did not increase *RIG-I* or *OAS1* expression. NHBE cells, briefly transfected with the indicated plasmids for 2 days, were infected with USSR H1N1 virus at 1.0 MOI for 24 h. Total RNA was extracted from each sample for type I dependent IFN gene expression, normalised to 18S rRNA. Significance determined by one way ANOVA, calculated using the comparative ct method. Gene expression compared to the uninfected DMSO control awarded a relative expression of 1. YFP = yellow fluorescent protein

In addition, facilitating SOCE activation through *CRACR2A* or *STIMATE* over-expression did not enhance expression of type I IFN dependent genes, *RIG-I* or *OAS1* (Figure 3-44). The expression pattern was inconsistent across cell types. In particular, type I IFN dependent gene expression was reduced in H1N1 virus infected NPTr cells (Figure 3-44A).

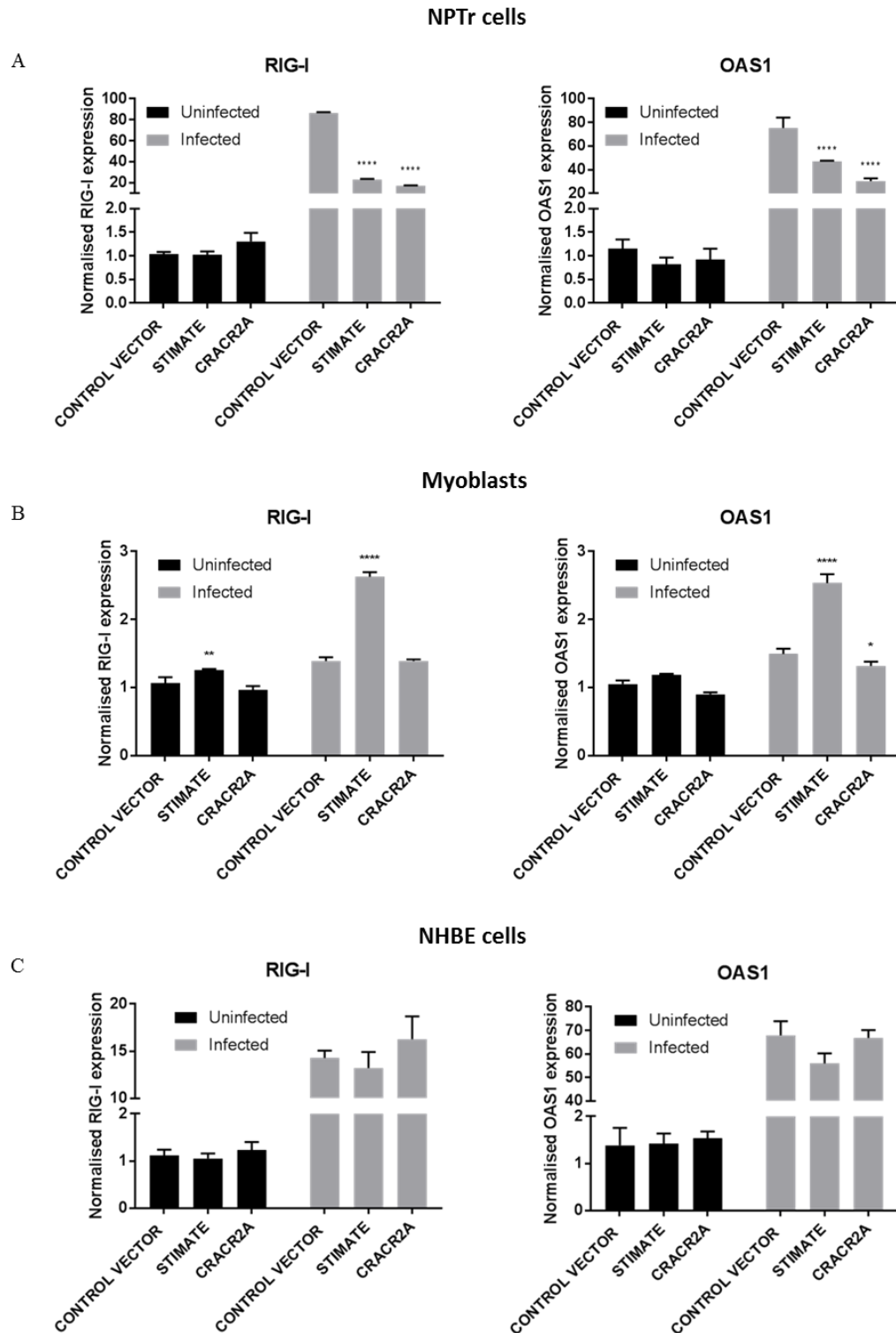


Figure 3-44. Over-expression of SOCE regulators, CRAC2RA and STIMATE, reduced type I dependent gene expression. (A) NPTr cells, stably transfected with indicated plasmids, were infected with USSR H1N1 at 0.5MOI for 24 h. (B) Porcine

myoblasts, transiently transfected with indicated plasmids for 48 h, were infected with USSR H1N1 at 2.0 MOI for 24 h. (C) NHBE cells, transiently transfected with indicated plasmids for 48 h, were infected with USSR H1N1 at 1.0 MOI for 24 h. Total RNA was extracted from each sample for type I dependent IFN gene expression, normalised to 18S rRNA, calculated using the comparative ct method. Fold gene expression relative to the uninfected control vector (mCherry), awarded a relative value of 1. Significance determined by a one way ANOVA, comparing to the corresponding control vector.

However, the process of transfection had an impact on the basal immune state of the cells. Transfected NPTr cells and porcine myoblasts displayed higher *RIG-I* and *OAS1* expression in uninfected and influenza virus infected cells, in comparison to DMSO primed cells (Figure 3-45). The unintended side effect of transfection on basal type I IFN expression is reflected by the significant increase in type I IFN dependent gene expression in uninfected cells expressing the negative control vector (pcDNA3.2 YFP) relative to DMSO primed cells. Activation of negative regulatory feedback mechanisms inhibiting further gene expression may account for the lack of elevated *RIG-I* or *OAS1* expression in H1N1 virus infected cells overexpressing *STIM1*, *ORAI1*, *ORAI2*, *ORAI3*, *STIMATE* or *CRACR2A*. Activation of the type I IFN response following over-expression of SOCE components may be better reflected at the protein level, rather than the transcriptional level.

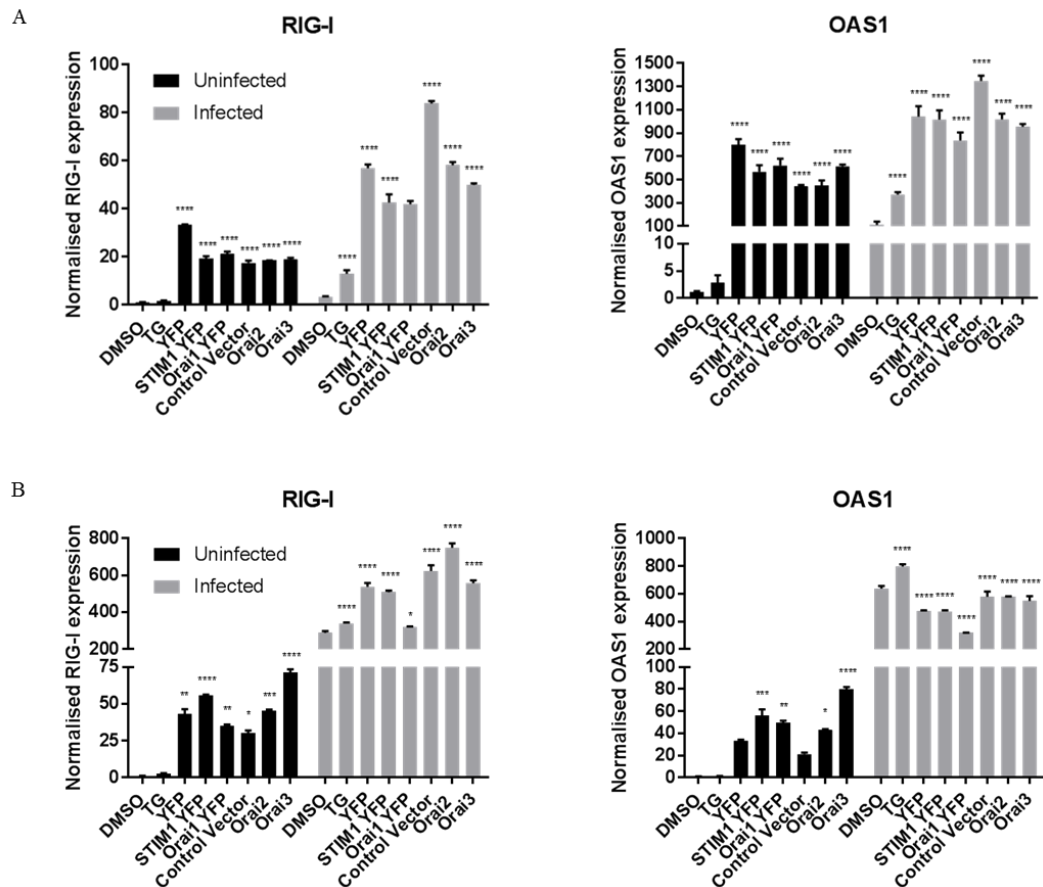


Figure 3-45. Transfection induced the type I IFN response in uninfected cells. Normalised expression of type I IFN dependent gene expression in uninfected (A) NPTr cells and (B) porcine myoblasts, relative to the uninfected DMSO control. The cells were briefly transfected with the indicated plasmids for 2 days or primed with DMSO or TG at (A) 1.0 μ M and (B) 0.5 μ M for 30 min. The cells were subsequently infected with USSR H1N1 virus at (A) 0.5 MOI and (B) 2.0 MOI for 24 h. Total RNA was extracted from each sample for type I IFN dependent gene expression, normalised to 18s rRNA, calculated using the comparative Ct method. Significance determined by 2 way ANOVA, relative to the corresponding TG treatment within uninfected and infection groups.

Alternatively, these results may indicate a distinct TG mediated antiviral response dependent on activation of the type I IFN response independent of SOCE.

3.3.19 Knockdown of STIM1 and ORAI1 reduced type I IFN dependent gene expression

Due to potential unintended effects of overexpressing SOCE components on basal immune gene expression, siRNA mediated knockdown may provide an accurate reflection of the contribution of SOCE to the type I IFN signalling pathway. Knockdown of *STIM1* and *ORAI1* in NHBE cells significantly impaired USSR H1N1 virus induced expression of *RIG-I* and *OAS1*, thereby supporting the proposed contribution of SOCE to activation of the type I IFN signalling pathway (Figure 3-46).

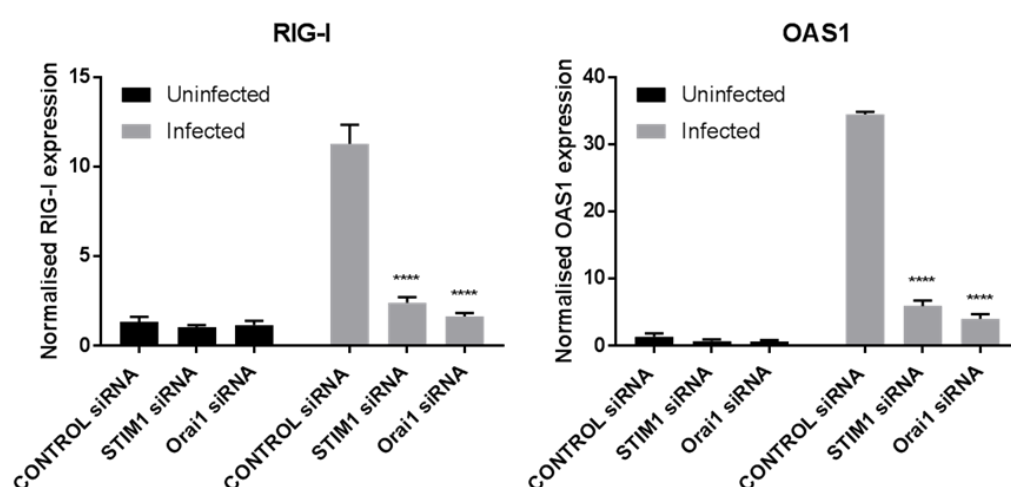


Figure 3-46. Type I IFN dependent gene expression of infected NHBE cells was impaired by STIM1 and ORAI1 knockdown. NHBE cells, transiently transfected with the indicated siRNAs for 48 h were subsequently infected with USSR H1N1 at 1.0 MOI for 24 h. Total RNA was extracted from each sample for type I dependent IFN gene expression, normalised to 18S rRNA, calculated using the comparative Ct method.

Fold gene expression relative to the uninfected control siRNA. Significance determined by a two way ANOVA, comparing to the corresponding control vector.

3.3.20 ER stress partially contributed to TG mediated induction of the type I IFN response

The ER stress induced unfolded protein response (UPR) can regulate the expression of multiple components of the immune response (Iwakoshi *et al.*, 2003, Hosoi *et al.*, 2013, Liu *et al.*, 2012a). TG mediated depletion of the Ca^{2+} ER store induces ER stress (Mekahli *et al.*, 2011, Yoshida *et al.*, 2006). Tunicamycin is utilised as an inducer of ER stress independent of Ca^{2+} depletion (Osowski & Urano, 2011, Kamiya *et al.*, 2013). Comparison between Ca^{2+} dependent and Ca^{2+} independent ER stress, mediated by TG and tunicamycin respectively, allowed the contribution of calcium mobilisation on ER stress response and type I IFN dependent gene expression in influenza virus infected cells to be determined.

TG was a potent inducer of ER stress, demonstrated by the significant upregulation of DNA Damage Inducible Transcript 3 (*DDIT3*), heat shock 70 kDa protein 5 (*HSPA5*) and Heat Shock Protein 90 Beta Family Member 1 (*HSP90B1*) in NPTr cells, NHBE cells and myoblasts (Figure 3-47). H1N1 virus infection alleviated TG mediated ER stress, which may be an influenza virus mediated mechanism for promoting viral protein translation and processing. However, TG priming still induced a significant increase in the expression of ER stress markers in USSR H1N1 virus infected cells, an effect that was particularly pronounced in NHBE cells (Figure 3-47C).

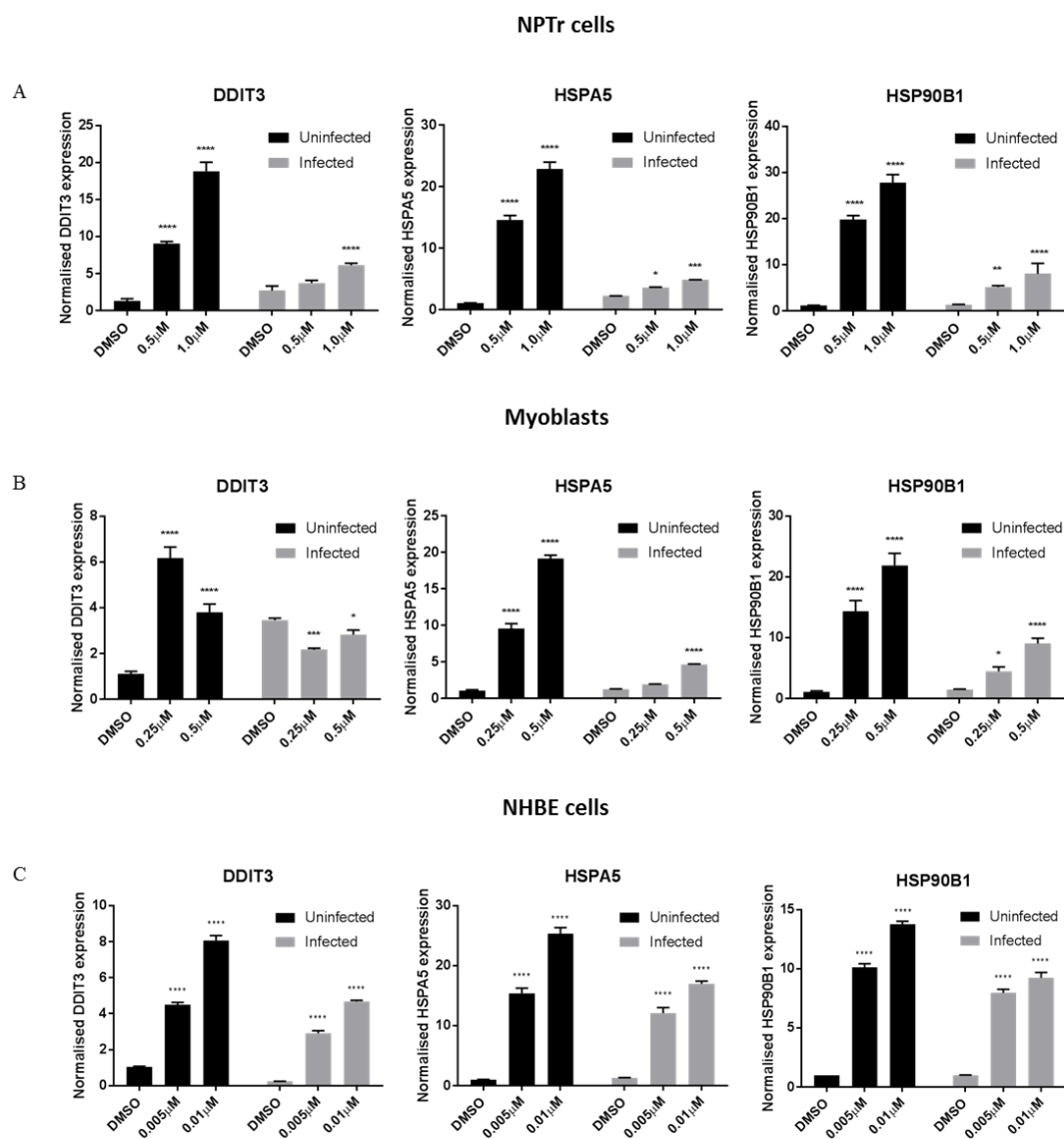


Figure 3-47. TG priming induced expression of endoplasmic reticulum stress genes.

(A) NPTr cells, pre-treated with 0.5 μ M or 1.0 μ M TG for 1 h, (B) porcine myoblasts, pre-treated with 0.25 μ M or 0.5 μ M TG for 30 min and (C) NHBE cells, pre-treated with 0.005 μ M or 0.01 μ M TG for 30 min were subsequently infected with USSR H1N1 at 0.5 MOI, 1.0 MOI and 2.0 MOI, respectively, for 24 h. Total RNA extracted 24 h later. Fold expression of ER stress markers, normalised to 18s rRNA, calculated using the comparative Ct method. Gene expression compared to the uninfected DMSO

control awarded a relative expression of 1. Significance determined by two way ANOVA, relative to the corresponding DMSO control.

Cytosolic Ca^{2+} was measured following TG or tunicamycin priming to confirm tunicamycin did not induce an increase in cytosolic Ca^{2+} . Tunicamycin did not induce an increase in cytosolic Ca^{2+} in pig or human tracheal epithelial cells, in comparison to the significant increase following TG treatment (Figure 3-48A and 3-48B). However, tunicamycin and TG treatment significantly induced an increase in intracellular Ca^{2+} in porcine myoblasts (Figure 3-48C).

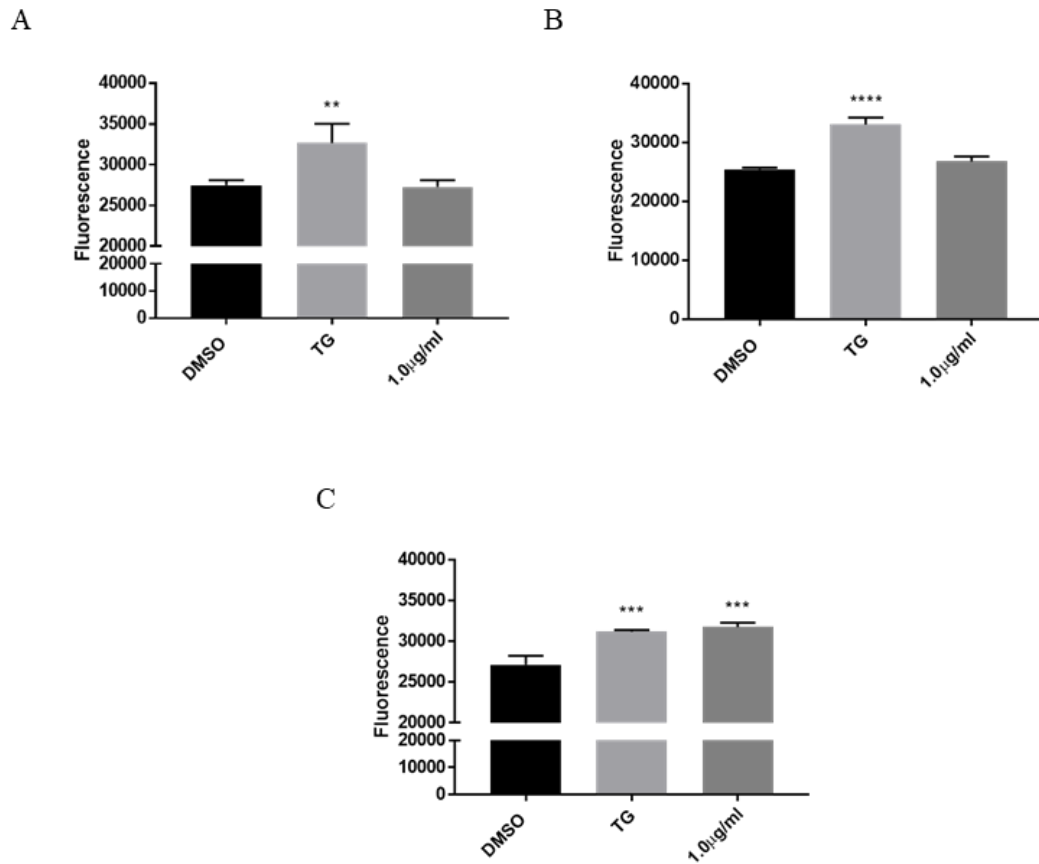


Figure 3-48. Tunicamycin did not induce an influx of Ca^{2+} into the cytosol in epithelial cells. Fluo-8 loaded (A) NPTr cells, (B) NHBE cells and (C) porcine myoblasts were incubated with 1.0 µg/ml tunicamycin for 30 mins. TG, at 1.0 µM, 0.5 µM and 0.5 µM respectively, served as a positive control. Significance determined by one way ANOVA, comparing the treated cells to the DMSO control.

Subsequent analysis of tunicamycin mediated Ca^{2+} independent ER stress was limited to NPTr cells. Porcine myoblasts were not considered for further analysis due to the consistent increase in intracellular Ca^{2+} following tunicamycin exposure, indicating potential cell type specific responses (Figure 3-48C). NHBE cells were not suitable for study due to their sensitivity to tunicamycin priming (cell viability data not shown).

Priming NPTr cells with non-toxic concentrations (Figure 3-49A and 3-49B) of tunicamycin for 30 min significantly reduced progeny virus output (Figure 3-49C and 3-49E). Tunicamycin inhibited influenza virus replication post-transcriptionally, indicated by a lack of significant reduction in M gene expression (Figure 4-49D and 4-49F).

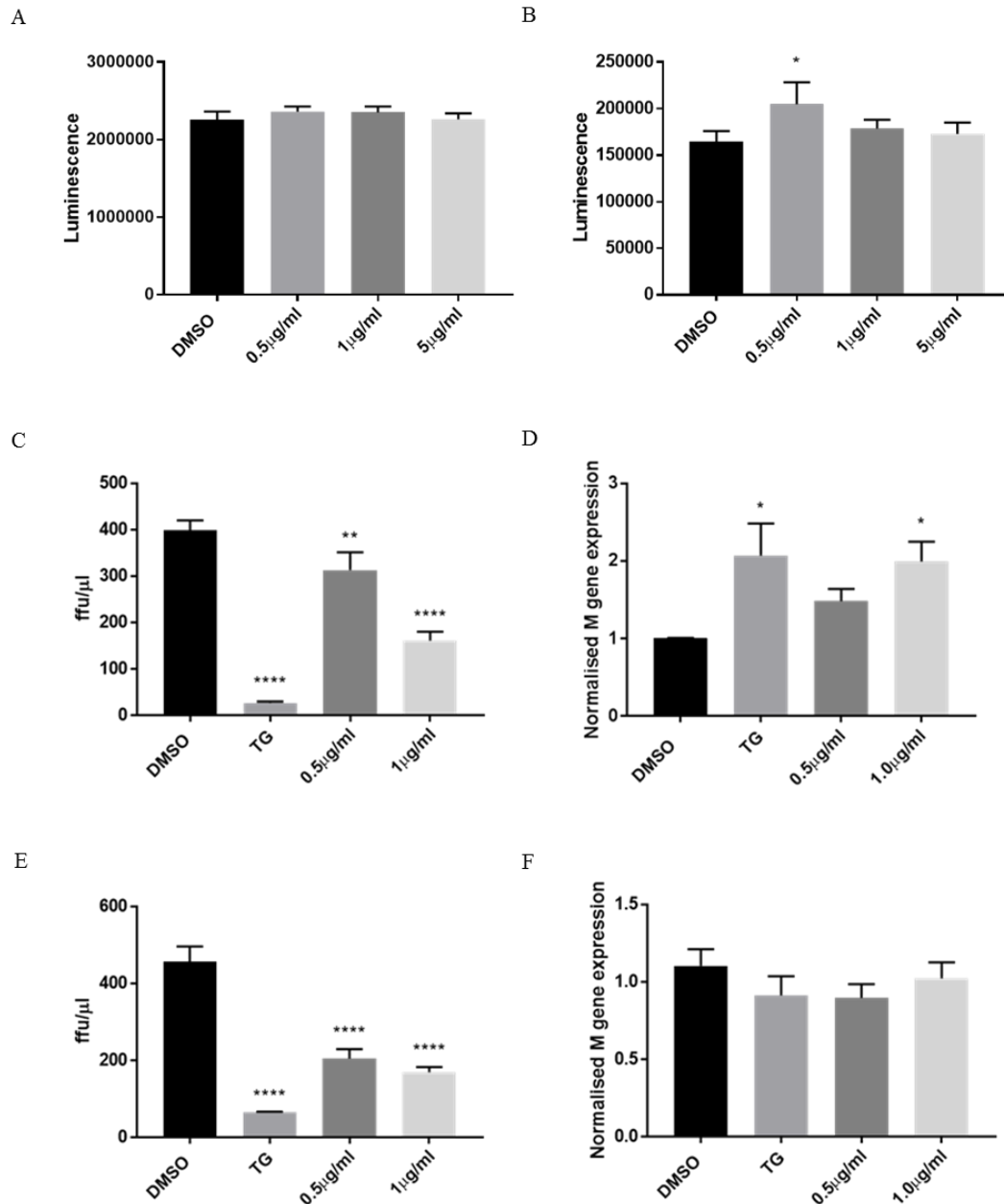


Figure 3-49. Tunicamycin treatment of NPTr cells reduced progeny viral output.

Cell viability of NPTr cells transiently exposed to indicated concentrations of tunicamycin measured using (A) CellTiter-Glo® Luminescent Cell Viability Assay and (B) Caspase3/7 Glo assay. NPTr cells exposed to the antibiotic for 30 mins before washing and media replacement. Cell viability measured 24 h later. NPTr cells, incubated with 0.5 µg/ml and 1.0 µg/ml tunicaymin for 30 mins were subsequently

infected with (C, D) USSR H1N1 or (E, F) pandemic H1N1 virus at 0.5 MOI for 24 h. TG exposure, 0.5 μ M for 30 mins prior to infection, served as a positive control. (C, E) Spun supernatants were used to infect MDCK cells for 6 h in focus forming assays (FFAs). (D, F) Total RNA was extracted from each sample for fold viral M gene expression, normalised to 18s rRNA, calculated using the comparative ct method. Gene expression compared to the DMSO control, awarded a relative expression of 1. Significance determined by one way ANOVA, comparing the treated cells to the DMSO control.

Tunicamycin priming induced expression of ER stress markers *DDIT3* and *HSPA5* in uninfected NPTr cells (Figure 3-50). Influenza A virus mediated attenuation of the ER stress response was also reflected in tunicamycin primed NPTr cells.

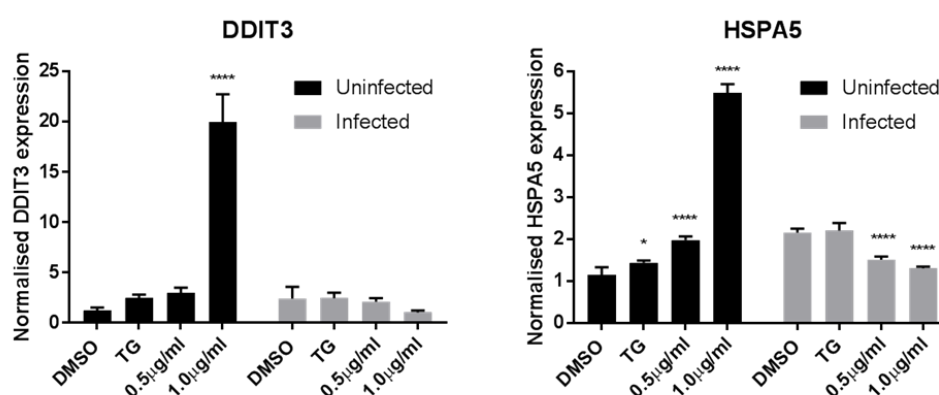


Figure 3-50. Tunicamycin enhanced endoplasmic reticulum stress marker gene expression in uninfected NPTr cells. NPTr cells, incubated with 0.5 μ g/ml and 1.0 μ g/ml tunicamycin for 30 mins were subsequently infected with USSR H1N1 virus at 0.5 MOI for 24 h. TG exposure, 0.5 μ M for 30 mins prior to infection, served as a positive control. Total RNA was extracted from each sample for fold ER stress marker gene expression, normalised to 18s rRNA, calculated using the comparative ct method.

Gene expression compared to the uninfected DMSO control, awarded a relative expression of 1. Significance determined by two way ANOVA, relative to the corresponding DMSO control.

However, the tunicamycin mediated ER stress response did not induce type I IFN signalling. Tunicamycin priming did not elevate *RIG-I* or *OAS1* expression, while TG priming significantly elevated type I IFN dependent gene expression in infected NPTr cells (Figure 3-51). This emphasised the distinct contribution of ER stress response and underlined the importance of Ca^{2+} mobilisation in ER stress mediated regulation of the type I IFN response.

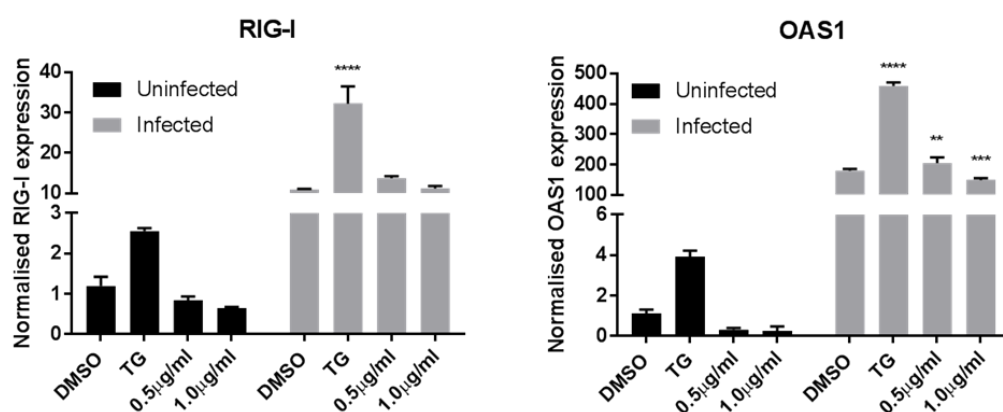


Figure 3-51. Tunicamycin did not elevate type I IFNs dependent gene expression.

NPTr cells, incubated with 0.5 µg/ml and 1.0 µg/ml tunicamycin for 30 mins were subsequently infected with pandemic H1N1 virus at 0.5 MOI for 24 h. TG (TG) exposure, 0.5 µM for 30 mins prior to infection, served as a positive control. Total RNA was extracted from each sample for fold type I IFN dependent gene expression, normalised to 18s rRNA, calculated using the comparative ct method. Gene expression compared to the uninfected DMSO control, awarded a relative expression of 1.

Significance determined by two way ANOVA, relative to the corresponding DMSO control.

3.3.21 Over-expression of SOCE components did not facilitate activation of the ER stress response

Over-expression of SOCE components revealed facilitating SOCE did not induce ER stress in NPTr cells (Figure 3-52), myoblasts (Figure 3-53) and NHBE cells (Figure 3-54).

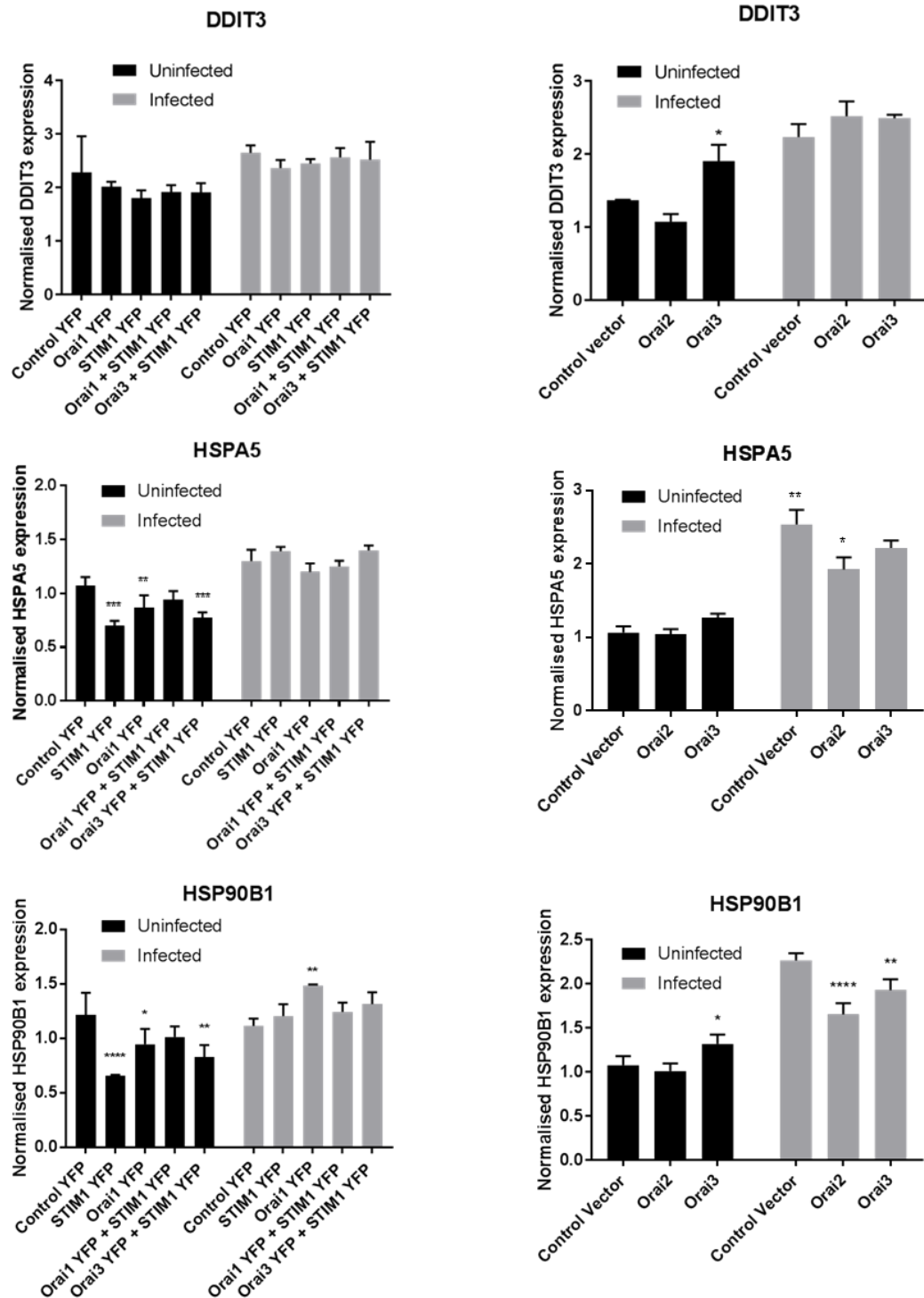


Figure 3-52. Over-expression of STIM1 and ORAI in NPTr cells had little effect on expression of ER stress markers. NPTr cells, transiently transfected with the indicated plasmids for 2 days, were infected with USSR H1N1 virus at 0.5 MOI for 24 h. Total RNA was extracted from each sample for ER stress marker expression,

normalised to 18S rRNA, calculated using the comparative ct method. Gene expression compared to the uninfected DMSO control, awarded a relative expression of 1. Significance determined by two way ANOVA, relative to the corresponding DMSO control.

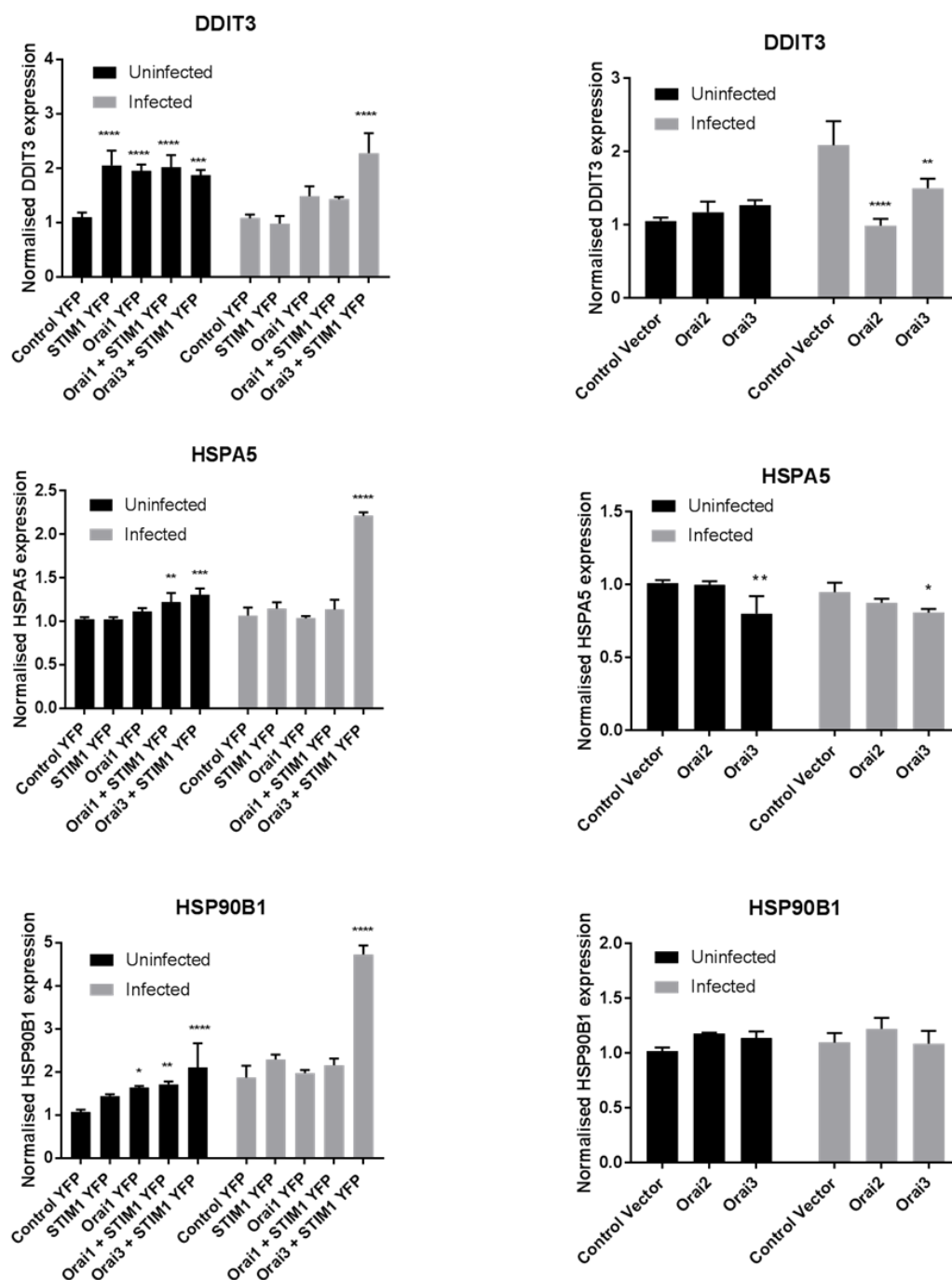


Figure 3-53. Over-expression of STIM1 and ORAI in porcine myoblasts had little effect on expression of ER stress markers. Porcine myoblasts, transiently transfected with the indicated plasmids for 2 days, were infected with USSR H1N1 virus at 2.0 MOI for 24 h. Total RNA was extracted from each sample for ER stress marker expression, normalised to 18S rRNA, calculated using the comparative ct method.

Gene expression compared to the uninfected DMSO control, awarded a relative expression of 1. Significance determined by two way ANOVA, relative to the corresponding DMSO control.

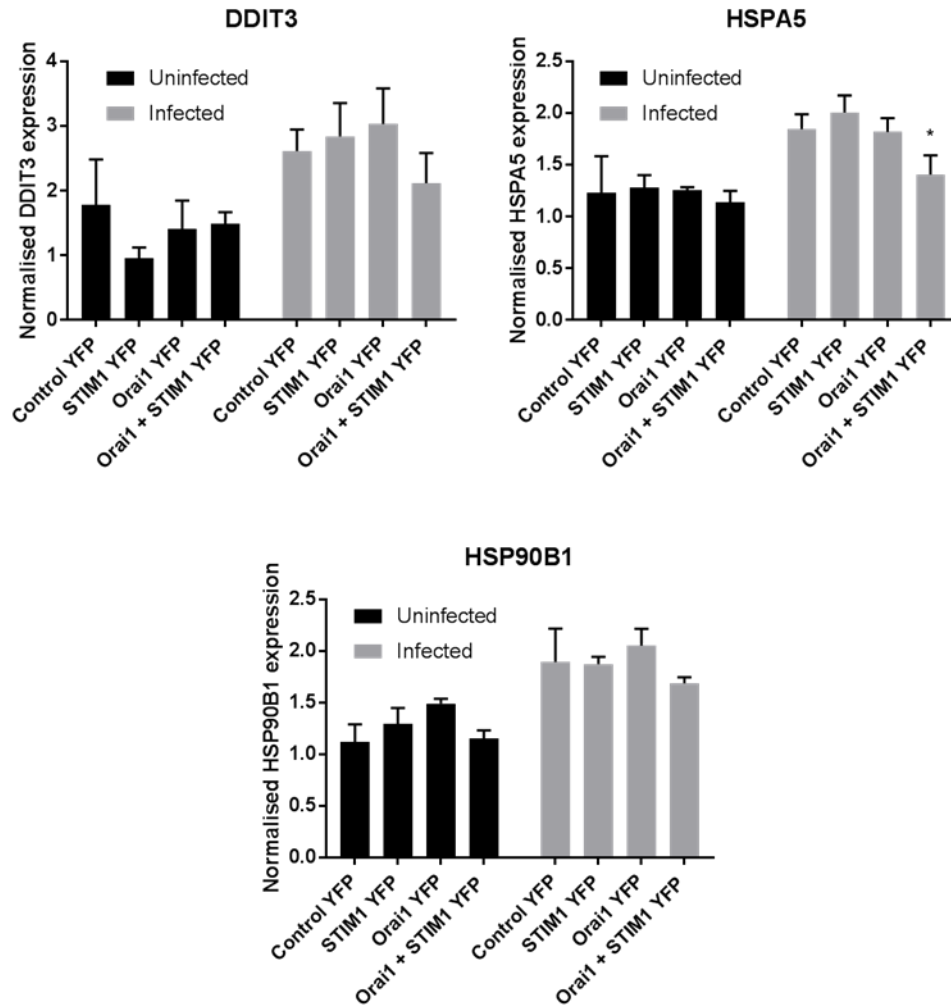


Figure 3-54. Over-expression of STIM1 and ORAI in NHBE cells had little effect on expression of ER stress markers. NHBE cells, transiently transfected with the indicated plasmids for 2 days, were infected with USSR H1N1 virus at 1.0 MOI for 24 h. Total RNA was extracted from each sample for ER stress marker expression, normalised to 18S rRNA, calculated using the comparative ct method. Gene expression compared to the uninfected DMSO control, awarded a relative expression of 1. Significance determined by two way ANOVA, relative to the corresponding DMSO control.

Over-expression of *CRACR2A* and *STIMATE* did not induce the ER stress response across all cell types (Figure 3-55).

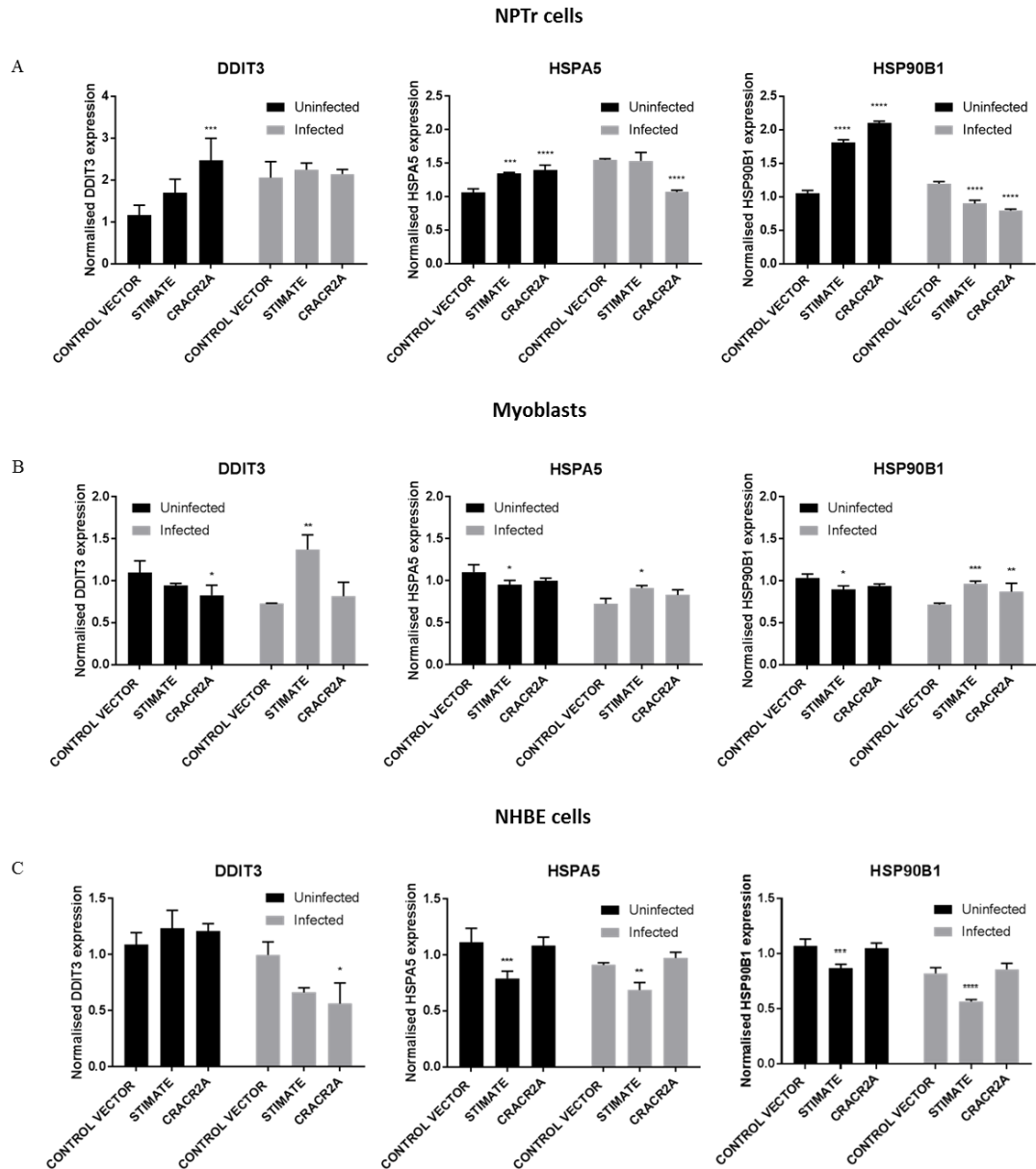


Figure 3-55. Over-expression of *CRACR2A* or *STIMATE* did not induce ER stress.

(A) NPTTr cells, stably transfected with indicated plasmids, were infected with USSR H1N1 at 0.5MOI for 24 h. (B) Porcine myoblasts, transiently transfected with indicated plasmids for 48 h, were infected with USSR H1N1 at 2.0 MOI for 24 h. (C)

NHBE cells, transiently transfected with indicated plasmids for 48 h, were infected with USSR H1N1 at 1.0 MOI for 24 h. Total RNA was extracted from each sample for ER stress dependent gene expression, normalised to 18S rRNA, calculated using the comparative ct method. Fold gene expression relative to the uninfected control vector (mCherry), awarded a relative value of 1. Significance determined by a one way ANOVA, comparing to the corresponding control vector.

Facilitating SOCE through overexpressing the core components or the positive regulators facilitates extracellular Ca^{2+} influx but does not induce store depletion. Therefore, activation of Ca^{2+} store depletion mediated ER stress response was not expected.

3.3.22 TG mediated an antiviral response dependent on STIM1

Despite evidence supporting a TG mediated a post-transcriptional antiviral response dependent on SOCE activation, thereby facilitating an influx of extracellular Ca^{2+} into the cytosol detrimental to influenza virus replication, other lines of evidence suggest TG activates antiviral signalling pathways independent of SOCE. Knockdown of *STIM1* in TG primed NHBE cells resulted in a significant increase in progeny virus output, confirming SOCE activation is a component of the TG mediated inhibition of influenza virus replication (Figure 3-56).

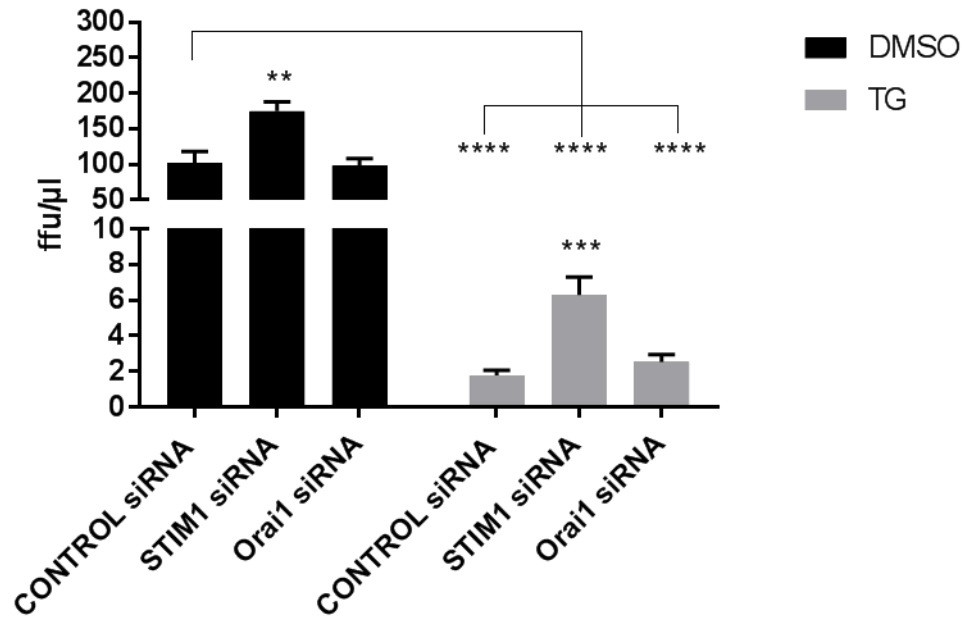


Figure 3-56. Knockdown of STIM1 in NHBE cells increased progeny virus output in the presence of TG. NHBE cells, transiently transfected with the indicated siRNAs for 48 h were subsequently pre-treated with DMSO or 0.01μM TG for 30 mins, washed with PBS and infected with USSR H1N1 at 1.0 MOI for 24 h. Spun supernatants were used to infect MDCK cells for 6 h in focus forming assays (FFAs). Significance determined by a two way ANOVA, comparing to the corresponding control vector unless otherwise indicated.

3.4 Discussion

Here a novel innate immune function of store operated calcium entry (SOCE) is reported and its potential as a therapeutic target for pharmacological activation to inhibit influenza A virus replication demonstrated. SOCE agonist thapsigargin (TG) orchestrated a potent antiviral response dependent on regulating host immunity and stress signalling pathways. SOCE mediated extracellular Ca^{2+} influx and Ca^{2+} dependent ER stress underlies the dual mechanism of the TG induced antiviral response. By not targeting the virus directly, TG could circumvent the issue of drug resistance suffered by current antivirals which operate by directly targeting specific viral components (McKimm-Breschkin, 2013).

Initial observations revealed that the level of extracellular Ca^{2+} corresponded with progeny virus titre, with an increase in extracellular Ca^{2+} associated with a decrease in progeny virus output. One pathway known to facilitate extracellular Ca^{2+} entry, SOCE, was subsequently analysed as a potential antiviral signalling pathway for influenza A virus infection. Activation of SOCE by increasing extracellular Ca^{2+} concentration has been reported in some cell types (Hu *et al.*, 2014). The tracheal epithelial cells are an initial barrier to influenza A virus infection; therefore they are a relevant cell type for analysing the contribution of SOCE to innate immunity (Wu *et al.*, 2016). In skeletal muscle, a major component of our body mass, SOCE regulates myoblast differentiation and facilitates contraction in myotubes (Darbellay *et al.*, 2009, Chen & Sanderson, 2017). Furthermore, influenza viruses may infect cells in other tissues as they may be capable of reaching the blood circulatory system and replicating outside of the respiratory tract (Taubenberger & Morens, 2008, Oughton

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et al., 2011, Tse *et al.*, 2011). Therefore, SOCE mediated regulation of immune signalling in muscle may also significantly contribute to the overall innate immune response. In order to interrogate the antiviral potential of this pathway, three strategies were used (i) chemical activation through TG, (ii) over-expression of SOCE components (ORAI homologues and STIM1) and SOCE facilitators (CRACR2A and STIMATE) and (iii) inhibition of SOCE through knockdown of ORAI1 and STIM1, and small molecule inhibitors of ORAI1.

Brief TG priming at non-cytotoxic concentrations immediately prior to infection reduced progeny virus output in porcine and human cells. TG induced a significant increase in cytosolic Ca^{2+} that was dependent on extracellular Ca^{2+} entry. A greater inhibitory effect on virus replication was observed in tracheal epithelial cells, with NHBE cells particularly sensitive to TG doses as little as 5nM (0.005 μM). Another activator of SOCE, cyclopiazonic acid (CPA), that could not induce calcium influx at non-toxic doses did not inhibit influenza A virus replication. This suggests the importance of extracellular calcium ion influx as necessary for the antiviral activity of SOCE agonists. However, a previous report on the effect of TG on influenza A virus infection proposed a pro-viral effect for TG priming. Priming MDCK cells with TG for 30 min significantly increased progeny virus output at a concentration of 0.001 μM , while priming with 0.1 μM and 1 μM of TG significantly reduced viral titre (Fujioka *et al.*, 2013). The higher TG doses used in this study could have induced SOCE activation while the lowest TG concentration was ineffective in SOCE activation. Further work may be necessary to establish the range of TG concentrations capable of activating SOCE in each cell type considered, in addition to determining the sensitivity of the Fluo-8 assay in detecting increases in intracellular Ca^{2+} concentration.

Facilitating SOCE through over-expression of the ORAI homologues (*ORAI1*, *ORAI2* and *ORAI3*), *STIM1* and over-expression of positive SOCE regulators (*CRACR2A* and *STIMATE*), significantly reduced progeny virus output in tracheal epithelial cells and skeletal muscle cells. This effect was consistent in human and porcine cells. Knockdown of *STIM1* in human epithelial cells increased progeny virus output, indicating an impaired antiviral response in the reduced presence of *STIM1*. *ORAI1* knockdown had no significant effect on progeny virus production but the presence of other isoforms may compensate for *ORAI1* silencing through functional redundancy. Expression of *ORAI1* was lower than *ORAI2* in NHBE cells. Due to technical issues, the impact of TG priming on extracellular Ca^{2+} influx on cells overexpressing the SOCE components or following *ORAI1* or *STIM1* knockdown was not explored. Pharmacological inhibition of the SOC channel, through BTP2 and Synta66 priming, significantly increased progeny virus output from NHBE cells and NPTr cells. BTP2 and Synta66 are able to indiscriminately inhibit SOC channels, thus accounting for the discrepancy between SOC channel inhibition and *ORAI1* knockdown. Overall, the results presented here provide overwhelming evidence in favour of an anti-influenza mechanism mediated by SOCE.

Virus replication may be inhibited post-translationally by Ca^{2+} mobilisation. This was supported by a lack of reduction in M gene expression in NPTr cells or NHBE cells primed with TG or overexpressing *CRACR2A* or *STIMATE*. However, while overexpression or knockdown of *STIM1* or *ORAI1* had no effect on M gene expression in NHBE cells, a significant reduction in M gene expression in NPTr cells and myoblasts overexpressing the overexpressing *ORAI1-3* or *STIM1* was reported. This may be attributable to species specific differences. However, the majority of

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inconsistencies in M gene expression appear in the porcine myoblasts when compared to the tracheal epithelial cells. TG priming or overexpression of the SOCE components in porcine myoblasts significantly reduced viral M gene expression. This suggests there may be some cell type specific variation between muscle cells and tracheal epithelial cells. This may be due to differences in Ca^{2+} regulation and the different functions of SOCE in muscle cells, such as differentiation and contraction, in comparison to tracheal epithelial cells. Virus protein M1 and NP levels in the host cells were also considered. Viral protein levels were not reduced in NPTr cells primed with TG and minimal alteration in myoblasts and NHBE cells supports a post-translational mechanism of inhibition. M1 in TG primed myoblasts and NP in TG primed NHBE cells infected with USSR H1N1 displayed a modest significant reduction in virus protein but virus protein was otherwise unaffected by TG priming. Furthermore, no reduction in M1 was measured in NPTr cells or porcine myoblasts overexpressing *STIM1* or *ORAI1* and NP was unaffected in the former. The reduction in M gene expression in NPTr cells and porcine myoblasts was not accurately or consistently reflected at the protein level. The reasons behind this are unclear but discrepancies between mRNA and corresponding protein levels are not uncommon (Greenbaum et al., 2003, Koussounadis et al., 2015). Overall, the collective evidence supports a post-translational antiviral mechanism in tracheal epithelial cells and suggests additional clarification of the mechanism of action in muscle cells may be necessary.

The integral finding underlying the TG mediated antiviral response is the regulation of host immune signalling pathways. TG priming dramatically elevated type I IFN

dependent gene expression in human and porcine cells during H1N1 virus infection. Interestingly, TG priming did not induce *RIG-I* or *OAS1* expression in uninfected cells. As aberrant activation of the immune response is detrimental to host health, the inability of TG to induce activation of the immune response in uninfected cells (i.e. non-target cells) may support its therapeutic use and safety. *IFN β 1* expression was only detected in infected NHBE cells and was significantly enhanced by TG priming. This is consistent with other reports of TG and Ca^{2+} dependent activation of type I IFN signalling. Following co-treatment with LPS, TG elevated *IFN β* and *IFN α* expression (Zeng *et al.*, 2010, Smith *et al.*, 2008). TG enhanced the recruitment of the transcriptional machinery (RNA polymerase II and the TATA binding protein) and significantly increased enhanceosome protein (CBP and IRF3) occupancy at the *ifnb1* promoter in the presence of LPS (Zeng *et al.*, 2010). Intracellular Ca^{2+} mobilisation, mediated by phospholipase C γ 2, elevated IRF3 activation in response to LPS (Chiang *et al.*, 2012). Over-expression of SOCE components (*STIM1* and the *ORAI* homologues) and SOCE facilitators (*CRACR2A* and *STIMATE*) did not elevate *RIG-I* or *OAS1* expression. This suggests SOCE activation does not induce type I IFN dependent gene expression. However, basal expression of the type I IFN dependent genes was elevated in transfected cells prior to influenza virus infection. This may account for the subdued response to influenza virus infection. Transfected cells displayed elevated *RIG-I* and *OAS1* expression relative to uninfected cells primed with DMSO or TG. The cells were transfected for 2 days before H1N1 virus infection, thus type I IFN signalling would have been activated prior to infection. The gene expression observed in influenza infected cells may reflect activation of negative feedback mechanisms; therefore the effect of SOCE on the innate immune signalling pathways may not be reflected in the mRNA profile in cells overexpressing the *ORAI*

or *STIM1*. This is supported by *ORAI1* and *STIM1* knockdown in NHBE cells. *STIM1* and *ORAI1* silencing significantly impaired influenza virus dependent activation of *RIG-I* and *OAS1* expression. This suggests SOCE activation may positively regulate the type I IFN response. Additionally, SOCE may activate other immune signalling pathways, such as NF- κ B. Although, the evidence presented here for TG mediated regulation of NF- κ B signalling conflicts with previous reports. TG activation of NF- κ B signalling has been reported. TG treatment induced phosphorylation and subsequent degradation of I- κ B α , as well as nuclear translocation of NF- κ B (Kaneko et al., 2003, Tam *et al.*, 2012, Tonnesen *et al.*, 2009). TG dependent activation of NF- κ B was inhibited by pre-treatment with Ca^{2+} chelators such as BAPTA-AM, suggesting ER Ca^{2+} efflux was essential for activation of NF- κ B (Pahl & Baeuerle, 1996). SOCE dependent activation of NF- κ B activity has also been discovered. *STIM2* was essential for expression of cytokines *TNF- α* , *IL-10* and *IL-6* through Ca^{2+} regulation of NF- κ B and calmodulin dependent kinase II activation in mouse neutrophils (Clemens et al., 2017). Expression of a dominant negative *ORAI1*_{E106A} suppressed NF- κ B activation (Yue et al., 2012). Thus, SOCE may mediate an immune response dependent on the calcium sensitive immune signalling pathways, such as NF- κ B.

TG may inhibit influenza virus replication by a dual mechanism, dependent on regulating type I IFN signalling and inducing the ER stress response. This may underlie a TG specific antiviral mechanism independent of extracellular Ca^{2+} influx. ER stress is strongly induced by TG priming, indicated by significantly elevated *DDIT3*, *HSPA5* and *HSP90B1* expression in tracheal epithelial cells and myoblasts.

Disruption of ER function due to cellular stress can interfere with protein folding and

processing. Accumulation of misfolded protein induces the unfolded protein response (UPR) to regain ER homeostasis or induce apoptosis. The UPR is mediated by three ER receptors: activating transcription factor 6 (ATF6), inositol requiring kinase 1 (IRE1) and double-stranded RNA-activated protein kinase (PKR)-like endoplasmic reticulum kinase (PERK) (Chakrabarti *et al.*, 2011a). The ATF6 UPR regulates expression of ER stress response elements (ERSE) and ER chaperones (Yoshida *et al.*, 1998, Yamamoto *et al.*, 2007). IRE-1 α and ATF6 induce activation of the transcription factor X-box binding protein-1 (XBP-1) to regulate ER stress responsive genes. IRE-1 α enhances XBP-1 activity by removing an unconventional 26-nucleotide intron through its endoribonuclease activity (Lee *et al.*, 2002). PERK-mediated UPR attenuates protein synthesis through phosphorylation of eukaryotic initiation factor-2 α (eIF2 α) and regulates ER stress sensitive genes through activation of the transcription factor ATF4 (Harding *et al.*, 1999, Harding *et al.*, 2000a). Influenza virus replication is dependent on virus protein folding and oligomerisation in the ER. Folding, oligomerisation and addition of post-translational modifications of influenza membrane glycoproteins, HA and NA, occurs in the ER (Saito *et al.*, 1995). The M2 protein is inserted in to the ER membrane for trafficking to the PM (Manzoor *et al.*, 2017). As induction of ER stress interferes with ER function and inhibits protein translation through the activity of kinase activity of PERK (Harding *et al.*, 2000b, Chakrabarti *et al.*, 2011a), it is possible TG mediated store depletion interferes with virus protein processing.

The antiviral activity of TG induced ER stress may also mediate the type I IFN response. Activation of the antiviral immune response due to ER stress induced proteins has been described for RNA viruses (Ge *et al.*, 2017). TG mediated ER stress was found to induce phosphorylation and nuclear translocation of IRF3 (Liu *et al.*, 198

2012a). IRF3 is a key transcription factor regulating type I IFN dependent gene expression (Honda et al., 2006). Activation of IRF3 was dependent on phosphorylation by the TBK1 serine/threonine kinase following its association with STING, an ER associated membrane protein (Liu et al., 2012a). Ca^{2+} mobilisation has been implicated in regulating STING signalling by others (Kim *et al.*, 2017, Shu *et al.*, 2012). Calcium independent ER stress stimulated by tunicamycin was independent of STING, instead acting via the ATF6 dependent UPR pathway (Liu et al., 2012a). Thus, TG may induce IRF3 activation via Ca^{2+} dependent regulation of the STING-TBK1 pathway. In contrast, ionomycin, which induced extracellular Ca^{2+} influx but not ER stress, did not significantly induce IRF3 phosphorylation, implying calcium influx alone was not sufficient for activating IRF3 (Liu et al., 2012a). The dependence of IRF3 activation on store depletion mediated ER stress rather than Ca^{2+} influx may account for differences in type I IFN dependent gene expression observed between TG and *ORAI/STIM* over-expression. Over-expression of SOCE components simply facilitates extracellular Ca^{2+} influx and does not induce store depletion. The dual antiviral mechanism of TG appears to hinge on Ca^{2+} , inducing a SOCE antiviral response but also a Ca^{2+} dependent ER stress mediated antiviral response.

The later stages of influenza virus replication, such assembly of the vRNP complex or virus budding, were also considered in the TG antiviral response. The former was explored through immunofluorescence detection of viral NP. The increased cytosolic localisation of NP may be due to reduced nuclear import of the translated protein for vRNP assembly in the nucleus (Ozawa *et al.*, 2007, Neumann *et al.*, 1997). NP is imported into the nucleus via the importin- α/β pathway (Labaronne et al., 2017, Melen *et al.*, 2003). The importin- α/β pathway is inhibited by high intracellular Ca^{2+}

concentration (Kaur et al., 2014). Ca^{2+} regulates interaction between importin- α and the nuclear localisation signal of the target proteins (Takata *et al.*, 2010). Therefore, TG mediated SOCE activation may interfere with vRNP assembly by impeding NP nuclear import through Ca^{2+} dependent inhibition of the importin- α/β pathway. Additional work is required to determine if extracellular Ca^{2+} influx or an increase in cytosolic Ca^{2+} due to inhibition of Ca^{2+} uptake into the intracellular stores mediates importin- α/β pathway inhibition.

The final stages of the virus lifecycle require assembly of the virus components beneath the PM and subsequent budding of the progeny influenza virus. The increase in ER stress marker expression in TG primed cells may indicate interference with protein folding and post-translational modifications of viral proteins. Interfering with viral protein folding may subsequently impair viral protein packaging and budding of viruses from the PM (da Silva *et al.*, 2015, Mehta *et al.*, 1998). Reduced progeny virus output as determined by FFA could indicate reduced overall production of infective virions or an increase in the proportion of defective progeny virus. The reduction in M gene expression in viruses collected from the supernatant, could reflect a reduction in production of progeny virus. Electron microscopy potentially allows for the visualisation of morphologically defective viruses (Barman & Nayak, 2007). TG priming of A549 or NPTr cells did not result in virus particles with aberrant morphology. Expression of HA or NA alone was sufficient to stimulate budding of virus-like particles (Lai *et al.*, 2010, Chen et al., 2007). This indicates that while HA and NA play an integral role in influenza virus budding from the host PM, the vRNPs are not essential for the formation of virus like particles. The reduction in M gene expression in RNA extracted from the supernatant may reflect impaired vRNP

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formation and packaging, resulting in the formation of virus-like particles which may have been observed by TEM. This is supported by aberrant localisation of NP observed following TG priming. Alternatively, TG mediated interference in post-translational modifications may compromise viral protein function and reduce virus production or infectivity.

In conclusion, an increase in cytosolic Ca^{2+} mediated by intracellular store depletion and an influx of extracellular Ca^{2+} is detrimental to influenza virus replication. SOCE is a well-characterised mechanism facilitating Ca^{2+} influx across the PM in response to depleted intracellular Ca^{2+} stores (reviewed by (Smyth et al., 2010)). The evidence presented here supports an antiviral function for SOCE in non-immune cells capable of inhibiting influenza virus replication. This was supported by a significant inhibition of virus replication through facilitating SOCE activation and conversely an increase in viral titre following SOCE inhibition. Priming with TG, an agonist of SOCE, induced a potent antiviral response through a Ca^{2+} dependent dual mechanism, orchestrated by activation of SOCE and the ER stress response. Further work is required to differentiate between the contribution of ER store depletion or extracellular Ca^{2+} influx to the antiviral response. The identification of a novel immune function for SOCE recommends it as target for antiviral therapy, potentially facilitating the development of new therapeutics, such as TG, for influenza A virus infection.

4. Chapter 4: TG mediated host antiviral properties and mechanisms

4.1 Introduction

Toxicity and lack of efficacy are the primary reasons for failure in the drug development process. A key component of drug discovery, or drug repurposing, is exploring its effect on biological processes within the cells through transcriptomics and proteomics (Bates, 2011). RNA sequencing (RNA-Seq) using high-throughput next generation sequencing (NGS), allows the transcriptome of the cells to be thoroughly assessed (Kukurba & Montgomery, 2015, Wang *et al.*, 2009). Genome wide screen may enable identification of potential antiviral host genes which could be targeted for antiviral therapy (Qian *et al.*, 2013, Shaw, 2011). In the early stages of drug development, gene expression data may be an integral component for identifying the predicted and novel pathways targeted by the compound (Kutalik *et al.*, 2008, Zainab *et al.*, 2014). Regulation of host signalling pathways may provide the next generation of drugs for influenza virus antiviral therapy (Marois *et al.*, 2014, Planz, 2013, Müller *et al.*, 2012). Targeting components of the host signalling pathways may be a critical step in overcoming the development of drug resistance (Marois *et al.*, 2014, Planz, 2013, Müller *et al.*, 2012). Drugs which modulate intracellular calcium concentration have already been identified as potential treatments for influenza virus infection (Marois *et al.*, 2014).

Describing the compound's mechanism of action (MoA) is necessary for identifying the key 'on-target' effects of the drug on biological pathways, in addition to detecting potential adverse side effects associated with toxicity (Verbist *et al.*, 2015, Iorio *et al.*, 2010). The selectivity index (SI) (*in vitro*) or therapeutic index (TI) (*in vivo*) is an essential component for assessing potential therapeutic drugs. The SI reflects the range

at which optimal drug efficacy may be achieved without unacceptable toxicity to the host (Muller & Milton, 2012). It is the ratio of the concentration of the compound which induces cell death in 50% of the host cells (50% cytotoxic concentration [CC₅₀]) to the minimum concentration of the drug required for 50% of its inhibitory activity *in vitro* (50% inhibitory concentration [IC₅₀]). Drugs with a low SI are not suitable for further development as the range at which the drug is effective without harming the host is too narrow, although lower SIs may be considered where limited treatments are available (Kissin, 2013, Zandi et al., 2011, Muller & Milton, 2012).

Influenza control strategies also include prophylaxis with the NAIs. Oseltamivir displays prophylactic activity, reducing the risk of symptomatic influenza by 55% in adults in one study and over 70% in a meta-analysis of two trials (Jefferson *et al.*, 2014, Cooper et al., 2003). In a human study, volunteers were orally administered oseltamivir 26 h prior to infection with H1N1 virus and this was associated with a significant reduction in infection and viral shedding (Hayden *et al.*, 1999). Zanamivir, when administered once daily for 10 days, was an effective prophylactic, conferring a household and individual protection of ~80% (Monto *et al.*, 2002, Hayden *et al.*, 2000). The prophylactic efficacy of peramivir treatment has been well established, with twice daily administration protecting mice against H1N1, H5N1, H9N2 and H3N2 influenza viruses (Govorkova *et al.*, 2001, Sidwell *et al.*, 2001, Abed *et al.*, 2010).

In this chapter, the therapeutic window and prophylactic properties of TG were assessed. The effectiveness of *in vitro* TG administration before and after influenza A virus infection was explored in order to characterise its potential as a prophylactic as

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well as therapeutic treatment. Furthermore, through analysing gene expression via high-throughput RNA expression profiling, the biological responses affected by TG were assessed and host-signalling pathways that may underlie the antiviral mechanism of action identified. This study may provide further insight into host cell signalling pathways regulated by Ca^{2+} mobilisation that target the virus.

4.2 Materials and Methods

4.2.1 Influenza A virus infection

At ~80-90% confluence, the cells were infected with USSR H1N1 virus (A/USSR/77) as described in Section 2.8. Owing to differences in permissiveness to influenza A virus infection, NPTr cells, NHBE cells and primary porcine myoblasts were infected with the indicated strain of H1N1 virus at 0.5 MOI, 1.0 MOI and 2.0 MOI, respectively for 12 or 24 h. Unless otherwise specified, infection media as described in Section 2.1.6 was used. Viral titre was quantified from collected supernatants by focus forming assays, described in Section 2.8.

4.2.1.1 TG priming prior to infection

NPTr cells were incubated in 0.5 μ M TG (Sigma Aldrich) for 30 min. The cells were washed with PBS and incubated for an additional 48 h or 24 h before infection with USSR H1N1 virus (A/USSR/77) at 0.5 MOI for 24 h.

4.2.1.2 TG exposure post infection

The cells were infected with the USSR H1N1 virus (A/USSR/77) at the appropriate MOI. After 6 h, the cells were incubated in IM containing the indicated concentration of TG or the equivalent volume of DMSO for 30 min. The media was removed, the cells washed with PBS and replaced with fresh IM for 24 h.

4.2.1.3 TG priming in the absence of extracellular Ca^{2+}

NPTr cells were primed for 30 min with TG in DMEM-Glutamax (high glucose) with 200mg/L CaCl_2 or calcium free media. Calcium free media was composed of DMEM high glucose, no glutamine, no calcium (Gibco) supplemented with L-glutamine to give a final concentration of 3.972mM. The NPTr cells were washed with PBS and infected with USSR H1N1 virus (A/USSR/77) at 0.5 MOI for 24 h in IM composed of UltraCULTURE media supplemented with 100U/ml P/S, 2mM L- glutatmine and 250ng/ml TPCCK trypsin.

4.2.2 Selectivity index

The selectivity index (SI) of TG was calculated as the ratio of CC_{50} over IC_{50} ($\text{SI} = \text{CC}_{50} / \text{IC}_{50}$). IC_{50} was determined by focus forming assay, described in Section 2.8. NPTr cells and NHBE cells were incubated in the indicated concentrations of TG for 30 min and infected with USSR H1N1 at 0.5 MOI and 1.0 MOI respectively. Supernatants collected 24 h later were subjected to virus titre quantification by focus forming assay. CC_{50} was determined with CellTiter-Glo® Luminescent Cell Viability Assay (Promega). NPTr cells and NHBE cells were incubated in the indicated concentrations of TG for 30 min and cell viability measured 24 h later.

4.2.3 Fluo-8 assay

The cells were cultured until near confluence in black Nunc MicroWell 96-well optical-bottom plates coated with cell culture treated polymer base (Thermo Fisher Scientific). The cells were loaded with Fluo-8 for 1 h at RT, using the Fluo-8 Calcium Flux Assay Kit (Abcam) according to the manufacturer's instructions. The cells were then treated with indicated concentrations of TG, an equivalent volume of DMSO serving as a negative control, and fluorescence measured 10 min after TG incubation at RT. To determine if the TG mediated increase in cytosolic Ca^{2+} was sustained for 24 h, NPTr cells and porcine myoblasts were incubated with TG for 30 min, washed with PBS and incubated for 24 h in fresh media prior to Fluo-8 loading. Fluorescence, indicative of cytosolic Ca^{2+} concentration, was measured using FLUOstar OPTIMA (BMG Labtech).

4.2.4 RT-PCR

Total RNA from treated NHBE cells and NPTr cells was isolated using the RNeasy Plus Mini Kit and reverse transcribed into cDNA. SYBR green RT-qPCR was performed with cycling conditions and volumes described in as described in Section 2.9. RT-qPCR relative fold expression was normalised to 18s rRNA, using 18s rRNA universal primers (F) 5' ACGGCTACCACATCCAAGGA 3' and (R) 5' CCAATTACAGGGCCTCGAAA 3' (Sigma-Aldrich). USSR H1N1 virus M gene (Eurofins) and porcine type I IFN dependent gene primers (Sigma-Aldrich) were designed using PrimerExpress 3.0.1 (Table 4-1). Human *RIG-I* and *OAS1* expression

was determined using pre-designed KSPQ12012 KiCqStart primer pairs (Sigma-Aldrich).

Gene	Organism	Primer	Sequence (5'-3')
M gene	USSR	F	AGATGAGCCTTCTAACCGAGGTCG
	H1N1 virus	R	TGCAAAAACATCTTCAAGTCTCTG
RIG-I	Pig	F	CCCTGGTTTAGGGACGATGAG
		R	AACAGGAACTGGAGAAAAAGTGATG
OAS1	Pig	F	CAAACCCAACCCGGAAATCT
		R	TTTCCCTCGGGACTTGCA

Table 4-1. Primer sequences for RT-PCR expression analysis

4.2.5 NGS

4.2.5.1 Library preparation and sequencing

At >80% confluence, NHBE cells were primed with 0.01 μ M TG or DMSO as control for 30 min, washed with PBS and subsequently infected with USSR H1N1 virus at 0.8 MOI. Twelve h post infection the cells were harvested and supernatants collected for FFA to confirm reduction in progeny virus output following TG treatment, as described in Section 2.8. Corresponding total RNA was extracted using an RNeasy Plus Mini Kit. Four RNA replicates were generated for each treatment type, consisting

of two technical replicates of two human donors (Table 4-2). TruSeq Stranded Total RNA libraries were generated from 18s rRNA depleted total RNA and sequenced using a HiSeq 3000 System at the SCIF Next Generation Sequencing Facility (University of Leeds, UK).

Treatment	Uninfected control	Influenza infected
DMSO primed	DM1_S1_L001_	DM13_S13_L002_
	DM2_S2_L001_	DM14_S14_L002_
	DM3_S3_L001_	DM15_S15_L002_
	DM4_S4_L001_	DM16_S16_L002_
TG primed	TG5_S5_L001_	TG17_S17_L002_
	TG6_S6_L001_	TG18_S18_L002_
	TG7_S7_L001_	TG19_S19_L002_
	TG8_S8_L001_	TG20_S20_L002_

Table 4-2. Treatment and influenza infection status of the TruSeq Stranded Total RNA library data sets. Samples from donor 1 in black and samples from donor 2 in blue.

4.2.5.2 Data analysis

Primary output files were provided in the form of fastq files. To remove any persisting adaptor sequences in the data, adaptor trimming was performed using skewer v0.2.2. ‘Read filtering by average quality’ was assigned a quality value of 20 and the ‘3’ end quality trimming’ was adjusted to a value of 3. The human transcriptome, containing human mRNAs, non-coding RNAs, was downloaded from Ensembl 92. Influenza virus sequences were downloaded from the OpenFlu database for the OpenFlu Isolate ID OFL_ISL_21018 (A/USSR/90/1977), last updated 2016-11-30. Sequence

alignment and read quantification were performed using the pseudo-alignment based tool Kallisto v0.43.0 (Bray *et al.*, 2016). Kallisto outputs the abundances of each identified transcript as transcripts per million (TPM). A package in R v3.4.4 ‘Someone to Lean On’ called Sleuth v0.28.1 (Pimentel *et al.*, 2017) was used to calculate differential expression. Differentially expressed genes (DEGs) were selected as those having a q-value (false detection rate corrected p-value) and a log₂ fold change (log₂FC) as described in Table 4-3, unless otherwise indicated. Fold change was always expressed as infected over uninfected or TG over DMSO.

Each gene list was subsequently analysed and enrichment analysis performed using the Gene Ontology Consortium (The Gene Ontology Consortium, 2017, Ashburner *et al.*, 2000) via Panther (version 11) utilising the PANTHER Overrepresentation Test (Released 20171205) (test type Fisher's Exact with false discovery rate (FDR) multiple test correction) and the GO Ontology database (Released 2018-06-01) (Mi *et al.*, 2017, Thomas *et al.*, 2003, Mi *et al.*, 2013, Thomas *et al.*, 2006). The enriched biological process (BP) gene ontology (GO) terms and enriched KEGG pathways were processed via NIPA (version 0.6.5) (available from <https://github.com/ADAC-UoN/NIPA>). KEGG pathways used the KEGG pathways database (Kanehisa *et al.*, 2017, Kanehisa *et al.*, 2016, Kanehisa & Goto, 2000). Genes of upregulated transcripts were analysed separately to downregulated genes in order to confer greater power to the analysis (Hong *et al.*, 2014).

The gene lists of DEGs were also analysed by Ingenuity pathway analysis (IPA) (QIAGEN, <https://www.qiagenbioinformatics.com/products/ingenuitypathway-analysis>, accessed 08.2018.) and interrogated using the causal analytic tools (Kramer

et al., 2014). IPA takes into account fold change in expression and does not require separate analysis of up- and down-regulated genes.

Infection status*	Control	Test	Analysis name	Parameters
U vs U	DMSO	TG	UUTG	$q < 0.05$, $\log_2FC \geq 1$ and ≤ -1
I vs U	DMSO	DMSO	IUDM	$q < 0.01$, $\log_2FC \geq 2$ and ≤ -2
I vs U	TG	TG	IUTG	$q < 0.01$, $\log_2FC \geq 2$ and ≤ -2
I vs I	TG	TG	IITG	$q < 0.05$, $\log_2FC \geq 1$ and ≤ -1
Additional analysis				
UITG less IUDMSO	IUDMSO	IUTG	IUTG-IUDM	$q < 0.05$, $\log_2FC \geq 1$ and ≤ -1

Table 4-3. Comparisons for differential expression analysis. DMSO= <DMSO control>, TG = <test drug>. *U = uninfected with <USSR H1N1>, I = infected with < USSR H1N1>; left-hand = control condition status, right-hand = test condition status. IUTG less IUDMSO gene list created by removing DEGs in IUDMSO from IUTG. Parameters = q-value and log2FC parameters applied to data set to generate gene list of DEGs, unless otherwise specified. FC= fold change.

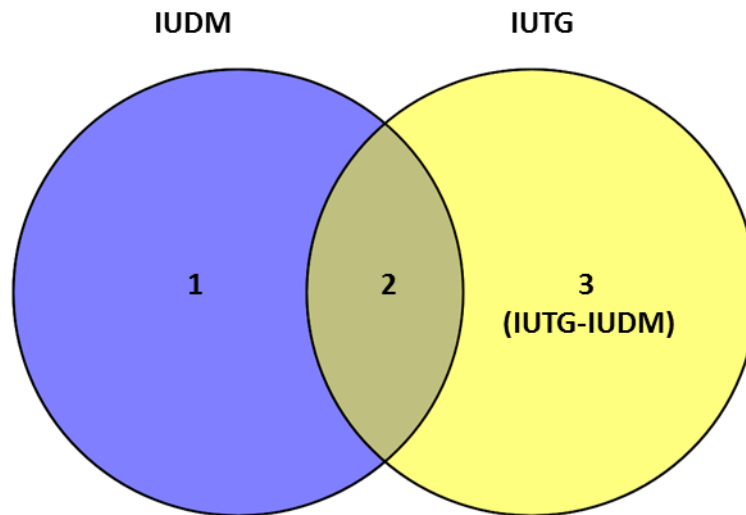


Figure 4-1. Venn diagram outlining differential expression analysis IUTG-IUDM.

Region 1 = transcripts differentially expressed in influenza virus infected relative to uninfected NHBE cells primed with DMSO. Region 2 = transcripts differentially expressed in influenza virus infected relative to uninfected NHBE cells primed with DMSO or primed with TG. Region 3 = transcripts differentially expressed in influenza virus infected relative to uninfected NHBE cells primed with TG. Differential expression analysis (IUTG-IUDM) was composed of differentially expressed transcripts unique to IUTG (region 3).

Graphical representations of the data (principle component analysis (PCA), multidimensional scaling (MDS) and volcano plot) were performed in R v3.4.4. PCA and MDS plot allow for the variation across the data set to be visualised on a two dimensional graph. PCA is a technique that reduces the number of variables within the data set through constructing a smaller number of artificial principles (aka the principle components) that summarise the variation in the data. It is dependent on

perceiving redundancy in the variables described for the data set, finding correlation between the observed variables and thereby reducing them into a smaller number of artificial principles. The first principal component accounts for the maximal proportion of the total variation within the data set and the second accounts for the maximum proportion of the remaining unaccounted for variance, providing it is uncorrelated with the first component. Overall, this allows the variability of large data sets, which would be expected to contain a high number of variables, to be reduced and displayed on a two-dimensional plot. Similarly, the MDS plot allows for grouping within the data set to be visualised. It creates a map where the relative distance between the points represents the similarities or dissimilarities and is not dependent on one or two dimensions, unlike the PCA plot. False detection rate correction was performed using the Benjamini-Hochberg method (Benjamini & Hochberg, 1995)

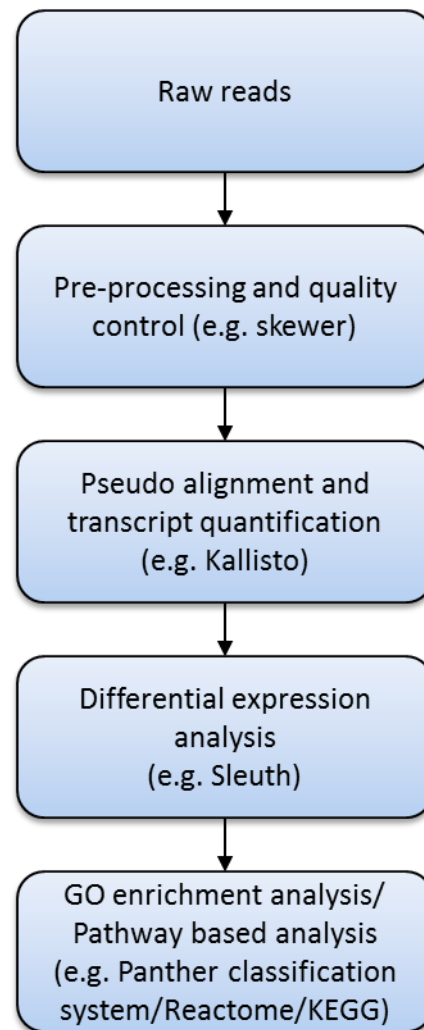


Figure 4-2. Overview of work flow for processing NGS reads.

4.3 Results

4.3.1 Selectivity index of TG

The selectivity index (SI) is defined as the ratio of $CC_{50}:IC_{50}$. In NHBE cells the cytotoxic concentration of 50% of cells (CC_{50}) was determined as $33.52\mu\text{M}$ and the corresponding inhibitory concentration for 50% inhibition of virus replication (IC_{50}) was $0.00391\mu\text{M}$. In NHBE cells, the SI for TG was 8572.89. In NPTr cells, CC_{50} was $56.58\mu\text{M}$ and IC_{50} was 0.007115. The resulting SI for TG in NPTr cells at 7952.21 was comparable to the SI of NHBE cells.

4.3.2 TG induced Ca^{2+} influx reduced progeny virus output in a dose-dependent manner

The relationship between TG mediated Ca^{2+} influx and virus output in tracheal epithelial cells was examined. TG induced a dose-dependent increase in cytosolic calcium influx in NPTr and NHBE cells (Figure 4-3A and 4-3C). Increasing concentration of cytosolic calcium ions corresponded with a greater reduction in progeny virus production (Figure 4-3B and 4-3D). Interestingly, the lowest doses of TG ($0.01\mu\text{M}$ in NPTr cells and 0.1nM ($0.0001\mu\text{M}$) in NHBE cells) that did not confer detectable increase in cytosolic Ca^{2+} were still able to induce significant reduction in virus production. This could be attributed to detection sensitivity of the Fluo-8 assay. However, this would require additional work to explore the sensitivity of the Fluo-8 assay and for the minimum increase in intracellular Ca^{2+} detectable to be established.

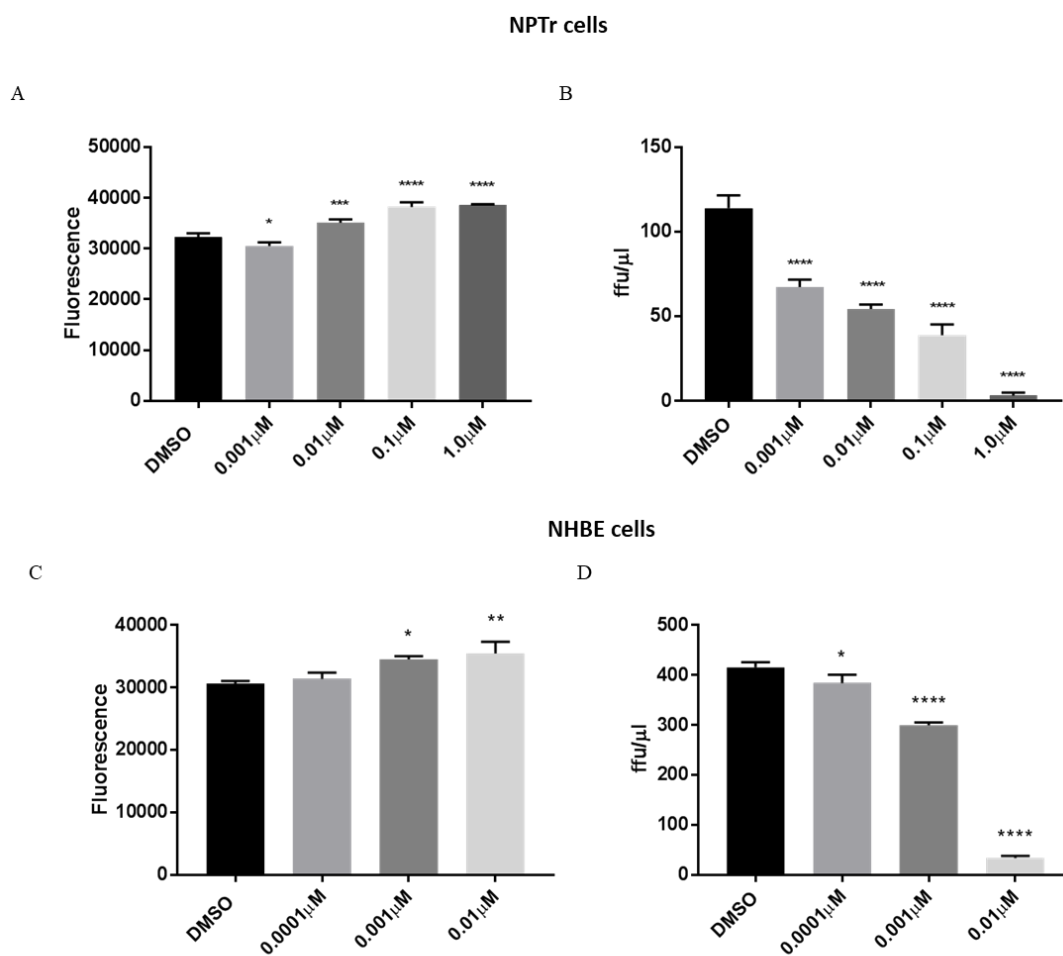


Figure 4-3. Increased cytosolic Ca^{2+} induced by TG corresponded with an increased reduction in progeny virus output. Fluo-8 fluorescence intensity measured in (A) NPTr cells and (C) NHBE cells incubated in the indicated concentrations of TG for 10 min. NPTr cells (B) and NHBE cells (D) were incubated in the indicated concentrations of TG for 30 min and subsequently infected with USSR H1N1 virus at 0.5 MOI for 24 h. Spun supernatants were used to infect MDCK cells for 6 h in focus forming assays (FFAs). Significance determined by one way ANOVA. Significance relative to corresponding DMSO treatment.

4.3.3 Absence of extracellular Ca^{2+} for duration of TG incubation had no effect on progeny virus output

Extracellular calcium was removed for the 30 min duration of TG priming to assess the contribution of extracellular Ca^{2+} to the TG induced antiviral response. TG mediated reduction progeny virus output was unaffected by the absence of extracellular Ca^{2+} throughout the duration of TG incubation (Figure 4-4).

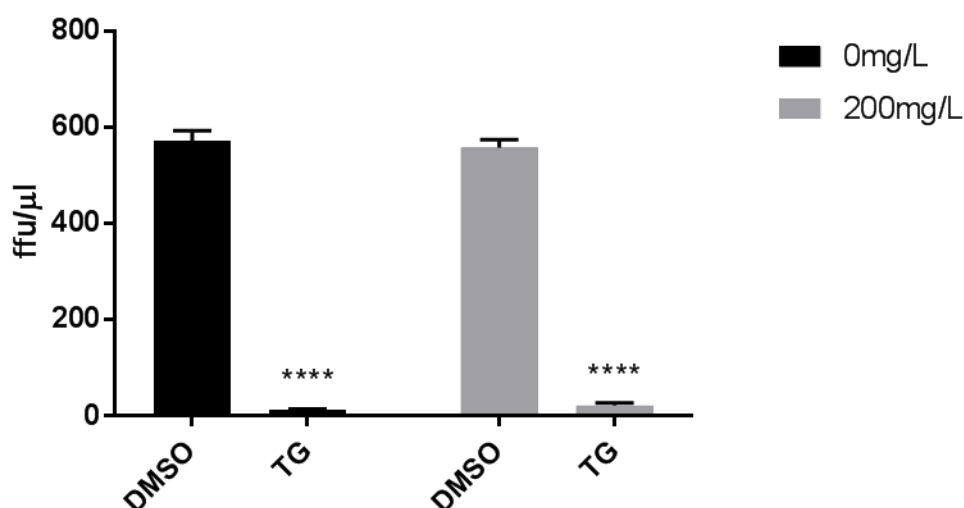


Figure 4-4. Absence of extracellular Ca^{2+} for duration of TG incubation had no effect on progeny virus output. NPTr cells incubated with $0.5\mu\text{M}$ TG for 30 min in calcium free DMEM (0mg/L CaCl_2) or DMEM containing 200mg/L CaCl_2 . The cells were subsequently washed in PBS and infected with USSR H1N1 at 0.5 MOI for 24 h. Spun supernatants were used to infect MDCK cells for 6 h in focus forming assays (FFAs). Significance determined by one way ANOVA, relative to the corresponding DMSO control.

The findings need careful qualification. The influx of Ca^{2+} may have merely been delayed until the re-introduction of Ca^{2+} after the 30 min incubation in calcium free media. The necessity of extracellular Ca^{2+} on cell viability precluded extended culture duration in the complete absence of Ca^{2+} .

4.3.4 TG priming induced an extended antiviral state of up to 48 h

The duration of the protective antiviral effect of TG priming was assessed. NPTr cells incubated with TG for 30 min retained their antiviral state for at least 48 h, evidenced by a significant reduction in progeny virus output from virus infection made 48 h post-TG priming (Figure 4-5).

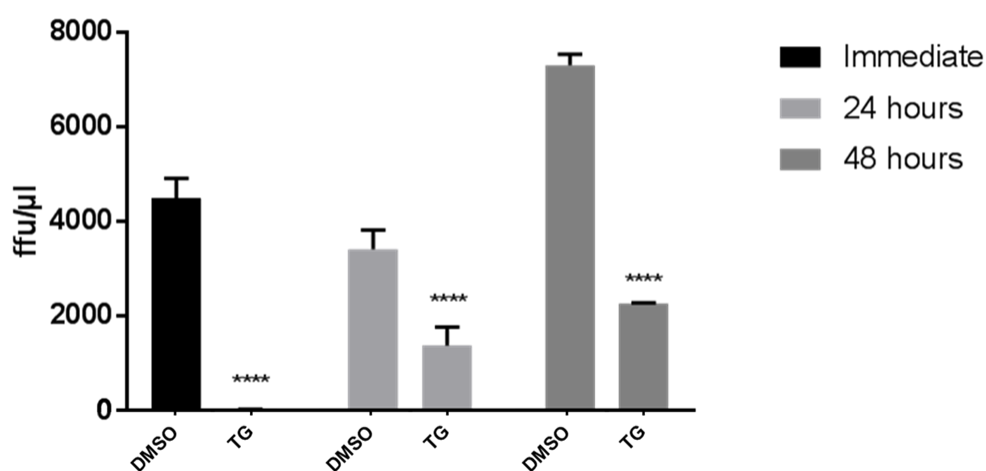


Figure 4-5. TG priming 48 h before infection reduced progeny viral output. NPTr cells incubated with $0.5\mu\text{M}$ TG for 30 min, washed with PBS and subsequently infected immediately or after an additional incubation of 24 or 48 h. NPTr cells were infected

with USSR H1N1 virus at 0.5 MOI for 24 h. Spun supernatants were used to infect MDCK cells for 6 h in focus forming assays (FFAs) to determine progeny virus output. Significance determined by two way ANOVA, relative to the corresponding DMSO control.

The antiviral state, in terms of reduced progeny virus output, maintained 24 h post-TG priming in NPTr cells and porcine myoblasts (Figure 4-6A and 4-6C), was accompanied by increased intra-cellular accumulation of viral M gene RNA (Figure 4-6B and 4-6D); this is highly suggestive of post-transcriptional viral inhibition.

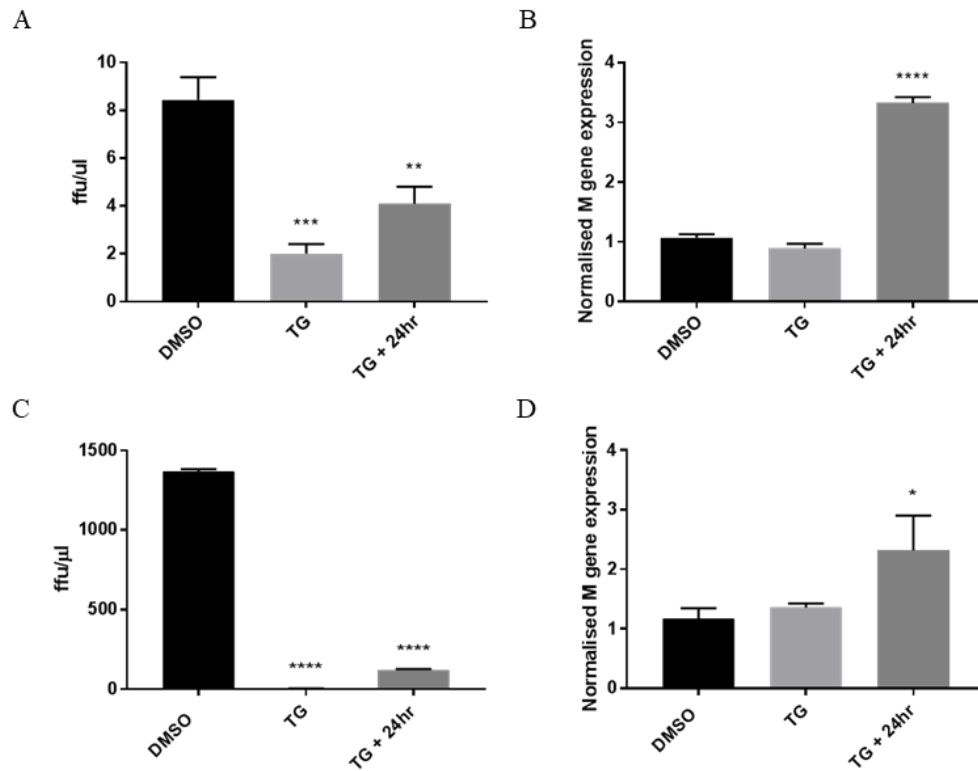


Figure 4-6. Immune response following TG treatment was sustained for 24 h after exposure. (A, B) NPTr cells incubated with 1.0 μ M TG and (C, D) porcine myoblasts incubated in 0.5 μ M TG for 30 min. The cells were infected with USSR H1N1 at 0.5 MOI and 2.0 MOI respectively for 24 h. (A, C) Spun supernatants were used to infect MDCK cells for 6 h in focus forming assays (FFAs). (B, D) Total RNA extracted 24 h later. Fold M gene expression, normalised to 18s rRNA, calculated using the comparative Ct method. Significance determined by one way ANOVA, relative to the corresponding DMSO control.

4.3.5 The TG mediated increase in cytosolic Ca^{2+} concentration was not sustained for 24 h

The antiviral state induced by TG priming was sustained for up to 48 h in NPTr cells and at least 24 h in porcine myoblasts. However, the TG mediated increase in cytosolic Ca^{2+} was not sustained for 24 h (Figure 4-7). This suggests the antiviral state induced by TG may be attributable to a brief increase in cytosolic Ca^{2+} .

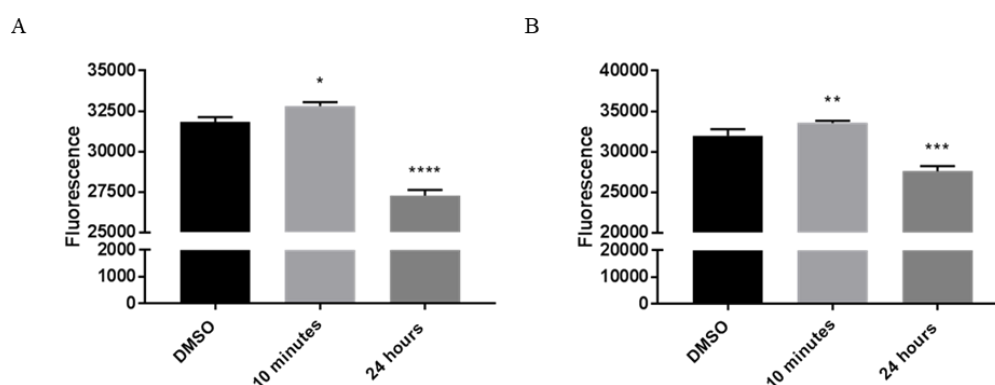


Figure 4-7. Exposure of cells to TG induced a brief increase in cytosolic Ca^{2+} . Fluo-8 fluorescence intensity measured in (A) NPTr cells incubated in 1.0 μM TG and (B) porcine myoblasts incubated in 0.5 μM TG. Fluo-8 fluorescence intensity was assessed 10 min after TG addition, or assessed after an initial 30 min TG priming, PBS washing and an additional 24 h incubation in fresh media at 37°C in 5% CO_2 . Significance determined by one way ANOVA, comparing Fluo-8 fluorescence intensity to the DMSO control.

4.3.6 TG exposure after 6 h of initial infection impaired virus replication

As TG priming prior to infection was able to induce a sustained antiviral state, its ability to inhibit virus production during infection was examined. NPTr cells, NHBE cells and porcine myoblasts were infected with USSR H1N1 virus for 6 h prior to TG exposure, the approximate time for a full cycle of virus replication (Abdoli *et al.*, 2013). Priming with TG at 6 h post-infection impaired virus replication without consistent effect on viral M gene expression, again indicative of post-transcriptional viral inhibition. (Figure 4-8).

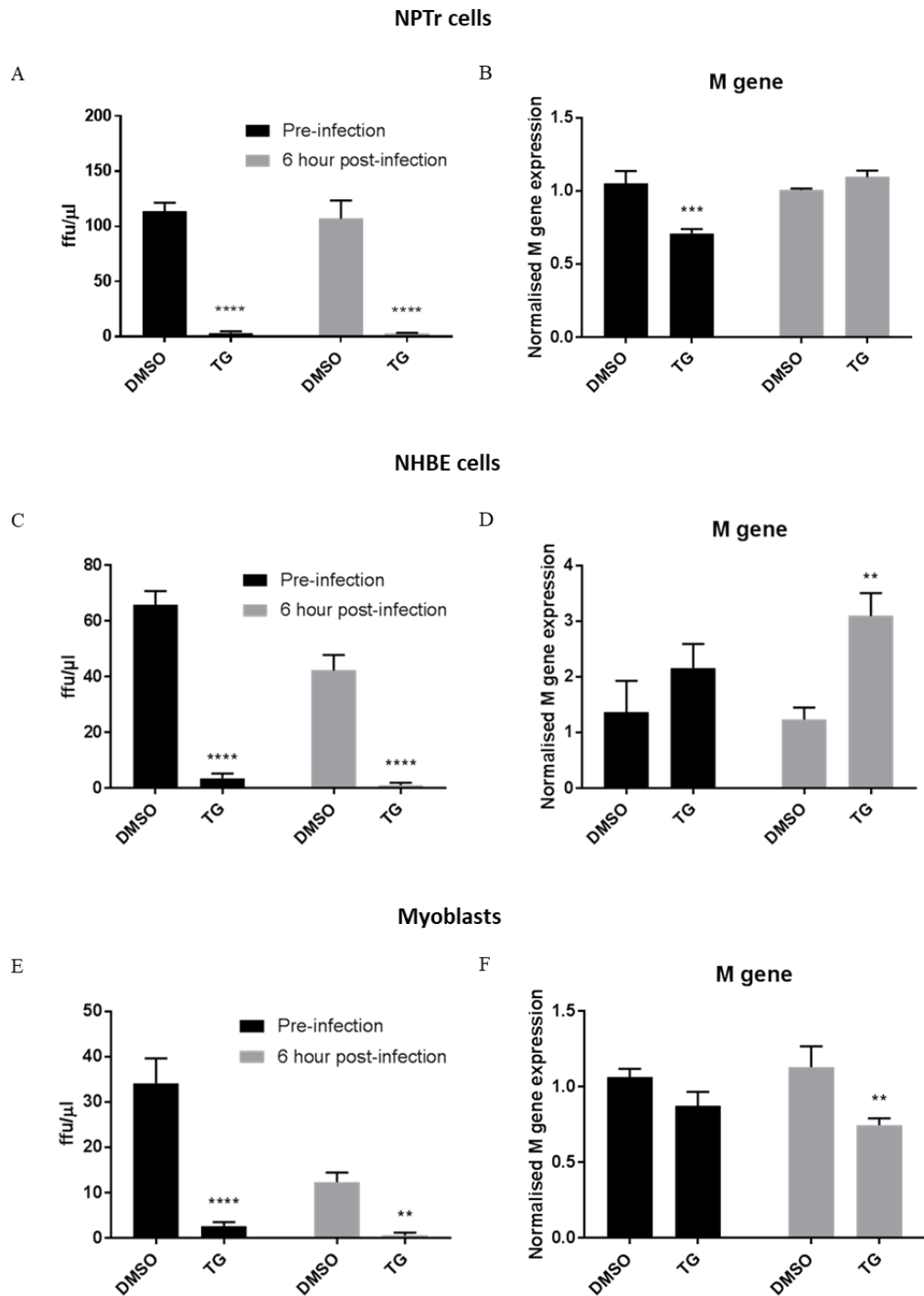


Figure 4-8. TG priming after the first 6 h of infection reduced progeny viral output.

(A, B) NPTr cells, (C, D) NHBE cells and (E, F) porcine myoblasts incubated with TG at $1.0\mu\text{M}$, $0.01\mu\text{M}$ and $0.5\mu\text{M}$ respectively for 30 min. TG incubation was followed by immediate infection (TG pre-infection) or the cells were first infected for 6 h before

TG exposure for 30 min (TG post-infection). NPTr cells, NHBE cells and porcine myoblasts were infected with USSR H1N1 virus for 24 h at 0.5 MOI, 1.0 MOI and 2.0 MOI respectively. (A, C, E) Spun supernatants were used to infect MDCK cells for 6 h in focus forming assays (FFAs) to determine progeny virus output. (B, D, E) Total RNA extracted 24 h later. Fold M gene expression, normalised to 18s rRNA, calculated using the comparative Ct method. Gene expression compared to the corresponding DMSO control, awarded a relative expression of 1. Significance determined by two way ANOVA, relative to the corresponding DMSO control.

TG priming pre-infection and 6 h post-infection enhanced type I IFN associated gene expression, *RIG-I* and *OAS1*, in all cell types examined (Figure 4-9).

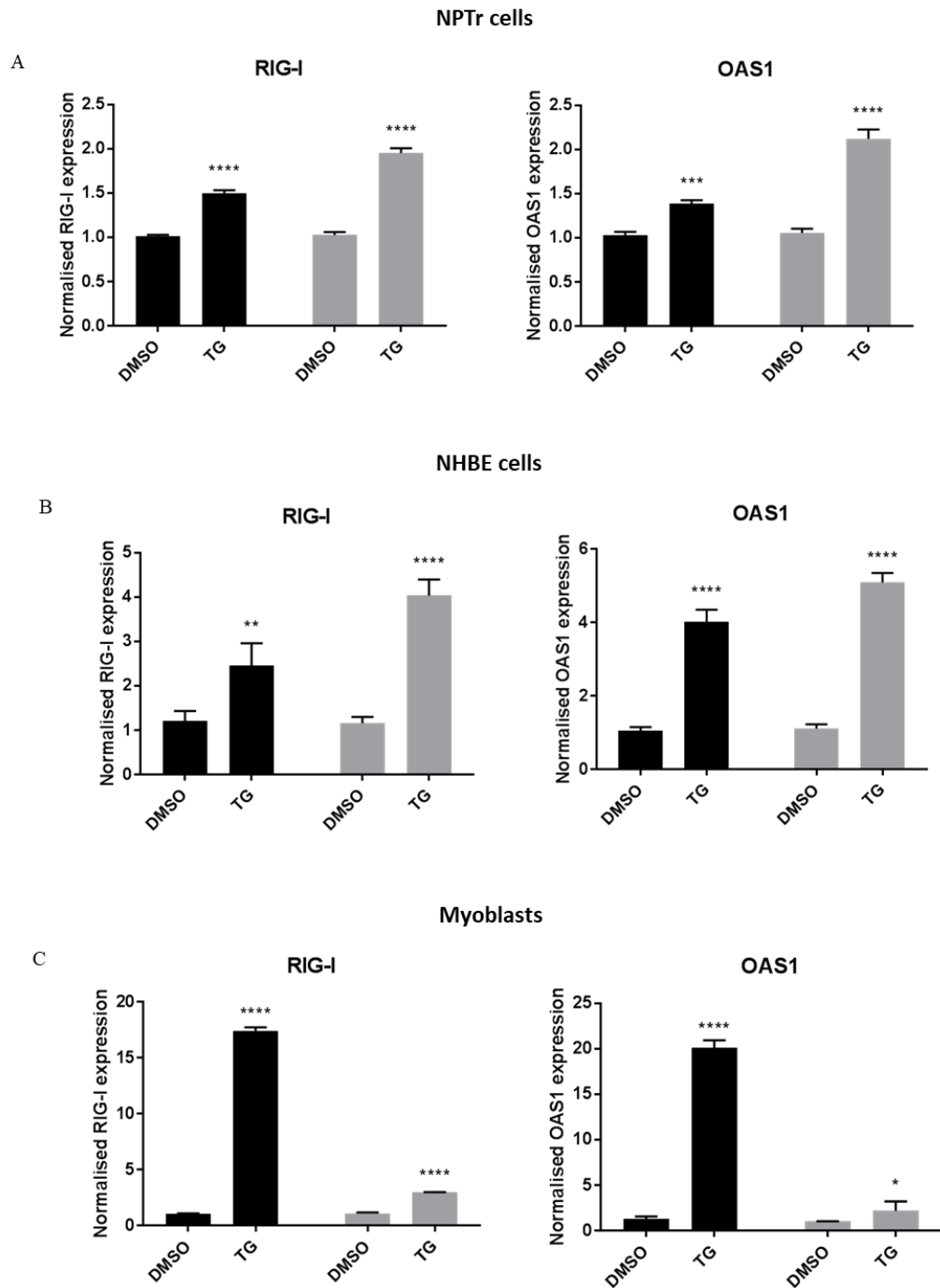


Figure 4-9. TG before infection or after 6 h of infection elevated type I IFN dependent gene expression. (A) NPTr cells, (B) NHBE cells and (C) porcine myoblasts incubated with TG at $1.0\mu\text{M}$, $0.01\mu\text{M}$ and $0.5\mu\text{M}$ respectively for 30 min. TG incubation was followed by immediate infection (TG pre-infection) or the cells were first infected for 6 h before TG exposure for 30 min (TG post-infection). NPTr

cells, NHBE cells and porcine myoblasts were infected with USSR H1N1 virus for 24 h at 0.5 MOI, 1.0 MOI and 2.0 MOI respectively. Total RNA extracted 24 h later. Fold RIG-I and OAS1 gene expression, normalised to 18s rRNA, calculated using the comparative Ct method. Gene expression compared to the corresponding DMSO control, awarded a relative expression of 1. Significance determined by two way ANOVA, relative to the corresponding DMSO control.

RNA-seq

4.3.7 TG priming significantly reduced progeny virus output for each human donor of NHBE cells

The inhibitory effect of TG priming on progeny virus output from NHBE cells isolated from the two human donors was confirmed. Priming with 0.01 μ M TG for 30 min significantly reduced virus output from primary tracheal epithelial cells from donor 1 and donor 2 (Figure 4-10).

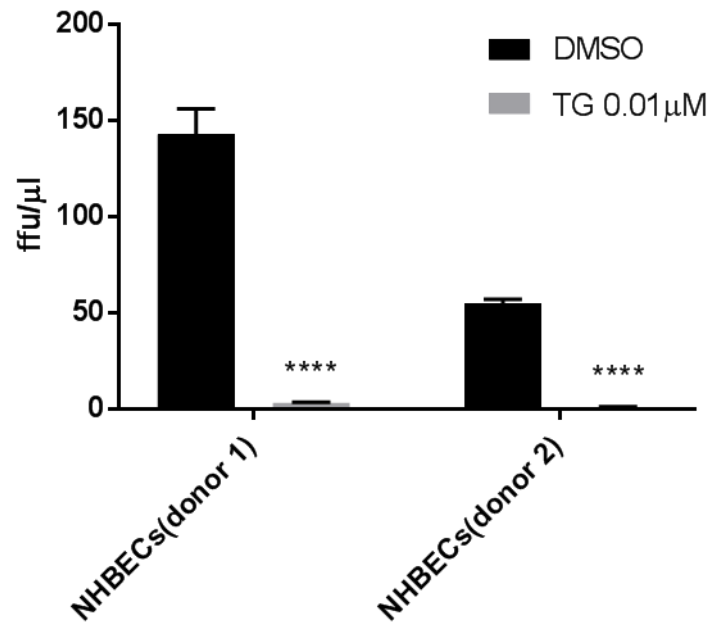


Figure 4-10. TG priming of NHBE cells reduced progeny virus output from NHBE cells of two donors. NHBE cells incubated with 0.01 μM TG for 30 min, were subsequently infected with USSR H1N1 at 0.8 MOI for 12 h. Spun supernatants were used to infect MDCK cells for 6 h in focus forming assays (FFAs). Significance determined by paired t-test in relation to corresponding DMSO control.

4.3.8 Quality metrics of the NGS dataset

4.3.8.1 Percentage alignment of the RNA-seq reads to the reference transcriptome

The filtered reads of the RNA-seq data were mapped to the human transcriptome downloaded from Ensembl 92 and influenza virus sequences from the OpenFlu database for the OpenFlu Isolate IDOFL_ISL_21018 (A/USSR/90/1977), last updated

2016-11-30. Sequence alignment and read quantification were performed using Kallisto v0.43.0 (Bray et al., 2016). The percentage sequence alignment per sample is displayed in (Figure 4-11). High similarity can be observed in the percentage alignment between experimental replicates. Percentage sequence alignment was lower in the uninfected samples (DM1-4 and TG5-8) as the human transcriptome was supplemented with influenza virus sequences.

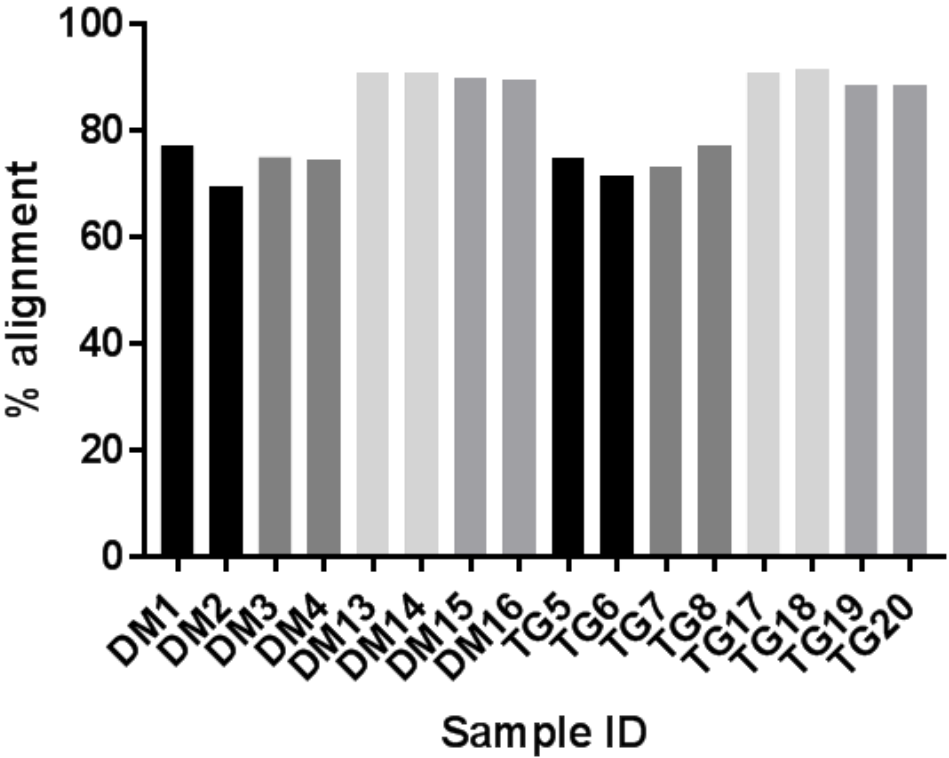


Figure 4-11. Percentage alignment of filtered reads to the transcriptome.

4.3.8.2 Global expression comparison between USSR H1N1 virus infected and uninfected samples pre-treated with TG or DMSO control

Principle component analysis (PCA) and multidimensional scaling (MDS) were applied to the data sets (assigned sample names described in Table 4-2) to visualise the differences in the global expression patterns between: (i) DMSO primed influenza infected NHBE cells relative to DMSO primed uninfected cells (IUDM, Table 4-3) and (ii) TG primed influenza infected NHBE cells relative to TG primed uninfected cells (IUTG, Table 4-3). In DMSO primed NHBE cells (Figure 4-12) and TG primed NHBE cells (Figure 4-13), the PCA plot and MDS plot revealed distinctly segregated sample groups, indicated by the distinct grouping according to colour. This suggests distinct differences between the groups of data according to influenza virus infection. Furthermore, the plots also reveal the similarity between the replicates of each donor.

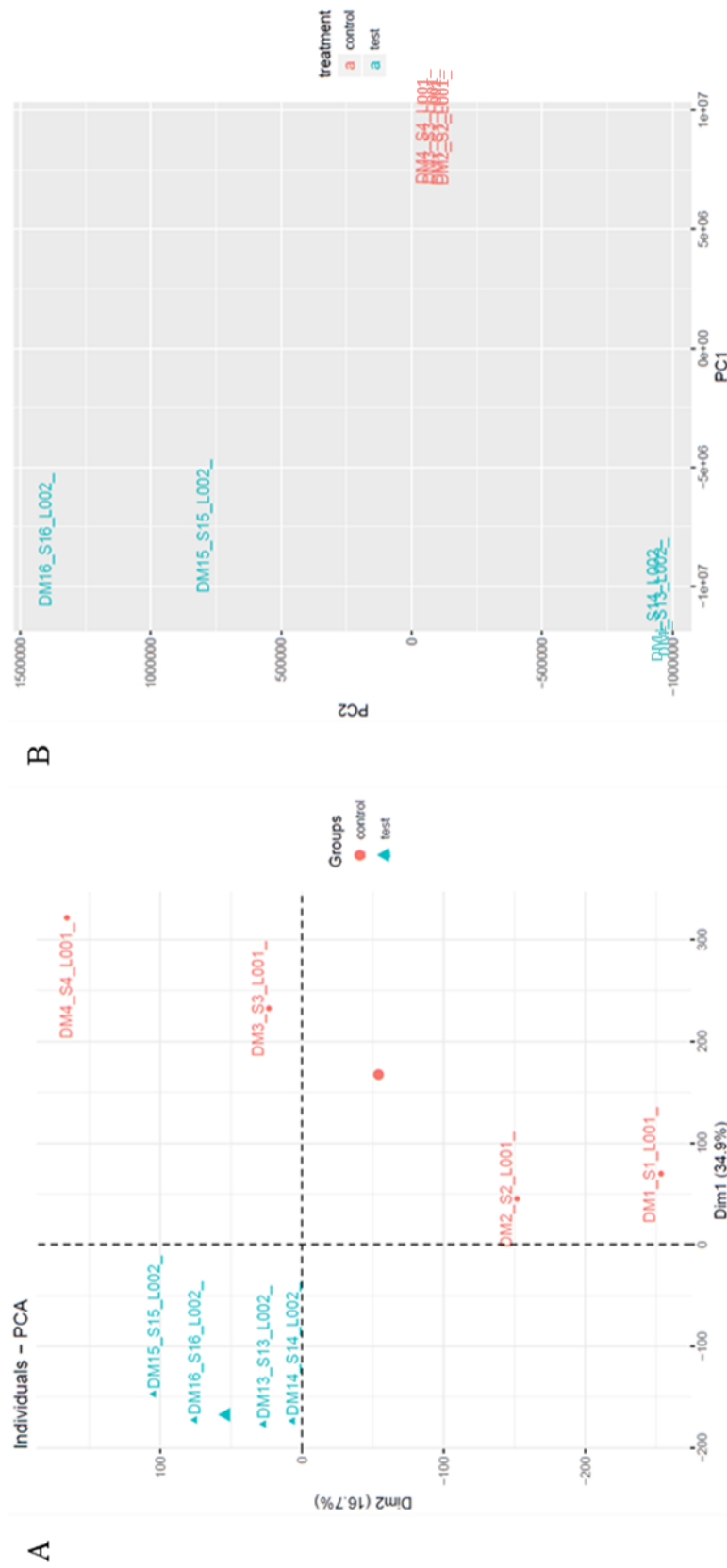


Figure 4-12. Distinct global expression pattern between USSR H1N1 virus infected and uninfected DMSO primed NHBE cells. (A) PCA plot for RNA-seq gene expression of DMSO pre-treated NHBE cells (IUDM). Orange circles (control) referring to the uninfected NHBE cell samples; blue triangles (test) representing the infected NHBE cell samples.

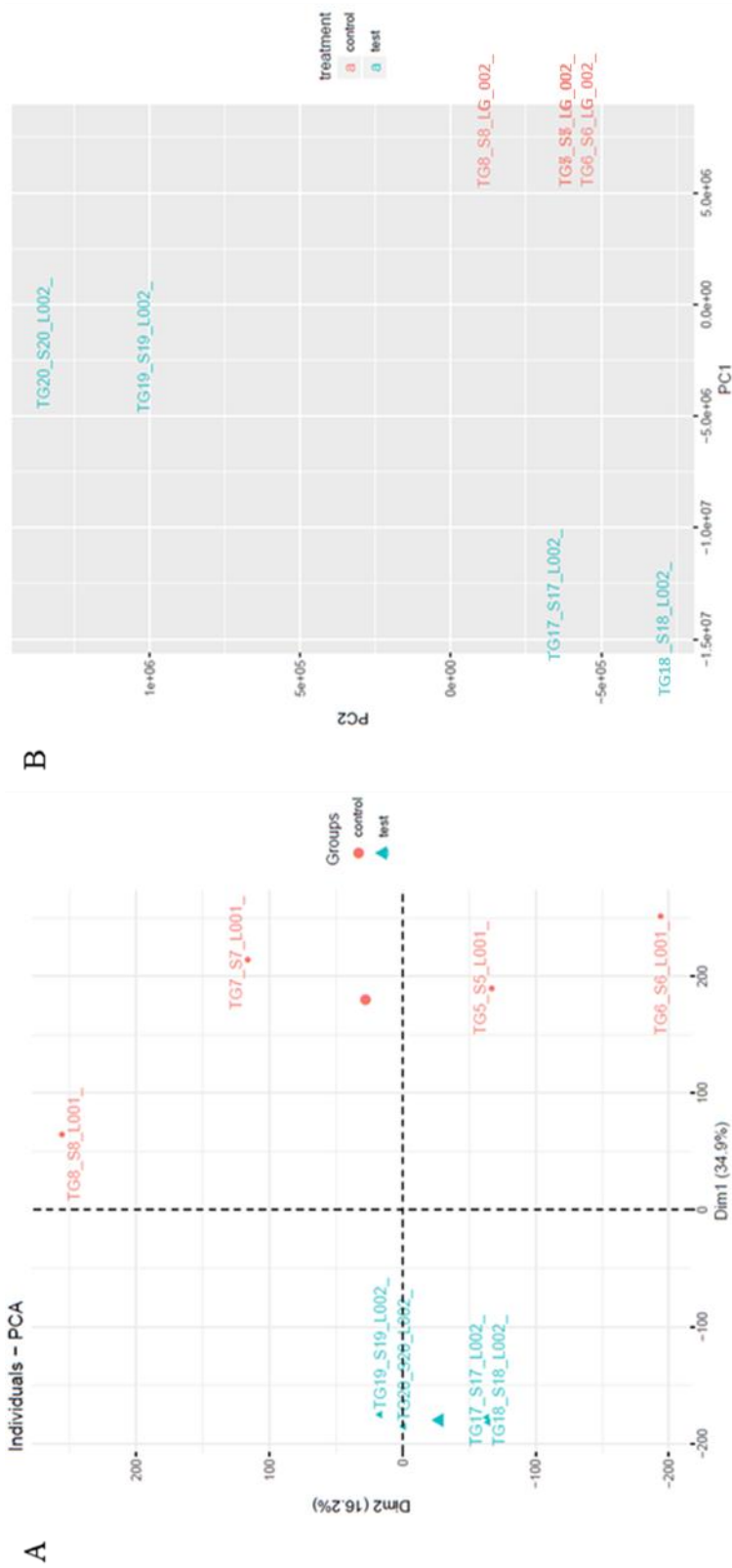


Figure 4-13. Distinct global expression pattern between USSR H1N1 virus infected and uninfected TG primed NHBE cells. (A) PCA plot and (B) MDS plot for RNA-seq gene expression of TG pre-treated NHBE cells (IUTG). Orange circles (control) referring to the uninfected NHBE cell samples; blue triangles (test) representing the influenza infected NHBE cell samples.

4.3.8.3 Global expression comparison between TG and DMSO primed NHBE cells within uninfected samples or influenza infected samples

The PCA plot and MDS plot for uninfected NHBE samples comparing differential expression in TG primed cells to DMSO primed cells (UUTG, Table 4-3), revealed the samples cannot be clearly separated according to their pre-treatment, as the samples of the two groups are interspersed on the plot (Figure 4-14). This implies the largest sources of variation are similar in each group. In this instance, variation due to TG priming relative to DMSO priming may not be the principal component which may be used to discriminate between the two groups. However, TG priming and DMSO priming resulted in two distinct groups in influenza infected NHBE cells (IITG, Table 4-3), reflected in the PCA and MDS plot (Figure 4-15).

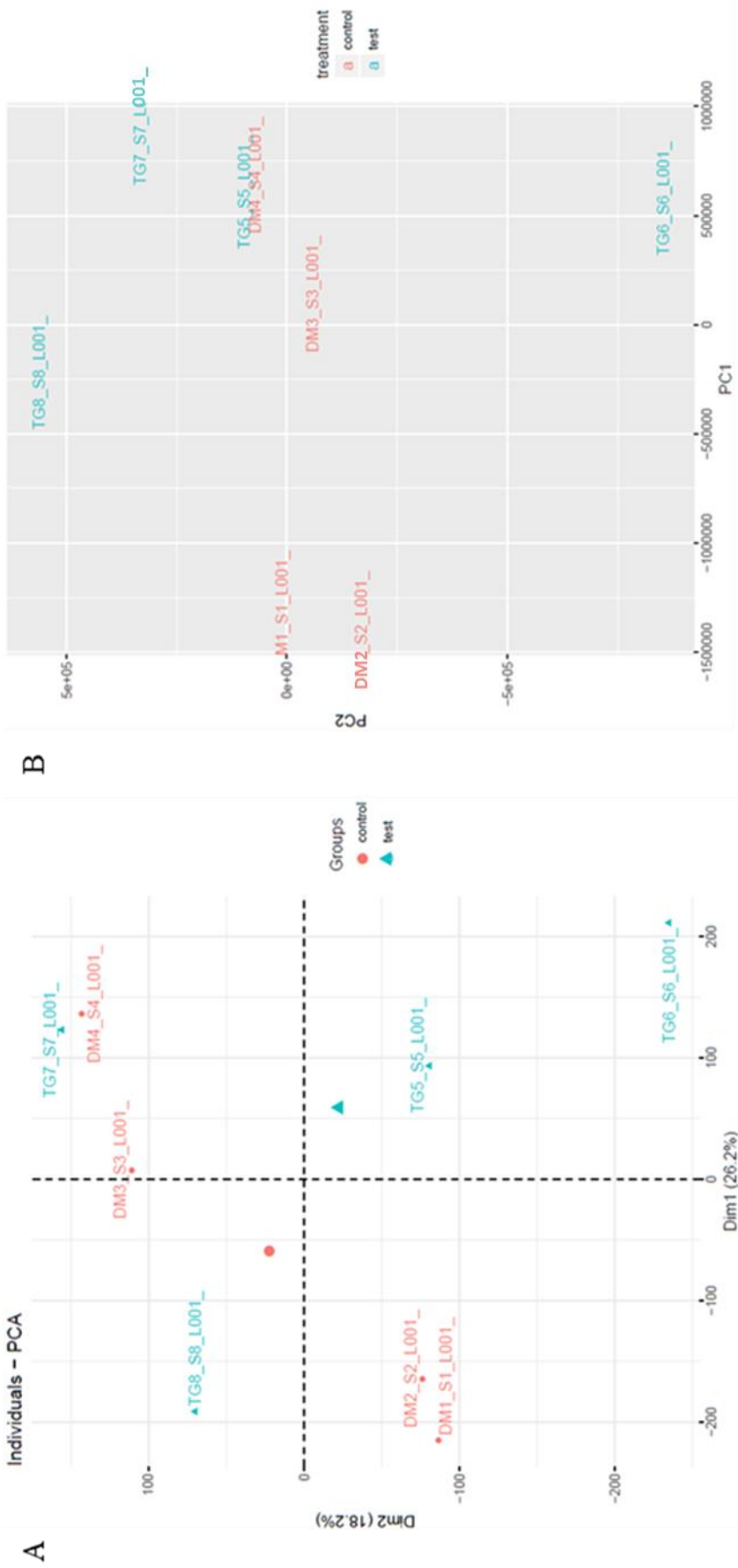


Figure 4-14. Similar global expression pattern between DMSO primed and TG primed uninfected NHBE cells. (A) PCA plot and (B) MDS plot for RNA-seq gene expression of uninfected NHBE cells primed with DMSO or TG (UUTG). Orange circles (control) referring to the DMSO primed NHBE cell samples; blue triangles (test) representing the TG primed NHBE cell samples.

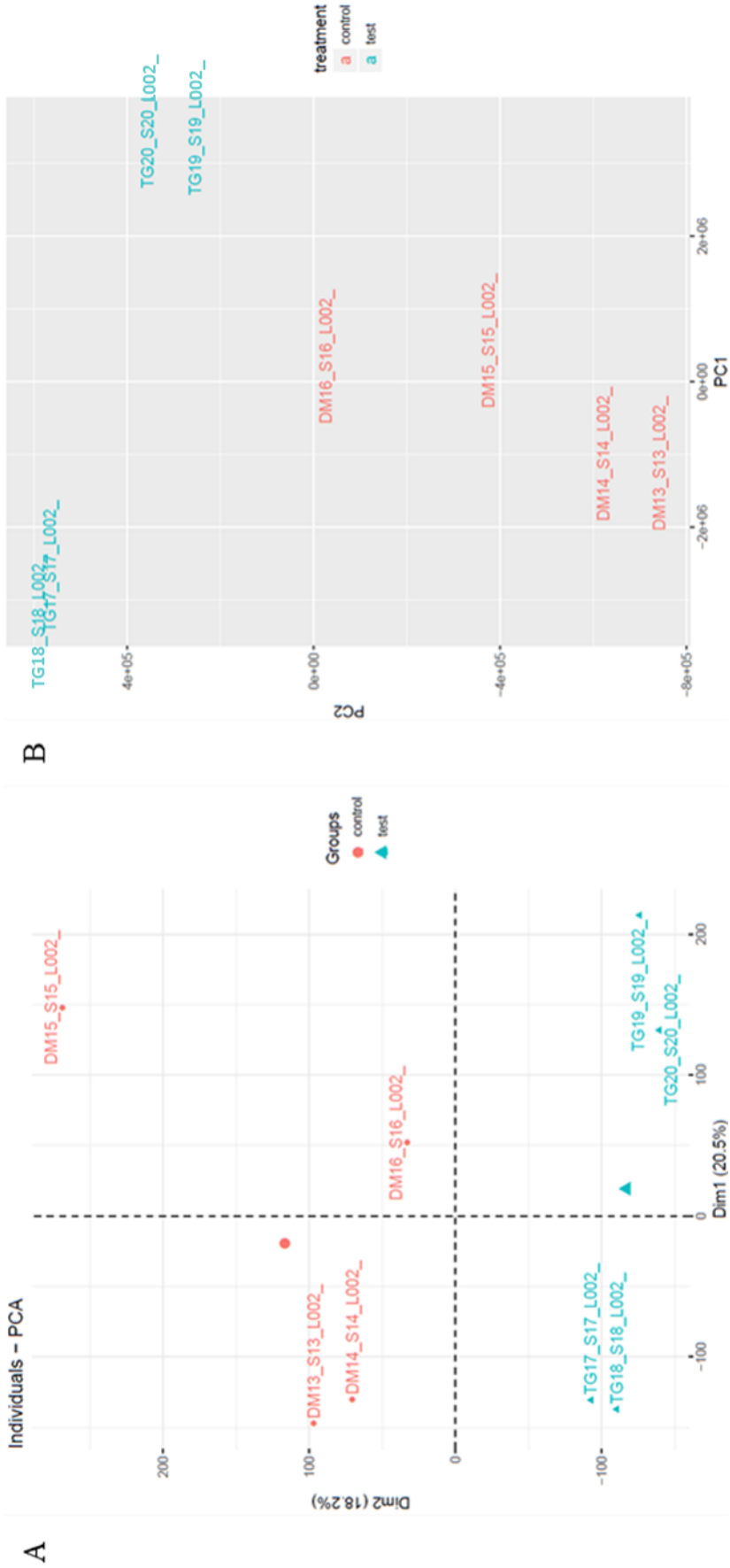


Figure 4-15. Distinct global expression pattern between DMSO primed and TG primed influenza infected NHBE cells. (A) PCA plot and (B) MDS plot for RNA-seq gene expression of H1N1 virus infected NHBE cells primed with DMSO or TG (IITG). Orange circles (control) referring to the DMSO primed NHBE cell samples; blue triangles (test) representing the TG primed NHBE cell samples.

4.3.9 TG priming reduced influenza A virus transcription in primary human tracheal epithelial cells.

The transcripts per million (TPM) for each influenza virus genome segment was compared between DMSO- and TG-primed NHBE cells. TG priming did not impair virus M gene expression in human and pig cells (Section 3.3.5). In contrast to previous results, M1/M2, HA and NS1/NS2 transcription was significantly downregulated by TG priming (Figure 4-16). A non-significant reduction in other virus transcripts, such as NP and PB1, in TG primed NHBE cells can also be observed, suggesting TG inhibits influenza virus transcription.

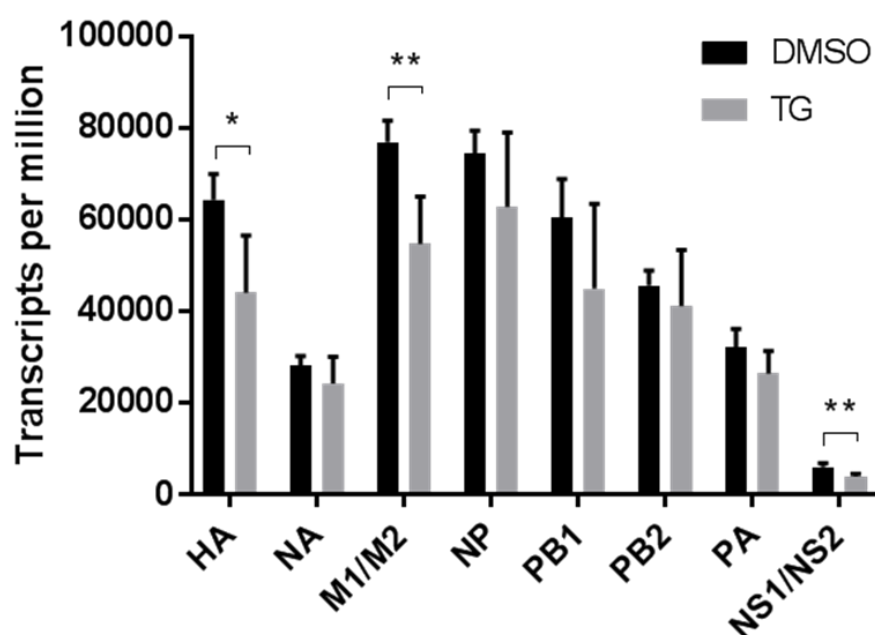


Figure 4-16. TG priming had an overall effect on influenza virus transcription. Transcripts per million of influenza virus transcripts compared between NHBE cells primed with DMSO or 0.01 μ M TG and infected with USSR H1N1 virus at 0.8 MOI for

12 h. Significance determined by paired t-test relative to the corresponding DMSO control.

4.3.10 Overview of transcripts differentially expressed in infected NHBE cells relative to uninfected NHBE cells

Volcano plots allow the significant differences in differentially regulated transcripts to be clearly visualised. The beta value, the regression coefficient measured in units of standard deviation representing the predicted change in the dependent variable for every 1 unit change in the independent variable, is plotted against the mean expression value of $\log_{10}(\text{p-value})$.

Based on pseudo-alignment utilising Kallisto v0.43.0 (Bray et al., 2016) and the differential gene expression calculated by the Sleuth v0.28.1 (Pimentel et al., 2017) in R v3.4.4 ‘Someone to Lean On’, the number of transcripts with significantly altered expression ($q < 0.01$) and a $\log_2\text{FC}$ greater than two or less than minus two ($\log_2\text{FC} \geq 2$ and ≤ -2), was determined. In NHBE cells primed with DMSO, 1347 transcripts meeting the stated criteria were significantly differentially expressed in infected NHBE cells relative to uninfected NHBE cells (UDM, Table 4-3) (Figure 4-17A). This describes the transcripts regulated by H1N1 virus infection, providing a baseline of comparison for differentially expressed genes (DEGs) following infection. In contrast, 1813 transcripts were differentially regulated in infected NHBE cells primed with TG (IUTG, Table 4-3), demonstrating the extensive alteration in transcript expression levels due to TG priming (Figure 4-17B).

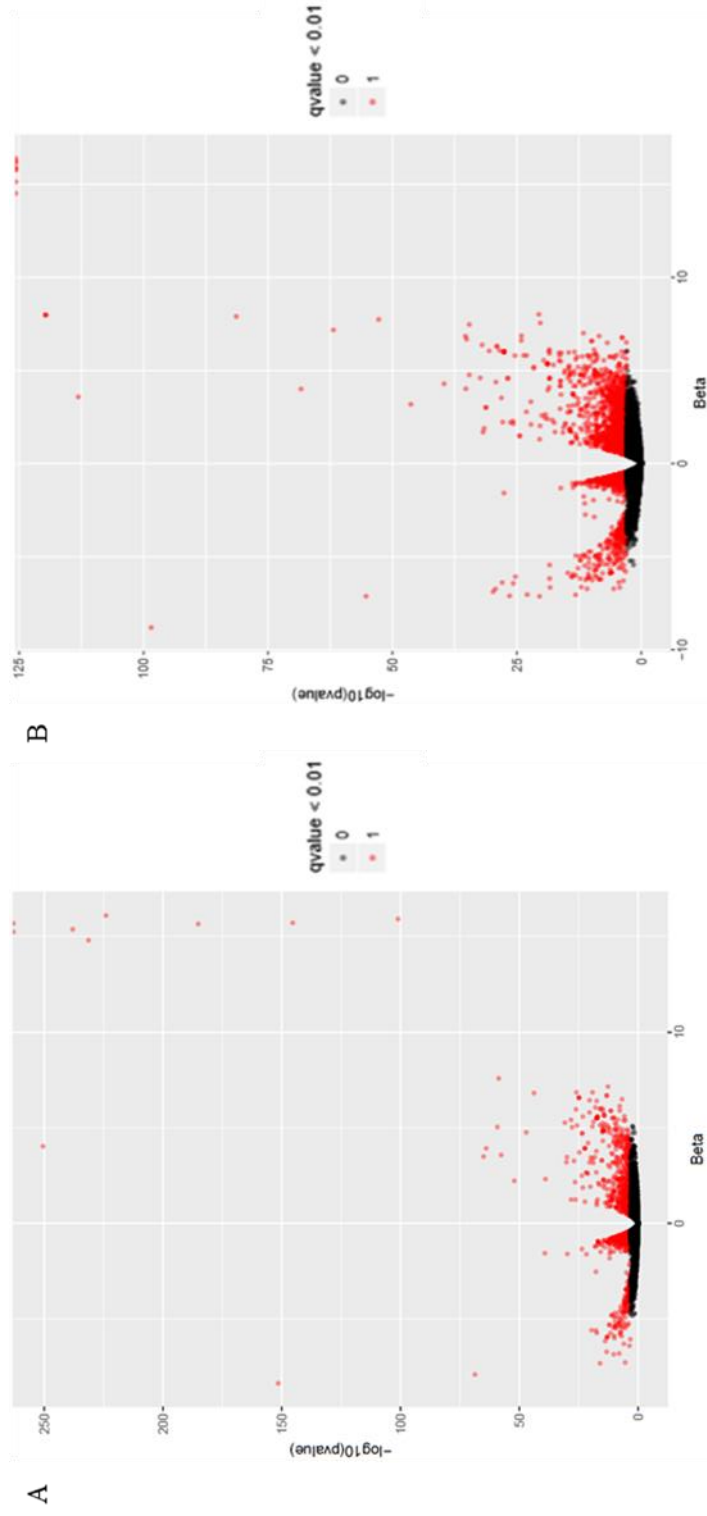


Figure 4-17. Volcano plots show a greater number of transcripts were differentially expressed in TG primed infected NHBE cells relative to TG primed uninfected NHBE cells. Differentially expressed transcripts in influenza infected NHBE cells relative to uninfected NHBE cells in (A) DMSO primed (IUDM) and (B) TG primed (IUTG) NHBE cells. NHBE cells infected with USSR H1N1 virus at 0.8 MOI for 12 h. The $-\log_{10}$ p-value was plotted against beta value, an estimator of fold change. The red dots denote the significantly differentially expressed transcripts ($q < 0.01$, $\log_2FC \geq 2$ and ≤ -2). The black dots signify the expressed transcripts which were not significantly differentially expressed.

4.3.11 Overview of transcripts differentially expressed in TG primed NHBE cells relative to DMSO primed NHBE cells

The effect of TG priming relative to DMSO priming on the transcriptome within uninfected NHBE cells (UUTG, Table 4-3) or influenza infected NHBE cells (IITG, Table 4-3) was assessed. The parameters were modified ($q < 0.05$, $\log_2FC \geq 1$ and ≤ -1) to increase the number of differentially expressed transcripts in the analysis. In uninfected NHBE cells (UUTG), 343 transcripts were significantly differentially expressed by TG priming relative to DMSO priming (Figure 4-18A). In comparison, 488 transcripts were significantly differentially expressed under the same criteria in influenza virus infected NHBE cells (IITG) (Figure 4-18B). This highlights the significant impact of TG treatment on the expression profile of tracheal epithelial cells, regardless of their infection status.

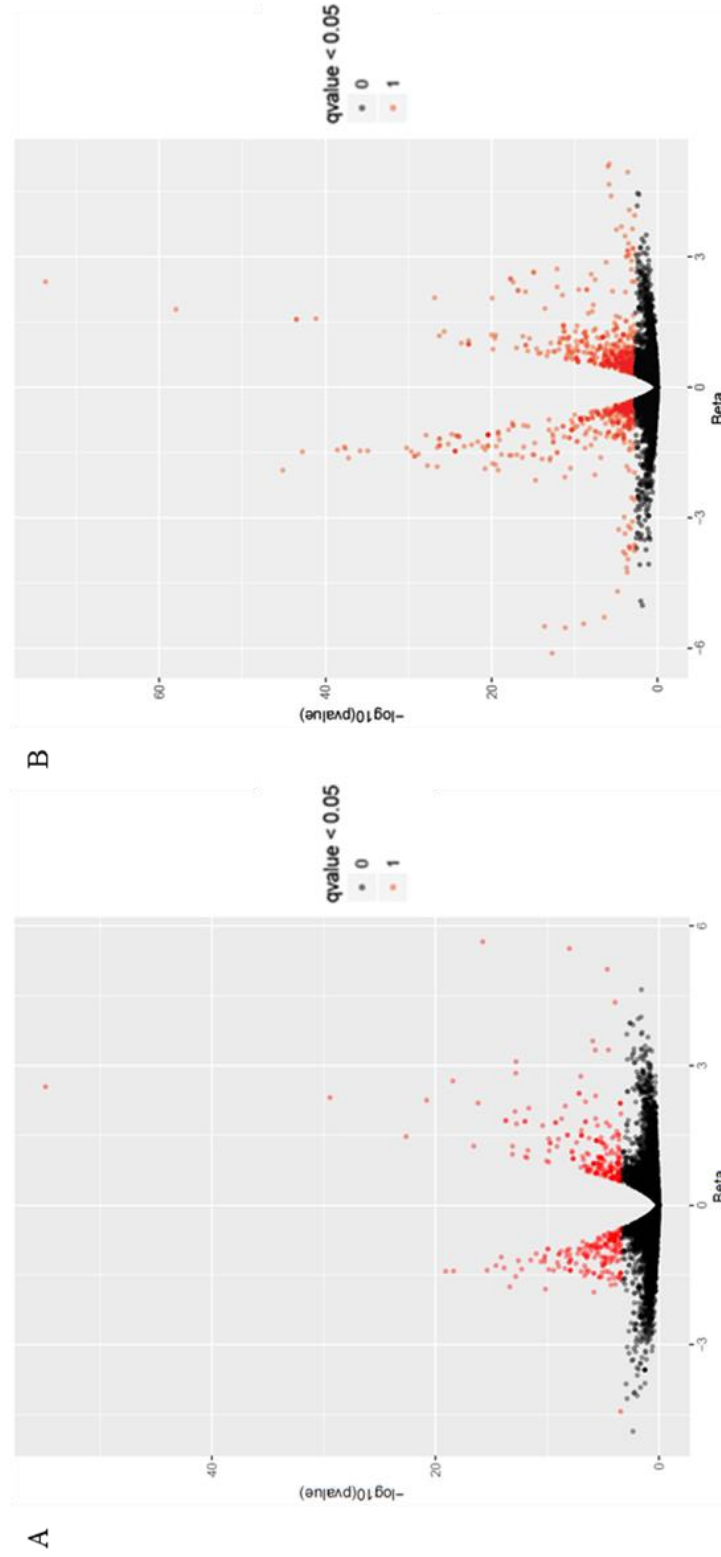


Figure 4-18. TG priming differentially regulated gene expression in uninfected and influenza virus infected NHBE cells.

Differentially expressed transcripts identified in (A) uninfected NHBE cells primed with $0.01\mu\text{M}$ TG relative to DMSO priming (UUTG) and (B) USSR H1N1 virus infected NHBE cells primed with $0.01\mu\text{M}$ TG relative to DMSO priming (IITG). The $-\log_{10}(\text{p-value})$ was plotted against beta value. The red dots denote the significantly differential expressed transcripts ($q < 0.05$, $\log_2FC \geq 1$ and ≤ -1). The black dots signify the expressed transcripts which were not significantly differentially expressed.

4.3.12 TG enhanced the innate immune signalling pathways in influenza infected NHBE cells

IPA identified a TG mediated antiviral response, based on the gene list of DEGs unique to TG primed NHBE cells upon infection (IUTG-IUDM, Table 4-3). Ingenuity Downstream Effects Analysis was performed to determine if there were any causal associations between the DEGs and biological functions or diseases. This is dependent on curated literature within the Ingenuity Knowledge Base and a corresponding z-score is assigned to the prediction. A z-score greater than two indicates the associated function is statistically significantly increased while a z-score less than minus two indicates a statistically significant decrease in function. Ingenuity downstream effects analysis strongly predicted a reduction in function in the category of infectious disease (Figure 4-19 and Table 4-4).



Figure 4-19. Heatmap of Ingenuity Downstream Effects Analysis within Infectious Diseases of DEGs in TG primed NHBE cells infected with influenza A virus. Colour intensity of the heat map represents the z-score of functions and diseases significantly enriched for DEGs ($q < 0.05$, $\log_2FC \geq 1$ and ≤ -1 , IUTG-IUDM analysis). Orange blocks indicate the z-score is positive and the associated biological function is activated, blue indicates the z-score is negative and the associated biological function is inhibited. Significance indicated if z-score ≥ 2 or ≤ -2 . z-scores < -2 outlined in green. Size of the square reflects the $-\log(p\text{-value})$ where the p-value is the likeliness of the association of the differentially expressed genes and the corresponding function due to random association. p-values < 0.05 ($-\log = 1.3$) indicate a significant, non-random association.

Categories	Diseases or Functions Annotation	p-Value	Predicted Activation State	Activation z-score
Infectious Diseases	Viral Infection	9.16E-19	Decreased	-6.352
Infectious Diseases	Infection of cells	5.94E-14	Decreased	-5.566
Infectious Diseases	Replication of virus	2.20E-11	Decreased	-4.581
Infectious Diseases	Replication of RNA virus	2.36E-11	Decreased	-4.22
Infectious Diseases	Infection by RNA virus	2.87E-11	Decreased	-5.609
Infectious Diseases	Infection by HIV-1	2.54E-10	Decreased	-5.563
Infectious Diseases	Infection of cervical cancer cell lines	7.92E-10	Decreased	-4.616
Infectious Diseases	Infection by Retroviridae	1.97E-09	Decreased	-5.356
Infectious Diseases	Infection of tumor cell lines	2.82E-09	Decreased	-4.502
Infectious Diseases	Infection by lentivirus	5.16E-09	Decreased	-5.664
Infectious Diseases	HIV infection	8.52E-09	Decreased	-5.762
Infectious Diseases	Infection of embryonic cell lines	7.71E-08	Decreased	-2.696
Infectious Diseases	Infection of epithelial cell lines	1.91E-07	Decreased	-2.956
Infectious Diseases	Infection of kidney cell lines	2.17E-07	Decreased	-3.05
Infectious Diseases	Replication of Influenza virus	5.37E-06	Decreased	-2.592
Infectious Diseases	Replication of Influenza A virus	9.82E-06	Decreased	-2.813
Infectious Diseases	Replication of Retroviridae	2.27E-05	Decreased	-2.859
Infectious Diseases	Replication of HIV	9.79E-05	Decreased	-2.547
Infectious Diseases	Replication of HIV-1	1.53E-04	Decreased	-2.243
Infectious Diseases	Production of Hepatitis C virus	1.72E-04		1.987
Infectious Diseases	Release of virus	6.31E-04		-0.746

Table 4-4. Predicted inhibition of infectious diseases in infected TG primed NHBE cells. Ingenuity Downstream Effects Analysis of DEGs ($q < 0.05$, $\log_2 FC \geq 1$ and ≤ -1 , IUTG-IUDM analysis) in TG primed NHBE cells infected with influenza A virus. Predicted activation state of diseases or functions within the category of ‘Infectious Diseases’ were assigned a z-score, significance indicated if $z\text{-score} \geq 2$ or ≤ -2 .

In particular, ‘Replication of virus’ and ‘Replication of RNA virus’ were predicted to be inhibited, displaying z-scores less than -2 (Table 4-4). This was associated with differential expression of 94 genes and 87 genes, respectively. This suggests TG may be a broad spectrum inhibitor of RNA virus replication. ‘Replication of Influenza A virus’ was also assigned a negative z score of less than -2, supporting the proposed TG mediated inhibition of influenza A virus replication.

TG priming may inhibit influenza virus replication through regulation of host innate immune signalling pathways. The 43 DEGs associated with a predicted decrease in influenza A virus replication are presented in Figure 4-20A. TG priming decreased expression of pro-viral molecules such as *ARCN1*, *GSK3B* and *CSE1L*, resulting in predicted inhibition of IAV replication.

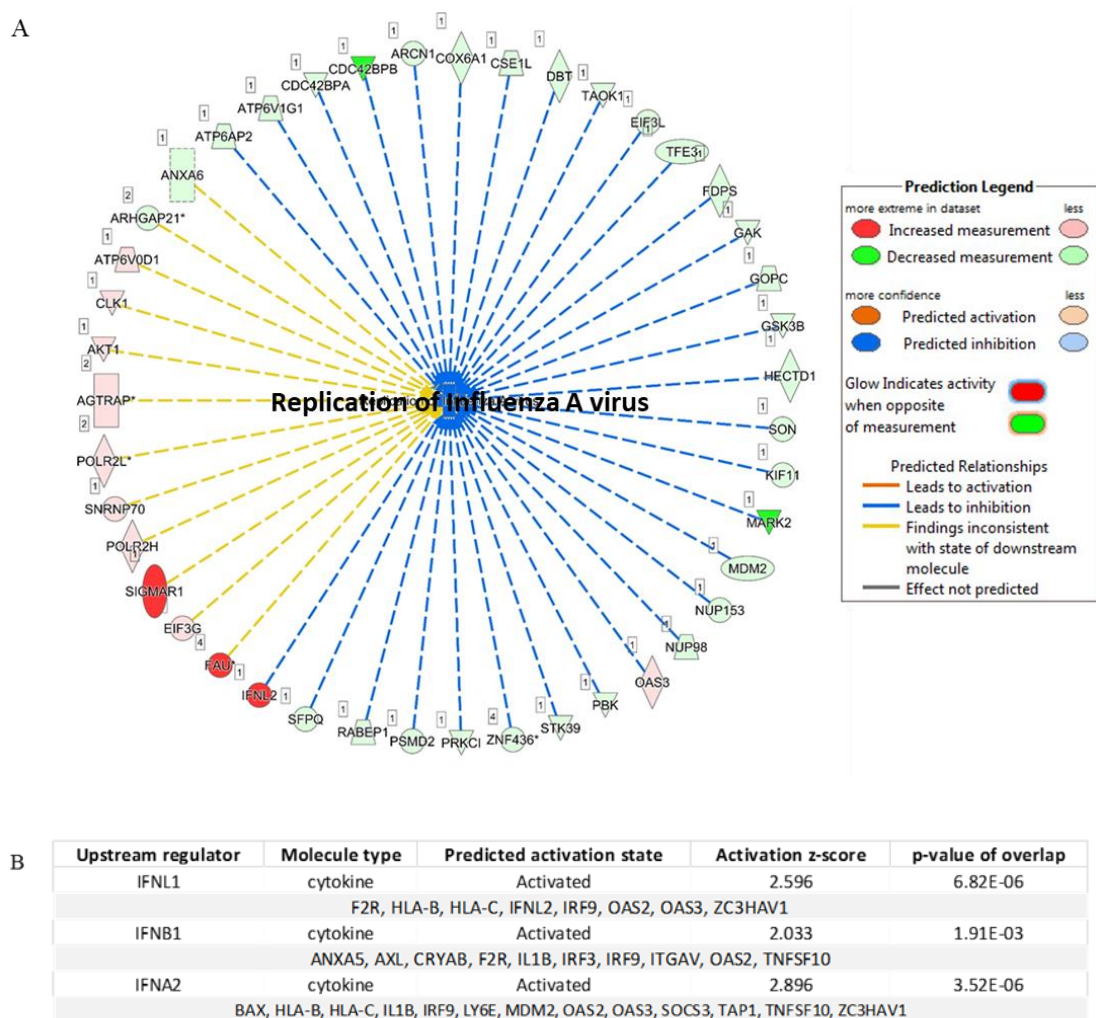


Figure 4-20. TG priming differentially regulated genes with anti-influenza virus effects. (A) Ingenuity downstream effects analysis of DEGs ($q < 0.05$, $\log_2 FC \geq 1$ and ≤ -1 , IUTG-IUDM analysis). Directional expression of molecules associated with IPA predicted inhibition of 'Replication of influenza A virus' shown with dotted blue lines. (B) TG priming was predicted to induce activation of upstream immune regulators. Ingenuity Upstream Regulator Analysis of DEGs ($q < 0.05$, $\log_2 FC \geq 1$ and ≤ -1 , IUTG-IUDM analysis), associated with 'viral infection' by Ingenuity downstream effects analysis. Shaded box lists target molecules in dataset corresponding to the upstream regulator.

TG priming did not only affect the expression of pro-viral host genes but induced activation of antiviral upstream regulators. IPA downstream effects analysis determined 249 molecules were associated with ‘viral infection’ (p-value 9.16E-19, z-score -6.352) (Table 4-4). Separate analysis of this subset of DEGs proposed TG induced activation of IFN- λ 1, IFN- β 1 and IFN- α 2 cytokine signalling pathways (Figure 4-20B). This suggests TG priming induced activation of innate immune signalling pathways, such as the type I and type III IFN responses.

Alternative analysis, using NIPA enrichment analysis of DEGs in infected relative to uninfected TG primed NHBE cells (IUTG, Table 4-3), confirmed TG priming elevated the expression of multiple components of the innate immune response. BP GO terms such as ‘*defense response to virus*’ and ‘*type I IFNs signalling pathway*’ were also significantly enriched in upregulated genes in TG primed NHBE cells (Figure 4-21). *IFNL2*, a type III IFN, was significantly upregulated in TG primed NHBE cells and associated with a predicted decrease in influenza A virus (Figure 4-20A). Furthermore, NIPA enrichment analysis identified the BP GO term ‘*regulation of type III IFN production*’ was significantly enriched in TG primed NHBE cells (Figure 4-21).

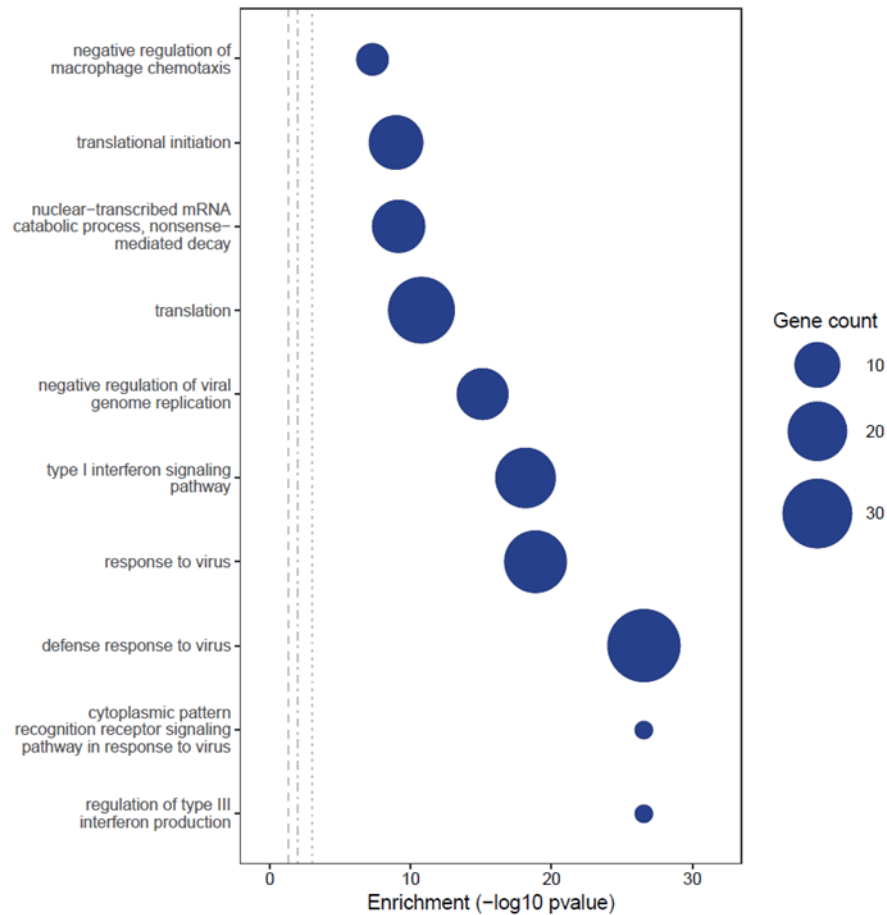


Figure 4-21. TG priming induced an antiviral state in NHBE cells. Top 10 significantly enriched BP GO terms of genes upregulated by influenza virus infection in TG primed NHBE cells ($q < 0.01$, $\log_2 FC \geq 2$, IUTG analysis). Circles representative of gene count within each GO term. Location along x-axis depicts the enrichment score ($-\log_{10} pvalue$).

TG priming was found to elevate *RIG-I* and *OAS1* expression in influenza infected tracheal epithelial cells and myoblasts (Section 3.3.17). RNA-seq analysis confirmed TG mediated elevation of innate immune gene expression. Directly comparing the fold change in gene expression induced by influenza virus infection in TG primed (IUTG,

Table 4-3) and DMSO primed (IUDM, Table 4-3) NHBE cells for the same transcripts revealed the potent effect of TG on the type I IFN response (Table 4-5). For example, in DMSO primed cells, *OASL* expression displayed an absolute fold change of 101.26 ($\log_2\text{FC} = 6.66$) following influenza virus infection. However, in TG primed NHBE cells, *OASL* expression displayed an absolute fold change of 471.2 ($\log_2\text{FC} = 8.88$) in expression. This was consistent for the majority of transcripts connected to the type I IFN immune response and demonstrates TG mediated their upregulation. However, the 270.58 fold change in *IFN β 1* expression in DMSO primed NHBE cells (p-value $8.79\text{E-}17$, q-value $3.35\text{E-}14$, $\log_2\text{FC}$ 8.08) was greater than the 89.3 fold change in TG primed NHBE cells (p-value $3.69\text{E-}16$, q-value $1.14\text{E-}13$, $\log_2\text{FC}$ 6.48). This may reflect activation of negative feedback regulatory mechanisms 12 h post infection. In order to explore this, quantifying IFN β production at different time points after infection with influenza A virus following TG or DMSO priming may be considered for future work.

Gene symbol	Gene ID	q-value	log ₂ FC
MX1	ENSG00000157601	6.61E-117	Inf
		1.22E-22	7.91146507
MX2	ENSG00000183486	3.38E-50	Inf
		5.00E-56	11.91320758
IFI27	ENSG00000275214	6.01E-17	9.530332805
		5.52E-13	7.018155589
IFI27	ENSG00000165949	6.01E-17	9.530332805
		5.52E-13	7.018155589
OASL	ENSG00000135114	4.70E-22	8.880201524
		2.44E-28	6.661887812
IFIT1	ENSG00000185745	2.36E-18	8.77483773
		4.67E-11	7.730770433
XAF1	ENSG00000132530	1.45E-09	8.248674708
		9.24E-06	6.347320971
IFIT2	ENSG00000119922	4.04E-10	8.226119287
		1.67E-15	8.061498991
IFI6	ENSG00000126709	2.56E-23	8.173364759
		9.09E-17	7.436403189
IRF7	ENSG00000276561	3.90E-11	7.07050091
		1.13E-07	4.242216632
IFITM1	ENSG00000185885	1.40E-16	5.14943908
		2.88E-20	4.481401289
OAS1	ENSG00000089127	1.12E-09	4.683762518
		0.00046941	3.755586578
OAS3	ENSG00000111331	2.29E-14	3.796845871
		7.16E-17	3.330555131
ISG20	ENSG00000172183	6.93E-05	3.214070089
		7.84E-06	2.48399167
OAS2	ENSG00000111335	0.000002398	3.203097204
		6.69E-11	3.195872443
IFITM3	ENSG00000142089	0.000656931	2.67646234
		0.003685709	2.25447784
IRAK1	ENSG00000184216	0.000855608	2.281469373
		0.001144382	2.984700518
IFIT3	ENSG00000119917	1.92E-14	5.010299832
		2.50E-21	5.604760418
OAS2	ENSG00000111335	2.72E-11	3.144200877
		1.76E-06	3.43682355
IFI35	ENSG00000068079	0.000356387	4.563062166
		0.002008904	4.601760197

Table 4-5. TG elevated type I IFN dependent gene expression in infected NHBE cells. Comparison of significantly upregulated genes ($q < 0.01$, $\log_2FC \geq 2$ and ≤ -2) in the type I interferon signalling pathway (GO:0060337) in TG primed (grey, IUTG) and DMSO primed (white, IUDM) NHBE cells infected with USSR H1N1 virus at 0.8

MOI for 12 h. If multiple transcripts were present for one gene, the differentially expressed transcript with the highest log₂FC in TG treated NHBE cells (IUTG) was selected. Genes in order of highest log₂FC in TG primed NHBE cells (IUTG). Bottom three genes displayed greater upregulation in DMSO primed NHBE cells (IUDM). Inf = infinity.

IPA analysis identified IRF3 and IRF7 as regulators of the TG dependent immune transcription. IRF3 transcript expression in infected relative to uninfected cells primed with DMSO (IUDM, Table 4-3) was downregulated (ENST00000596822: q value 0.0055, log₂FC -5.86 and ENST00000601809: q-value 0.0068, log₂FC -4.99). In contrast, in infected cells relative to uninfected cells primed with TG (IUTG, Table 4-3), IRF3 transcript ENST00000601809 was downregulated (q-value 0.016, log₂FC -4.18) while IRF3 transcript ENST00000601291 was upregulated (q-value 0.0083, log₂FC 1.32). Secondly, IRF7 (Transcript ID ENST00000631879) expression induced by influenza virus infection was elevated in TG primed cells (IUTG, q-value 3.90E-11, log₂FC 7.07) in comparison to DMSO primed cells (IUDM, q-value 1.13E-07, log₂FC 4.24). Activation of transcription factors such as IRF3 and IRF7 suggests TG priming extensively regulates host immunity, supported by IPA mapping of IRF3 direct interactions with immune signalling components (Figure 4-22).

Figure 4-22. IPA mapping of direct interactions of immune signalling components with IRF3.

4.3.13 The ER stress response was activated by TG priming

TG inducement of ER stress markers (*HSP90B1*, *HSPA5* and *DDIT3*) was considered in Section 3.3.20 but through RNA-seq analysis, the extent of TG priming on the ER stress response was revealed. TG priming inducement of ER stress was supported by multiple analysis, including GO ontology enrichment analysis, KEGG pathway enrichment and IPA. In order to explore the effect of TG dependent regulation of ER stress, genes differentially expressed due to influenza virus infection were not considered, due to the suppression of the ER stress response in influenza virus infected cells, as described in Chapter 3. Instead, genes differentially expressed in TG primed relative to DMSO primed NHBE cells were considered (UUTG and IITG, Table 4-3). The ER stress response may be divided into three branches; the ATF6, the IRE-1 and the PERK-mediated stress responses (Gardner *et al.*, 2013). Genes differentially expressed due to TG priming in uninfected NHBE cells (UUTG, Table 4-3) revealed significant enrichment of BP GO terms associated with multiple branches of ER stress response (Figure 4-23A and Table 4-6). The ER stress response was also induced by TG priming relative to DMSO in influenza virus infected NHBE cells (IITG, Figure 4-3) (Figure 4-23B and Table 4-6). However, genes associated with negative regulation of the IRE-1 stress response were upregulated, suggesting differential regulation of different components of the ER stress response (Table 4-6).

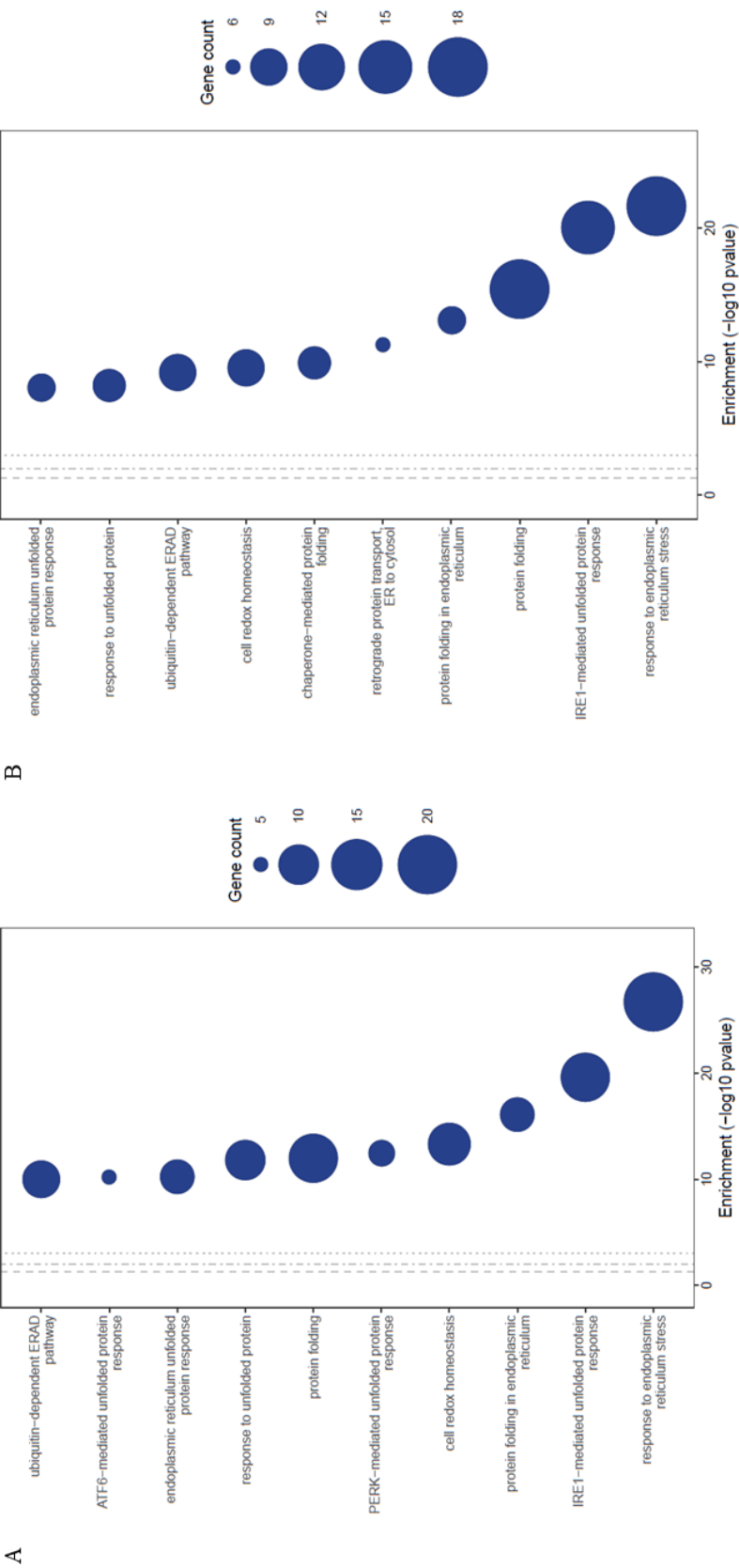


Figure 4-23. The ER UPR stress response is the top BP GO term significantly enriched with DEGs. BP GO terms significantly enriched for upregulated gene ($q < 0.05$, $\log_2 FC \geq +1$) in TG primed relative to DMSO primed (A) uninfected NHBE cells (UUTG) and (B) influenza infected NHBE cells (IITG). Top 10 enriched terms displayed. Circles representative of gene count within each GO term. Location along x-axis depicts the enrichment score ($-\log_{10} pvalue$).

Uninfected cells						
GO biological process	Reference gene list (Homo sapiens)	DEGs in GO BP	Expected	fold Enrichment	raw P-value	FDR
ATF6-mediated unfolded protein response (GO:0036500)		9	5	0.05	8.74E-09	3.35E-06
PERK-mediated unfolded protein response (GO:0036499)		12	6	0.07	4.19E-10	2.54E-07
IRE1-mediated unfolded protein response (GO:0036498)		53	13	0.29	3.25E-17	7.30E-14
negative regulation of IRE1-mediated unfolded protein response (GO:1903895)		3	2	0.02	2.95E-04	3.16E-02
Influenza infected						
GO biological process	Reference gene list (Homo sapiens)	DEGs in GO BP	Expected	fold Enrichment	raw P-value	FDR
ATF6-mediated unfolded protein response (GO:0036500)		9	4	0.06	61.93	1.68E-06
PERK-mediated unfolded protein response (GO:0036499)		12	4	0.09	46.45	4.22E-06
IRE1-mediated unfolded protein response (GO:0036498)		53	12	0.38	31.55	3.20E-14
negative regulation of IRE1-mediated unfolded protein response (GO:1903895)		3	2	0.02	92.9	4.97E-04

Table 4-6. TG priming upregulated genes associated with the multiple components of the ER stress response. Significantly enriched BP GO terms identified through PANTHER Overrepresentation Test ($FDR < 0.05$) of genes upregulated ($q < 0.05$, $\log_2 FC \geq 1$) by TG priming in uninfected (UUTG) and influenza infected (IITG) NHBE cells. BP GO terms associated with ER stress which were significantly enriched for

IPA supported TG mediated inducement of the ER stress response, specifically the UPR, suggested by the significantly enriched canonical pathways in uninfected NHBE cells (Figure 4-24A) and influenza infected NHBE cells (Figure 4-24B) for DEGs upregulated by TG priming.



Figure 4-24. The ER UPR stress response is the top canonical pathway represented by DEGs between TG primed and DMSO primed uninfected NHBE cells. IPA canonical pathways most significantly changed in DEGs ($q < 0.05$, $\log_2 FC \geq 1$ and ≤ -1) of (A) TG primed relative to DMSO primed uninfected NHBE cells (UUTG) and (B) TG primed relative to DMSO primed influenza infected NHBE cells (IITG). The

stacked bar chart displays the percentage of significantly DEGs that are upregulated (red) or downregulated (green) in each canonical pathway. The number of genes within the pathway recognised by the IPA knowledge database indicated by the numbers to the right of the bars. Orange line represents the $-\log p$ -value, indicating the statistical significance of the over-represented canonical pathway.

Activation of the ER UPR was dependent on TG priming and was not induced by influenza virus infection (Figure 4-25). In contrast to the extensive upregulation of genes associated with the canonical UPR pathway in TG primed NHBE cells (Figure 4-26), genes associated with the canonical UPR pathway (*SCAP*, *PDI*, *MBTPS*, *GRP94*, *VCP* and *OS9*) were downregulated in influenza virus infected cells (Figure 4-25).

IUDM

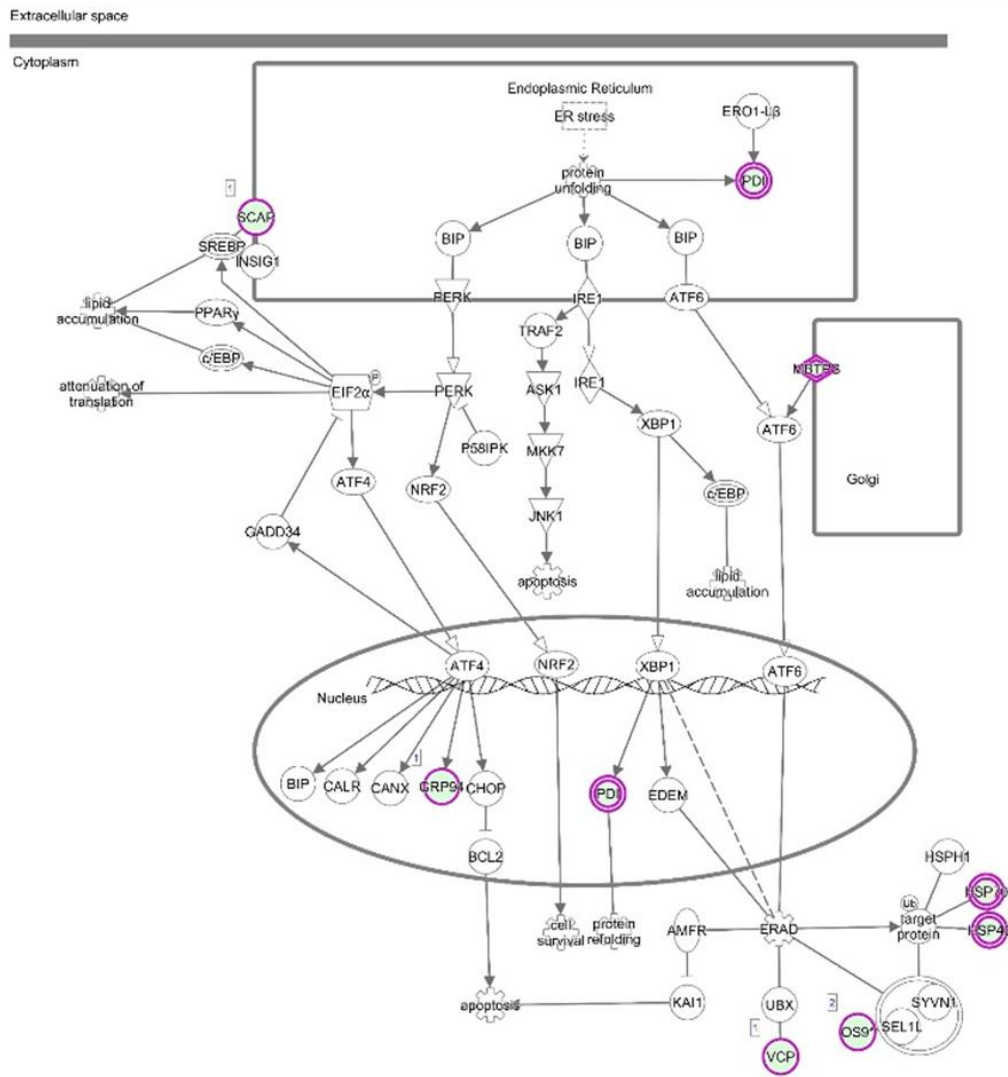


Figure 4-25. Influenza A virus infection did not induce activation of the ER UPR.

DEGs ($q < 0.05$, $\log_2 FC \geq 1$ and ≤ -1) in the Ingenuity UPR pathway in (A) TG primed relative to DMSO primed uninfected NHBE cells (UUTG), (B) TG primed relative to DMSO primed influenza infected NHBE cells (IITG) and (C) influenza infected relative to influenza uninfected NHBE cells primed with DMSO (IUDM). Red shading indicates upregulation of gene expression while green shading indicates downregulation. Colour intensity indicates the magnitude in fold change in gene expression.

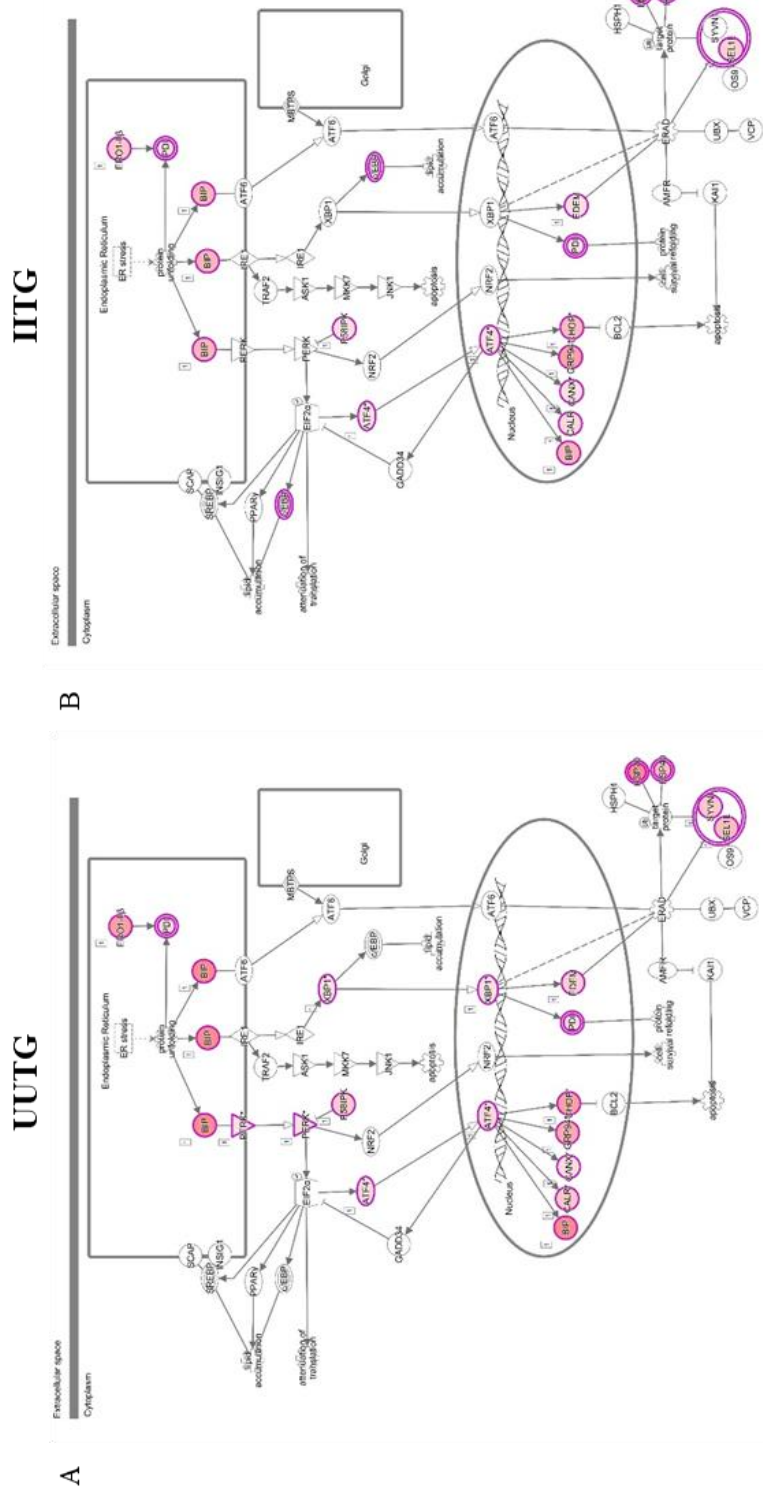


Figure 4-26. TG priming induced activation of the ER UPR. DEGs ($q < 0.05$, $\log_2FC \geq 1$ and ≤ -1) in the Ingenuity UPR pathway in (A) TG primed relative to DMSO primed uninfected NHBE cells (UUTG) and (B) TG primed influenza infected NHBE cells (IITG). Red shading indicates upregulation of gene expression while green shading indicates downregulation. Colour intensity indicates the magnitude in fold change in gene expression.

Induction of the UPR indicates impaired protein processing and an accumulation of misfolded proteins in the ER (reviewed by (Walter & Ron, 2011)). TG mediated interference of host protein synthesis was supported by the significant enrichment of the hsa04141 ‘protein processing in endoplasmic reticulum’ KEGG pathway for DEGs in TG primed infected (IITG) and uninfected (UUTG) NHBE cells (Figure 4-27).

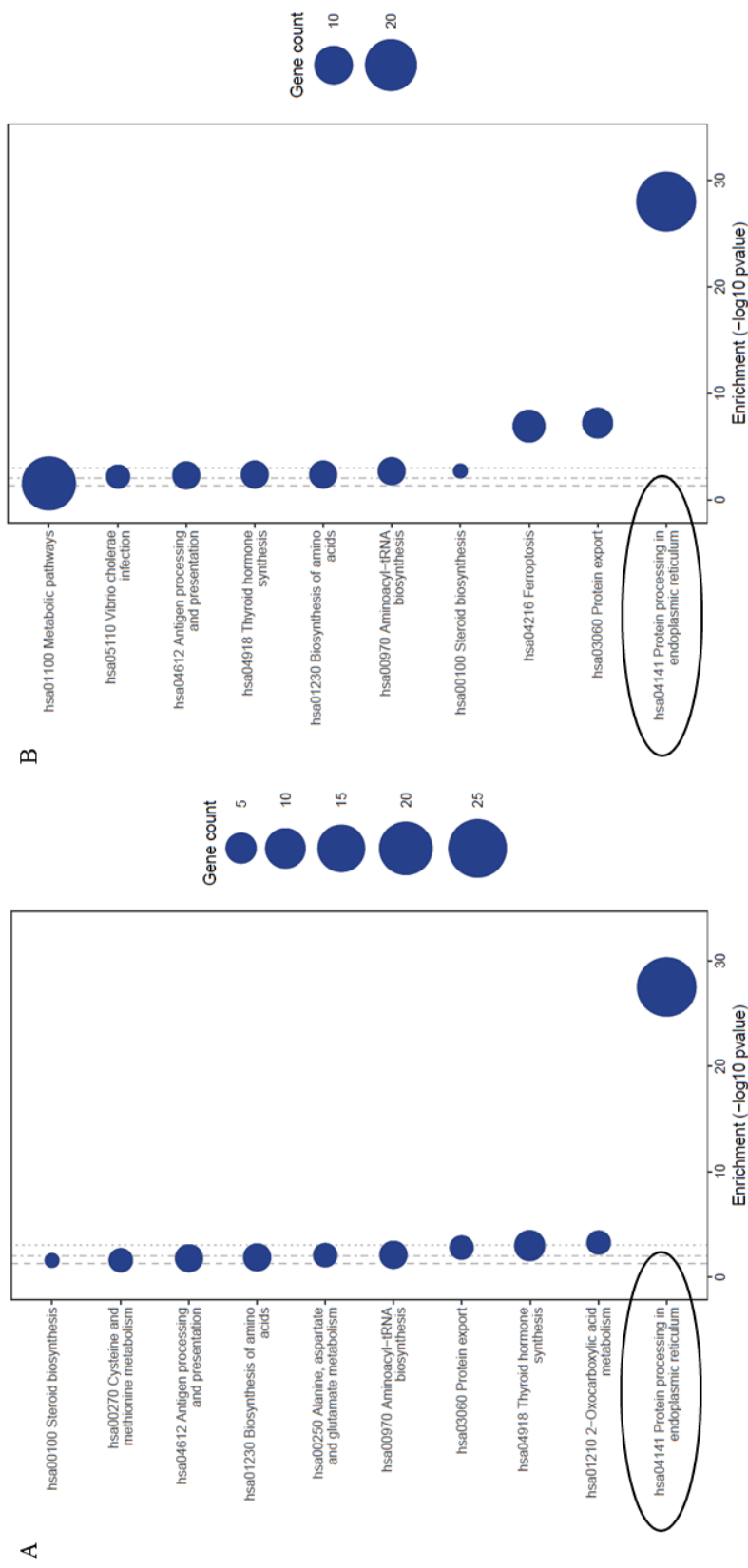


Figure 4-27. ‘Protein processing in endoplasmic reticulum’ is in the top 10 KEGG pathways significantly enriched for genes upregulated by TG priming. BP GO terms significantly enriched with differentially expressed genes ($q < 0.05$, $\log_2 FC \geq 1$) in TG primed relative to DMSO primed (A) uninfected NHBE cells (UUTG) and (B) influenza infected NHBE cells (IITG). Top 10 enriched terms displayed. Circles representative of gene count within each GO term. Location along x-axis depicts the enrichment score ($-\log_{10} pvalue$).

Upregulation of genes within the unfolded protein response (UPR) and may reflect impaired protein modifications and their retention in the ER. N-linked glycosylation is a key component of maintaining ER homeostasis and accumulation of hypoglycosylated misfolded glycoproteins induces the UPR (Cherepanova *et al.*, 2016). BP GO term '*protein N-linked glycosylation*' (GO:0006487) was significantly enriched for genes upregulated by TG priming in uninfected NHBE cells (UUTG, fold enrichment 15.12, p-value 4.44E-06, FDR 1.01E-03) and influenza infected NHBE cells (IITG, fold enrichment 11.61, p-value 1.99E-05, FDR 3.37E-03), indicated by PANTHER Overrepresentation Test. N linked glycosylated proteins enter the calnexin (CNX)/ calreticulin (CRT) cycle which promotes correct folding of the glycoprotein (Cherepanova *et al.*, 2016, Caramelo & Parodi, 2008a). IPA mapping of interactions between components of the UPR revealed upregulation of *CALR*, *CANX* and their upstream regulator *ATF4* in TG primed influenza infected NHBE cells (IITG) (Figure 4-28). This suggests TG priming may interfere with glycoprotein folding in the ER, requiring compensatory increases in gene expression to restore ER homeostasis.

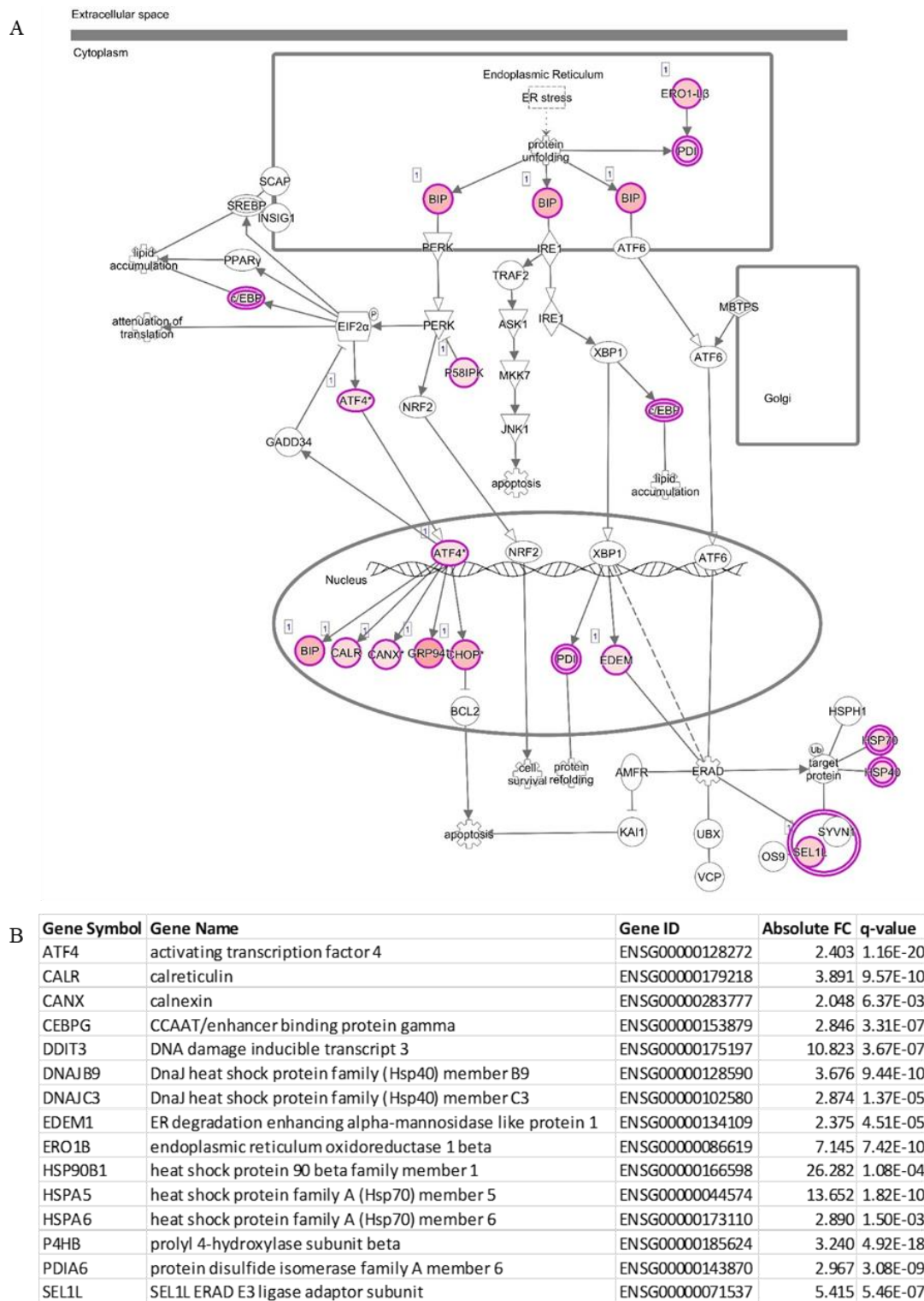


Figure 4-28. Upregulation of genes associated with glycoprotein folding in the UPR.

The UPR canonical pathway is significantly enriched with differentially expressed genes between TG primed and DMSO primed infected NHBE cells (IITG). (A)

Interaction of the products of the differentially expressed genes ($q < 0.05$, $\log_2 FC \geq 1$ and ≤ -1) in the Ingenuity canonical UPR pathway. Red shading indicating upregulated gene expression and green indicates downregulated expression. Intensity of colour is proportional to fold change in gene expression. (B) Associated absolute fold change (FC) in expression and q-value of the differentially expressed genes in the Ingenuity UPR pathway.

However, while downstream effects analysis identified differentially expressed genes implicated in protein folding (p-value 1.04E-06), no prediction on the activation of the function (z-score) was possible based on the available gene expression data.

4.3.14 Inhibition of protein transport in the Golgi apparatus may underlie a key feature of the TG mediated antiviral response

TG priming may interfere with transport of proteins from the ER through the Golgi and to the PM. Influenza glycoproteins HA and NA must travel through the secretory pathway to the apical PM (Ohkura et al., 2014). Pathway enrichment analysis of significantly downregulated genes ($q < 0.05$, $\log_2FC \leq -1$) in influenza virus infected relative to uninfected TG primed NHBE cells (IUTG, Table 4-3), revealed inhibition of genes associated vesicle transport to and within the Golgi apparatus (Table 4-7).

GO biological process	Reference gene list (Homo sapiens)	DEGs in GO BP	Expected	fold Enrichment	raw P-value	FDR
retrograde vesicle-mediated transport, Golgi to ER (GO:0006890)	84	21	6.82	3.08	3.03E-05	1.43E-03
ER to Golgi vesicle-mediated transport (GO:0006888)	207	45	16.81	2.68	4.80E-08	4.02E-06
Golgi vesicle transport (GO:0048193)	359	71	29.16	2.44	3.92E-10	4.60E-08
post-Golgi vesicle-mediated transport (GO:0006892)	94	18	7.63	2.36	1.85E-03	4.85E-02

Table 4-7. TG priming downregulated genes associated with the secretory pathway. Significantly enriched BP GO terms identified through PANTHER Overrepresentation Test ($FDR < 0.05$) of significantly downregulated genes ($q < 0.05$, $\log_2 FC \leq -1$) in influenza infection in TG primed NHBE cells (IUTG). BP GO terms associated with transport to, within and from the Golgi which were significantly enriched for downregulated genes displayed.

COPI-coated vesicles mediate intra-Golgi transport and retrograde Golgi to ER transport. (Rabouille & Klumperman, 2005). Downregulated genes within ‘*Golgi vesicle transport*’ (GO:0048193) significantly enriched the BP GO term ‘*COPI coating of Golgi vesicle*’ (GO:0048205) (PANTHER Overrepresentation Test, fold

Enrichment >100, raw P-value 2.91E-04, FDR 3.18E-02). Further analysis revealed differential expression of components of the COPI complex in TG primed NHBE cells (Table 4-8).

Gene ID	Gene name	Gene symbol	q-value	Absolute FC
ENSG00000129083	coatamer protein complex subunit beta 1	COPB1	9.36669E-06	0.127623347
ENSG00000184432	coatamer protein complex subunit beta 2	COPB2	0.008074738	0.170340529
ENSG00000111481	coatamer protein complex subunit zeta 1	COPZ1	0.033463867	0.17660538
ENSG00000095139	coatamer subunit delta	ARCN1	0.04724305	0.154887276

Table 4-8. COPI coatamer subunits were significantly downregulated in TG primed cells. Genes significantly downregulated ($q < 0.05$, $\log_2 FC \leq -1$) in influenza infection in TG primed NHBE cells (IUTG). FC = fold change.

In addition, expression of TRAPP complex subunits, forming vesicle tethers implicated in COPI transport, were also differentially regulated (Table 4-9).

Gene ID	Gene name	Gene symbol	q-value	Absolute FC
ENSG00000054116	Trafficking Protein Particle Complex 3	TRAPPC3	0.032909642	0.053100138
ENSG00000181029	Trafficking Protein Particle Complex 5	TRAPPC5	0.004606054	2.867017701
ENSG00000168538	Trafficking Protein Particle Complex 11	TRAPPC11	0.000000252	0.108838831

Table 4-9. TRAPP complex subunit gene expression was differentially regulated in TG primed cells. Genes significantly downregulated ($q < 0.05$, $\log_2 FC \leq -1$) in influenza infection in TG primed NHBE cells (IUTG). FC = fold change.

The stage at which TG inhibits vesicle transport requires further consideration. TG priming may disrupt vesicle transport at early and late stages, indicated by enrichment of BP GO terms ‘ER to Golgi vesicle-mediated transport’ (GO:0006888) and ‘post-Golgi vesicle-mediated transport’ (GO:0006892) (Table 4-7). Genes associated with the latter described in Table 4-10, emphasising the extensive effect of TG priming on genes associated with protein transport.

post-Golgi vesicle-mediated transport (GO:0006892)		Gene name	Gene symbol	q-value	Absolute FC
Gene ID					
ENSG00000124198		Brefeldin A-inhibited guanine nucleotide-exchange protein 2	ARFGEF2	0.0001393	0.143636266
ENSG00000113595		3 ubiquitin-protein ligase TRIM23	TRIM23	0.0130829	0.073620779
ENSG00000092108		Sec1 family domain-containing protein 1	SCFD1	0.00049	0.016222719
ENSG00000109113		Ras-related protein Rab-34	RAB34	0.0271237	0.147485218
ENSG00000107862		Golgi-specific brefeldin A-resistance guanine nucleotide exchange factor 1	GBF1	0.0299396	0.176279154
ENSG00000125124		Bardet-Biedl syndrome 2 protein	BBS2	0.0085973	0.123284463
ENSG00000131558		Exocyst complex component 4	EXOC4	0.0049004	0.164758851
ENSG00000178950		Cyclin-G-associated kinase	GAK	0.0443149	0.038090934
ENSG00000127603		Microtubule-actin cross-linking factor 1, isoforms 1/2/3/5	MACF1	0.0056697	0.0808616
ENSG00000119396		Ras-related protein Rab-14	RAB14	0.0012971	0.127949695
ENSG00000112685		Exocyst complex component 2	EXOC2	0.0010893	0.146758558
ENSG00000049245		Vesicle-associated membrane protein 3	VAMP3	0.0003805	0.007150582
ENSG00000115306		Spectrin beta chain, non-erythrocytic 1	SPTBN1	1.11E-07	3.63866E-10
ENSG00000047932		Golgi-associated PDZ and coiled-coil motif-containing protein	GOPC	0.0110221	0.16737205
ENSG00000131381		Rabenosyn-5	RBSN	0.0253848	0.046199472
ENSG00000090989		Exocyst complex component 1	EXOC1	0.0190243	0.1033074
ENSG00000123240		Optineurin	OPTN	0.0360984	0.421089538
ENSG00000076321		Kelch-like protein 20	KLHL20	0.0015459	0.136030287

Table 4-10. TG priming downregulated genes associated with post Golgi vesicle mediated transport.

Enrichment of BP GO term GO:0006892 for genes significantly downregulated ($q < 0.05$, $\log_2 FC \leq -1$) in influenza infection in TG primed NHBE cells (IUTG). Fold enrichment of BP GO terms determined by PANTHER Overrepresentation Test ($FDR < 0.05$).

4.3.15 TG priming induced Golgi stress and Ca^{2+} dysregulation in uninfected NHBE cells independent of SOCE

Having identified potential TG mediated inhibition of vesicular transport within the ER and Golgi complex to PM, the contribution of TG mediated disruption of Ca^{2+}

homeostasis on the Golgi apparatus was considered. Ca^{2+} dependent regulation of Golgi function is well established (Dolman & Tepikin, 2006). Similar to the ER, the Golgi is an intracellular Ca^{2+} store (Li *et al.*, 2013). Two types of Ca^{2+} ATPases are present in the Golgi membrane, belonging to the SERCA family and secretory pathway calcium ATPase (SPCA) family, both responsible for Ca^{2+} intake in the Golgi (Van Baelen *et al.*, 2004). Store independent calcium entry (SICE) facilitates extracellular Ca^{2+} entry through SPCAC2 interaction with the ORAI1 channels (Cross *et al.*, 2013). This merited analysis of the contribution of Ca^{2+} mobilisation on Golgi function.

The expression of the Ca^{2+} ATPases SPCA1 and SPCA2 (encoded by *ATP2C1* and *ATP2C2*, respectively) and Golgi stress markers (*WIP149*, *SERPINH1* and *GCP60*) following TG priming was analysed in uninfected and influenza infected NHBE cells. TG priming significantly induced *ATP2C2* (SPCA2) expression in uninfected and influenza infected NHBE cells but had no effect on *ATP2C1* (Figure 4-29). This suggests a potential enhancement of extracellular Ca^{2+} influx, mediated by ORAI1 channels, into the Golgi complex by TG.

Secondly, TG priming increased expression of markers of Golgi stress (Figure 4-29). TG priming at the higher concentration (0.01 μM) induced expression of *WIP149*, *SERPINH1* and *GCP60* in uninfected NHBE cells but Golgi stress was not as prominent in NHBE cells infected with USSR H1N1 for 24 h. As suggested for ER stress, this may reflect alleviation of the stress response by influenza A virus infection.

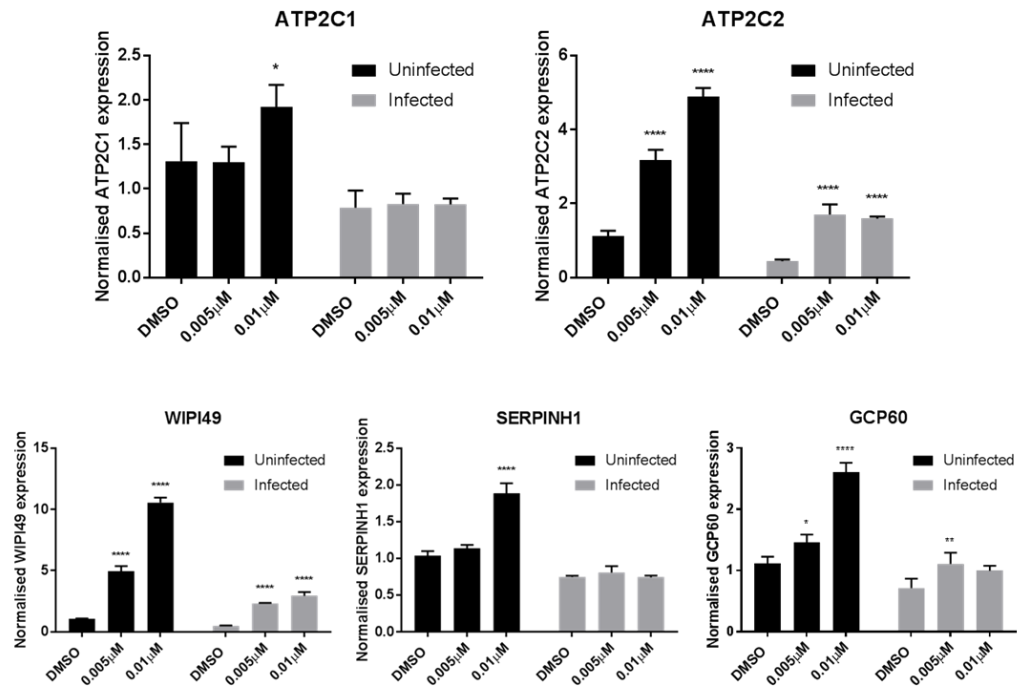


Figure 4-29. TG priming induced expression of Golgi stress genes in NHBE cells.

NHBE cells, pre-treated with 0.005 μM or 0.01 μM TG for 30 mins, were subsequently infected with USSR H1N1 virus at 1.0 MOI for 24 h. Total RNA extracted 24 h later. Fold expression of golgi receptors (SPCA1 and SPCA2) and golgi stress markers (WIPI49, SERPINH1 and GCP60), normalised to 18s rRNA, calculated using the comparative ct method. Gene expression compared to the uninfected DMSO control awarded a relative expression of 1. Significance determined by two way ANOVA, relative to the corresponding DMSO control

The effect of overexpressing components of SOCE was assessed to establish if facilitating SOCE mediated Ca^{2+} impacted Golgi function. However, no effect on WIPI49, SERPINH1 and GCP60 in cells overexpressing *STIM1* and *ORAI1* (Figure 4-30) was observed in NHBE cells, regardless of infection with influenza. Despite its

implied association with the Golgi complex Ca^{2+} ATPase, increasing expression of

ORAI1, thereby increasing the number of channels, did not facilitate an ATP2C2 mediated influx of Ca^{2+} into the Golgi apparatus.

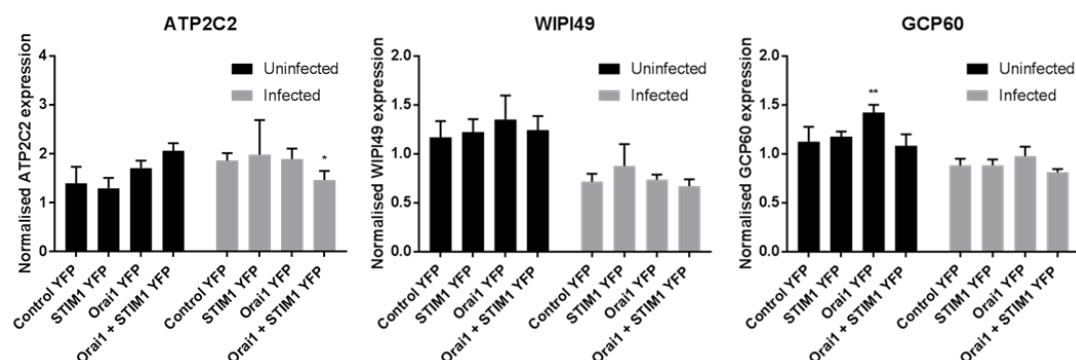


Figure 4-30. Over-expression of *STIM1* and *ORAI1* in NHBE cells had little effect on expression of Golgi stress markers. NHBE cells, transiently transfected with the indicated plasmids for 2 days, were infected with USSR H1N1 virus at 1.0 MOI for 24 h. Total RNA was extracted from each sample for ATP2C2, WIP149 and GCP60 expression, normalised to 18S rRNA, calculated using the comparative ct method. Gene expression compared to the uninfected DMSO control, awarded a relative expression of 1. Significance determined by two way ANOVA, relative to the corresponding DMSO control.

4.3.16 TG priming differentially regulated components of the ubiquitination pathway

The ubiquitination pathway is another host pathway essential for influenza A virus infection (Kirui *et al.*, 2016a, Su *et al.*, 2018, Košík *et al.*, 2015). In the genes significantly downregulated in influenza virus infected cells relative to uninfected

NHBE cells primed with TG (IUTG, Table 4-3), the GO term ‘*protein ubiquitination*’ (GO:0016567) was significantly enriched (PANTHER Overrepresentation Test, fold enrichment 2.23, p-value 3.42E-09, false discovery rate 5.01E-07). In addition, KEGG pathway ‘hsa04120 Ubiquitin mediated proteolysis’ was significantly enriched for downregulated genes (Figure 4-31).

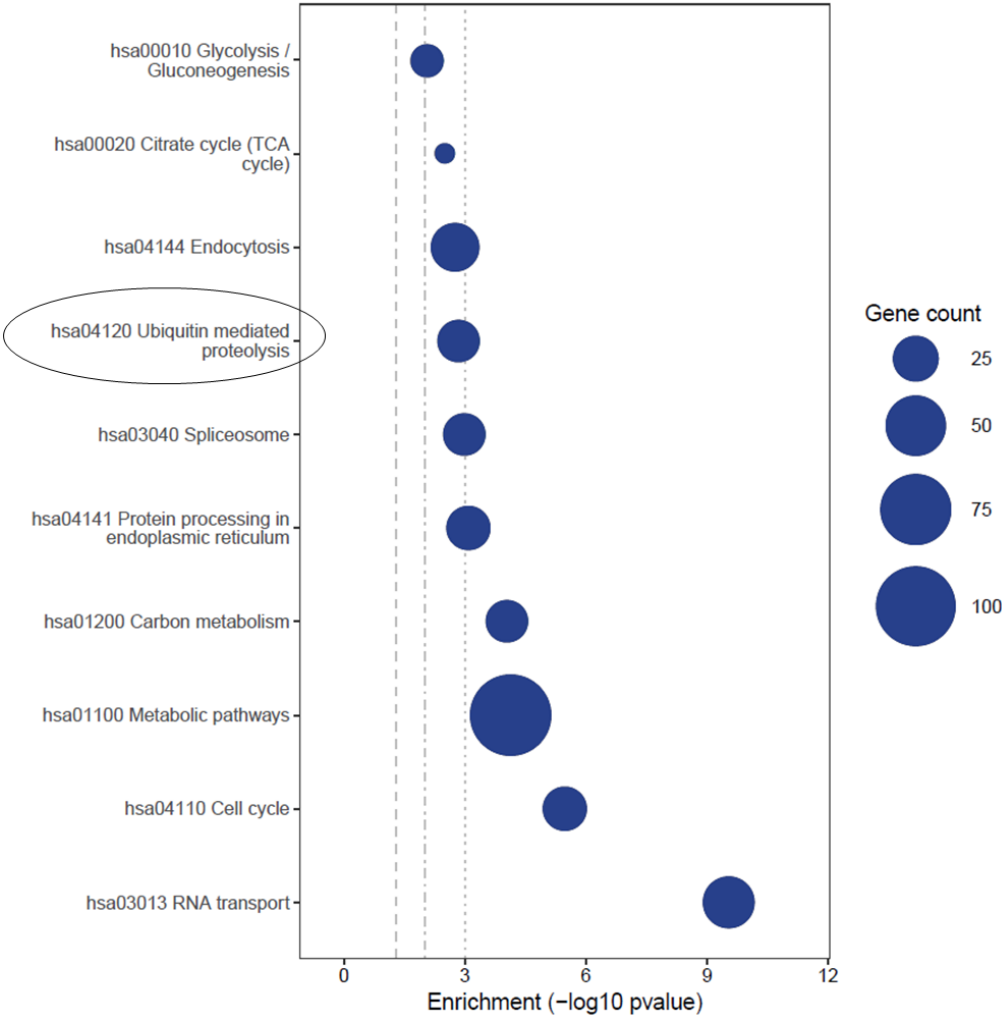


Figure 4-31. TG priming reduced expression of influenza virus infection sensitive genes implicated in ubiquitin mediated proteolysis. Top 10 significantly enriched KEGG pathways for downregulated genes ($q < 0.01$, $\log_2 FC \leq -2$) in TG primed NHBE

cells following influenza A virus infection (IUTG). Circles representative of gene count within each pathway. Location along x-axis depicts the enrichment score ($-\log_{10}pvalue$).

Potential inhibition of ubiquitination was supported by IPA analysis of significant DEGs ($q<0.05$, $\log_2FC\geq1$ and ≤-1) in the IUTG-IUDM analysis (Table 4-3). The ‘protein ubiquitination pathway’ was in the top 10 significantly enriched canonical pathways (Figure 4-32).

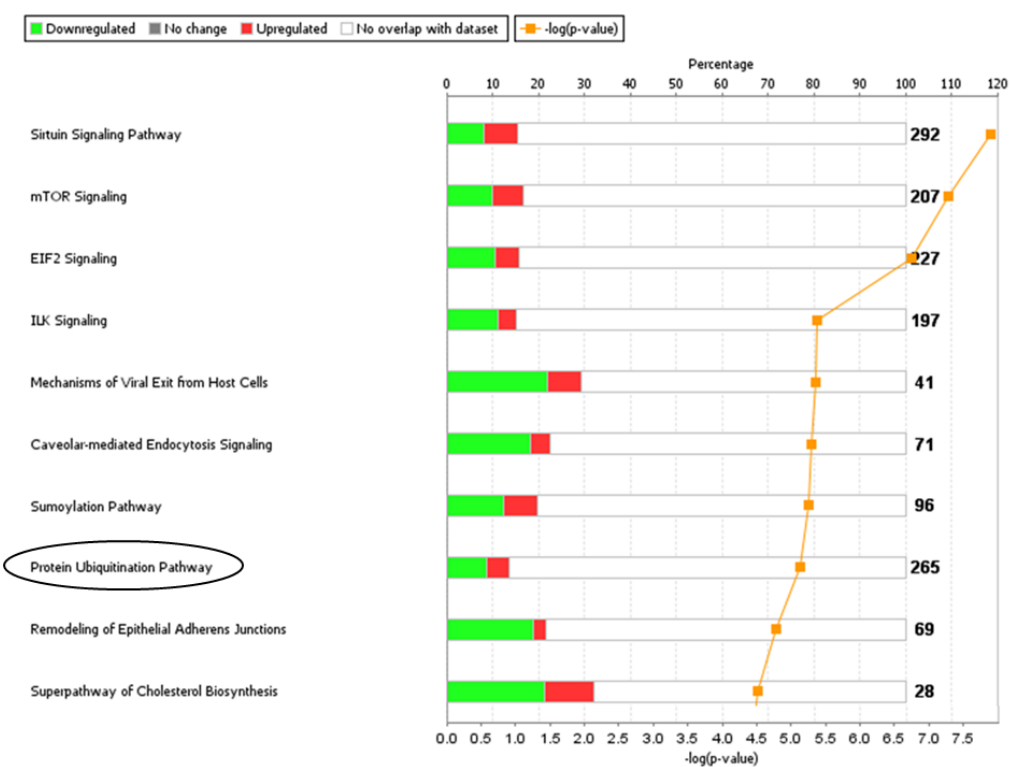


Figure 4-32. The top 10 canonical pathways represented by DEGs between uninfected and influenza infected TG primed NHBE cells. IPA canonical pathways most significantly changed in DEGs ($q<0.05$, $\log_2FC\geq1$ and ≤-1) of influenza virus

infected relative to infected NHBE cells primed with TG (IUTG-IUDM). Genes differentially expressed by influenza virus infection in DMSO primed NHBE cells removed. The stacked bar chart displays the percentage of significantly DEGs that are upregulated (red) or downregulated (green) in each canonical pathway. The number of genes within the pathway recognised by the IPA knowledge database indicated by the numbers to the right of the bars. Orange line represents the $-\log p$ -value, indicating the statistical significance of the over-represented canonical pathway.

Ingenuity Downstream Effects Analysis predicted function of the ubiquitin pathway may be decreased (activation z-score -0.085, p-value 1.61E-11). However, the IPA threshold for a z-score indicating inhibition of the indicated pathway is less than or equal to -2, therefore the z-score does not strongly support TG inhibition of the ubiquitination pathway. However, reduced expression of key components of this pathway may be sufficient to impair virus replication. Differential expression of molecules associated with the ubiquitination pathway were predicted to inhibit pathway activation (Figure 4-33). Further analysis to determine the biological significance of TG mediated inhibition of the ubiquitination pathway on influenza A virus replication is required.

4.3.17 TG priming downregulated expression of serine/threonine-kinases

Influenza A virus regulates kinase activity in order to promote virus replication (König et al., 2010, Hiyoshi *et al.*, 2015). Differential gene expression sensitive to influenza virus infection in TG primed NHBE cells was assessed (IUTG-IUDM, Table 4-3). IPA ‘Disease and Functions’ analysis revealed TG priming significantly inhibited protein phosphorylation (z-score -2.030, p-value 5.19E-06). The z-score was less than -2, therefore meeting the IPA threshold for predicted inactivation of the protein phosphorylation pathway. TG priming differentially regulates the expression of multiple kinases within the significantly enriched IPA pathway ‘phosphorylation of L-threonine’ (p-value 3.53E-04) (Table 4-11) and ‘serine phosphorylation of peptide’ (p-value 1.17E-04) (Table 4-12).

Gene ID	Gene symbol	q-value	Absolute FC
ENST00000555926	AKT1	0.001730238	3.996
ENST00000434813	CLK1	0.014440513	3.891
ENST00000355999	STK39	0.013606263	0.166
ENST00000295754	TGFBR2	0.017346628	0.141
ENST00000295754	TGFBR2	5.07977E-05	0.109
ENST00000339659	DYRK1A	0.014617823	0.122
ENST00000301905	PBK	0.041267881	0.122
ENST00000264235	GSK3B	0.010349369	0.118
ENST00000395284	CDK1	0.000626595	0.077
ENST00000513132	CAMK2D	0.003667939	0.044
ENST00000496808	NLK	0.035963392	0.037
ENST00000436817	HK1	1.77375E-05	0.008
ENST00000543220	MARK2	4.11E-07	-Inf

Table 4-11. TG priming inhibited expression of threonine kinases in infected NHBE cells. IPA function ‘phosphorylation of L-threonine’ was significantly enriched for DEGs ($q < 0.05$, $\log_2 FC \geq 1$ and ≤ -1) of influenza virus infected relative to infected NHBE cells primed with TG. Genes differentially expressed by influenza virus infection in DMSO primed NHBE cells removed. (IUTG-IUDM). FC = fold change.

Gene ID	Gene symbol	q-value	Absolute FC
ENSG00000142208	AKT1	0.001730238	3.996
ENSG00000013441	CLK1	0.014440513	3.891
ENSG00000183087	GAS6	0.012555281	0.492
ENSG00000198648	STK39	0.013606263	0.166
ENSG00000163558	PRKCI	0.002493128	0.156
ENSG00000159256	MORC3	0.026291268	0.154
ENSG00000163513	TGFBR2	0.017346628	0.141
ENSG00000163513	TGFBR2	5.08E-05	0.109
ENSG00000113712	CSNK1A1	0.031944961	0.128
ENSG00000157540	DYRK1A	0.014617823	0.122
ENSG00000168078	PBK	0.041267881	0.122
ENSG00000082701	GSK3B	0.010349369	0.118
ENSG00000170312	CDK1	0.000626595	0.077
ENSG00000145349	CAMK2D	0.003667939	0.044
ENSG00000050748	MAPK9	0.039755585	0.017
ENSG000000213923	CSNK1E	0.037919269	0.454
ENSG000000213923	CSNK1E	8.04E-05	0.012
ENSG00000067606	PRKCZ	0.013211991	0.056
ENSG00000067606	PRKCZ	0.004672259	0.010
ENSG00000156515	HK1	1.77E-05	0.008
ENSG00000071242	RPS6KA2	0.037142353	0.081
ENSG00000071242	RPS6KA2	0.000568753	3.71E-08

Table 4-12. TG priming inhibited expression of serine kinases in infected NHBE cells. IPA function ‘phosphorylation of L-serine’ was significantly enriched for DEGs ($q < 0.05$, $\log_2 FC \geq 1$ and ≤ -1) of influenza virus infected relative to infected NHBE cells primed with TG. Genes differentially expressed by influenza virus infection in DMSO primed NHBE cells removed. (IUTG-IUDM). FC = fold change.

4.3.18 Modification of viral proteins via the SUMO pathway may be impaired by TG priming

Small ubiquitin-related modifier (SUMO) is a post-translational modifier implicated in influenza A virus replication. Intracellular trafficking, assembly and function of viral proteins is dependent on reprogramming the host sumoylation machinery (Han *et al.*, 2014, Wu *et al.*, 2011, Xu *et al.*, 2011). IPA analysis of genes differentially

expressed by USSR H1N1 virus infection and sensitive to TG priming (IUTG-IUDM), indicated TG affected the sumoylation pathway (Figure 4-34).

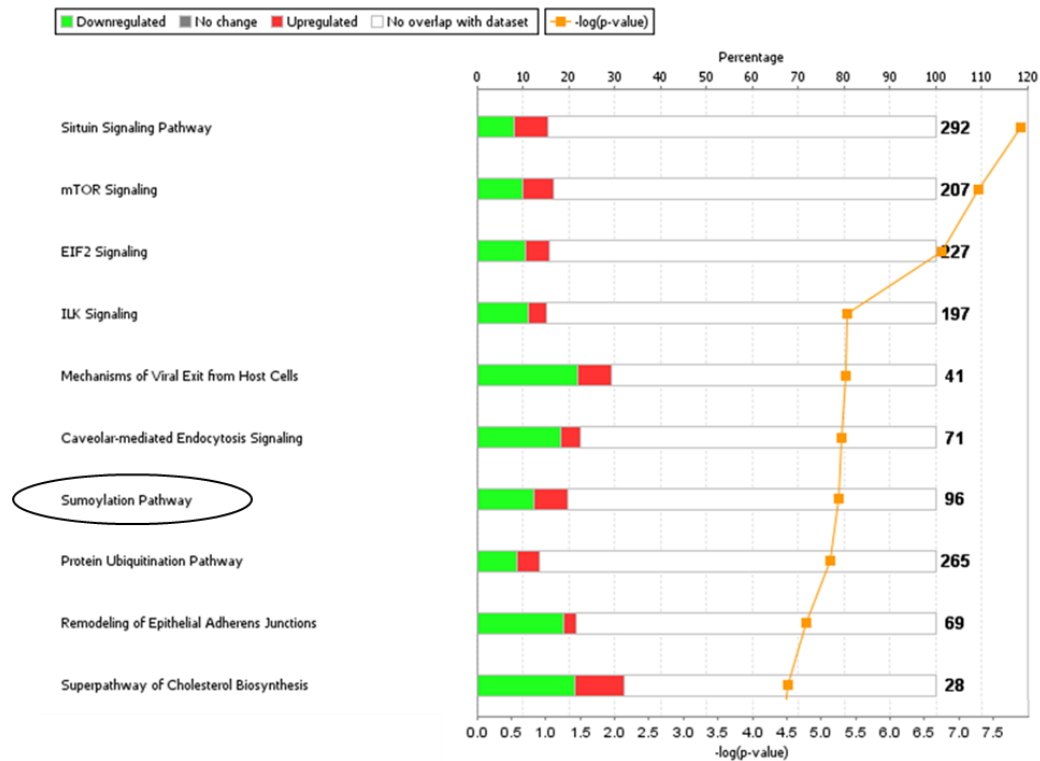


Figure 4-34. The top 10 canonical pathways represented by DEGs between uninfected and influenza infected TG primed NHBE cells. IPA canonical pathways most significantly changed in DEGs ($q < 0.05$, $\log_2 FC \geq 1$ and ≤ -1) of influenza virus infected relative to infected NHBE cells primed with TG. Genes differentially expressed by influenza virus infection in DMSO primed NHBE cells removed (IUTG-IUDM). The stacked bar chart displays the percentage of significantly DEGs that are upregulated (red) or downregulated (green) in each canonical pathway. The number of genes within the pathway recognised by the IPA knowledge database indicated by the numbers to the right of the bars. Orange line represents the $-\log p$ -value, indicating the statistical significance of the over-represented canonical pathway.

Ingenuity Downstream Effects Analysis predicted inhibition of the sumoylation pathway (z-score -0.775, p-value 5.39E-06), although this prediction was not significant as the z-score did not meet the IPA threshold. Closer analysis revealed the six DEGs associated with predicted pathway activation and the nine genes associated with pathway inhibition (Table 4-13).

Symbol	Entrez Gene Name	Ensembl	Expr Log Ratio	q-value	Direction of expression	Direction expected for pathway activation
ARHGDIA	Rho GDP dissociation inhibitor alpha	ENST00000580033	1000	1.26E-05 Up	Up	Down
CTBP1	C-terminal binding protein 1	ENST00000290921	1.202	2.04E-03 Up	Up	Down
CTBP2	C-terminal binding protein 2	ENST00000531469	-1.219	3.30E-02 Down	Down	Down
MDM2	MDM2 proto-oncogene	ENST00000258149	-2.532	3.54E-02 Down	Down	Down
PCNA	proliferating cell nuclear antigen	ENST00000379160	-2.742	1.19E-03 Down	Down	Down
RFC3	replication factor C subunit 3	ENST00000380071	-3.147	3.21E-02 Down	Down	Down
RHOC	ras homolog family member C	ENST00000369642	1.445	3.44E-03 Up	Up	Down
RHOF	ras homolog family member F, filopodia associated	ENST00000537265	2.682	1.64E-05 Up	Up	Down
STUB1	STIP1 homology and U-box containing protein 1	ENST00000566408	2.995	8.45E-04 Up	Up	Down
FOS	Fos proto-oncogene, AP-1 transcription factor subunit	ENST00000303562	1.178	1.76E-03 Up	Up	Up
MAPK9	mitogen-activated protein kinase 9	ENST00000523583	-5.912	3.98E-02 Down	Down	Up
SENP2	SUMO1/sentrin/SMT3 specific peptidase 2	ENST00000296257	-2.624	1.74E-02 Down	Down	Up
SENP3	SUMO1/sentrin/SMT3 specific peptidase 3	ENST00000582789	-4.665	1.76E-02 Down	Down	Up
SENP5	SUMO1/sentrin specific peptidase 5	ENST00000323460	-2.714	2.74E-02 Down	Down	Up
SUMO3	small ubiquitin-like modifier 3	ENST00000397898	1.246	1.82E-03 Up	Up	Up
NR3C1	nuclear receptor subfamily 3 group C member 1	ENST00000503201	-2.731	2.84E-02 Down	Down	Unknown
RANBP2	RAN binding protein 2	ENST00000283195	-3.058	1.24E-04 Down	Down	Unknown
RCOR1	REST corepressor 1	ENST00000262241	-3.143	5.99E-06 Down	Down	Unknown
XIAP	X-linked inhibitor of apoptosis	ENST00000434753	-2.607	4.53E-02 Down	Down	Unknown

Table 4-13. DEGs of the significantly enriched sumoylation pathway. IPA predicted direction of expression

expected for sumoylation pathway activation compared to actual direction of expression. Grey highlight indicates

genes differentially expressed associated with expected pathway inhibition.

Interaction between the differentially expressed components in the Ingenuity sumoylation canonical pathway, described in (Figure 4-35), reveals differential

Expression of *SUMO3* is significantly increased by TG priming (q value 0.0018, absolute FC 2.38) and this is associated with pathway activation. However, expression of *SENP2*, *SENP3* and *SENP5*, is decreased and this is associated with a predicted inactivation of the sumoylation pathway (Table 4-13 and Table 4-14). These genes encoded proteases which process the SUMO pre-cursors and mediated de-conjugation of the SUMO molecules from the target proteins (Kumar & Zhang, 2015).

Gene ID	Gene symbol	q-value	Absolute FC
ENSG00000163904	SENP2	0.017392388	0.162
ENSG00000161956	SENP3	0.017568574	0.039
ENSG00000119231	SENP5	0.027396957	0.152

Table 4-14. TG priming reduced expression of sentrin specific proteases. Differential expression ($q < 0.05$, $\log_2 FC \geq 1$ and ≤ -1) of proteases associated with the canonical sumoylation pathway sensitive to influenza virus infection in TG primed NHBE cells (IUTG-IUDM). FC= fold change.

4.3.19 Differential gene expression mediated by TG priming was not associated with toxicity functions

The specificity of TG for IAV sensitive differentially expressed genes may allay concerns regarding off-target effects and TG mediated cytotoxicity. TG priming extensively regulated expression of genes differentially regulated by influenza infection (IUTG-IUDM) (Figure 4-36 and Figure 4-37).

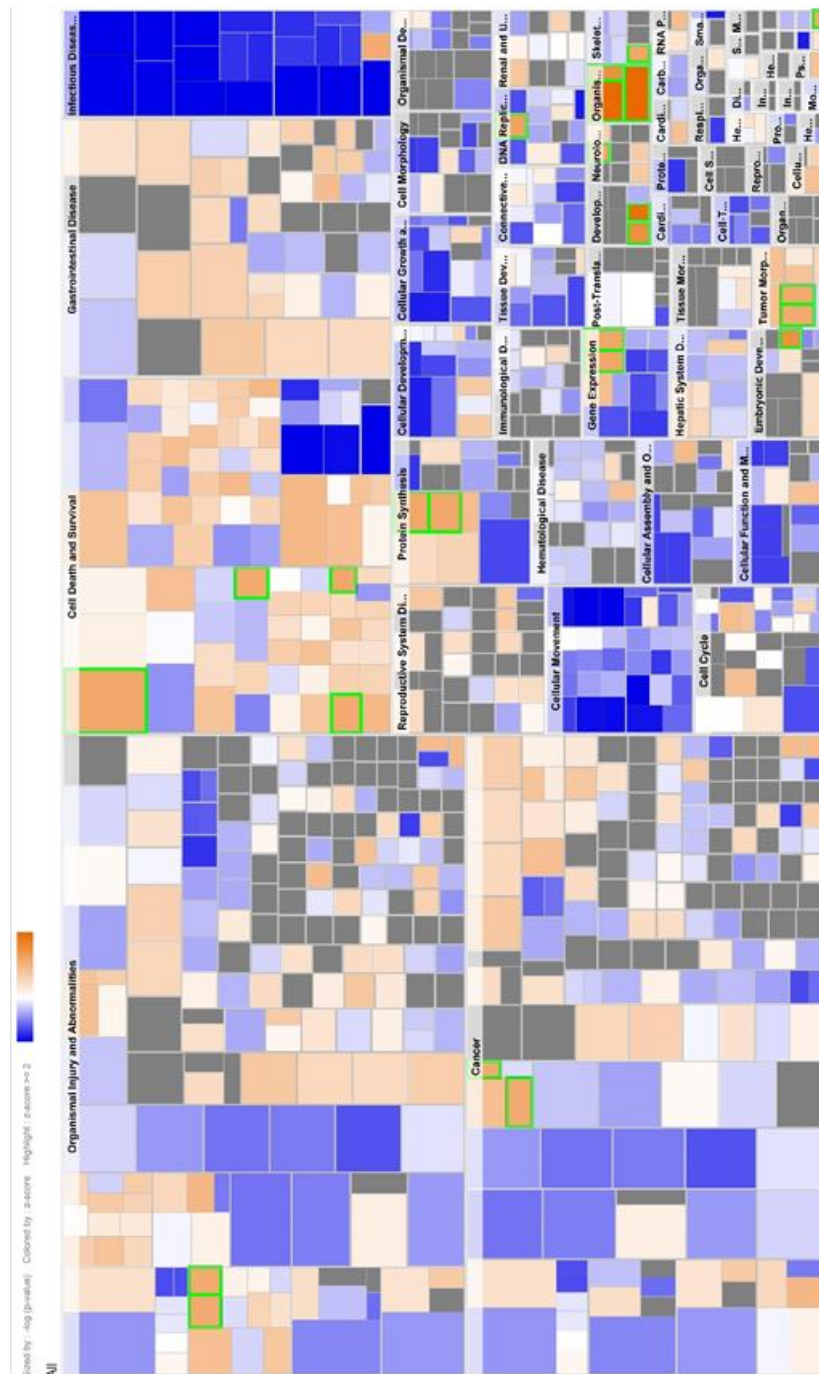


Figure 4-36. Heatmap of Ingenuity Downstream Effects Analysis of influenza virus infection mediated differentially expressed genes in TG primed NHBE cells. Differential gene expression due influenza virus infection in NHBE cells primed with TG (IUTG-IUDM). Colour intensity of the heat map represents the z-score of functions and diseases significantly enriched for DEGs ($q < 0.05$, $\log_2 FC \geq 1$ and ≤ -1). Orange

blocks indicate the z-score is positive and the associated biological function is activated, blue indicates the z-score is negative and the associated biological function is inhibited. Significance indicated if $-2 \geq z\text{-score} \geq 2$. Size of the square reflects the $-\log(p\text{-value})$ where the p-value is the likeliness of the association of the differentially expressed genes and the corresponding function due to random association. p-values < 0.05 ($-\log = 1.3$) indicate a significant, non-random association. z-scores > 2 highlighted green.

blocks indicate the z-score is positive and the associated biological function is activated, blue indicates the z-score is negative and the associated biological function is inhibited. Significance indicated if $-2 \geq z\text{-score} \geq 2$. Size of the square reflects the $-\log(p\text{-value})$ where the p-value is the likeliness of the association of the differentially expressed genes and the corresponding function due to random association. p-values < 0.05 ($-\log = 1.3$) indicate a significant, non-random association. z-scores < -2 highlighted green.

However, TG priming alone had a minimal effect on the host transcriptome regarding toxicity. TG priming did not significantly regulate toxicity functions (based on z-score analysis) in uninfected NHBE cells (UUTG, Table 4-3), indicated by IAP-Tox analysis tool (Figure 4-38).

activated, blue indicates the z-score is negative and the associated Tox function is inhibited. Function highlighted in green if z-score >2 or <-2. Size of the square reflects the $-\log(p\text{-value})$ where the p-value is the likeliness of the association of the differentially expressed genes and the corresponding function due to random association. p-values < 0.05 ($-\log = 1.3$) indicate a significant, non-random association.

In influenza infected cells (IITG, Table 4-3), ‘Damage of liver’ is predicted to be significantly decreased (z-score -2.2, overlap p-value 4.99E-01) due to differential expression of *HMGB1*, *GPX4*, *KRT8*, *DDIT3*, *ATF4* and *HSPA5* by TG priming (Figure 4-39). No other Tox function was significantly enriched in influenza virus infected NHBE cells.

Orange blocks indicate the z-score is positive and the associated Tox function is activated, blue indicates the z-score is negative and the associated Tox function is inhibited. Function highlighted in green if z-score >2 or <-2. Size of the square reflects the $-\log(p\text{-value})$ where the p-value is the likeliness of the association of the differentially expressed genes and the corresponding function due to random association. p-values < 0.05 ($-\log = 1.3$) indicate a significant, non-random association.

4.4 Discussion

Ca^{2+} dependent processes have been proposed to underlie the TG mediated antiviral response. TG induced a dose dependent increase in cytosolic Ca^{2+} concentration which corresponded to a greater decrease in progeny virus output. The increase in cytosolic Ca^{2+} was not maintained for 24 hours but the antiviral activity from brief TG exposure was sustained for up to 48 hours in NPTr cells. Removing extracellular Ca^{2+} for the duration of TG priming had no effect on the antiviral action of TG. However, SERCA inhibition may persist for longer than 30 minutes and facilitate Ca^{2+} influx upon re-introduction of extracellular Ca^{2+} . Progeny virus has been detected as early as 5 -6 hours post infection (Abdoli et al., 2013). TG priming 6 h after infection significantly reduced progeny virus output and elevated type I IFN dependent gene expression, suggesting the TG induced antiviral response is effective after infection. The sustained antiviral state of TG promotes its potential as a prophylaxis while its ability to reduced progeny virus production after infection supports its therapeutic potential. The antiviral activity of other small molecules capable of inducing an increase in cytosolic Ca^{2+} concentration may be worth consideration.

TG priming positively regulated the host type I/III IFN response

Ca^{2+} regulates multiple processes in the cell, including host immune signalling pathways (Bergmeier *et al.*, 2013, Feske *et al.*, 2015). The extensive role of Ca^{2+} as a second messenger emphasises the importance of identifying the pathways responsive for the antiviral activity of TG and potential off-target effects associated with

cytotoxicity. Next generation RNA sequencing supported the antiviral role proposed for TG and identified multiple potential antiviral mechanisms. Ingenuity downstream effects analysis suggested TG may serve as a broad spectrum inhibitor of RNA virus replication. This may be attributed to its regulation of host signalling pathways involved in the antiviral response rather than direct inhibition of virus proteins. Therefore, the antiviral activity of TG may not be specific for influenza virus and exploring the therapeutic potential of TG should be extended to other RNA viruses.

TG dependent regulation of the type I IFN response has been proposed as one of the mechanisms underlying its antiviral activity. RNA-seq confirmed the role of TG in regulating host innate immune signalling pathways. Multiple types of analysis, including BP GO term enrichment and Ingenuity effects analysis, supported the positive regulatory role of TG on the type I and type III IFN response. TG priming not only elevated immune genes expression but induced activation of upstream regulators IFN- λ 1, IFN- β 1 and IFN- α 2. In addition, TG extensively enhanced expression of immune genes associated with the GO ontology biological process '*the type I interferon signalling pathway* (GO:0060337)'. This provided further evidence in line with TG mediated elevation of *RIG-I* and *OAS1* expression in tracheal epithelial and muscle cells (Chapter 3). This may be due to the significant induction of the IFNs regulatory factor expression (*IRF3* and *IRF7*) by TG. Downregulation of *IRF3* transcripts may be attributed to their role in initiating early innate immunity while *IRF7* mediates the late phases (Honda et al., 2006).

TG priming regulated post-translational modifications

RNA-seq analysis supported TG mediated activation of the ER stress unfolded protein response (UPR). Three branches of the UPR, mediated by ER chaperones ATF6, PERK and IRE-1, are activated to restore ER homeostasis. The UPR may be split into two responses, the first mediates translation inhibition to prevent further accumulation of misfolded proteins while the second triggers transcription of ER stress sensitive genes such as ER chaperones to assist with protein folding. PERK regulates protein translation, inhibiting translation initiation through phosphorylation of eukaryotic translation initiation factor 2 α (eIF2 α). ATF6 regulates the expression of ER stress sensitive genes, cooperating with co-regulators such as NF-Y and through activation of X box-binding protein 1 (XBP1), to increase ER chaperone activity and degradation of misfolded proteins. IRE-1 also regulates expression of ER stress sensitive genes by activating the XBP1 transcription factor. In addition, IRE-1 activates host signalling pathways such as JNK and NF- κ B, but the contribution of these pathways is unclear (reviewed by (Chakrabarti et al., 2011a).

Each of three branches of the ER stress response (ATF6, PERK and IRE-1) were significantly enriched for upregulated genes, supported by BP GO term enrichment, KEGG pathway enrichment and IPA. Upregulation of genes within the UPR indicates an accumulation of misfolded proteins in the ER of TG primed cells. Interestingly, while TG upregulated expression of the ATF6 and PERK responses, BP GO term enrichment analysis identified upregulation of negative regulators of the IRE-1 stress pathway. IRE-1 has pro-viral function and inhibition of the IRE-1 stress response impaired influenza virus replication (Hassan *et al.*, 2012). Therefore, TG priming may

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activate antiviral ER stress pathways but also inhibit pro-influenza ER stress pathways. In support of the former, NGS analysis provided further evidence for TG mediated inhibition of influenza virus protein processing through dysregulating ER function. Upregulation of the UPR sensitive genes may reflect an accumulation of misfolded virus glycoproteins due to disrupted N-linked glycosylation. Protein folding and post-translational modifications, such as N linked glycosylation, occur within the ER and Golgi apparatus (Aebi *et al.*, 2010, Zhang & Wang, 2016). Ca^{2+} is essential for post-translational modifications of secretory and membrane proteins and depletion of the ER Ca^{2+} store impairs protein processing and induces the UPR (Brostrom & Brostrom, 2003). Glycosylation of the influenza virus HA is required for viral protein folding, trimerisation and trafficking to the PM (Mir-Shekari *et al.*, 1997, Roberts *et al.*, 1993, Gallagher *et al.*, 1992). Glycosylation of NA in the ER was required for tetramerisation and transport to the Golgi complex (Saito *et al.*, 1995, Wang *et al.*, 2008a). TG priming may inhibit influenza virus replication through inducing store depletion and interfering with post-translational modifications and folding of virus glycoproteins in the ER. Enrichment of BP GO term '*protein N-linked glycosylation*' (GO:0006487) for upregulated genes and significantly increased expression of genes associated with glycoprotein folding and processing, such as *CALR* and *CANX*, suggests an accumulation of misfolded glycoproteins. N linked glycosylation of HA and NA regulates their association with calnexin (CNX) and calreticulin (CRT) (Hebert *et al.*, 1997, Wang *et al.*, 2008a). Viral glycoprotein maturation is dependent on association with calnexin/calreticulin chaperones (Molinari *et al.*, 2004, Wang *et al.*, 2008a). CNX depletion reduced HA folding and transport to the PM (Molinari *et al.*, 2004). CNX/CRT may promote correct virus glycoprotein folding through preventing the formation of misfolded aggregates (Hebert *et al.*, 1996). Therefore,

upregulation of genes associated with these pathways may reflect an attempt to compensate for accumulated virus glycoproteins in the ER in TG treated cells. Ca^{2+} dependent inhibition of N linked glycosylation of viral proteins has previously been identified. Ca^{2+} depletion of ER calcium stores interfered with glycosylation of Bovine Virus Diarrhoea Virus (BVDV) structural proteins (Ashiru *et al.*, 2014). TG mediated depletion of ER Ca^{2+} interfered with N-linked glycosylation of rotavirus proteins VP7 and NSP4 (NS28) (Michelangeli *et al.*, 1995).

An antiviral mechanism dependent on inhibiting virus glycoprotein transport through the secretory pathway by a TG induced increase in cytosolic Ca^{2+} may also be proposed. Viral coat proteins HA, NA and M2 are synthesised at the ER, transported through the Golgi and to the apical PM for virus particle assembly (Zhu *et al.*, 2017a, Copeland *et al.*, 1986, Wang *et al.*, 2012). In support of this proposed mechanism of action for TG, genes associated with multiple steps of the secretory pathway were differentially regulated.

Downregulation of genes such as *TRAPPC3* and *SCFD1* may reflect TG mediated inhibition of ER to Golgi protein transport. The TRAPPI complex, with a TRAPPC3 subunit, is involved in the early component of the protein secretory pathway, tethering vesicles from the ER to the cis-Golgi membrane (Turnbull *et al.*, 2005, Sacher *et al.*, 1998, Sacher *et al.*, 2001). In yeast, Bet3p (TRAPPC3 in mammals) is essential for ER to Golgi vesicle binding to the Golgi complex (Barrowman *et al.*, 2000). TRAPPC3 has already been implicated in virus replication as inhibition of TRAPPC3 impaired vesicular stomatitis virus envelope glycoprotein transport from the ER and to the Golgi apparatus (Loh *et al.*, 2005). Downregulation of the gene encoding Sec1 family domain-containing protein 1 (SCFD1), also known as Sly1, may also impeded

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ER to Golgi protein transport. SCFD1 regulates ER to Golgi transport by positively regulating syntaxin 5 function, binding to a conserved N terminal domain (Dascher & Balch, 1996, Yamaguchi *et al.*, 2002). However, the limitations in assessing significantly enriched BP GO terms may be reflected by the presence of genes tenuously associated with the term or the absence of other genes. Downregulation of *STX18* may be an example of the latter. Syntaxin 18 (*STX18*) is required for protein export from the ER to the Golgi (Hatsuzawa *et al.*, 2000). However, it is not associated with the BP GO term '*ER to Golgi vesicle-mediated transport*' but with significant enrichment of '*Golgi vesicle transport*'. In addition, syntaxin 18 and Sly1 have been implicated in Golgi to ER retrograde transport (Laufman *et al.*, 2009, Li *et al.*, 2005). Their downregulation in TG primed cells may reflect inhibition of anterograde and retrograde transport between the ER and Golgi. Therefore, TG dependent downregulation of genes associated with ER to Golgi transport may inhibit virus glycoprotein transport at the early stage of the secretory pathway.

Furthermore, inhibition of intra-Golgi transport may underlie the antiviral mechanism of TG. Ca^{2+} dependent TG mediated inhibition of intra-Golgi transport has previously been reported (Porat & Elazar, 2000). Here, TG dependent inhibition of COPI-coated vesicles is proposed. COPI-coated vesicles mediate intra-Golgi transport and retrograde transport from Golgi to the ER (Rabouille & Klumperman, 2005, Popoff *et al.*, 2011). COPI vesicle transport has been implicated in influenza virus replication. Knockdown of COPI with siRNA silencing and pharmacological inhibitors of COPI impaired trafficking of viral proteins NA and M2 to the PM and reduced infectious progeny virus production (Sun *et al.*, 2013). In addition, COP-I coated vesicles have been implicated in vRNP trafficking to the apical PM (Yadav *et al.*, 2016)

The protein complex of COPI vesicles, the coatomer, is formed of seven subunits, such as coatomer subunit δ and ζ (Faulstich *et al.*, 1996, Kuge *et al.*, 1993). Coatomer subunit genes (*COPB1*, *COPB2*, *COPZ1*, *ARCN1*) were significantly downregulated in TG primed cells. In addition, downregulation of *Golgi specific brefeldin A-resistance guanine nucleotide exchange factor 1* (*GBF1*) and *ADP-ribosylation factor GTPase-activating protein 2* (*ARFGAP2*) in the significantly enriched BP GO term ‘*COPI coating of Golgi vesicles*’ (GO:0048205), suggests TG priming interfered with COPI coated vesicle transport. GBF1 is a guanine exchange factor for the ADP-ribosylation factor (ARF) which mediates the recruitment of COPI coats (Szul *et al.*, 2007). Depletion of GBF1 disrupted cis-Golgi structure and inhibited trafficking of some proteins (Szul *et al.*, 2007). While, ARFGAP2 is a key component in COPI coat assembly and knockdown of *ARFGAP2* interfered with COPI vesicle formation (Kartberg *et al.*, 2010). In addition, *trafficking protein particle complex subunit* (*TRAPPC*) 3 and 11 downregulation may also reflect COPI vesicle inhibition. The TRAPP II complex functions in late Golgi, and is formed of TRAPP 1 subunits (TRAPPC1, TRAPPC2, TRAPPC3, TRAPPC4, TRAPPC5 and TRAPPC6a,b) and three additional subunits (TRAPPC2L, TRAPPC9 and TRAPPC10) (Yip *et al.*, 2010). The TRAPP II complex is enriched on COPI coated vesicles, binding to coatomer subunit γ (Yamasaki *et al.*, 2009). Mutation of TRAPP II complex specific subunits, such as *TRS120*, impaired COPI vesicle trafficking (Cai *et al.*, 2005). Another of the TRAPPC proteins, TRAPPC6A, was essential for M2 post-translational trafficking to the PM (Zhu *et al.*, 2017a). Downregulation of *TRAPPC3* and *TRAPPC11*, subunits of TRAPP I and II, may contribute to TG mediated inhibition of COPI vesicle transport and thereby interfere with transport of influenza virus proteins.

Finally, downregulation of genes such as *VAMP3* and *RAB14* may mediate a post trans-Golgi dependent inhibition of influenza virus replication. SNAREs are essential drivers of membrane fusion in the trafficking pathways, forming tight complexes via complementary motifs in order to facilitate fusion (Jahn & Scheller, 2006). *VAMP3* is an R-SNARE implicated in post-Golgi trafficking to the PM (Gordon *et al.*, 2010). *Rab14* regulates transport between the trans-Golgi to the apical membrane of polarised epithelial cells (Kitt *et al.*, 2008). Targeting to the apical membrane by *Rab14* may occur through endosome intermediates (Kitt *et al.*, 2008, Junutula *et al.*, 2004). Influenza A virus buds from lipid raft domains located on the apical membrane (Rossman & Lamb, 2011). Therefore, TG mediated downregulation of the *Rab14* dependent trafficking pathway may impair correct transport of viral membrane proteins from the trans-Golgi to the apical PM.

Further exploration of the role of cytosolic Ca^{2+} and SOCE on Golgi complex function resulted in the identification of a potential store independent calcium entry (SICE) dependent antiviral response. The Golgi complex functions as a Ca^{2+} store with a distinct Ca^{2+} gradient across its compartments, the concentration decreasing towards to the trans-golgi compartment (Li *et al.*, 2013). Interaction between ORAI1 and the Ca^{2+} ATPases ATP2C1 (SPCA1) or ATP2C2 (SPCA2) facilitated Ca^{2+} intake into the secretory pathway (Smaardijk *et al.*, 2017, Smaardijk *et al.*, 2018, Cross *et al.*, 2013). Increased Ca^{2+} levels in the Golgi was associated with induction of Golgi stress (Smaardijk *et al.*, 2018). Golgi stress may subsequently impair Golgi complex function, thereby interfering with influenza protein processing and transport in the secretory pathway (Sasaki & Yoshida, 2015). TG priming may enhance SICE and Golgi stress through regulating expression of the Golgi complex ATPase (*ATP2C2*),

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thus increasing Ca^{2+} influx through the ORAI1 channels into the Golgi. Expression of markers of Golgi stress (*WIP149*, *GCP60*, and *SERPINH1*) was elevated in TG treated cells, suggesting TG priming induces a stress response in the Golgi. Additional work is required to clarify if elevating *ATP2C2* expression facilitates an increase in Golgi luminal Ca^{2+} and subsequently induces Golgi stress. Secondly, the impact of dysregulated Golgi luminal Ca^{2+} concentration on influenza protein processing and transport must be addressed. The contribution of ORAI1 mediated extracellular Ca^{2+} influx through store dependent (SOCE) and store independent (SICE) mechanisms to the antiviral response may be worth further consideration.

TG priming may regulate other post-translational modifications of viral proteins. The most common post-translational modification, protein phosphorylation, has been extensively connected to influenza A virus infection (Bakre *et al.*, 2013, Hutchinson *et al.*, 2012). Influenza virus proteins of the virus polymerase, glycoproteins, M proteins, NS1 and NEP are phosphorylated by host kinases (Hutchinson *et al.*, 2012). Ingenuity disease/function analysis predicted inhibition of protein phosphorylation in TG primed cells. TG inhibited expression of kinases implicated as host factors which promote influenza virus replication, such as *GSK3B*, *MARK2*, *PBK* and *STK39* (König *et al.*, 2010, Hiyoshi *et al.*, 2015).

Alternatively, TG priming may interfere with the ubiquitination pathway. The ubiquitin pathway is susceptible to Ca^{2+} regulation (reviewed by Mukherjee *et al.*, 2017). In particular, calmodulin may underly Ca^{2+} dependent regulation of protein ubiquitination (Mukherjee *et al.*, 2017, Shen *et al.*, 2005). BP GO terms and KEGG pathways associated with ubiquitination were significantly enriched for

downregulated genes in TG primed NHBE cells infected with influenza virus. In addition, 265 genes were differentially expressed in the significantly enriched protein ubiquitination canonical pathway, determined through IPA. Pro-viral functions have also been assigned to the host cell ubiquitination pathway. Defective ubiquitination altered M1 and M2 interaction and impaired vRNP incorporation into the virus particles (Su et al., 2018). Influenza nucleoprotein is ubiquitinated by an E3 ubiquitin ligase, Ccr4-Not transcription complex subunit 4 (CNOT4), at multiple lysine residues (Lin et al., 2017). Mutation of these lysine residues (K184, K227 and K273) impaired efficient virus RNA transcription/replication (Lin et al., 2017). Components of the ribonucleoprotein (PA, PB1 and PB2) are ubiquitinated and stimulated viral polymerase activity (Kirui et al., 2016b, Košík et al., 2015). In addition, ubiquitin regulation of host immune signalling pathways has been reported. Interferon-induced transmembrane protein 3 (IFIT3) has been implicated in activation of the host antiviral response and activation of immune regulators such as IRF3 and TBK1 (Liu et al., 2011b, Diamond & Farzan, 2013). *IFIT3* is significantly upregulated by TG priming. IFIT3 is inhibited by influenza induced ubiquitination by the E3 ubiquitin ligase NEDD4 (Chesarino et al., 2015). TG mediated downregulation of protein ubiquitination may interfere with virus protein modifications or influenza dependent regulation of host immunity. Although NEDD4 is activated rather than inhibited by calcium, so other enzymes within the ubiquitin pathway should be considered (Wang et al., 2010).

TG regulation of the sumoylation pathway may contribute to its antiviral activity. Sumoylation is a post-translational modification involving the addition of small ubiquitin like modifiers (SUMO) to the target protein. This requires a three step

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process involving the E1 heterodimer SAE1-SAE2, E2-conjugating enzyme Ubc9 and E3 ligases (Wilkinson & Henley, 2010). Four SUMO isoforms have been identified in humans (SUMO1, SUMO2, SUMO3 and SUMO4) (Saitoh & Hinchey, 2000, Bohren *et al.*, 2004). Influenza A virus replication is dependent on sumoylation of influenza virus proteins NP, NS1 and M1 (Domingues *et al.*, 2015, Pal *et al.*, 2011). NP sumoylation at conserved residues K4 and K7 regulated NP intracellular localisation and was essential for virus replication (Han *et al.*, 2014). Sumoylation of M1 at residue K242 was necessary for M1-vRNP complex formation and subsequent nuclear export of vRNP (Wu *et al.*, 2011). Aberrant sumoylation of NS1 impaired its neutralisation activity of IFN induced antiviral proteins (Santos *et al.*, 2013). Finally, knockdown of E2-conjugating enzyme Ubc9 reduced progeny virus production (Wu *et al.*, 2011). However, antiviral functions have been attributed to sumoylation of host proteins. Sumoylation of CDC73 (parafibromin) enhanced IFNs sensitive gene expression (Domingues *et al.*, 2015). ARF inhibition of PIAS1 through sumoylation was found to enhance the IFN- γ immune pathway (Alagu *et al.*, 2018). IPA predicted inhibition of the sumoylation pathway in TG primed cells, suggesting TG may interfere with expression of the sumoylation components and sumoylation targets. *SENP2*, *SENP3* and *SENP5* encode SUMO specific proteases and are downregulated in TG primed NHBE cells. The proteases cleave the SUMO pre-cursors to generate active SUMO molecules that can be conjugated to their targets (Mukhopadhyay & Dasso, 2007). In addition, the proteases mediate removal of SUMO, an additional mechanism regulating the availability of SUMO and the activity of the SUMO targets (Mukhopadhyay & Dasso, 2007). Downregulation of the proteases and the subsequent predicted inhibition of the sumoylation pathway, suggests TG priming may interfere with influenza virus regulation of sumoylation.

Interestingly, a gene associated with the ubiquitination, sumoylation and stress pathways was differentially regulated. *Mdm2* was downregulated in TG primed NHBE cells. Mdm2 is an E3 ubiquitin-protein ligase which suppressed p53 activity through ubiquitination and subsequent degradation (Meek & Knippschild, 2003). The post-translation modification of sumoylation regulated Mdm2 activity and promoted p53 accumulation (Meek & Knippschild, 2003). Mdm2 was implicated as a host factor necessary for influenza virus replication (König et al., 2010, Karlas *et al.*, 2010). Knockdown of the E3 ubiquitin ligases or small molecule inhibition facilitated influenza virus replication (Pizzorno *et al.*, 2018). TG mediated downregulation of *MDM2* was associated with a predicted decrease in influenza A virus replication. This may highlight cross talk between the post-translational modification pathways.

Finally, the potential host signalling pathways associated with potential TG mediated cytotoxicity were explored. The SI (or therapeutic index) of TG was determined in tracheal epithelial cells. The SI describes the ratio between toxicity and drug efficacy (Muller & Milton, 2012). The high SI for NPTr cells and NHBE cells (~8000) suggests TG may be a viable therapy for influenza virus infection. Off-target effects associated with toxicity were minimal. The IPA-Tox tool identified only one toxicity function with a z-score meeting the IPA threshold. In infected NHBE cells, TG priming was predicted to downregulate liver damage, thereby suggesting a protective effect for TG regarding toxicity.

In summary, TG may function as a broad spectrum inhibitor of RNA virus replication. Its safety and therapeutic potential was supported by its high SI and lack of enriched toxicity functions. Brief TG priming induced a sustained antiviral state, recommending its usage for prophylactic and therapeutic treatment of influenza virus infection. The antiviral response of TG is multifaceted and may interfere with multiple stages of influenza virus replication. TG extensively regulated the expression of influenza virus sensitive genes and induced activation of the type I/III IFN signalling pathways. TG dependent regulation of the host transcriptional response may be responsible for the sustained antiviral state induced by brief exposure to TG. RNA-seq analysis supported previous results suggesting TG mediated elevation of the type I/III IFN signalling pathway and induction of the ER stress response underlie its antiviral mechanism of action. Activation of the ER stress response by store depletion may interfere with host and virus protein synthesis. The ER UPR may impair folding, oligomerisation and N linked glycosylation of virus glycoproteins in the ER. In addition, an increase in cytosolic Ca^{2+} may impair virus protein transport through the secretory pathway through inhibition of vesicle transport in the secretory pathway. In addition, Ca^{2+} homeostasis within the Golgi complex was identified as a potential target for antiviral therapy. TG induced increases in cytosolic Ca^{2+} dependent on ER store depletion may interfere with Golgi function. However, store independent mediated influx of extracellular Ca^{2+} through the ORAI1 channels and into the Golgi complex has been reported. The ORAI1 channels may mediate Ca^{2+} dependent antiviral activity through store operated calcium entry and store independent calcium entry. The role of Ca^{2+} in regulating the Golgi complex and its potential function within influenza virus replication merits further consideration. The potential contribution of other post-translation modifications, such as phosphorylation and

sumoylation, to the TG dependent antiviral response was also identified. The precise contribution of TG mediated disruption of post-translational modifications to influenza virus replication inhibition requires further exploration. It is important to note that Ca^{2+} regulation of host post-translational modifications may not be reflected in the transcriptome. Ca^{2+} influx and store depletion may regulate protein activity directly, providing an immediate effect on the host cell response following TG priming. The contribution of TG mediated ER store depletion and extracellular Ca^{2+} influx to the TG mediated antiviral response needs to be addressed. However, RNA-seq analysis successfully identified multiple potential antiviral mechanisms which may be exploited for influenza virus therapy.

5. Chapter 5: The antiviral activity of the sesquiterpene lactone in response to influenza A virus

5.1 Introduction

Sesquiterpene lactones (SLs) are plant derived organic compounds with a wide range of biological activities, including anti-inflammatory, anti-parasitic, antiviral and anti-bacterial properties (Robles *et al.*, 1995, Siedle *et al.*, 2004, O'Neill *et al.*, 2010, Sinclair *et al.*, 2011, Duraipandiyan *et al.*, 2012, Ozcelik *et al.*, 2009, Wedge *et al.*, 2000a). The use of SLs in traditional Chinese medicine for a number of ailments and diseases, including diabetes, hepatitis and malaria, has been well documented (Lin, 2012, Mojab, 2012). Over 5000 structures of these secondary metabolites have been described, 3000 of which were derived from the *Asteraceae* plant family (Robles *et al.*, 1995, Wedge *et al.*, 2000b). The SLs are composed of a 15 carbon backbone, formed from condensation of three isoprene units into a *cis* or *trans*-fused lactone (Chaturvedi, 2011, Chadwick *et al.*, 2013). The structure of the hydrocarbon backbone may vary, giving rise to acyclic or cyclic compounds, although the latter includes the most functional forms (Kasymov, 1982, Chadwick *et al.*, 2013). Despite the diverse range of structures, the SLs share an α -methyl γ -lactone ring and its O=C-C-CH₂ moiety may be responsible for the biological activity of the SLs (Chadwick *et al.*, 2013, Bosco & Golsteyn, 2017). The cytotoxic activity of the sesquiterpene lactones can be attributed to alkylation of the thiols of host proteins by the α -methyl γ -lactone ring (Kupchan *et al.*, 1971, Hall *et al.*, 1980). The α -methyl γ -lactone ring can regulate gene expression through modifying the activity of transcription factors (Mazor *et al.*, 2000, Schomburg *et al.*, 2013, Wong & Menendez, 1999). In particular, the α -methyl γ -lactone ring and an α,β - or $\alpha,\beta,\gamma,\delta$ -unsaturated carbonyl group have been implicated in regulating the transcription factor NF- κ B (Rüngeler *et al.*, 1999). The presence of the ring and a carbonyl group in position 2 was essential for the anti-inflammatory

activity of sesquiterpene lactones. leukomisin and austricin, their activity was weakened by opening the lactone ring (Plutno *et al.*, 1995).

SL mediated antiviral activity has been described. The artemisinins display broad antiviral activity. Artesunate inhibited replication of human cytomegalovirus (HCMV) and herpes simplex virus type 1 (HSV-1) through inhibition of virus induced host signalling pathways, such as NF- κ B and Sp1 (Efferth *et al.*, 2002). Artemisinin and artesunate treatment inhibited hepatitis B virus replication *in vitro* without impairing host cell viability (Romero *et al.*, 2005). In addition, artemisinin inhibited hepatitis C virus replication and was amenable to co-treatment with hemin, resulting in a synergistic antiviral response (Paeshuyse *et al.*, 2006). Anti-bacterial properties have been attributed to other SLs. Dehydroleucodine (DHL) is a sesquiterpene lactone isolated from *Gynoxys verrucosa* and like other SLs, its biological activity was dependent on the lactone ring (Ordonez *et al.*, 2016, Ordóñez *et al.*, 2011). DHL displayed anti-microbial properties against a wide spectrum of bacteria, inhibiting methicillin-resistant *Staphylococcus aureus* (MRSA) and increasing the sensitivity of *Pseudomonas aeruginosa* to antibiotics (Mustafi *et al.*, 2015, Ordóñez *et al.*, 2011). DHL and other SLs may provide an alternative for the treatment of antibiotic resistant pathogens, indicated by the anti-microbial activity of DHL against antibiotic resistant strains of *Helicobacter pylori* (Vega *et al.*, 2009).

While antiviral activity has been described for a number of SLs, their effect on influenza A virus infection has not been extensively investigated. However, no effect on influenza A virus replication was reported following artseunate treatment (Efferth

et al., 2002). In contrast, extended exposure of MDCK cells infected with influenza A virus to TG induced an increase in progeny virus output (Fujioka et al., 2013).

The aims of this chapter are to (i) assess the antiviral activity of other sesquiterpenes and sesquiterpene lactones to determine if shared structures with TG are responsible for its antiviral activity, (ii) determine the dependence of the selected compounds in facilitating extracellular Ca^{2+} influx in comparison with TG and (iii) ascertain the antiviral mechanisms of the selected compounds, (+)-ledene and DHL.

5.2 Materials and Methods

5.2.1 Cell viability assay

NPTr cells and NHBE cells were cultured in 96 well plates near confluence. Culture conditions were described in Section 2.2. NPTr cells were exposed to 2.5 μ M or 10 μ M of DHL, (+)-ledene, valerenic acid, or 10 μ M artemisinin for 30 min (all from Sigma Aldrich). NHBE cells were incubated in 2.5 μ M or 5 μ M of dehydroleucodine or (+)-ledene for 30 min. Post-priming, the cells were washed with PBS and placed in the appropriate culture media and further cultured for 24 h after which cell viability was measured using the CellTiter-Glo® Luminescent Cell Viability Assay (Progenia) as described in Section 2.7.

5.2.2 Fluo-8 assay

NPTr cells and NHBE cells were cultured in black Nunc MicroWell 96-well optical-bottom plates with cell culture treated polymer base (Thermo Fisher Scientific) until near confluence. Culture media were replaced with DMEM-Glutamax (high glucose) supplemented with 1% FCS and the cells were loaded with Fluo-8 from the Fluo-8 Calcium Flux Assay Kit (abcam) and incubated for 1 h at RT. The cells were treated with the indicated concentrations of the sesquiterpenes and sesquiterpene lactones diluted in DMEM-Glutamax (high glucose) supplemented with 10% FCS. The cells were incubated for 10 min before fluorescence was measured using a FLUOstar OPTIMA (BMG Labtech).

5.2.3 Influenza virus infection of sesquiterpene lactone treated cells

NPTr cells and NHBE cells were cultured until ~80-90% confluent, as described in Section 2.2, and briefly incubated in the indicated concentration of sesquiterpenes or sesquiterpene lactones (Sigma Aldrich) for 30 min prior to infection. The cells were washed with PBS and immediately infected with USSR H1N1 (A/USSR/77) at the stated MOI (titration based on 6 h focus forming assay performed on MDCK cells) and viral titre analysed as described in Section 2.8.

5.2.4 RT-qPCR

Total RNA was isolated using the RNeasy Plus minikit (Qiagen) according to the manufacturer's instructions. One well of a 12 well plate sufficient for one RNA extraction. The extracted RNA was converted into cDNA as described in Section 2.10.2. SYBR green RT-qPCR, parameters described in Section 2.10.3, was performed. USSR H1N1 M gene expression was analysed using primers (F) 5' AGATGAGCCTTCTAACCGAGGTCG 3' and (R) 5' TGCAAAAACATCTTCAAG TCTCTG (Eurofins). Human *RIG-I*, *OAS1*, *DDIT3*, *HSPA5*, *ATP2C1*, *ATP2C2*, *GCP60*, *WIPI49*, *Orai1* and *STIM1* expression were determined using predesigned KSPQ12012 KiCqStart primer pairs (Sigma-Aldrich). RT-qPCR relative fold expression was normalised to 18s rRNA, using universal primers ((F) 5' ACGGCTACCACATCCAAGGA 3', (R) 5' CCAATTACAGGGCCTCGAAA 3') (Sigma-Aldrich).

5.2.5 Immunofluorescence confocal microscopy

NHBE cells were cultured in an eight well μ -slide (ibidi) and at ~70% confluence primed with 2.5 μ M (+)-ledene or DMSO for 30 min. The cells were washed with PBS and infected with USSR H1N1 at 1.0 MOI for 6 h. After 6 h, the samples were process according to Section 2.13, using the antibodies described in (Table 5-1). The cells were imaged with a Zeiss 710 confocal microscope in oil immersion with a 40x/1.3 objective.

1^o Antibody	Host species	Working concentration	Supplier
Anti-beta actin antibody	Rabbit	1µg/ml	Abcam
Anti-Influenza A Virus nucleoprotein antibody [AA5H]	Mouse	6.48µg/ml	Abcam
Anti-Influenza A H1N1 hemagglutinin antibody [C102 (IV.C102)]	Mouse	1µg/ml	Abcam
2^o Antibody	Working concentration		Supplier
Anti-Rabbit IgG H&L (Alexa Fluor® 488)	4µg/ml		Abcam
Anti-Mouse IgG H&L (Alexa Fluor® 647)	4µg/ml		Abcam

Table 5-1. Primary and secondary antibodies and their corresponding working concentrations used for confocal microscopy in human NHBE cells infected with USSR H1N1 virus.

5.3 Results

5.3.1 Pre-treatment of epithelial cells with non-toxic doses of sesquiterpenes reduced progeny virus output

The antiviral properties of a selection of sesquiterpenes (valerenic acid (VA) and (+)-ledene) and SLs (DHL and artemisinin) were assessed to determine if the lactone ring was important for the anti-influenza virus activity of TG. Oseltamivir was included for further comparison as one of the few commercially available antivirals approved for treatment of influenza A virus. Pre-treatment with a non-toxic concentration of VA, DHL and (+)-ledene at 10 μ M inhibited influenza A virus replication (Figure 5-1A and 5-1C). As artemisinin had no significant effect, it was excluded from further analysis (Figure 5-1A). Further interrogation of the selected compounds in NPTr cells revealed reduced concentrations (2.5 μ M) of VA, DHL and (+)-ledene significantly reduced viral titres, (Figure 5-1B).

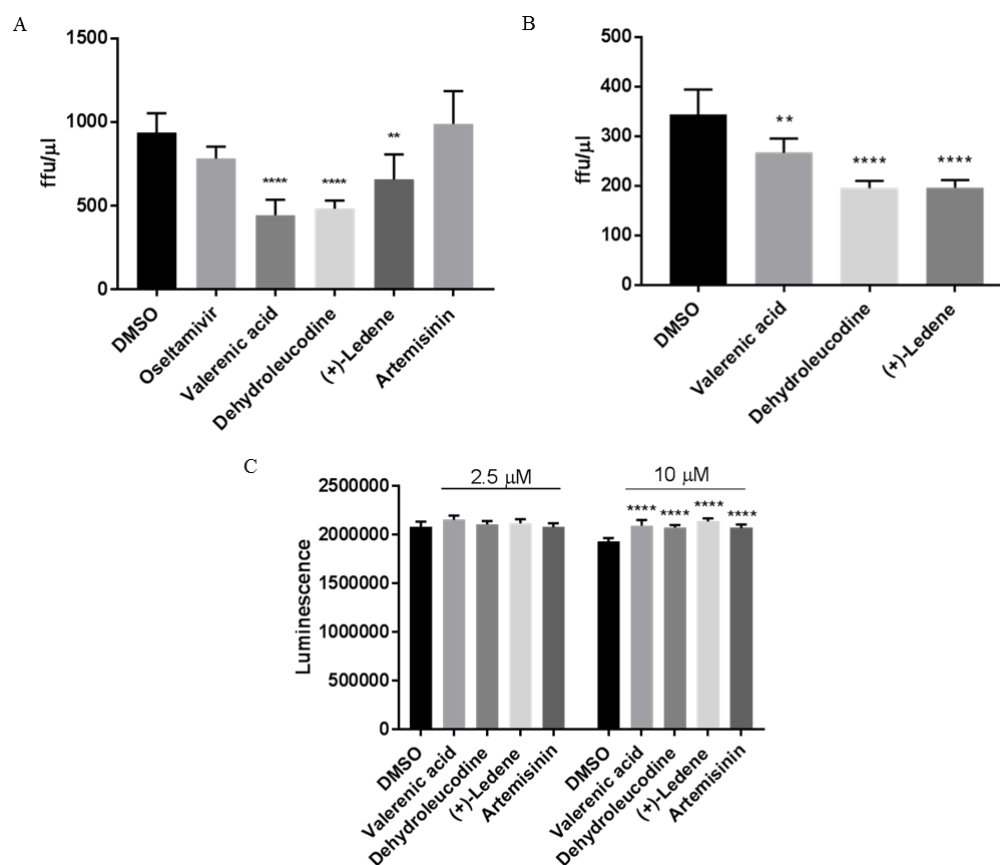


Figure 5-1. Brief exposure to non-toxic doses of sesquiterpenes reduces progeny virus output in NPTr cells. NPTr cells primed with (A) 10 μM or (B) 2.5 μM of the indicated compound unless otherwise stated for 30 min and subsequently infected with USSR H1N1 virus at 0.5 MOI for 24 h. Spun supernatants were used to infect MDCK cells for 6 h in focus forming assays (FFAs). (C) Cell viability of NPTr cells transiently exposed to the indicated sesquiterpene lactones at 2.5 μM or 10 μM for 30 min measured 24 h later using the CellTiter-Glo® Luminescent Cell Viability Assay. Significance determined by one way ANOVA, comparing treated cells to the corresponding DMSO control.

As DHL and (+)-ledene appeared to elicit the greatest reduction in progeny virus output in NPTr cells, their effect on influenza A virus replication in NHBE cells was subsequently examined. At a non-toxic concentration of 2.5 μ M, DHL and (+)-ledene significantly reduced progeny virus output (Figure 5-2). Of note, brief exposure of oseltamivir did not produce significant reduction in virus output from NPTr or NHBE cells (Figure 5-1A and 5-2A).

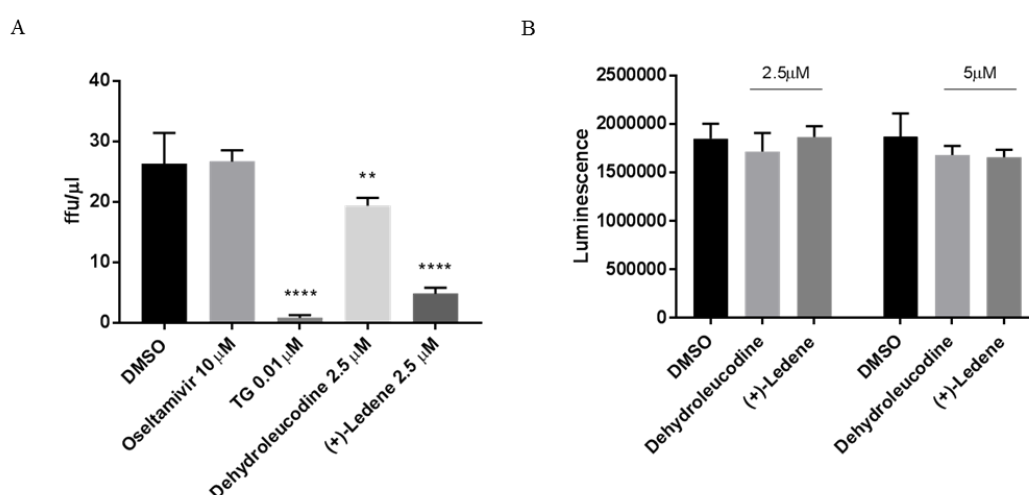


Figure 5-2. Brief exposure to non-toxic doses of sesquiterpenes reduced progeny virus output in NHBE cells. (A) NHBE cells primed with the indicated concentrations of the compounds for 30 mins. Primed cells were subsequently infected with USSR H1N1 virus at 1.0 MOI for 24 h. Spun supernatants were used to infect MDCK cells for 6h in focus forming assays (FFAs). (B) Cell viability of NHBE cells transiently exposed to the indicated sesquiterpene lactones, at 2.5 μ M or 5 μ M, for 30 min measured 24 h later using the CellTiter-Glo Luminescent Cell Viability Assay. Significance determined by one way ANOVA, comparing treated cells to the corresponding DMSO control.

Furthermore, as observed with TG, DHL and (+)-ledene priming inhibited influenza virus replication post-transcriptionally. This was reflected by a lack of reduction in M gene expression (Figure 5-3).

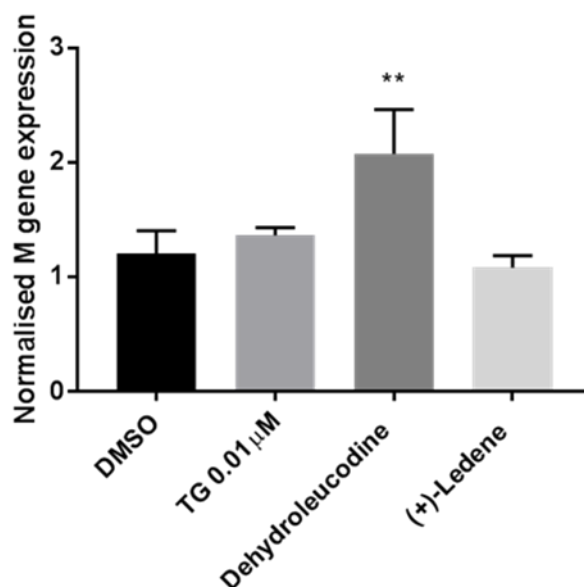


Figure 5-3. Viral M gene expression was not reduced in primed NHBE cells. NHBE cells primed with the indicated concentrations of the compounds for 30 mins. Primed cells were subsequently infected with USSR H1N1 virus at 1.0 MOI for 24 h. Total RNA was extracted for M gene expression, normalised to 18S rRNA, calculated using the comparative ct method. Fold gene expression relative to the DMSO control. Significance determined by one way ANOVA, comparing treated cells to the corresponding DMSO control.

5.3.2 Selected sesquiterpenes did not induce an influx of extracellular Ca^{2+} into the cytosol of epithelial cells.

TG induces an influx of extracellular Ca^{2+} into the cytosol, which may be partly responsible for its antiviral activity. Therefore, owing to the shared post-transcriptional inhibition of influenza virus replication of the compounds with TG, the ability of the selected compounds to induce extracellular Ca^{2+} influx was assessed. As expected, TG induced a significant increase in intracellular Ca^{2+} but this was not shared by any of the selected compounds for NPTr or NHBE cells (Figure 5-4).

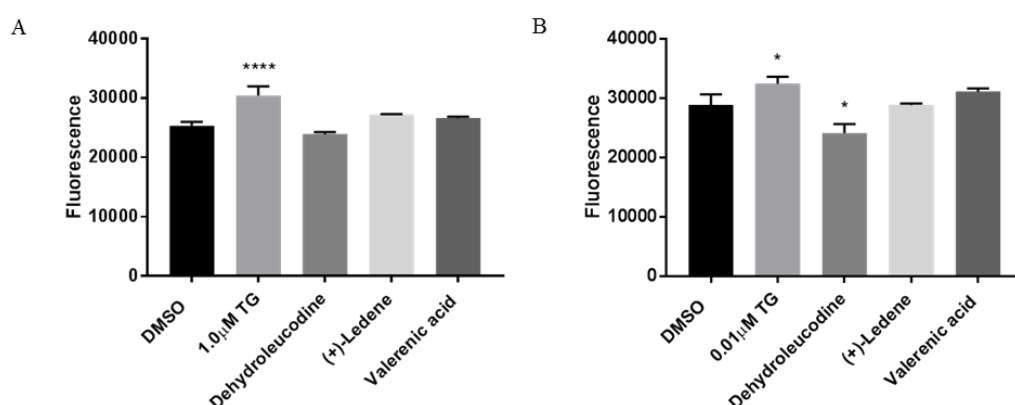


Figure 5-4. Sesquiterpene lactones did not induce an influx of extracellular Ca^{2+} into the cytosol of epithelial cells comparable to TG. Fluo-8 fluorescence intensity measured in (A) NPTr cells and (B) NHBE cells incubated in indicated sesquiterpene lactones at 2.5 μM for 10 min. 0.01 μM TG served as a positive control. Significance determined by one way ANOVA, comparing Fluo-8 fluorescence intensity of the treated samples with DMSO controls.

Brief pre-treatment with (+)-ledene induced a reduction in virus output in NHBE cells comparable to TG, thereby meriting further examination (Figure 5-2). A dose dependent inhibition of virus replication by (+)-ledene was clearly observed (Figure 5-5). However, the lowest concentration of (+)-ledene at 0.1 μM resulted in a ~50%

reduction in progeny virus output, while 0.01 μ M TG almost completely inhibited influenza A virus replication.

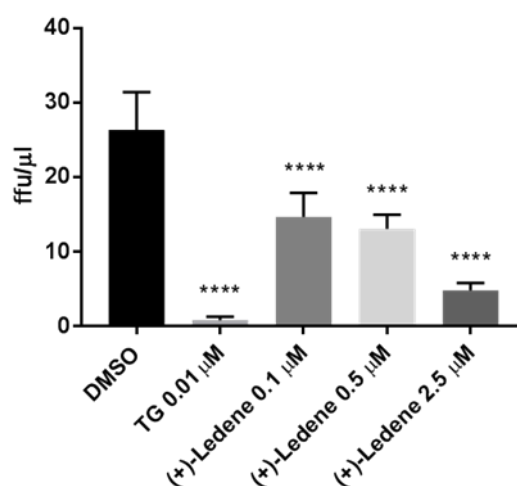


Figure 5-5. (+)-Ledene induced a dose dependent reduction in viral titre. NHBE cells primed with the indicated concentrations of (+)-ledene or TG for 30 mins and subsequently infected with USSR H1N1 virus at 1.0 MOI for 24 h. Spun supernatants were used to infect MDCK cells for 6h in focus forming assays (FFAs). Significance determined by one way ANOVA, relative to corresponding DMSO control.

In order to account for the possibility (+)-ledene induced an increase in intracellular Ca^{2+} at higher concentrations, Ca^{2+} influx for a range of concentrations extending up to 20 μ M was examined in NPTr and NHBE cells. (+)-ledene induced a dose dependent reduction in viral titre independent of extracellular Ca^{2+} influx in NHBE cells. The highest concentration of (+)-ledene, 10 μ M for NHBE cells and 20 μ M for NPTr cells, failed to induce any significant increase in intracellular Ca^{2+} , thereby suggesting the antiviral activity of (+)-ledene is not attributable to extracellular Ca^{2+} influx, (Figure 5-6).

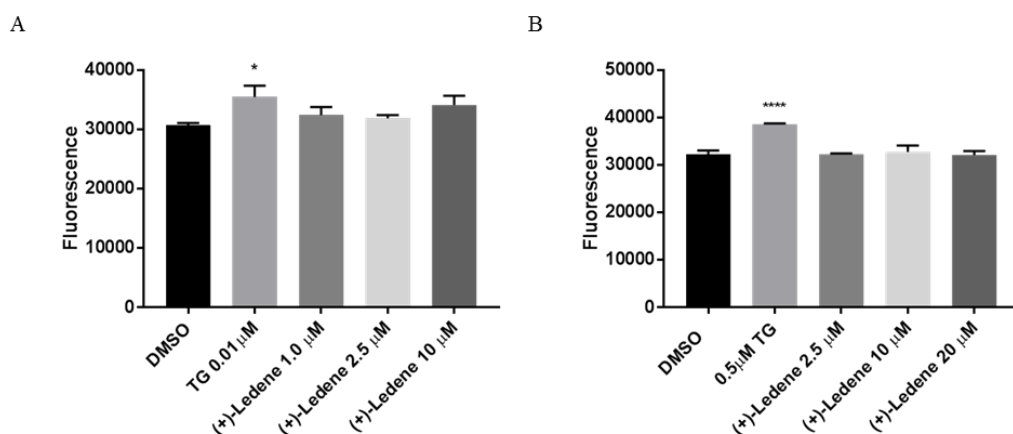


Figure 5-6. (+)-ledene did not induce a dose dependent influx of extracellular Ca^{2+} . Fluo-8 fluorescence intensity measured in (A) NHBE cells and (B) NPTr cells incubated with indicated concentrations of (+)-ledene or TG for 10 mins. Significance determined by one way ANOVA, comparing treated cells to corresponding DMSO control.

5.3.3 The sesquiterpenes did not facilitate extracellular Ca^{2+} entry through regulating expression of SOCE components

DHL and (+)-ledene did not actively induce extracellular Ca^{2+} influx but could enhance SOCE through regulating expression of key components of the pathway. TG induced expression of *STIM1*, while DHL and (+)-ledene significantly reduced *ORAI1* and *STIM1* expression in NHBE cells (Figure 5-7). This provides further evidence for an extracellular Ca^{2+} independent mechanism in the DHL and (+)-ledene mediated antiviral response. In contrast, TG not only induced extracellular Ca^{2+} influx but also enhanced expression of the SOCE components.

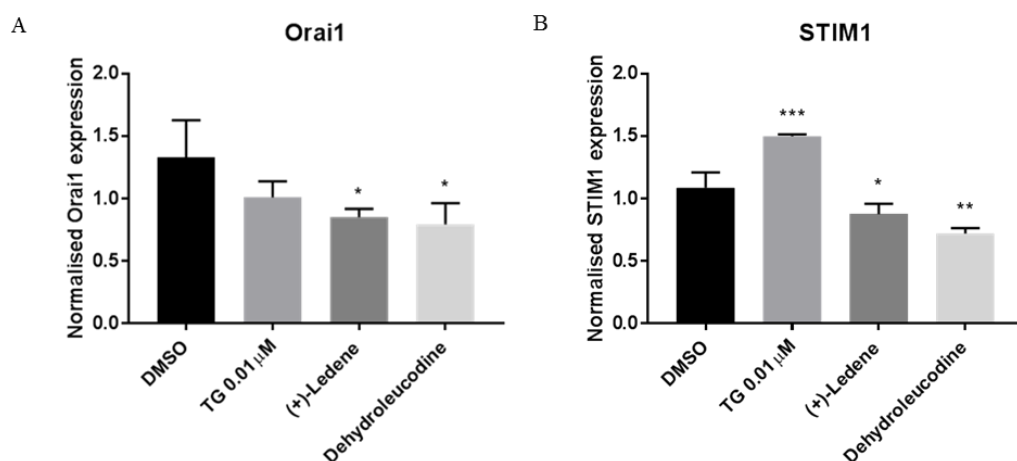


Figure 5-7. Selected sesquiterpenes did not alter the expression of SOCE members.

NHBE cells, pre-treated with 2.5μM of the indicated sesquiterpenes were subsequently infected with USSR H1N1 virus at 1.0 MOI for 24 h. Total RNA was extracted 24 h later. Fold expression of ORAI1 and STIM1, normalised to 18s rRNA, calculated using the comparative Ct method. Gene expression compared with the uninfected DMSO control. Significance determined by two way ANOVA, relative to the corresponding DMSO control.

5.3.4 Sesquiterpenes DHL and (+)-ledene did not reduce viral titre through regulation of type I IFN dependent gene expression

Expression of the type I IFN genes in uninfected and influenza virus infected NHBE cells following pre-treatment with the indicated compounds was assessed. *RIG-I* and *OAS1* expression was enhanced in TG pre-treated cells following USSR H1N1 virus infection, confirming previous reports of elevated expression of type I IFN dependent genes by TG (Figure 5-8). However, DHL and (+)-ledene pre-treatment had no effect

on *RIG-I* or *OAS1* expression in uninfected or infected NHBE cells, suggesting their antiviral activity was independent of type I IFN associated response (Figure 5-8).

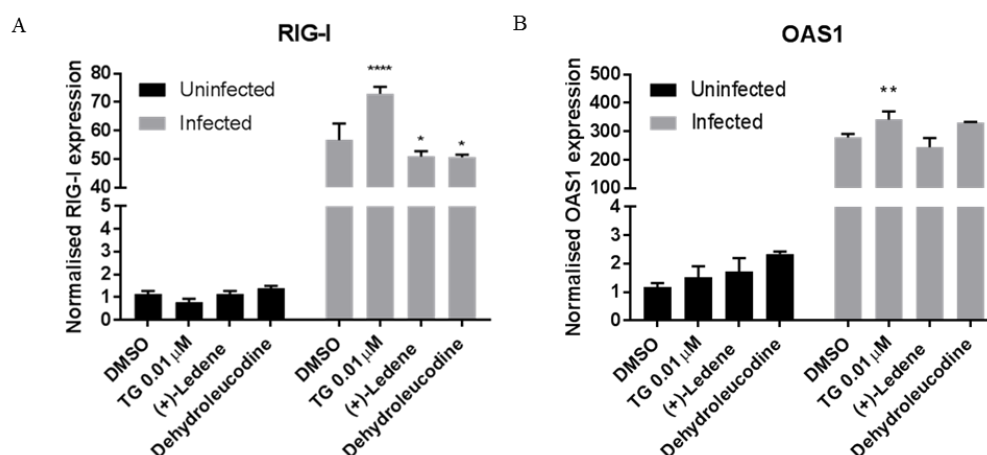


Figure 5-8. DHL and (+)-ledene did not induce type I IFN dependent gene expression in NHBE cells. NHBE cells, pre-treated with 2.5 μ M of DHL or (+)-ledene for 30 min were subsequently infected with USSR H1N1 virus at 1.0 MOI for 24 h. 0.01 μ M TG served as the positive control. Total RNA was extracted 24 h later. Fold expression of type I IFN dependent genes, normalised to 18s rRNA, calculated using the comparative Ct method. Gene expression was relative to the uninfected DMSO control. Significance determined by two way ANOVA, relative to the corresponding DMSO control.

5.3.5 ER and Golgi stress signalling pathways were not activated by brief exposure to DHL and (+)-ledene

The antiviral effect of DHL and (+)-ledene may be associated with the host stress signalling pathways. Expression of ER stress markers *DDIT3* and *HSPA5* were

strongly induced by TG in uninfected and USSR H1N1 virus infected cells but was unaffected by (+)-ledene or DHL pre-treatment, (Figure 5-9).

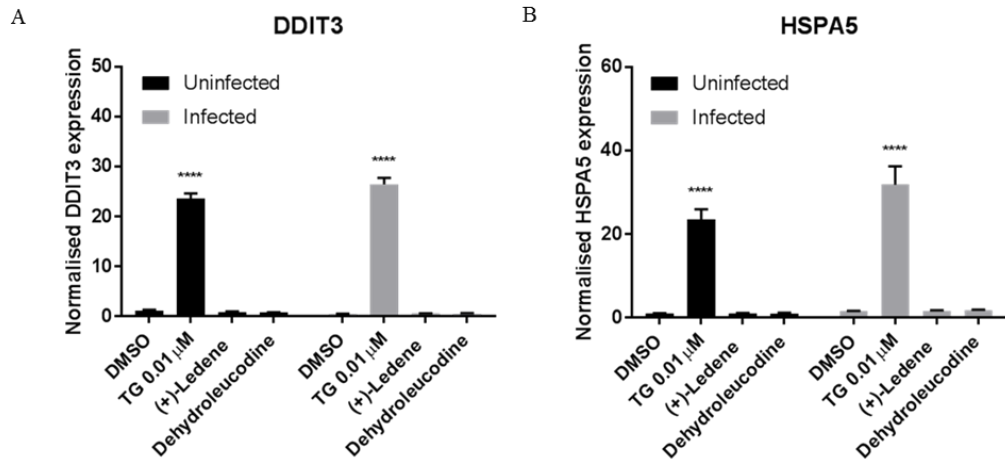


Figure 5-9. DHL and (+)-ledene priming did not mimic TG induced expression of ER stress markers in NHBE cells. NHBE cells, pre-treated with 2.5 μ M of DHL or (+)-ledene for 30 min were subsequently infected with USSR H1N1 virus at 1.0 MOI for 24 h. 0.01 μ M TG served as the positive control. Total RNA was extracted 24 h later. Fold expression of markers of Golgi stress, normalised to 18s rRNA, calculated using the comparative Ct method. Gene expression compared with the uninfected DMSO control. Significance determined by two way ANOVA, relative to the corresponding DMSO control.

Expression of markers of the Golgi stress response, *GCP60* and *WIPI49*, and Golgi Ca^{2+} ATPase pumps, *ATP2C1* and *ATP2C2*, were also not affected by DHL and (+)-ledene treatment (Figure 5-10). In contrast, expression of *WIPI49* and *ATP2C2*, the Golgi Ca^{2+} ATPase that associates with *ORAI1* to facilitate store independent Ca^{2+}

influx (Cross et al., 2013), was significantly elevated in TG primed cells (Figure 5-10B and 5-10D).

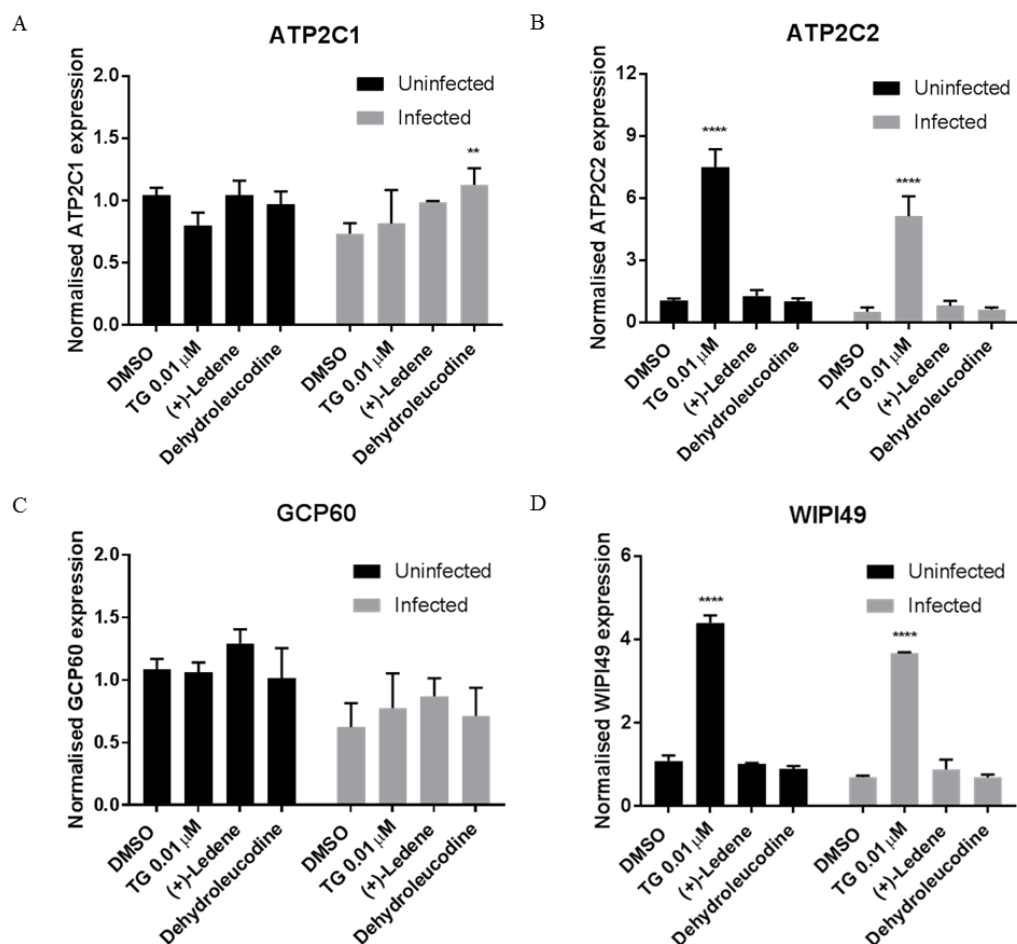


Figure 5-10. Selected sesquiterpenes did not mimic TG induced expression of Golgi stress markers in NHBE cells. NHBE cells, pre-treated with 2.5 μ M of DHL or (+)-ledene for 30 min were subsequently infected with USSR H1N1 virus at 1.0 MOI for 24 h. 0.01 μ M TG served as the positive control. Total RNA extracted 24 h later. Fold expression of markers of Golgi stress, normalised to 18s rRNA, calculated using the comparative Ct method. Gene expression compared to the uninfected DMSO control.

Significance determined by two way ANOVA, relative to the corresponding DMSO control.

5.3.6 Increased cytosolic accumulation of viral nucleoprotein in (+)-ledene primed NHBE cells

Since DHL and (+)-ledene neither regulate expression of the type I IFN genes nor the host cell stress signalling pathways, it appeared that the antiviral effect of the compounds was not dependent on regulating host gene expression in the same manner as TG. However, it was possible priming with DHL or (+)-ledene disrupted virus replication through altering virus protein processing or trafficking, rather than through regulating gene expression. Therefore, the localisation of NP 6 h post-infection following 30 min priming with (+)-ledene was analysed. (+)-Ledene priming induced a distinct pattern in NP localisation (Figure 5-11). NP was predominantly localised to the nucleus 6 h post infection in DMSO primed NHBE cells (Figure 5-11A). In contrast, increased distribution of NP in the cytosol and nuclei of the (+)-ledene primed NHBE cells can be observed (Figure 5-11B).

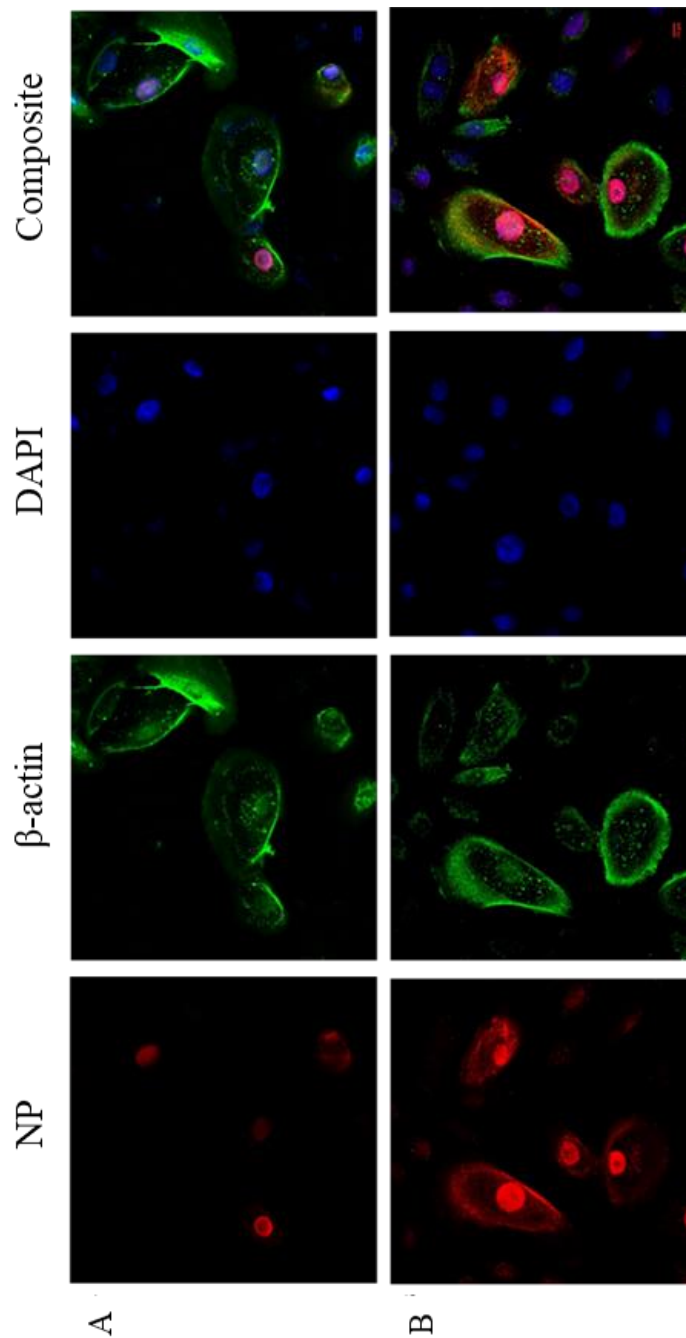


Figure 5-11. Increased cytosolic accumulation of viral nucleoprotein in (+)-ledene primed NHBE cells. NHBE cells were primed with (A) DMSO or (B) TG for 30 mins and subsequently infected with USSR H1N1 at 1.0 MOI for 6 h. Cells were fixed in 4% paraformaldehyde and stained for beta actin (rabbit anti-beta actin primary antibody detected by goat anti-rabbit IgG H&L conjugated to Alexa Fluor 488, green fluorescence) and viral nucleoprotein (mouse anti-influenza A virus nucleoprotein 325

primary antibody detected by donkey anti-mouse IgG H&L conjugated to Alexa Fluor 647, red fluorescence). DNA was stained with DAPI (blue fluorescence). NHBE cells imaged on Zeiss 710 confocal microscope. Scale bar on composite image, 10 μ M.

The ratio of NP in the nucleus to the cytosol for each NP positive cell was calculated. The intensity of NP fluorescence in the nucleus and cytosol was measured in ImageJ; DAPI staining marked the boundaries of the nucleus while immunostaining for beta actin determined the boundaries for cytosolic NP measurement. Despite the high variation in the DMSO primed cells, there was a significant difference in the distribution of NP, with a significantly greater proportion of NP located in the cytosol of (+)-ledene primed NHBE cells 6 h post infection (Figure 5-12). This suggests (+)-ledene may impair virus replication through interfering with virus protein transport, virus complex assembly (i.e. formation of the vRNP) or a combination of both.

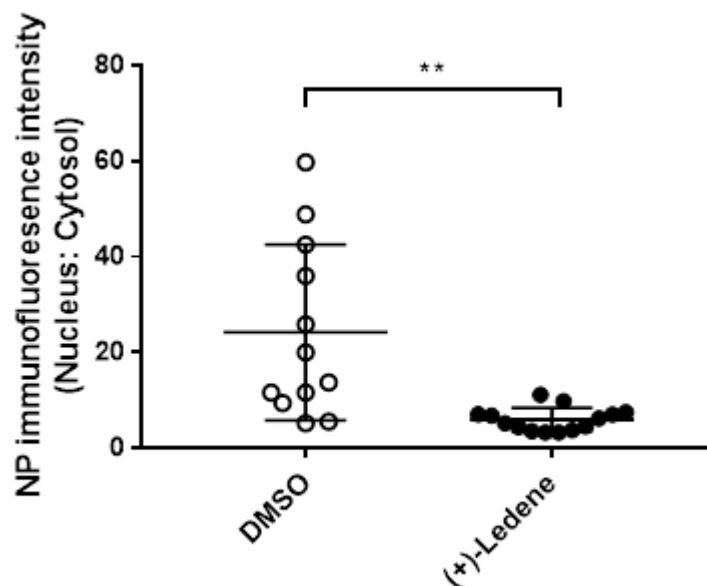


Figure 5-12. *The distribution of NP in the nucleus to the cytoplasm was reduced in (+)-ledene primed NHBE cells. Quantification of viral NP was performed on NHBE cells primed with DMSO or (+)-ledene for 30 min before infection with USSR H1N1 at 1.0 MOI for 6 h. Beta actin (rabbit anti-beta actin primary antibody detected by goat anti-rabbit IgG H&L conjugated to Alexa Fluor 488) was used to indicate the cytosolic portion of the cell and DAPI staining of DNA to indicate the nucleus. The distribution of viral NP (mouse anti-influenza A virus nucleoprotein primary antibody detected by donkey anti-mouse IgG H&L conjugated to Alexa Fluor 647) was quantified with Image J and the ratio of viral protein in the nucleus to cytosol calculated. Significance determined by unpaired t-test.*

5.4 Discussion

In the present study, the ability of selected sesquiterpenes to inhibit influenza A virus replication was examined. Since TG displayed such potent antiviral activity, compounds within the same structural family were assessed for antiviral properties. While antiviral activity has been described for a number of sesquiterpene lactones, their effectiveness in inhibiting influenza A virus replication had not been studied. The potential mechanisms behind the antiviral activity of the compounds, such as their ability to regulate immune gene expression, was compared with those of TG. Including sesquiterpenes and sesquiterpene lactones in the study allowed the contribution of the lactone ring to the biological activity of TG within the immune response to be assessed.

The lactone ring is proposed to be key to the bioactivity of the SLs. However, in this study, sesquiterpenes and SLs reduced progeny influenza A virus production in human and pig tracheal epithelial cells following transient priming with non-toxic concentrations. In addition, another sesquiterpene lactone (artemisinin) had no significant effect on progeny virus production in NPTr cells. This suggests the lactone ring, shared with TG, is not essential for the antiviral activity of the sesquiterpene lactones, summarised in Table 5-2.

Chemical	Lactone ring	Antiviral activity in NPTr cells	Antiviral activity in NHBE cells
Thapsigargin	Yes	Yes	Yes
(+)-ledene	No	Yes	Yes
Dehydroleucodine	Yes	Yes	Yes
Artemisinin	Yes	No	-
Valerenic acid	No	Yes	-

Table 5-2. Antiviral activity of the sesquiterpenes and sesquiterpene lactones was not dependent on the lactone ring. Antiviral activity defined by a significant reduction in progeny virus output, measured through FFAs, 24 h after priming with the indicated chemical. ‘-’ indicates the chemical was not assessed in the indicated cell type.

A lack of corresponding reduction in virus M gene expression was shared by DHL and (+)-ledene with TG, suggesting all of the compounds shared a post-transcriptional antiviral mechanism. However, key differences between the selected compounds and TG were subsequently identified. Comparison of the compounds on transcriptional regulation revealed distinct differences with TG on host gene expression. While TG elevated expression of the type I IFN regulated genes (*RIG-I* and *OAS1*), ER stress (*DDIT3* and *HSPA5*) and Golgi stress markers (*WIPI49* and *ATP2C2*), (+)-ledene and DHL pre-treatment had no comparable effect on gene expression. The difference in activity between the sesquiterpene lactones, TG and DHL, implies the lactone ring may not be the key structural feature responsible for the antiviral activity of TG. This is supported by the potent anti-influenza activity of (+)-ledene in this study and

another sesquiterpene, triptofordin C-2, which displays inhibitory activity against a range of viruses including influenza (Hayashi *et al.*, 1996). However, other structures essential for biological activity within the SLs have been identified which may be necessary for the antiviral activity of TG. For example, a second carbonyl group, in addition to the group within the lactone ring, may be responsible for the anti-microbial activity of DHL (Ordóñez *et al.*, 2011). The exact structure or combination of structures responsible for the biological activity of these compounds is a subject for potential further study

The differing characteristics of TG may be attributable to its ability to induce ER stress through Ca^{2+} store depletion. The lack of effect of the compounds on type I IFN dependent gene expression may be attributable to the lack of ER stress induced by DHL or (+)-ledene. Activation of the type I IFN response by the ER stress response has been reported in some detail and may account for TG mediated regulation of the type I IFN response in influenza A virus infected cells (Liu *et al.*, 2012a, Zeng *et al.*, 2010). Therefore, TG mediated store depletion may activate the type I IFN response through a combination of ER stress signalling pathways and an increase in cytosolic Ca^{2+} which is not shared by (+)-ledene or DHL.

Lastly, the mechanism of (+)-ledene mediated inhibition of virus replication was considered. TG priming altered the localisation of NP, increasing the proportion of cytosolic NP relative to nuclear NP (Section 3.3.14). As (+)-ledene priming dramatically reduced progeny virus production in NHBE cells, its effect on viral protein localisation was assessed. Priming the NHBE cells with (+)-ledene altered localisation of viral NP 6 h post infection in the same manner as TG, increasing the

proportion of NP protein in the cytosol relative to the nucleus. As with TG priming, the stage at which (+)-ledene interferes with NP localisation is unclear. Viral mRNA is translated in the cytosol, therefore translated NP must be transported to the nucleus (Ozawa *et al.*, 2007, Neumann *et al.*, 1997). Once NP is localised to the nucleus where it associates with viral RNA to form the viral ribonucleoprotein (vRNP) complex (Hutchinson & Fodor, 2012, Davey *et al.*, 1985). A number of factors have been implicated in regulating NP shuttling between the cytosol and nucleus. (+)-Ledene may reduce the efficiency of NP translocation to the nucleus after its synthesis in the cytosol, indicated by immunofluorescence microscopy. As proposed for TG, (+)-ledene may regulate post-translational modifications of influenza virus NP. The compound may interfere with the nuclear localisation of NP via impairing its sumoylation (Cros *et al.*, 2005, Han *et al.*, 2014). Alternatively, (+)-ledene may regulate phosphorylation of NP, another modification which regulates its transport between the nucleus and cytosol (Zheng *et al.*, 2015). Regulation of kinase activity by a sesquiterpene lactone, alantolactone, has been reported previously (Wang *et al.*, 2017).

Alternatively, the antiviral activity of (+)-ledene and DHL may be dependent on regulating other host cell pathways, such as the NF- κ B signalling pathway. The proposed anti-inflammatory activity of DHL and (+)-ledene is consistent with others within the sesquiterpene family (Rüngeler *et al.*, 1999). The sesquiterpene lactones negatively regulates the NF- κ B signalling pathway; inhibiting NF- κ B nuclear translocation and stabilising I- κ B α mediated inhibition of NF- κ B (Mazor *et al.*, 2000, Hehner *et al.*, 1998, Wong & Menendez, 1999). DHL dependent inhibition of the NF- κ B signalling pathway, through decreasing phosphorylation of p65, has been reported

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(Ordonez et al., 2016) but the activity of (+)-ledene has not been thoroughly described. The interplay between influenza A virus and the NF- κ B pathway is complex, with pro- and antiviral properties attributed to this immune pathway (Ludwig & Planz, 2008). Inhibition of NF- κ B has been found to impair influenza A virus propagation. Progeny virus output from MDCK cells and A549 cells was reduced following inhibition of NF- κ B by SC75741 or acetylsalicylic acid (Mazur *et al.*, 2007, Ehrhardt *et al.*, 2013). Therefore, taking into consideration the pro-viral function of NF- κ B signalling and the immunosuppressive activity of the sesquiterpenes, it is possible the compounds may mediate an antiviral response via suppressing virus induced NF- κ B activity.

Members of the SL family have already been considered for modern human medicine, such as the treatment of malaria by artemisinin derivatives (Ashley *et al.*, 2006, Li & Weina, 2010, Phan *et al.*, 2002). This study suggests the sesquiterpenes and sesquiterpene lactones display potential as antivirals, although further work is necessary to determine the post-transcriptional antiviral mechanism of DHL and (+)-ledene. In addition, this study provides further evidence for the role of extracellular Ca^{2+} influx, not shared by DHL or (+)-ledene, that may underlie the distinct TG antiviral response.

6. Chapter 6: General Discussion

The overall aim of this thesis was to explore the contribution of Ca^{2+} mobilisation and extracellular Ca^{2+} influx to host immunity and determine the impact of dysregulated Ca^{2+} homeostasis on influenza A virus replication. To achieve this, the contribution of store operated calcium entry (SOCE) to host innate immunity and its effect on influenza A virus replication was examined.

The influenza virus is a global challenge to public health. There have been approximately 14 pandemics in the last 500 years, five of which have occurred in the last 100 years alone (Taubenberger & Morens, 2009). The H1N1 virus pandemic in 1918, labelled Spanish influenza, was responsible for approximately 50 million deaths worldwide and less conservative estimates suggest this figure may be as large as 100 million (Johnson & Mueller, 2002). Seasonal influenza virus has been predicted to claim up to approximately 650 000 lives globally each year (Iuliano et al., 2018). The current strategies for inhibiting the spread of influenza virus throughout the population rely predominantly on vaccination. Owing to antigenic drift, point mutations accumulate over time resulting in novel influenza virus strains and new antigenic vaccines are needed each year. However, production of a vaccine requires up to 9 months and depends on reliable predictions of the likely influenza virus strains circulating in the target season (Carrat & Flahault, 2007). In contrast, antivirals can provide more immediate response to an influenza virus pandemic (Monto, 2006, Oh & Hurt, 2014). Three classes of antivirals have been developed; the adamantanes which inhibit the M2 channel, the neuraminidase inhibitors and the viral RNA

dependent RNA polymerase inhibitors (Zhou et al., 2018, Oh & Hurt, 2014). However, the development of influenza virus resistance presents a serious challenge to antiviral therapy (Hussain *et al.*, 2017). A strategy to overcome this recurrent problem may rely on targeting host signalling pathways, either inhibiting host factors required for influenza virus replication or enhancing protective host immune signalling pathways (Shaw, 2011, Crosse et al., 2018). Calcium dependent host signalling pathways have been identified as potential targets for host directed antiviral therapy (Marois et al., 2014). SOCE facilitates extracellular Ca^{2+} influx through channels in the PM in response to depletion of Ca^{2+} from the ER (Parekh & Putney, 2005). Some lines of evidence point to SOCE as a pro-viral host signalling pathway. Rotavirus infection induced SOCE through rotavirus nonstructural protein 4 (NSP4) mediated store depletion. Impairing SOCE through *STIM1* knockdown inhibited rotavirus replication (Hyser et al., 2013). Latent membrane protein-1 (LMP-1) of Epstein–Barr virus (EBV) promoted Ca^{2+} entry through the SOC channel (Dellis et al., 2011). Finally, the HBx protein of hepatitis B protein activated the SOC channels in hepatocytes (Casciano et al., 2017, Casciano & Bouchard, 2018). However, SOCE is an essential positive regulator of innate and adaptive immunity (Vig & Kinet, 2009, Feske et al., 2015). Patients suffering from combined immunodeficiency syndrome lacked SOCE and displayed greater susceptibility to viral infection (Feske, 2011, Feske, 2009). SOCE regulates T cell and B cell function, including proliferation and cytokine production (Vaeth *et al.*, 2017a, Vaeth et al., 2017b, Scharenberg *et al.*, 2007). Absence of STIM2 impaired TNF α , IL-10 and IFN- γ production in mouse neutrophils (Clemens et al., 2017). Spinal astrocyte TNF- α and IL-6 production was stimulated by SOCE activation and impaired by *STIM1* or *ORAI1* knockdown (Gao et al., 2016). Cytotoxic lymphocyte degranulation was impaired in natural killer cells lacking *ORAI1* (Maul-

Pavicic et al., 2011). Therefore the contribution of SOCE to host immunity requires further clarification.

The evidence presented here indicates that facilitating SOCE inhibits influenza virus replication. Two methods were utilised to induce SOCE activation. The first was the use of a chemical activator, thapsigargin (TG). TG activates SOCE through impeding ER/SR Ca^{2+} ATPase, preventing cytosolic Ca^{2+} uptake into the ER Ca^{2+} store and thereby inducing store depletion and STIM1-Orai activation. Secondly, components of the core molecular machinery essential for SOCE (STIM1 and the Orai homologues) and positive regulators (CRACR2A and STIMATE) were overexpressed. Chemical and genetic activation demonstrated activated SOCE significantly reduced total progeny virus release from the host cells. Conversely, an increase in progeny virus output from tracheal epithelial cells was observed following chemical inhibition of the SOC channels by BTP2 and Synta66, and knockdown of *STIM1*. TG mediated SOCE activation before and during infection induced a sustained antiviral state that was maintained for greater than 24 hours. This effect was consistent across a variety of porcine and human cells. Collectively, these results strongly suggest activation of SOCE facilitates a potent antiviral response to influenza virus infection.

The stage at which influenza virus replication was inhibited was considered. Overall M gene RNA expression in the host cell and viral protein levels were minimally affected or unaltered by TG priming or SOCE overexpression in tracheal epithelial cells, suggesting a post-translational mechanism of inhibition. TG priming did not appear to alter influenza progeny virus morphology, as indicated by transmission electron microscopy (TEM). The multifaceted antiviral mechanism orchestrated by

TG induced SOCE was dependent on activating host immune signalling pathways and inhibiting proviral host components. Regulation of the type I/III IFN response may underpin the former while the latter may be dependent on inhibition of host protein processing, post translation modifications and/or intracellular protein transport. The contribution of extracellular Ca^{2+} or ER Ca^{2+} store depletion to the antiviral mechanism of action must be considered throughout.

Firstly, SOCE dependent activation of innate immunity has been reported. The evidence presented here suggests that Ca^{2+} mobilisation regulates innate immune gene expression. TG priming significantly elevated IFN sensitive gene (ISG) expression in a dose dependent manner, demonstrated by RT-qPCR and RNA-seq. In particular, TG priming induced the type I/III IFN response in influenza infected cells through activation of the IRFs 3 and 7. Influenza specific antiviral genes such as *Mx1* and *IFN-induced transmembrane 3 (IFITM3)* were significantly elevated by TG priming and knockdown of *ORAI1* or *STIM1* inhibited type I IFN dependent gene expression. However, genetic activation of SOCE did not elevate type I IFN dependent gene expression. This may be due to elevated basal expression of type I IFN dependent genes in transfected cells prior to infection. The activation of negative feedback mechanisms on type I IFN regulated gene expression may account for the muted type I IFN response in cells overexpression *STIM1* or *ORAI*. Alternatively, extracellular Ca^{2+} may regulate other host immune pathways, such as the NF- κ B signalling pathway. *STIM1* and *ORAI1* mediated activation of NF- κ B signalling has been identified in T cells (Liu et al., 2016b).

Secondly, TG may inhibit influenza virus replication through the activation of the ER stress signalling pathways. This antiviral mechanism is dependent on Ca^{2+} store depletion. TG activates branches of the unfolded protein response (UPR). The UPR is split into three distinct responses mediated by activating transcription factor 6 (ATF6), inositol requiring kinase 1 (IRE1) and double-stranded RNA-activated protein kinase (PKR)-like endoplasmic reticulum kinase (PERK). Inducing ER stress through priming with non-toxic concentrations of TG or tunicamycin significantly reduced progeny virus output and induced expression of ER stress markers (*DDIT3*, *HSPA5*, *HSP90B1*), indicating ER stress alone was sufficient to inhibit virus replication. However, differences between the antiviral response in TG primed and tunicamycin primed cells were observed. TG mediated activation of the ER stress response may induce expression of the type I IFN dependent genes, in contrast to tunicamycin. ER stress has been demonstrated to activate IRF3 phosphorylation via the STING/TBK1 pathway. IRF3 activation by the STING/TBK1 pathway was dependent on Ca^{2+} depletion induced ER stress. Tunicamycin induced ER stress without inducing Ca^{2+} store depletion and did not induce IRF3 activation (Liu et al., 2012a). This is in agreement with the results presented here. Secondly, TG induced dysregulation of ER function may also inhibit influenza virus replication by disrupting influenza virus glycoprotein processing in the ER. TG induced activation of the ATF6 and PERK mediated unfolded protein response, indicating an accumulation of unfolded proteins in the ER. Dysregulated ER function may impair N-linked glycosylation of the HA and NA, thereby interfering with viral glycoprotein folding and oligomerisation. Calnexin (CNX) and calreticulin (CRT) are ER chaperones which mediate glycoprotein folding and quality control (Williams, 2006). Influenza glycoprotein folding and processing are mediated by CRX and/or CRT (Wang et al., 2008a, Hebert

et al., 1997). Upregulation of *CNX* and *CRT*, indicated by RNA-seq, may reflect an accumulation of misfolded viral glycoproteins.

Alternatively, TG may inhibit transport of viral components through the secretory pathway to the apical PM for assembly into progeny viruses. Newly synthesised HA, NA and M2 are transported through the ER and to the apical PM (Zhu et al., 2017a, Copeland et al., 1986, Wang et al., 2012). RNA-seq analysis suggested TG priming may inhibit transport within the secretory pathway, particularly ER to Golgi and intra-Golgi transport mediated by COPI coated vesicles and the TRAPP complex. In addition to reduced expression of genes associated with intracellular transport, TG mediated increase in cytosolic Ca^{2+} may inhibit intra-Golgi transport (Porat & Elazar, 2000). Dysregulation of the Golgi complex could inhibit influenza virus replication independently of TG mediated store depletion. ORAI1 channel mediated Ca^{2+} influx into the Golgi complex was facilitated by a Golgi secretory pathway Ca^{2+} -ATPase, SPCA2 (*ATP2C2*), termed store independent calcium entry (SICE) (Smaardijk et al., 2017, Smaardijk et al., 2018, Cross et al., 2013). Elevated Ca^{2+} in the Golgi complex increased Golgi stress (Smaardijk et al., 2018). An aberrant influx of Ca^{2+} through the ORAI1 channels may result in dysregulation of Golgi complex function independent from store depletion. This subsequently may interfere with influenza glycoprotein processing and transport in the secretory pathway. Despite its independence from ER store depletion, TG may enhance SICE by increasing expression of the Golgi apparatus Ca^{2+} ATPase, *ATP2C2*. TG priming also induced expression of Golgi complex stress markers, indicating disrupted Golgi function. However, additional work is required to determine if this was due to TG mediated increases in *ATP2C2* expression and subsequent elevation of Golgi complex luminal Ca^{2+} .

There is also evidence to suggest TG may impair vRNP assembly and intracellular transport. Recently the ER has been implicated in mediating vRNP transport to the PM, in contrast to the previously accepted mechanism dependent on Rab11-positive recycling endosomes (de Castro Martin et al., 2017). Therefore, TG priming may interfere with vRNP transport to the apical PM by inducing ER stress. In addition, TG may interfere with nuclear import of NP, indicated by increased cytosolic localisation of NP in TG treated cells during infection. TG mediated increases in cytosolic calcium may impair NP nuclear import through inhibiting the importin α/β nuclear import pathway (Kaur et al., 2014). Additionally, TG may inhibit vRNP assembly and incorporation into progeny virions through inhibition of posttranslational modifications. Sumoylation of NP was required for its nuclear import while ubiquitination of the matrix proteins was required for packaging vRNPs into progeny viruses (Han et al., 2014, Su et al., 2018). TG priming inhibited expression of components of the sumoylation and ubiquitination pathways.

In drug discovery or repurposing, it is essential to identify off target effects associated with toxicity. At high concentrations (μM) and exposure for an extended period of time (hours or days), TG exposure can be toxic to cells (Sehgal *et al.*, 2017, Gu *et al.*, 1995). However, for each cell type non-toxic doses of TG and brief priming durations (<60 mins) were sufficient to induce the potent antiviral response, as indicated by ATP cell viability and caspase 3/7 assays. The selectivity index (SI) of TG in tracheal epithelial cells was ~ 8000 , indicating the range at which TG optimal drug efficacy may be obtained without adverse toxicity is sufficiently high. The safety of TG was

further supported by the Ingenuity toxicity function which predicted no significant toxic effects.

Following identification of antiviral activity of TG at a safe range of concentrations, other chemicals with a similar molecular structure were considered. TG is a sesquiterpene lactone derived from *Thapsia garganica* (Xu et al., 2004). The sesquiterpenes are composed of acyclic or cyclic compounds with a 15 carbon backbone. The sesquiterpene lactones (SLs) are sesquiterpenes which share an α -methyl γ -lactone ring with a carbonyl moiety (Bosco & Golsteyn, 2017, Chadwick et al., 2013). Antiviral activity has been determined for some SLs. For example, replication of human cytomegalovirus (HCMV), herpes simplex virus type 1 (HSV-1) and hepatitis B virus was inhibited by artesunate and artemisinin (Romero et al., 2005, Efferth et al., 2002). Here inhibition of influenza A virus replication by sesquiterpene lactone dehydroleucodine and sesquiterpene (+)-ledene is reported. However, the antiviral mechanism of the sesquiterpenes differed to TG, as the compounds did not induce the ER stress unfolded protein response or elevate the type I IFN signalling pathway. (+)-Ledene may inhibit influenza virus replication post-translationally, indicated by unaltered M gene expression or viral protein levels but impaired NP intracellular localisation. The difference in the antiviral mechanism of action to TG may be dependent Ca^{2+} mobilisation, a characteristic that was not shared by (+)-ledene or DHL. Identifying the underlying antiviral mechanism of action for the sesquiterpenes may be considered for future work.

Host directed therapy is an alternative strategy proposed for treating a range of pathogens, including influenza virus (Kaufmann *et al.*, 2018, Marois et al., 2014).

Targeting the host signalling pathways, either enhancing immune signalling pathways or inhibiting host factors required for virus replication, may be a more effective in treating influenza virus infection. The challenge of drug resistance may be surmounted by not targeting virus proteins directly and thus applying selection pressure on the virus. SOCE mediates an antiviral response capable of inhibiting influenza virus replication in multiple cell types. SERCA inhibitor and SOCE agonist TG was identified as a possible therapeutic agent targeting the SOCE pathway to inhibit influenza virus. A patent application (United Kingdom Patent Application No. 1805665.5) for the use of TG and other sesquiterpene lactones for antiviral treatment of influenza A virus infection has been submitted. The extended antiviral response may be dependent on TG mediated regulation of host transcription, inducing expression of type I/III stimulated genes and inhibiting expression of proviral factors. However, TG may mediate a fast antiviral response through directly regulating host proteins independent from the transcriptional response. TG induced store depletion may dysregulate ER function and inhibit virus protein processing while the increase in cytosolic Ca^{2+} may inhibit intra-Golgi transport or nuclear import of viral proteins. The wide ranging effects of TG on host antiviral pathways, summarised in Figure 6-1, commend it as a broad antiviral inhibitor of RNA virus replication and not just specific to influenza A virus.

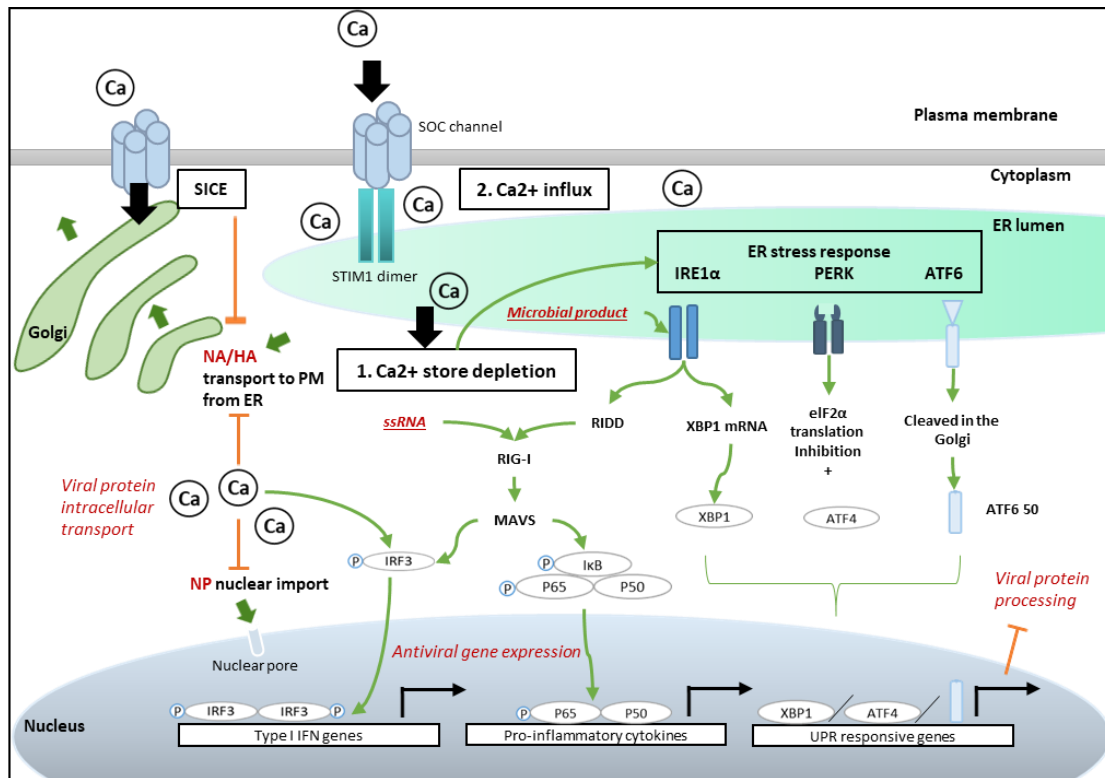


Figure 6-1. Summary of potential mechanisms underlying TG mediated inhibition of influenza A virus replication. TG priming may inhibit influenza A virus replication post-translationally by impairing viral protein folding and processing in the ER/Golgi and may interfere with viral glycoprotein transport in the secretory pathway (ER-Golgi-plasma membrane) or NP nuclear import. In addition, TG priming may induce expression of antiviral genes such as the type I IFNs. These responses may be dependent on the ER stress response or elevated cytosolic Ca^{2+} . SICE in the Golgi may contribute to Ca^{2+} dependent inhibition of influenza virus replication. HA, hemagglutinin; NA, neuraminidase; RIDD, IRE1-dependent decay of mRNA; ss, single stranded; SICE, store independent calcium entry; SOC, store operated calcium; UPR, unfolded protein response/

Future work exploring the impact of TG on viral protein processing and intracellular transport should be considered, especially considering the potential impact on intracellular Ca^{2+} mobilisation on influenza virus glycoprotein processing in the secretory pathway. While TG mediated inhibition of influenza A virus replication has been well established here, the exact contribution of the host cell stress pathways and increase in cytosolic Ca^{2+} to the antiviral activity of TG must be explored further. While an antiviral function has been attributed to SOCE during influenza A virus infection, there may be a role for SICE in mediating an antiviral response. Furthermore, the contribution of intracellular Ca^{2+} mobilisation, whether via SOCE or SICE, on the replication of viruses other than influenza virus must be established. Ca^{2+} mobilisation may underpin the activity of a new class of antivirals capable of inhibiting influenza A virus infection and other RNA viruses.

7. References

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