CHAPTER 6: INVESTIGATING APPROACHES TO SUPPRESS THE INNATE RESPONSE TO ROTAVIRUS TRANSCRIPTS USING PLASMID DERIVED ROTAVIRUS Wa AND SA11 PROTEINS

6

6.1 Introduction

To date, studies in cell culture involving rotavirus transcripts (mRNA) have been hampered by the aggressive innate immune response they elicit (Mlera, 2013, Uzri and Greenberg, 2013). Foregoing the adaptive immune response, the innate immune system is the initial defence against viruses (Samuel, 2001). In order for the cell to mount an effective antiviral offensive, most viral infections lead to the stimulation of a complex cascade of host cell signalling pathways ultimately resulting in the expression of IFN-stimulated genes (ISGs) which is able to directly inhibit viral replication (Edinger and Thompson, 2004, Levy et al., 2011, Stetson and Medzhitov, 2006). The secretion of cytokines belonging to the interferon (IFN) family (IFN type I and III in particular) play an important part in this innate immune response by activating the expression of IFN-stimulated genes (ISGs) (Randall and Goodbourn, 2008, Takeuchi and Akira, 2009). Viruses have adapted specific strategies to neutralize and exploit these immune responses to aid their own replication cycle (***Table 6.1***). The innate immune response to rotavirus transcripts is still not completely understood. At present, rotavirus NSP1 is the sole rotavirus protein known to be directly concerned with the evasion of the host's innate immune response (Bagchi et al., 2010, Feng et al., 2009). This non-structural protein is thought to be an E3 ubiquitin ligase which is able to bind to various IFN regulation factors (IRF3/5/7) and mark them for proteasomal degradation (Barro and Patton, 2007, Graff et al., 2007).

Table 6.1: Mechanisms of different viruses to evade the innate immune response of the host cell

Virus	Gene or encoded viral protein	Function	Reference(s)
Adenovirus	E1A	IKK suppression, Prevention of ISGF3 formation	(Kalvakolanu et al., 1991, Piguet et al., 1999)
Ebola Virus	VP35	Blocking of IRF3, dsRNA binding protein,	(Basler et al., 2003, Cardenas et al., 2006)
Influenza Virus	NS1	dsRNA binding protein	(Garcia-Sastre et al., 1998)
	Not known	Inactivation of PKR	(Melville et al., 1999)
Rabies Virus	P protein	Blocks phosphorylation of IRF3, JAK-STAT blocker	(Chelbi-Alix et al., 2006)
Reovirus	σ3	dsRNA binding protein	(Miller and Samuel, 1992)
Rotavirus	NSP1	Degrades IRF3 and IRF7	(Barro and Patton, 2007, Graff et al., 2007)
Simian Virus 5	V Protein	Degradation of STAT1	(Didcock et al., 1999)

NSP1 has also been shown to inhibit the expression of IFN-β by degrading β-TrCP, subsequently inhibiting the activation of NF-κB. This targeting of the NF-κB pathway by NSP1 has only been observed in the porcine rotavirus OSU (Graff et al., 2007). Apart from IRF degradation, evidence also exists that NSP1 down regulates the RNA sensitive retinoic acid-induced gene (RIG-I) (Qin et al., 2011). However, there is also some evidence indicating that NSP1 is not crucial for rotavirus replication. Rotavirus variants have been isolated with defective NSP1 proteins (C-truncated forms) which were still able to undergo normal viral replication *in vitro* (Taniguchi et al., 1996). siRNA suppression of genome segment 5 (NSP1) also had no detectable adverse effect on either gene synthesis, expression of viral proteins or assembly of the virion (Silvestri et al., 2004). Although NSP1's zinc-binding domain is well conserved among rotavirus variants, this viral protein in its entirety is the most varied protein among all rotavirus strains (Hua et al., 1994). Due to the affinity of NSP1 for multiple

interferon regulatory factors (IRF3 and IRF7), NSP1 is seen as a broad spectrum antagonist of type I IFN (IFNα and IFNβ) (Barro and Patton, 2007). In spite of the ability of NSP1 to orchestrate the degradation of IRFs, the expression of the type I IFNs, IFN-α and IFN-β, are still observed in significant quantities during rotavirus infections (Holloway and Coulson, 2013). It seems that NSP1 is incapable (or purposefully unwilling) to completely prevent the expression of type I IFNs.

In ***Chapter 5*** (***Table 5.1 and Table 5.4***) it was observed that transfecting certain individual rotavirus Wa plasmids or plasmid combinations led to pre-mature cell death in comparison to the transfection control. It was also noticed that rotavirus transcripts cause mass cell death short after transfection and that this cell death is delayed in the presence of certain viral proteins. In this chapter, the innate immune reaction to rotavirus transcripts and proteins will be investigated and the potential of specific plasmid derived rotavirus proteins to supress the interferon response provoked by rotavirus mRNA will also be examined. In excess of 2000 qRT-PCR samples were analysed during the course of this study in order to examine the interferon response to plasmid expressed rotavirus proteins and transfected transcripts.

6.2 Materials and methods

6.2.1 Cells, plasmids and transcripts

HEK 293H cells were chosen for the innate immune experiments due to their high transfection efficiency and compatibility with commercially available qRT-PCR human gene expression probes. MA104 cells were also tested but were found to be incompatible with the RT-PCR probes. Cells were propagated in 6-well plates as described in ***Chapter 4, section 4.2.1.1***. The rotavirus Wa-genome segment containing plasmids described in ***Chapter 5, section 5.2.1*****,** were utilized. Rotavirus SA11 transcripts were obtained from *in vitro* transcription as described in ***chapter 5, sections 5.2.7*****.**

6.2.2 In vitro transcription of GLYAT and RVFV mRNA

In order to determine if other *in vitro* derived transcripts elicit the same potent innate response as rotavirus transcripts, a human metabolic enzyme, human glycine N-acyl tranferase (GLYAT), and segments S and M of another RNA virus, Rift Valley fever virus (RVFV), were *in vitro* transcribed. The cDNA of glycine N-acyl transferase (GLYAT) and the S- and M segments of Rift Valley fever virus (RVFV) were used as templates for *in vitro* transcription. Plasmids containing the S- and M segments of RVFV were a kind gift from Prof. Christiaan Potgieter (Deltamune, South Africa) and GLYAT containing plasmids were a donation from Mr. Chris Badenhorst (NWU, South Africa). These plasmids were linearised with the appropriate restriction enzymes and used as a template for transcription. The DNA was then purified using the Qiagen Gel Extraction Kit (Qiagen) in accordance with the manufacturer's instructions. The purity and concentration of the excised DNA was determined using a NanoDrop® 1000 spectrophotometer. The mMESSAGE mMACHINE® T7 Ultra kit (Ambion®) was used for the *in vitro* transcription of the linearised cDNA as prescribed by the manufacturer.

Genome segments were transcribed individually in a 20 µl reaction. The *in vitro* transcription reaction was made up of T7 NTP/ARCA mixture (1X), T7 reaction buffer (5X) and T7 enzyme mix(0.5X) and 1µg of DNA template. After the removal of the DNA template with DNase (Ambion®), the transcripts were purified using a MEGAclear™ RNA purification kit (Ambion®) according to the manufacturer's instructions. The capping efficacy of the mMESSAGE mMACHINE T7 Ultra kit is between 70- 80%. Additional transcript capping was performed by the ScriptCap™ m7G capping system (Epicentre Biotechnologies). The capping reaction was done in a total volume of 20 µl and contained 1 µg of transcripts, capping buffer (1X), 1 mM GTP, 0.01 mM S-adenosyl methionine (SAM), 2 U/µl RNase inhibitor and 10 U capping enzyme. This additional capping reaction was performed at 37°C for 1 hour. After the capping procedure the transcripts were again purified with the MEGAclear™ kit.

6.2.3 Transfection of HEK 293H cells and quantitative RT-PCR

HEK 293H cells were grown to 70-80% confluence in 6-well plates (Nunc™) followed by transfection using XtremeGENE HP (Roche) as described in ***Chapter 4 (section 4.1.2.1)***. HEK

293H cells were transfected with 4 µg (4 µg per well of a 6-well plate) of either the pUC57 plasmids containing the individual rotavirus Wa genome segments, 4 µg of the pSMART plasmids containing the rotavirus SA11 cassettes or 0.5 µg of the rotavirus SA11 transcripts (mRNA). All experiments were done at least in triplicate. Depending on the type of transfection, cells were incubated for different time periods at 37°C in 5% CO_2. The relative expression of VP6 was used as a standard when determining the incubation times. The expression of VP6 from transcripts after 10 hours was generally comparable to the expression of plasmid derived VP6 after 24 hours (results not shown). On the other hand, the expression of VP6 from transcripts after 10 hours can be compared to the expression of VP6 during rotavirus SA11 infection (MOI of ~0.1) after 20 hours. The multiplicity of infection was adapted to match the expression levels of 0.5 µg of SA11 transcripts. After incubation, HEK 293H cells were harvested by scraping. The cells from duplicate wells were then pooled to a final volume of ~4 ml and total RNA was extracted using TRI-Reagent LS (Molecular Research Centre). cDNA was synthesised using the High Capacity RNA to cDNA kit (Life Technologies). The protocol was adapted from the instructions of the manufacturer. A reaction mixture was prepared containing 2 µl 10x RT buffer, 2 µl 10x random primers, 1 µl dNTP mix, 1 µl reverse transcriptase, 1 µl RNase inhibiter and 3 µl nuclease free water per reaction. This reaction mixture is thoroughly mixed and placed on ice. At least 1 µg of total RNA was required for a successful reverse transcriptase reaction. The RNA mixture contained at least 1 ug of RNA and was made up to 10 µl with nuclease free water. The reaction mixture and the RNA mixture were then mixed to obtain a final volume of 20 µl. The cDNA synthesis reaction was incubated at 25 °C for 10 minutes followed by 120 minutes at 37 °C and finally the enzyme was deactivated at 85°C for 5 minutes using a thermocycler (Biorad). PCR amplification of cDNA was performed using a TaqMan Gene Expression Master Mix buffer and commercial probes. It should be noted that the TaqMan Gene Expression Master Mix provides much better cDNA amplification than the TaqMan Universal II Master Mix buffer. The selected probes were *IFN-α1* (Hs00256882_s1), *IFN-1β* (Hs00277188_sl), *IFN-λ1* (assay ID: Hs00601677_gl), IFN-inducible *CXCL10* (Hs00171042_ml), *TNF-α* (Hs00174128_ml), Th1-type *IL12 p40* (Hs00233688_ml), anti-inflammatory *IL10* (Hs00961622m1) and the receptor interacting protein 1 (*RIP1*) kinase (Hs00169407_m1). All the TaqMan assays were purchased from Applied Biosystems (Life Technologies). Four independent quantitative PCR experiments were done, each at least performed in triplicate.

Analysis were done on a Applied Biosystem 7500 thermo cycler and relative quantities of specific cytokine's RNA was normalised to 18S rRNA (Applied Biosystems) using the $2^{-\Delta\Delta C}{}_{T}$ method (Kuchipudi et al., 2012). When comparing cytokine expression levels, the differences in CT values are the log2 of the relative starting concentrations of the 18S rRNA internal standard control.

6.2.4 Detection of the expression of RIG-I and MDA5 by flow cytometry

For flow cytometry, HEK 293H cells were transfected with pUC57 plasmids (4 µg) encoding either NSP1, NSP2, NSP5, VP1, VP6 individually or a combination of NSP2 and NSP5 (viroplasm-like structure) and incubated at 37°C. After 24 hours, a second transfection of 0.5 µg *in vitro* derived rotavirus SA11 transcripts was performed. HEK 293H cells also undergone a single transfection of rotavirus SA11 transcripts (0.5 µg) or were infected with rotavirus SA11 (MOI ~0.1). A mock transfection, wherein all the conditions were the same exept for the absence of any genomic material, was used as a control. HEK 293H cells transfected with rotavirus SA11 dsRNA served as a positive control. After 24 hours, cells were fixed using 4% paraformaldehyde (Merck) for 30 minutes at room temperature. Cells were then permeabilised by 0.25% Triton X-100 for 10 minutes at room temperature (Merck). The HRP/DAB detection kit (Abcam) was used to prepare cells for immunostaining. The primary antibodies (rabbit polyclonal RIG-I (H-300) (Santa Cruz) antibodies and mouse polyclonal MDA5 (C-5) (Santa Cruz) antibodies) were diluted 1:200 in PBS and incubated at room temperature for 2 hours. Cells were thereafter treated with the FITC (MDA5) (Santa Cruz) or PE (RIG-I) (Santa Cruz) secondary antibodies. The expression of RIG-I and MDA5 was determined by flow cytometry (FACSCalibur, BD Biosciences). The data was analysed by FCSExpress (version 4, De Novo Software).

6.2.5 Determining the effect pre-expressed plasmid derived rotavirus proteins have on the interferon response elicited by rotavirus transcripts

HEK 293H cells were transfected with pUC57 plasmids (4 µg) containing either the NSP1, NSP2, NSP5, VP1, VP6 or a combination of NSP2 and NSP5 (viroplasm-like structure) genome segments and incubate at 37°C. After 24 hours, a second transfection of 0.5 µg *in vitro* derived rotavirus SA11 transcripts was performed. Cells were harvested after 20 hours and

total RNA was isolated as described in ***Chapter 3 (section 3.2.2)***. cDNA was synthesized using the High Capacity RNA-to-cDNA master mix (Life Technologies) as prescribed by the manufacturer. Comparisons were done by determining the expression levels of selected cytokines using qRT-PCR with TaqMan® Gene Expression Assays (Applied Biosystems) as described in ***section 6.2.3***.

6.2.6 Statistical analysis of qRT-PCR data

The bulk of the statistical analysis was performed with the GraphPad Prism 6 software suit. qRT-PCR values were the arithmetic mean of four independent experiments (infections/transfections), done in triplicate, unless otherwise stated. Sample sizes appropriate for reliable statistical analysis were calculated with the 7500 software v2.0.6 (Applied Biosystems). Standard errors of the mean $2^{-\Delta\Delta C}{}_{\mathrm{T}}$ values were calculated for all samples. Statistically significant expression differences were calculated using the one-way ANOVA and multiple comparison methods calculated by the GraphPad Prism 6 software (***see Appendix F for analysed data***).

6.3 Results and Discussion

6.3.1 Comparison of the effect of rotavirus transcripts and plasmids encoding for rotavirus proteins on HEK 293H, MA104, BSR and COS-7 cells

The effect of transfecting rotavirus transcripts and plasmids encoding for rotavirus proteins on mammalian cells was investigated. HEK 293H, MA104 BSR and COS-7 cells were transfected with 4 µg of either pUC57 plasmids containing the 11 rotavirus Wa genome segments, pSMART plasmids containing the 11 rotavirus SA11 genome segments or the rotavirus SA11 transcripts. Numerous pilot transfections were performed in MA104, HEK 293H, BSR, COS-7 and co-cultured MA104/BSR, MA104/HEK 293H and MA104/COS-7 cells under different conditions to determine the best transfection conditions for rotavirus VP6 expression. Mass cell death was observed within 24 hours after the transfection of 1 µg *in vitro* derived rotavirus transcripts in all cell lines (***Table 6.2***). With a smaller amount (0.5 µg) of SA11 transcripts, cell death was slightly delayed.

Table 6.2: Comparison of cell death following the transfection of rotavirus SA11 transcripts and rotavirus encoding plasmids

Cell Type	Control[#]			0.5 µg SA11 Transcripts			1 µg SA11 Transcripts			4 µg Wa Plasmids			4 µg SA11 Plasmids		
	12h	24h	48h	12h	24h	48h	12h	24h	48h	12h	24h	48h	12h	24h	48h
HEK 293H	-	-	+	++	++++	∞	+++	∞	∞	-	+	+	-	-	+
	(-)	(-)	(-)	(••)	(••)					(•)	(••)	(•••)	(•)	(••)	(••)
MA104	-	-	-	++	+++	++++	+++	++++	∞	-	-	+	-	-	-
	(-)	(-)	(-)	(•)	(•••)	(•••)	(••)	(••)		(•)	(••)	(•••)	(•)	(••)	(•••)
BSR	-	-	+	+++	∞	∞	∞	∞	∞	-	+	++	-	+	++
	(-)	(-)	(-)	(•)						(-)	(•)	(•)	(-)	(•)	(•)
COS-7	-	-	+	+++	++++	∞	++++	∞	∞	-	+	+	-	+	++
	(-)	(-)	(-)	(-)	(•)		(•)			(-)	(•)	(•)	(-)	(-)	(•)
MA104/BSR	-	+	+	+++	∞	∞	∞	∞	∞	-	+	++	-	++	++
	(-)	(-)	(-)	(•)						(-)	(•)	(•)	(-)	(•)	(•)
MA104/HEK	-	+	+	+++	++++	∞	++++	∞	∞	-	+	+	-	+	+
	(-)	(-)	(-)	(•)	(••)		(•)			(-)	(•)	(••)	(•)	(•)	(•)
MA104/COS7	-	+	+	+++	∞	∞	∞	∞	∞	-	+	++	-	+	++
	(-)	(-)	(-)	(•)						(-)	(-)	(•)	(-)	(•)	(•)

[#]A mock transfection was used as a control.

0.5/1µg of SA11 *in vitro* derived transcripts were used for transfection per well of a 6-well plate (VP6 expression are indicated in brackets)The XtremeGENE HP transfection mixture was left on the cells for the duration of the experiment

CPE was gauged by visual inspection

- no/very little CPE
+ 10-20% CPE
++ 20-30% CPE
+++ 30-50% CPE
++++ 50-70% CPE
∞ complete CPE.

(-) no VP6 expression
(•) 10-20% of cells expressing VP6
(••) 20-40% of cells expressing VP6
(•••) >50% of cells expressing VP6
∞ no detectable VP6 expression due to extensive/total CPE

Transfection conditions were standardized with 0.5 µg of SA11 transcripts due to the relatively good expression of VP6 after 10 hours and low CPE (at 20-30% CPE, HEK 293H cells can still be harvested for further quantitative RT-PCR (qRT-PCR) analysis after 10 hours).

6.3.2 The interferon response of HEK 293H cells to rotavirus transcripts and plasmids containing rotavirus genome segments

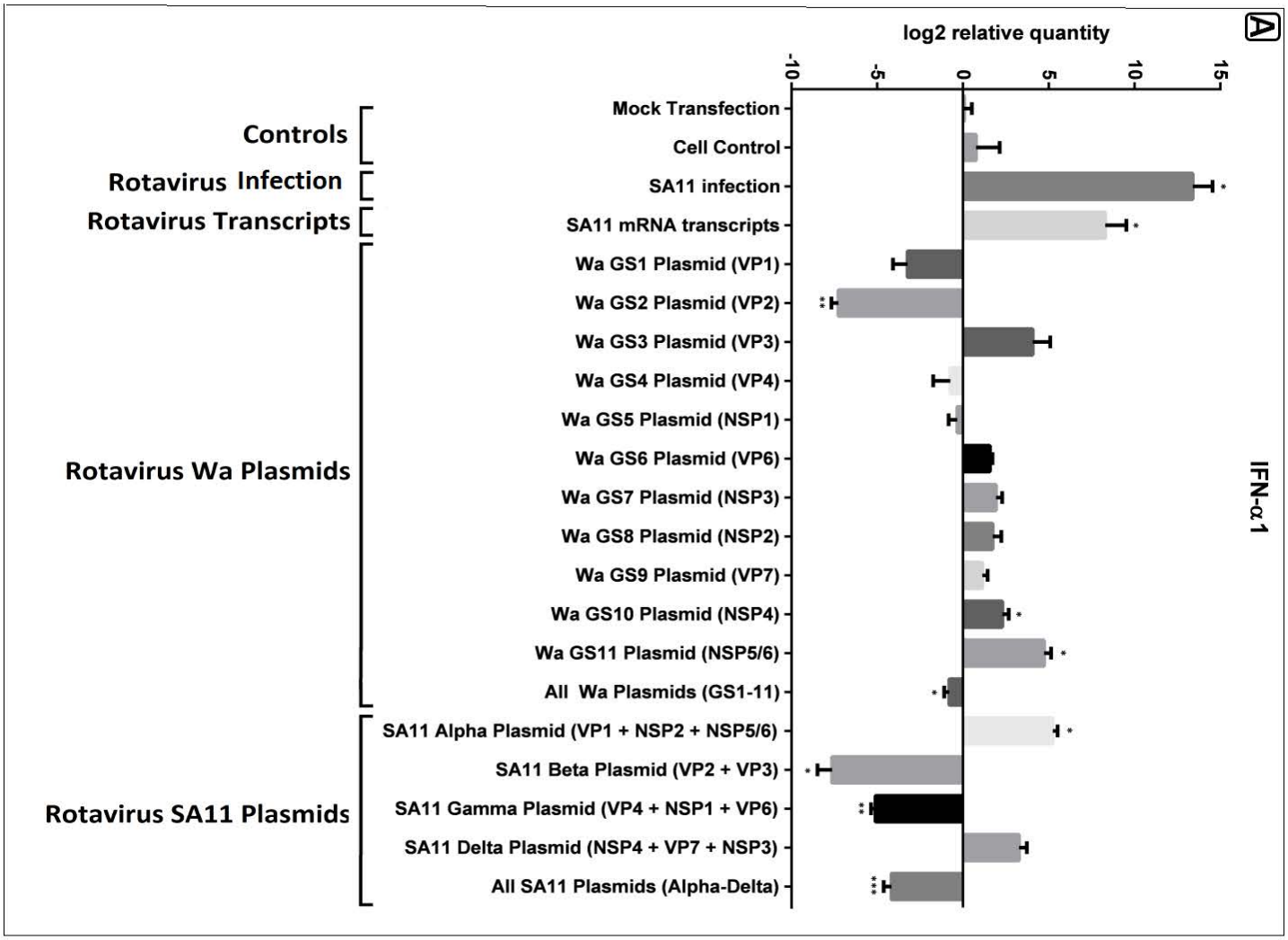
The mechanisms rotavirus use to counter the innate immune system of the host are not completely understood yet. With the lack of a true reverse genetics system for rotavirus, the suppression of the strong innate immune response elicited by rotavirus transcripts (Mlera, 2013, Uzri and Greenberg, 2013) is vital in the quest to better understand this virus's replication cycle and the development of a comprehensive reverse genetics system. The innate immune responses to rotavirus dsRNA and rotavirus particles have been described (Angel et al., 2012, Deal et al., 2010, Frias et al., 2012, Sen et al., 2011). These innate immune studies were primarily focused on the transfection of rotavirus dsRNA and particles into tissue culture or the infection of animals. However, the specific response to individual rotavirus proteins has not been fully characterised. Furthermore, the observation of mass cell death induced by rotavirus transcripts is also not completely understood although it has been documented (Mlera, 2013, Uzri and Greenberg, 2013). Mlera investigated the interferon response to 1.5 µg wild-type rotavirus SA11 mRNA, synthetic rotavirus DS-1 genome segment 6 mRNA and synthetic BTV-1 S3 mRNA (Mlera, 2012). When transfecting 1.5 µg of SA11 transcripts in this current study, mass cell death occurred within 10 hours and qRT-PCR analysis was not possible. For this study, 0.5 µg in vitro DLP-derived SA11 transcripts were transfected. In the previous study, relative quantities of cytokine RNA were normalised to β-actin (Mlera, 2013) which is known to be under expressed in kidney cells (Thellin et al., 1999, Zhu and Altmann, 2005) and the more suitable housekeeping gene, 18S rRNA, were used in this study. Analysis with the software *BestKeeper* and *NormFinder* found that 18S rRNA was the most stable and suitable housekeeping gene for RT-PCR in influenza virus infected cells (Kuchipudi et al., 2012). Observations following the transfection of plasmids containing rotavirus genome segments and rotavirus mRNA transcripts (***chapter 5***) stimulated this investigation into the

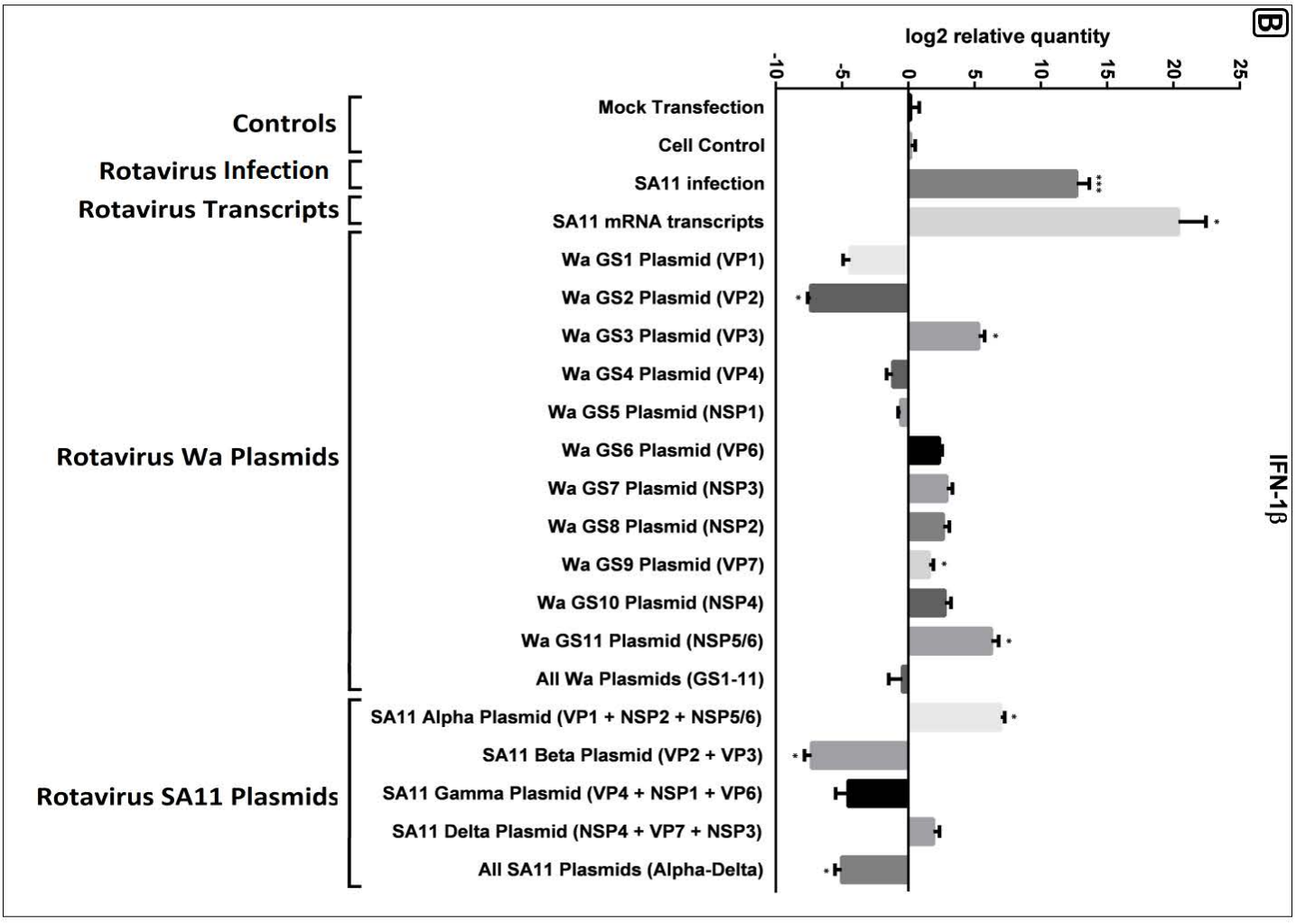
identification of the specific role players involved in this innate immune response. In the case of most viral infections, the secretion of cytokines belonging to the interferon (IFN) family (IFN type I and III in particular) play an important part in this innate immune response by activating the expression of IFN-stimulated genes (Arnold and Patton, 2011, Holloway and Coulson, 2013). The expression products of these IFN-stimulated genes are able to directly inhibit viral replication (Edinger and Thompson, 2004, Levy et al., 2011, Stetson and Medzhitov, 2006). Since the *in vitro* innate immune response to plasmid-derived expression of rotavirus genome segments has not been completely described, this section will also examine the expression of different cytokines (IFN-α, IFN-β, IFN-λ1, IFN-inducible CXCL10, *TNF-α,* and *RIP1* kinase) in HEK 293H cells following the transfection of a variety of expression plasmids containing cDNAs of individual rotavirus genome segments. MA104 cells are the cell-line of choice for rotavirus propagation. It was clear that MA104 cells are best suited for expressing VP6 (***Table 6.2***). However, in pilot quantitative RT-PCR experiments, no amplification of the targeted genes could be detected in MA104 cells. The commercial probes were designed for human genes and cannot be applied to rhesus monkey kidney cells. COS-7 and BSR cells can support the replication of Wa and SA11 rotaviruses. Both cell lines can be efficiently transfected and showed moderate VP6 expression. Unfortunately, these cell lines cannot be used with human TaqMan probes, either.

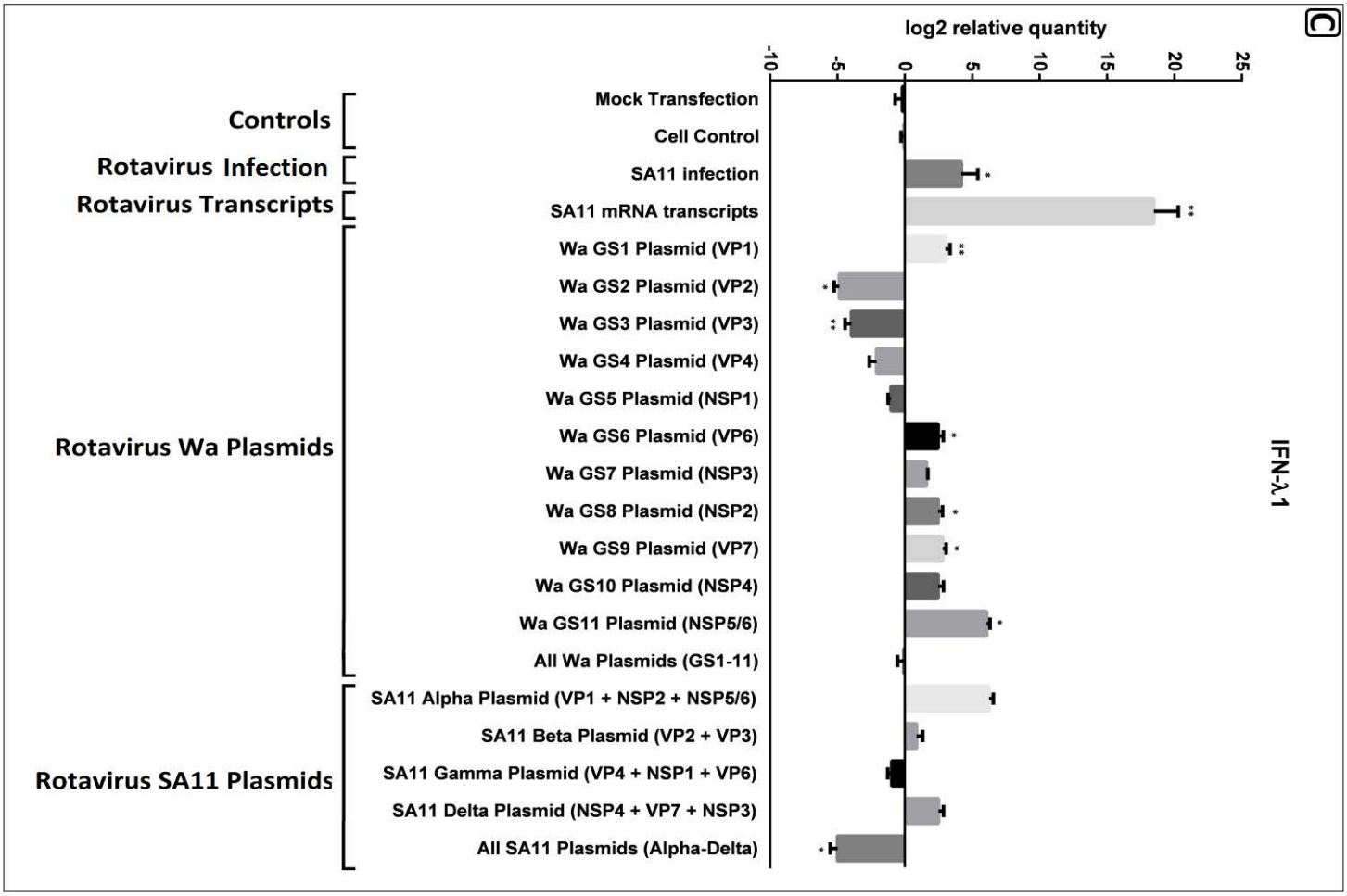
6.3.2.1 Cytokine response to rotavirus SA11 infection

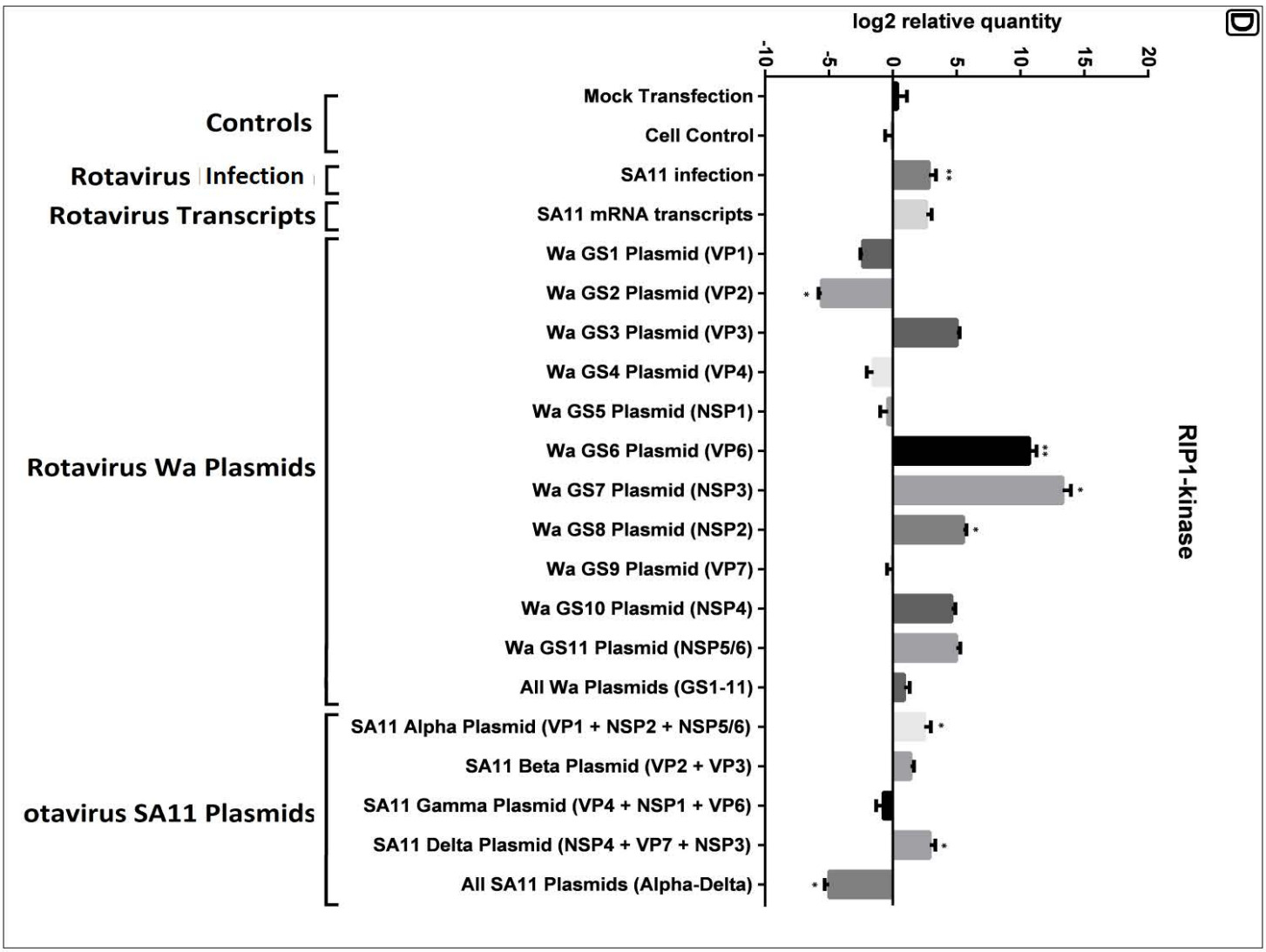
Expression of IFN-α1 and IFN-1β was both strongly induced by rotavirus SA11 infection after 20 hours (***Figure 6.1 A, B***) (significance = P value < 0.05 of a specific sample compared to the mock transfection which acts as the control - please see ***Appendix F*** for the multiple comparison test results and adjusted P values). The innate immune response to a rotavirus infection is dependent on the host cell type and specific rotavirus strain (Frias et al., 2012, Sen et al., 2011). Rotavirus SA11 infection stimulated a strong IFN type I (IFN-α; IFN-β) response in HEK 293H cells. It seems that an antiviral state was induced within 20 hours after infection. Extensive cell death, most likely apoptosis (Mlera, L. unpublished data), was observed after 30 hours. A moderate increase in expression of just over 9-fold was observed in CXCL10 (***Figure 6.1 F***). CXCL10 is an IFN-inducible cytokine which is

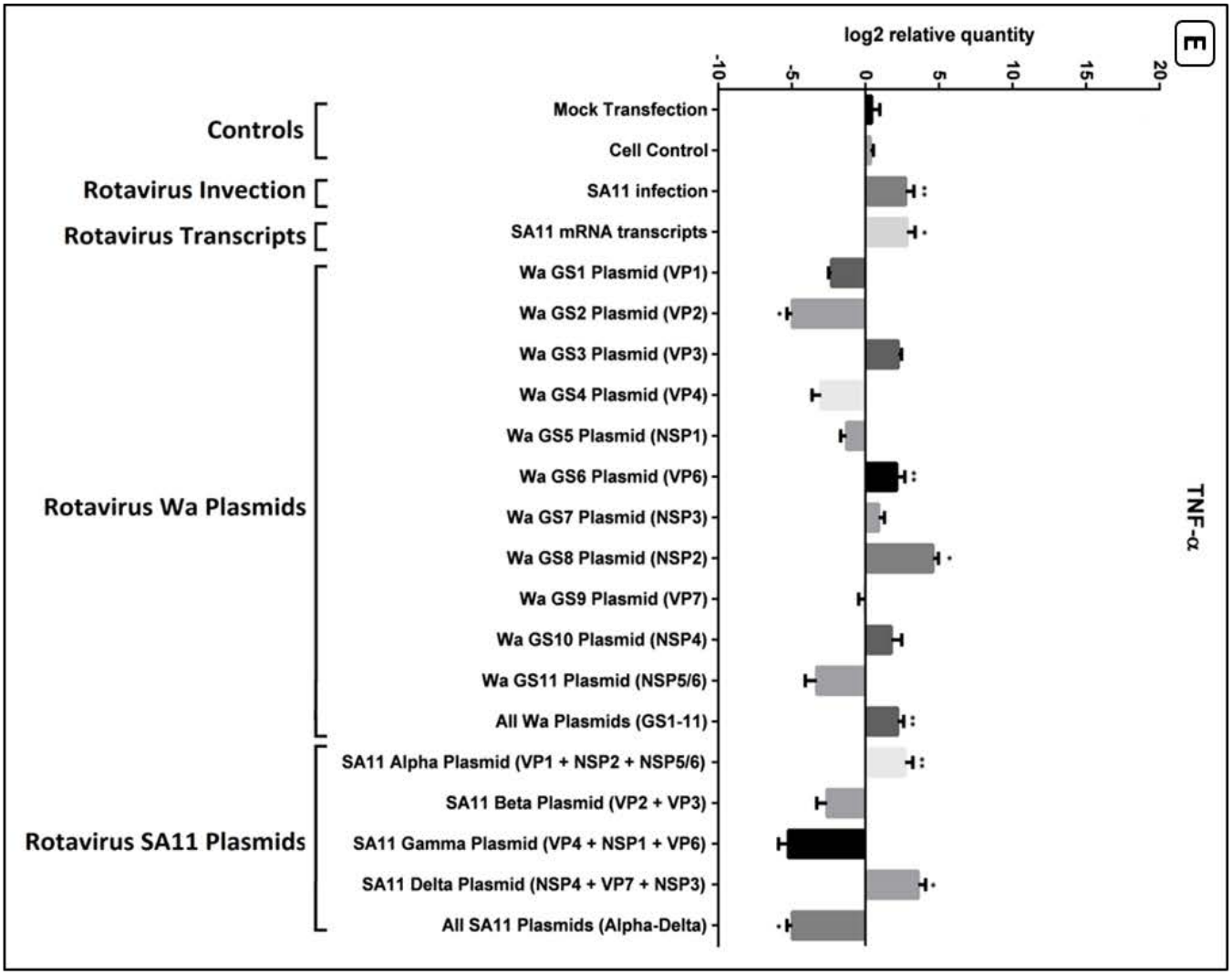
generally associated with anti-cell proliferation activity (Campanella et al., 2010). TNF-α (***Figure 6.1 E***) has potent antiviral activities and acts as a cytotoxic cytokine which can contribute to cell injury and death (Horiuchi et al., 2010, Ramshaw et al., 1997). RIP1 kinase (***Figure 6.1 D***) was tested due to its ability to act as an activator of necroptotic cell death which is a specialized pathway of programmed necrosis (Galluzzi and Kroemer, 2008). The expression activation of TNF-α and RIP1 kinase was relatively low with both exhibiting a smaller than 3-fold expression increase (***Figure 6.1 D, E***). Interestingly, the induction of IFN-λ1 (***Figure 6.1 C***) by rotavirus SA11 infection was relatively low (~4-fold increase) in comparison to that of IFN-α1 (~14-fold increase) and IFN-1β (~20-fold increase). ***Table 6.3*** summarises the effects different plasmids encoding rotavirus Wa and SA11 viral proteins have on cytokine mRNA expression/suppression induced in HEK 293H cells.


A
log2 relative quantity
-10
-5
0
5
10
15
Mock Transfection
Cell Control
SA11 infection
SA11 mRNA transcripts
Wa GS1 Plasmid (VP1)
Wa GS2 Plasmid (VP2)
Wa GS3 Plasmid (VP3)
Wa GS4 Plasmid (VP4)
Wa GS5 Plasmid (NSP1)
Wa GS6 Plasmid (VP6)
Wa GS7 Plasmid (NSP3)
Wa GS8 Plasmid (NSP2)
Wa GS9 Plasmid (VP7)
Wa GS10 Plasmid (NSP4)
Wa GS11 Plasmid (NSP5/6)
All Wa Plasmids (GS1-11)
SA11 Alpha Plasmid (VP1 + NSP2 + NSP5/6)
SA11 Beta Plasmid (VP2 + VP3)
SA11 Gamma Plasmid (VP4 + NSP1 + VP6)
SA11 Delta Plasmid (NSP4 + VP7 + NSP3)
All SA11 Plasmids (Alpha-Delta)
Controls
Rotavirus Infection
Rotavirus Transcripts
Rotavirus Wa Plasmids
Rotavirus SA11 Plasmids
IFN-α1


B
log2 relative quantity
-10
-5
0
5
10
15
20
25
Mock Transfection
Cell Control
SA11 infection
SA11 mRNA transcripts
Wa GS1 Plasmid (VP1)
Wa GS2 Plasmid (VP2)
Wa GS3 Plasmid (VP3)
Wa GS4 Plasmid (VP4)
Wa GS5 Plasmid (NSP1)
Wa GS6 Plasmid (VP6)
Wa GS7 Plasmid (NSP3)
Wa GS8 Plasmid (NSP2)
Wa GS9 Plasmid (VP7)
Wa GS10 Plasmid (NSP4)
Wa GS11 Plasmid (NSP5/6)
All Wa Plasmids (GS1-11)
SA11 Alpha Plasmid (VP1 + NSP2 + NSP5/6)
SA11 Beta Plasmid (VP2 + VP3)
SA11 Gamma Plasmid (VP4 + NSP1 + VP6)
SA11 Delta Plasmid (NSP4 + VP7 + NSP3)
All SA11 Plasmids (Alpha-Delta)
Controls
Rotavirus Infection
Rotavirus Transcripts
Rotavirus Wa Plasmids
Rotavirus SA11 Plasmids
IFN-1β


C
log2 relative quantity
-10
-5
0
5
10
15
20
25
Controls
Rotavirus Infection
Rotavirus Transcripts
Rotavirus Wa Plasmids
Rotavirus SA11 Plasmids
Mock Transfection
Cell Control
SA11 infection
SA11 mRNA transcripts
Wa GS1 Plasmid (VP1)
Wa GS2 Plasmid (VP2)
Wa GS3 Plasmid (VP3)
Wa GS4 Plasmid (VP4)
Wa GS5 Plasmid (NSP1)
Wa GS6 Plasmid (VP6)
Wa GS7 Plasmid (NSP3)
Wa GS8 Plasmid (NSP2)
Wa GS9 Plasmid (VP7)
Wa GS10 Plasmid (NSP4)
Wa GS11 Plasmid (NSP5/6)
All Wa Plasmids (GS1-11)
SA11 Alpha Plasmid (VP1 + NSP2 + NSP5/6)
SA11 Beta Plasmid (VP2 + VP3)
SA11 Gamma Plasmid (VP4 + NSP1 + VP6)
SA11 Delta Plasmid (NSP4 + VP7 + NSP3)
All SA11 Plasmids (Alpha-Delta)
IFN-λ1


D
log2 relative quantity
-10
-5
0
5
10
15
20
Controls
Rotavirus Infection
Rotavirus Transcripts
Rotavirus Wa Plasmids
otavirus SA11 Plasmids
Mock Transfection
Cell Control
SA11 infection
SA11 mRNA transcripts
Wa GS1 Plasmid (VP1)
Wa GS2 Plasmid (VP2)
Wa GS3 Plasmid (VP3)
Wa GS4 Plasmid (VP4)
Wa GS5 Plasmid (NSP1)
Wa GS6 Plasmid (VP6)
Wa GS7 Plasmid (NSP3)
Wa GS8 Plasmid (NSP2)
Wa GS9 Plasmid (VP7)
Wa GS10 Plasmid (NSP4)
Wa GS11 Plasmid (NSP5/6)
All Wa Plasmids (GS1-11)
SA11 Alpha Plasmid (VP1 + NSP2 + NSP5/6)
SA11 Beta Plasmid (VP2 + VP3)
SA11 Gamma Plasmid (VP4 + NSP1 + VP6)
SA11 Delta Plasmid (NSP4 + VP7 + NSP3)
All SA11 Plasmids (Alpha-Delta)
RIP1-kinase


E
log2 relative quantity
-10
-5
0
5
10
15
20
Controls
Rotavirus Invection
Rotavirus Transcripts
Rotavirus Wa Plasmids
Rotavirus SA11 Plasmids
Mock Transfection
Cell Control
SA11 infection
SA11 mRNA transcripts
Wa GS1 Plasmid (VP1)
Wa GS2 Plasmid (VP2)
Wa GS3 Plasmid (VP3)
Wa GS4 Plasmid (VP4)
Wa GS5 Plasmid (NSP1)
Wa GS6 Plasmid (VP6)
Wa GS7 Plasmid (NSP3)
Wa GS8 Plasmid (NSP2)
Wa GS9 Plasmid (VP7)
Wa GS10 Plasmid (NSP4)
Wa GS11 Plasmid (NSP5/6)
All Wa Plasmids (GS1-11)
SA11 Alpha Plasmid (VP1 + NSP2 + NSP5/6)
SA11 Beta Plasmid (VP2 + VP3)
SA11 Gamma Plasmid (VP4 + NSP1 + VP6)
SA11 Delta Plasmid (NSP4 + VP7 + NSP3)
All SA11 Plasmids (Alpha-Delta)
TNF-α

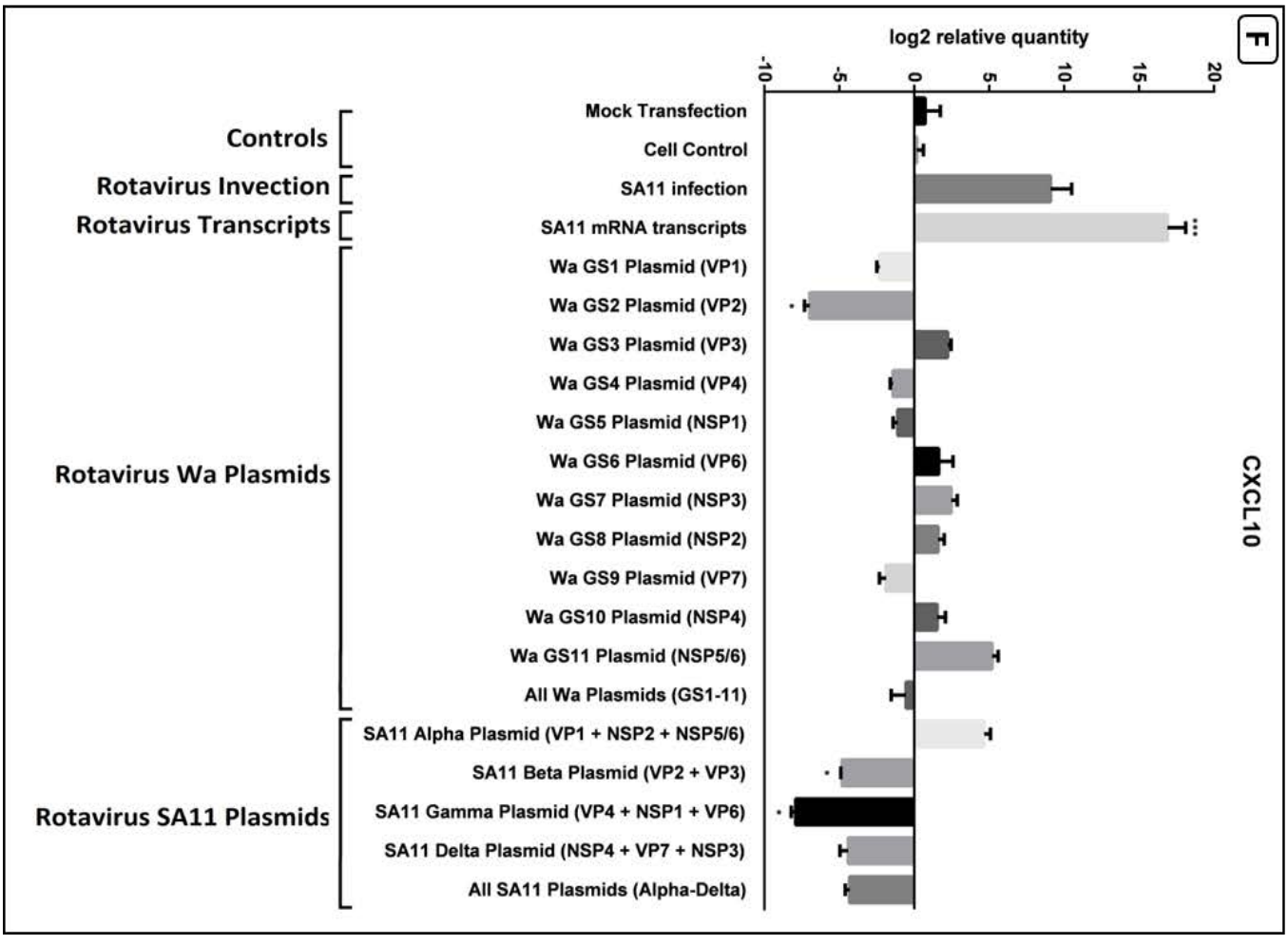

F
log2 relative quantity
-10
-5
0
5
10
15
20
Controls
Rotavirus Invection
Rotavirus Transcripts
Rotavirus Wa Plasmids
Rotavirus SA11 Plasmids
Mock Transfection
Cell Control
SA11 infection
SA11 mRNA transcripts
Wa GS1 Plasmid (VP1)
Wa GS2 Plasmid (VP2)
Wa GS3 Plasmid (VP3)
Wa GS4 Plasmid (VP4)
Wa GS5 Plasmid (NSP1)
Wa GS6 Plasmid (VP6)
Wa GS7 Plasmid (NSP3)
Wa GS8 Plasmid (NSP2)
Wa GS9 Plasmid (VP7)
Wa GS10 Plasmid (NSP4)
Wa GS11 Plasmid (NSP5/6)
All Wa Plasmids (GS1-11)
SA11 Alpha Plasmid (VP1 + NSP2 + NSP5/6)
SA11 Beta Plasmid (VP2 + VP3)
SA11 Gamma Plasmid (VP4 + NSP1 + VP6)
SA11 Delta Plasmid (NSP4 + VP7 + NSP3)
All SA11 Plasmids (Alpha-Delta)
CXCL10

Figure 6.1: ***The log_2 relative quantities of cytokine-encoding mRNA expression induced in HEK 293H cells following rotavirus infection and transfection of rotavirus SA11 transcripts and plasmids encoding rotavirus Wa and SA11 containing plasmids.*** *Graphs* ***A–F*** *indicate the effect of rotavirus infection and transfecting different rotavirus SA11 transcripts rotavirus Wa and SA11 plasmids on the induction of IFNα1 (A), IFN-1β (B), IFN-λ1 (C), RIP1-Kinase (D), TNF-α (E) and CXCL10 (F) in HEK 293H cells. A mock transfection serves as a control and a normal cell control (Untransfected/uninfected cells) are also included. Values were the arithmetic mean of four independent experiments (infections/ transfections), in triplicate, and bars indicate the standard error of the mean. Statistically significant expression differences are indicated by stars (*P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001).*

Table 6.3: Cytokine-encoding mRNA expression/suppression induced in HEK 293H cells following rotavirus infection and transfection of rotavirus SA11 transcripts and plasmids encoding rotavirus Wa and SA11 containing plasmids

	IFNα1	IFN-1β	IFN-λ1	RIP1-Kinase	TNF-α	CXCL10
Control[a]	-	-	-	-	-	-
RV SA11 Infection[b]	▲	▲	▲	▲	▲	-
RV SA11 Transcripts[c]	▲	▲	▲	-	▲	▲
RV-Wa GS1 (VP1)	-	-	▼	-	-	-
RV-Wa GS2 (VP2)	▼	▼	▼	▼	▼	▼
RV-Wa GS3 (VP3)	-	▲	▼	-	-	-
RV-Wa GS4 (VP4)	-	-	-	-	-	-
RV-Wa GS5 (NSP1)	-	-	-	-	-	-
RV-Wa GS6 (VP6)	-	-	▲	▲	▲	-
RV-Wa GS7 (NSP3)	-	-	-	▲	-	-
RV-Wa GS8 (NSP2)	-	-	▲	▲	▲	-
RV-Wa G9 (VP7)	-	▲	▲	-	-	-
RV-Wa GS10 (NSP4)	▲	-	-	-	-	-
RV-Wa GS11 (NSP5/6)	▲	▲	▲	-	-	-
RV-Wa All plasmids (GS1-11)	▼	-	-	-	▲	-
RV SA11 Alpha (VP1, NSP2/5/6)	▲	▲	-	▲	▲	-
RV SA11 Beta (VP2/3)	▼	▼	-	-	-	▼
RV SA11 Gamma (VP4/6, NSP1)	▼	-	-	-	-	▼
RV SA11 Delta (VP7, NSP3/4)	-	-	-	▲	▲	-
RV SA11 All plasmids (Alpha-Delta)	▼	▼	▼	▼	▼	-

[a] A mock transfection was used as a controll.
[b] Cells were infected with a MOI of ~0.1 of rotavirus SA11 that were comparable to the expression of VP6 from 0.5 µg of SA11 *in vitro* derived transcripts.
[c] 0.5 µg of SA11 *in vitro* derived transcripts were used for transfection
- No statistical significant change in gene expression
▲ Statistical significant increase in gene expression
▼ Statistical significant change in gene suppression

This weak response may be attributed to the cell type (human embryonic kidney cells) used in this study. The type III IFN response was weak or not observed in intestinal epithelial cells for which a strong IFN type I (IFN-α; IFN-β) response was reported (Frias et al., 2012). On the other hand, type III IFN response was strongly induced in neonatal gnotobiotic piglets (Gonzalez et al., 2010). Therefore, HEK 293H cells may have a physiologically weak type III IFN response to rotavirus SA11 infections.

6.3.2.2 Cytokine response to rotavirus SA11 transcripts

In vitro derived rotavirus SA11 mRNA induced significantly high expression levels of IFN-α1, IFN-1β, IFN-λ1 and IFN-inducible CXCL10 (***Figure 6.1 A, B, C and F, Table 6.3***) which confirmed results previously obtained in our laboratory (Mlera, 2012)**.** The relative gene expression of IFN-α1-encoding mRNA induced by rotavirus DLP-derived transcripts was up-regulated nearly 10x in comparison to the control. The induced expression levels of mRNA encoding for IFN-1β and IFN-λ1 increased 20x after exposure to SA11 mRNA, while CXCL10 induction increased almost 17-fold (***Figure 6.1 B, C and F, Table 6.3***). On the other hand, TNF-α and RIP1 expression induction was much less prominent and both cytokines exhibited a less than 3-fold increase after transfection with rotavirus transcripts. Mass cell death in HEK 293H cells was observed 24 hours after transfecting with 0.5 μg of SA11 transcripts (***Table 6.2).*** It is clear from the qRT-PCR results that rotavirus transcripts are a strong inducer of IFN type I and III responses. It must be noted that, in contrast to rotavirus infection, the transfection of rotavirus transcripts also elicited a strong IFN-λ1 response (***Figure 6.1 C, Table 6.3***). This may be due to the abundance of ssRNA fragments interacting with toll like receptors (TLR), especially TLR7 and TLR8, thus triggering a stronger IFN-λ1 response. Plus-sense ssRNA transcripts may also fold onto themselves creating secondary structures which could perhaps be mistaken by MDA5/RIG-I as dsRNA (Holloway and Coulson, 2013, Li et al., 2010). With the additional activation of the pathways induced by TLR7/8 and/or MDA5/RIG-I, expression of IFN-λ1 may be more prominent in cells transfected with rotavirus mRNA than observed in rotavirus infection.

6.3.2.3 Cytokine response to rotavirus Wa encoding plasmids

In ***Chapter 5, section 5.3.1***, expression of 4 for the 11 rotavirus proteins was confirmed. It was assumed that all viral proteins are expressed and that the effects observed are due to these proteins. HEK 293H cells were transfected with different rotavirus Wa expression plasmids in order to examine the effect of individually expressed viral proteins on the innate immune response. In ***chapter 5*** (***section 5.3.1***) it was observed that transfecting certain individual rotavirus Wa plasmids or plasmid combinations led to pre-mature cell death in comparison to the cell control (***Table 5.3***). The expression of the RNA-dependant RNA-polymerase, VP1, led to the active suppression of IFN-α, IFN-β, CXCL10, TNF-α, and RIP1 kinase (***Figure 6.1 A, B, D, E and ;, Table 6.3***). However, according to statistical analysis, the suppression effect of VP1 was not significant. VP1 has no known innate immune response suppression activity. The plasmid encoding for the inner capsid protein, VP2, also elicited a relatively strong, and significant ($P < 0.05$), ability to suppress all cytokines (IFN-α, IFN-β, IFN-λ1, IFN-inducible CXCL10, *TNF-α,* and *RIP1* kinase) examined (***Figure 6.1 A, B, C, D, E and F; Table 6.3***). As with VP1, VP2 is not a viral protein associated with the innate suppression. VP3, which performs the capping, induced a 4-fold and 5-fold increase in IFN-α and IFN-β expression, respectively (***Figure 6.1 A and B; Table 6.3***). Induction of the other cytokines was low. Mass cell death was observed within a 96 hour period in cells transfected with VP3 (***Table 5.1***), and the induction of IFN-α and IFN-β seems to be sufficient to trigger cell death, likely apoptosis. The spike protein, VP4, exhibited a very mild suppression effect on all cytokines examined. Moderate cell death (20-40% cell death) was observed 96h after the initial transfection of VP4 encoding plasmids in comparison to the cell control (***Table 5.1***). Thus it can be concluded that this observed cell death was not the result of over expression of any the tested cytokines. The rotavirus protein associated with the suppression of the innate immune response, NSP1, only slightly suppressed the expression of IFN-α, IFN-β, IFN-λ1, CXCL10, TNF-α, and RIP1 kinase (***Figure 6.1 A, B, C, E and F; Table 6.3***). Statistical analysis indicates that the cytokine suppression by NSP1 was not significant. This is a peculiar result due to the fact that NSP1 has been shown to suppress the type I IFN response by binding IRF3, IRF5 and IRF7 in order to orchestrate these regulatory proteins' degradation by the proteasome (Barro and Patton, 2007, Qin et al., 2011). Plasmid derived NSP1 of a wide range of rotaviruses have also been shown to

degrade IRF7 (Arnold and Patton, 2011). In addition, after 96 hours, transfected NSP1 encoding plasmids seemed to induce cell death in MA104 cells in comparison to the mock transfection (***Table 5.1***). This does not seem to be linked to the activation of any of the tested cytokines and may be to additional mechanical pressures placed on the cell through NSP1 functions. The plasmid middle capsid protein, VP6, and the non-structural protein which make out part of the viroplasm, NSP2, had no significant effect on the expression of the examined cytokines apart from the activation of RIP1 kinase. Neither VP6, nor NSP2 caused any extensive cell death after being transfected (***Table 5.1***) and it does not seem that the increased expression of RIP1 kinase (***Figure 6.1 D; Table 6.3***) had any noticeable biological effect. The non-structural protein responsible for virus specific 3' mRNA binding, NSP3, slightly, but significantly, induced IFN-β and IFN-λ1 expression. The plasmid encoding for NSP3 was also the strongest propagator of RIP1 kinase expression (***Figure 6.1 B and C; Table 6.3***). RIP1 kinase can act as an activator of necroptotic cell death which is a specialized pathway of programmed necrosis (Galluzzi and Kroemer, 2008), and in combination with increased expression of IFN-β and IFN-λ1, may explain the cell death observed at 48 hours after transfection with NSP3 encoding plasmids. It should also be kept in mind that NSP3 is responsible for the shut-down of protein synthesis mechanisms in the host cell, which may also have dire consequences to cell viability. The plasmid encoding for the outer capsid glycoprotein, VP7, significantly induced IFN-λ1 expression (***Figure 6.1 C; Table 6.3***) but had no real effect on any of the other cytokines. The expression of IFN-λ1 may play a role in the cell death that was observed after VP7 transfection. The plasmid encoding for the enterotoxin, NSP4, only significantly induced IFN-α expression (***Figure 6.1 A; Table 6.3***) which may also be responsible for the cell death observed. Interestingly, the plasmid encoding for NSP5 and NSP6, significantly induced IFN-α, IFN-β, IFN-λ1 and RIP1 kinase ***Figure 6.1 A, B, C and D; Table 6.3***). These two non-structural proteins interact with NSP2 to form viroplasm-like structures (Fabbretti et al., 1999). After transfection with NSP5/6 encoding plasmids, cell death can be observed 72 hours after the initial transfection (***Table 5.1***). The relative strong activation of type I and III IFNs (IFN-α, IFN-β, IFN-λ1) in association with RIP1 expression (***Figure 6.1 A, B, C and D; Table 6.3***) kinase may contribute to the observed cell death.

Finally, all 11 rotavirus Wa plasmids were combined in a single transfection. Interestingly, a minor suppression tendency was observed in IFN-α, IFN-β and IFN-λ1, while TNF-α and RIP1 kinase was slightly up regulated. It seems that when all genome segments are combined in a transfection, expression of type I and III IFNs (IFN-α, IFN-β, IFN-λ1) was marginally suppressed. This may suggest that a combination of viral proteins is important for successful suppression of the cellular innate response.

6.3.2.4 Cytokine response to rotavirus SA11 encoding plasmids

The innate response elicited in HEK 293H cells by the rotavirus SA11 plasmid set was also examined. The SA11 expression system described in ***chapter 5, section 5.2.2*** was used for these qRT-PCR experiments. Multiple SA11 genome cassettes were inserted into a single plasmid and the 11 genome segment cassettes were subsequently divided into 4 plasmids (**Alpha**- VP1, NSP2 and NSP5/6; **Beta**- VP2 and VP3; **Gamma**- VP4, NSP1 and VP6; **Delta**- NSP4, VP7 and NSP3) (***Figure 5.3***). Expression of the Alpha induced IFN-α, IFN-β, IFN-λ1, CXCL10, TNF-α, and RIP1 kinase (***Figure 6.1 A, B, C, E and F; Table 6.3***). It seems that the induction was similar to that seen with the rotavirus Wa NSP5/6 expression plasmid experiments. Although the rotavirus Wa VP1-encoding plasmid showed suppression characteristics in some of cytokines examined (IFN-α, IFN-β, CXCL10, TNF-α, and RIP1), it seemed that NSP5/6's induction nullifies the suppression effect completely. It must be stressed that different rotavirus strains have different immune effects and that the immune reaction elicited by the same Wa and SA11 viral protein, may not be directly comparable. The SA11 beta plasmid (VP2 and VP3) significantly supressed IFN-α, IFN-β, and TNF-α expression and appears to be similar to the suppression observed in the plasmid encoding rotavirus Wa VP2. Only IFN-λ1 seemed to be minutely induced by the Beta plasmid (Beta encodes for rotavirus SA11 VP2 and VP3). The largest significant suppression was seen by the expression of the SA11 Gamma plasmid (VP4, NSP1 and VP6). Expression of the Gamma plasmid substantially suppressed the expression of IFN-α, IFN-β, CXCL10 and TNF-α (***Figure 6.1 A, B, E and F; Table 6.3***). This suppression does not seem to follow any of the suppression tendencies of the individual rotavirus Wa proteins (VP4, NSP1 or VP6) examined. Generally, VP4 and VP6 rotavirus Wa encoding plasmids induced cytokine expression, while NSP1 only slightly suppressed cytokine expression. The innate immune

suppression viral protein, NSP1, seems to be more effective in supressing the examined cytokines in combination with VP4 and VP6. The SA11 Delta plasmid (NSP4, VP7 and NSP3) induced most of the cytokines examined (IFN-α, IFN-β, IFN-λ1, TNF-α, and RIP1 kinase) and was generally comparable with the expression of cytokines induced by the plasmid encoding rotavirus Wa NSP4. CXCL10 was the only exception and a significant suppression of this cytokine was observed. When transfecting all four of the SA11 plasmids (Alpha – Delta, encoding for all rotavirus SA11 proteins), an universal and significant suppression of all cytokines was observed. This strengthened the notion, as it was also observed in experiments with the rotavirus Wa plasmid system, that a combination of viral proteins are required for sufficient suppression of the innate immune response in HEK 293H cells.

It must be emphasized that although statistical analysis may give an indication of mathematical significance, it may not necessarily be applicable to biological relevance. In the innate immune response, seemingly insignificant expression levels of a cytokine may activate a cascade of immune reactions that, at the end, may have a substantial effect of the cell. This important difference between statistical significance and biological relevance must therefore be borne in mind when interpreting results (EFSA, 2011).

6.3.3 The interferon response of HEK 293H cells to in vitro *derived transcripts of GLYAT and RVFV*

In order to determine if other *in vitro* derived transcripts elicit the same potent innate response as rotavirus transcripts, a human metabolic enzyme, human glycine N-acyl tranferase (GLYAT), and segments S and M of another RNA virus, Rift Valley fever virus (RVFV), were *in vitro* transcribed. GLYAT is a human detoxification enzyme and would probably not be detected as a foreign transcript by the innate immune system of embryonic kidney cells. RVFV belongs to the Bunyavirus family which is negative single stranded RNA viruses. The RVFV consists of three segments, a small- (S), medium- (M) and large segment (L). Equivalent amounts (0.5 µg) of *in vitro* derived transcripts were transfected into HEK 293H cells and expression of selected cytokines was measured. A mock transfection was used as a control and the relative quantity of the RNA of a specific cytokine was normalised to the 18S rRNA internal standard (Applied Biosystems) using the $2^{-\Delta\Delta C}{}_{T}$ method. Neither

GLYAT mRNA nor mRNA of segments S and M of RVFV significantly induced cytokine-encoding mRNA expression in HEK 293H cells (***Figure 6.2***). Interestingly, transcripts of the S and/or M segments of RVFV did not significantly induce the expression of any of the tested cytokines (***Figure 6.2***). In contrast, rotavirus transcripts drastically induced expression of IFN-1β, IFN-λ1 and CXCL10 (~5000 – 20 000-fold increase in relative quantity) (***Figure 6.2***).

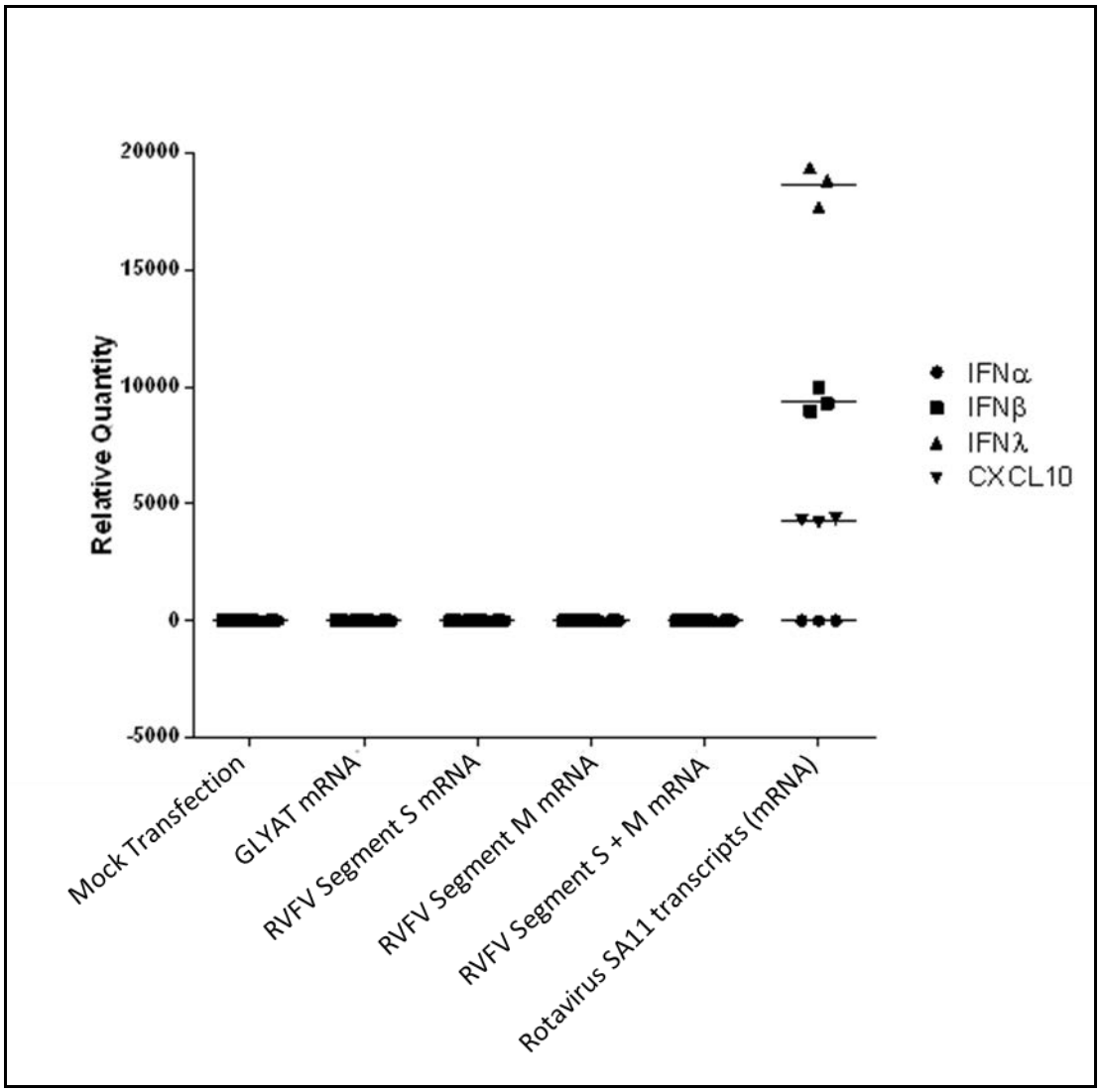


Figure 6.2: ***Comparison of the relative quantities of the expression of various cytokines induced in HEK 293H cells by* in vitro *derived GLYAT-, RVFV- and rotavirus transcripts****. Expression of IFN-α 1, IFN-1β, IFN-λ1 and CXCL10 was determined by qRT-PCR after the transfection of* in vitro*-derived transcripts encoding glycine N-acyl transferase (GLYAT), segments S and M of Rift Valley fever virus (RVFV) and rotavirus. All values are the arithmetic mean and standard deviation of at least three experimental replicates done in triplicate.*

6.3.4 Suppressing the innate immune response elicited by SA11 rotavirus transcripts in HEK 293H cells

As summarised in ***Table 6.2***, mass cell death was observed within 24 hours after the transfection of *in vitro* derived rotavirus transcripts. Transfecting plasmids containing genome segments encoding either NSP1, NSP2 or a combination of NSP2 and NSP5, 24 hours before transfecting rotavirus mRNA transcripts, noticeably reduced and delayed cell death (***Table 5.4, Chapter 5***). Furthermore, gene expression results indicated that cells pre-transfected with the plasmid encoding NSP1 significantly reduced the expression of type I IFNs (IFN-α1, IFN-1β) (***Figure 6.3 A and B***) in addition to decreasing the interferon induced cytokine (CXCL10) expression (***Figure 6.3 D***). Our results indicate that expressing NSP1 can also suppress the IFN-α1, IFN-1β response in cells transfected with rotavirus mRNA. As seen before, NSP1 had a limited ability to suppress type I IFNs when expressed on its own (***Figure 6.1 A and B***). In contrast, when rotavirus transcripts were introduced in cells expressing plasmid derived NSP1, a significant suppression of type I IFN (IFN-α1 and IFN-1β) and CXCL10 was observed (***Figure 6.3 A, B and D***). On the other hand, NSP1 failed to reduce expression of the type III IFN, IFN-λ1 (***Figure 6.3 C***). Type III IFNs (IFN-λ1, interleukin 28/29 or IL28/29) are the newly discovered members of an established IFN family (Ank et al., 2006, Kotenko et al., 2003). Although type III IFNs induce analogous subsets of ISGs as the type I IFNs, their action is exerted through a unique receptor complex (Kotenko et al., 2003, Zhou et al., 2007). Expressing NSP2 marginally, but significantly, reduced type I IFN expression in the presence of viral transcripts (***Figure 6.3 A and B***). Apart from being an integral part of the viroplasm, several other functions have been suggested for NSP2, including being involved in genome packaging, replication and non-specific ssRNA binding (Estes and Kapikian, 2007). It has been shown that type I IFN production is reduced in plasmacytoid dendritic cells expressing NSP2 (Deal et al., 2010), but the mechanism, or indeed function, of this suppression is still unclear.

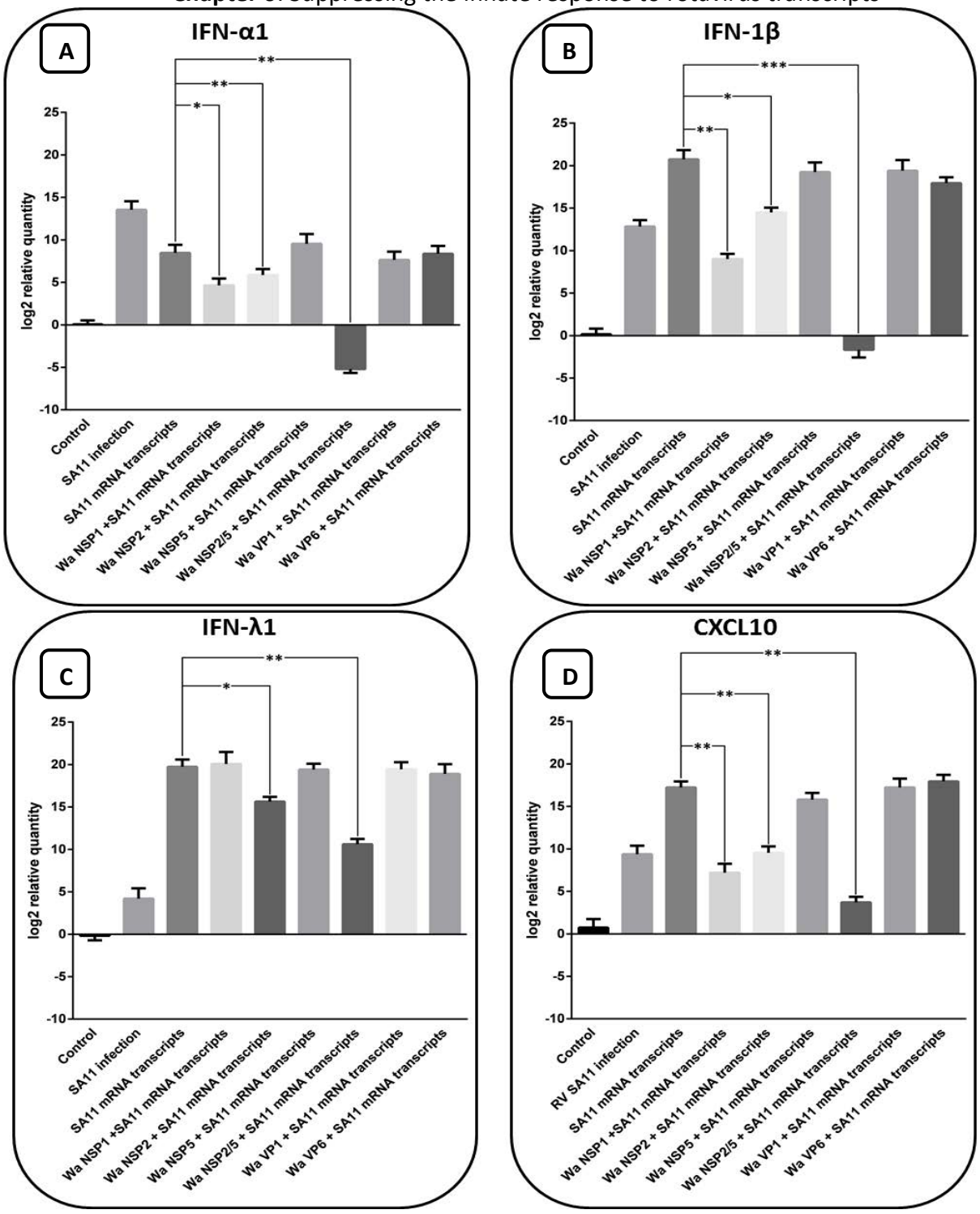


Figure 6.3: ***The log2 relative quantities of cytokine-encoding mRNA expression induced in HEK 293H cells 24 hours after transfection of rotavirus SA11 transcripts.*** *Graphs* ***A–D*** *indicate the effect of transfecting different rotavirus Wa encoding plasmids on the induction of IFNα1, IFN-1β, IFN-λ1 and CXCL10 in HEK 293H cells by rotavirus SA11 transcripts. Statistically significant expression differences are indicated by stars (*P ≤ 0.05,** P ≤ 0.01, *** P ≤ 0.001). All values are the arithmetic mean and standard deviation of at least three experimental replicates done in triplicate.*

Our results also indicated that NSP2 had some IFN suppression capability in embryonic kidney cells. The other major non-structural protein that is part of the viroplasm-like structure, NSP5, is central in orchestrating recruitment of viroplamsmic proteins and also seems to play an important role in viral replication (Campagna et al., 2005, Lopez et al., 2005b). In contrast to NSP2, pre-expressing NSP5 on its own did not lead to any noticeable reduction in the expression of the examined cytokines (***Figure 6.3 A-D***). Cytokine expression levels were comparable to that observed in cells only exposed to rotavirus transcripts, indicating that pre-expression of NSP5 had no innate immune suppression effects.

Interestingly, the most significant suppression of the IFN response resulted from the co-expression of NSP2 and NSP5 (***Figure 6.3 A-D***). Neither NSP2 nor NSP5 has any known direct influence on the host's innate immune system. Furthermore, the transfection of plasmids encoding for NSP5/6 induced significant expression of IFN-α1, IFN-λ1 and TNF-α (***Figure 6.1 A, C and E***). However, when co-expressed, NSP2 and NSP5 form viroplasm-like structures (Fabbretti et al., 1999) similar to the cytoplasmic inclusion bodies (viroplasms) found in normal rotavirus infections. Due to the association of viroplasms with the replication viral proteins (VP1, VP2, VP3 and VP6), synthesis of minus-sense strand ssRNA and genome replication are thought to take place in viroplasms, isolated from the host's innate immune system (Contin et al., 2010, Fabbretti et al., 1999). It is possible that the transfected rotavirus mRNA may be shielded from the innate immune system by the viroplasm-like structures. Although cells pre-transfected with NSP2 and NSP5 showed low expression of most cytokines examined, mass cell death was still observed after 40 hours following the transfection of mRNA transcripts. This may be due to the activation of alternative innate response pathways. The expression of the RNA sensitive retinoic acid-induced gene (RIG-I) and melanoma differentiation associated gene 5 (MDA5) was also tested. As mentioned before, RIG-I is best suited to recognize dsRNA between 21 b to 1 kb, whereas MDA5 detects dsRNA segments longer than 1 kb (Kato et al., 2008).

The expression RIG-I and MDA5 was investigated using flow cytometry. The data indicated that both RIG-I and MDA5 are expressed in rotavirus SA11 transcript transfected HEK 293H cells (***Figure 6.4***). The same levels of RIG-I and MDA5 expression was observed in cells pre-transfected with plasmids encoding for NSP1 or NSP2 and NSP5 as in cells only transfected

with rotavirus RNA transcripts. Rotavirus SA11 dsRNA was used as a positive control and induced relatively high expression levels of both RIG-I and MDA5.

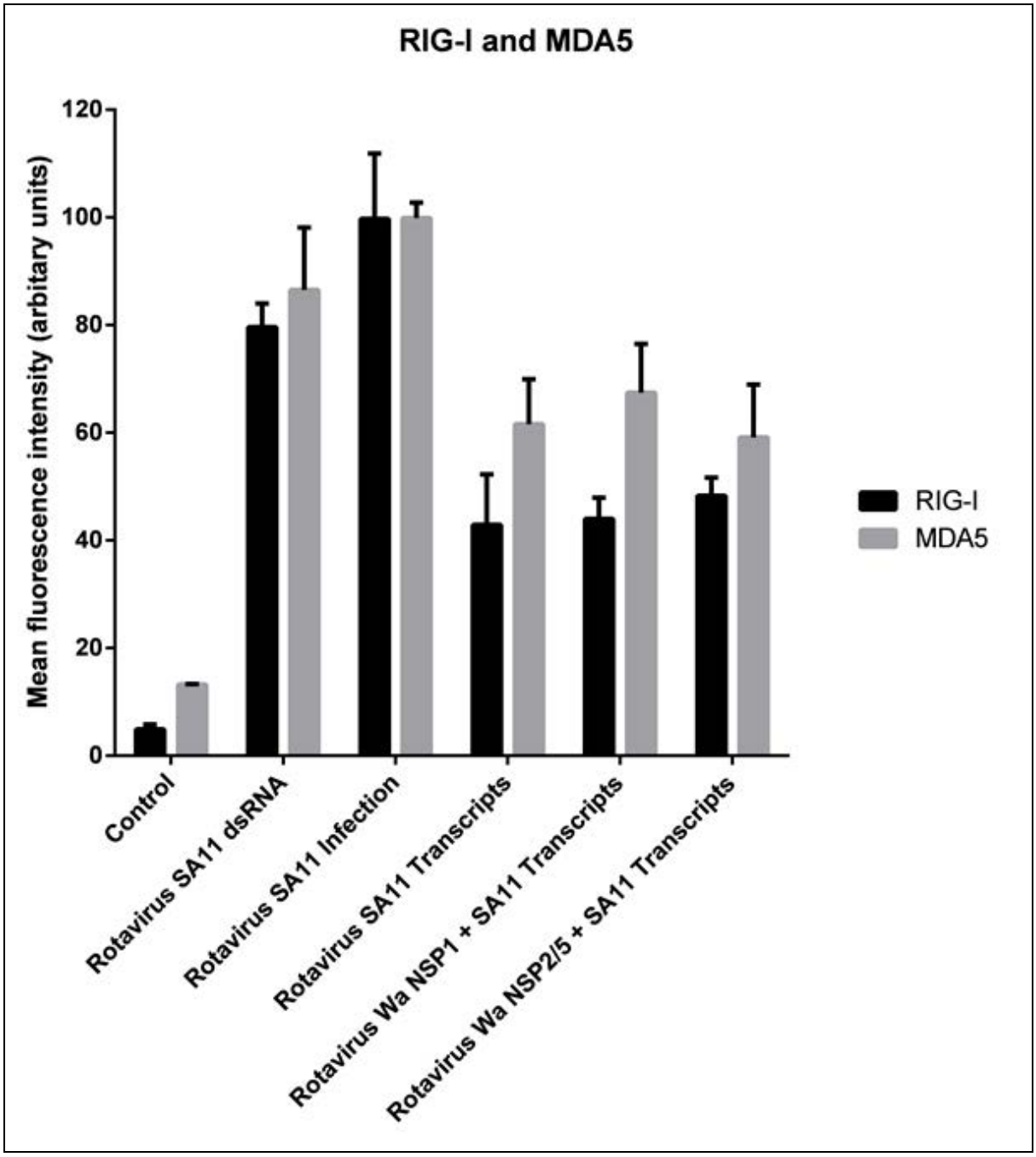


Figure 6.4: ***Comparison of flow cytometry detected expression of RIG-I and MDA5 induced in HEK 293H cells by rotavirus transcripts and plasmids****. HEK 293H cells were transfected with plasmids expressing different rotavirus Wa proteins before transfection with rotavirus SA11 transcripts. Rotavirus SA11 dsRNA was used as a positive control for the expression of RIG-I and MDA5. The expression of RIG-I and MDA5 in a normal rotavirus SA11 infection were examined. All values are the arithmetic mean and standard deviation of three experimental replicates (three independent transfection).*

HEK 293H cells infected with rotavirus SA11 had the highest RIG-I and MDA5 expression levels. Studies have reported that secondary structures of mRNA can also activate MDA5 (Li et al., 2010, Pichlmair et al., 2009). It seems feasible that both RIG-I and MDA5 will be able to recognize some rotavirus ssRNA, which has folded onto itself, as dsRNA-like secondary structures (Holloway and Coulson, 2013, Li et al., 2010). Another study showed that ssRNA produced during influenza A viral infections induced RIG-I expression (Pichlmair et al., 2009). dsRNA is not generated during influenza A viral infection and could not trigger RIG-I. Recently, two distinguished groups independently postulated that cytoplasmic (+) ssRNA may be ligands of RIG-I/MDA5 (Holloway and Coulson, 2013, Uzri and Greenberg, 2013). In the normal rotavirus life cycle, it is known that the viral RNA-dependant RNA polymerase, VP1, produces dsRNA containing a 5' triphosphate moiety (Imai et al., 1983). This is common among viral polymerases and is a strong activator of RIG-I (Plumet et al., 2007).

Rotavirus also employs other strategies to counter the innate immune system of the host. Rotavirus proteins are transcribed in the cytoplasm from plus-sense mRNA. These viral mRNA are exposed to RIG-I and MDA5 in the cytoplasm. In order to shield the 5' triphosphate end from RIG-I, the methyl-guanylyl transferase protein, VP3, of rotavirus "caps" the plus-sense strand of dsRNA (***Figure 6.5 A***) (Pizarro et al., 1991). This is a highly regulated process and the 5' cap consists of a guanine nucleotide connected to the mRNA via an unusual 5' to 5' triphosphate linkage (Huang and Yarus, 1997). Studies have found that RNA 5'-triphosphatase activity is deficient in open cores (Chen et al., 1999). The native function RNA 5'-triphosphatase is the removal of the γ-phosphate from the 5'-end of an emerging transcript. This process occurs just before the transfer of a GMP onto the targeted RNA by a guanylyltransferase (Chen et al., 1999, Patton and Chen, 1999). Triphosphatase activity is consequently critical to produce cap structures with a triphosphate linkage. The study also found that an excess of 70% of the RNAs capped in an open core system contained a tetra-phosphate linkage, indicating inefficient RNA 5'-triphosphatase activity during *in vitro* transcription. Flow cytometry data showed that rotavirus SA11 transcript induced the expression of both RIG-I and MDA5 in HEK 293H cells (***Figure 6.4***).

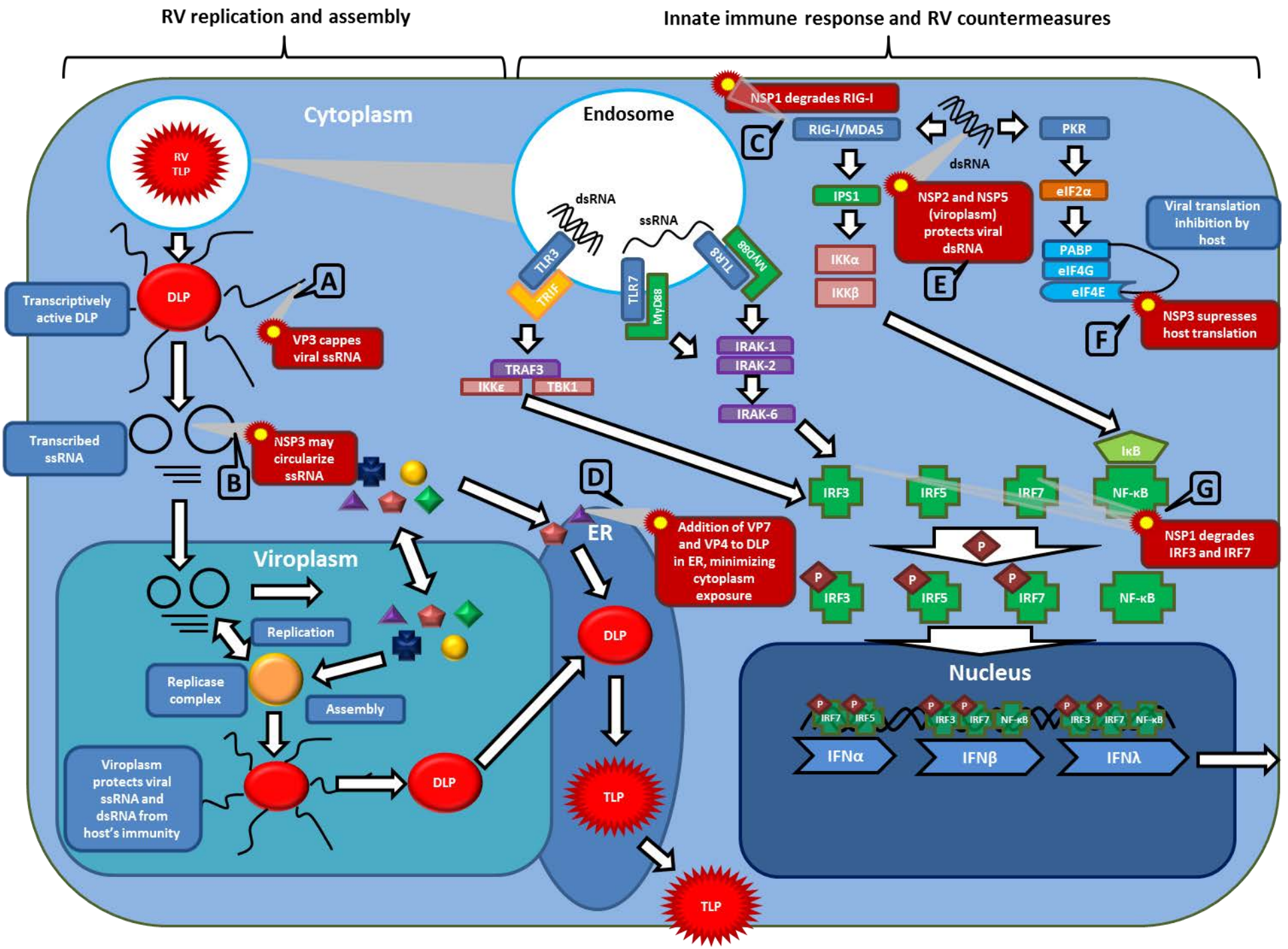

RV replication and assembly
Innate immune response and RV countermeasures
Cytoplasm
Endosome
RV TLP
DLP
Transcriptively active DLP
A
VP3 capes viral ssRNA
Transcribed ssRNA
B
NSP3 may circularize ssRNA
Viroplasm
Replication
Replicase complex
Assembly
Viroplasm protects viral ssRNA and dsRNA from host's immunity
DLP
dsRNA
ssRNA
TLR3
TRIF
TLR7
MyD88
TLR8
MyD88
TRAF3
IKKε
TBK1
IRAK-1
IRAK-2
IRAK-6
D
ER
Addition of VP7 and VP4 to DLP in ER, minimizing cytoplasm exposure
DLP
TLP
TLP
NSP1 degrades RIG-I
C
RIG-I/MDA5
IPS1
IKKα
IKKβ
dsRNA
NSP2 and NSP5 (viroplasm) protects viral dsRNA
E
PKR
eIF2α
PABP
eIF4G
eIF4E
Viral translation inhibition by host
F
NSP3 supresses host translation
IκB
NF-κB
G
NSP1 degrades IRF3 and IRF7
IRF3
IRF5
IRF7
NF-κB
P
Nucleus
IFNα
IFNβ
IFNλ

Figure 6.5: ***Innate immune response to rotavirus infection and likely strategies of rotavirus to subvert this immune response.*** *Rotavirus employs a range of strategies to counter the innate immune system of the host cell. (A) RNA is capped in order to shield the 5' triphosphate end from the innate immune system. (B) Circulation of ssRNA by NSP3 may help avoid complex secondary structures to form which can elicit an innate response. (C) NSP1 has been shown to degrade RIG-I in certain cell lines. (D) The addition of VP7 and VP4 to the DLP in the ER, minimises cytoplasm exposure. (E) Viroplasms may help to shield dsRNA from the detectors that trigger innate immunity. (F) NSP3 is able to shut-off the synthesis of host cell proteins. (G) NSP1 is able to bind to various IFN regulation factors (IRF3/5/7) and mark them for proteasomal degradation.*

The formation of rotavirus transcript secondary structures and uncapped 5' triphosphate ends are most probably responsible for the induction of RIG-I and MDA5 in this case. However, NSP1 contains a RNA binding domain that recognizes the 5' end of rotavirus mRNA that can possible mask the 5' triphosphate end of mRNA from RIG-I (Hua et al., 1994). In addition, it has been reported that NSP1 is able to bind to RIG-I in order to down regulate its activity (***Figure 6.5 C***) (Qin et al., 2011). Therefore, NSP1's potential to suppress the RIG-I/MDA5 responses was investigated. HEK 293H cells were pre-transfected with plasmids encoding for NSP1 followed by a transfection with rotavirus transcripts. Alas, no visible reduction in RIG-I or MDA5 expression were detected and NSP1 does not seem to supress the RIG-I/MDA5 activity in the innate response elicited by rotavirus mRNA in cultured HEK 293H cells.

NSP3 interacts with the eukaryotic initiation factor 2-α (eIF2-α) (Piron et al., 1999, Piron et al., 1998b) and is able to shut-off the synthesis of host cell proteins (***Figure 6.5 F***) (Montero et al., 2006). This machinery most likely provides an advantage for rotavirus propagation. VP4 and or VP7 can induce IFN-α in plasmacytoid dendritic cells (Deal et al., 2010). The addition of VP7 and VP4 to the double-layered particle takes place in the ER, most likely sheltering these viral proteins from the innate immune system (***Figure 6.5 D***). When co-expressed, NSP2 and NSP5 form viroplasm-like structures (Fabbretti et al., 1999), similar to the cytoplasmic inclusion bodies (viroplasms) found in normal rotavirus infected cells. Due

to the association of viroplasms with the core replication viral proteins, synthesis of minus-sense strand ssRNA and genome replication are thought to take place in viroplasms, isolated from the host's innate immune system (***Figure 6.5 E***) (Contin et al., 2010, Fabbretti et al., 1999).

The suppression potential of VP1, VP2, VP3 and NSP5/6 were also investigated but did not show visible reduction in the expression of RIG-I/MDA5 (not all results are shown). Pre-transfecting with the core structure (VP1, VP2, VP3 and maybe VP6) may have better suppression results and should be examined further.

Transfection of plasmids encoding for VP1, VP2, VP3, VP4, VP6 and NSP3 prior to rotavirus transcript transfection did not influence the expression of any examined cytokines and exhibited similar cell death patterns and levels of cytokine expression as seen in cells only transfected with rotavirus transcripts (results not shown). Although some structural proteins, notably VP1, VP2 and VP3, had some suppression effect when expressed on their own (***Figure 6.1***), pre-expressing these proteins had no protecting properties against the interferon response elicited by rotavirus transcripts.

6.4 Summary

The secretion of cytokines belonging to the interferon (IFN) family, IFN type I and III in particular, play an important part in the innate immune response by activating the expression of IFN-stimulated genes. Induced cytokines are able to directly inhibit viral replication or induce an apoptotic state in the virus-infected cells. In this chapter, the innate immune response elicited by plasmid derived rotavirus Wa and SA11 proteins and rotavirus transcripts was examined. The expression of certain viral proteins from plasmids (VP3, VP7, NSP2 and NSP5/6) seemed to stimulate specific IFN responses. On the other hand, some viral proteins (VP1, VP2, VP4 and NSP1) seem to be able to actively suppress the expression of certain cytokines. Remarkably, expressing different combinations of plasmids encoding for rotavirus genome segments, changed the cytokine expression profile drastically. Transfecting all the plasmids at the same time usually had a slight suppression effect on the cytokines examined. In the light of these suppression results, specific rotavirus proteins were expressed from transfected plasmids to investigate their potential in supressing the

interferon response provoked by rotavirus transcripts. Mass cell death after transfection of rotavirus transcripts was substantially delayed and reduced by transfecting cells with plasmids encoding NSP1, NSP2 or a combination of NSP2 and NSP5 24 hours before transfecting with rotavirus transcripts. qRT-PCR results indicated that cells transfected with the plasmid encoding the NSP1 and NSP2 reduce the expression of type I IFN and CXCL10. The transfection of plasmids encoding for the viroplasm proteins, NSP2 and NSP5/6, significantly suppressed the type I and III interferon responses elicited by transfected rotavirus transcripts. However, no visible reduction in RIG-I or MDA5 expression were detected in this study and NSP1 does not supress the expression of RIG-I/MDA5 induced by rotavirus mRNA in cultured human embryonic kidney cells. The IFN suppression findings point to other possible viral innate suppression mechanisms in addition to the degradation of IRFs by NSP1. With the lack of a true reverse genetics system for rotavirus, the suppression of the strong innate immune response elicited by rotavirus transcripts might prove to be vital in the quest to better understand the replication cycle of this virus.

The work performed in this chapter (***section 6.3.4***) has been submitted as a short communication to the Journal of General Virology (2012 IF: 3.127) (*see* ***Appendix G*** *for manuscript*)