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Temperature dependence in human Rhinovirus infection of human MRC-5

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Abstract

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Temperature has been known to be an important factor for in vitro studies where human cell cultures are infected with HRV (human Rhinovirus). The mechanisms behind the temperature effect on the struggle between virulence and cellular defense, are still largely unknown and may be a crucial part in finding a treatment to the common cold. In this study we focused on a few cellular key elements in this struggle and observed behavior changes in regards to the pre-infection growth temperature and the temperature during the viral infection.

Past studies have focused mainly on the temperature post inoculation, but here we also wanted to correlate virulence to the growth temperatures preceding the viral infection. We found that the growth temperature of the cell did indeed affect its response to the HRV. If the cells had been growing in an optimal body temperature of 37°C before getting virally infected at 33°C, the viability of the cells did decrease in comparison to cells that had been growing in 33°C from before the viral infection.

We could also observe a significant temperature dependence regarding IL-8 release upon HRV inoculation. HRV strive to block induction of inflammatory cytokines such as interferons and IL-1. It may be that impaired IL-8 release at lower temperatures will prevent important danger signals alerting the immune system when cytokine signaling is otherwise hampered by viral intervention.

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Sammanfattning

Alla har vi någon gång fått en förkylning, när näsan rinner och hostan inte vill sluta. Trots att förkylningar är så vanliga finns det fortfarande inga effektiva botemedel mot åkomman och en av huvudanledningarna är för att förkylningar oftast är orsakade av virus. Virus har historiskt sett varit svårare att skapa mediciner mot jämfört med bakterier. Eftersom bakterier har egna cellväggar och egna cellulära processer som skiljer sig från människans så kan vi skapa antibiotika som är specifika för dem. Virus använder mestadels vårt cellulära maskineri för att föröka sig. När cellerna slutligen går sönder och nya virus kommer ut så är de inte mer än partiklar laddade med skadlig kod. Vacciner är en möjlig väg eftersom virus skapar proteiner som skiljer sig från våra egna, men att vaccinera sig mot förkylningar har också visat sig vara svårt då det finns hundratals olika förkylningsvirus.

Förkylningar är oftast varken dödliga eller speciellt långvariga. Väntar man ett par dagar eller någon vecka försvinner de oftast. Varför är de då så viktigt att veta mer om dem? Förkylningar kan vara ekonomiskt mycket kostsamma för samhället. En forskargrupp från Universitetet av Michigan estimerade att den årliga kostnaden för förkylningar i USA var 17 miljarder dollar. Kostnaderna är beräknade på förlorade arbetsdagar och även om den förkylda personen dyker upp på jobbet så är resultatet ofta en minskad produktivitet. Rapporten inkluderade även alla läkarbesök och läkemedel som skrivs ut. Att öka kunskapen kring förkylningsvirus är viktigt både för individers välbefinnande och för samhällets ekonomi.

I vår studie undersöker vi ett virus som är en dominerande patogen när det gäller vanliga förkylningar, Rhinovirus, och vi valde att studera interaktionen mellan viruset och den humana cellen och närmare bestämt hur den interaktionen är temperaturberoende. Det har varit känt sedan 1950-talet att Rhinovirus lättare infekterar celler vid lägre kroppstemperaturer, i detta fall 33°, jämfört med den vanliga kroppstemperaturen, 37°C. Anledningen till detta är fortfarande inte helt utrett. Rhinovirus består av 160 varianter och de är indelade i tre kategorier, A, B och C. Vi valde att studera Rhinovirus 14 som är en kategori B variant.

Hur ser interaktionen mellan virus och humana celler ut? När en cell blir infekterad av ett virus så finns det ett par saker som cellen kan göra för att skydda oss. Den kan begå apoptos vilket betyder att cellen startar en systematisk nedstängning av alla funktioner, vilket i förlängningen betyder att cellen dör på ett ordnat sätt utan att skapa oordning. Eftersom viruset är beroende av att cellen replikerar viruset så blir detta en effektiv metod att stoppa viruset. Det andra är att bekämpa viruset med ett RNA degraderande enzym som kallas RNase L, RNase L kommer att degradera näst intill allt RNA i cellen och även om det sätter ett temporärt stopp i cellens egen produktion så fungerar detta som ett effektivt sätt att bli av med viruset. Det tredje är att skapa en inflammation via en grupp av protein som kallas interferoner. Detta kommer att förvarna granncellerna att det finns virus i närheten. Under de senaste 10 åren har det kommit mycket ny fakta om hur Rhinoviruset stör dessa mekanismer för att få ett övertag mot cellen. I det här projektet vill vi se om några av dessa interaktioner är temperaturberoende

på ett sätt som kan förklara varför viruset har så mycket lättare att infektera celler i kallare miljöer.

Experimenten gick ut på att infektera humana celler kallade MRC-5 efter att de hade fått växa i två olika temperaturer, 33° och 37°. Sedan analyserades mängden producerade proteiner och förändringar i proteinstorlekar för att möjligtvis hitta om vissa proteiner klyfts annorlunda. I vår studie kunde vi inte hitta några signifikanta förändringar i proteinuttrycken mellan temperaturerna, varken på basal nivå eller längre in i infektionen för de proteinerna valt att studera, RIPK1, RNase L eller OAS3. Alla dessa proteiner är medverkande i försvaren mot virus och förändringar i antingen kvantitet eller storlek kan tyda på att viruset aktivt motarbetar försvarsmekanismen som proteinet är inblandat i.

Överraskande var däremot att vi hittade en skillnad i överlevnad mellan celler som fick växa i en statisk temperatur och celler som växte i en varierad temperatur, oavsett om den slutgiltiga temperaturen var desamma under infektionen. De celler som hade fått växa i 33° både före och efter infektionen hade en högre överlevnadsgrad än de som fick växa i 37° fram till virus inokulering för att sedan odlas i 33° efter injektionen. Detta kan peka på att temperaturvariationer kan vara en negativ faktor för de humana cellernas välmående oavsett vad som är den mest gynnsamma temperaturen. Detta blir också en viktig aspekt när man planerar framtida experiment för att minska variationer i resultaten, samt om man skulle vilja jämföra resultat från olika tillfällen eller forskargrupper då de kan ha behandlat cellerna olika innan inokulationen.

En ny observation var också att koncentrationen av en inflammatorisk signalsubstans IL-8 varierade stort beroende på temperatur, den var tre till fyra gånger högre i mediet hos celler som blev infekterade i 37° jämfört med celler som blev infekterade på 33°C. Detta kan ha stor betydelse då viruset aktivt försöker förhindra produktion av inflammatoriska signaler från den infekterade cellen. IL-8 är en signal som kallar till sig immunceller som kan hjälpa till att bekämpa infektionen.

Slutligen kunde vi fastslå att man måste planera hur cellerna blir behandlade både före och efter tillsättningen av virus för att få konsistenta resultat i *in vitro*-försök med Rhinovirus.

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Abbreviations

ATP	Adenosine Triphosphate
CO_2	Carbon Dioxide
DMEM	Dulbecco's Modified Eagle's Medium
ELISA	Enzyme Linked Immuno-Assay
FADD	Fas-Associated protein with Death Domain
FBS	F. Bovine Serum
hpi	Hours Post Inoculation
HRP	Horse Radish Peroxidase
HRV	Human Rhinovirus
ICAM-1	Intercellular Adhesion Molecule -1
IFN	Interferon
IL -	Interleukin
IPS-1	IFN-ß promoter stimulator 1
IRF	Interferon Regulating Factor
kD	Kilo Dalton
MDA5	Melanoma Differentiation Associated protein 5
MRC-5	Medical Research Council - 5
NF-κB	Nuclear Factor - κB
NK Cell	Natural Killer Cell
OAS	Oligo Adenylate Synthetase
PBS	Phosphate Buffer Saline

pfu	Plaque- Forming Units
PI	Propidium Iodide
PS	Penicillin Streptomycin
RIG-1	Retinoic acid-Inducible Gene 1
RIPK1	Receptor Interacting Protein Kinase 1
RNA	Ribonuclease
RPMI	Roswell Park Memorial Institute
RV14	Rhinovirus 14
TBS-T	Tris-Buffer Saline with 0.1% Tween
TGF	Transforming Growth Factor - β
VRI	Viral Respiratory Infection

1 Introduction

1.1 Temperature dependence in HRV infection of human MRC5 cells

Human Rhinovirus (HRV) was first discovered in 1956 by Winston H. Price when he was isolating viruses from clinical respiratory infections (Price, 1956). HRV is an Enterovirus belonging to the family Picornavirus. They are single stranded RNA viruses that can transmit either via physical contact or by air. The symptoms of a HRV infection are mostly mild and lasts for a few days (Jacobs *et al.,* 2013). There are approximately 160 genotypes of Rhinovirus found today (Palmenberg *et al.,* 2010) and they account for more than half of all common cold cases (Mäkelä *et al.,* 1997).

The occurrence of HRV infections increases during the winter season when the temperatures are colder (Ikäheimo *et al*., 2016). This had led to the belief that it was the cold temperature that contributed to the increased HRV infections, giving it the name *The common cold*. In recent years after examining crowded student dorms with insufficient ventilation it was found that the rate of common colds were depending on the frequency of ventilation, concluding that being in an closed environment also increases the risk of getting respiratory infections (Sun *et al.,* 2011). When looking at cell culture studies the correlation between temperature and HRV potency is well established. After trying to optimize the growth rate of HRV in human cell lines Stott *et al*. showed that a lower temperature, 33° was the optimal replication temperature (Stott & Heath, 1970).

1.2 Economic impact of non-influenza related respiratory infections

It is important to persevere in the study of HRV as there are still no functional cures to common cold infections (Casanova *et al.,* 2016). This is not only unfortunate in regards to the wellbeing of individuals, but the economic cost of non-influenza related Viral Respiratory Infections (VRI) it was estimated to be up to 17 billion USD annually in US alone (Fendrick *et al.,* 2001). The public cost of VRI is due to decreased productivity, absence from school and work as well as medical care. This aspect is very important as the symptoms can continue for a week or two, which can make the burden on individuals significant as well (Bertino, 2002).

1.3 The general aim of this study

This study is aimed at finding what in the interaction between the virus and the host is responsible for the temperature dependence of human Rhinovirus virulence. The effect of how temperature alters protein expressions, as well as how the HRV influences the cells behavior to overcome certain challenges.

2. Mechanistic Background.

It is important to understand how the virus enters and replicates inside a host cell, what the host cell does in order to defend it self as well as its neighboring cells and how the virus attempts to avoid those defenses.

2.1 Rhinovirus in general

2.1.1 How does the virus infect the host?

When the HRV infects the human respiratory tract it enters the cells via binding to the surface receptor ICAM – 1 (Intercellular Adhesion Molecule -1) (Basnet *et al.,* 2019). Once inside the cell the capsids release their RNA and forces the cells own ribosomes to translate the viral RNA into a poly-protein. The HRV genome consists of one big open reading frame that is divided into three major parts, P1, P2 and P3 as shown in figure 1. The first part P1 codes for four capsid proteins (VPs). The parts P2 and P3 codes for all the non-structural proteins from priming proteins to several proteases (Royston & Tapparel, 2016). Once the HRV has multiplied it will force the host cell in to necrosis and escape to find the next host (Lötzerich *et al.,* 2018).

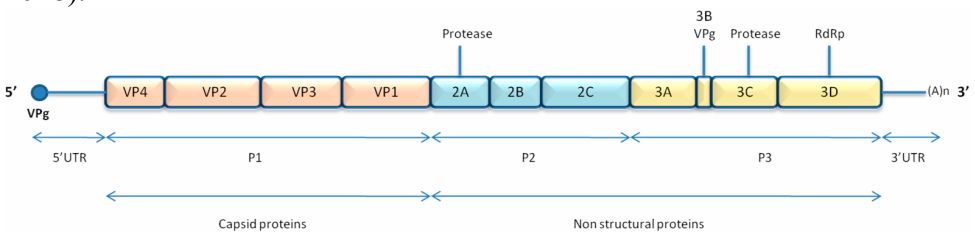

Figure 1: The genomic structure of human Rhinovirus. The genome is divided into three main parts: a structural part named VP (Virus proteins) and two non structural parts called 2 and 3. Part 2 and 3 codes for functional proteins, such as translation initiators and proteases. (Rouston and Tapparel, 2015)

2.2 The cells defenses and how the Rhinovirus escapes from them

2.2.1 The OAS/RNaseL response

The cell has several ways to fight the virus that enters. One of the main early responses is the OAS/RNaseL pathway. There are three OAS (Oligo A Synthase) proteins in the human cell, OAS 1, OAS 2 and OAS 3. Among these the OAS 3 protein acts as the main activator of the RNase L (Lia *et al.,* 2016). In this system the OAS proteins, in the presence of dsRNA, synthesizes a 2'-5'oligomer from ATP. These 2'-5' oligomers will in turn activate the RNase L that digests all ssRNA in the cell including self mRNA (Kimura *et al.,* 2018) (Figure 2). Without the RNA the virus has no template to replicate, while the host cell will only suffer a temporary halt in the protein production.

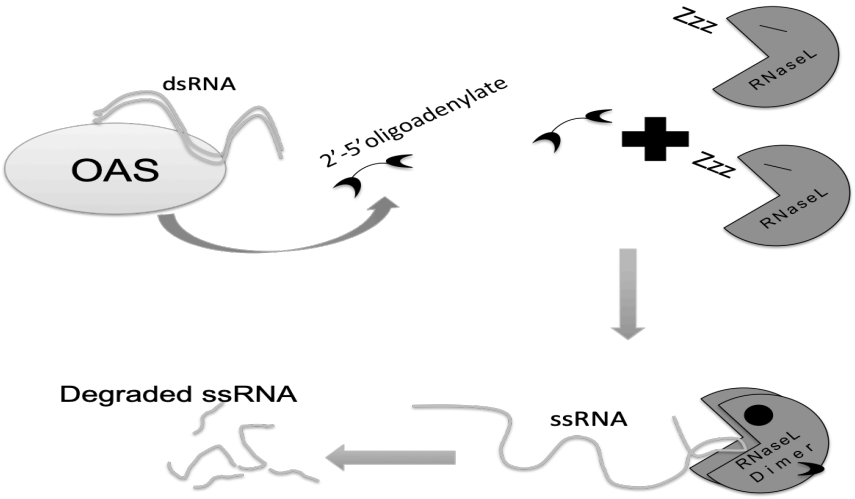

Figure 2: A schematic representation of the OAS/RNase L system. The circular light-grey symbol represents Oligo A Synthase and the dark-grey pac-man represents the RNase L.

To allow this system to react at an early stage there is a constant amount of RNase L in an inactive state present in the cell. The OAS levels however can be quite low if the cell is not stimulated and the basal level of these genes has been suggested to play a big role in how well the cell can fight off viral infections. The levels of the OAS protein will quickly increase in the presence of type I-IFN that greatly increases the initial defense against invading viruses (Birdwell *et al.,* 2015).

2.2.2 The Interferon (IFN) cytokines

The interferons (IFNs) are an important defense against viruses as they activate non-infected cells at risk. Type 1 interferons (IFN α/β) are rapidly produced in response to the infection of cells and will activate hundreds of genes in non-infected cells by binding to type 1 interferon receptors, thereby causing a cascade of intracellular signals (Biron, 2001)**.** The type 2 interferon (IFN γ) is mainly produced by NK cells and T cells and will initiate and regulate the immune responses (Lee & Ashkar, 2018). The IFN system can be activated through various pathways and among them the Toll like receptor 3 (TLR3). TLR3 is a cell surface receptor that reacts on viral RNA and activate the protein IPS-1 (IFN-ß promoter stimulator 1). IPS-1 then activates IKKε (Inhibitor of nuclear factor Kappa B[NF-ϰB] Kinase) and TBK1 (TANK-Binding Kinase) which are necessary for the phosphorylation and activation of IRF3 (Interferon Regulating Factor 3)(Figure 3)(Fitzgerald *et al.,* 2003).

2.2.3 Inactivation of IFN signals by virus genes

The 2A protease, which is synthesized from virus RNA, will first cut the protein IPS-1 disallowing the downstream to be activated, resulting in lowering of IFN-ß synthesis. The 2A protease will also prevent the IRF3 from forming dimers, which is necessary in order to make the IRF3 proteins active. This will then serve as a second inhibition of type I IFN expression (Drahos & Racaniello, 2009) (Figure 3). Subsequently this leads to the cells not being able to create an inflammation and thus the infected cell is not capable to send the danger signal on to the surrounding cells.

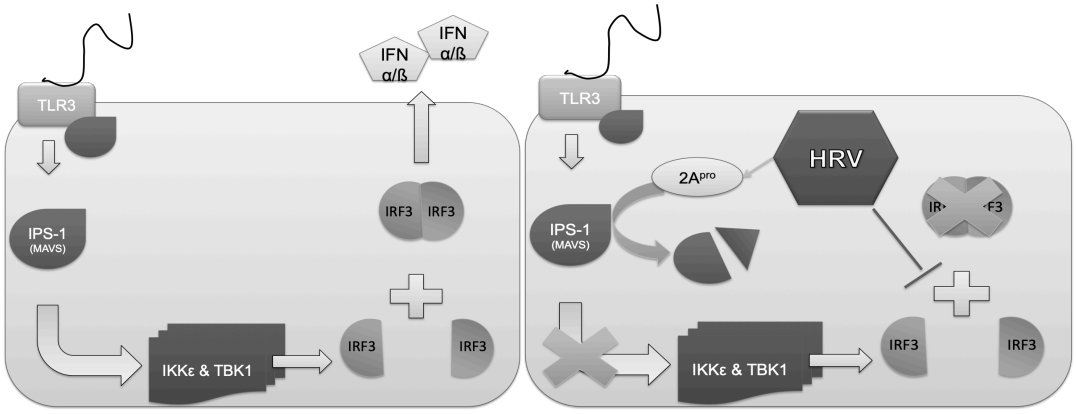

Figure 3: A schematic representation of the IFN type 1 expression in the presence of viral RNA. To the left is a representation of when the IFN expression is functioning. To the right is a representation of how the HRV inhibits the IFN expression.

2.2.4 Cell apoptosis and its inhibition by virus RV genes

Apoptosis is quite different from other types of cell deaths such as necrosis and involves several mechanisms ensuring that the de-mantling of the cell happens in a safe and orderly fashion. When the cell senses viral RNA through either RIG-1 or MDA5 a complex is formed consisting of RIPK1, FADD, IPS-1 and Caspase 8. The Caspase 8 will then be cleaved and in turn activate Caspase 9 and subsequently Caspase 3 to initiate the apoptotic mechanisms in a cascade manner (Elmore, 2007).

The virus depends on the infected cell to stay alive in order to have the virus replicated. If the cell commits apoptosis there will be no opportunity for the virus to replicate. To avoid the premature cell death of the host the virus disrupts the activation of the Caspase cascade (Croft *et al.,* 2018). The 3C protease produced from virus RNA (Figure 1) cuts the RIPK1 domain and efficiently inhibits the cells capability to activate Caspase 8. At a certain point in the viral replication necrosis will be activated breaking the cell membrane and allowing the virus to travel to the next potential host (Lötzerich *et al.,* 2018).

2.3 Cytokine production during HRV infections

As mentioned above virus-infected cells go to great length to produce interferons in order to warn surrounding cells of the virus and to alert the immune system that it needs to identify viral antigens and to produce antibodies capable of inactivating the virus. Furthermore, selective T cell clones with appropriate receptors needs to be developed that can kill of infected cells. In order to do this, a cascade of cytokines are produced (reviewed in Biron 2001). Chemokines like IL-8 attract immune cells and other cytokines like IL-1β and IFN-α induce inflammatory responses in macrophages and dendritic cells. Many more cytokines are involved, but in these experiments we measured these three. We also measured TGFβ that here was used as a marker of normal fibroblast activity.

2.4 The effect of temperature in HRV infection

It was previously shown that temperature played a crucial role for the mortality of the cells (Stott & Heath, 1970). Here we tried to determine if it is the temperature before or after infection that has the most impact on virulence.

Earlier studies by Foxman *et al.* found, using qPCR, differences in caspase 3 up-regulation depending on temperature, where the 37° sample gave a higher value than the 33° sample (Foxman *et al*., 2016). Later it was shown that the viral 3C protease would actively inhibit the activation of the Caspase pathway. (Lötzerich *et al.,* 2018 ; Croft *et al.,* 2018). The 3C protease was documented to have a maximum enzymatic speed at lower temperatures and that the efficacy of the C3 proteases slows down as the temperature rises in an extra cellular systems (Ruran-Kurussi *et al.,* 2013). Our hypothesis was that the reverse temperature dependency of the 3C protease would allow the Caspase cascade to be activated at 37° but not at 33°C.

The OAS/RNase L pathway is known to be one of the more important anti-viral defense systems inside the cell. The basal level of the OAS genes is one of the main contributing factors to the efficiency of this defense. Thus a high level of the OAS genes gave a higher activation of the RNase L proteins (Birdwell *et al.,* 2015). The OAS proteins consists of 3 variants OAS 1, 2 and 3 of which OAS 3 has been shown to be the main activator of RNase L (Lia *et al.,* 2016) and was hence chosen as the main candidate among the OAS genes in this experiment. The hypothesis is that there will be a difference in the basal level of the RNase L and OAS3 protein in correlation to the temperature and thus explaining the temperature dependence of virulence.

Furthermore, we attempted to find out whether cytokine and chemokine responses also varied with the temperature. The inflammatory cytokines INF-α, IL-1β and IL-8 (Kim *et al.,* 2015) were chosen as targets. TGF-β was also monitored as reference as it is produced by healthy fibroblasts.

2.5 Scope of this study

In this in vitro study we will try elucidate what in the interaction between the virus and the host is responsible for the temperature effect on human Rhinovirus virulence.

The mechanisms we choose to focus on are A) a cleavage of an apoptosis inducer RIPK1 by 3C protease, B) the basal levels of Oligo A Synthetase (OAS) 3 at different temperatures, C) the basal level of RNase L at different temperatures, D) the differences in cytokine productions and E) different survival rates depending on different temperatures.

There will be three different temperature conditions tested in this study. A) The cells will grow and be infected at the optimal body temperature, 37°C (denominated 37/37). B) The second condition is that cells will be allowed to grow in 37°C pre-inoculation and subsequently moved to 33°C after the virus inoculation (denominated, 37/33). C) In the last condition cells will be both grown and inoculation at 33°C (denominated, 33/33) to test if the basal conditions of the infection will have any effect on the cell cultures viability (figure 4).

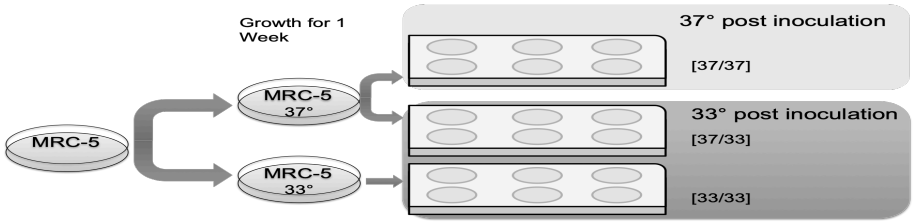

Figure 4: A visual description of the sample setups. The denominations are written in the hard brackets to the right.

3. Material & Methods

3.1 Cell cultivation

Human fibroblast lung cells called MRC-5 from ATCC were used. MRC-5 was cultivated in RPMI 1640 medium (Gibco, BRL, Life Technology Ltd. Paisley, Scotland) supplemented with 10 % FBS (Gibco) and Penicillin (50 units/mL)-Streptomycin (50 μg/mL) (1%PS, Gibco). The cultivation was at either 37°C or 33°C as indicated and in an atmosphere of 5% CO_2. 10 cm dishes (Thermo Fisher Scientific, Japan) were used for the cultivation.

3.2 Virus cultivation

The Rhinovirus 14 was kindly provided by Prof. M. Yamaya (Tohoku University, Sendai, Japan). The virus was cultivated using MRC-5 cells in capped polystyrene tubes. 1 ml of 10^6 MRC-5 cells were added to a tube tilted on a static stand with a 10° - 15° inclination to allow a bigger growth surface for the cells. When the cell culture had become 90% confluent the medium was removed and the cells were washed with 2ml PBS and inoculated with 1 ml virus solution at a concentration of 10^5 pfu/ml in 33°C for 48 - 56 h.

When harvesting the virus the tube was freeze-thawed once by placing the tube in a cold bath of –80°C EtOH (99%) until completely frozen and then transferred to a 37°C water bath until completely melted. The supernatant from the virus cultivation tube was stored using 1.8 ml tubes in -80°C. The viral concentration of the new stock was determined through titration (see 3.4).

3.3 MRC-5 cell inoculation

There were three different environmental sample setups tested. The high temperature sample (37/37) was both grown and kept in 37°C after inoculation. The low temperature sample (33/33) was both grown and incubated in 33°C post-inoculation. The varied temperature sample (37/33) was grown in 37°C but was moved to 33°C after inoculation. $5x10^5$ cells were added to each well in 6-well plates (Thermo Fisher Scientific, 130184) and incubated over night at the same temperature as they were grown. The cells were inoculated with a virus concentration of 10^5 pfu/ml, if not written differently, for two hours at the indicated temperature. After the two hour inoculation the cells were washed with 2 x 2 ml PBS and 2 ml fresh DMEM + 10% FBS + 1% PS was added for the remainder of the incubation.

3.4 Virus titration

MRC-5 cells cultivated in 96-well plate were used for virus titration. The virus solution was diluted 1:10 up to seven times in DMEM + 10% FBS + 1% PS unless otherwise indicated, creating a dilution range from 10^1 to 10^7 times the source solution. The cells were washed in PBS and later inoculated with the virus dilutions. The titration plate was put in 33°C for 6 days.

3.5 ELISA

ELISA assays for measurement of TGFβ, IL-8, IL-1β and IFNα (Mabtech, Stockholm, Sweden) were performed according to the manufacturer's instructions (www.mabtech.com). In short, high binding 96 well plates (Costar, Corning, NY, USA) were coated over night with 2 µg/ml capture antibodies in PBS with 0.02% sodium azide. The plates were blocked the next day with 0.1% BSA (Sigma-Aldrich, St. Louis, MO, USA) and 0.02% Tween20 in PBS sodium azide (incubation buffer) for further two hours at room temperature. Supernatants and standards were incubated for two hours. Secondary biotinylated antibodies were incubated for one hour followed by Streptavidin-HRP (SA-HRP) for one hour. For further details the protocols are available on the manufacturers homepage (see above).

3.6 Western Blot

The cells were washed twice with PBS and removed from the wells using RIPA Lysis Buffer (ATTA, lot 5600207). The Ripa buffer was supplemented with protease inhibitor cOmplete Mini (Sigma Aldrich, # 11836153001). Protein concentrations in the samples were measured using Pierce BCA Protein Assay Kit (product number # 23227), according to the manufacturers protocol. The samples were diluted to 1.2 µg protein/µl and mixed with non-reducing Sample Buffer (InVitrogen, ThermoSientific, Waltham, MA, USA) followed by boiling at 95° C for 5 min. 20 µl per lane was loaded on to E-R520L (e-PAGEL*,* AE- 6000). The electrophoresis was run for approximately one hour in 200V and 0.2 A in Running Buffer (InVitrogen). The separated proteins were transferred from the SDS-Page polyacrylamide gel to a PVDF membrane (Trans-Blot Turbo Transfer Pack, Bio-Rad Laboratories, # 1704157) using a Trans-Blot Turbo System (Bio-Rad), with the protocol 2.5 A, 25 V for 7 minutes. The membranes were blocked for one hour at room temperature with TBS-T (Tris-buffer saline + 0.1% tween) with 3% skimmed milk, except for RIP1 and GAPDH where TBS-T with 3% BSA was used. All primary antibodies were incubated at 4°C over night and with the secondary antibody anti-Rabbit IgG-HRP (Sigma) for one hour at room temperature. The membranes were washed three times with TBS-T for 15 minutes between each step. Blots were developed using RPN 2232 (Amersham, GE Healthcare, UK).

The primary antibodies used in the Western blot were rabbit anti-RIP1 (Cell Signaling, s3439), rabbit anti-OAS3 (Invitrogen, PA5-31090), rabbit anti RNase L (Invitrogen, PA5-76443), rabbit anti Caspase 3 (Cell Signaling), rabbit anti GAPDH(Cell Signaling).

3.7 AnnexinV and PI staining

The cells were grown at the indicated temperatures and inoculated according to the methodology *3.3 MRC-5 cell inoculation*. Cells were later removed from the incubators and washed twice with PBS. AnnexinV-FITC (Thermo Fisher Scientific*, V13242*), PI (Thermo Fisher Scientific*, V13242*) and Hoechts 33342 (Thermo Fisher Scientific) in 200µl water was added to each well. Images of the wells were taken in a fluorescent microscope and analyzed in the software Image J (National Institute of Health, USA).

4. Results

4.1 Looking for temperature dependence in the 3C protease activity towards RIPK1

To investigate if the 3C protease activity towards RIPK1 is temperature dependent at the cellular level the RIPK1 and the cleaved parts of RIPK1 were displayed and quantified (figure 5 and figure 6).

MRC-5 cells were inoculated at three different conditions (see methods) and harvested 9, 18 and 46 hours after inoculation. Cells were lysed and cleavage of RIPK1 was visualized by Western blotting.

RIPK1 is expected to be cleaved by the virus protease and give rise to two smaller proteins, one of them corresponding to 60 kD (*Lötzerich et al., 2018*). In figure 5, at the time point 9 hpi (hours past inoculation) there are faint bands at the size of the expected RIPK1 cleavage product for the RV infected samples 33/33 and 37/33. As these bands cannot be found in the control samples this result point towards that this reaction is induced by the HRV. The RV infected 37/37 sample does not exhibit this cleavage product either, suggesting a lower cleavage efficiency at higher temperatures. However, after 18 hours of incubation, control groups as well as the RV infected 37/37 samples exhibited degraded proteins and thereby contradicting the results shown in figure 5 (Figure 6). Figures shown are one of two representative results. In order to confirm the effect of inoculation temperature and 3C protease activities, more experiments need to be performed.

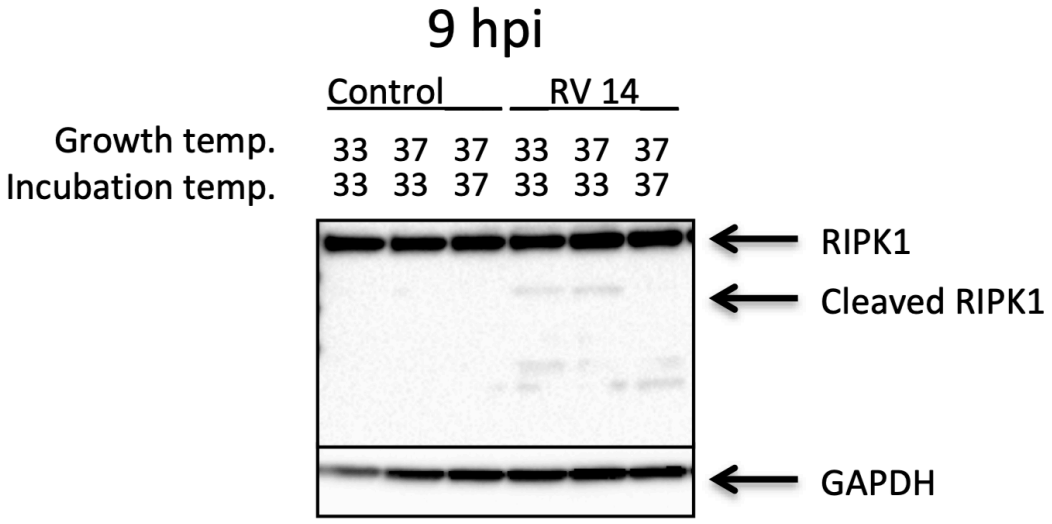

Figure 5: Western blot on RIPK1 on cells incubated for 9 hours. The cleaved RIPK1 can be visible on the HRV infected samples 33/33 and 37/33. But for HRV infected 37/37 and the control samples this cleaved band is not visible.

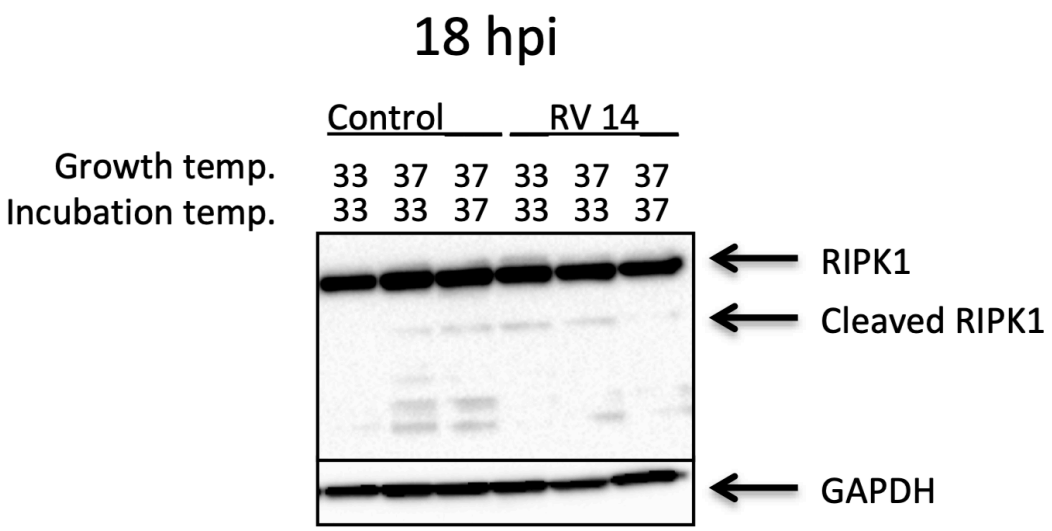

Figure 6: The RIPK1 western blot on samples incubated for 18 hours. The cleaved bands were very faint in all the samples but were present in all samples except the control of 33/33.

In figure 6 the 60 kD band corresponding to cleaved RIPK1 is visible in the control raising the question if this band is due to unspecific binding.

In order to correlate the RIPK1 analysis to Caspase activation, cleaved Caspase 3 was also measured. However, we did not find any stained band corresponding to cleaved or not cleaved Caspase 3. We suspected that the antibody used was likely to be faulty and turned out blank every time (Data not shown). Due to the shortage of my research time, we could not repeat the experiments with new antibodies.

4.2 Temperature dependence of basal levels of OAS3 & RNase L in MRC-5

To find out if the basal level of these genes would differ depending on temperature, cells were grown in both 33°C and 37°C. Because the regulation during the infection of these genes had not been mapped at a protein level, the OAS3 and RNase L protein levels were also measured at 9 hpi and 18 hpi with HRV-14 (Figure 7 and 8). The OAS3 levels were low and it was at times hard to obtain a good quantification. The RNase L levels were readily detected. However, there was no measurable difference between samples growing in 33°C and 37°C with or without HRV14 infection. We repeated these experiments and observed no significant differences of OAS3 and RV14 protein levels by temperature and virus infection.

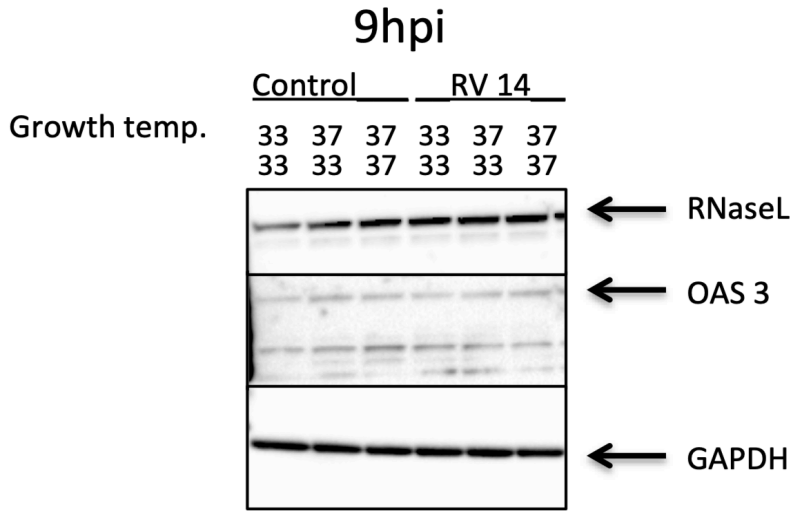

Figure 7: Both the RNase L and OAS3 levels displayed on western blots. There is a visible decline in color for the RNase L at the 33/33 control but this is most likely due to technical issues (too low volume when applying the anti-RNase L AB).

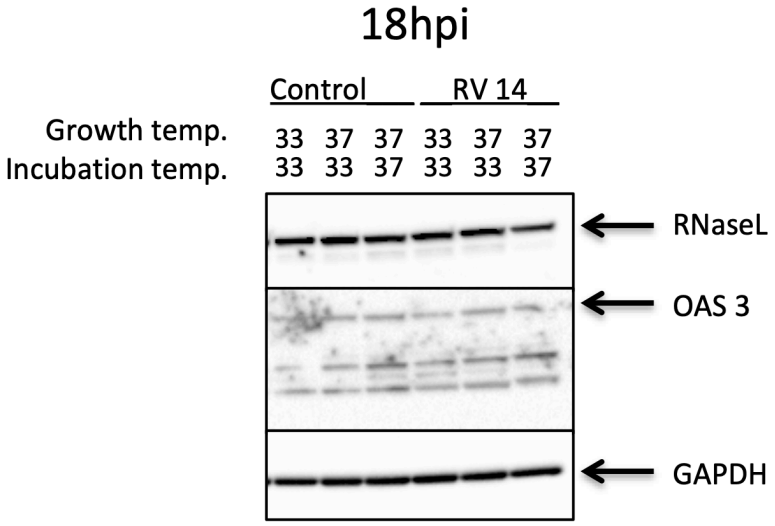

Figure 8: The RNase L and OAS 3 levels displayed after 18 hours of incubation. Once again the RNase L sample furthest to the right, HRV infected 37/37, is slightly lower than the other samples but this is likely due to staining error.

4.3 Viral replication depending on the temperature of the cell culture both before and after viral inoculation.

As described before, the HRV is known to replicate better in colder temperature (Stott & Heath, 1970). We tried to examine whether this observation is applicable in our experiment setup by measuring virus titrations of the culture supernatants after inoculation to MRC-5 cells. The viral replication in 37/37 was low as expected, only generating 10^4 or 10^5 pfu/ml after 46 hours incubation. Samples 37/33 and 33/33 had as expected significantly higher values. The sample 33/33 had a pfu/ml value around 10^6 while the sample 37/33 had constantly one degree higher, 10^7 pfu/ml. (Table 1)

Table 1: Viral replication in MRC-5 cells in different temperatures after 46 hours post inoculation.

Culture group	Replication (pfu/ml)
33/33	10^6
37/33	10^7
37/37	10^4 - 10^5

Cell viability of 37/33 and 33/33 was observed after 46 hours inoculation (Figure 9). Without virus infection, temperature changes of the culture did not affect cell viability (A vs C).

Virus inoculation to cells clearly increased dead cell number (A vs B or C vs D). In virus-infected conditions comparing the 33/33 and 37/33, the amount of dead cells in 37/33 was visibly higher (B vs D).

The 37/37 samples had no significant mortality after HRV infections and would increase in cell density as the experiment proceeded (Data in appendix (A.2), figure 14).

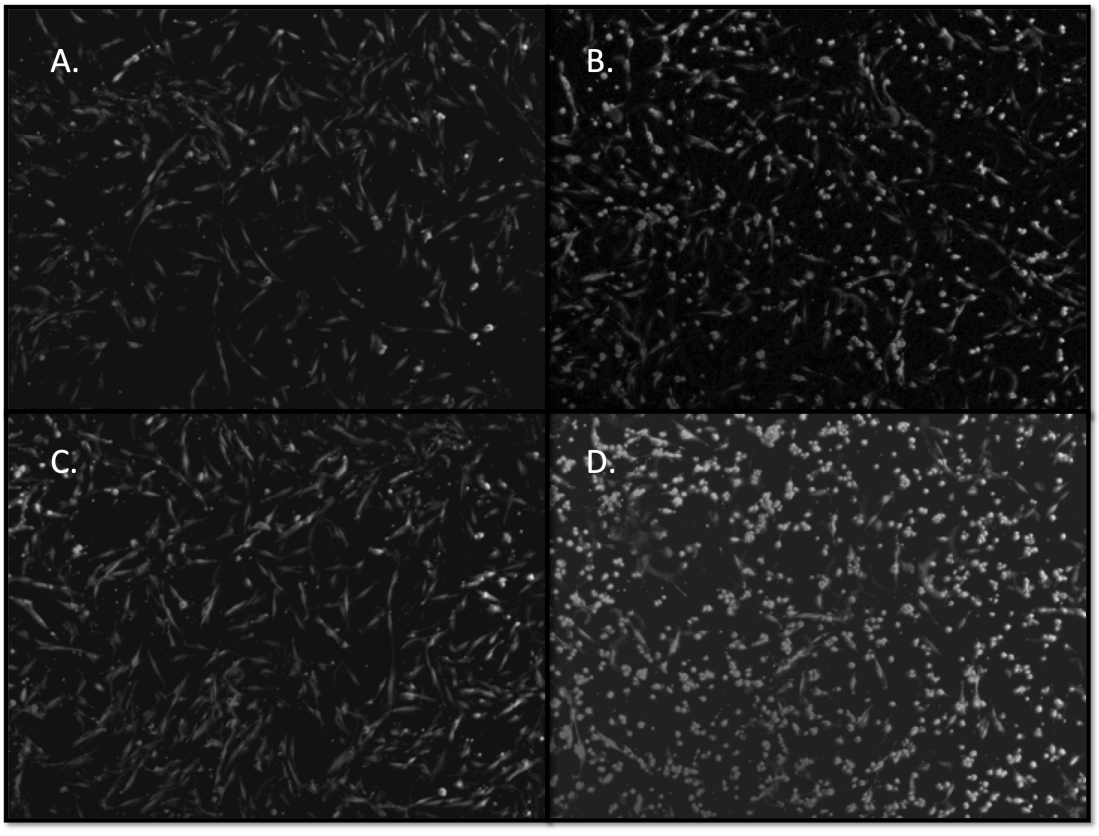

Figure 9: Images show a wider view on the samples 33/33 and 37/33 after 46 hours incubation. The living cells have a rod like structure and are a more grey in color. The dead cells have a round shape and can be seen as the white dots on the images. A) Control well of 33/33, there are mostly living cells. B) HRV infected sample of 33/33. About half of the cells are visually alive. C) Control well of sample 37/33, mostly healthy and living cells. D) The HRV infected 37/33 cells, majority of the cells are dead at this point.

4.4 Occurrence of Apoptosis and Necrosis depending on pre-inoculation cultivation temperature in MRC-5 infected by Rhinovirus

To get a conformation on whether setup 37/33 had a lower viability after HRV infection in comparison to 33/33, an AnnexinV and PI staining was conducted 24hpi. As can be seen in *Figure 10* the HRV infected 37/33 samples had twice the number of PI positive cells compared to the HRV infected sample of 33/33. AnnexinV single positive cells were apoptotic, which could be caused by culture condition or it could be a technical effect as HRV is mainly known to induce necrosis. Our results showed similar percentage of AnnexinV positive cells between HRV infected cells and control.

Many of dead cells were detached from the wells and had rounded shapes (Figure 9). In this examination, wells were washed with PBS before staining procedure. Much of the dead cells could have been removed by washing.

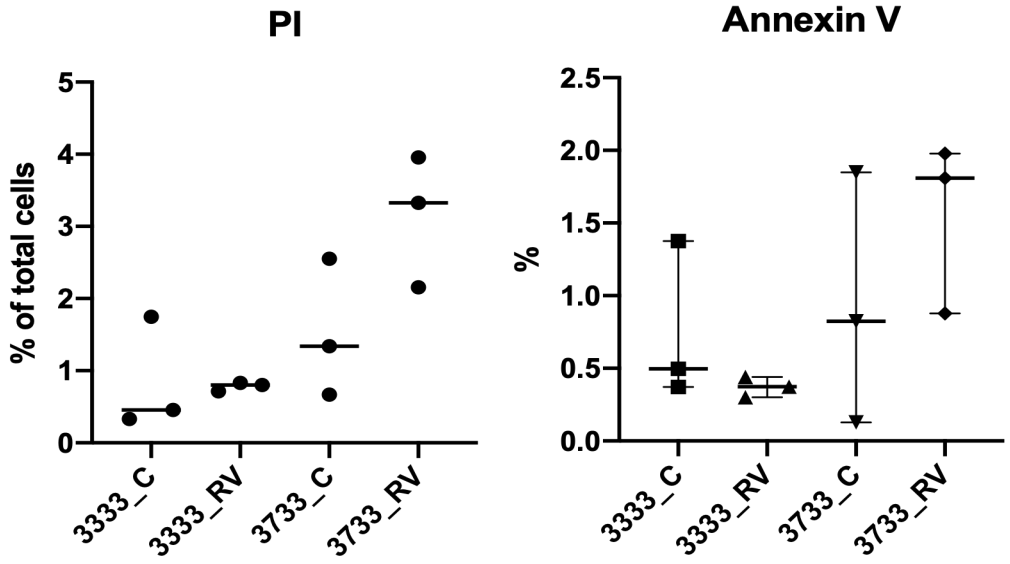

Figure 10: Measurement of the percentage of cells being PI and Annexin V positive after 24 hours incubation. All the samples displayed in the graphs were incubated at 33°C but samples 3733_C and 3733_RV were cultivated in 37°C prior to the experiment. The samples 3333_C and 3733_C were incubated with growth medium only, whilst samples 3333_RV and 3733_RV were incubated with 10^5 pfu/ml HRV14.

4.5 Temperature dependent Cytokine production in MRC-5

4.5.1 IL-8 (CXCL-8)

It was shown from previous studies that HRV infected cells up-regulate IL–8 mRNA expression (Etemadi *et al.,* 2017). In this study we examined the temperature effect on IL-8 production using an ELISA. In HRV infections the cell production of IL-8 is supposed to go up by a few magnitudes (Etemadi *et al.,* 2017), as was found in this study. What was also noticeable was that the uninfected cells would produce approximately the same amount of IL-8 independently of the temperature, but when the cells were inoculated with HRV the 37/37 sample increased 2-fold respectively 4-fold faster during the first 10 hours compared to the 37/33 or 33/33 samples and would later enter a decrease in value after the first day. In comparison the samples 33/33 and 37/33 would accumulate IL-8 throughout the experiment (*figure 11*).

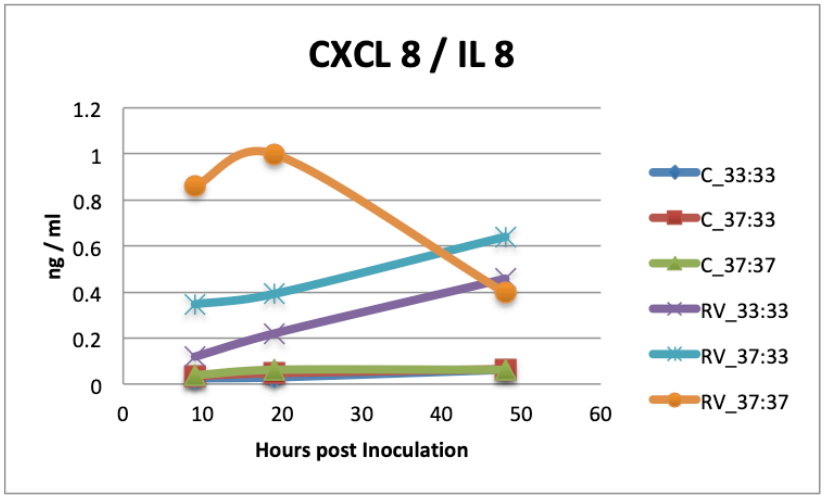

Figure 11: Graphs of the IL-8 production measured with an ELISA. The X-axis represents hours post inoculation and the Y-axis represents ng / ml. The samples that have a name beginning with "C_" (control) were only given new medium at time 0h. The samples that begin with "RV_" were given medium with 10^5 pfu / ml HRV 14.

4.5.2 TGF-ß

TGF-ß is constantly produced in MRC-5. The expression of TGF-ß is here used as a measure of normality. However, when infecting the MRC5 cells with HRV no decrease nor increase of TGF-ß could be found. The TGF-β expression had a slight tendency to increase with temperature. This tendency is however very minor in comparison to the temperature dependency in other cytokines and can be a result of a deviation in the cell count (*figure 12*).

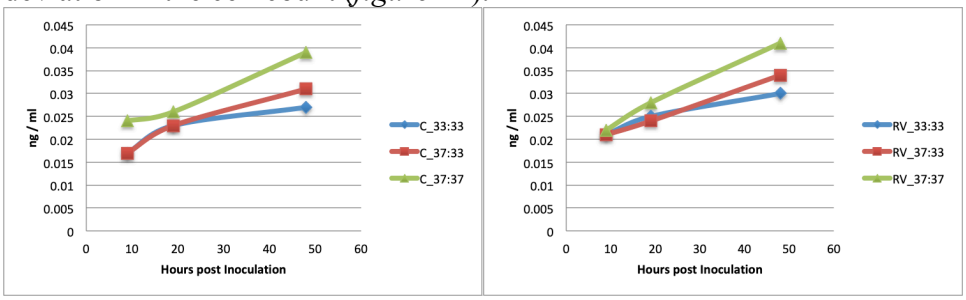

Figure 12: The TGF-ß productions in regards to temperature and HRV inoculation. The left graph shows un-inoculated MRC-5 cells. The right graph shows HRV 14 inoculated MRC-5 cells.

4.5.3 IL-1ß

No IL-1ß could be found in any of the samples even after 46hpi. (Data not shown)

4.5.4 IFN-alfa

The temperature dependence of the IFN expression has been examined a few times before but only on the RNA level (Foxman *et al.*, 2016). To ensure that the IFN expression remained silenced at the protein level an ELISA was conducted on the three setups 37/37, 37/33 and 33/33. No increase in IFN- alfa could be found on the protein level even after 46hpi in neither temperature setup (Data not shown). This was only done on the first experiment and was not done on the remaining experiments.

Discussion

During this project there were a few observations that were novel in regards to HRV infection. In the earlier studies there was no attention paid to what environment the cells had been in before the inoculation of the HRV, but here we found that the pre inoculation temperature did affect the response of the cells. We found that having the cells in a statically low temperature before and during infection (33/33) increased the viability and decreased the viral replication in comparison to the 37/33 samples. In AnnexinV & PI staining the death count was overall very low, under 4%, and there are a few possible explanations to why this was the case. First is the timing aspect, if cells would have been harvested on a later time point there is a high possibility that the ratios would have been higher. On the other hand there could have been a risk that a large part of the cells already died and hence would be washed away during the PBS wash step. The other possible explanation is that the virus inoculation titer was too low. The titer used here was the titer normally used in the literature (Stott & Heath, 1970) and in the laboratory. Modifications to the HRV titer could have allowed a bigger variation in the result. Because of the time constraint none of the above factors could be tested, but it would be necessary to do these follow up experiments in order to get a deeper understanding to how big of an impact this temperature variation has.

Other than the cell viability there was a big difference in CXCL-8 (IL-8) expression depending on temperature. At 37°C much more IL-8 was released into the cell culture supernatant after virus inoculation with a maximum value reached around the 20 hour mark and then decreasing at the later stages. Much lower amounts of IL-8 were produced at 33°C indicating an inability for cells at the lower temperature to communicate danger signals through this chemokine. IL-8 is pre-stored in membrane vesicles close to the outer cell surface (Utgaard *et al.,* 1988) and colder temperatures affect membrane fluidity negatively (Dymond, 2016 ; Hazel, 1995). Another possibility could be a difference in response between the temperatures 33° and 37° at the mRNA or translational level. We propose that the inability to communicate with certain cytokines/chemokines due to lower membrane fluidity might be an important factor to why the survival rates are so different depending on temperatures. In contrast to the IL-8, the TGF-β did not seem affected by neither HRV nor temperature. This is interesting as it means that TGF-β might be usable as a normalizer for the ELISA results.

In accordance to previous studies there was no IFNa protein expression, nor IL-β1 protein expression, found in the supernatant from the HRV infected cells. This confirms that, as expected, our HRV strain is blocking the pathways in the MRC-5 cells leading to production of these cytokines and that the results found in this study can be applicable to other studies as well.

Regarding the Western blots there are a few things that should be mentioned. There were interesting tendencies in the western blots developed. However, the quality of the Western blots in general was too low to give consistent, or in some cases, readable data. This in turn resulted in a too low number of replicates to draw any conclusions. The RNase L results were perhaps the only protein with clear stable results. It will be relatively safe to say that the RNase L levels did not vary significantly in relation to temperature, or after HRV infection. This would rule out the possibility that the basal level of RNase L or an up regulation of RNase L being

behind the temperature dependence which was suggested in an earlier study (Foxman *et al.,* 2016). The OAS3 quantification results have a slight tendency to be lower for the cells grown in 33°C in relation to the cells grown in 37°C, but this experiment has to be repeated. In the OAS3 western blot there was no visible up regulation in regards to HRV as well, which would be in agreement with previous findings stating that the OAS genes are regulated by IFN expression (Li *et al.,* 2015). It would have been good to have a positive control for the OAS3 experiments to see that the cells were indeed capable to up-regulate the OAS proteins. Adding IFN-α or IFN-β to the culture would most likely have been sufficient as a positive control.

The RIPK1 results were difficult to interpret. Sometime there would be bands of the expected sizes showing and other times there were not. This would happen almost independently of HRV or temperature making it very difficult to distinguish true positives or true negatives from false ones. There are a few possible explanations as to why this might be the case. Because of the low number of replicate experiments there is always the possibility of contamination between the wells. There could however be other reasons also. In the two successful Western blots the 60 k Da band on the HRV infected 37/37 sample were very weak in comparison to 37/33 and 33/33. The control samples of 33/33 would also consistently not show any lower weight band. To determine why and when these bands show up more replicates would be needed, as well as a positive control. The positive control could consist of a plasmid containing an inducible 3C protease to ensure that the cell is affected by the protease activity.

During the experiments all samples incubated at 37° post inoculation displayed no signs of necrosis nor apoptosis. This is in accordance to previous unpublished in-house data, suggesting that the different survival rates between the temperatures does not depend on the ability to activate apoptosis. This should be considered before pursuing further follow-up experiments.

Our findings are that suppression of IL-8 production at 33°C could hamper the necessary danger signal and thus explain some of the cold-induced sensitivity to HRV infections. We also found that cells grown in 37°C had a reduced resistance to HRV infection at 33°C. There could have been more conclusions to this study, but what needs to be addressed at a future study is the titer of inoculation. During the viral infections it seemed that not all cells got infected at the same time. It is likely that some cells were infected by a second generation virus and that only a part of the culture would have been under infection at any given time. This might give a blurred result when looking at changes over time. Furthermore, the fact that all dead cells were washed away before every measurement most likely amplified this effect. Increasing the titer of the virus to ensure that all cells get infected at the time of inoculation could have allowed a better view of the different stages of infection. It could also have been good to harvest and wash the dead cells and include them in the study.

Conclusions

The cells grown at 37°C had a higher susceptibility towards human Rhinovirus when infected at 33°C compared to cells constantly grown at 33°C. The 37/33 setup had a lower cell viability as well as a higher viral replication compared to the 33/33 setup.

There was a strong temperature dependency in IL-8 release from the cells when infected by the human Rhinovirus. The 37/37 setup had a 4 fold higher value than 37/33. The reason to this was not established.

No temperature dependence or viral dependence could be found in regards to TGF-β production.

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Appendix

A.1 Method for calculating the positive cells in Image J during AnnexinV and PI staining

1. To count the total amount of cells the Hoechts 33342 stain images were used.
   - 1.1 The colours was split in three channels, blue, green and red using *Image -> Colours -> split channels.*
   - 1.2 Discard the red and green split of the image and proceed with the blue.
   - 1.3 Remove the background using *Process / Subtract Background(input 10).*
   - 1.4 A binary image was created using *Image / Adjust / Threshhold, (input 25).*
   - 1.5 Double tops were removed using *Process / Binary / Watershed.*
   - 1.6 The cells were counted using *Analyse / Analyse particles.*

2. To count the PI stained cells the red filter was used.
   - 2.1. The colours was split in three channels, blue, green and red using *Image -> Colours -> split channels.*
   - 2.2. Discard the blue and green split of the image and proceed with the red.
   - 2.3. Remove the background using *Process / Subtract Background, (input 10).*
   - 2.4. If the noise is high, use *Process / Noise / Despeckle.*
   - 2.5. A binary image was created using *Image / Adjust -> Threshhold, (input 6.5).*
   - 2.6. The cells were counted using *Analyse / Analyse particles.*

3. To count the Annexin V stained cells the green filter was used.
   - 3.1. The colours was split in three channels, blue, green and red using *Image / Colours / split channels.*
   - 3.2. Discard the blue and red split of the image and proceed with the green.
   - 3.3. Remove the background using *Process / Subtract Background, (input 10).*
   - 3.4. Noise was removed using *Process / Noise / Despeckle.*
   - 3.5. A binary image was created using *Image / Adjust / Threshhold, (input 2).*
   - 3.6. The cells were counted using *Analyse / Analyse particles.*

A. 2 Images of HRV & mock infected MRC-5, 46 hpi

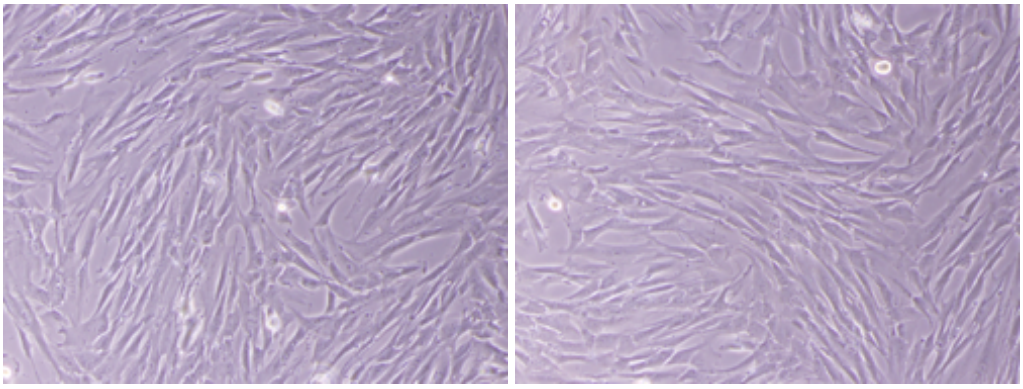

Figure 13: Images of sample 37/37 46hpi. The image to the left is the control well only receiving new medium at time 0 hours. The image to the right was inoculated with HRV 14 at time 0 hours.

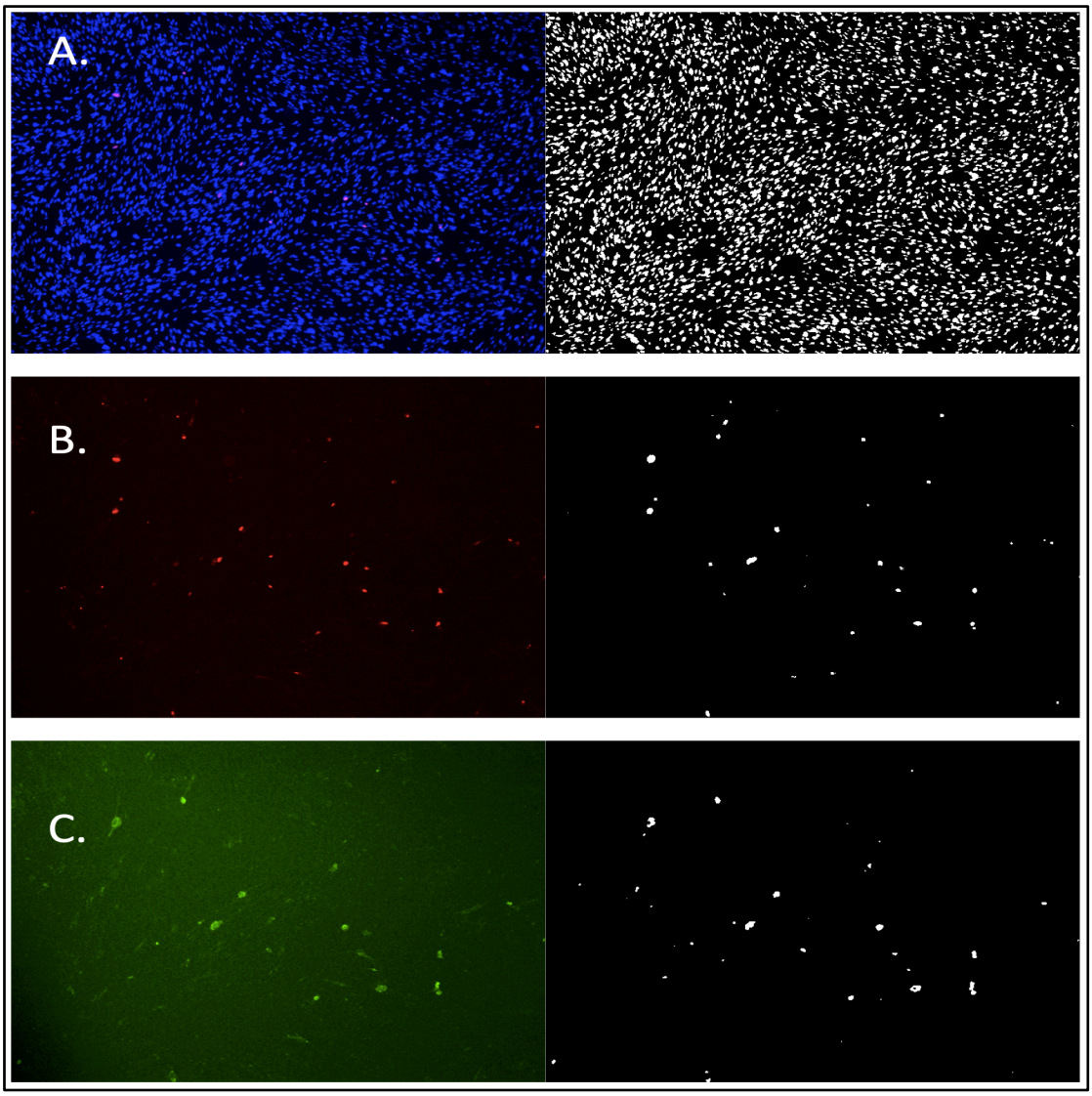

Figure 14: Examples of images and the final version of them after the processing, later the single dots were counted to give the results. A) Total amount of cells were counted using Hoechst. B) The PI stain calculation. C) The Annexin calculation.
Due to the calculation process it was difficult to separate single positives from double positives.