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ROLE OF SCHLAFEN FAMILY MEMBER 12 (SLFN12) IN HUMAN RHINOVIRUS (HRV) INFECTIONS

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Anna-Maria Kapsch, BSc

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Ass. Prof. Otto Majdic

Prof. Johannes Stöckl

Alexander Puck

Prof. Gerhard Zlabinger

Petra Cejka

Verena Berg

Madhura Modak

ABSTRACT

The mammalian Schlafen gene family (SLFN) has been implicated in the regulation of cell proliferation and differentiation as well as control of viral replication, such as of human immunodeficiency virus (HIV), vesicular stomatitis virus (VSV) and murine gammaherpesvirus 68 (MHV-68). SLFNs are inducible by type I interferons (IFNs).

Human Rhinovirus (HRV) infections are one of the most acute illnesses in humans worldwide and result in mild respiratory diseases like the common cold. HRV is a pathogen for humans and it is characteristic for this virus that its replication takes place only in the cytosol of epithelial cells.

Six human SLFN genes (SLFN5/11/12/12L/13/14) have been identified, whereby SLFN12 is the only human SLFN predicted to be located in the cytosol. Previously we found that SLFN12 is highly upregulated in dendritic cells (DCs) upon HRV stimulation. Therefore, we hypothesized whether SLFN12 might be involved in the regulation of HRV infection in human epithelial cells. HeLa-Ohio cells being highly susceptibility for HRV infections were chosen as a model for our in vitro studies.

The first finding of this study was that retroviral overexpression of SLFN12 in HeLa-Ohio cells, led to cell death and cells could not be further used for HRV infection experiments. Gene silencing impeded cell proliferation of HeLa-Ohio, but did not affect cell viability according to AnnexinV/PI staining. Moreover, we showed that silencing did not alter IL-6 production or expression of related cell markers such as intercellular adhesion molecule 1 (ICAM-1), myxovirus resistance gene A (MxA) and transferrin receptor (CD71). Most importantly, we found that replication of both HRV14, which uses ICAM-1 for cell entry, and HRV2, which enters via low density lipoprotein receptor (LDLR), was impaired in HeLa-Ohio cells upon SLFN12 silencing at the mRNA as well as at the protein level.

Thus, we conclude that SLFN12 is required for efficient HRV replication in HeLa-Ohio cells. We assume that SLFN12 is a novel cellular host factor for HRV replication, which might be involved in HRV translation as well as in transcription. Moreover, SLFN12 seems to be a rheostat that regulates the proliferation and viability of epithelial cells.

ZUSAMMENFASSUNG

Die Schlafen-Genfamilie (SLFN) ist in Säugetieren an der Regulation von Zellproliferation und Differenzierung, sowie der Kontrolle der viralen Replikation des humanen Immundefizienz-Virus (HIV), vesicular stomatitis virus (VSV) und murinen gamma-Herpesvirus 68 (MHV-68) beteiligt. SLFN-Gene sind durch Typ I Interferone induzierbar.

Infektionen durch humane Rhinoviren (HRV) sind eine der häufigsten akuten Krankheiten des Menschen weltweit und verursachen milde respiratorische Krankheitssypmtome, wie die Erkältung. HRV ist ein humanes Pathogen, dessen Replikation nur im Cytosol von Epithelzellen stattfindet.

Sechs humane SLFN (SLFN5/11/12/12L/13/14) konnten bereits identifiziert werden, wobei SLFN12 das einzige humane SLFN ist, welches im Cytosol lokalisiert ist. Wir konnten bereits zeigen, dass SLFN12 in Dendritischen Zellen (DZ) durch HRV Stimulation sehr stark hochreguliert wird. Daher nahmen wir an, dass SLFN12 möglicherweise an der Regulation von HRV-Infektionen in humanen Epithelzellen beteiligt sein könnte. Aufgrund ihrer Suszeptibilität für HRV Infektionen verwendeten wir HeLa-Ohio-Zellen als Modell für unsere in vitro-Experimente.

Wir konnten zeigen, dass retrovirale Überexpression von SLFN12 in HeLa-Ohio-Zellen zum Zelltod führte und daher nicht für weitere Experimente mit HRV genutzt werden konnte. „Silencing" des Gens hingegen hemmte die Proliferation der Zellen, beeinflusste jedoch nicht die Viabilität der Zellen laut AnnexinV/PI-Färbung. Darüber hinaus konnten wir zeigen, dass „Silencing" weder IL-6 Produktion noch die Expression verschiedener Zellmarker wie intercellular adhesion molecule 1 (ICAM-1), myxovirus resistance gene A (MxA) und des Transferrin-Rezeptors (CD71) beeinträchtigte. Vor allem aber haben wir festgestellt, dass die Replikation sowohl von HRV14, welches ICAM-1 als Rezeptor benutzt, um in die Wirtszelle zu gelangen, als auch von HRV2, das mittels low density lipoprotein receptor (LDLR) in die Zelle eindringt, in HeLa-Ohio-Zellen nach SLFN12 „Silencing" gehemmt war. Diese Hemmung konnte sowohl auf mRNA- als auch auf Proteinebene beobachtet werden.

Wir schließen daraus, dass SLFN12 für eine effiziente HRV Replikation benötigt wird. Wir nehmen an, dass SLFN12 ein neuartiger zellulärer Wirtsfaktor für die Replikation von HRV ist und womöglich sowohl in der Translation als auch in der

Transkription von HRV involviert sein könnte. SLFN12 scheint ein Regulator für die Proliferation und Viabilität von Epithelzellen zu sein.

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1 INTRODUCTION

1.1 The immune system: overview

The immune system (IS) is an organization of molecules, cells, tissues and organs that collectively function to provide immunity against foreign organisms [1]. Two types of immunity are used to protect the host from invading microbes [2]: innate immunity that provides first defense against infectious microbes and adaptive immunity that has to be established by activation of the innate one.

Epithelial barriers such as the skin or the mucosa of the gastrointestinal tract, and antimicrobial substances produced at epithelial surfaces are the first line of defense against entry of invaders [1]. Further components of innate responses include phagocytic cells (neutrophils, macrophages), natural killer cells (NK cells), the complement system and cytokines causing phagocytosis of microbes, activation of leukocytes or stimulation of inflammation [1]. Innate response occurs to the same extent however many times it is confronted with an infectious pathogen [3], whereas the adaptive immune system, which is composed of B-lymphocytes with their secreted antibodies (Abs) and T-lymphocytes, enhances on repeated contact to a given pathogen [3]. The main difference between innate and adaptive immunity is the way in which they recognize microorganisms [4]. Microbial sensors of the innate IS are the so-called pattern recognition receptors (PRR) [2], such as Toll-like receptors (TLR), which detect pathogen associated molecular patterns (PAMPs) of microbes. PAMPs can be structures/proteins, sugars or glycolipids [1], which are found in most microorganisms of a given class. Various cell types, such as macrophages, dendritic cells or epithelial cells, can express TLRs [1] either on their cell surface or on their endosomal membrane. Recognition of microbes through TLRs leads to induction of several events in innate and adaptive immunity [5]. Once TLRs are activated, signaling cascades are triggered resulting, in the production of inflammatory cytokines and chemokines, type I Interferon (IFN) and activation of diverse immune cells for elimination of the pathogen [6].

In contrast to innate immunity, the adaptive one is able to recognize specific parts (epitopes) of diverse proteins. Thus, adaptive immune responses are highly specific [2]. The receptors on B-lymphocytes recognize conformations of native antigen, whereas T-lymphocytes only recognize peptides [3].

1.2 Innate and adaptive immunity: antiviral response and viral counteractions

Viruses are obligate, intracellular parasites, which have to use the host cell machinery for their own replication. During decades of co-existence between viruses and host, viruses adapted and evolved strategies to escape the host´s immune response. However, several effective defense mechanisms of the host´s immune system have also evolved to detect and eliminate the invaders.

Innate immunity is mediated by type I IFNs and activation of NK cells, which are aimed against virally infected cells. Virus recognition by TLRs or cytosolic RNA helicases triggers signaling cascades, thereby generating type I IFNs, which induce an antiviral state in both infected and uninfected cells (see chapter 1.2.1). On the other hand, most viruses (i.e. Adenovirus, Human immunodeficiency virus-HIV) downregulate or alter major histocompatibility complex class I (MHC-I) resulting, in an activation of NK cells [1].

The effective mechanism of adaptive immunity is mediated by neutralizing Abs and cytotoxic T lymphocytes (CTLs). Abs either neutralize the virus by binding to the viral particle or opsonize it for phagocytosis/complement activation [1]. Cytotoxic $CD8^+$ T cells are the most important defense mechanism against viral infections. CTL activity requires antigen presentation via MHC-I molecules.

The immune system may provide several effective antiviral defense mechanisms, but viruses are continuously evolving to escape immune responses.

Antigenic variability of the viruses caused by infidelity of RNA polymerase avoids neutralization of Abs. Some viruses (i.e. Herpes simplex Virus-HSV, Mouse cytomegalovirus-MCMV, coronavirus) even encode Fc-receptors, which allow the Abs to bind to the infected cell, but inhibit Fc-dependent activation of the complement system and phagocytes [7]. Other mechanisms are encoding homologs of the complement system, thereby inhibiting its activation, or generating variable peptides that do not bind to MHC molecules [7].

Type I IFNs are the first line of defense against viruses; therefore various strategies, such as interference with diverse components of IFN-pathway or inhibition of IFN-stimulated antiviral genes, help the viruses to counteract this response.
In the recent years it has been observed that large DNA viruses encode viral cytokines (virokines) and soluble cytokine receptors (viroceptors) to modulate immune responses for their own benefits [7].

Infected cells recruit CTLs and NK cells, which induce apoptosis via secretion of cytokines, release of perforin and granzymes and activation of Fas in the target cell [1][7]. Viruses (i.e. Measles virus-MV, Human papillomavirus-HPV, Adenovirus) try to counteract this by inhibiting activation of caspases, inactivation of p53 or by encoding a homolog of the anti-apoptotic protein Bcl-2 [7].

Most viruses (i.e. Adenovirus, HPV, HSV, HIV) downregulate the MHC-I in order to avoid recognition by $CD8^+$ T cells. But this downregulation leads to NK cell activation as described by the missing "self" hypothesis [1]. To determine if a cell is infected or not, NK cells express two surface receptors: activating and inhibitory receptors, which recognize MHC-I alleles [1]. To avoid NK cell activation, viruses encode homologs of MHC-I as well as cytokine and/or chemokine antagonists/binding protein. Downregulation of activating ligands or expression of activating receptor antagonists are further mechanisms to escape NK cell eradication.

1.2.1 The Interferon-Pathway

Type I interferons (IFNs), belonging to the group of cytokines, are crucial components of innate immunity by indicating viral infections. In humans, 8 type I IFNs have been described, composed of IFN-α, which can be divided into 13 subtypes, IFN-β, IFN-ε, IFN-κ, IFN-τ, IFN-ω, IFN-ζ, and the pseudogene IFN-v [1][8]. All these IFNs have in common to bind to the same cell surface receptor consisting of two chains, IFN- alpha receptor 1 (IFNAR1) and IFNAR2 [1]. IFN-α is mainly produced by plasmacytoid cells and mononuclear phagocytes, IFN-β is produced by many cells such as fibroblasts [1]. These cytokines have the unique ability to inhibit viral growth by inducing an antiviral state in infected as well as in cells exposed to viruses. The IFN-pathway can be divided into two events: IFN-induction and IFN-signaling (Figure 1).

IFNs can be either induced via TLRs or cytosolic RNA helicases. Once these receptors are activated by viral products, they trigger a signal cascade resulting in induction of IFNs. TLRs signal either via myeloid differentiation protein 88 (MyD88) or Toll/IL-1 receptor domain containing adaptor inducing IFN (TRIF) [9]. MyD88 recruits TNF receptor associated factor 6 (TRAF6) and members of IL-1 receptor-associated

kinases (IRAK) family, thereby leading to phosphorylation of the IκB kinase (IKK) complex (consisting of IKKα/β/γ), an inhibitor of nuclear factor kappa light-chain-enhancer of activated B cells (NFκB) [9]. Moreover, MyD88 also phosphorylates mitogen-activated protein kinase (MAPK), which then activates the transcription factor activating protein 1 (AP-1) [9].

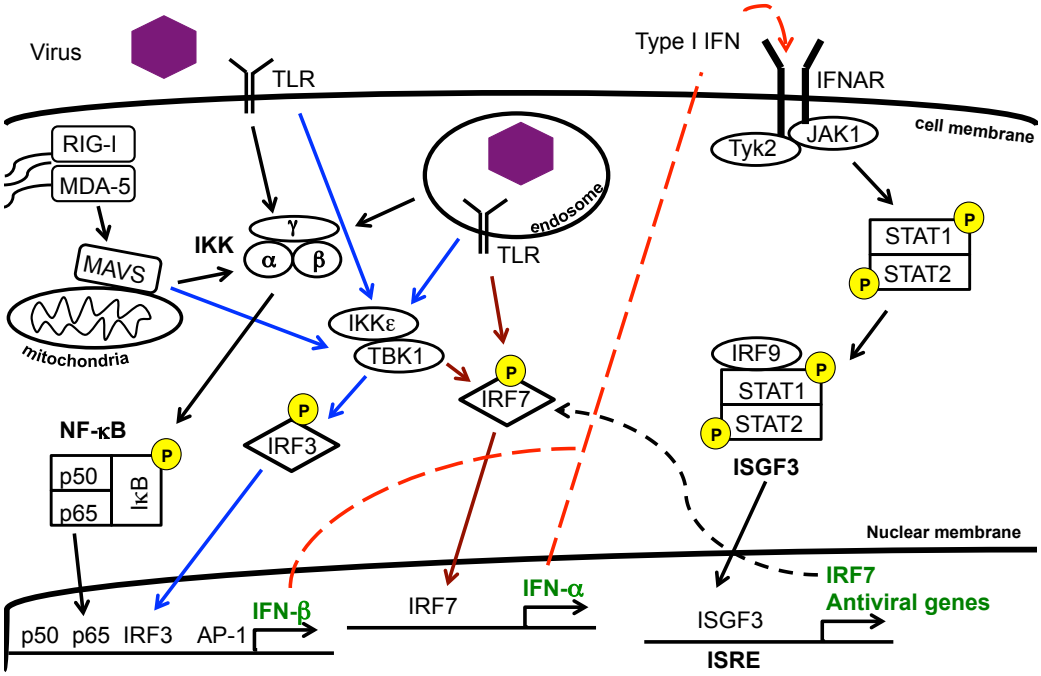


Figure 1: Type I interferon pathway
TLRs, which are located at the cell membrane or in the endosome, detect the virus and trigger signal cascades. Activation of IKK complex (IKKα/β/γ) leads to phosphorylation of the NFκB inhibitor IκB. Moreover, TBK1 and IKKε are recruited and phosphorylate IRF3 and IRF7. The cytosolic helicase RIG-I and MDA5 recognize dsRNA and recruit MAVS, leading in activation of NFκB and IRF3/7. IRF3, AP-1 and NFκB subunits p50/p65 translocate into the nucleus and induce expression of IFN-β. Type I IFNs are then released from the cell and bind to their receptor resulting in phosphorylation of Tyk2 and JAK1, which in turn phosphorylate STAT1/2. Heterodimers STAT1/2 form together with IRF9 the ISGF3 complex, which translocates into the nucleus to induce transcription of antiviral genes and IRF7. Based on [11]

TRIF recruits not only TRAF6 for NFκB activation, but also TRAF3, which in turn recruits the IKK related kinases TANK-binding kinase 1 (TBK1) and IKKε [9]. Both phosphorylates then IFN regulatory factor 3 (IRF3) and 7 to induce their nuclear translocation [9]. While TLR4 and endosomal TLRs (TLR3/7/8/9) lead to generation of type I IFNs (IFN-α and IFN-β), cell surface TLRs induce production of inflammatory cytokines [9]. However, it was shown that TLR2 triggers the generation of IFNs in inflammatory monocytes infected with vaccinia virus [9]. The endosomal TLRs 7/8/9

mediate induction of IFNs via MyD88 resulting in activation of IRF7, NFκB and AP-1 [9]. TLR3, an endosomal receptor, is the only TLR that signals via TRIF leading to activation of NFκB and IRF3 [9]. TLR4 is able to activate both MyD88- and TRIF dependent pathways [9].

The cytosolic receptors retinoic acid-inducible gene 1 (RIG-I) and melanoma differentiation-associated protein 5 (MDA5) bind via caspase recruitment domain (CARD)-CARD interactions to mitochondrial antiviral signaling (MAVS), also known as IFN-β promoter stimulator 1 (IPS-1), virus induced signaling adaptor (VISA) and CARD adaptor inducing IFN-β (Cardif) [8]. MAVS recruits TRAF proteins leading in activation/recruitment of MAPK, IKK complex and IKK related kinases [8]. The transcriptional factors NFκB, AP-1 and IRF3 initiate IFN-β production, while IRF7 induces IFN-α production. IRF7 in contrast to IRF3 is normally not present in most cell types. It is restricted to plasmacytoid DCs.

Type I IFNs are then released by the cell and bind to their receptor IFNAR1/2. Thereby phosphorylating the receptor associated kinases tyrosine kinase (Tyk2) and janus kinase 1 (Jak1) as well as the signal transducer and activator of transcription 1 and 2 (STAT1/2) [10]. The heterodimers STAT1/2 form then together with IRF9 the so-called IFN stimulating gene factor 3 complex (ISGF3), which translocates into the nucleus and activates IFN stimulating genes (ISGs) by binding to IFN stimulated response element (ISRE) [10]. One of these ISGs is IRF7, which has a positive feedback loop if viral infection is persistent. Some of these expressed genes have antiviral activity such as oligoadenylatesynthetase (OAS), myxovirus resistance (Mx) genes and Protein kinase R (PKR) [11]. In humans only MxA has antiviral activity by sequestering viral nucleocapsids for degradation [11]. In addition, the OAS proteins generate 2'-5' oligoadenylates, which activate ribonuclease L (RNase L) that degrades viral as well as cellular single stranded RNA (ssRNA) [11]. PKR phosphorylates translation initiation factor 2a (eIF2a) upon recognition of double stranded RNA (dsRNA), resulting in inhibition of cellular and viral translation [10].

1.3 HRV

1.3.1 Taxonomy

Human rhinovirus (HRV), the major cause of common cold, was isolated by Dr. Price at John Hopkins University in 1956 [12]. Belonging to the family *Picornaviridae*, genus *Enterovirus* [13], more than 160 different serotypes could be identified [14]. Whole genome sequencing and analysis of all known HRVs grouped them into three species: HRV A, HRV B and HRV C [15]. Recently, more HRVs of type C could be identified in clinical specimens [13]. The species can be further divided based on receptor specificity. The major group, including more than 90% of HRV A and B, binds via intercellular adhesion molecule 1 (ICAM-1) to the cell surface [16], while the remaining HRV A (minor group) uses low density lipoprotein receptor (LDLR) for cell entry [17]. Some major group viruses are also able to use heparin sulfate proteoglycans as a receptor [13]. The receptor for HRV C is still unknown.

1.3.2 Major cause of common cold

HRV is transmitted from human to human via direct contact, fomites or aerosols [18]. The optimal replication temperature for HRV is 33°C, the temperature of the nasal passages [19]. Therefore the HRV infection in normal individuals causes only mild symptoms like coughing, sneezing, rhinorrhea and nasal obstruction and is only limited to the upper respiratory tract [12]. However, it could be shown that some strains are not significantly hampered at 37°C [19]. Due to this, HRV is also capable of infecting the lower respiratory tract in patients who suffer from asthma or other chronic lung diseases [12]. HRV does not cause cytopathology like other respiratory viruses (i.e. influenza, respiratory syncytial virus). However, HRV is able to disrupt the epithelial barrier function, specifically it may cause the disruption of tight junctions, thereby increasing the vascular leakage and mucus secretion [12][18]. In most cell lines a cytopathic effect of the virus is visible, although non-cytopathogenic HRVs have been reported too [18][20].

1.3.3 Genetics

HRVs are small icosahedral (27-30 nm in diameter), non-enveloped viruses with a positive sense (+ve) ssRNA genome (ca. 7200 bp). After cell entry RNA is translated into a polyprotein that is proteolytically cleaved into structural (capsid) and non-structural proteins [13]. There are 60 copies (promoters) of each of the four capsid proteins (VP1, VP2, VP3, VP4) (Figure 2). The viral protein 1 (VP1), VP2 and VP3 are located on the viral surface and are the reason for antigenic diversity of the virus, whereas VP4 is located internally and is responsible for anchoring the RNA core to the capsid [18] as well as for assembly of the virus and infection of new cells [21]. Within the VP1 is a 20Å depression ("canyon"), which was hypothesized to be the site of receptor attachment in HRV14 [22,23]. The so-called canyon hypothesis suggests that only small cellular receptors would fit into this depression, whereas antibodies would be hindered to bind to the canyon [23] (Figure 2). This allows the virus to escape immune response. Colonno et al. and Pevear et al. confirmed the involvement of the canyon in receptor binding [23][24,25]. But, another crystal structure analysis revealed that antibodies exist that bind within the canyon [26].

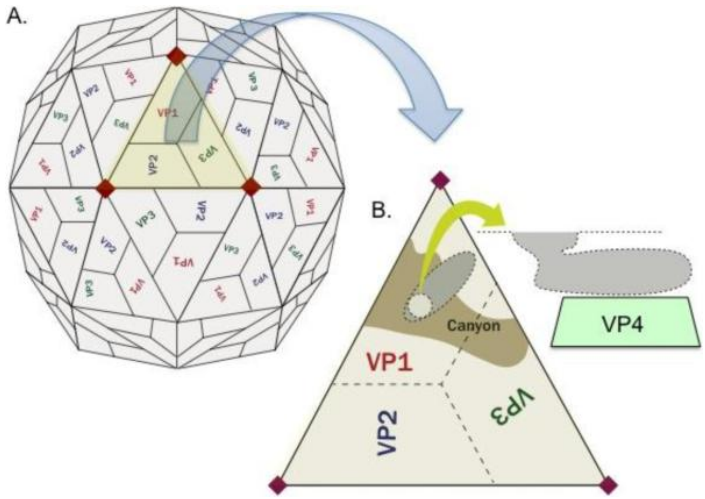


Figure 2: Capsid structure of HRV
(A) HRVs are small icosahedral, non-enveloped viruses with a positive sense ssRNA genome. There are 60 copies (promoters) of each of the four capsid proteins (VP1, VP2, VP3, VP4).
(B) Within the VP1 is a 20Å depression ("canyon"), which was hypothesized to be the site of receptor attachment in HRV14 [22,23]. VP4 is located internally and is responsible for anchoring the RNA core to the capsid [18] as well as for assembly of the virus and infection of new cells [21]. Figure was adapted from [12]

1.4 Life cycle

HRV has a short 8-12h life cycle, which involves following single step events: (1) attachment to the cell, (2) receptor mediated endocytosis, (3) uncoating (release of viral RNA), (4) RNA penetration, (5) synthesis and processing of viral proteins, (6) RNA replication, and (7) assembly and release of new, infectious virions (Figure 3) [13]. The replication cycle takes place in the cytosol of an infected cell.

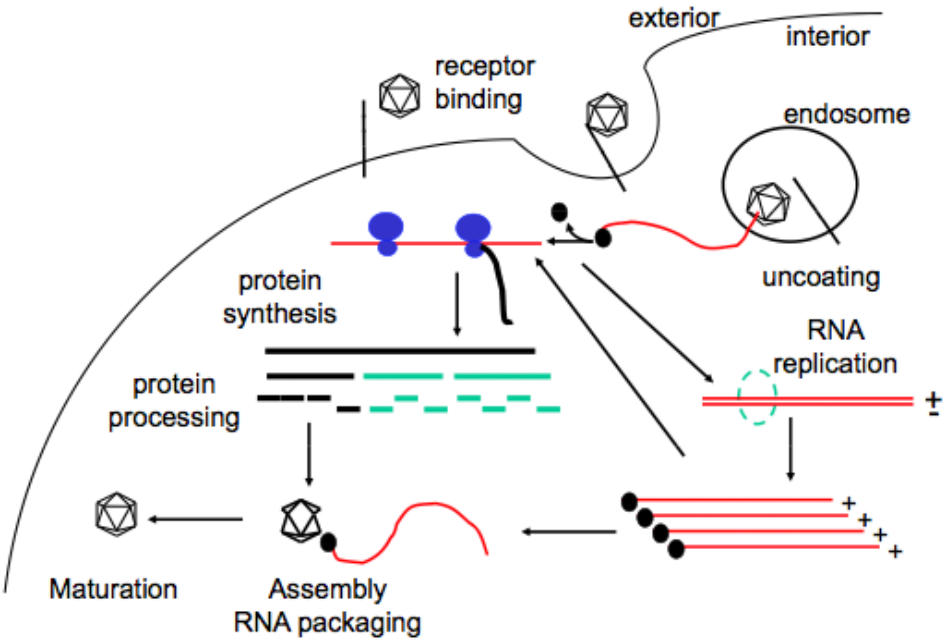


Figure 3: Life cycle of HRV2
Attachment of virus to its receptor leads to internalization of the virus into endosome. During development from early to late endosome, pH within the endosome drops and viral RNA is released into the cytosol. RNA is translated into a large polyprotein, which is further processed into mature proteins. If there are enough proteins produced, translation stops and RNA synthesis of pos. and neg. strand starts. Neg. strand serves as template for pos. strands. After replication pos. strands can either be used for translation/replication again or are packaged into viral particles. Viral particles are released by cell lysis. Figure was adapted from [83]

1.4.1 Attachment, cell entry and uncoating

HRV infection starts with attachment of virus to its cellular receptor. The two species HRV A and HRV B bind either intercellular adhesion molecule 1 (ICAM-1, also known as CD54) (major group) [16] or LDLR (minor group) [17].

Uptake of the virus is different for major and minor group. While major group viruses such as HRV14 enter the cell via clathrin and caveolin independent endocytosis, minor group viruses such as HRV2, enter via clathrin, dynamin dependent endocytosis [21]. In HeLa cells HRV14 uptake is indeed dependent on dynamin [21]. HRV2 has also the ability to enter HeLa cells via cytosol acidification or overexpression of non-functional dynamin, if clathrin-dependent endocytosis is inhibited [21].

Although HRV preferentially infects human bronchial epithelial cells, HeLa cells get widely used for in vitro studies of viral life cycle. The original HeLa cell line was derived from a cervical cancer patient, called Henrietta Lacks. It is the oldest human and first immortal cell line, which could be cultivated. Their quick doubling time (24h) and susceptibility to viruses (i.e. HRV, Coxsackie virus), makes HeLa cells an attractive model for in vitro studies of viral replication.

After cell entry, conformational changes in the viral capsid are either triggered by receptor binding or by low pH in the environment [21]. Uncoating of major group viruses are catalyzed by their receptor ICAM-1 depending on pH (5.5 to 6.0) [27] and temperature [13]. Internalization of HRV14 with the receptor in early endosomes was found to occur at 20°C [13], while penetration from late endosomes into the cytoplasm via membrane destabilization and rupture occurs at ≥ 26°C [13,21].

In contrast to the major group receptor, the minor group one does not catalyze uncoating [13]. In the early endosome the receptor of HRV2 dissociates from the virus and gets recycled to the plasma membrane [28]. Viral RNA is then translocated into the cytoplasm via a pore in the endosomal membrane [21,28].

1.4.2 Synthesis and processing of viral proteins

Once the viral RNA is in the cytosol, translation immediately takes place. Picornaviruses lack the 5’ cap structure and therefore translation has to be cap-independent. The 5’ untranslated region (UTR) contains an internal ribosome entry site (IRES), which initiates translation (Figure 4) [29].

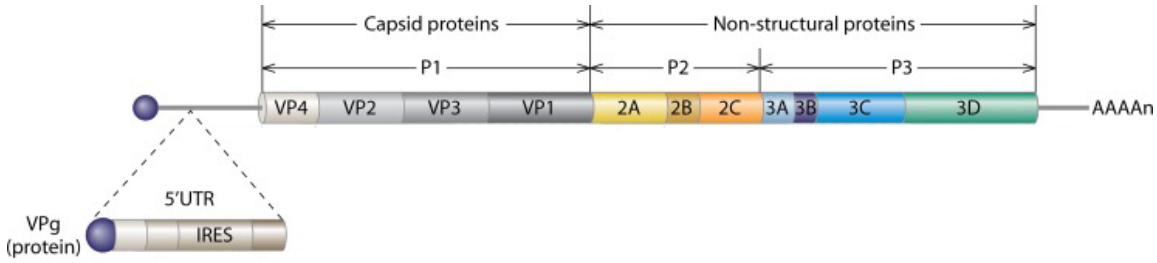


Figure 4: Genomic structure of HRV
HRVs have single stranded positive sense RNA genome (7,2 kb) with a single open reading frame. The 5' untranslated region (UTR) contains an internal ribosome entry site (IRES) and VPg. This large polyprotein is proteolytically cleaved into structural (capsid) and non-structural proteins. Figure was adapted from [18].

Entero- and rhinoviruses use a type I viral IRES [29]. The ribosome binds at an AUG of the IRES downstream of an oligopyrimidine tract of about 25 nucleotides (nt) [29]. However, initiation finally occurs at the next AUG further downstream at about 35 nt in rhinoviruses [29]. The produced single polyprotein is subsequently processed to precursor and later to mature proteins [30]. The proteolytic cleavage of the polyprotein into capsid (P1) and non-structural proteins (P2, P3) is performed by the 2A protease ($2A^{pro}$). The remaining cleavages are performed by $3C^{pro}$ [30][31]. These proteases are not only responsible for polyprotein processing, but they also alter several host cell proteins for enhanced viral translation and replication [30]. 2A protease cuts specifically between eIF4E binding site and eIF4G. The N-terminal domain of eIF4G binds the cap-binding protein eIF4E, while the C-terminal domain binds eIF4A, an ATP dependent helicase, eIF3 and the 40S ribosomal complex [32, 33]. Cleavage of both eIF4GI and eIF4GII leads to shut-off of host translation [32, 33]. It was also shown that $2A^{pro}$ cleaves nuclear pore complex (NPC) proteins known as nucleoporins (Nup62, Nup98, Nup153), which in turn lead to accumulation of nuclear proteins to the cytoplasm and to inhibition of their import to the nucleus [30,31][34]. However, IRES-mediated translation also requires canonical initiation factors and IRES trans-activating factors (ITAFs) to facilitate cap-independent translation [31]. Polypyrimidine tract-binding protein (PTB), a member of the hnRNP family, functions as splicing factor in uninfected cells and in infected cell as stimulator for IRES-mediated translation [30,31]. Other factors that are subverted for viral translation include poly (rC) binding protein 1,2 (PCBP1,2), lupus autoantigen (La) [30, 31] and the cellular protein upstream of N-ras (Unr) [35,36].

1.4.3 RNA replication

The virus does not only use the +ve RNA template for translation, but for replication too. Translation starts at the 5' end, whereas replication at the 3' end. But these two events do not occur at the same time. Increased levels of viral proteins for RNA replication and cleavage of host cell proteins lead to inhibition of translation. Two host proteins PTB and PCBP2 are hypothesized to mediate switch from translation to replication [30,31]. Cleavage of these two host proteins is performed by the viral 3C protease [30,31][37]. According to Gamarnik et al. binding of the viral precursor 3CD together with PCBP2 to the cloverleaf (CL) at the 5' end promotes viral replication [37]. VPg (3B, virion protein genome linked) acts as a primer for both positive- and negative strand RNA synthesis [31][38]. It is covalently linked to the 5' end of the viral RNA [38] and is cleaved by 5´-tyrosyl-DNA phosphodiesterase-2 (TDP2), termed "VPg unlinkase", in early stage of infection [39]. Before VPg can act as primer, it has to be uridylated by the 3D viral polymerase [31][40]. Recently, it was demonstrated that VPg is not required for translation or replication [40]. Why VPg is still released if it does not affect these two processes is not clear [40]. The generated negative strand serves then as template for positive-strand RNAs. All synthesized RNAs contain a VPg again that is covalently linked to the 5´end. After replication positive strands can either be used for translation/replication or are packaged into viral particles.

1.4.4 Assembly and release of new infectious virions

The final step of virion assembly already starts with the cleavage of P1 into VP0, 1 and 3. The 5S promoter, consisting of one copy of each VP0, VP1, VP3, assembles into 14S pentamer, which form together with the plus strand VPg RNA a 150S provirion [33]. The provirion is not infectious yet until auto-catalytically cleavage of VP0 in VP2 and VP4 has occurred [33]. After a final maturation step viral particles are released by cell lysis. Seipelt et al. showed that the 2A protease cleaves cytokeratin 8, which forms together with actin filaments and microtubules the cytoskeleton, in HeLa cells [41].

1.5 Immune response to HRV

HRV infects primary epithelial cells of the upper and lower respiratory tract, thus leading to an induction of innate and adaptive immune responses. During HRV infection, immune response is initiated by detection of viral components via TLRs and cytoplasmic RNA helicases [12]. TLRs and other PRRs trigger an enhancement of cytokine production [12], which in turn induce an accumulation of inflammatory cells to the site of infection [42]. Triantafilou et al. observed that HRV6 is recognized by TLR2, whereas, once internalized, ssRNA is detected by endosomal TLR7 and TLR8 [43]. TLR3 is known to recognize dsRNA and was shown to be upregulated in BEAS-2B epithelial cells upon HRV16 infection [44]. Viral dsRNA can be also detected by the RNA helicases RIG-I and MDA-5 [45,46]. MDA-5 recognizes long strands of dsRNA, while RIG-I recognizes ssRNA with a 5'-triphosphate and dsRNA not longer than 23 nt [43][47]. Several studies concluded that MDA-5 and not RIG-I is critical for picornavirus detection [43,47][48]. However, Slater et al. demonstrated that the coordinated role of both RNA helicases together with TLR3 are crucial for the induction of innate response, especially IFN-response, to HRV [45]. Further it was reported that not only MDA-5, but also RIG-I is cleaved during picornavirus infection [46]. Taken together, these findings indicate that these receptors might cooperate during infection.

Activation of these receptors result in the production of several cytokines including: IL-1β, IL-6, IL-11, IL-12, IL-15, tumor necrosis factor-α (TNF-α) and IFNs [12,18,42][49-52]. Additionally, growth factors, such as granulocyte-colony stimulating factor (G-CSF) and granulocyte monocyte-colony stimulating factor (GM-CSF), and chemokines, such as IL-8, epithelial cell-derived neutrophil activating peptide 78 (ENA-78), interferon-gamma induced protein 10 (IP-10) and RANTES, as well as vasoactive peptides bradykinin and lsylbradykinin were shown to be secreted by infected epithelial cells [12,18]. The cytokines IL-12, IL-15 and IFN are crucial for activation and recruitment of NK and $CD8^+$ T cells [12]. While IL-6 plays a role in Ab production and T-cell activation, IL-8 attracts neutrophils to the site of infection [51]. The proinflammatory cytokines IL-1β, IL-8, IL-6, TNF-α and IFN-γ are known to upregulate expression of ICAM-1 [50,51]. TLRs and RNA helicase proteins trigger a signal cascade leading to activation of NFκB. NFκB is associated with the induction of IL-6, IL-8 [52][53], TNF-α (secreted by airway macrophages) [49] and expression

of ICAM-1 [54]. Now it is known that common cold symptoms are caused by the burst of inflammatory cytokines responding to HRV infection and not by the virus itself [12].

Neutralizing Abs against HRV, especially secretory IgA (SIgA), are detectable in the serum after 1 week upon infection [48]. In contrast to SIgA, serum IgA (sIgA) and IgG do not increase before 6 weeks [48]. During secondary infections serum Abs may be detectable between 1 and 2 weeks after exposure, reaching their maximum levels after 5 weeks and can persist, above all IgG, for at least 1 year [48]. Neutralizing Abs do not play a role in recovery from infection due to their late appearance, when symptoms have already declined.

Lymphocyte and neutrophil infiltration can be found in nasal secretions during infection [18]. T cells are recruited by the secreted factors IP-10 and RANTES [18]. Gern et al. reported cross reactivity of T cells for different serotypes [55]. In vitro it was demonstrated that major group HRV16, bot not minor group (HRV1A), activates directly both $CD4^+$ and $CD8^+$ T cells independent of antigen presentation [56]. The role of $CD8^+$ T cells in HRV infection is still not completely understood.

1.6 HRV immune evasion

HRV uses the host cell machinery for its own translation/replication by altering cellular functions. Although the host´s immune system recognizes the invader, HRV is able to persist due to its different evading mechanisms. Diversity of serotypes and a high mutation rate caused by the infidelity of RNA-polymerase may be the two key mechanisms for a successful infection of HRV.

Modulation of different components of IFN-induction pathways is one of the major targets of viruses due to the high antiviral activity of IFNs. Certain types of HRV (HRV16, HRV1a) are able to degrade the PRRs, MDA-5 and RIG-I [46]. In another study cleavage of IFN-β promoter stimulator 1 (IPS-1), also known as MAVS, VISA or Cardif, was shown during HRV1a infection [57]. This cleavage might be concluded by either of the two viral proteases, 2A or 3C [57]. However, their findings also indicated that caspase-3 can cleave IPS-1 in vitro too [57]. It is known that certain HRVs induce apoptosis during infection, thus leading to activation of caspase-9 and caspase-3 [57]. Furthermore, it was observed that the protease 3C of HRV14 cleaves p65-RelA, a component of NFκB, thereby leading to interruption of NFκB-

dependent immune responses [38]. Recently, it was reported that HRV-induced IFN-stimulated gene of 15 kDa (ISG15) could modulate immune responses via RIG-I and regulation of IP-10 production [58].

Although leukocytes are not permissive for HRV, the virus is able to bind to these cells via ICAM-1 [59]. ICAM-1 does not only serves as receptor for binding of major group viruses, but play also a role in adhesion and migration of leukocytes through endothelial via ICAM-1/LFA-1 (CD11a/CD18) [59]. Interestingly, binding of HRV14 to ICAM-1 on human mononuclear phagocytes results in aggregation of monocytes and monocyte-derived DC, but not in lymphocytes [59]. These cells might be retained longer at the infected site, leading to a delayed emigration of these cells to lymph nodes, which in turn causes a delayed activation of lymphocytes [59]. In another study it was demonstrated that HRV16 inhibited antigen-specific T cell proliferation and cytotoxic T cell responses [59][60]. This effect only occurred when T cell proliferation was mediated by tetanus toxoid, Candida species or ragweed, but not by IL-2, irradiated allogeneic T cell line or mitogens [59][60]. Even UV-inactivated virus was able to inhibit proliferation [60].

Data from different studies suggest that both major and minor HRVs upregulate their own and the other group receptor on epithelial cells to increase susceptibility to HRV infection [51,54][61]. While HRV2 is reported to upregulate LDLR via NFκB and SP1 [61], HRV14 induces its receptor expression via NFκB [51,54] and phosphorylation of intracellular tyrosine kinases [62]. Whiteman et al. showed further that HRV modulates selectively expression of both forms, membrane-bound and soluble, of ICAM-1 [62]. While membrane-bound ICAM-1 gets upregulated, the virus simultaneously downregulates the soluble form due to its antiviral properties [62].

In addition, HRV increases IL-10 secretion, an anti-inflammatory cytokine, by monocytes, probably to counter the proinflammatory response [63]. IL-10 does not only prevent production of proinflammatory cytokines, but also leads to downregulation of CD86 as well as inhibition of IL-12, a T cell stimulating factor, upon HRV14 infection of monocytes [63].

On the other hand, modulating functions of DC, which lead to impaired T cell responses, is another popular strategy of viruses evading immunity. Major group viruses modulate DCs via upregluation of B7-H1 (CD274) and sialoadhesin (Sn, Siglec, CD169) resulting in induction of IL-35 producing regulatory T cells (Treg)

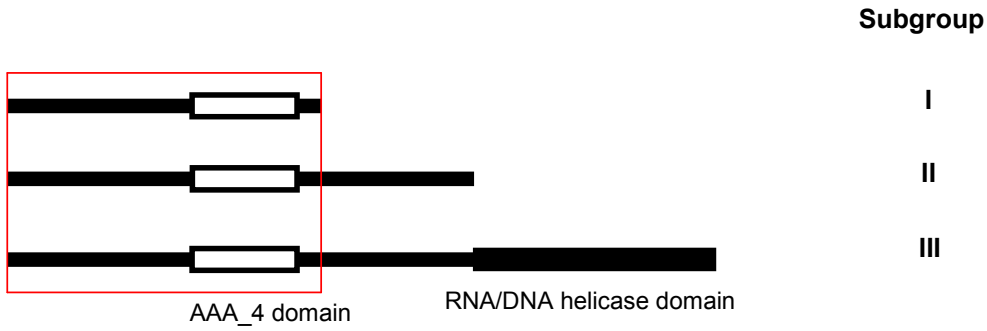
[59][64]. B7-H1, a member of the B7 molecule family, has inhibitory effects through its receptor PD-1 on T cells and is involved in the induction and maintenance of T cell anergy in IL-10 treated DCs [59]. Sn, a member of sialic acid binding lectin family of type I lectins, is known to bind to sialylated carbohydrate structures [59]. Induction of Tregs results in limitation of excessive immune responses.

In summary, these findings indicate that alteration of differential immune mechanism by HRV might cause adverse effects on local immunity, thereby making affected individuals more liable to secondary infections [59].

1.7 Schlafen gene family

The Schlafen (SLFN) gene family, first discovered in mouse by Schwarz et al. [65], includes several mouse and human member genes that are involved in immune processes [66-68], control of cell proliferation [65,67] and regulation of viral replication [67,68]. The genes are preferentially expressed in the lymphoid tissue and differentially transcribed during thymocyte maturation [65]. The expression levels vary during T cell and macrophage development as well as in response to infections [66]. While Slfn1, Slfn2 and Slfn4 were found to be expressed mainly in the thymus, spleen and lymph nodes, Slfn3 was detectable in testis [65,67]. The Schlafen genes are not only associated with several autoimmune disorders in human and mouse, but also with embryonic lethality, meiotic drive [66] and orthopoxvirus virulence [66][69]. The gene family can be divided into three groups based on their size (Figure 5) [67]. Group I includes proteins with a molecular mass between 37 and 42 kDa, group II between 58 and 68 kDa and group III between 100 and 104 kDa [67]. All SLFNs contain a SLFN box, which lies near an AAA domain that has ATP binding activity [66,68][70,71]. Group III of SLFNs also contains a motif that is homologous to the superfamily I of RNA helicases [66,68,70]. This suggests that SLFN gene domain is involved in RNA metabolism or RNA structure modeling activity [67,71]. Group III SLFNs contain a nuclear localization signal that might be involved in nuclear mechanisms [67,71]. Another SLFN-specific domain is the so-called SWADL domain, which is defined by a certain amino acid sequence (Ser-Trp-Ala-Asp-Leu), and is restricted to group II and III [67,70,71].

A



B

Subgroup	Murine Slfns	Human SLFNs
I	slfn1, slfn2	
II	slfn3, slfn4, slfn14	SLFN12, SLFN12L, SLFN14
III	slfn5, slfn 8, slfn9, slfn10	SLFN5, SLFN11, SLFN13

Figure 5: Domain structure and subgroups within SLFN family
(A) Schematic representation of domain structure
(B) Division of murine and human SLFNs into subgroups
Figure was adapted from [84]

10 known mouse SLFN genes could be identified (Slfn1, Slfn2, Slfn3, Slfn4, Slfn5, Slfn8, Slfn9, Slfn10 and Slfn14) [66,67]. Slfn1 and Slfn2 are the shortest ones and belong to group I, Slfn3 and Slfn4 belong to group II and group III includes Slfn5, Slfn8, Slfn9, Slfn10 and Slfn14 [66-68]. Slfn2 contributes to an immune response, to differentiation and to cell growth [67]. A mutation in mice renders them more susceptible to bacterial and viral infections [67]. Knockdown of Slfn2 leads to a decreased activation of c-jun and expression of NFATc, which in turn decreases osteoclastogenesis [67,68][72]. Furthermore knockdown increases anchorage independent growth [67,68,72,73]. Slfn2 is involved in the control of malignant cell proliferation too [67,72][73]. Slfn1 and Slfn2 control cell growth by regulating expression of cyclin D1 [67]. Slfn1 and Slfn8 transgenic mice reduce thymus size and thymocyte proliferation [65,66]. Ectopic expression of Slfn1 mouse fibroblasts leads to inhibition of cell growth, whereas induction of Slfn1 causes cell cycle arrest [65]. Slfn3 is upregulated during T cell activation, whereas it is downregulated upon activation and proliferation in $CD4^+$ $CD25^+$Tregs [67][74]. It has a major role in regulating intestinal differentiation, development and maturation [67,74]. It has been evidenced that Slfn3 can also be anti-proliferative and functions as negative regulator

of cell growth [67,74]. Neuman et al. found out that group I and group II SLFNs are localized in the cytoplasm, while group III is mainly localized to the nucleus [71].

6 SLFNs (SLFN5, SLFN11, SLFN12, SLFN12L, SLFN13 and SLFN14) could be identified in the human [67]. Human SLFN12 was found in the cytoplasm and belongs to group II [67]. Only little is known about SLFN12. It lacks the helicase domain compared to the other human SLFNs [67]. SLFN12 is upregulated in primary human melanocytes upon IFN-α treatment, but suppressed in all different malignant melanoma cell lines [72]. In a large screen SLFN12 was identified to moderately inhibit replication of Vesicular stomatitis virus (VSV) and murine gammaherpesvirus (MHV-68) [75].

SLFN5, SLFN11, SLFN13 and SLFN14 have all a helicase domain as well as a nuclear localization signal and they belong to group III [67]. Two of these SLFNs, SLFN5 and SLFN11, were studied extensively. SLFN5 knockdown causes increased anchorage independent malignant melanoma cell growth and increased invasion of malignant melanoma cells through collagen [67,72]. SLFN11 appears to be an attractive target for the development of antiviral drugs and anticancer agents. It was reported that SLFN11 blocks viral protein synthesis of retrovirus such as HIV on the basis of codon-usage discrimination [76]. Furthermore it plays a role in sensitizing malignant cells to topoisomerase inhibitors, alkylating agents and other DNA damaging agents [67]. Several studies have demonstrated that all SLFNs, human and mouse, are inducible by type I IFNs [67].

2 AIM OF THE STUDY

One of the most frequent infectious diseases worldwide is common cold caused by HRV. HRV is a specific pathogen, the replication of which takes place only in the cytosol of epithelial cells.

However, mammalian Schlafen gene family members have been implicated in the regulation of cell proliferation and differentiation as well as control of viral replication, such as of human immunodeficiency virus (HIV), vesicular stomatitis virus (VSV) and murine gammaherpesvirus 68 (MHV-68) [67,75]. SLFNs are inducible by type I interferons (IFNs). SLFN12 is a human specific gene and is the only human SLFN predicted to be located in the cytosol, where HRV replicates. Moreover, we have already demonstrated that SLFN12 is expressed in DCs and is highly upregulated upon HRV stimulation indicating antiviral properties of SLFN12 (unpublished data). SLFNs share similarities with Slfn like genes found in orthopoxviruses. Among the human SLFN family members SLFN12 is found to be the closest-related member to these viral Sfln like genes [66,69]. Therefore, SLFN12 seemed to be an attractive candidate to elucidate the role of this gene in HRV infections. The aims of the study were following:

1. To elucidate the effects of down-/upregulation of SLFN12 in HeLa-Ohio cells
2. To further study how this down-/upregulation in HeLa-Ohio affects the infections with HRV.

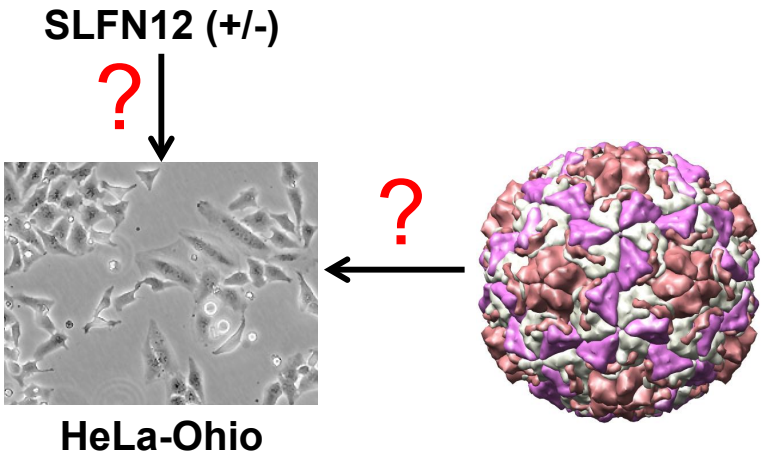


Figure 6: Aim of the study
Figures were adapted from [85] and [86]

3 MATERIAL AND METHODS

3.1 Antibodies, oligonucleotides, buffers and chemical reagents

Antibodies

species	specificity	clone	isotype	source
mouse	Calf intestine alkaline phosphatase	VIAP	IgG1	Otto Majdic, Institute of Immunology, Vienna, AT
mouse	CD11b	VIM12	IgG1	Otto Majdic
mouse	SLFN12	polyclonal (336/6E7)	IgM	Otto Majdic
mouse	SLFN12	polyclonal (314/6C5)	IgM	Otto Majdic
mouse	ICAM-1 (CD54)	5-341	IgG2a	Otto Majdic
mouse	MHC-I	W6/32	IgG2a	American Type Culture Collection (ATCC), Manassas, VA, USA
mouse	CD71	VIP-1	IgG1	Otto Majdic
mouse	MxA	383-7D4	IgG1	ADG, BioResearch GmbH, Kaumberg, AT
mouse	S100A4	8-9	IgG1	Otto Majdic
goat	human IgG-PE	polyclonal	IgG	Jackson Immunoresearch Lab., West Grove, PA, USA
goat	mouse IgG-Oregon Green	polyclonal	IgG	Life technologies, Carlsbad, CA, USA
goat	mouse IgG Dylight649	polyclonal	IgG	Jackson Immunoresearch Lab., West Grove, PA, USA
goat	SLFN12	polyclonal	IgG	Abgent, San Diego, CA, USA
goat	rabbit IgG-HRP	polyclonal	IgG	Dako Diagnostics AG, Glostrup, DK
goat	rabbit HRV14-VP	polyclonal	-	Dieter Blass, Max F. Perutz Lab., Vienna, AT
-	recombinant human Annexin V-FITC	-	-	Caltag Lab., Burlingame, CA, USA
-	propidium iodide	-	-	Sigma-Aldrich, St. Louis, MO, USA

Primers for qPCR

primer	sequence 5'-3'	source
HPRT_F	TCAGGCAGTATAATCCAAAGATGGT	van Zuylen et al [90]
HPRT_R	AGTCTGGCTTATATCCAACACTTCG	van Zuylen et al [90]
HRV2"A"_F	CTAGCAAACCAGCCTGAACC	Alexander Puck, Institute of Immunology, Vienna, AT
HRV2"A"_R	CCCCCAACATGTATTCCAAG	Alexander Puck
HRV14_F	GGCGCCATATCCAATGGTGT	Catharina Schrauf, Institute of Immunology, Vienna, AT
HRV14_R	TCCACCTGATCGAACGTCCA	Catharina Schrauf
SLFN12"A"_F	CATTACCTGCTCCCCACAGT	Alexander Puck
SLFN12"A"_R	GCCCTTTCTGACAGAGTCCA	Alexander Puck
SLFN5_F	AATTGCCCACAAGAGAATGG	Alexander Puck
SLFN5_R	AGCGTTTCTGCTGCTCTTTC	Alexander Puck
SLFN11_F	CCTCCCCTTAGCAGACCAGT	Alexander Puck
SLFN11_R	TTCCCCGAAAGAAAGGTTG	Alexander Puck
SLFN13_F	GACGCAGATCCAGAGTTTCC	Alexander Puck
SLFN13_R	AAATGTCCTGGTGGAACTGG	Alexander Puck
SLFN14_F	TCAGTCAGCTCCTCCCAGTT	Alexander Puck
SLFN14_R	CAAGGATGTATCAGGGTCTTCA	Alexander Puck
SLFN12L_F	TTGACCGAGAAGGAATGGAT	Alexander Puck
SLFN12L_R	GCAGAAGGTTTTTGGAGCAC	Alexander Puck
MxA_F	ACCTGATGGCCTATCACCAG	Alexander Puck
MxA_R	TTCAGGAGCCAGCTGTAGGT	Alexander Puck
GAPDH_F	CGACCACTTTGTCAAGCTCA	Maria Seyerl, Institute of Immunology, Vienna, AT
GAPDH_R	AGGGGAGATTCAGTGTGGTG	Maria Seyerl

All primers were synthesized by Sigma-Aldrich (Steinheim, DE).

siRNA

name	target	sense strand of duplex
siRNA_1	SLFN12	GCAATTCTGGTCTTAAATACAATCTCC
siRNA_1	SLFN12	CATCACACGGTTATCTTTCACATGCCA
siRNA_1	SLFN12	GTGAGCCTTCGACAAGATTTAAACATC
siRNA_2	SLFN12	CAAACTGCCCTAACCTTAA
siRNA_3	SLFN12	ATGAGTGACCTCGATGACTTA

SLFN12 siRNA_2 was purchased from Ambion (Houston, Tx, USA), siRNA_3 (#SI04132233) and Universal Scrambled Negative Control siRNA Duplex were obtained from Qiagen (Hilden, DE). SLFN12 siRNA_1 was purchased from Origene Technologies (Rockville, MD, USA).

Buffers

Freezing medium: RPMI 1640/ IMDM supplemented with 25% FCS and 10% DMSO

Heparin medium:

500 ml RPMI 1640
100 U/ml penicillin + 100 µg/ml streptamycin
2 mM L-glutamine
10 U/ml Heparin (Stock: 5000 U/ml, Baxter, Vienna, AT)

10x PBS solution (pH 7,2):

2 g KH_2PO_4
14,4 g Na_2HPO_4
80 g NaCl
2 g KCl
Dissolve in aquabidest, fill up to 10 litres.

20% PBS/BSA stock solution:

100 g BSA
10 g NaN_3
Dissolve in 500 ml 1x PBS buffer. For the 1% PBS/BSA wash buffer stock solution was diluted 1:20 with 1x PBS buffer.

4x running buffer (1L):

12 g Tris
57,6 g Glycine
4 g SDS
Fill up to 1L with aquabidest

1x Blotting buffer (1L):

250 ml 4x running buffer

200 ml methanol

Fill up to 1L with aquabidest. Degase before use.

0,05% PBST (1L):

PBS with 0,05% Tween20

Dry milk solution:

5% dry milk powder dissolved in PBST buffer.

2x Lämmli-sample Buffer:

4% SDS

20% Glycerol

120 mM Tris/HCl (pH 6,8)

Fill up with aquabidest.

Add 0,02% bromphenolblue

AnnexinV binding buffer (1L):

10 mM Hepes

140 mM NaCl

2.5 mM $CaCl_2$

Fill up to 1L with aquabidest and adjust pH to 7,4

NP-40 lysis buffer:

150 mM NaCl

1% Triton X-100

50 mM Tris/HCl (pH 7,4)

Fill up with aquabidest.

Cell lines

name	source
HeLa-Ohio	Flow laboratories, McLean, Virginia, USA
HeLa 91/1948	American Type Culture Collection (ATCC), Manassas, VA, USA
THP-1	ATCC, Manassas, VA, USA
Daudi	ATCC, Manassas, VA, USA
Jurkat	ATCC, Manassas, VA, USA
K562	ATCC, Manassas, VA, USA
Phoenix-SLFN12	Institute of Immunology, Vienna, AT
Phoenix-gag-pol	Institute of Immunology, Vienna, AT

Phönix-SLFN12 transductant was generated in our laboratory. The parental cell line Phönix-gag-pol was a gift from H. Strobl (IFI, Vienna, AT).

Viral stocks

virus	concentration/ml	source
HRV2	$2{,}15 \times 10^{11}$ $TCID_{50}$	Dieter Blaas, Max F. Perutz Lab., Vienna, AT
HRV14	7×10^{9} $TCID_{50}$	Dieter Blaas, Max F. Perutz Lab., Vienna, AT

Immunoglobulin

Immunoglobulin	concentration	final conc.	source
mIgA	10 mg/ml	1 mg/ml	Baxter, Vienna, AT
SIgA	5 mg/ml	100 µg/ml	Gerhard Zlabinger, IFI, Vienna, AT
Beriglobin (IgG)	20 mg/ml (in PBS/BSA)	1 mg/ml	Aventis Behring, Vienna, AT

3.2 Cell culture

Cells were maintained in RPMI 1640 medium (Institute of Immunology, Vienna, AT) supplemented with 10% heat-inactivated fetal calf serum (Gibco, Grand Island, NY, USA), 200 mM L-glutamine (Gibco, Grand Island, NY, USA), 100x penicillin (PAA, The cell culture company, Pasching, AT) and 100x streptomycin (PAA, The cell culture company). Phoenix cells were cultivated in IMDM-medium (PAA, The cell culture company, Pasching, AT) supplemented with 10% FCS, L-glutamine, penicillin

and streptomycin. Cells were incubated at 37°C with 5% CO_2.

3.3 Freezing and thawing of cells

Mammalian cells can be stored in liquid nitrogen. Cells were spun down, counted and resuspended in freezing medium to a concentration between 3-5x10^6 cells/ml. 1 ml aliquots were filled into cyrotubes (Nalgene Nunc International, Roskilde, Denmark) and kept overnight at -80°C in a freezing box filled with isopropanol before being transferred to liquid nitrogen.
For thawing, cyrotube were warmed in water until a clump of frozen cells was left in the tube. Cell suspension was transferred to a new tube and 1 ml cell culture medium (without FCS) was added in an interval of 1 min: Starting with five drops at 0', 10 drops at 1' etc. After two washing steps, cells were cultured in medium with FCS.

3.4 Cell preparation

Peripheral blood mononuclear cells were separated from whole blood of healthy donors by density gradient centrifugation using Ficoll-Paque (Amersham, Little Chalfont, UK). Blood was diluted 1:2 to 1:3 with heparin-medium. For density gradient centrifugation 15 ml of Ficoll-Paque were prepared in 50 ml tubes and a layer of heparinized blood was carefully pipetted onto. The cells were spun 30 minutes at 900g without brake. After centrifugation granulocytes and erythrocytes gathered at the bottom of the tube, mononuclear cells in the interphase.

3.5 Magnetic activated cell sorting (MACS)

Magnetic activated cell sorting (MACS) is a method for the selective enrichment or depletion of cells, which express a surface protein distinct for their cell type. Therefore cells can be labeled with antibodies directed against the specific molecule, which have been coupled to biotin. These labeled cells can be targeted with magnetic beads that contain a secondary antibody targeted against the biotin residues on the primary antibody. Therefore, specific cells in a mixture can be selectively retained in a magnetic column. The mAbs we used were labeled with biotin. These biotinylated antibodies recognizing cell-type specific surface molecules were mixed with the cells to be separated. Then the cells were washed twice with MACS-buffer. In a second incubation step paramagnetic beads (50 nm in diameter)

were coupled to the cells. The cells were applied onto a separation column placed in a strong permanent magnet. In this strong magnetic field the cells labeled with paramagnetic beads stack to the iron mesh and were retained while non-labeled cells passed through. Retained cells were eluted by removing the column from the magnetic field.

3.6 Purification of T cells and monocytes

For the isolation of monocytes up to $1x10^9$ PBMCs were incubated with 250µl of biotinylated CD14 (VIM13) and positively enriched (positive selection), whereas T cells were isolated by collecting the flow through of PBMCs depleted (negative selection) by using an antibody cocktail containing anti-CD14 (monocytes), anti-CD16 (monocytes, NK–cells), anti-CD19 (B cells), anti-CD36 (monocytes, thrombocytes), anti-CD56 (NK-cells) and anti-CD123 (progenitor cells, granulocytes, megakaryocytes). Freshly isolated PBMCs were resuspended in 750µl MACS buffer and incubated with 250µl of biotinylated antibodies for 15 minutes at 4 °C. To remove unbound antibodies the cells were washed with MACS buffer and again resuspended in 750µl buffer. Then 250µl of anti-Streptavidin MicroBeads (MiltenyiBiotec, BergischGladbach, Germany) were added to the suspension and incubated for 15 minutes at 4°C. In the meantime a CS column (Miltenyibiotec) was placed onto a VarioMACS apparatus and equilibrated with 40 ml MACS buffer. Afterwards the labeled PBMCs were loaded onto the column and washed with 40 ml MACS buffer. The flow through was collected as the monocyte-negative fraction. The column was further washed 4 times with 10 ml MACS buffer and monocytes were collected by removal of the column from the magnet and aspiration of the retained cells from the side valve using a syringe. For T cells the flow-through was collected, washed with MACS buffer and counted. 20µl of this solution were taken for cell counting with a Coulter Counter (Beckman Coulter, Miami, FL). Before measuring the cell number two drops of Zap-Oglobin® (Beckman Coulter) are added to the counting beaker to remove residual erythrocytes.

3.7 THP-1 derived macrophages

200.000 cells/well were seeded in 24-well cell culture plate. 10 nM PMA was added to the wells and incubated for 3 days at 37°C. After 3 days cells were treated with IFN-α (Stock: $2x10^6$ U/ml; 1:20.000) for 6 hours.

3.8 Generation of monocyte-derived dendritic cells

Monocyte-derived immature dendritic cells were generated by culturing peripheral blood $CD14^+$ monocytes for 7 days in the presence of 50 ng/ml GM-CSF (Novartis Research Institute, Vienna, AT) and 100 U/ml IL-4 (Novartis Research Institute, Vienna, AT).

3.9 Flow cytometry

3.9.1 Principles

Flow cytometry is a technique for counting and examining single cells or other particles. Cells in suspension are transported in a stream to the laser beam [77] and as they pass the beam, they scatter and emit fluorescence. The scattered light is measured as forward scatter light (FSC), which is approximately proportional to cell size, and side scatter light (SSC) giving information about granularity and complexity. The detectors also measure the fluorescence emitted by cells labeled with antibodies conjugated to fluorescent dyes.

3.9.2 Flow cytometric analysis

For intracellular staining, cells (100.000-500.000 cells/assay) were centrifuged for 5 minutes at 4°C at 1500 rpm. Cells were then resuspended in 50µl Beriglobin/assay (1:8 dilution, 20 mg/ml, Aventis Behring GmbH, Vienna, AT) and 50µl fixation medium (ADG, BioResearch GmbH, Kaumberg, AT) was added and incubated at RT for 20 minutes. Each assay was washed once with 1% PBS/BSA solution (resuspended in 1% PBS/BSA, centrifuged (5 minutes at 1500 rpm, 4°C), supernatant was discarded). 50µl permeabilisation medium (ADG, BioResearch GmbH, Kaumberg, AT) and 20µl/100µl primary antibody was added and incubated 20 minutes at RT. Cells were then washed twice with 1% PBS/BSA. 20µl secondary

antibody and 50µl permeabilisation medium were added and again incubated for 20 minutes at RT. Each assay was washed twice with 1% PBS/BSA. 50µl FACS fluid (BD, Biosciences, Franklin Lakes, NJ, USA) was added to each tube and was analyzed by using a FACScalibur Flow Cytometer (Becton Dickinson, Palo Alto, CA, USA).

For membrane staining, cells (100.000-500.000 cells/assay) were centrifuged for 5 minutes at 4°C at 1500 rpm. Cells were then resuspended in 50µl Beriglobin/assay and 20µl/100µl of primary antibody were added, mixed and incubated for 30 minutes at 4°C. Each assay was washed twice with 1% PBS/ BSA. 20µl of secondary antibody were added to the cells and again incubated for 30 minutes at 4°C. Each assay was washed twice. Cells were resuspended in 50µl FACS fluid before analyzed by FACScalibur Flow Cytometer.

For AnnexinV-PI staining, cells (200.000 cells/assay) were centrifuged for 5 minutes at RT at 1500 rpm. Cells were then resuspended in 50µl Beriglobin/assay and washed with 1 ml AnnexinV binding buffer. 20µl of AnnexinV (1:10- 1:12; Caltag Lab., Burlingame, CA, USA) were added and incubated in the dark at RT for 15 minutes. Each assay was washed again with AnnexinV binding buffer. Cells were resuspended in 50µl FACS fluid and 25µl PI before they were analyzed using a FACScalibur Flow Cytometer.

3.10 RNA-Isolation and cDNA synthesis

For RNA isolation 1-5x10^6 cells/ml were resuspended in 500µl peqGoldTriFast$_{TM}$ reagent (Peqlab, Erlangen, DE) and incubated for 5 minutes at RT (optional: -20°C until need). 100µl chloroform was added to separate RNA from DNA and proteins, mixed by vortexing for 15 seconds, incubated 5 minutes at RT and then centrifuged for 10 minutes at 13.000-14.000 rpm at 4°C. The aqueous phase was transferred into a new tube and 300µl isopropanol and 4µl glycogen (20mg/ml, ThermoScientific, Waltham, MA, USA) was added. Contents were mixed, incubated for 5 minutes on ice and again centrifuged for 10 minutes. The pellet was washed twice with 800µl 75% ethanol before dried (optional: after first washing step, RNA can be stored in 75% ethanol at -20°C). Dried pellet was resuspended in 12µl nuclease free water (NFW) and was shaking on a thermal mixer for 15 minutes at 56°C. RNA

concentration was then measured by using a Nano Drop 2000 spectrophotometer (Peqlab, Erlangen, DE). 1-2µg RNA was used for reverse transcription. RNA were mixed with 1µl Oligo(dT) 18 primers (0,5 µg/µl) and NFW was added up to 11µl. The mixture was incubated for 5 minutes at 70°C in a thermo cycler. To each reaction 4µl 5x Reaction Buffer RT (ThermoScientific, Waltham, MA, USA), 2.5µl NFW, 2µl dNTP (10 mM), 0.5µl RNase inhibitor (40 U/µl, ThermoScientific) and 1µl RevertAid H Minus RT (200 U/µl, ThermoScientific) were added and incubated for 5 minutes at 37°C, 60 minutes at 42°C and 10 minutes at 70°C. cDNA was stored at -20°C until use.

3.11 Quantitative Real-time PCR

Real-time PCR is the most sensitive method for the detection of low-abundance mRNA in different tissue samples [78]. Fluorescence dyes such as SYBR Green are used to detect and monitor in vitro DNA amplification [79]. SYBR Green binds to the minor groove of dsDNA during elongation resulting in an increase in the fluorescence signal and falls off when DNA is denatured [78]. A disadvantage of this type of detection is that SYBR Green also binds non-specific products and primer dimers if you do not completely optimize the assay [80]. The Taqman assay utilizes the 5´ nuclease activity of the DNA polymerase to hydrolyze a hybridization probe bound to its target amplicon [78]. The probe consists of a quencher at the 3´ end and a reporter dye at the 5´ end. As the Taq polymerase extends along the template, 5´ nuclease activity of the polymerase will degrade the probe so that the quencher dye is released from the reporter one resulting in a detectable fluorescence signal [78]. Fluorescence values get measured after every cycle and represent the amount of amplified product. The more template present at the beginning of the reaction, the fewer number of cycles it takes to reach the point in which fluorescence is deemed to be detectable above the background during exponential phase of amplification [78,80]. This point is termed cycle threshold (ct). Comparison of quantification of mRNA transcripts from different individuals requires an internal reference gene, so called housekeeping gene, against which the other mRNA values can be normalized. Housekeeping genes should be expressed at a constant level among different tissues of an organism and should be unaffected by the experimental treatment to make a normalization possible [78].

qPCR assays were analyzed by the CFX 96 realtime-PCR detection system (Bio-Rad Laboratories, Hercules, CA). For one PCR reaction 3µl NFW, 1µl cDNA, 5µl SYBR-Green I Mastermix (QuantaBioSciences, MD, USA)) and 1µl Primer-Mix (Forward and Reverse, each 5 µM) were mixed together and cDNA was amplified using a standard program (10 minutes at 95°C, 40 cycles of 15 seconds at 95°C/ 15 seconds at 60°C/ 45 seconds at 72°C, 5 seconds at 65°C, 50 seconds at 95°C).

3.12 RNA-Interference

RNA-Interference is a biological process causing the degradation of specific mRNA molecules. Double-stranded RNA (dsRNA) is processed to 21-23 nucleotide long dsRNA fragments by a ribonuclease III like nuclease, so called DICER [81]. These short dsRNAs also termed short-interfering RNAs (siRNAs) are bound by the RNA-induced silencing complex (RISC), which separates the double-strands. siRNAs base pair to their complementary mRNA sequence and induces degradation of this mRNA [82]. This phenomenon gets utilized in science to decrease the expression of genes of interest.

Three different SLFN12 siRNAs and a control siRNA (Qiagen, Valencia, CA, USA) at a final concentration of 5-10 nM were transfected into HeLa-Ohio cells. The transfection procedure was performed according to the manufacture´s protocol. After 72h samples for RNA-Isolation were taken.

3.13 Proliferation assay

Proliferation assays determine the amount of ^{3}H-labeled thymidine that is incorporated into the DNA of cultured cells. Measurement of DNA synthesis is directly proportional to the rate of cell division.

Proliferation assay of transfected HeLa-Ohio cells was performed in a 96-well cell culture plate. Transfection of HeLa cells was performed as described above. A total number of 10.000 cells/well were seeded in the plate and incubated at 37°C until cells were adherent again. 1,25 µCi of ^{3}H-labeled thymidine (Perkin Elmer, Waltham, MA, USA) was added to each well and again incubated at 37°C for 16 hours. Cells were then harvested onto a filter plate and dried for 2h at 37°C. Before radioactivity

was determined by using a microplate scintillation counter (Packard), 25µl/well of Microscint scintillation mix (Perkin Elmer) was added. Assays were performed in triplicates.

3.14 SDS-PAGE

Sodium-dodecyl sulfate polyacrylamide electrophoresis (SDS-PAGE) is a technique that allows the electrophoretic separation of proteins according to their size. SDS, an anionic detergent, masks the charged residues of proteins, so that all proteins apply a constant negative charge. It also denatures secondary and tertiary structures. β-mercaptoethanol, included in the 2x Lämmli-buffer, gets used to reduce the disulfide bonds between the molecules.

2x Lämmli sample buffer containing β-mercaptoethanol was used for lysis of cells. Samples were shock-frozen in liquid nitrogen and transferred to -20°C for storage. After denaturation (5-10 min, 95°C), proteins were separated on 10% acrylamide gels using Mini-PROTEAN® Tetra Cell System (Bio-Rad). As a size marker See Precision Plus Protein ™ Dual Color Standards (Bio-Rad, #161-0374, Richmond, CA, USA) was used. Gels were run at 60V until the marker was separated. Then the voltage was set to 100V.

Reagent	**10% Separation gel**	**4% Stacking gel**
H_2O	4,8ml	2ml
30% Acrylamide-solution	4ml	400µl
1.5M Tris/HCl pH 8,8	3ml	-
0.5M Tris/HCl pH 6.8	-	840µl
10% SDS-solution	100µl	35µl
10% APS	100µl	35µl
TEMED	8µl	4µl

3.15 Western Blot analysis

Western Blot relies on the detection of proteins in different samples via the use of specific antibodies. After gel electrophoresis native or denatured proteins are transferred to a membrane (nitrocellulose or PVDF membrane), where they are probed using primary antibodies specific to the target protein. A secondary antibody-enzyme conjugate binds then the primary one.

Proteins separated by SDS-PAGE were blotted onto Immobilion-PVDF membranes (Millipore, size 0,45 µm, Billerica, MA) using a Trans-Blot®SD Semi-Dry Transfer Cell (Bio-Rad) system for 45 minutes at 10V. Preventing Abs from unspecific binding, the membranes were shaken in 5% dry milk solution for 30 minutes and afterwards incubated with primary antibody (1 µg/ml) diluted in the same solution (overnight, 4°C). After washing (3x5 minutes) membranes were incubated with HRP conjugated goat anti mouse/rabbit IgG antibodies (1:2000; Dako, Glostrup, DK) for 1^{30}h. Before chemiluminescence detection (Bio-Rad, Richmond, CA, USA), membranes were given a final wash (3x5 minutes). Blots were developed on Kodak Biomax XAR films (Sigma Aldrich).

4 RESULTS

4.1 Expression of Schlafen gene family members in cell lines versus primary cells

Gene expression levels of all six human SLFNs in HeLa-Ohio and DCs were determined by qPCR. The expression levels of indicated mRNAs are presented as Ct-values between GAPDH and SLFN mRNAs (Table 1). The more template present in a sample, the fewer number of cycles it takes to reach the point, called cycle threshold (ct), in which fluorescence is deemed to be detectable above the background [78,80]. While SLFN5 and SLFN11 mRNA were higher expressed as SLFN12 in HeLa-Ohio, the remaining SLFNs (SLFN12L, SLFN13, SLFN14) were not detectable. In contrast all SLFNs except SLFN14 were expressed in DCs. Notably, we observed that SLFN12 was higher expressed in DCs compared to HeLa-Ohio.

ΔCt (target gene-GAPDH)	HeLa-Ohio	DCs
SLFN5	8,57 ± 0,18	10,00 ± 2,33
SLFN11	7,39 ± 0,21	9,90 ± 2,54
SLFN12	15,15 ± 0,42	11,05 ± 1,89
SLFN12L	N.D	15,66 ± 1,91
SLFN13	N.D	14,71 ± 2,85
SLFN14	N.D	N.D

Table 1: SLFN gene expression in HeLa-Ohio and DCs
Total cellular RNA was isolated from HeLa-Ohio and DCs, levels of indicated mRNAs and GAPDH were determined by qPCR. The expression of the indicated mRNAs is presented as ΔCt-values (delta Ct-values) between GAPDH and the SLFN mRNAs. Mean ±SD (n=2) is shown.

As already mentioned in the aim of the study, we focused our work on SLFN12.
To further study whether SLFN12 is also expressed in other cell lines, total RNA from Daudi, Jurkat, K562, undifferentiated and differentiated THP-1 cells was extracted and determined by qPCR. Differentiated THP-1 cells (THP-1 derived Mφ (macrophages)) were treated with 10 nM PMA for 3 days. Both, differentiated and undifferentiated cells were treated with IFN-α, due to the fact that all SLFNs are

inducible by type I IFNs [67]. SLFN12 was expressed in THP-1 cells, above all at a relatively high level in differentiated THP-1 cells treated with IFN-α, whereas the other cell lines contained no SLFN12 (Figure 7).

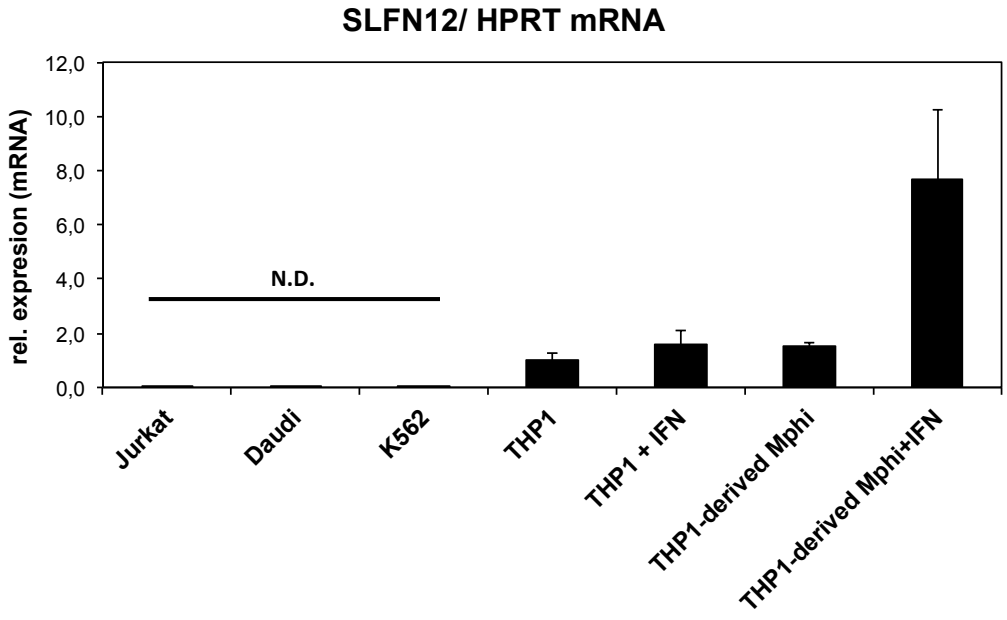


Figure 7: SLFN12 gen expression of diverse cell lines
Total cellular RNA was isolated from the indicated cell lines and mRNA levels were determined by qPCR. THP1 cells were either treated with IFN-α for 6h or with PMA for 3 days or with both. HPRT was used as reference gene. Mean ±SD (n=2) is shown.

4.2 Testing of novel antibodies against SLFN12

Two anti-SLFN12 Abs (314/6C5 and 336/6E7), generated in our laboratory, were tested for specificity of SLFN12 binding in intracellular stainings of cell lines and primary cells.

Regarding the qPCR data (Figure 7) that differentiated and undifferentiated THP-1 express SLFN12, we choose these cells for testing our anti-SLFN12 Abs. As controls CD11b, a differentiation marker, and MxA, a classical antiviral gene induced by type I IFNs, were used. As shown in figure 8 336/6E7 Ab did not seem to bind SLFN12 in differentiated as well as in undifferentiated cells. In contrast, 314/6C5 indeed showed a positive shift at least in THP-1 derived Mφ.

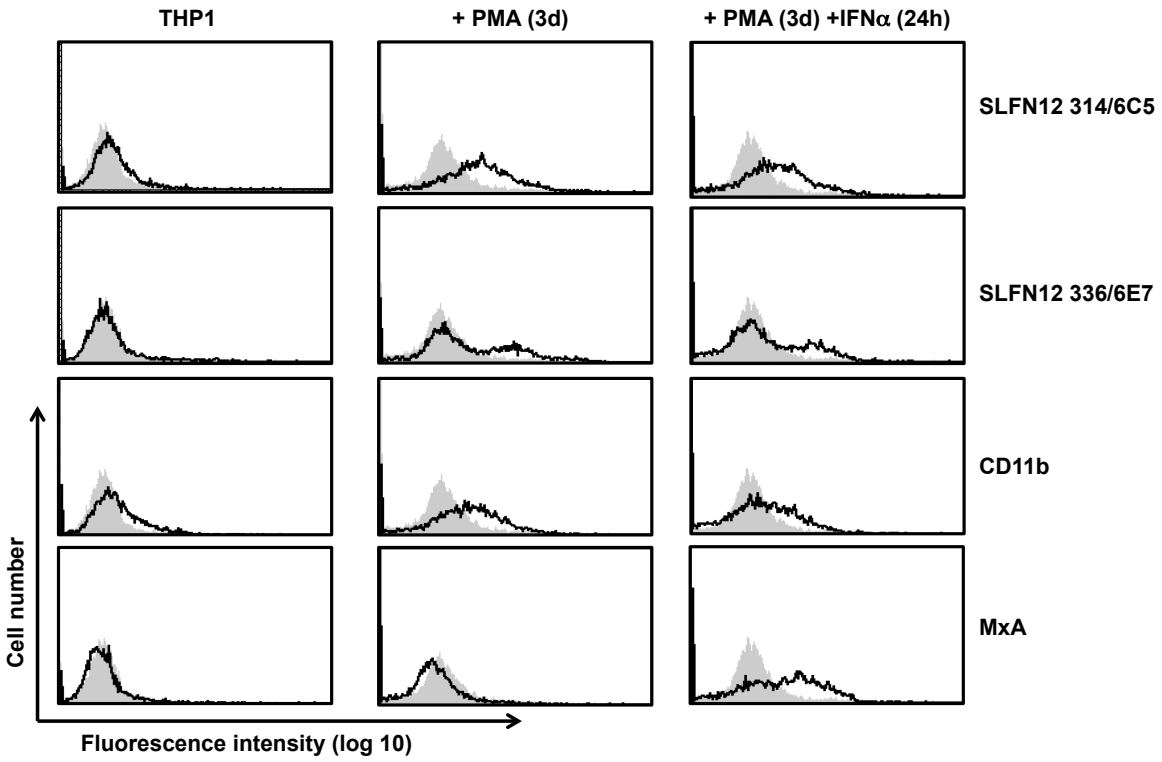


Figure 8: Flow cytometric analysis of THP1 cells
MOCK treated THP1 cells and THP1 cells treated with PMA (3d) or with PMA (3d) and IFN-α (24h) were used for flow cytometric analysis of the SLFN12 mAbs 314/6C5 and 336/6E7. As controls for IFN and PMA stimulation CD11b and MxA were used. The grey scale shows the isotype control mAb (VIAP).

Further stainings with primary cells were performed to examine the specificity of the Abs on other cells. Mononuclear cells (MNCs) from a donor were either treated with IFN-α or CD3/CD28 beads and incubated for 24 and 48h, respectively. MxA was again used as control for IFN-α production. While 336/6E7 did not work, 314/6C5 showed almost in all conditions except for treatment with beads for 48h a minimal shift (Figure 9).

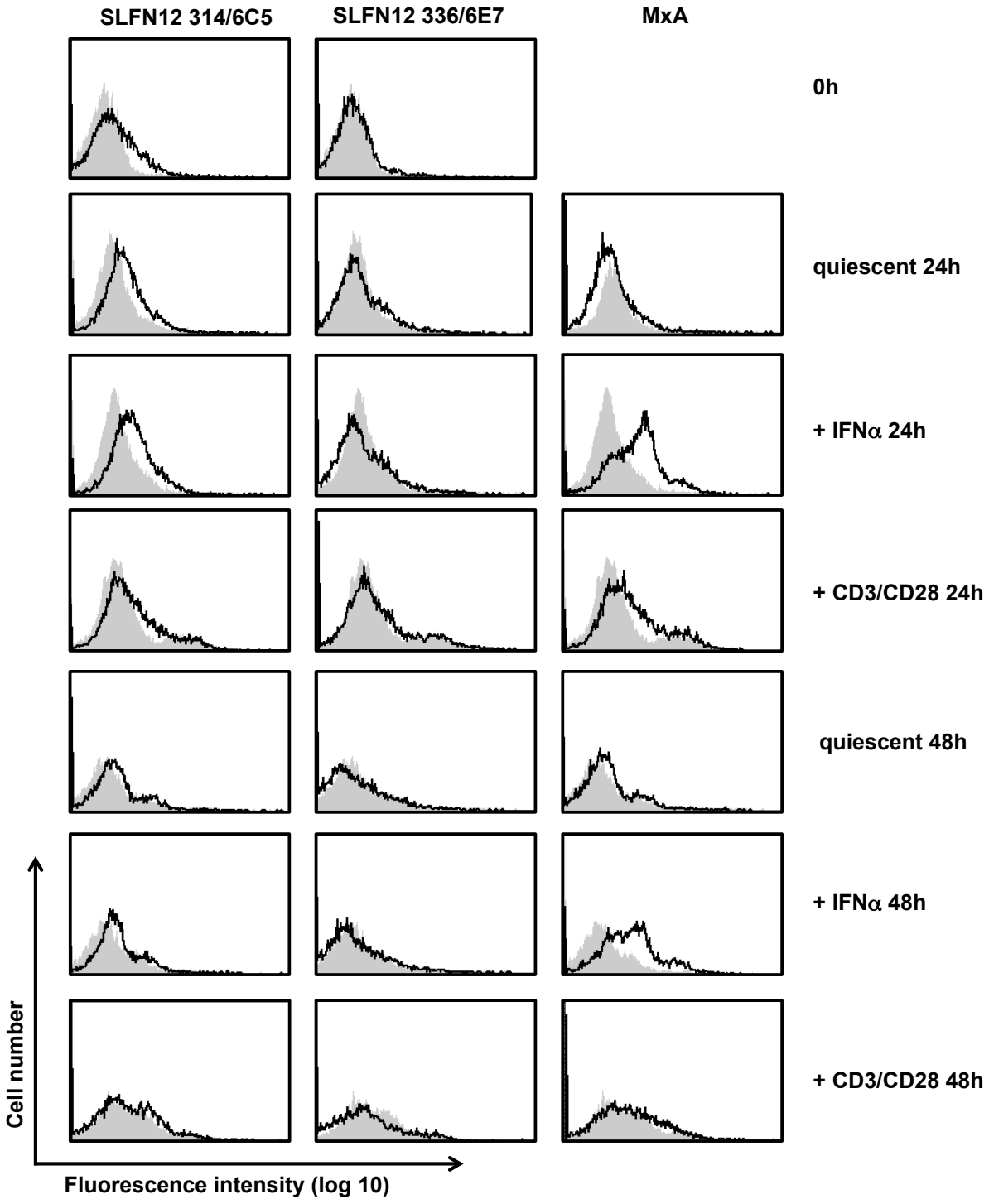


Figure 9: Intracellular staining of MNCs
SLFN12 and MxA production in MNCs (13/6143) incubated with IFN-α or CD3/CD28 beads for 24h and 48h were detected by intracellular staining. The grey scale shows the isotype control mAb (VIAP).

Staining of Phoenix cell lines, one of those stably transduced with SLFN12, was carried out to confirm the specificity of the 314/6C5 Ab. Success of transfection was controlled by qPCR (Figure 10 A). We expected that 314/6C5, if it was really able to bind to the protein as we have seen in previous stainings, should be higher in the transfected cell line. Unfortunately, flow cytometric analysis (Figure 10B) revealed that the transfectant (dotted line) seemed to express less SLFN12 compared to the non-transfected cell (black line).

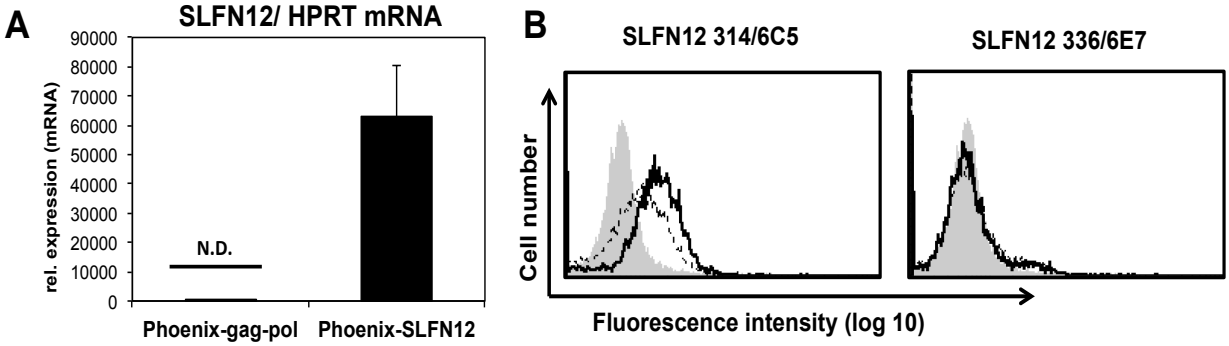


Figure 10: SLFN12 gene expression in Phoenix cell lines
(A) Phoenix-gag-pol (black line) and Phoenix-SLFN12 (dotted line) cell line were used for intracellular staining of the SLFN12 mAbs 314/6C5 and 336/6E7. The grey scale shows the isotype control mAb (VIAP).
(B) Total cellular RNA was isolated from the indicated cell lines and SLFN12 mRNA levels were determined by qPCR. HPRT was used as reference gene. Mean ±SD (n=2) is shown.

4.3 Overexpression of SLFN12 in HeLa-Ohio leads to cell death

Gene overexpression of SLFN12 in HeLa-Ohio cells was performed using retroviral gene transfer. A pMMP-vector, coexpressing SLFN12 and a fluorescent gene marker, EYFP, was transfected together with two plasmids, one of each encoding either gag-pol or env, into Phoenix cells by $CaPO_4$ precipitation. HeLa-Ohio cells were then “spininfected” with the supernatant collected 48h and 72h, respectively after transfection. A pMMP-vector expressing EYFP was used as control.
Unfortunately, only few viable cells were visible in comparison to untreated ones a few days after gene transfer (Figure 11A). Both forward and side scatter, reflecting cellular size and complexity are visualized in Figure 11B. Flow cytometric analysis confirmed our microscopy data that less SLFN12 transduced cells are detectable

compared to the respective controls. Transfection efficiency measured by EFYP via flow cytometry analysis was successful in terms of control vector, but in case of the pMMP-SLFN12 transfection less efficient (Figure 11C).

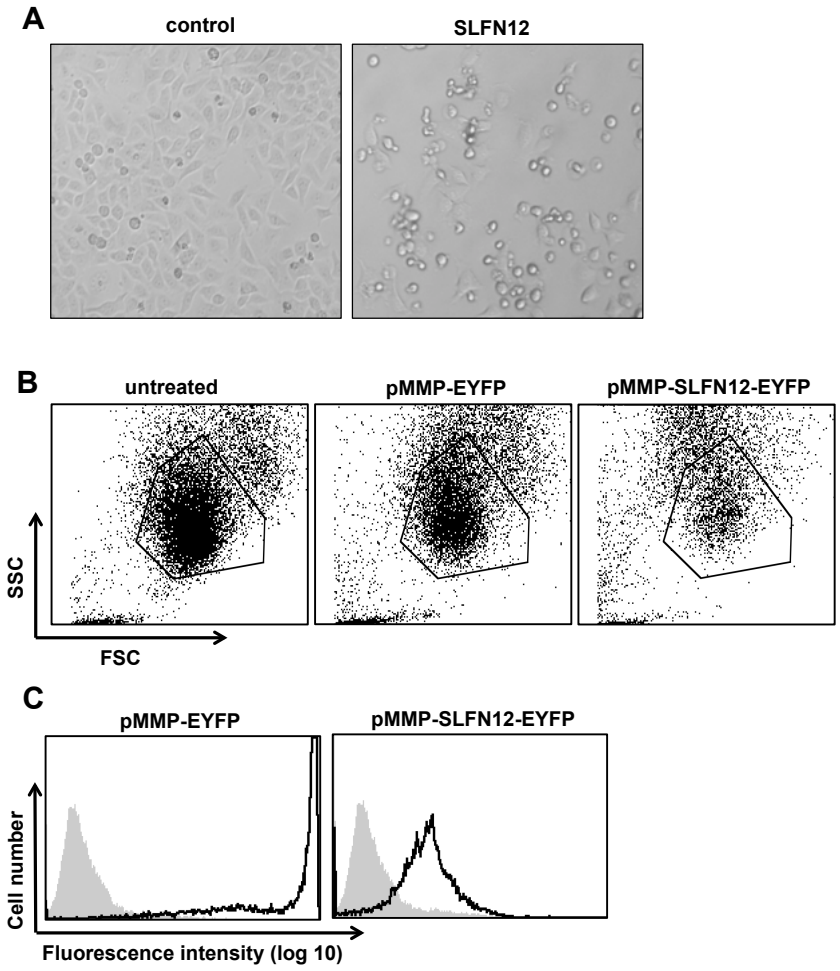


Figure 11: Overexpression of SLFN12 in HeLa-Ohio
(A) Light microscopic image of HeLa-Ohio cells, untreated and transfected with pMMP-SLFN12-EYFP vector.
(B) HeLa-Ohio cells were either transfected with a control vector (pMMP-EYFP) or with a pMMP-SLFN12-EYFP vector for several days. Cells were then analyzed by flow cytometry. Circularized cells visualize the gated cells.
(C) EYFP fluorescence of transduced cells was analyzed via flow cytometry. The grey scale shows the negative control (untreated HeLa-Ohio).

Considering that overexpression led to cell death, we concentrated our further work on gene silencing.

4.4 Effective knockdown via short-interfering RNAs (siRNAs) in HeLa-Ohio

To evaluate the temporal kinetics of SLFN12 knockdown in HeLa-Ohio by using 3 commercial siRNAs and one negative control siRNA (SCR), we performed a qPCR 48, 72 and 96h, respectively after transfection. As shown in figure 12, SLFN12 mRNA levels were only reduced half upon transfection with siRNA_1 and siRNA_3 for 48h. After 96h an effective knockdown was still visible, but 72h seemed to be the perfect time point (Figure 12, Figure 13A).

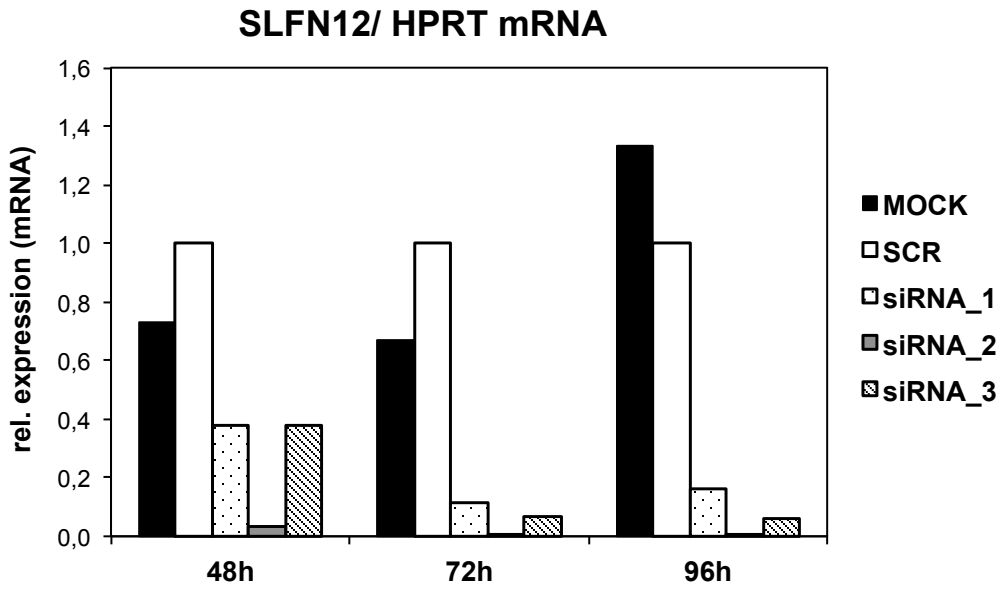


Figure 12: Temporal kinetics of SLFN12 knockdown
Total cellular RNA of MOCK treated HeLa-Ohio (black bar), HeLa-Ohio transfected with SCR (white bar), siRNA_1 (dotted bar), siRNA_2 (grey bar) and siRNA_3 (striped bar) was isolated after indicated time points and mRNA levels were quantitated by qPCR. HPRT was used as reference gene.

According to these results a 72h gene knockdown was chosen for further experiments. Screening of these siRNAs by Western Blot analysis (Figure 13B) demonstrated a nice knockdown on protein level too (34, 47 and 74% reduction respectively, based on band densitometry).

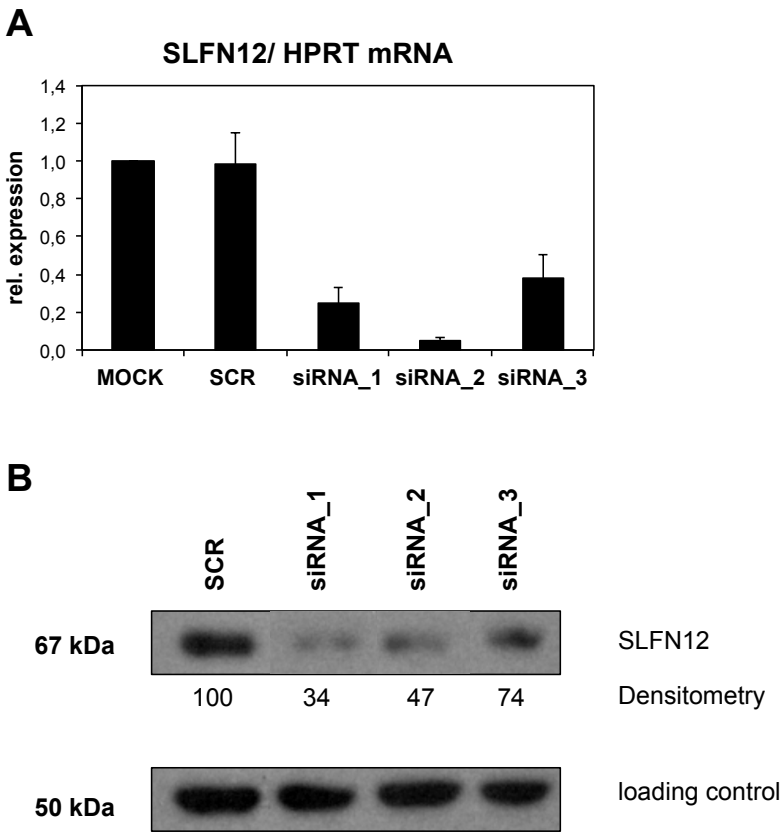


Figure 13: Effective knockdown of SLFN12
(A) Total cellular RNA of MOCK treated HeLa-Ohio, HeLa-Ohio transfected with SCR, siRNA_1, siRNA_2 and siRNA_3 was isolated 72h post-transfection and mRNA levels were quantitated by qPCR. HPRT was used as reference gene. Data show mean ±SD (n=2).
(B) HeLa-Ohio cells were transfected with SCR, siRNA_1, siRNA_2 and siRNA_3 for 72h. Cell lysates were prepared and analyzed with an Ab against SLFN12 by Western Blot.

4.4.1 SLFN12 knockdown is not compensated for by other human SLFNs

Next we asked whether other human SLFN genes compensate for this knockdown. For this purpose, knockdown cells (Figure 14A) were tested on the remaining SLFN expression levels as well as on MxA expression. SLFN12L, SLFN13 and SLFN14 were not detectable, whereas SLFN5 and SLFN11 expression was unchanged (Figure 14 B,C). MxA expression was unchanged too and according to the Ct-values (data not shown) only expressed in very low amounts (Figure 14D).

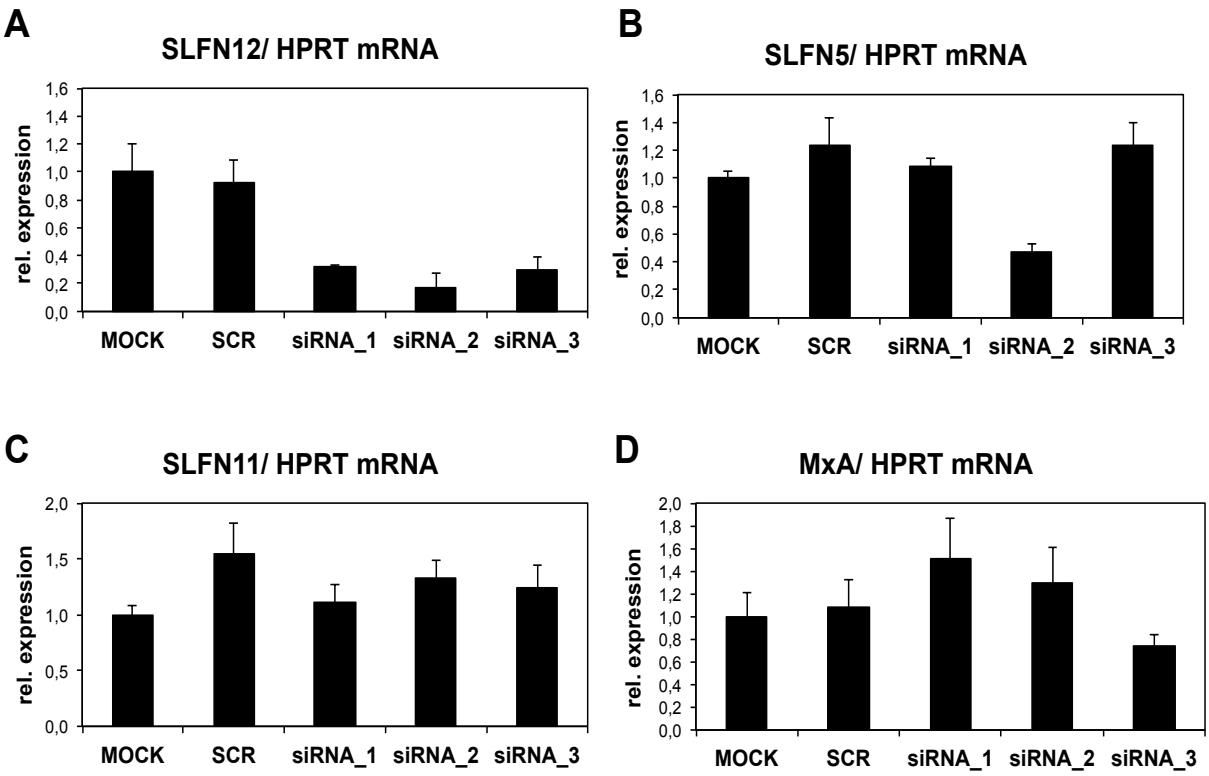


Figure 14: SLFN gene expression in knockdown cells
Total cellular RNA of MOCK treated HeLa-Ohio, HeLa-Ohio transfected with SCR, siRNA_1, siRNA_2 and siRNA_3 was isolated 72h post-transfection and mRNA levels were quantitated by qPCR. HPRT was used as reference gene. Data show mean ±SD (n=2). Cells were tested on following gene expressions: **(A)** SLFN12, **(B)** SLFN5, **(C)** SLFN11 and **(D)** MxA. The remaining human SLFNs, SLFN12L/13/14, were not detectable.

4.5 Proliferation of HeLa-Ohio cells was impeded by siRNAs

To determine the effects of siRNAs on HeLa-Ohio cell growth, cell proliferation was measured 72h after transfection by incorporation of ^{3}H-thymidine. SLFN12 overexpression led to cell death, therefore we expected that silencing would enhance proliferation of cells. Surprisingly, HeLa-Ohio proliferation was strongly impeded upon silencing with all three SLFN12 siRNAs as compared to the controls (Fig. 15A).
To further explore the effects of silencing on HeLa-Ohio cells, AnnexinV/PI staining was performed resulting in no significant differences in apoptosis between silenced and non-silenced cells (Figure 15B).

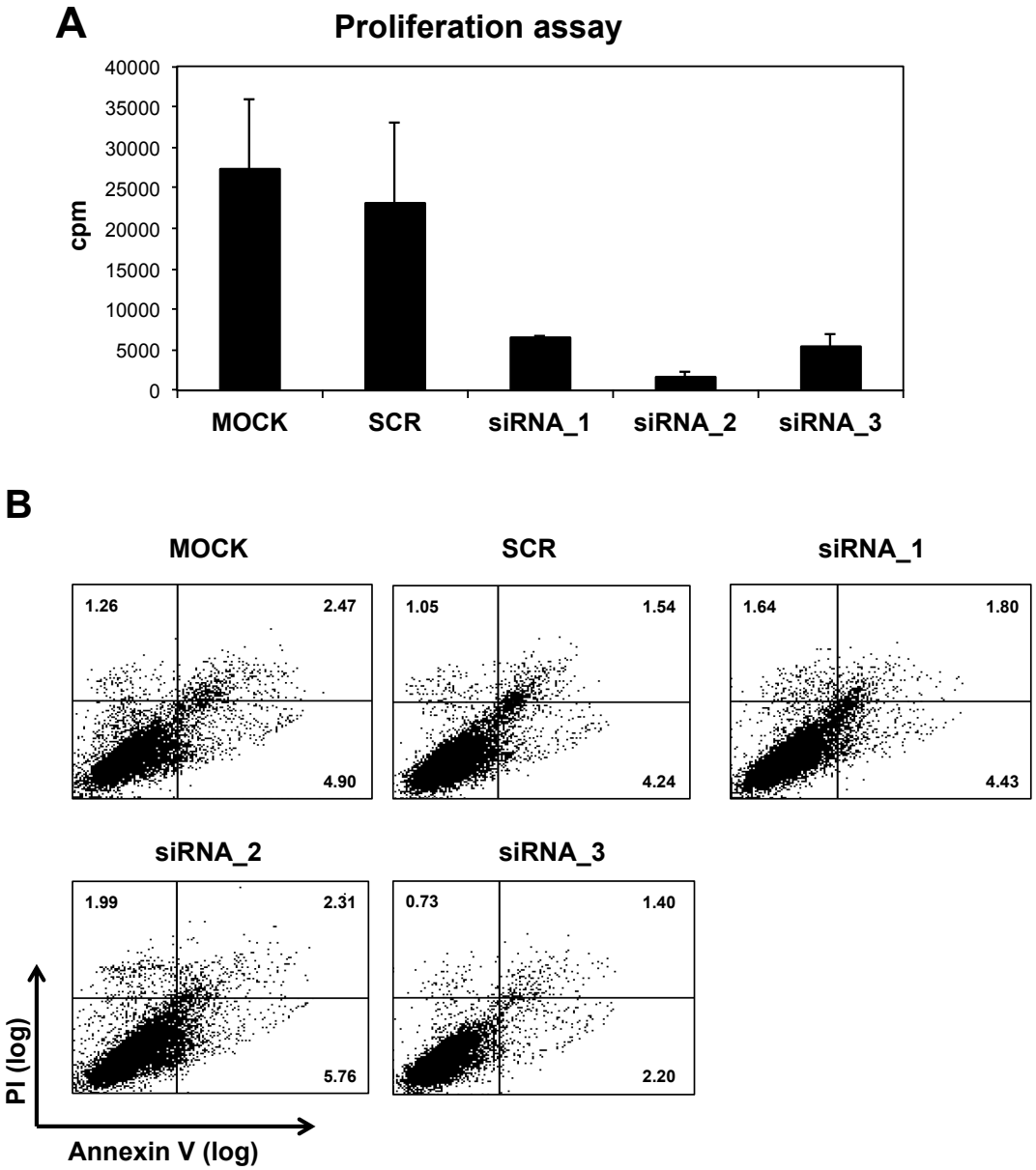


Figure 15: Knockdown cells are growth inhibited, but do not undergo apoptosis
(A) HeLa-Ohio cells were either MOCK transfected or transfected with SCR, siRNA_1, siRNA_2 and siRNA_3. 72h post-transfection proliferation was monitored by adding ^{3}H-Thymidine. Its incorporation was measured 16h later. Data show mean ±SD of two independent experiments.
(B) 72h post-transfected HeLa-Ohio cells were used for AnnexinV/PI staining. The percentage of single positives (bottom right, top left) and double positives (top right) is shown in each dot plot. One representative of two independent experiments is shown.

Furthermore, cytokine data demonstrated that even though cells are growth inhibited; they spontaneously produce IL-6 (Figure 16A). Less IL-6, but still a remarkable amount, was measurable in HeLa-Ohio cells infected with HRV14 (1 $TCID_{50}$/cell)

(Figure 16B). Interestingly, siRNA_1 transfected cells showed the highest amount of IL-6.

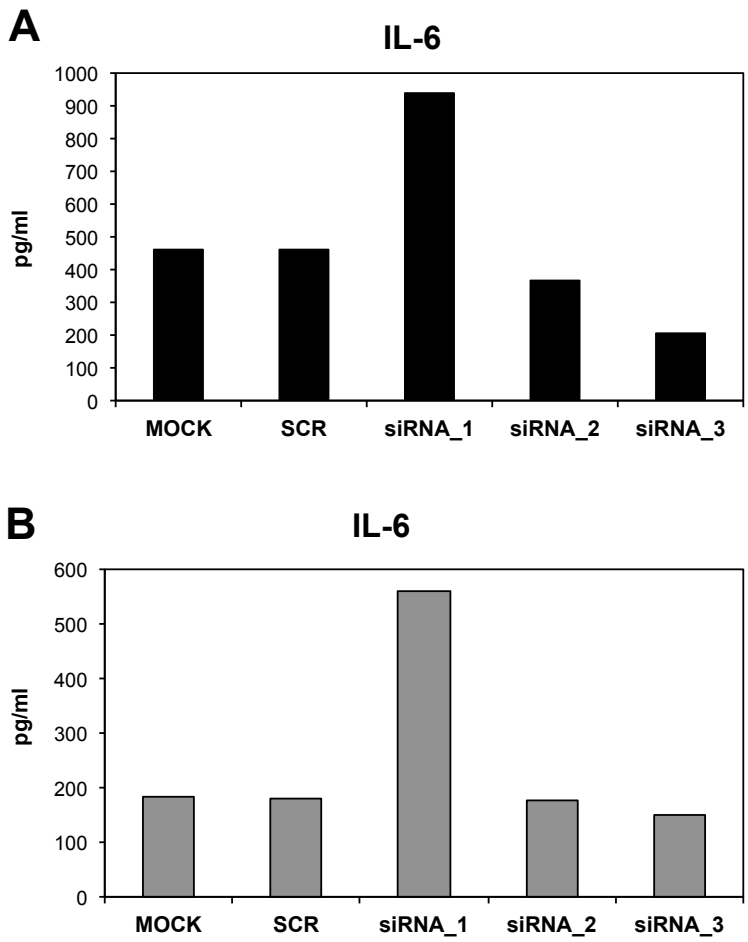


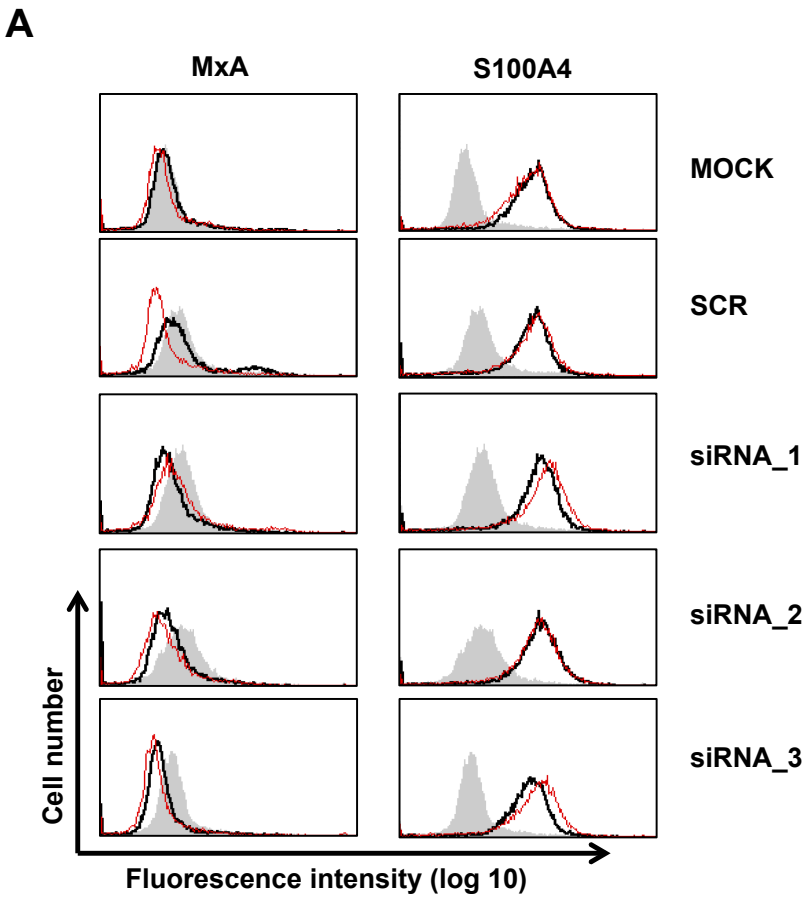
Figure 16: IL-6 production in knockdown cells
(A) Supernatants of MOCK transfected HeLa-Ohio or transfected with SCR, siRNA_1, siRNA_2 and siRNA_3 were analyzed for IL-6 production.
(B) MOCK transfected HeLa-Ohio or transfected with SCR, siRNA_1, siRNA_2 and siRNA_3 were infected with HRV14 (1 $TCID_{50}$/cell). 8h post-infection supernatants were analyzed for IL-6 production.

Based on these results, we concluded that SLFN12 knockdown inhibits cell growth, but cells do not undergo apoptosis.

4.6 ICAM-1 expression is unchanged in knockdown cells

Further evidence that transfected cells are “alive” was provided by intracellular as well as by extracellular flow cytometric analysis of related cell markers. MxA, a classical antiviral gene, was not expressed in all cells, while S100A4 expression, a

regulator of diverse cellular processes, was increased in all cells to the same extent (Figure 17A). MxA and S100A4 expression patterns did not change in presence of HRV14 (1 $TCID_{50}$/cell) (Figure 17A). MHC-I, which is known to be downregulated or modified by most viruses, was stably expressed in almost all cells even in response to HRV14 (1 $TCID_{50}$/cell) (Figure 17B). Less MHC-I expression was detectable in siRNA_3 transfected cells, while infection with virus upregulated this protein. In contrast, CD71 was slightly downregulated in siRNA_1 and siRNA_3 transfected cells, respectively, but highly upregulated in siRNA_2 for unexplainable reasons (Figure 17B). Most importantly, ICAM-1 expression was highly increased and unchanged in all cells, even in presence of virus.



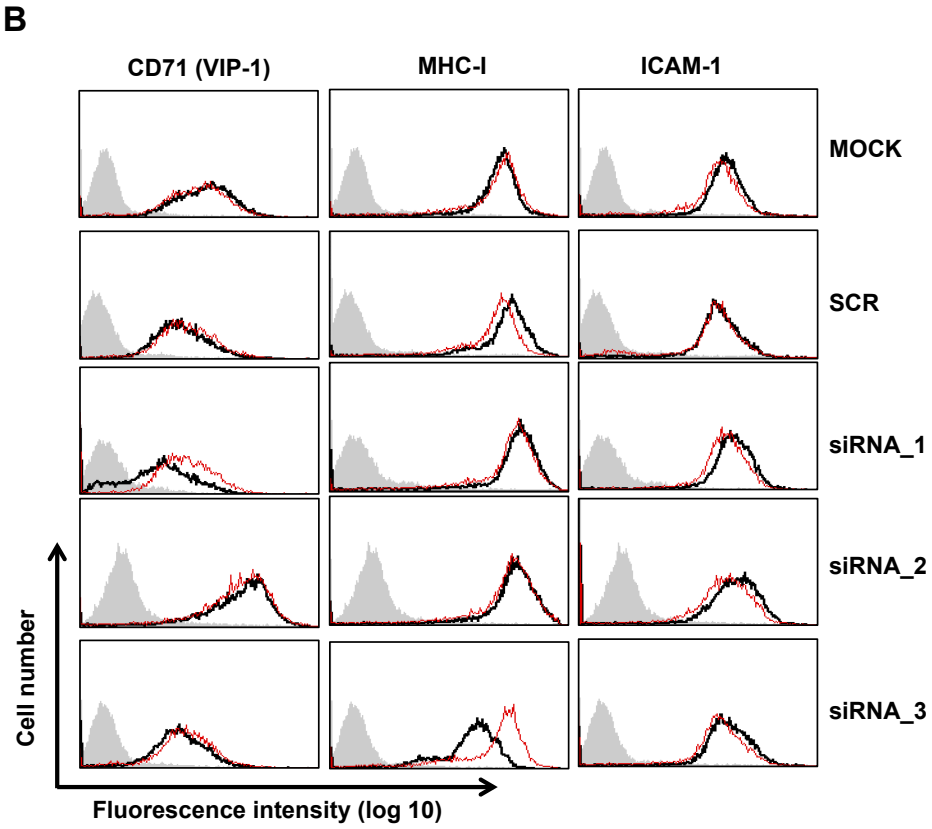


Figure 17: Flow cytometric analysis of diverse expression markers
(A) HeLa-Ohio cells either MOCK transfected or transfected with SCR, siRNA_1, siRNA_2 and siRNA_3 for 72h were used for intracellular staining of the Abs MxA and S100A4. Cells were cultured in the absence (black line) or in the presence (red line) of HRV14 (1TCID$_{50}$/cell) for 15h. The grey scale shows the isotype control mAb (VIAP). One representative of two independent experiments is shown.
(B) HeLa-Ohio cells either MOCK transfected or transfected with SCR, siRNA_1, siRNA_2 and siRNA_3 for 72h were used for cell surface staining of the Abs CD71 (VIP-1), MHC-I and ICAM-1. Cells were cultured in the absence (black line) or in the presence (red line) of HRV14 (1 TCID$_{50}$/cell) for 15h. The grey scale shows the isotype control mAb (VIAP). One representative of two independent experiments is shown.

4.7 HRV replication is impaired in knockdown cells

Having demonstrated an effective knockdown of SLFN12, we next carried out studies to address the role of this gene in HRV infected HeLa-Ohio cells. After 72h transfection, cells were infected with HRV14 (1 TCID$_{50}$/cell) for 1 and 7h. Analysis of HRV replication revealed that silenced SLFN12 clearly impaired viral replication in comparison to the controls (Figure 18A). Cells transfected with siRNA_1 and siRNA_2, respectively, showed the most dramatic effect considering HRV replication,

whereas siRNA_3 transfected cells the less dramatic one. This result perfectly correlated with the SLFN12 knockdown (see Figure 13A), in which siRNA_1 and 2, respectively caused the most effective knockdown. As shown in Western Blot analysis (Figure 18B) less viral (capsid) proteins (VP1-3) were detectable in transfected cells in response to HRV14 (1 $TCID_{50}$/cell). As controls HRV itself and non-infected cells were used.

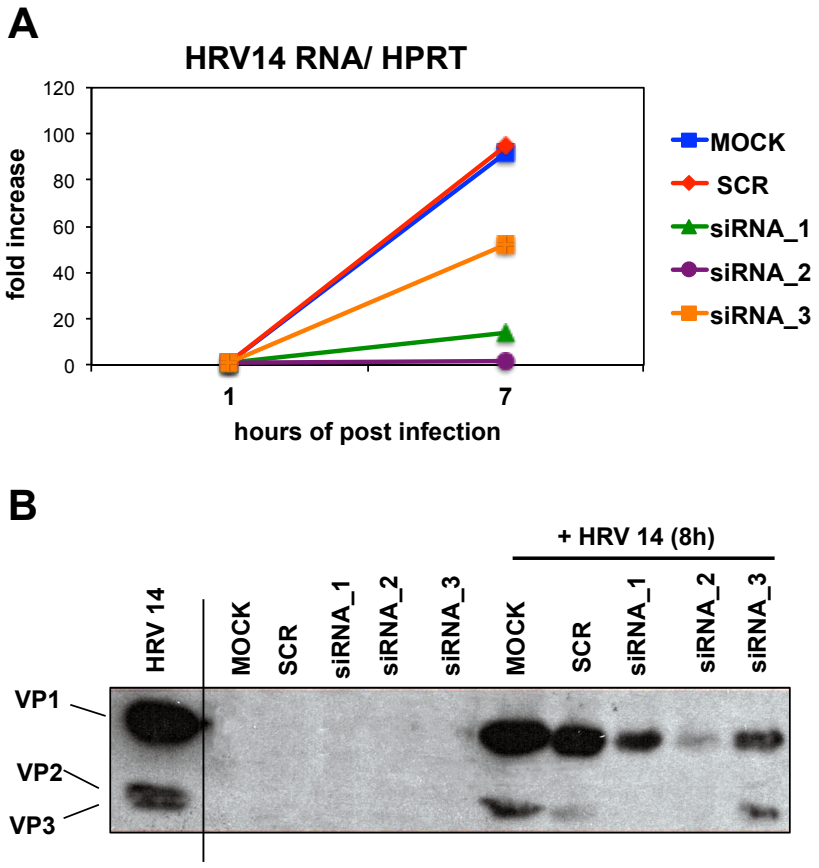


Figure 18: HRV14 replication is impaired in knockdown cells
(A) HeLa-Ohio were either MOCK transfected or transfected with SCR, siRNA_1, siRNA_2 and siRNA_3. 72h post-transfection cells were infected with HRV14 (1 $TCID_{50}$/cell). After 1 and 7h total cellular RNA was isolated and mRNA levels were determined by qPCR. HPRT was used as reference gene. One representative of three independent experiments is shown.
(B) Cells were treated as described in (A). Cell lysates were prepared and analyzed with an Ab against VP of HRV14 by Western Blot. One representative of two independent experiments is shown.

To study whether the virus is able to counteract SLFN12 silencing, we measured SLFN12 expression levels of infected cells by qPCR. As can be seen in Figure 19 gene expression was unchanged at 1 and 7h post-infection.

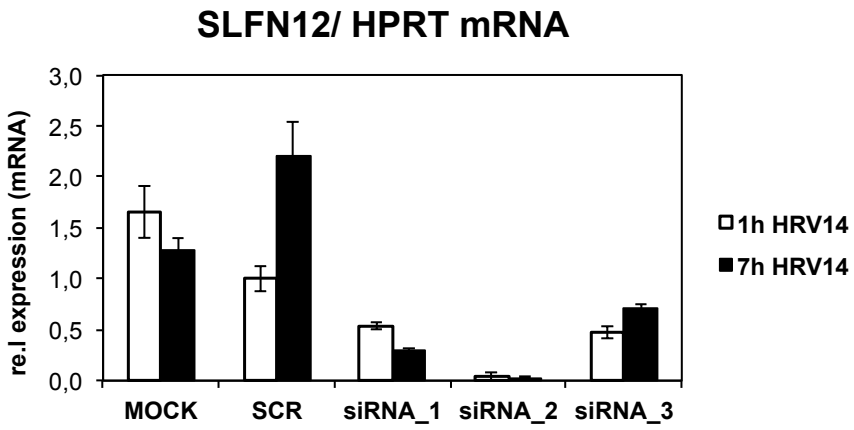


Figure 19: SLFN12 gene expression in knockdown cells infected with HRV14
72h post-transfection cells were infected with HRV14 (1 $TCID_{50}$/cell) for 1h (white bars) or 7h (black bars). Total cellular RNA was isolated and SLFN12 mRNA levels were determined by qPCR. HPRT was used as reference gene. Mean of ±SD (n=2) is shown. One representative of three independent experiments is shown.

As HRV14 belongs to the major group HRVs and uses a different receptor (ICAM-1) for cell entry as minor group HRVs (LDLR), silencing was performed in the presence of HRV2, a member of the minor group. The dose for HRV2 (100 $TCID_{50}$/cell) necessary to see comparable fold induction was higher than that for HRV14. A longer incubation time (1h and 9h) was chosen too for improvement of the induction. qPCR data clearly demonstrated that HRV2 replication is hindered too (Figure 20) and SLFN12 expression levels were not changed by the virus at the indicated post-infection time points (Figure 21), as expected.

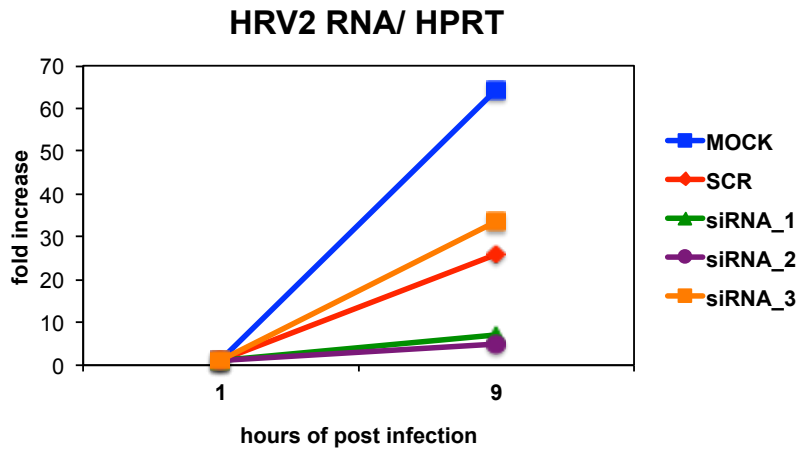


Figure 20: HRV2 replication is impaired in knockdown cells
HeLa-Ohio were either MOCK transfected or transfected with SCR, siRNA_1, siRNA_2 and siRNA_3. 72h post-transfection cells were infected with HRV2 (100 $TCID_{50}$/cell). After 1 and 9h total cellular RNA was isolated and mRNA levels were determined by qPCR. HPRT was used as reference gene. One representative of two independent experiments is shown.

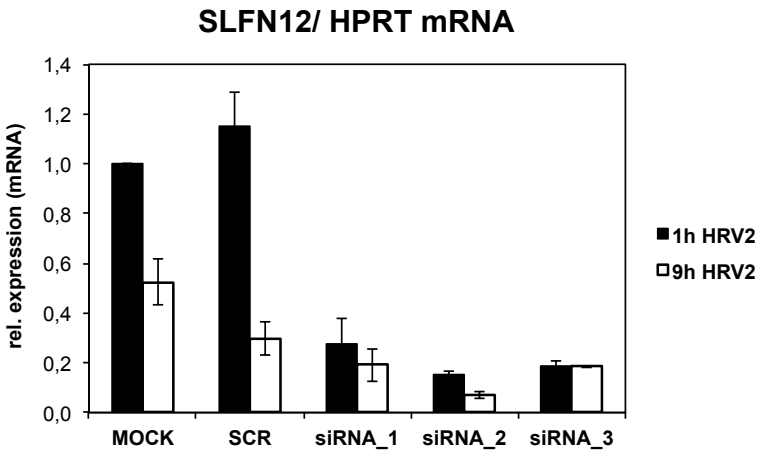


Figure 21: SLFN12 gene expression in knockdown cells infected with HRV2
72h post-transfection cells were infected with HRV2 (100 $TCID_{50}$/cell) for 1h (black bars) or 9h (white bars). Total cellular RNA was isolated and SLFN12 mRNA levels were determined by qPCR. HPRT was used as reference gene. Mean of ±SD (n=2) is shown. One representative of two independent experiments is shown.

Taken together, we could demonstrate that SLFN12 is required for HRV replication independent of receptor specificity of the viruses.

According to our HRV replication data upon SLFN12 silencing (Figure 18A), we next asked whether IgA and IgG might be able to neutralize the virus directly, thereby leading to decreased or complete inhibition of viral replication. It is known that these two Abs are the only ones, which are produced upon HRV infection. 1 mg/ml of mIgA and Beriglobin, which contains at least 95% IgG according to the manufacturer, was incubated together with HRV14 at 37°C for 1h before addition to HeLa-Ohio cells. 3 different viral concentrations ($TCID_{50}$/cell) were chosen. Analysis of the experiment was done by measuring cell proliferation. mIgA definitely protected the cells from HRV14 infection (Figure 22A) even at a higher viral titer. IgG was also able to neutralize the virus, but the protection was less effective at higher viral concentrations (Figure 23A). In a second experiment we replaced IgG by SIgA (secretory IgA) (100µg/ml) and choose a longer incubation time point. Cells were first incubated with mIgA and SIgA at 37°C for 1h before adding HRV14 ($TCID_{50}$/cell). Due to the limited used concentration of SIgA and the longer viral incubation time, SIgA did show little till no protection, whereas mIgA again neutralized the virus (Figure 22B).

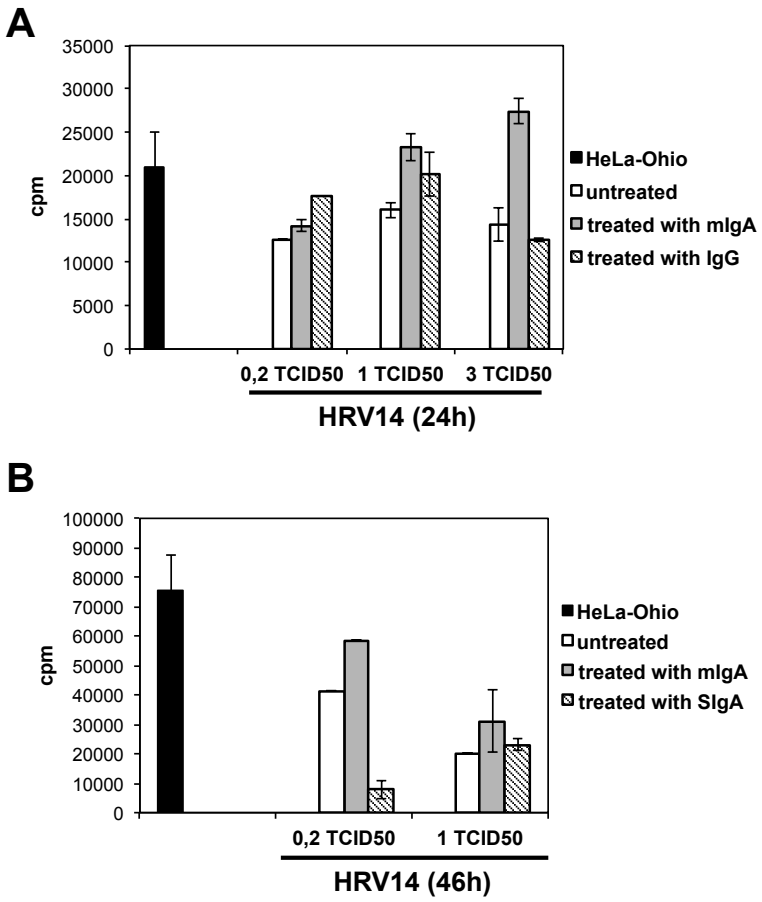


Figure 22: IgA protects cells from HRV infection
(A) HRV14 ($TCID_{50}$/cell) was incubated together with either mIgA or Beriglobin (IgG) at 37°C for 1h before addition to the cells. Proliferation of the cells was monitored 7h later by addition of ^{3}H-Thymidine. 17h later its incorporation was measured. Mean ±SD (n=3) is shown.
(B) Cells were first incubated with either mIgA or SIgA at 37°C for 1h before HRV14 ($TCID_{50}$/cell) was added. Cell proliferation was monitored 30h later by adding ^{3}H-Thymidine. Its incorporation was measured 16h later. Mean ±SD (n=2) is shown.
mIgA: monomeric IgA, SIgA: secretory IgA

Taken together, these results indicated that mIgA, SIgA and IgG do have a protective effect against HRV14, but this effect depends on the used viral titer, the Ab concentration and the viral incubation time.

4.8 SLFN gene expression in a HRV resistant HeLa-Ohio clone

During our study we generated a HeLa-Ohio cell line, called clone 6, which is resistant to HRV. When expression levels of all human SLFN genes were measured by qPCR in this cell line, clone 6 was found to express less SLFN12 even in response to HRV14 than HeLa-Ohio cells (Figure 23). SLFN5 mRNA level was

unchanged in both cell lines and in both cells SLFN12L/13/14 were not expressed. Although clone 6 is RV resistant, no immediate degradation of viral RNA was observable (Figure 24B), whereas viral RNA increased with time in HeLa-Ohio (Figure 24A).

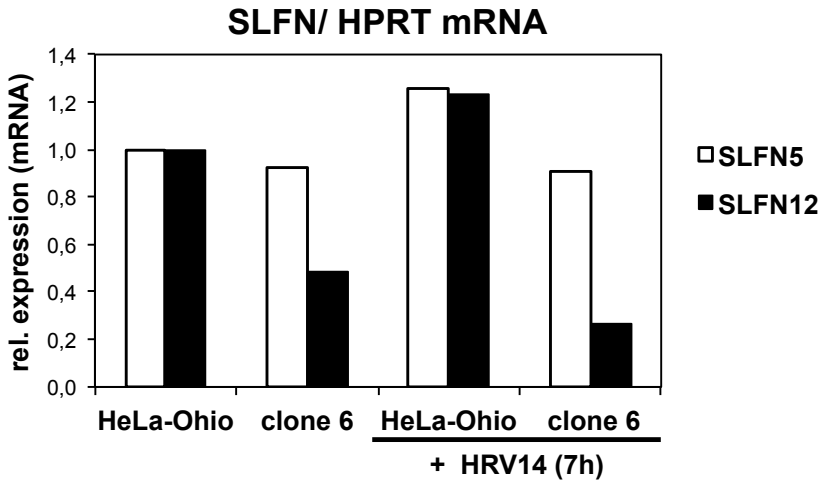


Figure 23: SLFN gene expression in clone 6
Total cellular RNA of HeLa-Ohio and clone 6 (RV resistant HeLa-Ohio cell line) was isolated and mRNA levels were determined by qPCR. Black bars show SLFN12 mRNA levels and white bars SLFN5. SLFN12L/13/14 were not detectable. As reference gene HPRT was used.

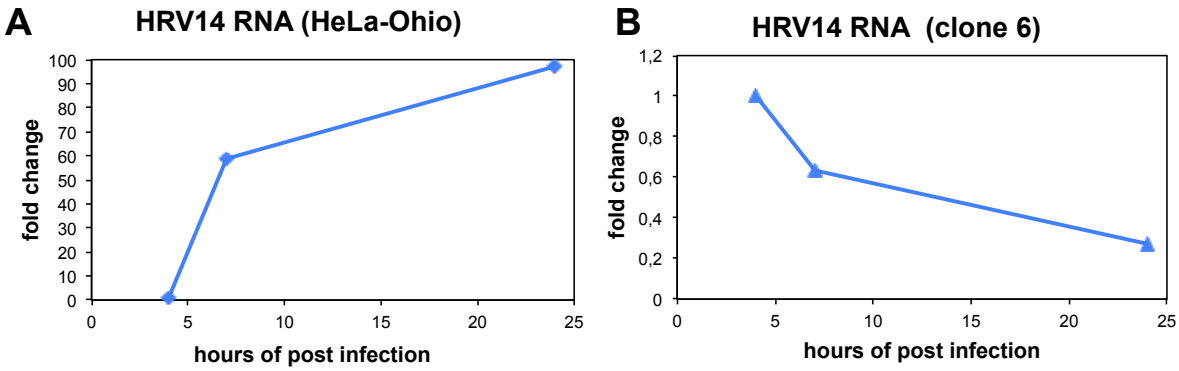


Figure 24: HRV14 replication in HeLa-Ohio and clone 6
(A) (B) Total cellular RNA of HeLa-Ohio and clone 6 infected with HRV14 was isolated and mRNA levels were determined by qPCR. As reference gene HPRT was used.

4.9 HeLa 91/1948 cells express slightly less SLFN12 than HeLa-Ohio cells

In order to examine if another HeLa strain, called HeLa 91/1948, shows the same human SLFN gene expression pattern, we isolated cellular RNA and quantitated indicated SLFN mRNA levels via qPCR. SLFN5 and SLFN11 mRNA levels were

quite similar in both cell lines, while SLFN12 was slightly decreased in HeLa 91/1948 cells (Table 2). The remaining human SLFN genes (SLFN12L, SLFN13 and SLFN14) were not detectable in both cell lines.

ΔCt (target gene-HPRT)	HeLa-Ohio	HeLa 91/1948
SLFN5	2,67 ± 0,21	2,76 ± 0,24
SLFN11	0,92 ± 0,33	1,47 ± 0,05
SLFN12	7,87 ± 0,14	8,75 ± 0,42
SLFN12L	N.D	N.D
SLFN13	N.D	N.D
SLFN14	N.D	N.D

Table 2: HeLa 91/1948 cells express slightly less SLFN12 than HeLa-Ohio cells
Total cellular RNA from HeLa-Ohio and HeLa 91/1948 was isolated and levels of indicated mRNAs and HPRT were determined by qPCR. The expression of the indicated mRNAs is presented as ΔCt-values between GAPDH and the SLFN mRNAs. Mean ±SD (n=2) is shown.

4.10 HeLa 91/1948 cells are less susceptible to HRV infections

Next we investigated whether this HeLa 91/1948 cell line is as susceptible to HRV infection as HeLa-Ohio cells. HeLa 91/1948 and HeLa-Ohio cells were infected with 4 different viral titers of HRV14 ($TCID_{50}$/cell) for 46h and analysis of the experiment was done by measuring cell proliferation. As seen in Figure 25A, cell survival of HeLa 91/1948 cells was not affected at viral concentrations of 0,2 and 1 $TCID_{50}$. A cpe was only observable at concentrations of 5 and 10 $TCID_{50}$ in these cells. In contrast HeLa-Ohio cells were already dead at those concentrations.

To further explore why such a high viral titer is necessary to observe any cpe in HeLa 91/1948 cells, we looked for ICAM-1 as well as MxA expression. Flow cytometric analysis demonstrated that this cell line does highly express the receptor (Figure 25B). MxA, which is a classical IFN-induced antiviral gene, was increased in HeLa 91/1948 cells compared to HeLa-Ohio cells (Figure 25C), suggesting an antiviral mechanism in those cells.

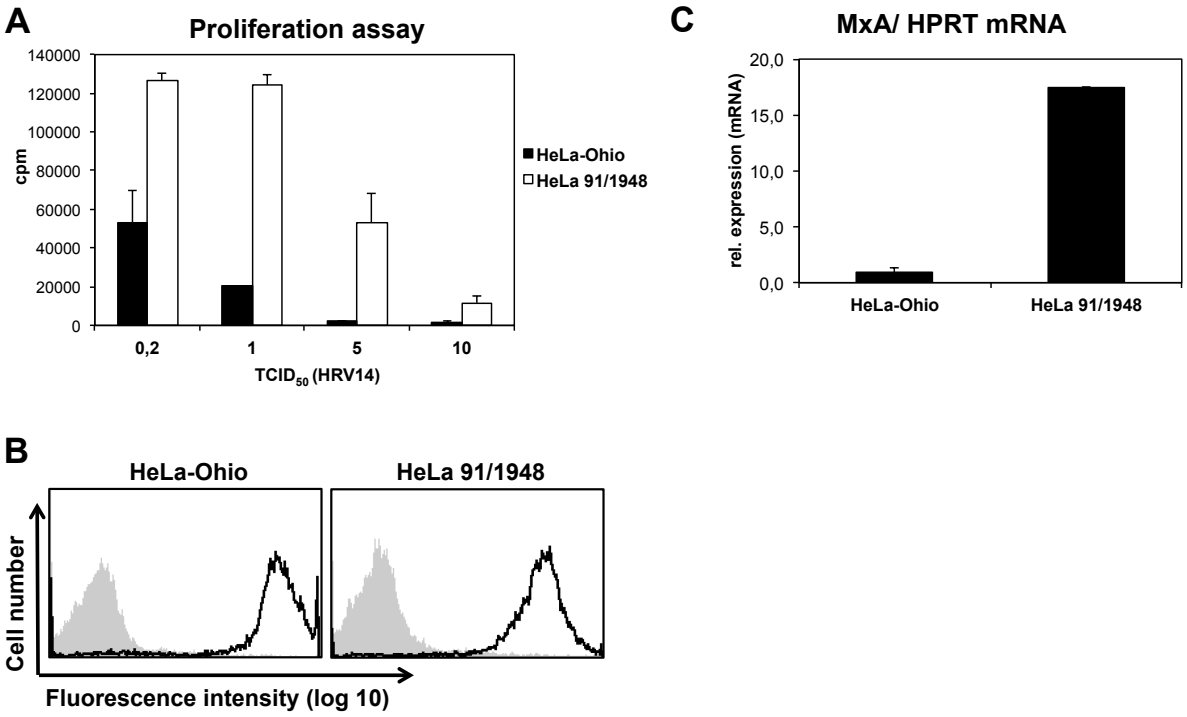


Figure 25: HeLa 91/1948 cells are less susceptible to HRV infections
(A) HeLa-Ohio and HeLa 91/1948 cells were infected with different viral titers ($TCID_{50}$/cell) of HRV14. Cell proliferation was monitored 30h later by adding ^{3}H-Thymidine. Its incorporation was measured 16h later. Mean ±SD (n=2) is shown.
(B) Total cellular RNA from HeLa-Ohio and HeLa 91/1948 was isolated and MxA mRNA levels were determined by qPCR. HPRT was used as reference gene. Mean ±SD (n=2) is shown.
(C) HeLa-Ohio and HeLa 91/1948 cells were used for cell surface staining of the mAb ICAM-1. Grey scale shows the isotype control mAb (VIAP).

4.11 Successful overexpression of SLFN12 in HeLa 91/1948

To further explore whether SLFN12 overexpression is possible in HeLa 91/1948 cells, a retroviral gene transfer as already described for HeLa-Ohio (see chapter 4.3) was performed. While overexpression of SLFN12 in HeLa-Ohio resulted in cell death (Figure 11A/B), no cell death was observable in HeLa 91/1948 cells (Figure 26A). Transfection efficiency measured by EYFP expression via flow cytometric analysis was again successful in terms of control vector, but in case of pMMP-SLFN12 vector less efficient (Figure 26B). To ensure that SLFN12 overexpression independent of EYFP expression results was successful, SLFN12 mRNA level was determined by qPCR (Figure 26C).

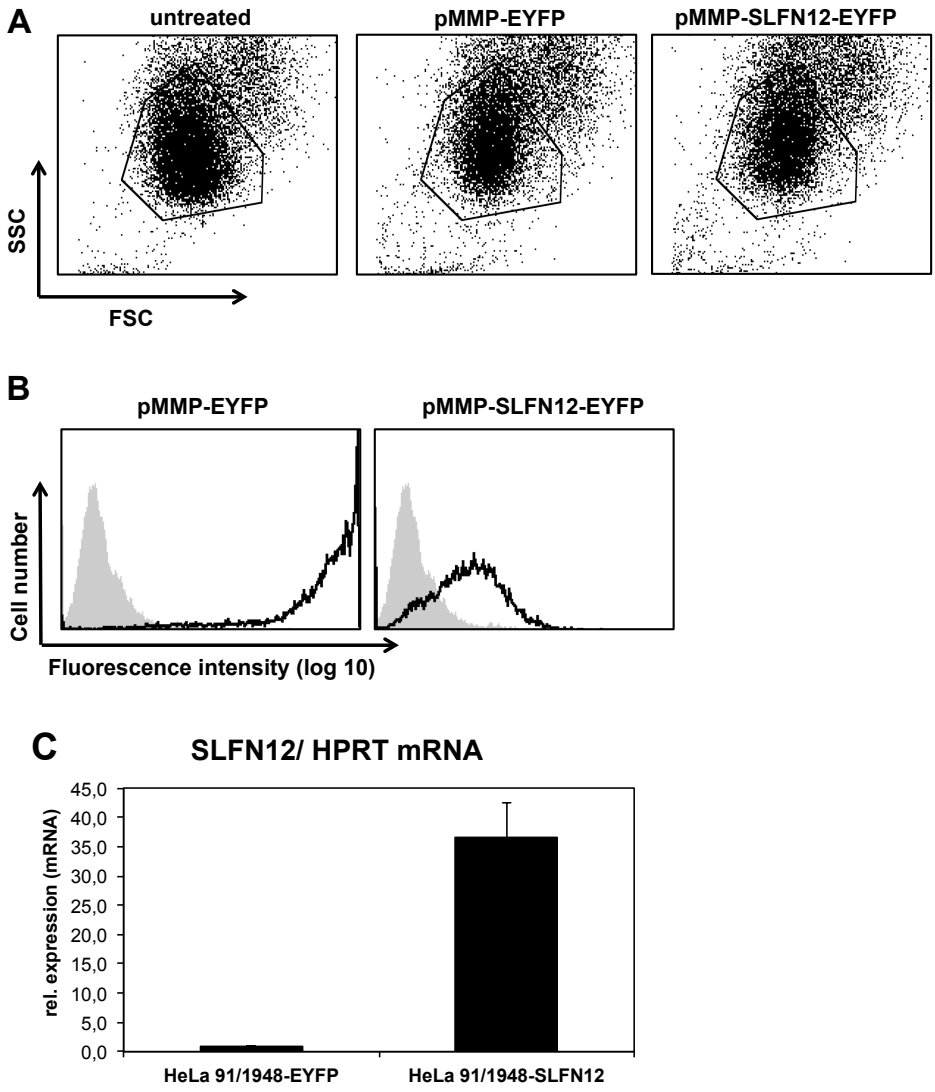


Figure 26: Overexpression of SLFN12 in HeLa 91/1948 cells
(A) HeLa 91/1948 cells were either transfected with a control vector (pMMP-EYFP) or with a pMMP-SLFN12-EYFP vector for several days. Cells were then analyzed by flow cytometry. Circularized cells visualize the gated cells.
(B) EYFP fluorescence of transduced cells was analyzed via flow cytometry. The grey scale shows the negative control (untreated HeLa 91/1948).
(C) Total cellular RNA from HeLa 91/1948 either transfected with pMMP-EYFP or pMMP-SLFN12-EYFP was isolated and SLFN12 mRNA levels were quantitated by qPCR. HPRT was used as reference gene. Mean of ±SD (n=2) is shown.

4.12 Overexpressed SLFN12 does not lead to a better HRV14 replication in HeLa 91/1948 cells

Due to the successful SLFN12 transfection in HeLa 91/1948 cells, we finally infected these cells with HRV14 (100 $TCID_{50}$/cell). Total cellular RNA was isolated 1h and 7h post-infection and determination of viral RNA was done by qPCR. Surprisingly, data demonstrated that there is no significant difference between overexpressed and non-overexpressed cells indicating that overexpression may not lead to a better replication (Figure 27A). To exclude that retroviral gene transfer had any impact on receptor expression, cells were analyzed for ICAM-1 via flow cytometric analysis (Figure 27B).

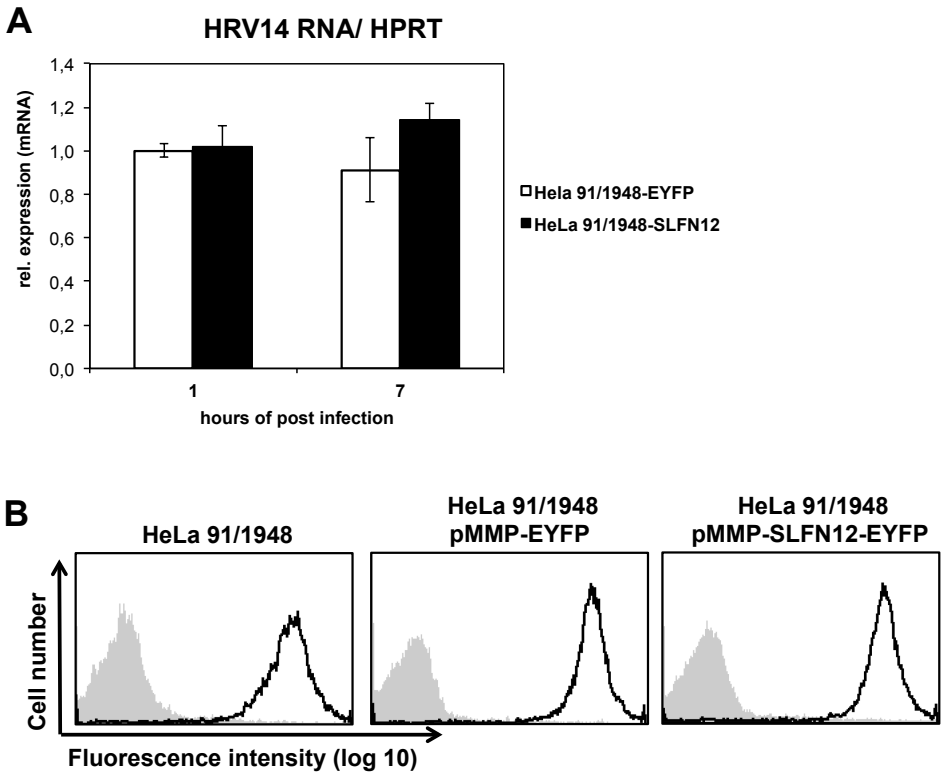


Figure 27: Overexpressed SLFN12 does not lead to a better replication of HRV14 in HeLa 91/1948

(A) HeLa 91/1948 cells transfected with pMMP-EYFP (white bar) or pMMP-SLFN12-EYFP (black bar) were infected with HRV14 (100 $TCID_{50}$/cell) for 1h and 7h. Total cellular RNA was isolated and mRNA levels were determined by qPCR. HPRT was used as reference gene. Mean of ±SD (n=2) is shown.

(B) HeLa 91/1948 either transfected with the control vector (pMMP-EYFP) or with SLFN12 were used for cell surface staining of the mAb ICAM-1. Grey scale shows the isotype control mAb (VIAP).

Recently, membrane hijacking was described for Hepatitis A virus (HAV), a non-enveloped virus, where the virus released from cells is cloaked in host-derived membranes [87]. Treatment with 1% NP-40, a nonionic detergent, destroyed this membrane without affecting the infectivity of the viral particles. Regarding this paper, we therefore asked whether HRV might be also encoated by a membrane and if treatment with NP-40 would make the viral particles more infectious.

10µl of the viral stock (HRV14) was incubated together with the same volume of 1% NP-40 buffer (1% Triton X-100) on ice for 1h, before infection of cells for 70h. To exclude that cells are more susceptible to the virus due to NP-40, virus/NP-40 mix was diluted 1:10.000. Analysis of cell proliferation demonstrated that treated NP-40 virus is less infectious in contrast to non-treated ones (Figure 28), thus leading to a higher rate of cell survival.

Our observations suggested that treatment of HRV14 with NP-40 makes the virus less infectious.

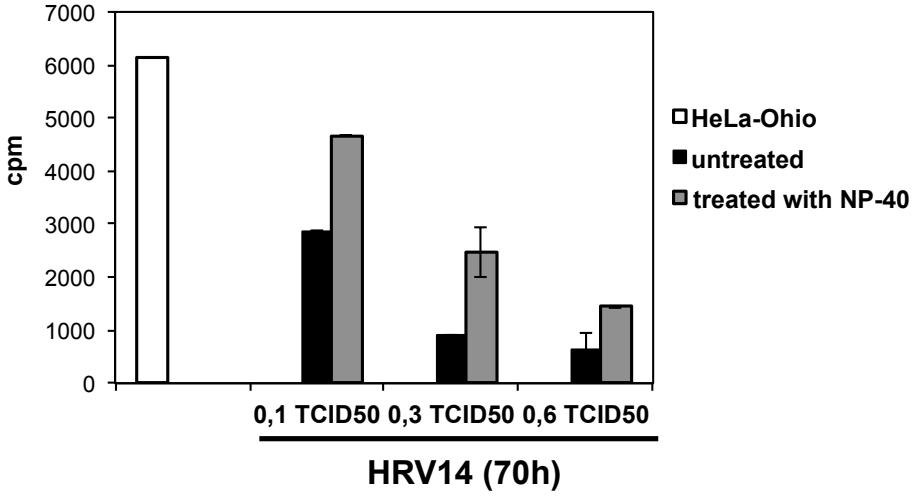


Figure 28: Treatment of HRV with NP-40 makes the virus less infectious
10µl of viral stock (HRV14) was incubated with the same volume of 1% NP-40 (1% Triton X-100) for 1h on ice. Virus/NP-40 mix was diluted 1:10.000 to get rid of NP-40, before infecting cells. Cell proliferation was monitored 54h later by adding ^{3}H-Thymidine. Its incorporation was measured 16h later. Mean ±SD (n=2) is shown.

5 DISCUSSION

HRV, the major cause of common cold, occurs worldwide and causes severe diseases in the lower as well as in the upper respiratory tract. Belonging to the *Picornaviridae* family, HRV species are divided into major and minor group HRVs based on receptor specificity. It is characteristic for these human pathogenic viruses that their replication takes only place in the cytosol of epithelial cells.

The SLFN gene family, first identified in the mouse, comprises several mouse and human member genes, which are involved in diverse immunological processes, such as regulation of cell proliferation and control of viral replication (i.e. HIV) [65-68]. All SLFNs are inducible by type I IFNs indicating antiviral properties of this gene family. Six human SLFN genes have been identified so far, of which only SLFN12 is predicted to be located in the cytosol, where HRV replicates. SLFN12 attracted our interest, because we observed that SLFN12 is expressed in DCs and is highly upregulated in DCs upon HRV infection (unpublished data). Recently, antiviral activity of SLFN12 was demonstrated for VSV and MHV-68 [75]. SLFN12 seemed therefore to be an attractive candidate to study its role in HRV infections.

Not much is known about SLFN12 yet, the more it is important to develop an Ab that specifically binds to this protein. Therefore, binding specificity of anti-SLFN12 Abs, generated in our laboratory, were analyzed in various cell lines and primary cells via flow cytometry. Unfortunately, none of these Abs showed specific reactivity with SLFN12.

Next SLFN12 mRNA levels were quantified in several cell lines, such as HeLa-Ohio, and primary cells via qPCR. According to our data (Table 1, Figure 7), only HeLa-Ohio, THP-1 cells and DCs expressed SLFN12. Interestingly, DCs and differentiated THP-1 cells treated with IFN-α showed the highest expression level indicating involvement of SLFN12 in antiviral defense. HRV does enter monocytes and DCs, but does not replicate due to the immediate induction of an immune response in these cells. In contrast to primary cells, HeLa-Ohio cells, an epithelial cell line, are highly susceptible to viral infection and were therefore chosen as model for our studies.

In order to elucidate the effects of overexpression and silencing, respectively, we first overexpressed SLFN12 in HeLa-Ohio cells, retrovirally. After transduction,

cell viability was dramatically reduced and could not be further used for HRV infection. In terms of SLFN12-transduced HeLa-Ohio cells, transfection efficiency measured by EYFP expression via flow cytometry was less efficient either due to the large size of the insert (~ 1,2 kb) or due to the conformation of the SLFN12-IRES-EYFP hybrid mRNA. However, considering that gene overexpression resulted in cell death, we concentrated on gene silencing.

Silencing efficiency of siRNA_1, siRNA_2 and siRNA_3, respectively was controlled by quantitation of mRNA as well as protein expression levels. In contrast to overexpression, knockdown did not affect cell viability. After transfection with siRNAs, not only morphological changes of HeLa-Ohio cells (data not shown) were observable, but also an antiproliferative effect of SLFN12 silencing (Figure 15A) was obtained. We demonstrated that SLFN12 siRNAs inhibited cell growth, but surprisingly no increased induction of apoptosis occurred according to our AnnexinV/PI staining (Figure 15B). Moreover, we showed that although transfected HeLa-Ohio cells are growth inhibited, silencing does not affect cytokine production or expression of related cell markers such as MxA or MHC-I. IL-6, an inflammatory cytokine, was spontaneously produced by transfected and non-transfected HeLa-Ohio cells in a remarkable amount even in response to HRV (Figure 16). Interestingly, siRNA_1 transfected cells had the highest measurable amount of IL-6. It is described in several studies that ds as well as ss siRNAs can induce inflammatory cytokines and IFN-responses in human peripheral blood mononuclear cells (PBMCs) [88,89]. This effect is sequence-dependent and requires endosomal localization [88,89]. Overall analysis indicated that siRNAs containing the dinucleotide GU activate the expression of inflammatory cytokines [88]. Our observation regarding IL-6 in case of siRNA_1 was either a coincidence due to the fact that this experiment was only performed once or a phenomenon caused by the siRNA itself. We also demonstrated that S100A4 and MHC-I are stable expressed in all cells (transfected and non-transfected), while MxA was not expressed as expected. In contrast, CD71 was slightly decreased in siRNA_1 and siRNA_3 transfected cells, respectively, but highly upregulated in siRNA_2 transfected cells. It is unclear why CD71 was increased, but maybe it was an off-target effect of the siRNA. Most importantly, ICAM-1, which is the receptor for cell entry of major group viruses, was unchanged and highly expressed in all cells.

The present results provide our hypothesis that SFLN12 has to be tightly regulated in HeLa-Ohio cells (Figure 29). Overexpression of the gene led to cell death, while silencing induced an impaired proliferation of HeLa-Ohio cells. Moreover, SLFN12 silencing did not induce enhanced apoptosis and was not compensated for by the other human SLFN genes (Figure 14). We could not find any similar results in the literature regarding our observations obtained during overexpression and silencing, respectively. It is reported that either gene overexpression led to cell death and silencing to an increased cell proliferation or vice versa.

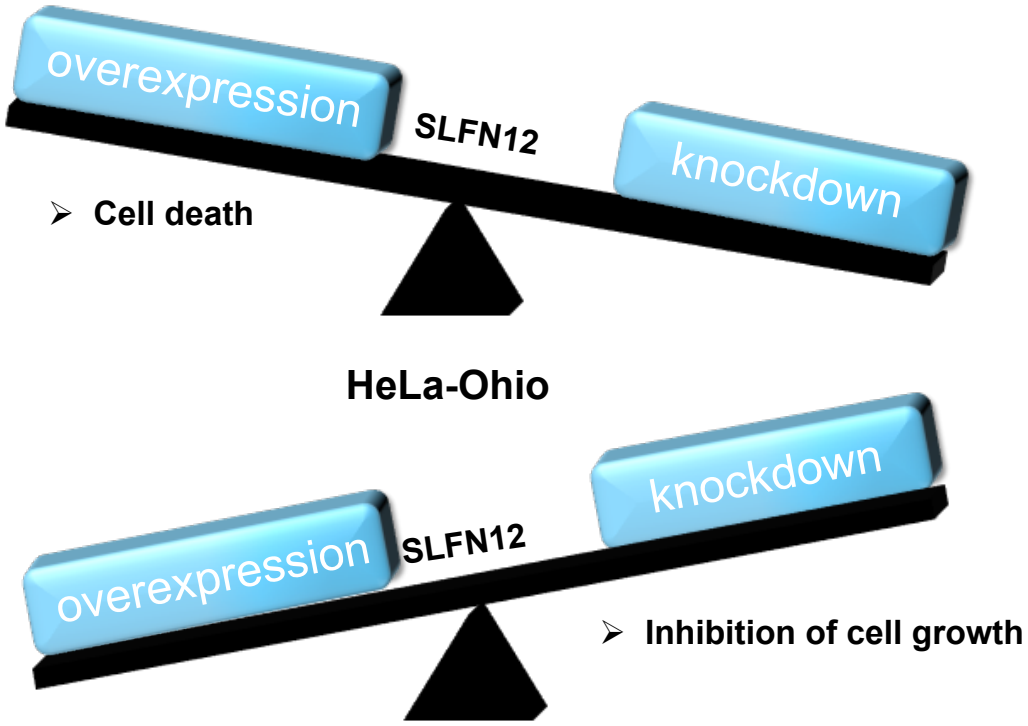


Figure 29: Hypothesis

Upon successful gene silencing, transfected cells were infected with HRV14 and HRV2, respectively. As major group HRVs, such as HRV14 enter via the receptor ICAM-1 and minor group HRVs (i.e. HRV2) via LDLR, we performed experiments with both viruses. As shown by qPCR and Western Blot, SLFN12 silencing was associated with an impaired viral replication as well as viral (capsid) protein (VP1-3) production and both viruses did not counteract this knockdown. This indicated that SLFN12 is required for a productive HRV replication in HeLa-Ohio cells independently of receptor specificity.

It is known that IgA and IgG are the only Abs produced during HRV infection, but do not play a role from recovery due to their late appearance. Therefore we asked whether these Abs might be able to neutralize HRV14 directly, thereby leading to a hindered viral replication or even a complete prevention of HRV infection in HeLa-Ohio cells. As shown by cell proliferation, mIgA, SIgA and IgG, respectively, had a protective effect, whereby IgG was less protective than mIgA at higher viral concentrations. We could demonstrate that these two Abs are able to neutralize the virus directly, but this protective effect was dependent on the viral titers used, Ab concentration and viral incubation time.

During our study we generated an HRV-resistant HeLa-Ohio clone, called clone 6, which expressed less SLFN12 compared to HeLa-Ohio cells.

Furthermore, we could demonstrate that another HeLa strain (HeLa 91/1948) expressed SLFN12 almost to the same extent as HeLa-Ohio cells, but was less susceptible to HRV14 infections. Surprisingly, gene overexpression in those cells did not affect cell viability as in HeLa-Ohio and cells were cultivable. Notably, a higher viral titer use was necessary to observe any cpe in HeLa 91/1948 cells. SLFN12 overexpression of HeLa 91/1948 cells infected with HRV14 did not result in a significant better replication of the virus, as expected. We could exclude that this HeLa strain did not express ICAM-1, but cells showed a higher MxA expression compared to control suggesting a still active antiviral defense against HRV in HeLa 91/1948 cells. Interestingly, although both HeLa strains express SLFN12 almost to the same extent, overexpression was successful only in HeLa 91/1948 cells. Moreover, these strains differed not only in MxA expression, but also in cell morphology (data not shown).

Recently, membrane hijacking was described for a non-developed picornavirus (HAV) [87] and therefore we asked whether HRV might be also encoated by a host-derived membrane and if treatment with a nonionic detergent, NP-40, would destroy the membrane, thereby leading to more infectious particles. Our cell proliferation results demonstrated that treatment of HRV14 with NP-40, a nonionic detergent, makes the virus less infectious, thereby leading to a higher rate of cell survival. This indicated that HRV14 is presumably not encoated by a host-derived membrane.

In summary, we could demonstrate that SLFN12 is required for productive HRV replication in HeLa-Ohio cells independently of receptor specificity. On the basis of our findings we assume that SLFN12 is a cellular host factor for HRV replication,

which might be involved in HRV translation as well as in transcription (Figure 30). Two host factors, PTB and PCBP2, are already known to be sufficient in both events [30,31]. The mechanism how SLFN12 supports HRV replication is not clear yet, but an EMSA (electrophoretic mobility shift assay) experiment would be a first step to elucidate if SLFN12 either directly interacts with viral RNA or is a cellular co-factor for another host factor of HRV replication, such as PTB or PCBP2. All viruses belonging to picornavirus family use host cell factors for their intracellular replication cycles. Therefore, it would be advisable to examine if SLFN12 may be also involved in the replication of other picornaviruses or may even have an inhibitory effect on viral replication as already shown for VSV and MHV-68 [75].

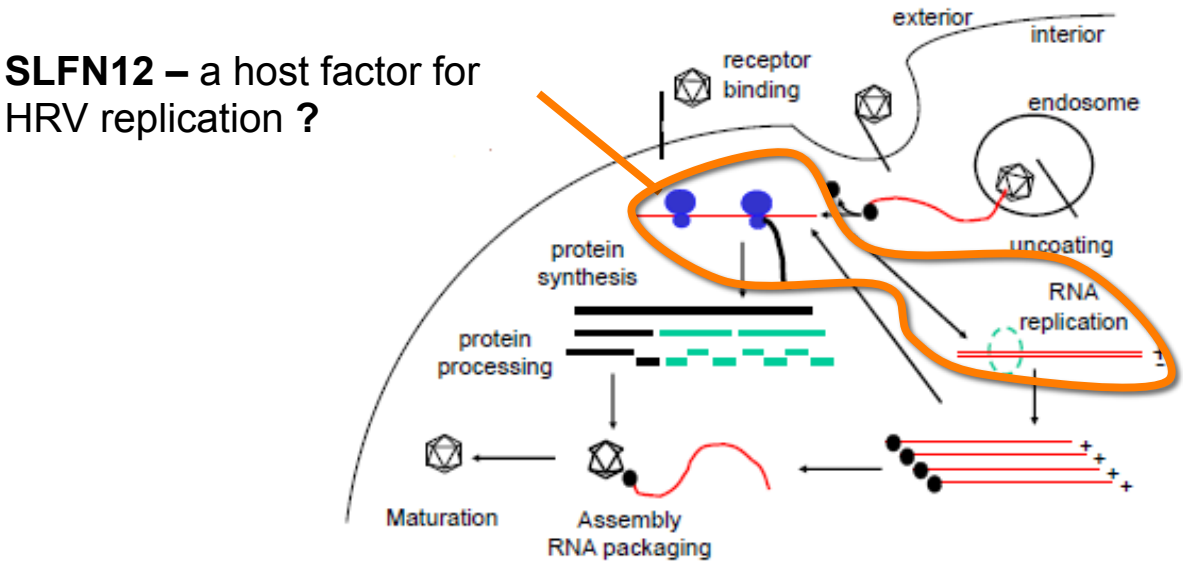


Figure 30: Outlook
Figure was adapted from [83]

6 ABBREVATIONS

(m)Ab	(monoclonal) antibody
ATCC	American Type Culture Collection
BSA	Bovine serum albumin
CD	Cluster of Differentiation
cDNA	Complementary DNA
CPE	Cytopathic effect
CPM	Counts per minute
Ct	Cycle threshold
DC	Dendritic cell
DMSO	Dimethyl sulfoxide
EYFP	Enhanced yellow fluorescent protein
FSC	Forward scatter
GAPDH	Glyceraldehyd 3-phosphate dehydrogenase
HPRT	Hypoxanthine phosphoribosyltransferase
HRP	Horseradish peroxidase
HRV	Human rhinovirus
ICAM-1	Intercellular adhesion molecule 1
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
LDL	Low density lipoprotein
MACS	Magnetic activating cell sorting
MHC	Major histocompatibility complex
mIgA	monomeric IgA
MNC	Mononuclear cells
mRNA	messenger ribonucleic acid
MxA	Myxovirus resistance gene A
Mφ	Macrophage
PCR	Polymerase chain reaction
PE	Phycoerythrin
PI	Propidium Iodide
PMA	Phorbol 12-myristate 13-acetate
q(RT)PCR	Quantitative (Real time) PCR
S100A4	S100 calcium binding protein A4
SD	Standard deviation
SIgA	Secretory IgA
siRNA	Small interfering RNA
SLFN	Schlafen gene familiy
SSC	Side scatter
$TCID_{50}$	Tissue culture infective dose
VP	viral (capsid) protein

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8 CURRICULUM VITAE

Personal data

Name:	Kapsch Anna-Maria
Date of birth:	February 4, 1989
Place of birth:	Klagenfurt, Austria
Nationality	Austria
Address:	A-9020 Klagenfurt
	A-1180 Wien
E-Mail:	am.kapsch@gmx.net

Education

1995-1999	elementary school VS 3, Klagenfurt
1999-2007	secondary school Europagymnasium, Klagenfurt
2007-2012	Bachelor degree in “Biology-Genetics and Microbiology” at the University of Vienna, Austria
Since 2012	Master study in “Molecular Microbiology and Immune Biology” at the University of Vienna, Austria
Nov. 2013 – Sept. 2014	Master thesis at the Institute of Immunology, Medical University Vienna, Austria Title: “Role of Schlafen family member 12 (SLFN12) in human rhinovirus (HRV) infections”

Spoken languages

German (native)

English