Innate Immune Mechanisms in the Recognition of Herpesviridae

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This thesis is dedicated to my mother and father.

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// Abstract

Throughout our life cycle, the human body is exposed to harmful microorganisms. The innate immune system is a fundamental factor in the human body, which helps eliminate foreign organisms through specific signalling pathways with the involvement of immune receptors and signalling molecules.

Viruses have evolved to infect the host and bypass the host immune responses, however a plethora of Pattern recognition receptors exist in the cell that are capable of detecting viral pathogens and mounting an innate immune response.

Herpes simplex Virus Type 2 and Cytomegalovirus are common human pathogens that cause genital ulcerations, organ failure and mental health problems like encephalitis. In this study, we have aimed to identify the host's innate immune response to HSV2 and HCMV infection in primary vaginal cells as well as Hela cells. Our data have shown that these viruses are recognized by TLR2 on the cell surface followed by intracellular PRRs such as TLR9, DAI, and IFI16, which trigger cytokine activation and release. Confocal imaging has revealed that these PRRs are located in different cell compartments and during viral cell entry and replication they can identify viral presence at specific parts of the cell. Therefore it seems that different PRRs are strategically placed in different cell locations to detect virus invasion and replication in order to activate cytokine secretion and protect the host.

When different agonists for PRRs were used it was revealed that they were effective against Herpes virus infection thus indicating that a combination of PRRs agonists especially ones that trigger different cytokines could provide a wider spectrum prophylaxis to the host and they can be used to generate efficient treatment against HSV2 and HCMV infection.

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Abbreviations:

A	
ADAR1	Adenosine deaminase RNA-specific 1
AIM2	Absent in melanoma 2
ASC	Apoptosis-associated speck-like protein containing a CARD
ATCC	American Type Culture Collection
AP-1	Activator protein 1
APS	Ammonium persulfate
B	
BFA	Brefeldin A
C	
c-di-AMP	Cyclic diadenylate monophosphate
c-di-GMP	Cyclic diguanylate monophosphate
C-terminal	Carboxyl-terminal
CARD	Caspase recruitment domain
CBA	Cytometric bead array
CD14	Cluster of differentiation
CHO	Chinese hamster ovary
cm^2	Square centimetre
CMV	Cytomegalovirus
CpG	Cytosine-phosphate-guanosine
CREB	Cyclic AMP-responsive element-binding protein
CTD	Carboxyl tail domain
D	
DAI	DNA-dependent activator of IFN-regulatory factors
DAMP	Damage-associated molecular pattern
pDC	Plasmacytoid dendritic Cells

DD	Death domain
DDX	DEAD box
dH_2O	Distilled water
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DNase	Deoxyribonuclease
dsDNA	Double-stranded deoxyribonucleic acid
dsRNA	Double-stranded ribonucleic acid
dUTPase	Deoxyuridine-triphosphatase
E	
EBV	Epstein-Bar virus
E.coli	Escherichia coli
ECL	Enhanced chemiluminescence
EEA-1	Early endosome antigen 1
EGFR	Epidermal growth factor receptor
ER	Endoplasmic reticulum
F	
FACS	Fluorescence activated cell sorter
FCS	Foetal bovine/calf serum
FITC	Fluorescein isothiocyanate
FSC	Forward scattered light
G	
g	glycoprotein
GFP	Green fluorescent protein
GMK	Green monkey kidney cell
H	
HCE	Human cervical epithelial cells

HCMV	Human cytomegalovirus
HEK293	Human embryonic kidney 293
HHV	Human herpes virus
HMGB	High mobility group proteins
HRP	Horse radish peroxidase
HS	Heparan sulfate
HSE	Herpes encephalitis
HSP	Heat shock protein
HSV	Herpes simplex virus
hTLR	Human TLR
HVEM	Herpesvirus entry mediator
I	
ICP	Infected cell protein
IE	Immediate early
IgV	V-like immunoglobulin
IFI16	Interferon inducible protein 16
IFN	Interferon
IKK	IκB-kinase
IL	Interleukin
IL-1R	Interleukin-1 receptor
IRAK	Interleukin-1 receptor-associated kinase
IRF	Interferon regulatory factor
ISGF3	Interferon-stimulated gamma factor 3
ISRE	IFN-stimulated response elements
IκB	Inhibitor of NF-κB
J	
JAK1	Janus kinase 1

JNK	Jun kinases
K	
kbp	Kilo-base pair
KSHV	Kaposi's sarcoma herpesvirus
L	
LAL	Limulus amebocyte lysate
LAT	Latency-associated transcript
LB	Lysogeny broth / luria broth
LGP2	Laboratory of genetics and physiology 2
LPS	Lipopolysaccharide
LRR	Leucine rich repeat
LRRFIP1	Leucine rich repeat (in FLII) interacting protein 1
LTA	Lipoteichoic acid
M	
MAPK	Mitogen-activated protein kinase pathway
MAVS	Mitochondrial antiviral-signaling protein
MCMV	Murine cytomegalovirus
MD2 (LY96)	Lymphocyte antigen 96
MDA-5	Melanoma-differentiation-associated gene 5
MHC	Major histocompatibility complex
MOI	Multiplicity of infection
MW	Molecular weight
MyD88	Myeloid differentiation primary response gene 88
N	
N.A.	Non-essential amino acids
N-terminal	Amino-terminal
NEMO	NF-κB essential modulator

NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B-cells
NIEPS	Non-infectious enveloped particles
NLR	Nod-like receptor
NLS	Nuclear localization signal
nm	Nanometer
NPC	Nuclear pore complex
O	
OB	Oligonucleotide/oligosaccharide binding
OD	Optical density
ORF	Open reading frame
P	
Pac 1	5'-TAAAAA-3'
Pac 2	5'-TTTTAT-3'
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PE	Phycoerythrin
PFA	Paraformaldehyde
PFU	Plaque-forming unit
PYD	Pyrin domain
PYHIN	Pyrin and HIN domain-containing protein
PMT	Photomultiplier tube
Poly (Poltorak et al.)	Polyinosine-polycytidylic acid
PRR	Pattern recognition receptor
R	
RIG-I	Retinoic acid-inducible protein 1
RIP	Receptor-interacting protein
RHIM	RIP homotypic interaction motif

RLR	RIG-I-like receptor
RNase	Ribonuclease
RNAi	RNA interference
rRNA	Ribosomal RNA
S	
SD	Standard Deviation
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEAP	Secreted embryonic alkaline phosphatase
shRNA	Small hairpin RNA
STAT	Signal transducers and activators of transcription
SSC	Side scattered light
STING	Stimulator of interferon genes
T	
TAB	TAK1-binding protein
TAK1	Transforming-growth factor-β-activated kinase
TBK1	TRAF-family-member-associated NF-κB activator (TANK) binding kinase 1
TEMED	N,N,N’,N’-tetra- methylethylenediamine
TGF	Transforming-growth factor
TIF	Transinducing factor
TIR	Toll/interleukin-1 receptor
TIRAP	TIR associated protein
TLR	Toll-like receptor
TMV	Tobacco mosaic virus
TNF	Tumour necrosis factor
TRAF6	Tumour-necrosis-factor receptor-associated factor 6
TRAM	TRIF related adaptor molecule
TREX1	Three prime repair exonuclease 1

TRIF	TIR domain-containing adaptor protein-inducing IFN-β
TRITC	Tetramethyl rhodamine iso-thiocyanate
TyK2	Tyrosine kinase 2
U	
UBC13	E2 ubiquitin-conjugating protein UBC13
UEV1A	Ubiquitin-conjugating enzyme E2 variant 1
UL	Unique long
US	Unique short
V	
VP	Viral protein
VZV	Varicella-zoster virus
Z	
ZnF	Zinc finger

Chapter 1: Introduction

1.1 The Immune System

Immunology is a branch of biology and medicine, which is concerned about the immune system. This system gives the ability to the organism to be able to distinguish between self and non-self entities. From the beginning of human life, the human body has to defend itself from life-threatening organisms to stay alive. This highly specific defense mechanism against foreign microorganisms, which is provided by the immune system, is called *immunity.* The word *immunity* is derived from the Latin word *immunitas* that means, “to be exempt from”. The human body will be protected from particular *disease causing infectious microorganism*, known as *pathogen*, by triggering the certain mechanisms to eliminate this foreign organism. The immune system is divided into two different groups; innate immunity and adaptive (acquired) immunity.

1.1.1 Innate Immunity

In innate immunity the basic resistance will be exerted against the foreign organisms, innate immunity is present in the body since the birth of the person. It is the first line of defence, which has no self/nonself discrimination. This nonspecific immunity functions through three stages; atomic (mechanical) barriers, physiological (humoral) barriers and cellular barriers respectively. Anatomical barriers are skin and mucous membranes, whereas physiological barriers are temperature, pH and various soluble factors. If the pathogen is still able to survive, immune molecules phagocytes such as macrophages, dentritic cells appear and reactions like phagocytosis, endocytosis and inflammation takes place in order to destroy the infectious particle (Medzhitov, 2007). The innate immunity is able to identify vast variety of microorganisms from their particular patterns through innate immune receptors that distinguish these motifs. *Pathogen-associated molecular patterns (PAMP*s) and *pattern recognition receptors*

(*PRR*s) were first referred by Janeway during Cold Spring Harbor Symposia on Quantitative Biology held in 1989. Primitive immune system, the evolvement of clonally distributed immunity from the innate immunity and how innate immunity can possibly operate, were the main topics that Janeway pointed out and discussed in depth (Janeway, 1989). He had a theory that other than foreign molecules modified proteins could also initiate antibody production in the host. He suggested that immune system cannot only identify self/non-self, but also infectious and non-infectious molecules. He speculated that innate immunity and its' receptors were responsible for this self/non-self discrimination. This idea was in opposition to the current belief of immunologists in those years. They believed that B and T-cells, were responsible for the recognition of these microbes. Janeway's idea was supported by studies on the the immune mechanisms of invertebrates, since they can initiate an immune response against pathogens even though they lack the adaptive immunity. Lemaitre et al. supported Janeways' hypothesis in a study in 1996, he showed that the'Toll' gene mutant drosophila was susceptible to fungal infection (Lemaitre et al., 1996). Innate immunity receptors of the host organism recognize these highly conserved motifs and activate the downstream signalling cascades. Each pathogen-associated molecular pattern is unique to that class of infectious microorganism and they are vital for the microbe survival. For this reason they are always conserved on the pathogen and they do not change structurally, resulting in the constant recognition by the innate immune system of the host cell.

1.2 Pattern Recognition Receptors

Pattern recognition receptors (PRRs) are proteins expressed by cells of the innate immune system to identify pathogen-associated molecular patterns (PAMPs), which are associated with microbial pathogens or cellular stress. PRRs can be divided into 4

groups, which are: Toll-like Receptors (TLRs), Retinoic acid-inducible Gene-like Receptors (RLRs), Cytosolic DNA Sensors and Nod-like Receptors (NLRs).

Toll-Like Receptors are the first pattern recognition receptors that have been identified in innate immunity. They are transmembrane proteins possessing a leucine rich repeated-domain (LRR-domain) at the extracellular amino-terminal (N-terminal), followed by a cysteine-rich domain that is a cytosolic carboxyl-terminal (C-terminal). Currently there are 13 TLRs that have been distinguished by the scientists (*Figure 1.1*). Human TLRs are TLR1-10, mice have TLR1-9, TLR11-13 but not TLR10. They recognise different pattern associated molecular patterns such as bacterial motifs, fungal proteins and viral protein structures.

RLRs are cytosolic receptors that recognise viral RNA molecules. Retinoic-acid-inducible protein 1 (RIG-I) and melanoma-differentiation-associated gene 5 (MDA-5) are responsible for dsRNA recognition (Yoneyama and Fujita, 2007). Even though LGP2 (laboratory of genetics and physiology 2) is a member of RLRs, it functions as a RIG-I and MDA-5 negative feedback regulator by acting on RIG-I repressor domain or by interacting with MAVS adaptor molecule (Komuro and Horvath, 2006;Saito et al., 2007;Yoneyama et al., 2005).

Cytosolic DNA sensors are responsible for the cytosolic DNA recognition. DNA-dependent activator of IFN-regulatory factors (DAI) interacts with cytosolic dsDNA to trigger type I IFN production (Takaoka et al., 2007), whereas another DNA sensor IFI16 (Interferon inducible protein 16) in the nucleus leads to IFN-β production by interacting with a signalling adaptor protein STING (Stimulator of interferon genes) (Unterholzner et al., 2010). Besides of IFI16, during cytosolic DNA detection, DDX41 (DEAD box polypeptide 41) signals through STING as well (Zhang et al., 2011). Absent in melanoma 2 (AIM2) is a cytosolic DNA that causes inflammasome pathway

activation via adaptor molecule ASC (Apoptosis-associated speck-like protein containing a CARD), leading to caspase-1 activation and IL-1β, IL-18 secretion from the cell (Hornung et al., 2009). LRRFIP1 (Leucine rich repeat (in FLII) interacting protein 1) can sense cytosolic nucleic acids and start type I IFN production through beta-catenin-dependent pathway (Yang et al., 2010). P202 binds cytoplasmic dsDNA to inhibit caspase activation (Roberts et al., 2009).

NLRs are in the cytosol and are responsible for the recognition of PAMPs and endogenous molecules from bacteria. Depending on their N-terminal structures, NLRs can be divided into groups: NLRA, NLRB, NLRC, NLRP, and NLRX.

1.3 The Discovery of Toll-like Receptors

In 1985 Anderson and her group have discovered the gene called Toll in the Drosophila, which was playing an important role during the dorsal-ventral polarity development of the Drosophila embryo (Anderson et al., 1985). The relation of the toll-gene with the immunity was introduced in 1991 by Gay and Keith, mentioning that there were matching amino acid sequences between the cytoplasmic domain of Toll protein and the human interleukin-1 receptor (IL-1R) (Gay and Keith, 1991). In 1996 Lemaitre et al discovered that Toll gene in Drosophila, was functioning as a defence mechanism against fungal infections in the organism. Flies lacking the toll-gene died in a couple of days after the infection (Lemaitre et al., 1996). This was followed by the discovery of hToll, the first human homologue of Toll, in 1997 by Medzhitov and Janeway and the discovery of 5 homologues of Drosophila Toll by Rock et al in 1998 (Medzhitov et al., 1997;Rock et al., 1998). Those five human toll-like receptors were named TLRs 1-5, and it was TLR4 that was named as hToll by Medzhitov & Janeway. Experiments, carried out by Poltorak et al on TLR4 deficient mice, have shown that lipopolysaccharide (LPS) from gram-negative bacteria its the

ligand for TLR4 (Poltorak et al., 1998a). New studies indentified new TLRs thus after screening the murine genomic library in 1999 Takeuchi & Akira discovered a novel TLR that showed similarities to human TLR1, so they called it TLR6 (Takeuchi et al., 1999b). Chuang and Ulevitch discovered TLR 7-9 in 2000 and TLR10 in 2001 (Chuang and Ulevitch, 2001;Chuang and Ulevitch, 2000). TLRs are highly expressed in cells and tissues where immune response takes place. This includes macrophages, neutrophils, dentritic cells, cells in the lung, placenta, peripheral blood leukocytes, bone marrow, spleen, lymph node, thymus and tonsils. TLRs are either expressed on the cell surface or in the intracellular compartments of the cell. Receptors that are expressed on the cell surface are TLR1, TLR2, TLR4, TLR5 and TLR6. TLR3, TLR7, TLR8 and TLR9 sit in the intracellular part of the cell (*Figure 1.1*). TLRs are transmembrane proteins, which adopts two parts; ectodomain, made up of leucine rich repeats (LRR) at N-terminus where the ligand interacts with and intracellular cytoplasmic IL-1R homologous domain at C-terminus that initiates the inflammatory response in the cell.

1.3.1 Toll-like Receptor Structure

TLRs are type I transmembrane proteins, with pathogen binding domain forming the ectodomain, a transmembrane domain and Toll/IL-1R (TIR) domain that forms the endodomain. Leucine-rich-repeat (LRR) motifs sit at the ectodomain part where the N-terminal is, whereas C-terminal owns TIR domain that is a homologue of that of IL-1R and this signalling domain triggers the downstream signalling cascade in the cell (Bowie A. 2000). A horseshoe shaped LRR domain, which is made of 19-25 tandem copies of LRR motifs, is approximately 20-30 amino acid residues long. LRR domain can be found in structures where protein-protein interaction is needed, it owns LxxLxLxxNxL conserved motif and xxLPxxxFx variable region. LxxLxLxxNxL forms parallel β-strands via "N" asparagine residue. Asparagine network is important

to create hydrogen bonds with neighbouring carbonyl groups, cysteine, threonine and serine residues can also form hydrogen bonds. Formation of H-bonds in the protein is important, because it maintains the shape of the ectodomain. Variable region xxLPxxxFx forms α helices and β-turns. This extracellular domain part is connected to cysteine rich residue and then to the TIR domain via a single transmembrane α helix. Toll/IL-1R (TIR) domain is approximately 160 amino acid residues long and it has 3 sequence boxes; Box 1, 2, and 3. The functional sides where the interaction of the TIR domain with its' adaptor molecule occurs are at Box 2 and 3.

1.3.2 Toll-like Receptor Signalling Pathways

During the recognition of PAMPs, TLRs function as a homodimer or heterodimer to generate the signalling cascade in the cell. TLR 1, 2, and 6 form heterodimers with each other whereas rest of the TLRs form homodimers. During this process adaptor molecules play an important role between the TIR domain of the TLRs' and downstream signalling molecules. Adaptor molecules which are: Myeloid differentiation primary response gene-88 (MyD88), TIR associated protein (TIRAP), TIR-domain-containing adaptor protein-inducing IFN-β (TRIF) also known as TIR-domain-containing molecule 1 (TICAM1), TRIF-related adaptor molecule (TRAM) functions in the downstream signalling cascades of TLRs. Once the LRR-domain recognizes and binds the pathogen, this leads to the conformational change of TIR-domain that enables the adaptor molecules to associate with the TIR-domain in the cytosol. There are two pathways that TLRs are signalling through, which are MyD88-dependent and TRIF-dependent signalling pathway (*Figure 1.1*). TLR 1, 2, 4, 5, 6, 7, 8, and 9 initiates the signalling cascades via adaptor molecule MyD88, whereas TLR 3 signals through TRIF. TLR 4 uses both of the signalling pathways.

MyD88 molecule functions together with IRAK family members which are IRAK-4, IRAK-1, IRAK-2 and IRAK-M, they contain death domain at the N-terminus and a serine/threonine kinase domain (Janssens and Beyaert, 2003). IRAK family members act through MyD88 pathway and also triggering mitogen-activated protein kinase pathway (MAPK). MyD88 recruits IRAK-4 (IL-1 receptor-associated kinase-4) and IRAK-1 via their death domains, IRAK-4 is the one that phosphorylates IRAK-1, which leads to the activation of TRAF6 (TNF receptor associated factor 6) (Burns et al., 2003;Cao et al., 1996). TRAF6 and UBC13/UEV1A functions as a ubiquitin protein ligase E3 and E2 respectively. They catalyze the polyubiquitin chain on TRAF6 itself and on IKK-γ/NEMO (NEMO: NF-κB essential modulator) by creating a complex made of TRAF6, TAK1/TAB1/TAB2/3, NEMO/IKK-γ/IKK-β/IKK-α. Besides of this, IRF-5, which is the transcription factor, moves into the nucleus to trigger synthesis of cytokine genes. TAK1 (TGF-β activated kinase 1) gets activated via interaction of TAK1 binding proteins TAB1, TAB2/3 complex (Jiang et al., 2002;Takaesu et al., 2000). TAB2 and TAB3 use their ZnF (zinc finger) at their C-terminal to be able to bind K-63 linked polyubiquitin chains in order to activate TGF-β activated kinase 1 and IKK (Kanayama et al., 2004). After Lysine 63-linked polyubiquitin chains on NEMO have been catalyzed by E3 and E2, in IKK complex, which consists NEMO/IKK-γ/IKK-β/IKK-α, TAK1 phosphorylates IKK-β. This is followed by the phosphorylation of IκB which is bound to NF-κB, K48 ubiquitinates IκB, leading to its' degradation. When IκB is degraded NF-κB is free, so it gets translocated in to the nucleus to initiate the proinflammatory cytokine gene expression. TAK1 also activates MAPK pathway (mitogen-activated protein kinase pathway) which causes the phosphorylation of Jun Kinases (JNKs), p38 and CREB (cyclic AMP-responsive element-binding protein). At the end AP-1 (activator protein 1)

transcription factor also moves into the nucleus and function with NF-κB and IRFs to trigger transcription of proinflammatory cytokine genes.

TRIF-dependent signalling pathway is achieved via TRAM, which is known as TRIF-related adaptor molecule, functioning between TIR-domain of TLR and TRIF. TRIF is an essential adaptor molecule during TRIF dependent signalling pathway of TLR3 and TLR4 (Yamamoto et al., 2003;Yamamoto et al., 2002). TRAM and TRIF are TIR-domain containing molecules, which leads to production of IFN-β and IFN-inducible genes (Yamamoto, M., 2002; Oshiumi H., 2003). TRIF acts together with TRAF6 and RIP1 (receptor-interacting protein 1) on IKK-i and TBK1 (TRAF-family-member-associated NF-κB activator (TANK) binding kinase 1). TRAF interacts with N-terminal of TRIF, whereas RIP interaction takes place at the C-terminal of a RIP homotypic interaction motif (RHIM) of TRIF (Meylan et al., 2004). IRF-3 phosphorylation by IKK-i/TBK1, leads to the translocation of IRF-3 into the nucleus to trigger type I interferon production.

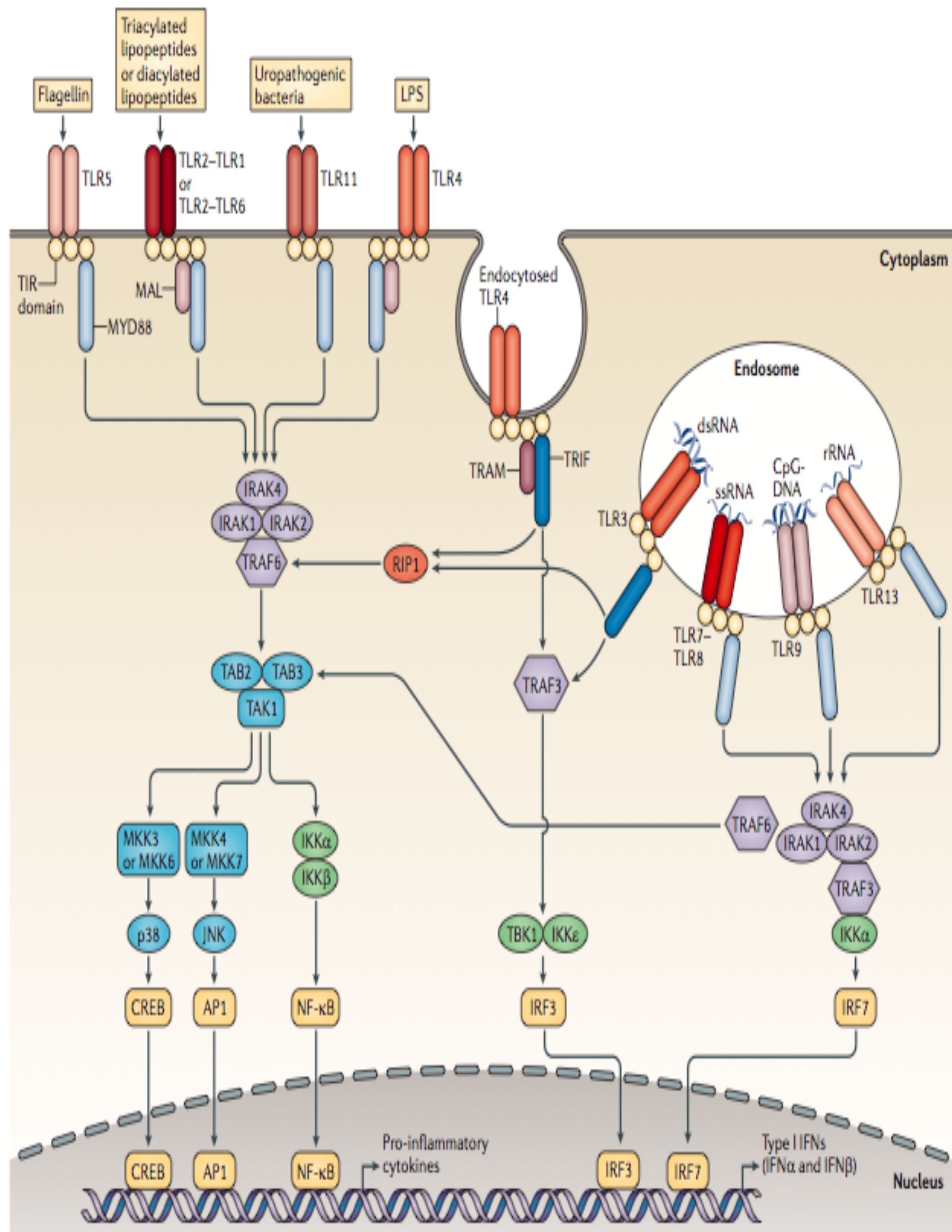


Figure 1.1: The diagram shows the distribution of the TLRs on the surface and inside of the cell in mammals. Specific ligands for each TLR and signalling cascade pathways they activate, is clearly represented in the diagram. Adapted from a nature review O'Neill et al. (2013).

1.3.3 Toll-like Receptors

1.3.3.1 TLR2, TLR1 AND TLR6

TLR2 can form heterodimers with TLR1 or with TLR6 to recognize a wide range of PAMPs and DAMPs on the cell surface where they are expressed (*Figure 1.1*). When TLR2 pairs up with other receptors, they can recognize bacterial PAMPs like triacyl lipopeptides from bacteria and mycobacteria, diacyl lipopeptides from mycoplasma, atypical lipopolysaccharides (LPS), lipoarabinomanan from mycobacteria, soluble factors, OspA, Porin PorB, proteoglycans, heat shock proteins (HSP 60, 70, GP96), high mobility group proteins (HMGB1), glycolipids, lipoteichoic acid (LTA) and peptidoglycans from Gram-positive bacteria. Fungal structures like yeast/zymosan, glycosylphosphatidyl inositol like proteins that belong to parasites. TLR2 can recognize viral particles from cytomegalovirus and from HSV which trigger downstream signalling cascades (Compton et al., 2003;Kurt-Jones et al., 2004).

TLR2 and TLR6 can associate together to recognize diacyl lipopeptides from bacteria and lipoteichoic acid (LTA) from gram-positive bacteria cell wall, yeast/zymosan from fungi, glycosylphosphatidyl inositol linked proteins from parasites (Triantafilou et al., 2006). The association of the TLR2 and TLR6 forms an ‘m’ shaped heterodimer. This heterodimer structure has two N-terminals that extends away from each other to the opposite direction. Ligand-binding sites sit at the central parts and C-terminals of the receptors overlapping parts. The difference in the structure of the ligand binding sites of TLR1 and TLR6 lead to the discrimination between the diacylated and triacylated lipopeptides. The structural difference of TLR1 and TLR6 lies in the ligand-binding site of TLR6 where side chain residues of Phenylalanine partially block the binding site. Thus the TLR6 ligand-binding site is half the length of the TLR1. TLR2 and TLR1 heterodimer ligands

1.3.3.2 TLR3

TLR3 forms a dimmer structure to recognize its' ligands. TLR3 is another receptor that recognises viral dsRNA as well as the synthetic dsRNA poly (I:C) (polyinosine-polycytidylic acid) (Alexopoulou et al., 2001). TLR3 resides in endosomes and triggers type 1 interferon and NF-κB production after the recognition of dsRNA, TLR3 is expressed in B cells, T cells, natural killer cells, dendritic cells at the endosomal/lysosomal compartment of the cell. TLR3 triggers IFNα/β and inflammatory cytokine secretion through the TRIF-dependent signalling pathway via TRIF and TRAM signalling adaptor molecules.

1.3.3.3 TLR4

TLR4 recognizes bacterial lipopolysaccharide (LPS), which is found in the structure of a Gram-negative bacteria cell wall and is the main cause of septic shock (Poltorak et al., 1998b;Takeuchi et al., 1999a). (Bryant et al., 2010;Mullarkey et al., 2003). Recognition of LPS is achieved with the help of lipopolysaccharide-binding protein, CD-14 and MD-2 (Schumann et al., 1990;Shimazu et al., 1999;Wright et al., 1990). (Miyake, 2007;Shimazu et al., 1999). The main PAMP of TLR4 is bacterial LPS, but it has been shown to interact with several different viruses as well. Using TLR4-deficient mice, it was found that TLR4 is involved in the innate immune response to RSV, through an interaction with the viral envelope fusion protein Mouse mammary tumour virus (MMTV) and Coxsackievirus (Kurt-Jones *et al.*, 2000; Haynes *et al.*, 2001].

1.3.3.4 TLR5

TLR5 is synthesized on the cell surface of epithelial and endothelial cells where it detects bacterial flagellin, leading to the activation of MyD88 pathway (Hayashi et al., 2001;Zhang et al., 2005).

1.3.3.5 TLR7 and TLR8

TLR7 and 8 are expressed in the cells of lungs, placenta, spleen, lymph node and bone marrow (Chuang and Ulevitch, 2000). They are located in the endosomal compartments where they recognize single-stranded RNA which leads to the activation of the MyD88 pathway, the synthesis of NF-κB followed by inflammatory cytokine and type 1 interferon secretion. These endosomal receptors have their pathogen recognition site inside the endosome whereas the TIR domain is in the cytoplasm of the cell. They detect the uridine-rich or both uridine and guanosine-rich single-stranded RNA from viruses like HIV, VSV, Influenza virus as well as imiquimod and resiquimod (R-848) analogues which are derived from imidazoquinoline (Diebold et al., 2004;Heil et al., 2004;Hemmi et al., 2002).

1.3.3.6 TLR9

Endosomal compartments of B and T cells, dentritic cells, monocytes and macrophages express TLR 9. This receptor size is 1032 a.a. residues long and it has a molecular weight of 115.9 kDa (Chuang and Ulevitch, 2000). TLR 9 plays an important role in the recognition of viral unmethylated CpG DNA from HSV-2 and bacterial unmethylated dinucleotide CpG DNA (Hemmi et al., 2000;Krieg, 2002;Lund et al., 2003). TLR9 can detect A/D type CpG DNA from synthetic oligonucleotides to initiate synthesis of type 1 interferon α\β in the plasmacytoid dentritic cells and IFNα production in human peripheral blood cells (Krug et al., 2001;Verthelyi et al., 2001). TLR9 can bind its' ligand directly which lead to conformational changes on the ectodomain of the TLR9 (Latz et al., 2004;Latz et al., 2007).

Studies have shown that recognition of CpG DNA from HSV 1 and HSV-2 leads to IFNα secretion in plasmacytoid dentritic cells (Krug et al., 2004a;Krug et al., 2004b;Lund et al., 2003). TLR9 can activate the immune response in the cell when it

detects viral DNA but not self-DNA; this recognition of "self" and "non-self" is because its localisation (Stetson et al., 2006). Conformational change of TLR9 is achieved when the CpG DNA binds to its' LRR domain, this structural change leads to the activation of the TLR9 dimer by bringing the TIR-domains closer which allows the signalling adaptor molecule to bind to the receptor.

TLR9 signalling is through the adaptor molecule MyD88 which recruits transcription factor IRF-7 leading to type 1 IFN production (Honda et al., 2005a). Activation of this MyD88\ IRF7 pathway, leads to the expression of TNFα, IL-6, IL-1 and type 1 IFNs.

1.3.3.7 TLR10

TLR 10 does not exist in mice but in humans, it is expressed on the cell membrane but the ligand for TLR10 is unknown. Human TLR10 is closely related to human TLR1 with 50% amino acid equality and to human TLR6 with 49% amino acid equality (Chuang and Ulevitch, 2001).

1.3.3.8 TLR11 and TLR12

TLR 11 and TLR12 exist in mice whereas humans do not have these receptors. Studies have shown that TLR11 knockout mice, die from kidney infection from uropathogenic bacteria, thus confirming its importance (Zhang et al., 2004). Toxoplasma gondii is also recognized by TLR11, leading to the production of IL-12 through MyD88 pathway (Yarovinsky et al., 2005). (Andrade et al., 2013).

1.3.3.9 TLR13

TLR13 exists in mice and is located in the endosome and functions during recognition of bacterial 23S ribosomal RNA, a binding side for antibiotics (Oldenburg et al., 2012). Moreover at the recent experiments done by Hidmark et al. on CHO (Chinese hamster ovary) cells, it was clear that TLR13 could bind to the bacterial RNA to activate NF-

κB, when TLR7 did not exist in the cell (Hidmark et al., 2012). TLR13 triggers IL-1β production after the recognition of rRNA from bacteria via the adaptor molecule MyD88 pathway and through endoplasmic reticulum transmembrane protein UNC93b (Li and Chen, 2012).

1.3.4 Toll-like Receptors Viral recognition and Modulation in the Cell

TLRs are expressed in variety organs and tissue types and have different location in the cell. Innate immunity cells like macrophages, dentritic cells, mast cells, neutrophils have these TLRs as well. Once viruses manage to pass through the physical barriers it will be recognized since it displays PAMPs, which are non-existent in eukaryotic cells. The innate immune cells will recognize the glycoproteins on the surface of the virus, as well as the nucleic acid in the nucleocapsid of the virus. Virus cell entry will trigger activation of signalling cascades leading to the interferon and proinflammatory cytokine secretion from the host. Secretion of these, leads to the magnification of the immune response, by initiating chemokine synthesis, cell apoptosis, by gathering immune effector cells, memory cells to the point of infection. They can either bind to the specific receptors in the same the cell or on other cells.

The first transmembrane receptors that will come into a contact with a virus are the ones that are expressed on the cell membrane such as TLR1, TLR2 and TLR6 It was shown that TLR2 binds to gH/gL and gB glycoproteins of HSV, binding of gh/gL leads to the activation of the NF-κB signalling cascade (Leoni et al., 2012). In addition HSV gB binds to TLR2 and initiates NF-κB via MyD88 adaptor molecule and TNF receptor associated factor 6 (TRAF6) dependent pathway (Cai et al., 2013).

After the internalisation of the virus TLRs that are located in the endosomal compartments of the cell take action. TLR3 is one these receptors and it recognizes the

dsRNA molecule of the virus, which lead to further activation of the NF-κB and IRF3 transcription factors, followed by pro-inflammatory cytokine and type I interferon synthesis (Alexopoulou et al., 2001). TLR3 uses TRIF-dependent signalling pathway for initiating the immune response in the cell.

Other receptors that function during the antiviral response are TLR7 and TLR8 which recognize the single stranded viral RNA and activate pro-inflammatory cytokine and type I interferon secretion (Heil et al., 2004;Hemmi et al., 2002;Lund et al., 2004).

Un-methylated CpG motifs of viral and bacterial genome are recognized by endosomal TLR9. TLR9 will bind to its ligand and interact with its adaptor molecule MyD88 and activate type I IFN and pro-inflammatory cytokine production (Hemmi et al., 2000;Krug et al., 2004a;Krug et al., 2004b;Lund et al., 2003).

1.4 Innate Immunity and DNA sensors

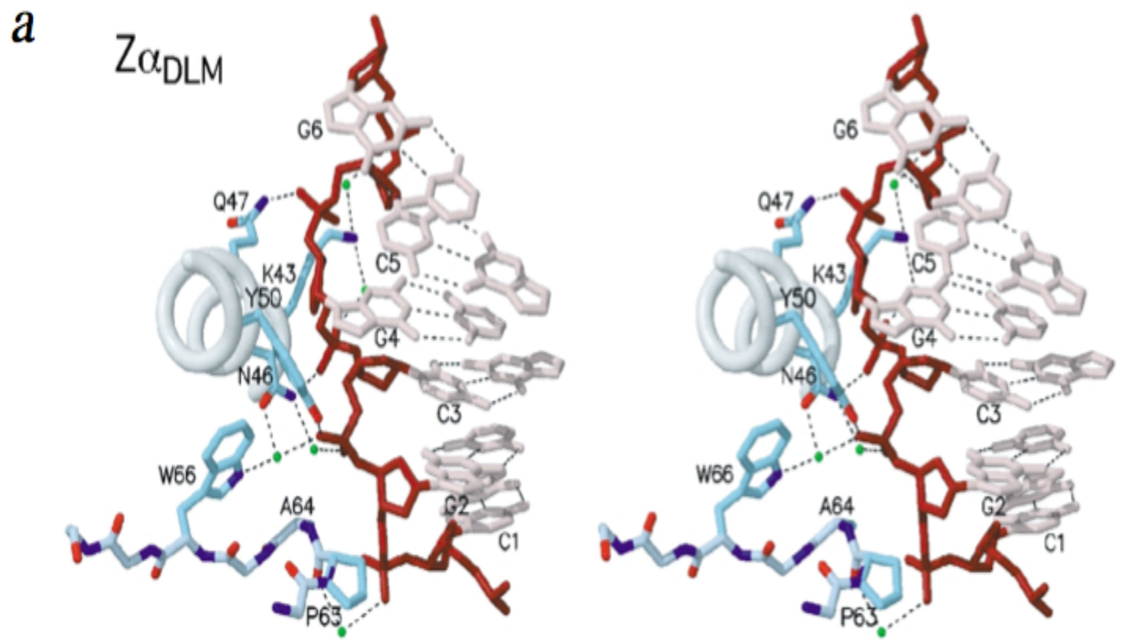
The viral and bacterial PAMP and genome recognition takes place in the endosomes as well as in the cytosol. The DNA of the pathogen is released into the cytosol, either through lysosomes, or through TREX1 (three prime repair exonuclease 1). When the pathogenic DNA has to be metabolized it ends up in the lysosomes where it is digested by DNase II. However in cells which lack the mechanism of DNase II, digestion the DNA enters into the cytoplasm (Okabe et al., 2005). TREX1 is a cytosolic DNase that sits on the endoplasmic reticulum and functions during the endogenous DNA degradation. The cells lacking TREX1 protein have pathogenic protein build up in the cytosol, which initiates the immune response (Stetson et al., 2008;Yang et al., 2007) When DNA virus replication takes place in the nucleus degradation is prevented. However in the cytoplasm ubiquitinalation of the HSV capsid occurs leading to degradation and viral genome exposure, leading to recognition by DNA sensors in the

cytosol (Horan et al., 2013). Cytosolic DNA sensors give a new insight in pathogen recognition in the cytosol and the DNA sensing mechanisms of the host.

1.4.1 DAI

This receptor was first discovered by Fu et al. and was named DLM-1 (Fu et al., 1999). DAI is a dsDNA sensor protein in the cell cytoplasm that initiates interferon production in the cell after HSV-1 infection (Takaoka et al., 2007). Besides HSV-1, DAI can also detect HCMV as well (DeFilippis et al., 2010a). DAI can also form complexes with RIP-3 to initiate necrosis in fibroblasts (Upton et al., 2010;Upton et al., 2012). The murine cytomegalovirus can interact with DAI through its M45 protein RHIM domain and inhibit DAI functioning to stop the immune response (Rebsamen et al., 2009). There are two RIP (receptor-interacting protein) homotypic interaction motifs (RHIMs) in DAI, which are conserved in the genome. Deletions or mutations on RHIM domains cannot initiate NF-kβ expression. RHIMs are the motifs which RIP kinases RIP1 and RIP3 can interact with. RIP2 and RIP4 lack RHIMs thus DAI cannot interact with them directly. In 2001 it was shown that DLM-1/DAI has Z-DNA binding domain at amino terminus where the receptor binds to Z-DNA. This domain is homologous to Zα and Zβ domains of ADAR1 (adenosine deaminase RNA-specific 1) (Schwartz et al., 2001) (*Figure 1.2*).

a



b

c

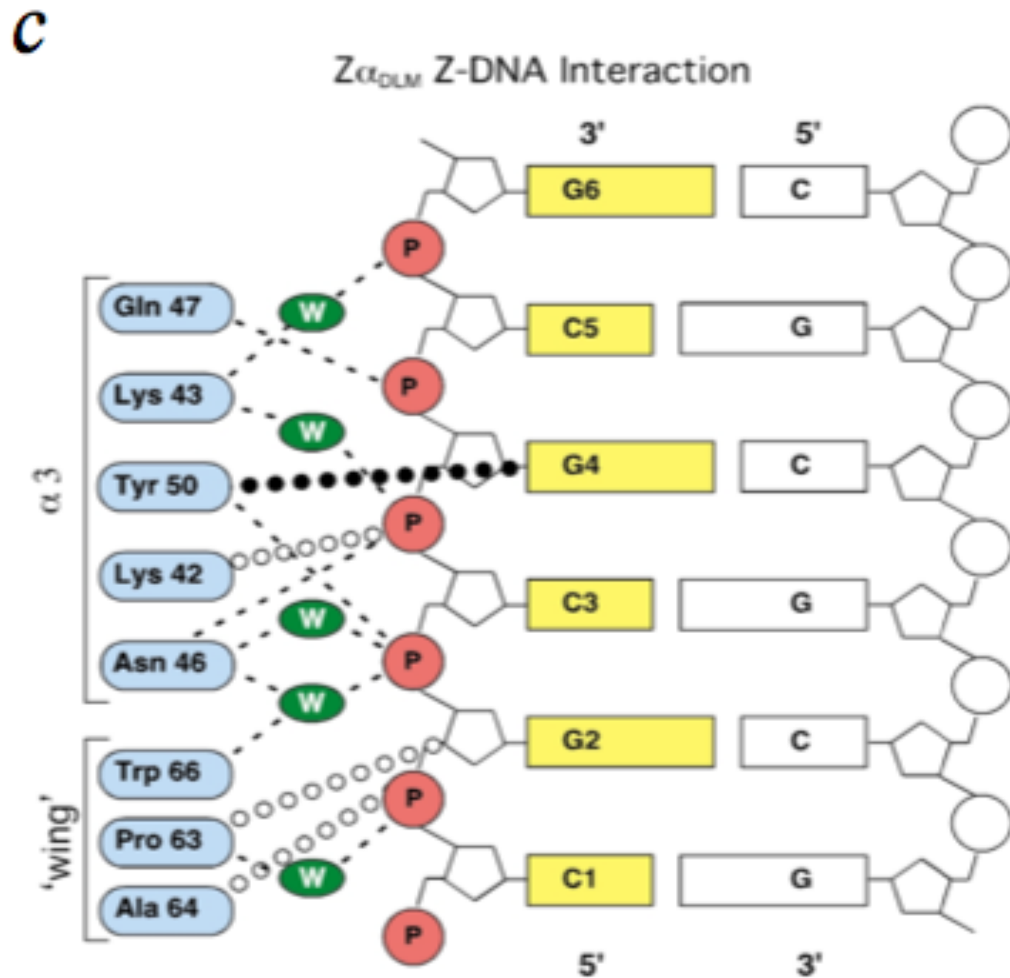


Figure 1.2: The detailed structure of the Z-DNA binding domain of DAI/DLM-1/ZBP-1 to Z-DNA. a**)** The structure of Z-DNA and Zα-DLM complex, grey helix is the DNA molecule attached to the domain, green dots are water molecules and hydrogen bonds between two proteins are shown as dashed lines. **b)** Representation of the Zα and Zβ domains of DAI (blue) with a C-terminal domain (grey). **c)** The detailed structure showing the residues involving and the type of the bonds forming between the binding domain and Z-DNA. The binding domain is represented on the left hand side and the Z-DNA is represented on the right hand side. Green 'W' are water molecules, white circled lines are Van der Waals, pink circles are phosphate groups of the DNA, dashed lines are hydrogen bonds and black circled line is CH-π which is located at the centre of the hydrophobic core Schwartz et al. (2001).

During the detection of the dsDNA, IRF3 (Interferon Regulatory Factor 3) transcription factor and the TBK1 serine/threonine kinase is recruited by DAI cytosolic receptor. Activation of IRF3 leads to the activation of type 1 interferon synthesis without initiating NF-κB and MAP kinase pathway (Stetson and Medzhitov, 2006). According to the experiments done on bone marrow pDCs, macrophages, viruses like HSV type 1 and type 2 can trigger type 1 IFNα and type 1 IFN-β production through TLR dependent or independent manner (Hochrein et al., 2004;Malmgaard et al., 2004). HSV-2 was shown to signal through IRF3 and NF-κB to initiate the IFNα/β in macrophages and type 2 triggers higher amounts of IFNα/β synthesis compared to type1 Herpes Simplex Virus.

1.4.2 AIM2 and IFI-16

AIM-2 and IFI-16 are from the PYHIN protein family; other members of this group are IFIX and MNDA. This family shares structural common features, which is a pyrin domain and two DNA binding HIN-200 domain. However AIM-2 pyrin domain shares more similarities to those of NLRPs and of ASC. After the sequence analysis it was understood that other PYHIN family members are located in the nucleus whereas AIM is located in the cytosol (Bürckstümmer et al., 2009;Hornung et al., 2009). AIM-2 gene was first discovered in 1997 and they named it Absent In Melanoma gene 2, the name was representing the gene that was missing in malignant melanoma chromosome 1 which was coding for a protein that had common amino acid residues as the

interferon-inducible gene family (DeYoung et al., 1997). AIM-2 recognizes the dsDNA and interacts with ASC to initiate IL-1β secretion in THP-1 monocytic cells. Moreover the inflammasome response was attained in unresponsive HEK293 cells by introducing AIM-2, ASC, caspase-1 and IL-1β (Bürckstümmer et al., 2009). The detection of the cytosolic bacterial, viral and host DNA takes place independent of NALP3 but not ASC, as a result activating the inflammasome response (Muruve et al., 2008). The AIM-2 protein interacts with the cytoplasmic DNA directly through its HIN-200 domains to oligomerize, followed by binding to its adaptor molecule ASC (also known as PYCARD, CARD5) forming pyroptosome to activate Caspase-1 leading to pro-inflammatory cytokine expression also leading to pyroptosis (Fernandes-Alnemri et al., 2009;Roberts et al., 2009b). HEK293 cells gain responsiveness to cytoplasmic DNA after the continuous expression of the AIM-2 inflammasome, in addition to that silenced AIM-2 results in reduced inflammasome and pyroptosis in macrophages (Fernandes-Alnemri et al., 2009). ASC has PYD (pyrin domain) at the N-terminal and CARD (caspase recruitment domain) domain at the C-terminal, which is the effector domain for the caspase-1 activation (Srinivasula et al., 2002). AIM2 interacts with this adaptor molecule through their pyrin domain via homotypic interactions. The AIM-2 and ASC complex is called AIM-2 inflammasome (Hornung et al., 2009). Pro-caspase-1 is the inactive form of the Caspase-1 and the interaction with the ASC through the CARD domains, activates Caspase-1. This active protein cleaves the inflammatory cytokines pro-IL-18 and pro-IL-1β into IL-18 and IL-1β, which are then secreted out of the cell (Hornung et al., 2009). The AIM-2 inflammasome complex formation can trigger pyroptosis through ASC and caspase-1. Pyroptosis is a programmed cell death that is triggered as a result of infectious or non-infectious agent entry by an inflammasome complex and caspase-1 interaction and activation.

IFI-16 is another innate immune receptor that recognizers viral DNA in the cell. This PYHIN protein binds to dsDNA to induce the IFN-β expression by recruiting STING adaptor molecule (Unterholzner et al., 2010). HIN A and HIN B domains of IFI-16 are formed from α helices and β strands, each domain possess two oligonucleotide/oligosaccharide binding (OB) sites (Liao et al., 2011). OB1-OB2 linker that links the OB sites also OB2 owns most of the DNA binding-residues in the HINB domain of IFI-16, forming hydrogen bonds, Van der Waals with the phosphates groups of the DNA backbone (Jin et al., 2012) (*Figure 1.3*).

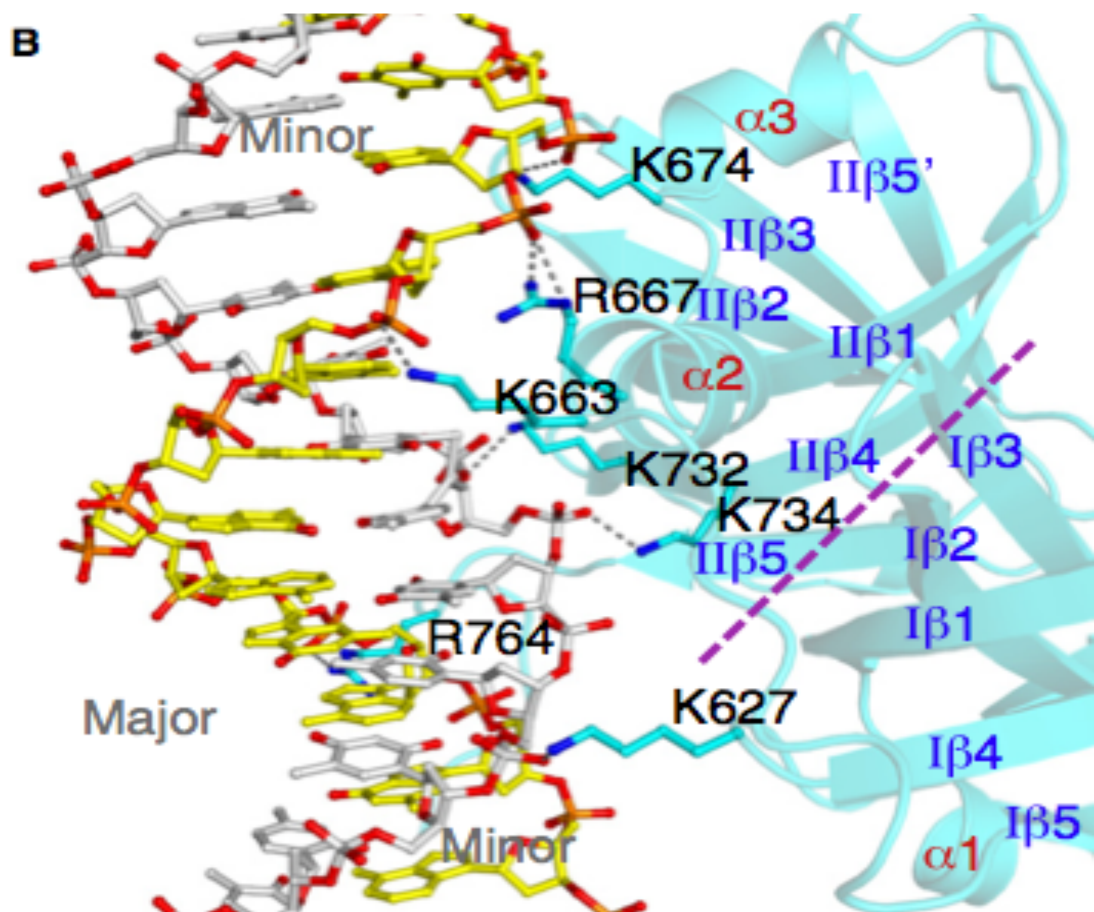


Figure 1.3: The detailed representation of IFI-16 HINb (on the right) and DNA complex (on the left) interaction. Grey and yellow DNA strands on the left-hand side are forming main and side chain hydrogen bonds (grey dashed lines) with the HINb domain of IFI-16. Pink coloured dashes indicate the boundary of OB1 and OB2. Minor and major grooves are illustrated in grey. Residues that are forming bonds with the DNA molecules are written in black. Adapted from Jin et al. (2012).

Experiments using dsDNA vaccinia virus motifs and HSV DNA fragments showed that IFI16 directly binds to the DNA of the virus. This interaction takes place via its' HIN domains HINa and HINb colocalize with DNA, leading to IFN-β production (Unterholzner et al., 2010). In HSV-1 infection the IFN-β expression is dependent on STING, which is a signalling adaptor. Imaging experiments tagging HSV-1 DNA showed that while the viral capsid was in the cytosol, HSV-1 replication took place in the nucleus (Ishikawa et al., 2009;Unterholzner et al., 2010). Since IFI-16 is expressed in the cytoplasm and the nucleus, the viral DNA can be recognized by IFI-16 either in the cytoplasm or in the nucleus where the replication takes place (Li et al. 2012)

NSL acetylation has been identified as a mechanism underlying the dynamic localization of IFI16 that extended its range of DNA surveillance. Phosphorylation clusters occur within two regions of IFI16: the linker region and C-terminus and lysine acetylation sites (S724) The two major NLS motifs, Δmotif-1 and Δmotif-2, contained acetylations at K99 and K128. It was shown that the phosphorylations within the linker region (S95, S106 and S153) had only minor roles in IFI16 localization. Since both lysine sites are highly conserved among IFI16 homologs and HIN-200 family members that suggests that acetylation is a common regulator of subcellular localization (Li et al. 2012).

In a recent study, HCMV and HSV-1 were shown to colocalize with the IFI-16 receptor in the cytoplasm, followed by induction of IFN-β expression in human macrophages (Horan et al., 2013). Ubiquitinalation of the HSV capsid triggered the proteasomal degradation and the release of the HSV DNA to the cytoplasm, which was recognized by IFI-16 in the cytoplasm (Horan et al., 2013). Another study has shown that Kaposi Sarcoma related herpesvirus infection leads to the IFI-16/ASC inflammasome complex formation in the nucleus of the endothelial cells (Kerur et al., 2011).

1.4.3 STING

STING (also known as MPYS, ERIS, TMEM173, MITA) signalling molecule has an important role during the type 1 interferon induction after the cytosolic DNA sensing (Ishikawa and Barber, 2008;Sun et al., 2009;Zhong et al., 2008). This membrane protein was first described by Jin et al in 2008 interacting with MHC class 2 molecules to initiate a death signal in B lymphoma cells by activating the extracellular signal regulated kinase (ERK) pathway (Jin et al., 2008). STING is 4 transmembrane regions where it binds to the ER membrane and it has an N-terminal, which is 130 amino acids long with 250 amino acids long globular shaped C-terminal. Inactive state of STING CTD is a dimmer and this structure is not dependent on the ligand binding (Ouyang et al., 2012;Shang et al., 2012;Shu et al., 2012;Yin et al., 2012). C terminal globular region of STING is located in the cytoplasm, where it responds to the cytosolic dsDNA and cyclic dinucleotides. The recognition of the B-DNA, HSV-1 DNA, bacteria Listeria monocytogenes, cytomegalovirus, vaccina virus and baculovirus in the macrophages, dentritic cells, acquires STING to trigger an immune response and initiate IFN production (Ishikawa et al., 2009). During this research it was observed that STING was relocalizing with the TBK1 from ER to the perinuclear vesicles. Besides of STING functioning as a downstream signalling molecule during the intracellular DNA recognition, STING was shown to be the direct immune sensor for the cyclic dinucleotides, such as cyclic di-GMP (Burdette et al., 2011). The ligand interaction enables the TBK1 and IRF3 binding at the globular C-terminal for IRF3 phosphorylation by TBK1 (Tanaka and Chen, 2012). The mutations at the residues Ser^{366} and Leu^{374} in the binding site of CTD-STING destroyed the IRF3 binding to the CTD, but did not affect the TBK1 interaction, thus stopping the IRF3 phosphorylation and activation by TBK1 (Tanaka and Chen, 2012). However these mutations did not

affect the TBK1 binding to the CTD-STING. The nucleic acid binding is followed by recruitment and activation of TBK-1, leading to relocalization of STING moves from the endoplasmic reticulum to the different part of the cytoplasm. TBK1 phosphorylates IRF3 transcription factor, which enters into the nucleus to initiate IFN-β production.

1.5 Signalling Pathways of Intracellular DNA Sensors

Several signalling receptors have evolved to detect nucleic acids in viruses and other microorganisms. DNA is not unique to pathogens and so it is more difficult to distinguish microbial nucleic acids from self nucleic acids; multiple sophisticated mechanisms have evolved to carry out this task. Recent advances in the field have identified DNA sensors IFI-16, DAI, AIM2 inflammasome and DExD/H box helicases. As well as the STING adaptor molecule, which recognize viral DNA.

IFI-16 can be found in the cytoplasm or at the nucleus and initiates IFNα/β production via the adaptor molecule STING. STING resides at the endoplasmic reticulum membrane where it is in its' inactive form. Viral invasion and detection of the nucleic acids in the cytoplasm leads to the colocalization of STING with TBK1 to the perinuclear vesicles. TBK1 phosphorylates IRF3, which moves to the nucleus to initiate type I interferon response.

The recognition of the viral DNA by the Z-DNA binding domain of DAI, also includes TBK1 and IRF3 molecules, HSV1 and HSV2 infection in macrophages, triggers IFN α/β production through IRF3.

AIM2 links with ASC via homotypic interactions, to form an inflammasome complex, which binds to the pro-caspase1 and cleaves it. Active caspase 1 cleaves the inflammatory cytokines pro-IL-18 and pro-IL-1β into IL-18 and IL-1β. Besides of the inflammatory response, this inflammasome complex formation triggers pyroptosis (cell death).

1.6 The Discovery of Viruses

The word virus comes from the Latin, which means poison, a potent juice. These small infectious entities were first discovered in 1892, by a Russian botanist Dmitri Iosifovich Ivanovsky, in a tobacco plant. Former to this discovery, a German chemist Adolf Mayer was working on tobacco mosaic disease, where he reported the juice extract of the infected plants was infectious for the healthy ones. He also used a term 'mosaic' to name the disease, because of the mosaic of dark and light spots on the leaves of an infected tobacco plants. In 1892 Ivanovsky mentioned that, a filterable infectious agent caused the tobacco mosaic disease. He used a filter, to filter the extract from the infected tobacco plants, then he injected this liquid to the healthy plants. As a result healthy tobacco plants were infected with the mosaic disease as well. Besides of Ivanovskys' studies, Martinus van Beijerinck has performed similar experiments to that of Ivanovskys'. He came to a conclusion that, this infectious substance was multiplying in living plants where there is a continuous cell division, the extract had living contagious fluid inside and this fluid could keep its' virulent property for years. Beijerinck was not able to see this infectious filterable agent under the light microscope and with the findings of Ivanovsksy, he was convinced that it was a 'contagium vivum fluid' (contagious living fluid). After many years in 1935, 1936 and in 1939, the crystallization, X-ray crystallography and the electron microscopy images of the tobacco mosaic virus (TMV), lead to a better understanding of the structure of this filterable virulent particles.

Viruses are small infectious pathogens that can only replicate in the cells of living organism that they infect. Their size and complexity varies but they all have common features. The viral genome has either single stranded or double stranded DNA or RNA, and depending on the genome the replication process varies from virus to virus.

A typical virion structure is made up of a core that consists of a nucleic acid, a capsid protein that encloses the nucleic acid, a tegument, which is the part between the core and the envelope which has all essential enzymes and proteins for the first stages of viral replication, a lipid envelope that is used forl entry into the host cell.

Electron microscopy studies showed that viruses have different complexity, shapes and sizes, thus in the past they were classified using the Linnaean hierarchical system or the Baltimore classification system, according to their nucleic acids in the core, the lipid membrane, the size and the symmetry of the capsid and the virus. They were also separated according to the disease type they were causing and the type of cells they were infecting. Nowadays viruses are classified with the help of sequencing technologies, depending on their genomic sequences (Table 1.1).

DNA				RNA			
Double stranded		Single stranded		Double stranded		Single stranded	
Enveloped	Non-en.	Enveloped	Non-en.	Enveloped	Non-en	Enveloped	Non-en.
Baculoviridae	Adenoviridae	Hepadnaviridae	Circoviridae	Cystoviridae	Birnaviridae	Coronaviridae Flaviviridae	Astroviridae
Herpesviridae	Caulimoviridae		Parvoviridae		Reoviridae	Togaviridae Arterivirus	Caliciviridae
Iridoviridae	Myoviridae					Retroviridae Orthomyxoviridae	Picornaviridae
Poxviridae	Phycodnaviridae					Filoviridae Paramyxoviridae	Potyviridae
African Swine Fewer Viruses	Tectiviridae					Rhabdoviridae Arenaviridae	
	Papovaviridae					Bunyaviridae	

Table 1.1: Virus classification according to their nucleic acid

1.6.1 Herpes Simplex Viruses

Herpesviridae family members are linear DNA viruses that have a core enclosed in a capsid, a tegument, an envelope and glycoproteins (*Table 1.1*). These large viruses have a large genomic size. Herpesviruses are divided into three subfamilies, depending on their tissue tropism. These are alpha-Herpesviridae which infect the nervous tissues (neurotropic), beta-Herpesviridae and gamma-Herpesviridae which infect the lymphatic system (lymphotropic). Herpes simplex virus types 1 and 2 (HSV-1 and -2),

and varicella-zoster virus (VZV) belong to alpha-Herpesviridae, (McGeoch et al., 1995). Beta-Herpesviridae include cytomegalovirus (CMV, also known as HSV-5), human herpes virus 6 (HSV-6), human herpes virus 7 (HHV-7). The gamma-Herpesviridae subfamily consists of Epstein-Bar virus (EBV) and Kaposi's sarcoma herpesvirus (KSHV) (human herpesvirus-8 (HHV-8)).

Herpes simplex viruses type 1 and type 2 both belong to the alpha-Herpesviridae subfamily, both types share almost half of their DNA. HSV 1 causes facial herpetic lesions, whereas HSV 2 leads to genital herpetic lesions, but both types infect mucosal surfaces and they have the ability to stay latent in the nervous system. The viral genes that are expressed at very first stages of the viral replication cycle maintain continuous latency of these viruses. Latency associated transcripts are the viral proteins sustaining the latency. LATs trigger the reactivation of the virus, however this cycle can be triggered due to a stress as well. The difference between these types is the tissue and organ that they infect in the organism. Usually the latency period of HSV-1 takes place in the nerve cells near the ear. HSV-1 can cause serious damage depending on the part it is infecting. Viral infection can either take place in the eye (ocular herpes) causing blindness or in the brain (herpes encephalitis), which can be lethal. However HSV-1 usually takes place on the face, chest and lips. HSV-2 stays latent in the base of the spine, and then moves to the genital area. This type does not spread as much as type-1 and usually it is not lethal, unless it is affecting newborns. Most people do not realize that they actually have the virus until the symptoms of the viral infection appear. Both types can be transmitted orally or sexually.

HSV-1 has a total genome length of 152,260 residues with 68.3% G + C base composition (McGeoch et al., 1988) (*Table 1.2*).

HSV-2 has 70.4% G + C composition with a total genome size 154,746 bp (Dolan et al., 1998) (*Table 1.2*).

Region	Virus	Length (bp)	% G+C	No. of genes[a]
R_L (as TR_L)	HSV-1	9,212	71.6	2
	HSV-2	9,297	75.4	2
U_L	HSV-1	107,947	66.9	58
	HSV-2	108,689	68.9	58
U_S	HSV-1	12,980	64.3	13
	HSV-2	14,329	66.2	13
R_S (as TR_S)	HSV-1	6,677	79.5	1
	HSV-2	6,711	80.1	1
Whole genome	HSV-1	152,261	68.3	74
	HSV-2	154,746	70.4	74

[a] Estimates of the number of genes encoding distinct proteins, as discussed in the text.

Table 1.2: Genome length and composition of different regions of HSV-1 and HSV22. Adapted from Dolan et al. (1998).

There are twelve glycoproteins in the lipid bilayer of the herpesviruses, not all of their functions are understood, however the main glycoproteins that function during viral infection are gB, gC, gD, gH and gL. Glycoproteins B, D, H and L play an important role during the fusion of the virus with the host cell membrane (Cai et al., 1988;Forrester et al., 1992;Roop et al., 1993) (*Table 1.3*).

GLYCOPROTEIN	GENE	AMINO ACIDS	MOLECULAR WEIGHT (kDA)	FUNCTION
gB	UL27	904	100.3	Mediates Fusion
gC	UL44	511	55	Host Cell Recognition
gD	US6	394	43.3	Viral Entry
gE	US8	550	59.1	Fc Receptor
gG	US4	239	25.3	Viral Spreading
gH	UL22	838	90.4	Viral Entry complex with gL
gI	US7	390	41.4	Viral Spreading
gJ	US5	92	9.5	Partially Prevents apoptosis
gK	UL53	338	37.6	Virus exocytosis
gL	UL1	224	24.9	Viral Entry complex with gH
gM	UL10	473	51.4	Unknown
gN	UL49A	91	9.2	Unknown

Table 1.3: List of glycoproteins in the lipid bilayer of herpesviruses and their functions

Glycoprotein B is needed during the viral entry of herpes simplex virus. All herpesviruses do have glycoprotein B and it works together with gH/gL for fusion of the virus in to the host cell. These glycoproteins are highly conserved in almost all herpesviruses. Glycoprotein B does not have fusion peptide, however the research on cytoplasmic domain mutant gB protein does suggest that this protein does have an effect on the fusion of the virus (Cai et al., 1988). The ectodomain of glycoprotein B consists of three promoters A, B and C coiled around each other with a left-handed twist and each promoter has 5 different domains (Heldwein et al., 2006) (*Figure 1.4*).

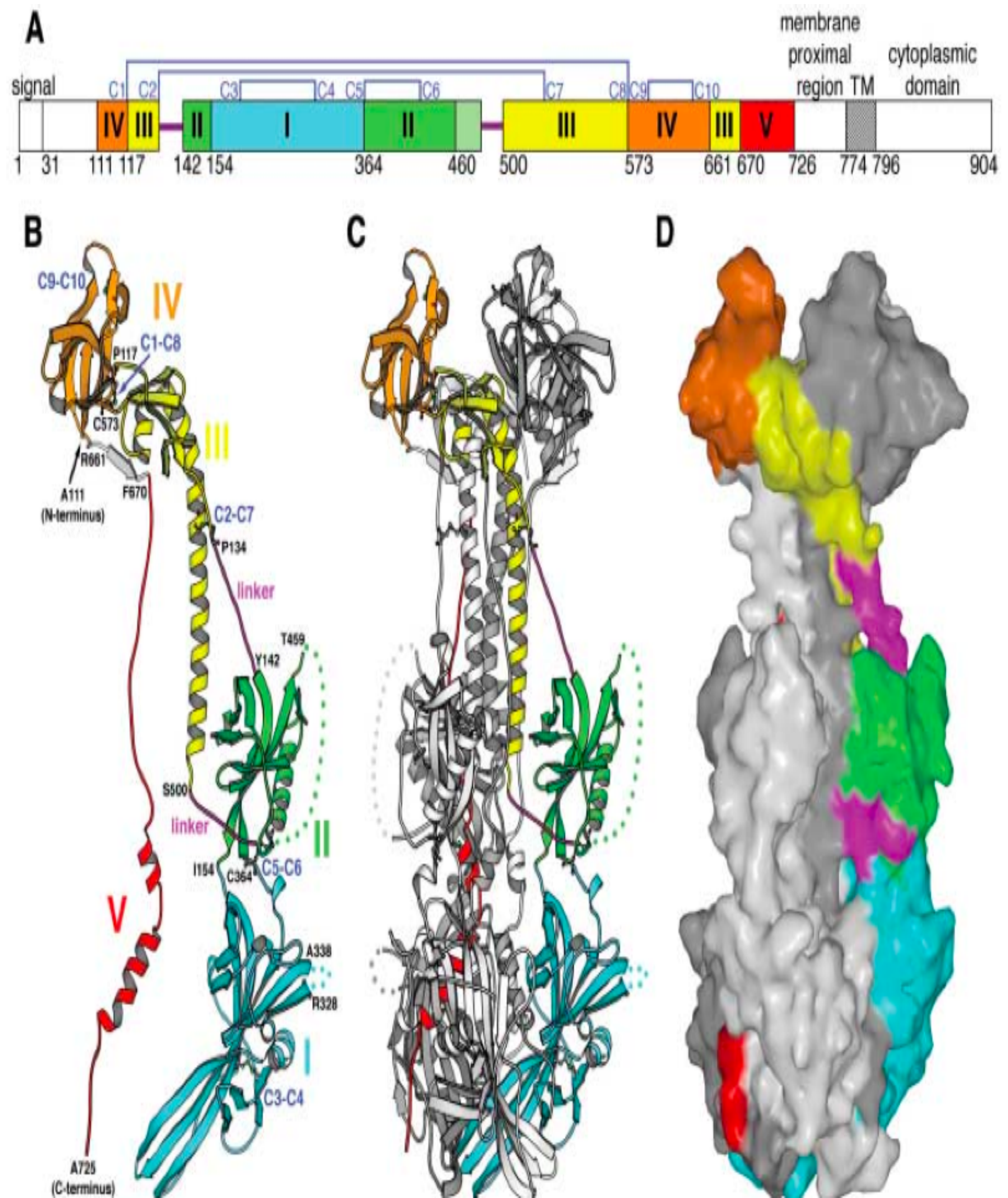


Figure 1.4: The structure of glycoprotein B. A) Is the crystal structure of full-length of ectodomain of one promoter of gB. Different colours are given for each domain. B) Single gB promoter represented in ribbon diagram. Colours and numberings are referring to the promoter in figure A. Dots are disordered segments. C) Trimer structure of gB, white and grey structures are referring to other two promoters B and C respectively. D) gB trimer. Adapted from Heldwein et al. (2006)

Glycoprotein C functions during the host cell recognition, and this non-essential binding of the virus is supported with the cell surface receptor heparan sulphate. It does not have a big effect on virus binding however when gC and gB are both absent, the viral binding to the host cells is severely reduced (Herold et al., 1994).

The interaction of the virus with the host cell and to initiate the endocytic pathway in some cells glycoprotein D is essential. This glycoprotein works with one of the three cellular receptors, which are nectin-1, herpesvirus entry mediator (HVEM also known as HveA; ATAR; TR2; TNFRSF-14), or heparan sulfate (Carfí et al., 2001). 50 residues long C-terminus of gD has an important role during the viral entry, this terminus is attached near the N-terminal to block the receptor-binding sites. 18 residues of C-terminal occupy the space at the N-terminal where HVEM-binding takes place (Carfí et al., 2001). When gD is bound to HVEM, first residues at the N-terminus forms a hairpin and it is supported by an α-helix and by the IgV (V-like immunoglobulin) core, this hairpin structure binds to the nectin-1 receptor (*Figure 1.5)*. However the primary entry receptor for HSV-1 and HSV-2 on neurons, keratinocytes and on epithelial cells is nectin-1 (HveC, CD111) (Galen et al., 2006;Huber et al., 2001;Krummenacher et al., 2004;Simpson et al., 2005). Nectin-1 and nectin-2 are both from nectin family, which have three extracellular immunoglobulin (Ig)-like structures, a transmembrane region and cytoplasmic region. Both Nectin-1 and HVEM are used by both HSV-1 and HSV-2. Nectin-2 is preferred by HSV-2 but not by HSV-1.

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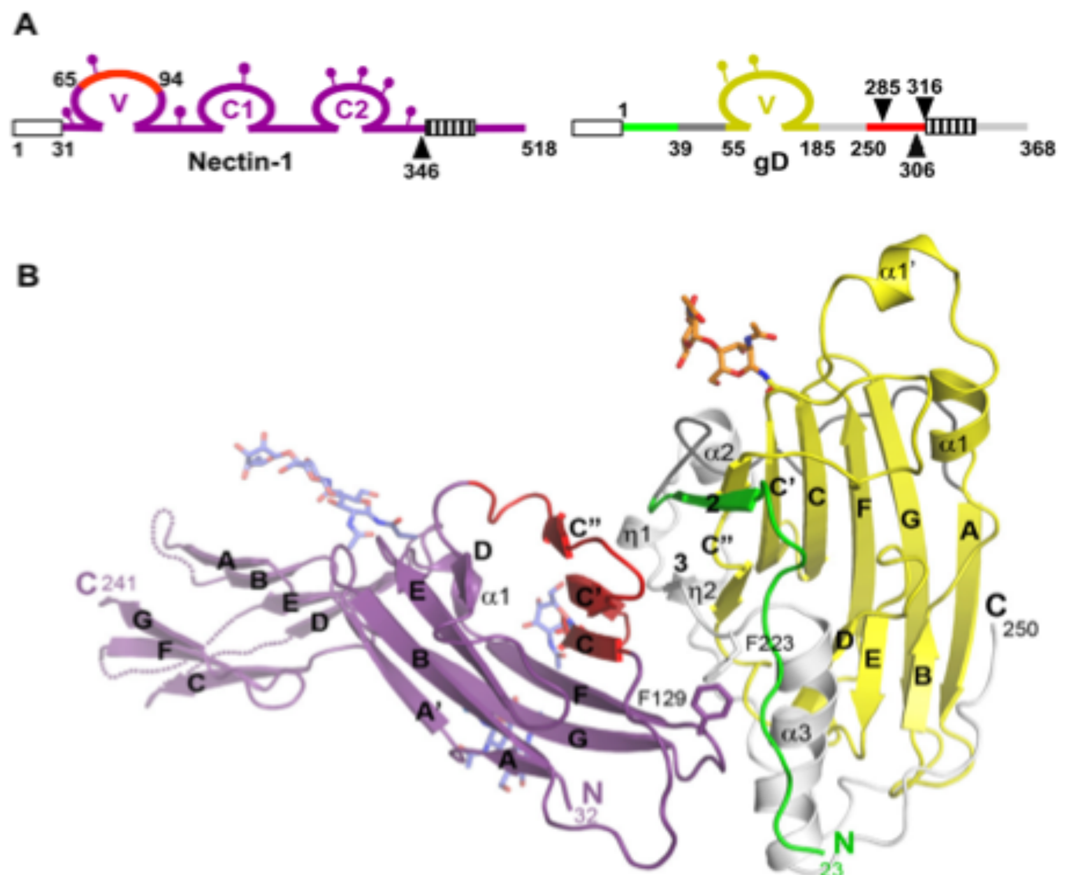


Figure 1.5: Glycoprotein D and human Nectin-1 complex. a) Nectin-1 structure is represented in pink on the left-hand side; herpes simplex virus type 1 glycoprotein D is yellow coloured on the right. Transmembrane regions are black-white boxes, N-glycosylation parts are dotted lines, and white boxes are signal peptides. The interaction point of Nectin-1 and gD is shown in red. **b)** The same colour coding was applied as in **a).** Hairpins that bind to HVEM are shown in green. Adapted from Di Giovine et al. (2011).

The last known receptor that gD binds to is heparan sulfate (HS) and again this is achieved by the first N-terminal residues of gD. After the interactions with glycoproteins B and C, heparan sulfate is modified by multiple D-glucosaminyl 3-O-sulfotransferase isoforms. This includes enzymatic reactions like: de-acetylation of glucosamine, sulphation of the amino group, epimerization, O-sulphations in the iduronic acid and in the amino sugar. At the end of this, the high affinity binding sites appear for gD to interact with. UA-GlcNS-IdoUA2S-GlcNAc-UA2S-GlcNS-IdoUA2S-GlcNH$_2$3S6S is the octasaccharide structure that is represented on heparan

sulphate (Liu et al., 2002), which gD can actually bind to increase the susceptibility of the cells to the HSV (Shukla et al., 1999).

Glycoprotein H and Glycoprotein L is found on the envelope as hetero-oligomers (Hutchinson et al., 1992;Kaye et al., 1992) (*Figure 1.6*). In the research done by Hutchinson et al., it was mentioned that for gL to be processed and expressed on the cell surface the expression of gH is needed, when they are expressed together they are carried to the cell surface at the end. Thus to achieve the fusion of the virus with the cell, the expression of these two glycoproteins together is needed. Moreover the experiments, which were done with the shortened gH that does not have trans-membrane and cytoplasmic domain, showed that the gL was still transformed out of the cell with this gH mutant (Dubin and Jiang, 1995). For this reason gH trans-membrane domain is the main part that attaches gL glycoprotein on to the surface as gL lacks trans-membrane domain. After the experiments that have been done on HSV type 2 gH-gL protein complex, it was revealed that glycoprotein H contains three domains, which are H1, H2 and H3 (*Figure 1.6*). H1 domain enables gH binding to the glycoprotein L, H2 domain is a central helical domain and the last domain H3 is C-terminal β-sandwich domain. H1 domain is divergent whereas H2 is the second most conserved domain after H3 (Chowdary et al., 2010). Disulfide bonds link the cysteine residues with each other both in gH and in gL. Glycoprotein L binding site (H1 domain) has two subdomains, which are H1A and H1B, connected with Gly116 to Pro136 residues. Subdomain H1A is the part where gL β-sheets interacts with that of gH. H1B subdomain interacts with H1A subdomain and H2 domain only.

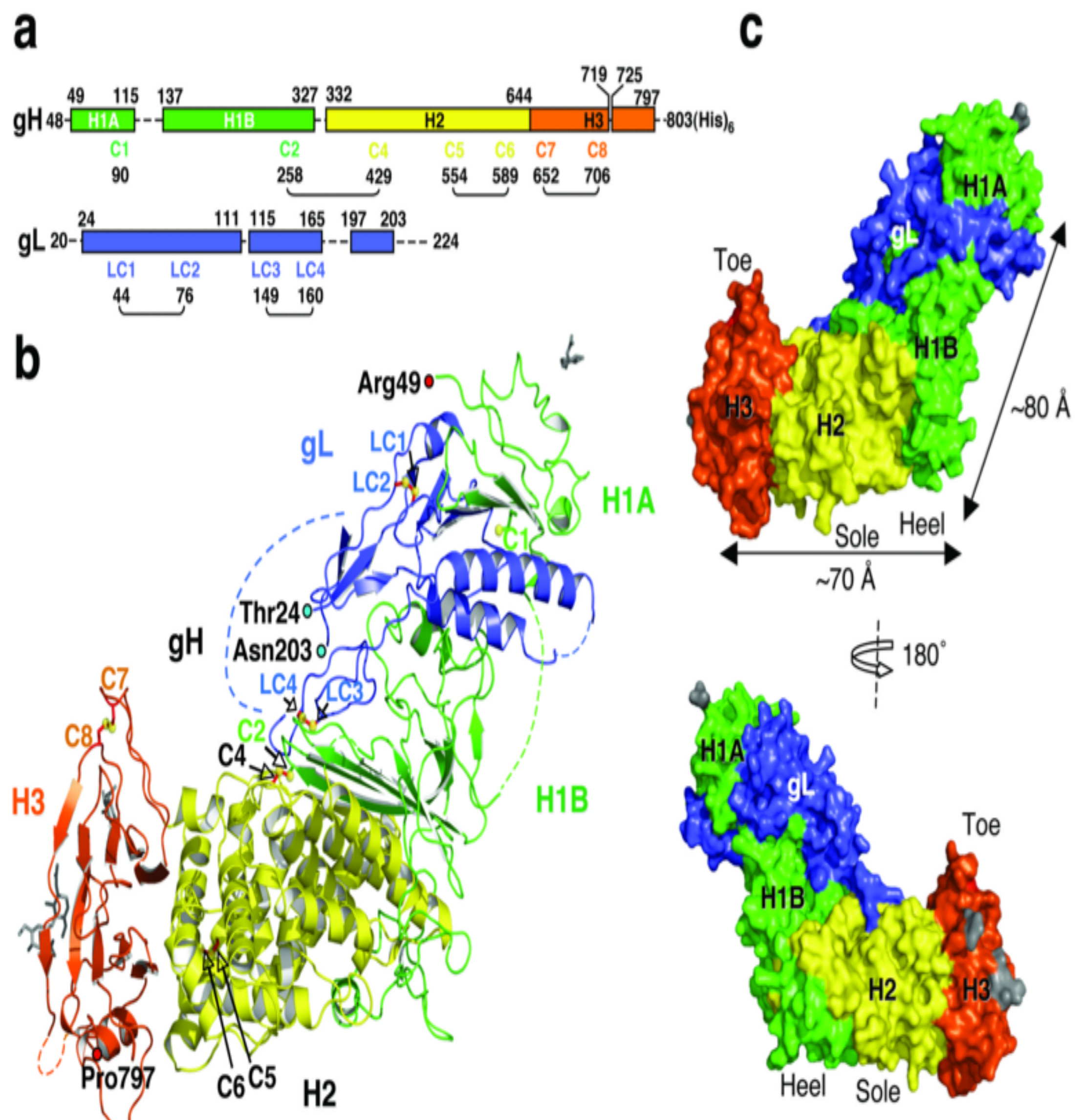


Figure 1.6: The individual structures of gH and gL and 180° representation of gH-gL complex of HSV type 2. **a)** Different colours are given to individual domains of gH and gL. Cysteines residues numbering of gH complex was done according to HSV type 1 structure, however in this representation C3 was not shown, because HSV type 2 does not have cysteine residue 3. **b)** The gH-gL complex structure; including the disulfide bonds (red sticks & spheres in yellow), disordered segments (dashed lines) and sugars (grey colour). The same colour coding of part **a)** applies in part **b)** as well. **c)** 180° surface representation of gH-gL complex, the same colour coding of part **a)** applies in part **c)** as well. Adapted from Chowdary et al. (2010) .

During the replication of the herpes simplex virus, proteins in the tegument part of the viruses play an important role. Once the gB, gD and gH/gL complex interacts with the cell surface receptors, they lead the fusion of the membrane and tegument part to be

transferred into the host cell cytoplasm. Tegument part of the HSV1 virus has 23 tegument proteins (Loret et al., 2008), which functions during the outer tegument disconnection, the early and late replication cycle of the virus (*Table 1.4*). These viral proteins were named depending on their open frame readings, unique short (US), unique long (UL) and infected cell protein (ICP) are the names given to them. Most of these tegument proteins are preserved in the viruses within the same subfamily whereas it can show some variation with the other subfamilies. In 1991 novel L particles of HSV1, which lacks the nucleocapsid and the core, indicated that tegument part could still form independently of the viral capsid structure (Szilágyi and Cunningham, 1991).

TEGUMENT GENE	TEGUMENT PROTEIN	A.A & MWT (kDa)	ESSENTIAL	FUNCTION
RL1	ICP34.5	248/26.2	NO	Controls host translation
RS1	ICP4	1298/132.8	YES	Controls viral transcription
UL7	EEP	296/33.1	NO	Controls Mitoc. Function
UL11	CETP	96/10.5	NO	Secondary Env.
UL13	VPK	518/57.2	NO	Protein kinase
UL14	ECP	219/23.9	NO	Nuclear Import
UL16	CETPbp	373/40.4	NO	Secondary Env.
UL21	CEF2	535/57.6	NO	Secondary Env.
UL23	pUL23	376/41.0	NO	Thymidine Kinase
UL36	VP1/2	3139/335.9	YES	Deubiquitinase
UL37	ICP32	1123/120.6	YES	Virus Transcription
UL41	VHS	489/54.9	NO	Host and viral translation reg.
UL46	VP11/12	718/78.2	NO	Modulate TIF-dependent transcription
UL47	VP13/14	693/73.8	NO	Modulate TIF-dependent transcription
UL48	αTIF/VP16	490/54.3	YES	Viral transcription
UL49	VP22	301/32.3	NO	Controls microtubule assembly in RNA transport
UL50	pUL50	371/39.1	NO	dUTPase
UL51	pUL51	244/25.5	NO	Unknown
UL55	pUL55	186/20.5	NO	Unknown
US2	pUS2	291/32.5	NO	Unknown
US3	pUS3	481/52.8	NO	Controls actin assembly
US10	pUS10	312/34.1	NO	Unknown
US11	pUS11	161/17.8	NO	Controls host translation and capsid transport

Table 1.4: Tegument genes were tabulated in the table. The first column represents the tegument gene followed by the protein product it encodes, the number of the amino acids it is made of & the molecular weight, the requirement of these tegument genes, and their function during the invasion of the host cell and the replication of the virus in the cell cytoplasm. Table content information was obtained from Kelly et al. (2009).

The viral linear dsDNA is enclosed in a spherical shaped capsid, which has symmetry with 2-5 fold axes. This icosahedral capsid of HSV1 contains viral proteins (VP) that are VP5, VP19c (50 kDa), VP23 (34 kDa), VP24 and VP26 (12 kDa) (Zhou et al., 2000) . The major capsid protein of them all is VP5, which has a molecular weight of 149 kDa and is encoded by UL19 gene. This structure has to be stable to make sure the viral genome is protected until it is released to the host nuclear cytoplasm. Pentons and hexons are the structures that form the capsid, which are made of VP5 monomers. These capsid structures interact with each other to form triplex with the help of other capsid proteins VP23 and VP19C (Bowman et al., 2003;Spencer et al., 1998). In a research done by Zhou et al in 1999, these pentons and hexons were shown to interact with the teguments proteins of HSV1 (Zhou et al., 1999).

1.6.1.1 HSV binding and entry

This process includes gC, gD, gB and gH/gL complex. During the binding of the virus to the cell surface, interaction of the cell surface proteoglycans with the glycoprotein C is followed by the gD binding to the cell surface receptors like HVEM which strengthens the binding of the virus to the cell surface. The fusion of the virus membrane with the cell membrane occurs after the involvement of the gB and gH/gL complex. The viral capsid is transferred next to the nucleus where the viral genomic DNA can be released into the nucleus through the nuclear pores. The phosphorylation of the tegument proteins by pUL13, pUS3 and by cellular kinases triggers the dissociation of the tegument part from the viral capsid (Morrison et al., 1998a;Morrison et al., 1998b). HSV-1 viral capsid was shown to colocalize with cytoplasmic microtubules, dynein and dynactin, dynein-dependent viral capsid transportation takes place possibly with the help of pUL36 and pUL37 (Antinone et al., 2006;Döhner et al., 2002;Wolfstein et al., 2006). Besides of pUL36 and pUL37, a research done in 2004, has shown that HSV1 viral capsid protein pUL35 interacts with

RIP3 and Tctex1, which are the light chains of the cellular dynein (Douglas et al., 2004). pUL36 was also needed for the transportation of the viral capsid to the nucleus, for the interaction between the capsid and the nuclear pore complex (NPC) (Copeland et al., 2009;Ojala et al., 2000).

1.6.1.2 Immediate-early Gene Expression

The protein synthesis and the viral gene expression is attained with the help of the tegument protein α-TIF (pUL48). It interacts with the transcription factors of the cell to initiate the early α gene expression in the nucleus. Once the viral nucleocapsid enters the nucleus, viral immediate early (IE) gene expression starts with the α-TIF (pUL48; α-transinducing factor) (Campbell et al., 1984;Pellett et al., 1985;Post et al., 1981). Binding of pUL48-induced transcriptional complex to immediate early gene promoters leads to the expression of IE genes like ICP0, ICP4, ICP22 and ICP27 in Alphaherpesvirinae. These genes trigger early and late gene expression in the cell which are β and γ (Roizman et al., 2005). This stage does not take place during the latent infection, before the transcription of the early genes genomic DNA interacts with histones to shut down the transcription except from the latency-associated transcript (LAT) expression. LAT expression is important for the reactivation of the viral cycle by initiating immediate early gene transcription.

The transcriptional machinery initiates the expression of the early genes, which are β, βγ and γ genes. These genes encode for the enzymes that will function during the viral DNA replication and they encode for the virion structures. This process takes place in the nucleus (Field and Wildy, 1978;Pyles et al., 1992).

1.6.1.3 HSV Assembly and Release

This process is achieved first by the primary envelopment of the virus, followed by the de-envelopment outside of the nucleus and secondary envelopment of the virus after the attainment of the tegument parts of the virion. The structure is transported to the Golgi for the addition of the glycoproteins and it leaves the cell by exocytosis. UL31 and UL34 form a complex with each other during the budding of the nucleocapsid into the perinuclear parts of the nucleus (Fuchs et al., 2002b;Roller et al., 2000). The mutation in these genes can stop the primary envelopment leading to the accumulation of the viral capsids in the nucleus. Deenvelopment of the virus includes pUS3, gB and gH proteins (Farnsworth et al., 2007;Klupp et al., 2001;Reynolds et al., 2002;Ryckman and Roller, 2004). As a result it was shown that deletion of these proteins from HSV-1 virion resulted in primary enveloped virion accumulation at the perinuclear parts of the cell. Secondary envelopment involves several proteins, which are pUL11, pUL16, pUL21, pUL36, pUL37, pUL46, pUL47, pUL48 and pUL49. The membrane associated protein UL11 has been shown to interact with pUL21 and pUL16 proteins and this tegument complex function during the secondary envelopment of the virus. The deletion of pUL11 stopped the secondary envelopment in HSV-1 and in HCMV cycle (Baines and Roizman, 1992;Seo and Britt, 2007). During the tegumentation of the capsid pUL36 and pUL37 proteins are needed (Roberts et al., 2009a). pUL36 is needed for the pUL37 trafficking to the Golgi apparatus, deletion of this protein resulted in the disruption in the pUL37 trafficking to the Golgi (Desai et al., 2008). pUL48 is needed for joining all of the viral parts together to initiate the secondary envelopment (von Einem et al., 2006). Interactions between pUL48 with pUL35, pUL38, pUL36, pUL41, pUL46, pUL47 and pUL49, which are capsid, inner tegument proteins and outer tegument proteins, have been shown (Elliott et al., 1995;Lee et al., 2008;Vittone et al., 2005). These interactions in

between the tegument proteins are completed by the interaction of pUL48 and pUL49 tegument proteins with the glycoproteins' cytoplasmic tails (Chi et al., 2005;Fuchs et al., 2002a;O'Regan et al., 2007).

1.6.2 Cytomegalovirus

Cytomegalovirus belongs to the beta-Herpesviridae subfamily of the Herpesviridae family. Cytomegalovirus infection symptoms are flu; sore throat, tiredness, high temperature, muscle pain can be the result of the viral infection. The transmission routes for the virus are through saliva, urine, milk, semen and cervical secretions. The virus can enter the body but stay latent without any distinct symptoms. More serious symptoms can occur when there is a congenital infection or when the infected person has an immune deficiency leading to organ damage such as pneumonia, hepatitis. Congenital infection is caused by the transmission of the virus from the mother to the unborn baby through placenta (Pass, 1985). The transmission of the virus can be through the placenta from the mother to the baby. The congenital infections can result in rare physical health problems such as blindness, hearing loss, epilepsy. Same as other herpesviruses, HCMV does stay latent in the host organism after the primary infection. During this latent phase the detection of the virus is not possible. The reactivation of the virus depends on the viral machinery that includes latency-associated transcript (LAT), triggering early genes expression. The viral genome is the largest among the herpesviruses, with 230 kbp long linear double-stranded DNA, enclosed in an icosahedral nucleocapsid which is surrounded by a tegument part and by a lipid bilayer. The viral genome has open reading frames (ORFs) like HSV-1; they include unique long (UL) and unique short (US) regions as well. The lipid bilayer is coated with more than 25 glycoproteins but most important ones are only 6 of them, which are gB, gH, gL, gM, gN and gO. Glycoprotein B, H and L are conserved in all herpesviruses, different from herpes simplex virus cytomegalovirus has glycoprotein

O encoded by UL74 gene that forms a trimer structure with glycoprotein H and L (Huber and Compton, 1998). After the viral entry the tegument part of the virus is released into the cell with the viral capsid. Same as HSV the HCMV viral capsid is transported to the host nucleus where the viral genome is released into the nucleus. The viral replication starts with the immediate early gene expression. The viral replication cycle follows the same cascade of gene expression of herpes simplex virus, however the viral proteins that function during this process are either conserved in all herpesviruses or just belong to betaherpesvirus subfamily.

The interaction of the glycoprotein B with the host cell is achieved via the interaction with the cell surface heparan sulfate glycosaminoglycans (Compton et al., 1993). HCMV was unable to bind the Chinese hamster ovary cells, which were deficient in heparan sulfate glycosaminoglycans. Further binding of glycoprotein B with the epidermal growth factor receptor (EGFR) and with β1-integrin through its disintegrin-like domains leads to the internalisation of the cytomegalovirus (Feire et al., 2010).

The UL74 gene encodes for the membrane bound glycoprotein O and it interacts with glycoproteins L/H to form a trimeric complex, which attains the fusion of the virus into the host cell (Huber and Compton, 1998). Viral nucleocapsid is carried next to the nuclear pore via microtubules, where it can release the viral genomic DNA. Early gene expression, viral genome replication, virus assembly and release follow the immediate early gene expression. Viral tegument proteins such as pUL86 (major capsid protein), pUL46 (minor capsid protein), pUL85 (minor capsid protein) are the viral capsid proteins (Chen et al., 1999). pUL80 functions during the nucleosomal translocation of the capsid proteins and cleavage of the viral capsid. pUL104, pUL77 and pUL93 is another set of viral proteins that are essential for the viral genome packing (Toropova et al., 2011). The HCMV genome cleavage starts with the pac1 and pac 2 signals, which are located in the viral genome (Wang and McVoy, 2011). Genome cleavage is

followed by the DNA encapsidation and nuclear egress. Primary envelopment of the virion capsid takes place in the perinuclear compartment of the cell. The egress occurs with the nuclear egress complex components, in the case of HCMV, pUL50 associates with pUL53 forming a heterodimer, pUL97 and RASCAL also function within the nucleus, to initiate the translocation of the nucleocapsid (Milbradt et al., 2007;Miller et al., 2010;Sam et al., 2009). Viral protein kinase (pUL97) controls the host cell kinases and DNA synthesis of the virus (Prichard, 2009). Assembly compartment is formed for the tegumentation of the nucleocapsid, envelopment and release of the mature virions from the host cell. It was observed that tegument proteins pUL32, pUL99, pUL83 and envelope glycoproteins gB and gL was located in this assembly compartment of the infected host cell during the late infection (Sanchez et al., 2000). After formation of the mature virus, they are released from the host cell. During the viral replication infectious and non-infectious viral particles are produced. The functions of these non-infectious viral particles are not well understood. Non-infectious enveloped particles (NIEPS) do not have the viral genome only, whereas dense bodies do not have the viral nucleocapsid and only possess the tegument part with the lipid bilayer (Irmiere and Gibson, 1983).

1.7 Receptors Involved in Generation of an Innate Immune Response to Herpesvirus Infection

The entry of the herpesviruses can be achieved through two pathways, which are either the endocytic or non-endocytic pathway (Akhtar and Shukla, 2009). The endocytic pathway causes the exposure of viral DNA in the endosomes where TLR9 is present. This can trigger the MyD88 dependent downstream signalling pathway causing the interferon and pro-inflammatory gene transcription in the nucleus (*See Sec 1.3.2*). On the non-endocytic entry, the direct interaction of the virus with the cell

surface receptors is followed by the release of the virial capsid into the cytoplasm. The first interaction with the cell surface PRRs does initiate a downstream signalling cascade through the MyD88-dependent signalling pathway (See *Sec. 1.3.2*). The viral capsid can be sensed, which results in the ubiquitylation of the capsid, this leads to the proteasomal activity and exposure of the viral genome to the intracellular DNA sensors. Once the DNA is exposed IFI-16 and DAI work with the signalling adaptor molecule STING for further activation of the interferons and pro-inflammatory cytokines. Unstimulated STING sits on the endoplasmic reticulum and viral sensing leads to the TANK-binding kinase 1 binding at the globular C-terminal of STING. The complex then colocalizes at the perinuclear compartments, IRF3 is phosphorylated by the STING/TBK-1 complex while phosphorylated IRF3 moves into the nucleus. Moreover the viral DNA is detected by the novel receptors, known as DExD/H box helicases, initiating either the MyD88 dependent signalling pathway or the STING/TBK1 signalling pathway. Therefore HSV recognition is through a plethora of PRRs.

TLR2-dependent recognition. Cell surface pattern recognition receptor TLR2 can form a heterodimeric structure with TLR1 to detect herpesviruses like HCMV (Boehme et al., 2006). TLR2 detects the hydrophobic bonds in the viral glycoproteins, which are exposed due to the conformational changes during the viral entry and fusion (Triantafilou et al., 2006). Followed by signalling via MyD88, which initiates the NF-κβ and proinflammatory cytokine secretion (Gaudreault et al., 2007;Kurt-Jones et al., 2004;Wang et al., 2005). Studies have shown that TLR2 deficient mice were more susceptible to HSV-2 infection and they had disrupted cytokine and natural killer cell activation (Sørensen et al., 2008) (Szomolanyi-Tsuda et al., 2006). Studies have shown a relation between the TLR2 polymorphism and increased number of vaginal lesions

as a result of HSV-2 infection, as well as increase in the viral replication during the HCMV infection (Bochud et al., 2007;Kijpittayarit et al., 2007).

TLR9-dependent recognition. The recognition of the intracellular viral or bacterial DNA is attained by the TLR9 in the endosomes (Ahmad-Nejad et al., 2002). UNC93B is a membrane spanning protein that is located on the endoplasmic reticulum, responsible for the endosomal TLR trafficking from endoplasmic reticulum to the endosome. Mutation in the gene Unc93b1 that encodes for UNC93B protein and a single point mutation at H412R, leads to impaired major histocompatibility complex (MHC) class 1 and class 2 presentation by disrupting the TLR3, TLR7 and TLR9 signalling pathway, H412 R mutation causes the disruption of the direct UNC93B binding within the transmembrane domains of TLR3 and TLR9 (Brinkmann et al., 2007;Tabeta et al., 2006). TLR9 can recognize all herpesviruses and IRF7 activation by TLR9 leads to type I interferon activation in plasmacytoid dentritic cells (Delale et al., 2005;Honda et al., 2005b;Lim et al., 2007;Lund et al., 2003;Varani et al., 2007).

TLR7-dependent recognition. This single stranded RNA sensor can IFN α response as a result of murine cytomegalovirus infection, TLR7 and TLR9 deficient mice had high susceptibility to the MCMV infection (Zucchini et al., 2008).

MDA5-dependent recognition. It was reported that HSV-1 infection could lead to MDA5 and MAVS dependent interferon production in macrophages (Melchjorsen et al., 2010;Melchjorsen et al., 2006). The expression of the HSV-1 viral RNA structure is detected by MDA5 n in human macrophages.

DAI-dependent recognition. This cytoplasmic DNA sensor can initiate type I interferon production via IRF3 (Takaoka et al., 2007). In addition to HSV, it can recognize HCMV and trigger type I interferon secretion (DeFilippis et al., 2010a;DeFilippis et al., 2010b).

AIM2-dependent recognition. Absent in Melanoma 2 is an inflammasome that can activate caspase-1 and trigger IL1β and IL18 production (Bürckstümmer et al., 2009;Fernandes-Alnemri et al., 2009;Hornung et al., 2009;Roberts et al., 2009b). This cytosolic DNA sensor has HIN domains where it interacts with the viral DNA. AIM2 and DNA interactions cause formation of the inflammasome complex that leads to the secretion of the IL-1β and IL-18 from the cell. Murine CMV infection can trigger the activation of the AIM2 inflammasome and its deficiency lowers the IL-18 and interferon-γ levels. HSV-1 dependent inflammasome activation is not dependent on AIM2 but on NLRP3 (Rathinam et al., 2010).

IFI-16-dependent recognition. This intracellular receptor is also a DNA sensor that triggers the IFN-β and pro-inflammatory cytokines due to the HSV-1 DNA detection. It localizes to the cytoplasm where it detects the viral genome. Low levels of IRF3 and NF-κβ were observed due to the reduction in IFI-16 expression (Unterholzner et al., 2010).

DHX41, DHX9 and DHX36-dependent recognition. These novel receptors are known as DExD/H box helicases, they are located in the cell where they detect CpG DNA and stimulate the IRF7 and NF-κβ activation through the adaptor molecule MyD88. HSV-1 infection of plasmacytoid DC results in the activation of IFNα and proinflammatory cytokine expression (Kim et al., 2010). DHX41 DEAD domain interacts with synthetic dsDNA and initiate the type I interferon production through the STING/TBK1 complex (Zhang et al., 2011).

1.8 Project Hypothesis, Aims and Objectives

The innate immune system has evolved many molecular sensors and signalling pathways to activate host-defence mechanisms that counteract viral infection. The

host's immune response senses viral infection largely through germline-encoded PRRs that recognize evolutionarily conserved structures known as PAMPs.

Herpesviruses are a large family of DNA viruses that establish chronic infections in the host. The purpose of this study was to shed light on how herpesviruses, are initially recognized during cellular infection by PRRs. Surprisingly, this involves multiple PRRs both on the cell surface and within endosomes and the cytosol.

The aims of this project:

- To distinguish the specific PRRs involved in herpesviruses recognition (HSV2 and HCMV) in HeLa and primary vaginal epithelial cells.
- To identify the downstream signalling cascades and cytokine responses that have been activated due to viral infection in HeLa cells and in primary vaginal epithelial cells.
- To determine the virus internalization process and how viral entry activates signalling pathways and PRRs detection.
- To test the effect and efficacy of PRR agonists on viral replication.

Chapter 2: Materials &Methods

2.1 Antibodies:

Receptor	Species	Company
TLR 2	Goat	Santa Cruz Biotechnology sc-8690
TLR 3	Rabbit	Santa Cruz Biotechnology sc-10740
TLR 4	Goat	Santa Cruz Biotechnology sc-8694
TLR 9	Goat	Santa Cruz Biotechnology sc-13218
DAI/ ZBP-1	Goat	Santa Cruz Biotechnology sc-50686
AIM-2	Rabbit	Santa Cruz Biotechnology sc-137970
IFI-16	Rabbit	Santa Cruz Biotechnology sc-6050
Phospho-IKappaB	Rabbit	Cell Signaling Technology #2859L
STING	Rabbit	Abcam

Secondary Antibody	Label	Company
Rabbit anti goat	FITC	DAKO (Code Nr. F0250)
Swine anti rabbit	FITC	DAKO (Code Nr. F0205)
Rabbit anti goat IgG	FITC	DAKO (Code Nr. F0250)
Rabbit anti goat IgG	FITC	DAKO (Code Nr. F0250)
Rabbit anti goat IgG	FITC	DAKO (Code Nr. F0250)
Swine anti rabbit IgG	FITC	DAKO (Code Nr. F0205)
Swine anti rabbit IgG	FITC	DAKO (Code Nr. F0205)
Goat anti rabbit IgG	HRP	Jackson-Immunoresearch (Cat 111-035-045-JIR)
Rabbit anti goat IgG	HRP	DAKO (Code Nr. P0449)
Goat anti rabbit IgG	Alexa 647	Molecular probes (Code Nr-A21071)
Donkey anti rabbit IgG	Alexa 546	Molecular probes (Code Nr-A10040)
Isotype control IgG	Alexa 647	BD (code Nr 558053)
Isotype control IgG	Alexa 488	BD (code Nr - 558055)
Mouse IgG isotype control	Alexa 546/TRITC	BD (code Nr 558595)

Table 1: This table lists the primary and secondary antibodies used during this project and their sources.

2.1.1 Chemicals:

All fine chemicals were purchased from Sigma (UK). TLR2, TLR4, TLR9, IFI16 and DAI specific polyclonal antibodies were obtained from Santa Cruz Biotechnology Inc. (Germany).

The anti-HSV2 ICP5 antibodies, as well as the rabbit anti STING antibody, were purchased from Abcam. Secondary antibodies Ig Alexa -546 and Ig-Alexa 633, Ig Alexa -488 were obtained from Molecular Probes, Netherlands. Manufacturer's instructions were taken into account during the viral DNA labelling with the Alexa-Fluor-488-Ulysis reagent (MolecularProbes Inc.; Cambridge Biosciences, Cambridge, UK).

Pam2CSK4, Pam3CSK4, FSL-1, CpG DNA, c-di-GMP and c-di-AMP were obtained from Invivogen USA.

2.2 Tissue Culture:

Tissue culture (TC) was performed in Microflow Advanced Biosafety Class 2 laminar flow cabinet under sterile working area. All the cell lines that were used in this project were cultivated under the same conditions and were kept in a 37°C 5% CO_2 incubator (Sanyo Biomedical). Sanyo Biomedical incubator has a distilled water open container at the bottom, which is essential for creating the humidified air in the incubator. Distilled water quality was achieved by adding 2-3 ml of an aqueous prothermal solution from Lucerna-Chem AG (Switzerland). The sterility of the cabinet and the equipment was attained, by using aqueous ultimate disinfectant Virkon (Du Pont™ Company). Before entering the tissue culture room, fundamentals like lab coats and overshoes were worn to keep the sterile environment to the maximum, and during the tissue culture procedure disposable gloves were used. Gilson pipettes were sterilised

by using industrially methylated spirit before and after the tissue culture process. Autoclaved pipette tip boxes and disposable plastic serological pipettes were kept separately in a sterile environment; used plastic pipettes and pipette tips were discarded by a technician. 25 cm^2 Nunclon™Δ Surface and 175 cm^2 BD Fulcon™ cell culture surfaces, Fisher Brand sterile centrifuge conical tubes, were utilised for the cultivation of the cells.

2.2.1 Cell Lines:

2.2.1.1 HeLa – Human Cervical Cell Line:

The human cervical cell line was obtained from ATCC (American Tissue Culture Collection); they were derived from cervical cancer cells of a 31 year-old black female patient in February 1951. The cell line is an adherent epithelial first immortalised cell. The cells were maintained in 25 cm^2 flasks with a medium consisting of DMEM-Dulbecco's Modified Eagle's Medium (Sigma-Aldrich) + 10% of FCS (foetal bovine/calf serum) + 1% N.A. (non-essential amino acids). A volume of 2.0 - 3.0 ml of proteolytic enzyme trypsin (Sigma) was applied to the confluent cells for the sub culturing and 1:2 to 1:6 sub cultivation ratios were used as recommended by the supplier.

2.2.1.2 GMK-African Green Monkey Kidney Cell Line:

African Green Monkey (*Chlorocebus sabaeus*) Kidney cell line (ATCC-American Tissue Culture Collection) is an adherent epithelial cell line, which was kept in the liquid nitrogen in 10% (v/v) dimethyl sulfoxide (DMSO) and FCS growth medium. The cells were generated in 175 cm^2 BD Falcon™ cell culture flasks with DMEM-Dulbecco's Modified Eagle's Medium (Life Technologies™) + 10% of FCS (foetal bovine/calf serum) + 1% N.A. (non-essential amino acids). Sub culturing of the cell

line was accomplished by the use of proteolytic enzyme trypsin (Sigma). Green monkey kidney cells used to propagate viruses.

2.2.1.3 Human Embryonic Kidney (HEK) 293 Cell Line:

<u>HEK-Blue IFN-α/β Cells</u>

HEK-Blue IFN-α/β cells and HEK-Blue-IL-1β as well as HEK-IL6 reporter cell lines were purchased from Invivogen USA DMEM, 4.5 g/l glucose, 2-4 mM L-glutamine, 10% (v/v) fetal bovine serum, 50 U/ml penicillin, 50 µg/ml Blasticidin, 10 µg/ml Zeocin were used to maintain these cells.

ISGF3 pathway activation can be analysed via HEK-Blue IFN-α/β cells, which makes it possible to observe human type I Interferon production (Alain et al., 2010;Bego et al., 2012). After steady transfection of HEK293 cells with the human STAT2 and IRF9 genes, HEK blue cells were produced. IFN-α/β inducible IFN-stimulated gene (ISG54) promoter is activated by ISGF3, phosphorylated STAT1 and STAT2 and IRF9 complex. This complex triggers ISRE (IFN-stimulated response elements) on the promoters to initiate the SEAP (secreted embryonic alkaline phosphatase) production. Interaction of Janus kinases; JAK1 and TyK2 with the complex is JAK/STAT/ISGF3 pathway (*Figure 2.1*).

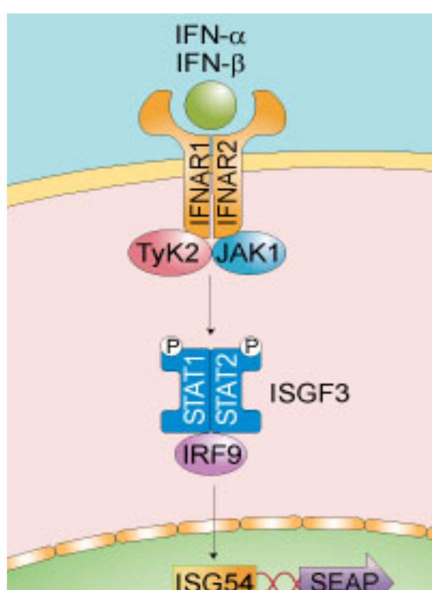


Figure 2.1: The mechanism behind HEK-Blue IFN-α/β cells. Once the HEK293 cells are stimulated with type I IFN-α and IFN-β, this leads to ISGF3 pathway activation, which ends up in ISG54 promoter activation and SEAP production. *Adapted from*: www.invivogen.com (03/05/14).

HEK-Blue™ IL-1β cells can only respond to IL-1β secretion. They consist of an NF-κB/AP-1-inducible SEAP reporter gene, which gets activated once IL-1β binds to the IL-1R receptor that is located on the surface of HEK-Blue™. Once the receptor initiates the signalling cascade because of the IL-1β binding, this interaction leads to the activation NF-κB through MyD88/MAPK/TRAF6 pathway (Neerincx et al., 2010).

HEK-Blue IL-6 cells allow the detection of bioactive IL-6 by monitoring the activation of the STAT-3pathway. These cells were generated by stable transfection of HEK293 cells with the human IL-6R gene and a STAT3-inducible SEAP reporter gene. Upon IL-6 stimulation, HEK-Blue IL-6 cells trigger the activation of STAT3 and the subsequent secretion of SEAP. SEAP can be readily monitored using QUANTI-Blue.

QUANTI-Blue is a colorimetric enzyme detection medium developed to determine the quantity of the secreted embryonic alkaline phosphatase (SEAP). This assay has been widely used in many inflammasome studies. The colour of the assay transforms from pink to purple/ dark blue, the concentration of the blue colour represents the AP promoter activity. The quantitative analysis of levels of AP promoter activity can be achieved by using a spectrophotometer at 620-655 nm.

2.2.1.4 Human Vaginal Epithelial Primary Cells:

Primary vaginal epithelial cells were purchased from Celprogen, CA, USA. Human Vaginal Epithelial Primary Cell Culture medium was used as recommended by the supplier. The cells were positive for ESA, cytokeratin-4, 8, & 18 and grown at 37C in 5% CO_2 humidified incubator. The cells were used up to eight passages.

2.2.2 Thawing Cells:

A small tube of cells was taken out of the liquid nitrogen, and when they were fully defrosted, cells were transferred into 15 ml centrifuge tubes with 5 ml of DMEM growth medium. Cells were centrifuged at 626,258 g for five minutes, and then the pellet was re-suspended with a new 5ml of fresh DMEM Dulbecco's Modified Eagle's Medium (Life Technologies^{TM}) and transferred into a 25 cm^2 Nunclon^{TM} Δ surface flask. Flasks were placed in a 37°C 5% CO_2 incubator (Sanyo Biomedical).

2.2.3.1 Propagating Adherent Cell Lines:

The supernatant was removed from the flask and the adherent cells were washed with 2ml of 1X PBS to remove dead cells or cell debris. The cells were then incubated with 2ml of trypsin, followed by the addition of 2ml of the appropriate growth medium to neutralise the trypsin. Equal amounts of cells were distributed into the flasks and fresh medium was added (to a total of 4ml). The flasks were placed back into 37°C 5% CO_2 incubator.

2.3 Viruses:

Towner strain of cytomegalovirus (CMV) and Herpes simplex virus 2 (strain G) were originally obtained from the American Type culture collection (ATCC). HSV2 and HCMV were propagated either at GMK or Hela cells. Virus stocks were kept frozen at -80°C.

2.3.1 Virus Purification:

HSV2 was purified as described by A. G. Vahlne and J. Blomberg in 1974. Cell monolayers were infected at a multiplicity of 5 PFU/cell and incubated at 37°C and 5% CO_2 for approximately 72 hours. The virus was harvested once the cells had been killed. The flasks were freeze-thawed three times, to break open the cells and release

the virus. The supernatant fluid containing free virus particles was loaded to a sucrose gradient and purified by sedimentation through a 10% to 60% sucrose gradient using a Beckmann SW28 ultra-centrifuge for 90 min, followed by a brief centrifugation through a preformed gradient of cesium chloride (density, 1.16 to 1.37 g/cm3).

2.3.2 Double-Stranded DNA (dsDNA) from Purified HSV2 and HCMV

Viral genomic DNA was isolated from purified virus (HSV2 or HCMV) according to the protocol described by Ling et. al in 1996.

Viral purified DNA was used to stimulate cells at 20 μg/ ml. All procedures, for DNA extraction, were performed in a class I cabinet, using sterile endotoxin free plasticware and endotoxin free water. Endotoxin content using LAL assay was also performed. The test showed low levels of endotoxin <0.1 EU/ug DNA (considered endotoxin free).

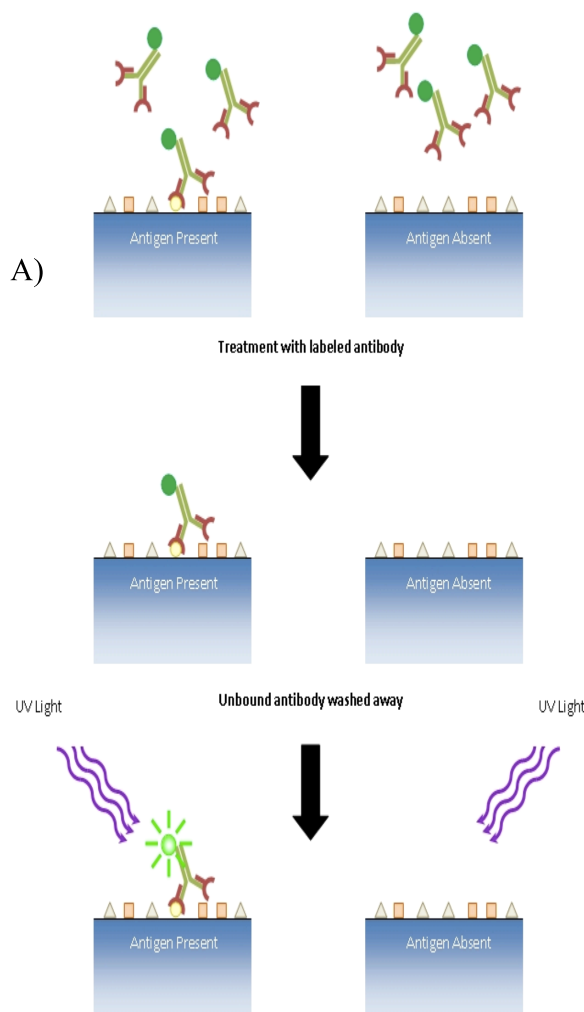
2.4 Immunofluorescence:

Immunofluorescence is a method that works with fluorescence antibody tags to be able to observe the protein molecule of interest. The light emitted by fluorescence microscope and light microscope cause and excitation of the fluorophore on the cell. These fluorescent tags go through excitation and emission stage where their excited electrons emit light until they reach the ground state. There are different kinds of fluorescent tags that are used during immunofluorescence. During this research, FITC (Fluorescein Isothiocyanate), Alexa 546 and Alexa 633, Alexa 488, TOPRO were used for quantifying or for visualizing the pattern recognition receptors (PRRs). FITC has maximal absorbance (excitation) wavelength at 490 nm and maximal emission at 525 nm, which is in the green spectrum. Alexa 546 has 561nm excitation and 572 emission

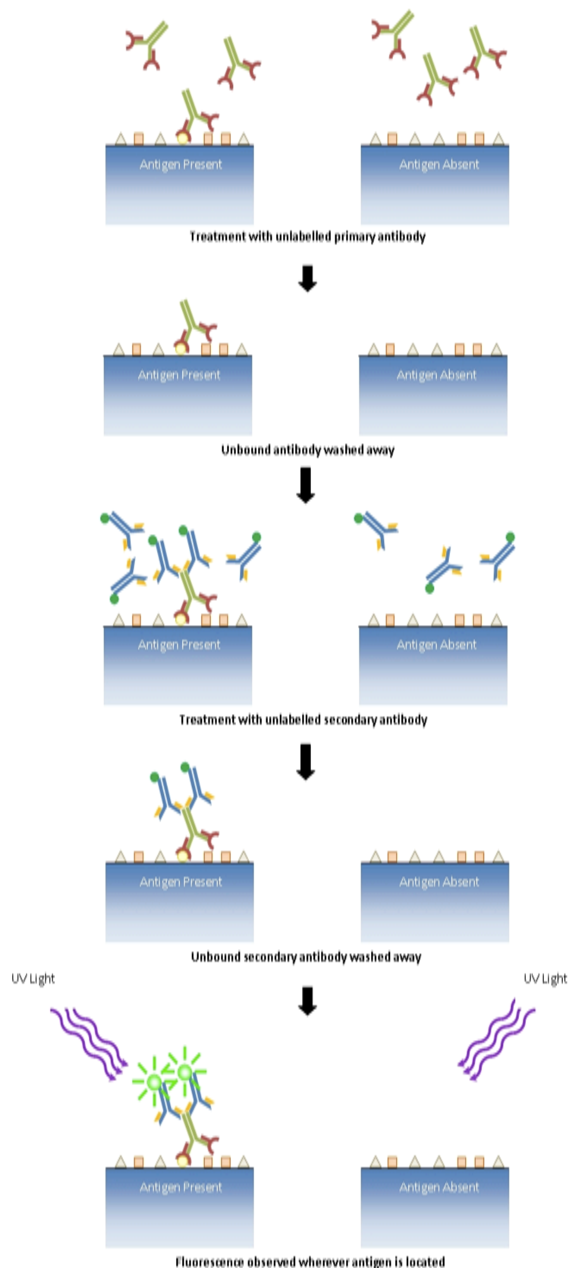
wavelengths, for Alexa 633 this is 632nm and 648nm, for Alexa 488 this is 499 and 519. Except from fluorescein isothiocyanate, there are more fluorescent tags that are used with different absorbance and emission levels. This large scale makes it easier for the scientists to examine different molecules at the same time. There are two types of immunofluorescence techniques, which are direct and indirect immunofluorescence. During direct immunofluorescence technique it is the primary antibody that has the fluorescent label, whereas in indirect immunofluorescence fluorescent tag is on the secondary antibody (USBiological, URL).

2.4.1 Indirect Immunofluorescence:

Throughout both of the direct and indirect techniques, antibody-antigen specificity plays the main role. These tags attach to the functional groups of the protein; antibodies used in the immunofluorescence are specific for the favourite protein. In direct immunofluorescence the primary antibody has a fluorescent tag attached to it and when the primary antibody binds to the specific molecule, that molecule can be visualized with microscopy or flow cytometry (*Figure 2.2 A*). That is why; antibody-antigen specificity plays an important role. Indirect immunofluorescence includes a primary and a secondary antibody with a conjugated fluorophore tag. While



in indirect immunofluorescence the secondary antibody has the fluorescent tag covalently attached to it. In order to visualise the fluorophore, the laser light will hit the tag and which will emit coloured light, (*Figure 2.2 B)*.



B)

Figure 2.2: Direct Immunofluorescence is depicted. The primary antibody has a fluorescence-conjugated tag. When the UV light hits the fluorophore, it emits a fluorescence light. On the right side of the diagram the antigen is absent thus there is no antibody antigen binding and no fluorescence light is observed. Adapted from: http://www.di.uq.edu.au/directif (A). Indirect Immunofluorescence is depicted. s. In both of the diagrams unbound antibodies were washed away with PBS Tween. Adapted from: http://www.di.uq.edu.au/indirectif (28/1/13) (B)

2.5 Flow Cytometry:

Flow cytometry is a technique used for assessing single cell characteristics. The method is used in immunophenotyping, ploidy analysis, cell counting and GFP expression analysis. The ability of the machine, to move thousand cells in a second through a laser light, provides information about the structural properties of the cell like the dimension, the composition, the phenotype and the health. Cells go through a stream by hydrodynamic focusing, passing through the laser beam one by one. In this project, cells were assessed by the flow cytometer called the Becton Dickinson (BD) Fluorescence-Activated Cell Sorting (FCASCalibur^{TM}) System (*Figure 2.3*).

Figure 2.3: Becton Dickson (BD) Fluorescence-Activated Cell Sorting System (FACSCalibur). *Adapted from:* http://www.bdbiosciences.com/instruments/facscalibur/index.jsp (25/1/13)

2.5.1 Principles of Flow Cytometry:

This method is a complex of fluidics system, laser, a lens, the optics, the filters and the detectors, which transmits the data onto the computer screen (*Figure 2.4*). Fluidics system operates with the hydrodynamic focusing leading to a stream of cells in front of the laser; cells have to pass through the laser one at a time. Sheat fluid reservoir passing through the central core, forces the cells to be in a line. The central core is quite narrow to make sure that cells moves through the laser beam uniformly. The laser light is needed for the illumination of the cells during their movement through the fluidics system. The light travels through the lens and hits every single cell reflecting a scattered light on to the detectors. Excitated electrons in the laser hits the fluorescence conjugated tagged antibody on the cells and produces scattered light that has fluorescence light coming from our fluorophore of interest. The light scattered forward provides information about the size of the cell. When the dimension of the particle or the cell is small, then the scattered light is less, as the size gets bigger the light scattered on the detector gets bigger as well. The side scatter provides information about the structural complexity granularity of the cell (Rahman, 2006). A different detector detects light that is scattered to the sides. The fluorescence light is directed to the detectors by optics, where intensity of the light is converted into a voltage pulse. Every optic reflects individual fluorescence at the right wavelength and the intensity. When the fluorophore-labelled antibody hits the laser beam, the fluorescence intensity and the wavelength will be detected by a detector, which only detects that particular range of wavelength. The filter in front of a particular detector catches the precise light wavelength range. The detectors are photomultiplier tubes (PMT), which convert the light intensity into voltage. Photomultipliers are forward scattered detector, side scattered detector, FL-1, FL-2, FL-3, FL-4. All the detectors convert the light into a voltage pulse; the scattered data is plotted on a one-

dimensional histogram graph. However the scattered data only includes the results from cells or particles of interest, this is achieved by setting a threshold on the scattered histogram, so that the samples scatter light above a certain voltage will dictate on the plotted histogram. Thus the particles that are smaller in size will not be taken into account in the scattered histogram.

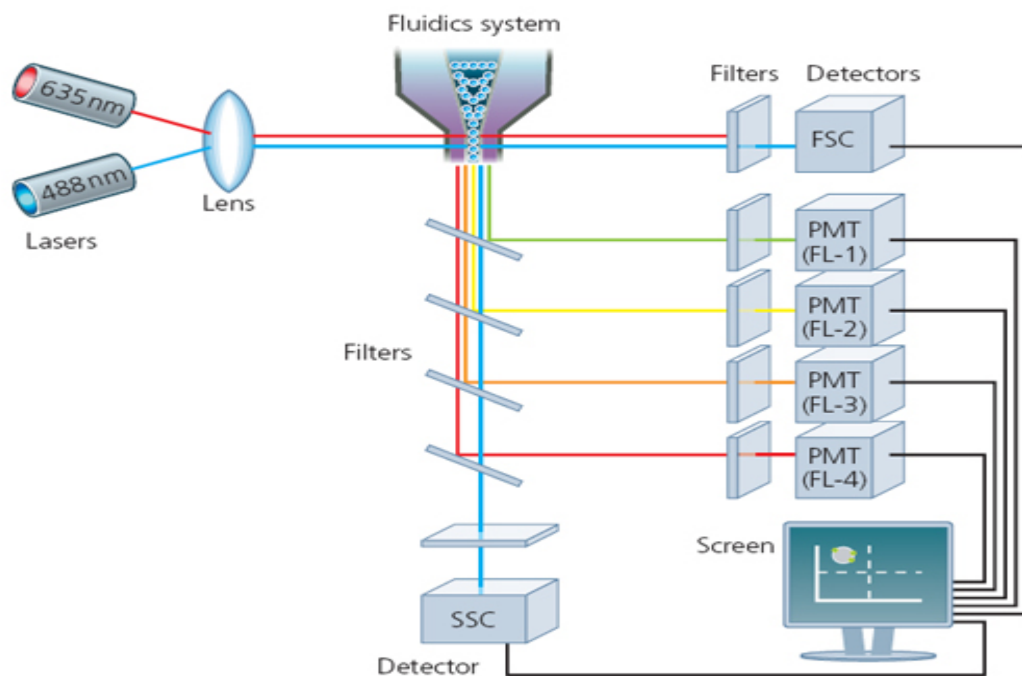


Figure 2.4: The diagram shows the flow cytometer mechanism. Fluidics system forces the cells to pass through the laser beam one at a time. The scattered light splits apart and depending on the wavelength of the scattered light they pass through the filters to a specific detector. FSC detector detects forward scattered light; SSC detector detects side-scattered light, as it is shown on the diagram. The other tubes detect certain wavelengths of the fluorescence. Adapted from: http://static.abdserotec.com/uploads/Flow-Cytometry.pdf (25/01/13)

2.6 SDS-PAGE:

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis known as SDS-PAGE separates macromolecules in an electric field depending on their Molecular Weight (MW) (Carpette, 2011). There are two types of SDS-PAGE; continuous and discontinuous (*Figure 2.5*).

2.6.1 Continuous SDS- PAGE:

Continuous SDS-PAGE includes identical gel and running buffer concentrations, which is easy to prepare. This protocol is adequate for small molecules but not for the big molecules.

2.6.2 Discontinuous SDS-PAGE:

Discontinuous SDS-PAGE uses different solution for the tank and for the gels. The gel made of two different gels. The upper stacking gel with a larger pore dimensions with a pH of 6.8 and the lower resolving gel with a smaller pore dimensions with a pH of 8.8. The protein samples are stacked into very thin, sharp zones at the interface between the stacking gel and resolving gel, and are then separated according to their MW as they pass through the resolving gel (Aldroubi et al., 1995).

During this procedure samples were placed on a discontinuous gel and were run in an electric field. Sodium dodecyl sulphate solution is an anionic detergent, denaturing protein molecules without affecting the peptide bonds (Schmieg, 2011). Thus the protein samples are in their primary linear structure. SDS adds negative charges to the amino acids leading to the disruption, whereas β-mercaptoethanol breaks the disulphate bonds. Denaturation of the proteins can be speeded up by boiling the sample at 90°C-100°C. Proteins that are now negatively charged will be attracted to the anode, passing through stacking into resolving part of the gel. When the proteins

are moving on this polyacrylamide gel under the effect of an electric field, the larger molecules will migrate slower than the smaller molecules. The stacking gel has a lower concentration compared to the resolving gel, thus until the end of the stacking gel the samples move at the same rate. When they reach the point where the resolving gel starts, the molecules start to move to the positive pole according to their molecular mass creating different levels of banding patterns.

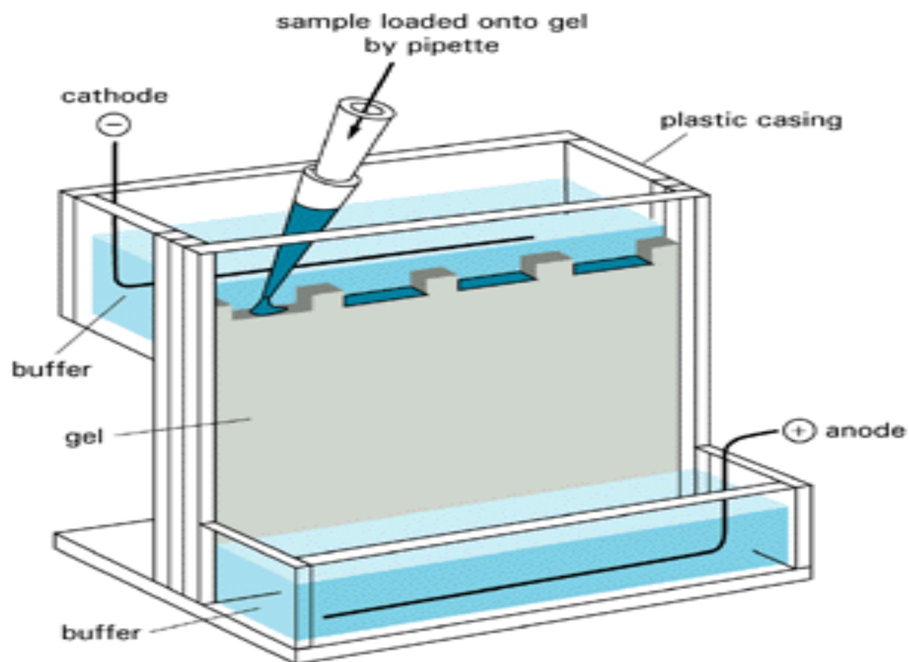


Figure 2.5: The diagram shows sodium dodecyl sulphate (SDS)-Polyacrylamide Gel Electrophoresis. The gel stands in a gasket electrode assembly. Samples are loaded onto the stacking gel and then when they reach to the resolving gel level, proteins start to separate according to their molecular weight. Negatively charged samples move from cathode end to the anode end. Adapted from: http://www.imbjena.de/~rake/Bioinformatics_WEB/proteins_purification.html (28/1/13)

2.6.2.1 Discontinuous SDS-PAGE Preparation:

10% of resolving and 4% of stacking gel were prepared (see Appendix).

After the preparation of the resolving gel solution, the solution was poured through the glass plates. Few drops of isopropanol were added in order to get rid of any bubbles on top and to make the gel even. After approximately 45-60 minutes when the resolving gel was polymerised, the isopropanol was washed away with dH_2O and the stacking gel was poured on top. The well combs (1.00mm) were added on top of the stacking gel and were left to set for approximately 30 minutes. Once polymerized, the gel was placed into a tank. The tank was filled up with approximately 200 ml of running buffer.

The samples were re-suspended in x2 SDS-PAGE Reducing Sample Buffer (See Appendix) and were boiled in a water bath for 10 minutes. 40μl of each sample was loaded in each well as well as a molecular weight marker. The gels were run for 45 minutes at a constant voltage of 200V.

2.7 Western Blot:

The proteins that were separated by SDS-Page gel electrophoresis were transferred onto the membrane via western blotting. The membrane that was used was nitrocellulose membrane (Whatman®). During this process a sandwich of the gel was made by packing the gel with the nitrocellulose membrane in a cassette between two layers of blotting paper and pads. Pads, blotting paper and nitrocellulose membrane were pre-soaked in a transfer buffer before preparing the sandwich. In the following step, the cassette was placed into a tank transfer system. A block of ice was placed into the tank to keep the system cool enough and the tank was filled up with transfer buffer. Electroblotting procedure lasted for 60 minutes at a constant current of 210mA.

2.7.1 Blocking and Primary Antibody Incubation:

Blocking the membrane prevents any other proteins from binding onto the surface as well as preventing any background signal coming from the unspecific antibody binding. This was achieved by 5% blocking reagent (milk powder) in 0.1% of PBS-Tween. The membranes were left on a rocking table for 60 minutes with the blocking reagent. Approximately 5-6ml of blocking reagent was used for each membrane, to make sure that the surface of the membrane is completely covered. This was followed by the washing of the membranes with a 0.1% PBS-Tween for 30 minutes. Appropriate primary antibody was added on to the membranes with a dilution of 1:1000 in 0.1% PBS-Tween. The membrane can be incubated in the primary antibody for 60 minutes or overnight in 4°C.

2.7.2 Secondary Antibody Incubation:

After the incubation with the primary antibody, three washes were performed with x0.1% PBS-Tween to remove any excess primary binding. The appropriate secondary antibody and a streptavidin-HRP conjugate were added with a dilution of 1:6000. The membranes were incubated with the secondary antibody for 45-60 minutes on a rocking table and washed with 0.1% PBS-Tween for 2-3 hours to make sure that excess antibody had been removed (changes every 15 minutes), before enhanced chemiluminescence detection.

2.7.3 Enhanced Chemiluminescence:

The termed chemiluminescence is a chemical reaction that occurs when an excited substance emits light. During this procedure phenols are used to increase the light output and extend the light emission duration. Detection of the bands was performed in a dark room, to avoid exposure of the autoradiography film to light (Quan et al., 2006). During this process, the nitrocellulose membranes were probed with ECL

detection reagents (GE Healthcare, Amersham^{TM}). Equal amounts of ECL solutions were added for each membrane (2ml), which is an excited state of this reaction, in the dark room (*Figure 2.6*). After the incubation of the membrane with the ECL reagent, the membrane was wrapped in a cling film, and put in a cassette. A sheet of autoradiography film was put on top of the membrane and then left in a cassette for 2-5 minutes. The film was then developed immediately, and depending on the intensity of the bands seen, a new exposure time could be tried.

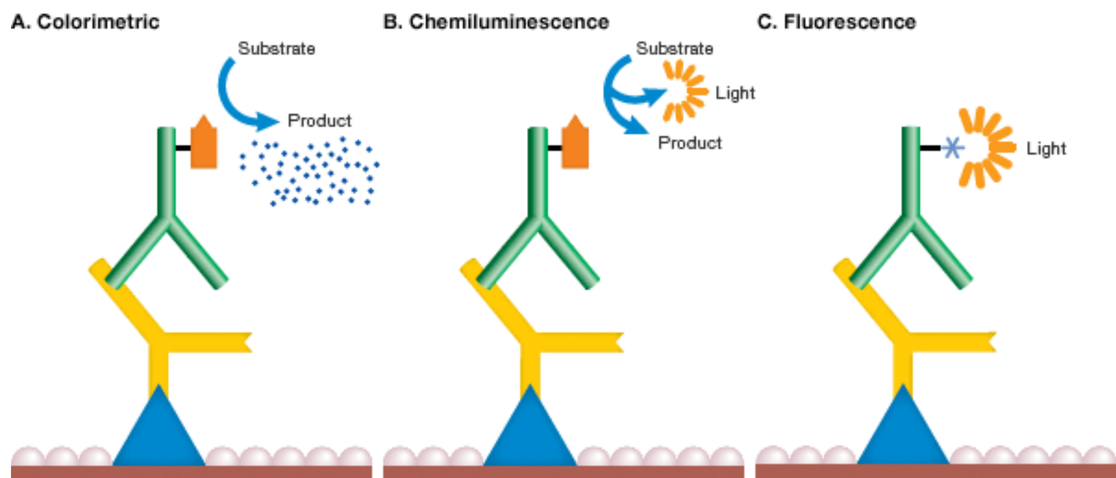


Figure 2.6: The diagram shows different western blot detection mechanisms. The diagram B represents the mechanism of how chemiluminescent signals are created with the use of streptavidin-biotinylated horseradish peroxidase complex. Adapted from: http://www.bio-rad.com/evportal/en/UK/evolutionPortal.portal?_nfpb=true&_pageLabel=SolutionsLandingPage&catID=LUSQ6KKG4 (27/1/13)

2.8 Confocal Microscopy:

This study enables the visualisation of the specific molecules in the cell to identify the location, and the interaction with the other molecules. The images obtained with the confocal microscopy have a better contrast and are three-dimensional, which is attained by excluding any light except from the microscope's focal plane (Triantafilou et al., 2012). This is achieved by using a spatial pinhole. Laser excitation source creates a beam of light through the pinhole aperture, which will hit the dichromatic

mirror and direct to the objective. Illumination is focussed on the objective lens, which is then reflected onto the detector pinhole aperture. Detector is connected to a photomultiplier where the data is shown as an image and can be displayed on the computer screen (*Figure 2.7*).

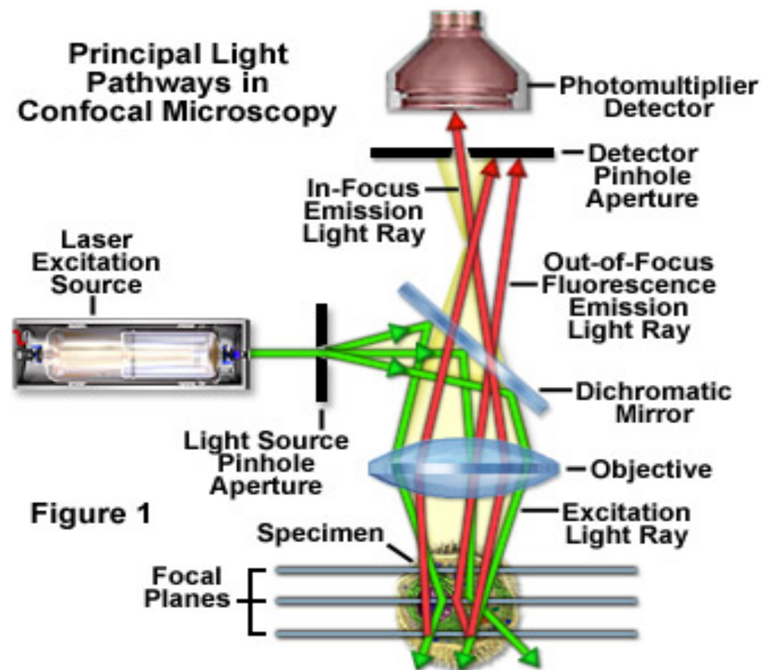


Figure 2.7: The diagram represents the principal light pathways in confocal microscopy. Laser source creates the light that is directed onto the objective for focusing. When the focussed light hits the cells emission light ray is created that hits the detector which creates an image on the computer system. Adapted from: http://www.microscopyu.com/articles/confocal/confocalintrobasics.htm (27/1/13)

2.8.1 Slide Preparation:

Human primary vaginal epithelial cells were stimulated with HSV-2 virus. The colocalization of TLR2, DAI, STING, TLR9 and IFI16 was investigated. HSV2 internalisation and the intracellular trafficking of DAI and IFI16 were also studied. Lab-Tek microchamber culture slides were used and all of the samples were examined at certain time points, three wells were used per time point. Cells were incubated overnight at 37°C 5% CO_2 incubator overnight until they are 80%-90% confluent, followed by the viral stimulations either with HSV2 (50 PFU) or with purified HSV2

DNA (20μg). Incubation times with virus were 1h, 4h and 6h. The cells were fixed with 4% PFA for 15 minutes, washed twice with 300μl of PBS and then treated with 150μl PBS/BSA/Saponin/NaN_3. Samples were incubated with primary antibody followed by the appropriate secondary antibody conjugated to Alexa 488 and Alexa 546. To preserve the fluorescence intensity of the secondary antibody, SlowFade Gold Antifade Reagent was used before placing the cover slip on top of the slide. The air bubbles were removed and the slides were sealed with a nail varnish. The samples were visualised under LSM510 confocal microscope.

2.8.2 Confocal Image Analysis:

During this procedure it is important to get accurate quantitative data thus the user bias was eliminated, then by automatically creating a threshold the interdependence of the red and green channels were calculated with the use of the correlation coefficient.

2.8.2.1 LSM Image Browser and AxioVision LE:

There are three software systems in confocal microscopy that enables the user to adjust the brightness and the contrast of the images and it saves the images as a LSM file. AxioVision LE software (Carl Zeiss, Inc.) is used to convert the image data to TIF files. Otherwise the images cannot be analysed with the next software system ImageJ and JACoP.

2.8.2.2 ImageJ and JACoP:

This software is used to analyse the TIF files that are converted by AxioVision LE software (Carl Zeiss, Inc.). This program is capable of analysing the confocal images in Java software. It automatically thresholds the images and removes the user bias.

2.9 Plasmid DNA:

2.9.1 Plasmid DNA Preparation:

2.9.1.1 Transformation:

Several agar plates were prepared with a selection antibiotic (1 μg/ml), either Zeocin, puromycin or ampicillin. pshRNA-TLR2, pshRNA-TLR4, pshRNA-TLR9, pshRNA-DAI, pshRNA-STING and pshRNA-RIG-I were transformed into a competent *E.coli* strain (*E.coli* GT116). Zeocin is a selection antibiotic for pshRNA TLR2, STING and TLR9 (Invivogen). Puromycin is the selection antibiotic for ZBP1 pshRNA plasmid. Ampicillin is for pshRNA RIG-I and was used as a negative control. The lyophilized pshRNA plasmid DNA (in powder form) was diluted in 200μl of sterile endotoxin free deionized water. The liquid solution was vortexed and 5μl of plasmid was introduced into tubes with 100μl of E.coli competent cells. The tubes were placed on ice for 30 minutes. The E.coli and pshRNA plasmid complex was placed on a hot plate for 1 minute at 42°C, which causes a heat shock to the bacteria to initiate the intake of the plasmid DNA. The tubes were then placed back on ice for 2 minutes and 0.5ml of Luria Broth was added in the eppendorf tubes, followed by incubation in a shaking incubator at 37°C for 2 hours.

All procedures for DNA extraction were performed in a class I cabinet. 100μl of transformed E.coli was added onto the agar plates containing the selection antibiotic and evenly distributed onto the agar surfaces using a glass spreader. The agar plates were incubated in an incubator at 37°C overnight. The plasmids contain the antibiotic resistance gene, so only the *E.coli* that have taken up the plasmid will survive on the agar plates containing the specific antibiotic.

After growing up the *E.coli* colonies, the transformed *E.coli* and plasmids were expanded. 25μl Zeocin, or Puromycin or Ampicillin depending on the plasmid used was added to 25ml LB in bottles, and individual colonies were added to the broths, as well as being added to the reference plate, and incubated overnight in a shaker incubator at 225 rpm at 37°C.

2.9.1.2 DNA Isolation:

Luria Broth solutions were spun at 3000rpm for 30 minutes; the pellets were treated with 400μl of STET Buffer (see Appendix). The samples were vortexed and transferred into autoclaved tubes. The pellets were placed on ice and 10μl of lysozyme was added. The tubes were boiled for a minute and placed back on ice bucket for another 5 minutes. The tubes were centrifuged at 13000 rpm for 30 minutes; the pellets were removed with autoclaved toothpick. 5μl of 20mg/ml RNAse A was added and incubated at a temperature between 37°C-42°C for 30 minutes. An equal amount of phenol/chloroform/isoamyl alcohol was added into the solution and vortexed. The tubes were centrifuged again for 20 minutes and the supernatant of the solution was kept. 400μl of chloroform/isoamyl alcohol was added in each tube. The tubes were centrifuged again for 20 minutes and the supernatant was kept and was placed in new tubes. The nucleic acids were precipitated by the addition of 1/20th of the original volume of sodium acetate 2M (pH 6.5) and 2.5 of the original volume of absolute ethanol in each tube. The components were mixed and the eppendorfs were placed into -80°C for at least 60 minutes. Afterwards, the eppendorfs were quickly removed from the -80°C to be centrifuged at 13,000 rpm for 20 minutes at room temperature. The excess supernatant was removed, and the eppendorfs were centrifuged at 13,000 rpm for a further minute, and the remaining supernatant was removed. 80μl sterile water (ddH_2O), or LAL water, was added to each eppendorf, and the samples frozen at -20°C.

2.9.1.3 Agarose Gel Electrophoresis:

Agarose gel electrophoresis was applied after the DNA isolation. The purpose of this electrophoresis technique was to examine the purity of the samples for the plasmid preparation procedure. An agarose gel was prepared with a concentration of 1g in 100ml of x1 ELFO buffer (see Appendix). 15μl of the samples were mixed with 7μl of ELFO Loading Buffer (see Appendix) were added in each well. The tank was filled up with ELFO running buffer. 5μl of 1kb DNA ladder with 10μl of ELFO Loading Buffer were added into the well of the gel, to examine the banding patterns of the samples. The system was set to run for 45 minutes at a constant voltage of 100V.

2.9.1.4 RNA Interference:

The silencing of a particular gene includes the inhibition or suppression of the expression of that molecule that the gene is coding for in the cell mechanism (Czauderna et al., 2003;Koper-Emde et al., 2004). Thus affecting the downstream signalling cascades that are related to that particular molecule. This is achieved by introducing a plasmid plus hairpin RNA to knock down the gene expression (ter Brake et al., 2008;Triantafilou et al., 2005). Short hairpin RNA interacts with a Dicer/Argonaute 2 complex, where dicer cleaves it. Passenger strand is cleaved by Argonaute 2 and is realeased, whereas RNA Interference Specificity Complex (RISC) uses guide strand for targeting our mRNA of interest. RISC translocates mRNA into processing bodies. Processing bodies contain proteins, which degrade the mRNA. In this project transfections of shRNAs were carried out using Lipofectamine. The target sequence of shRNA used were:

for TLR2 5'GTCAATTCAGAACGTAAGTCA3',

for TLR4 5'GCUUAUAUCCUUAAAGAAATT3'

for ZBP1, 5'GGCCACCUUGAACAAAGAAtt-3',

for STING, 5’GCAUCAAGGAUCGGGUUU3’

for TLR9 5’CCGCATCGTCAAACTGGCG3’.

Transfections with the specific shRNAs resulted in an approximately 90% decrease in receptor expression as determined by western blotting whereas transfection of cells with the scrambled shRNA did not show any decrease in TLR or STING and DAI expression.

2.10 Cytometric Bead Array:

The Human inflammation BD^{TM} Cytometric Bead Array (CBA (BD Biosciences)) kit was used for determining the cytokine concentrations in the sample. Interleukin-8 (IL-8), Interleukin-1β (IL-1β), Interleukin-6 (IL-6), Interferon-β (IFN-β), Tumour Necrosis Factor (TNF) and Interleukin-12p70 (IL-12p70) are the cytokines that the cytometric bead array can detect. These cytokines function during the human inflammatory response. The beads are coated with antibodies specific for each cytokine, thus they are capable of capturing the cytokines in a solution. This method is very specific to quantify the amount of the cytokines. Each of them has different fluorescent intensities. Detection antibody, which is phycoerythrin (PE) conjugated, is added to the cell samples. Each bead will give out different intensity of a signal; this makes it easier to identify the cytokines in the samples.

2.10.1 Assay Procedure:

This procedure was achieved by adding 50μl of the tissue culture supernatant into the flow tubes, followed by the addition of the cytokine beads (50μl) into the solution, mixed thoroughly. The complex was incubated for 60 minutes at room temperature. PE detection reagent (50μl) was injected into the tube, mixed and incubated in the

dark for two hours at room temperature. The pellets were washed with PBS-Tween and then centrifuged for five minutes. The supernatants were removed, and the pellets were re-suspended in a 300µl PBS-Tween. They were examined by using flow cytometry.

2.11 TLR Agonists:

Tests were carried out on primary human vaginal epithelial cells, which were obtained from healthy donors (Celprogen, Torrance, CA). They were cultured in human vaginal epithelial primary cell culture complete growth medium, which was obtained from Celprogen; the culture media were replaced with a fresh media every 24-48 hours. During this procedure the primary cells were cultured in a six well plate with the density of 1.5×10^6, cells were used up to 8 passages only. Subculturing was performed every 24-48 hours in a microflow cabinet and then placed back to 37°C in 5% CO_2 humidified incubator. Pam_2CSK_4, Pam_3CSK_4, FSL-1, CpG DNA (type C), cyclic diguanylate monophosphate (c-di-GMP), and cyclic diadenylate monophosphate (c-di-AMP) were obtained from Invivogen (San Diego, CA). Pam_2CSK_4, Pam_3CSK_4, FSL-1 were used as TLR2 agonists. CpG DNA was used as a TLR9 agonist and c-di-GMP and c-di-AMP for STING. Cells were stimulated with different concentrations of agonists and incubated for 60 minutes in 37°C in 5% CO_2 humidified incubator. 50ng/ml, 100 ng/ml and 150 ng/ml of Pam_2CSK_4, 100 ng/ml, 200ng/ml, 300ng/ml of Pam_3CSK_4, 50ng/ml, 100ng/ml, 150ng/ml of FSL-1 agonists were used for receptor TLR2. 100nM, 200nM and 300nm of CpG DNA (type C) were used for TLR9. 2µg/ml, 4µg/ml, 6µg/ml of c-di-GMP and c-di-AMP were used to test on STING (Triantafilou et al., 2014). 5MOI of HSV2 was introduced into the cells and they were left for 18 hours incubation in 37°C in 5% CO_2 humidified incubator. Indirect immunofluorescence technique was applied onto the cells to be able to examine the

HSV2 replication levels before and after the agonists, specific antibody known as anti-HSV2 ICP5 antibody from Abcam (Cambridge, UK) was used against ICP5 major capsid protein of HSV2 (Part 2.4.1). This was followed by indirect immunofluorescence using a FACSCalibur to determine the PRR expression levels.

Chapter 3: Results

CHAPTER 3: A SYNERGIC INVOLVEMENT OF DIFFERENT PRRs IN HERPESVIRUS INNATE IMMUNE RESPONSE

3.1 Introduction

Herpesviruses are a large family of dsDNA viruses. Alphaherpesviruses include two subtypes, which are HSV 1 and HSV 2. HSV-2 is the most common subtype seen in genital infections (Cunningham et al., 2006;Molina et al., 2011;Wald and Link, 2002).

Immunological control of herpesviruses by the infected host is achieved by both the innate and the adaptive immune systems. In the innate antiviral immune response, type I interferons (IFNs) and cytokines have key roles in the containment of herpesvirus infections. This response is governed by a variety of PRRs that recognize PAMPS and DAMPS and activate downstream signaling molecules. Cytokines and chemokines aid in mediating this response. Nucleic acids that are released into the cytosol during HSV infection are recognized by a range of intracellular DNA sensors that activate the type I interferon response.

To date, many TLRs and cytosolic DNA sensors have been described, however it is unclear how these receptors work in concert to mediate HSV clearance Previous studies show that TLRs can recognize herpesvirus glycoproteins. This recognition controls cytokine and IFN expression in various cell types (Kurt-Jones et al., 2004;Sato et al., 2006). Furthermore DAI recognizes HSV DNA (Takaoka et al., 2007). Herpesviruses are also detected by IFI16, which stimulates the expression of IFN-β (Unterholzner et al., 2010). Our understanding of how herpesviruses are detected by PRRs and the innate immune system and stimulate antiviral activities has grown tremendously over the past decade however it is essential to establish their roles

and mechanisms of action in different tissue types and cells. This part of the study aims to investigate the role of different PRRs in HSV2 infection, which is one of the most common pathogens in genital infections and how the innate immune system recognizes this virus in HeLa cells. Utilising siRNA knockdown of different PRRs as well as bead array assays to determine cytokine secretion our results have shown that TLR2 and TLR9 recognize HSV2 and trigger IL-6 secretion. While the DNA sensors DAI, IFI16 recognize the viral DNA and trigger type I IFN production through STING. Inflammasome activation and IL-1β secretion occurs through AIM2 in HSV2 infection.

3.2 Results

3.2.1 PRR Expression Levels in Response to HSV2 and HSV2 DNA

To determine the expression levels of PRRs, during the initial HSV2 infection in HeLa cells, we used indirect immunofluorescence and determined the fluorescence intensity by flow cytometry. HeLa cells were stimulated with HSV2 and also exposed to purified viral DNA at specific time points (*Fig.* 3.1 and *Fig.* 3.2 respectively). During the first hours of the viral entry there is an increase in the expression levels of TLR2, TLR9, DAI and a slight increase in the TLR4 (*Fig.* 3.1). TLR2 the cell surface receptor upregulation is probably due to detection of the glycoproteins on the envelope, which is consistent with the other studies (Compton et al., 2003;Kurt-Jones et al., 2004). TLR9 and DAI are intracellular receptors which recognize viral genomic dsDNA thus the upregulation of these receptors observed in the first few hours is due to the presence of HSV2 DNA after cell entry, these receptors seem to increase significantly again from 10hr onwards when the virus begins to replicate and the new

synthesized DNA is detected by DAI and TLR9 (Lund et al., 2003;Takaoka et al., 2007).

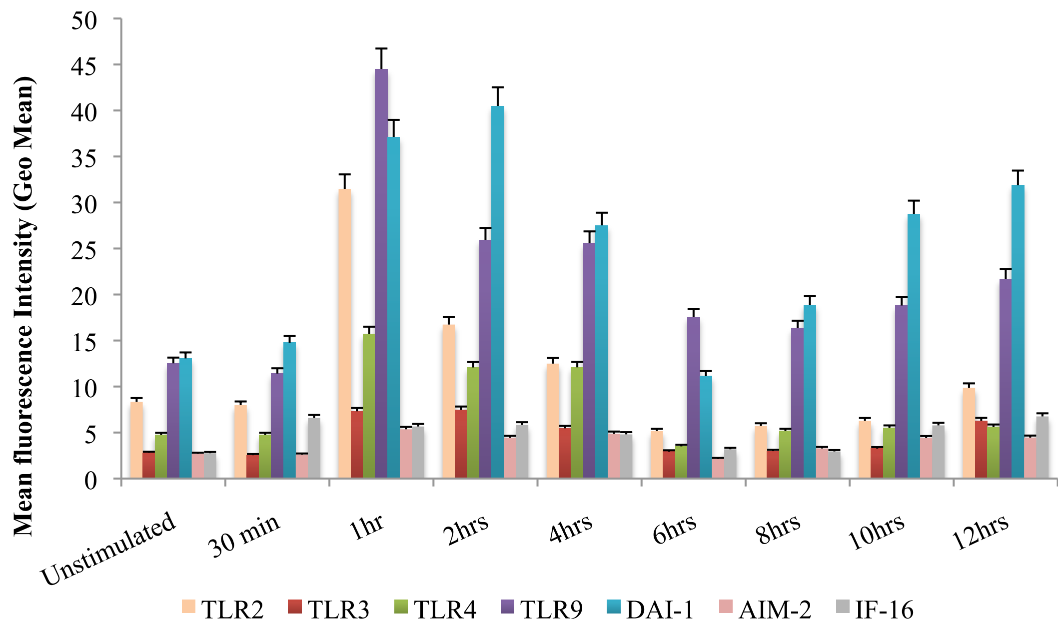


Figure 3.1: PRR responses to HSV2 infection. HeLa cells were stimulated with 5 MOI HSV2 at different time points and then PRR expression was determined by using a specific primary antibody for each receptor of interest followed by the appropriate secondary conjugated to FITC. The expression levels were represented by the mean fluorescence intensity determined by flow cytometry. The data represents the mean ± SD of three independent experiments.

HeLa cells were also stimulated with HSV2 DNA (*Fig*. 3.2).

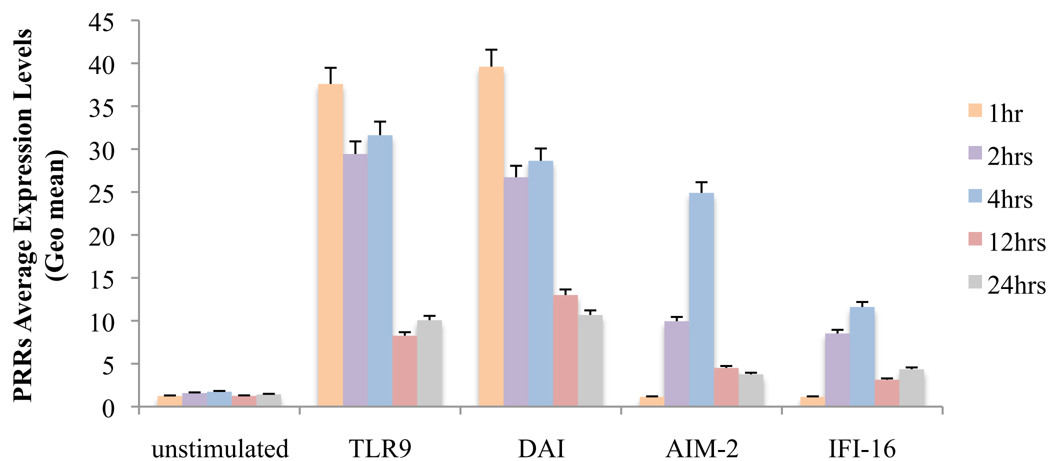


Figure 3.2: PRR responses to HSV2 DNA infection. HeLa cells were stimulated with 20 μg/ml of purified DNA at different time points and then PRR expression was determined by using a specific primary antibody for each receptor of interest followed by the appropriate secondary conjugated to FITC. The expression levels were represented by the mean fluorescence intensity determined by flow cytometry. The data represents the mean ± SD of three independent experiments.

HSV2 dsDNA seems to be recognized by a plethora of DNA sensors (*Fig.* 3.2). At first there is an upregulation of TLR9 which is logical since it is known that TLR9 recognizes viral DNA in the endosomal compartments during viral entry and DAI is abundant in the cytoplasm so they are the first DNA sensors picking up the HSV2 dsDNA inside the cell. Initially AIM-2 and IFI-16 expression levels do not change in the cell. However when TLR9 and DAI expression in the cell decrease there is an increase in the expression levels of AIM-2 and IFI-16 compensating for the loss of DAI. This possibly signifies that these receptors are triggered later on in the virus replication cycle when the virus replicates and there is an increase of viral DNA. In the 4th hour AIM-2 inflammasome and IFI-16 expression reaches to the maximum. IFI-16 is localized in the cytoplasm and in the nucleus (Unterholzner et al., 2010) and my data suggests that the viral genome accumulation in those compartments leads to the detection by IFI-16. During the 12th hour the expression levels of these sensors are diminished massively, which can be either linked to the lytic phase of the viral cycle or the inhibition of the detection mechanism by the viral components.

3.2.2 Host response and Signalling upon Infection with HSV2 and viral DNA

After the detection of the PRRs that were involved in HSV2 detection in HeLa cells, the downstream signalling cascades of those receptors were examined to verify their involvement in HSV2 and HSV2 dsDNA recognition.

TLRs when activated by their ligand induce the recruitment of downstream signalling molecules. This results in IκB being released from the NF-κB complex and getting phosphorylated, marking it for ubiquitination and degradation by proteosomes. The transcription factors NF-κB and IRF3 transcriptionally upregulate type I IFNs to mediate induction of the innate immune response. The detection of phospho-IκB therefore corresponds to NF-κB activation.

HeLa cells were infected with HSV2 and the cell lysate was used to detect phospho-IkkB via western blotting (*Fig.* 3.3).

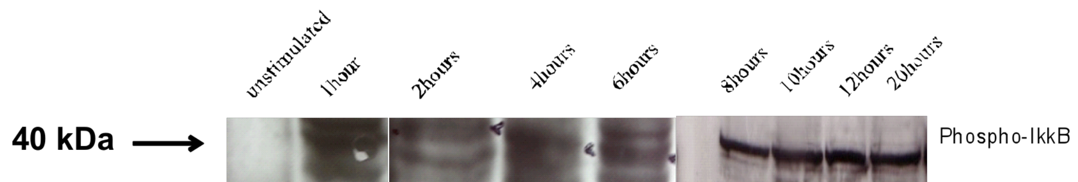


Figure 3.3: Immunostimulatory effect of HSV2 on HeLa cells. HeLa cells were stimulated with 5 MOI of HSV2 at different time points. The cell lysate obtained from each stimulation was used for SDS-PAGE gel electrophoresis and western blot analysis using primary antibodies for phospho-IkkB followed by the appropriate secondary antibodies conjugated to HRP. The data represents one set from three independent experiments.

As expected after the HSV2 infections as a result of immunostumulatory effect TLRs trigger signalling cascades leading to the activation of the transcription factor NF-κβ in HeLa cells. As a result of the viral stimulation the transcription factor production is quite high during the virus infectious cycle thus leading us to hypothesize that elevation in the PRR expression levels, which recognize the virus, causes activation of NF-κβ and their downstream signalling cascades.

To verify the PRR involved in HSV2 host immune response, DAI was also examined (Fig. 3.4).

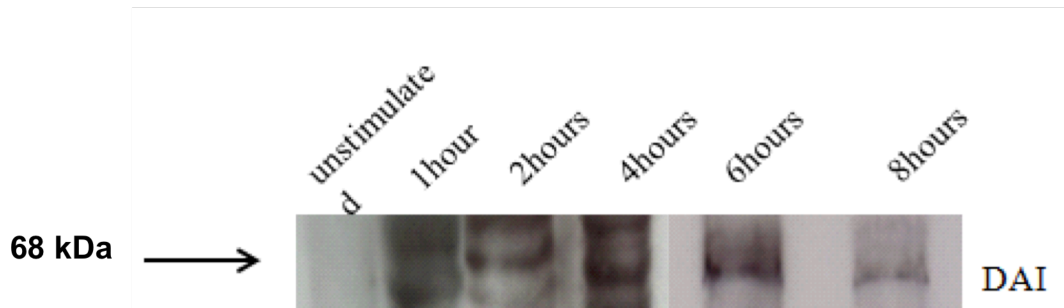


Figure 3.4: DAI synthesis after HSV2 stimulation. HeLa cells were stimulated with 5 MOI of HSV2 at different time points. The cell lysate obtained from each stimulation was used for SDS-PAGE gel electrophoresis and western blot analysis using primary antibodies for DAI, followed by the appropriate secondary antibodies conjugated to HRP. The data is a representative gel of three independent experiments.

The western blot analysis of the HeLa cell lysates shows DAI production in the presence of HSV2. DAI expression seems to increase up to the first 6hrs as the band becomes denser as time progresses. After 6hrs there is a decrease in DAI production.

HSV2 is a DNA virus so the presence of the adaptor STING that is crucial in DNA sensor signalling was also investigated (*Fig.* 3.5).

Figure 3.5: The presence of the adaptor STING in HeLa cells. HeLa cells were stimulated with 5 MOI of HSV2 at different time points. The cell lysate obtained from each stimulation was used for SDS-PAGE gel electrophoresis and western blot analysis using primary antibodies for STING, followed by the appropriate secondary antibodies conjugated to HRP. The data is a representative gel of three independent experiments.

Stimulator of IFN genes-STING (also known as MITA, MPYS, ERIS), is also activated in response to the cytosolic viral DNA and acts as an adaptor for DNA sensors DAI, AIM2 and IFI16 (Hornung and Latz, 2010). The amount of STING in the cell lysates was quite high during the first four hours of the infection since STING acts as an adaptor molecule in the downstream signalling cascades of DAI, AIM2 and IFI16 during the HSV2 infection.

In addition to the live virus infection cells were stimulated with HSV2 DNA as well and NF-κβ production was investigated by phospho-IkkB detection via western blotting (*Fig.* 3.6).

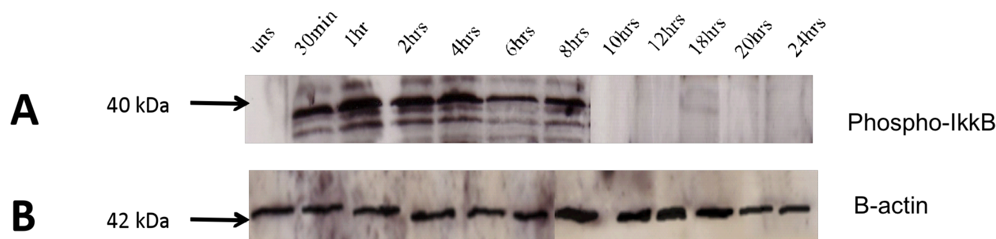


Figure 3.6: Immunostimulatory effect of HSV2 DNA on HeLa cells. HeLa cells were stimulated with 20µg/ml of HSV2 dsDNA at different time points. The cell lysate obtained from each stimulation was used for SDS-PAGE gel electrophoresis and western blot analysis using primary antibodies for phospho-IkkB followed by the appropriate secondary antibodies conjugated to HRP (A). Loading control for β-actin is also displayed (B). The data is a representative gel of three independent experiments.

NF-κβ production after HSV2 DNA stimulation was not as strong as the stimulation with the whole viral structure. The viral DNA is being recognized by the DNA sensors during the first few hours of cell entry, as time progresses there will be some degradation of the inserted DNA which could lead to diminished host response and recognition especially since there is no live virus present to replicate and produce more viral DNA.

DAI synthesis after HSV2 DNA stimulation was also examined (*Fig.* 3.7).

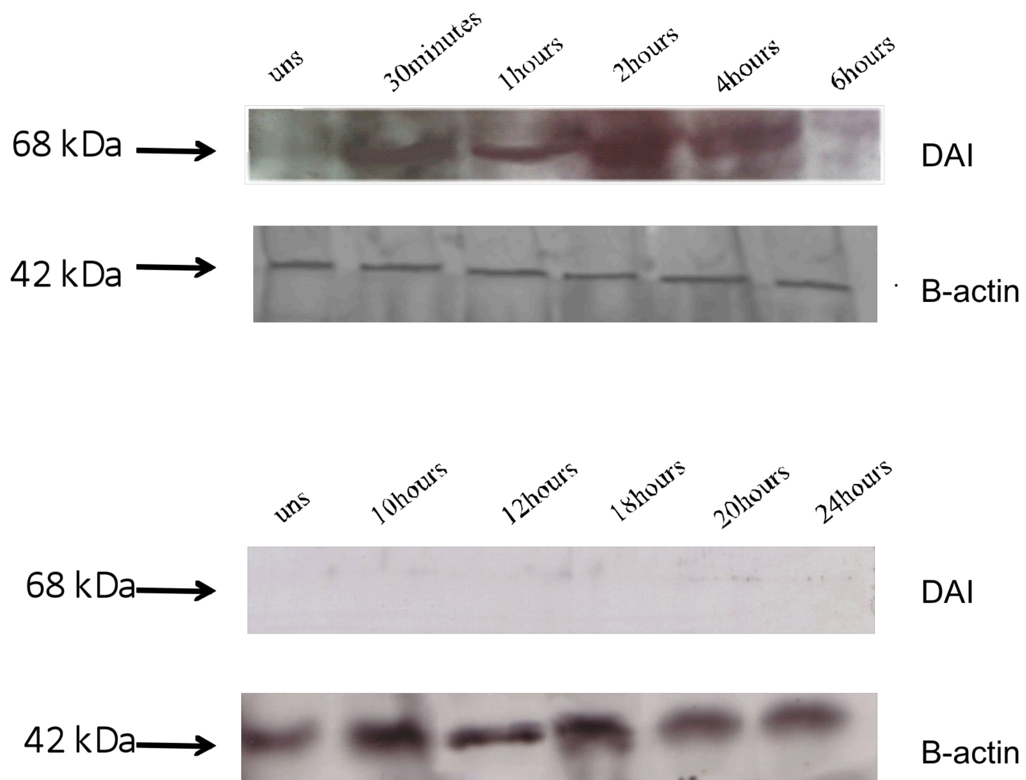


Figure 3.7: DAI involvement in HSV2 DNA detection. HeLa cells were stimulated with 20µg/ml of HSV2 dsDNA at different time points. The cell lysate obtained from each stimulation was used for SDS-PAGE gel electrophoresis and western blot analysis using primary antibodies for DAI, followed by the appropriate secondary antibodies conjugated to HRP. Loading controls for β actin are also depicted T he data is a representative gel of three independent experiments.

As seen with NF-κB activation DAI synthesis after the viral DNA entry increases for the first few hours but decreases gradually as the viral DNA begins to degrade. Finally STING expression after HSV2 DNA stimulation was also investigated (*Fig.* 3.8).

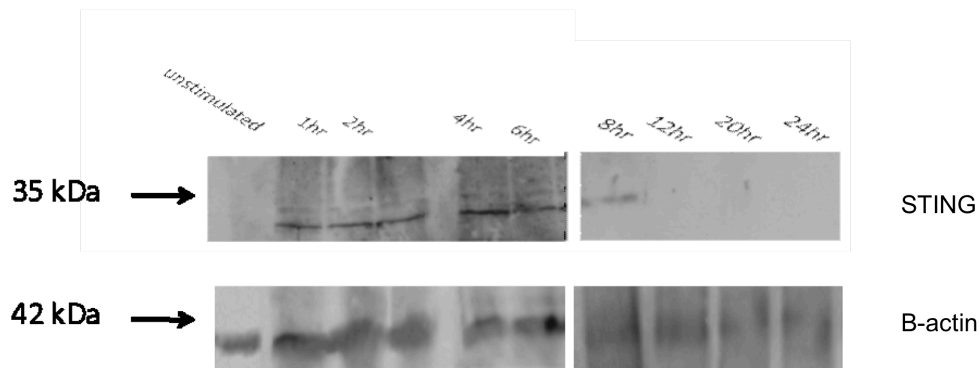


Figure 3.8: STING involvement in HSV2 DNA detection. HeLa cells were stimulated with 20µg/ml of HSV2 dsDNA at different time points. The cell lysate obtained from each stimulation was used for SDS-PAGE gel electrophoresis and western blot analysis using primary antibodies for STING, followed by the appropriate secondary antibodies conjugated to HRP. Loading controls for β actin are also depicted. The data represents the mean of three independent experiments.

The activation of STING is the highest during the first hours of entry. From the 8th hour there is decreased activation of STING, perhaps due to DNA degradation.

3.2.3 IFN-β, IL-6 and IL-1β Production

HEK IFN-β, HEK-IL-6 and HEK-IL-1β reporter cell lines were used to determine cytokine production in the cells during HSV2 infection. Supernatants of cell cultures, which had been infected with HSV2 or stimulated with HSV2 DNA at different times points, were incubated with the HEK-Blue cells between 18-24 hrs. After the addition of Quanti-Blue and incubation period between 30min-6hrs, samples were tested with the spectrophotometer and the SEAP production levels were determined (*Fig.* 3.9, *Fig.* 3.10, *Fig.* 3.11).

HEK IFN-β cells incubated with HSV2 stimulated HeLa cell supernatants (*Figure* 3.9).

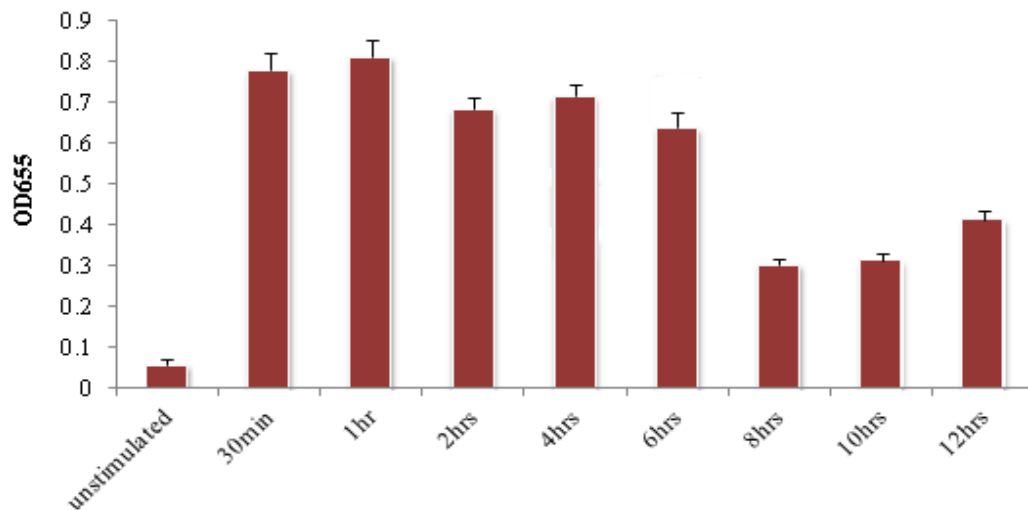


Figure 3.9: Levels of IFN-β secretion in HeLa cells. HeLa cells were stimulated with 5 MOI of HSV2, at different time points. The supernatants were harvested, incubated with HEK IFN-β cells between 18-24hr and assayed for IFN-β secretion by measuring the levels of SEAP in the supernatant using the Quanti-Blue assay. The SEAP levels were determined via spectrophotometer at 655nm. The data represents the mean ± SD of three independent experiments.

The specific times points were compared with the unstimulated cell sample and it was observed that there was a high IFN-β production in the cells due to the HSV2 stimulation. This is due to the recognition of the virus by TLR9 and DAI triggering their downstream signalling cascades to initiate the type I IFN secretion in the cell. In support of these findings another study using human cervical epithelial cells (HCE) infected with HSV2, observed an increase in TLR4 and TLR9 expression which lead to an increase in the IFN-β production (Li et al., 2009). The cytokine production increases for the first hours of the virus replication cycle then once a new replication cycle begins at the 12th hour there is an upregulation again.

HEK IL-6 cells incubated with HSV2 stimulated HeLa cell supernatants (*Figure* 3.10).

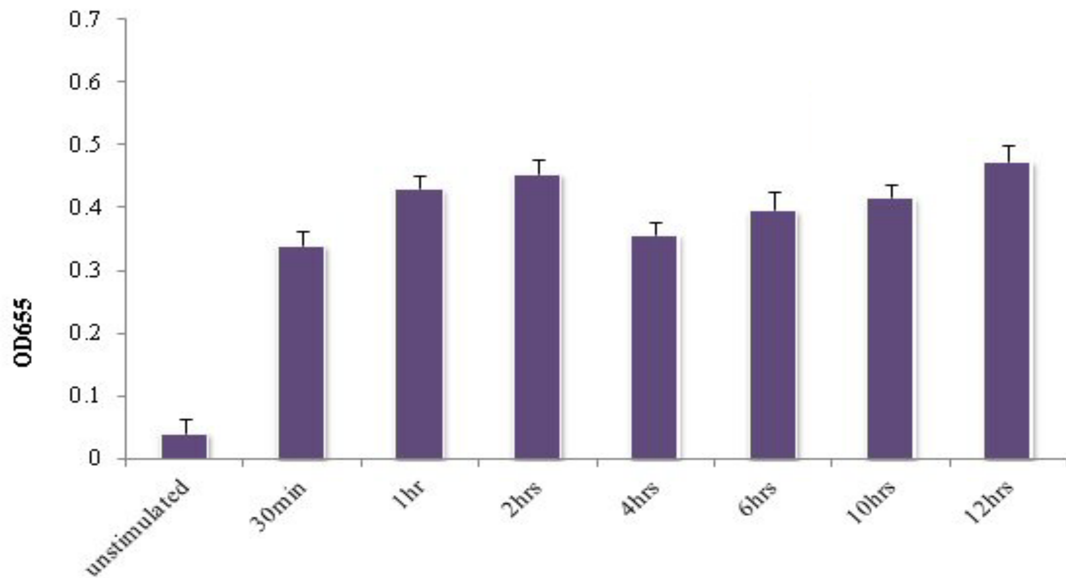


Figure 3.10: Levels of IL-6 secretion in HeLa cells. HeLa cells were stimulated with 5 MOI of HSV2, at different time points. The supernatants were harvested, incubated with HEK IL-6 cells between 18-24hr and assayed for IL-6 secretion by measuring the levels of SEAP in the supernatant using the Quanti-Blue assay. The SEAP levels were determined via spectrophotometer at 655nm. The data represents the mean ± SD of three independent experiments.

Once cells become infected with HSV2 there was an increase in IL-6 production. TLR upregulation leads to IL-6 expression in the cell (Li et al., 2009;Sato et al., 2006). These results show a marked increase in IL-6 production thus verifying PRR activation by HSV2.

HEK IL-1β cells incubated with HSV2 stimulated HeLa cell supernatants (*Figure* 3.11).

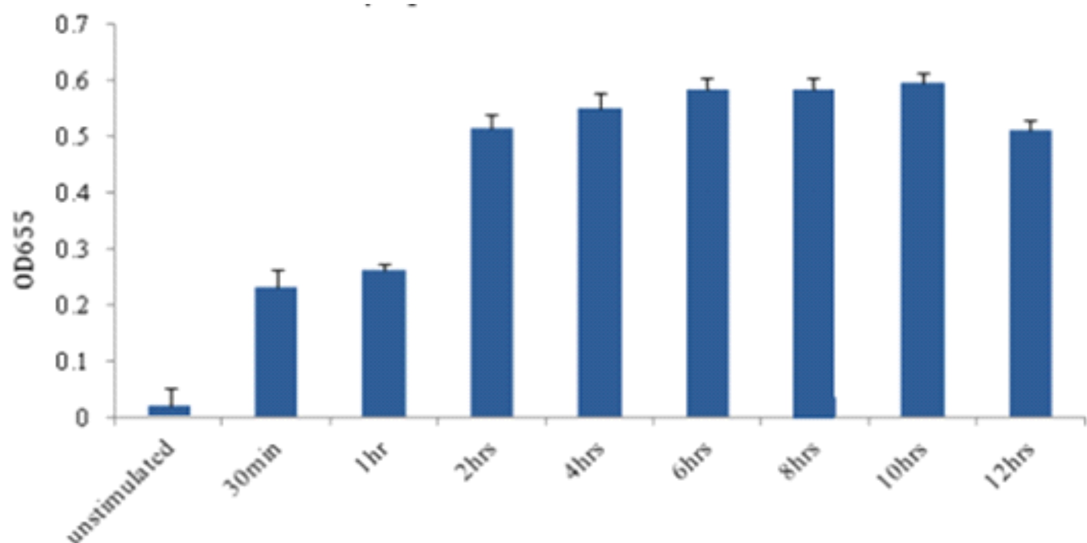


Figure 3.11: Levels of IL-1β secretion in HeLa cells. HeLa cells were stimulated with 5 MOI of HSV2, at different time points. The supernatants were harvested, incubated with HEK IL-1β cells between 18-24hr and assayed for IL-1β secretion by measuring the levels of SEAP in the supernatant using the Quanti-Blue assay. The SEAP levels were determined via spectrophotometer at 655nm. The data represents the mean ± SD of three independent experiments.

From the data obtained there was an increase in the IL-1β beginning at the 2nd hour, reaching a plateau at the 6th hour.

To determine the immunostimulatory effect and cytokine production of viral DNA, cells were stimulated with HSV2 DNA and cytokine secretion was determined (*Fig.* 3.12, *Fig.* 3.13, *Fig.* 3.14).

HEK IFN-β cells incubated with purified HSV2 DNA stimulated HeLa cell supernatants (*Figure* 3.12).

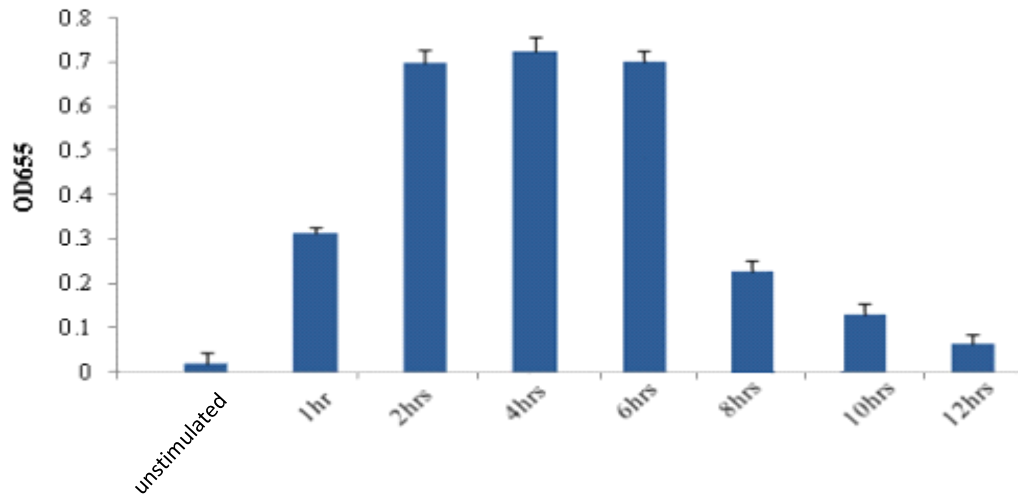


Figure 3.12: Levels of IFN-β secretion in HeLa cells in response to HSV2 DNA. HeLa cells were stimulated with 20μg/ml HSV2 DNA, at different time points. The supernatants were harvested with HEK IFN-β cells incubated between 18-24hr and assayed for IFN-β secretion by measuring the levels of SEAP in the supernatant using the Quanti-Blue assay. The SEAP levels were determined via spectrophotometer at 655nm. The data represents the mean ± SD of three independent experiments.

There is a gradual increase in the IFN-β secretion as a result of HSV2 genomic DNA between the 1st hour and 6th hour, following a drop in IFN-β production later on.

HEK IL-6 cells incubated with purified HSV2 DNA stimulated HeLa cell supernatants (*Figure* 3.13).

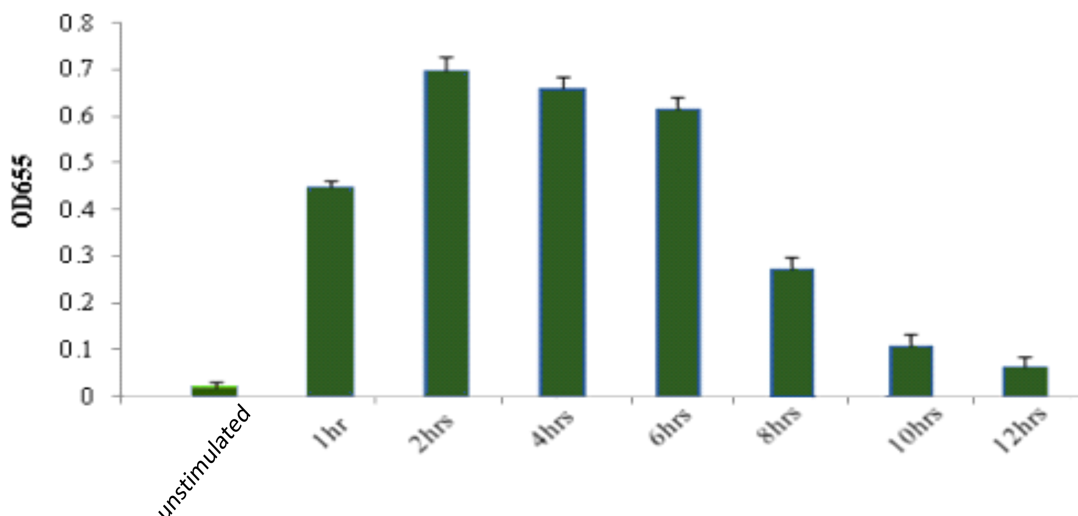


Figure 3.13: Levels of IL-6 secretion in HeLa cells in response to HSV2 DNA. HeLa cells were stimulated with 20μg/ml HSV2 DNA, at different time points. The supernatants were harvested with HEK IL-6 cells incubated between 18-24hr and assayed for IL-6 secretion by measuring the levels of SEAP in the supernatant using the Quanti-Blue assay. The SEAP levels were determined via spectrophotometer at 655nm. The data represents the mean ± SD of three independent experiments.

IL-6 production, due to HSV2 DNA stimulation, is quite high and it shows an increase between the 1st hour and the 6th hr, which can be dependent on the rise of TLR9 expression levels.

HEK IL-1β cells incubated with purified HSV2 DNA stimulated HeLa cell supernatants (*Figure* 3.14).

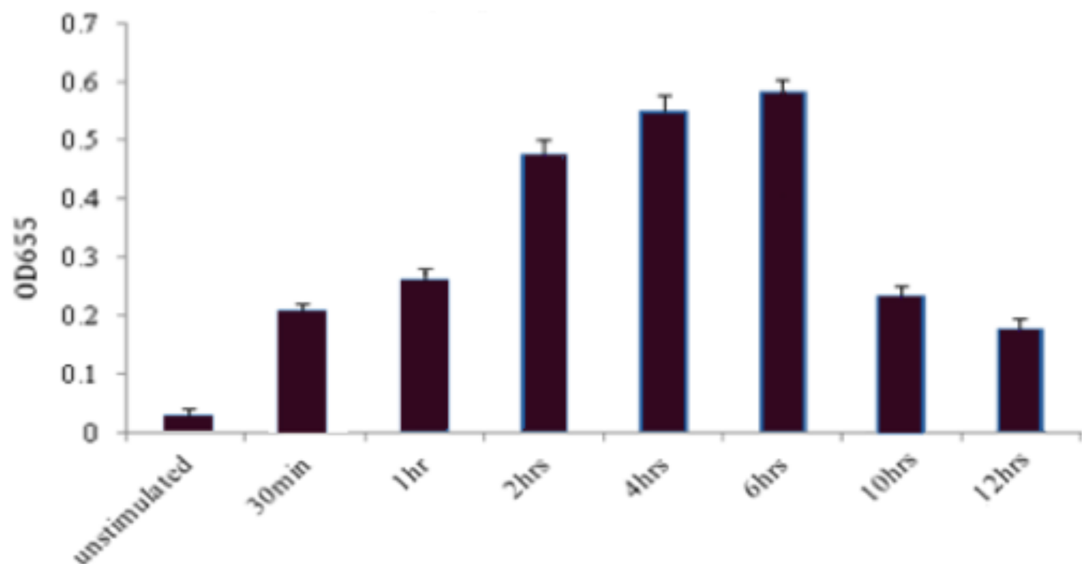


Figure 3.14: Levels of IL-1β secretion in HeLa cells in response to HSV2 DNA. HeLa cells were stimulated with 20μg/ml HSV2 DNA, at different time points. The supernatants were harvested with HEK IL-1β cells incubated between 18-24hr and assayed for IL-1β secretion by measuring the levels of SEAP in the supernatant using the Quanti-Blue assay. The SEAP levels were determined via spectrophotometer at 655nm. The data represents the mean ± SD of three independent experiments.

As expected HSV2 DNA entry has an effect on the IL-1β synthesis probably via AIM2, which triggers IL-1β secretion.

3.3 Silencing of TLRs, DAI and RIG-I

3.3.1 The effect of TLRs and DNA sensors in HSV2 and HSV2 DNA detection

To determine the importance of innate immunity in HSV2 infectious cycle, TLR2, TLR9, DAI, AIM2 as well as an independent irrelevant PRR, RIG-I that recognizes RNA instead of DNA, were silenced. The knockdown efficiency was at 80% and was determined by western blotting (*Fig.* 3.15). The pro-inflammatory response was examined to see the knockdown effect.

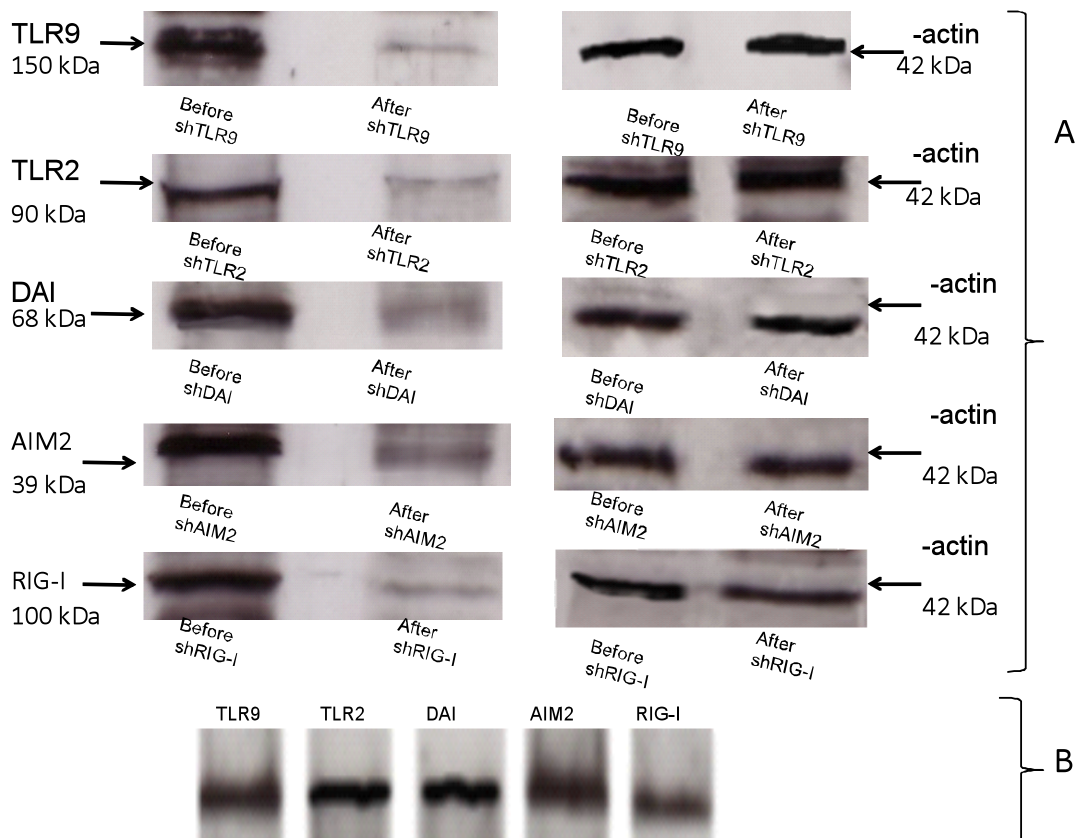


Fig 3.15: PRRs knockdown. TLR9, TLR2, DAI AIM2 as well as RIG-I expression was knocked down by shRNA and the expression levels were determined by western blotting using primary antibodies for TLR2, TLR9, DAI, AIM2 or RIG-I, followed by the appropriate secondary antibodies conjugated to HRP. Loading controls with β-actin are also depicted (A) pshRNA-h7SKGFPzeo-derived plasmid with scrambled sequence was used as a control. It had no effect on TLR9, TLR2, DAI, AIM2 or RIG-I expression (B). These gels are representatives of three independent experiments.

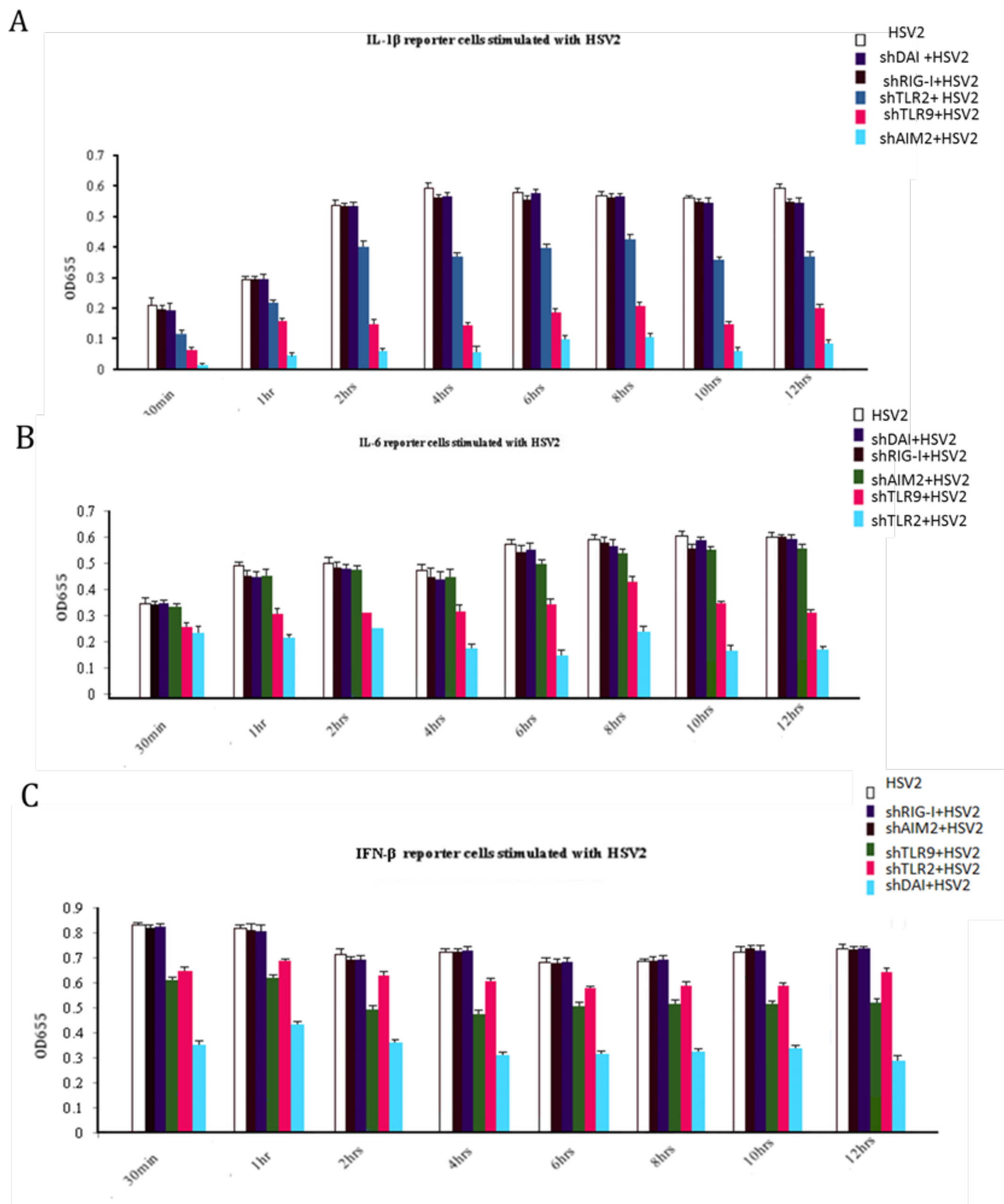


Figure 3.16: HeLa cells were either infected with 5moi of HSV2 or TLR2, TLR9, DAI, RIG-I or AIM2 expression was knocked down by shRNA and the silenced cells were infected with 5moi of HSV2. Supernatant was collected at 12hr post infection and analysed for IL-1β (A), IL-6 (B) and IFN-β (C). The data represent the mean of three independent experiments.

From the results we can see that by knocking down TLR2 and TLR9 there is a significant decrease in IL-1β production (*Figure 3.16*). The inflammasome is activated

via a two signal mechanism which leads to IL-1β secretion (Latz, 2010) thus leading us to the conclusion that TLR2 and TLR9 must provide signal 1 for caspase 1 activation and subsequent IL-1β production, thus by eliminating signal 1 we have a reduction in IL-1β. AIM2 is part of the DNA sensing inflammasome (Rathinam et al., 2010) thus by knocking down AIM2 we are now knocking down signal 2 even though signal 1 is triggered from the TLRs without AIM2 for the second signal we have no IL-1β secretion. As expected knocking down RIG-I or DAI has no effect in IL-1β secretion.

IL-6 is triggered by the TLRs, (Kawai and Akira, 2010) so we can see a considerable reduction in IL-6 secretion when TLR2, which recognizes the virus glycoproteins, is knocked down as well as a reduction when TLR9 is knocked down, since TLR9 recognized the HSV2 DNA (*Figure 3.16*). DAI as well as AIM2 silencing have no effect in IL-6 secretion since although they can recognize DNA they do not play a role in IL-6 activation and secretion. RIG-I silencing as expected has no effect (*Figure 3.16*).

Finally in respect to IFN-β production, the knockdown of DAI and TLR9 reduced IFN-β production (*Figure 3.16*). Knockdown of TLR2 had an effect as well by reducing IFN-β production albeit much less than the effect that DAI and TLR9 had on HSV2 infected HeLa cells. AIM2 and RIG-I had no role in IFN-β secretion.

Identifying the importance of PRRs in HSV2 DNA sensing

AIM2, DAI, TLR2, TLR9 and RIG-I were knocked down by shRNA. Cells were then stimulated with HSV2 DNA (*Fig.* 3.17) and cytokine secretion was measured.

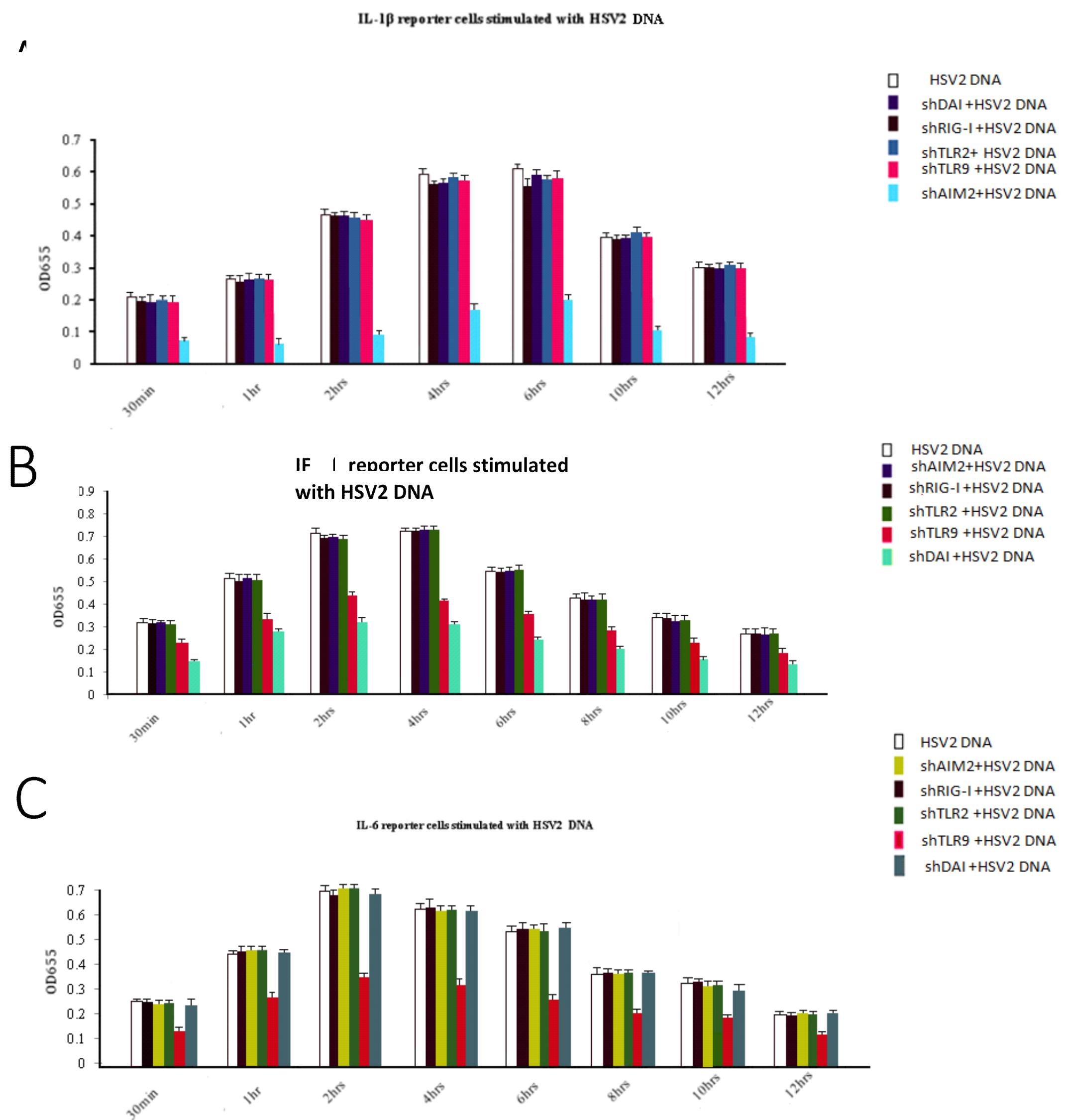


Figure 3.17: HeLa cells were stimulated with 20µg/ml of HSV2 DNA also TLR2, TLR9, DAI, RIG-I or AIM2 expression was knocked down by shRNA and the silenced cells were stimulated with 20µg/ml of HSV2 DNA. Supernatant was collected at 12hr post infection and analysed for IL-1β (A), IFN-β (B) and IL-6 (C). The data represent the mean of three independent experiments.

The data show that IL-1β secretion was reduced only when AIM2 was knocked down, thus confirming that AIM2 inflammasome recognizes HSV2 DNA and triggers IL-1β production (*Figure 3.17*). IFN-β was reduced when DAI, and to a lesser extent TLR9, were knocked down verifying their role in type I IFN production. Finally, IL-6 was only reduced when TLR9 was knocked down, which is expected since TLRs have been shown to trigger IL-6 secretion and TLR9 recognizes viral DNA.

3.4 Conclusion

From this data we can see that HSV2 is recognized by different PRRs such as TLR2 and TLR9 initially which trigger IL-6 secretion followed by the DNA sensors in the cytoplasm DAI, IFI16 and AIM2 where cytosolic STING‑dependent DNA signalling occurs which leads to type I IFN production through STING, while AIM2 triggers inflammasome activation and IL-1β secretion. We have seen that these receptors are upregulated and trigger a strong pro-inflammatory host response during the viral and DNA stimulations. Since the discovery that intracellular DNA could elicit a TLR independent IFN response in a variety of mammalian cells (Ishii et al., 2006;Okabe et al., 2005;Stetson and Medzhitov, 2006) much work has focused on finding the DNA receptors that initiate this response. The involvement of more than one PRR shows a complexity in HSV2 recognition, this is possibly a defense mechanism of the host, due to redundancy at the receptor level, co-operation of several DNA binding receptors upstream of STING activation, or access to different DNA receptors in different compartments, as has been proposed for the inflammasome activation by AIM2 in the cytosol and IFI16 in the cytosol and the nucleus (Kerur et al., 2011;Singh et al., 2013) gives the host a better chance to combat and control viral infections such as HSV2 which if not controlled by the immune system could become life threatening.

As a result of these findings we can conclude that virus uses cell surface receptor TLR2 while entering the cell, followed by interaction with TLR9 and with other DNA sensors. Infections with purified HSV2 DNA have shown that viral DNA does not interact with TLR2 on the cell surface. Internalisation of the virus is achieved by endocytic pathway that mainly leads to TLR9 and DAI activation straightaway. These interactions lead to MyD88 pathway, adaptor molecule STING activation, followed by activation of type I interferon and pro-inflammatory cytokine production.

Thus these findings identify TLRs and DNA sensors as PRRs that recognize HSV-2 infection, and also show that cytosolic receptors act in concert with TLR9 to induce cytokine expression.

Two previous studies have shown that two different PRRs RIG-I and TLR3 coordinately activate the host response against influenza A virus and respiratory syncytial virus infections (Liu *et al.*, 2007), and our work further supports the idea that a full innate antiviral response is induced through activation of several PRRs that act in concert to mediate host defense.

Collectively, our work has identified PRRs recognizing HSV2 infection, and shown that simultaneous stimulation through two or more PRRs impacts on the nature of the antiviral response.

CHAPTER 4: HERPES SIMPLEX VIRUS 2-INDUCED ACTIVATION IN VAGINAL CELLS INVOLVES TOLL-LIKE RECEPTORS 2 & 9 AND DNA-SENSORS DAI & IFI16

4. 1 Introduction

Genital infections caused by HSV2 are the most frequent cause of genital ulcerations (Beauman, 2005;Steben M, 1997). HSV infection has a high prevalence and represents a large economic burden. In industrialized countries, the prevalence rate for HSV-2 infection in adults is up to 20% (Benedetti et al., 1994). In a subset of people with more than 10 lifetime partners, the rate of genital herpes was 50% (Malkin et al., 2002). Although some infections are self-limiting, HSV2 genital infection has a relapsing pattern of illness (staying latently in sensory ganglia before reactivation) which impacts of patients' quality of life both psychologically and socially, as well as increasing susceptibility to other infections such as Human Immunodeficiency Virus (HIV) (Molina et al., 2011).

There is currently no vaccine available, and although many antivirals such as acyclovir have been developed to reduce duration and severity of infection, there are no therapies, which prevent initial infection. Innate immune responses are crucial during the period of acute infection to limit early virus replication and to facilitate the development of an appropriate specific acquired immunity. Knowledge of these innate immune mechanisms is therefore vital if a new therapeutic approach is to be developed. Thus experiments on primary cells with TLR agonists can provide us an important data about the effect of these novel therapies on viral replication cycle. These data can be used during the animal studies and pre-clinical studies for development of a vaccine against herpesvirus initial infections.

The pathway by which HSV2 triggers the innate immune system in the urogenital system has not as yet been fully elucidated. We have already seen that HSV2 is detected by a plethora of pattern recognition receptors (PRRs) in Hela cells in order to limit host infection. TLR2 recognizes virus lipoproteins and TLR9 as well as DAI and to a lesser extent IFI16 recognize the viral DNA and mount a strong inflammatory response.

4.2 Results

In this study I have utilized primary vaginal cells and TLR2 and TLR9 agonists as well as agonists for STING which is a crucial signaling adaptor for DAI and IFI16, in order to limit HSV2 infection in vaginal cells. The aim of this study was to identify whether these compounds could have therapeutic utility for urogenital HSV2 infection, also to spot out the cell compartments HSV2 is interacting with. The invasion pathway of HSV2 should be the same as in HeLa cervical cells, thus an increase in the synthesis of TLR2, TLR9, IFI16 and DAI is expected, furthermore the production of adaptor molecules and cytokines that are related to these immune receptors should increase. If TLR agonists are successful then there should be a decrease in the viral replication. Receptors that function in the recognition of HSV2 are located on the cell surface, in the endosomes, endoplasmic reticulum, cytoplasm and in the nucleus. For this reason localization of HSV2 or purified HSV2 DNA in these parts of the cell is expected.

4.2.1 PRRS Involved in HSV2 Recognition

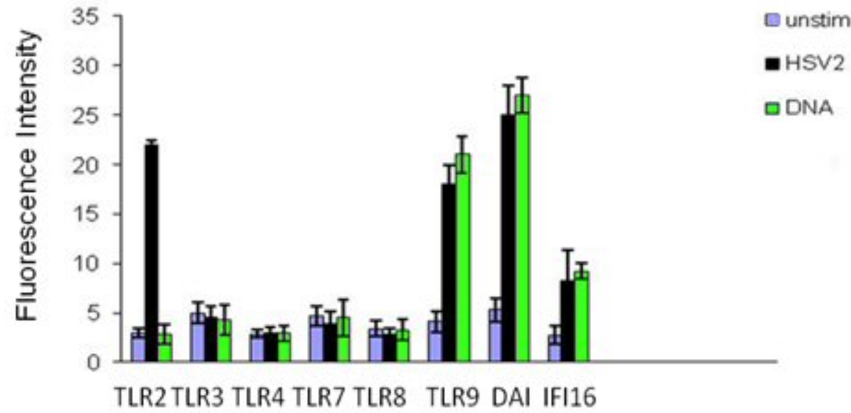
Herpes simplex viruses can trigger a strong innate response that involves a synergy of several pattern recognition receptors. Studies have shown that TLR2 recognises HSV glycoproteins and initiates a signalling cascade which leads to NF-κB activation and

cytokine secretion (Leoni et al., 2012). In addition DNA sensors TLR9 as well as DAI and IFI16 have been shown to play a key role in recognizing genomic DNA from herpesviruses and trigger IFN type I production (Fortin et al., 1999;Krug et al., 2004b;Lund et al., 2003;Unterholzner et al., 2010). However it has been shown that innate immune responses to viral infections can be tailor made depending on specific tissues or subsets of cells where TLR9 can be redundant as well as DAI (Ishii et al., 2008).

To determine which PRRs contribute to HSV2 recognition in vaginal cells, HSV2 as well as purified DNA from HSV2 (20 mg) was used to stimulate vaginal cells at different time points and the PRR expersion levels were investigated by indirect immunofluorscence and flow cytometry (*Figure* 4.1A). Isotype controls were also performed, which showed values similar to unstimulated samples (*Figure* 4.1B).

From the data obtained there was TLR2 upregulation when cells were infected with HSV2. There was also an upregulation in DAI and TLR9 in both HSV2 and HSV2 DNA stimulation suggesting that DAI and TLR9 play a role in HSV2 DNA recognition in the first hours of the infection probably when the DNA is released from the endosomes in the cytoplasm. A small upregulation in IFI16 DNA sensor is also detected in both HSV2 and DNA stimulation (*Figure* 4.1).

A



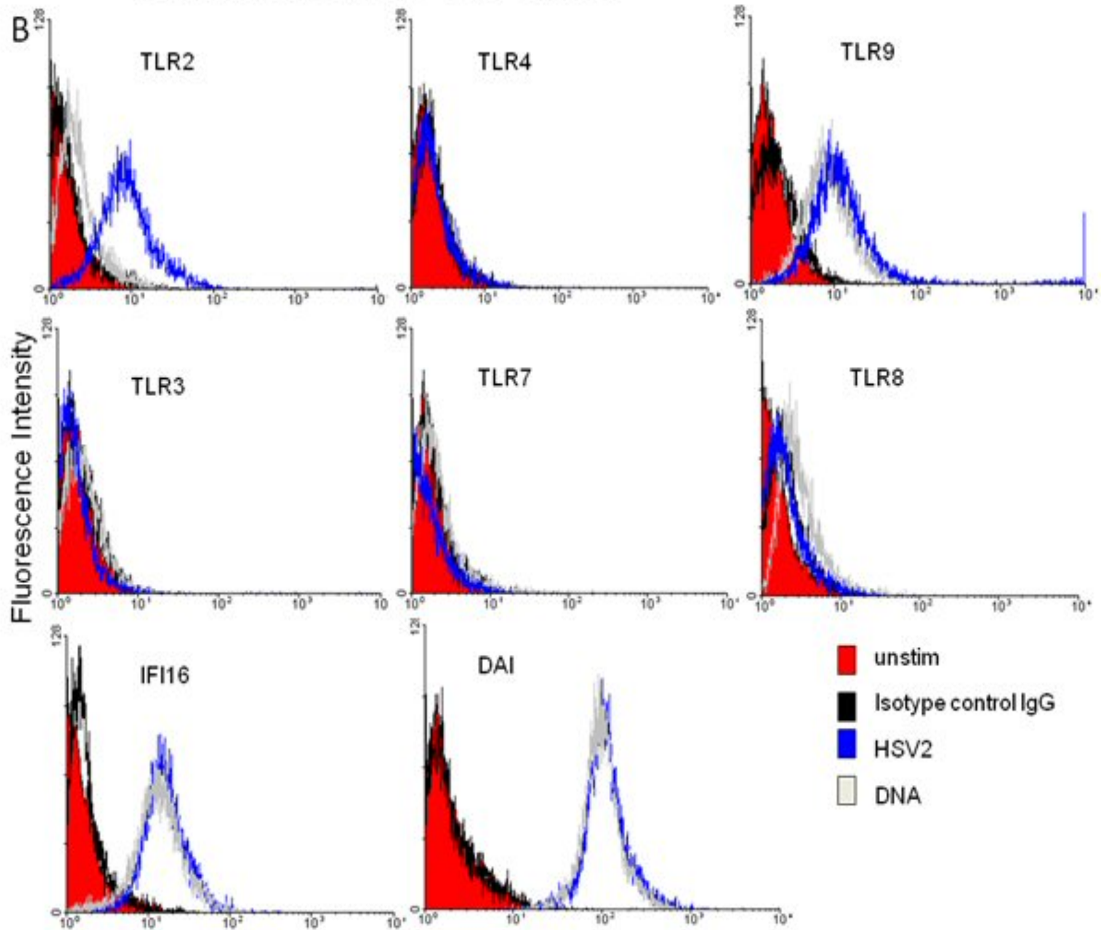


Figure 4.1: PRRs expression levels in vaginal cells during HSV2 infection
Vaginal cells were infected with 5 MOI of HSV2 or transfected with 20 mg HSV2 DNA for different time points. The 2 hr time point is depicted in this graph. The cells were stimulated, harvested, fixed and permeabilised, followed by incubation with TLR2, TLR4, TLR3, TLR7, TLR8, TLR9, DAI and IFI16 primary antibody and the appropriate secondary antibody conjugated to FITC. PRR expression levels are depicted as bar charts (A). Flow cytometric diagrams of individual PRR are also depicted (B). Fluorescence intensity was detected using a FACSCalibur (Becton Dickinson) counting 10,000 cells not gated. Isotype controls were performed, with values similar to unstimulated samples. The data represents the mean ± SD of three independent experiments.

4.2.2 TLR2, TLR9 and DAI Play a Role in The Innate Immune Response of Vaginal Cells

In order to determine the significance of TLR2 and DNA sensors in the host response to HSV2, we silenced TLR2, TLR9, DAI and IFI16 and then we stimulated with either HSV2 or HSV2 DNA (*Figure* 4.2A) and cytokine production was measured. TLR4 was silenced as a control since it is not involved in HSV2 recognition. Our data showed that IL-6 secretion was severely impaired when TLR2 was knocked down and cells were stimulated with HSV2. TLR9 silencing also affected IL-6 secretion when cells were stimulated with HSV2 or viral DNA. Furthermore when both TLR9 and TLR2 were silenced there was a significant inhibition in IL-6 secretion when cells were stimulated either with HSV2 or DNA (*Figure* 4.2A). As expected, DAI and IFI16 silencing had no effect on IL-6 secretion since they trigger IFNα/β production.

Knocking down DAI and TLR9 reduced IFN-β secretion, however it was only minimally affected when IFI16 was knocked down. TLR2 silencing had no effect on IFN-β secretion. When TLR9 and DAI were simultaneously silenced there was a significant reduction indicating that they are the main sensors, which activate IFN-β production (*Figure* 4.2B).

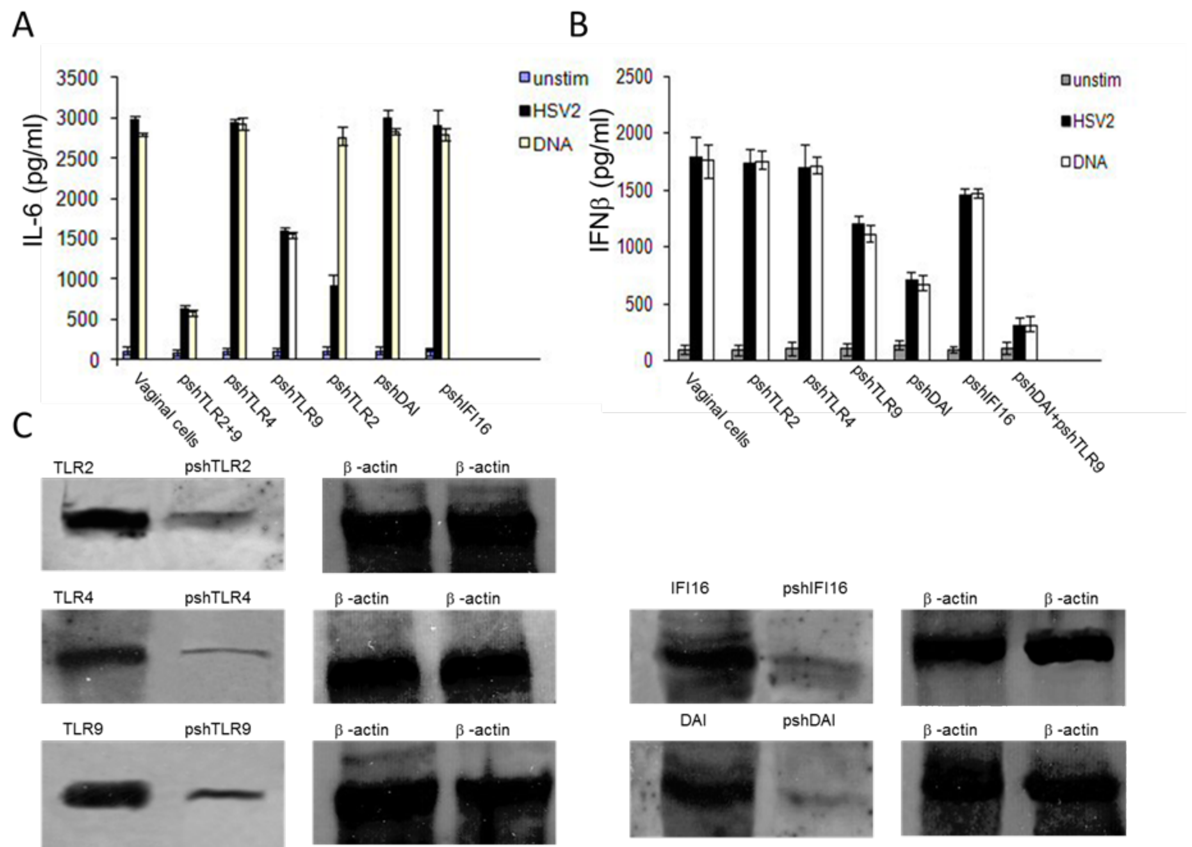


Figure 4.2: TLR2, TLR9 and DAI trigger HSV2 recognition
Vaginal cells were infected with 5 MOI of HSV2 or transfected with 20 mg HSV2 DNA. Supernatants were collected at 12 hr post infection and analysed for IL-6 (A) and IFN-β production (B) using the CBA bead array system on a FACSCalibur (Becton Dickinson). TLR2, TLR4, TLR9, DAI and IFI16 expression was knocked down by shRNA (the expression levels of these receptors as well as b-actin before and after pshRNA are depicted) (C) and the cells were again infected with 5 MOI of HSV2 or transfected with 20mg HSV2 DNA. Supernatant was collected at 12 hr post infection and analysed for IL-6 or IFN-β using the CBA system. The data represents the mean ± SD of three independent experiments.

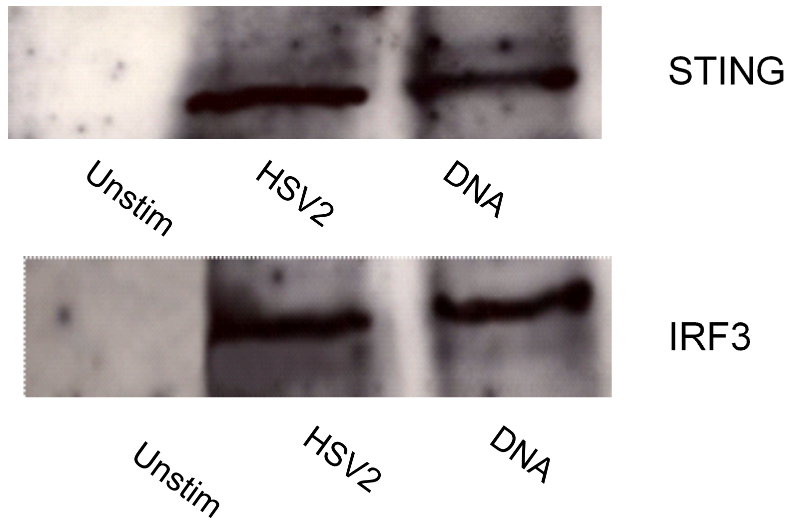
4.2.3 Activation of Signal Transduction in Response to Intracellular DNA Sensing

Cytosolic DNA recognition leads to the activation of TANK-binding kinase 1 (TBK1) and IRF3, and the production of type I IFNs and proinflammatory cytokines (DeFilippis et al., 2010a;Schröder et al., 2008). TBK1 interacts with the endoplasmic reticulum (ER) adaptor molecule STING (also known as TMEM173) (Ishikawa and Barber, 2008). STING is essential for activation of the signalling pathway upstream of TBK1 following HSV1 infection and has been shown to associate with IFI16 and to

relay signals downstream of DAI (DeFilippis et al., 2010a;Ishikawa et al., 2009;Unterholzner et al., 2010).

In order to determine the activation of signal transduction in response to intracellular HSV2 DNA sensing, vaginal cells were infected with HSV2 and the presence of STING as well as IRF3 was determined via western blotting (*Figure* 4.3A). The data showed an increased expression of STING and IRF3 in vaginal infected cells. To demonstrate the importance of STING in HSV2 innate immune responses, STING expression was knocked down by pshRNA in vaginal cells and then cells were infected with HSV2, HSV2 DNA as well as Cyclic diadenylate monophosphate (c-di-AMP), which is a second messenger molecule produced in bacteria but not in mammals. STING has been shown to directly sense this cyclic dinucleotide and induce type I interferon production, therefore cyclic dinucleotides were used as a positive control (Burdette et al., 2011). The data showed a significant decrease in IFN-β production in the absence of STING, confirming the importance of STING mediation in HSV2 DNA detection and host response. Overall these data verify that STING is essential for activation of the signalling pathway in response to HSV2 DNA and IFN-β production (*Figure* 4.3B).

A



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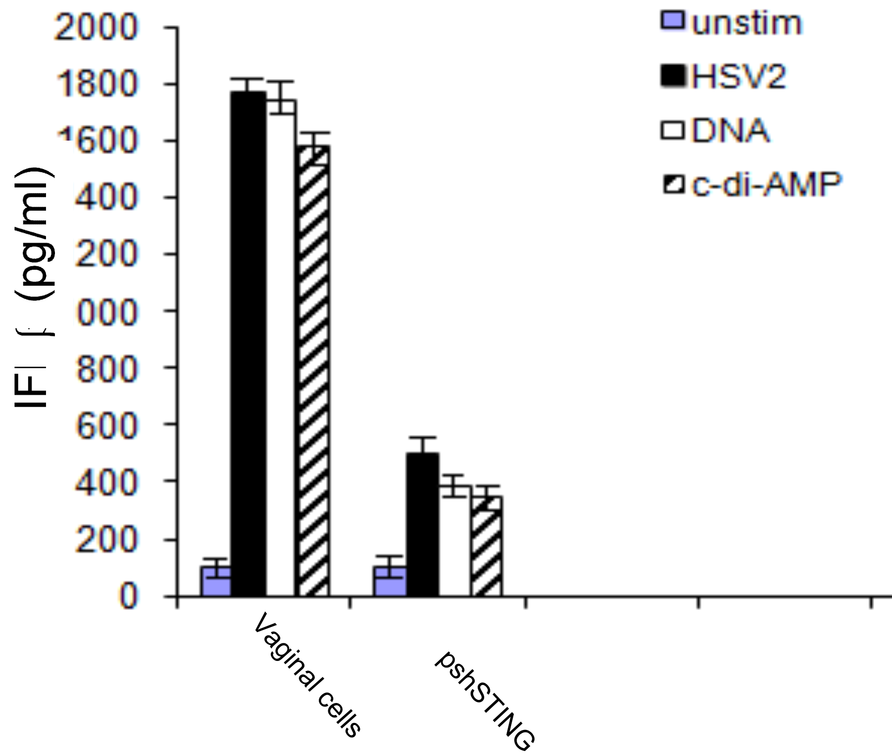


Figure 4.3: STING involvement in HSV2 DNA recognition
Vaginal cells were infected with 5 MOI of HSV2 for 4 hrs or transfected with 20mg HSV2 DNA. Cells extracts from unstimulated and also stimulated vaginal cells were analysed for the presence of IRF3 and STING by western blotting, using rabbit primary antibody specific for IRF3 or rabbit primary antibody specific for STING followed by swine anti-rabbit Ig-HRP (A). To determine the effect of STING in IFN-β production, STING expression was knocked down by pshRNA and the cells were again either infected with HSV2 or transfected with HSV2 DNA or c-di-AMP as a control and examined for IFN-β secretion (B). The data represents the mean ± SD of three independent experiments.

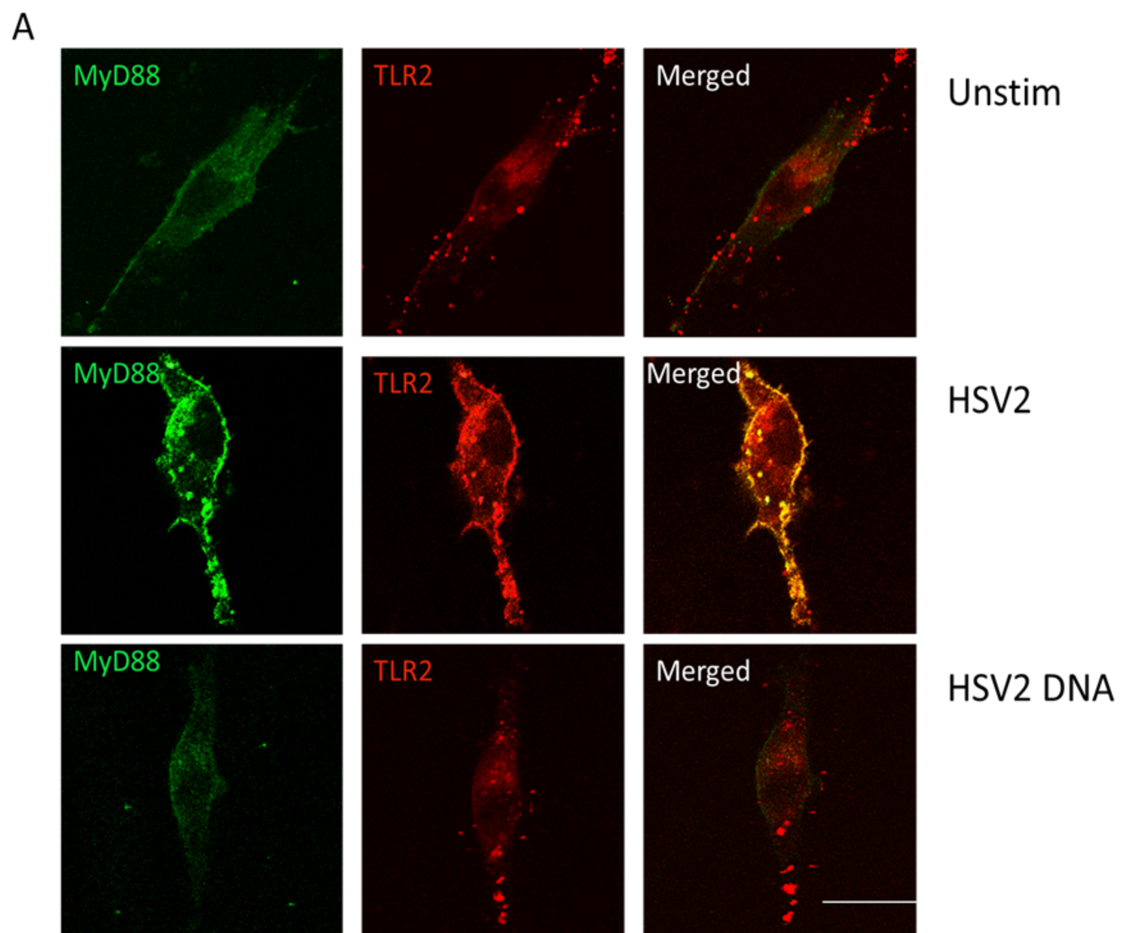
4.3 PRR Trafficking in Response to HSV2 Infection

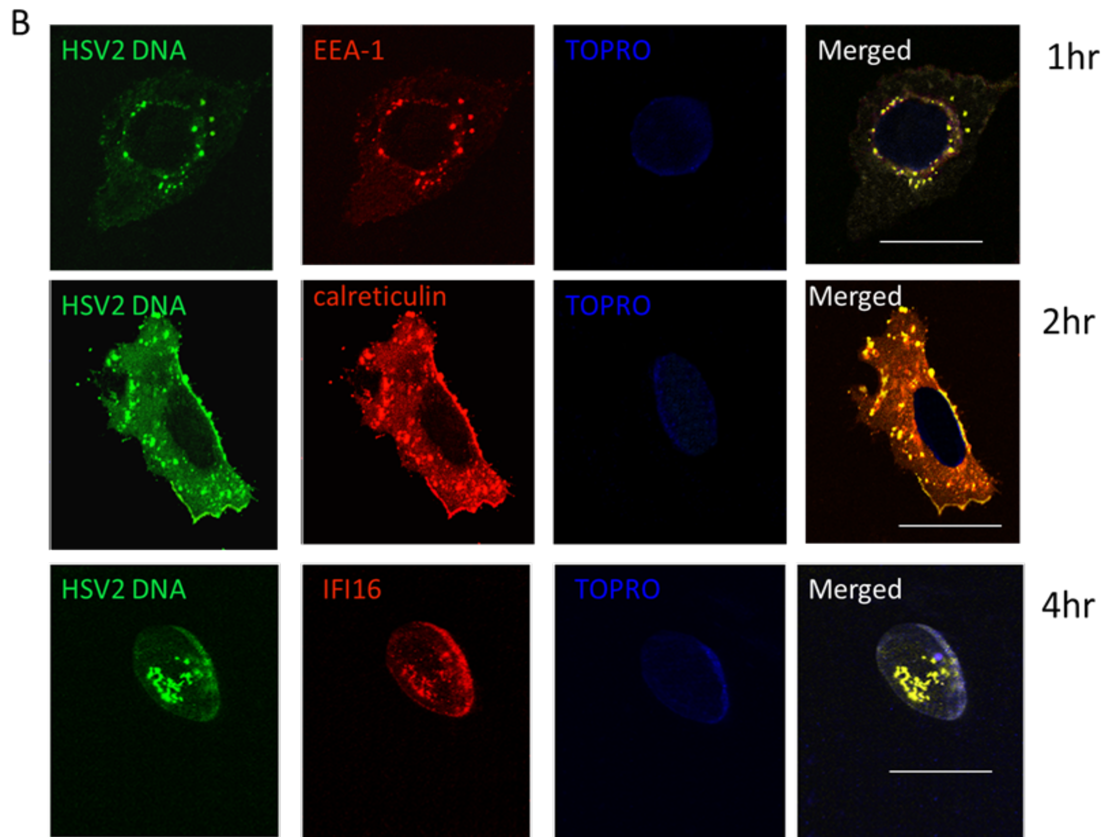
To verify the involvement of TLR2 in HSV2 infection confocal microscopy was used. Co-localisation of HSV2 with MyD88, a signalling adaptor essential for TLR signalling was observed when cells were stimulated with HSV2, whereas there was no TLR2 and MyD88 co-localisation in unstimulated cells or when cells were stimulated with DNA confirming TLR2 signalling in the presence of virus particles (*Figure* 4.4A). In order to detect the mechanism of HSV2 detection by DNA sensors, purified HSV2 DNA conjugated to Alexa-488 was transfected into vaginal cells. The subcellular localization of HSV2 DNA over time was observed using confocal microscopy. Endosomal compartments were labelled with EEA-1 specific antibody followed by goat-anti mouse Ig-Alexa546. Calreticulin specific antibody followed by goat-anti mouse Ig-Alexa546 was used to label the endoplasmic reticulum (ER), whereas TOPRO nuclear stain was used to stain the cell nucleus. The viral DNA was showed to co-localise within the endosomes within 1h. This was confirmed by the statistical analysis, which showed an R(obs) of 0.915, indicating that there was significant correlation between HSV2 DNA and the endosomes. The HSV2 DNA subsequently translocated to the cytoplasm (statistical analysis revealed an R(obs) of 0.858, which suggests strong co-localisation between HSV2 DNA and calreticulin). Finally it reached the cell nucleus within 4 hrs. Data shows strong co-localisation between HSV2 DNA and IFI16 (*Figure* 4.4B).

To gain functional insights into the viral DNA specificity we used drugs that disrupted endosomal pathways, such as monensin, an ionophore that neutralizes acidic intracellular compartments and leads to defects in vesicular budding as well as Brefeldin A (BFA), which blocks transport between the rough endoplasmic reticulum (ER) and Golgi complex (*Figure* 4.4C). The data showed that disruption of the endosomes as well as the ER and Golgi complex disrupted the viral DNA

colocalization and resulted in re-distribution of the viral DNA in the cell cytoplasm, thus verifying that its intracellular trafficking and localisation into intracellular organelles is specific and that there was no significant correlation between HSV2 DNA and the endosomes nor the ER.

PRR distribution was also investigated in the presence of DNA, co-localisation of DNA with TLR9 in the endosomes and MyD88 recruitment was initially observed followed by colocalization with STING and DAI in the ER. IFI16 was shown to be located in the cell nucleus in vaginal cells. Within 3hrs HSV2 DNA accumulated in the cell nucleus and localised with IFI16 (*Figure* 4.4D). Therefore revealing that HSV2 DNA can be recognised by different DNA sensors located in different cell compartments, which contribute to a synergic innate immune response to this virus.




B
HSV2 DNA
EEA-1
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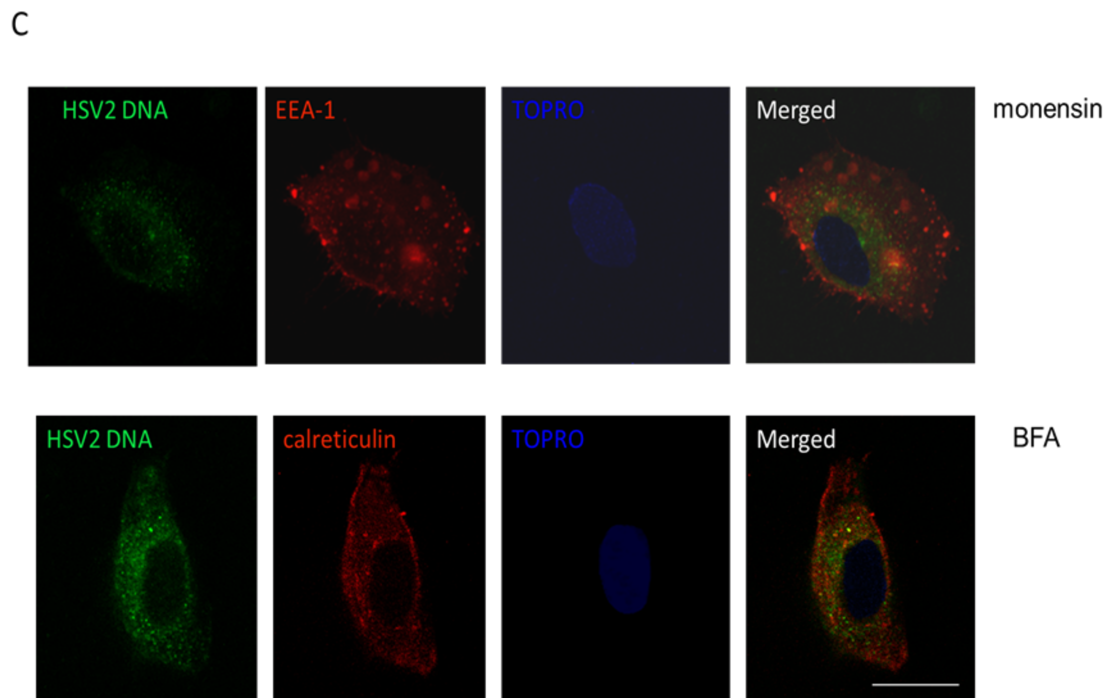
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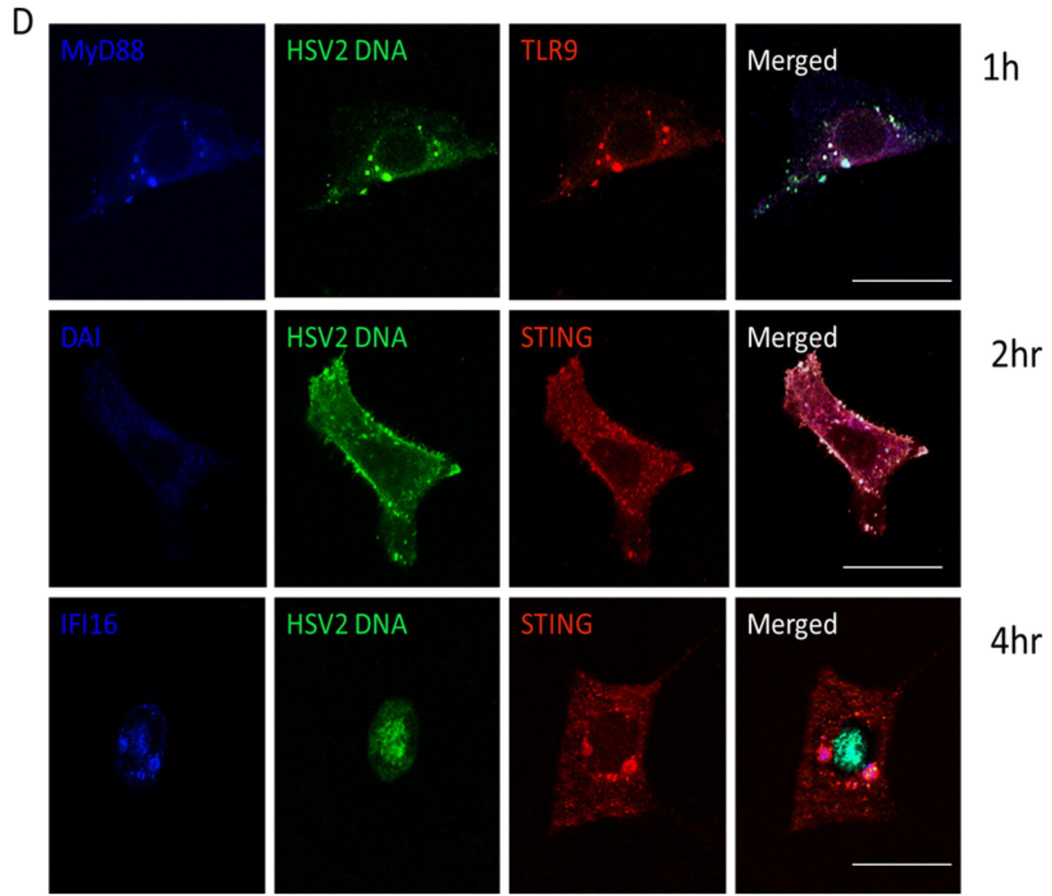

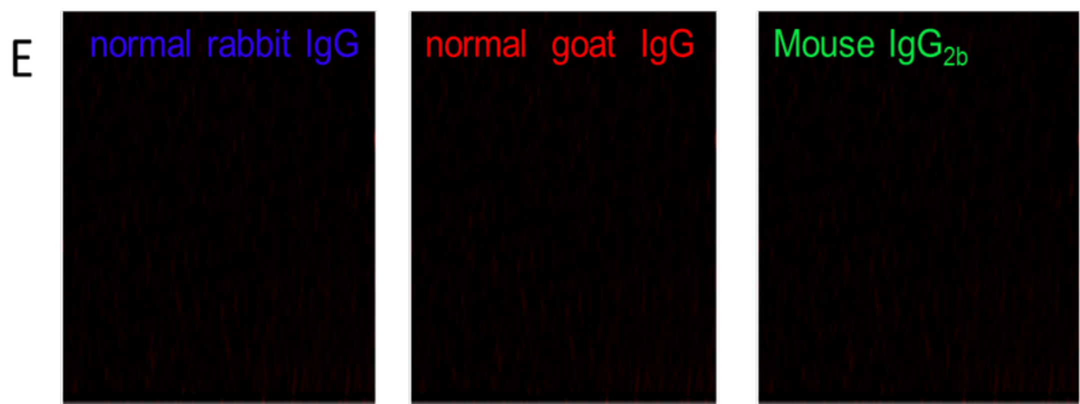

Figure 4.4: PRR trafficking in response to HSV2 infection Vaginal cells were either unstimulated or stimulated with HSV2 (50 PFU) or transfected with HSV2 DNA. The cells were fixed 1 h post stimulation and labelled with antibody specific for TLR2 followed by Alexa 546-Fab goat specific Ig and MyD88 specific antibody, followed by Alexa 488-Fab rabbit specific Ig (A).
Vaginal cells were transfected with HSV2 DNA-Alexa 488 for 1 h, 2 h, and 4 h post transfection cells were fixed and permeabilised using PBS/0.02% BSA/0.02% Saponin. The endosomes were stained with an anti EEA-1 primary antibody (EEA-1 is an early endosomal marker). The Endoplasmic reticulum (ER) was stained with an antibody specific for calreticulin conjugated to Alexa 546. IFI16 was labelled with an anti IFI16 specific antibody followed by Alexa 546-Fab goat specific Ig. TOPRO nuclear stain was also were used for nuclear visualisation (B).

In order to gain functional insights into the viral DNA specificity, cells were pre-treated with monensin, (10 µM) or Brefeldin A (BFA) (10 µg/ml) and stimulated with DNA-Alexa 488 in the presence of these drugs. The endosomes and ER were labelled as described above (C).

In addition vaginal cells were transfected with HSV2 DNA-Alexa 488 for 1 h, 2 h, and 4 h post transfection cells were fixed and permeabilised using PBS/0.02% BSA/0.02% Saponin. TLR9, STING, were labelled with specific primary antibodies followed by the Alexa 546-Fab goat specific Ig. IFI16, DAI and MyD88 were labelled with specific primary antibodies followed by the Alexa 633-Fab rabbit specific Ig (D). Isotype controls normal rabbit IgG followed by Alexa 633-Fab rabbit specific Ig, normal rabbit IgG followed by Alexa 546-Fab goat specific Ig and mouse IgG_2 followed by Alexa 488-Fab mouse specific Ig are also shown (E). Cells were imaged using a Zeiss 510 confocal microscope. Bars 10 mm. The data presented are representative images from four independent experiments.

4.4 Immunomodulation of HSV2 Infection Using PRR Agonists

Herpes simplex virus (HSV) infections are efficiently treated with antiviral drugs such as acyclovir (ACV), however resistance has been reported mainly among immunocompromised patients therefore there is an urgent need of alternative potential therapies. New studies such as Krepstakies et al, using synthetic peptide inhibitors as alternative therapies have shown that they can effectively block attachment and entry of herpes viruses, hepatitis B virus (HBV), and hepatitis C virus (HCV), and human immunodeficiency virus (HIV) (Krepstakies et al., 2012).

TLR agonists could also show some promise in reducing HSV2 infection in the genital tract. Thus TLR2 agonists Pam_2CSK_4 synthetic diacylated lipoprotein, FSL-1 synthetic diacylated lipoprotein and Pam_3CSK_4 synthetic triacylated lipoprotein were used, as well as CpG DNA in order to activate TLR9. The use of cyclic dinucleotides, c-di-AMP and c-di-GMP were also used to activate STING.

Therefore we screened these agonists to determine whether they could specifically inhibit HSV2 infection. Two quantitative methods were utilized to determine the effect of these agonists on HSV2 replication. Flow cytometry was initially used to determine HSV2 replication. Vaginal cells were seeded at a density of 1.5×10^6/well in a six well plate and incubated overnight until cells were confluent. Cells were cultured in media containing different concentration of Pam_2CSK_4, Pam_3CSK_4, FSL-1, CpG DNA, c-di-GMP or c-di-AMP for 60 min and subsequently challenged with HSV2. After virus internalisation for 1hr at 37 C in 5% CO_2 incubator, free virus was removed and

replaced with fresh medium and cells were incubated for 18 h. HSV2 replication was determined by flow cytometry staining using anti-HSV2 ICP5 antibody (*Figure* 4.5A). Plaque assay was also performed in a 6-well plate format. Vaginal cells were seeded at density of 1.5 $\times 10^6$/well and incubated overnight until cells were 100% confluent. Cells were similarly treated with different agonists. Control cells were not treated. After treatment, cells were challenged with HSV2 at 50 pfu/well.

After adsorption for 1 h at 37 ◦C in 5% CO_2 humidified incubator, free virus was removed and replaced with a mixture of complete medium and 1% agarose. Cells were incubated for 72 hours and then stained with 1% crystal violet and plaques were counted. Results were shown as number of plaques (*Figure* 4.5B). Our data showed that all agonists used reduced viral replication. However the most significant inhibition was when Pam_3CSK_4 was used as well as CpGDNA and cyclic dinucleotides thus indicating that PRR ligands could possibly have a therapeutic use in HSV2 genital infection.

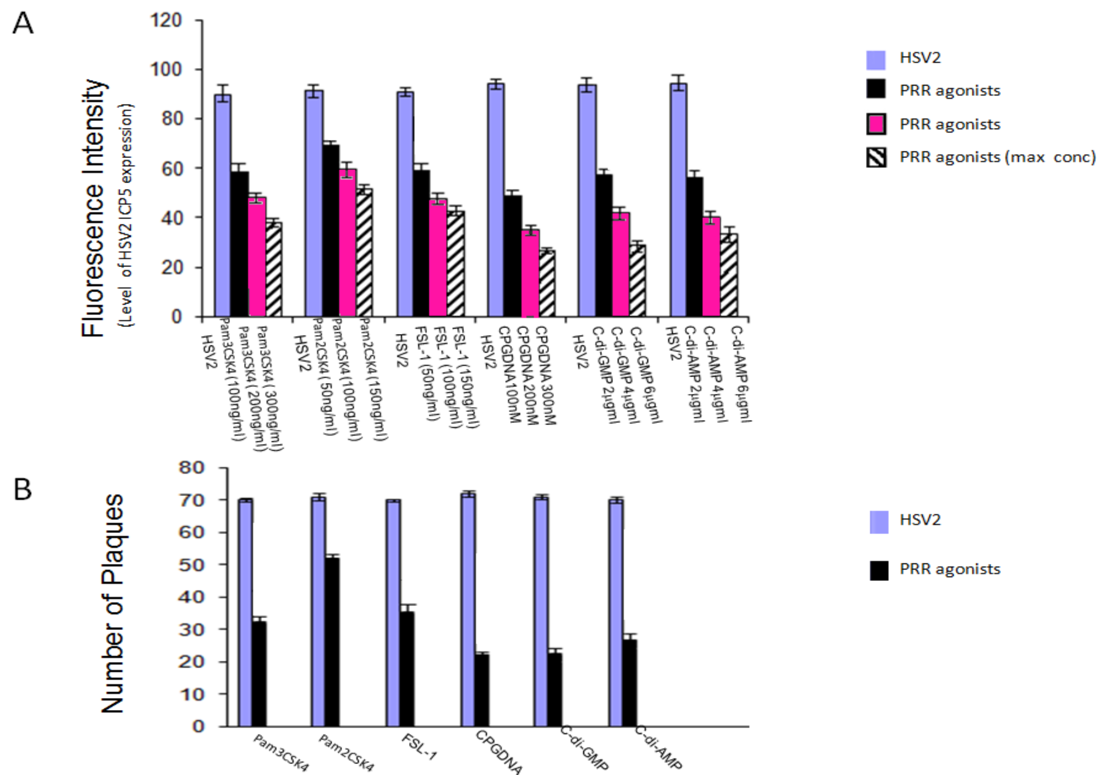

Figure 4.5: TLR agonists block HSV2 replication.
Two quantitative methods were utilized to determine the effects of different PRR agonists on HSV2 replication. Pa_3CSK_4 was used at 100ng/ml to 300 ng/ml. Pa_2CSK_4 was used at 50ng/ml to 150ng/ml, FSL-1 was used at 50ng/ml to 150ng/ml. CPG DNA was used at 100nM to 300nM and c-di-GMP as well as c-di-AMP at 2mg/ml to 6mg/ml. Vaginal cells were seeded at density of 1.5×10^6/well in a six well plate and incubated overnight until cells were confluent. Cells were cultured in media containing different concentrations of agonists for 60 min. Control cells were cultured in just media. Then the cells were challenged with HSV2 at [5 moi] and were incubated for 18 h. HSV2 replication was determined by flow cytometry staining using anti- anti-HSV2 ICP5 antibody and the appropriate secondary conjugated to FITC. Isotype controls were also used. Fluorescence was detected using a FACSCalibur counting 10, 000 cells not gated (A).
In addition, the replication of HSV2 was also determined using the classical plaque assay in vaginal cells as described in the results section. The data were counted as number of plaques (B). The data represents the mean ± SD of four independent experiments.

4.5 Conclusions

HSV-2 infects the genital tract of men and women, resulting in self-limiting lesions that can serve as portals for acquisition of secondary infection. In rare cases, primary HSV infection can also lead to encephalitis, hepatitis, and ocular keratitis and be

transmitted to newborns with considerable morbidity and mortality (Beauman, 2005;Gupta et al., 2007).

There is currently no vaccine available against HSV2. HSV2 infections are treated with antiviral drugs such as acyclovir (ACV), however, resistance has been reported, mainly among immunocompromised patients. Strains resistant to ACV are almost always cross-resistant to other TK-dependent drugs such as penciclovir and famciclovir (Morfin and Thouvenot, 2003). Therefore there is a developing need for alternative therapeutic strategies to the traditional HSV antiviral therapy in order to suppress or restrict the infection.

In this study primary human vaginal cells were used and we looked at the involvement of different PRRs in HSV2 vaginal innate immune responses. The results showed that TLR2 recognizes HSV2 and shuttles from the cell membrane to the Golgi where it co-localises with MyD88 a signalling adaptor essential for TLR signalling and cytokine production. In addition the DNA sensors TLR9 as well as DAI and IFI16 also recognise the viral DNA and trigger IFN α/β secretion. TLR9 moves from early endosomes and re-distributes in the endoplasmic reticulum (ER) upon HSV2 infection. While STING is located in the cytoplasma and ER in unstimulated vaginal cells and upon HSV2 DNA stimulation it co-localises with ER translocon components TRAP and seC61β (Ishikawa and Barber, 2008). In the experiments, IFI16 was shown to be located in the cytoplasm and cell nucleus in vaginal cells. Upon HSV2 DNA stimulation IFI16 accumulates more in the cell nucleus. Therefore it seems that different PRRs are strategically placed in different cell locations to detect virus invasion and replication in order to activate cytokine secretion and protect the host.

Thus it was investigated whether agonists/ligands for these specific PRRs could provide protection against HSV2 infection. Previous studies using TLR agonists have shown promise in reducing infection in mice (Gill et al., 2006;Rose et al., 2009).

In this study experiments were performed in human vaginal epithelial cells as a model of HSV2 infection instead of mice or human dendritic cells like previous studies. Contrary to Ashkar et al. there was no TLR3 upregulation nor IFN inhibition when poly I:C was used possibly the effect observed in their study on dendritic cells was tissue/cell specific and since we are using vaginal epithelial cells it seems that TLR3 was not involved.

Lipopeptides were used as TLR2 agonists, CpG DNA as TLR9 agonist as well as for the first time cyclic dinucleotides for STING, a signalling adaptor essential for DAI and IFI16 activation and triggering IFN type I. The data showed a significant viral inhibition especially in the presence of TLR2/6 agonists Pam_3CSK_4 and FSL1 as well as in the presence of cyclic dinucleotides, thus indicating that a combination of different agonists especially ones that trigger different cytokines could provide a wider spectrum prophylaxis to the host.

As a result of my findings, it is obvious that the agonists/ligands are successful in diminishing the HSV2 replication in vaginal cells. Cells, which were treated with certain type of agonist/ligand, had less HSV2 ICP5 expression and the number of the plaques caused by the virus was decreased. This also means virus could infect less neighbouring number of cells. These agonists magnify the immune response in the cell by acting on the immune receptors only, thus they do not cause any harm to the host unlike current anti-viral drugs. During the treatment of primary HSV2 infections combination of these agonists/ligands will provide an efficient and less harmful theraphy for the patients.

CHAPTER 5: DNA SENSOR INVOLVEMENT IN CYTOMEGALOVIRUS RECOGNITION

5.1 Introduction

Cytomegalovirus (human herpes virus 5(HHV-5)) is from the Herpesviridae family it belongs to the beta-Herpesviridae subfamily. Beta-herpesviruses are double-stranded DNA viruses as well however they target the lymphatic system unlike alpha-herpesviruses (Hewlett, 2004). Human cytomegalovirus (HCMV) infection symptoms can be flu like symptoms, which might be not distinguishable by the infected person. Moreover muscle pain; sore throat and high temperature are other symptoms due to the HCMV. However the damage of the viral infection can be more serious, if the person has a weak immune system or if there is a congenital transmission, this can lead to the organ failure, blindness and mental disabilities in the infected person. Thus the transmission of the virus from mother to the unborn causes more severe effects (Pass, 1985).

The symptoms of the virus are very flu like symptoms thus it can be hard to identify the viral infection, however the immune system detects the virus in the body, and initiates the antiviral response. HCMV engagement of pattern recognition receptors (PRR), such as TLRs and cytosolic DNA sensors (DeFilippis et al., 2010a;Varani et al., 2007), initiates the host immune response through activation of elaborate signalling programs. The ensuing inflammatory response is further sustained and amplified through cytokines, such as IL6, IFNα/β and IL-1β (Boehme et al., 2006;Boyle et al., 1999;Compton et al., 2003), activating signalling pathways greatly overlapping those utilized by TLRs. The central hypothesis of this study is that a HCMV is detected by specific TLRs as well as DNA sensors and triggers IFNα/β and IL-6-induced

signalling along the MyD88 to NF-κB pathway. To test this hypothesis HCMV innate immune responses was initially investigated in Hela cells. It was demonstrated that TLR2 plays a role in IL6 secretion During the viral entry TLR2 picks up the viral glycoproteins gB and/or gH on the lipid bilayer and triggers NFkB activation and IL6 secretion After the entry of the virus TLR9 and DAI recognize the genomic DNA and initiate the STING pathway to start type I interferon release.

5.2 Results

After examining the immune response against HSV2 infection, another member of the Herpesviridae family was used to compare the host's innate immune response We wanted to determine whether the same immune receptors that recognize HSV2 also play a role in HCMV innate immune recognition. Indirect immunofluorescence was use to determine receptor expression levels in infected cells or cells stimulated with genomic HCMV DNA. Thus HeLa cells were stimulated with HCMV or HCMV DNA at different time points beginning from 1h up to 32h which is a complete virus cycle since HCMV has a lengthy infectious cycle compared with other viruses. The expression levels were compared to unstimulated samples. If HCMV was detected there would be an upregulation in receptor expression levels.

5.2.1 PRR Expression Levels in Response to HCMV and viral DNA

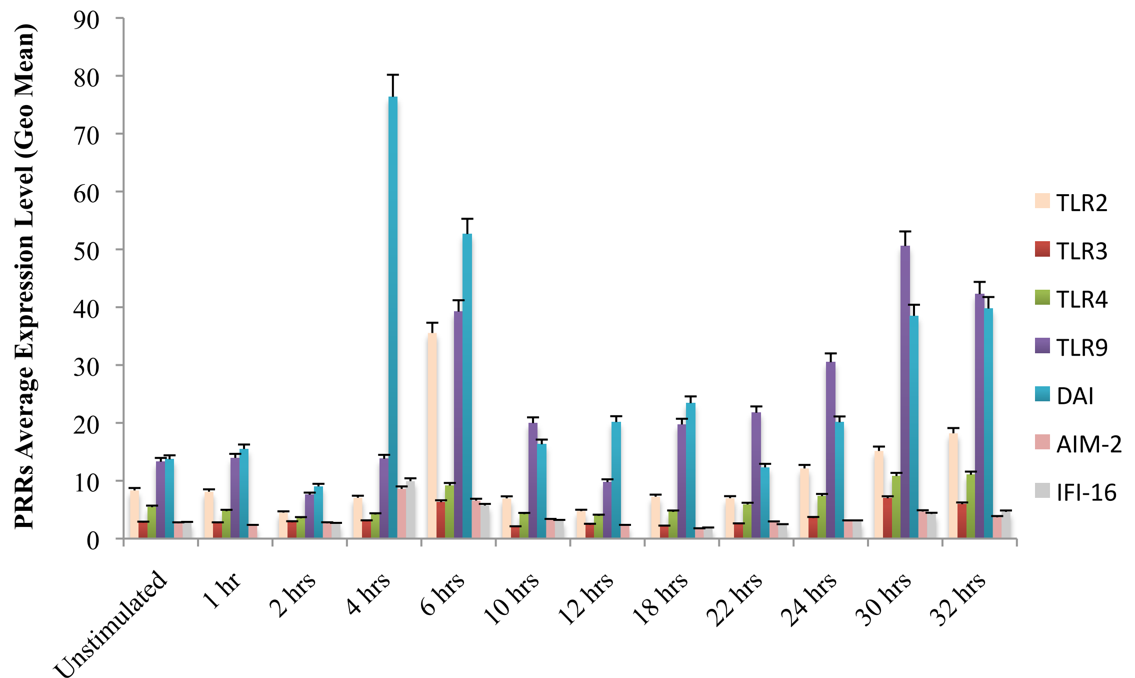

Figure 5.1: Pattern recognition receptor responses to HCMV infection. HeLa cells were stimulated with 5 MOI of HCMV at different time points and then PRR expression was determined by using a specific primary antibody for each receptor of interest followed by the appropriate secondary conjugated to FITC. The expression levels were represented by the mean fluorescence intensity determined by flow cytometry. The data represents the mean ± SD of three independent experiments.

The HeLa cells were stimulated with HCMV and it was observed that there was a gradual increase in the expression level of TLR2 where it reaches to the maximum at the 6th hour. This increase is probably due to the recognition of the glycoproteins on the lipid bilayer of HCMV. This level drops off to the minimum at the 12th hour, which can be during the latent phase of the virus when the replication cycle is shut. In addition to the upregulation of TLR2, there is an upregulation in the expression levels of DAI and TLR9 probably because they detect the HCMV genomic DNA, which is consistent with other studies showing the involvement of these receptors in viral DNA

recognition (DeFilippis et al., 2010a;Varani et al., 2007). DAI expression increases from the 4th hour until 32 hours where we have a complete virus cycle. TLR9 expression is also upregulated. However as a result of HCMV stimulation we did not see any increase in the AIM-2 inflammasome expression as it was reported in other studies (Rathinam et al., 2010).

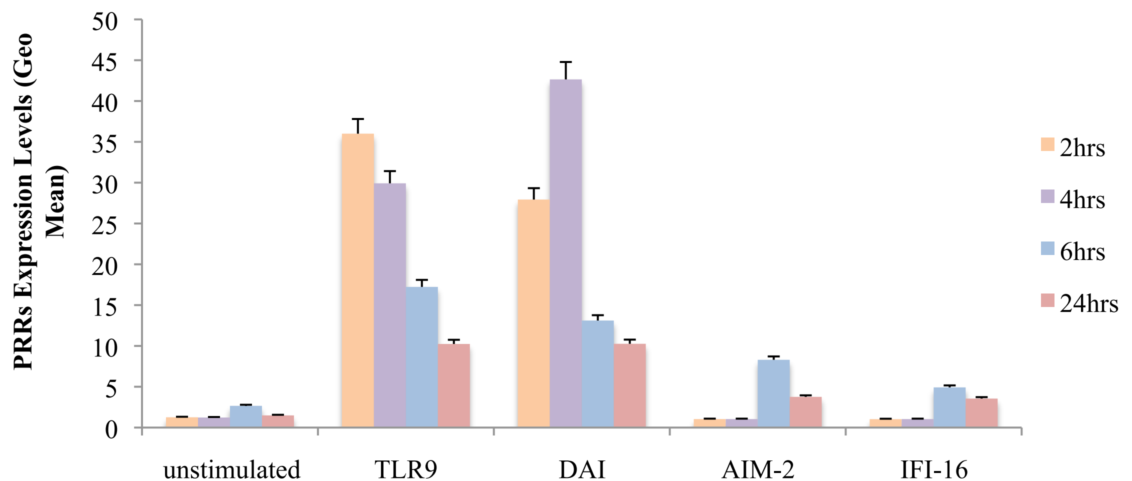

Figure 5.2: Pattern recognition receptor responses to HCMV DNA stimulation. HeLa cells were stimulated with 20 μg/ml of purified DNA at different time points and then PRR expression was determined by using a specific primary antibody for each receptor of interest followed by the appropriate secondary conjugated to FITC. The expression levels were represented by the mean fluorescence intensity determined by flow cytometry. The data represents the mean ± SD of three independent experiments.

HCMV DNA triggers TLR9 expression as well as DAI expression but to a lesser effect during the first hour of the infection, however it does not have an effect on AIM-2 and IFI-16. Possibly the viral DNA reaches to the endosomes first and then in the 2nd hour of the infection the viral DNA is accumulated in the cytoplasm leading to high expressions of DAI in HeLa cells. Furthermore the expression levels of DNA sensors AIM-2 and IFI-16 seems to become slightly increased after the 4th hour however they are not really significant when compared with TLR9 and DAI.

5.2.2 Signalling cascades upon Infection with HCMV and viral DNA

After the expression of PRRs was upregulated at specific time points during the viral entry in HeLa cells, the downstream signalling cascade molecules of those receptors were examined to see which mechanism does HCMV and HCMV dsDNA trigger in the cell.

NF-κβ production after HCMV stimulation

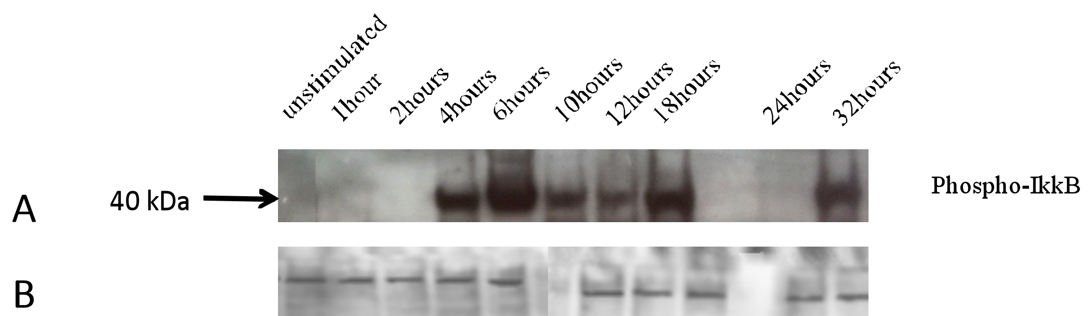

Figure 5.3: Immunostimulatory effect of HCMV on HeLa cells. HeLa cells were left unstimulated or were stimulated with 5 MOI of HCMV at different time points. The cell lysate obtained from each stimulation, was used for SDS-PAGE gel electrophoresis and western blot analysis using primary antibodies for phospho-IkkB followed by the appropriate secondary antibodies conjugated to HRP (A). Loading controls for equal loading are shown by Ponceau S staining (B). The gels are representatives of three independent experiments.

Cell lysate from different time points of HCMV infection were used to determine phosphor-IkkB, which signifies NF-κβ production. From the data above it is obvious that there is NF-κβ activation which results from TLR recognition, with highest activation between 4th -18th hour time interval of the infectious cycle.

HCMV DNA stimulation results in NF-kB activation

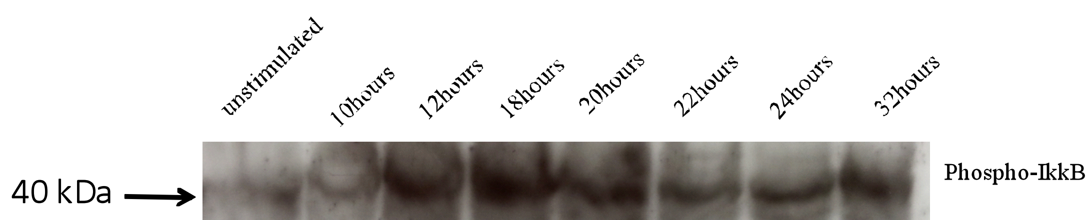

Figure 5.4: Immunostimulatory effect of HCMV DNA on HeLa cells. HeLa cells were left unstimulated or stimulated with 20µg/ml HCMV dsDNA at different time points. The cell lysate obtained from each stimulation was used for SDS-PAGE gel electrophoresis and western blot analysis using primary antibodies for phospho-Ikkβ followed by the appropriate secondary antibodies conjugated to HRP. The gels are representatives of three independent experiments.

Hela cells were stimulated with HCMV DNA and the cell lysate was examined for the presence of phosphor-IkkB. The results show that phosphor-IkkB was present thus confirming NF-kB activation and TLR involvement.

DAI synthesis after HCMV DNA stimulation

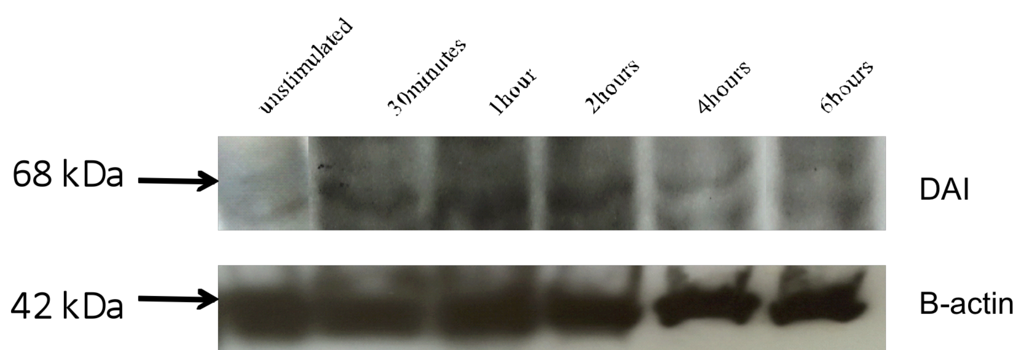

Figure 5.5: Immunostimulatory effect of HCMV DNA on HeLa cells. HeLa cells were left unstimulated or stimulated with 20µg/ml HCMV dsDNA at different time points. The cell lysate from each stimulation was used for SDS-PAGE gel electrophoresis and western blot analysis using primary antibodies DAI, followed by the appropriate secondary antibodies conjugated to HRP. Loading control for β- actin is also shown. The gels are representatives of three independent experiments.

Cells were stimulated with HCMV DNA and then DAI expression was examined. The DAI expression due to HCMV DNA was the highest during the first and the second hour of the infection. This decreases in the 4th and the 6th hour however it is not stopped completely.

5.2.3 IFN-β, IL-6 and IL-1β Production

HEK IFN-β, HEK-IL-6 and HEK-IL-1β reporter cell lines were used to determine whether HCMV or HCMV DNA could trigger cytokine secretion. Supernatants of cells which were infected with HCMV or with HCMV DNA at different times points, were incubated with the HEK-Blue cells between 18-24 hrs. After the addition of Quanti-Blue and incubation period between 30min-6hrs, samples were tested with the

spectrophotometer and the SEAP production levels were determined (*Figures* 5.6, 5.7, 5.8, 5.9, 5.10, 5.11).

HEK IFN-β cells incubated with HCMV stimulated HeLa cell supernatants (*Figure* 5.6).

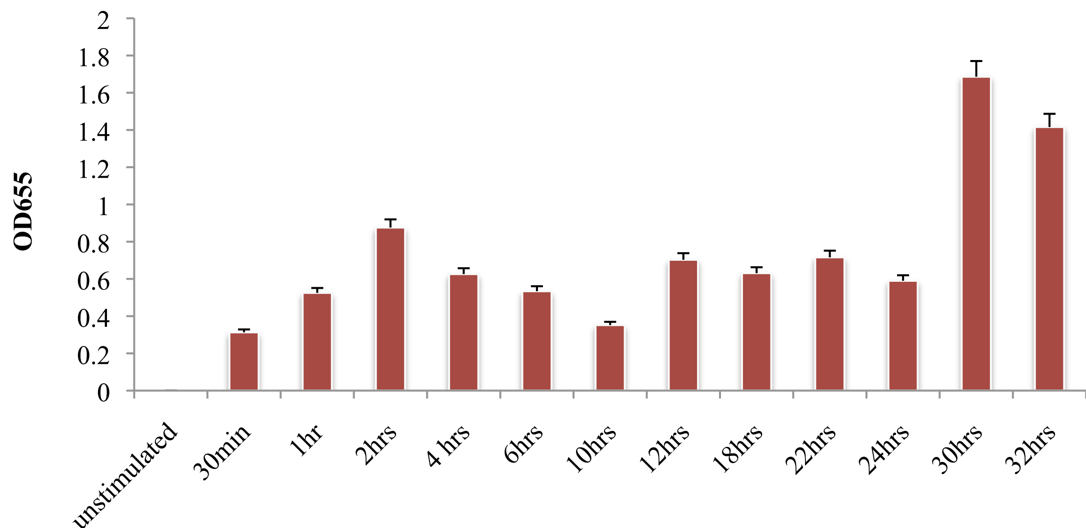

Figure 5.6: Levels of IFN-β secretion in HeLa cells. HeLa cells were stimulated with 5 MOI of HCMV, at different time points. The supernatants were harvested with HEK IFN-β cells incubated between 18-24hr and assayed for IFN-β secretion by measuring the levels of SEAP in the supernatant using the Quanti-Blue assay. The SEAP levels were determined via spectrophotometer at 655nm. The data represents the mean ± SD of three independent experiments.

Due to HCMV infection signalling cascades can trigger type I interferon production in the cell. Our results show that there is an increase in IFN-β production starting from the first 30 minutes of viral entry. This reaches to the highest in the 2nd and 30th hour. The increase is due to the increase in the synthesis of TLR9 and DAI, as they are the main PRRs which trigger the signalling pathway to initiate type I interferon production in the cell. In the 10th hour lower levels of IFN-β production might be because of the viral proteins trying to inhibit the interferon pathway to stop the antiviral response in the cell.

HEK IL-6 reporter cells incubated with supernatant from HCMV infected HeLa cells (*Figure* 5.7).

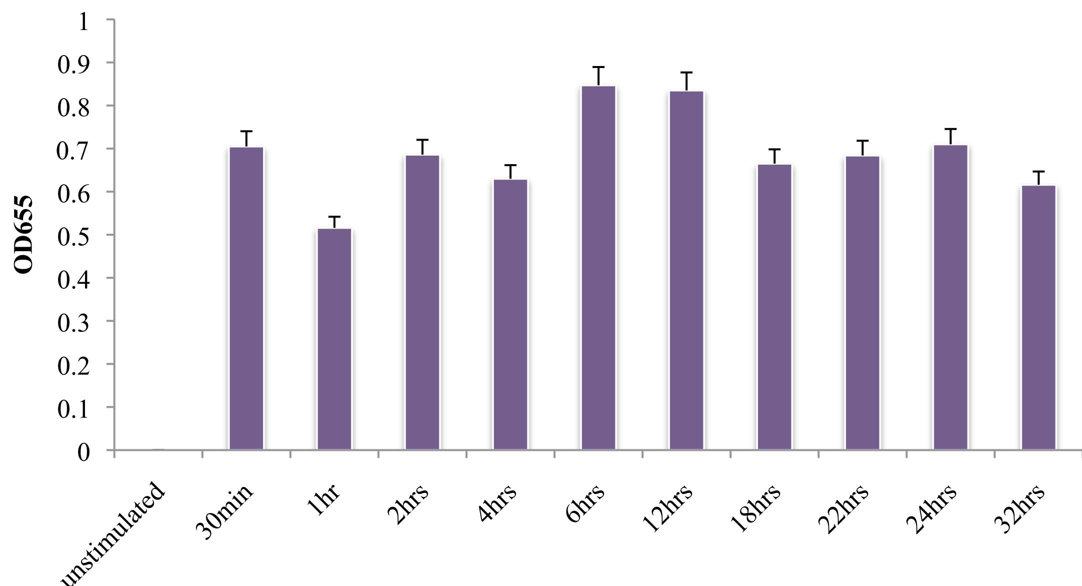

Figure 5.7: Levels of IL-6 secretion in HeLa cells. HeLa cells were stimulated with 5 MOI of HCMV, at different time points. The supernatants were harvested with HEK IL-6 cells incubated between 18-24hr and assayed for IL-6 secretion by measuring the levels of SEAP in the supernatant using the Quanti-Blue assay. The SEAP levels were determined via spectrophotometer at 655nm. The data represents the mean ± SD of three independent experiments.

The IL-6 production in the cell is mainly dependent on the TLR2 and TLR9. The expression levels of IL-6 is quite high especially in the beginning (30 minutes) which is logical since TLR2 is on the cell surface and will immediately detect the virus, followed by increases in the 6th and 12th hour, throughout these time points TLR9, TLR2 and DAI expression is the highest between the 4th- 6th hour, which possibly means that viral detection is the highest in between these time points. In addition to TLRs there is the possibility that other innate immune sensors such as DEAD box

proteins might function during the recognition of the HCMV leading to the production of IL-6.

HEK IL-1β cells incubated with HCMV stimulated HeLa cell supernatants (*Figure* 5.8).

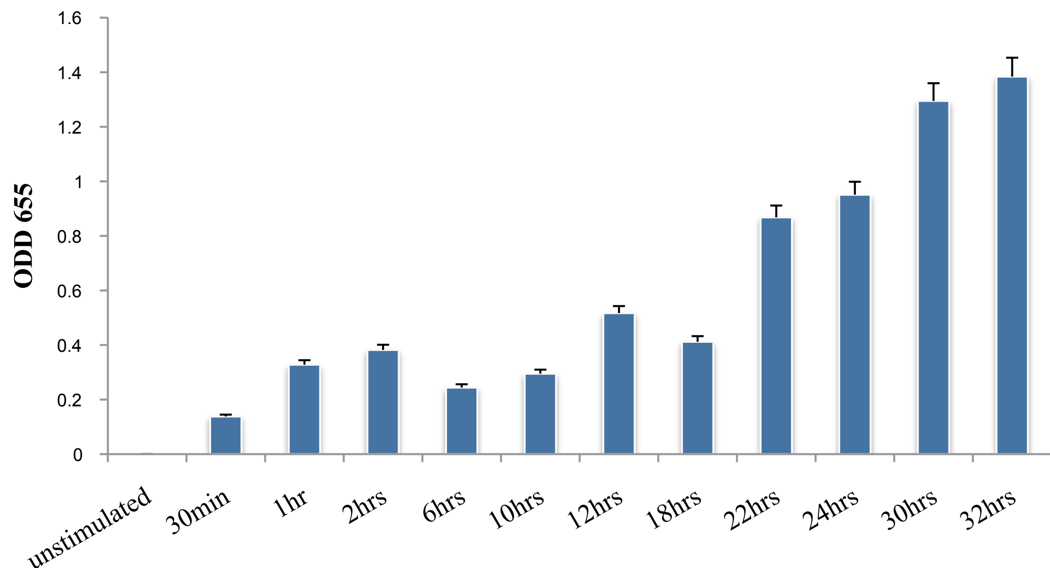

Figure 5.8: Levels of IL-1β secretion in HeLa cells. HeLa cells were stimulated with 5 MOI of HCMV, at different time points. The supernatants were harvested with HEK IL-1β cells incubated between 18-24hr and assayed for IL-1β secretion by measuring the levels of SEAP in the supernatant using the Quanti-Blue assay. The SEAP levels were determined via spectrophotometer at 655nm. The data represents the mean ± SD of three independent experiments.

The IL-1β production is triggered during HCMV infection as well. However we have not seen any AIM2 upregulation. It is possible that IL-1β production could be triggered by a different inflammasome to AIM2. The amount of IL-1β reaches the highest during the 32 hours when there is a viral accumulation in the cell cytoplasm, which is around 1.5 pg/ml.

HEK IFN-β cells incubated with purified HCMV DNA stimulated HeLa cell supernatants (*Figure* 5.9).

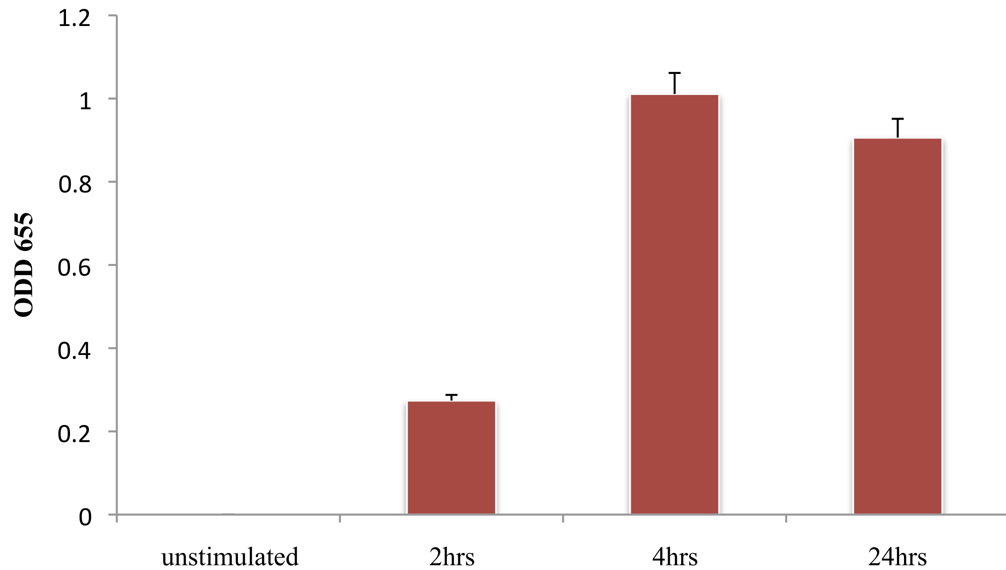

Figure 5.9: Levels of IFN-β secretion in HeLa cells. HeLa cells were stimulated with 20μg/ml HCMV DNA, at different time points. The supernatants were harvested with HEK IFN-β cells incubated between 18-24hr and assayed for IFN-β secretion by measuring the levels of SEAP in the supernatant using the Quanti-Blue assay. The SEAP levels were determined via spectrophotometer at 655nm. The data represents the mean ± SD of three independent experiments.

When we looked at IFN-β production in response to viral DNA we saw that it reaches the maximum during the 4th hour and decreases to around 0.9 pg/ml in the 24th hour of the stimulation with the viral DNA. We see almost the same pattern of the IFN-β production from the viral stimulation thus the IFN-β production is triggered mainly by the recognition of the viral DNA.

HEK IL-6 cells incubated with purified supernatants from Hela cells stimulated with HCMV DNA (*Figure* 5.10).

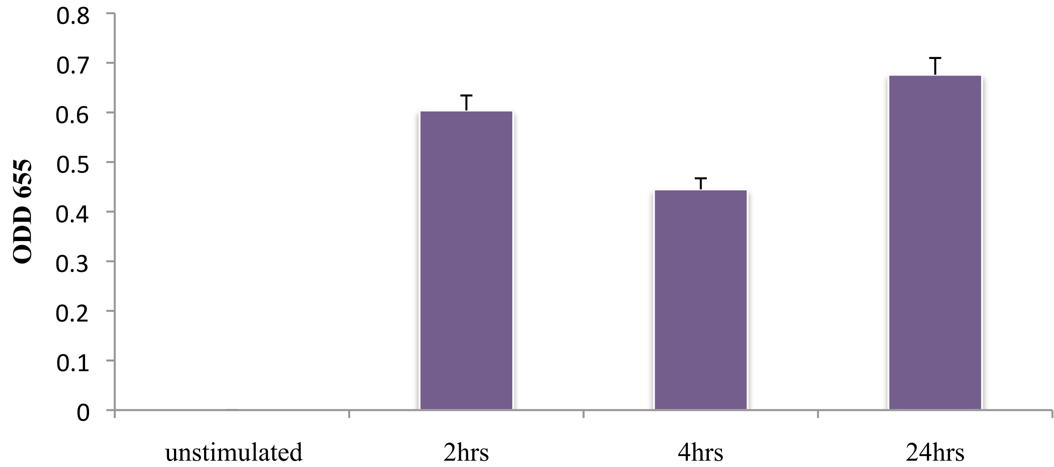

Figure 5.10: Levels of IL-6 secretion in HeLa cells. HeLa cells were stimulated with 20μg/ml HCMV DNA, at different time points. The supernatants were harvested with HEK IL-6 cells incubated between 18-24hr and assayed for IL-6 secretion by measuring the levels of SEAP in the supernatant using the Quanti-Blue assay. The SEAP levels were determined via spectrophotometer at 655nm. The data represents the mean ± SD of three independent experiments.

From the data we can see that IL-6 production is also triggered by viral DNA stimulation. This is probably due to TLR9 detection.

HEK IL-1β cells incubated with supernatants from HeLa cell supernatants stimulated with HCMV DNA (Figure 5.11).

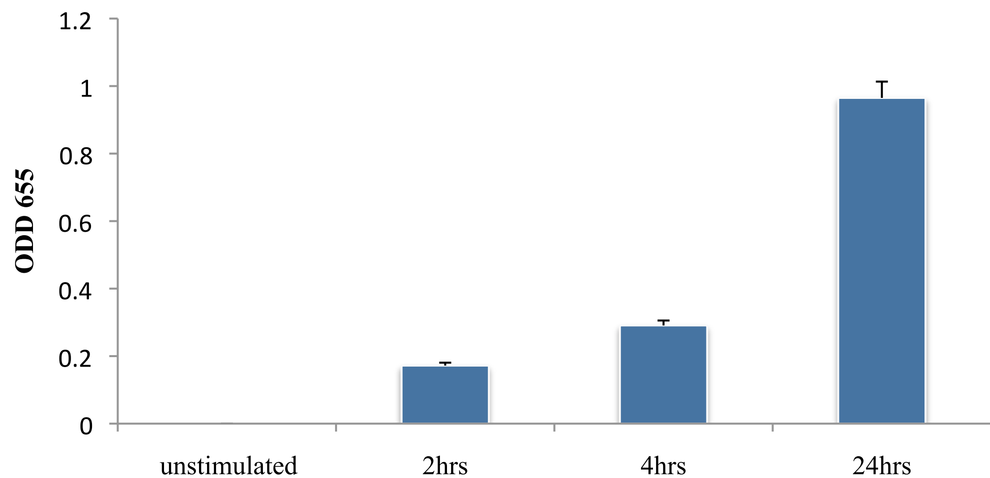

Figure 5.11: Levels of IL-1β secretion in HeLa cells. HeLa cells were stimulated with 20μg/ml HCMV DNA, at different time points. The supernatants were harvested with HEK IL-1β cells incubated between 18-24hr and assayed for IL-1β secretion by measuring the levels of SEAP in the supernatant

using the Quanti-Blue assay. The SEAP levels were determined via spectrophotometer at 655nm. The data represents the mean ± SD of three independent experiments.

Surprisingly, IL-1β production is triggered by HCMV DNA. This shows that inflammasome activation occurs in response to HCMV DNA.

5.3 Silencing of TLRs, DAI and RIG-I

5.3.1 TLRs and DAI innate immune Response to HCMV and HCMV DNA

PRRs were silenced in HeLa cells to determine the effect that they had in HCMV innate immune recognition by investigating cytokine secretion in HEK IFN-β, HEK-IL-6 and HEK-IL-1β reporter cell lines. TLR2, TLR9, DAI were knocked down in HeLa cells. The level of knockdown efficiency was 90%. Cells were stimulated with either HCMV or HCMV DNA. Supernatants of the cell cultures were obtained from different times points, and they were incubated with the HEK-Blue cells between 18-24 hrs. After the addition of Quanti-Blue and incubation period between 30min-6hrs, samples were tested with the spectrophotometer and the SEAP production levels were determined.

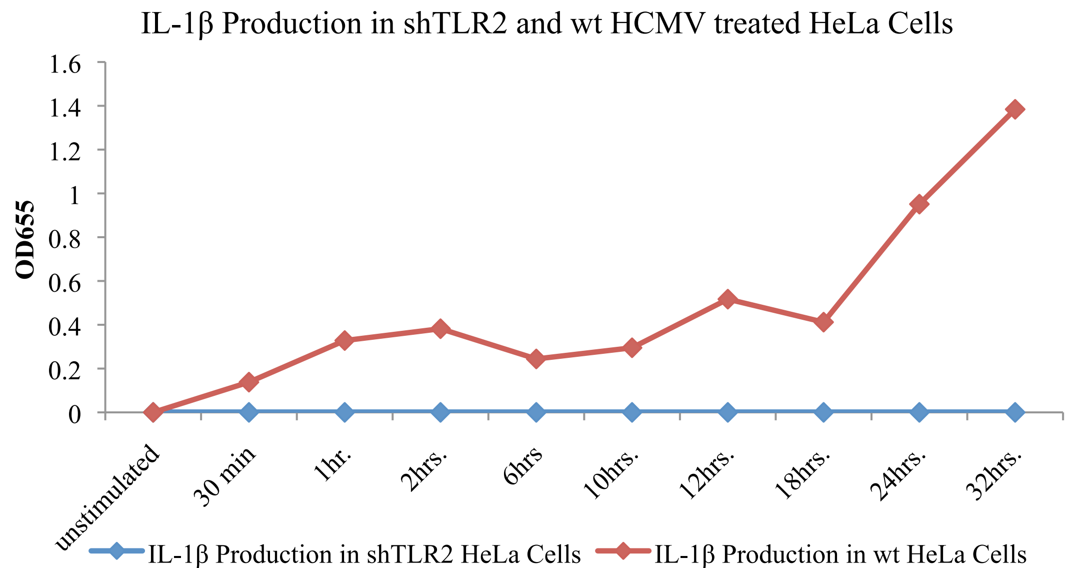

Figure 5.12: IL-1β synthesis of wt HeLa cells as well as shTLR2 HeLa cells infected with HCMV (moi 5), at different time points. The supernatants were harvested with HEK IL-1β cells incubated between 18-24hr and assayed for IL-1β secretion by measuring the levels of SEAP in the supernatant using the Quanti-Blue assay. The SEAP levels were determined via spectrophotometer at 655nm. The data represents the mean ± SD of three independent experiments.

When TLR2 expression is knocked down in Hela cells we can see a complete inhibition of IL-1β secretion thus revealing that TLR2 is essential for IL-1β secretion and provides signal 1 for inflammasome activation in HCMV infection.

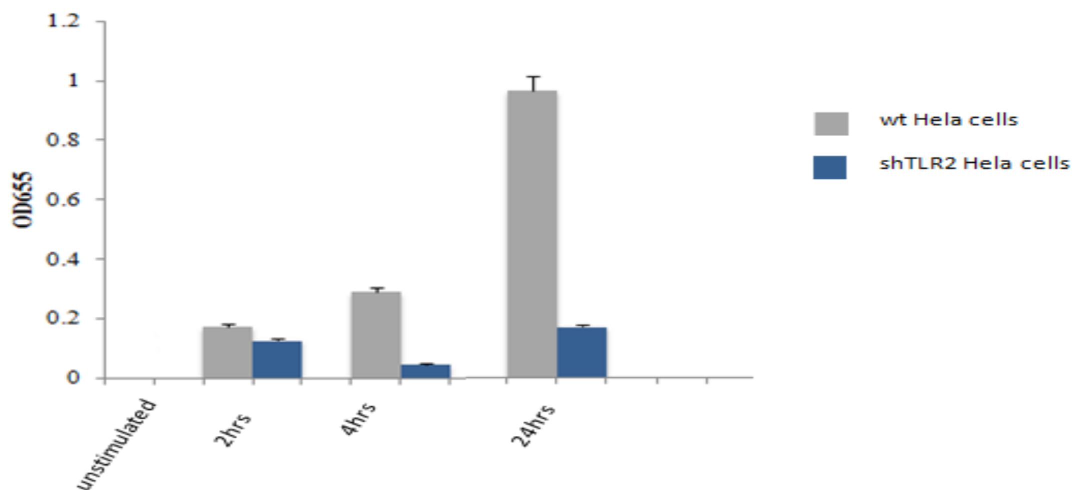

Figure 5.13: IL-1β synthesis of wt HeLa cells as well as shTLR2 HeLa cells stimulated with HCMV DNA. HeLa cells were stimulated with 20μg/ml HCMV DNA, at different time points. The

supernatants were harvested with HEK IL-1β cells incubated between 18-24hr and assayed for IL-1β secretion by measuring the levels of SEAP in the supernatant using the Quanti-Blue assay. The SEAP levels were determined via spectrophotometer at 655nm. The data represents the mean ± SD of three independent experiments.

Unexpectedly there is some IL-1β production triggered by HCMV DNA in shTLR2 HeLa cells. This is probably because in high concentrations of viral DNA other PRRs might compensate for signal 1 in the absence of TLR2 for inflammasome activation.

IL-6 synthesis in shTLR2 after HCMV stimulation in HeLa cells

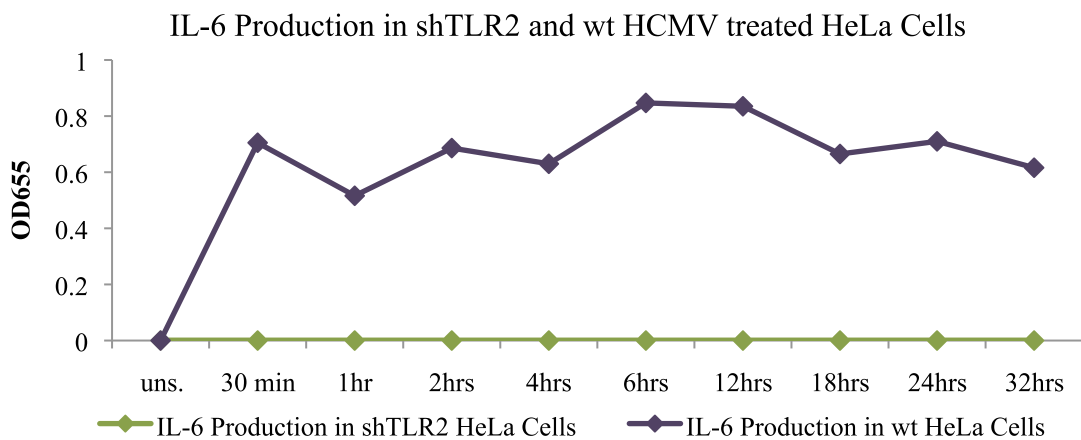

Figure 5.14: IL-6 synthesis of shTLR2 HeLa cells infected with HCMV. HeLa cells were infected with HCMV, (moi 5) at different time points. The supernatants were harvested with HEK IL-6 cells incubated between 18-24hr and assayed for IL-6 secretion by measuring the levels of SEAP in the supernatant using the Quanti-Blue assay. The SEAP levels were determined via spectrophotometer at 655nm. The data represents the mean ± SD of three independent experiments.

When TLR2 was knocked down IL-6 secretion was completely abrogated thus IL-6 production in HSV2 infection is TLR dependent.

In order to see the involvement of TLR9, HeLa wt cells as well as shTLR9 HeLa cells (*Figure* 5.15) were stimulated with viral DNA and IFN-β production was measured.

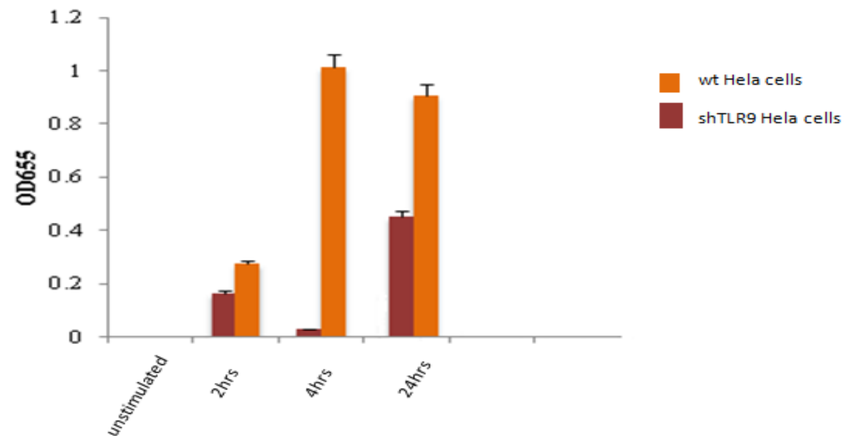

Figure 5.15: IFN-β synthesis of wt HeLa cells and shTLR9 HeLa cells stimulated with HCMV DNA. Cells were stimulated with 20μg/ml HCMV DNA, at different time points. The supernatants were harvested with HEK IFN-β cells incubated between 18-24hr and assayed for IFN-β secretion by measuring the levels of SEAP in the supernatant using the Quanti-Blue assay. The SEAP levels were determined via spectrophotometer at 655nm. The data represents the mean ± SD of three independent experiments.

From the results it is shown that inhibition of TLR9 in HeLa cells, does not have such a pronounced effect on IFN-β production, TLR9 silencing does not stop IFN-β secretion completely. With the involvement of other PRRs during the detection of the viral DNA, low levels of IFN-β are still observed.

When DAI is inhibited there is a pronounced IFN-β reduction thus DAI is the main PRR triggering type I IFNs (*Figure* 5.16).

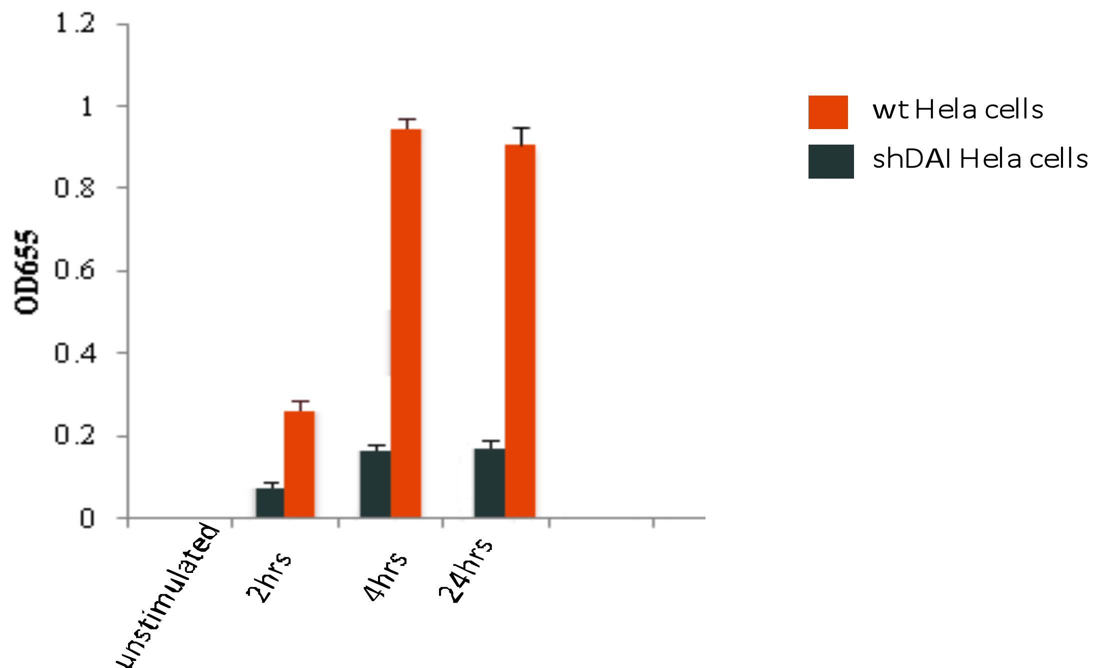

Figure 5.16: IFN-β synthesis of wt HeLa cells and shDAI HeLa cells stimulated with HCMV DNA. Cells were stimulated with 20µg/ml HCMV DNA, at different time points. The supernatants were harvested with HEK IFN-β cells incubated between 18-24hr and assayed for IFN-β secretion by measuring the levels of SEAP in the supernatant using the Quanti-Blue assay. The SEAP levels were determined via spectrophotometer at 655nm. The data represents the mean ± SD of three independent experiments.

5.3.2 Signalling cascades in TLR and DNA sensor Silenced HeLa cells

To determine the effect of TLR9 and DAI in HCMV DNA recognition, NF-kB activation was investigated.

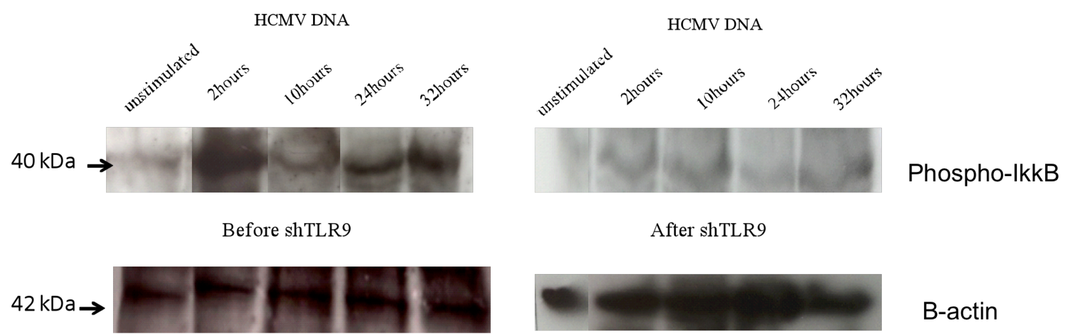

Figure 5.17: Immunostimulatory effect of HCMV DNA on HeLa cells before and after TLR9 silencing. Cells left unstimulated or were stimulated with 20µg/ml HCMV dsDNA at different time points. The cell lysate obtained from each stimulation was used for SDS-PAGE gel electrophoresis and western blot analysis using primary antibodies for phospho-IkkB followed by the appropriate secondary antibodies conjugated to HRP. Loading controls for β-actin are also shown. The data represents the mean of three independent experiments.

The data suggests that silencing of TLR9 has an effect on the NF-κβ activation since we can see less phospho-IkkB in the shTLR9 cells. TLR9 seems to be an important sensor for HCMV DNA.

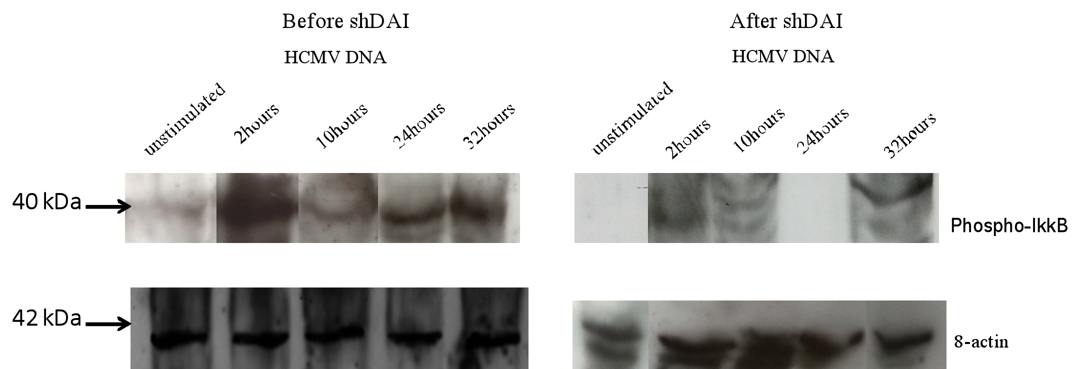

Figure 5.18: Immunostimulatory effect of HCMV DNA on HeLa cells before and after DAI silencing. Cells were unstimulated or stimulated with 20µg/ml HCMV dsDNA at different time points. The cell lysate obtained from every stimulation was used for SDS-PAGE gel electrophoresis and western blot analysis using primary antibodies for Phospho-IkkB followed by the appropriate secondary antibodies conjugated to HRP. Loading controls for β-actin are also shown. The data represents the mean of three independent experiments.

From the data above we can see that silencing of DAI had an effect on NF-κβ activation but not as pronounced as TLR9. Thus overall the data lead us to believe that TLR9 and DAI recognize HCMV DNA and can trigger a synergic NF-kB activation.

5.4 Conclusion

The results have shown that HCMV is recognized by a plethora of PRRs, which are TLR2, TLR9 and DAI. The viral DNA stimulations have shown that TLR9 and DAI detected HCMV genome, whereas there was almost no detection by AIM-2 and IFI-16.

According to the western blot results, HCMV was a strong activator of the transcription factor NF-κβ, especially the genomic HCMV DNA.

DAI production was also observed in the cells after stimulations with HCMV genomic DNA, this was quite high especially during the 2nd and 4th hours.

As a result of this activation the production of IFN-β, IL-6 and IL-1β was observed.

From silencing experiments we can see that IL-6 production is TLR2 dependent as well as IL-1β secretion, probably TLR2 triggers signal 1 in inflammasome activation. However, there was no indication that the inflammasome recruited was AIM2 dependent. Thus, NLRs may play a role in HCMV detection. This could be addressed in a future study.

Finally, from the silencing experiments we can conclude that type I IFN secretion is TLR9 and DAI dependent.

The innate immune system plays an important role in controlling herpesvirus infections and maintaining the balance between the virus and the host. This study revealed the host sensing mechanism in HCMV infection. It is clear that detection of classical PAMPs is central to immune surveillance. Recognition of the virus starts

from the beginning as soon as it interacts with the cell surface receptors, then it y moves to the endosomes, endoplasmic reticulum and in to cytoplasm.

Recognition of foreign DNA in intracellular compartments or in the cytoplasm sounds an alarm bell to the host cells signalling pathogenic invasion. In response, innate immune DNA-sensing pathways launch an antimicrobial cytokine and type I IFN response. It seems that TLR9 and DAI are major players in viral DNA detection and triggering host response. It remains to be determined, however, how the virus counteracts TLR and DAI activity and shifts the balance toward viral evasion and its consequent growth.

Chapter 6: Discussion

Immunity is the mechanism that organisms use to defend themselves against foreign microorganisms. This system gives the chance to identify between self and non-self particles and to trigger a signalling cascade to eliminate the specific unknown substance from the organism. Skin and mucosal membranes are the very first protective parts of the human body, which pathogens have to pass. These barriers are supported by the second defensive mechanism, which includes temperature, pH and soluble factors. The final mechanism is far more specific than the first two barriers, which is the innate immune system. This mechanism includes a broad spectrum of receptors, signalling adaptor molecules, transcription factors that cause a big immune response in the cells to eliminate the pathogen. Innate immunity is specific however it doesn't have a memory whereas acquired immunity has a memory (Kumar et al., 2011).

Janeway in the Cold Spring Harbor Symposia was the first one to refer to innate immune receptors and pathogen associated molecular patterns and since 1989 scientific research has revealed extensive information about the immune system and about these receptors (Janeway, 1989). PRR were discovered one after another and the way 'how' the immune system operates in response to viral infection was beginning to be understood. The Toll gene was first discovered in drosophila, (Anderson et al., 1985;Chuang and Ulevitch, 2001;Chuang and Ulevitch, 2000;Gay and Keith, 1991;Lemaitre et al., 1996;Medzhitov et al., 1997;Rock et al., 1998;Takeuchi et al., 1999b). Ligands from different pathogens were identified for every TLR and the signalling pathways were studied in detail (Yamamoto et al., 2003;Yamamoto et al., 2002). It was shown that TLR9 has evolved to detect CpG DNA commonly found in bacteria and viruses and to initiate the production of IFN and other cytokines (Hemmi et al., 2000). In addition several other PRR were discovered which were involved in DNA virus detection such as DAI; (previously also known as DLM1 and ZBP1) was

the first molecule to be reported that might function as a cytoplasmic DNA receptor (Takaoka et al., 2007). This IFN-inducible protein increased the production of pro-inflammatory cytokines and type I IFNs when overexpressed. AIM2 has also been shown to be essential for mediating inflammatory responses involving IL-1β following the sensing of microbial DNA (Hornung et al., 2009). IFI16, another PYHIN family protein, has been shown to be an intracellular sensor of HSV‑1 DNA that stimulates the expression of IFN-β and pro‑inflammatory genes during infection with this virus (Unterholzner et al., 2010). Recently, a molecule referred to as STING was demonstrated as being vital for recognizing cytoplasmic DNA and for activating the production of innate immune genes in response to a variety of DNA pathogens and even certain RNA viruses (Ishikawa et al., 2009).

Herpesviruses are a large family of DNA viruses, which can cause diseases in humans. Although TLRs have been shown to have a role in the sensing of herpesvirus infections, the fact that viruses replicate and persist intracellularly suggested that PRRs that function in the intracellular environment to detect viral DNA PAMPs also contribute to innate immune recognition of these viruses (Malmgaard et al., 2004). In this project the mechanism of HSV2 and HCMV in innate immune recognition and the host's innate immune response to herpes urogenital infection were studied. HCMV is an opportunistic pathogen associated with significant morbidity and mortality in immunocompromised hosts and HSV2 is the most frequent cause of genital ulcerations and infections.

Our data revealed that these viruses are detected by a plethora of PRRs, TLR2 detects both viruses and in addition to TLR2, TLR9 is involved in viral dsDNA sensing. In addition DAI and to a lesser extent IFI16 in primary vaginal cells can trigger cytokine secretion to protect the host from these viruses. There is currently no vaccine available

against neither HSV2 nor HCMV. HSV2 infections are treated with antiviral drugs such as acyclovir (ACV) and HCMV infections with ganciclovir however, resistance has been reported, mainly among immunocompromised patients (Morfin and Thouvenot, 2003). Therefore there is a developing need for alternative therapeutic strategies to the traditional Herpes virus antiviral therapy in order to suppress or restrict the infection.

Innate immune activation is crucial during the period of acute infection to limit early virus replication and to promote the development of an appropriate specific acquired immunity. Knowledge of these innate immune mechanisms is therefore of the outmost importance if new therapeutic approaches are to be developed.

In this study we used HeLa cells and primary human vaginal cells and looked at the involvement of different PRRs in HSV2 or HCMV vaginal innate immune responses. Our results showed that TLR2 recognizes HSV2 and HCMV and shuttles from the cell membrane to the Golgi where it co-localises with MyD88 a signalling adaptor essential for TLR signalling and cytokine production. In addition the DNA sensors TLR9 as well as DAI also recognises the viral DNA and trigger IFN α/β secretion. TLR9 moves from early endosomes and re-distributes in the endoplasmic reticulum (ER) upon HSV2 infection. While STING is located in the cytoplasma and ER in unstimulated vaginal cells and upon HSV2 DNA stimulation it colocalizes with ER translocon components TRAP and seC61β (Ishikawa and Barber, 2008). In our experiments, IFI16 was shown to be located in the cytoplasm and cell nucleus in vaginal cells. Upon HSV2 DNA stimulation IFI16 accumulates more in the cell nucleus. Therefore it seems that different PRRs are strategically placed in different cell locations to detect virus invasion and replication in order to activate cytokine secretion and protect the host.

Inflammasome activation and IL-1β secretion is also seen in innate immune responses to HCMV and HSV2 in HeLa cells. Surprisingly however this response is AIM2 independent leading us to speculate that IL-1β secretion maybe triggered by an NLR instead.

HCMV and HSV2 cause chronic persistant infection acyclovir, penciclovir, ganciclovir and foscarnet are current antiviral drugs used for herpesvirus-infected patients. These drugs target the viral DNA polymerase inhibiting the viral replication in the host cell. Acyclovir, valacyclovir and penciclovir are used as antiviral treatment for HSV infections. The active form of acyclovir inhibits the DNA polymerase leading to the termination of the viral DNA strand formation. The drug is first activated selectively by viral thymidine kinase in the cells, infected with HSV (Elion, 1993;Elion et al., 1999). Penciclovir and valacyclovir function the same way as acyclovir. Unfortunately these drugs that are activated through thymidine kinase, do not work with HCMV infections as this type of virus lacks thymidine kinase in its structure. However another antiviral agent ganciclovir targets HCMV UL97 kinase and can be used for HCMV infections. Ganciclovir slows down the elongation process of the viral DNA strand by interacting with the viral proteins as well, whereas foscarnet does not need this interaction with these viral proteins for activation (Gilbert et al., 2002). It directly binds to the DNA polymerase pyrophosphate-binding site to stop the elongation of the viral DNA molecule. Foscarnet is utilised as a second-line drug during herpes virus infections. Unfortunately high doses of these antiviral drugs can lead to side effects in infected patients. High doses of acyclovir, cidofovir and foscarnet can be destructive to the kidney cells (nephrotoxicity), furthermore they can damage the brain cells if taken for long periods (Haefeli et al., 1993;Lowance et al., 1999;Wagstaff and Bryson, 1994;Wagstaff et al., 1994). These drugs can also weaken the immune system by reducing the neutrophil levels in the blood (Safrin et al., 1997).

Circulation problems can also occur for example valacyclovir can lead to thrombi formation in small blood vessels (Ormrod et al., 2000) . However drug toxicities are not the only clinical limitation; antiviral drug resistance is another problem that clinicians are facing.

Antiviral drug resistance is common in HSV infections due to mutations in TK, which can cause loss of function or change in the functioning of the protein (Coen and Schaffer, 1980;Morfin and Thouvenot, 2003). Additions or deletions at guanine and cytosine residues are the reason for TK dysfunctioning (Gaudreau et al., 1998;Morfin et al., 2000). Although acyclovir and penciclovir are no longer effective however it is possible for these emerging mutants to respond to foscarnet and cidofovir as these drugs work via DNA polymerase. DNA polymerase is a highly conserved protein, which has an important function during the replication cycle and thus mutations in this protein is less common (Gilbert et al., 2002). However some cases have been reported about foscarnet and cidofovir resistance due to possible DNA polymerase mutations (Chen et al., 2000;Wyles et al., 2005). Vidarabine has selectivity for HSV DNA polymerase inhibiting the HSV activity, however clinical trials have shown that this drug was unsuccessful in HIV-infected patients (De Clercq, 1984;Safrin et al., 1991).

Ganciclovir and valganciclovir are also drugs used for HCMV infections, however resistance to these drugs has been reported as well (Erice et al., 1989). This resistance against the drugs is due to mutations in HCMV UL97 kinase gene (Chou et al., 2002). Unfortunately chronic HCMV antiviral therapy can induce multiple mutations of UL97 gene and of UL54 DNA polymerase, causing cross-resistance not only to ganciclovir but also to foscarnet and cidofovir (Chou et al., 1997;Smith et al., 1997). Frequent numbers of severe and fatal cases of drug resistant HCMV have been reported.

In order to treat immunocompromised patients infected with resistant HCMV strains, drugs that are used for other diseases are also being tested. Hematopoietic stem cell transplant patients, who were under immunosuppressive therapy, had less chance of getting HCMV infection (Cutler et al., 2004;Marty et al., 2007). Leflunomide is a drug used in rheumatoid arthritis therapy, which showed an inhibitory effect on HCMV replication both in vitro and in vivo (Waldman et al., 1999). Artesunate is used for malaria treatment and its' inhibitory effect on HCMV was demonstrated in vivo and in vitro studies (Efferth et al., 2008;Kaptein et al., 2006). These drugs could be effective on HCMV infection however the drug efficacy or potential side-effects in chronic patients are not yet fully determined

Targeting TLRs or other PRRS involved in viral infections is a promising field for HCMV and HSV management and infection control There are clearly many options and possibilities for targeting TLRs or other PRRs, because the key function of TLRs is to induce cytokines, which could be very potent in clearing an infection

TLR agonists are widely used to optimize vaccine efficacy, taking advantage of their powerful adjuvancity.

Monophosphoryl lipid A (MPL), a TLR4 agonist is used as a vaccine adjuvant because it binds to TLR4 triggering the TRAM/TRIF pathway in the cell (Mata-Haro et al., 2007)., Pam3CSK4 and MALP-2 are also TLR2 agonists that can be utilised to enhance TLR-2 activity (Ishii and Akira, 2007;Lombardi et al., 2008). The data from our study also sheds light into the effect of these TLR2 agonists in viral infections. Agonists of TLR3 and TLR7-9 have also yielded very promising results for treating viral infections. TLR7/8 agonists have reached phase 3 trials. When monocytes from HIV patients were stimulated with resiquimod a TLR7 agonist, IL-12 secretion was

augmented while TNF production was decreased compared to the control group. Additionally, HIV replication in cultured monocytes was inhibited (Nian et al., 2012).

Another TLR7/8 agonist imiquimod leads to cytokine production and can be used for treating genital and perianal human papilloma virus lesions. It is also effective as a topical treatment in recurrent acyclovir-resistant HSV sores and lesions and unlike other anti-viral drugs it is nontoxic for the patient (Martinez et al., 2006).

TLR9 agonists cytosine-phosphate-guanosine oligodinucleotide (CPG-ODN) are being used as vaccine adjuvant for cancer and infectious diseases (Vollmer and Krieg, 2009). Agatolimod is a CPG-ODN aiming at TLR9, triggering the dentritic cell and B cell activity, leading to cytotoxic T cell activation initiating anti tumor activity in the host. Therapeutic strategies focusing on TLR9-mediated immunomodulation are currently being implemented for chronic viral infections, such as chronic hepatitis C (HCV). TLR9 agonists stimulate plasmacytoid dendritic cells to produce large amounts of type I IFN, especially IFNα, which is the backbone of therapy for HCV. Indeed, IFNα powerfully inhibits viral replication and promotes innate and adaptive host immune response (Hartmann et al., 2003;Libri et al., 2009).

The use of antagonists is less successful compared to TLR agonists, as they might block the activity of other TLRs as well, very few could reach phase 3 trials. E5564 (eritoran) a TLR4 antagonist, which blocks TNFα synthesis, was tested for sepsis and septic shock therapy, after promising results in phase 1 and phase 2 trials, it failed in phase 3 clinical trials (Opal et al., 2013)

Thus all these new studies reveal that targeting TLRs is a promising field for bacterial and viral management and infection control. Therefore we also proceeded to investigate whether agonists/ligands for specific PRRs involved in Herpes virus recognition could provide protection against infection. Previous studies using TLR

agonists had shown promise in reducing infection. It had been previously shown that TLR2 agonists vaginally-applied prior to infection increased resistance to HSV-2 infection in mice (Rose et al., 2009). Surprisingly Poly I:C a synthetic RNA that is a TLR3 agonist applied 24 h prior to viral inoculation significantly increased resistance to genital HSV2 infection in mice, however TLR4 agonists had no effect (Ashkar et al., 2004). In addition, TLR9-stimulating CpG oligonucleotides have been shown to induce strong local and systemic immune responses in laboratory rabbits.

In this study we performed experiments in human vaginal epithelial cells as a model of HSV2 infection instead of mice or human dendritic cells like previous studies. Contrary to Ashkar et al. we found no TLR3 upregulation nor IFN inhibition when poly I:C was used possibly the effect observed in their study on dendritic cells was tissue/cell specific and since we are using vaginal epithelial cells it seems that TLR3 was not involved. We have used lipopeptides as TLR2 agonists, CpG DNA as TLR9 agonist as well as for the first time cyclic dinucleotides for STING. Our data showed a significant viral inhibition especially in the presence of TLR2/6 agonists Pam_3CSK_4 and FSL1 as well as in the presence of cyclic dinucleotides, thus indicating that a combination of different agonists especially ones that trigger different cytokines could provide a wider spectrum prophylaxis to the host. However, the intricate network of cytokine signalling in response to agonist stimulation needs to be well defined and these agonists would ideally be more effective prior to or just after a high-risk sexual encounter to prevent herpesvirus infection. Overall the use of PRRs agonists for prophylactic and therapeutic use is likely to increase in the future and should continue to be at the forefront of immunotherapy research.

Our data thus identify that vaginal and HeLa cells express both robust levels of TLRs as well as DNA sensors which have evolved to complement TLR recognition either

through distinct ligand recognition or cell location and thus protect the host from herpesvirus infection.

Novel therapies can be generated based on TLR agonists. The dosage of these PRR agonists, mono or combined therapies with different drugs should be determined based on experimentations on animal models and after clinical studies. This will also be helpful for determining toxicity of these drugs as well as taking into a consideration the patients' health history, and genetic information.

Additional genetic investigation is needed about drug resistant viruses, so that this data can be used for further improvement of these drug therapies. Detailed information about the viral mechanism and genetic mutations on these viral proteins will be useful to create drugs aiming at different parts of the viral replication cycle also increasing the selectivity for conserved viral proteins that are functioning during this process. The priority should be a substance with less toxicity and more antiviral efficiency, as the majority of the current antiviral drugs are harmful for the patient in the long term. Several therapies can be combined together to increase the success of the treatment and the precision of a novel therapy can be determined via clinical trials.

Glossary:

A

Agonist: chemical which binds to the receptor to activate it leading to a biological response.

α-TIF: Viral protein functioning during the transcription of the viral genome. Association of this protein with host cell proteins initiates immediate-early gene synthesis.

Antibody: Produced by B-lymphocytes, made up of two regions; Fc region and Fab region. Fab region recognizes the antigen and Fc binds to Fc receptors.

Antigen: Molecule that induces the immune response.

Antiviral drug: Drugs that have an inhibitory effect on the replication of the virus without damaging the host cell.

Apoptosis: Programmed cell death, initiated by an inflammasome complex.

Apoptosis-associated spec-like protein containing a CARD (ASC): Protein encoded by PYCARD gene. Pyrin domain functions in the receptor interaction and CARD domain functions during the caspase activation leading to inflammasome complex formation and apoptosis.

Assay: test.

B

B-lymphocytes: Immune cells that produce antibodies.

Baltimore scheme: Virus classification system created by David Baltimore that seperates the viruses depending on the type of genome they consist of.

bp: Specific nucleobases pair up with each other in the DNA by forming hydrogen bonds.

Brefeldin A: Antibiotic produced by fungi that inhibits the protein transportation between the endoplasmic reticulum and the golgi.

C

Capsid: Viral protein structure surrounding the viral genome and the core.

Capsomere: Viral capsid protein subunits.

Centrifugation: Process which involves rapid rotation creating a centrifugal force, leading to seperation of the different molecules from each other.

Cytokine: Proteins which function in the behaviour of the cell.

Cytometric bead array (CBA): A test used to quantify specific molecule in a sample with the help of an antibody.

D

Denature: A change in the structure of a molecule.

Dendritic cells: Antigen presenting cells, received from bone marrow.

Dimethyl sulfoxide (DMSO): obtained by oxidation of dimethyl sulfide, it dissolves polar and nonpolar bonds. It is used as a cryoprotectant to reduce cell death during freezing.

DNA ligase: Enzyme that functions in the DNA replication and repair, by forming a phosphodiester bond.

DNA polymerase: Enzyme that create the new DNA strand.

dsDNA: Double-stranded DNA

dsRNA: Double-stranded RNA

E

Encephalitis: brain inflammation

Endonuclease: enzyme that initiates the hydrolysis of nucleic acids.

Endocytosis: Engulfing of the molecules by the cells.

Endoplasmic reticulum: A eukaryotic cell organelle that is responsible for lipid, membrane and protein synthesis.

Envelope: Lipid layer surrounding the viral capsid and tegument part.

F

Fc region: C-terminal of an antibody, where it interacts with the Fc receptors.

Fluorescein isothiocyanate (FITC): Derivative of fluorescein molecule, with the excitation and emission spectrum wavelengths of 495mn and 519 nm respectively.

G

Genome: The genetic information of the organism either present as a DNA or RNA.

Glycoproteins: Polypeptide chains and oligosaccharide chains form membrane proteins. Viral glycoproteins function in the cell adhesion and are located on the lipid bilayer outer part of the virus.

Golgi apparatus: A eukaryotic cell organelle located near the cell nucleus, responsible for vesicular protein transport.

Growth factors: Proteins that signals the growth, proliferation of the cell.

H

Helix: A spiral

Hepatitis virus: Viruses that cause liver inflammation.

Herpesvirus: Large dsDNA viruses that cause latent, recurring infections.

Horseradish peroxidase (HRP): An enzyme obtained from horseradish that catalyzes the chemiluminescent substrate (ECL). This reaction produces light; this is used in enhanced chemiluminescence.

Human immunodeficiency virus: A member of retroviruses, ssRNA virus that causes AIDS.

I

Icosahedron: A polyhedron with 20 faces and 30 edges.

Immunofluorescence: Microscopic observation of a protein of interest through facilitating a specific fluorescent-conjugated antibody for that protein.

Incubation period: The period of time taken after the initial infection of the cells until the appearance of the symptoms in the host cell.

Inducible genes: Gene that is expressed due to environmental change or at the end of an activated signalling cascade in the cell.

Interference: The disruption of a specific gene expression by a different gene, via interfering with messenger RNA molecules.

Interferon (IFN): Proteins that are secreted by an infected cell to trigger the antiviral response in other cells.

Interleukin (IL): Proteins that are secreted by immune cells to stimulate the T and B-cell development and differentiation.

K

Kb: Kilobases is used to specify the size of the DNA or RNA molecule. 1 Kilobases is equal to 1000 bases.

L

Latency-associated transcripts (LATS): Transcripts that interact with the cellular histones to initiate latency period of the virus.

Latent infection: Infection that cannot be detected in the cell or no apparent clinical signs are observed on the patient.

Limulus amebocyte lysate (LAL) test: Test is used to determine the amount of endotoxins in a solution. LAL is obtained from horseshoe crab and it reacts with LPS and endotoxin from gram-negative bacteria.

Luria/Lysogeny broth (LB): Medium that is rich in nutrients, used for the growth of bacteria.

M

Macrophage: Mononuclear cells that are phagocytes. They function in the innate immunity secreating inflammatory cytokines and they can initiate adaptive immunity.

Major histocompatibility antigen: Cell membrane molecules, which are responsible for presenting the antigen sequences to T-cells.

Meningitis: inflammation of the protective tissue around the brain and the spinal cord.

Monensin: Polyether antibiotic obtained from bacteria, it can transport the ions through cellular and subcellular membranes. It blocks the protein transport and destroys the vesicular budding.

Monoclonal antibodies: Antibodies, which are the same, because the same immune cells have produced them.

Multiplicity of infection (MOI): The ratio of the infectious agents to the cells.

N

Neurotropic virus: Viruses that infect nerve cells.

O

Open reading frame (ORF): The part of the genome that doesn’t have stop codon.

P

Paraformaldehyde (PFA): Polyoxymethylene, which is depolymerised to formaldehyde to be used as a fixative for microscopy examinations.

Passage: Subculturing that is achieved by separating fully confluent cells into a new flask with a fresh growth medium, extending the cell life and increasing the number of the cells.

Pathogen: Disease causing microorganisms that trigger the immune system in the host.

Phosphate buffered saline (PBS): Isotonic buffer made of salt, it is non-toxic to the cells.

Phycoerythrin (PE): Pigment that is obtained from algae and cryptophytes. They consist of phycobiliproteins that give off light.

Plaque-forming unit (PFU): The number of the infectious particles that can form plaques per unit volume.

Polyclonal: Antibodies, which are clones of multiple immune cells.

Primary cells: Cells that are obtained from the tissue origin.

R

Reactivation: Reoccurrence of an infection after a latency period.

Receptor: A protein that sits on the cell surface or inside the cell, responsible for the recognition of certain sequences on other specific proteins (cellular or extracellular) to trigger certain signalling cascades in the host.

S

Standart deviation (SD): A measure to determine the total variation between certain set of data.

Secreted embryonic alkaline phosphatase (SEAP): Short form of human placental alkaline phosphatase, which is secreted out of the cell. It can be detected through HEK Blue detection Media.

Symptoms: Change of a normal state to unusual condition which can be diagnosed by the patient.

T

T lymphocytes: A type of white blood cell that function in the host immunity against certain antigen after an infection.

Tegument: The section between the viral capsid and envelope, consists of viral proteins.

TEMED: A chemical compound used in polyacrylamide gel electrophoresis to initiate the polymerization for the formation of the gel.

Thymidine kinase: An enzyme that functions in the synthesis of DNA, found in herpesviruses and some other DNA viruses.

Transcription: Synthesis of primary transcript RNA from DNA or RNA template by RNA polymerase.

Transfection: Introducing nucleic acids into the eukaryotic cells by using transfection chemical reagents.

Transformation: Genetic change of the cell as a result of the uptake of an exogenous DNA.

Translocation: The transport of the viral genome to a different part of the cell.

U

Ubiquitin: A small amino acid sequence tag on a protein, which marks the degradation point by proteosome.

V

Vaccination: Initiating an immune response in humans via introducing an inactive or a part of a pathogen, before the individuals are infected with that specific pathogen.

Vesicle: Small circular organelles made of lipid bilayer, consisting of fluid or infectious particles during an infectious disease.

Virion: A whole virus particle.

Virulence: an amount of the pathogenicity of an organism.

Virus passage: A number of the rounds of virus replication to generate viral stocks in the laboratory.

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Appendix

X10 PBS (500ml)

- 50g NaCl
- 1.25g KCl
- 7.2g Na_2HPO_4
- 1.25g KH_2PO_4
- 400ml distilled water (dH_2O)
- pH 7.2
- Top up to 500ml with dH_2O

For PBS-Tween, add 10ml Tween-20 (rinse tip in buffer).

X1 PBS (500ml)

- 50ml x10 PBS
- 450ml dH_2O

X2 PBS (500ml)

- 100ml x10 PBS
- 400ml dH_2O

X1 PBS/0.02%$_{(w/v)}$ BSA/0.02%$_{(w/v)}$ Saponin/0.02%$_{(w/v)}$ Sodium Azide (NaN_3) (500ml)

- 0.02% = 0.02g in 100ml
- In 500ml need 0.1g
- 0.1g of each added to 500ml x1 PBS

10% SDS (100ml)

- 10.0g SDS
- In 100ml dH_2O
- Mix on a heater / stirrer

4% Paraformaldehyde (4% PFA) (200ml)

- Add 8.0g PFA powder to approx. 80ml of dH_2O in a glass beaker.
- Heat to 60°C in a fume hood on a heater / stirrer

- Add a few drops of 1M NaOH to help dissolve, whilst still on a heater / stirrer
- When the solid has completely dissolved, top up to 100ml with dH_2O, let the solution cool to room temperature, add 100ml of x2 PBS

0.5M Tris pH 6.8 (250ml)

- 60.5g in 1000ml (M = Mass / M.wt therefore 0.5 = Mass / 121 therefore Mass = 60.5)
- 15.1g Tris in 250ml dH_2O
- pH to 6.8 with concentrated HCl

X2 SDS-PAGE Reducing Sample Buffer (approx. 40ml)

- 20ml 0.5M Tris pH 6.8
- 16ml 10% SDS
- 10.0g Glycerol
- 4ml 14.3M β-mercaptoethanol
- Small spatula with a bit of Bromophenol Blue
- Stir with spatula till mixed, transfer to bottle

Phenol / Chloroform / Isoamyl Alcohol

- In fume hood, in a 500ml sterile glass bottle
- Add 100ml Phenol + 100ml Chloroform / Isoamyl Alcohol + 100ml dH_2O
- Leave to settle
- Place in fridge

Stripping Buffer (200ml)

- 1.4ml β-Mercaptoethanol (100mM)
- 40ml 10% SDS (=2% in 200ml)
- 1.52g Tris HCl pH6.7 (62.5mM)
- Top up to 200ml with PBS-T

DNA Isolation Solutions:

STET Buffer (100ml)

- 8g Sucrose
- 500µl Triton
- 10ml EDTA (500mM) pH 8 (adjust with HCl)
- 1ml Tris pH 8 (1M) (adjust with HCl)
- Make up to 100ml with dH_2O

1M Tris (100ml)

- 12.1g Tris
- In 100ml dH_2O
- pH to 8 with HCl

500mM EDTA (=0.5M EDTA) (100ml)

- 37.22g EDTA
- In 200ml dH_2O
- pH to 8 with HCl

<u>Sucrose Gradient Buffers:</u>

60% / 30% / 10% in PBS

- 60g / 30g / 10g Sucrose (powder) into 3 separate conical flasks
- Top each up to 100ml with PBS
- Fridge

<u>SDS-PAGE / Western Blot Solutions:</u>

1.5M Tris-HCl pH 8.8 (500ml)

- 90.85g Tris
- In 500ml dH_2O
- pH to 8.8 with conc. HCl

X2 Transfer Buffer (1000ml)

- 4.88g Tris
- 20ml 10% SDS
- 400ml Isopropanol (= Propan-2-ol)
- Make up to 1000ml with dH_2O
- pH to 8.3 with Acetic Acid
- To get X1 TB → split into 2 beakers, add 500ml dH_2O to each

Running Buffer (500ml)

- 50ml x10 Running Buffer
- 450ml dH_2O
- [Tris-Glycine pH 8.8] → 25mM Tris, 192mM glycine, 0.1% SDS
- For 10x running buffer:
- 288g glycine
- 60.4g tris base
- 20g SDS
- 1800ml dH_2O

PBS-Tween (2000ml)

- 200ml x10 PBS into a 1000ml bottle
- Top up to 1000ml with dH_2O
- Add 2ml PBS-Tween20, dropping the tips into the bottle
- Mix (shake)
- Decant 500ml into a new bottle, top both up with 500ml dH_2O = 2 x 1000ml bottles of PBS-T

10% Resolving Gel (for 2 gels)

- 4.02ml dH_2O
- 2.5ml 1.5M Tris-HCl pH 8.8
- 100µl 10% SDS
- 3.33ml Acrylamide/Bis
- To Polymerise, add:
    - 50µl 10% APS
    - 10µl TEMED

4% Stacking Gel (for 2 gels)

- 6.1ml dH_2O
- 2.5ml 0.5M Tris-HCl pH 6.8
- 100µl 10% SDS
- 1.3ml Acrylamide/Bis
- To Polymerise, add:
    - 50µl 10% APS
    - 20µl TEMED

Blocking Reagent (40ml)

- 2g Milk Powder
- 40ml PBS-T

<u>Agarose Gel Electrophoresis Solutions:</u>

50X ELFO (2M Tris, 50mM EDTA) (1000ml)

- 242g Tris base
- 100ml EDTA (0.5M)
- pH 7.7 with Acetic acid
- Make up to 1000ml with dH_2O

1X ELFO (1000ml)

- 20ml 50X ELFO in 1000ml dH_2O

ELFO Loading Buffer (200ml)

- 100ml Glycerol
- 20ml 50X ELFO
- 80ml dH_2O
- A few mg of Bromophenol Blue

The original amounts for resolving and stacking solutions are shown in the Table 2, and Table 3 respectively.

	Two Gels	**One Gel**
Distilled Water	6.1ml	3.05ml
0.5 M Tris-HCl pH 6.8	2.5ml	1.25ml
10% (w/v) SDS	100µl	50µl
Acrylamide/Bis	1.3ml	0.65ml
10% APS	50µl	50µl
TEMED	10µl	10µl
Total Monomer	10ml	

Table 2: This table shows the amounts of each solution used to prepare a resolving solution for one gel.

	10%	**12%**
Distilled Water	4.02ml	3.5ml
1.5 M Tris-HCl pH 8.8	2.5ml	2.5ml
10% (w/v) SDS	100µl	100µl
Acrylamide/Bis	3.33ml	4.0ml
10% APS	50.0µl	50.0µl
TEMED	5.0µl	5.0µl
Total Monomer	10.0ml	10.0 ml

Table 3: This table shows the amounts of each solution to prepare a stacking solution for one gel.