Alma Mater Studiorum – Università di Bologna

DOTTORATO DI RICERCA IN
BIOLOGIA CELLULARE E MOLECOLARE

Ciclo XXVI

Settore Concorsuale di afferenza: 05/I1

Settore Scientifico disciplinare: BIO19

Role of αvβ3 – Integrin and TLR2 in the innate response to Herpes Simplex Virus infection and Delivery of retargeted oncolytic Herpes Simplex via carrier cells

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Esame finale anno 2014

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1. Introduction

1.1 Brief history of Herpesviruses

Lesions caused by HSV have been described in the ancient literature millennia ago, in particular descriptions of genital lesions that resemble those caused by HSV were found in a Sumerian tablet (third millennium BC) and in the Ebers Papyrus (1500 BC). In the ancient Greece, Hippocrates used the Greek word "herpes" to describe lesions that seem to creep or crawl along the skin [1]. Herodotus was the first to describe an association between the cutaneous lesions and fever caused by HSV, and Galen recognized that recurrent HSV lesions develop at the same anatomical location. During the 18th century, Bateman accurately described the nature of HSV infection as a "restricted group of localized vesicles with a short, self-limiting course".

The first description of the link between HSV and the genital organs was reported by John Astruc, physician for King Louis XIV, after studying the afflictions of French prostitutes, in the "De Morbis Venereis" (1736). During the late 19th and early 20th century, human volunteers were often used to test the transmission of infectious agents, in this context Vidal showed that HSV was infectious by passing it from human to another (1873). Animal studies conducted by Gruter demonstrated that HSV could be transmitted from rabbit to rabbit, and the virology community attributes to him the merit for the isolation of HSV (1924). In the 1930s, Andrews and Carmichael observed that recurrent HSV infections occurred only in adults who carried neutralizing antibodies, in sharp contrast to the behavior of other known infectious agents at that time. This was one of the milestones of HSV biology, as their article stimulated an international debate that ultimately shaped the perception of HSV as a disease agent. In an article of 1939, Burnett and Williams described the nature of latency, noting that HSV seems to persist for life and can be reactivated under stressful conditions to produce visible lesions.

The advent of culture protocols in the first part of the 50s led a big development in herpesvirology allowing the isolation of other members of the human herpesvirus family. In the 70s genital herpes was recognized as a serious sexually transmitted infection able to threaten fetus. Vidarabine was the first antiviral therapy developed at the end of the 70s, that reduced the fatality of HSV encephalitis, but only with the discovery of acyclovir there was a real therapeutic advance. In 1988, Dr. Gertrude Eliot, one of the pioneer in the study of acyclovir, who demonstrated its mechanism of action, was awarded Nobel Prize in Physiology and Medicine for his studies.

The vaccines against herpesvirus have been of great interest since the early 20th century but their history has been uncertain: two vaccines have been evaluated for prophylaxis and therapy, both based on viral glycoproteins and an adjuvant, but they gave difficult to interpret results so their efficacy has still to be

proven. Herpes simplex viruses were the first of the human herpesviruses to be discovered and are among the most intensively investigated of all viruses.

The beginning of modern research on HSV can be placed between the 60s and the 70s, when scientists discovered the size and complexity of the structure of HSV DNA, and begun to study the large number of proteins that make up HSV particles. This period was the golden age of research on HSV and led to collection of a great amount of information on viral genes functions and virus-cell interaction. Herpesviruses were used as models and tools for the study of translocation of proteins, synaptic connections in the nervous system, membrane structure, gene regulation, gene therapy, cancer therapy, and a myriad of other biological problems, both general to viruses and specific to HSV.

In the last twenty years research focused on the therapeutic potential of HSV, as a matter of fact HSV has been taken into account as a promising viral vector to be used in immunoprophylaxis and gene therapy (gene delivery to the CNS and oncolytic viral therapy).

1.2 Herpesviridae family and classification

The herpesviruses are highly widespread in nature and several animal species are the natural host of at least one of this virus; in particular in human nine herpesvirus have been isolated (Table 1).

Table 1 – Human Herpesviruses [2].

Designation	**vernacular name**	**abbreviation**	**subfamily**	**genome size (bp)**	**genome accession no.**
Human Herpesvirus 1	Herpes simplex virus 1	HHV-1 (HSV-1)	α	152000	NC_001806
Human Herpesvirus 2	Herpes simplex virus 2	HHV-2 (HSV-2)	α	152000	NC_001798
Human Herpesvirus 3	Varicella-zoster virus	HHV-3 (VZV)	α	125000	NC_001348
Human Herpesvirus 4	Epstein-Barr virus	HHV-4 (EBV)	γ	172000	NC_00760
Human Herpesvirus 5	Cytomegalovirus	HHV-5 (HCMV)	β	229000	NC_006273
Human Herpesvirus 6A		HHV-6A	β	162000	NC_001664
Human Herpesvirus 6B		HHV-6B	β	162000	NC_000898

Human Herpesvirus 7		HHV-7	β	153000	NC_001716
Human Herpesvirus 8	Kaposi's sarcoma associated virus	HHV-8 (KSHV)	γ	230000	NC_009333

The current herpesvirus classification was defined in 1981 [2]. All Herpesviruses are capable of establishing a latent infection in their natural hosts in a specific set of cells, which varies from one virus to another. Moreover Herpesviruses produce large amount of enzyme engaged in nucleic acid metabolism, DNA synthesis and processing of proteins, they synthesize viral DNA and assembly capsids in the nucleus, and generate infectious viral progeny associated with the lysis of the infected cell. Other biological properties vary, such as the length of the reproductive cycle, and these were used as the basis of classification, before DNA sequences of the viruses were known.

The virion of the members of the Herpesviridae family has a characteristic architecture: it is composed of a central core containing linear double-stranded DNA, an icosahedral capsid of 100-110 nm, an asymmetrical and amorphous tegument which surrounds the capsid, and a lipid envelope with surface glycoproteins.

Members of the family Herpesviridae were classified by the Herpesvirus Study Group of the International Committee on the Taxonomy of Viruses (ICTV) into three subfamilies on the basis of biological characteristics (Alphaherpesvirinae, Betaherpesvirinae, Gammaherpesvirinae) and then viruses in each subfamily were divided into genera on the basis of molecular data.

Alphaherpesvirinae are characterized by short reproductive cycle, rapid spread in tissue culture, efficient destruction of infected cells in a wide variety of hosts, and the ability to establish latent infections primarily in sensory ganglia. The subfamily consists of the genera Simplexvirus (HSV-1, HSV-2) and Varicellovirus (VZV), which have mammalian hosts, and Marek's disease-like virus and Infectious laryngotracheitis-like virus, which have avian hosts.

Betaherpesvirinae are characterized by a limited host range, long reproductive cycle, and slow infection progression in tissue culture. Cells that are infected often become enlarged (cytomegalia), and the viruses can maintain latency in secretory glands, lymphoreticular cells, kidneys, and other tissues. The subfamily consists of the genera Cytomegalovirus (HCMV), Muromegalovirus, and Roseolovirus (HHV-6, HHV-7).

Gammaherpesvirinae were classified by a limited host range and ability to replicate in lymphoblastoid cells, with some viruses also causing lytic infection in some types of epithelial and fibroblastic cells. Viruses are usually specific for either B or T lymphocytes, and latent virus is frequently demonstrated in lymphoid tissue. The Gammaherpesvirinae subfamily consists of the genera Lymphocryptovirus (EBV), and Rhadinovirus (KSHV).

1.3 Epidemiology of HSV infection

HSV has a worldwide distribution, with no distinction between developed countries and underdeveloped countries, and no seasonal variation. HSV is transmitted only by human – human contact [3, 4], in the specific during close personal contact from an infected to a susceptible individual, and there are no known animal carriers for this virus. Since HSV infection rarely results in fatality, except for newborns, and it persists in infected individuals in a latent form, more than half of the world's population probably has a recurring HSV infection, enabling the transmission of HSV.

Initial HSV infection usually occurs in children and is most often asymptomatic. The most common location of infection is the mouth area. It is necessary a contact between HSV virus and mucosal surfaces or abraded skin to initiate infection; then virus is transported by retrograde flow along axons that connect the mucosa to the nuclei of sensory neurons [5]. Viral replication occurs in a small number of sensory neurons, while in other cells the viral genome remains in a latent state lifelong. Various stimuli, like physical or emotional stress, fever, ultraviolet light, tissue damage are the main events that cause viral re-activation. In a reverse process, the virus is transported in an anterograde flow to mucosal membranes, and visible sores become active for transmission [Fig. 1.1].

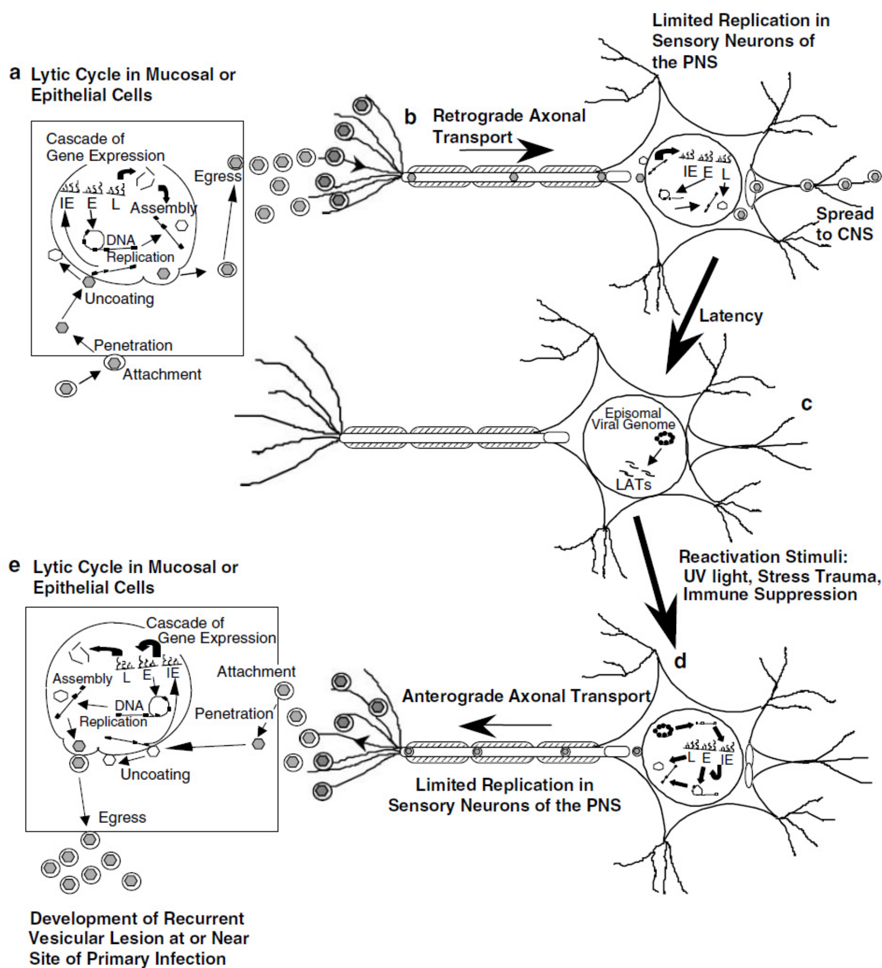

Fig. 1.1: **HSV-1 life cycle and routes of infection in the host.** (a) Primary lytic infection of epithelial or mucosal cells of the lips or eye results from the attachment and penetration of HSV particles to host cells. (b) Progeny virion particles can encounter and bind to axonal termini that innervate the site of the primary infection; viral capsids are transported in a retrograde manner to the nerve

cell body in the trigeminal ganglia. At this point, limited expression of virion proteins may lead virus spread to the CNS or (c) the circular viral genome can persist as an episomal molecule in a latent state within the neuron. (d) Periodically, various stimuli cause virus reactivation, and the virus enter the lytic cascade resulting in the production of progeny virus particles that travel to the neuron termini by anterograde axonal transport. This process does not result in lysis of the nerve cell. (e) The virus again initiates a productive recurrent infection that may result in lysis of these cells and formation of a lesion at or near the site of the primary lesion [5].

HSV replicates in many cellular types, including neurons where it carries out a lytic cycle and is capable of aggressive spread and tissue destruction.

At their first exposure to HSV-1 (facial) or HSV-2 (genital), seronegative individuals contract primary infection. Instead an "initial infection" occurs when a person with antibodies against HSV-1 contracts HSV-2 or vice versa. The clinical manifestations caused by the two serotypes are similar, but they are usually transmitted by different routes and infection localizes at different areas of the body.

HSV-1 seroprevalence is much more common than its counterpart HSV-2: while most genital infections are caused by HSV-2, there is an ever-increasing proportion attributable to HSV-1, but genital HSV-1 infections are usually both less severe than HSV-2 and less prone to recurrence. HSV-1 and HSV-2 have an incubation period of about 4 days and it ranges from 2 to 12 days. The diagnostic assessment is based on virus isolation in cell culture and PCR detection of viral DNA. HSV-1 causes symptomatic oropharyngeal disease which is characterized by sores of the buccal and gingival mucosa (lasting 2-3 weeks) and by fever between 38 to 40°C. Primary infection occurs with intraoral ulceration lesions whereas lip lesions suggest recurrent infection. Primary genital herpes by HSV-2 appears as macules and papules, followed by vesicles, pustules and ulcers. During pregnancy the infection is rarely transmitted to the fetus (5% in utero), while mother-to-baby transmission occur mainly during vaginal delivery (80%) or after birth, resulting in encephalitis and disseminated infection, which can cause the death of the newborn. Caesarian section is applied to prevent this type of transmission. Complications in men are rare; aseptic meningitis and urinary retention are more common in women. Non primary initial genital infection cause milder symptoms than primary infection.

Immunocompromised patients are at increased risk of severe HSV infection, they can develop progressive disease involving respiratory tract, esophagus or gastrointestinal tract [4].

The treatment for HSV infection is the topical, oral or intravenous administration of acyclovir (ACV), a purine-nucleoside analogue prodrug. Viral thymidine kinase activates ACV, which is incorporated into viral DNA and acts as a chain terminator. This treatment keeps symptoms under control and has been successfully employed to treat other HSV infection such as encephalitis, hepatitis, pulmonary infection, proctitis or eye infection [4].

1.4 Herpes Simplex Virus 1

HSV-1 was the first human herpes virus discovered, it is considered the prototype virus in the family, and it is still one of the most investigated herpes viruses [6]. Its biological properties, like latency, capacity to reactivate and to cause several infections, make it an interesting research subject. HSV-1 has been and is used as tool and model to study translocation of proteins, synaptic connections in the nervous system, membrane structure, gene regulation, gene therapy, cancer therapy and a lot of other biological problems [4].

1.4.1 Virion Structure

HSV virion consists of four elements [4, 6]: an electron-dense core, an icosahedral capsid around the core, an amorphous tegument and an outer envelope containing glycoprotein spikes. Recently the virion structure of HSV has been described in great detail, thanks to cryo-electron tomography data [7] (Fig. 1.2).

The virion consists of a pleiomorphic membrane-bound particle. It is generally spherical. The bilayer membrane was visualized as a continuous smoothly curved surface (5 nm thick). The virion has a diameter that ranges from 170 to 200 nm, averaging 186 nm and an array of spikes protrude from each viral particle, making the full diameter on average, ~ 225 nm.

The core contains the double stranded DNA genome in a liquid crystalline state; it is densely coiled and complexed with the polyamines spermidine and spermine that neutralize the DNA's negative charges and confer a toroidal shape to the viral genome [4]. The capsid consists of 162 capsomers arranged in a T=16 icosahedral symmetry and an intermediate layer organized in a T=4 lattice. The outer and the intermediate layers are organized so that channels along their icosahedral twofold axes coincide, forming a direct pathway and potential channel between the DNA layer and the exterior of the virion [4, 8]. The outer shell of the capsid is composed of four viral proteins, VP5 (UL19), VP26 (UL35), VP23 (UL18), and VP19C (UL38). The nucleocapsid occupy an eccentric position: on one side (the proximal pole), it is close to the envelope; on the other side (the distal pole), it is separated by 30 to 35 nm of tegument. The capsid occupy about one-third of the volume enclosed in the envelope [7]. The tegument is largely unstructured and it appears to consist of a reticulum of particulate density. Some tegument components appear polymeric; some cellular filaments resembling actin were incorporated [7]. Tegument is composed of at least 20 viral proteins, among which the most notable are VP16 (UL48) the virion transactivator protein, the virion host shut-off (VHS) protein (UL41), VP1-2 (UL36) a very large protein. Highly purified HSV virions contain also cellular and selected viral gene transcripts [4]. The envelope consists of a lipid bilayer with approximately 11 different viral glycoproteins (gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL and gM) embedded in it and the copy number of each glycoprotein can well exceed 1,000 per virion. HSV acquires the envelope lipids from its host, but the process by which HSV acquires its lipid envelope has given rise to disputes. Some studies

suggested that virion lipids are similar to those of cytoplasmic membranes and different from those of nuclear membranes of uninfected cells [9]. The envelope acquisition and the subsequent viral egress have been proposed to follow different mechanisms [10]: a) single nuclear envelopment model, b) the dual envelopment pathway (de-envelopment-re-envelopment pathway), c) single cytoplasmatic envelopment. Currently, the third mechanism is the most popular model of HSV maturation and exit. The envelope surface contains numerous spikes, which are arranged in a non-random way: they usually are few and scattered at the proximal pole and densely packed around the distal pole. This distribution could reflect functional associations such as local clustering of glycoproteins which have to make contact with several receptors during cell entry [11].

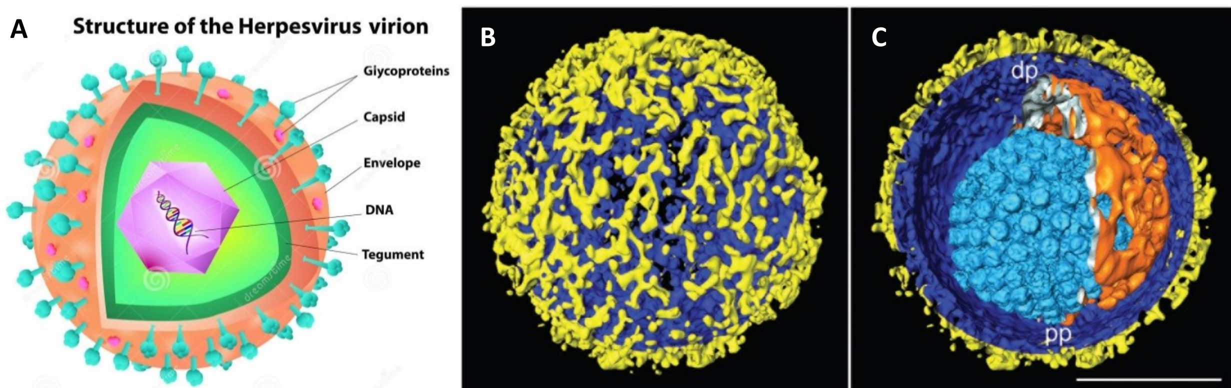

Fig. 1.2: **Structure of HSV1 virion.** A) Schematic representation of HSV-1 virion and its component: the viral circular dsDNA genome,the icosahedral capsid, the amorphous tegument, the external envelope containing glycoproteins (dreamstime.com). B-C Segmented surface rendering of a single virion tomogram. B) Virion outer surface with glycoproteins (yellow) protruding from the membrane (blue). C) Virion section. The capsid (light blue), the tegument (orange) and the envelope (blue and yellow). Scale bar, 100 nm [7]..

1.4.2 HSV-1 genome organization

The HSV-1 genome, enclosed in the nucleocapsid, is linear, double stranded DNA wrapped as a toroid or spool [12]. In vivo, the HSV-1 genome can assume at least three different conformation: linear, circular, and concatemeric. In the virion, genome is linear, but circularizes rapidly in the absence of protein synthesis after it enters the nuclei of infected cells [12, 13]. Complete sequencing of the HSV-1 genome described the genome as 152,261 bp, with a G+C content of 68%.

The HSV-1 genome consists of two unique sequences covalently joined, designated as U_L (long) and U_S (short); each region is flanked by inverted repeats: the repeats of the L component are designated ab and b'a', while those of the S component are a'c' and ca. The number of a sequence repeats at the L-S junction

and at the L terminus is variable and their sequences are highly conserved. The HSV genome can then be represented as:

aL an b – U_L - b′ a′m c′ - U_S – c aS

where aL and aS contain direct repeats [10]. In the viral genome there are three origins of replication: oriL located in UL, and two copies of oriS located in the flanking region of US.

The HSV-1 genome undergoes inversions that result from recombination events mediated by the viral DNA replication machinery. These events generate four genomic isomers with L and S sequences inverted relative to one another (prototype) in equimolar amounts [10, 14]. Herpes simplex virus DNA contains about 90 unique transcriptional units, at least 84 encode proteins, several stable non coding RNAs and miRNAs [15]. The majority of herpesvirus genes contain: a promoter region 50 to 200 bp upstream of a TATA box, a transcription initiation site 20 to 25 bp downstream of the TATA box, a 5′ untranslated leader sequence of 30 to 300 bp, a single major open reading frame (ORF) with a translation initiation codon meeting the host requirement for efficient initiation, 10 to 30 bp of 3′ untranslated sequence, and a polyadenylation signal with standard flanking sequences. Each viral transcript encodes a single protein with three known exceptions (ORF P - ORF O, UL26, UL3). Many clusters of transcriptional units are 3′ coterminal (arranged either head-to head, head-to-tail, or tail-to-tail). Several examples exist of transcriptional units wholly embedded in the coding sequence contained in the larger transcript. Some of the expressed ORFs are antisense to each other, few of the transcripts accumulating in infected cells arise as a consequence of splicing of RNA and several transcripts appear not to encode proteins (latency associated transcripts - LAT) [10].

1.4.3 Functional organization of HSV genome

HSV-1 genes are divided into three kinetic classes, expressed sequentially: α or immediate early; β or early and γ or late (Fig. 1.3).

The α genes map near the termini of the L and S components, with the exception of α0 and α4, which map within the inverted repeats of the L and S components, respectively. Immediate early genes are expressed shortly after infection, approximately from 2 to 4 hours post infection. It is not required prior protein synthesis, but it is necessary the viral tegument protein VP16, which promotes α genes transcription: in the nucleus VP16 interacts with host cell octamer binding protein 1 (Oct1) and host cell factor (HCF1) at the GnTAATGArTTC response element in the promoters of α genes. Moreover, the VP16/Oct1/HCF1 complex recruits the lysine specific demethylase 1 (LSD1), belonging to the dynamic repressor complex CoREST/REST, allowing the transcription initiation of α genes [16]. The α genes encode six proteins

designated as infected cell protein (ICP) ICP0, ICP4, ICP22, ICP27, ICP47 and US1.5. They subvert the cellular machinery by inhibiting transcription, immune response (block of interferon pathway), RNA splicing, RNA transport out of the nucleus and protein synthesis. Futhermore some of the immediate early gene promote the transcription of β and γ genes, facilitating the transition from cellular to viral gene expression.

β and γ genes are distributed in UL and US sequences, with the exception of ICP34.5, located in the repeated sequences flanking the L component. The expression of the β genes takes place from 4 to 8 hours post infection and requires at least the presence of functional ICP4, which acts both as a repressor and as a transactivator. The β genes encode proteins involved in replication of the viral DNA and nucleotide metabolism and they can be divided into two subgroups: β1 and β2. β1 genes are expressed within a short time after, or almost concurrently with, the onset of synthesis of α proteins (for exemple ICP8, UL29, single strand binding protein and ICP6, UL39, the large subunit of ribonucleotide reductase). The β2 genes are expressed later after α protein synthesis and are exemplified by UL23 encoding TK. Other essential β genes are UL30, UL42, UL52, UL9, UL8 and UL5 which encode proteins of the helicase–primase complex and DNA polymerase. Most of β proteins localize to the nucleus and assemble onto the parental viral DNA molecules in structures called prereplicative sites located near ND10 bodies, dynamic structures consisting of a large number of proteins [16]. Prereplicative sites attract a variety of histones, histone-modifying enzymes, coactivators and corepressors and then they develop in the replication compartment.

The γ genes expression occurs after viral DNA synthesis has started and their expression is enhanced by viral DNA synthesis. They are subgrouped into γ1 genes, early/late or leaky late, and γ2 genes, late or true late. The difference between β2 and γ1 genes is that inhibitors of DNA synthesis reduce γ1 but not β2 gene expression. The γ genes encode structural proteins of mature virions and tegument components. γ1 genes (ICP5, glycoproteins gB, gD and ICP34.5) are expressed early in infection and are stimulated by viral DNA synthesis whereas the γ2 genes (UL44, glycoproteins gC, UL41, UL36, UL38 and UL11) are expressed late in infection and are not expressed in the presence of effective concentrations of viral DNA synthesis inhibitors.

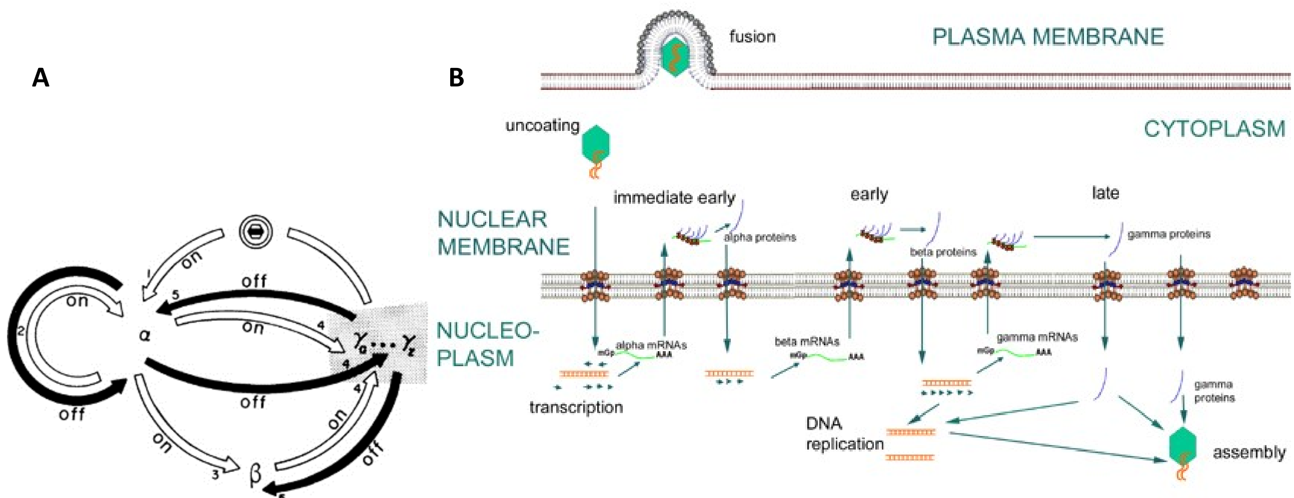

Fig. 1.3: **Schematic representation of the regulation of HSV gene expression.** A) white arrows: events that turn gene expression "on"; black arrows: events that turn gene expression off. (1) VP16 - αTIF stimulates α gene expression (2) α proteins autoregulate α genes expression and (3) stimulate transcription of β gene. (4) α and β proteins turn on γ genes transcription. (5) Late infection, γ proteins turn off α and β gene expression. B) HSV replication in host cell.

1.4.4 Brief overview of virus replication, assembly and egress

To initiate infection, the virus must attach receptors on the cell surface. Fusion of the envelope with the plasma membrane rapidly follows the initial attachment. HSV can follow two pathways to enter cells: 1) binding to cell membrane receptors, followed by fusion at the plasma membrane; 2) endocytosis of the enveloped capsid and receptor-dependent fusion of the enveloped virus with the membranes of the endocytic vesicle [17]. The de-enveloped tegument-capsid structure is released into the cytoplasm and then transported to the nuclear pores, taking advantage of the cellular microtubule network, where DNA is released into the nucleus and it is circularized; in this compartment viral genome is transcribed, replicated and new capsids are assembled. The viral DNA is transcribed by host RNA polymerase II with the participation of viral factors at all stages of infection. The synthesis of viral gene products are tightly regulated as described in the previous section. After α and β gene expression, UL9 binds to specific elements in either oriL or oriS and begins to unwind the viral DNA (Fig. 1.4). Then UL9 recruits the ssDNA binding protein ICP8 to unwound portion of viral DNA and this complex recruit to replication forks the remaining five proteins essential for the viral replication: the helicase-primase complex of three proteins, UL5, UL8, and UL52, viral DNA polymerase catalytic subunit (UL30) and its processivity factor (UL42). The helicase-primase and viral DNA polymerase complexes assemble at each replication fork and initiate theta form replication. Through an unknown mechanism, replication switches from theta form to the rolling circle form. The rolling circle replication forms long head-tail concatamers of viral DNA, which become cleaved into individual units during packaging of viral DNA into capsid [10].

Assembly occurs in several stages. After the γ capsid protein synthesis, the capsid assembly occurs in the infected cell nucleus. At the initial stages, VP5, the major capsid protein, VP26, located on the outer tips of hexons, and VP23, a triplex protein, are located in the cytoplasm. Other two viral proteins, pre VP22, a scaffolding protein, and VP19C participate in this process. When the complex enters the nucleus, the capsid proteins are added as hexons and pentons to form a partial capsid together with scaffolding proteins. First, there is the assembly of empty shells containing an internal scaffolding, then, during DNA encapsidation, the scaffold is lost in a process that requires HSV DNA concatemer cleavage into monomers and their packaging. The viral DNA has signals for cleavage and packaging, located in the a sequences, named pac1 and pac2. The assembled capsid can then proceed to the egress from the cell.

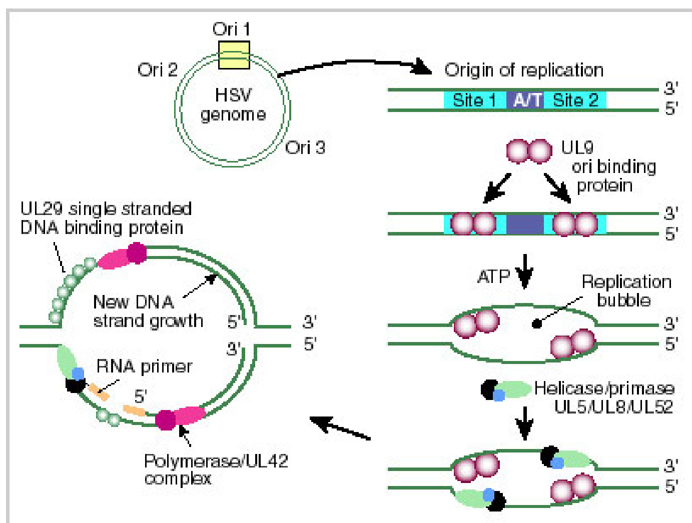

Fig. 1.4: **Schematic representation of the HSV genome replication in the nucleus.** UL9 binds to ori sequences and begins to unwind the viral DNA with the ssDNA binding protein ICP8; the complex recruit to replication forks the helicase-primase complex UL5, UL8, and UL52, viral DNA polymerase catalytic subunit UL30 and its processivity factor UL42. The rolling circle replication forms long head-tail concatamers of viral DNA

The maturation by addition of viral and cellular tegument proteins and the final envelopment occurs in the cytosol. Thus, herpesvirus nucleocapsids have to cross the nuclear envelope. Intranuclear herpesvirus capsids display a diameter of c.125 nm, which is too large to cross through intact nuclear pores. Two models of virus exit have been proposed (Fig. 1.5):

- the single envelopment model: virions acquire an envelope by budding of intranuclear capsids at the inner nuclear membrane followed by lumenal transport of these enveloped particles through the endoplasmic reticulum and the secretory pathway to the cell surface for release;

- the envelopment - de-envelopment - re-envelopment pathway: intranuclear capsids acquire a (primary) envelope by budding at the inner nuclear membrane, resulting in the formation of primary enveloped virions residing in the perinuclear space; their envelope fuses with the outer nuclear membrane (de-

envelopment), thus releasing the nucleocapsid into the cytoplasm for further maturation, like the acquisition of the tegument; finally, virions undergo a secondary envelopment (re-envelopment) by nucleocapsid budding into a trans-Golgi compartment, or trans-Golgi network, or into an endosomal compartment.

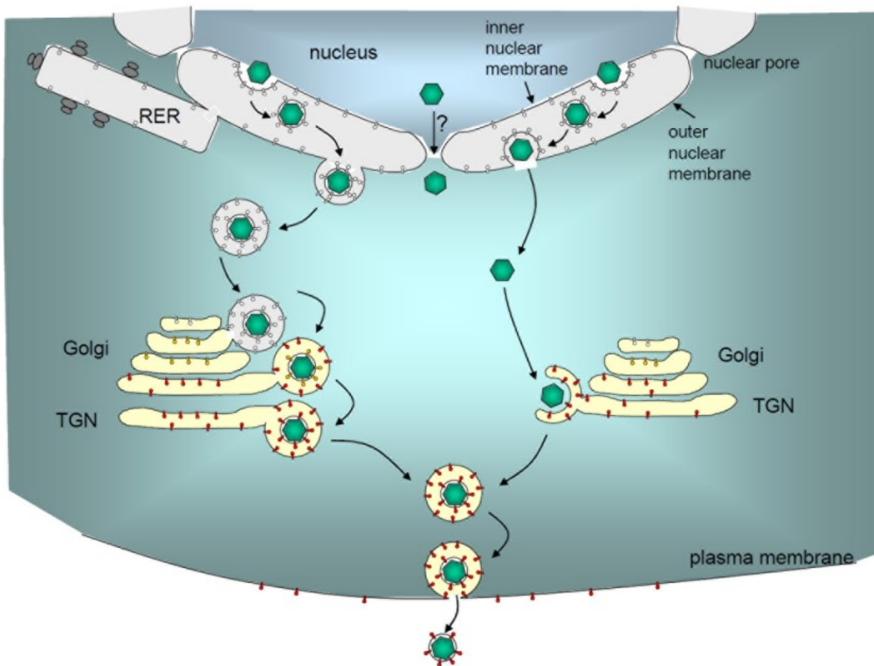

Fig. 1.5: **Schematic representation of the two alternative pathways of herpes virus egress.** On the left, the single envelopment pathway, on the right the envelopment - de-envelopment - re-envelopment model.

1.4.5 Latency

In neuronal cells, HSV-1 virions can either start lytic replication or enter the latent state. Latently infected neuronal cells remain alive, and HSV-1 latency lasts for lifetime. In neuronal cells the viral genome is maintained in episomal form, bound to histones: it is maintained in a repressed state with the exception of the latency-associated transcripts (LATs) and a set of microRNAs (miRNAs) derived from LAT or its precursor RNA, and no replicating virus is observed [16]. LAT gene is localized within the inverted repeat component that brackets the unique long segment. It is transcribed in an 8.3 kb precursor present at low copy (minor LATs) which, upon splicing, generates a series of stable non polyadenylated RNAs (major LATs, 2 kb and 1,5 kb in size) that accumulate at high levels in the nucleus [18]. LATs and miRNAs have a role in the maintenance of latency but not in its onset. No viral product expressed from LAT has been identified nor its role in HSV-1 latency. For these reasons LAT has been proposed to “protect” neurons from apoptosis. In latently infected cells VP16/HCF1 complex is not translocated to the nucleus, preventing the transcription of viral genes. Several lines of evidence show that HDAC-1 or HDAC-2/CoREST/LSD1/REST repressor complex have a role in silencing HSV DNA in neurons.

1.5 Entry of Herpes Simplex Virus 1

Entry in the host cell is a critical step in the HSV life cycle and it determines the tropism and pathology of each member of the herpesvirus family. Unlike smaller enveloped viruses, which possess only one or two fusion glycoproteins, HSV-1 encodes a multipartite entry machinery with distinct and specialized glycoproteins (gB, gC, gD, gH/gL), which act as ligands for receptors on the surface of the target cell; the complexity of this system prevents the indiscriminate activation of the fusion apparatus [19]. HSV enters cells by fusion of the envelope with the plasma or endocytic membranes. Membrane fusion is an energetically unfavorable and not spontaneus process. According to the stalk hypothesis of fusion, a highly curved bridge must form between the two bilayers for fusion to occur; following a local membrane bending, which creates a first site of contact: the monolayer rupture allows mixing of lipids, which develops in a hemifusion stalk. Subsequently, the radial expansion of the stalk leads to fusion pore formation, whose enlargement leads to complete fusion [20]. In virus entry, glycoproteins called fusion proteins provide the energy necessary for membrane deformation and bending. Upon appropriate triggering, the fusion protein interacts with the target membrane through a hydrophobic fusion peptide and undergoes a conformational change that drives the membrane fusion reaction.

The entry route of HSV differs from cell to cell, and it is the cell which is primarily responsible to choose the entry pathway for the virus and it routes HSV to the appropriate site on the cell membrane. For example, αvβ3-integrin, an epithelial cell integrin, routes HSV virions to cholesterol-rich rafts (lipid rafts); this pathway of entry requires dynamin2, proceeds to acidic endosomes, and is independent of caveolin [21].

The process of viral entry consists of four steps: attachment, recognition of cellular receptor by a viral glycoprotein, triggering of fusion and fusion execution.

The first interaction between the virus and the cell plasma membrane is represented by the binding of gC and gB to glycosaminoglycans (GAGs) portions of heparan sulfate (HS) or chondroitin sulphate [22, 23]. Heparan sulfate is a member of the glycosaminoglycan family of carbohydrates, closely related in structure to heparin, and widely distributed on cell surfaces in mammals, which consists of a repeating disaccharide unit, expecially glucuronic acid (GlcA) linked to N-acetylglucosamine (GlcNAc), variably sulfated. HSV infectivity is enhanced by the non-essential binding with heparan sulfate, and even if this binding is not specific, it helps to create multiple point of adhesion, concentrating virions at the cell surface; moreover this binding is reversible, since the detached virus is still infectious [19].

The role of glycoprotein C is to increase the efficiency of virus binding by approximately 10 fold and to confer greater efficiency for virus attachment to cell surfaces; gC is not essential for either virus entry or replication and if absent its function can be carried out by gB.

Attachment to the host cell may take place at filopodia-like membrane protrusions: in some cellular types, HSV-1 virions induce the formation of these actin-rich structures, in a process in which proteins of the Rho

GTPase family are involved, and virions are transported to the cell surface. This extracellular transport of virions is called “surfing” and gB seems to regulate it, as it binds to the heparan sulfate exposed on the filopodia, allowing the travel of virions to the cellular body. Although not essential, viral surfing increases infection efficacy by directing the virus to regions of cell membrane regions enriched in receptors, where gD can act [24].

gD interacts with at least three alternative receptors: nectin-1, herpesvirus entry mediator (HVEM or HveA, herpesvirus entry mediator A) and O-sulphated (3-O-S) moieties of HS (Fig. 1.6). These receptors are expressed in a wide variety of human cell types and tissues [25] and gD, after viral attachment, binds one of its specific receptors in an irreversible manner to initiate the fusion process. It is possible that the ability of HSV-1 to bind alternative receptors could explain the capacity of this virus to entry into a wide range of different cell types.

gD exibits a similar affinity to nectin-1 and HVEM (10^{-6} M), thus this property can not be considered discriminating for the preferential use of a receptor or another [26]. Probably the availability of multiple receptors allows the virus to increase the chance of a successful infection and spread in the human host. The binding of gD to one of its receptor induces a conformational change to gD, which triggers the fusion process [25] by recruiting the other three essential glycoproteins, gB, gH, gL. The fusion with the plasma membrane or endocytic vesicle of the target cell is executed by the three glycoproteins, which constitute the conserved fusion machinery across the herpesvirus family. There is an extensive literature about gH/gL and gB glycoproteins, their role in fusion execution and reciprocal interactions. For a long period, gH has been considered a potential HSV-1 fusogen, but its crystal structure reveals no homology with any known fusion proteins [27]; even the structure of gB has been solved and, unlike gH, it exhibits a considerable homology to gG, the vesicular stomatitis virus (VSV) fusogen, and to viral fusion glycoproteins in general [28, 29]. Considering the structure of the gH/gL complex, it is probable that gH/gL may activate gB for fusion rather than execute directly the fusion, but how the two glycoprotein cooperate is still unclear [28]. On this basis, given the number of proteins involved in the entry pathway, it is clear that a fine communication is necessary among all the glycoproteins.

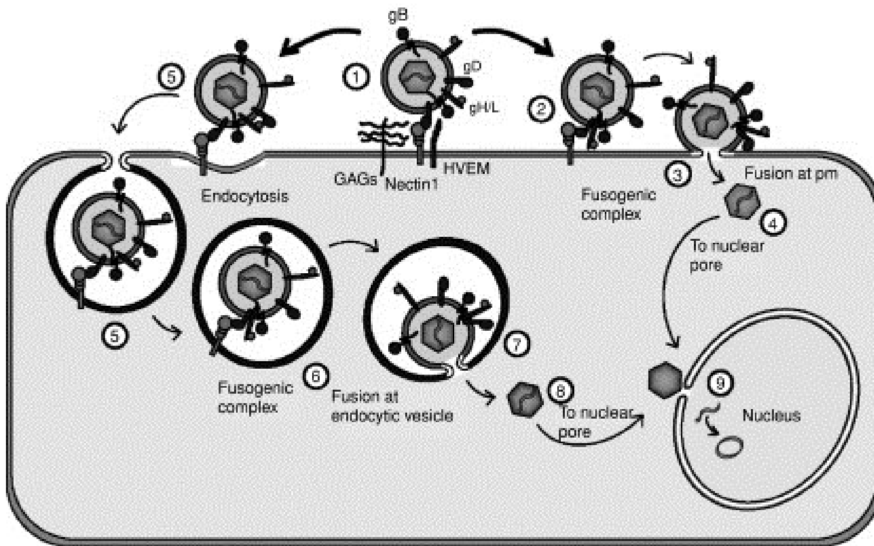

Fig. 1.6: **Schematic drawing of HSV entry.** Entry can occur either by endocytosis (left), or by fusion at the plasma membrane (right). Following attachment to cells, gD binds to a cellular receptor (1), and after a conformational change it recruits the gB, gH and gL in an active fusogenic complex (2), that executes the fusion between viral envelope and cellular plasma membrane (3). The nucleocapsids are transported to the nucleus (4, 9). Bound virions can also enter cells by endocytosis (5). Following acidification/maturation of the endocytic vesicles, it occurs the formation of a fusogenic complex (6), followed by membrane fusion (7). Finally nucleocapsids are transported to the nucleus (8, 9) [30].

1.5.1 Viral glycoproteins and cellular receptors involved in attachment and entry

Glycoprotein C and Heparan sulphate

The UL44 γ gene encodes glycoprotein C (gC), a 511 aa mucin-type glycoprotein, N- and O-glycosilated [31]. gC contains a 25 aa signal sequence at the N-terminus, a 453 aa extracellular domain, a 23 aa transmembrane anchoring domain and a short 10 aa C-terminal cytoplasmatic tail. As anticipated above, gC is the major actor during viral attachment, since it facilitates the adsorption of virions into cells by binding to heparan sulphate's glycosamminoglycans or to chondroitin sulphate [30]. Although gC is dispensable for the infection of cultured cells, its presence can increase the efficiency of virus binding about 10-fold, at least for HSV-1, so its main function seems to be the concentration of the virus on cell surfaces, enabling the more stable interaction of gD with an entry receptor. gC has long and slender projections, which are the most externally exposed structures of the virion [32]. gC role is confirmed by the fact that monoclonal antibodies for gB, gD, and gH inhibited HSV-1 penetration but had little or no effect on attachment; moreover, virions deleted of these glycoproteins are defective in penetration but bind to cells normally. Other property of gC is its ability to bind the C3b component of the complement and, in the context of the immune response, gC is a major viral antigen which elicits a strong humoral and cellular immune response [33].

Glycoprotein D and its receptors: nectin, HVEM, 3-O-S - HS

The US6 γ gene encodes a 394 aa precursor to glycoprotein D, which consists of a 25 aa signal peptide, a long 315 aa ectodoamin containing three glycosylation sites, 6 cysteine residues that form three disulfide bonds (Cys66 - Cys189, Cys106 - Cys202, and Cys118 - Cys127) and three N-linked oligosaccharide attachment sites, a 22 aa transmembrane domain, and a 32 aa C terminal cytoplasmic domain [34] (Fig. 1.7 A). gD has a key role in viral entry: it is the viral ligand for all known HSV-1 entry receptors and the main determinant of viral tropism [35], and consequently it is absolutely required for virus entry and virus-induced cell-to-cell fusion. Recent studies show that the interaction of gD with its receptors is not only required to activate the fusion machinery but, in some cell types, also to direct the virus to the endocytic pathway [36].

The receptors of HSV-1 gD are HVEM, nectin-1, nectin-2, and 3-O-sulphated heparan sulphate, and it has been demonstrated that cells expressing this glycoprotein are resistant to HSV infection in a dose-dependent manner due to the saturation of the corresponding entry receptor [37] (Fig. 7B).

The structure of gD has been solved both for gD alone, encompassing amino acids up to 259, and for gD bound to HVEM (up to aa 285 of gD) and nectin-1 (up to aa 285 of gD) [38-40] and this permitted a significant progress in our understanding of HSV entry.

The ectodomain of gD is required and sufficient to enable HSV entry into cells and structural studies show that it is composed of three regions with peculiar structural and functional characteristics: the N-terminal (res 1-37), carrying the receptor-binding sites, the central core (res 56-184), and the C-terminal carrying the pro-fusion domain [41] (Fig. 7C).

The N-terminal extension contains all the contact residues to HVEM and is disordered and flexible in the crystals of gD alone, but in the complex gD-HVEM it forms a hairpin loop and an intermolecular β-sheet, which is believed to stabilize the complex (Fig. 7D). Furthermore the first 32 amino acids are involved in binding 3-OS-HS but are dispensable for nectin-1 binding [42].

The core assumes an Immunoglobulin Variable (IgV) fold and is made of β-strands forming two antiparallel β-sheets with an unconventional disulfide-bonding patterns [38]. It is followed by a helix (α-helix 3) of 17 aa and a long flexible proline rich region.

The Ig core of gD seems to function as a scaffold, necessary to connect the functional C- and N-terminal regions: codons 61–218 do not encode executable functions required for viral entry into cells [35], and substitution of this region with a heterologous protein, such as a scFv to HER2, does not impair the functionality of the protein [43].

The pro-fusion domain (PFD), localized in the C-terminus of the ectodomain (res 250/260-310), is involved in triggering the membrane fusion and in the interaction with other viral glycoproteins [41]. PFD is responsible for the “switch-on” mechanism that allows activation of the virion near the cell surface and is

essential for virus entry, as a matter of fact insertions or deletions in this region impair infection and cell-cell fusion and among the soluble form of gD only gD285 and gD306 are able to rescue the infectivity of a gD null virus whereas gD260 is not able. In the gD-receptor complex the PFD domain has a flexible conformation, while in unbound gD it folds back around the core towards the N-terminus, in an auto-inhibited conformation [19]. This is also confirmed by the observation that the deletion of the C-terminal domain of gD increases the receptor affinity of the glycoprotein, compared to the full length molecule [39]. Concerning residues involved in Nectin-1 binding, the recent gD/Nectin-1 solved structure permitted to identify the contact region between the two molecules and show key residues (Y38, H39, Q132, D215, L220, P221, R222, F223) that make contact with the receptor [39] (Fig. 7E).

From the superimposition of crystal structure of gD with its receptors, it can be observed that in the complex gD/HVEM the residues involved in gD-nectin1 binding are hidden [39]; moreover soluble forms of each receptor can block virus entry mediated by the other receptor [44], so it is very probable that the two receptors interfere among themselves in the binding with gD.

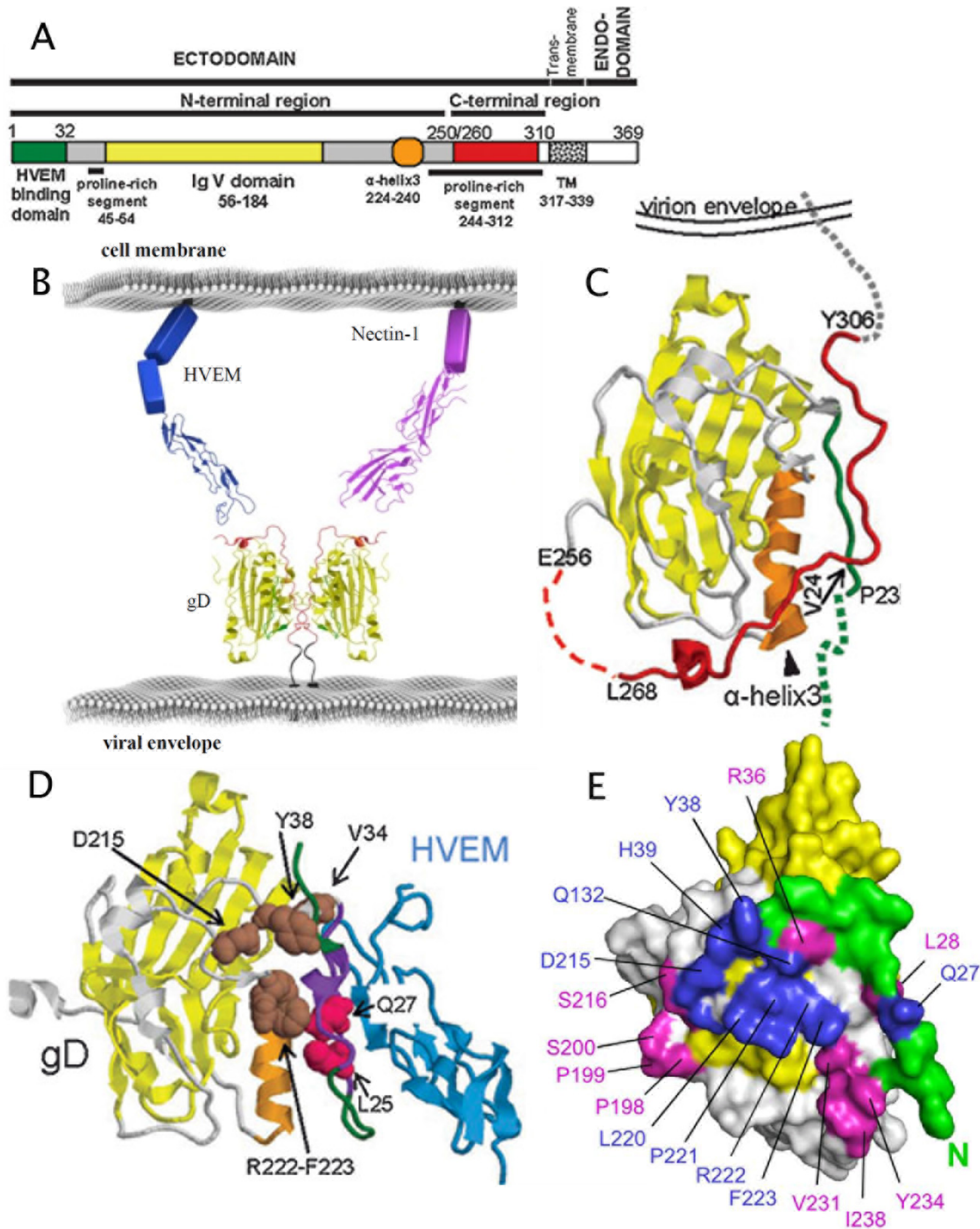

Fig.1.7: **Structure of HSV-1 glycoprotein gD.** A) Schematic representation of domains in mature gD. The colors are the same in panel A, C and D [Campadelli, 2007]. B) 3D structure of gD and its receptors HVEM and Nectin-1 [45]. C) Crystal structure of unbound gD ectodomain, where HVEM binding site (green) is unstructured. D) Crystal structure of gD/HVEM (light blue) complex; the N-terminal region of gD forms a hairpin that contacts the receptor; in this representation, critical residues for nectin-1 binding are represented as brown space fill (V34, Y38, D215, R222, F223), while arrows point to the contact residues to HVEM (Q27, L25). E) Surface representation of gD that shows the interface with nectin-1, colored in blue and magenta; mutation of “blue” and “magenta” residues affects nectin-1 binding; most of the contacts involve residues from the gD C terminal extension and, Y38 and Q27 from the N-terminal extension [39].

Nectins: Nectins are immunoglobulin (Ig)-like Ca2+ dependent cell adhesion molecules (CAMs) expressed in different cell types, like epithelial cells, neurons and fibroblast, and involved in a lot of cellular activities like cell-cell adhesion and polarization, differentiation, movement, proliferation and survival. It has been identified four distinct nectins, numbered from 1 to 4, which have two or three splicing variants indicated with Greek letters [30]. Originally nectin1 and nectin-2 were isolated as poliovirus receptor-related proteins and were called PRR1 and PRR2 respectively; only later it was observed their involvement as receptors for HSV, and so they were renamed HveC and HveB. Nectins form homo-cis-dimers on the plasma membranes and trans-dimers with nectins present on the adjacent cell.

Almost all nectins have an extracellular region with one Ig V-like domain, two C-like Ig domains and 8 potential sites for N-linked oligosaccharides, a single transmembrane region, and a cytoplasmatic tail region, that contains a conserved motif involved in the binding of the filamentous (F)-actin binding protein afadin [46]. The binding afadin – nectin is not necessary for nectin dimerization, but links nectins to the actin cytoskeleton and activates signalling pathways that involve several extracellular and intracellular factors like small GTPases (Ras, Rac, CDC42) [46].

From the crystal structure of gD bound to nectin-1 (Fig. 1.8) it was observed that the binding site of gD extends from aa 1 to 250, requiring exclusively the β sheets of the Ig V-like domain of the receptor [39]. Phe129 is a key residue in nectin-1, since it protrudes into a pocket formed by residue from the long α3-helix of gD and the side chain of Phe223, and the destruction of this interaction with mutation of Phe129 to alanine prevents nectin-1 binding to gD and HSV entry.

The crystal structure of the complex gD/nectin-1 highlighted similarities between this heterodimer and the structures of nectin-1 dimer, in particular it can be observed that gD contacts many of the same residues involved in nectin-1 dimerization, demonstrating that gD binding impairs normal cellular conformation of nectin-1 and thus interferes with its role in mediated cell adhesion.

In the context of viral entry, particularly interesting is the connection between nectin-1 and integrin: recently it has been observed the ability of the epithelial/endothelial αvβ3-integrin to relocalize the nectin-1 receptor to cholesterol-rich microdomains, platforms for a number of Toll-like receptors, independently by HSV-1 infection. In this way, αvβ3-integrin acts as the cellular factor that routes HSV to the acidic endosomal pathway, and moreover the presence of TLRs in these membrane regions opens the way to a possible involvement of αvβ3 in the innate immunity [21, 47].

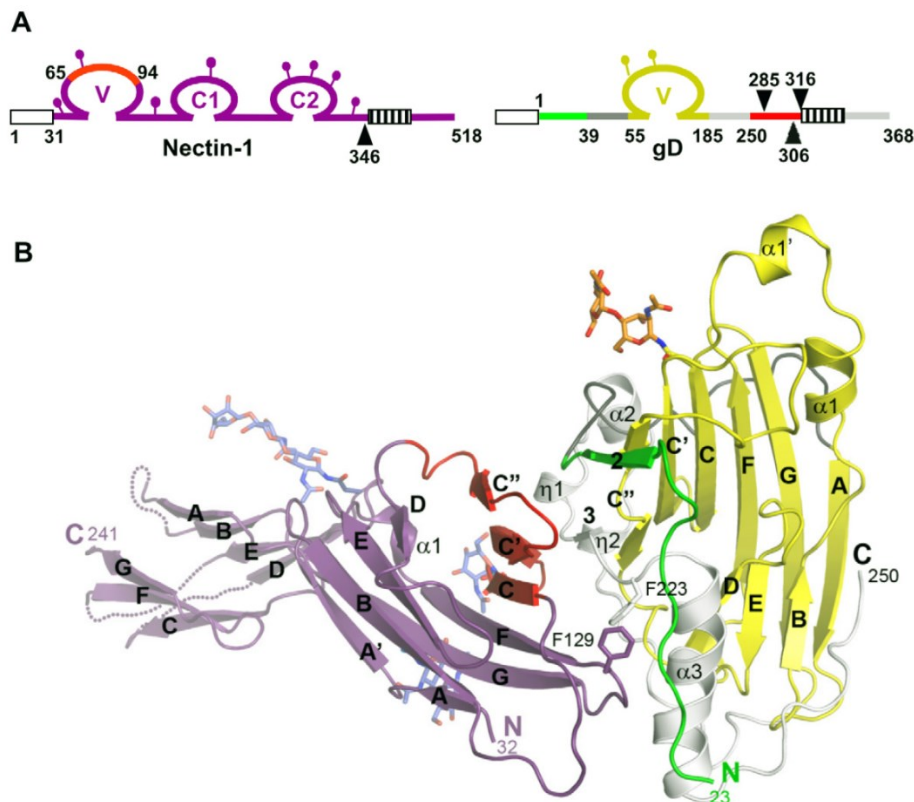

Fig. 1.8: **Structure of the gD/nectin-1 complex.** A) Schematic representation of human nectin-1 and HSV-1 gD. N-glycosylation sites are drawn as lollipops, signals peptides as white boxes, the transmembrane domains as hatched boxes; arrowheads indicate the location of truncations for production of soluble proteins. B) Ribbon representation of the gD/nectin-1 complex. Dotted lines represents unsolved loops [39].

HVEM: HVEM, or Herpes virus entry mediator A (HveA), was identified as a HSV receptor and subsequently was classified as a novel member of the tumor necrosis factor receptor (TNFR) superfamily [48], which groups signaling molecules involved in the regulation of cellular process like cell proliferation, differentiation and apoptosis, cytokine release, and expression of cell surface activation markers [30, 49]. Binding to HVEM triggers the signal and thanks to the cytoplasmic tail, which interacts with several members of the TRAF family (TNFR-associated factor), it leads to the activation of NF-κB, Jun N-terminal kinase, and AP-1 [50]. HVEM is expressed in several cultured cell line, but mainly in cells of the immune system, in particular in activated T-lymphocytes [30, 48]. HVEM binds wild-type gD. The affinity of the binding is of the same order of magnitude as that of nectin1/gD binding, and the interaction requires the same region of gD, i.e., the first 250 residues, or longer [51]. The structure of HVEM is very similar to that of other members of the TNFR family (Fig. 1.9): it consists of an ectodomain with four typical cystein-rich domains (CRD) of ~40 residues each and a cytoplasmic tail with signaling activity [38]. CRD 1 and 2 are involved in the contact with gD, in particular CRD 1, whose residues 35-37 form intermolecular antiparallel β-sheet with gD [38]. A monoclonal antibody that binds CRD1 blocks gD – HVEM interaction, but thanks to systematic structure-based mutagenesis approach 17 residues in CRD 1 and 4 in CRD 2 were observed in

the interface between the two protein, and their mutation abolish the HVEM binding to gD and its function as an HSV -1 receptor [52, 53].

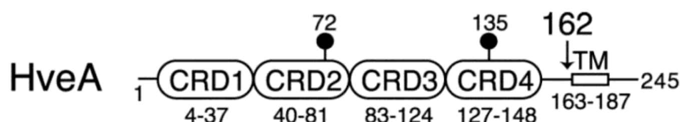

Fig.1.9: **Linear structure of HVEM/HveA.** Diagram of full-length HVEM, with indicated the positions of N-glycosylation sites (lollipops), transmembrane regions (TM), the four cystein rich domains (CRD), and the sites of truncation (arrows) [52].

<u>3-O-sulfated Heparan sulfate:</u> 3-O-sulfated heparan sulfates (3-OS HS) are polysaccharides, containing specific sulfated motifs added by a D-glucosaminyl 3-O sulfotransferases (3-OSTs) on heparan sulfates (Fig. 1.10). 3-O-sulphates are rare substitutions in heparan sulphate, generated by at least six 3-OSTs isoforms identified in humans and mice, some of which are broadly expressed in cells of different origins and tissues, while others are expressed mainly in the brain, or in the skeletal muscle [54].

Heparan sulphate are expressed in a great variety of cell types, but although they are used in the attachment by both HSV-1 and HSV-2, only HSV-1 can bind to the distinct modification sites on 3-OS HS, and this aspects could explain some of the differences in cell tropism exhibited by the two viruses [55].

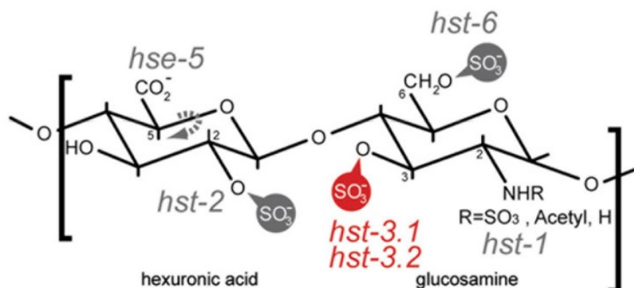

Fig. 1.10: **Chemical structure of 3-O-sulfated Heparan Sulfate.** Heparan Sulfate disaccharide comprising a hexuronic acid (glucuronic acid or iduronic acid) and glucosamine; the site target of HS 3-O-sulfotransferases activity is indicated in red [56].

<u>Glycoproteins gH/gL</u>

The γ genes UL22 and UL1 encodes respectively glycoprotein H (gH) and glycoprotein L (gL). gH is a type I membrane glycoprotein of 838 aa containing an 18 aa signal peptide, a long 785 aa ectodomain, a single 21 aa transmembrane hydrophobic domain close to the C-terminus, and a 14 aa C-terminal cytoplasmic tail. gL is a 224 aa protein with a 25 aa signal peptide, but it does not contain a transmembrane domain, as a matter of fact it is not an integral membrane protein, but it is associated to the membrane and incorporated into virus particles thanks to the interaction with gH [57]; specifically they form a stable 1:1 complex.

In transfected cells, in the absence of gL, gH polypeptide is neither folded nor processed correctly, it remains in the endoplasmic reticulum and undergoes self-aggregation [58, 59]. Similarly, cells infected with

a gL-null virus do not produce virions containing gH. The heterodimer gH/gL is highly conserved among Herpesviridae, it is essential for efficient viral entry and cell fusion, as its deletion produced non-infectious progeny and abolished cell–cell fusion [27, 60], but these virions are still able to attach to the cell surface. Numerous neutralizing antibodies directed to gH have been isolated and in infection their use blocks virus entry but not the binding to the host cell surface [61].

Recently the crystal structure of the complex gH/gL of HSV-2 has been resolved (Fig. 1.11): it has a "boot like" conformation and it is composed of three domains [27]. Domain H1 (res 49-327), in the upper part of the boot, is divided in two subdomains (H1A and H1B) connected by a short linker and it is the site of contact with gL. The central domain H2 (res 332-644) is globular, consist mainly of 13 alpha helices and corresponds to the terminal part of the boot. The C-terminal domain H3 (res 645-797) is located at the toe end of the boot and it is a 10-stranded β-sandwich, with five strands for each part, from which extend many loops. gL has not a regular conformation, in fact only 30% of the protein has a secondary structure, which includes three helices and two β sheets; moreover gL contains two disulfide bonds essential for folding and function of the complex.

Even if gL is necessary for the correct folding and trafficking of gH, it cannot be considered a chaperone protein, because it remains associated to gH also after its maturation. It seems that gL acts as a scaffolding protein for gH, interacting widely with its H1 domain, and so it was proposed that gH and gL need each other to stabilize their conformation.

Before gH/gL crystal was solved, it was proposed that this eterodimer could acts as a fusogen, since it contains structural elements associated with membrane fusion, specifically a hydrophobic α-helix 1 (res 377-397) with properties typical of a fusion peptide and two heptad repeats with propensity to form a coiled coil. Across the Herpesviridae family α-helix 1 is positionally conserved in all the gH orthologs, it is able to interact with biological membranes and to convert a soluble gD (gD 1-260) into a membrane-bound glycoprotein, it can be replaced by fusion peptides derived from glycoproteins of unrelated viruses [62], and finally a peptide with the sequence of α-helix 1 induces fusion of liposomes [63, 64]. Two heptad repeats are capable to interact with each other and to form coiled coils, moreover synthetic peptides homologous to them inhibit virus infection if present during virus entry into the cell [62-64].

According the crystal structure of HSV-2 eterodimer, gH/gL do not resemble any known viral fusogen; in addition the putative fusion peptides are buried helices or β-hairpins that take part in the formation of multistranded sheets, and their removal would affect complex stability. So it has been proposed that gB is the real fusogen and the complex gH/gL behaves as a positive regulator which coordinates the transition of gB into its fusion active state [27]. Since gL binds a region of gH around the hydrophobic α-helix, the role of gL in the eterodimer could be the shielding of the hydrophobic sequence of gH, allowing gH water solubility [62].

Several studies have been carried out to research a possible receptor for gH/gL, since indirect evidences suggest that it could exist, but it has not yet been found. For example, it was observed that CHO cells, which transiently overexpress some integrins, in particular αvβ3 integrin, better adhere to plastic wells functionalized with a soluble form of gH/gL [65]. However other data show that the interaction gH/gL - integrin is not critical since mutagenesis of the RGD motif present in gH did not alter virus entry and cell fusion [21, 66]. Despite this, there are multiple indirect evidences that gH/gL interacts with a cell surface related protein(s), not necessarily an αvβ3 integrin, and that this interaction is critical for entry and fusion of HSV-1 [21].

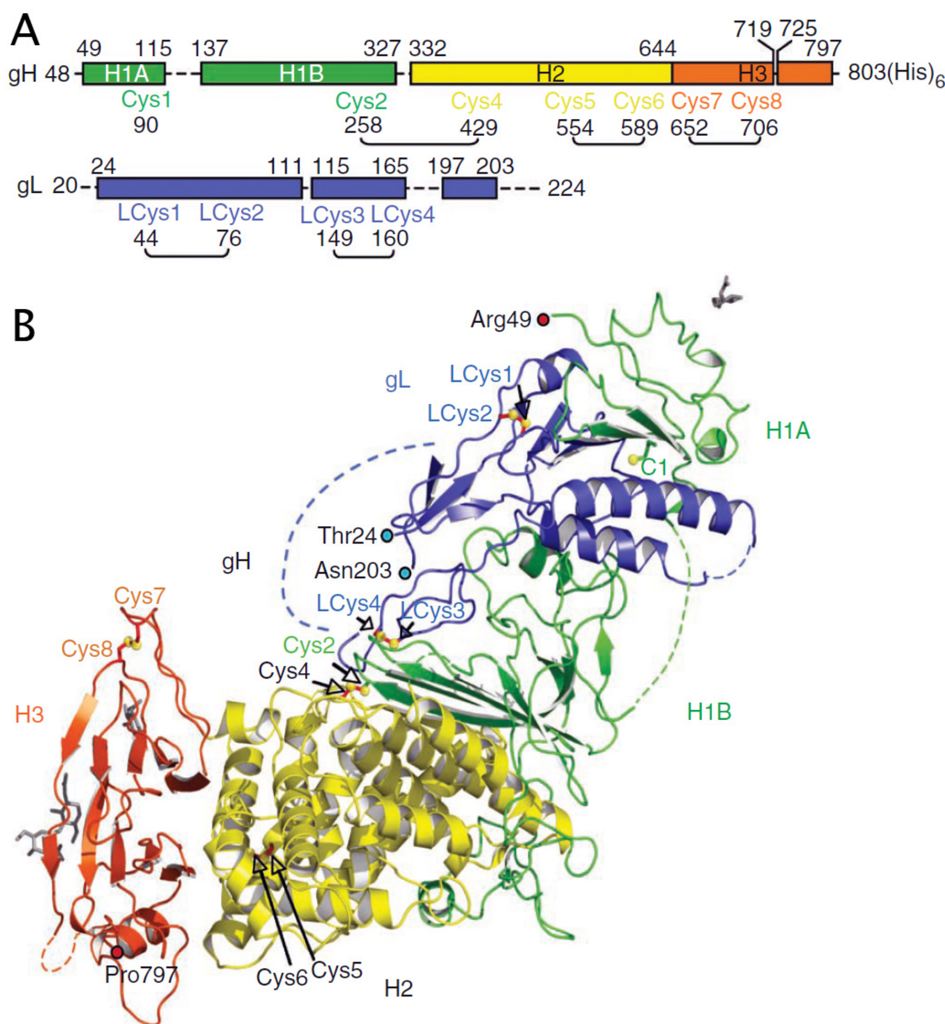

Fig. 1.11: **Structure of HSV-2 gH/gL complex.** A) Schematic representation of gH and gL domains, dashed lines represent unsolved structures, gH domains are in green (H1), yellow (H2) and red (H3), and gL is blue. B) Ribbon diagram of gH/gL showing disordered segments (dotted lines), sugars (gray), cysteins and disulfide bonds (yellow spheres and red sticks) [Chowdary, 2010 – gH/L crystal].

<u>Glycoprotein B and its receptors: PILRα, MAG and NMHC-IIA</u>

The γ gene UL27 encodes glycoprotein B (gB), a 904 amino acid trimeric protein, highly conserved across all subfamilies of herpesviruses. gB is one of the essential glycoproteins in HSV entry and it is involved both in the attachment of the virus to the cellular membrane through the interaction with heparan sulphate, and in

the fusion of the virus with the host membranes, in a process that requires also gH and gL; moreover gB has an anti-fusion activity, which is located in the cytoplasmic tail. The gB binding site for heparan sulphate is located between aa 68-76, but this glycoprotein is also able to interact with other receptors. The first evidence of the existence of specific gB receptors comes from Cohen's laboratory, where it was observed that a soluble form of gB binds to cells lacking heparan sulphate and this binding inhibits HSV-1 infection in some cell lines [67]. Currently, three gB receptors have been identified: the paired immunoglobulin like-type 2 receptor (PILR α), the myelin-associated glycoprotein (MAG), the non muscle myosin heavy chain IIA (NMHC-IIA).

HSV-1 gB is a homotrimeric type I membrane glycoprotein and consists of a wide ectodomain of 696 amino acids, the longest among HSV-1 glycoproteins and N-glycosylated at multiple sites, a transmembrane of 69 aa and a cytoplasmatic tail of 109 aa [28]. The cytoplasmatic tail is involved in the negative regulation of the fusogenic process, specifically through the activity of two alpha helices, containing two endocytosis domains, named YTQV 889-892 and LL871; mutation or deletion of the two alpha helices cause an increase of fusion assay efficiency and the appearance of a syn phenotype due to a block in gB internalization.

From the analysis of gB crystal structure it results a remarkable homology with the postfusion conformation of protein G from vescicular stomatitis virus (VSV) [29], suggesting the involvement of gB in the fusion process. Although neither HSV-1 gB nor VSV gG show a canonical fusion peptide, both glycoproteins are able to trigger the fusion between viral and cellular membranes and this suggested that the two proteins may represent a novel class of fusion glycoproteins [28, 29].

Three gB protomers (residues 103–730) create a trimeric spike, which is stabilized by several points of contact, in particular are present 10 cysteins residues that create 5 disulfide intramolecular bonds [28]. Each subunit of the trimer consists of five domain (named I, II, III, IV, V) (Fig. 1.12).

Domain I (res 154-363), the "base", is a continuous polypeptide chain, which has the fold typical of a pleckstrin homology (PH) domain, that is a β sandwich composed of two nearly orthogonal β sheets, followed by a long loop and a short helix that covers one opening of the β sandwich. It is a typical domain presents in proteins of the cytoplasmic signaling pathways that bind phosphoinositide and peptide; the fundamental role of domain I is stressed by the observation that monoclonal antibodies against it are able to block HSV-1 entry [68]. Domain II, the "middle", is composed of two discontinuous fragments (142-153 and 364-459), that forms a six-strand β barrel similar to a pleckstrin homology domain. Domain III, the "core", contains three discontinuous segments (117-133, 500-572 and 661-669) and in particular it has a long α helix of 44 residues that oligomerizes and forms the central coiled-coil with the other protomers. Domain IV, the "crown", comprises two discontinuous segments (res 111-116, 573-660) linked by a disulfide bond, its structure is not similar to other known structure, and it is fully exposed on top of the trimeric complex. Domain V, the "arm" (res.670-725), is a long extension that stretches from top to bottom

of each monomer and it adjusts into the groove between the core domains of the two adjacent protomers, contributing to the stability of the trimer [28].

Numerous lines of evidences support the view that gB could have an essential role in cell-cell fusion: first of all gB deletion mutant virus produces non-infectious particles which present a post-attachment defect, particular mutations in gB C terminus cause a syncytial phenotype, moreover antibodies to gB have a neutralizing activity and finally gB is necessary in the cell-cell fusion assay, along gD, gH and gL [30]. Initially, thanks to mutational studies, temperature sensitive mutants for viral growth and mutants resistance to antibodies with high neutralizing activity were identified and they allowed to define functional domains in the gB ectodomain, involved in the execution of fusion [69]. However, it was only the determination of gB crystal structure that allowed the recognition of single residues or continuous regions which reside on the trimer surface, on the lateral faces of the spike or on the tip of the crown [28]. In particular, there are three long central α-helices, like class I fusion proteins, and two loops, residues 173 – 179 and 258 – 265, that are structurally homologous to the fusion loops of VSV G and class II fusion proteins, with three essential residues (W174, Y179, A261) identified through structure-based mutagenesis [70].

PILRα: Paired immunoglobulin-like type 2 α is a cell-surface receptor expressed by immune cells, belonging to the "paired receptor" family, a group of high related receptors, wherein a receptor presents inhibitory functions, while the other activating ones. PILR genes are present in most mammals and play an important role in the regulation of immune cell, in particular PILRα exerts an inhibitory signaling activity, carried out by its cytoplasmatic domain, that contains an immunoreceptor tyrosine-based inhibition motif (ITIM) [71]. The interaction of PILRα with gB mediates HSV-1 infection, antibodies anti-PILRα have an inhibitory activity on HSV-1 infection of monocytes, but the only gB-PILRα interaction, in the absence of gD-HVEM binding, is not sufficient to mediate membrane fusion. Accordingly, PILRα behaves as an indispensable co-receptor for HSV-1 entry [71].

MAG: myelin-associated glycoprotein is a cell-surface protein that is expressed in neural tissues, particularly on myelin sheath, and is involved in the regulation of axonal growth. It has been observed that MAG is able to associate with VZV - gB and HSV-1 – gB, it can enhance HSV-1 infection and promote cell-cell fusion when coexpressed with VZV gB and gH/gL. Therefore, it can be inferred that MAG is involved in the fusion of neurotrophic herpesviruses HSV-1 and VZV with cell membrane, in particular of neural tissue [72].

NMHC-IIA: non-muscle myosin heavy chain IIA, is a subunit of non-muscle myosin IIA (NM-IIA), an ATP powered motor protein, localized in the cytoplasm and expressed in several human tissues and cell types, that participates in the movement of actin filaments and in the regulation of cell shape, cell adhesion, vesicular traffic and cytokinesis. In the first step of virus-cell interaction, it was observed a rapid induction of NMHC-IIA expression on cell surface. NMHC-IIA can interact with gB and function as an entry receptor for HSV-1: its overexpression in a cell line resistant to HSV-1 makes these cells susceptible to herpes infection, viceversa knockdown of NMHC-IIA in permissive cells inhibited HSV-1 entry and cell-cell fusion

assay. Furthermore, the treatment of susceptible cells with antibody anti – NMHC-IIA blocks HSV-1 infection and the use of a specific inhibitor of myosin light chain kinase, which regulate NM-IIA redistribution, reduced NMHC-IIA movement and also HSV-1 entry [73]. So, the ubiquitously expressed non-muscle myosin heavy chain IIA can acts as a functional HSV-1 entry receptor that mediates HSV-1 infectivity.

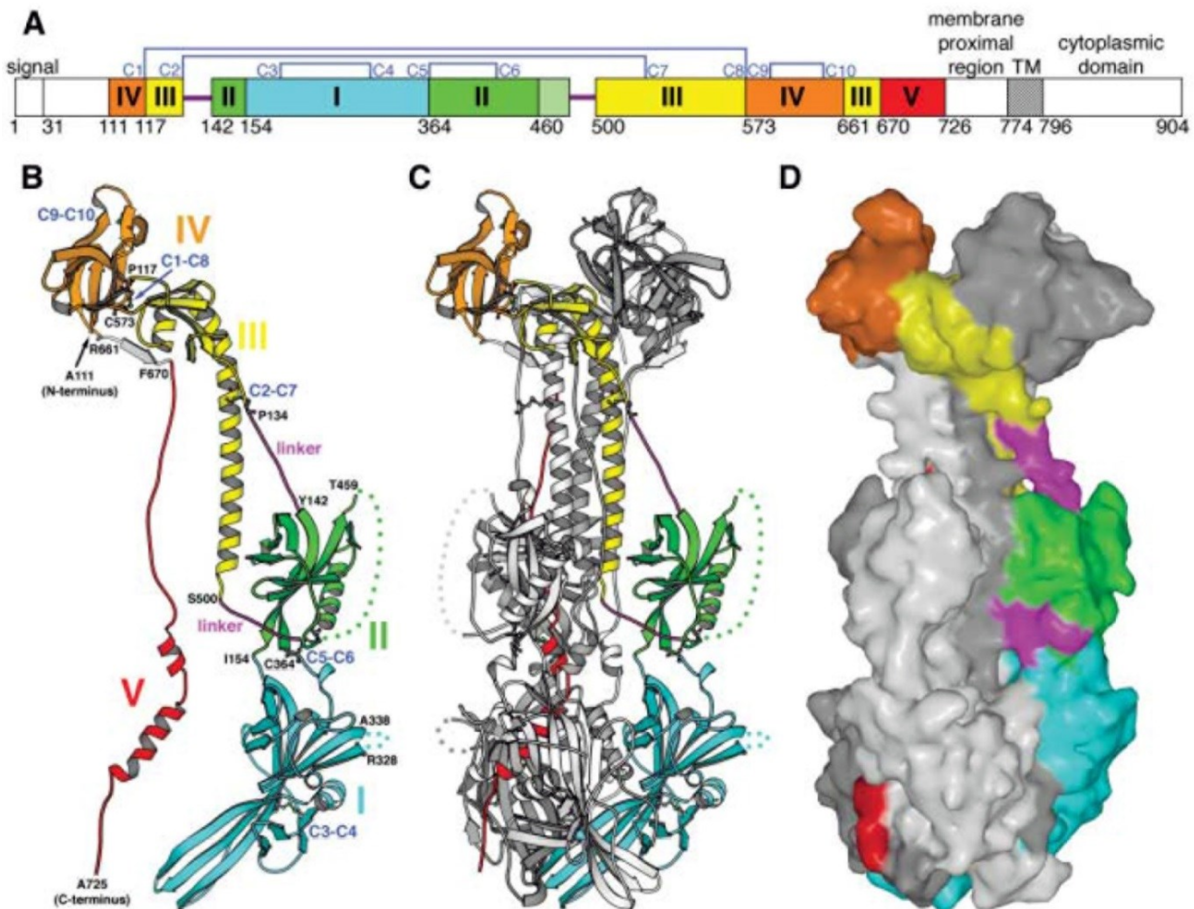

Fig. 1.12: **Structure of HSV-1 gB.** A) Schematic representation of gB domains. B) Ribbon diagram of a gB protomer, where each domain is colored as in A. C) gB trimer. D) Molecular surface of gB trimer, that shows the accessible area of the complex [28].

1.6 Innate Immunity

In vertebrates, immune systems is distinguished in adaptive and innate immunity: the first relies on clonally expanded T and B lymphocytes which express antigen-specific receptors, which are generated by gene rearrangements and hyper mutations; the second is evolutionally conserved, is the first line of defense utilized from organisms in the protection from invading microbial pathogens and is also critical in eliciting the adaptive response.

Both the innate and the adaptive immune systems participate in the immunological control of herpesviruses: in the adaptive immune response CD8+ T cells have a crucial role, while in the innate antiviral immune response, type I interferons (IFNs) and natural killer (NK) cells are essential in the containment of herpesvirus infections. Eukaryotic cells are equipped with receptors that sense the

presence of HSV virion, or some of its products, and initiate a signaling activity which culminates in the cell innate immune response [74].

IFNs are involved in the maturation of DCs: they increase the expression of costimulatory molecules, like CD80, CD86 and CD40, and antigen presentation through histocompatibility complex class I, which in addition promotes cross-presentation of viral antigens. Furthermore chemokines and antigen-specific CD8+ T cell responses are induced by type I interferon, and they lead to the stimulation and recruitment of lymphocytes and monocytes to the site of inflammation. So in general, type I interferons can alter significantly the cell to establish an "antiviral state" by the upregulation of hundreds of effector molecules that directly influence protein synthesis, cell growth and survival.

In the innate response to HSV it is possible to distinguish two temporal waves: the first immediate response activates NF-κB and other lines of defense within a few minutes after infection and it requires only the interaction with virion components, since it can be triggered by UV-inactivated viruses [75-78]; the second wave of NF-κB activation is more sustained, it takes place later, as it requires viral gene products [79-83].

The signaling activity is mediated by Pattern recognition receptors (PRRs) which sense pathogens by detection of pathogen-associated molecular patterns (PAMPs) [84], for example virion proteins, viral genomic DNA, or double-stranded viral RNAs. It can be distinguished PRRs on the plasma membrane and intracellular PRRs (Fig. 1.13): Toll-like receptors (TLRs), membrane-bound receptors localized in the plasma membrane and endosomal compartments; RIG-I-like receptors (RLRs), retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5), intracellular PRRs which detect 5′ triphosphate-panhandle RNA and higher order RNA structures, respectively; five intracellular DNA sensing proteins that is DNA-dependent activator of IFN-regulatory factors (DAI; also known as ZBP1), absent in melanoma 2 (AIM2), RNA polymerase III, leucine-rich repeat flightless-interacting protein 1 (LRRFIP1) and IFNγ-inducible protein 16 (IFI16) [74].

All TLRs and intracellular nucleic acid sensors, except AIM2, induce intracellular signalling pathways that lead to the expression of proteins with pro-inflammatory and microbicidal activities, including cytokines and type I IFNs (IFNα and IFNβ) [84]. The PRRs mediated activation of the transcription factors nuclear factor-κB (NF-κB) and activator protein 1 (AP1) stimulates the pro-inflammatory responses; IFN regulatory factor (IRF) family members (IRF3 and IRF7) are involved in the induction of IFNα expression, and IFNβ expression requires IRFs and NF-κB. AIM2 activates the inflammasome, a large multiprotein complex that stimulates a proteolytic caspase 1-dependent pathway that cleaves pro-interleukin-1β (pro-IL-1β) and pro-IL-18 into the mature bioactive pro-inflammatory cytokines.

It can be observed a substantial overlap between the downstream activities stimulated by PRRs so that some of the pathways that lead to IFN activation also drive activation of other cytokines and cell death.

1.6.1 Pattern Recognition Receptor involved in Herpesviruses recognition

The herpes virion is sensed by multiple pattern recognition receptor: TLR2, located in the plasma membrane, at or around cholesterol-rich membrane microdomains, is the first line of defense and recognizes proteic or lipidic PAMPs; TLR9, in endosomes, mainly in plasmacytoid cells, and a series of cytoplasmatic receptors, specifically DAI, DHX9, DHX36, AIM2 and IFI16, are able to recognize double-stranded DNA; TLR3 present in endosomes, and MDA5 in the cytoplasm, are able to sense viral RNA (Fig. 13).

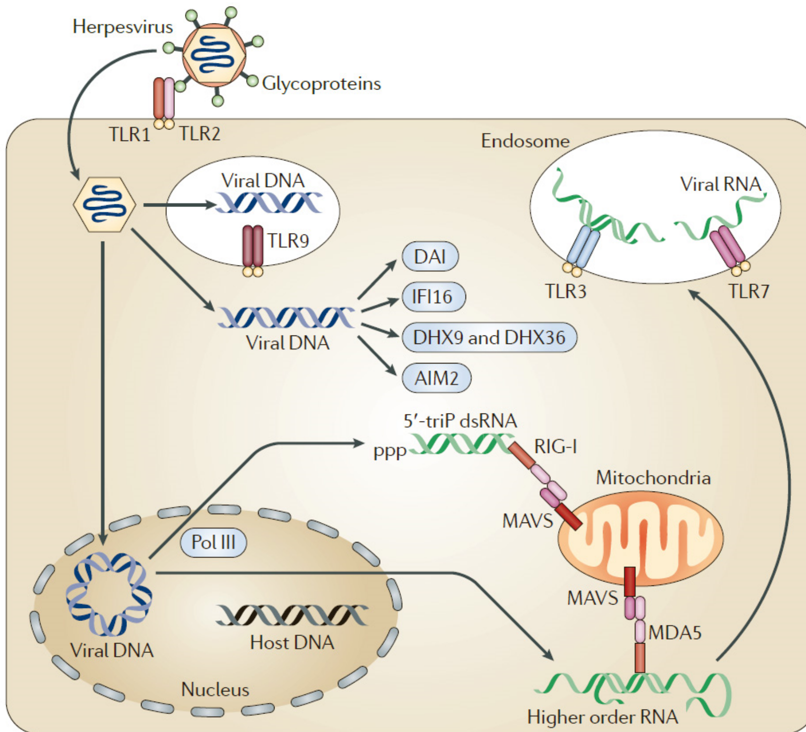

Fig. 1.13: **Innate immune recognition by herpesviruses.** Schematic representation of the cell with the known pattern recognition receptors (PRRs) involved in the recognition of herpesviruses and in the activation of the innate immune response [74].

<u>Toll-like receptors</u>

Toll-like receptors (TLRs) are the first discovered and best characterized PRRs; TLRs are transmembrane proteins located either at the plasma membrane or in endosomes, where they recognize hydrophobic molecules such as lipids and proteins, or nucleic acids, respectively. The name “Toll-like” derives from the structural and functional homology of these receptor to Toll, an essential receptor of Drosophila innate immunity, involved in host defense against fungal infection [85]. TLR4 was the first mammalian homolog of the Toll receptor discovered [86]; after that, several proteins structurally related to TLR4 were identified in mammals and actually they are grouped in a large family consisting of at least 11 members, and among these TLR1-9 are conserved between human and mouse [87].

Although the broadest repertoire and the highest levels of expression of TLRs have been observed in phagocytic cells such as macrophages, neutrophils, and dendritic cells, it seems that the majority of cells in the organism express at least a subset of TLRs [88].

TLRs are type 1 transmembrane proteins with an ectodomain, a single transmembrane domain, and a cytoplasmatic signaling domain. Several leucine-rich repeats (LRRs), that probably are the key elements in the interaction with microbes or microbial components, compose the ectodomain of TLRs.

Microbial recognition of TLRs promotes their dimerization, and this triggers activation of signaling pathways, which originate from a cytoplasmic domain. The cytoplasmic region of TLRs, required for initiating intracellular signaling, is termed Toll/IL-1 receptor (TIR) domain because of the high homology to that of the IL-1 receptor family. By contrast, the extracellular domain of the two family of receptors are unrelated, with TLRs bearing leucine-rich repeats (LRRs), while IL-1 receptors presents an immunoglobulin-like domain [87]. LLRs are hypothesized to directly interact with microbes or microbial components and the binding of TLRs with their PAMPs recruits citoplasmatic adaptors, that transmit the signal through a series of signaling events which culminates in the induction of inflammatory cytokines such as tumor necrosis factor (TNF)α, IL-6, IL-1b, IL-12, and type I IFN (IFN α and IFN β).

Furthermore, TLRs signaling participates in the dendritic cells (DCs) maturation inducing the upregulation of costimulatory molecules on these specialized antigen-presenting cells. This process links innate and adaptive immunity, since DC maturation is an essential step in the induction of pathogen-specific adaptive immune responses [89].

All TLRs activate conserved pro-inflammatory pathways, which culminate in the activation of NF-κB and activating protein-1 (AP-1). NF-κB is a dimeric transcription factor that belongs to the Rel-homology domain-containing protein family, and in most types of cells the prototypical NF-κB contains p65 and p50 subunits. In the inactive state, NF-κB is located in the cytoplasm in association with IκBα, an inhibitor protein; the activation of TLR2 leads to the phosphorylation of IKK (IκB kinase), the trimeric complex of IKKα, IKKβ, and the regulatory subunit IKKγ, also named NEMO, which in turn phosphorylates IκBα at serine residues and promotes its ubiquitination and proteasomal degradation by the 26S proteasome, with the subsequent release of the RelA-p50 dimer. This transcriptional factor is translocated to the nucleus where it regulates the transcription of NF-κB-responsive genes, in particular, cytokines, like IFNβ, and chemokines [90].

AP-1 is a dimeric complex of basic region leucine zipper (bZIP) proteins that belongs to the Jun, Fos, activating transcription factor (ATF) and the Maf subfamily, which recognizes either 12-O-tetradecanoylphorbol-13-acetate (TPA) response elements or cAMP-response elements, and regulates a wide range of cellular processes, like cell proliferation, death, survival and differentiation [91]. Many TRL ligands activate MAP kinases, such as c-Jun N-terminal kinase (JNK), p38 and extracellular signal regulated kinase (ERK), which in turn stimulate AP-1, and in particular c-Jun, that is one of the main characters in the inflammatory response.

TIR-domain-containing cytosolic adapters interact in a TIR-domain-dependent manner with TLR, thus starting intracellular signaling; the principal adapters are myeloid differentiation primary response protein

88 (MyD88), TIR domain-containing adapter protein (TIRAP)/Mal, TIR domain-containing adapter inducing IFNβ (Trif) (also known as TICAM1) and Trif-related adapter molecule (TRAM) (also known as TICAM2).

Among these factor, MyD88 is recruited by almost all TLRs and is a key factor in the control of inflammatory responses. MyD88 association with TLRs leads to the recruitment of IRAK (interleukin-1 receptor associated kinase) family proteins, specifically IRAK1 and IRAK2, which present a serine/threonine protein kinase activity, and IRAK4 and IRAK-M, that negatively regulate TLR-mediated signaling. Phosphorylation of IRAK proteins allows their dissociation from MyD88, and consequent activation of tumor necrosis factor receptor-associated factor 6 (TRAF6), an E3 ubiquitin ligases, which in turn activates transforming growth factor-b-activated protein kinase 1 (TAK1), in a ubiquitin-dependent manner. TAK1, which is a member of the MAP kinase kinase kinase (MAP3K) family, forms a complex with TAB1, TAB2 and TAB3, and this interaction stimulates its kinase activity, thus TAK1 phosphorylates the IKK complex with the final activation of NF-κB [90]. In TLR2 and TLR4 signaling mediated by MyD88, TIRAP/Mal is also required as an additional adapter.

Unlike the other TLRs, TLR3 signaling is not mediated by MyD88, but uses the adapter Trif; its C-terminal region contains Rip homotypic interaction motif (RHIM), which mediates interaction with members of the receptor interacting protein (RIP) family [92]. From studies on RHIM and RIP1, it was possible to infer that in TLR3 signaling Trif–RIP1 interactions are responsible for NF-κB activation. Moreover, Trif can interact with TRAF6 at the level of its N-terminal region, that contains typical TRAF6-binding domains, and even this interaction can lead to the NF-κB activation. Therefore, both Trif–RIP1 and Trif–TRAF6 pathways are able to converge at the IKK complex and to induce NF-κB [90].

In conclusion, TLR signaling occurs through two distinct pathway, a MyD88-dependent pathway and a MyD88-independent or TRIF-dependent pathway (Fig. 1.14).

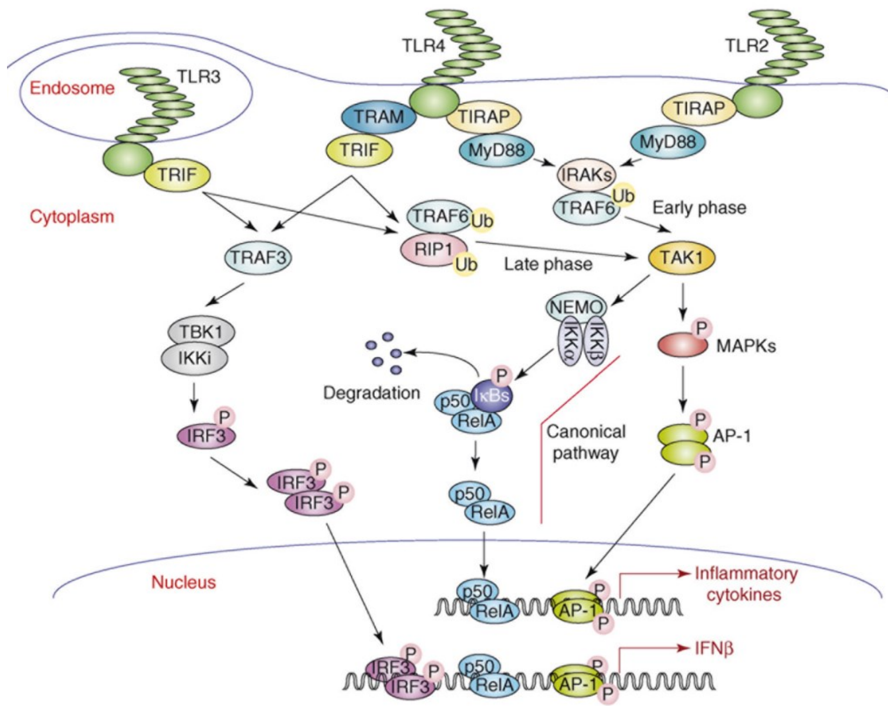

Fig. 1.14: **TLRs mediated signaling.** TLR2 and TLR9 (not shown in this scheme) trigger the MyD88-dependent pathway of activation of NF-κB; MyD88, associated with TIRAP, recruits TRAF6 and members of the IRAK family, then they activate TAK1, that in turn activates the IKK complex (IKKα, IKKβ and NEMO), which catalyzes the phosphorylation of IκB proteins. The proteasome-dependent pathway destroy IκBs, leading to the release of NF-κB (RelA–p50 heterodimer), that translocate into the nucleus (canonical pathway). TAK1 can also activate the MAPK pathway, with the consequent phosphorylation and activation of AP-1. NF-κB and AP-1 induce inflammatory cytokines triggering the inflammatory response. TLR3 interacts with TRIF, which recruits TRAF3, that in turn activate TBK1 and IKKi; these kinases mediate phosphorylation and dimerization of IRF3, which translocates into the nucleus and regulates gene transcription. Moreover, TRIF can interact with TRAF6 and RIP1, and so mediate NF-κB activation.

TLR2 is mainly expressed by myeloid cells and recognizes a variety of microbial components, in particular lipoproteins, lipopeptides, peptidoglycan, glycolipids, lipopolysaccharide (LPS) from various bacterial and fungal pathogens [87]; this property of TLR2 can be explained by its capacity to functional cooperate with several receptors, which are either structurally related, like TRL1 and TLR6, or unrelated.

TRL2, in complex with TLR1, is able to recognize HCMV, through the interaction with its glycoprotein gB and gH: gB and gH co-immunoprecipitate with TLR2 and TLR1, and antibodies specific for gB and gH block the inflammatory cytokine responses induced by the infection, both in model cell lines and in permissive human fibroblast [78, 93]. Regarding HSV-1, using both cell lines and animal model, it was observed that TLR2 mediates the inflammatory cytokine response to the virus [94-96]. Furthermore TLR2 partecipates in the inflammatory response to VZV infection [97] and it is involved in the activation of NF-κB and chemokine response against EBV infection [98].

It is not clear how herpesviruses stimulate TLR2, but since this PRR recognizes hydrophobic PAMPs, such as lipopeptides, a plausible hypothesis is that TLR2 detects hydrophobic peptides in gH and gB or a lipid component exposed during viral entry [74]. The importance of TLR2 in host defense against herpesviruses is

not completely clear, because in mouse model both beneficial and deleterious effects have been observed; certainly a lack of TLR2 leads to impaired expression of cytokines and reduced activation of NK cells [95, 96, 99]. In human, some genetic studies have pointed out the protective role of TLR2 during natural herpesvirus infections, suggesting the possible involvement of TLR in the control of HSV in latency [100, 101].

TLR9 is an endosomal receptor for CpG unmethylated DNA, which are typical of bacteria, but very rare in vertebrates, where CpG motifs are highly methylated [102]. It can be distinguished two types of CpG DNA, A/D-type CpG DNA and B/K-type CpG DNA, which are structurally different, but both able to induce inflammatory cytokines such as IL-12 and TNF-a, even if with different efficiency, in a TLR9 dependent way. TLR9 activation is strongly influenced both by CpG content and level of methylation of the motif [103]. TLR9 expression differs between human and mouse: in human it is expressed only in B cells and plasmacytoid DCs (pDCs), while in mice TLR9 can be find in a wide range of cell types [84]. The genomic DNA is probably the most potent immune-stimulating component of herpesvirus particles and TLR9 is able to recognize both alpha, and beta and gamma herpesviruses; this interaction leads to the activation of IRF7 [104], and results in expression of type I IFNs in human and mouse pDCs [105, 106]. Several animal studies have been performed and it results that TLR9 role in the immune response to herpesvirus is specialized but often redundant, and moreover the TLR9 dependent response becomes more active when pathogens reach lymphoid organs and blood, where pDCs are abundantly present [74].

TLR3 recognizes dsRNAs [107], which accumulate intracellularly with viral replication [108], and it is implicated in the identification of this structures during Herpesviruses infection. Consequently TLR3 stimulates a signaling pathway mediated by TRIF/TICAM1 that stimulates IRF3 and NF-κB and finally leads to the synthesis of type I interferons (IFN-α/β) and other pro-inflammatory cytokines, which exert anti-viral and immunostimulatory activities. An example of viral dsRNA able to induce TLR3 is represented by the latency-associated EBV-encoded small RNAs (EBERs), which are non-polyadenylated, non-coding dsRNAs with stem-loop structures [109]; instead for alpha and beta Herpesviruses specific dsRNAs have not yet been characterized. TLR3 is constitutively expressed by numerous cell types, as well as epithelial cells and CD8α+ DCs, and moreover, in most cell types, its expression is strongly induced by type I IFNs and viral infections [110]. CD8α+ DCs participate in the cross-presentation of viral antigens, contributing to development of an efficient CD8+ T cell response against HSV-1. The mechanism that allows endosomal TLR3 to detect cytoplasmatic viral dsRNA is unknown, but it is assumed that phagocytosis of infected cells or autophagy could be involved [74]. In vivo studies support the hypothesis of an important role for TLR3 in stimulation of the adaptive immune response [111] and have confirmed its role in the IFN response to HSV-1 [112].

Non-TLR receptors

In addition to TLRs, the organism avails itself of a set of intracellular cytoplasmatic PRRs involved exclusively in the recognition of nucleic acids, like RNA replication intermediates, but especially incoming genomic DNA, which have an important role in the activation of innate immune responses against herpesviruses.
Regarding viral genomic DNA, it is not entirely clear how it could become accessible to PRRs, since it is enclosed in the viral capsid and delivered directly to the nucleus. While it is easier to understand how the presentation of viral DNA could occur in the endocytic pathway of entry, namely via degradation followed by translocation to the cytosol, the situation is not clear in the non-endocytic entry and it must be present an alternative way, that probably involves autophagy or proteasomal degradation of the nucleocapsid [74].
The signaling pathway linking cytosolic DNA sensors to final effector are still not entirely known: DNA sensing leads to the activation of TANK-binding kinase 1 (TBK1) and IRF3, that stimulates the production of type I IFNs and pro-inflammatory cytokines. TBK1 associates with DDX3, a DEAD box RNA helicase [113], and interacts with SEC5 (EXOC2), an exocyst protein, in a complex that includes the endoplasmic reticulum adaptor STING (stimulator of IFN genes), which has an essential role [114], but all the steps of this pathway of signaling are not defined.

RNA. RIG-I like receptors (RLRs), similar to TLR3, are able to sense intracellular herpesvirus RNA; RIG-I is a DExD/H box RNA helicase that interacts with dsRNA through its helicase domain, and activates IRF3, NF-κB and MAP kinases through a signaling activated by its CARD-like domains. For example, in EBV infected cells it has been observed the induction of IFNs and IL-10 triggered by EBERs and mediated by RIG-I [115, 116].
MDA5 is structurally similar to RIG-I, as a matter of fact it also presents a single helicase and two CARD-like domain. In primary human monocyte-derived macrophages, MDA5 and its adaptor protein mitochondrial antiviral signalling protein (MAVS) induce IFN responses following HSV-1 infection and production of replication-induced higher-order RNA structures [117].
Since RLRs are expressed by many cell types in the body and are also strongly induced by IFNs, these receptors could be involved in the recognition of herpesvirus in permissive cells, especially at later stages of infection, when their expression is upregulated; in this context, it is much more probable that RLRs contribute to the control of productive infections, rather than partecipate in the initial antiviral IFN response [74].

DNA. The ISG DAI (DNA-dependent activator of IFN-regulatory factors) was the first cytoplasmic DNA sensor to be identified able to activate IRF3 and promote type I IFN expression in response to HSV-1 and HCMV infection in vitro [118, 119].
DEAH box protein 9 (DHX9) and DHX36, which belong to the family of DExD/H box helicases, are able to recognize CpG-containing DNA in pDCs and to induce a signaling pathway MyD88 dependent which leads to the activation of NF-κB and IRF7, and the consequent expression of pro-inflammatory cytokines and IFNα, after HSV-1 infection [120].

AIM2, which belongs to the pyrin and HIN domain-containing protein family (PYHIN family), is another cytosolic dsDNA sensor that stimulates caspase 1 activation through inflammasomes. AIM2 binds DNA through its HIN domain and interact with ASC/PYCARD (apoptosis-associated speck-like protein containing a CARD) through pyrin domain and consequently recruits pro-caspase 1, which is activated and leads to the maturation of IL 1β and IL 18 [121].

IFI16, another PYHIN family protein, bound directly to isolated viral DNA motifs and stimulates the expression of IFNβ and pro-inflammatory genes during HSV-1 infection [122]; IFI16 is present both in the nucleus and in the cytosol of the cell, so it is still not clear where exactly the DNA recognition takes place.

Besides AIM2 and IFI16, other PYHIN family protein that recognize intracellular DNA have been identified and collectively are termed ALRs, AIM2-like receptors.

1.6.2 Viral evasion of Innate Immunity

Considering the numerous systems that the organism and the cell possess to counteract pathogens, viruses had to evolve strategies to modulate host cells, evade host immune response and finally establish infection. For herpesviruses, evasion from the innate immune system and its modulation are essential both to initiate replication and successfully colonize the host, since herpes are some of the larger viruses, present slow replication cycle and are able to establish latent infections, with periodic reactivations.

Herpesviruses follow several ways to evade innate immune response: they can (I) avoid to be sensed by PRRs, or (II) block the action of these receptors, or still (III) inhibit the signaling pathway and gene expression that cells activate to counteract the infection.

(I) HSV-1 UL41 gene encodes a structural tegument protein called *vhs*, virion host shut-off protein, that acts as an mRNA-specific RNase and triggers rapid shutoff of host cell protein synthesis, with the disruption of preexisting polyribosomes and degradation of viral and host mRNAs [123]. Moreover, with the destruction of viral RNA in the early stages of the infection, *vhs* is able to make less effective the activation of the TLR3 and RLRs dependent RNA-sensing pathways in dendritic cells [124]. Another viral factor involved in the innate immune evasion is the HSV-1 – encoded neurovirulence protein ICP34.5: it antagonizes the antiviral, interferon-inducible PKR signaling pathway and directly binds to the mammalian autophagy-inducing protein Beclin 1, inhibiting its function, and therefore contributing to viral neurovirulence. Through this mechanism, HSV-1 counteracts the delivery of PAMPs from the cytoplasm to the endosome and counteract the detection of viral nucleic acids by endosomal TLR3 and TLR9 [125].

(II) The inhibition of the activity of specific PRRs is a second possible strategy followed by viruses to evade innate immunity and it can be exemplified by ICP0 and pp65.

ICP0 is one of the most studied HSV-1 proteins: it is an immediate early multifunctional protein able to enhances viral replication and to block chromatin silencing of viral lytic genes, it presents an E3 ubiquitin

ligase activity that promotes degradation of certain host proteins, such as promyelocytic leukemia (PML) in nuclear bodies, SUMO-1, and the catalytic subunit of DNA protein kinase [126]. In the context of evasion from innate immunity, ICP0 reduces the TLR2-mediated inflammatory response to HSV-1 infection, likely by promoting the degradation of MYD88 and MAL/TIRAP thanks to its E3 ligase activity [127]. Moreover there are several evidences that some herpesvirus protein are able to inhibit cytosolic DNA sensors like DAI, DHX9 and IFI16; one of these viral factors is the HCMV tegument protein pp65/UL83. It was already known the capacity of pp65 to inhibit virus-induced expression of ISGs [128, 129], but recently it was observed that UL83 directly interacts with IFI16, inhibiting the antiviral response mediated by this sensor [130], and that its deletion mitigate the virus activity in vivo [131]. The activity of MCMV M45 protein and HHV8 viral protein kinase (v-PK) are similar to the one of UL83: the RHIM-containing factor M45 disrupts the RHIM-based DAI–RIP1/3 interaction and so blocks DAI signaling [132], while v-PK inhibits signaling activities of DHX9 by the direct interaction with the receptor [133].

(III) The third strategy adopted by viruses to evade innate immunity consists in the inhibition of signaling activated by PRRs and consequently of antiviral gene expression programs.

The IRF-IFN pathway is targeted by all classes of herpesviruses, since type I IFNs plays a fundamental role in innate defense against herpesviruses. ICP0 participates even in this type of immune evasion: it is able to inhibit nuclear accumulation of IRF3 and induction of IFNs by degrading IRF3 or by sequestrating the complex IRF3-CBP/p300 [134, 135]. ICP27, another immediate early protein of HSV-1, binds the inhibitor of κB (IκBα), blocks its phosphorylation and ubiquitination, and stabilizes this factor, and therefore in this way represses NF-κB activity [136].

Particularly interesting is the strategy adopted by HHV8: its genome contains a cluster of ORFs with homology to the cellular transcription factors of the IRF family that encodes viral interferon regulatory factors (vIRF-1, vIRF-2 and vIRF-3). Among these viral proteins, vIRF-3, a 73 kDa protein homolog to cellular IRF-4 and vIRF-2, targets IRF-3, IRF-5 and IRF-7, avoids their DNA binding and accordingly down-regulates virus-mediated activation of IFN gene promoters and diminishes the early inflammatory response [137-139].

In conclusion, the presence of so many and various strategies implemented by herpesviruses to evade innate immune system, stress the importance of this type of immunity in the control of herpesvirus infections (Fig. 1.15).

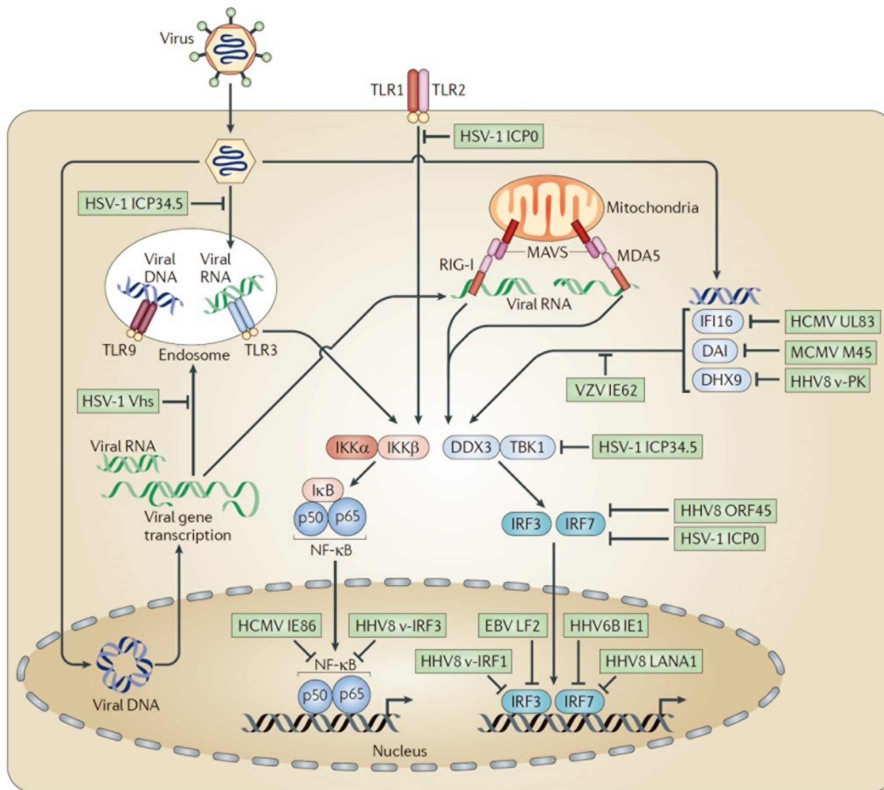

Fig. 1.15: **Herpesvirus evasion of Innate immune system.** Scheme of the viral proteins encoded by herpesvirues to evade from PRRs recognition. ICP34.5 and *Vhs* of HSV-1 prevent the recognition of viral nucleic acids; HSV-1 ICP0 inhibits TLR2 signalling by stimulating the degradation of TLR adaptor molecules; MCMV M45 protein inhibits the recruitment of RIP1 to DAI; HHV8 ORF45 protein interacts with IRF7 and inhibits its nuclear translocation; several viral proteins, such as HHV8 v-IRF3, interact with NF-κB, IRF3 and/or IRF7 and inhibit transcription of genes involved in the immune response [74].

1.7 Herpes as oncolytic agent

Since the beginning of the nineteenth century, viruses have been taken into consideration as possible agents for tumor destruction. The use of viruses in the treatment of cancer originated from the observation that, occasionally, cancer patients who contracted an infectious disease went into brief periods of clinical remission. In particular in patients affected by leukemia, it was observed that contraction of influenza sometimes produced beneficial effects [140].

In the first clinical trials, body fluids containing human or animal viruses were used to transmit infections to cancer patients; most often the viruses had no effects on tumor growth, but sometimes, particularly in immunosuppressed patients, the persistence of the infection lead to tumors regression. In any case, morbidity due to the infection of normal tissues was too high. In 1950s and 1960s the advent of rodent models and the introduction of new methods for virus propagation permitted the first attempts to force the evolution of viruses with greater tumor specificity, but there was no great success. So, it occurred a near-abandonment of the fields in the 1970s and 1980s [141]. Thanks to advance in technology, in

particular in reverse genetics, in the past two decades a renewal of interest in virotherapy occurred, that allowed the generation of more potent, tumor-specific oncolytics, culminating in the first marketing approval of an oncolytic virus, granted by Chinese regulators for the genetically modified oncolytic adenovirus H101 in November 2005 [142].

Oncolytic virotherapy is an emerging treatment modality that makes use of replication-competent viruses as therapeutic agents in order to infect and limit the growth of the tumor mass and to treat tumors and metastases for which currently there are no effective therapies, without causing harm to normal tissues. The advantages of oncolytic virotherapy are multiple: oncolytic viruses can kill selectively only mitotically active tumoral cells, infection can spread to tumoral cells distal from the injection site, viruses can be "armed" to potentiate their oncolytic activity through the introduction of heterologous genes, virotherapy can be combined with standard clinical therapy.

Even though each virus has a specific cellular tropism, a number of viruses exhibit a preferential tropism for tumor cells. This feature is probably due to tumor biology, as most tumors evolved strategies to avoid the key responses used by normal cells to limit virus infection, like immune response, apoptosis and translational suppression [143].

Oncolytic viruses can kill cancer cells in many different ways: the virus typically controls the molecular cell death machinery of the infected cancer cell to exploit cellular resources for the synthesis and assembly of new viruses, and only at this point it kills the infected cells. Moreover, oncolytic viruses can lead to the death of uninfected cancer cells by indirect mechanisms such as destruction of tumor blood vessels, amplification of specific immune responses or through specific activities of transgene-encoded proteins expressed from engineered viruses [143].

The specific targeting of cancer cells is fundamental in oncolytic virotherapy: some viruses, such as parvovirus H1, reovirus, Newcastle disease virus, mumps virus and Moloney leukemia virus, have a natural preference for cancer cells, whereas viruses such as measles, adenovirus, vesicular stomatitis virus (VSV), vaccinia and herpes simplex virus (HSV) can be engineered to increase their cancer specificity.

Two strategies to improve oncolytic viruses consist in using surface markers of cancer cells (such as epidermal growth factor receptor, Her2-neu, folate receptor, prostate-specific membrane antigen and CD20) or tumor specific nuclear transcription factor (such as estrogen receptor, androgen receptor, GATA factors and hypoxia-inducible factor 1) respectively as receptors for virus entry or as essential cofactors for viral gene expression [144].

An alternative strategy consists in the exploitation of the defective antiviral defenses of tumor cells: unlike normal cells, cancer cells are generally deficient in apoptotic and antimetabolic responses. Thus, viruses can be rendered nonpathogenic, but still able to propagate in tumor cells, by inactivation of the viral proteins deputed to evasion of the innate response. Examples of viral immunomodulatory proteins include the matrix protein of VSV, the NS1 protein of influenza virus, the C and V proteins of paramyxovirus family

members, the HSV γ34.5 protein and the proteins encoded in the E1 and E3 regions of the adenovirus genome [145].
A recent strategy to eliminate undesirable tropisms of oncolytic viruses and to "target" cancer cell is the use of tropism-regulating miRNAs: it was showed that it is possible to control the tropisms of viruses through the incorporation of miRNA targets into vector genomes, so that the viral life cycle is selectively blocked in the relevant target tissue [146].

1.7.1 Strategies for reprogramming: retargeting, arming, shielding

Retargeting to tumor receptors

The first challenge in oncolytic virotherapy is to target viruses with either a natural or partial tropism for the tumor cells. Until now the approaches used are: virus retargeting to cancer-specific surface molecules, virus activation by cancer-specific proteases, control of viral transcription and replication by tissue-specific promoters and exploitation of cancer cell defects [144].
A strategy to retarget viruses consist in engineering virion surface glycoproteins such that they only interact with specific cancer receptors, or are activated by molecules present in the cancer environment (Fig. 1.16 b).
For example, hemaglutinin of measles virus (MV) was manipulated with the insertion of a ligand to the epidermal growth factor (EGF) or the insulin-like growth factor 1 (IGF1), generating a recombinant virus that infects cells expressing EGR and IGF-1 receptor [147].
In the case of orphan receptors, it is possible to use single-chain fragment variable (scFv) antibodies. These include the antigen variable regions of the heavy and light chains of antibodies, but are much smaller than an antibody tetramer. In the last years MV, vaccinia virus and HSV-1 have been engineered with the insertion of scFv, specifically anti-EGFRvIII and anti-prostate specific membrane antigen (PSMA) were inserted in MV attachment protein H [148, 149], or human epidermal growth factor receptor 2 (HER-2) in HSV glycoprotein gD [43, 150].
A further strategy exploits the activity of cancer-specific proteases, highly expressed by nearly all cancer cells, such as metalloproteinases (MMPs), proteolytic enzymes that degrade the extracellular matrix and participate in tissue remodeling and tumour metastasis [144] (Fig. 1.16 a). Fusion protein F of MV has been fused with an hexameric sequence recognized by an MMP instead of furin, its natural activator; the recombinant MV obtained was strongly restricted to primary human hepatocytes, and moreover lost the capacity to infect cells that did not express MMP in the extracellular matrix [151].
To achieve full retargeting, detargeting is the big challenge in the construction of safe oncolytic viruses. The strategies adopted consist in the mutagenesis of residues that are involved in the interaction between virus

and its natural receptor, or simply the exogenous elements introduced to retarget virus can mask the normal site of virus attachment.

The cancer-specific transcriptional control envisions the manipulation of viral genome with the insertion of cancer specific promoters which regulate the expression of essential viral gene products (Fig. 1.16 c). This approach best fits to DNA viruses, since they can tolerate large DNA insertions, and adenovirus is an example: the key transactivator E1A, which regulates viral promoters to start the replication cycle, has been put under the control of noncollagenous bone matrix protein osteocalcin (OC) promoter, a prostate specific promoter [152] or the survivin promoter [153], leading to a tumor-specific replication.

Finally to increase safety and diminish viral toxicity it is possible to exploit some cancer specific defects like their increased replicative potential, particular mutation in p53, IFN and/or cell-cycle deficiencies, to attenuate viral replication in normal cells, but not in cancerous ones (Fig. 1.16 d). Several o-HSV-1 have been obtained by the deletion of $\gamma_1$34.5 gene, the viral factor involved in neurovirulence, or genes with a role in nucleotide metabolisms, with the aim to create viruses able to replicate only in dividing cancer cells (see next session). ONYX-015, a conditionally replicating adenovirus, is deleted in E1B gene, which blocks apoptosis by interacting directly with p53 in infected cells, and consequently it can replicate only in cells with a mutated p53, as occurs in some kinds of cancer cells [154]. ONYX-015 showed poor antitumor activity when employed alone; it is administrated in combination with chemotherapy with encouraging results [155].

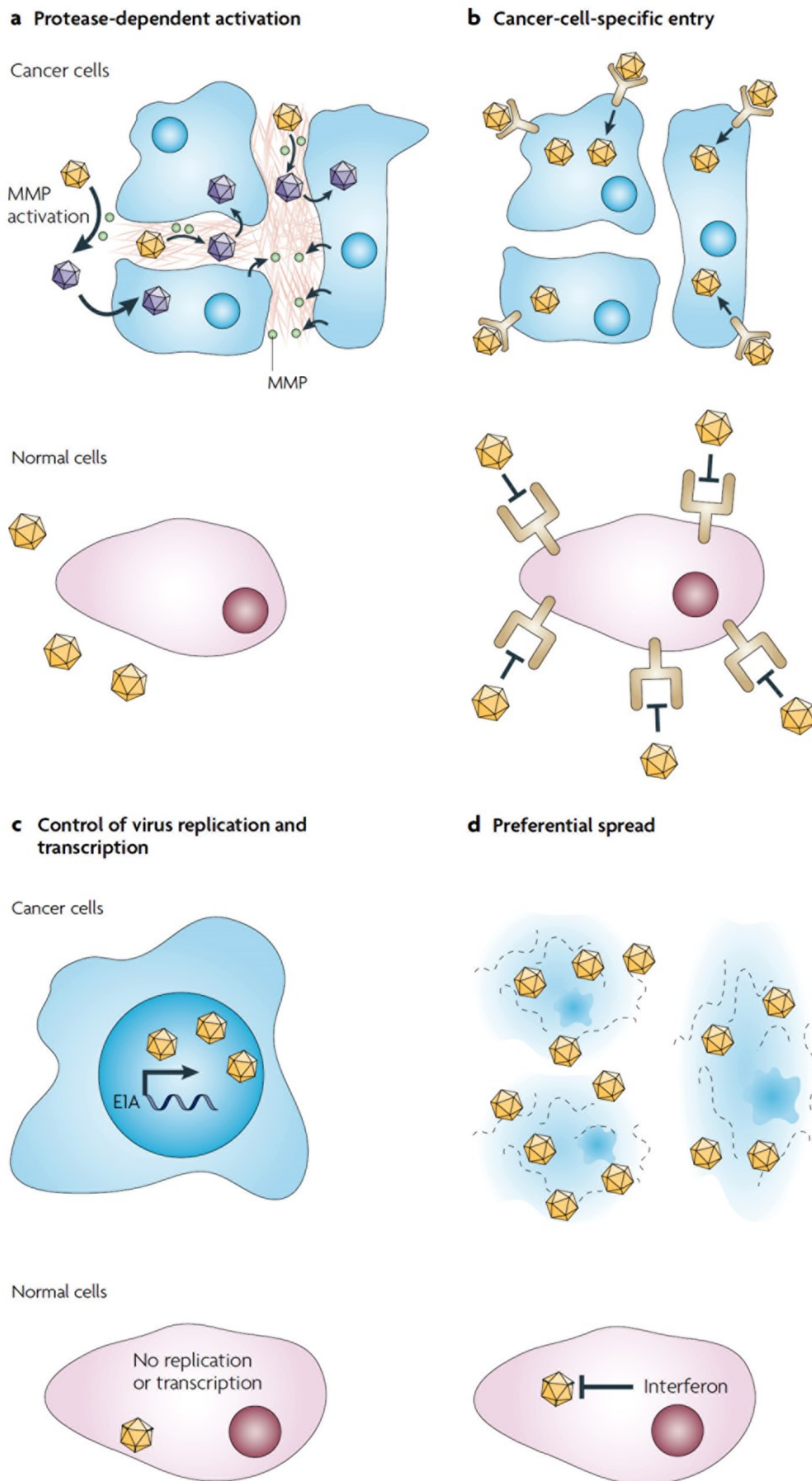

Fig. 1.16: **Viral retargeting to tumor receptors.** Virus particle activation can be engineered in several ways: a) to be activated by proteases, like matrix metalloproteases (MMPs), secreted by cancer cells in the tumor matrix; b) to enter cells through a tumoral specific receptor rather than through their natural attachment protein; c) to activate viral transcription and replication only in cancer cell, by the use of tissue- or cancer-specific promoters; d) to replicate preferentially in transformed cells, as a result of modifications or deletions of their immune-evasion proteins [144].

Arming with immunomodulator genes

In order to enhance the efficacy of oncolytic viruses, additional strategies were developed and applied also to retargeted viruses. The arming of viruses consists in the insertion in viral genome of genes that encode factors able to promote cell death or to stimulate the immune system. For example transgenes inserted can convert a prodrug into a harmful compound, or they can express immunomodulatory molecules able to recruit the immune system cells, or can induce apoptosis only at late stages of infection.

Vaccinia virus, a promising candidate oncolityc agent, thanks to biological properties, like intravenous stability, has been armed with granulocyte-macrophage colony stimulating factor (GM-CSF) and deleted of viral thymidine kinase gene. The resulting virus JX-594 was used in clinical trials on human metastatic solid tumors and was well-tolerated [156, 157].

Adenovirus has been engineered to express the TNF-related apoptosis inducing ligand (TRAIL), a pro-apoptotic gene able to induce apoptosis in a wide range of tumor cells and its effect is independent of p53 status. This oncolytic virus was effective both in vitro and in vivo, specifically against human tumor cell lines and in mice tumor models, where it was able to replicate efficiently and eliminate metastases [158].

Shielding and carrier cells

The optimal route to administrate oncolytic viruses in order to hit tumor metastasis otherwise unreachable is represented by the systemic administration. This approach is still problematic because the presence of prior immunity against viral agent can lead to its inactivation and of defense serum system, complement, etc.

The rapid sequestration of oncolytic viruses by the mononuclear phagocytic system (MPS) in the liver and spleen occurs after opsonization of viral particles by antibodies, complement and other serum proteins.

On the other hand the local immune response induced by therapeutic oncolytic viruses can contribute to the elimination of tumoral cells [144]. A way to limit the activity of neutralizing antibodies in the blood consists in the combination of virotherapy and chemiotherapy, for example treatment with cyclophosphamide (CPA), which is able to induce an immune-suppression state in the organism, acting on T and B lymphocytes. The combined use of CPA with different o-viruses showed an enhanced viral delivery and efficacy thanks to a decreased immune clearance of oncolytic viruses, due to a decrease in neutralizing antibodies or depletion of T cells [159]. In some cases the use of CPA had counterproductive effects, impairing the oncolysis in treatment with VSV [160].

A further approach designed to avoid the activation of immune system involves the use of biocompatible polymers, like polyethylene glycol (PEG), to construct a chemical shield on the virus. In this way, o-viruses can overcome the attacks of macrophages and reach their targets; for example PEGylation of adenovirus gave good results in therapy [161]. However, the presence of polymer on the viral surface can destroy or decrease virus infectivity, which can be restored with the engineering of the polymers with receptor-binding ligands.

A promising strategy for virus shielding consists in the use of carrier cells to hide oncolytic viruses inside them and to deliver them to the tumor mass and metastases. Two types of cells have been used and showed good results in preclinical models: tumor cell lines and specific types of primary cells. Carrier cells need to be susceptible to virus infection, but they showed not be rapidly lysed after the infection; moreover, they need to home to cancer cells and spread the infection directly there. Tumor cells lines have the advantage to be easily cultivable, expandable and engineerable; in a preclinical study lethally irradiated myeloma cells infected with an oncolytic measles virus and administered intravenously gave good therapeutic results in a mouse model of myeloma with high levels of anti-measles antibodies [162].

Some of the cells of the immune system well adapt to be used as carrier because of their feature: monocytes and macrophages extensively colonize solid tumors, professional antigen presenting cells, like dendritic cells, and NK cells are able to respond to the environment and reach site of inflammation thanks to integrins and chemokines, and antigen specific T cells traffic intensely in the organism [163].

Mesenchymal stem cells (MSCs) are another example of primary cells used as carrier: MSCs are non–haematopoietic stem cells, readily isolated from bone marrow, adipose tissue, and other sources, recognized by a panel of specific antigens (CD105+, CD73+, CD90+, CD14-, CD34-, CD19-, HLA-DR-, CD45-), and with the ability both to self-renew and to differentiate into multiple lineages, such as osteoblasts, chondrocytes and adipocytes. MSCs have two considerable properties that have aroused interest in their use as tumor-specific delivery vehicles: 1) if systemically administrated in healthy animals, MSCs show to home preferentially to the lung, liver and bone, while in mouse model of cancer or after an injury, they show a natural tropism for tumor and metastases or for the injury site; 2) MSCs are immunoprivileged cells, thanks to the low expression of MHC class 1 and other co-stimulatory molecules, and the secretion of factors that negatively regulate the T-cell immune response [164]. MSCs have been used both in preclinical and small clinical trials to deliver oncolytic viruses, like measles virus, to tumoral sites.

Oncolytic HSV

Among the viruses which are being evaluated in oncolytic virotherapy, HSV presents multiple advantages. First of all, HSV-1 is able to infect and replicate in many cell lines, including neuronal cells, thanks to its wide host range. HSV-1 has a short replication cycle (about 20 hours), compared to other oncoviruses, like adenovirus (48 – 72 hours), and it is able to spread cell to cell and to disseminate in the extracellular space, useful feature to promote an efficient viral diffusion within solid tumors. Its large DNA genome (about 152 kbp) does not integrate into the cellular genome and contains non essential genes that can be removed and replaced with different mutagenesis technologies; this is possible even in other DNA viruses, like adenovirus, but for example not in measles virus (MV) and adeno-associated virus (AAV). By manipulating HSV-1, attenuated, replication competent HSVs have been constructed, and tested in clinical trials, where they exerted efficacy against several tumor types. Finally, the possibility to use specific anti HSV-1 drug

therapy (Acyclovir, Ganciclovir etc.) in case of undesired infections is a great advantage compared to other oncolytic viruses [6, 25, 165].

However the use of HSV-1 in oncolytic virotherapy presents some disadvantages, including the prior immunity, given the high incidence of HSV in the human population, even if it seems not interfere in animal model [166]. Another important aspect is the route of delivery: actually in the majority of clinical trials o-HSV-1 was administered directly into the tumor or loco-regionally, even though the systemic route of administration is the ideal one. Even if the non-specific virion binding to heparan sulfate could distract virus from the targeted tumor cells, available results indicate that HSV administered through the blood stream does target the tumor [165]. Since HSV-1 remains latent in infected people, rare events of recombination between wt-HSV-1 and o-HSV-1, that could take place after the infection of a same cell at same genome copy numbers, may occur but the result will be the two starting viruses. Regarding spontaneous reversions, they do not represent a problem because deletion mutants cannot revert, but in the worst case scenario they can acquire compensatory mutations, producing mutants still highly attenuated [165].

An ideal oncolytic virus should be able to kill selectively tumor cells and not normal ones, and finally it should disappear from the organism together with tumor.

The main strategies adopted in the years to generate cancer-specific o-HSV-1 are: i) generation of conditional replicating mutants with the deletion of viral genes that counteract the IFN response or involved in the efficient replication in non-dividing cells; ii) potentiation of tumor clearance through the arming of o-HSV-1 with cytokines, like IL-12 or GM-CSF; iii) development of engineered viruses that are de-targeted from their natural target cells, and specifically re-targeted to tumor-specific receptors [165].

Conditionally replicating oncolytic HSVs. The conditionally replicating mutants are the first o-HSV studied, used in phase I/II clinical trials, and research on these viruses is still ongoing. They are characterized by the deletion of some non-essential genes, that cause attenuation of virulence and a replication restricted only to some conditions, typical of tumor cells. One of the deleted genes is $\gamma_1$34.5, a gene responsible for neurovirulence, present in two copies in HSV-1 genome. $\gamma_1$34.5 counteract the antiviral response mediated by cellular PKR and its deletion originates HSV-1 mutant that are still able to replicate in cells with a low level of PKR activation, like human and murine glioma cell lines, but present a significant attenuation in normal cells of the CNS [167]. A common limitation of $\gamma_1$34.5 HSVs is represented by a low protein synthesis and replication, due to the heterogeneous nature of tumors, which can have low or high level of PKR activity.

HSV-1716 and NV1020 were some of the first o-HSV-1 developed. HSV-1716 derives from strain 17 of HSV-1 and is deleted for both copies of $\gamma_1$34.5, in clinical trials it showed to be well tolerated, but presented a limited replication [168]. NV1020 (also called R7020) presents the replacement of a 15-kb region containing $\gamma_1$34.5 with a fragment of HSV-2 DNA (US2, US3, gJ and gG); it has been used in clinical trials in various non

CNS tumors, administrated through vascular infusion, and it showed to be replication competent, but highly attenuated.
Another deleted gene is UL39, that encode ICP6, a ribonucleotide reductase essential for DNA metabolism in non-replicating cells, but dispensable in rapidly dividing cells. G207 is a double deleted mutant that lacks both of $\gamma_1$34.5 UL39 [169]. It can replicate in tumors, but not in healthy tissues, it presents an high safety profile and specificity, and not showed serious adverse events in clinical trials.

Cytokines armed o-HSV-1. A significant limit of oncolytic virotherapy is the incapacity of viruses to infect and kill all cancer cells, and a possible solution is represented by the stimulation of the local immune response via cytokines introduced in viral genome. IL-4, IL-12 and granulocyte macrophage colony-stimulating factor (GM-CSF) are some of the cytokines inserted in HSV-1. The mutant obtained by the substitution of $\gamma_1$34.5 gene with IL-4 gene is able to stimulate, in mice bearing gliomas, the tumor infiltration, of macrophages, CD4+ and CD8+ T cells and shows higher antitumor activity [170]. The anti-tumor response elicited by IL-12 is stronger and more durable: it activates the Th-1 response, NK and CD8+ T cells. M002 is a mutant HSV-1 with $\gamma_1$34.5 genes replaced by murine IL-12, which is able to attract NK, CD4+ and in particular CD8+ T cells in tumor sites of neuroblastoma or glioma mice models, increasing their survival [171].
The useful feature of GM-CSF are the recruitment of macrophages and dendritic cells, and the stimulation of their differentiation, activation and proliferation, with the consequent enhance of the immune response. OncoVex is an attenuated HSV-1 derived from a fresh clinical isolate (JS-1), deleted of the two copies of $\gamma_1$34.5 and of ICP47, which normally block MHC class I antigen presentation and prevents the recognition from T cell of infected cells, and with the insertion of GM-CSF gene [172]. It resulted well tolerated and showed positive results both in phase I and II trials against solid tumors, and activation of systemic anti - tumor immunity [173, 174].A phase III trial was closed; results are under communication.

Retargeted o-HSV-1. Although deleted o-HSV-1 are relatively safe viruses, they present the limit of an attenuated virulence and lower replication than wt-HSV-1, which results in a reduced killing capacity. A strategy developed to overcome this problem consists in the manipulation of HSV-1 entry machinery of non attenuated viruses. Specifically, glycoprotein gD, which provides the binding sites for HSV-1 natural receptors (HVEM, nectin-1 and 3-OS-Heparan sulphate), was modified by insertion of heterologous ligand able to interact with a tumor-specific receptor.
R5111 was the first retargeted HSV-1 mutant: it carries an engineered gD with IL-13 (134 aa) inserted in the N terminus, between aa 24-25, a gB lacking a HS-binding sequence, and a second copy of IL-13 in place of gC, in order to target the tumor specific receptor IL-13Rα2, expressed in malignant gliomas, and to reduce binding to aspecific target through HS interaction [175]. However this mutant still retains the ability to

infect cells through HVEM and nectin-1. Only with further mutation, specifically the replacement of gD first 32 aa with IL-13 and the amino acid substitution V34S, it was possible to obtain a fully retargeted o-HSV-1, R5141 [176].

Another example of retargeted virus is represented by R5181, whose gD was engineered with the insertion of urokinase- plasminogen activator (uPA), between aa 24-25, to target uPA receptor (UPAR), expressed in glioma cells [177].

In case of orphan tumor receptors, which lack a natural ligang, a possible solution consists in the use of a single chain antibody (scFv) specific for the receptor. This strategy has been adopted in our laboratory to construct o-HSV-1 retargeted to the HER2 receptor (see next session).

It is possible to obtain a retargeted o-HSV-1 even without viral genome manipulation, by means of bifunctional molecules, able to bind from a side gD and from the other the target of choice [178]. Unlike genetic retargeted HSV-1, only the inoculated viruses target specifically the tumoral cell, while their progeny does not. Consequently, for safety reasons, they must be attenuated.

A further strategy consists in the regulation of the transcription of essential viral genes, inserting cellular promoter controlled by tumor specific factors upstream of the viral gene of interest; consequently viral replication will be restricted only to tumoral cells. rQNestin34.5 is a o-HSV-1 expressing ICP34.5 under control of a synthetic nestin promoter, that is upregulated in human glioma lines and primary glioma cells. Its replication is highly specific for glioma cell and it proved to be effective in cell culture and in vivo models [179].

1.7.2 R-LM249: HER-2 retargeted HSV-1

In the context of retargeted HSV-1, R-LM249 was constructed by inserting in gD a single chain antibody (scFv) directed to HER2 receptor.

HER2, the human epidermal growth factor receptor 2, also called ERBB 2, belongs to a sub-family of 4 cell surface receptors (ERBB 1, ERBB 3, ERBB 4), closely related each other, which is part of the epidermal growth factor receptor family. HER2 is a type 1 transmembrane tyrosine kinase, that consists of an extracellular ligand binding domain, subdivided into four domains, an α-helical transmembrane region and a cytoplasmic kinase domain. Unlike other ERBB receptor, because of its ectodomain conformation, HER2 is unable of binding to a ligand, feature that makes it an orphan receptor, but can heterodimerize with other family members, in particular with ERBB 3. HER2-containing heterodimers have a strong signaling activity, which participates in the regulation of cell proliferation, survival, invasion and angiogenesis; it plays a fundamental role in heart embryogenesis, while in normal adult tissues it is expressed at low levels, except for heart [180].

HER2 activation triggers a potent mitogen signal, mediated by MAPK cascade. HER2 is overexpressed in about 18-25% of human breast cancers, and also in ovarian cancers, gastric carcinoma, a small proportion

of nonsmall cell lung tumors and salivary gland tumors [181]. In breast cancers, which are characterized by high cellular proliferation, CNS and viscera metastasis, and a poor prognosis, it can be observed an amplification of copies of HER2 gene (up to 25 to 50 copies), with a relative increase in HER2 protein levels (up to 40 to 100 fold), feature that makes ERBB 2 an optimal target for cancer therapy.

At present, HER2-positive cancers are treated pharmacologically with two drugs approved by FDA, Trastuzumab and Lapatinib. Other molecules are under investigation.

Trastuzumab, a humanized monoclonal antibody, recognizes the extracellular domain of HER-2 and blocks intracellular signaling, with a still unclear mechanism; preclinical studies showed good results in therapies with different combination of trastuzumab and chemotherapy drugs [180].

Lapatinib is an oral small inhibitor of HER1 and HER2 tyrosine kinase activity, and so it results useful in tumors resistant to trastuzumab [182]. A novel therapeutic approach is represented by scFv antibody specific for HER2 fused to active molecules, like recombinant immunotoxins [183].

R-LM11 and R-LM11L were the first recombinants obtained by the insertion of scFv against HER2 between aa 24-25 of gD [150]. They are retargeted to HER2, but not detargeted from natural receptor, since they are still able to enter in J-nectin-1 cells. However, these recombinants had the merit to demonstrate that it is possible to retarget HSV-1 exploiting the capacity of antibody to bind an antigen, and surprisingly that gD can tolerate large insertions, since scFv is 250 aa long, about twice IL-13 size, and almost as big as gD ectodomain itself.

R-LM39 was generated inserting multiple mutation in residues responsible for nectin-1 binding, specifically D215G, R222N, F223I and V34S substitutions; however this mutant HSV-1 was still able to infect cells expressing nectin-1 [184].

The resolution of gD crystal structure allowed to manipulate gD in a more focused way: scFv was inserted in the deletion of aa 6-38, supposing that such a large insert, put in this position rather than between 24-25, could better sterically mask the nectin-1 binding site and make it inaccessible.

R-LM113 was generated in this way and resulted fully retargeted to cells expressing HER2 as sole receptor [184], and furthermore resulted fully detargeted from both nectin-1 and HVEM [43].

In in vivo experiment performed in a glioma model in adult mice, R-LM113 resulted safe, since it did not cause encephalitis when intracranially injected in mice, extremely sensitive to wild-type HSV-1, and effective in contrasting tumor growth [185].

R-LM249 was generated replacing the entire Ig-folded region (aa 61-218) with the scFv, maintaining only the minimal sequences of gD necessary to trigger fusion (Fig. 1.17) [35, 184]. This recombinant HSV-1 resulted functional, retargeted to HER2 and detargeted from natural gD receptors, and unable to spontaneous reverse mutation to wt ‐ genome and tropism.

In vivo experiments showed the anti-tumor efficacy of intratumorally administered R-LM249 [43]: in HER-2 positive SK-OV-3 tumors, both single and repeated injection induced a persistent therapeutic effect, with a high proportion (60%) of tumor free mice, in the second case.

Systemic i.p. administration of R-LM249 resulted effective against disseminated tumors, derived from SK-OV-3 cells, in mouse models that recapitulate tumor spread to the peritoneum in women: it strongly inhibited carcinomatosis reducing the growth of ovarian and breast carcinoma disseminated to the peritoneal cavity [186].

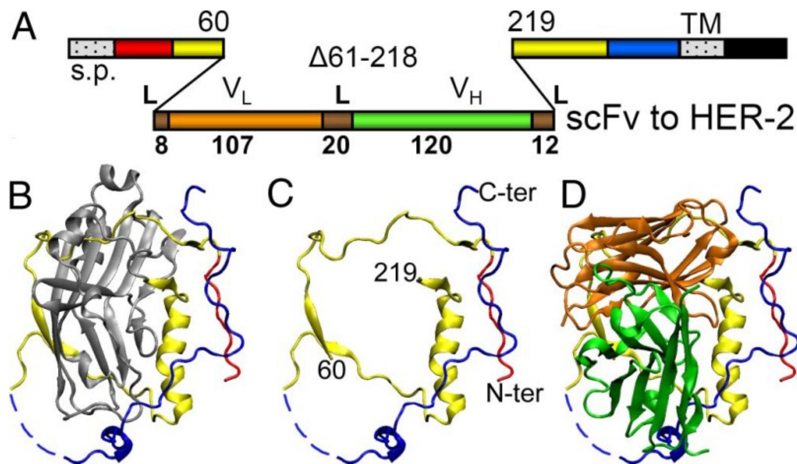

Fig. 1.17: **Linear map and 3D structure of chimeric R-LM249 gD.** A) Linear map of R-LM249 gD, with the indication of components of the chimeric protein: L, linkers; s.p., signal peptide; TM, transmembrane domain; VL and VH, variable light and heavy chains; numbers indicate the length in amino acid residues. Color code as in B–D. B) Monomeric crystal structure of gD ectodomain; in red and blue are indicated N terminus (res 23–32) and C terminus (res 257–307), respectively, while in gray (res 61-218) the Ig core and in yellow other portions [40]; C) the same structure as in B, but without the Ig core; D) arbitrary superimposition of the VL (orange) and VH (green) structure of trastuzumab scFv to gD as in C [43][Menotti, 2009 - o-HSV1 target HER2].

2. Role of αvβ3 – Integrin and TLR2 in the innate response to Herpes Simplex Virus infection

Data reported in this part of my thesis were published in:

Gianni Tatiana, **Leoni Valerio**, Campadelli-Fiume Gabriella. Type I interferon and NF-κB activation elicited by herpes simplex virus gH/gL via αvβ3 integrin in epithelial and neuronal cell lines. J Virol. 2013 Dec;87(24):13911-6. doi: 10.1128/JVI.01894-13. Epub 2013 Oct 9.

Gianni Tatiana**, Leoni Valerio**, Chesnokova Liudmila S., Hutt-Fletcher Lindsey M., Campadelli-Fiume Gabriella. αvβ3-integrin is a major sensor and activator of innate immunity to herpes simplex virus-1. Proc Natl Acad Sci U S A. 2012 Nov 27;109(48):19792-7. doi: 10.1073/pnas.1212597109. Epub 2012 Nov 12.

Leoni Valerio, Gianni Tatiana, Salvioli Stefano, Campadelli-Fiume Gabriella. Herpes simplex virus glycoproteins gH/gL and gB bind Toll-like receptor 2, and soluble gH/gL is sufficient to activate NF-κB. J Virol. 2012 Jun;86(12):6555-62. doi: 10.1128/JVI.00295-12. Epub 2012 Apr 11.

2.1. Objective

The aim of the first part of this thesis was to shed light on the mechanism of initiation of the innate response to HSV. Specifically we wanted: 1) to identify the HSV-1 PAMPs that activate TLR2 and lead to the initiation of innate immune response; 2) to define whether the HSV glycoproteins involved in entry and fusion can activate the TLR2 - dependent NF-κB signaling; 3) to study the possible role of αvβ3-integrin in the activation of innate response to viral infection.

Innate immune response is the first line of defense set up by the cell to counteract pathogens infection and it is elicited by activation of a number of membrane or intracellular receptors and sensors, collectively indicated as PRRs, Patter Recognition Receptors.

Numerous lines of evidence have shown the involvement of TLR2 in the activation of the first response to HSV-1 infection. However, while for HCMV it was defined which virion glycoprotein elicit this response, it was still unknown which was the HSV-1 component that is involved in this response.

Starting from the observation that entry-defective virions devoid of one of the essential glycoproteins, in particular gD-null virions, elicited an NF-κB response, we examined in details the capacity of the HSV-1 glycoproteins to elicit the innate immune response. Subsequently, we studied the NF-κB activation mediated by soluble glycoproteins and the physical interaction between them and TLR2.

Previous work from our laboratory showed the involvement of integrin αvβ3 in HSV-1 entry, in particular in the relocalization of virions to the cholesterol-rich microdomains where TLR2 resides. In this context, we asked whether αvβ3 participates in the innate response to HSV.

We studied the NF-κB response and the expression of a set of inflammatory cytokines by gain- and loss-of-function assays in cellular models expressing or not αvβ3 integrin. Next, we evaluated the physical interactions among the three key factors of this process: αvβ3 integrin, virion glycoproteins and TLR2.

Finally the data were confirmed in experimental models of epithelial, including keratinocytic, and neuronal cells, which are the major targets of HSV infection in vivo.

2.2 Materials and methods

2.2.1 Cells and viruses

The cells used were:

293T - a highly transfectable derivative of human epithelial cell line 293;

K562 cells – the human erythroleukemia cell line wt-K562 ($\alpha v\beta 3^-$ integrin) and their $\alpha v\beta 3^+$ integrin stable transformants were grown in Iscove's modified Dulbecco's medium supplemented with 10% FBS (Gibco) and medium containing 750 µg/ml neomycin G418 [187];

HeLa - an epithelial cell line derived from cervix adenocarcinoma;

SK-N-SH - a human neuroblastoma cell line;

HaCaT - immortal human keratinocyte line HaCaT, frequently used as a model for skin keratinocytes because of its highly preserved differentiation capacity;

Cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 5-10% FBS (Gibco), PenStrep (1%; Euroclone), unless otherwise stated. All cells were maintained at 37°C in 5% of CO2 atmosphere.

Wild-type HSV-1(F) and the mutant R7910, which carries the deletion of the α0 gene (ΔICP0) were described previously [188, 189].

R8102 carries the reporter Lac-Z under the α27 immediate-early promoter and was described [190].

The $gD^{-/+}$, $gH^{-/+}$, and $gB^{-/+}$ mutant viruses, which are deleted in the coding sequences of glycoprotein gD, gH, or gB respectively, were described previously [60, 191, 192].

2.2.2 Plasmids

gH-MTS, gL-MTS, and gB-MTS express HSV-1 gH, gL, and gB respectively, in mammalian cells under the control of the cytomegalovirus promoter, and they were described previously [193, 194].

gD-pcDNA3.1 and HVEM-pcDNA3.1 (pBEC) carry the nucleotide sequence of HSV-1 gD and human HVEM under the control of the cytomegalovirus promoter; they were described previously [193, 194].

pcDNA3.1 empty vector from Invitrogen used as a negative control in transfection.

pCMV6-XL-TLR2-Flag carry the nucleotide sequence of human TLR2 fused at the N terminal with Flag tag, and pNF-κB-luc express luciferase under the control of NF-κB promoter; the two plasmids were a gift from E. Kurt-Jones and were described previously [127].

pcDNA3-Flag-TOM1 encodes target of Myb protein 1 (TOM1) fused with FLAG tag and was a gift from A. Calistri.

pFUGW-ITGB3-1 or pFuGW-ITGB3-2 are a modified version of the lentiviral expression vector pFUGW49, which carry sequence expressing short hairpin RNA (shRNA) targeting integrin β3, able to silence this protein, under the control of the promoter of the small nuclear RNA U6; furthermore they express GFP under control of the promoter human ubiquitin C. The two plasmids are a generous gifts from [195].
pΔ8.9 and pVSV-G packaging vector that carry genes, like RNA polymerase and glycoprotein G of VSV, necessary for the production and assembly of lentiviral vector; they are a generous gifts from [195].

2.2.3 Antibodies

Monoclonal Ab (MAb) H1817 to gB recognizes a conformation-independent N-terminal epitope;
MAb H233 to gB recognizes a conformation-dependent epitope, neutralizes infectivity, and was purchased from the Goodwin Institute;
MAb 52S to gH recognizes a conformation-dependent epitope and neutralizes virion infectivity [196];
polyclonal Ab (PAb) to $gH_{t\text{-}st}$/gL was derived by standard techniques by PRIMM (Milan, Italy) [21];
Anti-Iκ-Bα MAb recognizes human Iκ-Bα and was from Cell Signaling;
Anti-tubulin Ab recognizes human β-tubulin and was from Sigma-Aldrich;
MAb AP3 and PAb AB1932 (from Chemicon) recognizes human Beta 3;
MAb L609 recognizes the αvβ3-heterodimer;
PE (phycoerythrin)-conjugated Strep-Tactin was from IBA GmbH (Göttingen, Germany);
anti-Flag M2 and anti-β-tubulin MAbs recognize Flag tag and β-tubulin respectively, and they were from Sigma-Aldrich.

2.2.4 Soluble glycoproteins

Soluble $gB_{730t\text{-}st}$ was constructed by dr. Arianna Cerretani and described [197]. It was produced in 293T cells and was purified by means of Strep-Tactin columns according to the IBA protocol at the IBA facility (IBA GmbH, Göttingen, Germany).
Soluble $gH_{t\text{-}st}$/gL was produced in insect cells as described previously [21].
Soluble gD290-299 was a generous gifts from G. H. Cohen and R. Eisenberg [198].

2.2.5 Infection

Cells were infected at the indicated multiplicities with extracellular virions, previously pelleted by ultracentrifugation. Following virus absorption to cells for 90 min at 37 °C, virus inoculum was removed, and monolayers were rinsed twice with medium. Infection with β-gal–expressing R8102 was usually stopped at 6 h after infection and revealed by X-gal staining [190]. For virus yield determinations, cells were frozen at 4 h (0 time) or 24 h after virus absorption. The virus was titrated by plaque assay in Vero (wt - HSV-1) or U2OS (R7910) cells.

2.2.6 Production of β3-Integrin silenced cell lines

Lentiviruses expressing two different sh-RNAs to β3-integrin (ITGB3), pseudotyped with vesicular stomatitis virus G glycoprotein, were produced by transfection of pFuGW-ITGB3-1 or pFuGW-ITGB3-2, packaging vector pΔ8.9, and pVSV-G into 293T cells harvested 48 h later, and immediately used to transduce 293T cells, HeLa, SK-N-SH and HaCaT cells. Cells trypsinized were mixed with lentiviruses virions and polybrene, and thus spinoculated to improve the transduction. The transduced (sh-β3) cells were monitored for effective silencing 7-10 d later. Two to three independently generated cell pools were obtained for each cell line. They were assayed for extent of silencing, and one for each cell line was then used for following assays. The sh-β3 cells remained stably silenced for several months. The control mock-silenced cells were generated similarly by means of plasmid pFuGW-control. Cell surface expression of αvβ3-integrin was measured by cell-ELISA: wt- or sh-β3 cells were grown in 96 well plates, reacted with MAb L609 to the αvβ3-heterodimer or with MAb AP3 to β3-integrin subunit for 2 h at 4 °C, paraformaldehyde-fixed, and then reacted with anti-mouse peroxidase, followed by o-phenylenediamine (Sigma-Aldrich) at 0.5 mg/mL and reading the optical density at 490 nm. Furthermore the extent of β3 integrin silencing was determined by quantitative real-time PCR (qRT-PCR) and the silencing was confirmed at the protein level by immunoblot analysis, carried out by means of PAb AB1932.

2.2.7 Transgene expression and NF-κB activity

Control or sh-β3 cells, or K562 cells negative or positive for αvβ3-integrin, were transfected by means of Arrest-in (Thermo Scientific) or Lipofectamine 2000 (Invitrogen) with plasmids encoding firefly luciferase under a NF-κB regulated promoter (pNF-κB-luc) (800 ng/2 × 10^5 cells) and Renilla luciferase (firefly : renilla ratio 130:1), plus TLR2-FLAG or pcDNA3.1 empty vector (50 ng/2 × 10^5 cells), as indicated. When indicated, pBEC encoding HVEM was added. After transfection with NF-κB-luc plasmid, cells were incubated with pre-exhausted medium for 2-3 d before use. To prepare the pre-exhausted medium, the respective cells were grown, and the medium was replaced with fresh medium containing 1% or 5% (vol/vol) FCS, incubated with the cells for 2-3 d, and thereafter filtered twice through 0.22 μm filters. The transfected cells were then exposed to the indicated viruses (UV-irradiated or not) for the indicated times or stimulated with lipopolysaccharide (LPS; #L2630 - Sigma-Aldrich) at 100 ng/mL, which induced a strong TLR2 response, $gD_{t290-299}$ at 1.5 μM, $gB_{730t-st}$ at 0.75 μM, or increasing concentrations of gH_{t-st}/gL for 4 h. When indicated, gH_{t-st}/gL were heat inactivated for 15 min at 100°C or pre-incubated with MAb 52S for 1 h at 37°C before addition to cells. Luciferase activity was quantified by means of Dual-Glo luciferase reporter assay system (Promega) and expressed as luciferase/renilla ratio. For detection and quantification of endogenous Iκ-Bα, wt- or sh-β3 293T cells, transfected or not with TLR2, were infected with UV-inactivated wt-HSV (wt- HSV-UV) or R9710 (20 PFU/cell). Lysates were harvested at 2, 4, and 6 h after infection in buffer containing 20

mM Hepes, 250 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.5% Nonidet P-40, plus protease and phosphatase inhibitors (Sigma-Aldrich), and separated by SDS PAGE. Iκ-Bα was detected by WB with anti–Iκ-Bα Ab. NF-κB activation leads to degradation of Iκ-Bα, visible as a decreased band. The blots were also reacted with anti-tubulin Ab. Quantification of the bands, expressed as DUs, was performed by Image-J software, and expressed as ratio of the DU of band of interest relative to that of tubulin, in the same sample. Data were expressed as percentage of the Iκ-Bα/tubulin value in uninfected cells.

2.2.8 Co-immunoprecipitation experiments

293T cells were transfected by means of Arrest-In with a TLR2-Flag-encoding plasmid (1.5 μg DNA/10-cm^2 dish) plus plasmids encoding full-length gH (1 μg) and gL (0,5 μg) or full-length gB (1 μg). Negative-control cells were transfected with the empty vector or with a plasmid encoding Flag-TOM1, an irrelevant protein. Eighteen hours after transfection, cells were lysed in PBS buffer containing 1% NP-40, 1% sodium deoxycholate, and protease inhibitors. TLR2 was immunoprecipitated with anti-Flag MAb M2. The proteins retained by protein G-Sepharose (Sigma-Aldrich) were lysed directly in SS (solubilizing solution) containing SDS and β-mercaptoethanol and boiled for 5 minutes. Hence they were separated by denaturing polyacrylamide electrophoresis PAGE and transferred onto Hybond-ECL nitrocellulose-membrane (Amersham Biosciences). The membranes were blocked with 5% non-fat dry milk in PBS for 30 minutes at 37°C, washed and reacted with PAb to gH/gL [21], MAb H1817 to gB, or MAb M2 to Flag, followed by peroxidase-conjugated anti mouse IgG horseradish peroxidase linked whole antibody (GE Healthcare) and enhanced chemioluminescence (ECL;Western blotting detection reagents; GE Healthcare). In a reverse co-immunoprecipitation experiment, the glycoproteins were first immunoprecipitated with MAb H1817 or PAb to gH/gL and the retained proteins were probed with anti-Flag MAb. In a different experiment, cells were transfected with plasmids encoding αvβ3-integrin, TLR2-Flag, or both. TLR2 was immunoprecipitated 24 h later by means of anti-Flag MAb. The proteins retained by protein A/G Sepharose were separated by SDS/PAGE and reacted with anti– β3-integrin PAb AB1932.

2.2.9 Reverse Transcription and q-RT-PCR

Total RNA was purified with Total RNA Isolation kit (Macherey-Nagel) according to the manufacturer's instructions; the procedure included digestion with DNase. Total RNA (1 μg/20 μL reaction mix) from mock- or HSV-infected cells, positive or negative for TLR2, wt, or sh-β3, was reverse transcribed with a high-capacity cDNA reverse transcription kit (Applied Biosystems). Real-time PCR primers were the inventoried TaqMan gene expression assays (Applied Biosystems). Reactions were performed in triplicates in a 20 μL volume that included cDNA (150 ng), gene expression assay (1 μL), and TaqMan gene expression master mix (10 μL) (Applied Biosystems). Real-time PCR reactions were performed on an Applied Biosystems Prism 7300 sequence detection system. Samples were normalized relative to GADPH Ct. To compare mock and

infected cell samples, relative changes in gene expression were determined using the $2^{-\Delta\Delta Ct}$ method. Data represent average of triplicates and were expressed as fold-variations relative to the uninfected cell value. Variations ≥ twofold relative to the corresponding value of mock-infected cells were considered significant.

2.2.10 Cytokine quantification

To determine the amount of IFN-β and IL10 produced by wt- or sh-β3 cells, negative or positive for TLR2 as indicated, the cell culture media were harvested 24 or 48 h after infection with R7910. IFN-β was detected by means of VeriKine kit (Pestka Biomedical Laboratories, PBL IFN Source) and human IL10 by Elisa kit (Thermo Scientific, Pierce). Media were added to the pre-coated wells in a 1:1 ratio with the kit dilution buffer for 1–2 h, according to the manufacturer’s instructions. The bound cytokines were revealed with appropriate antibodies conjugated to peroxidase plus substrate and reading the optical density at 450 nm. Standard quantities of the purified cytokines were run in parallel for relative quantification.

2.3 Results

2.3.1 Entry-defective virions devoid of one of the essential glycoproteins elicit the first wave of NF-κB activation dependent on TLR2.

First of all we confirmed the abilities of UV-inactivated virions and of the ΔICP0 R7910 mutant [189] to activate NF-κB, by transfecting 293T cells with TLR2 or an empty vector, plus the NF-κB-luc plasmid. This plasmid encodes an NF-κB-driven firefly luciferase reporter. Cells were co-transfected with a Renilla luciferase-encoding plasmid as a transfection control (Firefly/Renilla luciferase ratio, 130:1). The activation of NF-κB was expressed as units of Firefly luciferase relative to Renilla luciferase. In Figure 2.1 A it can be observed that the overall NF-κB activity was higher in the presence of TLR2 than in its absence, as expected, given that TLR2 is a sensor of HSV-1 [94]. UV-inactivated HSV-1(F) virions, able to penetrate cells but unable to direct expression of viral genes, account for the first wave of the NF-κB response. As expected, the R7910 ΔICP0 mutant exerted a greater effect than the wild-type virus at 4 to 6 h after infection, in agreement with recent findings which indicate that ICP0 counteracts the TLR2 signaling pathway [127].

Next, in the same cellular model transfected, or not, with TLR2 along with NF-κB-luc- and Renilla luciferase, we measured NF-κB activation by virions devoid of gD, gH, or gB. These viruses lack the indicated genes, they are usually grown in complementing cells, generating the corresponding $gD^{-/+}$, $gH^{-/+}$, and $gB^{-/+}$ virions, but when they are grown in non-complementing cells, they produce $gD^{-/-}$, $gH^{-/-}$, and $gB^{-/-}$ virions, which attach to cells but fail to carry out virion-cell fusion and entry. Figure 2.1 B shows that the glycoprotein-null virions elicited reporter NF-κB activation; in particular the greatest effect was observed with the $gD^{-/-}$ virions in TLR2-positive cells. Although the differences among the different mutant virions were not necessarily significant, these findings point to entry glycoproteins as candidate inducers of TLR2-dependent NF-κB activation.

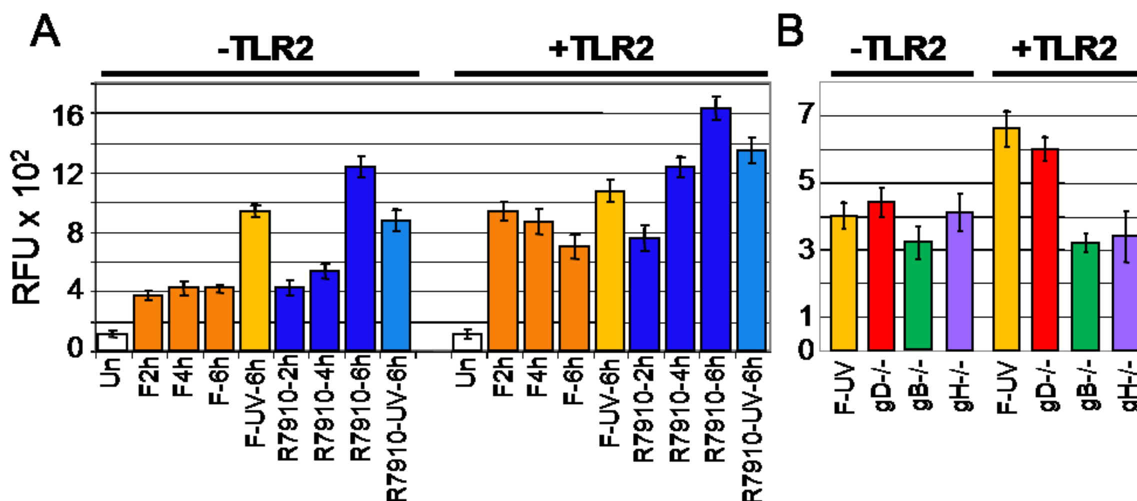

Fig. 2.1: **NF-κB activation by wild-type HSV-1(F), an ICP0$^-$ recombinant (R7910), or virions devoid of gD (gD$^{-/-}$), gB (gB$^{-/-}$), or gH/gL (gH$^{-/-}$).** 239T cells were transfected with NF-κB-luc and Renilla luciferase, and with TLR2 as indicated. At 18 h after transfection, cells were exposed to the indicated viruses and harvested at the indicated times (hours) for panel A or at 6 h for panel B. UV-inactivated virions, F-UV and R7910-UV. Results are the average of triplicate experiments and are expressed as relative fluorescence units (RFU)x10^2 +/- the standard deviation. Un, untreated.

2.3.2 Soluble forms of gH/gL, but not of gB, suffice for TLR2-dependent NF-κB activation.

Next, on the basis of these results, we investigated if soluble forms of the virion glycoproteins suffice to mediate NF-κB activation. $gH_{t\text{-}st}$/gL is a biologically active soluble form, produced in insect cells and carrying a C-terminal Strep tag for purification; it binds conformation-dependent neutralizing MAbs, binds to cell surfaces in a saturable manner, and inhibits virus infection [21]. In the same 293T cellular model used in the previous experiments, we evaluated the NF-κB response to increasing amounts of $gH_{t\text{-}st}$/gL. In Figure 2.2 A it results that $gH_{t\text{-}st}$/gL induced NF-κB activation in a TLR2-dependent way. Since preparations of soluble proteins may contain heat-stable LPS, lipopeptides, or other unspecific activators that impair NF-κB activation, we analyzed the response after heat inactivation of $gH_{t\text{-}st}$/gL and observed a reduced TLR2-dependent NF-κB activation by 60 to 70%, suggesting that most of the activity was specific to gH/gL. The preincubation of $gH_{t\text{-}st}$/gL with MAb 52S reduced NF-κB activation by about 60% and this confirmed the conclusion on this glycoprotein (Figure 2.2 B).

Next we evaluated the effect of $gB_{730t\text{-}st}$: our preparations of soluble gB were at much lower concentrations and so needed to be concentrated by centrifugal filter devices, and in general we employed larger volumes. We used at least three independent batches, but they all failed to elicit an NF-κB response, in agreement with a previous report [78]. A representative result is shown in Figure 2.4 C, along with NF-κB activation induced by R7910 or by a commercial LPS (100 ng/ml) preparation, which elicited a TLR2-dependent response. However some batches of $gB_{730t\text{-}st}$ elicited an NF-κB response, but this was mostly heat resistant and not decreased by preincubation of the glycoprotein with MAb H233 (data not shown). From these data, it can be concluded that soluble gH/gL, but not soluble gB, is able and sufficient to induce NF-κB activation in a TLR2-dependent fashion.

To complete the study on HSV-1 glycoproteins we analyzed the effects of gD. It was reported previously that gD induces NF-κB activation following binding to HVEM, one of its receptor [75]. We transfected 293T cells with HVEM, exposed them to soluble $gD_{290\text{-}299}$, and measured NF-κB activity (Figure 2.2 D). We confirmed the HVEM dependent NF-κB activation, and furthermore we noted that this activation did not vary whether cells were positive or negative for TLR2.

These data support the idea that HSV virions can elicit NF-κB activation through at least two independent signaling pathways, one initiated by gH/gL via TLR2 and one initiated by gD via HVEM and independent of TLR2. Afterwards to verify gH/gL-induced NF-κB activation at the endogenous protein level, we detected Iκ-

Bα, which is first phosphorylated and then degraded during the process of NF-κB activation. 293T cells, negative or positive for TLR2, were infected with R7910 or exposed to $gH_{t\text{-}st}$/gL or the commercial LPS preparation for 4 h. Cells were lysed, subjected to SDS-PAGE and Iκ-Bα was revealed by WB; the activation of NF-κB is indicated by a decrease in Iκ-Bα band intensities. The same blots were also reacted with anti-tubulin, band intensities were measured by means of ImageJ software, and their quantification was expressed in densitometric units (DUs). We calculated the ratio of the DUs of the Iκ-Bα band relative to the DUs of tubulin in the same sample and expressed it as a percentage of the ratio in the uninfected-cell sample. Figure 2.3 A shows a typical WB example and the average relative quantification of band intensities from two experiments is represented in Figure 2.3 B.

These data highlight that in TLR2-positive cells $gH_{t\text{-}st}$/gL induces a decrease in Iκ-Bα levels similar to that caused by R7910, confirming its ability to activate NF-κB.

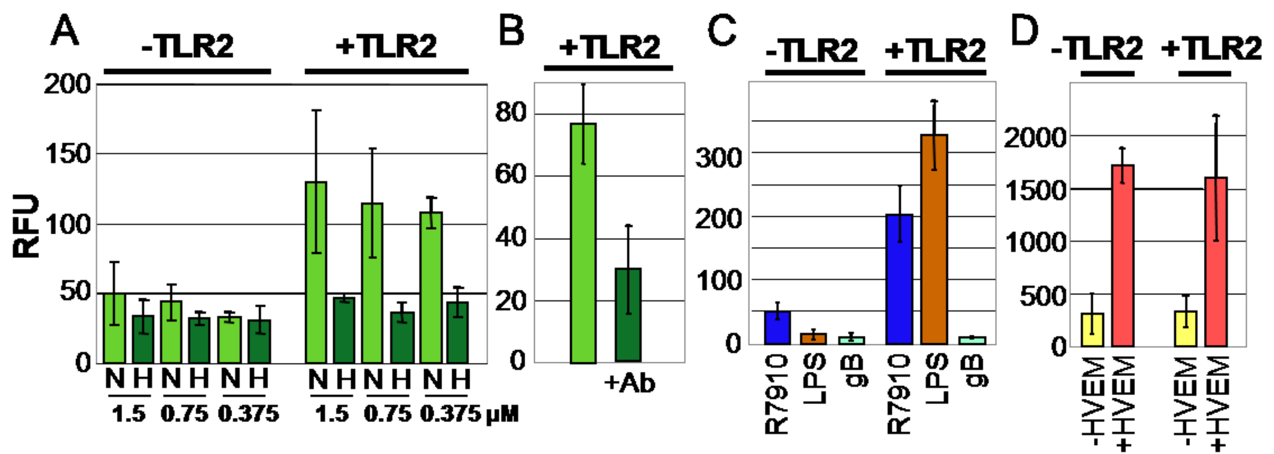

Fig. 2.2: **NF-κB activation by soluble glycoproteins $gH_{t\text{-}st}$/gL, $gB_{730t\text{-}st}$, and $gD_{290\text{-}299}$.** (A) 239T cells were transfected with NF-κB-luc, Renilla luceiferase and TLR2 as indicated. At 48 h after transfection, cells were exposed to the indicated amounts of native (N) or heat-denatured (H) $gH_{t\text{-}st}$/gL for 4 h. The results are the average of three experiments with two different batches of $gH_{t\text{-}st}$/gL. (B) 239T cells were transfected as in panel A; gH/gL was pre-incubated for 1 h with MAb 52S (+Ab) prior to administration to cells for 4 h. (C) 293T cells positive or negative for TLR2 were exposed to R7910, $gB_{730t\text{-}st}$, or LPS for 4 h as indicated in panel A. (D) 239T cells were transfected with NF-κB-luc, Renilla luciferase, TLR2, and HVEM as indicated. At 48 h after transfection, cells were exposed to $gD_{290\text{-}299}$ for 4 h. All results are averages of triplicate experiments.

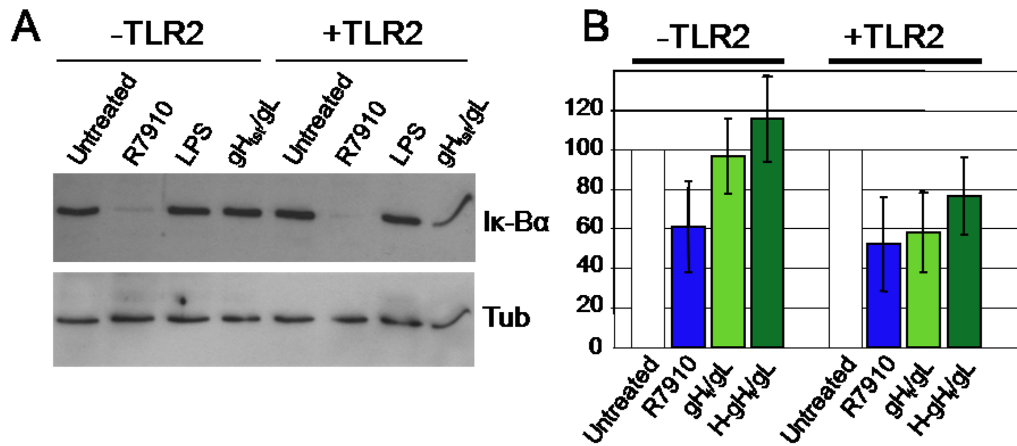

Fig. 2.3: **Validation of NF-κB activation by $gH_{t\text{-}st}$/gL through detection of Iκ-Bα degradation.** 239T cells were transfected with TLR2 as indicated for 48 h and exposed to R7910 or native or heat-denatured (H) $gH_{t\text{-}st}$/gL for 4 h. Cellular proteins extracted in the presence of protease and phosphatase inhibitors (Sigma-Aldrich) were separated by SDS-PAGE and identified by WB with Abs to Iκ-Bα and tubulin (Tub) (A). For each lane, quantification is expressed as the ratio of Iκ-Bα to tubulin (B).

2.3.3 TLR2 physically interacts with gH/gL and gB.

Taking into account that we have observed a TLR2-dependent NF-κB activation both from virions and soluble gH/gL treatment, and since previous works on HCMV showed that gB and gH/gL induce NF-κB activation by binding TLR2 [78], we investigated whether HSV gH/gL and gB physically interact with TLR2. We transfected 293T cells with TLR2-Flag or an irrelevant Flag-tagged protein (TOM1) as a negative control, together with full-length gH/gL or gB and carried out co-immunoprecipitation assays. In the first series of experiments (Figure 2.4 A and B), TLR2 was immunoprecipitated with anti-Flag MAb (IP Flag); the retained proteins were analyzed by WB with PAb to gH/gL or MAb H1817 to gB (panel A). As shown, TLR2-Flag co-immunoprecipitated gH (lane 3) and gB (lane 6). The glycoproteins were not immunoprecipitated by the protein TOM1-Flag (lanes 2 and 5).

The immunoprecipitation of TLR2 was ascertained by blotting the same strips with anti-Flag MAb (lanes 3' and 6' in panel B). TOM1 is not visible in lanes 2' and 5' because it has a much lower Mr than TLR2 and hence migrates faster. To validate the above results, we carried out the reverse co-immunoprecipitation (Fig. 2.4 C). gH and gB were first immunoprecipitated with specific Abs (IP-gH, lane 3; IP-gB, lane 6); co-immunoprecipitated TLR2 was revealed with anti-Flag MAb. Figure 2.4 C shows that immunoprecipitation of gH (lane 3) or gB (lane 6) pulled down TLR2-Flag. We noted that in the gB immunoprecipitations (Fig. 2.4 C, lanes 5 and 6), an unspecific band shows up, with a migration slower than that of TLR2-Flag. The lanes marked "Lys" contain aliquots of the lysates prior to immunoprecipitation and were included to enable identification of the migration position of the proteins of interest.

Therefore our data show the capacity of TLR2 to physically interact with the viral glycoprotein gH/gL and gB.

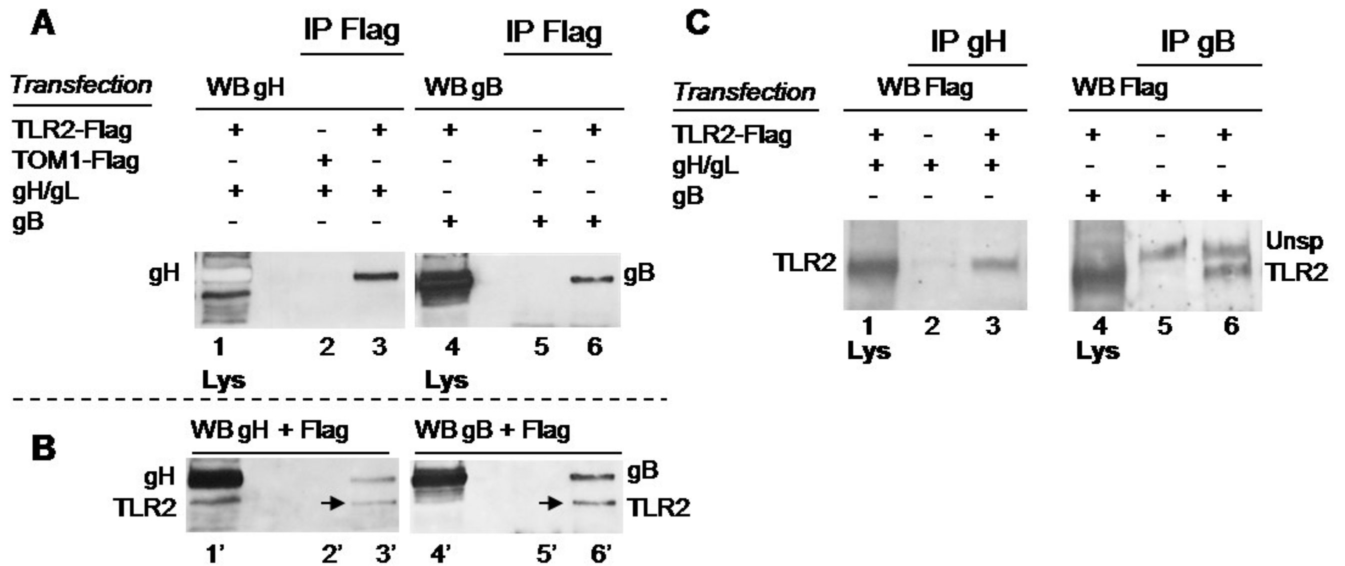

Fig. 2.4: **gH/gL and gB interact physically with TLR2 in co-immunoprecipitation assays.** 293T cells were transfected with TLR2-Flag or TOM1-Flag and full length gH/gL or gB, as indicated to the left in italics. (A and B) TLR2-Flag or TOM1-Flag was immunoprecipitated with anti-Flag MAb (IP Flag); complexes were harvested with protein G-Sepharose. Co-immunoprecipitated gH or gB was separated by SDS-PAGE and revealed by WB with PAb to gH or MAb H1817 to gB (A). Immunoprecipitated TLR2 was identified with anti-Flag MAb (B). (C) 293T cells were cotransfected as indicated to the left in italics. gH or gB was immunoprecipitated with PAb to gH (IP gH) or MAb H1817 to gB (IP gB). Co-immunoprecipitated TLR2, retained by protein A-Sepharose, was separated by SDS-PAGE and identified with anti-Flag MAb (TLR2 band). Unsp denotes an unspecific band which showed up only in immunoprecipitations with MAb H1817. In all panels, aliquots of lysates (Lys) were run in parallel for identification of the migration positions of the proteins of interest that were identified by WB.

2.3.4 β3-Integrin–silenced 293T cells support HSV replication.

In the context of innate immune response to HSV-1, the issue we addressed centered on the role of αvβ3-integrin. We performed loss-of-function experiments by silencing β3-integrin in 293T cells by means of lentiviruses encoding sh-RNA to β3 integrin. The stably silenced 293T cell line was designated as sh-β3 and the β3 reduction was verified both at mRNA level, by q-RT-PCR, and at protein level, by cell-ELISA and WB; mock-silenced cells, generated by a control non targeting sh-RNA lentivirus, were used as a control. We observed a decrease of about 70% relative to control cells in mRNA and surface expression of β3-integrin (Figure 2.5 A, B), also confirmed by WB (Figure 2.5 C). Subsequently we verified that sh-β3 293T cells remained susceptible to HSV-1, to rule out that any decrease in innate responses was due to reduced infection: wt- and sh-β3 293T cells were infected with R8102, which expresses the reporter β-gal under the immediate-early α27 promoter [190]. Levels of infection were quantified as β-gal expression. Figure 2.5 D shows that sh-β3 293T cells support HSV infection with similar efficiency as wt-cells at 5-10 PFU/cell. Replication in wt- and control mock-silenced cells was indistinguishable (Figure 2.5 E). Moreover sh-β3 and wt-293T cells produced similar amounts of immediate early, early, and late viral proteins (data not shown).

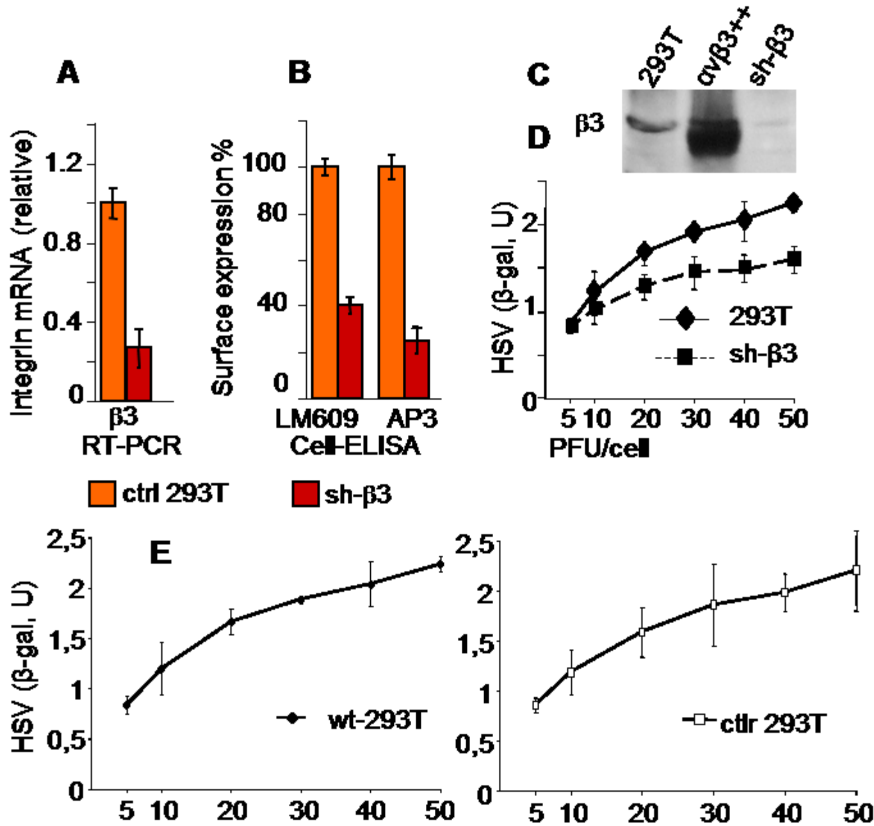

Fig. 2.5: **β3-Integrin-silenced 293T cells support HSV-1 infection.** Silencing of β3-integrin in 293T cells by lentivirus encoding shRNA to generate sh-β3 cells was quantified by q-RT-PCR (A), cell-ELISA (B), and WB (C) relative to control mock-silenced cells (ctrl 293T). (A) Relative changes in gene expression between control mock-silenced and sh-β3 cell samples were determined by q-RT-PCR using the $2^{-\Delta\Delta Ct}$ method. The results were normalized relative to those of control mock-silenced cells. (B) Cell-ELISA was performed by MAb LM609 to αvβ3-integrin or MAb AP3 to β3-integrin. (C) WB of lysates of control mock-silenced, 293T cells overexpressing αvβ3-integrin (αvβ3++), or sh-β3 cells performed by polyclonal antibody (PAb) to β3-integrin. (D) sh-β3 and wt-293T (293T) cells were exposed to R8102, β-gal expression was measured at 6 h p.i. and expressed as arbitrary units (U). (E) Replication of R8102 in wt- and control mock-silenced 293T cells. Wt-293T cells (Left) and control mock-silenced 293T cells (Right) were exposed to R8102, β-gal expression was measured at 6 h p.i and expressed as arbitrary units (U). In panels A, B, D, and E, data represent the average of triplicates or quadruplicates ± SD.

2.3.5 NF-κB response is inhibited in β3-integrin-silenced 293T cells, particularly in the presence of TLR2.

As shown above, the NF-κB response induced by incoming HSV-1 is detectable at 2 h p.i., and more sustained at 6-8 h p.i. It is best seen with R7910, which lacks ICP0, a viral protein that counteracts part of the host defense [127]. Control mock-silenced or sh-β3 cells were transfected or not with TLR2, NF-κB-luciferase and Renilla-luciferase and then infected with wt-HSV, UV-inactivated wt-HSV, or R7910. In β3-integrin–silenced cells, both the TLR2-independent and –dependent NF-κB activation was dramatically reduced (Figure 2.6 A and B). Interestingly, the inhibition was seen also when NF-κB was activated by a

commercial LPS, able to activate TLR2, implying that αvβ3-integrin–mediated innate response was activated also by bacterial components. Afterwards, we measured the endogenous NF-κB, specifically the levels of Iκ-Bα inhibitor, to validate the results observed. Lysates of control or sh-β3 cells, positive or negative for TLR2, infected with R7910 or UV-HSV-1, were subjected to SDS/PAGE and Iκ-Bα was detected by WB. In Figure 2.6 C a WB from a representative experiment is shown and the quantification is reported in Figure 2.6 D: in TLR2^{+} mock-silenced cells, Iκ-Bα was decreased by about 65% in infected cells, relative to uninfected cells, while in sh-β3 cells, Iκ-Bα did not significantly decrease, as exemplified by the 2 and 4 h samples.

These loss-of-function experiments provide the first line of evidence that αvβ3-integrin plays a prominent role in NF-κB response induced by HSV or bacterial components in 293T cells, particularly in the presence of TLR2.

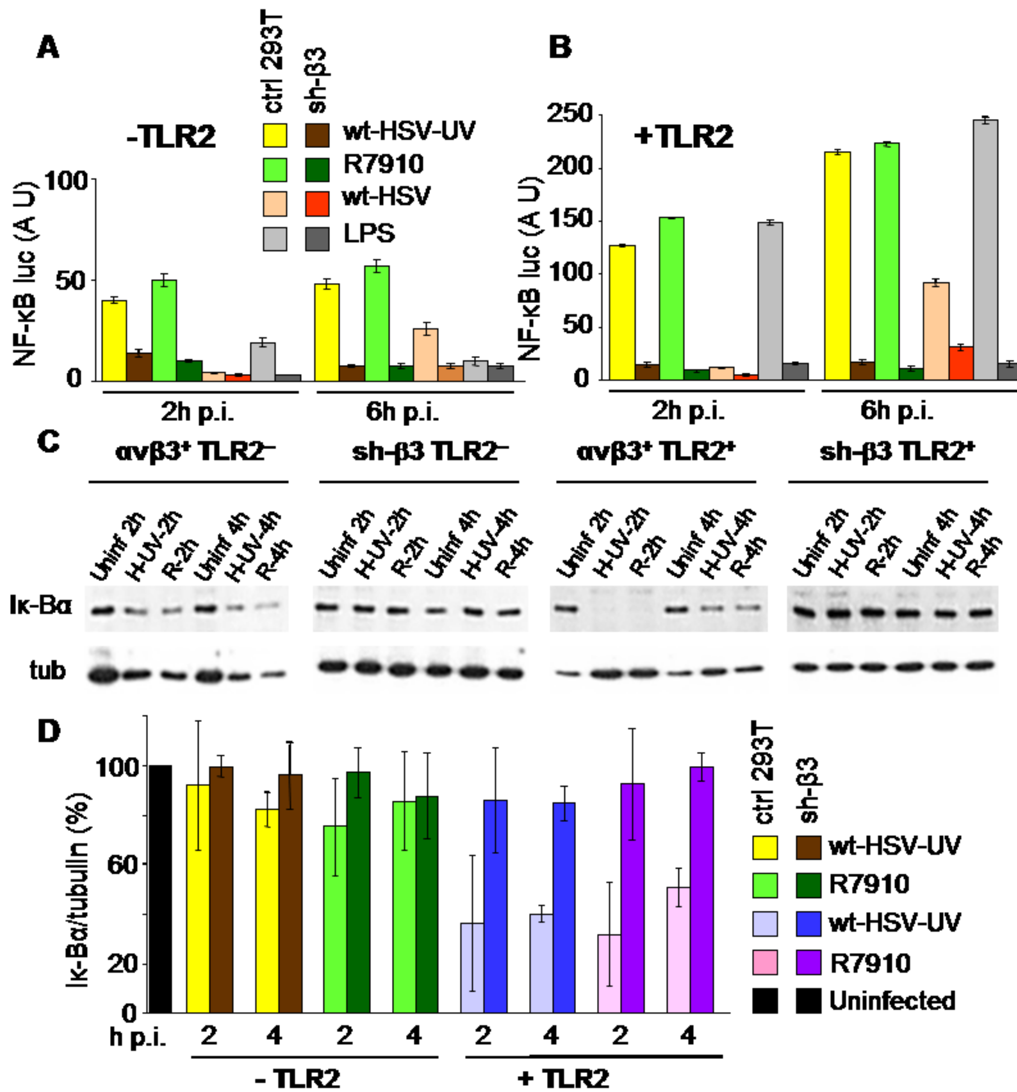

Fig. 2.6: **HSV-induced NF-κB activation is inhibited in sh-β3 293T cells.** (A and B) Control (ctrl) or sh-β3 293T cells were transfected with TLR2 (panel B) or empty plasmid (panel A) plus NF-κB-luc and Renilla luciferase. At 12 h after transfection cells were cultured in pre-exhausted medium for 48-72 h. Cells were then infected with 20 PFU/cell of wt-HSV, UV-inactivated wt-HSV (wt-HSV-UV),

R7910, or exposed to LPS (100 ng/mL) for 2, 4, or 6 h. NF-κB-luc was measured and expressed as arbitrary units (U) of luciferase:renilla ratio. The histogram shows the NF-κB-luc activity following infection or LPS treatment, and the decrease in sh-β3 cells relative to control cultures, at 2 and 6 h after infection. Each value represents the average of triplicate samples from two independent experiments. (C and D) Quantification of endogenous NF-κB activity through detection of Iκ-Bα. Control (ctrl) or sh-β3 293T cells, transfected with TLR2 (+TLR2) or an empty plasmid (–TLR2), were infected with UV inactivated wt-HSV (wt-HSV-UV) (H-UV) or R9710 (R) (20 PFU/cell) for 2, 4, and 6 h. Lysates were subjected to SDS PAGE; Iκ-Bα and tubulin were detected by WB. (D) Quantification of bands, expressed as densitometric units (DUs), was performed by Image-J software. Shown are the 2 and 4 h Ik-Bα/tubulin ratios, expressed as percentage, relative to the uninfected cell value taken at each time point (black column). Vertical bars represent SD.

2.3.6 Expression of IFNβ, IFNα, IL2, and IL10 (Group 1) cytokines is strongly inhibited in TLR2^{+} β3-Integrin-silenced 293T cells.

In light of the data we obtained, we decided to investigate the signaling pathways affected by αvβ3-integrin in the first hours of infection. The NF-κB-related genes in sh-β3 293T cells, positive or negative for TLR2, were preliminarily profiled by dr. T. Gianni by microarray. In collaboration with dr. T. Gianni, the relevant genes were validated by q-RT-PCR. Variations ≥ twofold were considered as significant. Based on the effect of β3-integrin silencing, the genes were clustered into three groups. The expression of group 1 cytokine genes -IFNB1, IFNA1, IL2, and IL10- was dramatically decreased in sh-β3 293T cells, independent of whether they were negative or positive for TLR2 (Figure 2.7 A-D). As it can be deducted from the fold increase, these genes were among the most highly induced by HSV, particularly in the presence of TLR2. Thus, β3-integrin silencing exerts an opposite effect on IL2 and its receptor, suggesting a fine-tuning capability. IFNβ and IL10 decreases were validated at the protein level (Figure 2.7 E,F). These results show that αvβ3-integrin dictates the HSV-induced production of IFNα, IFNβ, IL2, and IL10, and since this signaling is enhanced by TLR2, the transcription factor NF-κB can behave as its master regulator. The down-regulation of group 1 cytokine genes seen in β3-integrin-silenced and TLR2^{-} cells may reflect the TLR2-independent ability of αvβ3-integrin to activate NF-κB, observed through antibody cross-linking (data not shown, [199]).

The expression of group 2 genes (SRC, SYK, IRF3, IRF7, TRIF, and CARD9) was moderately affected by β3-integrin silencing. Collectively, these genes identify a TLR2-independent signaling pathway, distinct from the one defined by group 1 genes (data not shown).

The expression of group 3 genes (TNF, LTBR, IL1A, IL6, BCL3, FASLG) was not modified in sh-β3 cells, irrespective of TLR2 (data not shown). Beyond its intrinsic significance, group 3 genes show that β3-integrin silencing selectively affects the cell transcriptome.

Altogether, the profile of affected genes indicates that αvβ3-integrin-mediated induction of cytokines is highly polarized.

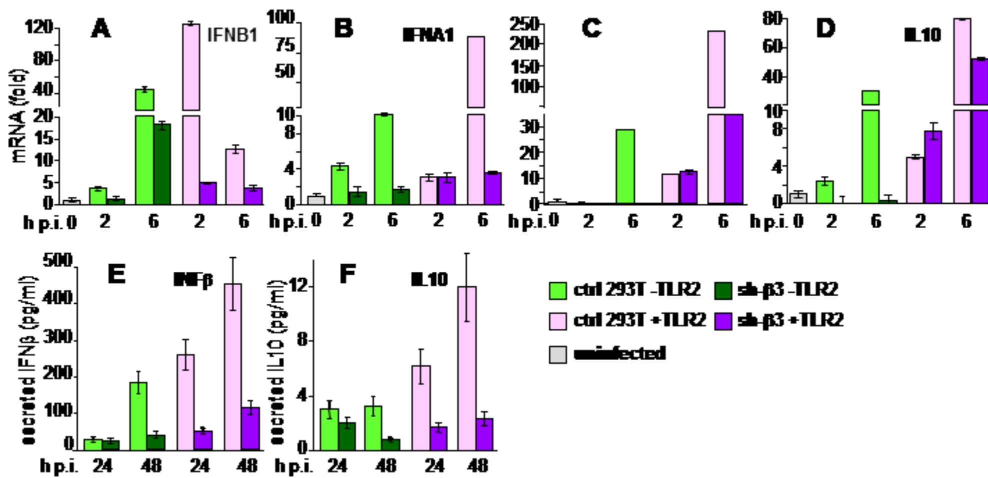

Fig. 2.7: **IFN-α and -β, IL2, and IL10 expression are selectively decreased in β3-integrin-silenced 293T cells.** (A–D) Control mock-silenced (ctrl) or sh-β3 293T cells, transfected with irrelevant plasmid (–TLR2) or TLR2 (+TLR2), were cultured in pre-exhausted medium from12 to 48-72 h after transfection and then infected with UV inactivated wt-HSV-1(F) (50 PFU/cell) and harvested at 2 h p.i. or infected with R7910 (20 PFU/cell) and harvested at 6 h p.i. RNA was extracted and retrotranscribed by standard techniques for q-RT-PCR. To compare infected versus uninfected cell (uninfected) samples, relative changes in gene expression were determined using the $2^{-\Delta\Delta Ct}$ method. Data were expressed as relative mRNA levels relative to the uninfected cell value, taken as 1 (column, uninfected). Each value represents the average of triplicate samples ± SD. (E and F) Secretion of IFN-β and IL10 by control or sh-β3 293T cells, infected with R7910. The media were harvested 24 or 48 h after infection. Each value represents average of triplicates ± SD.

2.3.7 Gain-of-function experiments: the NF-κB and type-1 IFN response is increased in K562 cells expressing αvβ3-integrin.

To verify the above conclusion on the role of αvβ3-integrin in the innate immune response, we performed a gain-of-function experiment on the myelomonocytic K562 cells, which do not express αv- and β3-integrins [21, 187]. Stable transformants expressing αvβ3-integrin were described [187] and the αv- and β3-integrin expression profile in these cells was previously assessed by RT-PCR, immunofluorescence, and FACS [21]. We transfected wt-K562 cells and $\alpha v\beta 3^{+}$ K562 with NF-κB-luc, Renilla-luciferase, and TLR2 and infected them with R7910 or treated with LPS. In $\alpha v\beta 3^{+}$ cells the NF-κB response induced by HSV or LPS was several-fold higher than in wt-K562 (Figure 2.8 A).

Next, we studied the expression of IFNB and IL10 induced by R7910 infection: the cytokines expression and secretion were significantly higher in the $\alpha v\beta 3^{+}$ than in the $\alpha v\beta 3^{-}$ K562 cells (Figure 2.8 B-E). Thus, even in K562 cell system, αvβ3-integrin was critical for the induction of IFNB1 and IL10.

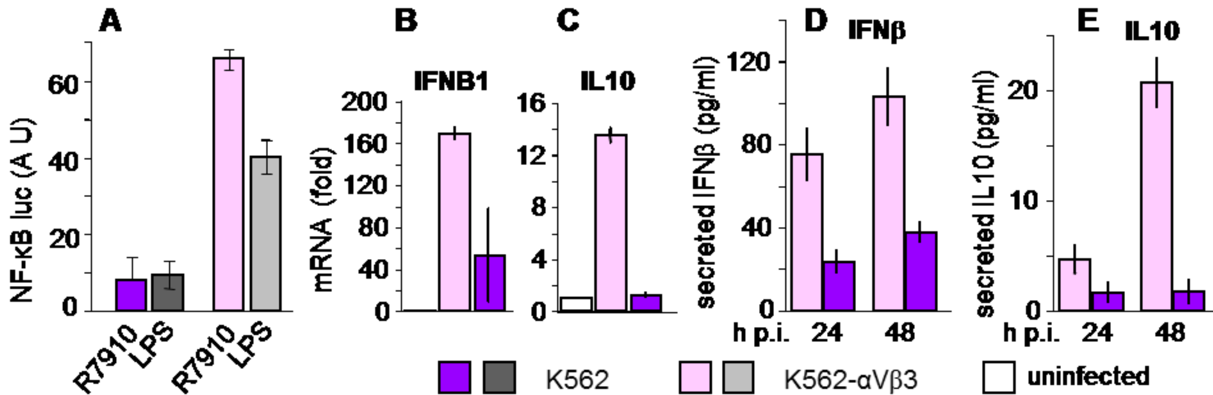

Fig. 2.8: **Gain-of-function assay.** HSV-induced NF-κB activation and IFNβ1 and IL10 expression and production are increased in K562 cells expressing αvβ3- integrin, relative to wt-integrin–negative-K562. (A) NF-κB activity in K562 cells expressing or not αvβ3-integrin. The integrin-negative K562 cells (K562) and their counterparts expressing αvβ3-integrin (K562-αvβ3) were transfected with NF-κB-luc and Renilla luciferase plus TLR2 and infected 96 h later with R7910 (20 PFU/cell) or exposed to LPS, both for 6 h. NF-κB activity was expressed as luciferase:renilla ratio, as in Fig.2.6 (B and C) Expression of IFNB and IL10 in K562-αvβ3 and K562 cells infected with R7910 (50 PFU/cell) for 6 h. Results are the average of two independent experiments. (D and E) Production of IFN-β1 and IL10 in the media of K562-αvβ3 and K562 cells infected with R7910 (50 PFU/cell) for 24 or 48 h. Each value represents the average of triplicate samples ± SD.

2.3.8 αvβ3-integrin physically interacts with the virion glycoproteins gH/gL and with TLR2.

Since a physical interaction between αvβ3-integrin and virions or TRL2 can explain its involvement in the innate response to HSV, we studied the binding of integrin to the these factors. Regarding the virion components, we focused on the envelope glycoproteins gH/gL because, in other herpesviruses, they interact with integrins [200, 201], and because gH/gL from HSV interacts with TLR2, as we showed above.

Chesnokova and Hutt-Fletcher in a collaborative study assessed the αvβ3-integrin - gH/gL physical interaction by Biacore. The thermodynamic K_D was $K_D = (4.2 \pm 1.8) \times 10^{-6}$M, which indicates that αvβ3-integrin binds gH/gL at relatively low affinity. Data obtained in our laboratory [21] showed that αvβ3-integrin does not play an essential role in HSV entry, and cells that lack β3-integrin are readily infected.

We studied the interaction between αvβ3-integrin and TLR2 by co-immunoprecipitation experiments: we cotransfected 293T cells with TLR2-Flag and αvβ3-integrin, immunoprecipitated TLR2 with anti-Flag MAb, and the retained proteins were analyzed by WB with PAb AB1932 to β3-integrin. We observed that TLR2-Flag co-immunoprecipitated β3-integrin (Figure 2.9 A), in agreement with a previous report [195].

In the above results, we reported that soluble gH_t/gL was sufficient to induce NF-κB activation; data obtained on silenced cells showed that this activation is αvβ3-integrin-dependent, as it was strongly decreased in sh-β3 293T cells. The gH_t/gL-mediated activation of NF-κB was specific, as it was inhibited by gH_t/gL pre-incubation with its neutralizing MAb 52S or heat-denaturation (Figure 2.9 B).

We further demonstrate that gH_t/gL was sufficient for IFNB induction and that this induction is αvβ3-integrin-dependent, in that it was strongly decreased in sh-β3 293T cells (Figure 2.9 C and D).

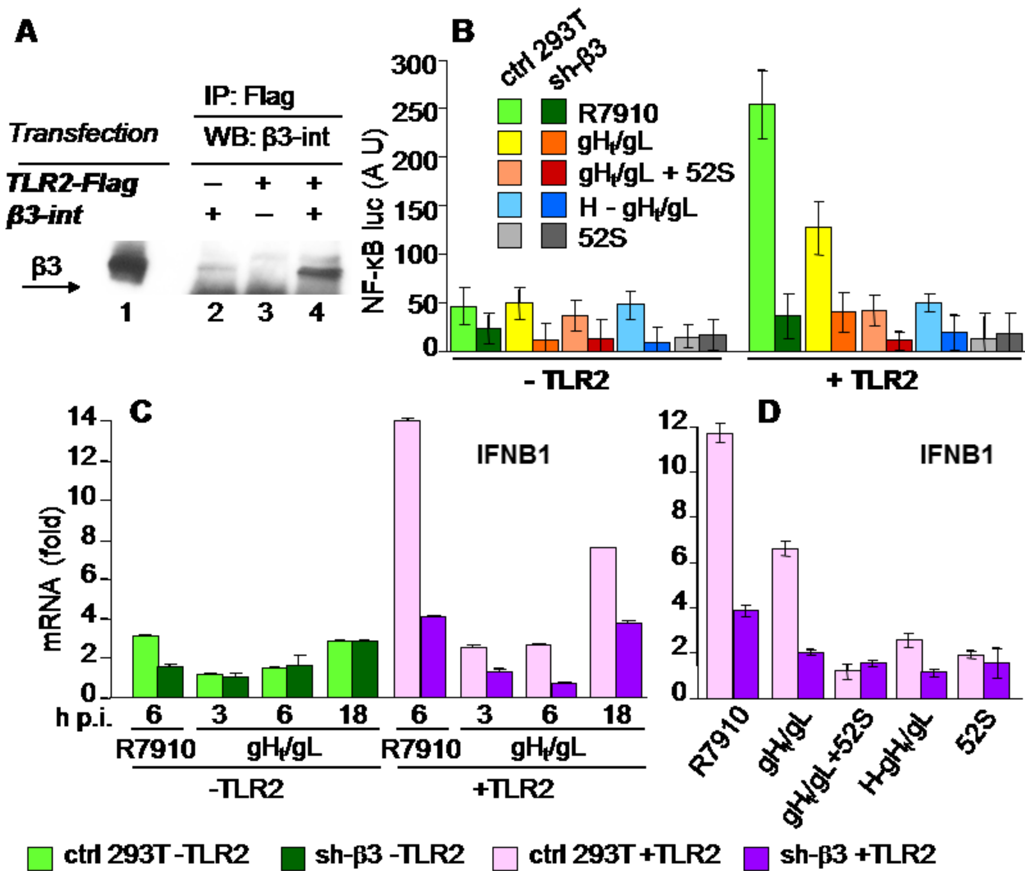

Fig. 2.9: **The virion gH/gL glycoproteins physically interact with αvβ3-integrin and induce NF-κB and IFNB activation in wt-293T cells but not in sh-β3 293T cells.** (A) Co-immunoprecipitation of β3- integrin by TLR2-Flag. Cells were transfected with αvβ3-integrin (1, 2), TLR2- Flag (3), or cotransfected with αvβ3-integrin + TLR2-Flag (4). At 24 h post-transfection TLR2 was immunoprecipitated by anti-Flag MAb (Sigma-Aldrich). The proteins retained by Protein A/G Sepharose were separated by SDS/PAGE and analyzed by WB with anti–β3-integrin PAb. Lane 1 shows the migration position of β3-integrin. (B) Control (ctrl) or sh-β3 293T cells were transfected with TLR2 (Right) or an empty plasmid (Left) plus NF-κB-luc and Renilla luciferase plasmids. Cells were infected with R7910 or exposed to gH_t/gL (1.5 µM) for 6 h. In the "gH/gL + 52S" sample, gH_t/gL was pre-incubated with neutralizing MAb 52S for 1 h at 37 °C. In the "H–gH_t/gL" sample, gH/gL was heat-inactivated for 1 h at 95 °C. In the "52S" sample, cells were exposed to MAb alone. NF-κB-luc was measured as detailed in Fig. 2.8 (C and D) Control mock-silenced (ctrl) or sh-β3 293T cells, transfected with irrelevant plasmid (–TLR2) or TLR2 (+TLR2), were infected with R7910 (6 h) or exposed to gH_t/gL (1.5 µM) for 3, 6, and 18 h (C) or for 18 h (D), as detailed in B. RNA extraction and IFNB1 mRNA expression were as detailed in Fig. 2.7. Each value represents the average of triplicate samples ± SD.

2.3.9 Defective replication of the ΔICP0 R7910 mutant is partially rescued in β3-integrin-silenced 293T cells.

It is well established that some of the innate responses limit viral replication within the initially infected cell and ΔICP0 mutants are highly impaired in replication [10]. Therefore we asked if ΔICP0 R7910 replication can improve in sh-β3 cells compared to wt cells, and for this reason we measured the yield of wt HSV-1 and R7910 in β3-integrin-silenced and mock-silenced 293T cells. Surprisingly, the growth defect of R7910, apparent in αvβ3^{+} TLR2^{+} cells, was partly rescued in the sh-β3 cells, particularly when TLR2 was absent (Figure 2.10 A and B), that is a condition in which the NF-κB response was severely decreased. Of note, the replication of wt-HSV was not increased in β3-integrin silenced cells relative to the non-silenced cells and was either unmodified or slightly decreased.

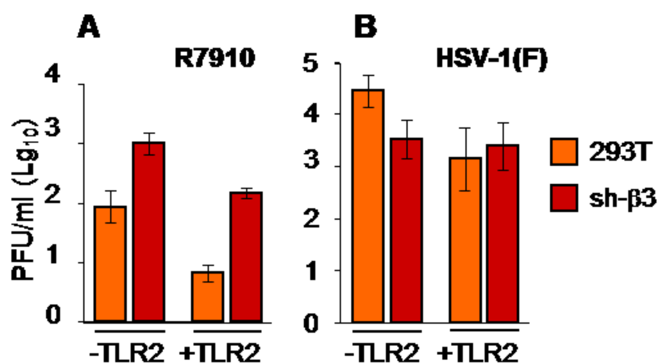

Fig. 2.10: **Yield of wt HSV-1(F) and R7910 in wt or sh-β3 293T cells, negative or positive for TLR2.** (A and B) Replicate cultures were infected (1 PFU/cell) and harvested 4 h (0 time) or 24 h after virus adsorption. The virus present in cell debris plus medium was titrated in Vero [HSV-1(F)] or U2OS (R7910) cells. Columns show the 24 h yield (PFU/mL) after subtraction of 0-time values. Results are average of three experiments ± SD.

2.3.10 β3-Integrin–silenced HaCaT, HeLa, and SK-N-SH cells support HSV replication

The next step was the study of αvβ3 integrin role in innate immunity using cellular models of HSV-1 cellular targets. We chose keratinocyte cell line HaCaT, epithelial line HeLa and neuronal line SK-N-SH. Dr. T.Gianni observed that these cells express β3 integrin at similar levels, as measured by qRT-PCR, and stably silenced β3 integrin by means of lentiviruses encoding shRNA to β3, as described above. The silenced cells were indicated as sh-β3 cells. Mock-silenced cells were generated by control non targeting sh-RNA lentivirus.

Through qRT-PCR mRNA level of β3 integrin were determined and we observed that the extent silencing in the three cell lines ranged between 80 and 95% (Figure 2.11 A,D, and G); silencing of β3 was confirmed at the protein level by WB (Figure 2.11 B, E, and H).

We ascertained that β3 integrin silencing did not grossly hamper HSV infection: β3 integrin-silenced and control cells were infected with increasing MOI of R8102 and levels of infection were quantified as β-gal expression. Figure 2.11 C, F, and I show that in β3 integrin-silenced cells, infection was not significantly inhibited in any cell type up to 20 PFU/cell, except for a modest increase in HaCaT cells.

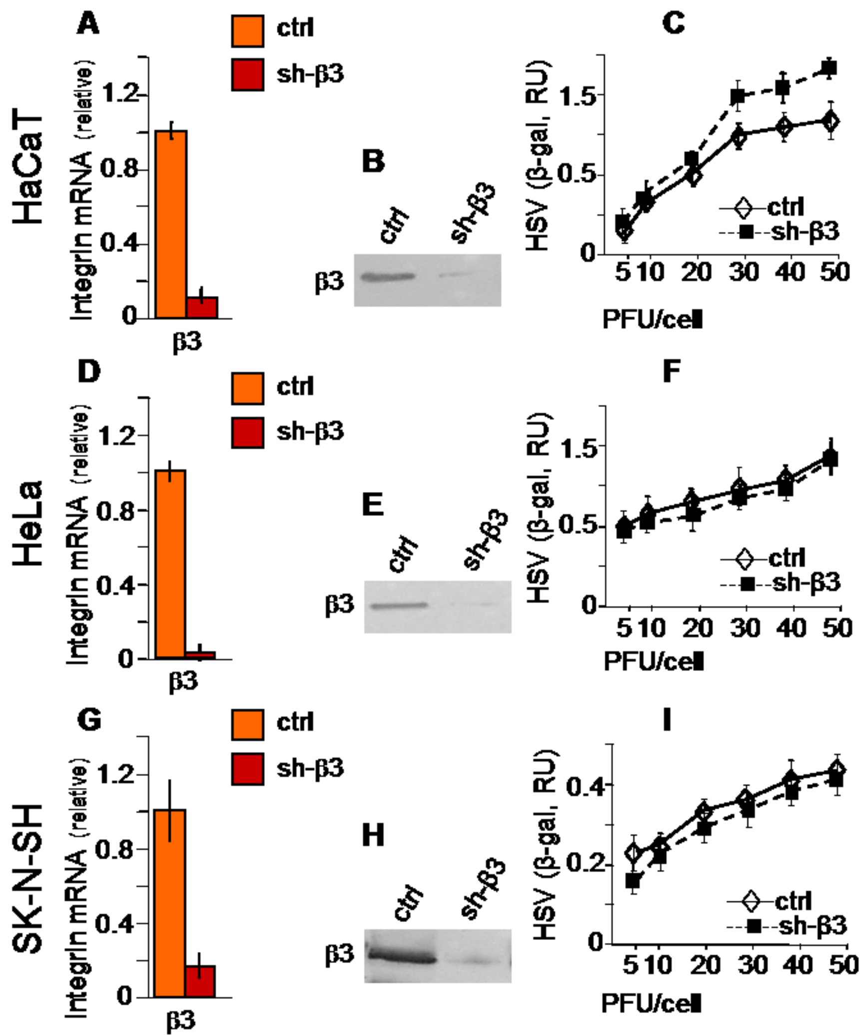

Fig. 2.11: **β3-Integrin-silenced HaCaT, HeLa, and SK-N-SH cells support HSV-1 infection.**

Silencing of β3-integrin in HaCaT, HeLa, and SK-N-SH cells by lentivirus encoding shRNA to generate sh-β3 cells (sh-β3) was quantified by q-RT-PCR (A, D, and G) relative to control mock-silenced cells (ctrl), as described in Fig. 2.5. (B, E, and H) The silencing was confirmed at the protein level by WB with polyclonal antibody (PAb) AB1932 to β3 integrin. (C, F, and I) Infection of HaCaT, HeLa, and SK-N-SH was not grossly altered following β3 integrin silencing. The extent of infection was determined as β-gal expression following R8102 infection as described in Fig. 2.5. Each point represents the average of triplicates +/- standard deviation.

2.3.11 Silencing of β3 integrin decreases NF-κB response in HaCaT, HeLa, and SK-N-SH cells.

As a first measure of the effect of β3 integrin silencing on innate response, we studied the NF-κB activation after infection with mutant HSV R7910 or treatment with commercial LPS. Control and sh-β3 HaCaT, HeLa, and SKN-SH cells were transfected with plasmids NF-κB–luc and Renilla luc, as described above, and thereafter maintained for 3 days under pre-exhausted medium. They were infected with R7910 (20 PFU/cell) for 6 h or exposed to LPS (100 ng/ml) for 4 h and in silenced cells it resulted a strong inhibition of NF-κB induction by both treatment (Figure 2.12 A, D, G). NF-κB induction was not suppressed in sh-β3 cells

and this aspect can be justified by the presence of several innate sensing systems that lead to NF-κB activation [74].

Next we focused on the production of cytokines in response to R7910 infection, by measuring the effect of β3 integrin silencing on the expression and secretion of IFN-β and of IL-10. In HaCaT, HeLa, and SK-N-SH cells, by qRT-PCR, dr. T Gianni evaluated mRNA levels of IFNB1 and IL-10, and we observed in wt cells a strong increase upon cell infection, but a decreased expression in sh-β3 cells. This inhibition resulted in a dramatic reduction in secretion of IFN and IL-10 (Figure 2.14 B, C, E, F, H, I). Thus, the production of IFN-β and IL-10 in epithelial and neuronal cell lines in response to R7910 infection is dependent on β3 integrin.

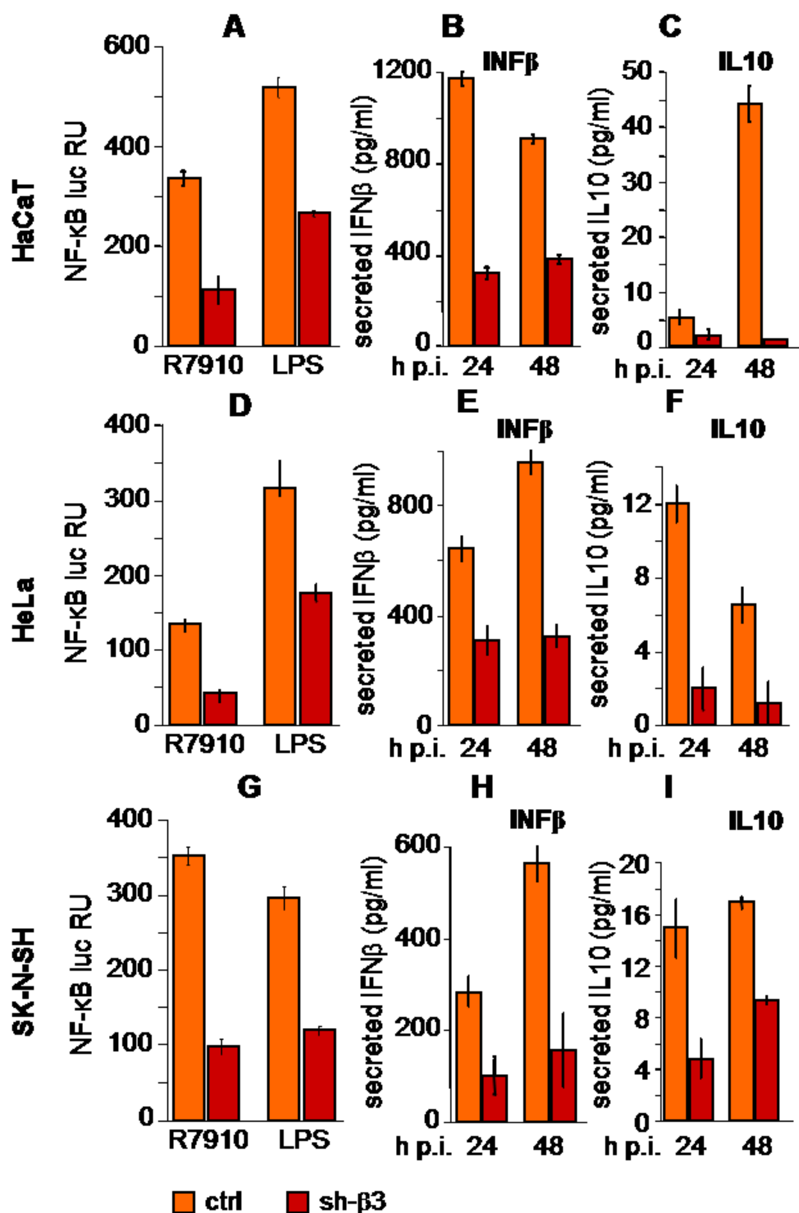

Fig. 2.12: **Innate response in β3 integrin-silenced or control HaCaT, HeLa, and SK-N-SH cells.** (A,D,G). sh-β3 and control (ctrl) cells were transfected as described in Fig. 2.1 and kept under pre-exhausted medium for 3 days. Then they were infected with R7910 (20 PFU/cell) or exposed to LPS (100 ng/ml) for 4 h, and NF-κB activity was determined. (B,C, E,F, H,I) sh-β3 and ctrl cells were infected

with R7910 (20 PFU/cell) and the infected cell media were harvested 24 or 48 h after infection for determination of secreted IFN-β and IL-10. Each column represents the average of triplicates +/- standard deviation.

2.3.12 Soluble gH/gL suffices to induce the β3 integrin-dependent NF-κB response in HaCaT, HeLa, and SK-N-SH cells.

Above we have demonstrated that in 293T cells gH/gL physically interact and cross-links αvβ3 integrin and TLR2 activating the innate response. So we asked whether soluble gH/gL suffices to induce this response even in HaCaT, HeLa, and SK-N-SH cells. The control and β3 integrin silenced cells, transfected with NF-κB–luc and Renilla luc, were exposed to soluble gH_t/gL (1.5 µM) for 4 h. This treatment elicited in wt cells an NF-κB response comparable to that induced by R7910, while in sh-β3 cells the response was decreased by 40 to 50% (Figure 2.13 A to C). Afterwards we verified the authenticity of this response either by pre-incubating gH_t/gL with MAb 52S to gH/gL, or by heat denaturing the glycoprotein: both treatments reduced the NF-κB response to the background level (Figure 2.13 A to C). Finally we studied the IFN response elicited by gH_t/gL in control and sh-β3 HaCaT, HeLa, and SK-N-SH cells exposed to soluble gp or R7910, for 6 h. We quantified by qRT-PCR IFNB1 expression and the results in Figure 2.13 D to F show a dramatic decrease in IFNB1 expression in β3 integrin-silenced cells.

Cumulatively, the experiments show that gH_t/gL was sufficient to elicit an IFNB1 response dependent on β3 integrin in epithelial and neuronal cell lines.

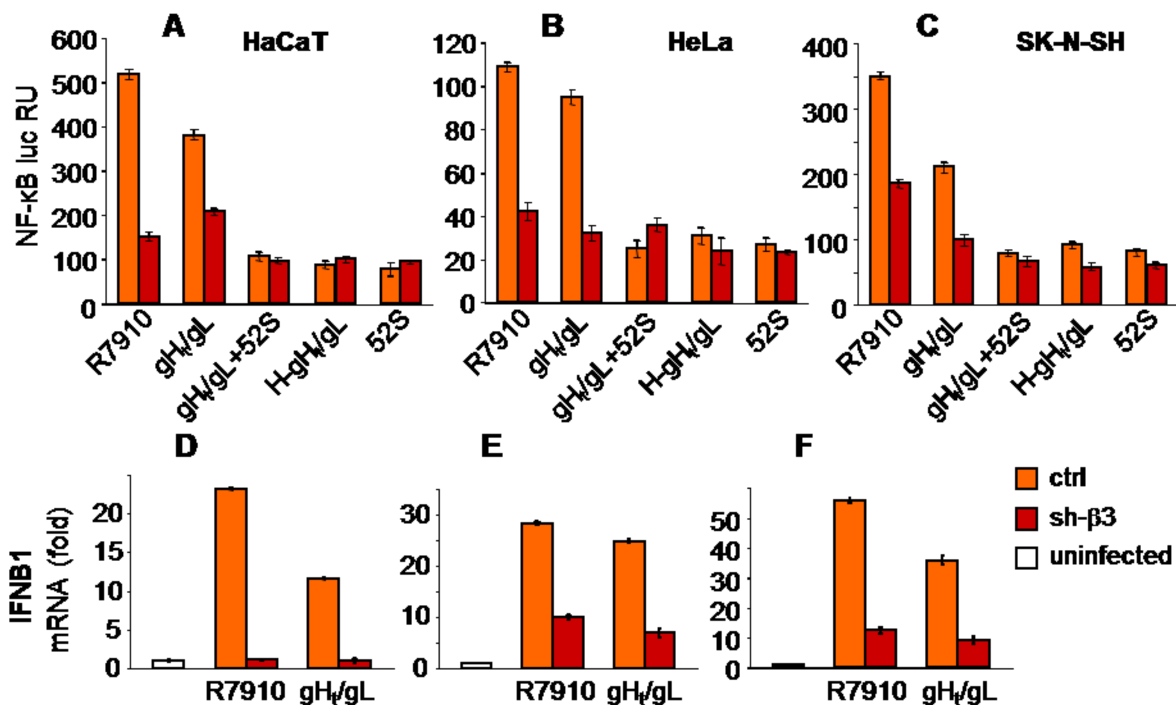

FIG 2.13: **A soluble form of HSV gH/gL (gH_t/gL) suffices to induce NF-κB activation and IFNB1 production.** (A to C) sh-β3 and control (ctrl) cells, transfected with NF-κB–luc and Renilla luc, as described in Fig. 2.1, were exposed to gH_t/gL (1.5 µM) for 4 h or infected with R7910 (20 PFU/cell). Where indicated, gH_t/gL was pre-incubated for 1 h with MAb 52S to gH/gL (gH_t/gL+52S) or heat inactivated for 15 min at 100°C (H-gH_t/gL). Each column represents the average of triplicates +/- standard deviation. (D to F) sh-β3

and control cells were exposed to gH_t/gL (1.5 µM) for 6 h, infected with R7910 (20 PFU/cell), or left uninfected. IFNB1 mRNA levels were determined 6 h after infection. The mRNA level is expressed as fold increase in infected cells relative to that of uninfected cells (set as 1). Each column represents the average of triplicates +/- standard deviation.

2.4 Discussion

2.4.1 Herpes simplex virus glycoproteins gH/gL and gB bind Toll-like receptor 2, and soluble gH/gL is sufficient to activate NF-κB

In the context of the innate immune response to HSV-1, TLR2 is one of the critical PRRs, Patter Recognition Receptors, which triggers the antiviral response [94, 95]. TLR2 is located in the plasma membrane, represents the first line of defense against invading HSV. However while for other herpesvirus it is known its PAMP, Pathogen Associated Molecular Pattern [78], until now the HSV-1 virion components which interact with TLR2 have remained elusive [74].

In the first part of my thesis, we provided evidence that HSV gH/gL and gB are TLR2 ligands and that soluble gH/gL suffice to elicit TLR2 signaling, which leads to NF-κB activation. First we observed that virions devoid of essential fusion glycoproteins, able to attach to cells but defective in fusion/entry, are sufficient to elicit an immediate NF-κB response to HSV. Among these mutant viruses, the most effective were the $gD^{-/-}$ virions, positive for gH/gL and gB. Second, in co-immunoprecipitation assays, we proved that gH/gL and gB physically interact with TLR2. Third we studied the NF-κB response induced by soluble truncated forms of HSV glycoproteins, and observed that gH/gL, but not gB, were sufficient to elicit an NF-κB response, while gD did so only when HVEM was also expressed. Results on gD are in accordance with previous data, which indicate that gD can signal to NF-κB through its HVEM receptor [75, 202], and moreover gD response was independent of TLR2, thus showing a signaling distinct from that of TLR2, a feature previously unknown. It is unclear why soluble gB failed to elicit an NF-κB response: it could be due to postfusion conformation adopted by gB in solution [203], or signaling might require the presentation of envelope-bound gB, or it could be an intrinsic property of gB, which alone does not suffice to elicit the NF-κB response. However, in a recent publication [204] it was demonstrated that even HSV-1 gB is able to activate NF-κB response through TLR2, so the scenario is not clear at all.

In the light of these data, HSV-1 behaves similarly to HCMV, where gH/gL, but even gB, are ligands to TLR2 and initiate a signaling cascade [78] HSV-1 differs from EBV and KSHV, in which the NF-κB response is elicited by the attachment glycoproteins gp350 or K1 [205, 206].

From our data, some interesting observations arise: first, the same glycoproteins which mediate virus entry into the cell also initiate the NF-κB response; second, each of the major entry/fusion glycoproteins, gD, gH/gL, and gB, can initiate such a response independently of one another.

2.4.2 αvβ3-integrin is a major sensor and activator of innate immunity to HSV-1

In the second part of my thesis, we showed that αvβ3-integrin is a major sensor and activator of specific components of the innate response to HSV-1.

First we developed several cell lines, including cells which are models of natural epithelial and neuronal HSV targets of in vivo HSV-1 infection, negative for the expression of β3-integrin, by means of lentiviruses encoding sh-RNA directed to β3. In these cells we studied the NF-κB response and production of cytokines induced by viral infection. Our data showed that αvβ3-integrin is a major determinant in NF-κB activation and it is critical for the production of type 1-IFNs and of a specific set of cytokines, in particular IFNβ, IFNα, IL2, and IL10. Through gain-of-function experiments in K562, we demonstrated that the NF-κB and type-1 IFN response is increased when these cells express αvβ3-integrin. Next we showed that soluble gH/gL suffices to induce the β3 integrin-dependent NF-κB response and finally that αvβ3-integrin physically interacts with the virion glycoproteins gH/gL and with TLR2.

The cumulative picture emerging from current and previous reports [21, 47] is that αvβ3-integrin relocates the HSV receptor nectin1, and thus HSV, to cholesterol-rich membrane microdomains, from where the virus is endocytosed, and simultaneously initiates the innate response.

The ability of αvβ3-integrin to activate innate immunity in response to viral or bacterial elements that we observed are features typical of PRRs and thus we proposed that it could be considered a non-TLR PRR.

We classified genes related to the innate response to HSV into three groups based on how they respond to β3-integrin silencing. The specificity of the αvβ3-integrin-mediated response was evident from the observation that β3-integrin silencing resulted in a highly polarized effect, that is, in strong decrease of group 1 cytokines (IFNβ, IFNα, IL2, and IL10), a moderate effect on group 2 genes (SRC, SYK, IRF3, IRF7, TRIF, and CARD9), and no effect on group 3 genes (TNF, LTBR, IL1A, IL6, BCL3, FASL), even though some of them are known to be dependent on NF-κB [207].

Collectively, group 1 and group 2 genes delineate two distinct αvβ3-integrin-mediated signaling pathways. The dependence of the activation of type 1-IFNs on αvβ3- integrin and TLR2 was unexpected and really interesting. To date, the sensors recognized as important for HSV-induced type-1 IFN production have been cytoplasmatic RLRs, TLR9, and TLR3, detected mainly in specific cell types, e.g., dendritic, NK, plasmacytoid, or 293T model cells [74, 208].

Clearly, this response coexists with other branches of innate immunity and contributes to the overall type I IFN production elicited by HSV, but is not the only one responsible for it. In agreement with this view, silencing of β3 integrin led to inhibition but not a complete abolishment of the NF-κB response.

The observation that the αvβ3 integrin-elicited signaling leads simultaneously to activation of type I IFN and of IL-10 suggests that this pathway is devoted mainly to establishing an antiviral state and to negatively controlling inflammation through IL-10.

Furthermore, until recently TLR2 was thought to be involved mainly in antibacterial defense and inflammatory cytokine induction, not in the activation of type-1 IFN [209]; only in the last few years it was documented a role for TLR2 in type-1 IFN induction by murine cytomegalovirus and vaccinia virus [210].

The signaling pathway of group 1 cytokine genes highlighted that it overlapped in part but was independent of the pathway regulated by TLR2. In fact, β3-integrin was required for the activation of group 1 cytokines in both TLR2+ and TLR2− cells; hence, TLR2 enhanced but was not the determinant factor in up-regulation of the cytokine response.

The signaling pathway defined by group 2 genes (SRC, SYK, IRF3, IRF7, TRIF, and CARD9) differed from that of group 1 cytokines in that it was activated exclusively by β3-integrin and not by TLR2. It included TRIF, a MYD88-independent adaptor in TLR3 signaling, which leads to activation of IRF3 and IRF7, major transcription factors of type-1 IFN.

αvβ3-integrin sensed HSV by binding the virion glycoproteins gH/gL, which, in turn, bind TLR2; furthermore αvβ3-integrin physically interacted with TLR2 and thus bridged the virion and TLR2. Remarkably, soluble gH/gL was sufficient for the αvβ3-integrin/TLR2-dependent induction of IFN-1, IL-10 and NF-κB activation, testifying a signaling capability of this glycoprotein. Thus, gH/gL represents also the PAMP of the αvβ3 integrin-dependent innate response.

2.4.3 Final considerations

In the light of the data that were presented in this first part of the thesis, it is possible to draw several conclusions.

First, this finding strongly argues for the coevolution of HSV with its human host.

A second consideration regards the entry mechanism of HSV-1: gD, gH/gL, and gB act in concert to enable virus entry, through a series of interactions that start with the binding of gD with its receptor and lead to the formation of glycoproteins complexes, and finally to the fusion-active conformation [19, 211, 212]. In this context, the simultaneous interaction of gH/gL and gB with a same molecule (TLR2) may favor complex assembly/stabilization and inter-glycoprotein signaling. However TLR2 effects on entry may not be essential, since cells that lack TLR2 can still be infected.

Third, it has been debated whether the NF-κB response is detrimental or favorable to HSV. Because gH/gL are essential for HSV entry, it follows that entry of the virus into the cell inevitably induces an innate immune response. By deploying PRRs whose PAMPs are the very same virion glycoproteins essential for virus entry, the cell ensures that the virus cannot escape its first line of defense. HSV has evolved a number of proteins which counteract the innate response, including the tegument protein *vhs*, which selectively degrades some of the NF-κB-dependent and -independent RNAs induced after infection, and the immediate-early proteins ICP0 and ICP27, which act immediately after the viral protein synthesis onset [124, 127, 136]. Our data indicate that ICP0 is the viral factor involved in the counteraction of the αvβ3-

integrin defense mechanism, since silencing of β3-integrin enabled the ΔICP0 mutant to better replicate, particularly when even the TLR2 defense arm was absent. The activity of these proteins is carried out when HSV-1 is already entered into the cell, raising the possibility that the immediate response is not totally detrimental to the virus. Beneficial effects of the NF-κB response may include some of the stress response RNAs elicited by NF-κB and/or the ability to promote the synthesis of proteins able to block apoptosis [10, 124, 213].

Fourth, our results support the conclusion that αvβ3-integrin is an important non-TLR PRR for HSV and possibly for a number of other viral and bacterial pathogens. Integrins are evolutionarily ancient and highly conserved. They recognize specific molecular patterns in many viral and bacterial pathogens [214, 215]. They exhibit signaling activities that lead to a polarized cytokine production. Monocyte integrins, such as αM (CD11b), are already considered as non-TLRs PRRs and regulators of the innate response [216]. A number of viruses, including the herpesviruses cytomegalovirus, Epstein Barr virus and Kaposi's sarcoma herpesvirus, adenoviruses, reoviruses, HIV, and hepatitis C virus, make use of integrins, in most cases as portals of entry into cell, and induce a strong innate response [200, 201, 214, 217-221]. With respect to bacteria, β-integrins physically interact with bacterial pathogens and β3-integrin delivers bacterial lipopeptide to TLR2, inducing inflammatory cytokines [195, 215].

Fifth, the innate response represents the first building block of adaptive immunity. A number of efforts have been made in recent years to generate HSV vaccines based on the HSV glycoproteins, unfortunately with modest protective and prophylactic results. A better understanding of the innate response to HSV glycoproteins may be key to the design of better HSV subunit vaccines.

3. Delivery of retargeted oncolytic Herpes Simplex via carrier cells

***** The results presented in section 3 are unpublished and should be considered as confidential communication. *****

3.1. Objective

In the second part of this thesis I addressed the issue of delivery of oncolytic viruses to target tumoral cell by means of carrier cells. In our laboratory several retargeted viruses have been engineered, and two of them, R-LM113 and R-LM249, have been tested for anticancer efficacy in mouse models. R-LM113 and R-LM249 are both retargeted to HER-2 receptor, which is highly overexpressed in breast and in ovarian cancers. In *in vivo* experiments, when administrated by intraperitoneal or intratumoral route, they showed the capacity to reduce tumor growth. The systemic administration of these viruses is marred by the fact that virions are taken up by liver and only a small fraction reaches their target.

For this reason we studied the possibility to use human mesenchymal stromal cells (hMSCs) as carriers for R-LM249 to deliver it to cancers cells.

Among the different sources of hMSCs known, one is represented by fetal membrane MSC (FM-hMSCs), cells isolated from placenta at the time of delivery: fetal membrane. These MSCs grow fast and are more abundant than bone marrow MSCs.

First of all we evaluated the FM-hMSC membrane expression of HER-2 receptor. Subsequently, we tried to infect FM-hMSC with R-LM249 virions at different multiplicity of infection and with the addition of polyethylene glycol (PEG) or polybrene, to enhance the efficiency of infection. Finally, we checked the capacity of infected cells to produce infectious viral particles and to spread them to cancer cells.

3.2. Materials and methods

3.2.1 Cells and viruses

In this series of experiments, the following cells were used:

Mesenchymal stromal cells were isolated and kindly provided by our collaborators from laboratory of Prof. Bonsi. According to the policy approved by the local Ethical Committee (S.Orsola-Malpighi University Hospital), all tissue samples used for cells isolation were obtained after informed consent.

FM-hMSCs (MF3619, MF3620, MF3356)- fetal membrane human mesenchymal stromal cells are primary cells isolated from term amniotic membrane, with a wide differentiation potential and great expansion potential; their isolation and characterization is described in [222].

AMSCs (ASC MEMB3) - human mesenchymal stromal cells isolated from adipose tissue.

DPSCs (DP36) – dental pulp stem cells, human mesenchymal stromal cells isolated from dental pulp.
BM-hMSCs (BM3476) - human mesenchymal stromal cells isolated from bone marrow.
WJ-hMSCs (WJ-MSC2) - human mesenchymal stromal cells isolated from the wharton's jelly of the umbilical cord.
J1.1-2 cells - a derivative of BHK-TK-cells (Baby Hamster Kidney) that lacks gD receptors, Nectin-1 and HVEM, and so are resistant to HSV-1 infection; they are described in [190].
293T - a highly transfectable derivative of human epithelial cell line 293.
SK-OV-3 - a derivative of human ovarian adenocarcinoma, that expresses HER2 at high levels.
RH4 - a derivative of alveolar rabdosarcoma.
J cell, 293T and RH4 were grown in DMEM supplemented with 5-10% FBS (Gibco), PenStrep 1% (Euroclone), SK-OV-3 were maintained in RPMI medium plus GlutaMAX (Gibco) with 10% FBS, hMSC were grown in DMEM High Glucose medium supplemented with 10% FBS (Gibco). All cells were maintained at 37°C in 5% of CO2 atmosphere.
The recombinant HSV-1-BACs was derived from pYEbac102, which carries pBeloBAC11 sequences inserted between UL3 and UL4 [223].
R-LM249 was described in [43]. Briefly it was obtained by means of 2-step replacement recombination in Escherichia coli DH10B strain, starting from the recipient genome gD minus EGFP-HSV-BAC and carried scFv to HER2 in place of gD residues 61 to 218.

3.2.2 Antibodies

MAb 9G6 (Santa Cruz) and MAb MGR2 (Enzo Life Sciences) are monoclonal antibodies directed to the ectodomain of HER2.
Anti-mouse IgG-FITC (fluorescin-isothiocyanate) conjugated antibodies were from Jackson Immunoresearch fragment of goat anti-mouse IgG (H+L) antibody is labeled with green-fluorescent Alexa Fluor® 488 dye (Life Technologies).

3.2.3 Infection assay and virus yield assay

FM-hMSCs, SK-OV-3 and J cells were counted and seeded in 12 or 24 well plates, at a subconfluent density, and 24 h post seeding they were infected with R-LM249 virions at a multiplicity of infection (moi) of 1 or 10 PFU/cell. Following virus absorption to cells for 90 min at 37 °C, virus inoculum was removed, and monolayers were treated, or not, with a solution of polyethylene glycol - 6000 (PEG) 40% in PBS for 10-60 seconds, then washed quickly and sequentially with PEG 20%, PEG 10%, PEG 5% and PBS, and finally covered with their growth medium with low FBS (1-2,5%). Infection was monitored as EGFP expression 24 or 48 hpi (hours post infection) and infected cells were examined and photographed with a Zeiss Axioplan fluorescence microscope connected to a Kodak camera. Alternatively, the same cells were infected in

suspension with the addition of polybrene and then spinoculated for 30 minutes at 2300 rpm; in this case the inoculum was not removed; at 24 or 48 hpi cells were examined at the fluorescence microscope.
For virus yield assay, replicate cultures of FM-hMSCs or SK-OV-3 cells in 12-well plates, were infected with R-LM249 at 1 or 10 PFU/cell for 90 min at 37°C and then treated or not with PEG 40%. Replicate cultures were frozen at 0, 16, 24, 48 h after infection, and the virus progeny (cell associated and supernatant) was titrated on SK-OV-3 cells for R-LM249.
The titer of extracellular virion preparations was determined in SK-OV-3 cells monolayers, overlaid with 1% SeaPlaque Agarose in RPMI medium plus GlutaMAX, supplemented with 6% FBS.

3.2.4 Infectious center assay.

FM-hMSCs donor cells were infected at a MOI of 10 PFU/cell at 37°C for 1,5 h and treated with PEG 40% for 20 seconds. The cells were incubated at 37°C for 3-4 h, trypsinized, resuspended in culture medium and counted. Serial dilutions of donor cells (10, 30, 100, 300, 1000, 3000, 10000 cells) were seeded onto monolayers of acceptor cells SK-OV-3 in a 12-well plate, incubated overnight at 37°C, and then overlaid with 1% SeaPlaque Agarose in RPMI medium plus GlutaMAX, supplemented with 6% FBS. In the following days, the cells were observed at the fluorescence microscope to count the infectious centers.

3.2.5 Flow cytometric analysis

FM-hMSCs, SK-OV-3 and RH4 cells, grown in T25 flasks, were trypsinized, resuspended in complete medium, counted. 200.000 cells were pelleted 30 seconds at 12000 rpm in 1,5 ml tubes. Pellets were resuspended in 50 µl of cold RPMI plus 5% FBS and incubated with anti-HER2 MAb MGR2, diluted 1:100, or no antibody, for 30 minutes in ice. Cells were rinsed with RPMI 5% FBS, and incubated with Alexa Fluor® 488 F(ab')2, diluted 1:100 in RPMI plus 5% FBS, for 30 minutes in ice. Cells were washed with RPMI containing 5% FBS, resuspended in 600 µl of PBS plus 1µg/ml EtBr and then analyzed with a FACSCalibur cytometer (BD).

3.3 Results

3.3.1 HER2 is expressed at low levels in FM-hMSCs

First of all, we analyzed the surface expression of HER2 receptor in human mesenchymal stromal cells via Fluorescence-activated cell sorting (FACS). Two different isolates of FM-hMSC, MF3619 and MF3629, were examined and in parallel SK-OV-3 cells, that express the receptor at high levels, and RH4 cells, negative for HER2. There is no difference between the two isolates of stromal cells, they are not negative for HER2, but present a level of expression significantly lower than SK-OV-3 (Fig. 3.1).

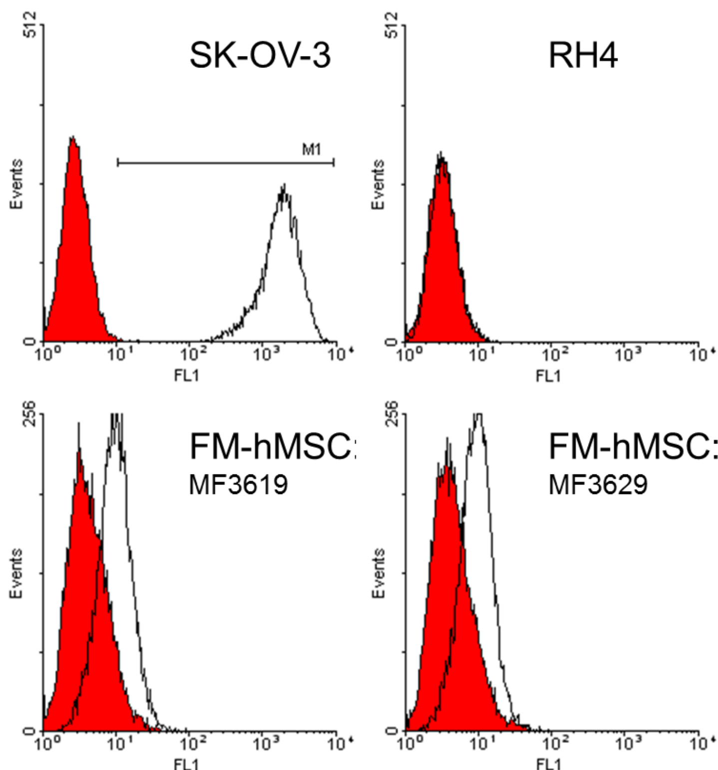

Fig.3.1: **FACS analysis of HER2 expression in FM-hMSC, SK-OV-3 and RH4.** FM-hMSC, SK-OV-3 and RH4 cells were trypsinized and 200.000 cells were incubated with anti-HER2 MAb MGR2, or no antibody, and then with Alexa Fluor® 488. Cells were analyzed with a FACSCalibur cytometer (BD). Red curve represents the background fluorescence level of cells incubated only with secondary antibody.

3.3.2 Infection of FM-hMSCs by R-LM249 is enhanced by the use of PEG and yields infectious progeny

Preliminarely we analyzed whether mesenchymal stromal cells can be infected with the retargeted recombinant R-LM249. In a previous work [224] the susceptibility of FM-hMSCs to human Herpesviruses infection was studied and stromal cells resulted permissive to wt HSV-1. We infected low passage (< 10 p) FM-hMSCs, SK-OV-3 and J cells (J1.1-2), respectively positive and negative control, with R-LM249 virions at a multiplicity of infection (MOI) varying from 1 to 10 PFU/cell.

We decided to use polyethylene glycol - 6000 (PEG) to enhance viral fusion to stromal cells, since this chemical is used to force membrane fusion for example in the generation of hybridomas or in infections with defective viruses [225]. Following virus absorption, virus inoculum was removed and monolayers were treated, or not, with a solution of PEG 40% in PBS for 10 or 20 seconds, washed and covered with their medium. In parallel, the same cells were infected in suspension with the addition of polibrene and spinoculated. 24 and 48 hours p.i. cells were examined at the fluorescence microscope, by monitoring expression of EGFP, the reporter gene present in R-LM249 genome. As expected, in absence of any treatment, FM-hMSCs present low levels of infection by the retargeted R-LM249, compared to permissive SK-OV-3 cells (Fig. 3.2 A). The use of PEG led to a significant increase in the percentage of infected cells.
In subsequent experiments, we assayed different time of exposure to PEG and optimized infection conditions, reaching about 70-80% of R-LM249 infected cells, upon treatment with PEG (Fig. 3.2 B).
Polibrene treatment was not well tolerated by cells and did not give positive results.
Next, we determined virus yield in the stromal cells: FM-hMSCs and SK-OV-3 were seeded in 12 well plates, infected with R-LM249 at 1 or 10 PFU/cell, and exposed or not to PEG 40%. Replicate cultures were frozen at 0, 16, 24, 48 h p.i., and the virus progeny was titrated on SK-OV-3 cells. In stromal cells the peak of virions production was observed between 16 and 24 h p.i. In cells treated with PEG virus replicated to 10 times higher yield than in untreated cells. The virus yield obtained in FM-hMSCs was about 20 times lower than in SK-OV-3 cells (Fig. 3.3).

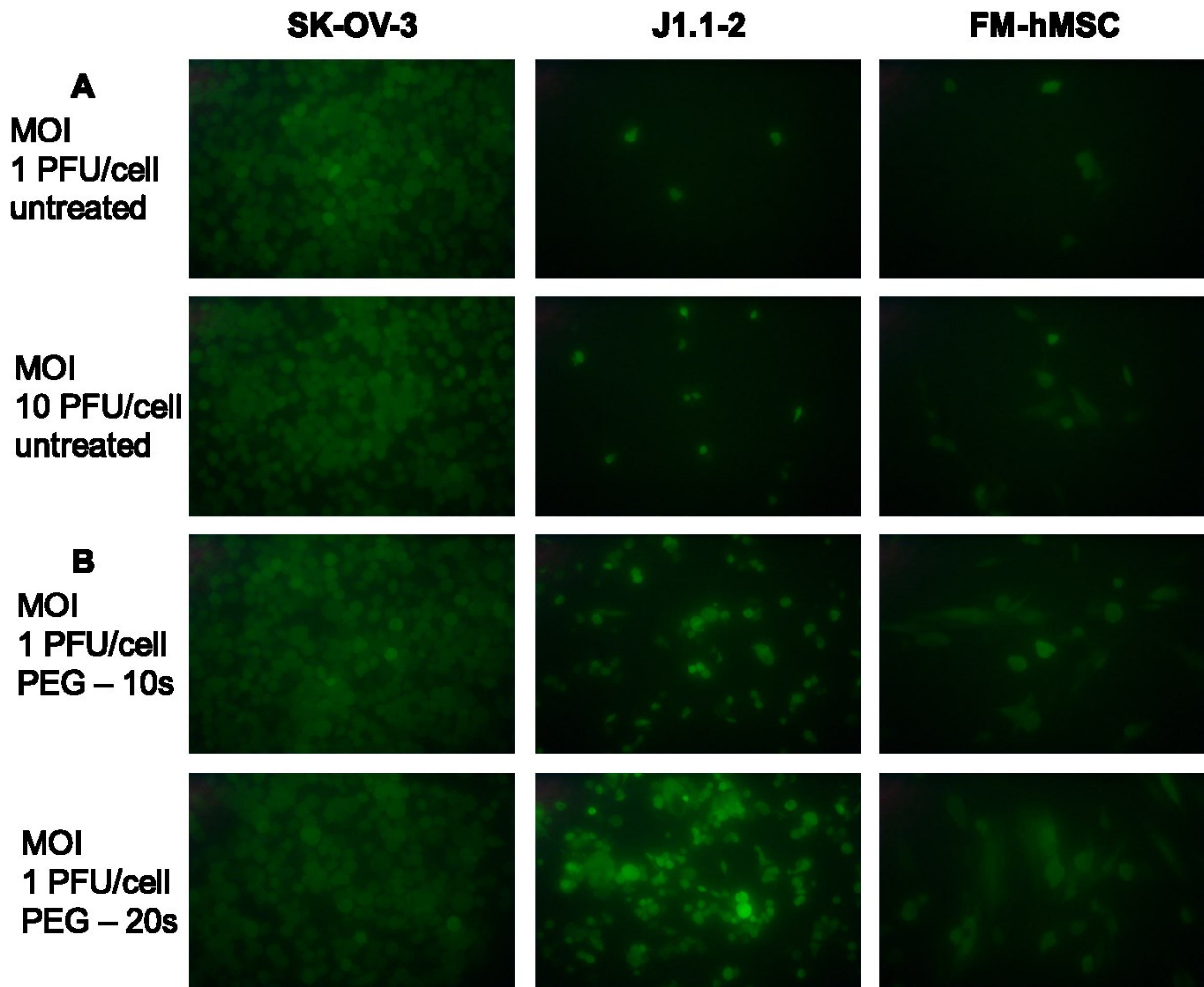

Fig. 3.2: **Infection assay in FM-hMSCs.** FM-hMSCs, SKOV3, and J1.1-2 cells were counted and seeded in 24 well plates, and 24 h post seeding they were infected with R-LM249 virions at a multiplicity of infection (MOI) of 1 or 10 PFU/cell. Following virus absorption to cells for 90 min at 37 °C, virus inoculum was removed, and monolayers were treated, or not, with a solution of polyethylene glycol - 6000 (PEG) 40% in PBS for 10 or 20 seconds, washed, and covered with their growth medium. Infection was monitored as EGFP expression 24 h p.i. and infected cells were examined and photographed with a Kodak camera connected to a Zeiss Axioplan fluorescence microscope.

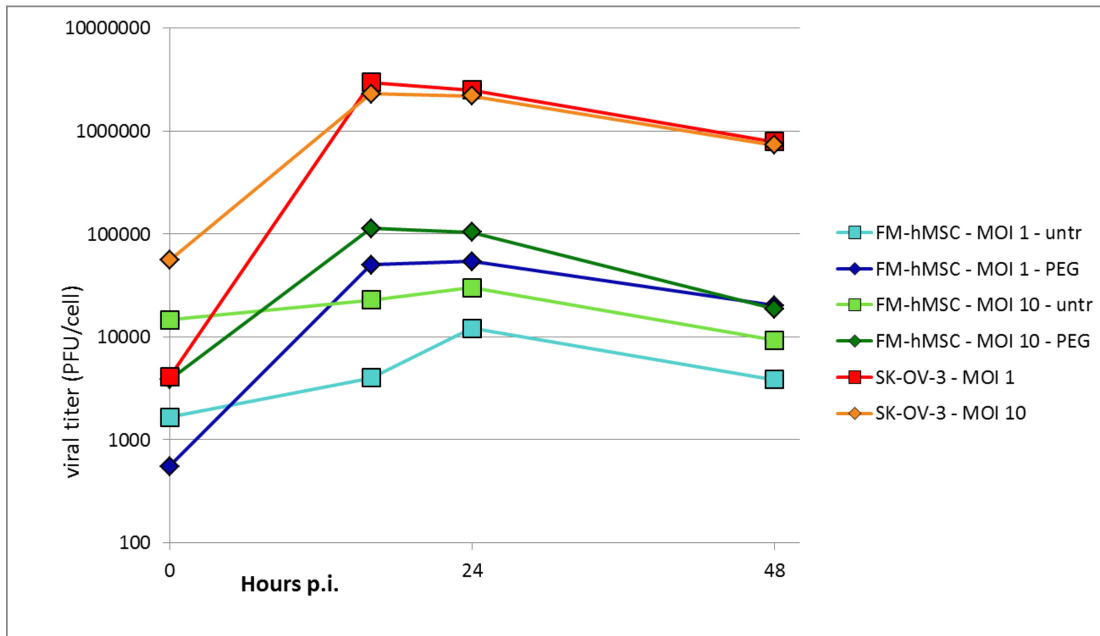

Fig. 3.3: **Viral yield in FM-hMSCs.** FM-hMSCs and SK-OV-3 cells were seeded in replicate in 12-well plates, were infected with R-LM249 at 1 or 10 PFU/cell for 90 min at 37°C and then treated or not with PEG 40%. Replicate cultures were frozen at 0, 16, 24, 48 h after infection, and the viral progeny (cell associated and supernatant) was titrated on SK-OV-3 cells monolayers, overlaid with 1% SeaPlaque Agarose in RPMI medium. About a week after the titration, plaques were counted using a stereomicroscope and title for each sample was determined.

3.3.3 Infected FM-hMSCs are able to transmit R-LM249 to permissive SK-OV-3 cells.

A critical issue for the employment of FM-hMSCs as carriers cells is whether they enable the spread of progeny virus to cancer cells. To evaluate this property, we made use of infectious center assay.

FM-hMSCs donor cells were infected at an MOI of 10 PFU/cell and treated with PEG 40%. At 3-4 h p.i. donor cells were detached, resuspended in culture medium, counted and seeded onto monolayers of acceptor SK-OV-3 cells. In the following days, cells were observed at the fluorescence microscope to assess the formation of infectious centers. 24 hours post the seeding on acceptor cells, we observed the presence of small aggregates of EGFP positive R-LM249 infected SK-OV-3 cells, which grew in size at 48 and 72 h p.i. (Fig. 3.4).

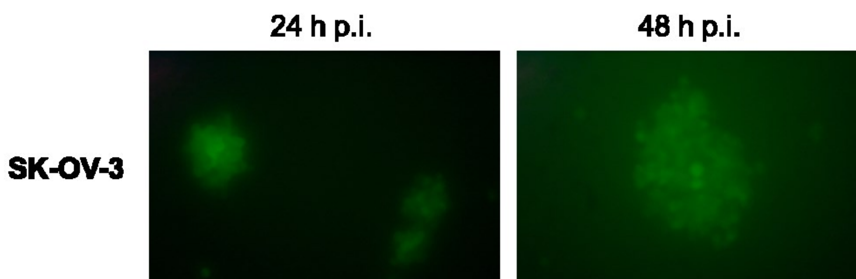

Fig. 3.4: **Infectious centers generated by infected FM-hMSCs on SK-OV-3 cells.**

FM-hMSC were infected at MOI 10 PFU/cell by means of PEG 40% for 20 seconds. They were incubated at 37°C for 3-4 h, trypsinized, resuspended in culture medium and counted. A quantity of donor cells ranging from 10 to 10000 was seeded onto

monolayers of acceptor cells in a 12-well plate. The formation of infectious centers in SK-OV-3 donor cells was monitored at the fluorescence microscope 24 and 48 hours post treatment.

3.3.4 Mesenchymal stromal cells of different sources can be infected by R-LM249 by means of PEG.

Mesenchymal stromal cells can be isolated from different sources, such as bone marrow [226], adipose tissue [227], dental pulp [228], wharton's jelly of the umbilical cord [229], amniotic fluid [230] and term placenta [231]. We assayed the capacity of recombinant R-LM249 to infect hMSCs isolated from various tissues, and FM-hMSCs obtained by different term placenta.

Stromal cells were seeded in 24 wells, infected with R-LM249 at a MOI of 1 PFU/cell and exposed or not to PEG 40%. 24 hours post infection they were examined at the fluorescence microscope. We observed no differences among the three batches of FM-hMSCs assayed, and even WJ-hMSCs and DPSCs gave good levels of infection post PEG treatment (Fig. 3.5). On the other hand, AMSCs and BM-hMSCs resulted much more resistant to R-LM249, and even PEG treatment did not lead to an high increase in the number of infected cells (Fig. 3.5).

Further advantages of FM-hMSCs compared to other stromal cells were their easiness of cultivation and high proliferative rate. For this reason they were chosen for *in vivo* experiments.

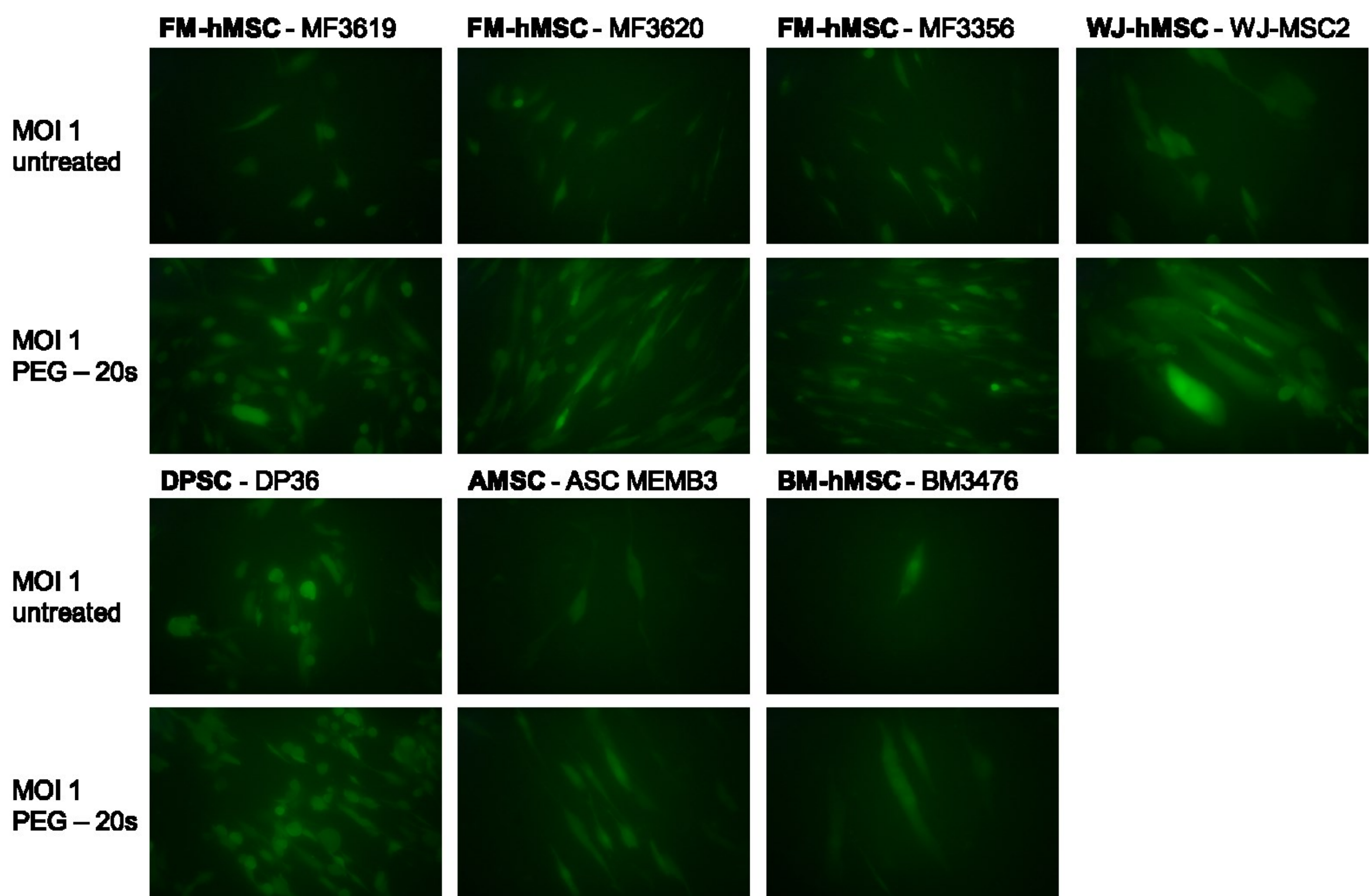

Fig. 3.5: **Infection assay on human mesenchymal stromal cells isolated from different tissues.** Three different batches of FM-hMSCs (MF3619, MF3620, MF3356), Wharton's jelly MSCs (WJ-MSC2), dental pulp MSCs (DP36), adipose MSCs (ASC MEMB3), bone

marrow MSCs (BM3476) were seeded in 24 well plates, infected with R-LM249 virions at a MOI of 1 PFU/cell and treated, or not, with PEG 40%. EGFP expressed by R-LM249 in infected cells was used to evaluate the level of infection at fluorescence microscope.

3.4 Discussion

In the last decade research in oncolytic virotherapy progressed thanks to the advance in the techniques to manipulate viral genomes and in the general knowledge of cancers and viral biology. Several strategies have been developed to manipulate viruses, target them to cancer cells and exploit their cytolytic activity to selectively destroy tumors otherwise difficult to eradicate with classical treatment (surgical resection, chemotherapy, radiotherapy). In this context, in our laboratory R-LM249was developed: it is a retargeted HSV-1 that has been engineered to target HER2 receptor expressing tumor cells and detargeted from other cells where HSV-1 normally replicates [43, 184].

In mouse model of subcutaneous HER2 positive tumor, intratumor administration of R-LM249 resulted effective in the reduction of tumor size [43]. Furthermore its efficacy was demonstrated even in a model of peritoneal disseminated tumors, after intraperitoneal (i.p.) administration of R-LM249 virions [186].

However cancers harder to treat, like metastatic ones, are also difficult to reach by these route of administration. In general, the systematical administration of oncolytic viruses is another possible alternative, but in most cases virions are retained in the liver and so are not able to reach their target. To overcome this problem, one of the solutions involves the use of carrier cells, that is eukaryotic cells that can be infected by the oncolytic viruses, carrier cells transport viruses to the tumoral targets and release them. Among the cell types used, mesenchymal stem cells are very promising, due to their biological properties and the possibility to use autologous cells.

In my thesis, we assayed the capacity of human mesenchymal stromal cells isolated by fetal membranes (FM-hMSCs) to be used as carrier cells for retargeted R-LM249 virus. First of all we evaluated HER2 expression and observed that FM-hMSCs express the receptor at low levels, compared to SK-OV-3 cells, a derivative of human ovarian adenocarcinoma, that express HER2 at high levels and are used to propagate R-LM249.

We tried to infect FM-hMSCs and observed that R-LM249 could infect them at low levels using high multiplicity of infection, so we added a treatment with polyethylene glycol (PEG) to force the viral entry. Optimizing the infection conditions with PEG, we reached a percentage of infected cells higher than 80%, even without the exogenous introduction of HER2 receptor. We assayed even hMSCs isolated from other sources, both from fetal tissue, like other term placenta or umbilical cord, and from adult tissues, such as bone marrow, dental pulp, adipose tissue. FM-hMSCs resulted the best candidates, because they better infect than the other ones and furthermore can be isolated and cultivated easily and in large quantities.

In *in vitro* assay we evaluated the capacity of FM-hMSCs to produce infectious viral particles and to transmit the infection to target tumoral cells, by viral yield assay and infectious center assay respectively. Stromal cells resulted suitable to be used as carrier cells.

From these preliminary data FM-hMSCs resulted suitable to be used as carrier cells. They can be easily and abundantly isolated from waste material, like term placenta, and cultivated. Moreover in several studies it was demonstrated their immunomodulatory properties and capacity to home towards damaged sites or tumor sites. We observed that they can be infected, sustain the replication of retargeted R-LM249 and spread it to target cells. In the light of these data, we will study *in vivo* the efficacy of FM-hMSCs to deliver retargeted R-LM249 to target tumor cells and to reduce the incidence of tumor in mouse model.

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