Aus dem Nationalen Centrum für Tumorerkrankungen (NCT) der Universität Heidelberg, (Geschäftsführende Direktoren: Prof. Dr. Dirk Jäger und Prof. Dr. Stefan Fröhling)

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Immunotherapy Development to Target Herpes Simplex Virus Infections

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Narges Seyfizadeh

aus

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Prodekan: Prof. Dr. med. Hans-Georg Kräusslich

Doktorvater: Prof. Dr. med. Jürgen Krauss

To My Parents.

Contents

Abbreviations

3-O-S-HS: 3-O-sulfated heparan sulphate

ACV: Acyclovir

ADCC: Antibody dependent cell cytotoxicity

ADCP: Antibody dependent cell phagocytosis

ATF/cJun: Activating transcription factor/cJun

CDC: Complement dependent cytotoxicity

CMC: Complement mediated cytotoxicity

CMV: Cytomegalovirus

CTL: Cytotoxic T lymphocytes

DAI: DNA-dependent activator of IRFs

DC: Dendritic cell

DRG: Dorsal root of ganglion

EBV: Epstein–Barr virus

FOC: Foscarnet

gB, D, C, H, L, E, I: Glycoprotein B, D, C, H, L, E, I,

GMP: Guanosine monophosphate

GTPases: Guanosine triphosphatases

HHV: Human herpes virus

HIV: Human immunodeficiency virus

HLV: Herpes lymphotropic virus

HSK: Herpes stromal keratitis

HSV: Herpes simplex virus

HVEM: Herpes virus entry mediator

IFN: Interferon

IKKε: Inhibitor of NFκB kinase epsilon

IRF-3: IFN regulatory factor 3

IRL: Inverted repeat of the L segment

IRS: Inverted repeat of the S segment

ISGs: IFN stimulated genes

IP: Intraperitoneal

IV: Intravenous

IVIG: Intravenous immunoglobulin

KSHV: Kaposi's sarcoma-associated herpesvirus

LCs: Langerhans cells

MHC: Membrane histocompatibility complex

MYD88: Myeloid differentiation primary response 88

Mφ: Macrophages

NFκB: Nuclear factor kappa B

NLPs: Nod-like receptors

ORFs: Open reading frames

PCV: Penciclovir

pDCs: Plasmacytoid dendritic cells

PKR: Protein Kinase R

PRRs: Pattern recognition receptors

PTLD: Post-transplant lymphoproliferative syndrome

RAG: Recombination activating gene

RIG-I: Retinoic acid inducible gene I

RLRs: RIG-I-like receptors

ScFv: Single chain fragment variable

SC: Subcutaneous

TBK-1: Tank binding kinase 1

TG: Trigeminal ganglion

Th1 ,2, 17 cells: T helper 1, 2, 17 cells

TK: Thymidine Kinase

TLRs: Toll-like receptors

TNF-α: Tumour necrosis factor-α

TRL: Terminal repeats long

TRS: Terminal repeats short

VSV: Vesicular stomatitis virus

VZV: Varicella zoster virus

1. Introduction

1.1 Herpes simplex infection

Herpes simplex is a widespread and chronic viral infection caused by the herpes simplex virus type 1 and type 2 (HSV-1 and HSV-2) which affects both genders (Looker *et al.*, 2008; Looker *et al.*, 2015). Although infection with herpes simplex virus is usually asymptomatic and subclinical, clinically apparent HSV-1 and HSV-2 infections can manifest as orolabial herpes and anogenital herpes, respectively. However, both viruses can cause a broad spectrum of diseases depending on the anatomical sites of replication including neonatal herpes, encephalitis, corneal blindness, meningitis, herpetic whitlow and many others (Figure 1) (Klein, 2016). Similar to other viral infections, HSV can also be treated effectively with different nucleoside analogues such as Aciclovir (ACV), Penciclovir (PCV) and their derivatives, or DNA polymerase inhibitors like Foscarnet (FOS) (Table 1) (Kimberlin and Whitley, 2007). However, long-term treatment with nucleoside analogues may result in drug resistance. This phenomenon is more frequent in immunocompromised patients, including HIV positive patients and bone marrow (hematopoietic stem cell) transplant recipient (3.5% up to 36% of immunosuppressed individuals infected with HSV)(Morfin and Thouvenot, 2003) (Langston *et al.*, 2002) (WILLIAMSON *et al.*, 1999) (Wade *et al.*, 1983) (Frangoul *et al.*, 2007) (Danve-Szatanek *et al.*, 2004) (Erard *et al.*, 2007) (Morfln *et al.*, 2000) (Chen *et al.*, 2000) (Chakrabarti *et al.*, 2000). That is mainly because they experience long-term anti-HSV therapy. In contrast, immunocompetent patients only require short-term anti-HSV therapy and resistance occurs more rarely (range from 0.1% to 0.6% of immunocompetent HSV infected individuals) (Sarisky *et al.*, 2002) (Bacon *et al.*, 2002) (Bacon *et al.*, 2003) (Boon *et al.*, 2000) (Christophers *et al.*, 1998) (Danve-Szatanek *et al.*, 2004) (Reyes *et al.*, 2003) (Stránská *et al.*, 2005).

A large percentage of clinical isolates, approximately 95% of ACV-resistant HSVs, have thymidine kinase (TK)-deficient phenotypes (which includes TK-negative and TK-low-producer mutants) and since most of the antiviral drugs have similar mode of action, cross-resistance for several antiviral drugs are common (Pottage and Kessler, 1995). TK- deficient phenotypes are developed due to the long-term application of ACV or its derivates. ACV is a guanosine analogue,

which upon its entry into the infected cells undergoes phosphorylation steps by the virus-encoded TK and host cellular guanosine monophosphate (GMP) kinase (Miller and Miller, 1980) and nucleoside diphosphate kinase, , resulting in ACV triphosphate (Miller and Miller, 1982). ACV-triphosphate integrates itself into DNA during DNA synthesis at its 3′ terminus. It blocks further virus DNA elongation as well as virus DNA replication (Reardon and Spector, 1989).

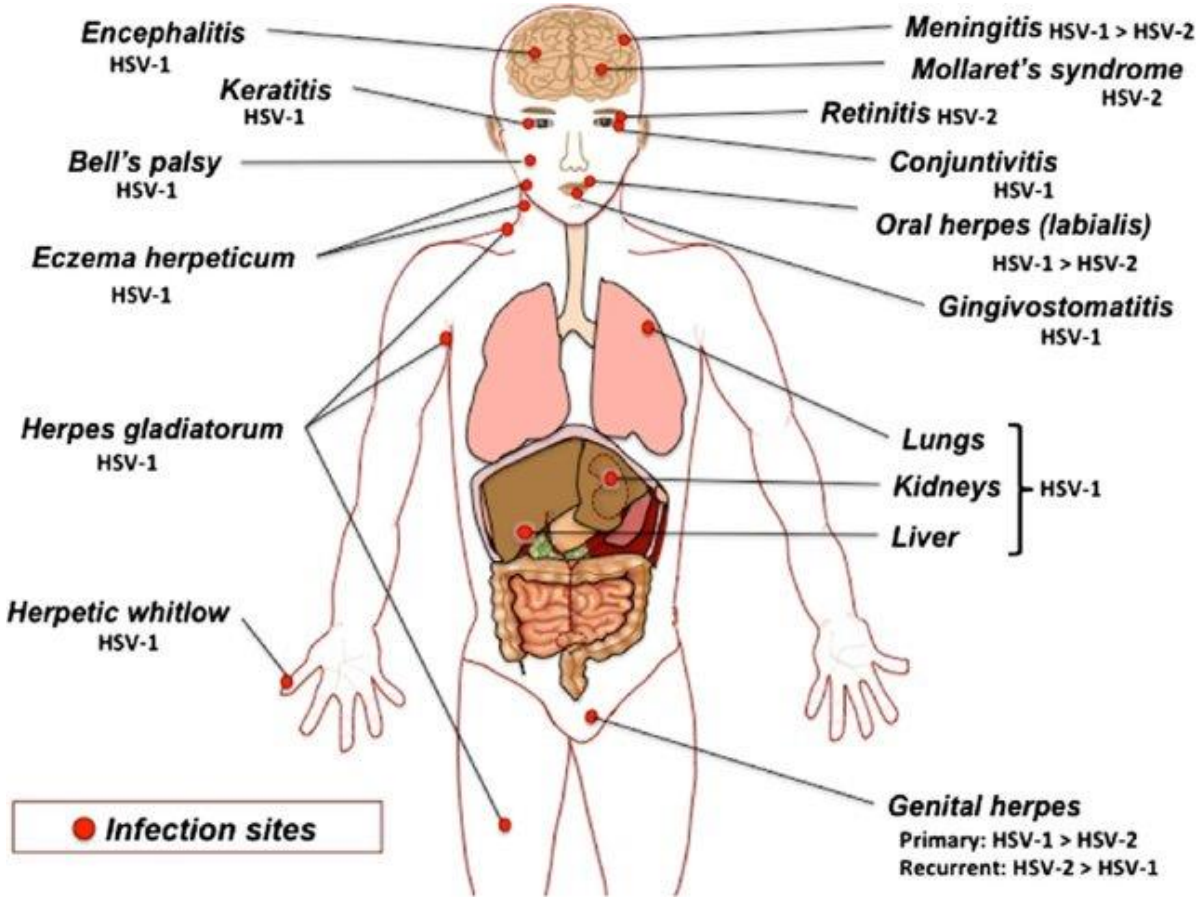


Figure 1. Clinically apparent herpes simplex viruses (HSV-1 and HSV-2).

Herpes simplex viruses infecting different anatomical sites of the human body can cause corresponding disease: **Brain** → meningitis or encephalitis, **Ocular area** → keratitis, retinitis and conjunctivitis, **Mouth** → orolabialis and gingivostomatitis, **Genital area**→ anogenitalis**, Fingers, Thumb, Toes** → herpetic whitlow, **Entire face or body (skin)** → herpes gladiatorum or eczema herpaticum and **internal organs**, such as lungs, kidneys and liver also can be a replication site for herpes simplex viruses. The most prevalent serotype of herpes simplex virus causing each pathology is specified in the picture (The picture is adapted from Klein et al. 2016) (Klein, 2016).

Table 1. Herpes simplex virus conventional therapy

Anti-herpes simplex agents	Mechanism of action	Class of drugs
Aciclovir	Nucleoside analogue	Acyclic guanosine analogues
Adefovir dipivoxil	Nucleoside analogue	Acyclic nucleotide analogues
Cidofovir	Nucleoside analogue	Acyclic nucleotide analogues
Foscarnet	Inhibitor of DNA polymerase	Pyrophosphate analogues
Famciclovir	Pro-drug of Penciclovir	Acyclic guanosine analogues
Penciclovir	Nucleoside analogue	Acyclic guanosine analogues
Valaciclovir	Pro-drug of Aciclovir	Acyclic guanosine analogues

1.2 Herpes simplex virus

Herpes simplex virus type 1 and type 2 are members of the *Herpesviridae* family. The family of Herpesviridae can be divided into three subfamilies owing to their replication properties, pathogenicity and the cell tropism: α-Herpesvirinae, β-herpesvirinae and γ-herpesvirinae. Herpes simplex virus type 1 and type 2 are part of the α-herpesvirinae subfamily and simplex virus genus (Arvin *et al.*, 2007). Humans are the host of at least eight herpes viruses called human herpes viruses (HHVs), which were named chronologically after their discovery (HHV-1 to HHV-8) (Table 2) (Ryan and Ray, 2004; Whitley, 1996).

Table 2. Human herpes virus classification.

Type	Name	Subfamily	Primary Target	Primary pathophysiology
HHV-1	HSV-1	α	Mucoepithelial	Orogenital infections
HHV-2	HSV-2	α	Mucoepithelial	Orogenital infections
HHV-3	VZV	α	Mucoepithelial	Chickenpox and shingles

HHV-4	EBV	γ	B cells and epithelial cells	Infectious mononucleosis, Burkitt's lymphoma and more
HHV-5	CMV	β	Monocytes and epithelial cells	Infectious mononucleosis-like syndrome, retinitis and more
HHV-6A	Roseolo virus	β	T cells	Sixth disease
HHV-6B	HLV	β	T cells	Sixth disease
HHV-7	-	β	T cells	Drug-induced hypersensitivity syndrome, encephalopathy
HHV-8	KSHV	γ	Lymphocyte	Kaposi's sarcoma

VZV: Varicella zoster virus, KSHV: Kaposi's sarcoma-associated herpesvirus, CMV: Cytomegalovirus, EBV: Epstein–Barr virus, PTLD: Post-transplant lymphoproliferative syndrome, HLV: Herpes lymphotropic virus

Herpes simplex virus type 1 and type 2, also known as HHV-1 and HHV-2, are neurotropic viruses which mainly target mucoepithelial cells and transmit through direct contact (oral secretions, sores on the skin, sexual contact) with a person with active HSV infection or virus shedding. HSV-1/2 are enveloped viruses with approximately 150-200 nm diameter and a relatively large linear double-stranded DNA around 152 kbps in length (Figure 2). The linear genome is encased within an icosahedral capsid, which is wrapped in a lipid bilayer envelope. The envelope and capsid are linked by means of a tegument (Mettenleiter *et al.*, 2006). HSV-1/2 encompass at least 74 open reading frames (ORFs) in the genome, leading to the expression of approximately 84 unique protein codings (Rajčáni *et al.*, 2004), which are involved in the replication and infectivity of the virus, formation of the capsid, envelope and tegument. The HSV-1/2 genome comprises two unique regions, namely the long unique region (UL) and the short unique region (US) containing 56 and 12 viral genes (Figure 2), respectively (McGeoch *et al.*, 2006).

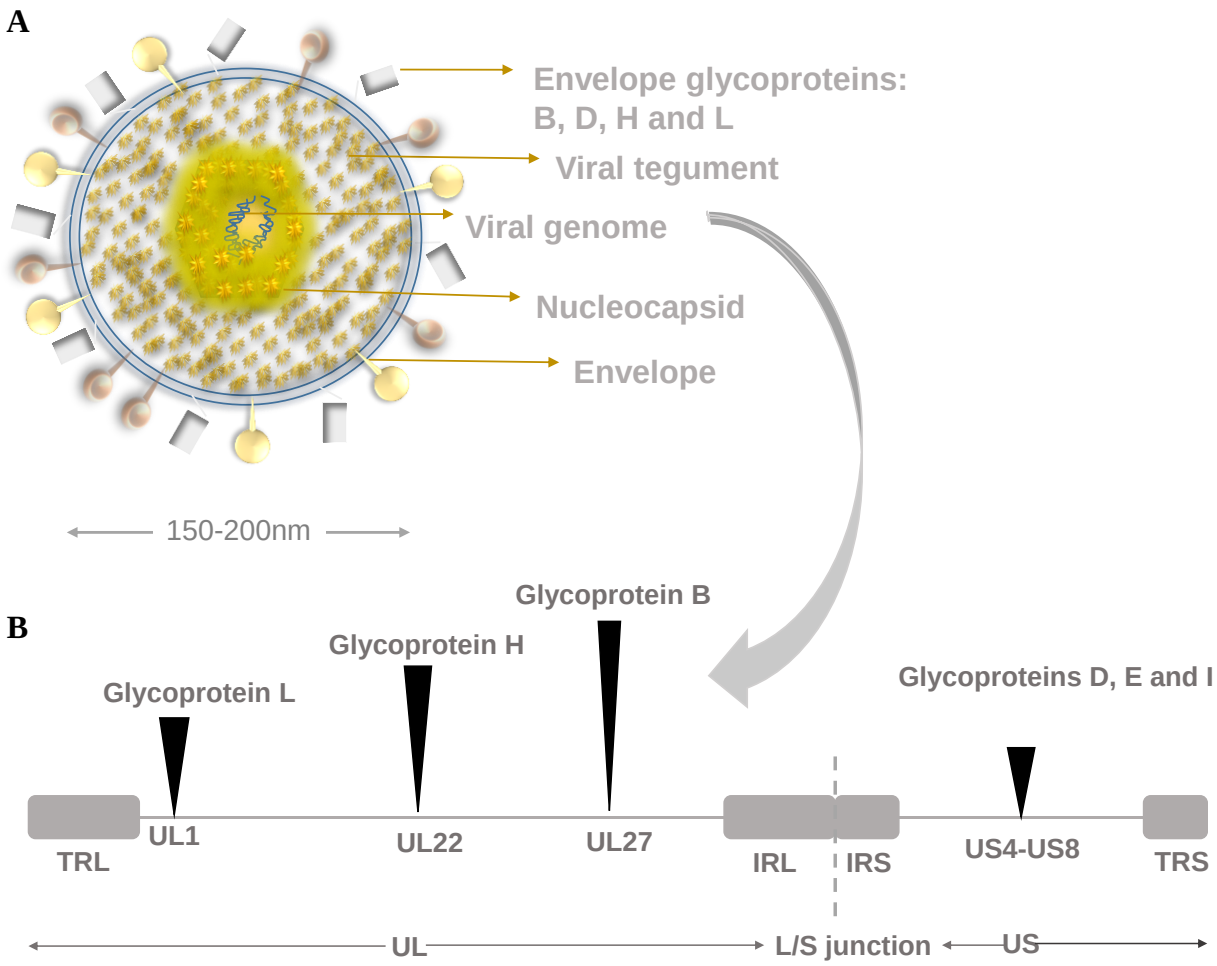


Figure 2. Schematic HSV virion structure and genome organization.

A) HSV is an enveloped virus with 150-200 nm diameter. The structure of HSV is defined by three different entities: icosahedral capsid which contains genome, envelope which surrounds the capsid and tegument which links envelope and capsid. B) Simplified map of the genome of herpes simplex virus. The HSV genome is a linear double-stranded DNA with approximately 152 kbps length. It has two unique regions called long unique region (UL) and short unique region (US) which regulate HSV replication, packaging and infectivity (some of the essential glycoproteins for HSV fusion are shown). TRL: terminal repeats long, TRS: terminal repeats short, IRL: inverted repeat of the L segment, IRS: inverted repeat of the S segment.

1.3 Herpes simplex virus life cycle

HSV is able to establish lytic or latent infections, depending on the host cell type. Lytic infections occur in different cell types while latency is highly specific for neurons. The **lytic phase of the HSV** life cycle includes the following phases: 1) HSV attachment and entry into the host cell

(which starts with skin epithelial cells or mucosa), 2) transport to the nucleus and expression of viral genes (immediate early, early and late genes), 3) DNA replication, 4) nucleocapsid assembly and capsid maturation, 5) primary envelope formation and at the end egress of the new generation of virions from the endoplasmic reticulum (ER). In permissive cell lines in a laboratory setting, this cycle takes approximately 18 to 20 hours (Heming *et al.*, 2017) (Homa and Brown, 1997).

A specific feature of herpes viruses is their ability to persist after a primary lytic infection and establish a life-long latent infection. In **latent HSV infection**, HSV enters sensory neurons via their termini, and retrograde transport takes the genome to the neuronal nuclei in the sensory ganglia during a lytic infection. Depending on the virus primary infection site different sensory ganglia can get infected and be involved in the virus latency. Normally, primary infection of the orofacial or anogenital areas later establishes latent infection of the trigeminal ganglion (TG) and dorsal root of ganglion (DRG), respectively (Margolis *et al.*, 2007). In a latent state the genome persists in neurons where it can be reactivated spontaneously or upon stress stimuli to resume replication and induce lytic infection. In a lytic infection the virus is exposed to the immune system and this usually can clear the infection. However, in a latent infection, due to the low level of viral gene expression, it is not readily recognized by the immune system. This gives the virus the advantage of avoiding the activation of the immune system (Maroui *et al.*, 2016).

Two **HSV entry mechanisms** have been proposed. The most widely accepted mechanism suggests the fusion of the viral envelope with the plasma membrane. This occurs through the interaction of surface glycoproteins located on the virion with specific cell surface receptors and intracellular transport of the viral capsid into the nucleus of the infected cells. The alternative pathway is the entry via endocytosis and/or phagocytosis-like uptake of the enveloped virion followed by the fusion of the envelope within intracellular vesicles. In contrast to a typical endocytosis or phagocytosis, this unique type of uptake is not mediated by clathrin-coated caveolae and it is not clear if it is pH-dependent or not. It is shown that phagocytosis-like uptake involves activation of cellular GTPases and cytoskeletal reorganization. Regardless of the uptake mechanism, the viral envelope finally fuses with the vesicular or plasma membrane and then releases the viral capsid into the cytoplasm (Nicola and Straus, 2004) (Clement *et al.*, 2006).

Virion attachment to the host cell surface is facilitated by the interaction of viral glycoproteins C (gC) and B (gB) with cell surface glycosaminoglycans, in particular heparan sulphate. Fusion of the viral envelope with the plasma membrane and subsequently viral entry into the host cells require interaction of four glycoproteins, glycoprotein D (gD), glycoprotein B (gB), and the heterodimer glycoprotein H/glycoprotein L (gH/gL) (Spear, 2004). The majority of the studies suggest that gD functions as a trigger, as a signal for fusion complex assembly. Its conformational changes upon binding to the cellular receptors (i.e. nectin-1 and nectin-2, herpes virus entry mediator (HVEM), or 3-O-sulfated heparan sulphate (3-O-S-HS)) triggers membrane fusion by interaction with the gB and gH/gL complex.

The exact mechanism and players of this process are poorly understood, however, it is known that upon gD interaction with cellular receptors via its N-terminal region, C-terminal displacement exposes domains required for the activation of gH/gL and finally activation of gB; thereby triggering membrane fusion (Figure 3) (Agelidis and Shukla, 2015) (Carfí *et al.*, 2001). Unlike the other viral fusogens, gB requires the gH/gL complex to get activated and functions as a fusogen. In general, gH/gL heterodimer is known as gB activator which functions as an adaptor to transmit the activating signals from gD to the gB although, in some herpesviruses such as KSHV, the gH/gL complex plays a role in fusion by binding to the cellular receptors (Hahn *et al.*, 2012). The gH/gL heterodimer consists of gH with a large ectodomain and a transmembrane anchor and gL, which unlike gH lacks a transmembrane region and strongly binds to the domains H1A and H1B of gH (Chowdary *et al.*, 2010). The mechanism by which gH interacts with gB and triggers gB activation is not well understood. However, there are some evidences suggesting, the prefusion structure of gB is stabilized by the gB cytoplasmic domain. This prevents conformational changes into the postfusion form (Wanas *et al.*, 1999). Therefore, the gH cytotail is believed to trigger conformational changes in gB by destabilizing the cytodomain clamp (Wilson *et al.*, 1994).

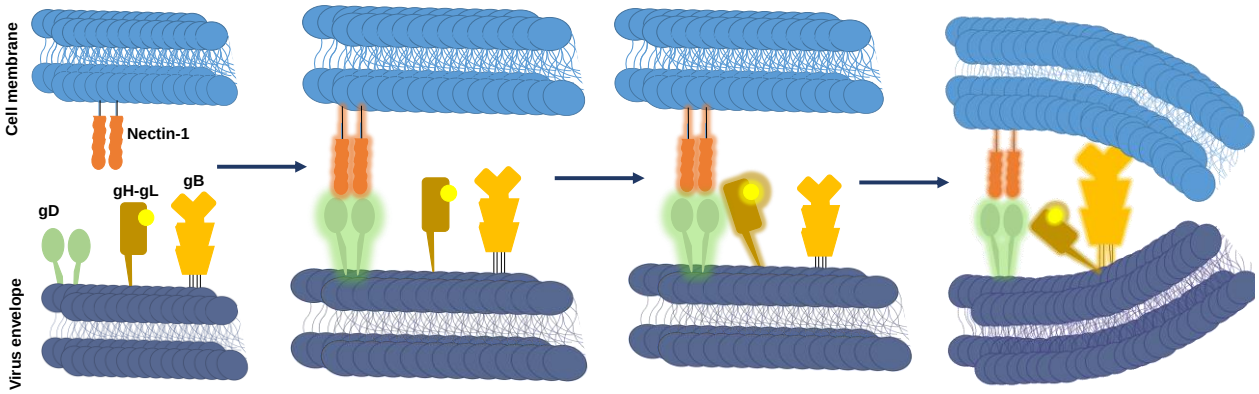


Figure 3 Schematic representation of the sequential events of HSV fusion process.

The fusion process initiates with the binding of gD to specific receptors (e.g. nectin-1), gD-gD receptor interaction triggers conformational changes by the C-terminus displacement of gD and exposing a hidden area of gD to interact with gH/gL. Formation of the gD /gH/gL complex activates gB to a fusogenic state and results in the insertion of gB fusion loops into the opposing membrane and fusion of the viral envelope with the cell membrane.

1.4 Glycoprotein B of HSV as membrane fusogen

Regardless of the mechanism by which the fusogenic machinery is initially triggered, herpesvirus fusion mechanisms converge on gB as HSV fusogen. Glycoprotein B of HSV is a multidomain trimeric fusogenic protein (Figure 4) which has been described as the most conserved component of the HSV fusion machinery (Heldwein *et al.*, 2006). Glycoprotein B of HSV belongs to class III of viral fusion proteins. Class III fusion proteins represent a complex multidomain organization with an α-helical coiled-coil core together with two long β-hairpins tipped with bearing hydrophobic loops. The transition between pre- and post-fusion configurations is then a result of domains' rotation and refolding (Harrison, 2008). There are several controversial models for conformational changes of gB during membrane fusion, the prefusion structure of gB HSV in particular is not known. However, there are some studies describing post- and pre-fusion structures of human cytomegalovirus (HCMV) (Si *et al.*, 2018), Epstein-Barr virus (EBV) glycoprotein B as well as vesicular stomatitis virus (VSV) glycoprotein G (Backovic *et al.*, 2009) which all are very similar to HSV gB. Glycoprotein B of HSV, EBV and HCMV and also glycoprotein G of VSV (Zeev-Ben-Mordehai *et al.*, 2016) share strong homology of amino acid sequences. They also present similarities in their 3D-structure (Cooper and Heldwein, 2015), as shown by X-ray crystallography. However, they have a distinct domain arrangement (Burke and Heldwein, 2015).

On the basis of the homology between gB of HSV and glycoprotein G of VSV, it has been hypothesized that the prefusion structure of gB might be similar to a proposed fusion model of glycoprotein G of VSV. According to this hypothesis Zeev-Ben-Mordehai et al. (Zeev-Ben-Mordehai *et al.*, 2016) and Gallagher et al. (Gallagher *et al.*, 2014) propose two different models for prefusion and postfusion configurations of HSV-1 gB (Figure 5).

In addition, Fontana et al. (Fontana *et al.*, 2017) performed cryo-electron tomography and subtomogram averaging to further investigate gB(HSV) prefusion to postfusion transition and envision a proper fusion model. They showed that gB has a compact conformation in its prefusion state, with the fusion loops in proximity of the viral membrane pointing down (similar to Gallagher et al.'s model), and then transition to a compact conformation with fusion loops pointing up (inversion), similar to Zeev-Ben-Mordehai et al.'s model. This series of conformational changes finally leads to membrane fusion. These fusion models are largely built up on assumptions for conformational rearrangements of gB during fusion and may help to explain how neutralizing antibodies can inhibit the gB structural transitions which is essential for viral entry and therefore infectivity.

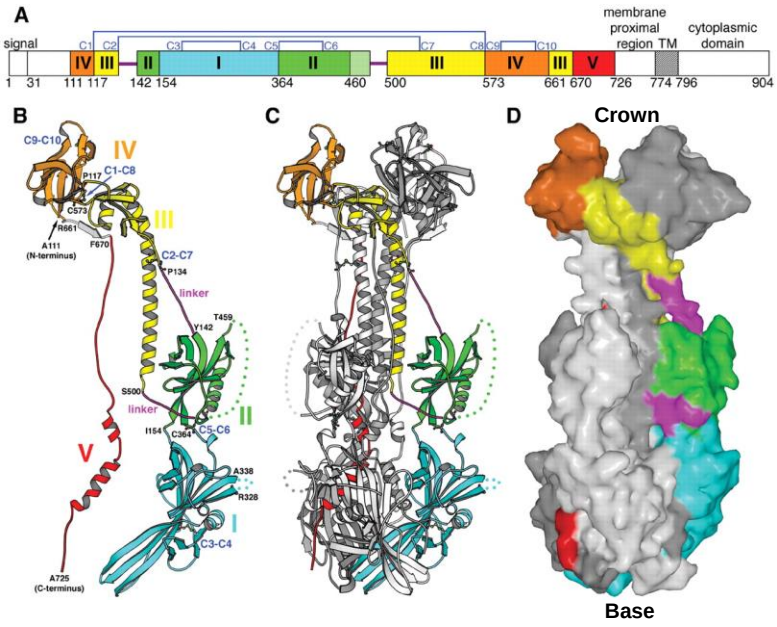


Figure 4. Schematic representation of the HSV-1 gB postfusion structure.

A) Colour coded full length gB domain organization according to the crystal structure. Amino acid numbering is shown below. B) A single gB protomer of the postfusion structure shown in ribbon diagram. C) gB postfusion trimer

in ribbon diagram showing one protomer in the same colour coding as (B). D) Surface representation of the crystal structure of the HSV-1 postfusion gB trimer (Image is adopted from Heldwein et.al. 2006, Science).

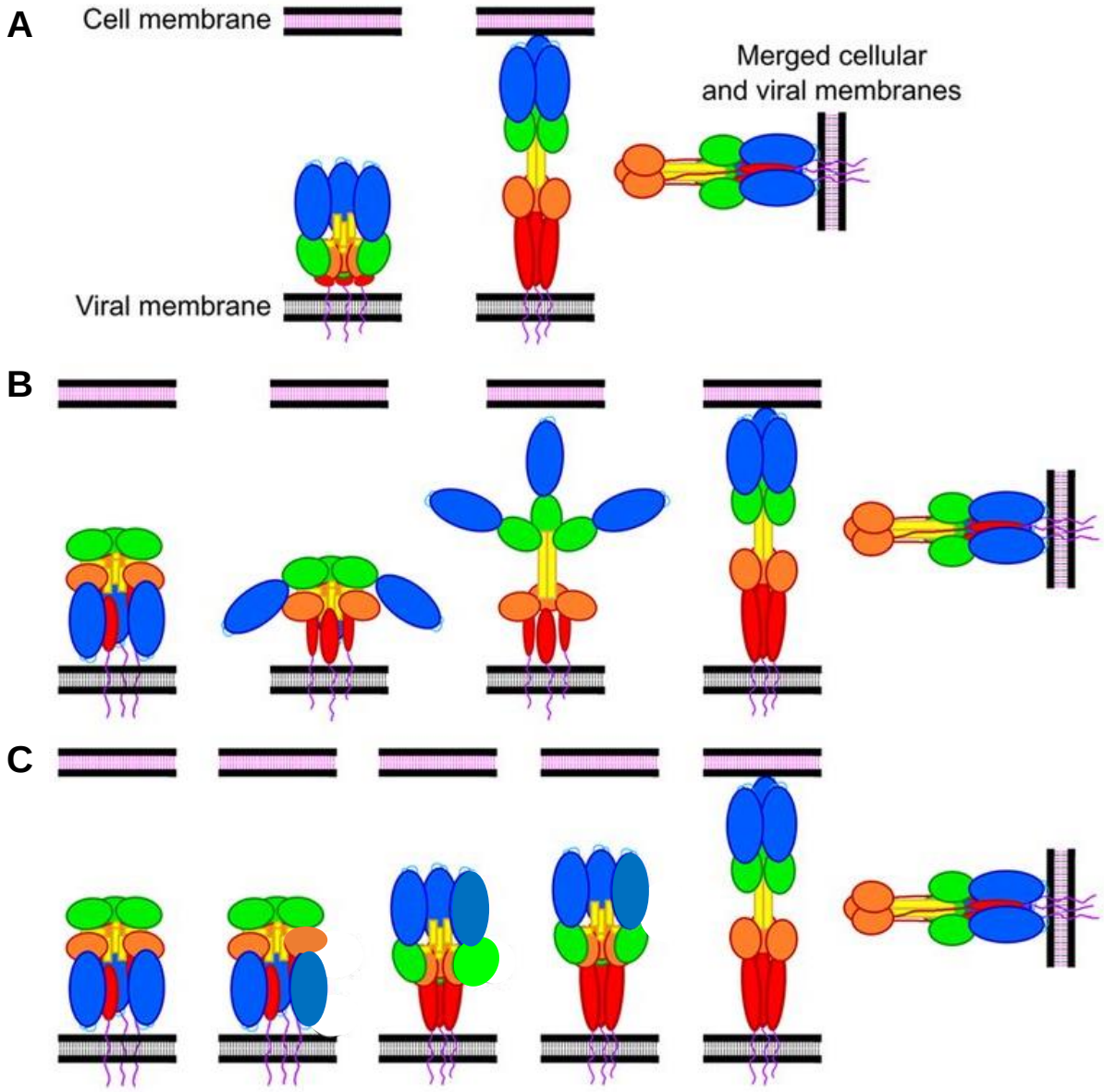


Figure 5. Glycoprotein B (HSV) transition models from the prefusion to postfusion state.

The image is showing three-hypotheses of pre- to postfusion transition of gB(HSV) trimers proposed by Fontana et al. A) Model in which the prefusion state contains fusion loops at the top of the gB trimer in a compact form and then undergoes an extension to reach out to the host cell membrane and finally experiences an inversion to fuse the membranes which fits to the model of Zeev-Ben-Mordehai et al. B) Showing the second hypothesis which fits to the model by Gallagher et al., in which the fusion loops point towards the viral membrane and extend to reach out to the host cell membrane, finally experience folding back upon itself into the postfusion structure to fuse the membranes.

C) Showing the hypothesis proposed by Fontana et al. in which the fusion loops are in virus membrane proximity pointing towards the viral membrane in the prefusion state. There is an initial inversion in the compact state and then extension to contact the cellular membrane following a second inversion and fusion (the image is adapted from Fontana et al. 2017 American Society for Microbiology).

1.5 HSV and Immune Responses

Herpes simplex viruses are known as highly successful human pathogens due to the multiple immune evasion mechanisms that they have developed over the evolution in interaction with the immune system. The immune response to Herpes simplex virus is multifaceted and recovery from HSV-1/2 infection by an intact immune system is gained by contribution of both innate and adoptive (cellular and humoral) immune response and main players (see below) might differ in the response to lytic and latent infections. Studies on the HSV latency in animal models showed that latency can be "leaky" and HSV protein or RNA might be found occasionally (Feldman *et al.*, 2002). The latency is maintained by HSV-1-specific memory/effector CD8(+) T lymphocytes that are retained in the ganglion in close apposition to the neurons preventing full reactivation and virion formation through IFN-gamma production (supressing immediate-early protein ICP0 of HSV) and with a possible contribution of CD4+ T cells (Khanna *et al.*, 2004).

The innate immune response against HSV infection play a critical role in the outcome of the infection. On the other hand, the adaptive immune response regulates disease progression, virus spread and latency.

1.5.1 Innate immunity: HSV recognition by cellular sensors

A variety of HSV-derived products, such as DNA, proteins, and lipoproteins, can trigger antiviral responses via binding to specific cellular sensors including Toll-like receptors (TLRs), scavenger receptors, retinoic acid inducible gene I (RIG-I)-like receptors (RLRs), Nod-like receptors (NLRs), and DNA-dependent activator of IRFs (DAI) (Cheng *et al.*, 2007). For example, TLR3, TLR9, and the TLR2/6 heterodimer were shown to play role in the resistance to HSV-1 and controlling the infection (Finberg and Kurt-Jones, 2004). It has been shown that the IFN response against HSV-1 requires TLR adaptor protein MyD88, and TLR9 (Krug *et al.*, 2004). However according to Kurt-Jones et al. (Kurt-Jones *et al.*, 2004) there might be an immune pathology mediating role for TLRs such as TLR2 which induce production of inflammatory cytokines and result in developing lethal

encephalitis following HSV-1 infection. Besides to the viral products, which serve as triggers to initiate anti-HSV immune responses, the virus entry event appears to be another (and the earliest) trigger of innate immune responses to viruses compared to the classical pathogen associated molecular patterns (PAMPs). Indeed, this immediate early response does not depend on PAMPs (e.g. TLRs) and begins with cellular changes associated with the membrane fusion event which may serve as a danger signal for anti HSV immune response induction (Collins *et al.*, 2004) (Netterwald *et al.*, 2004) (Noyce *et al.*, 2009) (Paladino *et al.*, 2006) (Prescott *et al.*, 2005) (Preston *et al.*, 2001).This also has been seen for other viruses, such as HCMV (Boyle *et al.*, 1999).

1.5.2 Innate immunity: Type I IFN response

The type I IFN pathway initiates upon the recognition of viral ligands by pattern recognition receptors (PRRs) which ultimately leads to activation of Tank binding kinase 1 (TBK-1) and/or Inhibitor of NFκB kinase epsilon (IKKε) (Cheng *et al.*, 2007). The activation of these kinases regulates the activation of IFN regulatory factor 3 (IRF-3) which along with other transcription factors including ATF/cJun and NFκB, results in the production of type I IFN and in many tissues, particularly epithelia, also type III IFN (Fitzgerald *et al.*, 2003). The type I IFNs play a key role in the host anti HSV immune response. Strong correlation between developing a rapid type I IFN response and resistance to infection with HSV is shown through in-vivo experiments (Noisakran and Carr, 2001).

Type I IFNs mediate these antiviral responses via JAK/STAT pathway and induce the expression of effector molecules such as IFN stimulated genes (ISGs) (Chew *et al.*, 2009). Studies involving mice with interrupted type I IFN signalling or IFNα/β receptor knockout mice show increased susceptibility to HSV infection and decreased survival, however, mice with impaired recombination activating gene (RAG), which were lacking T cell or B cell responses showed resistance to HSV-1 infection due to the intact type I IFN response (Vollstedt *et al.*, 2004). These effector molecules play a key role in the establishment of anti HSV immunity by inhibiting viral protein expression, inducing apoptosis, and recruitment of immune cells to the sites of infection. For example, IFN-induced Protein Kinase R (PKR) prevents viral gene expression by phosphorylating and inhibiting the activity of the host cells' translation initiation factor eIF2α

(Farrell *et al.*, 1978). Hence, mice with deficient PKR showed enhanced susceptibility to both HSV-1 and HSV-2 infections (Al-Khatib *et al.*, 2004).

1.5.3 Innate immunity: cell type specific anti HSV response

NK cells are important cellular components of the innate immune response. They play a crucial role in viral recognition by elimination of HSV infected cells and producing cytokines such as IFNγ (Biron and Brossay, 2001). In support of the role of NK cells in anti HSV responses, several studies showed that using animal models lacking NK cells or depleting NK cells as well as CCR5 deficiency (functions in NK cells recruitment to the site of infection) make the mice more susceptible to infection with HSV (Thapa *et al.*, 2007). However, there are some evidences highlighting that NK cells are not sufficient to overcome the HSV infection since increased NK cell activity did not protect mice from HSV induced mortality (Brandt and Salkowski, 1992). On the other hand, several studies suggested an important role of NK cells in contributing to anti HSV immunity. For instance, low NK cell activity or low NK cell counts were observed in patients with severe HSV infections (Dalloul *et al.*, 2004) (Ching and Lopez, 1979). In addition, lack of NK cell activity was found in a case suffering from recurrent severe HSV infections as the only detectable defect in the immune response (Biron *et al.*, 1989). In conclusion, the data suggest an important role of NK cells in controlling HSV replication and protecting from HSV-induced pathologies.

Owing to the significance of type I IFN in the anti HSV innate response, an anti HSV role has been proposed for plasmacytoid dendritic cells (pDCs) due to their ability to produce large amount of IFNα both in mouse models and in humans (Siegal *et al.*, 1999) (Honda *et al.*, 2005). There is an association between low pDC counts (e.g. in patients with atopic dermatitis) and severe recurrent HSV infections or higher susceptibility to HSV infection in human patients (Wollenberg *et al.*, 2002). Besides, severe inflammation and tissue damage was observed in a mouse model lacking pDC after genital inoculation with HSV-2 (Kittan *et al.*, 2007). Conclusively, as for many viruses, pDCs also have an important function in controlling HSV replication *in-vivo*.

1.6 Adaptive immunity: anti HSV cellular responses

The lytic phase of HSV infection in skin or mucosa, mount an inflammatory response (Eidsmo *et al.*, 2009) which initiates neutrophil influx (Stock *et al.*, 2014) and activation of dendritic cells and

Langerhans cells (LCs). Then, damaged cells/infected cells are taken up by migratory dendritic cells and carried to the nearby lymph nodes by afferent lymphatic vessels (the infected DCs are forced to stay at the lesion site and undergo apoptosis) (Nguyen and Blaho, 2006). There the HSV antigens can be passed to lymph node resident dendritic cells or CD8+ DCs and presented with MHC I (partially due to the cross presentation) (Bachem *et al.*, 2010) to naive cytotoxic T lymphocytes (CTLs) (Allan *et al.*, 2006). MHC II-presented HSV antigens by migratory DC, induce CD4+ T cell activation and proliferation which is responsible for priming CTL (CD8+ T cells) (Lee *et al.*, 2009).

CD8+ T cells play a key role in the host adaptive immunity against HSV infection. Early in the HSV infection, CD8+ T cells are recruited to the infection site and contribute significantly to the immune regulation and cytolysis (Mueller *et al.*, 2002). Following antigenic stimulation, CD8+ T cells experience three phases: 1) activation and proliferation, 2) contraction, and 3) the memory establishment. Upon antigen recognition by CD8+ T cells, they increase the expressions of perforin and granzymes and become cytolytic. It has been proposed that the anti HSV response of CD8+ T cells may be implicated in an IFN-γ dependent way and IFN-γ plays a crucial role in anti HSV response mediated by CD8+ T cells (Cantin *et al.*, 1995) (Dobbs *et al.*, 2005). It was also shown that CD8+ T cells are able to control HSV-1 infection in the trigeminal ganglion and prevent neurologic damage (Sheridan *et al.*, 2009). Indeed, several studies suggested that IFNγ-producing CD8+ T cells persist during neuronal latency and stay in the T cell infiltration area (Wakim *et al.*, 2008a) (Wakim *et al.*, 2008b). During HSV-1 infection, CD8+ T cells can establish a memory in ganglia and mucosa and also non-lymphoid tissue compartments. Virus-specific tissue-resident memory CD8+ T cells (resident memory T cells are the third class of memory T cells (Central and effector memory T cells are classified as first and second class of memory cells). They are found particularly in secondary lymphoid organs and barrier tissues and they never recirculate into the blood. They can raise a prompt immune response following pathogen reactivation in the local tissue sites) (Woon *et al.*, 2016) (Wu *et al.*, 2018) can trigger protective innate and adaptive immunity upon HSV reactivation (Torti and Oxenius, 2012).

CD4+ T cells as another branch of adaptive cellular immunity, contribute in eliminating acute HSV-1 infection and modulating the functions of innate immune cells in addition to adaptive immunity. CD4+ T cells are involved in mitigating genital herpes infection during HSV-1 infection

in a mouse model and depletion of CD4+ T cells show increased susceptibility to HSV infection. CD4+ T cells contribute to generate and expand primary CD8+ T cell response against HSV infection. It was shown that in CD4+ T cells depleted mice, CD8+T cells have reduced ability to produce IFN-γ and tumour necrosis factor-α (TNF-α) (Rajasagi *et al.*, 2009). During HSV-1 infection in the cornea Th17 and Th1 cells are orchestrating protective response against herpes stromal keratitis (HSK) (Suryawanshi *et al.*, 2011). Johnson et al. showed an important role for HSV-specific $CD4^+$ T cells in the clearance of HSV-1 from neural tissues after genital HSV-1 infection (Johnson *et al.*, 2008). Conclusively, both, CD4+ and CD8+ T cells play crucial roles in controlling HSV infection.

1.6.1 Adaptive immunity: The humoral anti-HSV response

Concerning the antibody responses targeting virus antigens following HSV infection in the protection against HSV infection, an antibody-mediated protection (reduced clinical scores of disease and HSV titres at the site of infection) has been shown in mouse models of HSV-2 (Zeitlin *et al.*, 1996) (Sherwood *et al.*, 1996). The importance of neutralizing antibodies in the protection against HSV infection were highlighted by intraperitoneal (IP) administration of a gD-specific mAb which prevented disease onset and reduced HSV-2 viral load in the vaginal area of intravaginal infected mice (Chu *et al.*, 2008). In addition, the humoral response against HSV has shown long-term effects. In a vaginal inoculated HSV-2 mouse model, HSV-2-specific plasma cells persist in the bone marrow and spinal cords of infected mice(Milligan *et al.*, 2005). The importance of humoral immunity in HSV infection protection was supported by studying B cell deficient mice (Milligan *et al.*, 2005) (Morrison *et al.*, 2001). In the cyclophosphamide-induced immunosuppressed mice, passively applying serum containing anti-HSV antibodies led to 100% survival in a mouse model compared to the control group, highlighting the role of antibodies in protection against infection and disease progression (Mitchell and Stevens, 1996). In the human Herpevac trial for women, which is a double-blind, randomized, controlled phase III study to evaluate the safety and prophylactic efficiency of a gD-vaccine in the prevention of genital herpes, surprisingly a strong correlation between the humoral response raised against gD(HSV-2) and protection against HSV-1 was observed. This supports the importance of antibodies and demonstrates protecting immune responses across the two different herpes simplex viruses (Belshe *et al.*, 2014).

1.7 Monoclonal antibody therapy against HSV infection

In-vivo studies using anti-herpetic polyclonal immunoglobulins show promising results in controlling HSV infection. However, there are several drawbacks which makes their translation into the clinic very complicated. Infusion of polyclonal immunoglobulins bears the risk of contamination with blood-derived products. Substantial variability in potency and specificity has been observed in different polyclonal immunoglobulin preparation (Pierce and Jain, 2003). The relatively small fraction of 'naturally elicited' antibodies as polyclonal immunoglobulin preparation is truly effective in clearing the viral infection. The application of human monoclonal antibodies (mAbs) can improve the disadvantages of polyclonal preparations and may replace the need to the classical anti-herpetic drugs in patients that usually develop resistances.

Antiviral mAbs are promising therapeutics against a variety of viral infectious diseases and the number of developed mAbs targeting viral antigens has increased exponentially during the recent years (Grilo and Mantalaris, 2019). Antiviral mAbs are mainly used to restrict viral propagation and transmission through direct effects. However, there is an emerging field focusing on the induction of long-lasting protective and vaccine-like effects. The application of mAbs to directly target infectious particles are a form of passive immunotherapy. Adamiak et al. showed that the monoclonal antibody B1C1 targeting the cell binding domain of glycoprotein C of herpes simplex virus type 1 neutralized virus infectivity and extended survival time of HSV-1 infected mice (Adamiak *et al.*, 2010). Several neutralizing mAbs targeting HSV glycoprotein D and B (as immunodominant antigens) have been developed (Sanchez-Pescador *et al.*, 1992). Table 3 summarizes a list of developed monoclonal antibodies targeting gB(HSV-1). As an example the mAb E317 (targeting gD) prevents HSV-1 and HSV-2 cell entry by interacting with Nectin-1 and herpes virus entry mediator binding domains in gD *in-vitro* (Burioni *et al.*, 1994b). Systematic administration of mAb AC-8 (a Fab fragment targeting gD) in mice challenged with corneal and intracutaneous HSV infection, result in a strong neutralization efficiency against HSV-2 (more potently) and HSV-1(Lee *et al.*, 2013). Recently developed HDIT101 (hu2c) and its parental murine antibody targeting a conserved epitope on gB (HSV-1/2) showed an even higher neutralization efficiency *in-vitro* and *in-vivo.* HDIT101(hu2c) and mAb2c showed highly efficient neutralization and blocking of HSV-1/2 cell-to-cell spread in-vitro by binding to a discontinuous epitope possibly by cross linking gB trimmers in its prefusion structure. HDIT101 (hu2c) and mAb

2c were capable of protecting infected BALB/c as well as severely immunodeficient NOD/SCID mice upon vaginal HSV-1/2 challenge. Notably, HDIT101 (hu2c) and mAb 2c showed similar neutralization efficiency toward drug resistant HSV-1/2 isolates (Krawczyk *et al.*, 2011b) (Krawczyk *et al.*, 2013a). Moreover, systemic treatment with HDIT101(hu2c) and mAb 2c prevented the HSV-1 induced HSK and acute retinal necrosis (ARN) in a BALB/c mouse model (Bauer *et al.*, 2017c) (Krawczyk *et al.*, 2015) (Bauer *et al.*, 2017a).

Table 3. Monoclonal antibodies directed against gB(HSV-1) and respective characteristics.(adapted from Sanchez-Pescador et al. (Sanchez-Pescador *et al.*, 1992))

	Domain (recognition site) *	**Isotype**	**Epitope type**	**Reactive Serotype**	**ELISA titre**	**Neutralizing**	**ADCC**
H1817	D1a (1-20)	IgG1	Con	TC	14,400	>640	100
H1830	(1-20)	IgG2a	Con	TS	320,000	<20	<20
H1839	(1-20)	IgG2a	Con	TS	42,000	<20	<20
H1392	D1b(Ala-32)	IgG2a	Con	TS	386	80	<20
H1396	(16-50)	IgG1	Con	TS	115	40	<20
H1397	(Thr-47)	IgG1	Con	TS	7573	<20	100
H1838	D1c (1-190)	IgG1	Con	TC	2265	80	20
H126	D2a(Asn-273)	IgG2b	Dis	TC	491	>640	100
H1375	(Gln-274)	IgG1	Dis	TC	1190	320	100
H1435	(Tyr-278)	IgG1	Dis	TC	78,125	>640	100
H1815	(263-283)	IgG2a	Dis	TC	491	640	20
H233	D2b(His-298)	IgG2a	Dis	TC	1928	>640	100
H1819	(273-298)	IgG2a	Dis	TC	6447	>640	100
H1828	(1-457)	IgG2a	Dis	TC	6200	<20	<20

H352	D3a (1-475)	IgG2a	Dis	TC	100+	<20	<20
H1693	D3b (1-475)	IgG2a	Dis	TC	14,500	<20	<20
H1708	(1-475)	IgG1	Dis	TC	400*	<20	<20
H1376	(1-475)	IgG1	Dis	TC	6000	<20	<20
H1359	D3c (457-475)	IgG1	Con	TC	290	<20	<20
H1385	(457-475)	IgG1	Con	TC	3500	<20	<20
H1393	(457-475)	IgG1	Con	TS	35,000	<20	<20
H1783	Dd6 (475-600)	IgG2a	Dis	TC	210	<20	<20
H1798	(475-600)	IgG2b	Dis	TC	16,000	<20	<20
H120	(475-600)	IgG1	Dis	TC	5500	<20	<20
H146	(475-600)	IgG1	Dis	TC	64,000	<20	<20
H1394	Dc4a (600-690)	IgG1	Con	TC	210	<20	<20
H1411	(600-690)	IgG2a	Con	TC	2500	<20	<20
H1382	(600-690)	IgG1	Con	TS	425	<20	<20
H1757	(600-690)	IgG1	Con	TS	3200	<20	<20
H1399	(600-690)	IgG1	Con	TC	500	<20	<20
H1316	Dc4b (600-690)	IgG2b	Con	TC	1600	<20	<20
H1163	(600-690)	IgG1	Con	TC	600	-690)	<20
H336	(600-690)	IgG1	Con	TC	7500	<20	<20
H1695	Dd5a (600-690)	IgG2a	Dis, d	TC	41,039	>640	<20
H1373	(600-690)	IgG2a	Dis, d	TC	13,000	<20	<20
H420	(600-690)	IgG1	Dis, d	TC	9500	<20	<20

H121	Dd5b (600-690)	IgG2b	Dis, d	TC	6500	<20	<20
H1457	(600-690)	IgG2a	Dis, d	TC	17,500	<20	<20
H157	(600-690)	IgG1	Dis, d	TC	300,000	<20	<20
H1814	(600-690)	IgG1	Dis, d	TC	35,000	<20	<20
H1807	(600-690)	IgG2a	Dis, d	TC	110	<20	<20
H1823	(600-690)	NT	Dis, d	TC	95	<20	<20
H1727	(600-690)	IgG2b	Dis, d	TC	4500	<20	<20
H1711	(600-690)	IgG2a	Dis, d	TC	700	<20	<20
H189	(600-690)	IgG2a	Dis, d	TC	90	<20	<20

ADCC, antibody-dependent cellular cytotoxicity; Con, continuous; HE, strain of HSV-1; NT, not tested; TC, type-common (reactive with both HSV-1/2); TS, type specific (reactive with gB of HSV-1 only). * MAb epitopes grouped in antigenic domains and by amino acids within. Recombinant monoclonal antibodies are able to reduce viral infection and viral cell–to-cell transmission via direct antigen-binding or via effector functions mediated by their Fc domain. To date, most of the therapeutic antiviral mAbs are initially chosen due to their efficiency to directly inactivate viral particles and to inhibit infection. Additional effector functions mediated by the Fc domain of the antibody such as complement-dependent cytotoxicity (CDC), antibody-dependent cellular phagocytosis (ADCP), and antibody-dependent cell-mediated cytotoxicity (ADCC) contribute to eliminate viruses and virus infected cells presenting viral antigens at their surface (Figure 6).

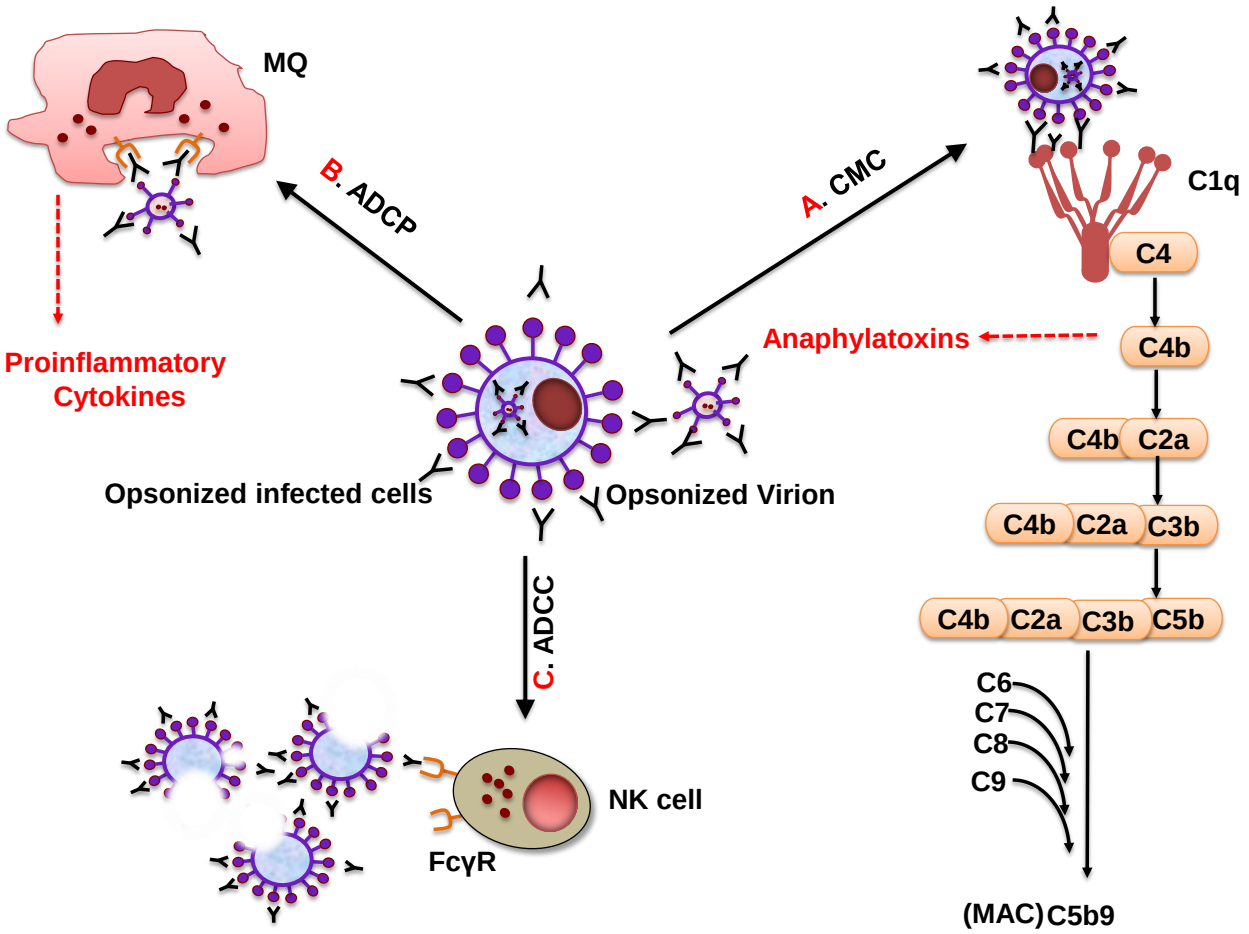


Figure 6. Effector functions mediated by the Fc fragment of IgG1.

A) Clustered mAbs on the surface of infected cells or virions activate the classical pathway of complement starting with C1q (one of the complement components) and finally forming membrane attach complex (C5b-9). B, C) Anti-viral mAb opsonizes virion/infected cells expressing viral antigens and targets them for killing by FcγR-mediated antibody-dependent cellular cytotoxicity or phagocytosis (ADCC (C) or ADCP(B)). Mϕ; Macrophages, MAC; Membrane attach complex, NK cells; Natural killer cells, ADCC; antibody dependent cell cytotoxicity, ADCP; antibody dependent cell phagocytosis, CMC; complement dependent cytotoxicity.

However, Fc-mediated effector functions may raise practical safety issues during passive prophylaxis with antiviral mAbs and regulatory requirements may limit the usability of Fc effector function. For example, in cases where mAbs target cell surface receptors or are intended to block ligand-receptor interactions it may be desirable to decrease or abolish Fc effector function in order to avoid undesired side effects. These can include the secretion of large amount of pro-inflammatory cytokines, also known as cytokine storm, which can cause severe clinical symptoms.

In order to engineer inert IgG Fc regions or reduce Fc mediated effector functions, the most common approach is modifying glycosylation patterns of the Fc region such as an aglycosylation approach by introducing a N297A substitution, which has been shown to diminish CDC and ADCC (Wang *et al.*, 2018). Interestingly, one study on neutralizing antibodies showed that effector functions of mAbs were associated with the protection against infection when the antibodies had weak neutralizing capacity, but for strongly neutralizing antibodies, the protection against infection in most cases was due to the neutralization potency of antibodies and not their effector functions (Gunn *et al.*, 2018). For this reason, it is very important to select the antiviral mAbs with strong neutralization efficiency avoiding effector functions to exert their ability to control virus infection without implementing side effects.

1.8 Aim of the Project

The clinical manifestation and pathogenesis of Herpes simplex infections depend at least on the site of primary infection, age, immune status of the host and the type of HSV. Although, the frequency and severity of infection usually decreases in the immunocompetent population with standard antiviral therapies (such as acyclovir/valacyclovir or penciclovir/famciclovir), there is a growing concern to induce selection of therapy-resistant HSV strains in immunocompromised patients, stem cell transplant recipients and HIV/HSV co-infected patients, which requires the development of novel anti-HSV therapeutics with different mechanism of action. In addition, conventional standard therapies are often not very effective and not long-lasting. Antibody immunotherapy has been demonstrated to be efficacious for the treatment and prevention of viral infections (Salazar *et al.*, 2017). There is a convincing number of evidences showing the protective role of antibodies against herpes simplex virus (HSV) and amelioration of severity of HSV-related diseases through neutralizing antibodies in *in-vivo* models. This may portend a promising future for antibody therapy of HSV infection.

Despite developing several anti HSV antibodies targeting mostly gD and gB as the most immune dominant antigens, so far only two antibodies (HDIT101(anti gB(HSV)), intravenous (IV) injection and UB-621 (anti gD (HSV)), subcutaneous (SC) injection))) ended up in clinical trials. A dose-ranging phase II trial to evaluate the safety and efficacy of UB-621(anti-HSV-gD antibody, SC injection on patients with recurrent genital HSV-2 shedding (NCT03595995) has been also

approved. Given the fact that there is always a risk of HSV resistance development against a specific therapy, there is still need to develop new monoclonal antibodies with better efficacies and with longer protection.

In this project, it was aimed to develop fully human therapeutic IgGs targeting glycoprotein B of HSV as a novel therapy. Compared to conventional drugs they could be useful in the treatment of drug-resistant HSV infections since they function via a completely different mechanism of action as compared to current drugs. We have previously selected fully human gB-specific single chain Fv (scFv) from patient-specific antibody library repertoires that were generated from lymph nodes of head and neck cancer patients. The project aimed to analyse the potential of selected antibodies for future therapeutic interventions in patients with HSV infections. For this purpose, selected scFv were reformatted into IgG type molecules and produced, followed by functional in-vitro as well as in-vivo characterization.

Antigen specificity of antibodies were analysed against HSV-1/2 by using cell associated HSV glycoproteins (infected Vero cells and 293T cells ectopically expressing gB). The neutralization capability of cell-free virus and cell to cell transmission of the antibodies were investigated (in-vitro and in-vivo). In addition, Fc effector functions (ADCC, CMC and ADCP) were characterized using both infected Vero cells as well as HEK293T stably expressing gB. With the purpose to find the epitopes that are targeted by the identified antibodies, antibody-resistant mutants were propagated in-vitro and the capability of the antibodies to bind to resistant mutants, to neutralize cell-free virus infection and to block virus transmission via cell-to-cell spread were investigated. The viral fitness of generated resistance mutants was evaluated in-vivo. The capability of neutralization and blocking cell-to-cell transmission of an aglycosylated anti-gB antibody was tested and compared with wild type antibody. The cross reactivity of the generated antibodies with viral antigens of other members of Herpesviridae family was determined.

The results of this thesis suggest the possibility to translate the identified fully human antibodies into a clinical therapy and hence likely provide a novel way to combat HSV infection.

2. Materials and Methods

2.1 Materials

2.1.1 Laboratory Equipment

Table 4. List of utilized laboratory equipment.

Instrument	Type	Supplier
Absorbance / Fluorescence plate reader	Infinite F200Pro	Tecan
Agarose gel station	Mini Sub-Cell GT	Bio-Rad Laboratories
	Sub-Cell GT	Bio-Rad Laboratories
Automatic dispenser pipette	Multipette Stream	Eppendorf
Automatic multichannel pipette	Xplorer	Eppendorf
Bacteriological incubator	BE 2000 32I	Memmert
	Heraeus B6	Thermo Fisher Scientific
Balance	AJ-2200CE Vibra	Shinko Denshi
Shaker incubator (cell and bacterial culture)	Multitron Pro	Infors HT
Centrifuge	5424 R	Eppendorf
	C5-6R	Beckmann
	Heraeus Megafuge 40R	Thermo Fisher Scientific
	Megafuge 1.0	Heraeus
	Mikro 200R	Hettich
	Multifuge 4KR	Thermo Fisher Scientific
	Sorvall RC 6+	Thermo Fisher Scientific
Centrifuge mini	3-1810	neoLab
Chemiluminescence visualization system	INTAS Advanced Fluorescence Imager	Intas Science Imaging

CO_2 incubator (cell culture)	Heracell 150 NU-5510E	Thermo Fisher Scientific Ibs Tecnorama
Electroblotting system	Trans Blot Semi-Dry Transfer Cell	Bio-Rad Laboratories
Inverted Confocal microscope	LSM 710	Carl Zeiss
Flow cytometer	BD FACS Celesta BD FACS Verse	BD Biosciences BD Biosciences
FPLC system	AEKTAFPLC system AEKTApure FPLC system	Amersham Bioscience GE Healthcare
Gel visualization system	Gel Jet Imager	Intas Science Imaging
Heating block	HBT-2 131 TH 21	HLC Biotech HLC Biotech
Hemocytometer	Neubauer improved	Karl Hecht
Inverted microscope	CKX41	Olympus
Magnetic stirrer	MR2000 2mag Mix 1 XL	Heidolph Instruments 2mag AG
Multichannel pipette	Pipetman neo P200	Gilson
PCR thermocycler	peqSTAR 96 Universal Gradient	peqLab
pH meter	PB-11	Sartorius
Photometer	NanoDrop ND-1000	Thermo Fisher Scientific
Pipette controller	Pipetus	Hirschmann
Pipettes	Pipetman neo P1000, P200, P20, P10	Gilson
Power supply	PowerPac Basic PowerPac HC peqPower	Bio-Rad Laboratories Bio-Rad Laboratories peqLab

Real time PCR	LightCycler 480 II	Roche Life Science (05015278001)
Rocking plate shaker	DSG 304|M4	Heidolph Instruments
Scanner	Perfection V750 Pro	Epson
SDS gel station	Novex Mini-Cell	Invitrogen
Sterile bench	SAFE2020 HERAsafe KSP 12	Thermo Fisher Scientific Thermo Fisher Scientific
Inverted microscope	IRBE	Leica
Suction Pump	LC 16	ATMOS
Vortex	L46 D-6012	GLW neoLab
Water bath	T100	Labortechnik Medingen

2.1.2 Chemicals, Reagents and Commercial Media

Table 5. List of utilized chemicals, reagents and commercial media.

Product	Supplier (Cat. No.)
10x Cut Smart buffer	New England Biolabs (B7204S)
10x T4 DNA ligase buffer	Thermo Fisher Scientific (B69)
6x DNA Loading dye	New England Biolabs (B70245)
Acetic acid	Carl Roth (7332.1)
Agar	Sigma-Aldrich (05039)
Agarose, universal	VWR Chemicals (35-1020)
Ammonium peroxodisulphate (APS)	Carl Roth (9592.2)
Ampicillin sodium salt	Carl Roth (K029)

Aqua ad iniectabilia	Braun (2351744)
Epiglu Tissue Adhesive	Meyer-Haake (L214ED52421)
Buffer solution pH 10	Carl Roth (P716.2)
Buffer solution pH 4	Carl Roth (P712.3)
Buffer solution pH 7	Carl Roth (A518.1)
Citric acid	Carl Roth (X863.2)
Coomassie Brillant Blue R 250	Carl Roth (3862.1)
Depo-Clinovir (Medroxyprogesteron)	Pfizer (2405793)
Isoflurane (FORANE)	Baxter (1001936040)
Dimethyl sulfoxide (DMSO)	Serva (20385.01)
12-O-tetradecanoylphorbol-l3-acetate (PMA)	Sigma-Aldrich (P1585)
Dithiothreitol (DTT)	Carl Roth (6908.3)
Dulbecco's Modified Eagle Medium (DMEM)	Sigma-Aldrich (RNBG3787)
Paraformaldehyde	Sigma-Aldrich (P6148-5KG)
Dulbecco's Phosphate Buffered Saline (DPBS)	Sigma-Aldrich (D8537)
Ethanol	Carl Roth (5054.4)
Ethylenediaminetetraacetic acid disodium salt dihydrate (EDTA)	Carl Roth (8043.2)
FACS clean	BD Biosciences (340345)
FACS flow	BD Biosciences (342003)
FcR blocking reagent, human	Miltenyi (130-059-901)
Fetal calf serum (FCS)	Sigma-Aldrich (F0804)
Freestyle F17 Medium	Life technologies (A13835)
GeneRuler 1 kb DNA Ladder	Thermo Fisher Scientific (SM0311)
Geneticin disulfate (G418)	Carl Roth (CP11.3)

Glycerol	Carl Roth (7530.4)
Glycine	Carl Roth (3908.2)
Hydrochloric acid, 2N (HCl)	Carl Roth (T134.1)
Isopropanol	Carl Roth (9866.2)
Kolliphor P188	Sigma-Aldrich (K4894)
L-Glutamine	Sigma-Aldrich (G7513)
Methanol	Carl Roth (8388.2)
Penicillin Streptomycin Solution (Pen/Strep)	Sigma-Aldrich (P0781)
PeqGREEN DNA/RNA Dye	peqLab (37-5000)
Polyethylenimine (PEI)	Polysciences (23966)
Potassium chloride (KCl)	Carl Roth (6781.1)
Powdered milk	Carl Roth (T145.2)
Rotiphorese 10x SDS PAGE	Carl Roth (3060.2)
Rotiphorese Gel A	Carl Roth (3037.1)
Rotiphorese Gel B	Carl Roth (3039.2)
Roswell Park Memorial Institute 1640 medium (RPMI-1640)	Sigma-Aldrich (RNBG6386)
Sodium acetate	Carl Roth (6773.1)
Sodium dihydrogen phosphate (NaH_2PO_4)	Carl Roth (K300.3)
Sodium dodecyl sulfate (SDS)	Carl Roth (2326.1)
Sodium azide (NaN_3)	Sigma-Aldrich (52002)
Sodium chloride (NaCl)	Carl Roth (9265.2)
Sodium hydroxide (NaOH)	Carl Roth (6771.1)
Spectra BR Multicolor Broad Range Protein Ladder	Thermo Fisher Scientific (26634)

Sulfuric acid (H_2SO_4)	Carl Roth (X873.1)
SYTOX Blue Dead Cell Stain	Invitrogen (S34857)
Tetramethyl ethylenediamine (TEMED)	Fluka (87689)
Tris Base	Calbiochem (648510)
Tris hydrochloride	Carl Roth (9090.3)
Tris Pufferan	Carl Roth (4855.2)
Trypan blue	Life Technologies (15250-061)
Trypsin/EDTA	Life Technologies (25300)
Tryptone N1 (TN1)	Organotechnic SAS (19553)
Tryptone/Peptone from casein	Carl Roth (8952.2)
Tween 20	Carl Roth (9127.1)
Yeast extract	Carl Roth (2363.2)
Mowiol	Sigma-Aldrich (81381-50G)
Human IgG depleted serum	Hoelzel (CSI20186A)
UltraPure Salmons sperm DNA	Invitrogen (15632011)

2.1.3 Consumables

Table 6. List of utilized consumables.

Consumable	Supplier
Chamber Slide (4well)	Nunc Lab-Tek
Cover glasses circular (12 mm, 0,13-0,16 mm)	R. Langenbrinck
Microscopic slides	Thermo Fisher Scientific
1.5 mm SDS PAGE cassette	Invitrogen
24-well plate polystyrene non-treated cell culture plate	Corning

96-well culture plate (U Bottom)	Cellstar
96-cell culture plate (Flat Bottom)	Falcon
Amicon Ultra Centrifugal Filters	Merck
96-well Maxisorp microtitre plate	Nunc
96-well White Round Bottom Polystyrene	Corning
96-well White Flat Bottom Polystyrene	Corning
480 white Multiwell Plate 96(Light Cycler)	Roche
AeraSeal breathable sealing film	Excel Scientific
Bacterial culture tube (12 mL)	Greiner Bio-One
Bottle top filter 50 mm	Thermo Fisher Scientific
Cell culture flask for adherent cells (T25, T75, T175)	Greiner Bio-One
Cell culture flask for suspension cells (T75, T175)	Greiner Bio-One
Cryogenic vial with screw cap (2.0 mL)	Greiner Bio-One
Dialysis membrane ZelluTrans	Carl Roth
Tris-Glycine Precast Protein Gel	Thermo Fisher Scientific
Erlenmeyer cell culture flask with filter cap (500 mL)	BD Biosciences
Erlenmeyer cell culture flask with filter cap (125 mL)	Corning
Extra thick blot paper / Mini blot size	BioRad
Flow cytometry round-bottom tubes	Falcon
Nitrocellulose western blot membrane	neoLab
PCR tube strip with 8 tubes (0.2 mL)	neoLab
PCR tubes, flat cap (0.2 mL)	AHN Biotechnology
Pipette filter tip (10, 20, 200, 1000 µL)	Greiner Bio-One
Plastic syringe (20 mL, 50 mL)	BD Biosciences
Polypropylene conical tube (15 mL, 50 mL)	Falcon

Polystyrene round bottom tube (5 mL)	Falcon
Reaction tubes (1.5 mL, 2.0 mL)	neoLab
Reagent reservoir (50 mL)	Corning
Serological pipette (1, 2, 5, 10, 20 and 50 mL)	Corning
Syringe filter 0.22/0.45 µm	Merk Millipore
U-100 Insulin syringe 0,3 x 8 mm	BD Micro-Fine
ESwab	Copan

2.1.4 Chromatography columns

Table 7. List of used chromatography columns

Column	Supplier (Cat. No.)
HiTrap rProtein A FF (1 mL)	GE Healthcare (17507901)
Superdex 200 Increase 10/300 GL column (20 mL)	GE Healthcare (28990944)

2.1.5 Kits

Table 8. List of applied kits

Kit	Supplier (Cat. No.)
EndoFree Plasmid Maxi Kit	QIAGEN (12362)
EndoFree Plasmid Midi Kit	QIAGEN (12362)
NucleoBond Xtra (EF) Maxi/Midi kits	MACHEREY-NAGEL
KAPA HiFi PCR Kit	Roche (KK2101)

QIAamp DNA Blood Mini Kit	QIAGEN(51306)
ADCC Reporter Assay	Promega (G7010)
Pierce ECL Western Blotting Substrate	Thermo Fisher Scientific (32106)
Pierce TMB Substrate Kit	Thermo Fisher Scientific (34021)
QIAprep Spin Minprep Kit	QIAGEN (27106)
QIAquick Gel Extraction Kit	QIAGEN (28706)
QIAquick PCR purification Kit	QIAGEN (28106)
Zenon Alexa Fluor 488 Human IgG Labeling Kit	Thermo Fisher Scientific (Z25402)
Enzygnost anti-EBV IgG ELISA	Siemens (46784)
Enzygnost anti-CMV IgG ELISA	Siemens (46881)
Enzygnost anti-VZV IgG ELISA	Siemens (46695)
Enzygnost anti-HSV IgG ELISA	Siemens 46371
Supplementary reagent for Enzygnost /TMB	Siemens (46793)
Human C5b-9 Terminal Complement Complex / TCC ELISA Kit	Blue Gene (e01t0508)
Cell Fixation & Cell Permeabilization Kit	Thermo Fischer
primaQUANT 2x qPCR-CYBR-Green-Blue-MasterMIX	Steinbrenner Labor system

2.1.6 Buffers, Solutions and Media

Table 9. List of utilized buffers, solutions and media

Buffer / Solution / Medium	Components
Mowiol	20 ml Glycerol, 40ml PBS, 10 g of Mowiol

10x PBS	1.4 mM NaCl, 27 mM KCl, 101 mM Na_2HPO_4, 18 mM KH_2PO_4 in ddH_2O
Ampicillin stock solution	100 mg/mL ampicillin sodium salt in ddH_2O
Coomassie staining solution	0.06% Coomassie Brilliant Blue G-250, 35 mM HCl in ddH_2O
Flow cytometry buffer	2% (v/v) FCS, 1% (w/v) NaN_3 in PBS
Freezing medium	10% DMSO (v/v) in FCS
LB-Amp agar	1.5% (w/v) agar in LB medium, 0.1% (v/v) ampicillin stock solution
LB-Amp medium	1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl in ddH_2O, 0.1% (v/v) ampicillin stock solution
MPBS	2-4% (w/v) milk powder in 1x PBS
PBST	0.05% (v/v) Tween 20 in 1x PBS
PEI transfection reagent	0.1% (w/v) PEI dissolved at pH 2.0 in ddH_2O, adjust pH to 7.0
Protein A binding buffer	100 mM NaH_2PO_4, 100 mM NaCl, 10 mM EDTA in ddH_2O, adjusted to pH 7.0, Filtered
Protein A elution buffer	0.1 M citric acid in ddH_2O, adjust to pH 3.0, Filtered
Protein A neutralization buffer	1 M Tris Pufferan in ddH_2O, adjust to pH 9.0, Filtered
Semi-dry blot buffer	48 mM Tris Pufferan, 39 mM glycine, 1.3 mM SDS, 20% (v/v) ethanol in ddH_2O, adjusted pH to 9.0-9.4, Filtered
Separating gel buffer	1 M Tris Pufferan in ddH_2O, adjust to pH 8.8, Filtered
SOC medium	2% (w/v) tryptone, 0.5% (w/v) yeast extract, 2.5 mM KCl, 10 mM NaCl, 20 mM $MgSO_4$, 20 mM glucose in ddH_2O
Stacking gel buffer	650 mM Tris Pufferan in ddH_2O, adjust to pH 6.8, Filtered
Supplemented DMEM medium	10% (v/v) Heat inactivated FCS, 1% (v/v) pen/strep in DMEM medium

Supplemented F17 medium (+ G418)	4 mM L-glutamine, 0.1% (w/v) Kolliphor P188, (+ 0.05% (v/v) Geneticin)
Supplemented RMPI-1640 medium	10% (v/v) Heat inactivated FCS, 1% (v/v) pen/strep in RPMI-1640 medium
TN1 feeding medium	20% (w/v) TN1 in supplemented F17 medium w/o G418
Transfection medium	Supplemented F17 medium without G418
Human IgG depleted serum	

2.1.7 Cell Lines

Table 10. List of used cell lines

Cell line	Organism	Description	Supplier (Cat. No.)
HEK293-6E	*Homo sapiens*	Suspension cell line established from embryonic kidney cells	National Research Council Canada
HEK293T	*Homo sapiens*	Adherent growing cell line established from embryonic kidney cells, expressing the SV40 large T antigen	American Type Culture Collection (CRL-3216)
Raji	*Homo sapiens*	Suspension cell line established from Burkitt's lymphoma B lymphocytes	American Type Culture Collection
Vero	*Cercopithecus aethiops*	Adherent cell line established from normal epithelial kidney cell	American Type Culture Collection
THP-1	*Homo sapiens*	Suspension cell line established from acute monocytic leukemia	American Type Culture Collection
Modified Jurkat	*Homo sapiens*	engineered Jurkat cells stably expressing the FcγRIIIa receptor, V158 (high affinity) variant, and an NFAT	ADCC Promega Kit

	response element driving expression of firefly luciferase	

2.1.8 Bacterial Strains, Viruses and Phage library

Table 11. List of used Bacterial strains, viruses and phage library

	Description	Supplier (Cat. No.)
LYNDAL library	Immune repertoire derived from lymph nodes of head and neck cancer patients, diversity approx. $5x10^9$	Internal, AG Krauss (NCT Heidelberg)
XL-1 Blue	Escherichia coli, Bacterial strain for transformation and DNA amplification	Stratagene (200228)
HSV-1/2	Herpes simplex virus 1F/2G	ATCC

Table 12. List of used vectors

Vector	Description
pConPlusIgG1za	contains the IgG1 constant region allotype G1m(za) (also known as G1m1,17), used for cloning heavy chain variable regions into VH-CH format (pConPlus GS Expression Vectors, Lonza)
pConPlusKappa vector	The pConPlusKappa vector contains the human kappa constant region allotype Km (3), which was previously known as Inv(3), used for cloning light chain variable regions into VL-CL format (pConPlus GS Expression Vectors, Lonza)
pConPlusLambda vector	The pConPlusLambda vector contains the human lambda constant region isotype 2, used for cloning light chain variable regions into VL-CL format (pConPlus GS Expression Vectors, Lonza)
pCSxW	Lentiviral self-inactivating (SIN) vector plasmid (also known as pHR'SEW). Lacks expression of HIV-1 proteins Gag, Pol, Env, Vpr, Vpu, Vif, Rev, Tat, Nef. Contains Rev-response element (RRE), central

	poly-purine tract (cPPT) and packaging signal Ψ. Internal SFFV promoter drives internal gene of interest (x) expression. Presence of woodchuck hepatitis post-transcriptional regulatory element (WPRE) to stabilize mRNA. Reference: PMID: 11895005
pCS-HSV-1gD-W	CSxW expressing gD from HSV-1 F
pCS-HSV-1gL-W	CSxW expressing gL from HSV-1 F
pCS-HSV-1gH-W	CSxW expressing gH from HSV-1 F
pCS-E2C-W	CSxW expressing far red fluorescent protein E2-Crimson
pCS-G-W	CSxW expressing enhanced GFP (also known as pHR'SEW)

2.1.9 Oligonucleotides

Table 13. List of used primers for cloning and sequencing

Name	Sequence 5'→3'	Description
MR0063	ATCAACTTCGACTGGCCCTT	qPCR (Quantification of HSV copy numbers)
MR0064	CCGTACATGTCGATGTTCAC	qPCR (Quantification of HSV copy numbers)
pConPlusG1mzaCH1-rev(800306)	TCAGGGCTCCGCTGTTCC	Sequencing heavy chain variable region in pConPlus vector
pConPlusKappa-rev(787832)	TCCTGCTCGGTGACGCTC	Sequencing of Light chain variable region in pConPlus vector
OTS2054-fw	ATCGGATCCACCATGCGCGGGGGGGGCTTGATTTGCGCGCTGGTC	Amplification of HSV-2 gB
OTS2055-rv	TGAGCGGCCGCTTAGAGCTCGTCTTCGTCTCCGGCCTCG	Amplification of HSV-2 gB
MR0005-fw	CCGGGACGACCACGAGACCG	Sequencing of HSV-1/2gB

MR0007-rv	CGCGTACTCCTCGAAGTACACG	Sequencing of HSV-1/2gB
MR0001-fw	ATGCGCCAGGGCGCCCCCGCG	Sequencing of HSV-1gB
MR0006-fw	CCGGGACGACCACGAGACCG	Sequencing of HSV-1/2gB
MR0016-rv	CCACCCGCGAGGGGT	Sequencing of HSV-1/2gB
OTS2054-fw	ATCGGATCCACCATGCGCGGGGGGGGCTTGATTTGCGCGCTGGTC	Amplifying HSV-2G gB from total DNA of infected Vero cells (afterwards, digestion with *BamHI-NotI* and cloning into pCSxW)
OTS2055-rv	TGAGCGGCCGCTTAGAGCTCGTCTTCGTCTCCGGCCTCG	Amplifying HSV-2G gB from total DNA of infected Vero cells (afterwards, digestion with *BamHI-NotI* and cloning into pCSxW)
RE_fwd_11	CTCACGACAAAGGCCCAGGCAACAGCTCCAACAACCC	Mutant gB(HSV-1) R335Q by SDM
RE_rev_11	GGGTTGTTGGAGCTGTTGCCTGGGCCTTTGTCGTGAGATCG	Mutant gB(HSV-1) R335Q by SDM

2.1.10 Services

Table 14. Services

Service provided by:	Description
Sequencing	Plasmids and corresponding primers were sent to MGT Eurofins Scientific for sequencing
DNA synthesize	Desired antibody variable chain genes with leader peptide and restriction sites were produced by Eurofins Scientific. Codon optimized HSV-1 gB synthesized by MWG Eurfins

Primer synthesize	Primers were purchased from Thermo Fisher Scientific

2.1.11 Antibodies and Antibody Conjugates

Table 15. List of used antibodies and antibody conjugates

Target structure, clone/clonality	Host	Target	Conjugate	Supplier (Cat. No.)
gB HSV (HDIT101), monoclonal	Human	Viral		Heidelberg Immunotherapeutics GmbH, NCT Heidelberg
Hen egg lysozyme, monoclonal (isotype control)	Human	Chicken		CrownBio (C0001)
gB HSV-1/2 (H126), monoclonal	Mouse	Viral		Virusys (p1122)
gB HSV-1 (H1817), monoclonal	Mouse	Viral		Virusys (p1105)
CD22 (hrfb4); monoclonal	Human	Human		NCT Heidelberg
Fcγ IgG, polyclonal	Goat	Human	PE	eBioscience (12-4998-82)
Fcγ IgG, polyclonal	Rabbit	Human	FITC	Jackson ImmunoResearch (309-096-008)
Fcγ IgG, polyclonal	Rabbit	human	HRP	Jackson ImmunoResearch (309-035-008)
Fcγ IgG, polyclonal	Goat	Mouse	HRP	Jackson ImmunoResearch (115-035-008)
gD HSV, (A18S) monoclonal	Mouse	Human	FITC	MA5-18211

gB HSV, (H4) monoclonal, protein A chromatography purified	Human	Viral		Narges Seyfizadeh NCT Heidelberg
gB HSV, (H28) monoclonal, protein A chromatography purified	Human	Viral		Narges Seyfizadeh NCT Heidelberg
HDIT101-FITC	Human	Viral	FITC	Labeled by BioRad
gH HSV-1 (H6), monoclonal	Mouse	Viral		Virusys (H1A258-100)
gD HSV-1/2 (2C10), monoclonal	Mouse	Viral		Virusys (HA025-1)
gL HSV-1 (L4), monoclonal	Mouse	viral		Virusys (H1A259-100)
HDIT101 LoA	Human	Viral		AG Arndt/Krauss
HDIT101 LoAEM	Human	Viral		AG Arndt/Krauss
HDIT101 LoNEM	Human	Viral		AG Arndt/Krauss
Mab2C	Mouse	Viral		AG Arndt/Krauss
CMV, CMV-Gyn (Cytotect 70), polyclonal	Human	Viral		Biotest (BT-084)

2.1.12 Enzymes and Proteins

Table 16. List of used enzymes

Enzymes / Proteins	Supplier (Cat. No.)
***Apa*I**	New England Biolabs (R0114S)
***Avr*II**	New England Biolabs (R0174S)
***Bam*HI-HF**	New England Biolabs (R3136S)
***Bsi*WI-HF**	New England Biolabs (R3553L)

***Eco*RI-HF**	New England Biolabs (R3101S)
***Hind*III-HF**	New England Biolabs (R3104L)
Kapa HF Polymerase	Roche (KK2101)
***NotI*-HF**	New England Biolabs (R3189S)
Pfu DNA Polymerase	Thermo Scientific (P0502)
Proteinase K	QIAGEN
***Pvu*I-HF**	New England Biolabs (R3150S)
T4 DNA Ligase	Thermo Scientific (EL0016)
***Xba*I**	New England Biolabs (R0145S)
***Xho*I**	New England Biolabs (R0146S)

2.1.13 Mouse Strains

Table 17. List of used mouse strains

Description	Supplier (Cat. No.)
BALB/cOlaHsd	Envigo RMS GmbH
NOD.CB17-Prkdcscid/NCrHsd	Envigo RMS GmbH

2.1.14 Software and Online Tools

Table 18. List of applied software and online tools.

Software / Online tool	Description	Supplier/Homepage
AxioVision 4.5	Microscopic imaging	Carl ZEISS

BD FACS Diva	Acquisition and analysis of flow cytometric data	BD Biosciences
BD FACS Suite	Acquisition and analysis of flow cytometric data	BD Biosciences
Chemo Star Imager 3.14	Fluorescence and ECL visualization	Intas Science Imaging
EndNote X9	Reference manager	Clarivate Analytics
Epson Scan	Image scan and digitalization	Epson
ExPASy ProtParam	Computation of physical and chemical parameters of amino acid sequences	http://web.expasy.org/protparam/
FlowJo 10.3	Flow cytometric data evaluation	Tree Star
FlowJo 9	Flow cytometric data evaluation	Tree Star
Geneious 10.1	Cloning manager	Biomatters
Graph Pad Prism 7.0	Statistical analysis and graph illustrator	GraphPad Software
IMGT/V-Quest	Database containing human antibody gene sequences	http://www.imgt.org/IMGT_vquest/vquest
Intas GDS 3.3.9	DNA visualization and documentation	Intas Science Imaging
Microsoft Office 2010	Data evaluation, illustration and text processing	Microsoft
NanoDrop 1000 3.8.1	Spectrophotometer control	Thermo Fisher Scientific
Photoshop Elements 10	Image editing	Adobe Systems
Tecan i-control 3.7.3	Absorbance/fluorescence reader control	Tecan Austria
Unicorn 5.10	FPLC control	GE Healthcare
Unicorn 6.3	FPLC control	GE Healthcare

VBASE2	Database containing human antibody gene sequences	http://www.vbase2.org/vbquery.php
ZEN 2012	Microscopic imaging	Carl ZEISS
PyMol	an open source molecular visualization system	DeLano Scientific LLC

2.2 Methods

2.2.1 Molecular, biological and microbiological methods

2.2.1.1 IgG Cloning- summary

To select human Herpes Simplex Virus Type 1 (HSV-1) specific single chain fragment variables (scFvs), a Phage Display Library, the Human Lymph Node Derived Antibody Libraries (LYNDAL), has screened against the recombinant expressed ectodomain of glycoprotein B from HSV-1 (strain KOS). According to the binding profile and HSV neutralization capacity, the two most promising candidates (H4, H28) were chosen for further investigation and downstream development (Diebolder *et al.*, 2014).

The Variable Heavy chain (VH) and Variable Light chain (VL) genes of the selected scFvs were synthesized as codon optimized cDNA by and suitable restriction sites for cloning into pConPlus vectors encoding IgG1 heavy/light-chain constant regions. A Kozak consensus sequence as a translation initiator (Kozak, 1987) and an Azurocidin signal peptide as one of the best secretion signal peptides were implemented in the variable region gene sequences(Olczak and Olczak, 2006). First, pConPlus IgG1 vectors were amplified by transforming chemically competent E. coli XL-1 blue. Then, the synthesized VH gene was cloned into pConPlus IgG1 containing (heavy-chain constant region gene). For this purpose, both insert and vector were cut by *Hind* III-HF and *ApaI* and the digested inserts and vectors were assembled by ligation.

Similarly, amplified VL cDNAs were cloned into pConPlus Lambda/Kappa encoding light-chain constant region, in brief; both insert and vector were digested by *Hind*III-HF (NEB) and *AvrII*/ *BsiwI*, purified through agarose gel electrophoresis and gel purified inserts and vectors were

assembled by ligation. Afterwards, pConPlus Lambda/Kappa and pConPlus IgG1 containing inserts were cut by *Not* I (NEB) and *PvuI*-HF and ligated to make a double gene vector expressing full human IgG1. The double gene vector was amplified by transforming chemically competent E. coli XL-1 blue. For this purpose, several colonies were screened by colony PCR to check the integrity of variable fragments and then the correct colony was amplified and afterwards, purified by endo free Maxi kit (QIAGEN).

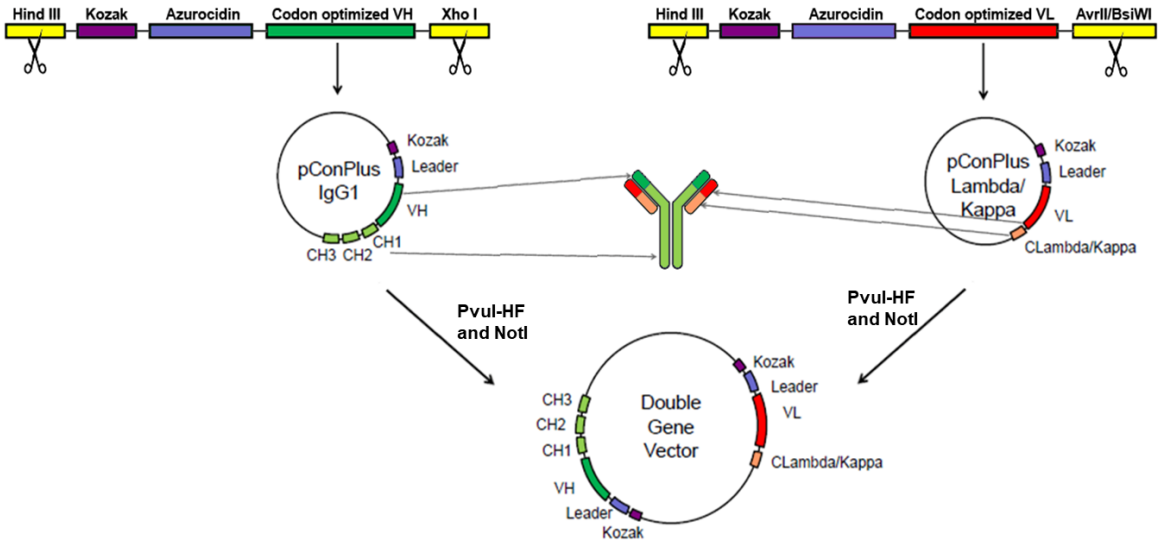


Figure 7. Schematic representation of cloning strategy (variable fragments to full IgG).

2.2.1.2 LYNDAL antibody fragment selection and codon optimized gene synthesize

LYNDAL is a combinatorial antibody library which was build based on the phage display technology from B cell repertoires of patients with squamous cell head and neck carcinoma. To generate gB-specific scFvs, recombinant ectodomain of HSV-1 gB (KOS) was chosen for panning the LYNDAL(Diebolder *et al.*, 2014).

The binding specificity of scFvs to HSV-1/2 gB was measured with a flow cytometry-based assay on infected Vero cells expressing cell surface HSV-1/2 gB. To assess the possible antiviral potency of the selected scFvs, a standard plaque neutralization assay was employed (scFvs cross-linked with myc-tag-specific IgG were representing a bivalent construct). According to their binding specificity and their ability to neutralize viral spreading, LYNDAL- derived scFvs H4 and H28 were chosen and subjected to full IgG cloning. The original variable gene sequences (VH, VL

Kappa and VL Lambda) were codon optimized for Homo Sapiens and synthesized by Eurofins Scientific, along with a 5' KOZAK consensus sequence followed by a DNA sequence encoding Azurocidin signal peptide and 5' and 3' terminal restriction sites (*HindIII, AvrII, Bswi* and *ApaI*) for cloning into pConPlus vectors containing constant IgG cDNA were synthesized by MWG Eurofins Scientific.

2.2.1.3 Transformation of *E. coli* for plasmid DNA amplification

For transformation by heat shock, frozen chemically competent *E. coli* XL-1 Blue (100 µL) (Stratagene) were thawed on ice, then 10 pg - 100 ng of plasmid DNA or the ligation reaction were mixed with the bacterial suspension and incubated in a 1.5 mL Eppendorf tube on blue ice for 30 minutes. Afterwards, the bacteria suspension was heat shocked at 42 °C for 45 seconds and immediately taken up into 1 mL prewarmed SOC medium and incubated for 1 hour in a shaking incubator at 37 °C, 180 rpm. Thereafter, the bacterial suspension was plated on prewarmed, ampicillin supplemented LB Agar plates and incubated overnight in a bacterial incubator at 37 °C.

2.2.1.4 Plasmid DNA isolation

The plasmid DNA was isolated either for analytical purposes (restrictive digests, sequencing) or on a preparative scale. For analytical purpose, amplification was carried out by culturing the transformed bacterial colony in 5 ml of Ampicillin supplemented LB medium (0.1mg/mL Ampicillin) in a shaking incubator at 37 °C, 180 rpm overnight. Isolation and purification of amplified plasmid DNA was carried out with a Mini preparation kit (QIAGEN) according to the manufacturer's instructions. To isolate the plasmid DNA on a preparative scale, 100 ml (Midi) or 250 ml (Maxi) bacterial cultures were processed using the Midi or endotoxin free Maxi preparation kit (QIAGEN) according to the manufacturer's protocol and the DNA was stored at -20 °C.

2.2.1.5 DNA digest with restriction enzymes

PCR products/synthesized DNA fragments (insert) and vectors were digested with restriction endonucleases according to Table 19 at the optimal enzyme working temperature (usually 37 °C but depending on enzymes it might differ) for 1-2 h. At half time incubation, 1 µL calf intestinal alkaline phosphatase was added to the vector digestion reaction to prevent religation. Digested DNA was purified as described in section 2.2.1.6.

Table 19. DNA digestion reaction mix.

Component	Final amount/ concentration	Volume (µL)
DNA (PCR product / vector)	up to 5000 ng	X
Digestion buffer (Cut Smart Buffer (5x))	1x	10
Enzyme I	10 U	1
Enzyme II	10 U	1
ddH_2O		50
∑		50

2.2.1.6 Gel electrophoresis, DNA extraction and purification

Digested DNA products were separated from residual DNA by preparative agarose gel electrophoresis. 1.5% agarose gels were prepared by adding 1.5 g agarose to 1x TAE buffer, boiling until agarose was completely dissolved and supplementing with peqGREEN (1:20000, peqLab) which intercalates in DNA and is UV excitable. DNA loading dye was added to the samples and the mix was loaded onto the agarose gel in parallel with a DNA marker (Thermo Fisher Scientific). Gel electrophoresis was performed at 75 volts for 1-2 h in 1x TAE running buffer. Afterwards, DNA bands were visualized with UV light in a INTAS Science Gel Imaging System and target DNA bands were excised with a scalpel. Corresponding DNA was extracted using QIAquick gel extraction kit (QIAGEN) according to the manufacturer's instructions and the concentration was determined by photometry.

2.2.1.7 DNA fragment ligation

Digested inserts and vectors were assembled by ligation process for 1 hour at RT according Table 20. DNA ligation reaction mixTable 20. A religation control was included in the absence of insert.

Table 20. DNA ligation reaction mix

Component	Final amount/ concentration	Volume (µL)
Digested vector	10-100 ng	X
Digested insert	>3-fold molar excess	Y
T4 Ligase Buffer (10x)	1x	2
T4 Ligase (1 U/ µL)	1 U	1
ddH_2O		20
∑		20

2.2.1.8 Colony PCR screening

To analyse variable fragments integrity in the double gene vector, colony polymerase chain reaction (PCR) was performed. Several plasmid transformed colonies were picked from LB agar plates and were transferred to 25 µL PCR reaction mix Table 21 and subjected to PCR Table 22. PCR products were analysed by agarose (1.5%) gel electrophoresis as described 2.2.1.6.

Table 21. Colony PCR reaction mix

Component	Volume (µL)
Template DNA	Single colony
2x KAPA2G Fast Ready Mix with Dye	12.5
NB_fw (25 µM)	0.5
NB_rv (25 µM)	0.5
ddH_2O	11.5
∑	25

Table 22. Colony PCR conditions.

Step	Temperature [°C]	Time [s]	Cycles
Initial denaturation	95	300	1
Denaturation	95	15	35
Annealing	49	15	35
Elongation	72	15	35
Final elongation	72	300	1
Cooling	4	∞	1

2.1.1.1 Sequencing

To confirm the variable genes' sequence, purified DNA from correct colonies were sent to MWG Eurofins scientific for Sanger sequencing by using primers shown in Table 23 .

Table 23. Employed primers for sequencing scFvs inserted into pConPlus vectors.

Name	Sequence 5'→3'	Description
pConPluslambda-rev(2166184)	TCCTGCCCAATTAACGCT	Sequencing of light chain variable region in pConPlus vector
pConPlusG1mzaCH1-rev(800306)	TCAGGGCTCCGCTGTTCC	Sequencing heavy chain variable region in pConPlus vector
pConPlusKappa-rev(787832)	TCCTGCTCGGTGACGCTC	Sequencing of Light chain variable region in pConPlus vector

2.2.2 Cell biological and Protein biochemical methods

Cell culture was done under sterile conditions at 37 °C, 5% CO_2 and cells were counted by 1:1 mixing with trypan blue and using a Neubauer counting chamber.

2.2.2.1 Cell freezing and thawing

For preparing cryostocks of cells and cell lines, $1x10^7$ cells were pellet by centrifugation for 10 min at 300 *g*, washed one time with cell culture medium and resuspended in freezing medium (10% DMSO in heat inactivated FCS) and transferred to 1 mL cryovials and frozen at -80 °C using a freezing container. For long-term storage, cryovials were transferred to liquid nitrogen. Cryo-conserved vials containing cells were thawed gradually in a water bath at 37 °C and transferred to a 50 mL tube containing 10 mL of prewarmed cell culture medium. The volume was increased to 20 mL and transferred to T75 cell culture flasks. HEK293-6E were handled in a different way, after thawing the cryovial, cells were transferred to a 125 mL shaking flask with filter caps containing 16 mL prewarmed F17 medium without antibiotics and incubated in a shaker incubator at 37 °C, 5% CO_2, 135 rpm. After overnight incubation the medium was adjusted to 30 mL with G418 supplemented F17 medium.

2.2.2.2 Cultivation of adherent cell lines

The adherent cell lines Vero and HEK293-T were cultured using DMEM supplemented with 10% FCS and 1% penicillin/streptomycin. Cells were passaged by discarding the media, rinsing the cells once with PBS, adding 0.2% Trypsin/EDTA and incubating at 37 °C until cells were detached from the flask. Cells were seeded at the required cell number in a new cell culture flask/plate with fresh medium. Passaging occurred every two to four days.

2.2.2.3 Cultivation of suspension cell lines

The suspension cell lines THP-1 and Raji were cultivated using RPMI-1640 medium supplemented with 10% FCS and 1% penicillin/streptomycin. Cells were passaged by simply transferring the required cell number to a new cell culture flask/plate with fresh medium. Passaging occurred every two to four days. HEK293-6E cells were cultured using F17 medium supplemented with 2% L-glutamine, 0.1% Kolliphor and 0.05% G418. Cells were seeded at density of $0.5x10^6$/mL and were cultivated in a shaking incubator at 37 °C, 5% CO_2, 135 rpm and were expanded every two to three days.

2.2.2.4 Antibody production and preparation

2.2.2.4.1 Transient transfection of HEK293-6E cells to produce IgG

To produce anti HSV gB IgG1 antibodies, HEK293-6E cells were transiently transfected with the double gene vector encoding constant and variable region of heavy and light chain. In brief: at the day of transfection, HEK293-6E cells were at density of 1.5 – 2.0x10^6/mL in 144 mL culture volume. 1 µg per mL of cell culture volume of eukaryotic expression vector and 2 µg per mL of cell culture volume of PEI were added to two separate 50 mL tubes containing prewarmed F17 medium without G418 (1/20 of the final culture volume). The tubes were vortexed thoroughly, the PEI solution was added to the DNA and mixed by vortexing and were incubate 3 minutes at RT. Afterwards, the transfection mix was added to the HEK293-6E culture. Cells were incubated for 24 hours and then fed with TN1 feeding medium (1/40 of the cell culture volume). Protein production were monitored for 72-96 hours with counting the living cells and calculating viability (cells were harvested when the viability dropped below 70-80%). The cell culture was harvested by two-step centrifugation for 10 min at 250 *g* and 2250 *g*, respectively. The supernatant was dialyzed against protein A binding buffer as described in section 522.2.2.4.2.

2.2.2.4.2 Dialysis of harvested supernatant

The production (cell culture supernatant) was dialyzed against 5 L (>30x volume excess) protein A binding buffer in a 12 kDa dialysis membrane overnight at 4 °C under gentle stirring. This allowed buffer exchange and exclusion of proteins with a molecular size lower than 12 kDa until equilibrium was reached. Dialyzed supernatant was sterile filtered using a 0.22 µm low protein binding filter and stored at 4 °C until purification.

2.2.2.4.3 Protein A affinity chromatography

IgG1 protein purification was carried out by protein A affinity chromatography using the AEKTApure fast protein liquid chromatography (FPLC) system. The dialyzed and sterile filtered supernatant (IgG1) was loaded onto the 1 mL protein A column (which was washed with water and was equilibrated with protein A binding buffer), then bound IgG1 was eluted with protein A elution buffer as 1 mL main fraction. The flow rate was 1 mL/min for all steps the collecting tubes contained protein A neutralization buffer. The main fraction was dialyzed against 500 mL PBS

and sterile filtered with 0.22 µm low protein binding filters. Protein concentration was measured by spectrometry at 280 nm (the extinction coefficient was determined by the ExPASy ProtParam online tool).

2.2.2.4.4 Analytical size exclusion chromatography

An analytical size exclusion chromatography (SEC) was performed on the AEKTA FPLC system to analyse the possible aggregates of produced IgGs. A Superdex 200 Increase 10/300 GL column (20 mL column volume) was washed and used for this purpose. The column was washed first with 40 mL water and then with PBS (0.3-1mL/min). A volume of protein A eluted and dialyzed suspension corresponding to 30-50 µg of produced IgG were loaded onto the column by injection through a 100 µL loop and the sample passed the column at a flow rate of 0.5 mL/min. Protein elution was monitored at 280 nm. The elution time was compared to standard runs with proteins of known molecular size.

2.2.2.4.5 Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Purified IgGs were separated according to molecular weight by reducing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE). A 12% acrylamide separating gel and a 5% acrylamide stacking gel were prepared as described in Table 24Table 1. The separating and the stacking gel were poured one after the other into a 1.5 mm gel cassette. After the gel polymerized, 2 µg protein was added to 4 µL 5x Laemmli buffer, 5 µL 1 M DTT and complemented with ddH_2O to a final volume of 20 µL. Samples were denaturated at 90 °C for 3 min and loaded into SDS gel wells. Together with a protein standard containing proteins of known molecular weight, the gel was run at 150 V for 2 h.

Table 24. Components for 12% acrylamide separating and 5% acrylamide stacking gel

Component	Separating gel (12%)	Stacking gel (5%)
Rotiphorese A	3110 µL	490 µL
Rotiphorese B	1300 µL	200 µL
Separating gel buffer	1920 µL	-

Stacking gel buffer	-	750 µL
ddH_2O	1520 µL	1500 µL
10% SDS	80 µL	30 µL
TEMED	6.5 µL	2.2 µL
10% APS	64 µL	25 µL
∑	8000 µL	3000 µL

2.2.2.4.6 Coomassie Blue staining

Proteins on the SDS gel were visualized with Coomassie Blue staining solution by incubation for at least 2 h on a shaker at RT followed by overnight destaining with water under the same conditions. Stained gels were scanned for documentation.

2.2.2.4.7 Western blot

To visualize specific proteins separated on an SDS gel, proteins were first transferred to a nitrocellulose membrane (western blot) and then immunostained with protein-specific antibodies. After gel electrophoresis, the SDS gel, a nitrocellulose membrane and filter blot papers were equilibrated with semi-dry blot buffer for 5 min on a shaker. Filter blot papers, nitrocellulose membrane and SDS gel were stacked on a blot transfer chamber. The transfer was carried out at 20 volts for 30 min and the membrane was subsequently blocked with 2% MPBS at RT for 60 min on a shaker. A specific HRP coupled antibody (human IgG Fc specific) was appropriately diluted in 2% MPBS and incubated with the membrane at RT for 30 min or at 4 °C overnight under shaking. The membrane was washed three times with PBST and once with PBS for 5 min while shaking. ECL substrate reagents were mixed in equal volumes and incubated with the membrane for 1 min in the dark. Target protein bands were visualized with an ECL imager at an exposure time of 1-10 min.

2.2.2.5 HSV large scale production

HSV stocks were produced by infecting Vero cells. For this purpose, Vero cells were cultured with complete DMEM until reaching 100% confluency(T175). Subsequently, the culture medium was removed and after one wash with PBS infected with HSV-1F/HSV-2G at MOI of 0.01 in DMEM without FCS for 2h at 37°C. Subsequently 30 ml medium with 10% heat inactivated FCS was added and the infected cells were incubated 2-3 days to observe maximal cytopathic effect (CPE), indicating viral replication. Afterwards, the contents were subjected to three freeze-thaw cycles (-80 °C, room temperature (RT)), to lyse the cells and release virus into the supernatant. Cell debris was removed by centrifugation (15 min, 300 g) and the virus-containing supernatant filtered using a 0.45 µm filter, followed by ultracentrifugation at 20k rpm speed for 1 hour. The virus pellet (from nine T175 flasks (270ml)) was resuspended in1ml PBS, aliquoted and stored at -80 °C.

2.2.2.6 Determination of the virus titre by endpoint titration (TCID50)

The titre of HSV stocks was determined by TCID50 assay (tissue culture infectious dose 50%). For this purpose, 1×10^5 Vero cells per mL were plated in a 96-well plate. The following day, a dilution series of virus (each step 1:10 dilution), starting with 1:1000 dilution was prepared and 100 µL pipetted onto the cells (the supernatant was already removed), infecting one row of a 96-well plate with one dilution. After 2 h incubation at 37°C, virus inoculum was removed and replaced with fresh DMEM supplemented with 10% heat inactivated FCS and the plates were incubated at 37°C. After 3 days, the plates were examined under the light microscope for positive cytopathic effect. The calculation was made using the following formula:

Calculation: (% of wells infected at dilution above 50%)-50%

(% of wells infected at dilution above 50%-% of wells infected at dilution below)

50% endpoint titre=10^log total dilution above 50%-(Ixlog h)

I= interpolated value of the 50% endpoint

TCID50/ML*0:7=PFU/ML

2.2.2.7 Flow Cytometric Determination of Antibody affinity

The affinity of an antibody is a measure of the strength of the interaction between an antibody and its antigen which is described by the equilibrium dissociation constant (KD) (Reverberi and Reverberi, 2007). The binding properties between the test antibodies and infected Vero cells expressing the HSV-1 gB antigen, was characterized by a flow cytometry-based assay. In short, Vero cells (~ 80% confluent) were infected with a MOI of 1. After an incubation period of 16-20 hours, the virus-containing medium was discarded, and the cells were washed once with PBS and then resuspended in PBS and harvested. Afterwards, the cells were pelleted at 300 × g for 5 min and resuspended in FACS buffer at a cell density of 5 x 10^6 cells / ml. The suspension was evenly distributed in a volume of 100 µl / well on a 96-well plate.

To determine the Equilibrium constant, the HSV infected Vero cells were incubated in triplets with a 1: 2 dilution series (0.03-500 nM) of the unconjugated primary antibody for 45 min at RT. After two times washing with FACS buffer, a FITC-conjugated detection antibody was added, targeting the Fc domain of the primary antibody. After an incubation period of 30 min cells were washed twice and taken up in 500 µl FACS buffer. Following this, the mean fluorescence intensity (MFI) was determined for each sample by flow cytometry. As a negative control for staining, infected cells were incubated with only secondary antibodies. As a negative control for specificity, uninfected Vero cells were stained in the same way. The calculation of the equilibrium dissociation constant (half maximal binding or half maximal saturation of antibody) was carried out under application of the one site specific binding method for nonlinear regression with Graph Pad Prism. The calculation principal follows as: $Y=Bmax \times X/ (Kd+X)$, based on a curve which is known as a rectangular hyperbola, binding isotherm, or saturation binding curve. Y is zero initially and increases to a maximum plateau value Bmax. The equation describes the equilibrium binding of a ligand to a receptor as a function of increasing ligand concentration. X is the concentration of the ligand, Y is the specific binding, Bmax is the maximum number of binding sites, expressed in the same units as the Y-axis. Kd is the equilibrium dissociation constant, expressed in the same units as the X-axis (concentration). When the drug concentration equals Kd, half the binding sites are occupied at equilibrium.

2.2.2.8 TCID50 based Neutralization assay

To examine the antiviral activity of the test anti-HSV antibodies, different antibody dilutions were incubated with a constant viral load (100 TCID50 HSV-1 /HSV-2) for 1 h at 37 °C. The antibody-virus mixtures were applied to 80-90% confluent Vero cells in 96-well plates (2×10^4 cells per well) in a volume of 100 µl per well. As a control, Vero cells were infected with a viral dose of 100 TCID50 without prior incubation with neutralizing antibodies. The extent of the cytopathic effect was examined by light microscopy three days after infection. The neutralization titre was determined to be the highest antibody dilution at which the virus was completely neutralized and the formation of a CPE in the inoculated cell cultures was completely prevented. In addition, the neutralization titre at which 50% of the cell culture wells were protected from infection could be calculated as follows (Krawczyk *et al.*, 2011b):

$$T = x + (b/10) \cdot x$$

T = neutralization titre at which 50% of the infected cell cultures are protected from infection.

x = Lowest antibody dilution at least 50% of cell cultures are infected

b = number of infected cell cultures exceeding the 50% infection rate at dilution x

2.2.2.9 Cell to cell spread blocking assay

Herpes viruses, like other enveloped viruses, are able to spread directly from an infected cell to an adjacent uninfected cell ("cell-to-cell spread"). The potential for H4 and H28 to inhibit the "cell-to-cell spread" of HSV-1 in cultivated Vero cells was investigated by immune-fluorescence microscopy. For this purpose, initially 2×10^5 Vero cells were seeded in each well of a 4-well chamber slide. The following day, the culture medium was discarded, and the cells were inoculated with a viral load of 200 TCID50 in a volume of 500 µl DMEM per well. Four hours after infection, the supernatant containing virus was discarded, unbound virus particles were removed by washing one time with 500 µl PBS, and the cells were incubated with 500 µl culture medium containing 10% FCS and 500nM of neutralizing antibodies. As a control for inhibition of cell to cell spread, human polyclonal anti HSV antibodies (Enzygnost) was used with a dilution of 1:20 in culture

medium. After two days of incubation, the medium was discarded. The cells were washed once with PBS and then fixed for 30 seconds with 5% paraformaldehyde solution (longer fixation led to decreased antibody binding to gB and losing signal) and washed again with PBS. Subsequently, HSV-infected cells were stained using HDIT101-FITC, a FITC-conjugated anti-gB antibody. After 1hour, the supernatant was discarded, and cells were washed with PBS remove excess antibodies. Afterwards, the cells were subjected to staining with Hoechst, DNA staining, for 15 minutes and then fixed with 5% paraformaldehyde for 15 minutes. The evaluation was carried out by fluorescence microscopy using the confocal Leica DM IRE2 microscope at a magnification of 200X (see Figure 8 for workflow).

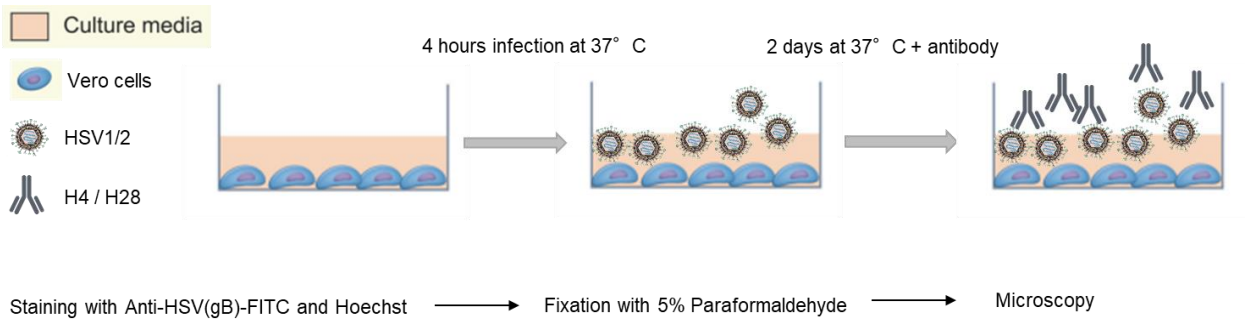


Figure 8. Schematic representation of cell to cell spread blocking assay.

2.2.2.10 Antibody-dependent cell cytotoxic (ADCC) assay

ADCC is a defence mechanism of the immune system whereby immune effector cells lyse the target cells via an antibody-dependent process. In this assay, antibodies bind to gB that is expressed on the surface of infected Vero cells and the Fc effector portion of gB-bound antibodies binds to Fcγ receptors on the cell surface of effector cells (engineered Jurkat cells stably expressing the FcγRIIIa receptor, V158 (high affinity) variant, and an NFAT response element driving expression of firefly luciferase (Promega, Figure 9) after antibody binding to FcR occurs. Subsequently, the antibodies' biological activity in activating ADCC can be quantified by measuring the luciferase produced as a result of FcγRIIIa receptor binding and NFAT activation.

In brief; Vero cells were plated and a day after were infected with MOI of 1(HSV-1F/2G). 20 hours after infection, infected Vero cells were harvested and distributed in white flat bottom 96 well plates (1.25×10^4 cells per well) and incubated 6 hours together with effector cells (The

effector: Target ratio was 6:1) and a serial dilution of test antibodies. Uninfected Vero cells were included as a negative control. Raji cells incubated with Rituximab served as positive control for the assay. Subsequently luciferase substrate was added to the plates and incubated 15 minutes. Afterwards the luminescence intensity was measured using a plate reader. Triplicate reads were performed. Plate Background was determined by calculating the average RLU (relative light unit) from control wells (empty). Fold of ADCC induction was calculated accordingly: Fold of ADCC induction = RLU (induced–background) /RLU (no antibody control–background) and data was graphed as Fold of ADCC induction versus Log10 [antibody].

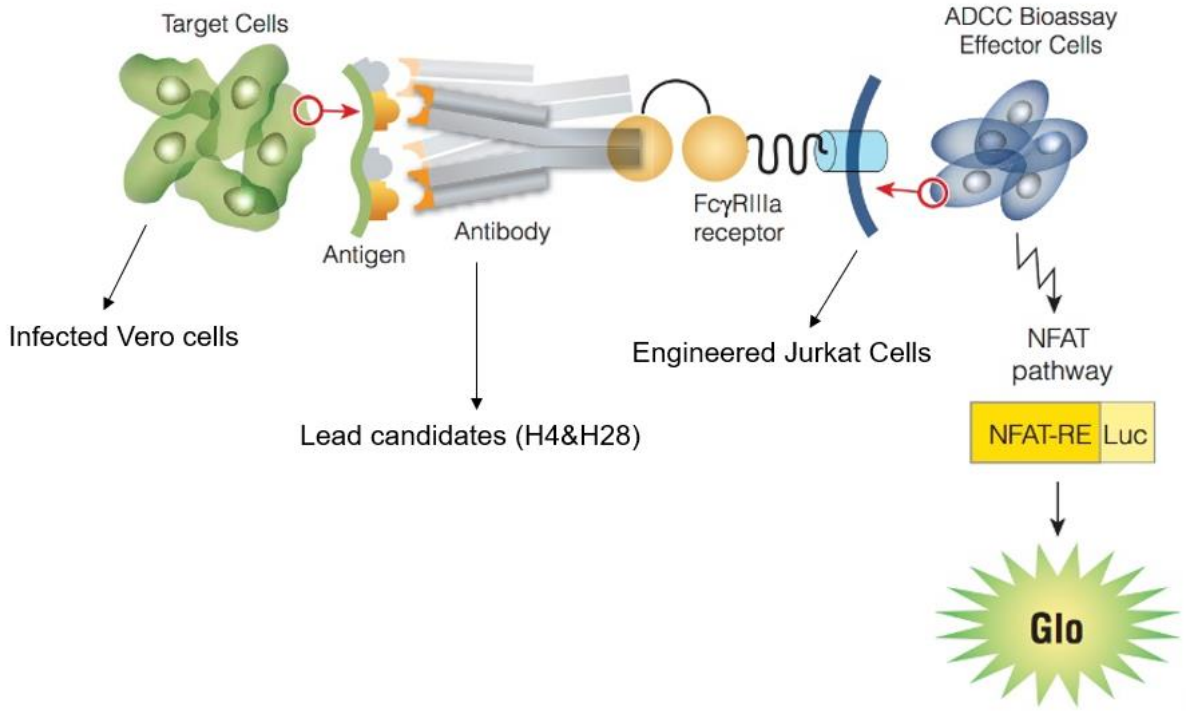


Figure 9. Schematic representative of ADCC assay (Promega ADCC assay guideline, modified)

2.2.2.11 Antibody-dependent cellular phagocytosis (ADCP) Assay

Antibody-dependent cellular phagocytosis (ADCP) is a mechanism by which professional phagocytes, such as macrophages and neutrophils contribute to antitumor or antimicrobial (e.g. antiviral) potency of monoclonal antibodies via the engagement of Fcγ receptors by antibody-

opsonized material. Here, a microscopy-based assay is described to define the phagocytic activity of gB-specific monoclonal antibodies. The assay was optimized with undifferentiated THP-1 cells, a human monocytic cell line derived from an acute monocytic leukemia patient. For this purpose, 100 TCID50 of HSV-1/2 were prepared in RPMI 10% IgG depleted human serum and mixed with 500 nM of test antibodies (which was also prepared in RPMI 10% IgG depleted human serum). The antibody and virus mixtures were incubated 1 hour at 37 °C. Afterwards 10^6 THP-1 cells per sample were washed with PBS and incubated with a mix of virus and antibody at 37 °C for 18 hours (total volume of mixture 500 µl, 250 µl of virus with 250 µl). After 18 hours of incubation, THP-1 cells were harvested into 1.5 ml tubes and centrifuged 5 minutes at 300×g at RT and subsequently washed once with PBS.

THP-1 cells were resuspended in 200 µl of fixation buffer (Perm/Fix Kit, BD) and incubated 30 minutes at RT. Then, fixed THP-1 cells were washed twice with 200 µl of 1X permeabilization buffer (Perm/Fix Kit, BD) and were resuspended in 200 µl of 1X permeabilization buffer (containing conjugated antibody or primary antibody) and incubated for 45 minutes at RT and protected from light. Afterwards, the samples were pelleted by centrifugation as above and washed once with 1X permeabilization buffer and stained with Hoechst (diluted in 1X permeabilization buffer) for 3minutes at RT. Samples were washed with PBS and resuspended in 20 µl of water. Samples were mounted with a drop of Mowiol on a microscopy glass slides. 24 hours later microscopic analysis was performed (see Figure 10 for ADCP assay layout and staining strategies). All centrifugation steps were carried out at RT and 300×g, for 5 minutes. For detecting virus which underwent phagocytosis, HDIT101 or H4 were conjugated with AlexaFluor 488 (by using Alexa Fluor 488 Antibody Labeling Kit) and for detecting antibodies, which were phagocytosed, anti-Fc antibodies were used. An isotype control antibody was also used in parallel.

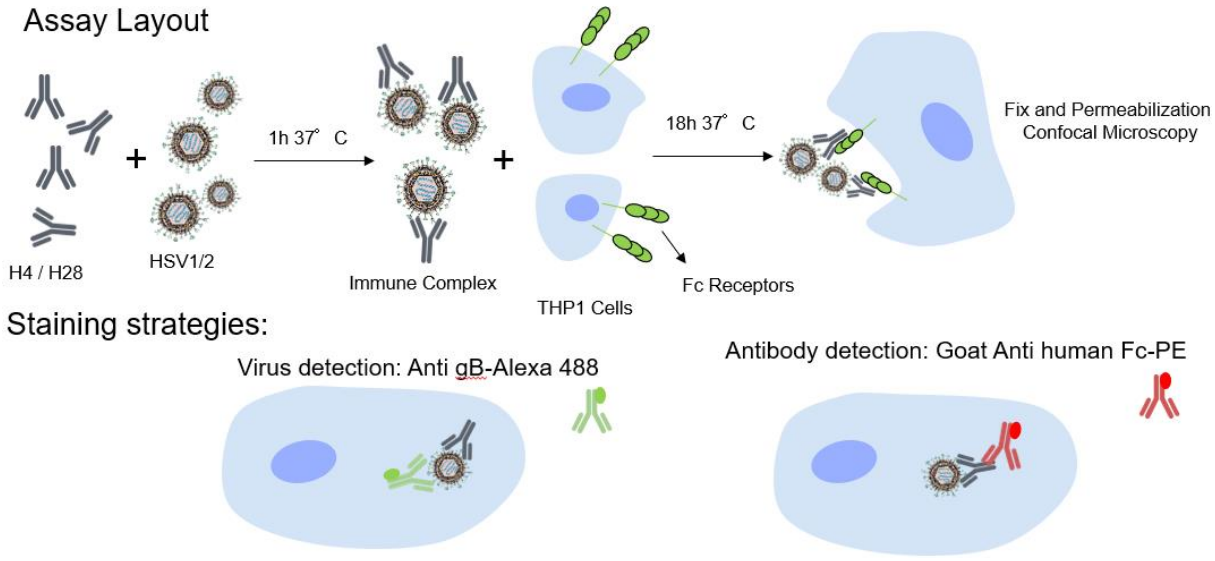


Figure 10. ADCP assay layout and staining strategies.

2.2.2.12 Complement-dependent neutralization effect of antibody

To clarify the question, whether antibody neutralization potency of the gB specific antibodies are dependent to complement or not, a TCID50 based neutralization assay was performed (see 2.2.2.8). In this assay, DMEM was supplemented with 10% heat- inactivated or not heat-inactivated IgG-depleted human serum as complement source. The human polyclonal IgG preparation targeting human cytomegalovirus (Cytotect) was used as a positive control. The gB specific antibodies' neutralization potency were compared in the presence of complement and heat inactivated complement.

2.2.2.13 Antibody-dependent complement-mediated cytotoxicity (CMC)

To measure and compare complement mediated cytolysis in the presence and absence of neutralizing antibodies, Vero cells were infected with HSV-1F/2G at an MOI of 1 for 20 hours in a T75 flask (containing 4×10^5/ml) (see). After the incubation time the infected Vero cells were harvested and washed once with PBS. Afterwards, 75µg of neutralizing antibodies were prepared in DMEM (RPMI) 20% heat-inactivated or not heat-inactivated human IgG-depleted serum and were added to 5×10^5 of infected Vero cells and incubation was carried out for 4 hours at 37 °C. Then, infected Vero cells were washed with PBS once and stained with SYTOX Blue (Dead Cell Stain) for 15 Minutes at RT to discriminate dead and viable cells. The extent of lysis was measured

by flow cytometry analysis of SYTOX stained cells. CMV-Gyn (Biotest) (human polyclonal anti CMV antibodies), Rituximab/Raji were used respectively as antibody positive control, negative control and an additional positive control for the experiment.

2.2.2.14 Quantifying complement activity by measuring terminal complement complex C5b-9 (TCC C5b-9)

For the quantitative determination of complement activity, human terminal complement complex C5b-9 (TCC C5b-9) concentration was measured by following the protocol of a commercial kit (Human terminal complement complex C5b-9 ELISA, Blue Gene for life science). The assay is based on a sandwich enzyme immunoassay technique. In brief: antibodies specific for TCC C5b-9 are pre-coated on a microplate. TCC C5b-9 in the samples or standards is bound by the immobilized antibody. After removing any unbound substances, a biotin-conjugated antibody specific for TCC C5b-9 is added to the wells. After washing, avidin conjugated Horseradish Peroxidase (HRP) is added. Following a wash to remove any unbound avidin-enzyme reagent, a substrate solution is added to the wells and colour develops in proportion to the amount of TCC C5b-9 bound in the initial step. The colour development was stopped, and the intensity of the colour was measured with an absorbance / fluorescence plate reader (Tecan) at wavelength of 450 nm.

2.2.2.15 Generation and characterization of gB(HSV)- specific mAbs resistant HSV-1/2 strains

To address the question whether propagation of HSV-1/2 in cultured cells in the presence of neutralizing antibody results in evolutionary adaptations that mediates resistance to antibodies and, an in-vitro assay was established

For this purpose, confluent Vero cells (4×10^5 cells/ml) were infected with HSV-1/2 (MOI 0.01) and passaged in the presence of increasing concentration of neutralizing mAbs in multiple round steps. After each round of the virus propagation, virus was harvested (see 2.2.2.5) and an aliquot was used to inoculate fresh Vero cells. After 4 rounds of passaging the virus, harvested virus was characterized using to antibody neutralizing potency assay (see 2.2.2.6) and antibody binding

potency assay (see 2.2.2.7) to confirm resistance against mAbs. Furthermore, isolated total DNA from infected cells were subjected to PCR by using MR0002-rv, OTS2054-fw and OTS2055-rv and PCR products were sequenced with primers MR0001, MR0005, MR0006, MR0007 and MR0016 to identify possible mutations in the gB coding region. For isolating a monoclonal virus population, a limiting dilution cloning approach was applied. For this approach, Vero cells were cultivated in 96 well plates (1×10^4 cells per each well). Vero cells were infected with serial dilution of virus stocks and after two-days incubation at 37°C, supernatant of wells with only one plaque were considered to be infected by one single clone and supernatant from these wells were harvested and used to expand the virus stock. Afterwards, a single clone of resistant viruses was sequenced and further analysed by antibody binding and neutralization assays, as described before.

2.2.2.16 Reversibility of HSV antibody-resistance to evolutionary adaptation

To investigate, the stability of the escape mutations in the HSV gB coding region and the number of passages required for the resistant clones to revert back to wild-type, resistant mutant viruses were passaged on Vero cells (8×10^6 cells per T75) in the absence of neutralizing antibodies in multiple rounds. After every passage, viral DNA was isolated from infected cells followed by PCR amplification and sequencing of gB (HSV-1/2) coding region as above.

2.2.2.17 Herpesviridae family cross reactivity (ELISA based assay)

To check for H4 and H28 cross-reactivity with other common members of Herpesviridae family, commercial Enzygnost anti-HSV/VZV/CMV/EBV IgG kits were used. First, the antibody-containing samples were applied to special 96-well plates coated with the target antigens (HSV/VZV/CMV/EBV infected cell lysate). After an incubation period of 1 hour at 37 °C, samples were washed three times with 200 µl washing buffer. The bound antibodies were detected with specific secondary antibody (Rabbit anti human Fcγ IgG- HRP conjugated polyclonal antibody (Jackson ImmunoResearch)). Except for the above-mentioned modifications, the test was carried out according to the manufacturer's manual. Quantification of the bound antibodies was carried out with an absorbance / fluorescence plate reader (Tecan) at wavelength of 450 nm, 650 nm were set as a reference wavelength.

2.2.2.18 Generation of 293T cell lines stably expressing HSV-1 gB and HSV-2 gB

To create HEK 293T cell line stably expressing HSV-1 gB or HSV-2 gB, respectively, 1×10^6 HEK293T cells were plated in a 6-well plate. The next morning, cells were transduced with VSV-G pseudotyped lentiviral CSxW vectors encoding codon optimized HSV-1 or non-codon optimized HSV-2 gB. The transduced cells were incubated for 2 days and expression of HSV-1/2 gB was analysed using HDIT101-FITC by flow cytometry.

Single cell cloning was performed by trypsinizing the transduced cells, counting them and plating 100 µl of cell dilutions (5 cells/ml) into wells of 96 well plate, using HEK293T conditioned media (50% fresh DMEM and 50% HEK293T conditioned media). Single cell clones were expanded, the expression of HSV-1 and HSV-2 gB on the single cell clones was confirmed using HDIT101 antibody by flow-cytometry, the clone with the highest gB expression was chosen for further cultivation and all experiments in this thesis were done using these selected clones.

2.2.3 Animal experiments

In-vivo efficacies of the antibodies were investigated in HSV-1/2 infected NOD.CB17-Prkdcscid/NCrHsd and BALB/cOlaHsd mouse models. One week prior to the experiment, 6-week-old Female mice (NOD/SCID or BALB c) (16-19gr) were purchased from ENVIGO company and one week prior to the viral inoculation, they were pre-treated with Medroxyprogesteron (SC injection). On the day of intravaginal inoculation, the experimental animals were anesthetized by isoflurane. During the short anaesthesia, the vaginal mucosa was cleaned from the vaginal secretions by using a sterile ESwab and the experimental animals were infected by intravaginal inoculation of the respective herpesvirus by applying 10-20 µl from the herpes virus stock diluted to reach a specific dose to the vaginal mucosa using a pipette. Afterwards, a small amount of Epiglu tissue adhesive was applied on the surface to glue the vagina (avoids inoculum to flow out). Normally the vagina was closed for few hours and the glue was lost within 1 day after inoculation.

The efficiency of the test antibodies for protecting mice from HSV infection was assessed by post-exposure IP/ IV administration of different antibody doses. The control group was usually treated with PBS. The experimental animals were regularly inspected for weight loss and the occurrence of perineal hair loss (HL) redness (R) and swelling (S) and neuronal damage (hind limb paralysis,

gastrointestinal track blockage) over a 30-60 days of observation period. Visible inspection was graded from slight to severe symptoms accordingly + / ++ / +++. Viral shedding was checked on days 1, 3 and 6 post-inoculation by taking vaginal swabs in 200 µl PBS (stored in -80°C for subsequent qPCR). Experimental animals were sacrificed in case of severe signs of herpes encephalitis or visible lesions to prevent undue suffering. All experiments were done in line with ethical approval (animal applications G-183/12 and G-128/18).

2.2.3.1 HSV-1/2 (WT) dose finding for animal experiments

Longacting progestin Depo-Clinovir were prepared at 25 mg/ml in PBS and 100 µl per mouse were administered SC one week prior to virus infection.

Table 25. The table shows grouping and experiment detail for lethal dose finding of HSV-1F infection in the NOD/SCID mouse model.

Groups	Virus	Viral dose	Animals
1	HSV-1F	$5x10^5$ $TCID_{50}$	4
2	HSV-1F	$1x10^5$ $TCID_{50}$	4
3	HSV-1F	$5x10^4$ $TCID_{50}$	4
			12

Table 26. The table shows grouping and experimental details for lethal dose finding of HSV-1F infection in the BALB/c mouse model.

Groups	Virus	Viral dose	Animals
1	HSV-2G	$5x10^5$ $TCID_{50}$	6
2	HSV-2G	$1x10^5$ $TCID_{50}$	6
3	HSV-2G	$5x10^4$ $TCID_{50}$	6
4	HSV-2G	$1x10^4$ $TCID_{50}$	6
5	HSV-2G	$5x10^3$ $TCID_{50}$	6
			30

2.2.3.2 HSV-1/2 (HDIT101-Resistant Mutant) dose finding for animal experiments

Table 27. Table shows grouping and experiment detail for lethal dose finding of HSV-1F resistant mutant infection in NOD/SCID mouse model.

Groups	Virus	Viral dose	Animals
1	HSV-1Rc1	$5x10^5$ $TCID_{50}$	4
2	HSV-1Rc1	$1x10^5$ $TCID_{50}$	4
3	HSV-1Rc1	$5x10^4$ $TCID_{50}$	4
			12

Table 28. Table shows grouping and experiment detail for lethal dose finding of HSV-2G resistant mutant infection in NOD/SCID mouse model.

Groups	Virus	Viral dose	Animals
1	HSV-2Rc1	$5x10^5$ $TCID_{50}$	4
2	HSV-2Rc1	$1x10^5$ $TCID_{50}$	4
3	HSV-2Rc1	$5x10^4$ $TCID_{50}$	4
4	HSV-2Rc1	$1x10^4$ $TCID_{50}$	4
5	HSV-2Rc1	$5x10^3$ $TCID_{50}$	4
			20

2.2.3.3 NOD/SCID HSV-1 infected mouse model and therapeutic application of antibodies

Table 29. Table shows grouping and experiment details for therapeutic application of antibodies in NOD/SCID HSV-1F infected mouse model

Groups	Virus	Viral dose	H4 dose (µg)	Animals
1	HSV-1F	$5x10^5$ $TCID_{50}$	0	6
2	HSV-1F	$5x10^5$ $TCID_{50}$	150	9
3	HSV-1F	$5x10^5$ $TCID_{50}$	300	9
4	HSV-1F	$5x10^5$ $TCID_{50}$	600	9
				33

2.1.1.2 BALB c HSV-2G infected mouse model and therapeutic application of antibodies

Table 30. Table shows grouping and experiment details for therapeutic application of antibodies in BALB c HSV-2G infected mouse model

Groups	Virus	Viral dose	Antibody dose (µg)	Animals numbers
1	HSV-2G	$5x10^5$ $TCID_{50}$	0	10
2	HSV-2G	$5x10^5$ $TCID_{50}$	600 HDIT101 i.v.	12
3	HSV-2G	$5x10^5$ $TCID_{50}$	300 HDIT101 i.v.	12
4	HSV-2G	$5x10^5$ $TCID_{50}$	150 HDIT101 i.v.	12
5	HSV-2G	$5x10^5$ $TCID_{50}$	300 H4 i.p.	10
6	HSV-2G	$5x10^5$ $TCID_{50}$	600 H4 i.p.	10

2.2.3.4 Quantification of HSV copy numbers in vaginal swabs (qPCR)

To analyse absolute copy numbers of HSV-1 and HSV-2 in vaginal swabs/TGs of infected mice, a quantitative polymerase chain reaction (qPCR), using a Roche Light Cycler 480 with prima QUANT 2x qPCR-SYBR-Green-Blue-Master MIX was performed. In principle, qPCR works by measuring the fluorescence signal (SYBR Green) of amplified DNA in each sample after each PCR cycle and comparing the signal to a reference standard with known DNA copy numbers. For this purpose, viral DNA from vaginal swabs/TGs were isolated using the Qia Amp DNA Blood Kit according to the manufacturer's recommendation for samples with low DNA yield (adding 5-10 µg of ultrapure salmon's sperm DNA(Invitrogen) as carrier DNA) and eluted in 50 µl ddH_2O. For preparation of standards, solutions with serial dilution of known copy numbers of target DNA were prepared. Briefly, the target region spanning the primers used in qPCR was amplified by PCR and cloned into pJet vector. The plasmid was amplified in bacteria, isolated, and the DNA concentration was determined by spectrometry. The DNA was diluted to $2x10^7$ copies/µl and further by using 10-fold dilution steps down to 2 copies/µl. The qPCR primers were described previously (Jabs, 1996) (Lakeman *et al.*, 1995)and are specific for a 179bp sequence in the HSV polymerase gene (UL30), which is conserved between HSV-1 and HSV-2, hence can be used for quantification of both viruses. The assay was performed using a Roche LightCycler480 with the following 2-step program (Table 31). Prior to each run, 5 µl of standard or sample was mixed with 15 µl of a mix containing 10 µl of 2x master mix and 0.6 µl of each of the specific primers (final concentration 300 nM each). Enzymes, Buffer and SYBR Green dye is provided in the 2x master mix (see Table 32).

Table 31. qPCR conditions.

Step	Temperature [°C]	Time [s]
Initial Denaturation	95	120
Denaturation	95	15
Annealing and elongation	65	30
	45 cycles	

Table 32. qPCR reaction mix

Component	Final amount/ concentration	Volume [µl/well]
PCR 2x Master Mix	1X	10
Primer forward	300 nM	0.6
Primer reverse	300 nM	0.6
Template DNA		5
ddH_2O		3.8
∑		20

Table 33. Employed primers for qPCR.

Name	Sequence 5'→3'	Description
MR0063	ATCAACTTCGACTGGCCCTT	qPCR (Quantification of HSV copy numbers)
MR0064	CCGTACATGTCGATGTTCAC	qPCR (Quantification of HSV copy numbers)

Absolute quantification was performed using the "abs quanti/2nd derivative Max" method using the Light Cycler 1.5 Software. By comparing the cycle, in which a defined fluorescence signal was reached in each individual sample with the standard the exact copy numbers of HSV genomes in every sample was calculated. All samples were run in duplicates and samples with a lower than 2-fold variation between the individual wells were considered valid. Data was analysed using Microsoft Excel and plotted using Graph Pad Prism.

3 Results

3.1 Selecting herpes simplex virus glycoprotein B specific scFvs from a human lymph node derived antibody library

To generate human monoclonal antibodies targeting herpes simplex virus with therapeutic potential, the human lymph node derived antibody library (LYNDAL) (phage display derived from the B cell repertoire of head and neck cancer patients) was screened against recombinant insect cell-derived trimeric glycoprotein B of HSV-1 (KOS) (kindly provided by Prof. Roselyn J. Eisenberg and Prof. Gary H. Cohen, Department of Microbiology, University of Pennsylvania) by a previous PhD student, Philipp Diebolder (AG Arndt/Krauss) (Diebolder *et al.*, 2014). According to characterization assays, promising gB-specific scFvs were selected and were subjected for further investigation (see Figure 11and for more detail refer to Diebolder et al. (Diebolder *et al.*, 2014)).

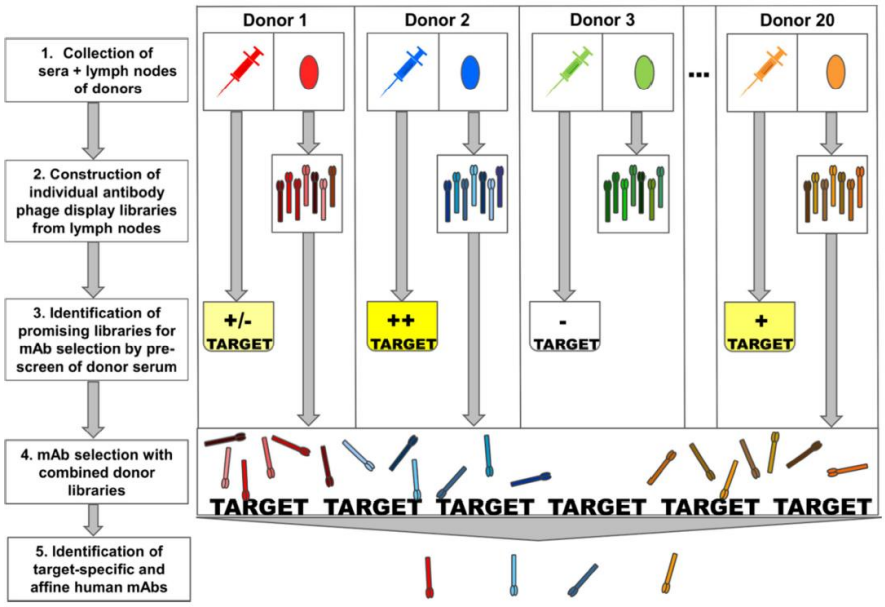


Figure 11. Schematic representation of the human lymph node derived antibody library (LYNDAL) concept.

Lymph nodes were sampled from patients with squamous cell head and neck carcinoma. Antibody gene information were obtained by amplifying relevant regions from lymph node-derived B cell mRNA and cloned as individual antibody phage display libraries. Finally, scFv libraries from donors with target-specific immune response were combined for subsequent scFv selection (Figure is adapted from Diebolder, Philipp, dissertation).

As the first characterizing assay the scFvs were subjected for the analysis of binding to gB (Figure 12Figure 11). In this assay binding specificity of scFvs targeting glycoprotein B of HSV-1 strain F or HSV-2 strain G were assessed by flow cytometry of infected Vero cells. Watson et al. in a comparative analysis sequenced the genome of seven different HSV-1 strains including McKrae, F, KOS (two different ones included in this analysis), HF10, H129 and strain17 and compared their coding protein (74) sequences. They showed 63 of the 74 predicted protein sequences were conserved and only eleven protein genomes were less than 98% conserved (Watson *et al.*, 2012). Therefore, due to the very small variations between strains, presumably strain KOS selected scFvs can bind to strain F as well. In addition, although LYNDAL was screened with the recombinant ectodomain of gB-1, the cross-reactivity of the selected scFvs to HSV-2G gB is likely due to the homology of more than 87% (767 out of 882 aa). As shown in Figure 12, many scFvs bound specifically to HSV-1F/2G gB expressed on the surface of Vero cells while no binding was found on uninfected Vero cells. . Some scFvs did not bind better to infected Vero cells than uninfected (e.g. H17). The mostly higher fluorescence intensities observed in HSV-2G infected Vero cells could result from higher viral genome copy numbers compared to that of HSV-1 infected cells (Krawczyk *et al.*, 2011b). 22 scFvs out of 34 scFvs showed relatively high reactivity toward both HSV-1 and HSV-2 infected cells (e.g. H1, H4, H25, H28) which might be the suitable candidates for developing therapeutic to target both HSV-1 and HSV-2 infections. However, their therapeutically relevant antiviral potentials had to be tested.

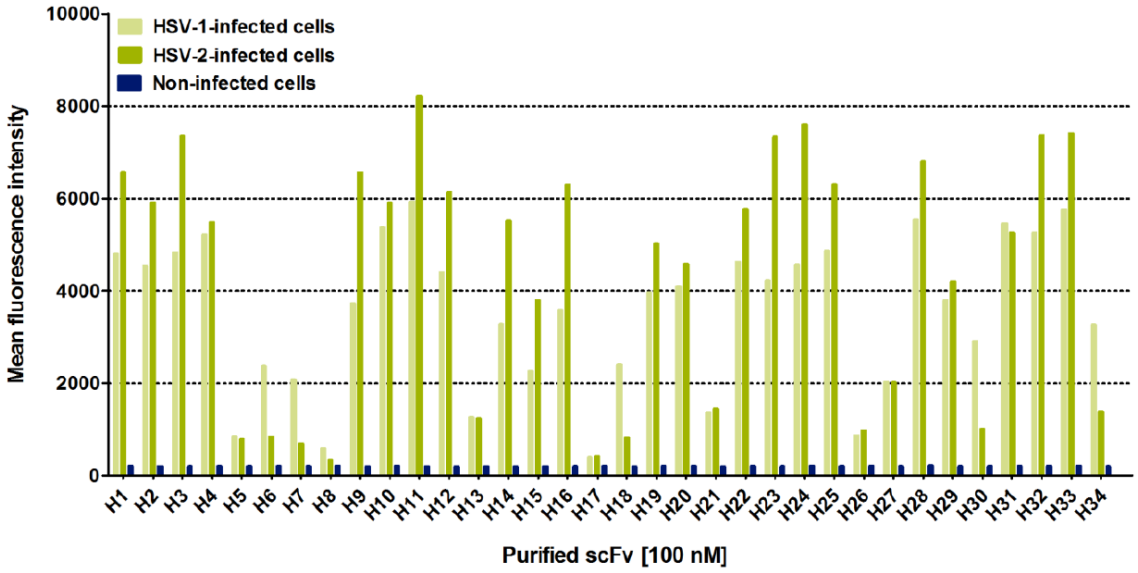


Figure 12. Binding specificity of LYNDAL selected scFvs.

Binding specificity of selected scFvs was analysed against cell membrane associated glycoprotein B (HSV-1F and HSV-2G infected Vero cells) by a flow cytometry based assay. Bound myc-tagged scFvs were detected with myc-specific mouse antibody, followed by anti-mouse FITC conjugate. Un infected Vero cells were also included as negative control.

The neutralizing potency of selected scFvs was evaluated by a standard plaque reduction assay. As shown in Figure 13, eight scFvs showed more than 10% HSV-1 neutralizing activity at 2 µM concentration. According to Krawczyk et al. (Krawczyk *et al.*, 2011b) who reported that the valency of gB(HSV) specific antibodies has a strong impact on the HSV neutralizing potency, scFvs were cross-linked using an anti-myc tag specific IgG. As it is illustrated several cross-linked scFvs showed augmented neutralizing potency and H4 and H31 which were derived from the same B cell clone (99% homology) had highest neutralization potential after crosslinking. However, scFv H28 completely loose its antiviral potential after crosslinking possibly because the viral epitopes were not readily accessible anymore, due to the size of antibody complex.

While many scFvs showed binding towards HSV-1 or HSV-2 infected cells, only a few of them showed the potential to neutralize HSV-1 infection. Some of the studied antibodies neutralized

HSV-1 efficiently through monovalent binding but less efficiently via bivalent binding through crosslinking (e.g. H22, H28), while others more strongly inhibited HSV-1 infection when cross-linked (e.g. H4, H31), suggesting for different binding sites within gB. According to the binding profile and HSV neutralization capacity, two antibodies, H4 and H28 with high affinity and highest neutralizing capacity were selected as lead candidates (Diebolder *et al.*, 2014).

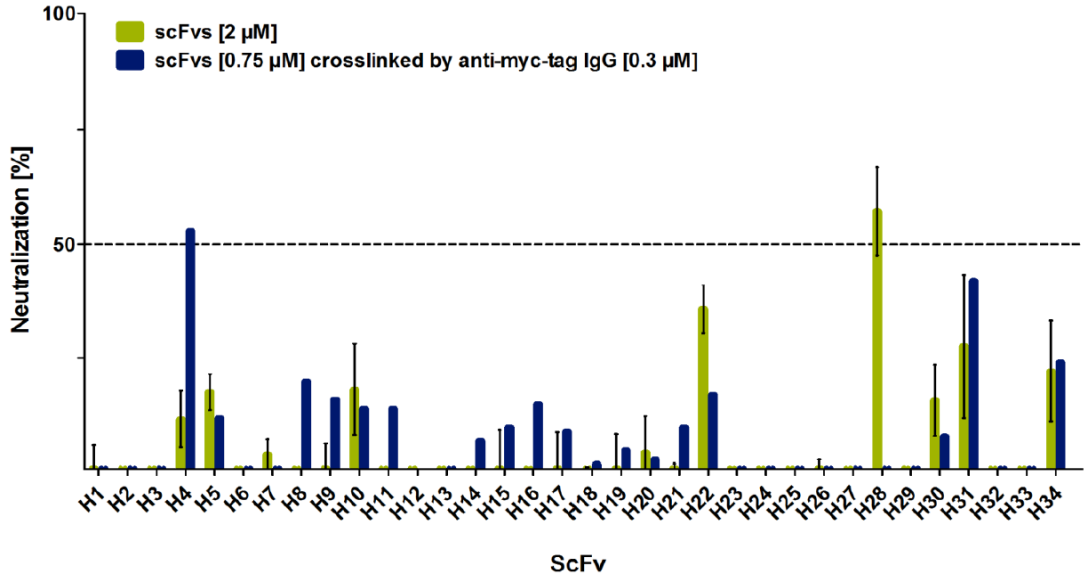


Figure 13. Neutralizing potency of gB-specific scFvs.

Standard plaque reduction assay was employed to test the neutralization potency of selected scFvs using HSV-1F infected Vero cells and applying constant concentration of scFvs (green columns) and scFvs cross-linked with myc-tag-specific antibody (blue columns). Error bars are representing the standard error of the mean (N=4).

3.2 scFvs sequence analysis with the database IMGT/V-Quest

According to www.imgt.org/V-Quest, the lead candidate scFv (H4) belongs to the VH1 antibody heavy chain family, VL3 antibody light chain family and lead candidate scFv (H28) belongs to VH3 antibody heavy chain family, VKIII antibody light chain family (Table 34).

Table 34. Genetic features of gB-specific LYNDAL selected scFvs and corresponding antibody families

	H4	**H28**
Clone Number	gB 4	gB 29
Heavy chain		
Heavy chain length	122 aa	122 aa
Heavy chain family	VH1	VH3
V-GENE and allele	Homsap IGHV1-8*01 F identity = 89,24%	Homsap IGHV3-23*01 F (or) Homsap IGHV3-23D*01 F identity = 91,67%
J-GENE and allele	Homsap IGHJ4*03 F (or) Homsap_IGHJ6*02 identity = 72,34%	Homsap IGHJ6*02 F (or) Homsap_IGHJ3*01 identity = 70,97%
D-GENE and allele	Homsap IGHD1-1*01 F	Homsap IGHD6-6*01 F
CDR-H1	GHTFRTFD	GFTFSSYA
CDR-H2	MSPNSGNT	ISANGLRT
CDR-H3	ARGPGSTGTTGSMDV	AKHSRMATRDDPMDV
Light chain		
Light chain length	106 aa	107 aa
Light chain family	VL3	VKIII
V-GENE and allele	Homsap IGLV3-21*01 F identity = 96,42%	Homsap IGKV3-20*01 F identity = 91,40%
J-GENE and allele	Homsap IGLJ2*01 F (or) Homsap IGLJ3*01 F	Homsap IGKJ1*01 F identity = 94,59%

	identity = 97,14%	
CDR-L1	NIGSKS	ESVSRS
CDR-L2	YDS	DAS
CDR-L3	QVWDSGSVL	QQYGHSPWT

3.3 Synthesis of codon optimized LYNDAL selected gB(HSV)-specific variable genes.

The optimal conditions to increase the antibody expression level were determined. For this purpose, the Kozak consensus sequence GCCRCCATGR, which has a main role in the initiation of the eukaryotic translation process, was modified to CGGCCACCATGA as it is reported that in the strongly expressed genes, the − 3 and the + 4 nucleotides should match exactly(Sakai *et al.*, 2001) In addition, the human Azurocidin signal peptide (corresponding nucleotide sequence: CCAGACTGACCGTGCTGGCCCTGCTGGCCGGCCTGCTGGCTTCTTCTA) which is known as the most appropriate leader peptide to generate stable cell lines with improved antibody production rates(Kober *et al.*, 2013), was used. According to the plasmid map of pConPlus IgG (H/k, λ) vectors, restriction sites including *HindIII* (AAGCTT) and *ApaI* (GGGCCC) for variable heavy chain genes and *HindIII* (AAGCTT), *AvrII* (CCTAGG) and *BsiWI* (CGTACG) for variable light chain genes were included. Designed variable genes were synthesized by Eurofins scientific as plasmids. The sequences were verified by standard Sanger sequencing (Table 35).

Table 35. Synthesized codon-optimized heavy/light variable genes with KOZAK sequence (underlined) and Azurocidin signal peptide coding region and respective amino acid sequences.

Name	Sequences
H4 (heavy chain)	(*HindIII*) AGCTTCGGCCACCATGACCAGACTGACCGTGCTGGCCCTGCTGGCCGGCCTGCT GGCTTCTTCTAGAGCCGAAGTCCAGCTCGTTCAGTCTGGAGCTGAGGTCAAAAC CCCAGGAGCCAGTGTTCGCGTGTCCTGCAAGGCTTCAGGACACACCTTCAGGAC CTTCGACATCAACTGGGTACGTCAGGCTGCAGGCCAAGGGCTGGAATGGATGG GCTGGATGAGCCCCAATTCCGGCAACACAGGGTATGCCCGACAGTTTCAGGGTA GAGTGACCATGACACGGAACATTAGCGCGAATACGGCCTATATGGAGCTGAGA GGGCTTCGGTTTGACGATACAGCCGTGTACTACTGTGCAAGGGGACCTGGCTCT ACAGGCACGACTGGTAGCATGGATGTGTGGGGTCAAGGGACTACCGTGACTGT CTCGAGTGCCAGCACCAAGGGCCC (*Apa I*) Corresponding amino acid sequence: ASATMTRLTVLALLAGLLASSRAEVQLVQSGAEVKTPGASVRVSCKASGHTFRTFD INWVRQAAGQGLEWMGWMSPNSGNTGYARQFQGRVTMTRNISANTAYMELRGL RFDDTAVYYCARGPGSTGTTGSMDVWGQGTTVTVSSASTKG
H4 (λ chain)	(*HindIII*) AAGCTTCGGCCACCATGACCAGACTGACCGTGCTGGCCCTGCTGGCCGGCCTGC TGGCTTCTTCTAGAGCCCAGGCTGGACTGACACAGCCACCTAGCGTTAGCGTGG CTCCTGGCAAAACTGCGCGCATTAGCTGTGGCGGGAACAACATTGGCAGCAAG AGTGTCCACTGGTATCAGCAGAAACCCGGACAAGCACCAGTGCTGGTGATCTAC TATGACTCCGACAGACCGTCAGGTATACCCGAGAGGTTCAGTGGGTCCAATTCC GGCAATACAGCCACTCTCACCATCTCTCGGGTTGAAGCCGGAGATGAGGCCGAC

	TACTACTGCCAAGTGTGGGATTCTGGGTCAGTGGTCTTTGGAGGCGGTACCAAG CTGACGGTCCTAGG (*AvrII*) Corresponding amino acid sequence: ASATMTRLTVLALLAGLLASSRAQAGLTQPPSVSVAPGKTARISCGGNNIGSKSVH WYQQKPGQAPVLVIYYDSDRPSGIPERFSGSNSGNTATLTISRVEAGDEADYYCQV WDSGSVVFGGGTKLTVL
H28 (heavy chain)	(*HindIII*) AAGCTTCGGCCACCATGACCAGACTGACCGTGCTGGCCCTGCTGGCCGGCCTGC TGGCTTCTTCTAGAGCCGAGGTTCAGCTGCTCGAAAGTGGCGGAGGTCTGGTGA AGCCAGAAGGGTCCTTGAGGCTCTCTTGCGCTGCATCAGGGTTCACCTTTAGCT CCTATGCCATGAACTGGGTGAGACAGGCTCCTGGAAAAGGCCTGGAGTGGGTA AGCTCCATTAGTGCCAATGGGCTTAGGACGTACTATGCGGATAGCGTCAAAGGA CGCTTCACCATAAGCCGGGACAATTCTGGCAACACTCTGCATCTGCAGATGAAC TCTCTGAGAGCTGAGGACACAGCCATCTACTACTGTGCCAAGCACTCACGAATG GCAACACGTGACGATCCCATGGATGTGTGGGGTCAAGGCACAACTGTCACCGTC TCGAGTGCCAGCACCAAGGGCCC (*ApaI*) Corresponding amino acid sequence: ASATMTRLTVLALLAGLLASSRAEVQLLESGGGLVKPEGSLRLSCAASGFTFSSYA MNWVRQAPGKGLEWVSSISANGLRTYYADSVKGRFTISRDNSGNTLHLQMNSLRA EDTAIYYCAKHSRMATRDDPMDVWGQGTTVTVSSASTKG

H28 (κ chain)	(*HindIII*) AAGCTTCGGCCACCATGACCAGACTGACCGTGCTGGCCCTGCTGGCCGGCCTGC TGGCTTCTTCTAGAGCCGAGATTGTCCTCACTCAGTCTCCAGGCACTCTGAGCCT TGCTCCTGGAGAGGAAGCGACATTCAGTTGTCGAGCATCCGAAAGCGTTTCCCG CTCTTTGGCCTGGTATCAGCAGAAACCCGGACAAGCACCCAGACTGCTCATCTA CGATGCCTCCAGTAGGGCTACAGGGATTCCGGATCGGTTTTCTGGCAGCGTATC AGAGACAGACTTCACCCTGACGATCAGCTCACTGGAACCTGAGGACTTTGCCGT GTACTTCTGCCAGCAGTATGGCCACAGTCCATGGACCTTTGGGCAAGGTACCAA GGTGGAGATAAAGCGTACG (*BsiWI*) Corresponding amino acid sequence: ASATMTRLTVLALLAGLLASSRAEIVLTQSPGTLSLAPGEEATFSCRASESVSRSLA WYQQKPGQAPRLLIYDASSRATGIPDRFSGSVSETDFTLTISSLEPEDFAVYFCQQYG HSPWTFGQGTKVEIKRT

Colour code: Restriction sites, KOZAK sequence, Azurocidin coding region, antibody variable gene

3.4 Cloning the variable coding regions of the codon-optimized sequences into pConPlus vectors containing constant domain of IgG1

As explained before (see 2.2.1.1) antibody variable domain genes H4 (heavy chain), H4 (lambda chain), H28 (heavy chain) and H28 (Kappa chain) were cloned into pConPlus IgG1, pConPlus λ pConPlus IgG1, pConPlus K, respectively. Afterwards, vectors containing heavy chain open reading frames were ligated with vectors containing light chain open reading frames to generate plasmids named double gene vectors of_H4 and_H28 encoding the entire antibody. Sequences were confirmed by Sanger sequencing (Eurofins scientific).

3.5 gB(HSV)-specific IgG1 (H4 & H28) production and purification

After transfection of HEK293-6E cells with the respective IgG expression vectors (double gene vectors of_H4 and_H28 encoding the entire antibody), the cell culture supernatant was collected and antibodies present in the supernatant purified using a protein A chromatography column (2.2.2.4.3). The yields of purified antibodies ranged from ≈ 1 to 15 mg per 144 mL culture volume (Figure 14). The purity and integrity of produced IgG proteins was confirmed by size exclusion chromatography (SEC) and SDS PAGE, staining with Coomassie blue and parallel immunoblotting. The produced antibodies showed full IgG profile after their separation by size exclusion chromatography; elution peak at 12 mL corresponding to a molecular size of approximately 158 kDa according to the standard (Figure 15). Coomassie blue staining visualized 2 bands for each antibody, one at 55 kDa and one at 25 kDa corresponding to the molecular weight of heavy chain and light chain, respectively. Unexpectedly, a fade additional band was observed at 55 kDa for H4 and H28 (Figure 16), which might indicate differences in posttranslational modifications such as different glycosylation pattern. It was observed in immunoblots stained with anti-human Fc IgG antibody, bands with the molecular weight of 55 kDa, confirming the heavy chain of purified IgGs **(**Figure 16).

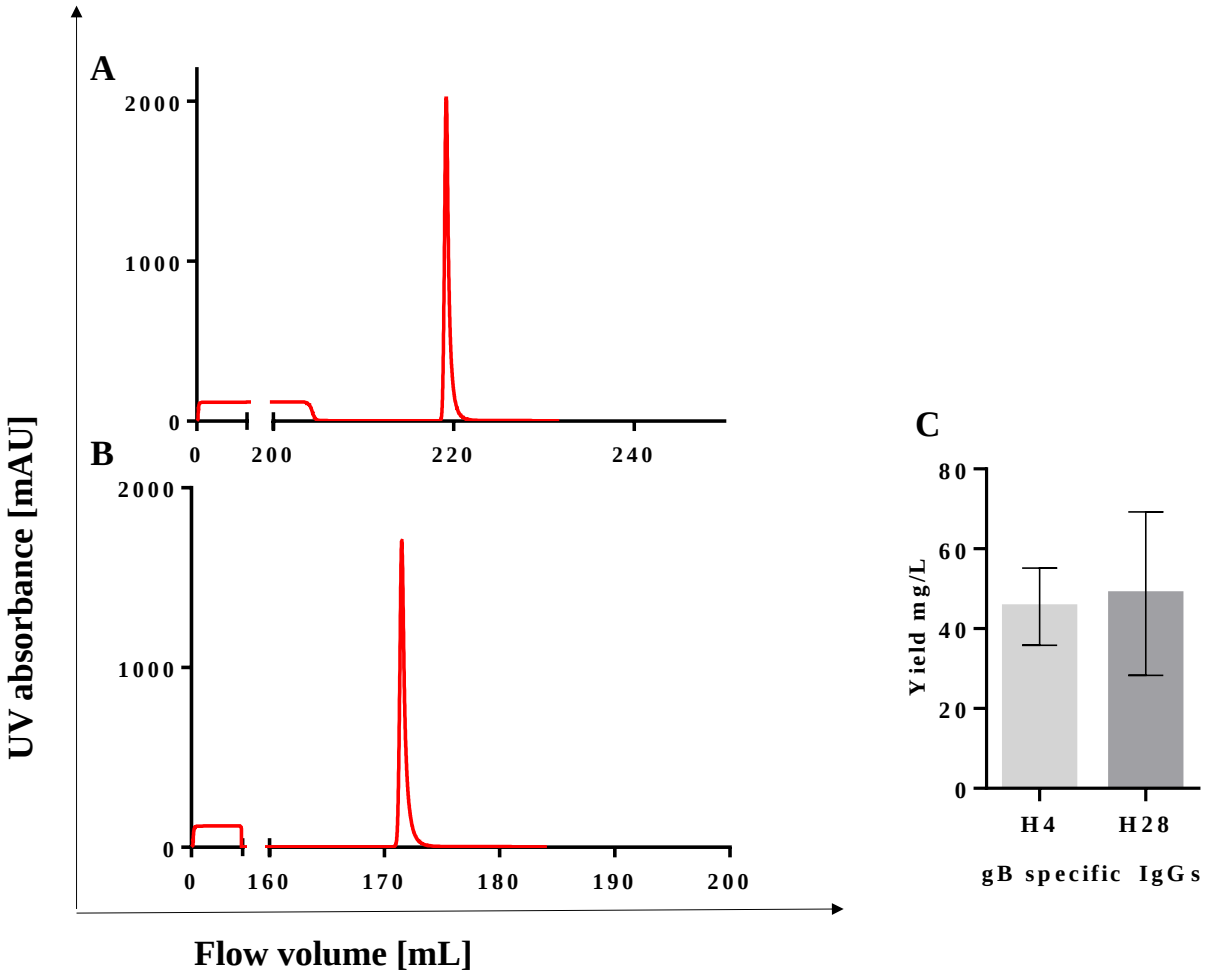


Figure 14.gB(HSV) specific IgG1 (H4 & H28) production and purification.

A, B) Representative protein A purification chromatograms, of gB(HSV) specific IgG1 H4 (A) & H28 (B). **C)** Expression yield of gB(HSV)-specific IgGs produced in HEK293-6E cells, Protein A purified. Yield is shown as mg antibody per litre of cell culture supernatant. Error bars represent the standard deviation of mean (N=8 independent production runs).

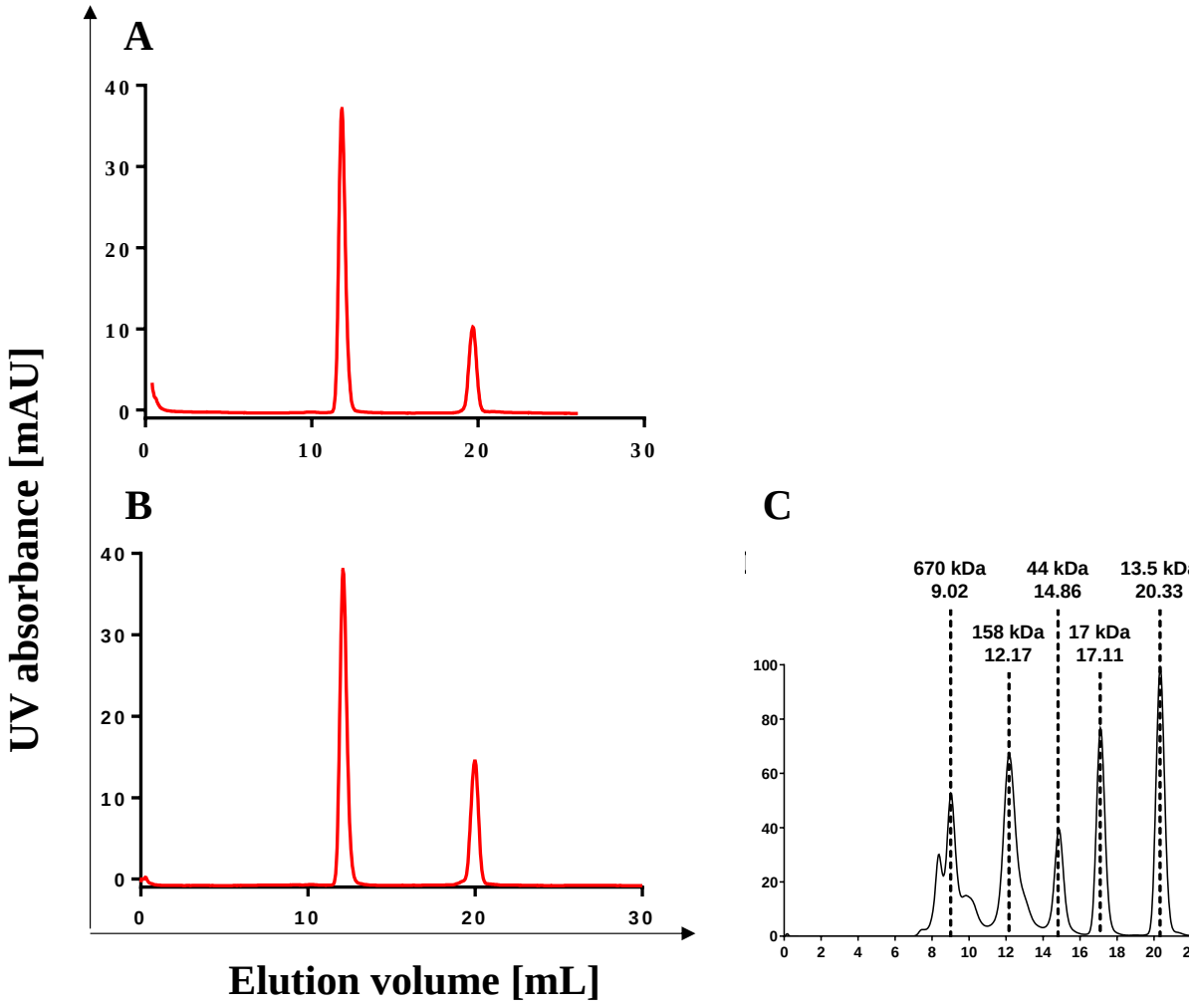


Figure 15. Size exclusion chromatography profiles of gB(HSV)-specific IgGs.

One example of gel filtration chromatogram per each antibody is shown. 40 µg of (A) H4, (B) H28, gB(HSV)-specific IgGs were loaded onto a 20 mL gel filtration column (Superdex 75 10/300 GL). The x-axis shows the elution volumes with main peaks corresponding to full IgG. C) size exclusion chromatography was done using standard proteins (by Dr. Valentino De Leo) with known molecular weight to determine the molecular weight of corresponding flow volumes.

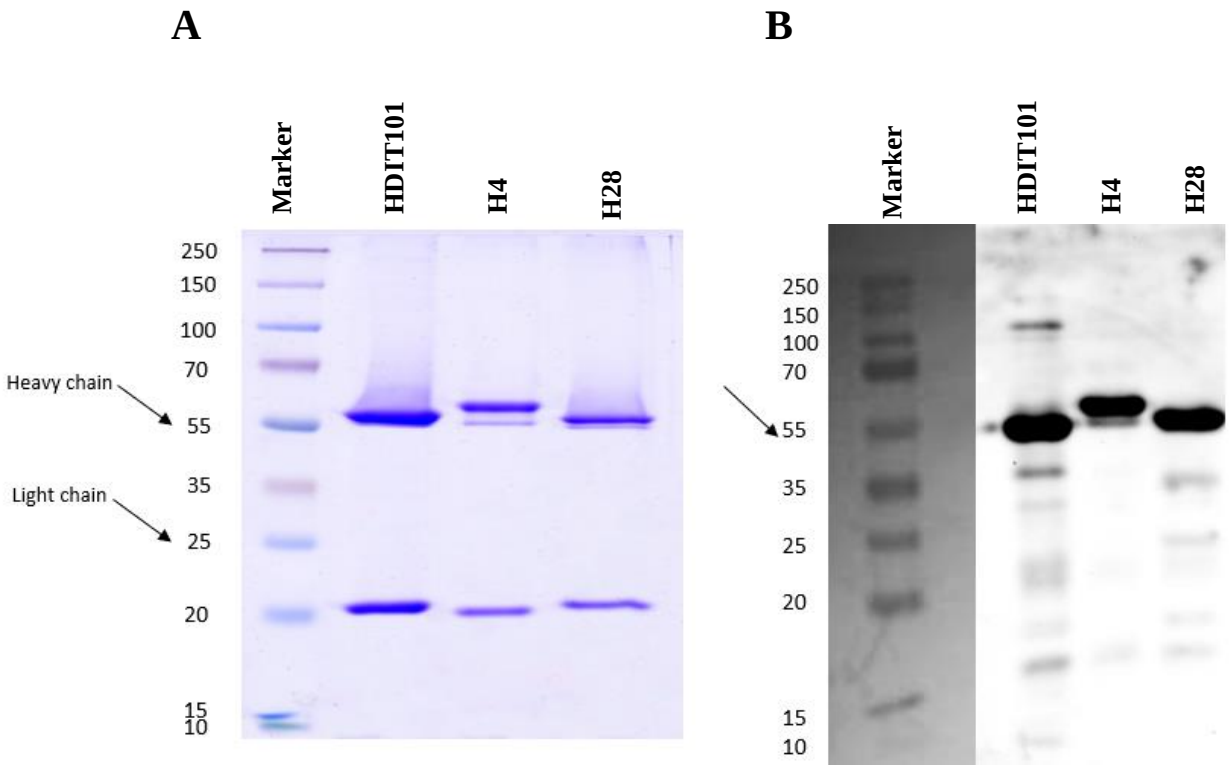


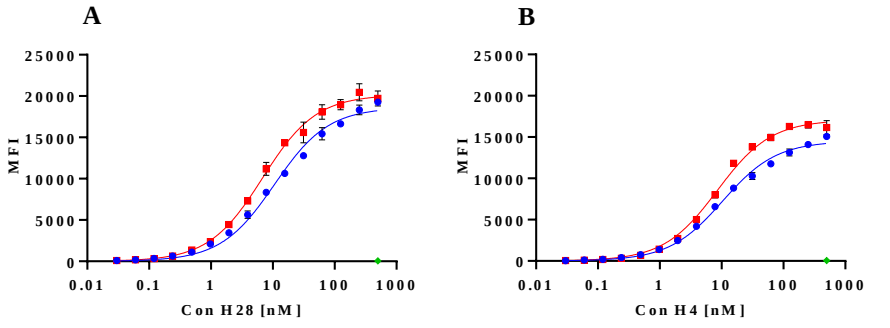
Figure 16. Coomassie Blue staining and Western blot picture of produced IgGs after SDS PAGE.

The produced antibodies were separated by SDS PAGE and visualized by (A) Coomassie Blue staining (arrows pointing the heavy and light chain, respectively) as well as by (B) Western blot using a HRP coupled anti-human IgG Fc antibody (representative picture for one production is shown). HDIT101 is a humanized gB(HSV) specific IgG1 developed in the company Heidelberg Immunotherapeutics GmbH and the AG Arndt/Krauss with high neutralization capacity and was included in A and B as control.

3.6 *In-vitro* characterization of gB(HSV)-specific IgGs

3.6.1 Analysis of the binding specificity of gB(HSV)-specific IgGs and determination of their functional affinity (avidity)

Generating efficacious and safe therapeutic mAbs highly relies on the mAbs' affinity for the target antigen. Therefore, binding of gB(HSV)-specific IgGs to their target antigen was analysed. The binding specificity and functional affinity of the gB(HSV)-specific antibodies were assessed by flow cytometry employing HSV-1 F or HSV-2 G infected Vero cells expressing membrane associated glycoprotein B. In this assay, glycoprotein B expressed on the cell surface was stained with the antibodies using various concentrations (0.03 nM-500 nM). Half-maximal effective binding (half maximal effective concentration (EC50)) was calculated based on the Marquardt-Levenberg method (Marquardt, 1963). According to these results, the EC50 for binding of H4 to HSV-1 F- and HSV-2 G-infected Vero cells was 8.5 nM and 10.9 nM, respectively. The EC50 for H28 to HSV-1 F and HSV-2 G infected Vero cells was 6.7 nM and 9.95 nM, respectively (Figure 17). Both candidates show very high affinity against HSV-1/2, which indicates specificity of binding and consequently safety of their application in the clinical setting. However, affinity is not representing the neutralization efficacy of antibodies and vice versa. There are several antibodies with very high affinities, which do not neutralize viral infection. Therefore, it is important to evaluate the neutralizing potency of selected antibodies in addition to their affinities.



C

Anti gB IgG	EC50 (HSV-1F)	EC50 (HSV-2G)
H28	6,7 nM	9,95 nM
H4	8,5 nM	10,9 nM

Figure 17. Equilibrium-binding curves of gB(HSV) specific IgG1 (H4 & H28).

Functional affinity of IgGs to membrane-associated gB was measured by flow cytometry. IgGs were titrated in triplicate on HSV-1F and HSV-2G infected Vero cells as well as uninfected Vero cells as control and binding was detected using a FITC-conjugated anti-human IgG Fc antibody. Nonlinear fitted curves for H28 **(A)** and H4 **(B)** were calculated based on averaged median fluorescence intensities (MFI).**C)** Summary of H4 and H28 EC50 against gB of HSV-2G and HSV-1F. The error bars showing standard deviation of mean (N=3). EC50; half maximal effective concentration. For uninfected cells the MFI of the maximal antibody dose did not reach the MFI of the lowest antibody dose (not shown), indicating specificity of the detection.

3.6.2 HSV-neutralization efficiency of gB(HSV)-specific IgG1s *in-vitro*

The efficiency of H4 and H28 to neutralize HSV-1F and HSV-2G infection in cell culture was investigated by determining the Tissue Culture Infection Dose 50 (TCID50) (2.2.2.8). HSV infection of Vero cells was confirmed visually by monitoring plaques using a light microscope. Figure 18 shows HSV-1 F infected Vero cells and plaques in different magnification which was stained by anti-gB(HSV)-FITC (HDIT101-FITC) and Hoechst. In this assay, for quantitative comparison, the amount of antibody required for complete (100%) or half (50%) neutralization of virus (at a dose of 100 TCID50 on 2×10^4 Vero cells in each well of a 96-well plate) was determined (2.2.2.8). The results of the neutralization studies showed that gB(HSV)-specific IgG1 H4 neutralized HSV-1F and HSV-2G with a substantially higher efficiency than IgG1 H28. For complete neutralization of HSV-1F and HSV-2G a concentration of 62.5 nM and 31.25 nM of H4 was required, respectively (Figure 19).

To completely neutralize the same viral load of HSV-1 and HSV-2, a concentration of 250 nM and > 500 nM of IgG1 H28 was necessary (Figure 19). HSV also like other enveloped viruses has two distinct routes of infection: through the cell-free environment (which was evaluated by TCID50 test) or directly through cell-to-cell contacts and both routes contribute to the success of viral infections *in-vivo* (Dingwell *et al.*, 1995). In the latter, the virions remain cell-associated and are passed from one cell-to-the neighbouring cells via existing or newly established contacts through tight junctions (Torres-Flores and Arias, 2015). It has been reported that cell-to-cell transmission is more relevant for virus propagation *in-vivo* as compared to cell-free virus infection (Mothes *et*

al., 2010). Therefore, for developing an efficient protective anti-viral mAb it is important that the antibody is capable of blocking cell-to-cell transmission.

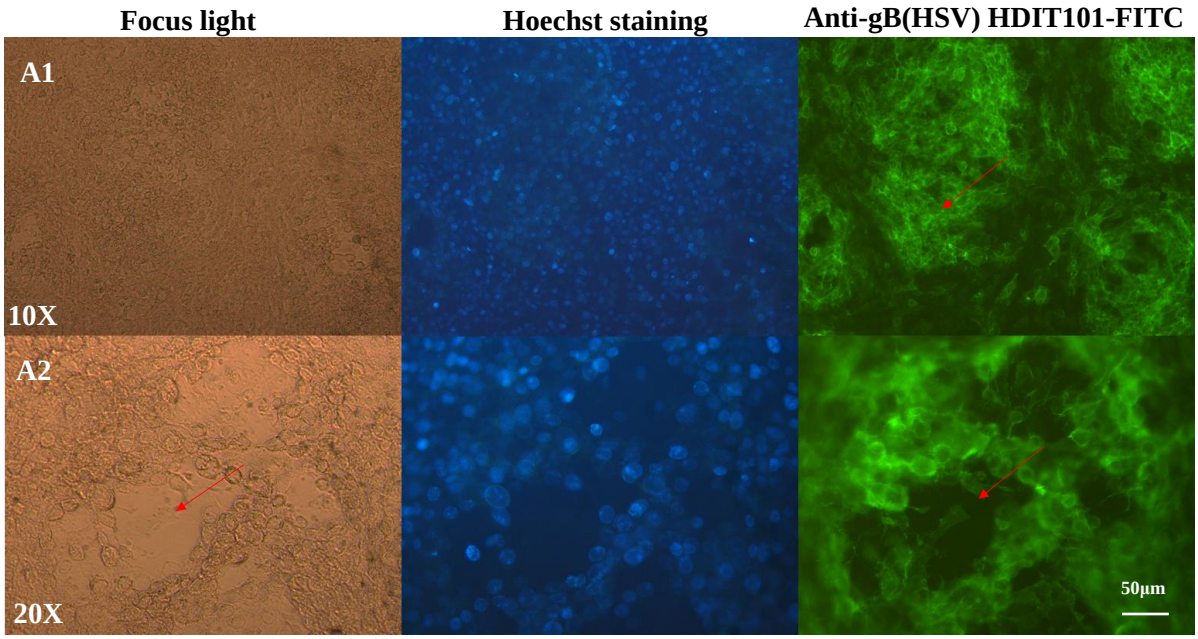


Figure 18. HSV-1F infected Vero cells and plaque formation.

1×10^{5} Vero cells in each well of 4-well chamber slide were seeded and one day later were infected with 100 TCID50 of HSV-1F, three days post-infection cells were stained with anti-gB antibody HDIT101-FITC and Hoechst as nuclear staining. Images were taken at **A1)** 10X and **A2)** 20X magnification from different fields of the same slide with inverted fluorescence microscope. Arrows point out individual plaques.

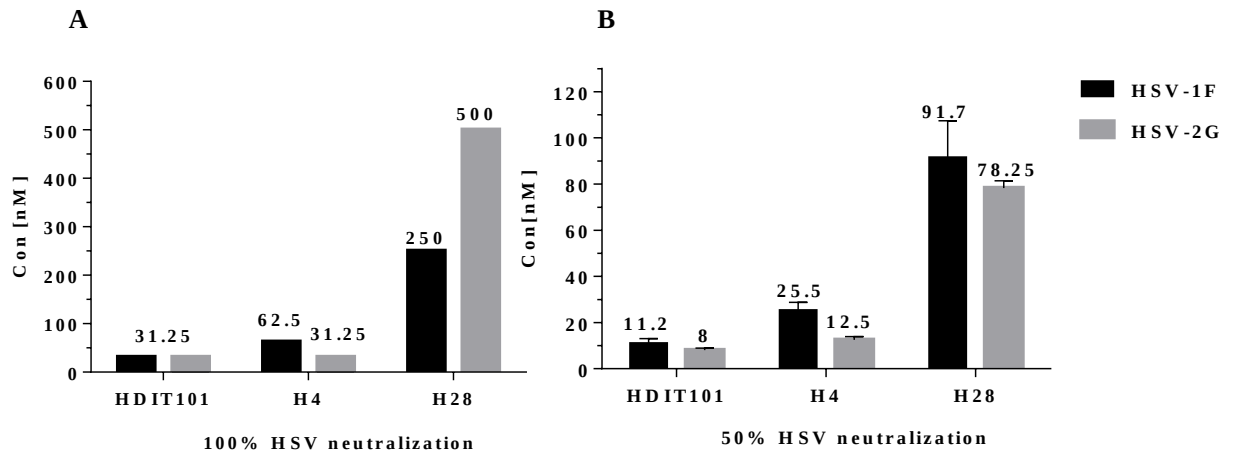


Figure 19. *In-vitro* neutralization efficiency of gB(HSV)-specific IgGs.

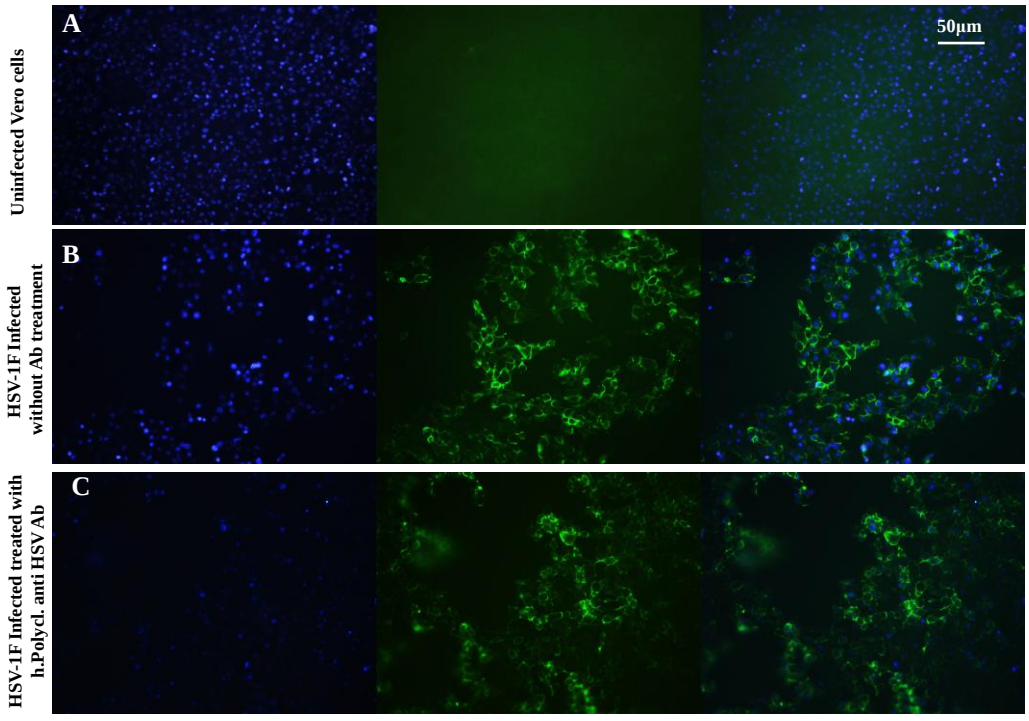
Efficacy of produced IgGs to neutralize HSV infections was evaluated by TCID50 assay using Vero cells. According to this assay required concentration of the gB(HSV)-specific IgGs to neutralize HSV-1F/HSV-2G infection by 100% **(A)** or 50% **(B)** was determined. HDIT101 was included as a positive control. Error bars, showing standard deviation of mean, calculated based on three independent experiments. Three independent experiments (biological replicates on three different days) resulted in identical EC50s in (**A)**, hence no error bars could be shown.

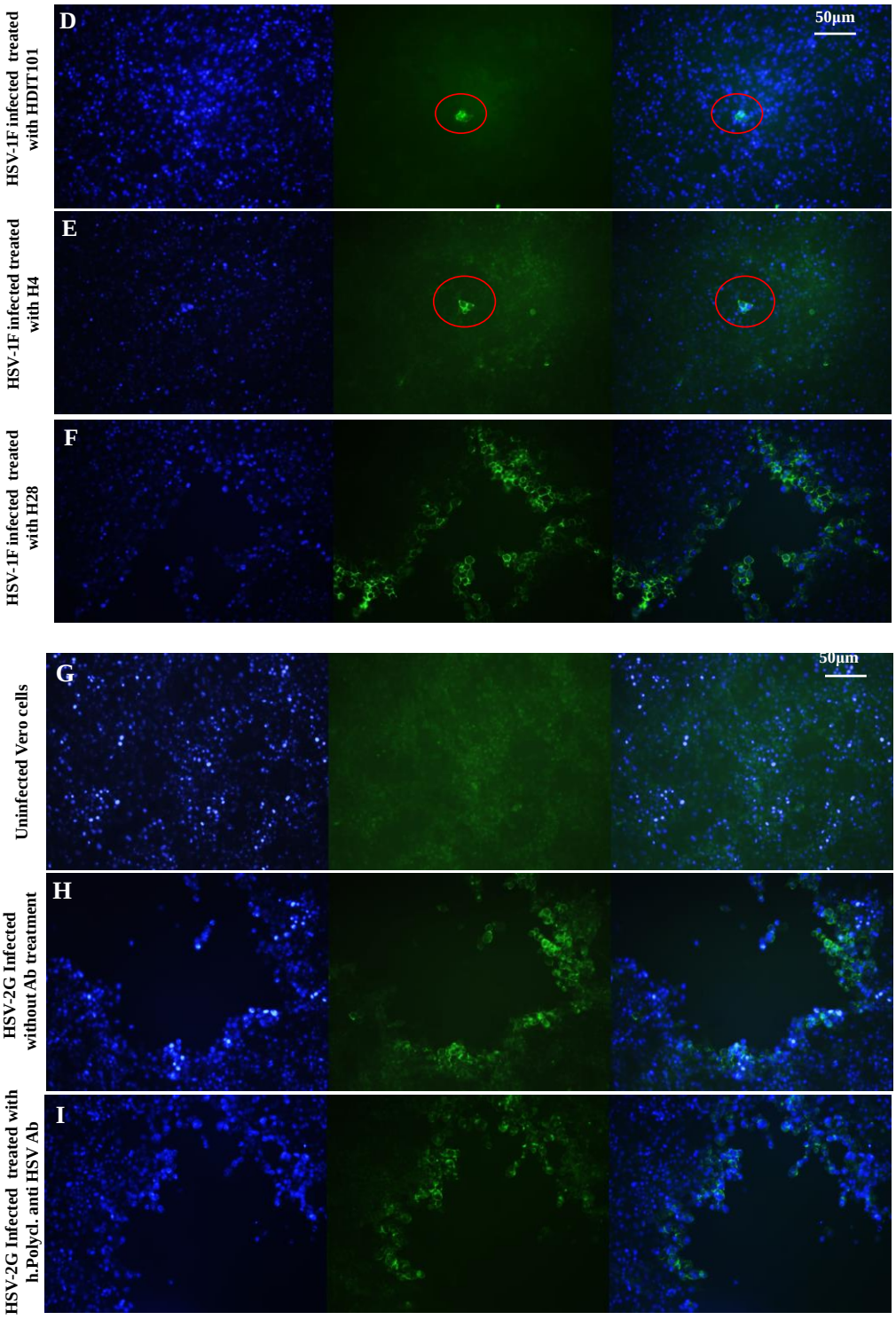
3.6.3 Antibody-mediated inhibition of cell-to-cell spread

All herpes viruses have the ability to pass through cell junctions (Sattentau, 2008) and infect neighbouring cells without being released into the extracellular matrix. Cell-to-cell transmission gives the virus the privilege to escape the humoral immune system (Mothes *et al.*, 2010) because the humoral system is mainly useful in neutralizing extracellular pathogens. In contrast, intracellular pathogens such as viruses are vulnerable to the humoral system during the initial entry into the body and while infecting the cells. Afterwards, by virus cell-to-cell spread without being released in to the extracellular matrix, the humoral immune response has only a small chance to clear the infection. This mechanism is also used by HSV to spread between epithelial cells and neurons (cell-to-neuron spread during primary infection, neuron-to-cell spread during reactivation) or within the infected peripheral tissues (Sattentau, 2008). This intercellular viral dissemination is critical for viral pathogenesis and usually it is not readily accessible for neutralizing antibodies

(Favoreel *et al.*, 2006). Recently developed humanized anti gB(HSV) hu 2c (HDIT101), showed an effective inhibition of the cell-to-cell spread of HSV-1 and HSV-2 for the first time (Krawczyk *et al.*, 2011a) (Bauer *et al.*, 2017b). Thereby to investigate the ability of H4 and H28 antibodies to inhibit direct virus spread from an infected cell to the uninfected neighbour cells, a fluorescence microscopy-based assay was established (Krawczyk *et al.*, 2011b). In order to prevent infection of neighbouring cells by virus released into the cell culture supernatant, media containing the inoculum was removed and replaced by fresh media with excess amounts of neutralizing antibody (75µg/ml).

Analysis by immunofluorescence microscopy confirmed that cell-to-cell spread was completely inhibited in the presence of gB(HSV) specific mAb H4 and HDIT101 (75µg/ml) and the infection was limited to the initial infection of single cells via virions in the inoculum. In contrast, IgG H28 did not show efficient plaque-reducing effects at any used concentration up to 75µg/ml, which corroborates the limited neutralization efficiency of cell-free virus with this antibody (Figure 19). Virus transmission in wells treated with H28 was comparable to the wells treated with human polyclonal anti-HSV antibodies (Figure 20).




D
50µm
HSV-1F infected treated with HDIT101
E
HSV-1F infected treated with H4
F
HSV-1F infected treated with H28
G
50µm
Uninfected Vero cells
H
HSV-2G Infected without Ab treatment
I
HSV-2G Infected treated with h.Polycl. anti HSV Ab

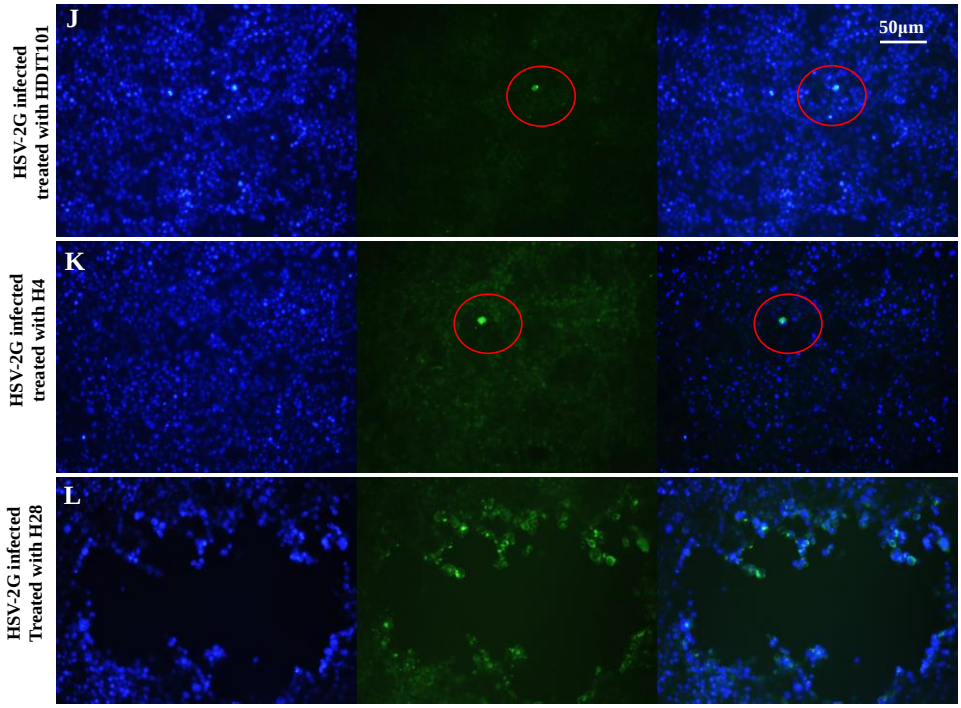


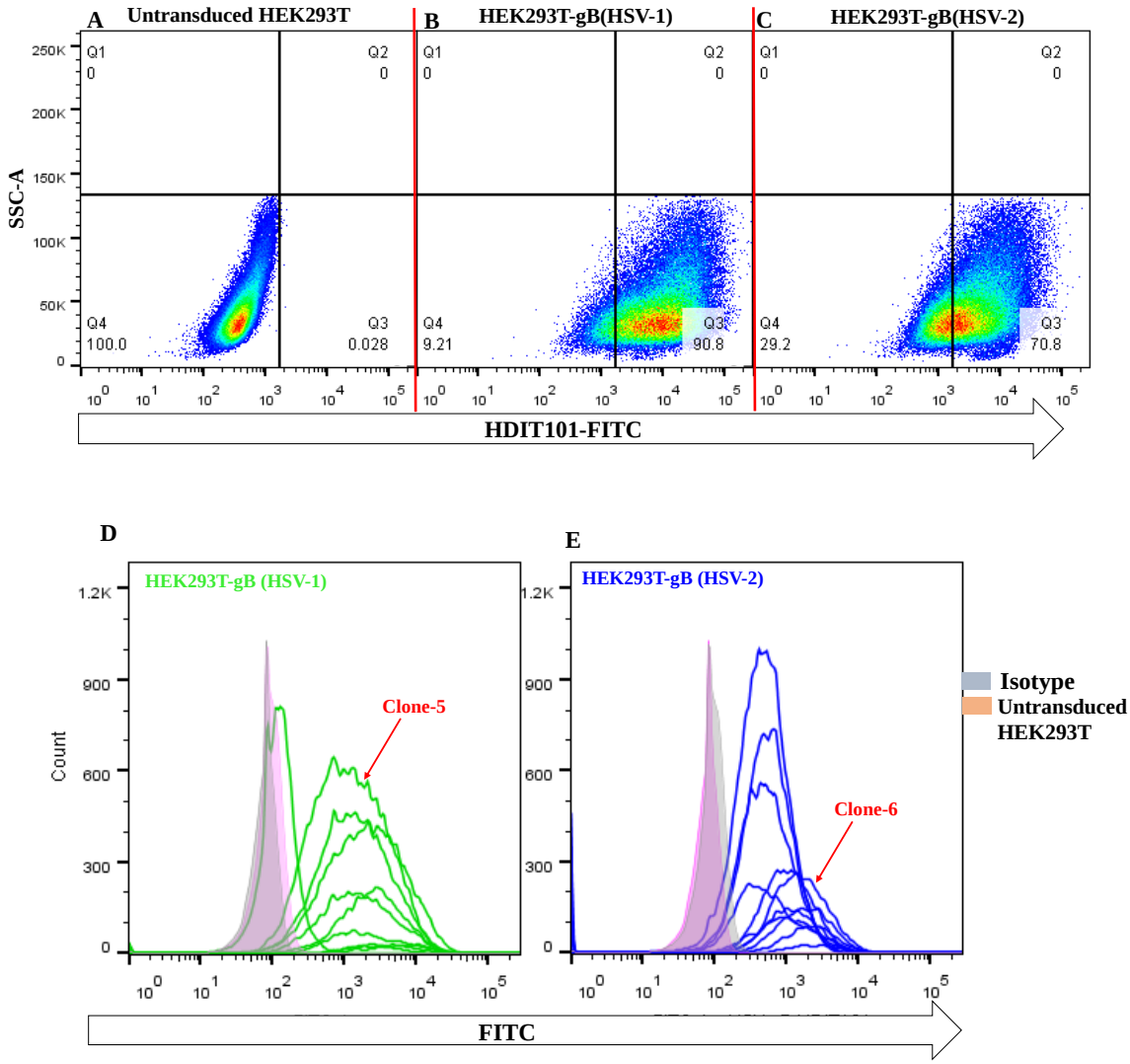
Figure 20. Inhibition of HSV-1F/2G cell-to-cell spread by various gB-specific antibodies.

A-L) Fluorescence micrographs of Vero cells infected with HSV1F/2G and treated with anti HSV antibodies and stained with Hoechst (in blue, left column) and HDIT101-FITC (in green, middle column). Images on the right side of the figure show the overlay of Hoechst and HDIT101-FITC. The cells were infected with HSV-1F/2G and incubated with 500 nM (75 µg) of test antibody for 48 h, washed, stained with HDIT101-FITC (5 µg/ml) and Hoechst (1:5000) and then fixed with 5% paraformaldehyde before imaging (20X, Inverted microscope, Leica). Results are showing one representative image per condition. Human polyclonal anti-HSV IgGs and HDIT101 were used as control.

3.6.4 Establishing HEK 293T stably expressing HSV-1 gB or HSV-2 gB as a model for HSV gB expression and a tool to study anti-gB antibodies

To further characterize binding of the H4 and H28 antibodies to HSV-1 or HSV-2 gB and also to study interaction of anti-gB antibodies outside the context of infectious virus and interaction of the other surface glycoproteins we generated HEK293T ectopically expressing gB. In this way, we could genetically manipulate gB without complicated mutagenesis of a large virus DNA. HEK 293T cells were transduced using a lentiviral vector (LV) to stably express glycoprotein B of HSV-1 and HSV-2 (Provided by Dr. Torsten Schaller). The expression of HSV-1/2 gB was confirmed

by FACS-analysis three days later and showed that 90.8% of cells (HSV-1 gB) and 70.8% (HSV-2 gB) of HEK293T cells were transduced (Figure 21). These cell cultures were subcloned by limiting dilution and more than eight clones per transduction were screened for gB expression. In the case of HSV-1 gB, all screened clones expressed gB, whereas one clone transduces with LV expressing HSV-2 gB did not show a positive staining. Following this result, one clone with the highest expression of HSV-1 gB (clone 6) or HSV-2 gB (clone 5), respectively, was picked and used in further assays. These clones were also tested for binding of H4 and H28 antibodies (Figure 21) (done by Dr. Moritz Ries).



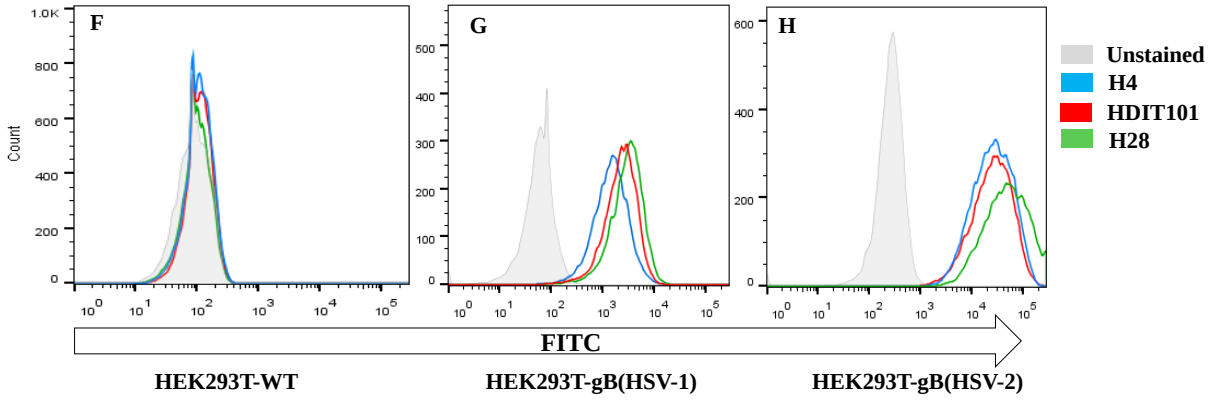


Figure 21. FACS analysis of lentiviral-gB (HSV-1/2) transduced HEK 293T cells.

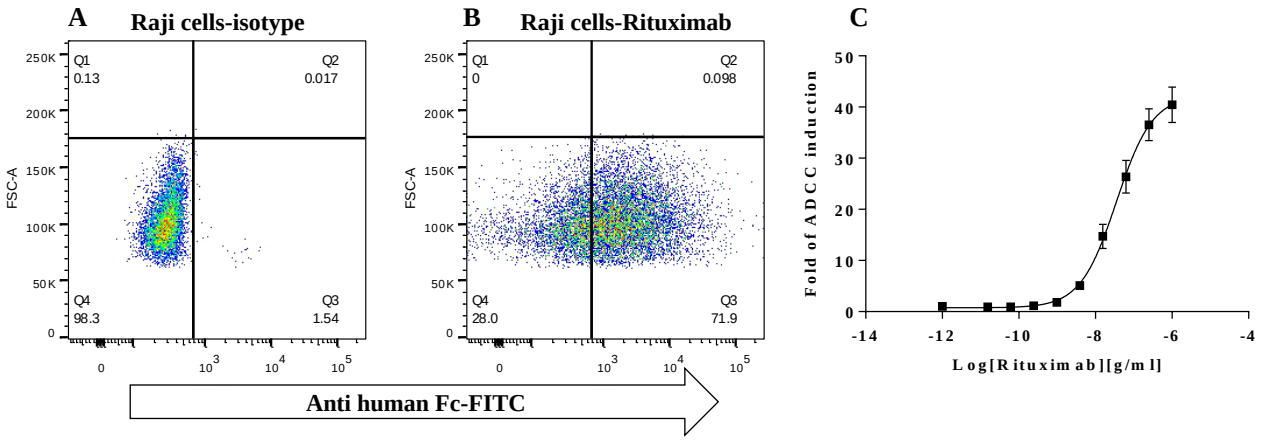
A) Untransduced HEK293T cells as negative control for staining and FACS-gating. **B, C)** HEK293T cells transduced with lentiviral vectors coding for HSV-1F gB **(B)** or HSV-2G gB (**C),** stained for gB expression using HDIT101-FITC. **D, E)** FACS-analysis of glycoprotein B expression (HSV-1F gB in D) and HSV-2G gB in E)) on the single cell clones generated by limiting dilution, clone-5 HEK293T gB (HSV-1) and clone-6 HEK293T gB (HSV-2) (which is shown by arrows in the graphs) with highest gB expression were chosen for the upcoming experiments. Grey and pink histograms represent respective cells stained with isotype control (human anti CD22 IgG1) and unstained cells respectively. Staining was performed as in **(A)**. **F-H)** Comparison of various gB(HSV)-specific antibodies on HEK293T clone-5 and clone-6 stably expressing HSV-1F **(G)** or HSV-2G **(H)** gB, respectively. Grey histograms represent unstained respective cells. Grey histograms represent unstained respective cells.

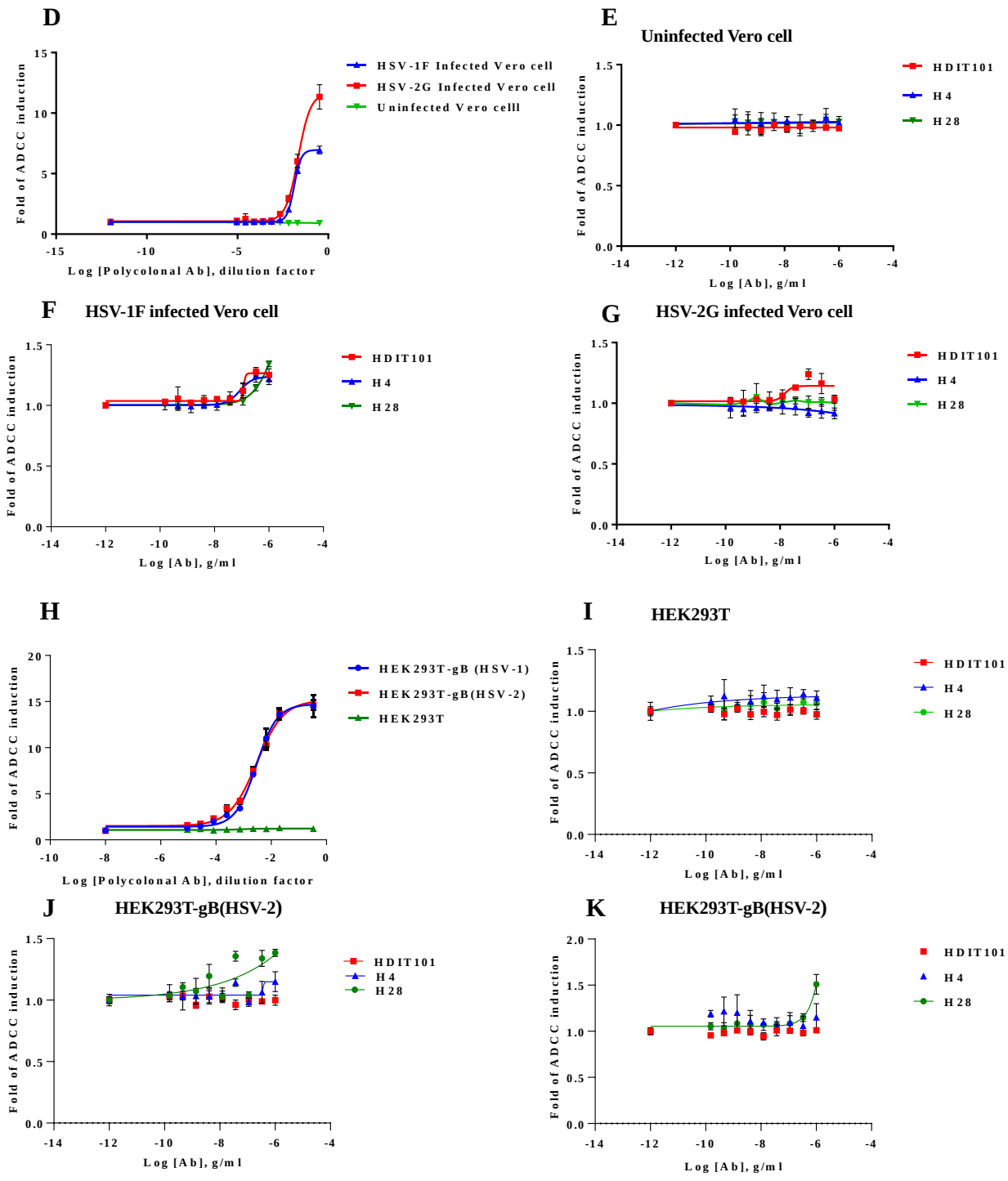
3.6.5 gB(HSV)-specific monoclonal antibodies H4 and H28 are not inducing ADCC

Antibody-mediated protection from viral spread *in-vivo* might be due to direct binding via the Fab domain and inhibition of e.g. interaction of gB with cellular or viral proteins or Fc effector functions such as ADCC, ADCP, CDC, maybe a combination of both. To find out whether the gB(HSV)-specific IgGs can act by the effector function domain (Fc), in-vitro assays were set up to test the Fc effector functions. Monoclonal antibodies can induce cell cytotoxicity by interacting with Fcγ-receptors expressed on immune cells (Hashimoto *et al.*, 1983). The FcγRIIIA receptor (CD16a), expressed by the variety of the effector cells, including macrophage cells and NK cells, interact with the Fc domain of IgGs and mediates cell cytotoxicity (Vidarsson *et al.*, 2014). Since *in-vitro* detection of ADCC often needs very high effector to target cell ratios and also peripheral

blood mononuclear cells often exert heterogeneous effects dependent on the donor, genetically engineered Jurkat cells (Jurkat-CD16-NFAT) were used, stably expressing the FcγRIIIa receptor (V158) which activates an NFAT response element inducing the expression of firefly luciferase (see section 2.2.2.10).

Luciferase activity was quantified by luminescence readout. For these assays, human polyclonal serum containing anti-HSV IgGs was used as positive control for HSV-infected cells and Rituximab bound to Raji cells served as positive control for the assay. According to the results, gB(HSV)-specific monoclonal antibodies H4, H28 as well as HDIT101 did not induce ADCC while Rituximab in presence of cells expressing the target antigen (CD20 on Raji cells) and Human polyclonal serum containing anti-HSV IgGs targeting HSV-1F or HSV-2G infected Vero cells showed strong ADCC induction (Figure 22). Since Rituximab and the studied anti-gB antibodies are derived from the same allotype (G1mza), differences in ADCC activation are likely due to differences in the interaction with the target antigen. IgG1 kappa targeting Hen Egg Lysozyme was used as an isotype control on infected as well as uninfected Vero cells, parental HEK293T and HEK293T expressing gB(HSV) and no ADCC induction was observed (data are not shown).




D
Fold of ADCC induction
Log [Polycolonal Ab], dilution factor
HSV-1F Infected Vero cell
HSV-2G Infected Vero cell
Uninfected Vero celll
E
Uninfected Vero cell
Fold of ADCC induction
Log [Ab], g/ml
HDIT101
H4
H28
F
HSV-1F infected Vero cell
Fold of ADCC induction
Log [Ab], g/ml
HDIT101
H4
H28
G
HSV-2G infected Vero cell
Fold of ADCC induction
Log [Ab], g/ml
HDIT101
H4
H28
H
Fold of ADCC induction
Log [Polycolonal Ab], dilution factor
HEK293T-gB (HSV-1)
HEK293T-gB(HSV-2)
HEK293T
I
HEK293T
Fold of ADCC induction
Log [Ab], g/ml
HDIT101
H4
H28
J
HEK293T-gB(HSV-2)
Fold of ADCC induction
Log [Ab], g/ml
HDIT101
H4
H28
K
HEK293T-gB(HSV-2)
Fold of ADCC induction
Log [Ab], g/ml
HDIT101
H4
H28

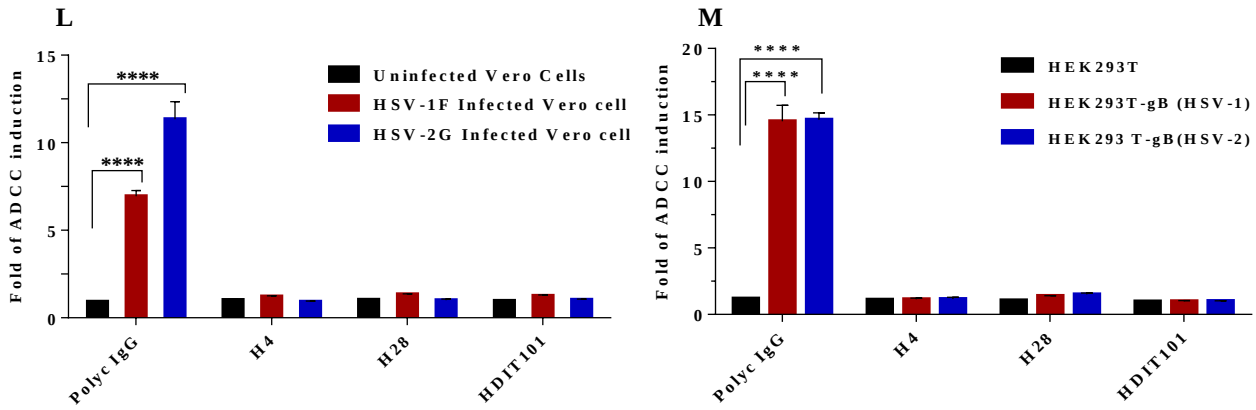


Figure 22. Analysis of ADCC activation by gB(HSV)-specific mAbs using HSV-infected Vero target cells and genetically engineered Jurkat-CD16-NFAT effector cells.

HSV-1F or HSV-2G infected Vero cells were co-incubated with Jurkat-CD16-NFAT cells at an effector: target cell ratio of 15:1 for 6h at 37°C with a serial dilution of HSV gB-specific antibodies (starting from 1000ng/ml, serial dilution 1:3, 8 points). After 6 hours of incubation at 37°C, luciferase substrate was added to the samples, and luminescence was determined using an Infinite F200Pro, Tecan. The RLU values were normalized by formula, Fold of ADCC induction = RLU (induced–background) /RLU (no antibody control–background). The Fold of ADCC induction were plotted against antibody concentration and were fitted to a 4PL (or linear) curve and using GraphPad Prism software. In this assay, Rituximab was used as a positive control. **A)** Raji cells were stained with isotype control (IgG1 kappa targeting Hen Egg Lysozyme). **B)** CD20 on the surface of Raji cells was detected using Rituximab as primary antibody and secondary FITC conjugated antibody. **C)** ADCC induction by Rituximab. Error bars represents standard deviation of mean, N=3. **D)** ADCC induction by human polyclonal anti-HSV IgGs against HSV-1F/2G infected Vero cells. **E, F, G)** ADCC activity of anti-gB antibodies HDIT101, H4 and H28 against uninfected, HSV-1F and HSV-2G infected Vero cells. **H, I, J)** ADCC activity of anti-gB antibodies HDIT101, H4 and H28 against parental HEK293T and HEK293T expressing gB(HSV-1/2) **L, M)** ADCC induction at highest antibody concentrations that were utilized. The differences were calculated with one-way ANOVA, **** P-value <0.0001, N=3 technical replicates.

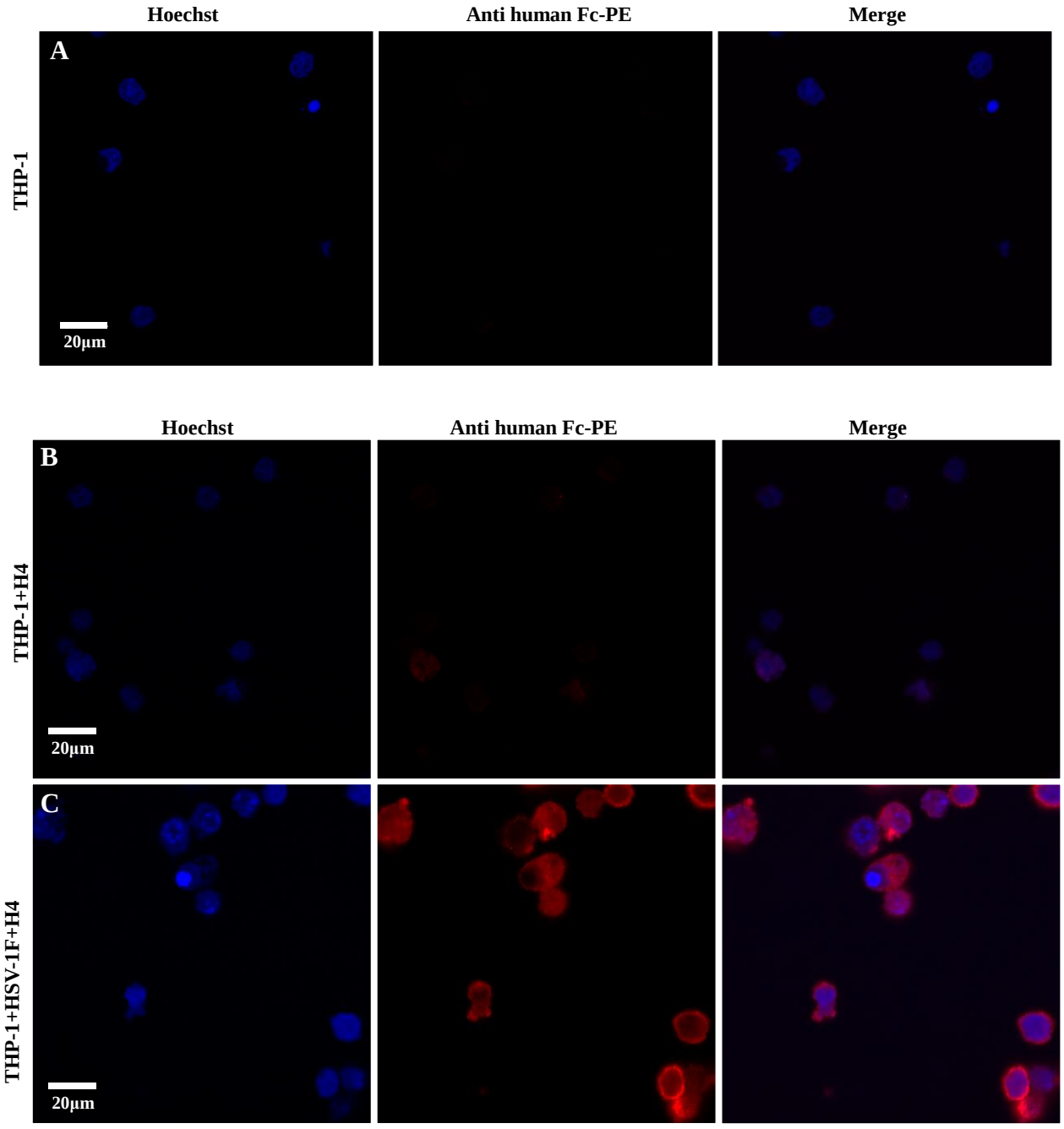
3.6.6 gB(HSV)-specific mAbs H4, H28 and HDIT101 induce cellular phagocytosis

Antibody-dependent cellular phagocytosis (ADCP) is another Fc-mediated function of antibodies which is a common immune response elicited in viral infections. ADCP mediates the clearance of the virus or virus-infected cells and at the same time facilitates antigen presentation which can lead to an activation of downstream adaptive immune responses(Huber *et al.*, 2001). In this regard, the

capacity of HDIT101, H4 and H28 to induce ADCP in-vitro was analysed using THP-1 cells as phagocytic cells. THP-1 cells are a monocytic acute myeloid leukemia cell line that is often used as a surrogate in phagocytic assays since they can be activated by reagents such as phorbol-myristate acetate (PMA) and differentiate into a macrophage-like phenotype (Bosshart and Heinzelmann, 2016). ADCP was determined by microscopic analysis of THP-1 cells incubated with antigen-antibodies complex. Using microscopic imaging, we distinguished virus/antibody attachment from actual engulfment. THP-1 cells were incubated with HSV-1F/2G in the presence of excess amount of antibody (75µg/mL), permeabilized after 20 hours, fixed with paraformaldehyde and stained with anti-human Fc-PE to detect the internalized antibody (Figure 23) or anti gB(HSV)-FITC/Alexa488 to detect the virus localization (Figure 24).

In the first approach (antibody localization), first THP-1 cells were incubated with mAbs in absence of virus to exclude the possible spontaneous antibody binding on the surface of THP-1 cells since they express variety of Fc receptor. Imaging THP-1 cells/mAbs resulted in a weak signal in the cellular periphery probably derived from few attached but not phagocytosed antibodies as it is shown in Figure 23, H4**(B)**, H28**(D)** and HDIT101**(F)**. When this was repeated in the presence HSV-1F a clear intracellular staining (anti-huFc-PE) could be observed for all three of test antibodies (Figure 23), H4**(C)**, H28**(E)** and HDIT101**(G)**. The results suggest that in the presence of virus, complexes of antibody-virus were formed and internalized by THP-1 cells. In the second approach, internalized virus was localized using HDIT101-FITC/H4-Alexa flour 488 to detect glycoprotein B of HSV-1F/2G (Figure 24). First THP-1 cells were incubated with HSV-F/HSV-2G in the absence of antibody to exclude none-Fc driven virus internalization, as it is demonstrated in Figure 24**(B,G)**, no signal were detected in absence of antibody suggesting no spontaneous virus endocytosis. However, when THP-1 cells were incubated with HSV-1F/2G in the presence of gB(HSV)-specific mAbs very strong signal were observed, but not for isotype antibody (human anti CD22 IgG1, Figure 24**(F/K)**), suggesting that complexes of antibody-opsonized viruses were engulfed in target specific way by the THP-1 cells (H4(**C/H**), H28(**D/I**) and HDIT101(**E/J**)Figure 24). We conclude that all of the gB(HSV)-specific mAbs were capable

of mediating ADCP against the target antigen, since we were able to detect virus-antibody complex in the THP-1 cells.



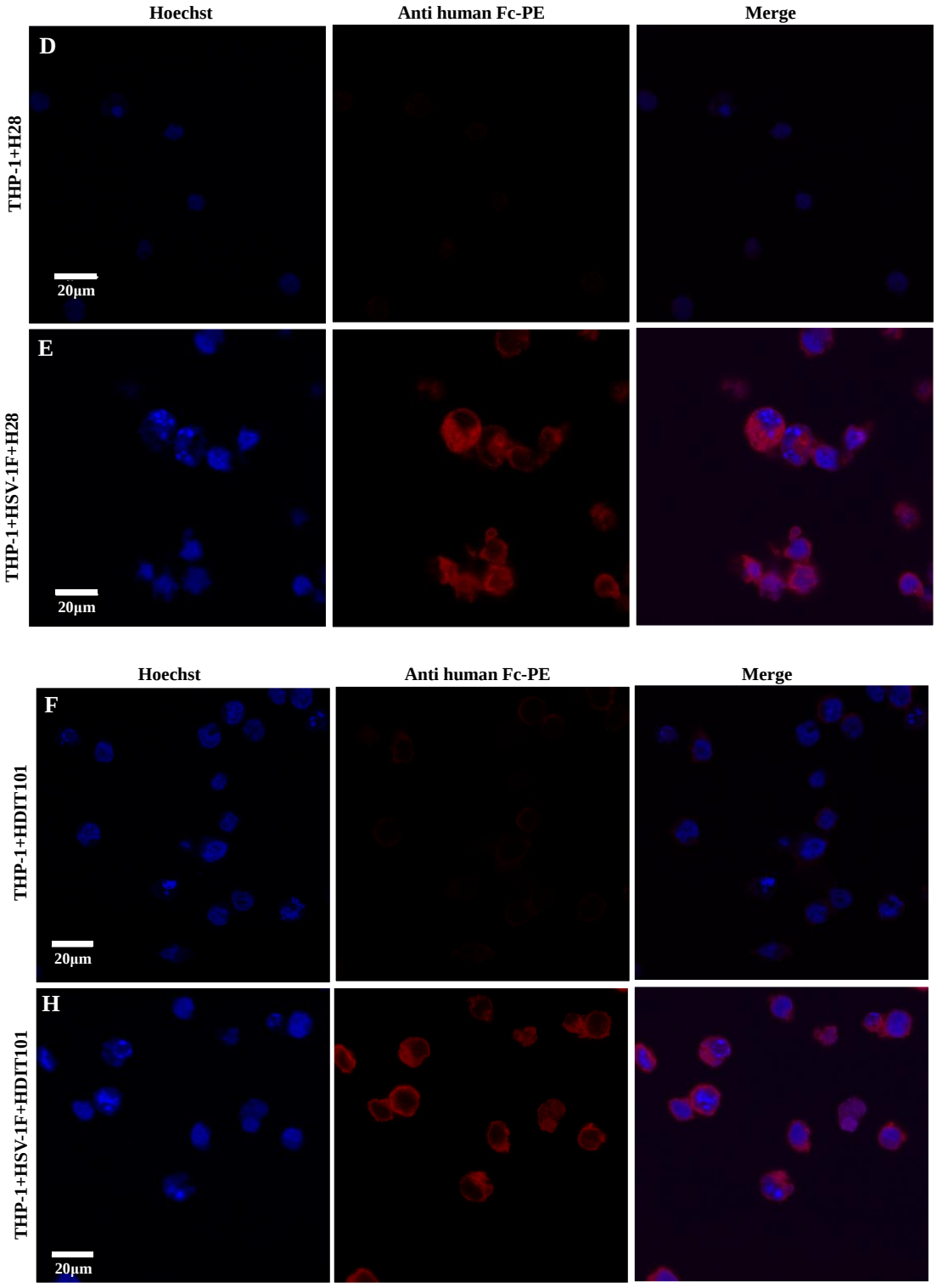
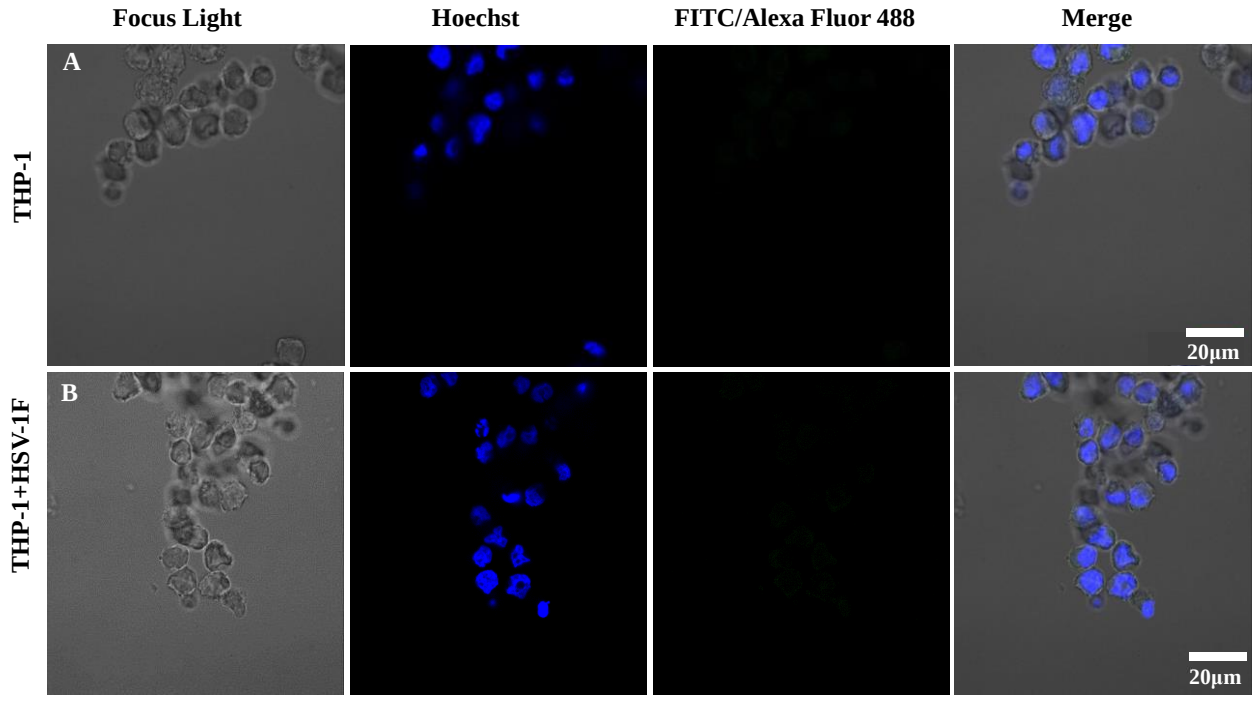
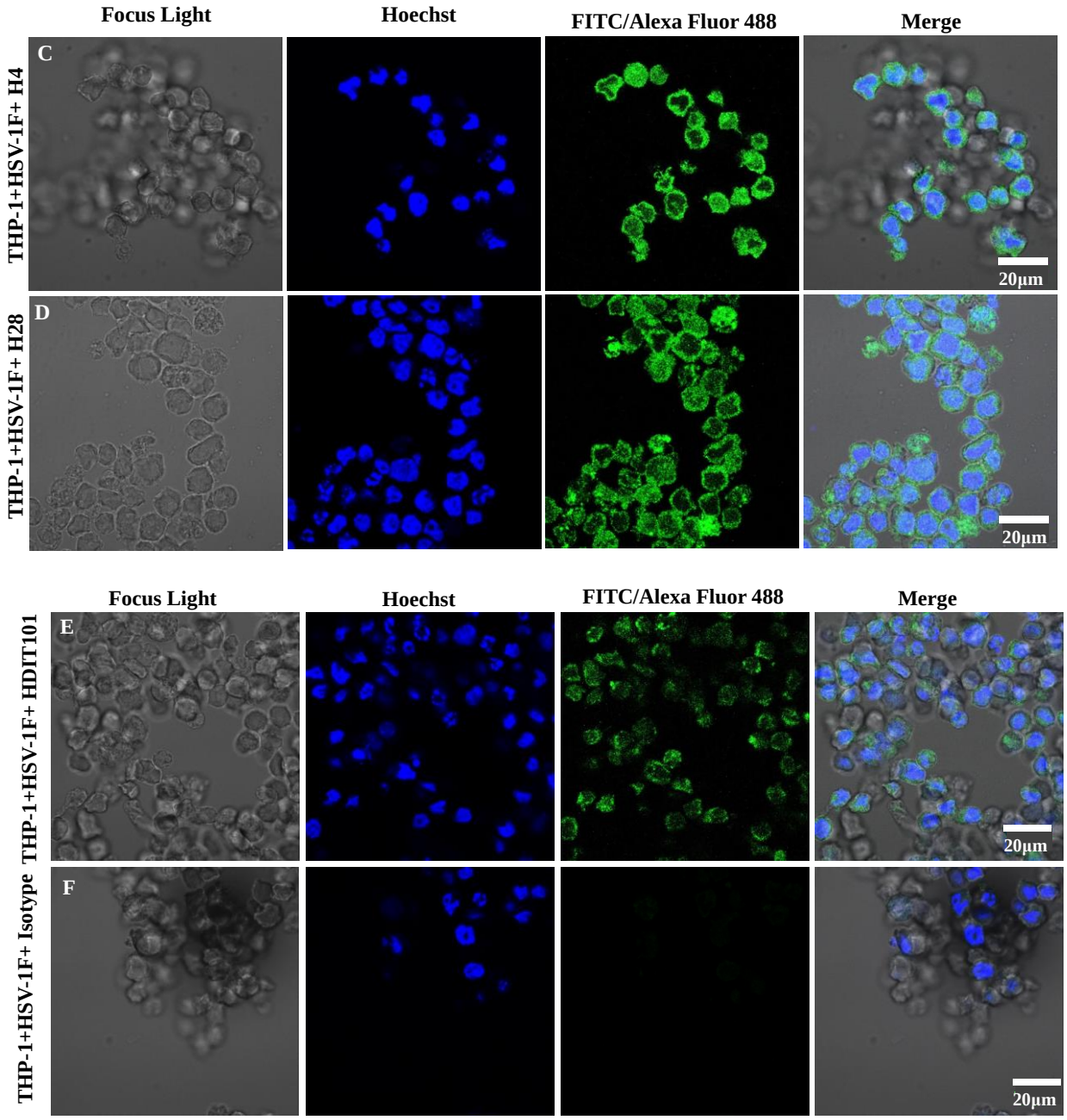

Hoechst
Anti human Fc-PE
Merge
D
THP-1+H28
20μm
E
THP-1+HSV-1F+H28
20μm
Hoechst
Anti human Fc-PE
Merge
F
THP-1+HDIT101
20μm
H
THP-1+HSV-1F+HDIT101
20μm

Figure 23. Antibody-dependent cell phagocytosis (ADCP) induced by gB(HSV)-specific monoclonal antibodies H4, H28 and HDIT101: antibody localization.

ADCP was analysed using a microscopy-based assay in which THP-1 cells were co-incubated with virus and mAbs for 20h (samples without antibody or virus also included as control). Afterwards, the cells were stained, harvested and spun onto glass slides for imaging. The internalized antibodies were detected by mouse anti human Fc–PE antibodies (left). Samples also were stained with Hoechst (middle). **A)** THP-1 cells without antibody-antigen complex. **B)** THP-1 cells incubated with H4 in the absence of HSV-1F. **C)** THP-1 cells incubated with H4/HSV-1F. **D)** THP-1 cells incubated with H28 in the absence of HSV-1F. **E)** THP-1 cells incubated with H28/HSV-1F. **F)** THP-1 cells incubated with HDIT101 in the absence of HSV-1F. **G)** THP-1 cells incubated with HDIT101/HSV-1F. Images were taken by confocal microscopy with 63X magnification. The images are representative of 2 different independent experiments with 3 technical replicates each.




Focus Light
Hoechst
FITC/Alexa Fluor 488
Merge
C
THP-1+HSV-1F+ H4
20µm
D
THP-1+HSV-1F+ H28
20µm
Focus Light
Hoechst
FITC/Alexa Fluor 488
Merge
E
THP-1+HSV-1F+ HDIT101
20µm
F
THP-1+HSV-1F+ Isotype
20µm

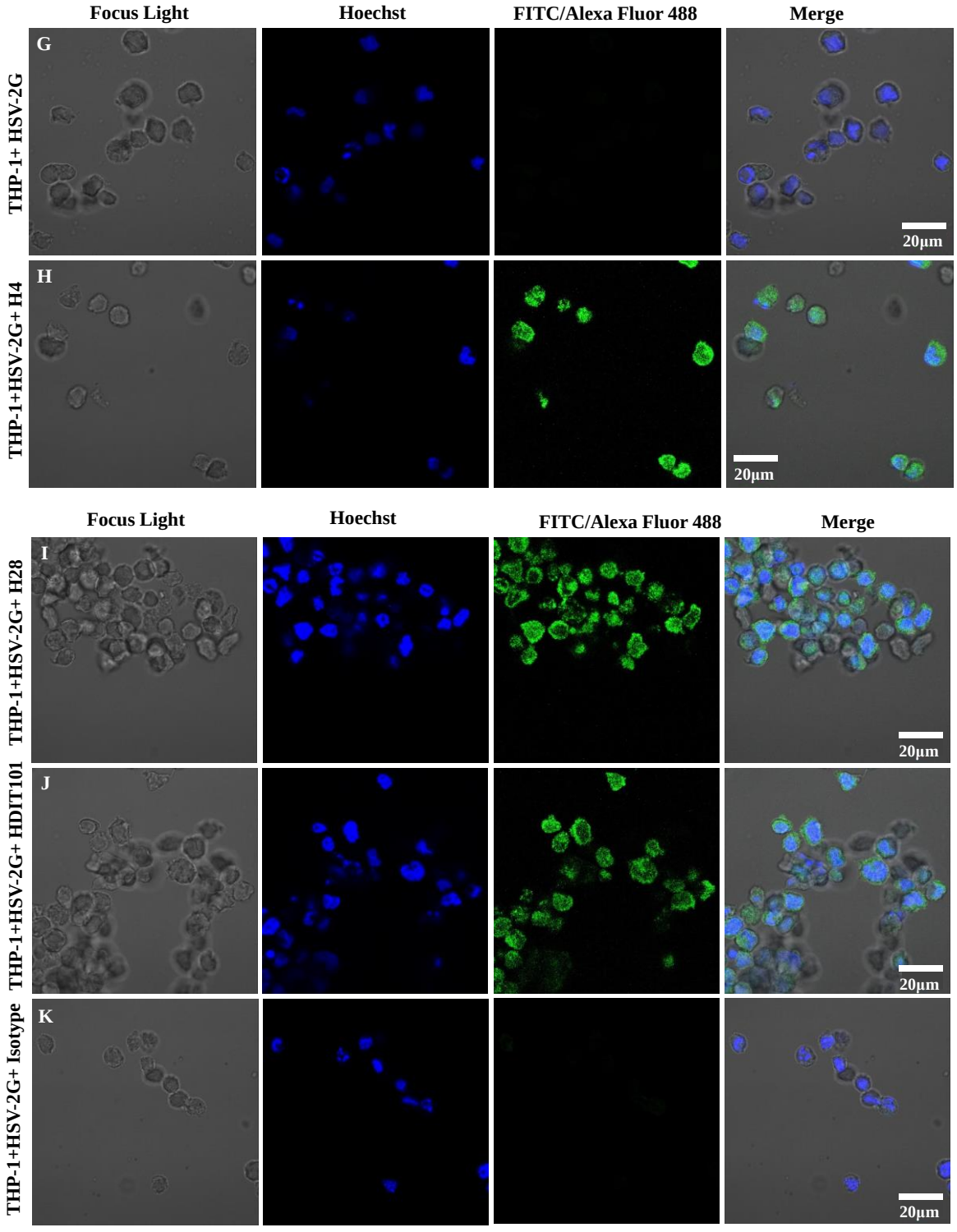

Focus Light
Hoechst
FITC/Alexa Fluor 488
Merge
G
THP-1+ HSV-2G
H
THP-1+HSV-2G+ H4
I
THP-1+HSV-2G+ H28
J
THP-1+HSV-2G+ HDIT101
K
THP-1+HSV-2G+ Isotype
20µm

Figure 24. Antibody-dependent cell phagocytosis (ADCP) induced by gB(HSV)-specific monoclonal antibodies H4, H28 and HDIT101: virus localization.

ADCP was analysed using a microscopy-based assay in which THP-1 cells were co-incubated for 20h with virus in presence of the mAbs. Afterwards, the cells were stained, harvested and spun onto glass slides for imaging. Visualization of opsonophagocytic uptake of HSV-1F/2G by THP-1 cells were performed by staining with H4-Alexafluor 488 or HDIT101-FITC. **A)** THP-1 cells without antibody-antigen complex. **B)** THP-1 cells incubated with HSV-1F in the absence of antibodies. **C)** THP-1 cells incubated with H4/HSV-1F. **D)** THP-1 cells incubated with H28/HSV-1F. **E)** THP-1 cells incubated with HDIT101/HSV-1F. **F)** THP-1 cells incubated with isotype (human anti CD22 IgG1)/HSV-1F. **G)** THP-1 cells incubated with HSV-2G in the absence of antibodies. **H)** THP-1 cells incubated with H4/HSV-2G. **I)** THP-1 cells incubated with H28/HSV-2G. **J)** THP-1 cells incubated with HDIT101/HSV-2G. **K)** THP-1 cells incubated with isotype (human anti CD22 IgG1)/HSV-2G. Images were taken by confocal microscopy with 63X magnification. The images are representative of at least 2 different independent experiments with 3 technical replicates each.

3.6.7 The neutralizing efficacy of gB(HSV)-specific mAbs does not depend on complement activation

Several studies so far showed complement-dependent neutralization of naturally developed antiviral antibodies and/or recombinant monoclonal antibodies. To understand whether the presence of complement could augment the antiviral activity of gB(HSV)-specific mAbs, the neutralization efficiency of the antibodies was tested in the presence of human IgG-depleted serum as source of complement or heat inactivated human IgG-depleted serum, in which complement is inactivated. As a control, the polyclonal human IgG preparation Cytotect® was used, which is characterized by a high proportion of anti-HSV-specific antibodies(Krawczyk *et al.*, 2013b) (Figure 25).

According to the TCID50 assay under with and without complement conditions, both gB(HSV)-specific mAbs H4 and HDIT101 neutralize HSV-1F/2G with similar efficacies regardless of presence or absence of complement (Figure 25). However, this does not necessarily rule out the possibility that the Fc domain of gB(HSV)-specific mAbs may activate complement and induce complement mediated cytotoxicity/cytolysis (CMC) which is a potent mechanism of antibody-dependent cell killing (Hirsch, 1982). Therefore, CMC was measured by a FACS-based assay in which Vero cells were infected with HSV-1F/2G and incubated by gB(HSV)-specific mAbs and

cell lysis was analysed in the presence or absence of complement. In the presence of Cytotect, complement mediated cell lysis of infected Vero cells, since more than 30% of cells were found to incorporate SYTOX, a specific fluorochrome that stains dead/dying cells, however there was no lysis induced by gB(HSV)-specific mAbs neither in the presence of complement nor in its absence (Figure 26).

In addition, to quantify complement activity, the supernatants of antibody-treated infected-cells (+/- complement) were subjected to C5b-9 measurement (see section 2.2.2.14). C5b-9 is the membrane attack complex, which is an end stage compartment of the complement cascade. The data showed no difference in C5b-9 levels of cells treated with the gB(HSV)-specific mAbs or untreated cells in presence of complement, suggesting no complement activity, however Cytotect as positive control showed statistically significant (one-way ANOVA, P-value<0.0001) difference in C5b-9 levels when the complement was present compared to the samples supplied with heat inactivated serum (Figure 27).Unlike murine antibodies, human antibodies can bind to the HSV gE /gI complex. These are the proteins that mimic cellular Fc receptors and can bind Fc domains of IgGs (Johnson and Feenstra, 1987). This could inhibit binding of the C1q protein to the IgG Fc domain and may therefore suppress complement-mediated cytotoxicity (Lubinski *et al.*, 2011).

To exclude a possible effect of gE/gI binding to the Fc domain of the studied antibodies and thereby reducing CMC, the CMC assay was repeated with HEK293T-gB(HSV1/2) cells (Figure 26). In contrast to Cytotect, which induced complement activation in 293T cells expressing gB but not in parental 293T cells, we did not observe complement activation in the presence of H4, H28, or HDIT101 in 293T cells expressing gB. This indicates that even in the absence of HSV gE/gI, CMC is absent and suggests that the negative result in infected Vero cells was not the cause of a masked CMC by gE/gI but rather that the studied anti-gB antibodies are incapable of inducing CMC at least under the studied conditions and in the studied models.

In conclusion, the anti-gB specific antibodies H4, H28 and HDIT101 are all capable of mediating ADCP, while an activity in ADCC or CMC was not detected. Since we also observed a potent neutralizing activity at least for H4 and HDIT101 and to a lesser extent also for H28 in infection

assays in Vero cells, in which phagocytosing cells were absent, we speculate that *in-vivo* these antibodies may function in at least two ways, by directly inhibiting the viral spread and by ADCP-induced immune responses.

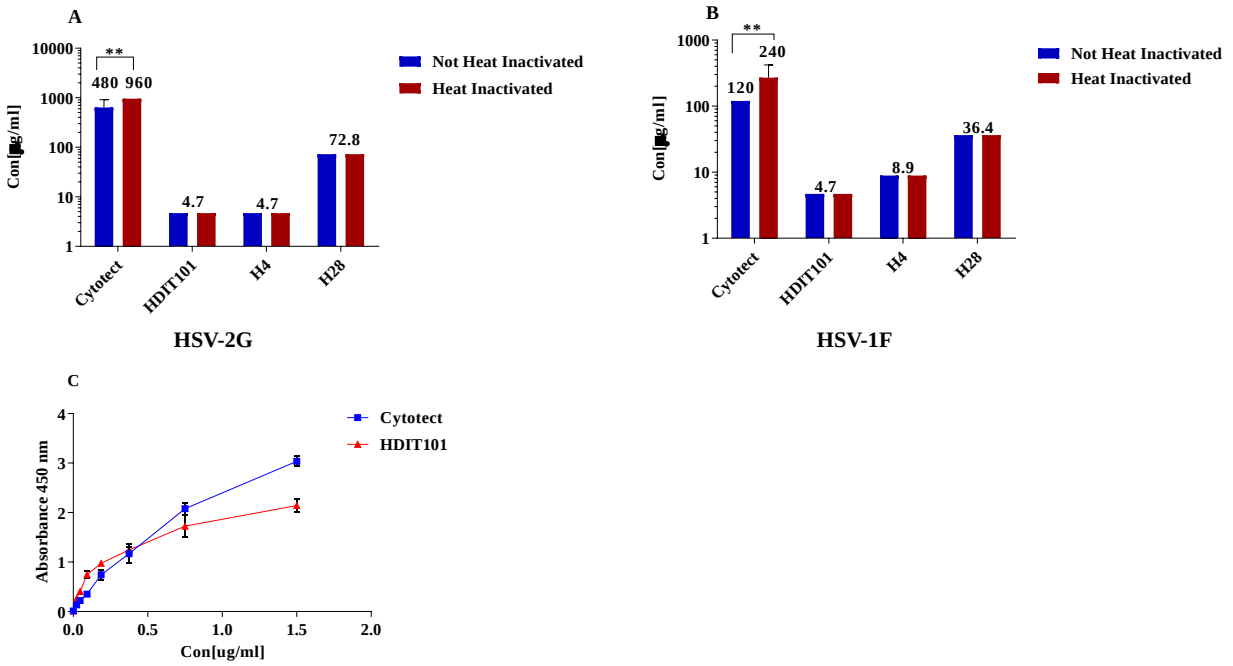
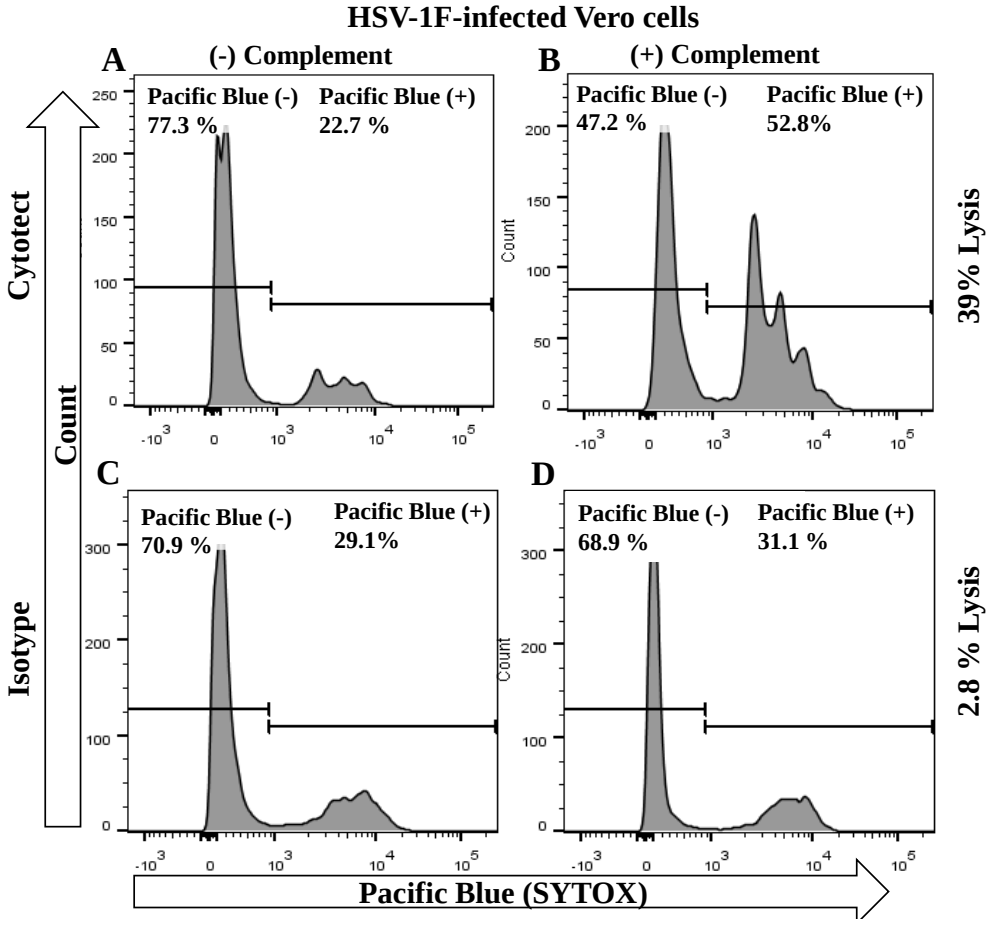
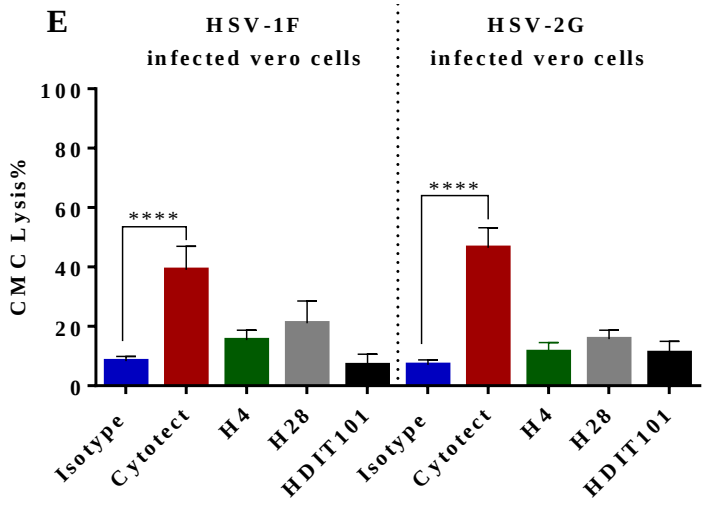
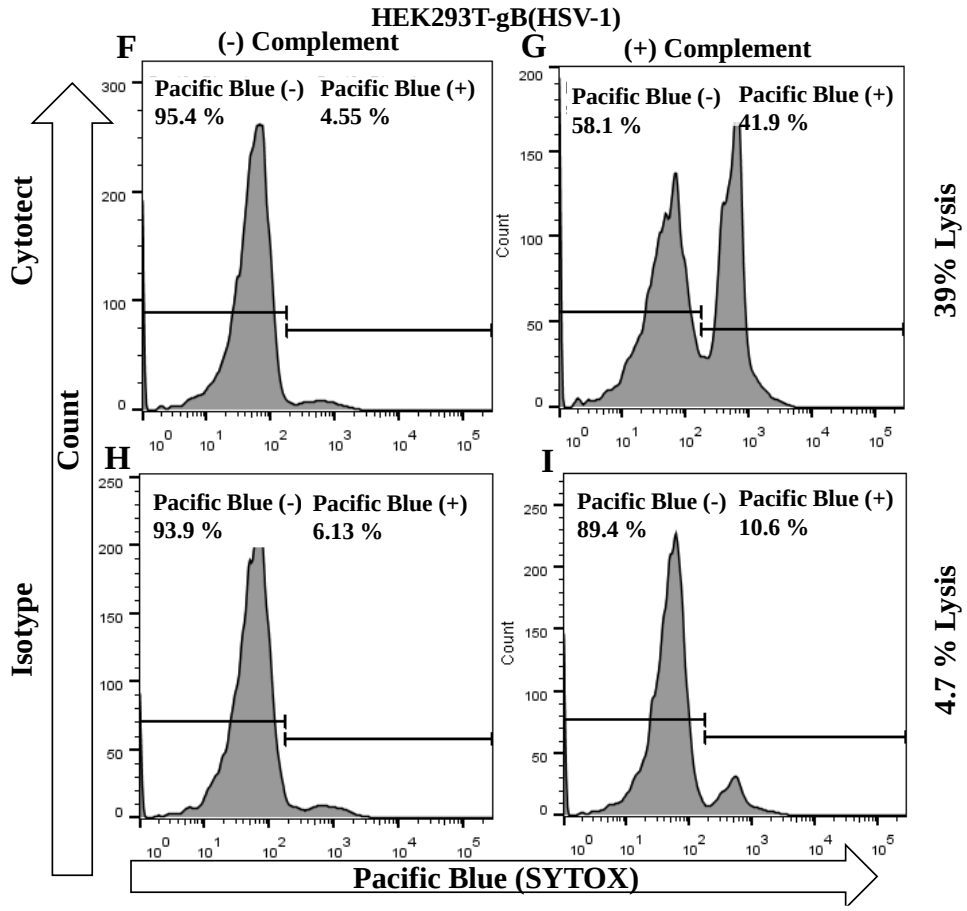


Figure 25. Investigation of complement dependency of H4, HDIT101 and H28' neutralization activity.

Vero cells were incubated with 100TCID50 of HSV-1F **(A)** HSV-2G **(B)** and gB(HSV)- specific mAbs. Neutralization activity was tested with (not heat inactivated) or without (heat inactivated) complement using IgG-depleted human serum. The resulting half maximal effective doses of antibodies to neutralize viral infection were determined 48 h after infection. (TCID50 based assay, the differences were calculated by one-way ANOVA, error bars represent standard deviation of the mean, N=3, ** P-value<0.005). **C)** Cytotect (human polyclonal IgG preparation against CMV) was used as a positive control. As previously shown, Cytotect strongly bound to HSV1F/2G-infected cells and complement enhanced the antiviral effect of Cytotect against HSV-1F/2G ((Krawczyk *et al.*, 2013a)). Error bars representing standard deviation of mean, N=2.


HSV-1F-infected Vero cells
A
(-) Complement
B
(+) Complement
Pacific Blue (-)
77.3 %
Pacific Blue (+)
22.7 %
Pacific Blue (-)
47.2 %
Pacific Blue (+)
52.8%
39% Lysis
Cytotect
Count
C
D
Pacific Blue (-)
70.9 %
Pacific Blue (+)
29.1%
Pacific Blue (-)
68.9 %
Pacific Blue (+)
31.1 %
2.8 % Lysis
Isotype
Pacific Blue (SYTOX)


E
HSV-1F
infected vero cells
HSV-2G
infected vero cells
CMC Lysis%
****
****
Isotype
Cytotect
H4
H28
HDIT101
Isotype
Cytotect
H4
H28
HDIT101


HEK293T-gB(HSV-1)
F
(-) Complement
G
(+) Complement
Pacific Blue (-)
95.4 %
Pacific Blue (+)
4.55 %
Pacific Blue (-)
58.1 %
Pacific Blue (+)
41.9 %
39% Lysis
H
I
Pacific Blue (-)
93.9 %
Pacific Blue (+)
6.13 %
Pacific Blue (-)
89.4 %
Pacific Blue (+)
10.6 %
4.7 % Lysis
Cytotect
Isotype
Count
Pacific Blue (SYTOX)

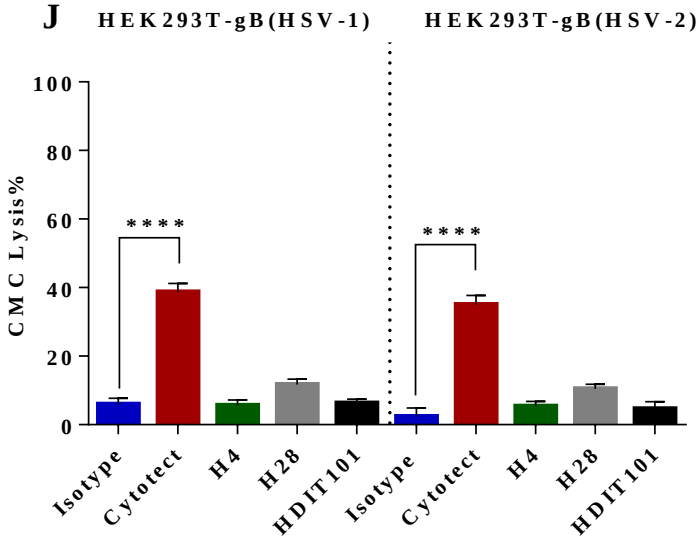

J
HEK293T-gB(HSV-1)
HEK293T-gB(HSV-2)
CMC Lysis%
****
****
Isotype
Cytotect
H4
H28
HDIT101
Isotype
Cytotect
H4
H28
HDIT101

Figure 26. Complement mediated cytotoxicity (CMC) induction by the combination of test antibodies (gB(HSV)-specific mAbs) and media with 20% IgG-depleted human serum.

CMC was determined by FACS analysis of HSV-1F/2G-infected Vero cells or HEK293T-gB(HSV-1/2), incubated with gB(HSV)-specific mAbs using live/dead staining in presence or absence of complement (20% IgG depleted-human serum (not/heat inactivated)) for 20 hours. Examples of SYTOX intensity histogram of **A)** HSV-1F infected Vero cell incubated with Cytotect (75 µg/mL, human polyclonal IgGs produced from human plasma targeting CMV) in absence of complement, **B)** HSV-1F infected Vero cell incubated with Cytotect in presence of complement, **C)** HSV-1F infected Vero cell incubated with human anti CD22 IgG1 as isotype control in absence of complement. **D)** HSV-1F infected Vero cell incubated with human anti CD22 IgG1 as isotype control in presence of complement. **E)** The cell lysis differences were calculated for different groups by one-way ANOVA, **** P-value<0.0001, error bars represent the standard deviation of mean, N=3. Cell lysis was calculated based on SYTOX incorporation (dead cell staining) and according to the formula: Lysis = 100*(% viable cells with inactivated serum - % viable cells with native serum)/ (% viable cells with inactivated serum). **F-J)** CMC assay was repeated with HEK293T-gB(HSV-1/2) as it was performed for HSV-1/2 infected Vero cells.

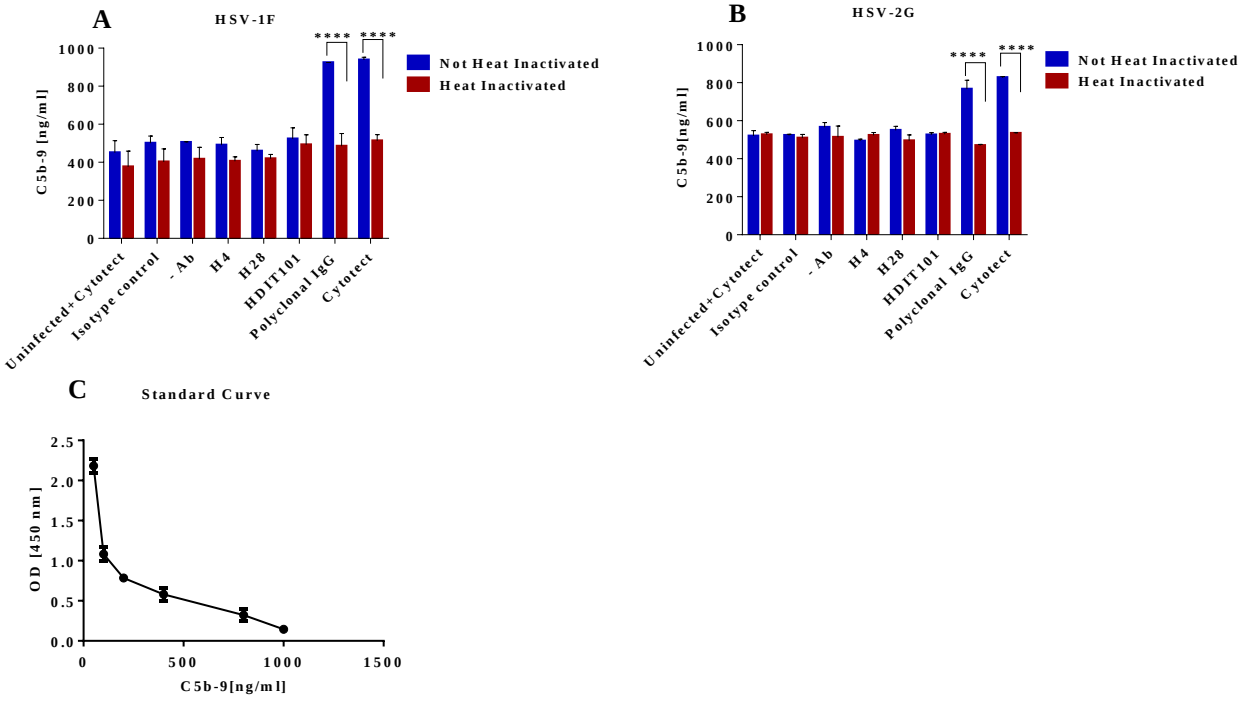


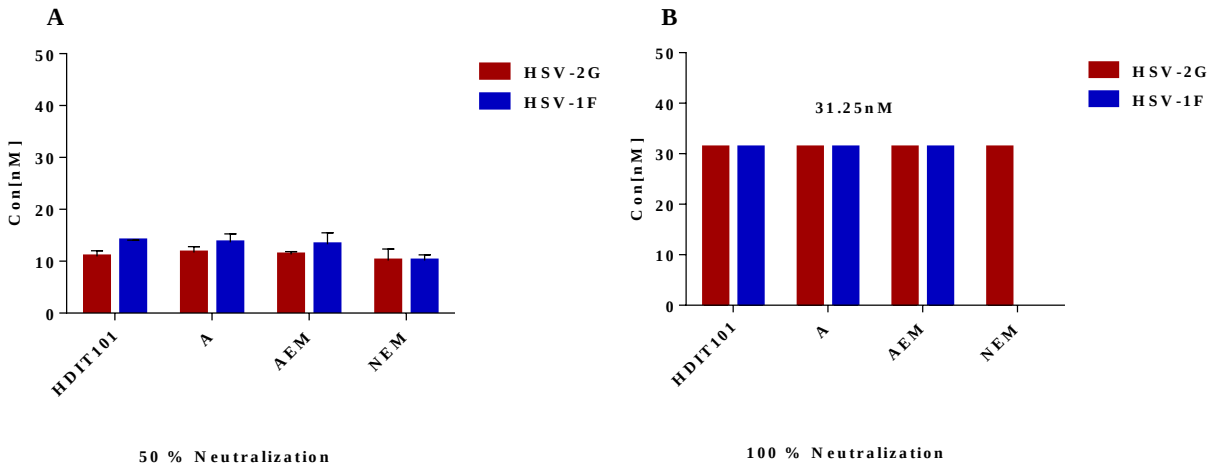
Figure 27. Quantitative evaluation of the terminal C5b-9 complement complex.

An ELISA based assay was employed to measure the levels of C5b-9 complex as terminal complement complex to quantify the complement activation in presence of gB(HSV)-specific mAbs. HSV-1F/2G infected or uninfected Vero cells (A, B) were incubated with not/heat inactivated IgG-depleted human serum in presence or absence of test antibodies. The supernatants were collected and subjected for an ELISA assay using plates coated with an anti C5b-9 antibody. Polyclonal human anti-HSV preparation and Cytotect were used as positive controls. C) the standard curve was provided by using standards prepared in the kit according to the manufacturer's instructions.

3.7 Aglycosylated gB(HSV)-specific mAb HDIT101 has similar neutralization efficiency as the WT antibody

Fc glycosylation of IgG can have a critical role in the antibody effector function (Saxena and Wu, 2016). Therefore, antibody glycoengineering to produce antibodies with specific desired therapeutic efficacy is one of the emerging strategies to tailor immune effector function. According to the *in-vitro* Fc function analysis, the gB(HSV)-specific mAbs H4, H28 and HDIT101 did not induce complement activation or ADCC, but mediated ADCP. On the other hand, gE/gI expressed on the HSV virus envelope and as well as host cell membrane play a role as FCγR which might bind to Fc region of gB(HSV) specific mAbs and limit the antibodies' flexibility to bind to the target and consequently change the neutralization potency of antibodies. Therefore, a deep understanding of the impact of glycosylation on the functionality of the antibodies *in-vitro* may help to explore the mode of action in more detail.

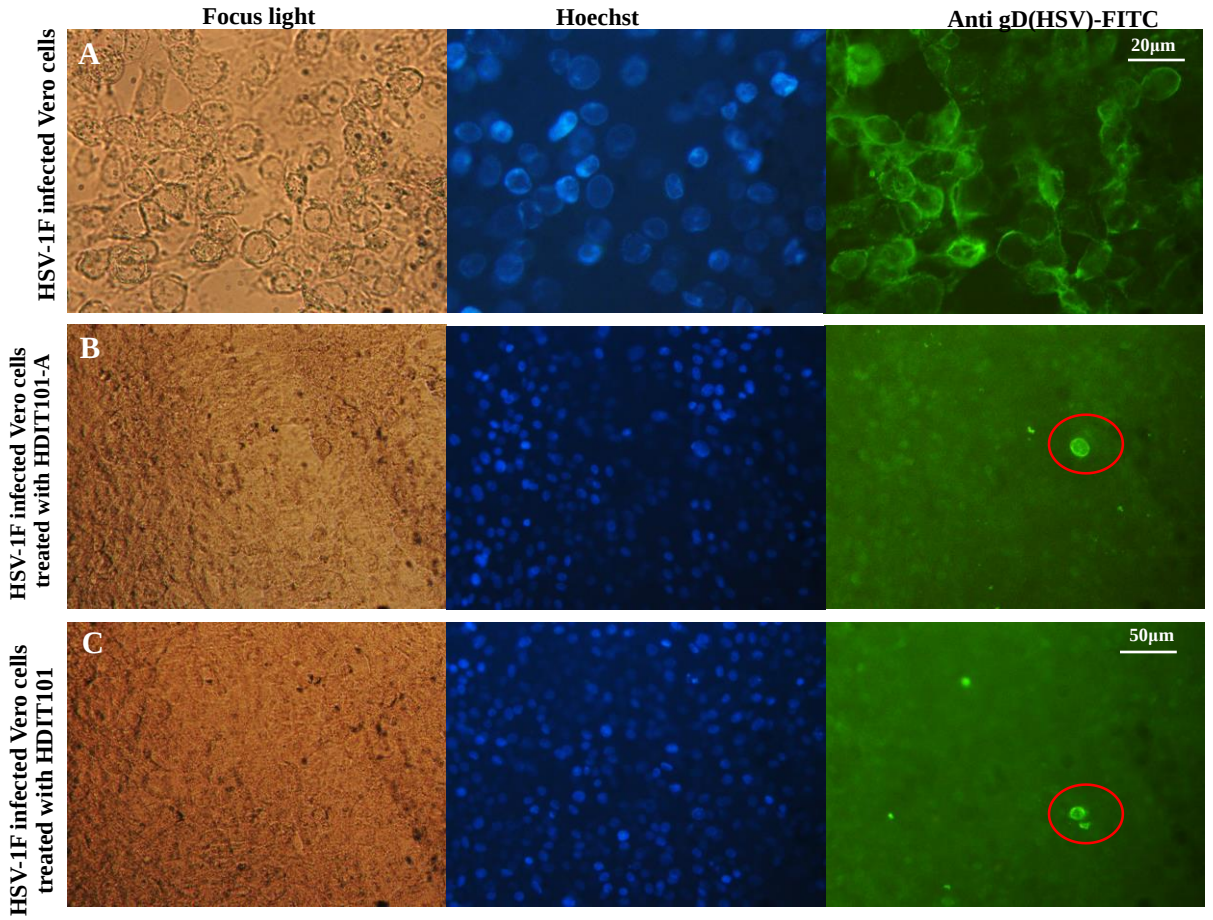
We created different Fc mutants of HDIT101 (N297A (A), N297A+D366E+L358M (AEM) and D366E +L358M (NEM)) and we tested the neutralization in Vero cells *in-vitro* (**Figure 28**) as well as cell-to-cell spread inhibition efficiency for HDIT101 N297A (Figure 29) of these mutants. We compared these mutants to the HDIT101 antibody without Fc modifications. Regardless of changes in the Fc domain we observed the same potency in neutralizing HSV-1F with the mutant variants as compared with parental HDIT101 antibody was observed. Similar to WT antibody, 31.25 nM of the either one of the Fc mutants was sufficient for 100% neutralization of HSV-1F/2G. This suggests that glycosylation does not impact the neutralization efficiency of HDIT101 *in-vitro*.

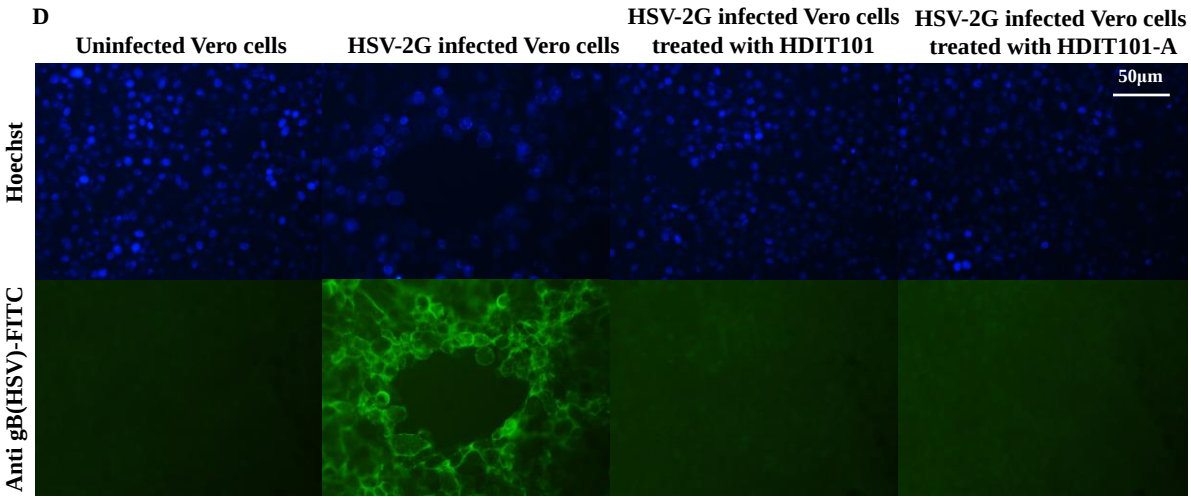


A: N297A
AEM : N297A+D366E +L358M
NEM : D366E +L358M

Figure 28. Assessment of the neutralization efficiency of Fc –modified HDIT101.

Three different Fc mutants of HDIT101 were tested: A: N297A, AEM: N297A+D366E +L358M, NEM: D366E +L358M. Required concentration of antibodies to neutralize 100 TCID50 of wild type HSV-1F and HSV-2G up to 50% neutralization (A) and 100% neutralization (B) were determined. Error bars are representing standard deviation of mean (N=3). Three independent experiments (biological replicates on three different days) resulted in identical EC50s in **(B)**, hence no error bars could be shown.


Focus light
Hoechst
Anti gD(HSV)-FITC
A
B
C
20μm
50μm
HSV-1F infected Vero cells
HSV-1F infected Vero cells treated with HDIT101-A
HSV-1F infected Vero cells treated with HDIT101


D
Uninfected Vero cells
HSV-2G infected Vero cells
HSV-2G infected Vero cells treated with HDIT101
HSV-2G infected Vero cells treated with HDIT101-A
50μm
Hoechst
Anti gB(HSV)-FITC

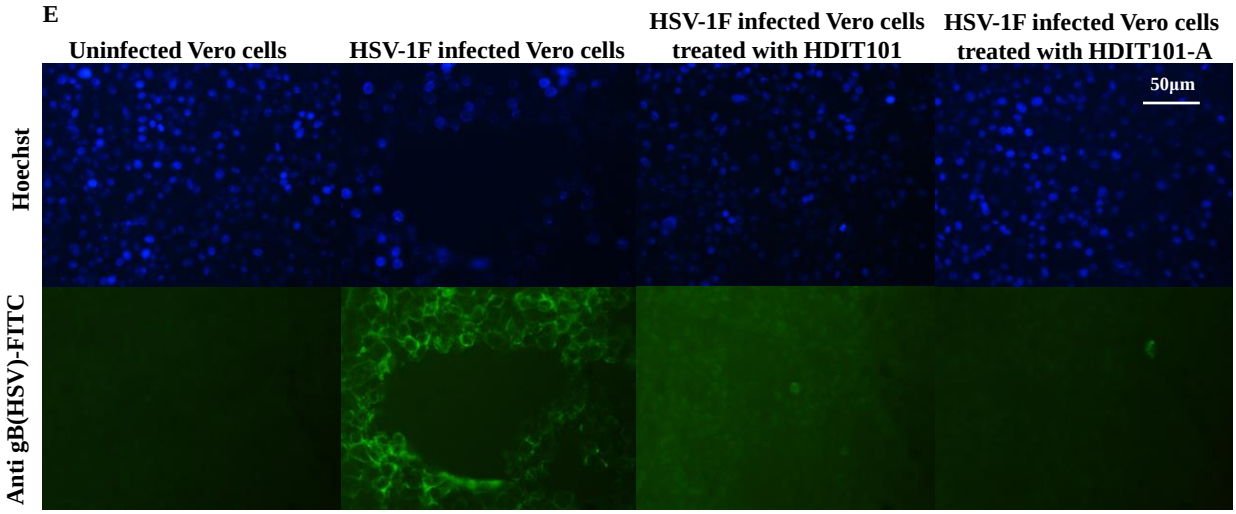


Figure 29. Assessment of the cell-to-cell transmission block using Fc mutated antibody via a microscopy-based assay.

HSV-1F/2G infected Vero cells were treated with WT antibody(HDIT101) or Fc modified variant (HDIT101, N297A) antibody, stained with mouse anti gD(HSV1/2)-FITC antibody (panel A, B, C) or human anti gB(HSV1/2) HDIT101-FITC antibody (panel D, E). Similar to WT antibody, 500 nM of the Fc mutated was sufficient for blocking cell-to cell transmission. Images were taken with inverted fluorescence microscope with 40x (A) and 20x objectives.

3.7.1 Neutralization of escape mutants

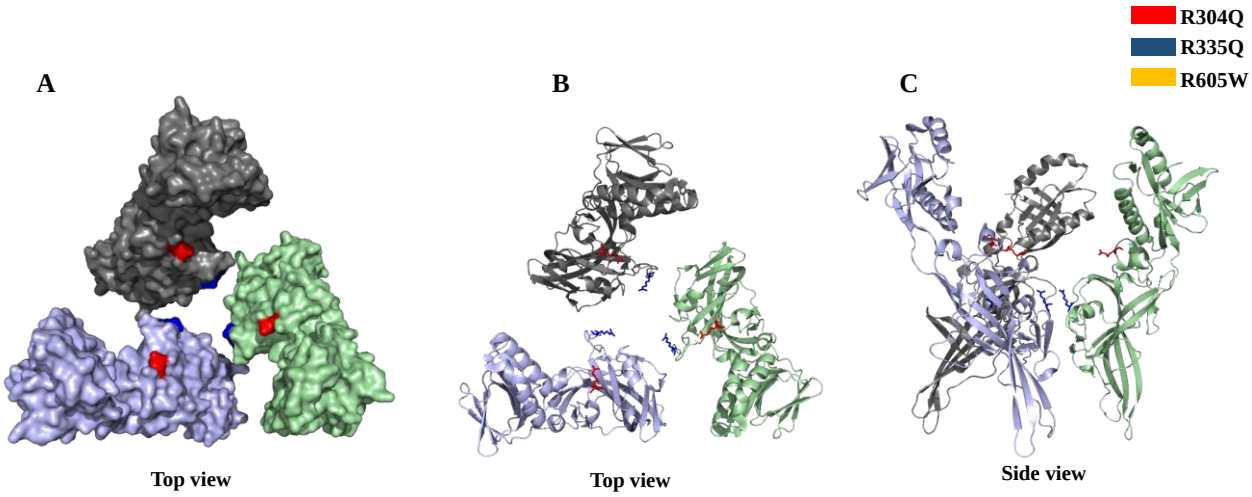
To identify the epitopes targeted by the gB(HSV)-specific monoclonal antibodies, we aimed to generate viral escape mutants by serial passages of HSV-1F/2G on Vero cells in the presence of suboptimal doses of neutralizing gB(HSV)-specific monoclonal antibodies. After sequential HSV-1F/2G passages in the presence of increasing concentrations of HDIT101, H4 or H28, the resulting resistant viruses were cloned by limiting dilution and infection of Vero cells. The resulting viral clones showed resistance towards neutralization by the respective mAbs (data are not shown).

To identify the escape mutations that conferred resistance, the glycoprotein B coding region of H4 and H28-resistant HSV clones were amplified from total DNA of infected Vero cells and sequenced. Sequencing analysis of these escape mutants showed single mutations leading to amino acid substitution in the gB (HSV-1F/2G) protein sequence. According to several sequences' results, R304Q, R296Q, R335Q, R327W, R605W amino acid substitutions were confirmed in

HDIT101-resistant HSV-1F (HDIT101RHSV1), HDIT101-resistant HSV-2G (HDIT101RHSV2), H4-resistant HSV-1F (H4RHSV1), H4-resistant HSV-2G (H4RHSV2), H28-resistant HSV-1F (H28RHSV1), respectively (Figure 30)

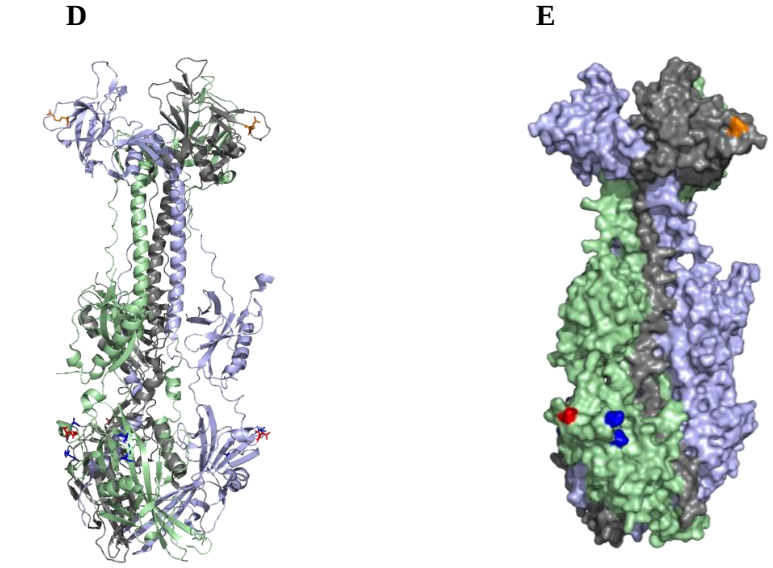
Interestingly, escape mutation against antibodies H4 and HDIT101 in HSV-1 and HSV-2 gB conferred amino acid substitutions in epitopes conserved in HSV-1 and HSV-2 gB. We were unable to generate an H28-resistant HSV-2G mutant. To define the site of the amino acids that conferred resistance to gB(HSV)-specific mAbs, these residues were mapped onto the existing post-fusion gB(HSV-1 F) protein and predicted prefusion gB(HSV) 3D structure (Figure 30). HSV-1 gB amino acid R304 that conferred escape from neutralization by HDIT101 is located in domain I and is exposed at the surface of the postfusion structure (figure). However, amino acid R335 which conferred resistance to H4 induced neutralization is not resolved in the current gB postfusion structures. Amino acid R605, which is found in domain IV of gB and which likely conferred resistance to HSV-1 neutralization by H28 upon change was most distantly located at the end of the gB structure (Figure 30). All amino acids that conferred neutralization escape appear hydrophobic or solvent accessible and highly exposed or hidden.

We showed that the grown viruses were resistant to the specific antibody that they were raised against. However, whether this was a result of escaping the neutralization of cell-free virus or whether this was an escape from antibody-induced block of cell-to-cell spread was unclear. Hence, the gB(HSV)-specific mAbs' capacity to block cell-to-cell spread of mutants also were evaluated by microscopic based assay. The data shows that escape mutants can spread by cell-to-cell transmission even in the presence of the antibodies that they were evolved against (Figure 31). In addition, Vero cells were infected with wild type HSV-1F/2G and treated with gB(HSV)-specific mAbs (as a control) to compare the antibodies potency against wild type and resistant virus in the same experiment (data are not shown).



Surface structure of predicted gB(HSV) prefusion

Ribbon structure of predicted gB(HSV) prefusion



Ribbon structure of gB(HSV) post-fusion, side view

Surface structure of gB(HSV) post-fusion, side view

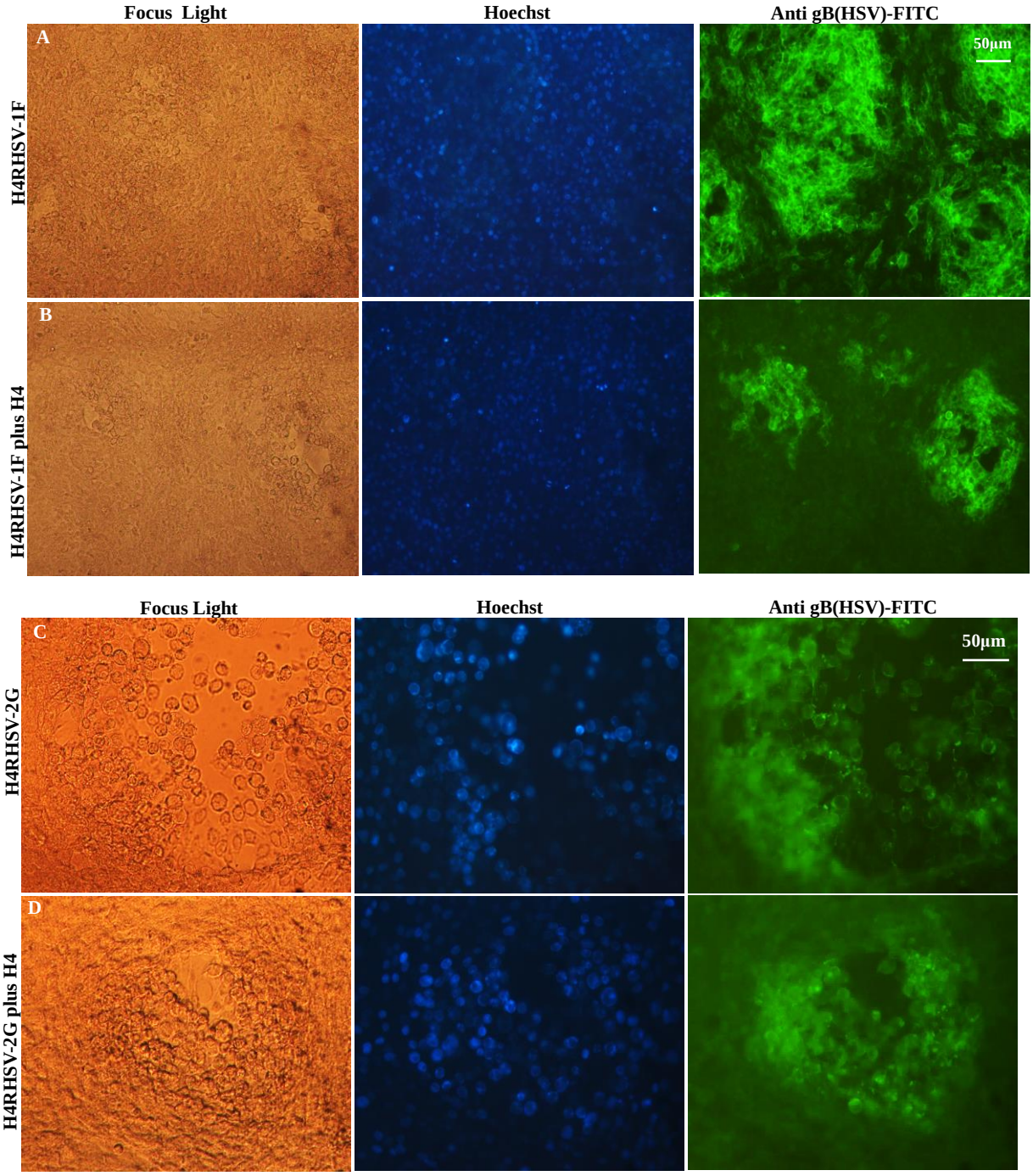
Figure 30. The escape mutant residues mapped onto the existing post-fusion gB(HSV-1 F) protein and predicted prefusion gB(HSV) 3D structure.

A) Top view surface structure of predicted trimer gB(HSV) prefusion structure (471 amino acids). gB promoters are shown in three different colours, green, grey and blue. Escape mutation residues: R335 (H4) (R335Q is not resolved in the postfusion structure hence, the area around this mutation was mapped on the postfusion structure), R304 (HDIT101) are highlighted in dark blue and red, respectively. **B)** Top view ribbon structure of predicted trimer gB(HSV) prefusion structure (471 amino acids). **C)** Side view ribbon structure of predicted trimer gB(HSV) prefusion structure (471 amino acids). **D)** Side view ribbon structure of existing trimer gB(HSV) post-fusion structure (700 amino acids). R335 (H4), R304 (HDIT101) are highlighted in dark blue and red, respectively. R605 residue is shown in orange highlight on top of the gB crown. **E)** Side view surface structure of existing trimer gB(HSV) post-fusion structure (700 amino acids). The 3D structures are adapted from published gB structures and modified using PyMol software.

Table 36. Summary of amino acid substitutions found in escape mutants.

	H4	**HDIT101**	**H28**
HSV-1F	R335Q H4-resistant HSV-1F	R304Q HDIT101-resistant HSV-1F	R605W H28-resistant HSV-1F
HSV-2G	R327W H4-resistant HSV-2G	R296Q HDIT101-resistant HSV-2G	NF

NF: Not found


Focus Light
Hoechst
Anti gB(HSV)-FITC
A
50µm
H4RHSV-1F
B
H4RHSV-1F plus H4
Focus Light
Hoechst
Anti gB(HSV)-FITC
C
50µm
H4RHSV-2G
D
H4RHSV-2G plus H4

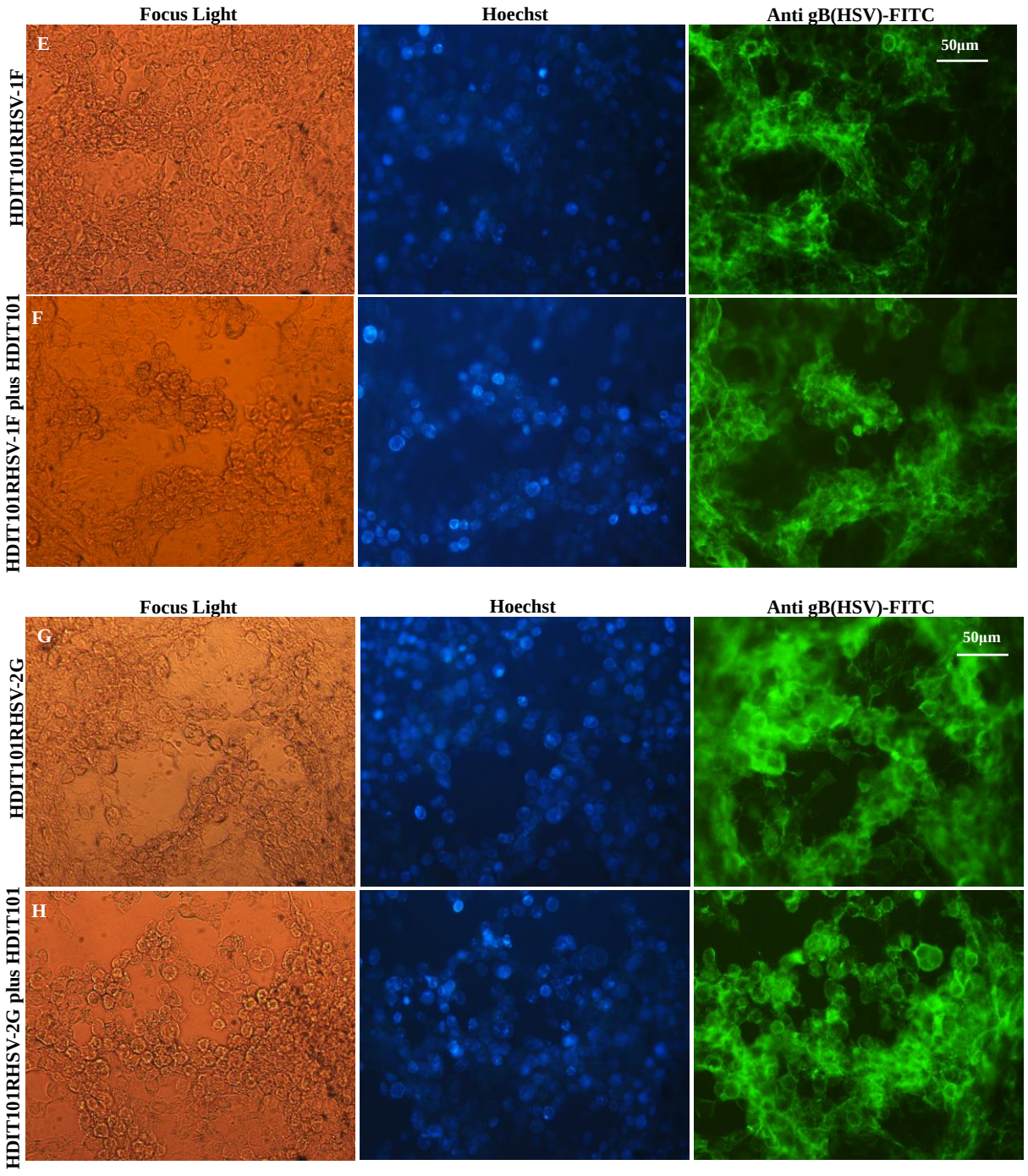
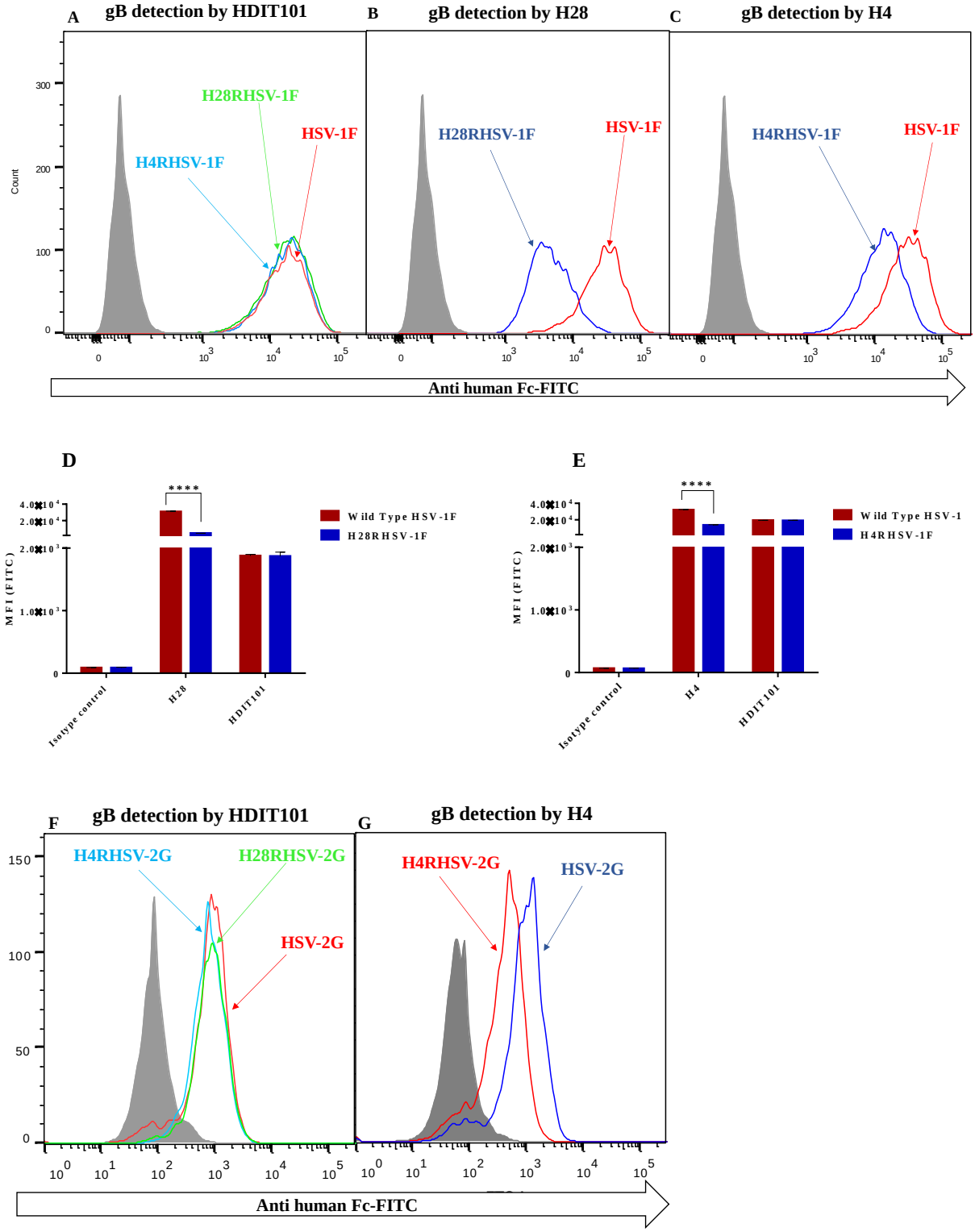


Figure 31. gB(HSV)-specific mAbs do not block cell-to-cell transmission of resistant viruses.

A cell-to-cell spread inhibition assay was performed with resistant viruses obtained after four passages under suboptimal concentrations of HDIT101 and H4. Vero cells were infected with resistant viruses as indicated. Infected

cells were treated post-infection with gB(HSV)-specific mAbs (HDIT101 and H4) (75μg/mL), fixed, stained with HDIT101-FITC/H4-Alexa flour 488 (right) (and Hoechst, middle). Bright-field microscopy images (left) were included to visualize plaques. Images are representative of two independent experiments. (Magnification 20 X). A) H4RHSV-1F infected cells without treatment. B) H4RHSV-1F infected Vero cells treated with H4. C) H4RHSV-2G infected Vero cells without treatment. D) H4RHSV-2G infected Vero cells treated with H4. E) HDIT101RHSV-1F infected Vero cells without treatment. F) HDIT101RHSV-1F infected Vero cells treated with HDIT101. G) HDIT101RHSV-2G infected Vero cells without treatment. H) HDIT101RHSV-2G infected Vero cells treated with HDIT101.

The data shows that all of the specific HSV resistant mutants escaped from neutralization by the respective antibody that they were evolved against and retained the ability to infect the cell junctions and spread in the entire cell monolayer without being released in to the supernatant of Vero cell culture (Figure 31). While antibody-induced neutralization was inhibited by the evolution of viral resistant mutations, this assay did not address the question whether the evolved escape mutants would have disrupted antibody binding. To assess the binding of gB(HSV)-specific mAbs to resistant mutants, Vero cells were infected with WT or resistant mutant virus and subsequently infected cells were subjected to staining using gB(HSV)-specific mAbs as primary antibody and were detected by secondary anti human Fc-FITC conjugated antibody. The binding of the gB(HSV)-specific mAbs was assessed by flow cytometry. All the escape mutants including HDIT101RHSV-1F, HDIT101RHSV-2G, H4RHSV-1F, H4RHSV-2G, H28RHSV-1F bound weaker to the corresponding gB(HSV)-specific mAbs which they were evolved against (Figure 32).


A
gB detection by HDIT101
B
gB detection by H28
C
gB detection by H4
H28RHSV-1F
HSV-1F
H4RHSV-1F
Count
Anti human Fc-FITC
D
E
MFI (FITC)
Wild Type HSV-1F
H28RHSV-1F
H4RHSV-1F
Isotype control
H28
H4
HDIT101
****
F
gB detection by HDIT101
G
gB detection by H4
H4RHSV-2G
H28RHSV-2G
HSV-2G
Count
Anti human Fc-FITC

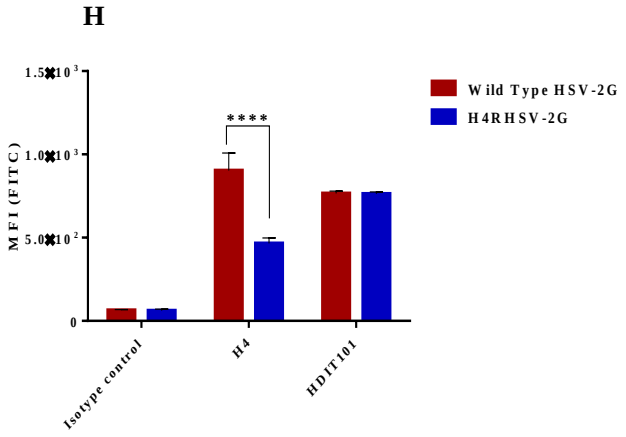


Figure 32. gB(HSV)-specific mAbs show reduced binding capacity to the resistant mutant viruses.

Binding analysis of resistant variants was performed on Vero cells infected with a resistant clone of HSV-1F/2G obtained after consistent passages under selection of HDIT101, H4 and H28. Vero cells were infected with either wild type HSV-1F/2G or resistant mutant viruses and the infected cells were subjected to FACS measurement using gB(HSV)-specific mAbs (HDIT101 were used for staining all mutants and wild type viruses, H4 were used for staining H4 resistant and wild type viruses and H28 were used for staining H28 resistant and wild type viruses) for analysing binding of the antibodies to the cell membrane associated gB. Anti-human Fc-FITC antibody was used as secondary antibody to detect bound antibodies. FITC intensity histogram showing HDIT101**(A)**, H28 **(B)** and H4 **(C)** interaction with gB of wild type (curves in red) and resistant HSV-1F viruses (curves in blue). For all measurements, background signal (of unstained sample) were subtracted and grey histograms display infected Vero cells stained with isotype control (human anti CD22 IgG1). **D, E)** Median florescence intensity (MFI) of bound anti human Fc-FITC (which reflect respective gB(HSV)-specific antibodies' binding to virus infected Vero cells) are plotted as Mean ± SD of technical triplicated. Differences of signal intensity were calculated using one-way ANOVA, **** P-value < 0.0001. FITC intensity histogram showing HDIT101**(F)** and H4 **(G)** interaction with gB of wild type and resistant HSV-2G viruses and controls were performed as it was explained for HSV-F resistant analysis. **H)** MFI of bound anti human Fc-FITC are plotted as Mean ± SD, N=3 technical replicates for H4RHSV-2G and wild type HSV-2G. Differences of signal intensity were calculated using one-way ANOVA, **** P-value < 0.0001.

Notably, selection with combinations of gB(HSV)-specific mAbs (e.g. HDIT101+H28 or HDIT101+H4) failed to produce viral escape mutants despite several independent attempts. The same results were observed when one escape mutant was cultured in the presence of another gB(HSV)-specific mAbs (e.g. H4-resistant mutant was passaged in presence of suboptimal doses

of HDIT101). Notably, several independent attempts to generate double or triple resistant strains were unsuccessful, which might indicate synergistic effects and suggests that targeting a combination of critical residues in gB prevents the emergence of resistance mutants. While *in-vivo* data show a lower fitness of viral single point resistance mutants and sequence analysis of primary isolates shows strong conservation of the epitope-associated regions in gB - both suggesting that individual point mutants will have reduced viral fitness and/or enhanced sensitivity to the host immune system *in-vivo* - a combination of two antibodies targeting two independent critical regions in gB in clinical application may even further reduce the risk for the emergence of resistance mutants.

The data above showed that H4-resistant HSV-1 mutant could still bind HDIT101 and vice versa the HDIT101-resistant HSV-1 mutant could still bind H4 (Figure 32). We assessed whether the escape mutants generated in the presence of one anti gB(HSV)-specific monoclonal antibody remained sensitive to neutralization by the other mAbs for both HSV-1 and HSV-2 with a TCID50-based assay. The HDIT101RHSV-1F/2G were neutralized efficiently by H4. Analogously, the H4RHSV-1F escape variant was inhibited by HDIT101. In contrast, HDIT101 could not neutralize H4RHSV-2G. In summary HDIT101, H4 and H28RHSV-1F escape variants remained sensitive to neutralization by one another which potentially can be considered as second line therapy. In contrast, H4RHSV-2G escape mutants was resistant to HDIT101 (Table 37). Following the result of neutralization assay, HDIT101 and H4 escape mutants were passaged for several round in absence of antibodies. After each passage the DNA was extracted and the gB coding sequence of the variants were sequenced. The results showed that the H4 resistant mutants reverted to the wild type after seven passages, but this was not the case for the HDIT101-resistant mutant at least after nine round of passaging in the absence of antibody. This suggests that the fitness defect induced by the HDIT101-resistant mutation is less pronounced in-vitro as compared to the H4-resistance mutation. Given that R335 is located centrally within the gB trimmer and hence may be involved in interprotomer interactions within the prefusion structure (refer to prefusion structure picture), it is possible that the change of this amino acid induces a stronger fitness defect and hence revertants may arise more readily.

Table 37. Summary of escape mutant sensitivity to be neutralized by the other gB(HSV)-specific mAbs.

H4 and HDIT101 were used in their efficient neutralization concentration (H4; 31.25 nM and 61.5 nM for RHSV-1F and RHSV-2G, respectively and HDIT101; 31.25 nM for both RHSV-1F/2G). ND, Not Determined + means sensitive to antibody neutralization, - means insensitive to antibody neutralization.

	H4RHSV-1F	H4RHSV-2G	H28RHSV-1F	HDIT101RHSV-1F	HDIT101RHSV-2G
H4	-	-	ND	+	+
HDIT101	+	-	+	-	-

To compare wild type HSV-2G infectivity with Ab-RHSV-2G and evaluate the viral fitness of the Ab-resistant virus, an *in-vivo* experiment was performed using the NOD/SCID immunodeficient mouse model. The aim was to understand the correlation between viral replicative fitness and the host survival in the absence of antiviral treatment comparing the wild type virus with the Ab-resistant mutant. For this approach, NOD/SCID mice were infected with various doses, 5×10^5 TCID50 of HDIT101RHSV-1F and 5×10^3 of HDIT101RHSV-2G and corresponding doses of wild type HSV-1F/2G. Results showed that mice infected with resistant mutants survived substantially longer as compared to similar doses of wild type viruses. This indicates that the resistance mutants had a substantially reduced viral fitness as compared to the respective wild type virus. Statistical comparison of survival curves was done by Log-rank (Mantel-Cox) test (Figure 33Figure 33). HSV-1FR infected mice showed significantly longer survival (P value < 0.001) and for HSV-2GR infected mice also showed substantially reduced viral fitness as compared to HSV-2G WT. The median survival of test groups infected with 5×10^2 HSV-2GR were 10.5 days and for the groups infected with 2.7×10^3 TCID50 or 5.5×10^2 TCID50 of HSV-2G WT were 7 days and 8 days, respectively. The median survival of test groups infected with 5×10^5 HSV-1FR and HSV-1F WT were 15 days and 8 days, respectively (Figure 33), suggesting a reduced viral fitness for antibody resistant escape mutants *in-vivo*.

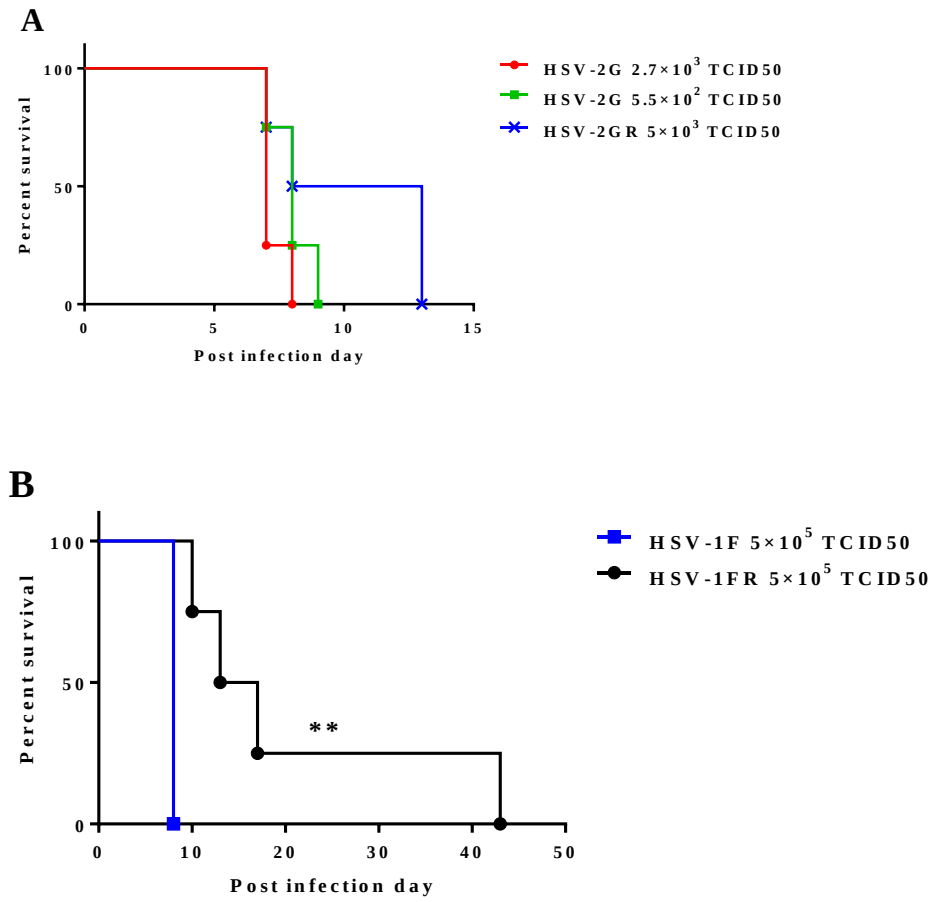


Figure 33. Antibody escape mutants showed reduced viral fitness compared to the wild type virus *in-vivo*.

To understand the effects of acquisition of gB mutations on viral fitness an *in-vivo* experiment was performed using an immunocompromised NOD/SCID mouse model. **A)** The mice were infected with two different doses of wild type HSV-2G, 2.7×10^{3} TCID50 or 5.5×10^{2} TCID50 as well as 5×10^{3} TCID50 HDIT101RHSV-2G (HSV-2GR). **B)** The mice were infected with 5×10^{5} TCID50 HSV-1F or HDIT101RHSV-1F (HSV-1FR). Comparison of survival curves was done by Log-rank (Mantel-Cox) test. ** P-value < 0.001.

3.7.2 gB(HSV)-specific mAbs target different epitopes

The fact that we raised escape mutants with different amino acid substitution for the specific mAbs suggests different epitopes for HDIT101, H4, H28 within gB. Relying on the data from HSV neutralization capacity of the mentioned glycoprotein B escape mutants, a competitive binding

assay was established to confirm that gB is targeted via different epitopes by H4, HDIT101 and H28. In this assay, Vero cells were infected with wild type HSV-1F. Membrane associated HSV gB was stained with a serial dilution of unlabelled HDIT101, H4 OR H28 in combination with 5µg/mL of FITC-labelled HDIT101. We took this approach because if H4 or H28 would compete for the same or proximal epitope, a reduction in the FITC-signal could be expected. As expected, the HDIT101-FITC signal decreased as the concentration of unlabelled HDIT101 was increased (Figure 34). However, different concentration of H4/H28 did not show any changes in the HDIT101-FITC signal, suggesting that H4 and H28 were unable to compete with HDIT101 binding to gB. According to the localisation of the amino acid residues conferring resistance and the results of the competition assay we speculated that gB(HSV)-specific mAbs H4, H28 and HDIT101 have different epitopes (Figure 34). Therefore, the concept of combining several mAbs targeting different epitopes to increase their therapeutic efficacy appears to be very attractive. However, strong evolutionary conservation in the putative epitope regions as well as a reduced viral fitness of resistance mutants *in-vivo* suggest that even targeting gB with a single antibody is likely sufficient to combat viral infections in the clinics.

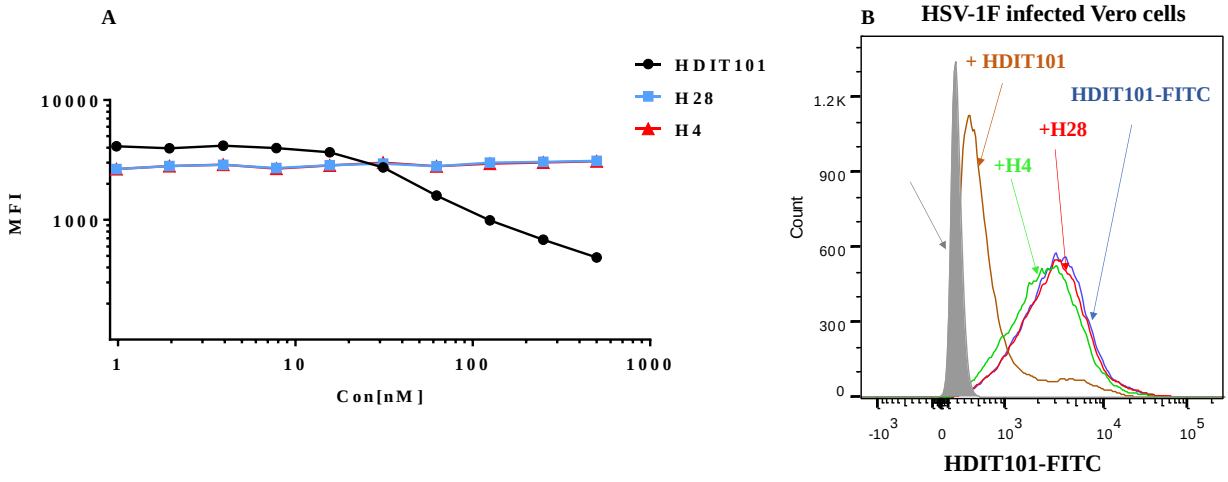


Figure 34. H4 and H28 bind to a different epitope of gB than HDIT101.

Following determining different escape mutations under selection of each gB(HSV)-specific mAbs, to verify that H4 and H28 target different epitopes on gB compared to HDIT101, a competitive FACS based experiment was done. A)

5 µg/mL of FITC labelled HDIT101 were incubated with HSV-1F infected Vero cells in combination with a serial dilution of unlabelled HDIT101, H4 or H28. B) highest concentration of antibodies in combination with HDIT101-FITC in histogram plot. The grey histogram is measure of FITC signal in an uninfected Vero cell control sample stained with HDIT101-FITC.

3.7.3 gB(HSV)-specific mAbs do not cross-react with other members of the Herpesviridae family

According to the crystal structures of post-fusion gB from HSV-1 (Heldwein *et al.*, 2006) and EBV (Backovic *et al.*, 2009) there is a strong protein conformational folding similarity between the two ectodomains. The gB protein of HSV-1/2 is also structurally very similar to the viral fusion protein G from vesicular stomatitis virus (VSV-G). We therefore analysed whether the mAbs targeting HSV gB would cross-react with viral proteins of other members of the Herpesviridae family. Different ELISA assays (Enzygnost) with pre-coated lysates of VZV, HCMV or EBV infected cells were employed and the binding properties of the gB(HSV)-specific antibodies H4, H28 and HDIT101 were tested. As positive control we used Cytotect and also the human polyclonal serum targeting CMV, EBV and VZV. Cytotect showed increased ODs indicating cross-reactivity between the viruses. In contrast, H4, H28 and HDIT101 did not show increased ODs. We conclude that H4, H28 and HDIT101 do not cross-react with similar fusion proteins or other viral proteins of other Herpesviridae, hence are specific to HSV-1 and 2 (Figure 35).

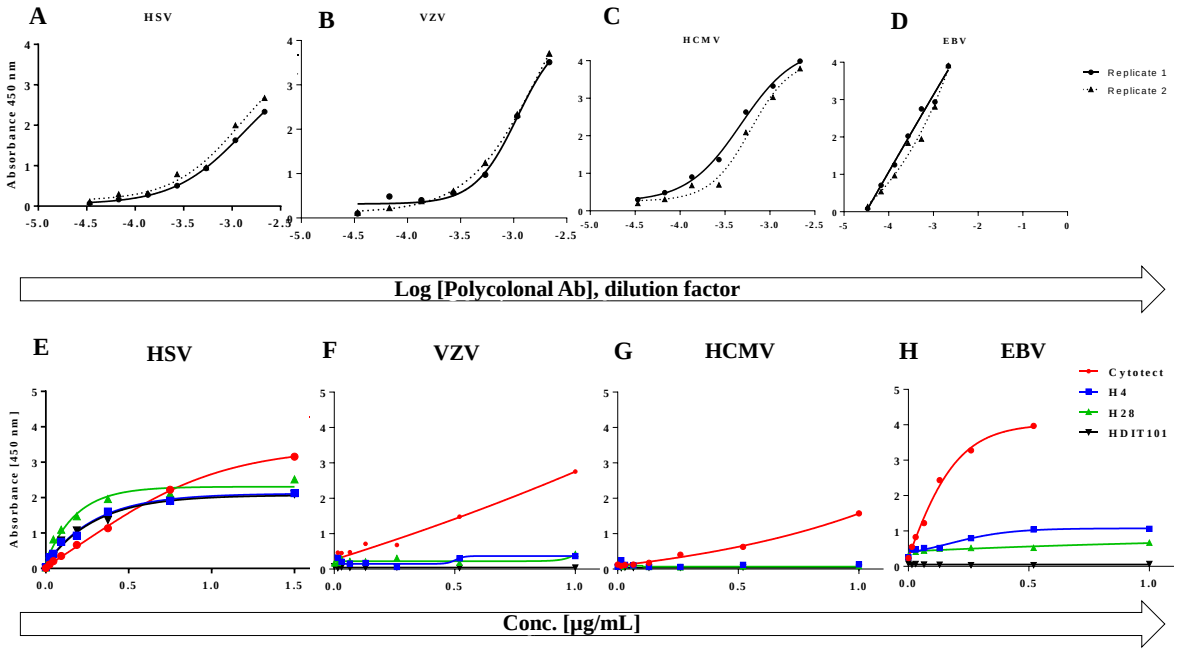


Figure 35. gB(HSV) specific mAbs (HDIT101, H4 and H28) do not cross react with common strains of other subfamilies from Herpesviridae.

To assess the binding properties of gB(HSV)-specific mAbs (HDIT101, H4 and H28) to homologous proteins of VZV, HCMV and EBV a simple ELISA assay was employed by using pre-coated plates with cell lysate of cells infected with VZV, HCMV or EBV. Standard curves are provided using HSV **(A)** VZV**(B)**, HCMV**(C)** and EBV**(D)** human seropositive serum samples. Using HSV **(E)** VZV **(F)** HCMV **(G)** EBV **(H)** pre-coated plate binding of the anti-gB antibodies was verified. Cytotect (anti-CMV polyclonal antibody preparation) was used as an additional positive control. Curves were plotted with mean of technical duplicates. The curves are fitted with non-linear regression.

3.8 *In-vivo* protection of mice by the gB(HSV)-specific mAbs

To evaluate the dose-dependent protective efficiency of H4, we examined whether this mAb protected mice from a lethal challenge with HSV-1F/2G virus. The lethality of different doses of HSV-1F was determined by including groups of each 4 NOD/SCID mice infected with 5×10^5, 1×10^5 or 5×10^4 TCID50 of HSV-1, respectively. The mice were infected by intravaginal inoculation. The results showed that mice of the group infected with $5x10^5$ TCID50 HSV-1F died within the first week after infection, while mice infected with 1×10^5 or 5×10^4 TCID50 HSV-1 survived longer (median: 9 days for $1x10^5$ and 15 days for $5x10^4$ TCID50). To investigate the

effects of antibody treatment within an acceptable time frame, for further experiments in NOD/SCID mice $5x10^5$ TCID50 HSV-1F were used.

To investigate the therapeutic effect of H4 treatment 30(600µg), 15(300µg) or 7.5 (150µg) mg/kg of H4 antibody in 100µl PBS, were administrated intraperitoneal to the mice in groups of 9 (NOD/SCID) 4 h post-infection (intravaginal HSV-1F 5×10^5 TCID50 in 10µl). We observed a dose dependent survival. H4 protected 44.4 % of HSV-1F infected NOD/SCID when dosed at 30 mg/kg for a 55-day observation period (Figure 36). With 15 or 7.5 mg/kg of H4 treatment, partially protection was observed. Median survival of the groups dosed at 7.5 mg/mL and 15 mg/mL was 20 and 17 days, respectively. All untreated control mice died within the first 9 days of the challenge. Furthermore, we assessed copy numbers of HSV-1F genome at 1, 3 and 6 days post-infection in vaginal swabs of infected mice. Total DNA from vaginal swab samples was subjected to qPCR to determine the HSV genome copy number. On day 3 and 6 post-infection, viral genome copy numbers in the vaginal swabs of infected mice that received H4 were significantly lower than those without treatment (Figure 36). We observed an anti-correlation between the H4 dose (30, 15, 7.5 mg/kg) and viral genome copy numbers in the vaginal swabs obtained on day 1, 3 and 6. These results demonstrate that H4 is able to suppress virus propagation in a lethal infection with HSV-1F. We next assessed the therapeutic activity of H4 in immunocompetent BALB/c mice against HSV-2G infection. For this similarly, we first determined the lethality of different doses of HSV-2G by including groups of each 6 BALB/c mice infected with 5×10^4, 1×10^4, 5×10^3 TCID50 of HSV-2G. 5×10^4 with 8 days median survival were chosen as HSV-2G lethal dose in BALB/c for upcoming experiments (Figure 37).

To that end, mice were challenged with a lethal dose of 5×10^4 TCID50 of HSV-2G and 4 h after infection treated intraperitoneal with 30 or 15 mg/kg of H4. All mice without treatment had severe symptoms of HSV infection (weight loss, redness of vagina, swelling and vaginal lesions, as well as hair loss within 13 days post-infection, with the notable exception of one mouse which had decreased viral genome copy numbers in vaginal swabs over the time. 50% of H4 treated mice of the 30 mg/kg dose group suppressed the infection up to the end of the observation period (60 days), with 54 days median survival (Figure 37). Mice in the group treated with 15 mg/kg of H4 were

also partially protected and during monitoring period (60 days post-infection) 30% of the mice in this group were still alive (14.5 days median survival) and did not show any obvious symptoms of HSV infection. Analysing virus copy numbers in vaginal swabs by qPCR showed decreased viral genome copy numbers over the time in both control and H4-treated groups which was more substantial for the treated group, which decreased viral copy number in control group, without treatment explains why one of the control mice in the experiment could survive (Figure 37).

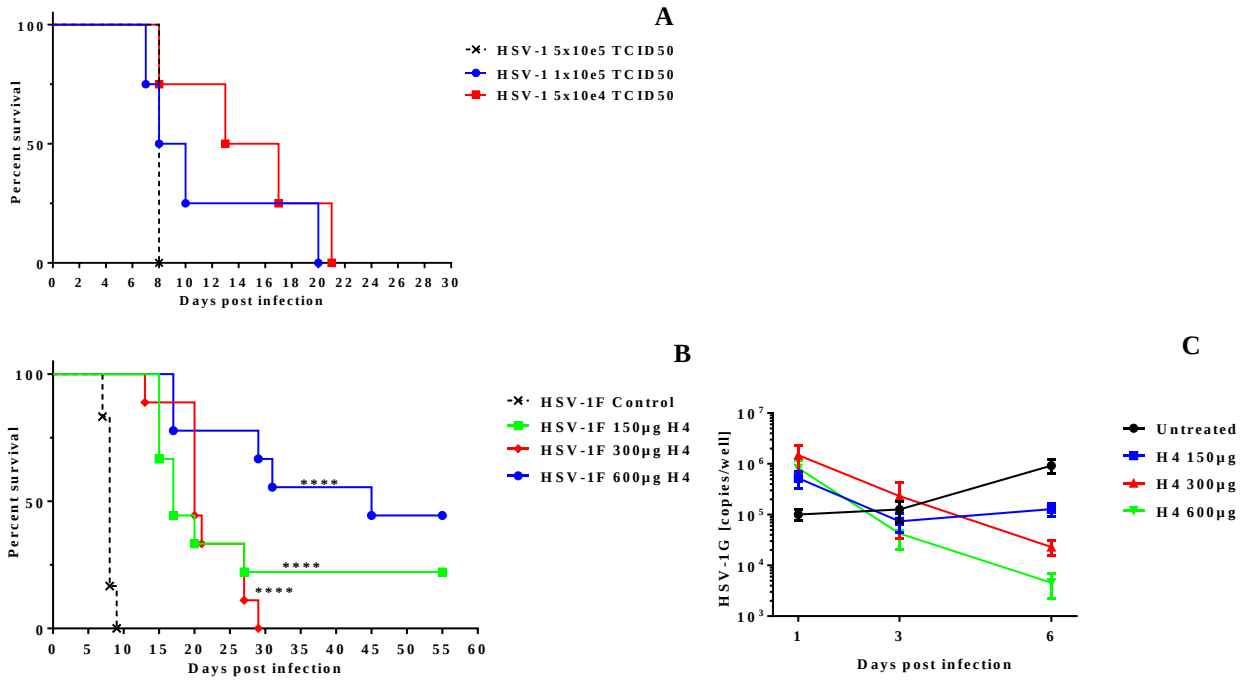


Figure 36. gB(HSV)-specific mAb (H4) protects NOD/SCID mice from lethal challenge with HSV-1F.

A) Minimal lethal doses of HSV-1F were determined by infecting groups of NOD/SCID mice with three different HSV-1F doses: 5×10^5, 1×10^5 or 5×10^4 TCID50. **B)** Therapeutic activity of H4 was evaluated by infecting (intravaginal inoculation with 5×10^5 TCID50) 8-week old Female NOD/SCID mice (N = 10 for treatment group and N=6 for control). Then, 4 h post infection the mice were treated i.p. with 30, 15 or 7.5 mg/kg of H4. Mice were monitored daily for 55 days post infection for body weight loss, symptoms of HSV infection, such as hair loss, redness and swelling of the vagina and lesions as well as survival. The differences between survival curves were calculated using Logrank Mantel Cox test ****P-value < 0.0001 **C)** Viral genome copy numbers in the vaginal swabs obtained on day 1, 3 and 6, were assessed with qPCR, shown as mean and standard deviation of mean.

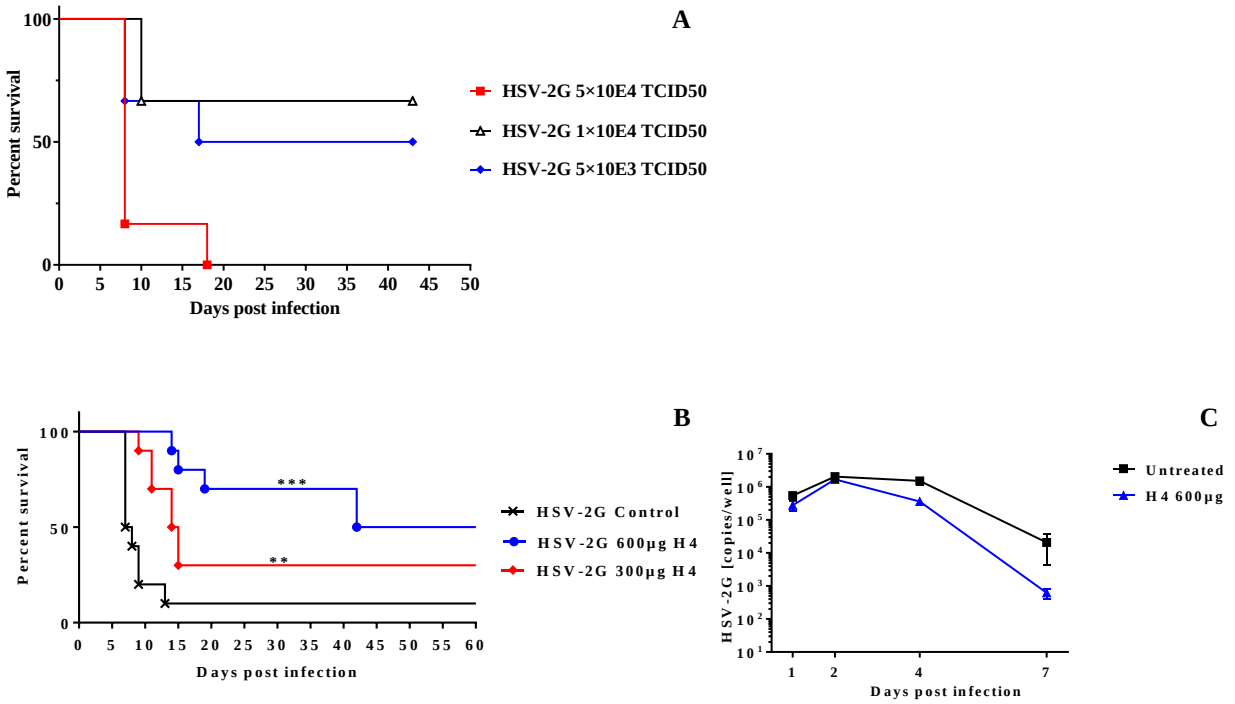


Figure 37. Survival of H4 treated immunocompetent mice after infection with HSV-2G.

A) Eight-week-old BALB/c mice (4 animals/group) were infected with three different HSV-2G titre (5×10^4, 1×10^4, 5×10^3 TCID50) to find the minimal lethal dose and 5×10^4 TCID50 were selected for future experiments in BALB/c. **B)** H4 protection *in-vivo* from lethal HSV-2G infection was examined using immunocompetent BALB/c. The mice were infected with the lethal dose determined in A) and 4h later 30 or 15 mg/kg of H4 (12 mice per group) were injected IP, while the control group received PBS. The statistical differences between survival curves were calculated using Logrank Mantel Cox test. ** P-value <0.05, *** P-value <0.001. **C)** Vaginal swabs were obtained on day 1, 2, 4 and 7 after infection and HSV-2G copy numbers derived from vaginal swabs were measured by qPCR. Error bars represent standard deviation of mean.

4 Discussion

Development of drug-resistant HSV infections is no longer an uncommon occurrence. Long-term Herpes Simplex treatment with antiviral drugs, which were generated over the last 40 years to treat HSV infections, can lead to resistance emergence, particularly in immunocompromised individuals (Bacon *et al.*, 2003) (Morfin and Thouvenot, 2003) (Langston *et al.*, 2002) (Pan *et al.*, 2013) (Kakiuchi *et al.*, 2013) (Turner and Beckingsale, 2013) (Piret and Boivin, 2011; Toriyama *et al.*, 2014).

The prevalence of drug-resistant HSV infections significantly varies between immunocompetent and immunocompromised patients. Prolonged replication of virus in an impaired immune system which allows persistence of low pathogenic viruses (resistant viruses have reduced pathogenicity) (Omura *et al.*, 2017), is one plausible reason that the incidence of drug-resistant HSV infections is higher in immunocompromised compared to immunocompetent patients (Piret and Boivin, 2011). Extensive screenings over the past 40 years showed a very low and steady prevalence of HSV resistance to common anti-herpetic drugs in immunocompetent patients (0.3%-0.7% (Collins and Ellis, 1993), 0.1-0.7% (Bacon *et al.*, 2002) (Bacon *et al.*, 2003) (Boon *et al.*, 2000) (Christophers *et al.*, 1998) (Danve-Szatanek *et al.*, 2004) (Reyes *et al.*, 2003) , 0.6% (Duan *et al.*, 2008), 0.19% (Sarisky *et al.*, 2002), 0.3% (Shin *et al.*, 2003)) which is usually not associated with adverse clinical outcome. However, there has been some case reports showing recurrent genital herpes (Kriesel *et al.*, 2005) (Swetter *et al.*, 1998) (Kost *et al.*, 1993) (Gupta *et al.*, 2005) (Ellis *et al.*, 1987), keratitis (Duan *et al.*, 2008) (Sarisky *et al.*, 2001) (Nugier *et al.*, 1992), disseminated HSV infection (Czartoski *et al.*, 2006), and encephalitis (Schulte *et al.*, 2010) in immunocompetent patients with drug-resistant HSV infections.

In contrast, a large number of studies report a higher prevalence of drug-resistant HSV infections in immunocompromised patients (3.5%-10%)(Stránská *et al.*, 2005) (Englund *et al.*, 1990) (Bacon *et al.*, 2003) (Christophers *et al.*, 1998) (Nugier *et al.*, 1992). The highest prevalence of drug-resistant HSV infections is reported among recipients of hematopoietic stem cell transplants

(4.1%-36%) (Langston *et al.*, 2002) (WILLIAMSON *et al.*, 1999) (Wade *et al.*, 1983) (Morfln *et al.*, 2000) (Frangoul *et al.*, 2007) (Erard *et al.*, 2007) (Danve-Szatanek *et al.*, 2004) (Chakrabarti *et al.*, 2000) (Chen *et al.*, 2000) (Morfin and Thouvenot, 2003). The rate of drug-resistant HSV infection is also growing in HIV-positive patients (3.5% -7%) (Danve-Szatanek *et al.*, 2004) (Englund *et al.*, 1990) (Levin *et al.*, 2004) (Reyes *et al.*, 2003; Ziyaeyan *et al.*, 2007). In addition to the patients receiving bone marrow transplant, in solid organ transplant recipients also a large percentage of resistance (2.5% -10%) has been reported (Danve-Szatanek *et al.*, 2004) (Christophers *et al.*, 1998). Drug-resistant HSV mutants may result in more severe and disseminated infections with high morbidity in immunocompromised patients. For example, the ACV-resistant isolates which were found in AIDS patients were associated with widespread mucocutaneous lesions and there have also been some case reports with lethal disseminated visceral HSV infections in patients receiving bone marrow transplants (Ljungman *et al.*, 1990) as well as meningoencephalitis in an AIDS patient (Gateley *et al.*, 1990).

However, it was shown that most of the drug-resistant HSV isolates result from a specific mode of action of a group of drugs against a particular target, e.g. thymidine kinase or DNA polymerase evolve resistance mutations during treatment with acyclic nucleotide analogues, or pyrophosphate analogues (Jiang *et al.*, 2016). Therefore, to overcome the drug-resistant HSV infections, it is crucial to identify novel approaches and develop new efficient anti-herpetic molecules with distinct modes of action and less cytotoxicity.

A plethora of technologic and scientific advances over the past 30 years paved the way for the development of therapeutics antibodies, which resulted in the approval of more than 90 recombinant monoclonal antibodies. Antibodies are the key components of adaptive host immune responses against viral pathogens. Due to their unique maturation process, they can be extremely specific to viral antigens and hence harbour less toxic side effects; in this aspect they have the privilege over the nucleoside analogues which show more side effects (Kaplon and Reichert, 2019). Nowadays, monoclonal antibodies are considered a feasible therapeutic modality for targeting viral infections (Salazar *et al.*, 2017). The key role of antibodies in limiting herpetic infections was shown by many studies and the number of anti-herpetic recombinant antibodies

with prophylactic and/or therapeutic purposes are rising (Krawczyk *et al.*, 2013a; Krawczyk *et al.*, 2015; Krawczyk *et al.*, 2011a) (Shiraki *et al.*, 2011) (Birlea *et al.*, 2013) (Tangye *et al.*, 2017) (Patel *et al.*, 2016) (Martins *et al.*, 2019) (Krampe *et al.*, 2010) (Hügler *et al.*, 2002) (Nigro *et al.*, 2005) (Adler *et al.*, 2007) (Clementi *et al.*, 2017).

In very early applications of monoclonal antibodies against HSV infection it was shown that glycoprotein D and B are the most immuno-dominant antigens and targeting these antigens with monoclonal antibodies resulted in a very efficient protection against HSV infection (Metcalf *et al.*, 1988). Human mAb E317 targeting HSV glycoprotein D was shown to prevent HSV-1 and HSV-2 entry via preventing interaction of gD with Nectin-1. The scFv E317 significantly inhibited HSV-1 and HSV-2 replication *in-vitro* and *in-vivo* (Lee *et al.*, 2013) (Burioni *et al.*, 1994a). The mAb AC-8 (targeting gD of HSV) neutralizes HSV-1 and more potently HSV-2, and protects mice from corneal, intracutaneous or vaginal HSV challenge (De Logu *et al.*, 1998) (Berdugo *et al.*, 2012). The humanized mAb hu2c targeting a common epitope of HSV-1/2 glycoprotein B showed significant neutralization efficacy in lethal vaginal HSV challenge (protective against both WT and ACV-resistant HSV infections) in a NOD/SCID mouse model (Krawczyk *et al.*, 2013b) (Krawczyk *et al.*, 2011b). Furthermore, mAb hu2c effectively prevented herpes simplex keratitis in corneal HSV-1 (WT/ ACV-resistant isolates) by inhibiting the neuronal spread of HSV (Bauer *et al.*, 2017c) (Krawczyk *et al.*, 2015). Systemic or topical application of HSV8, a human recombinant IgG1 targeting HSV glycoprotein D, has shown effective HSV neutralization *in-vitro* and *in-vivo* (De Logu *et al.*, 1998) (Zeitlin *et al.*, 1996) (SANNA *et al.*, 1996) (Burioni *et al.*, 1994b). Another monoclonal antibody targeting glycoprotein D of HSV-2 named m27f efficiently neutralized both HSV-1 and HSV-2 *in-vitro* and also protected mice infected with a lethal dose of HSV-2 (Du *et al.*, 2017). The monoclonal antibody HD1 targeting gB of HSV-1 prevented acute virus-induced neurological disease after footpad lethal challenge with HSV-1 in mouse model (Dix *et al.*, 1981) (see Table 38. Monoclonal antibodies targeting gB and gD HSV).

Table 38. Monoclonal antibodies targeting gB and gD HSV

Name	Target	Strain	Neutralization potency
E317	gD	HSV-1/HSV-2	+
AC-8	gD	HSV-1/HSV-2	+
hu2c	gB	HSV-1/HSV-2	+
HSV8	gD	HSV-1/HSV-2	+
m27f	gD	HSV-1/HSV-2	+
HD1	gB	HSV-1/HSV-2	+
HD2	gD	HSV-1/HSV-2	+
HD3	gD	HSV-1	+

Despite attempts to develop therapeutic HSV mAbs for >50 years (see Table 3), no monoclonal antibody has been licensed for clinical usage and only two antibodies are currently in clinical trials: 1) HDIT101(hu2c), a humanized gB(HSV)-specific antibody, is currently undergoing phase I/II clinical trials (intravenous (IV) application of genital HSV-2 infection) in patients with chronic recurrent anogenital HSV-2 infections (DRKS00014678). 2) UB-621 (anti-HSV-gD antibody) is tested in a dose-ranging phase II trial to evaluate the safety and efficacy (subcutaneous (SC) injection) on patients with recurrent genital HSV-2 shedding (NCT03595995).

While monoclonal antibody treatment may be highly efficient to neutralize HSV infections the fact that HSV persist lifelong in the patients suggests that patients may require lifelong antiviral therapy. Similar to the classical antiviral treatments, the single use of an antibody may induce only transient viral suppression and long-term application may induce viral resistance. Depending on the antigen that is targeted by the monoclonal antibody therapy viral resistance mutants may evolve relatively fast. Hence, the combinations of mAbs directed to different antigens or different epitopes of the same antigen may prevent the development of resistance as shown for other antiviral therapeutic agents (Mendoza *et al.*, 2018) (Jaworski, 2018). In accordance with this, the aim in

this project was to develop fully human antibodies as an alternative therapy regimen for the treatment of HSV-1/2 infections.

For this purpose the human lymph node derived antibody library was screened with ectodomain of gB HSV-1 (KOS) and based on binding profile and neutralization potency of the scFvs, two promising candidates namely H4 and H28 were chosen for further characterization (Diebolder *et al.*, 2014).

In-vivo application of scFvs requires a suitable route of administration and maintenance of sufficient concentration due to the small size and physiological drawback of rapid elimination from the body (Winthrop *et al.*, 2003). ScFvs interact monovalently with their target which results in low retention on the target and rapid off-rates (Krawczyk *et al.*, 2011b). Hence, this requires the application of high concentrations to reach a therapeutic dose and this may lead to enhanced side effects or cytotoxic effects *in-vivo*. This may be of importance given that scFvs do not exist as such in nature and hence are strictly speaking artificial constructs with the potential to induce immune responses.

In conclusion, the short *in-vivo* half-life as well as the monovalent binding and their non-natural structure restrict the application of scFvs in the clinics. Full-length therapeutic antibodies might improve all the limitations of scFvs mentioned above. The Fc region of a full-length antibody can interact with the neonatal Fc receptor that can decelerate the degradation of the IgG, hence prolonging its serum half-life (Vidarsson *et al.*, 2014). In addition, the Fc region can improve the efficacy of mAbs by mediating effector functions such as ADCC, ADCP and CMC. However, some of these effector functions might induce undesired and uncontrolled inflammatory responses like in the TeGenero (TGN1412) case (Horvath and Milton, 2009). TGN1412 was a humanized IgG4κ mAb targeting CD28 and stimulating T cells expansion, which were developed for treatment of B cell chronic lymphocytic leukemia with T cell deficiency, and for autoimmune diseases, such as rheumatoid arthritis, in which Treg cell expansion might be beneficial. TGN1412 dramatically failed in phase I resulting in severe and life threatening inflammatory responses in healthy volunteers in part resulting from a cytokine storm derived from Fc-Fc receptor interaction

(Horvath and Milton, 2009). These side effects can be modified by Fc engineering resulting in an efficient antibody with modulated effector functions according to therapeutic purposes (Saxena and Wu, 2016). Another advantage of reformatted scFvs into full IgGs is the increase in the valency of the antibody thereby inducing avidity effects. Several studies have shown the beneficial outcome of scFvs reformatting into IgG to improve affinity through avidity effects (Saerens *et al.*, 2008). In addition, increasing the valency might also enhance the neutralizing potency of antiviral antibodies by increased flexibility to crosslink two different sites on different protomers. Hultberg et al. showed that bivalent constructs targeting the respiratory syncytial virus (RSV) F fusion protein, increased the RSV neutralizing potency by 4000 fold compared to the monovalent construct (Hultberg *et al.*, 2011). Moreover, bivalent anti vesicular stomatitis virus (VSV) G protein constructs (Schepens *et al.*, 2011) and bivalent/trivalent anti influenza H5N1 HA antibodies (Ibanez *et al.*, 2011) showed a significantly higher neutralizing capacity than their monovalent counterparts *in-vitro* and *in-vivo*. Krawczyk et al. (Krawczyk *et al.*, 2011b) also demonstrated that the neutralization potency of mAb 2c most likely was connected with cross-linking of gB trimers and only bivalent derivatives of mAb 2c displayed potent neutralizing activity *in-vitro* and *in-vivo*, while monovalent constructs lost the antiviral effect. One possible explanation for an increased neutralization efficacy is that multi-valent interactions mask low affinities by inducing avidity effects (Wang and Yang, 2010).

The avidity effect may also reduce the risk of emergence of antibody-resistant escape mutants *in-vitro*. Palomo et al. (Palomo *et al.*, 2016) showed that by passaging RSV in the presence of the monovalent or multivalent antibodies it was much more difficult for the virus to escape from the multivalent construct as compared to the monovalent counterpart and the multivalent antibody still was able to neutralize the mutants escaping the monovalent antibody.

Considering the advantages of full IgG over the scFvs, LYNDAL-selected scFvs targeting gB(HSV-1/2), as one of the most immunodominant antigens of HSV and preserved protein of the HSV fusion machinery, were subjected for reformatting into full IgG1(G1mza) antibody.

As mentioned above, efficacy of monoclonal antibodies is achieved through their Fab or Fc domains or both. In order to characterize the binding specificity of the reformatted scFvs (IgGs H4 and H28), a FACS based experiment was performed using HSV infected Vero cells to detect cell associated gB. gB is the fusogenic protein of HSV and is involved in the docking of the virus particles to the host cells as well as the fusion of the host cell membrane with the viral membrane (Harrison, 2008). Despite several studies on the elucidation of the fusion mechanism, the details of the fusion process are not clearly understood. The current fusion model, which is widely accepted, proposes that gD undergoes a conformational change after binding to one of the corresponding cellular receptors, thereby initiating the fusion process by transferring the signal to the gH/gL heterodimer (Chowdary *et al.*, 2010). The cytoplasmic domain of gH disturbs the stability of the gB cytoplasmic domain which leads to gB inversion to extend the fusion loop toward the host cell membrane finally resulting in a second inversion of gB upon itself so that both membranes are brought in close proximity and the fusion can occur (Cooper and Heldwein, 2015).

The results presented here showed that the antibodies H4 and H28 bound with high affinity to HSV-1F/2G. EC50 for binding of H4 to HSV-1F or HSV-2G infected Vero cells was 8.5 nM and 10.9 nM, respectively, while H28 bound to HSV-1F or HSV-2G infected Vero cells with a functional affinity of 6.7 nM and 9.95 nM, respectively.

Neutralizing antibodies are the best correlate of protection from viral infection. To characterize the mechanism of action of the antibodies described in here, their neutralization efficiency was evaluated by a TCID50-based assay and was compared with HDIT101 and human polyclonal anti HSV antibodies. The *in-vitro* neutralization assay showed that gB(HSV)-specific IgG1 H4 neutralized HSV-1F or HSV-2G with a higher efficiency than gB(HSV)-specific IgG1 H28. For complete neutralization of HSV-1F or HSV-2G a concentration of 62.5 nM and 31.25 nM of H4 was required, respectively. To completely neutralize the same viral load of HSV-1 and HSV-2, a concentration of 250 nM and > 500 nM, respectively of the gB(HSV) specific IgG1 H28 was necessary. Of note, the human polyclonal anti-HSV antibodies failed to protect the cells from HSV infection, arguing that neutralizing anti-HSV antibodies do not appear frequently.

It has been shown frequently that extensive affinity maturation is required for providing a protective humoral response against viruses that persist in the body and usually, the germline repertoire does not provide high affinity antibodies to be protective(Hangartner *et al.*, 2006). The theory implies that the potency of neutralizing antibodies closely correlates with their affinity toward the native conformational form of viral envelope antigens specifically this is largely holds up for HIV-1 (Sanders *et al.*, 2013) (Parren *et al.*, 1998). However, there are cases described in which neutralizing antibodies directed against the membrane proximal region in the transmembrane antigens do not show potent neutralization efficacy despite high affinity (Kim *et al.*, 2014) (Huang *et al.*, 2012). Of note, the affinity of neutralizing antibodies for native entry-mediating antigens in general correlates well with their neutralization potency, but in some cases the affinity towards an induced transient form of the viral protein determines the neutralization potency (Klasse, 2014). In the data presented here H28 bound strongly with an EC50 of 6.7 nM and 9.95 nM for HSV-1 and HSV-2 respectively, however failed to neutralize virus particles and avoid the dissemination of infection *in-vitro*.

Since herpes viruses use both, the cell-free as well as cell-associated mode of virus spread, the later likely provides a physiologically more important way since virions can spread through the existing cell contacts without being released into the extracellular matrix, most likely a way to escape humoral immune responses (Cocchi *et al.*, 2000) (Carmichael *et al.*, 2018). There is a large number of examples for neutralizing antiviral antibodies which are not capable of blocking virus cell-to-cell transmission (Phillips, 1994) (Merz *et al.*, 1980) (Gupta *et al.*, 1989) (Ganesh *et al.*, 2004). We therefore investigated the neutralizing effects of the H4, H28 and HDIT101on cell-to-cell transmission of HSV. The results showed that, similar to HDIT101, for which the inhibition of cell-to-cell spread was already shown (Bauer *et al.*, 2017b) (Krawczyk *et al.*, 2011a), H4 was able to block cell-to-cell transmission and limited infection to the initial single cells that were infected by HSV-1/2. H28 failed to inhibit HSV cell-to-cell transmission correlating with its weak neutralization efficacy of cell-free virus. The binding of HDIT101 and H4 to gB presumably interferes with the fusion process between virus and cytoplasmic membrane of the host cell more efficiently than H28. Even though cell-to-cell viral spread can be replicated in cell culture, the

exact mechanism by which the viruses actually spread *in-vivo* is not well understood. Therefore, there might be another effector function deriving the possible protective effect of antibody *in-vivo*.

In addition to the direct virus neutralization, antibodies can employ Fc-mediated effector functions to suppress viral infections. Recent studies have highlighted the importance of antibody Fc-mediated effector functions in protection from pathogenesis of different infectious disease. Binding of Fc antibody domains to the human Fcγ receptors can in principle activate human NK cells or other cells expressing Fcγ receptors and can induce different effector functions like ADCC or ADCP.

For some infectious diseases, ADCC was shown to play a critical role in an effective immunity against the infection. For example, it was demonstrated that in HIV infections, Fc-mediated effector functions such as ADCC play a critical role in controlling HIV viremia (Bonsignori *et al.*, 2012) (Corey *et al.*, 2015) (Haynes *et al.*, 2012) (Bournazos *et al.*, 2014). Aiba et al. showed that antiviral activities of intravenous immunoglobulins (IVIG) targeting VZV can be attributed to ADCC effector function but IVIG targeting CMV failed to show significant ADCC toward CMV-infected cells (Aiba *et al.*, 2017). Animal models immunized with EBV-gp350 purified from EBV-infected cells or plasmid expressing gp350 developed EBV-neutralizing antibodies that mediated ADCC (Qualtiere *et al.*, 1982; Sojin *et al.*, 2001).

ADCC was also shown to regulate the protection from HSV infection (Petro *et al.*, 2015) (Balachandran *et al.*, 1982). Wang et al. showed that human mAbs from individuals immunized with the HIV RV144 vaccine, (a fusion protein of HIV gp120 and a region of HSV-1 gD that contains most of the HVEM binding domain) could control HSV infection *in-vivo* and the protection of HSV-1 infected mice was correlated with the ability of these mAbs to induce ADCC (Wang *et al.*, 2017). Kohl et al. investigated a set of well-characterized monoclonal antibodies directed against gB(HSV) and polyclonal antibodies against HSV glycoprotein D to determine their functional activity and association with protection in a murine model of neonatal HSV infection. They found that the protection was only guaranteed when human mononuclear cells were present and showed that protection was strongly associated with ADCC effector function

(Kohl *et al.*, 1990). Petro et al. observed that a single-cycle HSV vaccine (HSV-2 deleted in glycoprotein D (ΔgD-2)) elicited a high titre of ADCC HSV-specific Abs, that were protecting naïve mice when a single dose of immune serum was injected, but not in Fc receptor (FcR) KO or Fc neonatal receptor KO mice (Burn *et al.*, 2017) (Petro *et al.*, 2015) (Petro *et al.*, 2016). It was also shown that in neonates, high ADCC-inducing Ab levels were associated with less severe neonatal disease (Kohl, 1991). In general, it is believed that naturally derived antibodies against HSV infection in human are able to induce ADCC towards some of the epitopes of gD and gB (Sanchez-Pescador *et al.*, 1992).

Accordingly, the capability of H4, H28 and HDIT101 to induce ADCC *in-vitro* was evaluated using engineered Jurkat cells expressing Fcγ receptor as effector cells and employing infected Vero cells expressing target antigen or HEK-293T cells stably expressing gB(HSV) on their cell membrane. Compared to human polyclonal anti HSV antibody which induced ADCC when in contact with target antigen, none of the tested antibodies H4, H28 or HDIT101 initiated ADCC. Conclusively, considering the neutralization potency of the antibodies (H4 and HDIT101) *in-vitro* and in immunocompromised animal models with impaired NK cells and macrophages functions, presumably the protective effects and neutralizing potency of the antibodies tested here are not attributed to an ADCC activity.

However, Fc-mediated effector functions may raise practical safety issues during passive prophylaxis with antiviral mAbs and regulatory requirements may limit the usability of antibodies exerting Fc effector functions. For example, in cases where mAbs target cellular surface receptors or are intended to block ligand-receptor interactions it may be desirable to decrease or abolish Fc effector function in order to avoid undesired side effects. These can include the secretion of large amounts of pro-inflammatory cytokines, also known as cytokine storm, which can cause severe clinical symptoms, or the killing of the target cell.

To further elucidate the role of the Fc domain of the antibodies H4, H28 or HDIT101, their potency to induce ADCP was examined. ADCP is a mechanism for clearance of virus and virus-infected cells and functions as a linker between innate and adaptive immunity by facilitating antigen

presentation and cytokine production. Despite the complexity of ADCP in immune responses against viruses, a number of studies highlight the importance of ADCP in the antiviral immune response. Accordingly, a possible role of the Fc domain of the antibodies H4 and H28 in activating ADCP was examined. The data shows that the Fc domain of the tested antibodies complexed with HSV, were able to bind to the Fc receptors on the surface of THP-1 cells and triggered ADCP. THP-1 is a monocyte-like cell line, derived from a patient with acute myelogenous leukemia and represent a widely used model to study monocytes (Bosshart and Heinzelmann, 2016). THP-1 express a variety of FcγR, similar to primary monocytes from healthy donors. To exclude complement-mediated phagocytosis and also unspecific IgGs interference in ADCP, heat inactivated, and IgG depleted human serum was utilized.

We showed that H4, H28 and HDIT101 induced ADCP only in the presence of the gB target protein on HSV particles. The molecular differences on how FcγR discriminates between free antibody and antigen bounds antibody in ADCP is unclear. One model that could explain this is that free antibody interacts with FcγRs and is constantly circulating, while antigen-bound antibody is maintained in the cell to degrade the antigen. Interestingly, the potency of Abs to induce ADCP does not necessarily depend on their affinity or their potential to neutralize, as shown for different antibodies against HIV-1 Env (PMID: 27579713). This suggests that there are different yet unknown features of antigen-antibody interactions that dictate the potential to induce ADCP.

According to the performed *in-vitro* assays all three antibodies showed ADCP stimulation, but whether this activity plays a role in the protection *in-vivo* needs to be investigated in the future. The importance of ADCP in the protection from different viruses is highlighted by results from several preclinical studies (including studies on non-human primates), for example in clinical trials a key role of ADCP in the antiviral immune responses against HIV infection has been suggested before (Bournazos *et al.*, 2014) (Parsons *et al.*, 2019) (Ackerman *et al.*, 2013a) (Lassaunière *et al.*, 2016) (Ackerman *et al.*, 2013b). Collective data suggests the contribution of ADCP in the protection from influenza virus infection(Huber *et al.*, 2001) (DiLillo *et al.*, 2016) (Mullarkey *et al.*, 2016) (DiLillo *et al.*, 2014). Several studies have shown the ADCP contribution in immune responses against herpesvirus infections.

Nelson et al. (Nelson *et al.*, 2018) found ADCP as part of the antibody response elicited by vaccination with CMV glycoprotein gB/MF59 conducted in postpartum women. They found ADCP-mediating antibodies as the main portion of the produced antibodies upon CMV vaccination and only modest neutralizing antibody responses which was in agreement with the study by Cui et al. (Cui *et al.*, 2008). In addition, the antibodies elicited by the CMV vaccine mostly were of IgG3 isotype, which is superior to IgG1 in mediating ADCP, and contributes to the robust ADCP response (Tay *et al.*, 2016). Monoclonal antibodies targeting RSV G protein derived from healthy individuals (exposed to RSV before) showed ADCP activity *in-vitro* (Cortjens *et al.*, 2017). Moreover, Ebola virus glycoprotein-specific monoclonal antibodies derived from vaccinated individuals were shown to exert ADCP activity (Duehr *et al.*, 2017).

Wang et al. reported that ADCP activity contributes to the protection against vaginal HPV infection using passive transfer of monoclonal antibodies directed against HPV in mouse models, since they couldn't observe the same protection when they applied $F(ab')_2$ instead of whole IgG or in Fcγ-deficient mice. Collectively, these observations suggest that for many viral infections in which neutralizing antibodies control the infection, ADCP may also play a major role and can contribute to their protective function.

Many studies suggest that the complement system is of fundamental importance in the host antiviral immune response. Viral neutralization by human antiviral antibodies is enhanced primarily by the activation of the classical complement cascade which is mediated by the Fc domain of antibodies bound to their target antigens (Hirsch, 1982). Some of the proteins contributing to the complement cascade such as C3a and C5a, function as opsonins and facilitate phagocytosis (Scieszka *et al.*, 1991). In addition, the terminal complement complex, which is the result of the complement activation acts as membrane attack complex and lyses infected cells, so called complement mediated cytolysis, or even free virions, preventing further spread (Stoermer and Morrison, 2011).

We showed here by comparing neutralization potency of antibodies in the presence or absence of complement system that the complement system does not contribute to the neutralization

efficiency of the gB(HSV) specific mAbs H4, H28, as has been described before for HDIT101. Furthermore, measurement of C5b-9 levels, which constitutes the terminal complex of complement cascade and executes the CMC activity, showed that H4, HDIT101 or H28, all did not promote CMC, since the C5b-9 levels did not change upon presence of the antibodies. The absence of complement-induced cytolysis was also verified by FACS analysis of cell death in 293T cells expressing HSV-1 or HSV-2 gB. While 293T cells ectopically expressing gB represent an attractive model for this type of studies, infectious virions harbour a complex of glycoproteins consisting of gB, gD, gH/L. In addition, gB is thought to be the glycoprotein with the least abundance of those four on virions (Heldwein *et al.*, 2006). Hence, the results from ectopic expression of gB outside its context of a fusion complex with gD, gH/L should be interpreted with caution, since it is possible that interaction of H4, H28, or HDIT101 with gB may be slightly different when gB is present alone and in abundance, or in a complex with gD, gH/L and with a limited number of proteins. However, separation of gB from this context by expression in 293T cells allows the investigation of the molecular details underlying the ADCP, e.g. by generating gB mutants that show reduced binding to the investigated antibodies or mutants with trimerization defects etc.

The described gB(HSV)-specific human antibodies are capable of full complement-independent virus neutralization and this represents a tremendous advantage over human serum antibodies, which are largely dependent on complement. One advantage is that HSV neutralization can also be achieved in patients with complement deficiencies. In the course of evolution, herpes simplex viruses have evolved mechanisms to evade a human immune response. For example, glycoprotein C can protect the virus from complement-mediated neutralization by binding to and inactivating the complement protein C3b. In addition the HSV gE/gI heterodimer expressed on the surface of virions and infected cells has been shown to function as IgG Fc receptor and by binding to the Fc domains of antibodies prevents effector functions mediated by the respective antibodies (Fries *et al.*, 1986). Since we did not see an association between neutralization potency elicited by H4 or HDIT101 and a contribution of ADCC or CMC, presumably the natural evasion mechanisms

developed by HSV like countermeasures gC and gE/gI may not affect the potency of the therapeutic antibodies described here.

To investigate whether H4 neutralize independently of a Fc-mediated mechanism *in-vivo*, we employed a NOD/SCID mouse model, which in addition to the severe combined immunodeficiency of T- and B-cell, macrophage and NK cell functions as well as the ability to stimulate the complement pathway are disrupted. We could demonstrate that H4 antibody treated mice survived significantly longer as compared to the control group after a lethal infection with HSV-1 and HSV-2 with P values <0.0001 and <0.001 respectively. The data show that H4 was protective in immunocompromised NOD/SCID-mice and this suggests that a large amount of the antiviral effect can be explained by Fc-independent virus neutralization. However, for HDIT101 it is known/we have shown that disruption of the Fc domain function by introduction of specific amino acid substitutions interfering with the Fc-receptor engagement, leads to loss of neutralization capacity *in-vivo* (in NOD/SCID mouse model). This result suggests that there may be some functional cells remaining even in a NOD/SCID background which contribute to the antibody-mediated antiviral effects. Further studies should reveal whether H4 Fc-mutants exert a similar phenotype *in-vivo*.

Among the Herpesvirus envelope glycoproteins, glycoprotein B is the most conserved one. HSV-1 gB shares 29%, 24.2% and 49% identity with EBV gB (Backovic *et al.*, 2009), HCMV gB (Burke and Heldwein, 2015) and VZV gB, respectively (Cohen, 2006). Giving to the high similarity in amino acid sequence and also conformational folding we investigated cross reactivity of tested gB(HSV) specific antibodies with EBV, HCMV and VZV. The data showed that none of test antibodies cross-reacted with homologous members of the Herpesviridae family.

In-vivo neutralization potency of H4 were investigated using BALB/c or NOD/SCID mice infected with HSV-1/2. Passive immunization (therapeutic application) of H4 gB(HSV)-specific mAb after intravaginal HSV-1 lethal dose exposure in the NOD/SCID mouse model conferred 50% protection after 55 days post infection while all mice without treatment had severe symptoms of HSV infection (weight loss, redness of vagina, swelling and vaginal lesions, as well as hair loss

and death within 10 days post infection). Control mice had to be sacrificed due to virus replication in central nervous system resulting in paralysis and megacolon (due to the paralysis of the peristaltic movements of the bowel).

We observed an anti-correlation between the H4 dose (30, 15, 7.5 mg/kg) and viral genome copy numbers in the vaginal swabs obtained on day 1, 3 and 6 and there was a dose dependent survival. 600μg (30 mg/kg) of H4 per mouse suppressed infection and conferred 50% survival. H4 protection *in-vivo* from lethal HSV-2G infection was examined using immunocompetent BALB/c. 30 mg/kg H4 showed 44% survival over the 60-day observation period. In addition, 15 mg/kg also conferred to a partial protection. HSV-2G copy numbers derived from vaginal swabs were dramatically decreased for the H4-treated group however the viral genome copy numbers were also decreased in the control group. Since Balb/c mice are immunocompetent, it is likely that endogenous immune responses can control viral replication at least to a certain degree and even completely in a few cases (one control mouse survived the lethal challenge). The H4 mAb showed potent HSV neutralization in the preclinical animal models investigated in here and represents an effective alternative to the prophylaxis and therapy of HSV infections. Of note, H28 *in-vivo* experiments were not performed since in-vitro results suggest that the neutralization efficiency is much lower as compared to H4 and HDIT101.

Epitope mapping is a key step in characterizing antibodies that bind a potential therapeutic target. Determining epitopes of therapeutic antibodies significantly enhances the understanding of the host immune response interplay and provides insights into the molecular details of the interaction. This information can be helpful to improve the binding efficiency by rational computer-based drug design but may also help to predict the likelihood of emergence of drug-resistant escape mutants. To elucidate the mechanism of binding in more detail, we first performed a competitive assay between H4 and HDIT101. The rational of this method is that should antibodies share a common epitope or should the epitopes overlap, a saturating, competing effect can be expected when both antibodies are mixed.

The results showed that H4 did not compete with HDIT101 binding to glycoprotein B of HSV, suggesting their epitopes are not identical and probably do not overlap. Irrespective of the epitope type (conformational, linear), this result can be helpful since the HDIT101 epitopes has already been proposed. Mapping of the antigenic site recognized by the murine counterpart of HDIT101 by using overlapping peptide screens revealed a possible discontinuous epitope within structural domain I of the gB (located within residues 31 to 487) (Krawczyk *et al.*, 2011b). In particular, residues within two distant regions in domain I (region A: F186, E187, D188 and region B: F300, Y301, G302, Y303, R304, E305) have been suggested to contribute together to binding of the parental murine Ab. Another approach to identify residues within epitopes that play a role in antibody binding is, to grow viral escape mutants and to identify the amino acid substitutions that confer resistance to neutralization. This approach has been used to characterize antibody binding to viral proteins before (PMID:29643370; 28288189; 19052239). We identified for HDIT101 HSV-1 escape mutant R304Q and HSV-2 escape mutant R296Q, for H4 HSV-1 escape mutant R335Q and HSV-2 escape mutant R327W and for H28 HSV-1 escape mutant R605W. We were unable to grow resistant mutant against H28, likely reflecting the weak neutralization efficiency of H28. The data suggest that H4 binds to the structural domain I of gB HSV and that R335 in HSV-1 as well as R327 in HSV-2 are critical residues for H4 binding.

We attempted to identify the epitopes also by peptide mapping (JPT technologies) and unfortunately, the CD22 control antibody showed a strong background for many gB-derived peptides and H4 or H28 showed no specific interaction with any of the peptides, hence the results were uninterpretable. Indeed, epitope identification based on peptide screens often fails due to the fact that many antibodies recognize a complex target (such as a gB trimer) through structural interfaces rather than linear epitopes.

By running lysates of HSV-1/2 virions and also of exosomes from HEK293T stably expressing gB(HSV) on a reducing SDS gel electrophoresis and subsequent western blotting, we investigated the reactivity of H4 or H28 with gB under reducing (denaturing) conditions and observed that a weak to moderate signal was present at the size of monomeric gB. Since both, H4 as well as H28 bound to denatured glycoprotein B we suggest that the epitope may be a continuous epitope, or

that the epitope is located in a pseudocontinous region that is only moderately denatured under reducing conditions, as has been described before (Krawczyk *et al.*, 2011b). However, to precisely determine the H4 and H28 epitopes further studies will be required.

In addition to the epitope determination, the growth of escape mutants to HDIT101, H4 or H28 opened the opportunity to cross apply the antibodies on the resistant variants to analyse their neutralization efficiency toward heterologous gB-resistant mutants. We were able to completely neutralize HDIT101RHSV-1/2 mutants with H4 at similar EC50s as compared to wild type virus. Likewise, HDIT101 neutralized H4-resistent or H28-resistent HSV-1 escape mutants but not the H4-resistent HSV-2 mutant. We ruled out the impure population by generating single clones for the HDIT101 and H4-resistant mutants and repeating the experiment but still there was no neutralization potency of HDIT101 against H4-resistent or H28-resistent HSV-2 which we cannot explain at the moment given that the same amino acid substitutions are observed for HSV-1 and HSV-2 when resistant mutants to HDIT101 or H4 are grown out (HSV-1 gB R304Q and HSV-2 gB R296Q for HDIT101 and HSV-1 gB R335Q and HSV-2 gB R327W for H4, respectively). It is possible that HSV-2 gB, which is very similar but not identical to HSV-1 gB, obtains a slightly different trimeric structure, in which HDIT101 and H4-epitopes may be closer to each other than in HSV-1. Development of resistance towards H4 (HSV-2 gB R327W) may then also interfere with HDIT101 binding, while development of HDIT101 resistance (HSV-2 gB R296Q) may not affect H4 binding and neutralization. A more detailed molecular analysis will be required to untangle the mechanistic insights in these processes.

Despite several attempts to generate escape variants with multiple mutations to escape from neutralization by the two most potent antibodies (H4 and HDIT101), HSV-1/2 failed to grow over several passages. The most likely explanation for this is that gB only tolerates few amino acid changes, i.e. is genetically robust. The combination of these two antibodies as a combinatorial therapy therefore appears an attractive idea. In addition, the *in-vivo* data showed that HDIT101-resistant virus has a reduced fitness since survival was enhanced when comparing a challenge with an equal dose to wild type virus in NOD/SCID mice *in-vivo*. Taken together, the chances of developing resistance towards HDIT101 (which is already in clinical trials) is very low and even

if resistant virus would occur, the immune response may be able to control better a virus that replicates with less fitness. In line with this, the region around the predicted HDIT101 epitope is very strongly conserved in circulating primary HSV isolates, suggesting that the in-vivo changes in this region are not well tolerated. In addition, if developing resistance with single monoclonal antibody therapy *in-vivo* should become problematic and may require additional therapeutic intervention, applying a combination of the antibodies H4 and HDIT101 might be a potential alternative to avoid emerging resistance to therapies. The *in-vitro* data showed that over nine passages in the absence of H4 antibody, H4RHSV-1 reverted back to wild type. Regarding HDIT101 HSV-1 reversion there might be a need to continue virus passages longer because up to nine passages the escape mutation leading to gB R304Qsubstitution was still detectable.

The increasing development of multidrug-resistant virus strains in immunocompromised patients and also the absence of effective alternative antivirals can lead to serious complications in the clinic. According to the *in-vitro* and *in-vivo* results mAb H4 offers a potential therapeutic feature for clinical usage. The fact that H4 was able to neutralize HDIT101 resistant mutant by targeting a different epitope suggests H4 as a second-line regimen for HSV infection therapies. Owing to the loss of viral fitness in the presence of combinatorial antibody application and not emerging resistance in presence of multiple antibodies targeting gB *in-vitro*, we suggest using a combination of H4 and HDIT101 for severe HSV infections with or without resistance to the classical antiviral drugs (e.g. Aciclovir).

However, there is a need to understand the mode of action of the antibodies in more detail and *in-vivo*. Furthermore, it needs to be explored to which extend the antibodies H4 or HDIT101 may also induce changes in the T cell responses, i.e. possibly inducing a vaccine-like effect. For some antibodies it has been proposed that binding to their target would reveal MHC peptides that are normally hidden and hence not processed for MHC presentation. To reveal such antibody-induced new MHC epitopes that induce unique T cell responses may be an exciting and novel way to improve therapeutic strategies for HSV infection but also for other morbidities. To further drive the clinical development of H4, we suggest to investigate: 1) epitope mapping and characterization, 2) human tissue cross reactivity studies, and 3) verification of the *in-vivo* efficacy.

5 Summary

The clinical manifestation and pathogenesis of Herpes Simplex infections depend on at least the site of primary infection, age, immune status of the host and the type of HSV. The frequency and severity of infections usually decrease in the immunocompetent population with standard antiviral therapies (e.g. acyclovir/valacyclovir or penciclovir/famciclovir). However, there is a growing concern to induce selection of therapy-resistant HSV strains in immunocompromised patients, stem cell transplant recipients and HIV/HSV co-infected patients, which requires the development of novel anti-HSV therapeutics with different mechanisms of action. In addition, the result of conventional standard therapies is often neither long-lasting nor very effective. Antibody immunotherapy has been demonstrated to be efficacious for the treatment and prevention of viral infections. There is a convincing number of evidence showing the protective role of antibodies against herpes simplex virus (HSV) and amelioration of the severity of HSV-related diseases through neutralizing antibodies in *in-vivo* models. This may portend a promising future for antibody therapy of HSV infection. Given the fact that there is always a risk of HSV resistance development against a specific therapy, there is still a need to develop new monoclonal antibodies with better efficacies and longer protection. This project's aim has been to develop fully human therapeutic IgGs targeting glycoprotein B of HSV as a novel therapy.

Fully human gB-specific single chain Fvs (scFvs) have been previously selected from patient-specific antibody library repertoires that were generated from lymph nodes of head and neck cancer patients. The project aimed to analyse the potential of selected antibodies for future therapeutic interventions in patients with HSV infections. For this purpose, selected scFvs were reformatted into IgG type molecules and produced, followed by functional *in-vitro* as well as *in-vivo* characterization.

Antigen specificity of antibodies against HSV-1/2 was analysed by using cell associated HSV glycoproteins (infected Vero cells). The EC50 for binding of H4 (H28) to HSV-1 F- and HSV-2 G- infected Vero cells was 8.5(6.7) nM and 10.9 (9.95) nM, respectively. The neutralization

capability of cell-free virus and cell to cell transmission of the antibodies was investigated. Analysis by immunofluorescence microscopy confirmed that cell-to-cell spread was completely inhibited in the presence of gB (HSV) specific mAb H4 and HDIT101 (75µg/ml) and the infection was limited to the initial infection of single cells via virions in the inoculum. In contrast, IgG H28 did not show efficient plaque-reducing effects at any used concentration up to 75µg/ml, which corroborates the limited neutralization efficiency of cell-free virus with this antibody.

With the purpose of finding the epitopes targeted by the investigated antibodies, antibody-resistant mutants were propagated *in-vitro* and the capability of the antibodies to bind to resistant mutants, to neutralize cell-free virus infection and to block virus transmission via cell-to-cell spread were investigated. Escape mutations against antibodies H4 and HDIT101 in HSV-1 conferred amino acid substitutions including R304Q in HDIT101-resistant HSV-1F and R335Q in H4-resistant HSV-1F. According to the data from competitive binding assays and amino acid substitution found in generated viral escape mutants, H4 and H28 bind to different epitopes of gB than HDIT101. In addition, R304Q and R335Q are defining critical amino acids of HDIT101 and H4 epitopes respectively. In addition, Fc effector functions (ADCC, CMC and ADCP) were characterized using infected Vero cells as well as HEK293T stably expressing gB. According to the results, the anti-gB specific antibodies H4, H28 and HDIT101 are all capable of mediating ADCP, while an activity in ADCC or CMC was not detected. Both gB (HSV)-specific mAbs H4 and HDIT101 neutralize HSV-1F/2G with similar efficacies regardless of presence or absence of complement.

Conclusively, high affinity, specificity and high neutralization capacity of gB(HSV) specific mAbs (HDIT101, H4) *in-vitro* and *in-vivo* independent to Fc functions of antibodies make them potentially promising anti HSV therapies. The results of this thesis suggest the possibility to translate the identified fully human antibodies into a clinical therapy and hence likely provide a novel way to combat HSV infection.

Die klinische Manifestation und Pathogenese von Herpes Simplex Virus (HSV) Infektionen hängt hauptsächlich von Faktoren wie dem primären Ort der Infektion, dem Alter und Immunstatus des Wirts sowie dem HSV Typ ab. In der Regel nimmt die Inzidenz und Schwere der Infektion bei immunkompetenten Populationen mit antiviralen Standardtherapien (z.B. Acyclovir/Valacyclovir oder Penciclovir/Famciclovir) ab. Allerdings werden bei immungeschwächten Patienten, Empfängern von Stammzell-Transplantationen und HIV/HSV Co-Infizierten, durch herkömmliche Therapien die mit zunehmender Dauer der Therapie resistente HSV Stämme zu induziert. Daher ist die Erforschung neuer anti-HSV Therapien, denen neue Wirkmechanismen zugrunde liegen, dringend nötig. Hinzu kommt, dass das Ergebnis konventioneller Standardtherapien oftmals weder lang anhaltend noch sehr effektiv ist. Antikörper Immuntherapien haben gezeigt, dass sie sowohl zur Behandlung als auch zur Vorbeugung viraler Infektionen geeignet sind. Vieles deutet darauf hin, dass neutralisierende Antikörper sowohl vor Herpes Simplex Viren schützen, als auch den Krankheitsverlauf in in vivo Experimenten verbessern. Dies verspricht eine potenzielle Zukunft der Antikörper als Therapie bei HSV Infektionen. In Anbetracht der Tatsache, dass stets die Möglichkeit einer HSV Resistenzbildung gegenüber einer bestimmten Therapie besteht, gibt es auch weiterhin Bedarf an neuen monoklonalen Antikörpern mit erhöhter Effektivität und längerem Schutz. Das Ziel dieser Arbeit war es, komplett humanisierte therapeutische IgGs gegen Glykoprotein B des HSV als neue Antikörper Therapie zu entwickeln.

Im Vorfeld dieser Arbeit wurden komplett humane gB-spezifische single chain Fv Fragmente (scFv) aus Antikörper Libraries, welche aus Lymphknoten von Kopf- und Nacken-Krebs Patienten generiert worden waren, selektiert. In diesem Projekt sollte das Potential einer Auswahl an Antikörpern als zukünftige Therapiemöglichkeit für Patienten mit HSV Infektionen getestet werden. Dazu wurden die ausgewählten scFv Fragmente in IgG Moleküle re-formatiert und produziert, um in Folge dessen durch funktionelle in vitro und in vivo Experimente charakterisiert zu werden.

Die Antigen Spezifizität der Antikörper gegen HSV-1/2 wurde mittels Zell-assoziierter HSV-Glykoproteine (in Form infizierter Vero Zellen) bestimmt. Die EC50 Werte der Bindung von H4

und H28 an HSV-1 F- bzw. HSV-2 G-infizierte Vero Zellen betrug 8,5 und 6,7 nM (HSV-1 F) bzw. 10,9 und 9,95 nM (HSV-2 G), respektive. Darüber hinaus wurden die neutralisierenden Eigenschaften der Antikörper bezüglich freiem Virus und der viralen Zell-zu-Zell Transmission getestet. Hierbei ergab die Analyse mittels Immunfluorenszenz-Mikroskopie eine komplette Inhibition der Zell-zu-Zell Transmission für die gB (HSV) spezifischen monoklonalen Antikörper H4 und HDIT101 (bei 75µg/ml). Im Gegensatz zu IgG H4 zeigte IgG H28 für keine der getesteten Konzentrationen bis 75µg/ml eine effektive Reduktion von Plaques, was die limitierte neutralisierende Wirkung dieses Antikörpers auf freien Virus bestätigt.

Um die Epitope der untersuchten Antikörper zu bestimmen, wurden Antikörper-resistente Mutanten in vitro gezüchtet und mit Hilfe dieser, die Antikörper auf ihre Bindungsfähigkeit an die Mutanten und ihre neutralisierende Wirkung in Bezug auf freien Virus sowie die Zell-zu-Zell Transmission hin untersucht. Die ausgebildeten Resistenzen des HSV-1 F gegen die Antikörper H4 und HDIT101 zeigten sich in Form von Mutationen der Aminosäuren R335Q im Fall des H4 Antikörpers und R304Q für HDIT101. Anhand der Daten aus kompetitiven Bindungs-Assays und den unterschiedlichen Aminosäure Substitutionen der resistenten Virus Stämme lässt sich schließen, dass H4 und H28 unterschiedliche Epitope auf gB besitzen, welche sich auch von den Epitopen des IgG HDIT101 unterscheiden. R304Q und R335Q stellen jeweils essentielle Aminosäuren der Epitope für HDIT101 bzw. H4 dar. Um die Antikörper weiter zu charakterisieren, wurde außerdem mit Hilfe infizierter Vero Zellen und stabil gB-exprimierender HEK293T Zellen die Fc Effektor Funktionen (ADCC, CMC und ADCP) getestet. Die Ergebnisse zeigen, dass alle gB-spezifischen Antikörper H4, H28 und HDIT101 in der Lage sind, ADCP Aktivität zu induzieren, während ADCC oder CMC Aktivität nicht festgestellt werden konnten. Die beiden gB(HSV)-spezifischen monoklonalen Antikörper H4 und HDIT101 neutralisieren HSV-1F/2G mit vergleichbarer Effektivität und zudem unabhängig vom Komplementsystem.

Somit lässt sich zusammenfassen, dass die hohe Affinität, Spezifität und eine hohe neutralisierende Wirkung der gB(HSV)-spezifischen monoklonalen Antikörper (HDIT, H4) in vitro und in vivo, diese als vielversprechende Kandidaten für eine HSV Therapie auszeichnen. Die Ergebnisse dieser

Arbeit sprechen für eine potenzielle Translation der charakterisierten vollhumanen Antikörper in die Klinik und somit für einen neuen Weg HSV Infektionen zu bekämpfen.

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