Identification of the cleavage site of herpes simplex virus type 2 glycoprotein G (gG) and its involvement in gG activities

THESIS
Submitted in partial fulfilment of the requirements for the degree

Doctor rerum naturalium
(Dr. rer. nat.)

awarded by the University of Veterinary Medicine Hannover

by
Sangar Srivaratharajan
Bremen
Hannover, Germany 2019
Supervisor: Prof. Dr. Abel Viejo-Borbolla

Supervisor Group: Prof. Dr. Abel Viejo-Borbolla
Prof. Dr. Paul Becher
Prof. Dr. Eike Steinmann

1st Evaluation: Prof. Dr. Abel Viejo-Borbolla
(Institute of Virology, Hannover Medical School)
Prof. Dr. Paul Becher
(University of Veterinary Medicine Hannover)
Prof. Dr. Eike Steinmann
(Ruhr-University Bochum)

2nd Evaluation: Prof. Dr. Alí Alejo Herberg
(CBMSO, Madrid)

Date of final exam: 30th of October 2019

Sponsorship: Niedersachsen-Research Network on Neuroinfectiology (N-RENNT)
Sonderforschungsbereich 900 (SFB900)
Marie Skłodowska-Curie Actions
Table of content

List of abbreviation ........................................................................................................... VII
List of figures ....................................................................................................................... X
List of tables ......................................................................................................................... XIV
List of collaborators ............................................................................................................ XVII
Identification of the cleavage site of herpes simplex virus type 2 glycoprotein G (gG) and its involvement in gG activities .................. XIX
Identifizierung der Spaltstelle des Glycoproteins G (gG) von Herpes Simplex Virus Typ 2 und der Einfluss auf gG Funktionen XXIV

1. Introduction ..................................................................................................................... 1

1.1. General facts of herpesviruses .................................................................................. 1
  1.1.1. Characteristics of herpesviruses ................................................................. 1
  1.1.2 HSV Cycle ........................................................................................................ 5
  1.1.3 Genome characteristics among the Herpesviridae members 14
  1.1.4 Latency and reactivation of herpesviruses .................................................. 18
1.2 Current status of HSV-2 ......................................................................................... 24
  1.2.1 Prevalence and HSV-2-associated pathogenesis .................................. 24
  1.2.2 Present and future treatment .................................................................... 27
1.3 Immune Response ................................................................. 31
  1.3.1 First steps against HSV-2 ................................................. 31
  1.3.2 Adaptive immune system response to HSV ....................... 40
  1.3.4 The role of chemokines during HSV-2 infection ............... 43
1.4 Immune evasion of HSV ................................................................. 51
  1.4.1 Immune evasion on the intracellular and cellular level ...... 51
  1.4.2 Immune evasion on the cytokine level .............................. 56
1.5 Characteristics of glycoprotein G ............................................. 62
  1.5.1 Functions of glycoprotein G in different Herpesviruses ..... 62
  1.5.2 Glycoprotein G from HSV-1 and HSV-2 ......................... 67
1.6 Secretory pathway and its role in gG2 processing .................. 70
1.7 Objectives ........................................................................... 79

2. Material and Methods ................................................................. 80
  2.1 Materials ............................................................................ 80
  2.2 Methods ............................................................................. 97
  2.2.1 Generation of cleavage deficient gG2............................... 97
    2.2.1.1 Site-directed mutagenesis (SDM) ............................. 97
    2.2.1.2 Overlap PCR for the generation of cleavage site knockout
            (CSKO) variants ....................................................... 99
    2.2.1.3 Generation of rSgG2GSm and rSgG2ΔCS constructs .... 105
    2.2.1.4 Cloning of the recombinant full-length gG2 (rFLgG2) into
            the pAV41 vector ................................................... 106
  2.2.2 Generation of deletion rSgG2 constructs for binding assays . 108
    2.2.2.1 Terminal deletion of residues in rSgG2 ....................... 108
2.2.2.2 Generation of intramolecular deleted rSgG2 constructs.. 109
2.2.3 Cloning of the cleavage site region into a reporter plasmid... 113
  2.2.3.1 Cloning of H2S between mCherry and Gaussia Luciferase (GLUC).................................................................................................................. 113
  2.2.3.2 Cloning of the H2S between eGFP and mCherry............ 114
2.2.4 Plasmid production, isolation and ligation......................... 116
  2.2.4.1 Transformation and plasmid isolation from DH5α (and DH10Bac) bacteria .......................................................... 116
  2.2.4.2 Restriction digest control of pFastBac-Mel and pcDNA3.1Zeo......................................................................................... 117
  2.2.4.3 Agarose gel DNA electrophoresis .................................... 118
  2.2.4.4 Ligation of amplified and digested inserts into the desired expression vector ............................................................... 118
2.2.5 Baculovirus Expression System ........................................ 119
  2.2.5.1 Transformation and Bacmid isolation of DH10Bac to produce recombinant baculovirus.................................................... 119
  2.2.5.2 Generation and production of recombinant baculovirus . 120
  2.2.5.3 Recombinant SgG2 production and purification ............. 121
2.2.6 General techniques to investigate protein expression and size ................................................................................................. 123
  2.2.6.1 Separation of proteins according to their molecular weight using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) ......................................................................................... 123
  2.2.6.2 Western Blot ..................................................................... 124
  2.2.6.3 Coomassie staining .......................................................... 125
2.2.7 Functional experiments ..................................................... 126
2.2.7.1 Heparin-binding assays................................................................. 126
2.2.7.2 Chemotaxis assay using Jurkat E6.1 cells ......................... 127
2.2.7.3 Immunofluorescence of the rgG2 variants in transfected HeLa cells .......................................................................................................................... 128
2.2.7.4 Preparation of dorsal root ganglion dissociated neurons in RND450 microfluidic chambers (MFC)....................................................... 130
2.2.7.5 Luciferase assay................................................................................. 139

3. Results................................................................................................. 140

3.1 Substitution of potentially cleavage-involved residues of gG2 140
   3.1.1 Arginine to alanine substitution leads to partial cleavage inhibition........................................................................................................... 140
   3.1.2 Cleavage site region residues to alanine substitution lead to partial inhibition of cleavage in rSgG2 constructs .......................... 146
3.2 Production of recombinant SgG2 for mass spectrometry analysis .................................................................................................................... 148
   3.2.1 Baculovirus Expression System led to high quantities of rSgG2.............................................................................................................. 148
   3.2.2 Mass spectrometry analysis identified Pro317 and Phe319 as potential cleavage sites .............................................................. 153
3.3 Cleavage is highly inhibited through deletion or glycine-serine replacement of the cleavage site region from Pro314 to Leu343.... 158
3.4 Identification of proteases involved in cleavage....................... 164
   3.4.1 Furin protease is not involved in the cleavage of gG2 .... 164
   3.4.2 Batimastat inhibition of MMPs does not affect gG2 cleavage and secretion .............................................................. 168
3.4.3 BrefeldinA inhibition did not induce unconventional protein secretion of rSgG2 .......................................................... 173

3.5 Transfer of H2S into reporter plasmids ........................................ 175

3.5.1 The cleavage site region H2S enables separate expression of two fused proteins ................................................................. 175

3.5.2 In silico analysis led to internal alternative secretion signals inside the reporter plasmid ....................................................... 181

3.5.3 Deletion of the signal peptide in the rSgG2 construct did not indicate an unconventional secretion pathway .............................. 185

3.6 Immunofluorescence of the wild type full-length gG2, SgG2 and cleavage deficient variants showed different patterns in expression ........................................................................................................... 188

3.6.1 Expression patterns of rSgG2 and retention of the C-terminal HA-tagged domain in permeabilised HeLa cells ........................... 190

3.6.2 Expression patterns and localisation of both signals inside rFLgG2 permeabilised HeLa cells ...................................................... 192

3.6.3 Co-localization of V5-tagged N-terminal and HA-tagged C-terminal domain in FLgG2ΔCS and rFLgG2GSm permeabilised HeLa cells ................................................................. 194

3.6.4 Characterisation of the MgG2 construct in permeabilised HeLa cells ......................................................................................... 197

3.7 Terminal and internal deletions of rSgG2 do not lead to lack of GAG-binding .............................................................. 199

3.8 The absence of the cleavage site region reduced the chemokine-enhancing activity compared to the wild type SgG2 .................. 204

3.9 The absence of the cleavage site region enhances the neurite outgrowth of disassociated dorsal root ganglion derived neurons in microfluidic chambers ................................................................. 210
4 Discussion ................................................................................................................................. 222

4.1 Attempts to identify the gG2 cleavage site led to the H2S region .................................................. 222

4.2 Several N- and C-terminal deletions within the rSgG2 constructs do not prevent heparin binding .......................................................... 237

4.3 The enhancement of chemokine activity by gG2 does not depend on cleavage in the context of the rSgG2 cleavage variants ............ 240

4.4 Cleavage deficiency in the context of the full-length gG2 increases neurite outgrowth ................................................ 242

4.5 Conclusions ............................................................................................................................. 246

5. References ........................................................................................................................................ 248

6. Supplement ..................................................................................................................................... 275

Affidavit ........................................................................................................................................ 277

Acknowledgements .......................................................................................................................... 279
List of abbreviation

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAV</td>
<td>adeno-associated viral vector</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen-presenting cells</td>
</tr>
<tr>
<td>ATF</td>
<td>activator transcription factor</td>
</tr>
<tr>
<td>BHV1 and 5</td>
<td>Bovine herpesvirus 1 and 5</td>
</tr>
<tr>
<td>cGAMP</td>
<td>Cyclic guanosine monophosphate–adenosine monophosphate</td>
</tr>
<tr>
<td>cGAS</td>
<td>Cyclic GMP-AMP synthase</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>DAI</td>
<td>DNA-dependent activator of IRFs</td>
</tr>
<tr>
<td>DRG</td>
<td>Dorsal root ganglion</td>
</tr>
<tr>
<td>dsDNA</td>
<td>double-stranded DNA</td>
</tr>
<tr>
<td>E</td>
<td>Early genes</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein-Barr virus</td>
</tr>
<tr>
<td>EHV1</td>
<td>Equine herpesvirus 1</td>
</tr>
<tr>
<td>FeHV-1</td>
<td>Felid herpesvirus 1</td>
</tr>
<tr>
<td>FNE</td>
<td>free nerve ending</td>
</tr>
<tr>
<td>gB</td>
<td>Glycoprotein B</td>
</tr>
<tr>
<td>gC</td>
<td>Glycoprotein C</td>
</tr>
<tr>
<td>gD</td>
<td>Glycoprotein D</td>
</tr>
<tr>
<td>GDNF</td>
<td>Glial cell line-derived neurotrophic factors</td>
</tr>
<tr>
<td>gG1</td>
<td>Glycoprotein g1 (HSV-1)</td>
</tr>
<tr>
<td>gG2</td>
<td>Glycoprotein g2 (HSV-2)</td>
</tr>
<tr>
<td>gH</td>
<td>Glycoprotein H</td>
</tr>
<tr>
<td>gL</td>
<td>Glycoprotein L</td>
</tr>
<tr>
<td>gM</td>
<td>Glycoprotein M</td>
</tr>
<tr>
<td>gN</td>
<td>Glycoprotein N</td>
</tr>
<tr>
<td>HHV-6A/B</td>
<td>Human herpesvirus 6A and 6B (Roseolovirus)</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>HHV-7</td>
<td>Human herpesvirus 7</td>
</tr>
<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>HSV-1</td>
<td>Herpes simplex virus type 1</td>
</tr>
<tr>
<td>HSV-2</td>
<td>Herpes simplex virus type 2</td>
</tr>
<tr>
<td>HVEM</td>
<td>Herpesvirus entry mediator</td>
</tr>
<tr>
<td>ICP</td>
<td>Infected cell protein</td>
</tr>
<tr>
<td>IE</td>
<td>Immediate early genes</td>
</tr>
<tr>
<td>IFI16</td>
<td>gamma-interferon-inducible protein 16</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IKK</td>
<td>Inhibitor of NFkB epsilon</td>
</tr>
<tr>
<td>ILTV</td>
<td>Infectious laryngotracheitis virus</td>
</tr>
<tr>
<td>IRF</td>
<td>IFN regulatory factor</td>
</tr>
<tr>
<td>ISG</td>
<td>Interferon-stimulated genes</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus kinase</td>
</tr>
<tr>
<td>kb</td>
<td>Kilobases</td>
</tr>
<tr>
<td>KIF3A</td>
<td>Kinesin family member 3A</td>
</tr>
<tr>
<td>KSHV</td>
<td>Kaposi's sarcoma-associated hepresvirus</td>
</tr>
<tr>
<td>L</td>
<td>Late genes</td>
</tr>
<tr>
<td>LAT</td>
<td>Latency-associated transcripts</td>
</tr>
<tr>
<td>LAT</td>
<td>Linker for activation of T-cells</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Bertani</td>
</tr>
<tr>
<td>MDA5</td>
<td>melanoma differentiation associated gene 5</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MHV-68</td>
<td>murine herpesvirus 68</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>MTOC</td>
<td>microtubule organising centre</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mechanistic Target of Rapamycin</td>
</tr>
<tr>
<td>Nalp3/Nlrp3</td>
<td>Nucleotide-binding oligomerisation domain, Leucine-rich Repeat and Pyrin domain-containing protein 3</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>NFkB</td>
<td>nuclear factor ‘kappa-light-chain-enhancer’ of activated B-cells</td>
</tr>
<tr>
<td>NK cells</td>
<td>Natural killer cells</td>
</tr>
<tr>
<td>NKR</td>
<td>NK-cell receptors</td>
</tr>
<tr>
<td>NLR</td>
<td>nod-like receptors</td>
</tr>
<tr>
<td>NPC</td>
<td>Nuclear pore complex</td>
</tr>
<tr>
<td>PRR</td>
<td>Pathogen recognition receptor</td>
</tr>
<tr>
<td>RET</td>
<td>Rearranged during transfection</td>
</tr>
<tr>
<td>RHVP</td>
<td>Rodent herpesvirus Peru</td>
</tr>
<tr>
<td>RIG-I</td>
<td>Retinoic acid-inducible gene-I</td>
</tr>
<tr>
<td>RLR</td>
<td>RIG-I-like receptors</td>
</tr>
<tr>
<td>RT</td>
<td>Room temperature</td>
</tr>
<tr>
<td>SARS-CoV</td>
<td>Severe acute respiratory syndrome-related coronavirus</td>
</tr>
<tr>
<td>SOT</td>
<td>Solid-organ transplantation</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription proteins</td>
</tr>
<tr>
<td>STD</td>
<td>Sexually transmitted diseases</td>
</tr>
<tr>
<td>STING</td>
<td>Stimulator of interferon genes</td>
</tr>
<tr>
<td>TBK1</td>
<td>Tank binding kinase 1</td>
</tr>
<tr>
<td>TG</td>
<td>Trigeminal ganglion</td>
</tr>
<tr>
<td>TGN</td>
<td>Trans-Golgi network</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptors</td>
</tr>
<tr>
<td>vhs</td>
<td>viral host shutoff protein</td>
</tr>
<tr>
<td>VZV</td>
<td>Varicella-zoster virus</td>
</tr>
</tbody>
</table>
List of figures

Fig. 1 Representative particle of *Herpesviridae* family members .......... 5
Fig. 2 Schematic representation of the HSV replication cycle .......... 9
Fig. 3 Infection pathway and latency site of HSV .......................... 20
Fig. 4 Schematic representation of all four chemokine subclasses ...... 44
Fig. 5 Schematic representation of leukocyte migration to the site of infection .................................................................................................................................................. 46
Fig. 6 Expressional pattern of gG2 ................................................. 78
Fig. 7 Schematic representation of the predicted cleavage sites ........ 98
Fig. 8 Schematic overview of the basic rgG2 constructs ............... 141
Fig. 9 Potential cleavage site sequence .......................................... 142
Fig. 10 Site-directed mutagenesis of region 2 and 0 ...................... 145
Fig. 11 Western Blot of cleavage deficient rSgG2 constructs ............ 147
Fig. 12 Schematic overview of the insect cell expression vector pFastBacMel .................................................................................................................................................. 150
Fig. 13 Supernatant and cell lysate of rSgG2-His transfected adherent Hi5 insect cells .................................................................................................................................................. 152
Fig. 14 SDS-PAGE of filtered and purified rSgG2 ......................... 153
Fig. 15 rSgG2 amino acid sequence coverage after AspN digestion and MALDI TOF analysis ................................................................. 154
Fig. 16 rSgG2 amino acid sequence coverage after Trypsin digestion and MALDI-TOF analysis ................................................................. 155
Fig. 17 rSgG2 amino acid sequence coverage after Trypsin and AspN digestion and MALDI TOF analysis ................................................................. 157
Fig. 18 Cleavage site knockout (CSKO) of rSgG2 ........................................ 159
Fig. 19 Western Blot of cleavage deficient rFLgG2 constructs ........ 161
Fig. 20 ProP 1.0 – Prediction for propeptide cleavage sites for furin in the rSgG2 ........................................................................................................ 165
Fig. 21 Potential furin consensus motifs within the SgG2 protein .... 167
Fig. 22 rFLgG2 transfection of LoVo cells ........................................ 168
Fig. 23 Pre-analysis of potential ADAM proteases involved in rSgG2 cleavage ........................................................................................................ 170
Fig. 24 Batimastat inhibition assay ..................................................... 172
Fig. 25 BrefeldinA reduced rSgG2 secretion in HEK293T transfected cells ........................................................................................................ 174
Fig. 26 H2S cleavage sequence within the reporter plasmid pM157 . 177
Fig. 27 H2S cleavage sequence within the reporter plasmid pGL3.... 180
Fig. 28 Secretome Server 2.0 - Alternative secretion signal analysis. 183

Fig. 29 Western blot of rSgG2ΔCS .............................................. 186

Fig. 30 Schematic representation of gG2 constructs used for immunofluorescence .......................................................... 189

Fig. 31 Immunofluorescence of rSgG2 transfected HeLa cells ....... 191

Fig. 32 Immunofluorescence of rFLgG2 transfected HeLa cells ...... 193

Fig. 33 Immunofluorescence of rFLgG2ΔCS transfected HeLa cells 195

Fig. 34 Immunofluorescence of rFLgG2GSm transfected HeLa cells 196

Fig. 35 Immunofluorescence of rMgG2 transfected HeLa cells....... 198

Fig. 36 Heparin-binding Assay of N- and C-terminal deleted rSgG2 201

Fig. 37 Schematic representation and Western Blot analysis of sequentially deleted rSgG2 ......................................................... 203

Fig. 38 Schematic presentation of the plate used in the migration assay .......................................................................................... 205

Fig. 39 Standard curve of JURKAT cells ............................................. 206

Fig. 40 Migration assay of JURKAT cells in the presence of HEK293T cells transfected supernatant ................................................. 209

Fig. 41 MFC experimental setup .......................................................... 211
List of tables

Tab. 1 Affinities of soluble gG1 (HSV-1) and gG2 (HSV-2) towards human chemokines................................................. 64
Tab. 2 Animals, Cell lines and Viruses................................................. 80
Tab. 3 Antibiotics and selection markers................................................. 82
Tab. 4 DNA vectors ...................................................................... 83
Tab. 5 Enzymes........................................................................ 84
Tab. 6 Kits............................................................................. 85
Tab. 7 Cell culture media and reagents................................................. 86
Tab. 8 Conditions for eukaryotic and prokaryotic cell culture .......... 87
Tab. 9 Additional reagents.............................................................. 88
Tab. 10 Primary antibodies.............................................................. 90
Tab. 11 Secondary conjugated antibodies ......................................... 91
Tab. 12 Laboratory Equipment.......................................................... 92
Tab. 13 Software tools.................................................................. 96
Tab. 14 Primer list for the introduction cleavage site mutations into region 2 and 0............................................................ 99
Tab. 15 Primer list for the introduction cleavage site mutations into region 2 and 0 ................................................................. 103

Tab. 16 Primer list for the introduction of three additional alanine residues upstream of region 2 in the construct CSKO0+2/6AA ........ 105

Tab. 17 Primer list for introduction of the Glycine-Serine motif (GSm) instead of the cleavage site and deletion of the cleavage site .......... 106

Tab. 18 Flanking primers for the transfer of full-length gG2 from pGEM-T into the pcDNA3.1Zeo plasmid ............................................. 107

Tab. 19 Flanking primers for terminal deletion of residues in rSgG2 109

Tab. 20 Primer list for the generation of sequential intramolecular deleted SgG2 ........................................................................... 111

Tab. 21 Primer list for the introduction of the H2S region into the pM157 reporter plasmid (replacing P2A in mCherry-P2A-GLUC) through overlap PCR .................................................................................................................. 113

Tab. 22 Primer list for the introduction of the H2S region into the pGL3 reporter plasmid (replacing P2A in eGFP-P2A-mCherry) through overlap PCR .................................................................................................................. 115

Tab. 23 Reagents and concentrations for SDS gel ......................... 124

Tab. 24 Reagents for the pull-down of GAG-binding proteins .......... 126

Tab. 25 Reagents for dissociation of mice neurons .......................... 132
Tab. 26 Reagent Composition.......................................................... 137
List of collaborators

The following persons collaborated in this project by providing expertise, samples and reactives:

<table>
<thead>
<tr>
<th>Name</th>
<th>Institution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prof. Abel Viejo-Borbolla</td>
<td>Hannover Medical School, Germany</td>
</tr>
<tr>
<td>Prof. Andreas Pich</td>
<td>Hannover Medical School, Germany</td>
</tr>
<tr>
<td>Prof. Antonio Alcamí-Pertiño</td>
<td>Centro de Biología Molecular Severo Ochoa, Madrid, Spain</td>
</tr>
<tr>
<td>Prof. Beate Sodeik</td>
<td>Hannover Medical School, Germany</td>
</tr>
<tr>
<td>Prof. Christine Goffinet</td>
<td>Charité - Universitätsmedizin Berlin</td>
</tr>
<tr>
<td>Dr. Claus-Henning Nagel</td>
<td>Richter-Helm BioLogics, Hamburg, Germany</td>
</tr>
<tr>
<td>Prof. Eike Steinmann</td>
<td>Ruhr-University Bochum, Germany</td>
</tr>
<tr>
<td>Dr. Felix Polten</td>
<td>Hannover Medical School, Germany</td>
</tr>
<tr>
<td>Dr. Kai Kropp</td>
<td>Hannover Medical School, Germany</td>
</tr>
<tr>
<td>Lisa Rosemeyer</td>
<td>Leibniz University Hannover, Germany</td>
</tr>
<tr>
<td>Prof. Paul Becher</td>
<td>University of Veterinary Medicine Hannover, Germany</td>
</tr>
<tr>
<td>Pengfei Yu, MD</td>
<td>Department of Hematology, Shanghai East Hospital, Tongji University, Shanghai 200120, China</td>
</tr>
<tr>
<td>Shuyong Zhu, PhD</td>
<td>Hannover Medical School, Germany</td>
</tr>
<tr>
<td>Simon Krooss</td>
<td>Hannover Medical School, Germany</td>
</tr>
<tr>
<td>Victor Gonzalez Motos, PhD</td>
<td>Institute of Viral and Liver Disease, Strasbourg, France</td>
</tr>
</tbody>
</table>
Identification of the cleavage site of herpes simplex virus type 2 glycoprotein G (gG) and its involvement in gG activities

Sangar Srivaratharajan

The neurotropic herpes simplex virus type 2 (HSV-2) is a highly prevalent human virus, occurring commonly in the industrial as well developing part of the world. HSV-2 is transmitted easily through mucosal contact between individuals. Subsequently primary infection, the virus establishes latency in neurons of the peripheral nervous system. Both primary infection and reactivation are normally linked to disease. However, asymptomatic reactivation also occurs and may result in increasing transmission rates due to the lack of awareness of the affected and naïve individuals. HSV-2 causes several diseases, including cold sores and herpes labialis and genitalis, eczema herpeticum, herpes simplex encephalitis, herpetic keratitis and disseminated disease in the
neonate. The outcome of infection depends on the interaction between HSV-2 and the immune and nervous systems.

Chemokines and neurotrophic factors are involved in the interplay between the immune and nervous systems. Chemokines orchestrate leukocyte migration to the site of infection. They also induce neurite outgrowth and pain. Neurotrophic factors, like nerve growth factor (NGF), are required for neuronal development, survival and neurite outgrowth. They are also involved in inflammation and the maintenance of HSV latency. HSV-1 and HSV-2 glycoprotein G (gG1 and gG2, respectively) bind chemokines with high affinity and enhance chemokine-dependent leukocyte migration. Similarly, gG1 and gG2 bind NGF with high affinity, but only gG2 enhances NGF-dependent neurite outgrowth. The reasons for this difference between gG1 and gG2 are unknown. Finally, both gG1 and gG2 bind to the cell plasma membrane through glycosaminoglycans (GAGs). The functional meaning of this interaction is unknown. We hypothesise that binding to GAGs is important to modulate chemokine and NGF functions.
gG is the most divergent glycoprotein between HSV-1 and HSV-2. gG1 and gG2 have 212 and 673 amino acids, respectively. Both are type I transmembrane proteins present on the plasma membrane and viral envelope. However, gG2 is proteolytically cleaved, releasing an N-terminal domain to the supernatant of infected cells. This N-terminal domain is responsible for modulating chemokine and NGF activity. It is not known whether cleavage and secretion of gG2 have any functional relevance on gG2 activities.

The first objective of this thesis was to identify the gG2 cleavage site. So far, studies indicated that cleavage in gG2 occurs between the residues Arg321Ala322 and between Arg342Leu343, although an experimental proof is lacking. To identify the cleavage site, we performed extensive mutagenesis studies. We conclude that amino acids 314 and 343 are responsible for gG2 cleavage. Deletion or replacement of this sequence (termed here H2S) with a glycine-serine motif (GSm) inhibited nearly completely gG2 cleavage. Moreover, the transfer of the H2S sequence between two proteins resulted in cleavage. Interestingly, deletion of the
H2S sequence from full-length gG2 resulted in release of the protein, containing the transmembrane domain, to the supernatant. We are currently exploring the mechanism(s) leading to this phenomenon. Our working hypothesis is that full-length gG2 is released within extracellular vesicles.

The second objective was to determine whether gG2 cleavage is relevant for gG2 activities. Our results indicate that deletion of the H2S sequence did not dramatically affect gG2 functions. However, its substitution by the GSmed abrogated gG2 activities.

The third and final objective was to identify the GAG, chemokine and NGF binding sites. To do so, we generated truncated gG2 constructs. Unfortunately, many of these constructs showed aberrant expression precluding us from reaching any conclusions within this objective.

In summary, gG2 cleavage in the context of the short SgG2 construct seems to be not necessary for gG2 activity, since leukocytes migration was increased compared to CK alone. Inhibition of the cleavage site in the context of the full-length protein led to longer neurites compared to
the cleaved protein. This may be due to the secretion of the full-length gG2, possibly within extracellular vesicles. The latter observation indicates a novel potential secretion feature of cleaved WT-SgG2, which may improve viral infection and modulation of the immune and nervous systems.
Identifizierung der Spaltstelle des Glycoproteins G (gG) von Herpes Simplex Virus Typ 2 und der Einfluss auf gG Funktionen

Sangar Srivaratharajan


Chemokine und neurotrophe Faktoren sind in der Wechselwirkung zwischen dem Immunsystem und dem Nervensystem beteiligt. Chemokine koordinieren Leukozyten Migration zum Infektionsherd, diese können außerdem für das Wachstum von Neuriten und Schmerzen verantwortlich sein. Die neurotropfen Faktoren, wie z.B. der Nervenwachstumsfaktor (NGF), sind für das Neuriten Wachstum und neuronale Entwicklungen, sowie deren Überleben verantwortlich. Sie spielen ebenfalls eine wesentliche Rolle bei Entzündungen und der Aufrechterhaltung der HSV Latenz. HSV-1 und HSV-2 Glycoprotein G (gG1 bzw. gG2) binden Chemokine mit einer hohen Affinität, und steigern die Chemokin-abhängige Leukozyten Migration. Genauso ähnlich, binden gG1 und gG2 mit hoher Affinität NGF, wobei nur gG2 das NGF-abhängige Neuriten Wachstum steigert. Die Gründe hierfür
sind bisher unbekannt. Des Weiteren binden gG1 und gG2 die Glykosaminoglykane (GAGs) auf Zelloberflächen. Die funktionelle Relevanz ist auch hier unbekannt. Es wird jedoch vermutet, dass das Binden an GAGs, die Funktionen von Chemokinen und NGF beeinflussen.

Das gG Protein weist die meisten Unterschiede zwischen HSV-1 und HSV-2 auf, welches sich bereits durch die Größe der jeweiligen Peptide feststellen lässt (212 bzw. 673 Aminosäuren). Beide sind Typ I Transmembranproteine, welche sich auf den Zelloberflächen oder viralen Hüllen befinden. Jedoch sei zu erwähnen, dass das gG2 proteolytisch gespalten wird, was zur Sekretion in den Zellüberstand führt. Bisher bekannt ist die funktionale Steigerung von Chemokinen und NGF durch den sekretierten Teil. Ob die proteolytische Spaltung in diesem Zusammenhang eine relevante Funktion hat, ist bisher unbekannt.

Die erste Zielsetzung dieser Thesis war die Identifizierung der gG2 Spaltstelle. Trotz fehlender Beweise wurde in einer bisherigen Publikation auf die Spaltstellen Arg321Ala322 und Arg342Leu343
verwiesen. Um die Spaltstelle zu identifizieren wurden ausgedehnte Mutationsstudien durchgeführt. Daraus lässt sich folgern, dass die Aminosäuren zwischen Pro314 und Leu343 verantwortlich für die Spaltung von gG2 sind. Die Deletion oder das Ersetzen mit einem Glycine-Serine Linker (GSm) dieser Region (definiert als H2S) inhibierte beinahe komplett die gG2 Spaltung. Interessanterweise führte die Deletion der H2S Sequenz im Kontext der vollen Länge des gG2 (FLgG2ΔCS) zur Sekretion des Proteins, inklusive der Transmembrandomäne, in den Zellüberstand. Momentan versuchen wir diese Art der Sekretion im Kontext mit dem FLgG2ΔCS zu verstehen. Unsere Hypothese ist, dass das FLgG2ΔCS in extrazelluläre Vesikel abgegeben wird.

Die zweite Zielsetzung beschäftigte sich mit der Überlegung, ob die gG2 Spaltung eine funktionelle Relevanz hat. Die Ergebnisse wiesen darauf hin, dass die Deletion der H2S Sequenz nicht die gG2 Funktionen beeinflusste. Die Substitution mit dem GSm Linker hingegen beeinträchtigte die gG2 Funktion.
Die dritte und finale Zielsetzung war die Identifizierung von GAG, Chemokin und NGF Bindedomänen. Um diese zu identifizieren, wurden gekürzte gG2 Konstrukte generiert. Jedoch zeigten einige Konstrukte anomale Proteinexpressionen, was zu einem Ausschluss jeglicher Schlussfolgerungen für diesen Teil des Projekts führte.

Zusammenfassend lässt sich festhalten, dass die gG2 Spaltung im Zusammenhang mit dem kurzen SgG2 Konstrukt nicht notwendig für die gG2 Aktivität ist, da die Chemokin-abhängige Leukozyten Migration im Vergleich zum alleinigen Chemokin erhöht war. Die Inhibierung der Spaltung im Zusammenhang mit dem FLgG2 führte, im Vergleich zum spaltbaren WT-FLgG2, zu einem erhöhten Neuriten Wachstum. Diese Erkenntnis hat gegebenenfalls eine alternative virale Infektion und Modulierung des Immune- und Nervensystems zur Folge.
1. Introduction

1.1. General facts of herpesviruses

1.1.1. Characteristics of herpesviruses

James Lovelock once said: “An inefficient virus kills its host. A clever virus stays with it.” James Lovelock highlights with this sentence the members of the *Herpesviridae* family. Human herpesviruses and their hosts have a long co-evolution, where the existence of the virus is not recognised by, or it is controlled in healthy humans by the immune system. This quiescent existence of the viral genome is called latency, which in turn, is crucial for the virus to remain inside the host and, at some point, the contagious individuals can transmit viruses through blisters or cold sores to other hosts. In contrast, Ebola, human immunodeficiency virus (HIV), severe acute respiratory syndrome coronavirus and avian influenza usually display an enhanced virulence in humans due to their short co-evolution, more precisely recent zoonotic
background\textsuperscript{1}. The herpes simplex virus type 2 (HSV-2) belongs to the \textit{Herpesviridae} together with herpes simplex virus type 1 (HSV-1), varicella-zoster virus (VZV), human cytomegalovirus (HCMV), human herpesvirus 6A, B and 7 (HHV-6A, B and HHV-7, respectively), Epstein-Barr virus (EBV) and Kaposi’s sarcoma-associated herpesvirus (KSHV).

The family of \textit{Herpesviridae} evolved its three main subfamilies probably 180-220 million years ago. The evolution of the sublineages within each subfamily occurred before the mammalian radiation, 80-60 million years ago\textsuperscript{2}. Depending on the cell tropism, mainly due to their site of latency, family members are divided into three subfamilies, where HSV-1, HSV-2 and VZV belong to the subfamily \textit{\alpha-Herpesvirinae}\textsuperscript{1}. These members are neurotropic viruses and establish latency in peripheral nerve ganglia after primary infection in the mucosa or epithelial cells. They have a short replicative cycle with a broad range of infection sites. On the other side, members of \textit{\beta-Herpesvirinae}, like HCMV, HHV-6A, B and HHV-7, tend to infect and establish latency in lymphocytes, secretory glands and
kidney cells. Further, they are characterized by a longer replicative cycle. The members of the $\gamma$-Herpesvirinae have restricted viral replication properties and are mainly lymphotropic. Therefore, these viruses tend to infect T or B lymphocytes. The human herpesvirus KSHV and EBV belong to the last subfamily$^3$.

Structurally, members of Herpesviridae have many elements in common. The virion diameter of Herpesviridae members varies from 186 nm to 225 nm$^4$. The inner core of the capsid contains the double-stranded DNA (dsDNA), which has a size of approximately 129-230 kilobases (kb), encoding for 60-120 genes. The Cryo-EM analysis of purified B-capsids revealed the DNA resides in a liquid-crystalline form and is arranged in a toroid or spool like structure within the nucleocapsid$^5,6$. The nucleocapsid consists of 162 capsomeres, which are arranged in an icosahedral structure. This structure is composed of 140 hexagons, 11 pentons and one portal$^7$. The nucleocapsid is surrounded by the tegument, which is made of several proteins that are indispensable for several viral and cell regulations. Different numbers of tegument proteins are essential
for the virion during assembly. Many studies investigated the content of these teguments and revealed their functional purposes, like before and after de novo protein synthesis, structural and non-structural regulations during virion assembly. More precisely targeting viral elements to or away from the nucleus, hijacking cellular, molecular transporters, regulation of host and virus gene expression, and viral assembly while egress\textsuperscript{8–10}. A lipid bilayer forms the envelope that covers the tegument, a typical characteristic for the \textit{Herpesviridae} family. The host-membrane derived envelope contains several viral and host-derived proteins. Human herpesviruses have an estimated number of 12 (HSV-1) or even 20 viral integral membrane proteins. The six conserved glycoproteins on the viral envelope are glycoprotein C (gC), glycoprotein B (gB), glycoprotein H (gH), glycoprotein L (gL), glycoprotein M (gM) and glycoprotein N (gN). They have different functions depending on the infection process, e.g., in many human herpesviruses gC is necessary for attachment and gB, gH and gL are necessary for the fusion of the viral envelope with the hosts membrane\textsuperscript{11,12}. 
Fig. 1 **Representative particle of Herpesviridae family members.** Characterised by the dsDNA inside the nucleocapsid, followed by the inner and outer tegument and the lipid bilayer envelope. The lipid bilayer is host membrane derived and characterised by different glycoproteins

### 1.1.2 HSV Cycle

HSV-2 (and HSV-1 and VZV) infects primary mucosal or skin cells and tries to reach sensory nerve endings. Then, HSV-2 travels retrogradely along the axons towards the neuronal cell body, where the virus establishes latency. Depending on the health status of the host or
epigenetic influences, HSV induces its lytic cycle gene expression to produce new virus progeny that migrates in an anterograde manner towards the periphery, where HSV particles shed through the infected tissue and spread to other hosts. For the infection of mucosal or skin tissue, HSV requires different glycoproteins to attach, bind (gC, gB, gD) and mediate membrane fusion (gB, gH, gL). The initial attachment is dependent on glycosaminoglycans (GAGs), which are located on host cell membranes and show a variety of different long unbranched, polar and hydrophilic polysaccharides. The binding to GAGs increases the chance of several virus particles to attach to the host cell membrane and bind to their corresponding entry receptors\textsuperscript{13–15}. The soluble forms of glycoprotein G from HSV-1 (gG1) and HSV-2 (gG2) are also able to bind to GAGs through a non-canonical motif\textsuperscript{16}. A cell line derived from Chinese hamster ovaries lacking heparan sulfate is infected by HSV-1 with less efficiency than the wild-type cells\textsuperscript{17}. HSV particles can be uptaken through three different known ways: (I) direct fusion of the viral envelope with the plasma membrane, (II) endocytosis into acidic or neutral endosomes followed by fusion or (III) macropinocytosis\textsuperscript{18–20}. 


The uptake depends on the tissue and the members of the *Herpesviridae* family. Some viruses require different co-receptors than other members; for example, EBV requires additional glycoprotein 42 to fuse with lymphoid cells\(^{21}\). In the case of HSV-1 and HSV-2, initial binding to heparan sulfate proteoglycans occurs through gB or gC followed by interaction with specific receptors mediates infection. Studies have shown that gC increases HSV-1 infectivity tenfold\(^{11}\). Besides, HSV-2 requires gC for initial binding to the cell surface heparan sulfate facilitating infection\(^{22}\). These results are in agreement with the work of Herold *et al.*, where they define beside gB, gC as an essential factor for binding heparan sulfate and entry\(^{23}\). gD is the key for viral entry and fusion initiation in HSV-1 and HSV-2. Fusion starts with binding to potential receptors herpesvirus entry mediator (HVEM), nectin-1 or 3-O-sulfotransferase\(^{24-26}\). gD undergoes conformational changes and mediates activation of the neighbouring glycoproteins gB and gH/gL, which in turn initiate the formation of the fusion machinery with the host membrane. This mechanism leads to cell fusion and entry of the viral capsid into the host cell\(^{13,27}\). The fusion of α-herpesviruses depends on
the interplay of gB, gD, gH and gL because previous studies revealed sequential deletions of these glycoproteins lead to a severe reduction in viral titre and deletion of gC lead to less viral productivity\textsuperscript{28,29}. In this complex, gH function and surface localisation require gL\textsuperscript{30,31}. Other viral proteins of HSV show additional modulating properties during fusion, i.e. gK and gM. On the other side, the host cell membrane provides attachment possibilities with the lipid-raft relocated receptor nectin-1, which is initiated by gB and integrin Vβ3 interplay. In the same context, gG1 is involved in infection of apical surfaces of polarised cells, through an unknown mechanism\textsuperscript{32–35}. 
**Fig. 2 Schematic representation of the HSV replication cycle.** Virus particle attachment occurs through the GAG interaction. This step is followed by specific interaction(s) between viral glycoprotein(s) and cellular receptor(s) and glycoprotein-mediated fusion. The capsid is then transported through the microtubules towards the nucleus (NC), where the dsDNA is injected through the nuclear core complex into the NC. The lytic replication starts together with the sequential expression of immediate early genes (IE), early genes (E) and late genes (L). Encapsulation of viral DNA occurs, which is then followed by the first envelopment through the inner nuclear membrane and following de-envelopment through the outer nuclear membrane and capsid release into the cytosol. The secondary envelopment occurs at the Golgi Apparatus (GA), and full enveloped virus particles are released. Cell membrane located viral glycoproteins facilitate cell-to-cell spread.
During initial HSV attachment, fusion and capsid release into the cell, actin-filament bundle rearrangements close to the capsid occurs. Primarily, these bundle filaments should prevent the entry of virus particles, but herpesviruses counteract these mechanisms in order to continue the infection process\textsuperscript{36}. Upon capsid release into the cytoplasm, the virus particle also enables the release of many tegument proteins. The tegument proteins pUL36 and pUL37 remain on the capsid after release into the cytoplasm. These proteins play a role in capsid transport along the microtubule towards the microtubule organising centre (MTOC) and its final destination, the nuclear pore complex (NPC)\textsuperscript{37–40}. Generally, the microtubule transport machinery consists of kinesins (+)-end-directed motor proteins, which are involved in the transport of cargos away from the nucleus. On the other hand, dyneins are (-)-end-directed motor proteins that are involved in transport towards the nucleus\textsuperscript{41}. Due to the neuronal tropism, HSV has evolved mechanisms to overtake the cellular transport machinery to bypass the relatively long way from the axonal infection site towards the cell body. Once the capsid reaches the MTOC, they are close to the nucleus, able to attach to the NPC. Once the capsid
is attached to the NPC through pUL36, the capsid injects the DNA with the help of CAN/Nup214 and pUL25 with high pressure into the nucleus, where the DNA changes its form from linearised to the circularised viral genome. This conformation enables the safe maintenance of the viral genome as an extrachromosomal circular episome inside the nucleus of an infected cell, which excludes the requirement for the chromosomal integration\textsuperscript{42,43}. Since HSV establishes latency in post-mitotic cells, it does not require to bind its episome to the cellular chromosomes for segregation to the progeny, as other herpesviruses do. Establishment of latency in neurons allows HSV to settle inside the host in long-living cells and have a suitable replicative environment. The evolutionary adaptation of \textit{α-Herpesvirinae} to neurons has several advantages and starts with the immune-privileged status, cell cycle arrest (except the absence of neurotrophic factors, DNA damage, oxidative stress and excitotoxicity), continuous persistence and viral-preservation. So, it is a smart evolutionary move to establish latency, and it is an even smarter move to target neurons to establish latency (illustration of lytic HSV replication cycle, see figure 2).
Researches identified several pathways involved in inducing neuronal growth and survival of peripheral sensory and sympathetic nerve cells. The interplay of neurotrophins and their corresponding receptors is essential for axonal guidance and neuronal survival. There are several neurotrophic factors involved in the previously mentioned processes, like nerve growth factor (NGF), brain-derived neurotrophic factor, neurotrophin-3 (NT3), NT4/5 and their corresponding receptors, tropomyosin-related kinase A (TrkA), TrkB and TrkC. In the context of Trk receptors, another receptor involved in neuronal maintenance is the p75 neurotrophin receptor, belonging to the TNF receptor superfamily. P75 is a co-receptor for all neurotrophins and has a dual role during neuronal development and regulation. Moreover, the glial cell line-derived neurotrophic factors (GDNF) family ligands are to the same degree necessary for the neuronal regulation, and their ligands are involved in the activation of the co-receptor GDNF family receptor α family, which in turn activates tyrosine kinase receptor RET (rearranged during transfection). The latter interaction complex is necessary for the embryonal neurons and kidney cell development. Neurotrophic factors
are also involved in the maintenance of HSV latency since their removal induces reactivation \textit{in vitro}. Moreover, HSV-1 and HSV-2 establish latency in different neuronal subtypes that express different receptors for neurotrophic factors.

Some neurotrophic factors, such as NGF, are involved in immune-stimulatory processes as well, characterised by the interplay between the nervous, immune and endocrine systems and the resulting reception of pain, itch and inflammation\textsuperscript{46}. Due to the relevance of neurotrophic factors in neuronal survival, neurite outgrowth and HSV latency and reactivation, the group of Antonio Alcamí investigated their possible modulation by HSV. The work of Cabrera and colleagues revealed that gG1 and gG2, the focus of this thesis, bind NGF with high affinity\textsuperscript{47}. gG1 and gG2 are type I transmembrane proteins. The N-terminal domain of gG2 is cleaved and secreted to the supernatant. However, gG1 is not cleaved and remains as a type I transmembrane protein. The soluble gG1 ectodomain and the N-terminal secreted gG2 domain are responsible for NGF interaction. Interestingly, only gG2 modulates NGF activity,
enhancing NGF-dependent neurite outgrowth of sympathetic neurons ex vivo. The mechanism of action involves redirection of TrkA to lipid rafts, reduced ligand-mediated internalisation and subsequent retrograde transport. Remarkably, in vivo studies revealed enhanced growth of peptidergic neurons into the hind paw epidermis\textsuperscript{47}. This enhancement in neurite outgrowth may facilitate the infection of NGF-responsive neurons. Interestingly, HSV-2 also increases neurite outgrowth following reactivation from latency in humans. Reactivation leads to enhanced expression of IL-17c in human keratinocytes in the genital mucosa, leading to enhanced neurite outgrowth\textsuperscript{48}. The authors hypothesise that this is a protective effect to avoid neurite damage and neuronal death upon HSV-2 reactivation.

1.1.3 Genome characteristics among the Herpesviridae members

The genome of herpesviruses contains unique regions edged by direct or inverted repeats. The unique regions may appear in different orientations as a result of recombination during replication. The orientation of unique regions does not have any consequences on viral replication\textsuperscript{49,50}. 


Genomic studies have revealed the relationship among all members of *Herpesviridae*, where they determine centrally located 43 core genes within the human herpesviruses subfamilies that suggests the existence of a common ancestor. Despite the different orientations of core genes, human herpesviruses show the presence of the same core genes\textsuperscript{51}. In contrast, recently evolved non-core genes are more located at the termini of the genome\textsuperscript{52}. The comparison of core and non-core genes shows a high number of core genes, due to their importance in viral replication. The non-core genes are involved in host-interaction and cell adaptation. From an evolutionary point of view, all genes have established their importance within the viral genome, and others were sorted out or lost during evolution\textsuperscript{53}.

Further investigations revealed similarities among the *Herpesviridae* family towards the terminase complex that is important during the DNA packaging process. Additionally, similar genes are found in the region encoding for DNA polymerase, deoxyuridine triphosphatase and nucleoside kinase\textsuperscript{53,54}. The genome size varies from 129 – 230 kb and
every 1.5-2 kb contains one gene, which is transcribed via the host RNA polymerase II\textsuperscript{55}. Each gene has its promoter for expression (except EBV, common 5’-leader). It is also known that some genes share the same 3’-polyadenylation site, which results in 3’-coterminal transcripts\textsuperscript{56}. The number of introns differs among the \textit{Herpesviridae} subfamilies. The subfamilies β- and γ-\textit{Herpesvirinae} show more intron-containing genes than members of α-\textit{Herpesvirinae}\textsuperscript{53}. Among the genes, some members of \textit{Herpesviridae} include non-coding regions, like the latency-associated transcripts (LAT).

The initial viral replication cycle starts with an expression of immediate-early (IE) genes, which is defined by their ability to be transcribed without newly synthesised proteins. The IE genes encode regulatory proteins for the expression of early (E) genes. In this step of viral replication, further regulatory protein expression occurs. Here, the expressed proteins maintain the continuous viral genome replication, accumulation of early and late mRNA and express the late (L) genes for main structural components that in turn lead to assembly and release of
infectious particles. Altogether, the viral replication, gene expression and capsid expression happen in the intranuclear loci$^{57-59}$. For initial assembly, nuclear import protein importin-α1 plays a vital role in infected cell protein 4 (ICP4) import into the nucleus, capsid assembly inside the nucleus and capsid transfer into the cytoplasm$^{60}$. Besides ICP4, importin-α1 is essential to import other viral proteins (ICP0, ICP8, pUL30, pUL42, pUL31) into the nucleus as well$^{60-62}$. During the viral assembly and compartment formation, the nucleus changes upon viral replication. Here, G-actin is responsible for this event and necessary for chromatin and lamina disruption, capsid movement towards the nuclear membrane, together with the viral proteins pUL31 and pUL34$^{63}$. After approaching the nuclear membrane, the capsids go through the inner nuclear membrane (1st envelopment), which is then removed once it goes through the outer nuclear membrane and released to the cytoplasm. The whole process is depending on the nuclear egress complex, which contains several viral (pUL31, pUL34, γ134.5, pUS3, pUL47) and cellular proteins (p32, protein kinase C)$^{64}$. The whole envelopment of the
capsid is accompanied with coverage of the inner tegument consisting pUL36 and pUL37 within the cytoplasm, followed by binding to the trans-Golgi network (TGN) derived outer tegument, together with the secondary envelopment, when viral glycoproteins are incorporated into the viral particle\textsuperscript{65} (illustration of lytic HSV replication cycle, see figure 2).

1.1.4 Latency and reactivation of herpesviruses

The main differences between HSV-1 and HSV-2 are the prevalence level in the population, the site of infection and the target for latency. Historically, HSV-1 was considered to infect the orolabial mucosa and establish latency in the trigeminal ganglion (TG), whereas HSV-2 was associated with genital infections and latency in dorsal root ganglia (DRG) of the sacral region. However, recent epidemiological data show that HSV-1 infects both the orolabial and genital mucosa and establishes latency in TG and DRG. However, HSV-2 is still mostly restricted to the genital mucosa and sacral ganglia with a low number of individuals being infected by HSV-2 in the TG\textsuperscript{66}. Although both viruses establish latency
in sacral ganglia, HSV-2 reactivation is linked to more severe disease than that of HSV-1. This may be due to the fact that HSV-1 and HSV-2 seem to establish latency in different neuronal subtypes. In contrast to the lytic replication, latency is characterised by the suppression of viral gene expression, which enables evasion of the host immune system and persistence through the life of the host\textsuperscript{66}. The primary infection and the proximity towards the free nerve endings (FNE), allows HSV to reach the cell body and its nucleus of sensory neurons. The infection process enables the viral DNA injection to establish its genus-specific latency inside the nucleus. Furthermore, latency relies on epigenetic influence like packaging by histones since its composition and modifications indicate a regulatory factor in latency\textsuperscript{43,67,68}. Another passive regulatory factor for latency establishment is induced through the axonal transport itself, where lytic regulatory factors, like viral protein 16 (VP16) remains in the axon, and lytic gene expression is not active. Studies have also shown that distal infections favour latency, whereas infections in proximity to the nucleus initiate the lytic programm\textsuperscript{69}. 
Fig. 3 **Infection pathway and latency site of HSV.** Infection of epithelial cells leads to productive replication and virus production inside the nucleus (NC), which in turn infects neighbouring epithelial cells. Once the virus particles are in proximity of free nerve endings (FNE), virus fuses with membrane and gets transported through the axon towards the nucleus of the neuron. The dsDNA gets injected and establishes latency. Reactivation initiates lytic gene expression and produces capsid that is transported towards the axonal termini. Fully enveloped particles are released and infect surrounding epithelial cells. Image adapted from Knipe and Cliffe.  

The highly abundant viral transcript called LAT, encoded within the viral genome, controls the latency. The transcripts originate from the flanking
region of the unique long region of the HSV genome and express different length of transcripts\textsuperscript{66,71}. Publications confirmed these findings and identified a minor LAT transcript (8.5 kb) that is spliced into transcript variants with different lengths called major LATs (2.2 kb [HSV-2] and 1.5 + 2 kb [HSV-1])\textsuperscript{71}. The LAT transcripts are present during latency, and they overlap (antisense) with several viral and host transcripts (ICP0, ICP34.5, eukaryotic initiation factor 2 dephosphorylase)\textsuperscript{72,73}. Stevens and colleagues first revealed that antisense transcripts of ICP0 (lytic initiator) are the most abundant transcripts in their experiments (compared to ICP4, ICP27, VP5), and it is repressed through the LAT-derived miR-H2-3p\textsuperscript{73,74}. In another case, ICP4 is controlled by miR-H6, which is essential for the initiation of viral replication\textsuperscript{73}. During the lytic replication, HSV-1 and HSV-2 induce apoptotic pathways in cells. Therefore they have evolved different strategies to counteract these signal transduction through the expression of different viral proteins (gD, gJ, protein kinase US3, ICP27, ICP10)\textsuperscript{75–78}. The same observation shows LATs in context of rabbit trigeminal ganglia and apoptosis, where LAT(-) HSV induced more apoptosis in
neuronal cells compared to HSV-LAT(+)\textsuperscript{79}. The latency state is suggested to be controlled through the host’s immune system via CD8\textsuperscript{+}-T-cell\textsuperscript{80}. Studies of Freeman and St. Leger supports these results by the association and endocrine influence of CD8\textsuperscript{+}-T-cell towards infected neurons, thus the maintenance of HSV latency (as reviewed in St. Leger and Hendricks\textsuperscript{81}).

Reactivation from latency is also poorly understood, and only a few internal and external stimuli are recognised as triggers for unbalance shift towards HSV replication. A few are investigated more precisely, like sunlight, psychological stress, fever, menstruation and surgical removal of organs\textsuperscript{82}. The first evidence for stress-dependent reactivation is shown 100 years ago, where the nerve responsible for chronic pain was treated directly and led to a herpes outbreak in its respective dermatome\textsuperscript{83}. Further, investigations show on the \textit{in vitro} and \textit{in vivo} level reactivation during ganglia explantation, CD8\textsuperscript{+}-T-cell depletion, hyperthermia, skin damage, UV irradiation or hormone treatment\textsuperscript{84–88}. Many studies focus on HSV-1 latency, where viral proteins like VP16 are identified as
promoters for the viral lytic program. Experiments show an increased VP16 protein level, which in turn induces the expression of IE genes, followed by viral replication in neurons\textsuperscript{89}. Moreover, VP16 forms a complex with host cell factor 1 and Octamer transcription factor 1, which in turn initiates expression of viral genes leading to lytic replication. An additional viral protein involved in reactivation is ICP0. As already mentioned its downregulation during latency seems to be controlled via the LAT-derived miR-H2-3p\textsuperscript{73}.

During the reactivation, ICP0 contributes an environment for transcription and subsequent degradation of cellular repressors (i.e., nuclear domain 10)\textsuperscript{90–92}. There are also evidences that ICP0 interacts with histone deacetylases changing chromatin status to make the DNA more accessible for the transcription machinery\textsuperscript{93,94}. Once the reactivation occurs, the assembled capsid is transported in an anterograde manner to the FNE’s, where they are released to the tissue and transmitted to new host’s (illustration of the infection pathway and latency site of HSV, see figure 3).
1.2 Current status of HSV-2

1.2.1 Prevalence and HSV-2-associated pathogenesis

Nowadays, there is an estimated number of 536 million people (15-49 years old) seropositive for HSV-2, which is nearly 10% of the total number of HSV-1 seropositive people. Worldwide are more women (315 mio.) than men (221 mio.) seropositive for HSV-2, which might be due to anatomical differences, since the genital area of women is more receptive to infections. The distribution depends on the age and starts at a young age and increases until 35-39 years and afterwards the number of seropositive people stagnates. Furthermore, the studies from Looker et al. reveal a higher prevalence in developing than in developed countries. The broad dissemination of both HSV’s is due to their easy transmission that occurs through droplet infection during oral-to-oral or oral-to-genital contact. Besides the physiological symptoms, seropositive patients suffer psychological distress that results in more dissemination of the viruses. As previously mentioned, the primary infection site of HSV-2 is the genital area, where it infects the mucosa or enters the skin.
through lesions. Due to the different sexual practices or individual viral dissemination, transmissions can cause cross infections in the oral or genital area\textsuperscript{98,99}. The acute clinical manifestation after primary infection starts with regional pain, itches, burning, which usually starts 24 hours post-infection. In the context of primary infection, HSV-2 causes the development of numerous and local vesicles. In the following infection process, HSV-2 can cause symptoms like headache, swollen lymph nodes, anorexia, malaise and continue with pneumonitis, but severe cases of keratitis and hepatitis are possible, too\textsuperscript{100}. Additional symptoms can complicate the disease process, by rising fever, local lymphadenopathy, dysuria, paresthesia and aseptic meningitis. Another severe infection process can happen during birth, where HSV-2 can spread to neonates and cause severe cases of keratitis, encephalitis and disseminated disease since new newborns have not developed full immunity. The mortality rate of HSV-2 exposed and untreated newborns are up to 85%. 75% of HSV-2-exposed babies suffer from encephalitis or neurological disorder\textsuperscript{97,101,102}. Another complication is the asymptomatic reactivation of HSV-2 in patients that facilitates easy transmission to seronegative
individuals\textsuperscript{103}. Once the virus establishes latency in neurons, reactivation leads to lesions on the primary infection site. Next to the genital or anal area, where the primary infection occurs, the reactivation causes cysts and sensitive pruritus that result in purulent cysts. The healing process is accompanied by crust and heals up without any scar.
1.2.2 Present and future treatment

Nowadays, HSV-2-caused disease is an underestimated health problem in society, since there is an unawareness of HSV-2 seropositive individuals, facilitating transmission between individuals. Additionally, there is also an ignorance followed by negligent behaviour in our society since talking about sexually transmitted diseases (STD) is a taboo topic. Even the consultation of a doctor is in many societies not well accepted by affected individuals, only in severe cases, seropositive patients consider visiting a doctor. However, it is not always ignorance, individuals also stay under the psychological burden that implies frustration, anxiety, wrath, fear of rejection and following isolation, guiltiness, discomfiture, shame, feeling of disgust and contagious. HSV-2 and other STDs have an impact on further health status and even increase the risk of HIV (3-fold or more) and other infections. The urgent need to understand HSV-2 and its consequences is also underlined through neonatal infections. HSV-2 neonatal infections occur in at least two different states of pregnancy: intrapartum (90%) or congenital (5%). The incidence of severely infected areas starts downward from skin, eye
and mouth, followed by the central nervous system and viral shedding throughout the body, whereby viral dissemination has the highest lethality\textsuperscript{108}. As previously mentioned, the HSV-2 transmission also happens during the asymptomatic state, which facilitates transmission to new and unaware hosts. Another field of interest is patients with solid organ transplantation (SOT), due to their high number of morbidity and mortality rate caused by HSV-2 primary infections or reoccurrences. SOT patients are more affected by HSV compared to immunocompetent patients\textsuperscript{109,110}. Most reactivations occur directly after transplantation or during immunosuppressive treatment to prevent transplant rejection\textsuperscript{111}. Rare cases of primary infection after transplantation are also reported\textsuperscript{112,113}.

After the initial antiviral response, further ectopic treatment is required to slow down the viral replication and shedding into neighbouring tissues. Nucleoside analogues, like acyclovir and further developments like valacyclovir and valganciclovir, target the HSV replication phase. They function as a deoxyguanosine analogue that lacks 3’-hydroxyl group of
natural nucleosides\textsuperscript{114}. In replicating cells, the viral thymidine kinase phosphorylates and activates the analogue, which is further phosphorylated by cellular kinases and introduced into the viral genome by the viral DNA polymerase, causing strand termination and stopping viral genome replication. Since the nucleoside analogue competes with the host deoxyguanosine and lacks the 3’-hydroxyl group, the viral polymerase is not able to continue the replication. The nucleoside analogue binds with higher affinity to the viral than the host polymerase reducing its incorporation into the host genome\textsuperscript{115}. Despite the fact of the first line and second line anti-viral treatment, viruses have evolved different resistance mechanisms to overcome the inhibition of viral replication. Some strains of HSV-1 and HSV-2 developed mutations in the viral thymidine kinase that result in lack of acyclovir phosphorylation (as reviewed in Piret and Boivin\textsuperscript{116}).

There is an urgent need to develop novel therapeutic measures, but so far, there are no licensed vaccines on the market. Even though certain companies stopped the development of vaccines during clinical trials in
2017, other approaches are still going on\textsuperscript{117}. One step is to inhibit HSV primary infection and develop subunit vaccines like recombinant glycoproteins gB and gD in combination with adjuvant MF59\textsuperscript{118}. Moreover, gD plays a crucial role in attachment and may a suitable target against HSV primary infection\textsuperscript{119}. Further, efforts are made in the development of live virus vaccine that lacks genes involved in immune evasion, such as gC blocking the complement cascade, blocking interferon (IFN) responses and antigen presentation towards CD8\textsuperscript{+}-T-cells\textsuperscript{120}. Another alternative is the usage of DNA vaccine, where they use different modulatory chemokines and show an increase of HSV-2 specific immune response. More precisely, in the presence of IL-8 and RANTES plasmid DNA lead to antigen-specific Th1 type CD4\textsuperscript{+}-T-cells response\textsuperscript{121}.

The key of HSV to survive undetected inside the host is through its latency state, and the previously mentioned treatments are targeting the active replicating virus. Therefore, approaches that target the genome inside the nucleus of HSV positive neurons are necessary. The delivery
of genome editing enzymes is one approach. Aubert and colleagues investigate an approach using an engineered homing endonuclease called HE-HSV1m5 that recognises the sequence within UL19, which encodes for VP5. Here they combine HE, the exonuclease Trex2 and adeno-associated viral vector (AAV) to target the latently infected cells and, as a result, they reveal low cell toxicity and reactivation. These effects are increased by pre-treatment with a histone deacetylase inhibitor. The same group investigated the effect of endonucleases in HSV latent infected mice. They introduce via AAV vectors mutations into the latent HSV genome in vivo. This effect is also increased in the presence of histone deacetylase inhibitors\textsuperscript{122,123}. Overall, there is an urgent need to understand HSV evasive mechanisms and attack the latent HSV genome directly.

1.3 Immune Response

1.3.1 First steps against HSV-2

The virus encounters two main parts of the host immune system: innate and adaptive immune systems. The innate immune system involves
immediate, non-specific, antiviral mechanisms against the pathogen. The expression of different cytokines initiates the innate immune system, which in turn activates several cells that function as the first barrier against pathogens. Pathogens like HSV replicate inside the nucleus, and each cell can present antigens by major histocompatibility complex (MHC) to activated lymphocytes such as natural killer cells (NK-cells). The MHC groups consist of MHC-I, MHC-II and MHC-III and appear on different cell types. Structurally, MHC-I is encoded by the BCA region on chromosome 6 and is made of α1, α2 and α3 domains. Next to the α3 domain, β2-microglobulin is covalently bound. The central part is built up by α1 and α2 domain and has a pocket, where the antigen binds and is presented to lymphocytes.

In contrast to MHC-I, the D-region encodes MHC-II and has two domains called α-polypeptide domain (α1 and α2) and β-polypeptide domain (β1 and β2). As MHC-I, MHC-II has a binding pocket between α1 and β1 for antigen presentation to lymphocytes. The last MHC-III class does not function in the same manner as MHC-I and MHC-II, the
genome region instead encodes proteins, involved in immune stimulation and many involved in cell-to-cell communication (complement factors; cytokines). MHC-I and -II differ from each other in their functions, i.e. MHC-I is expressed on all cells and presents endogenous antigens that show any alterations in the cell physiology. On the other hand, MHC-II’s are expressed on antigen-presenting cells (APC), such as B-cells and macrophages\textsuperscript{124}. Before the MHC antigen presentation, the viral infection needs to be detected. Here, different pattern recognition receptors (PRR) induce signal transduction pathways, which sequentially secrete type I IFN. Recognition starts with protein or nucleic acid binding on PRR that induces downstream signalling molecules like tank binding kinase 1 (TBK1) or inhibitor of nuclear factor ‘kappa-light-chain-enhancer’ of activated B-cells (NFκB) epsilon (IKK). These events activate the key regulator IFN regulatory factor 3 (IRF3) together with NFκB and activating transcription factors (ATF)/cJun, which in turn lead to expression and secretion of type I IFN. Following paracrine and autocrine signalling IFN induces the Janus kinases/signal transducer and activator of transcription proteins (JAK/STAT) pathway, followed by expression
of IFN-stimulated genes (ISG) encoding for additional effector molecules\textsuperscript{125}. In case of HSV recognition, ligands like HSV-derived DNA can activate several PRR depending on the structure and localisation (retinoic acid-inducible gene-I [RIG-I]; RIG-I-like receptors [RLRs]; toll-like receptors [TLR]; nod-like receptors [NLR]; DNA-dependent activator of IRFs [DAI]). In the case of protein recognition, some TLRs identify viral lipopeptides. Since HSV recognition occurs mainly through nucleic acid, scavenger receptors on cell surfaces bind and internalise dsRNA from apoptotic cells to process for intracellular recognition. Within the cell, endosomal TLR3 detects dsRNA, whereas TLR9 identifies common CpG-rich DNA. In the case of cytoplasmic RNA, cytoplasmic RIG-I and melanoma differentiation-associated gene 5 (MDA5) is responsible for detection and subsequent signal transduction\textsuperscript{125}.

On the other hand, DAI, NACHT-LRR-PYD domains-containing protein 3 (Nalp3/Nrlp3) and absent in melanoma 2 identify DNA in the cytoplasm, too\textsuperscript{126}. Another well-known sensor is cGAS that detects viral
DNA within the cytoplasm as well. This event leads to the production of cGAMP, which in turn activates the ER-located STING dimer. The activated STING dimer activates TBK1, followed by IRF3 phosphorylation and final transfer into the nucleus, where it induces type I IFN\textsuperscript{125}. Upon infection and absence of replication, new events of immune response occur inside the cell. Here, IRF-3 seems to be part of the early cellular immune response, because virus entry stimulates ISGs expression. The counter experiments, where IRF3 is downregulated, confirm the importance in this context\textsuperscript{127,128}. For HSV recognition, some of the previously mentioned receptors play an essential role in the detection of nucleic acids and proteins. Wang and colleagues show that HSV detection occurs through DAI, which is necessary for IFN\beta expression\textsuperscript{129}. Further, HSV recognition occurs also through Nalp3, which induces inflammasomes\textsuperscript{130}. Other receptors like RIG-I and TLR9 show implications in HSV detection\textsuperscript{131}. Beside the TLRs, adaptor protein MyD88 also plays a crucial role in HSV response, because MyD88-deficient mice show high levels of lethal encephalitis\textsuperscript{132}. However, specific TLRs are not always crucial for immune response, e.g. TLR9
deficient mice do not change the viral load inside the neuronal system and mortality rate upon HSV-1 infection\textsuperscript{133}. Moreover, the TLR2-mediated cytokine response even mediates severe viral-induced encephalitis in mice\textsuperscript{134}.

The host's innate immune system has a range of different cells involved in pathogen control, like NK-cells, which are involved in the differentiation between foreign and host own antigens. Therefore, NK-cells express three families of NK-cell receptors (NKR) that recognise different counterparts, called killer cell inhibitory Ig-like receptor, C-type lectin receptors, natural cytotoxicity receptors and Ig-like transcripts\textsuperscript{135}. Among the cytolytic receptors (CD2, NKR-P1, CD28), CD16 is the best-studied receptor. After binding to CD16, downstream signalling through phosphorylation and activation of different kinases and specific motifs, leads to cytokine secretion, antibody-dependent cellular cytotoxicity and apoptosis\textsuperscript{135}. NK-cells express cytokines, defensins and nitric oxide and are involved in the recognition and elimination of infected cells. Rager-Zisman and colleagues showed the necessity of NK cells in HSV-1-
infected and NK cell-deficient mice and following transfer of NK cells (NK1.1+; asialo GM1+). Another experiment shows that the absence of NK cells and the presence of T-cells induce a significant mortality rate136,137. Another study reveals further pieces of evidence supporting NK and NKT cells as an essential part since their absence in IL15−/− deficient mice results in higher susceptibility compared to T- and B-cell absence during HSV-2 infection138. Here, it is necessary to mention that NK-cells do not always have a protective role during infection. Researches point out that virulent HSV strains and an over activation of NK cells lead to severe pathology and high mortality rate in mice139. The differences in these experiments may be due to the different set up of experiments and model mouse organism. However, clinical cases of patients confirm the need for NK cells again. For instance, a patient lacking NK cell activity suffers severe HSV outcomes. Lack of plasmacytoid dendritic cells (pDCs) and NK cells in blood results in the severe outcome of HSV infection in patients140,141.
In the context of HSV, infection induces in NK-cells a strong expression of IL-12 and type I responses\textsuperscript{142,143}. Furthermore, during acute HSV-2 infection in the central nervous system, NK-cells recruitment occurs through CCR5\textsuperscript{144}. Moreover, experiments with NK-cells-IFNγ\textsuperscript{−/−} in mice, reveal the importance of NK-cells and their IFNγ expression, since their absence results in severe HSV infection\textsuperscript{145}. This dependency is also essential in terms of IL12/IL23-IFNγ axis deficiency in mice, and humans since any lack or mutation of one element lead to a high vulnerability during HSV infection\textsuperscript{145}. The importance of IFNγ in protection during HSV-2 infection is further affirmed through Harandi et al. because HSV-2 re-infection is blocked by IFNγ-secreting CD4\textsuperscript{+} T-cells, while B-cells show in these experiments anti-viral as well as anti-inflammatory effects\textsuperscript{146}. Further studies of Harandi and colleagues reveal the importance of interleukins, which are involved in the primary response in HSV-2 infections. Lack of IL-12, IL-18 or both leads to higher mortality rates because the IFNγ-mediated immune response is impaired during HSV-2 genital infection\textsuperscript{147}. Another critical interleukin
is IL-15, whose absence in mice leads to a 100-fold more susceptibility genital HSV-2 infection\textsuperscript{148}.

Another set of immune cells are pDCs, active type I IFN producers. They constitutively express IRF-7, which is essential for type I IFN (IFN\textalpha) production during viral infection\textsuperscript{149}. During infection, pDCs sense RNA and DNA viruses through TLR7 and TLR9, inducing IFN-I production by the MyD88-IRF7 signalling pathway. Due to this ability, pDCs can also express proinflammatory cytokines and are essential for antigen presentation and an increase in immune responses in the presence of viruses. These tasks give pDCs a central role within the immune system\textsuperscript{150,151}. Humans with atopic dermatitis and decreased numbers of pDCs are more susceptible to HSV than healthy individuals\textsuperscript{152}. In patients with low-functional pDCs HSV-1 causes acute retinal necrosis\textsuperscript{153}.

pDCs are present in the vaginal mucosa in a stationary manner, where infection of HSV-2 increases their migration to the site of infection and induces type I IFN expression in a TLR9-dependent manner. However, the absence of pDCs does not influence the viral burden or survival after
vaginal HSV-2 infection in these individuals since Th1-mediated immunity regularly develops without these cells, as shown in pDC-depleted transgenic mice (CLEC4C-DTR)\textsuperscript{154}. During HSV-1 and HSV-2 infection, pDCs are essential in early immune response (early IFN-I and proinflammatory cytokine expression; NK cell activation; CD8-T-cell response) during systemic infection rather than in reducing viral load in acute local infections\textsuperscript{155}. Moreover, during reactivation of HSV-2, pDCs tend to co-localize with CD3\textsuperscript{+} lymphocytes and NK-cells (CD69\textsuperscript{+}) and initiate virus-specific proliferation of T-lymphocytes. These results allocate a vital role to pDCs during HSV-2 reoccurrence\textsuperscript{156}. Therefore, pDCs have implications in the innate and adaptive immune system.

1.3.2 Adaptive immune system response to HSV

Antibodies represent the first barrier in adaptive immune response after infection. Studies reveal that intravenous transfer of anti-HSV antibodies induces protection against HSV-2 vaginal transmission in mice. In other studies, the intraperitoneal application of gD-specific antibodies reduces the viral load as well as the disease outcome in mice\textsuperscript{157}. These findings
are affirmed by observation of Jiang et al., where they identify HSV-specific IgGs in human foetal TG\textsuperscript{158}. These results support a passive transfer of maternal immunity to the foetus. Further investigations from the same research group confirm the previous results in mice, showing that maternal immunity protects against neurological disease and death of the offspring\textsuperscript{158}. Milligan and colleagues show long-term HSV-2 specific antibody production in mice and guinea pigs, and speculate that this may be protective against HSV reinfection or even during latency and reactivation\textsuperscript{159}. The importance of long-term immunity and protection after HSV-2 challenge is supported by other studies\textsuperscript{160}. The antibody response is also protective against HSV-1 in mice\textsuperscript{161}.

Adaptive immune cells, mainly CD4\textsuperscript{+}, CD8\textsuperscript{+} T-cells and certain NK-cells control the reoccurrence of HSV-2. In particular, CD8\textsuperscript{+} T-cells react early in infection and have an impact on immune control and cytolysis. Even during HSV-2 lesions, clearance is not always depending on the viral epitope specificity of CD8\textsuperscript{+} T-cells. To a certain degree, unspecific CD8\textsuperscript{+} T-cells show similar activities compared to specific CD8\textsuperscript{+} T-cells during
HSV-1 challenge\textsuperscript{162}. The expression of IFN\(\gamma\) is an integral part of CD8\(^+\) T-cell-mediated antiviral response. The inhibition of IFN\(\gamma\) via antibodies leads to lower HSV-2 clearance than when IFN is present\textsuperscript{163}. IFN\(\gamma\) expression via CD8\(^+\) T-cells and not NK cells is essential for controlling genital HSV-2 infection\textsuperscript{164,165}. Another functional role that can be assigned to CD8\(^+\) T-cells is after the latency establishment. Resident CD8\(^+\) T-cells locate within the junctions of epidermis and dermis, next to the free nerve endings, probable due to previous infection and latency establishment. In biopsies of patients with an unusually high HSV reoccurrence rate, HSV-DNA is present together with HSV-specific CD8\(^+\) T-cells\textsuperscript{166}. Moreover, in a co-culture system, CD8\(^+\) T-cell response prevents HSV reactivation in TG and following spread into the surrounding area containing fibroblasts\textsuperscript{162}. In addition to the CD8\(^+\) T-cells, HSV-specific CD4\(^+\) T-cells can induce HSV-1 clearance in CD8\(^+\) depleted mice. Therefore, CD4\(^+\) T-cells are also probably able to block reactivation through an alternative non-lytic pathway\textsuperscript{167}. These experiments indicate that the production of IFN\(\gamma\) by CD4\(^+\) T-cells controls HSV infection. Furthermore, secretion of IFN\(\gamma\) and other
cytokines by resident CD4+ T-cells promotes the migration of CD8+ T-cells towards the infection site\textsuperscript{168}.

1.3.4 The role of chemokines during HSV-2 infection

Generally, chemokines orchestrate immune cell migration towards infected or damaged tissues. Chemokines influence the leukocytes directly by changing their morphological shape that results in polymerisation and depolymerisation of actin. This cellular behaviour enables leukocytes to form or retract lamellipodia and facilitate migration. This cellular event occurs mainly through heptahelical receptors and their coupled heterotrimeric GTP-binding proteins (G-proteins). Additional cellular changes upregulate integrin expression, to enable the rolling, adhesion onto endothelial cells and finally transmigration to the site of infection or cellular stress (as described in Figure 5)\textsuperscript{169}.
Fig. 4 Schematic representation of all four chemokine subclasses. Chemokines are characterised by the cysteine (C) residues and followed by any residue (X)

The chemokine network is promiscuous with most receptors interacting with more than one chemokine and most chemokines binding to several receptors. There are 20 known receptors that belong to the seven-transmembrane domain G-protein coupled receptors (GPCRs). The binding of chemokines to the extracellular loops stabilises the receptor
and triggers the C-terminal domain, which in turn activates intracellularly signal transduction\textsuperscript{170}.

Chemokines are low molecular weight proteins with essential roles in immune system orchestration. There are 50 known chemokines, and they are divided by their conserved cysteines into four main groups, named as CXC(α), CC(β), C(γ) and CX3C(δ) (Figure 4)\textsuperscript{171}. Besides the structural differences, there are different roles among the chemokines. Several chemokines are expressed by damaged tissues or leukocytes and are critical for inflammation steps, while some of them are constitutively expressed homeostatic chemokines\textsuperscript{170,172,173}. However, some chemokines can have different activities depending on the cellular and tissue environment.
Fig. 5 Schematic representation of leukocyte migration to the site of infection. Chemokines are secreted upon infection. Chemokines bind to glycosaminoglycans (GAGs) on the cell surface, which causes a chemokine gradient near the infection site. Leukocytes recognise the chemokines and start rolling on the endothelial surface, followed by arrest, crawling and transmigration (paracellular or transcellular) to the site of infection. Viral chemokine binding proteins (vCKBP) interfere with the chemokine presentation via the GAG binding site or through the GPCR binding site (see bottom middlebox). Image adapted from Gonzalez Motos et al. 174.

Maintenance of healthy tissues requires homeostatic chemokines. Practical examples are B- and T-cells since they differentiate through different compartments. Partially, few constitutively expressed CC(β) chemokines (CCL17; CCL18; CCL19; CCL20; CCL21) are identified and show roles in T- and B-cell attraction through their specific receptors (CCR4; CCR6; CCR7)175. More precisely, the function of CCL20 is
narrowed down to the attraction of naive T-cells into the germinal centres via dendritic cells. Furthermore, thymic dendritic cells can secrete CCL25, which in turn can direct macrophages, dendritic cells and thymocytes in mice.

In contrast, inflammatory chemokines navigate effector leukocytes to the site of infection and induce inflammation near tissue damage or cancer cells. Leukocytes undergo adhesion on the luminal side of the blood or lymphatic vessels, which enables the transendothelial migration towards the site of infection. During this process, the chemokine receptor activation is essential for increasing avidity and temporary attachment. Once leukocytes migrate through the endothelial cells, they can navigate towards the chemokine gradient and identify the site of infection or tissue damage (Figure 5).173,176

The proper functions of chemokines depend on cell surface located sulphated glycans, called glycosaminoglycans (GAG). Functionally, GAGs have many essential roles in cell growth, differentiation, morphogenesis, cell migration, bacterial and viral infections177.
Structurally, they are composed of repetitive disaccharide backbone with either glucose (GlcNAc) or galactose (GalNAc) and uronic acid (glucuronic or iduronic acid). Based on the different structural compositions, GAGs are classified as heparin/heparan sulphate, chondroitin sulphate/dermatan sulphate, keratan sulphate and hyaluronic acid. Early studies of Rot show that neutrophil migration is induced through NAP-I/IL-8 bound to polycarbonate filter, which in turn suggests that chemokines bound to endothelial cell surfaces act in the same manner as in vitro. Chemokine and GAG interaction and their resulting immobilisation after secretion are functionally relevant in vivo. In this way, chemokines can form a gradient and counteract the bloodstream to let attach immune cells near the site of infection.

During HSV-2 infection, several chemokines and cytokines are involved in pathogenesis. These initial steps are accompanied by infiltration of immune cells to the site of infection. The expression of chemokines in the vaginal mucosa upon HSV-2 infection leads to solid mobilisation of polymorphonuclear cells, macrophages and NK-cells. One of the
chemokines, which is involved in initial immune response towards HSV-2 infection, is CXCL1. Studies show that vagina epithelial cells express CXCL1 upon HSV-2 challenge. Chemokines CXCL9 and CXCL10 recruit T-lymphocytes and NK cells, thus controlling infection and reoccurrences. The response towards HSV-2 genital infection requires CXCL9 and CXCL10 expression in mice. The absence of these chemokines results in significantly higher viral titres inside the central nervous system. Even the recruitment of immune cells within the spinal cord is reduced compared to the wild type mice. Therefore, both chemokines play a crucial role in surveillance as well as infection control. The single observation of CXCL9 during viral infection and especially during HSV-2 infection, also indicate a role during CNS and viral challenge. However, the absence of CXCL9 is replaced to a certain degree by CXCL10 and CXCL11, which are also able to recruit NK cells. Additionally, CXCL10 is involved in the control of other infections such as murine hepatitis virus, MCMV, dengue virus, HIV and Toxoplasma gondii. CCL2 and CCL3 are also involved in anti-viral responses and act in two different ways upon infection. CCL2 can attract
CCR2 positive cells, like macrophages, monocytes and T-cells to the site of infection\textsuperscript{190}. CCL5 shows the same protective ability as described for CCL2. Effective CCL5-mediated protection against HSV-2 is higher than those of CCL2 or CCL3\textsuperscript{191}. Actual protection against vaginal HSV-2 infection is established through CCR5 and their corresponding ligands CCL3 and CCL5\textsuperscript{144}. Chemokines and their corresponding receptors can also induce a disproportional inflammatory response. CCR2 and CCL2 are accountable for experimental autoimmune encephalomyelitis in mice. In line with CCL2, CCL3 increases the acute inflammatory state and is responsible for recruitment and activation of polymorphonuclear leukocytes\textsuperscript{192,193}. Furthermore, the deficiency in CCR5 and consequently lack of chemokine binding leads to reduced control genital HSV-2 infection, inflammation and increase in viral shedding. The CCR5 deficiency goes along with less NK cell expansion, recruitment and activity, especially within the central nervous system\textsuperscript{144}.
1.4 Immune evasion of HSV

1.4.1 Immune evasion on the intracellular and cellular level

On one side, the host's immune system is preventing viral expansion inside the host. On the other side, viruses can counteract the immune response, here, especially the members of the *Herpesviridae*. As previously mentioned, less well-adapted viruses induce an exacerbated immune response that may be accompanied by severe and lethal outcomes. The control of the immune system by the virus is a necessary step to invade the host and bypass the immune response. During the initial infection of the mucosa or epithelial cells, one of the evading mechanisms of HSV is the inhibition of transporter associated with antigen presentation 1/2 by ICP47\(^{194}\). Another HSV protein called viral host shutoff protein (vhs) controls the antigen presentation through its RNase activity and degrades host mRNA shifting the transcription towards the viral mRNA synthesis\(^{195}\).

Several other identified proteins interact and disturb immune system-related pathways. The work of Johnsen and colleagues show that HSV-1
induces an immune response by gamma-interferon-inducible protein 16 (IFI16), NLRP3 inflammasome induction and IL-1β maturation. HSV-1 interrupts this mode of action by proteasomal degradation of IFI16 via ICP0 at a later time point of infection. During this infection process, caspase-I is blocked, which usually cleaves different precursors involved in immune response and following lytic cell death\textsuperscript{196}. Another key player in the immune response is NFκB, and here HSV US3 encodes a kinase that hyperphosphorylates p65 and results in nuclear translocation impairment and a reduction of the level of IL-8\textsuperscript{197}. At the same level, the protein encoded by UL24 retains p65 and p50 inside the cytoplasm\textsuperscript{198}. However, previous studies show contradictory results regarding NFκB localisation. Patel \textit{et al.} indicate that HSV-1 increases the virus replication through continuous nuclear translocation of NFκB\textsuperscript{199}. These contradictory results are probably due to the interplay of different determinants and different cell types.

HSV-2 is known to directly encounter immune cellular defensins, nitric oxide and cytokines such as IFN\textsuperscript{182}. Generally, HSV influences the
activity of NK cells upon infection. The gD protein mediates degradation of CD112, a ligand for NK receptor DNAX accessory molecule 1, resulting in less binding onto virus-infected and gD-transfected cells\textsuperscript{200}. Moreover, the NK-activating ligand MHC class I polypeptide-related sequence A is suggested to play an essential role in the anti-viral response, and upon HSV infection, this element is downregulated\textsuperscript{201}. Further downregulations of NKG2D ligands in infected cells seem to be implicated in a favourable viral replication environment for VZV (ULBP2; ULBP3) and HSV-1 (MICA; ULBP2; ULBP3)\textsuperscript{202}. Beside NK cell evasion, HSV-1 triggers directly the expression of FasL, which sequentially induces apoptosis in interacting CD4\textsuperscript{+}, CD8\textsuperscript{+} and NK cells\textsuperscript{203}. Along with this study, NK cells have contradictory roles during infection with HSV. NKT cells are responsible for glycolipid recognition of CD1d molecules in infected cells. During this initial anti-viral response, HSV can suppress the CD1d expression through phosphorylation of kinesin family member 3A (KIF3A) by US3 encoded kinase. By this, HSV circumvents the glycolipid presentation towards
NKT cells, facilitating HSV spread, since NKT cells seem to be important in the production of HSV-specific IgG antibodies\(^\text{204–206}\).

During viral infection, dendritic cells represent the transition between innate and adaptive immunity. Consequently, HSV induces cell death after successful infection of these cells\(^\text{207}\). An additional counter function is the antigen processing, where \(\gamma 34.5\) impairs the autophagosome maturation\(^\text{208}\). Remarkably, HSV blocks the expression of MHC-I (HLA-C) that causes the absence of MHC-I on the surface of dendritic cells, consecutively NK cells recognise the absence and induce cell death reducing antigen presentation\(^\text{209}\). Overall, dendritic cells have a central role, and HSV has developed different counter mechanisms to overcome it and delay the pathogen recognition and facilitate effective viral spread.

During adaptive immunity, there is an expression of HSV-specific antibodies, mainly targeting envelope proteins of HSV\(^\text{210}\). To counteract antibody response gE functions as Fc\(\gamma\) receptor (Fc\(\gamma\)R) and inhibits IgG Fc-mediated complement activation and antibody-mediated cellular cytotoxicity\(^\text{211}\). The T-cell receptor (TCR) signal pathway is inhibited
partially in HSV infected fibroblasts, more precisely linker for activation of T-cells (LAT) is less phosphorylated and following steps like calcium influx and activation of several mitogen-activated protein kinase are inhibited\textsuperscript{212}. Another study shows changes in LAT1 functions, which results as expected in restricted TCR signalling and IL-2 expression\textsuperscript{213}. As previously mentioned, the induced cell death takes place in T-cells as well. The study of Vanden Oever and Han shows that HSV-2 influences the intrinsic cell death pathway, which is defined by caspase 9\textsuperscript{214}. Besides the above stated immune cells, regulatory T-cells (T\textsubscript{reg}) control the response towards infection, but in some cases, T\textsubscript{reg}’s have a more suppressing ability than favoured from the immune system. T\textsubscript{reg}’s in neonatal mice contribute to the inflammatory immune reduction and favourable viral conditions. In contrast, HSV infection in T\textsubscript{reg}-depleted neonatal mice results in a higher number of HSV-specific CD8\textsuperscript{+} T-cells and lower viral titre, suggesting that T\textsubscript{reg}’s suppress the effective anti-viral response against HSV\textsuperscript{215,216}. Similar to HSV clearance, T\textsubscript{reg}’s suppress IFN\textgreek{y} expression by CD8\textsuperscript{+} T-cells and increase retroviral persistence\textsuperscript{217}. On the other hand, studies allocate still a significant role
in the immune response to $T_{\text{reg}}$, since the migration of effector immune cells towards the site of infection is still necessary for an effective antiviral response$^{218}$.

1.4.2 **Immune evasion on the cytokine level**

The coevolution between human and herpesviruses forced both sides to develop strategies to overcome the immune mechanisms and viral modulation, respectively. The interaction between host and virus drives the viruses to develop additional modulation features against the host to evade the immune system and establish latency. Cytokines are regularly expressed during homeostasis and infection. The stimulation of cytokine expression upon infection plays a central role in immune response and coordination of cells. Upon infection, different cytokines (tumour necrosis factor; IFN) activate intracellular pathways or initiate cellular processes like apoptosis. Additionally, cytokines drive immune responses and recognition, which in turn, recruit immune cells to control the viral spread through the initiation of cell death$^{219}$. This central role of cytokines makes these modulatory proteins a perfect target for viral
adaptation. Among the repertoire of immune modulation, herpesviruses are also able to express viral chemokine homologues. The development of viral chemokine homologues evidences the importance of broad immune system modulation. To modulate the immune system, viruses express soluble cytokines, soluble cytokine receptors and transmembrane cytokine receptors\textsuperscript{219}. An example of the soluble cytokine receptor is the expression of vIFN-\(\gamma\)R by myxoma virus, which inhibits the extracellular activity of host IFN-\(\gamma\)\textsuperscript{221}. So far, no soluble cytokine receptors have been discovered in herpesviruses. There are only membrane-bound transmembrane cytokine receptors, which is vTNFR expressed by HCMV. This type of a receptor homologue instead activates the NF\(\kappa\)B pathway, which in turn upregulates CCL22 and results in leukocyte migration. The advantage of HCMV-vTNFR mediated recruitment is the evasion of immune surveillance\textsuperscript{222}.

An example of soluble cytokine is the vIL10 expressed by EBV. Usually, host IL10 is known to reduce the expression of pro-inflammatory cytokines and activate immunostimulatory pathways. The vIL10 from
EBV does not cover the latter effect\textsuperscript{223}. In the same category, the U83 protein from human herpesvirus 6 enhances the migration of monocytes; thus, it might facilitate viral spread during infection\textsuperscript{224}.

In the case of soluble chemokines, HCMV is known to express vCXC1, which is a CXCR2 agonist and a neutrophil chemoattractant, which facilitates the spread of the virus. KSHV also expresses viral chemokines, such as macrophage inflammatory protein I and II (vMIP-I and II, respectively), which inhibit CD4\textsuperscript{+} T-cells activity and induce angiogenesis as an additional feature\textsuperscript{225}. In addition to the soluble chemokine receptors, viral chemokine receptors (vCKR) in herpesviruses have an essential part in immune modulation as well. KSHV and HCMV encode viral G-protein coupled receptors (vGPCR), involved in cell-to-cell spread, proliferation and apoptosis. More detailed, US28 from HCMV contributes to the cell-to-cell spread at later stages of the viral cell cycle, whereas the ORF74 from KSHV activates proliferation and anti-apoptosis related signals through a broad spectrum of G-proteins\textsuperscript{226,227}. Besides, vGPCR of the KSHV strain mECK36 leads to
initiation of angiogenesis as well as self-driven proliferation, which probably facilitates effective viral spread and pathogenesis\textsuperscript{228,229}. KSHV’s K7 protein is also able to interfere with vGPCR and retain the receptor within the endoplasmic reticulum (ER)\textsuperscript{230}.

The previously mentioned viral chemokines and vGPCRs have a high degree of sequence identity with host proteins. However, viral chemokine binding proteins (vCKBP), have low or no similarity to any host proteins or between themselves\textsuperscript{231}. Nevertheless, vCKBPs share common structural features. The structural studies from Lubman and Fremont show R17 protein of rodent herpesvirus Peru (RHVP) that binds CCL3 and has the same core folding as the chemokine decoy receptor M3 encoded by MHV-68\textsuperscript{232}. Further, studies in poxviruses reveal similarities among the vCKBP, more precisely the common conserved beta-sandwich scaffold of the poxvirus immune evasion domain (PIE) superfamily\textsuperscript{233}. This similarity in structure is believed to have occurred through parallel evolution\textsuperscript{232}.
In general, vCKBPs are divided into different subclasses that are secreted, found on the viral envelope or infected cells. The gammaherpesvirus murine herpesvirus 68 (MHV-68) encodes the first identified vCKBP expressed by a herpesvirus, called M3. Studies from Parry et al. show first insights regarding the vCKBP M3, which inhibits a broad spectrum of chemokines from the four subfamilies CC, CXC, C and CX₃C. The mechanism is basically through prevention of chemokine binding to its receptor and blockage of signal transduction and calcium mobilisation, and chemotaxis (see figure 5)²³⁴–²³⁶.

RHVP R17 interferes with CC and C subclass chemokines and inhibits chemokine-mediated chemotaxis and changes in calcium level in vitro due to the lack of chemokine and receptor interaction. Also, R17 can bind chemokines on the cell surface and modulate probably the chemokine gradient. However, this protein is also retaining chemokines on the cell surface and prevent secretion. This modulation affects the chemokine gradient and the leukocyte migration towards the infection site²³⁷. Further, HCMV is also known to encode pUL21.5 that has a high affinity
towards CCL5. Through this interaction, CCL5 is no longer able to induce cyclin D1 and MMP-9, which is known to mediate chemotaxis of leukocytes\textsuperscript{238,239}.

Furthermore, some vCKBPs interact with GAGs (R17 (RHVP), E163 (ECTV) and M-T1 (MYXV)\textsuperscript{237,240,241}), allowing binding of vCKBP to cells and probably inhibiting the formation of the chemotactic gradient in vivo. Further, R17 and M-T1 are multi-binding vCKBPs, because studies from Seet et al. and Lubman et al. show binding of chemokines and GAGs at once\textsuperscript{237,241}. Moreover, M3 is known to compete with chemokines on the level of GAG binding, which indicates another evasive mechanism to control the gradient near the site of infection\textsuperscript{242}. All in all, these viruses have developed several evasive mechanisms to replicate undercover inside the host’s cells.
1.5 Characteristics of glycoprotein G

1.5.1 Functions of glycoprotein G in different Herpesviruses

The glycoprotein G (gG) belongs, like the previously mentioned proteins, to the group of vCKBP and does not show similarities to host proteins or other known vCKBPs. The gG is coded by the US4 gene, present in most α-herpesviruses except VZV and Marek’s disease virus. gG from several animal herpesviruses binds chemokines and inhibits their activity through inhibition of chemokine-receptor interaction. This includes animal pathogens like equine herpesvirus 1 (EHV-1), bovine herpesvirus 1 and 5 (BHV-1 and BHV-5, respectively). Similar to the previously mentioned vCKBP, Bryant et al. also showed the interaction of gG with GAGs, suggesting that it may interfere with chemokine presentation in vivo. Similarly, the gG from Felid herpesvirus 1 (FeHV-1), infectious laryngotracheitis virus (ILTV) and pseudorabies virus (PRV) are vCKBPs that inhibit chemokine activity. gG’s derived from different animal species have different specificities. In the case of EHV-1 and ILTV, gG can inhibit
chemokine function and obstruct the receptor interaction in vitro and in vivo (PRV gG only shown in vitro). On the domain level, gG from EHV-1 contains a hypervariable region that plays a crucial role in chemokine binding beside the necessity of N-terminal glycosylation of gG.

An interesting shift in the paradigm that all vCKBP inhibit chemotaxis came with the discovery that gG expressed by HSV-1 and HSV-2 enhances chemokine function. Purified, recombinant soluble gG1 and gG2 (SgG1 [HSV-1] and SgG2 [HSV-2], respectively) bind chemokines from the CC and CXC families with nanomolar affinity (see Table 1).
Tab. 1 Affinities of soluble gG1 (HSV-1) and gG2 (HSV-2) towards human chemokines, measured by surface plasmon resonance; affinity based on the disassociation constant (KD) measured in molarity (M)\textsuperscript{251}

<table>
<thead>
<tr>
<th>Chemokine</th>
<th>SgG1 KD (M)</th>
<th>SgG2 KD (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>hCCL18</td>
<td>9.02 x 10\textsuperscript{-8}</td>
<td>2.8 x 10\textsuperscript{-8}</td>
</tr>
<tr>
<td>hCCL22</td>
<td>n.b.</td>
<td>5.22 x 10\textsuperscript{-9}</td>
</tr>
<tr>
<td>hCCL25</td>
<td>4.7 x 10\textsuperscript{-9}</td>
<td>1.6 x 10\textsuperscript{-9}</td>
</tr>
<tr>
<td>hCCL26</td>
<td>5.5 x 10\textsuperscript{-8}</td>
<td>1.72 x 10\textsuperscript{-9}</td>
</tr>
<tr>
<td>hCCL28</td>
<td>6.8 x 10\textsuperscript{-8}</td>
<td>3.2 x 10\textsuperscript{-9}</td>
</tr>
<tr>
<td>hCXCL9</td>
<td>3.8 x 10\textsuperscript{-8}</td>
<td>1.23 x 10\textsuperscript{-8}</td>
</tr>
<tr>
<td>hCXCL10</td>
<td>4.57 x 10\textsuperscript{-7}</td>
<td>5.5 x 10\textsuperscript{-9}</td>
</tr>
<tr>
<td>hCXCL11</td>
<td>1.09 x 10\textsuperscript{-8}</td>
<td>6 x 10\textsuperscript{-9}</td>
</tr>
<tr>
<td>hCXCL12a</td>
<td>3.15 x 10\textsuperscript{-8}</td>
<td>6.5 x 10\textsuperscript{-9}</td>
</tr>
<tr>
<td>hCXCL12b</td>
<td>7.7 x 10\textsuperscript{-9}</td>
<td>2.2 x 10\textsuperscript{-9}</td>
</tr>
<tr>
<td>hCXCL13</td>
<td>1.3 x 10\textsuperscript{-8}</td>
<td>4.3 x 10\textsuperscript{-9}</td>
</tr>
<tr>
<td>hCXCL14</td>
<td>4.2 x 10\textsuperscript{-7}</td>
<td>4.3 x 10\textsuperscript{-9}</td>
</tr>
</tbody>
</table>

Binding resulted in enhancement of chemokine activity \textit{in vitro} and \textit{in vivo}. These results indicate that we do not fully understand the relationship between viruses and the chemokine network. HSV gG does
not block the interaction of the chemokine with its receptor. Instead, it binds chemokines through their GAG-binding domain. It also binds GAGs, facilitating interaction with cellular surfaces, a characteristic that may be relevant for its mechanism of action. To enhance chemokine activity, gG enhances the concentration of the chemokine at the proximity of the receptor, reduces its internalisation, increases its oligomerisation and location in lipid rafts leading to higher signalling and activation of MAPKs\textsuperscript{252}. As mentioned above, VZV does not encode the US4 gene required for gG expression. In this virus, glycoprotein C (gC) binds chemokines with high affinity and enhances their activity. Binding takes place through the C-terminal immunoglobulin-like domain of gC. VZV gC activity was shown using purified protein and viral particles. Since VZV infects and replicates inside leukocytes, being transmitted systemically, gC-mediated enhancement of chemokine function and thereby leukocyte migration may facilitate virus spread within the host\textsuperscript{253}.
At the structural level, gG from HSV-2 is a type I transmembrane protein, where the N-terminal part is secreted into the cellular environment (SgG), where it shows chemokine binding activity. The C-terminal membrane-anchored domain (MgG) remains on the infected cell. However, gG from HSV-1 only encodes an MgG variant. Nevertheless, the MgG proteins of other viruses are able to bind chemokines on the cell surface or at the viral envelope. Bryant and colleagues suggest that the MgG version may play a noteworthy function in chemokine modulation. In this conformation, MgG may act as catching basin, for chemokines that delay the usual immune response mediated through secreted chemokines, which is similar to the previous mentioned HCMV encoded US28 receptor that sequesters chemokines and changes the inflammatory micro-environment to a virus beneficial one. Another suggestion of MgG from HSV is its involvement in entry inducing GPCR signalling and activation of mitogen-activated protein kinases facilitating productive infection. The remaining portion of gG2 (MgG2) is also suggested to be responsible for initial attachment to the cell surface glycosaminoglycans. Until now, the functional relevance of
chemokine enhancement by gG is not identified, although the lack of gG1 leads to virus attenuation\textsuperscript{260}. There is no information on the relevance of gG2 during \textit{in vivo} infection.

### 1.5.2 Glycoprotein G from HSV-1 and HSV-2

gG is the most divergent coding gene between HSV-1 and HSV-2, with HSV-2 \textit{US4} containing 1469 bp more than that of HSV-1\textsuperscript{261}. Altogether, both glycoproteins share 30\% amino acid identity, and the highest homology is found in the membrane-anchored C-terminal region. gG1 contains 238 and gG2 673 amino-acids. As previously mentioned, these glycoproteins appear on viral particles and infected cells. Despite the differences in amino acid sequence, both SgG1 and SgG2 interact with chemokines, as shown by surface plasmon resonance and crosslinking experiments, and enhance their activity. In case of gG2, additional modifications on the N-terminal part occurs, which leads to the secretion of the N-terminal \textasciitilde282 residues to the extracellular medium\textsuperscript{262}. The cleaved portion of gG2 (SgG2) binds chemokines in the supernatant of HSV-2 infected cells. In HSV-1 infected cells, chemokines are bound and
located on the cell surface. Binding to chemokines occurs through their GAG binding motif, as shown with chemokine mutants and GAG competition studies. SgG2 does not reduce the binding of hCXCL12α or hCCL25 to their corresponding receptors compared to the mock control. gG is capable of binding to GAGs despite lacking putative GAG-binding motifs, suggesting that this glycoprotein must have a different way of GAG interaction.

The role of gG2 during infection had not been properly addressed. In unpublished studies from Kropp et al., we generated recombinant reporter viruses expressing or lacking the expression of gG2 and compared them on their ability to induce neurite outgrowth and infect sensory neurons. In these experiments, the use of microfluidic chambers separated the somal and neurite sides. Infected HEK293T cells are seeded on the axonal chamber. Both compartments are separated by microgrooves, where neurites can grow from one compartment to another. It is known that HEK293T cells express repellents that inhibit neurite growth. However, HSV-2 infection overcomes this repulsion,
inducing neurite outgrowth towards the infected cells. This may facilitate infection of neurites from the HEK293T cells. However, the HSV-2ΔgG2, lacking gG2 expression, cannot overcome the repelling effect, resulting in less neurite outgrowth towards the infected cells. These results suggest that, as shown with purified gG2, this protein enhanced neurite outgrowth during infection\textsuperscript{47}. The mechanism is unknown at present.

Other studies show that HSV-2 infection of keratinocytes in the human genital mucosa following reactivation leads to the expression of interleukin 17c (IL-17c)\textsuperscript{48,264}, promoting the growth of peripheral nerve endings during the HSV-2 reactivation. We do not know at present whether the results observed with HSV-2 infection of HEK293T are due to a reduction in the expression of repellents, an increase in the expression of growth factors such as IL-17c or a direct effect of gG2 on NGF.

Therefore, HSV-1 and HSV-2 gG modulate the immune system through chemokine enhancement. However, only gG2 enhances NGF-mediated
neurite outgrowth and manipulates the retraction of neurotropic receptors to induce neurite outgrowth. This mode of viral action of HSV-2 may facilitate the viral spread and enable FNE infection and establishment of latency inside specific subtypes of neurons in the ganglion.

1.6 Secretory pathway and its role in gG2 processing

Viral replication and protein expression depend on the host machinery and cellular compartments like endoplasmic reticulum (ER) and the Golgi apparatus (GA/Golgi). Signal sequences within the proteins direct them towards the regular secretory pathway, following translocation of translation products into the rough ER. Depending on the signal sequence within the protein, translation occurs either on the free or ER-attached ribosomes. In the case of the latter type of ribosomal translation, the protein crosses the membrane co-translationally and enters the ER lumen. From this point, proteins are sorted throughout to other organelles. Structurally, the rough ER reminds of flattened sacs interconnected with each other. Within these sacs, proteins are sorted and transported to their
destination inside the cells. This process goes along for transmembrane proteins, too\textsuperscript{265}.

The transport inside the cells occurs in transport vesicles that either fuse with the cis-Golgi compartment or develop through vesicle fusion to another stack of cis-Golgi reticulum. Once the vesicles fuse with the cis-Golgi, the proteins are either redirected to the ER (ER-localized proteins) or undergo cisternal progression inside the Golgi apparatus. Luminal proteins move from the cis-compartment to the medial- and finally trans-compartment\textsuperscript{265}. Proteins predetermined to be secreted move from the trans-Golgi reticulum towards the plasma membrane. Some proteins are constitutively secreted, whereas some secretory vesicles remain inside the cell, waiting for a stimulus\textsuperscript{265}.

In this context, glycoproteins follow the same process of maturation as described in the natural secretory pathways. Glycoproteins are by definition proteins with covalently attached carbohydrates. Glycosylation is the most used conjugation of post-translational modifications in eukaryotes and prokaryotes. These modifications can be
different forms of carbohydrates: monosaccharide, oligo- and polysaccharide, sulfo or phosphoro substitute, aminosugars or acidic sugars. Glycosylation provides different properties for the protein, for example, the presence of OH groups that enables hydrophilic conditions and the proper folding of the protein. The primary site for glycosylation modification is the ER through the glycosyltransferase. Glycosylation is defined as O-linked glycosylation or N-linked glycosylation depending on whether the glycan is incorporated into OH groups of serine and threonine, or into asparagine, respectively. As already mentioned, the hydrophilicity plays a role in functional relevance in the folding process of the protein itself. Many misfolded proteins show clinical relevance, for example, in neurodegenerative diseases like Alzheimer or Parkinson. Further, modifications in glycosylation occur as well in tumour development, being involved in severe invasive or metastasis stages. More involvements are found in rheumatoid arthritis and immunodeficiency diseases. On the diagnostic level, these changes in glycosylation patterns in diseases are the basis for different markers.
As mentioned in the beginning, during viral infection, assembly of viral parts takes place mainly in the ER and Golgi. Therefore, many HSV proteins rely on the conserved secretory pathway. The initial translation of the N-terminal signal peptide leads to the recruitment of signal recognition particle (SRP), which in turn immediately stops further translation and directs ribosome and template to the rough ER (SRP receptor; GTP hydrolysis). The following tight interaction with the translocon components and SRP release leads to a conformational change of the translocon itself that widens and allow translation to continue. The chaperone (Grp78/BiP) is responsible for the proper folding of the protein, while signal peptidase cleaves the signal peptide. Secretory proteins are continuously translated into the lumen, whereas the translation of integral transmembrane proteins stop at the hydrophobic anchor domain within the translocon and undergo a lateral movement into the lipid bilayer. From the ER lipid bilayer the gG’s from HSV-1 and HSV-2 start their journey through the secretory pathway\textsuperscript{268}. During the folding process, the quality control of the ER lumen retains unfolded proteins.
Regarding herpesviruses, where the envelope contains receptors and fusion proteins, the oligomerisation of these proteins is characteristic for ER export. Beside the oligomerisation, glycosylation is the essential posttranslational modification for the proper functioning of translated proteins. As previously mentioned, N-linked modifications occur inside the ER lumen defined by mannose-rich oligosaccharides bound to NXS/T motifs through oligosaccharyltransferase delivered by dolichol. Further trimming of terminal glucose and mannose happens still in the ER, which is accompanied by protein quality control. Following ER modifications, Golgi modifies the protein by further trimming, the addition of sugars (N-acetylglucosamine; galactose; fucose; sialic acid) and O-linked glycosylation.\[269\]

In the case of gG2, during infection of BHK-21 cells, two major bands (108 kDa and 120 kDa) are detected. Radiolabeled sugars (glucosamine; mannose) show positive signalling for both sizes in immunoprecipitations. gG2 undergoes two main steps of modifications characterised by N-linked mannose glycosylation that is sensitive to
endoglycosidase H (EndoH), which cleaves at the chitobiose core of the high mannose structure (120 kDa gG2 sensitive to EndoH). This is followed by further attachment of other modifications, like fucose, galactose, sialic acid and other posttranslational additions (producing 108 kDa), which are resistant to EndoH. The precursor 120 kDa shows in chase label experiments the transformation into the mature 108 kDa polypeptide. The mature 108 kDa protein is resistant to EndoH treatment that affirms the role of the 120 kDa product as the precursor. In the same study, they identified that the mature protein undergoes further changes, which results in a 74 kDa intermediate from the 120 kDa precursor. This product appears only briefly during infection and especially under usage of monensin, which accumulates the intermediate within the cells. However, it does not always depend on monensin treatment, because in untreated BHK-21 cells, the 74 kDa product is still present as well.

The following work from Su et al. reveals a more in-depth look into the gG2 processing, including the involvement of the Golgi during gG2 synthesis. Here they identified the precursor of gG2, which is co-
translationally modified and results in a 120 kDa product. This product is then followed by the mature 104 kDa gG2 product. The following high-mannose product is then further modified by cleavage into a 74 kDa and 31 kDa product, whereas the larger product is glycosylated into the mature membrane-bound 108 kDa product (MgG2). The report from Su et al. determines the presence of the smaller 31 kDa product of gG2 with and without monensin, which is a secretion blocker (Golgi). The cleaved 34 kDa product appears in the supernatant of HSV-2 infected, non-monensin treated HEp-2 cells, which means SgG2 undergoes further modifications within the Golgi apparatus. Findings in the same study show that the presence of monensin disrupts the Golgi functions that leads to a less glycosylated 31 kDa product inside the cell lysate.

Further studies aimed at the in vitro translation of gG2, using the reticulocyte lysate system. The work from Weldon et al. indicates the actual size of gG2 is 100 kDa, which correlates with the 104 kDa in lysates of infected cells. Further, the absence of the cleaved products
(72 kDa and 31 kDa) leads to the suggestion that cleavage occurs in later stages of the secretory pathway and N-linked glycosylation occurs co-translationally inside the ER\textsuperscript{271}. Apart from the localisation of the cleavage event, experiments from Weldon \textit{et al.} approach the cleavage site by using different antibodies, aiming for the MgG2, gG2 precursor and secreted gG2 portion. During the studies, Su \textit{et al.} clarify the origin of the secreted portion (31 kDa) towards the N-terminal site and ~72 kDa portion correlates with the C-terminal portion of the 104 kDa intermediate. The same studies narrow down the cleavage site within the amino acid 260 and 437\textsuperscript{272}. The latest study from Liljeqvist and colleagues indicates that the cleavage site is located between amino acids 320 to 323, the most C-terminal residues that are recognised by a monoclonal antibody. Additionally, the same study mentions that a personal communication from Richard J. Courtney indicates that cleavage occurs between Arg321 and Ala322 along with Arg342 and Leu343\textsuperscript{273}. 

77
Fig. 6 Expressional pattern of gG2. The figure shows selected expressional pattern studies of gG2. gG2 is detected in the endoplasmic reticulum as a pre-cursor (120 kDa). Cleaved intermediate can be detected in the presence of Golgi apparatus inhibitor Monensin (*) [Balachandran et al., 1985]. gG2 is suggested to be cleaved co-translationally inside the ER and before the Golgi apparatus (GA) [Su et al., 1987]. However, in vitro translation does not confirm co-translational cleavage in the ER [Weldon et al., 1990]. Location of each detected gG2 variant is based on the Endoglycosidase H resistance (after GA) or sensitivity (before GA).
1.7 Objectives

In this project, we want to investigate the functionality and importance of cleavage and secretion of HSV-2 gG encoded by the *US4* gene. The aim is based on the fact that gG2 (and gG1) has the unique feature of enhancing chemokine function in contrast to other inhibitory vCKBP$^{16}$. gG2 but not gG1, also modifies NGF activity, increasing neurite outgrowth. The mechanism of action involves modulation of TrkA signalling, inhibition of TrkA internalisation and retrograde transport$^{47}$. Whether cleavage and secretion of the N-terminal domain of gG2, termed SgG2, is required for these activities is not known.

The main objectives of this thesis are:

1) To identify the cleavage site of HSV-2 gG

2) To determine the functional role of HSV-2 cleavage and secretion

3) To identify the gG2 domains involved in the interaction with GAGs, chemokines and NGF
# 2. Material and Methods

## 2.1 Materials

<table>
<thead>
<tr>
<th>Animal</th>
<th>Cell type</th>
<th>Genotype</th>
<th>Source/Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mice</td>
<td>BALB6c</td>
<td></td>
<td>Animal facility, MHH</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Cell type</th>
<th>Genotype</th>
<th>Source/Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEK293T</td>
<td>Human embryonic kidney 293 cells</td>
<td>Abel Viejo Borbolla</td>
<td></td>
</tr>
<tr>
<td>HeLa</td>
<td>Cervical cancer cells</td>
<td>Abel Viejo Borbolla</td>
<td></td>
</tr>
<tr>
<td>JURKAT T-cell E6.1</td>
<td>Human leukaemia T-lymphocyte cells</td>
<td>Martin Messerle</td>
<td></td>
</tr>
<tr>
<td>LoVo</td>
<td>Colon adenocarcinoma derived epithelial cells</td>
<td>Christine Goffinet</td>
<td></td>
</tr>
<tr>
<td>Adherent Hi5</td>
<td>Ovarian cells from Trichoplusia ni</td>
<td>Antonio Alcamí</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Bacterial strains</th>
<th>Cell type</th>
<th>Genotype</th>
<th>Source/Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Virus</td>
<td>Cell type</td>
<td>Genotype</td>
<td>Source/Reference</td>
</tr>
<tr>
<td>---------------</td>
<td>-----------</td>
<td>-----------------------------------------------</td>
<td>------------------</td>
</tr>
<tr>
<td>Baculovirus</td>
<td>Insect cell Bacmid-derived recombinant virus</td>
<td>Antonio Alcamí</td>
<td></td>
</tr>
<tr>
<td>Antibiotic</td>
<td>Concentration used</td>
<td>Source</td>
<td></td>
</tr>
<tr>
<td>------------------------------------------------</td>
<td>--------------------</td>
<td>------------------</td>
<td></td>
</tr>
<tr>
<td>Ampicillin</td>
<td>100 μg/ml</td>
<td>Sigma-Aldrich</td>
<td></td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>17 μg/ml</td>
<td>Carl Roth</td>
<td></td>
</tr>
<tr>
<td>Gentamicin</td>
<td>7 μg/mL</td>
<td>Panreac Applichem</td>
<td></td>
</tr>
<tr>
<td>Kanamycin</td>
<td>50 μg/mL</td>
<td>Serva</td>
<td></td>
</tr>
<tr>
<td>Tetracyclin</td>
<td>10 μg/mL</td>
<td>Sigma-Aldrich</td>
<td></td>
</tr>
<tr>
<td>5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside</td>
<td>50 mg/ml</td>
<td>Calbiochem</td>
<td></td>
</tr>
<tr>
<td>(X-Gal)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isopropyl β-D-1-thiogalactopyranoside</td>
<td>0.5 mM</td>
<td>Panreac Applichem</td>
<td></td>
</tr>
<tr>
<td>Vector</td>
<td>Selective antibiotic</td>
<td>Source</td>
<td></td>
</tr>
<tr>
<td>------------</td>
<td>--------------------------------------</td>
<td>-----------------</td>
<td></td>
</tr>
<tr>
<td>DH10Bac</td>
<td>Tetracyclin, Gentamycin, Kanamycin</td>
<td>Antonio Alcamí</td>
<td></td>
</tr>
<tr>
<td>pFastBacMel</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pcDNA3.1Zeo</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pM157</td>
<td>Ampicillin</td>
<td>Abel Viejo Borbolla</td>
<td></td>
</tr>
<tr>
<td>pGL3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SPV5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pGEM-T</td>
<td></td>
<td>Promega</td>
<td></td>
</tr>
<tr>
<td>Enzymes</td>
<td>Company</td>
<td></td>
<td></td>
</tr>
<tr>
<td>----------------------</td>
<td>------------------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EcoRI</td>
<td>New England Biolabs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HindIII</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NcoI</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NheI</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NsiI</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PpuMI</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SfiI</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SphI</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>XbaI</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Taq DNA Polymerase</td>
<td>Thermo Fisher Scientific</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ligase T4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phusion High-Fidelity DNA Polymerase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kits</td>
<td>Company</td>
<td></td>
<td></td>
</tr>
<tr>
<td>---------------------------------</td>
<td>----------------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lipofectamine 2000</td>
<td>Thermo Fisher Scientific</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lipofectamine 3000</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LTX</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nucleospin Plasmid</td>
<td>Machery &amp; Nagel</td>
<td></td>
<td></td>
</tr>
<tr>
<td>QIAquick Gel Extraction Kit</td>
<td>QIAGEN</td>
<td></td>
<td></td>
</tr>
<tr>
<td>QIAquick PCR Purification Kit</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>QuickChange II Site-directed</td>
<td>Agilent</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mutagenesis Kit</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TransIT-X</td>
<td>Mirus Bio</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reagents</td>
<td>Media</td>
<td>Company</td>
<td></td>
</tr>
<tr>
<td>--------------------------</td>
<td>-------------------------------</td>
<td>--------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>DMEM</td>
<td></td>
<td>Gibco (Thermo Fisher Scientific)</td>
<td></td>
</tr>
<tr>
<td>DMEM-F12</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fetal bovine serum (FBS)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Insect Xpress</td>
<td></td>
<td>Lonza</td>
<td></td>
</tr>
<tr>
<td>L-Glutamine</td>
<td></td>
<td>Cytogen</td>
<td></td>
</tr>
<tr>
<td>Opti-MEM serum-free medium</td>
<td></td>
<td>Gibco (Thermo Fisher Scientific)</td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td></td>
<td>Cytogen</td>
<td></td>
</tr>
<tr>
<td>Penicillin+Streptomycin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RPMI 1640</td>
<td></td>
<td>Gibco (Thermo Fisher Scientific)</td>
<td></td>
</tr>
<tr>
<td>Trypan blue 0,4%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trypsin-EDTA Solution</td>
<td></td>
<td>Merckmillipore</td>
<td></td>
</tr>
<tr>
<td>Cell line</td>
<td>Medium</td>
<td>Additional ingredients</td>
<td>Incubation</td>
</tr>
<tr>
<td>-------------------</td>
<td>--------------</td>
<td>----------------------------------------</td>
<td>------------</td>
</tr>
<tr>
<td>HEK293T and HeLa</td>
<td>DMEM</td>
<td>8% FBS</td>
<td>37°C</td>
</tr>
<tr>
<td>LoVo</td>
<td>DMEM-F12</td>
<td>1% L-Glutamine</td>
<td>5% CO₂</td>
</tr>
<tr>
<td>JURKAT T-cell E6.1</td>
<td>RPMI 1640</td>
<td>Penicillin/Streptomycin</td>
<td></td>
</tr>
<tr>
<td>Adherent Hi5</td>
<td>Insect-XPRESS™</td>
<td>10% FBS (2% for infection)</td>
<td>28°C</td>
</tr>
<tr>
<td>Suspension Hi5</td>
<td>Insect-XPRESS™</td>
<td>1% L-Glutamine</td>
<td>28°C</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1% Penicillin/Streptomycin</td>
<td>120 rpm</td>
</tr>
<tr>
<td>DH5α</td>
<td>LB</td>
<td>Appropriate antibiotics</td>
<td>37°C</td>
</tr>
<tr>
<td>DH10Bac</td>
<td></td>
<td></td>
<td>200 rpm</td>
</tr>
</tbody>
</table>
Tab. 9 Additional reagents

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic acid</td>
<td>J.T. Baker</td>
</tr>
<tr>
<td>Agar</td>
<td>Carl Roth</td>
</tr>
<tr>
<td>Agarose</td>
<td>Bio-Rad</td>
</tr>
<tr>
<td>All blue prestained protein standard</td>
<td></td>
</tr>
<tr>
<td>Ammonium chloride</td>
<td>Carl Roth</td>
</tr>
<tr>
<td>Ammonium persulfate (APS)</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Aqua bidest. bottled</td>
<td>Braun</td>
</tr>
<tr>
<td>Bacto yeast extract</td>
<td>Becton Dickinson</td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>Sigma Aldrich</td>
</tr>
<tr>
<td>Cell titer 96</td>
<td>Promega</td>
</tr>
<tr>
<td>Coomassie Brilliant Blue R-250</td>
<td>Panreac Applichem</td>
</tr>
<tr>
<td>Dimethyl sulfoxide (DMSO)</td>
<td>Carl Roth</td>
</tr>
<tr>
<td>Deoxynucleotides (dNTPs)</td>
<td>Promega</td>
</tr>
<tr>
<td>Ethanol</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Ethidium bromide solution (10mg/ml)</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Glycerol</td>
<td>Carl Roth</td>
</tr>
<tr>
<td>Glycin</td>
<td></td>
</tr>
<tr>
<td>Heparin</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Chemical Name</td>
<td>Supplier</td>
</tr>
<tr>
<td>------------------------------------</td>
<td>---------------------------</td>
</tr>
<tr>
<td>Heparin Agarose</td>
<td>Panreac Applichem</td>
</tr>
<tr>
<td>Imidazole</td>
<td>0.6 M Tris-HCl pH: 6.8, 0.715 M β-mercaptoethanol, 25% glycerol, 2% SDS and 0.1% bromophenol blue</td>
</tr>
<tr>
<td>Laemmli buffer</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Methanol</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Milk powder</td>
<td>Carl Roth</td>
</tr>
<tr>
<td>Ni-NTA Agarose</td>
<td>Qiagen</td>
</tr>
<tr>
<td>Oligonucleotides</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Sepharose CL-4B</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Sodium dodecyl sulfate (SDS)</td>
<td>Carl Roth</td>
</tr>
<tr>
<td>Sodium hydroxide (NaOH)</td>
<td>Carl Roth</td>
</tr>
<tr>
<td>Tetramethylethylenediamine (TEMED)</td>
<td>Panreac Applichem</td>
</tr>
<tr>
<td>Tryptone</td>
<td>Carl Roth</td>
</tr>
<tr>
<td>Tris</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Tween 20</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>Unstained protein standard</td>
<td>Bio-Rad</td>
</tr>
<tr>
<td>2-Mercaptoethanol</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>2-Propanol</td>
<td>Sigma-Aldrich</td>
</tr>
<tr>
<td>30 % Acrylamide/BIS 29:1</td>
<td>Bio-Rad</td>
</tr>
<tr>
<td>Antibody</td>
<td>Dilution</td>
</tr>
<tr>
<td>-------------------</td>
<td>-----------</td>
</tr>
<tr>
<td>αV5</td>
<td>WB 1:1000</td>
</tr>
<tr>
<td></td>
<td>IF 1:500</td>
</tr>
<tr>
<td>αV5</td>
<td>IF 1:500</td>
</tr>
<tr>
<td>αHA</td>
<td>WB 1:2000</td>
</tr>
<tr>
<td></td>
<td>IF 1:1600</td>
</tr>
<tr>
<td>αHis</td>
<td>WB 1:2000</td>
</tr>
<tr>
<td>αmature-MgG2</td>
<td>IF 1:100</td>
</tr>
<tr>
<td>αTUJ1 (Class III tubulin AB)</td>
<td>ICC 1:200</td>
</tr>
<tr>
<td>αMAP2</td>
<td>1:1100</td>
</tr>
<tr>
<td>Antibody</td>
<td>Dilution</td>
</tr>
<tr>
<td>------------------</td>
<td>----------</td>
</tr>
<tr>
<td>Alexa Fluor 488</td>
<td>IF 1:1000</td>
</tr>
<tr>
<td>GFP</td>
<td></td>
</tr>
<tr>
<td>Alexa Fluor 555</td>
<td>IF 1:1000</td>
</tr>
<tr>
<td>dsRed</td>
<td>ICC 1:1000</td>
</tr>
<tr>
<td>Alexa Fluor</td>
<td>IF 1:1000</td>
</tr>
<tr>
<td>Cyan5</td>
<td>ICC: 1:1000</td>
</tr>
<tr>
<td>IRDye 680</td>
<td>WB 1:10000</td>
</tr>
<tr>
<td>IRDye 800</td>
<td>WB 1:5000</td>
</tr>
<tr>
<td>Tab. 12 Laboratory Equipment</td>
<td></td>
</tr>
<tr>
<td>-----------------------------</td>
<td></td>
</tr>
<tr>
<td><strong>Equipment</strong></td>
<td><strong>Purpose</strong></td>
</tr>
<tr>
<td>Accu-Jet Pro</td>
<td>Pipetting &gt; 2 mL</td>
</tr>
<tr>
<td>Avanti J-25</td>
<td>Centrifuge</td>
</tr>
<tr>
<td>Axiovert 40 CFL</td>
<td>Light microscope</td>
</tr>
<tr>
<td>Centramate T-Series Cassette 10 and 30 kDa cut-off</td>
<td>Tangential flow filtration system</td>
</tr>
<tr>
<td>Certomat BS-1</td>
<td>Insect cell incubator</td>
</tr>
<tr>
<td>ChemoTx Disposable Chemotaxis System</td>
<td>Chemotaxis migration plate</td>
</tr>
<tr>
<td>Digital dry block heater</td>
<td>Heat Block</td>
</tr>
<tr>
<td>Erlenmeyer 150 and 500 ml</td>
<td>Cell culture</td>
</tr>
<tr>
<td>Balance</td>
<td>Measure out substances</td>
</tr>
<tr>
<td>Falcon tubes 15 and 50 ml</td>
<td>Regular work</td>
</tr>
<tr>
<td>Gel iX20 Imager</td>
<td>DNA gels Imaging</td>
</tr>
<tr>
<td>Glass plates</td>
<td>Acrylamide gels</td>
</tr>
<tr>
<td>-----------------------</td>
<td>--------------------------</td>
</tr>
<tr>
<td>Hera Cell 240</td>
<td>Mammalian cell incubator</td>
</tr>
<tr>
<td>Hera safe 377 and 423</td>
<td>Cell culture hood</td>
</tr>
<tr>
<td>Heraeus Fresco 17</td>
<td>Table centrifuge</td>
</tr>
<tr>
<td>Heraeus B-5060 EK-CO₂</td>
<td>Insect cell incubator</td>
</tr>
<tr>
<td>Heraeus Multifuge 3SR+</td>
<td>Table centrifuge</td>
</tr>
<tr>
<td>Agarose gel chamber</td>
<td>DNA gel electrophoresis</td>
</tr>
<tr>
<td>Imager LAS3000</td>
<td>Coomassie gels Imaging</td>
</tr>
<tr>
<td>Innova 40</td>
<td>Bacteria incubator</td>
</tr>
<tr>
<td>KMO 2 basic</td>
<td>Magnetic stirrer</td>
</tr>
<tr>
<td>Masterflex peristaltic pump</td>
<td>Tangential flow filtration system</td>
</tr>
<tr>
<td>Micropipette tips</td>
<td>Regular work</td>
</tr>
<tr>
<td>Mini Trans-Blot Cell</td>
<td>Blotting chamber</td>
</tr>
<tr>
<td>Minitron</td>
<td>Bacteria incubator</td>
</tr>
<tr>
<td>Equipment/Method</td>
<td>Application</td>
</tr>
<tr>
<td>------------------------------------------------------</td>
<td>------------------------------------</td>
</tr>
<tr>
<td>Nanodrop 1000</td>
<td>DNA/RNA quantification</td>
</tr>
<tr>
<td>Neubauer counting chamber</td>
<td>Cell counting chamber</td>
</tr>
<tr>
<td>Nitrocellulose blotting membrane</td>
<td>Western blot</td>
</tr>
<tr>
<td>Observer Z1 Axio</td>
<td>Immunofluorescence</td>
</tr>
<tr>
<td>Odyssey</td>
<td>Western blot Imaging</td>
</tr>
<tr>
<td>PCR SingleCap 8er-SoftStrips 0.2 ml, colourless</td>
<td>Cloning</td>
</tr>
<tr>
<td>Petri dish 92x16 mm with cams</td>
<td>Bacteria culture</td>
</tr>
<tr>
<td>Pipet</td>
<td>Micropipette</td>
</tr>
<tr>
<td>PowerPac Basic Power</td>
<td>Supply Electrophoresis</td>
</tr>
<tr>
<td>Safeseal tubes 0.5, 1.5 and 2 ml</td>
<td>Laboratory work</td>
</tr>
<tr>
<td>Serological pipette tip</td>
<td>Standard work</td>
</tr>
<tr>
<td>Short plates</td>
<td>Acrylamide gels</td>
</tr>
<tr>
<td>Synergy 2</td>
<td>Plate reader</td>
</tr>
<tr>
<td>TC Dish 100 and 150, Standard</td>
<td>Cell culture plate</td>
</tr>
<tr>
<td>Equipment</td>
<td>Description</td>
</tr>
<tr>
<td>---------------------------</td>
<td>-----------------------------------------</td>
</tr>
<tr>
<td>TC Flask T25 and T75</td>
<td>Cell culture flask</td>
</tr>
<tr>
<td>Standard, vent. Cap</td>
<td></td>
</tr>
<tr>
<td>TC-plate 6 well Standard</td>
<td>Cell culture plate</td>
</tr>
<tr>
<td>TE412</td>
<td>Balance</td>
</tr>
<tr>
<td>Thermomixer Comfort</td>
<td>Shaking heat block</td>
</tr>
<tr>
<td>Water bath</td>
<td>Sample and media heating</td>
</tr>
<tr>
<td>Software</td>
<td>Company/Source</td>
</tr>
<tr>
<td>--------------------------</td>
<td>-----------------------------------------------------</td>
</tr>
<tr>
<td>AxioVision Rel. 4.8</td>
<td>Zeiss</td>
</tr>
<tr>
<td>BioEdit</td>
<td>Tom Hall</td>
</tr>
<tr>
<td>Clone Manager</td>
<td>Sci-Ed Software</td>
</tr>
<tr>
<td>GraphPad Prism 5</td>
<td>GraphPad Software</td>
</tr>
<tr>
<td>ImageJ</td>
<td>National Institute of Health</td>
</tr>
<tr>
<td>Microsoft Office</td>
<td>Microsoft</td>
</tr>
<tr>
<td>ProP 1.0</td>
<td>Center For Biological Sequence Analysis, Technical University Denmark</td>
</tr>
<tr>
<td>PROSPER</td>
<td>Monash Bioinformatics Platform, School of Biomedical Sciences, Faculty of Medicine, Monash University, Australia</td>
</tr>
<tr>
<td>SecretomeP 2.0a Server</td>
<td>Center For Biological Sequence Analysis, Technical University Denmark</td>
</tr>
<tr>
<td>Serial Cloner</td>
<td>Serial Basics Softwares</td>
</tr>
</tbody>
</table>
2.2 Methods

2.2.1 Generation of cleavage deficient gG2

2.2.1.1 Site-directed mutagenesis (SDM)

The cloning of these constructs was done in the plasmid pAV11 (pFastBac Mel), which encoded for His-tagged rSgG2 from base pair (bp) 4167 to 5402 (total length 1242), lacking the gG2 signal peptide (provided by Viejo Borbolla, see Figure 7). The pAV11 contains the honeybee melittin signal peptide facilitating secretion in insect cells, upstream the His-tag. The primers to introduce the desired residues were created on the Agilent web page (https://www.agilent.com/). Here, the DNA sequence was uploaded in FASTA format, and the target residues were changed to the desired residues. The method was done according to the QuickChange Site-Directed Mutagenesis Kit Protocol (Catalogue: 200519). Once region 0 and 2 were mutated separately, both mutations were combined, having a plasmid SgG2 construct containing both mutations (SDM0+2). In a later step, the mutations were cloned via PpuMI and KpnI into the pcDNA3.1Zeo (details see following section).
Fig. 7 Schematic representation of the predicted cleavage sites. The corresponding definition called Region 2 stands for Arg321Ala322 and region 0 for Arg342Leu343. This definition continues through the whole thesis.
<table>
<thead>
<tr>
<th>Primer</th>
<th>Used for</th>
<th>Oligo sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>O-110</td>
<td>SDM0 Rev</td>
<td>GCCATGAGGGCCGCCCCCGGT TGCIGGGGGCCGCAACGTCG GGCT</td>
</tr>
<tr>
<td>O-111</td>
<td>SDM0 Fwd</td>
<td>ACCCCGACGTGCGGCCCCC GCACCCGCGGCGGCCCTCAT GGC</td>
</tr>
<tr>
<td>O-112</td>
<td>SDM2 Rev</td>
<td>TCGGGGTCTCTGCGCCAGGGC GGCTGCAAACGCCGGGGCG GGTGGGAAGGG</td>
</tr>
<tr>
<td>O-113</td>
<td>SDM2 Fwd</td>
<td>CCCTTCCCCACCCGCCCCCGG GTTTCGACCGCCCTGGCGCAGCACCCCCGA</td>
</tr>
</tbody>
</table>

2.2.1.2 Overlap PCR for the generation of cleavage site knockout (CSKO) variants

Due to cloning problems with the Site-Directed Mutagenesis (high GC content within the cleavage site regions), we decided to change the method to introduce mutations, using overlap PCR. The plasmid that was used as template was pcDNA3.1Zeo (pAV41), containing nucleotides 1060 to 2301 encoding for rSgG2 (provided by Abel Viejo-Borbolla).
The first PCR step included two independent PCR reactions, amplifying an upstream and a downstream part of the insert. The PCR reaction was done according to the protocol from Thermo Fisher Scientific using Phusion High Fidelity DNA polymerase (Catalogue: F-530S). Depending on the melting temperature ($T_m$) of the primers, the PCR reaction was adjusted accordingly. For all PCR and overlap PCR reactions, the buffer Combinatorial Enhancer Solution (CES) was used additionally to the commercially available GC buffer. The primer concentration was always 10 µM.

In some cases, the PCR reaction was done with a temperature gradient before the primary amplification. The temperature gradient was adjusted to the lowest $T_m$, for example, 2°C steps upwards and downwards from 56°C (52°C-54°C-56°C-58°C-60°C). As an example reaction, the reverse primer for the upstream part and the forward primer of the downstream part of the amplified insert contained the desired mutations for region 2. Afterwards, both PCR products were isolated and pooled for the overlap PCR. Here, the previously taken flanking primers were used
(T7 Forward + BGH Reverse). The PCR program was set according to the Thermo Fisher Scientific protocol and was slightly changed. Here, the first three cycles were done with a 2-step program, i.e. three cycles had the denaturation phase, followed by the elongation phase. Then the regular PCR program continued according to the Phusion HF protocol.

**PCR program for the overlap PCR**

<table>
<thead>
<tr>
<th>Step</th>
<th>Cycles</th>
<th>°C</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>98</td>
<td>30s</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>98</td>
<td>10s</td>
</tr>
<tr>
<td>3</td>
<td>32</td>
<td>72</td>
<td>30s/kb</td>
</tr>
<tr>
<td></td>
<td></td>
<td>98</td>
<td>30s</td>
</tr>
<tr>
<td></td>
<td></td>
<td>individual T&lt;sub&gt;m&lt;/sub&gt;</td>
<td>30s/kb</td>
</tr>
<tr>
<td></td>
<td></td>
<td>72</td>
<td>2m</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>72</td>
<td>10m</td>
</tr>
</tbody>
</table>

The products were separated and extracted with Gel Extraction Kit following the manufacturer’s instructions, digested with corresponding restriction enzymes (5’NheI and 3’HindIII), and inserted into the mammalian expression vector pAV41 backbone.

The following primer list was used for the cloning of CSKO variants. For the amplification of the upstream and the downstream part of the gG2
insert, each reverse primer was used in combination with the T7 forward primer (amplifying upstream part), whereas all forward primers were used in combination with BGH reverse primer (amplifying downstream part). Then the full insert (by pooling upstream and downstream part) was amplified in the overlap PCR using the flanking primers (227 + 228) only.
<table>
<thead>
<tr>
<th>Primer</th>
<th>Used for</th>
<th>Oligo sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>O-362</td>
<td>CSKO2/4AA Rev</td>
<td>GGGTCTGTCCGCGCGCCGGC TGCAAAACC GCGGGCGGGGTGG</td>
</tr>
<tr>
<td>O-363</td>
<td>CSKO2/4AA Fwd</td>
<td>CCACCCGCCCCGCGGGTTTGC AGCCGGCGCGCGGACAGAC CC</td>
</tr>
<tr>
<td>O-364</td>
<td>CSKO2/6AA Fwd</td>
<td>CTTCCCACCCCCGCCCCGCGGG CTGCAGCGCGCGCGACGAC AGACCCCCGAGGGGGTGC</td>
</tr>
<tr>
<td>O-365</td>
<td>CSKO2/6AA Rev</td>
<td>GACCCCCCTCGGGGTCTGTCG CCGCGCGCGCTGCAGCCCGC GGGCGGGGTGGGAAG</td>
</tr>
<tr>
<td>O-370</td>
<td>CSKO0/4AA Rev</td>
<td>TGTCCTCGGTCAGGCGCGCCGGTGTCG CCGGCCGCCGCCGGGCCGGG</td>
</tr>
<tr>
<td>O-371</td>
<td>CSKO0/4AA Fwd</td>
<td>GGGCCCCCAGACCGCGGGGC GGCGCGCGCGCCCTTGACCG AGGACA</td>
</tr>
<tr>
<td>O-372</td>
<td>CSKO0/6AA Rev</td>
<td>GGACGTGTGTCCTCGGTCGACGGTGCAATG CCGCGCGCGCCGGCGCCGGGT CC CGGGCGGGCCCGAA</td>
</tr>
<tr>
<td>O-373</td>
<td>CSKO0/6AA Fwd</td>
<td>TTCGGGCCCCCGGAACCGCGCG GCGGCCGCCGCGGCATTGACCG CGAGGACACGTCC</td>
</tr>
</tbody>
</table>
(4AA and 6AA refers to the number of mutated residues in the predicted cleavage site region; each primer pair with grey or white background were used for the upstream [Rev+227] and downstream part [Fwd+228])

<table>
<thead>
<tr>
<th>Flanking primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>O-227</td>
</tr>
<tr>
<td>O-228</td>
</tr>
</tbody>
</table>

Since these mutations were introduced in only one cleavage site region (CSKO2 refers to region 2; CSKO0 refers to region 0), the second step included another overlap PCR step that combines the previously made mutations into one construct called CSKO0+2.

Three additional alanines were introduced upstream of cleavage site region 2 in the CSKO0+2/6AA construct. The previously described method was also applied for cloning the construct CSKO0+2/6+3AA. The following primers were used in combination with the flanking primers O-227 and O-228, respectively.
**Tab. 16 Primer list for the introduction of three additional alanine residues upstream of region 2 in the construct CSKO0+2/6AA**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Used for</th>
<th>Oligo sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>O-532</td>
<td>CSKO0+2/6AA Fwd</td>
<td>CCCTTCCCACCCGCGCCGGC GGCGGGTGGGAAGGG</td>
</tr>
<tr>
<td>O-533</td>
<td>CSKO0+2/6AA Rev</td>
<td>CCGGCTGCAGCCGCCGC GGCGGGTGGGAAGGG</td>
</tr>
</tbody>
</table>

### 2.2.1.3 Generation of rSgG2GSm and rSgG2ΔCS constructs

The cloning of rSgG2 Glycerin-Serine motif (rSgG2GSm) and rSgG2 Δcleavage site (rSgG2ΔCS) was based on the pAV41-rSgG2 and using the overlap PCR procedure. Therefore, the following primers were used to generate these constructs. Additionally, the same flanking primers, as previously described, were used here as well (O-227 and O-228).
Tab. 17 Primer list for introduction of the Glycine-Serine motif (GSm) instead of the cleavage site and deletion of the cleavage site

<table>
<thead>
<tr>
<th>Primer</th>
<th>Used for</th>
<th>Oligo sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>O-669</td>
<td>SgG2GSm Fwd</td>
<td>GGATCTGGAGGAGGAGGATCTGGAGGAGGAGGATCTGGAGGAGGAGGATCTGGAGGAGGATCTATGGCCTTGACCGAGGACACGTCCTCCGAT</td>
</tr>
<tr>
<td>O-670</td>
<td>SgG2GSm Rev</td>
<td>TCCTCCAGATCCTCCTCCTCCTCCAAGATCCTCCTCCTCAGATCCTCTCCTCCTCCGAAGGGGGGCGTACGACCGTCATCTAGGGC</td>
</tr>
<tr>
<td>O-697</td>
<td>SgG2ΔCS Fwd</td>
<td>CCGTACGCCCCCTTCTCATGGCCTTGACCGA G</td>
</tr>
<tr>
<td>O-698</td>
<td>SgG2ΔCS Rev</td>
<td>CTCGGTCAAGGCGCATGAAGGGGGCGTACGGGCGTACCG</td>
</tr>
</tbody>
</table>

2.2.1.4 Cloning of the recombinant full-length gG2 (rFLgG2) into the pAV41 vector

pGEM-T-FLgG2 (provided by Sophie Luther) was used to amplify the rFLgG2 using primers upstream from gG2 (SgG2) (O-702) and downstream from gG2 (cytoplasmic domain) (O-703). The PCR reaction was set up according to the Phusion HF protocol by Thermo Fisher Scientific (+ CES buffer). The insert was then cloned via NheI and
HindIII into the pAV41 backbone, which was cut with same restrictions enzymes.

**Tab. 18 Flanking primers for the transfer of full-length gG2 from pGEM-T into the pcDNA3.1Zeo plasmid**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Used for</th>
<th>Oligo sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>O-702</td>
<td>rFLgG2 Rev</td>
<td>CTTAAGCTTTGGTACCGCAT GTTAAGCGTAATCTGGAAC ATCGTATGGGTACAAATCC CGCTCGGGTGGCAGACA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>O-703</td>
<td>rFLgG2 Fwd</td>
<td>GGTAAGCCTATCCCTAACC CTCTCCCTCGGTCTCGATTCT ACGATGCATAACAGCGATG TTGTTTTCCC CGGG</td>
</tr>
</tbody>
</table>

The restriction enzymes AccIII and KpnI were used to transfer the full ectodomain (+ transmembrane and cytoplasmic domain) from pAV41rFLgG2 into the deletion mutants (pAV41rSgG2GSm and pAV41rSgG2ΔCS), resulting in recombinant full-length gG2 (pAV41rFLgG2GSm and pAV41rFLgG2ΔCS). The restriction enzymes were used according to the NEB protocol (http://nebcloner.neb.com/). Depending on the buffer, which sometimes allowed 100% activity for
one enzyme, the second restriction enzyme (with <100% activity) was either added before and incubated longer or used after the first restriction enzyme digest. The latter case required the cleaning with the PCR Purification Kit from QIAGEN (digested sample was purified according to the protocol from QIAquick PCR Purification Kit, Catalogue: 28104).

2.2.2 Generation of deletion rSgG2 constructs for binding assays

2.2.2.1 Terminal deletion of residues in rSgG2

Initial experiments started with deletions (10 and 20 residues) at the N-terminal and C-terminal sides. Here, the following primers were used to create the N-terminal and C-terminal deletions. We used for the 10 residues C-terminal deletion 008+009 and 20 residues C-terminal deletion 008+027. The N-terminal deletions were established with the primer pairs 031+029 and 031+030, deleting 10 and 20 residues, respectively. The inserts were cloned with NcoI and SphI. Once the clones were established, each terminal deletion was combined.
<table>
<thead>
<tr>
<th>Primer</th>
<th>Used for</th>
<th>Oligo sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>O-008</td>
<td>gG2delC-Fwd</td>
<td>ATTGCCATGGGATCCACAGCGATGTTGTT</td>
</tr>
<tr>
<td>O-009</td>
<td>gG2delC1-Rev</td>
<td>TTTGCATGCTAGGCCTACGGACCGTCATCTAGGG</td>
</tr>
<tr>
<td>O-027</td>
<td>gG2delC3-Rev</td>
<td>TTTGCATGCTAGGCCCAATGGGGGCAGGAC</td>
</tr>
<tr>
<td>O-031</td>
<td>gG2delN-Rev</td>
<td>TTTGCATGCTAAACGCGGGCGGGGTGGGAA</td>
</tr>
<tr>
<td>O-029</td>
<td>gG2delN1-Fwd</td>
<td>ATTGCCATGGGTTCCCCTGTCCTCAAATATTGCCG</td>
</tr>
<tr>
<td>O-030</td>
<td>gG2delN2-Fwd</td>
<td>ATTGCCATGGACGATCCCGGGCTTTGGGTTC</td>
</tr>
</tbody>
</table>

### 2.2.2.2 Generation of intramolecular deleted rSgG2 constructs

DNA fragments of gG2 carrying deletions were cloned into the pAV41rSgG2 plasmid. The rSgG2 residues were deleted from the N-terminus in steps of 20 residues. As previously described, the PCR was
done through the overlap PCR. The inserts were cloned via NheI and HindIII.
<table>
<thead>
<tr>
<th>Primer</th>
<th>Used for</th>
<th>Oligo sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>O-927</td>
<td>new_del 1.1 Rev</td>
<td>GGACGACGCGGGGATAG GCATAACAATATT</td>
</tr>
<tr>
<td>O-928</td>
<td>new_del 1.2 Fwd</td>
<td>TGCCTATCCCCCGCGTCGT CCGTCACGAGCC</td>
</tr>
<tr>
<td>O-929</td>
<td>new_del 2.1 Rev</td>
<td>CCGCGCGGCCGGGCGGCGGAGGTCTTGCCGCC</td>
</tr>
<tr>
<td>O-930</td>
<td>new_del 2.2 Fwd</td>
<td>CCTGCCGCCGGCGCGCCGCCGGTACCGGATT</td>
</tr>
<tr>
<td>O-931</td>
<td>new_del 3.1 Rev</td>
<td>TGAGCCCGGTACAGCAAA ACCAGCCCCCCC</td>
</tr>
<tr>
<td>O-932</td>
<td>new_del 3.2 Fwd</td>
<td>GGTATTGTCTGTACCAGGTCC CACCCGCGGCTG</td>
</tr>
<tr>
<td>O-933</td>
<td>new_del 4.1 Rev</td>
<td>GCTCGCCGCCGTACGTCA CACGGGCCCGCAT</td>
</tr>
<tr>
<td>O-934</td>
<td>new_del 4.2 Fwd</td>
<td>TGTGACGTACCGGCACGGA GCCGCCGTCCCC</td>
</tr>
<tr>
<td>O-935</td>
<td>new_del 5.1 Rev</td>
<td>TCGGAGGCCCGCGACACC CTCCTACTGCC</td>
</tr>
<tr>
<td>O-936</td>
<td>new_del 5.2 Fwd</td>
<td>AGGGTGTCGCGGGCTCCG ACCCCTCGGTACGC</td>
</tr>
<tr>
<td>O-937</td>
<td>new_del 6.1 Rev</td>
<td>TCTCCCGCGCGCGCGCCCT GGTACGTCGTACG</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td><strong>O-938</strong></td>
<td><strong>new_del 6.2 Fwd</strong></td>
<td><strong>CCAGGGCGGCGCCGCGG</strong>&lt;br&gt;<strong>AGACATTCGAGTA</strong></td>
</tr>
<tr>
<td><strong>O-939</strong></td>
<td><strong>new_del 7.1 Rev</strong></td>
<td><strong>CGGTGGGGCCGATCTGGT</strong>&lt;br&gt;<strong>ACTCGAATGTCT</strong></td>
</tr>
<tr>
<td><strong>O-940</strong></td>
<td><strong>new_del 7.2 Fwd</strong></td>
<td><strong>GTACCAGATCGGCCCCAC</strong>&lt;br&gt;<strong>CGCCCCCCCCACA</strong></td>
</tr>
<tr>
<td><strong>O-941</strong></td>
<td><strong>new_del 8.1 Rev</strong></td>
<td><strong>GGCCCCGCGGCGGGCCCT</strong>&lt;br&gt;<strong>CCCAGCCCACCT</strong></td>
</tr>
<tr>
<td><strong>O-942</strong></td>
<td><strong>new_del 8.2 Fwd</strong></td>
<td><strong>GGAGGGCCCCCCCCCGCGG</strong>&lt;br&gt;<strong>GCCGCCCCGTGGCG</strong></td>
</tr>
<tr>
<td><strong>O-943</strong></td>
<td><strong>new_del 9.1 Rev</strong></td>
<td><strong>GGCCACGAAAGACCGGG</strong>&lt;br&gt;<strong>GGGACGCACCGGC</strong></td>
</tr>
<tr>
<td><strong>O-944</strong></td>
<td><strong>new_del 9.2 Fwd</strong></td>
<td><strong>CCCCCCCGGTCTTTTGTGG</strong>&lt;br&gt;<strong>CCTGCGTTTCCG</strong></td>
</tr>
<tr>
<td><strong>O-945</strong></td>
<td><strong>new_del 10.1 Rev</strong></td>
<td><strong>GTGGTAGGTCCCCCGGGG</strong>&lt;br&gt;<strong>TGGGGCCGGAAT</strong></td>
</tr>
<tr>
<td><strong>O-946</strong></td>
<td><strong>new_del 10.2 Fwd</strong></td>
<td><strong>CAACCCCCGGGACCTACC</strong>&lt;br&gt;<strong>ACGCGTCGTCTTT</strong></td>
</tr>
<tr>
<td><strong>O-947</strong></td>
<td><strong>new_del 11.1 Rev</strong></td>
<td><strong>TCCGAATAAACGCTGGGG</strong>&lt;br&gt;<strong>CGGCGGGCGTCT</strong></td>
</tr>
<tr>
<td><strong>O-948</strong></td>
<td><strong>new_del 11.2 Fwd</strong></td>
<td><strong>CGCCCCCCAGCTTTTATTCG</strong>&lt;br&gt;<strong>GATGGCACGACC</strong></td>
</tr>
<tr>
<td><strong>O-949</strong></td>
<td><strong>new_del 12.1 Rev</strong></td>
<td><strong>CTAGGCCCCCGTGCAGGG</strong>&lt;br&gt;<strong>CCGTAATCCCCA</strong></td>
</tr>
<tr>
<td><strong>O-950</strong></td>
<td><strong>new_del 12.2 Fwd</strong></td>
<td><strong>GGGCCCCACGCGGGGCCC</strong>&lt;br&gt;<strong>CTAGGACGGTCC</strong></td>
</tr>
</tbody>
</table>
2.2.3 Cloning of the cleavage site region into a reporter plasmid

2.2.3.1 Cloning of H2S between mCherry and Gaussia Luciferase (GLUC)

The cleavage site region H2S was cloned between the 5’ mCherry and 3’ GLUC. Therefore following primers were used for the overlap PCR:

<table>
<thead>
<tr>
<th>Primer</th>
<th>Used for</th>
<th>Oligo sequence</th>
</tr>
</thead>
</table>
| O-795    | Rev_Pm157_US           | CGAACGTCGGGGTGCACCTAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCGTCAGATCAAGCTCA
The amplified insert was digested (5’EcoRI; 3’XbaI) and ligated into the pM157 backbone.

2.2.3.2 Cloning of the H2S between eGFP and mCherry

The cleavage site region H2S was cloned between 5’eGFP and 3’mCherry. The following primers were used for the amplification of eGFP from pGL3, H2S from pAV11 and the mCherry from pM157:
Tab. 22 Primer list for the introduction of the H2S region into the pGL3 reporter plasmid (replacing P2A in eGFP-P2A-mCherry) through overlap PCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Used for</th>
<th>Oligo sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>O-873</td>
<td>CR_P1_GFP_Fwd</td>
<td>GCGCAAGCTTTATGGTGAGCAAGG GCG</td>
</tr>
<tr>
<td>O-874</td>
<td>CR_P2_GFP_Rev</td>
<td>GGCGGGGTGGATCCTTGTACAGCT CGTCCA</td>
</tr>
<tr>
<td>O-875</td>
<td>CR_P3_H2S_Fwd</td>
<td>GTACAAGGATCCACCCCGCCGC GGGTTTCG</td>
</tr>
<tr>
<td>O-876</td>
<td>CR_P4_H2S_Rev</td>
<td>TGTCACCATATGGAGGCGCCGC CGGTTC</td>
</tr>
<tr>
<td>O-877</td>
<td>CR_P5_mCh_Fwd</td>
<td>GCGCCTCCATATGGTGAGCAAGG GCGAGGA</td>
</tr>
<tr>
<td>O-879</td>
<td>CR_P6.2_mCh_Rev</td>
<td>GCGCTCTAGATTACTTGTACAGCT CGTC</td>
</tr>
</tbody>
</table>

Afterwards, three parts of the inserts were amplified. The amplified eGFP and H2S were pooled and used for another amplification using O-873 and O-876. The H2S and mCherry product were pooled and used for another PCR using O-875 and O-879 parallel to the previous one. The result of both amplification contains now 5’HindIII_eGFP with 3’H2S overlap and 5’H2S overlap with 3’mCherry_XbaI, respectively. These
both products were pooled again and used for the last PCR containing the primers O-873 and O-879. The full insert containing eGFP-H2S-mCherry was cloned via HindIII and XbaI into the pGL3 vector.

2.2.4 Plasmid production, isolation and ligation

2.2.4.1 Transformation and plasmid isolation from DH5α (and DH10Bac) bacteria

DH5α competent bacteria were thawed slowly on ice, and 1 µL of plasmid DNA was pipetted into 200 µL bacteria. This reaction was incubated for 30 minutes on ice and heat-shocked in a water bath for 45 seconds at 42°C. After the short incubation, the bacteria were cooled down on ice for 5 minutes. 400 µL (800 µL for DH10Bac) of Luria-Bertani broth (LB, at room temperature [RT]) was added to the bacteria, and the tube was shaken at 200 rpm for 30 minutes (4 hours for DH10Bac). After incubation, bacteria were plated on LB agar plates containing appropriate antibiotics. To identify bacteria, where recombination of the bacmid and the plasmid DNA occurred, we used
IPTG + XGal for blue-white screening (see Table 3 for concentration) and incubated overnight at 37°C. On the following day, colonies were picked and incubated overnight in 5 mL LB media containing selective antibiotic(s) (for most plasmids Ampicillin; for DH10Bac Gentamycin + Kanamycin + Tetracyclin; concentrations, see table 3) at 200 rpm and 37°C. The next day, the plasmid (Bacmid, see section “Transformation and Bacmid isolation of DH10Bac to produce recombinant baculovirus”) was isolated according to the protocol from the Machery & Nagel Nucleospin Plasmid kit (Catalogue: 740588). The concentration of the obtained DNA was determined with the Nanodrop 1000.

**2.2.4.2 Restriction digest control of pFastBac-Mel and pcDNA3.1Zeo**

Prior to sequencing, the plasmids were digested with the flanking restriction enzymes to determine the presence of the insert via Agarose DNA gel electrophoresis. The digestion was done according to the NEB protocol (http://nebcloner.neb.com/).
2.2.4.3 Agarose gel DNA electrophoresis

For the amplified insert and digestions, the DNA was separated in a 1% agarose gel via electrophoresis at 80V. The agarose gel was prepared in TAE buffer (Tris 40 mM; acetic acid 20 mM; EDTA 500 mM; ethidium bromide 0.5 µg/mL). After the gel electrophoresis, bands were visualised on a UV light bench, cut and cleaned according to the protocol from the Gel Extraction Kit from QIAGEN (Catalogue: 28704).

2.2.4.4 Ligation of amplified and digested inserts into the desired expression vector

The insertion of the gene of interest occurred through ligation. Here, the T4 Ligase from Thermo Fisher Scientific was used and pipetted according to the corresponding protocol (https://assets.thermofisher.com). The amount of insert was calculated according to the following equation:

\[
ng\ insert = \frac{ng\ vector * \ km\ insert}{\ km\ vector} * ratio\ vector:\ insert \ (3:1)
\]
2.2.5 Baculovirus Expression System

2.2.5.1 Transformation and Bacmid isolation of DH10Bac to produce recombinant baculovirus

See section “Transformation and plasmid isolation of DH5α (and DH10Bac) bacteria” for previous steps of DH10Bac transformation. After growing the bacterial clones with selective antibiotics, the bacmid isolation was done according to the protocol from the Machery & Nagel Nucleospin Plasmid kit (Catalogue: 740588) and was slightly adapted as followed. After the steps resuspension, lysis, neutralisation and centrifugation, the supernatant was treated further without any columns. After centrifugation, to precipitate the DNA, the supernatant was treated with ice-cold isopropanol and cooled on ice for 10 minutes and then centrifuged for 15 minutes at 11000 g. Afterwards, the pellet was washed two times with ice-cold 70% EtOH and centrifuged in between at 11000 g for 2 minutes. The EtOH was discarded, the pellet was air-dried and resuspended gently in 50µL sterile bi.dest. water. The obtained DNA was measured with the Nanodrop 1000.
2.2.5.2 Generation and production of recombinant baculovirus

For the production of the recombinant baculovirus, $1 \times 10^6$ adherent Hi5 cells were seeded into wells of 6-well plates, 24 hours before the transfection. The medium was changed prior to the transfection. The transfection mix was pipetted and applied to the Hi5 cells according to the Lipofectamine 2000 transfection protocol from Thermo Fisher Scientific (https://assets.thermofisher.com). The supernatant of the transfected cells was collected at 72 hours post-transfection, centrifuged at 200 g to remove debris and stored at 4°C in a light opaque box, due to the light sensitivity of the baculoviruses. Additionally, an aliquot of the supernatant and cell lysate were mixed with Laemmli buffer (LB; 0.6 M Tris-HCl pH: 6.8, 0.715 M β-mercaptoethanol, 25% glycerol, 2% SDS and 0.1% bromophenol blue) and stored at -80°C for western blotting. The supernatant collected from the transfected Hi-5 cells contains recombinant baculovirus, stock P0. The recombinant virus was amplified by infecting subconfluent Hi-5 cells (in P100 and then P150 culture dish) at low multiplicity of infection obtaining stocks P1 and P2, respectively.
All inoculations were incubated for several days until Hi-5 cells showed cytopathic effect, i.e. floating and round-shaped cells.

2.2.5.3 Recombinant SgG2 production and purification

For large-scale production of recombinant SgG2, 500 mL of Hi5-suspension cells containing 1 x 10^6 cells/mL were infected with 35 mL of P2 stock. The cells were incubated together with the virus for 72 hours on a shaker at 120 rpm and 28°C and checked daily for viability via trypan blue staining, where dead cells have no intact cell membrane and turn dark under the microscope. Next, the infected Hi-5 suspension cells were harvested and centrifuged at 4°C for 10 minutes at 200 g in a Heraeus Multifuge 3SR+ centrifuge to remove all floating cells followed by centrifugation of the supernatant at 6360 g for 45 minutes, 4°C with the Avanti J-25 centrifuge to remove cell debris.

The supernatant was then used for tangential filtration using Centramate T-Series Cassettes with a 10 kDa cut off for SgG2. During this process, the supernatant was dialysed with 100 mM phosphate buffer (20 mM NaH₂PO₄ + 81 mM Na₂HPO₄ + H₂O), 10 mM imidazole and
concentrated up to 40 mL. The concentrated sample was then incubated with Ni-NTA overnight, which allows the binding of the His-tag present at the N-terminus of rSgG2 to the nickel beads. Next, the concentrated Ni-NTA incubated sample was washed in a column using 100 mM phosphate buffer and 20 mM imidazole.

The elution of the Nickel beads bound rSgG2-His was done with 100 mM phosphate buffer containing 40 mM, 60 mM, 100 mM and 250 mM imidazole. The eluted samples were run in a sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) together with the unstained ladder from Bio-Rad, followed by a coomassie staining. If the purity was not higher than 90%, the samples were bound again to the Ni-NTA overnight in phosphate buffer and increased imidazole (30 mM), washed the following day with phosphate buffer and 40 mM imidazole and eluted with 60 mM, 100 mM, 250 mM and 500 mM.

When required, the eluted SgG2 was further concentrated using an Amicon Ultra-15 with a 10 kDa cut off. The final concentrated sample was rechecked via SDS-PAGE together with defined BSA concentration
curve to determine the final concentration of rSgG2. A picture of the stained gel was taken with the LAS3000 imager, and the concentration of the purified rSgG2 was quantified with ImageJ.

2.2.6 General techniques to investigate protein expression and size

2.2.6.1 Separation of proteins according to their molecular weight using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

All samples were mixed with LB-buffer. Before western blotting, the samples were boiled for 5 minutes, briefly centrifuged and loaded into the gel. The SDS-PAGE gels were prepared in two parts made of the stacking gel (5% acrylamide) for concentrating the samples and the separating gel for separation of the samples. Depending on the protein of interest and its size, different percentage of acrylamide and running time were used for the SDS-PAGE (usually 12% acrylamide).
<table>
<thead>
<tr>
<th>Compound</th>
<th>Separating gel (12%)</th>
<th>Stacking gel (5%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 % Acrylamide/BIS 29:1</td>
<td>2 mL</td>
<td>0.425 mL</td>
</tr>
<tr>
<td>Tris HCl</td>
<td>2 mL (pH 8.8, 1 M)</td>
<td>0.65 mL (pH 6.8, 0.5 M)</td>
</tr>
<tr>
<td>SDS 10%</td>
<td>0.05 mL</td>
<td>0.025 mL</td>
</tr>
<tr>
<td>APS 10%</td>
<td>0.025 mL</td>
<td>0.0175 mL</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.005 mL</td>
<td>0.0025 mL</td>
</tr>
<tr>
<td>Dist. H₂O</td>
<td>0.92 mL</td>
<td>1.425 mL</td>
</tr>
</tbody>
</table>

The gel was run in a buffer (1% SDS, 25 mM Tris-HCl and 192 mM Glycine) at 25 mA/gel inside a Mini-PROTEAN Tetra Cell chamber. The size of the protein was determined using All-blue pre-stained standard ladder from Bio-Rad.

### 2.2.6.2 Western Blot

Proteins separated by SDS-PAGE were transferred onto a nitrocellulose membrane using a Mini Trans-Blot Cell Blot chamber containing blotting
buffer (25 mM Tris + 192 mM Glycin + 20% methanol) and run at 250 mA for 1 hour. After protein transfer onto the membrane, the blot was blocked with 5% milk in PBS with 0.1% Tween-20 at RT for 1 hour. Next, the membrane was incubated with the primary antibody in 3% milk in PBS plus 0.1% Tween-20 at 4°C overnight. The dilutions of primary antibodies used are indicated in table 10. Then the blot was washed three times with PBS, 0.1% Tween-20 before incubation with the conjugated secondary antibody (see Table 11 for dilution) in 3% milk in PBS plus 0.1% Tween-20 during 0.75-1 hour and washed two times with PBS, 0.1% Tween-20 and two times 1xPBS. The proteins were visualised with the Odyssey LI-COR imager.

2.2.6.3 Coomassie staining

Proteins within the SDS-PAGE were stained with Coomassie staining (45% methanol + 10% acetic acid + 2.5 g/L Coomassie brilliant blue R-250). Here, the SDS-PAGE gel was soaked with Coomassie stain for 1-2 hours, followed by a wash with 45% methanol + 10% acetic acid. Since this method causes shrinking of the SDS gel, the gel was rehydrated
(5% methanol + 7% acetic acid) and visualised with the LAS3000 imager.

2.2.7 Functional experiments

2.2.7.1 Heparin-binding assays

Supernatants obtained from transfected HEK293T cells were used for heparin pull-down assay. Here, the supernatant was incubated with heparin-sepharose beads. Additionally, the mixture was treated with soluble heparin (0.1 mg to 2 mg) to compete with the heparin beads during the pull-down. For each mixture the following volumes were used:

<table>
<thead>
<tr>
<th>Components for each mixture</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Binding buffer*</td>
<td>390 µL</td>
</tr>
<tr>
<td>Heparin beads</td>
<td>10 µL</td>
</tr>
<tr>
<td>HEK293T obtained supernatant</td>
<td>25 µL</td>
</tr>
<tr>
<td>Soluble heparin</td>
<td>X mg**</td>
</tr>
</tbody>
</table>

*0.2% BSA in PBS

**0.1 mg – 2 mg
Agarose beads without any heparin were used as a negative control. The mixture was incubated for 1 hour at 4°C on a spinning wheel. Afterwards, the beads were washed twice in ice-cold PBS and centrifuged in between with 11000 g. For the third and last wash step, the beads were transferred into a new 1.5 mL tube to reduce unspecific binding of rSgG2 to the tube, followed by the last centrifugation step. The beads were incubated with LB-buffer for 1 minute at 95°C to elute bound proteins followed by SDS-PAGE and western blot. As a control, the input was checked in another western blot.

2.2.7.2 Chemotaxis assay using Jurkat E6.1 cells

The migration of JURKAT cells in the presence of different recombinant gG2 constructs was analysed. This experiment used a 96-well ChemoTx System plate, where 30 µL of supernatant from transfected cells with different recombinant gG2 was added into the bottom wells. Then, the plate was covered by a filter of 5 µm and 1.25 x 10^5 cells were pipetted on top. We used chemokine alone, mock-transfected and WT-rSgG2 as controls.
The plate was incubated for 1-2 hours at 37°C and observed during this time. At the end of the experiment, the droplets on the membrane were removed, and the filter was washed with 1x PBS. Next, the plate together with the membrane was centrifuged in Heraeus Multifuge 3SR+ at 150 g for 5 minutes. The membrane was removed carefully, and free wells were filled with known numbers of Jurkat cells for the standard curve (5 x 10^6 to 0.0391 x 10^6 in eight 1:2 dilution steps). Each well was then mixed with 5 µL Promega CellTiter96 AQueous One Solution and incubated for 1 hour at 37°C. Absorbance at 490 nm was measured with the Synergy 2 plate reader. The number of migrated cells was calculated using the standard curve generated by Excel.

2.2.7.3 Immunofluorescence of the rgG2 variants in transfected HeLa cells

0.1 x 10^6 HeLa cells were seeded 1 day before transfection into wells of 12 well plate containing coverslips (Ø 10 mm). Prior to transfection, the medium was changed with fresh medium. The transfection reagent and DNA were adjusted to the size of the well, using 0.5 µg DNA per
construct and well and 2 µL of transfection reagent (1:4 ratio) in a total volume of 100 µL Opti-MEM. Depending on the confluence, the HeLa cells were fixed 1 day or 2 days after transfection. For fixation, the medium was removed, the cells were washed once with PBS, treated with 3% PFA in PBS and incubated for 20 minutes at RT. After incubation, the cells were washed three times with PBS. Then ammonium chloride (50 mM in PBS) was added to the samples for 10 minutes to neutralise the remaining PFA. For permeabilisation, HeLa cells were treated with 0.2% Triton X-100 in PBS for 10 minutes, followed by treatment with 0.75% glycine in PBS for 10 minutes. Samples without permeabilisation were directly blocked. All samples were treated with blocking buffer (3% BSA in PBS) for 1 hour. After blocking, all samples were stored upside upon a parafilm piece, covered and incubated overnight with the primary antibodies diluted in 70 µL of 3% BSA in PBS (see Table 10 for dilution). After the overnight incubation, the coverslips were washed three times with PBS, stained with DAPI and the specific secondary antibodies, which were diluted in 3% BSA in PBS (see Table 11 for dilution). The whole chamber was incubated at RT for 1 hour in a light
protected environment. After incubation, the cells were washed three times with PBS and mounted on a slide with a drop of Prolong Gold antifade and stored at 4°C for a short time or -80°C for a longer period.

2.2.7.4 Preparation of dorsal root ganglion dissociated neurons in RND450 microfluidic chambers (MFC)

[Day 1] Coverslips were sorted into brackets to store them into a vessel, which was sonicated for 10 minutes in the following order: 1 x bi.dest. H₂O with dishwashing reagent → 1 x with Extran AP12 (0.4% in H₂O) → 2 x rinsing with bi.dest. H₂O without sonicating → 1 x with bi.dest. H₂O only → 1 x EtOH. Next, the coverslips were taken out of the vessel and dried under the cell culture hood. After drying, three coverslips were arranged inside a petri dish and covered with 100 µL of Poly-L-Lysin (0.01%) and incubated overnight at 4°C. Next, the coverslips were washed two times with bi.dest. H₂O and incubated with water for 3 hours. After incubation, the coverslips were washed three times with bi.dest. H₂O and dried under the hood for at least 2 hours.
MFCs were soaked in Extran AP12 (0.4% in H₂O) for several days. Then they were washed with a regular dishwashing reagent, then with distilled H₂O and finally soaked in 80% EtOH. After the EtOH step, the MFCs were transferred into a glass petri dish containing Whatman paper. These chambers were autoclaved and directly transferred onto the previously cleaned coverslips. To determine whether the chambers were properly attached to the coverslips, CO₂ independent medium was pipetted into the somal well and checked, whether the microgrooves were filled with medium after 5 minutes of incubation at 37°C. Once the grooves were filled, the medium was removed and replaced with 20 µL laminin (20 µg in Tris-HCL (pH 7.4), 0.15 M NaCl) solution and incubated overnight in a petri dish with wet paper at 37°C.

[Day 2] The preparation and collection of mouse DRGs required 1 x HBSS media at RT. The other following reagents were thawed and prewarmed directly before use:
Tab. 25 Reagents for dissociation of mice neurons

<table>
<thead>
<tr>
<th>Reagent</th>
</tr>
</thead>
<tbody>
<tr>
<td>1x complete HBSS</td>
</tr>
<tr>
<td>0.5 mL papain stock solution</td>
</tr>
<tr>
<td>1 mL collagenase/dispase mix</td>
</tr>
<tr>
<td>4.5 mL papain activation solution</td>
</tr>
<tr>
<td>F12 medium + 10% FCS + Pen/Strep (1:100)</td>
</tr>
<tr>
<td>CO₂-independent medium complete + 10% FCS + Pen/Strep (1:100)</td>
</tr>
</tbody>
</table>

The mouse was sacrificed through CO₂ sedation followed by cervical dislocation. The spine was separated from the mouse body, and the surrounding tissues were removed. The backbone was longitudinally cut, and the spinal cord was removed stepwise while collecting the DRGs in 1 x HBSS media (14 mL). Once the DRGs were collected, the tube was centrifuged for 1 minute at 381 g to remove the supernatant. Next, the DRGs were treated with the papain stock (0.5 mL) and the papain activation solution (4.5 mL), which was then incubated for 20 minutes at 37°C. After incubation, the DRGs were spun down again for 1 minute at 381 g to discard the supernatant. Next, the DRGs were treated with
collagenase/dispase (1 mL) dissolved in 1x complete HBSS (4 mL) for 20 minutes at 37°C. Here again, the supernatant was removed to resuspend the digested DRGs/neurons in 1x HBSS through three fire-narrowed glass Pasteur pipettes with decreasing diameters. This suspension was treated with 5 mL percoll/CO₂-independent-medium mix (1 mL + 4 mL) and centrifuged again for 8 minutes at 381 g. Afterwards, the supernatant was removed carefully, and the pellet containing the dissociated neurons was resuspended with 2 mL CO₂-independent medium, which was then centrifuged for 2 minutes at 1029 g to remove the supernatant. The pellet was resuspended in complete F-12 medium (volume used depending on the number of MFCs, i.e. 5 µL neurons/MFC were required, which means 9 MFCs require 45+5 µL neuron suspension).

The laminin was removed carefully from the MFCs, and 5 µL of neuron suspension was added into the upper somal well and transferred by capillary force through the channel. The MFCs were incubated for 10 minutes at 37°C. Then 100 µL and 50 µL of complete F-12 medium
were pipetted into the somal and axonal wells, respectively. This difference in volume generates a pressure gradient towards the axonal site, preventing the flow of neurons back to the somal wells. The MFCs were incubated overnight at 37°C. On the following day, HEK293T cells were seeded for transfection with recombinant gG2 variants.

[Day 3] The next day, MFCs were checked for neurite outgrowth and leakage. The HEK293T cells were transfected for at least 4 hours before adding them to MFCs. After 4 hours, transfected HEK293T cells were resuspended in complete F-12 (+ NGF [1:25,000] + Aphidicolin [1:250]). The Aphidicolin inhibits nuclear DNA replication and prevents overgrowth of HEK293T cells inside the chamber. Before the addition of HEK293T cells, the medium of the MFCs was removed first from the somal and then from the axonal site. Then 100 µL complete F-12 (+ NGF + Aphidicolin) was pipetted onto the top axonal well followed by addition of 20 µL of transfected HEK293T cells. This gradient allows the flow-through of cells from the upper axonal well to the lower axonal well. After attachment of the HEK293T cells (30 minutes are required), first,
the somal (200 µL) and then axonal site (170 µL) were filled with complete F-12 medium (+ NGF + Aphidicolin). The MFCs were observed for at least 3 days to detect neurite outgrowth on the axonal site, and the cells were fixed and subjected to IF staining.

The IF staining was separated in two days and started with removing the medium from the MFCs. Then 100 µL 3% PFA in PBS was added to the somal site and 50 µL to the axonal site of the chamber to create a pressure gradient and fix neurites inside the microgrooves. The devices were incubated for 30 minutes and washed with PBS twice for 5 minutes each.

The permeabilisation took place by incubating during 30 minutes at RT the cells with PBS + 0.2% Triton X-100 and creating a pressure gradient (somal: 100 µL / axonal: 50 µL) from the somal to the axonal site. After permeabilisation, the solution was removed from the chambers and replaced with blocking solution PBS-B (PBS + BSA 3% + 5% goat serum) for 30 minutes at RT. After blocking, the buffer was removed, and the primary mouse anti-TUJ1 antibody (1:1000 in PBS-B + 5% serum) and rabbit anti-MAP2 antibody (1:1100 in PBS-B + 5% serum)
were added to the chambers, creating a pressure gradient (somal: 150 µL / axonal: 100 µL) overnight.

The following day, the primary antibodies were removed, and the MFCs were rinsed 3 times with PBS-B. Next, the secondary antibodies (Alexa Fluor 555 anti-rabbit and Alexa Fluor 647 anti-mouse) were mixed 1:1000 in PBS-B without serum and pipetted into the chamber. Different volumes were applied to the somal and axonal sides to create a pressure gradient and enable staining of neurites through the microgrooves and at the axonal side as well. The chamber was incubated for 1-2 hours and washed three times with PBS for 5 minutes each. The chamber was mounted with Prolong Gold Antifade and pictures were taken with the fluorescence microscope Zeiss Observer Z1 Axio. The following reagents were used for the MFC experiment:
<table>
<thead>
<tr>
<th>Tab. 26 Reagent Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Poly-L-Lysine</strong></td>
</tr>
<tr>
<td><strong>1x HBSS (complete)</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td><strong>Papain stock</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td><strong>Papain Activation Solution</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td><strong>Collagenase/Dispase</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Item</td>
</tr>
<tr>
<td>-----------------------------</td>
</tr>
<tr>
<td>600 mg Dispase II (Sigma, P4762)</td>
</tr>
</tbody>
</table>
| Complete F-12-Medium        | 35.6 mL aliquots + 4 mL FCS  
On the day of the experiment add:  
0.4 mL Pen/Strep  
2 µg NGF |
| Complete C02-ind.-Medium    | 500 mL CO2-independent Medium (GIBCO 18045070)  
2.84 mL HEPES stock 1 M  
5.68 mL D-Glucose stock 1 M  
add HEPES and glucose, adjust the pH to 7.35, filter sterilise and prepare 36 mL aliquots.  
Day of the experiment add: 4mL FCS (final 10%) and 0.4 mL Pen/Strep (GIBCO BRL# 15140-122) per 36 mL aliquot |
| Laminin                    | Invitrogen 23017-015 ~1mg/mL | Aliquot 20 µL/20 µg |
| FCS                        | 4mL aliquots                                                               |
| Penicillin-Streptomycin     | Penicillin 10000 unit/mL and streptomycin 10 mg/mL (Sigma P4333)  
Stored in 0.4 mL aliquots |
| Percoll                     | Percoll (Sigma P1644)  
Stored in 1 mL aliquots |
| D-Glucose                  | 1M stock solution                                                         |
| CaCl2 dihydrate             | 1M stock solution                                                         |
2.2.7.5 Luciferase assay

1 x 10^6 HEK293T cells were seeded into wells of a 6 well plate 24 hours prior to transfection. After 24 hours, 1 µg of plasmid DNA was transfected according to the Lipofectamin2000 protocol from Thermo Fisher Scientific (https://assets.thermofisher.com/). After 2 days of incubation, cell supernatants were harvested, centrifuged at 7500 g for 2 minutes and used directly for the luciferase assay.

Steady-Glo Luciferase Assay was used to quantify the amount of GLUC present in the supernatant of transfected cells. The assay was performed according to the protocol from Promega Steady Glo Luciferase Assay System (promega.de). The measurement occurred with the luminometer and values were compared with the reporter plasmid containing the P2A sequence.
3. Results

3.1 Substitution of potentially cleavage-involved residues of gG2

3.1.1 Arginine to alanine substitution leads to partial cleavage inhibition

To identify the cleavage site (CS) and address its functional relevance during infection, we cloned the WT HSV-2 US4 gene encoding for gG2 in a mammalian and insect cell expression plasmid (Figure 8). We also generated a construct termed recombinant soluble gG2 (rSgG2), containing residues from 33 to 444, lacking residues from 445 to 673 of the ectodomain (MgG2/ED), the transmembrane and cytoplasmic domains (TMB and CD, respectively). All the constructs in the mammalian expression plasmid contain an N-terminal V5-tag located downstream of the HSV-2 signal peptide (SP, residues 1-32) and a C-terminal HA-tag. The constructs used in the baculovirus expression system contain honeybee melittin (HM) signal peptide, followed by the
N-terminal histidine tag (His). Additionally, this construct has a C-terminal HA-tag.

Fig. 8 Schematic overview of the basic rgG2 constructs. For cleavage site identification and mutagenesis. SP: HSV-2 signal peptide; V5 = N-terminal peptide V5 tag; HM = honeybee melittin signal peptide for Baculovirus expression system; His = N-terminal peptide His tag; SgG2 = soluble domain of gG2; CS = predicted cleavage site; MgG2 = membrane bound portion of gG2; TMB = transmembrane domain; CD = cytoplasmic domain; HA = C terminal peptide HA tag.

As previously mentioned, the studies by Liljeqvist and colleagues suggest that gG2 is cleaved between Arg321 and Ala322 along with Arg342 and Leu343\(^273\) (Figure 9), generating a soluble N-terminal fragment that is released to the supernatant of infected cells and a type I transmembrane protein termed MgG2 that remains on the plasma...
membrane\textsuperscript{272}. Figure 9 shows amino acid sequence 309 to 348, containing the residues predicted to be involved in gG2 cleavage. Additionally, the cleavage occurs in the extracellular N-terminal region of the full-length gG2, between the SgG2 and MgG2/ED region (termed as CS and later H2S).

\begin{verbatim}
   CCG TAC GCC CCC TTC CCA CCC CGC CCG CGG TTT CGA CGC GCC CTG CGG ACA
   309 - P Y A P F P P R P R F R R A L R T
   GAC CCC GAG GGG GTC GAC CCC GAC GTT CGG GCC CCC CGA ACC GGG CGG CGC CTC
   D P E G V D P D V R A P R T G R R L
   ATG GCC TTG ACC GAG
   M A L T E - 348
\end{verbatim}

Fig. 9 Potential cleavage site sequence. The region of interest spanning 90 base pairs [red letters] encoding for 30 amino acids [blue letters]. Potential cleavage sites according to R. Courtney are marked with a red box\textsuperscript{272}

Initially, we mutated R321 and R342 independently and in combination, based on the previous publications\textsuperscript{273}. Additionally, we changed all arginines near the cleavage site that might be involved in cleavage as well. In the beginning, we considered mutating only all arginines since past studies reveal complications in the expression of the protein as a
result of a few mutations. These regions were termed, depending on the cleavage site position, region “2” referred to the residues Arg321Ala322 and the region “0” referring to the amino acids Arg342Leu343.

The transfection of the parental and mutated constructs in 293T cells resulted in different expression patterns. As shown in figure 10, the cleaved and secreted N-terminal portion of rSgG2-SDM0+2 was slightly reduced compared to the other constructs and the WT-SgG2 (SN blot, figure 10). Additionally, a significant difference in the supernatant was seen in the appearance of the larger band around 90 kDa (rSgG2-SDM0+2, Figure 9). This band was detected by both anti-V5 and anti-HA tag antibodies, suggesting that this product represents the uncleaved rSgG2 including the short HA-tagged ectodomain. Nevertheless, the insertion of these mutations reduced but did not inhibit the cleavage. In case of the cell lysate, cleaved products tagged with V5 were seen only in WT-rSgG2, rSgG2-SDM0 and rSgG2-SDM2. However, the double mutant rSgG2-SDM0+2 did not show any bands similar to the single mutants and WT (CL blot, figure 10 B). Another
difference could be emphasised regarding the additional band spotted in single and double mutants compared to the parental construct around 90 kDa, which indicated a delayed cleavage and therefore a weaker processing efficiency in the presence of cleavage mutations. This phenotype could not be seen in the WT sample (CL blot, single green band around 90 kDa, figure 10 B). Mutation of Arg321, Arg342 and all additional arginines did not inhibit cleavage and release of the N-terminal domain SgG2 to the supernatant.
Fig. 10 Site-directed mutagenesis of region 2 and 0. (A) Schematic representation of WT-rSgG2 construct, the amino acid sequence spanning the predicted cleavage site (WT-CS) and the ones present in the mutated gG2 constructs SDM0, SDM2 and SDM0+2. (B) Western blot of HEK293T cells transfected with WT-rSgG2, SDM0, SDM2 and SDM0+2 (two clones per plasmid shown). Antibodies used: αV5 (green;1:1000) and αHA (red;1:2000) in the supernatant and αV5 (green) in the cell lysate. Cleaved and secreted SgG2 runs at ~40kDa [*]; uncleaved product running at ~90kDa [**]. Bottom blot (CL): cleaved N-terminal gG2 portion [at 40 kDa]. Retained SgG2+MgG2 construct in SDM0, SDM2, and SDM0+2 resulting in ~90kDa product [additional band at 90 kDa]. Almost no cleaved product in the double mutant SDM0+2 [40 kDa]
3.1.2 Cleavage site region residues to alanine substitution lead to partial inhibition of cleavage in rSgG2 constructs

Since the arginine-to-alanine mutations only partially inhibit the cleavage of SgG2, we substituted according to R. Courtney, the Arg321/Ala322 and Arg342/Leu343, and we also substituted stepwise the flanking residues with alanine. The residues were changed as followed for the upstream cleavage region 2: Phe319→Ala319, Arg320→Ala320, Arg321→Ala321, Ala322→Gly322, Leu323→Ala323, Arg324→Ala324. The downstream cleavage region 0 was mutated as followed: Gly340→Ala340, Arg341→Ala341, Arg342→Ala342, Leu343→Ala343, Met344→Ala344. A schematic representation of the mutated residues is shown in figure 11 A.
<table>
<thead>
<tr>
<th>WT-rSgG2</th>
<th>309PYAPF1PPPRFRRALRTDPEGVDVPRAPRTGRER</th>
<th>MALTE348</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) rSgG2-CSKO-0/4</td>
<td>314</td>
<td>-------</td>
</tr>
<tr>
<td>2) rSgG2-CSKO-2/4</td>
<td>314</td>
<td>-------</td>
</tr>
<tr>
<td>3) rSgG2-CSKO-0+2/4</td>
<td>314</td>
<td>-------</td>
</tr>
<tr>
<td>4) rSgG2-CSKO-0/6</td>
<td>314</td>
<td>-------</td>
</tr>
<tr>
<td>5) rSgG2-CSKO-2/6</td>
<td>314</td>
<td>-------</td>
</tr>
<tr>
<td>6) rSgG2-CSKO-0+2/6</td>
<td>314</td>
<td>-------</td>
</tr>
</tbody>
</table>

**Fig. 11 Western Blot of cleavage deficient rSgG2 constructs.** (A) Schematic presentation of the rSgG2 construct tagged with V5 and HA; overview of inserted mutations in the cleavage site region called as mentioned in the figure. (B) Western blots represent supernatant and cell lysate of transfected HEK293T cells. Blots were stained with αV5 (1:1000). Abbreviations: SN, supernatant; CL, cell lysate
The substitution of potentially involved residues (Phe319 to Arg324 and Gly341 to Met344) did not inhibit the cleavage. The observations seen in figure 11 B is comparable to the phenotype, as seen in figure 10 B. Therefore, further investigations regarding most-farthest C-terminal residue identifications through the mass spectrometry analysis are required.

3.2 Production of recombinant SgG2 for mass spectrometry analysis

3.2.1 Baculovirus Expression System led to high quantities of rSgG2

Due to the previous results, we decided to use a different approach to identify the cleavage site since the initial mutations did not inhibit or reduce the cleavage to a high degree at least. Therefore, the mass spectrometry identifies the most farthest C-terminal amino acids present in the cleaved SgG2 domain of the rSgG2. This should provide information on the residues that are involved in the cleavage. To obtain sufficient amounts of purified rSgG2, we took advantage of the baculovirus expression system available in the laboratory. As previously
mentioned, the 1.4 kB insert coding for SgG2 and a small C-terminal portion of the ectodomain (MgG2) was cloned into the pFastBacMel containing an N-terminal His-tag (Figure 12). This system allowed the introduction of the gene of interest into a bacmid coding for the baculovirus. This method allowed the expression of the protein of interest in high quantity. After expression, concentration and purification, the SgG2 construct was used for mass spectrometry analysis.
Fig. 12 Schematic overview of the insect cell expression vector pFastBacMel. The vector contains the gene of interest SgG2 (red), N-terminally flanked by a His-tag (green) and HM signal peptide.

The construct represented in figure 12 was used for the transformation of DH10Bac bacteria, which contain the bacmid coding for the baculovirus. The gene of interest was flanked by the upstream and downstream Tn7 transposon regions that allowed to target the mini-attTn7 site within the baculovirus shuttle vector bacmid. Additionally, the transfer and
recombination were enabled through the helper plasmid of the DH10Bac bacteria. Bacterial clones were cultivated and then plated onto selective LB plates. The additional supplement of X-Gal and IPTG allowed the differentiation between positive (successfully inserted gene = white), and negative clones (no inserted gene = blue), where the LacZ gene coding for β-Galactosidase was or was not disrupted, respectively. Positive clones were cultivated, and the bacmid was isolated for transfection of Hi5 insect cells. The supernatants of these cells were taken, and western blot was performed to detect His-tagged protein (Figure 13).
Fig. 13 Supernatant and cell lysate of rSgG2-His transfected adherent Hi5 insect cells. Different band sizes of rSgG2 are the result of different glycosylations during expression in Hi5 insect cells (CL). rSgG2 was detected with αHis (1:1000) antibody.

The harvested supernatant of P2 dishes containing the baculovirus was used for the infection of Hi5 suspension cells to produce rSgG2 in a large quantity. The duration of infection was based on the previous observations\textsuperscript{255,253}. Based on these results, we decided to harvest the supernatant containing rSgG2 between 72 and 84 hours. The obtained and cleared supernatant was used for the filtration and purification (see section 2.2.5.3 Recombinant SgG2 production and purification).
Fig. 14 SDS-PAGE of filtered and purified rSgG2. The gel shows different steps of the purification procedure. Hi5 suspension cell supernatant was centrifuged, filtered, concentrated and incubated with Nickel beads to capture the His-tagged rSgG2. These beads were treated with increasing concentrations of imidazole, which is separated in 3 fractions. The highest yield was obtained at 250 mM and fraction 1.

The concentration of the rSgG2 eluted with 250 mM was >5µg/mL based on the BSA standard curve and ImageJ quantification (data not shown).

3.2.2 Mass spectrometry analysis identified Pro317 and Phe319 as potential cleavage sites

Three samples of purified SgG2 separated in an additional SDS gel were handed to the Toxicology department (led by Prof. Pich, MHH). Prior to MALDI-TOF analysis, all samples were treated with different proteases to produce small SgG2 peptides. These peptides enable to identify the
last amino acids of the farthest highly abundant C-terminal peptide of rSgG2. In this experiment, three different digestions were applied using two peptidases. Initially, the peptidase AspN was applied onto the purified SgG2 sample. This peptidase was chosen since its number recognition site (usually N-terminal of an aspartic acid) is low. Figure 15 shows the abundance of specific peptides after digestion with peptidase AspN.

AspN digestion of the rSgG2 protein construct

NSDVVFPGGS PVAQCYAYP RLDDPGFLGS ADAGRQDLPR RVVRHEPLGR SFLTGLVLL
APPVRGFGAP NATYAARVTY YRLTRACRQP ILLRQYGGCR GGEPPSPKTC GSYTYTYQGG
GPPTYALVN ASLLVPIWDR AAETFYEQIE LGGELHVGLL WVEVGGEGPG PTAPPQAARA
EGGPCVPPVP AGRPWRVSPPV WWSAEPNGF RGLRFRESCL PPRQTAAPSD LPRVAFAPOS
LLVGITGRTF IRMARPTEDV GVLPPHWAPG ALDGPYAPF PPRPRFRRAL RTDPEGVDPD
VRAPRTGRRL MALTEDTSSD SPTSAEKTP LPVSATAMAP SVDPSAEPTA PATTPPDEM
ATQAATVAVT PEETAVASPP ATASVSSPL PAAAAATPG AHTNTSSASA A

Fig. 15 rSgG2 amino acid sequence coverage after AspN digestion and MALDI TOF analysis. Bold letters show coverage of detected peptides. Red letters represent the predicted cleavage site, according to R. Courtney272. Phe319 [blue letter F] is according to the analysis, the last amino acid of the farthest C terminally located peptide and where cleavage probably occurred according to this analysis.
After digestion, SgG2 was used for MALDI-TOF analysis, and peptides with high sequence coverage were identified (Figure 15). The results suggest that cleavage occurs before the suggested cleavage site from R. Courtney\textsuperscript{273}. This result suggested that cleavage occurs two amino acids upstream of Arg321 and Ala322, in Phe319 (compare putative cleavage site Arg321Ala322 [red] and Phe319 [blue] in figure 15).

**Trypsin digestion of the SgG2 protein construct**

\[
\text{NSDVFPGGS PVAQCYAYP RLDDPGPLGS ADAGRQDLPR RVVRHEPLGR SFLTGGVLL}
\]

\[
\text{APPVRGFAGP NATYAARVTY YRLTRACRQP ILLRQYGCCR GGEPPSPKTC GSYTYTYQGG}
\]

\[
\text{GPPTRYALVN ASLLVPIWDR AAETFYEQIE LGGELHVGLL WVEVGGEGPG PTAPPQAARA}
\]

\[
\text{EGGPCVPPVP AGRPWRSVPP VWYSAPNPGF RGLRFRECL PPQTPAAPSD LPRVAFAPQS}
\]

\[
\text{LLVGITGRGF IMARP TEDV GVLPPHWAPG ALDDGPYAPF PPRPERRAL RTDPEGVDPD}
\]

\[
\text{VRAPRTGRRL MALTEDTSSD SPTSAEKTP LPVSATAMAP SVDPSAEPTA PATTTPDEM}
\]

\[
\text{ATQAATVAVT PEETAVASPP ATASVESSPL PAAAAATFGA GHTNTSSASA A}
\]

Fig. 16 rSgG2 amino acid sequence coverage after Trypsin digestion and MALDI-TOF analysis. Bold letters show coverage of detected peptides. Red letters represent the putative cleavage site, according to R. Courtney\textsuperscript{273}. Arg335 [yellow underlined letter R] was according to the analysis the last amino acid of the farthest C-terminally located peptide and where cleavage probably occurs according to this analysis.
As a standardised digestion Trypsin was used compared to the AspN digestion. Trypsin cleaves at the carboxyl site of lysine and arginine, and the latter amino acid is highly present within the predicted cleavage region of SgG2. Therefore, this digestion produced a large number of peptides covering more amino acids than the AspN digestion revealing that the farthest identified peptide was Arg335 instead of Phe319 (underlined Arg335 [yellow] in figure16), although this latter residue was also identified.
Trypsin and AspN digestion of the SgG2 protein construct

NSDVFPFGGS PVAQCYAYP RLDDPGFLGS ADAGRQDLPR RVVHERPLGR SFLTGLVLL
APPVRGFAGP NATYAARVTY YRLTRACRQP ILLRQYYGCR GGEPPSPKTC GSHTYTYQGG
GPPTRYALVN ASLLVPIWDN AAETFHEYQIE LGGELHVGLL WVEVGGEGPG PTAPPQAARAA
EGGPCVPPVP AGRPWRSVPP VWYSAPNPFG RGLRFERCL PPPQTPAAPSD LPRVAFAPQS
LLVGITGRTF IRMARPTEDV GVLPPHWAAP ALDDGPYAPF PPRPFRRAL RTDPEGVDPD
VRAAPTGRRL MALTEDTSSD SPTSAPEKTP LPVSATAMAP SVDPSAEPTA PATTTPPDEM
ATQAATVAVT PEETAVASPP ATASVESSPL PAAAAATPGA GHTNTSSASA A

Fig. 17 rSgG2 amino acid sequence coverage after Trypsin and AspN digestion and MALDI TOF analysis. Bold letters show coverage of detected peptides. Red letters represent the putative cleavage site, according to R. Courtney\textsuperscript{272}. Phe319 is according to the double digest and MALDI-TOF analysis the last amino acid of the farthest C-terminal located peptide and where cleavage probably occurs.

The final digestion was performed with both peptidases together. The combined usage of AspN and trypsin showed the same farthest C-terminal peptide that ends before Phe319 (see blue Phe319 in Fig. 17).
Phe319 is according to the double digest and MALDI-TOF analysis the last amino acid of the farthest C-terminal located peptide and where cleavage probably occurs. In summary, the individual and combined digestion defined phenylalanine at 319 as a potential cleavage site for gG2. Beside Phe319, Pro317 was suggested through its presence in the mass spectrometry results as a potential cleavage site, too.

### 3.3 Cleavage is highly inhibited through deletion or glycine-serine replacement of the cleavage site region from Pro314 to Leu343

The presence of mutations in the area of both cleavage sites, included in the single and double CSKO and SDM constructs, did not inhibit the cleavage of rSgG2 nor its expression. Here again, the mutation of both suggested cleavage sites led to a partial cleavage inhibition that resulted in expression and secretion of two major bands. Both constructs, SDM0+2 and CSKO0+2, showed the presence of the cleaved SgG2 and the uncleaved SgG2+MgG2 portion (SN blot at ~90 kDa uncleaved and 40 kDa cleaved, see figure 10 B and 11 B). This confirms at least the
importance of both sites for cleavage but also indicates that they are not the only cleavage sites in gG2.

The following step includes a more harsh way to influence the cleavage in SgG2 and MgG2. One of the approaches was based on the mass
spectrometry results. According to these results, Phe319 and Pro317 could be involved in the cleavage. Therefore, mutations of these residues and its upstream residue Arg316 and downstream residue Arg318 were included (CSKO0+2/6+3AA). We also generated two constructs, where we removed the sequence from residue Pro314 to Leu343 (ΔCS) and replaced it with a glycine-serine motif (GSm) (Figure 18 A). Glycine-serine motifs are commonly used to substitute protein regions with flexible linkers that maintain correct protein folding\textsuperscript{290}.

Expression of these constructs in HEK293T cells showed that the CSKO0+2/6+3AA construct was cleaved and a protein of 90 kDa was released to the supernatant, as seen before for SDM0+2, CSKO0+2/4 and CSKO0+2/6 (SN of figure 18 B compared to figure 10 B and figure 11 B). However, the replacement (GSm) or knockout (ΔCS) of the cleavage site led to the expression of one dominant band of 90 kDa in the supernatant. Moreover, the cleavage and release into the supernatant of the N-terminal SgG2 40 kDa was highly reduced compared to the WT-SgG2 (see SN blot, at 40 kDa in figure 18 B). Within the cell lysate,
the ΔCS and GSm variant show additionally the absence of cleaved SgG2 compared to the WT-rSgG2 (see CL blot, at 40 kDa in figure 18 B). Summarised, the removal (ΔCS) or the replacement with GSm of the cleavage site from Pro314 until Leu343, spanning 30 residues, resulted in a drastic reduction of the cleaved N-terminal SgG2.

Fig. 19 Western Blot of cleavage deficient rFLgG2 constructs. (A) Schematic presentation of the WT rFLgG2 and the cleavage deficient constructs rFLgG2ΔCS and rFLgG2GSm tagged with V5 and HA. Overview of inserted mutation in the cleavage site region called as mentioned in the figure. (B) Western blots represent supernatant and cell lysate of transfected 293T cells used for migration assay. Blots were stained with αV5 (1:1000) and αHA (1:2000). Abbreviations: SN, supernatant; CL, cell lysate.
We decided to confirm the results obtained in the context of the rSgG2GSm and rSgG2ΔCS construct in the background of full-length gG2 (rFLgG2). Therefore, the missing part of the ectodomain of MgG2 (residue 445 to 673), the transmembrane domain and the cytoplasmic domain were cloned together with the cleavage site mutations.

Expression of rFLgG2 resulted in cleavage and release of SgG2 (40 kDa; residue 33 to 321) to the supernatant of 293T transfected cells, similar to the results obtained when the rSgG2 was used (see figure 19 B). The expression pattern observed in the cell lysate is different for rSgG2 and rFLgG2. The different sizes of rFLgG2 were due to different construct, i.e. rFLgG2 has the full ectodomain (see yellow bands in CL in figure 19 B). A similar band pattern was also observed in the constructs containing the GS motif and the ΔCS (see yellow bands in CL blot figure 19 B), except for the V5 positive band at around 40 kDa, which was missing in the GSm and ΔCS constructs. This indicates that cleavage did not take place. The cleaved protein of about 40 kDa was detected in the supernatant of cells transfected with the rSgG2 and FLgG2, as expected.
However, this protein was not detected in the supernatant of cells transfected with the GSm and ΔCS constructs, again indicating that cleavage was abolished. A 90 kDa band detected by the anti-HA antibody (red) in the supernatant corresponds to the uncleaved gG2, which was prominent in the cells transfected with the ΔCS and GSm variant. These results indicate that the cleavage in the context of the full-length gG2 was inhibited. The 90 kDa protein was also positive for V5, although the signal intensity was much weaker. This 90 kDa protein was not detected at all in the sample from cells transfected with the FLgG2, due to the cleavage of the protein. The detection the 90 kDa in the supernatant was not expected since the HA tag is located in the cytoplasmic tail of gG2. Therefore, this result suggests that the full-length gG2 containing the transmembrane domain was released when cleavage was inhibited (see SN blot, red signal in figure 19 B).
3.4 Identification of proteases involved in cleavage

3.4.1 Furin protease is not involved in the cleavage of gG2

Based on these results and recommendations from Prof. Pich and Dr. Polten from the mass spectrometry department, it was hypothesised that furin is involved in the processing and cleavage of gG2. To support these mass spectrometry results, the ProP 1.0 furin cleavage prediction tool analysed the amino acid sequence from rSgG2\textsuperscript{274}. The comparison with the initial results from R. Courtney\textsuperscript{273} and the mass spectrometry analyses revealed that it recognised four cleavage sites, Arg193, Arg83 and Arg74 not mentioned in the literature regarding gG2 cleavage (Figure 20).
Fig. 20 ProP 1.0 – Prediction for propeptide cleavage sites for furin in the rSgG2. This analysis includes the small ectodomain referring to the MgG2 as well. Each blue bar crossing the threshold (0.500) predicted a cleavage site, according to ProP1.0. The highest score had the Arg321Ala322 cleavage site region. The score was written next to the predicted cleavage site residues.

At the cellular level, furin concentration is high within the Golgi apparatus. Furin belongs to the family of subtilisin-like proprotein convertase and is described as the first of its kind in 1990\textsuperscript{275,276}. Drewinko and colleagues discovered furin-deficient human carcinoembryonic antigen-producing colon carcinoma cell line (LoVo), whereas the work from Mondino identified the involvement of deficient furin indirectly, through the abrogated expression of the precursor single-chain hepatocyte growth factor and the insulin receptor\textsuperscript{277,278}. The work from Takahashi finally identified the single nucleotide polymorphism within
the homo B domain, involved in the proteolytic activity of furin within the secretory pathway\textsuperscript{279}. Usually, furin is involved in several crucial processes, and the primary mechanism of action is to cleave domains in order to facilitate the function of the proteins. For instance, the proteolytic function of furin activates bone morphogenetic protein 4 during embryogenesis\textsuperscript{280}. Furin is also involved in the cleavage of pathogen-derived proteins like anthrax protective antigen from \textit{Bacillus anthracis} or the hemagglutinin from the avian influenza virus\textsuperscript{281,282}. A common feature among these proteins is the presence of the furin consensus motif, which is characterised by the repetitive presence of arginines separated by few amino acids (X stands for any amino acid: \textit{R-X-X-R, R-X-K/R-R, and R-X-R/K-R-X-R})\textsuperscript{273}.
Fig. 21 **Potential furin consensus motifs within the SgG2 protein.** Two out of three consensus motifs were identified through manual recognition of the furin consensus motifs within the amino acid sequence. The green motif is highly abundant throughout the rSgG2 amino acid sequence.

These motifs are present in the gG2 sequence, also in the region between Arg321Ala322 or Arg342Leu343, suggesting that they may be involved in the cleavage of the gG2 protein (Figure 21). To test this, we transfected FLgG2-expressing constructs into the furin-deficient LoVo cells. The bands for the wild type FLgG2 showed typical expression within the cell lysate of LoVo cells (Figure 18 B).
Fig. 22 rFLgG2 transfection of LoVo cells. (A) Hypothetic schematic representation of intracellular non-functional furin, which is not able to process the FLgG2 protein. (B) Western blot of cell lysates from transfected LoVo cells. Full processed WT-rFLgG2, including the presence of cleaved SgG2 [~40 kDa]. gG2 variants were detected with αV5 (1:1000)

3.4.2 Batimastat inhibition of MMPs does not affect gG2 cleavage and secretion

It is not known where the cleavage of gG2 occurs. The work from Weldon et al. indicates that cleavage does not occur co-translationally but in later stages of the secretory pathway\textsuperscript{271}. Secretory pathway steps that include the Golgi are not involved in cleavage\textsuperscript{270}. Therefore, it is possible that specific cell surface anchored proteins, such as disintegrin
and metalloproteinase (ADAM), are involved in the cleavage. These proteases belong to the family of zinc protease superfamily, which contains transmembrane and secreted proteins, catalysing functions such as cell adhesion and proteolytic processing of surface receptors or signal molecules\textsuperscript{283}. The subclass sheddases, called matrix metalloproteinase (MMP) may be involved in processing of gG2 intracellularly or extracellularly. For example, MMP9 shows intracellular activity in different types of cells; e.g. in leukocytes, it is responsible for AMP-activated protein kinase α degradation, following mTOR complex 1 activation and hypoxia-inducible factor 1-α expression upon lipopolysaccharide-stimulation\textsuperscript{284}. Similarly, MMP2 also functions intracellularly during the proteolysis of troponin I that causes acute contraction defects in rat hearts\textsuperscript{285}. The first described extracellular metalloproteinase, MMP1, is involved in degradation of native fibrillar collagens to create native collagen type 1, 2 or 3, crucial for the extracellular matrix\textsuperscript{286}. MMP2 and MMP9 are involved in the extracellular activation of TGF\textsuperscript{287}. 
Fig. 23 Pre-analysis of potential ADAM proteases involved in rSgG2 cleavage (A) 
*In silico* analysis through PROSPER to identify potential MMP cleavage sites within the rSgG2. MMP2, MMP3 and MMP9 were chosen based on the potential cleaved products that result theoretically in the cleaved 40 kDa SgG2 protein. (B) Hypothetical schematic representation of FLgG2 processing.
We performed an *in silico* analysis with gG2 to narrow down potential cleavage sites for metalloproteases (Figure 23 A)\textsuperscript{288}. The amino acid sequence of the rSgG2 construct lacking the transmembrane domain was the template for this analysis. Three of the proposed MMPs produced cleavage patterns similar to the secreted gG2 product with the size of 40kDa. There is one potential cleavage site for MMP2, different from the one suggested by R. Courtney\textsuperscript{273}, located C-terminally from the second predicted cleavage site (red I, figure 23 A).

In contrast to MMP2, there are more potential hits for MMP9, which are closer to the first predicted cleavage site (Arg321Ala322) and directly within the second one (Arg343Leu344, red I, figure 23 A). The program also identified four additional recognition sites C-terminal to the second predicted cleavage site (MMP9 analysis, red I, figure 23 A). There is only one potential MMP3 cleavage site, which is the farthest potential cleavage recognition site of all three MMPs (MMP3 analysis, red I, Figure 23 A). To determine whether MMPs were involved in gG2
cleavage, we used the broad-spectrum inhibitor of MMPs, batimastat, which mimics their substrate\textsuperscript{289}.

After the treatment with increasing concentrations of batimastat (4 hours prior transfection with 0, 20, 200 and 2000 nM), we transfected 293T cells with rSgG2 tagged with V5. The IC\textsubscript{50} of batimastat is around 3-20nM\textsuperscript{289}. We harvested the supernatant and cell lysates after 24 and 48 hours of batimastat treatment and performed western blot.

Fig. 24 Batimastat inhibition assay. Western blot of supernatant and cell lysate from rSgG2 transfected HEK293T cells to detect gG2 products. The cells were treated with batimastat 4 hours prior transfection of rSgG2-V5. Then, the cells were treated with the indicated concentration of batimastat for 24 or 48 hours (h). rSgG2 was detected with αV5 (1:1000) antibody. Abbreviations: SN, supernatant; CL, cell lysate.
The presence of batimastat did not change the cleavage pattern of rSgG2 (40 kDa for the cleaved protein and 90 kDa for the uncleaved one, see figure 24) at the different time points or concentrations tested. There was also no accumulation of uncleaved SgG2 protein compared to the untreated sample. These results suggest that MMPs are not involved in gG2 cleavage.

3.4.3 **BrefeldinA inhibition did not induce unconventional protein secretion of rSgG2**

Grieve and Rabouille described a transmembrane protein that reaches the membrane through non-COPII coated vesicles or by bypassing the Golgi compartment\textsuperscript{294}. Another example of unconventional protein secretion was described in Jung \textit{et al.}, where a mutated pendrin can be rescued through Hsp70 and its co-chaperone DNAJC14\textsuperscript{295}. BrefeldinA results in the collapse of Golgi followed by membrane fusion with the ER, which in turn inhibits the secretion of proteins\textsuperscript{296}. Prior to the collapse, BrefeldinA blocks the vesicle transport and fusion between both mentioned cellular compartments\textsuperscript{297}. To initiate UPS, BrefeldinA was
applied onto rSgG2 transfected HEK293T cells for 4 hours before transfection with rSgG2. Afterwards, the cells were incubated with BrefeldinA for 24 and 48 hours. As a control, we transfected a plasmid expressing Factor IX since it is secreted through the regular secretory pathway (Figure 25).

Fig. 25 **BrefeldinA reduced rSgG2 secretion in HEK293T transfected cells.** Western blots showing the effect of BrefeldinA on the cleavage and secretion of mock, rSgG2 and Factor IX. Samples were harvested after 24 and 48 hours and the cell lysate (CL) and supernatant (SN) were checked for the presence of cleaved SgG2 with an antibody targeting V5. FactorIX was used as a secretion control and detected using αFactorIX. Abbreviations: SN, supernatant; CL, cell lysate.
Cleaved SgG2 and Factor IX were detected in the cell lysates and supernatant of non-treated cells. Addition of BrefeldinA did not impair cleavage of both proteins drastically whereas it inhibited their secretion, indicating that SgG2 is not secreted through the UPS pathway. Unfortunately, BrefeldinA also reduced the expression of SgG2 and Factor IX, especially when the drug was incubated for 48 hours, precluding us from clearly concluding that the H2S sequence is involved in secretion through the UPS pathway.

3.5 Transfer of H2S into reporter plasmids

3.5.1 The cleavage site region H2S enables separate expression of two fused proteins

Our results suggest that amino acids Arg314 to Leu343 are responsible for the cleavage and release to the supernatant of the N-terminal SgG2 domain. We then addressed whether this region spanning 30 amino acids (termed from now on H2S; Pro314 to Leu343) acts as a cleavage motif
in other proteins. We transferred this sequence into a reporter plasmid, expressing mCherry and Gaussia luciferase (GLUC) under the control of the CMV promoter. The control reporter plasmid contains open reading frames (ORFs) of mCherry and GLUC, separated by the 2A peptide (P2A) sequence. The P2A sequence appears in different viruses as a functional feature to mediate “auto-cleavage” through ribosomal skipping of polypeptides during translation. We substituted the P2A sequence by that of the H2S (see Figure 26 A for schematic representation of both reporter plasmids).
Fig. 26 **H2S cleavage sequence within the reporter plasmid pM157.** (A) Schematic presentation of the reporter plasmids containing N-terminal mCherry, P2A or H2S sequence and C-terminal GL. (B) Luciferase assay showing the signal obtained in the supernatant of cells transfected with 3 different clones containing the H2S sequence and one clone containing the P2A sequence. (C) Western blot analysis showing the detection of mCherry in the cell lysate and supernatant of the transfected cells stained with αmCherry. Abbreviations: SN, supernatant; CL, cell lysate.
The results of the luciferase assay revealed that the WT construct containing the P2A showed the highest luciferase activity (see the rose curve in figure 26 B). However, we could also detect similar high levels of luciferase in the supernatant of cells transfected with plasmids expressing the construct containing the H2S sequence instead of the P2A one (Figure 26 B). These results indicated that insertion of H2S sequence did not interfere with luciferase expression and secretion to the supernatant. We also performed western blot to detect mCherry in the cell lysate and supernatant. As shown in figure 26 C, mCherry was not detected in the cell lysate when the construct containing H2S was used. It was only detected in the supernatant running at an apparent molecular weight of about 26 kDa, similar to that of mCherry expressed from the plasmid containing P2A. Therefore, the molecular weight is identical in both situations, indicating that mCherry is not fused to luciferase when the H2S sequence is present, clearly showing that cleavage or another process leading to the independent translation of both genes occurred. The detection of mCherry in the supernatant was not expected since its
gene does not contain a signal peptide and it is expressed and translated upstream the GLUC gene, which contains a signal peptide.

To confirm that the H2S sequence allows cleavage of two proteins, and to address whether the N-terminal protein would be secreted, we cloned the H2S between 5’ enhanced green fluorescent protein (eGFP) and 3’ mCherry and compared its effect with that of P2A (Figure 27). We transfected the pGL3 plasmid into HEK293T cells and detected eGFP and mCherry. As shown in figure 27 B, both proteins expressed by the pGL3-eGFP-H2S-mC were present in different cellular locations, suggesting they were cleaved. We also addressed the expression of eGFP by western blot analysis of the cell lysate. The eGFP expressed by the pGL3-eGFP-H2S-mC was detected with specific antibodies and appeared as a single band with the corresponding size of 26 kDa, confirming the lack of a fusion protein between the two fluorophores (Figure 27 C). This indicates that the cleavable or processing activity of the H2S sequence can be transferred to another construct.
Fig. 27 H2S cleavage sequence within the reporter plasmid pGL3 (A) Schematic presentation of reporter plasmid containing N-terminal eGFP, separated by the cleavable peptide and C-terminal mCherry. (B) Immunofluorescence pictures from HEK293T cells, showing separated GFP and mCherry signals within the cells. (C) Western blot analysis shows the cell lysate and supernatant of the transfected HEK293T cells stained with αeGFP (1:2000). Abbreviations: SN, supernatant; CL, cell lysate.
The results indicated a novel phenotype, where the N-terminal mCherry was secreted despite the absence of an upstream N-terminal signal peptide (see SN blot, lane mC-H2S-GL in Figure 26 C). The same observation can be made in the context of the second reporter plasmid as well. However, eGFP was also detected in the supernatant when transfected alone, suggesting that overexpression may lead to the secretion of eGFP, despite the lack of H2S (see SN blot, lane eGFP-P2A-mCherry and lane eGFP only, in Figure 27 C).

3.5.2 *In silico* analysis led to internal alternative secretion signals inside the reporter plasmid

The results obtained with the mC-H2S-GLUC reporter plasmid suggested that H2S may induce secretion of N-terminal proteins. The H2S sequence lacks a classical signal peptide. Therefore, we hypothesised that H2S might induce secretion of N-terminal proteins through the unconventional protein secretion (UPS) pathway. Therefore we analysed the sequence of the mC-H2S-GLUC with the Secretome...
Server 2.0, which is provided by the center for biological sequence analysis CBS, University of Denmark\textsuperscript{292,293}, to determine whether this sequence has at least a signal peptide for type IV UPS.
Fig. 28 **Secretome Server 2.0 - Alternative secretion signal analysis.** Schematic representation of the reporter plasmids containing H2S and P2A. The analysis always required 40 residues. Sample 1 and 4 represent the C-terminal residues of mCherry and N-terminal residues of GLUC. Sample 2, 3, 5 and 6 contain the 10 C-terminal residues of mCherry and 10 N-terminal residues of GLUC together with the cleavage sites (H2S [orange]; P2A [purple]). The NN-score indicates the potential of a non-secretory pathway signal of the corresponding peptide (grey box). The NN-score should exceed the threshold of 0.6 and should not detect any signal peptide motifs (within the grey box, “Warning”)
Figure 28 shows the schematic representation of the reporter plasmid and the region around the cleavage site and the cleavage site itself. At least 40 residues were required for the analysis and predicted any internal alternative secretion signal. The area of the cleavage site was separated into four parts for the H2S construct and two parts from the P2A construct. The NN score represents the potential secretion signal within the analysed peptide. The peptides 1 and 4, representing C-terminal part of mCherry and N-terminal part of GLUC, respectively, show the required threshold to indicate a secretion signal. The same observations were made with the other analysed peptides, which represent the area around the cleavage site. However, the highest values were obtained from the analysed peptides containing the cleavage site (H2S) (peptide [2] 0.857; peptide [3] 0.855). It is also necessary to mention that GLUC indicates a high NN score, which also indicates a potential alternative secretion signal. However, this analysis did not detect the signal peptide from GLUC within the 40 residues. The P2A construct and its analysed peptide did not show a high NN score as seen for the H2S construct.
3.5.3 Deletion of the signal peptide in the rSgG2 construct did not indicate an unconventional secretion pathway

An additional experiment to prove whether H2S is involved in secretion of the N-terminal protein through an unconventional pathway and without a signal peptide, was the knockout of the regular gG2 signal peptide. We wanted to observe whether the presence of the H2S sequence induces secretion of the SgG2 protein independently from the signal peptide and functions as an internal signal peptide. Therefore, we removed the signal peptide from rSgG2 and addressed whether this construct would be cleaved and secreted upon transfection of 293T cells (Figure 29 A).
The results showed the expression of all rSgG2ΔSP clones within the cell lysate. The expression pattern was different in the rSgG2ΔSP clones compared to the WT-rSgG2 (compare green bands of rSgG2ΔSP and WT-rSgG2 in CL blot in figure 29 B). Intracellularly, the rSgG2ΔSP is not processed in the same manner as WT-rSgG2, resulting in a larger band at 60 kDa (see green bands in clone 1 to 5 in Figure 29 B). These results indicated that rSgG2 was cleaved in the presence of its putative signal peptide. We detected the cleaved rSgG2 N-terminus in the supernatant of the transfected cells. However, this N-terminal SgG2
domain was not secreted when the rSgG2ΔSP clones were transfected in HEK293T cells. These results clearly show that the gG2 signal peptide was required for processing and secretion of the cleaved N-terminal polypeptide.
3.6 Immunofluorescence of the wild type full-length gG2, SgG2 and cleavage deficient variants showed different patterns in expression

Another field of interest is the subcellular localisation and expression patterns of the cleavage deficient constructs and the wild type gG2. The observation of gG2 inside the cell is not well studied so far. Therefore, HeLa cells were transfected with the WT full-length gG2 (rFLgG2) and the cleavage deficient variants (rFLgG2ΔCS; rFLgG2GSm), the short version SgG2 including a small portion of the ectodomain (rSgG2) and a construct that contains only the ectodomain together with the transmembrane and cytoplasmic domains (rMgG2; see figure 30 for schematic constructs). The HeLa cells were transfected for 48 hours and fixed with 3% PFA/PBS, followed by immunofluorescence with specific antibodies. Representative pictures were chosen and shown in figure 31 to 35.
Fig. 30 **Schematic representation of gG2 constructs used for immunofluorescence.** All recombinant constructs were tagged with N-terminal V5 and C-terminal HA.
3.6.1 Expression patterns of rSgG2 and retention of the C-terminal HA-tagged domain in permeabilised HeLa cells

The red HA-tag signal in the permeabilised cells of the construct containing the rSgG2 was not detected at specific loci of the cell (HA only in figure 31). The red HA signal was rather all over the cytoplasm and around the nucleus. Additionally, the red HA signal seems to be less abundant than the green V5 signal (compare HA and V5 in figure 31). The green V5 signal was located in the perinuclear region of the cell since these accumulations were most of the time near the nucleus (V5 in figure 30). In most cells there was no co-localization of the HA and V5 signals, suggesting that cleavage had occurred.
Fig. 31 Immunofluorescence of rSgG2 transfected HeLa cells. Cells were stained with: 1:500 αV5 (green channel), 1:1600 αHA (red channel), 1:1000 DAPI. Representative dsRed and GFP channel area (white frame) were chosen and shown as single channels underneath. Scale (red line) = 100 μm. Cells were magnified 40 times and visualised in Observer Z1 Axio microscope.
3.6.2 Expression patterns and localisation of both signals inside rFLgG2 permeabilised HeLa cells

The detailed observation of the rFLgG2 tagged with N-terminal V5 and C-terminal HA showed additional differences compared to the previous short construct. As previously mentioned, the full length is made of the SgG2 and the full ectodomain (MgG2) containing the transmembrane domain and short cytoplasmic domain. This construct showed partial coexistence of both terminal domains (merged figure 32). However, in most cells, there was no co-existence between the V5 and HA signals.
Fig. 32 Immunofluorescence of rFLgG2 transfected HeLa cells. Cells were stained with 1:500 αV5 (green channel), 1:1600 αHA (red channel), 1:1000 DAPI. Descriptive dsRed and GFP channel area (white frame) were chosen and shown as single channels underneath. Scale (red line) = 100 µm. Cells were magnified 40 times and visualised in Observer Z1 Axio microscope.
3.6.3 Co-localization of V5-tagged N-terminal and HA-tagged C-terminal domain in FLgG2ΔCS and rFLgG2GSm permeabilised HeLa cells

Both V5 and HA signals show co-existence in cells transfected with the FLgG2ΔCS and rFLgG2GSm, indicative of lack of cleavage (see V5 and HA in figure 33 and figure 34). Moreover, both signals were visible in the whole cell compartment, which was different from the wild type variants (compare figure 33 and 34 with figure 31 and 32).
Fig. 33 Immunofluorescence of rFLgG2ΔCS transfected HeLa cells. Cells were stained with 1:500 αV5 (green channel), 1:1600 αHA (red channel), 1:1000 DAPI. Descriptive dsRed and GFP channel area (white frame) were chosen and shown as single channels underneath. Scale (red line) = 100 µm. Cells were magnified 40 times and visualised in Observer Z1 Axio microscope.
Fig. 34 **Immunofluorescence of rFLgG2GSm transfected HeLa cells.** Cells were stained with: 1:500 αV5 (GFP channel), 1:1600 αHA (dsRed channel), 1:1000 DAPI. Descriptive dsRed and GFP channel area (white frame) were chosen and shown as single channels underneath. Scale (red line) = 100 μm. Cells were magnified 40 times and visualised in Observer Z1 Axio microscope.
### 3.6.4 Characterisation of the MgG2 construct in permeabilised HeLa cells

In cells transfected with the MgG2 construct, which should also lack a cleavage sequence, the V5 and HA tag were separated from each other. The red HA-tagged signal was rather concentrated and probably located in the perinuclear region (compare HA and V5 with the merged channel, in Figure 35). The green V5 signal was present and diffuse in the whole cell compartment (V5 channel, Figure 35).
Fig. 35 Immunofluorescence of rMgG2 transfected HeLa cells. Cells were stained with 1:500 αV5 (green channel), 1:1600 αHA (red channel), 1:1000 DAPI. Descriptive dsRed and GFP channel area (white frame) were chosen and shown as single channels underneath. Scale (red line) = 100 µm. Cells were magnified 40 times and visualised in Observer Z1 Axio microscope.
3.7 Terminal and internal deletions of rSgG2 do not lead to lack of GAG-binding

GAGs are important for chemokine immobilisation and presentation onto the endothelial cells. This mechanism facilitates the generation of a gradient to counteract the bloodstream and enable leukocyte migration towards the damaged or infected tissue\textsuperscript{180,181}. Moreover, GAGs are important for viral attachment and for the envelope glycoproteins to approach their corresponding receptors. As previously mentioned, the lack of GAGs leads to less viral infection compared to the infection of GAG-positive Chinese hamster ovary derived cells\textsuperscript{13–15,17}. Several vCKBPs bind to GAGs as well, inhibiting the chemokine gradient formation\textsuperscript{237,240,241}. HSV gG is known to enhance chemokine function by increasing local chemokine concentration in the proximity of the receptor and by modifying GPCR biology. Therefore, we hypothesise that the ability of gG to bind to cells through GAGs is important for gG activity. Thus, to understand how important the GAG-binding domain is for gG function \textit{in vitro} and \textit{in vivo}, we attempted to identify the GAG-binding
domain. Therefore, we deleted initially 10 and 20 residues at the N-terminus and C-terminus of gG. Then each terminal deletion was combined together. Despite the aberrant expression of some gG2 constructs, we performed GAG pull-down assays (see method section 2.2.7.1). The results revealed that deletion of either terminus did not affect the GAG binding of SgG2 (see figure 36 B). However, only the construct rSgG2ΔC20 shows lack of binding during the heparin-binding assay compared to the input.
Fig. 36 **Heparin-binding Assay of N- and C-terminal deleted rSgG2.** (A) Schematic presentation of N- and C-terminal deleted rSgG2. (B) Top blot shows the input of deletion construct and the bottom blot shows the results of the heparin-binding assay. Blots were stained with αHis (1:1000). Abbreviations: SN, supernatant; CL, cell lysate.
Therefore we decided to delete intramolecular, i.e. sequential deletion of 20 residues per construct starting from the N-terminus and ending at the C-terminus (Figure 37 A and B). Transfection of 12 constructs into HEK293T cells resulted in expression as shown by western blot of cell lysates (Figure 37 C). However, SgG2 was detected in the supernatant only for constructs 5, 9, 11 and 12, indicating that cleavage and secretion occurred. No secreted protein was detected in the supernatant of the other clones, despite their presence in the cell lysate (see figure 37 C).
Fig. 37 Schematic representation and Western Blot analysis of sequentially deleted rSgG2. (A) Deletion constructs lacking 20 residues each, starting from the N-terminus to the C-terminus. (B) rSgG2 amino acid sequence. Blue sequence represents the already deleted constructs (see figure 36). The yellow sequence represents the cleavage site. The green numbered section represents the sequentially deleted rSgG2. (C) rSgG2 expression of 12 different sequential deleted constructs in HEK293T cells. Blots represent supernatant and cell lysate. Blots were stained with αV5 (1:1000) and αHA (1:2000). Abbreviations: SN, supernatant; CL, cell lysate.
3.8 The absence of the cleavage site region reduced the chemokine-enhancing activity compared to the wild type SgG2

The following experiment focussed on the functional relevance of SgG2 cleavage, i.e. is cleavage necessary for enhancing chemokine activity? As previously mentioned, gG2 was the first described vCKBP to enhance chemokine activity. The studies revealed several binding partners and increasing activity for different chemokines, like hCXCL12β that increased leukocyte migration in the presence of rSgG of HSV-1 and HSV-2\(^{251}\). Since gG from HSV-2 is secreted, and gG from HSV-1 is not, we attempted to determine whether the cleaved protein (rSgG2) had different function as the non-cleaved one (rSgG2ΔCS; rSgG2GSm).
Fig. 38 **Schematic presentation of the plate used in the migration assay.** Supernatant with or without chemokine was pipetted into the well. The drop was then covered by a membrane containing pores with a determined size. On top of the membrane, $1.25 \times 10^5$/mL leukocytes were pipetted and incubated. When the leukocytes detect the chemokine they migrate to the bottom chambers. The number of migrated leukocytes was determined by measuring an enzymatic activity and comparing the results to those obtained with known cell numbers.
To determine the functional activity of these constructs we transfected HEK293T cells with rSgG2, SgG2ΔCS and SgG2GSm, collected the supernatant 3 days post transfection and incubated them with chemokine in the bottom chamber of the transwell plate. Following incubation, Jurkat T cells were added to the top chamber and incubated at 37°C (see materials and methods for more details). These constructs were compared with chemokine alone (hCXCL12β), mock-transfected 293T cells plus chemokine and WT-SgG2 alone (Figure 38).

![Standard curve of JURKAT cells.](image)

Fig. 39 **Standard curve of JURKAT cells.** Cells per mL (X-axis) and absorbance (Y-axis). The formula of the linear regression and $R^2$ within the graph

$y = 0.3943x + 0.2555$

$R^2 = 0.9817$
After 1 hour of incubation, the membrane was washed with PBS to stop further migration of Jurkat T cells. Prior to measurement, a dilution series of Jurkat T-cells was applied into empty wells starting with $5 \times 10^6$ cells/mL. This cell number was continuously diluted 1:2 until it reached the dilution 1:128. This dilution series served as a standard curve for the calculation of migrated Jurkat T cells in the presence of different conditions (Figure 40 A). The measured values were transferred to the graph shown in figure 39. Based on the formula shown in the graph, the number of migrated cells in the presence of gG2 variants with or without chemokine was calculated, using the absorbance value. The chemokine concentrations used were 0 nM and 1 nM. Figure 40 shows the number of migrated Jurkat T cells following incubation with supernatants obtained from transfected HEK293T cells incubated with 1 nM and 0 nM hCXCL12β.

As previously described, SgG2 enhanced hCXCL12β activity (Figure 40 A). The direct comparison with WT-rSgG2 and rSgG2ΔCS, lacking the cleavage site domain indicated a lower enhancement compared to the
WT-rSgG2, whereas no differences could be seen between rSgG2ΔCS and rSgG2GSm. The direct comparison of WT and rSgG2ΔCS construct indicated an enhanced influence on migration of Jurkat cells when compared to hCXCL12β alone. The comparison of the hCXCL12β alone, together with rSgG2GSm indicated no differences in both samples. This result led to the partial conclusion that the absence of the cleavage site negatively affects the chemokine-enhancing activity of gG2, despite higher levels of gG2 protein levels (Figure 40 B).
Fig. 40 Migration assay of JURKAT cells in the presence of HEK293T cells transfected supernatant. Clones used: WT-rSgG2, cleavage deficient variants in the presence of CXCL12β, CXCL12β alone, WT-rSgG2 alone and Mock transfected. (A) Graph showing columns for the number of cells that migrated towards supernatant of HEK293T transfected cells and 1 nM and 0 nM CXCL12β, respectively. Measurements were done in triplicates. The experiment was performed once. Significance: *P<0.05; **P<0.005; ***P<0.0005. ns = not significant. Error bars represent standard deviation. (B) Western blot represents supernatant of transfected 293T cells used for migration assay. 1) rSgG2; 2) rSgG2ΔCS; 3)rSgG2GSm Blots were stained with αV5 and αHA
The absence of the cleavage site region enhances the neurite outgrowth of disassociated dorsal root ganglion derived neurons in microfluidic chambers

Next, we aimed at investigating the influence of the cleavage site in the enhancement of NGF-dependent neurite outgrowth. To this end, we used dissociated mouse dorsal root ganglion (DRG) neurons. Therefore, mice were decapitated after CO\textsubscript{2} sedation, and the spinal column was extracted from each mice to harvest DRGs. After disassociation, 5 x 10\textsuperscript{3} neurons were seeded into the somal well of microfluidic chambers (MFC; Figure 41). The use of MFC allows the separation of the cell bodies and neurites of the sensory neurons in two chambers connected by microgrooves of 450 \textmu m. A volume gradient was generated to induce neurite outgrowth towards the neurite chamber. Since NGF was the only neurotrophic factor used most of the neurites correspond to NGF-dependent neurons.
Fig. 41 **MFC experimental setup.** DRGs were dissected and disassociated from mice. After disassociation, neurons were seeded to the somal site and incubated for 1 day. The next day, transfected HEK293T cells either containing rFLgG2, rSgG2, rFLgG2ΔCS or rFLgG2GSm were seeded into to the axonal site. Both cell types were incubated for 2 days, depending on the neurite outgrowth. After incubation, cells were fixed and stained with Tuji1 (neuronal marker) and DAPI. The MFCs were analysed with immunofluorescence.

HEK293T cells express repellents of neurite outgrowth such as class A ephrins and semaphorin3A\(^{47}\). Incubation of neurites with HEK293T cells or supernatant from HEK293T cells inhibits neurite outgrowth (Kropp *et al.* 2021).
al., manuscript in preparation). To determine the role of HSV-2 gG during infection, I generated a recombinant HSV-2 lacking gG expression in the bacterial artificial chromosome (BAC)-containing MS strain, by deleting the start codon, introducing a stop codon and a frameshift together with an XhoI restriction site, which allowed to control the insertion of the mutations via digest (unpublished work, MSc Thesis^298).

<table>
<thead>
<tr>
<th>AAG</th>
<th>CACGCC</th>
<th>TAG</th>
<th>G</th>
<th>CTCG-AG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Start codon deletion</td>
<td>-</td>
<td>Stop codon</td>
<td>-</td>
<td>XhoI restrictions site + frameshift</td>
</tr>
</tbody>
</table>

Fig. 42 Introduced mutations into the US4 gene encoding for gG2 of HSV-2. Mutations abrogate expression of gG2 during HSV-2 infection.

Then, a reporter cassette expressing mCherry and GLuc under the control of the HCMV promoter was inserted into the intergenic locus between UL55 and UL56. Lack of gG2 expression did not affect HSV-2 growth in HEK293T cells. Infection of HEK293T cells with parental, reporter HSV-2 strain MS, reduced the inhibitory effect of HEK293T cells towards neurite outgrowth (Kropp et al., manuscript in preparation,
figure 43). However, HEK293T cells infected with the HSV-2ΔgG virus inhibited neurite outgrowth as efficiently as the mock-infected ones. These experiments show that HSV-2 infection of HEK293T cells reduced their inhibitory effect on neurite outgrowth. Moreover, they suggest that gG2 is important for this activity. To confirm the relevance of gG2, we transfected HEK293T cells with rSgG2 or FLgG2 prior to infection with HSV-2ΔgG virus. These transfected and infected cells were incubated with neurites from DRG neurons, and neurite outgrowth was measured as explained in Materials and Methods. As shown in figure 43, SgG2 and FLgG2 complemented in trans the lack of gG2 expression during infection with HSV-2ΔgG. The presence rSgG2 or rFLgG2 in HEK293T cells infected with HSV-2ΔgG leads to longer neurite length compared to HSV-2ΔgG without complementation. Therefore, our results show that HSV-2 gG2 is required to inhibit the repelling effect of HEK293T cells.
Fig. 43 Neurite outgrowth experiment HSV-2 WT and HSV-2 ΔgG2. Growth of DRG dissociated neurons in the presence of HEK293T cells mock, WT-HSV2 and HSV2ΔgG2 infected (1 dpi). HEK293T cells with mock, SgG2 and FLgG2 transfection were infected with HSV2ΔgG2. The neurite outgrowth and gG2 complementation were observed and compared to WT-HSV2 infected HEK293T cells and their neurite lengths. Significance: *P<0.05; **P<0.005; ***P<0.0005. Bars represent the standard error of the mean.
The next question was to determine whether the H2S sequence was important to inhibit the repelling effect of HEK293T cells and induce neurite outgrowth. To do so, we transfected HEK293T cells with WT-rSgG2, WT-rFLgG2, rFLgG2ΔCS and rFLgG2GSm. This step was followed by seeding HEK293T cells into the neurite chambers of MFC 1 day post-transfection. After 2 days, the cells were fixed, and immunofluorescence was performed. The neurons and their neurites at both sites were detected through the Tuji1 antibody that allowed to differentiate the neurite from the 293T cells at the axonal side of the chamber.
Western blots of recombinant WT-gG2 and cleavage-deficient mutants. Blots were showing cell lysate and supernatant of HEK293T transfected cells used for neurite outgrowth experiment. Blots were stained with αV5 [1:1000] and αHA [1:2000].

Figure 45 shows the neurite growth in the presence of different HEK293T transfected cells that were plotted. This experiment shows that FLgG2 and SgG2 had the highest number of grown neurites (99;44;89 and 81;142;24, respectively), which was followed by the FLgG2ΔCS (95;36;12) and the lowest number for FLgG2GSm (5;7;17). The longest...
neurites was found in presence of rFLgG2ΔCS transfected cells ([#1]1444, [#1]1149 and [#1]786 µm), followed by rFLgG2GSm ([#3]1282, [#2]736 and [#1]445 µm), rSgG2 ([#2]873, [#2]837 and [#2]661 µm) and rFLgG2 ([#3]779, [#3]683, [#3]668 µm). The tendency of rFLgG2 is showing an increasing mean value of grown neurites, whereas the rSgG2 has a constant mean value among the chambers. This observation went along for the rFLgG2GSm as well. The highest difference was observed for the chambers from rFLgG2ΔCS (Figure 45).
Fig. 45 Neurite outgrowth experiment. Growth of DRG dissociated neurons in the presence of transfected HEK293T cells with different wild type rFLgG2, rSgG2 and full-length cleavage variants (ΔCS and GSm). Transfected HEK293T cells were applied together with dissociated DRG-derived neurons into the axonal and somal compartment of the MFC, respectively. Neurites that grew into the axonal department were measured using ImageJ and the scale bar within the pictures. Each column represents one MFC, where the corresponding construct was applied to the 293T cells. Each dot per column represents one measured neurite that grew from the somal through the microgrooves to the axonal site. The number above each column represents the number of counted neurites per MFC. This experiment was performed once. Bars represent the standard error of the mean. #1, #2, #3 refers to three chambers for each clone.

The closer look on the median neurite length of all three chambers per clone revealed a tendency regarding the neurite length. rFLgG2ΔCS had
the highest impact on neurite length, resulting in a high mean for chamber #1 (190 µm) and #2 (183 µm). These mean values were followed by rSgG2 ([#2]126 µm; [#3]69 µm; [#1]94 µm), rFLgG2GSm ([#1]111 µm; [#2]76 µm; [#3]57 µm) and rFLgG2 ([#3]78 µm; [#2]117 µm; [#1]53 µm) (Figure 46).

Fig. 46 Median neurite length from each column summarised into one column [based on Fig.45]. Each column represents three independent microfluidic chambers per clone. The red dot represents a value that is out of range. Median lengths are represented in µm. Bars represent standard error of the mean.
The focus on the number of neurites revealed another distribution for each clone. The highest number of neurites that grew from the somal to the axonal site was seen in for the rFLgG2 and rSgG2, 76 and 80, respectively. The mean value of rFLgG2ΔCS was less than the previously mentioned clones and their chamber, which was around 48. The lowest number was found in the chambers, where rFLgG2GSm transfected cells influenced the growth of neurites (10) (Figure 47). The red median value of rFLgG2ΔCS ([#3]48 µm) represent a value, which was an outliner compared to the other both chambers, due to lack of sufficient neurons compared to the other chambers.

The results in figure 47 indicate that the clone rFLgG2ΔCS had an increased neurite length into the axonal compartment when compared to rFLgG2, rSgG2 and rFLgG2GSm. However, the number of neurites was reduced in rFLgG2ΔCS transfected HEK293T cells, when compared to rFLgG2 and rSgG2, but not rFLgG2GSm.
Fig. 47 **Mean number of neurites from each column summarised into one column** [based in Fig.45]. Each column represents three independent microfluidic chambers per clone. Bars represent standard error of the mean. The red dot represents an outliner due its reduced presence of neurons at the somal site.
4 Discussion

4.1 Attempts to identify the gG2 cleavage site led to the H2S region

The functional role of cleavage was one of the central focuses of this project. The first step was to identify the cleavage site to be able to obtain non-cleaved gG2 constructs that could be used in functional assays \textit{in vitro}. With this knowledge, we would have generated recombinant HSV-2 virus lacking gG2 cleavage and secretion to determine the role of these processes \textit{in vitro} and \textit{in vivo}. Many viral proteins require cleavage to be functionally active during infection. One example is influenza A virus, whose cleavage of the viral nucleoprotein and M2 proteins depends on the Bik-mediated caspase activation\textsuperscript{299}. Another example is HIV, where the cleavage and activation of the glycoprotein gp160 depend on furin activity\textsuperscript{300}. The herpesviruses also express several proteins, which need to be cleaved to get fully activated. Glycoprotein B (gB) is conserved in most herpesviruses, and it is involved in a crucial process like viral fusion and cell-to-cell spread. Some gB’s are proteolytically
cleaved through cellular proteases. In bovine herpesvirus 1 (BHV-1), the proteolytical cleavage of gB is essential for the cell-to-cell spread, but not for viral replication\textsuperscript{301}. Similar observations can be found in murid herpesvirus, where cleavage deficient gB lead to lower infection of myeloid cells, like macrophages and bone marrow-derived dendritic cells. \textit{In vivo} studies support the necessity of gB, since murid herpesvirus 4 lacking gB cleavage is deficient in the viral spread in lungs and macrophages, but not in the infection of epithelial cells and fibroblasts\textsuperscript{302}. Another example is the glycoprotein 42 (gp42) of EBV, where the cleavage and secretion are required for membrane fusion. More detailed, the mutation of the cleavage site leads to less secretion and less gp42-mediated B-lymphocyte fusion, which underlines the importance of the secreted part beside the full-length gp42 in infection.

Therefore, the approach to understanding how important cleavage is in the context of function was crucial in this project. Especially the structural difference between gG1 (HSV-1) and gG2 (HSV-2) regarding the presence of the additional secreted domain in gG2 is interesting. From
an evolutionary point of view, HSV-2 has an extra N-terminal secreted domain probably for a particular purpose. Both predicted cleavage sites, including four amino acids Arg321/Ala322 and Arg342/Leu343, are mentioned in the publication of Liljeqvist as personal communication from R. Courtney. So far, there is just only this prediction known and no other studies have aimed for this region nor any other cleavage sites within gG2 were identified.

In this study, we attempted to inhibit the cleavage site without affecting US4 gene expression in mammalian cells. Previous studies showed that deletions of large domains lead to lack of expression of gG2 (Viejo-Borbolla, personal communication), which underlines the sensitivity of insertion of mutations in the US4 gene. Therefore, we initially performed side-directed mutagenesis to minimise the impact in expression or folding of gG2. Our results contradict the prediction from R. Courtney since mutation of Arg321/Ala322 and Arg342/Leu343 has a minor impact on cleavage and secretion of SgG2 (SDM0+2; Figure 10 B). The continuous addition of more alanine residues near the cleavage site did
not decrease the cleavability from gG2, which led to the suggestion that several other residues are also involved in the cleavage. However, the suggestion that both sites are involved in cleavage is confirmed to a certain degree, because both mutations are necessary to affect the cleavage partially.

Since the mutations did not affect the cleavage, we decided to produce high quantities of the WT-rSgG2 construct with the help of the Baculovirus Protein Expression System. Here we produced the WT-SgG2 protein to identify the last C-terminal residue after cleavage. The MALDI-TOF mass spectrometry analysis showed that cleavage occurred upstream of Arg321Ala322, more precisely in Phe319 and probably Pro317 as well. Based on the recommendations from Prof. Pich and Dr. Polten from the toxicology department, we addressed whether furin was the main protease involved in the processing of gG2. Their suggestion is funded through the consensus motif from furin, which is characterised by some arginines separated by unspecific and specific
residues (Lys). Even the analysis through the ProP 1.0 predicts additional cleavage sites that can be cleaved theoretically by furin (Figure 20).

To investigate the involvement of furin in gG2 processing, we analysed the processing of gG2 in the context of furin deficient LoVo cell line. The optimal control was the gp160 envelope protein from HIV, which is not cleaved into their final forms (gp41 and gp120). However, in this experiment, the primary antibody showed a high degree of unspecific binding (blot not shown). However, our results indicated that rFLgG2 was still cleaved, indicated through the presence of cleaved SgG2 (see 40 kDa product in figure 22). This result indicates that rFLgG2 is not cleaved by furin or furin-like protease; it is instead processed through another protease.

Since it is not clear where exactly the cleavage occurs, which is also supported by other studies, we checked whether other proteases acting at later stages of glycoprotein expression were involved. The aim was to block the activity of ADAM proteases and identify an extracellular mechanism in SgG2 cleavage and secretion. The online program
PROSPER from the Monash University, Australia, runs several proteases onto the amino acid sequence and predicts cleavage sites and the resulting N-terminal and C-terminal fragment\textsuperscript{288}. Based on the location of the proteases and the size of the N-terminal fragment, we narrowed the proteases down to three ADAM proteases (MMP2, MMP3, MMP9, see figure 23 A). The usage of Batimastat did not reveal any further insights into the processing of gG2 (Figure 24). These results suggest at least that the most common involved ADAM proteases were not responsible for cleavage of SgG2.

Our main objective was not to identify the protease involved in gG2 cleavage but to obtain gG2 constructs lacking cleavage. Therefore, we continued to investigate the cleavage site further and mutated the cleavage site region 2 and 0 as a whole. Therefore, the next step included the direct change of the Arg321Ala322 and Arg342Leu343 and residues around the predicted cleavage site to alanine (Figure 11 A), resulting in the generation gG2 constructs with four or six residue changes of the cleavage site (CSKO0+2). We also substituted residues Phe319 and
Pro317 by alanine (CSKO0+2/6+3AA), based on the mass spectrometry data. These mutations did not prevent cleavage completely, and the cleavage phenotype was somewhat similar to CSKO0+2 and SDM0+2 (compare figure 18 B with figure 10 B and figure 11 B). Even the additional mutations of proline and phenylalanine did not prevent or highly reduced the cleavage.

Due to the lack of any cleavage site consensus motif or cleavage inhibition, we decided to remove the whole region potentially involved in the cleavage. The area spans 30 residues (Pro314 to Leu343) and includes cleavage region 2 and region 0 (Figure 18 A). In this approach, the deletion had the highest impact in cleavage, which resulted in a larger uncleaved band in the supernatant and cell lysate (Figure 18 B). However, the full-length gG2 lacking this sequence is present in the supernatant. The hypothesis was that rFLgG2GSm and rFLgG2ΔCS would remain in the cell, but the expression of the cleavage deficient construct shows clearly its presence in the supernatant (Figure 19 B). This protein contained both N-terminal V5 and C-terminal HA tag,
clearly indicating that the released protein has its transmembrane domain. This observation supports the hypothesis that rFLgG2GSm and rFLgG2ΔCS are released in extracellular vesicles (EV) and a potential new feature of modulation towards the host by HSV-2.

EVs function as cargo for several proteins and act on local or distant targets. On the cellular level, exosomes play a role in organ-specific metastasis, where exosome uptakes lead to a pre-metastatic niche formation and the preparation for the invasion of primary tumour cells. Targeting these integrins-incorporated EVs lead to less exosome uptake and less metastasis\textsuperscript{303}. The focus on the viral level shows more insights in the involvements of EVs. Studies from Arakeylan and colleagues show that HIV-1 produces exosomes containing gp120. Furthermore, they showed that depletion of gp120-EVs leads to decrease in infectivity of HIV-1 in the context of human lymphoid tissue ex vivo\textsuperscript{304}. Studies on CMV support the production and secretion of EVs. Here they analysed the separated EVs through an iodixanol gradient and detected typical EV markers (tetraspanin CD63 and Rab27A). Flow analysis of EVs show
incorporation of gB and gH, which are known to be a part of membrane fusion complex and facilitate viral entry. Based on these publications, it is plausible to hypothesise that cleaved SgG2 from full-length gG2 is released in EV ([3], Figure 19 B). Whether this is the case and its potential functional implications need to be confirmed.

Fig. 48 Different models of secretion. Based on the previous observations made with rFLgG2ΔCS Figure 23. [1] Current view on the SgG2 and MgG2 processing. [2] Observed and suggested model of uncleaved rFLgG2, producing exosomes. [3+4] Showing the secretion pathway based on the previously made observation of uncleaved rFLgG2; Moreover, the secretion of SgG2 probably occurs through the EV release.
whereas the MgG2 remains on the surface of transfected cells due to its transmembrane domain.

Based on the western blot of the rFLgG2ΔCS and the presence of the HA and V5 tag within the supernatant, the secretion of MgG2 together with SgG2 may indicate a similar expression pattern as shown for some HIV and CMV proteins regarding EVs. This observation is underlined by the fact that rFLgG2ΔCS secretion occurred in the presence of the transmembrane portion and the C-terminal HA tag (rFLgG2ΔCS scheme in figure 19 A). The presence of HA together with V5 tag can probably explain the existence of membranes, i.e. the potential formation of EVs initiated here through the expression of rFLgG2ΔCS (see alternative rFLgG2ΔCS secretion [2] in figure 48). This kind of phenotype may also be the reason for the significant increase in neurite outgrowth. The SgG2 and the MgG2 (ectodomain) may act in a paracrine manner, similar to the affirmed enhanced HIV infectivity. Secretion pathway [1] of rFLgG2 in figure 48 represents the expression and secretion of gG2 according to the current view. gG2 is translated in the ER and transported in vesicles that fuse with the Golgi and undergoes additional modifications. At later steps
gG2 vesicle fuses with the membrane and releases the N-terminal SgG2 portion into the supernatant, whereas the MgG2 remains probably on the membrane.

Based on the observation from rFLgG2ΔCS, we suggest two separated, at the same time occurring, secretion pathways for the cleaved SgG2 and the membrane-bound portion MgG2 (see alternative secretion pathway [3+4] in figure 48). Initially, after translation the SgG2 is cleaved and processed through the ER and Golgi, acquiring all modifications. After the Golgi, there is SgG2 specific enrichment of SgG2-exosomes in multi-vesicular body (MVB). This step requires free cytoplasmic cleaved SgG2, but the process is unknown (see alternative secretion pathway [3], the step after the Golgi, red text in figure 48). Nevertheless, the MVB containing the exosomes fuse with the cell membrane and release SgG2 containing exosomes. The retained MgG2 portion is probably delayed in its processing and fuses with other vesicles together, which fuses at the end with the cellular membrane, where the MgG2 portion remains on the cell surface (see alternative secretion pathway [4] in figure 48). On the
cellular level, it would also be interesting to see, whether SgG2 cleaved from rFLgG2 and the cleavage deficient rFLgG2ΔCS have the same phenotype and colocalisation with EV markers during the expression. This would support the experiment using gradient-based EV separation and flow cytometry.

Since we successfully determined the cleavage site and inhibited its cleavage nearly completely, we considered whether the region might be transferable into other constructs and function similar to cleavable peptides in bi- or polycistronic constructs. The 2A sequences (GDVEXNPGP) are known to cause ribosomal skipping during translation, which is based on the presence of three specific residues following the order proline-glycine-proline. More detailed, during translation the glycine and the following proline inhibits the peptidyl transferase, which initiates the release of the nascent chain, continuing translation of the new N-terminal proline and the following peptide. The results are two different products during one single translation process. The comparison to the H2S sequence did not lead to a similar 2A site.
However, the transfer of these 30 residues encoding sequence into the reported plasmid containing N-terminal mCherry and C-terminal GLUC, led to the expression of a functional Luciferase and the expression of mCherry (Figure 26 B and C). The activity of the luciferase is nearly close to the activity from the Luciferase expressed together with P2A sequence. mCherry was also present as an independent protein, not fused to GLUC.

Interestingly, we observed mCherry in the supernatant of the transfected cells. Our experiments indicated that this secretion of mCherry was not due to the existence of an internal signal peptide in H2S since deletion of the signal peptide from rSgG2 did not result in the secretion of the cleaved, N-terminal domain into the supernatant. The presence of mCherry within the supernatant of the mCherry-H2S-GLUC construct can be probably explained through the signal peptide of the C-terminal GLUC. In previous studies, the GLUC was used in a reporter construct for bicistronic and non-conventional secretion. The experiment showed that N-terminal GFP fused with GLUC (GFP$_{sp}$GLUC) induces secretion
of GFP due to the cleavage of the internal signal peptide of GLUC. Therefore, the secretion of the N-terminal GFP can be explained through the cleavage of the internal signal peptide of GLUC\textsuperscript{301}. Summarised, the internal signal peptide of GLUC gets cleaved and initiates secretion of the N-terminal mCherry within the H2S construct. Introduction of the H2S sequence between the cDNAs of eGFP and mCherry also resulted in the cleavage of the two proteins (Figure 27 B). These transfer experiments confirm the functionality of the H2S sequence as a cleavable peptide, although through different mechanisms. One characteristic of the P2A sequence is that it facilitates the equimolar expression of the two proteins that are translated from the same mRNA. Whether this is the same for the H2S remains to be investigated.

Another experiment that confirmed the cleavage deficiency of rFLgG2ΔCS transfected HeLa cells was the immunofluorescence (Figure 33). The coexistence of green V5 signal and red HA affirmed the presence of the cleavage deficient full-length construct in comparison with the WT variants. Additionally, the V5 signal corresponding to the
SgG2 domain of WT-rFLgG2 was less abundant than in rFLgG2ΔCS, which is probably because SgG2 from WT-rFLgG2 gets processed and secreted faster than the cleavage deficient construct. The same observation was also made for the rFLgG2GSm construct. There were no differences seen between both cleavage deficient constructs (compare Figure 33 and Figure 34), which would mean the cleavage region spanning the proline 314 and leucine 343 is essential for cleavage.

For future experiments, it is necessary to identify cell compartments through specific markers. It would be interesting to know whether rFLgG2ΔCS coexist with specific compartments inside the cell compared to the wild type variants. Moreover, it is necessary to observe how the cleavage variants behave in comparison with the wild type variants of gG2 (rSgG2 and rFLgG2). Additionally, it is necessary to use confocal microscopy to narrow down the potential cellular site of each construct. As previously suggested, EV markers in this regards are required as well, to confirm the previously described gG2 secretion pathway with EVs. The Society for Extracellular Vesicles cites a range
of different markers, which are highly abundant in EVs, which would help in combination with gG2 antibodies\textsuperscript{305}.

4.2 Several N- and C-terminal deletions within the rSgG2 constructs do not prevent heparin binding

The initial N- and C-terminal deletions were done to identify potential GAG binding sites, and we considered small deletion due to problems in expression in the presence of larger deletions. Previous studies show that GAGs are essential for chemokine binding and presentation in a gradient manner upon infection and tissue damage\textsuperscript{173,176}. Moreover, GAGs are also essential for viral binding on host cells, which allows approach to their corresponding entry receptor\textsuperscript{13–15}. As previously described some vCKBP interfere with GAG-binding on the cell surface and gG from HSV-2 does the opposite. It rather binds the chemokines through GAG binding motif and stabilises the interaction with their corresponding receptors\textsuperscript{252}.
Therefore we wanted to identify the GAG-binding motif and study its functional relevance. Despite the mutations of potential GAG-binding consensus motifs within the SgG2 domain, binding was still possible (unpublished work, MSc Thesis²⁹⁸). In this experiment, we considered the deletion of N- and C-terminal residues and combined them (10 and 20 residues). In the binding assay, each deleted construct was still able to bind to heparin beads, which indicates that each terminal part is not involved in GAG binding (Figure 36). Noteworthy, the rSgG2ΔC20 construct seems to abrogate the binding to heparin beads. However, this phenotype can be explained through the low input of the protein itself. The additional brightness adjustment revealed a green signal of this construct (blot not shown). The next step was to sequentially introduce small 20 residues deletion as described in figure 37 A. Expression studies in HEK293T-transfected revealed interesting expression patterns (Figure 37 C). On the first sight, deletion of the residues in region 5, 9, 11 and 12 leads to the expression and secretion of mutated rSgG2 constructs. Due to time restriction, we were not able to obtain heparin-binding results of these constructs. Therefore, these constructs need to be further
investigated in initial heparin-binding assays. Ideally, we expect to see that binding to heparin beads is abrogated and additional purification of these constructs enables studies on binding with chemokines using surface plasmon resonance. This method allows the interaction of the previously mutated and secreted rSgG2 variants with chemokines that were used in the studies from Viejo-Borbolla et al.16.

However, the other constructs did not show any secreted bands, whereas the expression in the cell lysates shows similar levels of protein as the secreted ones (compare clone 5, 9, 11, 12 with the other clones in figure 37 C). This observation is remarkable since secretion of the short rSgG2 construct (without transmembrane domain) is inhibited, which either means processing is abrogated, and the mutants accumulate within cells or secretion is blocked since the corresponding deleted 20 residues refers to a secretion signal. It is known that specific localisation signals direct the newly expressed proteins to specific sites of the cell and maybe gG2 encodes another internal signal peptide that directs the protein through a novel pathway during the secretory pathway269. To determine the
subcellular retention of these non-secreted constructs, additional immunofluorescence studies are required with specific cellular markers and compare them with the paternal construct. Moreover, markers related to degradation, like lysosome markers (LAMP1; LAMP2B) could give first insights in the absence of non-secreted gG2 deletion variants.

4.3 The enhancement of chemokine activity by gG2 does not depend on cleavage in the context of the rSgG2 cleavage variants

Previous studies showed that soluble forms of gG1 and gG2 enhance chemokine activity in vitro (transwell experiments, time-lapse assays using monocytes) and in vivo (air-pouch model in mice). Here, we investigated the functional relevance of uncleaved rSgG2 (GSm and ΔCS) in comparison with WT-rSgG2 using transwell experiments with Jurkat T cells and hCXCL12.

Our results suggest that the cleavage site is not necessary to enhance chemokine activity. The absence of the cleavage site (rSgG2ΔCS), rather induces a lower level of migration compared to WT rSgG2. However, the
rSgG2ΔCS still induced more chemokine-dependent migration than the chemokine alone, whereas the rSgG2GSm construct did not (Figure 40A). These results suggest that cleavage is not essential for chemokine enhancement, whereas the insertion of the glycine-serine motif negatively affected chemokine enhancement. This observation relies probably on the fact that the motif causes structural changes, which results in different folding and accessibility to chemokines compared to the WT and cleavage deficient construct (rSgG2ΔCS). Usually, glycine residues provide flexibility, and the serine is a polar group facilitating solubility, but the introduction of these groups may change the folding of the C-terminal part of rSgG2GSm. Another evidence for an alternative folding is the fact that proline is usually present inside the cleavage site and this residue is known to be a rigid residue, and the presence of glycine and serine could cause another possible folding product. This alternative folding could allow the C-terminal domain to act on the intra-molecular protein level and interfere with chemokine binding and enhancement, whereas the proline featured region prevents an interaction prior to the cleavage of the SgG2 in the WT-rSgG2.
Summarised, we can conclude that the absence of the cleavage site in rSgG2ΔCS does not affect the chemokine-enhancing function of gG2. However, once the GS linker replaced the region, the folding of the protein probably changed and allowed lower accessibility for chemokines. To determine whether this is the case, binding and chemotaxis assays using purified WT-rSgG2, rSgG2ΔCS and rSgG2GSm should be performed.

### 4.4 Cleavage deficiency in the context of the full-length gG2 increases neurite outgrowth

Cabrera and colleagues show clearly the involvement of SgG2 in context of increased NGF-dependent axonal growth of neurons. Additionally, the \textit{in vivo} studies show that SgG2 facilitate peptidergic neurite growth. SgG2-NGF induced re-direction of TrkA to lipid rafts, and its ligand-mediated internalisation is abrogated upon SgG2 presence. Even the unpublished studies from Kropp \textit{et al.} from our group indicate that HSV-2 lacking gG2 expression leads to reduced neurite outgrowth of DRG dissociated neurons. Therefore it would be interesting to know
whether cleavage and secretion of SgG2 may play a functional role in neurite outgrowth using microfluidic chambers. The results in the microfluidic chamber experiment and the direct comparison of all constructs, including the WT-gG2 (i.e. WT-rSgG2 and WT-rFLgG2), indicate a trend among the constructs. The scattered dot plot showed clearly that the expression of rFLgG2ΔCS induced the longest neurite outgrowth compared to the wild type versions (Figure 46). We hypothesise that the higher neurite outgrowth is due to the secretion of the full-length construct lacking the H2S sequence. The exosome secretion hypothesis is described in figure 48. Here, the vesicles arrive after modifications the trans-Golgi part and based on our interpretation the vesicle fuse with endosomes and build up multivesicular bodies (MVB), which fuse with the membrane and release their cargo. Based on this current event, we suggest that MVBs may be present in the regular secretion of SgG2 as well. Here, the endosomal development fuses at later stages with secretory pathway derived vesicles containing SgG2, which fuses with the membrane to release the EVs and act probably in a paracrine manner towards the NGF together with its TrkA receptor. So
far it is not clear, how SgG2 is secreted in context of rFLgG2ΔCS and if the secretion has similarities with HIV and CMV, which are producing exosomes that facilitate infection of lymphoid tissue and presence of viral entry mediators (gB/gH), respectively\textsuperscript{304,306}.

Interestingly, the mean neurite number of rFLgG2ΔCS that grew to the axonal site was lower than the other constructs, which was due to the lower presence of seeded neurons at the somal site (Figure 47). However, the mean length was the highest in rFLgG2ΔCS among the samples. The exclusion (due to low number of neurons and probably blocked microgrooves) of one sample (red dot in figure 46) increases the mean of neurite length even more, which would suggest secretion of uncleaved rFLgG2 together with the C-terminal ectodomain induce longer neurites (from less neurons) than the other constructs (Figure 46). The chambers containing rFLgG2GSm transfected cells and the corresponding reduced number of neurites and lengths occurred probably through reduced amount of seeded neurons into the somal site of chambers (see supplement figure 50). However, to confirm or exclude this reduced
neurite phenotype of rFLgG2GSM, additional neurite outgrowth experiments are required. The presence of the MgG2 ectodomain may induce longer neurites than SgG2 and, thereby, facilitate infection of FNEs. However, the rSgG2 construct induces longer neurites that grew through the microgrooves compared to the rFLgG2 (Figure 45 and 46). These experiments must be repeated to confirm or not confirm the current observations and determine the effect of the ectodomain in the uncleaved rFLgG2 construct.

However, there is theoretically a novel secretion mechanism of HSV-2 gG, which acts in a paracrine manner in neurite outgrowth. This consideration is underlined with the secretion mechanism of rFLgG2ΔCS. However, it is still necessary to do further experiments, which includes the analysis of the EVs in the supernatants of transfected as well as infected cells. First, the analysis via the iodixanol gradient and FACS analysis could differentiate EVs from the supernatants of transfected cells. Additionally, markers allow the separation of specific
EVs and unspecific vesicles. Moreover, FACS analysis using anti-V5-magnetic beads allow better differentiation of gG2 positive EVs.

4.5 Conclusions

The identification of the cleavage site to determine its functionality in the context of leukocyte migration and neurite outgrowth was the aim of this project. Using this information, we will generate recombinant HSV-2 lacking gG2 cleavage and determine the functional relevance of this process during infection.

Our results show that a sequence of 30 residues contains a transferable cleavable region. This contradicts the suggestion from R. Courtney, who determined specific residues responsible for cleavage (Arg321Ala322 and Arg342Leu343). However, the removal of this specific region led to release to the supernatant of full-length gG2, suggesting that it is present in EV. This raises further questions as to the possible role of EV in the secretion of SgG2 and, potentially, full-length gG1. Addressing these questions require further investigations that may provide information on
the ability of these viral proteins to act in an autocrine and paracrine manner to facilitate neurite growth and infection of FNEs.
5. References


31. Cairns, T. M. *et al.* N-Terminal Mutants of Herpes Simplex Virus Type 2 gH Are Transported without gL but Require gL for Function Downloaded from. *J. Virol.* **81**, 7328 (2007).


33. Gianni, T., Avitabile, E., Lombardi, G., Campadelli-Fiume, G. & Capri, M.
Coexpression of UL20p and gK Inhibits Cell-Cell Fusion Mediated by Herpes Simplex Virus Glycoproteins gD, gH-gL, and Wild-Type gB or an Endocytosis-Defective gB Mutant and Downmodulates Their Cell Surface Expression. *J. Virol.* **78**, 8015–8025 (2004).


57. Honess, R. W. & Roizman, B. Regulation of herpesvirus macromolecular


81. St Leger, A. J. & Hendricks, R. L. CD8 + T cells patrol HSV-1-infected trigeminal ganglia and prevent viral reactivation. doi:10.1007/s13365-011-0062-1


91. Boutell, C., Sadis, S. & Everett, R. D. Herpes Simplex Virus Type 1 Immediate-Early Protein ICP0 and Its Isolated RING Finger Domain Act as
Ubiquitin E3 Ligases In Vitro Downloaded from. J. Virol. 76, 841–850 (2002).

92. Sant, C. Van, Hagglund, R., Lopez, P. & Roizman, B. The infected cell protein 0 of herpes simplex virus 1 dynamically interacts with proteasomes, binds and activates the cdc34 E2 ubiquitin-conjugating enzyme, and possesses in vitro E3 ubiquitin ligase activity.


103. Wald, A. et al. Reactivation of Genital Herpes Simplex Virus Type 2 Infection


106. WHO. *SEXUALLY TRANSMITTED INFECTIONS (STIs)* The importance of a renewed commitment to STI prevention and control in achieving global sexual and reproductive health sexually transmitted infections: large burden and serious consequences little progress in reducing. (2012).


120. Coleman, J. L. & Shukla, D. Recent advances in vaccine development for herpes simplex virus types i and II. Hum. Vaccines Immunother. 9, 729–735 (2013).


127. Paladino, P., Cummings, D. T., Noyce, R. S. & Mossman, K. L. The IFN-Independent Response to Virus Particle Entry Provides a First Line of


131. Rasmussen, S. B. *et al.* Herpes simplex virus infection is sensed by both Toll-Like Receptors and RIG-like receptors, which synergize to induce type I interferon production. *J Gen Virol** **90**, 74–78 (2009).


186. Liu, M. T., Armstrong, D., Hamilton, T. A. & Lane, T. E. Expression of Mig (Monokine Induced by Interferon- 


196. Johnson, K. E., Chikoti, L. & Chandran, B. Herpes Simplex Virus 1 Infection Induces Activation and Subsequent Inhibition of the IFI16 and NLRP3
Inflammasomes. 87, 5005–5018 (2013).


207. Jones, C. A. *et al.* Herpes Simplex Virus Type 2 Induces Rapid Cell Death and


220. Same as reference 219


242. Webb, L. M. C., Smith, V. P. & Alcamí, A. The gammaherpesvirus chemokine binding protein can inhibit the interaction of chemokines with


260. Balan, P. *et al.* An analysis of the in vitro and in vivo phenotypes of mutants of herpes simplex virus type 1 lacking glycoproteins gG, gE, gI or the putative gJ. *Journal of General Virology* 75, (1994).


263. Same as reference 47.

264. Zhu, J. *et al.* IL-17c promotes peripheral nerve growth during human herpes simplex virus type 2 (HSV-2) infection. *J. Immunol.* 198, 61.9 LP-61.9


Mondino, A., Giordano, S. & Comoglio, P. M. Defective Posttranslational


298. Srivaratharajan, S. & Viejo-Borbolla, A. Generation and Characterization of Glycoprotein g2 and Glycoprotein g2 Deficient Herpes Simplex Virus Type 2. (Medical School Hannover, 2014).


300. Hallenberger, S. et al. Inhibition of furin-mediated cleavage activation of


Fig. 49 **Immunofluorescence of mock-transfected HeLa cells.** Cells were stained with: 1:500 αV5 (GFP channel), 1:1600 αHA (dsRed channel), 1:1000 DAPI. Descriptive dsRed and GFP channel area (white frame) were chosen and shown as single channels underneath. Scale (red line) = 100 μm. Cells were magnified 40 times and visualised in Observer Z1 Axio microscope.
Fig. 50 **Immunofluorescence of Tuji1 stained neurons.** Pictures were taken from the somal site of the well. Each frame represents one chamber. Top left, top right and bottom left represents the seeded neurons at the somal site. rFLgG2GSm transfected HEK293T cells were seeded into the axonal site (not shown). Bottom right, representative somal site of dissociated neurons with evenly distributed neurons. The axonal site was seeded with rSgG2 transfected cells. Cells were magnified 20 times and visualised in Observer Z1 Axio microscope.
Affidavit

I herewith declare that I autonomously carried out the PhD-thesis entitled

“Identification of the cleavage site of herpes simplex virus type 2 glycoprotein G (gG) and its involvement in gG activities”

No third party assistance has been used.

I did not receive any assistance in return for payment by consulting agencies or any other person. No one received any kind of payment for direct or indirect assistance in correlation to the content of the submitted thesis.

I conducted the project at the following institution(s)*: University of Veterinary Medicine Hannover (TiHo) and Hannover Medical School (MHH), Institute of Virology.

The thesis has not been submitted elsewhere for an exam, as thesis or for evaluation in a similar context.
I hereby affirm the above statements to be complete and true to the best of my knowledge.

______________________________
City, date

______________________________
Signature
Acknowledgements

At first, I would like to say thank you to Prof. Abel Viejo Borbolla, who gave me this opportunity to do this project and experience the scientific field for almost 5 years. I remember, when I started the gG2 project back in 2014 as a master student and now, I was glad to be a member of his group. Of course, during my doctoral studies, I was confronted with some challenges, but he was always patient (and still is) and gave me the space and time to approach and solve these challenges. Besides the scientific part, it was always nice talking with him about current off-topic news in science, politics, society and of course football.

Next, I would like to say thank you to Prof. Thomas F. Schulz, to accomplish my doctoral studies at the Institute of Virology, MHH. I want to thank my co-supervisors Prof. Paul Becher and Prof. Eike Steinmann, who guided me through this project together with Prof. Viejo Borbolla. Additionally, I want to say thank you to Prof. Beate Sodeik and her group members, where I had an additional “supervisor-meeting” with additional
and helpful input to my project. This applies also to all collaborators, who supported me with results and further information, suggestions and materials, thank you. My appreciation goes also to the Andreas Pich Lab, Goffinet Lab, Alcamì Lab and Dr. Claus Henning Nagel.

Moreover, I want to express my gratitude to the N-RENNT network and the administration of the TiHo. Here, I would like to mention Apl. Prof. Beatrice Grummer, Dr. Tina Selle and Tanja Czeslik, thank you all. Further, I want to express my appreciation to the SFB900 and the Marie Skłodowska-Curie Actions, who supported several projects of our group.

As previously mentioned, I was glad to be a part of Prof. Viejo Borbolla’s group a.k.a. the HERPES ULTRAS. Here, I would like to mention especially Birgit Ritter (despite she supports Hannover 96), who was in all these years always the same funny, kindly, open and cheerful person, who lightened even cloudy and rainy days. Thanks for all the conversations, especially about politics and football. Moreover, I want to say thank you for being always the central gear in our lab organisation and maintenance. Of course, I want to say thank the current staff Kai
Kropp, Shuyong Zhu, Carina Jürgens, Guorong Sun, Jingjing Shi, Daniel Prüß and Renzo Bruno and all former members like Victor Gonzalez Motos, Pengfei Yu, graduate and undergraduate students Lisa, Sophie and Freddy. It was always funny and pleasant with you during work and cooking, wine tasting or football events. Moreover, it was always fun during our annual company excursions and bowling evenings with the Sodeik and Krey group. Additionally, I want to thank our former office members Penelope Kay-Jackson and Jasmin Zischke for all the previous conversations about anything work-related or off-topic, especially about Werder Bremen, Lebenslang Grün Weiß!

Furthermore, I want to thank all institute groups and their lovely current and former members, for a great time and atmosphere in the department and thanks for the cheerful and amazing Christmas parties. Also many thanks to all the administrative, sequencing and cleaning staff, Annemarie Meyer, Carmen Schohr, Michael Scharr, all the federal volunteer service employees, Sandra Flucht, Jenny Witthuhn and Nina Heinrich (and colleagues), who kept the institute running.
I want to express my appreciation to Simon Krooss and Katharina Andersson who created a pleasant and comfortable home, where we had delicious dinners, chats and pleasant evenings or had just a beer or whiskey to escape the stress sometimes. Thank you both for the support during my studies. Thank you to Julio César Villalvazo Guerrero as well for all the amusing, funny and MARVEL conversations.

At last, I want to say thank my family, Mona and her family and my friends from Bremen and Hamburg, who also brought diversion during the last five years. In particular, thanks to my mother, who supplied me always with Kottu Rothi. Many thanks to my sisters and my nephew, who were always there, cheered me up and supported me. This also extends to Mona’s family, who also became a huge part of my life with all the annual get-togethers. Finally, I want to say thank you to Mona, who lightened the last years and just started discovering the world with me.

Thank you all!