General Materials & Methods

Several analysis and experimental techniques were used repeatedly throughout this work. Whilst the general protocols can be found below, the exact parameters for each individual experiment can be found in methodology sections of corresponding chapters.

1. Sequence analysis
   1. Multiple sequence alignment assembly

Amino acid sequences of HCMV gB were downloaded on 14.02.2024 from GenBank NIH protein sequence database ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)). The search query “((glycoprotein B[Protein Name] OR (glycoprotein[All Fields] AND B[All Fields])) AND ("Human betaherpesvirus 5"[Organism] OR human herpesvirus 5[All Fields]))” was used, and all search results were filtered by sequence size to be between 850 and 950 amino acids in length. Sequences of non-gB proteins, sequences with >1% of unknown amino acids, as well as sequences obtained from synthetic viruses were manually removed.

Aliview sequence viewer and editor was used for sequence visualisation and processing and MAFFT online server was used for sequence alignment (ref, ref).

BLASTp-p online tool was used on the relevant protein seuqences for identifying percentage identity (link).

* 1. Phylogenetic analysis

Maximum likelihood phylogenetic trees with ultrafast bootstrap approximation were generated with the use of one of three IQTree online servers (description of 3, link). Further tree processing, including rooting by midpoint, as well as visualisation was performed in FigTree software (ref).

* 1. Protein structure generation

Where available, protein structure files were downloaded from the publicly available PDB database (link). Otherwise, ColabFold platform was used for generating predicted protein structures (ref). To assess the plausibility of ligand binding or oligomer assembly the multimer prediction feature was used while setting up ColabFold, given more than one input sequence.

* 1. Protein structure visualisation

UCSF ChimeraX was then utilised for visualising predicted or database-derived protein structures, and screenshots from the program were generously implemented in the figure generation process (ref). “Matchmaker” option was used for overlaying two or more structures against a relevant reference. “H-bonds” structure analysis feature was used to predict plausible hydrogen bonds between amino acid residues. Additionally, ChimeraX “Molecule display” features were implemented to display the hydrophobicity and electrostatic profiles of the structure of interest.

* 1. Immunogenicity prediction

Immune Epitope DataBase (IEDB) online tool BepiPred-2.0: Sequential B-Cell Epitope Predictor was used for epitope quantifying and visualising immunogenicity throughout my work. Due to the context-aware nature of the prediction algorithm the protein sequences were given in its whole form, as opposed to just the domain of interest (<http://tools.iedb.org/bcell/help/#Bepipred-2.0>) <- also a 2017 paper tht’s usful is cited here.

1. In vitro work
   1. Cell line maintenance and plating

Unless specified otherwise, all media for cell maintenance were supplied with 10% foetal bovine serum (FBS, manufacturer) and 1% Penicillin-Streptomycin (manufacturer).

Primary human foreskin fibroblasts (HFF, manufacturer), HFF-TET cell line (gift from colleagues in Prof Rich Stanton group at Cardiff University), owl monkey kidney cells (OMK, gift from colleagues in Dr Robert White’s group at Imperial College London) were grown and passaged in Dulbecco's modified eagle's medium (DMEM, manufacturer).

Epithelial cell line (spontaneously arising retinal pigment epithelia, ARPE-19, manufacturer) were grown and passaged in Dulbecco's modified eagle's medium (DMEMF/12, manufacturer). All cells were grown in T175 flasks, monitored and passaged appropriately at 90-95% confluence.

Passaging adherent cells would be performed by first treating with trypsin (manufacturer) for 5 minutes at 37°C, removing trypsin by centrifuging at 500g for 5 minutes, and resuspending the cell pellet with excess media, followed by an appropriate split of the cell suspension and transfer into a new flask. Primary HFF were discarded around passage 25 or once it would take longer than 7 days for a T175 flask to become confluent after a 1:3 split.

B cell hybridoma cells (gift from colleagues in Dr Marco Thomas’ group at Erlangen University, Germany) were maintained in suspension in RPMI (RPMI, manufacturer). and split 1:8 every 3 days.

(THP-1 cells if I end up using NL data)

Passaging cells in suspension would include swapping some amount of the cell suspension for complete media to achieve the desired split ratio.

All cells were kept in a 37°C (Oxygen and CO2 %) incubator. Cells would be plated out a day before the assay. After being trypsinised (manufacturer) from a confluent T175 flask (manufacturer) they were spun at 500g for 5 minutes to remove trypsin and then made up in 30mL of media, before being seeded at 100µL/well into a 96-well plate (TPP manufacturer).

For long term storage 1\*106 cells were frozen down in culture-specific freezing media as advised by the manufacturer and kept first kept at -80°C in a Mr Frosty (manufacturer) for slow temperature reduction before being transferred to liquid nitrogen.

* 1. Virus production

All viral strains, unless a specific protocol is cited, were grown and harvested as described below, in the cell type of intended further use (e.g., virus that will be used for infecting epithelial cells will be grown in epithelial cells).

A small amount of virus, aiming at MOI 0.01, would be introduced to a nearly confluent T175 flask. After 30minutes on orbital shaker (30 RPM) at RT the flask was transferred to a 37°C incubator. During the following few days, the infected flask would be monitored for CPE. Once more than 50% of cells in the flask have detached all media in the flask would be collected, spun down at 500g for 5 minutes to remove cell debris, and aliquoted into cryovials. The vials of harvested virus would be transferred into a -80°C freezer for storage until further use.

2.2.1 HCMV strains

Some experiments either required an HCMV strain with a specific phenotype or were set up to compare different strains under the same conditions. Most commonly, a clinical isolate Merlin strain that has been extensively laboratory-passaged was used. Whenever an assay required infecting both epithelial cells and fibroblasts, I used TB40E-Gata-MCherry strain as it retained tropism to both cell types. For spread assays, where a virus would need to stay cell-associated rather than spreading through the monolayer extracellularly, I used a modified IE2-GFP Merlin strain engineered by colleagues at Prof Rich Stanton’s group at Cardiff university.

* 1. Neutralisation assays

I have completed several assays determining if a certain compound is able to block cell-free virus. The general principle can be found below, and many variations added to the protocol are discussed further within specific chapters.

For all assays involving infection the optimal amount of virus needed for the desired end proportion of infected cells was first determined by a mock experiment with varying viral titres.

On the day of the assay the needed amount of virus was combined with the relevant dilution of the inhibitor of interest. The virus-inhibitor cocktail was then inoculated at 37°C for 1 hour and then introduced onto cells. For HCMV infection, after 3 hours at 37°C all liquid above cells was removed and changed for plain media, and the plate was fixed at 24hpi. For HSV-1 infection all liquid above the cells was changed for plain media after 1 hour at 37°C and the plate was fixed at 18hpi, to adjust for the shorter replication cycle of the virus.

* 1. Fixing cells

Cells were fixed at the desired time of end of experiment with ice-cold ethanol for at least 20 minutes at -20°C. Experiments where HSV-1 was used were kept in ethanol for no longer than 30 minutes to avoid weakening the GFP signal.

* 1. Infection visualisation and quantification
     1. Immunostaining

Immunostaining was performed with the appropriate mouse primary antibody and an anti-mouse secondary antibody (Alexa Fluor 568, A11004, Thermo Fisher).

Cells’ nuclei were stained for with Hoechst 33342 (4082S, Cell Signalling Technology) or DAPI (recipe, manufacturer) depending on availability, both made up to 0.01mg/mL with PBS.

HCMV infection was visualised using a primary antibody specific to immediate early protein IE2 (manufacturer). VZV infection was visualised using a primary antibody specific to immediate early protein IE62 (manufacturer). HSV-1 infection was visualised without staining, using the associated viral green fluorescence due to the VP26-GFP fusion protein. HVS infection was quantified through obsering the CPE of the virus, as no known HVS-specific antibody existed at the time of the experiments.

* + 1. Fluorescent microscopy

The infection was visualised with the use of Hermes Wiscan automated fluorescent microscope (manufacturer) and further quantified with Metamorph software (manufacturer). Imaging protocols, acquired pictures and montages, as well as imaging protocols and all other associated files can be found at (thesis github).

Unless a great loss of cells was anticipated in the experiment, data obtained from wells with less than 1000 total detected cells were removed as an outlier.

* 1. Peptide preparation

Custom peptides were purchased from Peptide2.0 US and Genscript UK as lyophilised powder and made up with 100% DMSO to a concentration of >20mg/mL and then aliquoted for long-term storage at -20°C. Some peptides were ordered with a GGC tag to improve their binding to an ELISA plate in further experiments (do I need a ref for that??). Peptide sequences can be found in [Peptide\_table].

* 1. ELISA

While all ELISA I have conducted throughout my thesis were different from one another, the principle stayed the same.

The 96-well plate (NUNC Immuno manufacturer) was coated with 100µL of prepared substrate of interest made up to needed concentration in coating Carbonate-Bicarbonate buffer pH 10 and left at 4°C overnight.

Each step on the day of the assay apart from the final addition of acid was preceded by washing the plate at least 3 times with excess PBS-T (PBS with 0.1% Tween-20, manufacturer) and then patting it dry on tissue paper.

On the day of the assay the plate was blocked with 2% FBS in PBS blocking buffer, before the addition of the relevant primary antibody diluted to the desirable concentrations in blocking buffer. This was followed by introducing the secondary antibody, specific to the Fc domain of the primary antibody, made up 1:10000 in blocking buffer. Blocking buffer, primary antibody and secondary antibody were each incubated for 1 hour at 37°C after being added onto the plate

Finally, 50µL of TMB substrate (manufacturer) was added onto a thoroughly washed and dried plate and left in the dark to develop. The reaction was stopped with 50µL of 1M phosphoric acid and quantified immediately by measuring optical density at 450nm, with the use of MultiscanFC ELISA plate reader (Thermo Fisher). During the data analysis the negative control values were subtracted from all others to account for background fluorescence.

* 1. DNA extraction

Unless specified otherwise, DNA extraction from a cell monolayer or directly from viral harvest was performed as follows.

First solutions A and B were prepared, with solution A comprised of 100mM KCl (manufacturer), 10mM Tris-HCL (manufacturer), 2.5mM MgCl (manufacturer) in nuclease-free H2O (manufacturer), and solution B comprised of 10mM Tris-HCL, 2.5mM MgCl, 1% Tween-20, 1% Nonidet P-40 (manufacturer), 0.4mg/mL proteinase K (manufacturer) in nuclease-free H2O.

After washing the plate with the cell monolayer with sterile PBS, 50µL of both solutions A and B were added to each well one after another, each followed by a 5-minute incubation. In case of DNA extraction from a vial of viral harvest, the washing step was omitted, and solution volume was increased to 100µL each. After the final incubation, the mixture was transferred onto a 60°C heat block for 60 minutes, followed by a 95°C heat block for 10 minutes. Extracted DNA was stored at -20°C between assays.

* 1. Polymerase Chain Reaction (PCR)

Reactions were set up using a PCR Mastermix (Thermo Fisher), relevant primers, and template DNA on a thermocycler (manufacturer). The reaction conditions were set up in accordance with the manufacturer’s protocol.

Where needed, the PCR products were separated with a gel electrophoresis on a 1% agarose TAE gel (tank manufacturer) and purified using a kit (manufacturer).

Quantitative Real Time PCR (q-RT PCR) was set up using Power-Up SYBR Master Mix (Thermo Fisher) in accordance with the manufacturer’s protocol using a QuantStudio3 (Applied Biosystems by Thermo Fisher) thermocycler. All oligonucleotides and primers were ordered from Thermo Fisher. Sequences and melting temperatures for primers used for PCR and q-RT PCR can be found in [Peptide\_table] and are given appropriate annotations in text. Primers marked as “designed by self” were picked using Primer3Plus online toolkit ([www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi](http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi)) and then further optimised through PCR troubleshooting if needed.

* 1. Concentration

Protein (IgG) and DNA (double stranded) concentrations were determined using a Nanodrop mini (manufacturer) with relevant settings.

1. Data processing and figures

Processing the raw data was done primarily in Microsoft Excel, Python, R and Rstudio (ref, ref, ref).

Whenever microscope images required colour correction or enhancement, the pictures of control (and all other) conditions were edited at the same time as part of one whole figure to avoid bias.

Figures and graphs were generated using Python, R and RStudio , GraphPad Prizm 10, as well as Biorender.com (ref, ref, ref). Figures generated with Biorender were downloaded under paid subscription.