Chapter 2 (AD6) Methods

1. Experimental rationale

I was very lucky to have the beginning of my PhD project coincide with a discovery of a novel antigenic domain in HCMV gB. also outlined a potential mechanism with which the gB vaccine can induce protection.

Excited to work within the new unexplored world of AD-6, I set to characterise and investigate the domain’s function and immune towards it. Aiming to have BLAH my experiments informative while keeping the process cost- and time-efficient

the exact function of AD-6 remains a mystery. In this chapter I investigated AD-6 function and immune response towards it using several approaches, varying in their degree of sophistication.

Chronologically, I first focused on the computational analysis of the known conservation of gB structure and function across HHV. Sequence and structure analysis has identified putative location of AD-6 analogues and further outlined the possibility of a conserved cross-herpesvirus epitope.

The first line of action was to repeat the initial viral spread assays against existing AD6 polyclonal antibody with the use of a virus other than HCMV. With the help of multiple groups from several institutes, I was able to replicate the experiment and show the striking protective effect of AD-6 pab on two alphaherpesviruses and at least one gammaherpesvirus. The striking similarity of AD-6 and its HHV analogues drove me to the conclusion that whatever function the domain has, it is most likely also conserved across HHV.

After dissecting the activity of the polyclonal antibody specific against AD-6, during the final year of my PhD, I have come in contact with a group who have produced monoclonal antibodies against the same target. Utilising this collaborative unique opportunity, I were able to use and characterise the monoclonal antibodies, which has proven to be an incredibly useful asset in understanding AD-6 function, and its involvement in cell-to-cell spread of HCMV, as well as dissecting the exact BLAH of AD-6 constituents.

I then took a step back and computationally investigated AD-6 in the context of its native virus with the hope that my findings for HCMV would be applicable at a wider scale.

With a great help of Alexander Hargreaves, a rotation PhD student at the time, I expanded the peptide neutralisation assays of the original AD-6 discovery paper were repeated and expanded on using HSV-1 virus and peptide, as well as multiple cell types.

The final question, first raised in the AD-6 identification paper, was whether the target of native AD-6 peptide is viral or cellular. As it was a “fishing expedition” with no presumed or expected outcome in mind, I wanted to keep the experiments simple, cost-effective, yet still conclusive. To do that I developed a binding assay by modifying the existing protocol for the purification of His-tagged proteins. Using a modified AD-6 peptide as bait, I asked the question of whether running the virus against it would reduce the number of infectious particles and viral DNA in the flow-through.

1. *In silico* work
   * 1. Sequence analysis within HHV and CMV of different species

Reference gB sequences for four HHV were obtained from Uniprot as follows: P13201:GB\_HCMVT (HCMV), P03188: GB\_EBVB9 (EBV), P06437:GB\_HHV1K (HSV-1), and Q4JR05:GB\_VZVO (VZV). Reference gB sequences for animal CMV that were analysed alongside HCMV were reverse transcribed from gB gene??? Look up

* 1. Structure conservation analysis

Four gB structures were downloaded from the open source PDB database – 7KDD (CMV), 3FVC (EBV), 3NWF (HSV-1), and 6VLK (VZV). Putative amino acid coordinates of AD-6 structural analogues in HHV gB were identified visually after overlaying the aforementioned structures with the HCMV gB as a reference. For HVS gB, due to the lack of an available PDB structure in the database, the file was generated with the use of Alphafold, in parallel to an equivalent job of HCMV gB structure prediction. Such intricacy was needed because the alphafold-generated structures could not be directly aligned with structures obtained through Xray – crystallography due to low similarity. More detailed description of methods and software used for analysing protein structures can be found in [General Methods].

* 1. Molecular frustration

Both database-derived and alphafold-generated PDB structures were analysed using the frustratometer server (<http://frustratometer.qb.fcen.uba.ar/>). Plots produced by the server were annotated with correct coordinates accounting for the missing structure.

* 1. Linear epitope prediction and database matching

B cell epitope prediction was performed on several reference sequences of HHV gB (github). Additionally, the database was searched for direct linear epitope matches to the reference HCMV Merlin gB sequence with desired BLAST similarity set at >90%, only including epitopes that were obtained through positive assays. The final search and file export was conducted on 21st of May 2024. Epitopes recorded with an indication of PTM were removed and their non-adulterated counterparts were used further. Out of 185 output epitopes, Y had the PTM notation altered. The epitopes were then aligned to the reference sequence both with the use of MAFFT and manual sequence editing where needed. Finally, any identical epitope sequences were omitted. The resultant MSA can be found in (github).

1. *In vitro* work
   1. VZV production and harvest

While HCMV, HSV-1, and HVS were all amplified and harvested in a similar manner described in detail in [generic methods viral harvest], VZV required some special adjustments due to a variety of biological differences in viral lifecycle [rewrite that?]. VZV was amplified in epithelial cells from a vial gifted to us by colleagues in Prof Judith Breuer group (UCL). Upon removing the media from the T175 flask, the virus vial was made up to 2mL with warm media containing 2% FBS and introduced onto the cells. The flask was transferred into a 37°C incubator for 2 hours, and then into a 34°C incubator for further culture. The flask was then monitored for CPE and plaque formation and the virus was harvested when about 60% of the cell monolayer was comprised of plaques.

Low titres were first amplified in a T75 flask until 60% CPE. After removing the media from the flask, the cells were scraped with a sterile scraper and resuspended in 2mL of 2% FBS media. The suspension was introduced onto a T175 flask with nearly confluent epithelial cells. Similarly to the previously described process, the new production flask was first kept in a 37°C incubator for 2 hours before being moved into a 34°C incubator for further culture.

Since VZV is highly cell-associated, I was not able to obtain the virus simply by harvesting the media above the infected monolayer, like with HCMV or HSV-1. To do that I introduced 5mL of mitomycin-C (manufacturer) made up to 0.05mg/mL in FBS-free media to the flask, before incubating it at 37°C for 3 hours. After the incubation, the cells were washed with PBS and trypsinised similarly to a regular cell splitting protocol. After 5 minutes, trypsin was deactivated with excess media and then removed by centrifuging for 5 minutes at 500g. The cell pellet was resuspended with 2mL of freezing media (45% FBS, 45% complete media, 10% DMSO), aliquoted, and kept at -80°C for further use.

* 1. Monoclonal antibody production and harvesting

Two (maybe even more????) hybridomas were available for murine AD-6 monoclonal antibody production, 12B2 and 14F71, both produced by colleagues from Dr Marco Thomas’ group (Marco’s uni) with the methods described in detail in (ref to marco’s paper if its published). The cells were transferred into RPMI supplemented with IgG-depleted FBS (Capricorn manufacturer) and monitored for cell death for several days. Once more than 50% of cells became permissive to Trypan blue (manufacturer) all liquid in the flask was harvested. Cells and cell debris were removed by centrifuging at 1000g for 5 minutes, followed by sterilising via running through a 0.22mm filter ( filter manufacturer, vacuum pump manufacturer). Preliminary analysis to confirm presence of antibodies specific to HCMV AD-6 was done on unpurified supernatant. For all other analyses the supernatant containing the monoclonal antibodies was stored at 4°C before antibody purification through a proteinG column (manufacturer) as described below.

Prior to the purification of antibody harvest, the column (manufacturer) was set up with 500µL of homogenised protein G beads (manufacturer). The column was then washed with sterile PBS before introducing the previously harvested supernatant. After adjusting the flow-through rate to be slow enough, the column was left overnight to let the antibody bind to the beads. The following day the column was washed again with excess of sterile PBS before eluting the bound antibody with glycine buffer (pH 2.0) into a prepared volume of Tris-Base (ask Liv who the manufacturer is). All steps up to this point were done at 4°C.

The mixture was then transferred into a 50KDa filter (manufacturer), centrifuged for 10 minutes at 3200rpm (look up g) and washed at least 3 times with sterile PBS until the retentate volume was below 500µL. Retentate was collected, and the concentration of the purified monoclonal antibody was estimated.

Each of the fractions was collected and had the concentration of IgG estimated.

Optimisation process involved upscaling the production process to multiple harvest flasks at a time, as well as [changing the beads? ] as the original harvest would seemingly not bind the Protein G beads.

* 1. Polyclonal antibody production

Custom rabbit polyclonal antibody against HCMV AD-6 peptide was purchased from Genscript UK. The amino acid sequence of the peptide immunogen used for rabbit immunisation can be found in the [Peptide table].

* 1. Spread assays

While the four of the analysed viruses each required slightly different experimental conditions to assess their cell-to-cell spread, the core principle of spread assays stayed the same.

First, the monolayer of relevant and virus-appropriate cells were infected at low MOI (around 1%). After a short period of time (time A in table X) allowing the infection to be established, the virus was removed and swapped for a relevant dilution of AD-6 polyclonal antibody with or without added semi-viscous overlay. The infection was then left to spread over a longer period (time B) followed by fixing the plate using a standard protocol. For HCMV spread assays or in other instances of long-lasting experiments (> 5 days) the AD-6 polyclonal antibody and media would be refreshed on day 5 by removing the old supernatant and adding an identical dilution of the antibody.

For relevant viruses a semi-viscous carboxymethyl cellulose (CMC) overlay was introduced to the assay in order to encourage the virus stay cell-associated and reduce the formation of *de novo* plaques. CMC stock was prepared by slowly adding 3g of high viscosity CMC (manufacturer) and 3g of low viscosity CMC (manufacturer) to a beaker of 80mL ddH2O and 120mL PBS. The mixture was shaken and warmed up continuously until homogenous. After autoclaving, the mix would be aliquoted to 50mL portions. When needed for the assay, 50mL aliquots would be made up with 325mL of complete media and mixed well until homogenous.

Table X . Protocol specifications for herpesvirus spread assays. Exact values for both A and B were obtained through protocol optimisation and repeat experiments with varied parameters.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Virus | Cells | Time A | Time B | CMC overlay needed? |
| HCMV | HFF | 24h | 9 days | No (cell-associated strain was used) |
| HSV-1 | ARPE-19 | 6h | 2 days | Yes |
| HVS | OMK | 1h | 5 days | Yes |
| VZV | ARPE-19 | 4h | 3 days | No (VZV is naturally highly cell-associated) |

[biorender of generic protocol showing where A and B are? ]

* 1. Neutralisation assays

For HCMV and HSV-1 neutralisation assays I followed the protocol described in [Generic Neutralisation Methods]. The AD-6 polyclonal antibody as well as several monoclonal antibodies were tested on their ability to block viral entry.

Additionally, HCMV AD-6 peptide and HSV-1 AD-6 analogue peptide were used as the neutralising agent for both HCMV and HSV-1 assays in order to assess the role of AD-6 in viral entry.

2.6 Quantification and validation

For all cellular assays the level of virus-associated fluorescence (with immunostaining where applicable) was measured as the primary quantitative result. For some assays, the plate was processed further by running a validation q-RT PCR with relevant primers on extracted DNA.

2.7 ELISA (include pab and mab and different proteins?)

The protocol described in [Generic Methods ELISA] was followed with the parameters specified below.

2.7.1 Polyclonal AD-6 antibody

The plate was coated with HCMV AD-6 peptide (ref to peptide table), AD-6 analogue peptides from HHV (ref to peptide table), and AD-6 analogue peptides from animal CMV (ref to peptide table), made up to 1µg/mL in coating buffer. AD-6 polyclonal antibody was used as a primary antibody, and an anti-rabbit HRP as a secondary antibody (manufacturer).

2.7.2 Monoclonal AD-6 antibodies

While the plate was coated in identical manner as in [2.6.1], the respective murine AD-6 monoclonal antibody produced from B-cell hybridoma cultures was used as a primary antibody, and an anti-mouse HRP was used as a secondary antibody (manufacturer).

2.8 Binding assay

I modified an existing protocol for His-tag protein purification on a Ni column (HisPur™ Ni-NTA Purification Kit, Thermo Fisher).

Custom His-tagged AD-6 peptide was (ref to peptide table) ordered. Protein structure prediction confirmed that the tagged AD-6 peptide forms a secondary structure similar to the untagged peptide.

Equilibration (10mM imidazole), washing (25mM imidazole), an elution buffers (250mM imidazole) were made according to the manufacturers’ protocol with the pH further adjusted to 7.5 using diluted HCl.

Upon equilibrating the 0.2mL column to room temperature, 400µL of equilibration buffer was loaded before its removal via centrifuging at 700g for 2 minutes. Then, the column was washed with 400µL of washing buffer and centrifuging in a similar manner.

1mg (25µL at 40mg/mL) of the His-tagged peptide was made up to 400µL with equilibration buffer and loaded onto the column with the bottom cap on. The peptide was left to bind the resin for 30 minutes on an orbital shaker at 4°C, after which the cap was removed and excess buffer removed by centrifuging like before. The cap was replaced and 50µL of viral harvest was loaded onto the column. The virus was left to bind the peptide for 30 minutes on an orbital shaker at 4°C. Upon removing the cap for the last time, the column was centrifuged again and the flow-through fraction was collected. Lastly, the elution fraction was collected by adding 400µL of elution buffer onto the column and centrifuging. All collected fractions were stored on ice before desalting.

An identical procedure was performed side-by-side using a negative control column without any bound peptide.

[biorender visual ?]

All collected fractions, as well as the plain virus control, were further desalted with the use of Desalting Columns (manufacturer) to remove excess imidazole for further cellular assays, without any deviations from the manufactrurer’s protocol.

Finally, immediately after desalting 10µL of relevant fractions were then used to establish infection in a cell monolayer in 96 well plate. Additionally, all desalted fractions were collected for a further DNA extraction and an HCMV-targeted q-RT PCR. Meanwhile, the cells were fixed at 24hpi and standard immunostaining was implemented to visualise infection.

Optimisation of this protocol included increasing the quantity of peptide and reducing the amount of virus introduced onto the column. Due to the ideal quantity of peptide being very high for a single experiment (20% of the 5mg original peptide order) and the need for protocol optimisation, the assay was only performed with HFF cells and a Merlin HCMV strain.