Chapter 2 (AD2) Materials and Methods

1. Experimental rationale

Phylogenetic analysis of a multiple sequence alignment (MSA) compiled from up-to-date HCMV gB amino acid sequences has confirmed the AD-2 variation presenting itself in a form of set alleles, mirroring the multiallelic region XX in Charles et al (Oscars paper). Further exploration of the differences between the AD-2 alleles discovered that the predicted patterns of post-translational modifications (PTM). I focused on the different glycosylation profiles of AD-2 alleles, as AD-2 site 2 is one of the most heavily glycosylated parts of gB, potentially providing a glycan shield to AD-2 site 1 - the well-known target of highly neutralising antibodies.

The “ideal experiment” to uncover the effect of AD-2 multiallelicity on gB function, AD-2 antibody binding, and subsequent viral phenotype, would focus on isolating AD-2 site 2 variation whilst keeping all other conditions identical. This goal can be achieved using bacterial artificial chromosome (BAC) recombineering of the HCMV genome. The system developed by Prof Rich Stanton from Cardiff University allows for introducing variation into a BAC containing HCMV genome, using the ability of host bacteria to mediate homologous recombination. While the principle seemed straightforward, BAC recombineering proved to be a lot more challenging to perform, ultimately yielding no quantifiable results.

That leads me onto the next-best experiment for investigating AD-2 variation and its effect on AD-2 epitope access: a series of neutralisation assays using the available HCMV strains that possess the representative sequences for AD-2 alleles, and a potent AD-2-specific antibody.

Moreover, the role of PTM and specifically varied glycosylation patterns that are associated with the AD-2 amino acid variation was explored via a variety of enzyme-linked immunoassay (ELISA) experiments. The AD-2-specific antibody binding was quantified against the variable AD-2 peptides obtained via chemical synthesis (no PTM present), in the context of a native whole virion (all PTM present), and finally, in the context of a native whole virion after de-glycosylation (no glycans present, while all other PTM remain).

1. *In silico* work
   1. Main and validation MSA assembly

The amino acid MSA was acquired using the methods described in [General methods].An equivalent MSA was assembled using genomic sequences of HCMV isolated from seropositive solid organ transplant recipients as a part of UCL17-0008 Analysis of Cytomegalovirus Pathogenesis in Solid Organ Transplant Patients (Wellcome collaborative grant 204870/Z/16/Z UKRI) at Royal Free Hospital London, approved by the NRES Committee London Queens Square Ethics Committee (REC reference 17/LO/0916).

First, the genomic sequences plus one reference sequence (Merlin AY446894.2) were aligned using the MAFFT software downloaded locally on my machine. Then, ul55 gene was identified within the resultant alignment using the gene boundaries reported for the reference sequence. The new, smaller, ul55 alignment was then converted to its reverse-complement counterpart to account for the direction of gene transcription. The nucleotide sequences were then converted to the corresponding amino acid sequences, and the resultant gB amino acid alignment was finally re-aligned locally with MAFFT.

* 1. Phylogenetic analysis

Maximum likelihood phylogenetic trees were constructed using IQTree software with WAG substitution matrix and 1000 bootstrap

* 1. PTM prediction

Glycosylation sites within gB amino acid sequence were predicted using a MusiteDeep online tool (link) with default parameters.

* 1. Strain selection for *in vitro* work

Selection of strains for experimental work was determined by the availability of viruses that carry representative variants of AD-2. Four strains were chosen at the end. The nucleotide sequence of ul55 coding for a region that contains AD-2 was amplified via PCR and then sequenced by XXX with the help of colleagues at Breuer lab, UCL. Primers used for the PCR are listed in table Primers\_table

1. *In vitro* work
   1. Recombineering

2.1.1 First round

2.1.2 Second round

2.1.3 BAC Maxiprep

2.1.4 Transfection

MAXIPREPS

Optimising ^^^^^^^

Introducing an extra spin step

* 1. Neutralisation assays

The amount of the virus needed to obtain a consistent level of infection across 4 strains was determined through a series of infection assays with varying viral titres.

On the day of the assay the needed amount of each virus was combined with the relevant dilution of the ITC88 monoclonal antibody (manufacturer) that targets AD-2 site 1. The virus-antibody cocktail was inoculated at 37°C for 1 hour and then introduced onto cells. After 3 hours at 37°C all liquid above cells was removed and changed for plain media.

Online resource “Very Simple IC50 Tool Kit” (link) extensively was used to calculate IC50 values.

* 1. ELISA

Optimisation steps included washing with excess of 6M urea (manufacturer) PBS-T solution after the primary antibody incubation. Ultimately, this step was not improving the outcome quality and was therefore removed for simplicity.

* + 1. Peptide preparation and coating

Custom-made AD-2 site 2 peptides (Peptide table) were purchased from Peptide2.0 US and made up from lyophilised powder with 100% DMSO (manufacturer) to 10mg/mL stock concentration. A -GGC tag was added to each sequence’s C terminus to improve coating efficiency and reduce non-specific binding (ref???).

Each peptide was made up from stock with the coating buffer to a concentration of 1µg/mL. An equivalent dilution of 100% DMSO in coating buffer was used as a negative control.

* + 1. Whole virion preparation and coating

An optimisation ELISA was conducted to determine the amount of virus needed to coat the plate in excess.

* + 1. De-glycosylated whole virion preparation and coating

De-glyc kit (manufacturer)

Additionally, de-glycosylated virions were tested for infectivity [and what happened next???]