A temperature dependent virus binding assay reveals the presence of neutralising antibodies in human cytomegalovirus gB vaccine recipients' sera

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Abstract

Human cytomegalovirus (HCMV) remains an important cause of mortality in immune compromised transplant patients and following congenital infection. Such is the burden an effective vaccine strategy is considered highest priority. The most successful vaccines to date have focused on generating immune responses against glycoprotein B (gB) – a protein essential for HCMV fusion and entry. We have previously reported that an important component of the humoral immune response elicited by gB/MF59 vaccination of patients awaiting transplant is the induction of non-neutralising antibodies that target cell associated virus which is concomitant with little evidence of concomitant classical neutralizing antibodies.

Here we report that a modified neutralization assay which promotes prolonged binding of HCMV to the cell surface reveals the presence of neutralizing antibodies in sera taken from gB vaccinated patients which cannot be detected using standard assays. We go on to show that this is not a general feature of gB neutralizing antibodies suggesting specific antibody responses induced by vaccination could be important. Although we can find no evidence that these neutralizing antibody responses are a correlate of protection in vivo in transplant recipients their identification demonstrate the utility of the approach in identifying these responses. We hypothesise that further characterization has the potential to aid the identification of functions within gB important during the entry process and could potentially improve future vaccine strategies directed against gB if they prove to be effective against HCMV at higher concentrations.

Introduction

Human cytomegalovirus[1] (HCMV) remains a major clinical burden in the population. The problem is exacerbated by the manifold threat posed by primary infection, reinfection as well as reactivation of latent infections in the host[2]. Indeed, HCMV remains the most common viral cause of pathogenesis in the congenital infection setting and is responsible for substantial morbidity in immune compromised hosts[2, 3]. This burden led the US National Institute of Medicine to designate HCMV the highest priority status for the development of a vaccine[4]. Despite substantial progress by a number of research groups no vaccines against HCMV have been licensed.

The most successful vaccination strategy against HCMV to date is based on the viral fusion protein glycoprotein B (gB)[5, 6] which was administered with an MF59 adjuvant[7-9]. The natural host immune response against gB is prodigious and thus gB is considered highly immunogenic. A number of elegant studies have identified and characterised five antigenic domains (ADs) that predominate in the humoral response to gB in naturally infected individuals[10-16]. A proportion of these antibody responses against ADs of gB are neutralizing (nAb) although this is highly variable between HCMV seropositive individuals and complex to measure because virion proteins other than gB are also important targets for neutralizing antibody responses. Indeed, the overall contribution of neutralizing antibodies directed against gB to control of HCMV is an area of debate: neutralizing activity associated with sera from HCMV seropositive individuals is argued to be involve a significant proportion of antibodies that recognize components of additional glycoprotein complexes present in the virion envelope (e.g. the pentameric and trimeric complexes containing gH and gL)[17-22]. Furthermore, it has been hypothesized that aspects of the humoral immune response against gB may actually be detrimental through direct competition for binding epitopes with responses considered protective[23]. In addition to a prodigious humoral response against gB, there is also a strong T cell response against this antigen with responses detected against peptides that span the breadth of the

gB protein[24, 25]. It is this broad immunogenicity coupled with the pivotal role that gB plays in viral infection that has supported the inclusion of gB in multiple HCMV vaccine preparations[26].

Our laboratory is interested in identifying correlates of protection associated with the gB/MF59 vaccine[7, 8, 27] – with the ultimate goal of providing mechanistic correlates of protection to inform future vaccine strategies. We have previously reported on our analyses of sera taken from HCMV seronegative individuals subsequently vaccinated with the gB/MF59 vaccine whilst on the waiting list for solid organ transplant. In these analyses we could not detect evidence of anti-gB antibodies with neutralizing activity using standard assays prior to organ transplant[28]. This was despite clear evidence of a robust humoral response to gB overall in these vaccine recipients – with the magnitude of the response directly correlating with reduced CMV viraemia post-transplant[7]. An inability to detect evidence of a robust neutralizing antibody response was surprising but was independently observed in a separate study of sera obtained from a different gB/MF59 vaccine cohort which addressed the ability of the gB vaccine to reduce HCMV acquisition in women of child-bearing age[29]. Furthermore, the response to the gB vaccine in HCMV seronegative and HCMV seropositive individuals is likely to be different. Initial analyses of the sera from HCMV seropositive individuals suggested the gB vaccine largely boosted pre-existing immune responses against specific epitopes within gB identified in studies of natural infection whereas, intriguingly, the response in HCMV seronegative gB vaccinees revealed that the humoral response was distinct from that seen in response to natural infection[28, 30, 31]. Most recently, we have identified one such vaccine-specific response was directed against epitopes overlapping within Domain V of gB – a response we have called AD6[32].

In direct contrast to our ability to detect nAbs in the sera of taken **pre-transplant**, we noted that we could identify clear evidence of nAbs directed against gB in a number of patients within the same HCMV seronegative vaccine cohort when sera were taken and analysed early **post-transplant**[33].

Two immediate interpretations were potentially valid for these observations: 1) Even though immune-suppressed, patients could rapidly generate novel gB antibody responses post-transplant including neutralizing antibodies or 2) HCMV seronegative vaccine recipients did generate low level nAb responses and these were enhanced upon infection with HCMV following the transplant of an organ from a seropositive donor – essentially a two-step prime:boost event elicited by vaccine and subsequent infection post-transplant with an organ from a seropositive donor.

To investigate the hypothesis that patient sera taken pre-transplant from HCMV seronegative individuals who receiving gB/MF59 vaccine contained low levels of anti-gB neutralizing antibodies we employed a cold neutralization assay that alters the kinetics of HCMV infection at the plasma membrane by allowing virus to bind but not enter the cell but potentially promoting conformational changes in gB required for fusion and entry. Using this approach, we detect evidence of neutralizing antibodies present in the sera of HCMV seronegative individuals who have been vaccinated with the gB/MF59 vaccine **prior to transplant**. Using a panel of HCMV gB antibodies we demonstrate that this is not a general feature of the assay and, in some cases, nAbs from standard assays actually display reduced activity in the modified assay arguing that the protocol does not just 'improve' all neutralizing antibody responses against HCMV non-specifically. Since some of the vaccine recipients proceeded to transplant we could investigate whether this novel activity correlated with any clinical outcomes post-transplant. However, we could find no evidence that the detection of neutralizing antibodies in pre-transplant sera was a statistically significant correlate of protection when tested against a number of clinical parameters. Although in this case no clinical correlation was observed in these limited samples available from the clinical study, they do suggest the presence of nAbs that recognize gB in certain states during the binding and fusion activity of gB during entry and were induced by gB vaccination. Characterisation of these responses that neutralize infection in these assays have the potential to provide new insights in to regions of gB important for function and entry of HCMV.

Materials & Methods

Ethics statement

The study was approved by the UCL Research Ethics Committee and all patients whose samples were investigated here gave written informed consent[7].

Patient Population

The population from whom samples have been evaluated and described in this work is the highest risk cohort of seronegative solid organ transplant patients who were enrolled in a Phase II randomised and double-blinded placebo controlled cytomegalovirus glycoprotein-B vaccine with MF59 adjuvant trial[7]. For post-transplant analyses, HCMV seronegative patients who received an organ from seropositive individuals were selected and have been described elsewhere[33]. In the original study this cohort represents 11 vaccine recipients and 5 placebo, however, for this study removal of consent for future studies and a patient death mean the data presented is from the remaining 9 vaccine and 4 placebo recipients. No other selection criteria were applied. In short, the vaccine or placebo was given in three doses: at day 0 (baseline), 1 month and 6 months later. Following vaccination, blood samples from patients were obtained consecutively. The patients who subsequently underwent transplantation were followed up and tested by real-time quantitative PCR (rtqPCR) for cytomegalovirus DNA[7]. CMV PCR was done on a routine basis with an in-house TaqMan (ABI)–based method as described in detail in Atabani et al [34]. HCMV viraemia was defined as one or more positive HCMV PCR results (cut-off, 200 genomes/mL of whole blood, equivalent to 168 IU/mL). If viraemia higher than 3000 genomes per mL was detected (equivalent to 2520 IU/mL), the patient was treated with antiviral drugs as described in [7]. Exclusion criteria included: pregnancy (a negative pregnancy test was required before each vaccine dose); receipt of blood products (except albumin) in the previous 3 months, and simultaneous multi-organ transplantation[7] .

Cells, virus and media

Human foreskin fibroblasts were routinely cultured in DMEM supplemented with fetal bovine serum (10%) plus penicillin and streptomycin (100ug/ml). For neutralisation assays, cells were harvested by tryspinisation and seeded at 80% confluency 1 day prior to infection in 96 well plates.

The HCMV strain Merlin was propagated in HFFs and purified by density dependent centrifugation. Merlin was used at an MOI of 1-5 in an experiment dependent manner.

Neutralisation assays

To assess sera for neutralising capacity by standard assay, HCMV was pre-incubated with heat inactivated sera for 1 hour and then used to infect HFFs. Alternatively, virus was incubated with anti-gB antibodies HCMV37 (abcam) or QG1 IgG and IgA monoclonal antibodies targeting AD-2 of gB and have been characterised previously[35]. After 24 hours cells were fixed and stained for IE gene expression using anti-IE (Millipore; 1:2000) and goat anti-mouse Alexafluor 568nm (Life Technologies; 1:1000). Nuclei were counterstained with DAPI (SIGMA). Percentage infection was enumerated using Hermes WiScan instruments and software.

To perform the modified neutralisation assay, HFFs were infected at +4^{o}C for 1 hour with HCMV. Then antibody or sera was added directly to the target well and incubated at +4^{o}C for an additional 1 hour. Cells were then shifted to 37^{o}C to promote virus internalisation, washed after 2 hours with PBS, and infection scored as described for a standard assay (above).

For experiments testing the effect of complement 5% guinea pig complement (SIGMA) was added to the heat inactivated sera at the time of incubation of sera with the virus:cell infections.

Results

Routinely, we interrogate sera for the presence of neutralizing antibodies using a standard assay that incubates cell free HCMV with sera for 1 hour prior to infection of permissive cells. Using this approach we could find little evidence for neutralizing antibody activity in the sera of a cohort of individuals who were HCMV seronegative prior to gB/MF59 vaccination[7, 28]. However, in an unrelated study of an inhibitor of viral entry (DIDS) we noted that pre-absorption of HCMV virions to the cell at +4^{o}C dramatically changed the antiviral profile of DIDS[36] and thus we decided to test what impact pre-absorption of the virus had on neutralizing antibody activity against HCMV. To do this, HFFs were first inoculated with HCMV at +4^{o}C for 1 hour then incubated with sera from seronegative vaccine recipients either pre vaccine (day 0) or post vaccine (day of transplant) for a further 1 hour also at +4^{o}C. The tissue culture plates were then shifted to 37^{o}C to trigger viral entry and infection scored by immunostaining for IE positive cells 24hpi (Fig. 1). As expected, the sera taken pre vaccine had no impact on virus infection but, interestingly, the data show clear evidence of virus neutralization is observed under these experimental conditions in some but not all patient sera taken post vaccination (Fig. 1A). A more detailed analysis of each individual serum sample taken from renal (Fig. 1B) and liver (Fig. 1C) transplant candidates revealed that the neutralization reached significance compared to seronegative control sera. In contrast, and consistent with a previous report[28], these same sera did not neutralize HCMV infection in a standard assay in both pre and post gB vaccination samples (Fig. 1D,E) suggesting that +4^{o}C assay could be revealing potential neutralizing antibody responses.

Before investigating this further we decided to test whether the ability to detect evidence of neutralizing antibody activity in our vaccine recipients pre-transplant sera using this modified assay could simply reflect an artefact of an in vitro experimental approach that amplifies the activity of low-level neutralizing antibody responses against gB. Thus we tested whether this approach enhanced the ability of known gB antibodies when they were used at concentrations which elicited partial neutralization of viral infection. Our first analyses focused on the gB antibody HCMV37[37]. First we identified a concentration at which HCMV37 partially neutralized HCMV infection under our standard assay conditions (Fig. 2A). Next, we tested whether HCMV37 was able to neutralize HCMV under our modified conditions. To do this, cells were incubated with HCMV for 1 hour at +4^{o}C and then incubated with HCMV37 for a further hour at +4^{o}C before shifting to 37^{o}C to promote internalisation. As expected, HCMV37 (10ug/ml) partially neutralized HCMV which was lost at a higher dilution in a standard assay (1ug/ml; Fig. 2B). However, neutralizing activity associated with HCMV37 was completely lost in our modified assay (Fig. 2B) and thus opposite to our observations with vaccine patient sera (Fig. 1). To investigate whether this was HCMV37 specific, we took advantage of two monoclonal antibodies directed against AD2 that we have previously reported to neutralize HCMV infection[35]. Using the same experimental approach, we incubated HCMV with QG1 IgG and IgA monoclonal antibodies directed against the AD2 epitope as before at concentrations known to promote partial neutralisation. Similar to the data with HCMV37, we observed that the neutralizing activity of both the IgG and IgA of QG1 was more efficient if pre-incubated with HCMV virions in a standard neutralization assay. Specifically, for both antibodies the neutralizing activity was again lower when added to cells post virus absorption at +4^{o}C although, unlike with HCMV37, neutralizing activity was not completely abolished (Fig. 2C). Thus whilst the pre-binding of HCMV to the plasma membrane at +4^{o}C was potentially revealing neutralizing antibody activity in our vaccine sera it was not enhancing the activity of all the gB neutralizing antibodies used routinely in the laboratory.

To understand the relative importance of the detection of antibodies with neutralizing activity in our vaccine recipient sera under these modified experimental conditions we investigated whether one explanation for the observed neutralization in some patients' sera was that individuals who made large overall responses to gB[7] were also the individuals with evidence of neutralizing antibodies under these experimental conditions. To investigate this possibility the 'neutralising activity' and total gB antibody titre was determined from the previous clinical study[7] and analysed for correlations (Fig. 3). A very simple analysis of the two cohorts where sera was stratified into those with and without evidence of neutralizing antibodies pre transplant (based on a significant decrease in viral infection in the neutralization assay in Fig. 1A-C) showed that the mean gB antibody titre between the two groups was significantly higher in the individuals we detected nAbs although there was a substantial range to the gB IgG titres in both groups (Fig. 3A). Next we investigated whether we could find any correlation between antibody titre and neutralizing activity specifically in the sera of patients we identified as displayed neutralizing activity. Neutralising activity (0% infection = 100% neutralizing activity) was plotted against gB antibody titre (Fig. 3B). Inspection of the plot suggested that with increasing antibody titre there was an increase in neutralizing activity in the sera (Fig. 3B). However, this correlation was non-significant by both Pearson and Spearman Rank analyses although we noted that the p value for the Spearman Rank analysis was approaching a value of $p<0.05$ (Fig. 3B). Although not conclusive, it does suggest the possibility of a monotonic relationship between titre and neutralizing activity. Thus whilst total gB IgG titre was an important factor it may not necessarily be the sole determinant of neutralizing activity in the assay.

Previously, we have observed that HCMV seronegative individuals who were vaccinated prior to transplant displayed a rapid increase in gB antibody titre if the organ received was from an HCMV seropositive donor[33]. Additionally, we observed that this increase in antibody titre also revealed the presence of gB neutralizing antibody responses in post-transplant sera by our standard assays[33].

Thus we next asked whether the individuals with detectable neutralizing antibodies post-transplant were the same individuals in who's sera we detected neutralizing antibodies pre-transplant using our modified cold binding neutralization assay (i.e. in Fig. 1). To do this, we analysed the data from the vaccinated R- individuals who received a D+ organ who had been assessed for neutralizing activity in their pre and post-transplant sera. Ten patients fitted the criteria for the comparison. We plotted the relative neutralizing activity of the pre-transplant (modified assay) and post-transplant (standard assay) sera to test whether there was any correlation. Interestingly, the data show that individuals with evidence of neutralizing activity pre-transplant did not necessarily display the strongest neutralizing antibody response post-transplant (Fig. 4). Consistent with this, there was no direct correlation between the levels of neutralizing activity observed from paired sera taken pre and post transplant from the same patient (Fig. 4). For example, three individuals with high levels of neutralizing antibodies post-transplant had no detectable neutralizing antibodies pre-transplant in the cold modified assay.

The utility of these serum samples is that retrospective analyses of parameters measured in the clinical study of the gB vaccine can be performed. Thus we investigated whether the detection of neutralizing antibodies in our modified assay was an indicative correlate of protection post-transplant. To investigate this, patient sera were again stratified into those that did and did not have detectable neutralizing antibodies in our modified assay as described previously (i.e. in Fig. 3). We focused our analysis on seronegative individuals who had received the gB vaccine and then received an organ from an HCMV seropositive donor. In all, 13 patients fitted these criteria – with 7 individuals displaying significant evidence of nAbs pre-transplant and 6 with no significant evidence of nAbs pre-transplant. A tabular analysis showed that 6/7 individuals with detectable nAbs had evidence of viraemia and 3/6 of individuals without nAbs had evidence of viraemia with two requiring anti-viral treatment (summarized Table 1). An analysis of specific clinical parameters provided no evidence that the

presence of nAbs pre-transplant was a predictive correlate of protection (Fig. 5). Specifically, no statistical significant difference in peak viral load, length of anti-viral treatment and duration of viraemia was observed between the two cohorts (Fig. 5A-C). However, we have also observed that gB IgG titre correlated with neutralizing antibody levels (Fig. 3A)and thus a lack of correlation was puzzling given that gB IgG titre was the correlate of protection in the previous phase II study [7]. However, when we analysed the HCMV seronegative vaccine recipient sera just from patients who received a D+ organ there was no statistical difference between those displaying neutralizing antibodies versus those that did not (Fig. 5D) arguing the correlation of gB IgG titre with clinical outcome is much stronger than we report for neutralizing antibody activity here.

These studies thus far had focused on the capacity of antibodies to 'neutralize' HCMV infection in an absence of complement. Thus in effect are acting as blocking antibodies that limit gB activity and function. However, it is well established that the repertoire of gB neutralizing antibody responses incorporate both complement independent [15] and complement dependent [38, 39] activities and it has also been demonstrated that complement can enhance neutralizing activity of antibodies produced in response to gB/MF59 challenge [40]. Thus we tested whether the addition of complement to the assay could enhance any neutralizing activity we observed (Fig. 6). First we confirmed complement did not specifically promote neutralization with HCMV seronegative sera and also did observe it had a minor enhancing effect on seropositive sera at the dilution used (Fig. 6A). Next we then performed a side-by-side analysis of all 30 heat inactivated patient sera samples with or without the addition of complement (Fig. 6B-E). The data suggest that addition of complement increases the general level of neutralizing activity observed but just failed to reach significance (Fig. 6B) when the means of the two conditions were compared. However, when the data was de-convoluted into 10 paired analyses it was clear that complement enhanced some but not all patient sera which would be

consistent with patient sera containing repertoires of complement and complement independent neutralizing antibodies(Fig. 6C-E).

Discussion

Standard neutralization assays (which we and others employ routinely) incubate cell free HCMV with sera or antibodies and then measure infection either via immuno-staining for viral gene expression or the gold standard plaque reduction assay. In this short report, we demonstrate that inclusion of a +4^{o}C virus binding step to the assay has the potential to detect low levels of neutralizing antibodies directed against HCMV. In this case this was demonstrated using sera obtained from a phase II study of the gB/MF59 vaccine in individuals on the waiting list for organ transplantation[7].

The motivation for the development of this approach was not to replace existing assays – the +4^{o}C step to promote viral binding is clearly not directly applicable in vivo – but driven by our desire to better understand the composition and ontogeny of the immune response in our gB vaccine recipients. Previously, we and others have reported that the gB/MF59 vaccine does not induce readily detectable neutralizing antibody responses in cohorts from two phase II clinical trials[28, 29] and thus concluded that there was no evidence that neutralizing antibodies could be claimed as a correlate of protection. However, following natural infection, gB neutralizing antibodies are clearly made thus why the vaccine was comparatively poor at inducing neutralizing antibodies remained unclear[26]. A possible explanation is that the mutations introduced into the gB vaccine alter the structure. For example, the transmembrane domain has been deleted (to facilitate secretion for purification) and the furin cleavage site also has been mutated. Furin cleavage is considered important for the formation of the native gB trimer [41, 42] and removal likely leads to an increase in the gB monomer in the vaccine[43]. Indeed, work from Liu and colleagues demonstrated that the pre-fusion structure

of gB could dramatically change the availability of known antigenic domains of gB when compared with the post fusion structure of gB [44] which again would be consistent with different humoral responses against the gB vaccine versus native gB.

Despite this it was still surprising that neutralizing antibodies were largely undetectable in the sera and we remained intrigued that this is not a universal observation: other studies of gB vaccine preparations clearly demonstrate evidence of neutralizing antibody responses directed against other forms of gB[26, 29, 43]. Furthermore, in our own follow up study of sera taken from **post-transplant** gB vaccine recipients we could also detect evidence of an increase in the gB antibody titre which was concomitant with the detection of IgG neutralizing antibodies directed against gB as early as 7 days post HCMV challenge (i.e. 7 days post organ transplant)[33].

When considering the implications of our later study[33] of the gB antibody response in our post-transplant cohort two immediate explanations for the appearance of detectable neutralizing antibody responses directed against gB could be considered: either challenge with the virus (at the time of transplant with HCMV-positive organ) was boosting a small but pre-existing response generated against the vaccine or de novo responses against gB were being formed upon challenge with HCMV. We noted that IgM responses were rarely detected early post-transplant and IgG responses against other HCMV antigens not included in the vaccine developed much later with no difference between vaccine recipient and placebo controls. Furthermore, the development and maturation of IgG immune responses against HCMV is reported to take much longer than 30 days post transplant[45, 46]. This led us to hypothesize that the vaccine primes the humoral immune response against gB which is then rapidly boosted following challenge with HCMV and that a small component of the initial priming response may include gB neutralizing antibodies which become detectable in post-transplant sera using our conventional assays due to an increase in titre.

A clear contradiction to our prime:boost hypothesis was our failure to detect neutralizing antibody responses pre-transplant in the same vaccine recipients[28]. However, we hypothesized that if the initial neutralizing antibody response was low it was plausible that a response was made but below the level of detection in our assays in vitro. Potentially, neutralising antibodies were present but not at sufficient concentrations to be effective against high titres of cell free HCMV used in vitro. This led us to investigate alternative strategies to assess for neutralization including cold neutralization. Using this approach, we identified low but reproducible levels of neutralizing antibodies directed against gB in some, but not all, of our vaccine recipients. The ability to detect neutralizing antibodies in our vaccine sera by this approach prompted a number of potential interpretations.

One interpretation of the data from the vaccine sera was that this method was potentially more sensitive for the detection of neutralizing antibodies. Binding at +4^{o}C changes the association and dissociation rates of antibody binding with a decrease in temperature favouring higher avidity antibodies[47]. Thus, potentially the low proportion of neutralizing antibodies in the vaccine sera are high avidity and the +4^{o}C step favours the binding of these high avidity antibodies over competing low avidity antibodies present in the sera. However, we are cautious of this interpretation as in our original neutralization assays when we incubated HCMV with sera at +4^{o}C prior to infection no overt evidence of neutralization was detected and thus likely does not wholly explain the differences[28]. Furthermore, known neutralizing antibodies against gB were much more effective when used at 37^{o}C compared to +4^{o}C arguing that the protocol itself does not non-specifically enhance neutralizing antibody responses – although of course the assays using purified gB antibodies were not performed in the presence of competing antibodies. That said, we note that it has been demonstrated that a bivalent AD2 antibody (but not an AD4 targeted antibody) can still neutralize HCMV infection post binding using a similar approach[48]. This appears to be partially in contrast to our data since we have previously shown that QG1 monoclonal antibodies used in our study are targeted against AD2[35].

That said, here we demonstrate that our AD2 antibodies are less effective at +4°C compared to 37°C but without detailed side-by-side characterization of different AD2 antibodies it is not sensible to infer too many conclusions apropos the prior study.

A tantalizing alternative explanation for the vaccine sera data presented here is the presence of an antibody response directed against an epitope of gB that is only exposed transiently during the fusion process. The gB trimer undergoes multiple structural changes as it transitions from a pre-fusion to a post fusion form during the entry process[49] with recent evidence suggesting gB antibodies directed against AD-5 display different activity against different forms[50]. At 37°C the exposure of these epitopes is possibly more transient if compared to infection at +4°C where the virus is stalled at the plasma membrane and thus gB could be in a transitioning state for longer. Thus one possibility is that an epitope is being presented for longer in this assay that is being recognized by neutralizing antibodies in the pre-transplant vaccine sera. If this is coupled with the possibility that the gB/MF59 vaccine presented these antigens more effectively then it may explain their detection in the sera of vaccine recipients.

It is tempting to speculate that possibly strategies that could increase the concentration of these antibodies may serve to make them functional against HCMV under normal infection conditions – a hypothesis we considered when trying to explain the neutralizing activity of post-transplant vaccine sera where gB antibody titres are increased. That said, the lack of a correlation between the neutralizing activity of pre and post-transplant sera argues that this is not the explanation here but instead the neutralizing antibodies detected pre and post-transplant may be against different regions of gB. Indeed, neutralizing activity in the post-transplant sera was detected by conventional assays. If the cold neutralization approach identifies antibodies with different neutralization profiles then a lack of correlation, arguably, is unsurprising. Why these responses occur at all is less clear but it may reflect differential presentation of gB epitopes by the vaccine which has been modified[28, 29, 31, 32]. For

instance, the presence of gB monomers in the preparation could lead to responses against epitopes hidden in normal virion associated gB. Additionally, these studies have only been performed in fibroblasts. It could be informative to assess the activity of these neutralizing antibody responses to gB in the context of infection of non-fibroblast cells. Upon infection of epithelial or endothelial cells the presentation of gB epitopes could be different during the process of endocytic entry compared to fibroblasts where binding and fusion occurs at the plasma membrane. Further characterization of specific responses responsible for these observations could lead to the development of antibodies that can be used to probe differential changes in gB structure during the entry into multiple cell types.

Even though these humoral responses are generated against a vaccine preparation of gB, and thus may not be so common in natural infection, it does not mean they could not be important. Pathogens are adept at masking epitopes to limit de novo immune responses against them or to prevent recognition if they are made [51-53]. However, that does not mean if a humoral (or T cell) response can be generated (by vaccination, for example) that such responses could be biologically or clinically important. Our work identifying AD6 is direct example of this [32].

From a clinical perspective, we could find no evidence of neutralizing activity in the sera pre-transplant correlating with protection. Indeed, the trend was actually towards poorer clinical outcomes post-transplant – but we highlight a major caveat that this is a retrospective study not powered to formally address this question and so remain cautious in our interpretation of the clinical implications of this. For example, once we stratified for specific parameters the number of patients eligible for inclusion in the analysis was small. That said, in our previous study of post-transplant sera we did observe a correlation between the detection of neutralizing antibodies post-transplant and better outcomes post-transplant[33] – thus the differential impact as a correlate on outcome of pre and post-transplant

sera neutralizing activity likely explains the lack of a strong correlation between levels of neutralizing activity in paired sera pre and post-transplant.

In summary, we report data from a modified approach for the characterisation of neutralizing antibody responses present at low levels in the sera of gB vaccine recipients and for some patient sera the addition of complement enhanced the neutralizing activity. In doing so, we identify evidence of low levels of neutralizing antibodies in HCMV seronegative patients who had received the HCMV gB vaccine. The importance of these neutralizing antibody responses is not fully understood as we observed no correlation between ability to detect neutralizing responses pre-vaccine with better outcomes post-transplant. This is consistent with the increasing evidence that a component of humoral immunity important for the control of HCMV by the gB vaccine includes non-neutralising antibody effector functions[26, 28, 29, 32, 54, 55]. That said, our drive to present the data from this study is to demonstrate the potential utility of this approach to enrich our understanding of clinical samples and also possibly for the study of HCMV entry and the role of glycoprotein conformation. For example, a panel of antibodies directed against known epitopes within a glycoprotein may show differential neutralizing activity at +4^{o}C and 37^{o}C which could aid the identification of regions important for the function of glycoproteins during the different stages of the complex entry process of HCMV.

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Competing Interests

I.A.B., P.D.G. and M.B.R. are co-inventors on WO Patent WO 2022/129937A1 – HCMV vaccine and antibody target. The other authors declare no competing interests.

Table 1: Thirteen HCMV seronegative individuals who received gB/MF59 vaccine and proceeded to transplant with a D+ organ

Patient	nabs Pre-tx	Days Viraemia	Peak viral load	Anti-viral treatment (days)
2	**N**	**47**	**357470**	**49**
7	**Y**	**147**	**132585**	**151**
8	**N**	**53**	**69051**	**70**
5	**N**	**0**	**0**	**0**
16	**Y**	**102**	**279387**	**115**
9	**N**	**0**	**0**	**0**
19	**Y**	**1**	**206**	**0**
25	**Y**	**14**	**4952**	**15**
28	**Y**	**28**	**23217**	**28**
14	**N**	**0**	**0**	**0**
6	**N**	**23**	**2711**	**0**
22	**Y**	**27**	**195774**	**36**
17	**Y**	**0**	**0**	**0**

References

1. **Gatherer D, Depledge DP, Hartley CA, Szpara ML, Vaz PK et al.** ICTV Virus Taxonomy Profile: Herpesviridae 2021. *J Gen Virol* 2021;102(10).
2. **Griffiths P, Reeves M**. Pathogenesis of human cytomegalovirus in the immunocompromised host. *Nat Rev Microbiol* 2021.

3. **Lazzarotto T, Guerra B, Gabrielli L, Lanari M, Landini MP**. Update on the prevention, diagnosis and management of cytomegalovirus infection during pregnancy. *Clin Microbiol Infect* 2011;17(9):1285-1293.
4. **Arvin AM, Fast P, Myers M, Plotkin S, Rabinovich R**. Vaccine development to prevent cytomegalovirus disease: report from the National Vaccine Advisory Committee. *Clin Infect Dis* 2004;39(2):233-239.
5. **Isaacson MK, Compton T**. Human cytomegalovirus glycoprotein B is required for virus entry and cell-to-cell spread but not for virion attachment, assembly, or egress. *J Virol* 2009;83(8):3891-3903.
6. **Isaacson MK, Juckem LK, Compton T**. Virus entry and innate immune activation. *Curr Top Microbiol Immunol* 2008;325:85-100.
7. **Griffiths PD, Stanton A, McCarrell E, Smith C, Osman M et al.** Cytomegalovirus glycoprotein-B vaccine with MF59 adjuvant in transplant recipients: a phase 2 randomised placebo-controlled trial. *Lancet* 2011;377(9773):1256-1263.
8. **Bernstein DI, Munoz FM, Callahan ST, Rupp R, Wootton SH et al.** Safety and efficacy of a cytomegalovirus glycoprotein B (gB) vaccine in adolescent girls: A randomized clinical trial. *Vaccine* 2016;34(3):313-319.
9. **Pass RF**. Development and Evidence for Efficacy of CMV Glycoprotein B Vaccine with MF59 Adjuvant. *Journal of clinical virology : the official publication of the Pan American Society for Clinical Virology* 2009;46(Suppl 4):S73-S76.
10. **Meyer H, Masuho Y, Mach M**. The gp116 of the gp58/116 complex of human cytomegalovirus represents the amino-terminal part of the precursor molecule and contains a neutralizing epitope. *J Gen Virol* 1990;71 (Pt 10):2443-2450.
11. **Meyer H, Sundqvist VA, Pereira L, Mach M**. Glycoprotein gp116 of human cytomegalovirus contains epitopes for strain-common and strain-specific antibodies. *J Gen Virol* 1992;73 (Pt 9):2375-2383.
12. **Spindler N, Diestel U, Stump JD, Wiegers AK, Winkler TH et al.** Structural basis for the recognition of human cytomegalovirus glycoprotein B by a neutralizing human antibody. *PLoS Pathog* 2014;10(10):e1004377.
13. **Spindler N, Rucker P, Potzsch S, Diestel U, Sticht H et al.** Characterization of a discontinuous neutralizing epitope on glycoprotein B of human cytomegalovirus. *J Virol* 2013;87(16):8927-8939.
14. **Potzsch S, Spindler N, Wiegers AK, Fisch T, Rucker P et al.** B cell repertoire analysis identifies new antigenic domains on glycoprotein B of human cytomegalovirus which are target of neutralizing antibodies. *PLoS Pathog* 2011;7(8):e1002172.
15. **Ohlin M, Sundqvist VA, Mach M, Wahren B, Borrebaeck CA**. Fine specificity of the human immune response to the major neutralization epitopes expressed on cytomegalovirus gp58/116 (gB), as determined with human monoclonal antibodies. *J Virol* 1993;67(2):703-710.
16. **Wagner B, Kropff B, Kalbacher H, Britt W, Sundqvist VA et al.** A continuous sequence of more than 70 amino acids is essential for antibody binding to the dominant antigenic site of glycoprotein gp58 of human cytomegalovirus. *J Virol* 1992;66(9):5290-5297.
17. **Ai Y, Wu C, Zhang M, Jaijyan DK, Liu T et al.** Neutralization Epitopes in Trimer and Pentamer Complexes Recognized by Potent Cytomegalovirus-Neutralizing Human Monoclonal Antibodies. *Microbiol Spectr* 2022;10(6):e0139322.
18. **Vanarsdall AL, Chin AL, Liu J, Jardetzky TS, Mudd JO et al.** HCMV trimer- and pentamer-specific antibodies synergize for virus neutralization but do not correlate with congenital transmission. *Proc Natl Acad Sci U S A* 2019;116(9):3728-3733.
19. **Chandramouli S, Malito E, Nguyen T, Luisi K, Donnarumma D et al.** Structural basis for potent antibody-mediated neutralization of human cytomegalovirus. *Sci Immunol* 2017;2(12).
20. **Kschonsak M, Rouge L, Arthur CP, Hoangdung H, Patel N et al.** Structures of HCMV Trimer reveal the basis for receptor recognition and cell entry. *Cell* 2021;184(5):1232-1244 e1216.

21. **Fouts AE, Comps-Agrar L, Stengel KF, Ellerman D, Schoeffler AJ et al.** Mechanism for neutralizing activity by the anti-CMV gH/gL monoclonal antibody MSL-109. *Proc Natl Acad Sci U S A* 2014;111(22):8209-8214.
22. **Chiuppesi F, Wussow F, Johnson E, Bian C, Zhuo M et al.** Vaccine-Derived Neutralizing Antibodies to the Human Cytomegalovirus gH/gL Pentamer Potently Block Primary Cytotrophoblast Infection. *J Virol* 2015;89(23):11884-11898.
23. **Speckner A, Glykofrydes D, Ohlin M, Mach M**. Antigenic domain 1 of human cytomegalovirus glycoprotein B induces a multitude of different antibodies which, when combined, results in incomplete virus neutralization. *J Gen Virol* 1999;80 (Pt 8):2183-2191.
24. **Sylwester AW, Mitchell BL, Edgar JB, Taormina C, Pelte C et al.** Broadly targeted human cytomegalovirus-specific CD4+ and CD8+ T cells dominate the memory compartments of exposed subjects. *J Exp Med* 2005;202(5):673-685.
25. **Jackson SE, Sedikides GX, Mason GM, Okecha G, Wills MR**. Human Cytomegalovirus (HCMV)-Specific CD4(+) T Cells Are Polyfunctional and Can Respond to HCMV-Infected Dendritic Cells In Vitro. *J Virol* 2017;91(6).
26. **Nelson CS, Baraniak I, Lilleri D, Reeves MB, Griffiths PD et al.** Immune Correlates of Protection Against Human Cytomegalovirus Acquisition, Replication, and Disease. *J Infect Dis* 2020;221(Suppl 1):S45-S59.
27. **Pass RF, Zhang C, Evans A, Simpson T, Andrews W et al.** Vaccine prevention of maternal cytomegalovirus infection. *N Engl J Med* 2009;360(12):1191-1199.
28. **Baraniak I, Kropff B, Ambrose L, McIntosh M, McLean GR et al.** Protection from cytomegalovirus viremia following glycoprotein B vaccination is not dependent on neutralizing antibodies. *Proc Natl Acad Sci U S A* 2018;115(24):6273-6278.
29. **Nelson CS, Huffman T, Jenks JA, Cisneros de la Rosa E, Xie G et al.** HCMV glycoprotein B subunit vaccine efficacy mediated by nonneutralizing antibody effector functions. *Proc Natl Acad Sci U S A* 2018;115(24):6267-6272.
30. **Baraniak I, Kern F, Holenya P, Griffiths P, Reeves M**. Original Antigenic Sin Shapes the Immunological Repertoire Evoked by Human Cytomegalovirus Glycoprotein B/MF59 Vaccine in Seropositive Recipients. *J Infect Dis* 2019;220(2):228-232.
31. **Baraniak I, Kropff B, McLean GR, Pichon S, Piras-Douce F et al.** Epitope-Specific Humoral Responses to Human Cytomegalovirus Glycoprotein-B Vaccine With MF59: Anti-AD2 Levels Correlate With Protection From Viremia. *J Infect Dis* 2018;217(12):1907-1917.
32. **Gomes AC, Baraniak IA, Lankina A, Moulder Z, Holenya P et al.** The cytomegalovirus gB/MF59 vaccine candidate induces antibodies against an antigenic domain controlling cell-to-cell spread. *Nat Commun* 2023;14(1):1041.
33. **Baraniak I, Gomes AC, Sodi I, Langstone T, Rothwell E et al.** Seronegative patients vaccinated with cytomegalovirus gB-MF59 vaccine have evidence of neutralising antibody responses against gB early post-transplantation. *EBioMedicine* 2019;50:45-54.
34. **Atabani SF, Smith C, Atkinson C, Aldridge RW, Rodriguez-Peralvarez M et al.** Cytomegalovirus replication kinetics in solid organ transplant recipients managed by preemptive therapy. *Am J Transplant* 2012;12(9):2457-2464.
35. **Siddiqui S, Hackl S, Ghoddusi H, McIntosh MR, Gomes AC et al.** IgA binds to the AD-2 epitope of glycoprotein B and neutralizes human cytomegalovirus. *Immunology* 2021;162(3):314-327.
36. **Murray MJ, Bonilla-Medrano NI, Lee QL, Oxenford SJ, Angell R et al.** Evasion of a Human Cytomegalovirus Entry Inhibitor with Potent Cysteine Reactivity Is Concomitant with the Utilization of a Heparan Sulfate Proteoglycan-Independent Route of Entry. *J Virol* 2020;94(7).
37. **Kirchmeier M, Fluckiger AC, Soare C, Bozic J, Ontsouka B et al.** Enveloped virus-like particle expression of human cytomegalovirus glycoprotein B antigen induces antibodies with potent and broad neutralizing activity. *Clin Vaccine Immunol* 2014;21(2):174-180.

38. **Britt WJ, Vugler L, Butfiloski EJ, Stephens EB**. Cell surface expression of human cytomegalovirus (HCMV) gp55-116 (gB): use of HCMV-recombinant vaccinia virus-infected cells in analysis of the human neutralizing antibody response. *J Virol* 1990;64(3):1079-1085.
39. **Britt WJ, Vugler L, Stephens EB**. Induction of complement-dependent and -independent neutralizing antibodies by recombinant-derived human cytomegalovirus gp55-116 (gB). *J Virol* 1988;62(9):3309-3318.
40. **Li F, Freed DC, Tang A, Rustandi RR, Troutman MC et al.** Complement enhances in vitro neutralizing potency of antibodies to human cytomegalovirus glycoprotein B (gB) and immune sera induced by gB/MF59 vaccination. *NPJ Vaccines* 2017;2:36.
41. **Britt WJ, Vugler LG**. Oligomerization of the human cytomegalovirus major envelope glycoprotein complex gB (gp55-116). *J Virol* 1992;66(11):6747-6754.
42. **Lopper M, Compton T**. Disulfide bond configuration of human cytomegalovirus glycoprotein B. *J Virol* 2002;76(12):6073-6082.
43. **Cui X, Cao Z, Wang S, Lee RB, Wang X et al.** Novel trimeric human cytomegalovirus glycoprotein B elicits a high-titer neutralizing antibody response. *Vaccine* 2018;36(37):5580-5590.
44. **Liu Y, Heim KP, Che Y, Chi X, Qiu X et al.** Prefusion structure of human cytomegalovirus glycoprotein B and structural basis for membrane fusion. *Sci Adv* 2021;7(10).
45. **Kelsey SM, Newland AC**. Cytomegalovirus seroconversion in patients receiving intensive induction therapy prior to allogeneic bone marrow transplantation. *Bone Marrow Transplant* 1989;4(5):543-546.
46. **Humar A, Mazzulli T, Moussa G, Razonable RR, Paya CV et al.** Clinical utility of cytomegalovirus (CMV) serology testing in high-risk CMV D+/R- transplant recipients. *American Journal of Transplantation* 2005;5(5):1065-1070.
47. **Johnstone RW, Andrew SM, Hogarth MP, Pietersz GA, McKenzie IF**. The effect of temperature on the binding kinetics and equilibrium constants of monoclonal antibodies to cell surface antigens. *Mol Immunol* 1990;27(4):327-333.
48. **Ye X, Su H, Wrapp D, Freed DC, Li F et al.** Recognition of a highly conserved glycoprotein B epitope by a bivalent antibody neutralizing HCMV at a post-attachment step. *PLoS Pathog* 2020;16(8):e1008736.
49. **Burke HG, Heldwein EE**. Crystal Structure of the Human Cytomegalovirus Glycoprotein B. *PLoS Pathog* 2015;11(10):e1005227.
50. **Chandramouli S, Ciferri C, Nikitin PA, Calo S, Gerrein R et al.** Structure of HCMV glycoprotein B in the postfusion conformation bound to a neutralizing human antibody. *Nat Commun* 2015;6:8176.
51. **Kwong PD, Doyle ML, Casper DJ, Cicala C, Leavitt SA et al.** HIV-1 evades antibody-mediated neutralization through conformational masking of receptor-binding sites. *Nature* 2002;420(6916):678-682.
52. **Levitskaya J, Coram M, Levitsky V, Imreh S, Steigerwald-Mullen PM et al.** Inhibition of antigen processing by the internal repeat region of the Epstein-Barr virus nuclear antigen-1. *Nature* 1995;375(6533):685-688.
53. **Murin CD, Wilson IA, Ward AB**. Antibody responses to viral infections: a structural perspective across three different enveloped viruses. *Nat Microbiol* 2019;4(5):734-747.
54. **Jenks JA, Nelson CS, Roark HK, Goodwin ML, Pass RF et al.** Antibody binding to native cytomegalovirus glycoprotein B predicts efficacy of the gB/MF59 vaccine in humans. *Sci Transl Med* 2020;12(568).
55. **Gomes AC, Griffiths PD, Reeves MB**. The Humoral Immune Response Against the gB Vaccine: Lessons Learnt from Protection in Solid Organ Transplantation. *Vaccines (Basel)* 2019;7(3).

Figure Legends

Figure 1 Detection of low levels of neutralizing antibodies in gB vaccine sera pre-transplant A-E) Sera from 30 HCMV seronegative transplant recipients at time of transplant was heat inactivated (HI) and then either incubated with cells pre-infected with HCMV at +4^{o}C (A-C; A=all samples and B&C reflect same analyses but separated into renal (18) and liver (12) transplant cohorts) or with HCMV prior to infection (D,E). Cells were then incubated at 37^{o}C and analysed for IE gene expression by indirect IF staining 24 hours later. Infection was quantified by Hermes wiscan automated counting. For studies of human sera each dot represents the mean of three independent experiments performed in duplicate on individual patient sera. In B&C) a red dot denotes patients with sera that significantly ($p<0.05$) neutralized infection at +4^{o}C compared to the same sera analysed at 37^{o}C (D,E) in a standard assay. Also in B&C control HCMV seronegative sera (Lanes 19 & 13, respectively and control HCMV seropositive sera (Lanes 20 and 12, respectively) are shown.

Figure 2 Neutralising activity of known gB nAbs is not enhanced at +4^{o}C A) HCMV was incubated with HCMV37 antibody at 100, 10 and 1ug/ml for 1 hour prior to infection and scored for neutralizing activity by indirect IF for IE protein expression. **B&C)** HCMV was incubated with HCMV37 (B) or QG1 IgG or IgA (C) prior to infection (Pre) or HFFs were infected at +4^{o}C for 1 hour then incubated for a further hour with HCMV37 (B) or QG1 IgG or IgA (C) before shifting to 37^{o}C to promote infection (Post). At 24hpi, infection was scored by indirect IF for IE protein expression. The mean of four independent experiments performed in duplicate is shown.

Figure 3 Total gB response does not completely predict those with neutralizing antibodies A) The total gB antibody titre in patient sera was calculated for pre-transplant sera from 31 HCMV seronegative vaccine recipients that had been defined as possessing neutralizing (nAb pre-tx) or no neutralizing (no nAb pre-tx) antibodies. A comparison of the means was performed using Mann-U-Whitney test assuming a non-parametric distribution. **B)** Sera from patients identified as possessing

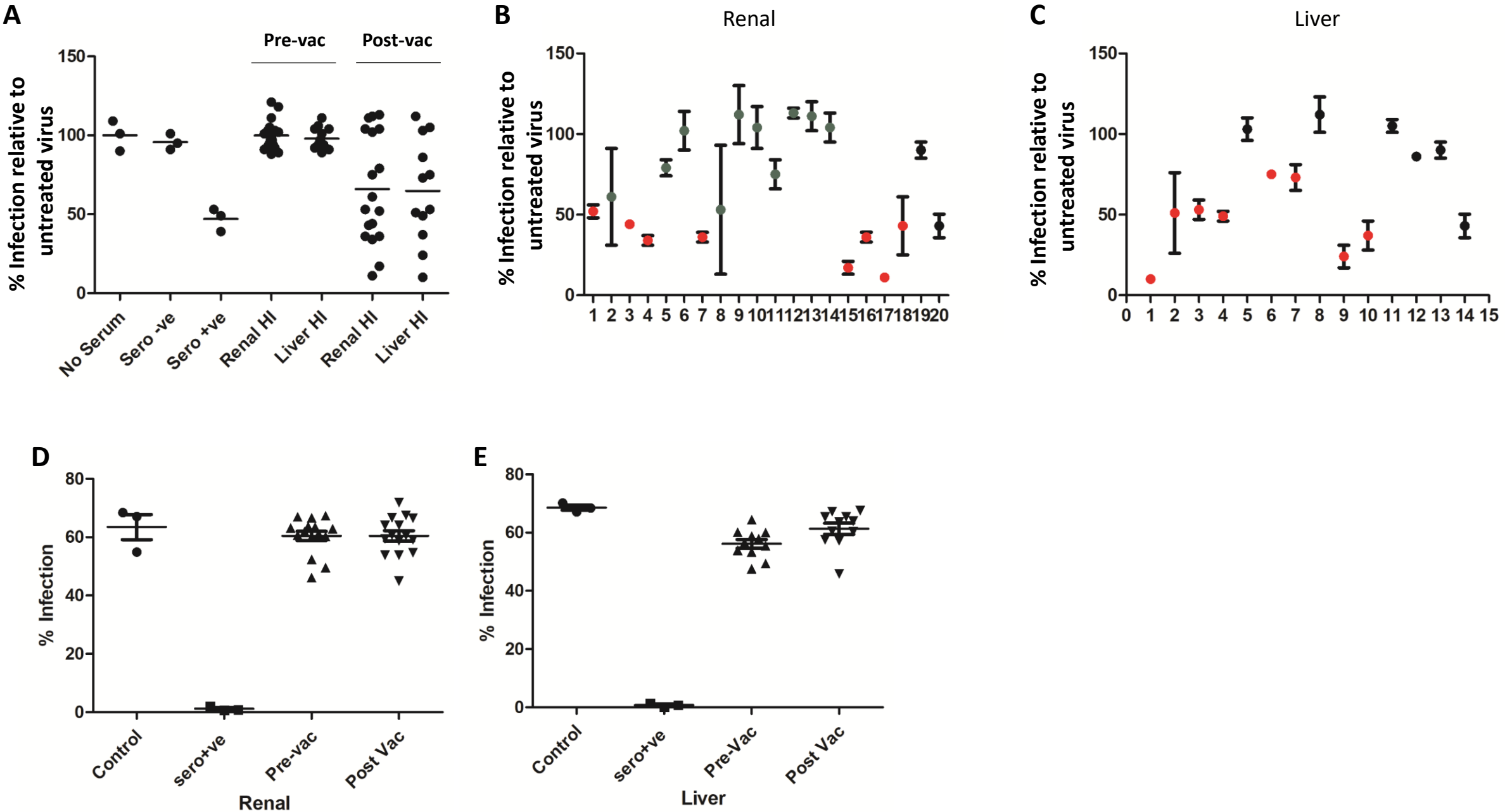
neutralizing activity were then analysed by linear regression comparing neutralizing activity versus antibody gB antibody titre by Pearson and Spearman Rank analysis.

Figure 4 No correlation between detection of neutralizing activity in paired sera from pre and post transplant samples The comparative neutralizing activity of sera of 10 seronegative patients receiving gB/M59 vaccine who proceeded to transplant with a seropositive organ was assessed pre and post-transplant and subject to linear regression analysis for correlation.

Figure 5 Presence of neutralizing antibodies pre-transplant does not predict better outcomes post transplant A-C) Thirteen HCMV seronegative individuals who received gB/MF59 vaccine and then proceeded to transplant with an organ from an HCMV seropositive donor were stratified into those with (nabs pre-tx) and without (no nabs pre-tx) and assessed for peak viral load (A), duration of anti-viral treatment (B) and Total days viraemic (C). Means were compared using Mann-U-Whitney test assuming a non-parametric distribution. **D)** The total gB antibody titre in sera taken from 13 HCMV seronegative patients receiving gB/MF59 vaccine who went onto receive an organ from a HCMV seropositive donor was calculated for pre-transplant sera defined as possessing neutralizing (nAb pre-tx) or no neutralizing (no nAb pre-tx) antibodies. A comparison of the means was performed using Mann-U-Whitney test assuming a non-parametric distribution.

Figure 6 Complement enhances the neutralizing activity of vaccine patient sera in the cold neutralization assay A) Sera from healthy HCMV seronegative (circle) or HCMV seropositive (triangle) was incubated with HFFs previously infected for 1 hour at +4^{o}C in the presence or absence of 5% Guinea Pig complement and then assayed for infection by IE IF and quantified by Hermes WiScan. The % infection is expressed relative to a virus only control (no serum). **B)** Sera from 30 HCMV seronegative

individuals vaccinated with gB was analysed pre-transplant for neutralizing activity as described in (A) with (square) or without (circle) 5% guinea pig complement. A comparison of the means was performed by Mann U Whitney test (B). **C-E)** The cumulative data in (B) was presented as paired samples of sera showing the impact of complement on the activity of individual patient sera (C-E).


A
Pre-vac
Post-vac
% Infection relative to untreated virus
150
100
50
0
No Serum
Sero -ve
Sero +ve
Renal HI
Liver HI
Renal HI
Liver HI
B
Renal
% Infection relative to untreated virus
150
100
50
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
C
Liver
% Infection relative to untreated virus
150
100
50
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
D
% Infection
80
60
40
20
0
Control
sero+ve
Pre-vac
Post Vac
Renal
E
% Infection
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40
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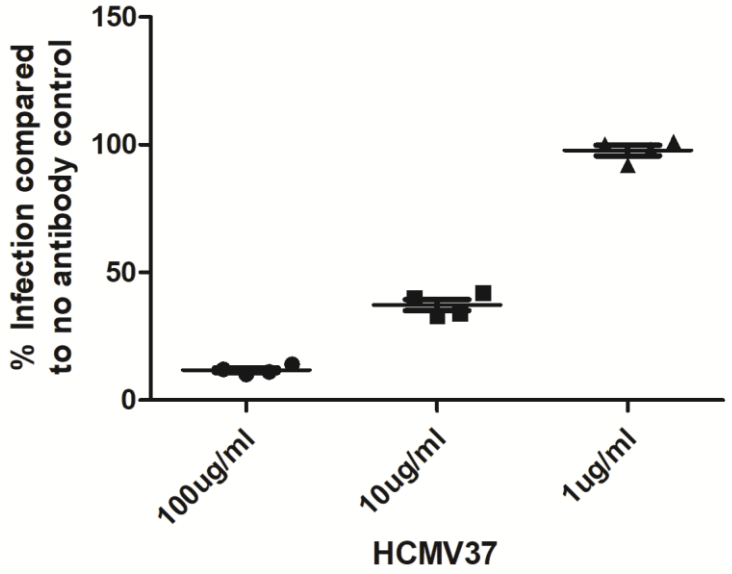
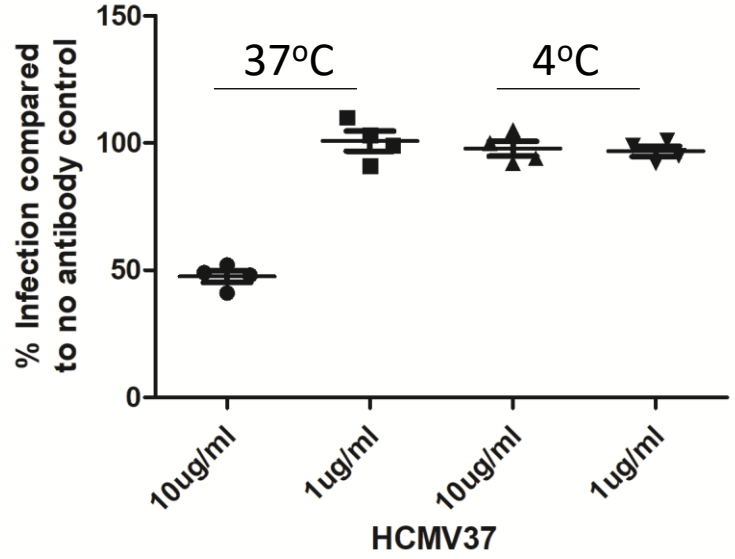
Control
sero+ve
Pre-Vac
Post Vac
Liver

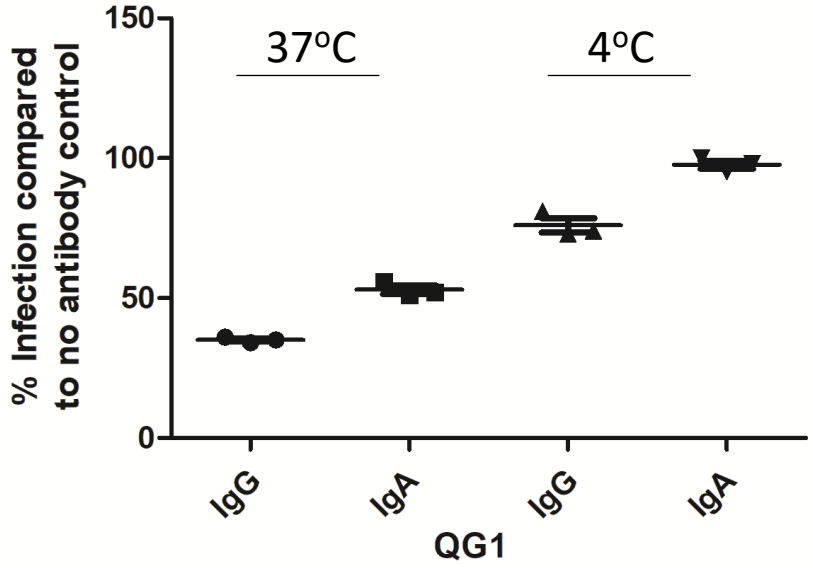
A


% Infection compared to no antibody control
150
100
50
0
100ug/ml
10ug/ml
1ug/ml
HCMV37

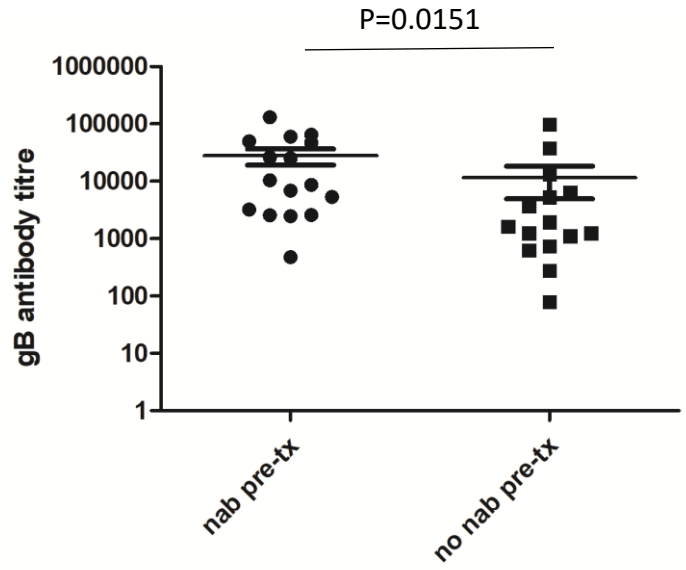
B


% Infection compared to no antibody control
150
100
50
0
37ºC
4ºC
10ug/ml
1ug/ml
10ug/ml
1ug/ml
HCMV37

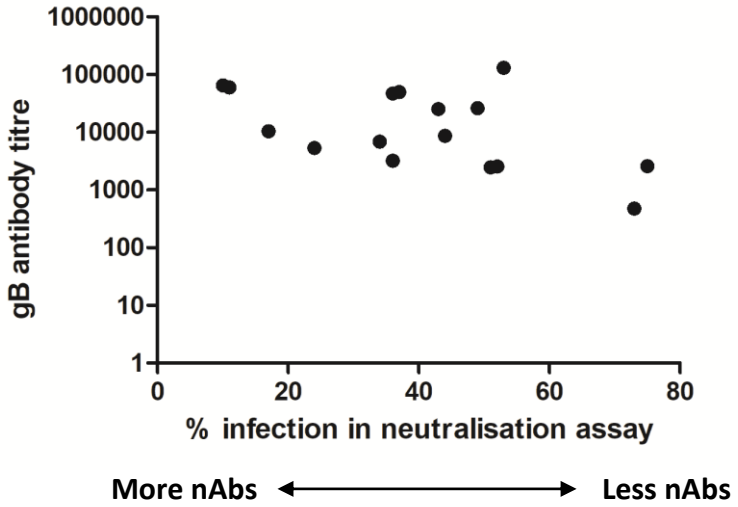
C


% Infection compared to no antibody control
150
100
50
0
37ºC
4ºC
IgG
IgA
IgG
IgA
QG1

A



B



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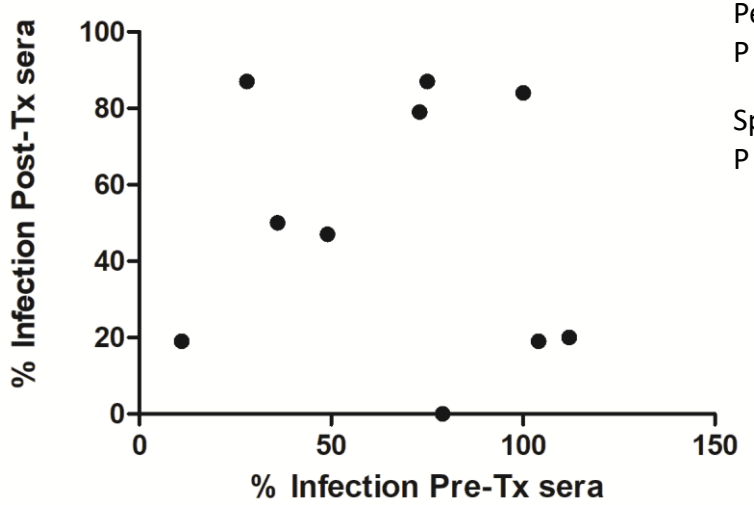
P value	0.3908
Spearman r	-0.4577
P value	0.0746

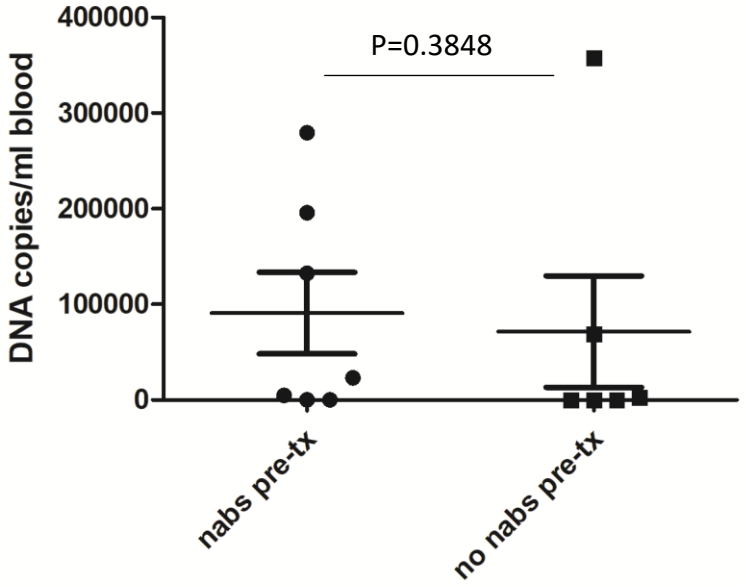
Less nAbs



Pearson r	-0.1120
P value	0.7580
Spearman r	-0.2134
P value	0.5603

More nAbs **Less nAbs**

A


Peak Viral Load
DNA copies/ml blood
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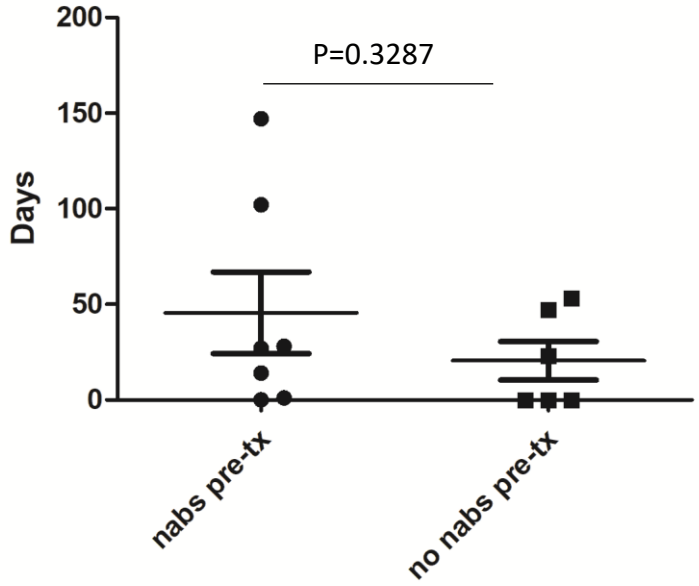
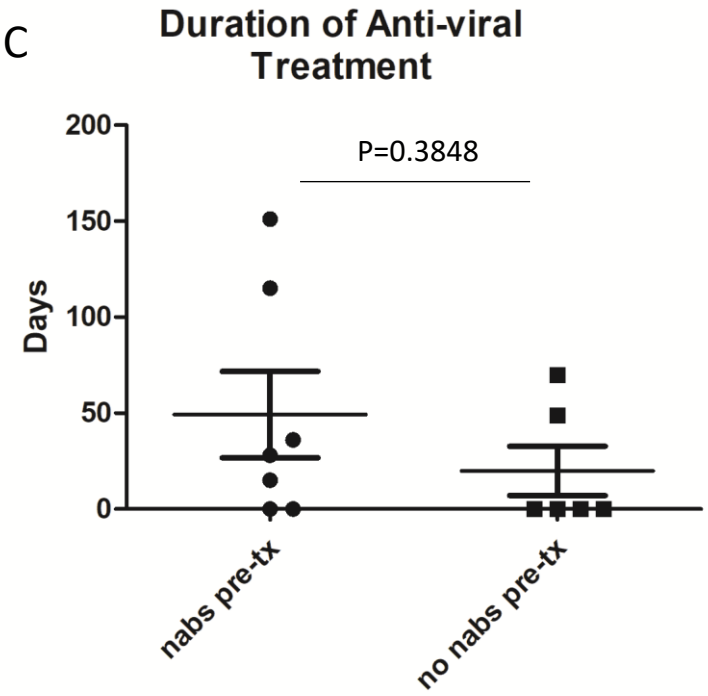
300000
200000
100000
0
P=0.3848
nabs pre-tx
no nabs pre-tx

B


Total Days Viraemic
Days
200
150
100
50
0
P=0.3287
nabs pre-tx
no nabs pre-tx

C


Duration of Anti-viral Treatment
Days
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150
100
50
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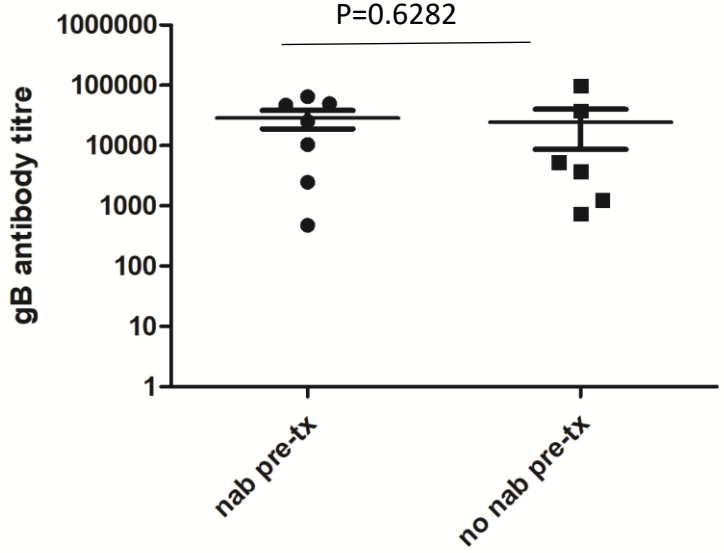
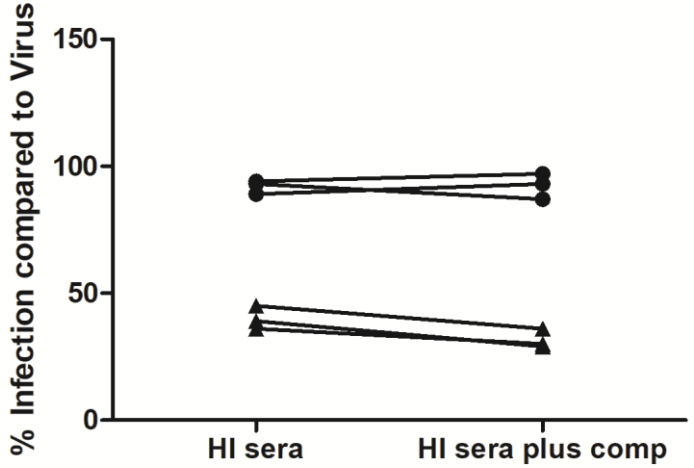
P=0.3848
nabs pre-tx
no nabs pre-tx

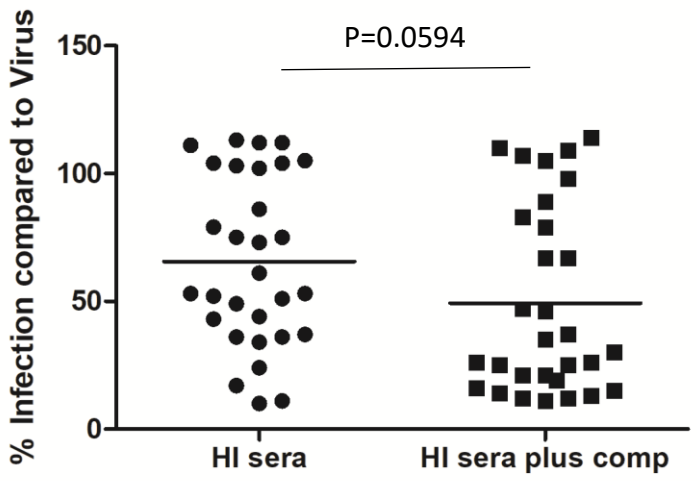
D


gB antibody titre
1000000
100000
10000
1000
100
10
1
P=0.6282
nab pre-tx
no nab pre-tx

A


% Infection compared to Virus
150
100
50
0
HI sera
HI sera plus comp

B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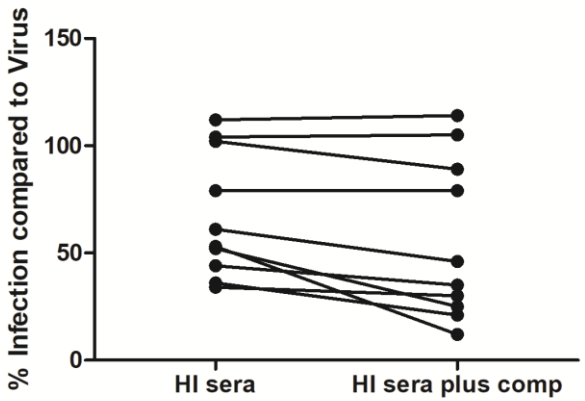
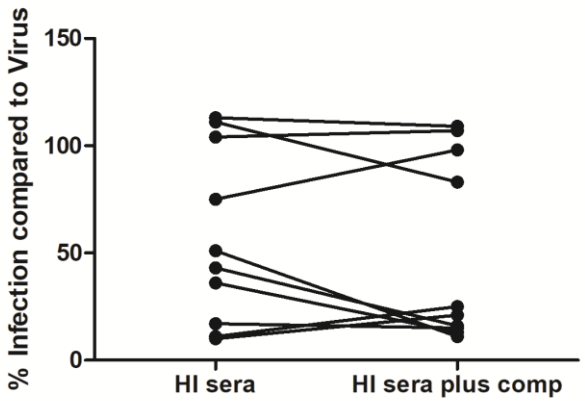
100
50
0
HI sera
HI sera plus comp

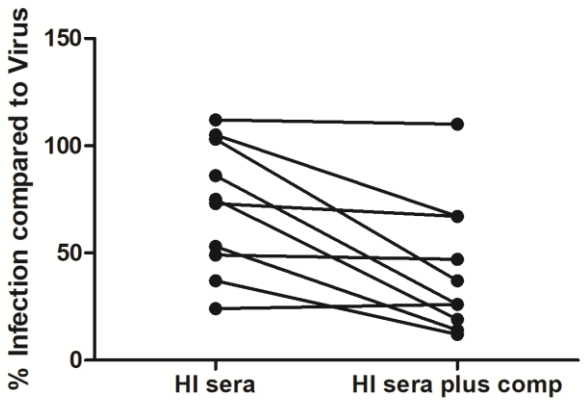
C


% Infection compared to Virus
150
100
50
0
HI sera
HI sera plus comp

D


% Infection compared to Virus
150
100
50
0
HI sera
HI sera plus comp

E


% Infection compared to Virus
150
100
50
0
HI sera
HI sera plus comp