Research paper

Detection of antibody-secreting cells specific for the cytomegalovirus and herpes simplex virus surface antigens

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ABSTRACT

Infections with the herpes simplex virus (HSV) and the human cytomegalovirus (HCMV) can lead to life-threatening diseases, particularly in immunosuppressed patients. Furthermore, HSV infections at birth (herpes neonatorum) can result in a disseminated disease associated with a fatal multiorgan failure. Congenital HCMV infections can result in miscarriage, serious birth defects or developmental disabilities. Antibody-based interventions with hyperimmunoglobulins showed encouraging results in clinical studies, but clearly need to be improved. The isolation of highly neutralizing monoclonal antibodies is a promising strategy to establish potent therapy options against HSV and HCMV infections.

Monoclonal antibodies are commonly isolated from hybridomas or EBV-immortalized B-cell clones. The screening procedure to identify virus-specific cells from a cell mixture is a challenging step, since most of the highly neutralizing antibodies target complex conformational epitopes on the virus surface. Conventional assays such as ELISA are based on purified viral proteins and inappropriate to display complex epitopes.

To overcome this obstacle, we have established two full-virus based methods that allow screening for cells and antibodies targeting complex conformational epitopes on viral surface antigens. The methods are suitable to detect surface antigen-specific cells from a cell mixture and may facilitate the isolation of highly neutralizing antibodies against HSV and HCMV.

1. Introduction

Neutralizing antiviral antibodies have become a potent tool for the treatment and prevention of severe viral infections during the past decades (Marasco and Sui, 2007). Polyclonal immunoglobulin G (IgG) preparations derived from immunized human donors are used against a wide range of viral infections, such as the human cytomegalovirus (HCMV), respiratory syncytial virus (RSV), hepatitis B virus (HBV), rabies and other viral infections (Both et al., 2013). However, the efficacy of such preparations is limited since virus-specific neutralizing antibodies are only a minor proportion of the total pool of polyclonal serum-derived antibodies (Marasco and Sui, 2007). The development of the hybridoma technology for the production of monoclonal antibodies by Köhler & Milstein in 1975 was a mile stone in the antibody field and led to the isolation of numerous monoclonal antibodies (mAbs) (Kohler

and Milstein, 1975). From then on, numerous potent antiviral monoclonal antibodies against H5N1 influenza virus, human immunodeficiency virus (HIV), hepatitis C virus (HCV), Ebola virus, severe acute respiratory syndrome-related coronavirus (SARS-CoV) and other viruses causing dangerous infections were isolated and are currently in clinical studies or even approved for antiviral treatment (Bornholdt et al., 2016; Both et al., 2013; Caskey et al., 2016; Kwong et al., 2013; Pelegrin et al., 2015; Traggiai et al., 2004). Human monoclonal antibodies can be isolated from the B-cells of patients who have recovered from disease (e.g. SARS or Ebola) or are long-term controllers of chronic infections (e.g. HIV or herpesviruses). Several technologies are available including the immortalization of human B-cells with the Epstein-Barr virus (EBV) (Ali et al., 2015; Traggiai et al., 2004), generation of stable human hybridomas (Yu et al., 2008), direct cloning of the heavy and light antibody chains (VH and VL genes) into

phage expression libraries (Diebolder et al., 2014; Marks et al., 1991; McCafferty et al., 1990) or the generation of monoclonal antibodies from single human B-cells by single cell PCR (Tiller et al., 2008; Wardemann et al., 2003). Many of these strategies are time-consuming and laborious. In the case of working with EBV-immortalized B-cells or hybridoma cells, the sorting of antigen-specific donor B-cells prior to culturing can significantly facilitate the identification of rare B-cells secreting neutralizing antibodies (Kodituwakku et al., 2003; Morris et al., 2011; Potzsch et al., 2011; Zhang et al., 2016). For this purpose, donor B-cells are presorted by flow cytometry techniques using recombinant viral glycoproteins. This strategy is highly promising if the target antigen of the neutralizing antibodies is known or if the virus incorporates only one or two surface glycoproteins as potential targets of neutralizing antibodies (e.g. Ebola) (Zhang et al., 2016). In the case of complex viruses like the human cytomegalovirus (HCMV) or the herpes simplex viruses (HSV), an enrichment of antigen-specific B-cells may be highly challenging, particularly if the target antigen is not exactly known. Herpesviruses use complex entry machinery consisting of numerous glycoproteins (Connolly et al., 2011; Sathiyamoorthy et al., 2017). Four viral glycoproteins (gB, gD, gH and gL) are required and sufficient for HSV-1 and 2 entry into host cells (Agelidis and Shukla, 2015). HCMV uses two distinct pathways to enter host cells. While gB represents the fusion protein, two different glycoprotein complexes control the cell tropism of the virus: the gH/gL/gO trimer is involved in the infection of all cell types, while the gH/gL/pUL128/pUL130/pUL131A pentamer is additionally required for the infection of endothelial, epithelial and myeloid cells (Kabanova et al., 2016; Vanarsdall and Johnson, 2012; Zhou et al., 2015). Neutralizing antibodies targeting such complexes often bind to conformation-dependent epitopes (Bender et al., 2007; Chandramouli et al., 2017; Ciferri et al., 2015; Gardner et al., 2016; Macagno et al., 2010; Ohlin et al., 1993; Wussow et al., 2014). Obviously, a critical factor for the successful isolation of monoclonal antibodies from blood donors is a proper screening strategy. The most crucial element of the detection strategy is a highly specific antibody-antigen interaction. A prerequisite to identify such antibodies is the use of a correctly folded antigen or antigen complex. This issue may be simple for viruses using only one glycoprotein for cell entry (e.g. Ebola), but challenging for herpesviruses using highly complex entry machinery. Therefore, conventional screening strategies based on single recombinant proteins may be unsuitable to identify antibodies/B-cells specific for such antigens (de Alwis et al., 2012; Jones et al., 2016). Prior studies have shown that some dengue virus neutralizing antibodies target complex epitopes that exist in a correct conformational state only on the surface of the virion particle, but not on a recombinant soluble antigen (Smith et al., 2014). A promising strategy to improve the recovery of rare neutralizing antibodies to complex epitopes can be the inclusion of a whole virus-based screening of EBV-immortalized B-cells or hybridomas into the standard antibody isolation protocol prior to the amplification of VH/VL genes for antibody production.

In the present study, we describe two distinct methods for the identification of antigen-specific cells secreting virus-specific antibodies directed against HSV and HCMV. These methods may facilitate the isolation of highly neutralizing antibodies targeting complex conformational epitopes.

2. Material and methods

2.1. Cells

The hybridoma cell line secreting the HSV-1/2-glycoprotein B (gB) specific monoclonal antibody mAb 2c (2c-hybridoma cells) was generated by fusion of B-cells from BALB/c mice hyperimmunized with HSV-1 strain 342 hv with murine myeloma cells and maintained in Iscove's modified Dulbecco's medium (IMDM, Life Technologies Gibco, Darmstadt, Germany) as previously described (Eis-Hubinger et al.,

1993). H34 hybridoma cells producing a murine, Friend virus specific monoclonal antibody H34 were kindly provided by Ulf Dittmer (Institute for Virology, Essen, Germany). The cells were cultured in Roswell Park Memorial Institute 1640 medium (RPMI, Thermo Fisher Scientific, Waltham, MA USA). Vero cells (American Type Culture Collection, ATCC, CCL81, Rockville, MD) were cultured in Dulbecco's Modified Eagle Medium (DMEM, Thermo Fisher Scientific). All media were supplemented with 10% (v/v) fetal bovine serum (FBS, Thermo Fisher Scientific), 100 U/ml penicillin and 0.1 mg/ml streptomycin (Thermo Fisher Scientific).

The hybridoma cell line H10 secreting an HCMV-gH specific monoclonal antibody (see supplementing fig. S1) originated from a hybridoma cell library generated by fusing HCMV-immunized mouse B-cells with the myeloma cell line Sp2/0 according to standard polyethylene glycol (PEG) fusion procedures (Falk et al., 2016). The hybridoma cell line 28–77 secreting a monoclonal antibody specific for the HCMV-tegument phosphoprotein (pp65) was described previously (Britt and Auger, 1985). Both cell lines were cultured in RPMI medium supplemented with 10% fetal bovine serum (PAN-Biotech, Aidenbach, Germany), 100 µg/ml gentamicin (Sigma-Aldrich, Darmstadt, Germany), 25 µM 2-Mercaptoethanol (Sigma-Aldrich), 2% murine interleukin 6 (IL-6, 100 U/µl, PeproTech, Hamburg, Germany). Primary human foreskin fibroblasts (HFFs) were cultured in MEM supplemented with GlutaMAX (Life Technologies Gibco), 5% fetal bovine serum (PAN-Biotech), 0.5 ng/ml basic fibroblast growth factor (bFGF, Life Technologies Gibco) and 100 µg/ml gentamicin (Sigma-Aldrich). During infection, bFGF was omitted from HFF-medium (denoted as MEM5). Stably transfected Sp2/0-Ag14 cells secreting an HSV-1/2 specific, humanized antibody mAb hu2c were cultured in DMEM containing 10% (v/v) fetal bovine serum (FBS, Thermo Fisher Scientific), 100 U/ml penicillin and 0.1 mg/ml streptomycin (Thermo Fisher Scientific). Multiple myeloma IM9 cells were kindly provided by Ralf Küppers (Institute for Cell Biology (Tumor Research), Essen, Germany). The IM9 cells, which secrete a human monoclonal antibody not specific for HSV-1/2 were cultured in RPMI containing 10% (v/v) fetal bovine serum (FBS, Thermo Fisher Scientific), 100 U/ml penicillin and 0.1 mg/ml streptomycin (Thermo Fisher Scientific). For the generation of cells transiently expressing a human anti-HCMV antibody, HEK-293 T cells (highly transfectable derivative of the human primary embryonic kidney cell line 293, DSMZ) were transfected (K2 Transfection System, Biontex, Munich, Germany) with two plasmids encoding the heavy and light chain of human IgG 8 J16, a neutralizing antibody directed against the HCMV pentameric complex (Macagno et al., 2010). An eGFP-encoding plasmid (pEGFP-N3, Takara, Mountain View, USA) was used to control transfection efficiency, which was around 90%. Supernatants of 8 J16-transfected cells were specifically neutralizing endothelial cell infection, as intended (not shown). HEK-293 T cells were cultured in DMEM (Life Technologies Gibco) supplemented with 10% fetal bovine serum (PAN-Biotech) and 100 µg/ml gentamicin (Sigma-Aldrich).

2.2. Virus

Recombinant, Fc-receptor (ΔgE) deleted herpes simplex virus 1 (HSV-1 ΔgE) was described previously (Farnsworth et al., 2003), kindly provided by Hartmut Hengel (Institute of Virology, Freiburg, Germany) and propagated and titrated on Vero cells. Virus stocks containing 2×10^7 TCID$_{50}$/ml HSV-1 ΔgE were UV inactivated for 30 min (UV analysis lamp, 254 nm, Herolab, Wiesloch, Germany) and stored at −20 °C. Thawed stocks were then used for coating of microtiter plates.

The GFP-expressing reporter virus RV-TB40-BAC$_{KL7}$-SE-EGFP was generated as described previously, has a self-excisable BAC-cassette, and GFP expression is controlled by a shortened IE-promotor (Sampaio et al., 2017).

2.3. Antibody production and purification

For antibody production, the cells were cultured under serum-free conditions in EX-CELL Sp2/0 Serum-Free Medium (Sigma-Aldrich) supplemented with 100 U/ml penicillin and 0.1 mg/ml streptomycin (Thermo Fisher Scientific). Monoclonal antibodies (mAb) 2c (IgG2a), mAb hu2c (IgG1), H34 and IM9 were purified from serum-free cell-culture supernatants by protein A chromatography (Thermo Scientific, Worcester, MA, USA) as previously described (Krawczyk et al., 2013; Krawczyk et al., 2011) and dialyzed against phosphate-buffered saline (PBS).

2.4. Identification of HSV-specific hybridoma cells and antibodies with a virus-based ELISA assay

Microplates (96-well plates, Greiner Bio One, Kremsmünster, Austria) were coated with an UV inactivated HSV-1 ΔgE overnight at 4 °C. The immobilized virus was then fixed with 2% paraformaldehyde (PFA, Carl Roth, Karlsruhe, Germany) for ten minutes and washed three times with PBS. Non-specific binding was blocked with PBS containing 0.5% BSA (Life Technologies Gibco) for 1 h at room temperature. Subsequently, the plates were washed three times with PBS. Immobilized HSV-1 ΔgE was then incubated with different numbers (500, 100, 50, 25, 10, 5 and 1) of murine hybridoma cells secreting the monoclonal antibody mAb 2c and SP2/0 cells secreting the humanized counterpart mAb hu2c. Both antibodies recognize a conformational epitope on the HSV-1 gB. The cells were diluted in culture medium supplemented with 10% FBS, 100 U/ml penicillin and 0.1 mg/ml streptomycin, respectively. As control, either murine H34-hybridoma cells or human IM9 multiple myeloma cells were added as an unspecific control to reach a total number of 500 cells/well. The cultures were then incubated for 48 h at 37 °C. The plates were washed five times with PBS containing 0.2% Tween 20 thereby lysing the cells in order to remove cells and unbound antibodies. Bound antibodies were detected using an ultra-sensitive ABC mouse IgG staining kit (Thermo Fisher Scientific, cat. no. 32052). The kit includes a goat-anti-mouse (GAM) IgG-specific, biotin-conjugated secondary antibody. The bound secondary antibody was then incubated with avidin-horseradish peroxidase (HRP) complexes to maximize the signal. The secondary antibody in combination with the avidin-peroxidase complex was incubated for 1 h at room temperature, followed by washing three times with PBS 0.2% Tween 20. The same kit was used to detect bound humanized mAb hu2c, but the secondary antibody was replaced by a biotin conjugated goat-anti-human (GAH) IgG-specific secondary antibody (cat. no. 109-065-008, JacksonImmunoReseacrh, Cambridgeshire, UK). TMB-ELISA substrate (Thermo Fisher Scientific) was then incubated for up to 15 min, until a characteristic colour change was detectable. The reaction was stopped with 2 M sulfuric acid (AppliChem, Darmstadt, Germany) and the signal was measured by spectrophotometry (Berthold Technologies, Bad Wildbad, Germany) at 450 nm. Cut-off was defined as a 3-fold value of the PBS control.

To determine the detection limit of this assay, serial dilutions (500–0.03 nM) of purified monoclonal antibody mAb 2c or mAb hu2c were incubated on HSV-1 ΔgE coated plates for 1 h at 37 °C. Bound antibodies were detected as described above.

To determine the sensitivity of the virus-based ELISA assay, decreasing concentrations (250–0 nM) of mAb 2c or mAb hu2c were incubated on HSV-1 ΔgE coated plates and the bound antibodies were measured as described above. A murine, Friend virus-specific control antibody H34 or a human antibody IM9 not specific for HSV-1 was used to generate background values at each dilution (250–0 nM).

2.5. Identification of HCMV-specific cells with an antibody-footprint assay

2.5.1. Detection of HCMV-specific hybridoma cells

Microplates (96-well µClear high-bind black, Greiner Bio One) were coated with a goat F(ab´)₂ anti-mouse IgG, specific for Fcγ (13 µg/ml, Jackson ImmunoResearch, West Grove, PA, USA) in 100 mM carbonate buffer (pH 9.6) overnight at 4 °C. Non-specific binding was blocked with PBS containing 5% milk powder (Sigma Aldrich) for 2 h at room temperature. The blocking solution was removed. Trypan blue (Sigma-Aldrich) was used for counting the hybridoma cells, hence excluding dead cells. Varying numbers of HCMV gH-specific H10– hybridoma cells (e.g. 500, 100, 50, 25, 10, 5 and 1) were mixed with 28–77-hybridoma cells, serving as unspecific control to reach a total number of 500 cells/well. The hybridoma cells were seeded in RPMI medium (Life Technologies Gibco) supplemented with 10% fetal bovine serum (PAN-Biotech), 100 µg/ml gentamicin (Sigma-Aldrich), 25 µM 2-mercaptoethanol (Sigma-Aldrich), 2% murine interleukin 6 (IL-6, 100 U/µl, PeproTech) on the plates with the immobilized goat anti-mouse capture antibody. The cultures were then incubated overnight at 37 °C. In order to lyse the cells, the plates were washed three times with 0.2% Tween 20 in H_2O. The reporter virus RV-TB40-BAC$_{KL7}$-SE-EGFP was diluted in MEM5 to 5×10^6 IU/ml (infectious units per ml) and incubated on the plates for 2 h at 37 °C. The unbound virus was removed by three washing steps with MEM5. HFF cells were seeded on the plate in MEM5 with a density of 2.5×10^5 cells per well. After two days of incubation at 37 °C, foci were visualized with an Axio Observer D1 microscope (Zeiss, Oberkochen, Germany). To visualize all footprints in one well, six pictures were taken at a 40-fold magnification and merged.

2.5.2. Identification of HEK-293 T cells secreting an HCMV specific human antibody

Microplates (96-well µClear high-bind black, Greiner Bio One) were coated with a goat F(ab´)₂ anti-human IgG, specific for Fcγ (15 µg/ml, Jackson ImmunoResearch, West Grove, 216 PA, USA) in 100 mM carbonate buffer (pH 9.6) overnight at 4 °C. Non-specific binding was blocked with PBS containing 5% milk powder (Sigma Aldrich) for 2 h at room temperature. The blocking solution was removed and HEK-293 T cells, transiently expressing an HCMV specific human IgG 8 J16, were seeded on the microplate in a serial dilution. In parallel, stably transfected Sp2/0-Ag14 cells secreting a humanized antibody mAb hu2c were seeded serving as unspecific control. Cells were incubated for two days at 37 °C. In order to lyse the cells, the plates were washed with 0.2% Tween 20 in H_2O and further three times with MEM5. The reporter virus RV-TB40-BACKL7-SE-EGFP was diluted in MEM5 to 5×10^6 IU/ml (infectious units per ml) and incubated on the plates for 2 h at 37 °C. The unbound virus was removed by three washing steps with MEM5. HFF cells were seeded on the plate in MEM5 with a density of 2.5×10^5 cells per well. After two days of incubation at 37 °C, foci were visualized with an Axio Observer D1 microscope (Zeiss, Oberkochen, Germany). To visualize all footprints in one well, six pictures were taken at a 40-fold magnification and merged.

3. Results

Identification of antigen-specific antibody-secreting cells, such as B-cells, hybridomas or cells transfected with plasmids encoding IgG-genes, is a crucial step during the process of isolating highly neutralizing antibodies. To facilitate this step and thereby promote the generation of novel broadly neutralizing antibodies against HSV and HCMV, we established two screening approaches to identify virus-specific antibody-secreting cells. Since the entry of HSV and HCMV is a highly complex process involving several viral surface proteins, we used intact virus particles for the screening procedure. This strategy allows identifying antibodies directed against the complex entry machinery including miscellaneous conformational epitopes.

3.1. Virus-based ELISA for identification of HSV-specific cells

3.1.1. General description

For the identification of HSV-specific cells, we established an ELISA
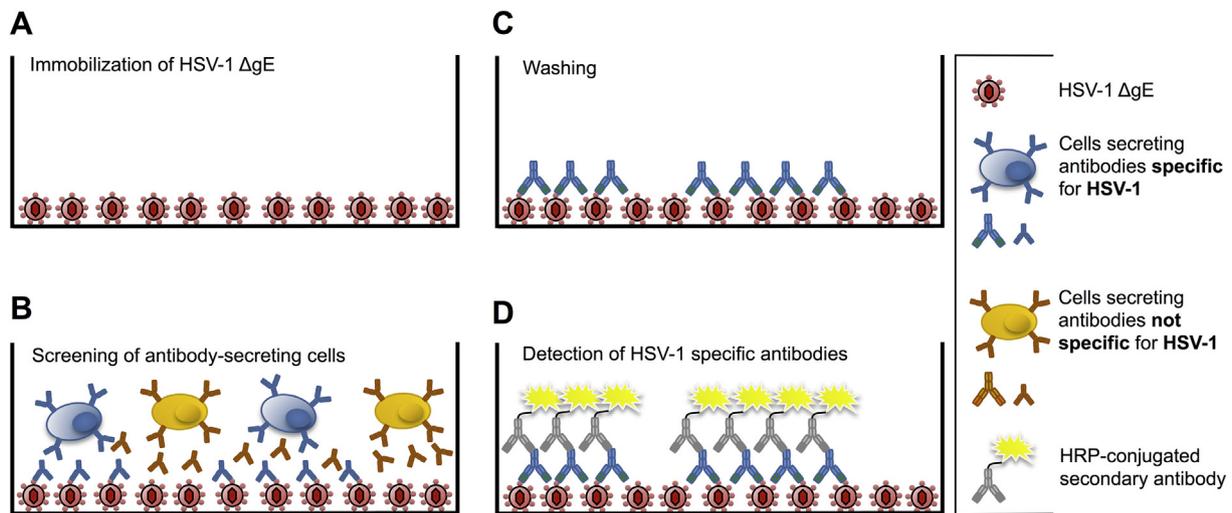
Fig. 1. Virus-based ELISA assay for the identification of cells secreting HSV-specific antibodies. (A) Microtiter plates are coated with HSV-1 ΔgE. (B) Immobilized virus is then incubated with a mixture of HSV-1 specific and unspecific B-cells. (C) After 48 h of incubation, the antibody-secreting cells are lysed and (D) bound virus-specific antibodies are detected with an appropriate peroxidase conjugated secondary antibody.

that is based on the detection of HSV-specific antibodies bound to immobilized virus (Fig. 1). The assay is designed for the screening of antibody-secreting cells or cell culture supernatants for HSV-specific antibodies. Microtiter plates are coated with HSV-1 ΔgE lacking the HSV-Fc-receptor (gE/gI complex) to minimize unspecific binding of antibodies (Fig. 1A). The binding of the IgG-Fc domain to the HSV-Fc-receptor is species-specific. In contrast to murine antibodies, human IgGs bind to the HSV-1 Fc-receptor (Sprague et al., 2006). To exclude unspecific binding when working with human antibody-secreting cells or antibodies in subsequent studies, we decided to establish this assay with the HSV-1 ΔgE mutant. The immobilized virus is then fixed and overlaid with murine human antibody-secreting cells. The cells are incubated for 48 h with the immobilized virus (Fig. 1B). Subsequently, antibody-secreting cells and HSV-unspecific antibodies are removed (Fig. 1C). Bound HSV-specific antibodies can be detected with a peroxidase conjugated secondary antibody (Fig. 1D). Cell cultures positive for virus-specific antibodies can subsequently be diluted to single cell level, expanded, retested for specific antibodies and selected for the production of monoclonal antibodies.

3.1.2. Detection of cells secreting HSV-specific antibodies with a virus-based ELISA assay

The virus-based ELISA assay is suitable for identifying cells (hybridoma cells, B-cell clones or transfected cells) secreting HSV-specific antibodies directed against viral surface antigens. The method was established for the detection of cells secreting murine and human antibodies. As a model system for cells secreting murine or human antibodies, we used a murine hybridoma cell line 2c and a stably transfected Sp2/0-Ag14 cell line producing the humanized antibody mAb hu2c. Both antibodies are specific for a conformational epitope on the glycoprotein B of HSV-1/2 (Eis-Hubinger et al., 1993; Krawczyk et al., 2013; Krawczyk et al., 2011). Microtiter plates are coated with HSV-1 ΔgE. Immobilized HSV-1 ΔgE was then incubated with distinct numbers of either 2c-hybridoma cells or Sp2/0-Ag14 cells (500, 100, 50, 25, 10, 5 and 1) secreting murine or humanized HSV-1 specific antibodies. The cells were mixed with the respective number of murine, Friend-Virus-specific H34 hybridoma cells or human IM9 cells secreting a monoclonal antibody not specific for HSV-1 as an unspecific control to reach an equal number of 500 cells per well. H34 or IM9 cells alone served as a background control. The cut-off was defined as the twofold value of the unspecific background measured for 500 H34 or IM9 cells, respectively.

The assay proved to be reliable to detect five murine hybridomas

(Fig. 2A) or ten cells secreting a humanized antibody specific for HSV-1 (Fig. 2C) from a total number of 500 cells. The test was performed three times in triplicates with similar results, which demonstrated the reproducibility of the assay. Only a slight background signal was observed when using H34 or IM9 cells alone. We conclude that the screening procedure can be performed as a high throughput assay to identify HSV-specific antibody-secreting cells.

3.1.3. Sensitivity of the HSV-1 ΔgE based ELISA

To determine the detection limit and sensitivity of the virus-based ELISA, we incubated various concentrations (500–0 nM) of the murine mAb 2c or the humanized mAb hu2c on immobilized HSV-1 ΔgE. The binding of these HSV-specific antibodies to the immobilized virus was quantified by the activity of the peroxidase-conjugated secondary antibodies. The cut-off was defined as a threefold value of the PBS signal. We found that bound mAb 2c was detectable at concentrations ≥ 0.98 nM (Fig. 2B) and mAb hu2c at concentrations ≥ 3.91 nM (Fig. 2D). However, unspecific binding of antibodies that are not directed against HSV might lead to false positive results, particularly at the low concentration range.

To investigate the impact of unspecific antibody binding, we repeated the measurements and compared the binding properties of mAb 2c and mAb hu2c to those of the murine Friend virus-specific antibody H34 or the human antibody IM9 that is not specific for HSV-1. The detection limit was defined as a threefold value of the PBS control. HSV-1 specific binding could be detected at a concentrations ≥ 1.95 nM mAb 2c (Fig. 3A) or mAb hu2c (Fig. 3B).

A slightly background signal was observed only at a concentrations of 250 nM H34 or IM9. However, it was considerably lower than the mAb 2c or mAb hu2c signal at this concentration. These data demonstrate that the virus-based ELISA is a reliable method to detect cells secreting murine or human/humanized antibodies specific to HSV-1 surface antigens, including those recognizing conformational epitopes. The method is also suitable for detecting antibodies in cell culture supernatants.

3.2. GFP reporter virus-based assay for the detection of HCMV-specific B-cells

3.2.1. General description

To identify novel broadly neutralizing monoclonal antibodies against HCMV, we have established a HCMV-GFP-reporter virus-based assay. This assay was designed to identify cells secreting antibodies
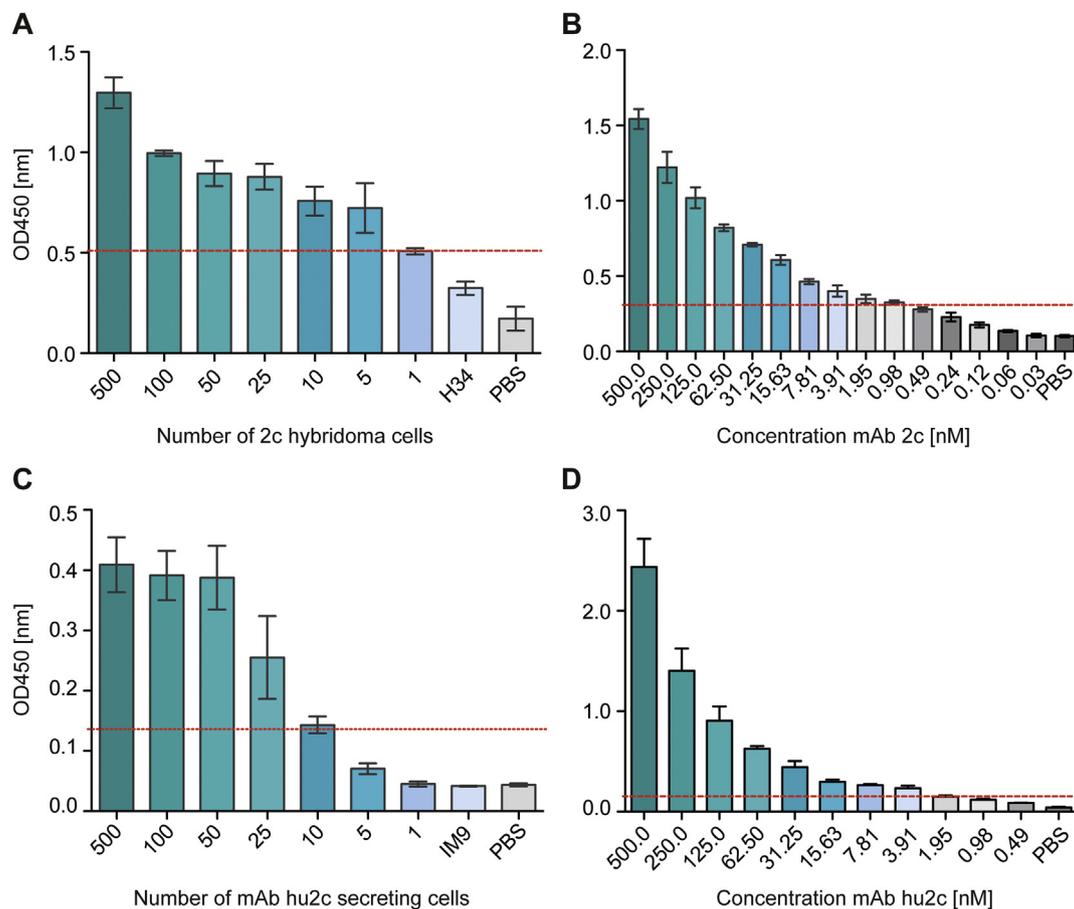
Fig. 2. Identification of cells secreting HSV-specific antibodies. Microtiter plates were coated with HSV-1 ΔgE. Immobilized virus was incubated with various numbers (500–0) of cells secreting HSV-1 specific antibodies. (A) Murine 2c hybridoma cells secreting an HSV-1/2 gB-specific antibody were mixed with H34 hybridoma cells secreting a Friend virus-specific murine antibody. (C) Stably transfected Sp2/0-Ag14 cells (500–0) secreting an HSV-1/2 gB-specific, humanized antibody mAb hu2c were mixed with IM9 cells, which secreted a human antibody not specific for HSV-1/2. The total cell number was 500 cells/well. H34 or IM9 cells alone served as background control. After 48 h of incubation, the cells were lysed and virus-bound antibodies were detected with a peroxidase conjugated secondary antibody specific for murine (A) or human (C) IgG-Fc-fragment. (B) Serial dilutions of purified mouse antibody mAb 2c or (D) the humanized counterpart mAb hu2c were incubated on immobilized HSV-1 ΔgE. Bound antibodies were detected as described above. The experiments were performed as triplicates. The detection limit is given as a threefold value of the PBS signal (red line). Values are given as the averages of triplicates. Error bars indicate the standard deviation of the mean (SDM). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
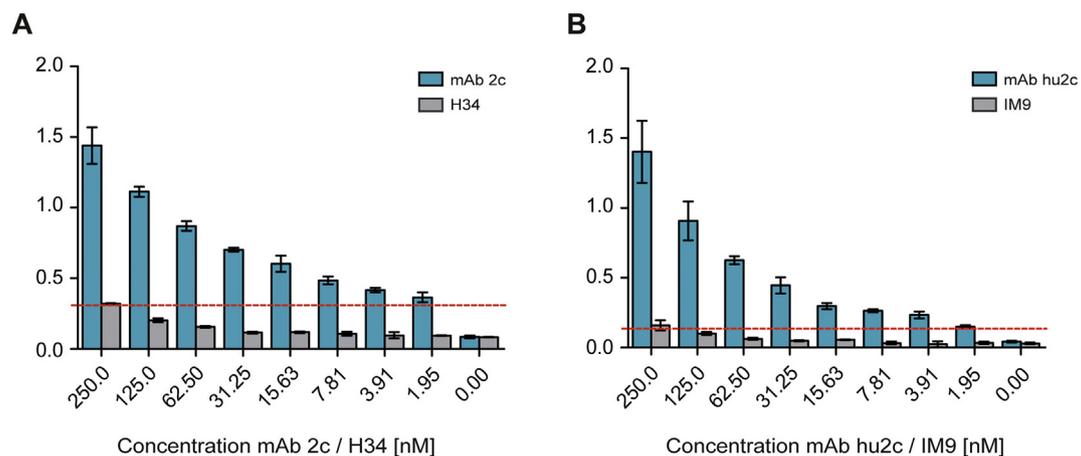
Fig. 3. Sensitivity of the HSV-1 ΔgE based ELISA. Decreasing concentrations (250–0 nM) of purified HSV-1/2 gB specific murine antibody mAb 2c (A) or humanized antibody mAb hu2c (B) were incubated on immobilized HSV-1 ΔgE. A murine, Friend virus-specific control antibody H34 (A) or a human antibody IM9 that is not specific for HSV-1 (B) was used to generate background values at each dilution (250–0 nM). The experiments were performed in triplicates. The detection limit is given as a threefold value of the PBS signal (red line). Error bars indicate the standard deviation of the mean (SDM). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
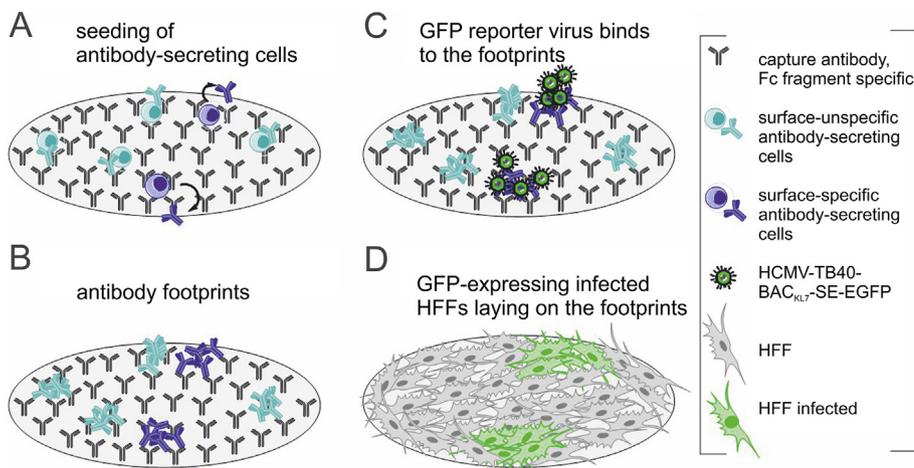
Fig. 4. HCMV-GFP reporter virus-based footprint assay for the detection of cells secreting HCMV-specific antibodies. (A) Antibody-secreting cells are seeded on microtiter plates coated with Fcγ-specific capture antibodies. (B) Antibodies secreted by the cells (B-cells, hybridoma cells or transfected cells) are bound by the immobilized capture antibody (hybridoma "footprints"). (C) The antibody-secreting cells are lysed and bound antibodies are incubated with an HCMV-GFP reporter virus. The virus exclusively binds to cell footprints if the captured antibodies recognize an HCMV surface-antigen. (D) Subsequently, HFF indicator cells are seeded onto the microtiter-plates, thereby allowing infection at the positions of bound reporter virus. Green fluorescent cells indicate the presence of HCMV-specific cells in the cell culture. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

directed against HCMV surface glycoproteins from a comprehensive cell repertoire such as murine cells from a hybridoma cell library or EBV-immortalized B-cells of preselected blood donors. Basically, the assay relies on the fact that the indicator cells will only become infected by the fluorescent HCMV reporter virus at sites where cells secreting HCMV-specific antibodies have left "antibody footprints" (Fig. 4).

Microtiter plates are coated with GAM- or GAH-Fcγ-specific capture antibodies and overlaid with cell cultures including cells secreting anti-HCMV specific antibodies (Fig. 4A). The secreted antibodies will be bound by the solid phase-fixed capture antibodies. Thereby, the cells leave an antibody-footprint at the place where they were sedimented (Fig. 4B). After removing the cells, the captured antibodies are overlaid with cell culture medium containing the HCMV-GFP reporter virus. The virus binds to the captured antibodies only if they are specific for HCMV surface antigens (Fig. 4C). Subsequently, the unbound virus is removed by washing. Indicator HFF cells are then seeded onto the immobilized antibody/virus complexes (Fig. 4D). Thereby, only those cells are infected that attach at the cell-footprints that had bound virions in the previous step (Fig. 4D). Subsequently, HFF-cells that are infected with the HCMV reporter virus produce GFP and hence can be visualized and counted by fluorescence microscopy (Fig. 4D). Cell cultures that are identified to produce virus-specific antibodies can subsequently be diluted to single cell level, expanded, retested and selected for the production of HCMV-specific monoclonal antibodies.

3.2.2. GFP-reporter virus based detection of HCMV-specific hybridoma cells

The assay was established using the murine hybridoma clone H10 secreting antibodies specific to the HCMV envelope glycoprotein H (gH) (Falk et al., 2016). In a first series of three experiments, we tested whether our concept of detecting HCMV-specific antibody footprints is valid and foci of infected cells only form if the seeded hybridoma cells

were specific for HCMV surface proteins.

Microtiter plates were coated with a GAM-Fcγ-specific capture antibody. Hybridoma cells were then seeded at a density of 500 cells per well and incubated overnight. The hybridoma cells are expected to secrete antibodies that will be bound by the capture antibodies, thus leaving an antibody-"footprint" at the position where they sedimented. HCMV-gH-specific hybridoma cells (clone H10) served as a positive control as the respective antibodies can bind to the surface of virions. Hybridoma cells of clone 28–77 served as a background control as they release antibodies directed against an HCMV tegument protein (pp65), which is not accessible at the surface of virions. In addition, a mixture of both hybridoma cells was applied, seeding 10 cells of clone H10 together with 490 cells of clone 28–77 per well. After the overnight incubation, the hybridoma cells were removed by detergent lysis, and the captured antibodies were overlaid with the reporter virus RV-TB40-BAC$_{KL7}$-SE-EGFP for 2 h. The virus is expected to bind exclusively to the captured hybridoma-"footprints" of clone H10. In contrast, antibodies of clone 28–77 are expected not to bind to the surface of virions and therefore not cause infected foci in the indicator cell layer. HFF indicator cells were then seeded onto the plate under the assumption that only those cells would be infected that lie at the "footprint"-bound virions. Two days later, GFP-producing infected cells were visualized by fluorescence microscopy (Fig. 5). As expected, foci of infected cells were not detected in wells where only cells of clone 28–77 were seeded, indicating that nonspecific focal binding of reporter virions did not occur. Under this condition, only very few dispersed GFP-positive cells could be detected (Fig. 5C). In contrast, the seeding of hybridoma clone H10 always resulted in the formation of infectious foci. When 500 cells of clone H10 were seeded, numerous foci were visible that could not be distinguished from each other (Fig. 5A). When 10 cells of clone H10 were seeded together with an excess of clone 28–77, foci of infected
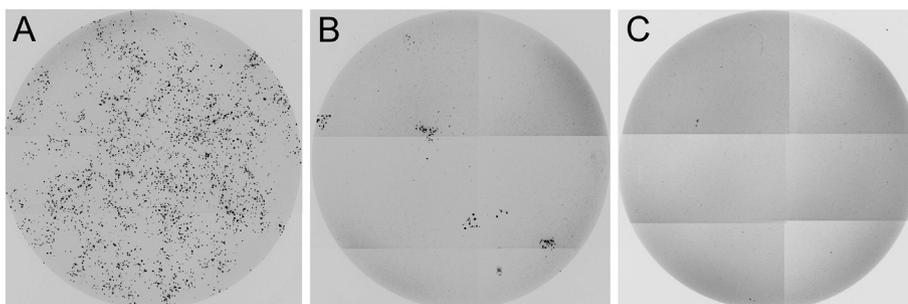
Fig. 5. Detection of HCMV-specific hybridoma cell footprints by fluorescence microscopy. Microtiter plates were coated with a goat anti-mouse Fcγ-specific capture antibody. (A) Hybridoma cells (clone H10) specific for the HCMV surface protein gH were seeded at a density of 500 cells per well. (B). H10 cells were seeded at a density of 10 cells per well together with 490 hybridoma cells of clone 28–77, which is specific for the HCMV tegument protein pp65. (C) Wells seeded with 500 cells per well of clone 28–77 served as a negative control. After 24 h of incubation, hybridoma cells were removed by lysis and medium containing the HCMV-BAC$_{KL7}$-eGFP reporter virus was added. After 2 h, unbound reporter virus was removed and HFF indicator cells were seeded. After 48 h, HCMV-infected GFP-producing HFFs were detected by fluorescence microscopy. Six images were compiled to visualize the whole well. To facilitate the evaluation, photos are shown in an inverted mode, i.e. dark dots represent GFP-expressing cells. Each focus of GFP-expressing cells is assumed to represent one hybridoma cell footprint.
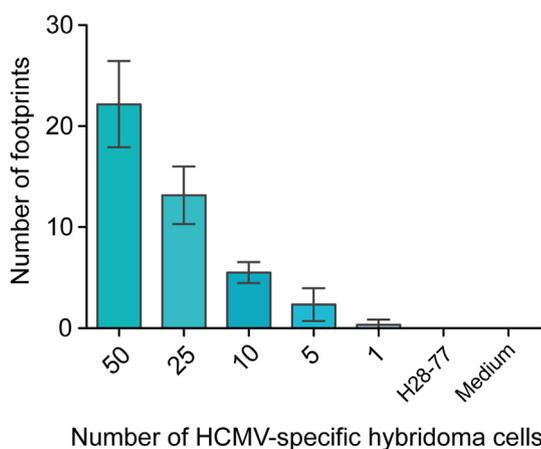
Fig. 6. Detection of HEK-293 T cells secreting an HCMV-specific human IgG.

cells were clearly distinguishable and the number of foci was below 10, fitting with the assumption that reporter virus would focally adhere at sites where H10 cells had been sedimented before (Fig. 5B).

Taken together, hybridoma cells secreting antibodies against an HCMV envelope protein reliably induced the formation of infected foci within the indicator cell layer, whereas only few dispersed infected cells were found with the background control, most probably due to individual virions remaining on the plate after the washing procedure.

3.2.3. Detection of HEK-293 T cells secreting an HCMV specific human antibody

In order to demonstrate that the antibody-footprint assay is also suitable for the detection of human cells secreting HCMV specific human IgGs, we used transiently transfected HEK-293 T cells expressing an HCMV specific human antibody IgG 8 J16 (Macagno et al., 2010). This antibody is directed against the pentameric glycoprotein complex on the surface of HCMV. As control, stably transfected Sp2/0-Ag14 cells secreting a HSV-1 gB-specific humanized antibody mAb hu2c were used. In wells containing 500 of HEK-293 T cells secreting IgG 8 J16 numerous foci could be found, but could not be distinguished from each other (Fig. 6A). In wells containing 15 HEK-293 T cells secreting IgG 8 J16, the foci of infected cells were distinguishable (Fig. 6B). As expected, the foci of infected cells were not detected in wells where only mAb hu2c-secreting cells were seeded, indicating that there was no unspecific binding of the reporter virus. Under this condition, only a few dispersed GFP-positive cells could be detected (Fig. 6C). These results clearly demonstrate that the method presented here is suitable for the detection of cells secreting human IgGs specific for complex epitopes on HCMV surface.

3.2.4. Sensitivity of the GFP-reporter virus based detection of HCMV-specific hybridoma cells

Since hybridoma cells are more robust compared to transiently transfected HEK-293 T cells, the sensitivity of the assay was determined using hybridoma cells as follows. Therefore, we tested whether the assay is reliable for detecting a single HCMV surface-specific hybridoma cell within a great number of surface-unspecific hybridoma cells. To simulate a situation where HCMV-envelope-specific B-cells are hidden in an excess of nonspecific cells, we mixed distinct numbers (50, 25, 10, 5 and 1) of HCMV gH-specific H10 hybridoma cells with a surplus of 28–77 hybridoma cells specific for HCMV tegument protein pp65 to reach a total number of 500 cells per well. After 24 h of cultivation, cells were lysed with detergent solution, and antibody footprints of the hybridoma cells were overlaid for 2 h with the GFP-reporter virus to allow the binding of virions at sites where H10 cells have been located. After washing and the addition of indicator fibroblasts and further incubation for two days, cultures were screened for GFP-expressing cells

with a fluorescence microscope and the number of infectious foci was counted (Fig. 7). Expectedly, the number of visible footprints increased with the number of H10 hybridoma cells per well. The probability that seeded hybridoma cells would form a focus of infected cells was around 50% at all concentrations, i.e. seeding of 5 hybridoma cells reliably resulted in the formation of 2–3 foci in three repeated experiments. Even if only a single H10 cell was seeded among 500 28–77 cells, a focus was detected in 1 out of 3 experiments. This indicates that the assay is suitable for the detection of HCMV surface antigen-specific specific B-cells in a cell mixture. For example, if only 1 out of 10,000 B-cells of a donor is directed against an envelope protein of HCMV and 100,000 cells are screened (= 200 wells, each containing 500 cells), the probability of finding HCMV-specific B-cells can be calculated as > 99%. This is easily feasible with this assay.

4. Discussion

In the present study we have established two distinct methods for detecting cells secreting antibodies specific to viral surface-antigens of HSV and HCMV.

Infections with HSV or HCMV may lead to severe or even life-threatening diseases in immunocompromised patients or when acquired at birth (HSV) or during pregnancy (HCMV) (Berrington et al., 2009; Bhat et al., 2015; Boppana et al., 2013; Thompson and Whitley, 2011). Antivirals are available but may lead to the development of resistance (Minces et al., 2014; Morfin and Thouvenot, 2003) or are contraindicated for the treatment during pregnancy due to potential teratogenic side effects (Kimberlin, 2001). Antibody-based strategies revealed to be highly promising to fight viral infections. Numerous potent neutralizing antibodies against HIV, SARS-CoV, Ebola virus and other viral pathogens have been isolated within the past years (Bornholdt et al., 2016; Caskey et al., 2016; Traggiai et al., 2004). Preclinical and early phase clinical trials with hyperimmunoglobulins or monoclonal antibodies targeting HSV or HCMV also have shown promising results (Krawczyk et al., 2013; Masci et al., 1995; Nigro et al., 2005; Revello et al., 2014). However, the identification of novel, highly neutralizing antibodies against these viruses would be of great benefit to significantly improve the antiviral treatment.

Commonly, monoclonal antibodies are derived from EBV-immortalized human B-cells collected from seropositive patients recovered from infection or from immunized mice (Marasco and Sui, 2007). Therefore, the screening and selection process is a crucial step to identify antigen-specific B-cells from the whole B-cell repertoire of seropositive humans or immunized mice in a limited time, particularly when looking for B-cells secreting antibodies targeting complex discontinuous epitopes on viral surface antigens. Functional assays such as a microneutralization assay are useful and contribute to the isolation of potent neutralizing antibodies against HCMV (Macagno et al., 2010). Alternatively to performing neutralization assays, which may require high amounts of purified antibodies, B-cells or cell-culture supernatants can be screened for antigen binding. Conventional ELISA assays are well established for routine diagnostics and usually based on purified viral antigens (Liermann et al., 2014). High throughput screening techniques based on antigen microarrays were developed to facilitate the screening process (De Masi et al., 2005; Tickle et al., 2015). These methods allow for the simultaneous screening of B-cells for antibody specificity to one particular antigen or to several antigens. However, since these assays are based on purified recombinant peptides or proteins, they are inadequate to detect neutralizing antibodies targeting complex surface-antigen epitopes.

With respect to identifying novel highly neutralizing antibodies against HSV and HCMV, we established two screening methods based on full viruses, which allow for the detection of antibodies targeting the complex entry machinery of these viruses including miscellaneous conformational epitopes. Notably, prior studies reported that highly potent antibodies against HSV or HCMV bind to such complex
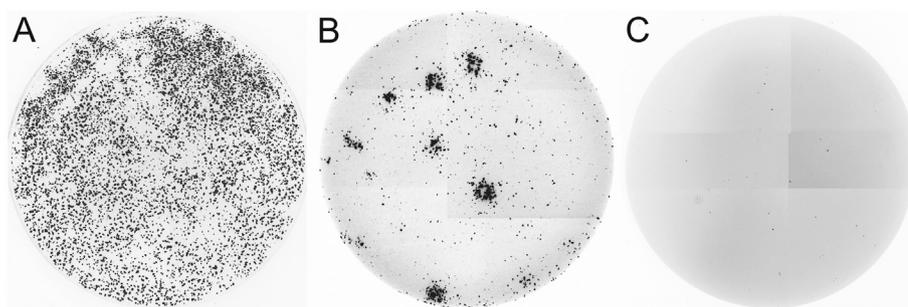
Fig. 7. Sensitivity of the HCMV-GFP reporter virus-based footprint assay. (A) Microtiter plates were coated with a goat anti-mouse-Fcγ-specific capture antibody. Varying numbers (50, 25, 10, 5 and 1) of clone H10 hybridoma cells specific for the HCMV surface antigen gH were mixed with HCMV surface antigen-unspecific hybridoma cells 28–77 and seeded on the microtiter plates. The total cell number was 500 cells/well. The cells were removed after 24 h of incubation and captured antibodies were overlaid with medium containing the HCMV-BAC$_{KL7}$-eGFP reporter virus. After 2 h, unbound reporter virus was removed and HFF indicator cells were seeded and cultured for 48 h to allow the onset of infection. The total number of GFP-positive footprints/well was counted by fluorescence microscopy. Data are given as means of two independent experiments, each performed in triplicates. Error bars represent the standard error of the mean.

discontinuous epitopes on one or more than one viral protein (Cairns et al., 2014; Krawczyk et al., 2013; Macagno et al., 2010). Accordingly, Dengue virus (DENV) immune serum depletion studies have shown that the antibodies responsible for serum neutralizing activity following primary DENV infection target complex epitopes that exist in a correct conformational state only on the virion particle, but not on recombinant soluble forms of the viral surface antigen E (Smith et al., 2014). These data indicate that it might be of great benefit to work with full viruses when screening B-cell clones for neutralizing antibodies.

When working with herpesviruses such as HSV or HCMV, one should keep in mind that these viruses can bind antibodies via viral Fc-receptors (Atalay et al., 2002; Ndjamen et al., 2016; Sprague et al., 2006). The interaction between the viral Fc-receptors and immunoglobulins is species-specific. IgGs from humans exhibit stronger binding to the HSV-FcR and HCMV-FcR than mouse IgGs (Antonsson and Johansson, 2001; Johansson et al., 1985). The Fc-related unspecific binding can be eliminated by using an Fc-receptor-lacking virus or by blocking the viral Fc-receptors with human Fc-fragments or sera from seronegative donors. The HSV-1 Fc-receptor consisting of the glycoprotein dimer gE/gI is well characterized, and a gE-deletion mutant lacking the viral Fc-receptor is available (Farnsworth et al., 2003). In the present study we worked with the HSV-1 ΔgE mutant to exclude unspecific binding related to the HSV-1 Fc-receptor. There was no significant unspecific binding observed when using a human antibody not specific for HSV-1, indicating that the assay is reliable for detecting virus-specific antibodies in particular. Since there are a number of HCMV proteins described that can bind the human IgG-Fc-domain, and no appropriate deletion mutant was available, we decided to use an HCMV-GFP reporter virus. Although we could not observe any background related to HCMV-receptor binding of HCMV-unspecific antibodies in our study, such binding can easily be inhibited by pre-incubation of the reporter virus with human Fc-fragments as previously described (Antonsson and Johansson, 2001) when indicated. A critical point for identifying HSV or HCMV specific antibodies with the assays described here might be the antibody affinity. Cells secreting low-affinity HSV or HCMV specific may induce only weak signals and thus remain undetected. However, we know from the HIV, HSV or influenza field, that the best neutralizing antibodies bind the target antigens with a high affinity, at least at nanomolar range (see e.g. (Mascola and Haynes, 2013)). The assays described here were developed to support the identification of novel, highly neutralizing antibodies that can be further developed for clinical use. In line with prior studies, we expect that such antibodies will bind the target antigens with a high affinity. The affinity of the HSV-1 specific mAbs 2c and hu2c used in this study are at nanomolar range (Krawczyk et al., 2013). The affinity of the HCMV-specific IgG 8 J16 could not yet be determined, but based on a prior study we estimate that it is also in the nanomolar range (Macagno et al., 2010). Therefore, we conclude that the methods described in the manuscript are suitable for the detection of high-affinity antibodies targeting HSV or HCMV.

In summary, the whole virus-based screening assays described in this study were highly sensitive and allow the detection of between one and ten cells secreting murine or human/humanized antibodies specific to HSV-1 or HCMV from a cell mixture. After a positive selection of high-affinity binders, the newly identified antibodies need to be further characterized for potent virus neutralization to select potential candidates for clinical use. These methods described here may facilitate the screening procedure of B-cells specific for HSV and HCMV surface antigens, including those targeting highly complex epitope structures, and contribute towards identifying novel potent antibodies against HSV and HCMV.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jim.2018.07.010.

Microtiter plates were coated with a goat anti-human Fcγ-specific capture antibody. (A) HEK-293 T cells, transiently transfected with plasmids coding for the heavy and light chain of IgG 8 J16, a human IgG specific for an HCMV glycoprotein complex, were seeded at a density of 500 cells and (B) at a density of around 15 cells per well. (C) As control, 1000 Sp2/0-Ag14 cells secreting an HSV-1/2 specific, humanized antibody which was mAb hu2c seeded. After 24 h, cells were removed by lysis and medium containing the HCMV-BACKL7-eGFP reporter virus was added. After 2 h, unbound reporter virus was removed and HFF indicator cells were seeded. HCMV-infected GFP-producing HFFs were detected by fluorescence microscopy after 48 h of incubation. Six images were compiled to visualize the whole well. To facilitate the evaluation, photos are shown in an inverted mode, i.e. dark dots represent GFP-expressing cells. Each focus of GFP-expressing cells is assumed to represent a footprint of one HEK-293 T cell secreting IgG 8 J16.

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