Cell-to-cell spread inhibiting antibodies constitute a correlate of protection against Herpes Simplex Virus Type 1 reactivations

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Abstract (200 words)

Herpes simplex viruses (HSV) cause ubiquitous human infections. For vaccine development, knowledge concerning correlates of protection against HSV is essential. Therefore, we investigated if humans principally can produce highly protective cell-to-cell spread inhibiting antibodies upon natural infection and whether such antibody responses correlate with protection from HSV reactivation. We established a high-throughput HSV-1 GFP reporter virus-based assay and screened 2496 human plasma samples for HSV-1 cell-to-cell spread inhibiting antibodies. We conducted a survey among the blood donors to analyze the correlation between the presence of cell-to-cell spread inhibiting antibodies in plasma and the frequency of HSV reactivations. In total, 128 of 2496 blood donors (5.1 %) exhibited high levels of HSV-1 cell-to-cell spread inhibiting antibodies in the plasma. Such individuals showed a significantly lower frequency of HSV reactivations compared to subjects without sufficient levels of HSV-1 cell-to-cell spread inhibiting antibodies. This study provides two important findings: (I) a fraction of humans produce HSV cell-to-cell spread inhibiting antibodies upon natural infection and (II) such antibodies correlate with protection against recurrent HSV. Moreover, these elite neutralizers can provide promising material for hyperimmunoglobulin, the isolation of superior antiviral antibodies and information for the design of a vaccine against HSV.

Importance:

Herpes simplex virus 1 infections can cause painful mucosal lesions at the oral or genital tract and severe, life threatening disease in immunosuppressed patients or neonates. There is no approved vaccine available, and the emergence of drug resistances especially in long time treated patients makes the treatment increasingly difficult. We tested 2496 people for HSV-1 cell-to-cell spread inhibiting antibodies. Five percent exhibited functional titers such antibodies and showed significantly lower risk of reactivations, uncovering cell-to-cell spread inhibiting antibodies as a correlate of protection against Herpes simplex virus reactivations. Isolation of the cell-to-cell spread inhibiting antibodies from B-cells of these donors may contribute to

56 develop novel antibody-based interventions for prophylactic and therapeutic use and provide
57 starting material for vaccine development.
58

Introduction (Main body text: 2671 words)

Herpes simplex viruses (HSV) types 1 and 2 are among the most common human infections worldwide. Globally, more than 3.7 billion people are infected with HSV-1 [1] and nearly 500-million with HSV-2 [2]. Both viruses cause a broad range of disease manifestations ranging from painful and irritating but self-limiting oral or genital lesions to severe disseminated and life-threatening infections in immunocompromised patients [2-5]. Serious complications can also be observed in patients suffering from ocular herpes infections, which may result in irreversible damage of the eye or even blindness [6, 7].

Until today, there is no approved vaccine available [8]. Numerous animal studies investigating the efficacy of distinct vaccine candidates such as inactivated virus particles, live- or genetically attenuated viruses or recombinant subunit vaccines yielded promising results [9, 10]. However, none of the vaccine candidates being tested in clinical trials has been effective [8]. The GlaxoSmithKline (GSK) Herpevac trial using a recombinant HSV-2 glycoprotein D (gD2) subunit vaccine was largest clinical trial performed so far [11]. In strong contrast to prior animal studies, the vaccine failed to protect against the acquisition of HSV-2 infection [11]. The discrepancy between promising results of animal studies and the failure of clinical trials in humans indicated a fundamental difference in the immune response to HSV in mice or guinea pigs and humans. Retrospective studies uncovered differences in antibody responses between humans and rodents concerning virus-specific antibodies, neutralizing antibodies, and cell-to-cell spread inhibiting, neutralizing antibodies (CCSi-NAbs). Most recently, the antibody responses to the gD2 subunit vaccine were analyzed in humans and guinea pigs [12]. Antibodies produced by vaccinated humans recognized significantly fewer crucial gD2 epitopes as compared to guinea pig antibodies [12, 13]. The crucial gD2 epitopes are targets of neutralizing or cell-to-cell spread inhibiting antibodies [14]. The cell-to-cell spread of HSV is known as a mechanism of immune evasion, and markedly facilitates the spread of HSV upon reactivation [14]. Antibodies, which can block this route of viral transmission, are associated with protection from disease [12, 15]. We developed a highly neutralizing and cell-to-cell spread inhibiting monoclonal antibody called mAb 2c. This antibody mediates almost complete

protection from lethal genital HSV-1 infection - even in highly immunodeficient NOD/SCID mice [15, 16]. Moreover, mAb 2c protects mice from the development of severe ocular infections [17-19]. Importantly, mAb 2c is significantly more effective in protecting from disease than polyclonal human neutralizing antibodies used at a similar neutralizing titer, highlighting the importance of the inhibition of cell-to-cell spread in protecting from disease [20]. These *in vitro* and *in vivo* data strongly suggested that neutralizing antibodies, which inhibit the cell-to-cell spread are superior to antibodies that "just" neutralize but do not inhibit the cell-to-cell spread [20]. These findings raise the apparent question, if the inhibition of the cell-to-cell spread might contribute to protection from primary and/or recurrent disease. Intriguingly, the re-evaluation of the GSK Herpevac trial revealed that gD2 immunized individuals only barely produced antibodies that targeted gD2 epitopes associated with cell-to-cell spread [13], raising the fundamental question whether humans are in principle able to produce cell-to-cell spread inhibiting antibodies against HSV.

To address this question, we established a HSV-1 GFP reporter virus-based high-throughput screening assay and tested 2496 plasma samples for cell-to-cell spread inhibiting antibodies. We show for the first time that a small fraction of humans is indeed able to produce functional levels of cell-to-cell spread inhibiting antibodies (elite responder) and - even more striking - that the presence of sufficient concentrations of such antibodies correlated with protection from HSV reactivation.

Results

To evaluate whether humans are able to produce potent antiviral antibodies upon natural HSV-1 infection, we established a high-throughput assay to test human plasma and serum samples for HSV-1 cell-to-cell spread inhibiting properties.

HSV-1-ΔgE-GFP reporter virus-based screening assay for cell-to-cell spread inhibiting antibodies

The screening assay is based on the quantification of the progressing plaque expansion, which correlates with the extent of cell-to-cell spread. By using the HSV-1-ΔgE-GFP reporter virus, plaque formation, which is proportional to the GFP expression level, can be quantified using a fluorescence reader and visualized by fluorescence microscopy (Fig. 1). Confluent Vero cell monolayers were infected with the HSV-1-ΔgE-GFP reporter virus at a low multiplicity of infection (MOI = 0.001). Thereby, only scattered cells within the cell layer become infected. Afterwards, the infected cell cultures were overlaid with medium containing the sample to be tested, e.g. serum, plasma or purified antibodies (Fig. 1). The test was evaluated 3 days after infection in a quantitative manner by assessing the GFP signal or in a qualitative manner by fluorescence microscopy (Fig. 1). Single infected cells accompanied by a low GFP signal represent a complete inhibition of the cell-to-cell spread. Unrestricted plaque formation and strong GFP signals at levels similar to those of the HSV-1 seronegative control were scored as no inhibition of the cell-to-cell spread. Small plaques and moderate GFP signals indicated the presence of cell-to-cell spread inhibiting antibodies in the sample, even if there was no complete inhibition of the cell-to-cell spread (Fig. 1).

Evaluation of an HSV-1-ΔgE-GFP-based high-throughput screening assay for cell-to-cell spread inhibiting antibodies

The HSV-1-ΔgE-GFP-based high-throughput screening assay was first evaluated using the humanized antibody mAb hu2c that completely inhibits HSV-1 and HSV-2 cell-to-cell spread (Fig. 2). Confluent Vero cell cultures were infected with HSV-1-ΔgE-GFP and subsequently

overlaid with medium containing graded concentrations (0 - 500 nM) of mAb hu2c. Plasma or serum from an HSV-1 and HSV-2 double seronegative donor was added at a 1:40 dilution. Complete inhibition of the cell-to-cell spread could be observed at mAb hu2c concentrations between 125 and 500 nM (Fig. 2A). Almost complete inhibition was observed at 62.5 nM. At this concentration, only very small plaques with a maximum of 4 infected cells/plaque were visible (Fig. 2A) and the quantitative analysis showed an almost unchanged GFP signal compared to higher mAb hu2c concentrations (Fig. 2B). This concentration represents the lowest mAb hu2c concentration that almost completely inhibits the cell-to-cell spread (Fig. 2A, dashed line). At concentrations between 2 and 7.8 nM of mAb hu2c there was no visible reduction of the cell-to-cell spread (Fig. 2A) and the GFP-signal was notably higher when compared to concentrations above 62.5 nM mAb hu2c (Fig. 2B). Interestingly, plaques were smaller at mAb hu2c concentrations between 15.6 and 31.3 nM mAb hu2c (Fig. 2A) accompanied by only slightly increased GFP-signals (Fig. 2B), indicating a partial inhibition of the cell-to-cell spread at these concentrations.

These data clearly show that the quantitative measurement of the GFP signal correlates with the plaque expansion observed in cell-culture, which obviously represents the extent of the HSV-1 cell-to-cell spread. Furthermore, our assay distinguishes complete, partial and no inhibition of the HSV-1 cell-to-cell spread.

Identification of HSV-1 elite responders with cell-to-cell spread inhibiting antibodies

To investigate whether humans can produce HSV-1 cell-to-cell spread inhibiting antibodies, plasma samples from 2496 blood donors were screened for cell-to-cell spread inhibiting properties. All samples were tested using the high-throughput screening assay described above. A mAb hu2c positive control was included on each 24-well plate. The efficacy of the plasmas regarding cell-to-cell spread inhibition was determined by dividing the GFP-signal of a culture containing the plasma of interest through the GFP-signal of mAb hu2c-treated control exhibiting “complete inhibition”. This quotient was termed inhibitory quotient (IQ) and represents the x-fold value of the GFP-signal measured for mAb hu2c. Plasmas were stratified

according to their cell-to-cell spread inhibiting activity as completely inhibiting (IQ ≤ 1.5), partially inhibiting (IQ = 1.51 to < 2.8) and non-inhibiting (IQ ≥ 2.8). In total, 128 (5.1 %) of the plasmas showed complete and 1061 (42.5 %) exhibited partial inhibition (Fig. 3). The fold change of the remaining 1307 (52.4 %) plasmas was in a similar range as plasmas derived from HSV-1 seronegative donors and had no effect on the cell-to-cell spread. None of the 147 HSV-1 seronegative control plasmas exhibited partial or complete cell-to-cell spread inhibition, demonstrating the specificity of the assay.

Assessment of the frequency of HSV reactivations in plasma donors

Next, we assessed the frequency of HSV reactivations in blood donors to investigate whether there is a correlation between the presence of cell-to-cell spread inhibiting antibodies and the frequency of reactivations. For this purpose, we conducted a retrospective survey including 158 blood donors that were randomly selected from the complete inhibition group (n = 47), partial inhibition group (n = 58) and the no inhibition group (n = 53). The HSV-seropositive status was confirmed by a diagnostic IgG ELISA. The biometric characteristics of the three different inhibition groups are summarized in Table 1. All three groups were comparable regarding mean age, gender, smoking behavior as well as the mean body mass index (BMI). Next, the three different groups were evaluated regarding the frequency of HSV reactivations. The frequency of HSV reactivations was recorded according to the observed occurrence of oral or genital lesions with less than one time per year or one or more symptomatic reactivations per year (< 1 or ≥ 1 reactivation per year). Interestingly, study participants from the complete inhibition group showed significant lower frequencies of HSV reactivation as compared to the groups that show only partial or no cell-to-cell spread inhibition capacity (Fig. 4). Only 17 % of individuals from the complete inhibition group reported one or more reactivation per year, whereas the frequency of at least one reactivation per year was 38 % in the partial inhibition group and 36 % in the no inhibition group. These data clearly demonstrate a significant correlation between the presence of cell-to-cell spread inhibiting antibodies and a lower rate of HSV-reactivation, providing a strong argument for their functional relevance in

preventing recurrent herpes disease.

Determination of the neutralizing antibody titers of blood donor plasmas

To investigate whether antibodies capable to neutralize HSV-1 but incapable to block the cell-to-cell spread of the virus have an impact on the frequency of HSV-1 reactivations, we determined the neutralizing titers of the donor plasmas that completed the survey.

Although there was a significant difference in the frequency of reactivations between the complete inhibition group and partial inhibition group (Fig. 4), the neutralizing antibody titers were at similar levels (Fig. 5). Only the no inhibition group was shown to have significantly lower neutralization titers compared to the complete inhibition and partial inhibition group.

These results indicate that the significantly lower HSV-reactivation likelihood observed for the complete inhibition group correlates with cell-to-cell spread inhibiting antibodies but not with antibodies that neutralize the virus but fail to counteract the cell-to-cell spread.

In conclusion, we showed for the first time that (i) about five percent of HSV-1 seropositive blood donors (elite responder) are able to produce HSV-1 cell-to-cell spread inhibiting antibodies and (ii) that the presence of these antibodies correlate with a significantly lower risk of HSV-reactivation.

Discussion

In the present study, we investigated whether humans are able to produce antibodies that effectively block the HSV-1 cell-to-cell spread upon natural infection. We demonstrated that humans are principally able to produce such cell-to-cell spread inhibiting antibodies against HSV-1.

In our large cohort of 2496 blood donors, we identified a small proportion of 128 (5.1 %) that had a sufficiently high antibody-concentration to block the cell-to-cell spread of HSV-1 in cell culture (elite responder). Most importantly, these individuals reported a significantly lower frequency of symptomatic HSV-reactivations when compared to people with lower or no detectable cell-to-cell spread inhibiting antibodies.

Interestingly, 42.5 % of the plasmas showed a partial inhibition of the cell-to-cell spread, indicating that there might be cell-to-cell spread inhibiting antibodies in the plasmas, but at lower concentrations. The average concentration of antibodies in human sera/plasma was described with 11 mg/ml [21]. In the present study, we tested the plasma samples at a 1:40 dilution, which corresponds to a median IgG concentration of 0.28 mg/ml. At least in 5 % of individuals whose plasmas showed complete cell-to-cell spread inhibition in our high-throughput, this concentration was sufficient to prevent reinfections.

However, we found that there was no correlation between neutralizing antibody titers and the frequency of HSV reactivation. Despite similar neutralizing antibody titers, people who had cell-to-cell spread inhibiting antibody concentrations in plasma reported significantly fewer rate of HSV-reactivations than people with insufficient concentrations of such antibodies did. These data provide evidence for the unique protective role of cell-to-cell spread inhibiting antibodies in HSV infection.

These data support prior findings. Neutralizing antibodies, which are not necessarily inhibiting the cell-to-cell spread, contributed to protect from a severe course of disease [22]. The presence of HSV-specific antibodies in HSV-infected mothers has been suggested to decrease the risk of acquisition of HSV-2 by newborns [23, 24]. Similar findings were reported in mice. Maternal antibodies were shown to access neural tissues of the fetus or neonate, thereby

protecting neonatal mice against HSV-1 neurological infection and death [24]. Notably, in animal studies neutralizing antibodies blocking virus entry and cell-to-cell spread were superior to normal neutralizing antibodies that did not inhibit the cell-associated viral spread [20]. These data are in line with our here presented findings.

In conclusion, by using a high-throughput HSV-1-ΔgE-GFP reporter assay we have demonstrated that HSV-1 infected humans are able to produce cell-to-cell spread inhibiting antibodies. In addition, we were able to show that the presence of these antibodies directly correlates with a significantly lower frequency of HSV reactivations, representing a correlate of protection. Plasmas of these individuals may be used for passive immunization strategies. Isolation of the cell-to-cell spread inhibiting antibodies may contribute to develop novel antibody-based interventions for prophylactic and therapeutic use. Moreover, characterizing of epitopes recognized by these antibodies may contribute to optimize the target antigens for novel vaccine approaches.

Material and methods

Ethics statement

The study was performed in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) and was approved by the ethical committee of the University of Ulm and the University Hospital Essen.

Antibodies, Sera and Plasma

Sera and plasma samples were harvested during routine blood donations at the Institute of Transfusion Medicine, University of Ulm, Germany. The humanized monoclonal antibody mAb hu2c was produced and purified as described previously [15].

Viruses

HSV-1 strain F, HSV-2 strain G and HSV-1-ΔgE-GFP reporter virus were propagated in Vero cells and stored at −80 °C. HSV-1-ΔgE-GFP was kindly provided by Hartmut Hengel (Institute of Virology, Freiburg, Germany) and initially described by Farnsworth et al. [21]. Viral titers were determined by a standard endpoint dilution assay and calculated as 50 % tissue culture infectious dose ($TCID_{50}$)/ml as previously described [22].

Cells

Vero cells (American Type Culture Collection, ATCC, CCL81, Rockville, MD) were cultured in Dulbecco's Modified Eagle Medium (DMEM, Life Technologies Gibco, Darmstadt, Germany) containing 10 % (v/v) fetal calf serum (FCS; Life Technologies Gibco), 100 U/ml penicillin and 0.1 mg/ml streptomycin.

HSV-1-ΔgE-GFP based screening for cell-to-cell spread inhibiting antibodies

To investigate whether humans are able to produce cell-to-cell spread inhibiting antibodies, we established an HSV-1-ΔgE-GFP reporter virus based assay for the high-throughput

276 screening of HSV-1 seropositive human serum or plasma samples. The assay was evaluated
277 using the HSV-1 and HSV-2 cell-to-cell spread inhibiting antibody mAb hu2c [15].
278 Highly permissive Vero cells were seeded on 24-well plates at a density of 1 x 105 cells/well.
279 Confluent cell cultures were infected with 200 $TCID_{50}$ HSV-1-ΔgE-GFP/well (MOI = 0.001).
280 After 2 hours of incubation, the inoculation medium was removed and the cell cultures were
281 incubated with serial dilutions of mAb hu2c (0 - 500 nM). Commercial polyclonal human
282 antibody preparations, Cytotect and Intratect (Biotest, Dreieich, Germany), were used as
283 controls at a concentration of 1 or 2 mg/ml. To standardize the background levels, all purified
284 antibodies were applied in medium containing serum or plasma from an HSV-1 and HSV-2
285 seronegative donor at a 1:40 (v/v) dilution. After 3 days of incubation, the plaque formation
286 was examined by fluorescence microscopy (Axio Observer D1, Zeiss). Additionally, the
287 fluorescence levels were quantified. For this purpose, the cell culture medium was removed,
288 the cells washed with PBS, detached with Trypsin/0.5% EDTA (Life Technologies Gibco),
289 resuspended and transferred to 96-well plates. GFP-signals were quantified using the Mithras²
290 LB 943 microplate multimode reader (Berthold Technologies).
291
292 **High throughput screening of plasmas for the inhibition of HSV-1 cell-to-cell spread**
293 A total number of 2496 human plasmas were screened for the inhibition of the HSV-1 cell-to-
294 cell spread using the high throughput assay as described above. Human plasma samples were
295 applied at 1:40 dilutions. The monoclonal humanized antibody mAb hu2c served as positive
296 control at a concentration of 500 nM (75 µg/ml) diluted in plasma from an HSV-1/2
297 seronegative donor (1:40 in cell culture medium). At this concentration of mAb hu2c, the HSV
298 1 cell-to-cell spread is completely blocked. After 72 hours of incubation, the GFP-signal was
299 measured using the Mithras² LB 943 microplate multimode reader (Berthold Technologies).
300 Fluorescence-values for individual plasma samples were compared with the GFP-intensity
301 measured for mAb hu2c for each plate. The values obtained for the plasma samples were then
302 normalized to the mAb hu2c control and calculated as the x-fold value of the mAb hu2c signal.
303

304 **Identification of HSV-seropositive plasmas by ELISA**

305 The HSV-seropositivity status of donors completing the survey was confirmed by ELISA using
306 the anti-herpes simplex virus type 1 and 2 IgG human ELISA kit (abcam, Cambridge, United
307 Kingdom) according to manufacturer's instructions.

308

309 **Retrospective survey to determine the frequency of HSV-reactivations**

310 To investigate the role of HSV-1 cell-to-cell spread inhibiting antibodies in HSV-seropositive
311 people, we assessed the frequency of symptomatic HSV reactivations in the frame of a
312 retrospective survey. The data acquisition comprised the annual numbers of symptomatic oral
313 or genital HSV-reactivations characterized by the occurrence of characteristic lesions.
314 Furthermore, data on general topics like age, gender, body mass index (BMI) as well as
315 smoking behavior were collected. The survey enrolled a total number of 158 blood donors
316 stratified in three comparable groups according to the presence of cell-to-cell spread inhibiting
317 antibodies as complete inhibition (n = 47), partial inhibition (n = 58) and no inhibition (n = 53).

318

319 **Neutralization assay**

320 To investigate the neutralizing antibody titers in blood donor plasmas, a neutralization assay
321 was performed as previously described [16]. Briefly, serial dilutions (1:20 to 1:2560) of the
322 respective plasma samples were pre-incubated with 100 TCID50 of HSV-1 F for one hour at
323 37 °C and added afterwards to confluent Vero cells cultured in 96-well microtiter plates. After
324 72 hours, the cytopathic effect was analyzed by microscopy and the reciprocal neutralization
325 titer was determined.

326 **Footnote Page**

327

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337 **Author Contributions**

338 SW, MA, RD, MD and UWA performed the experiments. SW, MA, RD, MD, UWA, BG, MR,
339 UD, OW, MT, CH and AK analyzed the data. KR conducted the survey. RL, MR and AK
340 planned the study. SW, MA, CH and AK wrote the manuscript. All authors approved the final
341 version of the manuscript.

342

343 **Conflict of interest**

344 The authors declare that the research was conducted in the absence of any commercial or
345 financial relationship that could be construed as a potential conflict of interest.

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References

1. Looker KJ, Magaret AS, May MT, et al. Global and Regional Estimates of Prevalent and Incident Herpes Simplex Virus Type 1 Infections in 2012. PLoS One **2015**; 10:e0140765.
2. Looker KJ, Magaret AS, Turner KM, Vickerman P, Gottlieb SL, Newman LM. Global estimates of prevalent and incident herpes simplex virus type 2 infections in 2012. PLoS One **2015**; 10:e114989.
3. Bernstein DI, Bellamy AR, Hook EW, 3rd, et al. Epidemiology, clinical presentation, and antibody response to primary infection with herpes simplex virus type 1 and type 2 in young women. Clin Infect Dis **2013**; 56:344-51.
4. Shin H, Iwasaki A. Generating protective immunity against genital herpes. Trends in immunology **2013**; 34:487-94.
5. Whitley RJ, Roizman B. Herpes simplex virus infections. Lancet **2001**; 357:1513-8.
6. Burrel S, Boutolleau D, Azar G, et al. Phenotypic and genotypic characterization of acyclovir-resistant corneal HSV-1 isolates from immunocompetent patients with recurrent herpetic keratitis. J Clin Virol **2013**; 58:321-4.
7. Lau CH, Missotten T, Salzmann J, Lightman SL. Acute retinal necrosis features, management, and outcomes. Ophthalmology **2007**; 114:756-62.
8. McAllister SC, Schleiss MR. Prospects and perspectives for development of a vaccine against herpes simplex virus infections. Expert Rev Vaccines **2014**; 13:1349-60.
9. Johnston C, Corey L. Current Concepts for Genital Herpes Simplex Virus Infection: Diagnostics and Pathogenesis of Genital Tract Shedding. Clin Microbiol Rev **2016**; 29:149-61.
10. Dasgupta G, Chentoufi AA, Nesburn AB, Wechsler SL, BenMohamed L. New concepts in herpes simplex virus vaccine development: notes from the battlefield. Expert Rev Vaccines **2009**; 8:1023-35.
11. Belshe RB, Leone PA, Bernstein DI, et al. Efficacy results of a trial of a herpes simplex vaccine. The New England journal of medicine **2012**; 366:34-43.
12. Hook LM, Cairns TM, Awasthi S, et al. Vaccine-induced antibodies to herpes simplex virus glycoprotein D epitopes involved in virus entry and cell-to-cell spread correlate with protection against genital disease in guinea pigs. PLoS Pathog **2018**; 14:e1007095.
13. Whitbeck JC, Huang ZY, Cairns TM, et al. Repertoire of epitopes recognized by serum IgG from humans vaccinated with herpes simplex virus 2 glycoprotein D. J Virol **2014**; 88:7786-95.
14. Sattentau Q. Avoiding the void: cell-to-cell spread of human viruses. Nature reviews Microbiology **2008**; 6:815-26.
15. Krawczyk A, Arndt MA, Grosse-Hovest L, et al. Overcoming drug-resistant herpes simplex virus (HSV) infection by a humanized antibody. P Natl Acad Sci USA **2013**; 110:6760-5.
16. Krawczyk A, Krauss J, Eis-Hubinger AM, et al. Impact of valency of a glycoprotein B-specific monoclonal antibody on neutralization of herpes simplex virus. Journal of virology **2011**; 85:1793-803.
17. Bauer D, Alt M, Dirks M, et al. A Therapeutic Antiviral Antibody Inhibits the Anterograde Directed Neuron-to-Cell Spread of Herpes Simplex Virus and Protects against Ocular Disease. Frontiers in microbiology **2017**; 8:2115.
18. Bauer D, Keller J, Alt M, et al. Antibody-based immunotherapy of aciclovir resistant ocular herpes simplex virus infections. Virology **2017**; 512:194-200.
19. Krawczyk A, Dirks M, Kasper M, et al. Prevention of herpes simplex virus induced stromal keratitis by a glycoprotein B-specific monoclonal antibody. PLoS One **2015**; 10:e0116800.
20. Eis-Hubinger AM, Schmidt DS, Schneweis KE. Anti-glycoprotein B monoclonal antibody protects T cell-depleted mice against herpes simplex virus infection by inhibition of virus replication at the inoculated mucous membranes. J Gen Virol **1993**; 74 (Pt 3):379-85.

400 21. Gonzalez-Quintela A, Alende R, Gude F, et al. Serum levels of immunoglobulins (IgG,
401 IgA, IgM) in a general adult population and their relationship with alcohol consumption,
402 smoking and common metabolic abnormalities. Clin Exp Immunol **2008**; 151:42-50.
403 22. Belshe RB, Heineman TC, Bernstein DI, et al. Correlate of immune protection against
404 HSV-1 genital disease in vaccinated women. J Infect Dis **2014**; 209:828-36.
405 23. Brown ZA, Benedetti J, Ashley R, et al. Neonatal herpes simplex virus infection in relation
406 to asymptomatic maternal infection at the time of labor. N Engl J Med **1991**; 324:1247-52.
407 24. Jiang Y, Patel CD, Manivanh R, et al. Maternal Antiviral Immunoglobulin Accumulates in
408 Neural Tissue of Neonates To Prevent HSV Neurological Disease. mBio **2017**; 8.
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410

411 **Figure legends**

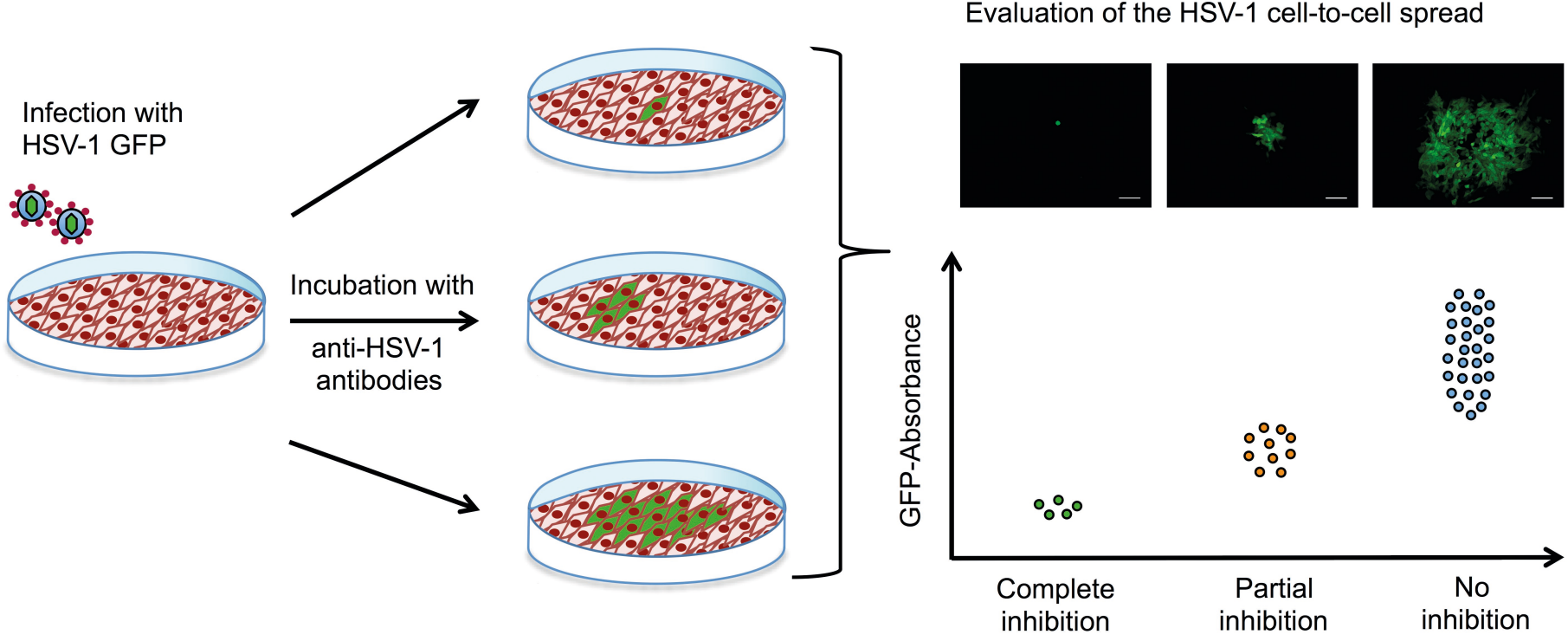
412 **Figure 1: Assessment of the HSV-1 cell-to-cell spread inhibiting antibodies of human**
413 **plasma or serum samples using the HSV-1 ΔgE GFP reporter virus-based screening**
414 **method.** The procedure was based on assessing the extent of plaque formation, which was
415 proportional to the GFP-signal emitted by the infected cells. Confluent Vero cells were infected
416 with HSV-1 ΔgE GFP reporter virus at low MOI. Infected cell cultures were overlaid with a
417 medium containing either sera or plasma samples from HSV-seropositive humans at a 1:40
418 dilution. After 72 h hours of incubation, plaque formation was qualitatively assessed by
419 fluorescence microscopy and simultaneously the GFP-signal was quantified as relative
420 fluorescence units (RFU).

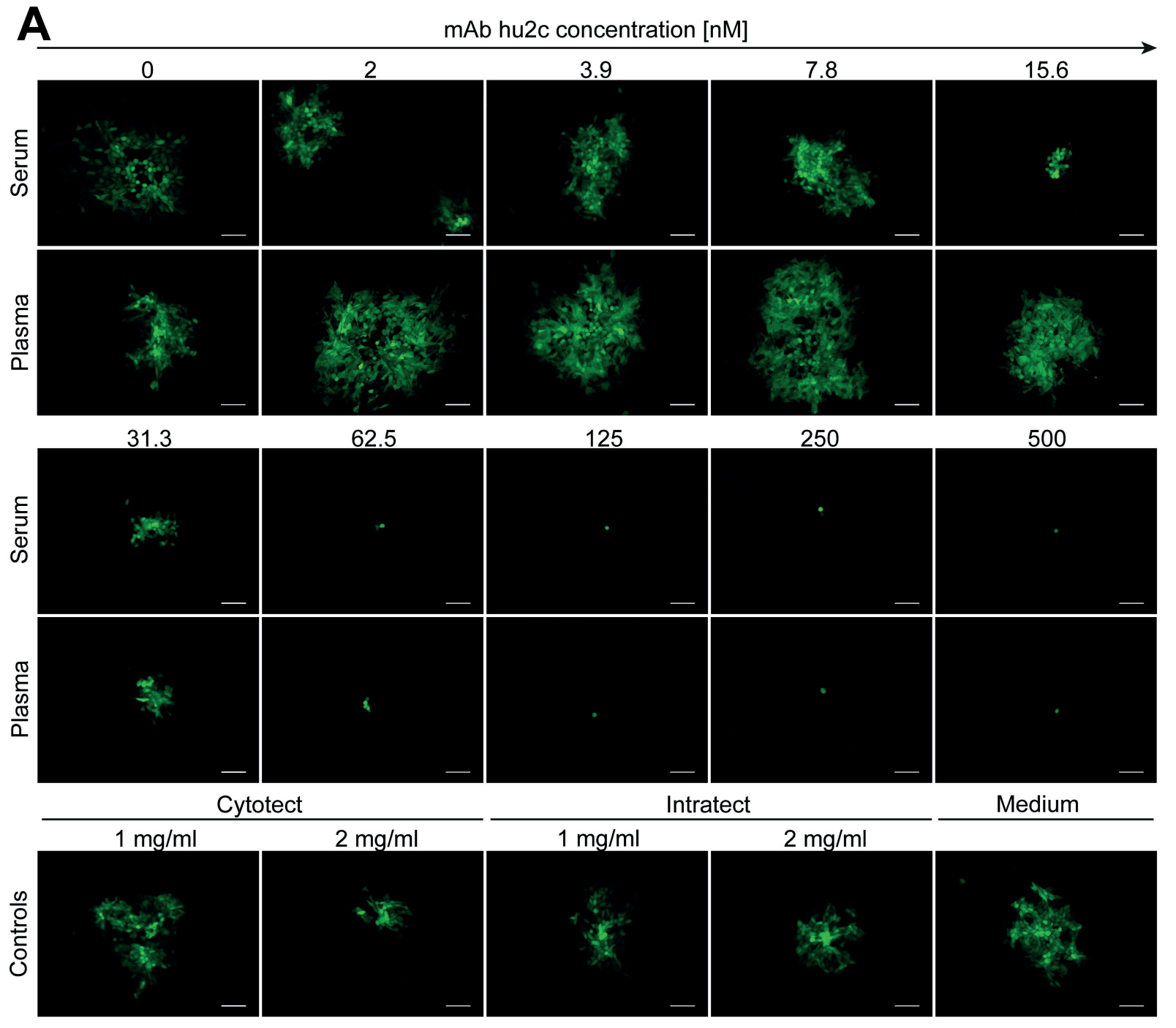
421

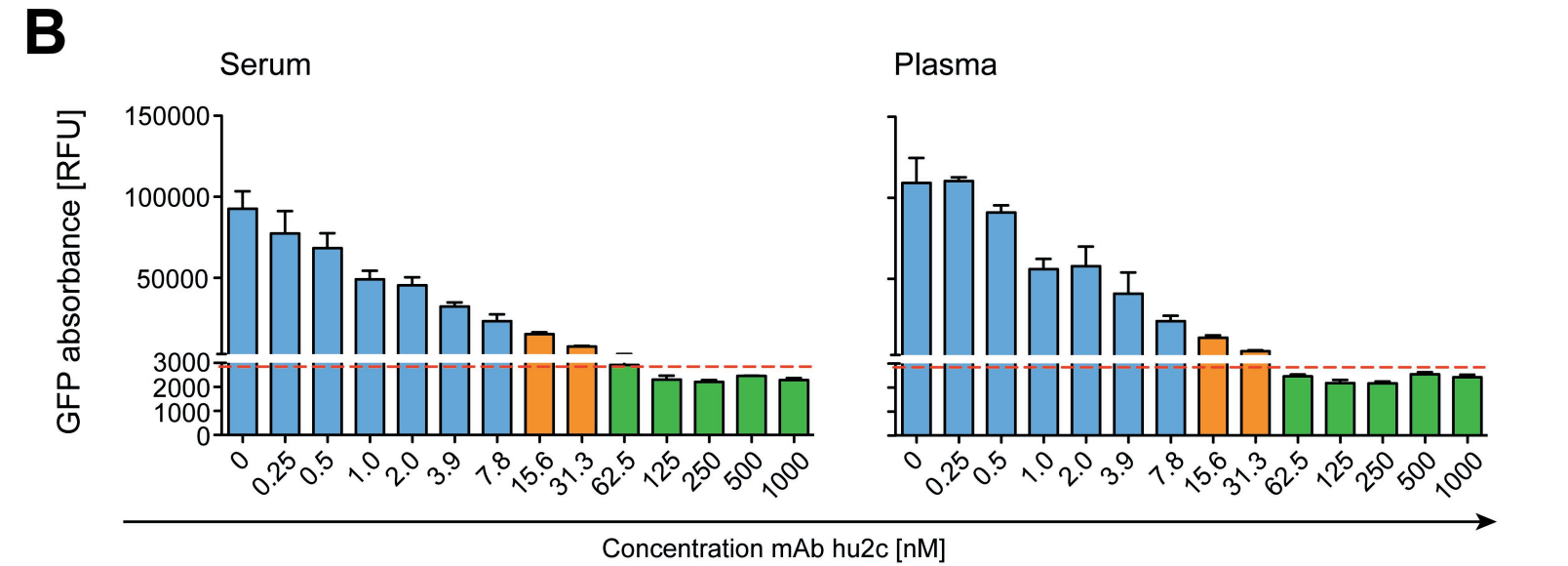
422 **Figure 2: Evaluation of the HSV-1 cell-to-cell spread inhibition by mAb hu2c.** The
423 performance of the HSV-1 ΔgE GFP reporter virus-based assay was evaluated for the
424 screening of sera and plasma that contain various concentrations of HSV-1 cell-to-cell spread
425 inhibiting antibodies. (A) Confluent Vero cells growing on 24-well plates were infected with 200
426 $TCID_{50}$/500 µl of the HSV-1 ΔgE GFP reporter virus. After 2 h of incubation, the inoculating
427 medium was removed and the cells were overlaid with a medium containing sera or plasma
428 from a HSV-1 seronegative donor at a 1:40 dilution. Additionally, the monoclonal, HSV-1/2
429 cell-to-cell spread inhibiting antibody mAb hu2c was added at a final concentration ranging
430 from 0 to 1000 nM. After 72 h hours, plaque formation, which indicates HSV-1 spread via the
431 cell-to-cell spread, was qualitatively assessed by fluorescence microscopy. 100x
432 magnification, scale bar = 100 µm. (B) Additionally, the cell cultures were transferred to 96-
433 well plates to quantify the GFP-signal as relative fluorescence units (RFU). Dashed line = cell-
434 to-cell spread inhibiting concentration of mAb hu2c. Green bars = complete inhibition,
435 orange = partial inhibition, blue = no inhibition of HSV-1 cell-to-cell spread.

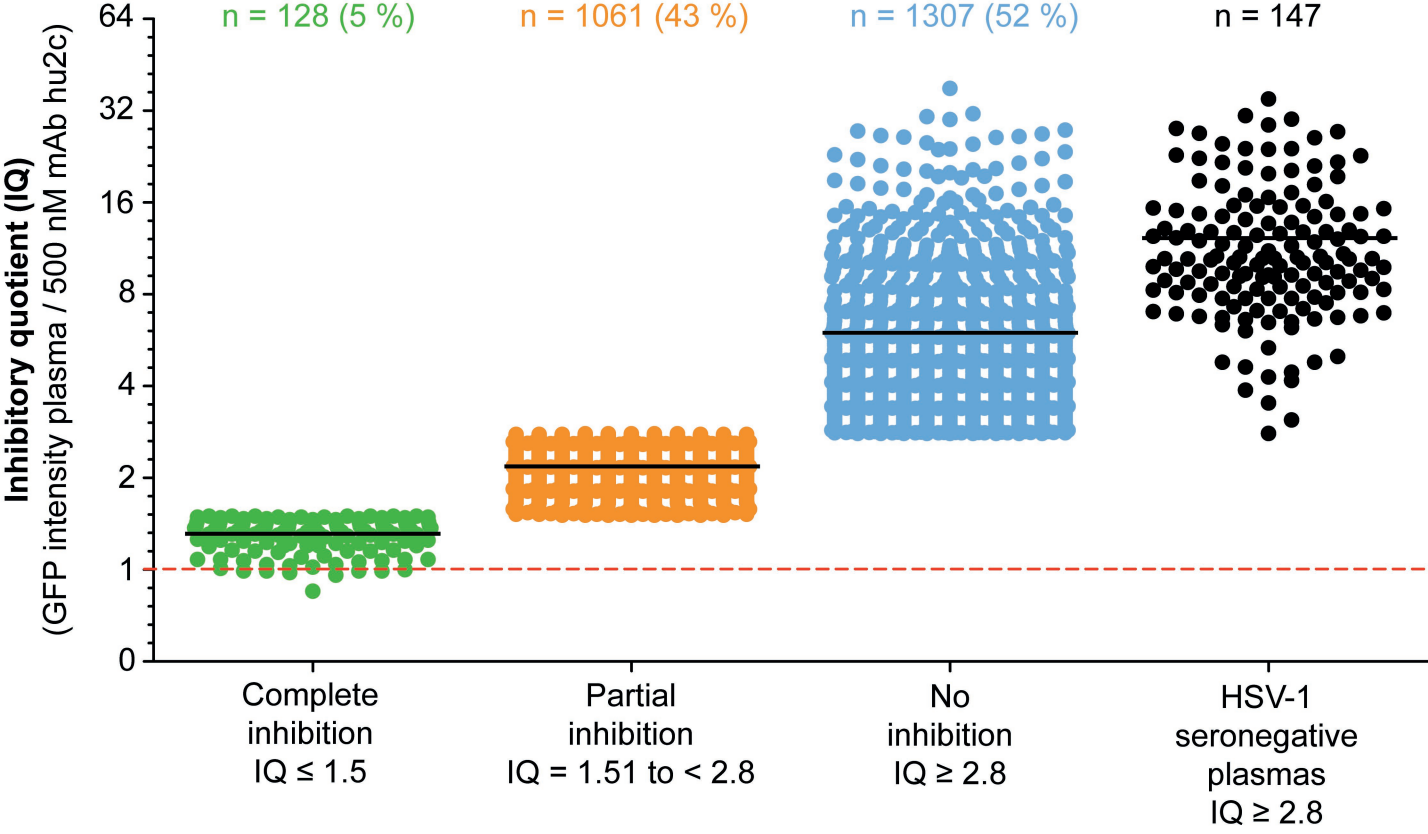
436 **Figure 3: Assessment of the HSV-1 cell-to-cell spread inhibition capacity of plasma**
437 **samples from HSV-1 seropositive blood donors.** A total number of 2496 plasma samples
438 from blood donors were investigated for HSV-1 cell-to-cell spreading properties using a
439 HSV-1 ΔgE GFP reporter virus-based assay as described above. The efficacy of the plasma
440 samples regarding cell-to-cell spread inhibition is shown as a fold change of the 500 nM mAb
441 hu2c threshold (dashed line). At this concentration mAb hu2c completely inhibits the HSV-1
442 cell-to-cell spread. The efficacy of the plasma samples regarding cell-to-cell spread inhibition
443 was determined by dividing the GFP-signal of a cell culture treated with a plasma sample
444 through the GFP-signal of mAb hu2c control. This quotient was termed inhibitory quotient (IQ)
445 and represents the x-fold value of the GFP-signal measured for mAb hu2c. The plasma
446 samples were classified as completely cell-to-cell spread inhibiting (green dots, IQ ≤ 1.5),
447 partially inhibiting (orange dots, IQ = 1.51 - 2.8) and non-inhibiting (blue dots, IQ ≥ 2.8). Each
448 point represent the IQ for each donor, horizontal bars represent the median value.
449
450 **Figure 4: Correlation between protective antibody response and the frequency of HSV**
451 **reactivation.** A total number of 158 HSV seropositive blood donors previously being tested for
452 cell-to-cell spread inhibiting antibodies were retrospectively interviewed for the frequency of
453 symptomatic HSV reactivations per year. The donors were divided into the three groups
454 (complete inhibition, n = 48; partial inhibition, n = 58 and no inhibition, n = 53) according to the
455 performance of the donor plasmas on the HSV-1 cell-to-cell spread inhibition. The total
456 numbers of donors are depicted as a bar chart and the percentages as a pie chart above.
457 Differences in the annual frequency of HSV reactivation were analyzed using the Fischer´s
458 exact test. Significant changes ($*p < 0.05$) are indicated by asterisks and non-significant
459 changes ($p > 0.05$) are labeled as “n.s.”.

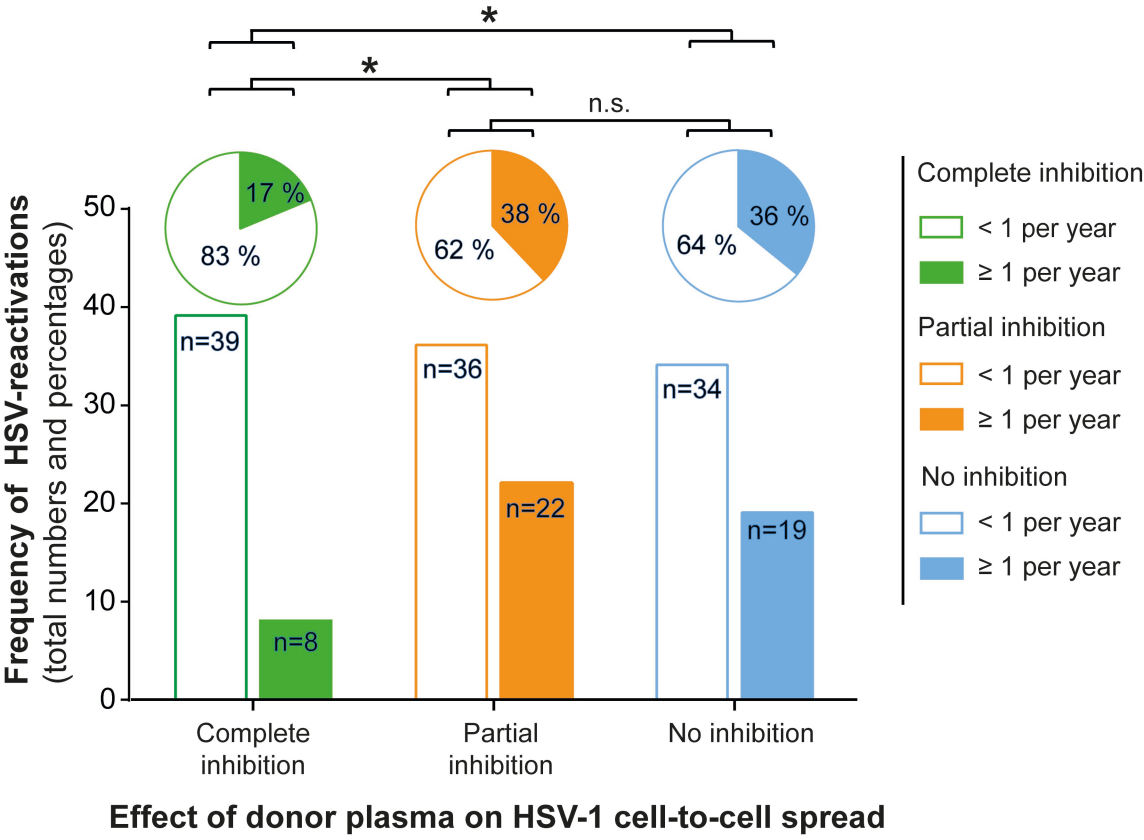
460 **Figure 5: HSV-1 neutralizing antibody titers of the plasma samples of the three inhibition**
461 **groups (complete, partial and no inhibition group)**. Serial dilutions of the respective plasma
462 samples (1:20 to 1:2560) were preincubated with 100 $TCID_{50}$ HSV-1 F for one hour and
463 subsequently added to Vero cells in 96-well microtiter plates. After 48 h of incubation, the
464 cytopathic effect was analyzed and the respective neutralization titers were determined. Data
465 sets were statistically analyzed using the One-way ANOVA followed by the Dunn's multiple
466 comparison *post-hoc* test. Significant changes ($^{**}p < 0.01$, $^{****}p < 0.0001$) are indicated by
467 asterisks and non-significant changes ($p > 0.05$) are labeled as "n.s.".
468

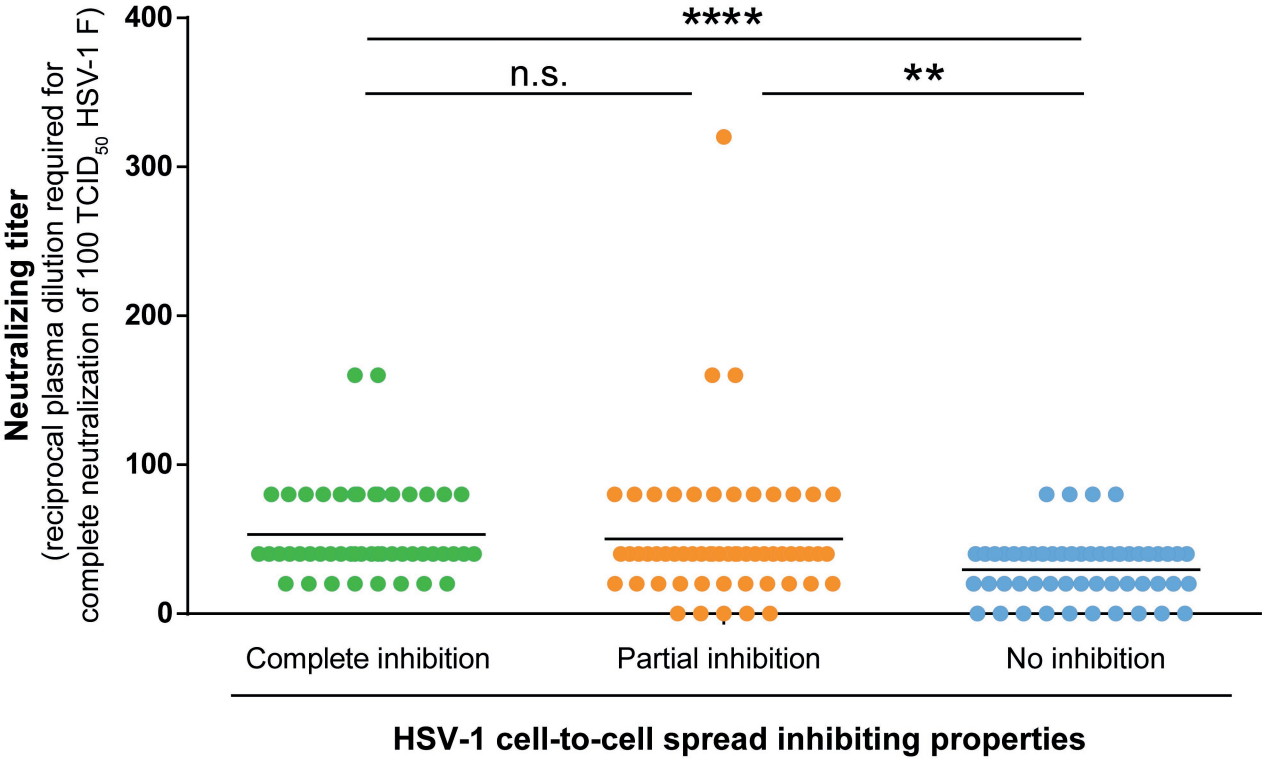

Infection with
HSV-1 GFP
Incubation with
anti-HSV-1
antibodies
Evaluation of the HSV-1 cell-to-cell spread
GFP-Absorbance
Complete
inhibition
Partial
inhibition
No
inhibition


A
mAb hu2c concentration [nM]
0
2
3.9
7.8
15.6
Serum
Plasma
31.3
62.5
125
250
500
Serum
Plasma
Cytotect
Intratect
Medium
1 mg/ml
2 mg/ml
1 mg/ml
2 mg/ml
Controls


B
Serum
Plasma
GFP absorbance [RFU]
150000
100000
50000
3000
2000
1000
0
0
0.25
0.5
1.0
2.0
3.9
7.8
15.6
31.3
62.5
125
250
500
1000
Concentration mAb hu2c [nM]


n = 128 (5 %)
n = 1061 (43 %)
n = 1307 (52 %)
n = 147
Inhibitory quotient (IQ)
(GFP intensity plasma / 500 nM mAb hu2c)
64
32
16
8
4
2
1
0
Complete inhibition IQ ≤ 1.5
Partial inhibition IQ = 1.51 to < 2.8
No inhibition IQ ≥ 2.8
HSV-1 seronegative plasmas IQ ≥ 2.8


*
*
n.s.
17 %
83 %
38 %
62 %
64 %
36 %
n=39
n=8
n=36
n=22
n=34
n=19
Frequency of HSV-reactivations
(total numbers and percentages)
0
10
20
30
40
50
Complete inhibition
Partial inhibition
No inhibition
Effect of donor plasma on HSV-1 cell-to-cell spread
Complete inhibition
< 1 per year
≥ 1 per year
Partial inhibition
< 1 per year
≥ 1 per year
No inhibition
< 1 per year
≥ 1 per year


Neutralizing titer
(reciprocal plasma dilution required for complete neutralization of 100 TCID50 HSV-1 F)
400
300
200
100
0
****
n.s.
**
Complete inhibition
Partial inhibition
No inhibition
HSV-1 cell-to-cell spread inhibiting properties