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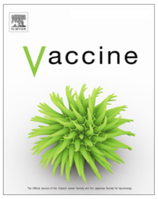

A trivalent gC2/gD2/gE2 vaccine for herpes simplex virus generates antibody responses that block immune evasion domains on gC2 better than natural infection

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ABSTRACT

Vaccines for prevention and treatment of genital herpes are high public health priorities. Our approach towards vaccine development is to focus on blocking virus entry mediated by herpes simplex virus type 2 (HSV-2) glycoprotein D (gD2) and to prevent the virus from evading complement and antibody attack by blocking the immune evasion domains on HSV-2 glycoproteins C (gC2) and E (gE2), respectively. HSV-2 gC2 and gE2 are expressed on the virion envelope and infected cell surface where they are potential targets of antibodies that bind and block their immune evasion activities. We demonstrate that antibodies produced during natural infection in humans or intravaginal inoculation in guinea pigs bind to gC2 but generally fail to block the immune evasion domains on this glycoprotein. In contrast, immunization of naïve or previously HSV-2-infected guinea pigs with gC2 subunit antigen administered with CpG and alum as adjuvants produces antibodies that block domains involved in immune evasion. These results indicate that immune evasion domains on gC2 are weak antigens during infection, yet when used as vaccine immunogens with adjuvants the antigens produce antibodies that block immune evasion domains.

1. Introduction

A vaccine for prevention or treatment of genital herpes has remained an elusive goal. The largest human trials for prevention of genital herpes have involved subunit antigen vaccines aimed at blocking virus entry [1–3]. These vaccine efforts have failed to achieve their primary endpoints of preventing genital herpes lesions or preventing both genital lesions and evidence of subclinical infection [1–3]. The Friedman laboratory is pursuing a vaccine strategy for prevention and possibly treatment of genital herpes that involves blocking virus entry by targeting gD and blocking immune evasion of antibody mediated by gE and of complement by gC [4,5]. Herpes simplex virus (HSV) gC and gE are immune evasion molecules that are expressed on the virion envelop and on the surface of infected cells [6–11]. Therefore, these glycoproteins are potentially targets for antibodies produced during infection or by immunization [12–14].

Viral proteins that are key targets of antibodies may remain hidden from host immunity under a carbohydrate shield, protection provided by neighboring proteins, or frequent mutations in antigenic structure [15–19]. A hallmark of human herpesviruses is their ability to produce recurrent infections. We evaluated whether antibodies produced to one HSV-2 immune evasin, gC2 block immune evasion domains on this glycoprotein and whether immunization is more effective than infection at producing antibodies that bind and block sites involved in immune evasion. Our results indicate that immune evasion sites on gC2 are weakly immunogenic, yet when gC2 is combined with adjuvants, antibodies are produced in immunized guinea pigs that bind and block immune evasion sites.

Abbreviations: HSV-1, herpes simplex virus type 1; HSV-2, herpes simplex virus type 2; gC, gD, gE, Herpes simplex virus glycoproteins C, D or E; gC2, gD2, gE2, HSV-2 glycoproteins C, D or E; IM, intramuscularly.

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2. Materials and methods

2.1. Human ethics statement

The Institutional Review Board of the University of Pennsylvania Perelman School of Medicine approved the use of the human sera/plasma for these studies (protocol 818596). Plasma samples were de-identified by Genocea Biosciences prior to shipment to the University of Pennsylvania to ensure the privacy of subjects [20].

2.2. Guinea pig ethics statement

Guinea pig studies were performed in accordance with protocol No. 805187 approved by The Institutional Animal Care and Use Committee of the University of Pennsylvania. The protocol followed recommendations in the Institute for Laboratory Animals Research's "Guide for the Care and Use of Laboratory Animals." Saline (10 ml) was given subcutaneously to guinea pigs that appeared dehydrated (>15% weight loss), while meloxicam (0.3 mg/kg intraperitoneally) was used to control pain upon the development of genital lesions. Mock-immunized animals and animals infected prior to immunization that developed more than two genital lesions during the acute infection (days 1–14) were treated once daily with acyclovir intraperitoneally (50 mg/kg) until recovery from acute infection or until humanly euthanized. Animals that lost >20% body weight, that developed bloody urine, or hind limb weakness were humanly euthanized using Euthasol in accordance with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association.

2.3. Source of human sera/plasma

Sera from 10 human subjects infected with HSV-2 (HSV-1 seronegative, HSV-2 seropositive) and 3 double seronegative controls (HSV-1/-2 seronegative) were kindly provided by Dr. Anna Wald. Plasma was provided by Genocea Biosciences from 75 human subjects, including: (i) 4 HSV-1 and HSV-2 naïve individuals with no known exposure to genital herpes; (ii) 17 HSV-2 seronegative subjects who were exposed to a partner with HSV-2 infection; (iii) 4 HSV-1 seropositive/HSV-2 seronegative subjects; (iv) 18 HSV-2 seropositive individuals with no genital recurrent infections; (v) 19 HSV-2 seropositive subjects with 1–3 recurrent infections per year; and (vi) 13 HSV-2 seropositive subjects with $\geq$4 recurrences per year [20].

2.4. Source of guinea pig sera for the studies

Sera were obtained from two previous studies performed in guinea pigs [4,5]. Animals assigned to the Infected group were infected intravaginally with HSV-2 strain MS and mock immunized intramuscularly (IM) 19 days post infection with 3 doses of CpG oligonucleotide (5′-TCGTCGTTGTCGTTTTGTCGTT-3′) and alum spaced approximately 2 weeks apart [5]. Sera from this infected group were obtained 90 days post infection. Animals assigned to the Immunized group received 3 immunizations 2 weeks apart of the trivalent vaccine IM in the gastrocnemius muscle containing gC2, gD2, and gE2 subunit antigens mixed with CpG and alum. Sera from this immunized group were obtained 2 weeks after the final immunization, prior to intravaginal infection [5]. Animals assigned to the Naïve group were uninfected and not immunized. In a second series of experiments, animals were infected with HSV-2 strain MS first and then randomized to receive either the mock or trivalent vaccines: Animals in the Infected & mock immunized group were infected with HSV-2 strain MS and then immunized 3 times at 2-week intervals with the mock vaccine CpG oligonucleotide and alum [4]. Animals in the Infected & trivalent immunized group were infected with HSV-2 strain MS and then immunized 3 times at 2-week intervals with the trivalent vaccine consisting of gC2, gD2, and gE2 subunit antigens mixed with CpG and alum [4]. Sera were obtained from these Infected and Mock or Trivalent Immunized animals on day 35 post-infection, just prior to immunization to reflect their antibody response to gC2 following infection and 2 weeks after the final immunization with either the mock or trivalent vaccine [4].

2.5. IgG purification

IgG was purified from human sera/plasma using a Protein G Spin Plate and from guinea pig sera using a Protein A Spin Plate according to the manufacturer's instructions (Thermo Scientific, Pierce Biotechnology, Rockford, IL). The protein concentration of purified IgG was determined using a Micro BCA Protein Reagent Kit according to the manufacturer's instructions (Thermo Scientific, Pierce Biotechnology, Rockford, IL). Fractions containing IgG were stored at −80 °C.

2.6. gC2 ELISA assays

Purified bac-gC2(426t) was added to 96-well High Binding Costar microtiter plates (Corning Incorporated, Corning, NY) at 50 ng/well in binding buffer (50 mM sodium bicarbonate pH 8.5), incubated for 1 h at room temperature then overnight at 4 °C, and blocked for 1 h with 5% (wt/vol) nonfat milk in PBS 0.05% Tween 20 (PBST) [21]. Purified IgG at 1 µg/well or a 1:1000 dilution of sera/plasma in PBST was added to protein-coated wells. Bound IgG was detected at an optical density of 405 nm using horseradish peroxidase-conjugated anti-human or anti-guinea pig IgG at 1:2000 dilution followed by ABTS.

2.7. gC2 blocking assays

An ELISA-based assay was used to evaluate antibody responses to gC2 that blocked the interaction of gC2 with C3b following infection or immunization [5]. Wells of a 96-well High Binding Costar microtiter plate were coated with purified C3b (kindly provided by Dr. John Lambris) at 200 ng/well in sodium bicarbonate binding buffer (pH 8.5), incubated for 1 h at room temperature, then overnight at 4 °C, and blocked for 2 h at room temperature with 5% (wt/vol) nonfat milk in PBS 0.05% Tween 20 (PBST). Purified human or guinea pig IgG at the concentrations indicated was incubated with 50 ng bac-gC2(426t) for 1 h at 37 °C and added to C3b coated wells for 1 h. Bound gC2 was detected with rabbit anti-gC2 serum (UP2152) followed by HRP-conjugated goat anti-rabbit IgG.

2.8. Statistical analysis

The D'Agostino & Pearson normality test (GraphPad Prism software) was performed to evaluate data normality. Student's t and Mann-Whitney tests were used to determine statistical significance depending upon normality. Possible correlations among data sets were evaluated using Spearman's correlation test. Fisher's exact test was used to evaluate proportional differences between variables. Results were considered significant at a P value <0.05.

3. Results

3.1. Antibodies produced following HSV-2 infection in humans generally fail to block immune evasion domains on gC2

We evaluated sera from 10 HSV-2 infected subjects that were HSV-1 seronegative and HSV-2 seropositive (HSV-1-/2+) and three uninfected subjects (HSV-1-/2-) by ELISA for antibodies to gC2. Nine of ten HSV-2 infected subjects produced gC2 antibody titers that were higher than titers in the seronegative subjects (Fig. 1a). We evaluated whether the antibodies produced by infection block gC2 binding to C3b. Antibodies from 8/10 subjects failed to block this binding (Fig. 1b), while antibodies from 2/10 blocked (marked in red). Serum #2 blocked gC2 binding to C3b by >50% at 6.3 µg/100 µl (representing ~ 1:160 dilution of serum) while sample #9 blocked binding by >50% at 25ug/100ul (~ 1:40 dilution of serum). Both sera #2 and #9 had the highest ELISA titers, suggesting high titer may correlate with the development of blocking antibodies. Most HSV-2 infected subjects however, fail to produce antibodies that block immune evasion domains on gC2.

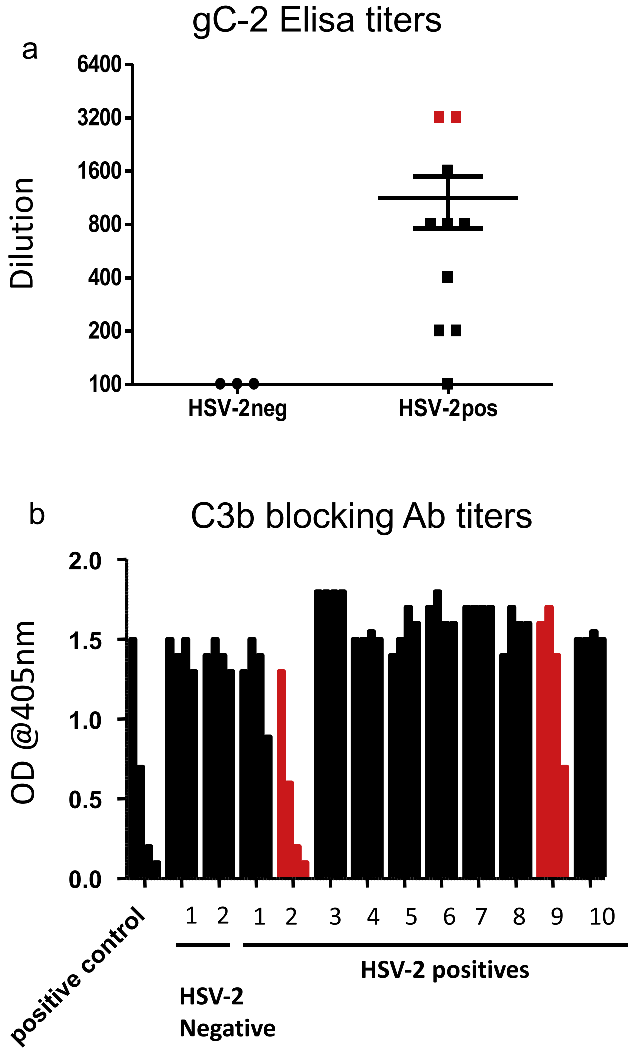

Fig. 1. ELISA antibody responses to gC2 in human sera. (a) gC2 ELISA antibody levels in seronegative and seropositive subjects. Sera from seronegative (n = 3) and HSV-2 seropositive (HSV-1-/2+, n = 10) subjects were evaluated for reactivity to gC2. (b) Antibodies that block gC2 binding to C3b. The positive control represents mouse polyclonal sera to gC2 produced by immunization with bac-gC2(426t). Sera from 2 of the 3 seronegative subjects and all 10 seropositive subjects from "a" were evaluated. Serial two-fold concentrations of purified IgG at 3.1, 6.3, 12.5, and 25 µg/100 µl of IgG were incubated with gC2 protein and added to wells coated with C3b. Samples that blocked C3b binding are shown in red. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. Antibodies produced in humans with frequent outbreaks of genital herpes fail to block immune evasion domains on gC2 despite high ELISA titers

We evaluated a larger panel of 75 plasma samples, including 13 from subjects with frequent recurrences of genital herpes to determine whether the antibodies produced to gC2 after HSV-2 infection block domains involved in immune evasion and whether development of high titer antibodies to gC2 correlates with the development of blocking antibodies [20].

3.2.1. gC2 ELISA titers

Comparing the gC2 ELISA titers in naïve subjects with the other groups (Fig. 2a): the gC2 ELISA titers were higher than the highest gC2 titer in the naïve group (above the dotted line) in 4/17 HSV-2 exposed subjects, 3/4 HSV-1 infected subjects, 11/18 HSV-2 infected subjects with no recurrences, 18/19 HSV-2 infected with 1–3 recurrences and 11/13 HSV-2 infected with ≥4 recurrences. The mean HSV-2 gC2 ELISA titers were significantly higher in subjects with 1–3 or ≥4 recurrences than HSV-1 infected subjects ($p < 0.01$) or the exposed ($p < 0.01$) or naïve groups ($p < 0.05$). No significant differences in titers were detected among HSV-2 infected subjects with no, 1–3, and ≥4 recurrences.

3.2.2. Blocking C3b binding to gC2

We evaluated whether the antibodies produced by infection or exposure block gC2 binding to C3b. Each sample was tested at a single IgG concentration of 10 µg/100 µl. No significant differences were detected among the various groups by comparing the reduction in OD at 405 nm (Fig. 2b). A weak, yet non-significant correlation was observed between gC2 ELISA and blocking C3b binding (Fig. 2c). Therefore, despite high ELISA titers, most HSV-2 infected subjects fail to produce antibodies that block gC2 domains that mediate immune evasion.

3.3. Immunization produces antibodies that block gC2 binding to C3b better than infection

Guinea pigs were uninfected (n = 2), infected intravaginally with HSV-2 (n = 9), or immunized with gC2/gD2/gE2 subunit antigens given with CpG/alum (n = 9) [5]. Serum was evaluated for gC2 antibody titers ~90 days after infection in the infected group or 14 days after the 3rd immunization in the immunized group.

3.3.1. gC2 ELISA titers

The gC2 ELISA titers in the infected guinea pigs were higher than in the naïve animals in 8/9 animals, while in the immunized guinea pigs, ELISA titers were higher than naïve animals in 9/9 animals (Fig. 3a). The mean ELISA titers were significantly higher in the immunized than infected animals (Mann-Whitney, p = 0.0008).

3.3.2. Blocking immune evasion domains on gC2 in infected and immunized guinea pigs

We evaluated whether immunization produces antibodies that bind gC2 and block the interaction between gC2 and C3b more effectively than infection. IgG at 10 µg/100 µl from immunized animals significantly blocked gC2 binding to C3b as measured by a reduction of OD at 405 nm compared with infected animals (Mann-Whitney, p = 0.0028) (Fig. 3b). The data indicate that antibody responses produced through immunization block immune evasion domains better than natural infection.

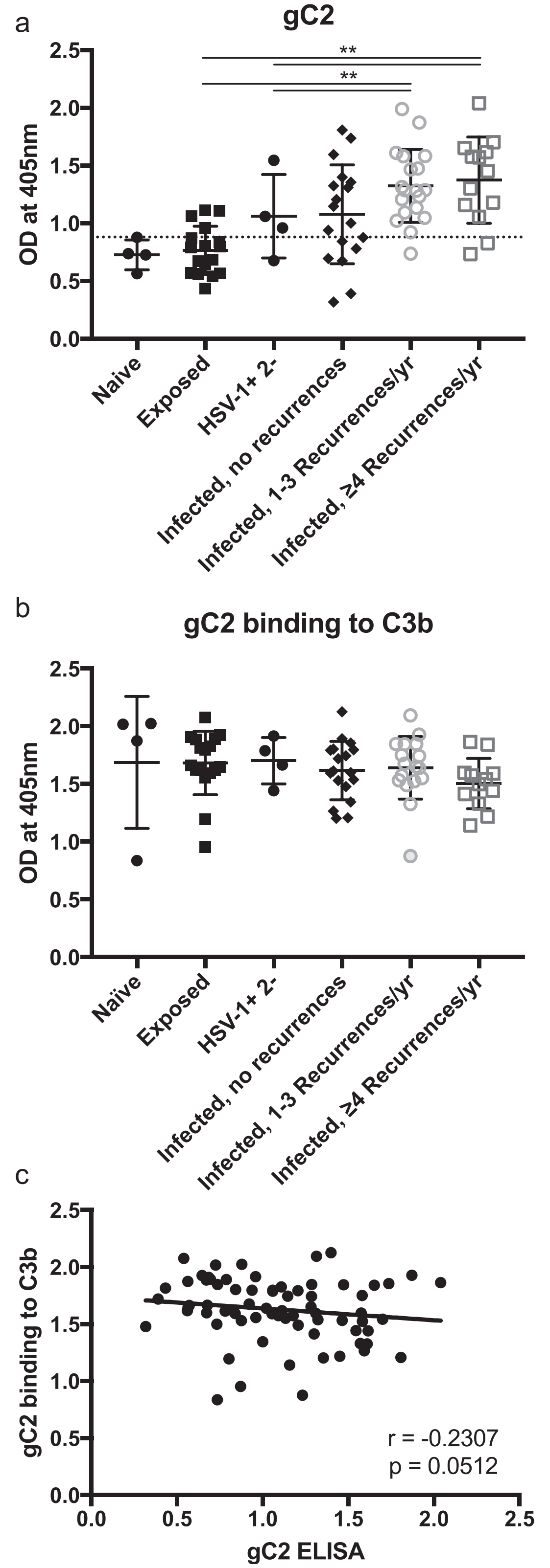

Fig. 2. Antibodies to gC2 in a broad panel of human plasma. (a) gC2 ELISA antibody levels in naïve, HSV-2 exposed, HSV-1+/2−, and HSV-2 infected humans with no recurrences, 1–3 recurrences annually or ≥4 recurrences annually. Plasma was evaluated at a dilution of 1:1000 for reactivity to gC2 by ELISA. The dotted line was set just above the highest value obtained in the naïve subjects. Lines represent mean ± SD. **, p < 0.01. (b) Assessing antibodies that block gC2 from binding C3b. 10 µg/100 µl of IgG was incubated with gC2 protein and added to wells coated with C3b. Lines represent mean ± SD. (c) Lack of correlation between gC2 antibody and blocking gC2 binding C3b. P and rho (r) values were calculated by Spearman's correlation.

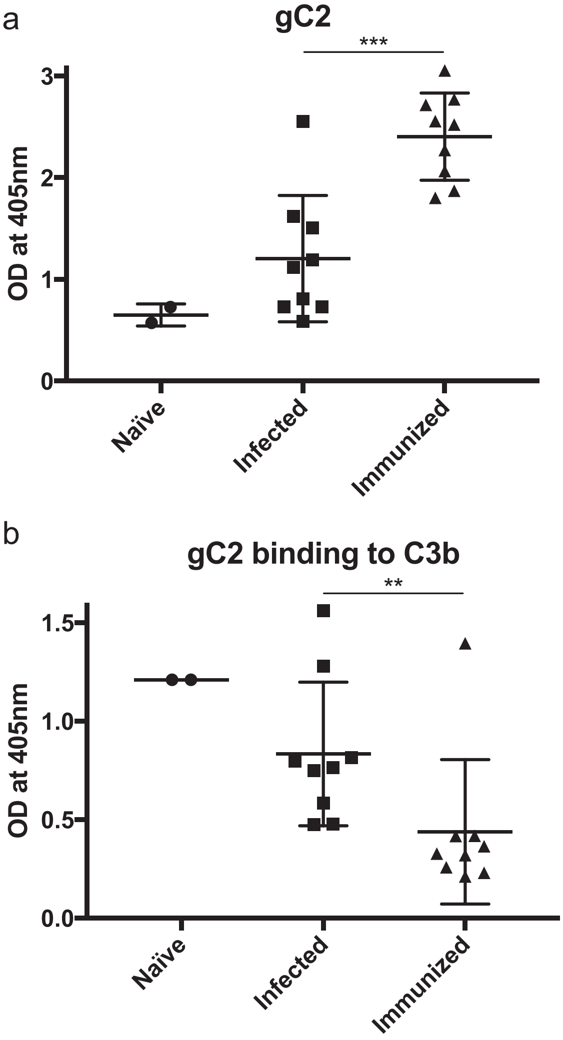

Fig. 3. Comparison of antibody responses to gC2 in infected and immunized guinea pigs. (a) gC2 ELISA antibody levels in naïve, HSV-2 vaginally infected and gC2/gD2/gE2 immunized but uninfected guinea pigs. Purified IgG isolated from naïve, HSV-2 vaginally infected and gC2/gD2/gE2 immunized guinea pig sera was evaluated for reactivity to gC2 using IgG at 1 µg/ml. Lines represent mean ± SD. ***, p < 0.001. (b) IgG from immunized guinea pigs blocks gC2 binding to C3b. gC2 was incubated with 10 µg/100 µl of purified IgG from naïve, HSV-2 vaginally infected and gC2/gD2/gE2 immunized but uninfected guinea pigs and then evaluated for binding to C3b. Lines represent mean ± SD. **, p < 0.01.

3.4. Immunization of previously infected guinea pigs produces antibodies that block domains on gC2 that bind C3b

3.4.1. gC2 ELISA

We evaluated gC2 ELISA responses in guinea pigs that were infected intravaginally with HSV-2 and then randomized to receive either CpG/alum (labeled as Infected & mock immunized) or gC2/gD2/gE2 with CpG/alum (labeled as Infected & trivalent immunized) [4]. Animals in each group were bled 35 days post-infection (labeled Post-infection) and then the same animals were bled 8 weeks later after either mock-immunization or immunization with the trivalent vaccine (labeled Post-infection & immunization). Animals in the Infected & mock immunized group had an average of 4.8 days with recurrent genital lesions from the time of the first mock immunization until the final bleed [4]. Despite these frequent recurrences, antibody titers to gC2 were not significantly different from antibody levels observed in the Infected group, which was evaluated on sera obtained from the same animals at an earlier time post-infection (Fig. 4a). In contrast, the Infected and trivalent immunized animals had an average of 0.8 recurrences after the first immunization [4]. Despite the low number of recurrences, immunization with the trivalent vaccine significantly boosted ELISA antibody titers to gC2 in infected animals (Mann-Whitney, $p < 0.0001$) (Fig. 4a).

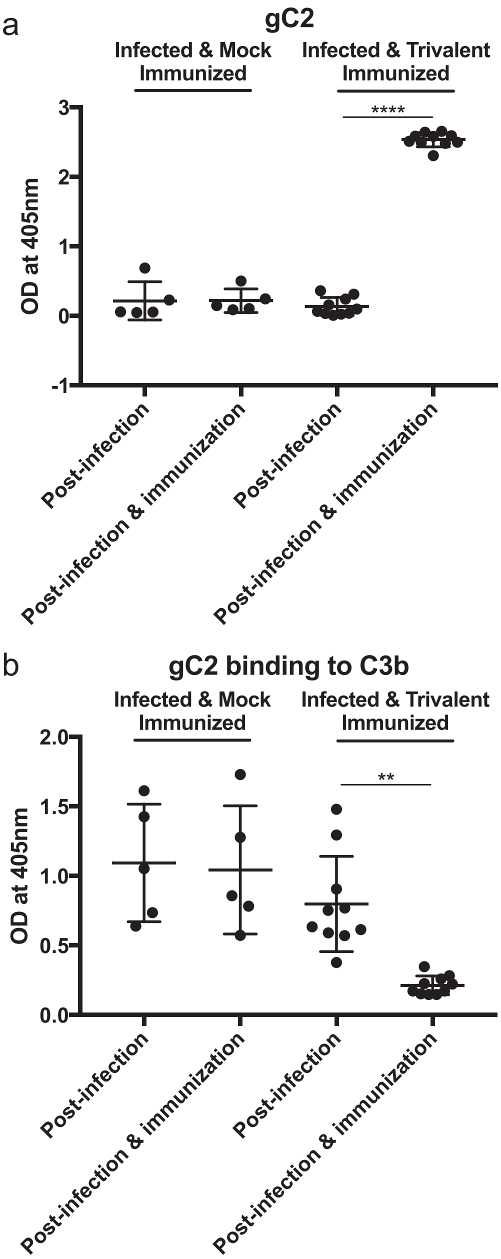

Fig. 4. Immunization of previously infected guinea pigs boosts gC2 ELISA antibody titers and produces antibodies that block gC2 binding to C3b. HSV-2 vaginally infected guinea pigs were mock immunized with CpG/alum (labeled as Infected & mock immunized) or immunized with gC2/gD2/gE2 with CpG/alum (labeled as Infected & trivalent immunized). Sera were obtained from the infected guinea pigs just prior to immunization (labeled as Post-infection) and approximately 3 weeks after the final immunization (labeled as Post-infection & immunization). The same 5 animals are evaluated pre- and post-immunization in the Infected & mock immunized group, and the same 9 animals are in the Infected and trivalent immunized group. (a) ELISA antibody titers to gC2 and (b) blocking gC2 binding to C3b were evaluated as in Fig. 3. Lines represent mean ± SD. **, $p < 0.01$; ****, $p < 0.0001$.

3.4.2. Blocking immune evasion domains on gC2 in infected guinea pigs that are subsequently immunized

Immunization of previously infected guinea pigs with the trivalent vaccine produced antibodies that blocked C3b binding to gC2 as measured by a significant reduction of OD at 405 nm compared with mock-immunized animals (Mann-Whitney, $p = 0.007$) (Fig. 4b). No differences were observed in C3b blocking in mock-immunized animals pre and post immunization (Mann-Whitney). Therefore, immunization of infected animals produces antibodies that block immune evasion domains on gC2.

4. Discussion

The central observation from these studies is that HSV-2 infection in most humans fails to produce antibodies that block immune evasion domains on gC2, including subjects with frequent recurrences of genital herpes. Individuals with frequent recurrences boost their gC2 ELISA titers but do not produce antibodies that block immune evasion. Our results confirm a prior study that detected no differences in gC2 antibody responses in humans with either frequent or no genital recurrences, although assays were not performed to identify antibodies that block C3b binding [13]. In guinea pigs, despite frequent recurrences, most infected animals failed to produce antibodies that blocked immune evasion domains on gC2. In contrast, immunization of naïve or previously infected guinea pigs produced antibodies that bind to gC2 and block its ability to bind C3b. The key conclusions are that: (i) immune evasion epitopes on gC2 are not very immunogenic during natural HSV-2 infection in humans or in HSV-2-infected guinea pigs, and (ii) immunization of naïve guinea pigs or previously infected guinea pigs with gC2 subunit antigen administered with CpG/alum as adjuvants enhances immunogenicity of otherwise weakly immunogenic epitopes.

We previously demonstrated that humans naturally infected with HSV-1 or HSV-1 infected mice produce antibodies to gC1 that block C3b binding infrequently and only at high concentrations, while gC1 immunized mice develop high titers of gC1 antibodies that block [22]. We now extend these observations to include HSV-2 with the added information that despite frequent recurrences, humans and animals fail to produce antibodies that block gC2 immune evasion domains. Nevertheless, the immunogenicity of these weak antigens is enhanced by immunization.

Others have measured epitope specific antibody responses to gD2 in infected humans or subjects immunized with the gD2 subunit antigen vaccine in the Herpevac Trial for Women [23,24]. Using a similar approach, we will attempt to identify the specific epitopes on gC2 that are immunogenic in infected and immunized guinea pigs. A comparison of the epitope specific antibody responses in immunized and infected animals will help identify crucial epitopes on gC2 that produce antibodies that block immune evasion.

Our initial hypothesis when planning these studies was that subjects with frequent recurrences would have lower titers of antibodies that block immune evasion domains on gC2 than subjects with less frequent or no recurrences based on antibodies that block gC immune evasion being helpful in host defense [25]. Our hypothesis was not validated since the number of recurrent infections did not correlate with producing antibodies to gC2 immune evasion domains in humans or guinea pigs. Antibodies that blocked gC2 immune evasion domains were only consistently produced after immunization of naïve or previously infected guinea pigs suggesting that gC2 immune evasion domains may be hidden from host immunity under a carbohydrate shield or protected by neighboring proteins during infection, and only become exposed to host immunity following immunization with gC2 subunit antigen given with adjuvants. The production of blocking antibodies provides a possible mechanism for the observation that a therapeutic vaccine consisting of gC2/gD2/gE2 with CpG alum reduced recurrent genital lesions and genital shedding of HSV-2 DNA [4].

5. Conclusions

We demonstrate in humans and guinea pigs that infection with HSV-2 generally fails to produce antibodies that block immune evasion domains on gC2, suggesting that these domains are weak immunogens. Nevertheless, immunization of guinea pigs with gC2 subunit antigen administered with CpG/alum can overcome the weak immunogenicity and produce antibodies that bind to these glycoproteins and block their immune evasion domains. These results represent an important example of how subunit antigens may provide better protection against the antigen selected than immunity acquired after natural infection or vaccine-induced immunity provided by attenuated live virus vaccines.

Author contributions

Conceptualization: LMH, SA, HMF; Data Curation: LMH, SA, JD, JF, DL; Experimentation: LMH, SA, JD; Formal Analysis: LMH, SA, HMF; Supervision: HMF; Writing original draft: LMH, HMF; Funding Acquisition: SA, HMF.

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Conflict of interest statement

None of the authors has competing interests. JF and DL are employed by a commercial company Genocea Biosciences that is not currently working on an HSV vaccine.

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