Vaccine induced HIV-1 envelope gp120 Constant Region 1-specific Antibodies Expose a CD4-inducible Epitope and Block the Interaction of HIV-1 gp140 With Galactosylceramide

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Running title: Blocking of HIV-1 gp140 binding to Galcer.
Abstract

Mucosal epithelial cell surface galactosylceramide (Galcer) has been postulated to be a receptor for HIV-1 envelope (Env) interacting with mucosal epithelial cells. Disruption of HIV-1 Env interaction with such alternate receptors could be one strategy to prevent HIV-1 entry through the mucosal barrier. To study antibody modulation of HIV-1 Env-Galcer interactions, we have used Galcer containing liposomes to assess whether natural- and vaccine-induced monoclonal antibodies can block HIV-1 Env binding to Galcer. HIV-1 Env gp140 proteins bound to Galcer liposomes with $K_d$ (Dissociation constant) in nM range. Several HIV-1 ALVAC/AIDSVAX vaccinee-derived mAbs specific for gp120 first constant (C1)-region blocked Galcer binding of a transmitted/founder HIV-1 Env gp140. Among the C1-specific mAbs that showed Galcer blocking, the ADCC-mediating CH38 IgG and its natural IgA isotype were the most potent blocking antibodies. C1-specific IgG monoclonal antibodies that blocked Env binding to Galcer induced up-regulation of the gp120 CD4-inducible (CD4i) epitope bound by mAb 17B, demonstrating that a conformational change in gp120 may be required for Galcer blocking. However, the mAb 17B itself did not block Env-Galcer binding suggesting that the C1-antibody induced gp120 conformational changes resulted in alteration in Galcer binding site distant from the CD4i 17B mAb binding site.

Importance

Galactosyl ceramide (Galcer), a glycosphingolipid has been postulated to be a receptor for HIV-1 envelope glycoprotein (Env) interacting with mucosal epithelial cells. Here we have mimicked this interaction using artificial membrane containing synthetic Galcer and recombinant HIV-1 Env proteins to identify antibodies that would block HIV-1 Env-Galcer interaction. Our study
reveals that a class of vaccine induced human antibodies potently blocks HIV-1 Env-Galcer binding by perturbing the HIV-1 Env conformation.
Introduction

Understanding the interaction of HIV-1 virions with epithelial cells at mucosal sites of HIV-1 entry is critical for investigating any modulatory effect of vaccine induced antibodies on such interactions and to design strategies for prevention of HIV-1 transmission (1-3). Entry of HIV-1 through both columnar and squamous epithelial surfaces allows virions to reach tissue depths and potentially infect target T cells and macrophages (4). Virions have been suggested to be trancytosed through epithelial cells via endocytic vesicles as well as migrate through epithelial cell layers by movement through mucosal epithelial cell intercellular spaces (5, 6). Previous studies have shown that CD4 independent infection of epithelial or neural cells by certain strains of HIV-1 can occur, suggesting that HIV-1 can employ alternate receptors for mucosal entry (7-10). The interaction of HIV-1 with such alternate receptors can be adhesive in nature, like those described for cell-cell transmission (11, 12), and therefore, could potentially facilitate the mucosal entry of HIV-1. Thus, any mode of disruption of viral adhesion to epithelial cells could be a strategy to hinder mucosal entry of HIV-1.

One proposed alternate receptor for HIV-1, the glycosphingolipid Galactosyl ceramide (Galcer), is expressed on the surface of colonic epithelial cells (13-15), and vaginal epithelium (16, 17), as well as primary mammary epithelial cells (18). Antibodies against Galcer have been reported to inhibit epithelial cell-HIV-1 virion binding (7, 8, 19) and previous biochemical studies have shown that HIV-1 envelope (Env) gp120 interacts with Galcer supporting the view that Galcer might play the role of an alternate receptor for HIV-1 (7-10, 20-24). Enhanced expression of Galcer on an endocervical epithelial cell line, following infection with C. trachomatis, increased binding of HIV-1 and levels of virus in co-cultures (25), and endosomal
uptake of HIV-1 by Galcer expressing mammary epithelial cells have been reported to facilitate infection of CD4 T cells (18).

While the other HIV-1 vaccine efficacy trials were unsuccessful in demonstrating efficacy (26-33), the RV144 ALVAC/AIDSVAX trial showed a 31.2% efficacy (34). Subsequent immune correlate analysis revealed that antibodies targeting HIV-1 envelope (Env) glycoprotein gp120 variable region 1 and 2 (V1/V2) correlated inversely with infection risk whereas Env specific plasma IgA antibodies directly correlated with infection risk (35). Further studies raised the hypothesis that the observed protection in RV144 trial could partially be due to Antibody Dependent Cellular Cytotoxicity (ADCC) mediating antibodies (35); and the majority of those isolated from vaccine recipients mapped to epitopes within the gp120 first constant (C1) region (36). The gp120 C1 specific IgA antibodies derived from RV144 vaccinee plasma blocked the binding and the ADCC mediating ability of anti-gp120 IgG antibodies providing rationale for the direct correlation observed between infection risk and high plasma Env specific IgA (37). Furthermore, HIV-1 antibodies isolated from RV144 vaccinees were demonstrated to capture infectious virions (38). However, the ability of these antibodies to inhibit HIV-1 interactions with mucosal surfaces (2, 3) has not been tested. Therefore, we were interested to determine whether RV144 vaccinee derived antibodies would block HIV-1 Env binding to Galcer and subsequently whether such antibodies would be effective in hindering mucosal entry of HIV-1.

In this report we have used Galcer containing liposomes and HIV-1 Env gp140 proteins as mimics of epithelial cells and HIV-1 virions/HIV-1 infected cells, respectively, to examine the HIV-1 Env-Galcer interaction and the ability of several natural and ALVAC/AIDSVAX vaccine induced HIV-1 Env antibodies to inhibit this interaction. While earlier studies have implicated
the third variable region (V3) loop (39) and the gp41 membrane proximal external region (MPER) in Galcer binding (40, 41), our results showed that many natural neutralizing and non-neutralizing antibodies that target gp120 and gp41 regions did not block Env-Galcer interaction. Remarkably, the ALVAC/AIDSVAX vaccine induced antibodies targeting the gp120 first constant region (C1) blocked a clade C transmitted/founder (T/F) virus gp140-Galcer interactions with varying potency. In particular, the ADCC mediating CH38 IgG and its IgA2 isotype (36) antibodies were the potent blockers of a T/F virus gp140-Galcer interactions. Furthermore, our results demonstrated that the Galcer blocking potency of the gp120 C1-specific antibodies derived from ALVAC/AIDSVAX vaccinees directly correlated with their ability to induce conformational changes on the Env that expose the CD4 inducible epitope (CD4i). The CD4i-binding mAb 17B did not block T/F virus gp140-Galcer binding either before or after CD4 triggering of gp140, and neither did the C1-binding and CD4i-upregulating mAb A32. These data indicate that the ALVAC/AIDSVAX vaccine regimen induced a novel class of gp120 C1-region binding antibodies that alters HIV-1 Env conformation and disrupt Env-Galcer interactions. These antibodies can be tested in passive protection trials against SHIV challenge in non-human primates to determine any protective potential of gp120-Galcer blocking antibodies (42).

Materials and Methods

Lipids: Lyophilized powder of D-galactosyl-β-1,1' N-octanoyl-D-erythro-sphingosine (Galcer) and chloroform stock of 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) were purchased from Avanti Polar Lipids (Alabaster, AL).
HIV-1 Env Proteins: The consensus, chronic and T/F type Env gp140 proteins were produced recombinantly as described previously (43).

Monoclonal antibodies: The mouse monoclonal antibody anti-galactocerebroside (anti-Galcer mAb (44)) was obtained from Millipore. The 2F5, 4E10 and 2G12 mAbs were purchased from Polymun Scientific (Vienna, Austria). The PG9 mAb was a gift from D. Burton (Scripps Research Institute, La Jolla, CA) and VRC01 mAb was a gift from J. Mascola (National Institutes of Health, Vaccine Research Center, Bethesda, MD). The b12 mAb was procured from Quality Biological (Gaithersburg, MD). The gp41 MPER specific mouse monoclonal antibody 13H11 (45) was recombinantly produced as described previously (46). The HIV-1 Env IgG antibodies from RV144 vaccinees were generated as described earlier (36). The CH38 IgA2 and 7B2 IgA2 antibodies were made recombinantly using protocols published earlier (37). The HIV-1 nonreactive human plasma IgA was obtained from Lee Biosolutions (St. Louis, MO). The Synagis mAb (Palivizumab®), a humanized mAb directed against the F protein of respiratory syncytial virus (RSV) (47), purchased from MedImmune (Gaithersburg, MD), was also used as negative control in some of the experiments.

Liposome preparation: The POPC liposomes and Galcer liposomes were made using extrusion technique. Appropriate volumes of stock solutions of POPC (for making POPC liposomes) or Galcer (dissolved in chloroform:methanol 70:30 v/v) and POPC (in chloroform) were mixed together in a 1:1 molar ratio. This composition was chosen based on earlier reports that the apical membranes of epithelial cells contained 37±9.1 mol% of glycosphingolipids (15) and Env binding to Galcer liposomes reached maximum at Galcer content above 55 mol% (22). The lipid mixture was dried in a stream of gaseous nitrogen and any residual solvent was removed by drying the lipids film under vacuum for overnight. The dried lipid mixture was hydrated with...
appropriate volume of PBS buffer (pH 7.4) and incubated at 60°C for 45 minutes. The hydrated mixture was then sonicated in a bath sonicator (Misonix 3000) and extruded through 0.4 µm and 0.1 µm polycarbonate membranes using a mini-extruder obtained from Avanti Polar Lipids.

**Biolayer interferometry (BLI) assay:** The Galcer binding of HIV-1 gp140 proteins was performed with Biolayer Interferometry technique using a ForteBio OctetRed 96 instrument and Aminopropylsilane (APS) biosensors. Briefly, the Galcer and POPC liposomes were loaded onto APS biosensors by dipping them into sample plate wells containing 250 µM of liposomes for 10 minutes. As a control, an APS sensor was dipped into a well containing PBS buffer. The blank and liposome-loaded APS sensors were washed in PBS for 1 minute. In order to minimize non-specific interaction of HIV gp140 proteins with the blank and liposome-loaded APS sensors, they were coated with bovine serum albumin (BSA) by dipping into wells containing 0.1% BSA for 5 minutes followed by a wash with PBS for 1 minute. The interaction of HIV gp140 proteins with Galcer liposomes was measured by monitoring wavelength shift (in nanometer). After 2 minutes dip in PBS to obtain baseline, the blank and liposome-loaded APS sensors were dipped into wells containing gp140 proteins at 0.71 µM (or at the indicated) concentration for 30 minutes followed by a 10 minutes wash in PBS. The signal from blank APS sensor was subtracted to obtain signal specific to Galcer and POPC liposome binding. For the blocking of JRFL gp140 binding to Galcer by pre-coated anti-Galcer mAb (Figure 1F), Galcer liposomes were loaded onto three sensors. One of the Galcer liposome sensors was dipped into well with PBS buffer (control) and the remaining two were dipped into anti-Galcer mAb (150 µg/ml) for 30 minutes to follow the association of anti-Galcer mAb. The PBS treated Galcer liposome sensor and an anti-Galcer pre-coated Galcer liposomes sensor were moved to dip into a well containing JRFL gp140 (50 µg/ml) for 30 minutes to monitor Env-binding to Galcer. As a
control another anti-Galcer pre-coated Galcer liposome sensor was dipped in to PBS.

Subsequently all these sensors were dipped into PBS to follow dissociation for 30 minutes. Blank APS sensors were used in parallel to subtract out binding due to non-specific interactions.

For Galcer blocking assay, the Galcer liposome binding of 1086.C gp140 at 50 µg/ml was monitored for 30 minutes and dissociation was monitored for 1 hr. In parallel Galcer liposome binding of 1086.C gp140 (50 µg/ml) incubated with 3 molar excess of antibodies was monitored. The Galcer liposome binding responses (after subtracting the signal from blank sensors) at the end of 1 hr dissociation phase were averaged (20s). The percentage Galcer blocking was calculated using the equation; % Blocking = [(A-B)/A]×100, where A is the Galcer binding response of 1086.C gp140 and B is the Galcer binding of 1086.C gp140 in presence of 3 molar excess of antibody of interest.

The binding of 1086.C gp140 to CH38 IgG and IgA2 antibodies pair was measured by coupling the antibodies to amine reactive sensors (AR2G sensors) as per manufacturer’s instructions. The antibody coupled AR2G sensors were dipped into wells containing 1086.C gp140 at varying concentrations and subsequently in PBS buffer for monitoring association and dissociation respectively. CH65 IgG (a broadly Influenza neutralizing antibody (48)) immobilized AR2G sensors were used as blank sensors to subtract out non-specific binding signal. The experiments were performed in triplicate. Titration curves were fitted to a Langmuir 1:1 binding model using ForteBio Data Analysis software.

Surface plasmon resonance (SPR) assay: All SPR assays were performed using a Biacore 3000 instrument at 25°C and data analyses were done using BIAEvaluation 4.1 software. The 17B mAb upregulation assay was performed using a CM5 chip immobilized with 17B mAb
(6000-7000 RU) by standard amine coupling procedure in three flow cells. The fourth flow cell was immobilized with 6000 RU of Synagis (47), and used as a negative control surface to subtract out responses due non-specific interactions. The T/F HIV Env 1086.C gp140 (40 µg/ml) was flowed over the antibody surfaces at a 20 µl/minute flow rate for 2 minutes. Dissociation was followed for 500 s after the injection of Env protein was over. In order to measure 17B upregulation 1086.C gp140 (40 µg/ml) was mixed with the antibodies (100 µg/ml) listed in Table 1 and injected over antibody surfaces. The binding data were processed to obtain specific binding response by subtracting out binding response on RSV specific mAb Synagis surface. The specific binding responses from three 17B surfaces were averaged and presented in Table-1. The percentage of 17B upregulation was calculated using the equation: % Upregulation = [(D-C)/C]×100 where C and D are the 17B binding responses of 1086.C gp140 in the absence and in the presence of antibody respectively.

**Isothermal Titration Calorimetry (ITC):** All ITC measurements were performed using a MicroCal iTC200 instrument (GE Healthcare). Prior to titration the antibodies and 1086.C gp140 protein were extensively dialyzed against PBS buffer (pH 7.4). The mAbs CH38 IgG or CH38 IgA2 were placed in the syringe at a 19.4 µM concentration and injected (2.5µl per injection) into a cell containing 1086.C gp140 at a 1.9 µM concentration for duration of 5s with 180s spacing between injections. Reference power was kept at 6 µCal. Experiments were performed in duplicate at 25°C. Using Origin 7 program, the titration data were analyzed by fitting the integrated titration peaks with a single site binding model. The determined stoichiometry (N), association constant (K) of the reaction, reaction enthalpy (ΔH) and the derived entropy change (ΔS) are displayed in Figure 6C-D along with the titration data.
**Statistical Analysis:** Spearman correlation analysis was used to determine the nonparametric statistical dependence between 1086.C gp140 binding and Galcer blocking ability. The apparent outliers were not included in this analysis.

**Binding to Env on the surface of HIV-1 infected CD4+ cells.**

Primary CD4+ T cells were isolated from an HIV-1 seronegative donor, activated, and infected with an infectious molecular clone that encodes the HIV-1 subtype C Env from isolate 1086.C (GenBank No. ACS67968) in an isogenic backbone that contains the *Renilla* luciferase reporter gene and all viral open reading frames (49). Cell activation and infection were conducted as previously described (50). Mock-infected and HIV-1 1086.C-infected cells were incubated with the RSV-specific negative control Palivizumab, or mAb CH38 at 1 μg/ml for 2 hours at 37°C. Primary Ab binding was detected by secondary labeling with FITC-conjugated goat anti-human IgG (KPL Inc., Gaithersburg, MD). Live and HIV-1-infected cells were identified by staining with a viability dye, and for intracellular expression of p24 by standard methods.

**ADCC Assays**

ADCC activity was determined by a luciferase-based assay as previously described (51). Briefly, CEM.NKRCCR5 cells (NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: CEM.NKR-CCR5 from Dr. Alexandra Trkola.) infected with the HIV-1 1086.C infectious molecular clone were used as targets. NK effector cells were isolated by negative selection with magnetic beads (Miltenyi Biotec GmbH, Germany) from cryopreserved PBMC collected from a healthy HIV-1 seronegative donor. The NK cells were added to the HIV-1-infected target cells at a ratio of 5:1. Serial dilutions of Palivizumab and CH38 (concentration range 40 to 0.4 μg/ml) were added to wells of a 96-well plate containing the targets and effector cells. Plasmas from an
HIV-1 seronegative and HIV-1 seropositive donor were used as negative and positive controls, respectively, at final dilutions of 1:1,000. The assay plates were incubated for 6 hours at 37°C in 5% CO₂. ADCC activity (% killing) was calculated from the change in luciferase activity resulting from the loss of intact target cells in wells containing effector cells, target cells, and Ab compared to control wells containing target cells and effector cells alone.

Results

HIV-1 Env JRFL gp140 binds to Galcer liposomes. Since the ectodomain of HIV-1 envelope glycoprotein gp160 consists of both gp120 and gp41 portion, we reasoned that gp140 proteins (i.e. gp160 without the transmembrane domain and cytoplasmic tail) would be an appropriate mimic and used them in examining their binding to Galcer liposomes that optimally present Galcer in correct orientation (Figure S1). We first tested the binding of JRFL gp140, an HIV-1 Env protein from a chronically infected individual. Figure 1A-B display the time courses of binding and dissociation of JRFL gp140 at various concentrations to Galcer liposomes (Figure 1A) and Galcer-free POPC liposomes (Figure 1B). The near steady-state binding level of JRFL gp140 interaction with Galcer liposomes was markedly higher than its binding to the POPC liposomes (Figure 1C-D). The low avidity binding of gp140 to POPC liposomes reached saturation at a relatively lower concentration of Env protein. This lower level of binding of gp140 to POPC liposomes is due to non-specific binding of gp140 to lipids as it has been reported before that gp41 segments such as fusion peptide proximal region, immunodominant loop and membrane proximal region interact with membranes of different lipid composition (52,
Analysis of kinetics of JRFL gp140 binding to Galcer liposomes (Figure 1E) yielded an apparent affinity ($K_d$) value of 74.7 nM with an association rate constant ($k_a$) $1.9 \times 10^3$ M$^{-1}$ s$^{-1}$ and a dissociation rate constant ($k_d$) of $1.4 \times 10^{-4}$ s$^{-1}$.

Next, to validate whether the binding of JRFL gp140 to Galcer liposomes was via interaction with Galcer, we pre-coated the Galcer liposomes with anti-Galcer mAb (44) and then tested the binding of JRFL gp140. Figure 1F shows the binding profiles of JRFL gp140 binding to Galcer liposomes immobilized sensors that were first dipped in either PBS buffer or in anti-Galcer mAb. Galcer liposome binding of JRFL gp140 was completely blocked by the pre-treatment of Galcer liposomes with anti-Galcer mAb thus demonstrating that the binding of JRFL gp140 to Galcer liposomes is indeed via Galcer interaction. In order to understand whether gp140 interaction with Galcer liposomes involve only the galactose head group, we tested the binding of gp140 protein to Galcer liposomes in the absence and in the presence of varying concentrations of galactose. No blocking by galactose was detectable (Figure S2) suggesting the importance of linkage of galactose to the ceramide moiety for gp140-Galcer interaction.

**Galcer binding of chronic, consensus and transmitted/founder virus envelope gp140s.**

Since new infections from a transmitting chronic HIV+ individual via mucosal route is established typically by a single variant, the transmitted/founder (T/F) virus, there is considerable interest in understanding the differential behavior of T/F virus (54-56). In order to investigate whether T/F virus Env proteins possess Galcer binding ability different from chronic virus Env proteins and gp140 proteins having consensus sequences, we carried out a study of Galcer liposome binding of a large panel of Env proteins. The Galcer liposome binding of this large panel of Env proteins is summarized in Figure 2 and shows that Env proteins in each group bind...
to Galcer liposomes. All of the tested consensus gp140 proteins bound to Galcer and there was no clear distinction between the ability of chronic and T/F virus Env gp140 proteins to bind to Galcer. There was no significant clade differences in Galcer binding of Env gp140 proteins studied here (Table S1). The majority of the chronic virus Env gp140 we have tested (4/7) showed strong binding to Galcer liposomes while others were relatively weaker. The Galcer liposome binding varied among the T/F virus Env proteins, with 4/9 showing strong binding.

Among the T/F virus Env we have tested, the clade C 1086 gp140 bound to Galcer with the highest affinity (Figure 3) with a $K_d$ of 17 nM (Figure 3). Taken together, these data indicated that many HIV-1 Env gp140 proteins bind Galcer liposomes and there was no significant difference between Galcer binding of chronic and T/F virus Env.

**Ability of HIV-1 Env IgG antibodies to block Galcer binding of a transmitted/founder virus Env.** One of the major goals was to apply this assay for identification of HIV-1 Env antibodies that would block T/F virus Env-Galcer interaction with high potency. To accomplish this we chose a clade C T/F virus Env 1086.C gp140 that showed highest affinity Galcer binding and tested the ability of various HIV-1 Env antibodies to block this interaction. Figure 4A shows the % blocking of Galcer binding of 1086.C gp140 by soluble CD4 (sCD4) and several monoclonal antibodies targeting gp120 and gp41 regions. Neither sCD4 nor the CD4i mAb 17B with and without CD4 triggering of 1086.C gp140 blocked its Galcer binding. Other tested mAbs that target different gp120 regions also did not block Galcer binding of 1086.C gp140 (Figure 4A). The gp41 mAb 7B2, and including the MPER bNAbs 2F5 and 4E10 also did not block (Figure 4A). Next we screened several RV135/144 vaccinee derived mAbs that target gp120 C1 region (blockable by A32 mAb (36) which recognizes a discontinuous epitope from C1 and C4 regions (57)), V3 region and V2 region (Figure 4B). The two V2 specific mAbs CH58 and
CH59 did not show Galcer blocking ability. The V3 specific tier-1 neutralizing mAb CH23 (36) also did not block Galcer binding of 1086.C gp140. However, the C1 region specific CH38 IgG showed the strongest blocking (~60%) among all the vaccinee antibodies tested (Figure 4B).

Several other C1 region specific antibodies blocked the Galcer binding of 1086.C gp140 with relatively weak or moderate efficiency (5-35%). Interestingly, a positive correlation (Spearman correlation coefficient \( \rho = 0.8349 \) and \( P = 0.0014 \)) between the strength of A32 mAb blockable C1-specific antibody (36) binding to Env and the blocking of Env-Galcer binding was observed (Figure 4C). Thus, CH29 and CH92 mAbs that did not bind 1086.C gp140 showed no Galcer blocking ability. On the other hand, the strongly 1086.C gp140 binding mAbs CH40, CH91 and CH38 exhibited high Galcer blocking. However, an outlier group of 3 antibodies (CH77, CH78 and CH94), bound to Env gp140 strongly but did not block Galcer binding. Taken together, these data showed that while Galcer blocking ability was restricted to A32 mAb blockable C1-specific vaccinee derived mAbs, not all high affinity C1-specific vaccinee derived mAbs blocked Galcer binding of 1086.C gp140.

Bonsignori et al reported that the RV135/144 vaccinee derived C1-specific mAbs target distinct but overlapping epitopes that are A32 blockable (36). The observation that some A32 mAb-blockable C1-specific antibodies (CH77, CH78 and CH94) that strongly bind to 1086.C gp140 (the group of outliers in Figure 4C) but did not block Galcer binding, suggests that either the antibodies bind to distinct sites on C1 and/or have differential effects upon Env binding. One explanation of such an effect would be that C1 antibodies that induce conformational changes in gp120 might alter Env reactivity with Galcer. Since a previously described C1-specific mAb A32 induced conformational change in gp120 resulting in the exposure of the co-receptor binding site (CD4i epitopes) (58), we tested whether the Galcer blocking and non-blocking C1
antibodies would differentially upregulate the CD4i epitope upon binding to 1086.C gp140. For this purpose we monitored the binding of 17B, a mAb specific for co-receptor binding site that becomes exposed upon CD4 binding of Env (59), to 1086.C gp140 alone and in the presence of Galcer blocking or non-blocking C1 antibodies (Table 1). As previously observed for other Env proteins (58), 17B mAb binding of 1086.C gp140 was enhanced when the Env was bound by A32 mAb. Interestingly, several gp120 C1-specific RV135/144 vaccinee antibodies also upregulated 17B mAb binding of 1086.C gp140 (Table 1). However, there were a few antibodies that did not upregulate 17B binding. These results suggested that the gp120 C1-specific RV135/144 vaccinee antibodies are of two types; one type that induces rearrangement of Env resulting in exposure of CD4i epitope and another type that binds to overlapping C1 region but does not induce this gp120 conformational rearrangement.

However, we found that differential 17B upregulation behavior showed by gp120 C1-specific RV135/144 vaccinee antibodies correlated with their ability to block Galcer binding of 1086.C gp140 (Figure 4D). Remarkably, the three gp120 C1 antibodies (CH77, CH78 and CH94) that failed to block Galcer binding (outliers in Figure 4C) did not upregulate 17B binding of 1086C gp140 (circled in Figure 4D). The CH29 and CH92 mAbs did not bind 1086C gp140 (Figure 4C) and therefore showed no upregulation of 17B binding. There was a linear correlation between 17B upregulation of 1086C gp140 by RV135/144 C1 antibodies and their ability to block 1086C gp140-Galcer interaction (Spearman correlation coefficient (\(\rho\)) = 0.6496 and P = 0.0119). Although one exception was CH40, which exhibited moderate Galcer blocking but did not upregulate 17B binding. Thus, these results showed that the RV135/144 vaccinee-derived C1 antibodies that bind strongly to 1086.C gp140 and blocked Galcer binding of 1086.C gp140 also induce gp120 conformational changes associated with upregulation of 17B binding.
Ability of monomeric IgA2 antibodies to block Galcer binding of a transmitted/founder virus Env. Since the RV144 vaccinee derived CH38 mAb was originally an IgA isotype (36), we tested additional monomeric IgA2 Env antibodies for their ability to block Galcer binding of 1086.C gp140. As observed with the CH38 IgG isotype previously (36), CH38 IgA2 also blocked A32 mAb binding to Env (Figure S3). The concentration dependence of blocking of Galcer binding of 1086.C gp140 by CH38 IgA2 along with gp41 immunodominant region specific 7B2 IgA2 and human plasma IgA non-reactive for HIV-1 (as a negative control) is presented in Figure 5. The negative control IgA did not block Galcer binding of 1086.C gp140.

As observed with its IgG isotype, 7B2 IgA2 did not block the binding of 1086.C gp140 to Galcer liposomes. Like its IgG isotype, the CH38 IgA2 also blocked 1086.C gp140 binding to Galcer (60% at saturation).

Since both IgG and IgA2 isotypes of CH38 blocked Galcer binding of 1086.C gp140 with approximately equal potency we asked whether the binding affinities of this antibody pair towards 1086.C gp140 were similar. Figure 6A-B shows the binding interaction of 1086.C gp140 with CH38 IgG and CH38 IgA2. Global fitting of the binding curves revealed that there was no significant difference in 1086.C gp140 binding affinities. We have also measured the thermodynamics parameters of CH38 IgG and IgA2 isotypes binding to 1086.C gp140 by performing isothermal titration calorimetry (ITC). The ITC study indicated that both the IgG and IgA2 isotypes of CH38 bind with similar enthalpy and entropy changes (Figure 6C-D). The stoichiometry and affinity of 1086.C gp140 binding were also similar for CH38 IgG and IgA2 isoforms even though the ITC determined affinities were weaker than the BLI determined $K_d$ values. Thus, CH38 IgG and IgA2 isotypes have similar 1086.C gp140 binding affinity, thermodynamic profiles, and block Galcer binding of 1086.C gp140 with an equal efficiency.
Recognition of HIV-1 infected T cell surface Env and ADCC mediation by CH38 mAb. We next asked whether CH38 IgG, the potent Galcer blocking antibody identified above would recognize Env presented on 1086.C HIV-1 virions and on CD4+ 1086.C HIV-1 T infected cells.

In virus capture assays, the CH38 IgG did not capture 1086.C virions (Figure S4). In contrast, CH38 IgG bound well to 1086.C HIV-1 infected CD4+ T cells but not to mock-infected cells (Figure 7 A-B). The CH38 IgG also weakly mediated ADCC of 1086.C HIV-1 infected CD4+ T cells (Figure 7C). These data indicate that CH38 IgG is capable of recognizing Env on the 1086.C virus infected CD4+ T cells but not on the 1086.C virions suggesting that any effect of CH38 IgG inhibiting Galcer-gp120 binding in vivo would necessarily be targeted to gp120 on virus infected cells interacting with epithelial cell Galcer.

In summary, we have shown that Galcer binding to Env gp140 is blocked by select IgG antibodies that target the gp120 C1- epitope and also induce CD4i mAb binding. Among the C1-specific mAbs that blocked Galcer, both the IgG and IgA2 isotypes of CH38 mAb strongly induced 17B mAb binding, and gave the strongest blocking of T/F Env gp140 to Galcer. These data suggest that the blocking of Galcer is allosterically mediated by C1-specific mAbs that induce CD4i epitope.

Discussion

The mechanism of transmission of cell-free HIV-1 viruses and HIV-1 infected cells across the genital epithelia remains unclear, although earlier studies demonstrated the ability of one or more strains of HIV-1 to infect CD4 negative cell lines (7, 9, 19). The ability of HIV-1 gp120 to interact with Galcer supports the view that Galcer might play the role of an alternate receptor for HIV-1 (7-10, 20-24). With Galcer being present in the apical membrane of the epithelial cells
lining the mucosal surfaces, including colonic (10), vaginal and mammary epithelium (16-18),
the HIV-1 Env-Galcer interaction could play an important role in establishing initial adhesion of
HIV-1 virions/infected cells. Thus, if Galcer plays a role in HIV-1 transmission, we hypothesized
that antibodies that block Galcer-Env interactions could potentially be protective at the mucosal
site of HIV-1 entry. Only a few studies have attempted to develop agents or identify Env
antibodies that inhibit Env-Galcer interaction (39, 60). In this study we have used a Biolayer
interferometry assay for detecting HIV-1 Env glycoproteins binding to Galcer liposomes,
screened several antibodies against HIV-1 Env glycoprotein and identified gp120 C1-specific
antibodies that blocked Galcer binding of a T/F HIV-1 Env glycoprotein.

We have examined the time-resolved binding of HIV-1 Env gp140 with Galcer presented
in a physiologically-relevant bilayer form. Previous studies have used Galcer in multilamellar
liposomes (22) and planar mono- or bilayer forms (20, 21, 24) for detecting Env-Galcer
interaction and did not provide time resolved binding data. In our assay, the Galcer binding on
rate was in the order of $10^3 \text{ M}^{-1}\text{s}^{-1}$ and the off rate in the order of $10^{-4}$ to $10^{-5} \text{ s}^{-1}$ with $K_d$ values in
nanomolar range (17 to 75 nM). These rate constants derived $K_d$ values are consistent with the
earlier studies that have used different techniques in determining rgp120-Galcer interaction
quantitatively (8, 20, 21).

Earlier studies that used HP-TLC assay and recombinant gp120, monolayer binding assay
with gp120 and synthetic peptides derived from gp120 suggested that the Galcer binding site is
in or proximal to the V3 loop (24, 39). In our studies, using the V3 mAbs 19B and F39F and Env
1086.C gp140, we did not observe any blocking of Galcer-Env interactions. Neither did we
observe Galcer blocking by V1/V2 mAbs or the CD4i mAb 17B, with or without CD4 triggering
of gp120. Instead, Galcer blocking was observed only for antibodies specific for the gp120 C1
region, suggesting that the Galcer binding site could involve the gp120 C1 region. However, not all C1-specific mAbs showed blocking of Galcer. Interestingly we found a positive correlation between Galcer blocking and the extent of CD4i upregulation by C1-specific antibodies. These results provide two important features about the binding properties of C1–specific antibodies. First, that select C1 mAbs can induce CD4i epitopes, similar to that of the previously described CD4i-inducible mAb A32. Second, that upon binding to gp120, C1 specific antibodies can differentially alter Env reactivity. Thus, based on the ability to upregulate CD4i and to block Galcer binding, we have found three different classes of C1-specific mAbs (Figure 8). The first class is represented by Galcer blocking C1-specific mAbs that alter Env reactivity such that CD4i epitope is exposed while the Galcer binding site is simultaneously masked/disrupted (Figure 8B). A second class of C1-specific antibodies include the Galcer non-blocking mAbs—those that neither induce CD4i nor mask Galcer binding site (Figure 8C). A32 mAb, the third class of antibody, is an exception in that it up-regulates CD4i epitope but does not block Galcer. Conversely CH40 can block Galcer without inducing CD4i epitope. Taken together, these data suggest that conformational changes outside of the CD4i epitope are required for Galcer blocking. Recently emerging data revealed that some RV144 vaccinee derived C1-specific antibodies synergize the ADCC activity of V2 antibodies by upregulating V2 epitope (61). Thus, while each class of C1 mAbs can differentially affect Env gp120, our data suggest that Galcer blocking is mediated not through steric effects upon binding to gp120 C1, but rather by an allosteric effect on the Galcer binding site and such an effect can be associated with induction of CD4i epitope. However, to date, there is no evidence to indicate that vaccine-induced Galcer-blocking antibodies contributed to reduction of acquisition of HIV-1 in RV144 trial.
Earlier studies have shown that cell associated SIV establishing infection of rhesus macaques upon vaginal exposure (62, 63) and transmission from HIV-1 infected cells to uninfected CD4+ T cells (11, 64). Our results showed that the Galcer blocking CH38 IgG bound well to the 1086.C virus infected cells even though it failed to capture the cell free 1086.C virions. These results suggest that by binding the Env expressed on the virus infected cells CH38 could potentially inhibit the interaction of virus infected cells and epithelial cell Galcer. In the immune correlate analysis of RV144 high levels of plasma IgA antibodies against HIV-1 Env correlated with increased infection risk (35), suggesting that certain class of plasma IgA compete with IgG for binding to Env and could block effector function (37). The Galcer binding assay we have described here is aimed at mimicking the interactions of HIV-1 virions or HIV-1 infected cells with epithelial cells at the mucosal surfaces and in this regard we have identified blocking ability of an originally isolated IgA antibody from vaccinee. Thus, certain IgA antibodies could have divergent functions, depending on the form of IgA (i.e. monomeric, secretory, dimeric) and the location (systemic versus mucosal). Thus, while IgA antibodies cannot mediate ADCC via NK cells, they can block transcytosis of HIV-1 virions and CD4+ T cell infection by potentially hindering HIV-1 Env binding to Galcer (40, 65, 66).

In summary, we have identified a class of C1-specific vaccine induced IgG and IgA2 antibodies that block HIV-1 Env-Galcer interaction and such antibodies could be potential candidates for blocking the binding of virus infected cells to epithelial cells in vivo. Further studies such as testing the effect of Galcer blocking antibodies on inhibition of epithelial cell binding of HIV-1 infected cells, cell to cell transmission of HIV-1 and infection of rhesus macaques by SHIV infected cells in a passive protection trial would help understanding whether Galcer-Env interactions play a role in HIV-1 transmission.
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Disclaimer: The views expressed in this article are those of the authors and should not be construed as official or as representing the views of the Department of Defense or the Department of the Army.


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CD4 and CCR5 receptors with equal efficiency and are not inhibited by blocking the integrin alpha4beta7. PLoS Pathog 8:e1002686.


Figure legends

Figure 1: HIV-1 Env JRFL gp140 binds to Galcer liposomes and anti-Galactocerebroside antibody blocks this interaction. A-B: The time courses of binding of JRFL gp140 at various concentrations to Galcer (A) and POPC (B) liposomes are shown. The background binding of JRFL gp140 to blank sensors was subtracted out to obtain the specific binding shown in panel A and B. C-D: The last 25 seconds of the association phase (in panels A and B) were averaged and plotted to compare the Galcer liposome binding (C) and POPC liposome binding (D) as a function of concentration of JRFLgp140. E: The Galcer specific binding (POPC binding subtracted) time courses of JRFL gp140 at 0.36-1.43 µM concentrations (red lines) were globally fitted (black lines) using a 1:1 binding model yielding k_a and k_d and the derived K_d values. F: The time courses of pre-binding of anti-Galcer mAb at 150 µg/ml (black line) or PBS (red line) followed by JRFL gp140 (0.71 µM) binding to Galcer liposomes and the subsequent dissociation phases are shown. The PBS treated Galcer liposome binding curve was normalized to the anti-Galcer binding endpoint. Signal from blank sensor treated similarly was subtracted out to obtain the specific binding signal shown here.

Figure 2: Comparison of Galcer liposome binding of various HIV-1 Envelope glycoproteins. Galcer liposome binding of gp140 proteins are shown for a group of chronic HIV-1 Env, consensus sequences and transmitted/founder HIV-1 Env. The binding values displayed are an average of last 20 seconds of the 30 minutes association phase. The binding data and the error bars shown are from two separate measurements. JRFL gp140 was included in
every experiment and its Galcer binding was used as reference to normalize data obtained from different experiments.

Figure 3: Kinetics of Galcer specific binding of a transmitted/founder HIV-1 Env protein.
Galcer specific binding (POPC binding subtracted) time courses are shown for T/F HIV-1 Env 1086.C gp140 (red lines) at concentrations 0.1 to 1.43 µM. The time courses were globally fitted (black lines) using a 1:1 binding model yielding $k_a$ and $k_d$ and the derived $K_d$ values. Representative data of two independent experiments is presented.

Figure 4: The majority of HIV-1 Env antibodies do not block Galcer binding of a T/F virus Env but few RV135/144 vaccinee derived antibodies do partially block. A: A comparison of %Blocking of Galcer liposome binding of 1086.C gp140 by soluble CD4 (sCD4), gp120 CD4 binding site specific mAbs VRC01 and b12, CD4 induced (CD4i) epitope binding mAb 17B with and without sCD4, gp120 V3 loop specific mAbs 19B and F39F, glycan specific gp120 mAb 2G12, glycan dependent gp120 V1V2 mAbs PG9 and CH01, gp120 first constant (C1) region specific mAb A32 and gp41 immunodominant loop specific mAb 7B2 and gp41 membrane proximal external region specific mAbs 2F5, 4E10 and 13H11 is shown. B: The ability of blocking of Galcer binding of 1086C gp140 by RV135/144 vaccinee antibodies that are specific to gp120 V3 region (CH23), gp120 C1 region (CH29-CH94) and specific to gp120 V1V2 region (CH58 and CH59) is compared. The %Blocking data and the error bars shown are from two separate measurements. C: The correlation between blocking of Galcer binding and 1086.C gp140 binding is shown for RV135/144 C1 IgG antibodies. The solid line is the linear fit
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**D:** The % Blocking of Galcer binding 1086.C gp140 is correlated with % upregulation of 17B
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**Figure 5:** Selected monomeric IgA2 gp120 antibodies block Galcer binding of a T/F virus

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**Figure 6:** Kinetics and thermodynamics of CH38 IgG-IgA2 antibody pair interaction with
1086.C gp140. **A-B:** 1086.C gp140 binding titration time courses (red lines) are shown for the
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Figure 8: Schematic diagram depicting the allosteric masking of Galcer binding site by some gp120 C1 specific antibodies derived from RV135/144 vaccinees. A: A pictorial representation of the binding of HIV-1 Env to Galcer liposome bilayer is shown. In the Galcer liposome bilayer depiction, filled and open black circles with tails represent Galcer and POPC molecules respectively. For simplistic reason, only gp120 is shown as an ellipse. B: A few RV135/144 vaccinee derived C1 mAbs bind gp120 and induce conformational change (gp120 shown as cloud shaped) and block Galcer binding. C: Some RV135/144 vaccinee derived C1
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Table-1: 1086.C gp140 binding to CD4i epitope specific mAB 17B in the absence and presence of various C1 specific antibodies and control antibodies.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>17B binding response (RU)</th>
<th>% Upregulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>357±37</td>
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</tr>
<tr>
<td>Synagis</td>
<td>363±38</td>
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</tr>
<tr>
<td>A32</td>
<td>636±40</td>
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<td>CH29</td>
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<tr>
<td>CH38</td>
<td>756±44</td>
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<tr>
<td>CH40</td>
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<td>CH51</td>
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<tr>
<td>CH52</td>
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<tr>
<td>CH38 IgA2</td>
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<td>88.7</td>
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</table>

The 17B binding experiment was performed and %upregulation was calculated as mentioned in methods. An average of three measurements of 17B binding and the estimated standard deviations are presented.