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Bovine alpha-2-HS-glycoprotein functions as a booster antigen for efficiently stimulating humoral immune responses to CCR5 and SIVmac239 envelope glycoprotein

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ABSTRACT

The presence of anti-CCR5 and anti-HIV-1 envelope glycoprotein (ENV) gp41 antibodies (Abs) at sites of HIV-1 exposure was effective in preventing its transmission to HIV-1-exposed seronegative (ESN) subjects. Here, we design an immunogen that can induce Abs against CCR5 and SIVmac239 ENV simultaneously and show that bovine alpha-2-HS-glycoprotein (bAHSG) functions as a booster antigen for efficiently stimulating humoral immune responses to CCR5 and ENV. Initially, we generated a rhesus CCR5-derived cyclopeptide (cDDR5) conjugated with a recombinant trimeric SIVmac239 Env. When inguinally administered to rhesus macaques, the immunogen simultaneously induced both anti-CCR5 and anti-ENV Abs in sera, and the purified serum IgG fraction exerted an inhibitory effect on SIVmac239 infection *in vitro*. When further boosted with bAHSG, the responses of both Abs were significantly enhanced. To examine the cross-reactivity of bAHSG, it was administered to naïve cynomolgus macaques. The results showed a statistically significant increase in IgG response against cynomolgus CCR5 and SIVmac239 ENV, and the induction of neutralizing activity against SIVmac239. These findings suggest that bAHSG is useful for immune strategies aimed at generating Abs against CCR5 and ENV simultaneously to confer HIV-protective immunity.

1. Introduction

HIV sequence diversity has presented a major hurdle for the development of AIDS vaccines. Since conventional vaccines have not been proven successful against HIV-1 infection, it is necessary to explore other strategies including mucosal vaccination. Clinical studies have sought to identify correlates of mucosal protection in HIV-1 infection. Some studies showed that mucosal Ab responses could contribute to the apparent resistance to HIV-1 infection. The studies, in which humoral responses against HIV-1 in the vaginal secretions of ESN subjects were analyzed, indicated the presence of CCR5-specific mucosal autoantibodies [1] or secretory anti-gp41 IgA [2]. As attempts to reproduce some of the functional aspects of humoral immunity in ESN subjects, some vaccination strategies of inducing anti-CCR5 or anti-gp41 Abs have been reported. Our previous attempts were to induce CCR5-specific autoantibodies with anti-HIV-1 activity by inoculating cDDR5 into Balb/c mice and cynomolgus and rhesus macaques [3–5]. On the other hand, mucosal vaccination with gp41-peptide immunogens has produced sera that can neutralize some HIV-1 strains [6] and block viral transcytosis *in vitro* [7]. Results of these studies indicate that vaccines aimed at inducing anti-CCR5 or anti-gp41 Abs can be developed to mimic the natural humoral responses to HIV-1 infection in ESN subjects.

The mucosal transmission of HIV-1 infection established in a short term seems to have presented another hurdle for vaccine development. Although the events between HIV-1 exposure and establishment of infection in humans are poorly understood, it has been established in nonhuman primates that mucosal infection can occur following 30–60 min exposure to the infectious virus [8,9], suggesting that the time for the induction of protective immunity during HIV-1 transmission must be critically short, or

Abbreviations: SIV, simian immunodeficiency virus; HIV, human immunodeficiency virus.

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vaccine-induced immune responses should be maintained before exposure to HIV-1. Therefore, exposure to nonpathogenic cross-reacting antigens capable of boosting HIV-1-vaccine-induced immune responses seems to be required for the success of an HIV-1 vaccine candidate.

In this study, we investigated the immunogenicity of a synthetic vaccine candidate containing a rhesus cDDR5 conjugated with a recombinant trimeric SIV Env protein. Moreover, we showed that bAHSG functions as a booster antigen for efficiently stimulating humoral immune responses to CCR5 and ENV.

2. Materials and methods

2.1. gp140 production

pERK-SIVmac239 gp140(R512E, K523E) was prepared with a Qiagen HiSpeed Plasmid Maxi kit. DNA was linearized with *Not* I, and RNA was transcribed *in vitro* using an Ambion mMESSAGE mMACHINE T7 kit. This RNA (14 μg) was used to transfect Vero cells (1.2×10^7 cells). Soluble gp140-containing culture supernatant was collected at 72–144 h postelectroporation and dialyzed in 100,000-molecular-weight-cutoff tubing (Spectrum Laboratories, Inc.) at 4 °C and concentrated by pressing the tubing. This process was repeated five times. The expression of SIVmac239 gp140(R512E, K523E) was examined by reducing SDS–PAGE [10]. Trimers were analyzed by blue native polyacrylamide gel electrophoresis (BN-PAGE, Invitrogen) and western blotting with murin anti-gp130 SIVmac251 Ab (Immuno Diagnostics, Inc.).

2.2. Preparation of HIV multiantigen vaccine (HMV)

To induce Abs against CCR5 and ENV simultaneously, rhesus cDDR5 was conjugated via a Hubantigen with AB-NTA (Dojindo Molecular Technologies, Inc.). The recombinant trimeric SIV Env protein was bound to the Hubantigen via Ni-binding NTA (NTA-Ni). Furthermore, the M-cell-targeting molecule (TGDK) [5] and 6-aminohexanol-labeled phosphorothioate-modified CpG ODN 2006 (Japan Bio Services Co., Ltd.) were also conjugated with the Hubantigen for application as a mucosal vaccine in a future study. Briefly, SUNBRIGHT HGEO-200NP (NOF Corporation; 1 equivalent) was mixed with SUNBRIGHT HGEO-200PA (NOF Corporation; 7.2 equivalents) in DMF for 16 h, as described in Ref [5]. The resulting product was dialyzed in dialysis bags (Spectrum Laboratories; molecular-weight-cutoff = 12–14 kDa) against Milli-Q water for 2 days. The dialysate was lyophilized and used as a Hubantigen. To prepare rhesus cDDR5, a CCR5-derived linear dodecapeptide (H_2N-KRSQREGLHYTG-COOH) was synthesized and cyclized as previously described [3]. To bind TGDK, cDDR5, AB-NTA, and CpG-ODN to the Hubantigen, the amino group of ethylenediamine in TGDK (two equivalents), that of Lys^1 in the deprotected cDDR5 (two equivalents), that of AB-NTA (two equivalents), or that of 6-aminohexanol-labeled CpG-ODN (two equivalents) was conjugated to SUNBRIGHT PTE-100NP (NOF Corporation; 1 equivalent). Finally, the Hubantigen (168 mg) was coupled to the PEGylated TGDK (6 μmol), cDDR5 (6 μmol), AB-NTA (6 μmol), and CpG-ODN (1.1 μmol) in DMF for 48 h. HMV was dialyzed for 48 h against PBS(−), and the dialysate was lyophilized. The resulting HMV (10 mg) included TGDK (56 nmol), cDDR5 (90 nmol), AB-NTA (36 nmol), CpG-ODN (20 nmol), and proteins (183 μg). In contrast, a control Ag included only the Hubantigen.

2.2.1. Immunization schedule

This study was approved by the Institutional Animal Care and Use Committee of Shin Nippon Biomedical Laboratories, Ltd., and conducted in accordance with the by laws of the committee. Three 4- to 6-year-old rhesus macaques (Nos. 4–6) were inguinally administered HMV (10 mg/2.5 ml in PBS(−)) at 0, 1, and 6 week. The other control macaques (Nos. 1–3) were immunized with a control Ag (10 mg/2.5 ml in PBS(−)) following the same immunization schedule. Serum samples were obtained at 0, 1, 2, 4, 6, 7, and 8 week postinitial immunization (wpim), which were then subjected to cDDR5-coupled-multipin ELISA [4] and anti-ENV Ab ELISA.

2.2.2. cDDR5-coupled-multipin ELISA

The ε-amino group of Lys in cDDR5 was conjugated to MultiPin-block (Mimotopes). The block was used for detecting anti-CCR5 Abs in serum samples in accordance with the method of Misumi et al. [3].

2.2.3. Anti-ENV Ab ELISA

Abs against SIVmac239 ENV in sera were detected by ELISA. Each well of a COVALINK™ NH MODULE (Nunc) was coated with 50 μl of anhydrous DMSO (pH 8.0) containing AB-NTA (150 nmol) and SUNBRIGHT PTE-100NP (75 nmol) and incubated for 2 h. The wells were washed with 20 mM Tris–HCl (pH 7.4) and incubated with 200 mM $NiSO_4$ (50 μl/well). Subsequently, 50 μl of MAT-tag-containing SIVmac239 gp140(R512E, K523E) (1 nmol) was added to each well and incubated for 16 h. Finally, 200 μl of blocking buffer (0.5% skim milk and 0.05% Triton X-100) was added to each well and incubated for 1 h. The plate was washed with blocking buffer three times, followed by the addition of 60 μl of a serum or purified serum IgG to each well. The plates was incubated for 2 h and then washed with blocking buffer four times. 100 μl of peroxidase-conjugated anti-monkey IgG (diluted 1/4000) was added to each well and incubated for 1 h. 50 μl of 3,3′,5,5′-tetramethylbenzidine solution (Wako Pure Chemical) as the substrate was added to each well and incubated at RT. Absorbance was measured at 450/630 nm using a microplate reader.

2.2.4. MAGIC-5 assay

The antiviral activity of serum IgG obtained after immunization with HMV was determined using MAGIC-5 cells, as previously described [3]. MAGIC-5 cells were plated at 1×10^4 cells/well and incubated overnight in RPMI 1640 containing 5% FCS (200 μl); the medium was then replaced with suspensions (40 μl) of SIVmac239 (5 ng of p27 antigen) and serum IgG purified by Byzen Pro® (Nomadic Bioscience Co., Ltd.) in the presence of 20 μg/ml DEAE dextran, and then cocultured in the medium (160 μl) for 48 h. The cells were fixed, and SIV-infected cells identified by their blue staining were counted by conventional methods.

2.2.5. Depletion of anti-cDDR5 serum IgG

Purified serum IgG was diluted in PBS to 100 μg/ml. An aliquot of diluted sample was added to cDDR5-coupled multipins, which were then incubated at 4 °C for 1 h. This process was repeated seven times and the anti-cDDR5-IgG-depleted sample was recovered. Ab titer was measured by cDDR5-coupled multipin ELISA using POD-conjugated anti-monkey IgG (Cappel Laboratories), and anti-HIV activity was analyzed by MAGIC-5 assay.

2.2.6. Booster immunization

Three HMV-immunized macaques (Nos. 4–6) were inguinally administered 1 mg of bAHSG (1 mg/ml in PBS(−)) at 106 wpim because the titers of anti-cDDR5 and anti-ENV sera nearly declined. The other control macaques (Nos. 1–3) were also immunized with 1 mg of bAHSG at 106 wpim. The Ab titer in sera (1/100 dilution) was measured by anti-ENV Ab ELISA and cDDR5-coupled-multipin ELISA. Furthermore, the activity of #6 antiserum at 108 wpim was determined by western blotting using recombinant gp130 (#2322) obtained from the NIH AIDS Research and Reference Reagent.

2.2.7. Preparation of TGDK-Hubantigen-bAHSG (THbA) and immunization schedule

bAHSG was conjugated via the Hubantigen with TGDK. Briefly, the amino group of ethylenediamine in TGDK (1 equivalent) was conjugated to SUNBRIGHT PTE-100NP (1.5 equivalents). The PEGylated-TGDK (12 μmol) was coupled to the Hubantigen (168 mg) in DMF for 12 h. The resulting TGDK-Hubantigen was further coupled to bAHSG (50 mg in PBS(-)) at pH 7.8 overnight. The resulting THbA was dialyzed for 24 h against PBS(-) and for 36 h against deionized water, and the dialysate was lyophilized. The resulting THbA (5.4 mg) included bAHSG (1 mg). In contrast, the control Ag included only the Hubantigen. Three 4- to 6-year-old cynomolgus macaques (Nos. 4–6) were inguinally administered THbA (5.4 mg/1 ml in PBS(-)) at 0 wk. The other macaques (Nos. 1–3) were immunized with the control Ag. Serum samples were obtained at 0, 4, 8, 10, 12, and 14 wpim. Serum IgG was purified using Byzen Pro® and then subjected to cDDR5-coupled-multipin ELISA, anti-ENV Ab ELISA, and MAGIC-5 assay.

2.2.8. Flow cytometry

SIV-infected CEMx174 and HSC-F cells [11] were suspended in a washing buffer (PBS containing 2% FBS and 0.02% NaN_3) at 1×10^6 cells/ml. The cells were preincubated with anti-SIVmac251 gp130 Ab (ImmunoDiagnostic, Inc.) or anti-CCR5 Ab (MARS8) [12] for 30 min. After preincubation, the cells were further incubated with 0- and 4-wpim-serum IgGs from #2 or #5 cynomolgus macaque, which were purified using Byzen Pro® and washed with a washing buffer. The cells were resuspended in the washing buffer containing FITC-conjugated anti-monkey IgG (Sigma Chemical Co.,). After 30 min of incubation at 4 °C, the cells were analyzed using an EPICS XL flow cytometer (Beckman Coulter).

The specificity of 4-wpim-serum IgG from #5 macaque for SIVmac239 ENV or native CCR5 expressed on SIVmac239-infected CEMx174 or HSC-F cells was determined by examining the ability of 4-wpim-serum IgG from #5 macaque to block the binding of an anti-CXCR4 Ab (44717.111; R&D Systems, Inc.) or an anti-CD4 Ab (Leu-3a; BD Biosciences). A total of 1×10^6 cells was incubated with 4-wpim-serum IgG from #5 macaque for 30 min on ice. The cells were stained with an anti-CXCR4 Ab or an anti-CD4 Ab for 30 min on ice. After washing, the cells were incubated with PE-labeled anti-mouse IgG and then analyzed using a flow cytometer. Control experiments were conducted with isotype control IgG_{2b} or IgG_{1k}.

3. Results

3.1. Design and synthesis of HMV

HIV-1 ENV is a heterotrimeric protein complex composed of three gp120-gp41 heterodimers that are noncovalently linked in their ectodomain, which is difficult to obtain as a recombinant protein for a vaccine antigen. Therefore, the common means of obtaining it has been to mutate the cleavage site between gp120 and gp41 [13–15]. Furthermore, Kovacs et al. have recently demonstrated that HIV-1 ENV trimers more accurately mimic the antigenic properties of the native ENV on the surface of virions than gp120 monomers [16]. In this study, both primary and secondary furin-like protease cleavage sites in recombinant SIVmac239 gp140 were eliminated by the mutation of Arg^{512} and Lys^{523} to Glu. Furthermore, the MAT-tag sequence was introduced at the C-terminaus of gp140(R512E, K523E) to bind to the Hubantigen via NTA-Ni (Supplementary Fig. 1). The band of gp140(R512E, K523E) was observed at about 165 kDa by reducing SDS–PAGE (Fig. 1A). Western blotting with an anti-gp130 Ab showed that the 165 kDa band correspond to gp140(R512E, K523E) (Fig. 1A). By further assessment using BN-PAGE and western blotting with an anti-gp130 Ab, we found that

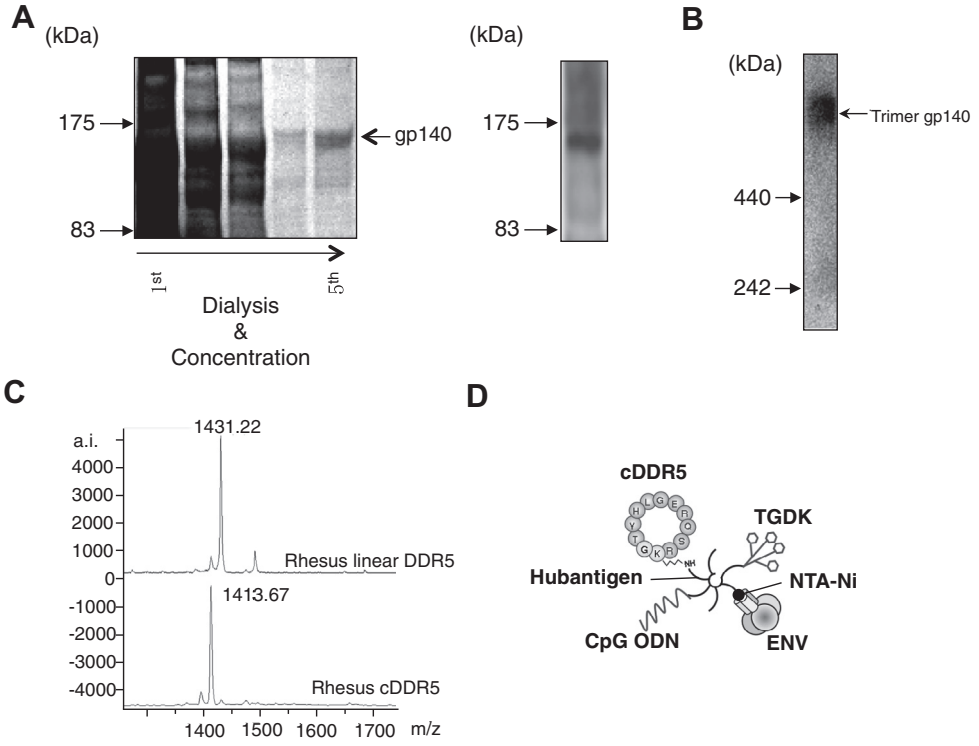

Fig. 1. *Design and synthesis of HMV.* (A) Reducing SDS–PAGE, western blotting, and (B) BN-PAGE analysis of purified SIVmac239 gp140(R512E, K523E). Proteins in each dialysate (100 μl) were precipitated with ethanol and the resulting precipitates were subjected to SDS–PAGE (Fig. 1A left panel) and western blotting with an anti-gp130 Ab (Fig. 1A right panel). (B) The lyophilized gp140(R512E, K523E) was dissolved in BN sample buffer and then subjected to BN-PAGE. (C) MALDI-TOF MS spectra of linear DDR5 and cDDR5. The spectra exhibited two peaks at *m/z* 1431.22 and 1413.67: the upper peak is that of the ion derived from linear DDR5, and the lower peak is that of the ion derived from cDDR5. (D) Schematic representation of HMV.

the gp140 was predominantly trimeric after being secreted from Vero cells (Fig. 1B).

To mimic the undecapeptidyl arch (Arg^{168} to Cys^{178}) of rhesus CCR5, a linear side-chain group-blocked oligopeptide (H_2N-KRSQREGLHYTG-COOH) was synthesized and then cyclized by peptidyl bond formation between the amino group of Lys^1 and the carboxyl group of Gly^{12}. After the removal of the side-chain-blocking group, cDDR5 was purified, and its molecular mass was determined by MALDI-TOF-MS. The spectrum of purified cDDR5 exhibited major peaks at m/z 1413.67 (Fig. 1C).

To induce Abs against CCR5 and ENV simultaneously, cDDR5 was conjugated with gp140(R512E, K523E) via the Hubantigen and NTA-Ni. Furthermore, both TGDK and CpG ODN 2006 were also conjugated with the Hubantigen to design an HIV vaccine capable of reconstructing the immune response induced in ESN subjects and to confirm its immunogenicity before its application for mucosal vaccine (Fig. 1D).

3.2. Vaccination of rhesus macaques with HMV and induction of anti-cDDR5 and anti-ENV Abs

To verify whether HMV could induce anti-cDDR5 and anti-ENV Abs simultaneously, an experiment was performed using rhesus macaques immunized following the time schedule shown in Fig. 2A. Three macaques (Nos. 4–6) were inguinally immunized with HMV. cDDR5- or ENV-specific Abs were significantly induced in the macaques (Nos. 4–6) at 7 wpim (Fig. 3B) or 2 wpim (Fig. 3C). In contrast, the immunization of the macaques (Nos. 1–3) with control Ag did not induce both Abs (Fig. 3B and C). Furthermore, the anti-SIVmac239 activity of the purified serum IgG from #6 macaque was determined using MAGIC-5 cells. The cells were inoculated with SIVmac239 in the presence of purified serum IgG derived from #3 or #6 macaque. As expected, 7-wpim-serum IgG from HMV-immunized #6 macaque markedly suppressed infection by SIVmac239 in a dose-dependent manner (Fig. 2D right panel) because cDDR5- and ENV-specific Abs are induced in 7-wpim serum (Fig. 2B and Supplementary Fig. 2). In contrast, the serum IgG from #3 macaque did not prevent SIVmac239 infection (Fig. 2D left panel). To further determine the relative importance of Abs specific for cDDR5 and ENV in SIVmac239 neutralization, 0- and 7-wpim-serum IgGs from #3 and #6 macaques were selectively depleted of Abs that bound to cDDR5-coupled multipins. To test the efficiency of the depletion using the cDDR5-coupled multipin, the titers of Abs reactive with cDDR5 were determined by ELISA (Fig. 2E left panel). The resulting anti-cDDR5-IgG-depleted fraction was evaluated for its neutralizing capacity against SIVmac239. Although most of the cDDR-specific serum IgG from #6 macaque was efficiently removed by immobilized cDDR5 (Fig. 2E left panel), the remaining anti-ENV Ab retained about 50% of its neutralizing activity (Fig. 2E right panel).

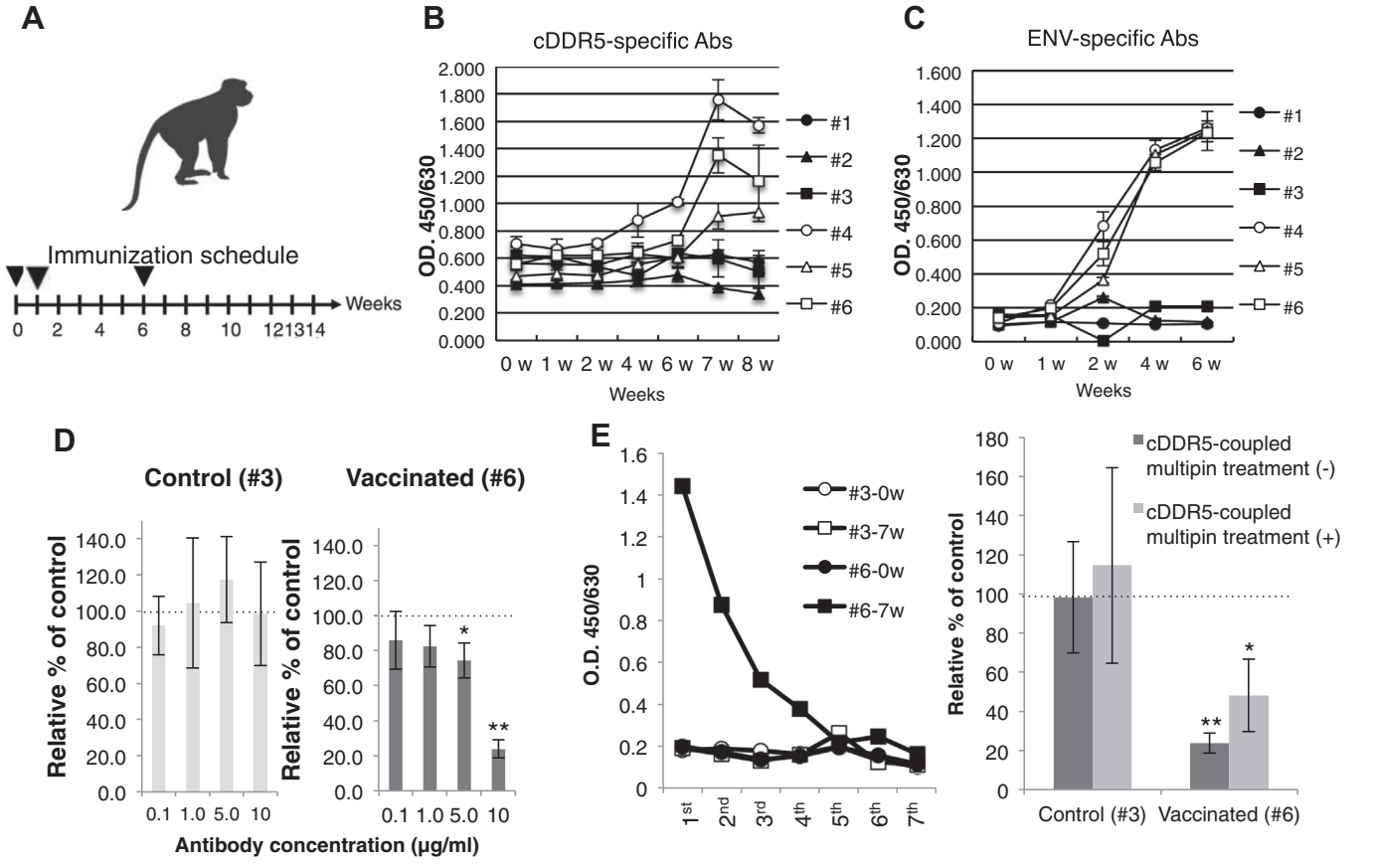

Fig. 2. *Immunization schedule and inhibitory effects of postimmunization sera on SIVmac239 infection.* (A) Immunization schedule for rhesus macaques. (B, C) Sera (1/100 dilution) obtained after immunization with HMV or control Ag were examined by cDDR5-coupled-multipin ELISA and anti-ENV ELISA to investigate whether the anti-cDDR5 (B) and anti-ENV Ab (C) can be raised. (D) Antiviral activities of purified 7-wpim-serum IgGs from #3 and #6 macaques. MAGIC-5 assay was carried out as described in *Materials and Methods*. Values are means of triplicate determinations. $^{*}P < 0.05$, and $^{**}P < 0.01$ by Mann–Whitney *U*-test. (E) 0- and purified 7-wpim-serum IgGs from #3 and #6 macaques were selectively depleted of Abs that bound to cDDR5-coupled multipins. The Ab titers against cDDR5 and antiviral activities were measured by cDDR5-coupled multipin ELISA (left panel) and MAGIC-5 assay. Values are means of triplicate determinations. $^{*}P < 0.05$ and $^{**}P < 0.01$ by Student's *t-test*.

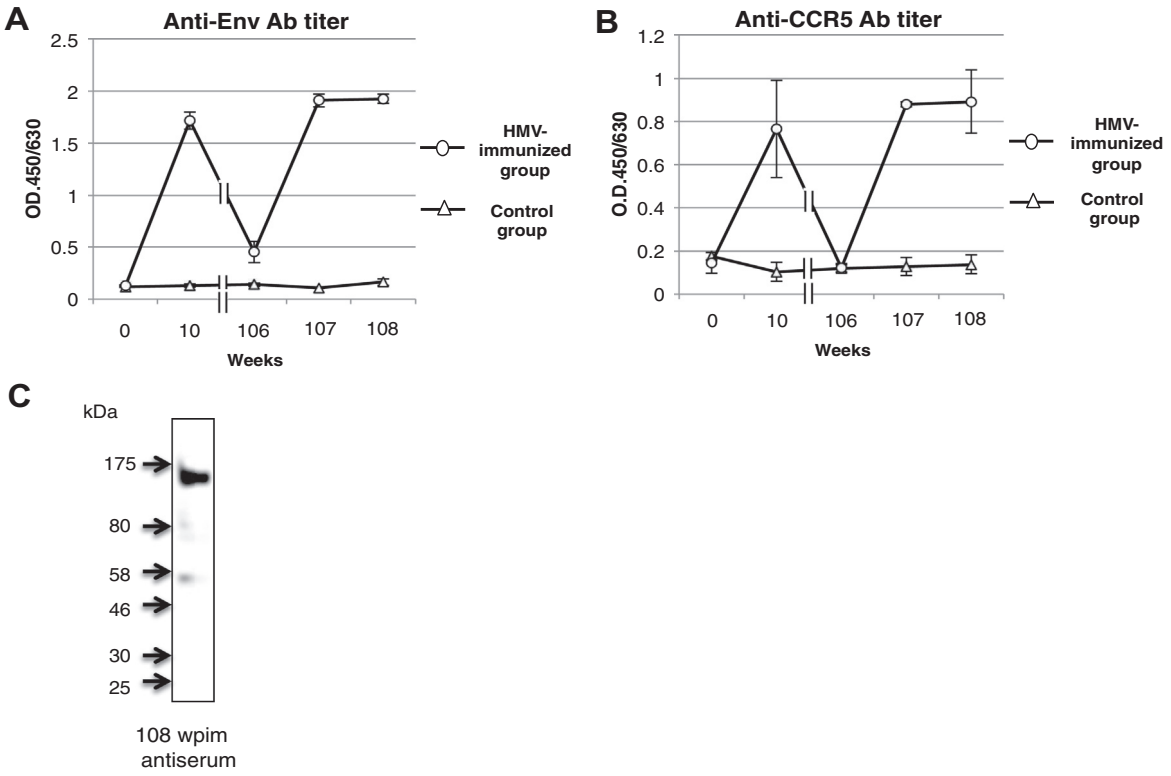

Fig. 3. *Booster effect of bAHSG.* (A) Three HMV-immunized macaques (Nos. 4–6) and control Ag-immunized macaques (Nos. 1–3) were inguinally administered 1 mg of bAHSG. The induction of anti-ENV and Anti-CCR5 Abs in sera (1/100 dilution) was analyzed by anti-ENV Ab ELISA (A) and cDDR5-coupled-multipin ELISA (B). The activity of bAHSG-boosted antisera at 108 wpim was analyzed by western blotting with SIVmac239 gp130 (5 μg) (C).

3.2.1. Booster effect of bAHSG

Because it is difficult for host immunity to eradicate HIV-1 in a body once the virus has infected the host, an immune response induced by an at least partially successful vaccination should be boosted to induce a sufficiently large immune response to be effective in protecting against HIV-1 infection. Therefore, we explored a booster antigen that can efficiently stimulate humoral responses to CCR5 and ENV. The results of Western blotting using antiserum against HMV and proteome analysis suggest that bAHSG is a non-pathogenic cross-reacting antigen. To confirm whether bAHSG functions as a booster antigen that simultaneously induces humoral immune responses to CCR5 and ENV, three HMV-immunized macaques (Nos. 4–6) and control macaques (Nos. 1–3) were inguinally administered 1 mg of bAHSG (1 mg/ml in PBS(−)) at 106 wpim. Interestingly, cDDR5-coupled-multipin ELISA and anti-ENV Ab ELISA demonstrated that the HMV-induced immune response was boosted by bAHSG (Fig. 3A and B). Furthermore, to determine whether the anti-ENV Ab is induced in the antisera at 108 wpim, western blotting was carried out. As shown in Fig. 3C, bAHSG-boosted antisera recognized the recombinant SIVmac239 gp130.

3.3. Vaccination of cynomolgus macaques with THbA and induction of anti-cDDR5 and anti-ENV Abs

To verify the immunogenicity of THbA capable of boosting Abs against CCR5 and ENV, an experiment was performed using cynomolgus macaques immunized following the time schedule (Fig. 4A). Three macaques (Nos. 4–6) were inguinally immunized with THbA. As expected, cDDR5- and ENV-specific Abs were significantly induced in the macaques (Nos. 4–6) at 4 wpim (Fig. 3B and C) or 2 wpim (Fig. 3C). In contrast, the immunization of the macaques (Nos. 1–3) with control Ag did not elicit cDDR5- or ENV-specific Abs (Fig. 3B and C). To further rule out the possibility that 4-wpim-serum IgG derived from #5 macaque reacts with native CCR5 and SIVmac239 ENV as expressed on HSC-F and SIVmac 239-infected CEMx174 cells, we examined the ability of anti-CCR5 Ab [15] and a commercially available anti-gp130 Ab to block the binding of 4-wpim-serum IgG derived from #5 macaque. The incubation of HSC-F with anti-CCR5 Ab reduced the percentage of HSC-F cells that were stained by 4-wpim-serum IgG from #5 macaque (Fig. 4G). In addition, the incubation of SIVmac239-infected CEMx174 cells with anti-gp130 Ab also reduced the percentage of SIVmac239-infected CEMx174 cells that reacted with 4-wpim-serum IgG from #5 macaque (Fig. 4E). However, 4-wpim-serum IgG from #2 macaque did not bind to HSC-F or SIVmac239-infected CEMx174 cells (Fig. 4D and F). On the other hand, 4-wpim-serum IgGs from #2 and #5 macaques did not decrease the binding activity of anti-CXCR4 and anti-CD4 Abs (Fig. 4H–K), suggesting that the observed binding of 4-wpim-serum IgG from #5 macaque was specific. Taken together, these results support the conclusion that THbA is capable of boosting specific Abs against CCR5 and ENV. Furthermore, the anti-SIV activity of the purified serum IgG from #5 macaque was determined using MAGIC-5 cells. As expected, 4-wpim-serum IgG from THbA-immunized #5 macaque markedly suppressed SIVmac239 infection (Fig. 4L).

4. Discussion

It has been found that HIV sequence diversity presents a major obstacle to the development of AIDS vaccines. Furthermore, the mucosal transmission of HIV-1 infection established in a short term seems to have presented another obstacle. Therefore, the study of unconventional mucosal immunity in ESN subjects needs to be promoted to identify potential strategies of protective immunity or functional control of HIV infection. Lopalco et al. have reported that anti-HIV Abs such as anti-gp41 IgA are simultaneously induced with

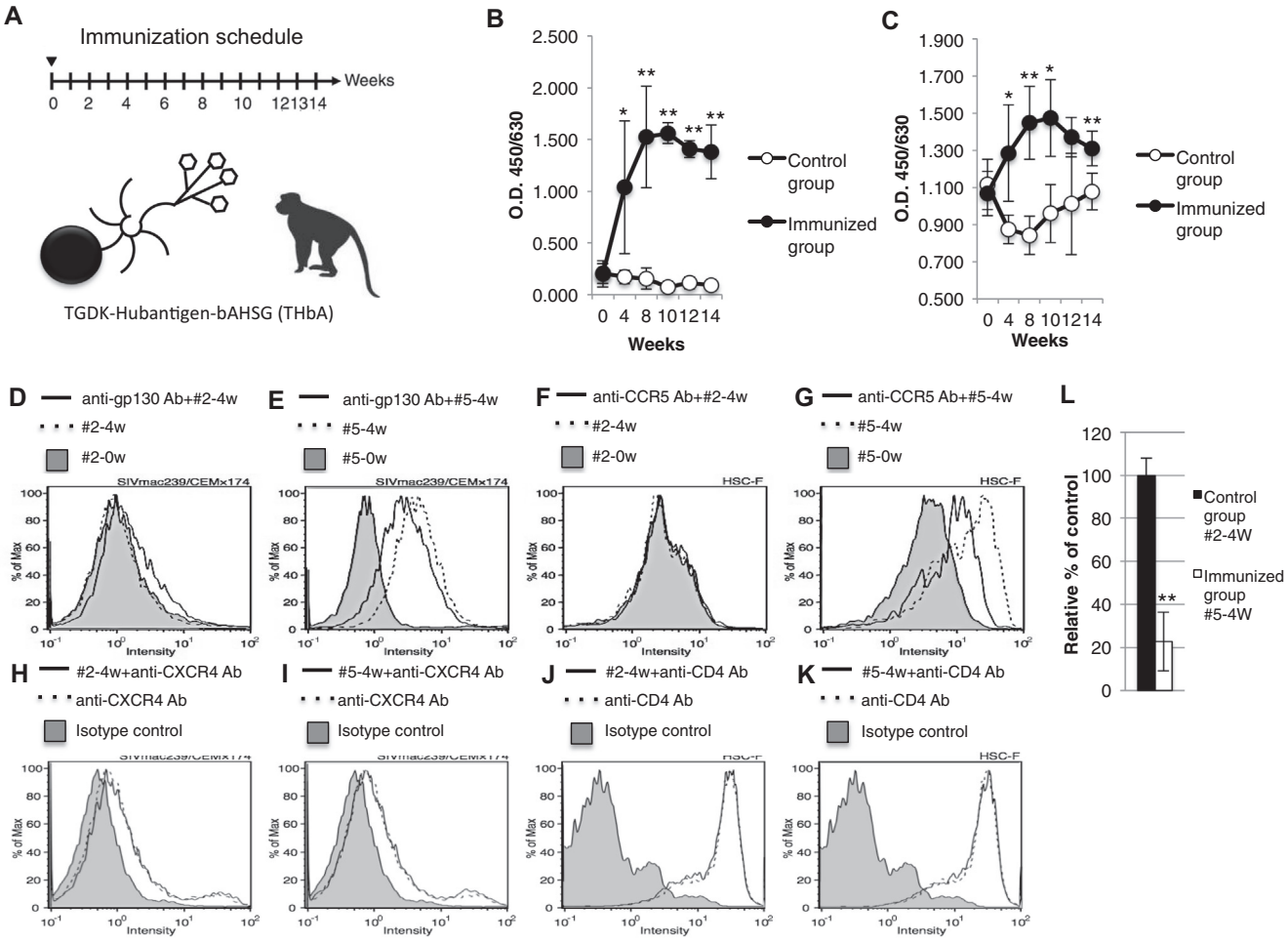

Fig. 4. *Immunogenicity of THbA and induction of anti-cDDR5 and anti-ENV Abs.* (A) Immunization schedule for cynomolgus macaques. (B and C) Serum IgG (100 μg/ml) obtained after immunization with THbA or control Ag were examined by cDDR5-coupled-multipin ELISA and anti-ENV ELISA to investigate whether the anti-cDDR5 (B) and anti-ENV Abs (C) can be raised. (D–G) Inhibition of binding of 4-wpim-serum IgG from #2 or #5 macaques to SIVmac239-infected CEMx174 or HSC-F cells by anti-gp130 (D and E) or anti-CCR5 Ab (F and G) was analyzed by flow cytometry. (H–K) Inhibition of binding of anti-CXCR4 (H and I) or anti-CXCR4 Ab (J and K) to SIVmac239-infected CEMx174 or HSC-F cells by 4-wpim-serum IgG from #2 or #5 macaques was also examined. SIVmac239-infected CEMx174 cells were used as SIVmac239 ENV- and CXCR4-positive cells. HSC-F cells were used as CCR5- and CD4-positive cells. (L) The antiviral activities of 4-wpim-serum IgGs from #2 and #5 macaques were measured by MAGIC-5 assay. Values are means of triplicate determinations. $^{**}P < 0.01$ by Student's *t-test*.

anti-CCR5 and anti-CD4 IgGs in some Italian ESN subjects [17]. These humoral immune responses contribute to an extremely low level of viral replication below the detection limit of a standard assay in ESN subjects, suggesting that more than one type of immunity such as anti-CCR5 and anti-HIV humoral responses must be induced by a vaccine if that vaccine is to be effective for HIV-1 infection. In this study, HMV could induce anti-cDDR5 and anti-ENV Abs simultaneously in nonhuman primates. However, it is important that the vaccine-induced immune responses are maintained before HIV-1 exposure owing to HIV-1 mucosal infection established in a short term. Therefore, exposure to nonpathogenic cross-reacting antigens capable of boosting the vaccine-induced immune responses against HIV-1 infection seems necessary for the success of an HIV-1 vaccine candidate. Interestingly, we demonstrated that bAHSG functions as a booster antigen to efficiently stimulate humoral responses to CCR5 and ENV. Furthermore, we showed that bAHSG conjugated via the Hubantigen with TGDK is capable of inducing specific Abs against CCR5 and ENV (Fig. 4); this was demonstrated by our finding that only the TGDK-binding Hubantigen could not boost humoral immune responses to CCR5 or ENV in HMV-immunized macaques (unpublished data). In addition, the similarity of the primary sequences among bAHSG, CCR5 and SIVmac239 gp140 is not high. These findings suggest that the conformational epitope of highly glycosylated bAHSG is associated with the booster effect. The determination of the crystal structure of bAHSG may reveal the as yet unknown booster mechanism.

In conclusion, it is possible to reconstruct the immune response in ESN subjects before HIV-1 exposure if a vaccine-induced immune response is boosted by an antigen such as bAHSG through daily food intake after the mucosal administration of an immunogen such as HMV.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.11.098.

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