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Enhancing the Quality of Antibodies to HIV-1 Envelope by GagPol-Specific Th Cells

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The importance of Fc-dependent effector function of Abs induced by vaccination is increasingly recognized. However, vaccination of mice against HIV envelope (Env) induced a skewed Th cell response leading to Env-specific Abs with reduced effector function. To overcome this bias, GagPol-specific Th cells were harnessed to provide intrastructural help for Env-specific B cells after immunization with virus-like particles containing GagPol and Env. This led to a balanced Env-specific humoral immune response with a more inflammatory Fc γ profile. The increased quality in the Ab response against Env was confirmed by FcγR activation assays. Because the Env-specific Th cell response was also biased in human vaccines, intrastructural help is an attractive novel approach to increase the efficacy of prophylactic HIV Env-based vaccines and may also be applicable to other particulate vaccines. The Journal of Immunology, 2015, 195: 000–000.

Immumization is among the most effective measures to prevent infectious diseases, and the majority of vaccines protect by the induction of sufficiently strong humoral immune responses (1). Unfortunately, despite tremendous efforts, a prophylactic vaccine against HIV-1 remains elusive. Comparative analyses of clinical HIV-1 vaccine trials indicate that the isotype and subclass distribution rather than the magnitude of induced humoral immune responses may be critically important for protection (2–4). The Ab subclass determines its IgG1 and the FcR-dependent effector func-
internal proteins of influenza or hepatitis B virus can support the development of Ab responses to their respective surface proteins after virus infection or upon immunization with viral particles (21–23). The molecular mechanism explaining these observations is based on BCR-dependent uptake of entire virus particles by B cells specific for the surface protein of the virion and subsequent presentation of Th cell epitopes from the surface protein and internal viral proteins on MHC-II molecules of these B cells. Thus, Th cells specific for internal viral proteins may provide intracellular help (ISH) for B cells specific for the surface protein. Although ISH is not observed postinfection with every virus (24), we have recently demonstrated its relevance to SIV (25). Yet, it remains to be determined if ISH can also affect the Ag-specific IgG subclass distribution, provided that the Th cell responses between internal and surface proteins of a given virus differ.

We report in this study the applicability of ISH to HIV-1 and its impact on the Env-specific IgG subclass distribution and glycosylation. The improved Ab response against Env showed an enhanced FcR activation profile, which should lead to an increased efficacy in vivo. Because we also demonstrate differential regulation of Env- and Gag-specific CD4+ T cells in vaccinated human volunteers, our observations may support the development of a prophylactic vaccine against HIV-1 and provide a more general strategy to modulate the IgG subclass response in a predictable manner.

Materials and methods

Cell culture

The human embryonic kidney cell line 293T (HEK 293T; ATCC CRL-3216) and the mouse mastocytoma cell line P815 (ATCC TIB-64) were maintained in DMEM (Life Technologies, Darmstadt, Germany) supplemented with 10% FCS (Life Technologies) and 250 μg/ml gentamicin (Applichem, Darmstadt, Germany). The retrovirally transduced P815Env cell line was kept under selective pressure with 500 μg/ml G418 (Applichem). BWS5147 (ATCC TIB- 47) cells stably transduced to express an mFcRIV-CD3zeta, an mCD32-CD3zeta, or an mCD16-CD3zeta chimera were maintained in RPMI 1640 (Life Technologies) supplemented with 10% FCS (Life Technologies), 1% penicillin/streptomycin (Life Technologies), and 35 μmol 2-ME.

Plasmids

The codon-optimized HIV-1 expression plasmids HgpSyn (26) encoding for GagPol and pConBgpl40GCD/ (25, 27) encoding for Env that carries the cytoplasmic domain of VSV-G were used for the virus-like particle (VLP) production and as DNA immunogens. Additional DNA immunogens included the codon-optimized consensus clade B full-length Env expression plasmid pConBgpl60opt (27) and the expression plasmids pV-sgp140 and pV-sgp140Trim, which are based on the pVax1 vector (Invitrogen, Life Technologies) and encode the complete extracellular domain of the cleavage defective ConB gppl60-UNC (27) with or without an additional C-terminal trimerization motif from the fibrinogen T4 bacteriophage (28). The murine IL-12 (mIL-12) expression plasmid pVax1-IL2 (Addgene) and the mouse mastocytoma cell line P815 (A TCC TIB-64) were maintained in RPMI 1640 (Life Technologies) supplemented with 10% FCS (Life Technologies), 1% penicillin/streptomycin (Life Technologies), and 35 μmol 2-ME.

VLP preparation

HIV-1 VLPs were prepared as described before with slight modifications (25, 29). Briefly, 293T cells were transfected with 35 μg HgpSyn and 35 μg pConBgpl40GCD/ in 175-cm2 flasks (Greiner Bio One, Frickenhausen, Germany) with 1.5 μg polyethylenimine per 1 μg DNA. Two days later, VLPs were purified from the supernatant of transfected cells by ultracentrifugation through a 20% sucrose cushion for 2.5 h at 90,000 × g and 4˚C. Finally, the purified VLPs were resuspended in sterile PBS and stored at −80˚C until further use.

Immune precipitation of VLPs

To show that our VLPs incorporate Env and Gag into the same particles, we performed an immune precipitation. To this end, we coated 50 μl protein G Dynabeads (Invitrogen) with 12 μg monoclonal human anti-Env Ab 2G12 (Polymun, Klosterneuberg, Austria) in PBS + 0.05% Tween-20 (PBS-T) for 30 min at room temperature. Following removal of excess 2G12 and washing steps with PBS-T and PBS, we incubated the Dynabeads with our VLPs with and without Env diluted in PBS for another 30 min at room temperature. After extensive washing with PBS, bound VLPs were eluted with reducing SDS sample buffer by boiling for 5 min at 95˚C and analyzed by Western blot. HIV-1 Env was detected with a polyclonal goat anti-gp120 Ab (Acris, Herford, Germany) and Gag proteins with the monoclonal murine anti-p24 Ab 183-H12-5C (National Institutes of Health AIDS Reagent Program) and their respective HRP-conjugated secondary Abs (DakoCytomation, Hamburg, Germany).

Ethics statement

All animal experiments performed during this study were approved by an external ethics committee authorized by the North Rhine-Westphalia Ministry for Environment and Nature Protection, Agriculture and Consumer Protection (project licenses AZ 84-02.04.2011.A111; AZ 84-02.04.2012.A149; AZ 84-02.04.2012.A210; AZ 8.87-50.10.37.09.280; and AZ 8.87-50.10.32.08.064). Animals were anesthetized by isoflurane inhalation for blood collection and by i.p. ketamine/xylazine (100/15 mg/kg) injection for immunizations. Before collection of spleens, animals were sacrificed by cervical dislocation. All animals were handled according to the Federation of Laboratory Animal Science Associations.

The RV138 study was approved by the Institutional Review Board of the Division of Human Subjects Protection, Walter Reed Army Institute of Research, and was conducted under the auspices of the U.S. Food and Drug Administration, IND #BB12207 (registered with the National Institutes of Health, clinicaltrials.gov as NCT #00013572). All volunteers provided written informed consent following discussion and counseling by the clinical study team prior to enrollment and before any study-related procedures were performed (30).

Mice and immunizations

Six- to 8-wk-old BALB/cJr and C57BL/6J mice were purchased from Janvier Laboratories (Saint-Beauregard, France) and housed in individual ventilated cages in accordance with the national and institutional guidelines at the animal facility of the Faculty of Medicine, Ruhr University Bochum (Bochum, Germany). For the DNA immunizations, the animals were anesthetized by i.p. injection of 100 mg/kg body weight ketamine (CP-Pharma, Burgdorf, Germany) and 15 mg/kg body weight xylazine (CP-Pharma). The TriGrid electrode array (Ichor Medical, San Diego, CA) with 2.5-mm electrode spacing bearing the centered injection needle was inserted into the shaved hind legs of mice. A volume of 50 μl PBS containing 2.5–20 μg plasmid DNA was injected i.m. in each hind leg immediately followed by the local application of electrical signals of 63 V amplitude and 40 mS total duration. The VLPs were diluted in sterile PBS to a final concentration of 4 μg/ml Env. All animals received a total volume of 100 μl by s.c. injection distributed to both hind footpads.

Determination of humoral immune responses in mice

To monitor the humoral immune responses mice were bled by puncture of the retro orbital sinus with a heparinized 10 μl hematocrit capillary (Hirschmann Laborgeraete). The sera were obtained after 5 min centrifugation at 2600 × g in a tabletop centrifuge and stored at −20˚C until further use. Ab responses against HIV-1 Env and Gag were determined by Ag-specific ELISA essentially as previously described (25). Ninety-six–well high binding microtiter plates (Greiner Bio-One) were coated with 100 ng ConBg120 or 150 ng GST-Gag in 0.1 mol bicarbonate buffer (pH 9.6) per well at 4˚C overnight. After washing with PBS-T, the wells were blocked with 5% skimmed milk powder in PBS-T and washed again before they were incubated with the various sera at different dilutions in blocking buffer. Bound Igs of the IgG1 or IgG2a subclasses were detected, after another washing step, with the respective HRP-conjugated Abs (X56 and R19-15; BD Biosciences) given amount of the respective subclass. The other two subclasses were detected with a biotinylated anti-mouse IgG2b Ab (X56 and R19-15; BD Biosciences) followed by Streptavidin-HRP (BD Biosciences) or an HRP-conjugated unlabeled anti-mouse IgG2b Ab (DakoCytomation). Subsequently, the plates were washed and developed with an ECL solution in an Orion microplate reader (Berthold, Bad Wildbad, Germany). Humoral immune responses are expressed as log10-transformed relative light units.

Determination of cellular immune responses in mice

HV-1 Env- and Gag-specific CD4+ T cell responses in spleens from mice were determined by intracellular cytokine staining (ICS) or cytokine-
specific ELISA 2 or 5 wk after immunization. Briefly, mice were sacrificed, the spleens were removed, and single-cell suspensions were prepared using a 70-μm cell strainer (BD Biosciences). After RBC lysis, spleenocytes were resuspended at 10^6/ml in RPMI 1640 (Life Technologies) supplemented with 10% FCS (Life Technologies), 1% penicillin/streptomycin (Life Technologies), 10 mM HEPES, 4 mM l-glutamine (Life Technologies), and 50 μmol 2-ME.

For the ICS, 10^5 spleenocytes/well were seeded in a 96-well U-bottom microtiter plate (Nunc, Thermo Scientific, Braunschweig, Germany) and stimulated with 5 μg/ml MHC–II–restricted peptides PVGIEYKRWILGLN and SVEVIPMFSLASEGFA for HIV-1 Gag and GVPVKWEATTLFCASDAKA for HIV-1 Env in the presence of 2 μg/ml anti-CD28 (37.51; eBioscience, Frankfurt am Main, Germany) and 2 μmol monensin for 6 h at 37°C in a humified 5% CO2 atmosphere. After stimulation, the cells were surface stained with anti-mouse CD4-PE, anti-human CD4-APC (RM4-5), and Fixable Viability Dye eFluor 780 (both from eBioscience). Following fixation with 2% paraformaldehyde, cells were permeabilized with 0.5% saponin in the presence of 1.7 μg/ml anti-mouse CD16/CD32 (93; eBioscience) and subsequently stained with anti-mouse TNF-α PE-Cy7 (MP6-XT22), anti-mouse IFN-γ PE (XMG1.2; both from eBioscience), and anti-mouse IL-2 APC (JES6-5H4; BD Biosciences). Data were acquired on an FACS Canto II (BD Biosciences) and analyzed with FlowJo (Tree Star, Ashland, OR). The gating strategy is depicted in Supplemental Fig. 1.

For the cytokine-specific ELISA, 5 × 10^5 cells/well were seeded into 48-well plates and stimulated with 5 μg/ml MHC–II–restricted peptides PVGIEYKRWILGLN and SVEVIPMFSLASEGFA for HIV-1 Gag and GVPVKWEATTLFCASDAKA for HIV-1 Env in the presence of 2 μg/ml anti-CD28 (37.51; eBioscience) for 48 h at 37°C in a humified 5% CO2 atmosphere. After the stimulation, the supernatants were analyzed for the presence of IL-4, IL-5, IL-10, and IL-13 by the cytokine-specific ReadySET-Go! ELISA (eBioscience) according to the manufacturer’s protocol and measured in a Sunrise ELISA reader (Tecan, Crailshaim, Germany) at 450 with 620 nm as the reference wavelength.

Study participants
Six participants immunized by four canarypox ALVAC-HIV (vCP205) injections encoding HIV-1 env/gag/pro at weeks 0, 4, 12, and 24 were included in our analysis (30). Cryopreserved PBMCs were obtained from the i.m. arm of the RV138 vaccine study at ~2 wk after the final immunization (week 26).

Ex vivo stimulation of human PBMCs
Cryopreserved PBMCs were thawed and allowed to rest overnight at 37°C and 5% CO2 at a concentration of 1 to 2 × 10^6 cells/ml in R10 medium (RPMI supplemented with 10% heat-inactivated FCS, 2 mM l-glutamine, penicillin [100 U/ml], and streptomycin [100 μg/ml]). The following day, PBMCs were stimulated in 5-ml FACS tubes for 6 or 48 h, for polychromatic flow cytometric and luminex analyses, respectively, with 15-mer overlapping peptide pools comprising HIV Gag or Env Potential T cell Epitopes (National Institutes of Health AIDS Research and Reagents Program) at a concentration of 1 μg/ml in the presence of anti-CD28/49d costimulatory Abs (1 μg/ml; clones L293 and L25; BD Biosciences). Media alone served as a negative control. PBMC stimulated with Staphylococcus enterotoxin B served as a positive control.

ICS of human PBMCs
A pretitred amount of LEAF anti-CD40 (HB14; BioLegend) and APC-Cy7–conjugated anti-CD40L (24-31; BioLegend) Abs were included in the stimulation mixture. The transport inhibitors brefeldin A (Sigma-Aldrich) and monensin (BD Biosciences) were added during the final 5 h of incubation to facilitate detection of intracellular cytokine production. After stimulation, PBMCs were washed with PBS and stained with an amine reactive dye (LIVE/DEAD Aqua Blue; Invitrogen) to exclude nonviable cells, washed in cold staining buffer (PBS with 0.5% BSA) and surface stained for 30 min at 4°C. Surface markers included CD14-PE–CD48 (RDA-T8; BD Horizon) and CD45-BV 711 (UCHL1; BioLegend). After staining, cells were fixed and permeabilized using 2% parafomaldehyde and 1× CytoPerm Wash Buffer (BD Biosciences). Subsequently, cells were resuspended in 100 μl 1× CytoPerm containing IFN-γ–PE-Cy7 (4S-B3; BD Pharmingen), TNF-α–Alexa Fluor 700 (MAb 11; BD Pharmingen), CD4-BV 421 (RPA-T4; BioLegend), and CD3-Qdot 605 (UCHL1; Life Technologies).

Stimulated events were acquired on an LSR II with a special-order four-laser FACSDiva software (BD Biosciences). Compensation was performed with single-stained capture beads (CompBeads; BD Biosciences) and amine dye-reactive beads (ARc; Invitrogen). Cytometer settings were standardized between experiments using multicolor fluorescent calibration beads (Rainbow Fluorescent particles; Spherotech). The total number of cells in each condition was collected for analysis. Data were analyzed with FlowJo version 9.4.1 (Tree Star). Background values were subtracted from all cytokine responses on the basis of the unstimulated control for each patient/stimulation peptide combination.

Luminex analysis of human PBMCs
CDS T cells were positively depleted from PBMC samples following manufacturer’s protocol (Life Technologies) and subsequently stimulated as described above. Depletion was confirmed by polychromatic flow cytometry analysis using LIVE/DEAD Aqua Blue viability dye and CD8–PE–CD594 (RDA-T8; BD Horizon), CD4–BV 421 (RDA-T4; BioLegend), and CD3–Qdot 605 (UCHL1; Life Technologies) Abs as described above. Cytokine production in CDS-depleted PBMC cultures was assessed after 48 h using the Milliplex MAP Human High Sensitivity T Cell Magnetic Bead Panel according to the manufacturer’s protocol (Millipore). Briefly, cell–culture supernatants were cleared by centrifugation at 10,000 rpm for 10 min prior to use. Samples were analyzed on a BioPlex 200 instrument using a five-parameter logistic standard curve with a standard acceptance range of 80–120%.

Adoptive transfer
Five weeks after the DNA immunization, the mice were sacrificed, and their spleens as well as their popliteal and inguinal lymph nodes were collected. Single-cell suspensions were prepared with a 70-μm cell strainer (BD Biosciences). RBCs were removed from the spleenocyte suspensions by ACK lysis. Lymphocytes were pooled by groups, and the CD4+ T cells were purified by negative selection using the CD4+ T cell isolation kit II (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer’s protocol. Successful purification was verified by flow cytometry. Subsequently, the cells were resuspended in sterile PBS and adoptively transferred into syngeneic recipients via the tail vein. Each animal received one mouse equivalent of purified CD4+ T cells.

Env-specific Ab purification and glycan analysis
AssayMAP 5 μl streptavidin cartridges (Agilent Technologies) were conjugated with 70 μg biotinylated pConSgp140CFI (31) and used for purification of Env-specific Abs from individual murine immune sera. To restore physiological pH immediately, 0.5 mol potassium phosphate (pH 8.9) was provided into the wells of the receiver plate before acidic elution at pH 2.8 was performed with 0.1 mol sodium citrate.

Glycan analysis was performed essentially as described before (32, 33). Briefly, 20 μg purified Abs were treated with 20 U PNGase F (New England Biolabs, Ipswich, MA) for 3 h at 37°C. Subsequently, proteins were precipitated with ethanol, and the supernatant containing the glycans was concentrated using a centrifuge vacuum concentrator. Labeling solution was prepared by dissolving 5 mg 2-amino benzamide and 6 mg cya noborohydride in 100 μl 30% (v/v) acetic acid in DMSO. Glycans were fluorescently labeled for 1 h at 37°C. After labeling, glycan was removed using excess dye with microcrystalline cellulose in 0.45-μm GMP filter plates ( Pall, Port Washington, NY), the glycans were analyzed using a 150-nm Amide-80 column (Tosoh Bioscience, King of Prussia, PA) with 30% (v/v) acetic acid in DMSO. Glycan analysis was confirmed using an Agilent 1100 series HPLC device (Agilent Technologies, Santa Clara, CA), and peak identities were confirmed via use of a glycan standard (Laugder, Oxfordshire, U.K.). Quantification by area-under-the-curve analysis was performed with ChemStation software (Agilent Technologies).

Cloning of FcγR-ζ constructs and generation of FcγR-ζ BW5147 reporter cells
The cloning of the mouse FcγR-ζ constructs was performed according to Corrales-Aguilar et al. (34). Briefly, the extracellular portion of FcγRs was amplified from cDNA prepared from mouse spleenocytes by PCR using a pair of primers, which comprise an SpeI restriction site at the 5′-end and the primer 5′-AAG ACT AGT CTT AGC GAG GGG CCA GGG TCT G-3′ including an Spel restriction site at (underlined) at the 5′-end. The two amplified fragments were combined, and double-template PCRs were performed with the 5′ Spel primer and the 3′ Spel primer for the generation of the respective chimeric FcγR-ζ constructs. The FcγR-ζ constructs were cloned into pcDNA3.1 expression vector (Invitrogen). For the lentiviral transduction, the FcγR-ζ constructs were inserted into pue2CL6IwpO, a lentiviral transfer plasmid. All constructs were verified by restriction digest and DNA sequencing.
Lentiviral particles were produced in HEK 293T cells by cotransfection of plasmids coding for VSV-G (envelope plasmid), gagpol (packaging plasmid), and the FcγR--expression by flow cytometry.

the coculture was incubated at 37˚C in a humidified 5% CO2 atmosphere. The FcγR activation assay

To analyze whether Env-specific Abs from sera of immunized mice activate murine FcγRII, III, and IV, to 2 × 109 parental P815 cells and P815Env cells expressing ConBgp1400CD3 were incubated with 1:100 dilutions of the different sera in 100 μl PBS for 30 min at room temperature in a 96-well V-bottom plate. After a washing step with PBS, 2 × 106 mFcγR-CD3β, mCD32-CD3ε (mFcγRII-CD3ε), or mCD16-CD3ζ (mFcγRIII-CD3ζ) transduced BW5147 cells were added in 100 μl culture medium, mixed, and the coculture was incubated at 37˚C in a humified 5% CO2 atmosphere.

Sixteen hours later, 100 μl PBS supplemented with 10% FCS and 0.1% Tween-20 were added to each well and thoroughly mixed to support the release of murine IL-2 from the BW5147 cells. After 15 min incubation at room temperature, the cells were pelleted. FcγR activation was measured as the mIL-2 content in the supernatants by a specific sandwich ELISA with purified capture and biotinylated detection Ab (BD Biosciences). The ELISA was developed using streptavidin-conjugated HRP and TMB solution (BD Biosciences) and measured in a Sunrise ELISA reader (Tecan) at 450 nm with 620 nm as the reference wavelength.

Statistical analysis

Two-tailed Mann–Whitney tests, two-tailed paired and unpaired t tests, one-way ANOVA with Tukey posttest, Kruskal–Wallis test with Dunn posttest, Wilcoxon matched-pairs signed-rank test, or Pearson correlation calculation were performed as indicated in the figure legends using GraphPad Prism software (GraphPad).

Results

Subclass distribution of HIV-1–specific Abs

Previous publications have reported a predominance of the Th2-associated IgG1 Ab subclass in the humoral immune response of mice vaccinated against HIV-1 Env (15–17). To verify this bias and ascertain its Ag specificity, we immunized mice with a combination of HIV-1 Env- and GagPol-encoding plasmids by i.m. DNA electroporation. Genetic vaccines avoid a bias in the immune response imparted by the Ag production system (35), and DNA electroporation has been shown to favor the induction of immune responses with a Th1 phenotype (36). Nevertheless, the Env-specific humoral immune response induced by our vaccine was highly dominated by the Th2-associated IgG1 subclass (Fig. 1A). In contrast, the very same animals developed a balanced Ab response against Gag, although both DNA vaccines were coinfected. Furthermore, Th1-associated IgG2b and IgG3 responses were also stronger against Gag than against Env (Supplemental Fig. 2A). The bias became even more obvious when we calculated the individual Ag-specific IgG2a/IgG1 ratios (Fig. 1B). Because the BALB/c genotype has been reported to develop Th2-prone immune responses under certain circumstances, we also immunized mice of the C57BL/6 genotype. Despite their ability to mount Th1 responses, these animals exhibited the same IgG1 predominance in the Env-specific Ab response as the BALB/c mice (Fig. 1B). Furthermore, this bias was not dependent on the quaternary structure of the Ag, because DNA vaccines encoding soluble or membrane-bound monomeric and trimeric forms of Env induced a similarly IgG1-dominated Env Ab response, suggesting that it is a biochemical rather than a conformational property of the Env Ag (Supplemental Fig. 2B).

A classical way to modulate immune responses induced by vaccination is the incorporation of a suitable adjuvant. This approach has been pursued for HIV-1 Env-based Ags before (16, 17, 20). Although Env-specific immune responses could be enhanced, only minor alterations of the quality of immune responses were observed. We also tried to enhance Env-specific Th1 responses by coapplication of a plasmid encoding the prototypical Th1 cytokine IL-12. Although an increase in IgG1 responses against Env could be detected after the priming immunization, the enhancing effect of IL-12 vanished after the booster immunization (Fig. 1C). Gag-specific Ab responses were not affected (Fig. 1D). As a consequence, no significant differences in the Ag-specific IgG2a/IgG1 ratios were observed (Fig. 1E).

Th cell responses against HIV-1

CD4+ T cells orchestrate humoral immune responses and play a crucial role in the induction of different Ab isotypes and subclasses. To investigate their impact on the Env-specific IgG1 predominance, we analyzed the CD4+ T cell responses against HIV-1 Env after immunization and compared these to the Gag-specific Th cell responses.

ICS revealed Env- and Gag-specific IFN-γ–producing CD4+ T cells in the spleens of mice 2 wk after immunization (Fig. 2A). Most of these cells were polyfunctional because they also produced TNF-α and IL-2. Although coimmunization against Env and GagPol only had a minor impact on Env-specific CD4+ T cell responses, Gag-specific responses were reduced. Because the cytokines analyzed by ICS are associated with a Th1 phenotype of CD4+ T cells, we were surprised to see that Env-specific responses were substantially stronger than the ones against Gag. The same analyses were performed 5 wk after the DNA immunization, but despite a substantial contraction in Env- and Gag-specific CD4+ T cells, no further differences compared with the results from day 14 were observed (data not shown).

Th2-specific cytokines are generally expressed at lower levels, complicating their detection by ICS. Therefore, we also investigated Ag-specific secretion of Th2 cytokines by ELISA 2 and 5 wk after the immunization. Restimulation of splenocytes with Env- and Gag-derived MHC-II–restricted peptides induced significant IL-4 secretion in the Env and Gag immunization groups, respectively (Fig. 2B). Although the Env-specific secretion remained stable until 5 wk after immunization, the Gag-specific IL-4 production vanished. These results already indicate a minor shift toward a Th2 cell response against Env. This shift became more obvious when the Th2 cytokines IL-5 and IL-13 were analyzed. In this study, Env-specific restimulation induced substantially stronger and more sustained cytokine secretion as compared with Gag. Finally, the same was true for the Th2-associated regulatory cytokine IL-10. Together, these results suggest that Env-specific CD4+ T cells with a Th2-shifted phenotype are responsible for the IgG1 bias in the humoral immune response against Env.

Although much of the current knowledge about immunology was first uncovered in mice, not all observations can necessarily be translated to the human system (37). To verify that the immune response to HIV-1 Env and Gag also differs in vaccinated human volunteers, we thoroughly analyzed the CD4+ T cell responses in RV138 participants (30). Many other HIV-1 vaccine studies either only included an Env immunogen or used only Env for additional booster immunizations (38–40). In contrast, the vaccine in RV138 consisted of Env and Gag immunogens that were delivered by the same poxviral vector. This allows to exclude that potential differences in the CD4+ T cell responses to Env and Gag are due to differences in the delivery mode or adjuvants.

ICS of PBMCs after stimulation with Gag and Env overlapping peptide pools revealed higher numbers of IFN-γ– and TNF-α–producing CD4+ T cells specific for Gag as compared with Env (Fig. 3A). The difference became statistically significant when the
Gag-specific CD4+ T cells observed in mice and humans, we may overcome the Ab bias. Given the dominant Th1 phenotype of CD4+ T cells are also differentially regulated in humans. differences in Th1 responses and particularly CD40L expression in with Env- and Gag-derived peptides (Fig. 3B). Nevertheless, the associated cytokine detectable, did not differ upon restimulation than for Gag (Fig. 3A). Secretion of IL-13, which was the only Th2-
cytokine secretion was analyzed (Fig. 3B). In contrast, the frequency of Ag-specific CD4+ T cells expressing CD40L was higher for Env than for Gag (Fig. 3A). Secretion of IL-13, which was the only Th2-associated cytokine detectable, did not differ upon restimulation with Env- and Gag-derived peptides (Fig. 3B). Nevertheless, the differences in Th1 responses and particularly CD40L expression in vaccinated human volunteers demonstrate that Env- and Gag-specific CD4+ T cells are also differentially regulated in humans.

**Overcoming the Env-specific IgG1 bias**

Although IL-12 enhanced the IgG1 Ab response after a single DNA immunization, it failed to modulate the subclass distribution. Because Th cells are probably responsible for the IgG1 predominance, we hypothesized that a substitution of Env-specific CD4+ T cells may overcome the Ab bias. Given the dominant Th1 phenotype of Gag-specific CD4+ T cells observed in mice and humans, we reasoned that they should be able to modulate the humoral immune response against Env if harnessed by ISH for Env-specific B cells. To this end, we produced VLPs containing cores of HIV-1 Gag and Env on their surface as verified by immunoprecipitation (Supplemental Fig. 3). These VLPs were used as booster Ags 5 wk after an initial DNA prime immunization with Env- or GagPol-expressing plasmids given either alone or in combination. Priming against Env or against Env and GagPol induced Env-specific IgG1, but no IgG2a Abs, recapitulating the previously observed bias (Fig. 4B). The subsequent VLP immunizations boosted the anamnestic Env-specific Ab responses, but did not change their IgG1 dominance. In addition, the mock-primed group developed an Env-specific humoral immune response after the VLP immunization that was also dominated by IgG1. Thus, Env DNA and VLP immunization regimens, as well as their combination, induced the same biased Env-specific Ab response. In contrast, induction of GagPol-specific immune responses prior to the VLP booster immunizations led to a significant increase in Env-specific IgG2a Abs, whereas IgG1 titers remained unchanged (Fig. 4C). The striking difference became even more obvious when the individual Env-specific IgG2a/IgG1 ratios were calculated. Only the GagPol-primed group reached a ratio above one. This increase was also significantly different from animals that received an Env or mock prime immunization (Fig. 4D). Only a minor effect was observed for the IgG2b and IgG3 subclasses, although a slight, but significant increase in Env-specific IgG3 was conferred by the GagPol priming immunization (Supplemental Fig. 4A, 4B).

As expected, DNA immunization against GagPol induced a balanced Gag-specific humoral immune response with minor effects of the coapplication of the Env expression plasmid (Fig. 4E). An anamnestic Gag-specific Ab response was observed after the VLP immunizations without an effect on the subclass pattern (Fig. 4F, Supplemental Fig. 4C, 4D). Thus, in GagPol-primed animals boosted with the VLPs, the subclass pattern of the Ab response against Env resembles the one against Gag, indicating that GagPol-specific CD4+ T cells imprint their phenotype onto the Env-specific humoral immune response by ISH.

**GagPol-specific CD4+ T cells mediate the increase in Env-specific IgG2a**

To verify that GagPol-specific Th cells determine the subclass pattern of Env-specific Abs after the VLP immunization, an adoptive transfer experiment was performed (Fig. 5A). Mice were immunized with the GagPol expression plasmid or the empty vector control. The induction of Ag-specific immune responses was verified by Ab ELISA and ICS, the results of which mirrored our previous observations (Fig. 5B, 5C). Five weeks after the immunization, CD4+ T cells were purified from spleens and popliteal and inguinal lymph nodes by negative selection and adoptively transferred into syngeneic mice. Recipient mice re-
received HIV-1 VLP booster immunizations 2 h after transfer and 3 wk later. After the second VLP immunization, Env-specific IgG2a Ab levels were significantly higher in the recipients of CD4+ T cells from GagPol-primed mice compared with the control group (Fig. 5D). In contrast, IgG1 responses against Env were not affected. Consequently, Env-specific IgG2a/IgG1 ratios were significantly increased in animals that received the GagPol-primed CD4+ T cells (Fig. 5E). Therefore, it can be concluded that the selective increase of Env-specific IgG2a Abs by priming against GagPol is indeed mediated through ISH provided by GagPol-specific CD4+ T cells for Env specific B cells.

**CD4+ T cell help impacts Fc glycosylation**

In addition to the subclass, glycosylation of the Fc domain affects its ability to mediate effector functions. Therefore, we wanted to know if a different kind of CD4+ T cell help also influences the
glycosylation profile of the humoral immune response. To this end, mice were primed with GagPol or Env expression plasmids by i.m. DNA electroporation and boosted 5 and 8 wk later with VLPs. Two weeks after the final immunization, the animals were sacrificed for final bleeding. Env-specific Abs from individual sera samples were purified and successful purification was verified by ELISA (data not shown). IgG glycans were enzymatically released, fluorescently labeled with 2-aminobenzamide, and analyzed by hydrophilic-interaction HPLC, with peaks identified via comparison with glycan standards (Fig. 6A, 6B). Glycoforms were grouped into classes according to fucosylation, sialylation, and N-acetylglucosamine bisection levels, as each of these hexoses is associated with functional modulations of Ab activity. Env-specific Abs from Env-primed mice showed significantly higher fucose levels (Fig. 6C). In contrast, sialic acid containing glycans were more prevalent in sera from animals that received a GagPol prime immunization (Fig. 6D). Env-specific Abs from these animals also demonstrated a significantly increased prevalence of bisected glycan species (Fig. 6E). Thus, the CD4+ T cell help afforded by GagPol priming affected the glycosylation pattern of Abs produced by Env-specific B cells, generally shifting glycoforms toward species with enhanced affinity for FcγR4.

**Enhancement of FcγR activation**

The Ab subclass and the Fc glycan profile are the major determinants of Fc-dependent effector functions. Because both were significantly affected for Env-specific Abs by GagPol-specific CD4+ T cells, we wanted to know if this also translates into enhanced functionality. In lack of a reliable murine assay for Ab-dependent cell-mediated cytotoxicity (ADCC), we adapted a recently described (34) human FcγR activation assay that was shown to correlate with ADCC activity to our murine system. Env-expressing murine target cells were prepared by retroviral gene transfer of an Env expression cassette into P815 cells (P815Env). P815Env or the parental P815 cells were incubated with serum samples from nonimmunized mice or mice primed with either a GagPol or Env expression plasmid and boosted with VLPs. The opsonized target cells were subsequently cocultured with reporter cell lines expressing IL2 upon either FcγRII, FcγRIII, or FcγRIV activation. Compared to P815, the use of P815Env cells resulted in substantially stronger IL-2 secretion by all three reporter cell lines, verifying the Ag specificity of the assay. Env-specific Abs

![Image of graph](http://www.jimmunol.org/)

**FIGURE 3.** Env- and Gag-specific CD4+ T cell responses in vaccinated human volunteers. CD4+ T cell responses in PBMCs from RV 138 study participants after four i.m. immunizations with ALVAC-HIV. (A) Env- and Gag-specific CD4+ T cell responses as determined by ICS. Shown are the mean values with SEM (n = 6; ***p < 0.001, two-tailed paired t test). (B) Env- and Gag-specific cytokine secretion of PBMCs as determined by Luminex. Shown are interleaved box-and-whiskers plots (n = 6; *p < 0.05, Wilcoxon matched-pairs signed-rank test).

![Image of graph](http://www.jimmunol.org/)

**FIGURE 4.** ISH increases the Env-specific IgG2a response. (A) BALB/c mice were immunized with 20 μg pConBgp140GC (Env) and 20 μg HgpSyn (GagPol) alone or in combination (Mix) by i.m. DNA electroporation. Total amount of DNA was adjusted with pcDNA in Env- and Gag-primed groups, and the control-primed group received only the empty pcDNA vector (mock). Five and eight weeks later, animals were boosted with Env and Gag containing VLP. Ab responses against Env (B and C) and Gag (E and F) determined by ELISA 4 wk after the DNA prime (B and E) and 2 wk after the second VLP boost immunization (C and F). Shown are the mean values with SEM of 10–12 animals out of two independent experiments at a 1:100 (B and E) and 1:1000 (C and F) dilution. The dotted line represents the background of naive sera (*p < 0.05 versus mock, one-way ANOVA with Tukey posttest; lower p values are not further specified due to space constraints). (D) Env-specific IgG2a/IgG1 ratios of mice primed with the indicated DNA regimen 2 wk after the second VLP immunization at a 1:10,000 dilution. The bars represent the geometric mean values of 6 or 16 animals, respectively. Results for GagPol- and Env-primed groups are derived from two independent experiments (*p < 0.05, ****p < 0.0001, Kruskal–Wallis test with Dunn posttest).
DNA electroporation. Five weeks later, CD4+ T cells were isolated by negative selection from spleen and popliteal and inguinal lymph nodes and transferred into syngeneic recipients. Two hours after transfer and 3 wk later, recipients were boosted with Env- and Gag-containing VLPs. (B) Four weeks after the second VLP booster immunization, Ab responses against Env were measured by ELISA. Shown are the mean values with SEM of five to six animals at a 1:100 dilution (**p < 0.01, ****p < 0.0001 versus mock, two-tailed unpaired t test). (C) Gag-specific CD4+ T cell responses in the spleen on the day of transfer as determined by ICS. Shown are the mean values with SEM of six animals (**p < 0.01, ***p < 0.001 versus mock, two-tailed unpaired t test). (D) One week after the second VLP booster immunization, Ab responses against Env were measured by ELISA. Shown are the mean values with SEM of five to six animals at a 1:1000 dilution (**p < 0.01 versus mock, two-tailed unpaired t test). (E) Env-specific IgG2a/IgG1 ratios of individual mice. The bars represent the geometric mean values (**p < 0.01, two-tailed Mann–Whitney U test).

Discussion

The HIV-1 Env glycoprotein is the only viral target amenable for Abs induced by vaccination. The comparative analyses of humoral immune responses from the efficacious RV144 and the unsuccessful Vax003 trial (2, 3), which employed the same protein Ag, suggest that the quality rather than the quantity of the induced Ab response determined efficacy. In this regard, previous publications reported an intriguing bias of Env immunogens to induce Abs of the Th2-associated IgG1 subclass in mice, but no means are known to overcome such a bias if adjuvants fail to do so (15–17, 42).

We confirm in this study the marked IgG1 predominance in the humoral immune response against Env of mice immunized by i.m. DNA electroporation or with VLPs. Intramuscular DNA electroporation is generally known to promote Th1 responses (36). Accordingly, we previously observed IgG2a-dominated humoral immune responses after a similar immunization against the likewise glycosylated viral surface proteins hemagglutinin of influenza A or F of the respiratory syncytial virus (43). Intriguingly, IgG1 has been shown to be inferior to IgG2a with regard to protection from viral infections (44–46). Furthermore, even neutralizing Abs against influenza virus and HIV-1 demonstrate enhanced protection if expressed with an IgG2a Fe domain (8, 47). Although only in mice thoroughly analyzed, a similar bias toward less protective IgG subclasses may also be evident in humans. Our data from the RV138 trial clearly demonstrate a differential regulation of Gag- and Env-specific CD4+ T cell responses within the same vaccinees. Therefore, it is tempting to speculate that the HIV-1 Env has evolved to induce less protective Abs as an additional immune-evasion mechanism.

Gag-specific CD4+ T cells in RV138 vaccinees were polarized toward a Th1 phenotype, which should induce Abs with an enhanced antiviral Fc effector profile. Similarly, mice immunized by DNA electroporation also developed polyfunctional Th1 responses against the Gag Ag. In contrast, Env-specific CD4+ T cells demonstrated much stronger and more sustained Th2 cytokine expression as compared with Gag-specific CD4+ T cells, although Th1 cytokine responses were also evident. Intriguingly, the cytokines IL-4, IL-10, and IL-13 for which we observed enhanced secretion from Env-specific CD4+ T cells in mice are associated with the induction of the inhibitory IgG4 subclass in humans (48–52). Unfortunately, most Th2 responses of RV138 vaccinees were below the detection limit of our assays.
Even the strong CD40L expression by Env-specific CD4+ T cells may be unfavorable, despite its fundamental role in the induction of humoral immune response (53). It has been reported that increased CD40 stimulation, either by an anti-CD40 Ab or by transgenic CD40L overexpression, augments early Ab responses but significantly reduces their t1/2 due to premature germinal center regression (54, 55). In line with these observations is the remarkably short lifespan of Env-specific Abs induced by vaccination (56–58).

To enhance the quality of the humoral immune response against HIV-1 Env, we wanted to modulate the Ag-specific Ab subclass selection. Different adjuvants are known to affect the Ag-specific Th cell profile or to lead to a stronger induction of a specific Ab subclass, but several have failed to completely overcome the Env-specific IgG1 bias (16, 17, 20). Correspondingly, coapplication of an IL-12–encoding plasmid as a genetic adjuvant also failed in this regard. Therefore, we tried to modulate the Ab subclass distribution by employing ISH, which is known to increase humoral immune responses against viral surface proteins (21–23, 25). The exchange of Env-specific CD4+ T cells with GagPol-specific cells, which secreted little to no Th2-associated cytokines, imprinted the IgG2a-dominated phenotype of the Gag-specific humoral immune response.

**FIGURE 6.** The CD4+ T cell help affects the glycosylation pattern of Env-specific Abs. BALB/c mice were immunized with 30 μg HgpSyn (GagPol) or 30 μg pConBgp140GCD (Env) by i.m. DNA electroporation. Five and eight weeks later, animals were boosted with Env- and Gag-containing VLPs. Env-specific Abs were purified from immune sera collected 2 wk after the final immunization, and their glycosylation profiles determined by HPLC. (A) Representative HPLC spectrum with peaks annotated according to the glycan residues attached to their biantennary core (B, bisected; F, fucose; G0, no galactose; G1, one galactose; G2, two galactose; nd, not determined; S1, one sialic acid; S2, two sialic acids). (B) Area-under-the-curve (AUC) analysis of glycan peaks according to (A). Shown are the mean values with SEM of 8 (GagPol) and 10 (Env) successfully analyzed individual glycan profiles. Proportion of fucosylated (Fuc) (C), sialylated (Sia) (D), or bisected (E) glycan species from Env-specific Abs. Shown are the mean values with SEM of 8–10 individual glycan profiles (**p < 0.01, ***p < 0.001, ****p < 0.0001, one-tailed unpaired t test).

**FIGURE 7.** FcγR activation by Env-specific Abs from immune sera. Mice were immunized by a DNA prime VLP boost immunization as described in the legend to Fig. 4. The DNA-priming immunization is indicated on the x-axis. Env-specific Abs from sera collected 2 wk after the second VLP boost were adsorbed to Env-expressing P815 or parental P815 cells. Opsonized cells were then cocultured with FcγRII (A), FcγRIII (B), and FcγRIIV (C) reporter cell lines. The amount of IL-2 released from the reporter cells as determined by ELISA indicates activation of the respective FcγRs (34). Shown are the mean IL-2 concentrations with SEM for 7 naive and 14–15 immune sera per group derived from two independent experiments at a 1:100 dilution (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, one-way ANOVA with Tukey posttest).
response onto the one against Env. As a consequence, we were able to completely reverse the Env-specific IgG1 bias by ISH. Importantly, this reversal was only possible if no Env-specific CD4+ T cells were induced during the prime immunization. Again, the similarities in Gag-specific CD4+ T cell responses of mice and human vaccinees suggest that the ISH approach may be applicable to humans, as well. Whether this approach can be extended to Th cells with non-HIV specificities needs to be explored, but the idea to avoid possibly detrimental HIV-1-specific CD4+ T cell responses is appealing (58–61).

Another factor that is important for the functionality of Abs is their glycosylation profile within their Fc domain. Although signaling molecules from CD4+ T cells including IFN-γ and IL-21 have been shown to affect Ab glycosylation in vitro (62), this is, to the best of our knowledge, the first demonstration of such a relationship in vivo. The increased degree of fucosylation in Env-primed animals supports our observation that Env immunogens induce less protective Abs. Fucose residues interfere with Fc-dependent effector functions like ADCC and Ab-dependent cell-mediated viral inhibition, probably through sterical hindrance of FcγR engagement (63–66). In addition to the decreased levels of fucosylation, Env-specific Abs from GagPol-primed animals also showed an increased prevalence of bisected glycans, further supporting their enhanced capacity to mediate protective mechanisms dependent on FcγRs (67, 68). Given its regulatory property (69–71), only the concomitant increase in sialic acid could reduce the functionality of these Abs (72) but may as well be a compensatory mechanism to avoid Abs with an excessive inflammatory profile that could cause immune pathology.

Both the different glycosylation pattern and the increased IgG2a/IgG1 ratios of the Env-specific humoral immune response in GagPol-primed mice led to an enhanced engagement of the activating FcγRII and -IV. In contrast, engagement of the inhibitory FcγRII was not significantly different between the groups. Keeping in line with the importance of the activating-to-inhibitory FcγR affinity ratio for the functionality of Abs (73), the GagPol priming should therefore lead to an increased ADCC activity in vivo. Sera from GagPol-primed mice showed stronger Env-specific FcγRIV activation than sera from Env-primed mice (Fig. 7C), although the latter contained higher levels of IgG2a Abs (Fig. 4C). Thus the ratio of the Ag-specific IgG subclasses seems to be more important for FcγR activation than the overall level of an activating Ag-specific IgG subclass. IgG subclasses with lower affinity for the FcγR may actually displace IgG subclasses with higher FcγR affinity for Ag binding and thus reduce FcγR activation. Taken together, the increased IgG2a/IgG1 ratio, the more proinflammatory Fc glycosylation, and the enhanced engagement of activating FcγR after ISH immunization clearly indicate an increased functionality of Env-specific Abs, even in the absence of a reliable ADCC assay.

How the differences in the subclass pattern of Abs against the different viral proteins observed in mice relate to the Ab responses in humans remains to be determined. Unfortunately, Ab responses in RV138 have been too low to answer this question. Based on the affinity for FcRs, mlgG2a seems to be the functional analog to human IgG3, whereas the IgG1 subclasses seem to have differential functional activities in the two species (74, 75). Although IgG1 efficiently mediates Fc effector functions in humans, Env-specific IgG1 responses do not seem to correlate with vaccine efficacy, as exemplified by the Vax 003 and RV144 trials (2, 3, 38, 40). The protection observed in RV144 was likely mediated by Abs, although total humoral and IgG1 responses against Env were lower compared with Vax 003 (4). In contrast, the gp120-specific IgG3 response rates and proportions of total gp120-specific IgG Abs were significantly higher in the RV144 trial, despite the use of the same aluminum hydroxide–adjuvanted gp120 vaccine Ag in both trials (2, 3). These Env-specific IgG3 responses correlated with ADCC activity and, if directed against the V1-V2 region of HIV-Env, also with protection (2). Thus, Abs capable of mediating ADCC seem to be able to protect from HIV-1 infection as long as Lgs with lower functionality, like IgG4 and IgA, do not interfere (3, 4, 76, 77).

Broadly neutralizing Abs, which are probably the most potent mediator of sterilizing immunity from HIV infection, have not been induced by any vaccination approach so far. Sustained exposure to an evolving Ag may be required to achieve the high degree of somatic hypermutation observed for broadly neutralizing Abs (78–81). Because ADCC-mediating Abs are less mutated (77) and should therefore develop faster, they may be more amenable for induction by vaccination. The ISH approach described in this report offers a more general strategy to induce Abs with the desired Fc effector functions. By modifying the composition of the VLPs, we could harness, for example, tetanus toxoid–specific Th cells for Env-specific B cell responses or Gag-specific Th cells for B cell responses to hen egg lysozyme (G. Nabi, V. Temchura, M. Tenbusch, and K. Uberla, unpublished observations).

In summary, we demonstrate that heterologous Gag-specific T cell help can be used to promote the induction of more desirable Env-specific humoral immune responses. In a murine model, this ISH was able to reverse the Env inherent IgG1 bias and affect the glycosylation of the Lgs. Although overall Env-specific Ab titers were slightly lower, their quality was significantly increased, as demonstrated by the enhanced activation of the FcγRIV. Given that the quality rather than the quantity of Ab responses has been associated with HIV vaccine efficacy and that differential regulation of Env- and Gag-specific Th cell responses was also observed in vaccinated human volunteers, these results warrant studies to improve the efficacy of HIV vaccines by ISH.

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Disclosures

D.H. is an employee of the company Ichor Medical Systems. The other authors have no financial conflicts of interest.

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Figure S1: Gating strategy for the intracellular cytokine staining of splenocytes. Representative flow cytometric analysis of splenocytes from an Env-immunized mouse restimulated with the Env (top panel) or an irrelevant peptide control (bottom panel). After gating on live cells and singulettes, splenocytes were selected according to their front and sideward scatter. Within the splenocyte population, CD4+ T cells were then analyzed for the expression of IFNγ, IL2 or TNFα. Subsequently, CD4+ T cells secreting all three cytokines together, referred to as polyfunctional, were identified by Boolean gating.
Figure S2

Figure S2: Env and Gag-specific humoral immune responses after DNA immunization. A) BALB/c mice were immunized twice in a three week interval with a mixture of 10 µg pConBgp140GCD (Env) and 10 µg of HgpSyn (GagPol) by intramuscular DNA electroporation. One week after the boost immunization Env- and Gag-specific humoral immune responses were determined by an antigen-specific ELISA. Shown are the mean values with SEM of 5 animals at a 1:1000 dilution. The dotted line represents the background of naive sera. B) Env and Gag-specific IgG2a/IgG1 ratios of individual mice two weeks after the second immunization with different Env expression plasmids or the empty vector control together with the GagPol expression plasmid. The bars represent the geometric mean values (ns = not significant; * p<0.05; Wilcoxon matched-pairs signed rank test).
**Figure S3**

**A**

Figure S3: Virus-like particles incorporate HIV-1 Env and Gag into the same particles. Virus-like particles were prepared as described before (38). Protein G coupled dynabeads were loaded with the Env-specific monoclonal antibody 2G12 and incubated with VLPs containing Gag and Env (VLP) or Gag alone (ΔEnv). As a control, Dynabeads without 2G12 were used (Δ2G12). Precipitates were eluted with denaturing SDS sample buffer at 95°C and analyzed by SDS-PAGE and Western blot with A) a polyclonal antibody against gp120 or B) a monoclonal antibody against p24. For comparison, the same amounts of VLPs that were used for the immunoprecipitation were analysed directly by SDS-PAGE (VLP Input). In the absence of Env and/or 2G12, no VLPs were precipitated. In contrast, precipitation of VLPs with 2G12 resulted in quantitative recovery of Env as well as Gag indicating the incorporation of both proteins into the same particle.
Figure S4: Env- and Gag-specific IgG2b and IgG3 responses after DNA prime and VLP booster immunizations. BALB/c mice were immunized with 20 μg pConBgp140GCD (Env) and 20 μg HgpSyn (GagPol) alone or in combination (Mix) by intramuscular DNA electroporation. Total amount of DNA was adjusted with pcDNA in Env and Gag primed groups and the control primed group received only the empty pcDNA vector (Mock). Five and eight weeks later animals were boosted with Env and Gag containing virus-like particles. Antibody responses against Env (A and B) and Gag (C and D) as determined by ELISA two weeks after the second VLP boost immunization are shown as mean values with SEM of 6 animals at a 1:1000 dilution. The dotted line represents the background of naive sera (**** p<0.0001; *** p<0.001; ** p<0.01; * p<0.05 vs. mock; one-way ANOVA with Tukey’s post test).