Intrastructural Help: Harnessing T-Helper Cells Induced by Licensed Vaccines for Improvement of HIV Env Antibody Responses to Virus-Like Particle Vaccines

Running title: Licensed vaccines improve HIV Env antibody responses

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Abstract

Induction of persistent antibody responses by vaccination is generally thought to depend on efficient help by T follicular helper cells. Since the T helper cell response to HIV Env may not be optimal, we explored the possibility to improve the HIV Env antibody response to virus-like particle (VLP) vaccines by recruiting T helper cells induced by a commonly-used licensed vaccines to provide help for Env-specific B cells. B cells specific for the surface protein of a VLP can internalize the entire VLP and thus present peptides derived from the surface and core proteins on their MHC-II molecules. This allows T helper cells specific for the core protein to provide intrastructural help for B cells recognizing the surface protein. Consistently, priming mice with an adjuvanted Gag protein vaccine enhanced the HIV Env antibody response to subsequent booster immunizations with HIV VLPs. To harness T helper cells induced by the licensed Tetanol®pur vaccines, HIV VLPs were generated that contained T helper cell epitopes of tetanus-toxoid. Tetanol-immunized mice raised stronger antibody responses to immunizations with VLPs containing tetanus-toxoid T helper cell epitopes, but not to VLPs lacking these epitopes. Depending on the priming immunization, the IgG subtype response to HIV Env after the VLP immunization could also be modified. Thus, harnessing T helper cells induced by other vaccines appears to be a promising approach to improve the HIV Env antibody response to VLP vaccines.

IMPORTANCE: Induction of HIV Env antibodies at sufficient levels with optimal Fc effector functions for durable protection remains a challenge. Efficient T cell help may be essential to induce such a desirable antibody response. Here, we provide proof-of-concept that T helper cells induced by a licensed vaccine can be harnessed to provide
help for HIV Env-specific B cells and to modulate the Env-specific IgG subtype response.

Keywords: Intrastructural help, T helper cells, HIV, Env, antibody, vaccine, VLP, IgG subtype.
Introduction

Virus-like particle vaccines have been shown to be highly immunogenic and to provide protection from a number of viral diseases (reviewed in (1)). Due to the repetitive nature of their surface proteins, virus-like particles (VLP) can trigger cross linking of B cell receptors specific for the surface protein, which may partially explain the good antibody responses observed after VLP immunization (2). Development of VLP vaccines against HIV faces a number of challenges. The diversity of circulating HIV strains requires Env immunogens that induce broadly-reactive antibody responses to the native Env spikes on the virion. Therefore, Env immunogen design is a major focus of current HIV vaccine development efforts (reviewed in (3)). In addition, induction of HIV Env antibodies at levels high enough to provide durable protection from HIV acquisition remains a challenge (4). Providing adequate T cell help may be a critical step for the development of vaccines inducing long-lasting antibody response protecting from HIV acquisition. In mice, we observed that induction of primary anti-Env antibody responses by VLP immunization depends on T-helper cells (5). However, T helper cell responses to HIV Env are generally weak during HIV infection (6) and HIV Env antibody responses in human vaccinees seem to rapidly decline (7). We could previously show that Gag-specific T helper cells induced by gene-based vaccines can provide help for HIV Env-specific B cells after booster immunizations with VLPs (8, 9). This intrastructural help (10–13) can be explained by uptake of entire VLPs by Env-specific B cells and subsequent presentation of Gag and Env-derived T helper cell epitopes on MHC-II molecules of the Env-specific B cells (Fig. 1). We now extend these observations to adjuvanted protein vaccines and provide proof-of-concept that T helper cells induced by
a commonly-used licensed protein vaccine can also be harnessed to deliver help for Env antibody responses. Thus, recruitment of pre-existing T helper cells induced by licensed vaccines seems to be a promising novel strategy to optimize antibody responses to surface proteins of VLP vaccines.

Results

Modulation of the HIV Env specific antibody response by an adjuvanted HIV Gag-protein immunization

We previously observed that T helper cells induced by DNA or adenoviral vector vaccines encoding Gag or GagPol provided intrastructural help for Env antibody responses after VLP booster immunizations in mice (8, 9). To explore whether an adjuvanted protein vaccine could similarly modulate the HIV Env antibody response by intrastructural help, mice were first immunized with recombinant Gag protein adjuvanted with polyICLC, a TLR3 and MDA-5 ligand (14). PolyICLC has been shown to be a strong inducer of T helper cell responses in mice (15) and non-human primates (16). Several reports (17, 18) also demonstrated that polyICLC induces strong CD4+ T cell responses but weak CD8+ T cell responses.

The Gag antibody response after a single immunization with the adjuvanted Gag protein vaccine was compared to an HIV-Gag DNA vaccine to define a suitable concentration of the adjuvant. Mice were primed with a constant amount of Gag protein in the presence of increasing concentrations of PolyICLC. Three weeks after the immunization, Gag-specific antibody levels directly correlated with the dose of the adjuvant. The minimal
dose of polyICLC required for significant enhancement of Gag-specific IgG1 and IgG2a antibody levels was 10 µg (Fig 2A). We therefore selected this dose for all subsequent experiments. The adjuvanted Gag protein and the Gag DNA vaccines also induced Gag-specific T helper cells producing Th1 cytokines (Fig. 2B). Interestingly, the cytokine profiles differed between the two groups. The response in the adjuvanted Gag group was dominated by TNF-α, while a more balanced INF-γ and TNF-α production was observed in the Gag DNA group (Fig. 2B).

To explore the extent to which Gag-specific T helper cells provide intrastructural help, mice received two priming immunizations with different Gag immunogens or controls prior to two booster immunizations with the same VLPs (Fig. 2C). After two priming immunizations with the adjuvanted Gag protein vaccine strong Gag-specific IgG1 and IgG2a antibody responses were observed that exceeded the ones induced by the Gag-DNA vaccine (Fig. 2D). Non-adjuvanted Gag only induced a weak Gag-specific IgG1 response. Looking at the HIV Env antibody responses after the two VLP booster immunizations, we noted that priming with adjuvanted Gag significantly enhanced HIV Env specific IgG1 and IgG2a antibody levels, while Gag DNA priming only enhanced HIV Env-specific IgG2a antibody responses (Fig. 2E).

Since Fc effector functions may depend more on the ratio of IgG subtypes than their absolute values we also determined the ratio of HIV-1 Env-specific IgG2a/IgG1 antibodies. Mice that were either primed by HIV Gag DNA or adjuvanted HIV Gag protein immunization had a significantly enhanced ratio of HIV-1 Env-specific IgG2a/IgG1 antibodies (Fig. 2F) confirming that intrastructural help can modulate IgG subtype ratios.
Generation of VLPs containing fragment C of tetanus-toxoid

Having shown that an adjuvanted Gag protein vaccine can provide intrastructural help for HIV Env antibody responses triggered by a VLP vaccine, we asked whether T helper cells induced by a licensed protein vaccine, such as Tetanol, could be harnessed to provide intrastructural help for HIV VLPs. Since fragment C of tetanus-toxoid (TT) has been shown to efficiently harness T cell help for antibody responses in the context of fusion proteins (19), we inserted the coding sequence of fragment C of TT in frame between MA and CA of Gag (Fig. 3A). Insertions at this site have been shown previously to be compatible with particle formation and budding (30). Transient co-transfection of the FrC-Gag and Env expression plasmids into 293T cells led to the release of particles (FrC-VLP) that could be pelleted through a 35% sucrose cushion (Fig. 3B). Although the Env content of wild type VLPs seemed to be higher, Env could also be detected readily in the FrC-VLPs (Fig. 3B). Western-blot analysis with antibodies to Gag and fragment C of tetanus toxoid revealed that the FrC-Gag fusion protein migrated at a molecular weight of approximately 105 kDa, while the parental Gag had the expected molecular weight of 55 kDa (Fig. 3B).

Influence of Tetanol immunization on the Env-specific IgG responses to VLP vaccines containing tetanus-toxoid T helper cell epitopes

To explore the effect of Tetanol immunization on the HIV Env antibody response to VLPs containing fragment C of TT, Balb/c mice were immunized twice with Tetanol
(week 0 and 4) prior to one or two injections of VLPs containing fragment C. After the booster immunizations VLPs containing fragment C, there was a trend to higher Env IgG1 antibody responses were higher in Tetanol-primed mice receiving VLPs containing fragment C than in unprimed Balb/c mice, although this difference did not reach statistical significance (Fig. 3C). No increase in Env-specific IgG2a antibody responses was observed. Since Balb/c mice were reported to have a Th2 bias favoring IgG1 antibody responses we also performed a Tetanol prime (week 0 and 4) and VLP boost (week 8) experiment in BL/6 mice. A strong increase in Env-specific IgG1 antibody levels was observed in BL/6 mice that had received prior Tetanol immunizations, while there was no effect on Env-specific IgG2a antibody levels (Fig. 3D). In both Balb/c and BL/6 mice no Tetanol priming effect on Env IgG responses was observed after boosting with VLPs lacking fragment C of TT (Fig. 3E-F).

Influence of Tetanus-toxoid specific CD4+ T cells on the in vivo proliferative response of antigen-specific B cells to VLPs containing tetanus-toxoid T helper cell epitopes

To corroborate our hypothesis, that TT-specific CD4+ T cells provided direct help to HIV-1 Env specific B cells, we used the hen egg lysozyme (HEL) model antigen and established an in vivo proliferation assay for antigen-specific B cell responses that are dependent on TT-specific T helper cells (Fig. 4A-F). Injection of VLPs displaying HEL on their surface into mice that received CFSE-labelled naïve HEL-specific B cells four hours prior to the VLP injection led to proliferation of the HEL-specific B cells (Fig. 4G). Priming mice with Tetanol prior to transfer of CFSE-labelled HEL-specific B cells and
injection of VLPs containing HEL on their surface and fragment C inside enhanced the proliferative response of the HEL-specific B cells in comparison to non-primed mice (Fig. 4G). Depletion of CD4+ T cells abolished the enhancement of HEL-specific B cell proliferation, while the T cell independent proliferation of HEL-specific B cells induced by HEL VLPs was maintained. The SW-HEL donor mice from which the HEL-specific B cells are derived, are on the wild type RAG background and only up to 40% of the transferred CFSE-labeled B cells carry HEL+BCR, while the rest of the B cells have undergone VH gene replacement (21) and did not bind HEL on their BCR (Fig. 4E). These HEL-negative B220+CFSE+ B cells did not proliferate in any of the experimental groups (data not shown) confirming the antigen-specific nature of the B cell proliferation after VLP immunization. These results clearly demonstrate that the initial proliferation of naïve B cells specifically recognizing VLP surface proteins was CD4+ T cell independent. However, Tetanol-induced CD4+ T cells were able to significantly increase this proliferation indicating an effect of intrastructural help already on the early steps of antigen-mediated primary B cell expansion. To exclude the possibility that the proliferative response induced by the Tetanol immunization led to cell death we also analyzed the effect of Tetanol priming on the expansion of HEL+ B cells in spleens on day 7 after HEL-VLP immunizations (Fig. 4H). Naïve B cells from SW-HEL mice were transferred into Tetanol-primed and non-primed mice which were then immunized with HEL-VLPs containing fragment C. As expected, priming with Tetanol led to a significant increase in the percentage of HEL+B220+ B cells in the spleen (Fig. 4H) confirming their expansion.
Since the yield of VLPs based on the FrC-Gag expression plasmid were low, we compared the efficiency of VLP formation by FrC-Gag with wild type Gag and a novel Gag expression plasmid, that only contained the p2 T helper cell epitope of fragment B and domain 1 of fragment C (Fig. 3A). Wild type VLPs, FrC-VLPs, and p2D1-VLPs were prepared by co-transfection of the HIV Env expression plasmid with expression plasmids for wild type Gag, FrC-Gag, or p2D1-Gag, respectively. As expected from the calculated molecular weight, the p2D1-Gag pelleted through a 35% sucrose cushion was detected by Western blot analysis with antibodies to Gag and fragment C of TT at 80kDa (Fig. 5A). Although additional weaker bands, presumably due to proteolytic cleavage, could also be observed, the intensity of the p2D1-Gag band was substantially stronger than the FrC-Gag band. Consistent with more efficient particle formation, the Env content of p2D1-VLPs was also higher than the Env content of FrC-VLPs (Fig. 5A).

To further confirm incorporation of HIV Env into the VLPs, the HIV Env expression plasmid was co-transfected with the p2D1-Gag expression plasmid. VLPs were purified from the supernatant of transfected cells and immunoprecipitated with the HIV Env antibody 2G12. Western blot analyses for HIV Env revealed the presence of HIV Env in the precipitates (Fig. 5B). Co-precipitation of Gag (Fig. 5C), that also reacted with an antiserum to fragment C (Fig. 5D), indicated that HIV Env, Gag and fragment C were part of the same VLPs. The specificity of the immunoprecipitation was confirmed using VLPs lacking Env (Fig. 5B-D). Based on the more efficient particle formation, p2D1-Gag was selected for all subsequent experiments.
Results shown in Figure 2F indicated that after priming with either adjuvanted Gag-protein or Gag-DNA intrastructural help increased the IgG2a/IgG1 ratio of anti HIV Env antibodies after the VLP immunization. In contrast, priming with Tetanol only enhanced the HIV Env IgG1 antibody responses to FrC-VLP immunizations (Figure 3C–D). To further confirm that the HIV Env-specific IgG subtype response was dependent on the type of T helper cells mediating intrastructural help, we compared the effect of Tetanol-priming on the HIV Env antibody responses to VLPS containing (p2D1-VLPs) or lacking (wt-VLPs) the p2D1 fragment. To reveal whether the formulation and/or the nature of the antigen can modulate the IgG subtype response, we also included tetanus-toxoid and Gag DNA vaccines for priming (Fig. 6A).

Priming with Tetanol or TT-DNA enhanced HIV Env specific antibody levels in response to p2D1-VLPs only, while the Gag-DNA priming immunizations modulated HIV Env antibody responses to both type of VLP vaccines to a similar extent (Fig 6B-F).

Interestingly, the HIV Env IgG subtype response to the same p2D1-VLP booster immunizations clearly differed for the different priming immunizations (Fig. 6C-F).

Priming with Tetanol significantly enhanced only HIV Env IgG1 antibody levels, while priming with TT-DNA enhanced IgG1, IgG2a, and IgG2b antibody levels. Priming with Gag-DNA led to an approximately 2000-fold higher mean Env-specific IgG2a antibody levels in comparison to the non-primed groups (Fig. 6D), while HIV Env IgG1 antibody levels increased 50 to 100-fold (Fig. 6C).
To correlate the differential IgG subtype responses to differences in T helper cells mediating intrastructural help, TT-specific CD4⁺ T cell responses were characterized after the priming immunization. ELISPOT analyses and intracellular cytokine staining after stimulation with the MHC-class II-restricted peptides, p30 and p2, revealed induction of T helper cells producing IFN-γ (Fig. 7A, B) which trended to be more pronounced in the TT-DNA vaccine group. For other Th1 cytokines (TNF-α and IL-2) no difference between the two groups was observed (data not shown). In contrast, secretion of the Th2 cytokines, IL-4 and IL-5, could only be detected in Tetanol immunized animals (Fig. 7C+D).

Influence of Tetanol priming on the quality of the HIV Env antibody response to virus-like particle vaccines containing tetanus-toxoid T helper cell epitopes

To further characterize the effect of Tetanol priming on the quality of the antibody response, we analysed Fc-effector functions of HIV Env-specific antibodies 4 weeks after the second p2D1-VLP booster immunization by reporter cell-based activation assays for Fc-γRII, Fc-γRIII and Fc-γRIV (9). Sera from Tetanol primed and p2D1-VLP-boosted mice led to a stronger activation of all three Fc-γ receptors compared to sera from mice only receiving the p2D1-VLP immunizations, although these differences did not reach statistical significance (Fig. 8A-C).

To exclude that intrastructural help is only transiently enhancing antibody responses, a 10 week serological follow-up was also performed. Clearly, HIV Env-specific IgG1
antibody levels induced by p2D1-VLP immunization continued to be significantly higher in Tetanol-primed animals than in non-primed controls (Fig. 8D).

**Discussion**

Similar to gene-based vaccines encoding Gag (8, 9), the adjuvanted Gag protein vaccine also induced T helper cells at levels sufficient to provide intrastructural help. However, for the protein vaccine this clearly required a strong adjuvant, such as polyICLC. Extending the concept of intrastructural help from virus-specific T helper cells, the results also constitute proof-of-concept for the strategy that T helper cells induced by licensed vaccines can be harnessed to provide help for B cell responses induced by VLP vaccines. The observation that the enhancement of antibody responses to HIV Env by prior immunization with tetanus vaccines is entirely dependent on the incorporation of tetanus-toxoid derived protein fragments into the VLP indicates that it is not a general modification of the inflammatory milieu during priming that persists and mediates the modulation of the Env antibody response at the time of the VLP immunization. Consistently, depletion of CD4+ T cells abolished the enhancement of the proliferation of BCR-transgenic B cells specific for the model surface antigen of the VLP by the Tetanol priming immunization.

Interestingly, both the antigen and the type of the priming immunization affected the IgG subtype responses to HIV Env after the VLP immunization (Fig. 2 and Fig. 6). Tetanol priming strongly enhanced the IgG1 Env antibody response and had no effect on the anti Env IgG2a, IgG2b, and IgG3 responses. Priming with a DNA vaccine encoding tetanus-toxoid fragment C led to a strong increase in IgG1 and a modest increase in
IgG2a and IgG2b responses to HIV Env. These IgG sub-class distributions may reflect
the Th1/Th2 cytokine profile of the antigen-specific CD4+ T cells induced by priming.
Priming with Tetanol raised TT-specific CD4+ T cells with a prominent Th2 phenotype.
Intrastructural help by these T helper cells during the VLP booster immunizations may
trigger expansion of B cells secreting Env-specific IgG1 and block class switching to
other IgG subtypes. On the contrary, TT-DNA immunization induced tetanus-specific
CD4+ T cells with a Th1-dominated phenotype most likely imprinting the Th1 phenotype
to Env-specific B cells during the VLP booster immunizations (Fig. 7). The difference in
the IgG subtype responses may directly affect Fc-effector functions of the HIV Env-
specific antibodies as observed previously (9). In the current study, intrastructural help
also enhanced Fc effector function of the immune sera (Fig. 8A-C). Whether this is due
to higher overall level of HIV-1 Env-specific antibodies, changes in the ratio of IgG
subtypes, or differences in the glycosylation of the antibodies, remains to be
determined. Intrastructural help provided by Tetanol priming also resulted in a long-
lasting increase of the IgG1-dominated humoral immune response (Fig. 8D), suggesting
formation of long-lived plasma cells.

The applicability of intrastructural help in humans will critically depend on the efficient
incorporation of protein fragments of licensed vaccines into the VLP. As shown by the
inefficient particle formation by FrC-Gag, the size of the fragment that can be
incorporated and/or it’s folding may be limiting. Therefore, it may be advantageous to
incorporate linear T helper cell epitopes rather than entire protein domains. However,
the allelic variation of MHC-II in the human population may require the identification of
MHC-II epitopes to which many individuals raise T helper cell responses or the
incorporation of a panel of T helper cell epitopes. Promiscuous or universal T helper epitopes that are binding on a broad range of MHC-II human alleles might be an elegant solution to overcome this problem. Tetanus-toxoid sequence offers even two of such epitopes: p2 and p30 (22). Although, each of the epitope demonstrates MHC-II binding on multiple MHC-II alleles among different species (from mice to human), helper T cell activity might strongly vary between the alleles (23–25). Our observation that VLP containing FrC with only one epitope (p30) were more potent in BL/6 (H-2b) than in Balb/c (H-2d) mice (Fig. 3) is consistent with previous reports (23). Reduction of total FrC size with additional incorporation of the second universal epitope (p2) led to efficient particle formation and improved humoral immune responses in Balb/c mice (Fig. 6). Since each HIV particle contains approximately 2000 Gag molecules we assume a similar number of molecules of the T helper cell epitopes. However, the precise number of epitope molecules required for efficient intrastructural help remains to be determined. The merit of incorporating protein fragments derived from the tetanus-toxoid is that most people have been previously vaccinated with Tetanol and therefore are likely to have pre-existing memory T cells. Follow-up studies will explore whether pre-existing memory T cells can be directly recruited by intrastructural help or whether Tetanol booster immunizations are required prior to the VLP immunizations. However, given the fact, that the type and persistence of antibody responses is thought to be largely regulated by T helper cells, the intrastructural help approach seems to offer plenty of opportunities to improve the antibody response to VLP vaccines.

Materials and Methods
Cell lines, plasmids and VLP preparation

HEK293T cells were cultured in Dulbecco’s modified Eagle Medium (DMEM) (Life Technologies, Darmstadt, Germany) with 10% heat-inactivated fetal calf serum (FCS) (Life Technologies, Darmstadt, Germany) and 250 µg/ml gentamicin (Applichem, Darmstadt, Germany). Plasmids: Frc-Gag and p2D1-Gag expression plasmids were constructed by inserting the coding sequence for fragment C (FrC; aa 865-1316 of tetanus toxoid (TT) according to GenBank entry number AAA23282.1) (23, 26, 27) or for the p2D1 fragment spanning the promiscuous Th epitope p2 (QYIKANSKFIGITE) and domain 1 of FrC (FrCD1; aa 865 to 1120 according to Genbank entry number AAA23282.1) by overlap extension PCR between the 3’ codons (DTGHSSQ) of matrix (MA) and the 5’ codons (VSQNYPI) of capsid (CA) of the Hgsyn expression plasmid (28). The plasmids, HgSyn (a codon-optimized HIV Gag expression plasmid) (28), pConBgp140GC/D (encoding codon-optimized HIV-Env clade B consensus sequence aa 1 to 703 of Genbank entry number ABG67916.1 fused to the intracellular domain of VSV-G (aa 97 to 122 of Genbank entry number: CAA24524.19) (8, 29), and pC-HEL-TM (encoding the membrane-anchored form of HEL) (30) have been described. All plasmids were prepared with the NucleoBond Xtra Maxi EF or the PC10000 EF Kit (Macherey-Nagel, Dueren, Germany). VLPs were prepared as described previously with slight modifications (8, 31). Briefly, 293T cells were co-transfected with 30 µg of one of the Gag expression plasmids HgSyn, FrC-Gag, or p2D1-Gag, and 30 µg pConBgp140GC/D to generate wild type VLPs (wt-VLP), FrC-VLPs, and p2D1-VLPs, respectively. HEL-VLPs containing HEL on its surface and FrC-Gag inside were produced by co-transfection of 30 µg of FrC-Gag and 30 µg pC-HEL-TM. Transfections
were performed in 175-cm² flasks (Greiner Bio One, Frickenhausen, Germany) with 1 µg polyethylenimine per 1 µg DNA. Six hours post transfection, the medium was replaced with fresh DMEM medium free of FCS and containing only 1% gentamicin (Applichem) and 1% GlutaMax (Life Technologies). Two days later, VLPs were purified from the supernatant of transfected cells by ultracentrifugation through a 35% sucrose cushion for 4 h at 90,000 x g and 4°C. Finally, the purified VLPs were resuspended in sterile PBS and stored at 80°C until further use. To determine the amount of Env and Gag in the VLP preparations, high binding microtiterplates (Greiner Bio One) were coated with the purified VLPs or serial dilutions of known amounts of p55 Gag (32) or HIVgp120 (HIV-1 IIIB, NIH Reference and Reagent Program) as standards. The amounts of p55 and HIVgp120 from the VLP preparations bound to the microtiter plate were then determined using an anti-HIV-1 p24 monoclonal antibody (183-H12-5C, NIH AIDS Reference and Reagent Program), the monoclonal gp120 antibody 2G12 (Polymun, Klosterneuberg, Austria) and matched HRP-conjugated secondary antibody reagents. The endotoxin levels of the immunogens were determined by the limulus Amebocyte Lysate assay, QCL-1000 (Cambrex, Walkersville, MD) to be below 0.1 units per injection dose.

**Immune precipitation of p2D1-VLPs**

To show that the p2D1-VLPs incorporate Env, Gag and the p2D1 fragment of TT into the same particles, we performed an immune precipitation with p2D1-VLPs as described before (9). To this end, we coated 50 µl protein G Dynabeads (Invitrogen, Life
Technologies) with 12 µg monoclonal human anti-Env Ab 2G12 (Polymun, Klosterneuburg, Austria) in PBS + 0.05% Tween-20 (PBS-T) for 30 min at room temperature. After removal of excess 2G12 and washing steps with PBS-T and PBS, we incubated the Dynabeads with p2D1-VLPs containing or lacking Env for 30 min at room temperature. Following extensive washing with PBS, bound VLPs were eluted with reducing SDS sample buffer by boiling for 5 min at 70°C and analyzed by Western blot. HIV-1 Env was detected with a polyclonal goat anti-gp120 Ab (Acris, Herford, Germany), Gag proteins with a monoclonal murine anti-p24 Ab (183-H12-5C, National Institutes of Health AIDS Reagent Program), and p2D1 and FrC with a polyclonal antiserum against TT (POL 016 Anti Tetanus Toxin; Statens Serum Institute, Copenhagen, Denmark) (33) and HRP-conjugated secondary antibodies, P0449, P0447, and P0399 (DakoCytomation, Glostrup, Denmark), respectively.

Mice and immunizations

BALB/cJRj (Balb/c) and C57BL/6J (BL/6) mice (6–8 weeks old) were purchased from Janvier Laboratories (Saint-Berthevin, France), 12 weeks old SW-HEL mice from in-house breeding were donors for HEL-specific B cells for adoptive transfer experiments. All mice were housed in individual ventilated cages in accordance with the national law and institutional guidelines at the animal facility of the Faculty of Medicine, Ruhr University Bochum (Bochum, Germany). For the DNA immunization, 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 the mice (23). A volume of 50 µl PBS containing 30 µg plasmid DNA was injected i.m into the gastrocnemius muscle of 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. All animals received 400 ng of Env per immunization in a total volume of 100 µl by s.c. injection distributed to both hind footpads. A volume of 50 µl PBS containing 1µg of Gag protein (p55) (32) alone or in combination with 0.3, 1, 3, or 10 µg polyICLC (Hiltonol-Oncovir, Inc) was injected i.m. into each hind leg. For Tetanol immunization, mice received a volume of 100 µl PBS containing 10 µl Tetanol®pur (Novartis Vaccines and Diagnostics, Marburg, Germany) by i.m. injection distributed to both hind legs.

Characterization of cellular immune responses

HIV Gag-specific CD4⁺ T cell responses and Tetanus-specific CD4⁺ T cell responses in spleens from mice were determined by intracellular cytokine staining (ICS), cytokine-specific ELISA as described before (9), and IFNγ ELISPOT. To this end, mice were sacrificed 2 weeks after a single immunization, the spleens were removed, and single-cell suspensions were prepared using a 70µm cell strainer (BD Biosciences, Heidelberg, Germany). After RBC lysis, splenocytes were resuspended at 10⁷ cells/ml in RPMI 1640 supplemented with 10% heat inactivated FCS, 1% penicillin/streptomycin, 10 µmol HEPES, 4 µmol L-glutamine (all from Life Technologies, Thermo Fisher Scientific).
For ICS, 10^6 splenocytes/well were seeded in a 96-well U-bottom microtiter plate (Nunc, Thermo Fisher Scientific) and stimulated with 5 µg/ml MHC-II restricted peptides: PVGEIYKRWIIL and SPEVIPMFSALSEGA for HIV-1 Gag or 2 µg/ml MHC-II–restricted peptides: p2 (QYIKANSKFIGITELK) and p30 (FNNFTVSFWLRVPVSASHLE) for tetanus toxoid in the presence of 2 µg/ml anti-CD28 (37.51; Life Technologies, Thermo Fisher Scientific) and 2 µmol monensin for 6 h at 37˚C in a humidified 5% CO_2 atmosphere. After stimulation, the cells were surface stained with anti-mouse CD4 PerCP-eFluor710 (RM4-5) and fixable viability dye eFluor780 (both from Life Technologies, Thermo Fisher Scientific). 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; Life Technologies, Thermo Fisher Scientific) and subsequently stained with anti-mouse TNFα PE-Cy7 (MP6-XT22), anti-mouse IFNγ PE (XMG1.2; both from Life Technologies, Thermo Fisher Scientific), and anti-mouse IL-2 APC (JES6-5H4; Life Technologies, Thermo Fisher Scientific). Data were acquired on an FACS Canto II (BD Biosciences) and analyzed with FlowJo (Tree Star, Ashland, OR USA). The gating strategy was reported previously (9). For the cytokine-specific ELISA, 5 x 10^6 cells/well were seeded into 48-well plates and stimulated with 2µg/ml MHC-II–restricted peptides of tetanus toxoid (p2 and p30) in the presence of 2 µg/ml anti-CD28 (37.51; Life Technologies, Thermo Fisher Scientific) for 48 h at 37˚C in a humidified 5% CO2 atmosphere. After the stimulation, the supernatants were analyzed for the presence of IL-4 and IL-5 by the cytokine-specific Ready-SET-Go ELISA (Life Technologies, Thermo Fisher Scientific) according to the manufacturer's protocol. For ELISPOT assay, IFNγ responses were detected by mouse IFNγ ELISPOT kit (88-7384; Life Technologies, Thermo Fisher Scientific).
Life Technologies, Thermo Fisher Scientific). Briefly, 96-well Multiscreen-IP filter plates (Millipore) were pre-treated with 20 µl of 70% methanol for 30-60 sec then washed three times in PBS (Ca^{2+}/Mg^{2+}-free) followed by coating with 100 µl of capture antibody. The plates were incubated over night at 4°C. Excess coating antibody was removed and plates were washed 2 times with 200 µl of ELISA/ELISPOT coating buffer (Life Technologies, Thermo Fisher Scientific) and then were blocked in complete RPMI 1640 medium for 1 h at 37 °C before cells were added. Medium was removed from the plates and splenocytes in single cell suspension were added at 2×10^5 cells in duplicates to the wells, then the cells were stimulated by tetanus p2 and p30 peptides at a final concentration of 2 µg/ml. Wells containing complete RPMI 1640 medium and 10% DMSO (% as in peptide stimulated cultures) served as negative controls. Plates were incubated at 37°C for 72 hours then washed 3 times with ELISA/ELISPOT washing buffer. Subsequently, 100 µl of biotinylated detection antibody was added to each well. Plates were then incubated at room temperature for 2 h before washing 4 times with ELISA/ELISPOT washing buffer. Next, 100 µl Avidin-HRP was added per well and incubated at room temperature for 45 min. After washing plates 3 times with ELISA/ELISPOT washing buffer, 100 µL of freshly-prepared AEC substrate was added to each well. Plates were left at room temperature until spots developed (approximately 3–5 min) and then washed with water to stop the reaction. Plates were allowed to dry and then read in an ELISPOT plate reader (Immunospot, CTL Europe GmbH, Bonn, Germany). Spots were counted using AID software (Autoimmune Diagnostika, Germany). For analysis, the number of background spots (DMSO treated) was
subtracted from the number of spots detected in wells containing the p2 and p30 peptides.

**CD4+ T cells depletion and in vivo B-Cell proliferation and expansion assays**

Tetanus-specific CD4+ T cells were depleted by 3 injection of purified anti-mouse CD4 antibody (GK1.5; BioLegend, Germany). As described before (30), SW-HEL B-cells (unlabeled or labeled with CFSE) were transferred to recipient BL/6 mice (3–5 × 10^6 cells per mouse) by i.v. injection. On the same day, recipient mice were immunized with FrC-VLP-HEL containing FrC by i.v. injection at a final dose of 100 ng HEL per mouse. After 72 hours, splenocytes were isolated and stained with HEL-Alexa647 and B220 APC-eFluor780. CFSE fluorescence intensity of CFSE+ HEL+B220+ cells was determined by flow cytometry.

**Analyses of humoral immune responses**

Mice were bled by puncture of the retro orbital sinus with a heparinized 10 µl hematocrit capillary (Hirschmann Laborgerate, Eberstadt, Germany). The sera were obtained after 5 min centrifugation at 2600 × g in a tabletop centrifuge and stored at -20°C until further use. Antibody responses against HIV-1 Gag and HIV-1 Env were determined by Ag-specific ELISA as previously described (8). Briefly, 96-well high binding microtiter plates (Greiner Bio-One) were coated with 200 ng GST-Gag or 200 ng ConB gp120 in 0.1 mol bicarbonate buffer (pH 9.6) per well at room temperature 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. After washing, bound antibodies were incubated with HRP-conjugated anti-mouse antibodies (P0447; DakoCytomation, Glostrup, Denmark) and washed extensively. Bound HRP-conjugated antibodies were detected with an ECL solution composed of 5 ml Luminol solution (3-aminophtolhydrazide, Sigma-Aldrich 09253-25G), 50 µl solution B (p-coumaric acid; Sigma-Aldrich C9008-5G) in addition to 1.6 µl H$_2$O$_2$ 30% (Merck KGaA, Darmstadt, Germany) in an Orion-96 microplate reader (Berthold, Bad Wildbad, Germany). Humoral immune responses for Gag and Tetanus toxoid are expressed as log$_{10}$-transformed relative light units (RLU). To determine Env-specific IgG1, IgG2a, IgG2b, and IgG3 antibody concentrations, recombinant b12 antibodies containing the different Fc-regions were expressed in 293T cells and purified by Protein G Sepharose® 4 Fast Flow from (GE Healthcare Amersham, Darmstadt, Germany). After quantification of the concentration of the different b12 IgG subtypes by a sandwich ELISA with Affinipure Goat Anti-mouse IgG (Jackson ImmunoResearch, United States) as capture antibody and HRP-labelled, polyclonal rabbit anti-mouse sera recognizing IgG1, IgG2a, IgG2b, and IgG3 (5300-01B, SouthernBiotech, Birmingham, USA) as detecting antibodies, respectively, they were used as standards in the IgG subtype specific Env ELISAs. Bound IgG1, IgG2a, IgG2b or IgG3 antibodies were detected, after a washing step, with HRP-conjugated Abs specific for each of the IgG subtypes respectively (X56 and R19-15 from BD Biosciences, Heidelberg, Germany and SB74g and SB76b from SouthernBiotech, Birmingham, USA).
*FcγR activation assay*

Activation of each murine Fcγ receptor by the different immune sera was analyzed as described previously (9). Briefly, parental P815 cells and P815 Env cells expressing ConBgp140GC/D 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, either mFcγRII-CD3ζ or mFcγRIII-CD3ζ or mFcγRIV-CD3ζ transduced BW5147 reporter cells (9) were added in 100 µl culture medium, mixed, and the co-cultures were incubated at 37°C in a humidified 5% CO2 atmosphere. Eighteen hours later, 100 µl PBS supplemented with 10% FCS and 0.1% Tween-20 were added to each well and utterly mixed to favor the release of murine IL-2 from the BW5147 cells. After 15 min incubation at room temperature, the cells were pelleted and the IL-2 concentration in the supernatants were determined by ELISA with purified capture and biotinylated detection antibodies (BD Biosciences), as previously described (9).

*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 licenses: 84-02.2011.A111, 84-02.04.2012.A220 and 84-02.04.2013.A052. All mice were handled according to the instructions of the Federation of European Laboratory Animal Science Association.

*Statistical analysis*
Quantitative summary data are shown as means ± SEM. Statistical evaluation was performed with GraphPad Prism software version 6 using the unpaired t tests, one-way ANOVA with Tukey posttest, or the Kruskal–Wallis test with Dunn posttest, as indicated in the figure legends.

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VT and KÜ conceived and designed the studies. HE, GN, VT and KÜ designed the experiments. HE, VT and GN performed and analyzed the experiments. MT helped with experiments and contributed to discussions. AMS provided pICLC and contributed to discussions and to the interpretation of the results. WJM, KKK and JM produced recombinant Gag protein and contributed to the interpretation of the results. HE and VT prepared the figures and drafted the manuscript. HE, VT and KÜ wrote the final manuscript.
AMS is the CEO of Oncovir, Inc., which provided the poly ICLC for the current study. He was not involved in the acquisition and analysis of the data. All other authors declare no financial or commercial conflict of interest.

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Figure legends

Figure 1. Mechanism of intrastructural help
Env-specific B cells encountering HIV virions or VLPs take-up the entire particle in a BCR-dependent manner and present Env and Gag-derived T helper cell epitopes on their MHC-II molecules. Therefore, Gag-specific T helper cells can provide help to Env-specific B cells.

Figure 2. Intrastructural help by Gag immunogens

(A) Balb/c mice (n=4 per group) were immunized once with 1 µg Gag and the indicated µg dose of poly-ICLC (pICLC) or with 25 µg Gag DNA vaccine by i.m. DNA electroporation. Three weeks later, the antibody responses against Gag were analyzed at 1:500 serum dilutions. Shown the mean values with the SEM for logarithmically transformed values for Gag IgG1 and IgG2a. *p<0.05, **p<0.01, ***p<0.001 vaccine groups versus non-primed, One-way ANOVA with Tukey posttest. (B) Gag-specific CD4+ T cell responses were analyzed by intracellular cytokine staining for the indicated cytokines two weeks after a single i.m. injection of 1 µg Gag, 10 µg polyICLC (pICLC), the combination of Gag and pICLC, or a single i.m. electroporation of 25 µg of a Gag-DNA vaccine into Balb/c mice. Shown are the mean values with SEM of four animals per group (*p<0.05 versus PBS, Gag and pICLC for IFNγ; *p<0.05 versus PBS for IL-2; **p<0.001 versus PBS and pICLC for TNFα; #p<0.05 versus Gag for TNF-α; one-way ANOVA with Tukey posttest). (C) Balb/c mice (n=11-12 per group) were immunized on week 0 and 4 with 1 µg Gag or 10 µg pICLC alone or in combination or with the Gag-DNA vaccine. All primed and non-primed mice were boosted on week 8 and 12 with the same VLP preparation containing Env and Gag, naive sera were taken.
one week before the first immunization. (D) Antibody responses to Gag three weeks
after the second priming immunization at a serum dilution of 1:1000. Shown are the
mean values with SEM of 11–12 animals from two independent experiments. The
dotted line represents the background of naïve sera for Gag antibodies (For IgG1:
*p<0.05, versus non-primed; ****p<0.001 versus non-primed, Gag, plICLC and Gag-
DNA. For IgG2a: ****p<0.0001 versus non-primed, Gag and plICLC;***p<0.001 versus
Gag-DNA. One-way ANOVA with Tukey posttest). (E) Env-specific antibody responses
two weeks after the second VLP booster immunization. Shown are the mean values
with SEM for logarithmically transformed HIV Env antibody concentrations of 11–12
animals from two independent experiments (for IgG1: *p<0.05 versus plICLC. For IgG2a:
**p<0.01 versus non-primed, Gag and plICLC; ***p<0.001 versus Gag, plICLC. Kruskal–
Wallis test with Dunn’s posttest). The dotted lower and upper lines represent the
detection limit of the HIV Env-specific IgG2a and IgG1 antibodies levels, respectively.
(F) Env-specific IgG2a/IgG1 ratios two weeks after the second VLP immunization. The
bars represent the median of the ratios of all animals of each group that were positive
for both Env-specific IgG1 and IgG2a antibodies (open symbols). Samples that were
under the limit of detection for IgG2a and/or IgG1 are shown by closed black symbols.
*p<0.05, **p<0.01, Kruskal–Wallis test with Dunnett’s posttest; vaccine groups versus
non-primed.

Figure 3. Intrastructural help by tetanus-toxoid-specific T helper cells
(A) Map of Gag expression plasmids encoding wild type Gag, an FrC-Gag fusion protein, or an p2D1-Gag fusion protein. (B) Western blot analyses of wild type VLPs (wt-VLP) and FrC-VLPs with antibodies detecting the indicated proteins. X marks the position of lanes removed from the Western blots. (C-F) Env-specific antibody responses in (C) Balb/c mice primed twice with Tetanol and boosted once or twice with FrC-VLPs, (D) BL/6 mice primed twice with Tetanol and boosted once with FrC-VLPs, (E) Balb/c mice primed twice with Tetanol and boosted once or twice with wt-VLPs, and (F) BL/6 mice primed twice with Tetanol and boosted once with wt-VLPs. The dotted line represents the background observed in naïve animals. Shown are the means and the SEM of logarithmically transformed RLUs of (C, E) 4 animals per group at a 1:250 serum dilution, and of (D, F) 6 animals per group at a 1:100 serum dilution, (D) ***p<0.001; unpaired T-test.

Figure 4. Enhancement of the proliferative response of naïve antigen-specific B cells by intrastructural help

(A) BL/6 mice were primed s.c. twice over a four-week interval with 10 µL of Tetanol per animal. Ten days after the last Tetanol injection CD4 cells were depleted. Two weeks after the last Tetanol injection naïve B cells from SW-HEL mice were labelled with CFSE (B-G) and adoptively transferred into non-primed or Tetanol-primed mice with or without CD4 cell depletion. The acceptor-animals were injected i.v. with FrC-VLPs containing membrane-anchored HEL (HEL-VLP). Three days later, splenocytes of these mice and of untreated controls were stained with Alexa647-conjugated HEL and anti-
(B) Gating strategy for the analysis of in vivo proliferating CFSE⁺HEL⁺B220⁺ B cells. (B) Gating on lymphoid cells according to their forward and sideward scatter. (C) Elimination of doublets by FSC-A vs. FSC-H gating. (D) A dump channel was used to exclude auto-fluorescent cells among B220 positive cell singlets. (E) HEL and CFSE positive cells were selected. (F) Gating on proliferating CFSE⁺HEL⁺B220⁺ B cells. Representative data are shown. (G) The columns represent mean values of percent proliferating cells for each experimental group; values of individual mice are also shown. *** p<0.001 one-way ANOVA with Tukey’s posttest. (H) The columns represent expansion of HEL-specific B cells (percentage of HEL⁺B220⁺ B cells among the total B220⁺ B cell population) in non-primed and Tetanol-primed animals versus controls. **p<0.001, ***p<0.001, one-way ANOVA with Tukey’s posttest, vaccine groups vs control.

Figure 5. Characterization of p2D1-VLP

(A) Wild type VLPs (wt-VLP), FrC-VLPs, and p2D1-VLPs were analyzed by Western blot with an antiserum against gp120 of HIV Env (α Env), a monoclonal antibody against p24 of Gag (α Gag), and a monoclonal antibody against FrC and p2D1 (α TT). (B-D) p2D1-VLPs containing or lacking Env (p2D1-VLPΔEnv) were immunoprecipitated with 2G12 or mock-treated (Mock IP). Precipitates were analyzed by Western blots with the antibodies described in (A). Equal amounts of p2D1-VLPs that were used for the immunoprecipitation were analyzed directly by SDS-PAGE (Input). The additional bands

33
at 25 kDa in immunoprecipitation samples are due to protein G that was co-eluted from the beads.

Figure 6. Requirements for the modulation of the Env-specific IgG subtype response by intrastructural help

(A) Balb/c mice were immunized on week 0 and 4 with Tetanol or DNA vaccines expressing tetanus-toxoid or HIV Gag. All primed and non-primed mice were boosted on week 8 and 12 with p2D1-VLPs or wild type VLPs (wt-VLP). (B) Total Env-specific antibody concentrations in sera of mice primed with the indicated immunogens two weeks after the second immunization with the indicated VLPs. Shown are the mean values with SEM of logarithmically transformed HIV Env antibody concentrations in the sera of 12–18 animals per group from three independent experiments. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, Kruskal–Wallis test with Dunnett’s posttest, vaccine groups vs non-primed. (C-F) Env specific IgG1 (C), IgG2a (D), IgG2b (E) and IgG3 (F) subtypes two weeks after the second VLP immunization in animals boosted with p2D1-VLPs versus those boosted with wt-VLPs. Shown are the mean values with SEM of logarithmically transformed concentrations of Env-specific IgG subtypes in sera of 12–18 animals per group from three independent experiments. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, Kruskal–Wallis test with Dunnett’s posttest, vaccine groups vs non-primed. (B, D, E) The dotted lines represent the background of naïve sera. (C, F) The background of naïve sera lies on X-axis at value of 1.
Figure 7. Characterization of tetanus-toxoid-specific cellular immune responses

Balb/c mice were immunized i.m. once with by PBS, Tetanol, or TT-DNA. (A) TT-specific T cell responses 2 weeks after immunization as determined by an IFNγ ELISPOT assay with splenocytes stimulated with MHC II restricted p2 and p30 T helper peptides derived from TT. Shown are the mean values with SEM of spot forming cells (SFC) producing IFNγ per 200,000 splenocytes for 8 animals per group out of two-independent experiments. **p<0.01, one-way ANOVA with Tukey’s posttest vaccine groups vs PBS. (B) Percentage of splenic CD4+ T cells cell producing IFNγ after in vitro stimulation with p2 and p30 T helper peptides as measured by intracellular cytokine staining. Mice (6 per group) were sacrificed two weeks after immunization. The cells were stained for surface expression of CD4 and for intracellular expression of IFNγ. The background values of unstimulated cultures were subtracted. Shown are the mean values with SEM. *p<0.05, one-way ANOVA with Tukey’s posttest vaccine groups versus PBS. Tetanus-specific IL-4 (C) and IL-5 (D) cytokine secretion as determined by ELISA. Two weeks after immunization splenocytes were stimulated with p2 and p30 T helper peptides for 48 h. (C, D) The mean values with SEM of 10 animals out of two-independent experiments are shown. *p<0.05 Tetanol vs PBS and **p<0.01 Tetanol vs TT-DNA.

Figure 8. Fcγ receptor activation assays and durability of the humoral immune response
Balb/c mice (6 per group) were immunized twice on week 0 and 4 with Tetanol. Primed and non-primed mice were boosted on week 8 and 12 with p2D1-VLPs. (A-C) FcγR activation by Env-specific Abs from sera taken 2 weeks after the second VLP immunization using FcγRII (A) FcγRIII (B), or FcγRIV (C) reporter cell lines secreting IL-2. Background values from parental P815 cells not expressing Env were subtracted from the values generated with P815Env cells to obtain the Env-specific Fcγ receptor activation. Shown are the mean IL-2 concentrations with SEM for 6 immune sera per group at a 1:100 dilution. *p<0.05, Tetanol-primed vs non-primed, unpaired T-test. (D) Env IgG1 antibody levels were monitored for 10 weeks after the second VLP immunization. Shown are the mean values with SEM of logarithmically transformed RLU in the sera of 6 animals per group (*p<0.05, **p<0.01, Tetanol-primed vs non-primed, one-way ANOVA with Tukey’s posttest).
Figure A: IL2 (pg/ml) for FcγRII

- Non-primed Tetanol
- FcγRII

p = 0.36

Figure B: IL2 (pg/ml) for FcγRII

- Non-primed Tetanol
- FcγRII

p = 0.05

Figure C: IL2 (pg/ml) for FcγRII

- Non-primed Tetanol
- FcγRII

p = 0.11

Figure D: Env IgG1 (Log RLU) vs. weeks after 2nd VLP immunization

- Non-primed Tetanol
- Tetanol

wks after 2nd VLP immunization:
- 2
- 4
- 6
- 8
- 10

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