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HIV-1 Envelope Mimicry of Host Enzyme Kynureninase Does Not Disrupt Tryptophan Metabolism

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Abstract

The HIV-1 envelope protein (Env) has evolved to subvert the host immune system, hindering viral control by the host. The tryptophan metabolic enzyme kynureninase (KYNU) is mimicked by a portion of the HIV Env gp41 membrane proximal region (MPER) and is cross-reactive with the HIV broadly neutralizing antibody (bnAb) 2F5. Molecular mimicry of host proteins by pathogens can lead to autoimmune disease. Here, we demonstrate that neither the 2F5 bnAb nor HIV MPER-KYNU cross-reactive antibodies elicited by immunization with an MPER peptide-liposome

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vaccine in 2F5 bnAb V_HDJ_H and V_LJ_L knock-in mice and rhesus macaques modified KYNU activity or disrupted tissue tryptophan metabolism. Thus, molecular mimicry by HIV-1 Env that promotes the evasion of host anti-HIV-1 antibody responses can be directed towards non-functional host protein epitopes that do not impair host protein function. The 2F5 HIV Env gp41 region is therefore a key and safe target for HIV-1 vaccine development.

Introduction

HIV-1 infection remains a serious threat to health world-wide, and developing an effective HIV-1 vaccine is a global priority. A major challenge in achieving an effective vaccine is the inability of vaccines to induce broadly neutralizing antibodies (bnAbs) that recognize conserved epitopes on a majority of circulating HIV-1 strains (1-4). To date, bnAbs have not been elicited by vaccination, yet up to 50% of chronically individuals infected with HIV-1 develop bnAbs (5). Isolation of bnAbs from infected individuals has revealed six conserved sites of vulnerability on the HIV-1 envelope protein (Env): the gp120 CD4 binding site (CD4bs) (6-11); gp120 V1/V2 loop epitopes (12-14); gp120 V3-glycan epitopes (15-17); the gp41 membrane-proximal external region (MPER) (18-21), the gp41 fusion domain (22) and; the gp120-gp41 interface (23, 24). At high concentrations, passive administration of bnAbs that target these conserved epitopes (2F5, 4E10, 2G12, b12, VRC01, 3BNC117 or 10-1074) have prevented simian-human immunodeficiency virus (SHIV) infection in monkeys (25-29).

Molecular mimicry of antigenic determinants between pathogens and host molecules can result in tissue damage, such as the antibodies against a region of the hepatitis B virus that cross-react with myelin basic protein that can lead to tissue damage in the central nervous system (30-32). Similarly, *Campylobacter jejuni* lipo-oligosaccharides mimic host nervous system gangliosides and cause Guillain-Barre syndrome following infection (33, 34). Studies in mice have demonstrated that these host ganglioside antibodies are under tolerance control (33). Autoantibodies have also been shown to penetrate the blood brain barrier and affect intracellular enzyme function of neuronal cells during autoimmune disease (35). In retinopathy anti-enolase antibodies can alter the function of the enolase enzyme in neuronal cells (36). Furthermore, in multiple sclerosis (MS) anti-hnRNP antibodies were found to traffic into neurons and lead to apoptosis, contributing to MS pathology (37, 38).

HIV-1 bnAbs have unusual features such as extensive somatic hypermutation (SHM) and long third heavy chain complementarity regions (CDRH3s) that are associated with poly- or autoreactive B-cell antigen receptors (BCR)---all traits that can make antibodies subject to immune tolerance control (39-41). We previously demonstrated that many HIV-1 bnAbs are autoreactive and/or polyreactive and the MPER-reactive bnAb, 2F5, binds to the tryptophan metabolism enzyme kynureninase (KYNU) (42, 43). A second MPER bnAb, 4E10, cross-reacts with the RNA splicing factor 3b subunit 3 and also with anionic lipids. Because of the lipid reactivity, the 4E10 bnAb has anticoagulant activity and when administered to humans, it was biologically active and prolonged the partial thromboplastin time (44). Knock-in (KI) mice expressing the 2F5 or 4E10 bnAb $V_HD_HJ_H$ and V_LJ_L rearrangements exhibit profound

deletion of immature B cells in the bone marrow, demonstrating first-tolerance checkpoint control of these autoreactive, HIV-1 bnAbs (45-50).

KYNU is a phylogenetically conserved enzyme of tryptophan metabolism that contains the 2F5 core epitope (ELDKWA) within the KYNU H4 domain; the 2F5 bnAb binds this site with nM affinity (42). A key goal of HIV vaccine design is to induce gp41 MPER 2F5-like antibodies, yet the effects of MPER antibodies on KYNU function are unknown. Interestingly, disregulated tryptophan metabolism has been implicated in AIDS-related dementia, thus it is of critical importance to determine if MPER antibodies that cross-react with KYNU disrupt tryptophan metabolism (51).

Here, we have determined the effect of 2F5 and 2F5-like anti-HIV gp41 Abs on KYNU enzymatic activity using an MPER peptide-liposome vaccine that activates anergic B cells in 2F5 bnAb double KI ($V_HD_HJ_H + V_LJ_L$) mice. This vaccine also elicited MPER-specific Abs in rhesus macaques (50, 52). Thus, it is essential to determine if antibodies to the KYNU cross-reactive gp41 epitope inhibit KYNU or are deleterious *in vivo*. We demonstrate that immunization with MPER peptide-liposomes in 2F5 double KI mice and in rhesus macaques elicited KYNU-reactive, 2F5 epitope-targeted Abs, but that these Abs did not inhibit KYNU enzymatic activity *in vitro* nor perturb tryptophan metabolism or tissue pathology *in vivo*.

Materials and Methods

In vitro Kynureninase enzyme assay

Recombinant human kynureninase (rndsystems #4877-KH) and 10nM 3-hydroxy-DL-kynurenine (Sigma #H1771) substrate are incubated in assay buffer (50mM tris, 0.05% (w/v) Brij-35, 5μM pyridoxal phosphate, pH 8.0 in a F16 black maxisorp plate (Nunc, #475515). Fluorescence is read at excitation and emission wavelengths of 315nm and 415nm, respectively, in kinetic mode for 5 minutes by a fluorescent plate reader (SpectraMax Genini EM). Calibration standard 3-hydroxyanthranilinic acid (Sigma, #H9391) is used as a calibration control to calculate the conversion factor. Specific activity is calculated (Fig. S2) (42).

Recombinant antibodies are added at 0, 6, 60, 600, or 6000 ng and immune sera added at a 1:10 dilution to determine the inhibition or enhancement of KYNU enzyme activity compared with no inhibitor control.

Recombinant proteins and binding

Polyclonal anti-KYNU antibody was purchased (rndsystems #AF4887) and the V(D)J gene fragments of antibodies 2F5, CH65 were synthesized and cloned (GenScript) into plasmids containing human or rhesus IgG1/IgK/IgL constant regions. Recombinant mAbs were produced in 293 F cells (Life Technologies) by cotransfection with plasmids expressing the Ig heavy and light chain genes and were purified from the culture supernatant by protein A column chromatography (53, 54). Human KYNU and human KYNU with a single aspartic acid to glutamic acid mutation genes were synthesized with a C-terminal 6-histadine tag and cloned into pcDNA3.1 (GenScript). Plasmids were transfected into 293F cells and culture supernatants purified by nickel and size exclusion chromatography (55). Biotin-labeled SP62

(^{652}QQEKNEQELLELDKWASLWN^{671}) peptide and Biotin-labeled MPER656 (^{656}NEQELLELDKWASLWNWFNITNWLWYIK^{683}) peptide were synthesized (CPC scientific).

Recombinant and serum antibody binding was determined using ELISA as described (55, 56).

Mouse immunization

The mature 2F5 (m2F5 DKI) and germline 2F5 (gl2F5 DKI) double knock-in mice were generated on the C57BL/6 background previously described (46, 50). Mature 2F5 DKI mice were immunized six times with intraperitoneal injections (200μl) administered every 14 days in 3 groups of 4 mice each with the MPER peptide-liposome (25μg) formulated with GLA (0.625μg), Alhydrogel (Alum;12.5μg;QD565) or a combination. MPER peptide-liposomes contained a version of the 2F5 epitope-containing MPER peptide 656 (^{656}NEQELLELDKWASLWNWNITNWLWIK^{683}) that was synthesized with the C-terminal hydrophobic membrane anchor tag GTH1 (YKRWIILGLNKIVRMYS) previously described (57). All mice were 8 to 12 weeks old at the start of the immunization study and were housed in the Duke University Vivarium in a pathogen-free environment with 12-hour light/dark cycles at 20-25°C in accordance with all the Duke University Institutional Animal Care and Use Committee (IACUC)-approved animal protocols.

Mouse tissue processing, chemistry and pathology

Serum was collected and analyzed 10 days post immunization and animals were necropsied after completion of the study. Two groups of 6 (12 total) non-immunized aged-matched B6 control mice and unimmunized 2F5 mature (5 mice) and 2F5 germline (6 mice) double knock-in mice were necropsied as unimmunized controls.

Serum and brain tryptophan, kynurenine and kynurenic acid levels were performed on immunized and naïve mice as previously described (58).

Prepared stained tissue sections were delivered to a board certified veterinary pathologist for assessment at Duke University Department of Pathology. The tissues were fixed in 10% neutral buffered formalin, paraffin embedded, cut on microtome at 5 microns, and stained with hematoxylin and eosin.

Rhesus macaque immunization and pathology

23 healthy, adult Chinese-origin rhesus macaques were housed at BIOQUAL Inc (Rockville, MD) in accordance with the standards of the American Association for Accreditation of Laboratory Animal Care. The protocol was approved by BIOQUAL's Institutional Animal care and Use Committee under OLAW Assurance Number A-3086-01. BIOQUAL is IAAALAC accredited. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH) and with the recommendations of the Weatherall report; “The use of non-human primates in research”. All procedures were performed under anesthesia using ketamine hydrochloride, and all efforts were made to minimize stress, improve housing

conditions, and to provide enrichment opportunities (e.g., social housing when possible, objects to manipulate in cage, varied food supplements, foraging and task-oriented feeding methods, interaction with caregivers and research staff). Animals were euthanized by sodium pentobarbital injection in accordance with the recommendations of the panel on Euthanasia of the American Veterinary Medical Association.

Macaques were immunized (250μl × 2 sites) with MPER peptide-liposomes formulated with GLA (8 animals), GLA + Alum (8 animals) or Alum alone (7 animals) 6 times in 6 week intervals. Blood was collected preimmunization and 2 weeks after each immunization. Animals were necropsied at the completion of the study animals.

Throughout the immunization study all animals were clinically monitored. That included Complete Blood Count (CBC), serum chemistry and hematology that were performed by the attending veterinarian using commercial automated hematology and serum chemistry analyzers (BIOQUAL Inc.). Prepared stained splenic tissue sections were delivered to a board certified veterinary pathologist for assessment at Duke University Department of Pathology. The tissues were fixed in 10% neutral buffered formalin, paraffin embedded, cut on microtome at 5 microns, and stained with hematoxylin and eosin.

Isolation and characterization of MPER-reactive antibodies

MPER-specific memory B cells of a macaque immunized 3× with the MPER peptide-liposome vaccine formulated in GLA + Alum were sorted by flow cytometry as described (21, 50). Briefly, ~ 1×10^7 PBMCs were decorated with B cell antibody panel: CD14 (BV570), CD3 (PerCPCy5.5), CD20 (FITC), CD27 (APC-Cy7), and IgD (PE) (BD Biosciences) and Alexa Fluor 647 and Brilliant Violet 421–tagged MPER.03 peptides (KKKNEQELLELDKWASLWNWFDITNWLWYIRKKK). HIV gp41-specific memory B cells were gated as $CD3^-CD14^-CD20^+CD27^+sIgD^-MPER.03\ (AF647)^+$ $MPER.03\ (BV421)^+$ and sorted into 96-well PCR AQ22 plates containing 20 μl of reverse transcription reaction buffer that included 5 μl of 5× first-strand complementary DNA (cDNA) buffer, 1.25 μl of dithiothreitol, 0.5 μl of RNaseOUT (Life Technologies), 0.0625 μl of Igepal (Sigma-Aldrich), and 13.25 μl of ultrapure distilled water (Life Technologies).

Rhesus macaque V_HDJ_H and V_LJ_L segments were isolated by single-cell reverse transcription PCR (RT-PCR) using the method as described (54). The isolated V(D)J gene fragments were used for the construction of linear expression cassettes for production of recombinant mAbs in 293T cells for small scale ELISA screening (59).

Cloanalyst (Cloanalyst. http://www.bu.edu/computationalimmunology/research/software/) was used to annotate isolated V_HDJ_H and V_LJ_L sequences with immunogenetic information and to test for clonal lineage membership (50, 54).

Select V(D)J gene fragments were synthesized, expressed and purified as rhesus IgG1 recombinant mAbs described above.

Rhesus macaque serum and recombinant mAbs were screened by ELISA for binding to SP62 and select SP62 alanine mutants, recombinant KYNU and KYNU Mut (55).

Polyreactivity analysis of antibodies

The polyreactivity of rhesus mAbs was assessed with the AtheNA Multi-Lyte System (ZEUS Scientific) and HEp-2 cells immunofluorescence assay (Inverness Medical Professional Diagnostics) as described (50, 60).

Statistical analysis

Statistical analysis was performed in SAS version 9.4 (SAS Institute). All statistical tests performed were Wilcoxon-Mann-Whitney tests with Benjamini-Hochberg false discovery rate correction for multiple testing. GraphPad Prism version 6.01 was used for graphical representation.

Results

The 2F5 bnAb does not inhibit KYNU enzymatic activity

We previously identified that KYNU cross-reacted with the HIV-1 gp41 membrane-proximal targeting bnAb 2F5 (42). 2F5 binds to the HIV-1 gp41 peptide (^{652}QQEKNEQELLEL**D**KWASLWN^{671}), but does not bind this peptide when position 664 is mutated within the 2F5 epitope (^{652}QQEKNEQELLEL**A**KWASLWN^{671}**;** Fig. 1A). The KYNU H4 domain in most mammals (ELDKWA) exactly replicates this MPER epitope, and this motif is conserved within humans, mice and rhesus monkeys (42). Opossums, however, carry a rare substitution in the KYNU H4 EL**D**KWA motif where aspartic acid (D) is replaced by glutamic acid (E) (42). The same replacement in human recombinant KYNU (mutKYNU) completely abrogates binding by the 2F5 bNAb (Fig. 1A).

KYNU catabolizes 3-hydroxy-kynurenine into 3-hydroxyanthranilic acid. We used a standard *in vitro* enzymatic assay to measure the production of 3-hydroxyanthranilic acid at saturating substrate concentrations (42) (Fig S1A). Addition of recombinant KYNU or mutKYNU resulted in identically increased rates of 3-hydroxyanthranilic acid production, whereas addition of substrate alone or assay buffer did not (Fig. 1B). As a control for Ab-mediated enzyme inhibition, we added increasing concentrations of polyclonal anti-KYNU IgG Ab and demonstrated a concentration-dependent inhibition of 3-hydroxyanthranilic acid production (Fig. 1C). Increasing amounts of the CH65 influenza IgG Ab had no detectable effect on KYNU/mutKYNU enzyme activity (Fig. 1D & S1E).Both KYNU and mutKYNU were identically inhibited by KYNU IgG and neither were affected by the CH65 rIgG (Fig. S1B-E).

In contrast, at no concentration did the addition of 2F5 bNAb inhibit KYNU or mutKYNU enzymatic activity (Fig. 1E & 1F). Likewise, the 2F5 bnAb variable region inserted into a rhesus macaque IgG1 F_c backbone showed no activity on KYNU or mutKYNU enzymatic activity (Fig. S1D).

Vaccine-elicited gp41 bnAbs in mutated 2F5 knock-in mice do not inhibit KYNU

We immunized mice that express only the mutated (m)2F5 bnAb V_H +V_L termed m2F5 double KI mice with MPER peptide-liposomes formulated with alum, glucopyranosyl lipid adjuvant (GLA;TLR4 agonist), or a combination of alum and GLA to determine which

adjuvant elicited the highest titers of 2F5 bnAbs. After two immunizations, GLA and GLA + alum groups had induced antibodies that targeted the HIV gp41 MPER peptide (^{652}QQEKNEQELLELDKWASLWN^{671}) with ELISA Log AUC values of 4 and 6, respectively, while no animal in the alum alone group had titers of MPER-binding mAbs (Fig. 2A). Similarly, after two immunizations, GLA and GLA + alum immunized mice had titers of anti-KYNU antibodies (Fig. 2A). Ab to MPER peptide and KYNU protein was boosted with subsequent immunizations for the three adjuvant groups, but GLA or GLA + alum immunized mice maintained higher antibody titers compared to alum immunized mice (Fig. 2A). Ab to MPER and KYNU were strongly correlated with one another in all adjuvant groups demonstrating the relatedness of the two responses (Fig. 2B). At bleed 6, plasma antibody neutralization was observed against the HIV virus MN in the TZM-bl neutralization assay (Fig. 2C). The magnitude of neutralization titers corresponded with the HIV binding antibody titers with the GLA+Alum and GLA adjuvant groups having the highest neutralization titers, while only 1 animal in the Alum immunized group had ID50 titers greater than 50 (Fig. 2C).

We then examined the effects of plasma IgG Ab from immunized m2F5 double KI mice on KYNU enzymatic activity. No significant inhibition of KYNU enzymatic activity was observed for any of the three immunization groups, including the GLA and GLA + alum groups with the highest titers of vaccine-elicited KYNU IgG Abs (Fig. 2D). Indeed, there was no significant difference in the inhibitory activity between pre- and post-immunization plasma IgG samples. Thus, vaccine-elicited 2F5 bnAbs from m2F5 double KI mice had no effect on KYNU activity.

Induction of KYNU Ab has no deleterious effects on m2F5 double KI mice

Although we did not detect any inhibition of KYNU activity by the 2F5 bNAb or plasma IgG in immunized m2F5 double KI mice (Fig. 1E & 2D), we recovered tissue samples from naïve B/6 control mice, 2F5 double KI germline mice (gl2F5) that expressed the 2F5 unmutated ancestor (UA), m2F5 double KI mature mice and from immunized m2F5 double KI mature mice, and compared brain and serum levels of kynurenic acid, kynurenine, and tryptophan, the products of tryptophan metabolism upstream of KYNU (Fig. 3A). Ab that inhibited KYNU activity *in vivo* would result in increased levels of these metabolites. No significant differences in kynurenic acid, kynurenine or tryptophan levels in the brains of naïve or immunized mice was observed (Fig. 3B & S2A). No significant differences were found in serum kynurenic acid levels, but slightly higher levels of kynurenine and tryptophan were present in mice immunized with all forms of MPER peptide-liposome *versus* control B/6 mice (Fig. 3C & S2A). The elevations of kynurenine and tryptophan in immunized mice were not specifically caused by elicited KYNU Abs, since the alum-formulated liposome-immunized group with the lowest levels of KYNU Ab titers among the three immunization groups exhibited levels of serum kynurenine and tryptophan that were indistinguishable from those animals receiving the more potent GLA and GLA + alum immunogens (Fig. 3C).

Histopathology assessment of spleen tissue sections from m2F5 double KI mice were performed. Consistent with the deletional tolerance characteristic of m2F5 double KI mice (46), both naïve and immunized KI mice exhibited comparably low numbers of splenic B

lymphocytes when compared to B/6 controls resulting in small spleens and diminished white pulp areas (Fig. 3D). Furthermore, spleens were analyzed for vaccine-related lesions and no pathologic abnormalities to marginal zone, periarteriolar lymphoid sheath areas or red pulp were observed in vaccinated animals with high titers of KYNU cross-reactive antibodies (Fig. 3D & 3E). Immunized germline (gl) V_H + V_L 2F5 double KI mice without anti-KYNU antibodies also exhibited B cell deletion and tolerance control (50) and our analysis revealed that similar to the m2F5 KI mice, gl2F5 KI mice also had small spleens that were similarly lymphodepleted (Fig. S2B). Thus, the lymphodepletion seen in m2F5 and gl2F5 KI mice was due to deletion in bone marrow of greater than 95% of B cells due to immune tolerance.

We also performed histopathology of the brains from m2F5 double KI, gl2F5 double KI and B/6 control mice, and there was no evidence of neural degeneration, demyelination or inflammation in any of the brain sections studied (Fig. S2C).We concluded that despite the induced 2F5 bnAb levels and high titers of KYNU binding IgG Ab resulting from induced 2F5 bnAb levels elicited by immunizing m2F5 double KI mice with MPER peptide-liposome, did not perturb tryptophan metabolism in the brain or serum and induced no pathological changes in peripheral lymphoid tissues (Fig. 3).

Adjuvanted MPER peptide-liposome vaccine-elicited, non-pathogenic 2F5-epitope targeting, KYNU-reactive antibodies in rhesus macaques

Three groups of rhesus macaques (RMs) were immunized six times with MPER peptide-liposomes formulated with GLA, alum, or GLA + alum. In contrast to immunization of 2F5 knock-in mice, GLA alone was poorly immunogenic in RMs and elicited the lowest MPER IgG plasma Ab (Fig. 4A). In contrast, MPER liposomes formulated in alum or GLA + alum elicited higher titers of MPER plasma Ab (Fig. 4A & 4B). Immunization with alum or GLA + alum MPER peptide-liposomes also elicited higher titers of cross-reactive KYNU antibodies in macaques when compared to the GLA alone group (Fig. 4C). The 2F5 core epitope is the DKW motif within the HIV-gp41 MPER peptide SP62 (^{652}QQEKNEQELLEL**DKW**ASLWN^{671}), and plasma antibody binding to SP62 mutated peptides within the D, K or W was reduced for all three groups, and significantly reduced for the SP62 D664A mutant in the GLA and GLA + Alum groups (Fig. 4D; $P < 0.05$; Wilcoxon-Mann-Whitney Test). Thus, MPER peptide-liposome-induced plasma antibody was targeted near the 2F5 bnAb epitope in all RMs, with alum alone or GLA + alum adjuvants eliciting the highest titers of MPER and KYNU-reactive antibodies.

In all three adjuvant groups, the KYNU RM plasma IgG had no effect on KYNU enzyme activity *in vitro* (Fig. 5A). We analyzed immunized macaque spleen histopathology and found it was normal in all groups (Fig. 5B). These data confirmed that the splenic lymphopenia of naïve gl2F5 and vaccinated m2F5 double KI mice is the consequence of B cell deletion due to immune tolerance and not a pathologic consequence of anti-KYNU antibody production.

RMs blood levels for electrolytes, glucose, renal and liver function and complete blood counts denoted no changes related to MPER peptide-liposome vaccination (Fig. S3A & S3B).

Vaccine-elicited MPER recombinant MAbs in RMs targeted the 2F5 epitope

Gp41 Env-reactive Memory B cells ($CD3^{-}CD14^{-}CD20^{+}CD27^{+}sIgD^{-}$) from PBMCs from a RM immunized with MPER-liposome formulated in GLA and alum that were decorated with MPER peptide tetramers (MPER.03) labeled with two fluorophores were sorted for sequencing of the B cell receptor (BCR $V_H + V_L$) genes. MPER-reactive memory B cells represented 0.26% of total memory B cells (Fig. 6A). BCR genes were RT-PCR amplified, sequenced and fused to cassettes containing a CMV promoter, Ig constant region and polyA tail by overlapping PCR. Heavy and light chain amplicon pairs were transiently transfected into 293F cells and supernatants containing recombinant antibody were screened for binding by ELISA. Using this approach, 31 MPER-reactive antibodies were identified that bound clade B MN gp41 recombinant protein, 27 bound recombinant HIV-1 gp140 Env (Con-S or B. JRFL gp140; Fig S4A), and 6 MPER-reactive rAbs that in addition to the MPER, also bound human KYNU (Fig. 6B).

VDJ sequence analysis revealed that the isolated MPER antibodies used diverse V_H gene segments, and 3 of the 6 KYNU-cross-reactive Abs used a VH gene segment orthologous to the human V_H2-5 gene used in the 2F5 bNAb (Table 1; Fig. S4B) (61). The remaining KYNU binders carried rearrangements of V_H4 family gene segments (Table 1; Fig. S4B).

The 31 VDJ rearrangements recovered from memory B cells that bound the MPER.03 tetramer exhibited varying levels of somatic hypermutation (SHM) and diverged from the germline RM VH gene segments by 0.38% to 11.8% ; rearrangements from KYNU-reactive Abs exhibited a similar range of mutation frequencies, 1.1% to 5.2% (Table 1; Fig. S4C). Most MPER bnAbs have long third heavy chain complementarity regions (CDRH3) that are greater than 15 amino acids, and the RM antibodies had CDRH3 lengths ranging from 12 to as long as 21 amino acids (Table 1; Fig. S4D).

We selected four mAbs, based on gp41 and KYNU cross-reactivity, including 2 that used the RM V_H2-5 gene segment orthologue, for further study. All 4 mAbs bound to HIV-1 gp41 peptide (SP62; ^{652}QQEKNEQELL**ELDKW**ASLWN^{671}) and 3 of the 4 mAbs had greater than 60% reduction in binding to peptides containing alanine substitutions within the 2F5 bnAb DKW epitope (Fig. 6C). The mAb DH563 showed a more moderate 20-50% reduction on the DKW alanine mutants (Fig. 6C). Similarly, all 4 antibodies bound to KYNU, and had reduced binding to mutKYNU that contains EL**E**KWA instead wild-type KYNU, EL**D**KWA (Fig. 6D). None of the mAbs were capable of neutralizing HIV-1 in the TZM-bl pseudovirus inhibition assay (data not shown).

In addition to KYNU-cross-reactivity, 3 of the 4 antibodies also were cross-reactive with other host proteins associated with autoimmune disease; DH653 reacted with dsDNA, DH654 reacted with SSA, SSB and Jo1 proteins and DH656 reacted with Jo1 protein (Fig. 6E). Three of the four KYNU reactive Abs also exhibited antinuclear antibody phenotypes by reacting with nuclear antigens in HEp-2 cells; DH654 decorated cytoskeletal proteins, DH655 bound to nuclear punctae (dots), and DH656 diffusely bound within the cytoplasm (Fig. 6F). Even though these KYNU binding Abs exhibited reactivity for HEp-2 cell components, none of them were capable of inhibiting KYNU enzymatic activity *in vitro* (Fig. 7). Thus, adjuvanted MPER-liposome immunogens were capable of inducing a

polyclonal and polyreactive IgG Ab responses to MPER epitopes in RMs, but whereas these responses included anti-KYNU antibodies, they did not result in tissue pathology nor did they inhibit KYNU enzyme function.

Discussion

Mimicry of host proteins by microbial pathogens can subvert the host immune response (30-34, 62, 63). In this study, we have demonstrated that the HIV-1 bnAb 2F5 had no inhibitory effect on KYNU enzyme activity. Moreover, a single-residue change within the 2F5 epitope in KYNU that abolished 2F5 binding also did not effect KYNU enzyme function. The 2F5 epitope (ELDKWA) within KYNU is in the H4 domain that mediates KYNU homodimerization (64), likely explaining the inability of anti-ELDKWA antibodies to be able to inhibit KYNU activity.

B cell development in 2F5 V_HDJ_H and V_LJ_L knock-in mice was blocked by clonal deletion in bone marrow, because of host cross-reactivity with both lipids and KYNU resulting in bnAb immune tolerance control (46, 50). MPER bnAbs 2F5, 4E10 and 10E8 all interact with the virion membrane as a part of their epitopes (47, 65, 66). We have developed a minimal peptide-liposome immunogen that has been designed to present MPER bnAb epitopes in the same manner as on an HIV-1 virion (57). Immunization of 2F5 knock-in mice with the MPER peptide-liposome immunogen rescued anergic B cells that escaped deletion to produce high-levels of bnAbs (46). Immunization of macaques showed that the MPER peptide-liposome can initiate antibodies with 2F5-like characteristics (50).

In this study, bnAb 2F5 knock-in mouse plasma antibodies recognized KYNU, but like the human 2F5 monoclonal antibody, did not inhibit KYNU activity. Moreover, we examined brain and serum levels of tryptophan and its metabolites after MPER peptide-liposome immunization *in vivo* and did not detect any tryptophan pathway metabolite perturbations.

Immunization of rhesus macaques with MPER peptide-liposomes formulated in Alum or GLA + Alum elicited high-titers of antibodies that co-recognized the 2F5 epitope and KYNU. In macaques, plasma antibody inhibition of KYNU was not observed and no significant pathology was observed in macaque spleen or blood hematology or chemistry tests. Isolation of monoclonal antibodies by antigen-specific single-cell flow cytometry revealed that the 2F5-epitope (ELDKWA) in HIV-1 gp41 and KYNU could be recognized by antibodies that used diverse immunoglobulin gene segments, and these mAbs did not inhibit KYNU activity. We did not detect plasma HIV-1 neutralization in the TZM-bl assay for any of the vaccine-induced MPER-KYNU cross-reactive antibodies, indicating that further immunizations with affinity maturation, selecting antibodies with recognition of lipid, will be required to achieve neutralization activity (50).

In summary, host mimicry is a strategy utilized by HIV-1 to escape antibody responses to functionally conserved bnAb epitopes. Efforts to induce bnAbs by vaccination and to use bnAbs as therapeutic antibodies are critical to control of the AIDS epidemic. Thus, determining the impact of potentially protective antibodies on host protein function is an important safety consideration. Our study demonstrates that the 2F5 bnAb and vaccine-

elicited 2F5-epitope targeted antibodies elicited in mice and macaques that cross-reacted with the host enzyme KYNU, did not inhibit enzymatic function *in vitro* nor cause tissue or enzyme activity abnormalities *in vivo*. While cross-reactivity with host antigens may impede bnAb development and impact therapeutic antibody pharmacokinetics, the 2F5-KYNU interaction does not impair host enzyme function or mediate adverse events. Thus, the 2F5 HIV-1 Env gp41 region therefore appears to be a safe target on HIV-1 Env for vaccine development.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used in this article

KYNU	kynureninase
MPER	membrane-proximal external region
bnAb	broadly neutralizing antibody
SHM	somatic hypermutation
VH	variable heavy chain
VL	variable light chain
KI	knock-in
UA	Unmutated ancestor

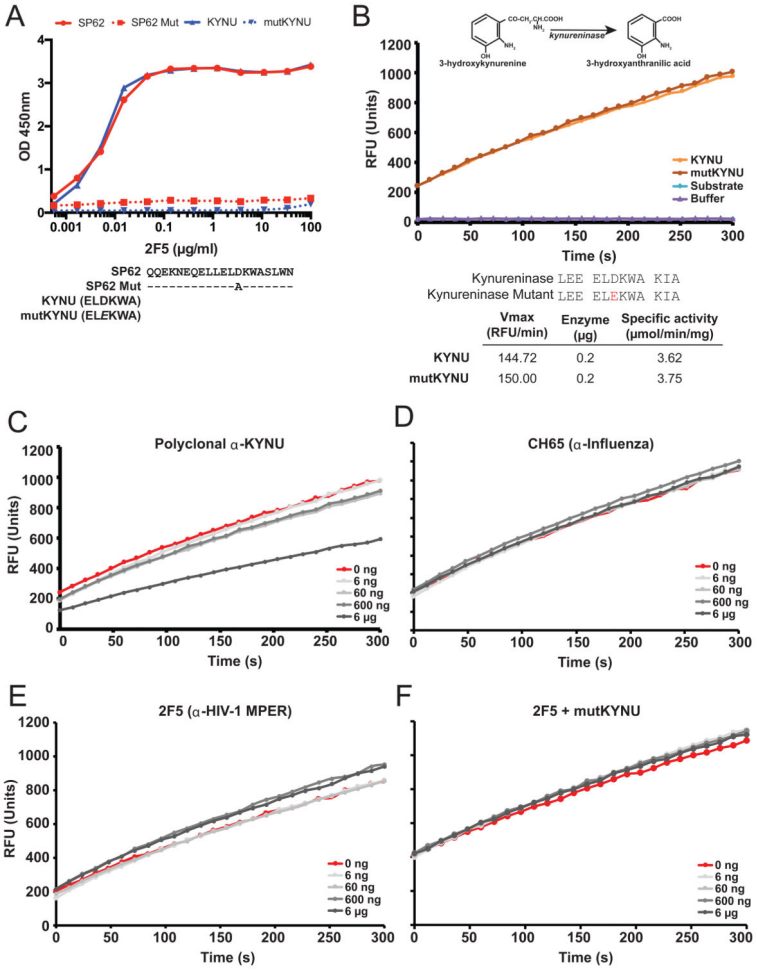

Figure 1. 2F5 does not inhibit KYNU enzymatic activity

(A) ELISA binding of 2F5 to the HIV-1 gp41 peptide SP62 that includes the 2F5 epitope (red), SP62 Mut (red dashed), KYNU (blue) and mutKYNU (blue dashed). (B) *In vitro* KYNU enzymatic assay measuring levels of 3-hydroxyanthranilic acid being converted by kynureninase from 3-hydroxykynurenine measured as RFU over time with the addition of KYNU, mutKYNU, substrate alone and buffer alone. Sequences of KYNU and mutKYNU that were tested in the *in vitro* KYNU enzymatic assay. Vmax and specific activity of wild-type KYNU and mutKYNU in the *in vitro* KYNU enzymatic assay using 0.2μg of each enzyme (C-F) KYNU enzymatic assay by adding increasing amounts of (C) polyclonal α-KYNU antibody (D) CH65 antibody (E) 2F5 antibody or (F) 2F5 antibody with mutKYNU enzyme.

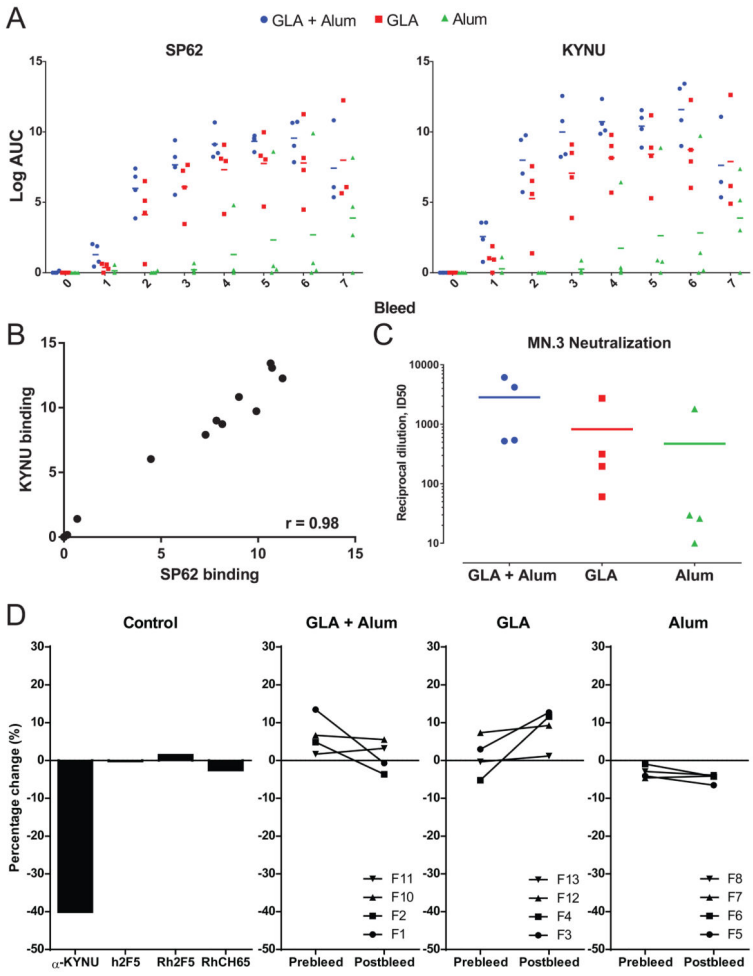

Figure 2. Immunization of m2F5 DKI mice with MPER peptide-liposomes

(A) Plasma antibody binding of SP62 and KYNU at pre-immunization (bleed 0) and after 7 immunizations (bleeds 1-7) with MPER peptide-liposomes formulated with Alum, GLA or GLA + Alum adjuvants measured by ELISA. Binding for each individual animal displayed, bar indicates group mean. (B) Plot of plasma antibody binding titers to SP62 and KYNU for each mouse in all adjuvant groups at bleed 6. Axis are Log AUC in ELISA. Spearman correlation shown (r = 0.98; $P < 0.0001$). (C) Plasma antibody HIV neutralization of MN virus measured in the TZM-bl neutralization assay at bleed 6 for mice immunized with MPER peptide-liposomes formulated with Alum, GLA or GLA + Alum adjuvants. (D) KYNU enzymatic assay indicating the percent change in KYNU activity for control antibodies (left panel; h = human antibody, Rh = rhesus macaque antibody) and mice from all three adjuvant groups pre and post 5th immunization (right three panels). Binding for each individual mouse displayed.

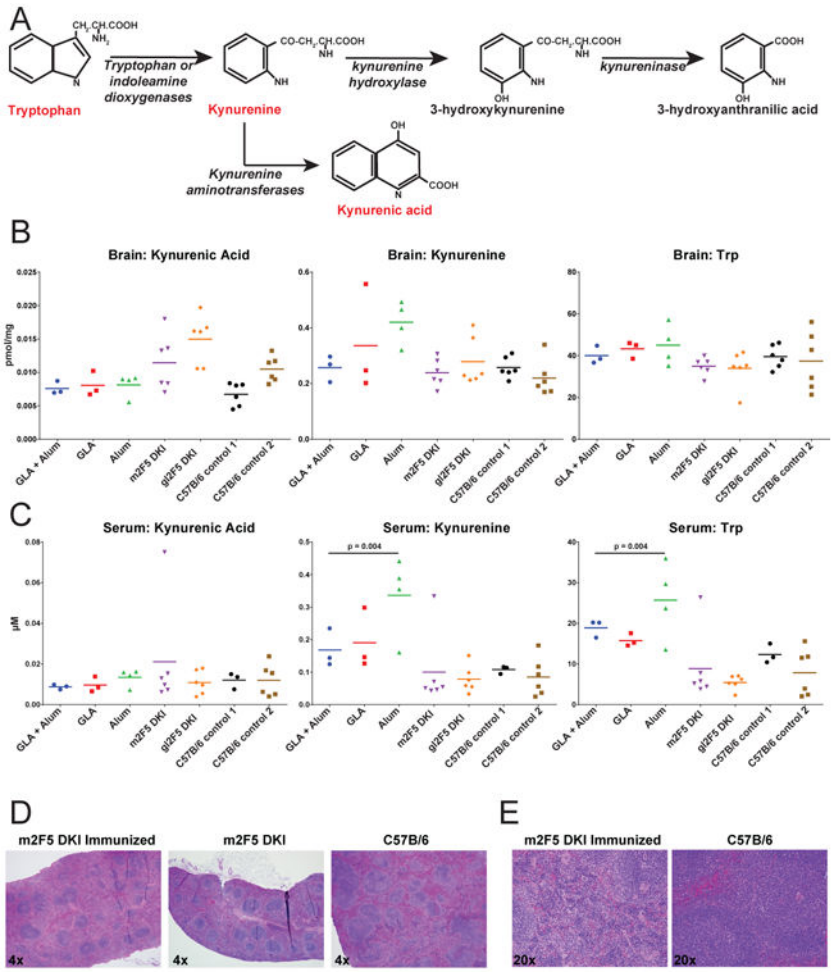

Figure 3. Tryptophan metabolites and spleen histology of 2F5 $V_HD_HJ_H$ + V_LJ_L knock-in mice

(A) The first 3 steps of tryptophan metabolic pathway with monitored metabolites highlighted in red. (B-C) Tryptophan metabolite levels in (B) brain and (C) serum of MPER peptide-liposome-immunized m2F5 DKI mice formulated with GLA + Alum, GLA or Alum, unimmunized 2F5 DKI mice with the mutated (m2F5 DKI) and germline 2F5 knocked-in (gl2F5 DKI) and two groups of C57B/6 control mice (*P*-values determined by Wilcoxon-Mann-Whitney). Levels of individual animals graphed. Line graphed at group mean. (D) HE stains at 4× magnification of spleens from m2F5 DKI MPER peptide-liposome-immunized mice, unimmunized m2F5 DKI mice and control C57B/6 mice. (E) HE stains at 20× magnification of spleens from m2F5 DKI immunized and C57B/6 mice.

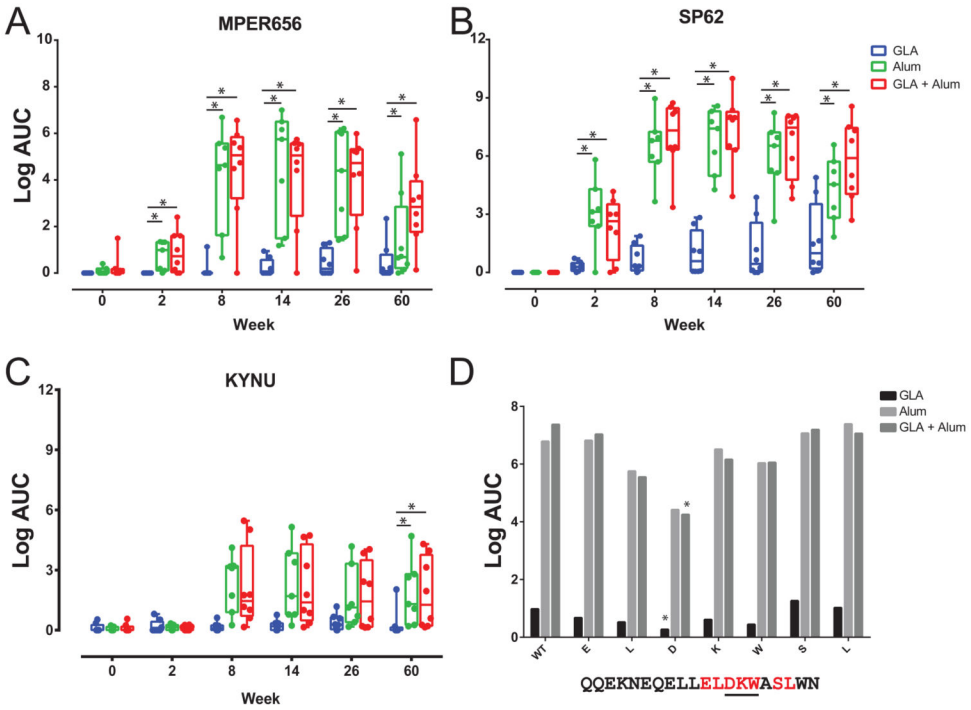

Figure 4. Adjuvanted MPER-peptide-liposome-immunized rhesus macaques elicits KYNU-reactive antibodies

(A-C) Plasma antibody levels measured by ELISA targeting (A) MPER656, (B) SP62 and (C) KYNU analyzed after immunization with MPER peptide-liposomes formulated with GLA, Alum and GLA + Alum adjuvants in rhesus macaques (*$P < 0.05$; Wilcoxon-Mann-Whitney). Animals were immunized at weeks 0, 6, 12, 18, 24, and 58. (D) Average plasma antibody binding at week 60 to wild-type SP62 and alanine mutated versions (red; core 2F5 epitope underlined) for all vaccine groups (*$P < 0.05$; Wilcoxon-Mann-Whitney).

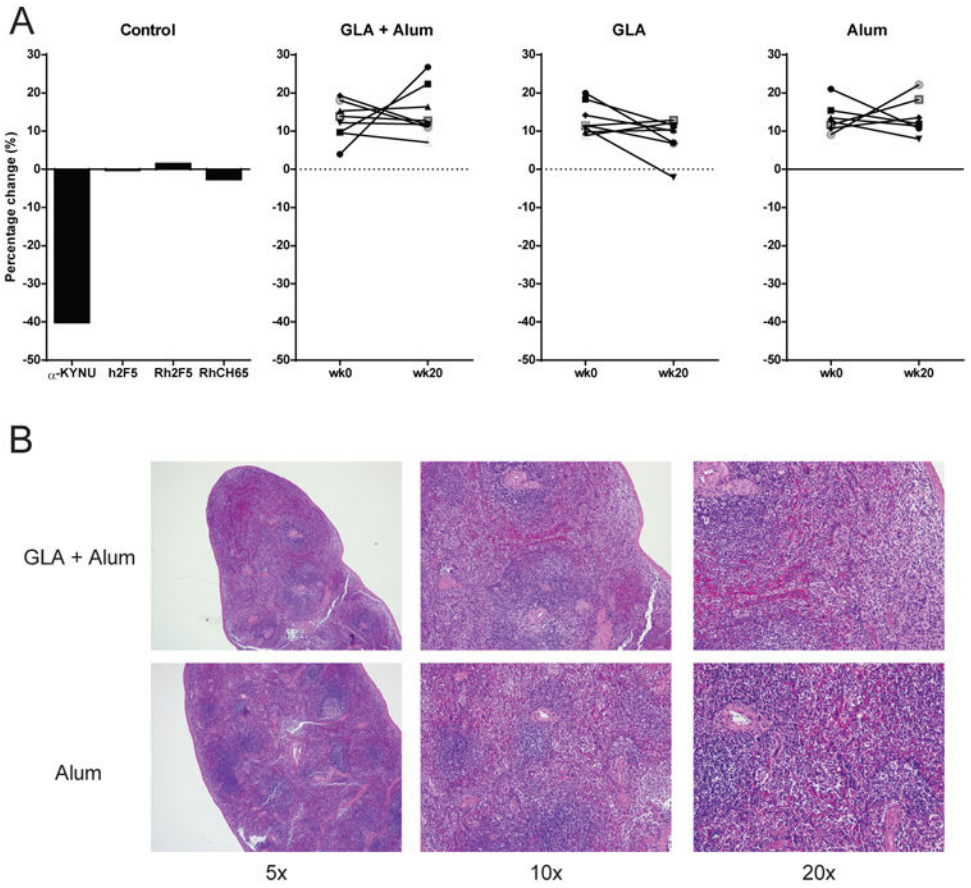

Figure 5. Adjuvanted MPER peptide-liposome-immunized rhesus macaques do not inhibit KYNU or display tissue abnormalities

(A) Percent inhibition of KYNU enzymatic activity measured by the *in vitro* KYNU enzyme assay for positive and negative control antibodies (left panel) and for plasma antibodies from rhesus macaques measured pre and post immunization with MPER peptide-liposomes formulated with GLA + Alum, GLA or Alum (right 3 panels). (B) HE stained spleens from MPER peptide-liposome formulated with GLA + Alum or Alum alone immunized macaques at 5×, 10× and 20× magnification.

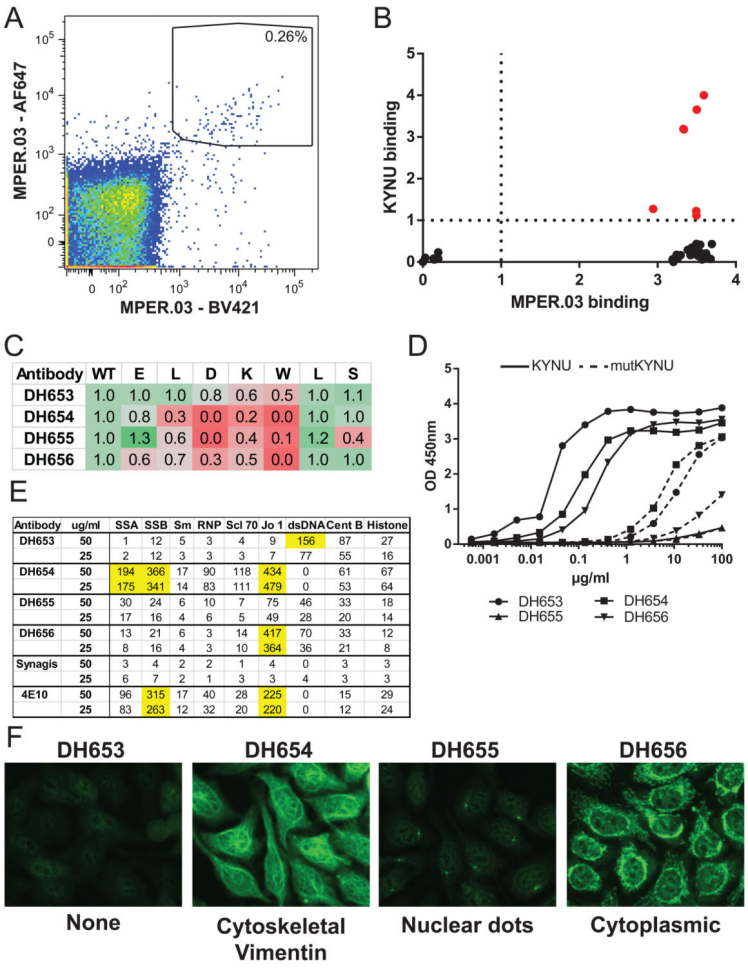

Figure 6. Isolation of MPER-targeting KYNU cross-reactive antibodies from MPER peptide-liposome-immunized rhesus macaques

(A) Memory B cells decorated with an MPER tetramer (MPER.03) conjugated with two fluorophores (AF647 and BV421) from PBMCs of an MPER peptide-liposome-immunized rhesus macaque 2-weeks after the third immunization. Single-cells were sorted into 96-plates for immunoglobulin gene amplification and sequencing shown within the black sort gate. (B) Individual wells with positive immunoglobulin sequences were amplified and transiently transfected for small-scale production of recombinant antibody and tested for MPER and KYNU binding by ELISA (O.D. at 450nm shown on X and Y axis). (C) Epitope mapping of MPER and KYNU reactive antibodies on the wild-type (WT) HIV-1 SP62 peptide and alanine mutants in the core 2F5 epitope (ELDKW) and 2 amino acids outside the 2F5 core epitope (LS). Values are ratio of binding of alanine mutants compared to WT. Red shading indicates decrease in binding. (D) Isolated antibody binding to KYNU (solid) and mutKYNU (dashed) measured by ELISA. (E) Autoreactivity of MPER antibodies isolated from rhesus macaques in the AtheNA autoantibody assay. Values >150 are positive and highlighted in yellow. Synagis and the MPER bnAb 4E10 were used as negative and positive controls, respectively. (F) Hep-C IFA staining of antibodies targeting the HIV-1 MPER and cross-reactive with KYNU. Antibody concentration was 50 µg/ml and exposed

time was 5 seconds. Original magnification 40×, representative section displayed. Cellular staining pattern for each antibody shown below.

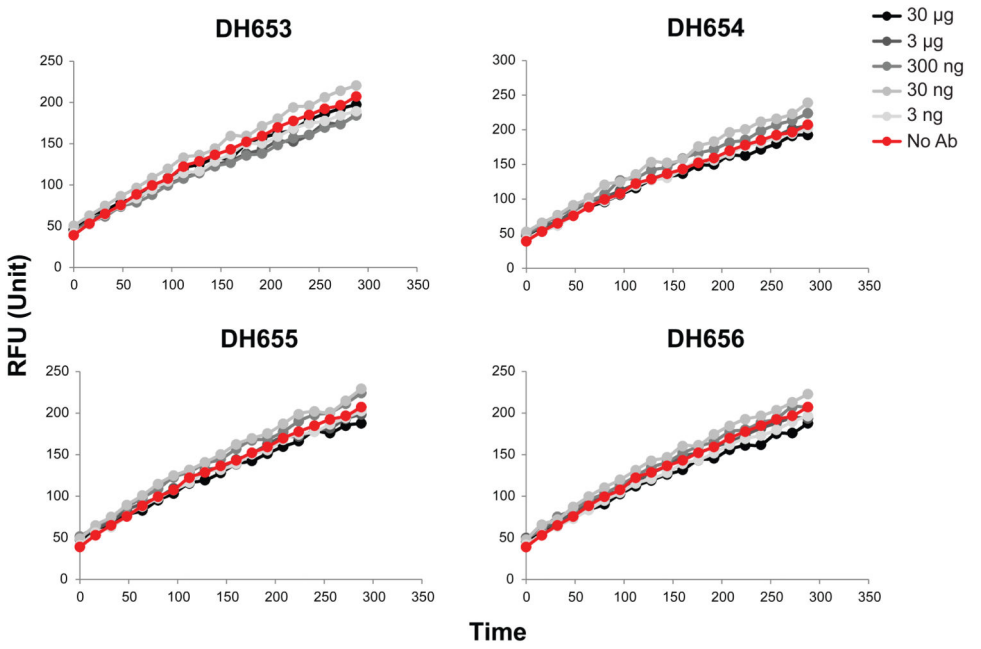

Figure 7. MPER and KYNU cross-reactive antibodies from MPER peptide-liposome immunized rhesus macaques do not inhibit KYNU enzymatic activity

KYNU enzyme activity over time with no Ab and increasing concentration of mAbs isolated from MPER peptide-liposome immunized rhesus macaques that cross-reacted with KYNU.

Table I

Immunogenetic characteristics of KYNU cross-reactive antibodies. DH653, DH654, DH655 and DH656 studied as purified mAbs. DH656.2 and DH673 identified by transient transfection

Antibody	VH (human)	HCDR3 (AA)	Mut. Freq (%)	VL (human)	LCDR3 (AA)	Mut. Freq (%)
DH653.2	2-A (2-5)	12	1.1	κ2-S10 (2-28)	9	8.7
DH653	2-A (2-5)	12	3.4	κ2-S10 (2-28)	9	7.5
DH656	2-A (2-5)	20	2.7	λ3-B (3-19)	11	2.7
DH655	4-H (4-39)	14	4.9	λ1-F (1-36)	11	1.1
DH654	4-L (4-4)	16	4.9	λ3-B (3-19)	11	2.7
DH673	4-L (4-4)	18	2.6	λ3-B (3-19)	11	4.6