Increased, durable B-cell and ADCC Responses Associated with T-helper Responses to HIV-1 Envelope in Macaques Vaccinated with gp140 Occluded at the CD4 Receptor Binding Site.

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Abstract

Strategies to improve the immunogenicity of HIV-1 envelope (Env) antigens for more long lived, efficacious HIV-1 vaccine induced B-cell responses. HIV-1 Env gp140 (native or un-cleaved molecules) or gp120 monomeric proteins elicit relatively poor B-cell responses which are short-lived. We hypothesized that Env engagement of the CD4 receptor on T-helper cells may result in anergic effects on T-cell recruitment and consequently a lack of strong robust and durable B-memory responses. To test this hypothesis we occluded the CD4 binding site (CD4bs) of gp140 by stable cross-linking with a 3kD CD4 miniprotein mimetic serving to block ligation of gp140 on CD4+T-cells while preserving CD4 inducible (CDi) neutralizing and epitopes targeted by antibody dependent cellular cytotoxic (ADCC) effector responses. Importantly immunization of rhesus macaques consistently gave superior B-cell ($p<0.001$) response kinetics and superior ADCC ($p<0.014$) in a group receiving the CD4bs-occluded vaccine compared to those animals immunized with gp140. Of the cytokines examined, Ag-specific IL-4 T-helper ELISpots in the CD4bs-occluded group increased earlier ($p=0.025$) during the inductive phase. Importantly CD4bs-occluded gp140 antigen not only induced superior B-cell and ADCC responses, the elevated B-cell responses proved to be remarkably durable lasting more than 60 weeks post-immunization.

Short title: Improved HIV vaccine B-cell responses using CD4bs occluded Env .

Importance

Attempts to develop HIV vaccines capable of inducing potent and durable B-cell responses have until now been unsuccessful. Antigen specific B-cell development and affinity maturation occurs in germinal centers in lymphoid follicles through a critical interaction between B-cells and T follicular helper cells. The HIV envelope binds the CD4 receptor on T-cells as soluble shed antigen or as antigen antibody complexes causing impairment in the activation of these specialized CD4 positive T-cells. We proposed that CD4-binding impairment may in part be responsible for the relatively poor B-cell responses to HIV envelope based vaccines. To test this hypothesis we blocked the CD4 binding site of the envelope antigen and compared it to currently used unblocked envelope protein. We found superior and durable B-cell responses in macaques vaccinated with an occluded CD4 binding site on the HIV envelope antigen, demonstrating a potentially important new direction in future design of new HIV vaccines.

Introduction

Antibody (Ab) responses directed to the HIV-1 envelope have been correlated with protection from viral infection; however, the ability to induce the B-cell responses necessary to generate long-lived protective antibodies by vaccination has proven difficult. Impressive protection from *in vivo* challenge has been achieved repeatedly using passive transfer of broadly neutralizing monoclonal antibodies (bnAb) and these approaches are now being advanced by multiple groups to clinical proof-of-concept. Such bnAbs have been cloned from memory B-cells from HIV-1 infected patients and sequence analysis has revealed substantial somatic hypermutation (SHM) from parental Ig germline (1) characteristic of high affinity maturation of antigen specific B-cells in germinal centers. CD4 positive T follicular helper cells (Tfh) play a fundamental role in Ab maturation by promoting Ig class switch recombination (CSR), SHM, B-cell selection and differentiation. A deeper understanding of these events may provide insights for improved HIV vaccine design.

The close interaction of activated Ag specific CD4 T-cells and MHC II B-cells within germinal centers is critical for optimal development of anti-HIV Ab responses. Priming of naïve CD4+ T-cells is initiated by MHC class II positive dendritic cells in lymph nodes to differentiate into Tfh cells prior to their migration to the T-B cell interfaces of germinal centers (2–4) (GC). This promotes their encounter with B-cells that share the same Ag specificity that reinforces their lineage commitment and coalescence, mutual activation and formation of GC. Importantly it is the intensity of the Tfh signal which is dictated by the quality and longevity of B cell interactions with molecules expressed on the surface of Tfh cells (5). Signals from Tfh are critical for differentiation of GC B cells into memory B cells and long-lived plasma cells and

their maturation of Ig affinity by CSR and SHM (6). Those B cells with the strongest Tfh cell interactions are those that become memory B cells or leave the GCs and differentiate into long-lived plasma cells (7) . Importantly the cytokines, IL-21, IL-6 and IL-4 play key roles in affinity maturation of Igs in B-cells. IL-21 which is central to Tfh development is augmented by IL-4 and together collaborate to promote Ig responses (8). Additionally, the tight regulatory program between Ag-specific B-cells in germinal centers is enhanced by the circulation and exchange of Tfh cells between B-cell rich germinal centers to ensure maximal diversification of CD4 T-cell help.

Factors which interfere with Tfh activation and collaboration with B-cell development have a negative response on maturation of Ig responses and ultimately on their effector function. Of viruses which cause persistent infection, HIV is unique in that it utilizes the CD4 receptor with a specific high affinity CD4 binding site (CD4bs) on the envelope subunit gp120. Through the CD4bs the HIV Env gp120 subunit can bind to the CD4 receptor in the absence of an intact infectious virion, either as a monomer or in its trimeric form (gp120 or gp140). Notably, HIV-1 and 2, and SIVs infect Tfh cells in GCs (9) and the Tfh population serves as the major T-cell compartment for HIV infection, replication, and production (10), ultimately contributing to the loss of CD4 T-cells and immune deficiency. However, very early in infection before numerical CD4 T-cell loss, HIV causes defects in CD4 T-cell and MHC class II APC cell function, defects which also affect B-cell responses infection. (11, 12)

A growing number of studies have confirmed that gp120 alone or immune complexed with antibodies are likely to decrease CD4 T cell function (13). HIV-1 replication is not only associated with virion-bound Env glycoprotein but with shedding of soluble gp120 or gp160 during replication in vivo (14,15). Soluble gp120

is found in plasma (16-18) and lymphoid tissues of HIV+ patients (19,20). This has been found to correlate with dysfunctional CD4+ T cell responses (18, 21, 22). A number of studies have demonstrated that gp120 binding to the CD4 receptor interferes with normal TCR-induced CD4+ T cell activation (19–21). The recent confirmation that gp120–immune complexes also engaged CD4 receptors and also prevent subsequent TCR-mediated activation of CD4+ T cells has raised concerns of over immunization with HIV envelope (13).

Here we set out to examine the hypothesis that in a vaccine setting, CD4 binding HIV-1 envelope immunogens could potentially be detrimental to achieving optimal vaccine induced B-cell responses. To study this, we compared an HIV-1 gp140 immunogen in which the binding to CD4 on the surface of T cell was abrogated by stable complexing of the antigen with a CD4 miniprotein mimetic that served to occlude the CDbs on the Env molecule. For this purpose we used scyllatoxin as a scaffold molecule from the scorpion, *Leiurus quinquestriatus* that mimics features of the CD4 reception which actually bind to the HIV Env glycoprotein (the β-hairpin of scyllatoxin can be superimposed on to positions 36 – 47 of the CDR2 loop of the CD4 molecule). Subsequent transfer of the side chains of the amino acids of CD4 on to their equivalent positions of scyllatoxin resulted in a minipeptide which specifically binds to the HIV-1 Env glycoprotein CD4 binding site (CD4bs) with affinity for HIV-1 equivalent to CD4 itself (22). Notably, structural data demonstrates that the M64U1 mimetic occludes the CD4 S-375 HIV-1 protein residue (23) which has been reported to enhance HIV-1 Env CD4 binding and virus replication in macaques (24).

Importantly our design provided both CD4bs occlusion blocking high affinity binding Env residues such as S-375, while allowing and preservation of CD4i nAb epitopes

(with putative ADCC epitopes), both which we have demonstrated in previous structural and small animal studies (22, 25, 26).

Since non-human primates have CD4 receptors on T-cells that are functionally and structurally very similar to human CD4, we studied this using 4 groups of 24 macaques to determine if vaccine induced B-cell responses and effector responses such as ADCC could be improved by preventing CD4 engagement. Furthermore, to determine if the effect was determined at the level of CD4 T cells, we enumerated antigen specific CD4 T cell subsets that secreted IFNγ, IL-2 or IL-4 to determine which of these subsets may correlate with the observed B-cell and/or ADCC responses.

153 **Materials and Methods.**

154

155 As a well characterised model HIV-1 immunogen that has been used in human and
156 non-human vaccine trials, we used the recombinant HIV-1 gp140 of the subclade B
157 SF162 (27, 28) from which pathogenic SHIV has been developed (29). To prevent
158 CD4 receptor engagement a mini CD4 peptide (M64U1-SH) was used to cross-link
159 the gp140dV2SF140, as described by Van Herrewege and Dereuddre-Bosquet (30,
160 31). Cross-linking of the mini-peptide mimicking the CD4 receptor binding site to
161 gp140 was described by Martin et al. (22) which effectively (Fig. 1; (23)) occludes
162 critical Env CD4bs sites such as residue 375 (32).

163

164 ***Animals and immunizations***

165 A total of 24 mature captive-bred male rhesus macaques (Macaca mulatta) were
166 housed at the Biomedical Primate Research Centre (BPRC), The Netherlands. The
167 rhesus macaques were negative for antibodies to SIV, simian type D retrovirus and
168 simian T-cell lymphotropic virus at the initiation of the study. The study protocol and
169 experimental procedures were approved by the institute's animal ethical care and use
170 committee and were performed in accordance with Dutch law and International
171 ethical and scientific standards and guidelines. Behaviour, discomfort, and appetite
172 were observed daily during the study by specially trained personnel. Body weight and
173 body temperature were measured before the start of the experiment and each time the
174 animals were sedated for immunization and/or blood sampling immediately following
175 sedation of each individual animal.

176 The study consisted of 24 animals divided into four groups of 6, randomized based on
177 age and weight. All groups received 0.5 ml of the MF59 adjuvant IM (upper leg) to

formulate the protein while the last group served as the adjuvant only control. Group 1 animals were immunized with gp140 with its CD4bs-blocked (gp140 CD4bs-x = 100 µg of $gp140_{\Delta V2SF162}$ with the CD4bs blocked by the mimetic M64U1-SH). Group 2 animals were immunized with the same but unblocked gp140 (100 µg of $gp140_{\Delta V2SF162}$), while group 3 received the mimetic M64U1 as a control, while group 4 was the adjuvant only control group (0.5 ml of the adjuvant MF59). All animals were immunized at weeks 0, 4, 24, 36. To assess the durability of vaccine-induced responses animals, immune responses were followed up to week 107 (71 weeks post 4^{th} Immunization (Fig. 2).

For immune assays serum, plasma and peripheral blood mononuclear cells (PBMC) were isolated from blood samples collected from sedated animals (ketamine hydrochloride anesthesia, 10mg/kg) at regular time intervals aseptically (Vacutainer, Becton Dickinson). To investigate possible adverse effects, body weight, rectal temperature, routine haematology and clinical chemistry were performed at regular intervals.

Humoral immune responses: Binding antibody titers and neutralization assay

Antibodies to HIV-1_{SF162} Env (gp140) in serum were measured by ELISA. Plates were coated overnight with Env in 100mM NaHCO3 and were blocked for 1 hour with 1% non-fat milk before application of serum, serially diluted in 1% BSA PBS buffer. After 1 hour 1µg/ml anti-human IgG-HRP conjugate was added for an additional hour before the addition of ultra-TMB ELISA development reagent. The reaction was stopped by addition of 0.5M H_2SO_4. Results were expressed as IgG endpoint dilution titers.

For the standardized and validated neutralization assays the TZM-bl cell line was used

(33, 34). It was obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH from Dr. John C. Kappes, Dr. Xiaoyun Wu and Tranzyme Inc. The HeLa cell line was engineered to express CD4 and CCR5 receptors. Following infection with SHIV pseudotyped virus the cells produce luciferase, the activity of which was detected by chemi-luminescence. Sera were diluted to give a 1 in 20 dilution and subsequently in a threefold series to a final dilution of 1 in 43,740. Each dilution was mixed with sufficient pseudovirus to give 500,000 counts per second in a Perkin-Elmer Victor 6016971 luminometer. The mixture included 15 μg/ml of DEAE and was then incubated for one hour before 10,000 TZM-bl cells were added. The cells were cultured for 48 hours, the supernatants removed and the cells lysed. The cell lysates were transferred to black / white plates, britelite reagent added and the luciferase activity quantified. Antibody titers are expressed as the dilution of serum required to reduce the luciferase activity in cultures exposed to pseudovirus alone by 50% (35–37). As a positive control for the detection of CD4i neutralizing antibodies, a modified neutralization assay using HIV-2_{7312A} pseudovirus was used as previously described (38).

B-cell Elispots

Antigen specific B-cell counts were performed as described by Crotty et al. (39). PBMC were plated in 48 well plates at $1x10^6$ cells/ml in complete medium (RPMI 1640 with L-Glutamine, P/S, HEPES buffer and 10% Fetal Bovine Serum) containing: PWM (Pokeweed Mitogen) at the dilution of 1:10,000, SAC (staphylococcus Aureus Cowan Strain –1) at the dilution of 1:10,000, β-mercapto-ethanol at the dilution of 1:000, 20U/ml IL2; IL4; IL5; IL6 and CpG oligonucleotide at a concentration of 5 μg/ml. Plates were incubated at 37ºC, 5% CO_2 for 5 days. To

enumerate Ag-specific B-cell Antibody Secreting Cells (ASC) or spot forming units (SFU), 96-well plates were coated with 50 µl/well $gp120_{SF162}$ Env antigen at 5 µg/ml final concentration. After 18 hours, plates were washed and blocked with 100 µl/well complete medium at 37ºC for 1-2 hours prior to use.

On day 6, the cells were washed thoroughly, plated onto the ELISPOT plates and incubated 37ºC 5% CO2 overnight. Plates were washed with phosphate buffered saline (PBS) followed by PBS containing 0.05% Tween-20 (PBST). Plates were then incubated overnight in 1 µg/ml Biotinylated-goat-anti-rhesus Ig (Hybridoma Reagent Laboratory) in PBST with 2% FCS. Plates were again washed and then developed using 5 µg/ml HRP-conjugated avidin dilution in PBST, incubate for 1-2 hours at 37º C. Plates were washed again and then developed using 3 amino-9 ethyl-carbazole (AEC, Sigma) and give spot formation. The reaction was stopped by washing the plates with tap water. Spots were counted using the AELVIS ELISPOT reader. Data are presented as number of Antibody Secreting Cells (ASC)/1x10^6 PBMC.

ADCC assays.

ADCC assays were performed as previously described by Pollara *et al*. (40), using $CEM.NKR_{CCR5}$ cells coated with recombinant HIV-1 SF162gp120 as target cells, and PBMC obtained from an HIV-seronegative donor as effector cells. The ADCC-mediating antibody titer was defined as the reciprocal of the highest dilution indicating a positive GzB response (>8% GzB activity) after background subtraction as previously described (40).

T-cell Elispots

Enumeration of antigen specific IFN-γ, IL-2 and IL-4 cytokines was measured using

an ELISpot assay as described by Koopman et al. (41). Separate peptide pools, consisting of 15mers with an 11 amino acid overlap, which covered the entire gp41 and gp120 of SF162 (NIH, AIDS reagent program) were used to measure antigen-specific immune responses after each immunization, during follow up and after challenge. Medium alone was used as negative control, whilst PMA (20ng/ml) plus ionomycin (1μg/ml) stimulation was used as positive control. In brief, $4x10^6$ cells/ml were stimulated in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS) in a 24-well tissue culture plate for 24 hours. For the enumeration of antigen specific cytokine production, non-adherent cells were collected and plated at $2x10^5$ cells/well in triplicates in a 96-well ELISpot plate with the same antigen. Microtiter plates were pre-coated with mAbs; anti-IFN-γ mAb (MD-1, U-Cytech, Utrecth, The Netherlands), anti-IL-4 mAb (QS-4, U-Cytech) and anti-IL-2 mAb (B-G5, Diaclone Laboratories, Besançon Cedex, France). Detection of the cytokine secreting cells took place after 15 hours for IL-4 and 4 hours for IFN-γ as well as IL-2. The cells were lysed and the debris was washed away before adding detector antibodies. IFN-γ, IL-2 and IL-4 were detected using biotinylated rabbit-anti-rhesus IL-2, biotinylated rabbit-anti-rhesus IFN-γ, or biotinylated mouse-anti-rhesus IL-4 (U-Cytech). Spots were visualized using streptavidin-HRP and an AEC (3-amino-9-ethylcarbazole) coloring system.

CD4 T-cell Proliferation inhibition assay

PBMC were incubated ON at 4 °C in RPMI with 10% FCS containing either SF162gp140 (CD4bs-open) (1 μg/ml/10^6 cells), SF162gp140CD4bs-occluded (1 μg/ml/10^6 cells) or no additions. Cells were subsequently harvested and labelled with CellTrace (CellTace Violet cell Proliferation Kit, Molecular Probes, Invitrogen,

Carlsbad, CA, USA, 20 min. incubation at 37 °C, 1 µl Cell Trace/ml/10^6 cells). Cells were then incubated for 72 hours on CD3 coated microwell plates (coated with 1 µg/ml CD3 clone SP34 (Becton & Dickinson), 2 hours incubation at 37 °C then 3 times wash with PBS) either without additions, with SF162gp140 (CD4bs-open) (1 µg/ml) or with SF162gp140CD4bs-occluded (1 µg/ml). Cells were then stained with $CD3^{APC}$ and $CD4^{PE\text{-}Cy7}$ and expression of Cell Trace Label was detected by FACS analysis.

Statistical analyses

The statistical significance of differences between responses induced by the different (CD4bs-occluded or open gp140) immunogens was determined by Dunnett's Multiple Comparison Test (one-way analysis of variance (ANOVAR)), Dunn's Multiple Comparison Test (non-parametric test) or Bonferroni post tests (two-way ANOVAR). For IFN-γ and IL-4 the area under the curve (AUC) was calculated for weeks 22-26, whereas week 22 data were used for IL-2 (week 26 data not available). Correlations between cytokine AUC, B-cell ELISpot and ADCC were then assessed using Spearman's Rho (Non-Parametric test). ELISpot AUC were calculated for the induction phase (weeks 22-38) and CD4bs-occluded or open gp140 immunized groups were compared non-parametrically (Mann-Whitney U test).

Results.

Immunisation kinetics of B-cell responses in Rhesus macaques immunized with HIV-1 Env occluded at the CD4 binding site.

We set out to test our hypothesis that CD4 binding HIV-1 envelope immunogens could potentially be detrimental to achieving optimal and durable vaccine induced B-cell responses. In addition to the structural evidence of occlusion the CD4bs on the Env protein (Fig. 1, (23)) we performed an exploratory FACS-based assay to confirm the inhibitory effect of SF162gp140 on rhesus CD4 T-cell proliferation in the presence of anti-CD3 in vitro (Fig. 3). This assay confirmed that gp140 inhibition of CD4 T-cell proliferation was abrogated by using the M48U1 CD4bs-occluded form of Env protein. The vaccine study was performed in 24 Rhesus macaques divided into the immunization-active and long-term follow-up or durability phase as indicated in Fig. 2. Animals were immunized four times over 40 weeks (Fig. 2A) and vaccine induced antigen specific B-cells in peripheral blood enumerated.

Throughout the immunization phase (Fig. 4A) of the study the gp140CD4bs-x (CD4bs-occluded) vaccine group developed significantly ($p < 0.001$) higher numbers of Ag-specific B-cells than the gp140 (CD4bs-open). Despite the expected individual variation in the number of circulating Env-specific B-cell ELISpots found, there were consistent and significant differences between the two Env vaccinated groups. Two weeks after the second immunization, these numbers were the highest in group 1 (gp140CD4bs-x) ranging between 250 and 1,865 ELISpots/10^6 PBMCs versus a lower range between 105 to 975 ELISpots/10^6 PBMCs for macaques immunized with gp140 alone. Both groups reached statistical significance with the gp140CD4bs-occluded group being consistently higher by the second immunization and increasing

further after the third immunization, reaching a peak plateau in the gp140CD4bs-occluded group, with a range from 3,630 to 11,823 ELISpots/10^6 PBMCs by week 26 (Fig. 4A). Macaques receiving gp140 (CD4bs-open) required a fourth immunization to reach a plateau, responses which were at a lower range; from 1,550 to 4,365 ELISpots/10^6 PBMCs by week 38 (Fig. 4A). The CD4bs-occluded group had significantly ($p < 0.001$) and consistently higher numbers of circulating Ag-specific B-cells than the CD4bs-open group throughout the entire immunization schedule. This demonstrated a significant and positive impact on the kinetics of priming and development of B-cell responses by simply altering the CD4bs on Env antigen to prevent CD4 receptor binding by Env vaccine antigen. A minor background response in the CD4 mimetic minipeptide and adjuvant control groups was detectable at only one time point (week 20) over the entire study).

Antibody titers to total Env are primed first and peak earliest in CD4bs-occluded Env immunized animals

During the active immunization phase of the study the early appearance of antibodies reflected the early appearance of Ag-specific B-cells in circulation in the CD4bs-occluded Env group compared to the CD4bs-open gp140 Ag (Fig. 5A). Total Env binding antibodies were detectable and higher within 6 weeks, two weeks after the second immunization in the CD4bs-occluded Env immunized group, already comparable to mean Env titers observed in the gp140 group after the third immunization (Fig. 5A). Titers again increased markedly after the third immunization, peaking at week 24 until a plateau was reached at week 40. Antibody titers in these immunized macaques were significantly ($p > 0.0001$ in a two-way ANOVAR) greater than those induced with gp140 (CD4bs-open) during the active immunization phase

(A), four weeks after the fourth immunization suggesting a positive impact of occluding the CD4bs of gp140 on priming and the magnitude of anti-Env titers reached during the immediate immunization period (Fig. 5A).

Neutralizing antibodies peak earlier and show activity against HIV-2 in the presence of soluble CD4.

To accurately assess neutralization of HIV-1 enveloped viruses in Rhesus macaques, we turned to *in vivo* Rhesus adapted SHIV viruses using the lentivirus pseudotype system to avoid differences in non-envelope encoded differences in their genomes. Importantly, early in the immunization protocol, CD4bs-blocked gp140 immunized animals induced the first neutralizing antibody response to the relatively homologous Tier 1 $SHIV_{SF162P4}$ observed at weeks 22 and 24 ($p < 0.001$ in one-way ANOVAR) (Fig. 6). Subsequently, neutralizing antibody titers increased after each immunization until the third immunization when slower developing neutralizing titers in the gp140 group had eventually caught up with the CD4bs-blocked group. By the third immunization (week 24) there was a boost in homologous neutralizing titers in the gp140 group that reached similar levels to the CD4bs-blocked group (Fig. 6). Given that globally, the Env neutralizing epitopes were otherwise identical in the CD4bs-blocked versus open gp140, this was not unexpected (with exception of fine specificity differences of CD4i epitopes caused by CD4bs-M48U1 cross-linking (42)). Due to the slower acquisition of heterologous neutralizing antibodies, later time-point sera collected at weeks 38 and 42 (2 or 6 weeks post 4^{th} Immunization) were measured for neutralization against clade B SHIV virus strains. In the pseudotype system these included Tier 1 $SHIV_{89.6}$, $SHIV_{W6.1D}$ and Tier 2 $SHIV_{SF162p3}$, as well as HIV-2.

Based on the observation that HIV-1 gp120 bound to CD4 gives a stable conformation that presents an increased affinity for the chemokine receptors and CD4i antibodies that also broadly neutralize HIV-2 (43–46). We used sCD4 in our assays to determine if CD4-inducible neutralizing antibody responses remained intact in the CD4bs-occluded gp140 group (38). The mean 50% neutralization titers against Tier 2 $SHIV_{SF162p3}$ were <20 (in the presence or absence of sCD4; Fig. 7). Against the $SHIV_{89.6}$ the mean titers were undetectable (without sCD4) and 1:125 (with sCD4) while against the Tier 1 $SHIV_{W6.1D}$ the mean titers were 1:355 (without sCD4) and even higher (with sCD4) in the gp140 immunized group. The mean titers in the CD4bs-blocked gp140 immunized group were <20 (both against the Tier 2 SF162p3 and 89.6 pseudo viruses), 1:259 against the Tier 1 W6.1D virus all without sCD4, while the mean titers in presence of sCD4 were <20, 1:135 and higher than 1:540 respectively. Of note, this gp140 was chosen for this proof-of-concept CD4bs-occlusion study because the protein was well characterised and used in many previous studies, not because of the broad-neutralising Ab epitopes it presented. It was also selected because of its neutralisation inducing potential for CD4i, one of the control features for the M48U1 occlusion. Importantly, we observed neutralizing responses against HIV-2 (Y720S) in the presence of sCD4 in the CD4-bound gp140 group, confirming that the CD4i epitopes were exposed and functional and induced by this immunogen (Fig. 7), and independently confirmed in Shen et al (42).

Superior Antibody Dependent Cellular Cytotoxicity (ADCC) responses elicited by immunization with CD4bs-occluded Env immunogen

"Non-neutralizing" antibodies are becoming recognized as important vaccine induced effector responses in protective HIV-1 immunity. Antibody dependent cellular

cytotoxicity (ADCC) responses have been correlated with slower disease progression (47–49) as well as vaccine efficacy (50–52). A high proportion of ADCC responses in patient sera are directed towards CD4i epitopes (53), those same epitopes we have preserved by stabilizing with our CD4bs-linked CD4 mimetic complex as we previously validated (22, 54). To determine if ADCC activity was induced by Env immunization with the CD4bs-occluded or open gp140, sera from immunized animals were assayed at weeks 0, 26 and after long-term follow-up. ADCC activity was measured as the serum titer for mediating Granzyme B release by PBMC upon incubation with target cells coated with SF162 gp120 protein. Group 1 immunized with the gp140 CD4bs-occluded had statistically superior ADCC responses compared to group 2 immunized with gp140 with the CD4bs open with a FDR_*p* value of 0.014 (Wilcoxon rank sum exact test *p* value controlled for false discovery rate (FDR) with the Benjamini-Hochberg method) at week 26 (Fig. 8).

Circulating antigen specific interleukin-4 (IL-4) CD4 T-cell responses increase early during immunization with CD4bs-occluded Env immunogen

Immunization with both HIV-1 Env immunogens induced T-cell ELISpots specific for peptides of the external HIV-1 envelope glycoprotein (gp120) that were detectable during the active phase of immunization (Fig. 9A) but which tapered off during the long-term follow-up period (Fig. 9D). These increases were observed with lymphocytes producing IL-4, IL-2 or IFN-γ and reached statistical significance relative to the 2 groups of control macaques (mimetic and adjuvant controls). These data were analysed in a two-way analysis of variance with all time-points included where data from all twenty-four macaques were available.

Importantly the earliest and most robust T-helper responses were observed in macaques immunized with gp140-CD4bs-occluded which induced the highest number of IL-4 producing ELISpots found at week 26 ($p=0.025$) (Fig. 9A) for both gp120 and gp41 sets of peptides (HIV-1 gp120, $p < 0.0001$(Fig 9A); HIV-1 gp41, $p < 0.0001$ (gp41 data not shown). The highest number of IL-2 producing ELISpots recognizing HIV-1 gp120 peptides was seen after three immunizations at week 34 ($p < 0.01$). The IFN-γ ELISpots peaked at week 38 (gp120, $p < 0.0001$; Fig. 9A). All six gp140 immunized macaques induced more than 50 IFN-γ producing lymphocytes per million PBMCs at week 38 while five of the macaques immunized with the CD4bs-occluded Ag induced this same level of ELISpot activity while four also had more than 50 IL-2 and IL-4 producing lymphocytes. While macaques immunized with gp140 only did not have increased numbers of HIV-1 gp41 specific, γ-interferon producing ELISpots overall, numbers were significantly increased, relative to control macaques, at weeks 38.

Immunizations at weeks 24 and 36 each produced an increase in the number of T-cell ELISpots recognizing either HIV-1 gp120 or gp41 peptides in both the gp140 alone and gp140 CD4bs-occluded groups (Fig. 9A; data not shown). This pattern was observed following each immunization with the exception of IL-2 ELISpots at week 36. Also with the exception of IL-2 at week 38, ELISpot numbers at weeks 26, and 38 were statistically significantly higher than controls at these time-points. Peak levels were found after four immunizations (week 38; γ-IFN, $p < 0.0001$ and IL-4, $p < 0.0001$). The gp140-CD4bs-occluded and gp140 groups induced statistically significant numbers of IL-4, but not IL-2 producing ELISpots recognizing peptides from the HIV-1 transmembrane envelope glycoprotein (gp41) (data not shown). The number of ELISpots responding to gp41 peptides were lower and delayed with their

highest levels seen after five immunizations for all three cytokines (data not shown). In summary, during the active immunisation phase (panel A Fig. 9), CD4bs-occluded gp140 immunisation induced earlier and more robust IL-4 (week 26 p=0.025), as well as trend for earlier IL-2 responses (p=0.065), suggesting a more vigorous and early recruitment of CD4+ T-helper cells during the inductive phase of the B-cell response (Fig. 4A).

Durability of B-cell responses

A key concern of HIV-1 vaccine development has been the very poor durability of HIV-1 vaccine induced responses, especially B-cell responses (52, 55–57). To address this, after an active immunization phase with 4 immunizations given over 40 weeks, we embarked on a long-term follow-up phase where animals were rested without sedation and protocol bleeds for approximately 70 weeks (1.5 years) to determine the durability of B and T-cell immune responses.

Importantly, while the plasma antibody titres gradually contracted after boosting, the superior numbers of Ag-specific B-cells in circulation were sustained in the CD4bs-occluded group throughout the long-term follow-up with a slight decline over the long 70 week period (Fig. 4 phase D). Despite these impressive and durable levels of Ag-specific B-cells during the active (Fig. 4 phase A), and the early higher titers and peak of total anti-Env antibodies in the CD4bs-occluded group, during the 70 weeks of observation in the durability phase (Fig. 5 phase D), total Env titers began to wane. The higher titer of total Env antibodies slowly decayed and after the 1 year endpoint (week 107) to levels similar to the gp140 “open” immunized animals (Fig. 5 phase D). This suggested that the global antibody response produced by the plasma cell pool that had accumulated during immunisation, had reached a maximum equilibrium

despite the impressive and sustained kinetics of the Ag-specific memory B-cells in circulation.

Control macaques immunized or with the CD4-mimetic or adjuvant alone had no specific antibody to gp140. Macaques immunized with gp140 CD4bs "open" produced low levels of antibodies that transiently cross-reacted with the CD4-mimetic peptide at week 26 (data not shown). Antibodies to the mimetic peptide were not detectable in other macaques.

Remarkably the long-term durability of Env-specific memory B-cells in circulation correlated with more robust ADCC responses ($p<0.001$, spearman 0.85) which were also found to be durable during the extended long-term follow-up of more than 70 weeks (Fig. 8).

Discussion.

This study set out to determine if rationale HIV-1 Env-antigen design could improve B-cell responses in primates. High affinity CD4 binding associated with HIV-1 Env residue 375 substitutions have been associated with increased virulence in macaques (32). We reasoned that if we could prevent CD4 binding by envelope antigens, yet preserve key CD4-inducible (CD4i) epitopes which were important for virus neutralization and rich in ADCC epitopes, that we could provide the basis for an improved HIV-Env antigen scaffold which could be suitably modified for future presentation of key broad neutralising epitopes, and ultimately deletion of dominant non-conserved antigen decoys. Our design criteria were two fold; CD4 binding site occlusion and preservation of CD4i Nab epitopes, both which we have demonstrated in previous structural and small animal studies (22, 25, 26). Our immunological

criteria required an *in vivo* primate CD4 T-cell system compatible with HIV-1 Env binding to study the *in vivo* inductive events in the presence of functional CD4 gp140 interaction *in vivo*. Our immunological criteria included; improved B-cell responses with respect to magnitude and durability, preservation of CD4i epitopes and induction of ADCC, and evidence for improvement of one of more of the antigen specific T-cell subsets (IFN-γ, IL-2 & IL-4).

When HIV-1 gp120 binds to CD4 it stabilize the virus envelope in a conformation that presents an increased affinity for the chemokine receptors and CD4i antibodies (43–46). The bound envelope glycoproteins offer therefore different targets both to induce and bind antibodies. Recent studies evaluating the evolution and specificities of broadly-neutralizing antibodies during HIV-1 infection (38, 58, 59) have provided important insights regarding the significance of CD4i antibodies and their potential role in vaccine against HIV-1. So far, recombinant monomeric gp120 or oligomeric/trimeric gp140 glycoproteins have failed to elicit broad and potent neutralizing antibodies in experimental animal models. Past studies based on gp120-CD4 (or CD4 mimic) complexes or constrained 'core' gp120 antigens have been evaluated as vaccine candidates, aiming at inducing CD4i antibodies (26, 60–63) Fouts et al. demonstrated that gp120 cross-linked to CD4 D1D2 domains raised antibodies that neutralized primary viruses regardless of co-receptor usage and genetic subtype in nonhuman primates (61). These findings were extended in a challenge study by DeVico et al. (60) where macaques immunized with a single chain complex containing gp120BaL-rhesus macaque CD4 D1D2 showed improved CD4i antibody response that correlated with the control of infection when challenged with $SHIV_{SF162P3}$. Although this correlation did not prove that efficacy was mediated by neutralizing CD4i antibodies, it demonstrated that the presence of CD4i Abs was

524 dependent on the CD4- bound conformation of HIV-1 envelope *in vivo*. These studies
525 demonstrated the potential importance of strategies directed to raising antibodies
526 against the CD4i site. Recently we used a practical approach of eliciting CD4i
527 epitope-directed virus neutralizing antibodies using a stably cross-linked complex of
528 recombinant oligomeric gp140 and miniCD4 (M64U1-SH) (22, 31) to target the
529 conserved co-receptor binding site of the HIV-1 Env. In those studies, two CD4
530 mimetic mini proteins (miniCD4) were cross-linked to various forms of HIV-1 Env
531 (M64U1-SH). Based on results from those studies, the M64U1-SH miniCD4 was
532 selected for generating the cross-linked gp140-miniCD4 complex.
533 Two important under appreciated issues were addressed in this study. First was the
534 observation that binding or cross-linking of the CD4 molecule of T-helper cells causes
535 functional impairment (11, 12, 64–66) and within germinal centres. The hypothesis
536 was that CD4 binding by antigen would impair critical interactions between Ag
537 specific CD4 Tfh cells and MHC II B-cells which are fundamentally important in
538 generating memory B-cell responses and functionally important antibody effector
539 responses such as neutralising (n)Ab and ADCC. The second was that CD4i epitope
540 regions are also rich in ADCC epitopes (53), thus stabilizing their presentation would
541 promote such ADCC in naive vaccinated individuals. Importantly, in this study, by
542 simple cross-linking of the small CD4 receptor mimetic to the CD4 binding site of
543 gp140 we have been able to demonstrate; 1) preservation of CD4i nAb, 2) improved
544 and long-term durable B-cell responses, 3) early induction of anti-HIV-1 binding and
545 nAbs, and 4) ADCC. The early and robust CD4 T-helper responses characterized by
546 IL-4 secretion correlated with the early induction of B-cell and antibody responses,
547 suggesting that preventing CD4 binding of the Env antigen in B-cell inductive sites
548 was an underlying and important feature of this antigen modification. These findings

beg mechanistic follow-up studies to prove this hypothesis and to understand the in vivo half-life and kinetics of gp140 in the CD4bs-occluded, M48U1-complexed versus the unbound forms, in lymph nodes draining vaccine injection sites. Most notability the induced B-cell responses were durable for more than 1.5 years post-immunization, representing a major advance in a key area of HIV vaccine development. Future modifications to further improve Env antigen structures with additional modifications to better present and recruit key bnAb and ADCC epitope rich regions are likely to ultimately contribute to more highly effective HIV-1 vaccines.

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References

1. **Scheid JF, Mouquet H, Ueberheide B, Diskin R, Klein F, Oliveira TY, Pietzsch J, Fenyo D, Abadir A, Velinzon K, Hurley A, Myung S, Boulad F, Poignard P, Burton DR, Pereyra F, Ho DD, Walker BD, Seaman MS, Bjorkman PJ, Chait BT, Nussenzweig MC.** 2011. Sequence and structural convergence of broad and potent HIV antibodies that mimic CD4 binding. Science **333**:1633–1637. https://doi:10.1126/science.1207227.
2. **Vinuesa CG, Linterman MA, Yu D, MacLennan IC**. 2016. Follicular Helper T Cells. Annu. Rev. Immunol. **34**:335-368. https://doi:10.1146/annurev-immunol-041015-055605.
3. **Zhang Y, Garcia-Ibanez L, Toellner KM.** 2016. Regulation of germinal center B-cell differentiation. Immunol. Rev. **270**:8–19. https://doi:10.1111/imr.12396.
4. **Butler NS, Kulu DI.** 2015. The regulation of T follicular helper responses during infection. Curr. Opin. Immunol. **34**:68–74. https://doi:10.1016/j.coi.2015.02.007.
5. **Kageyama R, Cannons JL, Zhao F, Yusuf I, Lao C, Locci M, Schwartzberg PL, Crotty S**. 2012. The receptor Ly108 functions as a SAP adaptor-dependent on-off switch for T cell help to B cells and NKT cell development. Immunity **36**:986–1002. https://doi:10.1016/j.immuni.2012.05.016.
6. **Victora GD, Nussenzweig MC.** 2012. Germinal centers. Annu. Rev. Immunol. **30**:429–457. https://doi:10.1146/annurev-immunol-020711-075032.
7. **Schwickert TA, Victora GD, Fooksman DR, Kamphorst AO, Mugnier MR, Gitlin AD, Dustin ML, Nussenzweig MC.** 2011. A dynamic T cell-limited checkpoint regulates affinity-dependent B cell entry into the germinal center. J. Exp. Med. **208:**1243–1252. https://doi:10.1084/jem.20102477.
8. **Zotos D, Coquet JM, Zhang Y, Light A, D'Costa K, Kallies A, Corcoran LM, Godfrey DI, Toellner KM, Smyth MJ, Nutt SL, Tarlinton DM.** 2010. IL-21 regulates germinal center B cell differentiation and proliferation through a B cell-intrinsic mechanism. J. Exp. Med. **207**:365–378. https://doi:10.1084/jem.20091777.
9. **Petrovas C, Yamamoto T, Gerner MY, Boswell KL, Wloka K, Smith EC, Ambrozak DR, Sandler NG, Timmer KJ, Sun X, Pan L, Poholek A, Rao SS, Brenchley JM, Alam SM, Tomaras GD, Roederer M, Douek DC, Seder RA, Germain RN, Haddad EK, Koup RA.** 2012. CD4 T follicular helper cell dynamics during SIV infection. J. Clin. Invest. **122:**3281–3294. https://doi:10.1172/JCI63039.
10. **Perreau M, Savoye AL, De Crignis E, Corpataux JM, Cubas R, Haddad EK, De Leval L, Graziosi C, Pantaleo G.** 2013. Follicular helper T cells serve as the major CD4 T cell compartment for HIV-1 infection, replication, and production. J. Exp. Med. **210:**143–156. https://doi:10.1084/jem.20121932.

11. **Heeney JL.** 2002. The critical role of CD4(+) T-cell help in immunity to HIV. Vaccine **20**:1961–1963.
12. **Heeney JL.** 1995. AIDS: a disease of impaired Th-cell renewal? Immunol. Today **16**:515–520.
13. **Zimmermann K, Liechti T, Haas A, Rehr M, Trkola A, Günthard HF, Oxenius A.** 2015. The orientation of HIV-1 gp120 binding to the CD4 receptor differentially modulates CD4+ T cell activation. J. Immunol. **194:**637–649. https://doi:10.4049/jimmunol.1401863.
14. **Pyle SW, Bess JW Jr, Robey WG, Fischinger PJ, Gilden RV, Arthur LO.** 1987. Purification of 120,000 dalton envelope glycoprotein from culture fluids of human immunodeficiency virus (HIV)-infected H9 cells. AIDS Res. Hum. Retroviruses **3:**387–400.
15. **Oh SK, Cruikshank WW, Raina J, Blanchard GC, Adler WH, Walker J, Kornfeld H.** 1992. Identification of HIV-1 envelope glycoprotein in the serum of AIDS and ARC patients. J. Acquir. Immune. Defic. Syndr. **5**:251–256.
16. **Popovic M, Tenner-Racz K, Pelser C, Stellbrink HJ, van Lunzen J, Lewis G, Kalyanaraman VS, Gallo RC, Racz P.** 2005. Persistence of HIV-1 structural proteins and glycoproteins in lymph nodes of patients under highly active antiretroviral therapy. Proc. Natl. Acad. Sci. U.S.A. **102:**14807–14812. https://doi:10.1073/pnas.0506857102.
17. **Gay D, Maddon P, Sekaly R, Talle MA, Godfrey M, Long E, Goldstein G, Chess L, Axel R, Kappler J, Marrack P.** 1987. Functional interaction between human T-cell protein CD4 and the major histocompatibility complex HLA-DR antigen. Nature **328:**626–629. https://doi:10.1038/328626a0.
18. **Veillette A, Bookman MA, Horak EM, Samelson LE, Bolen JB.** 1989. Signal transduction through the CD4 receptor involves the activation of the internal membrane tyrosine-protein kinase p56lck. Nature **338:**257–259. https://doi:10.1038/338257a0.
19. **Chirmule N, McCloskey TW, Hu R, Kalyanaraman VS, Pahwa S.** 1995. HIV gp120 inhibits T cell activation by interfering with expression of costimulatory molecules CD40 ligand and CD80 (B71). J. Immunol. **155:**917–924.
20. **Marschner S, Hünig T, Cambier JC, Finkel TH.** 2002. Ligation of human CD4 interferes with antigen-induced activation of primary T cells. Immunol. Lett. **82:**131–139.
21. **Rosenstein Y, Burakoff SJ, Hermann SH.** 1990. HIV-gp120 can block CD4-class II MHC-mediated adhesion. J. Immunol. **144:**526–531.
22. **Martin G, Burke B, Thai R, Dey AK, Combes O, Ramos OH, Heyd B, Geonnotti AR, Montefiori DC, Kan E, Lian Y, Sun Y, Abache T, Ulmer JB, Madaoui H, Guerois R, Barnett SW, Srivastava IK, Kessler P, Martin L.** 2011. Stabilization of HIV-1 envelope in the CD4-bound conformation through specific cross-linking of a CD4 mimetic. J. Biol. Chem. **286:**21706–21716. https://doi:10.1074/jbc.M111.232272.

23. **Acharya P, Luongo TS, Louder MK, McKee K, Yang Y, Do Kwon Y, Mascola JR, Kessler P, Martin L, Kwong PD.** 2013. Structural Basis for Highly Effective HIV-1 Neutralization by CD4-Mimetic Miniproteins Revealed by 1.5 Å Cocrystal Structure of gp120 and M48U1. Structure **21:**1018–1029. https://doi:10.1016/j.str.2013.04.015.

24. **Li H, Wang S, Kong R, Ding W, Lee F-H, Parker Z, Kim E, Learn GH, Hahn P, Policicchio B, Brocca-Cofano E, Deleage C, Hao X, Chuang G-Y, Gorman J, Gardner M, Lewis MG, Hatziioannou T, Santra S, Apetrei C, Pandrea I, Alam SM, Liao H-X, Shen X, Tomaras GD, Farzan M, Chertova E, Keele BF, Estes JD, Lifson JD, Doms RW, Montefiori DC, Haynes BF, Sodroski JG, Kwong PD, Hahn BH, Shaw GM.** 2016. Envelope residue 375 substitutions in simian-human immunodeficiency viruses enhance CD4 binding and replication in rhesus macaques. Proc. Natl. Acad. Sci. U.S.A. **113:**E3413-22. https://doi:10.1073/pnas.1606636113.

25. **Dey AK, Burke B, Sun Y, Hartog K, Heeney JL, Montefiori D, Srivastava IK, Barnett SW.** 2012. Use of a polyanionic carbomer, Carbopol971P, in combination with MF59, improves antibody responses to HIV-1 envelope glycoprotein. Vaccine **30:**2749–2759. https://doi:10.1016/j.vaccine.2012.02.027.

26. **Dey B, Svehla K, Xu L, Wycuff D, Zhou T, Voss G, Phogat A, Chakrabarti BK, Li Y, Shaw G, Kwong PD, Nabel GJ, Mascola JR, Wyatt R.** 2009. Structure-based stabilization of HIV-1 gp120 enhances humoral immune responses to the induced co-receptor binding site. PLos Pathog. e1000445. https://doi:10.1371/journal.ppat.1000445.

27. **Barnett SW, Lu S, Srivastava I, Cherpelis S, Gettie A, Blanchard J, Wang S, Mboudjeka I, Leung L, Lian Y, Fong A, Buckner C, Ly A, Hilt S, Ulmer J, Wild CT, Mascola JR, Stamatatos L.** 2001. The ability of an oligomeric human immunodeficiency virus type 1 (HIV-1) envelope antigen to elicit neutralizing antibodies against primary HIV-1 isolates is improved following partial deletion of the second hypervariable region. J. Virol. **75:**5526–5540. https://doi:10.1128/JVI.75.12.5526-5540.2001

28. **Srivastava IK, VanDorsten K, Vojtech L, Barnett SW, Stamatatos L.** 2003. Changes in the immunogenic properties of soluble gp140 human immunodeficiency virus envelope constructs upon partial deletion of the second hypervariable region. J. Virol. **77:**2310–2320. https://doi:10.1128/JVI.77.4.2310-2320.2003

29. **Hsu M, Ho SH, Balfe P, Gettie A, Harouse J, Blanchard J, Cheng-Mayer C.** 2005. A CCR5-tropic simian-HIV molecular clone capable of inducing AIDS in rhesus macaques. J. Acquir. Immune Defic. Syndr. **40:**383–387. https://doi:10.1097/01.qai.0000184857.39318.4f

30. **Dereuddre-Bosquet N, Morellato-Castillo L, Brouwers J, Augustijns P, Bouchemal K, Ponchel G, Ramos OH, Herrera C, Stefanidou M, Shattock R, Heyndrickx L, Vanham G, Kessler P, Le Grand R, Martin L.** 2012. MiniCD4 microbicide prevents HIV infection of human mucosal explants and

vaginal transmission of SHIV(162P3) in cynomolgus macaques. PLoS Pathog. e1003071. https://doi:10.1371/journal.ppat.1003071.

31. **Van Herrewege Y, Morellato L, Descours A, Aerts L, Michiels J, Heyndrickx L, Martin L, Vanham G.** 2008. CD4 mimetic miniproteins: potent anti-HIV compounds with promising activity as microbicides. J. Antimicrob. Chemother. **61:**818–826. https://doi:10.1128/AAC.01627-07.
32. **Li H, Wang S, Kong R, Ding W, Lee F-H, Parker Z, Kim E, Learn GH, Hahn P, Policicchio B, Brocca-Cofano E, Deleage C, Hao X, Chuang G-Y, Gorman J, Gardner M, Lewis MG, Hatziioannou T, Santra S, Apetrei C, Pandrea I, Alam SM, Liao H-X, Shen X, Tomaras GD, Farzan M, Chertova E, Keele BF, Estes JD, Lifson JD, Doms RW, Montefiori DC, Haynes BF, Sodroski JG, Kwong PD, Hahn BH, Shaw GM.** 2016. Envelope residue 375 substitutions in simian–human immunodeficiency viruses enhance CD4 binding and replication in rhesus macaques. Proc. Natl. Acad. Sci. U.S.A. **113:**3413-3422. https://doi:10.1073/pnas.1606636113.
33. **Derdeyn CA, Decker JM, Sfakianos JN, Wu X, O'Brien WA, Ratner L, Kappes JC, Shaw GM, Hunter E.** 2000. Sensitivity of human immunodeficiency virus type 1 to the fusion inhibitor T-20 is modulated by coreceptor specificity defined by the V3 loop of gp120. J. Virol. **74:**8358–8367. https://doi:10.1128/JVI.74.18.8358-8367.2000
34. **Wei X, Decker JM, Liu H, Zhang Z, Arani RB, Kilby JM, Saag MS, Wu X, Shaw GM, Kappes JC.** 2002. Emergence of resistant human immunodeficiency virus type 1 in patients receiving fusion inhibitor (T-20) monotherapy. Antimicrob. Agents Chemother. **46:**1896–1905.
35. **Li M, Gao F, Mascola JR, Stamatatos L, Polonis VR, Koutsoukos M, Voss G, Goepfert P, Gilbert P, Greene KM, Bilska M, Kothe DL, Salazar-Gonzalez JF, Wei X, Decker JM, Hahn BH, Montefiori DC.** 2005. Human immunodeficiency virus type 1 env clones from acute and early subtype B infections for standardized assessments of vaccine-elicited neutralizing antibodies. J. Virol. **79:**10108–10125. https://doi:10.1128/JVI.79.16.10108-10125.2005
36. **Montefiori DC.** 2009. Measuring HIV neutralization in a luciferase reporter gene assay. Methods Mol. Biol. 395–405. https://doi:10.1007/978-1-59745-170-3_26.
37. **Seaman MS, Janes H, Hawkins N, Grandpre LE, Devoy C, Giri A, Coffey RT, Harris L, Wood B, Daniels MG, Bhattacharya T, Lapedes A, Polonis VR, McCutchan FE, Gilbert PB, Self SG, Korber BT, Montefiori DC, Mascola JR.** 2010. Tiered categorization of a diverse panel of HIV-1 Env pseudoviruses for assessment of neutralizing antibodies. J. Virol. **84:**1439–1452. https://doi:10.1128/JVI.02108-09
38. **Decker JM, Bibollet-Ruche F, Wei X, Wang S, Levy DN, Wang W, Delaporte E, Peeters M, Derdeyn CA, Allen S, Hunter E, Saag MS, Hoxie JA, Hahn BH, Kwong PD, Robinson JE, Shaw GM.** 2005. Antigenic

conservation and immunogenicity of the HIV coreceptor binding site. J. Exp. Med. **201:**1407–1419.

39. **Crotty S, Aubert RD, Glidewell J, Ahmed R.** 2004. Tracking human antigen-specific memory B cells: a sensitive and generalized ELISPOT system. J. Immunol. Methods **286:**111–122.

40. **Pollara J, Bonsignori M, Moody MA, Liu P, Alam SM, Hwang KK, Gurley TC, Kozink DM, Armand LC, Marshall DJ, Whitesides JF, Kaewkungwal J, Nitayaphan S, Pitisuttithum P, Rerks-Ngarm S, Robb ML, O'Connell RJ, Kim JH, Michael NL, Montefiori DC, Tomaras GD, Liao HX, Haynes BF, Ferrari G.** 2014. HIV-1 vaccine-induced C1 and V2 Env-specific antibodies synergize for increased antiviral activities. J. Virol. **88**:7715–7726. https://doi:10.1128/JVI.00156-14.

41. **Koopman G, Mortier D, Hofman S, Mathy N, Koutsoukos M, Ertl P, Overend P, van Wely C, Thomsen LL, Wahren B, Voss G, Heeney JL.** 2008. Immune-response profiles induced by human immunodeficiency virus type 1 vaccine DNA, protein or mixed-modality immunization: increased protection from pathogenic simian-human immunodeficiency virus viraemia with protein/DNA combination. J. Gen. Virol. **89:**540–553. https://doi:10.1099/vir.0.83384-0.

42. **Shen X, Bogers WM, Yates NL, Ferrari G, Dey AK, Williams WT, Jaeger FH, Wiehe K, Sawant S, Alam SM, LaBranche CC, Montefiori DC, Martin L, Srivastava I, Heeney J, Barnett SW, Tomaras GD.** 2017. Crosslinking of a CD4 Mimetic Miniprotein with HIV-1 Env gp140 Alters Kinetics and Specificities of Antibody Responses against HIV-1 Env in Macaques. J. Virol. JVI.00401-17. https://doi:10.1128/JVI.00401-17.

43. **Kwong PD, Doyle ML, Casper DJ, Cicala C, Leavitt SA, Majeed S, Steenbeke TD, Venturi M, Chaiken I, Fung M, Katinger H, Parren PW, Robinson J, Van Ryk D, Wang L, Burton DR, Freire E, Wyatt R, Sodroski J, Hendrickson WA, Arthos J.** 2002. HIV-1 evades antibody-mediated neutralization through conformational masking of receptor-binding sites. Nature **420**:678–682. https://doi:10.1038/nature01188

44. **Kwong PD, Wyatt R, Robinson J, Sweet RW, Sodroski J, Hendrickson WA.** 1998. Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody. Nature **393:**648–659. https://doi:10.1038/31405

45. **Xiang SH, Wang L, Abreu M, Huang CC, Kwong PD, Rosenberg E, Robinson JE, Sodroski J.** 2003. Epitope mapping and characterization of a novel CD4-induced human monoclonal antibody capable of neutralizing primary HIV-1 strains. Virology **315:**124–134. https://doi.org/10.1016/S0042-6822(03)00521-X.

46. **Zhang W, Canziani G, Plugariu C, Wyatt R, Sodroski J, Sweet R, Kwong P, Hendrickson W, Chaiken I.** 1999. Conformational changes of gp120 in epitopes near the CCR5 binding site are induced by CD4 and a CD4

miniprotein mimetic. Biochemistry **38:**9405–9416. https://doi:10.1021/bi990654o.

47. **Baum LL, Cassutt KJ, Knigge K, Khattri R, Margolick J RC, Kleeberger CA, Nishanian P, Henrard DR, Phair J.** 1996. HIV-1 gp120- specific antibody-dependent cell-mediated cytotoxicity correlates with rate of disease progression. J. Immunol. **157:**2168–2173.

48. **Chung AW, Navis M, Isitman G, Wren L, Silvers J, Amin J, Kent SJ, Stratov I.** 2011. Activation of NK cells by ADCC antibodies and HIV disease progression. J. Acquir. Immune Defic. Syndr. **58:**127–131. https://doi:10.1097/QAI.0b013e31822c62b9.

49. **Wren LH, Chung AW, Isitman G, Kelleher AD, Parsons MS Amin J, Cooper DA, ADCC study collaboration investigators, Stratov I, Navis M, Kent SJ.** 2013. Specific antibody-dependent cellular cytotoxicity responses associated with slow progression of HIV infection. Immunology **138:**116–123. https://doi:10.1111/imm.12016.

50. **Tomaras GD, Ferrari G, Shen X, Alam SM, Liao HX, Pollara J B, M, Moody MA, Fong Y, Chen X, Poling B NC, Zhang R, Lu X, Parks R, Kaewkungwal J, Nitayaphan S, Pitisuttithum P, Rerks-Ngarm S, Gilbert PB, Kim JH, Michael NL, Montefiori DC, Haynes BF.** 2013. Vaccine-induced plasma IgA specific for the C1 region of the HIV-1 envelope blocks binding and effector function of IgG. Proc. Natl. Acad. Sci. U.S.A. **110:**9019–9024. https://doi:10.1073/pnas.1301456110.

51. **Bonsignori M, Pollara J, Moody MA, Alpert MD, Chen X HK, Gilbert PB, Huang Y, Gurley TC, Kozink DM, Marshall DJ, Whitesides JF, Tsao CY, Kaewkungwal J, Nitayaphan S, Pitisuttithum P, Rerks-Ngarm S, Kim JH, Michael NL, Tomaras GD, Montefiori DC, Lewis GK, DeVico A, Evans DT, Ferrari G, Liao HX, Haynes BF.** 2012. Antibody-dependent cellular cytotoxicity-mediating antibodies from an HIV-1 vaccine efficacy trial target multiple epitopes and preferentially use the VH1 gene family. J Virol **86:**11521–11532. https://doi:10.1128/JVI.01023-12.

52. **Haynes BF, Gilbert PB, McElrath MJ, Zolla-Pazner S TG, Alam SM, Evans DT, Montefiori DC, Karnasuta C, Sutthent R, Liao HX, DeVico AL, Lewis GK, Williams C, Pinter A, Fong Y Janes H, DeCamp A, Huang Y, Rao M, Billings E, Karasavvas N, Robb ML, Ngauy V, de Souza MS, Paris R, Ferrari G, Bailer RT, Soderberg KA, Andrews C, Berman PW, Frahm N, De Rosa SC, Alpert MD, Yates NL, Shen X, Koup RA, Pitisuttithum P, Kaewkungwal J, Nitayaphan S, Rerks-Ngarm S, Michael NL, Kim JH.** 2012. Immune-correlates analysis of an HIV-1 vaccine efficacy trial. N. Engl. J. Med. **366:**1275–1286. https://doi:10.1056/NEJMoa1113425.

53. **Veillette M, Coutu M, Richard J, Batraville LA, Dagher O BN, Tremblay C, Kaufmann DE, Roger M, Finzi A.** 2015. The HIV-1 gp120 CD4-bound conformation is preferentially targeted by antibodydependent cellular cytotoxicity-mediating antibodies in sera from HIV-1- infected individuals. J. Virol. **89:**545–551. https://doi:10.1128/JVI.02868-14.

54. **Lai RP, Seaman MS, Tonks P, Wegmann F, Seilly DJ, Frost SD, LaBranche CC, Montefiori DC, Dey AK, Srivastava IK, Sattentau Q, Barnett SW, Heeney JL.** 2012. Mixed adjuvant formulations reveal a new combination that elicit antibody response comparable to Freund's adjuvants. PLoS One **7:**e35083. https://doi:10.1371/journal.pone.0035083.
55. **Heeney JL, Plotkin SA.** 2006. Immunological correlates of protection from HIV infection and disease. Nat. Immunol. **7:**1281–1284.
56. **Corey L, Gilbert PB, Tomaras GD, Haynes BF, Pantaleo G, Fauci AS.** 2015. Immune correlates of vaccine protection against HIV-1 acquisition. Sci. Transl. Med. **7:**310. https://doi:10.1126/scitranslmed.aac7732.
57. **Kim JH, Excler JL, Michel NL.** 2015. Lessons from the RV144 Thai phase III HIV-1 vaccine trial and the search for correlates of protection. Annu. Rev. Med. **66:**423–437. https://doi:10.1146/annurev-med-052912-123749.
58. **Gray ES, Moore PL, Choge IA, Decker JM, Bibollet-Ruche F, Li H, Leseka N, Treurnicht F, Mlisana K, Shaw GM, Karim SS, Williamson C, Morris L.** 2007. Neutralizing antibody responses in acute human immunodeficiency virus type 1 subtype C infection. J. Virol. **81:**6187–6196. https://doi:10.1128/JVI.00239-07
59. **Vaine M, Wang S, Liu Q, Arthos J, Montefiori D, Goepfert P, McElrath MJ, Lu S.** 2010. Profiles of human serum antibody responses elicited by three leading HIV vaccines focusing on the induction of Env-specific antibodies. PLoS One **5:**e13916. https://doi:10.1371/journal.pone.0013916.
60. **DeVico A, Fouts T, Lewis GK, Gallo RC, Godfrey K, Charurat M, Harris I, Galmin L, Pal R.** 2007. Antibodies to CD4-induced sites in HIV gp120 correlate with the control of SHIV challenge in macaques vaccinated with subunit immunogens. Proc. Natl. Acad. Sci. U.S.A. **104:**174477–17482.
61. **Fouts T, Godfrey K, Bobb K, Montefiori D, Hanson CV, Kalyanaraman VS, DeVico A, Pal R.** 2002. Crosslinked HIV-1 envelope-CD4 receptor complexes elicit broadly cross-reactive neutralizing antibodies in rhesus macaques. Proc. Natl. Acad. Sci. U.S.A. **99:**11842–11847. https://doi:10.1073/pnas.182412199
62. **He Y, D'Agostino P, Pinter A.** 2003. Analysis of the immunogenic properties of a single-chain polypeptide analogue of the HIV-1 gp120-CD4 complex in transgenic mice that produce human immunoglobulins. Vaccine **21:**4421–4429.
63. **Varadarajan R, Sharma D, Chakraborty K, Patel M, Citron M, Sinha P, Yadav R, Rashid U, Kennedy S, Eckert D, Geleziunas R, Bramhill D, Schleif W, Liang X, Shiver J.** 2005. Characterization of gp120 and its single-chain derivatives, gp120-CD4D12 and gp120-M9: implications for targeting the CD4i epitope in human immunodeficiency virus vaccine design. J. Virol. **79:**1713–1723.https://doi:10.1128/JVI.79.3.1713-1723.2005
64. **Heeney JL.** 2004. Requirement of diverse T-helper responses elicited by HIV vaccines: induction of highly targeted humoral and CTL responses. Expert Rev. Vaccines **3:**S53-64.

65. **Mooij P, Nieuwenhuis IG, Knoop CJ, Doms RW, Bogers WM, Ten Haaft PJ, Niphuis H, Koornstra W, Bieler K, Kostler J, Morein B, Cafaro A, Ensoli B, Wagner R, Heeney JL.** 2004. Qualitative T-helper responses to multiple viral antigens correlate with vaccine-induced immunity to simian/human immunodeficiency virus infection. J. Virol. **78:**3333–3342. https://doi:10.1128/JVI.78.7.3333-3342.2004

66. **Heeney JL, Teeuwsen VJ, van Gils M, Bogers WM, De Giuli Morghen C, Radaelli A, Barnett S, Morein B, Akerblom L, Wang Y, Lehner T, Davis D.** 1998. beta-chemokines and neutralizing antibody titers correlate with sterilizing immunity generated in HIV-1 vaccinated macaques. Proc. Natl. Acad. Sci. U.S.A. **95:**10803–10808.

Legends.

Figure 1.

Zoomed-in view of M48U1 binding site showing close contact (pink dotted line) between M48U1 cyclohexylmethoxy group Phe23 and gp120YU2 Ser375. Gp120 is shown as a transparent surface. M48U1 is shown in cyan cartoon representation with its 23 residue represented as sticks. Illustration was prepared with the Pymol 1.8.2.1 Open-Source using 4JZZ PDB ID. The interaction between cyclohexylmethoxy (U1) in M48U1 with both main chain and side chain O atoms of Ser375 gp120 (23).

Figure 2.

Immunization schedule and long-term (17.5 months/70 week) follow-up depicting the immunization phase (box A), and durability phase (box D).

Figure 3.

SF162gp140 mediated inhibition of CD3/TCR induced CD4 T-cell proliferation is

prevented by CD4bs occlusion. In each graph bars are used to indicate the number of undivided cells (right hand bar) and the number of cells for each cell division, within the CD3CD4 population. Note the difference in the fraction of undivided cells, which is increased after addition of SF162gp140 (CD4bs-open), but not by SF162gp140CD4bs-occluded.

Figure 4: Env specific B-cells in peripheral blood (ASC/1x10^6 PBMC) per group during the immunization phase (box A) (at 0, 4, 24, 36 weeks) and after 70 weeks of long-term follow-up (box D). The frequency of Env specific memory B cells were determined by B cell ELISpots in animals immunized with gp140 CD4bs-occluded group 1 (black diamonds), gp140 CDbs open group 2 (=black squares). Control groups; group 3 (mini CD4 mimetic only lower value black triangle) and group 4 (MF59 only: open squares). Immunizations were given at weeks 0, 4, 24, and 36 (box A). The values (numbers of Antibody Secreting Cells (ASC) per 10^6 PBMCs) are means from 6 animals per group ± standard deviations (error bars).
* = points where $p > 0.001$ (two-way ANOVAR).

Figure 5: Kinetics of anti-Env titers per group during the immunization phase (at 0, 4, 24, 36 weeks) (box A) and after 70 weeks of long-term follow-up (box D). HIV-1_{SF162} gp140 specific binding antibody responses induced after immunization with gp140 CD4bs-occluded (group 1: black diamonds), gp140 CDbs open (group 2 =black squares), group 3 immunization mini CD4 mimetic (black triangles), and control group 4 immunized with MF59 only (group 4: open squares). The values (binding end-point titers) are means of 6 animals per group ± standard deviations (error bars).
*= $p > 0.0001$ (two-way ANOVAR).

Figure 6: Early induction of neutralization responses in gp140 CD4bs-occluded (black diamonds) and gp140 CD4bs-open immunized animals (black squares). Neutralization of relatively "homologous" $SHIV_{SF162p4}$ by sera from immunized animals. Antibody titers are expressed as the dilution of serum required to reduce the luciferase activity in cultures exposed to $SHIV_{SF162p4}$ pseudovirus alone by 50%. The values are means from 6 animals per group ± standard deviations (error bars). Elevated early responses detected 18 and 20 weeks after the second immunization (administered at 4 weeks); 22 and 24 weeks after first immunization. Subsequently homologous neutralization titers became similar in both Env immunized groups.
* = $p < 0.001$ (one-way ANOVAR).

Figure 7. Heterologous neutralizing activity in the presence or absence of soluble CD4 during the immunization phase. Heterologous neutralization of a panel of clade B SHIV pseudoviruses with sera taken at 2 or 6 weeks post 4^{th} vaccination. Comparison of neutralization activity of sera from animals immunized with gp140-CD4bs-x (occluded group: circles) in the absence (-sCD4) or presence of sCD4 (+sCD4) versus gp140 CD4bs open (squares). To confirm that the CD4i epitopes in the gp140-CD4bs-x immunized group (1) were exposed and functional, sera was tested against the HIV-2_{7312A} pseudovirus. IC50 neutralization titers are indicated against different viral isolates. The symbols represent values from individual animals while the horizontal bars are means of 6 animals per group ± standard deviations (error bars).

947 **Figure 8**. ADCC activity in CD4bs-x (occluded; black diamonds) Env immunized
948 animals versus CD4bs-open (black squares) Env immunized animals. Data shown are
949 two weeks after third immunization and after long-term follow up post 4^{th}
950 immunization (*p value of 0.014; LOD: limit of detection).
951
952 **Figure 9.** Env specific cytokine-secreting T-cell ELISpot responses following
953 immunizations at 0, 4, 24 and 36 weeks (immunization phase left panel A) and long-
954 term 70 weeks follow-up (durability phase, panel D). Shown are IL-4 (upper row), IL-
955 2 (middle row) and IFN-γ (lower row) secreting SFU over time. ELISpots from
956 individual animals immunized with gp140-CD4bs-x (occluded group) first column,
957 gp140-CD4bs open (2^{nd} column), mini CD4 mimetic only (3^{rd} column) and adjuvant
958 only (4^{th} column). Background responses (mean numbers of spots plus 2x the standard
959 deviations of triplicate assays with medium alone) were subtracted. Responses after
960 stimulation with overlapping SF162 gp120 20mer peptides are presented as the
961 number of spot forming cells (SFC) per 10^6 PBMCs. N.S.: no significance.
962

Figure 1

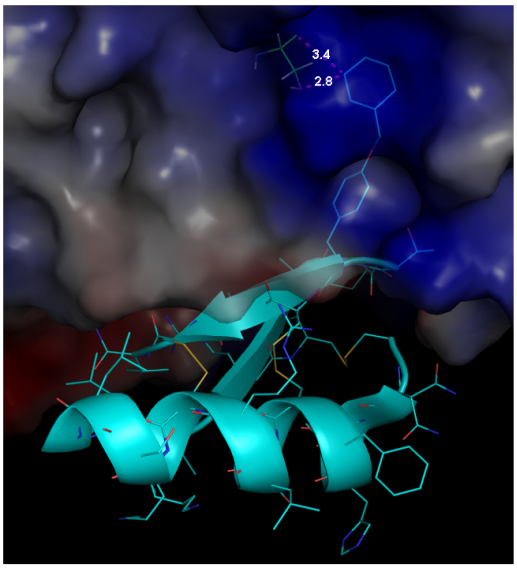


Figure 2

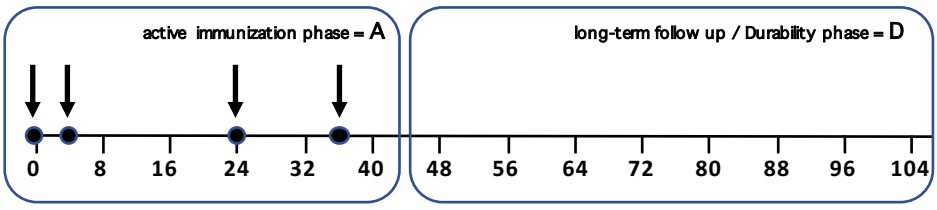
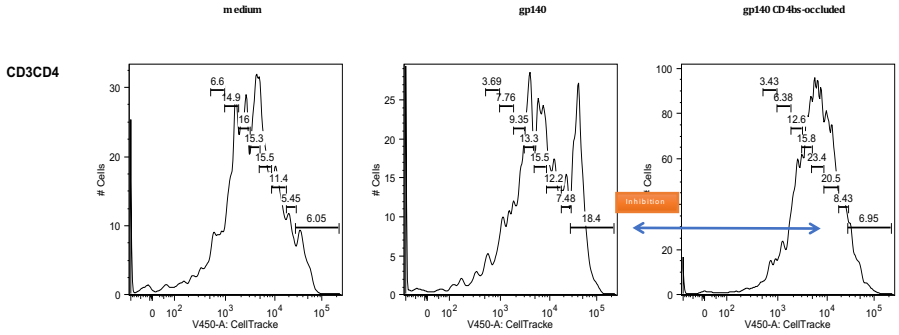


Figure 3



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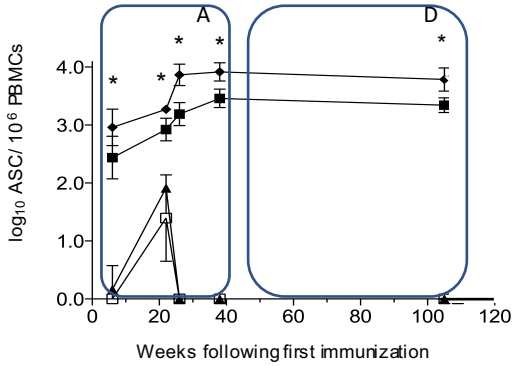


Figure 5

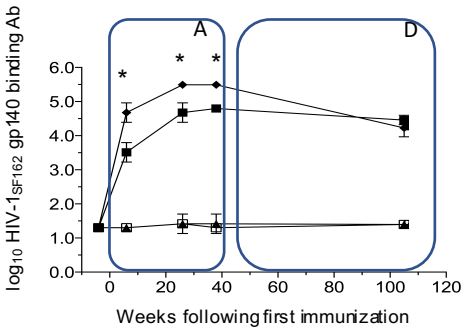
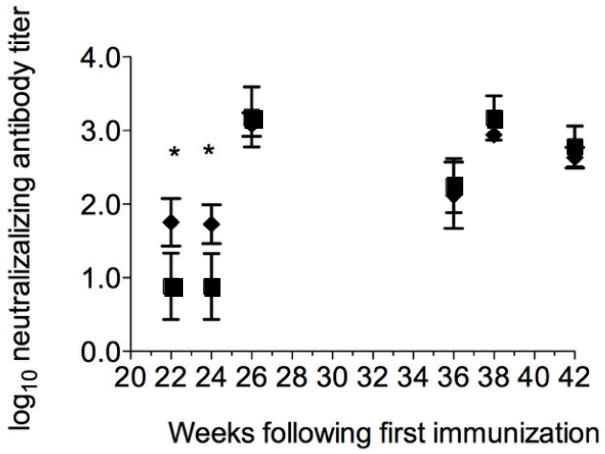


Figure 6



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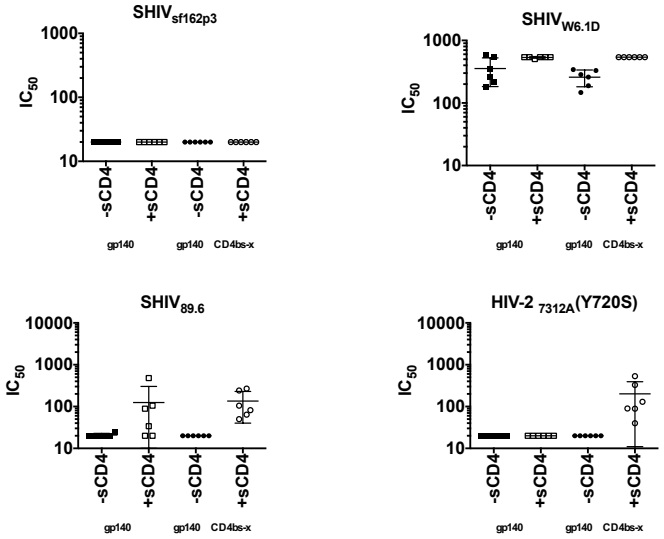


Figure 8

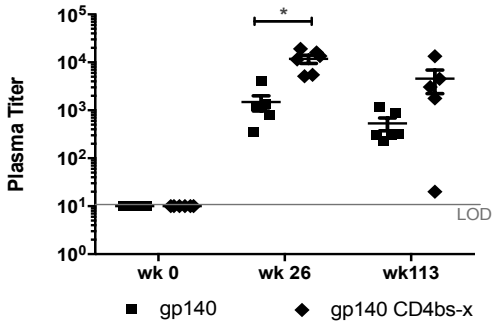
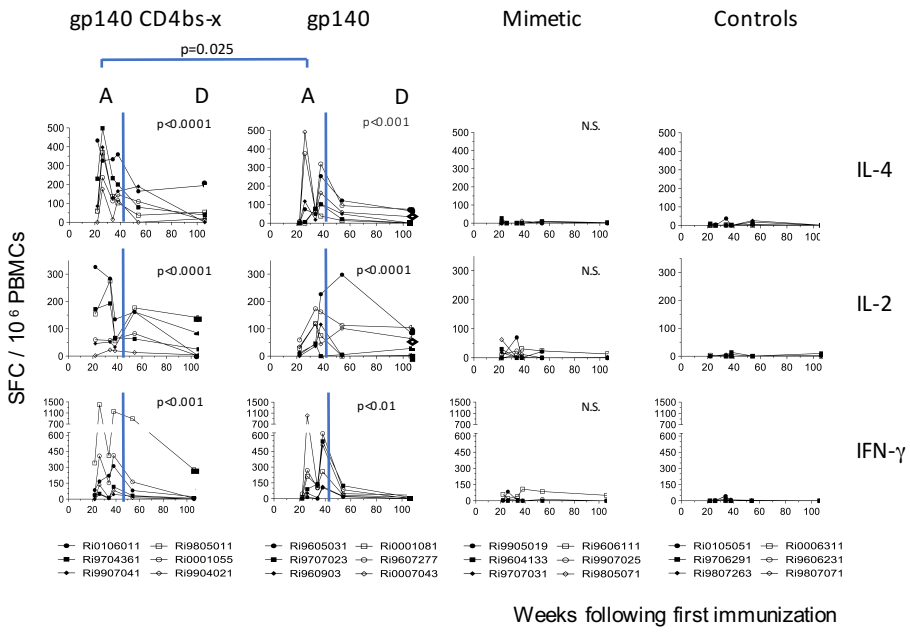


Figure 9


gp140 CD4bs-x
gp140
Mimetic
Controls
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A
D
A
D
p<0.0001
p<0.001
N.S.
IL-4
p<0.0001
p<0.0001
N.S.
IL-2
p<0.001
p<0.01
N.S.
IFN-γ
SFC / 10 6 PBMCs
Ri0106011
Ri9805011
Ri9704361
Ri0001055
Ri9907041
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Ri0001081
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Ri9807071
Weeks following first immunization