SUPPLEMENTAL DATA

EXPERIMENTAL PROCEDURES

MiniCD4 sequences:

M33: TpaNLHFCQLRCKSLGLLGKCAGSBipCACV

M33-F: TpaNLHFCQLRC(K-Fluo)SLGLLGKCAGSBipCACV

M48: TpaNLHFCQLRCKSLGLLGRCAdPTFCACV

M48-F: TpaNLHFCQLRC(K-Fluo)SLGLLGRCAdPTFCACV

M33PolyLys-F: TpaNLHFCQLRC(K-K^2-Fluo)SLGLLGKCAGSBipCACV

M48PolyLys-F: TpaNW(K-GKK)ACQ(K-Fluo)RC(K-GKK)SLGLLGRCAdPTFCACV

M48(N_3)-F: TpaNLHFCQLRC(K-Fluo)SLGLLGRCAdPT(F-N_3)CACV

For M48(N_3)-F, Phe23 was replaced by *p*-azidoPhe; for M33PolyLys-F, fluorescein was coupled at position 11 to the sidechain of the first branched lysinyl residue. K-GKK indicates a lysine with a two-fold-bifurcating polylysinyl ramification separated by an additional glycine. K-K^2 indicates a lysine modified by two additional lysinyl residues. K-X indicates a lysine derivatized at the ε-NH_2, where X = Fluo for fluorescein, Tpa for 3-mercaptopropanoyl (-$COCH_2CH_2SH$). Bip stands for biphenylalanine and dP for *(D)*-proline. *N.B.*: M33 and M48 were earlier called CD4M33 and [Phe23]M47 (1).

Glutaraldehyde crosslinking. Gp120SF162 was preincubated with an equimolar concentration of M33PolyLys-F, during 1 h at RT, and then incubated one hour in the presence of 0.02% glutaraldehyde (Sigma, St Louis, MO). The reaction was stopped by addition of 100 mM Tris/HCl (pH 8).

BS^3 crosslinking. M48PolyLys-F was incubated at a molar ratio of 1:1 with gp120SF162 in PBS, 1 h at RT. A second incubation was performed with a 100 molar excess of

bis(sulfosuccinimidyl)suberate BS^3 (Pierce, Rockford, IL) for 1 h at RT. Crosslinking reaction was quenched by addition of 50 mM Tris/HCl (pH 8).

Photoaffinity labeling. The irradiation device was composed of a 1000 W mercury lamp (Osram), an aperture, and a series of lenses focusing the polychromatic light to a monochromator (Jobin-Yvon), after which a series of lenses focused the monochromatic light to a quartz cuvette. The photolabeling procedure was carried out under argon atmosphere as oxygen contributed to damaging gp120 (data not shown). M48(N_3)-F was incubated in the presence of gp120SF162 in equimolar concentration. The complexes were then irradiated at 310 nm (E = 25 $\mu W.cm^{-2}$) for 1 h, at 10° C, under stirring in a quartz cuvette.

Crosslinking yield evaluation by size exclusion chromatography. Fluorescently labeled miniCD4 association with gp120 was measured by injection through a Bio-Silect column (particle size: 5 μm; 300 x 7.8 mm) calibrated with gel filtration standard of molecular mass ranging from 1,350 to 670,000 daltons (BIO-RAD laboratories, Hercules, Calif.). The column was pre-equilibrated with 5 volumes of sodium acetate buffer 0.05 M (pH 3.5), 0.3 M NaCl and 30% acetonitrile. Acidic conditions were used to separate non-covalently linked miniCD4 from envelope protein. High salt concentration and acetonitrile were shown crucial to prevent the nonspecific binding of miniCD4 onto the column. Elution was monitored by fluorescence detection with 445 nm excitation and 550 nm emission filters. All the fractions constituting the two peaks eluted were concentrated using a 5 ml vivaspin membrane with a 30,000 Da cut off (Vivascience AG, Hannover, Germany). Gp120 was identified by surface plasmon resonance using D7324, a C-terminus anti-gp120 mAb.

Crosslinking characterization by fluorescence polarization. Measurements were made in a LJL Analyst (LJL Biosystems, Sunnyvale, CA, USA) microplate reader, using the fluorescence polarization detection mode with 485 nm excitation and 530 nm emission filters, and an additional dichroic filter at 505 nm to limit blank response. A 1.2 G parameter was

used. Samples were diluted in PBS-Tween 0.05% to prevent sticky phenomena onto plates. The binding assay was performed in a final volume of 20 μl in black 384-well small-volume microtitration plates. Experiments were performed in triplicate. Fluorescence was measured after 40 min equilibrium at 25°C as previously described (1). Both crosslinked products and control samples were made with fluorescein labeled miniCD4 peptides. Complexes were diluted at 5 nM and incubated or not with a 1000 molar excess of unlabeled miniCD4 (5 mM) for 40 min, at RT, to chase non-covalently linked fluorescent miniCD4. Binding of the remaining labeled miniCD4 on gp120SF162 was followed at equilibrium by fluorescence polarization. The coupling yield was calculated as follows: 0% of coupling corresponds to 55 mP, which is the mP value for the fluorescein labeled miniCD4 measured in absence of HIV envelope; the mP value without competitor corresponds to 100% of fluorescein labeled miniCD4 bound to HIV envelope.

RESULTS

Glutaraldehyde, bis-succinimide and photolabeling crosslinking. Three standard crosslinking methods were initially assessed: chemical crosslinking with glutaraldehyde or bis(sulfosuccinimidyl)suberate (BS^3) and photoaffinity labeling (Fig. S1-3). Fluorescein-labeled miniCD4 peptides were used to estimate the coupling yield to gp120 by size exclusion chromatography (SEC) and fluorescent polarization (panels A and B, respectively). They also allowed detection of miniCD4 covalently linked to gp120 on SDS-PAGE (panel C). Crosslinked complexes were finally evaluated for their ability to expose CD4-induced epitopes by surface plasmon resonance (SPR) assays, through binding to X5 CD4i antibody (panel D). The results obtained for each method can be summarized as follows.

Glutaraldehyde is a homobifunctional crosslinker, which reacts with amino groups leading to Schiff base formation. In order to increase its reactivity towards glutaraldehyde, a

fluorescently modified version of M33 with two additional amino groups was used (M33PolyLys-F, see experimental procedures). Glutaraldehyde fixing led to high coupling yields (about 90%, Fig. S1A, B, C). Unfortunately, SPR showed that binding of the CD4i mAb X5 to the gp120-miniCD4 crosslinked complex was very low compared to the uncrosslinked one (Fig. S1D), demonstrating that glutaraldehyde treatment is not optimal to prepare such an immunogen, as it may partially obscure critical neutralizing epitopes unmasked by miniCD4 binding.

Then, we evaluated the use of BS^3 crosslinker for the preparation of gp120-miniCD4 covalent complex, as it had been reported that CD4-gp120 complexes crosslinked by this coupling reagent were able to elicit broadly cross-reactive neutralizing antibodies in several animal models (2,3). BS^3 is a water-soluble homobifunctional crosslinker, which also forms covalent bonds by reaction with amino groups. Actually, a high coupling yield was achieved (about 80%, Fig. S2A, B, C). However, crosslinked complexes also failed to bind X5 mAb indicating a strong deterioration of HIV gp120 epitopes due to BS^3 coupling (Fig. S2D).

At last, we chose photoaffinity labeling, a more specific covalent coupling method. Therefore, a miniCD4 called M48(N3) containing an azide group on the *para* position of the benzene ring of Phe23 was preincubated in the presence of gp120. Phe23 lateral chain has been described to penetrate into the deepest zone of the cavity formed by the CD4 binding site, entitled Phe43 cavity, which is a crucial area for CD4 binding to gp120 (4,5,6). Once bound, crosslinking was induced upon irradiation *via* nitrene formation. Photolabeling yielded a moderate coupling rate in comparison with the previous methods (about 50%, Fig. S3A, B, C), and photolabeled complexes exhibited a two fold lower binding activity towards X5 mAb compared to unirradiated gp120-miniCD4 complex (Fig. S3D), suggesting that the photocrosslinked complex was not able to bind the CD4i mAb. This result may be explained by (i) a deterioration of CD4i epitopes due to the irradiation step, (ii) a modification of

miniCD4 orientation in the CD4 binding site imposed by the linkage and/or (iii) a different rearrangement due to constraints applied by the linkage at the deepest region of the gp120 cavity leading to a reduction of CD4i epitopes accessibility.

FIGURE LEGENDS

Fig. S1. Characterization of miniCD4-gp120SF162 complex fixed by glutaraldehyde. (A) Analysis of the ratio of fluorescein labeled miniCD4 covalently linked or not to gp120SF162 by size exclusion chromatography. Separation was made on high-resolution sizing column in acidic conditions and high salt concentration (50 mM acetate buffer, pH 3.5, 300 mM NaCl and 30% acetonitrile). (B) Coupling yield determination by fluorescence polarization. 5 nM of fluorescein labeled miniCD4 crosslinked or not to gp120 were treated or not with 5 μM of nonlabeled miniCD4 as competitor (+/- comp). (C) Crosslinked complex profile on SDS-PAGE: Each sample was separated on SDS-PAGE and visualized by fluorescence. The faint band seen in CL corresponds to a dimer of gp120, which is classically obtained by recombinant expression (7). (D) Analysis of the binding to X5 antibody by surface plasmon resonance. Experiments were conducted in HBS (HEPES-buffer saline, 3 mM EDTA, 0.05% Biacore surfactant, pH 7.4). Each sample was used at 50 nM. Sample binding was tested on X5 immobilized conformational antibody. CL (covalently linked): M33PolyLys-F/gp120SF162 + 0.02% glutaraldehyde; NCL (not covalently linked): M33PolyLys-F + gp120SF162.

Fig. S2. Characterization of miniCD4-gp120SF162 crosslinked by BS^3. (A) Analysis of the ratio of fluorescein labeled miniCD4 covalently linked or not to gp120SF162 by size exclusion chromatography. Separation was made on high-resolution sizing column in acidic conditions and high salt concentration (50 mM acetate buffer, pH 3.5, 300 mM NaCl and 30%

acetonitrile). (B) Coupling yield determination by fluorescence polarization. 5 nM of fluorescein labeled miniCD4 crosslinked or not to gp120 were treated or not with 5 μM of nonlabeled miniCD4 as competitor (+/- comp). (C) Crosslinked complex profile on SDS-PAGE: Each sample was separated on SDS-PAGE and visualized by fluorescence. The fluorescent miniCD4 was detected by Kodak image station 2000 MM (Molecular Imaging Systems Eastman Kodak Company, New Haven, CT) using wavelengths of 465 nm for excitation and 570 nm for emission. The faint band seen in CL corresponds to a dimer of gp120, which is classically obtained by recombinant expression (8,9). (D) Analysis of the binding to X5 antibody by surface plasmon resonance. Experiments were conducted in HBS (HEPES-buffer saline, 3 mM EDTA, 0.05% Biacore surfactant, pH 7.4). Each sample was used at 50 nM. Sample binding was tested on X5 immobilized conformational antibody. CL (covalently linked): M48PolyLys-F/gp120SF162 + BS^3 crosslinker; NCL (not covalently linked): M48PolyLys-F + gp120SF162.

Fig. S3. Characterization of miniCD4-gp120SF162 photocrosslinked products. (A) Analysis of the ratio of fluorescein labeled miniCD4 free or covalently linked to gp120SF162 by size exclusion chromatography. Separation was made on high-resolution sizing column in acidic conditions and high salt concentration (50 mM acetate buffer, pH 3.5, 300 mM NaCl and 30% acetonitrile). (B) Coupling yield determination by fluorescence polarization. 5 nM of fluorescein-labeled miniCD4 crosslinked or not to gp120 were treated or not with 5 μM of nonlabeled miniCD4 as competitor (+/- comp). (C) Crosslinked complex profile on SDS-PAGE: Each sample was separated on SDS-PAGE and visualized by fluorescence. The fluorescent miniCD4 was detected by Kodak image station 2000 MM (Molecular Imaging Systems Eastman Kodak Company, New Haven, CT) using wavelengths of 465 nm for excitation and 570 nm for emission. (D) Analysis of the binding to X5 antibody by surface

plasmon resonance. Experiments were conducted in HBS (HEPES-buffer saline, 3 mM EDTA, 0.05% Biacore surfactant, pH 7.4). Each sample was used at 50 nM. Sample binding was tested on X5 immobilized conformational antibody. CL (covalently linked): M48(N_3)-F/gp120SF162 + irradiation; NCL (not covalently linked): M48(N_3)-F/gp120SF162 without irradiation.

Fig. S4. CD4i antibody titer. Rabbits were immunized with miniCD4. Four protein immunizations were administered intramuscularly, in the gluteus, at weeks 0, 4, 12, and 24. Serum samples were collected before the first immunization (pre) and two weeks post third (2wp3) and fourth (2wp4) immunizations, and titered for their CD4i antibodies.

References

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Fig. S1

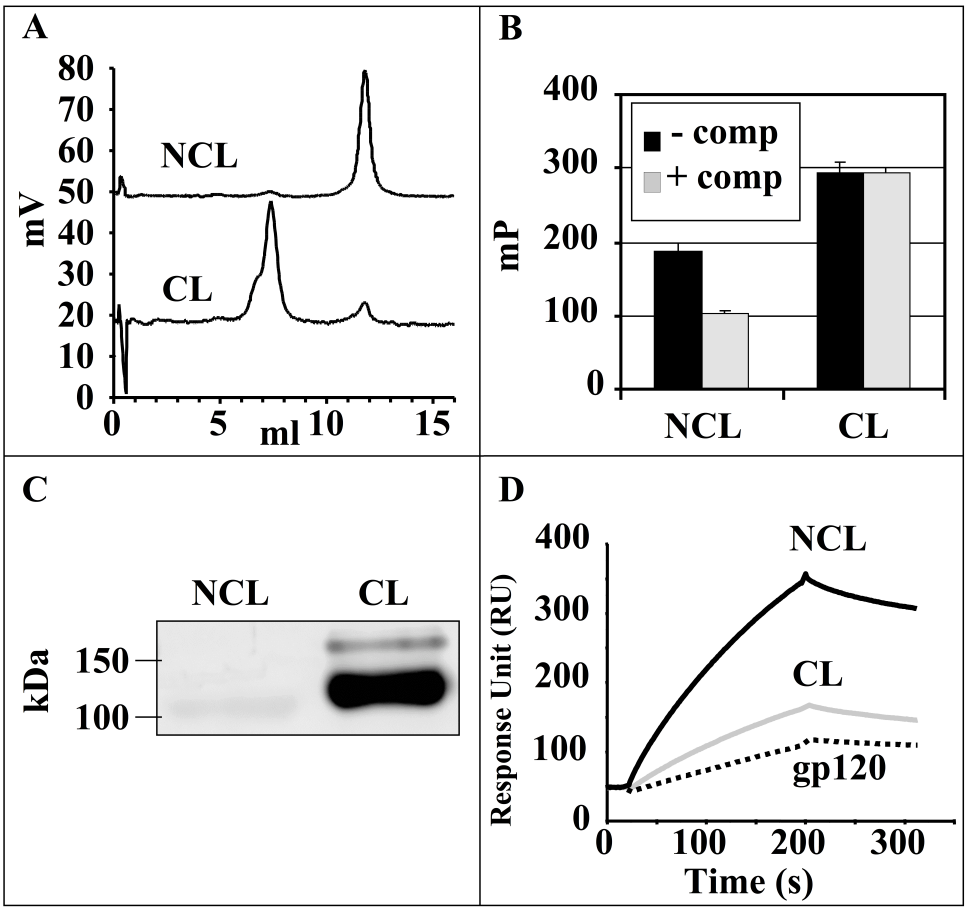
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Fig. S2

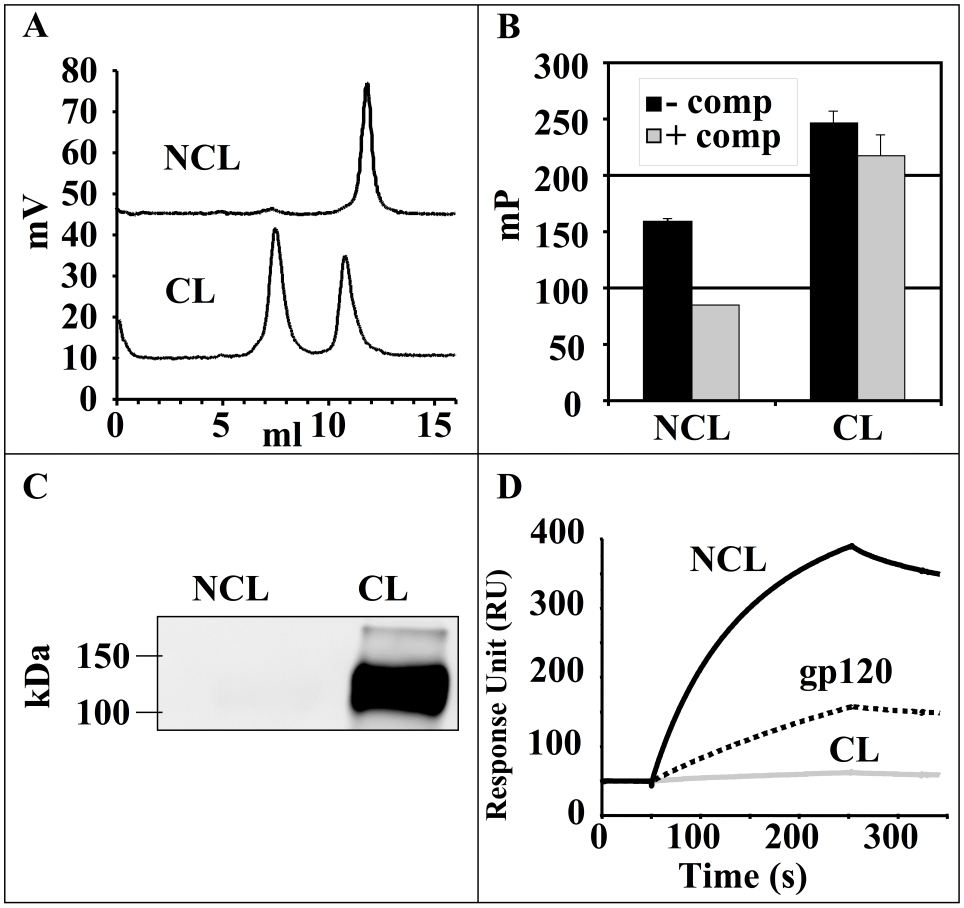
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Fig. S3

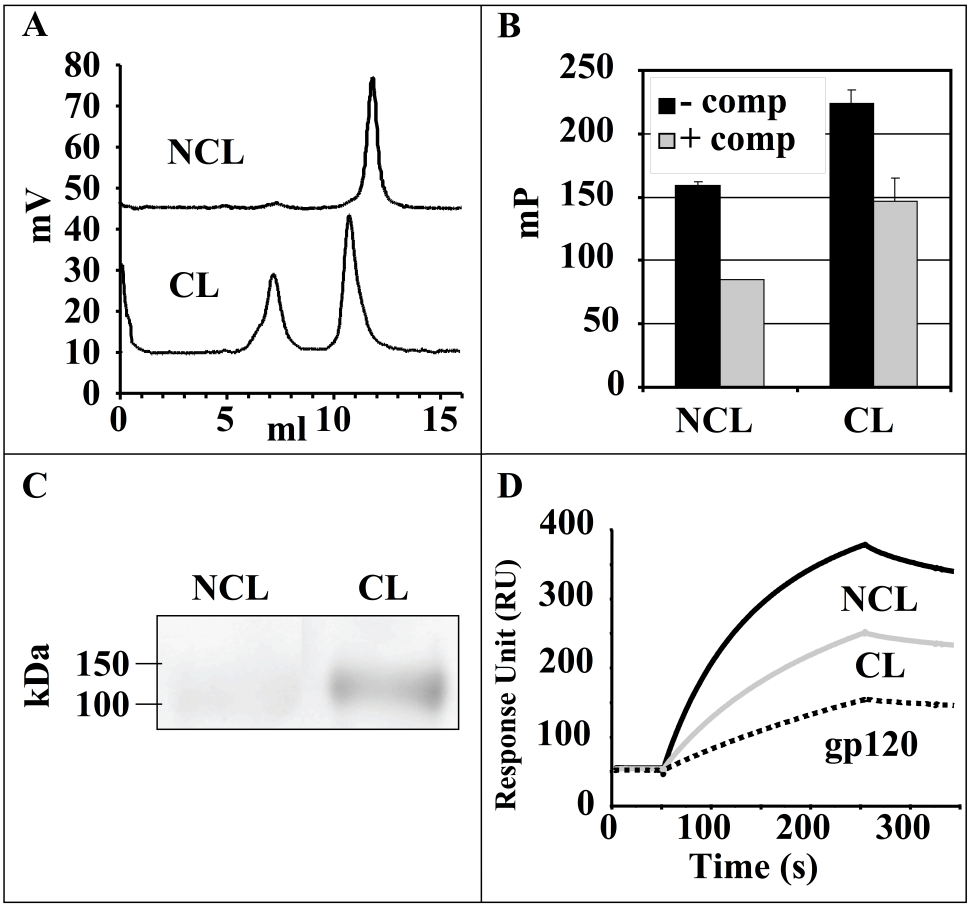
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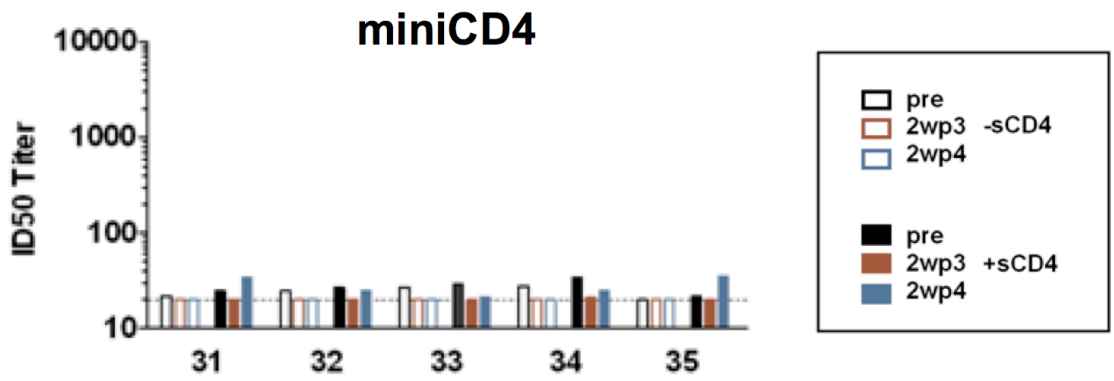
Fig. S4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