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Supplementary Information for

Natural Cystatin C fragments inhibit GPR15-mediated HIV and SIV infection without interfering with GPR15L signaling

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Supplemental Materials and Methods

Cell lines

GHOST BOB (GPR15), Bonzo (CXCR6), CCR5 and parental cells were obtained from the NIH AIDS Reagent Program. GHOST cells were maintained in in Dulbecco's modified Eagle medium (DMEM) supplemented with FCS (10% (v/v)), L-glutamine (2 mM), streptomycin (100 mg/mL) and penicillin (100 U/mL), geneticin G418 (500 µg/ml), 100 µg/ml hygromycin and 1 µg/ml puromycin. The GHOST parental cell line is puromycin sensitive, so medium was not supplemented with Puromycin. Cells were cultured at 37°C, 90% humidity and 5% CO2. The cells were regularly split 1:10 or 1:20 twice a week. HEK293Tcells were provided and authenticated by the ATCC. TZM-bl cells were provided and authenticated by the NIH AIDS Reagent Program, Division of AIDS, NIAID. HEK293T, TZM-bl and Hela cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with FCS (10%), L-glutamine (2 mM), streptomycin (100 mg/mL) and penicillin (100 U/mL). Cells were cultured at 37°C, 90% humidity and 5% CO2. CEM-M7 cells were cultured in RPMI 1640 medium with 10% fetal bovine serum, 1% penicillin-streptomycin, 1% 200 mM L-glutamine and 500 µg/ml G418.

Primary blood cells

Buffy coats (lymphocyte concentrates from 500 ml whole blood) were obtained from the blood bank (Ulm) and diluted 1:3 with PBS. Ficoll separating solution was overlaid with the diluted blood and centrifuged at 1,600 x g for 20 min without breaks. The white interface layer formed by peripheral blood mononuclear cells (PBMCs) was transferred into a fresh tube and washed twice with PBS. After separation and washing 1 x 106cells/ml were cultured in supplemented RPMI-1640 with 1 µg/ml PHA and 10 ng/ml IL-2 for 3 days.

Purification of CysC95-146

The peptide was purified from a 1000 L HF-equivalent sample (308 mg) of the active fraction CEX-Pool 4/RP-Fr19-20 (peptide bank HF020802), by a combination of reversed-phase and cation-exchange HPLC techniques followed by biological testing of collected fractions after every purification step. The sequence of chromatographic steps was: A) Source RPC15 column of dimensions 20 x 250 mm; flow rate: 13 mL/min; gradient (min/%B): 0/2, 5/20, 20/30, 60/40, 70/60, 75/100, being A: 0.1% TFA and B: 0.05% TFA in 80% ACN; detection wavelength: 280 nm. B) Source RPC15 column of dimensions 20 x 250 mm; flow rate: 13 mL/min; gradient (min/%B): 0/2, 10/20, 58/32, 65/40, 68/60, and 75/100; detection wavelength: 280 nm. C) Source RPC15 column of dimensions 10 x 125 mm; flow rate:3 mL/min; gradient (min/%B): 0/5, 10/20, 60/28, 70/40, 68/60, and 80/100; detection wavelength: 280 nm. D) Grace Vydac 218TP54 RP18 column of dimensions 4.6 x 250 mm; flow rate: 1 mL/min; gradient (min/%B): 0/5, 5/26, 65/31, 70/36, and 75/100; detection wavelength: 225 nm. E) PolySULFOETHYL A cation-exchange column of dimensions 4

x 150 mm; flow rate: 0.7 mL/min; gradient (min/%B): 0/0, 30/30, 45/50, and 55/100, being A: 10 mM phosphate + 20% acetonitrile, pH 3.2, and B: 10 mM phosphate + 1M KCl + 20% acetonitrile pH 3.2; detection wavelength: 220 nm.

MALDI-TOF analysis of the active fraction

The molecular mass measurement of the biologically active fraction after the latest purification step (cation-exchange HPLC) was performed with a Voyager-DE Pro matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI–TOF–MS) device (PerSeptive Biosystems, Framingham, MA, USA). The matrix solution was prepared with α-cyano-4-hydroxycinnamic acid dissolved at 5 mg/mL in mass buffer (0.1% TFA in 1:1 acetonitrile/water solution). The chromatographic fraction was desalted with an RPC18 cartridge (Waters, USA) prior to MS measurement. One microliter of the sample solution and matrix solution were mixed on a 100-well stainless steel MALDI plate. Measurements were performed in linear mode. Positive ions were accelerated at 20 kV, and up to 100 laser shots were automatically accumulated per sample position. Voyager RP BioSpectrometry Workstation version 3.07.1 (PerSeptive Biosystems, USA) was used as control and visualization software.

Synthetic peptides

Solid phase peptide synthesis was carried out using conventional Fmoc chemistry. Fmoc-protected amino acids with protected side chains were purchased from Novabiochem (Merck KGaA, Darmstadt, Germany). Synthesis was carried out using a preloaded Wang resin (Novabiochem) in a 0.1 mmol scale on a Liberty blue microwave peptide synthesizer (CEM Corporation, Matthews, NC, USA). After coupling reactions, deprotection and cleavage from the resin the raw peptides were precipitated and purified by reversed-phase HPLC. The correct molecular mass of the obtained purified synthetic peptides was analyzed by electrospray (ESI) and MALDI-mass spectrometry and their purity of 95% was confirmed by analytical HPLC. Human Cystatin C protein (purified, from human urine, BML-SE479-0100) was obtained from Enzo Life Sciences.

Quantitation of CysC95-146 in hemofiltrate by nanoLC-ESI-iTRAP-Orbitrap

CysC95-146 amount in hemofiltrate was estimated from the chromatographic fraction CEX-Pool 4/RP-Fr19-20 of the peptide bank HF020802, where the biological activity was initially found. For disulfide bridge reduction and carbamidomethylation, the standard (synthetic CysC95-146) and the HF sample were incubated with 50 mM NH4HCO3 + 5 mM DTT at RT for 20 min. Subsequently, iodoacetamide was added up to 50 mM to the samples and incubated at 37oC for 20 min. The reaction was stopped (quenching) with 10 mM DTT. The injection volume in the LC-MS/MS system was 15 µL. The amounts of injected peptide standards were (pmol) 62.5, 125, 250, 500 and 1000. Samples were measured using an LTQ Orbitrap Velos Pro system (Thermo Fisher Scientific) online coupled to an U3000 RSLCnano (Thermo Fisher Scientific) as described (1) with the following modifications: Separation was carried out using a binary solvent gradient consisting of solvent A

(0.1% FA) and solvent B (86% ACN, 0.1% FA). The column was initially equilibrated in 5% B. In a first elution step, the percentage of B was raised from 5 to 15% in 5 min, followed by an increase from 15 to 40% B in 30 min. The column was washed with 95% B for 4 min and re-equilibrated with 5% B for 19 min. Ion chromatograms were visualized by XCalibur Qual Browser (Thermo Fisher Scientific, Bremen, Germany). For the identification of the CysC-F52 in the samples, the total ion chromatograms were filtered by searching the m/z range corresponding to the m/z value of the most intense signal (± 20 ppm) according to the theoretical isotopic distribution at z=6. The peak areas were calculated by using the default parameters of the software. The peak areas were exported and the linear regression calibration curve (peak area vs standard amount) was constructed in Microsoft Excel 2016.

Molecular clones of HIV and SIV

Generation of the SIVmac239 proviral construct carrying a functional nef gene followed by an IRES element and the eGFP cassette has been described previously (2). A similar approach was used to generate the replication-competent SIVmac239 F-Luc construct. Therefore, PCR amplification with flanking primers introducing MluI and XmaI restriction sites of the IRES-FLuc cassette from an HIV-1 IRES-FLuc reporter construct was used and therewith the IRES-eGFP cassette in SIVmac239 proviral construct replaced. The integrity of the PCR-derived insert was confirmed by sequencing. See Key reagent table for details on additional proviral HIV-1 and SIV constructs.

Virus stocks

Virus stocks were generated by transient transfection of HEK293T cells using the calcium-phosphate precipitation method or the TransIT®-LT1 Transfection Reagent (Mirus). One day before transfection, 0.65 x 106 HEK293T cells were seeded in 6-well plates (Greiner Bio-one, Frickenhausen, Germany). At a confluence of 50-75% cells were used for transfection. For the calcium-phosphate precipitation method, 5 µg DNA was mixed with 13 µl 2 M CaCl2 and the total volume was made up to 100 µl with water. This solution was added drop-wise to 100 µl of 2x HBS. The transfection cocktail was vortexed for 5 sec and added drop-wise to the cells. The transfected cells were incubated for 8-16 h before the medium was replaced by fresh supplemented DMEM. Transfection with TransIT®-LT1 Transfection Reagent was performed according to the manufacturer's recommendation. 48 h post transfection, virus stocks were prepared by collecting the supernatant and centrifuging it at 1300 rpm for 3 min. SIVmac239 F-Luc virus stocks were additionally concentrated by ultracentrifugation. Virus stocks were stored at – 80°C.

Cell viability

GHOST Bob cells were seeded in 96-well F-bottom plates at a density of 10,000 cells per well one day prior to the experiment. Cells were washed once in PBS and incubated with increasing amounts

of CysC95-146 for 3 days at 37°C. Metabolic activity was analyzed by MTT and CellTiter-Glo assay (Promega) according to the manufacturer's recommendations.

Infectivity Assays

Effect of CysC95-146 and derivatives and GPR15L on GPR15-dependent SIVmac239 infection. GHOST Bob (GPR15) cells were seeded in 96-well plates at a density of 10,000 cells per well. The next day, cells were treated with Cystatin C derived peptides or GPR15L at the indicated concentrations and incubated for 1 – 2 hours at 37°C, 90% humidity and 5% CO2. Then, cells were infected with SIVmac239 F-Luc previously produced by transient transfection of HEK293T cells. Three days post infection, viral infectivity was determined using a Firefly-Luciferase Assay kit from Promega as recommended by the manufacturer. Firefly-Luciferase activities were quantified as relative light units (RLU) per second with an Orion Microplate luminometer (Berthold).

Co-receptor usage

GHOST-GPR15, CCR5 and CXCR6 cells were seeded 24h prior with a density of 6,000 cells/well in 96 well F-bottom plates. Cells were preincubated with increasing amounts of CysC95-146 for 2h at 37°C prior to virus addition. 3dpi, infection rates were analyzed by measuring GFP-positive cells via flow cytometry.

Inhibition by CysC95-146

GHOST-GPR15 cells were seeded 24h prior to infection at a density of 6,000 cells/well in 96-well F-bottom plates. Cells were incubated with increasing amounts of CysC95-146 from humans or agm/mac for 2h at 37°C prior to infection with SIVmac239 F-Luc. Three days post-infection, viral infectivity was determined using a Firefly-luciferase Assay kit from Promega as recommended by the manufacturer. Firefly-luciferase activities were quantified as relative light units (RLU) per second with an Orion Microplate luminometer (Berthold).

Infection of primary blood cells

1 million cells were incubated with various amounts of peptide or compound for 1 – 2 at 37°C. Then, cells were infected with virus stocks previously generated by transient transfection of HEK293T cells with the respective pro-viral constructs. PBMCs were cultured in the presence of 10 ng/ml IL-2 in RPMI-1640 supplemented with FCS (10% (v/v), L-glutamine (2 mM), streptomycin (100 mg/mL) and penicillin (100 U/mL). 3 days post infection cells were used for FACS analyses. Infected cells were first washed with 500 µl FACS buffer (PBS with 1% (v/v) FCS) and stained with 50 µl FACS buffer containing 1 µl FITC-conjugated p24 antibody. The cells were incubated for 30 min at 4°C and then washed with 1 ml FACS buffer to remove unbound antibody. Then, cells were fixed with 200 µl FACS buffer containing 2% PFA and incubated for 30 min at 4°C. PBMCs were gated based on forward and side scatter characteristics, followed by exclusion of doublets and then by the respective marker expression. Data were generated with BD FACS Diva 6.1.3 Software.

Replication kinetics in PBMCs

0.75 million cells were transferred into FACS tubes, washed twice in PBS and incubated with 10 µM GPR15L, 10 µM CyC95-146, 15 µM Maraviroc, 10 µg/ml AMD3100 or 500 ng/ mL CXCL16 in RPMI-1640 plus supplements for 1 hour at 37°C. Then, cells were infected with virus stocks previously generated by transient transfection of HEK293T cells with the respective pro-viral constructs. 16 hours post-infection, cells were washed and transferred to a 96 U-Well plate. At the indicated time points, cells were spun down and ~80% (v/v) of supernatants of the PBMC cultures were aspirated and frozen at -80°C. Medium was replaced with fresh RPMIxxx supplemented with 10 ng/mL IL-2. CysC95-146, GPR15L and the remaining compounds were added again to achieve the concentrations stated above.

Infectious virus yields from PBMCs

Infectivity of virions produced in primary cells in the presence and absence of CysC95-146, GPR15L, AMD3100, MVC, CXCL16. To determine the infectivity of virions produced in infected human and rhesus primary blood cells, TZM-bl cells were seeded in 96-well plates at a density of 10,000 cells/well and infected after overnight incubation with the supernatants collected from the PBMC cultures. Three days p.i., viral infectivity was determined using a galactosidase screen kit from Tropix as recommended by the manufacturer. β-Galactosidase activities were quantified as relative light units (RLU) per second with an Orion Microplate luminometer (Berthold).

GPR15 antibody competition assay

50,000 GHOST-GPR15 cells were counted, washed in FACS buffer (1x PBS with 1% FCS, 4°C) and transferred to FACS tubes. Cells were centrifuged at 4°C and 1,300 rpm (350 x g). Supernatants were discarded and cell pellets were resuspended in 100 µL master mix containing anti-GPR15 antibodies (ab8104 and ab188938) and CysC 95-146. Indicated concentrations of CysC 95-146 were achieved during this inoculation step. Antibody concentrations were 10 ng/mL. Cells were incubated at 4°C for 1 hour and washed 3 times in FACS buffer. Then, staining with a secondary antibody (Goat anti-rabbit IgG H&L PE, ab97070) was performed. For this, samples were incubated with secondary antibody at 10 ng/mL for 30 minutes at 4°C. After staining with the secondary antibody, cells were washed 3 times in FACS buffer, fixed in 4% PFA (1 h, 4°C) and kept at 4°C until analysis by flow cytometry. Cells were gated based on forward and side scatter characteristics, followed by exclusion of doublets and then by the respective marker expression. Data were generated with BD FACS Diva 6.1.3 Software before analysis with FlowJo 8.8 Software (Treestar).

Receptor downmodulation

GHOST-GPR15 or CEM-M7 cells were preincubated with increasing amounts of GPR15L or CysC95-146 for 30 min at either 37°C or 4°C (to prevent receptor internalization) prior to staining with anti-GPR15 or isotype control antibody. GPR15 expression was analyzed by flow cytometry.

Flow cytometric analysis

Human PBMCs were isolated from whole blood as described before. 250,000 cells were incubated with 10 µM of CysC95-146 or GPR15L in RPMI-1640 supplemented with 10 ng/mL IL-2 for 1h at 37°C, 90% humidity and 5% CO2. Then, cells were washed with PBS and stained for the respective markers using 1 µL of each antibody in 50 µL FACS buffer. Antibodies and isotype controls were obtained from BioLegend: Brilliant Violet 421™ anti-human CXCR4 (#306517), APC anti-human CD69 Antibody (#310910), PE anti-human CD25 Antibody (#302606), FITC anti-human CCR5 Antibody (#313705), APC anti-human GPR15 Antibody (#373006), Brilliant Violet 421™ anti-human CXCR6 (#356014). The recommended isotype control of each antibody described above was used at equal concentrations. Cells were incubated with the antibodies for 1 h at 4°C. Cells were washed and fixed in 4% (v/v) PFA in PBS. PBMCs were gated based on forward and side scatter characteristics, followed by exclusion of doublets and then by the respective marker expression. Data were generated with BD FACS Diva 6.1.3 Software before analysis with FlowJo 8.8 Software (Treestar).

Proteolysis of Cystatin C

100 µg (7.54 nmol) human Cystatin C protein (purified, from human urine, BML-SE479-0100) obtained from Enzo Life Sciences were digested with indicated proteases at 1:100 molar ratio (75.4 pmol protease). Digestions were performed in 0.2 M citrate buffer pH 4.0 (cathepsin D, G and E); 100 mM Tris HCl with 10 mM CaCl2 pH 8 (trypsin), 20 mM sodium acetate pH 3.5 (pepsin), 50 mM Tris HCl with 0.26 M NaCl pH 8 (chymase) or 0.1 M NaOAc with 0.2 M NaCl pH 3.5 (napsin A). Digestions with Cathepsin G and E were incubated for 24 h at 37°C, all other reactions for 2 h at 37°C. To remove proteases and exchange buffer, digestion products were diluted in PBS (75 µl digestions reaction mixture + 425 µl PBS) and applied to 30 kDa Amicon ultrafiltration units (Merck, #UFC5030). Samples were centrifuged for 30 minutes at 12,000 x g and room temperature. Columns were discarded and flow through 3 kDa Amicon ultrafiltration units (Merck, #UCF5003) was applied to retain CysC fragments (~ 5-7 kDa) and to remove salts and buffer components. Samples were centrifuged again as described above. Cystatin C peptides were eluated from the filter by inverting the unit in fresh 1.5 ml Eppendorf tubes. Samples were centrifuged for 2 minutes at 1,000 × g and eluate volumes adjusted to the starting volume of 75 µl. The amount of recovered digestion product used for the GHOST Bob infection assay was based on the molecular weight of the full-length Cystatin C and assuming complete digestion of the protein.

Coomassie staining

Digestion products were mixed with Protein Loading Buffer (LI-COR #928-40004) and TCEP (50 mM final concentration) and heated to 70°C for 10 min. Proteins were then separated on NuPAGE 4-12% BisTris gels, fixed with 50% methanol plus 7% acetic acid solution and stained with GelCode Blue (Thermo Fisher #24590) for 1 h at RT. After destaining with ultrapure water, the gel was imaged on the Gel Doc™ XR+ Gel Documentation System (BioRad). GPR15 expression constructs. For all PCRs, the Phire Hot Start II DNA Polymerase (Thermo Fisher Scientific, F122L) was used. GPR15 was PCR-amplified from the plasmid pCMV6-XL4 human GPR15 (OriGene, CAT# SC116846, ACCN: NM-005290). Thereby the single restriction sites NheI and HindIII were added to the sequences by the primers used (Primer: NheI-GPR15-for and GPR15-HindIII-rev). The amplified GPR15 was cloned in the empty pcDNA3.1 vector by using the introduced single restriction sites. Additionally, GPR15 constructs C- or N-terminal tagged with eGFP were cloned via overlap-extension PCR. For the N-terminal tagged pcDNA3.1-eGFP-GPR15 construct eGFP was fused to GPR15 by replacing the stop codon of eGFP and the start codon of GPR15 by the linker sequence CCCGGG (Primer: eGFP NheI-for, GPR15-HindIII-rev, GFP-GPR15-SOE-rev and GFP-GPR15-SOE-for) and afterwards ligated in the pcDNA3.1 construct by using NheI and HindIII. The C-terminal tagged construct pcDNA3.1-GPR15-eGFP was constructed in the same way by fusing eGFP to the C-terminal end of GPR15 by replacing the stop codon of GPR15 and the start codon of eGFP with the linker sequence GGCGCCGGCGCC (Primer: NheI-GPR15-for, eGFP-HindIII-rev, GPR15-GFP-SOE-rev and GPR15-GFP-SOE-for).

CCR5 genotyping

1x 107 Δ32/Δ32 PBMCs were transferred into a 1.5 mL Eppendorf tube and spinned down. Supernatants were discarded and cells were washed three times in 1x PBS. The cell pellet was lysed in M-PER Mammalian Protein Extraction reagent (Thermo Fisher # 78501) in presence of 100 µg/mL Proteinase K for 1 h at 56°C, followed by an inactivation step at 95°C for 10 minutes. Polymerase chain reaction (PCR) was carried out using the Phusion High-Fidelity DNA Polymerase according to the manufacturers' instructions using the primer pair p5CCR5fw 5`-TGG-TGG-CTG-TGT-TTG-CGT-CTC-3` and p3CCR5rev 5`-AGC-GGC-AGG-ACC-AGC-CCC-AAG-3`. A total of 35 thermal cycles per PCR were performed in a VeritiTM 96-Well Thermal Cycler (Applied Biosystems) in a total volume of 50 µl. Then, 25 µl of product were separated on a 3 % agarose gel (w/v, Sigma Aldrich) in 1x TAE buffer (Carl Roth) next to a 1 kb Plus DNA ladder (Thermo Scientific). The samples were run for 25 min at 140 V, stained using ethidium bromide (AppliChem GmbH) and visualized in a Bio-Rad Gel Doc XR+.

Confocal microscopy

5,000 Hela cells were seeded in 300 µL DMEM supplemented with FCS (10%), L-glutamine (2 mM), streptomycin (100 mg/mL) and penicillin (100 U/mL) in µ-Slide 8-well Ibidi microscopy chambers one day prior to the experiment. Cells were washed in PBS and medium was replaced

with fresh medium once. Cells were then transfected with 0.25 µg GFP-tagged GPR15 expression plasmids using the TransIT®-LT1 Transfection Reagent (Mirus) according to the manufacturer's protocol. 4 hours post-transfection, the medium was changed and cells were cultured for 2 days at 37°C, 90% humidity and 5% CO2. At 2 days post-transfection, cells were washed in PBS, fixed in 4% (v/v) PFA for 30 minutes at room temperature, permeabilized using Block and Perm Buffer (5% (v/v) FCS in PBS with 0.5% (v/v) Triton X100). Nuclei were stained using DAPI by incubating cells for 2 hours at 4°C. Cells were washed three times with PBS and once with HPLC water. Cells were covered with glycerol-based mounting medium to prevent bleaching of the fluorophores during microscopy. Confocal microscopy was performed using a LSM710 (Carl Zeiss). To examine the interaction of CysC95-146 with GPR15, Hela cells were transfected as described above. 2 days post-transfection, cells were treated with 5 µM Atto647-labeled CysC95-146 for 1 h at 4°C, then washed several times in ice-cold PBS, fixed in 4% (v/v) PFA for 30 minutes at 4°C. Nuclei were stained using Hoechst33342 (Merck) according to the manufacturer's instructions. Cells were washed with PBS, covered with glycerol-based mounting medium to prevent bleaching of the fluorophores during microscopy. Confocal microscopy was performed using an LSM710 (Carl Zeiss). Images were deconvoluted using Huygens Software (Scientific Volume Imaging) and processed in Fiji (Fiji is just ImageJ).

Antibody-mediated inhibition of GPR15-dependent virus infection

Antibodies targeting the N-terminus (ab8104) or the first extracellular loop (ab188938) were obtained from Abcam. An additional antibody against human GPR15 with unknown target site from R&D was also purchased (R&D #367902). GHOST-GPR15 cells were seeded 24h earlier at a density of 6,000 cells/well in 96 well F-bottom plates. Cells were preincubated with increasing amounts of anti-GPR15 antibodies for 2h at 37°C. Then, cells were infected with SIVmac239 F-Luc. At 3 dpi, infectivity was analyzed via firefly luciferase reporter assay.

CXCR4 Antibody competition assay

Competition of compounds with antibody binding was performed on SupT1 cells. Cells were washed in PBS containing 1 % FCS and 50,000 cells were then seeded per well in a 96 V-well plate. Buffer was removed and plates were precooled at 4°C. Compounds were diluted in PBS and antibody (clone 12G5, APC labeled) was diluted in PBS containing 1 % FCS. The antibody was used at a concentration close to its determined equilibrium dissociation constant (Kd). Compounds were added to the cells and 15 µl antibody immediately afterwards. Plates were incubated at 4°C in the dark for 2 hrs. Next, cells were washed with PBS containing 1% FCS and fixed with 2% PFA. Antibody binding was analyzed by flow cytometry (CytoFLEX; Beckman Coulter®).

Molecular Modeling

Based on the full CysC structure (Protein Data Bank identification code 3GAX), taken from the Protein Data Bank (4), the initial atomic positions of the CysC95-146 model were obtained. Possible initial GPR15 atomic coordinates were created with Phyre2 (5), while hydrogen termination was performed with Maestro (www.schrodinger.com/releases/release-2019-3) for pH 7. Initial equilibration (300K for 0.1 ns) was then performed by classical MD (molecular dynamics) simulations using the GROMACS MD engine and the non-reactive force field Amber99sb. Afterwards, ReaxFF (reactive molecular dynamic) simulations (6) were performed with the Amsterdam Modeling Suite 2019 (ADF2019, SCM, Theoretical Chemistry, Vrije Universiteit, Amsterdam, The Netherlands, http://www.scm.com) within NVT ensembles over 25 ps and coupling to a Berendsen heat bath (T=300 K with a coupling constant of 100 fs). Random interaction structures between GPR15 and CysC95-146 were generated and preselected using the overall system energy as selection criterion. For the most stable systems, reactive MD simulations were performed to evaluate the interaction energy and morphology. Afterwards, amino acid-resolved interaction energies were obtained by individually removing amino acids from the frustrated interaction model, followed by evaluating the energy changes. For all visualizations the Visual Molecular Dynamics program (VMD) (7) was used.

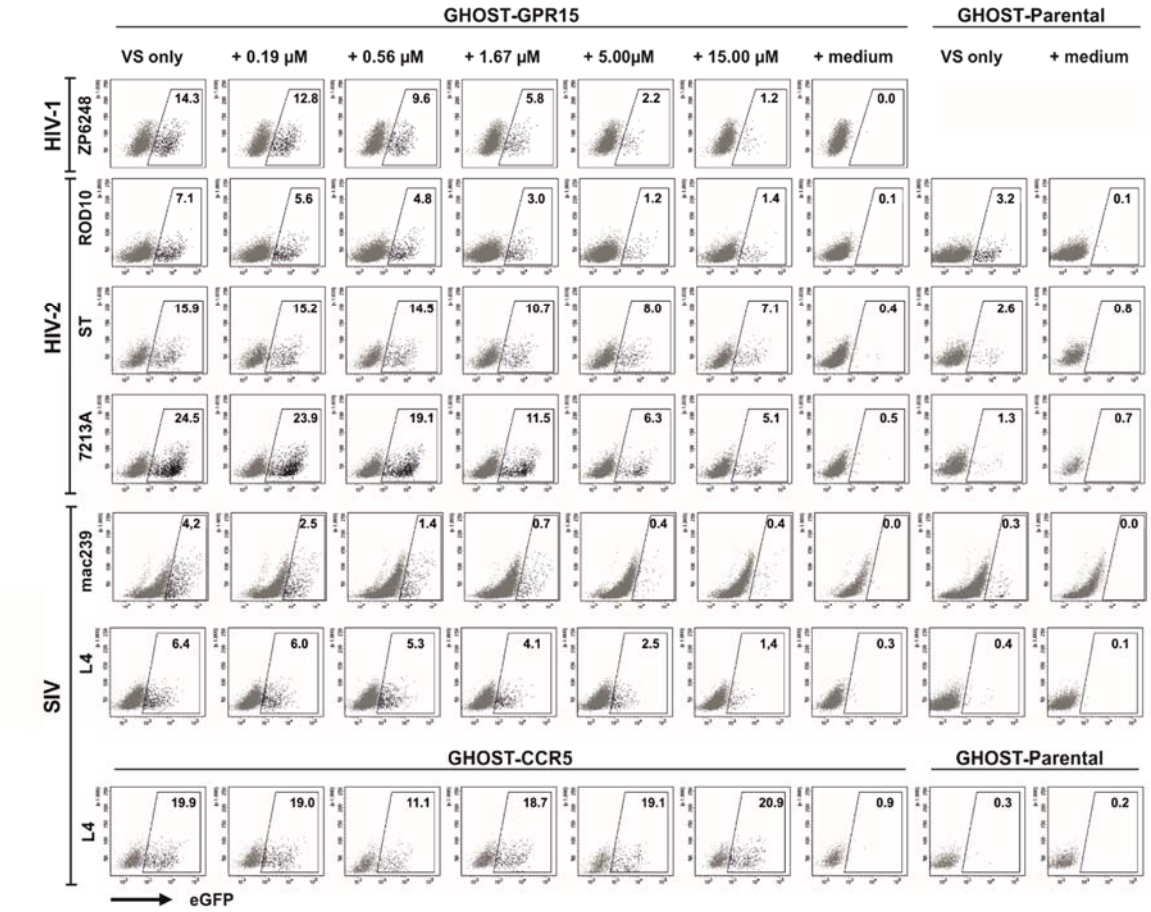

Fig. S1. Examples of primary FACS data for infection of GHOST cells in the presence of CysC95-146. GHOST-GPR15 or parental cells were infected with the indicated virus in the presence or absence of CysC95-146. Three days post-infection, infection rates were determined by flow cytometry. Exemplary primary data show the frequency of detected GFP-positive (infected) cells of the respective population.

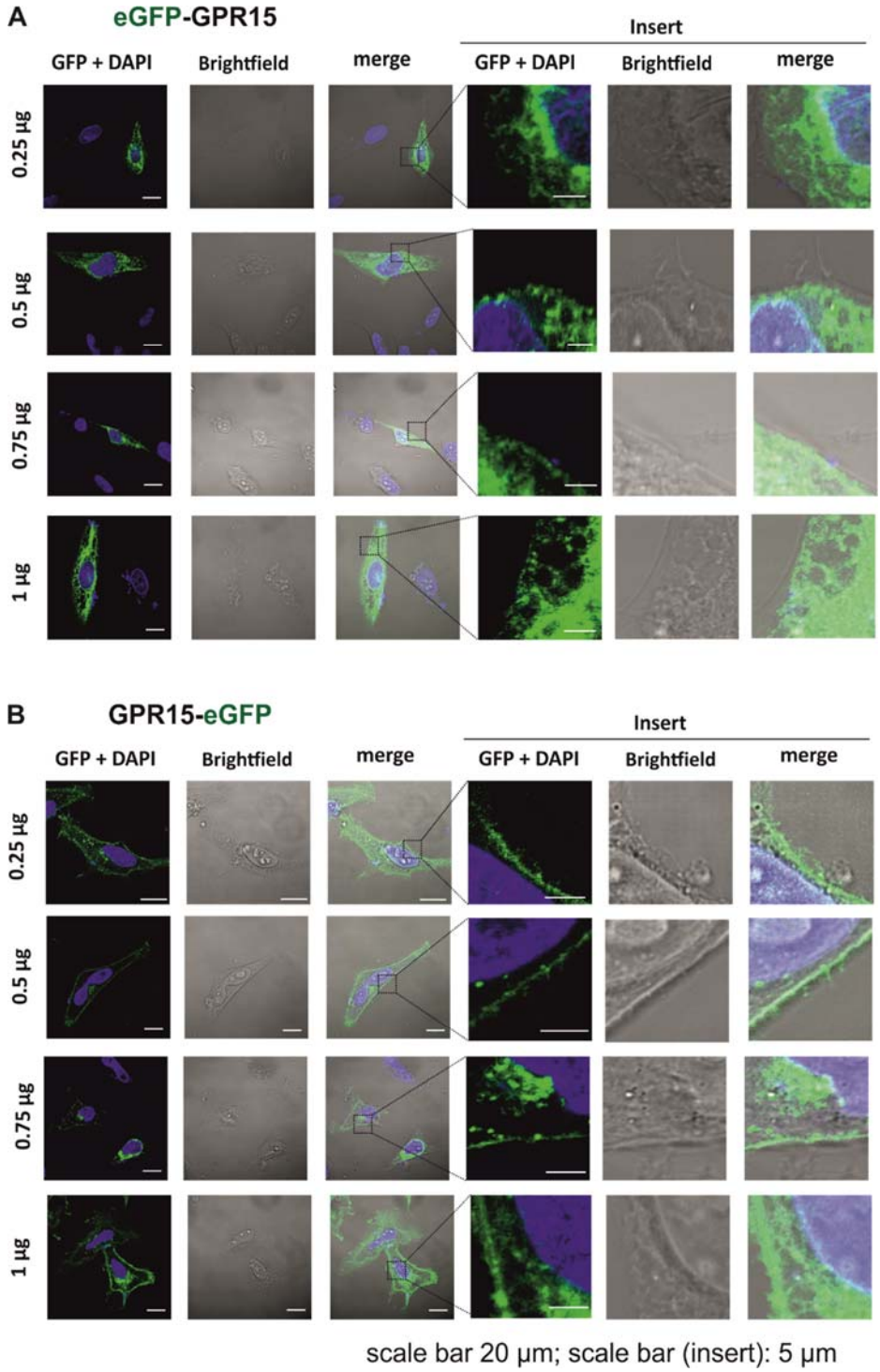

Fig. S2. Expression of eGFP-tagged GPR15. (A, B) Hela cells were transfected with the indicated amount of plasmid DNA of constructs expressing (A) N-terminally or (B) C-terminally eGFP-tagged GPR15. 2 days post-transfection, nuclei were stained using DAPI and analysed by confocal microscopy using an LSM710 (Carl Zeiss). Scale bar indicates 20 µm in the left panel and 5 µm in the insert panel.

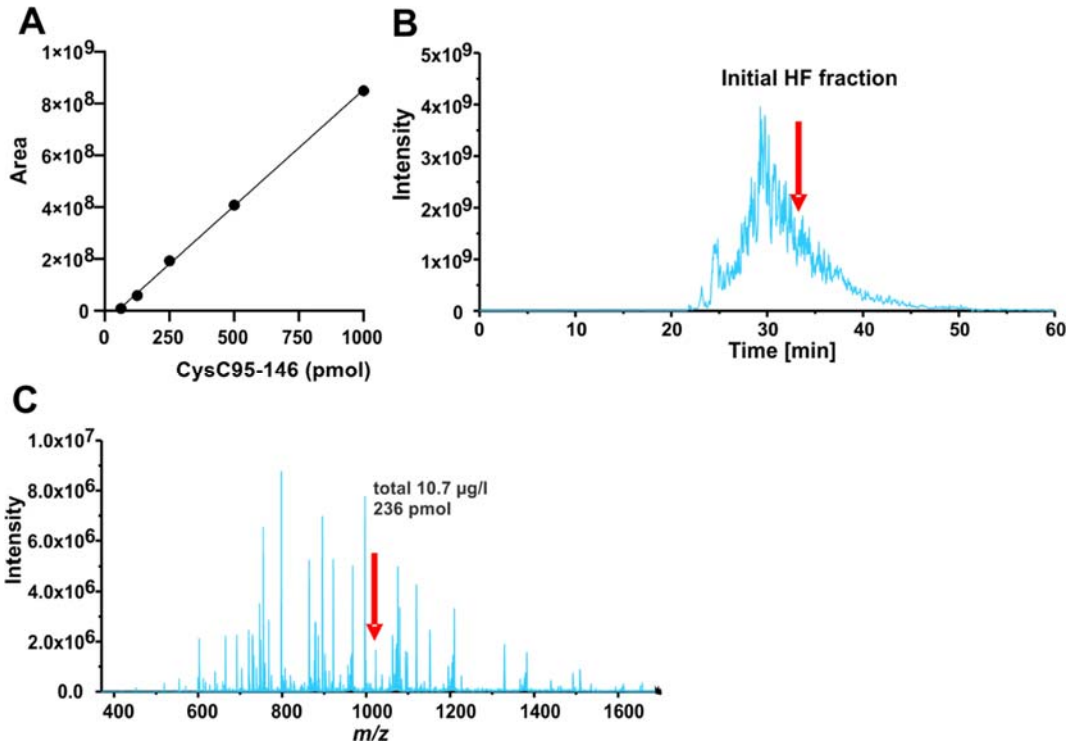

Fig. S3. Detection of CysC95-146 in the HF peptide library. (A) In "X" color: total ion chromatogram (TIC) of the starting HF sample (HF020802 P4 Fr19-20) visualized by XCalibur2.2 from the raw data obtained in the nanoLC-ESI-iTrap-Orbitrap system, and in "Y" color: ion chromatogram obtained after filtering TIC (m/z range 1025.3112±20 ppm; z=6) aiming to illustrate the CysC-F52 position in the starting HF chromatogram. (B) Mass spectrum of the components present within the time range where CysC95-146 is located (signal highlighted). (C) Calibration curve (peak area vs CysC95-146 amount) obtained by the analysis of CysC95-146 standards and the starting material HF 020802 P4Fr19-20, for the quantitation of CysC95-146 in hemofiltrate.

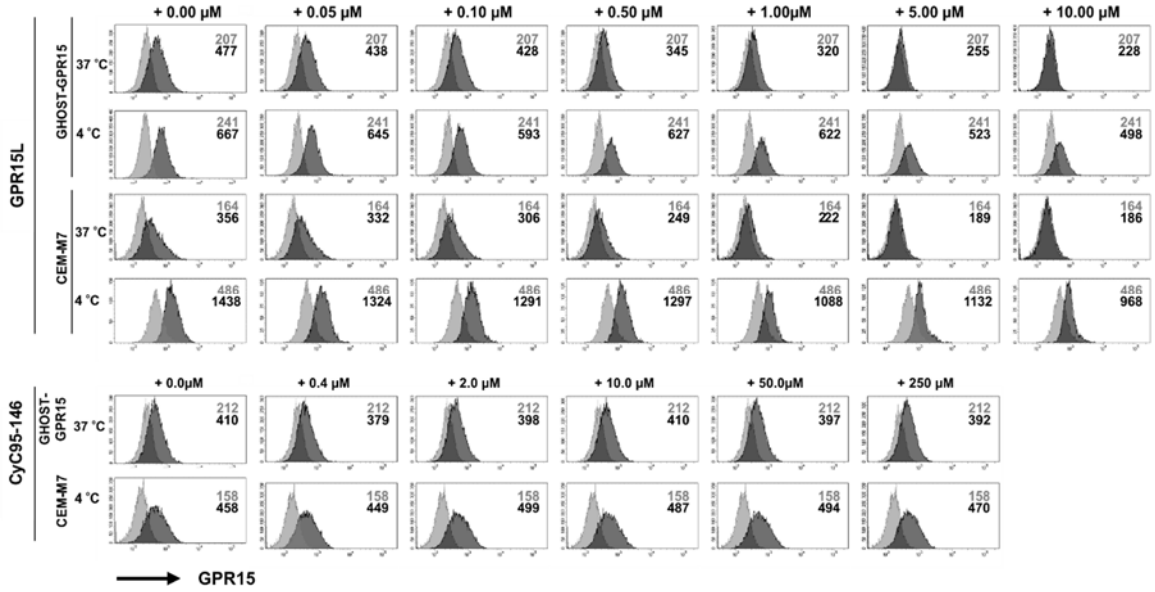

Fig. S4. Effect of GPR15L and CysC95-146 on GPR15 expression. GHOST-GPR15 or CEM-M7 cells were incubated with different concentrations of GPR15L or CysC95-146 for 30 min at either 37°C or 4°C (to prevent receptor internalization) prior to staining with anti-GPR15 or isotype control antibody. GPR15 expression was analyzed by flow cytometry. Shown are the histograms for GPR15 (dark gray) and the respective isotype control (light gray). Numbers indicate the MFIs.

GPR15 Interaction energy

Aa residue	energy (eV)
D2	-2,4
P3	-1,4
E5	-2,1
T6	-1,6
Y14	-4,4
A15	-1,2
T16	-3,0
S17	-1,5
P18	-1,3
S20	-1,3
D21	-1,3
H26	-1,2
T32	-1,3
G97	-1,4
L98	-1,1
L280	-1,2
L281	-1,4
L282	-1,2

Fig. S5. Predicted interaction energies of specific amino acid residues in GPR15 with CysC95-146.

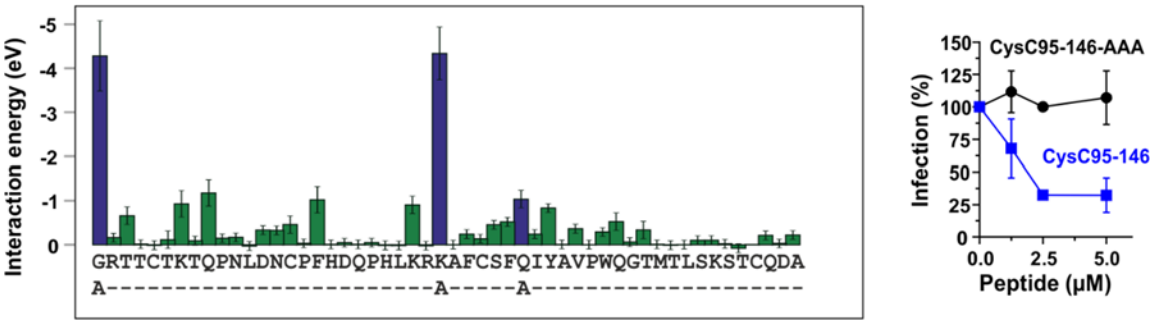

Fig. S6. Effect of amino acid changes of G69A, K94A and Q100A on the antiviral activity of CysC95-146. The left panel shows the predicted amino acid-resolved interaction energies and the position of amino acid changes whilst the right panel shows the effect of the parental and mutant CysC95-146 peptides on SIVmac239 infection in GHOST-GPR15 cells. Data shows mean values +/- SEM, ** $p < 0.01$, * $p < 0.05$ (Multiple t-test, unpaired, corrected for multiple comparisons using the Holm-Sidak method).

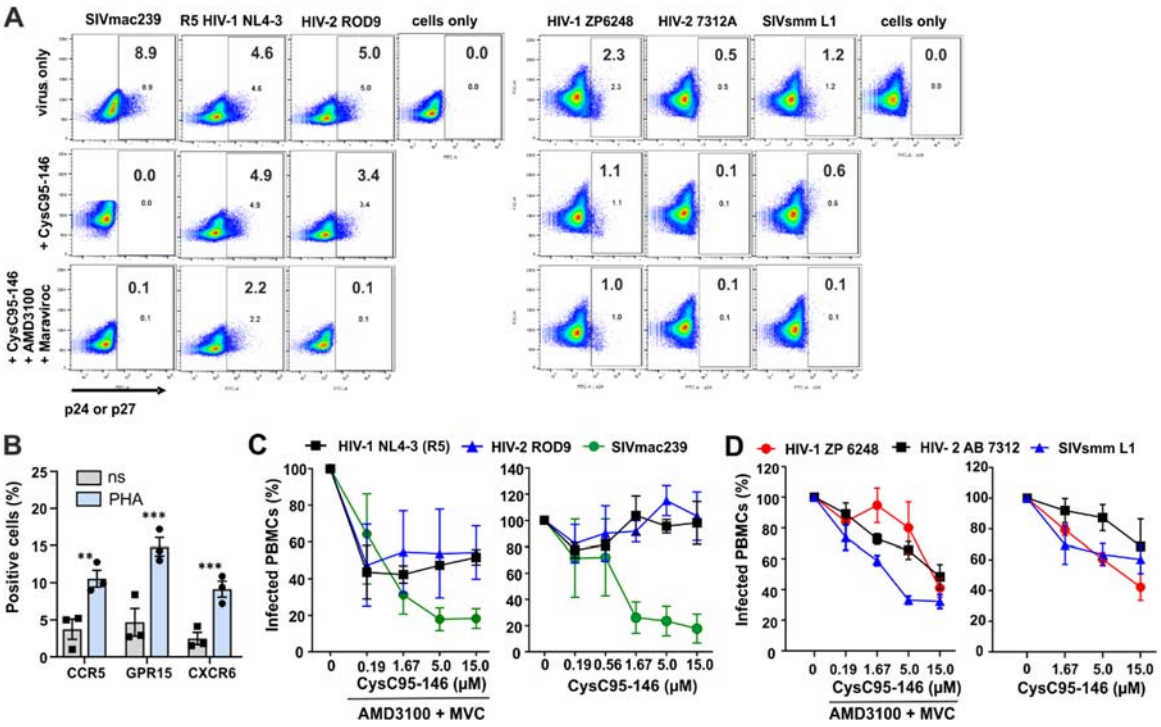

Fig. S7. Effect of CysC95-146 and GPR15L on virus infection and receptor expression in PBMC cultures. (A) CysC95-146 inhibits GPR15-mediated SIVmac239 infection in human PBMCs. Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats of three healthy donors, stimulated with PHA and IL-2. Cells were incubated with the indicated amounts of CysC95-146 or a combination of CysC95-146, AMD3100 and Maraviroc for 2h before infection with the indicated viruses. 3 dpi, PBMCs were stained for p24 and analyzed by flow cytometry. Shown are exemplary FACS data for one donor. Indicated with numbers are the frequency of infected cells. (B) Upregulation of CCR5, GPR15 and CXCR6 in human PBMCs upon stimulation with IL-2/PHA. PBMCs from three donors were isolated from buffy coats as described previously. Cells were then either analyzed for CCR5, GPR15 and CXCR6 expression immediately after the isolation or after a 3 days stimulation with IL-2/PHA. Shown are the mean values of n=3 donors. (C, D) Effect of CysC95-146 on SIV and HIV infection of primary human cells. Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats of three healthy donors, stimulated with IL-2/ PHA and treated with the indicated amounts of CysC95-146. Then, cells were infected with the indicated virus strains. 3 days post-infection, PBMCs were stained for p24 and analyzed by flow cytometry. Shown are the mean values for n = 3 donors +/- SEM and infection in cells obtained in the absence of peptide was set to 100%. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ (Multiple t-test, unpaired, corrected for multiple comparisons using the Holm-Sidak method).

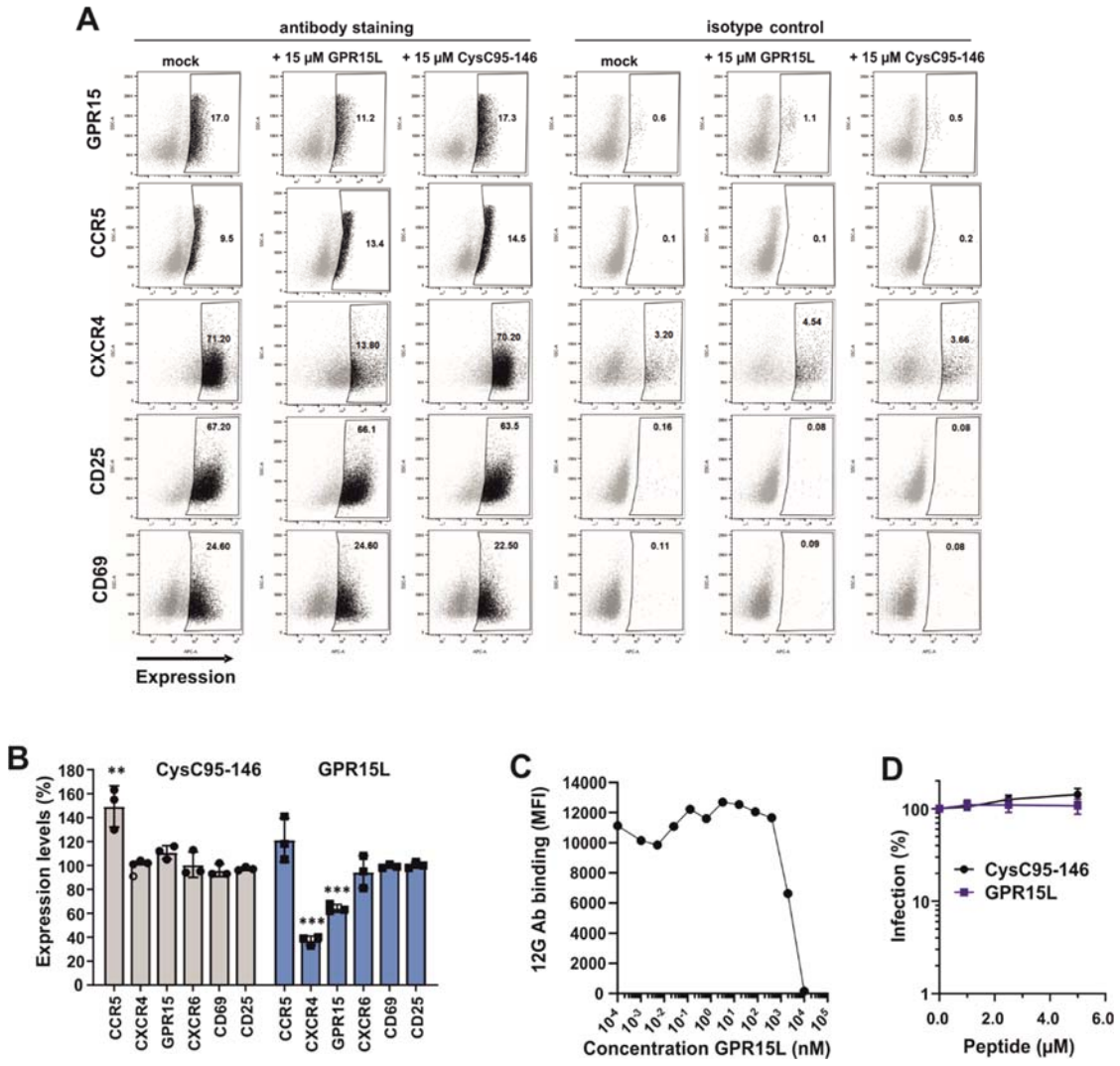

Fig. S8. Effect of CycC95-146 on surface expression of various GPCRs and X4-mediated HIV-1 infection (A) Exemplary FACS data of one donor showing changes in surface expression of GPR15, CCR5, CXCR6, CD25, CD69 and CXCR4 in the presence of CysC95-146 or GPR15L. (B) Changes in the expression levels of different chemokine receptors and/or activation markers in the presence of GPR15L or CysC95-146. Data show normalized expression levels detected in stimulated PBMCs relative to the absence of peptide treatment (100%) of n = 3 donors +/- SEM, *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ (Multiple t-test, unpaired, corrected for multiple comparisons using the Holm-Sidak method). (C) Competition of GPR15L with binding of Ab 12G5 targeting the 2nd extracellular loop of CXCR4. (B) Effect of CysC95-146 and GPR15L (left) or AMD3100 (right) on CXCR4-tropic HIV-1 NL4-3 infection of GHOST-CXCR4 cells. Results were derived from three experiments and show mean values (±SEM) compared to the infection rates measured in the absence of inhibitor (100%).

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