CD4-Specific Designed Ankyrin Repeat Proteins Are Novel Potent HIV Entry Inhibitors with Unique Characteristics

Andreas Schweizer¹, Peter Rusert¹, Livia Berlinger¹, Claudia R. Ruprecht¹, Axel Mann¹, Stéphanie Corthésy¹, Stuart G. Turville²*, Meropi Aravantinou², Marek Fischer¹, Melissa Robbiani², Patrick Amstutz³, Alexandra Trkola¹*¹

¹ Division of Infectious Diseases, University Hospital Zurich, Zurich, Switzerland, ² Center for Biomedical Research, Population Council, New York, New York, United States of America, ³ Molecular Partners AG, Zurich-Schlieren, Switzerland

Abstract

Here, we describe the generation of a novel type of HIV entry inhibitor using the recently developed Designed Ankyrin Repeat Protein (DARPin) technology. DARPin proteins specific for human CD4 were selected from a DARPin DNA library using ribosome display. Selected pool members interacted specifically with CD4 and competed with gp120 for binding to CD4. DARPin proteins derived in the initial selection series inhibited HIV in a dose-dependent manner, but showed a relatively high variability in their capacity to block replication of patient isolates on primary CD4 T cells. In consequence, a second series of CD4-specific DARPin proteins with improved affinity for CD4 was generated. These 2nd series DARPins potently inhibit infection of genetically divergent (subtype B and C) HIV isolates in the low nanomolar range, independent of coreceptor usage. Importantly, the actions of the CD4 binding DARPins were highly specific: no effect on cell viability or activation, CD4 memory cell function, or interference with CD4-dependent virus entry was observed. These novel CD4 targeting molecules described here combine the unique characteristics of DARPins—high physical stability, specificity and low production costs—with the capacity to potent block HIV entry, rendering them promising candidates for microbicide development.

Citation: Schweizer A, Rusert P, Berlinger L, Ruprecht CR, Mann A, et al. (2008) CD4-Specific Designed Ankyrin Repeat Proteins Are Novel Potent HIV Entry Inhibitors with Unique Characteristics. PLoS Pathog 4(7): e1000109. doi:10.1371/journal.ppat.1000109

Copyright: © 2008 Schweizer et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: Support was provided by the Swiss National Science Foundation (PP00B-102647 to AT), by a research grant of the Kanton Zurich, and by research grants from the following foundations: Velux, Vontobel, Novartis, and Hartmann Muller. AT is an Elizabeth Glaser Scientist supported by the Elizabeth Glaser Pediatric AIDS Foundation. Additional support was provided by NIH grant AI040877. MR was a 2002 Elizabeth Glaser Scientist. SGT was supported by a CJ Martin Fellowship of the NH&MRC of Australia.

Competing Interests: PA is affiliated with Molecular Partners AG (Switzerland), which is developing designed repeat proteins as binding agents for research, diagnostics, and therapy.

* E-mail: alexandra.trkola@usz.ch

* Current address: Center for Virus Research, Westmead Millennium Institute, Westmead Hospital and University of Sydney, Sydney, New South Wales, Australia

Introduction

The increasing need for a vaccine to control the HIV pandemic is undoubted, but recent failures of vaccine programs have made clear that it will be years to decades before a successful vaccination program can be installed [1]. In the meantime, drug based intervention strategies must be found to fill the gap and put the continuous spread of HIV at halt, particularly in resource poor settings where 90% of the estimated 33 million HIV infected individuals live [2].

HIV infection is predominantly acquired via heterosexual transmission across mucosal surfaces [3]. Strategies that prevent mucosal transmission are therefore considered to significantly impact on diminishing viral spread [4]. Microbicides, agents that by topical application on mucosal surfaces protect from HIV infection, are regarded as one of the most promising preventive intervention strategies in the absence of effective vaccination programs [2,4,5]. The sought for microbicides against HIV have to fulfill highly specific requirements: Besides promoting strong and reliable protection from HIV infection, these compounds have to be inexpensive, readily available, stable, well tolerated and easy to apply to allow a wide spread use. Recent efforts in microbicide research have mainly focused on chemical compounds of relatively simple composition that provide protection from HIV infection by largely nonspecific (non HIV specific) mechanisms as for instance charge-charge interactions [6]. Although in vivo efficacy of two such candidate microbicides, nonoxynol-9 [7] and cellulose sulfate [8], could not be established [9–12] several other pan-reactive molecules are in development that show promise [4,6,13]. As for all drug interventions against HIV, combination therapy will likely also be necessary in microbicide application to reach potent and broad efficacy. Thus microbicides that target HIV specifically and potentially can be used in combination with pan-reactive molecules are urgently sought for.

Prime targets for microbicide attack are the virus and cellular proteins involved in the early events in infection: the entry receptors CD4, CCR5 and CXCR4, the viral envelope proteins and compounds that interfere post entry with reverse transcription and integration of HIV into the host cell. Application of specific HIV inhibitors targeting these events as topical microbicides has proven effective in blocking mucosal HIV transmission in the SHIV macaque infection model underlining their potential in HIV prevention [4,14–18].
Author Summary

There is an increasing need to develop inhibitors of HIV entry into target cells for both application in therapy and prevention. The development of specific HIV inhibitors as microbicides, agents that by topical application prevent infection, is considered particularly important in limiting the spread of HIV in the absence of effective vaccines. To derive highly potent and specific inhibitors of HIV entry for potential use as microbicide, we employed the recently developed Designed Ankyrin Repeat Protein technology. Using this technique, Designed Ankyrin Repeat Proteins can be evolved that bind their target molecules as specifically and efficiently as antibodies. In the present study, we generated a panel of Designed Ankyrin Repeat Proteins that bind specifically to the cellular CD4 receptor, the main entry receptor of HIV. The obtained proteins are very potent and highly specific inhibitors of HIV entry and provide a broad reactivity against genetically different virus strains. Due to the high physical stability of Designed Ankyrin Repeat Proteins and their low cost production, these novel HIV entry inhibitors represent promising candidates for microbicide development.

To date only few small molecules that inhibit HIV entry have been defined [4]. While protein-based inhibitors are commonly more expensive in production, they can have functional advantages. Most importantly, they provide outstanding target specificity since the contact area between agent and target protein is formed by comparatively large surface patches as for instance in antibody-antigen interactions.

The aim of our study was to derive inhibitors of HIV entry that achieve the desired specificity and potency together with the high physical stability and low production costs required for the application as microbicide. To this end, we made use of the recently established Designed Ankyrin Repeat Protein (DARPin) technology which is based on the principle of naturally occurring ankyrin repeat proteins, a ubiquitously expressed family of proteins mediating specific protein-protein interactions across species [19]. DARPins were designed as an alternative to antibodies: they share the antibodies’ ability to be selected and achieve the desired specificity and potency together with the high physical stability and production costs [20,21]. Highly diverse DARPin DNA libraries, comprising at least $10^{11}$ different sequences per reaction, have successfully been encoded for a minimum of $10^{11}$ individual members [20]. The diversity of the library is further increased by introducing errors through the polymerase used in subsequent PCR cycles. Library selections were performed against the tetrameric CD4 fusion protein CD4-IgG2 (kindly provided by W. Olson, Progenics Pharmaceuticals; [30]) which was immobilized via a Fab-specific anti human IgG-antibody (Sigma). For selections, PCR-amplified libraries were transcribed and three standard ribosome-display selection rounds were performed as described [23,31,32]. Two alternate approaches were probed in the fourth selection round to achieve highly specific binders: i) a standard ribosome display selection round with more extensive washing (3 h in total) and ii) the use of purified gp120 of the R5-tropic virus JR-FL (1 nM; kindly provided by W. Olson Progenics Pharmaceuticals) to elute binders that compete with viral glycoprotein for binding to CD4. The RT-PCR products of the genes obtained after both fourth cycles were combined in a pool (termed 1st series binders) and then used for a single clone analysis as described below.

In a separate line of experiments we aimed to select binders with improved affinities for CD4. To this end, all round 3 and round 4 sublibraries were transcribed and translated in vitro as described [33]. Then the ternary complexes of ribosome, mRNA, and displayed proteins were equilibrated with 1 nM biotinylated CD4-IgG2 at 4°C for 1 h before 1 μM non-biotinylated CD4-IgG2 was added. The aliquots were incubated for 3 h at 4°C and the complexes were recovered by binding to streptavidin-coated magnetic beads (Roche Applied Science) for 30 min. The beads were washed five times, and the RNA was eluted and purified as described [33]. The pool of binders derived from this affinity selection was termed 2nd series binders and characterized as described below.

Detection of selected binders by ELISA

CD4-IgG2 was immobilized via a Fab-specific anti-IgG capture antibody (Sigma) on Maxisorp 96-well plates (Nunc). To screen for CD4 binders, 100 μl each of crude *Escherichia coli* extracts containing DARPins or purified DARPins were applied to wells containing immobilized CD4-IgG2 and to wells containing the capture antibody alone. Bound DARPins were detected upon incubation with anti-RGS-His antibody (Qiagen), anti-mouse-IgG-alkaline phosphatase conjugate (Sigma) and p-nitrophenylphosphate (Sigma) as substrate. Wells without CD4-IgG2 were used as negative controls to verify the binding specificity of the tested DARPins.

Competition ELISA

For the gp120 competition ELISA the same setup as described above was employed. CD4-IgG2 coated plates were incubated with JR-FL gp120 (0–800 nM; kindly provided by Progenics Pharmaceuticals) for 1 h at 25°C before pure DARPins (200 nM) were added. Detection and readout was carried out as described above. For the competition ELISA using CD4-directed monoclonal antibodies (mAbs) as competitors, soluble CD4 (20 nM, Progenics Pharmaceuticals) was biotinylated using EZ-link sulfo-NHS-LC-biotin (Pierce) according to the manufacturer’s instructions and immobilized via neutravidin (Pierce, 66 nM) on Maxisorp 96-well plates (Nunc). mAbs L222, Q4120, 13B82 [34,35] and 5A8 [36] were kindly provided by Q. Sattentau. DARPin (20 nM) plus different CD4-antibodies (66 nM) were added and incubated at

Methods

Ribosome display and selection for binders with improved affinities

An introduction into the DARPin technology and ribosome display is provided as Supporting Information (Protocol S1 and Figures S1 and S2). Detailed specifics on the use and generation of DARPin libraries has been described previously [20]. Here, N2C and N3C libraries encoding for DARPins consisting of an N- and a C-terminal capping repeat, and either two (N2C) or three (N3C) internal ankyrin repeat modules containing randomized amino acid residues, were used. The theoretical diversity of the N3C library is $3.8 \times 10^{22}$. Ligated library DNA used in the selection described here encoded for a minimum of $10^{11}$ individual members [20]. The diversity of the library is further increased by introducing errors through the polymerase used in subsequent PCR cycles. Library selections were performed against the tetrameric CD4 fusion protein CD4-IgG2 (kindly provided by Bili Olson, Progenics Pharmaceuticals; [30]) which was immobilized via a Fab-specific anti human IgG-antibody (Sigma). For selections, PCR-amplified libraries were transcribed and three standard ribosome-display selection rounds were performed as described [23,31,32]. Two alternate approaches were probed in the fourth selection round to achieve highly specific binders: i) a standard ribosome display selection round with more extensive washing (3 h in total) and ii) the use of purified gp120 of the R5-tropic virus JR-FL (1 μM; kindly provided by W. Olson Progenics Pharmaceuticals) to elute binders that compete with viral glycoprotein for binding to CD4. The RT-PCR products of the genes obtained after both fourth cycles were combined in a pool (termed 1st series binders) and then used for a single clone analysis as described below.

In a separate line of experiments we aimed to select binders with improved affinities for CD4. To this end, all round 3 and round 4 sublibraries were transcribed and translated in vitro as described [33]. Then the ternary complexes of ribosome, mRNA, and displayed proteins were equilibrated with 1 nM biotinylated CD4-IgG2 at 4°C for 1 h before 1 μM non-biotinylated CD4-IgG2 was added. The aliquots were incubated for 3 h at 4°C and the complexes were recovered by binding to streptavidin-coated magnetic beads (Roche Applied Science) for 30 min. The beads were washed five times, and the RNA was eluted and purified as described [33]. The pool of binders derived from this affinity selection was termed 2nd series binders and characterized as described below.

Detection of selected binders by ELISA

CD4-IgG2 was immobilized via a Fab-specific anti-IgG capture antibody (Sigma) on Maxisorp 96-well plates (Nunc). To screen for CD4 binders, 100 μl each of crude *Escherichia coli* extracts containing DARPins or purified DARPins were applied to wells containing immobilized CD4-IgG2 and to wells containing the capture antibody alone. Bound DARPins were detected upon incubation with anti-RGS-His antibody (Qiagen), anti-mouse-IgG-alkaline phosphatase conjugate (Sigma) and p-nitrophenylphosphate (Sigma) as substrate. Wells without CD4-IgG2 were used as negative controls to verify the binding specificity of the tested DARPins.

Competition ELISA

For the gp120 competition ELISA the same setup as described above was employed. CD4-IgG2 coated plates were incubated with JR-FL gp120 (0–800 nM; kindly provided by Progenics Pharmaceuticals) for 1 h at 25°C before pure DARPins (200 nM) were added. Detection and readout was carried out as described above. For the competition ELISA using CD4-directed monoclonal antibodies (mAbs) as competitors, soluble CD4 (20 nM, Progenics Pharmaceuticals) was biotinylated using EZ-link sulfo-NHS-LC-biotin (Pierce) according to the manufacturer’s instructions and immobilized via neutravidin (Pierce, 66 nM) on Maxisorp 96-well plates (Nunc). mAbs L222, Q4120, 13B82 [34,35] and 5A8 [36] were kindly provided by Q. Sattentau. DARPin (20 nM) plus different CD4-antibodies (66 nM) were added and incubated at
25°C for 1 h. Bound DARPin was detected by ELISA using an anti-poly-His-alkaline phosphatase conjugate (Sigma) as described above. Wells without added antibody where included as control and defined as 0% competition. Competition was rated as follows: −, +, ++, and +++ for signal decreases of 0–25%, 25–50%, 50–75% and 75–100%, respectively.

Protein purification and endotoxin removal
DARPin were produced in soluble form in E. coli and purified using Ni-NTA affinity chromatography as described [37]. Endotoxins (lipopolysaccharides) were removed using 0.1% Triton X-114 as described [30] and the DARPin were further purified using EndoTrap red columns (Propolis) according to the manufacturer’s recommendations. The remaining endotoxin content was determined using the kinetic chromogenic limulus amebocyte lysate assay (Endotell) according to the manufacturer’s instructions. All DARPin preparations used for investigation of cellular activation had endotoxin levels below 0.5 EU/mg.

Surface plasmon resonance (SPR)
All SPR measurements were performed at 25°C using a Biacore 3000 instrument and a SA sensor chip (Biacore). To immobilize CD4-IgG2, the protein was first chemically biotinylated using EZ-Link sulfo-NHS-LC-biotin (Pierce). The individual DARPin were applied in various concentrations (0.25–1’000 nM, depending on affinity) to a flow-cell with immobilized CD4-IgG2 for 180 s at 50 µl/min, followed by washing with buffer. The signal of an uncoated reference cell was subtracted from the measurements. The kinetic data of the interactions were evaluated with a global fit using the BIasevaluation 3.0 software (Biacore).

Generation of human mouse CD4 domain 1 chimera
A chimeric construct coding for human CD4, where the human domain 1 sequence is replaced by its murine homologue sequence, was constructed as follows: in pEYFP-N1-hCD4 (a kind gift from Jun-ichi Fujisawa [39]), an expression vector for human CD4, a ScaI restriction site was introduced at position 10 in CD4-domain 1 by two conservative nucleotide exchanges via site directed mutagenesis (QuikChange XL, Stratagene), resulting in plasmid pEYFP-N1-hCD4-Sca. The murine CD4-D1 domain was amplified by PCR from the plasmid pCMV-Sport6-mCD4 with primers mD1_fw: gtcactcaaggagcatgtagggaagggaggg and mD1_rev: ggtcaggctctgcccctgcagcaggtgggtacccggactgaagg. The PCR product was digested with BstEII and XhoI restriction sites, were digested with these two enzymes and the PCR-generated insert encoding the murine CD4-domain 1 was ligated into the human CD4 plasmid finally resulting in pEYFP-N1-hCD4mD1.

Immunofluorescent staining and analysis
Cells (100'000/well) were incubated with DARPin (200 nM) for 20 min at 37°C. Bound DARPin was detected using anti-RGS-His antibody (Qiagen) and goat-anti-mouse phycoerythrin labeled antibody (Caltag). Binding of DARPin to CD4+ A5.01 cells, CD4+ A2.01 cells (NIH AIDS Research & Reference Reagent Program, No. 2059 and 166), CEM5.25luc-gfp (CD4+; provided by N. Landau) and TZM-bl cells (CD4+; [40]) was investigated. Cells were washed three times between all incubation steps using PBS containing 0.1% azide and 1% BSA. After the last step, cells were fixed (in PBS, 0.1% azide, 1.25% formaldehyde) and subjected to flow cytometry using a FACSCalibur flow cytometer (BD Biosciences) and FlowJo software (Tree Star).

To measure the effect of DARPin on cellular CD4 expression, untouched CD4+ T cells were isolated from CD8-depleted peripheral blood mononuclear cells (PBMC) of healthy donors using the CD4+ T cell isolation kit II (Miltenyi Biotech) according to the manufacturer’s instructions. Purity of the isolated CD4+ T cells was routinely >97%. CD4+ T cells were cultured in the presence or absence of indicated DARPin (200 nM) for 1 h, 3 h, or 18 h. Thereafter, CD4+ T cells were washed twice, stained with PE-labeled anti-CD4 (Caltag) and analyzed for CD4 expression by flow cytometry.

To analyze overlapping binding patterns amongst the selected CD4 specific DARPins, competition of DARPin to bind to cellular CD4 was investigated. To this end, DARPin 29.2 and 57.2 were chemically modified with the HLX63 fluorescent dye (Invitrogen) according to the manufacturer’s recommendations and purified by size exclusion using NAP5 columns (GE Healthcare). CD4+ A3.01 cells were incubated with the fluorescently labeled DARPin at 20 nM (10+, 25+, 50+) followed by addition of the unlabeled DARPin (1 µM, 20+, 25+) after 1 h. Cells were washed thereafter and analyzed by flow cytometry.

Stimulated primary CD8-depleted PBMC
CD8+ T-cell depleted (Rosette Sep cocktail, StemCell Technologies Inc.) PBMC were isolated by Ficoll-Hypaque centrifugation ofuffy coats obtained from three healthy blood donors. Cells were adjusted to 4×10⁶/ml in culture medium (RPMI 1640 medium, 10% fetal calf serum, 10 U/ml interleukin-2, glatimine, and antibiotics), divided into three parts, and stimulated with 5 µg/ml phytohemagglutinin (Sigma), 0.5 µg/ml phytohemagglutinin, or anti-CD3 mAb OKT3 as previously described [42]. After 72 h, cells from all three stimulations were combined (referred to as three-way-stimulated PBMC) and used as a source of stimulated CD4+ T cells for infection and virus isolation experiments.

Replication competent viruses
Replication competent viruses were produced by infection of three-way-stimulated PBMC. The 50% tissue culture infectious dose (TCID₅₀) was determined by end point dilution. Infections were detected by p24 ELISA. In sum 10 subtype B viruses, including 7 R5 users (JR-FL, SF-162, Pat 17, Pat 20, Pat 111, Pat 114, Pat 120) and 3 X4 users (NL4-3, 2044 and Pat 19) were probed. Pat 17 is a R5 tropic primary isolate derived from plasma of a chronically HIV infected individual as described [43]. The origin of the other viruses has been described previously [42,43].

Env-pseudotyped HIV
Env-pseudotyped HIV was generated by transfection of 293T cells with plasmids encoding the reporter gene expressing virus backbone, pNLuc [44] (kindly provided by A. Marozsan and J. P. Moore) and the respective functional envelope clone using 25 kD polylethylamine as described [41]. Viral supernatants were harvested 2 days post transfection and the TCID₅₀ was determined by end point dilution. Infections were measured by firefly luciferase activity (Bright-Glo Luciferase Assay System, Promega). Plasmids encoding envelopes of R5 using viruses of subtype B [41] and stained 48 h post transfection with fluorescently labeled DARPin at a concentration of 5 to 50 nM and subsequently analyzed by flow cytometry.
DU422.1) were kindly provided by D. Montefiori [45,46]. Plasmids encoding envelopes of JR-FL and NL4-3 were provided by N. Landau and the plasmid encoding the envelope of SF162 was provided by L. Stamatatos.

Neutralization assays using env-pseudotyped virions on TZM-bl cells

Neutralization assays on TZM-bl cells using pseudotype viruses were performed as described [40]. Briefly, TZM-bl cells (10'000/well; 96well format) were preincubated for 1 h at 37°C with serial dilutions of DARPin and were then infected with aliquots of the viruses (100 TCID50) together with DEAE dextran [10 μg/ml] in a total infection volume of 200 μl. After three days, the cells were lysed using Glyc-lysis buffer (Promega) and luciferase activity determined upon addition of Glyc-substrate (Promega) on a Dynex Technologies Luminometer. The DARPin concentration causing 50, 70, 90% reduction in p24 antigen production was determined by regression analysis.

Potential synergistic effects of combinations of the CD4-specific DARPin 25.2 with other entry inhibitors were investigated with JR-FL pseudotyped virus on the TZM-bl reporter cell line. Combination indices [CI] were calculated using the Loewe additivity formula [47–49]:

\[
\frac{D_{A}(I)}{D_{A}(I)} + \frac{D_{B}(I)}{D_{B}(I)} = CI_{AB}(I)
\]

D_A(I) is the dose of drug A alone required to result in inhibition I and D_A(AB) / D_B(I) the dose of drug A in the combination of A+B required to give the inhibition I. CI of 1 indicates additivity, <1 synergy and >1 antagonism.

PBMC based neutralization assay

Inhibition of replication-competent virus infection of primary human CD4 T cells was assessed essentially as described [50]. Briefly, stimulated CD8 depleted PBMC (100'000/well) were preincubated for 1 h with DARPin at 37°C, followed by infection with the respective replication-competent virus (100 TCID50). After incubation for 6 to 8 days, p24 antigen production was determined in cellular supernatant by ELISA as described [49,51]. The DARPin concentration causing 70% reduction in p24 antigen production was determined by regression analysis as described [42].

For macaque PBMC based neutralization assays, macaque PBMC were cultured with 5 μg/ml of PHA-P (Sigma) for 3 days, before being plated at 2×10^5 cells per well of a 96 well plate (Becton Dickinson) in medium with 50 μU/ml IL-2. Graded doses of the CD4-specific DARPin 25.2 or the control E3_5 DARPin were added to each well (duplicates per dose) and incubated for 1 h at 37°C. After the incubation, 1000 TCID50 of SIVmac239 was added to each well (with 50 μU/ml IL-2). The cells were cultured for 7 days (adding more IL-2 every other day), after which the cells were collected and lysed for PCR. SIV infection was determined by regression analysis as described [42].

Assessment of T cell proliferation

Labeling of PBMC with CFSE (carboxy-fluorescein succinimidyl ester) was performed as described [54]. Briefly, CD8-depleted PBMC from a single donor were stained 8 min at room temperature with 3 μM CFSE (Molecular Probes). Staining was stopped by addition of an equal amount of FCS and cells washed three times with PBS containing 1% FCS. CFSE-labeled cells were incubated with 500 nM endotoxin purified DARPin (1 h at 37°C) and cultured for 4 days in RPMI 1640 containing 10% FCS, antibiotics, 100 U/ml interleukin-2 and anti-CD3 mAb OKT3. The cells were analyzed by flow cytometry using anti-CD3-PE and propidium iodide for gating. Proliferation of cells was assessed on the basis of the shifts in the CFSE-labeling intensity using the FlowJo software as described [54].

Assessment of T helper memory cell function in presence of CD4 specific DARPin

To assess whether CD4-specific DARPin interferes with T helper memory cell functions, activation of antigen-specific T cells in presence and absence of DARPin 55.2 and the control E3_5 using a standard interferon-γ ELISPOT assay was assessed [55]. Briefly, 96-well membrane plates (MAIP S45, Millipore) were coated overnight with anti-human IFN-γ antibody (1-D1K, MAbtech). CD8-depleted PBMC were isolated one day prior to the experiment and cultured in RPMI 1640 containing 10% FCS and antibiotics overnight. The next day cells were preincubated with 200 nM (streptokinase/streptodornase experiment) or 250 nM (cytomegalovirus experiment) endotoxin free DARPin 55.2 and E3_5 for 1 h at 37°C. Cells (2×10⁶) were then seeded into wells of the coated 96-well membrane plates and stimulated with either streptokinase/streptodornase (400 U/ml) or cytomegalovirus (CMV)-lysat (10 μg/ml) overnight at 37°C. Phytohaemagglutinin (10 μg/ml) was added as positive control. IFN-γ production was determined by sequential addition of a detection antibody cocktail containing a biotinylated anti-human IFN-γ antibody (7-B6-1, MAbtech), streptavidin alkaline phosphatase (MAbtech), followed by washing. AP (alkaline phosphatase) conjugate substrate kit (Biorad) was used and the resulting colored spots were quantified using an ELISpot reader (AID). Background reactivity observed in cultures without stimulation was subtracted and results are expressed as specific spot forming cells (SFC) per 10⁶ CD8-depleted PBMC.

Interference of DARPin with CD4:MHC class II interaction

To study if CD4 specific DARPin interfere with CD4:+MHC class II interaction, we performed a cell based binding assay based on rosette formation between CD4 and MHC class II expressing cells [56]. Briefly COS-7 cells (ATTC CRL-1651; cultivated in DMEM, 10% FCS) were seeded at a density of 200'000 cells per 6-well, and one day later transfection with the CD4 encoding plasmid pEYFP-N1-hCD4 ([39]) using the Ca-phosphate transfection system (Promega) according to the manufacturer’s instructions. Transfection medium was replaced 8 h later and two days post transfection cells were utilized in the rosette assay. To this end CD4 expressing and control COS-7 cells were treated with CD4 specific DARPin 23.2, 25.2, 27.2, 29.2, 55.2, 57.2, and a control DARPin (E3_5), buffer or the anti-CD4 E. coli lipopolysaccharide (2.5 EU/ml; Charles River Endosafe) was used as control. Finally, to assess the activation status of the cells, DC were stained with PE-labeled anti-CD80 (BD Biosciences) and with propidium iodide (BD PharMingen) and CD80 expression levels were quantified by flow cytometry.
antibody Q4120 specific for domain 1 (Sigma; 100 nM), which is known to block CD4 binding to MHC II, for 30 min at 37 °C at a concentration of 50 nM or 200 nM. Subsequently, medium was removed, and cells incubated with 1×10^6/well Raji B cells (NIH AIDS Research & Reference Reagent Program, No. 9944) cultivated in RPMI1640, 10% FCS containing identical concentrations of inhibitors. After 1 h incubation at 37 °C non-adherent cells were removed by washing the wells gently seven times with medium. Cells were then fixed with 1.5% paraformaldehyde (PFA) and rosette formation assessed microscopically.

Crossreactivity with macaque CD4
Crossreactivity with rhesus CD4 was investigated using PBMC from adult male and female rhesus macaques (Macaca mulatta) which were housed at the Tulane National Primate Research Center (TNPRC, Covington, USA). Animals were anesthetized with ketamine-HCl (10 mg/kg) prior to heparinized blood samples being taken (no more than 10 ml/kg/month/animal). Protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the TNPRC. Animal care procedures were in compliance with the regulations detailed in the Animal Welfare Act and in the “Guide for the Care and Use of Laboratory Animals.” PBMC were isolated using Ficoll-Hypaque density gradient centrifugation (GE Healthcare). Cells were washed twice in 1× PBS and resuspended in FACS wash (FW) buffer (1× PBS supplemented with 1% human serum and 1 mM EDTA, both from Sigma). For DARPin staining, 4×10^5 macaque PBMC were resuspended in 50 μl FW buffer in a 96 well plate (BD Biosciences). DARPins, 2 μl of each (5 μM), were added to the cells and incubated for 20 min at 4 °C. Cells were washed twice in FW buffer and CD4 T cells were identified using a 1/25 dilution of FITC-conjugated anti-CD3 (clone Sp34, BD Pharmingen) and PE-conjugated anti-CD4 (clone L200, BD Pharmingen). PE- and FITC-conjugated isotype Ig controls were included in all experiments and typically gave signals <1 log of fluorescence. To detect DARPin binding, cells were incubated with a 1/100 dilution of the anti-Penta-His Alexa Fluor 647 conjugate (Qiagen). The DARPin negative control was no DARPin with anti-Penta-His Alexa Fluor 647. Gates were set to include all mononuclear leukocytes based on the forward- and side-scatter characteristics (excluding any contaminating neutrophils). The gates used to define the CD3/CD4 cells were determined based on the isotype controls. All samples were acquired on a FACSCalibur (BD Biosciences) and analyzed using FlowJo software (Tree Star, Inc.). Mean fluorescent intensities (MFI) of DARPin staining in the CD3/CD4 population were adjusted by subtracting the MFI of the negative DARPin control. Standard deviations represent n = 4 animals, processed and stained in parallel.

Results
Selection and biochemical characterization of CD4-specific DARPins
DARPins specific for human CD4 were selected using PBMC from adult male and female Chinese rhesus macaques (Macaca mulatta) which were housed at the Tulane National Primate Research Center (TNPRC, Covington, USA). Animals were anesthetized with ketamine-HCl (10 mg/kg) prior to heparinized blood samples being taken (no more than 10 ml/kg/month/animal). Protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the TNPRC. Animal care procedures were in compliance with the regulations detailed in the Animal Welfare Act and in the “Guide for the Care and Use of Laboratory Animals.” PBMC were isolated using Ficoll-Hypaque density gradient centrifugation (GE Healthcare). Cells were washed twice in 1× PBS and resuspended in FACS wash (FW) buffer (1× PBS supplemented with 1% human serum and 1 mM EDTA, both from Sigma). For DARPin staining, 4×10^5 macaque PBMC were resuspended in 50 μl FW buffer in a 96 well plate (BD Biosciences). DARPins, 2 μl of each (5 μM), were added to the cells and incubated for 20 min at 4 °C. Cells were washed twice in FW buffer and CD4 T cells were identified using a 1/25 dilution of FITC-conjugated anti-CD3 (clone Sp34, BD Pharmingen) and PE-conjugated anti-CD4 (clone L200, BD Pharmingen). PE- and FITC-conjugated isotype Ig controls were included in all experiments and typically gave signals <1 log of fluorescence. To detect DARPin binding, cells were incubated with a 1/100 dilution of the anti-Penta-His Alexa Fluor 647 conjugate (Qiagen). The DARPin negative control was no DARPin with anti-Penta-His Alexa Fluor 647. Gates were set to include all mononuclear leukocytes based on the forward- and side-scatter characteristics (excluding any contaminating neutrophils). The gates used to define the CD3/CD4 cells were determined based on the isotype controls. All samples were acquired on a FACSCalibur (BD Biosciences) and analyzed using FlowJo software (Tree Star, Inc.). Mean fluorescent intensities (MFI) of DARPin staining in the CD3/CD4 population were adjusted by subtracting the MFI of the negative DARPin control. Standard deviations represent n = 4 animals, processed and stained in parallel.

Selection for improved affinities results in higher inhibition potencies
This relatively high variability in suppressing virus replication on primary CD4+ T cells suggested that DARPins with superior activity are needed to achieve potent and broad inhibition of genetically diverse isolates in vivo. We reasoned that increasing the affinity of the DARPins to CD4 is the most feasible strategy to boost their potency in inhibiting HIV entry. To enrich for DARPins with high affinity for CD4 we performed off-rate selections during ribosome display to specifically select for proteins with low dissociation rates [59]. To that end, we combined the DNA-sublibraries generated during the first selection rounds and performed a single round of off-rate selection where dissociation of DARPins with low affinity was induced by addition of excess CD4 analyzed. This pool of DARPins obtained after four rounds (referred to as 1st series pool) was screened for CD4 specificity directly from crude bacterial lysates by ELISA (Figure 1A). More than 50% of the examined candidate DARPins showed specific binding (signal/background >2), whereas unselected DARPins showed no interaction with immobilized CD4-IgG2 (data not shown). Out of this pool of CD4-specific DARPins, six candidate proteins with the most favorable binding properties in the ELISA screen were chosen (referred to as 1st series binders) and purified to homogeneity for further investigations. The six selected proteins were purified and their capacity to bind to CD4 in presence and absence of gp120 assessed (Figure 1B). Notably, all six selected DARPins interfered with gp120 binding to CD4. We further analyzed the ability of DARPins to interact with the native CD4 receptor in a cellular context. All probes selected DARPins bound to CD4+ cell lines and to primary CD4+ T cells but not to CD4− cell lines, whereas the unselected control DARPin, E3.5, did not interact with any of the tested cell lines (Figure 1C and data not shown).

As affinity and kinetics of the interaction with CD4 are anticipated to steer the efficacy of the DARPins as inhibitors of HIV entry, we investigated the interaction of one candidate from the 1st series pool, DARPin 3.1, with CD4 by kinetic SPR measurements. Association and dissociation experiments at various concentrations of DARPin 3.1 with immobilized CD4-IgG2 yielded a dissociation constant (K_D) value of 8.9 nM, which is in the range of high affinity antibodies (Table 1).

CD4-specific DARPins are potent HIV entry inhibitors
To explore the effect of CD4-specific DARPins on HIV entry we evaluated the inhibitory activity of our panel of CD4-DARPins in vitro using a standardized assay system based on infection of TZM-bl reporter cells with envelope pseudotyped HIV particles [58]. All tested DARPins inhibited HIV entry of JR-FL, SF-162 and NL4-3 env-pseudotype viruses in a dose-dependent manner with IC50 values ranging from 67.8 nM to 820 nM (Supporting Table S1). Importantly, none of the CD4-selected DARPins had an effect on CD4-independent virus entry as demonstrated by their inability to block entry of murine leukemia virus (data not shown). Equally, an unselected DARPin (E3.5) had no effect on HIV entry (Figure 2B and data not shown).

When we further explored the effects of the DARPins against a panel of 10 replication-competent R5 or X4 virus isolates of subtype B on primary lymphocytes (Figure 2A) we confirmed that all selected DARPins inhibited replication of the tested virus isolates, even over multiple rounds of replication. Notably though, we observed a considerable variability in the sensitivity of different viruses with IC70 values ranging from <24 nM up to >1 μM, with a relatively high resistance of the three probed X4 isolates to the DARPin inhibitors.
Figure 1. DARPin interact specifically with CD4 and compete with gp120 for binding to CD4. (A) Binding of DARPin, in the form of crude bacterial lysates, to CD4-IgG2 (black) is determined by ELISA and compared with binding to the capture antibody alone (gray). DARPin A–F show specific binding to CD4-IgG2, a property that was confirmed by further tests using the purified proteins. DARPins G and H reveal nonspecific binding whereas I and J are examples of library members that do not recognize the target protein. (B) Competition ELISA using soluble gp120 as competing ligand. Shown is binding of 200 nM of the CD4-specific DARPin 1.1 to 6.1 in competition with 0 nM, 50 nM and 800 nM gp120. Binding of DARPin alone was defined as 100% and background binding as 0%. (C) Binding of the DARPin to cellular CD4 was tested using A2.01 cells (CD4^+), the CD4 expressing lines A3.01 cells, TZM-bl cells, CEM 5.25 cells and CD8-depleted PBMC as source of primary CD4 T cells. CD4-specific DARPins D3.1 (blue), D23.2 (red), and of a control DARPin, E3_5 (black), an unselected library member binding to the various cell types are shown. PE-labeled CD4-antibody (clone Q4120, Sigma) (green) was used as positive control and a PE-labeled goat-anti-mouse antibody (shaded in gray) as negative control. The shifts in fluorescence intensity correspond to the differences in affinities of the DARPins for CD4 (see Supporting Table S1). Representative data from 2–4 independent experiments are shown.

doi:10.1371/journal.ppat.1000109.g001

Table 1. Dissociation constants of CD4 specific DARPin as determined by surface plasmon resonance (SPR).

| Binder | kon 1 [1/Ms]; kon 2 [1/s] | koff 1 [1/s]; koff 2 [1/s] | K_D [M] | fitting model | Chi^2/Rmax |
|--------|-----------------|-----------------|--------|-------------|----------|
| D3.1   | 9.43E+05 1.03E-01 | 8.93E-09 3.09E-03 | 2.25% |
| D23.2  | 2.96E+06 7.66E-04 | 2.59E-10 1.66E-03 | 4.04% |
| D27.2  | 1.39E+06 2.44E-03 | 1.75E-09 1.02E-03 | 4.70% |
| D29.2  | 1.11E+06 1.66E-03 | 1.49E-09 8.39E-10 | 1.64% |
| D55.2  | 1.43E+06 1.20E-03 | 8.39E-10 1.02E-03 | 4.70% |
| D57.2  | 1.39E+06 2.44E-03 | 1.75E-09 1.02E-03 | 1.64% |

^1Data required use of the two state model, which assumes a conformational change upon binding, for fitting.  
^2Data gave best fits using the standard 1:1 langmuir binding model.  
doi:10.1371/journal.ppat.1000109.g001
In solution. From this pool of binders we chose a panel of six DARPins, D23.2, D25.2, D27.2, D29.2, D55.2 and D57.2 (referred to as 2nd series binders), for further analysis.

When we assessed this panel of 2nd series binders using kinetic SPR measurements we found that off-rate selection had indeed resulted in selection of binders with dissociation constants ($K_D$) that were almost exclusively in the subnanomolar range (Table 1).

When compared to DARPin 3.1, the most potent inhibitor of the 1st series, this represents a 5 to 10-fold decrease in $K_D$ values. Importantly, this substantial increase in affinity was also reflected by a dramatic increase in HIV entry inhibition potency of the 2nd series over the 1st series binders (Figure 2B). The IC50 values of the six affinity improved binders against the reference strains JR-FL, SF162 and NL4-3 in the TZM-bl based assay were in the range of 1.1 to 5.1 nM, 1.2 to 7.7 nM, and 2.7 to 10.5 nM, respectively (Supporting Table S1). In sum, this represents about a 70-fold reduction in inhibitory concentrations ($p<0.0001$, unpaired t test) over the 1st series DARPin inhibitors and renders the 2nd series inhibitors equal in potency to the clinically approved entry inhibitor T-20 [60–62], which was probed alongside and inhibited replication of JR-FL, SF-162 and NL4-3 pseudotyped viruses with IC50 values of 1.1 nM, 3.1 nM and 8.1 nM, respectively.

While the 1st series DARPins displayed a relatively wide variability in their potency to inhibit infection of PBMC by replication-competent viruses (Figure 2A, Table S1), the 2nd series DARPins were significantly improved and blocked virus replication at IC70s in the very low nanomolar range (2.1 nM-30.9 nM; Figure 2B and Table S1). The most potent inhibitors of this pool, DARPin 55.2 and 57.2 blocked HIV replication of the three probed viruses, JR-FL, SF-162 and NL4-3, with IC70 values between 2.1 and 7.8 nM.

**Potency and breadth of CD4-specific DARPins**

To obtain more detailed information on potency and breadth of the CD4-specific DARPins we analyzed the activity of DARPin...
3.1, the most potent inhibitor of the 1st series pool, and DARPin 55.2, as representative of the 2nd series, against a reference panel of nine subtype B and four subtype C env-pseudotyped R5 viruses (Figure 3A). Notably, D3.1 only reached IC50 values between 20.2 and 144.8 nM (median: 67 nM) against clade B viruses and 11.3 to 52.5 nM (median: 20 nM) against clade C viruses while DARPin 55.2 inhibited both subtype B and C viruses very potently with IC50 values of 0.4–4.1 nM for subtype B (median: 1.3 nM) and 0.3–1.6 nM for subtype C viruses (median: 0.7 nM). The latter confirmed the result of the initial screen and verified that the 2nd series DARPin has a markedly improved capacity to inhibit HIV, irrespective of the genetic background of the virus. As with all inhibitors against HIV, effective application of CD4-specific DARPin for prevention or therapy will require their use in combination with other types of inhibitors. To probe potential effects of CD4-DARPin in drug cocktails, DARPin 25.2 was tested for its efficacy in inhibiting HIV entry in combination with a series of entry inhibitors: the neutralizing mAbs IgG-b12 [63], 2F5 [64], 4E10 [65] and 2G12 [66], the fusion inhibitor T-20 [60], the anti-CCR5 mAb PRO140 [67] and CD4-IgG2 [30].

To derive further information on their target specificity, we studied binding of a selection of 2nd series DARPin to CD4 in competition with a panel of CD4-binding mAbs. In general, strong competition with the three D1 binding mAbs L222, Q4120, 13B82 [34,35] was observed, while less interference was found with 5A8 [36], a D2 binding antibody (Table 2). Notably, this competition by mAb 5A8 was not observed with DARPin 23.2, but with all other tested DARPins.

In summary these experiments suggest that the selected DARPin have overlapping specificities mainly directed against D1. We confirmed these experiments in competition experiments in which binding of fluorescently labeled DARPin 29.2 or 57.2 to CD4 expressing cells was probed in presence of unlabeled competitor DARPin (Figure 4A). Both sets of experiments gave identical results: the labeled DARPin was competed off by all other CD4 specific but not the control DARPin E3_5, indicating that the probes CD4-specific DARPin have closely overlapping epitopes.

To more specifically define the binding domain of the DARPins we generated a chimeric CD4 molecule in which domain 1 of human CD4 was exchanged by the corresponding domain of mouse CD4. The chimeric CD4 molecule expressed well upon transfection in 293-T cells, and had the required specificities, as antibody 83.5, specific for human D1, failed to bind, whereas mAb G1K.3, specific for mouse D1, bound the chimeric molecule but not wild type human CD4 (data not shown). Likewise mAb OKT4, specific for human CD4 D3, bound equally well to both wildtype human CD4 and the chimeric molecule (Figure 4B).

Table 2. Competition between DARPin and CD4-specific antibodies for binding to CD4.

| mAb/DARPin | E3_5 | 23.2 | 27.2 | 29.2 | 55.2 | 57.2 |
|------------|------|------|------|------|------|------|
| 5A8        | -    | -    | ++   | +    | +    | +    |
| L222       | -    | +++  | +++  | +++  | ++   | +    |
| Q4120      | -    | +++  | +++  | +++  | ++   | +    |
| 13B82      | -    | ++   | ++   | +    | +    | +    |
| -Flag      | -    | -    | -    | -    | -    | -    |

doi:10.1371/journal.ppat.1000109.t002
binding pattern of mAb S3.5 and thus confirming their specificity for CD4 domain 1 (Figure 4B).

Probing the effect of CD4-specific DARPins on cell function

Since the action of CD4-specific DARPins is directed against the host cell, particular care has to be taken to assess their effect on cell function before these agents can be considered for further development as HIV inhibitors. In a first step, we investigated whether CD4-specific DARPins interfere with CD4 T cell proliferation, by probing the effect of a candidate CD4-specific DARPin (D55.2) and a nonspecific control DARPin (E3_5) on primary CD4 T cell proliferation over a four day period. As Figure 5A shows, addition of the CD4-binding DARPin had no noticeable impact on cell proliferation compared to the untreated control.

To explore the effects of CD4 engagement by DARPins on dendritic cells (DC), we assessed whether treatment of immature monocyte-derived DC with DARPin 55.2 for 24 h induced activation and maturation of these cells, which is reflected by increased expression of the costimulatory molecule CD80. Neither the CD4-specific DARPin 55.2 nor the control DARPin induced DC maturation (Figure 5B), whereas E. coli lipopolysaccharide (LPS), known to induce DC maturation via TLR-4, gave rise to a pronounced shift in CD80 expression (data not shown).

Notably, the DARPins did not reveal any cytotoxic effects: prolonged incubation of primary cells with DARPin - CD4-specific or unselected - did not result in increased cell death as measured by uptake of propidium iodide: Both the CD4+ T cells (incubated with DARPins, 500 nM, for 4 days) and the dendritic cells (incubated with DARPins, 375 nM, for 24 h) remained unaffected (Figure 5C).

Effect on CD4 receptor density

As our competition binding experiments with gp120 indicate (Figure 1B), CD4-specific DARPins most likely act by blocking viral attachment to the receptor. Theoretically, binding of the DARPin to CD4 could also induce receptor internalization and DARPins thus may exhibit their antiviral activity through decreasing CD4 receptor density on the target cells. To probe this, we explored the effect of DARPin binding on surface CD4 receptor levels of primary CD4 T cells. Treatment of CD4 T cells from healthy donors with DARPin for 0, 1, 3 and 18 h at 37°C (to
DARPins as Specific HIV Entry Inhibitors

allow receptor internalization) or at 4°C (to limit internalization) yielded identical results: Neither treatment with the CD4 specific nor the unspecific DARPin resulted in down- or upregulation of CD4 (Figure 5D). Recognition of CD4 by the CD4 mAb used in these FACS analyses was not impaired in the presence of CD4 specific DARPin. Most importantly, CD4 staining in presence of CD4 specific DARPin remained stable independently whether DARPin and mAb were added simultaneously or cells were pretreated with DARPin for extended time periods (Figure 5D).

Effect of CD4 specific DARPins on CD4 interaction with MHC class II

In the absence of T cell receptor interaction the binding of CD4 to MHC class II is of extremely low affinity (K_D = 200 μM; [68]). Using a previously established assay that allows to study this weak interaction based on rosette formation between CD4 and MHC-II expressing cells [56], we were able to show that all tested DARPins, 23.2, 25.2, 29.2, 55.2 and 57.2, as well as the CD4-D1 specific antibody Q4120 blocked rosette formation efficiently (Figure 6A and data not shown). Hence, in the absence of cognate T cell receptor (TCR) and peptide, the CD4 specific DARPins interfered with CD4 binding to MHCII.

To probe the effect on specific T cell functions, we assessed if the CD4-specific DARPin 55.2 affects activation of memory T helper cells specific for either streptokinase/streptodornase or cytomegalovirus antigens. When we quantified antigen specific helper cells specific for either streptokinase/streptodornase or the CD4-specific DARPin 55.2 affects activation of memory T cell receptor (TCR) and peptide, the CD4 specific DARPins interfered with CD4 interaction based on rosette formation between CD4 and MHC-class II expressing cells. Rosette formation was blocked by all tested CD4 specific DARPins and mAb were added simultaneously or cells were pretreated with DARPin for extended time periods (Figure 5D).

Efficacy of DARPins in blocking rhesus macaque CD4

To evaluate the potential of using these binders directly in non-human models, crossreactivity of the DARPins with CD4 from rhesus macaques was investigated. The sequence identity between human and macaque CD4 is 91% on the amino acid level, as opposed to 54% between human and murine CD4. Experiments using PBMC from macaques revealed that 4 out of 7 tested DARPins recognize also rhesus CD4 (Figure 7A), while none of them interacts with murine CD4 (data not shown and Figure 4B). This finding is intriguing as it opens the possibility to probe the potential of DARPins as candidate microbicides in the macaque infection model. To obtain an initial insight into the potential of these DARPins in inhibiting SIV infection, we probed the efficacy of DARPin 25.2 in blocking SIVmac239 infection of primary rhesus macaque cells. Results obtained in infection experiments with cells from three individual donors depicted in Figure 7B indicate that DARPin 25.2 potently inhibit SIV infection of these cells.

Discussion

Making use of the recently developed DARPin technology [19–21,23], we investigated here DARPins as HIV-specific inhibitors since they can be engineered to fulfill many of the sought for properties of a microbicide, namely high target specificity and affinity, high physical stability and comparatively low production costs. As proof of concept, we aimed to derive DARPin-based inhibitors that target CD4, the primary receptor for HIV. The technology employs highly diverse DARPin DNA libraries combined with ribosome display as selection technology, which allowed the selection of binders with specificity for the CD4 receptor in a relatively short time. The resulting DARPins interacted with very high affinity with human CD4 as reflected by dissociation constants in the lower nanomolar range, which upon off-rate selection even reached subnanomolar values. This high affinity has proven a common characteristic of DARPins: although monovalent binders, they routinely achieve affinities that are equal if not superior to most antibodies [23,26,28,59]. We subjected the derived CD4-specific DARPins to a careful assessment of the HIV inhibitory capacity. Notably, all probed CD4-specific DARPins from the 1st and the 2nd affinity improved series inhibited HIV entry both in cell line and primary cell based infection systems. Inhibition was achieved over both single round and multiple rounds of infection proving the stability of this effect. Particularly notable was the potency of the 2nd series DARPin, which were specifically selected for low dissociation rates. They exhibited potent and broad neutralization of HIV across subtypes

![Figure 6. Effect of DARPin on T cell function and MHC class II interaction.](image-url)

(A) The effect of the DARPin:CD4 interaction was assessed in a binding assay based on rosette formation between CD4 and MHC class II expressing cells. Rosette formation was blocked by all tested CD4 specific DARPins (200 nM, shown are D25.2 and D55.2) or by the CD4-specific mAb Q4120 but not by the control DARPin E3_5. One out of two representative experiments is shown. (B) ELISpot assay to detect IFN-γ production by activated T cells showed no interference of DARPin 55.2 with CD4+ T cell activation. The response of two donors against CMV or streptokinase/streptodornase (SKSD) antigen was tested without DARPin (gray) and with nonspecific (blue) or CD4-specific DARPin (red) at 200 or 250 nM. One out of two independent experiments is depicted.

doi:10.1371/journal.ppat.1000109.g006

PloS Pathogens | www.plospathogens.org 10 July 2008 | Volume 4 | Issue 7 | e1000109
targeting a cellular receptor, we found the actions of the selected DARPin inhibitors with topical application as a microbicide in mind, where comparatively low systemic exposure is expected, it is nevertheless critical to carefully assess their potential side effects on immune function. Despite interference with gp120 binding to CD4 being their mode of action (Figure 5D and data not shown), supporting the notion that direct interference with viral attachment. Competition between virus and inhibitor to an epitope on D1 that is different from the gp120 binding site, resulting in either conformational changes or in stabilization of an incompatible conformation of the entire domain.

The screening strategy should enrich for DARPin specific for D1, as competitive displacement from CD4 by gp120 was applied in the final ribosome display rounds. It also has to be considered that the D2 domain is probably less exposed in the tetrameric form of CD4-IgG2 and therefore likely not as accessible for DARPin binding during the screening. More detailed epitope mapping will reveal the distances between those regions in D2 or are dependent on a D2 steered conformation.

Our screening strategy should enrich for DARPin specific for D1 because these regions are involved in the interaction with the virus and targeting of these regions by specific antibodies have been shown to interfere with HIV infection [71–75]. D1 harbors the binding site for gp120 and interference is expected to abrogate this interaction. The role of D2 in the infection process appears to be more indirect, nevertheless important: the D2 specific antibody 5A8 blocks HIV infection efficiently and its humanized derivative TNX-355 is now under clinical investigation [69,76,77]. Notably, all tested DARPin selected against D1 and D2 of CD4 in our screen inhibited HIV entry. The most obvious concept of inhibiting HIV entry is blocking of the gp120 binding site within the D1 domain of CD4 and thus direct interference with viral attachment. Competition between virus and inhibitor could likewise arise from binding to an epitope on D1 that is different from the gp120 binding site, resulting in either conformational changes or in stabilization of an incompatible conformation of the entire domain.

Aiming strategy should enrich for DARPin specific for D1, as competitive displacement from CD4 by gp120 was applied in the final ribosome display rounds. It also has to be considered that the D2 domain is probably less exposed in the tetrameric form of CD4-IgG2 and therefore likely not as accessible for DARPin binding during the screening. More detailed epitope mapping will reveal the distances between those regions in D2 or are dependent on a D2 steered conformation.

We further found that engagement of CD4 by the DARPin 27.2, 29.2, 55.2, and 57.2 did not induce downregulation of CD4 (Figure 5D and data not shown), supporting the notion that direct interference with gp120 binding to CD4 is their mode of action (Figure 1B).

Although we developed the DARPins with topical application as a microbicide in mind, where comparatively low systemic exposure is expected, it is nevertheless critical to carefully assess their potential side effects on immune function. Despite targeting a cellular receptor, we found the actions of the selected CD4-specific DARPins to be highly HIV specific. No effect on CD4-independent virus entry was detected using murine leukemia virus. Equally important, we did not observe effects on cell viability, proliferation of T-cells, or activation of immature DC for the individual DARPin probed in these assay systems, indicating that these monovalent binders did not activate the receptor and initiated downstream signaling events. Moreover, although DARPin can interfere with the low affinity interaction between CD4 and MHC class II which occurs in the absence of cognate TCR and peptide (Figure 6A; [68]), DARPin treatment did not disturb activation of specific memory T helper responses (Figure 6B). The latter supports previous observations that CD4/MHC class II interaction is tightened on TCR engagement [68,78], which may explain why the inhibitory effect of the DARPin is overcome in this context.

The fact that targeting of CD4 by the high affinity DARPin can occur without loss of CD4 T cell function and unwanted side effects, holds great promise of their in vivo application. This is further underlined by our finding that CD4-specific DARPin act in synergy with several other HIV entry inhibitors directed to different targets on the virus or host cell. The DARPin technology is a relative young invention and the potential in vivo applications of DARPin still awaits proof. This notwithstanding, our in vitro analysis strongly suggests that DARPins have unique properties that render them promising candidates for microbicide development. Further assessment of their application as microbicides is clearly feasible, particularly as we selected several molecules that are specific for human and rhesus macaque CD4, which will allow future study of their efficacy in the macaque infection model.

**Accession numbers**

The nucleotide and the amino acid sequences of the 12 DARPins described here were deposited in the EMBL Nucleotide Sequence Data Base (www.ebi.ac.uk/embl) and are available under the accession numbers AM997259–AM997270.

**Supporting Information**

**Protocol S1** Designed Ankyrin Repeat Proteins (DARPins) and ribosome display.

Found at: doi:10.1371/journal.ppat.1000109.s001 (0.07 MB DOC)

**Figure S1** Repeat sequence motif of a DARPin repeat and X-ray structure of a randomly selected member of the N3C DARPin library, E3_5.
Christian Zahnd, Michael Stumpf, and H. Kaspar Binz are acknowledged for helpful discussions and Ingrid Niervogel and Christine Vogtli for administrative assistance. Additional thanks to Agenechu Gettie and the veterinary staff at TNFPC for providing the macaque blood samples. The use of the Population Council’s Flow Cytometry Facility is gratefully acknowledged. The following reagent was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: Human rIL-2 from Dr. Maurice Gately, Hoffmann - La Roche Inc. [79].

Author Contributions
Conceived and designed the experiments: AS PR LB CRR AM SGT MF PA AT.Performed the experiments: AS PR LB CRR AC SG ST MA. Analyzed the data: AS PR LB CRR AM SG ST PA AT. Wrote the paper: AS AT.

References
1. Berkley SF, Koff WC (2007) Scientific and policy challenges to development of an AIDS vaccine. Lancet 367: 94–101.
2. (2007) AIDS Epidemiol Update. December 2007. Joint United Nations Programme on HIV/AIDS and World Health Organization.
3. Belyakov IM, Berzofsky JA (2004) Immunobiology of mucosal HIV infection and the basis for development of a new generation of mucosal AIDS vaccines. Immunol Rev 201: 247–253.
4. Klass PJ, Shenderov J, Moore JP (2007) Antiretroviral drug-based microbics to prevent HIV-1 sexual transmission. Ann Rev Med 58: 453–511.
5. Stone A (2002) Microbics: a new approach to preventing HIV and other sexually transmitted infections. Nature Reviews Drug Discovery 1: 977–985.
6. Balzarini J, Van Damme L (2007) Microbicide drug candidates to prevent HIV infection. Lancet 369: 787–797.
7. Hughes LM, Griffith R, Aitken RJ (2007) The search for a topical dual action spermicide/microbicide. Curr Med Chem 14: 775–786.
8. Darzynkiewicz Z (2006) Exploring common targets in human fertilization and HIV infection: development of novel contraceptive microbics. Hum Reprod Update 12: 103–117.
9. Cheek E (2007) Scientists rethink approach to HIV gels. Nature 446: 12.
10. Hillier SL, Moench T, Shattock R, Black R, Rechberger P, et al. (2005) In vitro and in vivo: the story of nonoxynol-9. J Acquir Immune Defic Syndr 39: 1–8.
11. Rampe G, Govinden R, Moraz NS, Mbewu A (2007) South Africa’s Experience of the Closure of the Cellulose Sulphate Microbicide Trial. PLoS Med 4: e235. doi: 10.1371/journal.pmed.0040235.
12. Roberts JN, Buck CB, Thompson CD, Kines R, Bernardo M, et al. (2007) Prevention of vaginal SHIV transmission in rhesus macaques from vaginal SHIV challenge by vaginally delivered inhibitors of membrane fusion. J Virol 81: 533–539.
13. van de Wijgert JH, Braunstein SL, Morar NS, Jones HE, Madurai L, et al. (2005) The Immunology of mucosal HIV infection. Lancet 365: 787–797.
14. Vellonen I, Rekola R, Simell M, Soderlund V, Heikkinen T, et al. (2001) Analysis of a single-step protocol to purify recombinant proteins with low endotoxin contents. Protein Expr Purif 46: 483–488.
15. Healey D, Dianda L, Moore JP, McDougal JS, Moore MJ, et al. (1990) Novel single chain antibody fragment (scFv) with low picomolar affinity. J Biol Chem 265: 279: 18870–18877.
16. Davis SJ, Schoechel GA, Somozza C, Buck DW, Healey DG, et al. (1992) Antibody and HIV-1 gp120 recognition of CD4 undermines the concept of mimicry between antibodies and receptors. Nature 358: 76–79.
17. Healey D, Dianda L, Moore JP, McDougal JS, Moore MJ, et al. (1990) Antibody and HIV-1 gp120 recognition of CD4 undermines the concept of mimicry between antibodies and receptors. Nature 358: 76–79.
18. Healey D, Dianda L, Moore JP, McDougal JS, Moore MJ, et al. (1990) Antibody and HIV-1 gp120 recognition of CD4 undermines the concept of mimicry between antibodies and receptors. Nature 358: 76–79.
19. Jarvis P, Bax BY, Morris H, Patel RD, Pragay Z, et al. (2000) Fluorescence resonance energy transfer. Microbes Infect 8: 520–532.
20. Montefiori D (2004) Evaluating neutralizing antibodies against HIV, SIV, and the basis for development of a new generation of mucosal AIDS vaccines. Nature Rev Drug Discovery 1: 977–985.
21. Montefiori D (2004) Evaluating neutralizing antibodies against HIV, SIV, and the basis for development of a new generation of mucosal AIDS vaccines. Nature Rev Drug Discovery 1: 977–985.
22. Amstutz P,秘诀 HK, Parizk V, Stumpf MT, Kohl A, et al. (2005) In vitro escape from neutralizing antibodies 2G12, 2F5 and 4E10. J Virol 81: 8793–8808.
23. Belyakov IM, Berzofsky JA (2004) Immunobiology of mucosal HIV infection and the basis for development of a new generation of mucosal AIDS vaccines. Immunol Rev 201: 247–253.
24. Davis SJ, Schoechel GA, Somozza C, Buck DW, Healey DG, et al. (1992) Antibody and HIV-1 gp120 recognition of CD4 undermines the concept of mimicry between antibodies and receptors. Nature 358: 76–79.
25. Healey D, Dianda L, Moore JP, McDougal JS, Moore MJ, et al. (1990) Antibody and HIV-1 gp120 recognition of CD4 undermines the concept of mimicry between antibodies and receptors. Nature 358: 76–79.
26. Healey D, Dianda L, Moore JP, McDougal JS, Moore MJ, et al. (1990) Antibody and HIV-1 gp120 recognition of CD4 undermines the concept of mimicry between antibodies and receptors. Nature 358: 76–79.
27. Healey D, Dianda L, Moore JP, McDougal JS, Moore MJ, et al. (1990) Antibody and HIV-1 gp120 recognition of CD4 undermines the concept of mimicry between antibodies and receptors. Nature 358: 76–79.
28. Schweizer A, Loschitzki-Voser H, Amstutz P, Brander C, Gallo-Megeva A, et al. (2007) Inhibition of caspase-2 by a designed ankyrin repeat protein: specificity, structure, and mechanism of inhibition. Structure 15: 625–636.
29. Semnus A, Amstutz P, Brander C, Stoeckerbueger O, Gratrix MG (2006) Drug export pathway of multihistag exporter AcrB revealed by DARPin inhibitors. PLoS Biol 5: e7. doi:10.1371/journal.pbio.0050007.
30. Sennhiser G, Amstutz P, Brander C, Stoeckerbueger O, Gratrix MG (2006) Drug export pathway of multihistag exporter AcrB revealed by DARPin inhibitors. PLoS Biol 5: e7. doi:10.1371/journal.pbio.0050007.
31. Allaway GP, Davis-Bruino BL, Beaudry GA, Garcia EB, Wong EL, et al. (1995) Novel single chain antibody fragment (scFv) with low picomolar affinity. J Biol Chem 265: 279: 18870–18877.
32. Davis SJ, Schoechel GA, Somozza C, Buck DW, Healey DG, et al. (1992) Antibody and HIV-1 gp120 recognition of CD4 undermines the concept of mimicry between antibodies and receptors. Nature 358: 76–79.
33. Healey D, Dianda L, Moore JP, McDougal JS, Moore MJ, et al. (1990) Antibody and HIV-1 gp120 recognition of CD4 undermines the concept of mimicry between antibodies and receptors. Nature 358: 76–79.
