Materials and Methods

Preparation of CD8 T Lymphocytes. Peripheral blood mononuclear cells (PBMC) were obtained by standard Ficoll/Hypaque gradient centrifugation. CD8 T cells were isolated from PBMC by positive selection using magnetic beads coated with an anti-CD8 monoclonal antibody (Dynal, Lake Success, NY). Beads were then removed from cells by a Detach-a-Bead magnet (Dynal). The purity of the cells obtained by this procedure is >99.5% as judged by flow cytometry. Isolated CD8 T cells were then stimulated for 3 days at 37ºC in a humidified CO_2 incubator with anti-CD3 (12F6; 0.1 μg/ml) (a gift from J. Wong of Massachusetts General Hospital) and anti-CD28 (5 μl/ml; Becton Dickinson, San Diego, CA) antibodies, together with an equal number of allogeneic irradiated PBMC in serum-free RPMI 1640 medium (Cellgro, Herndon, VA), supplemented with recombinant IL-2 (20 U/ml), penicillin (100 U/ml) and streptomycin (100 μg/ml). Culture supernatants were collected and cleared by centrifugation at 3000 rpm for 5 min, and stored at –80ºC until use.

Ciphergen ProteinChip Analysis. Initially, multiple types of Ciphergen ProteinChip Arrays were used with various surfaces having distinct characteristics including weak cation exchanger (WCX2), strong anion exchanger (SAX2), immobilized metal affinity capture (IMAC-3) for protein molecules that bind divalent cationic metals, and reverse phase H4 for capturing proteins through hydrophobic interactions. We eventually chose the WCX2 array for the entire study, except as specified, due to its reproducibility in detecting protein/peptide species from the culture supernatant. The procedures were

carried out as follows. Culture supernatant (100 µl) was mixed with an equal volume of binding buffer (100 mM NaAc, pH 4.5; 0.2% Triton X-100 in PBS), and then applied onto a WCX2 array (Ciphergen) and incubated at 4ºC overnight with constant horizontal shaking. Unbound proteins/peptides were removed by washing with a buffer (50 mM NaAc. pH 4.5; 0.1% Triton X-100 in PBS) three times for 5 min each under identical conditions. The WCX2 arrays were then removed and rinsed in 1 mM HEPES (pH 4.5) for 30 sec, and air-dried before a saturated solution of 3,5-dimethoxy-4-hydroxycinnamic acid (SPA) in 50% acetonitrile and 0.5% trifluoroacetic acid was added to the chip surface. The arrays were analyzed with the Ciphergen ProteinChip Reader (model PBS II). In SELDI-TOF-MS analysis, a nitrogen laser (337 nm) desorbs the protein/SPA mixture from the array surface, enabling the detection of the proteins/peptides captured by the array. The mass spectra of proteins/peptides were generated using an average of 80 laser shots at a laser intensity of 220-240 arbitrary units. The mass to charge ratio (m/z) of each of the proteins/peptides captured on the array surface was determined according to externally calibrated standards: ARG-8-vasopressin (1,084.3 Da), somatostatin (1,637.9 Da), bovine insulin β chain (3,495.9 Da), human insulin (5,807.7 Da), and hirudin (7,033.6 Da).

Enrichment of Proteins of Interest for Identification. The low molecular weight proteins were enriched using an anion exchange column (BioSepra Q HyperD® SAX Resin, Ciphergen) followed by a reverse phase column (BioSepra RPC PolyBio^{TM} C18 Resin, Ciphergen). A BioSepra Q HyperD® SAX column was equilibrated and washed with 50 mM Tris HCl (pH 8.0) containing 10% acetonitrile (ACN) and 0.1%

trifluoroacetic acid (TFA). To enrich the proteins of interest, the culture supernatant was mixed with 50% ACN in 50 mM Tris HCl (pH 8.0) and applied to the column. Unbound material was collected, diluted five-fold in the same buffer and applied directly to a BioSepra RPC PolyBio™ C18 column. The bound proteins/peptides were eluted using 50 mM Tris HCl (pH 8.0) with 0.1% TFA and an increasing amount of ACN in the solution (30%, 40%, 50%, 60%, and 80%). The TFA in the eluate was neutralized by adding 10% v/v 100 mM ammonium bicarbonate buffer (pH 8.0). The neutralized eluate was concentrated via speed vacuum at room temperature for 30 min until the solution reached approximately 25 femtomoles of proteins per microliter. The concentrated material was reduced with 5 mM DTT in 50 mM Tris HCl (pH 8.6-9.0) for 5 min at 90ºC. The reduced mixture was cooled to room temperature and was digested with 0.6 μg/μl trypsin at 40ºC for 2 h and then stopped on ice. All of the above reactions were performed under inert argon gas. Trypsin-digested products were then directly applied to a Ciphergen NP-20 array, and protein identity was determined using a Micromass Q-TOF II instrument with a Ciphergen PCI-1000 ProteinChip Interface.

Antibody Depletion of α-Defensins. Dynal beads were coated with human-α-defensin-specific antibody or human-MIP-1α-specific antibody according to manufacturer's instructions. In brief, biotinylated monoclonal antibody specific for human α-defensins-1,2,3 (clone D21, HyCult Biotechnology, Norwood, MA) was mixed with Biotin-Binder Dynabeads (Dynal) in PBS containing 0.1% Tween 20, and rotated at room temperature for 30 min. Unbound antibodies were removed by washing three times with PBS containing 0.1% Tween 20. Separately, human-MIP-1α-specific monoclonal antibody

(R&D Systems, Minneapolis, MN) was directly coupled to Dynabeads M-450 Epoxy (Dynal) in 0.1 M borate buffer (pH 7.0-7.5) by rotating at 4ºC overnight. After 15-30 min of incubation, 0.5% BSA was added to ensure an optimal orientation of the antibody. Antibody-coated beads were then washed three times with PBS containing 0.1 % BSA (pH 7.4). For every 10^7 beads, approximately 2-5 μg of each antibody was used. The antibody-coated beads (approximately 4 x 10^7) were then mixed individually with 200 μl of culture supernatants from stimulated $CD8^+$ T cells in the presence of 0.01% of hexadecyltrimethylammonium bromide (Sigma, St. Louis, MO), and rotated at 4ºC overnight. The antigen-antibody complexes-coated beads were then removed from the supernatant by a Detach-a-Bead magnet (Dynal).

Virus Inhibition Assays. Virus inhibition assays were performed using replication-competent HIV-1 strains. PBMC were firstly stimulated with PHA at the concentration of 20 μg/ml for 3 days. Approximately 2 x 10^5 cells were seeded per well in a 96-well plate with 100 tissue culture infectious doses ($TCID_{50}$) of each virus in the presence of supernatant from stimulated CD8 T cells. In the antibody neutralization assay, various amounts of human-α-defensin-specific antibodies and/or human-β-chemokine-specific antibodies were added to the supernatant prior to mixing with the virus and PBMC. The antiviral activities of commercial defensins-1 and -2 were tested in the presence or absence of human-α-defensin-specific antibodies. After infecting for 2 h at 37°C, remaining viruses in the supernatant were removed by extensive washing with PBS. Fresh medium containing the same concentration of human-α-defensin-specific antibodies and/or human-β-chemokine-specific antibodies, or commercial defensins-1

and -2, were added. The p24 antigen levels in culture supernatants were measured on day 0, 3 and 7 post-infection using a standard protocol. The percent inhibition was determined by comparing p24 levels in the supernatant to that seen in cultures without inhibitors at the time of peak virus production, normally on day 5 after infection.

Immunofluorescence Staining, Microscopy and Flow Cytometric Analysis for α-Defensins. For microscopic and flow cytometric evaluation, neutrophils and purified CD8 T lymphocyte were stained for α-defensins and CD8 protein in suspension. Cells were first surface labeled using mouse anti-human CD8-APC (Caltag Laboratories) on ice according to manufacturer's recommendation. After surface labeling, cells were washed in iced PBS and fixed in fresh 1% formaldehyde for a minimum of 15 minutes. Fixed cells were washed in PBS and labeled internally using biotinylated anti-human – defensin monoclonal antibody (D21 from HyCult Biotechnology) at 5 ul/10^6 cells for 15 minutes, followed by washing and fluorescence labeling with streptavidin-FITC (Caltag Laboratories). For microscopic analysis, cells were first counterstained using Hoechst 33242 (0.1 ug/ml) and affixed to microscope slides using a cytocentrifuge. Slides were first prewetted with PBS and 1% fetal calf serum before affixing cells, after which coverslips were mounted using SlowFade Light antifade component A (Molecular Probes). Representative fluorescent cell images were acquired using a DeltaVision deconvolution microscopy system (Applied Precision, Inc.). Flow cytometry was carried out using a dual-laser FACScalibur (Becton-Dickinson) and data were displayed using either CELLQuest (Becton-Dickinson) or WinMDI (Joseph Trotter) software.

Legend for Supplemental Figures

Figure S1. Changes in molecular mass/charge before and after reduction with dithiothreitol (DTT).

Figure S2. Antiviral activity against HIV-1 before (solid) and after treatment of culture supernatants of LTNP-3 and LTNP-5 with anti-defensin-1,2,3 (hatched) or an irrelevant antibody (blank). This is the control for experiments shown in Fig. 3a.

Figure S3. Antiviral activity of culture supernatant from stimulated CD8 T cells from LNTP-3 in the presence of increasing amounts of irrelevant antibodies. This is the control for experiments shown in Fig. 3b.

Figure S4. Mass spectrometry profiles of commercial preparations of α-defensin-1 and -2 as compared to that found in the supernatant of stimulated CD8 T cells from Normal-2.

Figure S5. α-defensins-1, -2, and -3 purified from neutrophils of a normal person is virtually indistinguishable from those released from stimulated CD8 T cells.

Figure S6. Number of αβ CD8 T cells expressing α-defensins on day 0, 1, and 2 after stimulation. The Y-axis denotes the number of events detected.

Figure S7. Immunofluorescence staining of neutrophils and unstimulated CD8 T lymphocytes using irrelevant antibodies. These non-staining cells serve as negative controls for those shown in Fig. 5.

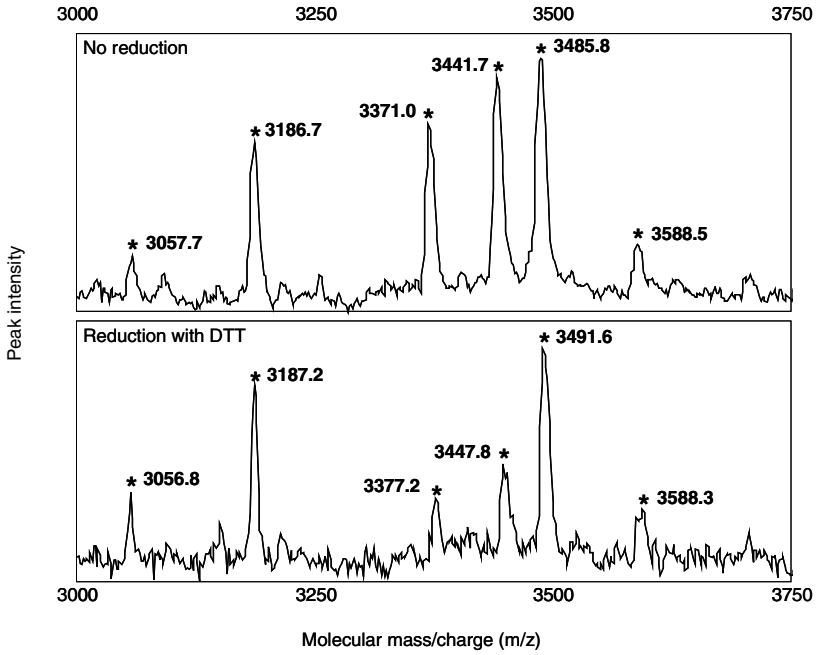
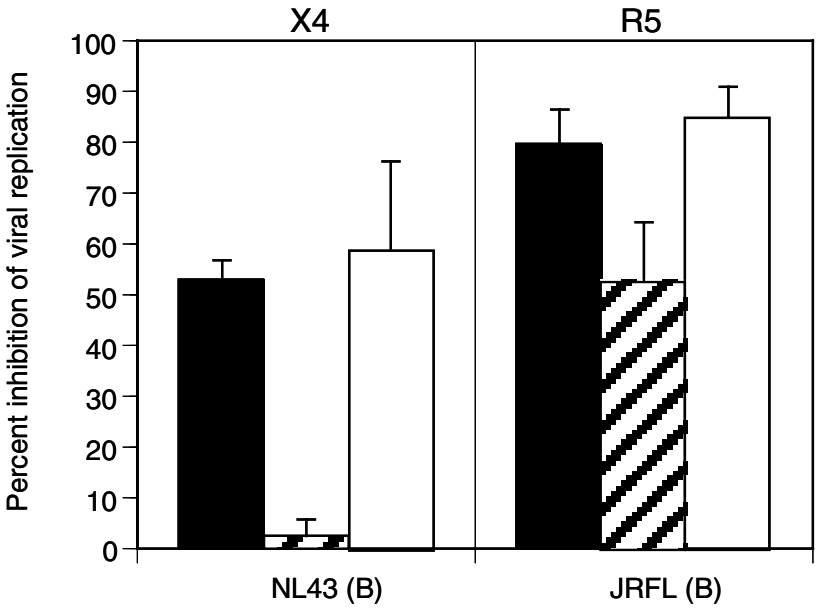

3000
3250
3500
3750
No reduction
3485.8
3441.7
3371.0
3186.7
3057.7
3588.5
Peak intensity
Reduction with DTT
3491.6
3187.2
3447.8
3377.2
3056.8
3588.3
3000
3250
3500
3750
Molecular mass/charge (m/z)

Fig. S1


X4
R5
Percent inhibition of viral replication
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90
80
70
60
50
40
30
20
10
0
NL43 (B)
JRFL (B)

Fig. S2

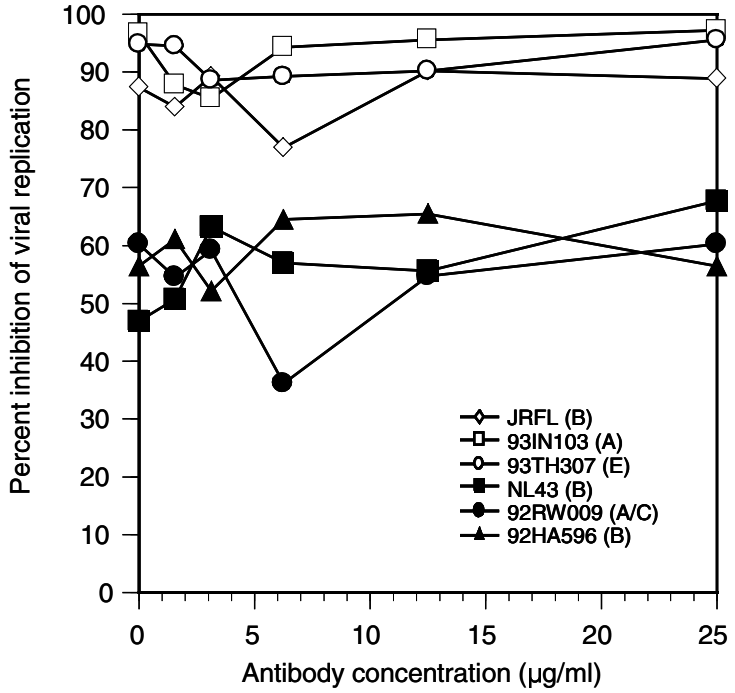
Percent inhibition of viral replication
100
90
80
70
60
50
40
30
20
10
0
0
5
10
15
20
25
Antibody concentration (µg/ml)
JRFL (B)
93IN103 (A)
93TH307 (E)
NL43 (B)
92RW009 (A/C)
92HA596 (B)

Fig. S3

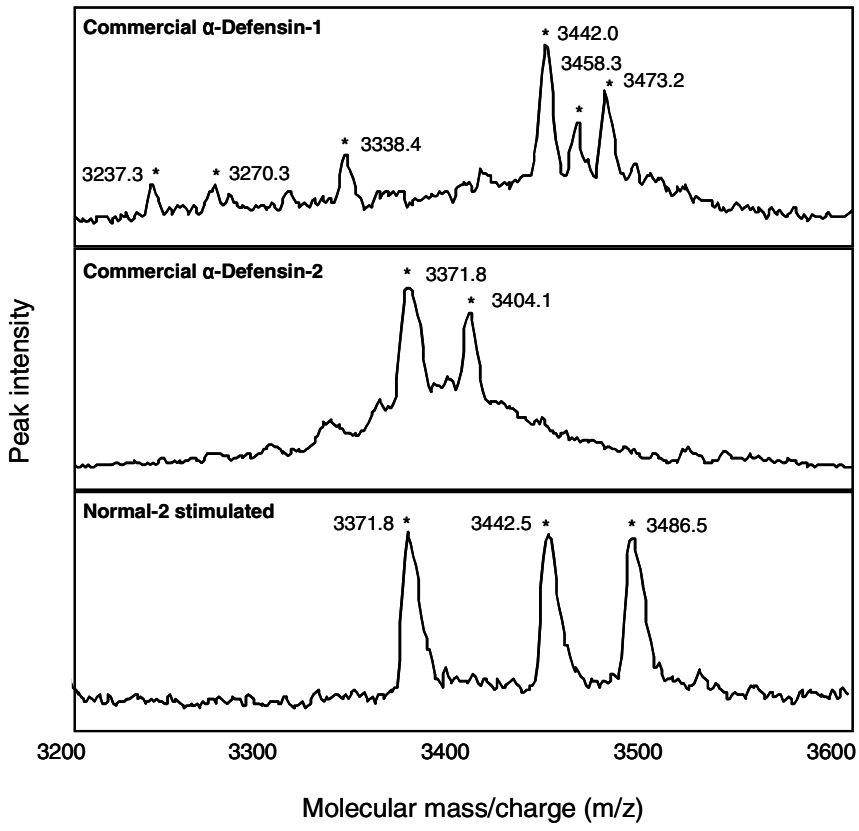
Commercial α-Defensin-1
3442.0
3458.3
3473.2
3338.4
3237.3
3270.3
Commercial α-Defensin-2
3371.8
3404.1
Normal-2 stimulated
3371.8
3442.5
3486.5
Peak intensity
3200
3300
3400
3500
3600
Molecular mass/charge (m/z)

Fig. S4

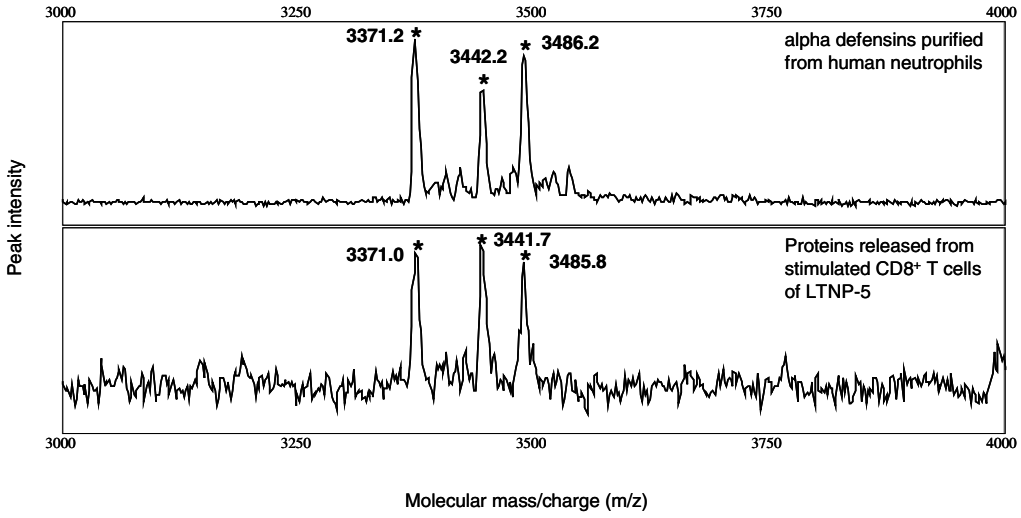
3000
3250
3500
3750
4000
3371.2
3442.2
3486.2
alpha defensins purified from human neutrophils
3371.0
3441.7
3485.8
Proteins released from stimulated CD8+ T cells of LTNP-5
Peak intensity
Molecular mass/charge (m/z)

Fig. S5

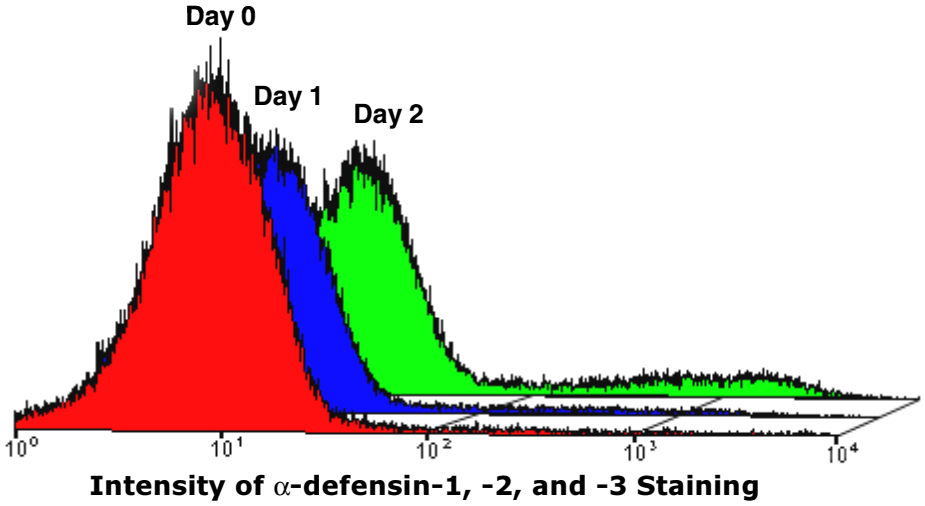
Day 0
Day 1
Day 2
10⁰
10¹
10²
10³
10⁴
Intensity of α-defensin-1, -2, and -3 Staining

Fig. S6

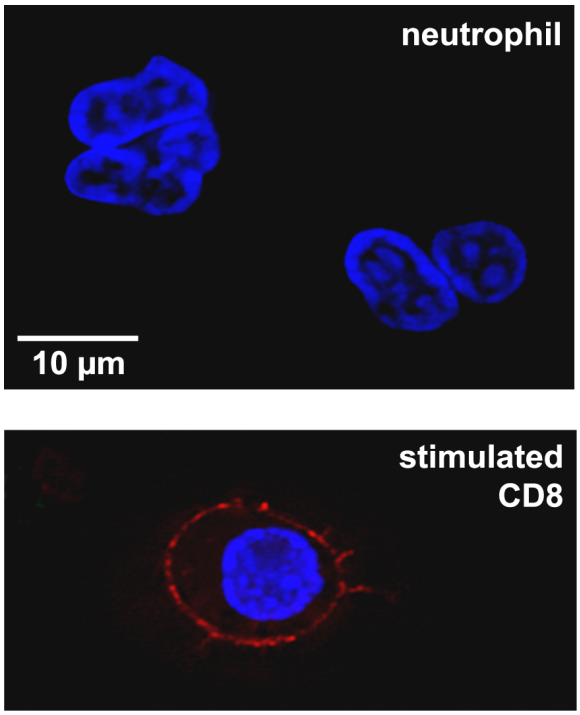

Fig. S7