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Contribution of Human α-Defensin-1, -2, and -3 to the Anti-HIV-1 Activity of CD8 Antiviral Factor

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It is known since 1986 that CD8 T lymphocytes from certain HIV-1-infected individuals who are immunologically stable secrete a soluble factor, termed CAF, that suppresses HIV-1 replication. However, the identity of CAF remained elusive despite an extensive search. By means of a protein-chip technology, we identified a cluster of proteins that were secreted when CD8 T cells from long-term non-progressors with HIV-1 infection were stimulated. These proteins were identified as α-defensins-1, -2, and -3 on the basis of specific antibody recognition and amino-acid sequencing. CAF activity was eliminated or neutralized by an antibody specific for human α-defensins. Synthetic and purified preparations of α-defensins also inhibited the replication of HIV-1 isolates in vitro. Taken together, our results indicate that α-defensins-1, -2, and -3 collectively account for the anti-HIV-1 activity of CAF that is not attributable to β-chemokines.

CD8 T lymphocytes play a critical role in controlling HIV-1 or SIV replication in vivo (1). The initial control of viremia following primary infection is temporally correlated with the onset of virus-specific CD8 cytotoxic T lymphocytes (CTL) (2, 3). SIV replication in macaques increases dramatically when a monoclonal antibody is used to deplete CD8 T cells (4, 5). Moreover, the strong pressure exerted by cellular immunity in vivo is apparent from the rapid emergence of CTL-escape viruses (6, 7). While the direct killing of infected cells by CD8 CTL is important in virus suppression (1), soluble factors secreted by CD8 T lymphocytes can also inhibit HIV-1 or SIV replication in vitro. In 1986, Walker et al (8) first described the CD8 antiviral factor (CAF), a diffusible molecule secreted by stimulated CD8 T cells from certain HIV-1-infected individuals. Unlike CTL, the antiviral activity of CAF is non-cytolytic and does not require restriction by major histocompatibility complex class-I molecules or cell-to-cell contact. Instead, the activity is believed to be mediated by a heat-stable, acid-stable protein (9) with m.w. <20 KDa (10) or <10 KDa (11). Importantly, CAF inhibits HIV-1 replication irrespective of viral phenotype or tropism (9), but its precise mechanism of action remains unknown, although there are indications that the effect may be at the level of viral transcription (12, 13).

CAF is released in greater abundance by stimulated CD8 T lymphocytes from HIV-1-infected persons who are doing well clinically, particularly those characterized as long-term non-progressors (LTNP) (9, 14-16). In contrast, it is uncommonly detected in CD8 T cells from infected patients with evidence of immunodeficiency (progressors). CAF-like activity has been detected in stimulated CD8 T cells from SIV-infected rhesus macaques (17) or African green monkeys (18), HIV-1-infected chimpanzees (19), and some healthy uninfected humans (20).

Despite tremendous efforts over the past 16 years (9), the identity of CAF has remained elusive. In 1995, Cocchi et al (21) showed that stimulated CD8 T lymphocytes can secrete β-chemokines (RANTES, MIP-1α and MIP-1β) that block HIV-1 infection in vitro. However, their antiviral activity was observed against macrophage-tropic viral isolates, but not against T-cell-line-tropic strains. This dichotomy was later explained by the discovery that the receptor for β-chemokines, CCR5, also serves as the co-receptor for HIV-1 entry into CD4 T cells (22-24). Thus, it became apparent that β-chemokines can competitively block so-called R5 viruses that use CCR5 as co-receptor, but not so-called X4 viruses that use an alternate co-receptor, CXCR4 (25). Such an antiviral profile clearly distinguished β-chemokines from CAF, which can inhibit both types of HIV-1. Moreover, CAF activity could not be eliminated by removing either β-chemokines (26, 27) or SDF-1α (28), the ligand for CXCR4, with specific monoclonal antibodies. Other cytokines have subsequently emerged as possible candidates for CAF, including macrophage-derived chemokine (29) and interleukin-16 (30), but none has stood the test of time (31, 32).

Identification of a Cluster of Small Proteins Secreted by Stimulated CD8 T Cells from LTNP and Normal Persons. We have long studied a cohort of LTNP, many of whom were good producers of CAF (14). Supernatant fluids were harvested from stimulated and unstimulated CD8 T-lymphocyte cultures derived from 3 of these LTNP, as well as from 4 progressors and 15 normal controls (33). Each sample was analyzed on the ProteinChip® System (Ciphergen Biosystems, Inc., Fremont, CA), which is based on the integration of chemically modified array surfaces with surface-enhanced laser desorption/ionization (SELDI) time-of-flight (TOF) mass-spectrometry (MS) detection (34). This technology was chosen because of its power of resolution, high reproducibility, ease of use, and femtomole level sensitivity (34, 35). As shown in Fig. 1a, representative protein mass spectra for two LTNP and one normal control revealed significant differences in peak pattern between stimulated and unstimulated CD8 supernatants. A cluster of two or three peaks, with m.w. of 3,371.9 Da, 3,442.5 Da, and 3,486.5 Da, was found in stimulated cultures. This cluster was detected in cultures of stimulated CD8 T-lymphocytes

from 3 of 3 LNTP and 11 of 15 normal individuals, but not from 4 progressors (Fig. 1a). A unique peak at 7,815.0 Da, later identified as MIP-1α (see below), was also detected in stimulated samples from two LTNP. Although plenty of peaks were observed from 8,000 to 200,000 Da, no significant differences were found between stimulated and unstimulated CD8 cultures for the three study groups (data not shown). In particular, no peak >8,000 Da consistently correlated with the presence of CAF activity.

To further characterize the cluster of peaks between 3,300 and 3,500 Da, culture supernatants from stimulated CD8 T cells from LTNP subject-3 (LTNP-3) and normal control number-2 (Normal-2) were enriched for these proteins as described (33). Enriched materials were then treated with dithiothreitol (DTT), acrylamide, or iodoacetamide to probe for existence of disulfide bonds within each protein in the cluster. The resultant materials were then analyzed by SELDI-TOF-MS. Table 1 shows the changes in molecular mass for the three peaks found in Normal-2 upon reduction with DTT. Each peak gained ~6 Da after reduction (Fig. S1), suggesting that every protein in the cluster contains three internal disulfide bridges, because DTT reduction would add two hydrogen atoms to form two free sulfhydryl groups for each disulfide bond broken. Furthermore, for peaks detected in the culture supernatant from LTNP-3, reduction and alkylation with acrylamide or iodoacetamide led to increases of ~434 Da or 349 Da, respectively (Table 1). Given the molecular weights of acrylamide (m.w. 71) and iodoacetamide (m.w. 57), the observed mass increases were again consistent with the addition of 6 acrylamide or iodoacetamide molecules to each protein through six free sulfhydryl groups. This result further confirmed the presence of three intramolecular disulfide bridges in each protein in the cluster. Taken together, these findings raised the possibility that these small proteins are similar and related.

Identification of the Protein Cluster as Human α-Defensins-1, -2, and -3. By searching through protein databases (NCBI: www.ncbi.nlm.nih.gov; Swiss-Prot: www.ebi.ac.uk/swissport), we found that the peaks of 3,371.9 Da, 3,442.5 Da, and 3,486.5 Da correspond precisely to the m.w. of human α-defensins-2, -1 and -3, respectively, which are peptide antibiotics made principally by human neutrophils (36-38). Each of these peptides is known to contain three internal disulfide bonds (39). Moreover, the absence of the 3,486.5 Da peak in some cases (e.g., LTNP-3 in Fig. 1a) is consistent with the lack of α-defensin-3 in about 10% of the population (40). To confirm the identity of these molecules, we first examined whether a monoclonal antibody specific for human α-defensin-1,2,3 would recognize the protein peaks of interest. Supernatants from stimulated CD8 T lymphocytes from LTNP-3 and Normal-2 were preincubated with beads only or beads coated with an anti-α-defensin-1,2,3 or anti-MIP-1α monoclonal antibody as described (33), prior to testing on protein-chip arrays using SELDI-TOF-MS. Pretreatment with an anti-defensin-1,2,3 antibody eliminated the cluster of proteins in the range of 3,300 to 3,500 Da (Fig. 2a), without significantly affecting other peaks. Preincubation with an anti-MIP-1α antibody did not affect the peaks of interest, but did result in the removal of a peak at 7815.0 Da. These findings strongly support the suggestion that this cluster of proteins represents members of the human α-defensin family.

To further confirm this conclusion, the enriched materials previously used for the reduction and alkylation experiments were digested with trypsin and analyzed by tandem mass spectrometry (33). Trypsin-digested materials from both LTNP-3 and Normal-2 yielded a unique 1060.50 Da (Fig. 2b, upper-right inset) fragment, which was further selected and fragmented into smaller ions by collision-induced dissociation in the MS-MS collision cell. The seven unique ions generated by this means (Fig. 2b) were then used in a protein search engine (see Legend to Fig. 2b) to look for theoretical fragments of the 1060.50 Da parent ion. The search yielded a perfect and unequivocal match with a trypsin-digest fragment of human α-defensins-1, -2, and -3. In fact, this peptide is conserved among these three molecules and corresponds exactly to the sequence YGTCIYQGR from amino-acid positions 16 to 24 (Fig. 2b). We therefore confirmed by protein sequencing that the proteins of interest are indeed members of the human α-defensin family.

Human α-Defensins-1, -2, and -3 Account for the HIV-1-Suppressive Activity of CAF That Is Not Attributable to β-Chemokines. To evaluate the relative contribution of α-defensins-1, -2, and -3 to CAF activity, culture supernatants from stimulated CD8 T cells from LTNP-3 and LTNP-5 were selectively depleted of these molecules using an affinity column or beads coated with a specific antibody (33). Fig. 3a compares the antiviral activity, before and after depletion of α-defensins-1, -2, and -3, against a panel of X4 and R5 HIV-1's from various genotypes (33). Before depletion, culture supernatants were able to inhibit ~50-60% of the replication of all X4 viruses tested. After depletion, however, the inhibitory effect against X4 viruses was completely eliminated, indicating that α-defensins-1, -2, and -3 account for most, if not all, of the suppressive activity of CAF against X4 viruses. For R5 viruses, there was an average of ~40% reduction in anti-HIV-1 activity after the removal of α-defensins (Fig. 3a). Control experiments using an irrelevant antibody did not results in the loss of antiviral activity (Fig. S2).

We next examined whether CAF activity could be neutralized in a dose-dependent manner by the addition of an anti-α-defensins-1,2,3 antibody to the culture supernatant, with and without the co-addition of antibodies to β-chemokines. Anti-HIV-1 activity of CD8 supernatants from LTNP-3 and LTNP-5 decreased as the concentration of an anti-α-defensin antibody increased (Fig. 3b). For all X4 viruses tested, the suppressive activity of CAF was virtually eliminated when antibody concentration reached 25 μg/ml, while a similar amount of a control antibody had no effect (Fig. S3). The inhibitory activity against R5 viruses was also reduced by the addition of an anti-α-defensin antibody, although the effect was not as profound (Figs. 3b and S3). To address the possibility that the residual activity against R5 viruses could be due to β-chemokines (21), culture supernatants from LTNP-3 and LTNP-5 were treated with increasing amounts of a mixture (1:1:1) of antibodies against MIP-1α, MIP-1β and RANTES, together with a fixed concentration (25 μg/ml) of an anti-α-defensin antibody. The residual antiviral activity against three R5 isolates was almost completely neutralized at the highest antibody concentration used (Fig. 3b, right panels). Collectively, these results suggest that α-defensins-1, -2, and -3 account for nearly all of the anti-HIV-1 activity in supernatants of stimulated CD8 T-lymphocyte cultures that is not attributable to β-chemokines.

Synthetic and Purified Human α-Defensins Can Inhibit HIV-1 Replication In Vitro. We next turned our attention to the testing of synthetic or purified forms of α-defensins. Two products are commercially available: α-defensin-1 and -2 (American Peptide Company, Sunnyvale, CA). With

increasing concentrations of a mixture (1:1) of these two synthetic α-defensins, a greater degree of inhibition was observed against 6 isolates of HIV-1 (Fig. 4), regardless of viral phenotype or genotype. The 50% inhibitory concentrations (IC_{50}) for the mixture ranged from ~11 to 24 μM, and there was no evidence of cytotoxicity associated with these peptides (data not shown). While the antiviral potency of this mixture was not great, it was noted that these commercial products were not pure (Fig. S4). Thus, to ensure the specificity of the anti-HIV-1 activity of commercial α-defensin preparations, virus inhibition assays (33) were repeated with these peptides but now in the presence of an anti-α-defensin antibody. Fig. 4 (left panel) shows that the antibody indeed neutralized substantially the anti-HIV-1 activity of commercial peptides. This result suggests that the antiviral effect is not mediated by non-specific contaminants in the commercial preparations; instead, the activity resides in elements that are recognized by the anti-α-defensin antibody.

We also examined the anti-HIV-1 activity of α-defensins-1, -2, and -3 purified from neutrophils of a normal person (36, 39). This preparation contained α-defensin peaks that are virtually indistinguishable by mass spectrometry from those found in supernatants of CD8 T cells from LTNP-5 (Fig. S5). It too inhibited HIV-1 replication with IC_{50} of ~0.5 to 2.2 μM (Fig. 4, right panel) and without cytotoxicity, suggesting that purified α-defensins are about 10-20 fold more potent against HIV-1 than commercial products. The antiviral effect of purified human neutrophil α-defensins was also substantially reduced or eliminated by the addition of the α-defensin-specific antibody.

A Subset of CD8 T Lymphocytes Express α-Defensins-1, -2, and -3. Neutrophils and CD8 T cells purified from several normal blood donors were studied by immunofluorescence (33) for intracellular expression of α-defensins-1, -2, and -3. A fraction of unstimulated CD8 T lymphocytes carried these molecules within small cytoplasmic granules, but in quantities considerably less than that found in neutrophils (Fig. 5). Upon stimulation, some of the CD8 T cells seemed to lose the α-defensin-positive granules, presumably due to secretion into the culture supernatant. On the other hand, a small percentage of CD8 T cells were activated to express a higher amount of α-defensins (Fig. 5; cell on the extreme right).
By flow cytometric analysis (33), about 2.3% of unstimulated αβ CD8 T lymphocytes expressed appreciable levels of α-defensins (Fig. S6). After one day of stimulation, some of the α-defensin-containing cells were no longer detectable. However, consistent with the immunofluorescence results, a sizeable population of CD8 T cells (21.1%) expressing higher amounts of α-defensins emerged after two days of stimulation. The α-defensin-positive CD8 cells were predominantly αβ T cells without γδ or NK markers. These findings further confirm that CD8 T cells do indeed harbor and secrete α-defensins-1, -2, and –3, establishing yet another linkage between innate and acquired immune systems.

DISCUSSION

The principal source for α-defensins-1, -2 and -3 is the neutrophil (36, 39). However, additional producers have been described, including NK cells, γδ T cells, B cells, and monocyte/macrophages (41), as well as certain epithelial cells (42). Thus, it is not too surprising that these molecules are also made by CD8 T lymphocytes, as shown here. Having identified certain α-defensins as CAF, it will now be easier to determine the precise subpopulation of CD8 T cells that is producing this family of antiviral factors.

α-defensins-1, -2, and -3 are cationic molecules, which contain three intramolecular disulfide bonds (39) (see Fig. 2b). These peptides can damage the membrane of bacteria, probably only when they are in a proper conformation constrained by disulfide linkages (39). We do not know whether this would be true of their anti-HIV-1 activity. Nor do we know the elements within α-defensins that mediate the antiretroviral effect. Likewise, the mechanism of action of defensins on HIV-1 is also unclear, although previous studies on CAF have suggested an effect on viral transcription (12, 13).

Our studies to date do not adequately quantify the potency of α-defensins against HIV-1. While the commercial products are active in blocking viral infection (Fig. 4), they also contain components with molecular masses that are incorrect for α-defensins (Fig. S4). In addition, there is no assurance that proper forms are synthesized even for those peaks that have the right approximate mass. In this regard, it is worth noting two additional points. First, when α-defensin-1 or -2 was individually tested against HIV-1, the inhibitory activity was rather low compared to results for the two defensins combined. It is possible that the anti-HIV-1 activity is mediated by different defensins interacting together, perhaps through the formation of heterodimers. Second, the commercial products together inhibited HIV-1 replication with IC_{50} of 11-24 μM. However, we found that α-defensins purified from human neutrophils blocked HIV-1 infection with a 10-20-fold greater potency. Additional studies are necessary to define the true antiviral potency of α-defensins, which in turn will determine their clinical utility in treating HIV-1.

In light of our new findings, it is interesting to note that several defensins have been reported to have antiviral activity in general and anti-HIV-1 activity in particular. Human α-defensin-1 can inactivate herpes simplex virus types 1 and 2, cytomegalovirus, vesicular stomatitis virus, and influenza virus (43). Modest anti-HIV-1 activity has been found for defensin-like peptides from guinea pigs, rabbits and rats (44), as well as from insect species (45). Lastly, a human homologue of the monkey θ-defensin, termed retrocyclin, has recently been demonstrated to block HIV-1 infection in vitro (46). More studies are required to relate these observations to ours. Nonetheless, we hope that the identification of α-defensins-1, -2, and -3 as the long-sought-after CAF will open up fruitful avenues of research.

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Figs. S1 to S7

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Figure 1. Representative protein mass spectra of culture supernatants from stimulated and unstimulated CD8 T cells from two LTNP, one normal individual, and one progressor. Protein peaks that are up-regulated after stimulation are highlighted and their masses are indicated.

Figure 2. (a) Identification of protein peaks with molecular masses of 3,371.9 Da, 3,442.5 Da, and 3,486.5 Da as human α-defensins-2, -1 and -3, using beads coated with an anti-α-defensins-1,2,3 antibody. (b) Protein sequencing of a unique 1060.50 Da peptide fragment after trypsin digestion (upper right), using tandem mass spectrometry. Unique peaks from collision-induced dissociation of 1060.50 Da parent ion are indicated. Protein Prospector MS-Tag search of NCBI and SwissProt database showed that the peptide fragment, YGTCIYQGR (highlighted in the upper left), from α-defensins-1, -2, and -3 was the best match with a Probability Based Mowse Score of 49 (Mascot software from Matrix Science). The next closest match (β-galactosidase precursor, 76,091 Da) had a score of only 17. In such analyses, a Mowse score over 38 is regarded as positive identification or extreme homology.

Figure 3. (a) Antiviral activity against a panel of X4 and R5 HIV-1's before (solid) and after (hatched) depletion of α-defensins-1, -2 and -3 from culture supernatants of LTNP-3 and LTNP-5. The names of the viral isolates are as indicated, and the HIV-1 genotypes are shown in parentheses. The error bars indicate the standard deviation from the mean of two independent experiments. (b) Antiviral activity of culture supernatants from stimulated CD8 T cells from LTNP-3 and LTNP-5 in the presence of increasing amounts of antibodies against α-defensins-1, -2 and -3 (left panels) or in combination with that against β-chemokines (right panels).

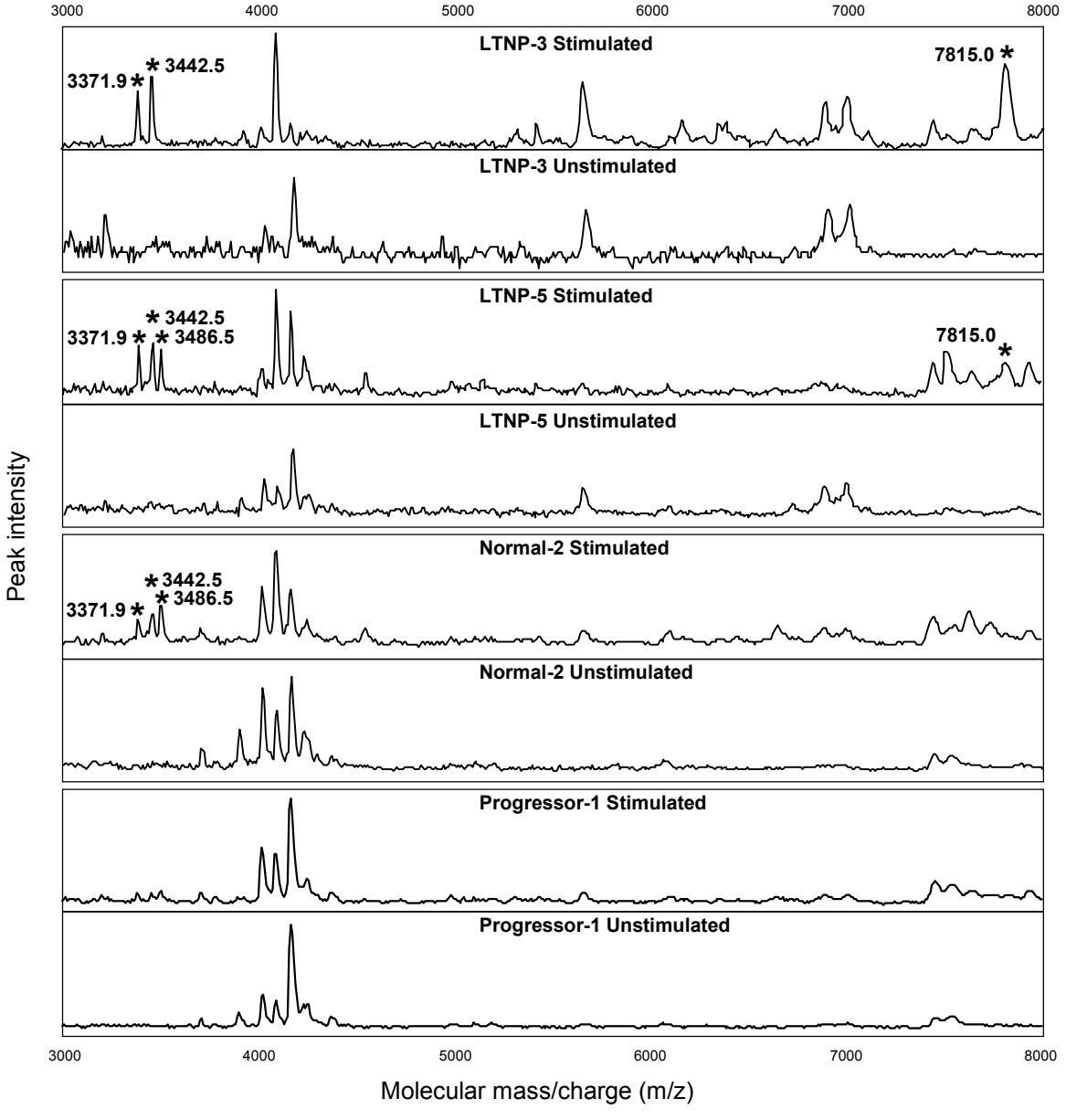
Figure 4. Anti-HIV-1 activity of commercially available α-defensins-1 and -2 peptides (left panel) and purified α-defensins-1, -2 and -3 (right panel). The unconnected symbols at the lower right corner of each panel denote the antiviral activity of the highest concentration of α-defensins when an anti-α-defensin monoclonal antibody (25 μg/ml) is also added.

Figure 5. Immunofluorescence staining of α-defensins-1, -2, and -3 in human neutrophils as well as in unstimulated and stimulated CD8 T lymphocytes. The procedure was carried out as described (33) such that α-defensins stain in green, CD8 proteins in red, and nuclei in blue. Cells stained with an irrelevant antibody are shown in Fig. S7.

Table 1. Changes in molecular mass (m/z) before [-] and after [+] reduction ± alkylation.

	dithiothreitol				acrylamide			iodoacetamide		
	[-]	[+]	net change		[-]	[+]	net change	[-]	[+]	net change
Normal-2				LTNP-3						
peak 1	3371.0	3377.2	6.2	peak 1	3370.0	3804.3	434.3	3371.5	3720.0	348.5
peak 2	3441.7	3447.8	6.1	peak 2	3441.3	3875.0	433.7	3442.1	3791.3	349.2
peak 3	3485.8	3491.6	5.8	peak 3	n/d	n/d	n/d	n/d	n/d	n/d
Average			6.0				434.0			348.9
# of disulfide bonds			3				3			3

n/d: not detected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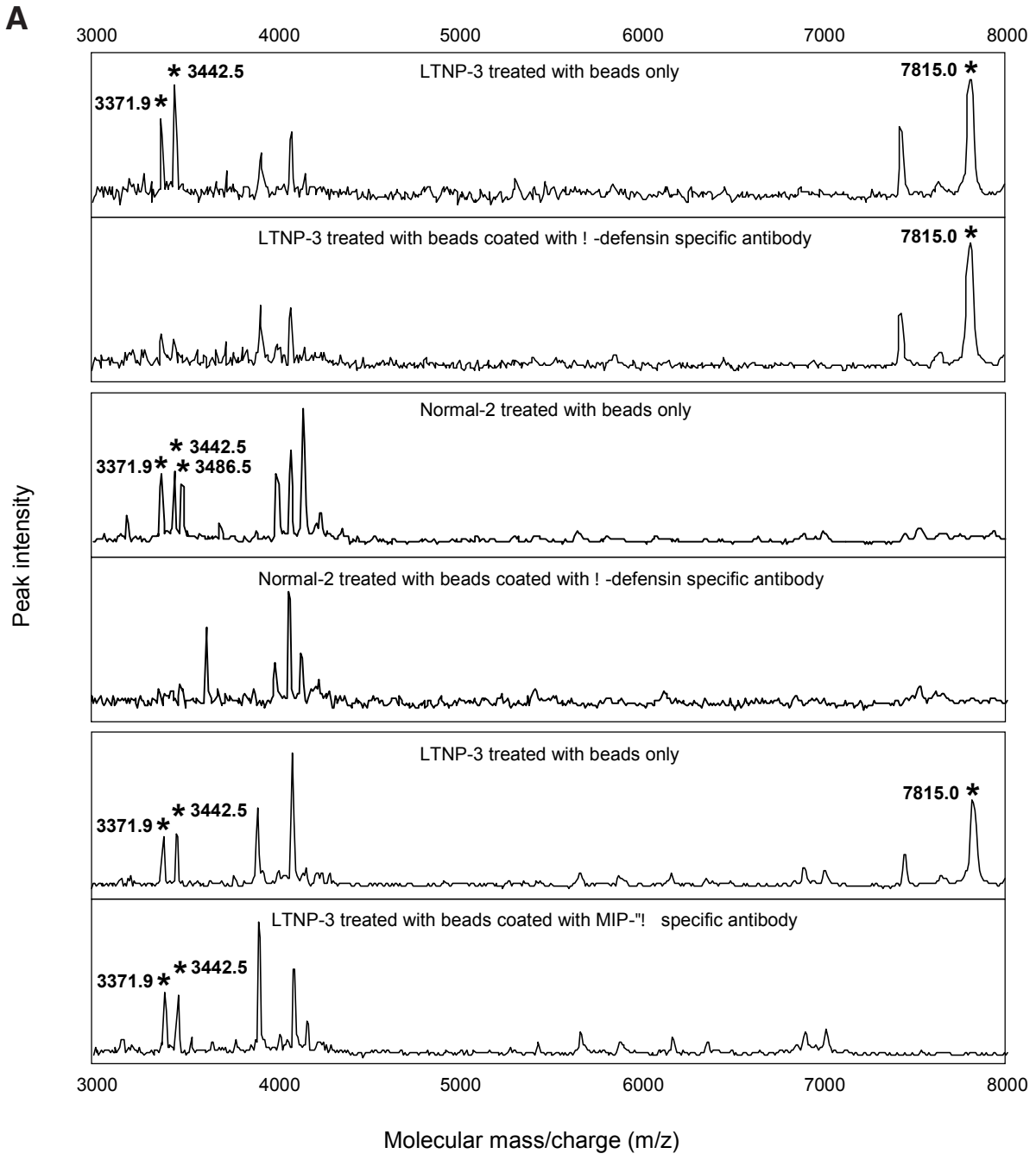
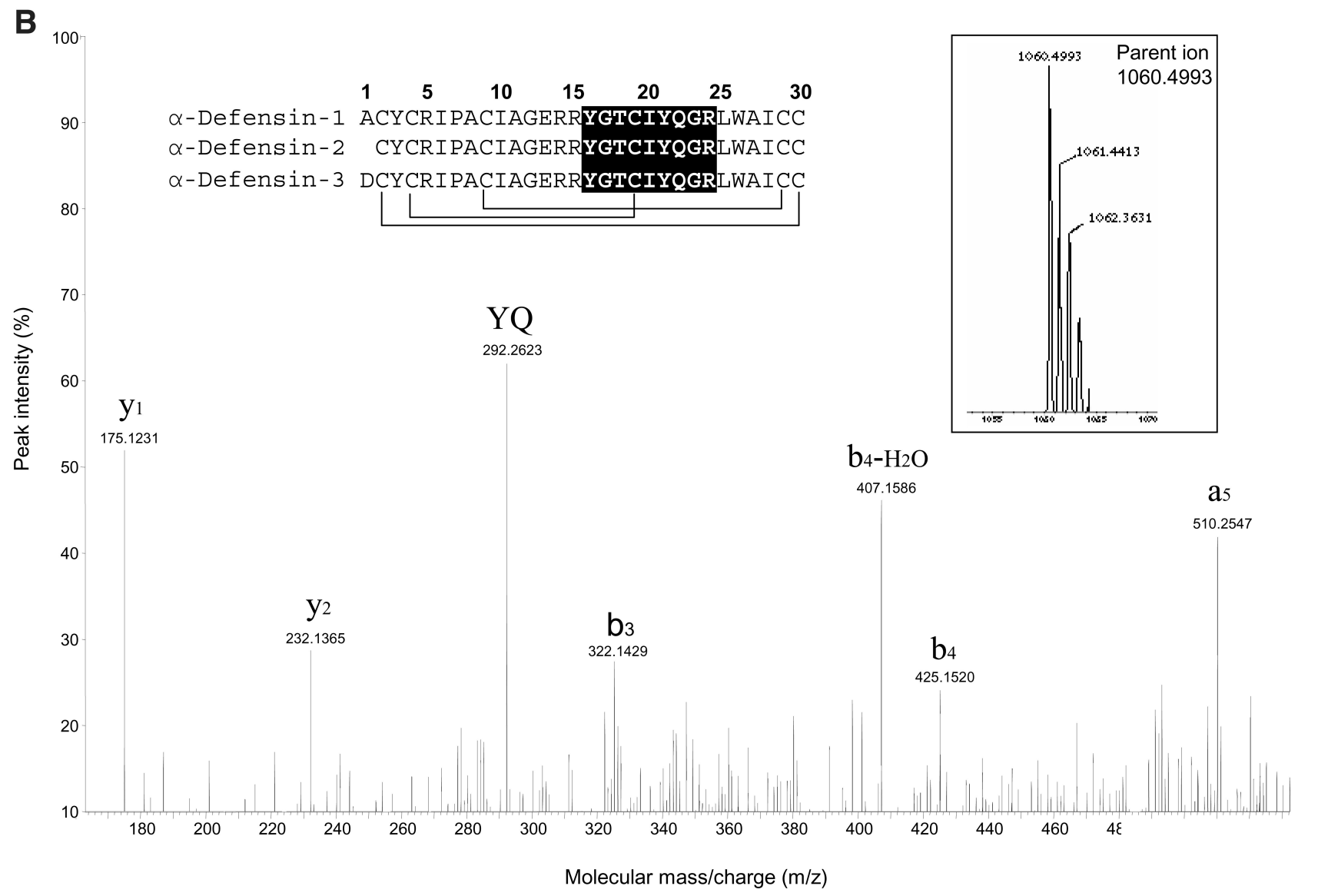
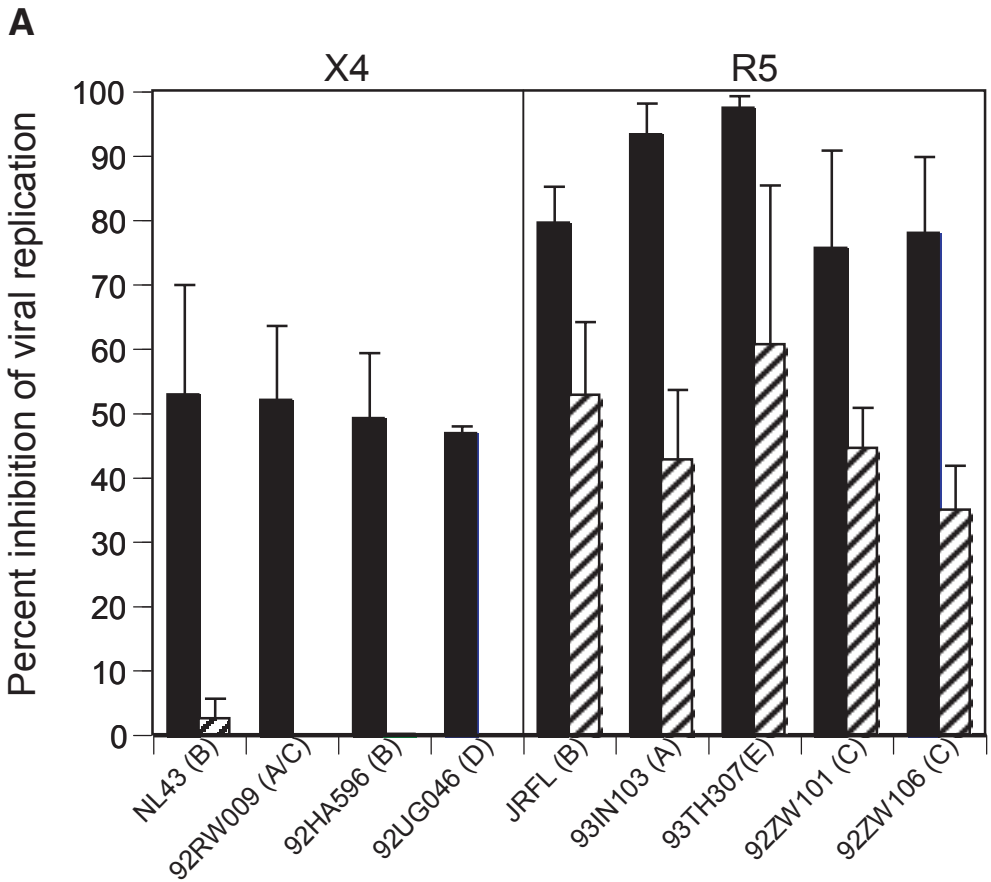
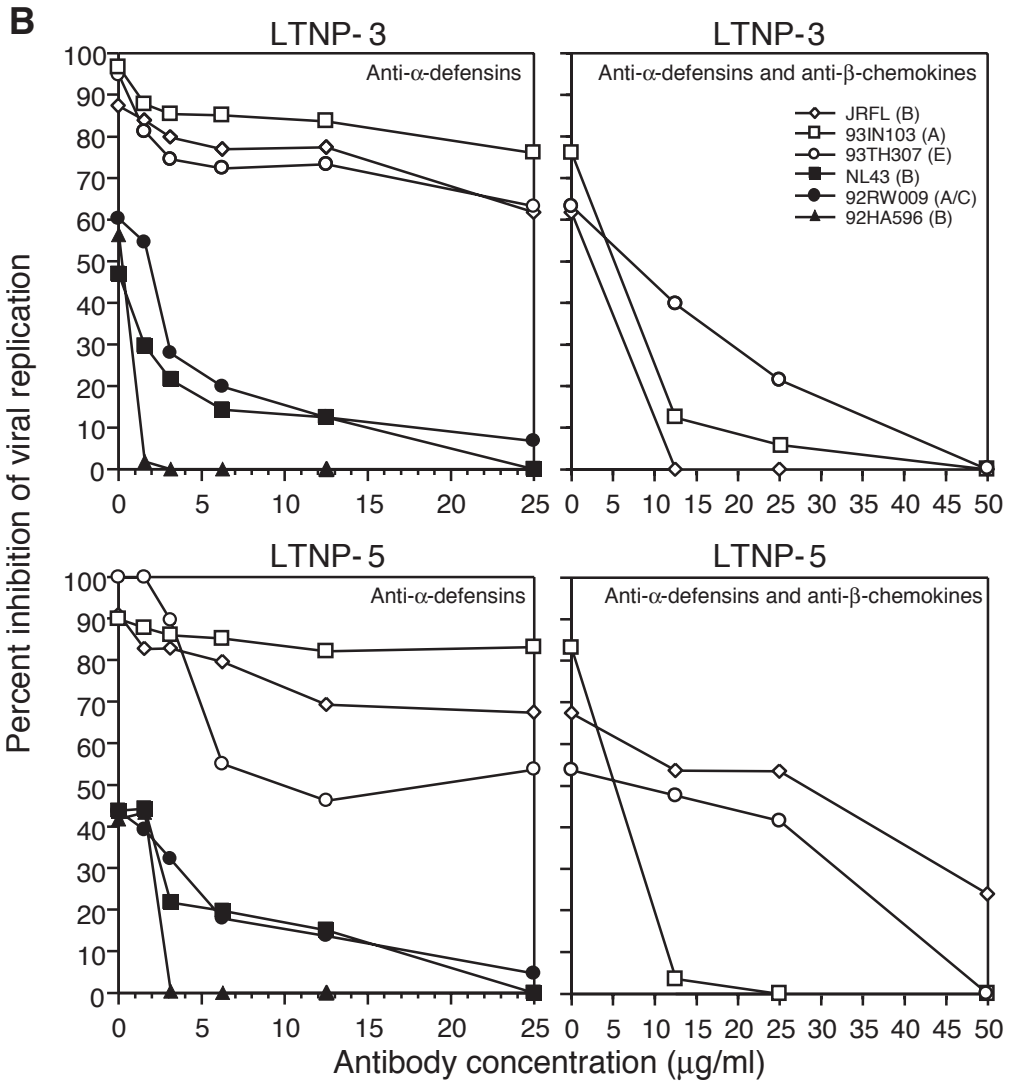
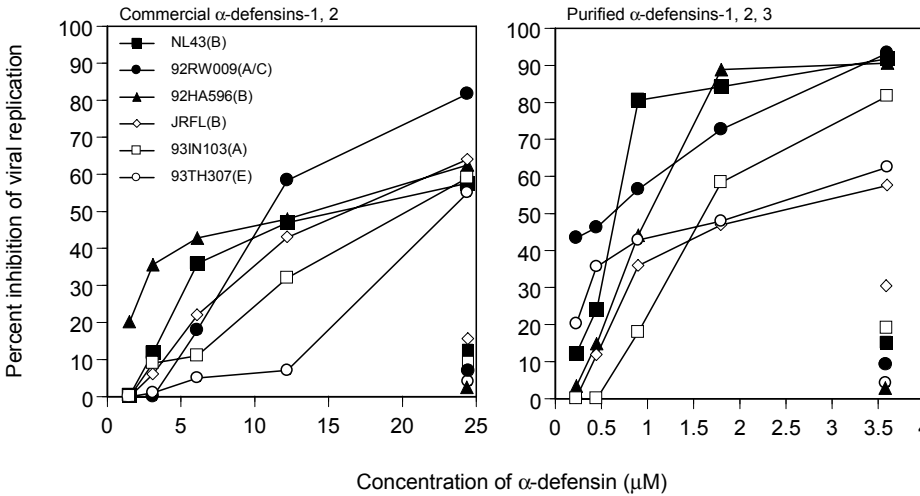
4000
5000
6000
7000
8000
LTNP-3 Stimulated
3371.9
3442.5
7815.0
LTNP-3 Unstimulated
LTNP-5 Stimulated
3371.9
3442.5
3486.5
7815.0
LTNP-5 Unstimulated
Normal-2 Stimulated
3371.9
3442.5
3486.5
Normal-2 Unstimulated
Progressor-1 Stimulated
Progressor-1 Unstimulated
Peak intensity
Molecular mass/charge (m/z)

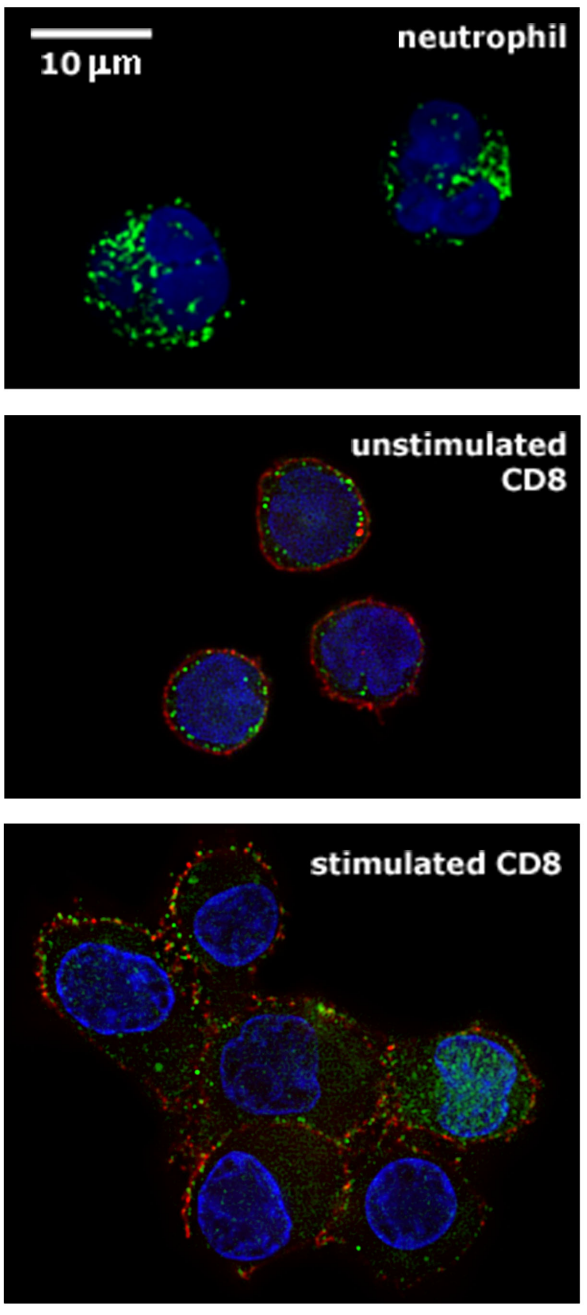

A
3000
4000
5000
6000
7000
8000
LTNP-3 treated with beads only
3371.9
3442.5
7815.0
LTNP-3 treated with beads coated with ! -defensin specific antibody
7815.0
Normal-2 treated with beads only
3371.9
3442.5
3486.5
Normal-2 treated with beads coated with ! -defensin specific antibody
LTNP-3 treated with beads only
3371.9
3442.5
7815.0
LTNP-3 treated with beads coated with MIP-"! specific antibody
3371.9
3442.5
Peak intensity
Molecular mass/charge (m/z)


B
1 5 10 15 20 25 30
α-Defensin-1 ACYCRIPACIAGERRYGTCIYQGRLWAICC
α-Defensin-2 CYCRIPACIAGERRYGTCIYQGRLWAICC
α-Defensin-3 DCYCRIPACIAGERRYGTCIYQGRLWAICC
Parent ion
1060.4993
1061.4413
1062.3631
YQ
292.2623
y1
175.1231
y2
232.1365
b3
322.1429
b4
425.1520
b4-H2O
407.1586
a5
510.2547
Peak intensity (%)
Molecular mass/charge (m/z)


A
X4
R5
Percent inhibition of viral replication
100
90
80
70
60
50
40
30
20
10
0
NL43 (B)
92RW009 (A/C)
92HA596 (B)
92UG046 (D)
JRFL (B)
93IN103 (A)
93TH307(E)
92ZW101 (C)
92ZW106 (C)


B
LTNP-3
Anti-α-defensins
LTNP-3
Anti-α-defensins and anti-β-chemokines
JRFL (B)
93IN103 (A)
93TH307 (E)
NL43 (B)
92RW009 (A/C)
92HA596 (B)
LTNP-5
Anti-α-defensins
LTNP-5
Anti-α-defensins and anti-β-chemokines
Percent inhibition of viral replication
Antibody concentration (μg/ml)


Commercial α-defensins-1, 2
Purified α-defensins-1, 2, 3
NL43(B)
92RW009(A/C)
92HA596(B)
JRFL(B)
93IN103(A)
93TH307(E)
Percent inhibition of viral replication
Concentration of α-defensin (μM)


10 µm
neutrophil
unstimulated CD8
stimulated CD8