Dominant Ex Vivo Cross-Stimulation of CD8+ T-Cells With Whole Soluble Gag Protein in HIV-Infected Subjects

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Background: Soluble HIV proteins are often used to detect HIV-specific $CD4^+$ T-helper cell responses in vitro. However, exogenous antigens can also indirectly stimulate $CD8^+$ T-cells and thus complicate assessment of $CD4^+$ T-cell responses.

Objective: To analyze the extent of in vitro HIV-1 Gag p55 protein cross-stimulation to $CD8^+$ T-cells in therapy-naive and highly active antiretroviral therapy (HAART)-treated HIV patients and to correlate this phenomenon with HIV disease progression.

Methods: Gag protein–stimulated T-cell responses were measured in total and CD8-depleted peripheral blood mononuclear cells (PBMCs) by interferon (IFN)-γ enzyme-linked immunosorbent spot (ELISPOT) assays in 20 therapy-naive and 60 HAART-treated HIV patients. Numbers of spot forming cells (SFCs) relative to $CD4^+$ and $CD8^+$ T-cell subsets were calculated. Gag protein–stimulated responses were correlated with markers of disease progression.

Results: Stimulation of PBMC with HIV-1 Gag protein induced higher $CD8^+$ T-cell responses than $CD4^+$ T-cell responses in both therapy-naive and HAART-treated HIV patients ($P < 0.001$). Gag protein cross-stimulation of $CD8^+$ T-cells was higher in therapy-naive than in HAART-treated HIV patients ($P < 0.001$). In HAART-treated HIV patients, we detected an inverse correlation between Gag protein cross-stimulation of $CD8^+$ T-cells and the CD4 count ($R = -0.311$; $P = 0.016$). Depletion of $CD14^+$ cells abrogated the responses, suggesting that Gag protein cross-stimulation of $CD8^+$ T-cells depends on antigen processing and presentation by antigen-presenting cells (APCs).

Conclusions: HIV protein cross-presentation to $CD8^+$ T-cells should be taken into account when detecting HIV-specific T-cell responses by stimulation of PBMCs with whole exogenous antigens.

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As a rule, soluble exogenous proteins are processed and presented by antigen-presenting cells (APCs) to $CD4^+$ T helper cells in the context of MHC class II molecules, whereas $CD8^+$ T-cells are stimulated by endogenous peptides in the context of MHC class I molecules. Several studies, however, observed that soluble exogenous proteins may also reach the MHC class I pathway and thereby stimulate $CD8^+$ T-cells in addition to $CD4^+$ T-cells. This process, referred to as antigen cross-presentation, seems to be an exclusive feature of dendritic cells and macrophages and may occur via various pathways.[1–8] A considerable number of studies have provided evidence for the importance of this alternative antigen presentation pathway (reviewed in Refs. [9,10]), and its role in CTL activation during the immune defense against infectious diseases has been proposed.[11,12] Recently, the CD91-dependent cross-presentation and the increased surface expression of CD91 on $CD14^+$ monocytes have been associated with the apparent HIV resistance that is observed in exposed seronegative subjects.[13] However, the degree of HIV protein cross-presentation in HIV patients after in vitro stimulation of peripheral blood mononuclear cells (PBMC) with entire soluble exogenous HIV protein has not yet been described.

HIV-specific cellular immune responses have been shown to play an important role in the containment of HIV replication (reviewed in refs.).[14–16] Unfortunately, HIV-specific T-cell responses ultimately fail to control the virus in most HIV-infected persons, mainly as a consequence of impaired $CD4^+$ T helper and $CD8^+$ cytotoxic T-cell function.[17–23] For the assessment of HIV-specific T-cell responses, recombinant vaccinia viruses, whole protein preparations or (overlapping) peptides are used for stimulation in "enzyme-linked immunosorbent spot" (ELISPOT) or "intracellular cytokine staining" (ICS) assays.[24–28] However, up to date, the correlates of protection against HIV infection and/or progression are still not well defined. One possible explanation may be that the currently used sensitive ELISPOT assays are unsuitable to clearly discriminate between $CD4^+$ and $CD8^+$ T-cell responses. On the other hand, ICS can distinguish $CD4^+$ and $CD8^+$ T-cell responses; however, this assay is less likely to detect low-level responses.[29]

In the present study, we analyzed the relative contributions of $CD4^+$ and $CD8^+$ T-cell responses upon in vitro HIV-1 Gag protein stimulation in therapy-naive and highly active antiretroviral therapy (HAART)-treated HIV patients. Furthermore, we studied the association between whole Gag protein cross-stimulation and markers of HIV disease progression in both patient groups.

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METHODS

Study Subjects and Samples

Ninety-five HIV patients who attended the clinic at the Institute of Tropical Medicine in Antwerp, Belgium, were enrolled. HIV Gag-specific T-cell responses were analyzed in 80 HIV patients. HIV Nef- and CMV-specific T-cell responses were analyzed in 10 HIV patients. Samples from 5 HIV patients were used in control experiments such as ICS, $CD14^+$ T-cell depletion, and $CD8^+$ cell enrichment. In addition, blood samples were collected from 16 HIV-negative blood donors. The study was approved by the ethical committee of the Institute of Tropical Medicine.

Blood Sample Preparation

Whole blood from HIV patients and uninfected controls was drawn into EDTA tubes. Plasma was separated from cells by centrifugation at $500\times g$. For each patient, the viral load (VL) (number of HIV-1 RNA copies/mL plasma; Cobas Amplicor HIV-1 Monitor assay, version 1.5, Roche) and the CD4 count (number of $CD4^+$ T-cells/μL blood, based on flow cytometric enumeration using a FACSCalibur instrument; Becton Dickinson, San Jose, USA) was determined. PBMC were obtained by density gradient centrifugation using Ficoll-Paque Plus (Amersham Biosciences, Uppsala, Sweden). Depletion of $CD8^+$ T-lymphocytes from PBMC was done by using Dynabeads (Dynal Biotech, Oslo, Norway) according to the manufacturer's instructions. Two rounds of depletion were applied with a $CD8^+$ T-cell/bead ratio of 1:5. This resulted in an average $CD8^+$ T-cell depletion of 97.5%. A similar protocol was used to deplete $CD14^+$ cells from total PBMC. Enrichment of $CD8^+$ T-lymphocytes from PBMC was performed by using Miltenyi microbeads and enrichment columns according to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany).

HIV Proteins

Recombinant HIV-1 subtype B p55 Gag protein expressed in yeast was obtained from the AIDS Research and Reference Reagent Program (Catalog number 5109, Division of AIDS, NIAID, NIH, Rockville, MD, USA), recombinant HIV-1 Nef and cytomegalovirus (CMV) pp65 proteins, both expressed in *Escherichia coli*, were obtained from Biodesign (Saco, ME, USA), and CMV lysate was obtained from Virion (Zürich, Switzerland).

Interferon (IFN)-γ ELISPOT Assay

ELISPOT assays were performed as previously described,[30] with minor modifications. Briefly, 96-well polyvinylidene fluoride-bottom plates (Millipore, Molsheim, France) were prewetted with sterile water containing 70% ethanol, washed 4 times with sterile water, and coated with 5 μg/mL anti-IFN-γ monoclonal antibody (Mabtech, Nacka, Sweden) overnight at 4°C. The next day, plates were washed 4 times with phosphate-buffered saline (PBS) (Cambrex, Verviers, Belgium) and blocked with 50 μL/well of RPMI containing 10% fetal bovine serum (Biochrom KG, Berlin, Germany), 100 U/mL penicillin, and 100 μg/mL streptomycin (Roche Diagnostics, Mannheim, Germany) (further referred to as medium). Total PBMC as well as CD8-depleted PBMC resuspended in medium were added in duplicate wells at a concentration of 200,000 PBMC per well. As negative control, PBMC were added to duplicate wells with medium alone. As positive control, PBMC were added to duplicate wells with 0.5 μg/mL staphylococcal enterotoxin B (SEB) (Sigma-Aldrich, Bornem, Belgium). HIV Gag p55 protein was used at a final concentration of 10 μg/mL in duplicate wells. Plates were incubated overnight at 37°C and 5% CO_2. The next day, plates were washed 4 times with PBS containing 0.05% Tween-20 (Merck, Hohenbrunn, Germany), incubated with 1 μg/mL biotinylated anti-IFN-γ (Mabtech) for 2 hours at 37°C and 5% CO_2, washed again and incubated with streptavidin-conjugated alkaline phosphatase (Mabtech) for 1 hour at room temperature. Plates were washed again and developed with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium substrates (Biorad Laboratories, Hercules, CA, USA) for 30 minutes at room temperature followed by rinsing in tap water. Spots were counted with an automated ELISPOT reader (Autoimmun Diagnostika, Strassberg, Germany). Average numbers of spots per well were normalized to spot forming cells (SFC) per million PBMC. Negative control SFC were subtracted from stimulated SFC and negative values were scored as zero.

Whole Protein Cross-Stimulation of $CD8^+$ T-Cells

Antigen cross-stimulation was analyzed by stimulating total and CD8-depleted PBMC from HIV patients and controls with the entire soluble HIV Gag p55 protein, resulting in numbers of HIV-specific T-cells per million PBMC and HIV-specific $CD4^+$ T-cells per million CD8-depleted PBMC, respectively. Numbers of HIV-specific $CD4^+$ and $CD8^+$ T-cells per million PBMC were calculated by adjusting for the relative changes in T-cell subset proportions after CD8 depletion (Eqs. (1a) and (1b)). The numbers of HIV-specific $CD4^+$ and $CD8^+$ T-cells per million $CD4^+$ and $CD8^+$ T-cells, respectively (Eqs. (2a) and (2b)), were also calculated. The correction factors applied in Eqs. (1a), (1b), (2a), and (2b) were derived from fresh whole blood $CD4^+$ and $CD8^+$ T-cell subset analysis using fluorochrome-labeled anti-CD45, anti-CD3, anti-CD4, and anti-CD8 monoclonal antibodies, a FACSCalibur flow cytometer and CellQuest software (all from Becton Dickinson).

$$\begin{aligned}&\#\text{ spec. CD4}^+\text{ T-cells/10}^6\text{ PBMC}\\&=\#\text{ SFC/10}^6\text{ PBMC}^{\text{CD8}-}\\&\quad\times\frac{\%\text{ CD4}^+\text{ T-cells in PBMC}}{\%\text{ CD4}^+\text{ T-cells in PBMC}^{\text{CD8}-}}\end{aligned}\qquad\text{(Eq.1a)}$$

$$\begin{aligned}&\#\text{ spec. CD8}^+\text{ T-cells/10}^6\text{ PBMC}\\&=\#\text{ SFC/10}^6\text{ PBMC}-\#\text{ spec. CD4}^+\text{T-cells/10}^6\text{PBMC}\end{aligned}\qquad\text{(Eq.1b)}$$

$$\begin{aligned}&\#\text{ spec. CD4}^+\text{ T-cells/10}^6\text{ CD4}^+\text{ T-cells}\\&=\frac{\#\text{ spec. CD4}^+\text{ T-cells/10}^6\text{ PBMC}}{\%\text{ CD4}^+\text{ T-cells in PBMC}^{\text{CD8}-}}\times 100\end{aligned}\qquad\text{(Eq.2a)}$$

$$\#\ \text{spec. CD8}^+\ \text{T-cells/10}^6\ \text{CD8}^+\ \text{T-cells} = \frac{\#\ \text{spec. CD8}^+\ \text{T-cells/10}^6\ \text{PBMC}}{\%\ \text{CD8}^+\ \text{T-cells in PBMC}^{\text{CD8}}} \times 100 \quad \text{(Eq.2b)}$$

Yearly Change in CD4$^+$ T-Cell Count

Linear regression of longitudinal CD4$^+$ T-cell count analyses per patient provided the yearly change in CD4$^+$ T-cell count as described by Oxenius et al.[31] Similarly, we calculated the yearly change in the ratio of the CD4$^+$ T-cell count with log VL. Regression analyses were performed over a maximum period of 2 years before blood sample collection and with a minimum of 4 follow-up time points. HIV patients on HAART with therapy interruptions of >1 month were excluded from this analysis.

Intracellular Cytokine Staining

Cryopreserved PBMC were thawed, washed twice, and stimulated with 10 μg/mL HIV Gag p55 protein for 20 hours at 37°C and 5% CO_2 in 96-well U-bottom plates. Brefeldin A (10 μg/mL) was added to the cell cultures after 4 hours of incubation. Intracellular staining was performed as previously described, with minor modifications.[32] PBMC were washed with PBS (Cambrex) containing 1% BSA and 0.05% sodium azide by centrifuging at 500×*g* for 10 minutes and decanting the supernatant. Next, PBMC were incubated for 10 minutes at 37°C and 5% CO_2 with 0.02% EDTA in PBS and washed again. Cells were stained by adding anti-CD3 allophycocyanin and anti-CD8 peridinin-chlorophyll-protein mAbs (Becton Dickinson) to the cell suspension for 15 minutes at 4°C. The cells were washed, fixed using Leucoperm reagent A (Serotec, Oxford, UK) for 15 minutes at room temperature, and washed again. Cells were then permeabilized using Leucoperm reagent B (Serotec) and stained with anti-CD69 fluorescein isothiocyanate and anti-IFN-γ phycoerythrin mAbs (Becton Dickinson) for 30 minutes at 4°C. The cells were washed twice, resuspended in PBS with 1% PFA, and stored at 4°C until flow cytometric analysis using a FACSCalibur flow cytometer and CellQuest software (Becton Dickinson). Percentages of HIV Gag p55-specific T-cells, noted as CD69/IFN-γ double positive cells, were analyzed within the CD8$^+$ and the CD8$^-$ (CD4$^+$) T-cell subsets.

TABLE 1. Clinical Characteristics of HIV Patients Enrolled in the Study

	Therapy-naive Patients (n = 20)	Patients on HAART (n = 60)
Sex (M/F ratio)	0.90	0.65**
Age (years)	38 (33 to 41)	41 (36 to 47)
Months on HAART	n.a.	47 (24 to 80)
CD4 count (cells/mL)	410 (359 to 496)	489 (335 to 675)
CD4 count > 200 (%)	100	95
Viral load (log_{10} HIV-1 RNA copies/mL)	4.7 (4.5 to 5.1)	1.7 (1.7 to 1.7)**
Detectable VL (%)	100	17
Yearly CD4 count change	−5 (−50 to +124)	+50 (+5 to +125)*
Yearly CD4 count change > 0 (%)	50	79
Yearly CD4 count/log VL change	−2 (−11 to +38)	+38 (+1 to +127)*
Yearly CD4 count/log VL change > 0 (%)	43	77

Median (interquartile range) *$P < 0.05$; **$P < 0.001$; n.a., not applicable.

Cutoff Values and Statistical Analyses

Negative cutoff values for p55-stimulated ELISPOT responses were calculated as the mean number of SFC among 6 HIV-negative blood donors plus 2 times the standard deviation. Nonparametric tests were used for statistical analyses. Mann–Whitney *U* tests were used for comparing 1 variable in 2 groups, Wilcoxon signed rank tests were used for comparing 2 variables in the same group. Spearman rank tests were used for correlations. For all analyses, the level of significance was set at $P < 0.05$.

RESULTS

Characteristics of the Study Population

Twenty of 80 (25%) HIV-positive patients had never received therapy, whereas 60 (75%) were on HAART. Treatment-naive and HAART-treated HIV patients had comparable CD4$^+$ T-cell counts ($P = 0.259$). A higher number of HAART-treated HIV patients showed positive yearly changes in CD4 counts compared with naive HIV patients ($P = 0.058$). As expected, VL levels were significantly lower among HIV patients on HAART than among treatment-naive HIV patients ($P < 0.001$) (Table 1).

In Vitro Stimulation of PBMC From HIV Patients With Soluble HIV Gag p55 Protein Elicits Dominant HIV-Specific CD8$^+$ T-Cell Responses

In vitro IFN-γ ELISPOT responses to HIV Gag p55 whole protein were measured in total and CD8-depleted PBMC from therapy-naive and HAART-treated HIV patients. Sixty-seven of 80 patients (83.8%) showed HIV Gag p55-specific T-cell responses above the negative cutoff value (naive: 95%; HAART-treated: 80%). Both treatment-naive and HAART-treated HIV patients showed significantly higher numbers of HIV Gag p55-specific CD8$^+$ T-cell responses than CD4$^+$ T-cell responses per million PBMC after stimulation with whole HIV Gag p55 protein (naive: median of 457 CD8$^+$ vs 37 CD4$^+$, $P < 0.001$; HAART-treated: median of 109 CD8$^+$ vs 43 CD4$^+$, $P < 0.001$; Fig. 1A). To correct for decreased CD4/CD8 ratios in HIV patients, we analyzed HIV Gag p55-specific ELISPOT responses within the CD4$^+$ and CD8$^+$ T-cell subsets. Numbers of HIV Gag p55-specific CD8$^+$ T-cells per million CD8$^+$ T-cells were still significantly higher than the numbers of HIV Gag p55-specific CD4$^+$ T-cells per million CD4$^+$ T-cells (naive: median of 1428 CD8$^+$ vs 102 CD4$^+$, $P < 0.001$; HAART-treated: median of 307 CD8$^+$ vs 141 CD4$^+$, $P < 0.001$; Fig. 1B).

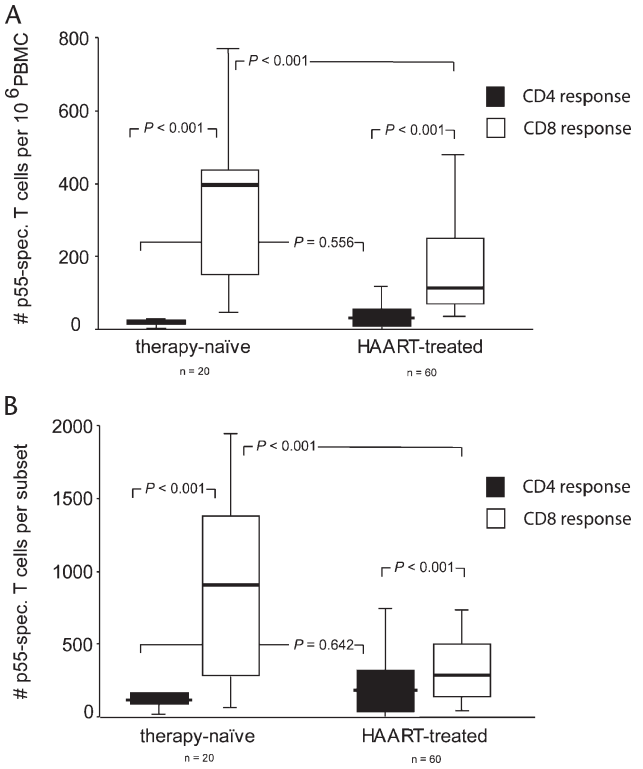

FIGURE 1. Comparison of HIV Gag p55-specific $CD4^+$ and $CD8^+$ T-cell responses in treatment-naive and HAART-treated HIV patients detected by IFN-γ ELISPOT assay. A, Number of $CD4^+$ and $CD8^+$ T-cell responses per million PBMC. B, Number of $CD4^+$ and $CD8^+$ T-cell responses per million $CD4^+$ and $CD8^+$ T-cells, respectively. Black box plots represent the $CD4^+$ T-cell responses, whereas white box plots represent the $CD8^+$ T-cell responses.

Treatment-Naive HIV Patients Showed Higher Levels of HIV Gag p55 Protein Cross-Stimulation of $CD8^+$ T-Cells Than HIV Patients on HAART

The $CD8^+$ T-cell ELISPOT responses after stimulation with whole Gag p55 protein were significantly higher among treatment-naive HIV patients than among HIV patients on HAART (median of 457 vs 109, $P < 0.001$; Fig. 1A). This difference also remained present for the number of HIV Gag p55-specific $CD8^+$ T-cells within the $CD8^+$ T-cell subset (median of 1428 vs 307, $P < 0.001$; Fig. 1B). HIV Gag p55-protein-specific $CD4^+$ T-cell responses were similar in naive and HAART-treated HIV patients ($P = 0.556$) (Figs. 1A–B).

Correlations Between HIV Gag p55-Protein-Specific T-Cell Responses and Markers of Disease Progression

In HAART-treated HIV patients, we detected an inverse correlation between the number of HIV Gag p55-specific $CD8^+$ T-cells per million PBMC and the CD4 count ($R = -0.311$; $P = 0.016$) (Fig. 2A). In naive HIV patients, no significant correlations between HIV Gag p55 protein cross-presentation and markers of disease progression were found. However, in naive HIV patients there was a negative correlation between the number of HIV Gag p55-specific $CD4^+$ T-cells per million PBMC and the VL ($R = -0.564$; $P = 0.010$) (Fig. 2B). No correlations were found between the HIV Gag p55-specific T-cell responses and the yearly changes in CD4 or VL (Table 2).

Detection of HIV Protein Cross-Stimulation of $CD8^+$ T-Cells

Intracellular IFN-γ staining and flow cytometric analyses for 5 HIV patients confirmed that HIV Gag p55 whole protein elicits high levels of HIV-specific $CD8^+$ T-cell responses in addition to the expected $CD4^+$ T-cell responses ($P = 0.043$; Figs. 3A–B). To investigate the APC-dependent nature of the $CD8^+$ T-cell responses, we compared HIV Gag p55-protein-stimulated T-cell responses in total PBMC, purified $CD8^+$ T-cells, and monocyte ($CD14^+$ cells)-depleted PBMC for 5 HIV patients using IFN-γ ELISPOT assays. For each of the 5 HIV patients tested, both $CD8^+$ T-cell purification (Fig. 3C) and $CD14^+$ cell depletion (Fig. 3D) abrogated the ELISPOT responses, suggesting that HIV Gag

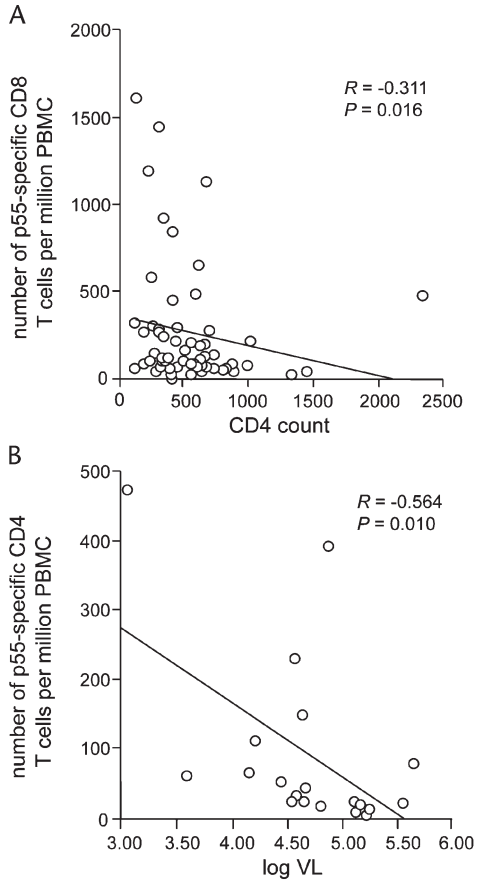

FIGURE 2. Correlations between HIV Gag p55-specific T-cell responses and markers of disease progression. A, Negative correlation between HIV Gag p55-specific $CD8^+$ T-cells and CD4 count in HAART-treated HIV patients. B, Negative correlation between HIV Gag p55-specific $CD4^+$ T-cells and log VL in therapy-naive HIV patients.

TABLE 2. Correlations Between HIV Gag p55-Specific T-Cell Responses and Markers of Disease Progression

	Therapy-naive HIV Patients (n = 20)				HAART-treated HIV Patients (n = 60)			
	HIV p55-specific $CD4^+$ T-cells/106 PBMC		HIV p55-specific $CD8^+$ T-cells/106 PBMC		HIV p55-specific $CD4^+$ T-cells/106 PBMC		HIV p55-specific $CD8^+$ T-cells/106 PBMC	
	R	P	R	P	R	P	R	P
CD4 count	−0.057	0.811	−0.050	0.833	0.179	0.171	−0.311	0.016
Log VL	−0.564	0.010	−0.083	0.726	−0.100	0.449	0.017	0.900
Yearly change in CD4 count	0.121	0.681	−0.077	0.794	−0.140	0.299	−0.087	0.521
Yearly change in CD4 count/log VL	0.235	0.418	−0.288	0.318	−0.047	0.727	0.207	0.113

R indicates correlation coefficient; *P*, *P* value.

p55 protein stimulation of $CD8^+$ T-cells occurs via protein processing and presentation by APCs.

In this study, $CD8^+$ T-cell ELISPOT responses were calculated indirectly by subtracting responses measured in CD8-depleted PBMC from responses measured in total PBMC, that is, relative to the accompanying $CD4^+$ T-cell ELISPOT response (see Eqs. (1a), (1b), (2a), and (2b)). Therefore, underestimation of $CD4^+$ ELISPOT responses would directly result in overestimation of $CD8^+$ ELISPOT responses. To exclude a potential negative bias of $CD8^+$ cell depletion on the $CD4^+$ T-cell responses, we analyzed the percentages of HIV Gag p55-protein-stimulated $CD4^+$ T-cells directly with ICS and flow cytometry for 5 HIV patients before and after $CD8^+$ T-cell depletion. These data show that HIV Gag p55-protein-stimulated $CD4^+$ T-cell responses were not affected by $CD8^+$ cell depletion ($P = 0.893$; Fig. 3E).

To assess whether cross-stimulation of $CD8^+$ T-cells occurred for other soluble HIV antigens than Gag, HIV Nef whole protein-specific T-cell responses were measured by IFN-γ ELISPOT assays in total and CD8-depleted PBMC. For 4 of 7 HIV patients with detectable HIV Nef-protein-specific T-cell responses, the HIV Nef-specific $CD8^+$ T-cell responses were higher than the $CD4^+$ T-cell responses. However, this difference was not statistically significant ($P = 0.374$; Fig. 4A).

Detection of Antigen Cross-Stimulation of $CD8^+$ T-Cells in HIV-Negative Subjects

Next, we evaluated whether cross-stimulation of $CD8^+$ T-cells also occurs in HIV-negative subjects. We stimulated PBMC from CMV-positive HIV patients and CMV-positive but HIV-negative controls with whole recombinant CMV pp65 protein and a CMV lysate. Most CMV-positive but HIV-negative controls showed a dominant pp65-specific $CD8^+$ T-cell response ($P = 0.144$; Fig. 4B). Although CMV pp65-specific T-cell responses were low among HIV patients, 2 HIV patients showed a dominant pp65-specific $CD8^+$ T-cell response. Two of 4 HIV patients showed a dominant CMV-lysate-specific $CD8^+$ T-cell responses compared with none of 4 CMV-positive but HIV-negative controls (Fig. 4C).

DISCUSSION

Usually, whole protein antigen preparations are considered to be ideal antigens to measure $CD4^+$ T helper cell responses in vitro. Taking into account the importance and different function of $CD4^+$ and $CD8^+$ T-cells during HIV infection, we are convinced that it is important to identify the phenotype of the in vitro responding T-cells during assessment of immune correlates of protection. In this study, we analyzed the occurrence and degree of whole HIV-1 Gag protein cross-stimulation of $CD8^+$ T-cells among treatment-naive and HAART-treated HIV patients.

The results of the present study show that in vitro stimulation of PBMC with the entire HIV Gag p55 protein in IFN-γ ELISPOT assays induces high HIV-specific $CD8^+$ T-cell responses in addition to the expected MHC class II restricted $CD4^+$ T-cell responses to exogenous soluble antigens in a total of 80 HIV patients tested. These differences persisted after controlling for the higher $CD8^+$ and lower $CD4^+$ T-cell counts among HIV patients. Our results are in agreement with data from Sester et al[33] who detected higher HIV-specific $CD8^+$ than $CD4^+$ T-cell responses by ICS upon whole blood stimulation with HIV Gag p55 virus-like particles (VLP) and VLP-derived soluble p55 protein. In a previous study, we showed that $CD8^+$ T-cells stimulated with CMV lysate are more frequent in HIV-positive than in HIV-negative individuals, suggesting a role for $CD8^+$ cross-stimulation in HIV infection.[34] In addition, the occurrence of cross-presentation of HIV antigens was demonstrated by stimulating T-cells with dendritic cells loaded with a variety of antigens, for example, noninfectious virions, VLP, apoptotic cells, and lipopeptides.[35–38] It has also been shown that multiple $CD4^+$ and $CD8^+$ T-cell epitopes, processed from an exogenously added HIV-1 Gag p24 peptide, complexed to heat shock protein (HSP) gp96 were efficiently processed and presented by APCs.[39] Together, these studies have provided evidence for the existence and mode of action of antigen cross-presentation. However, the present data show that, in a large group of HIV patients, in vitro HIV cross-stimulation of $CD8^+$ T-cells by using exogenous soluble entire protein may be much more prevalent than previously thought.

In this study, we found significantly higher levels of HIV Gag protein cross-stimulation of $CD8^+$ T-cells among treatment-naive patients than among HAART-treated patients. The decrease of p55 protein cross-stimulation during therapy could result from the lower numbers of HIV-specific $CD8^+$ effector cells in HAART-treated patients, secondary to suppressed viral replication.[40–43] In line with this, we detected an inverse correlation between the number of HIV Gag p55-specific $CD8^+$ T-cells per million PBMC and the CD4 count in HAART-treated HIV patients. In contrast, the HIV-specific $CD4^+$ T-cell

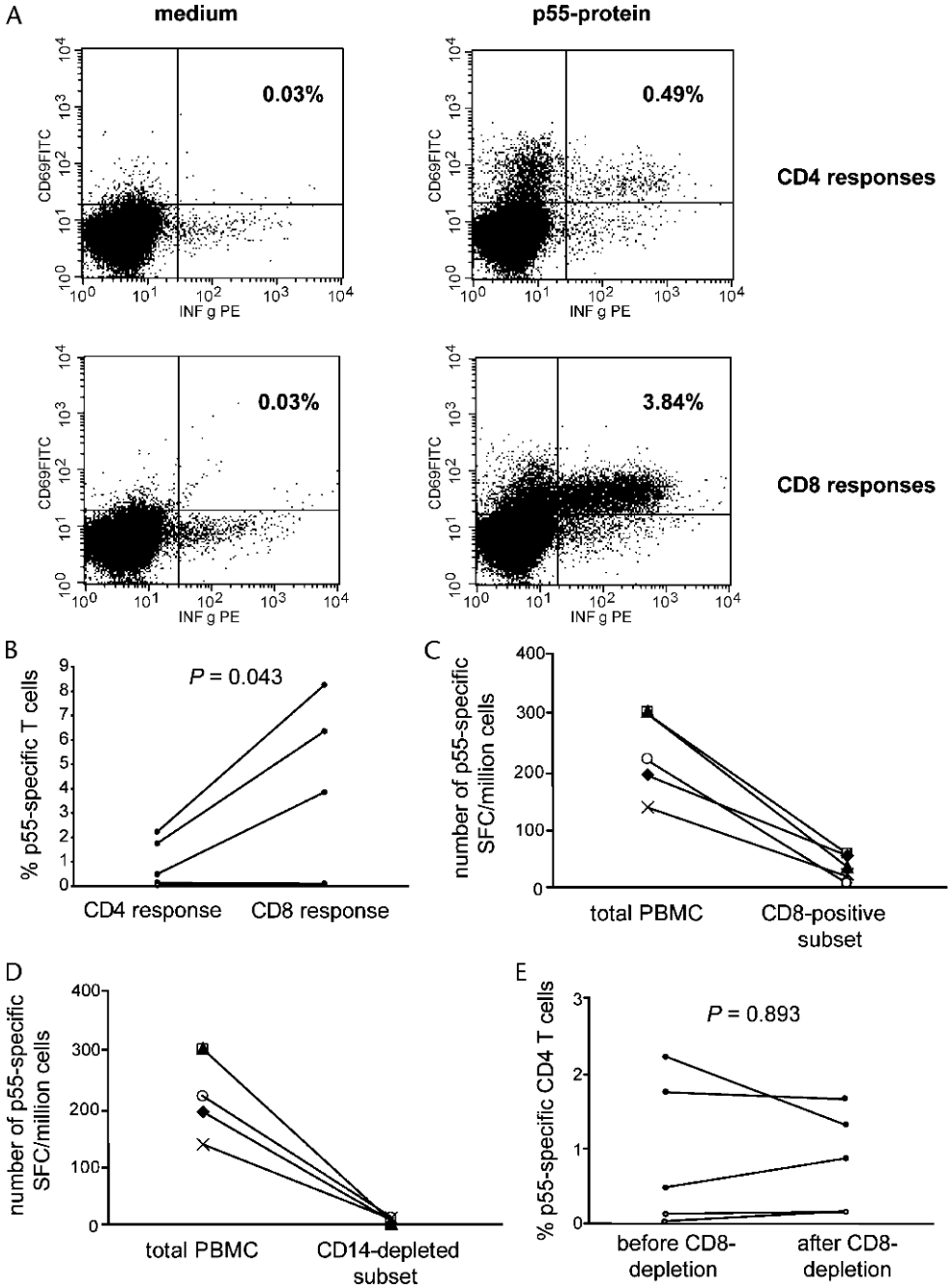

FIGURE 3. HIV Gag p55-protein-specific T-cell responses. A, $CD4^+$ and $CD8^+$ T-cell responses detected by ICS and flow cytometry upon stimulation with medium alone and HIV Gag p55 protein. Data are shown for 1 HIV patient representative for 5. B, Comparison of the percentage of HIV Gag p55-specific $CD4^+$ and $CD8^+$ T-cells detected by ICS and flow cytometry for 5 HIV patients. C–D, HIV Gag p55-protein-specific T-cell responses detected by IFN-γ ELISPOT assay in total PBMC, $CD8^+$ T-cell enriched subset, and $CD14^+$ cell-depleted subset. E, HIV Gag p55-specific $CD4^+$ T-cell responses detected by ICS and flow cytometry before and after $CD8^+$ T-cell depletion.

responses were similar among treatment-naive and HAART-treated HIV patients, suggesting that the effect of HAART on HIV-specific $CD4^+$ T-cell responses is much less pronounced, like previously proposed.[44,45]

Many studies have attempted to correlate the presence of HIV-specific T-cell responses with the VL or the $CD4^+$ T-cell count at time of analysis. To obtain a better indication of the recent history of disease progression of the HIV patients, we correlated the presence of HIV Gag p55-specific T-cell responses with the yearly change in $CD4^+$ T-cell count and the yearly change in the ratio of $CD4^+$ T-cell count and log VL. Our data do not corroborate the correlations detected

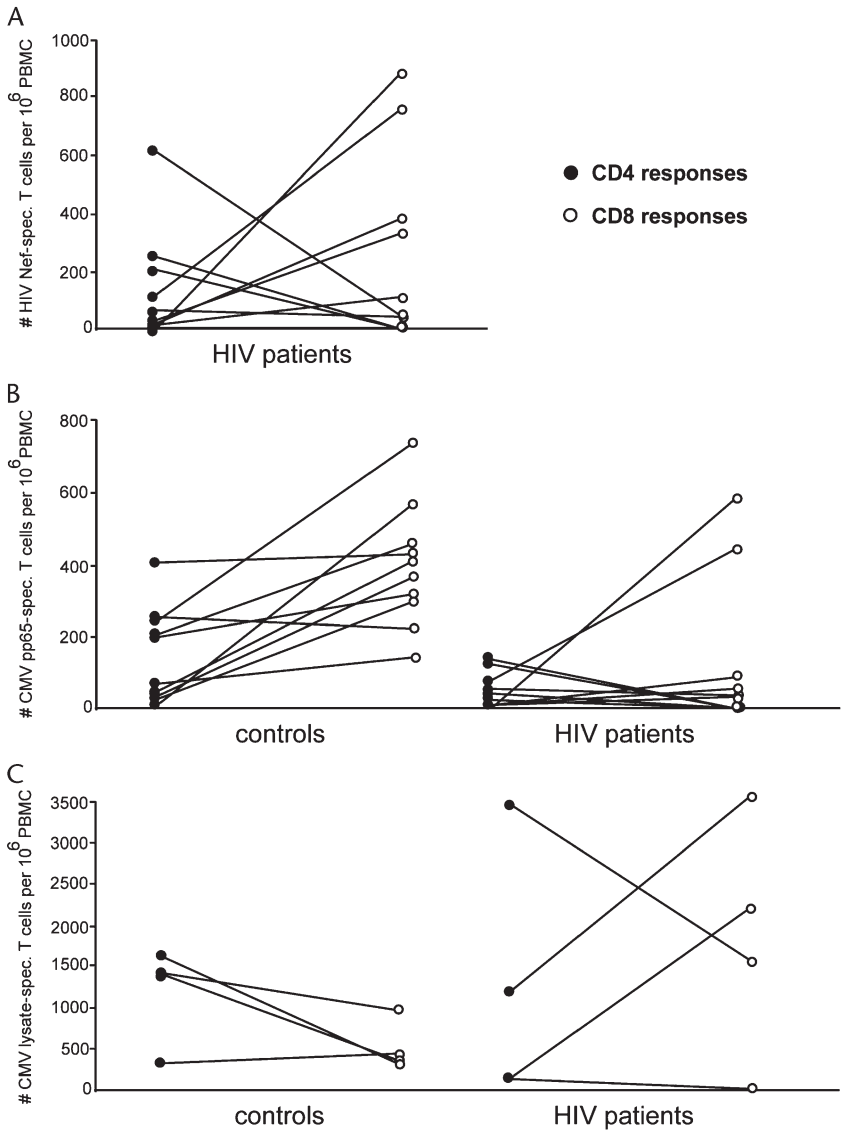

FIGURE 4. Cross-stimulation of $CD8^+$ T-cells by several antigens. A, Number of HIV Nef-protein-specific $CD4^+$ and $CD8^+$ T-cells in HIV patients. B, Number of CMV pp65-protein-specific $CD4^+$ and $CD8^+$ T-cells in CMV-positive controls and CMV-positive HIV patients. C, Number of CMV lysate-specific $CD4^+$ and $CD8^+$ T-cells in CMV-positive controls (frozen samples) and CMV-positive HIV patients.

by Oxenius et al,[46] who found positive correlations between HIV-peptide-specific $CD8^+$ T-cell responses and the yearly change in ratio CD4 count/log VL in treatment-naive patients. Interestingly, in our study, we found an inverse correlation between the number of HIV Gag p55-specific $CD4^+$ T-cells per million PBMC and the VL among treatment-naive HIV patients. This could either indicate that HIV Gag p55-specific $CD4^+$ T-cells are eliminated when viral replication increases, or that high levels of HIV-specific $CD4^+$ T-cells may help in maintaining low VL levels.[47] Finally, we could not detect any beneficial effect of increased HIV Gag p55 protein cross-stimulation of $CD8^+$ T-cells on the disease progression in our patient population.

To analyze $CD4^+$ and $CD8^+$ T-cell responses with ELISPOT assays, we have depleted the $CD8^+$ T-cell subset by using immunomagnetic beads as this method is generally used to differentiate between T-cell subsets.[48,49] We calculated the number of antigen-specific $CD4^+/CD8^+$ T-cells by using novel equations to correct for changes in cell subset populations after $CD8^+$ T-cell depletion. This allowed us to analyze $CD4^+$ and $CD8^+$ T-cell responses separately after stimulation with whole soluble protein. We showed that HIV Gag p55-protein-stimulated $CD4^+$ T-cell responses were not affected by $CD8^+$ cell depletion. Additionally, $CD14^+$ cell depletion and $CD8^+$ T-cell enrichment abrogated the ELISPOT responses, suggesting that HIV Gag p55 protein stimulation of $CD8^+$ T-cells occurs via protein processing and presentation by APCs. The fact that in vitro stimulation with whole protein does not only elicit HIV-specific $CD4^+$ T-cell responses but also stimulates a large population of HIV-specific $CD8^+$ T-cell responses should be taken into account for instance when immune responses in vaccine trials are evaluated with whole protein preparations.

We detected high levels of HIV Nef-protein-specific $CD8^+$ T-cell responses among HIV patients, suggesting that whole HIV protein cross-stimulation is not limited to Gag.

Although dominant CMV-specific cross-stimulation of $CD8^+$ T-cells could be detected in HIV-negative controls with a recombinant pp65 protein, this seemed not to be the case when PBMC were stimulated with a CMV lysate. This suggests that $CD8^+$ T-cell cross-stimulation, although depending on the nature of the whole soluble antigen, does not exclusively occur among HIV patients. The course of CMV infection may also have an influence on the occurrence of $CD4^+$ and $CD8^+$ T-cell responses, irrespective of HIV status. During primary infection, the cellular immune response is strongly dominated by CMV-specific $CD8^+$ T-cells, whereas during chronic CMV infection a dominant CMV-specific $CD4^+$ T-cell response is described.[50]

In conclusion, because of relatively high $CD8^+$ T-cell responses after in vitro stimulation with whole HIV Gag p55 protein and the variability per patient, it is highly recommended to identify the phenotype of the responding T-cells in ELISPOT to correctly assess $CD4^+$ and $CD8^+$ T-cell responses.

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