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Highly active antiretroviral therapy results in a decrease in CD8$^+$ T cell activation and preferential reconstitution of the peripheral CD4$^+$ T cell population with memory rather than naive cells

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Abstract

Objective: Highly active antiretroviral therapy (HAART) can produce marked increases in peripheral blood CD4$^+$ T cells and decreases in HIV plasma RNA copy numbers. However, it is not clear whether these absolute changes will be accompanied by a recovery in the known naive CD4$^+$ T cell depletion or a decrease in the marked CD8$^+$ T cell activation. Design: Twenty-nine patients were enrolled in studies of either nucleoside therapy alone or nucleoside therapy combined with a protease inhibitor (zidovudine + lamivudine + indinavir). One hundred and ninety-one examinations were carried out at three baseline time points and during 40 weeks of follow-up to evaluate the effect of HAART on CD4$^+$ memory/naive phenotype and CD8$^+$ T cell activation. Methods: CD4$^+$ and CD8$^+$ T cell number, CD62L/CD45RA expression on CD4$^+$ T cells and CD38 expression on CD8$^+$ T cells were measured by three-color flow cytometry. Results: Most protease inhibitor treated patients had a significant rise in CD4$^+$ numbers. The marked rise in the CD4$^+$ T cells seen in individuals in this study was not accompanied over a 40-week period by a change in the abnormally low CD4$^+$ naive compartment, and thus was almost completely of memory phenotype. The CD38 expression on CD8$^+$ cells fell during treatment, and decreased to a greater degree than the comparable rise in CD4$^+$ T cell counts. This decrease continued in many patients after the CD4$^+$ T cell rise or viral load decline had plateaued. Conclusion: HAART results in changes in activation to a greater extent than absolute changes in CD4$^+$ T cell numbers, but is not accompanied by an increase in naive CD4$^+$ T cells. Measurements of CD4$^+$ T cell numbers alone may not allow appropriate interpretation of immune activation or immune competence in patients receiving those drugs. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: CD38; CD45RA; CD62L; Flow cytometry; HIV-1

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1. Introduction

Although the use of the absolute $CD4^+$ T cell count or $CD4^+$ lymphocyte percent as a staging and prognostic marker in HIV infection has proven to be of great value over the past decade, the use of this lymphocyte subset as a surrogate marker of treatment effect has been less helpful (Choi et al., 1993, Jacobson et al., 1995). Of the numerous other potential surrogates that have been evaluated recently, the best one appears to be the quantitative measurement of HIV RNA in plasma (Luque et al., 1994, Mellors et al., 1995, O'Brien et al., 1996). Viral load magnitude correlates inversely with clinical outcome, and a decrease in viral load following therapy is usually associated with a positive treatment effect (Saag et al., 1996).

An important question regarding the use of highly active antiretroviral therapy (HAART) and the subsequent changes in these surrogate markers is whether the resultant increase in $CD4^+$ T cells produces T cell-dependent responses qualitatively similar to those in an individual with a similar CD4 value who was never treated. In other words, is a CD4 count of 300/μl produced by HAART as protective against infections and other clinical outcomes as a CD4 count of 300/μl in an untreated individual? Pending the results of ongoing clinical endpoint trials, one approach to answering this question is to evaluate the phenotype of the repopulating or redistributing $CD4^+$ T cells. An expansion in the peripheral blood of predominantly memory $CD4^+$ T cells may not be as protective as an expansion of true naive $CD4^+$ T cells, which require thymic processing and appear to be preferentially depleted in advanced HIV infection (Chou et al., 1994, Rabin et al., 1995, Roederer et al., 1995).

Another potential surrogate marker of T cell function which may correlate with the extent of viral load reduction would be the direct measurement of T cell activation. As recent studies have revealed that the level of viral load may be related directly to viral production rather than clearance, and since production may be directly correlated with overall T cell activation (Perelson et al., 1996), it is reasonable to hypothesize that viral load and activation markers may parallel each other (Kelleher et al., 1996). The expression of both DR and CD38 on the surface of T cells corresponds to the level of T cell activation (Radka et al., 1986; Yagi et al., 1992; Lund et al., 1995). The $CD8^+CD38^+DR^+$ subset contains the majority of $CD8^+$ cytotoxic T lymphocyte (CTL) activity in HIV infection, and in prior studies has been shown to correlate directly both with viral burden and with poor clinical outcome (Choi et al., 1993, Luque et al., 1994, Jacobson et al., 1995, Mellors et al., 1995, O'Brien et al., 1996, Saag et al., 1996). A clinical situation in which the correlation of this surrogate with other outcomes could clearly be shown is one in which large changes of viral load (and presumably therefore cell activation) may be taking place. We have taken the opportunity to study individuals with relatively low CD4 cell counts who are enrolled in a trial of HAART to evaluate these changes in surface expression of CD38 on $CD8^+$ T cells, as well as the memory and naive markers on $CD4^+$ T cells.

2. Materials and methods

2.1. Patients and samples

Samples from patients at the University of Rochester AIDS Clinical Trials Unit (ACTU) who were eligible for enrollment in AIDS Clinical Trials Group (ACTG) study 320 were evaluated by flow cytometry at several time points. ACTG 320 is a trial of zidovudine–lamivudine (AZT–3TC) versus AZT–3TC–indinavir in HIV-1 infected persons with an absolute $CD4^+$ T cell value of 200/μl (Hammer et al., 1997). For most of these patients, three separate flow cytometric determinations were made prior to receiving therapy (a screen, a pre-entry and an entry blood sample) which allowed for determination of the biologic and laboratory variability of the sample measurements. These determinations were usually made within a 1-month period. Viral load was measured using the Roche Amplicor HIV-1 Monitor assay at baseline (two measurements) and at four visits over the following 40-week period. To

increase the number of observations for this baseline evaluation, we also included specimens from patients enrolled in other trials of nucleosides alone who received multiple screening evaluations for study entry.

2.2. Flow cytometric measurements

At each of the immunophenotyping time points, two-color $CD4^+$ and $CD8^+$ T cell determinations were made using Simulset® reagents and software from Becton Dickinson (San Jose, CA) on a FACScan®. Three-color flow cytometry was carried out on these samples using CD38–FITC/HLA-DR–PE/CD8–CyC and CD62L–FITC/CD45RA–PE/CD4–CyC staining reagents (PharMingen, San Diego, CA; FITC, fluorescein isothiocyanate; PE, phycoerythrein). A single lot of each reagent was used throughout the study. The voltage gains of the fluorescent channels were set using defined calibration beads (PharMingen), and compensation was accomplished by setting orthogonal populations using a CD4–FITC/CD19–PE/CD8–CyC compensation control stain prepared by PharMingen. For each reagent, either the $CD4^+$ or $CD8^+$ cells were tightly gated. Dot plots of the FL1 versus FL2 fluorescence of the gated events were then displayed with quadrant markers set using an unstained sample. The $CD38^+$ population was further analyzed by displaying the $CD8^+$ gated population on a FL1 histogram, and the mean fluorescence intensity (MFI), median fluorescence intensity and geometric mean fluorescence intensity (GMFI) were displayed by Cellquest® (Becton Dickinson) software. The percentage of naive cells was defined as the percentage of the $CD4^+$ gated lymphocytes expressing both CD62L and CD45RA.

2.3. Statistical analysis

Measurements were obtained at up to four visits prior to treatment initiation and at several visits afterwards, up to 40 weeks. For each patient with three or more baseline samples, the mean, standard deviation (S.D.) and coefficient of variation (CV = S.D./mean) were calculated for the following values: the CD4 absolute cell counts, the absolute CD8 cell counts, the $CD8^+CD38^+$ MFI and GMFI, and the percent and absolute $CD45RA^+/CD62L^+/CD4^+$ (naive) populations. The mean CV of all patients was then determined for each of these values to allow estimates of overall sample-to-sample variability. Since the number of visits and duration of follow-up varied from patient to patient, for each cell subset of interest and for each patient we calculated the slope of the regression line over time expressed in weeks. All pretreatment visits were assigned time 0. Because the error on the slope was dependent on the number of observations and length of follow-up, both of which varied between patients, we divided the slope by its variance. These weighted slopes were our endpoints and were compared to zero by Wilcoxon's signed rank test to determine whether there was a change in the cell subset measurement over time.

A subgroup analysis was conducted using only those individuals whose CD4 increased at least 45% during the treatment period, and also by initial assignment. To examine whether the HIV RNA viral load affected the frequency of the different T cell populations, for each patient we calculated the slope of the regression line of the $CD4^+$, $CD8^+$, $CD38^+$ and $CD4^+$ naive percent and absolute values on the logarithm of the viral load. The departure of these slopes from zero was tested by Wilcoxon's signed rank test. All correlations between variables were measured by Spearman's rank coefficient of correlation. P values are reported two-sided and were considered statistically significant if equal to or less than 0.05. The analysis was conducted using STATISTICA/w version 5.1E software (StatSoft, Inc., Tulsa, OK).

3. Results

3.1. Baseline determinations

Three-color determinations were performed on specimens obtained from 29 patients at 196 different time points. In 23 of these patients (18 in ACTG 320 and five in other studies) there were three pretreatment determinations. The mean coefficient of variation ($\pm$S.D.) for these 23 sam-

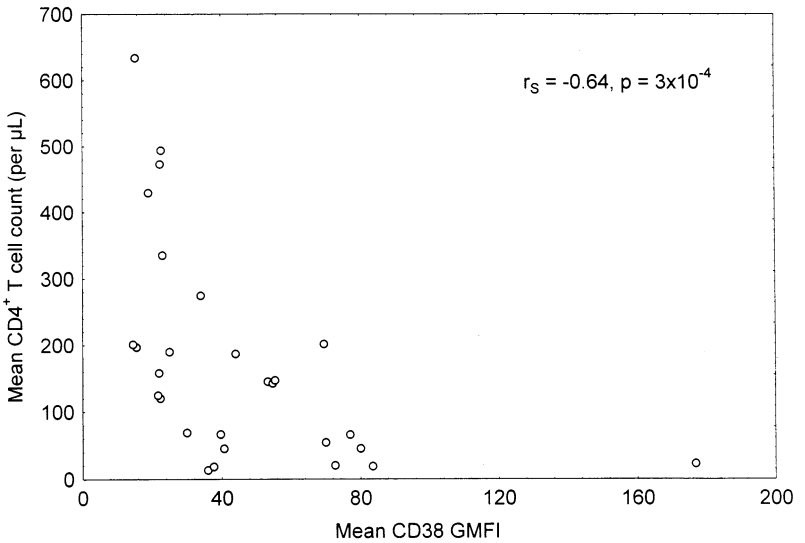

Fig. 1. The correlation at baseline between the geometric mean fluorescence intensity on $CD8^{bright}$ cells and the absolute number of $CD4^+$ T cells. These are shown as the mean values for each patient included in the analysis, rather than graphing all data points, in order to avoid overweighting of repeated values on given patients.

ples for the CD38 GMFI (0.12 ± 0.01) was actually smaller than that of the absolute $CD4^+$ T cell count (0.21 ± 0.03), the $CD8^+$ T cell count (0.19 ± 0.03), the naive $CD4^+$ T cell percent (0.20 ± 0.02) and the absolute naive $CD4^+$ T cell value (0.33 ± 0.04). As our intralaboratory variation on repeated $CD4^+$ T cell absolute values from a single sample is less than 5%, this greater fluctuation in the absolute CD4 values was likely due to biological changes from visit to visit. We used the geometric mean of the CD38 value for the slope analysis in preference to the arithmetic mean or median because of its smaller variability at baseline.

The overall relation between $CD4^+$ cell number and CD38 GFMI for all baseline measurements is shown in Fig. 1, in which the mean absolute CD4 is graphed versus the mean CD38 GMFI for each patient. As previously reported, there is an increase in the quantity of CD38 activation marker in the $CD8^+$ T cell population which increases as the $CD4^+$ value falls (Bouscarat et al., 1996). The percent of naive cells at baseline in our overall study is lower than in non-infected individuals measured in our laboratory ($51 \pm 6\%$) and reported in the literature (Rabin et al., 1995, Roederer et al., 1995). Almost all naive cell percents fall below the marked line delineating this mean normal value in Fig. 2. In addition, the graphic representation in Fig. 2 also reveals that the extent of naive T cell depletion actually increases as the $CD4^+$ T cell value declines, as previously reported (Rabin et al., 1995, Roederer et al., 1995).

3.2. Longitudinal studies

Flow cytometry specimens obtained at baseline and after at least 4 weeks of therapy were available for 22 patients enrolled in ACTG 320. Viral load determinations were available in 21 of these individuals. Table 1 summarizes the descriptive statistics of the weighted slopes for each endpoint for the protocol 320 subset, for the subset which includes only those individuals with a treatment response defined as a $>45\%$ rise in the absolute CD4 count, and for the subset receiving protease inhibitors. The graphical representation of the change in means of these values for all patients over the study period is shown in Fig. 3.

Of note, there was a significant decrease in viral load in all groups and an increase in CD4 cell

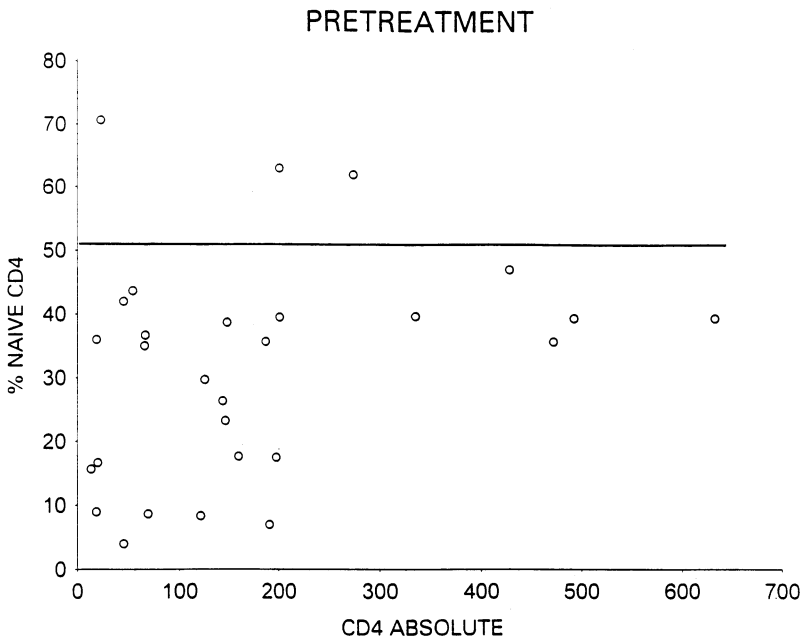

Fig. 2. The CD4$^+$ absolute T cell values plotted versus the percentage of the naive CD4$^+$ cell population in values prior to treatment.

counts. The CD8$^+$CD38 GMFI fell in all groups, but was not significantly negative in the protease inhibitor treatment group. This is likely due to the wide variability seen at late time points (see Figs. 4 and 5). There was an excellent correlation between the fall in CD38 and fall in viral load over the first 8 weeks of study ($P = 0.004$), but this correlation was lost with increasing time on the study. A correlation between change in CD4$^+$ T cells and change in CD38 GMFI was also not significant.

The slopes of both the CD8$^+$ T cells and the percentage of naive CD4$^+$ cells were not different from zero, indicating no effect of treatment in any group. The naïve CD4$^+$ T cell percent falls whereas the absolute naive cell number non-significantly rises from 28 to 43/μl. In addition, there was no indication of correlation between the increase in CD4$^+$ T cell counts and the percent of naive CD4$^+$ cells in any of the groups analyzed, including those receiving protease inhibitors. No correlation was found between the starting naive CD4$^+$ percentage and change in the absolute CD4$^+$ T cell count as measured by weighted slope ($r = -0.13$, $P = 0.56$).

Representative graphical examples of the changes in CD38 MFI, viral load and change in CD4$^+$ naive cells compared to the absolute CD4 cell changes are informative. Most individuals with marked increase in CD4$^+$ T cell counts were in the protease inhibitor arm (Fig. 4, A–F). Despite the rises in CD4$^+$ T cell values in Fig. 4 (A–F) and Fig. 5 (B,E), none had a significant increase in the absolute number of naive CD4$^+$ cells. Overall, most responders actually had a corresponding fall in the naive CD4$^+$ percentage. Many patients also continued to have a decline in the CD38 MFI despite a pronounced plateau effect seen in either the viral load or the CD4 cell count (Fig. 4, A,B,F Fig. 5, B,F). Most individuals in the dual therapy arm, which did not include a protease inhibitor, displayed a lesser fall (improvement) in the CD38 MFI (Fig. 5, A,C,D).

4. Discussion

In this study of phenotypic surface markers in patients treated with antiretroviral agents, we have shown that treatment is associated with a

Table 1
Median trend [a] of the CD36 GMFI, CD4, naive CD4 percentage and log viral RNA load over the time course of the study

	All patients ($n = 22$)		CD4 responders ($n = 12$)		Indinavir treatment group ($n = 11$)	
	Median	P value [b]	Median	P value	Median	P value
CD38	−4.8	0.02	−7.8	0.002	−4.7	0.18
CD4	2.3	0.002	5.1	0.002	6.9	0.006
Naive CD4 percent	−3.8	0.18	−3.4	0.14	12.1	0.93
Log viral load	−58.8	0.002	−58.8	0.04	−95.2	0.005

[a] Measured as the weighted slope of the regression line of the values over time (see text). A positive number indicates an average rise of the values over time, and a negative one a decrease over time.
[b] Comparing the values to zero (i.e. no change).

significant fall in CD38 activation markers, and that rises in CD4 cell counts seen during antiretroviral therapy are not accompanied by increases in the naive CD4 cell population. The fall in CD8 activation status as measured by the expression of CD38 after therapy has not been previously well described, as most reported studies have used cross-sectional data or natural history cohorts (Prince and Jensen, 1991, Giorgi et al., 1993, Ho et al., 1993, Bouscarat et al., 1996, Liu et al., 1996). By utilizing patients with advanced disease, many of whom have received HAART, we have biased our results to see such changes. The decrease in activation markers persists longer than the rise in CD4 cell levels in some individuals, and continues to fall despite plateau values in viral loads. The change in CD38 was highly correlated to changes of viral load early in therapy, but became more variable over treatment time. Thus, its potential use as a surrogate of viral load may be limited to circumstances in which large absolute changes may occur.

In our CD38 analysis we did not use a correction factor to calculate the actual number of CD38 receptors on $CD8^+$ T cells as discussed by Liu et al. (Bouscarat et al., 1996), because all of these studies were performed with one staining lot, and we concentrated on the changes in these values rather than on the absolute numbers. The samples were received and studies were performed over the entire study period, and our high reproducibility of the GMFI in samples drawn weeks apart, as well as those measured in normal controls in other experiments not described here, lead us to believe that these changes are accurate and real.

Despite the increases in absolute $CD4^+$ T cells seen in most participants, no increase in naive $CD4^+$ percents was noted over our 40-week time course. Recent reports have noted a similar phenomenon, with no early recovery in either naive T cells or expansion of T cell V beta chain subsets (Kelleher et al., 1996, Autran et al., 1997, Connors et al., 1997). As the $CD45RO^+$ isoform can recycle to the CD45RA isoform, the use of CD45RA or $CD45RO^+$ cells alone as a marker for memory could lead to erroneous interpretation (Rothstein et al., 1991). We chose the methodology of Roederer et al. to define the populations in this study, although other combinations of phenotypic markers could have been utilized (such as CD11a). The use of the 62L marker in conjunction with CD45RA in those studies was shown by flow cytometric, cell signaling and functional studies to accurately define the naive cell population (Rabin et al., 1995, Roederer et al., 1995). The lack of an increase in the naive $CD4^+$ cell population in our study is associated with an increase in the population of memory cells defined either as $CD45RA^-/CD62L^-$ or $CD45RA^+/CD62L^-$. Even when large increases are seen in the CD4 cell population, actual falls in the naive $CD4^+$ T cell percentage are often observed.

In a number of patients we and others have noted a trend toward a possible late increase in

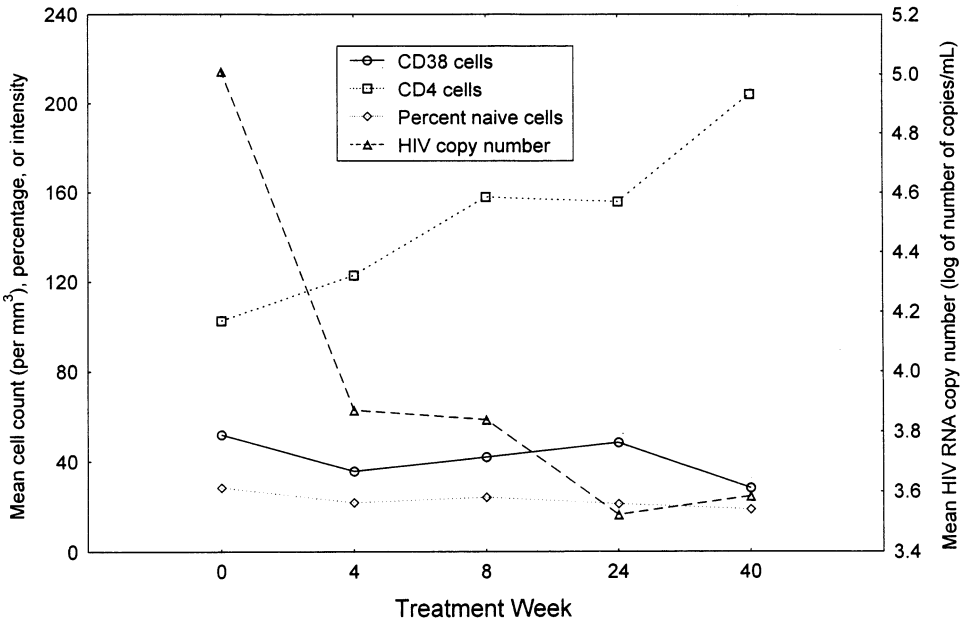

Fig. 3. The mean values of the CD4 cell count (dotted line, □), the CD4 naive cell percentage (light dotted line, ◇), the log viral load/ml (dashed line, Δ), and the CD38 GMFI (solid line, ○) are shown for the given time points in the study.

the naive CD4$^+$ T cell population. However, up to the 40-week prospective observations defined by our study, no statistical increase was seen. In non-prospective observations, late recoveries by CD4$^+$ naive cells have been seen in some of these individuals. This recovery is reminiscent of the prolonged recovery time required for naive cell repopulation seen in chemotherapy patients (Choi et al., 1993; Storek et al., 1995). In those patients, recovery of T cells was related to age and thymic processing ability, and often took up to a year to become apparent. Recent studies have shown that repopulation after transplantation typically involves a population of memory cells that are driven by antigen and are thymus-independent (Mackall et al., 1995, 1996). One approach to determining the potential importance of thymic precursors to T cell recovery would be to evaluate the repopulation dynamics in children versus adults, as the number of thymic repopulating cells is age-dependent (Mackall et al., 1995).

One interpretation of our memory cell repopulation data would be that the initial CD4$^+$ T cell rise is merely due to a redistribution of cells from lymphoid tissue due to the changes in adhesion and activation markers as typified by CD38 or CD62L. It is also possible that the decreased T cell activation leads to an improvement in the turnover of existing memory T cells. These early changes could then be followed by a later (as measured in years) recovery of overall immune function as the thymus-dependent cells might recover. If this were true, then one would hypothesize that response to new infection or neoantigens during the initial phase of HAART would be as fully impaired after as before therapy. Thus, in situations in which the patient may be confronted with a 'new' infection or neoantigen, rather than reactivation from a dormant infection (such as toxoplasma), protection may be lacking. Studies to differentiate the effects of this rise on protection from a variety of pathogens are needed to further delineate the nature of the HAART-related immunologic recovery. Another approach would be the use of immunization to a wide variety of neoantigens or recall antigens in such individuals and appropriate controls. If the rise in CD4$^+$ T cells seen in such patients is not accompanied by a sufficient response, then alternative methods of immune therapy, such as thymic transplant, may need to be considered.

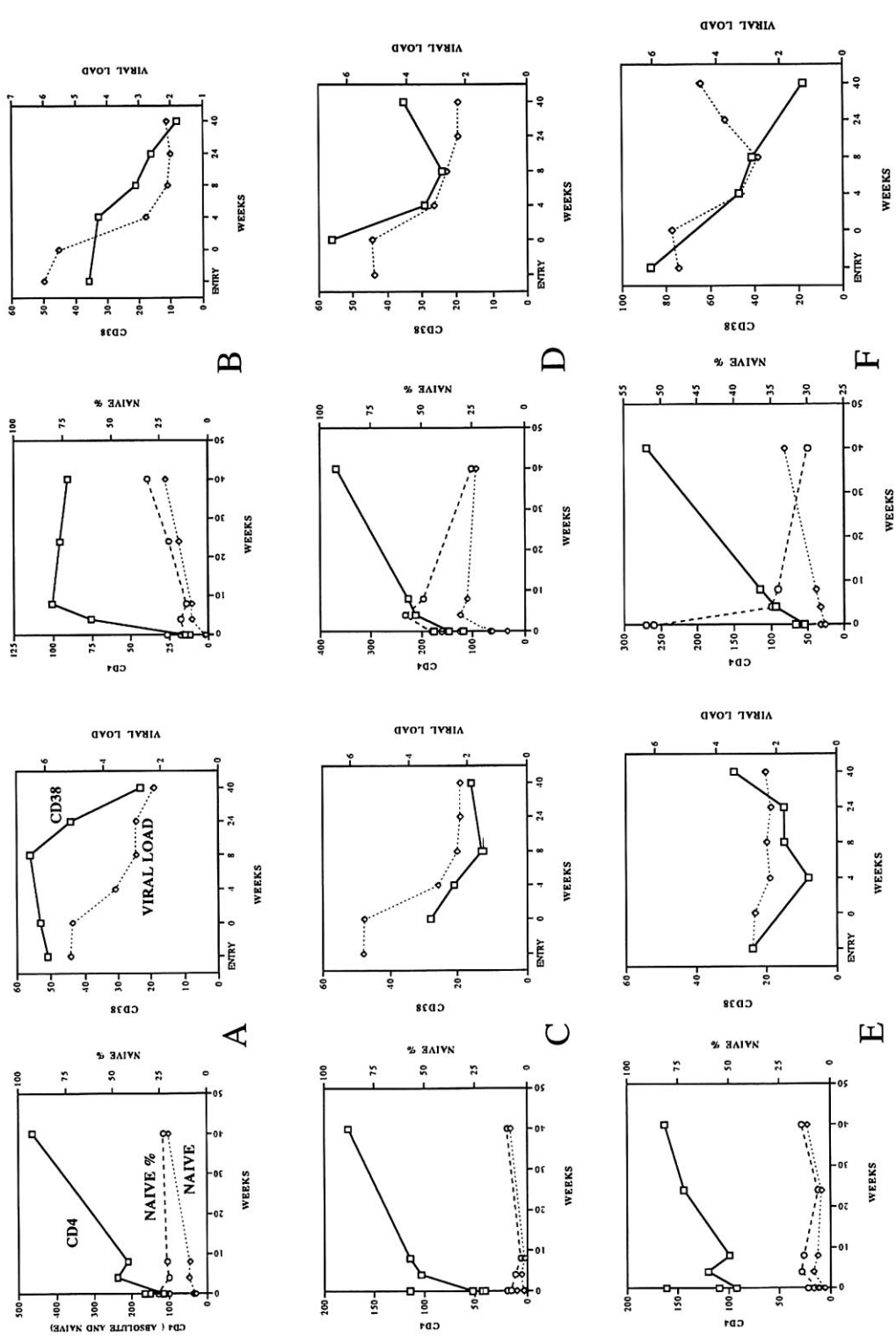

Fig. 4. The phenotypic response of six of the indinavir plus nucleoside analogue-treated patients (A–F) who were enrolled in ACTG 320 are shown. The figures show the simultaneous measurement of $CD4^+$ T cells (cells/μl; solid line, □), the absolute number of naive $CD4^+$ cells (cells/μl; dotted line, ◇), the naive CD4 percentage (dashed line, ○) in the first graph, and the CD38 intensity on $CD8^{bright}$ cells measured as the mean fluorescence intensity (solid line, □) and log viral load/ml (dotted line, ◇) in the second graph for the same patient.

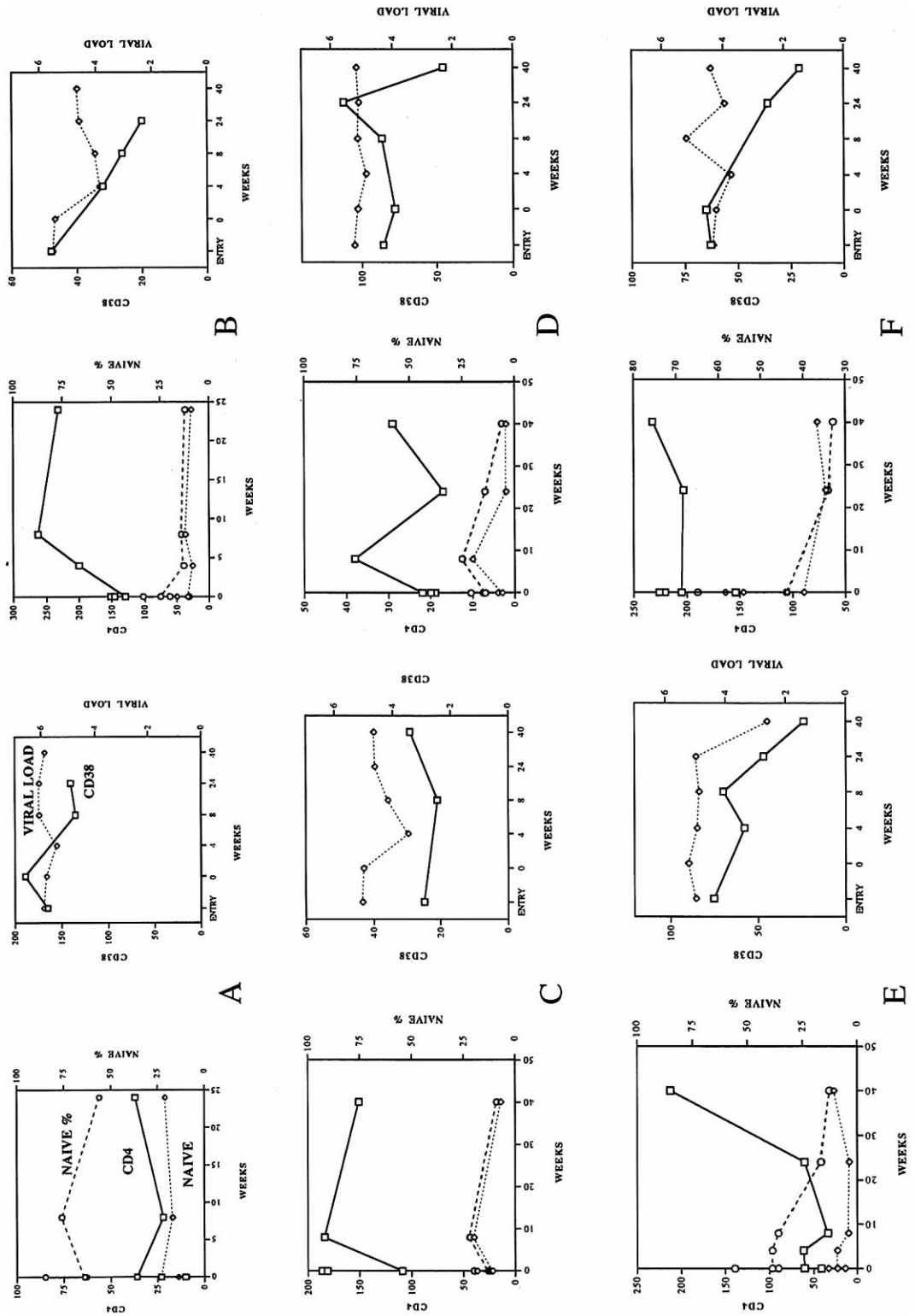

Fig. 5. The phenotypic response of six of the patients treated with nucleoside analogues alone (A–F) who were enrolled in ACTG 320 are shown. The figures show the simultaneous measurement of $CD4^+$ T cells (cells/μl; solid line, □), the absolute number of naive $CD4^+$ cells (cells/μl; dotted line, ◇), the naive CD4 percentage (dashed line, ○) in the first graph, and the CD38 intensity on $CD8^{bright}$ cells measured as the mean fluorescence intensity (solid line, □) and log viral load/ml (dotted line, ◇) in the second graph for the same patient.

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