Premature Aging of T cells Is Associated With Faster HIV-1 Disease Progression

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Objective: To determine if untreated HIV-1 infection and progression is associated with premature aging of memory CD8+ and CD4+ T cells and naive CD4+ T cells.

Methods: Twenty HIV-1–infected fast progressors and 40 slow progressors were included in our study, using risk set sampling. The expression of cell surface markers reflecting the differentiation stages of lymphocytes was measured using flow cytometry analyses performed on cryopreserved peripheral blood mononuclear cells.

Results: We found that HIV-1 disease progression is associated with a decreased CD28 median florescence intensity on CD4+ and CD8+ T cells; an increased proportion of intermediate- and late-differentiated CD8+ T cells and a decreased CD31 median florescence intensity on naive CD4+ T cells of recent thymic origin. A selective depletion of peripherally expanded naive CD4+ T cells was found to be associated with HIV-1 infection but not with HIV-1 disease progression.

Conclusions: The overall change during HIV-1 infection and progression is associated with a shift in the T-cell population toward an aged conformation, which may be further compromised by impaired renewal of the less-differentiated CD4+ T-cell population. Our results suggest that HIV-1 infection induces an accelerated aging of T lymphocytes, which is associated with the clinical progression to AIDS and death.

Key Words: disease progression, HIV/AIDS, premature aging, T cells

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INTRODUCTION

Increasing evidence has suggested that HIV-1–infected individuals experience similar immunologic changes as the uninfected elderly.[1,2] The phenotypic and functional alterations in T cells observed during human aging are often referred to as immunosenescence, which is thought to be a consequence of persistent T-cell activation and proliferation driven by repeated antigenic exposure experienced over the lifetime. Immunosenescence may lead to a generalized decline in immune responses and increased susceptibility of the elderly to infections.[3] By analogy, during HIV-1 infection, accelerated aging of T cells, or immunosenescence, may occur due to the continuous highly productive viral replication, which persistently stimulates immune cells.[4] Studies have suggested that T cells have a defined proliferative lifespan. In cell culture, they inevitably reach a state of replicative senescence after repeated antigen-driven cell divisions, with loss of proliferative capacity and other striking functional changes.[5]

One characteristic feature of senescent T cells is the complete and permanent loss of the CD28 costimulatory molecule.[6,7] The CD28 molecule is essential for effective T-cell activation, upregulating cytokine expression, augmenting proliferation, and providing essential survival signals for T cells.[8] CD28− T cells are functionally distinct from CD28+ T cells.[9,10] Both HIV-1 disease and aging[10–13] affect proportions of T cells that are CD28−, but there has been conflicting data regarding the relationship of these cells to HIV-1 disease progression. Some researchers report that the accumulation of CD28−CD8+ T cells is associated with a decline of CD4+ cell counts[14] or AIDS development,[15] whereas others have suggested that CD28 expression on CD4+ but not CD8+ T cells is independently associated with disease progression.[16,17] Furthermore, the expression of CD28 and CD57 on individual CD8+ T cells is thought to be inversely related.[18] The CD57+ T cells are late-stage cells derived from CD57− T cells.[19] A recent study[20] reported that the CD57 expression on HIV-1–specific CD8+ T cells defines the proliferative defects after antigenic stimulation in vitro better than the lack of CD28 expression. An elevated proportion of CD8+ T cells expressing CD57 has been observed in both aging[18] and HIV-1 infection,[21] but there is no clear evidence showing that this marker is associated with clinical progression in HIV-1–infected adults. Thus, it would be informative to assess the coexpression patterns of CD28 and CD57 in the context of HIV-1 infection and to identify which one is the more relevant marker for HIV-1 disease progression.

Another indicator for aging of the memory T-cell population is the distribution of T cells with distinct

differentiation phenotypes.[22] During T-cell differentiation, antigen-primed cells sequentially lose the expression of CD28 and CD27. Although the loss of CD45RA has long been associated with a memory T-cell phenotype, reexpression of CD45RA has been found among terminally differentiated T cells.[1,23,24] T-cell subsets may be positioned into the putative linear differentiation pathway mainly based on the expression of these markers.[25] Elevated proportions of highly differentiated T cells in both the CD4$^+$ and CD8$^+$ T-cell compartments have been observed over the different stages of HIV-1 infection.[24,26] It will therefore be informative to determine whether HIV-1–infected individuals with distinct disease progression rates have disparate distributions of differentiated T-cell subsets.

One well-known immunological alteration observed during normal aging is the homeostatic change in the naive CD4$^+$ T-cell compartment. The peripheral naive CD4$^+$ T-cell pool in adults is maintained by 2 following pathways: continued emigration of newly generated CD4$^+$ T cells from the thymus [so called recent thymic emigrants (RTE)] and expansion of existing naive CD4$^+$ T cells in the periphery.[27,28] CD31 is a differentiation antigen expressed on all RTE. These cells are thought to undergo peripheral homeostatic proliferation after emigrating from the thymus and during this process lose CD31 expression and become CD31$^-$-naive CD4$^+$ T cells.[29–31] Thus, CD31 expression provides a sensitive measure of the proliferation history of the naive CD4$^+$ T cells.[31,32] Studies have shown that CD31$^+$-naive CD4$^+$ cells also have a proliferative history, which is much shorter than their CD31$^-$ counterparts.[28,33] The use of RTE to represent CD31$^+$ cells herein is for ease only and reflects that in the differentiation scheme, these cells are less differentiated than the CD31$^-$-naive CD4$^+$ T cells. With human aging, the size of the peripheral naive CD4$^+$ T-cell pool remains fairly stable, despite the constant decline in the frequencies of naive CD4$^+$ T cells coexpressing CD31, reflecting a reduced thymic output and a counterbalancing increased postthymic naive cell proliferation.[28] As studies have shown that HIV-1 may induce thymic involution or atrophy,[34] we question whether HIV-1 infection induces a significant alteration in naive CD4$^+$ T-cell homeostasis similar to that observed during aging, due to either altered T-cell generation by the thymus and/or peripheral mechanisms such as increased homeostatic T-cell proliferation, and whether this change is related to HIV-1 disease progression.

To address the above proposed questions, we designed a unique study by directly identifying 2 groups of HIV-1–infected men who had similar age and CD4 level at early stage of HIV-1 infection and then progressed to AIDS in distinctly different rates. We conclude that HIV-1 infection induces premature aging of both memory T cells and naive CD4$^+$ T cells; in particular, the fast progressors (FPs) experience accelerated aging of lymphocytes, as evidenced by a greater accumulation of T-cell subsets associated with chronological aging.

MATERIALS AND METHODS
Study Subjects

A nested case–control study was carried out within the Multicenter AIDS Cohort Study (MACS), which enrolled 4954 homosexual men between March 1984 and April 1985 from 4 centers located in Baltimore, Chicago, Los Angeles, and Pittsburgh. Ethical approval was obtained from the institutional review boards at each of the clinical centers, and all participants provided informed consent. Semiannually, participants in the MACS cohort return to 1 of the 4 centers for follow-up visits and specimen collection. Other details regarding the recruitment and characteristics of the MACS cohort have been reported elsewhere.[35]

We selected 60 men from the MACS participants who were HIV-1 seropositive at the time of enrollment, using risk set sampling as follows: First, we identified 20 cases from those who developed AIDS within 4 years after enrollment (termed FPs); then for each case, at the time of his AIDS diagnosis, 2 controls were randomly selected from those who were then free of AIDS and had a total AIDS-free time of at least 8 years after enrollment [termed slow progressors (SPs)]. The 2 controls (SPs) were matched to the index case (FPs) on age (±2 years) and CD4$^+$ T-cell count (±100) at an early visit (visit 3 of MACS). Thus, our 60 seropositive samples consisted of 20 triplets, for which each set had 1 FP matched with 2 SPs. Immunologic parameters were measured on cryopreserved peripheral blood mononuclear cells (PBMCs) collected at a single time point, called the index visit, which was approximately 1 year before the AIDS diagnosis for each FP and the same calendar time for his 2 matched SPs. All participants were antiretroviral therapy naive at the time of sample evaluation. Our study also included 9 HIV-uninfected individuals (UIs) from the MACS, who were frequency matched with the 60 HIV-1–seropositive men on age. Their cryopreserved PBMC samples were collected during the same period as our HIV-1–seropositive samples. The ethnicity of the study participants did not differ among the groups ($P = 0.68$), 62 of the 69 study subjects were non-Hispanic white. All measurements in our study were made in a laboratory by 1 person, who was blinded to the progression status of the participants. Samples belonging to the same triplet (1 FP and 2 SPs) were always assayed in the same analytical batch.

Monoclonal Antibodies

Monoclonal antibodies purchased as conjugates of allophycocyanin (APC), cyanin (Cy), fluorescein isothiocyanate (FITC), phycoerythrin (PE), or peridinin chlorophyll protein (PerCP) from Becton Dickinson Immunocytometry Systems (BDIS, San Jose, CA) included anti-CD3 (PerCP), anti-CD4 (PE-Cy7), anti-CD8 (APC-Cy7), anti-CD45RA(FITC), anti-CD57 (FITC), anti-CD28 (PE), and anti-CD31(PE). Anti-CD27 (APC) was purchased from eBioscience (San Diego, CA), and anti-CD45RA (APC) was purchased from Caltag Invitrogen (Carlsbad, CA).

Flow Cytometry

The cryopreserved PBMC were thawed by the standard method.[36] The median viability of the thawed cells, assessed by trypan blue exclusion, was 82%. Aliquots of 5×10^5 cells were incubated with saturating amounts of specific antibodies for 30 minutes at 4°C in the dark. After staining, all aliquots were subjected to a brief NH$_4$CL lysis to remove any residual contaminating red cells. Cells were then washed once and

analyzed on a FACSCanto flow cytometer (BDIS), appropriately compensated daily using freshly stained PBMC. The sensitivity of the fluorescence detectors was standardized daily using fixed chicken red blood cells (Biosure, Grass Valley, CA). Data analysis was performed using FACSDiva software (BDIS). In addition to percentage data, CD28 and CD31 expression was also expressed as median fluorescence intensity (MFI). A separate aliquot of PBMCs were also stained with 7-amino actinomycin D (Calbiochem, La Jolla, CA) to guide in dead cell discrimination. Live cell gating yielded a purity of at least 98%. Six-color staining always included CD3, CD4, and CD8 monoclonal antibodies for optimal lymphocyte subsetting. To facilitate reading, we list in Table 1 the phenotypes as suggested by literature. Figure 1 showed the gating strategies in identifying the CD4 and CD8 T-cell subsets.

Statistical Analysis

Data are presented as medians and ranges. To assess changes associated with HIV-1 infection, we combined the HIV-1–infected FPs and SPs and compared their group medians with that of UIs using the Wilcoxon rank sum test. The 2 matched groups of HIV-1–infected men with different progression rates were compared pairwise using the Wilcoxon signed rank test. Fisher exact test was used to compare percentages. Bivariate correlations were determined by the Spearman rank correlation test. A regression line was calculated by the least squares method. Conditional logistic regression models were used to obtain odds ratios and 95% confidence intervals. The P values of 0.05 or less were considered significant. The analysis was performed using the SAS program.

RESULTS

Characteristics of Participants

As summarized in Table 2, all 20 FPs developed AIDS within 4 years after enrollment into the MACS, with a median AIDS-free time of 3.0 years; in contrast, only 19 patients of the

40 SPs had developed AIDS by January 2006, the time samples were withdrawn from the repository for this study, with a median AIDS-free time of 9.3 years. All 20 FPs died from AIDS-related conditions with a median survival of 4.2 years after enrollment, as compared with 16 AIDS-related deaths with a median survival of 10 years of the 40 SPs. Thus, the incidence of both AIDS and death were significantly higher in the FP versus the SP group after they entered the MACS. The FPs and SPs were well matched by set on age. Data regarding $CD4^+$ and $CD8^+$ T-cell counts, measured on fresh whole blood, were retrieved from MACS records. Comparing within each risk set, the CD4 count and proportion of FPs at study entry were similar to that of SPs. However, at the time of sample evaluation, that is, 1 year before AIDS onset for FPs, comparing within each risk set, the $CD4^+$ cell counts of FPs were lower than that of SPs by a median of 230 $CD4^+$ T cells ($P < 0.0001$) reflecting accelerated damage to the immune system in FPs.

HIV-1 Infection Induces Premature Aging in the Memory T-Cell Compartment, Mainly Within CD8+ T Cells

Aging of CD4+ and CD8+ T Cells During HIV-1 Infection as Is Indicated by the Accelerated Loss of CD28 Molecules

As shown in Figure 2A, the combined groups of HIV-1–infected men (n = 60) had higher proportions of $CD28^-CD4^+$ T cells ($P = 0.02$) and $CD28^-CD8^+$ T cells ($P < 0.0001$) than UIs (n = 9). Comparing FPs (n = 20) with SPs (n = 40), there is a further accumulation of $CD28^-$ cells in $CD8^+$ T cells ($P = 0.0007$) but not in $CD4^+$ T cells ($P = 0.85$). However, when comparing the CD28 MFI between $CD28^+$ T cells of the 2 HIV-1–infected groups (Fig. 2B), FPs had significantly lower CD28 expression on both $CD4^+$ ($P < 0.0001$) and $CD8^+$ T cells ($P < 0.0001$) than the SPs. As shown by both Figures 2A and B, the $CD4^+$ T-cell subset had a much higher baseline CD28 expression than $CD8^+$ T cells, as measured by either MFI or proportion. The CD28 MFI values between $CD4^+$ and $CD8^+$ T cells are closely correlated in both FPs ($r = 0.61$, $P = 0.004$) and SPs ($r = 0.62$, $P < 0.0001$), indicating a parallel loss of CD28 molecules among both T-cell subsets during HIV-1 infection (Fig. 2C). Our data revealed an inverse correlation between the proportions of $CD28^-CD8^+$ T cells and $CD4^+$ T cells in FPs ($r = -0.51$, $P = 0.02$) and SPs ($r = -0.45$, $P = 0.003$) (Fig. 2D), which is consistent with previous findings in HIV-1 infection[37] and in aging.[7]

Aging of the CD8 T Cells as Indicated by the Distribution of Different Phenotypic Subsets Defined by the Expression of CD28 and CD57 Together

Consistent with previous studies, our data revealed that there is an increase in the proportion of $CD57^+ CD8^+$ T cells among HIV-1–infected individuals ($P = 0.01$, Fig. 3A).[21] However, there was no difference between FPs and SPs ($P = 0.47$), indicating a lack of association of this marker with disease progression. We further assessed the distribution of $CD8^+$ T-cell subsets defined by the expression patterns of CD28 and CD57 (Fig. 3B). The expression of CD28 and

TABLE 1. Phenotypes of Different Stages or Types of T Cells

| Phenotypes	Stages or Types of T Cells as Suggested by Literature
$CD28^+CD57^-CD8^+$	Early-stage $CD8^+$T cells
$CD28^-CD57^-CD8^+$	Intermediate-stage $CD8^+$T cells
$CD28^-CD57^+CD8^+$	Late-stage $CD8^+$T cells
$CD45RA^+CD27^+CD28^+$	Naive $CD4^+$or $CD8^+$T cells
$CD45RA^-CD27^+CD28^+$	Early-stage memory $CD4^+$or $CD8^+$T cells
$CD45RA^-CD27^-CD28^+CD4^+$	Intermediate-stage memory $CD4^+$T cells
$CD45RA^-CD27^+CD28^-CD8^+$	Intermediate-stage memory $CD8^+$T cells
$CD45RA^-CD27^-CD28^-$	Late-RA$^-$stage memory $CD4^+$or $CD8^+$T cells
$CD45RA^+CD27^-CD28^-$	Late-RA$^+$stage memory $CD4^+$or $CD8^+$T cells
$CD45RA^+CD27^+CD31^+CD4^+$	RTE
$CD45RA^+CD27^+CD31^-CD4^+$	Naive $CD4^+$T cells that have undergone peripheral proliferation
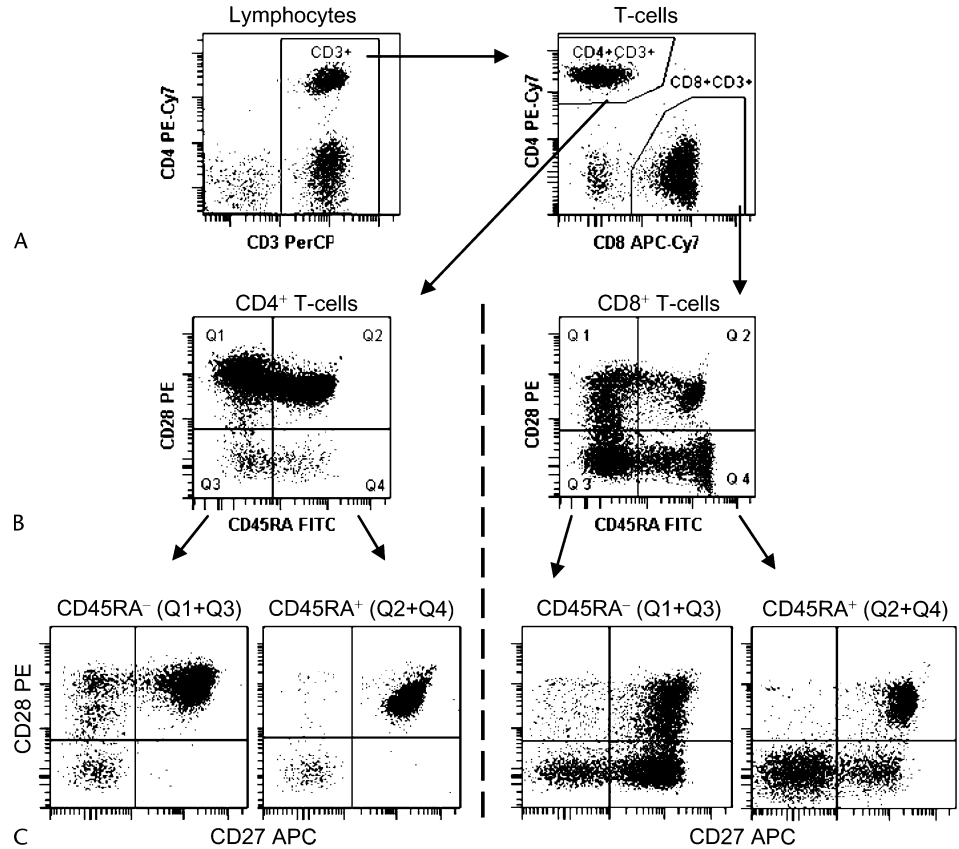
FIGURE 1. Gating scheme for measuring coexpression of CD45RA, CD27, and CD28 on CD4+ and CD8+ T cells from a representative FP. Row A shows CD3 gate on the left and CD4 and CD8 gate on the right. Row B shows CD45RA versus CD28 gated on CD4+ T cells (left) and CD8 T cells (right). Total CD28 and CD28 MFI were measured from these plots. Row C shows CD27 versus CD28 expression gated on CD45RA− and CD45RA+ T cells to illustrate all phenotypes defined by the 3 differentiation markers. These plots were used to enumerate naive and memory cell frequencies.

CD57 were thought to be mutually exclusive,[18] which means that CD28/CD57 double-positive or double-negative cells should be rare. Although this holds true among the UIs, we found that the 2 HIV-1–infected groups had a clear elevation of CD28−CD57− T cells ($P < 0.0001$), indicating that HIV-1 infection may have changed the inverse expression of CD57 and CD28 on CD8+ T cells. The CD28−CD57−CD8+ expression pattern is consistent with an intermediate-stage phenotype, as suggested by a previous study.[38] The

CD28−CD57+CD8+ T cells are most likely a terminally differentiated subset, as these cells have been associated with shortened telomeres, oligoclonal expansions,[39,40] and the inability to proliferate in response to stimuli in vitro.[41,42] As shown in Figure 3B, we found that HIV-1 infection is accompanied by a reduction of early-stage CD8+ T cells (CD28+CD57−, $P < 0.0001$) and an expansion of late-stage CD8+ T cells (CD28−CD57+, $P = 0.02$), similar to the changes described with aging.[18] Compared with SPs, FPs displayed

TABLE 2. Comparison of the Basic Demographic and Immunologic Parameters At the Index Visit

	FP (n = 20)	SP (n = 40)	UI (n = 9)	P*
Developed AIDS by January 2006 (%)	100	47.5	NA	<0.0001
AIDS-free time after enrollment among those developing AIDS (yrs)	3.0 (1.7–3.8)	9.3 (8.4–16.7)	NA	<0.0001
Death by 01/2006 (%)	100	40	NA	<0.0001
Survival time after enrollment among those dying (yrs)	4.2 (2.6–7.2)	10.0 (8.3–18.2)	NA	<0.0001
Age	36.2 (23.3–50.5)	35.5 (23.9–50.2)	36.6 (24.4–47.1)	0.97
CD4+T cells/mm3 at study entry (visit 3)	371 (204–619)	391 (198–707)	979 (387–1352)	0.36
CD4+% at study entry (visit 3)	24.5 (13–43)	28.0 (11–47)	45.0 (34–54)	0.17
Parameters at the index visit				
CD4+%	19.3 (2.9–29)	28.0 (7.5–52)	46.0 (34–59)	<0.0001
CD4+T cells/mm3	266 (13–675)	519 (213–1090)	995 (387–1195)	<0.0001
CD8+%	49.0 (24.2–74.0)	47.0 (23.7–78.4)	34.0 (23–45)	0.70
CD8+T cells/mm3	606 (112–2793)	826 (169–5673)	529 (323–857)	0.006

If unspecified, values are presented as medians (range).
*Wilxoxon signed rank test and Fisher exact test were used to compare group medians or percentages between 2 seropositive groups.
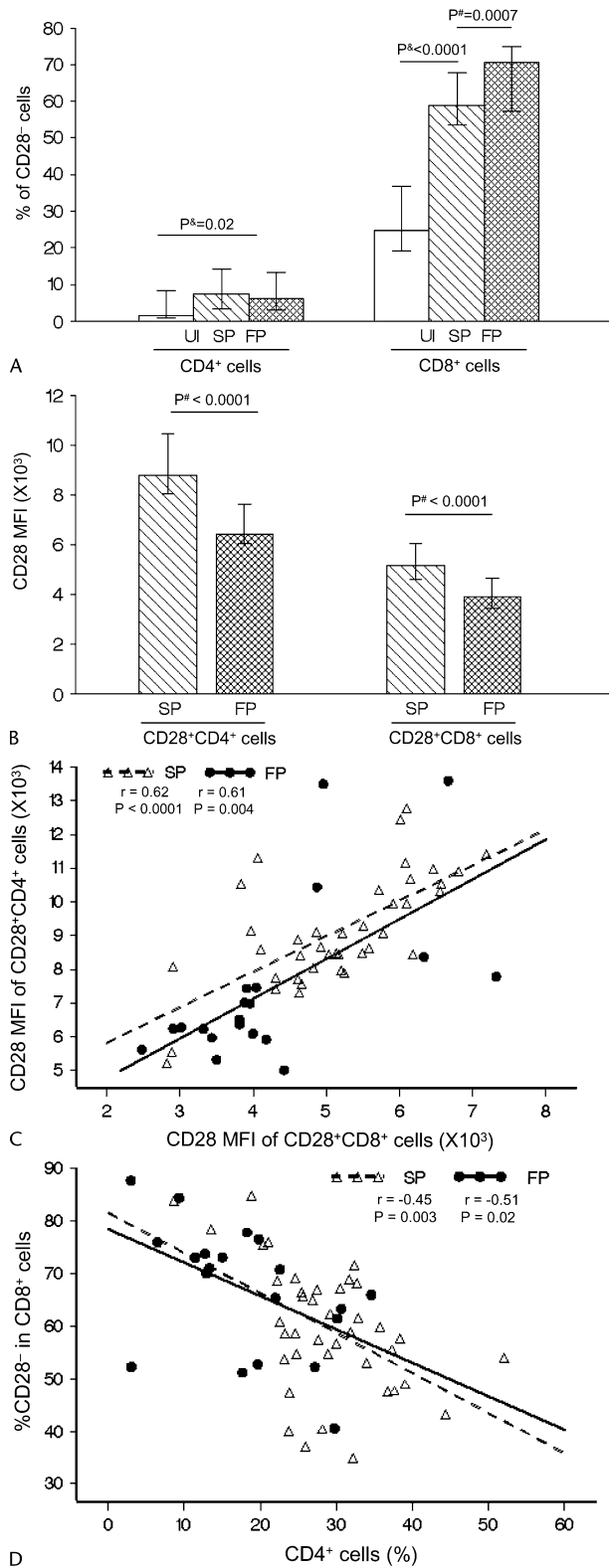
FIGURE 2. A, percentages of CD28$^-$ cells within the CD4$^+$ or CD8$^+$ T-cell populations in HIV-1–infected FPs, SPs, and UIs. B, CD28 MFI on CD28$^+$CD4$^+$ and CD28$^+$CD8$^+$ T cells in FPs and SPs. C, correlations between CD28 MFI values of CD28$^+$CD4$^+$ T cells and CD28$^+$CD8$^+$ T cells, in FPs and SPs. D, correlations

a further decline of early cells (CD28$^+$CD57$^-$, $P < 0.0001$) and an increase of intermediate-stage cells (CD28$^-$CD57$^-$, $P = 0.001$). Within the CD4$^+$ T-cell compartment, HIV-1 infection did not cause a significant change in terms of either the CD57 expression alone (Fig. 3A) or the coexpression of CD57 and CD28. In contrast to CD8$^+$ T cells, CD4$^+$ T cells consisted of more than 90% early-stage cells (CD28$^+$CD57$^-$, median, 93%, 90%, and 94%, for FPs, SPs, and UIs, respectively), followed by a small proportion of late-stage cells (CD28$^-$CD57$^+$, 5.1%, 5.5%, and 1.1%, for FPs, SPs, and UIs, respectively). Intermediate-stage CD4$^+$ T cells (CD28$^+$CD57$^+$ or CD28$^-$CD57$^-$) were rare. Correlation analysis revealed a close inverse relationship between the expression of CD28 and CD57 on CD4$^+$ T cells in all 3 groups (data not shown).

Loss of Naive and Enrichment of Intermediate- and Late-Differentiated T Cells During HIV-1 Infection

Various CD4$^+$ and CD8$^+$ T-cell subsets, corresponding to the major steps of differentiation, include naive (CD45RA$^+$CD27$^+$CD28$^+$), early- (CD45RA$^-$CD27$^+$CD28$^+$), intermediate- (CD45RA$^-$CD27$^-$CD28$^+$CD4$^+$ or CD45RA$^-$CD27$^+$CD28$^-$CD8$^+$), late-CD45RA$^-$ (CD45RA$^-$CD27$^-$CD28$^-$) and late-CD45RA$^+$ (CD45RA$^+$CD27$^-$CD28$^-$) differentiation subsets (Table 1). The difference in the phenotypes of intermediate-stage CD4$^+$ or CD8$^+$ T cells is due to their opposite sequence in losing CD27 and CD28.[24] We found that, among CD4$^+$ T cells, about 80% were naive or early-stage cells regardless of HIV-1 infection status; although naive CD4$^+$ T cells trend toward a decrease in proportion comparing infected with UIs, these changes are small and statistically insignificant (39%, 40%, and 50% for FPs, SPs, and UIs, respectively). In contrast, among CD8$^+$ T cells (Fig. 4A), HIV-1 infection is associated with a profound loss of naive cells ($P < 0.0001$) and a clear enrichment of intermediate- ($P < 0.0001$), late-CD45RA$^-$ ($P = 0.001$), and late-CD45RA$^+$ ($P = 0.047$) differentiation subsets. Comparing FPs with SPs, there is a further decline of naive cells ($P = 0.0002$) and an increase of intermediate ($P = 0.003$) and late-CD45RA$^-$ ($P = 0.003$) cells. Terminally differentiated (late-CD45RA$^+$) CD8$^+$ T cells are not associated with disease progression. We also found that CD4$^+$ T-cell depletion was correlated with a reduction in naive CD8$^+$ T cells ($r = 0.49$, $P = 0.03$ for FPs, $r = 0.5$, $P = 0.001$ for SPs) and an increase in late-CD45RA$^-$ CD8$^+$ T cells ($r = -0.51$, $P = 0.02$ for FPs, $r = -0.32$, $P = 0.04$ for SPs) (Fig. 4B). Additionally, we confirmed a previous report[24] that, although most of the CD45RA reexpression occurs on the

between the percentages of CD28$^-$ cells within the CD8$^+$ T cells and the percentages of total CD4$^+$ T cells, in FPs and SPs. The following statement applies to Figures 2–5: depicted in bar lots are medians and interquartile ranges. The P values for comparing group medians between the 2 HIV-1–infected groups or between the combined groups of HIV-1 infected and uninfected were calculated by the Wilcoxon signed rank test ($P^\#$) and the Wilcoxon rank sum test ($P^\&$), respectively. Spearmen rank correlation test was used to determine correlations.
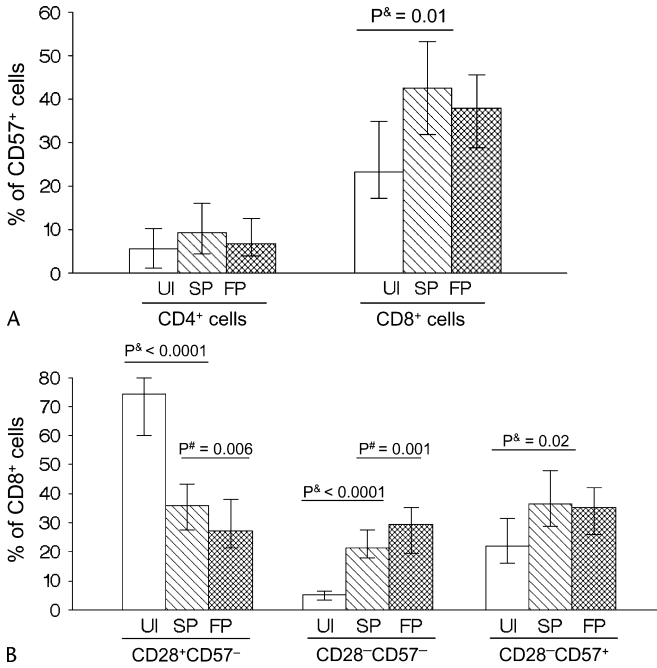
FIGURE 3. A, percentages of CD57+ cells within the CD4+ or CD8+ T cells. B, percentages of CD8+ T-cell subsets defined by expression patterns of CD28 and CD57.

late-differentiated CD8+ T cells (CD27−CD28−), there is a small population of intermediate CD8+ T cells (CD27+CD28−) that also regains CD45RA expression (6.3%, 8.2%, and 4.2%, for SPs, FPs, and UIs, respectively, data not shown). However, we cannot rule out the possibility that CD45RA

was never lost in this subset and CD28 was the first marker to be lost.

HIV-1 Infection Seems to Induce Accelerated Aging and Homeostatic Changes in the Naive CD4+ T-Cell Compartment

To address the question of whether a change in homeostasis was induced in the naive CD4+ T-cell pool by HIV-1 infection, we determined the frequencies of RTE (CD45RA+CD27+CD31+CD4+) and peripherally expanded naive CD4+ T cells (CD45RA+CD27+CD31−CD4+). Figure 5A showed gating scheme for measuring CD31 expression on naive CD4+ T cells. Our data revealed that HIV-1 infection is associated with a decrease in the proportion of CD31− cells within either the naive CD4+ T cells (31%, 35%, and 49% for FPs, SPs, and UIs, respectively) or the total CD4+ T cells (10%, 12%, and 20%, for FPs, SPs, and UIs, respectively, Fig. 5B), indicating a selective depletion of peripherally expanded naive cells. Though FPs seem to have less CD31−-naive CD4 cells than SPs, the difference is not statistically significant. The proportions of RTE remained stable across the 3 groups (27%, 25%, and 29%, for FPs, SPs, and UIs, respectively, $P = 0.68$, Fig. 5B), indicating that the decline of this cell population during HIV-1 infection is proportional to the total CD4+ T-cell reduction. We found that the CD31 intensity on RTE (Fig. 5C) was significantly lower in FPs (median 2523) versus SPs (median 3485, $P < 0.0001$), indicating a history of more peripheral proliferation in the RTE of FPs.[31,32]

Confirming previously described data,[28,32] we observed a decreasing trend in the proportion of RTE with an increase in age (data not shown). However, the age-related changes did not reach significance, which may be due to the narrow age range of our study subjects (90% of our participants were
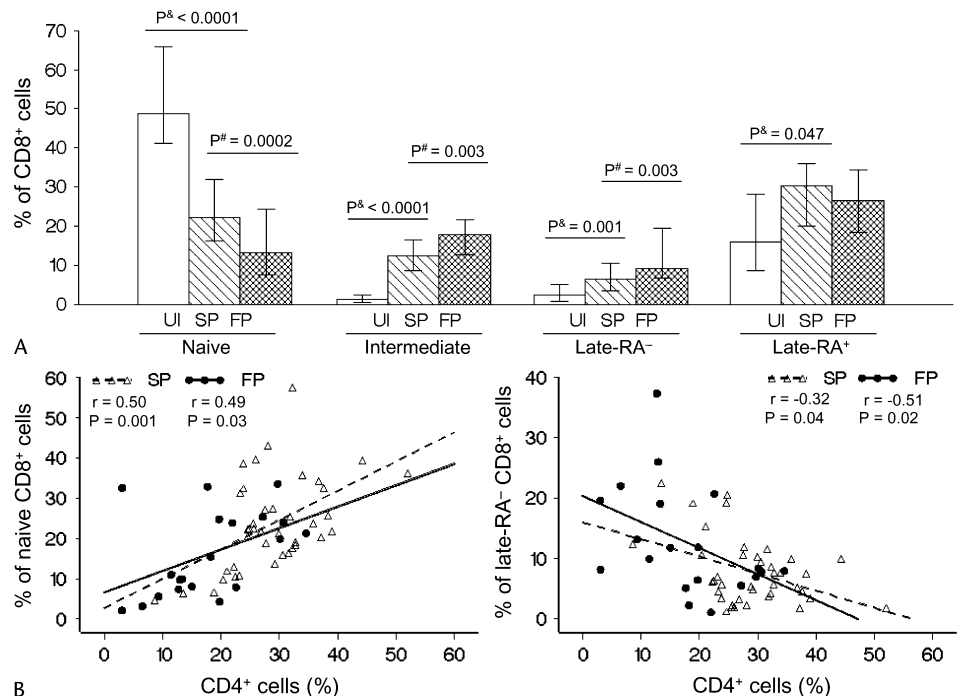

FIGURE 4. A, percentages of various CD8+ T-cell subsets representing differentiation stages defined by the expression of CD45RA, CD27, and CD28. B, percentages of CD4+ T cells are positively correlated with the percentages of naive CD8+ T cells (CD45RA+CD27+CD28+) and inversely correlated with the percentages of late-CD45RA− CD8+ T cells (CD45RA−CD27−CD28−) in HIV-1–infected individuals.
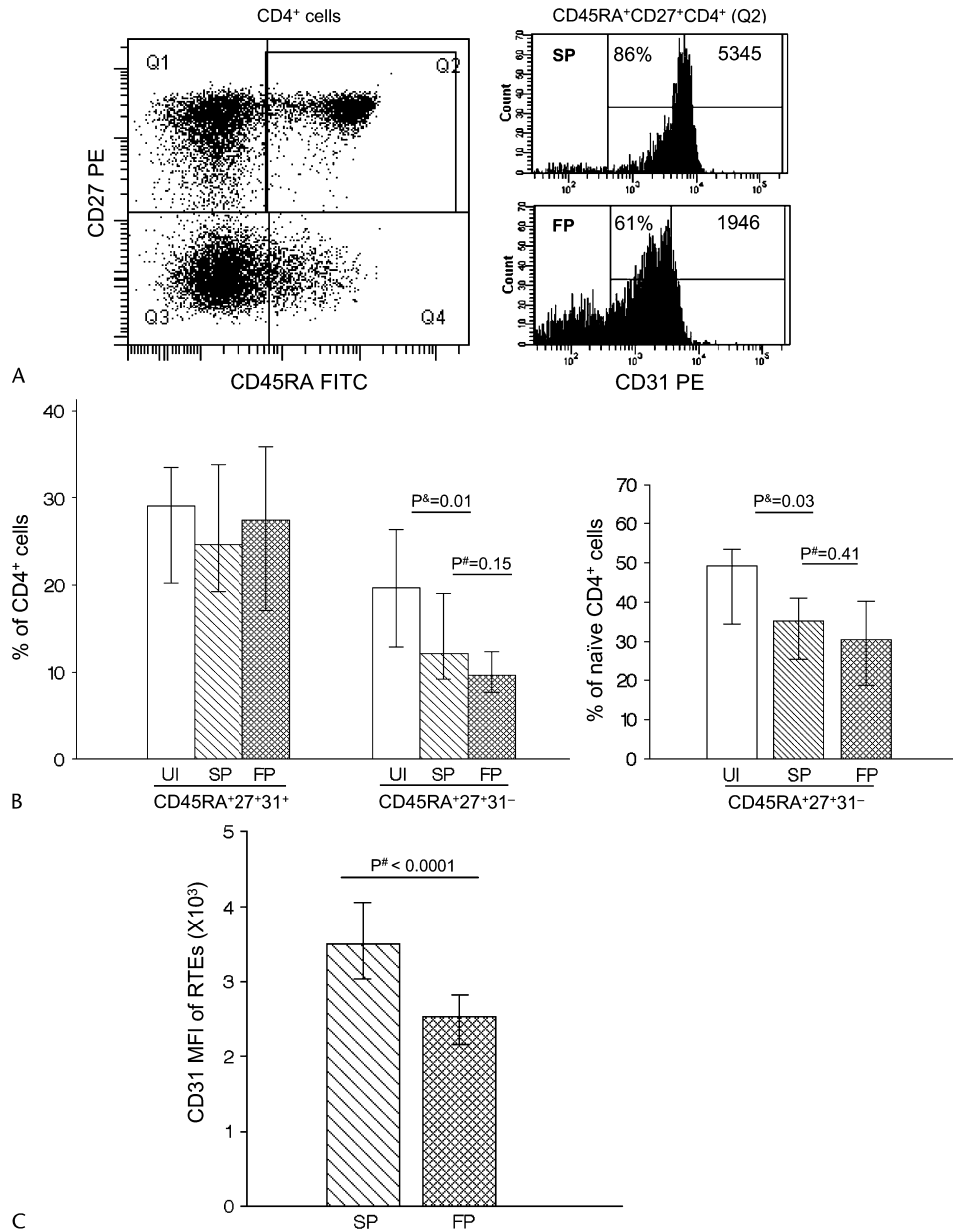
FIGURE 5. A, on the left, naive CD4+ T cells (CD45RA+CD27+) was gated in Q2. On the right, the CD31+ percentages and MFI on naive CD4+ T cells were shown for a paired SP and FP, respectively. B, percentages of RTE (CDRA+ CD27+CD31+CD4+) and peripherally expanded naive cells (CD45RA+CD27+CD31−CD4+) within the CD4+ T cells or the naive CD4+ T cells. C, CD31 MFI on RTE (CDRA+CD27+CD31+CD4+) in FPs is lower than that of SPs.

within a 15-year range). We also confirmed a previous report[31] that CD31 is expressed on the great majority of naive CD4+ T cells (69%, 65%, and 51% for FPs, SPs, and UIs, respectively) and on a small subset of CD45RA−CD27+CD4+ T cells that are at an early stage of maturation (16%, 8%, and 12% for FPs, SPs, and UIs, respectively).

Immunological Parameters Associated With HIV-1 Progression to AIDS and Death

Table 3 shows the association of different immunological parameters with HIV-1 progression calculated from conditional logistic regression. Controlling only for matching variables (age and CD4+ cell counts at the early visit), reduction in CD28 intensity on both CD4+ and CD8+ T cells was associated with faster progression, as is an increase in the proportion of CD28−CD8+ T cells. Within the CD8+ T cells, the proportion of naive cells (CD45RA+CD27+CD28+) was negatively associated with disease progression, whereas increases in the cells possessing phenotypes consistent with intermediate (either CD45RA−CD27+CD28− or CD28−CD57−) or late-stage CD8+ T cells (CD45RA−CD27−CD28−) were associated with faster HIV-1 progression. In addition, CD31 intensity on RTE was negatively associated with HIV-1 progression. After adjusting for both the matching variables and the CD4+ T-cell counts at the index visit, CD28 intensity on CD4+ T cells remained negatively associated with HIV-1

TABLE 3. Association of Immunological Parameters With HIV-1 Progression

Variable	Increment of Increase	OR* (95% CI)	P*	OR§ (95% CI)	P†
CD4$^+$T cells/mm^3	100	0.32 (0.14 to 0.71)	**0.005**	NA	NA
CD28 MFI of CD28$^+$CD4$^+$	500 units	0.74 (0.60 to 0.91)	**0.005**	0.70 (0.51 to 0.97)	**0.03**
CD28 MFI of CD28$^+$CD8$^+$	500 units	0.61 (0.43 to 0.87)	**0.006**	0.72 (0.49 to 1.06)	0.1
%CD28$^-$in CD8$^+$	5%	1.49 (1.05 to 2.10)	**0.024**	1.17 (0.82 to 1.68)	0.38
%CD45RA$^+$CD27$^+$CD28$^+$in CD8$^+$	5%	0.57 (0.36 to 0.90)	**0.015**	0.69 (0.41 to 1.18)	0.17
%CD45RA$^-$CD27$^+$CD28$^-$in CD8$^+$	5%	1.71 (1.06 to 2.76)	**0.027**	2.91 (1.04 to 8.12)	**0.04**
%CD45RA$^-$CD27$^-$CD28$^-$in CD8$^+$	5%	1.83 (1.10 to 3.05)	**0.020**	1.49 (0.75 to 2.97)	0.26
%CD28$^-$CD57$^-$in CD8$^+$	5%	1.57 (1.11 to 2.22)	**0.010**	1.46 (0.90 to 2.37)	0.13
CD31 MFI of CD45RA$^+$CD27$^+$CD31$^+$CD4$^+$	500 units	0.01 (0.00 to 0.66)	**0.029**	0.002 (0.00 to 8.6)	0.15

Bold *P* values are less than 0.05.

CI, confidence interval; NA, not applicable; OR, odds ratio.

*Conditional regression model 1, which was conditioned on matching variables, age and CD4 counts, at visit 3.

†Conditional regression model 2, which was conditioned on matching variables and CD4 counts at the index visit.

progression and the proportion of intermediate-differentiated CD8$^+$ T cells remained positively associated with HIV-1 disease progression.

DISCUSSION

Our study extends prior studies that have investigated the association between CD28 and HIV-1 progression by measuring the CD28 expression by both proportion and MFI. Study has shown that cells with low CD28 intensity have lower replicative capacity than those with high CD28 intensity.[43] Thus, loss of CD28 is a continuous process and it is more informative when CD28$^+$ T cells are not treated as a homogeneous population. In most of our HIV-1–infected men, the majority of CD8$^+$ T cells were CD28$^-$, as compared with less than 10% within the CD4$^+$ T-cell subset. A similar pattern of phenotypic change has been described with aging.[7,9] We found that faster HIV-1 progression was associated with an accumulation of CD28$^-$ T cells among CD8$^+$ T cells and an accelerated reduction in CD28 intensity in both CD4$^+$ and CD8$^+$ T cells. This lack of accumulation of CD28$^-$ cells within CD4$^+$ T cells, despite an accelerated loss of CD28, may be a consequence of several factors. First, the baseline CD28 intensity on CD28$^+$ cells is much higher within CD4$^+$ T cells than within CD8$^+$ T cells, as observed in our study. The fact that we used the same gate to classify CD28$^+$ and CD28$^-$ cells for both T-cell subsets means that CD4$^+$ T cells need to lose more CD28 molecules to shift below the cursor line and be classified as CD28$^-$ T cells. Second, a in vitro study has demonstrated that, in response to repeated antigenic stimulations, CD8$^+$ T cells lose CD28 expression remarkably faster compared with CD4$^+$ T cells which seem to be much more division resistant.[6] The third possible explanation might be that CD28-negative CD4$^+$ and CD8$^+$ T cells have different lifespan; as a study in mice has suggested that antigen-specific memory CD4$^+$ T cells seem to have a considerably shorter lifespan than primed CD8$^+$ T cells.[44] In summary, our data indicate that intensity measurements are more informative for representing changes in CD28 than proportion of positive or negative cells, especially for CD4$^+$ T cells.

This loss of CD28 expression is a feature of senescent T cells and has functional consequences. A number of studies have shown that in both chronic HIV-1 infection and normal aging, the loss of CD28 expression was coupled with a concurrent decline in telomerase activity, telomere length, and proliferative capacity.[6,10,40] Telomeres are chromosomal structures that shorten with each cell division and are the determinant of the cell's proliferative capacity.[45] Thus, the accelerated loss of CD28 on T cells observed in FPs signifies an impaired capacity to proliferate, which may lead to suppressed T-cell immune responses to antigens and result in faster disease progression to AIDS. This is further supported by studies showing that T cells from long-term nonprogressors, compared with progressors, maintain a high capacity to proliferate upon antigenic stimulation.[46,47] Possible deleterious effects of CD28 loss are more than just the inability to divide. The CD28$^-$ T cells have altered cytokine profiles such as decreased interleukin-2 production by CD4$^+$ T cells in response to stimuli.[11] Furthermore, they are associated with oligoconal expansions, a feature of both chronic viral infection and aging.[48] It has been suggested that the expansion of a few memory T-cell clones, directed at a restricted number of epitopes, fills up the "immunological space." Narrowing of the T-cell repertoire may subsequently impair the generation of new immune responses to infections[1] and may also account for the close correlation between the high proportions of CD28$^-$CD8$^+$ T cells and reduced vaccine responses in the elderly.[49–51] Therefore, despite a significant number of CD8$^+$ T cells observed during HIV-1 infection, the CD8$^+$ T-cell compartment is mostly comprised of functionally defective CD28$^-$ T cells.[52] The mechanism for the accelerated loss of CD28 expression during HIV-1 infection remains to be elucidated. Research has shown that CD28$^+$ T cells are differentiated from CD28$^+$ precursors. Their accumulation during HIV-1 infection may be a combined result of accelerated antigen-driven differentiation and prolonged survival due to the apoptosis resistance property ascribed to this T-cell subset.[53,54]

One unique component of our study is that we used multiple markers that allow precise identification of naive intermediate-stage and late-stage T cells. Aging of immune

cells during HIV-1 infection is supported by our observation that there is a depletion of naive and an enrichment of intermediate- and late-differentiated CD8$^+$ T cells, which were also found to be correlates of HIV-1 progression. Our finding is in keeping with a previous report, which documented that HIV-1–infected nonprogressors had fewer late-stage T cells than progressors.[26] Accumulating evidence has shown that the process of T-cell senescence is a component of the T-cell differentiation pathway, as terminally differentiated cells share common features of end-stage senescent cells, such as reduced replicative capacity, shortening of telomere lengths owing to reduced telomerase activity, and a restricted oligoclonal T-cell repertoire.[1,10,22] Intermediate- and late-differentiated CD8$^+$ T cells have been shown to possess a reduced proliferation potential in comparison with early-differentiated CD8$^+$ T cells.[55] Studies in the murine system revealed that the less-differentiated subset of virus-specific CD8$^+$ T cells is associated with protective immunity[56] and good proliferative capacity.[57] Due to homeostatic regulation of the immune system, the expansion of aged T-cell clones may lead to the contraction of the less-differentiated and more functional T-cell populations. Our finding that the expansion of late-differentiated CD8$^+$ T cells was associated with a decrease in CD4$^+$ T cells is also consistent with a deleterious role of these subsets in HIV-1 disease progression. Taken together, all the above observations suggested an overall structural change toward aged memory T cells during HIV-1 infection and progression, which may be partially due to accelerated differentiation driven by chronic antigenic stimulation and immune activation, and may eventually lead to the exhaustion of adaptive immune resources.

Another unique finding of study is that HIV-1 infection alters the composition of the naive CD4$^+$ T-cell pool by selective depletion of peripherally expanded (CD31$^-$) naive cells and induces accelerated loss of CD31 on naive CD4$^+$ T cells. The change is important as naive CD4$^+$ cells account for over 40% of the total CD4$^+$ cell population even in FPs, as shown by our data. The observation that CD31$^-$-naive CD4$^+$ T cells were depleted in HIV-1 infection does not resemble natural aging, in which CD31$^-$-naive CD4$^+$ T-cell enrichment has been observed. The most plausible explanation is that the rate of CD31$^-$ cell depletion (HIV-mediated cell death and/or recruitment into the memory cell pool) exceeds the recruitment of these cells from the CD31$^+$ subset (RTE). An ex vivo experiment has shown that HIV-1–infected, HIV-1–naive CD4$^+$ T cells are principally confined to those that have proliferated,[58] which, we speculate, are more likely to be the CD31$^-$-naive CD4$^+$ T cells. Our preliminary data also demonstrated the activation and infection of the naive CD31$^-$ subset during HIV-1 infection (data not shown). On the other hand, the increased recruitment of CD31$^-$-naive cells into memory cells induced by HIV-1 infection is supported by our preliminary studies (data not shown) demonstrating T-cell receptor (TCR) Vβ expansions within the CD31$^-$-naive subset during HIV-1 infection which mirror those present in the effector/memory compartment, although it should be noted that the number of TCR Vβ expansions in the CD31$^-$-naive subset is significantly reduced compared with the effector/-memory pool. Due to the distortion by HIV-1 infection, the

proportion of CD31$^-$ cells is not a good marker for T-cell age. Thus, CD31 intensity was used in our study as a surrogate for assessing differentiation/aging of CD31$^+$-naive cells. We found that the RTE from FPs had a significantly lower CD31 intensity than that of SPs. Previous observation from our group has shown that T-cell receptor excision circle (TREC) decrease within the RTE with aging, and the fluorescence intensity of CD31 on RTE, is highly correlated with the level of TREC.[59] Together, these findings support our contention that decreased CD31 intensity is consistent with a more extensive proliferative history as found with aging. Therefore, our data suggest that naive CD4$^+$ cells from FPs have increased proliferative history compared with the age matched SPs and UIs. This is consistent with a previous finding from our group showing that that RTE and peripherally proliferated naive CD4$^+$ T cells from HIV-1–infected individuals have significantly shortened telomeres compared with uninfected controls.[28]

In conclusion, our data suggest that HIV-1 pathogenesis involves an accelerated aging of both naive CD4$^+$ T cells and memory CD4$^+$ and CD8$^+$ T cells. The expansion of aged T-cell clones may lead to the homeostatic contraction of the less-differentiated and more functional T-cell populations, leading to a more rapid progression to AIDS and death. Whether highly active antiretroviral therapy can reverse or retard this process is not yet clear and needs to be investigated, although 1 study has shown that accumulation of aged T cells continues in highly active antiretroviral therapy–treated patients with increased CD4$^+$ T cells and long-term viral suppression.[60] Major advances in treatment have led to the significantly prolonged survival time for HIV-1–infected individuals,[61] but the continuous HIV stimulation and the natural aging process may act together to induce immunosenecence and raise a particular challenge for continued immune control of HIV-1 and long-term survival. Therefore, to retard the loss of immune function, strategies need to be developed specifically to prevent or reverse the accumulation of senescent cells or rejuvenate senescent T cells by repairing their functional defects. Therapeutic approaches, which have been proposed, include physically removing aged T cells to make room for the more functional earlier subsets and pharmaceutically enhancing telomerase to retard the shortening of telomere length.[62]

Our study has several following limitations: (1) We lack the data on HIV-1 viral load. The PBMC samples utilized were collected early in the course of the MACS, when the maximum number of antiretroviral-naive individuals was available; however, measures of HIV-1 RNA on heparinized plasma samples were not performed within the MACS at that time. We acknowledge that viral load could be a driving force for T-cell differentiation and lack of this data limits the interpretation of our results. (2) We chose our sample evaluation to be 1 year before AIDS diagnosis for FPs, that is, during late-stage disease, reasoning that if aging of T cells is part of the HIV-1 progression, alterations in T-cell subsets will be more prominent during late disease, increasing the likelihood of detecting differences between FPs and SPs with our limited sample size (20 FPs, 40 SPs). It would have been ideal to be able to examine samples at a time point before clinical differentiation; however, this would have required sample sizes

unobtainable even in a large cohort such as the MACS (total 4954 men), due to our strict selection criteria to ensure that the 2 participating groups have distinct progression rates. (3) We also acknowledge that HIV-specific CD4^{+} and CD8^{+} T cells make up a very small percentage of the total antigen-specific T-cell pool. As our measurements were performed on the total T-cell population, the results may not reflect changes in HIV-1–specific T cells. However, we believe that changes in the total T-cell population may be at least as important as changes in HIV-1–specific T cells in terms of progression to AIDS, as AIDS involves decreased immunity to a wide spectrum of infectious agents. Indeed, high proportions of CMV-specific CD8^{+} T cells have been documented during chronic HIV infection.[63] The presence of expanded populations of late-stage T lymphocytes has been documented in other viral infections (Epstein-Barr virus, cytomegalovirus, and influenza) and in cancer[11]; conditions commonly contracted by HIV-1–infected individuals, particularly in those at an immunosuppressed stage. A common feature of all these diseases is long-term chronic antigenic stimulation of the immune system by either virus or tumor-associated antigen. Thus the accelerated immunological aging observed in chronic HIV-1 infection, especially in those with an advanced disease status, may be a consequence of continuous HIV-1 infection and a multitude of other antigens and is best reflected by our assessment of the general T-cell population.

ACKNOWLEDGMENTS

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