Progressive CD4+ central–memory T cell decline results in CD4+ effector–memory insufficiency and overt disease in chronic SIV infection

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Primary simian immunodeficiency virus (SIV) infections of rhesus macaques result in the dramatic depletion of CD4+ CCR5+ effector–memory T (T_{EM}) cells from extra-lymphoid effector sites, but in most infections, an increased rate of CD4+ memory T cell proliferation appears to prevent collapse of effector site CD4+ T_{EM} cell populations and acute-phase AIDS. Eventually, persistent SIV replication results in chronic-phase AIDS, but the responsible mechanisms remain controversial. Here, we demonstrate that in the chronic phase of progressive SIV infection, effector site CD4+ T_{EM} cell populations manifest a slow, continuous decline, and that the degree of this depletion remains a highly significant correlate of late-onset AIDS. We further show that due to persistent immune activation, effector site CD4+ T_{EM} cells are predominantly short-lived, and that their homeostasis is strikingly dependent on the production of new CD4+ T_{EM} cells from central–memory T (T_{CM}) cell precursors. The instability of effector site CD4+ T_{EM} cell populations over time was not explained by increasing destruction of these cells, but rather was attributable to progressive reduction in their production, secondary to decreasing numbers of CCR5− CD4+ T_{CM} cells. These data suggest that although CD4+ T_{EM} cell depletion is a proximate mechanism of immunodeficiency, the tempo of this depletion and the timing of disease onset are largely determined by destruction, failing production, and gradual decline of CD4+ T_{CM} cells.

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Abbreviations used: ART, antiretroviral therapy; BAL, bronchoalveolar lavage; PID, postinfection day; PLN, peripheral LN; pvl, plasma viral load; RM, rhesus macaque; SHIV, simian immunodeficiency virus/HIV hybrid virus; SIV, simian immunodeficiency virus; T_{CM}, central–memory T; T_{EM}, effector–memory T.

The clinical manifestations of HIV infection typically ensue years after primary infection, despite continuous, often very high levels of viral replication (1–3). Although viral replication clearly drives the development of overt disease (1, 3, 4), the slow tempo of progression implies that host mechanisms must participate in the pathogenic sequence. Over years of investigation, a wide variety of pathogenic mechanisms have been proposed to play a role in AIDS pathogenesis in humans and AIDS-susceptible Asian macaques, including direct viral cytopathogenicity on CD4+ T cells and/or other target cells, indirect effects of infection such as persistent immune "hyperactivation," and other forms of T cell or accessory cell dysregulation (1, 5–12), but the degree to which these various mechanisms "connect" HIV or simian immunodeficiency virus (SIV) replication to immunodeficiency, as well as their interaction and regulation, remains poorly defined.

Primary CXCR4- or dual-tropic SIV/HIV hybrid virus (SHIV) infection can induce pan-CD4$^+$ T cell depletion and early immune deficiency in rhesus macaques (RMs), suggesting that acute, massive destruction of CD4$^+$ T cells can directly lead to AIDS (13, 14). However, pan-CD4$^+$ T cell depletion is unusual for typical CCR5-tropic SIV infection and is clearly not required for AIDS to develop (15, 16). The CCR5 coreceptor is selectively expressed on (CCR7$^-$) effector site (or effector site–seeking) effector–memory T (T_{EM}) cells (9, 17, 18), and in keeping with this, CCR5-tropic HIV/SIV infection dramatically depletes extra-lymphoid effector sites of their CD4$^+$ T_{EM} cell complement during acute infection (12, 19–23). In most infected monkeys and humans, this acute phase depletion does not result in overt immunodeficiency because in contrast to the pan-CD4$^+$ T cell-depleting SHIVs, CCR5-tropic viruses leave a substantial CD4$^+$ central–memory T (T_{CM}) cell population intact, and the CD4$^+$ T_{EM} cell depletion is accompanied by increased proliferation of these spared CD4$^+$ memory T cells that continuously provides an influx of new CD4$^+$ T_{EM} cells into extra-lymphoid effector sites (15, 23). Because failure of this CD4$^+$ memory T cell–proliferative response was a strong correlate of early AIDS onset (15), CD4$^+$ T_{EM} cell dynamics appear to be a major determinant of immunocompetence in acute CCR5-tropic SIV infection, with clinical immunodeficiency requiring both the destruction of extant T_{EM} cells and a failure to replenish these cells above a critical threshold (15, 23).

Whereas rapid progression results from a relatively abrupt, inadequately compensated alteration of a normal immune system, chronic AIDS is thought to result from a slow, progressive deterioration of a quasi-stable steady-state characterized by ongoing CD4$^+$ T cell death and regeneration, and in particular, a pervasive, chronic immune activation that has been shown to be a strong predictor of the tempo of progression (10–12, 24–26). The question therefore arises as to whether a sustained or increasing CD4$^+$ T_{EM} cell deficit continues to play a primary pathogenic role, and if so, whether and how direct viral cytopathogenicity, immune activation, and/or other factors contribute to this deficit. Here, we address these questions with a detailed analysis of the development of AIDS in RMs chronically infected with SIVmac239. Our results continue to strongly implicate the inability to maintain CD4$^+$ T cell populations in extra-lymphoid effector sites above a critical threshold as the common final pathway in the development of AIDS. However, direct viral destruction of effector site CD4$^+$ T_{EM} cell populations did not appear to be the determining mechanism of AIDS onset. Rather, we found that persistent immune activation renders these populations highly dependent on the proliferation and differentiation of (CCR5$^-$) T_{CM} cells for their homeostasis, and that this CD4$^+$ T_{CM} cell–derived, T_{EM} cell production progressively declines in chronic infection. Thus, although CD4$^+$ T_{EM} cells are the primary targets of HIV/SIV infection, our data suggest that the direct and indirect effects of infection on the less efficiently targeted CD4$^+$ T_{CM} cell population determine the tempo of disease progression during chronic infection.

RESULTS

CD4$^+$ memory T cell depletion in extra-lymphoid effector sites correlates with overt disease

SIVmac239 and related CCR5-tropic SIVs are highly pathogenic for Indian origin RMs, inducing rapid disease progression (AIDS in <200 d) in ~25% of infected animals and normal progression (AIDS in 1–3 yr) in the majority of the remainder (27). Fig. 1 A shows plasma viral loads (pvls) over the course of infection of 14 SIVmac239-infected RMs that did not receive antiretroviral therapy (ART) and were followed to an AIDS endpoint (4 rapid progressors and 10 normal progressors). As reported previously (15), rapid progressors manifest 1–2 log higher pvls than RMs destined for >350 d survival. However, among the latter group, plateau-phase pvls did not distinguish between RMs with considerably different survival periods. Indeed, when the entire study group was included in a correlation analysis of plateau-phase pvls versus time to endpoint, a significant association was observed (by either parametric or nonparametric analysis); however, when the four rapid progressors were excluded from the analysis, this association was no longer apparent (Fig. 1 B). Moreover, in all the normal progressors, pvls remained steady through the development of overt disease.

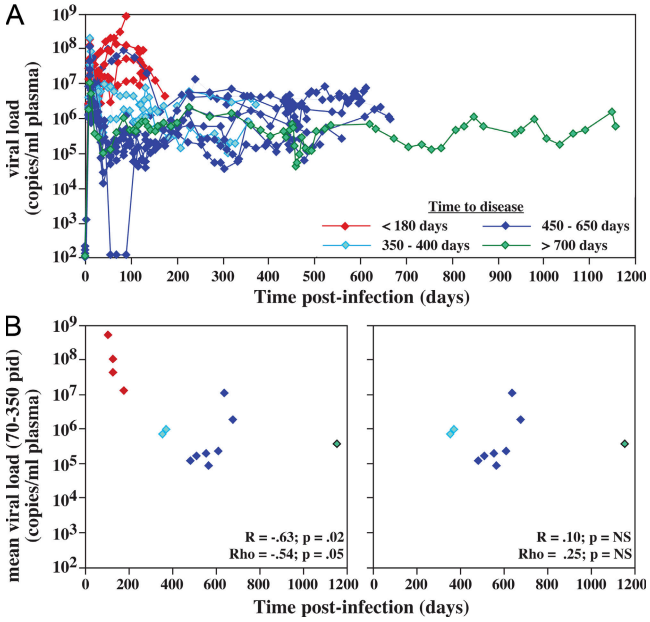

Figure 1. Relationship between pvl and survival in SIVmac239 infection. (A) Pvl data is shown for 14 SIVmac239-infected RMs that did not receive ART during their course and were followed to an AIDS endpoint. Profiles are color-coded according to time to endpoint as indicated. Note that the y axis starts at 10^2 copies/ml (the threshold sensitivity of the viral load assay) rather than 0 (acute-phase viral load data on these RM can be viewed in detail in reference 15). (B) The mean pvl for each RM from postinfection day (PID) 70 to either endpoint (rapid progressors) or PID 350 (normal progressors) is plotted against time to AIDS for all 14 RMs (left) or the 10 normal progressors alone (right). The graph configuration and color-coding are as described for A. Both linear regression (R) and Spearman rank (Rho) correlation coefficients and the p-values for these analyses are indicated in each plot. NS, nonsignificant.

Thus, within the range of viral replication manifested by typical SIV-infected normal progressors, the time to disease was not closely linked to mean pvls over time nor to any late acceleration of viral replication, observations suggesting that the rate of disease progression in these RMs was largely determined by their ability to handle the consequences of chronic viral replication, rather than by differences in viral replication itself.

A major consequence of acute CCR5-tropic SIV infection is destruction of $CD4^+$ memory T cells, particularly $CD4^+$ T_{EM} cells in extra-lymphoid effector sites, and we have previously shown that a strong immunologic correlate of rapid progression is the failure to follow this initial $CD4^+$ T_{EM} cell depletion with a sustained increase in the fraction of proliferating $CD4^+$ memory T cells (15). This increase provides "targets" for SIV (12, 20), but also contributes to the continuous production of new $CD4^+$ T_{EM} cells that limits extra-lymphoid tissue deficits and prevents immunological collapse (15). As it is possible that the same mechanisms extend to chronic progression, we asked first whether $CD4^+$ T_{EM} cell depletion was a specific and uniform feature of SIVmac239-mediated AIDS (Fig. 2). Because almost all bronchoalveolar lavage (BAL) T cells manifest the phenotypic signature, $CCR7^-$ and/or $CCR5^+$, of T_{EM} cells (18, 23), the fate of $CD4^+$ T cells in this site provides insight into the extra-lymphoid $CD4^+$ T_{EM} cell compartment. As shown in Fig. 2 A, analysis of 13 normal progressors and 4 rapid progressors demonstrated that overt disease in both settings was uniformly associated with a striking (>99%) decline in $CD4^+$ T cell representation among BAL T_{EM} cells. Because total lymphocyte recovery from BAL in later stages of infection was comparable to or reduced from preinfection (unpublished data), this decline predominantly represents $CD4^+$ T_{EM} cell depletion, rather than $CD8^+$ T_{EM}

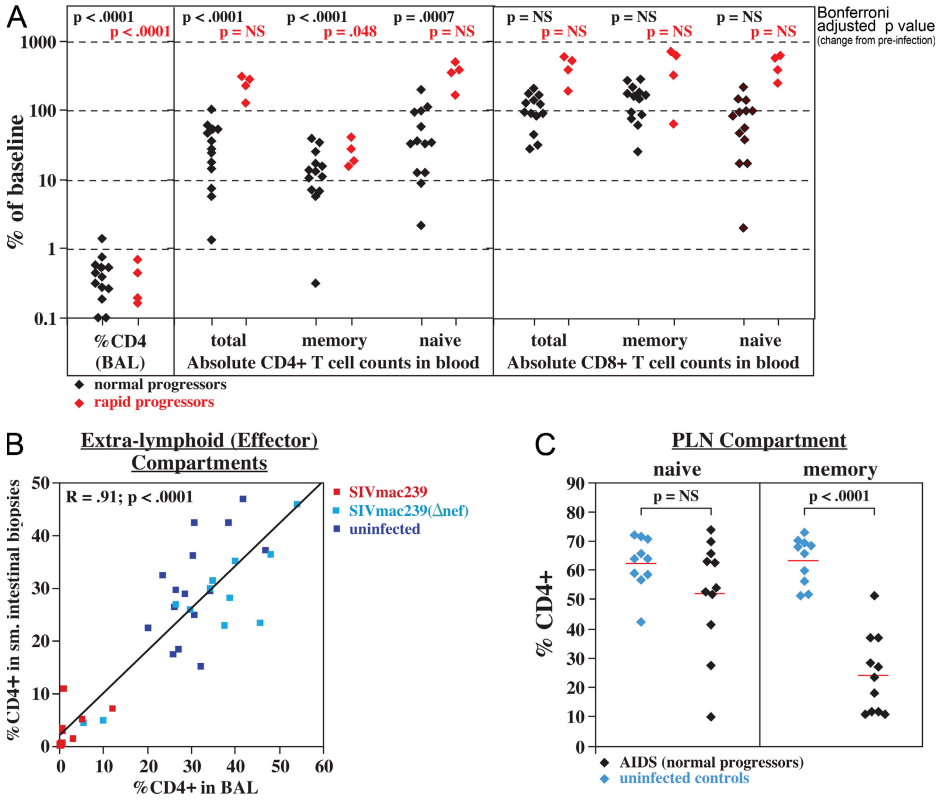

Figure 2. Systemic analysis of T cell populations in SIVmac239-infected RMs with overt immunodeficiency. (A) The absolute number of total, memory, and naive $CD4^+$ and $CD8^+$ T cells in the blood and the percent $CD4^+$ T cells (of total $CD3^+$) in BAL were evaluated by flow cytometry at the time of infection (baseline) and at an AIDS endpoint in 17 SIVmac239-infected RMs: 4 rapid progressors (red) and 13 normal progressors (black). Results are presented as percent change from baseline, with the significance of this change assessed separately for normal and rapid progressors using a mixed-effects statistical model. (B) The percent $CD4^+$ of total T cells in BAL is plotted against the same measurement in small intestinal (jejunal) biopsy cell preparations from RMs with pulmonary lavage examination and small intestinal biopsy within 7 d of each other. The analysis includes 12 plateau-phase SIVmac239-infected RMs (red; median pvl = 550,000 copies/ml), 12 SIVmac239(Δnef)-infected RMs (light blue; median pvl = 180 copies/ml), and 15 SIV^- normal control RMs (dark blue). The plot shows a linear regression line with the correlation coefficient and p-value of the correlation indicated in the top left corner. (C) PLNs from 11 SIVmac239-infected normal progressors with AIDS and 10 SIV^- control RMs were analyzed by flow cytometry for evidence of relative $CD4^+$ depletion in either the memory or naive compartments. $CD3^+$ T cells, including both $CD4^+$ and $CD8^+$ lineages, were first separated into naive and memory compartments by phenotypic criteria (see Materials and methods), and then the relative fractions of $CD4^+$ cells within these compartments were determined. Results are presented as percent $CD4^+$ (of total naive or memory T cells). The red lines designate the mean percent $CD4^+$ values, with significance of differences between SIV^- and SIV^+ RMs determined by unpaired *t* test.

cell expansion. Importantly, the representation of CD4+ T cells in BAL closely correlated with that of small intestinal lamina propria, another T_{EM} cell–populated effector compartment (18), suggesting that the BAL compartment was an accurate indicator of systemic effector site CD4+ T_{EM} cell representation (Fig. 2 B).

Decline in the absolute number of circulating total CD4+ memory T cells was also highly significant in both rapid and normal progression, albeit less dramatic than the loss of CD4+ T_{EM} cells in BAL (Fig. 2 A). In contrast, the numbers of CD4+ naive T cells in blood were not a strong correlate of AIDS as they were only variably and modestly diminished in chronic AIDS and, as described previously (15), were actually increased in rapid progression. Because of this CD4+ naive T cell heterogeneity, the absolute numbers of total CD4+ T cells in blood, the standard marker in disease progression in HIV infection (1), were significantly reduced only in the chronically SIV-infected RMs, and even in this setting, the extent of this loss was highly variable. The numbers of circulating CD8+ T cells, both memory and naive, also varied in overt AIDS but tended to be normal or elevated (Fig. 2 A). Cross-sectional analysis of the overall naive and memory T cell compartments of peripheral LNs (PLNs) in AIDS-stage normal progressors versus uninfected RMs revealed a statistically significant decline in CD4+ representation in the total memory, but not the naive, compartments (Fig. 2 C), providing further evidence that systemic CD4+ memory, but not naive, T cell depletion is a uniform T cell population correlate of AIDS.

Collectively, these data indicate that BAL CD4+ T_{EM} cell depletion is the most profound T cell population abnormality in AIDS ($P < 0.0001$), but as severe depletion has already occurred in acute infection, the resulting CD4+ T_{EM} cell deficit might not be expected to play a determining role in the much later onset of chronic-phase disease. However, as shown in Fig. 3, CD4+ T cell frequencies in BAL initially rebounded after acute depletion, but after day 100, they progressively declined in all normal progressors examined (with a mean population half-life of ~107 d; see legend to Fig. 3). This observation strongly supports the hypothesis that CD4+ T_{EM} cell depletion constitutes a primary mechanism of immunodeficiency, with overt disease ensuing when the representation of these cells in extra-lymphoid effector sites declines below a critical level. At the time of AIDS onset in our cohort, the average (± SEM) fraction of CD4+ T cells within the BAL T cell compartment was 0.42 ± 0.08%, a value that provides a substantive estimate for this critical threshold.

T cell populations in BAL are short-lived and dependent on continuous T_{EM} cell production and migration

To investigate the mechanisms underlying BAL CD4+ T_{EM} cell decline, we used short-term BrdU labeling (three doses over 24 h) to compare the dynamics of BAL T_{EM} cell populations in clinically stable, chronically SIVmac239-infected RMs versus uninfected RMs. Fig. 4 A shows a typical profile of a chronically infected RM. Note that peak labeling exceeds 25% of BAL CD4+ T cells, occurs 4 d after the last BrdU dose, and

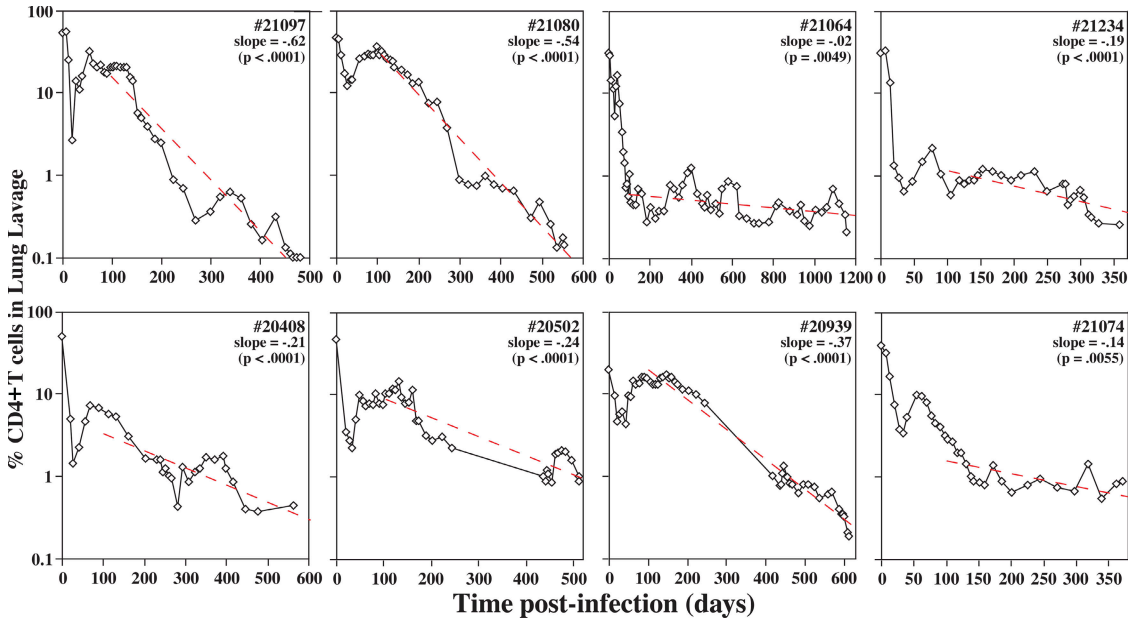

Figure 3. Kinetics of CD4+ T cell decline in the lung. BAL cell preparations were stained for CD3 and CD4, and the percent CD4+ T cells (of total CD3+ T cells) was determined by flow cytometry for eight SIVmac239-infected normal progressors that did not receive ART during their course and were followed to an AIDS endpoint (note that nearly all BAL T cells are T_{EM} cell phenotype [18]). For each RM, the $\Delta\log_{10}$(%CD4) per 100 d was determined from PID 100 to endpoint ("slope"), and the p-value of the regression line is designated in the top right corner of each profile. The pooled $\Delta\log_{10}$(%CD4) per 100 d plus SEM for all eight RMs was −0.28 ± 0.04 ($P = 0.0003$), which corresponds to an average CD4+ T cell population half-life of 107 d (using the formula $t_{1/2} = \log_{10}(0.5)/b$, where b is the pooled slope estimate).

includes cells that are almost entirely postproliferative (Ki-67$^-$). Collectively with previous observations (15), these data indicate that a substantial fraction of the BAL CD4$^+$ T_{EM} cell population is replaced daily from recently divided precursors, originating elsewhere, and emigrating to the lung via the blood. The vast majority of labeled cells disappear over the ensuing month, which, in the absence of proliferative dilution, is most likely due to in situ death (and replacement by nonlabeled cells). Both the peak labeling of CD4$^+$ T_{EM} cells in chronically infected RMs and the subsequent reduction in number of these labeled cells were two- to threefold greater than in uninfected RMs (Fig. 4 B), indicating a substantial increase in the fraction of short-lived cells within the BAL population of infected versus uninfected animals. Notably, the peak BrdU labeling and the magnitude of the subsequent decay of CD8$^+$ T_{EM} cells in BAL were similarly elevated in the infected versus uninfected RMs,

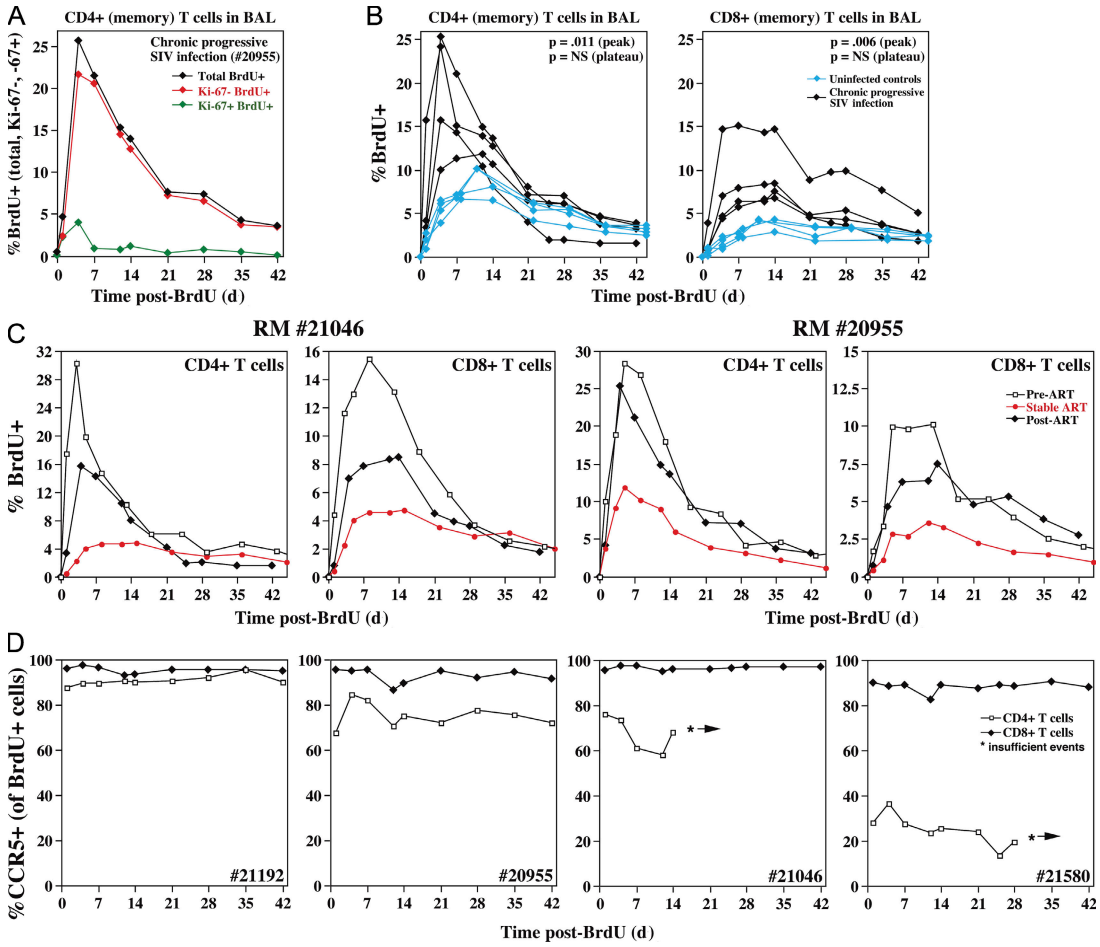

Figure 4. T_{EM} cell dynamics in the pulmonary airspace. (A) An SIVmac239-infected normal progressor (RM no. 20955: PID 1,125; pvl = 69,000 copies/ml) was administered three intravenous doses (30 mg/kg) of BrdU over 24 h, and BAL samples were obtained before BrdU administration (day 0) and at subsequent intervals for flow cytometric analysis of BrdU incorporation in CD4$^+$ T cells. The first post-BrdU sampling was 14 h after the last BrdU dose and is designated day 1. The percentage of total BrdU$^+$ cells, Ki-67$^-$ BrdU$^+$ cells, and Ki-67$^+$ BrdU$^+$ cells was determined at each time point. (B) BrdU was administered to and BAL samples were collected from four uninfected (control) RMs and four plateau-phase SIVmac239-infected RMs (RM no. 20955, as above; no. 21046: PID 1,125; pvl = 360,000 copies/ml; no. 21192: PID 456; pvl = 240,000 copies/ml; no. 21580: PID 876; pvl = 80,000 copies/ml) as described in A. The overall percent BrdU$^+$ in BAL CD4$^+$ and CD8$^+$ T cell populations are shown. Differences in peak and plateau (day 42) labeling were determined by unpaired *t* test (p-values shown in the top right corner of each profile). (C) BrdU was administered to and BAL samples were collected from RM numbers 21046 and 20955 three successive times over the course of their SIVmac239 infection: Pre-ART (PID 577; pvls = 200,000 and 500,000 copies/ml, respectively; percent CD4$^+$ in BAL = 0.41 and 1.7%, respectively); on stable ART (PID 764: pvls = 280 and <30 copies/ml, respectively; percent CD4$^+$ in BAL = 19.7 and 9.6%, respectively), and 7 mo after ART discontinuation (PID 1,125; pvls = 360,000 and 69,000, respectively; percent CD4$^+$ in BAL: 0.43 and 1.4%, respectively). The overall percent BrdU$^+$ of BAL CD4$^+$ and CD8$^+$ T cell populations is shown. (D) CCR5 expression was determined by flow cytometry on the BrdU-labeled CD4$^+$ and CD8$^+$ T cells of the SIVmac239-infected RMs described in B. The fraction of BrdU$^+$ CD4$^+$ and CD8$^+$ T cells expressing CCR5 is shown over time (*, insufficient cells for analysis). A random slope statistical model was used to determine the stability of CCR5 expression over time for the labeled CD4$^+$ T cells. This analysis showed that (a) none of the individual slopes (loss of CCR5-expressing, BrdU$^+$ CD4$^+$ T cells in BAL over time) was statistically significant after adjusting for multiple comparisons, and (b) the overall slope (average loss per day among four animals) was not statistically significant.

and effective ART normalized the BrdU labeling profiles of both CD4$^+$ and CD8$^+$ T_{EM} cells (Fig. 4 C). As viral cytopathogenicity would affect CD4$^+$ T_{EM} cells much more than CD8$^+$ T_{EM} cells, the essentially identical dynamics and ART response of the two subsets strongly suggest that the accelerated turnover of BAL T_{EM} cells primarily reflects immune activation–mediated apoptosis, rather than viral-mediated destruction. To further assess whether direct coreceptor-targeted, viral-mediated cell destruction contributes to the rapid decline of BrdU-labeled CD4$^+$ T_{EM} cells in BAL, we compared the frequency at which these cells expressed CCR5 over time to that of labeled CD8$^+$ T_{EM} cells (Fig. 4 D). Direct viral-mediated killing would be expected to preferentially target the CCR5$^+$ component of CD4$^+$ T_{EM} cells, resulting in decreasing frequencies of CCR5$^+$ cells over time among labeled CD4$^+$ T_{EM} cells, but not CD8$^+$ T_{EM} cells. Importantly, CCR5 expression was largely stable in the labeled CD4$^+$ T_{EM} cells in the BAL of infected RMs, further indicating that if direct viral killing of recently divided CD4$^+$ T_{EM} cells in BAL was occurring, its contribution to the decreased lifespan of these cells in infected RMs must be quite small relative to their overall rate of loss.

Progressive loss of CD4$^+$ T_{CM} cell homeostasis and T_{EM} cell production in chronic infection

The above data indicate that during chronic infection, the diminished BAL CD4$^+$ T_{EM} cell population is largely comprised of relatively short-lived cells that are highly dependent on new memory cell production and emigration for homeostasis, a finding suggesting that CD4$^+$ memory T cell production dynamics might play a role in the progressive decline of BAL CD4$^+$ T_{EM} cells in chronically infected RMs. As shown in Fig. 5 A, the proliferating (Ki-67$^+$) fraction of CD4$^+$ memory T cells in the blood may indeed fall during chronic infection (RMs nos. 20197 and 20408), but this decline is not uniform, coming very late in some RMs (no. 21064) and not at all in others (no. 20502). However, systemic production is more

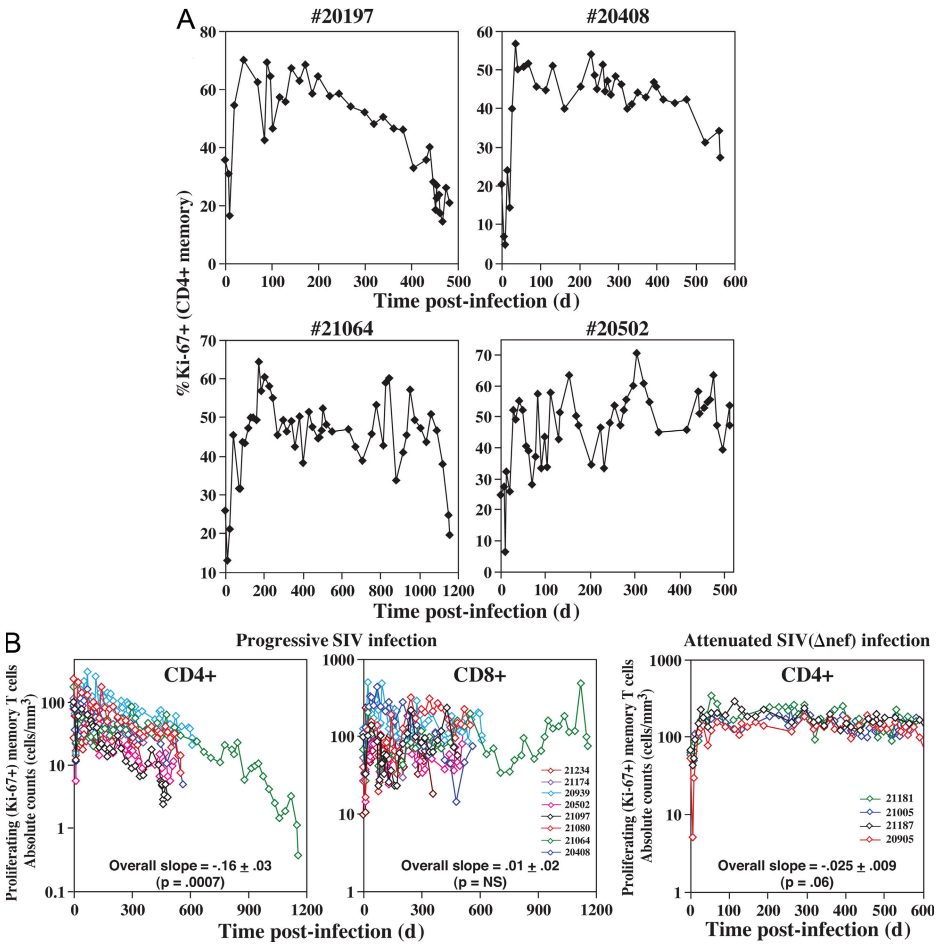

Figure 5. Proliferating CD4$^+$ and CD8$^+$ memory T cell dynamics in the blood. (A) The fraction of proliferating cells (Ki-67$^+$) is shown over the course of chronic/progressive SIVmac239 infection in four representative RMs that did not receive ART and were followed to an AIDS endpoint. (B) The absolute number of proliferating (Ki-67$^+$) CD4$^+$ and CD8$^+$ memory T cells is shown for the entire course of SIVmac239 infection in the same group of untreated normal progressors described in Fig. 3, and for comparison, a group of SIV(Δnef)-infected nonprogressors followed for 600 d. The pooled $\Delta\log_{10}$(%Ki-67$^+$ memory T cells) per 100 d (± SEM) from PID 100 to endpoint (progressors) or PID 100–600 (nonprogressors) is shown for each analysis, along with the p-value delineating the statistical significance of these pooled slopes.

directly related to the overall number of proliferating cells, and we therefore followed the absolute number of proliferating total memory T cells in the blood throughout the course of infection in eight untreated SIVmac239-infected normal progressors and in four RMs with attenuated (clinically nonprogressive) SIVmac239(Δnef) infection (Fig. 5 B). This analysis demonstrated a highly significant progressive loss of proliferating $CD4^+$ memory T cells in the normal progressors, but not of proliferating $CD8^+$ memory T cells in the same RM. In attenuated SIVmac239(Δnef) infection, the numbers of proliferating $CD4^+$ memory T cells showed a very slight decline over 600 d that did not quite achieve statistical significance.

The overall memory T cell population in blood is composed of three phenotypically distinguishable components: T_{CM} cells, transitional T_{EM} cells, and fully differentiated T_{EM} cells, with both T_{EM} cell subsets expressing CCR5 and being effector site directed (Fig. 6 A) (18). On average, approximately one third of the proliferating $CD4^+$ memory T cell compartment

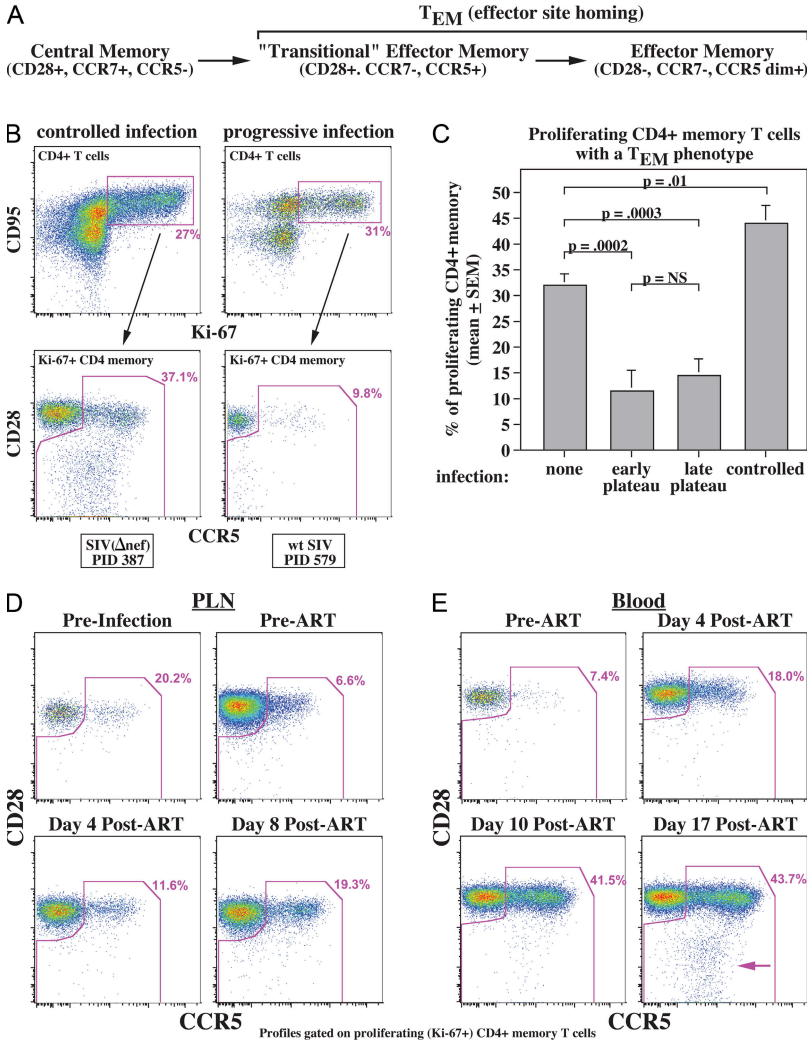

Figure 6. Effect of infection on $CD4^+$ T_{EM} cell differentiation from proliferating T_{CM} cell precursors. (A) Schema of memory T cell differentiation in RMs (18). (B) The top profiles show $CD4^+$ T cells from one RM with attenuated SIVmac239(Δnef) infection (PID 387; pvl = 5,200 copies/ml) and one with progressive SIVmac239 infection (PID 579; pvl = 3,800,000 copies/ml), indicating the gating of proliferating (Ki-67^+) $CD4^+$ memory T cells. The bottom profiles show the representation of T_{CM} cells ($CD28^+$; $CCR5^-$) versus total T_{EM} cells (including $CD28^+$, $CCR5^+$ transitional T_{EM} cells, and $CD28^-$/$CCR5^{dim+}$ mature T_{EM} cells) within the proliferating $CD4^+$ memory compartment. (C) The figure shows cross-sectional analysis of the fractional representation of total T_{EM} cells (as in A) in 10 SIV^- RMs, 7 RMs with early plateau-phase SIVmac239 infection (PID 105; median pvl = 5,300,000 copies/ml), 8 RMs with late plateau-phase SIVmac239 infection (PID 533–878; median pvl = 660,000 copies/ml), and 12 RMs with controlled SIV infection: 9 infected with SIVmac239(Δnef) (PID 154–390; pvls < 400 copies/ml) and 3 spontaneous controllers of SIVmac239 (PID 105–147: pvls < 4,000 copies/ml). Differences were assessed by unpaired *t* test. (D) The profiles show the fractional representation of T_{EM} cells among proliferating (Ki-67^+) $CD4^+$ memory T cells from PLNs from an RM 5 d before SIVmac239 infection, at PID 150 (immediately before ART; pvl = 4,400,000 copies/ml), and at 4 (pvl = 180,000) and 8 d (pvl = 87,000) after ART initiation. (E) The profiles show the fractional representation of T_{EM} cells among proliferating (Ki-67^+) $CD4^+$ memory T cells from the blood of an SIVmac239-infected RM at PID 105 (immediately before ART; pvl = 3,000,000 copies/ml) and days 4 (pvl = 220,000 copies/ml), 10 (pvl = 170,000 copies/ml), and 17 (pvl = 24,000 copies/ml) after ART. The arrow indicates the development of a fully mature $CD4^+$ T_{EM} cell population.

in the blood of uninfected RMs is comprised of CCR5-expressing T_{EM} cell subsets; however, in SIV-infected RMs, this fraction is highly dependent on the degree of pathogenesis. In attenuated SIV infections or wild-type SIV infections in RMs with spontaneous control, we observed a significant increase in the T_{EM} cell fraction of proliferating $CD4^+$ memory cells in comparison to uninfected RMs, consistent with the expected effect of chronic immune activation in driving effector differentiation (28). In contrast, the T_{EM} cell fraction was reduced approximately fourfold throughout pathogenic infection (Fig. 6, B and C). $CD4^+$ T_{EM} cells (predominantly transitional type) were also reduced in PLNs in chronic SIVmac239 infection (Fig. 6 D), indicating that the reduction in proliferating $CD4^+$ T_{EM} cells in the blood was not due to retention in secondary lymphoid tissues. Moreover, effective ART results in an immediate burst of proliferating or recently divided transitional $CD4^+$ T_{EM} cells in the PLNs and blood (Fig. 6, D and E), followed later by mature $CD4^+$ T_{EM} cells (Fig. 6 E), suggesting that although rapid $CD4^+$ T_{EM} cell generation and differentiation is continuously driven by infection-associated immune activation, these new T_{EM} cells and/or their immediate precursors are being destroyed almost as rapidly by direct virus-mediated cytopathogenicity. Importantly, the efficiency of this depletion does not appear to change over the course of chronic infection (compare early vs. late plateau-phase in Fig. 6 C), suggesting that this effect acts as a relatively constant impediment to $CD4^+$ T_{EM} cell production that does not, by itself, explain the progressive decline in CD4 T_{EM} cell populations in extra-lymphoid sites.

$CD4^+$ T_{CM} cells are susceptible to immune activation, infection, and direct virus-mediated destruction

Collectively, these data (an overall decrease in proliferating $CD4^+$ memory T cells and stable rates of $CD4^+$ T_{EM} cell differentiation) lead to the conclusion that the progressive decline in $CD4^+$ T_{EM} cell production in chronic SIV infection originates from a progressive decline in proliferating $CD4^+$ T_{CM} cells caused by a decreasing $CD4^+$ T_{CM} cell proliferation rate and/or a progressive decline in overall $CD4^+$ T_{CM} cell numbers (Fig. 2, A and C). Factors contributing to loss of $CD4^+$ T_{CM} cell homeostasis would therefore constitute primary determinants of immunodeficiency development. One such factor might be persistent immune activation with its previously demonstrated effect of increasing T cell proliferation and turnover (12, 34). Indeed, we found that in the chronically SIV-infected RMs studied here, $CD4^+$ T_{CM} cells manifest a higher fraction of proliferating cells with rapid turnover (Fig. 7 A), potentially leading to proliferative senescence and/or inadequate renewal of long-lived cells (11, 29). However, interpreting these factors as contributing to the progressive failure of $CD4^+$ T_{CM} cell homeostasis is not straightforward because $CD8^+$ T_{CM} cell populations, which are relatively stable during chronic infection, manifest similar, although somewhat less severe, changes in proliferation and turnover in infected RMs (Fig. 7 A).

A factor with perhaps greater potential to differentially affect $CD4^+$ T_{CM} cell versus $CD8^+$ T_{CM} cell homeostasis is direct SIV infection of the former subset. True $CD4^+$ T_{CM} cells lack detectable surface expression of the CCR5 coreceptor and thus would be predicted to be protected from infection; however, previous work has suggested that detectable surface expression of CCR5 may not be necessary for a $CD4^+$ memory cell to be infected (30). Indeed, as shown in Fig. 7 B, SIV DNA was readily detectable by real-time PCR in sorted, precisely defined ($CCR7^+$ $CCR5^-$) $CD4^+$ T_{CM} cells from both early plateau-phase RMs and normal progressors with AIDS. SIV DNA was either absent from $CD8^+$ T_{CM} cells or found at significantly lower levels. In normal progressors with AIDS, SIV DNA levels in $CD4^+$ T_{CM} cells were ~15% of those detected in $CD4^+$ ($CCR5^+$) transitional T_{EM} cells, consistent with a less efficient infection of the former subset. Interestingly, the SIV DNA content of $CD4^+$ T_{CM} cells from RMs with early plateau-phase SIVmac239 infection was significantly (threefold) higher than in the RMs with end-stage infection, whereas SIV content of $CD4^+$ T_{EM} cells was not different between these RM groups. This difference in $CD4^+$ T_{CM} cell infection was not related to overall SIV replication, as pvls were not significantly different in the two groups. However, $CD4^+$ T_{CM} cell proliferation (%Ki-67) was significantly higher in the early plateau-phase RMs, suggesting that $CD4^+$ T_{CM} cell infection can be driven by high activation/proliferation to levels approaching those of $CD4^+$ T_{EM} cells with overt CCR5 expression.

To determine the pathobiologic significance of this $CD4^+$ T_{CM} cell infection, we compared the dynamics of ($CCR7^+$ $CCR5^-$) $CD4^+$ and $CD8^+$ T_{CM} cells during acute SIVmac239 infection and through early plateau-phase ART administration. As illustrated in two representative RMs (Fig. 7 C), absolute numbers of $CD4^+$ T_{CM} cells in the blood decline ~70% in acute infection, concomitant with a 98–99% depletion of BAL $CD4^+$ T_{EM} cells in the same RM (not depicted). The observations that (a) $CD8^+$ T_{CM} cells show only a modest, very transient depletion during this period (Fig. 7 C), and (b) that CD4 representation in the PLN T_{CM} cell compartment declines to a similar degree as blood $CD4^+$ T_{CM} cell counts (not depicted) suggest that the $CD4^+$ T_{CM} cell depletion primarily reflects direct viral-mediated killing of $CD4^+$ T_{CM} cells, rather than a redistribution effect. Increased proliferation rates stabilize circulating $CD4^+$ T_{CM} cell numbers in plateau-phase infection, but continued viral-mediated killing is demonstrated by the observation that ART administration in plateau-phase results in a very rapid and lineage-specific increase in $CD4^+$ T_{CM} cell counts and in the fraction of proliferating $CD4^+$ T_{CM} cells (Fig. 7 C). A similar increase in proliferation with ART is observed in PLNs (Fig. 7 D), suggesting that ART is acting immediately to relieve direct viral-mediated destruction of rapidly proliferating $CD4^+$ T_{CM} cells. Thus, despite undetectable CCR5 expression, $CD4^+$ T_{CM} cells, particularly proliferating cells, are SIV infectable and directly killed by virus.

DISCUSSION

In the past few years, it has been widely documented that primary infection with pathogenic, CCR5-tropic HIV and SIV, though generally asymptomatic, has extensive immunologic

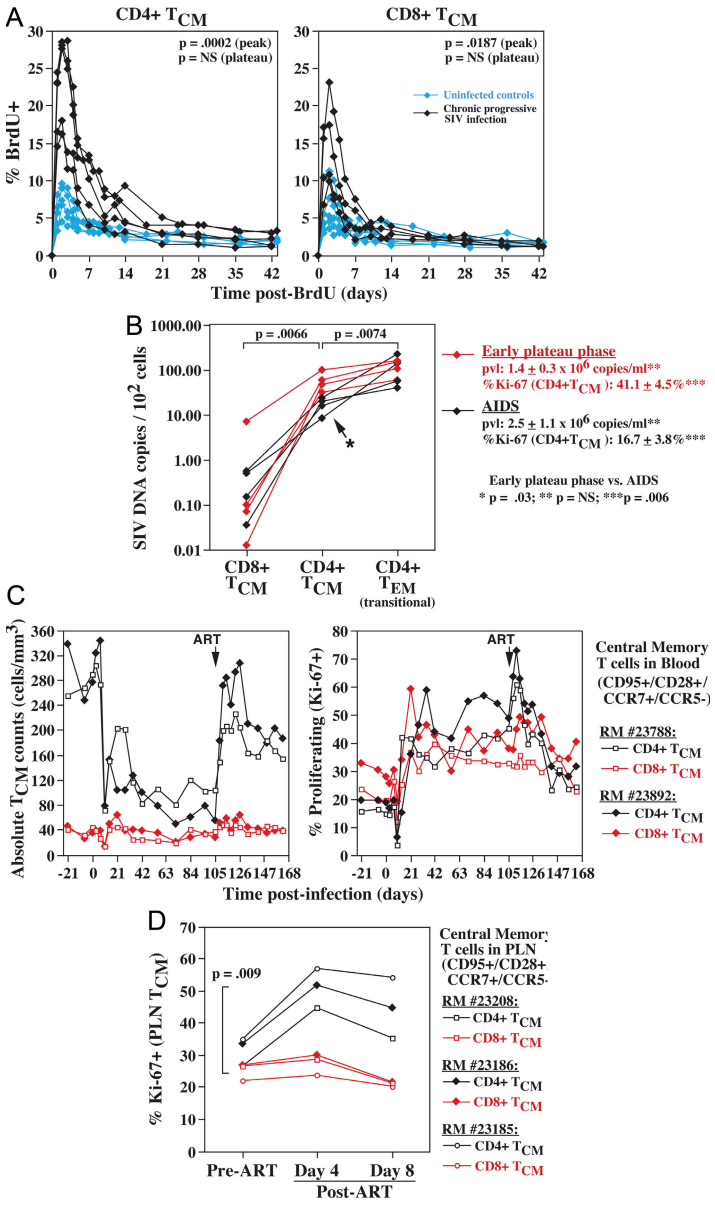

Figure 7. Effect of infection on CD4$^+$ T_{CM} cell homeostasis. (A) BrdU was administered to and blood samples were collected from five uninfected (control) RMs and five late plateau-phase SIVmac239-infected RMs (PID 466–1125; median pvl = 240,000 copies/ml) as described in Fig. 4. The overall percent BrdU$^+$ was determined by flow cytometry on CD95$^+$, CD28$^+$, and CCR5$^-$ T_{CM} cells, both CD4$^+$ and CD8$^+$, at the time points shown. Data from all 10 RMs are included in the CD4$^+$ T_{CM} cell analysis. As one SIV$^+$ RM had insufficient numbers of evaluable CD8$^+$ T_{CM} cells, only four SIV$^+$ RMs are included in the CD8$^+$ T_{CM} cell analysis. (B) CD4$^+$ and CD8$^+$ T_{CM} cells (CD95$^+$, CD28$^+$, CCR5$^-$, CCR7$^+$) and CD4$^+$ transitional T_{EM} cells (CD95$^+$, CD28$^+$, CCR5$^+$) were sorted from splenic mononuclear cell preparations taken at necropsy from eight SIVmac239-infected RMs (four with early [asymptomatic] plateau-phase infection [PID 78–85] and four with chronic-onset AIDS [PID 372, 483, 554, and 1,157]) and assessed for SIV DNA content. The average pvl and percent Ki-67$^+$ within the splenic CD4$^+$ T_{CM} cells from each RM group are also shown. The significance of differences was assessed by unpaired *t* test. (C) The absolute number and fractional proliferation (percent Ki-67$^+$) of highly defined CD4$^+$ and CD8$^+$ T_{CM} cells in the blood are shown for two representative SIVmac239-infected RMs through early plateau phase and after ART. The median pre-ART plateau-phase pvls in both of these RMs were ~4,000,000 copies/ml, with ART decreasing these values to 22,000 and 50,000 copies/ml for RM numbers 23788 and 23892, respectively. (D) Three SIVmac239-infected RMs were treated with ART at PID 132 (no. 23186) or PID 153 (nos. 23208 and 23186) with PLN biopsy immediately before and 4 and 8 d after ART (mean pre-ART and post-ART days 4 and 8; pvls = 6,000,000, 237,000, and 56,000 copies/ml, respectively). The proliferating fraction (percent Ki-67$^+$) of CD4$^+$ and CD8$^+$ T_{CM} cells from each of these PLN biopsies is shown (with differences between post-ART change in percent Ki-67$^+$ of CD4$^+$ and CD8$^+$ T_{CM} cells evaluated by paired *t* test).

consequences (12, 20, 21, 23). The high viral replication of primary infection rapidly destroys the bulk of the body's $CD4^+$ memory T cell population, particularly $CD4^+$ T_{EM} cells in mucosal extra-lymphoid effector sites (15, 19, 30, 31), and also initiates a state of persistent immune activation that has been widely implicated in immunodeficiency development (10–12, 21, 24, 25, 32). However, $CD4^+$ memory T cells are not equally susceptible to this acute destruction, and in SIV-infected RMs, we have previously demonstrated that spared populations (predominantly $CCR5^-$ T_{CM} cells) undergo a substantial increase in proliferative activity that sustains these populations and provides sufficient T_{EM} cell production and influx into effector sites to maintain clinical immune competence (15). Chronically SIV-infected RMs and HIV-infected humans establish a new immunologic steady-state associated with high $CD4^+$ memory T cell turnover and characterized by a substantially diminished $CD4^+$ T_{EM} cell compartment (11, 12, 15, 22, 33–35). Ultimately, this steady-state is degraded and immunodeficiency ensues, but the quantitative changes in rate-limiting parameters that underlie this degradation in the course of chronic infection, as well as the mechanisms responsible for these changes, have not been clearly defined.

Here, we systematically assessed the relationship between potential determinants of pathogenesis and AIDS onset in chronically SIVmac239-infected RMs. Although it remains possible that there are multiple paths to AIDS in this model, our data strongly support a common "primary" pathway and suggest a surprisingly cohesive mechanistic sequence. Two important virologic observations guided our investigation: first, that within the range of plateau-phase pvls manifested by SIVmac239-infected normal progressors, these pvls did not strongly predict time to disease, and second, that the plateau-phase pvls in these normal progressors remained steady through to disease onset. Regarding the first observation, it is clear that in HIV infection, plateau-phase pvls delineate broad groups of individuals with different rates of disease progression (3), but the predictive value of viral replication is limited (36), and host factors (such as the degree of immune activation) have been demonstrated to exert a major influence on prognosis (24–26). Similarly, although SIVmac239 replication rates predict rapid versus normal versus slow progression in RMs (15, 37–39), they did not account for the range of disease-free survival times exhibited by normal progressors in our study, strongly suggesting that in these RMs, host factors are the primary determinants of chronic-phase disease. The stability of plateau-phase pvls through disease onset in the normal progressors studied here further supports this hypothesis, suggesting that a diminishing ability of host immunity to control viral replication or the emergence of more efficiently replicating viral variants were unlikely to be the critical processes leading to AIDS.

We next established that BAL $CD4^+$ T_{EM} cell depletion was the strongest correlate of disease. Most importantly, having shown that changes in the $CD4^+$ T_{EM} cell representation in BAL closely paralleled changes in the small intestine, we demonstrated a highly consistent, gradual progression in BAL $CD4^+$ T_{EM} cell depletion from the initial post-acute steady-state to disease onset in chronic, progressive infection. The latter observation supports the existence of a threshold in effector site $CD4^+$ T_{EM} cell representation below which the SIV-infected host becomes highly susceptible to opportunistic infection. This interpretation is even more compelling given our previous observation that rapid progression to AIDS was associated with cessation of $CD4^+$ T_{EM} cell production and influx into extra-lymphoid sites (15), and the fact that the opportunistic infections found in our AIDS-afflicted RMs (CMV, cryptosporidia, pneumocystis) most commonly developed in such effector sites (unpublished data). We do not discount the possibility that functional or repertoire alterations of $CD4^+$ or $CD8^+$ memory T cells or abnormalities in accessory non–T cells might also contribute to immune deficiency, but would postulate that such qualitative abnormalities would serve to facilitate immune deficiency only in the setting of profound $CD4^+$ T_{EM} cell depletion, perhaps affecting the threshold of $CD4^+$ T_{EM} cell depletion at which overt immune deficiency ensues.

BrdU labeling of chronically SIVmac239-infected RMs was then used to explore the dynamics of pulmonary $CD4^+$ T_{EM} cells and provide insights into the mechanisms underlying their progressive decline. We found that this population is largely comprised of short-lived nonreplicating cells and consequently is highly dependent on the continuous emigration of recently divided T_{EM} cells from the blood. Notably, pulmonary $CD8^+$ T_{EM} cells manifested similar kinetic properties suggesting that (a) the short life span of the $CD4^+$ T_{EM} cells in BAL was not attributable to direct viral-mediated destruction, but rather was likely a result of chronic immune activation (12, 34), and (b) that the progressive decline in $CD4^+$ T_{EM} cells was determined elsewhere, probably by factors controlling the supply of these cells to extra-lymphoid effector sites. In keeping with the latter hypothesis, we found that absolute numbers of proliferating or recently divided $CD4^+$, but not $CD8^+$, memory T cells in the blood progressively declined in chronic SIVmac239 infection (but not in attenuated SIVmac239(Δnef) infection). The fraction of these proliferating or recently divided $CD4^+$ memory cells exhibiting T_{EM} cell differentiation (i.e., the immediate precursors to extra-lymphoid site T_{EM} cells) was considerably diminished in progressive infection by direct virus-mediated destruction (Fig. 6), but, importantly, this fraction did not appear to change over the course of chronic infection. Collectively, these findings strongly suggested that a progressive, systemic decline of the proliferating $CD4^+$ T_{CM} cell compartment resulted in a progressively diminished substrate for $CD4^+$ T_{EM} cell differentiation. This in turn reduced $CD4^+$ T_{EM} cell production and effector site emigration, leading to decreasing effector site $CD4^+$ T_{EM} cell populations and ultimately (when the latter fell below threshold) to AIDS (Fig. 8).

In contrast to a previous report (33), $CD4^+$ naive T cell populations were not generally depleted in the progressive infections studied here, as measured by both the absolute numbers of $CD4^+$ naive T cells in the blood and the relative representation of $CD4^+$ versus $CD8^+$ naive T cells in PLNs. Thus, at least in the SIVmac239 AIDS model, $CD4^+$ T_{CM} cell homeostasis can fail in the face of a relatively intact $CD4^+$ naive

cell compartment. Moreover, studies of thymectomized RMs infected with CXCR4-tropic (naive CD4+ T cell–depleting) SIVmac155T3 (15) have demonstrated that CD4+ T_{CM} cell homeostasis can be maintained long-term in the complete absence of a CD4+ naive T cell compartment (unpublished data). These data suggest that although naive CD4+ T cells are nominally the precursors for CD4+ T_{CM} cells, the naive population is not a major determinant of CD4+ T_{CM} cell homeostasis in chronic SIV infection.

What then is responsible for the progressive decline in CD4+ T_{CM} cell production? BrdU labeling studies demonstrate that both CD4+ and CD8+ T_{CM} cell populations manifest a sustained increase in cell turnover in chronic SIV infection, consistent with an immune activation effect (12) and potentially setting up these populations for slow proliferative senescence (10, 29). This increase in turnover, measured in the blood, is slightly more pronounced for CD4+ T_{CM} cells than CD8+ T_{CM} cells, but this difference, by itself, seems unlikely to explain the gross disparity in CD4+ versus CD8+ T_{CM} cell stability. However, we also demonstrate that, as opposed to CD8+ T_{CM} cells, CD4+ T_{CM} cells are readily infected by CCR5-tropic SIV in vivo (albeit less efficiently than CCR5+ T_{EM} cells), and are, in fact, continuously destroyed in vivo by a direct, virus replication-dependent mechanism (as revealed by their immediate post-ART kinetics). This killing clearly targets proliferating CD4+ T_{CM} cells in LNs (Fig. 7 D) before their emigration into the blood, therefore making their loss "invisible" to BrdU analysis in the blood (indicating that total CD4+ T_{CM} cell loss rates are underestimated by this analysis). This ongoing, virus-mediated CD4+ T_{CM} cell destruction, especially of the proliferating compartment in LNs, would clearly put CD4+ T_{CM} cell homeostasis under more stress than its CD8+ counterpart, potentially leading to imbalance in production versus destruction in the former, but not the latter, subset. Other factors could also contribute to a CD4+ T_{CM} cell homeostatic imbalance, including progressive damage to microenvironments supportive of T_{CM} cell homeostasis (9, 40) and progressive loss of regeneration capacity due to accelerated "aging-like" disorganization of the immune system (12). Even though such factors would potentially affect both CD4+ and CD8+ subsets, the effects need not be equal (9, 21).

Our data define a complex interplay between cell destruction by direct infection on the one hand and immune activation on the other in AIDS pathogenesis. With regard to the CD4+ T_{EM} cell compartment, direct viral-mediated destruction (either productive infection or virus-triggered apoptosis) is almost certainly responsible for the initial depletion of extra-lymphoid site CD4+ T_{EM} cells in acute infection (15, 30, 31) and for the continuous elimination of ~70% of the differentiating CD4+ T_{EM} cells arising from T_{CM} cell precursors in PLNs and blood (this study). In chronic infection, however, the fate of extra-lymphoid site T_{EM} cells, both CD4+ and CD8+, appeared to be governed by immune activation with a considerably larger proportion of cells of both subsets possessing shorter life spans (due to activation-induced apoptosis) than those observed in uninfected controls. Interestingly, although infected

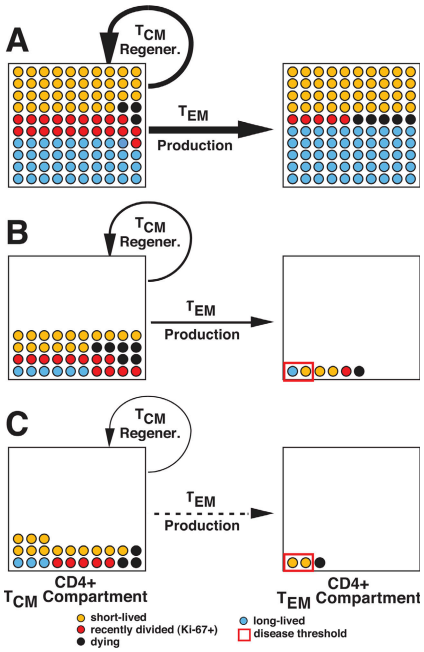

Figure 8. The descent to AIDS: decreasing numbers and changing proportions of CD4+ memory T cell populations in chronic SIVmac239 infection. The figure schematically illustrates the progressive decline in the regeneration of long-lived T_{CM} cells and, consequently, in the production of short-lived, tissue-seeking T_{EM} cells. The T_{CM} cell compartment consists of T_{CM} cells in secondary lymphoid tissues and recirculating in the blood. The T_{EM} cell compartment represents cells resident in extra-lymphoid effector sites. Estimated populations include: (a) recently divided (Ki-67+) cells (cells that have been in S-phase of the cell cycle in the preceding week), (b) short-lived cells (Ki-67− cells destined to die or resume division in <2–3 wk), (c) long-lived cells (Ki-67− cells destined to live >3 wk without cell division), and (d) dying cells (either apoptosis or direct viral destruction). The left to right horizontal arrows indicate the relative rate of production and effector site emigration of T_{EM} cells. The semicircular arrow adjacent to the T_{CM} cell compartment indicates the relative efficiency of T_{CM} cell regeneration (note that proliferating T_{CM} cells provide the substrate for both T_{CM} cell regeneration and T_{EM} cell production). (A) Pre-infection baseline. Both T_{CM} and T_{EM} cell compartments include relatively large proportions of long-lived cells. (B) Asymptomatic chronic phase—mid-course. Both compartments have experienced dramatic acute-phase depletion ($T_{EM} > T_{CM}$) and have established a quasi-stable steady-state with substantially reduced cell numbers, especially of long-lived cells, and increased proliferation and death rates. Both T_{CM} cell regeneration and T_{EM} cell production are reduced severalfold from baseline, despite the increased proportion of proliferating T_{CM} cells, but this fractional increase in a reduced compartment is still sufficient to maintain predominantly short-lived T_{EM} cell populations above the immune deficiency threshold. (C) End-stage chronic phase—AIDS onset. Progressive decline in the T_{CM} cell compartment has reduced T_{EM} cell production and delivery to effector sites below the minimum necessary to maintain T_{EM} cell populations above threshold, leaving these sites increasingly susceptible to opportunistic infection.

CD4+ T cells can be visualized by in situ hybridization in the extra-lymphoid effector sites of these RMs (unpublished data), we found no functional evidence that this infection further

shortened the already brief life span of most CD4+ T_{EM} cells. However, the initial viral-mediated depletion and the immune activation-mediated shortening in the average life span of extra-lymphoid site CD4+ T_{EM} cells render this population highly dependent on a continuous influx of new cells that are derived from the proliferation and differentiation of T_{CM} cells. Immune activation drives this CD4+ T_{CM} cell proliferation and differentiation, but at the same time it appears to increase the susceptibility of these cells to infection and destruction, reducing their steady-state production. Persistent immune activation is also thought to be responsible for the microenvironmental destruction and aging-like disorganization of the immune system mentioned above (12), and these indirect processes likely combine with the direct viral effects to destabilize CD4+ T_{CM} cell homeostasis, resulting in the slow, progressive decline of this precursor population.

Our model suggests that CD4+ T_{CM} cell decline may "set the clock" in regard to the timing of AIDS onset in RMs and therefore predicts that protection of CD4+ T_{CM} cell homeostasis may be the key to preventing AIDS, a notion supported by the recently reported positive correlation between the numbers of circulating CD4+ CD28+ T cells (predominantly T_{CM} cells) with long-term survival in vaccinated SIVmac-challenged RMs that fail to control viremia (41). It is also noteworthy that in the natural apathogenic SIV infections of African monkeys, viral loads are as high or higher than in pathogenic infections of humans and RMs and initial extra-lymphoid CD4+ T_{EM} cell depletion is as extensive, but in the vast majority of these infections, CD4+ memory T cell populations in the blood and LNs are maintained and CD4+ T_{EM} cell depletion is not progressive (references 42–46 and Silvestri, G., personal communication). Although the mechanisms underlying the lack of clinical progression in these highly active infections are likely complex and multi-factorial, the fact that preservation of the CD4+ T_{CM} cell compartment is associated with maintenance of a diminished, but stable, CD4+ T_{EM} cell compartment suggests that in these animals, the CD4+ T cell system may be able to tolerate the consequences of continuous viral replication because a preserved CD4+ T_{CM} cell compartment can stably maintain CD4+ T_{EM} cell populations at supra-threshold levels. Notably, these infections lack the chronic immune activation response that characterizes pathogenic infection (42–46), in keeping with our above described hypothesis that chronic immune activation undermines CD4+ T_{CM} cell stability.

Progression of typical SIVmac239 infection to AIDS (e.g., normal progression with AIDS onset at 1.5–2 yr after infection) is approximately fivefold accelerated relative to HIV infection, and the plateau-phase pvls in this model are ~10-fold higher (3), raising the question of whether the mechanisms proposed here for RMs apply to human infection. In arguing that this is indeed likely to be the case, we would submit that the crucial mechanisms that we have identified in SIVmac-infected RMs as driving disease progression have all been documented in human infection, including selective viral targeting of CCR5+ CD4+ T_{EM} cells and rapid destruction of this compartment in acute infection (22, 35) (but with the ability to infect CD4+ T_{CM} cells as well [47, 48]), the occurrence of generalized immune activation, and in particular, the effect of this immune activation on the turnover and differentiation of CD4+ memory T cells (including T_{CM} cells) and on prognosis (24–26, 34, 49, 50). Moreover, the clinical manifestations of AIDS in SIVmac-infected RMs are highly analogous to those of HIV-infected humans (27, 51, 52), consistent with a similar immune deficit. The generally lower viral replication rates of HIV-infected people deplete the CD4+ T_{EM} cell compartment less completely than in SIV-infected RMs, and the immune activation-driven production of CD4+ T_{EM} cells is clearly better able to initially keep up with direct and indirect destruction of these T cells, as both the fraction of memory T cells among total CD4+ T cells and the fraction of CCR5+ CD4+ T_{EM} cells among these memory cells in the blood are typically normal or elevated in HIV infection (35, 50, 53). However, although these differences may allow post-acute phase HIV-infected subjects to maintain their CD4+ T_{EM} cell tissue compartments above the threshold for clinical immunodeficiency for a longer period of time as compared with SIV-infected RMs, the progressive loss of total CD4+ T cell counts in the blood that is the hallmark of HIV infection (1), and the extensive tissue depletion of CD4+ T cells reported at AIDS (54, 55), clearly indicate that the ability of infected humans to maintain circulating CD4+ T_{CM} cells and their T_{EM} cell progeny declines with time, just as described herein in SIV-infected RMs. Whether in HIV infection a parallel depletion of CD4+ naive T cells is critical for the decline of CD4+ T_{CM} cells, or is only a correlate of the longer time to AIDS, is an open question. Thus, although the extent and/or tempo of CD4+ memory T cell depletion and viral replication are exaggerated in SIV infection compared with HIV infection, we believe the evidence strongly suggests that the fundamental pathogenetic processes are similar in these two situations.

One of the principal enigmas of AIDS pathogenesis, pertinent to both the human disease and the RM model, has been the paradoxical combination of the continuous (often high level) replication of pathogenic virus with long periods of clinical "latency" and delayed, but catastrophic, disease. Our data suggest that this paradox results from a dual nature of CCR5-tropic HIV/SIV infection: an efficient, highly destructive infection of easily replaceable CD4+ T_{EM} cells, and a less efficient/more selective infection and much slower net destruction of precursor CD4+ T_{CM} cells. It is the differential impact of HIV/SIV infection on the CD4+ T_{CM} and T_{EM} cell compartments that probably determines the typical pattern of chronic AIDS. When the T_{CM} cell compartment remains intact (e.g., infection of natural hosts), the infection is nonpathogenic; when both compartments are efficiently targeted (e.g., dual-tropic SHIV infection), disease onset is rapid. What may be most crucial for understanding the pathogenesis of typical chronic AIDS is that although the exquisite susceptibility of the CD4+ T_{EM} cell compartment to infection results in the most dramatic changes to the immune system, it is most likely the more covert dynamics of the CD4+ T_{CM} cell compartment that dictate the tempo of disease progression. If these concepts

are accurate, countering this second process, the slow erosion of $CD4^+$ T_{CM} cell homeostasis, may prove to be the most effective target of nonvirus-directed AIDS therapies. Interventions capable of preserving or rejuvenating $CD4^+$ T_{CM} cells might well "reset the clock" with respect to disease progression and provide significant increases in AIDS-free survival.

MATERIALS AND METHODS

Animals and viruses. A total of 71 purpose-bred male RMs (*Macaca mulatta*) of Indian genetic background and free of Cercopithicine herpesvirus 1, D type simian retrovirus, and simian T lymphotrophic virus type 1 infection were used in this study: 37 infected with wild-type SIVmac239, 19 with SIVmac239(Δ*nef*), and 15 uninfected controls (15, 56, 57). 14 SIVmac239-infected and 4 SIVmac239(Δ*nef*)-infected RMs were studied longitudinally for virologic or immunologic parameters or both; the remaining animals were used in one or more cross-sectional analyses. Early infection data (<200 d) on the longitudinally studied SIVmac239- and SIVmac239(Δ*nef*)-infected RMs have been reported (15). SIVmac239 and SIVmac239(Δ*nef*) infections were initiated with intravenous injection of 5-ng equivalents of SIV p27 ($2.7–3.0 \times 10^4$ infectious centers), as described previously (15). ART consisted of daily subcutaneous injections of 30 mg/kg 9-R-(2-phosphonomethoxypropyl)adenine (tenofovir) and 30 mg/kg β-2',3′-dideoxy-3′-thia-5-fluorocytidine (emtricitabine) (58). BAL and small intestinal biopsies were performed as described previously (15). BrdU (Sigma-Aldrich) was prepared as described previously (15) and administered intravenously in three separate doses of 30 mg/kg body weight over a 24-h period. All RMs were housed at the Oregon National Primate Research Center in accordance with standards of the Center's Animal Care and Use Committee and the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (59). RMs that developed disease states that were not clinically manageable were killed in accordance with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association (60). End-stage AIDS was defined by the presence of AIDS-defining opportunistic infections, wasting syndrome unresponsive to therapy, or non-Hodgkin lymphoma (52, 61).

Viral quantification. Plasma SIV RNA was assessed using a real-time RT-PCR assay (threshold sensitivity <100 SIV gag RNA copy Eq/ml of plasma; interassay CV ≤25%) (62, 63). Cell-associated SIV DNA was also quantified by real-time PCR (sensitivity = single copy of gag DNA) using parallel quantification of albumin gene copy number to define total cell number in a sample, as described previously (47). When no viral DNA was amplified from a given cell population, we report half the lower limit of detection (47).

Flow cytometric analysis and sorting. PBMCs, PLN cells, BAL cells, and intestinal mucosal cells were obtained and stained for flow cytometric analysis as described previously (15, 17, 64). Six-parameter flow cytometric analysis was performed on a Becton Dickinson FACSCalibur instrument using FITC, PE, peridinin chlorophyl protein-Cy5.5 (PerCP-Cy5.5), and allophycocyanin (APC) as the four fluorescent parameters. Polychromatic (8–12 parameter) flow cytometric analysis was performed on an LSR II Becton Dickinson instrument using Pacific Blue, AmCyan, FITC, PE, PE-Texas Red, PE-Cy7, PerCP-Cy5.5, APC, APC-Cy7, and Alexa 700 as the available fluorescent parameters. Cell sorting was performed on a Becton Dickinson FACS ARIA at 70 PSI using FITC, PE, PE-Cy5, PE-Texas Red, APC, APC-Cy7, and QD-705 as fluorescent parameters. Instrument set-up, data acquisition, and sorting procedures were performed as described previously (47, 65, 66). List mode multiparameter data files were analyzed using the FlowJo software program (version 6.3.1; Tree Star, Inc.).

Memory and naive T cell subsets were delineated based on CD28, β7-integrin, CCR7, CCR5, and CD95 expression patterns on gated $CD4^+$ or $CD8^+$ T cells, as described previously (18, 64, 66). In brief, naive cells constitute a uniform cluster of cells with a $CD28^{moderate}$, β7-$integrin^{moderate}$, $CCR7^{moderate}$, $CCR5^-$, $CD95^{low}$ phenotype, which is clearly distinguishable from the phenotypically diverse memory population that is predominantly $CD95^{high}$ and displays one or more of the following "non-naive" phenotypic features: $CD28^-$, $CCR7^-$, $CCR5^+$, β7-$integrin^{bright}$, or β7-$integrin^{-/dim}$. All analyses included at least two of these markers (usually CD28 vs. CD95) for memory subset discrimination. The T_{CM} and T_{EM} cell components of the memory subset in the blood and LNs were delineated based on the phenotypic criteria shown in Fig. 6 A, with T_{EM} referring to the combination of transitional and fully differentiated T_{EM} cells. As we have shown that T cells in effector sites such as BAL and jejunal lamina propria are almost exclusively of the T_{EM} cell phenotype (18), $CD4^+$ and $CD8^+$ T cells from these sites are generally referred to as T_{EM} cells. For determination of CD4 representation within the naive versus memory compartments of LNs, CD4 naive and memory and CD8 naive and memory subsets were individually delineated, and Boolean gating was used to create a total naive cell and a total memory cell gate from which the percent CD4 was determined.

mAbs. mAbs L200 (CD4; AmCyan, APC-Cy7), SP34-2 (CD3; PerCP-Cy5.5), SK1 (CD8α; APC-Cy7, PerCP-Cy5.5, PE-Cy7; unconjugated), CD28.2 (CD28; PE, PerCP-Cy5.5), B56 (Ki-67; FITC, PE), B44 (anti-BrdU; FITC; APC), DX2 (CD95; PE, APC, PE-Cy7), 3A9 (CCR5; PE, APC), and FIB504 (β7-integrin, PE, PE-Cy7) were obtained from BD Biosciences. mAb 2ST8.5h7 (CD8β; PE, PE-Cy7), CD28.2 (PE-Texas Red), and streptavidin–PE-Cy7 were obtained from Beckman Coulter. FN-18 (CD3) was produced and purified in house and conjugated to Pacific Blue or Alexa 700 using a conjugation kit from Invitrogen. mAb 150503 (anti-CCR7) was purchased as purified immunoglobulin from R&D Systems, conjugated to biotin using a Pierce Chemical Co. biotinylation kit, and visualized with streptavidin–Pacific Blue (Invitrogen). Purified anti-CD8 mAb was conjugated with QD705 (Invitrogen) using standard protocols (http://drmr.com/abcon).

Statistical analysis. Linear regression analysis was used to determine the association between (a) the percentages of $CD4^+$ T cells in BAL versus small intestinal lamina propria, and (b) percent $CD4^+$ T cells in BAL and absolute numbers of memory cells in blood versus time, and both linear regression and Spearman rank analysis was used to evaluate the association between mean plateau-phase viral loads and time to disease. A random slope model was used to estimate the overall decay rate of percent $CD4^+$ T cells in BAL and absolute numbers of memory cells in the blood in the study animals over time. A mixed effect model was used to determine whether the size or representation of T cell subsets in the blood or BAL significantly changed over the course of infection (preinfection to terminal disease), and if so, determination of which changes were most closely associated with disease. Unpaired *t* tests were used to determine the significance of differences in (a) CD4 representation in naive and memory compartments of PLNs, (b) peak and plateau BrdU labeling in SIV-infected versus uninfected monkeys, (c) the representation of cells with T_{EM} cell differentiation among proliferating total memory T cells in progressive SIV infection versus controlled infection versus uninfected RMs, and (d) the level of SIV DNA content within the $CD4^+$ T_{CM}, $CD8^+$ T_{CM}, and $CD4^+$ transitional memory T cell subsets of RMs in early plateau-phase versus end-stage SIV infection. Paired *t* tests were used to determine the significance of differences in the level of (a) SIV DNA content between the $CD4^+$ T_{CM} versus $CD8^+$ T_{CM} versus $CD4^+$ transitional memory T cell subsets, and (b) post-ART proliferation between $CD4^+$ and $CD8^+$ T_{CM} cells in PLNs. Statistical analysis was conducted with the SAS program (SAS Institute) and Statview (Abacus Concepts). p-values of <0.05 were considered significant with the Bonferroni adjustment used in situations with multiple comparisons.

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