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Impaired Induction of CD27 and CD28 Predicts Naive CD4 T Cell Proliferation Defects in HIV Disease

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Many immunological defects have been described in HIV disease, including a diminished capacity of naive CD4+ T cells to expand after TCR stimulation. The mechanisms underlying impaired naive CD4+ T cell expansion in HIV disease are not well described. Using a rigorous phenotypic definition of naive T cells, we found that cell cycle entry after TCR engagement was restricted to cells that increased surface expression of costimulatory molecules CD27 and CD28. Induction of these receptors, however, was not sufficient to result in cell cycle entry among the CD4+CD31− naive T cell subset. Analyses of cells from HIV-infected persons indicated that naive CD4+CD31+ T cells from these subjects were impaired in their ability to enter the cell cycle after stimulation and this impairment was predicted by the relatively poor induction of costimulatory molecules on these cells. Thus, failure to increase surface expression of costimulatory molecules may contribute to the naive T cell expansion failure that characterizes HIV infection. The Journal of Immunology, 2007, 179; 3543–3549.

Naive T cells are the critical underpinning of adaptive immune potential. These cells provide an essential resource both for mounting immune responses against novel Ags and also for maintaining T cell homeostasis. In HIV disease, the progressive depletion of naive CD4+ and CD8+ T cells (1) predicts clinical outcome (2) and responsiveness to immunization (3). Compounding the problems associated with the decline in naive T cell numbers, function of the remaining naive CD4+ T cells is impaired in HIV-infected individuals (4). In particular, naive CD4+ T cells from HIV-infected persons have a reduced potential to progress beyond G1 phase of the cell cycle in response to TCR activation (5) and this impairment is not predictably corrected by exogenous IL-2 (4). The mechanism that underlies naive CD4+ T cell proliferation failure in HIV disease is unknown.

Human naive CD4+ T cells can be subdivided into CD31+ and CD31− subsets. CD31+ naive T cells possess higher levels of TCR excision circles (TRECs)5 reflective of recent emigration from the thymus (6). Naive CD4+ T cells lacking CD31 expression have proportionally fewer TRECs, presumably a consequence of homeostatic proliferation. The possibility that CD31+ and CD31− naive CD4+ T cell subsets could have distinct proliferation potential in response to TCR stimulation has not been addressed previously, but seems reasonable because in mice, recently produced naive T cells proliferate better in response to TCR engagement than do aged naive T cells (7). We therefore considered the possibility that CD31 could distinguish naive CD4+ T cells with good or poor expansion potential in response to TCR activation.

At a single cell level, defects in cell cycle progression of activated naive T cells could stem from insufficient TCR or costimulatory signals. To investigate this possibility, we have examined the expression of two well-characterized costimulatory molecules, CD27 and CD28, on naive CD4+ T cells after TCR stimulation. CD27 provides costimulatory signals for naive CD4+ T cells (8) and is transiently increased on the cell surface following TCR triggering (9, 10). CD28 provides a critical costimulatory signal that enhances IL-2 production and augments cell cycle progression in response to TCR stimulation (11, 12). CD28 expression also can be transiently induced by TCR stimulation (13). In this study, we test the hypothesis that induction of CD27 and CD28 costimulatory molecule expression by TCR activation is important for cell cycle progression, and that this induction may be diminished in naive CD4+ T cells from HIV-infected individuals.

Our findings uncover a global impairment in the ability of CD31− naive CD4+ T cell subsets to proliferate in response to TCR stimulation, suggesting that heightened homeostatic division may impair subsequent T cell responsiveness. Moreover, in analyses restricted to the CD31− naive T cell subset, we found that cells from HIV-infected individuals proliferated poorly in response to TCR stimulation. Importantly, cells capable of cell cycle progression were identified by their expression of higher levels of costimulatory molecules, suggesting that increased sensitivity to costimulation may promote cell cycle progression in naive CD4+ T cells after TCR engagement. In addition, poor induction of costimulatory molecules in naive CD4+ T cells corresponded to poor proliferation responses after cells were activated with TCR agonists. Thus, impaired induction of costimulatory molecules in naive T cells from HIV-infected individuals may underlie naive T cell expansion failure in this disease.

Materials and Methods

Subjects

These studies were approved by the Institutional Review Board of Case Western Reserve University at University Hospitals of Cleveland. Samples were obtained from 20 HIV infected individuals and 9 healthy donors. The

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3 Abbreviations used in this paper: TREC, TCR excision circles.

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The significance of P and the resulting T cell populations were determined to be depletion beads. Depletion of these cells was confirmed by flow cytometry (AutoMACS; Miltenyi Biotec), was supplemented with CD45RO-depletion mixture, which depletes CD8^+ T cells. CD45RO-depleted PBMCs were incubated with surface-bound anti-CD3 mAb (BD Pharmingen) and soluble anti-CD28 mAb (5 μg/ml; BD Pharmingen). Ki-67 was measured 4 days poststimulation as outlined above. For analyses of alloimmune T cell responses, purified naive CD4^+ T cells were incubated with irradiated T cell-depleted PBMC. The T cell-depleted PBMC were a mixed population derived from five healthy donors that had been depleted of CD3^+ T cells by magnetic beads and had been incubated overnight with LPS (20 ng/ml). These cells were irradiated (500 rads) and frozen at −80°C for storage. On the day of the assay, the irradiated cells were thawed, washed, and incubated with purified naive CD4^+ T cells at a ratio of 1:10. Cells were surface stained with fluorochrome-labeled Abs mentioned previously and anti-Ki-67 intracellular Ab.

**Statistical analysis**

A comparison of the Ki-67 expression and induction of CD27 and CD28 between the groups was performed using the independent samples t test and ANOVA. The Shapiro-Wilk test was used for testing normality. Spearman rank correlation analysis was performed to investigate the relationships between induction of costimulatory molecules and induction of Ki-67. For the purified naive CD4^+ T cell experiments, groups were analyzed with the Mann-Whitney U test. Statistical significance was established at p < 0.05.

**Results**

Naive CD4^+ CD31^+ T cells obtained from HIV-infected individuals less frequently enter cell cycle after TCR engagement

Naive CD4^+ T cells were identified in CD45RO-depleted PBMC by coexpression of CCR7, CD27, and CD28. We found that such cells (CD4^+ CD45RO^+ CCR7^+ CD27^+ CD28^+) expressed IL-2 but not IFN-γ when stimulated with superantigen (Staphylococcus aureus Enterotoxin B) plus costimulatory agonists (anti-CD28 and anti-CD49d antibodies), consistent with a naive T cell phenotype (data not shown).

We hypothesized that naive T cell subsets defined by CD31 expression might have different potential to proliferate in response to TCR activation. CD45RO-depleted PBMC were incubated with...
that became CD31 induction after TCR stimulation (data not shown) confirming that from healthy donors resulted in nearly a complete loss of Ki-67 cycle after TCR engagement by anti-CD3 Ab stimulation. CD31 are independent of APCs. Purified naive CD4 cells from healthy donors. Because CD31 cells were simply a consequence of defective APC. Therefore, we asked whether the induction of CD27 or CD28 expression might be impaired in naive T cells from HIV-infected individuals. We found that naive CD4+ T cells obtained from healthy controls had increased expression of CD27 and CD28 following TCR activation (9, 10, 13). Therefore, CD27 and CD28 costimulatory molecules play important roles in T cell activation process and the surface expression of these molecules increases following TCR activation (9, 10, 13). Therefore, we asked whether the induction of CD27 or CD28 expression might be impaired in naive T cells from HIV-infected individuals. We found that naive CD4+ T cells obtained from healthy controls had increased expression of CD27 and CD28 following TCR stimulation. The increased expression of these costimulatory molecules was observed at 2 days (Fig. 3A) and also as early as 18 h after stimulation conditions, expressing less Ki-67 than cells from healthy donors (Fig. 2). Thus, the defect in cell cycle progression is an intrinsic property of naive CD4+ T cell populations in HIV disease.

Naive CD4+ T cells in HIV infection have impaired induction of cell surface expression of CD27 and CD28 after TCR engagement CD27 and CD28 costimulatory molecules play important roles in T cell activation process and the surface expression of these molecules increases following TCR activation (9, 10, 13). Therefore, we asked whether the induction of CD27 or CD28 expression might be impaired in naive T cells from HIV-infected individuals. We found that naive CD4+ T cells obtained from healthy controls had increased expression of CD27 and CD28 following TCR stimulation. The increased expression of these costimulatory molecules was observed at 2 days (Fig. 3A) and also as early as 18 h after stimulation.
Cell cycle entry is largely restricted to naive CD4+ T cells. Defects were intrinsic to the naive T cells and soluble anti-CD28 (data not shown), further indicating that the infected persons that had been activated by immobilized anti-CD3 Ab and TCR engagement associate with cell cycle progression. We gated on cells with elevated costimulatory molecule expression and evaluated the expression of Ki-67 in these cells 2 days poststimulation. Cells that increased surface expression of CD27 and CD28 after TCR activation were enriched for Ki-67+ cells, whereas cells that did not increase CD27/CD28 expression above the background of unstimulated cells, rarely expressed Ki-67 (Fig. 4). Interestingly, in three separate experiments, we found that naive CD4+ T cells stimulated with allogeneic APC (T cell-depleted PBMC) also increased surface expression of CD27/CD28, and again the induction of Ki-67 was restricted to the CD27/CD28 bright cells (representative experiment in Fig. 5). Thus, increased cell surface expression of CD27 and CD28 occurs in response to anti-CD3 Ab or in response to allogeneic stimulation, potentially facilitating cell cycle progression. The proportions of CD4+CD31+ naive T cells that increased surface expression of costimulatory molecules after TCR stimulation tended to be slightly reduced, but not significantly different from CD4+CD31+ naive T cells (Fig. 3B). The density of costimulatory molecule expression measured as the change in mean fluorescent intensity of CD27 or CD28 staining after TCR stimulation, however, was significantly lower among the CD4+CD31+ cells (mean fluorescent intensity CD31+ cells = 14886 and 7659 for CD27 and CD28, respectively; p < 0.01). These results demonstrate that CD4+CD31+ cells respond to TCR stimulation by increasing costimulatory molecule expression, however, the induction of these costimulatory molecules is not sufficient to permit cell cycle progression among these cells.

Next, we asked whether providing increased costimulatory signals with agonistic anti-CD28 Ab would enhance TCR-mediated recruitment of naive CD4+ T cells into the cell cycle. CD27 expression alone was used to quantify costimulatory molecule induction because CD28 was bound by the agonistic Ab. Interestingly, addition of the anti-CD28 agonistic Ab to cultures containing anti-CD3 agonist markedly enhanced cell cycle entry, even among naive CD4+ T cells that failed to increase surface expression of CD27 (Fig. 6). Thus, increasing costimulatory agonist activity might compensate for the lack of increased costimulatory molecule expression thereby promoting cell cycle progression of naive T cells that otherwise would be incapable of division.

Naive CD4+ cell cycle progression is related to induction of costimulatory molecule expression in health and in HIV infection

To ascertain whether the defects in cellular proliferation observed in TCR-activated naive CD4+ T cells from HIV-infected individuals were a consequence of impaired induction of costimulatory

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**FIGURE 4.** Ki-67 induction in cells that increase expression of costimulatory molecules CD27 and CD28. Expression of costimulatory molecules (CD27 and CD28) was analyzed among CD45RO-depleted CD4+ T cells after 2 days of incubation with anti-CD3 Ab. The cells were gated on CD4+CCR7+ cells and further analyzed for expression of CD27 and CD28 (dot-plots). A background gate was drawn around CD27/CD28 double-positive cells using cells from unstimulated cultures (lower gate) and a second gate (upper gate) was drawn around cells that increased expression of CD27 and CD28 above the background levels after TCR engagement. The cells in the lower gate and cells in the upper gate were analyzed for Ki-67 expression (frequency distributions histograms). Representative results are shown for a healthy control (C) and an HIV-infected patient (P). Numbers indicate the percentage of Ki-67+ cells.

**FIGURE 5.** Induction of costimulatory molecules and Ki-67 in naive CD4+ T cells after allograft stimulation. CD45RO-depleted PBMC were incubated in medium alone or with irradiated allogeneic T cell-depleted PBMC and induction of CD27 and CD28 was examined after 4 days of incubation. The CD4+ cells that increased expression of costimulatory molecules and the cells that did not increase surface expression of costimulatory molecules after allogeneic stimulation were compared for intracellular expression of Ki-67. The y-axis label for frequency distribution histograms represents cell number.
molecule expression, we assessed the relationship between the induction of Ki-67 expression and the induction of costimulatory molecules among these cells after activation (Fig. 7, A and B). Analyses of the whole naive CD4\(^+\) T cell population demonstrated a clear relationship between induction of costimulatory molecules and cell cycle progression only in cells from healthy donors but not...
in HIV infection. Nevertheless, by restricting the analysis to CD4\(^+\)CD31\(^+\) cells, we found that the magnitude of CD27/CD28 induction was directly and significantly related to the expression of Ki-67 (Fig. 7, C and D) in cells from both healthy donors and HIV-infected individuals. To further assess the importance of the costimulatory molecules in this model of T cell activation, we studied the effect of blocking costimulation with Abs that bind Abs are reflective of a defect that is apparently broadly shared molecules at increased levels are subsequently induced to express Ki-67 in circumstances where the anti-CD3 Ab alone is used to activate cells. In contrast, by increasing signaling through CD28 with agonistic Abs, cells that were less capable of increasing costimulatory molecule surface expression were better able to progress into the cell cycle. We propose that the additional agonistic signaling provided by anti-CD28 Abs replaced the requirement for heightened sensitivity normally provided by increasing the density of costimulatory molecules. Consequently, even cells without increased costimulatory molecule expression could undergo cell cycle progression. Further studies will be needed to ascertain whether increased surface expression of costimulatory molecules on activated naive T cells results in increased sensitivity to natural costimulatory ligands.

Importantly, this model may not be applicable to CD4\(^+\)CD31\(^-\) naive cells. These cells did not proliferate after TCR stimulation, even though they did increase the surface expression of CD28 and CD27. It could be argued that the CD4\(^+\)CD31\(^-\) cells express less surface density of costimulatory molecules compared with CD4\(^+\)CD31\(^+\) cells, thereby not reaching a critical threshold of sensitivity for costimulation, however, it should be noted that these cells failed to enter cell cycle even when additional costimulation was provided with anti-CD28 agonistic Abs (data not shown). Thus, CD4\(^+\)CD31\(^-\) cells appear to be intrinsically less able to expand in response to TCR activation even in the presence of additional costimulatory signals.

The poor induction of costimulatory molecule expression on the surface of naive CD4\(^+\) T cells from HIV-infected persons may be a critical determinant of cellular proliferation defects in these cells. We propose that the relative lack of costimulatory molecules may render the cells less able to receive these critical signals, resulting in cell cycle arrest instead of cell cycle progression. If this can be confirmed, then agents that can enhance CD28 and CD27 expression on T cells might be useful adjuvants in HIV disease. Alternatively, as we have shown in this study, use of costimulatory agonists may provide sufficient signal to bypass this limitation.

Finally, our previous studies demonstrated that CD4\(^+\) T cells (5, 17) and particularly naive CD4\(^+\) T cells (4) from HIV-infected persons failed to progress efficiently into the cell cycle after TCR activation. These studies relied on agonistic Abs targeted to the V\(\beta\)3 TCR chain for stimulation of a subset of naive T cells and used CD62L expression and CD45RO depletion to identify naive T cells. In the present study, we have used more restricted criteria for defining naive CD4\(^+\) T cells that, according to other published observations, should provide at least 98% confidence that the cells so identified are truly naive (18). This represents a substantial refinement of previous studies that relied on only one or two cell surface markers to define and examine the function of naive T cells in HIV infection. Moreover, because anti-CD3 Ab does not restrict activation to a single V\(\beta\) family, our current studies provide evidence that defects observed previously with anti-V\(\beta\)3 agonistic Abs are reflective of a defect that is apparently broadly shared among circulating naive CD4\(^+\) T cells. Thus, with this work, we provide more evidence that naive CD4\(^+\) T cell expansion defects are characteristic of HIV infection and we propose that these defects may play an important role in the immunodeficiency of HIV infection and AIDS.

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**Disclosures**

The authors have no financial conflict of interest.
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