Reduced Thymic Output Is a Major Mechanism of Immune Reconstitution Failure in HIV-Infected Patients After Long-term Antiretroviral Therapy

Taisheng Li, Ning Wu, Yi Dai, Zhifeng Qiu, Yang Han, Jing Xie, Ting Zhu, and Yanling Li
Department of Infectious Disease, Peking Union Medical College Hospital, and Chinese Academy of Medical Sciences, Beijing

Background. Approximately 20% of human immunodeficiency virus type 1 (HIV-1)–infected adults do not normalize their CD4+ T lymphocytes after long-term effective highly active antiretroviral therapy (HAART). The mechanistic basis for this failure is unclear.

Methods. Seventy-four patients were followed up regularly for 3–7 years. Patients with undetectable plasma viral load (<50 copies/mL) for over 12 months were further classified into 2 groups: (1) immunological nonresponders, whose CD4+ T-cell count was <200/μL or <20% compared with baseline; and (2) immunological responders, whose CD4+ T-cell count was >300/μL or >30% compared with baseline.

Results. Compared with 17 immunological responders, 13 immunological nonresponders had a lower magnitude of naive CD4+ T-cell increase, a lower percentage of recent thymic immigrants (CD31+%), and a higher percentage of activated CD8+ T cells. Furthermore, unlike CD4+ T cells, which increased along with the decrease of viral load, the percentage of recent thymic immigrants (CD31+%) had little change in the majority of patients. These data were fit into a mathematical model, \( N(t) = A + B [1 - \exp(-t/\tau)] \), from which we deduced that the initial rate of CD4+ T-cell restoration is associated significantly with the percentage of recent thymic immigrants (CD31+%).

Conclusions. Our data indicate that the failure to restore CD4+ T-cell count following HAART was associated primarily with a defect in recent thymic immigrants, which suggests the existence of thymus exhaustion.

Highly active antiretroviral therapy (HAART) is capable of dramatically reducing plasma viral load to undetectable levels in patients infected with human immunodeficiency virus type 1 (HIV-1). As a consequence, a majority of patients could reconstitute their immune system with respect to CD4+ T-cell counts in peripheral blood [1]. This CD4+ T-cell restoration is typically a biphasic process that includes an initial rapid increase in the first 3–6 months reflecting the redistribution of memory CD4+ T cells, followed by a slow increase reflecting the central regeneration of naive CD4+ T cells by the thymus [2, 3]. However, ∼20% of patients fail to significantly improve their CD4+ T-cell count even with a fully suppressed HIV-1 replication for a long time [4–7]. This phenomenon is known as immune reconstitution failure, and these patients are defined as immunological nonresponders (INRs) [8–10]. It is important to elucidate the mechanism of immune reconstitution failure because INRs with sustained low levels of peripheral CD4+ T-cell count have an increased risk of developing AIDS or non–AIDS-related complications including cardiovascular, liver, and renal diseases [11].

Many studies have been performed to elucidate the mechanisms of immunological reconstitution failure. Older age, male gender, lower baseline CD4+ T-cell count, longer duration of HIV-1 infection, and coinfection with other virus are known risk factors associated with prolonged low CD4+ T-cell count [4, 8, 12–14].
Poor CD4+ T-cell restoration was also linked to reduced production of naive CD4+ T cells, especially recent thymic immigrants as determined by quantification of T-cell rearrangement excision circles (TRECs), elevated rate of CD4+ T-cell apoptosis, and excessive activation of T cells [7, 8, 10, 15, 16].

In order to gain insight into the mechanisms of immune reconstitution and improve the treatment for INRs, we designed the current study to investigate the kinetics of T-cell subset changes in HIV-infected patients who had been followed up for 3–7 years. Based on the kinetic information obtained, we also constructed a mathematic model to interpret the heterogeneity of immune reconstitution patterns observed in different individuals.

METHODS

Study Population

HIV-1–infected adults had been enrolled in HIV/AIDS Clinic Center of Peking Union Medical College Hospital (PUMCH) since 2003. The criteria of patients’ recruitment were: (1) HIV-1 seropositive, (2) HAART-naive, (3) age 18–65, (4) both genders, (5) baseline CD4+ T-cell count <250/µL, and (6) no severe opportunistic infections (OIs) or other diseases. After enrollment, patients were started on HAART regimens including 2 nucleoside reverse-transcriptase inhibitors (NRTIs) and 1 nonnucleoside reverse-transcriptase inhibitor (NNRTI), or 1 protease inhibitor (PI) in combination with 1 protease inhibitor (PI) in combination with NRTIs and 1 nonnucleoside reverse-transcriptase inhibitor (NNRTI), or, 1 protease inhibitor (PI) in combination with either 2 NRTIs or 1 NNRTI. They were then regularly followed up for 3–7 years at month 0, 1, 3, 6, 9, 12, and every 6 months thereafter. Most data reported here were from a 3-year-long period. Those patients who maintained good viral response as defined by the plasma viral load decreased to an undetectable level (<50 copies/mL) and maintained <50 copies/mL at all time points thereafter, or, at only one time point there was a viral load flipped of >50 copies/mL. Patients were defined as INRs if the restoration of CD4+ T-cell count were <200/µL or 20% compared with baseline after the plasma viral load had become undetectable (<50copies/mL) for over 12 months; all other patients were defined as IRs [5]. Seventeen IRs with the restoration of CD4+ T-cell count >300/µL or >30% were selected to exclude patients on the borderline, and 17 healthy volunteers were also included as controls. All patients were enrolled after written informed consents were obtained.

Plasma HIV-1 Viral Load Assay

Plasma HIV-1 RNA viral load was measured by QUANTI-PLEXTM b-DNA System 340 (Bayer, version 3.0). The detectable range is 50 copies/mL ~500 000 copies/mL. When the viral load was under the lower detectable limit, 49 copies/mL was recorded for statistical analysis. Log10VL values were used for all analyses.

T-Cell Subsets Analyses by FAC

PBMCs were separated from freshly collected, EDTA-anticoagulated whole blood and stained with combinations of different fluorescent monoclonal antibodies followed by flow cytometry analysis (3-color EPICS-XL flow cytometer, Beckman-Coulter Inc) detecting T cells (CD3+), CD4+ T cells (CD4+CD3+), CD8+ T cells (CD8+CD3+), naive CD4+ T cells (CD4+CD45RA−CD62L+), memory CD4+ T cells (CD4+CD45RA-), activated CD8+ T cells (CD8+CD3+CD8+ or CD8+CD3+HLA-DR+). PBMCs thawed from previously cryopreserved samples were analyzed by using a BD FACS-Canto I flow cytometer (BD Biosciences) to detect recent thymic immigrants of CD4+ T cells (CD4+CD45RA+CD3+CD8+) [17] and early apoptotic CD4+ T cells (CD4+AnnexinV+PI). Immunofluorescent monoclonal antibodies FITC-CD3, PEcy5-CD4, PE-CD8, PE-CD45RA, and FITC-CD62L were purchased from Beckman-Coulter and Immunotech; PEcy5-CD8, PE-CD38, FITC-CD8, FITC-HLA-DR and FITC-AnnexinV apoptosis detection kit were from BD Pharmingen; APC-CD31 was from eBioscience.

Statistical Analyses

All statistical analyses were performed using SPSS for Windows (Statistical Package for Social Sciences, version 16.0). Normal variables were displayed as mean ± standard deviation according to Kolmogorov–Smirnov test. When comparing the means of normal variables, independent-samples t test was used for 2 groups and one-way analysis of variance (ANOVA) was used for multiple groups. Paired-samples t test was performed to compare different time points within certain group. The means of categorical data were compared with Pearson χ2 test. Associations between normal variables were assessed using Pearson bivariate correlations assay. All analyses were 2-tailed and when P value <.05 they were considered to have significant difference.

The Mathematical Model of Immune Reconstitution

After HAART, the CD4+ T-cell count at any given time point were calculated with

\[ N(t) = A + B \left[ 1 - \exp \left( -\frac{t}{\tau} \right) \right] \]  

(1)

\( N(t) \): CD4+ T-cell count at one given time point; \( A \): Baseline CD4+ T-cell count, which is a constant; \( B \): For a given patient, the CD4+ T-cell count would reach plateau after 4–6 years [4], and \( B \) refers to the theoretical maximum increase of CD4+ T-cell count that could be reached by one given patient, which is also a constant; \( e \) is natural logarithm; \( \tau \) is another constant introduced in this model, and it was defined as “restoration index.”
RESULTS

The Kinetics of Different T-Cell Subsets

Along with the successful suppression of HIV-1 replication, the plasma viral load decreased and CD4\(^+\) T-cell count increased dramatically in patients. CD4\(^+\) T-cell count increased much more slowly in the INR group than in the IR group, and fewer CD4\(^+\) T cells were gained after 36 months of treatment (Figure 1A and 1B). The increase of memory CD4\(^+\) T cells (the absolute cell count in each follow-up time points minus those in baseline, \(\Delta m\)) in the INR group was similar with the IR group, while the increase of naive CD4\(^+\) T cells (\(\Delta n\)) in the INR group was significantly less than the IR group (Figure 1C and 1D).

Instead of using TREC (T-cell rearrangement excision circles), we studied the kinetics of the recent thymic immigrant CD4\(^+\) T cells using the percentage of CD31\(^+\)CD4\(^+\) T cells (CD31\(^+\)CD4\(^+\)CD45RA\(^+\)CD31\(^+\)) [17]. Since the samples were not available for all patients, only 18 patients’ samples were measured for CD31\(^+\) (7 in the INR group and 11 in the IR group; Figure 2A). CD31\(^+\) was significantly less in the INR group than in the IR group, and significantly reduced in both patient groups in comparison to healthy volunteers at all 6 time points (Figure 2A). To our surprise, the CD31\(^+\) among CD4\(^+\) T cells, no matter in the INR group or in the IR group, had little change throughout the 36-month study period. The kinetics of CD31\(^+\) of CD4\(^+\) T cells did not change in the majority of patients (\(n = 12\), 67\%, including 5 INRs and 7 IRs; Figure 2B) although CD31\(^+\) in some patients did increase (\(n = 4\), 22\%, including 1 INR and 3 IRs; Figure 2C) or fluctuate (\(n = 2\), 11\%, including 1 INR and 1 IR; Figure 2D) in the initial phase of the HAART and remained constant afterwards.

The Kinetics of T-Cell Apoptosis and Activation

CD4\(^+\) T-cell early apoptosis percentage in the INR group was significantly higher than in the IR group at baseline and the early

### Table 1. Basic Characteristics of Patients

<table>
<thead>
<tr>
<th></th>
<th>INR n = 13</th>
<th>IR n = 17</th>
<th>(P) value between groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>40.9 ± 6.4</td>
<td>37.6 ± 9.1</td>
<td>.270</td>
</tr>
<tr>
<td>Gender (male/female)</td>
<td>8:5</td>
<td>10:7</td>
<td>&gt;.999</td>
</tr>
<tr>
<td>Coinfection: HCV</td>
<td>46.2% n = 6</td>
<td>29.4% n = 5</td>
<td>.454</td>
</tr>
<tr>
<td>Coinfection: TB</td>
<td>15.4% n = 2</td>
<td>5.9% n = 1</td>
<td>.685</td>
</tr>
<tr>
<td>Zidovudine (AZT) usage</td>
<td>69.2% n = 9</td>
<td>64.7% n = 11</td>
<td>.999</td>
</tr>
<tr>
<td>AIDS-defining disease</td>
<td>61.5% n = 8</td>
<td>29.4% n = 5</td>
<td>.136</td>
</tr>
<tr>
<td>Baseline pVL (log10VL)</td>
<td>4.77 ± 0.74</td>
<td>4.76 ± 0.48</td>
<td>.961</td>
</tr>
<tr>
<td>Duration for pVL to decrease to &lt;50 copies/mL, months</td>
<td>5.1 ± 2.0</td>
<td>5.6 ± 3.2</td>
<td>.662</td>
</tr>
<tr>
<td>Baseline CD4(^+) T-cell count</td>
<td>29 ± 33</td>
<td>164 ± 78</td>
<td>&lt;.001</td>
</tr>
</tbody>
</table>

Abbreviations: INR, immunological nonresponders; IR, immunological responders; pVL, plasma viral load.  

\(a\) *P* value between groups: independent-samples t test (2-tailed).  

\(b\) *P* value between groups: Pearson \(\chi^2\) test (2-tailed).

\[\text{When } t=0, 1-\exp(-t/\tau)=0, \text{ so } N(t)=A\]

\[\text{When } t\to \infty, 1-\exp(-t/\tau)=1, \text{ so } N(t)=A+B\]

**Nonlinear Regression**

Nonlinear regression based on the mathematical equation 1 was performed manually using Excel 2007. For the 2 variables \(B\) and \(\tau\), we set \(B\) from 0 to 5000 with a step of 10; we set \(\tau\) from 0 to 10 000 with a step of 5. In each patient, for every combination of \(B\) and \(\tau\), the sum of the square of regression value minus actual value was calculated:

\[
\text{Sum value} = \sum_{i=1}^{n} \left( \text{regression value} - \text{actual value} \right)^2
\]

The \((B, \tau)\) set with smallest \(\text{Sum value}\) was defined as the final result.

**RESULTS**

**Basic Characteristics of Patients**

In total, 74 patients were recruited. Among 39 patients who had good viral response, 13 (33.3\%) were found to be immunological nonresponders (INR); 17 selected immunological responders (IRs) were matched the INRs with baseline characteristics (Table 1). There was no statistically significant difference observed between the 2 groups with regard to age, gender, coinfection with HCV or TB, Zidovudine (AZT) usage, baseline plasma HIV-1 viral load, the duration for plasma HIV-1 viral load to decrease to undetectable level, or duration to AIDS-defining diseases using the CDC-revised 1993 AIDS case definition [18]. However, we did observe significant difference between INR and IR groups in baseline CD4\(^+\) T-cell count (29 ± 33/\(\mu\)L and 164 ± 78/\(\mu\)L for INR and IR groups, respectively, \(P < .001\)).
phase of HAART. However, this apoptosis percentage decreased in both groups, and decreased faster in the INR group. By the end of the 36th month, it was similar in both groups, although still higher than in healthy volunteers (Figure 3A). In the initial phase of HAART (within 6 months), the activation percentage of CD8\(^+\) T cells expressing CD38 (CD8\(^+\)CD38\(^+\))/CD8\(^+\) in the INR group was similar as in the IR group (Figure 3B). Then CD38\(^+\)CD8\(^+\) T-cell percentage decreased along with the HIV-1 viral load decrease in both groups. The decrease was slower in the INR group. At the end of 36 month, it was significantly higher in the INR group than in the IR group (43.6 ± 17.9% vs 28.6 ± 9.6%, \(P = .011\)). For the percentage of CD8\(^+\) T cells expressing HLA-DR, there was no statistical difference observed between the kinetics of these 2 groups except at the 3-month time point (data not shown).

### The Pathophysiological Meaning of the Coefficients

From the definition of \(A\) and \(B\), it was very easy to understand that \(A + B\) determines whether a patient could reconstitute his or her CD4\(^+\) T-cell count (Figure 4A).

If we did infinitesimal calculus for equation 1, 
\[
\frac{dN(t)}{dt} = \frac{B}{\tau} \exp\left(-\frac{t}{\tau}\right)
\]
When \(t = 0\), 
\[
\frac{dN(t)}{dt} = \frac{B}{\tau}.
\]

Thus, \(\frac{B}{\tau}\) could be termed as initial restoration slope (Figure 4A), which described the initial therapeutic response. We defined \(\tau\) as “restoration index,” because the bigger \(\tau\) is, the longer period of time a patients needed to restore his/her CD4\(^+\) T-cell count (or reach \(A + B\)).

### Initial Restoration Slope Is Correlated With Early CD31%

For both INRs and IRs, their CD4\(^+\) T-cell count were fit into the mathematic model. Here we displayed 2 patients’ regression curves as examples (Figure 4B; patient 25 was an IR and patient...
11 was an INR). More importantly, we found the initial restoration slope ($\frac{C_0}{B}$) was highly correlated with the percentage of CD31% ($r = 0.843, P < .001; $Figure 4C). Since some patients had changing CD31% in the early phase of HAART, here we used the means of CD31% at baseline and CD31% at the 6-month time point.

Figure 2. A, Immunological nonresponders group had significantly less CD31% than in immunological responders group at all time points, and in both patient groups CD31% remained similar over time. Individual patients’ CD31% kinetics: patients with few changes (B), increased (C) or fluctuated (D) CD31% in the initiation phase of the highly active antiretroviral therapy treatment and it remained constant later on. An asterisk indicates significant difference.

948 • CID 2011:53 (1 November) • HIV/AIDS

Figure 3. A, The percentage of CD4+ T cells undergoing apoptosis (CD4+Annexin V+/CD4+). Immunological nonresponders (INR) group had significantly higher percentage of cells undergoing apoptosis in the initial phase of highly active antiretroviral therapy, while decreased to similar level as immunological responders (IR) group in later phase of the treatment. B, The percentage of activated CD8+ T cells expressing CD38 (CD8+CD38+/CD8+). Both groups had very high proportion of activated CD8+ T cells at the beginning and decreased overtime. However, it decreased slower in INR group and in the later phase of the study it was significantly higher than in IR group. An asterisk indicates significant difference.


Recent guidelines for treatment of HIV-1–infected patients suggest starting treatment early [19–21]. Although early HAART is preferred, for various reasons, late diagnosis and therapy is inevitable in real clinical practice [22]. This leads to the consequence that a high percentage of patients being INRs, especially in resource-limited countries like China where patients start HAART relatively late. In our study, the INR group started HAART with extremely low baseline CD4+ T cells (29 ± 33/μL) and 1/3 of patients became INRs, which is very rare in Western countries. Therefore, it is important to understand the mechanisms of immune reconstitution failure and search for effective treatments for INRs. In our study, the risk factors of INRs (age, gender, AZT usage, et al) were matched and the 3-year kinetics of various T-cell subsets was compared between INR and IR groups. Consistent with other findings, we also found that baseline CD4+ T-cell counts were significantly different between the 2 groups, as expected by the study design [6].

The 3-year kinetics of naive and memory CD4+ T-cell increase (Δn and Δm) showed that the main determinant causing the differential restoration of CD4+ T cells is a significantly less naive CD4+ T-cell increase (An) in the INR group than in the IR group, while Δm was similar between the 2 groups (Figure 1C and 1D). The results suggest that CD4+ T-cell reproduction is more important than peripheral redistribution and/or proliferation. Furthermore, the less naive CD4+ T-cell increase was due to less thymus production in the INR groups, as demonstrated by a smaller percentage of recent thymic immigrant CD4+ T cell (CD31%) in the INR group than in the IR group, and significantly less percentages in both patients groups than that in the healthy volunteer group (Figure 2A). It also highlighted the importance of a central immune function: thymus function. These data are similar to other reports obtained using the TREC measurement [5–7, 14].

However, we found a novel and surprising result that the recent immigrant CD4+ T-cell percentage (CD31%) did not increase over time in both IR and INR groups (Figure 2A). It is possible that CD31% represents the thymus function that related to different levels of thymic damage caused by HIV-1 virus infection, which might not be reversible. In our study, application of HAART alone could not reverse the existing thymus damage in the majority of patients. Additionally, we did observe limited recovery of CD31% in 1/3 of patients but only in the early phase (Figure 2C and 2D), which is consistent with other observations of thymus recovery [15].

With CD31% remaining constant over time, one could easily make an assumption that IRs had more recent thymic immigrants released from the thymus than did INRs in a certain period of time, and that CD31% might be correlated with the rate of CD4+ T-cell increase (Δn). Thus, we proposed an empirical equation: \( N(t) = A + B \left[ 1 - \exp \left( -t/\tau \right) \right] \). Pakker et al [2] proposed a 2-stage model of CD4+ T-cell reconstitution in 1998, which described the early redistribution and later regeneration of CD4+ T cells. Pakker’s model was straightforward, easy to

**DISCUSSION**

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understand, and has been widely accepted despite some limitations. The 2-stage model was mainly descriptive and needed 2 different determinants. Our model has only one equation, but it could cover 2 stages at the same time. Also, it is suitable for both INR or IR patients (Figure 4B), and only one determinant CD31% was used. Thus, our model is simpler and more intuitive. More importantly, we found the initial restoration slope ($\frac{d}{dt}$) is highly correlated with CD31% with $r = 0.843$, $P < .001$ (Figure 4C). This result was interesting because the initial rapid increase of CD4+ T-cell restoration should reflect the redistribution of memory CD4+ T cells in the periphery, while CD31%, a marker of thymic function, should reflect the later slow increase of CD4+ T cells [2]. One possible explanation is that central thymus organ and peripheral lymphoid tissue are not separate from each other, and there is a very precisely regulated feedback loop, similar to the thymus-bone marrow loop [23, 24]. Thus, CD31% could not only reflect the central thymus function but also reflect the amount of CD4+ T-cell retention in peripheral lymphoid tissue.

All of the above data, no matter clinical or mathematical, yielded that same conclusion—that thymus exhaustion is a major mechanism of immunological reconstitution failure.

We also compared immune activation and cell apoptosis. The kinetics data on CD8+ T-cell activation showed that CD38% (CD8+CD38+/CD8+) in the INR group was higher compared with the IR group and healthy volunteers, despite significant decrease observed from baseline (Figure 3B). It suggests that the immune activation might play a role in immunological reconstitution failure, although the mechanism of continuous activation of immune system in the INR group is unknown. There could be other stimulations, such as (1) low level of ongoing HIV-1 replication due to the insufficient suppression [25]; (2) reservoir virus rebound every now and then; and (3) those other than antigen stimulation, such as translocation of microbial from gut to systemic immune system [26], Helicobacter pylori [27], or other coinfections.

The higher rate of CD4+ T-cell apoptosis was believed to be another important reason for the poor CD4+ T-cell restoration. Nevertheless, the kinetics data we collected here showed that even though the higher percentage of apoptosis was true in the early phase of immune reconstitution (within 1 year), it was not a problem anymore when HAART treatment was administered for a long time (Figure 3A). These data were not surprising, because the percentage of apoptosis was a dynamic variable, it could be reversed when no viral replication exists. Our data are in disagreement with some data in literature [7, 10]. The reason for this might be that we relied data from a lengthy study, while others performed only short-term follow-up that might not allow discovery of this phenomenon.

Another importance of our findings is its implication on the guidance for therapeutic strategy for INRs. (1) Who to treat? Now, we could have one more factor other than baseline CD4+ T-cell count to predict who would become INR—CD31%. If a HIV-infected patient had low CD4+ T-cell count and low CD31%, he or she might need additional treatment other than HAART. (2) When to treat? We believe it should be started as early as possible. In our study, the thymus damage would not improve over time even under long-term HAART. Thus, there was no reason to wait for additional treatment. (3) How to treat? We do not know the answers yet. However, the key point of the treatment should be focused on enhancing the function of thymus and helping thymus to produce more T cells. IL-7 is required for T-cell development and acts in the upstream of thymic seeding of T-cell progenitors [28]. Growth hormone (GH) was also shown to boost thymus size and function, increase naive CD4+ T cells, and enhance thymopoiesis [29]. All these might be good directions for additional studies and might give hope to INRs.

Of course, the current study has some limitations. The case number was small; the amount of TREC may need to be analyzed; thymus size may need to be examined by CT scan; and the mathematical equation is empirical and requires further verification.

**Notes**

**Acknowledgments.** We thank Professor Chongqing Wu, Beijing Jiaotong University School of Science, for his generous help with the proposal of mathematical model and the help with the nonlinear regression. We also appreciated Stephen J. Gould, Johns Hopkins University, Xia Jin, University of Rochester and Mengyu Zhou, Peking Union Medical College, for their help in revising the manuscript.

**Financial support.** This work was supported by the National Natural Science Foundation of China [grant 81071372 to L. T.]; National Key Technologies R&D Program for the 11th Five-year Plan [grant 2008ZX10001-006], and Key Clinical Program of the Ministry of Health [2010–2012].

**Potential conflicts of interest.** All authors: No reported conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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