T cell turnover and thymic function in HIV-1 infection
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10 Depletion of naive CD4+ T cells by syncytium inducing, CXCR4 using HIV-1 variants occurs mainly through increased peripheral T cell death rather than thymic dysfunction

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Abstract

Using SCID-Hu mice models and *in vitro* culture systems, it has been shown that syncytium inducing / CXCR4 (X4) using HIV-1 variants affect thymic function through infection and killing of CXCR4+ thymocytes. In the present longitudinal study we are the first to investigate the effect of the emergence of X4 using HIV-1 variants on naive, memory and effector CD4+ and CD8+ T cells *in vivo* in humans. Patients with X4 variants (n=18) had significantly lower numbers of CD4+ T cells than patients with R5 variants (n=74), due to increased loss of naive and CD27+ memory CD4+ T cells. In addition, emergence of X4 variants was associated with a small but significant loss of naive CD8+ T cells that might be related to a thymus-specific effect. Thymic dysfunction could however explain only part of the accelerated naive CD4+ T cell decline. With time, the number of TREC's decreased, but TREC content of sorted naive and CD27+ memory CD4+ T cells remained stable despite increased T cell division that was associated with the emergence of X4 variants. Mathematical modeling showed that this triad can only be explained by increased peripheral CD4+ T cell death rates. Taken together, our data suggest that even if X4 variants cause thymic dysfunction, the effect of such an event on T cell dynamics is only minimal and that the accelerated CD4+ T cell decline induced by X4 variants is mainly caused by increased peripheral naive and CD27+ memory CD4+ T cell death rates.
Introduction

Whereas primary HIV-1 infection is established by non-syncytium inducing (NSI) / CCR5 (R5) using viruses, approximately half of the patients experiences emergence of syncytium inducing (SI) / CXCR4 (X4) using variants, which is associated with a more rapid decline in CD4+ T cells (1:2). Recently, our laboratory showed that X4 viruses are able to establish productive infection in peripheral blood naive CD4+ T cells (3:4). Whereas R5 viruses were predominantly isolated from patient CD4+ memory T cells, X4 variants were distributed over naive and memory CD4+ T cells (3). X4 variants were therefore suggested to exercise their harmful effects through enhanced infection and killing of peripheral blood naive T cells, thereby also affecting their progeny, the CD4+ memory T cells. In addition, given the tropism of X4 viruses for thymocytes(5-7), the deleterious effect of X4 variants could also be related to impairment of thymic function.

The extend to which X4 variants affect naive, memory and effector T cell numbers in vivo however is not known. To study the effects of X4 variants on T cell kinetics, we analyzed changes in naive, memory and effector CD4+ and CD8+ T cell numbers, division rates, and T cell receptor excision circles (TRECs) longitudinally in patients harboring R5 variants only, and in patients who experienced conversion to X4 variants. It was recently confirmed that Ki67 is a good surrogate marker for in vivo T cell proliferation (8:9). TRECs are episomal circles that are formed as excision products during the process of T cell receptor gene rearrangements in the thymus (10-12). The majority of recent thymic emigrants contains a Signal joint (Sj) TREC, the excision product that derives from T cell receptor α gene rearrangements. Changes in TREC content, TREC number, T cell division rates and T cell numbers were used to model the effect of X4 variants. Our results suggest that the accelerated CD4+ T cell decline that is associated with the emergence of X4 variants is mainly related to increased CD4+ T cell death rather than to impairment of thymic function.

Methods

Patients
We used cryopreserved peripheral blood mononuclear cells (PBMC) from ninety-nine HIV-1 infected participants of the Amsterdam cohort studies on HIV-1 infection and AIDS. Ninety-two patients were studied before HIV-seroconversion and at one and five years after seroconversion for the effect of emergence of the X4 phenotype on the loss of naive, memory and effector CD4+ and CD8+ T cells and changes in cell division. Patient selection was based on the availability of cryopreserved samples at the indicated timepoints. All patients initially got infected with R5 variants. Emergence of X4 variants occurred in 18 out of these 92 patients during the period under study. Mean age of patients who harbored R5
variants and patients who would develop X4 variants was 36 and 35 years, respectively, at the start of the study. In addition, we selected seven HIV-1 infected cohort participants of whom cryopreserved samples were available of two timepoints before emergence of X4 variants, and of two timepoints thereafter, for TREC analysis. None of the patients received HAART at any timepoint. Syncytium inducing X4 variants were detected by co-cultivation of patient peripheral blood mononuclear cells (PBMC) with the MT2 cell line (13). Cryopreservation was performed using a computerized freezing device that results in optimal quality of viably frozen cells for functional studies (14), and frozen PBMC were stored in liquid nitrogen.

**Flow cytometry**

CD4⁺ and CD8⁺ T cells were subdivided into naive (CD45RO⁺/CD27⁺), CD27⁺ memory (CD45RO⁺/CD27⁺), CD27⁻ memory (CD45RO⁺/CD27⁻) and CD27⁻ effector (CD45RO⁻/CD27⁻) CD4⁺ and CD8⁺ T cell subsets as described previously (15:16). Ki67 is a protein that is exclusively expressed by cells that are in cell cycle (17). For flow cytometric analysis of T cell subset distribution and cell division, frozen PBMC were thawed and incubated with CD4⁻ or CD8-PerCP mAb, CD45RO-PE (Becton Dickinson (BD), San Jose, CA), biotinylated CD27 mAb (CLB, Amsterdam, The Netherlands), and, after washing, with Streptavidin-APC mAb (BD). Lymphocytes were fixed (FACS Lysing Solution, BD), permeabilized (FACS Permeabilization Buffer, BD) and incubated with Ki67-FITC mAb (Immunotech, Marseille, France). Cells were fixed using Cellfix (BD), and analyzed on a FACSCalibur (BD) with Cellquest software.

**Purification of naive and memory CD27⁺ CD4⁺ T cells**

Cryopreserved patient PBMC were thawed and incubated with CD4-TC mAb (Caltag, Burlington, CA), CD45RA-PE mAb (Caltag) and CD27-FITC mAb (CLB). CD45RA⁺ CD27⁺ naive CD4⁺ T cells and CD45RA⁻ CD27⁻ memory CD4⁺ T cells (15) were isolated through FACS-sorting on a FACStar (BD). With this technique, at least 94 % purity was reached. DNA was isolated from these naive and CD27⁺ memory CD4⁺ T cell fractions using the QIAamp Blood Kit according to manufacturer's instructions (Qiagen, Hilden, Germany).

**TREC measurements**

In order to detect Signal joint (Sj) TRECs, a real-time quantitative PCR method was used as described previously (18). The Cα constant region that remains present on TCR genes despite rearrangement processes was used as an internal control measurement, to normalize for input DNA, and the number of Sj TREC copies present in a given cell population was calculated by including a dilution series of a Sj standard (18) in each PCR experiment. For each sample the Ct-value, defined as the minimal number of cycles necessary to exceed threshold values, was measured and applied to the standardization curve created from the dilution series. From this average TREC content as measured per µgram DNA.
TREC content per naive or CD27+ memory T cell was calculated by dividing TREC content by 150,000 (assuming that 1 μgram DNA corresponds with 150,000 T cells). The numbers of TREC copies isolated from naive and CD27+ memory CD4+ T cells were calculated by multiplying the naive and CD27+ memory CD4+ TREC content by the number of naive and CD27+ memory CD4+ T cells, respectively.

Model analysis of TREC kinetics
We have previously developed a mathematical model that can be used in the interpretation of TREC data (18). In this model, changes in the number of TRECs can be described as dT/dt = cσ - δT - δT, and changes in the number of naive T cells as dN/dt = σ + αN - δN. T reflects the total number of TRECs in the naive T cell population, c the number of TRECs in recent thymic emigrants (RTE), σ the age dependent thymic output of naive T cells, δ the naive T cell death rate, δ the intracellular degradation of TRECs, N the number of naive T cells, and α the rate of naive T cell division. The average TREC content can be described as A = T/N. In quasi steady state, T = cσ / (δ + δ), and A = c / [1 + N(δ + α) / σ]. We assumed c and δ not to be affected by HIV-1 infection, and kept these parameters constant.

Statistical analysis
Differences in viral load, T cell numbers, TREC content and number of TREC copies between patients with R5 variants and patients with X4 variants were compared using non-parametric Mann Whitney U Test or independent samples T Test, based on Shapiro-Wilk W test for normality. Dependent samples were analyzed using the Wilcoxon Signed Ranks Test, and correlations were estimated by calculating Spearman’s correlation coefficients. P values lower than 0.05 were considered statistically significant.

Results

Effect of X4 variants on peripheral blood T cell numbers
Ninety-two participants of the Amsterdam Cohort Studies on HIV-1 infection and AIDS were analyzed for the effect of X4 variants on peripheral blood T cell numbers and T cell division. During the period under study (up to five years after HIV-1 seroconversion), 18 of these individuals experienced emergence of X4 variants and 74 did not. Before HIV-1 seroconversion, CD4+ and CD8+ T cell numbers were comparable between both groups (Figure 1a). One year after seroconversion, five patients had already experienced emergence of the X4 phenotype. At this timepoint, CD4+ and CD8+ T cell numbers in these patients were lower than in patients with R5 variants and in patients that would experience emergence of the X4 phenotype during the period under study, but differences were not significant (mean values (±SD) 390 (±239), 644 (±279) and 588 (±266) cells per μl, respectively; Figure 1a). Five years after seroconversion, eighteen
Figure 1. Emergence of X4 variants was associated with a decline in naive and CD27+ memory CD4+ and CD8+ T cell numbers. (a) Mean (±SD) CD4+ and CD8+ T cell numbers before (Pre) and at 1 and 5 years after HIV-1 seroconversion for patients with R5 variants (black bars), patients who during the period under study would but have not yet experienced emergence of X4 variants (white bars), and patients with X4 variants (gray bars), at the indicated timepoints. (b) The number of naive, CD27+ memory, CD27- memory and effector CD4+ and CD8+ T cells as measured five years after seroconversion. Symbols: N: naive; CD27+: M: CD27+ memory; CD27-: M: CD27- memory; E: CD27+ effector; *: significant difference between patient groups (p < 0.05).

Figure 2. X4 related accelerated naive T cell decline was most pronounced in the CD4+ T cell pool. (a) Five years after seroconversion, the number of naive CD4+ T cells was significantly lower than the number of naive CD8+ T cells in patients with X4 variants, whereas naive CD4+ and naive CD8+ T cell numbers in patients with R5 variants were not different. (b) The decline in number of CD4+ CD27+ memory T cells was most pronounced in individuals harboring X4 variants. Patients with R5 variants showed an expansion of the CD8+ CD27+ memory T cell pool that was absent in patients harboring the X4 phenotype. Depicted are mean (±SD) naive (a) and CD27+ memory (b) CD4+ (circles) and CD8+ (squares) T cell numbers of patients with R5 variants (black symbols), patients with X4 variants (grey symbols) and individuals that would but have not yet converted to the X4 phenotype (white symbols). Asterix indicates number of CD4+ T cells that is significantly lower than the number of CD8+ T cells of the same patient group at that timepoint (p<0.005).

patients had experienced X4 emergence, fourteen of whom were still alive at this timepoint. These patients had significantly lower numbers of CD4+ T cells compared with patients who harbored R5 variants only (mean values (±SD) 199 (±146) and 458 (±194) cells per μl, p<0.001; Figure 1a), confirming previous reports (1:2). Increased CD4+ T cell loss in these patients was associated with a
Increased T cell death after X4 HIV-1 emergence

significant reduction of naive and CD27+ memory CD4+ T cell numbers (mean values 166 (±126) and 39 (±49) cells per μl, p<0.001, for naive CD4+ T cells, and 188 (±71) and 67 (±51) cells per μl, p<0.001, for CD27+ memory CD4+ T cells: Figure 1b, left panel). No significant differences in total CD8+ T cell numbers were observed at this timepoint (Figure 1a), however, patients with X4 variants had significantly lower numbers of naive and CD27+ memory CD8+ T cells compared with patients harboring R5 variants (mean values 165 (±83) and 112 (±63) cells per μl, p<0.05, for naive CD8+ T cells; 364 (±250) and 244 (±166) cells per μl, p<0.05, for CD27+ memory CD8+ T cells: Figure 1b, right panel). Thus, patients with X4 variants showed an accelerated decline in naive T cell numbers, that was most pronounced for the naive CD4+ T cells. Whereas patients with R5 variants had equal numbers of naive CD4+ T cells and of naive CD8+ T cells 5 years after seroconversion, individuals who experienced emergence of X4 variants had significantly lower numbers of naive CD4+ T cells than of naive CD8+ T cells at this timepoint (p<0.005: Figure 2a).

X4 emergence was also associated with significantly lower numbers of CD27+ memory CD4+ and CD8+ T cells (Figure 1b). Whereas the difference in number of CD4+ CD27+ memory T cells was caused by a more rapid decline of these cells in patients with X4 variants, the difference in CD8+ CD27+ memory T cell numbers was mainly related to an expansion of this subset in patients with R5 variants that was absent in patients harboring the X4 phenotype (Figure 2b).

Effect of X4 variants on cell division and plasma HIV-1 RNA

HIV-1 infection is associated with a several fold increase in CD4+ and CD8+ T cell division rates (8:9:19:20). In the first year following seroconversion, Ki67 expression increased to the same extend in patients with R5 variants and in patients who at this timepoint harbored R5 variants only but would later convert to the X4 phenotype (Figure 3). The five individuals that had already experienced X4 conversion at this timepoint had significantly increased proportions of Ki67+ CD4+ T cells compared with patients with R5 variants and patients who would later convert to the X4 phenotype, but not of Ki67+ CD8+ T cells (Figure 3). Five years after seroconversion, the proportion of dividing CD4+ and CD8+ T cells was significantly higher in patients with X4 variants (mean values 13.1 (±7.2) and 8.4 (±5.0) %, p<0.05, for Ki67+ CD4+ T cells, and 9.2 (±4.4) and 6.3 (±3.3) %, p<0.05, for Ki67+ CD8+ T cells: Figure 3). At all timepoints, increased cell division was observed in naive, CD27+ memory, CD27+ memory and effector CD4+ and CD8+ T cells (p<0.05; data not shown), and the proportion of Ki67+ CD4+ T cells correlated significantly with the proportion of Ki67+ CD8+ T cells (p<0.001; data not shown).

Plasma HIV-1 RNA was higher in patients who had experienced X4 emergence, but the difference with patients who harbored R5 variants only was not significant (p>0.05). Neither group showed a significant increase in plasma HIV-1 RNA with time (Figure 3c).
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Figure 3. Longitudinal analysis of mean (±SD) Ki67 expression by CD4+ (a) and CD8+ (b) T cells, and HIV plasma RNA (c). Black bars: patients with R5 variants; white bars: patients with R5 variants that would develop X4 variants; gray bars: patients harboring the X4 phenotype; *: significant difference between patient groups (p < 0.05).

X4 conversion and TREC kinetics
To assess the effect of X4 variants on TREC kinetics over time, we measured TREC content and the number of TREC s isolated from sorted naive and CD27ṃ memory CD4+ T cells in parallel with CD4+ T cell numbers and cell division in a subset of patients at timepoints before and after the emergence of X4 variants. Characteristics of these patients are depicted in Table 1: none of these patients developed AIDS during the period under study.

TREC content (the average number of TREC copies per T cell) of sorted naive and of sorted CD27ṃ memory CD4+ T cells was variable but overall remained stable (Figure 4a). This was surprising because Ki67 expression by CD4+ and CD8+ T cells increased following X4 conversion (Figure 4b and Figure 3) which is expected to decrease TREC content through dilution of TREC s (18). The number

Table 1. Characteristics of patients selected for TREC analysis

<table>
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<tr>
<th>Patient ID</th>
<th>Age1 (years)</th>
<th>Follow-up2 (years)</th>
<th>CD4+ T cells Start3 (per μl)</th>
<th>CD4+ T cells End3 (per μl)</th>
<th>HIV-1 RNA Before4 (copies/ml)</th>
<th>HIV-1 RNA After5 (copies/ml)</th>
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<td>na</td>
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<tr>
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</tr>
<tr>
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<td>51</td>
<td>8.8</td>
<td>1350</td>
<td>170</td>
<td>13,000</td>
<td>95,000</td>
</tr>
</tbody>
</table>

1At the start and 2at the end of the period under study; 3Follow-up from HIV-1 seroconversion; plasma HIV-1 RNA measured 4before and 5after emergence of X4 variants.
of TREC copies in the naive CD4$^+$ T cell pool decreased over time and with the emergence of X4 variants in all patients, which is not surprising as it reflects the decline in naive CD4$^+$ T cell numbers (Figure 4c, left panel). Changes in the number of TREC$s in the CD27$^+$ memory CD4$^+$ T cell pool showed more variability, but overall also declined (Figure 4c, right panel).

**Analysis of TREC data**

In an attempt to account for all factors that may influence TREC kinetics in the interpretation of our data, and to analyze the effects of X4 emergence on TREC number and TREC content over a prolonged period of time, we applied our previously developed mathematical model (18) and studied it numerically. The emergence of X4 variants could lead to a decrease in thymic function, and / or an increase in T cell death rates, and / or an increase in T cell division rates. In Figure 5a, the effect of abolishment of thymic output (i.e. setting $\sigma = 0$ in our model) on the number of TREC$s and on TREC content of the total body naive T cell pool is presented in panel 1. Over a period of six years, TREC content and the number of TREC$s decreases gradually in parallel with a decline in naive T cell numbers. These relatively slow kinetics can be attributed to the longevity of naive
Conversely, a ten-fold increase in the death rate ($\delta$) of naive T cells leads to a significant, rapid decline in the number of TRECs and in the number of T cells, and to a rapid rise in TREC content, reaching a new steady state after approximately two years (panel 2). This increase in TREC content reflects rejuvenation of the naive T cell pool that results from continuous elevation of T cell death rates. A 10-fold rise in naive T cell division rates ($\alpha$) did not affect the number of TRECs, but induced a rapid decline in TREC content (panel 3) (18). Finally, we simultaneously increased naive T cell division and death rates 5-fold (panel 4). TREC content showed a biphasic pattern, such that the decline of the first two years was followed by a rise in TREC content. The number of TRECs initially also declined, but reached a plateau after two years (panel 4).

To allow comparison of this model analysis with our experimental data, total body numbers of TREC$^+$ naive T cells were calculated from the experimental data by multiplying the number of TREC$^+$ naive T cells per µl blood with $5 \times 10^8$, assuming that adult total blood volume is 5 liters and that in case of HIV-1 infection, 1% of the T cells resides in the blood (22;23). The number of TRECs over time was

![Figure 5](image-url)

**Figure 5.** Numerical mathematical analysis of TREC dynamics in HIV-1 infection. (a) In panel (1), thymic output of TREC$^+$ naive T cells is completely abrogated, that is, $\sigma = 0$. In panel (2), naive T cell death rate ($\delta$) is 10-fold increased and in panel (3) naive T cell division rate ($\alpha$) is 10-fold increased. Panel (4) shows the effect of simultaneously increasing naive T cell death ($\delta$) and naive T cell division ($\alpha$) 5-fold. Solid lines: total body number of naive T cells; dashed lines: naive T cell TREC content; dashed-dotted lines: the number of TRECs in the naive T cell pool. Parameters (11): $\alpha = 7.9 \times 10^{-4}$/day, $c = 1$, $\sigma = 5 \times 10^7$ cells / day, $\delta = 0$/day and $\delta = 10^{-3}$/day. (b) Individual patterns of total body naive T cell TREC numbers. Calculation of TREC numbers is described in the text. Symbols represent patients as depicted in Figure 2.
highly variable between patients and between individual timepoints, such that relatively stable periods alternated with periods of rapid decline (Figure 5b).

Discussion

In HIV-1 infection, the emergence of X4 HIV-1 variants is associated with a more rapid decline in CD4+ T cell numbers (1:2). Because immature progenitor T cells in the thymus and mature naive T cells in the blood express the CXCR4 coreceptor (5:6:24:25), the deleterious effect of X4 variants may be related to cytopathic effects directed at mature naive CD4+ T cells in the periphery, or at CD4+ and CD8+ T cell precursors in the thymus, or both. It has been shown in vitro and in the SCID-hu Thy/Liv mouse model that X4 and R5 variants affect different thymocyte subsets, related to distinct coreceptor expression by maturing thymocytes (6:7:25:26). Similarly, in vitro infection of mature peripheral blood T cells with R5 and X4 variants resulted in depletion of naive and memory CD4+ T cell subsets that corresponded with CXCR4 and CCR5 coreceptor expression (4:27). In vivo, naive CD4+ T cells were found to be exclusively infected by X4 variants whereas R5 and X4 variants could be isolated from memory CD4+ T cells which again corresponded with coreceptor expression by these subsets (3). Although these and other studies suggest distinct effects of R5 and X4 variants on T cell subsets, no information on the in vivo effect of the emergence of X4 variants on naive and memory T cell numbers is available to date.

Here, we show in a cohort study that the more rapid CD4+ T cell decline in patients harboring X4 variants was associated with a significant loss of naive and CD27+ memory CD4+ T cells. In addition, X4 emergence was associated with significantly lower numbers of naive and CD27+ memory CD8+ T cells. As CD27 is irreversibly downregulated upon stimulation (28), we consider CD4+ and CD8+ T cells that are CD27-positive and CD45RO-negative as non-reversed truly naive T cells that are derived directly from the thymus. The accelerated loss of T cells following X4 emergence involved naive CD8+ T cells and naive CD4+ T cells, suggesting that thymic output of naive T cells may be affected by X4 variants. However, naive CD4+ T cell decline was much more pronounced than naive CD8+ T cell decline. As CXCR4 is expressed by triple-negative, immature single-positive and triple-positive thymocytes (11), we assume that X4 variants would affect maturing intrathymic CXCR4+ progenitors of CD4+ and CD8+ T cells equally. The more rapid decline in naive CD4+ T cell numbers therefore demonstrated that additional factors play a role, such as direct infection and killing of CXCR4-positive naive CD4+ T cells (3:4).

The number of CD27+ memory CD4+ and CD8+ T cells was also significantly lower in patients with X4 variants. The decline in CD4+ CD27+ memory T cells may be related to the increased number of memory CD4+ T cells that can become infected with the enlargement of the target cell population after X4 emergence (3:4). In
addition, depletion of naive CD4+ and naive CD8+ T cells may also be important, as these cells now are unable to generate their progeny. CD27+ memory T cells (3).

To study the effect of X4 variants on T cell turnover in more detail, we measured TREC content and the absolute number of TREC's longitudinally in seven untreated HIV-1 infected individuals that were initially infected with R5 variants, but over time experienced X4 conversion. Mathematical modeling showed that abrogation of thymic output induced a relatively slow decline in TREC content of naive T cells, which can be attributed to the longevity of naive T cells. Similarly, an increase of T cell death rates was associated with an increase in TREC content followed by a plateau phase, and an increase of T cell division rates was associated with a decrease in TREC content. Despite declining naive CD4+ T cell numbers and increasing peripheral T cell division, we did not observe significant changes in naive CD4+ T cell TREC content after emergence of the X4 phenotype. This could be explained by increased rather than decreased thymic output, however, this would lead to elevated instead of declining numbers of TREC's and naive T cells. Thus, another factor has to be involved. To explain the stable TREC content per naive T cell in the presence of increased T cell proliferation (Figure 4), the effect of increased death (Figure 5) is required.

The number of TREC's did decline with time. Similar to the number of naive T cells, the number of TREC's depends on thymic output and death of TREC+ T cells (18). Numerical simulations with our model showed that complete abrogation of thymic output in the context of constant naive T cell death rates would lead to a relatively slow decline in TREC number, compared with the rapid decline in number of TREC's that was found to be associated with increasing T cell death rates (Figure 5). Thus, our model shows that the rapid effect of increased T cell death rates on TREC number would mask thymic impairment, if any.

In conclusion, emergence of X4 variants affected predominantly naive and CD27+ memory CD4+ T cell subsets. Our TREC data imply that an important aspect of X4 variants may be their capacity to infect and kill peripheral blood naive CD4+ T cells that, as a consequence, can then no longer generate progeny and are difficult to be replaced (3:4:29).

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