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The authors unfortunately deleted a reference during preparation of the manuscript that originally described the activation of resting CD4+ T cells with cytokines in a non-HIV setting. The omitted reference is as follows.

Induction of HIV-1 Replication in Latently Infected CD4+ T Cells Using a Combination of Cytokines

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Summary

Although it has been demonstrated that certain cytokines, particularly proinflammatory cytokines, can enhance ongoing viral replication in peripheral blood mononuclear cells (PBMCs) of HIV-1–infected individuals, it is unclear what role these cytokines play in the induction of HIV-1 replication in latently infected, resting CD4+ T cells. This study demonstrates that the in vitro combination of the proinflammatory cytokines interleukin (IL)-6 and tumor necrosis factor (TNF)-α together with the immunoregulatory cytokine IL-2 are potent inducers of viral replication in highly purified, latently infected, resting CD4+ T cells derived from HIV-infected individuals who are antiretroviral therapy–naive as well as those who are receiving highly active antiretroviral therapy (HAART). Viral replication induced by this combination of cytokines was completely suppressed in the presence of HAART in vitro. Given that an array of cytokines, including IL-6, TNF-α, and IL-2, are copiously expressed in the microenvironment of the lymphoid tissues, which harbor the latent viral reservoirs, induction of HIV by this combination of cytokines may in part explain the commonly observed reappearance of detectable plasma viremia in HIV-infected individuals in whom HAART was discontinued. Moreover, since it is likely that these infected cells die upon activation of virus and that HAART prevents spread of virus to adjacent cells, the observation that this combination of cytokines can markedly induce viral replication in this reservoir may have important implications for the activation-mediated diminution of the latent reservoir of HIV in patients receiving HAART.

Key words: HIV-1 • latency • resting CD4+ T cells • cytokines • antiretroviral therapy

Cytokines are important effector molecules in the initiation and propagation of inflammatory and immune responses; in addition, certain cytokines mediate a number of important immunoregulatory functions (1). The role of cytokines in the pathogenesis of HIV disease has been extensively studied (2–5). It has been demonstrated by in vitro experiments that HIV replication in PBMCs of HIV-infected individuals is finely regulated by a variety of endogenous cytokines acting in an autocrine and paracrine manner (5, 6). Cytokine-rich supernatants derived from cultures of PBMCs from normal individuals were first shown to induce the expression of HIV-1 in chronically infected cell lines (7, 8). Subsequently, a variety of individual cytokines, particularly proinflammatory cytokines, were demonstrated to induce HIV expression either endogenously or when added to acutely or chronically infected cell cultures (3, 4, 9). Of these, IL-1, IL-6, and TNF-α have been implicated in the pathogenesis of HIV-1 as direct modulators of virus expression (3, 10–13) and higher levels of these cytokines have been detected in the sera of infected individuals; furthermore, secretion of these cytokines is increased in PBMCs of infected individuals (14–18). For these reasons, selective cytokine inhibitors such as IL-1 receptor antagonist or agents that neutralize TNF-α have been suggested as potential therapeutic strategies aimed at the control of viral replication (13, 19–23).

Recently, a small but detectable reservoir of latently infected, resting CD4+ T cells has been shown to persist in patients receiving highly active antiretroviral therapy (HAART)1 and in whom plasma viremia had fallen below the level of detectability of commonly used assays (24–26). The presence of this latent reservoir of HIV is of considerable concern since these cells remain as a potential source of reactivation of viral replication. In this regard, these cells reside predominantly in the microenvironment of lymphoid tissue (27), where endogenous cytokine secre-
tion regularly occurs in response to normal antigenic stimuli (2, 28–32).

In this study, we demonstrate that the in vitro combination of cytokines IL-2, IL-6, and TNF-α has a profound effect on reactivation of HIV-1 replication in latently infected, resting CD4⁺ T cells both from antiretroviral naïve patients and from patients who were receiving HAART and in whom plasma viremia was markedly suppressed, including some patients in whom plasma viremia was below detectable levels. In vitro HAART prevents the spread of virus in cytokine-induced cultures. These findings may explain the reappearance of plasma viremia in patients in whom HAART is discontinued due to drug toxicity or lack of compliance (33). In addition, since cytokines alone can reactivate HIV-1 replication in latently infected, resting CD4⁺ T cells, and since these cells probably die upon reactivation, it is conceivable that a strategy of administration of cytokines together with HAART might result in a diminution of this reservoir of latently infected cells.

Materials and Methods

Patient Population. Six HIV-1-seropositive patients who were naive to antiretroviral therapy, and seven patients who were receiving HAART (Table 1) were subjected to apheresis in order to obtain PBMCs according to protocols approved by the National Institute of Allergy and Infectious Diseases and the University of Washington Institutional Review Boards.

Isolation of Resting CD4⁺ T Cells. Resting CD4⁺ T cells were isolated from peripheral blood of HIV-1–infected individuals using a combination of magnetic bead depletion and fluorescent activated cell sorting techniques as described previously (27).

Micrococulture Assay. To determine the frequency of latently infected, resting CD4⁺ T cells from patients carrying replication competent HIV-1, micro coculture assays were carried out as described previously (27)

Cytokines and Cell Cultures. After isolation of resting CD4⁺ T cells from HIV-1–infected individuals, cells (2.0–10⁶) were incubated with complete medium consisting of RPMI supplemented with 10% FCS, penicillin–streptomycin, and l-glutamine in a tissue culture plate with the following cytokines: IL-2 (100 U/ml; Boehringer Mannheim, Indianapolis, IN), IL-1β (5 ng/ml; R & D Systems, Minneapolis, MN), IL-4 (3 ng/ml; R & D Systems), IL-6 (5 ng/ml; R & D Systems), TNF-α (2.5 ng/ml; R & D Systems), or with the combination of IL-2, IL-6, and TNF-α.

Table 1. Profiles of HIV-1–infected Patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>CD4 count cells/μl</th>
<th>Plasma HIV RNA* copies/ml</th>
<th>Antiretroviral treatment</th>
<th>Months receiving HAART †</th>
<th>Infectious viral load ‡ infectious U/10⁶ cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>520</td>
<td>14,394</td>
<td>N/A</td>
<td>N/A</td>
<td>81.7</td>
</tr>
<tr>
<td>2</td>
<td>540</td>
<td>47,704</td>
<td>N/A</td>
<td>N/A</td>
<td>81.7</td>
</tr>
<tr>
<td>3</td>
<td>490</td>
<td>10,414</td>
<td>N/A</td>
<td>N/A</td>
<td>8.1</td>
</tr>
<tr>
<td>4</td>
<td>324</td>
<td>29,216</td>
<td>N/A</td>
<td>N/A</td>
<td>205.8</td>
</tr>
<tr>
<td>5</td>
<td>399</td>
<td>9,032</td>
<td>N/A</td>
<td>N/A</td>
<td>8.1</td>
</tr>
<tr>
<td>6</td>
<td>602</td>
<td>12,157</td>
<td>N/A</td>
<td>N/A</td>
<td>40.5</td>
</tr>
<tr>
<td>7</td>
<td>638</td>
<td>1,330</td>
<td>AZT, 3TC, Ritonavir</td>
<td>14.0</td>
<td>5.7</td>
</tr>
<tr>
<td>8</td>
<td>367</td>
<td>2,520</td>
<td>AZT, 3TC, Indinavir</td>
<td>15.0</td>
<td>40.1</td>
</tr>
<tr>
<td>9</td>
<td>1,176</td>
<td>1,034</td>
<td>AZT, 3TC, Indinavir</td>
<td>1.0</td>
<td>3.2</td>
</tr>
<tr>
<td>10</td>
<td>648</td>
<td>&lt;50</td>
<td>AZT, 3TC, Indinavir</td>
<td>2.5</td>
<td>0.5</td>
</tr>
<tr>
<td>11</td>
<td>790</td>
<td>343</td>
<td>AZT, 3TC, Indinavir</td>
<td>2.8</td>
<td>1.6</td>
</tr>
<tr>
<td>12</td>
<td>353</td>
<td>&lt;50</td>
<td>AZT, 3TC, Indinavir</td>
<td>18.0</td>
<td>0.5</td>
</tr>
<tr>
<td>13</td>
<td>689</td>
<td>&lt;50</td>
<td>ddI, 3TC, Indinavir</td>
<td>16.0</td>
<td>0.5</td>
</tr>
</tbody>
</table>

NA, not applicable; AZT, zidovudine; 3TC, lamivudine; ddI, didanosine.

*Plasma HIV RNA was measured using the ultrasensitive reverse transcriptase PCR assay (Roche Labs.) with a detection limit of 50 copies/ml.

† Indicates the number of months after the start date of protease inhibitor.

‡ Determined by limiting dilution micro coculture assay by a maximum likelihood method (47).
GA), and the protease inhibitor indinavir (10 μM; Merck, Rahway, NJ) were added to the media.

Flow Cytometric Analysis and Cell Proliferation Assays. To measure cytokine-induced cellular activation in latently infected, resting CD4⁺ T cells, flow cytometric analysis of the expression of activation markers and thymidine incorporation assays were carried out. Resting CD4⁺ T cells that had been incubated with various cytokines were stained with PE-conjugated anti-CD25 (Becton Dickinson, San Jose, CA) and PE-conjugated anti–HLA-DR antibodies (Becton Dickinson) along with FITC-conjugated anti-CD4 antibody (Becton Dickinson) 6 d after incubation. On day 6, [³H]thymidine incorporation was measured in resting CD4⁺ T cells that had been incubated with the above cytokines or with anti-CD3 antibody.

Results

Cytokine-induced Cellular Activation in Latently Infected, Resting CD4⁺ T Cells. The replication of HIV-1 is intimately associated with the state of cellular activation of susceptible cell populations (2, 28). In this regard, we first investigated the role of individual cytokines or combinations of cytokines on the activation of highly enriched, resting CD4⁺ T cells from HIV-1-seropositive patients by examining the expression of cell surface activation markers CD25 and HLA-DR. As shown in Table 2, in the absence of cytokines or any other activating stimuli, purified resting CD4⁺ T cells from infected individuals expressed no measurable CD25 or HLA-DR on their cell surfaces. After a 6-d incubation in vitro, only IL-2 and IL-4 induced slight increases in activation markers on resting CD4⁺ T cells. However, the combination of IL-2, IL-6, and TNF-α showed marked increases in CD25 and HLA-DR expression. The induction of cellular activation in purified resting CD4⁺ T cells by the combination of three cytokines was further confirmed by the cellular incorporation of DNA precursor thymidine (Fig. 1). With the exception of IL-2, which induced a slight degree of cellular proliferation, no individual cytokine resulted in active proliferation of resting CD4⁺ T cells. Cells stimulated with anti-CD3 antibody showed profound cellular activation as judged by both the expression of activation markers and [³H]thymidine incorporation, confirming that purified resting CD4⁺ T cells are fully capable of undergoing cellular proliferation.

Induction of HIV-1 Replication by the Combination of IL-2, IL-6, and TNF-α in Latently Infected, Resting CD4⁺ T Cells from Antiretroviral Drug-naïve Patients. Given the fact that the combination of IL-2, IL-6, and TNF-α potently activates resting CD4⁺ T cells as described above, we first examined the inducibility of replication-competent HIV-1 in latently infected, resting CD4⁺ T cells from antiretroviral

Table 2. Cytokine-mediated Cellular Activation in Latently Infected, Resting CD4⁺ T Cells

<table>
<thead>
<tr>
<th>Cytokine added</th>
<th>% CD4⁺/HLA-DR⁺</th>
<th>% CD4⁺/CD25⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0</td>
<td>No cytokine</td>
<td>0.7</td>
</tr>
<tr>
<td>Day 6</td>
<td>No cytokine</td>
<td>1.0</td>
</tr>
<tr>
<td>IL-1β</td>
<td>0.8</td>
<td>0.9</td>
</tr>
<tr>
<td>IL-2</td>
<td>5.7</td>
<td>3.3</td>
</tr>
<tr>
<td>IL-4</td>
<td>3.2</td>
<td>5.0</td>
</tr>
<tr>
<td>IL-6</td>
<td>1.5</td>
<td>1.0</td>
</tr>
<tr>
<td>TNF-α</td>
<td>0.5</td>
<td>0.4</td>
</tr>
<tr>
<td>IL-2/IL-6/TNF-α</td>
<td>23.7</td>
<td>8.3</td>
</tr>
<tr>
<td>anti-CD3-stimulated</td>
<td>50.6</td>
<td>92.2</td>
</tr>
</tbody>
</table>

Purified, resting CD4⁺ T cells from one HIV-1-seronegative and two HIV-1-infected individuals were incubated with the indicated single cytokine, combination of cytokines, or anti-CD3 antibody for 6 d and the expression of CD25 and HLA-DR was determined by FACS. Percentage of CD4⁺/CD25⁻ and CD4⁺/HLA-DR⁻ positive cells are indicated. A representative set of results from patient no. 2 is shown.

Figure 1. Cytokine-induced cellular proliferation in latently infected, resting CD4⁺ T cells. Highly enriched (>99%) resting CD4⁺ T cells from one HIV-1-seronegative and two HIV-1-infected individuals were incubated with the indicated individual cytokines, a combination of cytokines, or anti-CD3 antibody for 6 d, and [³H]thymidine incorporation by 10⁴ resting CD4⁺ T cells was measured. A representative set of results from patient no. 2 is shown.
Figure 2. Induction of HIV-1 replication by the combination of IL-2, IL-6, and TNF-α in latently infected, resting CD4+ T cells from antiretroviral drug-naive patients. Resting CD4+ T cells from infected patients were isolated and further incubated with no cytokine, individual cytokines as indicated, the combination of IL-2, IL-6, and TNF-α, or anti-CD3 antibody with irradiated PBMCs from HIV-1-seronegative individuals. In addition, cells were incubated with the combination of IL-2, IL-6, and TNF-α or anti-CD3 antibody in the presence of three drugs (AZT, 3TC, and Indinavir; see Materials and Methods). Supernatants from each culture were removed on days 3, 6, and 9, and HIV-1 p24 was measured by ELISA.

Figure 3. Induction of HIV-1 replication by the combination of IL-2, IL-6, and TNF-α in latently infected, resting CD4+ T cells from patients receiving HAART. Resting CD4+ T cells from infected patients were isolated and further incubated with no cytokine, individual cytokines as indicated, the combination of IL-2, IL-6, and TNF-α, or anti-CD3 antibody with irradiated PBMCs from HIV-1-seronegative individuals. In addition, cells were incubated with the combination of IL-2, IL-6, and TNF-α or anti-CD3 antibody in the presence of three drugs (AZT, 3TC, and Indinavir; see Materials and Methods). Supernatants from each culture were removed on days 3, 6, and 9, and HIV-1 p24 was measured by ELISA.
Patient 7

Patient 9

Patient 11

Patient 13

Patient 8

Patient 10

Patient 12

No Cytokine
IL-2
IL-18
IL-4
IL-6
TNF-α
IL-2/IL-6/TNF-α
Anti-CD3 stimulated
IL-2/IL-6/TNF-α in vitro HAART
Anti-CD3 stimulated in vitro HAART
Anti-CD3 stimulated

p24 (ng/ml)

Day 3
Day 6
Day 9
therapy–naive, HIV–1–seropositive individuals using individual cytokines and combinations of cytokines in vitro. Despite the fact that highly purified, resting CD4+ T cells from all patients carried replication-competent HIV-1 with a frequency determined by micro coculture assay ranging from 8.1 to 205.8 infectious units per 10^6 resting CD4+ T cells (Table 1), measurable p24 was not detected in cultures containing neither cytokines nor anti-CD3 antibody throughout the entire incubation period (Fig. 2). Among the individual cytokines, only IL-2 had a slight effect on the induction of HIV-1 expression in latently infected, resting CD4+ T cells (Fig. 2). However, cultures incubated with the combination of IL-2, IL-6, and TNF-α had a dramatic increase in HIV-1 replication. The level of viral production induced by this cytokine combination was equivalent to that of cells stimulated with anti-CD3 antibody. When a combination of three antiretroviral drugs was added to the cultures incubated with the three cytokine combination or with anti-CD3 antibody, viral replication was completely suppressed as shown in Fig. 2.

Induction of HIV-1 Replication by the Combination of IL-2, IL-6, and TNF-α in Latently Infected Resting CD4+ T Cells from Patients Receiving HAART. We and others have recently reported that HIV-1–infected individuals receiving HAART for prolonged periods of time with no detectable plasma viremia carry a small number of latently infected, resting CD4+ T cells that produce infectious HIV-1 upon mitogenic stimulation in vitro (24–26). In this regard, we sought to determine whether activation of HIV-1 replication can be achieved by cytokines alone in the absence of a potent mitogenic stimulus in latent infected, resting CD4+ T cells from patients who had been receiving HAART for considerable periods of time and in whom plasma viremia was markedly suppressed. The frequency of latently infected, resting CD4+ T cells carrying replication-competent HIV-1 from these patients ranged from 0.5 to 40.1 infectious units per 10^6 resting CD4+ T cells (Table 1). When purified resting CD4+ T cells from these patients were incubated with individual cytokines, including IL-2, no significant HIV-1 replication was detected in the supernatants (Fig. 3). However, the combination of IL-2, IL-6, and TNF-α effectively induced HIV-1 replication in this population of cells from seven out of seven patients receiving HAART, three of whom (patients nos. 10, 12, and 13) had no detectable plasma viremia as determined by the ultrasensitive reverse transcriptase PCR. Despite the fact that certain proinflammatory and immunoregulatory cytokines have been shown to upregulate the expression of HIV-1 in chronically infected cell lines and in the PBMCs of infected individuals (3, 10–13), it has been unclear what effect they have on latently infected, resting CD4+ T cells. This study clearly demonstrates a synergistic role of the combination of IL-2, IL-6, and TNF-α in the induction of HIV-1 replication in latently infected, resting CD4+ T cells. The fact that individual cytokines did not induce significant replication of HIV in these cells, whereas combinations of cytokines were potent inducers, is consistent with previous observations of the synergistic effects of certain cytokines in the induction of expression of HIV from chronically infected cell lines shown that glucocorticoids can block nuclear factor κB–mediated cellular activation in vitro (42). To examine the inhibitory role of glucocorticoids in the induction of HIV-1 replication in latently infected, resting CD4+ T cells by cytokines, dexamethasone was added to cultures containing the combination of three cytokines or anti-CD3 antibody. As shown in Fig. 4, cultures containing dexamethasone markedly inhibited replication of HIV-1 in latently infected, resting CD4+ T cells from antiretroviral drug–naive patients when cultures were stimulated with either the combination of IL-2, IL-6, and TNF-α or with anti-CD3 antibody.

**Discussion**

In this study, we have examined the synergistic role of cytokines in the reactivation of HIV-1 replication in latently infected, resting CD4+ T cells from patients who were receiving no antiretroviral therapy as well as from patients who were receiving HAART and in whom plasma viremia was markedly suppressed, and in certain patients even undetectable by the ultrasensitive reverse transcriptase PCR. Despite the fact that certain proinflammatory and immunoregulatory cytokines have been shown to upregulate the expression of HIV-1 in chronically infected cell lines and in the PBMCs of infected individuals (3, 10–13), it has been unclear what effect they have on latently infected, resting CD4+ T cells. This study clearly demonstrates a synergistic role of the combination of IL-2, IL-6, and TNF-α in the induction of HIV-1 replication in latently infected, resting CD4+ T cells. The fact that individual cytokines did not induce significant replication of HIV in these cells, whereas combinations of cytokines were potent inducers, is consistent with previous observations of the synergistic effects of certain cytokines in the induction of expression of HIV from chronically infected cell lines.

**Figure 4.** Suppression of induction of HIV-1 replication in latently infected, resting CD4+ T cells stimulated with the combination of cytokines or anti-CD3 antibody by in vitro glucocorticoids. Resting CD4+ T cells were incubated with IL-2, IL-6, and TNF-α, or with anti-CD3 antibody with irradiated feeders in the presence or absence of dexamethasone (10−8 M). Supernatants from each culture were removed on days 3, 6, and 9, and HIV-1 p24 was measured by ELISA.
HIV-1 replication in latently infected, resting CD4+ T cells induced by the three cytokine combination and anti-CD3 stimulation was effectively suppressed by dexamethasone at modest concentrations (10−8 M), which agrees with previous findings that glucocorticoids can block HIV-1 replication by inhibiting the secretion of certain cytokines, particularly proinflammatory cytokines (39-41) or by inhibiting nuclear factor κB-mediated cellular activation (42).

The findings in our study suggest that combinations of cytokines secreted in response to nonspecific stimuli or as a result of a specific antigenic stimulus could explain at least in part the reappearance of plasma viremia in patients receiving HAART in whom HIV-1 replication was successfully contained initially but in whom therapy was subsequently interrupted (33). Given the fact that the latently infected pool of resting CD4+ T cells resides predominantly in lymphoid tissue (27), and since cells situated in the microenvironment of lymphoid tissue are continually exposed to the secretion of proinflammatory and immunoregulatory cytokines (43), it is highly likely that these cytokines readily induce HIV replication in latently infected, resting CD4+ T cells in the absence of HAART or when HAART is ineffective in completely suppressing virus replication (33). The fact that latently infected, resting CD4+ T cells from individuals receiving HAART and in whom plasma viremia was markedly suppressed, and in certain patients even undetectable, could be readily induced to replicate virus when cells were taken ex vivo and cultured in the presence of the combination of cytokines is highly consistent with their ability to become reactivated when in vivo HAART is discontinued. The ability of in vitro HAART to again suppress cytokine-induced virus replication underscores this observation.

Finally, given the relatively long half-life of these latently infected, resting CD4+ T cells (24–26) and the fact that they are constantly within an environment capable of providing the stimuli for reactivation, it is not unreasonable to explore strategies aimed at deliberately diminishing the size of this pool of cells. In this regard, since cytokine-mediated induction of HIV-1 replication in these cells with subsequent release of virus probably results in death of the cell (44–46), and since the presence of HAART in vitro prevents spread of released virus, it is conceivable that in vivo administration of cytokines together with HAART may have such an effect.

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