Changes in Cytokine Secretion Patterns of CD4+ T-Cell Clones in Human Immunodeficiency Virus Infection

By Linde Meyaard, Sigrid A. Otto, Irenous P.M. Keet, René A.W. van Lier, and Frank Miedema

In addition to the loss of CD4+ T cells in later stages of human immunodeficiency virus (HIV) infection, functional defects of Th cells can already be observed in early infection. Decreased interleukin (IL)-2 and interferon (IFN)-γ production by CD4+ T cells and diminished delayed type hypersensitivity reactions are indicative for impaired Th1 responses. We studied the cytokine secretion patterns of T-cell clones (TCC) generated by mitogenic stimulation of CD4+ memory T cells. Compared with TCC from HIV-negative controls, TCC isolated from HIV-infected individuals consistently showed increased IL-4 production, often paralleled by increased IL-5 and decreased IFN-γ production. This resulted in a decreased percentage of Th1 clones with an increase in Th0 clones. To rule out the influence of interindividual differences, we studied two individuals from whom cells were available before and after infection with HIV. Indeed, an increase in Th2 cytokine secretion was observed after HIV-infection. Loss of Th1 and enhanced Th2 responses might further curtail cellular responses resulting in deficiency of cellular immunity in HIV infection.

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C O N C L U S I O N S  A N A L Y S I S of murine T cells has led to the identification of two types of T-helper cells, Th1 and Th2.1 Murine Th1 cells secrete interferon (IFN)-γ and interleukin (IL)-2, while Th2 cells produce IL-4, IL-5, IL-6, and IL-10. In addition, a third subset of helper cells, designated Th0, with a nonrestricted cytokine secretion profile has been recognized.2 Th1 cells are important mediators of delayed type hypersensitivity (DTH) responses, are involved in activation of macrophages, and provide help to B cells for IgG2a synthesis. In contrast, Th2 cells preferentially provide help for IgE and IgG1 synthesis and are mediators of eosinophilia. The two T-cell subsets were shown to be important in determining the outcome of certain parasitic infections in mice. The genetic determined resistance against infection with Leishmania major seems to be associated with Th1 responses, whereas fatal leishmaniasis in mice is associated with Th2 responses.3 Schistosoma mansoni infection in mice is associated with a shift to Th2 responses. Importantly, infection with S mansoni induces Th2 responses not only to the parasite itself, but also to nonparasite antigens.4

The distinction between the two subsets in humans is not as clear-cut as in mice, but in several pathologic conditions an unbalanced production of, in particular IL-4 and IFN-γ, has been observed. In tuberculosis leprosy5 and in response to Mycobacterium tuberculosis6 or Borrelia burgdorferi Th1-like responses can be demonstrated,7 which was also described for Yersinia enterolitica induced reactive arthritis.8 In contrast, in atopic individuals9-10 and in the conjunctiva of patients with vernal conjunctivitis,11 Th2-like T cells could be demonstrated. In human leishmaniasis, the distinction in the cytokine secretion by the T cells of the patients, Th1 versus Th2-like, is reflected by different clinical features, localized versus chronic, and destructive leishmaniasis.12

Before the loss of CD4+ T cells in later stages of infection, immunodeficiency caused by human immunodeficiency virus (HIV) infection is characterized by functional defects of capacities ascribed to Th1 cells that are observed in early infection. Proliferation of T cells in response to ligation of the T-cell receptor (TCR) CD3 complex is impaired14-16 and IL-2 production decreased.17,18 Furthermore, antigen-induced IFN-γ production19 and DTH reactions are decreased.20,21 Clinical symptoms associated with a relative dominance of Th2-mediated responses have been described in HIV-infected individuals and include eosinophilia,22 elevation of serum IgE levels23 and allergic manifestations.24,25

Here, we investigated whether HIV infection might be associated with a change in Th cytokine-secretion patterns. To study the potential of T cells from HIV-infected individuals to secrete lymphokines, we investigated the type of T cells that could be cloned during the asymptomatic phase of the infection.

MATERIALS AND METHODS

Subjects. Peripheral blood from both asymptomatic HIV-infected subjects and noninfected controls enrolled in the Amsterdam cohort study on HIV infection homosexual men19 was used for isolation of cells. All were in the asymptomatic phase of infection (Centers for Disease Control, class II) and did not receive medication. For all but one subject, the date of HIV-1 antibody seroconversion was known.

Cells from a seronegative participant of the cohort and cells from subjects no. 1160 and 0082, obtained before infection with HIV and stored by cryopreservation, were used as controls. None of the subjects had allergic or atopic manifestations before infection with HIV. Table 1 summarizes the characteristics of the subjects at the moment cells were taken for cloning. In follow-up, it showed that subject no. 0545 developed a syncytium-inducing virus variant26 and had 0.31 × 10^3/L CD4+ cells at 15 months from the time point studied here. Apart from idiopathic dermatologic manifestations, he remained asymptomatic until then. Subject no. 1160 still had normal CD4+ cell counts (0.45 × 10^9/L) at 40 months after the moment studied and remained asymptomatic. Subject no. 6032 suffered from eczema and 18 months after the date from which cells were used, AIDS was diagnosed because of a clinical manifest infection with

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M. tuberculosis. The subject responded with hypersensitivity reactions to the treatment he received. Subject no. 0082, from which cells were isolated from heparinized blood by Ficoll density gradient centrifugation and cryopreserved. After thawing the cells, they were stained with fluorescein isothiocyanate (FITC) labeled Leu-2A (CD8, Becton Dickinson, Sunnyvale, CA) and phycoerythrin (PE)-labeled 2H4 (CD45RA, Coulter, Hialeah, FL). After washings, cells were purified for CD8+CD45RA+ T cells by cell sorting (FACStar, Becton Dickinson).

Generation of T-cell clones. Purified CD8+CD45RA+ T cells were cultured under limiting dilution conditions (0.3 cells/well) in Iscove’s medium (IMDM) supplemented with 10% human pooled serum, 20 U/mL rIL-2 (Hoffman-La Roche, Nutley, NJ) and antibiotics. Clones from each donor were generated with similar cloning efficiency and passaged weekly by culturing equal numbers of either clone (0.3 x 10^6/mL) with PHA, feeders, and IL-2 as described above. Clones were analyzed for the expression of CD4+ and the absence of CD8+ by fluorescence-activated cell sorter (FACS) analysis using standard procedures. Functional studies were performed 7 days after restimulation.

Functional studies. 0.25 x 10^6/mL cells were stimulated with phorbol myristate acetate (PMA) (1 ng/mL) and CD3 monoclonal antibody (MoAb) (CLB-T3/4.5, 5 µg/mL) in IMDM supplemented with 10% fetal calf serum (FCS), 7 days after the last restimulation. At this time, no viable feeder cells producing cytokines were present, and the clones did not produce detectable levels of cytokines spontaneously. Supernatants were collected after 24 hours to measure IL-2 and at 72 hours for IL-4, IL-5, IL-10, and IFN-γ measurement, which in our hands were optimal time intervals from the production of these cytokines. Supernatants were stored in aliquots at -20°C and thawed once before analysis. IL-2 concentration in the supernatant was measured in a bioassay using the IL-2 dependent CTLL-2 line.6 IL-4 production was assayed in an IL-4-specific enzyme-linked immunosorbent assay (ELISA), generously provided by Dr T. van der Pouw-Kraan (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands).10 IL-5, IL-10,22 and IFN-γ33 production were determined by ELISA, developed and kindly provided by DNAx Institute (Palo Alto, CA). Cytokine production was measured in supernatants from three separate experiments.

As a control for the capacity of the clones to respond to the given stimulus, proliferation was measured by [3H]-thymidine incorporation.

RESULTS

Increased IL-4 production by T-cell clones from HIV-infected subjects. We characterized TCC derived from CD8+CD45RA+ T cells from asymptomatic HIV-infected subjects and seronegative homosexual men as controls. The randomly generated CD4+ TCC were analyzed for their cytokine secretion on stimulation with PMA and CD3 MoAb. Compared with two controls, PBMC from HIV-infected individuals yielded a decreased percentage of IFN-γ producing TCC, whereas the percentage of IL-4 producing clones was increased (Table 2).

Despite decreases in percentages of TCC producing IFN-γ (Table 2), mean IL-4 production by clones from HIV-infected subjects was not different from controls (Fig 1B). Importantly, mean IL-4 production by TCC from HIV-infected men as a group was significantly increased compared with TCC from controls (Fig 1C). When analyzed per individual, clones from all three seropositive subjects had increased IL-4 production, although in one case (no. 0545) this increase was not significant. IL-5 production paralleled IL-4 production as can be clearly observed in Fig 1D; however, the differences were not significant. The percentage of IL-2, IL-5 and IL-10 producing TCC

Table 1. Characteristics of HIV-Seropositive Individuals Analyzed

<table>
<thead>
<tr>
<th>Subject No.</th>
<th>Time After Seroconversion (mo)</th>
<th>CD4+ Cells (x10^6/L)</th>
<th>CD8+ Cells (x10^6/L)</th>
<th>CD3 Response (% of control)</th>
<th>PHA Response (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0545</td>
<td>45</td>
<td>0.38*</td>
<td>0.50*</td>
<td>38†</td>
<td>38†</td>
</tr>
<tr>
<td>1160</td>
<td>27</td>
<td>0.56</td>
<td>0.73</td>
<td>14</td>
<td>74</td>
</tr>
<tr>
<td>6032</td>
<td>&gt;34</td>
<td>0.62</td>
<td>1.48</td>
<td>102</td>
<td>133</td>
</tr>
<tr>
<td>0082 (1)</td>
<td>33</td>
<td>0.80</td>
<td>1.27</td>
<td>20</td>
<td>40</td>
</tr>
<tr>
<td>0082 (2)</td>
<td>51</td>
<td>0.49</td>
<td>1.79</td>
<td>11</td>
<td>11</td>
</tr>
</tbody>
</table>

* Absolute numbers of CD4+ and CD8+ cells determined by standard procedures.
† A total of 0.25 x 10^6/mL PBMC were stimulated with PHA or CD3 MoAb and proliferation was assessed by [3H]-thymidine incorporation on day 4. Data are expressed as percentage of mean proliferation of four HIV− controls measured simultaneously.

Table 2. Cytokine Secretion Profiles of TCC From HIV-Infected Individuals and Seronegative Controls

<table>
<thead>
<tr>
<th>Subject No.</th>
<th>No. of TCC</th>
<th>IL-2</th>
<th>IFN-γ</th>
<th>IL-4</th>
<th>IL-5</th>
<th>IL-10</th>
</tr>
</thead>
<tbody>
<tr>
<td>0177</td>
<td>33</td>
<td>37</td>
<td>94</td>
<td>12</td>
<td>12</td>
<td>9</td>
</tr>
<tr>
<td>1160 (−6)</td>
<td>40</td>
<td>35</td>
<td>95</td>
<td>13</td>
<td>23</td>
<td>38</td>
</tr>
<tr>
<td>HIV−</td>
<td>73</td>
<td>49</td>
<td>95</td>
<td>12</td>
<td>18</td>
<td>25</td>
</tr>
<tr>
<td>0545</td>
<td>40</td>
<td>95</td>
<td>80</td>
<td>20</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>1160 (≥7)</td>
<td>38</td>
<td>245</td>
<td>87</td>
<td>37</td>
<td>26</td>
<td>18</td>
</tr>
<tr>
<td>6032</td>
<td>36</td>
<td>53</td>
<td>86</td>
<td>36</td>
<td>56</td>
<td></td>
</tr>
<tr>
<td>HIV+</td>
<td>114</td>
<td>58</td>
<td>84</td>
<td>361</td>
<td>24</td>
<td>27</td>
</tr>
</tbody>
</table>

A total of 0.25 x 10^6/mL clone cells were stimulated with PMA and CD3 MoAb 7 days after the last restimulation. At day 1 (IL-2) and day 3 (IFN-γ, IL-4, IL-5, and IL-10), supernatants were collected and cytokines were measured.

* Data are percentages of clones producing indicated cytokine above two times the background level of the assay. Clones from two HIV− (0177 and 1160) and three HIV+ (0545, 1160, and 6032) subjects are displayed per subject and as a group (HIV− and HIV+). Seventy-three clones from two seronegative individuals are displayed as seronegative controls (HIV−). Each clone was analyzed in three separate experiments.

† From subject no. 1160, TCC were obtained 6 months before (−6) and 27 months after (+7) seroconversion.
‡ P < .005.
§ P < .05, compared with HIV− controls in χ² test.
‖ P < .005.
varied alike in infected and noninfected individuals (Table 2). Although the percentage of IL-2 producing TCC of subject no. 0545 was significantly higher (Table 2), the mean IL-2 production by the clones was comparable to HIV-negative controls because the quantity of IL-2 produced by TCC of this subject was relatively low (Fig 1A). Mean IL-10 production was increased in the clones from subject no. 6032. It is of interest to note that in this subject, TCC also produced high amounts of IL-4 and IL-5.

We grouped clones according to their Th1 (IL-2 and/or IFN-γ), Th2 (IL-4 and/or IL-5) or Th0 phenotype (clones producing a combination of Th1 and Th2 cytokines) (Fig 2). IL-10 was not used as a discriminatory cytokine. Although in mice the only T cells producing IL-10 are Th2 cells, IL-10 in humans is produced by Th0, Th1 and Th2 cells. Subject no. 0545 showed the same distribution of clones as the HIV− controls. Subjects no. 1160 and 6032, in contrast, had a decreased percentage of clones producing Th1 and an increased percentage producing Th2 cytokines. Thus, in two out of three of the seropositive subjects, a shift to Th0 clones was demonstrated.

Changes in cytokine secretion in two subjects followed over seroconversion. Interindividual differences in the cytokine secretion patterns might influence the comparison of TCC from HIV-infected subjects with controls. For this reason, we compared TCC from the same subject obtained before and after HIV infection by taking the opportunity to use cryopreserved patient material.

As displayed in Table 2, from subject no. 1160, TCC were generated from cells stored 6 months before and 27 months after seroconversion. Indeed, when TCC are compared within this subject, the percentage of IL-2 and IFN-γ producing clones decreases on HIV infection, while the percentage of IL-4 producing clones significantly increases (Table 2).
In addition, the mean IL-4 production of the TCC obtained after HIV infection increases (HIV−: 0.06 ng/mL to HIV+: 0.28 ng/mL; Fig 3).

In a second set of experiments, we generated TCC from CD8−CD45RA− cells from cryopreserved material from one subject obtained 8 months before and 33 and 51 months after seroconversion and were able to analyze the temporal changes of TCC characteristics in HIV infection. Also in this subject, the percentage of TCC producing Th1 cytokines: IL-2 and/or IFN-γ, Th2 cytokines: IL-4 and/or IL-5, and clones producing a combination of Th1 and Th2 cytokines (Th0) is indicated. Seventy-three T-cell clones from two seronegative subjects (HIV−) were compared with TCC from HIV-infected subjects no. 0545 (n = 40), 1160 (n = 38), and 6032 (n = 36).

![Diagram of cytokine secretion pattern of TCC from HIV-infected subjects compared with clones from two HIV− controls. The percentage of clones producing Th1 cytokines: IL-2 and/or IFN-γ, Th2 cytokines: IL-4 and/or IL-5, and clones producing a combination of Th1 and Th2 cytokines (Th0) is indicated. Seventy-three T-cell clones from two seronegative subjects (HIV−) were compared with TCC from HIV-infected subjects no. 0545 (n = 40), 1160 (n = 38), and 6032 (n = 36).](http://www.bloodjournal.org)

**Fig 2.** Cytokine secretion pattern of TCC from HIV-infected subjects compared with clones from two HIV− controls. The percentage of clones producing Th1 cytokines: IL-2 and/or IFN-γ, Th2 cytokines: IL-4 and/or IL-5, and clones producing a combination of Th1 and Th2 cytokines (Th0) is indicated. Seventy-three T-cell clones from two seronegative subjects (HIV−) were compared with TCC from HIV-infected subjects no. 0545 (n = 40), 1160 (n = 38), and 6032 (n = 36).

**DISCUSSION**

Our findings demonstrate that on HIV infection, T cells cloned from CD4+ memory cells, show changes in their cytokine profiles. Most marked was the consistently observed increase in IL-4 production after HIV infection, often paralleled by increased IL-5 and decreased IFN-γ production, resulting in an increase in Th0 clones. Importantly, we were able to compare TCC from two HIV-infected subjects with clones obtained from the same subjects before HIV infection. We demonstrated that the cytokine secretion pattern from TCC from two subjects changes on HIV infection to a Th0 pattern, excluding interindividual differences as a cause for the observations.

The change in cytokine secretion patterns was not observed in all subjects tested. We cannot correlate the Th0 shift with a specific stage of infection, but our results are suggestive for a relation with the stage of infection, because one subject followed over seroconversion displayed a gradual change in cytokine patterns. These observations are compatible with the findings from Clerici et al. They studied IL-2 production induced by recall antigen and mitogen-induced IL-4 production by PBMC from HIV-infected individuals. In the early phase of infection, both cytokines are produced at comparable levels as in negative controls. However, at later time points, IL-4 production increases and is accompanied by a decreased IL-2 production, supporting a shift from Th1 to Th2 type cells in asymptomatic HIV infection. In the more progressive stage of infection, both IL-2 and IL-4 production are decreased.

So far, analyses of human TCC have suggested Th1 or Th2 polarization in several pathological conditions. In studies revealing Th1 responses to M. leprae and Th2 cells in the conjunctiva of patients with vernal conjunctivitis, discrimination between the two subsets was mainly based on quantitative differences in IL-4 and IFN-γ secretion, rather than qualitatively different clones. In studies in atopic patients or Yersinia-induced reactive arthritis, production of IL-4 or IL-5 was considered discriminatory for Th2 cells. IL-2 in one study was reported as discriminatory. Thus, the extent of changes in cytokine secretion patterns we observed in HIV-infected subjects are comparable to those observed in other pathologic conditions in humans.

By analyzing TCC, Maggi et al. demonstrated a reduced production of IL-2 and IFN-γ in acquired immunodeficiency syndrome (AIDS) patients. Recently, the same group generated a large panel of CD4+ TCC specific for purified protein derivative (PPD) or T gondii, which are in vivo activated memory cells, and have observed a significant increase in the production of Th2 cytokines by clones generated from HIV-infected individuals, resulting in an increased percentage of Th0 type clones. At the same time Graziosi et al. concluded from mRNA levels in peripheral blood T cells and lymph nodes that no change in cytokine secretion can be observed on HIV infection. This might be explained by the report from Maggi et al, in which TCC, randomly generated and not specifically from the memory T-cell population, do not show a shift towards Th0, in agreement with their findings in bulk PBMC cultures.

The mechanism by which HIV infection leads to a change in cytokine secretion of CD4+ T cells remains to be elucidated. Allergen- or parasite-induced changes in cytokine profile in the individuals studied can be ruled out, because none of the patients had evidence of atopic manifestations before HIV infection or experienced clinically manifest parasitic infections. The outgrowth of Th0 cells might result from a disturbed Th1/Th2 balance following dysfunction of Th1 cells. The defective outgrowth of Th1 cells might be based on infection of accessory cells and thereby defective antigen presenting cell function resulting in Th1 anergy. Mono-
cytes from HIV-infected individuals are deficient for IL-12 production, a cytokine produced by antigen presenting cells, instrumental in the generation of Th1 type responses. Decreased IL-12 production might result in diminished outgrowth of Th1 cells and a subsequent increase of Th0 cells. Th1 nonresponsiveness will preferentially cause deficiency of cellular immunity and render the infected person susceptible to infection and disease induced by various pathogens, but may be equally important to control HIV replication and spread. Interestingly, subject no. 6032, of whom the majority of clones produced Th2 cytokines, suffered from tuberculosis, in which clearance of the pathogen is dependent on Th1 DTH responses. The reported resistance to murine AIDS of IL-4 deficient mice infected with LP-BM5 virus, is suggestive for a role of Th2 cytokines in disease development, probably by further downregulating Th1 and cytotoxic T-cell responses. Correction of the Th1/Th2 balance may provide a basis for therapeutic intervention in HIV-infected individuals. Restoration of in vitro T-cell responses by IL-4 antibodies and addition of IL-12 in HIV-
Table 3. Cytokine Secretion Profiles of TCC From Individual 0082 Before and After HIV Serocconversion

<table>
<thead>
<tr>
<th>Time From Serocconversion (mo)</th>
<th>Percentage Producing Clonesa (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 (n = 30)</td>
<td>IL-2 100  IFN-γ 77  IL-4 100  IL-5 100  IL-10 77</td>
</tr>
<tr>
<td>33 (n = 30)</td>
<td>IL-2 100  IFN-γ 68  IL-4 100  IL-5 100  IL-10 77</td>
</tr>
<tr>
<td>51 (n = 53)</td>
<td>IL-2 100  IFN-γ 68  IL-4 100  IL-5 100  IL-10 77</td>
</tr>
</tbody>
</table>

TCC were obtained from cryopreserved PBMC from one subject collected 8 months before and 33 and 51 months after seroconversion. A total of 1 x 10⁶ cells/ml were stimulated with PMA and CD3 MoAb. On day 1 (IL-2) and day 3 (IFN-γ, IL-4, IL-5, and IL-10) supernatants were collected and cytokines were measured.

* Data are percentages of clones producing indicated cytokine above detection level. Each clone was analyzed in three separate experiments.

P < .05 compared with clones from HIV− subjects in χ² test.

infected individuals was reported. If shortage of IL-12 production is indeed causing the defective outgrowth of Th1 cells, therapeutic use of this cytokine might be considered.

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