CD3ζ Down-Modulation May Explain Vγ9Vδ2 T Lymphocyte Anergy in HIV-Infected Patients

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The aim of the present study was to explain the observed anergy of Vγ9Vδ2 T cells from human immunodeficiency virus (HIV)-positive patients. CD3ζ expression and interferon (IFN)–γ production by Vγ9Vδ2 T cells from HIV-positive and HIV-negative subjects were analyzed. We demonstrated that Vγ9Vδ2 T cells from HIV-infected patients expressed a lower level of CD3ζ than did Vγ9Vδ2 T cells from healthy donors. A direct correlation was found between CD3ζ expression and IFN-γ production capability by Vγ9Vδ2 T cells. However, activation of protein kinase C by phorbol myristate acetate is able to restore CD3ζ expression and IFN-γ production. Our findings may contribute to clarification of the molecular mechanisms of Vγ9Vδ2 T cell anergy found in HIV-positive patients.

Vδ T cells constitute a small proportion (0.5%–10%) of circulating T lymphocytes, and most of them coexpress Vγ9 and Vδ2 T cell receptor (TCR) chains [1]. Vγ9Vδ2 T cells recognize non-peptide phosphorylated metabolites (phosphoantigens) without antigen uptake, processing, and presentation by classical major histocompatibility complex molecules [2]. On stimulation, Vγ9Vδ2 T cells proliferate, release Th1 cytokines, and acquire cytotoxicity capability against tumor or virus-infected cells.

Moreover, activated Vγ9Vδ2 T cells may enhance dendritic cells functions [3, 4], thus strengthening the adaptive immune response.

Although there is no evidence that HIV encodes antigens recognized by Vγ9Vδ2 T cells, efforts have been made to explore γδ T cell responses during HIV infection. In vitro recognition of HIV-infected cells by Vγ9Vδ2 T cells induces their expansion and a potent cytotoxicity [5]. Stimulated Vγ9Vδ2 T cells are able to suppress HIV replication by releasing β chemokines [6], suggesting antiviral function of Vγ9Vδ2 T lymphocytes. However, a significant decrease in the number of peripheral Vγ9Vδ2 T cells was observed in HIV-infected persons, and, in most of these patients, the remaining Vγ9Vδ2 T cells exhibited a functional anergy to phosphoantigens [7].

TCR is a multisubunit complex in which the invariant subunit CD3 (CD3γ–CD3ε, CD3δ–CD3ε, and CD3ζ–CD3ζ) couples antigen recognition with intracellular signal transduction. The CD3ζ chain is a 16-kDa transmembrane protein expressed as a disulfide-linked homodimer. After TCR engagement, CD3ζ is phosphorylated at the immunoreceptor tyrosine-based activation motifs, initiating a signal transduction pathway leading to T cell activation [8]. Therefore, the CD3ζ chain is indispensable for coupling antigen recognition by the TCR and the T cell response.

Various reports have shown that, in many pathologies characterized by chronic inflammation, such as HIV infection, CD8 T cells are functionally impaired, and this impairment is associated with lower expression of the CD3ζ chain [9, 10]. The association between the CD3ζ chain and the functionality of Vγ9Vδ2 T cells is not known. In the present study, the role of the CD3ζ chain on Vγ9Vδ2 T cell anergy during HIV infection was investigated.

Materials and methods. Thirty-seven blood samples obtained from HIV-positive patients at the National Institute for Infectious Diseases “Lazzaro Spallanzani” (Rome, Italy) were included in the present study. Anonymous blood count residual samples were used. Of the HIV-positive patients, 75% were male (age range, 20–78 years). The CD4 T cell counts ranged from 88 to 1474 cells/mm³, and the viral loads ranged from <50 to 107,363 viral RNA copies/mL. The percentage of circulating Vδ2 T cells ranged from 0.1% to 3.66% (mean, 0.59%). Blood samples obtained from 29 healthy, HIV-seronegative donors were used as a control and were processed under the same conditions used for samples obtained from HIV-infected donors. Of the HIV-negative donors, 50% were male (age range, 27–67 years).
The percentage of circulating \( V_\beta 2 \) T cells ranged from 0.26% to 9.63% (mean, 2.56%). Peripheral blood mononuclear cells (PBMCs) were isolated from peripheral blood by density gradient centrifugation (Lympholyte-H; Cederlane). The expression of \( CD3_\zeta \) on \( V_{\gamma 9} V_\beta 2 \) T cells was evaluated soon after isolation of PBMCs by flow cytometry. Cells were then resuspended in RPMI 1640 (EuroClone) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (EuroClone), 2 mmol/L L-glutamine, 10 mmol/L HEPEs buffer (N-2-hydroxyethylpiperazine-N’-2-ethane sulfonic acid), 2 mmol/L penicillin, and 50 mmol/L streptomycin (EuroClone). When indicated, PBMCs were stimulated with 20 \( \mu \)g/mL isopentenyl pyrophosphate (IPP) (Sigma-Aldrich), 100 U/mL interleukin (IL)-2 (Sigma-Aldrich), 4500 U/mL IL-15 (Sigma Aldrich), 50 ng/mL phorbol myristate acetate (PMA), and 1 \( \mu \)g/mL ionomycin (Serva) for 18 h. In selected experiments, PBMCs from healthy donors were stimulated with 20 \( \mu \)g/mL IPP for 30 s, 1 min, 3 min, 5 min, and 10 min. Cells then were fixed, and the expression of phosphorylated-CD3\_\( \zeta \) was evaluated by flow cytometry.

Immunophenotyping of \( V_{\gamma 9} V_\beta 2 \) T cells was accomplished using fluorescein isothiocyanate–conjugated anti-\( V_\beta 2 \), phycoerythrin (PE)–conjugated anti-\( CD3_\zeta \), allophycocyanin (APC)–conjugated anti-\( CD27 \), and peridinin–chlorophyll A protein–conjugated anti-\( CD45RA \) monoclonal antibodies (mAbs) (BD Biosciences). In brief, cells were incubated with 50 \( \mu \)L of antibodies at 4°C for 20 min and then were washed and fixed with 1% paraformaldehyde. For intracellular staining, cells were fixed with 1% paraformaldehyde, permeabilized, and stained with PE-labeled anti-\( CD3_\zeta \) (Immuno-tech), PE-labeled anti-\( p-CD3_\zeta \) (Santa Cruz Biotechnology), and APC-labeled anti-IFN-\( \gamma \) mAbs (BD Biosciences). Control for nonspecific staining was monitored by isotype-matched mAbs. Acquisition of 100,000 events in the lymphocyte-gated population and analysis were performed using Cell Quest software (Becton Dickinson). The number of CD3\_\( \zeta \) molecules per cell was evaluated using the Phycoerythrin Fluorescence Quantitation Kit (BD Biosciences) according to the manufacturer’s instructions.

Comparisons between the 2 groups of subjects were performed using nonparametric analysis (the Mann-Whitney \( U \) test). The nonparametric Wilcoxon matched-pairs test was used to compare unstimulated and stimulated cells. \( P < .05 \) was considered to denote statistical significance. The nonparametric Spearman’s test was used to describe correlation. GraphPad Prism software (version 4.00 for Windows; GraphPad) was used to perform the analysis.

The study was conducted in accordance with a protocol approved by the ethics committee at National Institute for Infectious Diseases “Lazzaro Spallanzani.”

**Results.** We investigated whether \( V_{\gamma 9} V_\beta 2 \) T cell anergy, previously noted in HIV-infected patients, was caused by an alteration of the proximal \( V_{\gamma 9} V_\beta 2 \) TCR signalling cascade. To address this issue, the expression of \( CD3_\zeta \) by \( V_{\gamma 9} V_\beta 2 \) T cells from 37 HIV-positive subjects and 29 healthy donors was analyzed by flow cytometry. We found that 100% of \( V_{\gamma 9} V_\beta 2 \) T cells, obtained from either HIV-negative or HIV-positive subjects, expressed \( CD3_\zeta \); however, the mean fluorescence intensity of \( CD3_\zeta \) in \( V_{\gamma 9} V_\beta 2 \) T cells from HIV-positive patients was significantly lower than that of \( CD3_\zeta \) in \( V_{\gamma 9} V_\beta 2 \) T cells from healthy donors (figure 1A and 1C). To confirm this result, we quantified the number of CD3\_\( \zeta \) molecules per cell by use of the Phycoerythrin Fluorescence Quantitation Kit (Becton-Dickinson). Accordingly, the number of CD3\_\( \zeta \) molecules on \( V_{\gamma 9} V_\beta 2 \) T cells was significantly lower in HIV-positive patients than in healthy, HIV-negative donors (figure 1D). Moreover, we did not find any differences in CD3\_\( \varepsilon \) (data not shown) and \( V_\beta 2 \) expression (figure 1B) on \( V_{\gamma 9} V_\beta 2 \) T cells between HIV-positive patients and healthy donors, suggesting that only the \( \zeta \) chain of the TCR complex is affected. A significantly lower expression of CD3\_\( \zeta \) on CD3/V\( \beta 2^- \)T cells in HIV-positive donors, compared with healthy donors, was also observed (mean fluorescence intensity, 94.91 vs. 175.0, respectively; \( P = .0001 \)) (data not shown).

Circulating \( V_{\gamma 9} V_\beta 2 \) T cells can be divided into 4 subpopulations: naive, central memory, effector memory, and effector cells. CD3\_\( \zeta \) expression on each \( V_{\gamma 9} V_\beta 2 \) T cell subpopulation was lower in HIV-positive patients than in healthy donors; moreover, no significant difference in CD3\_\( \zeta \) expression was found when the 4 subpopulations were compared to each other (data not shown).

We questioned whether the reduction in CD3\_\( \zeta \) expression was correlated with the lower capacity of \( V_{\gamma 9} V_\beta 2 \) T cells from HIV-positive patients to produce IFN-\( \gamma \). To investigate this possibility, we used IPP to stimulate PBMCs obtained from HIV-positive and HIV-negative subjects for 18 h, and IFN-\( \gamma \) production was analyzed by flow cytometry. As expected, a reduced frequency of \( V_{\gamma 9} V_\beta 2 \) T cells producing IFN-\( \gamma \) was observed in HIV-positive donors, compared with healthy donors (figure 1E). Moreover, a positive correlation was shown between ex vivo expression of CD3\_\( \zeta \) on \( V_{\gamma 9} V_\beta 2 \) T cells and the ability of such cells to produce IFN-\( \gamma \) after stimulation (figure 1F), suggesting that the low number of CD3\_\( \zeta \) molecules expressed on \( V_{\gamma 9} V_\beta 2 \) T cells from HIV-positive subjects may partially explain \( V_{\gamma 9} V_\beta 2 \) T cell anergy.

Because CD3\_\( \zeta \) phosphorylation was never studied in \( V_{\gamma 9} V_\beta 2 \) T cells, to support our assumption, we performed phosphoprotein flow cytometry analysis. PBMCs obtained from healthy donors were stimulated with IPP for 30 s, 1 min, 3 min, 5 min, and 10 min, and phosphorylated-CD3\_\( \zeta \) was evaluated by flow cytometry. We found that CD3\_\( \zeta \) is phosphorylated after IPP stimulation, reaching maximum phosphorylation after 1 min of stimulation. A representative panel is shown in figure 1G.
Figure 1. The expression of CD3ζ was evaluated by flow cytometry on gated Vδ2+ T cells from 29 HIV-negative and 37 HIV-positive subjects. A and B, Representative flow cytometry data on CD3ζ and Vδ2 expression on gated Vδ2 T cells from HIV-negative and HIV-positive subjects. The solid line denotes HIV-negative subjects, and the dashed line denotes HIV-positive subjects. C, CD3ζ mean fluorescence intensity (mfi). D, The number of CD3ζ molecules per Vδ2+ T cell. E, The percentage of Vδ2+ T cells from 13 HIV-negative and 20 HIV-positive subjects producing interferon (IFN)-γ after isopentenyl pyrophosphate (IPP) stimulation for 18 h, as evaluated by intracellular staining. Results are expressed as box and whisker plots. Statistical analysis was performed using a nonparametric test (the Mann-Whitney U test). F, The correlation between CD3ζ expression (the number of molecules/cell) and IFN-γ production is evaluated, including the results for HIV-negative (white diamonds) and HIV-positive (black diamonds) patients. The nonparametric Spearman’s test was used to describe correlation. P < .05 was considered to denote statistical significance. G, A representative dot plot showing the percentage of the phosphorylated CD3ζ (pCD3ζ) versus forward scatter (FSC) on gated Vδ2 T cells after 1 min of stimulation with IPP.
Peripheral Vδ2 T cells express IL-2 receptor after stimulation with phosphoantigen, and to evaluate the effect of IL-2 on CD3ζ expression, PBMCs obtained from HIV-negative and HIV-positive patients were stimulated with IPP and IL-2 for 18 h, and the expression of CD3ζ was analyzed by flow cytometry. We found that CD3ζ expression on Vγ9Vδ2 T cells from both healthy donors and HIV-positive patients was not affected by IL-2 treatment, regardless of whether IPP was used (data not shown). Because IL-15 binds the IL-2 receptor γ chain, and because it plays a role in Vγ9Vδ2 T cell activation and differentiation [12], we then investigated whether IL-15 may be involved in the regulation of CD3ζ expression. We found that IL-15 treatment had no effects on CD3ζ expression on Vγ9Vδ2 T cells, even after IPP stimulation (data not shown).

Interestingly, we found that PMA and calcium ionophore were able to induce a significant up-regulation of CD3ζ on Vγ9Vδ2 T cells from HIV-positive patients (figure 2A). However, up-regulation of CD3ζ was not the result of cytokine release, because it was found in the presence of brefeldine A, which blocks cytokines exocytosis. PMA alone—not the calcium ionophore—consistently was able to induce the up-regulation of CD3ζ (figure 2A). On the other hand, PMA did not affect CD3ζ expression on Vγ9Vδ2 T cells from healthy donors. Moreover, we questioned whether up-regulation of CD3ζ after PMA treatment is able to restore the capacity of Vγ9Vδ2 T cells to produce IFN-γ. To this aim, PBMCs obtained from HIV-positive patients were incubated with PMA for 3 h; IPP was then added, and production of IFN-γ was evaluated by intracellular staining. We found that IPP stimulation after PMA treatment, but not PMA alone, induced a higher amount of IFN-γ production, compared with IPP alone (figure 2B), indicating that the restoration of CD3ζ increased the response to phosphoantigens.

**Discussion.** A significant impairment of the subset of peripheral Vγ9Vδ2 T cells in HIV-infected persons has been recognized for a long time. In most of these patients, the remaining Vγ9Vδ2 T cells exhibit a functional anergy to phosphoantigen stimulation [6, 7]. However, the causes underlying Vγ9Vδ2 T cell depletion and anergy during HIV infection are still unclear. In the present study, we reported that Vγ9Vδ2 T cells obtained from HIV-infected subjects expressed CD3ζ levels that were lower than those noted in healthy subjects, and that this down-modulation occurred regardless of their differentiation status. We also observed down-modulation of CD3ζ in CD+ Vδ- T cells, indicating that all of the T cell compartment is affected. Moreover, we found a positive correlation between CD3ζ expression and IFN-γ production capability after in vitro stimulation of Vγ9Vδ2 T cells, suggesting that CD3ζ impairment has a role in Vγ9Vδ2 T cell anergy. To corroborate our assumption, we demonstrated that, after Vγ9Vδ2 TCR engagement by phosphoantigen, CD3ζ is phosphorylated, indicating its role in Vγ9Vδ2 T cell function.

CD3ζ down-modulation was previously observed in freshly isolated CD8 T cells and, to a lesser extent, in CD4 T cells from most HIV-infected patients, and it was correlated with disease progression, with the highest down-modulation noted in patients with AIDS [13]. Different mechanisms were suggested as explanations for CD3ζ down-modulation, including continuous exposure to antigenic stimulation or to proinflammatory cytokines [14, 15]. However, in patients in the present study, CD3ζ expression on Vγ9Vδ2 T cells did not correlate with the viral load or the CD4 cell count (data not shown), indicating that this modulation does not seem to be directly affected by HIV and that it is independent of CD4 functions. We found that, consistent with these data, exogenous IL-2 did not have any effect on...
the expression of CD3ξ molecules on Vγ9Vδ2 T cells. On the other hand, CD3ξ expression on αβ T cells is up-regulated after culture with IL-2 overnight [10], although, in CD8 T cells from late-stage HIV-infected individuals, the CD3ξ chain remains suppressed even after overnight incubation with IL-2 [11]. There is growing evidence that cytokines that bind receptors containing the common γ chain (other than IL-2) are involved in T cell maintenance and homeostasis. In particular, it was shown that IL-15 plays a role in proliferation and differentiation of γδ T cells [12]. We found that, because IL-2 or IL-15 stimulation with or without IPP did not have any effects on CD3ξ expression on Vγ9Vδ2 T cells, Vγ9Vδ2 T cells could demonstrate a response to these cytokines that is different from that of αβ T cells.

Impaired expression of CD3ξ on Vγ9Vδ2 T lymphocytes from HIV-positive patients is not irreversible, because in vitro PMA stimulation improved CD3ξ expression, and restored the functional capability of Vγ9Vδ2 T lymphocytes to produce IFN-γ after stimulation with IPP. It is probable that, throughout PKC activation, PMA triggers a signal transduction pathway that, in turn, may induce new CD3ξ mRNA synthesis or may overcome the inhibition of CD3ξ protein expression. These hypotheses are under scrutiny.

In conclusion, we found that the Vγ9Vδ2 T cell anergy observed in HIV-infected subjects may be explain ed by a low expression of the CD3ξ chain in the TCR complex and may be bypassed by PKC activation. These findings may contribute to an understanding of the molecular mechanisms of Vγ9Vδ2 T cell anergy, and they may have an implication in the design of effective immune-based therapies.

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References