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CD4$^+$IL-21$^+$T cells are correlated with regulatory T cells and IL-21 promotes regulatory T cells survival during HIV infection

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ABSTRACT

Introduction: IL-21 enhances T and natural killer cells survival and antiviral functions without promoting T cell activation during HIV infection, which makes it a better adjuvant in anti-HIV immunotherapy. Due to the pleiotropy and redundancy of cytokines, it is vital to have a comprehensive knowledge of the role of IL-21 in the regulation of immune responses. Regulatory T cells (Tregs) play an important role in immune regulation and are a determinant of immune therapeutic efficacy in certain circumstances. In this study, we explored the direct effect of IL-21 on Tregs during HIV infection, which has not been addressed before.

Methods: Thirty-four HIV treatment-naïve patients were enrolled and the relationship between CD4$^+$IL-21$^+$T cells and Tregs were studied. The effects of IL-21 on CD4$^+$CD25$^+$CD127^{low} Tregs' apoptosis, proliferation, and CTLA-4 and TGF-β expression in HIV-infected patients was investigated and compared with the effect of other common γ-chain cytokines.

Results: We found the percentage and absolute numbers of CD4$^+$IL-21$^+$T cells were positively related to the frequency or absolute numbers of CD4$^+$CD25$^+$ or CD4$^+$CD25$^+$CD127^{low} Tregs. Compared with the media-alone control, IL-21, IL-7, and IL-15 could significantly reduce apoptosis of Tregs ($p < 0.05$). IL-21 did not promote the proliferation of Tregs as compared with media alone, while IL-2, IL-7, and IL-15 could significantly increase the proliferation of Tregs ($p < 0.05$). IL-21 enhanced CTLA-4 expression by Tregs ($p < 0.05$), but could not induce TGF-β secretion of Tregs from HIV infected patients. There were no significant differences of the fold induction of apoptosis, proliferation, or CTLA-4 and TGF-β expression by Tregs from HIV-infected patients and normal controls after IL-21 treatment. *In vitro* experiment showed that pretreatment with IL-21 significantly enhanced the suppressive effect of Tregs on CD8+ T cells' IFN-γ expression.

Conclusion: We conclude that IL-21 promotes the survival and CTLA-4 expression of Tregs and enhanced the suppressive capacity of Tregs during HIV infection. These results broaden the understanding of HIV pathogenesis and provide critical information for HIV interventions.

1. Introduction

HIV infection is associated with a progressive decline in circulating CD4$^+$ T cells and a concomitant loss of immune function [1,2]. Although antiretroviral therapy (ART) regimens have proven to be effective in controlling active HIV replication, complete recovery of CD4$^+$ T-cell counts does not always occur, even among patients who display high levels of virologic control [3]. Furthermore, prolonged treatment comes with other problems such as drug resistance, side effects, and reduced adherence to the medication regimen. Accordingly, different adjuvant therapies, including immune modulation, are being tested in clinical trials or are under consideration in hopes of addressing these remaining challenges [4].

Common γ-chain (γc) cytokines – including interleukin (IL)-2, IL-4, IL-7, IL-9, IL-15 and IL-21 – regulate a variety of cellular responses such as proliferation, differentiation, and survival [5]. Of these, interleukin-21 was discovered relatively recently [6]. Mainly produced by CD4$^+$ T cells [7–9], IL-21 affects an extremely broad set of target cells including T cells, B cells, NK cells, NKT cells,

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and DCs [10]. During HIV infection, IL-21 promotes the survival of $CD4^+$ and $CD8^+$ T cells as well as NK cells; IL-21 also enhances $CD8^+$ T cell and NK cell antiviral function [11–13]. In addition, IL-21 directly suppresses HIV-1 replication through the promotion of microRNA-29 transcription [14]. Unlike other γc cytokines, IL-21 does not promote T cell activation or proliferation when it enhances the function of cytotoxic cells [11–13,15–19]. This prominent characteristic of IL-21 led to the hypothesis that IL-21, when used as an immunotherapeutic agent, was potentially capable of improving immune reconstruction without promoting immune activation and virus replication [11,12,15]. However, due to the pleiotropy and redundancy of cytokines [20], it is vital to have a comprehensive knowledge of the role of IL-21 in the regulation of immune responses.

Regulatory T cells (Tregs) play a prominent role in chronic viral infections by limiting viral-specific immune responses and immune activation [21]. Tregs constitutively express the high-affinity IL-2Rαβγ complex, therefore IL-2 plays a very important role in regulating the Treg population [22]. Two large clinical trials have revealed that IL-2 administration in HIV-infected patients induced a newly expanded population of immunosuppressive Tregs [23,24]. The induction of Tregs may be the most plausible explanation why IL-2 administration plus ART yielded no clinical benefit in either study when compared to ART alone [23,24]. These results highlight the importance of considering the effects of Tregs in potential immune interventions. Tregs express the IL-21 receptor [25,26], indicating that IL-21 has the potential to affect Tregs. The reported direct effects of IL-21 on the expansion, survival, and function of Tregs have been inconsistent [27–30]. Some reports indicate that IL-21 renders T cells resistant to Treg-mediated suppression, but has no direct effect on Treg viability, activation, or function [25,26,31–34]. By contrast, previous studies revealed that IL-21 was able to affect Tregs, either by decreasing the number and function of Tregs [35,36] or by increasing Tregs' frequency and expression of Foxp3 [37–40] during viral infection or tumor development. The inconsistent results suggest the effect of IL-21 on Tregs varies depending on the disease, the anatomic site, or the physiological condition of the patient. To our knowledge, the effect of IL-21 on Tregs' survival and function has not yet been clarified in HIV infection.

In this study, we investigated the relationship between $CD4^+$IL-21^+T cells and Tregs in HIV-infected patients. We then analyzed the effects of IL-21 on Treg apoptosis, proliferation, the expression of the key functional molecules, CTLA-4 and TGF-β and Treg's suppression activity. We found a positive relationship between $CD4^+$-IL-21^+T cells and Tregs in HIV-infected patients. Although IL-21 did not promote the proliferation of Tregs of HIV-infected patients in contrast to other γc cytokines, IL-21 could decrease Treg apoptosis and CTLA-4 expression and enhance Tregs' suppressive activity *in vitro*. Our results expand the knowledge of the function of IL-21 in HIV infection, which is helpful in the development of new immunotherapeutic strategies.

2. Materials and methods

2.1. Subjects

Thirty-four treatment-naïve HIV-infected patients, including 33 males and 1 female (median age: 36 years) were enrolled in this study. The absolute $CD4^+$ T cell numbers and Log10 viral loads of these patients were 405 ± 202 cells/μL and 4.11 ± 0.90 copies/mL (mean ± SD), respectively. Ethical approval for this study was obtained from the local ethical review committee and written informed consent for participation in the study was obtained from all patients.

2.2. Flow cytometric analysis

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by Ficoll centrifugation. The following monoclonal antibodies (mAbs) and reagents were used in this study: FITC-conjugated anti-CD8, APC-Cy7-conjugated anti-CD4, PerCP-conjugated anti-CD3, PE-Cy7-conjugated anti-CD25, PE-conjugated anti-TGF-β, APC-conjugated anti-CD127, PE-conjugated anti-CTLA-4, PE-conjugated anti-IFN-γ, PE-conjugated anti-Annexin V, 7-AAD and purified anti-CD3 mAb (BD Biosciences); PE-conjugated anti-IL-21 (eBioscience); Recombinant hIL-21 (Biosource), recombinant hIL-2, hIL-7, and hIL-15 (R&D Systems). For the detection of $CD4^+$IL-21^+T cells, 0.5×10^6 PBMCs were co-cultured for 6 h with phorbol 12-myristate 13-acetate (PMA, 50 ng/mL, Sigma-Aldrich, St Louis, MI, USA) and ionomycin (0.75 μg/mL, Sigma-Aldrich) at 37 °C, 5% CO_2; GolgiStop (500 μg/mL, BD Biosciences) was added after the first 1 h of culture. The cells were stained with anti-CD3-PerCP and anti-CD8-FITC followed by intracellular staining using Cytofix/Cytoperm Kit (BD Biosciences) with PE-conjugated IL-21. For the detection of Tregs, 1×10^6 cells were incubated with anti-CD3-PerCP, anti-CD4-APC-Cy7, anti-CD25-PE-Cy7, and anti-CD127-APC for 45 min on ice. The cells were washed, fixed in 1% paraformaldehyde in PBS prior to acquisition on LSR II (Becton Dickinson, San Jose, CA). $CD4^+$-$CD25^+$ Tregs or $CD4^+CD25^+CD127^{low}$ Tregs were identified. The absolute numbers of Tregs were calculated by multiplying the frequencies of Tregs by the absolute numbers of $CD4^+$T cells. Because PMA induced the down-regulation of the CD4 molecule, $CD4^+$IL-21^+T cells in our study were identified as $CD3^+CD8^-$IL-21^+ cells. The absolute numbers of $CD4^+$IL-21^+T cells were calculated by multiply the frequencies of $CD4^+$IL-21^+T by the absolute numbers of $CD4^+$T cells.

2.3. Proliferation and apoptosis of Tregs

For proliferation detection, PBMCs were labeled with CFSE at a concentration of 4 μM/5×10^6 cells for 10 min at 37 °C. After washing 1640 complete media supplemented with 10% FBS, cells were cultured in 96-well plates (200 μL) at 37 °C, 5% CO_2 with IL-21 (100 ng/mL), IL-2 (200 U/mL), IL-7 (50 ng/mL), IL-15 (50 ng/mL) or anti-CD3 (0.5 μg/mL) for 5 days. After culture, the cells were washed once and then stained with anti-CD3-PerCP, anti-CD4-APC-Cy7, anti-CD25-PE-Cy7, and anti-CD127-APC. For apoptosis detection, PBMCs were cultured for 3 days with IL-21 (100 ng/mL), IL-2 (200 U/mL), IL-7 (50 ng/mL), IL-15 (50 ng/mL), and anti-CD3 (0.5 μg/mL). After culture, the cells were stained with anti-CD3-APC-Cy7, anti-CD4-FITC, anti-CD25-PE-Cy7, and CD127-APC for 30 min followed by staining with 5 μL 7-AAD and anti-Annexin V-PE for 15 min before data acquisition. Cells were acquired on LSR II (Becton Dickinson) and analyzed using FACSDiva software.

2.4. Intracellular detection of the expression of CTLA-4 and TGF-β within Tregs

PBMCs were cultured with IL-21 (100 ng/mL), IL-2 (200 U/mL), IL-7 (50 ng/mL), IL-15 (50 ng/mL), and anti-CD3 (0.5 μg/mL). For the detection of CTLA-4 expression, the cells were stained with anti-CD3-PerCP, anti-CD4-APC-Cy7, anti-CD25-PE-Cy7, and CD127-APC after 3 days of culture followed by intracellular staining with anti-CTLA-4-PE. For TGF-β detection, PBMCs were cultured for 5 days with the cytokines mentioned above and GolgiStop was added during the last 10 h. The cells were then stained with anti-CD3-APC-Cy7, anti-CD4-FITC, anti-CD25-PE-Cy7, and CD127-APC followed by intracellular anti-PE-TGF-β