Published in final edited form as:

Hum Immunol. 2011 February ; 72(2): 115–123. doi:10.1016/j.humimm.2010.10.015.

IL-21 and cellular activation concurrently induce potent cytotoxic function and promote antiviral activity in human CD8 T cells

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Abstract

Infection with HIV-1 induces a progressive deterioration of the immune system that ultimately leads to AIDS. Mouse models indicate that the common gamma chain (γ_c)-sharing cytokine interleukin (IL)-21 and its receptor (IL-21R) play a crucial role in maintaining polyfunctional T cell responses during chronic viral infections. Therefore, we analyzed the ability of this cytokine to modulate the properties of human CD8 T cells in comparison to other γ_c -sharing cytokines (IL-2, IL-7 and IL-15). CD8 T cells from healthy volunteers were stimulated *in vitro* via T cell receptor signals to mimic the heightened status of immune activation of HIV-infected patients. The administration of IL-21 up-regulated cytotoxic effector function and the expression of the costimulatory molecule CD28. Notably, this outcome was not accompanied by increased cellular proliferation or activation. Moreover, IL-21 promoted antiviral activity while not inducing HIV-1 replication *in vitro*. Thus, IL-21 may be a favorable molecule for immunotherapy and a suitable vaccine adjuvant in HIV-infected individuals.

Keywords

IL-21; CD8 T lymphocytes; cytotoxicity; HIV; immunotherapy

Introduction

The main function of CD8 T cells, also referred to as cytotoxic T lymphocytes (CTL), is to kill virally infected and transformed tumor cells. Antigen (Ag) encounter of a CD8 T cell induces the acquisition of cytotoxic effector functions. These functions are predominantly mediated by the release of perforin, a membrane-disrupting protein, and granzymes, a family

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Authorship

A.P. performed experiments, analyzed results and wrote the paper; M.F.P. performed experiments; H.S. designed the virological experiments; S.P. designed the overall research; ML assisted in research design. ML and SP helped in writing the paper..

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of serine proteases [1,2]. After Ag encounter, CTL also undergo changes in their phenotype that reflect differences in homing, life span, proliferative capacity and ability to produce cytokines [3]. Changes in the expression of surface markers have allowed the classification of Ag-experienced CD8 T cells into central memory (T_{CM} ; CD27+ CD28+ CCR7+ CD45RA-), effector memory (T_{EM} ; CD27+ CD28- CCR7- CD45RA-) and effectors (T_E ; CD27- CD28- CCR7- CD45RA+/-) [4].

In CTL, T cell receptor (TCR) plus cytokine signals can induce the expression of perforin by activating key transcription factors, including Stat 3 and 5 [5,6], T-bet [7], Eomesodermin (EOMES) [8] and Runx3 [9,10]. The transcriptional regulation of the granzyme gene family is less understood, but it may involve overlapping as well as distinct signaling pathways [10,11].

Interleukin (IL)-21, the sixth member of the family of common gamma chain (γ_c)-sharing cytokines, is an important immunomodulator of CD8 T cells [12]. IL-21 on its own has little effect on mouse CTL *in vitro*, but it induces their expansion and cytotoxic activity in the presence of IL-15 and IL-7 [13]. Moreover, IL-21 enhances the capacity of CD8 T cells to mediate tumor regression upon adoptive transfer [14]. Due to its ability to promote antitumor CTL-mediated responses, IL-21 has entered human clinical trials in patients with metastatic melanoma and renal cell carcinoma, with demonstrable antitumor activity [15–17].

IL-21 may also play a pivotal role in controlling chronic viral infections. In fact, both *Il21*^{-/-} and *Il21r*^{-/-} mice chronically infected with lymphocytic choriomeningitis virus (LCMV) mount reduced antiviral CTL responses and fail to contain the infection as compared to wild-type animals [18–20].

We previously reported that *ex vivo* incubation of human peripheral blood lymphocytes with IL-21 enhanced perforin levels preferentially in CD8 T cells from virally controlled HIV+ patients under antiretroviral treatment (ART) as compared to uninfected donors [21]. Since HIV infection is accompanied by chronic immune activation [22], we tested in this report the hypothesis that cellular activation plays a key role in determining the responsiveness of CTL to IL-21 versus that to other γ_c -sharing cytokines. Furthermore, we studied the effects of IL-21 on HIV replication in infected CD4 T cells.

Materials and Methods

Reagents

The following anti-human monoclonal antibodies (MoAbs) were purchased from BD Biosciences (San Jose, CA, USA): Purified anti-CD28 (α CD28), anti-CD4 and anti-CD8 peridinin chlorophyll protein (PerCP), anti-CD3 allophycocyanin (APC), anti-perforin fluorescein isothiocyanate (FITC), anti-CD45RA FITC and V450, anti-CD62L APC, anti-CD25 APC, anti-CD38 FITC, anti-CD69 APC, anti-CD107a APC, anti-CD127 phycoerythrin (PE), anti-CD27 FITC, anti-CD28 PE, anti-PD-1 PE, anti-Ki-67 FITC, Annexin V FITC, anti-phosphoStat3 PE and anti-phosphoStat5 PE. The purified anti-CD3 (α CD3, clone OKT3) was obtained from eBioscience (San Diego, CA, USA). Anti-IL-21R PE MoAb was purchased from R&D Systems (Minneapolis, MN, USA) and anti-granzyme B PE MoAb from Serotec (Oxford, UK).

Recombinant human (rh)-IL-2 was obtained from Roche (Basel, Switzerland). Rh-IL-7 and rh-IL-15 were purchased from R&D Systems. Rh-IL-21, phytohemagglutinin (PHA) P form and Iscove's modified Dulbecco's medium (IMDM) were obtained from Invitrogen (Carlsbad, CA, USA).

Cell isolation and culture conditions

Peripheral venous blood from healthy volunteers was collected into heparinized tubes under an Institutional Review Board approved protocol at the University of Miami.

CD3, CD4 and CD8 T cells were isolated with > 95% purity using RosetteSep Enrichment Cocktails (StemCell Technologies, Vancouver, Canada), according to manufacturer's instructions. Naïve CD8 T cells (CD3⁺ CD8⁺ CD45RA⁺ CD62L⁺) were isolated with > 99.7% purity using a FACS Aria[™] cell sorter (BD Biosciences, USA). Purified cells were resuspended at 10⁶ cells/ml in IMDM containing L-glutamine, 10% heat inactivated fetal bovine serum (Atlanta Biologicals, Norcross, GA, USA) and 50 µg/ml gentamicin (Invitrogen, USA). Cells were cultured either in medium alone or in the presence of plate-bound αCD3 (1 µg/ml) and soluble αCD28 (1 µg/ml) for 2 days, then washed, resuspended in fresh medium and recultured in medium alone or supplemented with IL-2 (200 U/ml), IL-7 (10 ng/ml), IL-15 (50 ng/ml) or IL-21 (50 ng/ml).

Immunofluorescent staining and flow cytometry analysis

Following stimulation, cells were stained with MoAbs for surface immunophenotyping, then fixed, permeabilized with Cytotfix/Cytoperm buffer (BD Biosciences, USA) and stained with MoAbs specific for intracellular antigens. After washing, cells were fixed in 1% paraformaldehyde (PFA, Electron Microscopy Science, Darmstadt, Germany) and acquired on BD[™] LSR II or FACSCalibur (BD Biosciences, USA). Between 100,000 and 500,000 events were collected for each sample. Data were analyzed with FlowJo software version 6.3.3 (Tree Star, San Carlos, CA, USA).

Real-Time PCR

Total RNA was extracted from CD8 T cells using RNeasy kit (Qiagen, Valencia, CA, USA) and DNase-treated, according to the manufacturer's protocol. cDNA was synthesized using the Omniscript Reverse Transcription kit (Qiagen, USA) with random hexaprimers. Relative expression levels of the specific genes were quantified with the StepOne[™] Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Primers and probes were obtained from Applied Biosystems and used according to standard methodologies. *HPRT* and *18s* RNA levels were used to normalize the samples.

Redirected cytotoxicity assay (RCA)

CD3 T cells were activated with αCD3 + αCD28, cultured in the presence of different γ_c-sharing cytokines as previously described and used as effector (E) cells. The mouse cell line P815 served as target (T) cells. Target cells were labeled with the fluorescent cell linker PKH-26 Red (Sigma-Aldrich, St. Louis, MO, USA), extensively washed in complete medium, incubated for 30 minutes on ice with 10 µg/ml of αCD3 MoAb, washed and resuspended at 5×10⁵ cells/ml. Effector cells were added to 50,000 target cells in 96-well U-bottom plates to yield E:T ratios of 1:1, 2.5:1 and 10:1; controls included target and effector cells alone. The plates were incubated at 37°C for 4 hours. Cells were then harvested, washed and stained with Annexin V FITC. Target cells were gated according to side scatter and PKH-26 fluorescence; a total of 20,000 target cells was acquired for each sample. Percent lysis of target cells was calculated as: [(% Annexin V⁺ cells in sample – % Annexin V⁺ cells in negative control)/(100 – % Annexin V⁺ cells in negative control)] × 100, according to published methods [23].

Detection of granzyme B activity into target cells

Effector cells were generated as above and co-cultured at a ratio 10:1 with P815 cells (target) that were previously coated with αCD3 MoAb and labeled with the fluorescent cell

marker TFL4. The fluorogenic granzyme B substrate solution was added to the co-cultures and cells were incubated at 37°C for 2 hours, as per manufacturer's instructions (GranToxiLux, OncoImmunin, Gaithersburg, MD, USA). At the end of the incubation, cells were washed and acquired by flow cytometry. Target cells were gated according to side scatter and TFL4 signal (FL4 channel). The fluorescent signal generated by the cleavage of granzyme B substrate in the target cells was detected in the FL2 channel and the transfer of granzyme B activity was expressed as percentage of FL2+ target cells [24].

Degranulation assay

Purified CD8 T cells were stimulated with α CD3 + α CD28 and cytokines as described above. After 6 days of culture, cells were resuspended in fresh medium at 10^6 cells/ml, transferred to a 96-well plate pre-coated with α CD3 and cultured for 4 hours in the presence of soluble α CD28, anti-CD107a APC MoAb and monensin sodium salt (2 μ M), according to published methods [25]. Following stimulation, cells were washed, resuspended in 1% PFA and acquired on the flow cytometer.

Confocal microscopy

Purified CD8 T cells were pre-activated with α CD3 + α CD28 for 2 days, then cultured in medium or in the presence of IL-2 or IL-21 and harvested after 4 days of cytokine treatment. Cells were washed, permeabilized with Cytotfix/Cytoperm buffer and stained with anti-CD107a APC and anti-granzyme B PE, washed, fixed in 1% PFA and stained with the nuclear dye DAPI (4,6 diamidino-2-phenylindole). After another wash, cells were transferred to glass bottom petri dishes for acquisition. Samples were acquired with a Zeiss LSM 510 UV confocal microscope (63 $\times$ objective, water immersion). Images were analyzed with AxioVision LE software, version 4.7 (Zeiss, Göttingen, Germany). Images are presented as single confocal sections.

In vitro HIV-1 infection of CD4 T cells and evaluation of viral replication

Purified CD4 T lymphocytes were activated for 2 days with IL-2 (20 U/ml) and PHA (5 μ g/ml), then infected with primary R5-tropic HIV-1 at multiplicity of infection of 0.1 for 2 hours at 37°C. After 3 washes, cells were resuspended in complete medium supplemented with IL-2 (20 U/ml) and virus was allowed to replicate for 3 days. At this point cells were washed, split and cultured in medium alone or with IL-2 (200 U/ml) or IL-21 (50 ng/ml). Supernatants were collected every 3 days and replenished with medium and freshly added cytokines. Supernatants were filtered and assayed with HIV-1 p24 ELISA kit (PerkinElmer, Waltham, MA, USA).

To evaluate inhibition of viral replication by cytokine-treated CD8 T cells, CD4 T cells from healthy volunteers were infected *in vitro* as described above. Concurrently, autologous CD8 T lymphocytes were activated for 2 days with α CD3 + α CD28, then washed and cultured in medium alone or in the presence of IL-2 versus IL-21 for 3 days. At this point, CD4 and CD8 T cells were resuspended in fresh medium and co-cultured at 1:1 ratio; wells with only CD4 T cells served as control. Supernatants were collected every 3 days and filtered. The cells were replenished with fresh medium; no exogenous cytokines were added to the co-cultures. Viral infectious units were evaluated by infecting the reporter cell line TZM-bl with the culture supernatants [26]. Briefly, TZM-bl cells were seeded in 96-well plates 24 hours prior to infection, treated with 40 μ g/ml DEAE-dextran (Sigma-Aldrich, USA) and infected for 2 hours at 37°C with the supernatants obtained from the CD4/CD8 co-cultures. After this incubation, medium was added to the wells and TZM-bl cells were incubated for 4 days at 37°C. Cells were then washed and incubated for 30 minutes at 37°C with β -galactosidase assay reagent (Thermo Scientific, Waltham, MA, USA). The reaction was stopped by adding 1M Na₂CO₃ and plates were immediately read at 405 nm. Inhibition of

viral replication was calculated with the formula: $1 - (\text{O.D. for experimental wells} / \text{O.D. for control wells}) \times 100$, adapted from published methods [27].

Statistical analysis

Statistical analysis was performed with GraphPad Prism software version 5.0b (GraphPad Software, La Jolla, CA, USA), using Student's *t*-test and one-way ANOVA. Statistical significance was defined as $p < 0.05$. Results are presented as mean $\pm$ standard deviation (SD).

Results

IL-21 induces maximum cytotoxic activity in pre-activated T cells

T lymphocytes isolated from healthy donors were activated with $\alpha\text{CD3} + \alpha\text{CD28}$ in the presence of IL-21 versus other γ_c -sharing cytokines (IL-2, IL-7 or IL-15), as described in the Methods. TCR/CD28 stimulation of CD8 T cells significantly increased IL-21R expression, as compared to control cultures ($p < 0.01$) within the first 2 days (Supplementary Figure 1A). By day 6, the expression of IL-21R on CD8 T cells was induced by IL-21, both at the mRNA (data not shown) and at the protein level (Supplementary Figure 1B), consistent with previous observations [28]. To further validate the culture system, cytokine-induced phosphorylation of Stat molecules was analyzed. IL-21R signaling primarily involves Stat3, with partial activation of Stat5 [29]. IL-21 predominantly induced Stat3 phosphorylation also in our *in vitro* culture system of TCR/CD28-activated CD8 T cells. In contrast, the other γ_c -sharing cytokines tested preferentially activated Stat5 (Supplementary Figure 1C).

We analyzed the role of TCR/CD28 pre-stimulation and IL-21 treatment in the induction of cytotoxicity using redirected lysis assays. Killing of target cells by pre-activated IL-21-treated T cells was 25 times higher than that observed for non pre-activated IL-21-treated T cells ($p < 0.01$, Figure 1A). TCR/CD28 activation itself promoted cytotoxic activity as compared to unstimulated T cells, but this was significantly lower than that of $\alpha\text{CD3} + \alpha\text{CD28}$ -activated IL-21-treated T cells ($p < 0.05$, Figure 1A).

Comparison of the cytotoxic potential of T-cell blasts cultured with IL-21 versus other γ_c -sharing cytokines, namely IL-2, IL-7 and IL-15, was performed. Redirected lysis of target cells promoted by pre-activated IL-21-treated CD3 T lymphocytes was approximately two times higher than what was observed for the other cytokines tested ($p < 0.01$, Figure 1B–C). Addition of EGTA (ethyleneglycoltetraacetic acid) during the cytotoxicity assay reduced the lysis of target cells (data not shown), suggesting that the killing activity promoted by IL-21 was largely dependent on the Ca^{2+} -induced release of cytotoxic granules [30].

To confirm these data, we analyzed the transfer of granzyme B from CTL into target cells. Effector and target cells were co-cultured at a 10:1 ratio in the presence of granzyme B substrate solution [24]. Transfer of granzyme B activity promoted by IL-21-treated T-cell blasts was significantly higher than that promoted by IL-2-treated cells ($p < 0.05$, Figure 1D–E). This was consistent with the higher ability of IL-21-treated CTL to degranulate in response to TCR stimulation, as compared to IL-2-treated CTL ($p < 0.05$, Figure 1F).

Taken together, the results from these experiments show that TCR/CD28 plus IL-21 signals induce cytotoxic activity and granule release to levels exceeding the other γ_c -sharing cytokines tested.

IL-21 promotes CTL-mediated antiviral activity *in vitro* without enhancing HIV-1 replication

Since we observed that the combined IL-21 plus TCR/CD28 signals enhance cytotoxic mechanisms in CD8 T cells, we next investigated how this cytokine may influence CTL-mediated control of HIV replication. IL-2 can induce *in vitro* suppression of HIV replication via CTL-mediated lysis of infected CD4 T cells [31]. Thus, we compared the antiviral activity of CD8 T lymphocytes treated with IL-21 versus IL-2. CD8 T cells were activated with α CD3 + α CD28 and treated with IL-21 or IL-2, and subsequently co-cultured with autologous CD4 T cells that had been infected *in vitro* with HIV. Viral replication was assessed after 6 days of co-culture by measuring the amount of viral infectious units in the collected supernatants (as described in the Methods). IL-21-treated CTL inhibited viral replication similarly to IL-2-treated CTL (Figure 2A). As expected, HIV p24 levels in the same supernatants were proportionally reduced (data not shown). Several pro-inflammatory and immunoregulatory cytokines promote HIV replication in infected CD4 T cells and monocytes [32]. Among these, IL-2 induces HIV replication *in vitro*, probably as consequence of the increased cellular activation directly induced by the cytokine [32]. IL-21, unlike IL-2, did not induce expression of activation or proliferation markers in TCR-activated CD4 T cells (Figure 2B). In view of this, we speculated that IL-21, unlike IL-2, would not promote HIV replication. To verify this assumption, PHA-treated *in vitro*-infected CD4 T cells were cultured with these two cytokines and viral replication was assessed by measuring HIV p24 levels in the culture supernatants. p24 levels detected in IL-21-treated CD4 T-cell blasts were similar to the control condition and about ten times lower than the levels detected in cultures treated with IL-2 ($p < 0.01$, Figure 2C). With a combination of IL-2 and IL-21, viral replication was similar to that observed with IL-2 alone (data not shown).

Overall, these data indicate that IL-21 can induce antiviral activity in CTL without promoting HIV replication in CD4 T cells.

IL-21 induces maximal levels of effector molecules in TCR-activated CD8 T cells

Because TCR/CD28 signal combined with IL-21 induced maximum granule-mediated cytotoxicity (Figure 1), we analyzed the intracellular content of perforin and granzyme B in purified CD8 T cells by flow cytometry. The enhanced cytotoxic function of the IL-21-treated CTL was accompanied by maximal accumulation of cytotoxic molecules, as compared to the other cytokines tested (Figure 3A). Similar results were obtained when analyzing total CD3 T cells from the same individuals (data not shown), indicating that in this *in vitro* system IL-21 acts directly on CD8 T cells.

In time-course experiments conducted in activated, cytokine-treated CTL, the progressive accumulation of granzyme B preceded the accumulation of perforin (Figure 3B). This gradual acquisition of cytotoxic molecules appeared to be succinct and we arbitrarily divided CTL into six distinct granule stages according to the content of granzyme B and perforin (Stage 0: granzyme B- perforin-; stage 1: granzyme B+ perforin -; stage 2: granzyme B++ perforin+; stage 3: granzyme B+++ perforin +; stage 4: granzyme B+++ perforin++; stage 5: granzyme B+++ perforin+++). According to this staging, IL-21 was the only γ_c -sharing cytokine that pushed cells to the cytotoxic stage 5. IL-2 and IL-15 pushed CTL up to stage 4 and IL-7 up to stage 3 (Figure 3C).

To investigate whether IL-21 induced the number or the size of the cytotoxic granules, the cytokine-treated CTL were co-stained with antibodies specific for granzyme B and the lysosomal marker CD107a. Confocal microscopy revealed that the number of granules remained similar between the conditions tested, as determined by CD107a staining.

However, the filling of the granules, measured as content of granzyme B, was increased in the IL-21-treated CTL as compared to IL-2 (Supplementary Figure 2).

Similarly to what was observed in total CD8 T cells, the concomitant TCR/CD28 plus IL-21 signal promoted higher acquisition of cytotoxic molecules also in naïve CD8 T lymphocytes, than TCR/CD28 plus IL-2 (Figure 4A, representative histograms). At the mRNA level, however, IL-21 and IL-2 equally induced the expression of the genes encoding granzyme B and perforin (Figure 4B). Therefore, even if IL-21 and IL-2 similarly induced the expression of the genes encoding for cytotoxic molecules in CTL, the accumulation of cytotoxic molecules was greater in the IL-21-treated CD8 T cells. Moreover, IL-2 induced the expression of the transcription factors implicated in CTL differentiation (namely *EOMES* and *RUNX3*), while IL-21 only up-regulated *EOMES* (Figure 4B) [8, 9]. A preliminary analysis of gene expression in TCR-activated, cytokine-treated T_{CM} and T_{EM} CD8 T cells suggested similar results for *PRF1*, *GZMB* and the genes encoding for relevant cytotoxic transcription factors, indicating that TCR plus IL-21 signals induce a similar cytotoxic program in CD8 T cells at distinct stages of differentiation (unpublished observation).

IL-21 promotes the acquisition of a memory-like phenotype in TCR-stimulated CD8 T lymphocytes

Our results demonstrated that IL-21 promotes maximal accumulation of perforin and granzyme B in CTL. Of interest was the question whether this cytokine would also influence the expression of surface markers conventionally used to characterize the differentiation stages of Ag-experienced T cells [4]. When purified naïve CD8 T cells were activated with α CD3 + α CD28 and cytokine-treated, the expression of the costimulatory molecules CD27 and CD28 was increased after incubation with IL-21, but not with IL-2 ($p < 0.01$, Figure 5A). Similarly, CD62L and CD127, the alpha chain of IL-7R, were retained after incubation with IL-21, but markedly reduced after IL-2 treatment, as compared to the control condition (data not shown).

Moreover, IL-21 promoted the expression of BCL6, a transcription factor that is crucial for maintaining Ag-experienced memory CD8 T cells, in contrast to IL-2 (Figure 5B) [34–35]. Thus, naïve CD8 T cells primed in the presence of IL-21 acquire markers predominantly associated with a memory rather than effector phenotype.

IL-21 has a marginal effect on CTL activation and proliferation

To investigate how IL-21 would affect activation state and proliferation of CTL, the expression of several activation markers, namely CD25, CD69, CD38, PD-1 and the nuclear antigen Ki-67, that is expressed by proliferating cells, was investigated. Levels of activation markers in IL-21-treated CTL were comparable to those observed in the control (medium alone) and IL-7-treated cultures, while they were up-regulated by IL-2 and IL-15 (Figure 6A and B). This was similar to what was observed in the CD4 T cells isolated from the same donors (see Figure 2B). Ki-67 levels in IL-21-treated CTL remained similar to the medium control, while both IL-2 and IL-15 induced high expression of this molecule. This suggests that IL-21 promoted little or no cellular proliferation, consistent with the cell counts (data not shown).

Discussion

HIV-1 infection progressively induces loss of CD4 T helper lymphocytes, consequently affecting the major source for γ -sharing cytokines. The disruption of this cytokine network may account for the defects in T lymphocytes of infected patients [36]. In this context, exogenous cytokines, alone or in combination with antiretroviral drugs, have been

administered with the goal of promoting antiviral immune responses in HIV-infected patients. So far, IL-2 and IL-7 have been tested in clinical trials. The use of IL-2 induced toxicity [37] and showed no clinical benefit despite the induction of CD4 T cell proliferation [38], probably due to predominant expansion of two distinct T cell populations (CD4+ CD25^{low} CD127^{low} FOXP3⁺ and CD4+ CD25^{high} CD127^{low} FOXP3^{high}) with phenotypic and functional characteristics of T regulatory cells [39,40]. IL-7 was shown to support CD4 and CD8 T cell proliferation without toxicity and changes in viral load, thus ameliorating the lymphopenic status of HIV-infected individuals [41,42].

The interest in investigating IL-21 in the context of HIV infection arose from the original observation that IL-21 enhances perforin expression preferentially in CTL from HIV-infected individuals [21]. Remarkably, IL-21 plasma levels are reduced in HIV-infected individuals and ART only partially amends this defect [43]. Our study suggests that IL-21 has properties that may favor its use as immunotherapeutic agent for HIV-infected individuals. Demonstrable attributes of IL-21 include the promotion of cytotoxic function (Figure 1) without CD8 or CD4 T cell activation (Figures 2 and 6) or HIV replication (Figure 2), and the induction of the costimulatory molecule CD28 (Figure 5), as discussed below.

Recent findings about CD8 T cell-mediated antiviral activity suggest that non-cytolytic suppression of viral replication is an important mechanism of viral control in SIV chronically infected Rhesus Macaques [44,45]. Other studies indicate that efficacious cytotoxic functions is beneficial for HIV-infected individuals, because CTL from HIV controllers (HIV-infected subjects with persistently undetectable plasma viral load in the absence of ART) have a higher ability to eliminate HIV-infected CD4 T cells as compared to CTL from progressors [46]. These findings support the idea that vigorous CD8 T cell responses play a key role in controlling chronic viral infections. Here we describe for the first time that IL-21, compared to other γ_c -sharing cytokines, generates CTL with greater perforin and granzyme B loading, a superior ability to degranulate upon TCR stimulation and, ultimately, enhanced cytotoxic activity. Thus, it is possible that this cytokine has the ability to promote cytotoxic effector function *in vivo* in HIV-infected subjects. Importantly, the increase in cytotoxic activity was not accompanied by augmentation of PD-1 expression. PD-1, a negative regulatory molecule of the B7 family, is induced by TCR signal and it exerts its suppressive function by inhibiting TCR-mediated events in T cells. PD-1 expression is increased in HIV-specific CD8 T cells and it is associated with CTL dysfunction [47,48]. Although it has been shown that γ_c -sharing cytokines, especially IL-15 and IL-2, promote PD-1 expression in T cells [49], we observed only minimal induction of this molecule by IL-21. This suggests that IL-21 may promote cytotoxic function in CD8 T cells without compromising their responsiveness to TCR signals.

A previous study from Hogg et al. showed that the γ_c -sharing cytokines IL-21 and IL-15 induce granzyme, a molecule with broad-spectrum antimicrobial activity [50,51], in CD8 T cells through Stat3/Stat5 signaling pathway [52]. The study also demonstrated that the induction of perforin and granzyme by these two cytokines is reduced in PBMC infected *in vitro* with HIV as compared to non-infected PBMC, due to a diminished Stat3/Stat5 activation in the presence of HIV. Here we showed that IL-21 potently enhances cytotoxic function in CD8 T cells by up-regulating perforin and granzyme B. Taken together, these two studies suggest that IL-21 may increase the ability of CTL to eliminate cells infected by viruses and other microorganisms.

HIV replication requires CD4 T cell activation. For a molecule to be considered for immunotherapy, it is important to demonstrate that it does not enhance viral replication. This was the expectation with IL-21, as it did not promote cellular activation in our *in vitro*

model. Indeed, IL-21 only minimally induced viral replication, as opposed to IL-2. Concomitantly, IL-21-treated CTL displayed antiviral activity comparable to that induced by IL-2.

The acquisition of cytotoxic molecules in IL-21-treated CTL was accompanied by sustained expression of markers characteristic for memory T cells (i.e. the transcription factor BCL6 and the surface markers CD27, CD28, CD62L and CD127). This observation is consistent with previous studies that described *in vivo* accumulation of memory CD8 T cells in transgenic mice overexpressing IL-21 [53]. Memory T cells are capable of self-renewal and maintain the ability for secondary proliferation and differentiation [54]. In this regard, IL-21 would be favorably considered for adoptive immunotherapy of HIV infection (as already suggested by a murine tumor model [14]). CD28 is a key costimulatory molecule required for proper T cell activation. The expression of this receptor is reduced on HIV-specific CD8 T cells. This defect raises the threshold for TCR-induced cell activation, and possibly accounts for the altered CD8 T cell activation observed in the course of HIV infection [55,56]. Promotion of sustained levels of CD28 by IL-21 could overcome this defect in T cell activation.

How IL-21 induces greater accumulation of cytotoxic molecules in TCR/CD28-activated CD8 T cells than IL-2 has not yet been clarified. The results of this study indicate that IL-2 concomitantly up-regulated *RUNX3* and *EOMES*, known to orchestrate the expression of cytotoxic effector genes. In contrast, IL-21 only induced *EOMES*, suggesting that these two cytokines control perforin and granzyme B transcription in CD8 T cells, at least in part, through distinct mechanisms. Furthermore, although IL-21 and IL-2 induced mRNA expression of *GZMB* and *PRF1* equally, IL-21 promoted higher accumulation of granzyme B and perforin at the protein level. Under these circumstances, IL-21 did not inhibit spontaneous degranulation, which could account for the increased accumulation of effector molecules. Therefore, a post-transcriptional regulation mechanism, perhaps similar to that described for perforin expression by NK cells [57], may promote mRNA translation or protein stability of the cytotoxic molecules.

In conclusion, IL-21 appears to be a favorable immunomodulatory agent for induction of CTL function in HIV-infected individuals because it can boost CTL-mediated antiviral activity without promoting HIV replication. Furthermore, the fact that IL-21, unlike IL-2, promotes a memory-like phenotype in T cells, suggests that this cytokine may be a suitable adjuvant to therapeutic vaccines. Further studies in non-human primate models of SIV infection are warranted to investigate the clinical utility of IL-21.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr. O. Umland at Flow Cytometry Core Facility and Dr. G. McNamara at Analytical Imaging Core Facility at Diabetes Research Institute, University of Miami, for cell sorting and confocal microscopy analysis, respectively. We are especially grateful to Dr. L. Reidy for reviewing the manuscript. This work was supported by NIH grant AI077501 (S.P.) and by the Laboratory Sciences Core of University of Miami Developmental Center for AIDS Research grant 5P30AI073961 (SP).

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Figure 1. Cytotoxic activity in IL-21-treated T-cell blasts

(A) Non activated (α CD3/ α CD28 $^{-}$) versus activated (α CD3/ α CD28 $^{+}$) purified CD3 T cells were cultured with or without IL-21 for 6 days and used as effectors (E) in a redirected cytotoxicity assay (RCA) against α CD3-coated P815 cells (target cells, T) at a ratio E:T 10:1. Percent target lysis induced by pre-activated IL-21-treated CD3 T cells was set as 100% and other values were expressed accordingly. The graph summarizes the results of 3 independent experiments. (B–C) TCR/CD28-activated CD3 T cells cultured with γ_c -sharing cytokines for 6 days served as effectors in a RCA. Figure B shows histograms of annexin V staining in target cells of a representative experiment where E and T were co-cultured at a 10:1 ratio. Figure C summarizes the results of 5 independent experiments. Percentage of target cell lysis induced by IL-21-treated T cell blasts was set as 100%. (D–E) E and T cells generated as above were co-cultured at a ratio of 10:1. Transfer of granzyme B activity into target cells was measured as cleavage of the specific fluorogenic substrate. Histograms in panel D depict a representative experiment; Figure E shows results from 5 independent experiments. (F) Purified CD8 T cells were activated with α CD3 + α CD28 and subsequently treated with IL-21 or IL-2. On day 6 cells were re-stimulated with α CD3 + α CD28 and degranulation was measured by CD107a staining. The graph shows the percentage of CD8+ CD107a+ cells obtained in 4 independent experiments. * $p < 0.05$, ** $p < 0.01$.

Figure 2. Effects of IL-21 on HIV-1 replication *in vitro*

(A) TCR/CD28-activated cytokine-treated CD8 T cells from healthy donors were co-cultured with PHA-stimulated autologous CD4 T cells infected *in vitro* with HIV at a CD8:CD4 ratio of 1:1. Viral infectious units in the supernatants were measured after 6 days of co-culture using the reporter cell line TZM-bl. The percentage of inhibition of viral replication was measured as: $[1 - (\text{O.D. for experimental wells} / \text{O.D. for control wells})] \times 100$, as described in the Methods. (B) TCR/CD28-stimulated CD4 T cells were cultured in medium alone or in the presence of IL-21 versus IL-2. Expression of proliferation and activation markers was evaluated by flow cytometry after 2 days of TCR/CD28 activation (empty histograms) and after 4 more days of incubation with the different γ_c -sharing cytokines (day 6, filled histograms). Histograms are representative of 3 independent experiments. (C) PHA-stimulated CD4 T cells from healthy donors were infected *in vitro* with HIV-1 and cultured in medium alone or in the presence of IL-2 or IL-21. Viral replication was determined by measuring HIV-1 p24 levels in the culture supernatants at day 6 post infection. Graphs show mean $\pm$ SD of 3 independent experiments. * $p < 0.05$, ** $p < 0.01$.

Figure 3. Granzyme B and perforin levels in IL-21-treated CD8 T-cell blasts

(A) Purified CD8 T cells were activated with α CD3 + α CD28 and cultured with IL-21 versus other γ_c -cytokines; expression of cytotoxic molecules was evaluated by flow cytometry (MFI, mean fluorescence intensity) after 2 days of TCR/CD28 activation (empty histograms and bars) and after 4 more days of incubation with cytokines (day 6, filled histograms and bars). Graphs show mean $\pm$ SD of 6 independent experiments. (B–C) Time-course accumulation of cytotoxic molecules in purified CD8 T cells cultured as above. Cells were stained at baseline (day 0), then TCR/CD28-activated for 2 days and subsequently cultured in the presence of IL-21 or other γ_c -sharing cytokines. Cells were staged from 0 to 5 according to their content of granzyme B and perforin that was measured by flow cytometry. Dot plots are representative of 3 independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 4. Expression of cytotoxic molecules induced by IL-21 in naïve CD8 T cells upon TCR/CD28-mediated activation

Purified naïve CD8 T cells were stimulated with α CD3 + α CD28 and incubated with IL-2 or IL-21. Both protein and gene expression were determined on day 5 by flow cytometry (A) and Real-Time PCR (B), respectively. Real-Time PCR data are presented as fold increase over control cultures (med), with med RNA levels normalized to 1. Graphs show mean of 3 independent experiments. * $p < 0.05$, ** $p < 0.01$.

Figure 5. Expression of differentiation markers in IL-21-treated CTL

Purified naïve CD8 T cells were cultured for 2 days with α CD3 + α CD28 in medium alone or with IL-2 or IL-21, then washed and recultured in medium alone or with cytokines for 3 more days. Expression of differentiation markers was evaluated at the end of the culture (day 5) by flow cytometry (A; MFI, mean fluorescence intensity) and Real-Time PCR (B). Real-Time PCR data are presented as folds over control cultures (med). Graphs show mean $\pm$ SD of 3 to 4 independent experiments. * $p < 0.05$, ** $p < 0.01$.

Purified CD8 T cells were stimulated with α CD3 + α CD28 and cultured in the presence of IL-21 versus other γ_c -sharing cytokines. Expression of proliferation and activation markers was evaluated by flow cytometry after 2 days of TCR/CD28 activation (empty histograms and bars) and after 4 more days of incubation with different γ_c -sharing cytokines (day 6, filled histograms and bars). (A) Graphs show percentage of positive cells (mean $\pm$ SD of 5 independent experiments). (B) PD-1 expression was evaluated at the end of the culture (day 6). Histograms report the mean fluorescence intensity values for each culture condition. * $p < 0.05$, ** $p < 0.01$.