HIV-1-specific IL-21+ CD4+ T cell responses contribute to durable viral control through the modulation of HIV-specific CD8+ T cell function

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Abstract

Functional defects in cytotoxic CD8+ T cell (CTL) responses arise in chronic human viral infections, but the mechanisms involved are not well understood. In mice, CD4 cell mediated IL-21 production is necessary for the maintenance of CD8+ T cell function and control of persistent viral infections. To investigate the potential role of IL-21 in a chronic human viral infection, we studied the rare subset of HIV-1 controllers, who are able to spontaneously control HIV-1 replication without treatment. HIV-specific triggering of IL-21 by CD4+ T-cells was significantly enriched in these persons (p=0.0007) while isolated loss of IL-21 secreting CD4+ T-cells was characteristic for subjects with persistent viremia and progressive disease. IL-21 responses were mediated by recognition of discrete epitopes largely in the Gag protein, and expansion of IL-21+CD4+ T cells in acute infection resulted in lower viral set-points (p=0.002). Moreover, IL-21 production by CD4+ T cells of HIV controllers enhanced perforin production by HIV-1-specific CD8+ T cells from chronic progressors even in late stages of disease, and HIV-1-specific effector CD8+ T cells showed enhanced ability to efficiently inhibit viral replication in-vitro after IL-21 binding. These data suggest that HIV-1-specific IL-21+CD4+ T cell responses might contribute to the control of viral replication in humans and are likely to be of great importance for vaccine design.

Introduction

CD4+ T cell help is essential to generate long-lived antiviral CD8+ T cell memory (17-18). Although antigen-specific CD8+ T cells can be primed in the absence of CD4+ T cell help, secondary expansion upon antigen reencounter is inefficient under such circumstances (7, 11, 18, 22). Progressive loss of CD4+ T cells in HIV, HCV and HBV infection has been associated with dysfunction of virus-specific CD8+ T cells and ineffective containment of these chronic viral infections. Moreover, under repetitive antigenic stimulation, virus-specific CTL become increasingly impaired, exhibiting
decreased effector functions and up-regulation of negative immunoregulatory molecules (2, 19). This dysfunction is likewise more severe in the absence of CD4+ T cell help (21).

The nature of CD4 help required to control chronic human infections remains unclear. In mice, recent studies indicate that CD4+ T cell production of interleukin-21 (IL-21), a common γ-chain cytokine, is required for maintenance of CD8+ T cell function in persistent but not resolving viral infections (3-4, 23). During LCMV infection, expansion of CD4+ T helper cells producing IL-21 is required for sustained CD8+ T cell proliferation and control of viremia. In contrast, mice lacking either IL-21 or the IL-21 receptor are more susceptible to uncontrolled chronic LCMV infection, providing evidence that this cytokine is a key regulator of viral control in a murine model of chronic viral infection.

Material and Methods

Study subjects

HIV-1 seropositive subjects and uninfected healthy control volunteers were recruited at the Massachusetts General Hospital after providing written, informed consent for participation in the study (supplemental table 1). Persons were divided into four groups: HIV-1 controllers (viral loads <2000 copies/mL for at least 1 year in the absence of any antiretroviral therapy), HIV-1 chronic progressors (treatment naïve and >10,000 copies RNA/ml), subjects on antiretroviral therapy (>6 months treatment) with fully suppressed viral load (<50 copies/mL) and HIV- individuals (see table 1 for patient characteristics). Subjects with acute infection were enrolled at the HIV clinic Jessen, Jessen, Stein in Berlin, Germany or Fenway community Health Center, Boston, USA. Acute HIV-1 infection was defined as negative anti-HIV-p24 antibody ELISA or positive ELISA with less <3 bands in the Western blot. The present study was approved by MGH institutional review boards and was conducted in accordance with the organization’s human experimentation guidelines.
Measurement of IL-21 secretion using a combined transwell-microbead capture assay

CD8+ T cells were depleted from PBMCs using RosetteSep Technology (Stemcell Technology) or by magnetic beads (MACS). Efficiency of depletion was consistently above 95%. Depletion with lower efficiency was not used in subsequent assays. CD4+ T cells counts in comparison to other PBMCs after depletion was assessed but was not significantly different between the groups. 5x10^5 peripheral blood mononuclear cells (PBMCs) in R10 (RPMI 1640 supplemented with 10% heat-inactivated FCS, penicillin/streptomycin) were placed in the bottom chamber of 96-well HTS transwell permeable support (Corning Inc., 0.4 µm pore polycarbonate membranes) and stimulated with pooled HIV peptides (Gag, Pol, Nef, gp120, gp41 and a pool containing the proteins Vif, Vpr, Vpu, Rev, Tat (VVVRT) as well as PMA/ionomycin (AG Scientific) as a positive control. For the Gag epitope mapping, cells were stimulated using single overlapping peptides (OLPs) spanning the entire Gag protein (66 peptides synthesized by the MGH Peptide Core Facility). These peptides are 15- to 20-mers overlapping by 10 amino acids and were based on the HIV clade B consensus sequence available from the Los Alamos National Laboratory HIV immunology database (www.hiv.lanl.gov). Luminex anti-IL-21 microbeads (Milliplex MAP Kit, Millipore) were added to the upper chamber in the transwell assays. After 40h incubation at 37°C, 5% CO2, microbeads were harvested, washed twice and processed according to the manufacturer’s protocol. Plates were run in a Bio-Plex reader (Bio-Plex 100IS, Bio-Rad). Results were analyzed using a (5PL-fit) standard curve derived from recombinant cytokine standards.

Measurement of IL-21 secretion by flow cytometry

2x10^6 PBMCs were stimulated with respective HIV peptide pools in R10 containing 50ng/ml IL-21. SEB served as a positive control, and the respective media as a negative control. Cells were stimulated for 6hrs in the presence of brefeldin A and monensin at 37°C, 5% CO2. Cells were then stained with an amine-live/dead discrimination dye.
After a wash with 2%FCS/PBS cells were stained using anti-CD3, anti-CD4, anti-CD8, anti-CD19, anti-CCR4, anti-CCR5, anti-CCR6, anti-CXCR3, anti-CXCR4, anti-CXCR5 or anti-IL23R. Cells were then fixed and permeabilized using FixPerm A and B (Caltag, Invitrogen) according to manufacturer’s procedure. Cells were then stained for IFNγ, IL-21, IL17, IL5, IL4 and TNFα. Samples were acquired on a BD LSRRII (BD Biosciences) multicolor flow cytometer and analyzed using FlowJo 8.8.6 software (TreeStar). Initial gating was on the lymphocyte population; forward scatter width (FSC-W) versus height (FSC-H) plot was used to remove doublets. CD4+ T cells were defined as Viability dye-, CD19-, CD8-, CD3+ and CD4+ lymphocytes.

Quantitation of IL-21-Receptor (IL-21R) expression on antigen-specific CD8+ T-cells using multiparameter flow cytometry

Cryopreserved PBMCs were thawed, resuspended at 1×10^6 cells/mL in R10 medium and rested for 2 h at 37 °C; 5% CO2. Cells were then washed with PBS and stained with APC-labeled HLA class I tetramers/pentamers (Beckman-Coulter, Proimmune) refolded with the respective CD8+ T cell epitopes. Following 20 min incubation at room temperature, cells were washed with PBS/2%FCS, resuspended in R10, and stimulated for 5 h at 37 °C; 5% CO2 in the presence or absence of IL-21 (50ng/ml) and in the presence or absence of CD8+ T cell optimal epitopes (2µg/mL). Cells were then washed with PBS/2%FCS and stained for intracellular amino groups to discriminate between live and dead cells (violet viability dye, Invitrogen). After an additional wash the following surface antibodies were added: anti-IL-21R-PE (R&D), anti-CD3-Qdot (Invitrogen), anti-CD8-APC-Cy7 (BD Biosciences). Cells were washed and fixed with 1% paraformaldehyde. Samples were acquired on a BD LSRRII (BD Biosciences) multicolor flow cytometer and analyzed using FlowJo 8.8.6 software (TreeStar). Initial gating was on the lymphocyte population; forward scatter width (FSC-W) versus height (FSC-H) plot was used to remove doublets. Expression of IL-21R was measured on HIV-1-specific cells (tetramer+CD8+CD3+CD19-CD14-cells).

Functional analysis of antigen-specific CD8+ T-cells by flow cytometry
Functionality of CD8+ T cells were assessed as previously described (16). Briefly, cells were stimulated for 5h with IL-21 (50 ng/mL) and washed. Then, Gag peptide pool (each OLP 2 µg/mL) anti-CD28/anti-CD49d (BD Biosciences) and anti-CD107a-PECy5 (BD Biosciences) were incubated in the presence of monensin (BD Biosciences) and brefeldin A (Sigma-Aldrich) for 5h at 37°C. Cell were consecutively washed, stained with viability dye (Invitrogen) and anti-CD3-Qdot605 (Invitrogen), anti-CD8-APC-Cy7 (BD Biosciences), anti-PD1-APC (eBioscience) and anti-CD4-Qdot655 (Invitrogen). Cells were fixed with 1% paraformaldehyde, washed, permeabilized (Fix Perm B solution; Caltag Laboratories) and stained intracellularly using a panel of anti-IL2-FITC, anti-IFNγ-PEcy7, anti-TNFα-Alexa700, anti-MIP1β-PE (all from BD Biosciences). Samples were analyzed as described above.

Perforin expression in antigen-specific CD8+ T-cells by flow cytometry

PBMCs were incubated for 5h in the presence of IL-2, IL-4, IL-7, IL-15 or IL-21. Cells were stained as described above and intracellularly stained using anti-perforin-PE (Tepnel, Lifecodes Corp.).

Measurement of perforin expression by real-time quantitative PCR

CD8+ T cells were isolated from PBMCs by MACS sorting (Miltenyi Biotec) and cultured in R10 medium in the presence or absence of IL-21 for 5h at 37°C / 5% CO2. Cells were then resuspended in RNeasy lysis buffer (Qiagen) containing 1% β-Mercaptoethanol following manufacturer’s protocol. Briefly, total RNA was isolated using RNeasy kit (Qiagen) and treated with DNase (Invitrogen), followed by reverse transcription using a High Capacity RNA-to-cDNA Master Mix (Applied Biosystems). Quantitative real-time PCR was performed using Brilliant SYBR Green (Stratagene) on a Mx3005P real time thermocycler (Stratagene). Each sample included a no-reverse transcriptase control and all reactions were done in triplicate; results were normalized to GAPDH and expressed in relative mRNA expression level. Primers were designed to
span at least one intron and product specificity was verified by Tm measurement and
standard PCR. Primers included GAPDH (forward) 5’-ACCCACTCCTCCACCTTTGA-
3’ (reverse) 5’-TGGTGAGGCCAGGGTCTTAC-3’; IL-21 (forward) 5’-
AGCTCCAAGGTAAGATCG-3’ and (reverse) 5’-
CTGTCTTCTCCCTGCATTTGTG-3’; Perforin (forward) 5’-
CATGTAACCAGGGCCAAAGTC-3’ and (reverse) 5’-
ATGAAGTGGTGCCGGATAGTTG-3’; Granzyme A (forward) 5’-
CCTATTCAAGACCATCATGG-3’ and (reverse) 5’-
TGGCTCTTCCCTGGTTATTG-3’; Granzyme B (forward) 5’-
GGCTTCCTGATACGAGACGAC-3’ and (reverse) 5’-
TCCTGACTGTGTCATCTTACC-3’.

Viral inhibition assay

Autologous CD4 lymphocytes were prepared as described(8) and infected at day 3 with
the NL4-3 HIV-1 isolate at a multiplicity of infection (MOI) 0.01 for 4h at 37°C, washed
twice, resuspended in medium and plated at 10^5 cells/well. Effector cells, consisting of
purified CD8+ T cells from the same donor were separated, rested for 2d in medium
alone and pre-incubated in the presence or absence of IL-21 for 5hr. Cells were then
washed and added at an effector: target ratio of 1:1 and co-incubated in IL2 supplemented
media at 37°C 5%CO2. Infected autologous CD4+ T cells alone and uninfected CD4+T
cells served as positive and negative control, respectively. Supernatant was collected at
baseline, day 3, 5 and 7. Viral replication was quantified by p24 Elisa (Perkin&Elmer) in
duplicates.

Transwell co-incubation assay

Cryopreserved PBMCs from HIV controllers were CD8+ T cell depleted (MACS), either
rested or stimulated with Gag peptide pool (2µg/mL) for 1h at 37°C, washed, and 1
million cells were plated in the bottom chambers of transwell plates. 300,000 CD8+ T-
cells from non-controllers were added in the upper chambers and when indicated, IL-21 signaling was blocked using anti-IL-21 (10µg/mL) and anti-IL-21 receptor (25µg/mL) antibodies. Cells were co-incubated for 12h at 37°C, 5% CO2. CD8+ T-cells were harvested and perforin expression was measured by quantitative PCR.

Results and Discussion

To explore the potential role of IL-21 as a mediator of CD4+ T cell help in a chronic human viral infection, we analyzed this cytokine in persons infected with HIV-1, examining those with both progressive uncontrolled viremia—which is the usual outcome of infection—as well as the rare subset of HIV-1 controllers(13), who spontaneously maintain viral loads at low levels in the absence of antiretroviral therapy (<2000 RNA copies/ml plasma).

We first developed a novel technique to quantitate IL-21 secretion by CD4+ T-cells: co-culturing anti-IL-21 micro-beads separated by a 0.4µm transwell permeable membrane from CD8+ depleted PBMCs stimulated in vitro. Released IL-21 was captured by the beads and then measured by Multiplex Technology. Using this approach (Figure 1A), we found significantly elevated levels of IL-21 secretion by CD4+ T cells from HIV-1 controllers upon stimulation with phorbol myristate acetate (PMA) and ionomycin compared to HIV-1 infected persons with persistent viremia (p=0.02), subjects with persistent viremia successfully suppressed with antiretroviral therapy (ART; p=0.001), and HIV-1 uninfected individuals (p=0.003), as previously described (6). However, there was no significant difference in the IL-21 secretion between HIV chronic progressors and ART treated or HIV-1 uninfected individuals.

Given the above association between IL-21 production and disease outcome, we next evaluated whether IL-21 production might be triggered by virus-specific immune cells. We stimulated CD8–depleted T cells from each group with overlapping HIV Gag peptides, known to be the major stimulus for CD4+ T cell IFNγ production in chronic HIV-1 infection(9, 14). The same capture assay reproducibly detected HIV-1-specific IL-21+CD4+ T cell responses in HIV-1 controllers (Figure 1B) as well as a nearly
complete lack of these responses in HIV-1 progressors (p<0.001). In contrast, these HIV-
1 progressors exhibited robust IL-21+CD4+ T cell responses against the CMV peptide
pp65 at similar levels as observed in HIV controllers, pointing to an antigen specific loss
of IL-21 secreting CD4 T-cells in chronic, progressive HIV-1 infection (Figure 1B).
Although Gag was the strongest inducer, IL-21 production was occasionally triggered by
other viral proteins, including gp41, gp120 and Pol. These responses were present
particular in subjects on fully suppressive antiretroviral therapy; however, no significant
differences were observed between the patient groups (see supplemental Figure 1). In
addition, a small number of HIV controllers did not mount a detectable IL21+CD4+ T
cell response. While the reason for this occurrence is not straightforward, several
potential explanations are plausible, including viral escape from IL21+CD4+ T cell
responses, IL21 responses below the limit of detection, or alternative, IL-21
independent mechanisms of antiviral function in these subjects.
Given the prominent role of Gag in the induction of HIV-specific CD4 T-cell responses,
we next assessed the relative secretion of IL-21 and IFNγ upon antigenic stimulation with
individual overlapping 15-20mer Gag peptides in CD8-depleted T-cells of 5 HIV-1
controllers. In each individual tested, multiple different Gag peptides were able to induce
IL-21 secretion; although some peptides elicited both IL-21 and IFNγ, the majority of
immunogenic peptides were discordant for these responses, indicating that the effector
populations were largely non-overlapping (Figure 1C). These responses were enriched in
the Gag region p24 when overlapping peptides spanning the entire Gag region was tested
(Figure 1C and supplemental table 2).
Given the robust differences in IL-21 CD4+ T-cell frequencies between individuals with
controlled versus progressive disease, we were interested whether there is a causative link
between HIV-1-specific IL-21+CD4+ T cell response induction and viral control. We
therefore selected 12 individuals with acute HIV infection, following them over one year
into the chronic phase of infection. All subjects had been diagnosed with symptomatic
acute HIV-1 infection, with a negative or indeterminate Western Blot (<3 bands positive),
in the presence of detectable viremia (average viral load: 2,951,214copies/ml; range:
250,000-10,000,000copies/ml). **Gag-specific IL-21+CD4+ T cell responses were**
detectable in most patients within six months of acute HIV-1 infection. However, six subjects were able to maintain robust IL-21+CD4+ T cell responses in the first 12 months of the infection and had a significantly lower viral load compared to subjects lacking these responses (4,810-39,400 copies/ml versus 55,900-1,600,000 copies/ml \((p=0.002)\) (Figure 2A and Figure 2B), yet both groups did not differ in their viral loads at baseline level (1,780,667 versus 2,042,800 \((p=0.98)\)). Moreover, the same individuals maintained stable frequencies or decreasing IFN\(\gamma\) or IL-2 secreting Gag-specific CD4+ T-cell responses over the course of 1 year (Figure 2C and D), suggesting a preferential expansion of HIV-1-specific IL21+CD4+ T-cells in HIV-1 controllers. Interestingly, subjects able to maintain their IL21 response show a trend -but not significant peak- of HIV-specific IL2+CD4+ T cell responses, which warrants further investigation. The selective expansion of IL-21 secreting CD4+ T cells was HIV-specific, as CMV-specific IL-21+ CD4 T-cells remained unchanged from acute to chronic infection (Figure 2E). It is important to note, however, that most likely IL-21 responses alone do not explain enhanced control of viral replication, but rather contributes to enhanced ability to control viral replication. Interestingly, viral suppression by ART restored IL-21 secretion of Gag specific CD4+ T cells, although the levels were significantly lower than in HIV-1 controllers \((p=0.02)\). Longitudinal analysis following treatment in acute HIV-1 infection showed that these responses initially expanded but then declined (Figure 2F). Thus, our data indicate that the expansion of HIV specific IL-21+ CD4+ T-cells contributes to enhanced viral control early in infection and that the loss of IL-21 secreting CD4+ T-cells is characteristic of progressive HIV disease.

To further characterize the properties of the IL-21+CD4+ T cells we analyzed the phenotype of the HIV-1-specific IL-21+ CD4+ T cells in controllers by cytokine flow cytometry (Figure 3A). We were able to visualize clear but discrete Gag-specific IL-21+CD4+ T cell populations in these individuals after stimulation with Gag peptides in IL-21 supplemented media using intracellular cytokine staining. Addition of IL-21 was necessary to induce the previously described autocrine feedback regulation of IL-21 secretion(12). We compared HIV-1-specific CD4+ T cells that either secrete IFN\(\gamma\) or IL21 and observed that the IL21 secreting population exhibited higher expression levels of the HIV co-receptor CCR5, which has been linked to greater susceptibility to
infection in vivo(1). In addition, the IL21+ CD4+ T cells also showed increased expression of IL23R, CXCR3, CCR6 and CCR4, which have been previously described to also be increased on effector CD4+ T cells such as Th1 or Th17 cells (Figure 3A)(5, 12).

In addition, we confirmed our previous finding that IL21 and IFNγ are not necessarily co-secreted by flow cytometry for different Gag-specific CD4+ T cell responses (Figure 3B). To further characterize the functional properties of IL-21-secreting CD4+ T cells in humans and to investigate if IL-21+ CD4+ T-cells are able to secrete additional cytokines, we stimulated PBMCs from HIV-1 controllers for 6hrs with Staphylococcal enterotoxin B (SEB) in IL-21 supplemented media. Interestingly, while a fraction of the IL-21+CD4+ T cells were simultaneously secreting IFNγ or TNFα, a majority of these cells stained negative for both cytokines. Furthermore, we also detected IL-21+CD4+ T cells co-secreting the proinflammatory cytokine IL-17, confirming previous findings from the murine model (Figure 3C)(12). Although we cannot fully exclude that the addition of exogenous recombinant IL21 might skew the responses seen in vitro, these findings confirm via a separate approach the results of our multiplex bead based capture assay described above. Moreover, a recent publication by Iannello et al also suggests higher IL21 expression in CD4+ T cells from HIV controllers compared to progressors(6), which is in contrast to a publication by Yue et al. describing lower levels of IL-21 secreting CD4+ T cells in long-term non-progressors (<200 RNA copies/ml) (24). While the explanation for this discrepancy is not immediately apparent, it is interesting to note that Yue et al observe the highest level of HIV-specific IL21-secreting CD4+ T cells in individuals with “relative control” (defined as having viral loads between >200-20,000 RNA copies/ml). The divergent findings of the latter paper may also be due to detection limitations of the specific assay used.

As IL-21+ CD4+ T cells are major contributors to control in the setting of LCMV infection through the modulation of virus-specific CD8+ T cells, and because these cells
are markedly enriched in the setting of controlled HIV-1 infection, we next examined the influence of these responses on HIV-1-specific CD8+ T cells. We first determined IL-21 receptor (IL-21R) expression on tetramer positive HIV-specific CD8+ T cells of HIV-1 controllers compared to HIV-1 chronic progressors. We did not observe any differences in the expression levels of IL-21R at baseline (Figure 4A), but observed significant up-regulation of IL-21R on HIV-1-specific CD8+ T cells of chronic progressors following a 5 hr stimulation with the respective optimal peptide (p=0.02); the expression levels of IL-21R on HIV-1-specific CD8+ T cells of controllers did not significantly change. When we then added recombinant IL-21 together with the cognate peptide, the expression of IL-21R significantly decreased on HIV-1-specific CD8+ T cells of chronic progressors (p=0.02), but this had no impact on the expression level of IL-21R on HIV-1-specific CD8+ T cells of controllers. Thus, the expression levels of IL-21R on HIV-1-specific CD8+ T cells upon antigen stimulation is sensitive to external IL-21 and the presence of IL-21 secreting CD4+ T-cells in HIV controllers versus the lack of these cells in progressors might explain the discordant expression levels of IL-21R in both groups.

To further investigate whether HIV-1 specific IL-21+CD4+ T cell responses impact the functional profile of HIV-1-specific CD8+ T cells, we next stimulated PBMCs of HIV-1 controllers and progressors with pooled Gag peptides in the presence or absence of IL-21 supplemented media (Supplemental Figure 3). After a 5hr co-incubation period, we observed a significant increase of LAMP1 (CD107a) expression on HIV-1-specific CD8+ T cells from controllers as well as progressors, demonstrating the augmented ability of CD8 T cells to degranulate upon peptide stimulation in the presence of IL-21(p=0.0003; Figure 4B and Supplemental Figure 3); degranulation was more pronounced in HIV-1 progressors (black lines: p-value=0.002) compared to controllers (green lines: n.s.). In addition, we observed a similar effect for other effector cytokines such as IFNγ and TNFα. In contrast, no difference was observed in CD8+ T-cell secretion of IL-2 or MIP1β (data not shown), suggesting that IL-21 specifically increases the expression of antiviral effector molecules of HIV-specific CD8+ T cells.
Based on the observation of enhanced degranulation of HIV-specific CD8+ T cells in the presence of IL-21, we next determined whether IL-21 also changes the granule composition of perforin and granzyme in these cells. Previous reports have demonstrated that HIV-1-specific CD8+ T cells, under persistent antigenic stimulation, become dysfunctional and non-responsive to external signals(21). Thus, we assessed whether the mRNA expression levels of typical granule content, like granzyme A and B as well as perforin, change in HIV-1 specific CD8+ T cells after incubation with IL-21 (Figure 4C). Indeed, CD8+ T cells significantly upregulated both granzyme A and perforin mRNA expression levels after a short co-incubation with recombinant IL-21 (both p=0.002), while the upregulation of granzyme B was less pronounced but still significant (p=0.02). We next confirmed perforin upregulation on the protein level as measured by intracellular cytokine staining in tetramer+ HIV-1-specific CD8+ T cells (p=0.0004) (Figure 4D and Supplemental Figure 4). These changes were independent of whether the CD8+ T cells were derived from HIV-1 controllers or progressors (ratio controllers: 1.34±0.28 vs. progressors: 1.38±0.33, respectively).

To exclude the possibility that perforin upregulation is non-specifically mediated via γ-chain signaling, we repeated the experiment in three subjects, stimulating with a number of γ-chain cytokines: IL-2, IL-4, IL-7, IL-15 and IL-21 (see Supplemental Figure 5). Perforin was preferentially upregulated only following IL-15 and IL-21 incubation. Overall, these data demonstrate that IL-21 enhances the effector functions of HIV-1-specific CD8+ T cells independently of their exhaustion profile or the disease stage of the individual subjects.

We then further examined the IL-21 induced mechanism responsible for alterations in CD8+ T cell function. As previous reports have suggested that IL-21 can induce apoptosis, we simultaneously assessed the expression of perforin and Annexin V on HIV-1-specific CD8+ T cells over 24hrs in the presence of added IL-21. Both perforin loading and Annexin V expression peaked within 2 hrs on HIV-1-specific CD8+ T cells (Supplemental Figure 6). These findings suggest that IL-21 signaling can affect CD8+ T cells by inducing apoptosis or by increasing effector functions, supporting a model by
which CD4+ T helper cells can mediate the fine-tuned regulation of virus-specific CD8+ T cell responses through IL-21.

We next determined whether HIV-1-specific CD4+ T cells from controllers can directly exert the same effect as exogenous recombinant IL-21 on CD8+ T cells through IL-21 secretion. PBMCs from 6 controllers with strong IL-21 responses were depleted of CD8+ T cells and then pulsed with Gag peptide for 1hr. The remaining peptide was thoroughly removed by repetitive washes. We placed the peptide pulsed CD8 depleted PBMC from each individual in the bottom chamber of a transwell plate and compared their impact on CD8+ T cells from non-controllers, which were added to the upper chamber. Unstimulated CD8 depleted PBMC in the lower chamber with CD8+ T cells in the upper chamber served as a negative control (Figure 4E). The Gag-stimulated CD8 depleted PBMCs of controllers induced robust upregulation of perforin mRNA expression in the CD8+ T cells (p=0.01; Figure 4E). Addition of a neutralizing antibody to IL-21 significantly abrogated the effect (p=0.01) demonstrating the direct modulating role of IL-21 on CD8+ T-cell effector functions. However a potential inhibitory effect on the autocrine IL-21 secretion of CD4+ T cells might contribute as well. Taken together, these data demonstrate that HIV-1-specific IL-21-secreting CD8 depleted PBMC induce important effector functions in HIV-1-specific CD8+ T cells. This effect can be induced by the HIV-1-specific CD4+ T cells of HIV controllers and can improve the functionality of CD8+ T cells of progressors.

Finally, we assessed whether IL-21 secretion can improve the ability of HIV-1-specific CD8+ T cells to suppress viral replication in vitro. We selected 7 HIV-1 infected individuals for whom we had previously determined that the CD8+ T cells were minimally capable of inhibiting viral replication in vitro. CD8+ T cells were separated from PBMCs and rested in the absence of mitogens or cytokines for 2 days. Five hours before the co-culture experiment, CD8+ T cells were incubated in the presence or absence of IL-21 (50ng/ml). As expected, HIV-infected CD8+ T cells alone showed a high level of viral replication, and we observed no differences in the level of viral replication when autologous bulk CD8+ T cells were added (Figure 5A, representative result from one individual). However, addition of CD8+ T cells pre-incubated with low
levels of IL-21 for 5hrs resulted in a 1000 fold increase in the ability of the CD8+ T cells
to inhibit viral replication. We repeated this in 6 more subjects in whom we had
previously confirmed minimal inhibitory capacity of the peripheral CD8+ T cells. This
revealed heterogeneity among study subjects in their ability to suppress viral replication
after pre-incubation with IL-21 (Figure 5B). Although some individuals benefited
strongly from the IL-21 signal, others showed only minimal improvement in CD8+ T cell
mediated viral inhibition. Interestingly, those individuals who exhibited enhanced viral
inhibition after IL-21 pretreatment tended to be the subjects who upregulated perforin
expression the greatest as determined by flow cytometry (data not shown). Thus, IL-21
has the ability to substantially enhance the potential of HIV-1-specific CD8+ effector
cells to lyse HIV-1-infected cells by increasing the perforin content of cytolytic granules.

Understanding exactly how CD4+ T helper cells coordinate the immune system and
respond to immune challenges is crucial for the development of an effective HIV
vaccine(15, 20). Several previous studies have focused on a potential role of CD4+ T
cell help on HIV-specific CD8+ T cell responses. It has been demonstrated that in the
chronic phase of the infection, CD8+ T cells lose their ability to proliferate upon
antigen stimulation, but the presence of CD4+ T cells derived from cryopreserved
PBMCs was able to rescue their proliferative capacity (10) in a manner that has been
primarily attributed to the secretion of IL2. Interestingly, in the absence of other
cytokines such as IL15, IL21 does not induce proliferation of HIV-specific CD8+ T
cells(25). However, our data suggest that a different CD8+ T cell effector function –
namely cytolytic antiviral activity- can be rescued through the IL21-secretion by CD4+
T cells. Nevertheless, it is important to note that a limitation of both these studies is
that a singular helper function of CD4+ T cells has been assessed. It is likely that
several additional—and possibly unknown—helper functions act in concert to provide
optimal stimulation for CD8+ T cells in vivo.

Our data indicate that the ability of HIV-1-specific CD4+ T cells to improve the antiviral
efficiency of HIV-1-specific CD8+ T cells via IL-21 signaling is a central part of virus-
specific immunity. This effect is conserved early on in individuals with controlled
infection and is preferentially lost in subjects with chronic progressive HIV-1 infection
and likely contributes to the controller phenotype through augmentation of CD8 T cell effector function. Other factors, including epitope processing, antigen recognition, synapse formation, target cell adhesion, and signal transduction, are also critical elements in successful target cell lysis and are not likely to be affected by IL-21. This may help to explain not only the variability in IL-21 production in a small proportion of the controller patients, but also provides a basis for the heterogeneous effects of IL-21 on the ability of CD8+ T cells to kill infected CD4+ T cells in these subjects. However, the ability of IL-21 produced by HIV-1-specific CD4+ T cells of HIV-1 controllers to improve the effector functions of HIV-1-specific CD8+ T cells of HIV-1 progressors suggests that the induction and preservation of these responses is likely to be an important cornerstone for the generation of an effective HIV-1 vaccine.

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References


Figure Legends

Figure 1: IL-21 production by CD4 T cells in HIV-1 infected subjects.

CD8-depleted PBMCs from HIV-1 chronic progressors, ART treated patients, HIV-1 controllers, and HIV negative subjects were stimulated with PMA/Ionomycin (A) or with HIV Gag peptide pool or the CMV peptide pp65 (B) for 40h at 37°C in the bottom chamber of a transwell plate with anti-IL-21 Luminex beads co-incubated in the upper chamber. Beads were then used for the quantification of secreted IL-21 (pg/mL). Mann Whitney t-tests were performed; only significant p-values are indicated. Significantly higher IL-21 responses were detected in the controllers compared to the other groups. HIV-specific IL-21+ responses were directed against epitopes within the Gag protein in HIV-1 controllers whereas rare specific responses were detected in the progressors. (C) Analysis of the secretion of IL-21 and IFNγ in five HIV-1 controllers following stimulation with individual peptides spanning the HIV Gag proteins p17, p24 and p15. Color intensity indicates the relative magnitude of the IFNγ response and the IL-21 response to each peptide.

Figure 2: IL-21+ CD4+ T-cell responses in acute HIV infection
(A) 12 individuals with acute HIV infection were grouped based on their viral load set-points at 12 month after presentation (high: 55,900-1,600,000 copies/ml and low: 4,810-39,400 copies/ml). PBMCs from these individuals were obtained at baseline and 2, 6 and 12 month after initial presentation. PBMCs were CD8 depleted, stimulated with Gag peptide pool and IL-21 (B), IL-2 (C) and IFNγ (D) secretion was measured by Luminex. (E) Following the same protocol, CMV specific IL-21 secretion was assessed at the same time points in all Individuals. (F) shows longitudinal assessment of IL-21 production by Gag-stimulated CD4+ T cells in treated (n=4) and untreated (n=4) subjects during acute HIV-1 infection showing an initial increase in the early phase of acute infection in treated subjects which then decreased.

**Figure 3:** Phenotypic and functional characteristics of HIV-1-specific IL-21+ CD4+ T cells.

(A) Phenotypic (representative for 6 controllers) and (B) functional characterization of HIV-1-specific IL-21+ CD4+ T cells. For assessing intracellular cytokine secretion, cells of four HIV controllers were stimulated as indicated with Gag peptide pool or SEB for 5hrs in IL-21 supplemented media (50ng/ml). (C) Functional characterization of IL21+CD4+ T cells after SEB stimulation.

**Figure 4:** CD8+ T-cells responses to IL-21 stimulation.

(A) PBMCs from 8 HIV-1 progressors and 9 controllers were cultured for 5h in the presence or absence of HIV CD8+ optimal peptides and IL-21 (50ng/ml). Boxplots represent the IL-21R expression on antigen-specific (tetramer-positive) CD8+ T-cells detected by flow cytometry. (B) PBMCs of 16 HIV progressors (black) and 5 HIV controllers (green) were cultured with or without IL-21 and the percentage of CD8+ T-cells expressing CD107a, IFNγ or TNFα was assessed by flow-cytometry using intracellular cytokine staining. Figure C shows granzyme A, granzyme B and perforin mRNA up-regulation measured by qPCR on bulk CD8+ T-cells after IL-21 stimulation of...
PBMCs from 10 untreated HIV infected subjects. (D) IL-21 mediated perforin up-regulation in tetramer-positive CD8+ T-cells of 17 untreated HIV infected subjects was measured by flow-cytometry. These results together demonstrate the ability of IL-21 to increase both bulk and HIV-specific CD8+ T-cell cytotoxic potential of chronic progressors. (E) CD8 depleted PBMCs from 6 controllers were pre-stimulated with Gag peptides for 1h or left unstimulated. The cells were thoroughly washed and co-incubated for 12hrs in the bottom chamber of a transwell plate with CD8+ T-cells from a chronic progressor in the upper chamber. IL-21 blocking was performed by adding neutralizing anti-IL-21 and anti-IL-21-Receptor antibodies to the CD8+ T-cell in the upper chamber. After the incubation, CD8+ T-cells were harvested and perforin mRNA relative levels were assessed by qPCR (bar diagrams).

Figure 5. IL-21 induces suppression of HIV-1 replication by bulk CD8+ T cells.

In order to test if IL-21 can improve viral suppressive capacity of bulk CD8 T cells we selected subjects with a known lack of CD8+ T cell viral inhibition assessed prior to understand the impact of IL-21 on their inhibitory capacity. Figure A shows the representative results from one individual: Bulk CD8 T cells, isolated from frozen PBMCs by positive selection and rested for 3 days, failed to inhibit replication of HIV-1-virus NL4-3 (X4-tropic) in autologous CD4 T cells at a CD8 to CD4 T cell ratio of 1:1. However, pre-incubation of the CD8 T cells with IL-21 (50ng/ml) lead to a 1000 fold viral inhibition. Figure B: Comparing viral suppressive capacity of bulk CD8+ T cells with or without pre-incubation with IL-21 in seven subjects selected based on a previous assessment of their inhibitory capacity. First we confirmed that in the presence of CD8 T cells viral replication was not significantly reduced (log10 p24 (pg/ml): 5.18+0.36 versus 4.63±0.76, for CD4 controls and CD4:CD8 co-cultures, respectively; n.s.). However, pre-incubation of the CD8 T cells with IL-21 improved the inhibitory effect significantly (log10 p24 (pg/ml): 3.71±1.11; p<0.05).
Figure 3

A

- CCR5
- CXCR4
- CXCR5
- IL23R
- CXCR3
- CCR6
- CCR4

Legend:
- CD4+ T cells
- IL21+ CD4+ T cells

B

Gag-specific CD4+ T cell responses

- IFNγ
- IL21

C

- IFNγ
- TNFα
- IL17

Values:
- IFNγ: 21.9
- TNFα: 43.7
- IL17: 16.8

SEB control