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Effect of IL-7 Therapy on Phospho-Ribosomal Protein S6 and TRAF1 Expression in HIV-Specific CD8 T Cells in Patients Receiving Antiretroviral Therapy

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IL-7 therapy has been evaluated in patients who do not regain normal CD4 T cell counts after virologically successful antiretroviral therapy. IL-7 increases total circulating CD4 and CD8 T cell counts; however, its effect on HIV-specific CD8 T cells has not been fully examined. TRAF1, a prosurvival signaling adaptor required for 4-1BB–mediated costimulation, is lost from chronically stimulated virus-specific CD8 T cells with progression of HIV infection in humans and during chronic lymphocytic choriomeningitis infection in mice. Previous results showed that IL-7 can restore TRAF1 expression in virus-specific CD8 T cells in mice, rendering them sensitive to anti–4-1BB agonist therapy. In this article, we show that IL-7 therapy in humans increases the number of circulating HIV-specific CD8 T cells. For a subset of patients, we also observed an increased frequency of TRAF1$^+$ HIV-specific CD8 T cells 10 wk after completion of IL-7 treatment. IL-7 treatment increased levels of phospho-ribosomal protein S6 in HIV-specific CD8 T cells, suggesting increased activation of the metabolic checkpoint kinase mTORC1. Thus, IL-7 therapy in antiretroviral therapy–treated patients induces sustained changes in the number and phenotype of HIV-specific T cells. ***The Journal of Immunology*****, 2018, 200: 000–000.**

The factors that influence the number and function of Ag-specific CD8 T cells during persistent infection have been the subject of intensive investigation (1). Persistent stimulation of CD8 T cells is associated with sustained upregulation of the inhibitory receptor programmed death 1 (PD-1) (2–6), increased expression of T cell Ig and mucin protein 3 (Tim-3) (7), as well as impaired cytokine production (8).

Chronically stimulated T cells have decreased levels of TRAF1 (9), a prosurvival signaling adaptor that binds to the cytoplasmic tail of a subset of TNFR family members, including 4-1BB (CD137). TRAF1 contributes to effector and memory T cell survival and is essential for CD137-induced NF-κB and MAPK signaling (10–12). TRAF1 protein is lost from HIV-specific CD8 T cells with progression of infection, but maintained at higher levels in rare patients who spontaneously control HIV replication, also called viral controllers (9). TRAF1 is also lost from virus-specific T cells during persistent infection of mice with lymphocytic choriomeningitis virus (LCMV) clone 13, impairing the CD137 costimulatory pathway (9). Treatment of mice with rIL-7 increases TRAF1 levels in LCMV-specific CD8 T cells at the chronic stage of infection, thereby allowing anti-CD137 agonist therapy to reduce viral load (9).

IL-7 is a pleiotropic cytokine of critical importance in immune system development (13) and in the homeostasis of naive and memory T cells (14, 15). IL-7 therapy allows resolution of persistent LCMV infection (16, 17) and expands CD4 and CD8

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Abbreviations used in this article: ART, antiretroviral therapy; LCMV, lymphocytic choriomeningitis virus; MFI, mean fluorescent intensity; PD-1, programmed death 1; pS6, phospho-ribosomal protein S6; r-hIL-7, recombinant human IL-7; Tim-3, T cell Ig and mucin protein 3.

T cells in SIV-infected rhesus macaques, without increasing viremia (18, 19). IL-7 increases TRAF1 levels in HIV-specific CD8 T cells ex vivo (9). Thus, one benefit of IL-7 therapy may be to increase TRAF1 levels in chronically stimulated CD8 T cells.

The safety and efficacy of recombinant human IL-7 (r-hIL-7) in expanding T cells have been tested in refractory cancer patients (20), after hematopoietic stem cell transplantation (21), and in chronically HIV-1–infected patients whose CD4 T cell counts failed to recover after antiretroviral therapy (ART), despite successful suppression of plasma HIV load (22–25). Clinical studies have indicated that r-hIL-7 is well tolerated and induces sustained increases in naive and central memory CD4 and CD8 T cells, with intermittent blips in plasma HIV levels but without sustained increases in viremia (22–26). Central and transitional memory CD4 T cells contain a reservoir of latent HIV (27), and although the increase in T cell counts also increases the numbers of circulating CD4 central memory T cells containing proviral DNA, the clinical importance of this increase and whether it also reflects an increase in inducible infectious virus remains to be determined (28). Moreover, whether the benefit of r-hIL-7 in restoring CD4 and CD8 T cell counts outweighs the effect of increasing the number of cells harboring proviral DNA is unknown. Importantly, the effect of rIL-7 therapy on HIV-specific T cell responses has not been fully examined. In this study, we analyzed the quantitative and qualitative differences, including functional markers of chronically stimulated T cells, in HIV-specific $CD8^+$ T cell responses at baseline and 12 wk after the initiation of rIL-7 treatment. Although previous results showed that a single dose of IL-7 did not change the number of HIV-specific T cells (24), in this article, we report that 12 wk after the initiation of a single cycle of three weekly doses of systemic IL-7 administration (25) there is an overall increase in the number of HIV-specific CD8 T cells, as well as changes in their phenotype. Specifically, IL-7 treatment increased the level of the downstream mTORC1 target phospho-ribosomal protein S6 (pS6) in the HIV-specific T cells for all donors analyzed and increased the frequency of $TRAF1^+$ HIV-specific cells for a subset of donors.

Materials and Methods

HIV-infected study participants

Patients were participants in a substudy of the multicenter North American clinical trial CLI-107-13 INSPIRE-II, "An open-label, multi-center study of subcutaneous intermittent recombinant Interleukin-7 (CYT107) in chronically HIV-infected patients," sponsored by Cytheris, U.S. subsidiary of Cytheris, France (study registered at ClinicalTrials.gov, NCT01190111) (22, 23, 25). PBMCs were obtained by leukapheresis and cryopreserved until use. All patients gave informed consent as approved by the research ethics boards of all participating institutions. Patient characteristics are summarized in Table I.

Flow cytometry and reagents

Biotinylated MHC monomers (A2-SLYNTVATL, A2-ILKEPVHGV, B7-FPRIWLHGL, and B57-KAFSPEVIPMF) were obtained from the National Institute of Allergy and Infectious Diseases tetramer facility and conjugated to streptavidin allophycocyanin. PBMCs were surface-stained with fixable viability dye (eF506) from eBioscience (La Jolla, CA), and the Foxp3/Transcription Factor Staining Buffer Set (eBioscience) was used to fix and permeabilize cells for cytokine and TRAF1 intracellular staining. Human TRAF1-specific Ab 1F3 (29) (Serviceeinheit Monoklonale Antikörper; Institut für Molekulare Immunologie, Munich, Germany) was labeled with PE (Molecular Probes) or used unlabeled with goat anti-rat IgG (Poly4054)-PE secondary Ab (BioLegend, San Diego, CA). Anti–CD127 (A019D5)-AF488, anti–CD3 (OKT3)-Pacific Blue, anti–CD45RA (HI100)-BV605, anti–CD57-Pacific Blue, anti–KLRG-1 (2F1/KLRG1)-FITC, and anti–PD-1 (EH12.2H7)-PE-Cy7 were purchased from BioLegend. Anti–CD107a (H4A3)-FITC was purchased from BD Biosciences (San Jose, CA). BD Phosflow Perm/Wash Buffer I was used for Phosphoflow staining procedures. Phosphoflow Ab anti–pS6 S235/236 (D57.2.2E)-Pacific Blue was purchased from Cell Signaling Technology (Danvers, MA). Anti–TIM3 (344823)-AF700 was purchased from R&D Systems (Minneapolis, MN). Anti–CD8 (RPA-T8)-allophycocyanin-eF780, anti–granzyme B (GB11)-PE, anti–IFN-γ (4S.B3)-PE-Cy7, anti–IL-2 (MQ1-17H12)-allophycocyanin, and anti–pErk T202/Y204 (MILAN8R)-PerCPeF710 were purchased from eBioscience. All samples were fixed and acquired by flow cytometry on a BD LSR Fortessa X20 with FACSDiva software. Data analyses were performed on FlowJo v10.

Analysis of HIV-specific T cell populations

MHC-tetramers specific for immunodominant HIV epitopes were tested based on HLA type. Gating strategy is shown in Supplemental Fig. 1A. For analysis of cytokine production and degranulation, PBMCs were stimulated for 6 h with specific HIV peptides or no peptide control in the presence of GolgiStop and GolgiPlug (BD Biosciences).

Table I. Patient clinical data and summary of epitopes detected

SPC	Time Point	Age (y)	Sex	Rx	Coinfection	CD4	CD8	VL	HIV Tetramer	TRAF1 Increased?
16123	Baseline	50	M	Yes	No	364	699	<40	A2 (SL9)	Yes
	Week 12					981	1335	<40		
31209	Baseline	49	M	Yes	No	263	1118	<40	A2 (SL9, ILKE)	No
	Week 12					661	2663	<40		
31427	Baseline	30	M	Yes	No	274	293	<40	A2 (SL9, ILKE)	Yes
	Week 12					836	768	<40		
16206	Baseline	38	M	Yes	No	191	699	<40	B7 (FPRI)	Yes
	Week 12					441	1648	<40		
31210	Baseline	32	M	Yes	No	162	604	<40	A2 (SL9)	No
	Week 12					485	1292	<40		
31231	Baseline	42	M	Yes	No	365	588	<40	A2 (SL9)	No
	Week 12					838	979	<40		
31208	Baseline	41	M	Yes	No	191	350	<40	B7 (FPRI)	No
	Week 12					662	1342	<40		
31213	Baseline	45	M	Yes	No	457	342	<40	B7 (FPRI)	Yes
	Week 12					747	929	<40		
31206	Baseline	53	M	Yes	No	338	591	<40	B57 (KAFS)	No
	Week 12					451	729	<40		
16207	Baseline	55	M	Yes	No	155	1553	<40	B57 (KAFS)	Yes
	Week 12					282	2435	<40		

Summary of patient data including age, sex, treated (Rx), and CD4 and CD8 T cell counts before and 12 wk after first IL-7 treatment. HIV tetramer indicates population identified in the study participants. A2 epitopes were SL9 or ILKE, B7 (FPRI), and B57 (KAFS) as described in *Materials and Methods*; where both are listed, study participants had both HIV-specific CD8 T cell populations.

M, male; SPC, study participant code; VL, viral load.

Statistics

GraphPad software was used for statistical analyses. We used Mann–Whitney analysis for nonpaired analysis and Wilcoxon matched-pairs signed rank test for paired analysis; Spearman coefficient was used for analyses of correlation or linear regression. To determine whether TRAF1 expression in patients after IL-7 treatment is partitioned into two or more groups, we evaluated partition into cluster sets using the percentage of variance explained following K-means clustering, computed by dividing the between-cluster sum of squares statistic for each clustering by the total within-cluster sum of squares (for $K = 1$). The function *kmeans* in R was used to conduct the clustering analysis.

Results

Patient characteristics

All study participants had achieved plasma HIV levels <40 copies/ml for at least 6 mo after ART initiation and persistently low CD4 T cell counts (between 101 and 400 cells/μl) before IL-7 treatment as previously reported (22–25) (Table I). r-hIL-7, at a dose of 20 μg/kg, was given s.c. once weekly for 3 consecutive weeks. Most patients treated with r-hIL-7 maintained plasma viremia <40 copies/ml throughout the study, with occasional blips in a subset of patients of <100 copies/ml as previously described (25). We analyzed PBMCs at baseline and week 12 after the first cycle of r-hIL-7 (10 wk after last dose) (Fig. 1). T cells specific for known HIV epitopes were identified in 10 study participants using MHC tetramers. In two patients, two different epitope-specific T cell populations were identified, allowing the study of 12 HIV-specific CD8 T cell populations in 10 patients before and after r-hIL-7 treatment (Table I).

r-hIL-7 therapy increases the number of circulating HIV-specific CD8 T cells, but not the frequency of PD-1$^+$ or TIM3$^+$ cells

We observed a significant increase in the number of circulating HIV-specific (tetramer$^+$) CD8 T cells after r-hIL-7 treatment ($p = 0.027$; Fig. 1A, 1B), mirroring the overall increase in CD8 T cell numbers, whereas the frequency of HIV-specific CD8 T cells remained constant overall (Fig. 1C). Changes in total CD4 T cell counts did not correlate with the change in frequency of HIV-specific CD8 T cells posttreatment (Supplemental Fig. 1B), making it unlikely that the effect of r-hIL-7 on the CD8 T cells is linked to CD4 T cell expansion. We also found no correlation between the change in frequency of T cells specific for individual HIV epitopes and the level of CD8 T cell activation (measured by CD38 expression) (Supplemental Fig. 1C).

For a subset of donors, we also analyzed the level of IL-7R on HIV-specific CD8 T cells. About 20% of tetramer$^+$ CD8 T cells expressed CD127 at baseline, and this did not change after treatment (Supplemental Fig. 1D). The mean fluorescent intensity (MFI) for CD127 was also unchanged after treatment (data not shown).

PD-1 and TIM3 are cosignaling receptors that limit the survival and function of HIV-specific CD8 T cells (3–7). Following r-hIL-7 treatment, the frequency of PD-1$^+$ or Tim-3$^+$ HIV-specific CD8 T cells remained constant overall (Fig. 1D, 1E). There was also no correlation between the frequency of PD-1– and TIM3-expressing cells and the frequency of TRAF1$^+$ HIV-specific T cells (data not shown), or their level of activation as measured by CD38 (Supplemental Fig. 1E, 1F).

Thus, IL-7 therapy increases the number of HIV-specific CD8 T cells proportionally to the overall increase in T cell numbers in the patients, with similar levels of PD-1 and TIM3 before and after IL-7 treatment.

r-hIL-7 therapy increases TRAF1 expression in HIV-specific CD8 T cells in a subset of donors

TRAF1, a critical survival factor for activated and memory T cells (30, 31), is lost from HIV-specific CD8 T cells with progression of HIV infection (9). Because IL-7 increases TRAF1 levels in chronically stimulated CD8 T cells (9), we measured TRAF1 levels in the HIV-specific CD8 T cells before and 12 wk after initiation of r-hIL-7 therapy. Overall, there was no change in the level of TRAF1 per cell (MFI) or the frequency of TRAF1$^+$ cells in the HIV-specific CD8 T cells after IL-7 treatment (Fig. 2A, 2B). However, TRAF1 frequency data appeared to show two distinct responses to treatment. To determine whether TRAF1 expression in patients following IL-7 treatment was partitioned into two or more groups, we evaluated partition into cluster sets using the

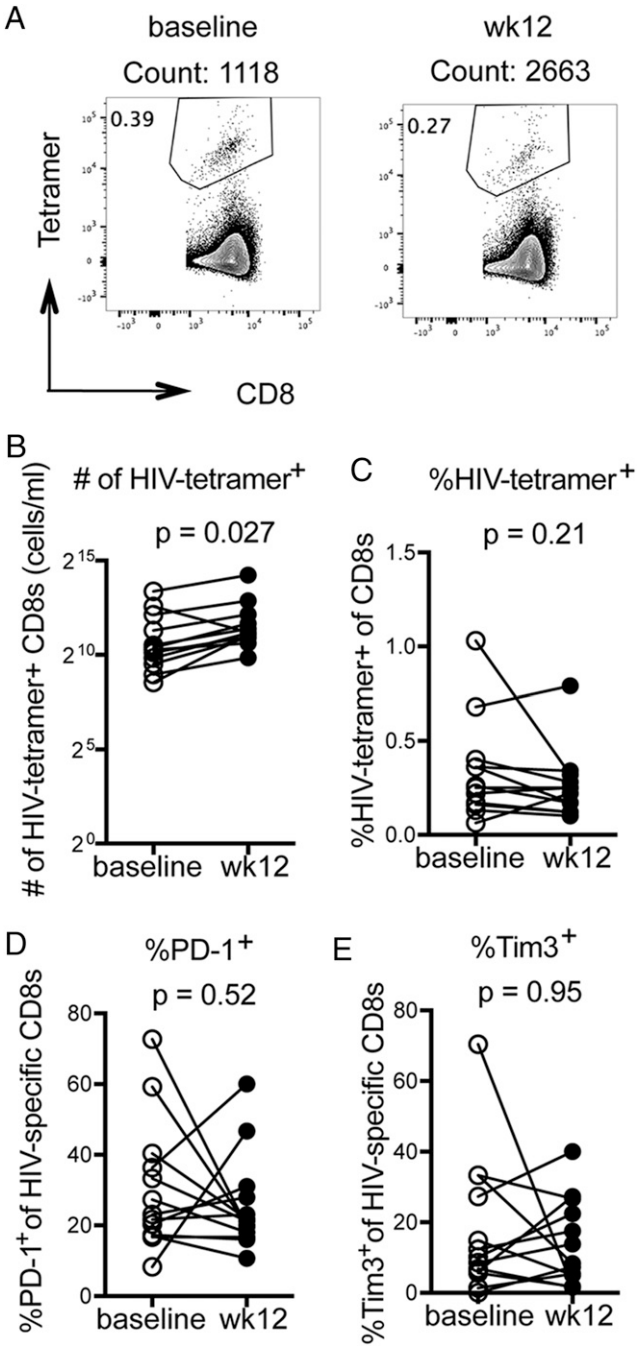

FIGURE 1. r-hIL-7 therapy increases the number of circulating HIV-specific CD8 T cells. HIV-specific T cells were enumerated by tetramer staining (**A**), for a total of 12 epitopes in 10 participants before and 12 wk after r-hIL-7 therapy. The number (**B**) and frequency (**C**) of HIV-tetramer$^+$ CD8 T cells are shown. Frequencies of HIV-specific PD-1$^+$ (**D**) and TIM3$^+$ (**E**) T cells before and after r-hIL-7 therapy. Each symbol represents one tetramer$^+$ population from a total of 10 donors, analyzed in the same experiment.

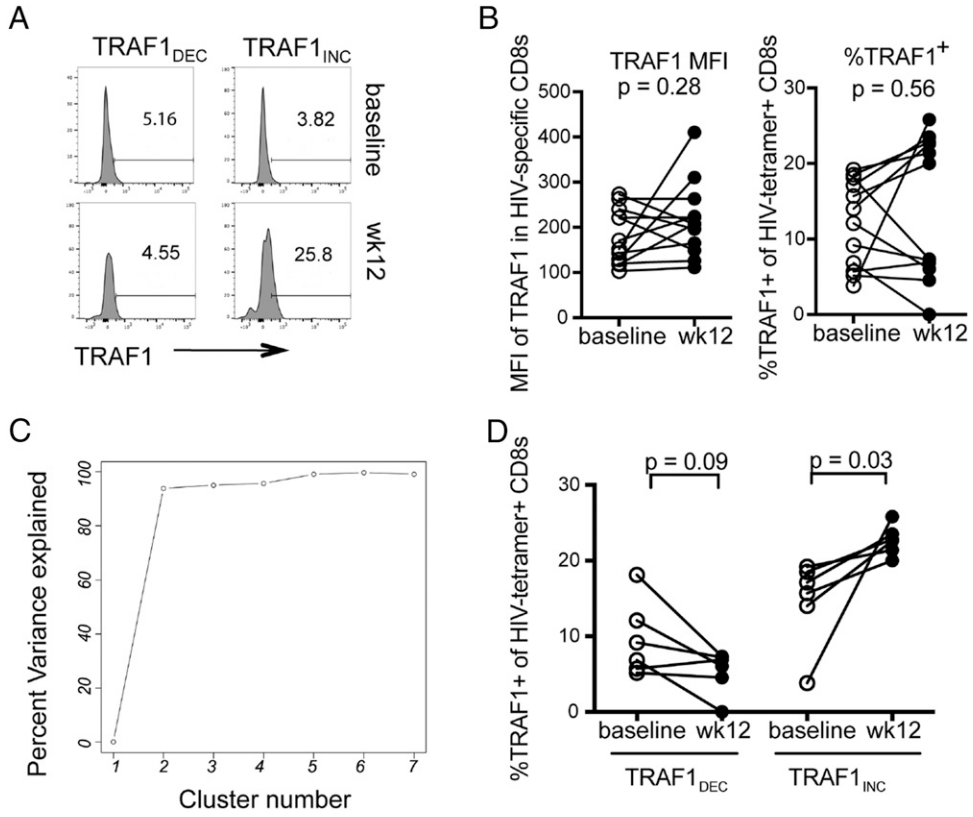

FIGURE 2. r-hIL-7 therapy altered TRAF1 expression in a subset of participants. Intracellular TRAF1 was measured by flow cytometry in HIV-specific CD8 T cells before and after r-hIL-7 therapy (**A**) and reported as either MFI or frequency of TRAF1$^+$ cells (**B**). (**C**) To determine the degree of variance in TRAF1 expression after IL-7 treatment, we evaluated partition into cluster sets using the percentage of variance explained following K-means clustering into different numbers of clusters. Based on the results in (C), data were grouped into two clusters: TRAF1$_{INC}$ refers to one cluster with epitopes showing an increased frequency of TRAF1$^+$ HIV-specific CD8 T cells posttreatment, whereas the other cluster, TRAF1$_{DEC}$, showed a trend toward decreased frequencies (**D**). Each symbol represents one tetramer$^+$ population from a total of 10 donors analyzed in the same experiment.

percentage of variance explained following K-means clustering. Partitioning into two clusters explained a large portion of the variance in the data (94%), and effects of additional clusters were incremental (95, 95.7, and 99% for three, four, and five clusters, respectively) (Fig. 2C). TRAF1 expression before treatment did not show a similar separation (data not shown). Based on this analysis, we reanalyzed the data after dividing into the two clusters after IL-7 treatment: those that showed an increase in TRAF1 expression (TRAF1$_{INC}$) in HIV-specific CD8 T cells and those that do not (TRAF1$_{DEC}$) (Fig. 2D, Table I). In contrast, a similar analysis for PD-1 or TIM3 would require four or more clusters to explain the data. These data show that a subset of patients respond to IL-7 therapy by upregulating TRAF1 in their HIV-specific CD8 T cells.

r-hIL-7 did not change the overall level of cytokine production per cell

Cytokine production by HIV-specific CD8 T cells was identified by comparing responses to HIV peptide stimulation ex vivo over no-peptide controls. Some donors from each group showed increases in the frequency of cytokine-producing or CD107$^+$GranzymeB$^+$ cells after treatment, but these increases did not reach statistical significance overall (Supplemental Fig. 1G, data not shown). Thus, IL-7 treatment did not appear to change the functionality of the T cells overall.

r-hIL-7 therapy increases levels of the downstream mTORC1 target pS6

The metabolic checkpoint kinase mTORC1 plays a critical role in regulation of cell size and metabolic activity and is highly active during the expansion phase of T cell responses, with reduced activity in quiescent T cells (32–35). Phosphorylation of S6, a downstream target of mTORC1, is often used as a readout of mTORC1 activity. There was a significant increase in pS6 MFI in the pS6$^+$ HIV-specific T cells (Fig. 3A), but not in the total population of pS6$^+$ CD45RA$^-$ CD8 T cells (Supplemental Fig. 2A). mTORC1 is regulated by a number of upstream signals, including negative regulation by TSC2. TSC2 is inhibited by Erk phosphorylation; thus, Erk activation can increase mTORC1 activity (36). Because TRAF1 is strongly linked to Erk activation in T cells (10), we measured pErk and pS6 in HIV-specific (Tetramer$^+$) T cells (Fig. 3B). There was a strong concordance between the frequency of TRAF1$^+$ HIV-specific T cells and the frequency of pErk$^+$pS6$^+$ HIV-specific T cells overall (Fig. 3C), but not with the frequency of pErk$^-$pS6$^+$ cells (Supplemental Fig. 2B). This correlation was significant only in the HIV-specific T cells (Fig. 3C), not in the bulk memory T cells (CD45RA$^-$CD8$^+$) (Supplemental Fig. 2C). The level of TRAF1 was also significantly higher in pS6$^+$ HIV-specific T cells (Supplemental Fig. 2D). Of note, the MFIs for pErk and pS6 were highly correlated in HIV-specific CD8 T cells (R^2 = 0.48 at baseline, R^2 = 0.96 post–IL-7; Supplemental Fig. 2E).

mTORC1^{hi} T cells are larger and have more proliferative potential than mTORC1^{low} cells (37), consistent with the role of mTORC1 in regulating mammalian cell size (34). mTORC1 is critical for T cells to expand and differentiate into effector cells (33). Conversely, inhibition of mTORC1 activity favors long-lived memory T cell formation (38). The pS6$^+$ HIV-specific CD8 T cells have significantly greater forward scatter as compared with

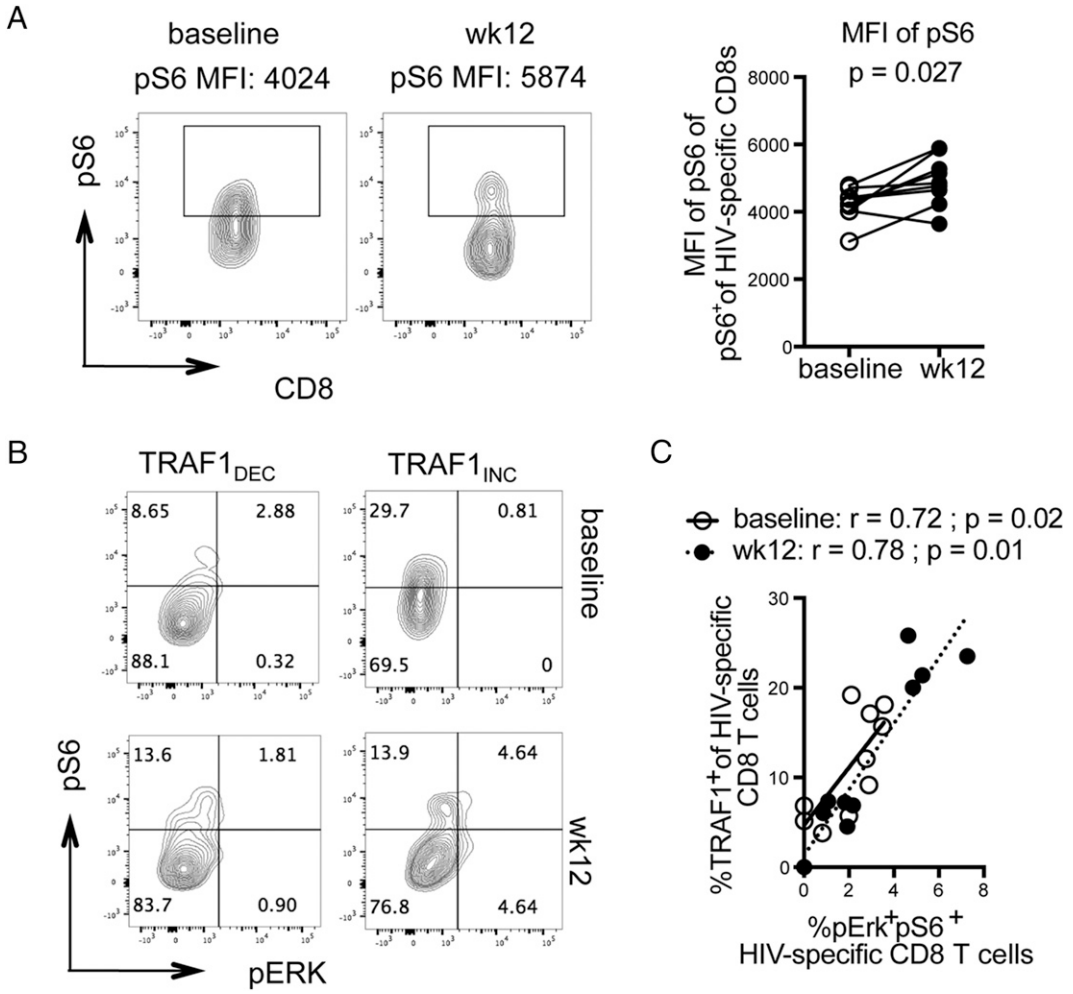

FIGURE 3. r-hIL-7 therapy increases pS6 levels in HIV-specific CD8 T cells. pS6 was analyzed in HIV-specific CD8 T cells before and after r-hIL-7 therapy by intracellular flow cytometry (**A**). pErk and pS6 were analyzed in HIV-specific CD8 T cells before and after r-hIL-7 therapy by intracellular flow cytometry (**B**). The frequency of $TRAF1^+$ cells is plotted against the frequency of $pERK^+$ $pS6^+$ (**C**). Each symbol represents one $tetramer^+$ population from a total of 10 donors analyzed in the same experiment.

$pS6^-$ cells, indicating greater cell size (Supplemental Fig. 2F). These data show that IL-7 therapy increases the level of pS6 in HIV-specific T cells, but not in bulk CD8 T cells, that pS6 expression in the HIV-specific T cells correlated with larger cell size, and that these changes persist up to 10 wk after the last IL-7 dose.

Discussion

Although ART has revolutionized the treatment of HIV infection, a fraction of patients do not regain normal CD4 T cell counts despite achieving control of plasma viremia. The potential of IL-7 to restore CD4 T cell counts in such patients has been demonstrated in clinical trials (22–25). In terms of evaluating the net impact of therapy, it is important to analyze not just the overall change in T cell numbers but also the impact of IL-7 therapy on HIV-specific T cells. In this study, we demonstrated that r-hIL-7 therapy in HIV-infected patients increased the overall number, but not the frequency, of HIV-specific CD8 T cells. Thus, HIV-specific CD8 T cells increase proportionally to the level of expansion of the T cell compartment overall.

We also analyzed the phenotype of the HIV-specific CD8 T cells before and after IL-7 therapy. The MFI or percentage expression of inhibitory receptors PD-1 and TIM3 were not changed overall by IL-7 therapy, nor did the potential of the HIV-specific T cells to produce cytokines or to degranulate following peptide stimulation change in the treated compared with pretreatment samples. However, we found that there was an overall increase in the MFI of pS6 staining in HIV-specific CD8 T cells, but not in the bulk CD8 T cells posttreatment. This measurement was made 10 wk after the last cycle of IL-7, suggesting a sustained effect on the HIV-specific T cells. Phospho-S6 regulates protein translation and can regulate cell size, proliferation, and metabolism (39). Moreover, loss of the *S6* gene in T cells inhibits their accumulation (40). Consistently, we found that the level of pS6 correlated with cell size in our studies. How this increase in pS6 contributes to control of HIV remains to be determined.

Phospho-S6 is often used as a downstream readout for the metabolic checkpoint kinase mTORC1 (34), but can also be phosphorylated directly by mitogen-activated protein kinases under some circumstances (41). Several upstream factors can induce mTORC1 activation including PI3K-AKT downstream of growth factor receptors (34). mTORC1 can also be negatively regulated by TSC2, and this inhibition can be relieved by Erk phosphorylation of TSC2 (36). TRAF1 is required for activation of Erk downstream of 4-1BB signaling (10). The strong correlation between the frequency of $TRAF1^+$ and $pErk^+$, but not $pErk^-$ $pS6^+$ cells that we observed in this study could reflect a role for TRAF1 through Erk (10) in activating pS6 in the HIV-specific T cells.

In this study, we observed an increase in the frequency of TRAF1$^+$ HIV-specific T cells, but not in bulk T cells, in a subfraction of study subjects using the percentage of variance explained following K-means clustering to define an IL-7 responder and non-responder group with respect to TRAF1 induction. Although it is debatable whether such a division of subjects has biological relevance with such a small sample size, the results suggest that TRAF1 levels may be a useful parameter to monitor in future HIV therapeutic trials. It is not clear why only a subset of patients respond to IL-7 by upregulating TRAF1. Because the starting frequency of PD-1$^+$TIM3$^+$ HIV-specific T cells was similar between the two groups, it is possible that the TRAF1 responders are generating new HIV-specific T cell responses, and that these new HIV-specific CD8 T cells have not yet become exhausted and lost TRAF1.

The finding that r-hIL-7 expands T cells containing proviral DNA raised concerns as to the appropriateness of this therapy for chronic HIV infection (28). For the trial described in this study, proviral DNA content of PBMCs increased from 1.96 (1.39–2.48) to 2.27 (1.65–2.85) $\log_{10}$ copies per milliliter at 12 wk posttreatment overall (25). The clinical significance of the apparent improvement in immunity (T cell expansion, increased pS6 per HIV-specific T cell) or of increases in the proviral DNA pool are unknown.

Overall, this study provides evidence that treatment of ART-treated HIV-infected patients with r-hIL-7 induces changes in HIV-specific CD8 T cells that are apparent 10 wk after the last treatment. HIV-specific CD8 T cell numbers in blood increased proportionately with the increased overall T cell pool and showed sustained increases in the level of pS6 suggestive of higher mTORC1 activity. These results suggest that TRAF1 and pS6 could represent useful parameters to monitor and relate to long-term outcomes.

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Disclosures

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