Cytokines that signal via the common γ chain (γc) represent promising therapeutics based on their potential to augment T cell expansion and increase the effectiveness of immune-based therapies (1–6). Within this family, IL-2 is a prototypic activating cytokine, secreted by and selectively signaling activated T cells, as well as up-regulating its own receptor (IL-2R). In contrast, IL-7 is a prototypic homeostatic cytokine, produced constitutively by nonlymphoid cells. Its receptor (IL-7Rα) is expressed on resting T cells, and then rapidly down-regulated after T cell activation or IL-7 signaling (7). IL-7 is essential for T cell development in mice (8–10) and humans (11, 12), as well as for T cell homeostasis, because it is required to maintain naive CD4+ and CD8+ T cells in vivo (13, 14). IL-7 levels rise in serum and tissues upon recovery (14–16).

In preclinical studies, IL-7 therapy exerts marked effects on T cell immune reconstitution in mice and primates. First-in-human clinical studies of recombinant human IL-7 (rhIL-7) provided the opportunity to investigate the effects of IL-7 therapy on lymphocytes in vivo. rhIL-7 induced in vivo T cell cycling, bcl-2 up-regulation, and a sustained increase in peripheral blood CD4+ and CD8+ T cells. This T cell expansion caused a significant broadening of circulating T cell receptor (TCR) repertoire diversity independent of the subjects’ age as naive T cells, including recent thymic emigrants (RTEs), expanded preferentially, whereas the proportions of regulatory T (T reg) cells and senescent CD8+ effectors diminished. The resulting composition of the circulating T cell pool more closely resembled that seen earlier in life. This profile, distinctive among cytokines under clinical development, suggests that rhIL-7 therapy could enhance and broaden immune responses, particularly in individuals with limited naive T cells and diminished TCR repertoire diversity, as occurs after physiological (age), pathological (human immunodeficiency virus), or iatrogenic (chemotherapy) lymphocyte depletion.
Figure 1. Effects of rhIL-7 therapy on circulating T cells and spleen. rhIL-7 was administered every other day on day 1–14 (8 injections, indicated by tick marks on X axis), at 4 dose levels: 3 μg/kg/d (dashed line with ●; n = 3); 10 μg/kg/d (dotted line with △; n = 3); 30 μg/kg/d (dashed line with □; n = 5); and 60 μg/kg/d (solid line with ○; n = 4). Mean value for each cohort (± the SEM) are plotted at the indicated time points. (A) The absolute lymphocyte count from complete blood counts (left) and flow cytometry–based frequency were used to determine circulating absolute CD3+/CD4+ and CD3+/CD8+ counts, absolute numbers ± the SEM (bottom graphs), and percent change in absolute numbers over baseline (top graphs) of the respective subsets shown on day 1 (total lymphocytes only), 7, 14, 21, and 28 for all treated subjects, as well as day 55–90 for subjects treated with 30 or 60 μg/kg/d. Baseline values represent the mean of four separate analyses performed in each subject within 2 wk before initiation of rhIL-7 therapy. (B) Spleen size: bidimensional product by CT scan obtained at the time points shown; percent changes from the pretherapy scan are plotted. (C–F) Baseline (1 value obtained on day 0) and day 7, 14, 21, and 28 data points were generated for CD3+/CD4+ (left) and CD3+/CD8+ subsets (right). (C) Ki-67 shows the percentage of cells in the respective subsets expressing Ki-67 via flow cytometry at the time points shown. (D) IL-7Ra expression...
augments antitumor responses, leading to improved survival when combined with antitumor vaccines (21, 22). The capacity for supranormal levels of IL-7 to augment T cell cycling in response to antigens with low affinity for the TCR appears largely responsible for homeostatic peripheral expansion, which involves increased T cell proliferation to self-antigens during lymphopenia (13, 23, 24). Many studies have documented a central role for homeostatic peripheral expansion in the immunorestorative effect of IL-7 therapy (19, 20, 25), whereas augmentation of thymic output has also been recently suggested (18). In regard to CD4+CD25high regulatory T (T reg) cells, IL-2 plays a major role in their development, maintenance, and expansion, whereas T reg cells express low IL-7Rα levels (26, 27) and recent work suggests that IL-7 therapy can expand CD4+ T cells without expanding T reg cells (28).

Lymphopenia induced by cytotoxic chemotherapy or other insults can significantly diminish immune function (29). In adults, CD4+ T cell recovery after severe immune depletion requires the reemergence of a pool of naive T cells, which requires 18–24 mo and may occur only in individuals younger than 40–45 yr (30, 31). A therapeutic approach to accelerate, or simply promote in older individuals, the recovery of a widely diverse T cell repertoire may find a multitude of clinical applications. If rhIL-7 therapy in humans can augment immune responses to weak antigens, hasten reconstitution of naive T cell populations, and spare T reg cell expansion in particular but not restricted to older individuals, it could improve the effectiveness of immune-based therapies for cancer or chronic infection in various populations with iatrogenic (chemotherapy), pathological (HIV), or physiological (aging) immune insufficiency. This study sought to characterize the immunobiologic effects of rhIL-7 therapy in humans and, specifically, its potential for immune rejuvenation of T cell subpopulations.

RESULTS
IL-7 therapy induces widespread T cell cycling and expands the T cell pool in humans in a dose-dependent manner, while preserving T cell function

To assess effects of IL-7 therapy on human lymphocytes in vivo, 16 subjects with refractory cancer (age 20–71) were enrolled on a phase I dose escalation trial of rhIL-7. Doses extrapolated from mouse and primate studies (3, 10, 30, and 60 μg/kg) were administered subcutaneously every other day for 14 d (8 doses). As shown in Fig. 1 A, after a very transient decrease, circulating numbers of lymphocytes and CD4+ and CD8+ T cells increased in a dose-dependent manner, with increases at the highest dose level approaching 300% for CD4+ and exceeding 400% for CD8+ T cells. The initial decrease in circulating lymphocytes is likely caused by early trafficking out of the circulation and is also seen with administration of IL-2 and, more recently, IL-21. Specifically, we have observed by FACS analysis an initial up-regulation of CXCR4 on circulating lymphocytes with rhIL-7 treatment (unpublished data), providing one plausible mechanism for T cell trafficking into SDF-1–rich tissues.

Like other γc cytokines, IL-7 also supports T cell survival by up-regulating members of the antiapoptotic bcl-2 family (32, 33), and rhIL-7 therapy induced significant increases in T cell bcl-2 expression in this study (Fig. 1 F); as bcl-2 has normalized/10⁴ copies of AXTB via Q-PCR in sorted CD4+ and CD8+ cells. (F) bcl-2 expression as MFI (after subtracting background staining for each subset) via flow cytometry for each subset.
rhIL-7 therapy leads to transient, widespread T cell cycling that is tightly regulated by IL-7Rα expression, as well as a more sustained increase in bcl-2 expression, resulting in dramatic increases in T cell numbers persisting for several weeks after cessation of therapy. These rhIL-7–expanded T cells retain robust proliferative capacity, even to low level of TCR triggering, a finding that is consistent with IL-7’s known capacity to costimulate TCR signaling and to mediate augmented responses to weak antigens (21).

Figure 2. PET-CT imaging of the self-limited lymphoid organs’ enlargement and increased metabolic activity after rhIL-7 therapy. Shown are representative images on PET-CT scan in one subject (42-yr-old female treated with 60 μg/kg/dose). (left) Imaging on day 14 (last day of rhIL-7 treatment). (right) Day 56 (6 wk after the end of treatment). The full ovals indicate areas of increased size (except for vertebral bodies) and activity of lymphoid organs (left and right axillary adenopathy, spleen, and lumbar spine) at the end of treatment. The dotted contours indicate the thymic area where no increased activity can be demonstrated at day 14. Increased metabolic activity is seen in pink and maximal in yellow areas.

Effects of IL-7 versus IL-2 administration in selected T cell subpopulations

rhIL-2, the only γc cytokine routinely administered therapeutically to humans, also increases CD4+ T cell numbers in vivo (34, 35). However, IL-2–induced expansion of CD4+ T cell populations in humans involve selective expansion of CD4+/CD25+, FOXP3+ T reg cells (34–36), as predicted by the CD25 expression that is the hallmark of this subset. Low level IL-7Rα expression on T reg cells (26, 27) suggests that IL-7 therapy may not selectively expand this subset. To assess whether CD4+ expansion was associated with preferential T reg cell expansion, FOXP3 mRNA was quantified in sorted CD4+ T cells before and after rhIL-7 therapy (Fig. 3 B). FOXP3 mRNA was not increased in CD4+ cells collected during the period of CD4+ expansion, nor was any significant FOXP3 mRNA detected in CD8+ cells at baseline or after therapy. These mRNA data are corroborated by multicolor flow cytometry analyses performed in selected patients from cohort 4, specifically addressing rhIL-7–induced proliferation in FOXP3+ and FOXP3− subsets. In summary, after 1 wk of treatment with rhIL-7, there is a mean 4.3-fold increase of the percentage of CD4+/FoxP3+/Ki67+ cells, whereas the mean fold increases for CD4+/FoxP3−/Ki67+ cells and for CD8+/FoxP3−/Ki67+ cells are 53.4- and 63.9-fold, respectively. After 2 wk of treatment, the mean fold increases are 1.6-, 24.1-, and 35.7-fold, respectively, whereas at week 3, 1 wk after the end of treatment, they are 0.2-, 5.9-, and 9.6-fold. Representative two-dimensional plots and overlay histograms in one patient are shown in Fig. 3 C.

Thus, in contrast to rhIL-2, rhIL-7 therapy in humans induces robust CD4+ T cell expansion without selective expansion of T reg cells, which is consistent with a recent study from a separate cohort of subjects in another clinical trial of rhIL-7 (28). Furthermore, multiple studies have also demonstrated that in vivo IL-2 administration in humans has minimal effects on CD8+ T cells numbers (37, 38). In contrast, we show that the in vivo effects of rhIL-7 on the expansion of CD8+ T cells are, at a minimum, comparable if not superior to the effects on CD4+ T cells.

rhIL-7 increases TCR repertoire diversity

Because rhIL-7 receptor expression is high on resting, naive populations, we postulated that the rhIL-7–induced increase in circulating T cells could preferentially expand naive cells,
and therefore lead to an overall increase in TCR diversity. TCR diversity spectratype analysis was performed on sorted CD4+ and CD8+ populations before and after (day 21) rhIL-7 therapy in 6 subjects treated at either 30 or 60 μg/kg, and the pre- and post-therapy spectratype divergence from a normal donor standard was assessed (see Materials and methods). Indeed, 4 of the 6 subjects tested had a statistically significant increase in repertoire diversity by 1 wk after the end of treatment.
Figure 4. rhIL-7 therapy increases TCR repertoire diversity by VB family spectratype analysis. (A) Schematic representation of the methodology. VB family spectratype analysis was performed for each of 6 subjects (3 of each cohort 3 and 4), separately on CD4+ and CD8+ cells by RT-PCR on RNA extracted from sorted T cells at baseline and at D21. The distributions of peaks for each VB family are graphically represented on a curve. (B) Schematic of divergence score calculation. Divergence from the normal donor standard was calculated for each VB family, separately for CD4+ and CD8+ cells, for each individual, at baseline and at day 21. Two VB families (VB14 on the left and VB23 on the right) are illustrated for the sorted CD8+ population in one individual (Pt 11), pre (rows 1 and 3), and post (rows 2 and 4) therapy. Overlays of histograms for the subject (no color) and the normal donor standard (in gray) illustrate how the divergence scores were calculated [see Materials and methods]. (C) Divergence scores for CD8+ cells in one subject. Divergence scores are shown at baseline (pre) and day 21 (post) for each VB family, and a diversity shift was obtained (Pre-Post). The median diversity shift (a positive value indicates a shift toward greater repertoire diversity) and the P value of Wilcoxon pair test comparing the pre and post divergence scores are shown,
rhl-L-7 therapy selectively expands CD4+ recent thymic emigrants (RTEs), naive cells, and central memory populations, but not effector T cells

IL-7Rα expression is tightly regulated during postthymic T cell differentiation, such that RTEs (25, 39), naive, and early memory populations (40) express high IL-7Rα levels, whereas effectors and late memory/senescent populations express lower levels (41, 42). To determine whether the effects of rhl-L-7 therapy on TCR repertoire diversity could be explained by varied effects among peripheral T cell subsets with differential IL-7Rα expression, we measured cycling rates, bcl-2 expression, peripheral blood frequencies, and absolute numbers of RTEs, naive, effector, and memory populations before, during, and after therapy. Fig. 5 illustrates representative changes in a single subject (treated at 60 μg/kg). Fig. 6 summarizes findings across subjects and dose levels; detailed kinetics for all subsets are included in Fig. S2 (available at http://www.jem.org/cgi/content/full/jem.20071681/DC1). Using CD45RA with CD27 or CCR7 coexpression to define the naive subsets, rhl-L-7 therapy preferentially expanded naive CD4+ and CD8+ T cells, leading to increased frequency (Fig. 5, A and C) and absolute numbers (Fig. 6, A and D). Similarly, CD4+ RTEs defined by coexpression of CD45RA and CD31 substantially increased both in frequency and number after rhl-L-7 therapy. In addition, modest expansion of the memory and effector subsets were observed (Fig. 6 A), which appeared to preferentially involve CD4+ central memory cells (Fig. 6 D). Of all subsets, the naive CD8+ expanded the most and significantly more than the CD8+ memory and effector subsets (P = 0.008), and these effects were similar regardless of subject age (Fig. S1 B). This preferential effect on naive cells, including RTEs, was not caused by differences in maximal Ki67 expression because rhl-L-7 similarly increased the cycling rate of nearly all subsets in a steep dose-dependent manner (Fig. 5 B and Fig. 6 B) by day 7 of therapy, followed by a gradual return to near baseline by day 21 (Fig. 5 B and Fig. S2). Interestingly, however, whereas memory and effector subsets showed a brisk decline in Ki67 frequency by day 14, there remained a distinct subset of Ki67+ cells at this time point in both CD4+ and CD8+ naive subsets (Fig. 5 B). rhl-L-7 also induced a dose-dependent increase in bcl-2 mean fluorescence intensity (MFI) in all subsets except CD8+ effectors (Fig. 6 C). Thus, rhl-L-7 therapy increased total body T cell mass with preferential expansion of the naive subsets, resulting in a T cell pool in this adult population that more closely resembles that seen earlier in life. This could potentially relate to fine differential kinetics of cell cycling, IL-7Rα expression, or bcl-2 up-regulation, although changes in these parameters of similar magnitude were observed in nonnaive subsets as well (Fig. S2). Alternatively, these results could suggest that, after similar levels of rhl-L-7 signaling, naive T cells undergo a greater proliferative burst than memory or effector subsets and/or that some component of the increase in naive subsets reflects input of cells from an alternative source.

rhl-L-7 increases circulating TCR excision circles (TRECs) numbers

Because rhl-L-7 preferentially increases RTEs and naive T cells, we investigated a potential thymic contribution to its effects. Peripheral blood TRECs have been used as an indirect measure of thymic output because they reflect TCR gene recombination events and are enriched within the RTE fraction (43). Because TRECs are nonreplicating DNA, however, they become diluted with T cell division and, in preclinical primate studies (19, 20), TREC frequencies diminished after rhl-L-7 therapy, presumably caused by induced cycling in RTEs (44). In this study, we observed diminished absolute numbers of circulating TRECs/milliliter on day 7 (Fig. 7 A), an expected reflection of the diminished total number of circulating phenotypically naive cells at that point, followed by an increase in the absolute number of TRECs/milliliter3 (doubling in the 2 highest dose cohorts) between days 7 and 21, which is consistent with the subsequent rise in naive T cell absolute number. Despite widespread rhl-L-7–induced T cell cycling, TREC+ frequencies were largely unchanged in sorted CD4+ and CD8+ peripheral T cells before and after therapy (Fig. 7 B). The absence of profound reductions in TREC frequencies in the face of significant T cell cycling raises the possibility that rhl-L-7 might also induce new thymic emigrants to enter the circulation. However, in other clinical settings such as after chemotherapy, where a substantial thymic output increase does occur, it occurs later, is preceded by a thymic size increase on CT scan, is accompanied by a >10-fold increase in TREC, and the magnitude of the effects diminishes with increasing age (30, 45). We observed no increase in thymic size on day 14 or 28 CT scans after rhl-L-7 therapy, and there was not a significant correlation

and then incorporated into the summary in D. (D) Summary of repertoire diversity analysis. For each tested individual [age], shown at baseline (D0) and D21 are as follows: the total naive CD4+ and naive CD8+ cells counts; the median diversity shift for each CD4 and CD8 subsets of each individual and the P values of the Wilcoxon pair test indicating the likelihood that the calculated change in divergence scores for a particular subject's CD4+ or CD8+ cells between day 21 and baseline occurred by chance; P values < 0.05 indicate a statistically significant increase in diversity toward normality between baseline and day 21 studies.

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Figure 5. rhIL-7 therapy induces cycling of most T cell subsets, but preferentially expands naive CD4+ and CD8+ cells and CD4+ central memory cells. Representative data from a single subject treated at 60 μg/kg/d are shown. (A) Two-dimensional plots illustrating the rhIL-7 therapy-induced increased frequency of CD4+ RTEs (CD31+/CD45RA+; red boxes in first row of graphs), and changes in CD4+ and CD8+ naive, memory, and effector subsets (respectively CD45RA+/CD27−, CD45RA−/CD27+, and CD45RA−/CD27− sections on second and third rows). (B) Overlay histograms of Ki-67 expression on CD4+ RTEs (CD31+/CD45RA+) and CD4+ and CD8+ naive (CD45RA+/-CD27−), memory (CD45RA−/CD27+), and effector (CD45RA−/CD27−) subsets. Percent Ki-67+ cells are indicated in each frame. Red lines, pretreatment; blue lines, day 7; green lines, day 14; orange lines, day 21. Note that...
between age and TREC increase (Fig. S1 B). We also performed a similar TREC analysis on sorted naive CD4+/CD31+/CD45RA+ cells and naive CD8+/CD27+/CD45RA+ cells before and after rhIL-7 therapy to probe for increased cycling of this subset resulting in TREC dilution. Indeed, in the two individuals studied, we observed pronounced dilution of TREC copies (reduction by 80 and 62% of TREC/200K RNaseP copies in sorted CD4+ RTEs, and reduction by 32 and 56% of TREC/200K RNaseP copies in sorted CD8+ naive T cells) after rhIL-7 therapy, which is consistent with intense proliferation in these subsets. Therefore, although rhIL-7–induced augmentation of thymopoiesis cannot be ruled out, the available data are most consistent with rhIL-7–induced entry of new thymic emigrants into the circulation via mobilization from lymphoid sites and/or enhanced thymic egress, as previously observed in an animal model of IL-7 administration (25), in concert with vigorous cycling of the naive and RTE subsets.

In summary, rhIL-7 induces increases in total body T cell mass accompanied by an age-independent overall increase in TCR repertoire diversity. Preferential expansion of naive subsets leads to an increased TCR diversity and changes in peripheral T cell subset compositions that reverse the aged phenotype. The timing of this increase, the lack of frank increase in the absolute number of circulating TREC, the marked dilution effect on TREC in the naive subsets of selected individuals, and the detailed phenotypic analysis of T cell subset kinetics indicate that this increase in TCR diversity primarily arises from the preferential peripheral expansion of the RTEs and naive T cells, the most diverse components of the T cell pool.

**DISCUSSION**

Administration of supraphysiologic doses of recombinant growth factors to augment hematopoietic cell recovery has had a substantial impact in medical practice, with recombinant proteins now available to enhance red cell, white cell, and platelet regeneration approved for clinical use. Thus far, however, no agent able to safely expand a broad repertoire of T cells has been identified, and impaired immune reconstitution remains problematic in many clinical settings (46). Several preclinical studies have implicated IL-7 as a primary modulator of peripheral T cell homeostasis with potent immunorestorative properties, raising the prospect that rhIL-7 may serve as a clinically effective T cell growth factor. This study demonstrates that rhIL-7 administration in humans can safely induce polyclonal T cell expansion in vivo, resulting in dramatic increases in T cell number. Although the clinical details associated with the therapy are the focus of a separate report (unpublished data), the agent was well tolerated on this trial. Mechanistically, the effect of rhIL-7 can be attributed to a combination of increased cell cycling, likely via TCR triggering to cross-reactive self-antigens, and diminished programmed cell death, which was self-limited by down-regulation of IL-7Rα in the face of continued drug administration. The rhIL-7–expanded T cells remained functional, retaining robust responsiveness to TCR triggering with even a suggestion of increased sensitivity to TCR ligation after a suboptimal stimulus, as seen in preclinical models.

rhIL-7 preferentially expanded naive CD4+ and CD8+ subsets, including the most naive circulating RTEs (CD4+/CD45RA+/CD31+), consistent with the results from mouse studies (44). The effects on CD4+ and CD8+ memory cells and CD4+ effectors were intermediate, whereas CD8+ effectors underwent little expansion. Notably, rhIL-7–expanded central memory CD4+ T cells, a subset that may be critically important in avoiding clonal exhaustion in the context of chronic persistent viral or tumoral antigenic exposure (47, 48). It is also noteworthy that rhIL-7 preferentially expanded the two subsets of T cells capable of initiating lymph node germinal center formation, the naive and central memory subsets.

Despite the clear evidence presented in this study that rhIL-7 therapy substantially increases TCR diversity by an increase in the size of the naive T cell pool, the contribution of the thymus to these rapid changes remains unclear. Preclinical studies provide evidence both for and against rhIL-7–induced augmentation of thymopoiesis (18–20). In this study, we observed increases in absolute numbers of TREC in sorted RVIs (albeit modest) and in numbers of peripheral blood phenotypic RTEs. However, we saw neither evidence for thymic enlargement nor significant correlation between age and rhIL-7 effects. Furthermore, our TREC analysis on sorted naive CD4+/CD31+/CD45RA+ cells and naive CD8+/CD27+/CD45RA+ cells shows a pronounced dilution effect of TREC consistent with the intense proliferation in these subsets, thus suggesting that the observed effect on repertoire diversity is in large part caused by decreased skewing by proliferation of RTEs.

Together, these data suggest that the observed changes reflected rhIL-7–induced increases in RTE cycling, as well as trafficking of RTEs from peripheral lymphoid tissue into the bloodstream as seen in the mouse model (25, 44), whereas no direct effect on thymopoiesis can be demonstrated in the short time span of this study. Indeed, in clinical settings, thymic participation in immune reconstitution in adults is delayed for several months after the depleting event (30), raising the possibility that more direct thymopoietic effects of rhIL-7, if induced, could require more chronic rhIL-7 administration. However, even if the observed rhIL-7 effects are thymic independent and reflect naive cell expansion rather than increased thymic throughput per se, the net effect of rhIL-7 therapy remains a substantial, measured, increase in...
TCR repertoire diversity in adult subjects, thereby reshaping their T cell pool. During the aging process, the accumulation of senescent and more oligoclonal CD8+ cells with a terminal differentiation phenotype has been associated with dysfunctional immune responses. The effects of rhIL-7 on naive and central memory subsets could restore balance in T cell subsets during immunological aging and restore a T cell profile resembling that seen earlier in life. Such “antiaging” effects could prove beneficial for T cell–depleted hosts or older hosts, where the combined loss of total T cell number, naive cells, and the corollary loss of repertoire diversity appears to contribute to diminished overall immune responsiveness.

In summary, in this first clinical trial of rhIL-7 initiated in humans, and after the brief study of another National Cancer Institute–initiated trial, we present evidence that rhIL-7 therapy in humans induces dramatic, polyclonal, prolonged CD4+ and CD8+ T cell expansion in vivo, with preferential increases in T cells bearing diverse TCR repertoire specificities. The effects are mediated primarily through increased peripheral T cell cycling and augmented cell survival. The rhIL-7–expanded T cells retain significant functional capacity, and the CD4+ T cell expansion is not accompanied by a disproportional increase in T reg cells, such as that which occurs after rhIL-2 therapy. rhIL-7 appears to be an effective T cell growth factor.
with “immune rejuvenating” properties that suggest it would be effective in augmenting immune reactivity in hosts with impaired immunity caused by physiological (age), iatrogenic (chemotherapy/transplantation), or pathological (HIV) lymphodepletion. In immunologically normal, as well as deficient hosts, rhIL-7’s capacity to augment responses to weak antigens and to increase T cell cycling without T reg cell expansion may be clinically exploitable in the context of immunotherapy regimens for cancer and/or chronic infection. Future clinical studies of rhIL-7 in the context of immune-based therapies are warranted.

MATERIALS AND METHODS

Subject population and trial design

We enrolled 16 subjects with nonhematologic, nonlymphoid cancer refractory to standard therapy on a standard phase I dose escalation study of rhIL-7 (National Cancer Institute, NCI protocol 03-C-0152). The study was approved by the Institutional Review Board of the NCI and by the Food and Drug Administration. All subjects signed a written informed consent before enrollment. Subjects received subcutaneous injections of rhIL-7 (provided by Cytheris Inc., Rockville, MD) at the doses noted every other day for eight doses. A maximum tolerated dose was not reached on this study, and two subjects experienced dose limiting toxicity. One patient treated at 30 μg/kg/d received only one injection before being removed from the study (because of a grade 3 transaminase elevation) and is not included in the analyses. Another subject was removed from study (because of grade 3 hypertension and chest pain) after receiving the first four injections at 60 μg/kg/d and is included in the relevant analyses. Significant differences in the pattern of total lymphocyte expansion were not seen when this subject was compared with the study subjects who completed the treatment. A detailed description of the eligibility criteria, subject population, and clinical results of this trial is the object of a separate report (unpublished data).

Flow cytometry and cell sorting

PBMC analysis. We used flow cytometry to assess the intracellular and cell surface expression of selected markers on PBMCs at the specified time points. We analyzed EDTA anticoagulated peripheral blood specimens on a FACSVantage (BD Biosciences). Real-time quantitative PCR products in plasmids using Platinum Taq and TOPO TA Cloning kit (Invitrogen) as per the manufacturer’s instructions. PCR conditions were a 94°C 2-min hot start followed by 94°C for 30 s, 58°C for 30 s, 72°C for 45 s for 30 cycles, and culminating in a final 72°C 7-min extension step. Primer sequences were as follows: ACTB, 5′-GAGCACAGAGCCTTCGCCCTTTG-3′ (sense), 5′-GCCGCCACATAGGAATCCTTCTTCTT-3′ (antisense); FOXP3, 5′-GACACTTCTCCCAACTCCCGT-3′ (sense), 5′-GACAGCAAGACATGGAAACC-3′ (antisense); IL-7RA proprietary (“HS00233682” from ABI).

T cell proliferation in response to anti-CD3 signaling

We thawed PBMC cryopreserved at each time point, and then incubated it with 2 d in 200 μl/well of 10% human AB serum/AIM-V medium on 96-well flat-bottomed plates (Nunc) coated with the designed concentrations of OKT3 (Orthoclone). We measured cell proliferation by incorporation of [3H]thymidine (1 μCi/well; ICN) after a 16–18-h pulse. Cpm reflects proliferation after anti-CD3 minus cell proliferation in the medium. We ran normal donor controls with each assay.

TREC analysis

For TREC analysis, we sorted CD3+ CD4+ and CD3+ CD8+ T cells and, in two subjects, CD4+ CD31+ and CD8+ CD45RA+ T cells from cryopreserved PBMCs, using a FACSVantage (BD Biosciences). Real-time quantitative-PCR was performed using the 5′-nuclease (Tagman) assay on cell lysates on an ABI7700 system (Applied Biosystems) according to the protocol of Douek et al. (43). Cells were lysed in 100 μg/ml proteinase K (Boehringer-Mannheim) in 10 mM TRIS, and heated for 1 h at 56°C, and then for 10 min at 95°C. PCR reactions contained Platinum Quantitative PCR SuperMix-UDG with ROX (Invitrogen) with 500 nM of each primer, and 150 nM probe. PCR conditions were 95°C for 5 min, 95°C for 30 s, and 60°C for 1 min for 40 cycles. The forward and reverse TREC primers were 5′-CACATCCCTTTACACCAT- GCT-3′ and 5′-GCCAGTCGAGGGTTAGTG-3′, respectively, and
the probe FAM-5′-ACACC/CTCGGTTTTTGAAGGTTGCCCCACT-TAMRA (Integrated DNA Technologies, Inc.). A standard curve (10^4–7 TREC copies/well) was plotted using a plasmid containing the TREC gene sequence (provided by D.C. Douek, National Institutes of Health, Bethesda, MD). We normalized TREC values to cell content by concurrent measurement of the single-copy gene, RNot P, using the TaqMan RNot P Control Reagents kit (Applied Biosystems). Samples were analyzed in duplicate, and the results were averaged and normalized as TREC/s/200,000 copies RNot P, equaling TREC/s/100,000 cells.

**T cell repertoire diversity evaluation and statistical evaluation of TCR β-chain variable region (Vβ) spectratypes**

We performed spectratype analyses separately on CD4+ and CD8+ T cell subsets from 6 subjects (3 each of cohorts 3 and 4). We sorted CD3+CD4+ and CD3+CD8+ T cells to 98% purity from cryopreserved PBMC on a FACS Vantage (BDIS). We used 10^5 cells of each to isolate RNA using Trizol (Invitrogen) in the presence of carrier tRNA and glycogen as carrier, and converted to cDNA using Oligo-dT as a primer for reverse transcription (First Strand, Invitrogen). We amplified cDNA in 22 separate PCR reactions (Vβ families 1–24, excluding Vβ 19 and 10), each containing 1 specific human Vβ subfamily primer coupled with an unlabeled consensus Cβ region primer, followed by a 10-cycle run-off nested PCR reaction, using a FAM-labeled internal BC primer (Integrated DNA Technologies, Inc.). Labeled run-off reaction CB primer and Vβ family-specific (forward) primers were as specified in Currier et al. (51), except for Vβ 5, 13, 15, and 16. These remaining Vβ primers and the CB primer were as from Puisieux et al. (52). The initial and run-off PCR used 0.75 U cloned Pfu DF polymerase (Stratagene), and PCR settings were hot start with 2 min at 95°C followed by 30 cycles of 95°C for 30 s, 55°C for 45 s, and 72°C for 1 min, followed by a final extension at 72°C for 10 min. Run-off reaction product was mixed at 1:9 ratio with loading buffer, consisting of Hi-Di Formamide (Applied Biosystems) containing size standards GeneScan-500 ROX, Red A (Applied Biosystems) mixed at a 1:20 ratio. Reactions were heated for 2 min at 95°C, and products were run on an ABI sequencer 3130xl (Applied Biosciences). Peaks were identified in the final spectratype histogram, and the area underneath was determined using GeneMapper v. 3.7 software (Applied Biosciences).

Using this same methodology, we also analyzed each Vβ family of 10–15 normal donor T cells (CD4+ and CD8+ combined), thereby defining “normal donor standards.” For each donor’s spectratype, we aligned the peaks by nucleotide base pair number, and used the area under each of 13 potential peak locations to calculate a total area under the spectratype curve, and then a proportional area for each peak. We then averaged the proportional areas to establish a normal donor standard spectratype. As expected, as we added more donors to the determination, the mean curves approached a more Gaussian-like distribution.

For each study subject, we then quantified the “diversity score” of each Vβ family from its counterpart Vβ normal donor standard as the sum of the absolute value of the divergence in proportional area at each peak. Therefore, divergence scores were established for each Vβ family, for each patient, separately for CD4+ and for CD8+ cells, and for both at baseline (pre) and day 21 (post). We then tested the hypothesis that, for a given CD4+ or CD8+ population in a given subject, the day 21 (post) divergence scores were significantly smaller than the baseline (pre) divergence scores in a non-parametric Wilcoxon analysis (2-tailed). A P value <0.05 indicates a statistically significant increase in repertoire diversity toward normality between the baseline and day 21 studies.

Finally, for each single Vβ family, we calculated a “diversity shift” as the difference between “pre” and “post” divergence scores, and we calculated the median of these diversity shifts (median diversity shift) for the set of 22 Vβ family of each T cell population (CD4+ or CD8+), a positive value indicating a shift toward greater repertoire diversity.

**Statistical analyses**

**Anti-CD3 proliferation.** We performed statistical analyses using GraphPad Prism version 4.0a for Macintosh (GraphPad Software). Significant differences when comparing two groups was determined by two-tailed unpaired nonparametric Mann-Whitney test. P values were considered significant if <0.05.

**Correlation with age.** We evaluated the statistical significance of the correlation of age with the percent increase of various lymphocyte populations, as well as with TREC s, with a Spearman’s correlation test with a significant 2-tailed P value of <0.05 (GraphPad Prism version 4.0a for Macintosh; GraphPad Software).

**Spectratyping analysis.** See previous section.

**Online supplemental material**

Fig. S1 shows time courses of various lymphocyte populations following rhl-7 therapy. Fig. S2 shows time course on percent change over baseline in circulating cells, K67 expression, and Bcl-2 up-regulation for all studied subsets. The online version of this article is available at http://www.jem.org/cgi/content/full/jem.20071681.DC1.

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Three co-authors have financial interest in Cytheris capital: M. Morre is the founder and Chief Executive Officer and J. Engels and R. Buffet are employees of Cytheris. All other co-authors have explicitly denied any conflict of interest.

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