Exogenous IL-7 Increases Recent Thymic Emigrants in Peripheral Lymphoid Tissue without Enhanced Thymic Function

Running Title: IL-7 Alters RTE Homeostasis

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Abstract

IL-7 is critical in maintaining thymic-dependent and thymic-independent pathways of T-cell homeostasis. TCR Rearrangement Circles (TREC) have been used as a marker for recent thymic emigrants (RTE) in assessing human thymic function. To study the thymic and peripheral effects of IL-7 on RTE, we measured TREC content and peripheral naïve T-cell subsets and turnover in IL-7 treated mice. Short-term administration of IL-7 into thymus-intact mice resulted in increased total TREC number consistent with RTE accumulation. Decreases in TREC frequency were attributable to dilution secondary to increased cell turnover. Significantly, IL-7 administration into thymectomized mice resulted in patterns of decreased TREC frequency and increased total TREC number similar to those in IL-7 treated thymus-intact mice. Distinct patterns of naïve cell and RTE distribution among peripheral immune organs and altered expression of CD11a were observed following IL-7 treatment in thymus-intact and thymectomized mice. These results demonstrate: 1) total TREC number and not TREC frequency accurately reflects quantitative changes in RTE; 2) short-term IL-7 administration results in preferential accumulations of RTE among peripheral immune organs, accounting for the increase in TREC in the total peripheral lymphoid pool; and 3) no evidence for regulation of thymic function by short-term IL-7 administration.
**Introduction**

Identifying factors regulating thymic function is critical in developing strategies to enhance and/or preserve immune function in immunodeficient states associated with HIV infection and hematopoietic stem cell transplantation (HSCT). An important parameter of thymic function is the generation of T-cells as recent thymic emigrants (RTE). In the absence of phenotypic markers distinguishing RTE, measurement of TCR rearrangement excision circles (TREC) has been developed for quantifying RTE in humans\(^1\). TREC are episomal DNA circles generated during TCR rearrangement. Most human TCR\(\alpha/\beta\) T-cells contain TREC formed by the rearrangement between the \(\delta\)-rec and \(\psi J\alpha\) loci during TCR\(\delta\) locus deletion and TCR \(\alpha\)-chain gene rearrangement\(^2\). Because episomal DNA does not replicate, TREC frequency, i.e. TREC molecules per cell number, decreases with successive cell divisions, permitting its use not only as a marker of thymic function, but also as a quantitative marker for RTE in the periphery.

Interpretation of human TREC data expressed as a frequency is problematic because this value can be influenced by changes in peripheral cell turnover in the absence of altered RTE generation\(^3\,4\,5\,6\). Importantly, sampling of human tissue for TREC analysis is almost exclusively limited to the peripheral blood. Even when quantitatively expressed, TREC number in blood cannot detect changes in RTE trafficking among multiple lymphoid compartments and extra-lymphoid tissue, nor does it provide information regarding the maintenance of RTE in these sites. Thus, changes in TREC frequency in many cases may not reflect thymic function, and measuring TREC in peripheral blood may not be representative of changes to peripheral RTE numbers as a whole. In this
regard, the development of a TREC assay in mice enhances the interpretative value of TREC data because unrestricted tissue sampling makes it possible to determine T-cell population size and turnover and total peripheral TREC number in peripheral lymphoid organs and peripheral blood. The murine homologs to \( \delta \)rec and \( \psi \)J\( \alpha \) have been cloned and sequenced \(^7\), and enumeration of this single murine TREC species has been demonstrated \(^8\)-\(^{10}\)\(^{11}\). However, to date, there have been no studies with murine TREC that examine the relative contributions of thymic output and peripheral T-cell homeostasis on TREC frequency and number.

Cytokines influence the production and turnover of peripheral T-cell populations and could also lead to alterations in RTE homeostasis. IL-7 is required for T-cell development in the thymus \(^{12}\)^{13}, and has been shown to enhance thymopoiesis following HSCT \(^{14}\)^{15}. Additionally, IL-7 has been shown to have potent effects on the proliferation and survival of peripheral T-cells \(^{16}\)^{17}^{18}. The importance of IL-7 as a regulator of peripheral T-cell homeostasis is highlighted by the observation that serum levels of IL-7 are elevated in states of peripheral T-cell depletion \(^{19}\). However, IL-7 effects on RTE homeostasis remain largely unexplored. Here, we demonstrate that exogenous administration of IL-7 into normal thymus-intact mice results in an increase in spleen and LN RTE numbers in the absence of detectable changes to thymic function, and that similar increases in spleen and lymph node RTE numbers are also observed in thymectomized mice. The changes in total spleen and LN RTE suggest a potential effect of IL-7 on lymphocyte trafficking resulting in accumulations of RTE in peripheral immune organs.
Materials and Methods

Animals

8-12 week old thymus-intact and thymectomized C57Bl/6 mice were purchased from the Animal Production Unit, National Cancer Institute. IL-7 knockout mice were acquired from DNAX Research Institute. Suction thymectomies were performed at 4-6 weeks of age. Completeness of the procedure was confirmed by visual inspection at the time of sacrifice. Studies were carried out under approved animal study protocols.

Administration of Murine IL-7

Recombinant murine IL-7 (rmIL-7) (Peprotech) was reconstituted according to manufacturer’s instructions and resuspended in 5% sucrose and 0.1% C57Bl/6 normal mouse sera in phosphate-buffered saline (PBS). rmIL-7 was administered by continuous infusion at 5 ug/day via subcutaneous osmotic pumps for 7 days (1007D) or 14 days (1002) (DURECT). Mice were anesthetized by i.p. injection of xylazine and ketamine. Pump were implanted according to institutionally approved manufacturer’s protocols.

Flow Cytometry

Single-cell suspensions were prepared from thymus, spleen, and pooled inguinal (two), axillary (two), cervical (two), and mesenteric (three) lymph nodes (LN) of mice sacrificed by CO2 asphyxiation. Red blood cells were lysed by treatment with ACK lysing buffer (Quality Biological). Peripheral blood lymphocytes (PBL) were isolated from whole blood after two rounds of ACK lysing buffer treatment. Cells were counted and prepared for flow cytometry. 1x10^6 cells were incubated with 2.4G2 followed by
staining with fluorescent antibodies. Intracellular staining of Ki-67 or BrdU was performed using the Cytofix/Cytoperm and BrdU Flow Kits (Pharmingen). Directly conjugated and biotinylated monoclonal antibodies (mAb) used included: CD25, CD69, CD71, CD45RB, CD103, Ki-67, CD3, CD4, CD5, CD8, CD11a, CD44, B220, IgM, HSA, Gr-1, DX5, Mac-1, H57, GL-3 and BrdU (Pharmingen); and Cy-5-conjugated CD4 and CD8 (Caltag). Biotinylated mAbs were developed with Cy5-conjugated streptavidin (Pharmingen). Isotype control mAb were Leu4 for surface staining, mouse IgG₁ for Ki67 staining, and mouse IgG₁κ (Pharmingen) for BrdU staining. Three-color flow cytometry was performed using a FACS Vantage and analyzed with Cell Quest software (Becton Dickinson).

For cell sorting, combined splenocyte and LN CD4+ and CD8+ cells were purified using immunomagnetic beads (Miltenyi Biotec). CD4+ cells were sorted into naïve (CD45RBhi, CD44lo) and memory (CD45RBlο, CD44hi) subsets; CD8+ cells were sorted into naïve (CD103hi, CD44lo) and memory (CD103lo, CD44hi) subsets. Live cells were selected based on light scatter and propidium iodide staining. The purity of all sorted populations was confirmed to be greater than 99%.

TREC Analysis

Cell lysates were prepared by incubation at 55°C for one hour in 100 ug/mL proteinase K (Boehringer Mannheim), followed by inactivation at 95°C for fifteen minutes. The lysate from 50000 cells was added to a real-time quantitative PCR reaction, containing mδRec primer (5’-GGGCACACACAGCAGCTGTG), ψJα primer (5’-
GCAGGTTTTTGTAAAGGTGCTCA), and mδRec-ψJα fluorescent probe (5’-FAM-CACAAGCACCCTGCACCCATGGA-TAMRA-3’). Lysates were separately amplified for the single-copy CD8β gene using forward primer (5’-CAGGACCCCAAGGACAAGTACT-3’), reverse primer (5’-CACTTTCAACCATAAAAACCTCTTTCG-3’), and probe (5’-FAM-TGAGTTCTGCTGCTGCCCTGAGTTCTTC-TAMRA-3’). Primers were synthesized by Invitrogen and fluorescent probes by Biosource. PCR reactions contained 0.5uM of each primer, 0.3 uM of fluorescent probe, 1x Platinum Quantitative PCR Supermix-UDG (Invitrogen), and Blue-636 reference dye (MegaBases). Amplifications were performed in triplicate on an ABI Prism 7700 Sequence Detection system (Perkin-Elmer) and analyzed using associated software. PCR conditions were 50°C for two minutes, 95°C for five minutes, then 40 cycles of 95°C for 15 seconds and 60°C for one minute.

Standard curves for murine TREC were generated by cloning a δRec-ψJα TREC PCR product into pCR-XL-TOPO (Invitrogen). Stock dilutions of 10^7, 10^6, 10^5, 10^4, 10^3, 10^2, 10^{1.5}, and 10^{1.25} plasmids per 5 uL were generated. Standard curves for the CD8β chain gene were similarly prepared. For each real-time PCR experiment, δRec-ψJα TREC and CD8β chain gene standards were run in duplicate per experiment to generate standard curves. TREC frequency, expressed as the number of TREC molecules per 50000 cells, was determined by normalizing the number of TREC amplified in the real-time PCR reaction to the number of amplified CD8β molecules. The total number of TREC in a given cell population was calculated by multiplying the TREC frequency by the number of cells in the population.
BrdU Labeling

Mice were given a continuous oral infusion of BrdU (Sigma) in the drinking water at a concentration of 0.8 mg/mL. BrdU treated water was made fresh and changed daily. Untreated mice were used as a negative control for BrdU staining.

Histology

5 um paraffin sections of thymus, spleen, and lymph nodes were generated, stained with hematoxylin and eosin, and visualized by light microscopy.
Results

Exogenous IL-7 Expands Combined Spleen/LN RTE Populations in Thymus-Intact and Thymectomized Mice

We sought to determine the effect of exogenous IL-7 on peripheral RTE and non-RTE T-cell populations in mice. Subcutaneous pumps were placed into young adult mice to deliver rmIL-7 by continuous infusion. Combined single-cell suspensions from spleen and LN were analyzed for their overall cellularity and total TREC number. Because RTE reside within the naïve cell compartment, sorted naïve CD4+ and CD8+ cells were quantified and analyzed for TREC frequency and total TREC. Cells with activated/memory phenotype were also collected to confirm that TREC resided predominantly in the naïve compartment (data not shown). Studies in our laboratory (data not shown) and those of others⁸,¹¹ have shown that measurable TREC levels persist following thymectomy. Parallel experiments were performed in thymectomized mice to determine the effect of absent thymic function on peripheral naïve cell and RTE populations.

rmIL-7 administration into thymus-intact mice resulted in significant increases in total spleen and LN cell number and TREC, and in the size of naïve CD4+ and CD8+ populations (Fig. 1A, C, and G). TREC frequencies in both naïve CD4+ and CD8+ cells were decreased by 20% with rmIL-7 treatment (Fig. 1E and I). Accounting for increases in naïve T-cell numbers, total TREC number was increased by roughly two-fold (Fig. 1F and J). The observed decrease in TREC frequency indicated a dilutional effect as a result of rmIL-7 induced cell proliferation. We therefore measured the rate of BrdU incorporation in naïve CD4+ and CD8+ cells. Mice were given BrdU in the drinking
water at the start of the rmIL-7 infusion. BrdU incorporation was measured in naïve T-cells during rmIL-7 and BrdU co-administration. rmIL-7 administration dramatically increased cell division in naïve T-cells. By day 8 of rmIL-7 infusion, 50% of naïve CD4+ cells were BrdU-positive in IL-7 treated mice, compared to 6% in diluent treated mice (Fig. 1D). Naïve CD8+ cells showed a more pronounced degree of cell proliferation with rmIL-7 treatment in that 70% of naïve CD8+ cells were BrdU-positive compared to 5% in the control mice (Fig. 1H). Additional evidence for increased proliferation in naïve T-cells was that expression of the cell proliferation marker Ki67 in these populations was increased by ten-fold after seven days of rmIL-7 treatment (data not shown).

Significantly, when rmIL-7 was administered into age-matched thymectomized mice, similar changes in naïve and RTE populations were observed. Compared to thymus-intact mice, total spleen and LN TREC numbers, as well as naïve T-cell and TREC numbers, were decreased in thymectomized animals, consistent with the expected loss of RTE production (Fig. 1). Similar to observations in thymus-intact mice, administration of rmIL-7 into thymectomized mice resulted in a marked increase in the number of naïve T-cells (Fig. 1C and G). TREC frequencies in naïve CD4+ and CD8+ cells from rmIL-7 treated mice were decreased by 40% from those of control diluent-treated mice (Fig. 1E and I). That the decrease in TREC frequency was due to the effects of rmIL-7 on cell proliferation was again demonstrated by the increased rate of BrdU uptake in naïve cells (Fig. 1D and H) corroborated by a ten-fold increase in the levels of Ki67 expression on the seventh day of rmIL-7 infusion (data not shown). Despite the decrease in TREC
frequency, total combined spleen and LN and naïve CD4+ and CD8+ TREC numbers were increased in rmIL-7 treated mice by a proportion comparable to that observed for thymus-intact animals (Fig. 1A, F and J). These results demonstrate that even in the absence of thymic function, exogenous IL-7 resulted in an accumulation of combined spleen and LN RTE. Furthermore, these results highlight the limitations of interpreting TREC frequency as a measure of thymic output during altered peripheral T-cell homeostasis, and that determination of total TREC in a broad sampling of peripheral lymphocytes provides a more accurate assessment of the size of the RTE population.

**Short-Term IL-7 Administration Has No Effect on Thymic Function**

Analysis of the thymuses from rmIL-7 treated mice revealed no evidence of altered thymic function, further supporting the concept of an extrathymic mechanism accounting for the accumulation of combined spleen/LN RTE in thymectomized mice. There were no differences in the size or cytoarchitecture of thymi taken from rmIL-7 treated mice. In contrast, their spleen and LN were noticeably enlarged, with evidence of cell proliferation in T-cell rich areas consistent with the previously described changes in cell numbers and rates of cell proliferation (Fig. 2). Thymocyte number and total intrathymic TREC content in the rmIL-7 treated mice were identical to those of control mice (Fig. 3A-B). Proportions of double negative (DN), double positive (DP), CD4+ single positive (CD4 SP), and CD8+ single positive (CD8 SP) thymocytes were unaltered in IL-7 treated mice (Fig. 3C). Because IL-7 has been shown to be critical in the development of DN thymocyte population 20, we examined the distribution of the DN subpopulations in IL-7 treated vs. diluent treated control mice based on dual CD25 and CD44 expression in
lineage thymocytes. No differences were seen in the numerical distribution of DN1 through DN4 thymocytes (Fig. 3D). Finally, comparing maturation marker expression patterns in DN, DP, CD4 SP, and CD8 SP thymocytes revealed no differences between IL-7 treated vs. diluent-treated mice (Fig. 3E).

To investigate the possibility that exogenous rmIL-7 enhanced thymic function through alterations in thymocyte development kinetics, without alteration of thymic size or cellularity, we measured the rate of BrdU uptake in each thymocyte subset in mice given continuous oral BrdU. Cell division in the thymus predominantly occurs at the late DN stage^21^, and these cells remain BrdU+ during subsequent maturation stages^21,22^. The rate of BrdU acquisition in DP thymocytes therefore reflects the rate of DN to DP thymocyte maturation. In the CD4 and CD8 SP populations, BrdU acquisition reflects a combination of three events: 1) proliferation of intermediate single positive cells that are the developmental precursors to DP thymocytes; 2) maturation of DP into mature SP populations; and 3) proliferation of mature SP thymocytes^23,24^.

In marked contrast to the rapid rate of BrdU incorporation in peripheral T-cell subsets described previously, the kinetics of BrdU incorporation in the four thymocyte subsets, reflected by changes in the percentage of BrdU+ cells (Fig. 3F) and the absolute number of BrdU+ cells (data not shown) over time, were similar between the rmIL-7 treated and control diluent treated mice. The identical rate of BrdU incorporation among DP thymocytes in the two groups demonstrated that the kinetics of the DN to DP transition was not affected by rmIL-7 administration. Likewise, given the identical rate of BrdU incorporation in CD4+ and CD8+ SP thymocyte populations between the rmIL-7 and control diluent treated mice,
none of the aforementioned events that promote BrdU incorporation in these populations were noticeably affected by rmIL-7 treatment.

A possible explanation for the aforementioned findings is that the dosage and/or route of administration of rmIL-7 used in these experiments were insufficient to effect any phenotypic or functional change in the thymus. Yet, rmIL-7 given in an identical dose schedule to young adult IL-7 knockout mice resulted in a seven-fold increase in both thymic cellularity and total intrathymic TREC (data not shown), demonstrating that the dose and route of administration of rmIL-7 used in these experiments was sufficient to alter thymic phenotype in IL-7 deficient states.

**Exogenous IL-7 Affects the Distribution of RTE among Peripheral Immune Organs**

Possible mechanisms to explain the accumulation of combined spleen and LN TREC in the absence of changes to thymic function with exogenous rmIL-7 include: 1) TREC duplication; 2) increased extrathymic T-cell development; 3) enhanced survival of RTE; and 4) preferential accumulation of RTE in individual lymphoid compartments due to an effect of exogenous IL-7 on RTE trafficking. No evidence supporting the first three mechanisms was found in multiple experiments. First, correlates of decreases in TREC frequency resulting from cell division in vitro showed no evidence of TREC duplication, even when rmIL-7 was added to the cultures (data not shown). Second, analysis of T-cell populations in thymectomized mice treated with rmIL-7 suggested that the induction of extrathymic T-cell development was not a major mechanism for accumulation of spleen and LN TREC, in that 1) aged thymectomized mice treated with rmIL-7, containing
negligible TREC levels, exhibited no increases in spleen/LN TREC; 2) there were no observed indications of increased extrathymic T-cell development with respect to increases in peripheral CD4+/CD8+ double positive cells, intra-epithelial lymphocyte derived CD8αα+ cells, or CD3lo-int T-cells typically seen in models of extrathymic T-cell development; and 3) histologic analysis of small intestine in rmIL-7 treated mice failed to show expansion of Peyer’s patches or IEL-associated areas in the lamina propria (data not shown). Moreover, while administration of IL-7 into athymic nude mice showed increases in the number of naïve cells and TREC indicative of IL-7 dependent extrathymic T-cell development, the magnitude of these increases was limited and could not account for the degree of naïve T-cell and TREC accumulation observed in identically treated thymectomized mice described earlier (data not shown). Third, whereas a principal mechanism of IL-7 on T-cells is enhanced cell survival through bcl-2 upregulation, administration of exogenous IL-7 into bcl-2 transgenic mice resulted in a similar accumulation of TREC in combined spleen and LN (data not shown), indicating that additional mechanisms accounting for the increases in combined spleen/LN TREC exist. Moreover, kinetic measurements of spleen and LN TREC numbers during rmIL-7 infusion into thymectomized mice showed a progressive increase in total TREC number over time (data not shown) rather than a persistent maintenance of TREC number that would be predicted if preferential survival of RTE was the predominant effect of IL-7.

To provide evidence that exogenous IL-7 can influence the accumulation of naïve cells and RTE in peripheral immune organs, we sought to determine if distributive changes in naïve cell number, cell proliferation, and TREC numbers among separate lymphoid
compartments occurred with IL-7 treatment. Following seven or fourteen days of continuous rmIL-7 administration, naïve T-cell and TREC numbers in each of three individual peripheral lymphoid compartments—spleen, LN, and PBL—were determined in thymus-intact and thymectomized mice. Cell proliferation was determined by assessing Ki67 expression. Evidence for preferential trafficking of RTE into an individual lymphoid compartment with IL-7 treatment would comprise a change in the distribution of total organ TREC number among the spleen, LN, and PBL that are independent of alterations in cell numbers and the degree of cell proliferation. In this regard, organ TREC frequency is useful because it is indicative of the relative contribution of RTE entry vs. cell proliferation to total T-naïve cell numbers within that organ.

Seven days of treatment with rmIL-7 resulted in an altered distribution of RTE among the peripheral immune organs, with patterns that were similar in thymus-intact and thymectomized mice. Expansion of both naïve CD4+ and CD8+ populations occurred almost exclusively in the LN and peripheral blood, with a three-fold increase in the number of naive CD4+ and CD8+ cells in LN and a two-fold increase in PBL. In contrast, the number of CD4+ naïve cells in the spleen was not increased and the number of CD8+ naïve cells was only modestly, though significantly, increased (Fig. 4A and G) in thymectomized mice. Coincident with the altered distribution pattern of naïve cells among the spleen, LN, and PBL, proliferation of naïve T-cells as measured by Ki67 expression was significantly higher in all three compartments of IL-7 treated mice compared to diluent controls but did not significantly differ among the three
compartments (Fig. 4B and H). Despite this, total TREC number in the LN was increased two-fold in rmIL-7 treated mice, whereas total TREC numbers in the spleen and PBL compartments were not as dramatically altered (Fig. 4D and J). In rmIL-7 treated thymectomized mice, spleen TREC number was significantly increased but less dramatically than LN TREC number. Consequently, after seven days of rmIL-7 treatment in thymus-intact and thymectomized mice, the LN constituted the largest reservoir of naïve T-cells and RTE, whereas in the diluent-treated groups, naïve cells and RTE were found in the spleen and LN in equal numbers. Further evidence of preferential RTE accumulation in the LN with seven days of rmIL-7 treatment is that despite equally elevated levels of Ki67 expression in naïve cells in all three compartments, TREC frequency in LN was only decreased to 80% of diluent control, whereas in the spleen and PBL TREC frequency was more substantially decreased to 20% of that of diluent controls (Fig. 4C and I).

Treatment with rmIL-7 for fourteen days resulted in shifting RTE distribution patterns that differed between thymus-intact and thymectomized mice. In thymus-intact mice, total TREC number in the LN was increased two-fold in rmIL-7 treated mice. Total TREC numbers in the spleen and PBL compartments were also significantly increased, but not to the same degree as that observed for LN (Fig. 4F). TREC frequency in LN was only decreased to 60% of diluent control, whereas in the spleen and PBL TREC frequency was more substantially decreased to 20% of that of diluent controls (Fig. 4E). In contrast to thymus-intact mice, thymectomized mice given rmIL-7 for fourteen days resulted in an accumulation of RTE in both spleen and LN, with both total spleen and LN
TREC significantly increased after IL-7 treatment. However, TREC frequency in the LN was not significantly decreased relative to the diluent control, whereas in the spleen and PBL the decrease in TREC frequency was to a level 40% and 8% of diluent control levels, respectively (Fig. 4E-F), suggesting that the accumulation of RTE relative to overall increases in naïve cell number may occur to a greater degree in LN than in spleen despite significant increases in total TREC number in both organs. Also of note, total PBL TREC was significantly decreased in mice treated with fourteen days of rmIL-7 compared to controls, in contrast to thymus-intact mice where PBL TREC numbers were increased (Fig. 4L).

Exogenous IL-7 Increases CD11a Expression on Naïve T-Cells

The ability of T-cells to traffic specifically into peripheral immune organs is dependent on the coordinate expression of cell adhesion molecules. Particularly relevant to the observed accumulation of naïve cells and RTE in the LN of rmIL-7 treated mice is the expression level of the adhesion molecule CD11a (LFA-1), a member of the β2 family of integrin receptors. CD11a expression on T-cells has been shown to be critically important in the migration of T-cells through the high endothelial venules of LN. We therefore measured the expression of CD11a on naïve T-cells isolated from rmIL-7 treated mice. Expression of the CD11a in naïve CD4+ and CD8+ cells of thymus-intact and thymectomized mice treated with rmIL-7 was significantly increased in naïve cells derived from LN (Fig. 5A), spleen, and PBL. Alterations in CD11a expression with rmIL-7 administration occurred in the absence of overt T-cell activation as measured by CD69, CD25, and CD71 expression (Fig. 5B).
Discussion

Our results indicate that increases in spleen/LN RTE populations in mice given short courses of rmIL-7 result from extrathymic effects of IL-7, and not from enhanced thymic function. The finding of increased total naïve T-cell and TREC numbers in spleen/LN cells in rmIL-7 treated thymectomized mice is compelling evidence in support of this hypothesis. Although residual thymic function following incomplete thymectomy is possible, all thymectomized mice were carefully inspected to verify the completeness of the procedure. In addition to establishing a mechanistic role of IL-7 in RTE homeostasis, the results confirm that TREC frequency is affected by changes in T-cell turnover and that only by determining total TREC numbers could changes in the size of the RTE population be accurately assessed.

rmIL-7 treatment of thymus-intact mice did not affect thymic function by multiple parameters. Clinical studies in HIV+ patients and in patients receiving chemotherapy suggest a direct relationship between thymic size and thymic output\textsuperscript{32,33}. Given the absence of changes to thymic size or phenotype, and the fact that thymocyte development kinetics and proliferation as measured by BrdU incorporation were unchanged, there is no evidence of enhanced thymic function with IL-7 administration in these experiments. Interestingly, the absence of altered thymic function with exogenous rmIL-7 is similar to that found in mice expressing the IL-7 transgene under the control of the MHC class-II E\textsubscript{a} promoter. Despite a marked increase in IL-7 production by thymic epithelium, there were no significant alterations in thymic phenotype, while at the same time, profound increases in the size of peripheral T-cell populations due to systematic IL-7 production by
Peripheral epithelium were observed. This is in contrast to mice expressing the IL-7 transgene driven by the Ig promoter/enhancer or the pLCK promoter (El Kassar and Gress, unpublished observations), where marked perturbations in thymocyte development were observed. The differences among these thymic phenotypes likely relate to different quantitative and temporal-spatial patterns of IL-7 expression and regulation. That such profound effects of exogenous IL-7 could be seen with respect to the changes in peripheral T-cell population size in the absence of changes to thymic function underlies the role of IL-7 administered short-term as an important regulator of peripheral T-cell homeostasis while minimizing its role as a dominant regulator of thymic output in otherwise normal mice, at least at the dose used in these experiments. These results support the role of IL-7 in T-cell reconstitution following HSCT in enhancing peripheral maintenance of RTE and other peripheral T-cell populations in addition to restoration of thymic function.

Examining the distribution of naïve T-cells and TREC among separate peripheral immune compartments supports the potential role of short-term IL-7 administration in increasing spleen/LN RTE number through altered trafficking of RTE into these organs. No clear evidence supporting other plausible mechanisms that could increase peripheral RTE/TREC with rmIL-7 treatment was found. Distribution patterns of RTE differed in thymus-intact and thymectomized mice depending on the duration of rmIL-7 treatment. In both thymus-intact and thymectomized mice, seven days of rmIL-7 treatment resulted in a predominant increase in LN RTE. In contrast, with fourteen days of rmIL-7 treatment RTE numbers in LN in thymus-intact mice remained high along with
significant, though more modest, increases in RTE in the spleen. In thymectomized mice, the patterns of RTE localization with exogenous IL-7 were different in that the RTE number was prominently increased in both spleen and LN, yet the absence of changes in TREC frequency in LN and the significant decreases in spleen TREC frequency suggests that RTE accumulation accounted for a greater proportion of overall T-cell population increases in the LN than in the spleen. The depletion of RTE from PBL in these mice further supports the role of IL-7 in redirecting circulating RTE into secondary lymphoid organs, in contrast to IL-7 treated thymus-intact mice, where continuous production of RTE by the thymus may compensate for the exit of RTE from the circulation. A similar pattern of preferential RTE accumulation in the LN has also been observed in HIV-infected patients\(^3^7\). Although not measured, it is possible that these findings were due in part to elevated IL-7 levels in this setting of T-cell depletion\(^1^9\).

The mechanisms by which IL-7 controls RTE trafficking require further characterization. Evidence exists that IL-7 plays at least an indirect role in controlling lymphocyte migration to the extent that it has been shown to act as a mobilizing agent for pluripotent hematopoietic stem cells in a murine bone marrow transplant model\(^3^8\). At the cellular level, IL-7 has been implicated in modulating expression of surface adhesion molecules and chemokine receptors \textit{in vitro} including CD11a expression\(^3^9\)\(^4^0\)\(^4^1\). Our data comparing relative expression levels of CD11a in naïve T-cells is evidence that short-term treatment with IL-7 may modulate CD11a expression \textit{in vivo}. It remains a possibility that increased CD11a expression in naïve cells and RTE with rmIL-7
treatment may play an important role in the preferential trafficking of these cells to LN. Completely consistent with our observations, adoptively transferred lymphocytes from CD11a deficient mice exhibited markedly impaired homing into LN, whereas their homing into the spleen was unaffected. Additional evidence for the role of CD11a in the homing of T-cells to LN is the observation that the aforementioned accumulations of naïve cells and TREC in the LN of HIV-positive patients were associated with increases in CD11a expression. Of note, in these experiments, alterations in CD11a expression occurred in the absence of overt T-cell activation as assessed by the expression of surface activation markers, although the possibility that this is the result of IL-7 induced homeostatic proliferation leading to a partially activated phenotype cannot be ruled out. Moreover, the relationship between IL-7 treatment and expression of other cell adhesion molecules involved with lymphocyte trafficking requires further experimentation.

While the redistribution of RTE among LN, spleen, and PBL after IL-7 treatment is clear, the aggregate number of RTE in these organs is greater in IL-7 treated thymectomized mice than in diluent treated controls. This suggests that RTE upon exit from the thymus not only migrate to spleen and LN, but must also traffic into other peripheral lymphoid or non-lymphoid organs. Upon treatment with IL-7, RTE that initially migrated to these sites would then traffic to LN and spleen. That RTE migrate to organs other than LN runs counter to the currently accepted notion that newly-generated naïve T-cells exclusively traffic to LN. Yet, adoptively transferred ⁵¹Cr-labelled naïve T-cells were found to migrate to non-lymphoid tissue in substantial numbers up to 24 hour after their injection. Together with the results reported here, extrathymic tissue may in fact...
represent a significant reservoir of RTE, and entry and exit of RTE from extrathymic
tissues may be controlled at least in part by cytokines such as IL-7. As these cell
populations were not analyzed in these experiments, it will be necessary to isolate T-cells
from lymphoid and non-lymphoid organs not included in this study and demonstrate a
decrease in RTE number by enumeration of total TREC in these organs concomitant with
an increase in RTE number in spleen and lymph node. Alternatively, trafficking patterns
of naïve T-cells and/or RTE with or without IL-7 administration could be monitored by
adoptively transferring appropriately labeled cells that would permit real-time in vivo
trafficking studies in whole animals 44 45.

In conclusion, short-term exogenous IL-7 administration affects RTE populations in
spleen and LN not by enhancement of thymic function, but by altering peripheral RTE
trafficking. These findings provide insights into the biology of RTE and interpretation of
TREC data.

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Naïve CD4+ (CD44lo; CD45RBhi)

Figure 1A-F
Figure 1. Short-term administration IL-7 similarly affects cell numbers, cell turnover, and TREC content in thymus-intact and thymectomized mice. Mice were given rmIL-7 by continuous infusion at a dose of 5 ug/day, or diluent only, for 14 days. Total combined spleen and lymph node cell number (A) and TREC number (B), naïve CD4+ cell number, defined by CD44lo and CD45RBhi co-expression (C), and CD8+ cells, defined by CD44lo and CD103hi co-expression (G), were determined. TREC frequency (#TREC/50 cells) was measured by real-time quantitative PCR (E and I), and total TREC number in sorted naïve CD4+ (F) and naïve CD8+ (J) cells were calculated. To assess changes in cell turnover as a result of rmIL-7 administration, rmIL-7 or diluent treated mice were given BrdU in the drinking water and the percentages of BrdU+ naïve (CD44lo) CD4+ (D) and CD8+ (H) cells were measured at the indicated time points. Data in (A) and (B) are data from a single experiment, representative of two experiments carried out, showing the mean ± S.D. of six mice per group. Data in (C) through (J) are representative data from single experiments, representative of two experiments carried out, showing the mean ± S.D. of three mice per group. *P < 0.05 when comparing rmIL-7 treatment to diluent control by the Mann-Whitney U test. **P = 0.09.
Figure 2. Short-term administration of IL-7 does not affect thymic cytoarchitecture, but greatly affects spleen and lymph node cytoarchitecture. H&E sections were prepared of thymus (cortex C and medulla M), spleen (arrows indicating arterioles within periarteriolar T-cell zones), and mesenteric lymph node (B-cell follicles B and T-cell parafollicular zones T). Mice treated with rmIL-7 (5μg/day x 14 days) were compared with diluent controls. Magnification: 100x in all sections. Sections shown here are representative of six mice in each group.
Figure 3A-E
Figure 3. Short-term IL-7 administration does not alter thymic phenotype or function. Mice treated with rmIL-7 (5ug/day x 14 days) or diluent were analyzed for thymocyte number (A), total intrathymic TREC (B), and distribution of thymocyte subsets (C). (D) Lin- negative thymocytes (CD3, CD4, CD8, B220, IgM, Mac-1, pan-NK, and Gr-1 negative) were analyzed for CD44 and CD25 expression that define the DN1 through DN4 subpopulations. Data shown in graphs A-D are data from a single experiment, representative of two experiments carried out, showing the mean ± S.D. of three mice per group. (E) Patterns of thymocyte maturation markers within each thymocyte subset were analyzed in IL-7 treated (dashed line) and diluent treated (solid line) mice. Shown are representative data from three separate experiments. (F) The kinetics of thymocyte development was determined in IL-7 and diluent treated mice by continuous oral administration of BrdU (see Materials and Methods). At the indicated time points, the percentage of thymocyte subset populations that incorporating BrdU was determined. Shown are data from a single experiment, representative of two experiments carried out, with the mean ± S.D. of three mice per group.
**IL-7 x 7 days: Thymus-Intact Mice**

**A)**

- CD4+/CD44lo
- CD8+/CD44lo

**B)**

- CD4+/CD44lo
- CD8+/CD44lo

**C)**

- Organ TREC Frequency (TREC/50000 Cells)

**D)**

- Total Organ TREC (x10^5)

**IL-7 x 14 days: Thymus-Intact Mice**

**E)**

- Organ TREC Frequency (TREC/50000 Cells)

**F)**

- Total Organ TREC (x10^5)

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**Figure 4A-F**
Figure 4. Short-term IL-7 administration results in preferential accumulation of naïve T-cells and TREC in the lymph nodes of thymus-intact and thymectomized mice. Thymus–intact (A–F) and thymectomized (G–L) mice were given rmIL-7 by continuous infusion at a dose of 5 ug/day, or diluent only, for seven days or fourteen days. Following the seven-day treatment course, numbers of naïve (CD44lo) CD4+ and CD8+ cells (A and G), Ki67 expression (B and H), organ TREC frequency (C and I), and total organ TREC number (D and J) were determined. TREC frequency (E and K) and total organ TREC number (F and L) following fourteen days of rmIL-7 treatment are also shown. Shown are combined data from two to three experiments, with mean ± S.D. of seven to twelve mice per group. *P < 0.05 when comparing rmIL-7 treatment to diluent by the Mann-Whitney U test. In both thymus-intact and thymectomized mice, statistically significant differences in naïve cell number and total TREC between the lymph nodes and spleen of rmIL-7 treated mice are indicated by brackets (A and D) and (G and J) after seven days of rmIL-7 treatment, and following fourteen days of rmIL-7 treatment in thymus intact mice (F).
Figure 5. Short-term IL-7 administration alters the expression of CD11a (LFA-1) in naïve cells in the absence of T-cell activation. Histogram plots of CD11a expression in lymph node derived naïve CD44lo CD4+ and CD8+ cells in diluent control and rmIL-7 treated (5 ug/day for seven days) thymus-intact mice are shown (A). Mean fluorescent intensity (MFI) of CD11a was consistently higher in cells from rmIL-7 treated mice. Numerical values represent the MFI ± S.D. of seven mice per group. Similar increases in CD11a MFI were observed in spleen and PBL derived naïve T-cells (data not shown). In contrast, administration of rmIL-7 did not induce expression of the T-cell activation markers CD69, CD25, or CD71 (B). Solid lines represent histograms of diluent-treated control mice, dashed-dotted lines represent histograms of rmIL-7 treated mice, and dashed lines represent histograms of normal splenocytes that were stimulated in vitro with anti-CD3 (2C11) antibody serving as a positive control for CD69, CD25, and CD71 expression. Shown are representative data from seven mice. Similar patterns of CD11a and T-cell activation marker expression were observed in rmIL-7 treated thymectomized mice and in thymus-intact and thymectomized mice treated with rmIL-7 for fourteen days (data not shown).
Exogenous IL-7 Increases Recent Thymic Emigrants in Peripheral Lymphoid Tissue without Enhanced Thymic Function

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