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The Interaction of LCK and the CD4 Co-receptor Alters the Dose Response of T-cells to Interleukin-7

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Abstract

CD8 and CD4 T-cells grow optimally under different concentrations of the cytokine, interleukin-7 (IL-7). While CD8 T-cells expand at high doses of IL-7, CD4 T-cells favor low doses. To examine the reason for the preference of CD4 T-cells for lower doses of the cytokine, we used IL-7-dependent T-cells to study signal transduction upon a range of IL-7 concentrations. We found that the high dose responsiveness of CD8 T-cells to IL-7 could be altered if these cells also expressed CD4. Using the phosphorylation of STAT5 as an indicator of growth, we found that the co-receptor associated kinase, LCK, contributed to phospho-STAT5 levels. Phospho-STAT5 was elevated at high dose IL-7 for CD8 T-cells and at low dose IL-7 for CD4 T-cells, which was reversed upon LCK inhibition. Examining the direct association of LCK with CD4 using a T-cell line that over expresses CD4, we determined that CD4 could directly sequester LCK. Non-CD4 T-cells were not restricted in this manner and levels of phospho-STAT5 increased proportionally to the IL-7 dose. Our studies, therefore, show that the response of a T-cell to IL-7 can be modulated by the availability of LCK.

INTRODUCTION

Cytokines provide support, growth and differentiation signals to the cells of the immune system and as such are targets of interest for therapeutic development. Of these, the cytokines that signal through the gamma common (γ c) chain, such as IL-2, IL-4, IL-7, IL-9 or IL-15, are among the most essential¹. Defects in γ c signaling can cause severe combined immunodeficiency (SCID) and its most common form, X-linked SCID (XSCID)². However, ablation of each of the γ c cytokines revealed that only IL-7 was non-redundant and absolutely required for normal lymphocyte development and growth. Mice lacking IL-7³, the IL-7 receptor unique α chain (IL-7R)⁴ or any of the components of its signaling pathway, JAK3⁵ or STAT5⁶, had severe lymphocyte deficiencies and were immunocompromised. Not only mice, but humans with defects in IL-7R developed T(−)B(+)NK(+) SCID⁷, indicating that IL-7 is an essential cytokine for development and maintenance of a normal immune response.

IL-7 is not produced by lymphocytes but rather by stromal or accessory cells. Circulating serum levels of IL-7 in healthy individuals are low (0.3–8.4 pg/ml)⁸; while serum levels of IL-7 inversely correlate with the depletion of T-cells, as has been shown in patients infected with HIV-1⁹. IL-7 production can be induced, as was shown with hepatocytes in the liver upon

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generation of an acute phase response¹⁰. Hence, the actual levels of IL-7, whether low (as in healthy individuals) or high (induced during infection) remain to be determined. While the effects of extreme ranges of IL-7 concentrations are well known: immunodeficiency in the absence and lymphoma when overexpressed¹¹, more subtle dose effects of IL-7 upon T-cell subset growth have also been reported. In one study, by controlling the expression levels of an IL-7 transgene (Tg) in mice, a concentration effect of IL-7 upon T-cell development was revealed¹². Only low doses of IL-7 could support $\alpha\beta$ T-cell development, while high doses effectively blocked development at an early intrathymic stage. We observed, using an IL-7 dependent T-cell line, that a low concentration of IL-7 provided survival signaling, while a high concentration of IL-7 was required to sustain proliferation^{13,14}. Such findings were translated into distinct signaling effects upon the proliferation of naïve versus memory CD4 T-cells, with naïve T-cells cycling less in response to IL-7¹⁵. In fact, transgenic expression of IL-7 could enhance the proliferation of both CD4 and CD8 T-cells but not B-cells¹⁶, however the proliferation of CD4 T-cells compared to CD8 T-cells was significantly slower. Moreover, injections with IL-7 resulted in CD4 T-cells increasing their numbers only 4-fold as compared to CD8 T-cells which increased 14-fold¹⁷. These studies suggest that there are IL-7 dose specific effects, and that lymphocyte subsets do not respond equally to IL-7, with CD4 T-cells lagging behind CD8 T-cells in their proliferative response. The molecular basis for this differential response to IL-7 remains to be elucidated but could depend on how the activation of the transcription factor, STAT5, is regulated.

A critical transducer of the IL-7 signal, STAT5 exists in two redundant forms of STAT5a and STAT5b and can be phosphorylated and activated by both JAKs and Src kinases, such as LYN and LCK^{18–20}. Recent studies showed that CD8 T-cells but not CD4 T-cells, with the exception of regulatory T-cells, are more dependent on STAT5 activity. Loss of STAT5 severely affected the survival of CD8 T-cells as compared to CD4 T-cells⁶, and mice expressing a constitutively active form of STAT5b displayed a striking expansion of CD8 T-cells²¹. Why CD8 T-cells thrive under conditions of high STAT5 activity, but not CD4 T-cells, remains to be fully understood. Answers may lie in a better understanding of the function of the CD4 and CD8 co-receptors and how their modulation of Src kinase activities affects cytokine signaling through STATs.

It is well known that homeostatic proliferation of naïve T-cells requires both an IL-7 signal, since these cells fail to grow when transferred to IL-7^{-/-} recipients²², and a T-cell receptor (TCR) signal transduced through the Src kinase, LCK²³. However, administration of IL-7 has a strong biological effect on CD8 T-cells, leaving unexplained why CD4 T-cells fail to grow as abundantly upon receiving the same IL-7 stimulus. One recent explanation is that MHC class II expression on dendritic cells, which also express an IL-7R, is down-regulated by IL-7 and inhibits the expansion of CD4 T-cells, at least *in vivo*²⁴. However CD4 T-cells still fail to thrive in STAT5b transgenic mice, in which the transgene was only expressed in lymphocytes²¹, suggesting that there also exists inherent differences in CD4 T-cells that account for their inability to respond to IL-7 in the same manner as CD8 T-cells. Our own observations with *in vitro* culture of isolated T-cells with IL-7¹⁴ support this conclusion. To explore the different dose responses of T-cell subsets to IL-7 and reveal a potential mechanism to account for such differences, we studied the expansion of CD4-expressing and non-CD4 expressing T-cells to high and low dose IL-7. We found that expression of the CD4 co-receptor and the Src kinase, LCK, modulated the responsiveness of CD4 T-cells to IL-7, enabling these to preferentially grow under conditions of low dose, but not high dose, IL-7. CD8 T-cells, in contrast, had no such restrictions. CD4 and CD8 T-cells are thus equipped to respond differently to IL-7, through the interaction of LCK and CD4 co-receptor, which has significant implications for the use of the cytokine in a therapeutic setting.

MATERIALS AND METHODS

Mice, cells and cell isolation techniques

C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, Maine) and OT-1/Rag1^{-/-} mice were purchased from Taconic. Mice were housed in the animal facility at the University of Central Florida and used at 2–3 months of age. STAT5b-CA mice were generously provided by Dr. Michael Farrar (Department of Laboratory Medicine and Pathology, University of Minnesota, Minneapolis, MN). IL-7R transgenic mice were generously provided by Dr. Alfred Singer (NEI, NIH). Animal studies were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Central Florida.

Lymph node and spleen cells were isolated by gentle crushing of organs through a 70µm pore filter (BD Falcon) and pooled. Spleen cells were further treated with ACK lysis buffer (Quality Biological, Inc.) to lyse red blood cells (RBCs) and enriched for T-cells by negative selection with a commercially available kit, the IMag Mouse T-lymphocyte Enrichment kit (BD Biosciences). CD4 or CD8 T-cell subsets were enriched by negative selection using the IMag T-cell enrichment kit supplemented with biotinylated CD4 or CD8 antibodies. Thymocytes were isolated from thymic lobes by gentle disruption to with a micropipette after treatment with 0.2% collagenase and double negative thymocytes (CD4^{-/-}CD8^{-/-}) isolated by negative selection using the IMag system as described above. Naïve T cells were isolated directly from the lymphoid organs of C57BL/6 mice, while CD44^{hi} memory T cells were isolated from OT1/Rag1^{-/-} mice after 6 weeks from receiving an interperitoneal injection 10 mg of ovalbumin (OVA). Isolation of T-cells was confirmed by analysis of surface markers by flow cytometry as described below.

The IL-7-dependent T-cell line, D1, was established from pro-T-cells isolated from a p53^{-/-} mouse as previously described²⁵.

In vitro culture with IL-7

Culture conditions, for the *ex vivo* expansion of IL-7-responsive cells, were previously described¹⁴. Briefly, primary T-cells isolated from lymphoid organs were maintained at a density of 3–5 × 10⁶ cells/mL in complete medium (RPMI 1640 Medium (Invitrogen) supplemented with 10% fetal bovine serum (Hyclone), 2-βmercaptoethanol (1000X, Life Technologies), and 50 U/mL penicillin/streptomycin) in 24 well plates. Recombinant human IL-7 (Peprotech) was added at the start of culture at a concentration of 10 or 150 ng/mL and refreshed every other day. Previously we established that murine T-cells grow well with human recombinant cytokine. Cultures were maintained with or without IL-7 through 14 days or until noticeable cell death was apparent and confirmed by Trypan Blue exclusion staining. In LCK inhibition experiments, cells were treated with LCK inhibitor II (Calbiochem) at 1µM or with a comparable volume of the vehicle control (DMSO) at the time points indicated in the figure legends. The D1 and D1-CD4 T-cell lines were maintained in complete medium as described above and cultured with 0, 10, or 50 ng/mL of IL-7 as indicated.

Cell surface protein analysis

Surface expression of membrane proteins expressed on T-cells was assessed by flow cytometry using the following fluorochrome-conjugated antibodies: PE-conjugated anti-CD4 (clone GK1.5), PerCP-conjugated anti-CD4 (clone RM4–5), PerCP-conjugated anti-CD8 (clone 53–6.7) (BD Biosciences) and PE-conjugated anti-CD127 (IL-7R) (clone A7R34)(eBiosciences). PE- or PerCP-conjugated isotype matched antibodies were used as controls for non-specific staining. Cells were incubated with saturating amounts of the appropriate antibodies for 20 minutes on ice, washed in buffer (PBS + 0.1% bovine serum albumin (BSA)) and analyzed by

flow cytometry on a FACSCalibur flow cytometer (BD Biosciences) or C6 flow cytometer (Accuri).

Viability and proliferation assays

T-cell viability was determined based on cell shrinkage and fragmentation by gating on FSC/SSC parameters acquired by flow cytometry. To measure DNA synthesis, 5×10^6 cells were pulsed with BrdU (10 μ M) for 48 hours, and BrdU incorporation was detected with a commercially available kit (BD Biosciences) according to manufacturer's protocol. Briefly, cells were typed for surface markers CD4 or CD8 as described above, washed, fixed, and permeabilized prior to incubation with a FITC-conjugated anti-BrdU antibody. The cells were analyzed by flow cytometry as described above. Statistical analysis was performed using Prism software (GraphPad).

Intracellular staining of phospho-STAT5 or LCK

For detection of intracellular phosphorylated STAT5 and total LCK, we used an optimized protocol designed to enhance detection of these intracellular proteins²⁶. Prior to fixation, cells were stained with PerCP-conjugated antibodies for CD4 (clone RM4-5) or CD8 (clone 53.6.7) (BD Biosciences) as described above. Cells were washed, fixed, and permeabilized with the Fix & Perm Cell Permeabilization kit (Caltag) following the manufacturer's protocol. Cells were stained with either PE-conjugated anti-phospho-STAT5 (clone 47), or PE-conjugated anti-LCK (clone MOL 171) or isotype matched control PE-conjugated antibodies (BD Biosciences). Cells were analyzed by flow cytometry using the C6 flow cytometer (Accuri) described above.

Plasmids and Transfection of Cells

To generate the D1-CD4 T-cell line, D1 T-cells were transfected with CD4 cDNA by electroporation using standard methodologies. Stable lines were selected by antibiotic resistance to Blastocidin. For transient expression of the CD4 co-receptor on CD8 T-cells, CD8 T-cells were enriched by negative selection with biotinylated anti-CD4 (clone GK1.5) (BD Biosciences). CD8 T-cells were then nucleofected with 4 μ g of plasmid encoding for the CD4 co-receptor, EGFP or the empty vector (pcDNA), using the Mouse T-cell Nucleofection kit (Amaxa) according to the manufacturer's protocol. Cells were analyzed after 24 hours.

Confocal microscopy and live cell imaging

T-cells were stained with the indicated fluorochrome-conjugated antibody as previously described for cell surface protein analysis. D1-CD4 T-cells were fixed after staining using the fixation protocol that is part of the Fix & Perm Cell Permeabilization kit (Caltag). Images were acquired with LSM 510 using 100x/1.4 Oil DIC objective. The composite images were processed and 3D projections of the Z-stacks were generated using the LSM image browser from Zeiss. For live cell imaging, images were acquired with Axio Observer Z1 using a Plan-Apochromat 63x/1.4 Oil objective. The images were processed with AxioVision LE (Zeiss). Scanned images were generated and modified within the Zen 2009 image processing program (Zeiss).

Immunoprecipitation and Immunoblotting

D1 and D1-CD4 T-cells were lysed using Cell Lysis Buffer (Cell Signaling) in the presence of protease inhibitors (Roche) at 4°C. Lysates were precleared with Preclearing Matrix E (sc-45056; Santa Cruz), following manufacturer's protocol, and incubated overnight at 4°C with CD4 antibody (clone 5B5) or CD3 (clone 5B2) (Santa Cruz) antibody complexed with an immunoprecipitation matrix according to the manufacturer's protocol (ExactaCruz E: sc-45042, Santa Cruz). Next day, the matrix was washed and resuspended in 2x loading buffer

(Invitrogen), heated to 94 C and centrifuged to pellet matrix. Supernatants were loaded onto a 12% SDS-PAGE gel and proteins transferred onto nitrocellulose membrane. Immunoblotting was performed with anti-LCK (clone 28/LCK) or anti-CD4 (clone C-18) (Santa Cruz) primary antibody followed by the appropriate ExactaCruz E secondary antibody (for immunoprecipitated lysates) or an HRP-conjugated secondary antibody (for pre- and post-precipitation lysates). Signal was developed by enhanced chemiluminescence (ECL) (Pierce) and visualized on BioMax ML film (Kodak). The relative intensities of the bands were quantitated using the Kodak Image Station 4000MM PRO with band detection and density determined using the Carestream Molecular Imaging SE software.

RESULTS

IL-7 has been described as a growth factor for T-cells; however the reported proliferative effects of IL-7 upon different lymphocyte subsets vary considerably. Consistent with this observation, we reported that *ex vivo* culture of T-cells with a high dose of IL-7 (in the absence of TCR stimulation) resulted in the preferential expansion of CD8, but not CD4, T-cells¹⁴. This bias of IL-7 on the proliferation of CD8 T-cells over CD4 T-cells under high dose conditions was also apparent upon IL-7 administration to humans²⁷ or mice¹⁷. In this report, we sought to examine the basis for the differential responses of CD4 T-cells versus CD8 T-cells to IL-7, focusing on why CD4 T-cells grow optimally at a lower dose of IL-7.

IL-7 Dose Responsiveness is Different Between CD4 and CD8 T-cells

Using the IL-7 *ex vivo* culture system that we previously developed¹⁴, we compared the growth of CD4 and CD8 T-cells cultured in different concentrations of IL-7. These doses of IL-7 was chosen after examining the effect of low and high concentrations of IL-7 upon the expansion of IL-7 dependent cells¹⁴. T-cells were isolated from the lymphoid organs of wild type mice and cultured with 150 or 10 ng/mL of IL-7 for 5–14 days. Figure 1A shows that expansion of T-cells occurred at both doses of IL-7 tested, with slightly more cells recovered in response to the higher 150 ng/mL dose. This was indicated by an approximate doubling of T-cell numbers from 5 through 14 days of culture (Fig. 1A). Assessment of the viability of the cell cultures revealed that after 5 days, cells grown with 10 or 150 ng/mL IL-7 were 62–64% viable (Fig. 1B). However, after 14 days of culture, more death was observed with cells grown at the 150 ng/mL dose of IL-7 (39% viable) compared to the lower dose of 10 ng/mL (53% viable) (Fig. 1B). Immunostaining revealed that the majority of the T-cells that expanded at the higher dose of IL-7 were CD8 T-cells (Figs. 1C, 1E). In contrast, the CD4 T-cell population, when cultured with 150 ng/mL of IL-7, was significantly diminished relative to CD8 T-cells (Figs. 1D, 1E). While CD8 T-cell numbers more than doubled when cultured with 150 ng/mL of IL-7, CD4 T-cells were maintained at the lower dose of 10 ng/mL of IL-7 (Figs. 1C, 1D). Initially, CD4 T-cells were detected in approximately equal ratio with CD8 T-cells (typical CD4:CD8 ratios for C57Bl/6 mice range from 1.3–1.0:1.0) (Fig. 1E), but after 5 and 14 days CD4 T-cells were increased only in culture with the lower dose of IL-7 tested (Fig. 1E). Hence, relative to CD8 T-cells, CD4 T-cell numbers decreased over time when maintained with the higher dose of IL-7. The apparent loss of the CD4 T-cells when cultured with 150 ng/mL of IL-7 could result from increased cell death or loss of proliferation.

Previously, we found that CD4 T-cells incorporated less BrdU (synthesized less DNA), compared to CD8 T-cells, under conditions of high dose IL-7¹⁴, suggesting that their proliferation was impaired. However, we also detected decreased viability in total T cell cultures grown with 150 ng/mL of IL-7 for 14 days that could be due to increased CD4, but not CD8, T-cell death (Fig. 1B). To examine this, we enriched T-cells isolated from lymphoid organs for either CD4 or CD8 subsets and assayed for viability of each subset grown under conditions of high dose IL-7. In Figure 1F are the results showing that enrichment yielded

cultures of either CD4 or CD8 T cells without measureable cross-contamination. After seven days of culture with high dose IL-7, we observed fewer viable CD4 T-cells (69% viable) and more viable CD8 T cells (90% viable), indicating that CD4 T-cells were dying (dead cells indicated by shrinkage and fragmentation) under conditions of 150 ng/mL of IL-7 (Fig. 1F). Moreover, an approximate doubling of enriched CD8 T-cells occurred at the higher dose of IL-7 that was not noted with CD4 T-cells (data not shown). These results suggested that CD4 T-cells were inhibited for both proliferation and survival when the amount of IL-7 was increased and are therefore intrinsically different from CD8 T-cells in their response to the cytokine.

To rule out potential competitive advantages due to differential expression of IL-7R, we isolated T-cells from mice transgenically engineered to express IL-7R (IL-7RTg). While IL-7RTg CD4 and CD8 T-cells express higher levels of IL-7R than their wild-type counterparts, both IL-7RTg CD4 and CD8 T-cells express similar levels of the receptor (data not shown). Despite the fact that IL-7R levels were elevated on both T-cell subsets, culture of IL-7RTg T-cells in high dose IL-7 still skewed the CD4:CD8 ratio to mostly CD8 T-cells (Fig. 1G). BrdU incorporation showed that, in contrast to CD8 T-cells, IL-7RTg CD4 T-cells proliferated poorly in response to IL-7 (Fig. 1G). We conclude therefore that CD4 T-cells were inhibited for growth in high dose IL-7 irrespective of the level of IL-7R.

Expression of CD4 Alters the IL-7 Dose Responsiveness of T-cells

As our findings showed that IL-7R expression could not fully account for the variation in responses of CD4 versus CD8 T-cells to high dose IL-7, we examined the contribution of the CD4 co-receptor and tested whether expression of CD4 on CD8 T-cells could reverse IL-7 high dose responsiveness. We transiently expressed the CD4 co-receptor on freshly isolated naïve CD8 T-cells, that is T cells that had not been exposed to exogenous antigen, using the technique of nucleofection and cultured the nucleofected naïve T cells in high dose (150 ng/mL) or low dose (10 ng/mL) IL-7 for 24hrs. Nucleofection efficiency, as measured by the expression of GFP that is independent of any IL-7 effects (Fig. 2A), averaged about 32%, and, at time 0, we detected the expression of CD4 in about 56% of the total cells nucleofected with the CD4 cDNA (Fig. 2A). Note, that we observed a small percentage of naïve CD8 T-cells (5–6%) that also endogenously expressed CD4 in cells nucleofected with the empty vector, pcDNA (Fig. 2B). In naïve CD8 T cells nucleofected with the CD4 co-receptor, we observed few CD8 T-cells (11%) cultured with 150 ng/mL of IL-7 (Fig. 2B). However, at 10 ng/mL of IL-7, these cells composed almost 34% of the total naïve T cell population (Fig. 2B).

To account for the loss of naïve CD8 T-cells co-expressing CD4 at the higher dose of IL-7, we examined cell viability. Note that the short nature of the nucleofection experiment would not allow for significant proliferation to have occurred. Overall viability for all nucleofected cells (pcDNA and CD4) ranged from 40–50% and equal numbers of cells were analyzed in each experimental set (data not shown). Figure 2C is gated on naïve CD4-expressing CD8 T-cells and shows that there were fewer viable cells (12%) in 150 ng/mL of IL-7 compared to 10 ng/mL of IL-7 (32%). This result shows that there was increased cell death (identified by shrinkage and fragmentation) of naïve CD4-expressing CD8 T-cells at the higher dose of IL-7. Furthermore, the amount of CD4-expressing CD8-T cells detected at 10 ng/mL of IL-7, 34% (Fig. 2C), correlated well with the percentage of CD8 T-cells that were nucleofected as indicated by EGFP expression (Fig. 2A). This data suggested that expression of the CD4 co-receptor altered the IL-7 dose response of naïve CD8 T-cells, adapting them for growth in low dose IL-7.

To determine whether the effect of CD4 altering the IL-7 dose responsiveness of naïve CD8 T cells could occur in other subsets of T cells, we isolated CD44^{hi} memory CD8 T cells from OT-1/Rag1^{-/-} mice previously primed with ovalbumin and nucleofected with CD4 cDNA.

Nucleofection efficiency and CD4 expression were optimal in these cells (Fig. 2D). Shown in Figure 2D are the results indicating that expression of CD4 on these memory CD8 T cells (note that OT-1 mice do not have endogenous CD4 T cells) also significantly shifted the dose response to the lower concentration of IL-7. We confirmed, therefore, the observation made with naïve T cells (Fig. 2B,C) that CD4 expression alters the capacity of CD8 T cells to respond to IL-7. We also examined a third subset of T cells that are highly responsiveness to IL-7 – these are the double negative (DN) thymocytes (CD4^{-/-}CD8^{-/-}) found in the thymus. DN thymocytes were very difficult to nucleofect with gene expression efficiencies of 11–13% (Fig. 2F). None-the-less we observed minimal differences in CD4-expressing cells cultured with high or low dose IL-7, suggesting that thymocytes at this stage of development may be independent of the effects of CD4 expression upon IL-7 signaling. Given the findings observed with naïve and memory peripheral CD8 T cells, we concluded that there were no significant intrinsic differences between CD4 and CD8 T-cells, in regards to IL-7 dose responsiveness, other than co-receptor expression. Our data thus far supported a role for the CD4 co-receptor in modulating the responses of T-cells to different concentrations of IL-7.

To examine how the expression of CD4 versus CD8 co-receptor could affect IL-7 signaling, we analyzed co-receptor distribution. Using confocal microscopy, we acquired images using unfixed, live cells to observe the distribution of the co-receptors. In Figure 3A are two representative images of multiple cells that were visualized showing that the CD8 co-receptor (green fluorescence) is evenly distributed on the surface of freshly isolated naïve T-cells. In contrast, when freshly isolated naïve CD4 T-cells were imaged, distinct patches or brighter aggregates of the co-receptor (red fluorescence) could be seen at or near the surface of these cells (Fig. 3A, right), perhaps localizing to membrane proximal vesicles. These results suggested that there were significant differences in the segregation of the membrane-associated CD4 co-receptor, but not the CD8 co-receptor, that could influence intracellular signaling cascades.

In order to examine the contribution of the CD4 co-receptor to IL-7 signaling at the mechanistic level, we used a T-cell line, called D1, that is IL-7 dependent and does not express any co-receptors but retains TCR:CD3 expression ²⁵. Previously, we showed that D1 T-cells begin to die between 24–48 hours after IL-7 withdrawal ²⁵. To study the role of the CD4 co-receptor, we stably transfected D1 T-cells with CD4 cDNA (D1-CD4) and selected for cells expressing the co-receptor. These experiments were possible because CD4, unlike CD8, is a single transmembrane protein that can be expressed from a single stably integrated plasmid.

We observed significant levels of CD4 expression on 70–80% of the D1-CD4 T-cells, but not D1 T-cells (Fig. 3B and data not shown). Figure 3B are a composite images showing surface expression of the CD4 co-receptor and IL-7R on D1-CD4 T-cells that were fixed and analyzed by confocal microscopy. We noted that while the IL-7R seemed equally distributed on the cell surface, the CD4 co-receptor appeared to aggregate within patches in the membrane (Fig. 3B) similar to that observed on naïve CD4 T cells (Fig. 3A, right). No significant overlay of the CD4 co-receptor and the IL-7R was noted (Fig. 3B, overlay). Interestingly, while the parental D1 T-cells grew optimally at an IL-7 dose of 50 ng/mL ²⁵, we observed decreased viability of D1-CD4 T cells at this dosage (data not shown). In fact, the D1-CD4 T cells grew best at a lower dose of 10 ng/mL of IL-7. We thus routinely maintained the D1-CD4, but not the D1 T-cells, at an IL-7 concentration of 10 ng/mL. As we observed for primary T cells (Fig. 1) and CD4-expressing CD8 T-cells (Fig. 2), expression of CD4 co-receptor enabled D1 T-cells to grow at a lower dose of IL-7 compared to the parental D1 cell line.

Association of LCK with CD4 is observed in an IL-7 dependent T cell line

Our data suggests that the CD4 co-receptor modulates the dose response of a T-cell to IL-7. We ruled out a contribution from the IL-7R by showing that transgenic expression of the IL-7R

did not generate comparable responses to IL-7 between CD4 and CD8 T-cells (Fig. 1G) nor did CD4 directly associate with the IL-7R (Fig. 3B). Therefore to understand the contribution of the CD4 co-receptor to the IL-7 signal, we examined the intracellular association of CD4 with the Src kinase, LCK²⁸. It is known that LCK has a different affinity for CD4 versus CD8²⁹. The proportion of LCK associated with the CD4 co-receptor was documented to be 10–20 fold higher than the amount associated with the CD8 co-receptor³⁰. In fact, a range of 75–95% of cellular LCK has been reported to be associated with CD4 co-receptor while only 5–10% is associated with CD8³¹. Such findings led us to determine whether the association of LCK with the CD4 co-receptor or the CD3/TCR complex could be detected in D1 and D1-CD4 T cells. We directly examined the association of LCK with either the CD4 co-receptor or the CD3/TCR complex by co-immunoprecipitation. Using D1 and D1-CD4 T-cells, we performed immunoprecipitation experiments using antibodies against either CD4 or CD3 and immunoblotted for the presence of LCK. In Figure 3C, representative pre-precipitation lysates are shown to demonstrate that LCK was present in all the lysates assayed. Figure 3D are the results of two representative experiments of six that were performed. In Figure 3D the amount of LCK that co-precipitated with either CD3 or CD4 is shown with results from a densitometry scan below. Results from two representative experiments (Precipitate #1 and Precipitate #2) are shown. Moreover, since multiple bands at the approximate size of LCK and CD4 immunoprecipitated, we used two methods to define the band for the protein of interest: (1) Whole lysates containing LCK or CD4 were run along size molecular marker lanes in an SDS-PAGE gel to determine upon immunoblotting the location of the protein of interest (data not shown) and (2) For an unbiased determination of the protein band of interest, we used the Carestream Molecular Imaging SE software to identify and calculate the density of the unique band corresponding to our protein of interest, LCK or CD4. In the panels shown in Figure 3D, arrows indicate the location of LCK or CD4 determined as described above. We observed that in D1 T-cells, detectable amounts of LCK associated with CD3 when cells were stimulated with 50 ng/mL of IL-7 (Fig. 3D). No CD3-bound LCK was measured in lysates from D1-CD4 T-cells (Fig. 3D). Likewise, no LCK was co-immunoprecipitated with CD4 in D1 cells, since these cells lack expression of CD4 (Fig. 3D). As a control, we only detected CD4 protein by immunoblot in D1-CD4 T-cells that were co-immunoprecipitated with anti-CD4 antibody. This co-immunoprecipitation experiment clearly demonstrated that LCK was associating with CD4 when the co-receptor was over-expressed. In cells, lacking CD4, LCK could be either “free” or associated with CD3, the latter more so in the presence of IL-7.

LCK Modulates the Dose Response to IL-7 in Primary T cells

IL-7 signaling involves activation through phosphorylation of JAK3 and STAT5. In addition, LCK has been shown to promote the direct or the indirect, through JAK3³², phosphorylation of STAT5. Phospho-STAT5 levels therefore serve as an indication of a cell's signaling status. To determine whether CD4 and CD8 T-cells differed in their ability to generate phosphorylated STAT5, we measured the intracellular levels of phospho-STAT5 using an optimized flow cytometry-based methodology²⁶ with an antibody specific for the Tyr694 phosphorylation on STAT5a and STAT5b. We found that a short 20 minute pulse with either high or low dose IL-7 could induce the phosphorylation of STAT5 in both CD4 and CD8 T-cells (data not shown).

Culturing primary T-cells for 5 days with high dose or low dose IL-7 enabled us to examine the basal levels of phospho-STAT5 and total LCK in response to the cytokine. We previously compared untreated samples to vehicle (DMSO)-treated samples and no differences were noted (data not shown); hence to minimize duplication of data we show the results for vehicle-treated cells. Extended culture of primary T-cells with either 150 ng/mL IL-7 or 10 ng/mL IL-7 did show a concentration dependent effect upon the level of phospho-STAT5. In vehicle-treated CD8 T-cells, we found increased levels of phospho-STAT5 when cultured with high dose IL-7

(peak median, 1830) compared to low dose IL-7 (peak median, 1108) (Vehicle, Fig. 4A). Consistent with the preferred growth of CD4 T-cells in low dose IL-7, we detected more CD4 T-cells with elevated phospho-STAT5 at 10 ng/mL of IL-7 (peak median, 1437) as compared to high dose IL-7 (peak median 1193) (Vehicle, Fig. 4A). Figure 4B shows the total intracellular LCK in the cultured primary cells. CD8 T-cells had higher levels of total LCK compared to CD4 T-cells, and these levels were independent of the concentration of IL-7 in the medium (Fig. 4B).

Next, we treated the high dose or low dose IL-7-cultured primary T-cells with an LCK inhibitor overnight and assayed for intracellular phospho-STAT5. We noted that LCK inhibition reversed the observed effects of high dose IL-7 upon phospho-STAT5 levels. In CD4 T cells, LCK inhibition restored responsiveness to high dose IL-7, with STAT5 levels increasing (peak median 1193, increased to 1889), while in CD8 T-cells, LCK inhibition decreased phospho-STAT5 (peak median 1830, decreased to 1411) (Fig. 4A). At low dose IL-7 (10 ng/ml), LCK inhibition effects were less significant with decreased phospho-STAT5 in CD4 T-cells, but increased phospho-STAT5 levels in CD8 T-cells (Fig. 4A).

CD4-Associated LCK Confers Responsiveness to Low Dose IL-7

Results from primary CD4 and CD8 T-cells indicated that LCK was influencing the response to an IL-7 dose (Fig 4A). To determine whether the CD4 co-receptor was sufficient to modulate the IL-7 dose response, we examined basal levels of phospho-STAT5 and LCK using D1 and D1-CD4 T-cells following culture with IL-7. Previously we established that a 20 minute pulse with high or low dose IL-7 could induce the phosphorylation of STAT5 in D1 and D1-CD4 T cells, indicating that both cells lines were equipped to respond to an IL-7 (data not shown). Figure 5A (vehicle control) shows that phospho-STAT5 levels were highest in D1 T-cells cultured with 50 ng/mL of IL-7 and in D1-CD4 T-cells cultured at 10 ng/mL of IL-7, paralleling the results obtained with primary cells in that phospho-STAT5 levels peaked in CD8 T-cells cultured at a higher dose of IL-7 and in CD4 T-cells cultured at a lower dose of IL-7 (Fig. 4A). In the absence of IL-7, we also detected significant basal levels of phospho-STAT5 in D1 cells but not D1-CD4 cells (Fig. 5A). Total LCK levels for D1 T-cells decreased in an IL-7 dose dependent manner, with the lowest levels detected in the absence of the cytokine (Fig. 5B). Minimal changes were detected in the total LCK levels for D1-CD4 T-cells, although these were also slightly decreased in cells deprived of IL-7 (Fig. 5B).

As observed with primary T-cells, the effect of LCK inhibition reversed the IL-7 dose responsiveness of D1 and D1-CD4 T-cells. When we compared the effect of LCK inhibition in D1 T-cells (Fig. 5A, D1), we detected decreased levels of phospho-STAT5 in response to 50 ng/mL of IL-7 (peak median 1898 decreasing to 1457 upon LCK inhibition) as well as a significant decrease in basal phospho-STAT5 in D1 cells cultured without IL-7. In D1-CD4 T-cells, LCK inhibition effectively eliminated most of the increased phospho-STAT5 detected at the 10 ng/mL dose of IL-7 (peak median 1655 decreasing to 887 upon LCK inhibition) (Fig. 5A, LCK INH). Therefore, we concluded that LCK modulated the levels of phosphorylated STAT5 and that the sequestering of LCK by the CD4 co-receptor could be in part responsible for the optimal growth of CD4-expressing cells at low dose IL-7.

In summary, our results suggest that the dose responsiveness of CD4 T-cells to IL-7 depends on the ability of the co-receptor to bind and sequester LCK. We propose that CD4 T-cells are engineered to attenuate a strong IL-7 signal and that this is achieved through the activity of LCK upon the phosphorylation of STAT5. One possibility is that CD4 co-receptor sequesters LCK in manner that optimizes IL-7 signal transduction at low doses, while conditions at high doses inhibits the activation of the STAT5 pathway. Support for the idea that a strong IL-7 signal is detrimental for CD4 T-cells comes from data acquired from STAT5b-constitutively active (CA) transgenic mice ²¹. We found that few CD4 T-cells localized to the lymphoid

organs of STAT5b-CA mice 14 and that *in vitro* culture of the remaining CD4 T-cells with IL-7 did not induce their growth (Supplemental Figure S1). In contrast to CD4 T-cells, CD8 T-cell may contain more “free” or CD3-bound LCK, are thus able to respond proportionally to the strength of an IL-7 signal. Therefore, the ability of a T-cell to respond to IL-7 is not only determined by expression of the IL-7R but may also depend on the localization of the Src kinase, LCK, and whether it is bound to a co-receptor.

DISCUSSION

We and others have shown that *in vitro* and *in vivo* conditions in which IL-7 concentrations are high favor the growth and proliferation of CD8 but not always CD4 T-cells. In this study, we found that T-cells, which express the CD4 co-receptor, preferentially expanded at lower doses of IL-7 relative to CD8 T-cells because the activity of LCK, which contributed to the phosphorylation of STAT5, was in part controlled through association with the CD4 co-receptor. Furthermore, we established a direct correlation between the amount of phospho-STAT5 and the optimal dose of IL-7 for growth and showed that the LCK-induced phosphorylation of STAT5 significantly contributed to the overall levels of the active transcription factor.

Evidence found in the literature indicates that JAK signaling is likely an evolutionarily recent addition to a primordial Src/STAT pathway¹⁸ and suggests that interactions between Src kinases and JAKs are at times necessary for the full activation of STATs³³. Older studies showed that the Src kinases, FYN, LYN and LCK, can directly associate with the IL-7 receptor complex^{34;35} and more recently that JAK3 can be phosphorylated upon TCR stimulation independently of γc receptor engagement³², indicating that direct associations between IL-7 signaling and Src kinases exist. Moreover, expression of a constitutively active LCK kinase induced the phosphorylation and activation of STAT5b and promoted proliferation²⁰. These studies and others support our findings that LCK contributes to the phosphorylation of STAT5 in T-cells. We found, in non-CD4 T-cells deprived of IL-7 or CD4 T-cells cultured with low dose IL-7, that increases in phospho-STAT5 were due to the activity of LCK. This is in line with what others have reported in that a proliferative response to an IL-7 signal does not require LCK³⁶. The conclusion that can be drawn from our findings is that repression of LCK activity is inherent in the IL-7 signal. Only when IL-7 is limiting does LCK contribute to the phosphorylation of STAT5 in a yet to be described manner.

The activity of JAKs and Src kinases can be inhibited by inactivating phosphorylation. For the Src kinases, this inactivation is mediated through the negative regulatory kinases Csk/Crk. LCK has a C-terminal tyrosine (Y505) that, when phosphorylated by Csk, inhibits its kinase activity, causing the protein to adopt a non-functional closed conformation³⁷. The transmembrane adaptor protein called Cbp (Csk-binding protein) serves the function of localizing Csk to the plasma membrane where LCK is found³⁸. Moreover, Cbp is known to localize to membrane lipid rafts³⁹.

Among other signaling molecules that also localize to lipid rafts is the CD4 co-receptor⁴⁰. The interaction of LCK with the co-receptors is mediated through an N-terminal dicysteine motif that forms a unique zinc “clasp” like structure⁴¹. Additionally, the longer CD4 tail provides a larger interface for interacting with LCK. In part this explains why 75%–95% of the intracellular LCK is associated with the CD4 co-receptor⁴². Using either HIV infected CD4 T cells or an anti-CD4 antibody, researchers found that the distribution of LCK to lipid rafts was inhibited upon pre-engagement of CD4⁴³. Such findings support a potential mechanism in which the CD4 co-receptor can “sequester” and negatively regulate the activity of LCK. This scenario was proven in an elegant study in which both the CD4 and CD8 co-receptors were deleted and such cells were found to respond to non-MHC ligands⁴⁴. Hence co-receptors

not only function to initiate TCR signaling but also to prevent inappropriate TCR-independent T-cell activation by binding and potentially inhibiting the kinase. Taken in total, such studies support our observations that T cells, in which CD4 is over expressed, had detectable CD4-bound LCK, and that the CD4 co-receptor itself localized to discrete patches on the cell surface – unlike the CD8 co-receptor that was more evenly dispersed. It is therefore likely that the negative regulation of LCK activity is mediated by signaling proteins such as the CD4 co-receptor or Csk/Cbp that are brought together in lipid rafts.

In earlier published work ²⁵, we performed a cDNA microarray using D1 T-cells and found that, after 2 hours of IL-7 readdition to deprived cells, a number of factors involved in regulating signal transduction activity were increased. Of significance to our current studies, results from the microarray revealed that IL-7 signaling induced synthesis of the negative regulator of LCK, Csk ²⁵, suggesting that a strong IL-7 signal could be expected to provide a negative regulatory mechanism for the inactivation of LCK. Given this information, we propose that, in CD4-expressing T-cells, a high dose of IL-7 would increase the levels of Csk found at the membrane lipid rafts associated with Cbp. At the same time, the CD4 co-receptor, also located in the lipid rafts, would recruit LCK to the membrane complex for phosphorylation by Csk. As a result, the level of LCK phosphorylated at Y505 would increase and the total amount of phospho-STAT5 available to transduce an IL-7 growth signal would decrease – thereby attenuating the response of the CD4 T cell to IL-7. This idea is described in model shown in Figure 6. Only when the concentration of IL-7 drops, and the level of Csk decreases, would active LCK be available to contribute to the phosphorylation of STAT5. In T cells lacking co-receptors (like D1 T cell line) little or no LCK could be brought to the membranes for phosphorylation by Csk, and thus more active LCK would be available to generate phospho-STAT5. An in between scenario likely occurs in CD8 T cells, with the IL-7 dose dependent increase in LCK inhibitory phosphorylation by Csk being balanced by reduced recruitment of the kinase to the membrane lipid rafts.

As a result of our studies, we have identified a novel role for LCK bound to the CD4 co-receptor in the dose response of T-cells to IL-7. Our findings on the relationship between LCK and STAT5 not only explain the differential response of CD4 and CD8 T-cells to IL-7 but could also be extended to explain the outcome of cytokine signaling in terms of cell cycling. We previously showed that the cell cycle inhibitor, p27kip, increased upon IL-7 loss ⁴⁵ and that the cell cycle activator, Cdc25A, decreased due to p38 MAP kinase-targeted degradation ⁴⁶. It remains possible that in CD4 T cells, exposed to high dose IL-7, cell cycle arrest could occur upon CD4-coupled LCK inactivation due to a combination of increased p27kip as well as the decreased stability of Cdc25A. As the clinical use of supraphysiological amounts of IL-7 (>10 µg/kg/dose) in the treatment of immunodeficiencies, transplants and cancer therapy increases ⁴⁷, the need for a better understanding of the effects that can be expected when a T-cell encounters higher than normal doses of IL-7 becomes absolutely necessary. Studies such as ours serve to provide select answers but also provoke questions that illustrate the complex nature of this unique and critically important cytokine.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. High dose IL-7 supports the growth of CD8 T-cells but not CD4 T-cells (A–D). Splenic T-cells were isolated from C57Bl/6 mice and cultured with 150 or 10 ng/mL of IL-7 for 5 and 14 days as described in Materials and Methods. Total number of T-cells recovered (A), viability of total T-cells (B), and the number of cells in the individual subsets of CD8 T-cells (C) and CD4 T-cells (D) were determined by acquiring forward scatter (FSC), side scatter (SSC) and cell number and volume using the Accuri CFlow cytometer and software. (E). CD4 versus CD8 T-cell subset distribution is shown for the IL-7 cultured cells quantitated in (A–D). Ratio of CD4 to CD8 T-cells (shown in the percentages in bold) was determined by staining with a specific PE-conjugated anti-CD4 antibody and a PerCP-conjugated anti-CD8 antibody and analyzed by flow cytometry as described in Materials and Methods. Results

shown were acquired from the viable cell gate. (F). CD4 T-cells and CD8 T-cells were enriched from spleens isolated from C57Bl/6 mice. CD4 or CD8 T-cells were cultured with 150 ng/mL of IL-7 for 7 days, and the ratio of CD4 to CD8 T-cells and viability of each subset were determined by flow cytometry as described above. (G). Splenic T-cells were isolated from IL-7R transgenic mice and cultured with 150 ng/mL of IL-7 for 7 days. Ratio of CD4 to CD8 T-cells was determined by flow cytometry as described above. Proliferation as indicated by the amount of DNA synthesis was determined by measuring BrdU incorporation as described in Materials and Methods. Representative experiments of three or more performed are shown. * $p < 0.05$.

Figure 2. Low Dose IL-7 maintains CD8 T-cells that also express the CD4 co-receptor (A–C). Naïve splenic CD8 T-cells were isolated from C57Bl/6 mice and nucleofected with empty vector (pcDNA) or the cDNA for murine CD4 as described in Materials and Methods. Nucleofected CD8 T-cells were cultured for 24 hours with either 10 or 150 ng/mL of IL-7 and assayed for cells retaining the CD4 co-receptor using specific antibodies by flow cytometry. The expression of CD4 on CD8 T-cells (shown in the percentages in bold) was determined by staining with a specific PE-conjugated anti-CD4 antibody and a PerCP-conjugated anti-CD8 antibody as described in Materials and Methods. (A) Naïve splenic CD8 T-cells were nucleofected, as described above, with the cDNA for EGFP as an indicator of nucleofection efficiency. (B). Naïve splenic CD8 T-cells were nucleofected, as described above, with the

cDNA for pcDNA or CD4 as indicated in the figure. (C) Viability (shown by percentage of encircled cells) was determined by examining morphological changes that accompany death, such as cell shrinkage and fragmentation, using forward (FSC) and side scatter (SSC) gating. (D–E) Memory CD44^{hi} CD8 T cells were isolated from OT-1 mice previously primed with ovalbumin as described in Materials and Methods. Cells were nucleofected with EGFP cDNA (nucleofection control) or CD4 cDNA and the expression of CD4 on CD8 T-cells cultured with IL-7 determined as described above. (F–G). DN thymocytes were isolated from WT mice by negative selection as described in Materials and Methods. Cells were nucleofected with EGFP cDNA (nucleofection control) or CD4 cDNA and the expression of CD4 on CD8 T-cells cultured with IL-7 determined as described above.

Figure 3. LCK associates with the CD4 co-receptor
(A). Surface expression of CD8 and CD4 on unfixed lymph node T-cells was visualized by confocal microscopy using a Plan-Apochromat 63x/1.4 Oil objective (Zeiss) as described in Materials and Methods. Arrows indicate aggregation of the CD4 co-receptor. The images were processed with AxioVision LE (Zeiss). Representative experiments of two or more performed are shown. (B) Surface expression of IL-7R and CD4 on D1-CD4 T-cells was visualized by confocal microscopy. Images were acquired using 100x/1.4 Oil DIC objective. Composite images were generated from Z-stacks using the LSM image browser from Zeiss. (C-D). Immunoblots display the results of co-immunoprecipitation of LCK with CD4. Whole cell lysates were prepared from D1 and D1-CD4 T-cells cultured with 50, 10 and 0 ng/mL IL-7 as

described in Materials and Methods. LCK levels in pre-precipitation D1 and D1-CD4 T-cell lysates were determined (C). Pre-cleared D1 and D1-CD4 T-cell lysates were incubated with anti-CD3 or anti-CD4 antibodies to immunoprecipitate CD3- or CD4-associated proteins and then precipitates were immunoblotted for LCK (D). Two representative experiments are shown. As control the immunoprecipitated amounts of CD4 are shown. Band detection and density were determined using the Carestream Molecular Imaging SE software and results displayed in the accompanying bar graphs. Arrows indicate protein of interest and * denotes non-specific protein bands.

Figure 4. Phospho-STAT5 levels in primary T-cells are dependent on IL-7 dose and LCK activity (A). Lymph node T-cells were isolated from C57Bl/6 mice and cultured for 5 days with 150 or 10 ng/mL IL-7. T-cells were treated with either vehicle (VEH) control, DMSO, or 1 μ M LCK inhibitor II (LCK Inhib) for 18 hours prior to analysis. Surface expression of CD4 or CD8 was determined using a PE-conjugated anti-CD4 antibody or a PerCP-conjugated anti-CD8 antibody and cells were gated for display as described in Materials and Methods. Intracellular phospho-STAT5 was measured using a PE-conjugated phospho-STAT5 (pTyr694) antibody. Levels of surface CD4, CD8 or intracellular phospho-STAT5 were determined by flow cytometry. Isotype matched antibodies were used as controls for non-specific staining. Shown is the matched isotype control (CTRL) antibody for PE-conjugated phospho-STAT5. (B.) Using the samples from (A), total intracellular LCK was measured using a PE-conjugated antibody that detects both unphosphorylated and phosphorylated forms of LCK. A matched isotype control (CTRL) for PE-conjugated LCK is shown. Surface expression of CD4 or CD8 was determined as described in (A) and cells gated for display as described above. Medians for each histogram peak were determined using FSC Express software and displayed in the table alongside each histogram set. Representative experiments of three or more performed are shown.

Figure 5. CD4-associated LCK modulates IL-7 dose responsiveness in D1 T-cell line
 (A). D1 and D1-CD4 T-cells were cultured in 50, 10 or 0 ng/mL IL-7 for 24 hours and treated with either vehicle (VEH) control, DMSO or 1 μ M LCK inhibitor II (LCK Inhib) for 18 hours prior to analysis. Surface expression of CD4 in D1-CD4 T-cells was confirmed using a specific PE-conjugated anti-CD4 antibody. Intracellular phospho-STAT5 was measured using a PE-conjugated antibody specific for phospho-STAT5 (pTyr694). Levels of intracellular phospho-STAT5 were determined by flow cytometry. Shown is the matched isotype control (CTRL) antibody for PE-conjugated phospho-STAT5. (B). D1 and D1-CD4 T-cells were cultured in 50, 10 or 0 ng/mL IL-7 for 24 hours as in (A) and total LCK measured using a PE-conjugated antibody that detects both unphosphorylated and phosphorylated forms of LCK. A matched isotype control (CTRL) for PE-conjugated LCK is shown. Medians for each histogram peak were determined using FSC Express software and displayed in the table alongside each histogram set. Representative experiments of three or more performed are shown.

Figure 6. Model for proposed high dose IL-7 signaling events in CD4 T cells

In CD4 T-cells, the co-receptor sequesters cytosolic LCK and brings it into the lipid raft membrane complex. Within the lipid rafts, Csk, anchored to the membrane by Cbp, is activated and negatively regulates the activity of LCK through inhibitory phosphorylation of Y505. The inhibition of LCK then decreases the total amount of active phospho-STAT5. IL-7 increases the amount of active Csk that inhibits LCK in a dose dependent manner. Solid gray lines indicate pathways supported by data and broken lines indicate proposed mechanisms.