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EX VIVO EXPANSION OF MEMORY CD8 T CELLS FROM LYMPH NODES OR SPLEEN THROUGH IN VITRO CULTURE WITH INTERLEUKIN-7*

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Abstract

Interleukin-7 (IL-7) increases lymphocyte numbers, a critical feature of immune reconstitution, through mechanisms that are still poorly understood. Part of the problem is that IL-7 is produced in limited amounts by non-lymphoid cells, making *in vivo* studies of the cytokine's activity a challenge. To overcome this, we developed an *in vitro* system by which lymphocytes from secondary immune organs could be cultured to produce IL-7 responsive cells. Using this method, we showed that CD8^{hi}CD44^{hi} T cells accumulate in culture with IL-7 from a population of lymph node or splenic cells. These results were validated when a similar lymphocyte subset was found in mice expressing a constitutively active form of STAT5b, a key transducer of IL-7 signals. Interestingly, IL-7-expanded cells also up regulated the activation marker, CD69. The IL-7-derived CD44^{hi}CD69^{hi} cells were not generated from naïve cells, but expanded from an existing population, since culture in IL-7 of naïve lymphocytes from OT-1/Rag1^{-/-} mice did not produce CD44^{hi}CD69^{hi} cells. Using the *in vitro* culture system to study lymphocytes from mice deficient in the apoptotic protein, BIM, we were able to attribute the expansion of CD8^{hi}CD44^{hi}CD69^{hi} T cells to the proliferative and not survival activity of IL-7. The *in vitro* culture system provides an important new methodology to examine the activities of this essential as well as immunotherapeutic cytokine.

Keywords

Proliferation; Cytokine; Lymphocytes; Immunotherapy; Activation

INTRODUCTION

The peripheral T cell pool in a healthy adult is maintained at a relatively constant level in terms of both overall size and subset composition by homeostatic mechanisms. The precise nature of these homeostatic mechanisms has not been unequivocally defined, but it is generally accepted that the cytokine, interleukin-7 (IL-7), is a critical growth factor. IL-7 has an essential role in early T cell development (Peschon et al., 1994; von-Frieden-Jeffry et al., 1995) and a

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mutation in the IL-7 receptor α chain is a main cause of severe combined immunodeficiency (SCID) syndrome in humans (Puel et al., 1998). Animal studies demonstrated that IL-7 increases thymic output (Pido-Lopez et al., 2002; Fry et al., 2001) and maintains both the naïve and memory T cell populations (Tan et al., 2001; Schluns et al., 2000). Based on its immunorestorative properties, IL-7 is a candidate for use in immunotherapy and is currently being tested in human clinical trials (Rosenberg et al., 2006; Sportes et al., 2008).

As an immunotherapeutic agent, IL-7 can augment T cell numbers by 1) increasing thymic output (Henson et al., 2004), 2) promoting T-cell survival (Kim et al., 1998; Khaled and Durum, 2003), and 3) inducing cell cycling (Geiselhart et al., 2001; Kittipatarin et al., 2006). While the activity of IL-7 as a survival factor promoting thymic development has been well documented (Maraskovsky et al., 1997; Khaled et al., 1999), its function as a proliferative factor remains poorly understood. The replicative function of IL-7 has been mainly examined through *in vivo* studies, and few *in vitro* systems have evaluated this activity of the cytokine. As example, primary T cells placed in single-cell suspensions die within a few days of standard culture (Tan et al., 2001) or poorly proliferate when these cultures are augmented with IL-7 alone (Armitage et al., 1990; Rathmell et al., 2001). In fact, many *in vitro* studies report that IL-7 promotes proliferation when in the presence of additional signaling factors or co-stimulation (Grabstein et al., 1990; Costello et al., 1993; Tan et al., 2001). Hence, in the absence of other stimuli, *in vitro* studies of IL-7 have revealed only its survival function, while it is the *in vivo* studies that suggest a proliferative activity.

Although *in vivo* experiments have yielded findings, analysis of the data can be problematic. For example, it is difficult to attribute the proliferation observed in *in vivo* experiments solely to IL-7. Contributions from other environmental components, such as co-stimulation or TCR:MHC signaling, must be taken into account. Moreover, most studies examining IL-7 proliferation utilize the technique of adoptive transfer into immunodeficient mice. Because of the paucity of lymphocytes in these animals, signaling by various cytokines, self-peptide/MHC complexes, or foreign antigens may be atypical due to reduced T cell competition (Bourgeois and Stockinger, 2006). Mice such as those lacking components of the IL-7 signaling pathway may also have abnormal lymphoid tissues, impacting lymphocyte trafficking (Kondrack et al., 2003; Singh and Schwartz, 2006). Finally, IL-7 treatment increases thymic output, making it difficult to distinguish recent thymic emigrants (RTE) from preexisting (proliferating) naïve T-cells (Swainson et al., 2007; Sportes et al., 2008).

In order to study the proliferative activity of IL-7 independent of other factors and identify specific subsets of T cells that respond to IL-7 by proliferating, we developed an *in vitro* culture system for the *ex vivo* expansion of primary T cells. Previous *in vitro* systems failed to reveal the proliferative activity IL-7 for multiple reasons. The dose of IL-7 was not optimal (usually 25 ng/ml or less). Cell concentrations varied but were usually low ($\sim 10^6$ cells/ml). Lastly, the duration of culture with IL-7 rarely exceeded 7 days. The methodology we developed relies on using a higher density of T cells with a high concentration of IL-7 under extended cell culture conditions. Using this method, we were able to distinguish subsets that proliferated in response to IL-7 from those in which only survival was maintained. This is an important distinction that needs to be made to fully develop the therapeutic uses of IL-7; application of IL-7 could result in the preferential expansion of a specific T cell subset, potentially leading to a significant skewing of the T cell repertoire.

MATERIALS AND METHODS

Mice used and cell isolation techniques

C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, Maine) and C57BL/6-Tg(OT-I)-RAG1^{tm1Mom} mice were purchased from Taconic (Hudson, NY). 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) and Bim deficient mice were a kind gift from Dr. Scott Durum (Laboratory of Molecular Immunoregulation, National Cancer Institute-Frederick, Frederick, Maryland). 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 70 μ M 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 Mouse T-lymphocyte Enrichment kit (BD Biosciences). T cell enrichment was confirmed by analysis of surface markers by flow cytometry.

In vitro culture with IL-7

Culture conditions, for the *ex vivo* expansion of IL-7-responsive cells, were established as follows: Cells were maintained at a density of $3\text{--}5 \times 10^6$ cells/ml in complete medium (RPMI 1640 Medium (Invitrogen) supplemented with 10% Fetal Bovine serum (Hyclone), 2- β mercaptoethanol (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 150 ng/ml and refreshed every other day. This concentration of IL-7 was established experimentally after testing a dose range from 10-200 ng/ml for optimal effects upon cell growth and viability. Cultures were maintained with or without IL-7 for >14 days or until noticeable cell death was apparent and confirmed by Trypan Blue exclusion staining.

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-CD8 (clone 53-6.7), FITC-conjugated anti-CD44 (IM7), and FITC-conjugated anti-CD69 (clone H1.2F3) (BD Biosciences). 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). Data was analyzed using FCS Express software (Ontario, Canada).

Proliferation assays

For CFSE labeling, 5×10^6 lymph node or splenic T cells were washed and resuspended in 2 μ M CFSE (Molecular Probes) staining solution (PBS supplemented with 0.1% BSA). After 10 minutes, labeling was stopped and cells washed. The division status of cells was determined by measuring CFSE fluorescence by flow cytometry at 48 hours after culture with or without IL-7. The generation number for the population was determined from a best fit of these data to a Gaussian curve using the Proliferation Add-on module for the FCS express software. Unstimulated, freshly-labeled CFSE-containing cells were used to verify the peak corresponding to the undivided population. 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.

RESULTS

Phenotype of T cells from STAT5b-CA transgenic mice

In order to develop an *in vitro* culture system for the *ex vivo* expansion of IL-7 responsive T cells, we first needed to identify the types of T cells that result from a constitutive IL-7 signal. Hence, we examined the distribution of T cells isolated from STAT5b-CA mice (Burchill et al., 2003). The signal transduced through the IL-7 receptor is typically mediated through the JAK/STAT pathway and expression of a constitutively activated STAT5 (STAT5b-CA) is sufficient to promote IL-7 dependent growth in the absence of the cytokine (Burchill et al., 2003). We analyzed the phenotype of T cells isolated from the spleen (by negative selection) and lymph nodes from STAT5b-CA mice. Results are shown in Figure 1. In agreement with the previous study (Burchill et al., 2003), we observed that the CD4:CD8 ratio of T cells either enriched from the spleen (Fig. 1A) or isolated from the lymph nodes (Fig. 1B) were significantly skewed towards CD8 T cells. Few CD4 T cells were detected (less than 16%). Moreover, the CD8 T cell subsets that was being generated by constitutive STAT5b signaling did not up regulate expression of the activation marker, CD69, but displayed elevated expression of CD44 (Figs. 1A and 1B). This was most prominent in the T cells enriched from the spleen (Fig. 1A). Therefore, these results indicate that IL-7 promotes the expansion of a specific subset of T cells, implicating that its replicative function may not extend to all T cell types.

IL-7 promotes the proliferation of CD8 T cells

To demonstrate that IL-7 can cause cell division under *in vitro* culture conditions, T cells were enriched from the spleens of wild-type (WT) C57BL/6 mice and labeled with the intracellular dye, CFSE. Splenic T cells were cultured with or without IL-7 for 48 hours. Cell division was assessed after two days of culture because beyond this significant cell death was observed in the IL-7 withdrawn cells. Figure 2A shows that a greater percentage of T cells divided in the presence of IL-7 compared to cells maintained in the absence of the cytokine. Forty-eight hours of CFSE labeling revealed that there were two major proliferating populations: (1) A slow dividing population (that underwent 1-2 cell division cycles) and (2) A rapidly dividing population (that underwent up to 4 more cell division cycles). The slow dividing population was IL-7 independent, and these were detected in both the presence and absence of the cytokine (Fig. 2A). In contrast, the rapidly dividing population was IL-7 dependent in that two fold more cells were detected in the presence of IL-7 (19%) compared to the absence (8.5%) and these cells underwent up to four more cell division cycles (Fig. 2A). Note that undivided cells only remained in the cultures lacking IL-7. Similar results were observed with cells isolated from lymph nodes, given that these cells, though not enriched for T cells like those from the spleen, still contained a major population of T cells (Fig. 2B).

We next determined whether the proliferation observed in *in vitro* cultures resembled that observed within the STAT5b-CA mice in terms of the preferential expansion of CD8 T cells. Since only a limited number of cell divisions can occur within 2 days, which may not be enough to detect a skew in CD4:CD8 ratios, we extended the culture period with IL-7 to 7 days and measured DNA synthesis by BrdU incorporation. Splenic T cells were pulsed with BrdU for the final 48 hours, and Figure 2C shows that 21% of the CD8 T cells as compared to 7% of CD4 T cells incorporated BrdU. These results along with the CFSE findings (Fig. 2A) formed the basis for development of the *in vitro* proliferative assay for the expansion of IL-7 responsive cells.

Ex vivo expansion of memory-phenotype CD8 T cells by IL-7

To identify the type of cell expanded by IL-7, we developed an *in vitro* culture system based on optimizing culture conditions and IL-7 dosage. Previously, we found that low doses of IL-7

only supported survival while high doses promoted cell division (Kittipatarin et al., 2006). Moreover, IL-7 proliferative effects were detected in cells from lymphopenic or IL-7 transgenic mice (Bosco et al., 2005), which are *in vivo* conditions of higher than normal IL-7 concentration. Using this information, we optimized the *in vitro* culture conditions and IL-7 dosage and assessed the phenotype of cells from C57BL/6 WT mice following long-term culture with the cytokine. Starting with cells isolated from lymph nodes, we examined the CD4:CD8 ratio. The starting CD4:CD8 ratio was approximately 1:1 and by day 7 had shifted to 1:6 and by day 14 to 1:8 (Figure 3A, top panels). Next the expression of activation and memory markers was examined. We observed an increase in the expression of CD69 on the expanding CD8 T cells that was sustained through 14 days of culture (Fig. 3A, middle panels). Similarly, the percentage of CD8^{hi}CD44^{hi} T cells increased upon *in vitro* culture with IL-7, becoming a predominant population by day 14 (Fig. 3A, bottom panels)

To rule out the contributions of additional factors possibly present in the lymph node cultures, we isolated T cells from the spleens of WT mice by negative selection. This enrichment typically yielded 95% or greater T cells (Fig. 3B, Day 0 data). Results from T cell cultures were comparable to those observed with the lymph node cells. After 14 days of growth with IL-7, CD8 T cells, expressing the memory-phenotype marker, accumulated (Fig. 3B). Note that a small population of CD8^{neg}CD44^{hi} cells, observed in lymph node cultures (Fig. 3A), were not present in splenic T cell cultures, suggesting that these CD44^{hi} lymph node cells were likely not lymphocytes. Our results with splenic T cells were consistent with those observed with the STAT5b-CA mice. We found that culture with IL-7 supported the expansion of CD8 T cells with a memory phenotype (CD44^{hi}). However, our results were also different since we detected up regulation of the CD69 activation marker; an outcome not observed with T cells from STAT5b-CA mice (Fig.1). Use of the *in vitro* culture system was successful in that we could expand, in the absence of other signals, the type of T cells, CD8 memory, supported by IL-7. However, unlike results from *in vivo* studies (Burchill et al., 2003; Geiselhart et al., 2001), the *ex vivo* amplified T cells had an activated phenotype, as indicated by increased CD69 expression, more typical of antigen-activated cells.

To determine whether the *in vitro* culture conditions with IL-7 were artificially inducing the expression of CD69 and CD44, we isolated lymph node cells from OT-1/Rag1^{-/-} mice. This mouse line is homozygous for a transgene that encodes a TCR specific for ovalbumin (OVA) and is also deficient in *rag1* (recombinase reactivating gene 1). These mice do not develop T or B cells expressing endogenous receptors, while peripheral CD8 T cells express the TCR-OVA transgene. In the absence of OVA, most CD8 T cells from the OT-1/Rag1^{-/-} mice are of a naïve phenotype. Shown in Figure 4 are lymph node cells from OT-1/Rag1^{-/-} mice cultured for 14 days with IL-7. Unlike the findings with WT cells (Fig. 2), the OT-1/Rag1^{-/-} cells did not proliferate to IL-7, as indicated by the absence of BrdU incorporation after 14 days of culture (Fig. 4A). These cells also did not substantially up regulate the expression of either CD69 or CD44 (Fig. 4B), as was observed with WT lymph node or splenic T cells (Fig. 3). We can therefore conclude from these findings that culture conditions with IL-7 do not result in spontaneous proliferation or the induction of activation or memory markers. Further, we can establish that the IL-7-driven accumulation of memory-phenotype CD8 T cells results not from the conversion of naïve cells to memory cells, but more likely from the expansion of an already existing population.

Use of the IL-7 *in vitro* culture system to differentiate survival from proliferative effects

To demonstrate the usefulness of the IL-7 *in vitro* culture system, we applied our methodology to determine whether the proliferative activity of IL-7 results as a default of its survival function. T cells were enriched from the lymph nodes and spleen of mice which were deficient for the apoptotic protein, Bim (Bim^{-/-}). Bim has a role in the death of antigen-activated T cells

(Snow et al., 2008) and deficiency of Bim rescued the T cell depleted phenotype of IL-7 receptor deficient mice (Pellegrini et al., 2004). Moreover, we observed that Bim^{-/-} deficient T cells, unlike WT T cells, survived but did not proliferate in cultures lacking IL-7 (data not shown), suggesting that we could replace the survival activity of IL-7 by ablating Bim. Moreover, unlike WT T cells, we could culture Bim^{-/-} T cells in the absence of IL-7 for extended periods of time. In Figure 5A, we show that the starting populations of Bim^{-/-} T cells from spleen or lymph nodes were predominantly small-sized cells with few larger blasts as indicated by forward scatter (FSC) and side scatter (SSC) analysis. This trend was reversed upon extended culture with IL-7 in that by day 7, and later day 14, blast-sized cells became the major population, and subsequently, when phenotyped, we found that these cells were mainly CD8 T cells (Fig. 5A).

Starting populations of Bim^{-/-} lymph node cells revealed a significant number of CD4 T cells (30%), which dropped to 4% by day 14 in culture with IL-7 (Fig. 5B, top panel). Conversely, we observed that CD8 T cells from Bim^{-/-} mice, cultured with IL-7, increased from 41% to 61% by day 14 (Fig. 5B, top panel). Hence, IL-7 *in vitro* culture conditions selectively expanded CD8 T cells from a mixed population of cells isolated from Bim^{-/-} mice in a manner similar to that observed with cells from WT mice. No such trend was observed with Bim^{-/-} lymph nodes cells cultured without IL-7 (Fig. 5B, top panel), indicating that maintenance of survival through Bim deficiency was not sufficient to promote the expansion of CD8 T cells. This also shows that there are no other factors present in IL-7 deprived lymph node cultures that could sustain the growth of CD8 memory T cells. In fact, the few CD8 T cells remaining in the absence of IL-7 also expressed low levels of CD4; a cell type not expanded in IL-7 containing cultures. Figure 5B (middle panel) showed that in the presence, but not absence of IL-7, there were more CD8 T cells expressing high levels of CD69 (32%) after a 2 week culture of Bim^{-/-} T cells. Surface staining also showed that the memory marker CD44 was up regulated only on CD8 T cells cultured with IL-7, resulting in a significant accumulation of these cells by day 14 of culture (Fig. 5B, bottom panel).

Next, we isolated T cells from the spleens of Bim^{-/-} mice. Initially, the starting population of Bim^{-/-} T cells enriched from the spleen was skewed toward a higher percentage of CD4 T cells (50%) compared to CD8 T cells (35%). Yet, by day 14 of culture in IL-7, the predominant population was CD8 T cells, while in the absence of IL-7, CD4 T cells predominated (Fig. 4C, top panel). Recall that analysis of cell size by FSC/SSC showed that after 14 days of culture with IL-7, the few remaining CD4 T cells were small sized and that the predominant blast-sized population was CD8 (Figure 4A, lower panels). In the absence of IL-7, few blast-sized were detected (data not shown). Similar to the results obtained from culture of WT T cells with IL-7 (Fig. 3), splenic T cells from Bim^{-/-} mice expressed high levels of CD69 (26%) and CD44 (42%) in contrast to cells cultured without IL-7 (1% and 3% respectively) (Figure 4C, middle and bottom panels).

In summary, our results show that *in vitro* culture with IL-7 results in the *ex vivo* expansion of CD8 memory-phenotype T cells. This expansion is due not to the survival function of IL-7, since Bim deficiency, in the absence of IL-7, could not recapitulate the effects of IL-7. Rather, our *in vitro* culture has demonstrated that IL-7 has a separate proliferative function, resulting in the expansion of a specific subset of T cells – CD8 memory-phenotype cells – and that this replicative activity may have the hallmark of antigen-activated proliferation.

DISCUSSION

We developed an *in vitro* culture system to specifically examine the proliferative activity of IL-7. Results obtained from this approach were independent of other variables that could affect cell growth, such as other cytokines or MHC interactions. The proliferative activity of IL-7

has been demonstrated by *in vivo* studies (Schluns et al., 2000; Tan et al., 2001), however, this activity could not be attributed exclusively to the cytokine given the complexity of the *in vivo* environment. Conversely *in vitro* studies that could more specifically study the function of IL-7 resulted in conflicting findings. In some *in vitro* experiments, IL-7 induced proliferation of T cells (Cassese et al., 2007), while in others it did not (Rathmell et al., 2001). One reason for the different findings made by *in vitro* studies could be that varying culture conditions were utilized. For example, we and others have shown that the concentration of IL-7 used in culture can influence the experimental outcomes- high doses of IL-7 induce proliferation while low doses of IL-7 promote survival (Kittipatarin et al., 2006; Swainson et al., 2007). Another factor that could affect *in vitro* results is the type of cell evaluated - naïve T cells may require multiple growth signals while memory T cells may not. Such variability in the *in vitro* assay conditions would significantly affect the response of T cells to IL-7, creating the ambiguous reports of the cytokine's proliferative function.

To address this problem, we developed an *in vitro* IL-7 culture method that would support the expansion of T cells as has been observed under *in vivo* lymphopenic conditions. Features of our system included an increased dose of IL-7 and extended culture periods. The concentration of IL-7 (150 ng/ml) utilized in our *in vitro* cultures is higher than the levels of IL-7 typically detected in serum (0.3-8.4 pg/ml) (Sasson et al., 2006). We found that this higher dosage was necessary to support the active cell cycling of CD8 T cells under our *in vitro* conditions. In previous studies we reported that the induction of proliferation by IL-7 required a stronger IL-7 dose (>50 ng/ml) than the maintenance of survival (<10 ng/ml) (Kittipatarin et al., 2006). It is possible that a strong proliferative stimulus (as we replicated in our culture system) could be delivered *in vivo* through synergy of IL-7 with additional signals such as TCR engagement of MHC/peptide as has been demonstrated with naïve T cells and CD4 memory T cells (Tan et al., 2001; Seddon et al., 2003). In this regard, IL-7 could function to lower the TCR activation threshold. Although less well-documented *in vivo*, the IL-7 signal could be enhanced by synergy with other cytokines to drive T cell proliferation. A recent report showed that IL-7 could synergize with IL-6 or IL-21 *in vitro* to promote the proliferation of CD8 T cells independent of TCR signaling (Gagnon et al., 2008) with the resulting enhancement of IL-7 dependent STAT5 activity. Keeping such possibilities in mind, *in vivo* experiments demonstrate that increasing IL-7 levels through the induction of lymphopenia (Napolitano et al., 2001; Schuler et al., 2004), expression of an IL-7 transgene (Kieper et al., 2002), or injecting high doses of IL-7 (relative to physiological doses) as has been done in human clinical trials lead to the expansion of T cells (Sportes et al., 2008). We found that using an elevated dose of IL-7 in our *in vitro* experiments mimicked the replicative function of this cytokine observed *in vivo*.

One specific goal was to use the culture method to identify the subset of T cells that actively proliferate to IL-7 in the absence of other signals. We observed that extended *in vitro* culture with IL-7 selectively expanded CD8 T cells with a memory phenotype, such as has been seen when T cells proliferate in IL-7 transgenic hosts (Kieper et al., 2002). Our *in vitro* culture likely promotes the specific expansion of memory-phenotype CD8 T cells for the following reasons: 1) The accumulation of CD44^{hi} CD8 T cells was not immediate but was detectable after 2 weeks of culture in IL-7, suggesting that these cells expanded from a small existing population and not from conversion of naïve cells to memory cells. This was confirmed when we observed that naïve T cells from OT-1/Rag^{-/-} mice did not generate CD44^{hi} cells after culture in IL-7. 2) After 14 days of culture, small populations of CD4 and CD44^{lo} T cells still remained, indicating that non-specific up regulation of CD44 was not occurring. 3) Only a subset of T cells, which were not naïve, rapidly proliferated to IL-7 as indicated by CFSE dilution and BrdU incorporation, demonstrating that IL-7 did not induce the proliferation of all cultured cells.

The proliferative activity of IL-7 has been characterized by others as non-activating in that the activation marker CD69 was not up regulated (Geiselhart et al., 2001; Burchill et al., 2003). In contrast, we observed that *ex vivo* expansion of T cells with IL-7 resulted in an increase in CD69 expression as well an increase in the percentage of CD8^{hi} CD69^{hi} T cells. This outcome is not an artifact of *in vitro* culture conditions. We found that CD8 T cells from OT-1/Rag^{-/-} mice did not up regulate CD69. Moreover, T cells expressing CD69 have been observed in different tissues. In the bone marrow, CD8 T cells expressing CD69 have been found (Zhang et al., 2006), and in the gut, intestinal epithelial cells (IELs) retain CD69 expression long after antigen has been cleared (Masopust et al., 2006). It follows that both the bone marrow and gut are anatomic sites where IL-7 production has been observed (Maeurer et al., 1997; Pillai et al., 2004).

What is unique with our IL-7 *in vitro* culture system is that we have revealed the presence of a population of cells that only rely on an IL-7 signal to induce its proliferation. This is significant because the cell types expanded *in vitro* in our IL-7 assay are phenotypically similar, with exception of CD69 expression, to the T cell subset that accumulates *in vivo* in the lymphoid organs of STAT5b-CA mice. Further, the expansion of memory CD8 T cells results not as a default effect of IL-7's survival function, but rather is a consequence of a distinct proliferative activity. This was shown by using the IL-7 *in vitro* system to culture Bim deficient T cells in the presence or absence of the cytokine. These conclusions could not have been reached by traditional adoptive transfer methods using immunodeficient mice. Therefore, the presented method for the *ex vivo* expansion of IL-7 responsive cells incorporates features from *in vivo* and *in vitro* studies to provide a novel methodology to study the diverse activities of this important cytokine.

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Figure 1. Phenotyping of T cells Isolated from STAT5b-CA Mice

T cells isolated from spleen (A) and lymph nodes cells (B) of STAT5b-CA mice were analyzed for changes in surface protein expression. Cells were stained with antibodies for CD4, CD8, CD69 and CD44 as described in Methods and analyzed by flow cytometry. FSC/ SSC gating was used to establish viable gates for lymphocytes. In each dot plot the numbers shown are the percentages of cells in the indicated quadrant relative to total cells. Representative data from one mouse is displayed

C.

Figure 2. In Vitro Proliferation of T cells by IL-7

(A-B) Splenic T-cells (A) and lymph nodes cells (B) of C57BL/6 mice were labeled with CFSE and placed in culture with or without IL-7 (150 ng/ml) for 48 hours. In the left panel are shown the histograms displaying the fluorescence profile of the viable CFSE-labeled lymphocytes. The percent of rapidly proliferating cells is indicated. Shown in the right panels are the same histograms subjected to analysis using FCS Express Proliferation software to determine generation time based on loss of CFSE due to cell division. Green lines indicate undivided (rightmost peak) and divided cells (leftmost peak). In the tables at the far right are displayed the percent of cells in each generation. A representative experiment of three is shown. (C) Splenic T cells were cultured with IL-7 for 7 days and pulsed with BrdU for the final 48 hrs to determine the percentage of cells synthesizing DNA. Cells were stained with PerCP-conjugated antibodies for CD4 or CD8 and a FITC-conjugated anti-BrdU antibody to detect BrdU incorporation and analyzed by flow cytometry. Percentages shown in the dotplots are gated on viable cells (based on FSC/SSC) and CD4 or CD8 T cells that incorporated BrdU. Representative results from three experiments are shown.

A.

B.

Figure 3. Expansion of Memory-Phenotype CD8 T cells by IL-7

Lymph node cells (A) and T cells enriched from spleens (B) of C57BL/6 mice were phenotyped for IL-7-dependent changes in surface proteins. Cells were cultured with IL-7 (150 ng/ml) for 0, 7 and 14 days. Cells were stained with antibodies for CD4, CD8, CD44 and CD69 as described in Methods and analyzed by flow cytometry. FSC/SSC gating was used to establish viable gates for lymphocytes. In each dot plot, the percentages of cells in each quadrant are shown relative to total cells. Representative results from four time course experiments are shown.

Figure 4. Naïve T cells Do Not Proliferate in Response to IL-7

(A.) To examine IL-7 dependent proliferation, cells from the lymph nodes of OT-I Rag^{-/-} mice were cultured with IL-7 (150 ng/ml) for 14 days and pulsed with BrdU for the final 48 hrs. Cells were stained with antibodies for CD8 and BrdU for analysis by flow cytometry. Displayed are the percentages viable CD8 T cells (based on FSC/SSC) that incorporated BrdU during the 48 hr pulse. Representative results from two experiments are shown. (B) Lymph node cells from OT-I Rag^{-/-} mice were cultured with IL-7 (150 ng/ml) for 0 and 14 days. For analysis by flow cytometry of surface protein changes, cells were stained with antibodies for CD8, CD44 and CD69 as described in Methods. FSC/SSC gating was used to establish viable gates for lymphocytes. In each dot plot, the percentages of cells in each quadrant are shown relative to total cells. Time course experiments were performed twice. Representative results are shown.

C.

Figure 5. Differentiation of the Proliferative from the Survival Effects of IL-7

(A) Changes in cell size, upon culture with IL-7, were examined by detecting differences based on FSC and SSC patterns. Lymph nodes cells and T cells enriched from spleens of *Bim*^{-/-} mice were cultured with IL-7 (150 ng/ml) (shown) or without IL-7 (not shown) for 0, 7 and 14 days. Formation of lymphocyte blasts was assessed by determining the FSC and SSC of cell populations by flow cytometry. In dot plots representing data from day 7 and 14 of culture with IL-7, the red gated populations, predominantly CD4 T cells, contain the smaller-sized cells, while blue gated populations, predominantly CD8 T cells, contain the larger blast-like cells. (B-C) To examine changes in surface marker expression upon extended culture with IL-7, lymph nodes cells (B) and T cells enriched from spleens (C) of *Bim*^{-/-} mice were cultured with IL-7 (150 ng/ml) or without IL-7 for 0 through 14 days. Cells were stained with antibodies for CD4, CD8, CD44, and CD69 as described in Methods and analyzed by flow cytometry. FSC/SSC gating was used to establish viable gates for lymphocytes. The percentages of cells in each quadrant are shown relative to total cells. Representative data is shown. Time course experiments were performed three times.