Exogenous Amino Acids Are Essential for Interleukin-7 Induced CD8 T Cell Growth

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Abstract

IL-7 signalling is important in regulating both survival and cellular size (growth) of T cells. While glucose metabolism has previously been implicated in the mechanism of IL-7 induced survival and growth, the role of amino acids has not before been reported. Here, we show IL-7 dependent T cell survival does not require either exogenous glucose or amino acids. In contrast, maintenance of cell size and IL-7 induced growth were specifically dependent on amino acids. Furthermore, cellular amino acid uptake was implicated in the mechanism of IL-7 induced growth. Analysis of IL-7 regulated gene expression revealed that neutral and cationic amino acid transporters were specific transcriptional targets of IL-7 signalling. In contrast, none of the four glucose transporters expressed in T cells were modulated. Taken together, these data reveal for the first time the central importance of amino acid homeostasis for IL-7 regulated T cell growth.

Results

Neither glucose nor amino acids are required for IL-7 induced survival of naïve CD8 T cells

To establish culture conditions compatible with analysing both survival and cellular growth of T cells, we examined the dose dependence of IL-7 for both growth and survival. CD8 T cells from C57Bl6/J wild type (WT) mice were cultured in complete medium at a range of IL-7 concentrations for 72 h, after which cell viability and size was assessed. Increasing concentrations of IL-7 promoted both cell survival and growth of CD8 T cells (Fig. 1). Maximal survival was achieved at a concentration between 25–50 ng/ml (Fig. 1A). Size of individual T cells, as assessed by normalised FSC profile on FACS, was also proportional to IL-7 dose. Maintenance of a cell size comparable to that of ex vivo CD8 T cells required that IL-7 was at least 10 ng/ml, while growth resulting in increased cell size was observed at doses above 10 ng/ml (Fig. 1B). For the studies here, we therefore chose a dose of 50 ng/ml as a compromise between the concentration required for optimal survival (~25 ng/ml) and the dose at which maximal growth was observed (100 ng/ml).

To investigate the role of cellular amino acid homeostasis in the function of IL-7 on naïve CD8 T cells, we first examined their
Figure 1. IL-7 mediated growth and survival is dose dependent in vitro. CD8 T cells were enriched from C57Bl6/J donors and cultured with a range of IL-7 concentrations. After 72 h, cells were stained for CD8 expression and with 7AAD and viability and cell size determined by flow cytometry. (A) Graph shows viability of CD8 cells in cultures with different IL-7 concentrations. Viable CD8 T cells were identified as large cells that excluded 7AAD dye. Viability in IL-7 free cultures was <1% at 72 h (data not shown). (B) Graph shows cell size in cultures with different concentrations of IL-7. Data are expressed as size relative to ex vivo CD8 T cell controls. Error bars indicate SD of technical replicates. Results are representative of two or more experiments.

doi:10.1371/journal.pone.0033998.g001

requirement for IL-7 dependent T cell survival in vitro. CD8 T cells were purified from WT mice and cultured with IL-7 in RPMI medium, or in RPMI medium specifically lacking either amino acids (aa) or glucose (Glu). Survival of naive CD8 T cells was determined by measuring viability of CD8 cells in the presence of Glu [13], that inhibits mTOR, impaired IL-7 dependent survival of either naive CD8 T cells (Fig. 4) or CD44hi memory phenotype CD8 T cells cultured either in the absence of aa and Glu (Fig. 3D).

These data suggested that IL-7 promotes survival of CD8 T cells independently of the need for exogenous nutrients. We therefore sought to reinforce these results by specifically blocking signalling pathways required for cell growth and measuring the ability of IL-7 to promote cell survival. Trophic effects of IL-7 are thought to be downstream of PI3K and mammalian target of rapamycin (mTOR) signalling [12]. Consistent with previous studies [12], neither phosphoinositide-3-kinase (PI3K) inhibitors nor rapamycin, that inhibits mTOR, impaired IL-7 dependent survival of either naive CD8 T cells (Fig. 4) or CD44hi memory phenotype CD8 T cells in the same cultures (Fig. S1D). Thus, IL-7 is able to promote T cell survival in vitro in the complete absence of FCS, aa and Glu, further suggesting that it is the upregulation of Bcl2 expression levels that is the key process by which IL-7 prevents T cell death.

Exogenous amino acids are essential for IL-7 induced CD8 T cell growth

As well as regulating T cell survival, IL-7 also plays a role in both maintaining cell size and inducing further T cell growth in size. CD8 T cells from WT mice undergo progressive growth in vitro in the presence of IL-7 (Fig. 1B), while in the absence of this cytokine, the remaining viable cells do not maintain their cell size [12]. Consistent with previous studies, IL-7 induced cell growth of naive CD8 T cells was dependent on the activity of PI3K and mTOR, since pharmacological inhibitors of these pathways specifically inhibited IL-7 induced cell growth (Fig. 5A). To investigate the requirement for aa and Glu in this growth response to IL-7, we cultured CD8 T cells with IL-7 in the presence or absence of exogenous aa or Glu. IL-7 induced identical growth of

Figure 2. Minimal fetal calf serum requirement for IL-7 dependent survival of CD8 T cells. CD8 T cells were enriched from C57Bl6/J donors and cultured for the times indicated, either alone (open symbols, dashed lines), or in the presence of 50 ng/ml of IL-7 (filled symbols, solid lines) and additionally either in presence (circles) or absence (squares) of foetal calf serum (FCS). Cells were stained with 7AAD and viability amongst CD44hiCD8+ cells determined by measuring the frequency of 7AAD+ live cells by flow cytometry. Percentage of surviving cells was normalized to the percentage of live cells on day 0. Results are pool of three independent experiments. Error bars indicate SD of biological replicates.

doi:10.1371/journal.pone.0033998.g002
naïve CD8 T cells when cultured in aa+ Glu+ and aa−Glu− medium, and it appears that Glu in culture medium is not required for IL-7 induced cell growth (Fig. 5B). In contrast, naïve CD8 T cells cultured in medium lacking aa, either in aa− Glu− or aa−Glu− medium, completely failed to grow in response to IL-7 stimulation, and even shrank in size as compared with the same cells at day 0 (Fig. 5B). Interestingly, there was a trend to suggest that naïve CD8 T cells cultured in the absence of IL-7 underwent even greater reduction in cell size specifically in aa deficient cultures (Fig. 5B). Analysis of growth of CD44hi memory phenotype cells in the same cultures revealed an identical specific requirement for aa for IL-7 induced growth (Fig. S2), as observed for naïve T cells. Hence, exogenous aa are essential for both maintenance of T cell size and IL-7 induced cell growth in vitro.

IL-7 regulates amino acid but not glucose transporter gene expression

Next, we asked whether IL-7 influenced cellular aa and/or glucose homeostasis at the level of specific transporter gene expression. In a previous study, we examined genes regulated by IL-7 signalling (Pears et al, in press). In this study, microarray analysis was used to identify gene expression changes in F5 TCR transgenic T cells stimulated with IL-7 for 24 h. We used this data set to ask whether IL-7 signalling regulated expression of either glucose or amino acid transporters. The solute carrier (SLC) family comprises a large number of membrane transporter proteins divided into different families. The SLC2 (14 member genes) and SLC5 (12 member genes) families are the facilitative GLUT transporters and sodium glucose co-transporters respectively. SLC7 (7 member genes) and SLC7C (14 member genes) families are high affinity glutamate and neutral amino acid transporters and the cationic amino acid transporter families respectively. The SLC7 family includes the glycoprotein associated light subunit that forms heterodimeric transporter complexes with SLC3A2, the 4F2 cell surface antigen heavy chain. Analysis of gene expression data revealed four amino acid transporter proteins expressed in F5 T cells: Slc1a4, S1cp1, Slc7a5 and Slc7a6 (Fig. 6A). Similarly, expression of four glucose transporters was detected: Slc2a1, Slc2a3, Slc2a9 and Slc5a2 (Fig. 6A). Significantly, this pattern of expression of SLC1, SLC2, SLC3, SLC5 and SLC7 families was virtually identical to that recorded in the Immunological Genome Project (http://www.immgen.org/index_content.html) for peripheral CD8 naïve T cells, with the exception of Slc7a6, whose expression was not detected in F5 T cells. Comparing gene expression between ex vivo and IL-7 stimulated F5 T cells revealed that transcription of three of the four amino acid transporters expressed in F5 T cells was substantially increased by IL-7 signalling (Fig. 6B). In contrast, none of the glucose transporter genes were affected by IL-7 signalling (Fig. 6B).

Finally, we examined CD98 protein expression in WT CD8 T cells by flow cytometry. CD98 is the heterodimeric amino acid transporter complex of Slc7a5 and Slc3a2 gene products that form the large neutral amino acid transporter (LAT1). In vitro, stimulation of WT CD8 T cells with IL-7 resulted in a substantial increase in CD98 levels as compared with cells from IL-7 free cultures or ex vivo CD8 T cells (Fig. 7A). To test whether CD98 expression could also be regulated in vivo, CD8 T cells from WT mice were adoptively transferred to lymphopenic Rag1−/− hosts and CD98 expression levels assessed three days later. CD98 expression was elevated on CD8 T cells from Rag1−/− hosts as compared with control T cells from WT mice to a similar extent as observed by T cells stimulated with IL-7 in vitro (Fig. 7B). Induction was found to be IL-7 dependent, since the same cells
IL-7 Regulates Amino Acid Dependent Growth

Discussion

The role of IL-7 in regulating metabolism by influencing glycolysis is well recognised. IL-7 signalling maintains glucose uptake and glycolysis, possibly by upregulation of Pim1 [13] that is implicated in increased glycolysis. Whether IL-7 signalling affects abundance or function of glucose transporters appears to depend upon circumstance. We found no evidence of transcriptional regulation of any of the glucose transporters expressed in F5 T cells. However, there is evidence that Glut1 is regulated by post-transcriptional control of protein trafficking to the cell surface [13]. One notable difference between our data and previous studies [13], is that we found glucose was not required for IL-7 mediated T cell survival in vitro. However, as previously reported [12], we found that IL-7 mediated survival and upregulation of Bcl2 expression did not depend on PI3K or mTOR activity. Since IL-7 induced glucose uptake is dependent on PI3K or mTOR pathways [13], the observations that CD8 T cell survival did not require either exogenous glucose or PI3K/mTOR pathways are consistent with one another. Together, these data strongly suggest that IL-7 dependent T cell survival does not absolutely depend upon increased glucose uptake stimulated by IL-7 signalling. The view that IL-7 promotes T cell survival independently of its influence on glucose uptake is also supported by other studies showing that conditional loss of IL-7R expression by T cells failed to upregulate CD98 in vivo [3,14], without affecting their uptake of glucose [14].

Remarkably, we found that IL-7 could promote T cell survival in vitro in a minimal RPMI medium lacking both exogenous glucose and amino acids. Significantly, IL-7 signaling still resulted in upregulation of Bcl2, which is likely to be a key anti-apoptotic mediator. However, since naive CD8 T cells are resting quiescent cells, it can be speculated that their requirements for such nutrients is minimal given their relative inactivity, and a combination of cellular reserves of amino acids and turnover of proteins may be sufficient to meet requirements for new protein synthesis, at least in the short term. This appeared to be the case over the 72 h of the experiments reported here. The dose of IL-7 used in the present study was chosen on the basis that it both promoted substantial and measurable cell growth but was below the dose required for maximal survival of CD8 T cells at 72 h. Future studies could determine whether the requirement for exogenous amino acids and glucose for T cell survival is different at lower levels of IL-7 stimulation.

In contrast to the requirements for IL-7 mediated CD8 T cell survival, we found that IL-7 induced growth was absolutely dependent on exogenous amino acids but not glucose. While CD8 T cell survival was maintained by IL-7, in media specifically lacking amino acids, T cells failed to grow in response to IL-7 stimulation. We also found evidence that IL-7 signalling was specifically regulating cellular homeostasis of amino acids. Of the four amino acid transporters expressed by T cells, we found that IL-7 stimulation specifically upregulated gene expression of three, suggesting that IL-7 signalling is tuning amino acid homeostasis by regulating expression of these transporters. While a passive nutritional requirement for amino acid uptake with respect to protein synthesis for cell growth is logical, our data suggest that amino acids also play an active role in regulating growth. Exogenous amino acids are known to inhibit autophagy [13], that would divert amino acids from cell proteins to essential processes. However, amino acids are also found to induce S6K1 activation that is dependent on mTOR activity [16], suggesting that they may also play an active signalling role in inducing T cell growth in response to IL-7 in cooperation with mTOR.

Figure 5. IL-7 induced T cell growth is strictly dependent on exogenous amino acids but not glucose. CD8+ T cells were enriched from C57B6/J donors and cultured for 3 days in RPMI containing standard nutrients, either alone (open symbols), or in the presence of 50 ng/ml IL-7 (filled symbols). Cultured cells were stained with 7AAD and size of viable CD44lo CD8+ cells determined by measuring FSc by FACS. (A) Where indicated, the inhibitors LY294002 (Ly, squares) (10 μM), rapamycin (Rap, triangles) (20 nM) or the vehicle control (DMSO, diamonds) were added to cultures. (B) Cells were cultured in RPMI medium containing standard nutrients (circles) or in RPMI specifically lacking amino acids (squares), glucose (triangles) or lacking both glucose and amino acids (diamonds). Cells were cultured in the presence (filled symbols) or absence (empty symbols) of exogenous IL-7 (50 ng/ml). Results are the pool of three or more independent experiments. Each point plotted is the mean of triplicate cultures for a single independent experiment. Error bars indicate SD of biological replicates. * p<0.001; ns, not significant. doi:10.1371/journal.pone.0033998.g005

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The potential influence of metabolic pathways by exogenous nutrients such as amino acids has important implications for immune responses. Blocking mTOR activity in vivo has been shown to enhance memory cell development [17]. Nutrient deprivation, either in draining lymph nodes or at the site of ongoing infections where there is high metabolic demand from large numbers of infiltrating cells, may therefore affect the fate of effector T cells, by tuning S6K1 activity to promote generation of resting memory cells, that are metabolically less demanding than active effectors that might otherwise undergo apoptosis. Th17 cell differentiation has also been shown to be affected by abundance of amino acids, since amino acid deprivation blocks Th17 differentiation [18], and aromatic amino acids are precursors of aryl hydrocarbon receptor ligands that also promote Th17 induction [19]. IL-7 promotes Th17 responses in vivo [20], therefore regulation of amino acid transport could play a role in this process.

In conclusion, we present clear evidence that amino acid uptake is crucial for IL-7 induced growth by normal T cells, and predict that perturbation of amino acid uptake will have a profound affect on normal T cell function.

Materials and Methods

Mouse strains used in this study

C57Bl6/J and F5 Rag1−/−, Rag2−/−, Il7−/− Rag1−/− mice were bred in a conventional colony free of pathogens at the NIMR, London. Animal experiments were performed according to institutional guidelines and United Kingdom Home Office regulations.

Flow cytometric analysis

Flow cytometry was carried out using lymph node or spleen cells. Cell concentrations were determined using a Scharfe Instruments Casy Counter (Scharfe System, Reutlingen, Germany). Cells were incubated with saturating concentrations of antibodies in 200 μl PBS-bovine serum albumin (0.1%)-azide.
Where used, LY294002 (Sigma) was used at 10 μM, and rapamycin (Calbiochem) was used at 20 nM.

**Statistics**

Statistical significance of changes in cell size (Figs. 5A, 5B and S2) were tested by One way Anova test with Dunnett’s multiple comparison test. *p<0.001; ns, not significant. Statistical significance of differences in gene expression level in IL-7 stimulated CD8 T cells (Fig. 6B) were tested by non-parametric Mann Whitney test. *p<0.05.

**Supporting Information**

Figure S1 Neither amino acids, glucose, PI3K nor mTOR are required for IL-7 dependent survival of memory CD8 T cells. CD8+ T cells were enriched from C57Bl/6j donors and cultured for the indicated time points, either alone (open symbols, dashed lines), or in the presence of 50 ng/ml of IL-7 (filled symbols, solid lines). Cultured cells were stained with 7AAD and frequency of viable 7AAD− cells amongst total CD44hi CD8+ memory phenotype T cells determined by flow cytometry. (A–C) Graphs show cell viability of cells cultured in RPMI medium containing standard nutrients (circles) or in RPMI specifically lacking (A) amino acids (aa−), (B) glucose (Glu−) or (C) glucose and amino acids (Glu−aa−, squares throughout). (D) Cells were cultured in RPMI containing standard Glu and aa nutrients and, where indicated, the inhibitors LY294002 (Ly) (10 μM) or rapamycin (Rap) (20 nM), or the vehicle control (DMSO) were added. Percentage of surviving CD44hi CD8+ T cells was normalized to the percentage of live CD44hi CD8+ T cells on day 0. Error bars indicate SD of biological replicates. Results are pool of three independent experiments. (TIF)

Figure S2 IL-7 induced T cell growth is strictly dependent on exogenous amino acids but not glucose. CD8+ T cells were enriched from C57Bl/6j donors and cultured for 3 days in RPMI containing standard nutrients, either alone (open symbols), or in the presence of 50 ng/ml IL-7 (filled symbols). Cultured cells were stained with 7AAD and frequency of viable CD44hi CD8+ T cells determined by measuring FSc by FACS. Cells were cultured in RPMI medium containing standard nutrients (circles) or in RPMI specifically lacking amino acids (squares), glucose (triangles) or lacking both glucose and amino acids (diamonds). Cells were cultured in the presence (filled symbols) or absence (empty symbols) of exogenous IL-7 (50 ng/ml). Results are the pool of three or more independent experiments. Error bars indicate SD of biological replicates. *p<0.001; ns, not significant. (TIF)

**Acknowledgments**

We thank S. Tung, and the Biological Services staff for assistance with mouse breeding and typing.

**Author Contributions**

Conceived and designed the experiments: BS AS CP. Analyzed the data: BS AS CP. Wrote the paper: BS.

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