Glutamine Uptake and Metabolism Are Coordinately Regulated by ERK/MAPK during T Lymphocyte Activation


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Activation of a naïve T cell is a highly energetic event, which requires a substantial increase in nutrient metabolism. Upon stimulation, T cells increase in size, rapidly proliferate, and differentiate, all of which lead to a high demand for energetic and biosynthetic precursors. Although amino acids are the basic building blocks of protein biosynthesis and contribute to many other metabolic processes, the role of amino acid metabolism in T cell activation has not been well characterized. We have found that glutamine in particular is required for T cell function. Depletion of glutamine blocks proliferation and cytokine production, and this cannot be rescued by supplying biosynthetic precursors of glutamine. Correlating with the absolute requirement for glutamine, T cell activation induces a large increase in glutamine import, but not glutamate import, and this increase is CD28-dependent. Activation coordinate enhances expression of glutamine transporters and activities of enzymes required to allow the use of glutamine as a Krebs cycle substrate in T cells. The induction of glutamine uptake and metabolism requires ERK function, providing a link to TCR signaling. Together, these data indicate that regulation of glutamine use is an important component of T cell activation. Thus, a better understanding of glutamine sensing and use in T cells may reveal novel targets for immunomodulation.

Materials and Methods

Abs and reagents

Anti-CD3 (mAb 145-2C11) and anti-CD28 (mAb 37.51) Abs, control hamster IgG, and PE-labeled anti-Thy1.2, anti-CD69, anti-CD25, and anti-CD98 Abs were purchased from eBioscience (San Diego, CA). HRP-conjugated anti-mouse IgG and anti-rabbit IgG were from Jackson ImmunoResearch Laboratories (West Grove, PA). The MEK inhibitor PD98059 was purchased from BIOMOL (Plymouth Meeting, PA) and used at a concentration of 40 μM. ADP, lactate dehydrogenase (Enzyme Commission [E.C.] 1.1.1.27), and malate dehydrogenase (E.C. 1.1.1.37) were purchased from Calbiochem (San Diego, CA). 1-Bromodecane, pyridoxal phosphate, NADH, triethanolamine- HCl, hydrazine dihydrochloride, α-ketoglutarate, glutaminase (E.C. 3.5.1.2), glutamate dehydrogenase (GDH; E.C. 1.4.1.3), glutamic-oxaloacetic transaminase (GOT; E.C. 2.6.1.1), and glutamic-pyruvic transaminase (GPT; E.C. 2.6.1.2) were purchased from Sigma-Aldrich (St. Louis, MO). 2-[3,4-3H]Glutamine and 2-[3,4-3H]Glutamic acid were from American Radiolabeled Chemicals (St. Louis, MO).

Animals

C57BL/6J mice (6-wk-old) were purchased from The Jackson Laboratory (Bar Harbor, ME). All of the mice were maintained in ventilated microisolator cages (Animal Care Systems, Centennial, CO) in the University of...
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Maryland animal facility. Animals received humane care in compliance with the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources). All of the mice were euthanized by carbon dioxide inhalation, as recommended by the American Veterinary Medical Association Panel on Euthanasia.

T cell purification

Murine T cells were purified from spleens using the EasySep negative selection system (Stem Cell Technologies, Vancouver, British Columbia, Canada) according to the manufacturer’s protocol. Purified T cells were generally >95% Thy1-positive, as determined by flow cytometry.

Cell lines and culture

The murine EL-4 thymoma cell line was purchased from American Type Tissue Collection (Manassas, VA). All of the cells were maintained in RPMI 1640 medium (Mediatech, Manassas, VA) supplemented with 10% FBS (HyClone, Logan, UT), penicillin/streptomycin, 10 mM HEPES buffer, and 55 μM 2-ME, with or without 2 mM glutamine, at 37 °C in a 5% CO2 atmosphere. For glutamine withdrawal experiments, the FBS was replaced with 10% dialyzed FBS (J R Scientific, Woodland, CA). For amino acid uptake experiments, glutamine-deficient and glutamate-deficient RPMI 1640 media were produced in-house, using the standard RPMI 1640 formulation but omitting either glutamine or glutamic acid.

T cell stimulation

Anti-CD3 and anti-CD28 Abs were covalently attached to tosyl-activated Dynabeads (Invitrogen, Carlsbad, CA) following the manufacturer’s instructions using 75 μg/ml of each Ab. Beads were mixed with T cells at a 3:1 ratio of beads/cells and incubated at 37 °C for the desired time. For unstimulated samples, T cells were mixed with control hamster IgG-linked Dynabeads and were then either used immediately or cultured in parallel with stimulated samples.

Proliferation and cytokine assays

Unfractionated C57BL/6J splenocytes were stimulated with soluble anti-CD3 Ab and either control IgG or anti-CD28 Abs for 24 h. Glutamine and glutamate uptake were measured using a modification of the amino acid uptake protocol of Edinger and Thompson (20). Briefly, murine splenocytes were labeled with [3H]glutamine or L-2,3,4-[3H]glutamic acid. Cells were allowed to take up the radiolabeled amino acid for 10 min at room temperature and then spun washed in 10 mM Tris-HCl, 40 mM NaCl, and 5 mM CaCl2 for 3 min at 4 °C. Flow cytometry was used to determine the percentage of cells stained with Annexin V and 7-AAD. For glutamine withdrawal experiments, the FBS was replaced with 10% dialyzed FBS (J R Scientific, Woodland, CA). For amino acid uptake experiments, glutamine-deficient and glutamate-deficient RPMI 1640 media were produced in-house, using the standard RPMI 1640 formulation but omitting either glutamine or glutamic acid.

Flow cytometry

Surface levels of activation markers were determined on unstimulated and stimulated purified T cells by flow cytometry. After harvest, cells were
(6.22 mmol⁻¹ cm⁻¹ l). The reported rates are representative of three independent experiments, and are averages of triplicate samples (±SD).

Statistical analysis

All of the statistical analyses were performed using Prism software, version 5 (GraphPad, San Diego, CA). The minimal level of confidence at which experimental results were considered significant was \( p < 0.05 \). Statistical significance between samples in proliferation assays and between glutamine and glutamate uptake was determined by two-way ANOVA with Bonferroni posttest analysis. Statistical significance of cytokine production was determined by one-way ANOVA with Bonferroni posttest analysis or by two-tailed \( t \) test. Statistical significance of enzyme activity assays in resting versus stimulated T cells was determined by two-tailed \( t \) test.

Results

**T cell activation is absolutely dependent on extracellular glutamine**

To define the amino acid requirements for T cell activation, splenic T cells were stimulated in tissue culture medium depleted of individual amino acids, and proliferation in culture was measured. Not surprisingly, removal of any of the essential amino acids (valine, leucine, isoleucine, phenylalanine, tryptophan, lysine, threonine, and methionine), which cannot be synthesized by mammalian cells, blocked T cell proliferation (data not shown). However, we also found that several nonessential amino acids were required for T cell proliferation and the removal of glutamine consistently gave the strongest inhibition of any amino acid. Reducing glutamine levels by as little as 50% produced a detectable impairment in T cell proliferation, and reduction below 10% of normal culture levels led to an absolute proliferation block (Fig. 1A). Similarly, removal of glutamine blocked secretion of IL-2 (Fig. 1B) and IFN-\( \gamma \) (Fig. 1C) by activated T cells. Depletion of other amino acids had variable effects on cytokine production, but glutamine was unique in being absolutely required for both IL-2 and IFN-\( \gamma \). Notably, no other single depletion inhibited IL-2 production by >50%, and the essential amino acid leucine was not required for either IL-2 or IFN-\( \gamma \) secretion (data not shown).

The concentration of glutamine in RPMI 1640 culture medium is 2 mM, which is >30% of the total amino acid pool. Thus, it is possible that glutamine depletion has such a dramatic effect because there are insufficient levels of other amino acids to compensate. We therefore tested whether supplementing the medium with glutamic acid, which is readily converted into glutamine, could restore T cell proliferation in glutamine-free medium. As shown in Fig. 2A, even medium supplemented with an extra 2 mM glutamic acid was unable to support T cell proliferation. Similarly, supplementing the medium with either 2 mM proline or 2 mM asparagine, each of which can serve as a precursor to synthesize glutamine, was unable to restore T cell proliferation in glutamine-free medium (Fig. 2B). Glutamate, asparagine, and proline were also unable to substitute for glutamine in supporting cytokine production (data not shown). Thus, T cells specifically require glutamine for activation, consistent with previous studies using mitogenic lectins to activate lymphocytes (1).

**Proliferation and cytokine secretion are relatively late events in T cell activation.** To determine whether glutamine depletion also inhibits earlier events, we examined the expression of T cell surface markers after 24 h of stimulation (Fig. 3A–C). T cells stimulated in complete or glutamine-free medium induced expression of CD69, CD25, and CD98/4F2hc comparably, indicating that cells are able to initiate activation events in the absence of glutamine. However, cell growth was severely impaired in glutamine-free medium, as seen by the reduced forward scatter values (Fig. 3D). Thus, T cells require glutamine for growth, cytokine secretion, and proliferation but not for expression of activation markers, largely consistent with the findings of Hörig et al. (17).

**T cell activation selectively increases glutamine uptake**

The requirement for glutamine during T cell activation and the inability of metabolic precursors to serve as substitutes suggested that activated T cells would consume glutamine at a substantially higher rate than resting cells. To test this, glutamine uptake rates in unstimulated and 24 h-stimulated T cells were compared. We consistently saw a 5- to 10-fold induction of glutamine uptake in activated T cells relative to that of unstimulated controls (Fig. 4A). Similar to previous findings with glucose uptake and metabolism
Glutamine uptake in T cells is regulated by ERK activity

The importance of glutamine for T cell function and the large increase in glutamine uptake during activation suggested that glutamine use might be regulated by TCR-initiated signals. TCR ligation results in activation of multiple signal transduction pathways, including members of the MAPK family (26). The MAPK family member ERK has been implicated in the control of SNAT expression and transport activity in response to amino acid starvation (27, 28). We therefore tested whether ERK activity was required for the increase in glutamine uptake during T cell activation. T cells were stimulated in the presence or absence of the MEK inhibitor PD98059, which blocks ERK activation, and glutamine uptake was determined. As shown in Fig. 6, 40 μM PD98059 [a concentration that inhibits MEK1 and MEK2 but not other kinases (29)] completely blocked the increased glutamine uptake. This was not due to a global block in activation, because PD98059 had little or no effect on the induction of several activation markers, including CD69 and CD98 (data not shown). Thus, ERK acts downstream of TCR/CD28 signaling to positively regulate glutamine uptake.

T cell activation induces enzymes involved in glutamine metabolism

One possible explanation for the dependence of T cells on glutamine is that T cells use glutamine as a primary energy source. To distinguish these possibilities, we compared the changes in glutamine and glutamate uptake during T cell activation. As seen in Fig. 4C, import of glutamine was induced by T cell activation, but glutamate uptake in activated T cells was comparable to uptake in resting cells. Thus, activated T cells selectively enhance glutamine uptake. Strikingly, the T lymphoma cell line EL-4 constitutively imported glutamine at an even higher rate than activated T cells (Fig. 4D) and had a similarly strong preference for glutamine over glutamate (Fig. 4E).

**T cell activation induces expression of glutamine transporters**

The increase in glutamine uptake during T cell activation suggested that expression of glutamine transporters was also induced. Although cells can import glutamine via multiple systems, the major glutamine transporters are members of the SNAT family (25). We therefore used quantitative real-time PCR to measure SNAT mRNA expression levels during T cell activation. As shown in Fig. 5A, stimulation of T cells through CD3 and CD28 induced expression of SNAT1 and SNAT2, with SNAT1 being induced more rapidly and to a higher level. mRNA expression peaked at 8 h and had begun to fall by 24 h. Thus, induction of glutamine transporter gene expression is consistent with the increased glutamine uptake after T cell activation.

In addition to overall transporter levels, nutrient transport can be regulated by cellular localization of the transporter proteins. For SNAT1 and SNAT2, it has been found that the bulk of the protein is located in intracellular vesicles (25), suggesting that increased transport may be due to a combination of increased protein expression and relocation of transporters from intracellular stores to the cell surface. We examined surface levels of SNAT2 during T cell activation by biotinylation of total surface protein, followed by immunoprecipitation of SNAT2 and detection by avidin–HRP. Immediately after stimulation, there was a moderate increase in surface SNAT2 levels, followed by a large increase after 15–18 h (Fig. 5B). Stimulation also induced changes in the pattern of surface proteins that associated with SNAT2 (seen as coprecipitating bands), suggesting that glutamine transport may be further regulated by proteins that interact with the transporters.

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One possible explanation for the dependence of T cells on glutamine is that T cells use glutamine as a primary energy source.
Although T cells are highly glycolytic, most pyruvate is converted to lactate rather than oxidized in the Krebs cycle (24, 30, 31). It has been suggested that glutamine serves as an alternate Krebs cycle substrate for lymphocytes (1, 3). We therefore examined the expression and activity of key enzymes in this metabolic pathway.

A major route of glutamine metabolism is initiated by the enzyme glutaminase, which hydrolyzes glutamine to produce glutamate. T cell activation induced glutaminase activity, consistent with an important role for glutamine in T cell function (Fig. 7A). As with glutamine uptake, glutaminase activity in EL-4 cells was constitutively high (Fig. 7A). Glutaminase mRNA was also induced early after T cell stimulation, peaking at 3 h (Fig. 7B). We have found that IL-2 mRNA also becomes readily detectable at 3 h poststimulation (data not shown), indicating that changes in glutamine metabolism occur with similar kinetics to other early T cell activation events.

The entry point into the Krebs cycle for glutamine is α-ketoglutarate. We therefore examined the enzymes GDH, GOT, and GPT, each of which converts glutamate to α-ketoglutarate. GDH catalyzes the oxidative deamination of glutamate, producing ammonia and reducing NAD⁺ to NADH. GOT and GPT transfer the amine group from glutamate to oxaloacetate and pyruvate, producing aspartate and alanine, respectively. The activities of all three enzymes were induced by T cell activation, but GOT activity was greater than the activities of GDH and GPT combined (Fig. 7C). This is consistent with analysis of glutamine metabolism in lymphocytes, because ammonia generation appears limited mainly to the initial step (deamidation to glutamate) and aspartate is a major product (1, 3). Notably, the activity of glutaminase was consistently lower than the activity of GDH or GOT, for both resting and activated cells, indicating that glutaminase activity is likely limiting for glutamine metabolic flux. EL-4 lymphoma cells demonstrated constitutively high enzyme activities, with GOT and GPT activities nearly 10 times the level found in activated T cells, but GOT remained the dominant enzyme (Fig. 7D). As with glutamine uptake, induction of all four enzymes during T cell activation was blocked by ERK inhibition (Fig. 7A–C). Thus, ERK signaling serves as a common regulatory pathway for both uptake and metabolism of glutamine in T cells.

Discussion
The role of metabolism as a regulator of cellular function has recently attracted increasing attention. There is a growing sentiment that metabolic pathways do not merely respond to changing conditions to maintain homeostasis but also are important participants in defining the activation state of a cell and can respond to signal transduction events. Much of this renewed attention has come from studies demonstrating that altered glucose and glutamine metabolism is important for tumor cell growth and survival (32–35). However, rather than being limited to transformed cells, similar changes in cellular metabolism are likely to be important for any rapidly dividing cells, included activated T cells (36, 37).

To investigate the regulation of amino acid use by T cells, we began by testing the importance of individual amino acids for T cell
activation. We found that T cells have an absolute requirement for a large external supply of glutamine and that even moderate reductions within the normal physiological range impair T cell function. Other amino acids, including direct biosynthetic precursors, cannot substitute for glutamine, indicating that the need for glutamine is not simply due to insufficient levels of other nutrients in tissue culture medium.

This dependence on glutamine is similar to results found for unfractionated human and rodent lymphocytes stimulated with the T cell mitogens Con A and PHA (16–19). However, in these systems, T cells represent ≤50% of the total cell population. This makes it difficult to assign metabolic effects directly to T cells, because activated T cells may in turn regulate the metabolism of other cells (e.g., via cytokines and/or direct cell–cell contact or by changing the metabolite composition of culture medium). In addition, Con A and PHA likely stimulate cells by clustering large numbers of surface receptors, including some that might not normally be involved in T cell activation. Our results confirm the importance of glutamine in a more defined system, using purified T cells and specific stimulation via CD3 and CD28. Notably, we were able to show a specific requirement for CD28 costimulation, similar to previous studies of glucose metabolism (24). It thus appears that CD28 is important for regulation of T cell metabolism more generally, possibly serving as a point of coordination for many metabolic pathways.

Glutamine is nonessential in the diet and is readily synthesized by mammalian cells (and is used by many as a mechanism of nitrogen excretion). Lymphocytes have been found to express glutamine synthetase activity (38), suggesting that they are also able to synthesize glutamine from glutamate. It is therefore unclear why T cells are unable to substitute other amino acids for glutamine. One possibility is that transport of other amino acids is insufficient to compensate for a lack of glutamine. We found that T cells increase glutamine uptake 5- to 10-fold upon activation but fail to increase glutamate import. This is consistent with studies of the anionic amino acid transport system $x_{\text{L}}^{-}$, which appears to have low activity in lymphocytes (39–41). Thus, even in the presence of high glutamate levels, T cells would be unable to import enough to replace glutamine. In addition, import of a given amino acid is frequently influenced by the intracellular concentration of other amino acids (42). It is therefore possible that other amino acids are unable to replace glutamine because T cells cannot import them efficiently in the absence of a glutamine supply.

An alternative (and complementary) possibility is that glutamine is directly sensed in T cells, and so other amino acids cannot substitute for it. Amino acid sensing by mammalian cells is still poorly understood, and sensor pathways for glutamine have not been well characterized. Two major signaling kinases involved in amino acid sensing are the general control 2 (GCN2) kinase (43) and the target of rapamycin (TOR, known as mTOR in mammalian cells) kinase (44). Although sensing by GCN2 has been established for several amino acids in mammalian cells (45–48), studies examining GCN2 responsiveness to glutamine have not been reported. Investigation of glutamine sensing by mTOR has
yielded conflicting results, with data indicating that mTOR activity may be dependent on (49), inhibited by (50), or insensitive to glutamine (51). Determining the roles of GCN2 and mTOR in glutamine responsiveness will require further study, and we are currently investigating this area.

Another attractive candidate for a glutamine sensor would be one or more of the proteins involved in glutamine transport (52). A transporter would have access to both intracellular and extracellular stores of glutamine and could therefore use either pool for sensing. Glutamine is a favored substrate for the SNAT (SLC38) family of transporters (25), and we found that expression of SNAT1 and SNAT2 mRNA as well as surface expression of SNAT2 protein are induced by T cell activation. Notably, SNAT2 has been identified as a glutamine-responsive amino acid sensor (53). Hyde et al. (53) have also implicated a separate, but unknown, JNK-dependent pathway for glutamine sensing, indicating that detection of individual amino acids may be complex, with overlapping and cooperating pathways contributing to the eventual response.

The absolute requirement of T cells for an external supply of glutamine and their high rate of T cell glutamine consumption suggest that T cells use glutamine for multiple roles beyond protein synthesis (14, 15, 54–56). The amide nitrogen of glutamine is used in the synthesis of nucleotides, NAD, and amino sugars, all of which are important for rapidly growing and dividing cells, such as activated T cells. However, it may be the production and further metabolism of glutamate that is of greatest importance. Although glutamate is a central component of many metabolic processes, the low glutamate uptake activity of T cells implies that they must generate glutamate intracellularly, most likely from the ample glutamine supply. We found that glutaminase gene expression is consistent with this hypothesis. A recent report suggests that activated T cells may also serve as important suppliers of glutamate for other cell types (57), similar to the function of APCs in providing glutamine and their high rate of T cell glutamine consumption.

Metabolism of glutamate that is of greatest importance. Although glutamate is a central component of many metabolic processes, the low glutamate uptake activity of T cells implies that they must generate glutamate intracellularly, most likely from the ample glutamine supply. We found that glutaminase gene expression and enzyme activity are both induced during T cell activity, consistent with this hypothesis. A recent report suggests that activated T cells may also serve as important suppliers of glutamate for other cell types (57), similar to the function of APCs in providing reduced cysteine for T cells (58). Significantly, glutaminase activity appears to be rate-limiting for glutamine metabolism in activated T cells. It is therefore likely that glutaminase is a critical enzyme for T cells, and we are currently investigating further the regulation of glutaminase activity during T cell activation.

T cell activation induces metabolic enzymes that allow glutamate to enter the Krebs cycle (as α-ketoglutarate), namely, GDH, GOT, and GPT. Our finding that GOT activity is the highest among these three enzymes suggests that synthesis of aspartate is an important outcome of glutamine metabolism in T cells. This is consistent with observed rates of aspartate secretion by T cells (1, 3) and may also indicate poor aspartate uptake capacity, analogous to the limited ability to import glutamate. Aspartate is itself important for nucleotide synthesis, and so glutamine can contribute both directly, as a nitrogen donor, and indirectly, via production of aspartate. The partial oxidation of α-ketoglutarate in the Krebs cycle also produces ATP, potentially allowing activated T cells to divert glucose to other biosynthetic pathways (36, 37). It will therefore be important to examine glutamine metabolism in a broader context, tracking contributions of nitrogen and carbon atoms to downstream metabolites and macromolecules and determining the interplay with other metabolic pathways.

The ability to coordinate the regulation of metabolic pathways is important for cellular homeostasis in the face of changing conditions but is also critical for cells undergoing changes in activation or differentiation state. The TOR signaling pathway is a well-established integrator of many cellular and extracellular cues, with both inputs and outputs connected to nutrient access and metabolism (reviewed in Refs. 44, 59). In this paper, we report that the ERK signaling pathway is another important regulator of cellular metabolism. ERK signaling is required for enhanced glutamine uptake and increased activity of key metabolic enzymes. ERK activation has also been found to be involved in the induction of system A transporter activity (mediated by expression of SNAT transporter proteins) in response to amino acid starvation in human fibroblasts (27) and Chinese hamster ovary cells (28). We have further found that ERK activity is required for TCR/CD28-induced changes in glucose metabolism (A. Marko, R. Miller, and K. Frauwirth, manuscript in preparation). Together, these results implicate ERK as another central player in cellular metabolic control.

Although ERK regulates glutamine uptake and metabolism in a coordinated fashion, it appears that multiple pathways are likely to be involved in these processes. Induction of glutaminase mRNA requires ERK activity, indicating that ERK regulates either transcription or stability of glutaminase mRNA. This differs from the induction of glutaminase via c-Myc, which blocks expression of regulatory microRNAs but does not appear to increase glutaminase mRNA itself (60). Thus, glutaminase expression is regulated by at least two distinct mechanisms.

The reliance of activated T cells on glutamine metabolism is strikingly similar to that of tumor cells and may represent a common feature of all rapidly dividing cells (37, 61). However, the pattern of enzyme activity that we observed (high GOT, low GPT) is distinct from the pattern recently reported for tumor (glioma) cells, in which GPT appeared to be the dominant activity (62). We found that the EL-4 lymphoma line showed a pattern of activity similar to that of activated T cells, albeit with higher levels for both enzymes, suggesting that GPT activity does not necessarily predominate in tumor cells. It remains unclear whether the differences between our findings and those of the Thompson group represent a divergence between tissue types or between the methods used to measure enzyme function, and analysis of a wider range of normal and tumor cells will be required to draw conclusions. However, this difference suggests the possibility that different aspects of glutamine metabolism may provide specificity for targeting therapeutics toward tumor cells versus normal cells or toward specific cell types (e.g., lymphocytes).

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Disclosures

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