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LAT1 Is a Critical Transporter of Essential Amino Acids for Immune Reactions in Activated Human T Cells

Keitaro Hayashi,* Promsuk Jutabha,* Hitoshi Endou,[†] Hironori Sagara,[‡] and Naohiko Anzai*

Activation of T cells accompanies remarkable enhancement of metabolism. Sufficient and continuous nutrient supply is therefore important to support immune reaction in T cells. However, the mechanism of the promotion of nutrient incorporation in activated T cells has not been elucidated. In this study, we show that L-type amino acid transporter 1 (LAT1) is a major transporter for essential amino acids into activated human T cells. CD3/CD28 stimulation in primary human T cells triggered dramatic induction of LAT1 expression mediated by NF- κ B and AP-1. Functional disturbance of LAT1 by a specific inhibitor and by small interfering RNA in human T cells suppressed essential amino acid uptake and induced a stress response mediated by DNA damage-inducible transcript 3 to attenuate cytokine production via inhibition of NF- κ B and NFAT activities. These results uncover the previously unknown mechanism by which T cells accelerate essential amino acid uptake upon activation and adapt to essential amino acid starvation. Our results also raise the possibility for application of an LAT1 inhibitor as a new drug for therapy of disease caused by exaggerated immune response. *The Journal of Immunology*, 2013, 191: 4080–4085.

Activation of T cells triggers a considerable metabolic switch (1–3). Sensing of a specific Ag by TCR on quiescent T cells induces the initiation of cytokine production and entry to the cell growth phase, which is crucial for immune response. Because those events are a process requiring high levels of energy and intracellular biosynthesis, T cells must shift the metabolic mode from housekeeping maintenance state to aggressive phase. Because facilitated metabolism is coupled to consumption of large amounts of intracellular materials such as amino acids and glucose, T cells must boost the rate of uptake of those nutrients by setting up special devices such as effective organic solute transporters. Supporting this notion, the glucose transporter 1 is upregulated upon activation of T cells (4–6). T cells also must enhance amino acid uptake rate to support increased protein synthesis accompanied by cytokine production and cell proliferation during activation. However, the molecular mechanism by which T cells elicit the incorporation of amino acids, especially essential amino acids, in response to Ag recognition has not been determined.

Sustained consumption of cellular resources without a supply from extracellular milieu leads to functional breakdown of cells.

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The microarray data presented in this article have been submitted to the Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/gds>) under accession number GSE48578.

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Abbreviations used in this article: DDIT3, DNA damage-inducible transcript 3; GCN2, general amino acid control nonderepressible 2; LAT1, L-type amino acid transporter 1; Luc, luciferase; mTOR, mammalian target of rapamycin; siRNA, small interfering RNA.

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Therefore, most cells have multiple systems to safeguard against nutrient deficiency. Growing evidence has indicated the existence of two major cascades in many cell types for response to amino acid starvation. Mammalian target of rapamycin (mTOR) senses environmental situations such as nutrient availability (7, 8). Many nutrients and growth factor activate mTOR, which phosphorylates its downstream molecules such as S6 kinase and eukaryotic initiation factor 4E-binding protein 1, leading to continuous protein synthesis, cell cycle progression, or inhibition of autophagy induction (9–12). However, when amino acids become deprived, mTOR is dephosphorylated, and the opposite reaction downstream of mTOR occurs. General amino acid control nonderepressible 2 (GCN2), which is activated by phosphorylation upon amino acid deprivation, induces expression of transcription factor 4, which regulates expression of multiple target genes that are crucial for stress response in a state of amino acid starvation (13, 14). Likewise, in T cells, because sustained activation is also strictly dependent on energy status, the availability of intracellular amino acids must be constantly monitored. If T cells cannot ensure that cytosolic amino acid resources are more than sufficient, they will activate an amino acid starvation response, which will downshift an intracellular immune response. However, although mTOR and GCN2 seem to have some functions in T cells (15–19), little is known about the molecular mechanisms leading to downregulation of immune response induced by amino acid deprivation in T cells.

In this study, we found that L-type amino acid transporter 1 (LAT1) is a major transporter for essential amino acid uptake in activated human T cells. LAT1 has been shown to be highly expressed in a wide range of cancer cells (20, 21), but the role of LAT1 in normal cells has not been determined because its expression in normal tissues is extremely low (20, 22, 23). In this study, we demonstrated that full activation of primary human T cells by TCR stimulation and costimulation triggers dramatic induction of LAT1 expression that is mediated by NF- κ B and AP-1. JPH203, a specific inhibitor of LAT1, suppressed leucine uptake, leading to decreased cytokine production in activated human T cells. Transfection of small interfering RNA (siRNA) for LAT1

reduction also exhibited similar effects of JPH203. Dysfunction of LAT1 as well as essential amino acid starvation in activated T cells facilitated the expression of DNA damage-inducible transcript 3 (DDIT3 also known as C/EBP homologous protein), which is known to be upregulated by GCN2. Overexpression of DDIT3 in T cells attenuated cytokine production through prevention of NF- κ B and NFAT activity, suggesting the potential mechanism for restraining immune reaction triggered by functional disturbance of LAT1 as a response to amino acid starvation. Thus, induction of LAT1 is the mechanism by which T cells incorporate the maximum amount of essential amino acid for full activation.

Materials and Methods

Reagents

Anti-LAT1 Ab was described previously (24). Anti-CD3 Ab (OKT3), anti-CD28 Ab (CD28.2), and FITC or allophycocyanin-conjugated anti-CD4 and anti-CD25 Ab were purchased from eBioscience (San Diego, CA). Anti-GAPDH was purchased from Medical and Biological Laboratories (Nagoya, Japan). NF- κ B Activation Inhibitor and JNK Inhibitor II were purchased from Calbiochem (Billerica, MA). JPH203 was described previously (25).

Cells

Human PBMCs were isolated from healthy volunteers by the Histopaque centrifugation method (Sigma-Aldrich, St. Louis, MO). The study was approved by the Dokkyo Medical University Bioethics Committee. For isolation of CD4-positive T cells, PBMCs were stained with fluorescence-labeled anti-CD4 and anti-CD25 Abs, and the CD4-positive CD25-negative cell population was sorted with FACS (BD Biosciences, Franklin Lakes, NJ). For stimulation of T cells, 1×10^5 cells were seeded on a plate bound with anti-CD3 (5 μ g/ml) and anti-CD28 (2 μ g/ml) Abs in RPMI 1640 medium with 10% FCS for the times indicated. For cytokine production, purified T cells were stimulated and cultured for 3 d, and cytokine concentration in the culture supernatant was measured by a cytometric bead array (BD Biosciences). For an experiment of a cell culture with a low concentration of essential amino acid, purified T cells were stimulated and cultured with HBSS (Invitrogen, Carlsbad, CA) including vitamin solution (Sigma-Aldrich) and MEM nonessential amino acid solution (Invitrogen) in the presence of standard amount (100%) or 1/10 amount (10%) of MEM amino acids solution (Invitrogen) for 3 d.

Transfection of siRNA and plasmid into T cells

Silencer select control siRNA (negative control number 1) and predesigned LAT1-specific siRNA (s15653) were purchased from Ambion (Austin, TX). siRNA (40 pmol) or plasmid (5 μ g) was transfected into PBMCs using a nucleofector (Lonza, Basel, Switzerland) with a human T cell nucleofection kit, according to the manufacturer's instructions. CD4⁺ T cells were then purified as described above, activated, and cultured for 3 d and then collected for the following experiment.

Western blot analysis

Cells were lysed in SDS sample buffer and boiled. Cell lysate samples were resolved by SDS-PAGE and transferred to a polyvinylidene difluoride membrane. Immunoblotting was performed with a standard protocol. Relative signal intensity of band was determined by Multi Gauge software (Fuji Film, Tokyo, Japan).

Reporter assay

Jurkat cells were provided by RIKEN Bio-Resource Center (Tsukuba, Japan). The cells were transfected by electroporation as described previously (26). NF- κ B-, AP-1-, and NFAT-luciferase (Luc) reporter constructs were described previously (26). Firefly Luc activity was assessed using a Dual Glo luciferase assay system (Promega, Madison, WI) and normalized by the activity of Renilla Luc derived from cotransfected pRL-TK (Promega).

¹⁴C-L-leucine uptake

Activated T cells were incubated with 1 μ M JPH203 for 5 min, and ¹⁴C-L-leucine uptake was initiated by incubating the cells in HBSS containing 1 μ M ¹⁴C-L-leucine (Moravsek, Brea, CA) at 37°C for 1 min. Uptake was terminated by washing the cells three times with ice-cold HBSS. Cells were filtered and lysed with 0.1 N NaOH, and radioactivity was measured using an LSC-5100 beta scintillation counter (Aloka, Tokyo, Japan).

Quantitative real time-PCR

Total RNA was extracted using an RNeasy mini kit (Qiagen, Valencia, CA). cDNA was synthesized from total RNA using a prime-script reverse transcriptase reagent kit (Takara Bio, Shiga, Japan). RT-PCR was performed with Sybr Premix Ex Taq (Takara Bio). The primers for RT-PCR (HA142581 for DDIT3 and HA067812 for GAPDH) were purchased from Takara Bio. The relative amount of DDIT3 mRNA was normalized to the amount of GAPDH mRNA.

Plasmid construct

Human DDIT3 cDNA was obtained by the PCR method using cDNA synthesized from human T cell mRNA as a template. The cDNA fragment was inserted into XhoI and BamHI site in pcDNA3.

Microarrays

Total RNA was isolated from T cells 3 d after activation as described above in the presence or absence of 1 μ M JPH203 and processed for microarray analysis by Takara Bio with an Agilent Expression Array chip.

Statistical analysis

All statistical significance was tested with a Student *t* test.

Results

Full activation of human T cells induces LAT1 expression

Nutrition consumption is prominent in both cancer cells and activated T cells. Evidence that cancer cells use LAT1 as a transporter for essential amino acids indicates the possibility that activated T cells also take advantage of LAT1 for efficient amino acid uptake. To understand the role of LAT1 in T cells, human primary T cells were activated by anti-CD3 and anti-CD28 Abs, and the expression of LAT1 protein was analyzed. Preactivated T cells express an almost undetectable level of LAT1 protein (Fig. 1A). Neither CD3 stimulation nor CD28 stimulation alone affected the expression of LAT1 protein. However, stimulation with anti-CD3 and anti-CD28 Abs dramatically induced LAT1 expression. These results indicate that full activation of T cells induces LAT1 expression.

The requirement of both TCR and costimulation for induction of LAT1 expression suggests that T cells use the same tool for induction of LAT1 expression and initiation of immune reaction. Such a system would be beneficial for T cells because they can promote immune reaction and nutrient supply at once. Therefore, we further examined the molecular mechanism of the induction of LAT1 expression in activated T cells. AP-1 and NF- κ B are representative transcription factors that are activated by CD3/CD28 and promote immune reaction in T cells (27–29). Because CD3/CD28 is required for LAT1 expression, we examined whether AP-1 and NF- κ B also participate in the regulation of LAT1 expression. An inhibitor of AP-1 or NF- κ B dramatically inhibited the induction of LAT1 expression by CD3/CD28 stimulation (Fig. 1B). These results indicate that upregulation of LAT1 expression in response to TCR/costimulation is mediated by AP-1 and NF- κ B.

LAT1 is crucial for essential amino acid incorporation in activated T cells

Because LAT1 expression is strongly induced in activated T cells, we investigated the functional significance of LAT1 as a transporter of essential amino acids in activated T cells. We initially assessed the influence of disturbance of LAT1 function by the use of JPH203, a LAT1-specific inhibitor (25), on the uptake of leucine, which is a representative neutral amino acid. Human T cells activated by CD3/CD28 Abs were pretreated with JPH203 for 5 min, and uptake of ¹⁴C-labeled L-leucine was determined. The uptake of leucine was impaired by JPH203 (Fig. 2). This result indicates that LAT1 functions as a central transporter of essential amino acids in activated T cells.

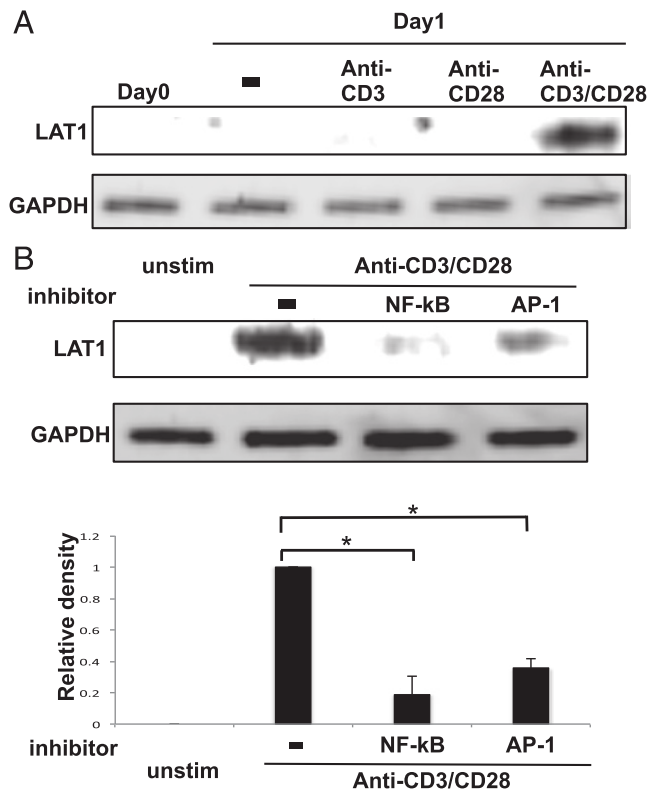


FIGURE 1. Induction of LAT1 expression by full activation of T cells. **(A)** Human primary T cells were activated with anti-CD3 and anti-CD28 Abs for 1 d. The expression level of LAT1 protein was determined by Western blot analysis. **(B)** Human primary T cells were activated with anti-CD3/CD28 Abs in the presence or absence of an NF- κ B (100 nM) or AP-1 (1 μ M) inhibitor for 1 d. LAT1 protein level was determined by Western blot analysis. Relative density of each band was determined. Data are representative of three separate experiments. Data are expressed as the mean \pm SD. * p < 0.01.

LAT1 is crucial for T cell function

We next wanted to evaluate the effect of inhibition of LAT1 on immunological reaction in T cell. Human T cells were activated in the presence or absence of JPH203, and production of IFN- γ , IL-4, and IL-17 was measured. A marked decrease in the production of all cytokines analyzed was observed in the T cells cultured with JPH203 (Fig. 3). These results indicate that insufficient uptake of essential amino acid caused by inhibition of LAT1 leads to attenuation of immunological function in T cells.

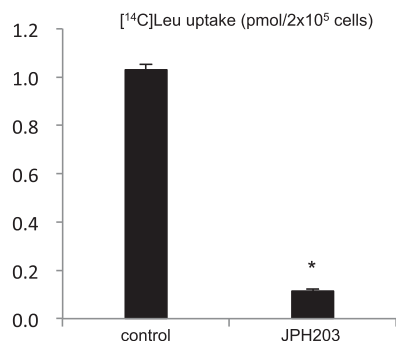


FIGURE 2. Effect of the LAT1 inhibitor JPH203 on uptake of amino acid in activated T cell. Activated human T cells were pretreated with 1 μ M JPH203 for 5 min, and [¹⁴C]leucine incorporation was determined. Data are representative of three separate experiments. Data are expressed as the mean \pm SD. * p < 0.01.

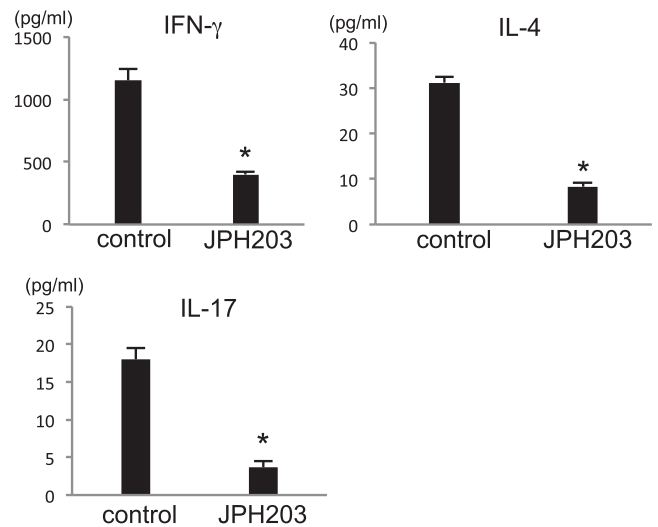


FIGURE 3. Effect of JPH203 on T cell function. Human primary T cells were activated with anti-CD3/CD28 Abs in the presence or absence of 1 μ M JPH203 for 3 d, and the concentration of cytokine in the culture medium was determined. Data are representative of three separate experiments. Data are expressed as the mean \pm SD. * p < 0.01.

LAT1-specific siRNA abrogates T cell function

JPH203 is a well-known LAT1-specific inhibitor. However, the possibility of this drug affecting some molecules other than LAT1 cannot be excluded. Therefore, we next verified that the effect of JPH203 exactly reflects the inhibition of the LAT1 function. We suppressed LAT1 expression using siRNA and performed functional analysis of T cells. LAT1-specific siRNA was transfected into primary human T cells. The cells were then stimulated by anti-CD3/CD28 Abs, and [¹⁴C]-labeled L-leucine uptake was analyzed. Transfection of LAT1 siRNA into T cells resulted in some deletion of LAT1 protein, although complete depletion of LAT1 expression was not achieved (Fig. 4A). Nevertheless, there was a significant decline of leucine incorporation in LAT1-specific siRNA-transfected T cells (Fig. 4A). We also observed defects in cytokines production in LAT1-specific siRNA-transfected human-activated T cells (Fig. 4B). These results are consistent with the results of the pharmacological inhibition experiment demonstrating the requirement of LAT1 for amino acid incorporation and immune response. Taken together, these results indicate that LAT1 plays a central role as a transporter of amino acids in activated T cells.

Alteration of gene expression is induced by LAT inhibition

We hypothesized that activated T cells with a defect in LAT1 function would modulate the pattern of gene expression profile to adjust to an environment with limited amino acids, because proceeding biosynthesis accompanied by immune reaction without nutrient supply could lead to a breakdown of the cells. To test this hypothesis, we used a microarray assay with RNA from T cells activated in the presence of JPH203. The microarray data are available in the Gene Expression Omnibus database (<http://www.ncbi.nlm.nih.gov/gds>) under the accession number GSE48578. Among those genes, DDIT3 attracted our attention because this protein has been demonstrated to be upregulated by various cellular stresses via GCN2 (30, 31), although its function in T cells has remained unknown. We therefore performed detailed analysis of DDIT3 function in LAT1-impaired T cells. Upregulation of DDIT3 in activated T cells treated with JPH203 as well as LAT1-specific siRNA was confirmed by using real-time PCR (Fig. 5A, 5B). We also confirmed that reduction of essential amino acid

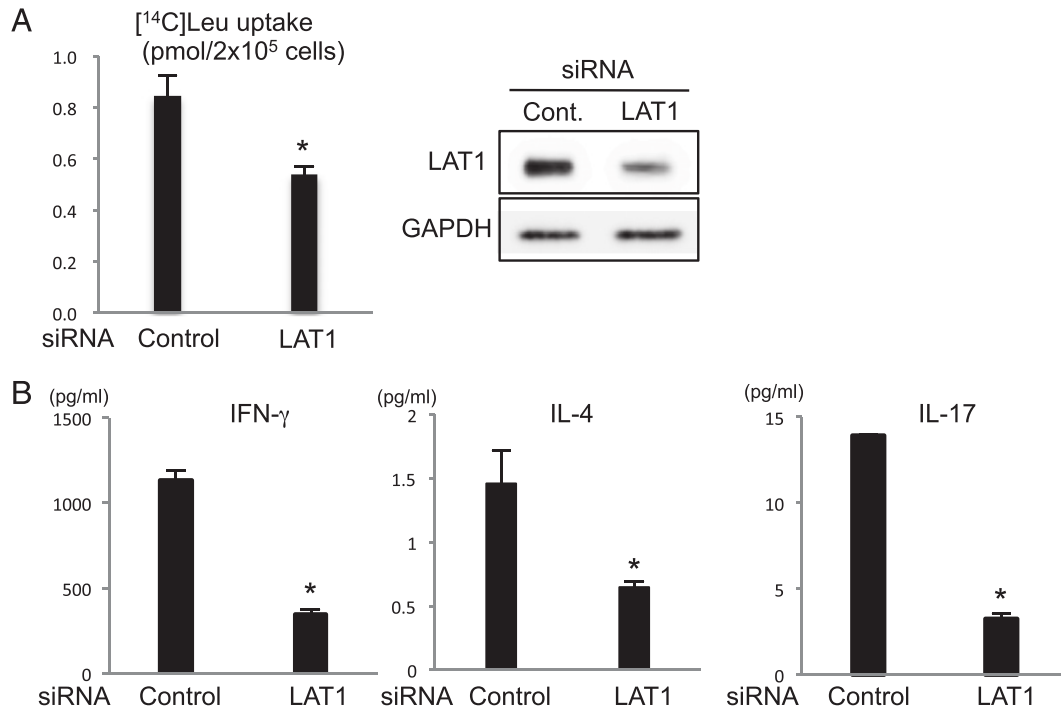


FIGURE 4. Effect of LAT1-specific siRNA on T cell function. **(A)** Human primary T cells were transfected with LAT1-specific siRNA or control siRNA and activated for 3 d. LAT1 protein was determined by Western blot analysis, and [¹⁴C]leucine incorporation in siRNA-transfected T cells was determined. **(B)** siRNA-transfected T cells were activated for 3 d, and the concentration of cytokine in the culture medium was determined. Data are representative of three separate experiments. Data are expressed as the mean \pm SD. **p* < 0.01.

concentration facilitated the expression of DDIT3 in activated T cells (Fig. 5C). These results prompted us to determine whether DDIT3 participates in the amino acid starvation response to downregulate immune reaction in T cells. Human primary T cells were transfected with a DDIT3 expression vector and stimulated

with anti-CD3/CD28 Abs, and the cytokine production level was measured. Overexpression of DDIT3 significantly reduced cytokine production (Fig. 6). These results indicate that primed DDIT3 expression is a response to an amino acid starvation caused by LAT1 dysfunction to suppress cytokine production.

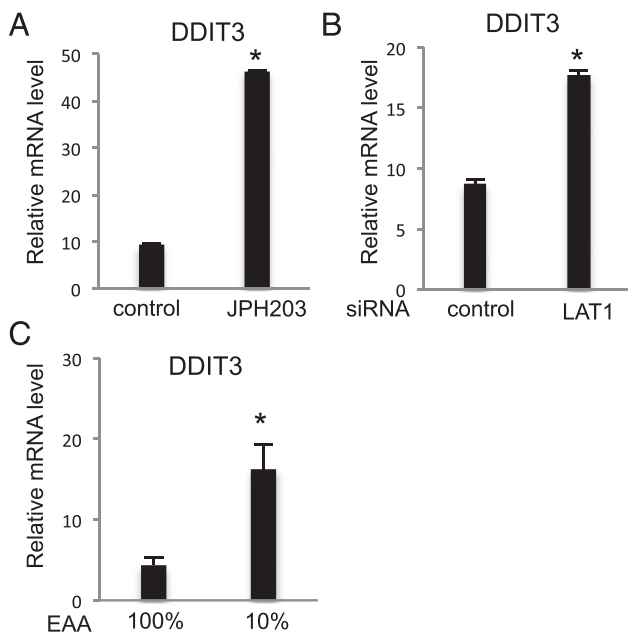


FIGURE 5. DDIT3 is induced in response to LAT1 dysfunction. Human T cells were activated for 3 d in the condition of 1 μ M JPH203 addition **(A)**, LAT1-specific siRNA transfection **(B)**, or a low concentration of essential amino acids (EAA) **(C)**. DDIT3 mRNA level was determined by RT-PCR. Data are representative of three separate experiments. Data are expressed as the mean \pm SD. **p* < 0.01.

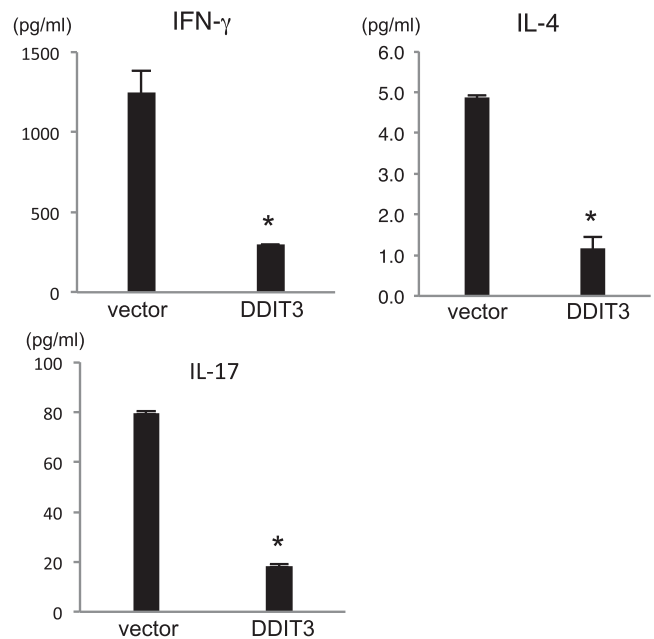


FIGURE 6. DDIT3 is a negative effector of T cell activation. T cells were transfected with a DDIT3 expression vector and activated for 3 d. The concentration of cytokine in the culture medium was determined. Data are representative of three separate experiments. Data are expressed as the mean \pm SD. **p* < 0.01.

DDIT3 inhibits NF- κ B, and NFAT activities but not AP-1 activity

DDIT3 was originally identified as a member of the family of CCAAT/enhancer-binding proteins (C/EBP) and as a dominant-negative factor of C/EBPs (31). Because C/EBP seems to upregulate some cytokines in response to T cell stimulation (32), it is possible that inhibition of cytokine production by DDIT3 is a result of attenuation of C/EBP function. However, induction of cytokine production is also mediated by multiple transcription factors, and we therefore studied in more detail the basis for inhibition of cytokine production in T cells with DDIT3 overexpression. We examined the effects of DDIT3 on NF- κ B, NFAT, and AP-1 functions, because these are key transcription factors for production of a number of cytokines upon T cell activation (33–35). Reporter genes for which expression is promoted by NF- κ B, NFAT, and AP-1 were cotransfected with a DDIT3 expression vector into Jurkat T cells, and the cells were activated by PMA/ionomycin to mimic signal transduction from TCR/costimulation. The activity of each transcription factor was determined by a reporter assay. We found that NF- κ B and NFAT activities were significantly inhibited by DDIT3 overexpression (Fig. 7). In contrast, DDIT3 did not affect AP-1 activity. These results suggest that DDIT3 functions as a potent factor for amino acid starvation response to interfere with cytokine production in LAT1-impaired T cells by selective inhibition of transcriptional regulators of cytokine expression.

Discussion

Sustained incorporation of more nutrients is necessary to ensure unimpeded biosynthesis in activated T cells. However, the mechanism by which uptake of amino acids is enhanced in activated T cells is not known. In this study, we showed that LAT1 is a critical factor for supply of essential amino acids to activated T cells. Our results revealed previously unknown facts that activation of T cells needs a special transporter to achieve maximum incorporation of essential amino acids for normal immune reaction and that LAT1 plays a central role as a transporter. LAT1 expression is induced by CD3

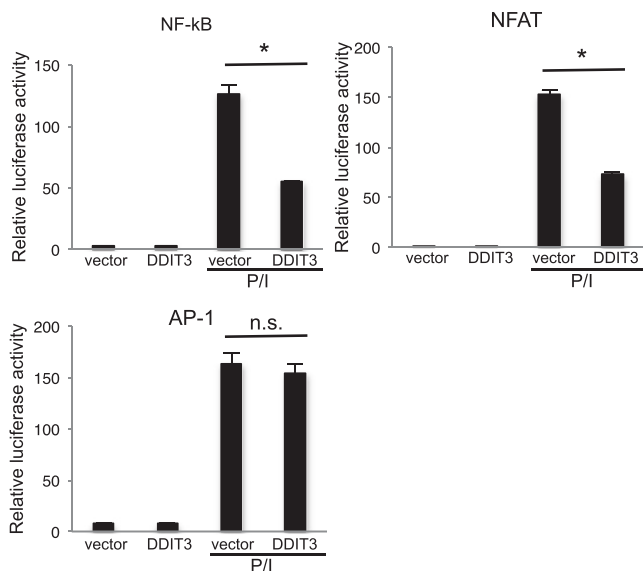


FIGURE 7. Effect of DDIT3 on the activity of NF- κ B, NFAT, and AP-1. NF- κ B-, NFAT-, or AP-1-Luc construct was transfected with the DDIT3 expression vector into Jurkat T cells. After 18 h, cells were treated with 100 nM PMA and 1 μ M ionomycin (P/I) for 6 h. Luciferase activity was measured and normalized with activity of pRL-TK-luc. Data are representative of three separate experiments. Data are expressed as the mean \pm SD. * p < 0.01.

and CD28 stimulation. Pharmacological inhibition and genetic inhibition of LAT1 expression resulted in a significant impairment of incorporation of amino acids and immune reaction in activated T cells. These results indicate that LAT1 is indispensable as an essential amino acid transporter to maintain the activation state of T cells. Although growing evidence has revealed the predominant expression and importance of LAT1 in a wide variety of cancer cells, the function of LAT1 in normal tissues is poorly understood. Our results showed that LAT1 has a substantial role not only in cancer cells but also in normal tissues.

Insufficient supply of essential amino acids leads to a restriction of cellular activity. Therefore, T cells must establish a system for inducing LAT1 expression to prevent a shortage of essential amino acids when stimulated. It is highly possible that cancer cells prefer using LAT1 because LAT1 can incorporate essential amino acids more efficiently than other transporters. Therefore, T cells would use LAT1 to satisfy the demand for a huge amount of biological resources when activated. We found that NF- κ B and AP-1 are essential for LAT1 expression upon T cell stimulation. These transcription factors are undoubtedly the most extensively studied factors for regulation of cytokine production in T cells. However, we also revealed a new role of those factors as crucial coordinators for the provision of nutrients. Our results suggest that the nutrient supply system is driven by molecular machinery that also induces cytokine production in T cells. Such a system would be advantageous for T cells because they could guarantee the concurrent promotion of immune reaction and nutrient supply. Interestingly, previous reports also showed that stimulation of Jurkat leukemia T cells with phorbol ester and calcium ionophore induces LAT1 mRNA expression (36). Because Jurkat T cells activate NF- κ B and AP-1 with stimulation by phorbol ester and calcium ionophore (Fig. 7), and the activation of both transcription factors is required to activate primary T cells stimulated by TCR/costimulation (33, 35), it is very likely that the upregulation of LAT1 in Jurkat T cells also is mediated by NF- κ B and AP-1, as we have shown with human T cells in Fig. 1B. Of note, we assume that the mechanism by which LAT1 expression is induced in T cells is different from the mechanism in cancer cells. Our previous study showed that enrichment of LAT1 expression in cancer cells is mediated by the proto-oncogene c-Myc (37). However, in the same cancer cell lines, we could not detect any effect of either an NF- κ B or AP-1 inhibitor on LAT1 expression (K. Hayashi and N. Anzai, unpublished observations). These results suggest that the molecular mechanisms for regulation of LAT1 expression are different in cancer and T cells.

In contrast, proper control of cellular metabolic activity depending on the nutrient condition is imperative for prolonged cell survival. We showed that impairment of LAT1 function leads to induction of DDIT3 expression. DDIT3 has been shown to be upregulated by GCN2 in some cells including T cells (19, 30, 38), but the role of DDIT3 in T cells is poorly understood. Our results suggest that one of the critical roles of DDIT3 in T cells is prompting a downshift of global cytokine production by inhibiting NF- κ B and NFAT activities in response to amino acid deprivation. These results suggest that T cells have a predesigned program to handle amino acid starvation and that when LAT1 expression is impaired, T cells turn on such a system for restraining the immune response to avoid waste of energy and resources. Interestingly, it has been shown that GCN2 also induces cell cycle arrest by suppressing cyclin D3 expression in arginine-starved T cells (39). This and our results suggest that GCN2 functions as a central factor for amino acid starvation response in T cells.

mTOR is one of the another major factor to monitor the nutrient condition. However, we could not detect clear suppression of mTOR activity in LAT1-impaired T cells. This result is similar to a prior

finding showing that halofuginone, an inducer of amino acid starvation response in T cells, does not alter mTOR signaling (40).

Although the immunological defect in LAT1-impaired T cells is thought to be a safeguard against cellular functional breakdown in a condition of restricted essential amino acids, our results using human tissues also indicate the possibility of a clinical benefit of an LAT1 inhibitor in therapy of immune disease. It is reasonable to assume that administration of JPH203 to patients would suppress excessive immune reaction by preventing T cell activity. This is attractive in that JPH203 could be expected to suppress symptoms with minimum adverse effects since LAT1 is expressed in activated T cells but not in resting T cells. Because the action mechanism of an LAT1 inhibitor is totally different from the action mechanism of any immunosuppressive drugs that are currently available, such an inhibitor is a potential alternative drug for patients in whom existing treatments are not effective.

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Disclosures

The authors have no financial conflicts of interest.

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