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SUPPORTING INFORMATION

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LAT1-specific inhibitor is effective against T cell-mediated allergic skin inflammation

To the Editor,

L-type amino acid transporter 1 (LAT1) is a predominant transporter of large neutral amino acids in human cancer cells,¹ and its specific inhibitor, JPH203, is currently undergoing clinical trials for treatment of malignancy.² Recent studies have demonstrated that LAT1 is critical for activating human and mouse T cells.^{3,4} Thus, LAT1 could be an attractive molecular therapeutic target for immunological diseases involving activated T cells. Here, we investigated the potential of JPH203 to treat T cell-mediated allergic skin inflammation.

Abbreviations: 2-DG, 2-deoxy-D-glucose; AD, atopic dermatitis; CDK, cyclin-dependent kinase; DAB, 3-3'-diaminobenzidine; ECAR, extracellular acidification rates; LAT1, L-type amino acid transporter 1; OCR, oxygen consumption rates; OVA, ovalbumin; OXPHOS, oxidative phosphorylation.

Keitaro Hayashi and Osamu Kaminuma equally contributed to this work.

Consistent with our previous Western blot data,³ immunostaining analysis revealed high LAT1 expression in anti-CD3/CD28 antibody (Ab)-stimulated, but not unstimulated, human peripheral CD4⁺ T cells (Figure 1A). Since atopic dermatitis (AD) is accompanied by T-cell activation,⁵ we assumed that LAT1 is expressed in T cells at the lesional sites of dermatitis patients. Indeed, DAB immunostaining revealed strong LAT1 expression in the skin with AD, which partly overlapped with the CD4 expression area (Figure 1B). Immunofluorescence staining demonstrated LAT1 expression in a substantial population of CD4⁺ T cells (Figure 1B,C). In contrast, very few LAT1⁺ cells were detected in normal skin (Figure 1B,C). These findings suggest that LAT1 expression is induced in CD4⁺ T cells in inflamed lesions of AD.

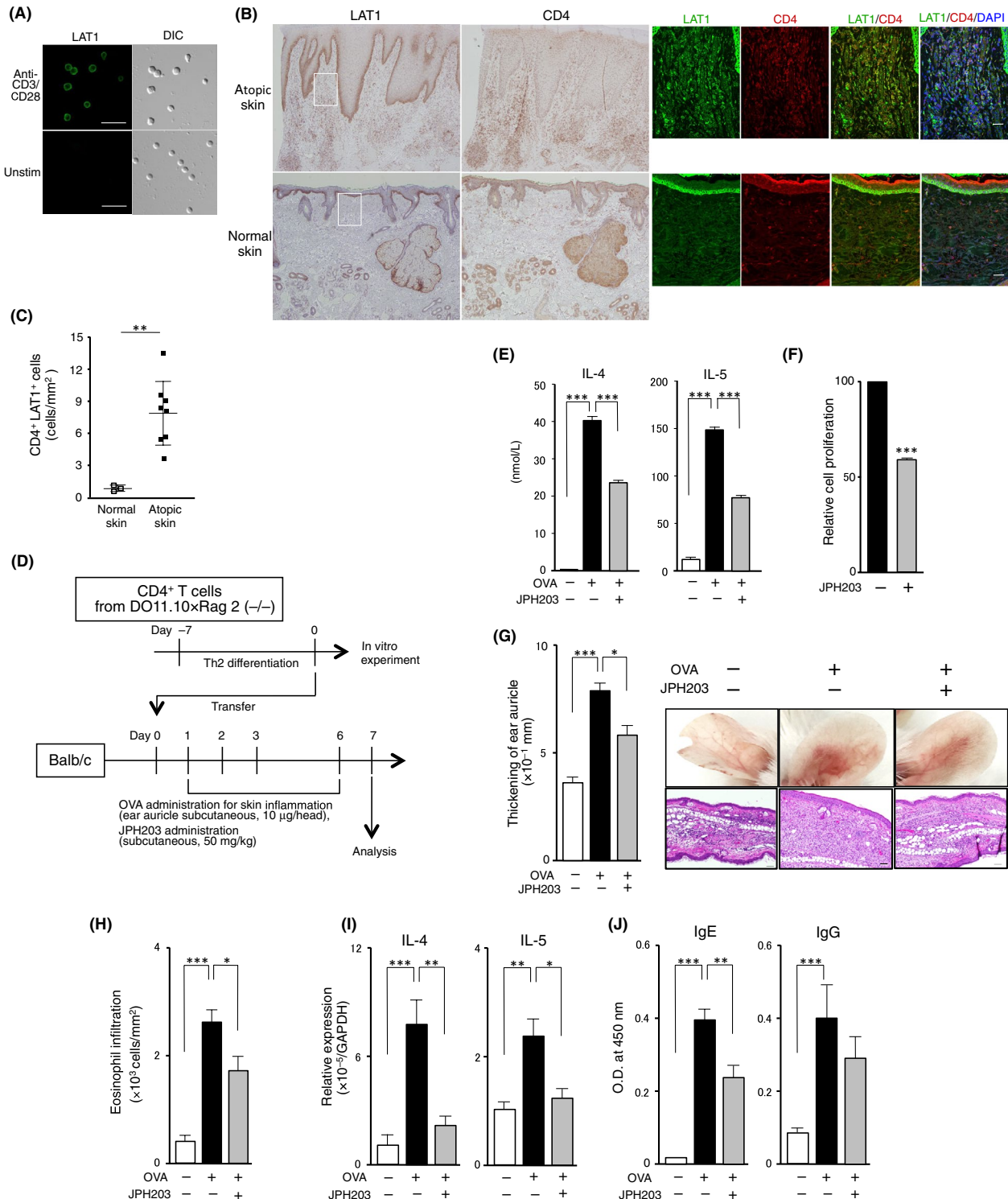


FIGURE 1 Effect of JPH203 on T cell-mediated allergic skin inflammation. A, LAT1 immunofluorescent staining (left panels) and differential interference contrast (DIC; right panels) for human peripheral blood CD4⁺ T cells stimulated with anti-CD3/CD28 antibody (Ab) for 48 h. Bars, 50 μ m; original magnification, $\times 200$. B, Left, DAB immunostaining with anti-LAT1 and anti-CD4 Abs in normal and atopic dermatitis skins. Right, immunofluorescent staining (white box in DAB staining). Bars, 50 μ m; original magnification, $\times 200$. C, Number of CD4⁺ LAT1⁺ cells in normal ($n = 3$ individuals) and atopic ($n = 8$ individuals) skins (per mm^2). Data are mean $\pm$ SEM. * $P < .01$ by Student's t test. D, Schematic diagram of allergic skin inflammation model experiments. E, F, Cytokine concentration in culture medium (E) and relative proliferation (F) of OVA323-339-stimulated Th2 cells with or without JPH203 for 72 h (mean $\pm$ SEM, $n = 3$). Data are representative of at least 3 independent experiments. (G-J) Ear thickness (G; left panel), ear images (G; right upper panels), ear sections stained with hematoxylin-eosin (G; right lower panels, bars, 50 μ m), number of eosinophils in ear sections (H), IL-4 and IL-5 transcript amounts in ear tissue (I), OVA-specific IgE and IgG serum concentrations (J) of Th2 cell-transferred and antigen-challenged mice (mean $\pm$ SEM, $n = 4$ -6 animals). * $P < .05$, ** $P < .01$, *** $P < .001$ by one-way ANOVA with Dunnett's multiple comparison test

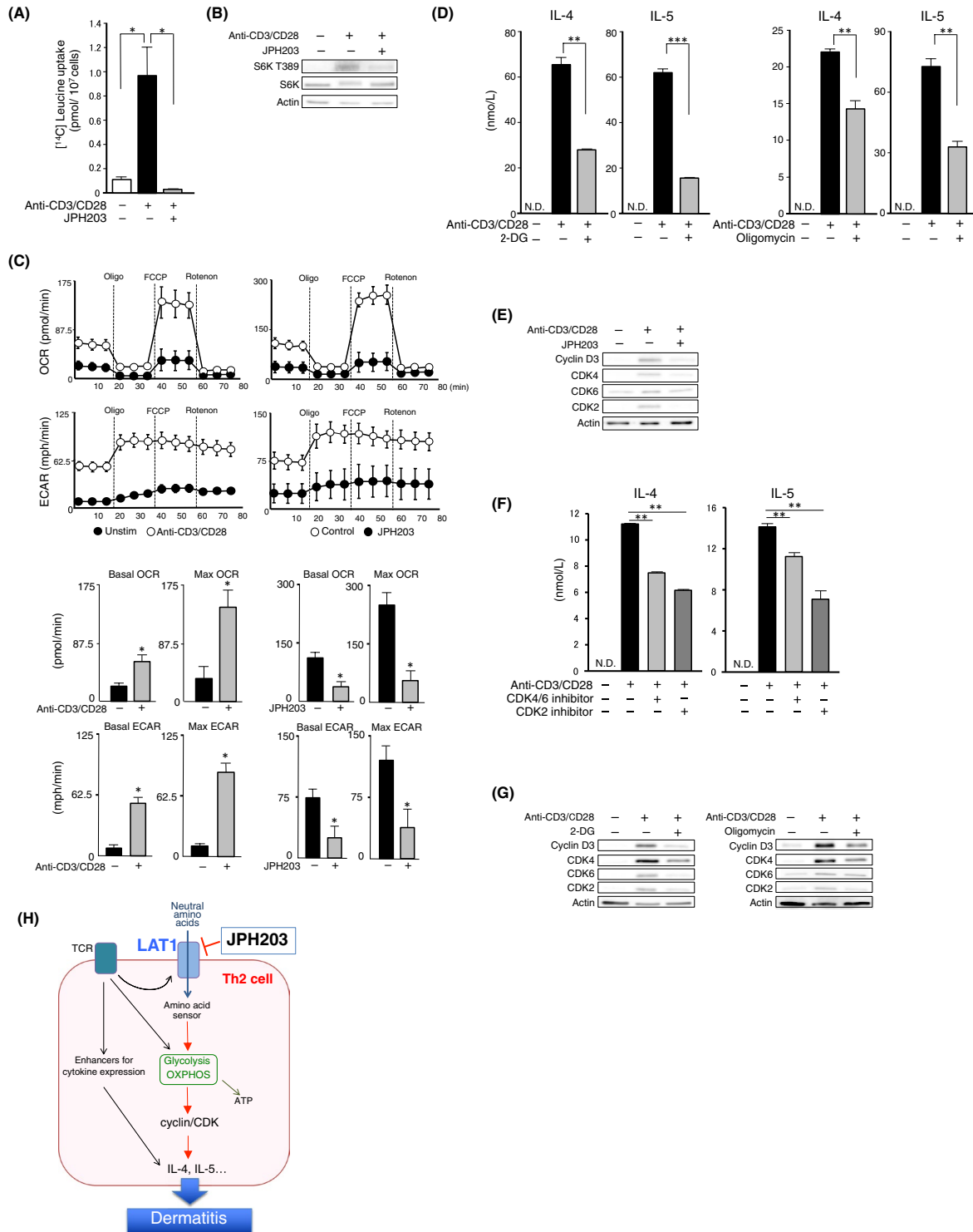


FIGURE 2 Molecular events elicited by JPH203 in Th2 cells. A, [¹⁴C]-leucine uptake with or without JPH203 for 1 min in OVA-specific Th2 cells after stimulation with anti-CD3/CD28 Abs for 24 h. B, Western blot analysis for phosphorylation of S6 Kinase (T389) in Th2 cells stimulated by anti-CD3/CD28 Abs with or without JPH203 for 24 h. C, Top, ECAR and OCR in Th2 cells stimulated as in (B). Bottom, bar graphs for basal OCR and ECAR (values before addition of oligomycin), maximum OCR (values after FCCP [carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone] addition) and maximum ECAR (values after oligomycin addition) derived from top panels. D, Cytokine concentration in the culture medium of Th2 cells stimulated in the presence of 2-DG (5 mmol/L) or oligomycin (1 μmol/L) for 24 h. E, Western blot analysis of cyclin-related proteins in Th2 cells stimulated as in (B). F, Cytokine concentration in the culture medium of Th2 cells stimulated with anti-CD3/CD28 Abs in the presence of CDKs inhibitor for 6 h. G, Western blot analysis of cyclin-related proteins in Th2 cells stimulated as in (E). H, Model of pathways regulated by LAT1 to induce T cell-mediated dermal inflammation. LAT1 inhibition alleviates T cell-dependent dermatitis by suppressing glycolysis/OXPHOS-cyclin/CDK axis in Th2 cells. Data are mean ± SEM (n = 3) for representative of at least 3 independent experiments. ND means not detected. *P < .05, **P < .01, ***P < .001

Based on the LAT1 expression pattern in AD patients, we hypothesized beneficial effects of JPH203 in an allergic dermatitis, and investigated the effect of JPH203 on a murine AD model induced by ovalbumin (OVA)-specific Th2 cells (Figure 1D).⁶ OVA-specific CD4⁺ T cells from DO11.10/Rag2(-/-) mice were developed into a Th2 lineage and re-stimulated by OVA with JPH203 (Figure 1D). JPH203 suppressed IL-4 and IL-5 production (Figure 1E) and cell proliferation (Figures 1F and S1), without causing apoptosis (Figure S1), suggesting that LAT1 inhibition reduces the functionality of mouse Th2 cells. We then examined the effect of JPH203 on allergic skin inflammation *in vivo* (Figure 1D). Th2 cells were adoptively transplanted into BALB/c mice, which were subcutaneously administered OVA into the ear. These mice developed prominent ear swelling, an indicator of dermal inflammation (Figure 1G), accompanied by eosinophil accumulation (Figure 1H); however, these responses were suppressed by JPH203 administration (Figure 1G,H). OVA-induced increases in IL-4 and IL-5 in the inflamed tissues were impaired by JPH203 (Figure 1I). OVA administration substantially increased antigen-specific serum IgE, but this was reduced by JPH203 (Figure 1J); antigen-specific IgG levels were not significantly affected by JPH203 (Figure 1J). In another AD model, in which mice were systemically immunized with OVA, antigen-induced ear swelling and inflammatory cell infiltration were also suppressed by JPH203 (Figure S2). These results suggest that LAT1 is critical for Th2 cell-mediated dermal inflammation.

To elucidate the mechanisms of attenuation of Th2 cell activation and the resultant allergic inflammation induced by LAT1 inhibition, we investigated the molecular events altered by JPH203 following T-cell receptor (TCR)/co-stimulation in Th2 cells. We first confirmed the inhibitory effect of JPH203 on amino acid incorporation, indicated by decreases in leucine uptake (Figure 2A) and phosphorylation of S6K (Figure 2B), a representative substrate of mTOR, which is a major sensor of amino acid sufficiency.

Oxidative phosphorylation (OXPHOS) and glycolysis are crucial for delivering energy for T-cell activation.⁷ Consistently, stimulated Th2 cells showed increased OXPHOS and glycolysis, which were reflected in basal level of oxygen consumption rates (OCR) and extracellular acidification rates (ECAR), respectively (Figure 2C). JPH203 decreased both parameters (Figure 2C), suggesting impairment of TCR-mediated rewiring of cellular metabolism. The maximum mitochondrial respiration (OCR after adding carbonyl cyanide-p-trifluoromethoxyphenylhydrazone [FCCP], a direct stimulator of respiratory chain complexes) in JPH203-treated Th2 cells did not reach the control level (Figure 2C), indicating the compromised mitochondrial functions. LAT1 substrate depletion also impaired OXPHOS and glycolysis (Figure S3), implying that the JPH203-mediated down-regulation of OXPHOS and glycolysis is caused by amino acid starvation. We verified the significance of glycolysis and OXPHOS in Th2 cell activation, by impairing IL-4 and IL-5 production using 2-deoxy-D-glucose (2-DG), a glycolysis inhibitor, and oligomycin, a mitochondrial ATP synthase inhibitor (Figure 2D). Thus, TCR-mediated glycolysis and OXPHOS restoration, achieved under normal LAT1 conditions, are necessary for full functionality of Th2 cells.

Since LAT1 was crucial for T cell proliferation (Figure 1F), we examined the effects of JPH203 on the expression of cyclins and cyclin-dependent-kinases (CDKs). Stimulated Th2 cells promoted the expression of CDK2 or cyclin D3 and its targets, CDK6 and CDK4 (Figure 2E). JPH203 repressed the expression of these proteins (Figure 2E), suggesting that LAT1 inhibition prevents TCR-evoked reassembly of factors related to cell cycle progression. Further, LAT1 substrate depletion also diminished cyclin/CDK expression (Figure S4), suggesting that the JPH203-mediated down-regulation of cyclin/CDK is caused by impaired LAT1 substrate incorporation. Although cyclin/CDKs are crucial for cell cycle progression, recent studies demonstrated that cyclin/CDKs could directly regulate cytokine expression.⁸ To determine whether aberrant cyclin/CDK expression causes cytokine suppression by JPH203, we stimulated Th2 cells with CDK2 and CDK4/6 inhibitors for 6 hours to exclude the influence of cell number. Both inhibitors impaired IL-4 and IL-5 production (Figure 2F), suggesting that a JPH203-mediated decrease in cytokine production is due, in part, to down-regulation of cyclin/CDKs. Lastly, we observed that 2-DG and oligomycin repressed expression of cyclin D3, CDK4, CDK6, and CDK2 (Figure 2G). Taken together with the suppressive effect of JPH203 on the expression of these proteins (Figure 2E) and on glycolysis/OXPHOS (Figure 2C), these results suggest new roles of glycolysis and OXPHOS in regulating cyclin/CDK expression to control immune reactions in response to amino acid availability. Potential mechanisms for preventing Th2 cell activation by JPH203 are shown in Figure 2H.

In conclusion, we demonstrated that LAT1 inhibition could be a novel therapeutic approach for T cell-mediated allergies. We also identified a novel pathway that regulates immune reactions according to amino acid availability in T cells. The LAT1-glycolysis/OXPHOS-cyclin/CDK axis could provide an advantage to T cells for the adaptive tuning of cytokine production and cell proliferation in response to nutrient shortages (either glucose or amino acids). Our findings contribute to improve the understanding of overactive immune responses and could promote the development of new therapeutic approaches for patients with such complications.

ACKNOWLEDGMENTS

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CONFLICTS OF INTERESTS

The authors declare that they have no conflicts of interest.

FUNDING INFORMATION

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SUPPORTING INFORMATION

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Figure S1

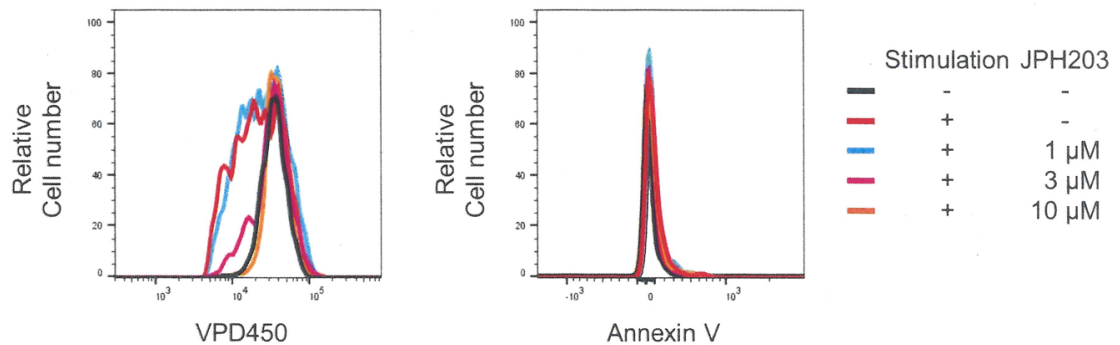


Figure S1. Effect of JPH203 on T cell proliferation and apoptosis. Th2 cells were stimulated with OVA323-339 with or without JPH203 for 4 days. The proliferation and apoptosis of CD4⁺ T cells were determined by flow cytometry with violet proliferation dye (VPD450; left panel) and Annexin V (right panel) staining, respectively. Data are representative of at least three independent experiments.

Figure S2

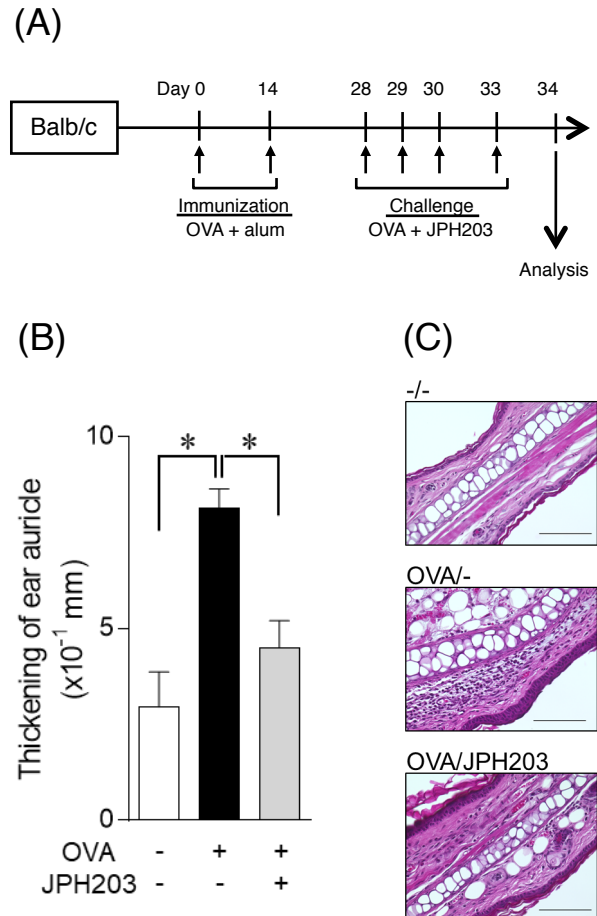


Figure S2. Effect of JPH203 on antigen-induced skin inflammation. (A) Schematic of the experimental design. (B) OVA-immunized mice were challenged by subcutaneous injection of 10 μ g OVA. JPH203 (50 mg/kg) was subcutaneously administered 30 min before each OVA injection. Twenty-four hours after the last challenge, ear thickness was measured (mean $\pm$ SEM, $n = 4$ animals). $*p < 0.05$ by one-way ANOVA with Dunnett's multiple comparison test. (C) Ear sections stained with hematoxylin-eosin (bars, 100 μ m).

Figure S3

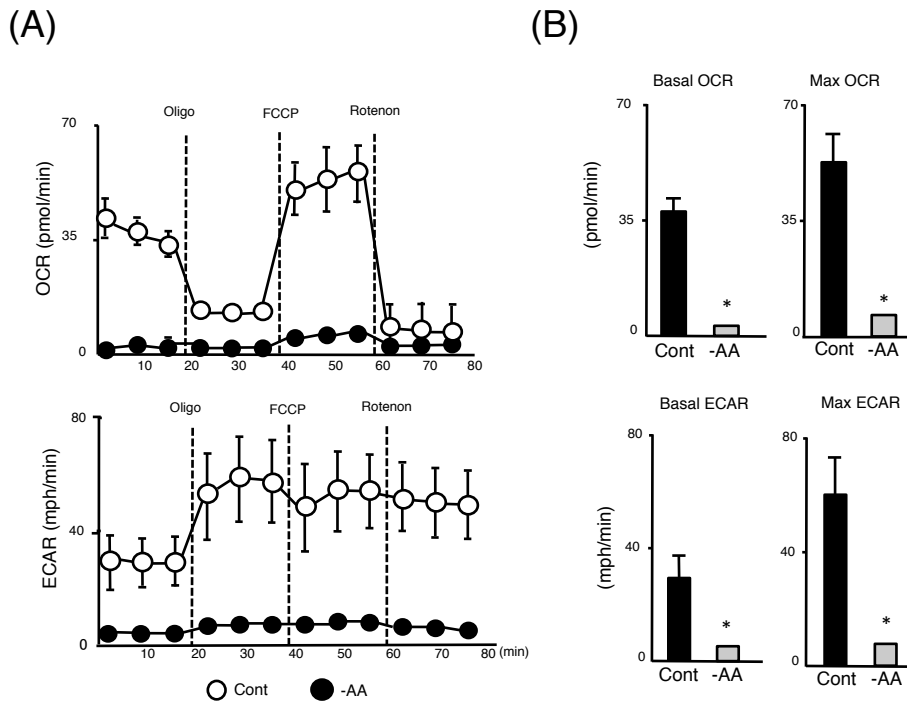


Figure S3. Influence of LAT1 substrate depletion on OXPHOS and glycolysis in Th2 cells. (A) ECAR and OCR in Th2 cells stimulated with anti-CD3/CD28 Abs for 24 hours in the complete (Cont) or LAT1 substrate-depleted (-AA) medium. (B) Bar graphs for basal and maximum OCR and ECAR derived from (A). Data are mean $\pm$ SEM ($n = 3$) representative of at least three independent experiments. * $p < 0.05$ by Student's t -test.

Figure S4

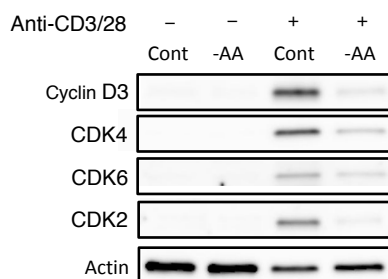


Figure S4. Influence of LAT1 substrate depletion on the expression of cyclin/CDKs in Th2 cells. Western blot analysis of cyclin-related proteins in Th2 cells stimulated with anti-CD3/CD28 Abs for 24 hours in complete (Cont) or LAT1 substrate-depleted (-AA) medium was performed. Data are representative of at least three independent experiments.

Methods

Reagents

JPH203 was produced and kindly provided by J-Pharma Co., Ltd (Yokohama, Japan).¹ CDK4/6 inhibitor (PD-0332991) was purchased from Selleck Chemicals (Houston, TX, USA). CDK2 Inhibitor III and Rotenone were purchased from Merck (Darmstadt, Germany). 2-deoxy-D-glucose (2-DG) was from Wako (Osaka, Japan). Oligomycin was from Abcam (Cambridge, UK).

Immunohistochemistry

The studies using human tissue samples were approved by the Bioethics Committee in Dokkyo Medical University and Fujita Health University. Informed written consent was obtained from each subject. Human peripheral blood mononuclear cells (PBMCs) were isolated from healthy volunteers using Histopaque centrifugation method (Sigma, St. Louis, MO, USA). CD4⁺ T cells were then purified by negative selection using Dynabeads untouched human CD4⁺ T cells kit (Invitrogen, Carlsbad, CA, USA) and activated for 48 hours by Dynabeads coated with anti-CD3/CD28 antibody (Ab) (Invitrogen) in RPMI1640 with 10% FCS. Cells were then fixed in cold methanol and subjected to the staining process. Biopsy specimens from patients with atopic dermatitis were obtained, along with specimens from donors without allergic diseases for use as normal control. Paraffin-embedded sections of skin tissues (4 µm thick) were deparaffinized and

dehydrated, and then endogenous peroxidase activity was blocked with 0.3% H₂O₂. For antigen retrieval, the slides were heated in citrate buffer using a microwave oven for 15 min (for LAT1 DAB [3-3'diaminobenzidine] staining) or in Tris-EDTA buffer at 98 °C for 40 min (for fluorescent staining and CD4 DAB staining). Anti-LAT1 Ab (J-Pharma) or anti-CD4 Ab (SP35, Roche, Basel, Switzerland) was applied to the slides at 4°C for overnight. For DAB visualization, slides were incubated with Histfine simple stain MAX-PO MALTI (Nichirei, Tokyo, Japan) and then reacted with DAB (Dojindo, Masushiro, Japan). The slides were counterstained with hematoxylin. For fluorescent staining, slides were incubated with Alexa Fluor 488 anti-mouse Ab or Alexa Fluor 568 anti-rabbit Ab and mounted with Vectorshield hardset anti-fade mounting medium with DAPI (Vector Laboratories, Burlingame, CA, USA). The slides were observed with a Zeiss LSM 780 confocal laser scanning microscope using a 20 × objective (Plan-Apochromat; 0.8 numerical aperture) and analyzed with ZEN software (Zeiss, Oberkochen, Germany).

***In vitro* Th2 differentiation**

Six-week-old female BALB/c mice were purchased from Japan SLC. DO11.10/RAG-2(-/-) mice were generated and maintained as described previously.^{2,3} Ovalbumin (OVA)-specific Th2 cells were prepared as described previously.² Briefly, naïve CD4⁺ T cells were isolated from splenocytes of DO11.10/RAG-2(-/-) mice by positive selection using CD4 microbeads and a magnetic cell sorting system (Miltenyi Biotec Bergisch, Gladbach, Germany). Then,

cells were cultured with X-ray-irradiated splenocytes in DMEM-F12/HAM medium (Merck) supplemented with 10% fetal bovine serum, 0.3 μ M synthetic OVA323-339 peptide, 10 U/ml recombinant IL-2, 10 U/ml recombinant murine IL-4 (PeproTech, Rocky Hill, NJ, USA) and 10 μ g/ml anti-IFN- γ monoclonal antibody (R4-6A2, eBioscience, San Diego, CA, USA). Seven days after stimulation, cells were harvested and used for further experiments. All animal experiments were approved by Animal care and use committee of Tokyo Metropolitan Institute of Medical Science.

Cell transfer, antigen immunization and tissue analysis in allergy model mice

Th2 cells were injected intravenously into BALB/c mice (2×10^7 cells/animal). For induction of allergic skin inflammation, mice were challenged by subcutaneous injection of 10 μ g OVA into their left ears at day 1, 2, 3, and 6 after transfer of Th2 cells. JPH203 (50 mg/kg) was administered in the subcutaneous tissue of the back immediately after Th2 cell transfer and 30 min before each OVA injection. In some experiments, BALB/c mice were immunized twice with biweekly intraperitoneal administration of 20 μ g OVA emulsified in 2.25 mg alum (Inject Alum; Thermo Fisher Scientific, Inc., Waltham, MA, USA) as described previously⁴. Two weeks after the last immunization, the mice were challenged with OVA following JPH203 administration as described above (Figure S2A). Twenty-four hours after the last challenge, ear thickness was measured with a dial thickness gauge. Then, ear sections (3 μ m thick) from the dermatitis mice were stained with hematoxylin/eosin

(HE). The number of infiltrated eosinophils were counted using a microscope. For cytokine mRNA measurement, total RNA was extracted from ear tissue. After reverse transcription using a random primer (Toyobo, Osaka, Japan) and transcriptase (Thermo Fisher Scientific, Inc.), quantitative real-time (qRT)-polymerase chain reaction (PCR) for IL-4 and IL-5 was performed using Assay-on-Demand Gene Expression Products (TaqMan MGB probes, Thermo Fisher Scientific, Inc.) with a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) as described previously.⁴ Serum levels of OVA-specific IgE and IgG were measured by enzyme-linked immune sorbent assay (ELISA) using HRP-conjugated anti-mouse IgE and IgG Abs (Serotech, Oxford, UK) as described previously.⁴

Cell proliferation assay, cytokine measurement, western blot

In vitro-differentiated Th2 cells were re-stimulated with 0.3 μ M OVA323-339 peptide plus X-ray-irradiated splenocytes for 72 hours in the presence of 10 μ M JPH203. The concentration of cytokines in the culture supernatants was measured by ELISA (BioLegend, San Diego, CA, USA), according to the manufacturer's instructions. Cell proliferation was determined by Cell Titer 96 Aqueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA). In some experiments, Th2 cells were labeled with a violet proliferation dye (VPD450; CellTrace Violet Cell Proliferation Kit) and then stimulated as described above. Four days after the stimulation, the proliferation and apoptosis of CD4⁺ T cells were analyzed by flow cytometry upon staining with anti-CD4-APC-Cy7 and

anti-Annexin V-FITC. For TCR/co-stimulation, cells were stimulated with plate-bound 2 µg/ml anti-CD3 Ab (2C11, Biolegend) and soluble 1 µg/ml anti-CD28 Ab (37.51, Biolegend) for 24 hours, unless otherwise indicated. One micro molar of PD-0332991, 5 µM of CDK2 Inhibitor III, 5 mM 2-DG, 1 µM oligomycin and 10 µM JPH203 were used for inhibiting their targets. For LAT1 substrate depletion experiments, the complete medium was made from Hanks' balanced salt solution (HBSS) with glucose (Wako), vitamin solution (Sigma), 10% FCS and amino acids (108 µM Ser, 120 µM Thr, 45.7 µM Asn, 585 µM Gln, 135 µM Pro, 207 µM Gly, 348 µM Ala, 222 µM Val, 26 µM Met, 62 µM, Ile µM, 120 µM Leu, 66 µM Tyr, 60 µM Phe, 80 µM His, 57 µM Trp, 190 µM Lys, 95 µM Arg, 50µM Glu). The medium without Leu, Val, Iso, Pro, Trp, Met, His and Phe was used as LAT1 substrate-depleted medium. For western blot, cells were lysed with 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 20 mM sodium phosphate, 5 mM sodium pyrophosphate, 1 mM Na₃VO₄, 10 mM NaF, 3 mM β-glycerophosphate and cOmplet protease inhibitor cocktail (Roche). After centrifugation (16,000 ×g) for 5 min at 4 °C and evaluation of the amount of proteins by the BCA assay, the supernatant containing 5 µg protein was used for western blotting. Electrophoresis with SDS-PAGE and immunoblotting were performed according to a standard protocol. Anti-cyclin D3 (DCS22), Anti-CDK4 (DCS156), Anti-CDK6 (DCS83), Anti-phospho S6 Kinase Thr389 (1A5) and Anti-S6K (#9202) Abs were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-CDK2 (8A12) and anti-β-Actin (6D1) Abs were from Medical and Biological

Laboratories (Nagoya, Japan).

[¹⁴C]-L-leucine uptake

Th2 cells were stimulated with anti-CD3/CD28 Abs for 24 hours as described above. Leucine uptake was initiated by incubating the 4×10^5 cells in HBSS containing 1 μ M [¹⁴C]-L-leucine (Moravek, Brea, CA, USA) with or without 10 μ M JPH203 at 37 °C for 1 min. The reaction was terminated by washing the cells 3 times with ice-cold HBSS. Cells were filtered and lysed with 0.1 N NaOH, and radioactivity was measured using an LSC-5100 β -scintillation counter (Aloka, Tokyo, Japan).

Metabolic profile

Real time analysis of extracellular acidification rates and oxygen consumption rates was performed using the Seahorse XFp analyzer (Agilent Technologies, Santa Clara, CA, USA) following the manufacturer's protocols. Briefly, Th2 cells were stimulated with anti-CD3/CD28 Abs in the presence of 10 μ M JPH203 for 24 hours, suspended in XF Base assay medium (Agilent Technologies) supplemented with 2 mM glutamine and 12 mM glucose, and seeded (5×10^5 /well) into the dedicated plates. Cells were then run on a XF analyzer to monitor ECAR and OCR. In this analysis 1 μ M oligomycin, 1 μ M carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP) included in XF Phenotype kit (Agilent Technologies), and 1 μ M rotenone were injected in order.

Statistics

Results are expressed as arithmetic mean $\pm$ SEM. Data were analyzed using the Student's *t*-test for comparisons between two groups and one-way ANOVA with Dunnett's multiple comparison test for three or more groups. $P < 0.05$ was considered indicative of statistical significance.

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