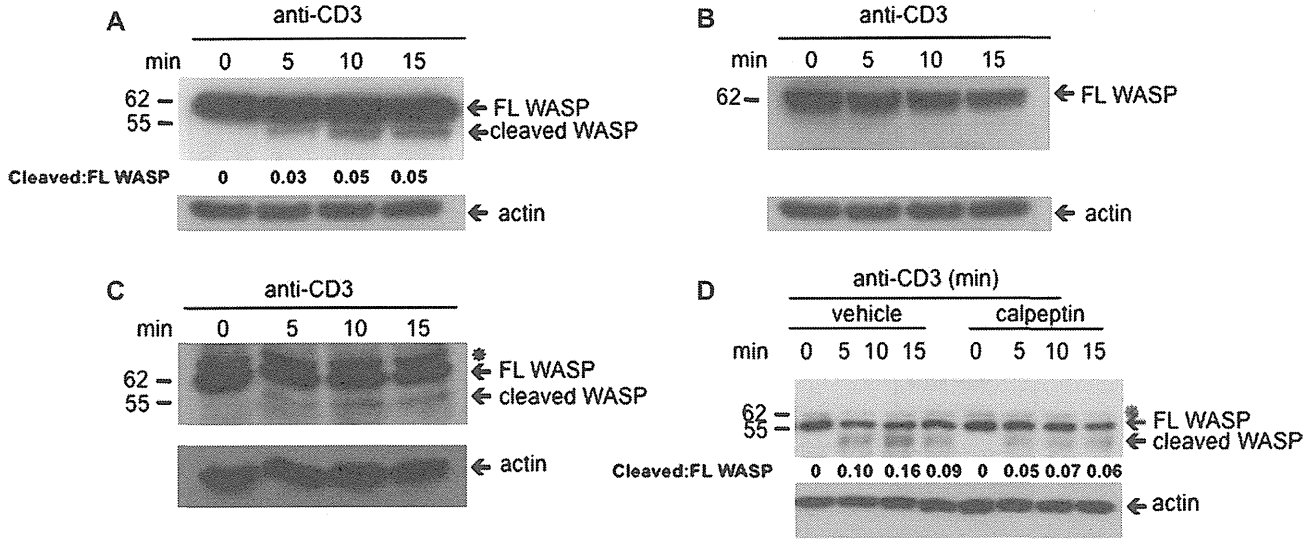

FIG 1. WASP is cleaved by calpain after TCR ligation. **A** and **B,** WASP immunoblot of peripheral blood T cells stimulated for 0 to 15 minutes with anti-CD3 mAb using mAb 5A5 (Fig 1, *A*) or polyclonal antibody K374 (Fig 1, *B*). **C,** WASP immunoblot of Jurkat T cells stimulated with anti-CD3 mAb using mAb 5A5. **D,** Effect of pretreatment for 6 hours with calpeptin on anti-CD3–driven WASP degradation in peripheral blood T cells. Lysates were immunoblotted with mAb 5A5. *Nonspecific band. The positions of molecular weight markers are indicated on the *left* in Fig 1, *A* to *D*. The ratio of cleaved WASP to full-length *(FL)* WASP in Fig 1, *A* and *D*, represents the mean of 5 experiments. Similar results were obtained in Fig 1, *A* to *D*, in 5 independent experiments.

WASP fragment at 5 minutes, which increased at 10 and 15 minutes after stimulation. Scanning densitometric analysis revealed that the intensity of the cleaved WASP band was approximately 5% that of the full-length WASP band at 10 and 15 minutes after stimulation. Similar results were obtained in Jurkat T cells (Fig 1, *C*).

Immunoblotting lysates of T cells with the rabbit polyclonal antibody K374 raised against the C-terminal 20 amino acids of WASP revealed the same 62- to 64-kDa band detected by using mAb 5A5 but did not detect the 55-kDa WASP fragment in anti-CD3–stimulated T cells that was detected by using mAb 5A5 (Fig 1, *B*). Similar results were obtained in Jurkat T cells (data not shown). This result indicates that the 55-kDa WASP fragment lacks the C-terminal VCA domains of WASP (amino acids 421-502) responsible for its actin-polymerizing activity.

Calpain cleaves WASP *in vitro*[29] and contributes to WASP degradation in WIP-deficient T cells.[16,30] To examine whether calpain was responsible for the cleavage of WASP after TCR ligation, T cells were pretreated with the calpain inhibitor calpeptin for 6 hours, washed, and stimulated with anti-CD3 mAb for 5 minutes. Preincubation with calpeptin attenuated by approximately 50% the generation of the 55-kDa WASP fragment in response to anti-CD3 stimulation (Fig 1, *D*), strongly suggesting that calpain mediates the C-terminal truncation of WASP after TCR/CD3 ligation, at least in part.

WASP is ubiquitinated and degraded by the proteasome in T cells after TCR ligation

In the absence of WIP, WASP is degraded by the ubiquitin-proteasome pathway.[16,30] To investigate whether WASP is a substrate for ubiquitination, we incubated *in vitro* transcribed and translated WASP with purified ubiquitin and ubiquitin-conjugating enzymes (mixture of E1, E2, and E3 enzymes), and the reaction mixture was immunoblotted with anti-ubiquitin mAb. WASP was polyubiquitinated in the presence of ubiquitin and ubiquitin-conjugating enzymes, as indicated by an intense high-molecular-weight smear (Fig 2, *A*). Addition of the 26S proteasome fraction to the ubiquitination mixture resulted in marked attenuation of the ubiquitinated WASP smear. These results indicate that after TCR/CD3 ligation, WASP is subject to ubiquitination, which targets it for destruction by the proteasome.

We next examined whether WASP is ubiquitinated in T cells after TCR ligation. Fig 2, *B*, shows the appearance of polyubiquitinated WASP after anti-CD3 mAb stimulation of Jurkat T cells. To examine whether WASP ubiquitinated after TCR ligation is targeted for destruction by the proteasome, Jurkat T cells were pretreated with the proteasome inhibitor MG132 for 6 hours and then stimulated with anti-CD3 mAb for 10 minutes, and WASP immunoprecipitates were prepared from their lysates and probed for ubiquitin. Fig 2, *C*, shows that ubiquitinated WASP was weakly detectable in unstimulated Jurkat cells, but its levels increased after TCR/CD3 stimulation. Pretreatment with MG132 modestly increased the amounts of ubiquitinated WASP in unstimulated Jurkat cells and strongly increased the amounts of ubiquitinated WASP detected after TCR/CD3 ligation. These results indicate that WASP is ubiquitinated and degraded by the proteasome after TCR ligation.

The Cbl family proteins c-Cbl and Cbl-b associate with WASP after TCR ligation and act as E3 ubiquitin ligases for WASP

Members of the Cbl family of E3 ubiquitin ligases are negative regulators in TCR signaling.[31,32] We tested the hypothesis that Cbl proteins might be involved in WASP ubiquitination. We first

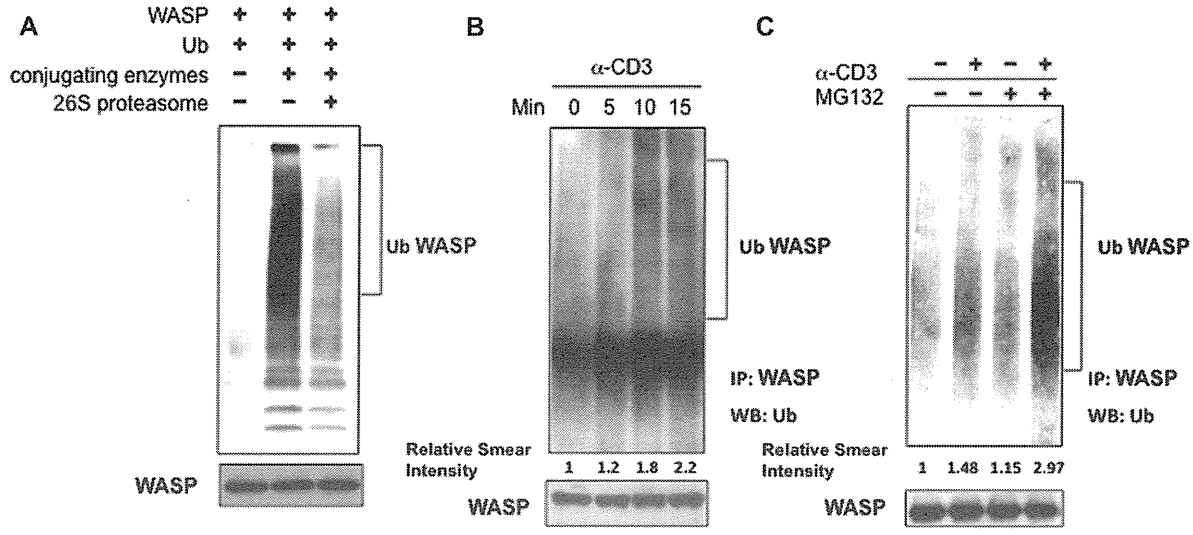

FIG 2. WASP is ubiquitinated and degraded by the 26S proteasome *in vitro* and in anti-CD3–stimulated Jurkat cells. **A,** Ubiquitination of *in vitro* translated purified WASP by ubiquitin-conjugating enzymes and its degradation by the 26S proteasome. Reaction mixtures were probed with anti-ubiquitin. **B,** Generation of ubiquitinated WASP in Jurkat T cells after stimulation with anti-CD3 mAb. WASP immunoprecipitates were probed with anti-ubiquitin mAb. Polyubiquitinated WASP appears as a smear. **C,** Protection of ubiquitinated WASP from degradation by the proteasome inhibitor MG132 in anti-CD3–stimulated Jurkat T cells. Similar results were obtained in Fig 2, *A* to *C,* in 4 independent experiments. The relative smear intensity in Fig 2, *B* and *C,* represents the mean of 4 experiments. *IP,* Immunoprecipitate; *Ub,* ubiquitin; *WB,* Western blot.

investigated whether Cbl proteins and WASP form a complex. WASP immunoprecipitates from Jurkat cell lysates were probed for c-Cbl and Cbl-b. c-Cbl, but not Cbl-b, coprecipitated weakly with WASP in unstimulated Jurkat T cells. TCR ligation increased the association of c-Cbl with WASP. It also induced the association of Cbl-b with WASP at 10 and 15 minutes after stimulation (Fig 3, *A*).

To investigate whether Cbl proteins act as E3 ubiquitin ligases for WASP, we transiently transfected 293T cells with plasmids coding for WT WASP, HA-tagged ubiquitin, FLAG-tagged c-Cbl, or FLAG-tagged Cbl-b. WASP coprecipitated with both c-Cbl and Cbl-b and was polyubiquitinated significantly more when co-transfected with ubiquitin, c-Cbl, and Cbl-b than with ubiquitin and empty vector (Fig 3, *B*).

To examine the role of Cbl-b in WASP ubiquitination after TCR ligation, we used purified T cells from spleens of *Cbl-b*$^{-/-}$ mice. Ubiquitination of WASP after TCR ligation was reduced, although not completely abrogated, in T cells from *Cbl-b*$^{-/-}$ mice (Fig 3, *C*), suggesting that WASP is a substrate for Cbl-b in antigen-stimulated T cells. We could not examine the role of c-Cbl on WASP ubiquitination after TCR ligation because we had no access to T cells from *c-Cbl*$^{-/-}$ mice.

WASP degradation after TCR/CD3 ligation limits TCR/CD3-driven F-actin assembly in T cells

WASP is important for F-actin assembly in T cells.[10] We examined whether WASP degradation after TCR/CD3 ligation regulates TCR/CD3-driven F-actin assembly. Purified T cells from WT and WASP-deficient mice were incubated for 6 hours with calpeptin or left untreated, washed and stimulated with anti-CD3 mAb, and cross-linked with a secondary antibody. The cells were then fixed, permeabilized, stained for F-actin with fluorescein isothiocyanate–conjugated phalloidin, and analyzed by means of flow cytometry. As previously reported, WASP-deficient T cells had a lower F-actin content than WT T cells.[16] TCR/CD3 ligation caused a parallel increase in F-actin levels in both WT and WASP-deficient T cells, which peaked at 5 minutes after stimulation and returned almost to baseline 10 minutes after stimulation. Pretreatment with calpeptin had no effect on F-actin content of the T cells at baseline or at 2 and 5 minutes after stimulation; however, it significantly increased the F-actin content of WT T cells at 10 minutes after anti-CD3 stimulation, maintaining it at almost the peak level achieved at 5 minutes after stimulation. In contrast, pretreatment with calpeptin had no effect on the F-actin content of WASP-deficient T cells 10 minutes after anti-CD3 stimulation. These results suggest that calpain-mediated WASP degradation limits the duration of F-actin assembly after TCR/CD3 ligation.

We next examined whether ubiquitination, which targets WASP for proteosomal degradation, regulates F-actin assembly after TCR/CD3 ligation. Because Cbl-b participates in WASP ubiquitination, we examined F-actin assembly in T cells deficient in Cbl-b. Baseline F-actin content and TCR-driven F-actin assembly were both significantly increased in T cells from c-Cbl-deficient mice compared with T cells from WT control animals (Fig 4, *B*). These results suggest that WASP degradation by ubiquitination regulates baseline and TCR-driven F-actin assembly.

DISCUSSION

Our results demonstrate that TCR ligation triggers the degradation of WASP by calpain-mediated cleavage and Cbl-mediated ubiquitination and subsequent proteasomal degradation. We present evidence that WASP degradation provides a mechanism for limiting the duration of TCR-driven assembly of F-actin.

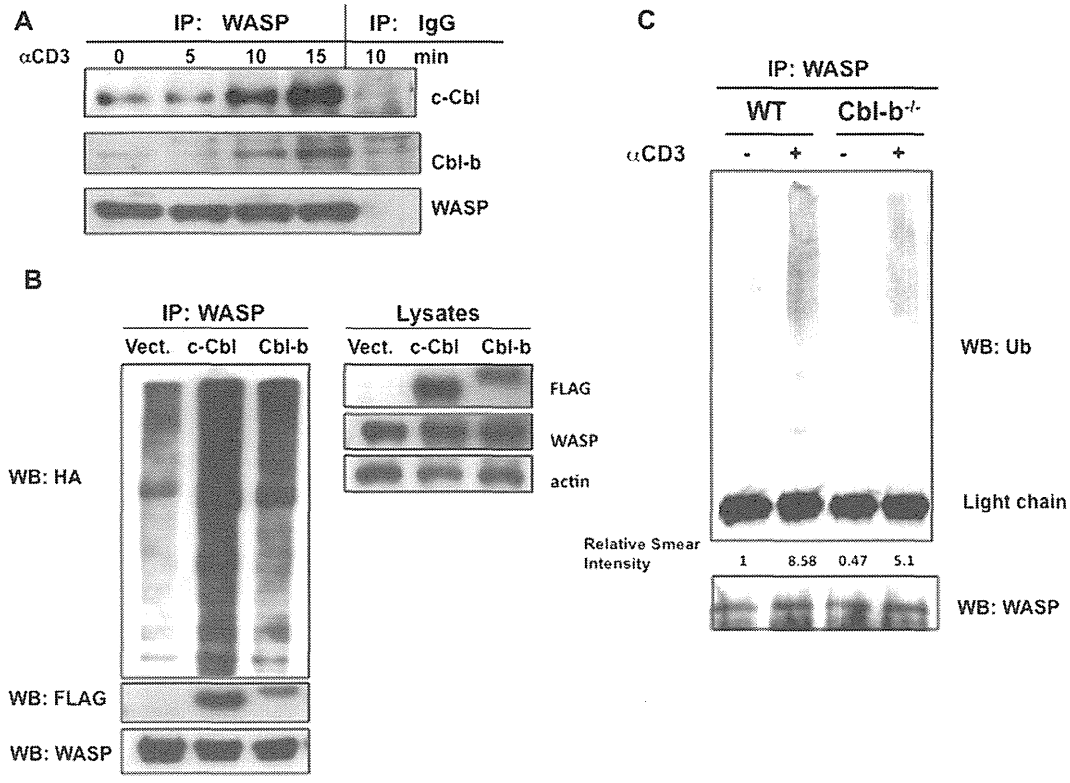

FIG 3. Cbl family E3 ubiquitin ligases associate with and ubiquitinate WASP after TCR ligation. **A,** Western blot analysis of WASP immunoprecipitates from anti-CD3–stimulated Jurkat T cells for c-Cbl and Cbl-b. IgG control antibody precipitates were prepared 10 minutes after anti-CD3 stimulation and used as controls. **B,** Ubiquitination of WASP in 293T cells transfected with WT WASP and HA-tagged ubiquitin plus either FLAG vector alone *(Vect.)*, FLAG-tagged c-Cbl, or FLAG-tagged Cbl-b. In the *left panel* WASP immunoprecipitates were probed for HA, FLAG, and WASP. In the *right panel* total lysates were probed for FLAG–c-Cbl or FLAG–Cbl-b, WASP, and actin. **C,** WASP ubiquitination after TCR ligation in T cells from *Cbl-b*$^{-/-}$ mice and WT control animals. WASP immunoprecipitates were probed for ubiquitin. Similar results were obtained in Fig 3, *A* to *C*, in 4 independent experiments. The relative smear intensity in Fig 3, *C*, represents the mean of 4 experiments. *IP*, Immunoprecipitate; *Ub*, ubiquitin; *WB*, Western blot.

TCR/CD3 ligation resulted in the degradation of a small fraction of WASP through calpain-mediated cleavage and the ubiquitin proteasome pathway. We estimated that approximately 5% of WASP is degraded after TCR/CD3 ligation. This is possibly an underestimate because the truncated 55-kDa WASP might be less stable than intact WASP. We could not detect a decrease in the levels of intact WASP in anti-CD3–activated T cells, probably because Western blotting is not sensitive enough to detect a small decrease in protein levels. We were unable to detect the cleaved, C-terminal, approximately 10-kDa fragment using an antibody to the C-terminus of WASP. This is most likely because such a small cleaved fragment would be rapidly degraded in the cell. Normally, WASP is protected from degradation by its partner, WIP.[16] The conformational changes in WASP induced by TCR signaling, which involve a change from an inactive to an active form capable of activating the Arp2/3 complex and F-actin polymerization, possibly increases the susceptibility of WASP to calpain cleavage and to ubiquitination and proteasomal degradation. The observation that WASP is degraded by calpain after TCR ligation is consistent with previous observations that WASP can be degraded in platelet lysates by calpain[29] and that *in vitro* translated WASP is a substrate for calpain I and II.[16] The increase in intracellular Ca^{++} concentration that follows TCR ligation could be the trigger for the Ca^{++}-dependent activation of calpain in anti-CD3–stimulated T cells.

Both c-Cbl and Cbl-b associated with WASP when overexpressed in 293T cells and acted as E3 ubiquitin ligases for WASP ubiquitination *in vitro*. More importantly, WASP ubiquitination after TCR ligation was impaired in Cbl-b-deficient T cells, implicating at least Cbl-b in WASP ubiquitination in T cells. Cbl family proteins act as negative regulators of TCR signaling by virtue of their ability to ubiquitinate LCK and ZAP-70,[33] which are upstream of WASP. Thus Cbl family members might regulate WASP activity indirectly by dampening TCR signaling upstream of WASP, as well as directly by ubiquitinating WASP and targeting it for degradation. Evidence has been presented that the activated WASP phosphorylated at Y291 is a target for ubiquitination.[34] We have also found that inhibition of the proteasome by MG132 increases the amount of tyrosine-phosphorylated WASP in anti-CD3–stimulated cells (see Fig E1 in this article's Online Repository at www.jacionline.org). This observation lends further support to the notion that activated WASP molecules are targets for degradation after TCR ligation.

It is not clear whether the interaction between WASP and c-Cbl and Cbl-b is direct or mediated by other proteins. It has been reported that c-Cbl associates with multiple proteins, which

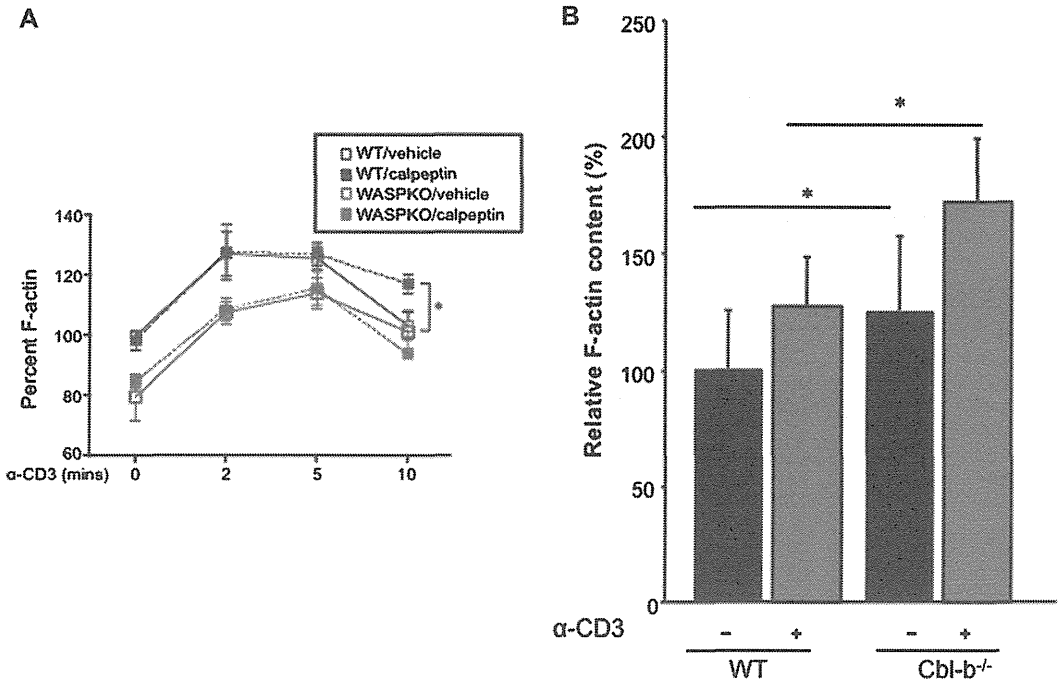

FIG 4. Effect of calpain inhibition and loss of Cbl on F-actin assembly in T cells. **A,** Effect of pretreatment of T cells from WT and WASP knockout mice with calpeptin on TCR-driven assembly of F-actin. Pretreatment with the vehicle dimethyl sulfoxide was used as a control. **B,** TCR-driven assembly of F-actin in T cells from *Cbl-b*$^{-/-}$ mice and WT control animals. T cells were stimulated with anti-CD3 for 10 minutes. Results are expressed as a percentage of the baseline F-actin content in unstimulated WT T cells and represent the means ± SDs of 3 independent experiments. *$P < .01$.

include tyrosine phosphorylated ZAP-70,[35] the adaptor proteins Nck[36] and Grb2[37] through their SH3 domains, CrkL through its SH2 domain,[38] Src tyrosine kinases through their SH3 domains,[39,40] and Vav and the p85 regulatory subunit of phosphatidylinositol-3-OH kinase through their SH2 domains.[41,42] Because these proteins are also reported to associate with WASP, its partner WIP, or both,[17,43] an indirect association of Cbl with WASP cannot be ruled out. Alternatively, c-Cbl and Cbl-b could directly interact with an activated form of WASP, such as tyrosine-phosphorylated WASP. Indeed, while this manuscript was in preparation, it was shown that WASP phosphorylation at tyrosine 291 after TCR activation results in recruitment of Cbl-b.[34]

Our data suggest that the degraded fraction of WASP includes activated WASP. This is supported by the observation that calpain inhibition and lack of the WASP-ubiquitinating E3 ligase Cbl-b resulted in more sustained F-actin assembly in WT T cells after TCR/CD3 ligation. The small fraction of WASP that is cleaved after TCR ligation could be important for F-actin polymerization because of its location close to the TCR. Indeed, we have shown previously that a fraction of WASP translocates together with a fraction of the TCR/CD3 complex to lipid rafts.[17] It is also well known that a fraction of WASP colocalizes with TCR molecules in the immune synapse (IS).[17,44,45] Cbl family molecules, which are also recruited to the IS, where they are activated by LCK and ZAP-70,[46,47] could ubiquitinate WASP molecules recruited to the IS, targeting them for degradation. The IS is a dynamic structure that constantly undergoes protein kinase Cθ–dependent dissolution and WASP/F-actin–dependent reformation of its peripheral supramolecular activation complex.[45] Protein kinase Cθ–dependent dissolution breaks the symmetry of the IS and allows T-cell motility. WASP/F-actin–dependent reformation of the IS is important for the sustained signaling that is necessary for IL-2 production. We speculate that cycles of TCR-triggered recruitment and activation of WASP in the IS followed by local degradation of the activated WASP might be important for IS dynamics and T-cell function.

The observation that baseline F-actin content was increased in *Cbl-b*$^{-/-}$ T cells, but not in calpeptin-treated T cells, suggests that under steady-state conditions, Cbl ubiquitination and proteasome degradation, but not calpain, degrade WASP molecules in activated in T cells. The observation that calpain inhibition had no effect on F-actin assembly in WASP-deficient T cells indicates that calpain regulates F-actin assembly by targeting WASP for degradation. These results strongly suggest that degradation of activated WASP by calpain and by the ubiquitin/proteasome pathway provide an important homeostatic mechanism for terminating signaling to the cytoskeleton after TCR ligation. Furthermore, WASP mutants that are resistant to ubiquitination are associated with enhanced T-cell activation, supporting the notion that WASP degradation limits TCR activation.[34]

Protein cleavage is used by prokaryotes and eukaryotes to activate or terminate signaling. Well-documented examples include the coagulation cascade, the complement activation cascade, degradation of the nuclear factor κB inhibitor IκBα, TNF receptor–associated factor 3, Argonaute, and voltage-gated calcium-channel proteins.[48-53] Degradation of activated WASP might regulate receptor signaling to the cytoskeleton not only in T cells but also in other hematopoietic cells. Such a control mechanism would avoid the potential pathology observed in patients with mutations that cause sustained WASP activation and manifest as X-linked neutropenia.

We thank K. A. Siminovitch, H. Gu, N. Ishii, and Y. Tanaka for reagents and mice.

Key message

- **TCR signaling causes WASP to be degraded by calpain and by Cbl-family members through ubiquitination and destruction by the proteasome, limiting TCR-driven assembly of F-actin.**

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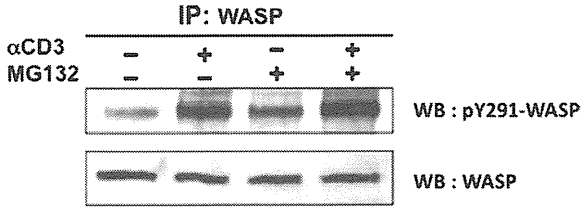

FIG E1. Tyrosine-phosphorylated WASP generated after TCR ligation is a target for proteasomal degradation. Effect of pretreatment of MG132 on the amount of tyrosine-phosphorylated WASP in anti-CD3–stimulated Jurkat T cells is shown. WASP immunoprecipitates were probed with anti-pY291-WASP antibody (Abcam). Similar results were obtained in 3 experiments. *IP*, Immunoprecipitate; *WB*, Western blot.

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Pediatrics 2014;133;e245; originally published online December 2, 2013;
DOI: 10.1542/peds.2013-0793

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KEY WORDS
abdominal pain, back pain, idiopathic stabbing headache, valproic acid

ABBREVIATIONS
ISH—idiopathic stabbing headache
VPA—valproic acid

Dr Kakisaka drafted the initial manuscript; Drs Ohara, Hino-Fukuyo, Uematsu and Kure reviewed and revised the manuscript; and all authors approved the final manuscript as submitted.

www.pediatrics.org/cgi/doi/10.1542/peds.2013-0793

doi:10.1542/peds.2013-0793

Accepted for publication Jul 8, 2013

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PEDIATRICS (ISSN Numbers: Print, 0031-4005; Online, 1098-4275).

FINANCIAL DISCLOSURE: The authors have indicated they have no financial relationships relevant to this article to disclose.

FUNDING: No external funding.

POTENTIAL CONFLICT OF INTEREST: The authors have indicated they have no potential conflicts of interest to disclose.

abstract

Idiopathic stabbing headache (ISH) is a primary headache syndrome characterized by transient, sharp, stabbing pains located in the first division of the trigeminal nerve. Reports of pediatric ISH are rare, and extracephalic pain in pediatric ISH is extremely rare. Here we report the case of a 7-year-old male patient suffering from frequent, short, stabbing headache, which was occasionally associated with abdominal and lower back pain. Various investigations were normal. He was diagnosed with ISH, and valproic acid was administered to relieve his headache and accompanying symptoms. Our case demonstrates that abdominal and lower back pain may occur in pediatric ISH. This case may provide new evidence linking ISH and migraine by showing that extracephalic symptoms accompanying ISH are similar to those of migraine. We hypothesize that the mechanism underlying the headache and abdominal and lower back pain associated with ISH may be similar to that of a migraine headache. Accumulating additional cases by asking specific questions regarding the presence of the unusual symptoms presented in our case may help to establish a detailed clinical profile of these unfamiliar and peculiar symptoms in the pediatric ISH population. *Pediatrics* 2014;133:e245–e247

Idiopathic stabbing headache (ISH) is a primary headache syndrome classified under "other primary headaches" in the *International Classification of Headache Disorders, Second Edition*.[1] The pain is characterized as a transient, sharp, stabbing pain located in the first division of the trigeminal nerve.[2] Few studies have investigated pediatric ISH.[3–6] Several peculiar symptoms, such as extracephalic pain, associated with ISH have been reported in adult cases[7] but not in the pediatric population. We report a case of a pediatric patient with ISH associated with abdominal and lower back pain.

CASE REPORT

A 7-year-old male patient presented at our hospital with severe stabbing headache in the left temporal region. Single episodes of stabbing pain several seconds in duration occurred about once a week and had begun 5 months before presentation. He was completely symptom-free between attacks. Before the initial visit, the attacks became more frequent, increasing to once or twice daily. Additionally, the headache was associated with antecedent abdominal pain in the epigastric to periumbilical region with or without bilateral lower back pain in approximately one-third of events. The extracephalic pain appeared ~20 seconds before headache onset and persisted in an intense manner, interfering with the patient's activities. He finally became incapacitated by simultaneous pain in multiple locations along with the stabbing headaches. Although he noted occasional hypersensitivity to sound during daily activity, his headache was not associated with nausea, vomiting, photophobia, osmophobia, or cranial autonomic symptoms (ie, lacrimation, conjunctival injection, eyelid edema, nasal congestion). The patient's mother had menstruation-related migraine headaches, but his history was not remarkable. The results of neurologic and physical examinations between attacks were normal. Laboratory examination and brain computed tomography results were normal. The patient was suspected to have idiopathic stabbing pain and administered valproic acid (VPA; 250 mg/day = 10 mg/kg/day) based on a previous report.[7] All symptoms resolved within a couple of days after VPA administration. He stopped the VPA after 2 weeks of medication and has been symptom free.

DISCUSSION

Our case demonstrates that abdominal and lower back pain may occur in pediatric patients with ISH. The clinical course of our case, the appearance and disappearance of headache, and the associated extracephalic pain before and after VPA administration suggest that the mechanism mediating the abdominal and lower back pain may share a common pathway with that underlying ISH. Given that migraines have been associated with cutaneous allodynia or corpalgia[8,9] and abdominal pain and that a genetic predisposition may underlie ISH,[2] we hypothesize that the mechanism underlying the pain in our case is similar to the increased responsiveness (sensitization) of central pain neurons reported in migraine headaches.[10]

Soriani et al examined the clinical profiles of 83 pediatric ISH cases. They reported that some ISH patients had (1) a history of symptoms suggesting the presence of cyclic vomiting syndrome or abdominal migraine or migraine equivalent and (2) associated symptoms such as photophobia and nausea.[3] Their findings support previous reports suggesting that patients with ISH have a genetic predisposition for migraines.[2] Our case may provide new evidence connecting ISH and migraine by showing that extracephalic symptoms accompanying ISH are similar to those of migraine.

In contrast to pediatric ISH patients like ours, only a few reports of visceral pain in adult patients with ISH are known. This may be related not only to the rarity of ISH but also to the peculiar age-dependent nature of migraine and its symptoms. In other words, pediatric patients with migraine suffer recurrent abdominal pain years before the typical migraine headache appears, although the details of this trajectory remain unclear.

We believe that accumulating cases by asking specific questions[11] regarding the presence of the unusual symptoms presented in our case may help to establish a detailed clinical profile of these unfamiliar and peculiar symptoms in the pediatric ISH population.

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Abdominal and Lower Back Pain in Pediatric Idiopathic Stabbing Headache

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Pediatrics 2014;133;e245; originally published online December 2, 2013;
DOI: 10.1542/peds.2013-0793

Pediatr Transplantation 2014: 18: E25–E30

Pediatric Transplantation
DOI: 10.1111/petr.12184

Selective expansion of donor-derived regulatory T cells after allogeneic bone marrow transplantation in a patient with IPEX syndrome

Horino S, Sasahara Y, Sato M, Niizuma H, Kumaki S, Abukawa D, Sato A, Imaizumi M, Kanegane H, Kamachi Y, Sasaki S, Terui K, Ito E, Kobayashi I, Ariga T, Tsuchiya S, Kure S. Selective expansion of donor-derived regulatory T cells after allogeneic bone marrow transplantation in a patient with IPEX syndrome.

Abstract: IPEX syndrome is a rare and fatal disorder caused by absence of regulatory T cells (Tregs) due to congenital mutations in the Forkhead box protein 3 gene. Here, we report a patient with IPEX syndrome treated with RIC followed by allogeneic BMT from an HLA-matched sibling donor. We could achieve engraftment and regimen-related toxicity was well tolerated. Although the patient was in mixed chimera and the ratio of donor cells in whole peripheral blood remained relatively low, selective and sustained expansion of Tregs determined as CD4+CD25+Foxp3+ cells was observed. Improvement in clinical symptoms was correlated with expansion of donor-derived Tregs and disappearance of anti-villin autoantibody, which was involved in the pathogenesis of gastrointestinal symptoms in IPEX syndrome. This clinical observation suggests that donor-derived Tregs have selective growth advantage in patients with IPEX syndrome even in mixed chimera after allogeneic BMT and contribute to the control of clinical symptoms caused by the defect of Tregs.

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Key words: allogeneic hematopoietic stem cell transplantation – enteropathy – Forkhead box protein 3 – immune dysregulation – polyendocrinopathy – reduced intensity conditioning – regulatory T cells – X-linked syndrome

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Accepted for publication 24 September 2013

Abbreviations: ALL, acute lymphoblastic leukemia; APC, allophycocyanin; ATG, antithymocyte globulin; BMT, bone marrow transplantation; CyA, cyclosporine A; DAB, 3, 3′-diaminobenzidine; DLI, donor leukocyte infusion; FITC, fluorescein isothiocyanate; GST, glutathione-S-transferase; GVHD, graft-vs.-host disease; HLA, human leukocyte antigen; HSCT, hematopoietic stem cell transplantation; IPEX, immune dysregulation, polyendocrinopathy, enteropathy, X-linked; IVIG, intravenous immunoglobulin; MLL, mixed lineage leukemia; PBMCs, peripheral blood mononuclear cells; PBSCT, peripheral blood stem cell transplantation; PE, phycoerythrin; PSL, prednisolone; RIC, reduced intensity conditioning; TBI, total body irradiation.

IPEX syndrome is primary immunodeficiency caused by the defects of regulatory T cells (Tregs). IPEX syndrome is often lethal in the first few months of life due to severe diarrhea associated with refractory enteropathy, infections, diabetes mellitus, dermatitis, and other autoimmune complications. This disorder is caused by mutations of Forkhead box protein 3 (*FOXP3*) gene located on chromosome Xp11.23. *FOXP3* encodes Forkhead box protein 3, which is essential for the development and maintenance of CD4+CD25+Foxp3+ Tregs (1, 2).

Established treatments for patients with IPEX syndrome include immunosuppressive therapy and allogeneic HSCT (3–6). Allogeneic HSCT serves as a curative therapy for patients with IPEX syndrome, and RIC regimens have been reported and resulted in better outcome than myeloablative conditioning regimen (7–11). In general, allogeneic HSCT with RIC regimen may increase the risk of rejection and mixed chimera. Some RIC regimens included the antibody against T lymphocytes such as alemtuzumab or ATG. However, these agents may increase the risk of viral reactivation after HSCT. To the best of our knowledge, only two cases of IPEX patients treated with allogeneic HSCT following RIC consisted of low-dose TBI instead of alemtuzumab or ATG have been reported (12).

Here, we report a patient with IPEX syndrome treated with RIC regimen consisted of fludarabine, cyclophosphamide, and low-dose TBI followed by allogeneic HSCT from an HLA-identical sibling donor. Although the patient was in mixed chimera, he was free from symptoms caused by the absence of Tregs. We could observe selective and sustained growth advantage of donor-derived Tregs and disappearance of anti-villin autoantibody in his serum, which was correlated with the improvement in refractory enteropathy.

Patient and methods

Patient

A Japanese male suffered from severe diarrhea at two months of age. He was diagnosed as IPEX syndrome by identifying a missense mutation of T1117G substitution in exon 10 of the *FOXP3* gene (13). We quantified CD4+CD25+Foxp3+ cells by flow cytometry, and positive cells were not identified at all in PBMCs. Autoantibodies examined were negative except anti-villin antibody in patient's serum. He was treated with immunosuppressive therapy of intravenous CyA and oral PSL. After complete remission was achieved, he was free from the symptom for six yr with oral low-dose CyA and PSL (14).

At the age of six, the patient suffered from severe diarrhea again and was referred to our hospital. Although he was treated with increased doses of CyA and PSL in addition to other immunosuppressive agents, these treatments were not effective enough to control his diarrhea completely. We next tried IVIG therapy, which resulted in the improvement in diarrhea, and we could taper immunosuppressive agents.

To control the disease without continuous immunosuppressive therapy, we considered to perform allogeneic BMT from an HLA-matched healthy sibling donor. The donor did not have the mutation in *FOPX3* gene. We used a RIC regimen consisted of 4 Gy (2 × 2 Gy) TBI (day 7), fludarabine at a dose of 30 mg/m^2 for five days (days 6 to day 2) and cyclophosphamide at a dose of 60 mg/kg for two days (days 3 and 2). Total nucleated bone marrow cells of 4.32×10^8/kg were transplanted. We selected CyA and short-term methotrexate as GVHD prophylaxis, and IVIG was continued weekly until autoimmune colitis was resolved.

Chimerism assay

Chimerism assay was performed by polymerase-chain-reaction-based assays analyzing polymorphic short tandem repeat markers (15). The chimerism was examined in each fraction of T cells, total lymphocytes, and granulocytes in bone marrow or peripheral blood. We evaluated the chimerism in bone marrow before day 100 and in peripheral blood after day 100, because we had similar results in both samples before day 100 in the patient and avoided frequent bone marrow aspiration after day 100.

Flow cytometry

PBMCs were stained with monoclonal antibodies of APC-conjugated human CD4, PE-conjugated human CD25, and FITC-conjugated human Foxp3 antibodies (BD Biosciences, San Jose, CA, USA) and analyzed by a FACSCanto II flow cytometer (BD Biosciences), as described previously (16).

Immunoblot analysis of anti-villin antibody

Anti-villin autoantibody in patient's serum was analyzed as described previously (17). Briefly, 500 ng of GST-villin recombinant protein (121 kD) was transferred to the membrane and incubated with diluted serum at 1:160. Anti-villin antibody bound to GST-villin was detected by horseradish peroxidase-conjugated antibody and DAB system.

Case report

Clinical improvement after RIC and allogeneic HSCT

The patient achieved an engraftment on day 11, and the last transfusion of platelets was on day 7 and that of red blood cells was on day 1. He was complicated with transient acute GVHD of the skin (grade I) on day 35 but this resolved without additional immunosuppressive therapy. He had no episodes of significant infection and other severe regimen-related toxicity during the course of RIC and allogeneic HSCT.

Severe and bloody diarrhea settled down on day 14 after engraftment. The patient was

consistently free from symptoms of enteropathy and any other autoimmune diseases. Laboratory findings showed improvement in hypoalbuminemia and anemia caused by severe enteropathy on day 21. Colonoscopy examination on day 60 revealed disappearance of mucosal inflammation, multiple ulcerations and hemorrhage that were observed before the HSCT.

After the discharge on day 120, we had followed the patient every two wk. He had no episodes of autoimmune disorders and infection, and we could taper and stop immunosuppressive agents at six months. Unfortunately, he suffered from MLL gene-rearranged ALL at 24 months after transplantation. The origin of precursor B lymphoblasts was recipient cells. We treated him with chemotherapy and allogeneic PBSCT from the same donor. We used myeloablative conditioning regimen consisted of busulfan at a dose of 4 mg/kg for four days and melphalan at a dose of 90 mg/m^2 for two days for the second transplant from the same sibling donor to cure this secondary ALL. He has been in complete remission for more than two yr. Chimerism completely changed to donor-type and the number of Tregs increased to normal after the second transplant.

Chimerism and immunological evaluation after first allogeneic HSCT

Because the ratio of donor T cells, total lymphocytes, and granulocytes in bone marrow was 74%, 48%, and 48%, respectively, on day 22 after HSCT, we reduced the dose of CyA immediately. The ratio of donor cells, however, was further declined to 5% on day 50, and the donor bone marrow was assumed to be rejected (Fig. 1). At that point, flow cytometric analysis of peripheral blood showed that 17.8% of PBMCs were CD4+CD25+Foxp3+ Tregs (Fig. 2a,b). This discordant result on day 50 was explained by selective expansion of donor-derived Tregs. After discontinuation of CyA on day 50, the ratio of donor T cells, total lymphocytes, and granulocytes was transiently increased up to 40% and then gradually decreased (Fig. 1). At 24 months after HSCT, donor cells were around 20% and CD4+CD25+Foxp3+ Tregs were at the range of 1.2–3.0% of PBMCs, which were comparable to healthy controls (Fig. 2a). We did not perform DLI because the ratio of donor cells was <50% and supposed that the patient was in high risk of bone marrow aplasia after repeated DLI.

The anti-villin autoantibody was detected by immunoblot analysis when the disease was active before HSCT. The antibody was under detectable levels both in clinical remission by immunosuppressive therapy before HSCT and after engraftment was achieved following HSCT even when immunosuppressive agents were not administrated (Table 1).

Discussion

The defect of Tregs in patients with IPEX syndrome causes symptoms related to

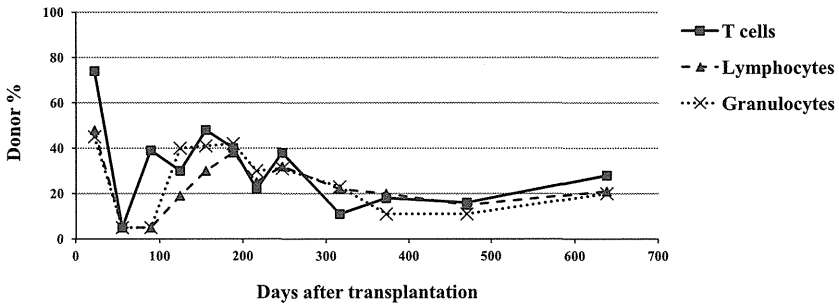

Fig. 1. Frequency of donor cells after allogeneic HSCT. Percentages of donor cells in each fraction of T cells, total lymphocytes, and granulocytes after HSCT are shown.

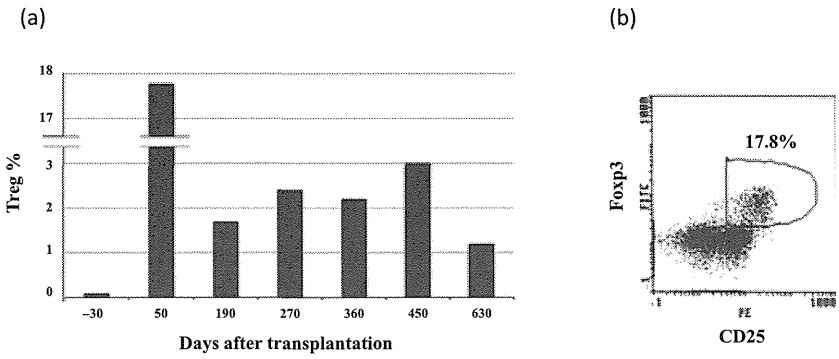

Fig. 2. Sustained expansion of donor Tregs after allogeneic HSCT. (a) Percentages of Tregs are evaluated as CD4+CD25+Foxp3+ cells in PBMCs. (b) Flow cytometric demonstration of CD25+Foxp3+ cells in CD4 gated PBMCs on day 50 after HSCT.

Table 1. Anti-villin autoantibody is correlated with clinical condition of enteropathy

Clinical condition of enteropathy	Anti-villin autoantibody
Disease onset	+
Remission before HSCT	−
Relapse before HSCT	+
After HSCT	−

GST-villin protein (121 kD) was transferred to the membrane, and immunoblot was performed with 1:160 diluted patient's serum at different disease condition of enteropathy as indicated. + indicates the presence of anti-villin antibody that recognizes GST-villin protein.

autoimmunity. However, clinical benefit of immunosuppressive therapy is often limited by its adverse effects and increased susceptibility to infection. At present, allogeneic HSCT is recognized as the curative therapy for patients with IPEX syndrome. We summarized all reported cases treated with HSCT in Table 2. Myeloablative regimen resulted in high fatality due to regimen-related toxicity or lethal infection (2, 5, 6). On the other hand, Rao et al. (7) first reported four patients who were successfully treated with non-myeloablative conditioning regimen consisted of fludarabine, melphalan, and alemtuzumab, and achieved high rate of donor chimerism above 84.6%. Non-myeloablative regimens with anti-T-lymphocyte antibody such as alemtuzumab or ATG have been used, and all patients are alive (7–11). However, it is known that alemtuzumab and ATG induce profound depletion of T cells and increase the risk of viral reactivation and fungal infection after HSCT. Therefore, we used low-dose TBI instead of anti-T-lymphocyte antibodies. The combination of low-dose TBI, fludarabine, and cyclophosphamide was well tolerated, and the patient was free from infections and severe regimen-related toxicities. Burroughs et al. (12) also reported that RIC regimen including low-dose TBI for IPEX syndrome resulted in stable engraftment of Tregs and better clinical outcome, proposing that this regimen was preferable for patients with IPEX syndrome.

The patient developed MLL-related secondary ALL in recipient cells. Although the dose of TBI was less than used in myeloablative conditioning, radiation and alkylating agents might cause DNA damage and increased the risk of secondary leukemia in recipient cells. Alternatively, the use of anti-T-lymphocyte antibody instead of low-dose TBI and/or dose reduction in alkylating agents should be carefully considered in IPEX syndrome.

Selected and sustained expansion of Tregs resulted in clinical improvement even though the patient was in mixed chimera after HSCT. Seidel et al. (11) reported a patient with IPEX

Table 2. Summary of IPEX patients treated with allogeneic HSCT reported in the literature

Case	Age	Donor	Conditioning regimen	Complications after HSCT	Outcome	% Donor after HSCT	Reference
1	13 yr	HLA-matched sibling	TBI 12 Gy + CY + ATG	Adenovirus infection, pneumonia	Dead	50%	2
2	9 yr	HLA-matched unrelated	TBI 12 Gy + CY + ATG	Cytomegalovirus infection, hemorrhagic cystitis, lymphoproliferative disorder	Dead	70%	2
3	4 months	HLA-matched sibling	BU + CY + ALG	Hemophagocytic syndrome	Dead	30% in T cell	5
4	1 yr	HLA-matched sibling	BU + CY + Flu + ATG		Alive	70% in T cell	6
5	7 yr	HLA-matched unrelated	Flu + L-PAM + alemtuzumab	Cytomegalovirus infection	Alive	100%	7
6	1 yr	HLA-matched unrelated	Flu + L-PAM + alemtuzumab	Acute respiratory distress syndrome	Alive	100%	7
7	4 yr	HLA-matched sibling	Flu + L-PAM + alemtuzumab	Histoplasma infection	Alive	89%	7
8	5 months	HLA-matched unrelated	Flu + L-PAM + alemtuzumab		Alive	84.6%	7
9	7 yr	HLA 5/6-matched cord blood	Flu + BU + ATG	Lymphoproliferative disorder	Alive	81 ~ 98%	8
10	7 months	HLA-matched unrelated	Flu + L-PAM + alemtuzumab	Sepsis of *Enterobacter cloacae*	Alive	100%	9
11	5 months	HLA-matched unrelated	Flu + L-PAM + alemtuzumab + anti-CD 45 monoclonal antibody		Alive	100%	10
12	11 months	HLA-matched unrelated	Flu + L-PAM + alemtuzumab		Alive	<10%	11
13	9 months	HLA-matched unrelated	TBI 4 Gy + Flu	Bacteremia	Alive	100%	12
14	16 yr	HLA-matched related	TBI 4 Gy + Flu	Bacteremia	Alive	20 ~ 60% in T cell	12

Case series transplanted with RIC regimens were highlighted.
CY, cyclophosphamide; ALG, antilymphocyte globulin; BU, busulfan; Flu, fludarabine; L-PAM, melphalan.

syndrome who showed selective engraftment of Tregs for six yr after non-myeloablative transplantation. It has been reported that partial BMT or injection of T-enriched splenocytes resulted in the rescue of autoimmunity in Scurfy mice, a mouse model for IPEX syndrome in which *FOXP3* gene is naturally mutated. Sustained engraftment of relatively high frequency of CD4+CD25+Foxp3+ Tregs was observed even though the frequency of donor cells in whole peripheral blood ranged from 1.7% to 50% (18). These observations illustrate that the paradigm in the generation of Tregs is reinforced by the requirement and growth advantage regardless of chimerism of other hematopoietic cells in IPEX syndrome. However, we should still consider the possibility that mixed chimerism may result in subsequent development of autoimmune diseases observed in other primary immunodeficiency, as previously reported in some patients with Wiskott–Aldrich syndrome (19).

Intractable diarrhea is a major symptom in patients with IPEX syndrome. Villin, an actin-binding protein, is expressed as the 95 kD antigen in the small intestine, which is frequently targeted by autoantibodies in patients with IPEX syndrome (17). Anti-villin antibody was clearly correlated with the severity of clinical symptoms in our patient. Therefore, monitoring of anti-villin antibody might serve as a useful examination for evaluating gastrointestinal complications in patients with IPEX syndrome.

We reported here a unique phenomenon of selective growth advantage of Tregs in a patient with IPEX syndrome who was in mixed chimera after RIC and allogeneic HSCT. Sustained expansion of donor-derived Tregs resulted in the significant improvement in enteropathy. To determine optimal RIC regimen to achieve complete chimera and avoid secondary malignancy in residual recipient cells, further analysis in more patients and long-term follow-up study after HSCT are required to conclude this issue.

Authors' contributions

Horino S and Sasahara Y designed the study, interpreted the data, wrote the paper, and treated the patient. Sato M, Kanegane H, Kamachi Y, and Kobayashi I performed experiments. All other authors treated the patient and collected clinical data.

Acknowledgments

We thank all of our colleagues in the Department of Pediatrics, Tohoku University, who contributed to the patient's care. This work was supported by the grants-in-aid from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (23591528 to YS), a grant for research on intractable diseases from the Ministry of Health, Labour and Welfare of Japan (to YS), grants from Japan Leukemia Research Fund and SENSHIN Medical Research Foundation (to YS).

Conflict of interest disclosure

The authors declare no conflict of interest.

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Molecular Genetics and Metabolism 109 (2013) 9–13

Contents lists available at SciVerse ScienceDirect

Molecular Genetics and Metabolism

journal homepage: www.elsevier.com/locate/ymgme

Fatigue and quality of life in citrin deficiency during adaptation and compensation stage

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ARTICLE INFO

Article history:
Received 13 December 2012
Received in revised form 30 January 2013
Accepted 30 January 2013
Available online 9 February 2013

Keywords:
Citrullinemia
Citrin
Aspartate–glutamate carrier
Fatigue
QOL
Mitochondria

ABSTRACT

Citrin-deficient children and adolescents between adult-onset type II citrullinemia and neonatal intrahepatic cholestasis by citrin deficiency do not have clear clinical features except for unusual diet of high-fat, high-protein, and low-carbohydrate food. The aims of the present study are to characterize fatigue and quality of life (QOL) in citrin-deficient patients during adaptation and compensation stage, and to define the relationship between fatigue and QOL. The study subjects were 55 citrin-deficient patients aged 1–22 years (29 males) and 54 guardians. Fatigue was evaluated by self-reports and proxy-reports of the PedsQL Multidimensional Fatigue Scale. QOL was evaluated by the PedsQL Generic Core Scales. Both scale scores were significantly lower in child self-reports ($p<0.01$ and $p<0.05$, respectively) and parent proxy-reports ($p<0.01$ and $p<0.01$, respectively) than those of healthy children. Citrin-deficient patients with scores of 50 percentile or less of healthy children constituted 67.5% of the sample for the Fatigue Scale and 68.4% for the Generic Core Scales. The PedsQL Fatigue Scale correlated with the Generic Core Scales for both the patients ($r=0.56$) and parents reports ($r=0.71$). Assessments by the patients and their parents showed moderate agreement. Parents assessed the condition of children more favorably than their children. The study identified severe fatigue and impaired QOL in citrin-deficient patients during the silent period, and that such children perceive worse fatigue and poorer QOL than those estimated by their parents. The results stress the need for active involvement of parents and medical staff in the management of citrin-deficient patients during the silent period.

1. Introduction

Citrin deficiency is caused by mutations of the SLC25A13 gene on chromosome 7q21.3, encoding a calcium-binding mitochondrial solute carrier protein [1]. Citrin functions as a liver-type calcium (Ca^{2+})-stimulated aspartate–glutamate carrier (AGC) by the electrogenic exchange of mitochondrial aspartate for cytosolic glutamate and proton [2]. AGC provides aspartate for the syntheses of urea, protein, and nucleotide, in addition to participating in gluconeogenesis from lactate and transporting cytosolic nicotinamide adenine dinucleotide (NADH) reducing equivalents into mitochondria as part of the malate–aspartate shuttle; thus, citrin (liver-type AGC) deficiency shows various symptoms [3–5]. The estimated prevalence of carriers of citrin deficiency in the Japanese population is 1 in 70, and that of patients is 1 in 17,000 [6,7].

Citrin deficiency is a recognized disorder that encompasses both adult-onset type II citrullinemia (CTLN2, OMIM 603471) and neonatal intrahepatic cholestasis by citrin deficiency (NICCD; OMIM 605814). The clinical characteristics of citrin deficiency vary with age. CTLN2 is characterized by frequent attacks of hyperammonemia, liver steatosis, and neuropsychiatric symptoms, such as disorientation,

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http://dx.doi.org/10.1016/j.ymgme.2013.01.020

delirium, mental derangement, sudden unconsciousness, and ultimately death within a few years of onset [8,9]. The onset is sudden and affected individuals vary in age at diagnosis from 11 to 79 years (usually between 20 and 40 years). Liver transplantation is remarkably effective.

NICCD causes intrahepatic cholestasis (jaundice and acholic stool), failure to thrive, hypoproteinemia, hypoglycemia, and multiple amino acidemias including citrullinemia. [10–15]. About half of the patients with NICCD in Japan were found by newborn mass screening such as hypergalactosemia, hyperphenylalaninemia, and/or hypermethioninemia [14,15]. The condition in almost all patients is self-limiting between 6 and 12 months of age. However, a few patients with this disorder develop severe hepatic dysfunction requiring liver transplantation [13,16].

Most citrin-deficient patients between NICCD and CTLN2 do not have clear symptoms except for unusual diet of low-calorie, high-fat, high-protein, and low-carbohydrate food [17]. Therefore, this stage is considered to be the silent period for adaptation and compensation to deficient metabolism.

Recently, a follow-up study of the patients with NICCD reported the appearance of symptoms and metabolic abnormalities during the silent period. A subgroup of patients showed nonspecific clinical features such as inappetence (lack of appetite), general fatigue, growth disturbance, abdominal discomfort, hypoglycemia, and hyperlipidemia. Especially, citrin deficient patients during the silent period complain of fatigue during school life and therefore are considered to have impaired quality of life (QOL). These patients also exhibit mild citrullinemia, high lactate/pyruvate rate, hypercholesterolemia, and oxidative stress state [18]. It is estimated that the patients in the silent period lack sufficient compensation for metabolism.

In the last decade, the term pediatric health-related quality of life (HRQOL) was frequently used and closely examined. HRQOL is currently recognized as an essential point of reference in clinical trials, health care settings, and school health care. The Pediatric Quality of Life Inventory (PedsQL), developed in the United States, has demonstrated satisfactory psychometric properties in both diseased and healthy children [19,20]. The PedsQL includes well-established methods of child self-reporting and parent proxy-reporting, which have been utilized in clinical trials in Japan and other countries worldwide [21]. The Japanese-language version of the PedsQL Multidimensional Fatigue Scale and PedsQL Generic Core Scales has good reliability and validity and can be useful for evaluation of Japanese children in school and health care settings [21,22]. Fatigue is a health problem that significantly impacts HRQOL, and is a frequent and ubiquitous complaint among patients with chronic disease. Therefore, the aim of any treatment or intervention in chronic diseases includes improvement of fatigue [23]. Not only in children with chronic disease, fatigue is reported to affect non-school attendance in school-age children [24,25].

The aims of the present study are to characterize fatigue and QOL in patients with citrin deficiency during the silent period, and to define the relationship between fatigue and QOL.

2. Methods

2.1. Patients

The subjects of the present study were 55 patients with citrin deficiency (age: 1–22 years, 29 males and 26 females) and 54 guardians. For patients aged 1–4 years, only proxy-reports were examined, and for patients aged 5–22 years, self-reports and proxy-reports were examined. Self-reports were provided by 40 patients (21 males and 19 females), and proxy reports by 54 guardians (45 mothers, 5 fathers, and 4 nonresponders). Three patients (5.7%) had chronic conditions, excluding citrin deficiency, which required medications or hospitalization for treatment. The diagnosis of citrin deficiency was based on gene analysis for the SLC25A13 in all patients.

The study protocol was approved by the Institutional Review Boards of Osaka City University Graduate School of Medicine and Hyogo College of Medicine. Signed consent forms were obtained from the study subjects and/or their guardians.

2.2. The PedsQL Multidimensional Fatigue Scale (Japanese-language version)

The PedsQL Multidimensional Fatigue Scale (Japanese version) was used to evaluate fatigue in citrin-deficient patients [22]. The PedsQL Multidimensional Fatigue Scale consists of 18 items forming parts of three subscales: (i) general fatigue (six items), (ii) sleep/rest fatigue (six items), and (iii) cognitive fatigue (six items). The PedsQL Multidimensional Fatigue Scale comprises parallel child self-report and parent proxy-report formats. Child self-reports are available for children aged 5–7, 8–12, and 13–18. Parent proxy-reports exist for children aged 2–4, 5–7, 8–12, and 13–18. The instructions for children aged 8–18 years and their parents (guardians) included reporting a score for each item during the past month, using a 5-point Likert scale. For the self-report for children aged 5–7 years, the recall time was shortened to a few weeks; the response choice was simplified to a 3-point scale.

Scores were calculated using a linearly transformed reversed score range from 0 to 100 (0:100, 1:75, 2:50, 3:25, 4:0). Higher scores indicate less fatigue status. Scale scores were calculated as the sum of items divided by the number of items answered; however, the scale score was not calculated when more than 50% of the items on the scale were missing.

2.3. The PedsQL Generic Core Scales (Japanese-language version)

The Japanese-language version of the PedsQL Generic Core Scales was used for evaluation of QOL of citrin-deficient patients [21]. The original PedsQL 4.0 Generic Core Scales were developed according to the definition of health given by the World Health Organization [26] and consist of 23 items classified into four subscales: (i) physical functioning (eight items), (ii) emotional functioning (five items), (iii) social functioning (five items), and (iv) school functioning (five items). Higher scores represent a better HRQOL [19].

2.4. Statistical analysis

Total and subscale scores of the PedsQL Multidimensional Fatigue Scale and the PedsQL Generic Core Scale in citrin deficient patients were expressed as mean values and compared with those in healthy controlled child provided by preliminary research [22] using the Mann–Whitney *U* test. The numbers of citrin-deficient patients were calculated for scores of less than 25 percentile, less than 50 percentile, less than 75 percentile, and more than 75 percentile of healthy controls, respectively, on PedsQL Multidimensional Fatigue Scale and the PedsQL Generic Core Scales.

Agreement between the PedsQL Fatigue Scale and Generic Core Scales was analyzed by Spearman rank correlation coefficient. According to Cohen [27], Spearman rank correlation coefficients effect sizes were considered small (0.10–0.29), moderate (0.30–0.49), and large ($\geq$0.50). Agreement between a child's self-report and a parent proxy-report was assessed with 36 to 38 pairs provided from both the citrin-deficient children and their guardians, by calculating Spearman rank correlation coefficients and intraclass correlation coefficients (ICCs). The ICC offers an index of absolute agreement given that it takes into account the ratio between subject variability and total variability [28,29]. Intraclass correlations are designated as 0.40 poor to fair agreement, 0.41–0.60 moderate agreement, 0.61–0.80 good agreement, and 0.81–1.00 excellent agreement [30].