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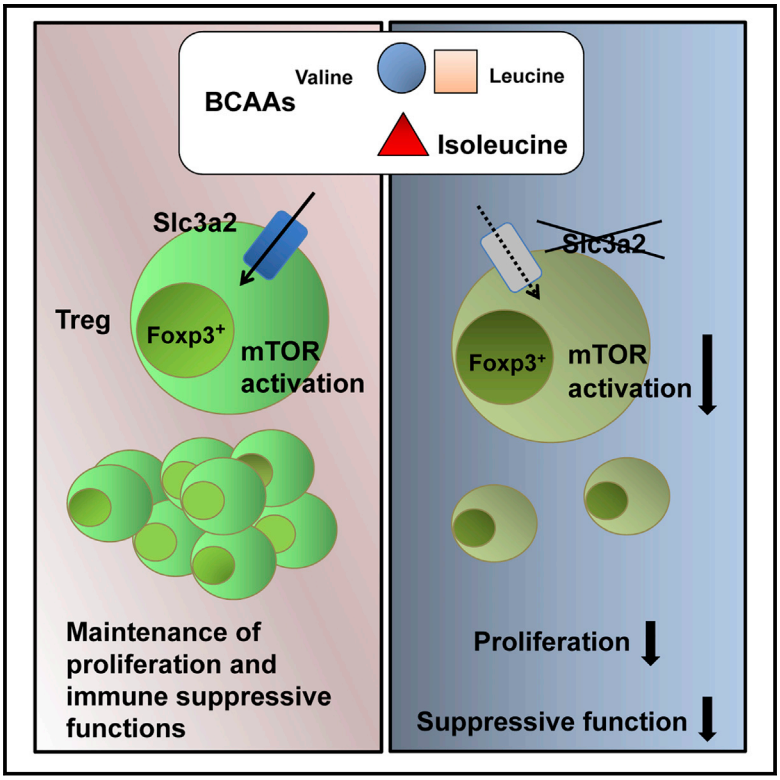
Slc3a2 Mediates Branched-Chain Amino-Acid-Dependent Maintenance of Regulatory T Cells

Graphical Abstract



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In Brief

Treg cells regulate excess immune responses and are highly proliferative *in vivo*. Ikeda et al. find that branched-chain amino acids (BCAAs) are essentially required to maintain expansion and the suppressive capacity of Treg cells via Slc3a2 and mTORC1.

Highlights

- Branched-chain amino acids are required for the *in vivo* maintenance of Treg cells
- Branched-chain amino acids activate the mTOR pathway via Slc3a2
- Slc3a2 is required for branched-chain amino-acid-dependent maintenance of Treg cells

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Slc3a2 Mediates Branched-Chain Amino-Acid-Dependent Maintenance of Regulatory T Cells

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SUMMARY

Foxp3$^+$ regulatory T (Treg) cells, which suppress immune responses, are highly proliferative *in vivo*. However, it remains unclear how the active replication of Treg cells is maintained *in vivo*. Here, we show that branched-chain amino acids (BCAAs), including isoleucine, are required for maintenance of the proliferative state of Treg cells via the amino acid transporter Slc3a2-dependent metabolic reprogramming. Mice fed BCAA-reduced diets showed decreased numbers of Foxp3$^+$ Treg cells with defective *in vivo* proliferative capacity. Mice lacking Slc3a2 specifically in Foxp3$^+$ Treg cells showed impaired *in vivo* replication and decreased numbers of Treg cells. Slc3a2-deficient Treg cells showed impaired isoleucine-induced activation of the mTORC1 pathway and an altered metabolic state. Slc3a2 mutant mice did not show an isoleucine-induced increase of Treg cells *in vivo* and exhibited multi-organ inflammation. Taken together, these findings demonstrate that BCAA controls Treg cell maintenance via Slc3a2-dependent metabolic regulation.

INTRODUCTION

Naturally occurring regulatory T (Treg) cells that express the transcription factor Foxp3 play a mandatory role in the maintenance of self-tolerance and prevention of autoimmunity (Sakaguchi, 2000; Wing and Sakaguchi, 2010). Treg cells are hyporesponsive to T cell receptor (TCR) stimulation *in vitro*. However, Treg cells are highly proliferative *in vivo* and highly express several T cell activation markers (Fisson et al., 2003; Vukmanovic-Stejic et al., 2006). The defective proliferation of Treg cells *in vivo* has been shown to correlate with autoimmune diseases in humans (Carbone et al., 2014). Thus, preservation of the *in vivo* replicative capacity of Treg cells is required for the maintenance of immune homeostasis. Interleukin-2 (IL-2) mediates the survival and proliferation of Treg cells *in vivo* (Sakaguchi et al., 2006). However, the proliferation of Treg cells is not induced by IL-2 *in vitro*, indicating the presence of unidentified environmental factors responsible for the *in vivo* maintenance of Treg cells.

Activated T cells are highly proliferative and undergo increased protein synthesis. Accordingly, activated T cells, including Treg cells, are metabolically regulated by mechanisms, including the enhanced uptake of amino acids (Newton et al., 2016). Several lines of evidence have indicated that amino acids, especially branched-chain amino acids (BCAAs), are required for maintaining the high metabolic status of activated T cells. Lymphocytes were shown to have the highest incorporation of isoleucine compared with eosinophils and neutrophils (Burns, 1975). Animals fed diets containing reduced valine, isoleucine, or leucine showed defective antibody responses or cytotoxic T cell responses (Calder, 2006; Jose and Good, 1973; Tsukishiro et al., 2000). Foxp3$^+$ Treg cells are highly activated *in vivo*; however, the requirement for amino acids to maintain Treg cells remains poorly understood.

Amino-acid-mediated cellular activation involves activation of the mammalian target of rapamycin (mTOR) pathway, which

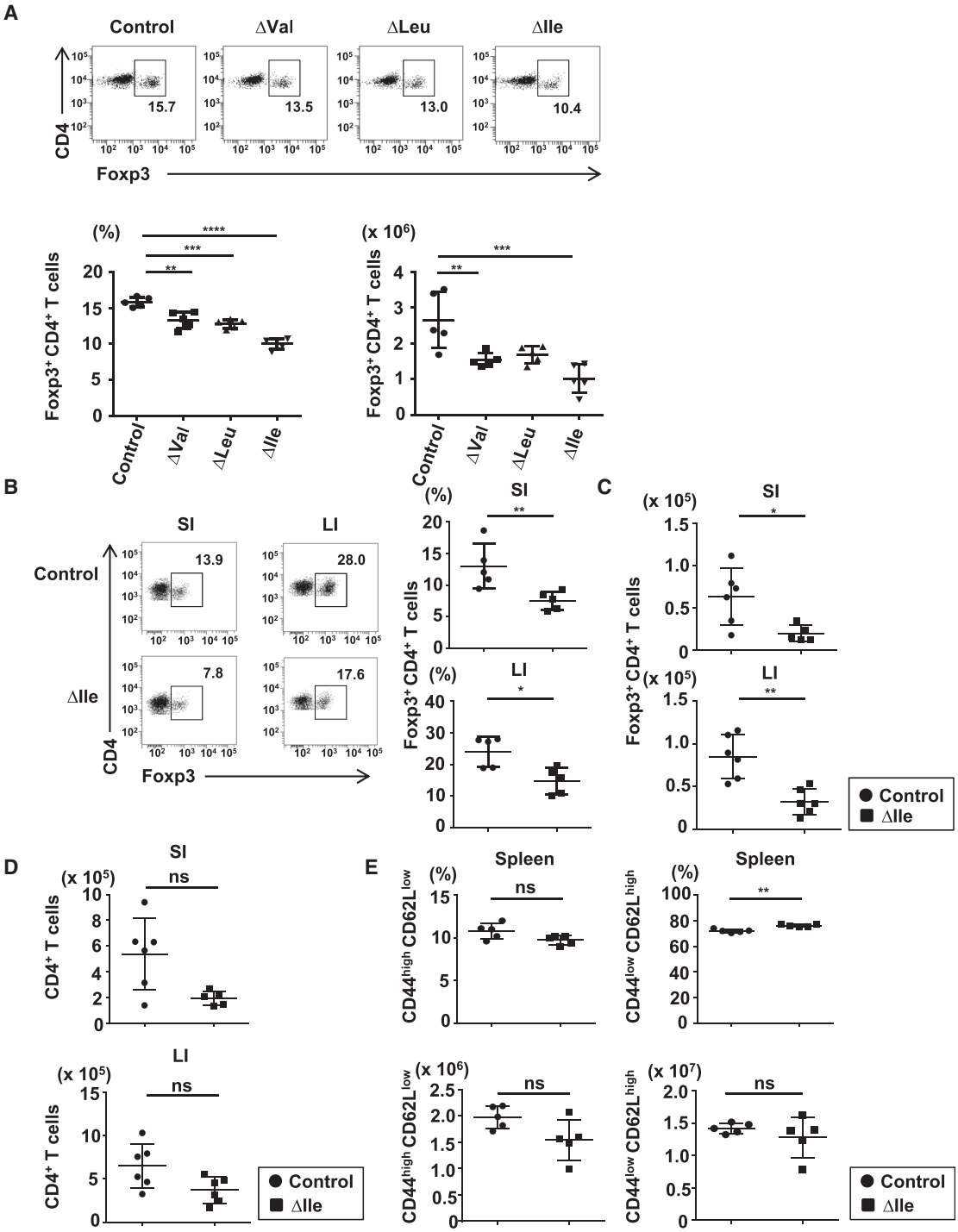

Figure 1. Decreased Number of Splenic Foxp3⁺ Treg Cells in Mice Fed a BCAA-Reduced Diet

(A) Frequency and cell number of splenic Foxp3⁺ CD4⁺ T cells in mice fed a control or the indicated BCAA-reduced diet. Representative dot plots (upper), percentage (bottom left), and total cell number (bottom right) of Foxp3⁺ CD4⁺ T cells are shown (n = 5 per group). p values were determined by Tukey-Kramer test. **p < 0.01; ***p < 0.005; ****p < 0.001.

(B) Frequency of Foxp3⁺ CD4⁺ T cells in the small intestine (SI) and the large intestine (LI) of the control or isoleucine-reduced diet groups. Representative dot plots (left) and percentage (right) of Foxp3⁺ CD4⁺ T cells are shown (n = 5 per group).

(C) Cell number of Foxp3⁺ CD4⁺ T cells in the SI and the LI of the control (n = 6) or isoleucine-reduced diet groups (n = 5 or 6) are shown.

(D) Total cell number of CD4⁺ T cells in the lamina propria of the LI (n = 6 per group) and the SI (n = 5 or 6) in BALB/c mice fed control or isoleucine-reduced diet for 2 weeks.

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mediates multiple cellular functions, including proliferation (Appuhamy et al., 2012; Laplante and Sabatini, 2012). mTOR forms two distinct complexes named mTORC1 and mTORC2, of which mTORC1 is activated by amino acids. The mTOR pathway is involved in several Treg cell functions as well as the differentiation of helper T (Th) cells (Chi, 2012; Procaccini and Matarese, 2012; Thomson et al., 2009). The transient stimulation of mTOR via the TCR and CD28 induced Foxp3 expression in T cells, but continuous stimulation led to a blockade of Foxp3 expression (Sauer et al., 2008). Similarly, the constitutive activation of mTOR by AKT inhibited Foxp3 expression in T cells (Haxhinasto et al., 2008). Control of Treg cell differentiation by mTOR was also demonstrated in mice with a T-cell-specific deletion of mTOR (Delgoffe et al., 2009). Thus, the mTOR pathway regulates Foxp3$^+$ Treg cell differentiation. The *in vivo* expansion of Treg cells has also been shown to be controlled by mTOR. Inhibition of the mTOR pathway by rapamycin resulted in the expansion of Foxp3$^+$ Treg cells in mice (Battaglia et al., 2005). A subsequent analysis of human Treg cells showed that the activation of mTOR inhibited Treg cell proliferation at early time points, but at later time points, it is required for sustained Treg cell proliferation over time (Procaccini et al., 2010). Thus, the oscillatory activity of mTOR controls the proliferation of Treg cells. Furthermore, the function and proliferation of Treg cells are controlled by mTORC1 through the promotion of biosynthesis (Zeng and Chi, 2015; Zeng et al., 2013). In addition, impaired suppressive functions of Treg cells were reported in mice with a T-cell-specific ablation of Tsc1, a key upstream regulator of mTORC1 (Park et al., 2013). Thus, multiple Treg cell functions are regulated by the mTOR pathway.

In line with the fact that the amino acid/mTOR axis regulates Th cell differentiation (Chi, 2012), transporters of amino acids, particularly Slc7a5 (also called L-type amino acid transporter 1 [Lat1]), that preferentially incorporate large neutral amino acids (LNAAs), were shown to be critical for the development of Th1 and Th17 cells (Sinclair et al., 2013). Slc7a5/Lat1 interacts with Slc3a2 (also called CD98 heavy chain [CD98hc]) to form the LNAA transporter (Kanai et al., 1998). Slc3a2/CD98hc is mandatory for the expansion of T cells as well as the differentiation of Th1 and Treg cells (Bhuyan et al., 2014; Cantor et al., 2011; Kurihara et al., 2015). However, it remains unclear whether the LNAA transporter, which mediates amino-acid-dependent activation of the mTOR pathway, regulates Treg cell proliferation and maintenance.

In this study, we analyzed the role of BCAA, particularly isoleucine, and Slc3a2 for the *in vivo* maintenance of Foxp3$^+$ Treg cells. Mice fed diets reduced in isoleucine, leucine, or valine showed reduced numbers of Foxp3$^+$ Treg cells in the periphery. The *in vivo* proliferation and suppressive ability of Treg cells were reduced in mice fed diets with reduced isoleucine. Isoleucine induced activation of the mTORC1 pathway in Foxp3$^+$, but not Foxp3$^-$ CD4$^+$ T cells. Foxp3$^+$ Treg cells expressed high levels of Slc3a2, which comprises the transporter complex that mediates isoleucine incorporation. Treg cells from mice lacking Slc3a2 specifically in Foxp3$^+$ cells showed decreased cell numbers, reduced proliferative activity, impaired isoleucine responses, and altered metabolic program. The mutant mice also showed defects in the suppressive activity of Treg cells and inflammatory changes in multiple organs. Thus, the BCAA/Slc3a2 axis is required for the maintenance of highly proliferative Treg cells in the periphery.

RESULTS

Reduced Numbers of Foxp3$^+$ Treg Cells in Mice Fed a Reduced BCAA Diet

In order to analyze the effect of BCAA for the *in vivo* maintenance of Treg cells, we fed 6-week-old BALB/c mice a diet reduced in valine (ΔVal), leucine (ΔLeu), or isoleucine (ΔIle) (10% of the standard amount) for 2 weeks. Then, we analyzed the frequency and number of Foxp3$^+$ CD4$^+$ cells as well as interferon (IFN)-γ$^+$, IL-17$^+$, or IL-10$^+$ CD4$^+$ T cells in the spleen by flow cytometry (Figures 1A and S1). The frequency and number of IFN-γ-, IL-17-, or IL-10-producing CD4$^+$ T cells were not altered in mice fed diets with reduced amounts of any BCAA. However, the frequency and number of Foxp3$^+$ CD4$^+$ T cells were decreased in mice fed diets with reduced BCAA. Especially, mice fed diet containing reduced Ile showed the most severe reduction in the number of Foxp3$^+$ CD4$^+$ T cells. Therefore, we analyzed the effect of Ile in the subsequent experiments. We analyzed the frequency and number of Foxp3$^+$ CD4$^+$ T cells and innate immune cell populations in the lamina propria of the small and large intestines (Figures 1B, 1C, and S2A). The frequency and number of Foxp3$^+$ CD4$^+$ T cells in these tissues were decreased in mice fed Ile-reduced diet, although total CD4$^+$ T cell number and innate myeloid cell populations in the intestine were not altered (Figure 1D). Because activated T cells require more amino acids than resting naive T cells, we analyzed the frequency and number of CD44^{high} CD62L^{low} effector CD4$^+$ T cells and CD44^{low} CD62L^{high} naive CD4$^+$ T cells in mice fed Ile-reduced diet (Figure 1E). The number of CD44^{high} CD62L^{low} effector CD4$^+$ T cells was not reduced in mice fed Ile-reduced diet. The number of Foxp3$^+$ CD4$^+$ T cells in thymus was not decreased in mice fed Ile-reduced diet (Figure S2B). Thus, the number of Foxp3$^+$ CD4$^+$ Treg cells in the periphery was selectively diminished in mice fed Ile-reduced food. Serum concentrations of Ile were selectively decreased among several amino acids analyzed in mice fed Ile-reduced diet, although concentrations of histidine and phenylalanine were slightly increased (Figure S3). Thus, the decreased number of Foxp3$^+$ CD4$^+$ Treg cells in the periphery correlated with the decreased serum concentration of Ile.

Defective *In Vivo* Proliferation of Foxp3$^+$ Treg Cells in Mice Fed a Reduced Isoleucine Diet

We analyzed whether Ile is required for Treg cell differentiation. Naive CD4$^+$ T cells were cultured in the presence of transforming

(E) Frequency among CD4$^+$ T cells (upper) and total cell number (bottom) of splenic CD44^{high} CD62L^{low} or CD44^{low} CD62L^{high} T cell subset in BALB/c mice fed control or isoleucine-reduced diet for 2 weeks (n = 5 per group).

In graphs, each symbol represents an individual mouse, and the means ± SD are shown by horizontal bars. p values were determined by Mann-Whitney U test. *p < 0.05; **p < 0.01. ΔIle, isoleucine-reduced diet; ΔLeu, leucine-reduced diet; ΔVal, valine-reduced diet; ns, not significant.

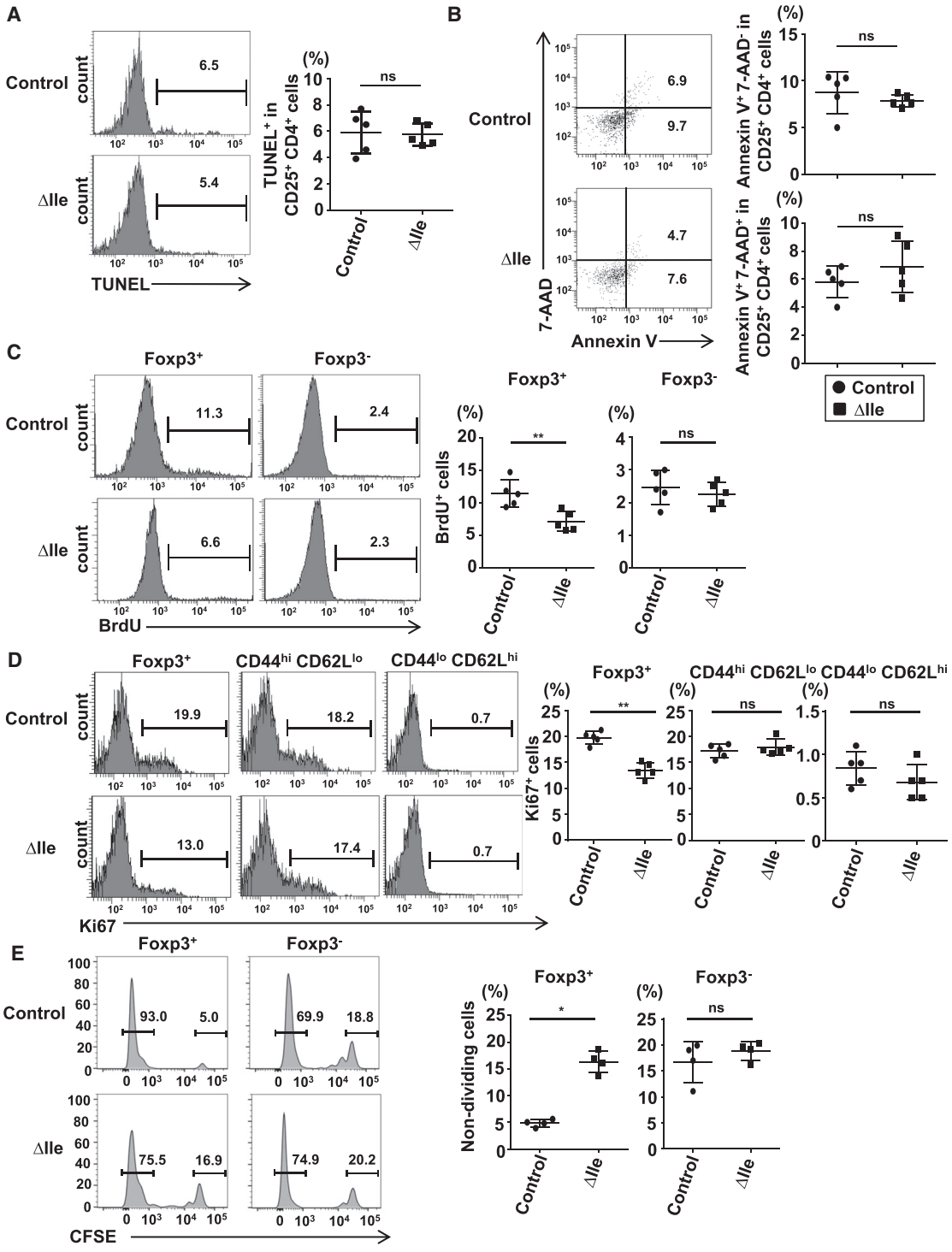

Figure 2. Impaired Proliferation of Foxp3⁺ Treg Cells by Isoleucine Insufficiency

(A) Representative histograms (left) and frequency (right) of TUNEL$^+$ cells among CD25$^+$ CD4$^+$ cells in the control or isoleucine-reduced diet groups (n = 5 per group).

(B) Representative fluorescence-activate cell sorting (FACS) dot plots (left) and frequency (right) of annexin V$^+$ 7-AAD$^-$ and annexin V$^+$ 7-AAD$^+$ cells among CD25$^+$ CD4$^+$ cells in the control or isoleucine-reduced diet groups (n = 5 per group).

(C) Representative histograms (left) and frequency (right) of BrdU$^+$ cells among the indicated cells in mice given a control or isoleucine-reduced diet (n = 5 per group).

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growth factor β (TGF-β) to induce Treg cells in media with or without Ile (Figure S4A). Foxp3$^+$ Treg cells were similarly induced in both conditions, indicating that Ile is not involved in the Treg cell differentiation.

We next analyzed whether Treg cells are prone to death in the absence of Ile. BALB/c mice were fed Ile-reduced or control diet for 2 weeks, and splenocytes were analyzed by terminal deoxynucleotidyl transferase deoxyuridine triphosphate (dUTP) nick end labeling (*TUNEL*) assay (Figure 2A). TUNEL-positive cells were not increased in the population of CD25$^+$ CD4$^+$ Treg cells from mice fed Ile-reduced diet. Splenocytes were also stained with annexin V and 7-amino-actinomycin D (7-AAD) to detect early (annexin V$^+$ 7-AAD$^-$) and late (annexin V$^+$ 7-AAD$^+$) apoptotic cells (Figure 2B). Annexin-V-positive CD25$^+$ CD4$^+$ Treg cells were not increased in mice fed Ile-reduced diet. We then isolated Foxp3$^+$ CD4$^+$ T cells and cultured them in control and Ile-free media for 6 hr. The number of annexin V$^+$ 7-AAD$^-$ and annexin V$^+$ 7-AAD$^+$ cells was not altered between control and Ile-free media (Figure S4B). Thus, the Ile insufficiency did not lead to the increased death of CD25$^+$ Treg cells. Next, we analyzed the *in vivo* proliferative capacity of Foxp3$^+$ CD4$^+$ Treg cells by measuring bromodeoxyuridine (BrdU) incorporation and Ki67 staining (Figures 2C and 2D). BrdU incorporation was markedly increased in splenic Foxp3$^+$ CD4$^+$ cells compared with Foxp3$^-$ CD4$^+$ cells, and the BrdU uptake was severely reduced in Foxp3$^+$ CD4$^+$ cells from mice fed Ile-reduced diet. The frequency of Ki67-positive Foxp3$^+$ CD4$^+$ cells was also decreased in mice fed Ile-reduced diet compared to those fed control diet. A reduction in the proliferative capacity of the Ile-reduced diet group was specific to Foxp3$^+$ CD4$^+$ cells, because the numbers of Ki67-positive cells were unaltered in the Foxp3$^-$ CD44^{high} CD62L^{low} effector CD4$^+$ T cell population and Foxp3$^-$ CD44^{low} CD62L^{high} naive CD4$^+$ T cell population. We then transferred carboxyfluorescein succinimidyl ester (CFSE)-labeled CD62L^{low} CD4$^+$ effector T cells into severe combined immunodeficiency (SCID) mice fed control or Ile-reduced diet. At 5 days after the transfer, CFSE intensity was analyzed in Foxp3$^+$ and Foxp3$^-$ populations of transferred CD62L^{low} CD4$^+$ T cells (Figure 2E). In the Foxp3$^+$ population, the percentage of reduced CFSE intensity was markedly decreased. In contrast, CFSE intensity was similar in Ile-sufficient and -reduced conditions in the Foxp3$^-$ population. Thus, during the Ile-insufficient condition, Foxp3$^+$ Treg cells showed a reduced proliferation *in vivo*, possibly causing a decrease in the number of Treg cells in the periphery.

mTOR-Dependent Maintenance of Foxp3$^+$ Treg Cell Functions

Amino acids activate the mTOR pathway (Laplante and Sabatini, 2012; Proud, 2007). In addition, the mTOR signaling pathway has been implicated in Treg cell function and development (Procaccini et al., 2010; Thomson et al., 2009; Zeng et al., 2013). Therefore, we next analyzed the Ile- or Leu-induced activation of the mTOR pathway by detecting the phosphorylation of S6 ribosomal protein (S6) and p70 S6 kinase (p70S6K), which are activated downstream of mTORC1. First, we analyzed the phosphorylation of p70S6K of splenic Foxp3$^+$ Treg cells in mice fed control or Ile-reduced diet (Figure 3A). In control-diet-fed mice, p70S6K was highly phosphorylated. However, the level of p70S6K phosphorylation was markedly decreased in mice fed Ile-reduced diet. Next, Foxp3$^+$ and Foxp3$^-$ CD4$^+$ cells were isolated from the spleen of mice fed standard diets, cultured for 12 hr in amino-acid-free media, and then stimulated with Ile or Leu for 20 min. After 12 hr of culture in the absence of amino acids, the phosphorylation level of S6 was markedly decreased. However, Ile and Leu induced the phosphorylation of S6 in Foxp3$^+$ CD4$^+$ cells, but not in Foxp3$^-$ CD4$^+$ cells (Figure 3B). The phosphorylation of p70S6K was also induced in Foxp3$^+$ CD4$^+$ cells, but not in Foxp3$^-$ CD4$^+$ cells, by Ile and Leu (Figure 3C). The majority of Foxp3$^-$ CD4$^+$ cells are resting naive T cells. Therefore, we analyzed Ile-mediated phosphorylation of S6 in Foxp3$^-$ CD44^{high} CD62L^{low} CD4$^+$ effector T cells (Figure 3D). In this experiment, cells starved in an amino-acid-free medium were stimulated with a full medium (containing essential amino acids) or an Ile-free medium (containing essential amino acids, but not Ile). The full medium induced the phosphorylation of S6 both in Foxp3$^+$ and Foxp3$^-$ effector T cells. In contrast, the Ile-free medium failed to induce the phosphorylation of S6 in Foxp3$^+$ cells but induced the phosphorylation of S6 in Foxp3$^-$ effector T cells. Thus, Ile is required for the phosphorylation of S6 in Foxp3$^+$ cells, but not in Foxp3$^-$ effector T cells. This result also indicates that mTOR activation in Foxp3$^-$ effector T cells was induced in an Ile-independent manner. The phosphorylation of S6 in Foxp3$^+$ CD4$^+$ cells was sustained after 12 hr of culture in the presence of Ile or Leu (Figure 3E). These findings indicate that Ile and Leu activate the mTOR pathway in Foxp3$^+$ Treg cells. Because mTORC1 has been shown to regulate the expression of surface molecules, such as cytotoxic T-lymphocyte antigen 4 (CTLA-4) (Zeng et al., 2013), we analyzed the expression of CTLA-4 and *glucocorticoid-induced TNF receptor-related* protein (GITR) on Foxp3$^+$ Treg cells of mice fed Ile-reduced diets (Figure 3F). The surface expressions of CTLA-4 and GITR on Foxp3$^+$ CD4$^+$ cells in the spleen were severely reduced in mice fed Ile-reduced diet compared to mice fed control diet. Because these molecules are required for Treg cell function, we analyzed the suppressive ability of CD25$^+$ CD4$^+$ cells in the spleen of mice fed Ile-reduced diet. CD25$^-$CD4$^+$ T cells were cultured with dendritic cells and anti-CD3 antibody in the presence of various numbers of

(D) Representative histograms (left) and frequency (right) of Ki67$^+$ cells among the indicated cells in mice given a control or isoleucine-reduced diet (n = 5 per group).

(E) SCID mice fed control or isoleucine-reduced diet were intraperitoneally transferred with CFSE-labeled CD62L^{low} CD4$^+$ cells from BALB/c. At 5 days after the transfer, splenic Foxp3$^+$ CD4$^+$ (Foxp3$^+$) and Foxp3$^-$ CD62L^{low} CD4$^+$ (Foxp3$^-$) cells were analyzed by flow cytometry. Representative histograms (left) and frequency (right) of non-dividing CFSE$^+$ cells among Foxp3$^+$ CD4$^+$ and Foxp3$^-$ CD62L^{low} CD4$^+$ cells in mice given control or isoleucine-reduced diet (n = 4 per group) are shown.

In graphs, each symbol represents an individual mouse, and the means ± SD are shown by horizontal bars. p values were determined by Mann-Whitney U test. *p < 0.05; **p < 0.01.

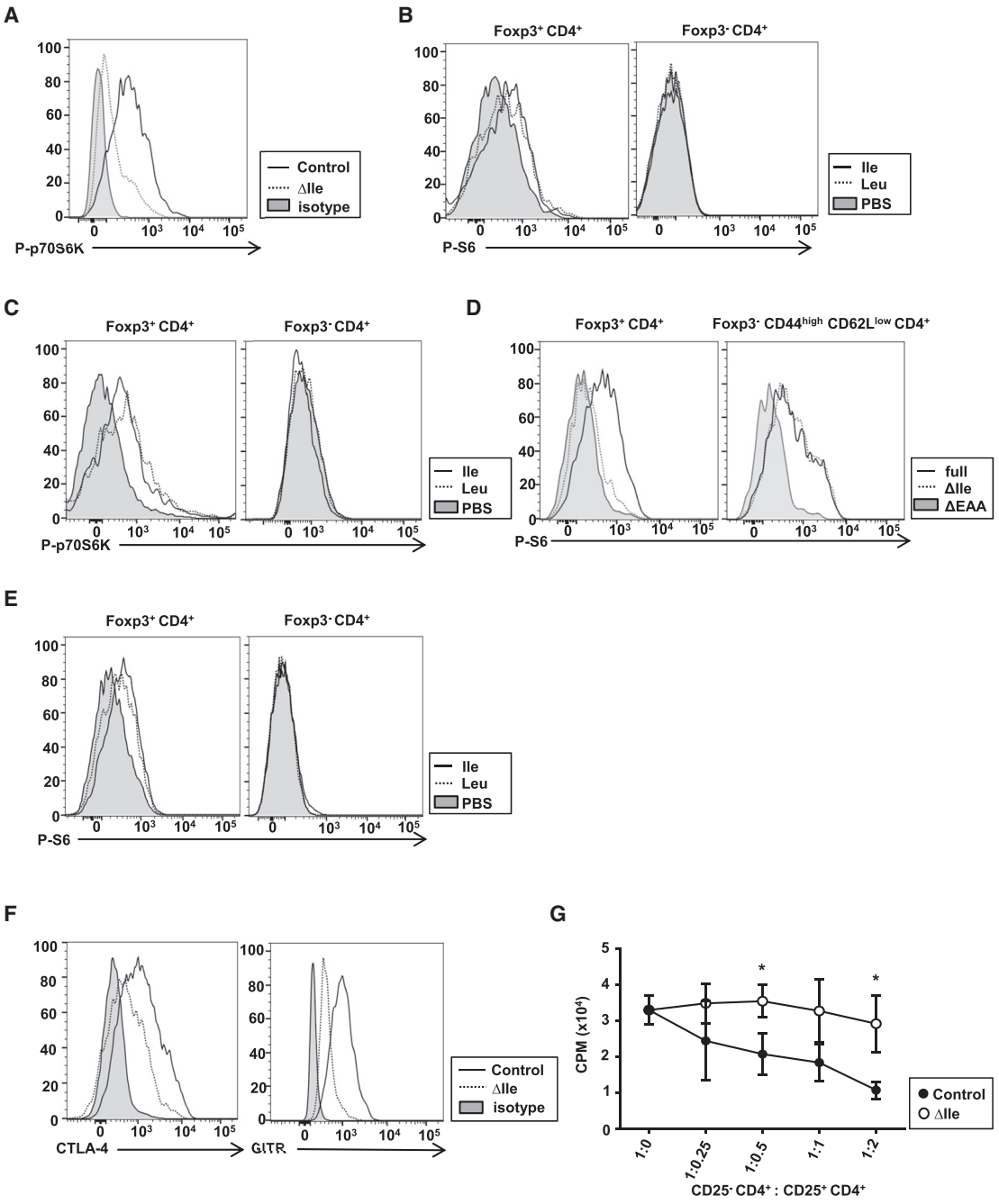

Figure 3. Isoleucine-Dependent Activation of mTOR Signaling in Foxp3$^+$ Treg Cells

(A) Phospho-p70 S6K (Thr389) of splenic Foxp3$^+$ CD4$^+$ Treg cells from BALB/c mice fed control or isoleucine-reduced diet were analyzed by flow cytometry. Gray histogram shows the isotype control.

(B and C) Foxp3$^+$ CD4$^+$ cells (left) and Foxp3$^-$ CD4$^+$ cells (right), cultured for 12 hr without amino acids, were stimulated with 5 mM isoleucine (solid lines), 5 mM leucine (dotted lines), or PBS (gray histograms) for 20 min and analyzed for phospho-S6 (P-S6) (B) and phosphor-p70S6K (P-p70S6K) (C). Data are representative of three independent experiments.

(D) Splenic Foxp3$^+$ CD4$^+$ cells (left) and Foxp3$^-$ CD44^{high} CD62L^{low} CD4$^+$ cells (right) from *Foxp3*-GFP mice were cultured without essential amino acids for 12 hr and then stimulated with full medium (solid line), isoleucine-free medium (dotted line), or essential amino acids (EAAs)-free medium (gray histogram) with 10% dialyzed fetal bovine serum (FBS), 5 μg/mL anti-CD3 antibody, and 5 μg/mL anti-CD28 antibody for 20 min. The cells were analyzed for phospho-S6 (P-S6) by flow cytometry.

(E) The cells were cultured for 12 hr in the presence of 5 mM isoleucine (solid line), 5 mM leucine (dotted line), or PBS (gray histogram) and analyzed for phosphor-S6 (P-S6). Data are representative of three independent experiments.

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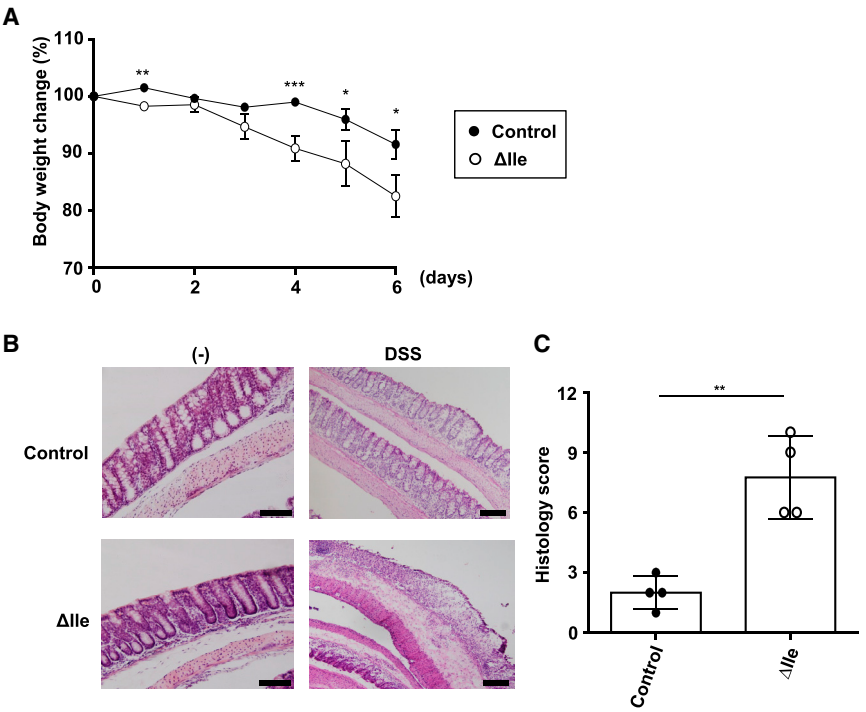

Figure 4. High Sensitivity to Intestinal Inflammation of Mice Fed an Isoleucine-Reduced Diet

(A) Body weight change (percent of initial weight) of control-diet-fed mice (closed circle; n = 17) or isoleucine-reduced-diet-fed mice (open circle; n = 16) per group. Data are the means ± SEM. p values were determined by Student's t test.

(B) H&E staining of colon sections without DSS (–) and after DSS administration on day 9. The scale bars represent 100 μm.

(C) Histological score of colonic inflammation of control-diet-fed mice (closed circle) or isoleucine-reduced-diet-fed mice (open circle; n = 4 per group) on day 9.

In graphs, each symbol represents an individual mouse and means ± SD are shown by horizontal bars. p values were determined by Student's t test. *p < 0.05; **p < 0.01; ***p < 0.005.

$CD25^+$ $CD4^+$ cells from mice fed Ile-reduced diet or control diet (Figure 3G). The suppression of T cell proliferation was impaired by the deprivation of Ile. Taken together, these findings indicate that the BCAA/mTORC1 pathway regulates the maintenance and function of $Foxp3^+$ Treg cells in the periphery.

The dysregulation of Treg cells leads to inflammatory disorders, including intestinal inflammation (Singh et al., 2001). However, after 2 weeks of feeding with Ile-reduced diet, mice did not show any obvious inflammatory changes. Therefore, we analyzed whether mice fed Ile-reduced diet were sensitive to dextran sodium sulfate (DSS)-induced intestinal inflammation. The DSS treatment of mice fed Ile-reduced diet resulted in severe intestinal inflammation characterized by pronounced weight loss and severe pathological changes of the colon compared with mice fed control diets (Figures 4A–4C). Thus, Ile insufficiency in diets resulted in high sensitivity to intestinal inflammation.

High Expression of Slc3a2 in $Foxp3^+$ Treg Cells

In order to analyze the mechanisms by which Ile and Leu control Treg cell homeostasis, we analyzed the expression of genes encoding molecules that mediate the transport of BCAAs. These included L-type amino acid transporters (Lat1–4) and system y^+ L-type amino acid transporters (y^+Lat1 and 2), Slc6a14 and Slc6a19 (Bodoy et al., 2005; Bröer et al., 2004; Ganapathy and Ganapathy, 2005; Kanai et al., 1998; Verrey et al., 2004). Because many of these molecules (Lat1 and 2 and y^+Lat1 and 2) associate with Slc3a2/CD98hc to form the transporter complex, we also analyzed the expression of *Slc3a2* (Figure 5A). The expression of several genes, including *Slc7a5* (encoding Lat1) and *Slc43a2* (encoding Lat4), was enhanced in $Foxp3^+$ $CD4^+$ cells compared to $Foxp3^-$ $CD4^+$ cells from the spleen. In particular, the expression of *Slc3a2* was markedly higher in $Foxp3^+$ $CD4^+$ cells than $Foxp3^-$ $CD4^+$ cells. We also analyzed protein expression of Slc3a2 (CD98hc) on $Foxp3^+$ $CD4^+$ cells, $Foxp3^-$ $CD44^{high}$ $CD62L^{low}$ effector $CD4^+$ T cells, and $Foxp3^-$ $CD44^{low}$ $CD62L^{high}$ naive $CD4^+$ T cells by flow cytometry (Figure 5B). Slc3a2 expression was the highest in $Foxp3^+$ $CD4^+$ cells. Thus, expression of subunits of amino acid transporters, Slc7a5, Slc43a2, and especially Slc3a2, was enhanced in $Foxp3^+$ Treg cells.

Decreased Numbers of $Foxp3^+$ Treg Cells in Treg-Cell-Specific Slc3a2-Deficient Mice

To analyze the role of Slc3a2 in Treg cells homeostasis, we generated mice lacking *Slc3a2* specifically in $Foxp3^+$ Treg cells (*Foxp3*^{cre}; *Slc3a2*$^{flox/flox}$; Figure S5A). The frequency of $Foxp3^+$ $CD4^+$ cells in the spleen of *Foxp3*^{cre}; *Slc3a2*$^{flox/flox}$ mice was markedly decreased (Figure 5C). We further analyzed the percentage and number of $Foxp3^+$ $CD44^{low}CD62L^{high}$ resting Treg cells and $Foxp3^+$ $CD44^{high}CD62L^{low}$ activated Treg cells in the

(F) Expression of CTLA-4 and GITR on splenic $Foxp3^+$ $CD4^+$ Treg cells in mice fed a control (solid line) or isoleucine-reduced diet (dotted line). The gray histogram represents the isotype control.

(G) [^{3}H]-thymidine uptake by $CD25^-$ $CD4^+$ T cells co-cultured with dendritic cells in the presence of increasing ratios of splenic $CD25^+$ $CD4^+$ cells derived from mice fed a control (closed circle) or isoleucine-reduced (open circle) diet.

Data are the means ± SD of triplicate well measurements and representative of three independent experiments. p values were determined by Student's t test. *p < 0.05. ΔEAA, essential amino-acid-free medium; ΔIle, isoleucine-free medium (D) or isoleucine-reduced diet (A, F, and G); CPM, counts per minute; Ile, isoleucine; Leu, leucine.

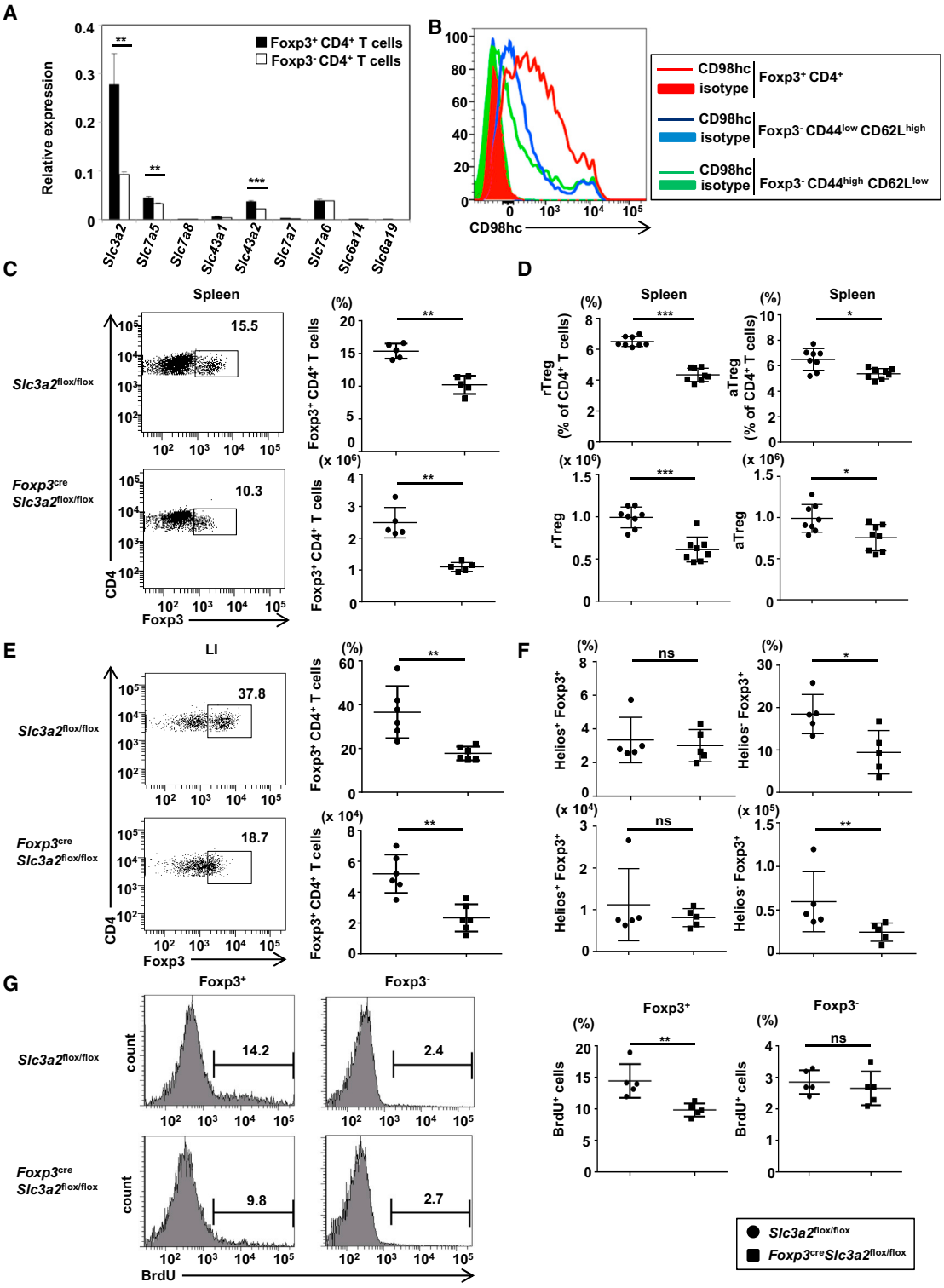

Figure 5. Decreased Number of Foxp3⁺ Treg Cells in Treg-Cell-Specific *Slc3a2*-Deficient Mice

(A) qRT-PCR analysis of the indicated amino acid transporter in splenic Foxp3$^+$ CD4$^+$ (closed bar) and Foxp3$^-$ CD4$^+$ T cells (open bar). Data are the means ± SD of triplicate samples in one experiment and representative of three independent experiments. p values were determined by Student's t test. $^{**}p < 0.01$; $^{***}p < 0.005$. (B) Expression of CD98hc on splenic Foxp3$^+$ CD4$^+$ cells (red), Foxp3$^-$ CD44^{low}CD62L^{high} cells (blue), and Foxp3$^-$ CD44^{high}CD62L^{low} cells (green) from *Foxp3*-GFP mice was analyzed by flow cytometry. CD98hc, CD98 heavy chain.

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spleen of control and *Foxp3*^{cre}; *Slc3a2*$^{flox/flox}$ mice (Figure 5D). The numbers of both activated and resting Treg cells were reduced in *Foxp3*^{cre}; *Slc3a2*$^{flox/flox}$ mice. We then analyzed the number of Foxp3$^+$ Treg cells in the large intestine (Figure 5E). The number of Foxp3$^+$ Treg cells was reduced in the large intestine of *Foxp3*^{cre}; *Slc3a2*$^{flox/flox}$ mice. In the large intestine, there exist two types of Foxp3$^+$ Treg cells: Helios$^+$ thymus-derived Treg (tTreg) cells and Helios$^-$ peripheral Treg (pTreg) cells. Therefore, we analyzed which type of Treg cells was affected by the *Slc3a2* deficiency (Figure 5F). The number of Helios$^+$ tTreg cells was not altered in the large intestine of *Foxp3*^{cre}; *Slc3a2*$^{flox/flox}$ mice. In contrast, pTreg cell number was reduced in the large intestine of *Foxp3*^{cre}; *Slc3a2*$^{flox/flox}$ mice. The number of Foxp3$^+$ CD4$^+$ T cells in thymus was not altered in *Foxp3*^{cre}; *Slc3a2*$^{flox/flox}$ mice (Figure S5B). In *Foxp3*^{cre}; *Slc3a2*$^{flox/flox}$ mice, the number of IFN-γ-, IL-17-, or IL-10-producing CD4$^+$ T cells was not altered (Figure S5C). Next, we analyzed the *in vivo* proliferative ability of Foxp3$^+$ Treg cells by measuring BrdU incorporation and found that BrdU uptake was decreased in Foxp3$^+$ CD4$^+$ cells, but not Foxp3$^-$ CD4$^+$ cells, in the spleen of *Foxp3*^{cre}; *Slc3a2*$^{flox/flox}$ mice (Figure 5G). Thus, Foxp3$^+$ Treg cells showed decreased numbers and reduced proliferation in the absence of Slc3a2. These findings suggest that Slc3a2 is required for the maintenance of Foxp3$^+$ Treg cells by supporting their *in vivo* proliferative ability.

Impaired Ile Response of Treg Cells in Treg-Cell-Specific Slc3a2-Deficient Mice

We next analyzed whether the decreased number of Foxp3$^+$ Treg cells in *Foxp3*^{cre}; *Slc3a2*$^{flox/flox}$ mice correlated with defective Ile responses. CD25$^+$ CD4$^+$ T cells, which mainly contain Foxp3$^+$ Treg cells, and CD25$^-$ CD4$^+$ T cells were isolated from the spleens of control and *Foxp3*^{cre}; *Slc3a2*$^{flox/flox}$ mice and cultured for 12 hr in amino-acid-free media. These cells were then stimulated with Ile or Leu for 20 min and analyzed for the phosphorylation of S6 (Figure 6A). The Ile- or Leu-mediated phosphorylation of S6 was not induced in CD25$^+$ CD4$^+$ T cells from *Foxp3*^{cre}; *Slc3a2*$^{flox/flox}$ mice. We next analyzed *in vivo* Ile responses by feeding control and *Foxp3*^{cre}; *Slc3a2*$^{flox/flox}$ mice Ile-reduced diets for 7 days. In control mice fed an Ile-reduced diet, the number of Foxp3$^+$ Treg cells was reduced and became comparable to that in *Foxp3*^{cre}; *Slc3a2*$^{flox/flox}$ mice fed with Ile-reduced diet. These mice were reared with an Ile-sufficient or -reduced diet for another 7 days, and the frequency of Foxp3$^+$ Treg cells in the spleen was analyzed (Figure 6B). In control mice, Ile supplementation increased the number of Foxp3$^+$ Treg cells. In sharp contrast, Ile did not increase Foxp3$^+$ Treg cells in the spleen of *Foxp3*^{cre}; *Slc3a2*$^{flox/flox}$ mice. Thus, the Ile response of Foxp3$^+$ Treg cells was abolished in the absence of Slc3a2. We also analyzed *in vivo* Leu responses (Figure S6A). Mice were fed a Leu-reduced diet for 7 days and then fed a Leu-sufficient diet or -reduced diet for another 7 days. In *Slc3a2*$^{flow/flox}$ mice, Leu supplementation increased the number of Foxp3$^+$ Treg cells in the spleen. However, Leu-mediated increase of Foxp3$^+$ Treg cells was not observed in *Foxp3*^{cre}; *Slc3a2*$^{flox/flox}$ mice, indicating that the Leu-mediated response was also dependent on Slc3a2.

Because Slc3a2 mediates the uptake of BCAAs, we measured intracellular concentration of Ile, Leu, and Val in CD25$^+$ CD4$^+$ and CD25$^-$ CD4$^+$ cells of *Slc3a2*$^{flox/flox}$ and *Foxp3*^{cre}; *Slc3a2*$^{flox/flox}$ mice (Figure 6C). Concentration of these BCAAs in CD25$^-$ CD4$^+$ cells was not altered between control and *Foxp3*^{cre}; *Slc3a2*$^{flox/flox}$ mice, rather somewhat increased in *Foxp3*^{cre}; *Slc3a2*$^{flox/flox}$ mice. Notably, concentration of Ile was higher in CD25$^+$ CD4$^+$ cells than CD25$^-$ CD4$^+$ cells of control (*Slc3a2*$^{flox/flox}$) mice. Furthermore, Ile concentration was decreased in CD25$^+$ CD4$^+$ cells of *Foxp3*^{cre}; *Slc3a2*$^{flox/flox}$ mice compared to control mice, although concentration of Val and Leu was not altered. We also analyzed Ile uptake by splenic CD25$^+$ CD4$^+$ Treg cells of *Slc3a2*$^{flox/flox}$ and *Foxp3*^{cre}; *Slc3a2*$^{flox/flox}$ mice (Figure 6D). Ile uptake by *Slc3a2*$^{-/-}$ Treg cells was decreased. Taken together, these findings indicate that Treg cells preferentially uptake Ile via Slc3a2.

Slc3a2 has also been shown to mediate integrin signaling to control migration, survival, and proliferation of cells (Fenczik et al., 1997; Feral et al., 2005; Suga et al., 2001). Therefore, we analyzed the β2-integrin (LFA-1)/ICAM-1-dependent adhesion of *Slc3a2*$^{-/-}$ Treg cells (Figure S6B). *Slc3a2*$^{-/-}$ Treg cells bound to ICAM-1 to a level comparable to control Treg cells. This binding was abrogated by addition of blocking antibody against LFA-1 (Figure S6C), indicating that the *Slc3a2* deficiency in Treg cells did not affect the integrin-mediated cell adhesion.

Because mTORC1 regulates the metabolic program of T cells, we analyzed whether *Slc3a2*$^{-/-}$ Treg cells displayed an altered metabolic status. The extracellular acidification rate (ECAR), which reflects aerobic glycolysis, was measured in CD25$^+$ CD4$^+$ T cells isolated from the spleen (Figure 6E). CD25$^+$ CD4$^+$ cells from *Foxp3*^{cre}; *Slc3a2*$^{flox/flox}$ mice showed a slight reduction of ECAR at baseline and displayed a reduced increase of ECAR after the addition of oligomycin, which blocks mitochondrial ATP production. CD25$^+$ CD4$^+$ T cells were also analyzed for the oxygen consumption rate (OCR), which indicates oxidative

(C) Representative dot plots (left), frequency (upper right), and cell number (lower right) of Foxp3$^+$ CD4$^+$ T cells in the spleens (n = 5 per group) of *Slc3a2*$^{flox/flox}$ and *Foxp3*^{cre}; *Slc3a2*$^{flox/flox}$ mice.

(D) Frequency (upper) and cell number (lower) of CD44^{low}CD62L^{high} resting Treg (left: rTreg) and CD44^{high}CD62L^{low} activated Treg cells (right: aTreg) in the spleen of *Slc3a2*$^{flox/flox}$ and *Foxp3*^{cre}; *Slc3a2*$^{flox/flox}$ mice (n = 8 per group).

(E) Representative dot plots (left), frequency (upper right), and cell number (lower right) of Foxp3$^+$ CD4$^+$ T cells in the large intestines (LIs) (n = 6 per group) of *Slc3a2*$^{flox/flox}$ and *Foxp3*^{cre}; *Slc3a2*$^{flox/flox}$ mice.

(F) Frequency among CD4$^+$ T cells (upper) and cell number (lower) of Helios$^+$ Foxp3$^+$ Treg (left) and Helios$^-$ Foxp3$^+$ Treg cells (right) in the large intestines of *Slc3a2*$^{flox/flox}$ and *Foxp3*^{cre}; *Slc3a2*$^{flox/flox}$ mice (n = 5 per group).

(G) Representative histograms and frequency of BrdU$^+$ cells in Foxp3$^+$ CD4$^+$ T cells (left) and Foxp3$^-$ CD4$^+$ T cells (right) derived from *Slc3a2*$^{flox/flox}$ or *Foxp3*^{cre}; *Slc3a2*$^{flox/flox}$ mice (n = 5 per group).

In graphs, each symbol represents an individual mouse and the means ± SD are shown by horizontal bars. p values were determined by Mann-Whitney U-test. *p < 0.05; **p < 0.01; ***p < 0.005.

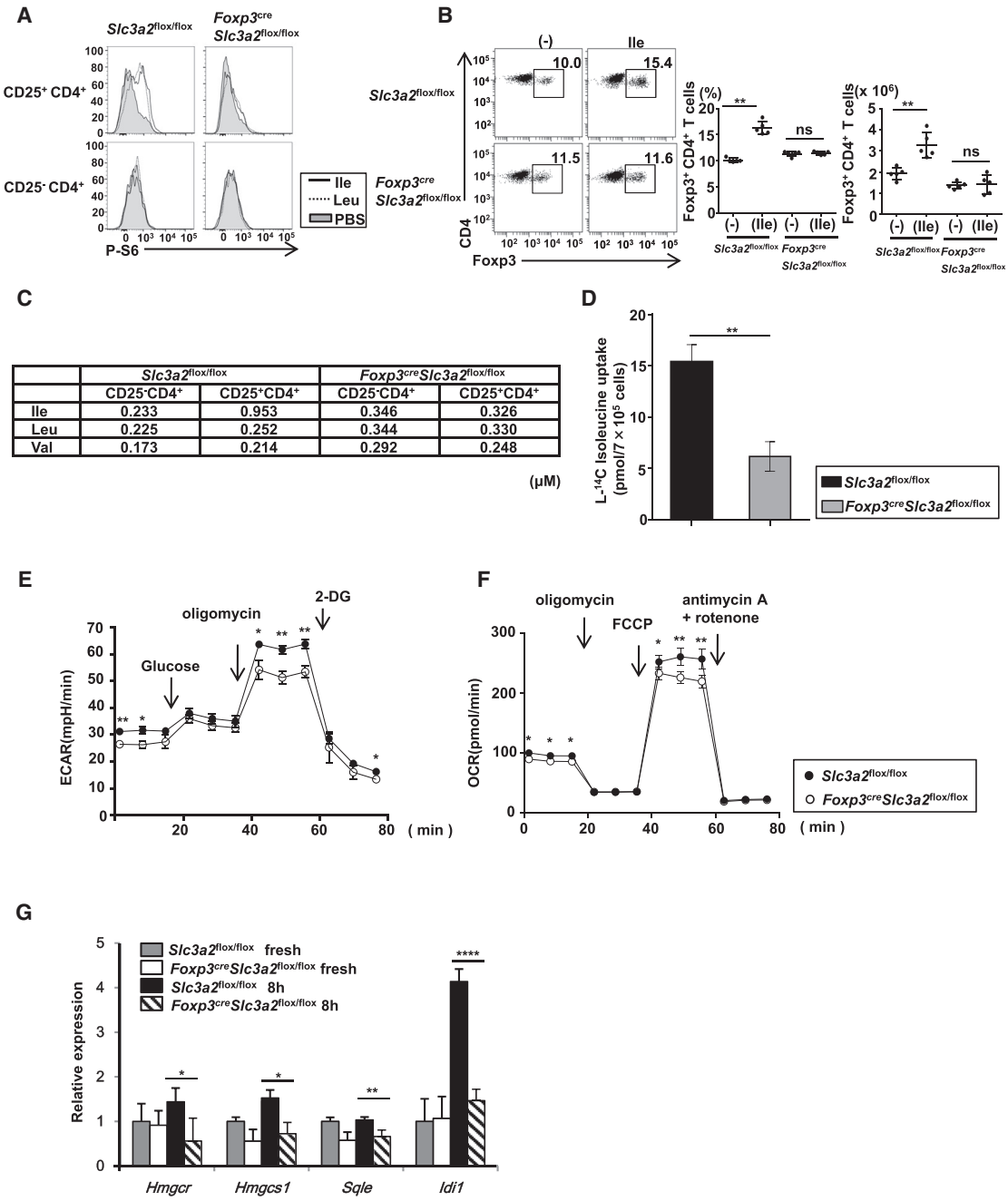

Figure 6. Impaired Ile Responses in *Slc3a2*-Deficient Treg Cells

(A) $CD25^+$ $CD4^+$ cells and $CD25^-$ $CD4^+$ cells from *Slc3a2*$^{flox/flox}$ or *Foxp3*^{cre}; *Slc3a2*$^{flox/flox}$ mice were cultured for 12 hr without amino acids. Then, cells were stimulated with 5 mM isoleucine (solid line), 5 mM leucine (dotted line), or PBS (gray histogram) for 20 min and analyzed for the phosphorylation of S6 ribosomal protein (P-S6).

(B) *Slc3a2*$^{flox/flox}$ or *Foxp3*^{cre}; *Slc3a2*$^{flox/flox}$ mice were fed an isoleucine-reduced diet for 7 days and then reared with isoleucine-sufficient (Ile) or -reduced diets (–) for another 7 days. The number of $Foxp3^+$ $CD4^+$ T cells in the spleen is shown. Representative FACS dot plot (left), frequency (middle), and the cell number (right) of splenic $Foxp3^+$ $CD4^+$ T cells in *Slc3a2*$^{flox/flox}$ or *Foxp3*^{cre}; *Slc3a2*$^{flox/flox}$ mice (n = 5 per group) is shown. Each symbol represents an individual mouse, and the means ± SD are indicated by horizontal bars.

(C) Intracellular BCAA concentrations of splenic $CD25^+$ $CD4^+$ Treg cells or $CD25^-$ $CD4^+$ T cells of *Slc3a2*$^{flox/flox}$ or *Foxp3*^{cre}; *Slc3a2*$^{flox/flox}$ mice were measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS) system. The concentrations of amino acids were adjusted by the recovery rate of Phe-d_5. Ile, isoleucine; Leu, leucine; Val, valine.

(D) L-[^{14}C] isoleucine uptake of splenic $CD25^+$ $CD4^+$ Treg cells from *Slc3a2*$^{flox/flox}$ mice or *Foxp3*^{cre}; *Slc3a2*$^{flox/flox}$ mice was measured. Data are the means ± SEM of 7 (*Slc3a2*$^{flox/flox}$ mice) or 6 (*Foxp3*^{cre}; *Slc3a2*$^{flox/flox}$ mice) wells measurements and are representative data of two independent experiments.

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phosphorylation (Figure 6F). An increase in OCR by the addition of carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), which uncouples oxidative phosphorylation in mitochondria allowing maximal respiration, was decreased in $CD25^+$ $CD4^+$ cells from *Foxp3*^{cre}; *Slc3a2*$^{flox/flox}$ mice compared to control cells. A previous report showed that altered metabolic program in *Rapter*$^{-/-}$ Treg cells correlated with the reduced expression of genes involved in the lipogenic program, which led to the impaired Treg cell function (Zeng et al., 2013). Therefore, we analyzed expression of genes involved in cholesterol biosynthesis, such as genes encoding 3-hydroxy-3-methylglutaryl-coenzyme A (CoA) reductase (HMGCR), 3-hydroxy-3-methylglutaryl-CoA synthase 1 (HMGCS1) squalene epoxidase (SQLE), and isopentenyl-diphosphate delta isomerase 1 (IDI1) (Figure 6G). Expression of these genes was all reduced in *Slc3a2*$^{-/-}$ Treg cells. Thus, the metabolic program was compromised in the absence of Slc3a2.

Defective Treg Cell Function in Treg-Cell-Specific Slc3a2-Deficient Mice

Because $Foxp3^+$ Treg cells in mice fed Ile-reduced diet had defective suppressive functions, we analyzed the surface expression of CTLA-4 and GITR and found that they were decreased in $Foxp3^+$ $CD4^+$ splenic T cells from *Foxp3*^{cre}; *Slc3a2*$^{flox/flox}$ mice compared to control mice (Figure 7A). We then analyzed the suppressive effect of Treg cells on T cell proliferation (Figure S7). $CD25^+$ $CD4^+$ splenic T cells from control mice suppressed co-cultured T cells in a cell-number-dependent manner. However, the suppression of T cell proliferation by $CD25^+$ $CD4^+$ splenic T cells from *Foxp3*^{cre}; *Slc3a2*$^{flox/flox}$ mice was impaired. In order to analyze the suppressive function of $Foxp3^+$ Treg cells more precisely, we generated *Foxp3*$^{YFP\text{-}cre}$; *Slc3a2*$^{flox/flox}$ mice (Rubtsov et al., 2008). We isolated YFP^+ ($Foxp3^+$) cells from *Foxp3*$^{YFP\text{-}cre}$; *Slc3a2*$^{+/+}$ and *Foxp3*$^{YFP\text{-}cre}$; *Slc3a2*$^{flox/flox}$ mice and analyzed for their suppressive activity on $CD4^+$ T cell proliferation (Figure 7B). YFP^+ ($Foxp3^+$) cells from *Foxp3*$^{YFP\text{-}cre}$; *Slc3a2*$^{flox/flox}$ mice did not suppress proliferation of $CD4^+$ T cells. We then analyzed Treg cell suppressive activity *in vivo*. We administered $CD4^+$ $CD45RB^{high}$ T cells together with $CD4^+$ $CD25^+$ cells from control (*Slc3a2*$^{flox/flox}$) or *Foxp3*^{cre}; *Slc3a2*$^{flox/flox}$ mice into *Rag2*$^{-/-}$ mice (Figures 7C–7E). *Rag2*$^{-/-}$ mice transferred with $CD4^+$ $CD45RB^{high}$ T cells developed severe intestinal inflammation. Cotransfer of wild-type Treg cells ameliorated colitis, but *Slc3a2*$^{-/-}$ Treg cells did not. In accordance with severe intestinal pathology, the number of IFN-γ-producing $CD4^+$ T cells was increased in mesenteric lymph nodes of *Rag2*$^{-/-}$ mice transferred with $CD4^+$ $CD45RB^{high}$ T cells. The number of IFN-γ-producing $CD4^+$ T cells was decreased by cotransfer of wild-type, but not *Slc3a2*$^{-/-}$, Treg cells (Figure 7E). Thus, Treg cells of *Foxp3*^{cre}; *Slc3a2*$^{flox/flox}$ mice showed a defective suppressive activity. Furthermore, although *Foxp3*^{cre}; *Slc3a2*$^{flox/flox}$ mice seemed healthy, focal lymphocyte infiltration was observed in many tissues, including the pancreas, thyroid, stomach, liver, and salivary gland of aged (40–45 weeks old) *Foxp3*^{cre}; *Slc3a2*$^{flox/flox}$ mice (Figure 7F). Taken together, these findings indicate that Slc3a2 is required for the function of Treg cells.

DISCUSSION

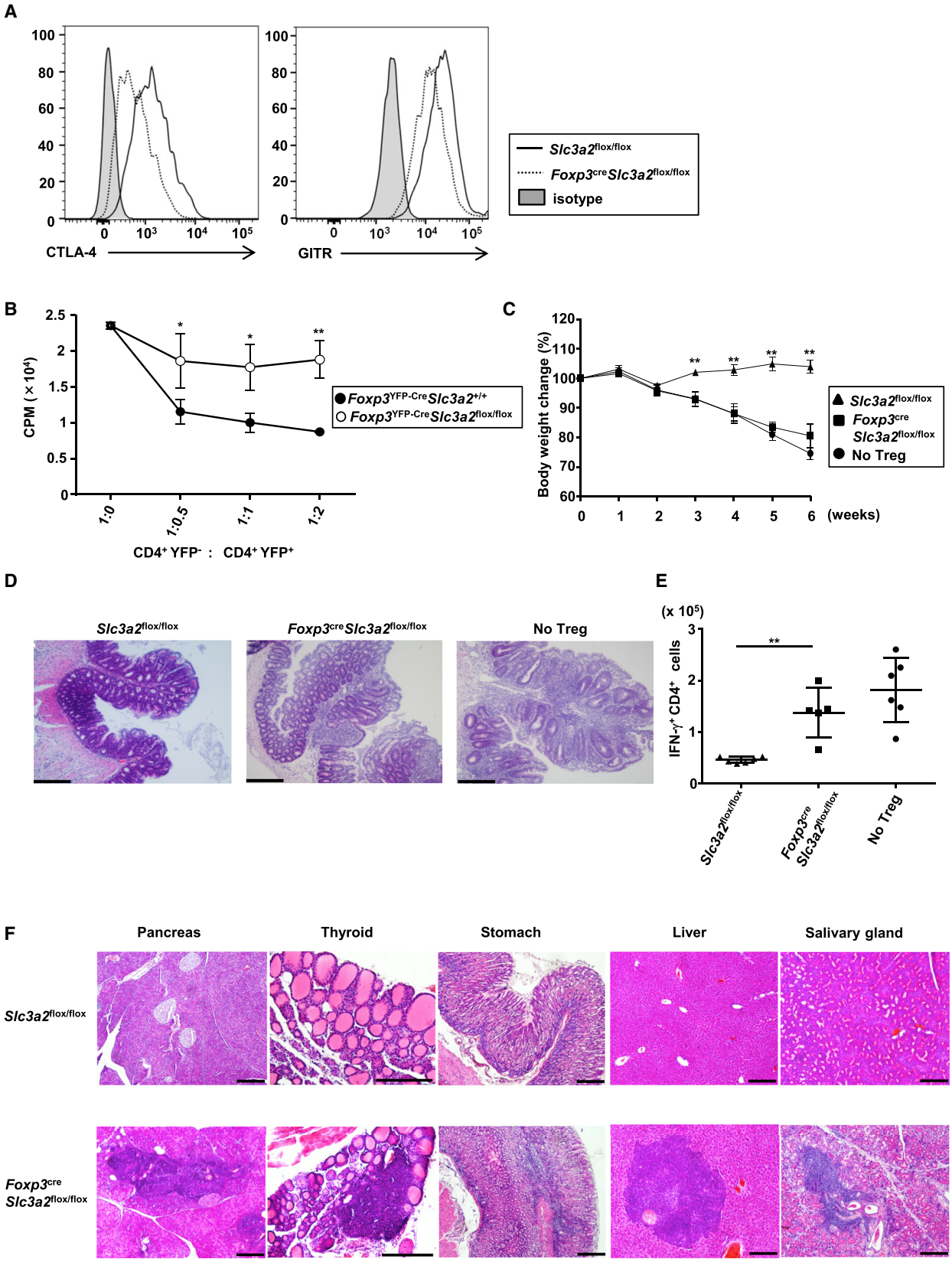
$Foxp3^+$ Treg cells are highly activated and proliferative *in vivo*. IL-2 is a critical growth factor required for Treg cell expansion. However, IL-2 cannot support Treg cell proliferation *in vitro*, indicating the requirement of other factors for the maintenance of Treg cells *in vivo*. Indeed, Treg cells require energy sources, such as glucose, amino acids, and fatty acids, for their *in vivo* maintenance (Newton et al., 2016; Procaccini et al., 2016). Amino acids, particularly glutamine and Leu, mediate the differentiation of Th1/Th17 cells and accordingly inhibit $Foxp3^+$ Treg cell differentiation (Klysz et al., 2015; Nakaya et al., 2014; Sinclair et al., 2013). However, the involvement of amino acids in the maintenance of differentiated Treg cells *in vivo* has not been characterized, although the requirement of glucose for Treg cell maintenance was suggested (Procaccini et al., 2016; Zeng and Chi, 2015; Zeng et al., 2013). In the present study, we demonstrated that BCAA controls the maintenance and function of Treg cells in the periphery. Ile and Leu similarly induced the phosphorylation of S6 in Treg cells and increased the number of Treg cells *in vivo*, but Treg cell number reduced more severely in mice fed Ile-reduced diet than mice fed Leu-reduced diet. In this regard, in mice fed Leu-reduced or Val-reduced diet, serum concentration of Ile was increased with unknown reason (unpublished data). Therefore, it is possible that Ile, Leu, and Val possess the similar activity on Treg cells and that the Leu or Val insufficiency might be compensated with the increased Ile. Alternatively, because the intracellular Ile concentration was higher in Treg cells than Leu or Val, and the Ile level decreased in *Slc3a2*$^{-/-}$ Treg cells, Treg cells utilize Ile more preferentially than Leu or Val. The precise comparison of Treg cell BCAA utilization will be an interesting future issue to be addressed.

The mTOR pathway is involved in multiple T cell functions, including differentiation, proliferation, survival, and gene activation, by sensing several cues in the environment, including amino acid levels (Chi, 2012; Laplante and Sabatini, 2012; Thomson et al., 2009). Accordingly, the mTOR pathway has been shown to control Treg cell differentiation and functions depending on the timing of mTORC1 activation (Procaccini et al., 2010). Indeed, mTORC1 is activated by immune signals, such as IL-2

(E and F) Extracellular acidification rate (ECAR) (E) and oxygen consumption rate (OCR) (F) of splenic $CD25^+$ $CD4^+$ cells from either *Slc3a2*$^{flox/flox}$ (closed circle) or *Foxp3*^{cre}; *Slc3a2*$^{flox/flox}$ mice (open circle) were analyzed by XF-96 extracellular flux analyzer in the presence of glucose, oligomycin, and 2-DG (ECAR) or oligomycin, FCCP, and antimycin A + rotenone (OCR). Data are the means ± SD of five (*Slc3a2*$^{flox/flox}$) or six (*Foxp3*^{cre}; *Slc3a2*$^{flox/flox}$) well measurements and are representative of three independent experiments. 2-DG, 2-deoxy-D-glucose; FCCP, carbonyl cyanide-p-trifluoromethoxyphenylhydrazone.

(G) qRT-PCR analysis of the lipogenic gene expression in freshly isolated splenic $CD25^+$ $CD4^+$ Treg cells or those stimulated with anti-CD3 antibody/anti-CD28 antibody and IL-2 for 8 hr.

Data are the means ± SD of three well measurements. p values were determined by Mann-Whitney U test (B and D–F) or Student's t test (G). $^*p < 0.05$; $^{**}p < 0.01$; $^{****}p < 0.001$.



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and TCR, leading to Treg cell activation via metabolic reprogramming (Procaccini et al., 2010; Zeng and Chi, 2015). In freshly isolated Treg cells exposed to many mTOR-activating environmental cues *in vivo*, the mTOR pathway was activated as evidenced by the phosphorylation of S6 and p70S6K (Procaccini et al., 2010 ; Zeng et al., 2013). However, the level of phosphorylation of S6 and p70S6K was markedly reduced after 12 hr of *in vitro* culture, in which mTOR-activating factors were absent. In this setting, the addition of Ile or Leu induced the phosphorylation of S6 and p70S6K, indicating that BCAAs directly activate the mTOR pathway in Treg cells. However, the BCAA-induced phosphorylation of S6 and p70S6K in Treg cells was observed at a low level. Currently, it is very hard to expand Treg cells *in vitro*. The identification of factors in addition to BCAA, the mixture of which effectively activates the mTOR pathway *in vitro*, will enable the establishment of *in vitro* culture protocols for Treg cell expansion.

The BCAA-mediated control of Treg cell maintenance and function was dependent on the subunit of amino acid transporter complexes, Slc3a2. Treg-cell-specific Slc3a2-deficient mice showed similar Treg cell phenotypes to those in mice fed Ile-reduced diet. Furthermore, Treg cells from *Foxp3*^{cre}; *Slc3a2*$^{flox/flox}$ mice showed defective Ile- and Leu-induced responses. Thus, Slc3a2 is required for the BCAA-dependent maintenance and function of Treg cells *in vivo*. Slc3a2 forms BCAA transporter complexes by associating with several molecules, including Lat1, Lat2, y^+Lat1, and y^+Lat2 (Bodoy et al., 2005; Kanai et al., 1998; Verrey et al., 2004). We analyzed *Foxp3*^{cre}; *Slc7a5*$^{flox/flox}$ mice (Treg-specific Lat1-deficient mice), but the decrease of Treg cell numbers was only marginal and those mutant mice did not show an inflammatory change in any organs, indicating that molecules other than Slc7a5, which associate with Slc3a2, also act as BCAA transporters on Foxp3$^+$ Treg cells (unpublished data).

The involvement of Slc3a2 on T cell functions has been studied previously. These studies all focused on effector T cell differentiation *in vivo* using *Cd4*^{cre} or *Lck*^{cre} mice, in which the targeted gene was deleted at the early stage of T cell development in the thymus (Bhuyan et al., 2014; Cantor et al., 2011; Kurihara et al., 2015). In these mutant mice, the number of Foxp3$^+$ Treg cells was not reduced. Because Treg-cell-skewed *in vitro* conditions induced the differentiation of naive CD4$^+$ T cells into Treg cells normally in the absence of Ile or Slc3a2, it is suspected that the Ile/Slc3a2 axis is not involved in the Treg cell differentiation process. The differential phenotypes between *Cd4*^{cre} (*Lck*^{cre}); *Slc3a2*$^{flox/flox}$ mice and *Foxp3*^{cre}; *Slc3a2*$^{flox/flox}$ mice might be caused because the time point of Cre action is different (in *Cd4*^{cre} or *Lck*^{cre} mice, Cre acts during T cell differentiation in thymus, whereas in *Foxp3*^{cre} mice, Cre works after Treg cell differentiation). Because Slc3a2 is essential for the BCAA-mediated maintenance of Treg cells, the *Slc3a2* deficiency during T cell differentiation might be compensated by other unknown BCAA-sensing molecules in *Cd4*^{cre} (*Lck*^{cre}); *Slc3a2*$^{flox/flox}$ mice. Similar to our results, *Slc3a2*$^{-/-}$ Treg cells isolated from *Cd4*^{cre}; *Slc3a2*$^{flox/flox}$ mice were unable to prevent intestinal inflammation in *Rag2*$^{-/-}$ mice transferred with naive T cells, indicating a defect in the suppressive activity of *Slc3a2*$^{-/-}$ Treg cells (Bhuyan et al., 2014). Thus, previous studies also support our findings that the Ile/Slc3a2 axis controls Treg cell functions.

In the large intestine, IL-10-producing pTreg cells are abundantly present. It is noteworthy that the number of pTreg cells rather than tTreg cells was reduced in the large intestine of *Foxp3*^{cre}; *Slc3a2*$^{flox/flox}$ mice. The mechanism by which intestinal pTreg cells require more BCAA than tTreg cells would be an interesting future issue to be addressed.

In conclusion, the present study demonstrates the importance of the BCAA/Slc3a2 axis in the *in vivo* maintenance of Treg cells. The proliferative potential of Treg cells is impaired in autoimmune diseases, such as type I diabetes and multiple sclerosis (Carbone et al., 2014; Wing and Sakaguchi, 2010). It would be interesting clinically to measure serum BCAA concentrations as well as Treg cell numbers in patients with autoimmune diseases. The supplementation of BCAAs might be useful for the treatment of autoimmune diseases with decreased numbers of Treg cells.

EXPERIMENTAL PROCEDURES

Mice

BALB/c mice were purchased from Japan SLC. Foxp3 bicistronic reporter knockin mice expressing EGFP (Foxp3-EGFP mice) on a C57BL/6J background and *Rag2*-deficient mice on a C57BL/6 background were purchased from The Jackson Laboratory. *Foxp3*-IRES-Cre mice on a C57BL/6J background were generated as described previously (Wing et al., 2008). *Foxp3*$^{YFP\text{-}Cre}$ mice were generated as described previously (Rubtsov et al., 2008). *Slc3a2*-floxed mice on a C57BL/6J background were generated previously (Liu et al., 2012). Mice deficient for *Slc3a2* specifically in Foxp3$^+$ Treg cells (*Foxp3*^{cre}; *Slc3a2*$^{flox/flox}$) were generated by crossing *Foxp3*-IRES-Cre mice with *Slc3a2*-floxed mice. The mice were used at 8–12 weeks old unless otherwise noted. All animal experiments were conducted in accordance with the guidelines of the Animal Care and Use Committee of Osaka University.

Figure 7. Defective Treg Cell Function in Treg-Cell-Specific *Slc3a2*-Deficient Mice

(A) Expression of CTA-4 and GITR on splenic Foxp3$^+$ CD4$^+$ Treg cells from *Slc3a2*$^{flox/flox}$ (solid line) or *Foxp3*^{cre}; *Slc3a2*$^{flox/flox}$ mice (dotted line). Gray histograms represent the isotype control.

(B) [^{3}H]-thymidine uptake by CD4$^+$ YFP$^-$ T cells co-cultured with CD11c$^+$ dendritic cells in the presence of increasing ratios of splenic CD4$^+$ YFP$^+$ Treg cells from *Foxp3*$^{YFP\text{-}cre}$; *Slc3a2*$^{+/+}$ mice (closed circle) or *Foxp3*$^{YFP\text{-}cre}$; *Slc3a2*$^{flox/flox}$ mice (open circle). Data were expressed as CPM. Data are the means ± SD of triplicate well measurements. p values were determined by Student's t test. *$p < 0.05$; **$p < 0.01$.

(C–E) *Rag2*$^{-/-}$ mice were transferred with CD4$^+$ CD45RB^{high} cells from *Slc3a2*$^{flox/flox}$ mice and CD25$^+$CD4$^+$ Treg cells from *Slc3a2*$^{flox/flox}$ mice or *Foxp3*^{cre}; *Slc3a2*$^{flox/flox}$ mice or no Treg cells intraperitoneally. Body weight changes (percent of initial weight) of *Slc3a2*$^{flox/flox}$ Treg cell group (closed triangle; n = 6), *Foxp3*^{cre}*Slc3a2*$^{flox/flox}$ Treg cell group (closed square; n = 5), or no-Treg cell group (closed circle; n = 6) were shown. Data are the means ± SEM (C). Representative photos of H&E staining of colon sections. The scale bars represent 200 μm (D). Cell number of IFN-γ$^+$ CD4$^+$ T cells in mesenteric lymph nodes is shown. Each symbol represents an individual mouse, and means ± SD are shown by horizontal bars (E). p values were determined by Mann-Whitney U test. **$p < 0.01$.

(F) H&E staining of the pancreas, thyroid, stomach, liver, and salivary gland from 40- to 45-week-old *Slc3a2*$^{flox/flox}$ and *Foxp3*^{cre}*Slc3a2*$^{flox/flox}$ mice. The scale bars represent 200 μm.

Diets

For amino-acid-reduced diet studies, 6-week-old BALB/c mice were fed with a control diet or amino-acid-reduced diets, in which either valine, leucine, or isoleucine was reduced by 90% (Oriental Yeast) for 2 weeks. In other experiments, mice were fed with a standard Oriental MF diet (Oriental Yeast).

Flow Cytometry

PerCp/Cy5.5-conjugated anti-CD4 (GK1.5), phycoerythrin (PE)-conjugated anti-mouse CD98 (RL388), PE-conjugated anti-mouse CX3CR1 (SA011F11), PE-conjugated anti-mouse CD45RB (C363-16A), fluorescein isothiocyanate (FITC)-conjugated anti-CD25 (7D4), FITC-conjugated anti-helios (22F6), PE/Cy7-conjugated anti-GITR (YGITR765), PE/Cy7-conjugated anti-mouse CD62L (MEL-14), FITC-conjugated anti-IFN-γ (XMG1.2), Pacific-blue-conjugated anti-mouse/human CD44 (IM7), and Pacific-blue-conjugated anti-mouse/human CD11b (M1/70) antibodies were purchased from BioLegend. Pacific-blue-conjugated anti-Foxp3 (MF-14) antibody was purchased from eBioscience. FITC-conjugated anti-CD103 (M290), PE-conjugated anti-CD152 (UC10-4B9), Alexa-Fluor-647-conjugated anti-IL-17A (TC11-18H10.1), PE-conjugated anti-IL-10 (JES5-16E3), and APC-conjugated anti-CD11c (HL3) antibodies were purchased from BD Biosciences. FITC-conjugated anti-CD98hc (RL388) antibody was purchased from Abcam. Alexa-Fluor-647-conjugated anti-Foxp3 (3G3) antibody and anti-CD11c (N418) antibody were purchased from Tonbo Bioscience. Single-cell suspensions were cultured for 4 hr in the presence of 5 μM calcium ionophore (Sigma-Aldrich), 50 ng/mL phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich), and Golgi Stop (BD Biosciences), and intracellular cytokine expression was analyzed with a Cytofix/Cytoperm plus kit with Golgi Stop according to the manufacturer's protocol (BD Biosciences). For Foxp3 staining, a Foxp3 Staining Buffer Set (eBioscience) was used. For nuclear Ki-67 staining, the cells were fixed and stained with anti-Ki-67-PE antibody (BD Biosciences) for 30 min at 4°C. Flow cytometric analysis was performed with a FACS Canto II flow cytometer (BD Biosciences) with FlowJo software (Tree Star).

Histological Analysis

Tissues were fixed with 4% paraformaldehyde phosphate buffer solution (Wako), and sections were stained with H&E (Wako). *Slc3a2*$^{flox/flox}$ and *Foxp3*^{cre}; *Slc3a2*$^{flox/flox}$ mice (40–45 weeks old) were used for the histological study. Images were taken using BX53 microscope system (Olympus).

qRT-PCR

RNA preparation from samples was performed with TRIzol reagent (Life Technologies). After treatment with RQ1 DNase (Promega), total RNA was reverse transcribed by Moloney Murine Leukemia Virus Reverse Transcriptase (Promega) and random primers (Toyobo). cDNA was subjected to qPCR using GoTaq qPCR Master Mix (Promega) in an ABI PRISM 7900HT sequence detection system (Applied Biosystems). All values were normalized to the expression of *Gapdh*. The following primer sets were used: *Gapdh*, 5′-CCTCG TCCCGTAGACAAAATG-3′ and 5′-TCTCCACTTTGCCACTGCAA-3′; *Slc3a2*, 5′-TGCTCAGGCTGACATTGTAGC-3′ and 5′-TCAGCCAAGTACAAGGGT GC-3′; *Slc7a5*, 5′-TTGAAGGCACCAATCTGGACG-3′ and 5′-GGAGATGATGA TGGCCAGGG-3′; *Slc7a8*, 5′-CTAGCCTCCAATGCAGTTGC-3′ and 5′-GGCT CCAGCAAAGAACAGC-3′; *Slc43a1*, 5′-TTCACATGGTCTGGCCTGG-3′ and 5′-TGTGGTCCAAGGCTAACCC-3′; *Slc43a2*, 5′-ACAGTTTGGTAGCCTCACT GG-3′ and 5′-CCGGTAGCAGATGAGGTAAAGG-3′; *Slc7a7*, 5′-ATGGGGGTG TGACTTCAGC-3′ and 5′-CCAGGGTCCTGTGTTTGC-3′; *Slc7a6*, 5′-TACATC CTGACCAACGTGGC-3′ and 5′-ATGCCGAATGTCTGGTCAGC-3′; *Slc6a14*, 5′-ATCCATTCCACTTGACCTCTG-3′ and 5′-GAAGTTTTTCGGCCAGGAC-3′; *Slc6a19*, 5′-AAGGATGAGGAATGGGATCA-3′ and 5′-CTTCCCCTACCTATGC CAGA-3′; *Hmgcr*, 5′-AGTCAGTGGGAACTATTGCAC-3′ and 5′-TTACGTCAA CCATAGCTTCCG-3′; *Hmgcs1*, 5′-TGTTCTCTTACGGTTCTGGC-3′ and 5′-AA GTTCTCGAGTCAAGCCTTG-3′; *Sqle*, 5′-TTGTTGCGGATGGACTCTTCTC CA-3′ and 5′-GTTGACCAGAACAAGCTCCGCAAA-3′; and *Idi1*, 5′-GGTTCAG CTTCTAGCGGAGA-3′ and 5′-TCGCCTGGGTTACTTAATGG-3′.

Statistical Analysis

p values were calculated with Student's t test or Mann-Whitney U test as specified in figure legends. Tukey-Kramer test was used to evaluate four groups with JMP Pro 10 software (SAS Institute). p values < 0.05 were considered significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and seven figures and can be found with this article online at https://doi.org/10.1016/j.celrep.2017.10.082.

AUTHOR CONTRIBUTIONS

K.I. performed experiments and wrote the paper. M.K., H.K., T.K., R.O., M.M., and E.U. analyzed BCAA-reduced-diet-fed mice. S.N., P.K., and Y.K. performed experiments for amino acid transporters. N.M. and S.S. analyzed *Foxp3*-GFP mice. S.S., Y.K., and K.O. provided scientific insights. S.U. and A.A. analyzed amino acid concentrations. M.I., H.T., and K.Y. generated and analyzed Treg-cell-specific *Slc3a2*-deficient mice. Y.S. and S.T. analyzed metabolic states. K.T. planned and directed the research. K.I. and K.T. wrote the paper.

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REFERENCES

Appuhamy, J.A., Knoebel, N.A., Nayananjalie, W.A., Escobar, J., and Hanigan, M.D. (2012). Isoleucine and leucine independently regulate mTOR signaling and protein synthesis in MAC-T cells and bovine mammary tissue slices. J. Nutr. *142*, 484–491.

Battaglia, M., Stabilini, A., and Roncarolo, M.G. (2005). Rapamycin selectively expands CD4+CD25+FoxP3+ regulatory T cells. Blood *105*, 4743–4748.

Bhuyan, Z.A., Arimochi, H., Nishida, J., Kataoka, K., Kurihara, T., Ishifune, C., Tsumura, H., Ito, M., Ito, Y., Kitamura, A., and Yasutomo, K. (2014). CD98hc regulates the development of experimental colitis by controlling effector and regulatory CD4(+) T cells. Biochem. Biophys. Res. Commun. *444*, 628–633.

Bodoy, S., Martín, L., Zorzano, A., Palacín, M., Estévez, R., and Bertran, J. (2005). Identification of LAT4, a novel amino acid transporter with system L activity. J. Biol. Chem. *280*, 12002–12011.

Bröer, A., Klingel, K., Kowalczuk, S., Rasko, J.E., Cavanaugh, J., and Bröer, S. (2004). Molecular cloning of mouse amino acid transport system B0, a neutral amino acid transporter related to Hartnup disorder. J. Biol. Chem. *279*, 24467–24476.

Burns, C.P. (1975). Isoleucine metabolism by leukemic and normal human leukocytes in relation to cell maturity and type. Blood *45*, 643–651.

Calder, P.C. (2006). Branched-chain amino acids and immunity. J. Nutr. *136* (*1, Suppl*), 288S–293S.

Cantor, J., Slepak, M., Ege, N., Chang, J.T., and Ginsberg, M.H. (2011). Loss of T cell CD98 H chain specifically ablates T cell clonal expansion and protects from autoimmunity. J. Immunol. *187*, 851–860.

Carbone, F., De Rosa, V., Carrieri, P.B., Montella, S., Bruzzese, D., Porcellini, A., Procaccini, C., La Cava, A., and Matarese, G. (2014). Regulatory T cell proliferative potential is impaired in human autoimmune disease. Nat. Med. *20*, 69–74.

Chi, H. (2012). Regulation and function of mTOR signalling in T cell fate decisions. Nat. Rev. Immunol. *12*, 325–338.

Delgoffe, G.M., Kole, T.P., Zheng, Y., Zarek, P.E., Matthews, K.L., Xiao, B., Worley, P.F., Kozma, S.C., and Powell, J.D. (2009). The mTOR kinase differentially regulates effector and regulatory T cell lineage commitment. Immunity *30*, 832–844.

Fenczik, C.A., Sethi, T., Ramos, J.W., Hughes, P.E., and Ginsberg, M.H. (1997). Complementation of dominant suppression implicates CD98 in integrin activation. Nature *390*, 81–85.

Feral, C.C., Nishiya, N., Fenczik, C.A., Stuhlmann, H., Slepak, M., and Ginsberg, M.H. (2005). CD98hc (SLC3A2) mediates integrin signaling. Proc. Natl. Acad. Sci. USA *102*, 355–360.

Fisson, S., Darrasse-Jèze, G., Litvinova, E., Septier, F., Klatzmann, D., Liblau, R., and Salomon, B.L. (2003). Continuous activation of autoreactive CD4+ CD25+ regulatory T cells in the steady state. J. Exp. Med. *198*, 737–746.

Ganapathy, M.E., and Ganapathy, V. (2005). Amino acid transporter ATB0,+ as a delivery system for drugs and prodrugs. Curr. Drug Targets Immune Endocr. Metabol. Disord. *5*, 357–364.

Haxhinasto, S., Mathis, D., and Benoist, C. (2008). The AKT-mTOR axis regulates de novo differentiation of CD4+Foxp3+ cells. J. Exp. Med. *205*, 565–574.

Jose, D.G., and Good, R.A. (1973). Quantitative effects of nutritional essential amino acid deficiency upon immune responses to tumors in mice. J. Exp. Med. *137*, 1–9.

Kanai, Y., Segawa, H., Miyamoto, Ki., Uchino, H., Takeda, E., and Endou, H. (1998). Expression cloning and characterization of a transporter for large neutral amino acids activated by the heavy chain of 4F2 antigen (CD98). J. Biol. Chem. *273*, 23629–23632.

Klysz, D., Tai, X., Robert, P.A., Craveiro, M., Cretenet, G., Oburoglu, L., Mongellaz, C., Floess, S., Fritz, V., Matias, M.I., et al. (2015). Glutamine-dependent α-ketoglutarate production regulates the balance between T helper 1 cell and regulatory T cell generation. Sci. Signal. *8*, ra97.

Kurihara, T., Arimochi, H., Bhuyan, Z.A., Ishifune, C., Tsumura, H., Ito, M., Ito, Y., Kitamura, A., Maekawa, Y., and Yasutomo, K. (2015). CD98 heavy chain is a potent positive regulator of CD4+ T cell proliferation and interferon-γ production in vivo. PLoS ONE *10*, e0139692.

Laplante, M., and Sabatini, D.M. (2012). mTOR signaling in growth control and disease. Cell *149*, 274–293.

Liu, Z., Hou, J., Chen, J., Tsumura, H., Ito, M., Ito, Y., Hu, X., and Li, X.K. (2012). Deletion of CD98 heavy chain in T cells results in cardiac allograft acceptance by increasing regulatory T cells. Transplantation *93*, 1116–1124.

Nakaya, M., Xiao, Y., Zhou, X., Chang, J.H., Chang, M., Cheng, X., Blonska, M., Lin, X., and Sun, S.C. (2014). Inflammatory T cell responses rely on amino acid transporter ASCT2 facilitation of glutamine uptake and mTORC1 kinase activation. Immunity *40*, 692–705.

Newton, R., Priyadharshini, B., and Turka, L.A. (2016). Immunometabolism of regulatory T cells. Nat. Immunol. *17*, 618–625.

Park, Y., Jin, H.S., Lopez, J., Elly, C., Kim, G., Murai, M., Kronenberg, M., and Liu, Y.C. (2013). TSC1 regulates the balance between effector and regulatory T cells. J. Clin. Invest. *123*, 5165–5178.

Procaccini, C., and Matarese, G. (2012). Regulatory T cells, mTOR kinase, and metabolic activity. Cell. Mol. Life Sci. *69*, 3975–3987.

Procaccini, C., De Rosa, V., Galgani, M., Abanni, L., Calì, G., Porcellini, A., Carbone, F., Fontana, S., Horvath, T.L., La Cava, A., and Matarese, G. (2010). An oscillatory switch in mTOR kinase activity sets regulatory T cell responsiveness. Immunity *33*, 929–941.

Procaccini, C., Carbone, F., Di Silvestre, D., Brambilla, F., De Rosa, V., Galgani, M., Faicchia, D., Marone, G., Tramontano, D., Corona, M., et al. (2016). The proteomic landscape of human ex vivo regulatory and conventional T cells reveals specific metabolic requirements. Immunity *44*, 406–421.

Proud, C.G. (2007). Amino acids and mTOR signalling in anabolic function. Biochem. Soc. Trans. *35*, 1187–1190.

Rubtsov, Y.P., Rasmussen, J.P., Chi, E.Y., Fontenot, J., Castelli, L., Ye, X., Treuting, P., Siewe, L., Roers, A., Henderson, W.R., Jr., et al. (2008). Regulatory T cell-derived interleukin-10 limits inflammation at environmental interfaces. Immunity *28*, 546–558.

Sakaguchi, S. (2000). Regulatory T cells: key controllers of immunologic self-tolerance. Cell *101*, 455–458.

Sakaguchi, S., Ono, M., Setoguchi, R., Yagi, H., Hori, S., Fehervari, Z., Shimizu, J., Takahashi, T., and Nomura, T. (2006). Foxp3+ CD25+ CD4+ natural regulatory T cells in dominant self-tolerance and autoimmune disease. Immunol. Rev. *212*, 8–27.

Sauer, S., Bruno, L., Hertweck, A., Finlay, D., Leleu, M., Spivakov, M., Knight, Z.A., Cobb, B.S., Cantrell, D., O'Connor, E., et al. (2008). T cell receptor signaling controls Foxp3 expression via PI3K, Akt, and mTOR. Proc. Natl. Acad. Sci. USA *105*, 7797–7802.

Sinclair, L.V., Rolf, J., Emslie, E., Shi, Y.B., Taylor, P.M., and Cantrell, D.A. (2013). Control of amino-acid transport by antigen receptors coordinates the metabolic reprogramming essential for T cell differentiation. Nat. Immunol. *14*, 500–508.

Singh, B., Read, S., Asseman, C., Malmström, V., Mottet, C., Stephens, L.A., Stepankova, R., Tlaskalova, H., and Powrie, F. (2001). Control of intestinal inflammation by regulatory T cells. Immunol. Rev. *182*, 190–200.

Suga, K., Katagiri, K., Kinashi, T., Harazaki, M., Iizuka, T., Hattori, M., and Minato, N. (2001). CD98 induces LFA-1-mediated cell adhesion in lymphoid cells via activation of Rap1. FEBS Lett. *489*, 249–253.

Thomson, A.W., Turnquist, H.R., and Raimondi, G. (2009). Immunoregulatory functions of mTOR inhibition. Nat. Rev. Immunol. *9*, 324–337.

Tsukishiro, T., Shimizu, Y., Higuchi, K., and Watanabe, A. (2000). Effect of branched-chain amino acids on the composition and cytolytic activity of liver-associated lymphocytes in rats. J. Gastroenterol. Hepatol. *15*, 849–859.

Verrey, F., Closs, E.I., Wagner, C.A., Palacin, M., Endou, H., and Kanai, Y. (2004). CATs and HATs: the SLC7 family of amino acid transporters. Pflugers Arch. *447*, 532–542.

Vukmanovic-Stejic, M., Zhang, Y., Cook, J.E., Fletcher, J.M., McQuaid, A., Masters, J.E., Rustin, M.H., Taams, L.S., Beverley, P.C., Macallan, D.C., and Akbar, A.N. (2006). Human CD4+ CD25hi Foxp3+ regulatory T cells are derived by rapid turnover of memory populations in vivo. J. Clin. Invest. *116*, 2423–2433.

Wing, K., and Sakaguchi, S. (2010). Regulatory T cells exert checks and balances on self tolerance and autoimmunity. Nat. Immunol. *11*, 7–13.

Wing, K., Onishi, Y., Prieto-Martin, P., Yamaguchi, T., Miyara, M., Fehervari, Z., Nomura, T., and Sakaguchi, S. (2008). CTLA-4 control over Foxp3+ regulatory T cell function. Science *322*, 271–275.

Zeng, H., and Chi, H. (2015). Metabolic control of regulatory T cell development and function. Trends Immunol. *36*, 3–12.

Zeng, H., Yang, K., Cloer, C., Neale, G., Vogel, P., and Chi, H. (2013). mTORC1 couples immune signals and metabolic programming to establish T(reg)-cell function. Nature *499*, 485–490.