Infectious tolerance via the consumption of essential amino acids and mTOR signaling

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Infectious tolerance describes the process of CD4$^+$ regulatory T cells (Tregs) converting naïve T cells to become additional Tregs. We show that antigen-specific Tregs induce, within skin grafts and dendritic cells, the expression of enzymes that consume at least 5 different essential amino acids (EAAs). T cells fail to proliferate in response to antigen when any 1, or more, of these EAAs are limiting, which is associated with a reduced mammalian target of rapamycin (mTOR) signaling. Inhibition of the mTOR pathway by limiting EAAs, or by specific inhibitors, induces the Treg-specific transcription factor forkhead box P3, which depends on both T cell receptor activation and synergy with TGF-β.

amino acid catabolism | foxp3 | mTOR inhibitor | regulatory T cells | rapamycin

Regulatory T cells (Tregs) maintain peripheral tolerance to both self and nonself antigens. Mice lacking the transcription factor foxp3, required for the generation of natural Tregs in the thymus, develop a range of autoimmune disorders (1, 2), whereas CD4$^+$foxp3$^+$ Tregs maintain and transfer tolerance to allografts (3, 4). An important component in the maintenance of peripheral tolerance is the ability of Tregs to convert naïve T cells or recent thymic emigrants into further cohorts of Tregs when they encounter antigens presented by the same antigen-presenting cell (APC), usually a dendritic cell (DC), in related phenomena known as linked suppression and infectious tolerance (3, 5). The molecular mechanisms by which Tregs mediate immune regulation and infectious tolerance in vivo remain to be defined, although the expression of TGF-β (6), the generation of extracellular adenosine (7), and catabolism of tryptophan (8) have been implicated.

Indoleamine 2,3-dioxygenase (IDO) is an enzyme with immune regulatory properties conferred by its ability to catabolize the essential amino acid (EAA) tryptophan (8). This was first clearly demonstrated as important in maintaining tolerance to paternal antigens expressed by the fetus, because specific inhibition of IDO activity by 1-methyl-DL-tryptophan in the placenta induced rejection of semiallogeneic, but not syngeneic, concepti in normal mice (9). IDO induction in APCs via CTLA4 ligation of CD80/CD86 is thought to represent an important effector pathway for Tregs to induce and maintain peripheral tolerance (8, 10, 11). It has been shown that the protein kinase general control nonderepressing-2 (GCN2), which has a predicted binding site for free acyl-tRNAs, can act as a molecular sensor for intracellular tryptophan (12), inducing the integrated stress response (ISR) pathway that acts to control cell growth and differentiation (13). Fallarino et al. (14) have suggested that this pathway, in the presence of the products of tryptophan catabolism (kynurenines) can lead to further induction of foxp3$^+$ Tregs (14).

As neither IDO$^{-/-}$ nor GCN2$^{-/-}$ mice show any gross immunological phenotype, is it possible that there is some redundancy in the requirement for this pathway? We tested the hypothesis that redundancy might be explained at 2 levels. First, do the mechanisms attributed to tryptophan catabolism by IDO apply to other EAAs and the enzymes that consume them? Second, can amino acid level sensing by T cells be performed by pathways other than GCN2? In particular, we wanted to test for any role for the mammalian target of rapamycin (mTOR) and phosphatidinylinositol-3-kinase (PI3K) pathways, because they have recently been implicated in Treg differentiation (15, 16) and are already known to control cellular responses to nutrients, including amino acids (17).

Results

Tregs Can Induce Amino Acid-Consuming Enzymes in Vivo. Antigen-specific Tregs are known to be enriched (18) and active (3) within transplanted tissues, so we generated foxp3$^+$ Tregs specific for the male antigen (DBY) by activation in the presence of TGF-β in vitro (6), and injected them into syngeneic female [either T cell-deficient RAG1$^{-/-}$ or CD4$^+$ T cell receptor (TCR) transgenic anti-DBY+E^k, A1.RAG1$^{-/-}$] mice given a male skin graft. We then looked within the grafts on day 7 by RT-PCR for the presence of CD3 and foxp3 to confirm that Tregs had entered the graft and identify any changes in the expression of candidate enzymes that might consume EAAs (Table S1). In T cell-deficient recipients we found that certain amino acid-consuming enzymes were up-regulated in 2 different ways (Fig. 1*A*). First, there were transcripts for 4 enzymes (*arg1*, *bcat1*, *ddc*, and *tdh*) that were clearly up-regulated in skin grafts when compared with fresh skin, suggesting an association with innate inflammation or wound healing. Second, 4 enzymes (*hdc*, *il4i1*, *indo*, and *nos2*) were further up-regulated in the grafts only when Tregs were present. In recipients that contained rejection competent T cells [under circumstances where we have shown that similarly injected Tregs can completely suppress graft rejection (6)], we observed that Tregs induced up-regulation of *arg1*, *il4i1*, *indo*, and *nos2* over and above the level observed in grafts destined for rejection (Fig. 1*B*). In addition, just as for the induction of tolerance and foxp3$^+$ T cells in the graft, this up-regulation partially depended on TGF-β, because it was inhibited with a neutralizing antibody, but not an isotype-matched control.

Tregs Induce Amino Acid-Consuming Enzymes in DCs. Tregs are known to secrete cytokines such as TGF-β and IL-10, which are able to convert DCs to a "tolerogenic" phenotype, and they also express CTLA4, which can induce IDO (*indo*) in particular DC

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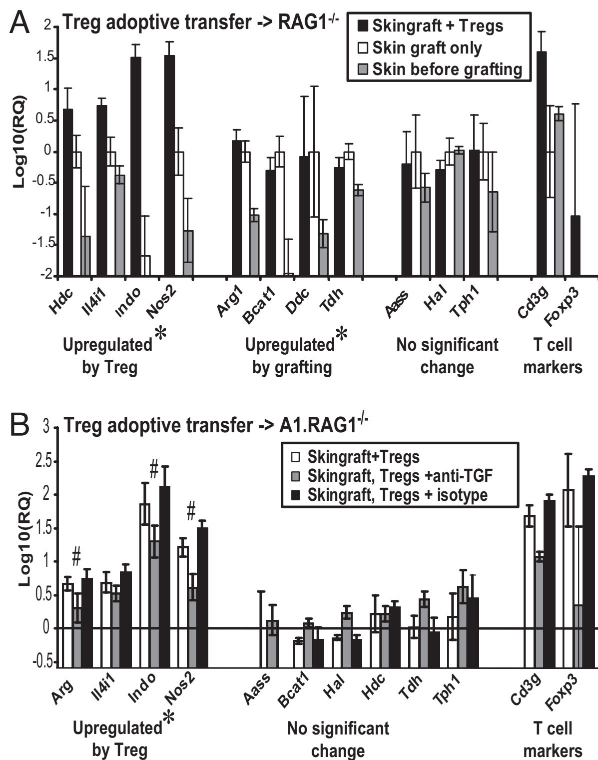

Fig. 1. The adoptive transfer of antigen specific, foxp3$^+$ Tregs induces multiple amino acid enzyme depleting transcripts within skin grafts. (*A*) In vitro-generated Tregs (1×10^7 DBYT cells) were adoptively transferred to CBA.RAG1$^{-/-}$ recipient female mice 1 day before grafting with CBA.RAG1$^{-/-}$ male tail skin ($n = 8$; black bars). Control RAG1$^{-/-}$ recipients received similar grafts, but no DBYT cells ($n = 4$; white bars). After 6 days, grafts were harvested and analyzed for the expression of gene transcripts by low-density TaqMan RT-PCR array. Grafted samples were compared with normal tail skin from CBA.RAG1$^{-/-}$ ($n = 4$; gray bars). Data are shown as mean ± SD of $\log_{10}$(RQ) values, where *n* indicates the number of recipient skin graft samples tested in independent experiments. Transcripts significantly ($P < 0.05$) up-regulated either by syngeneic grafting alone or Tregs in male grafts are indicated. Note that FoxP3 detection within grafts showed a wide variation, because it was close to the limit of detection, but was negative in all mice not given DBYT cells. (*B*) Tregs (1×10^7 DBYT cells) were adoptively transferred to A1.RAG1$^{-/-}$ female recipient mice given a male CBA.RAG1$^{-/-}$ skin graft 1 day later. One group of grafted mice received only Tregs (white bars), and other groups also received 2 mg of either a neutralizing anti-TGF-β (gray bars) or an isotype-matched control (black bars) mAb. Another group received no Tregs as a control for ongoing graft rejection (to which all data were normalized: indicated by the horizontal line at RQ = 0). All grafts were harvested and analyzed 6 days later for the expression of gene transcripts by low-density TaqMan RT-PCR array. Data are shown as mean ± SD of $\log_{10}$(RQ) values for independent biological replicate samples from 6 individually grafted mice per group. Transcripts significantly ($P < 0.05$) up-regulated by Tregs in male grafts are indicated by *, and those significantly dependent on TGF-β are indicated by #.

subpopulations (10). We tested whether 2 different types of Tregs (CD4$^+$foxp3$^+$ TGF-β-induced Tregs or CD4$^+$foxp3$^-$IL-10$^+$ Tr1) were able to up-regulate amino acid-consuming enzymes in bone marrow-derived DCs (bmDC). Foxp3$^+$ Tregs were found to induce 7 different enzyme transcripts (*arg1*, *hdc*, *il4i1*, *indo*, *nos2*, *tph*, and *tdh*) in bmDC, and it generally required the presence of the nominal antigen peptide (Fig. 2*A*); conventionally activated T cells (Tconv) were much less effective overall (Fig. 2*B*). Tr1 cells induced 5 of the 7 enzymes induced by Tregs, although they appeared to depend less on additional antigen (Fig. 2*C*). Because both Tregs and Tr1 cells express constitutive CTLA4 and can express TGF-β and IL-10, we tested whether these proteins could induce these enzymes themselves.

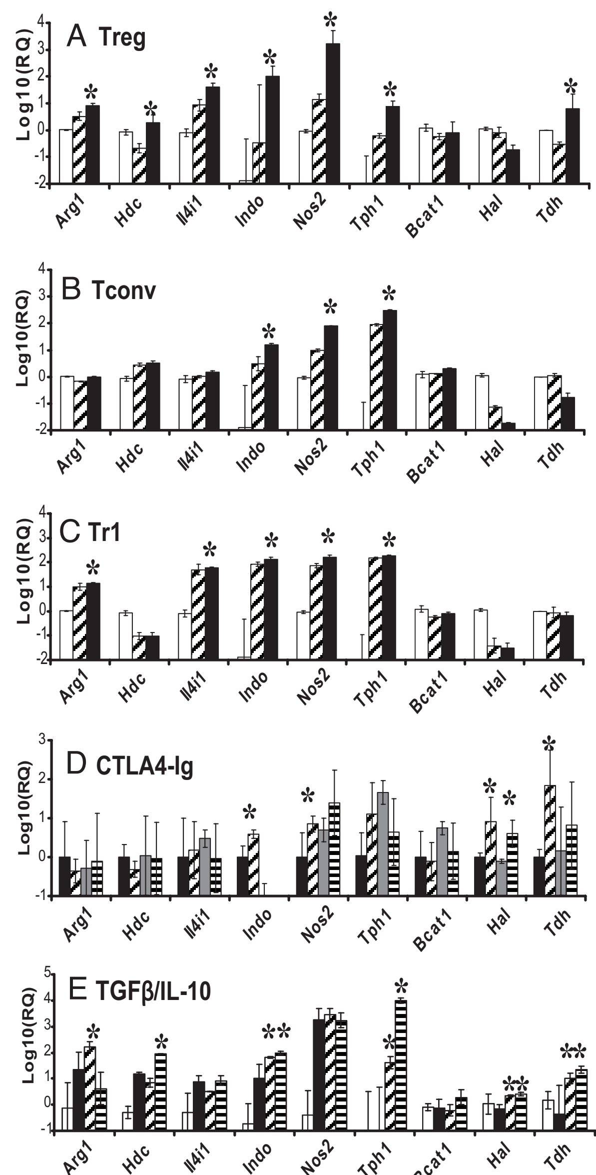

Fig. 2. Tregs induce amino acid-consuming enzymes in DCs as do CTLA4, IL-10, and TGF-β. (*A–C*) Immature bmDCs from female CBA/Ca mice were cultured either alone (white bars) or together (1:1 ratio) with male-specific TCR transgenic Tregs (*A*), similar, but conventionally activated, T cells (Tconv) (*B*), or Tr1D1 (a Tr1 cell clone) (*C*), both with (black bars) or without (striped bars) the cognate DBY antigen peptide, for 24 h at 37 °C. Samples were then analyzed for the expression of gene transcripts by low-density TaqMan RT-PCR array. * indicates gene transcripts significantly overexpressed only in the presence of T cells plus cognate peptide. (*D*) Splenic DCs (10^6/mL) from CBA/Ca (slashed and black bars) or CBA.IDO$^{-/-}$ (striped and gray bars) mice, as indicated, were cultured either with (slashed and striped bars) or without (black and gray bars) CTLA4-Ig (100 μg/mL) for 48 h, before analysis for the expression of gene transcripts by low-density TaqMan RT-PCR array. * indicates gene transcripts significantly overexpressed, in the presence of CTLA4-Ig, in either wild-type or IDO$^{-/-}$ T cells. (*E*) Gene transcripts were measured in immature DCs (white bars) or LPS-treated DCs that had been exposed to TGF-β (slashed bars), IL-10 (striped bars), or no additional cytokines (black bars) during their generation from bone marrow cultures, and analysis as above. * indicates gene transcripts up-regulated by TGF-β or IL-10 over and above that seen by LPS exposure alone. Data are shown as the mean ± SD $\log_{10}$(RQ) values of at least 3 independent biological replicates.

CTLA4-Ig induced *indo* (IDO) in wild-type DCs, but not IDO$^{-/-}$ splenic DCs, as expected, but it also induced *nos2*, *hal*, and *tdh* independently of IDO (Fig. 2*D*). TGF-β induced *arg1*,

indo, *tph*, *hal*, and *tdh*, whereas IL-10 up-regulated *hdc*, *indo*, *tph*, *hal*, and *tdh*, particularly in bmDCs after subsequent LPS exposure (Fig. 2*E*).

Enzymatic Consumption of Amino Acids by DCs Inhibits T Cell Proliferation. Is the expression of these enzymes functional, and does it act to control T cell proliferation? We tested these questions by taking LPS-matured or TGF-β+LPS-treated ["tolerogenic" (19)] bmDCs, shown to express these enzymes as above, and incubated them in tissue culture medium chosen to provide all of the amino acids in excess except for the one of interest, which was provided in amounts just limiting to T cell proliferation (Fig. S1). This process allowed us to isolate the effects of DC-expressed, enzyme-mediated consumption of a single amino acid at a time and to test the effects of specific enzyme inhibitors. We then assayed whether these conditioned media would support T cell proliferation in a standardized APC-independent, TCR cross-linking experiment (which eliminates any possible indirect effects of amino acid levels on antigen presentation). As expected, 1-methyl-tryptophan, a specific inhibitor of IDO, was able to reverse the weak suppression of T cell proliferation caused by tryptophan depletion by LPS-matured bmDCs (Fig. 3*A*), although it failed to reverse suppression by the TGF-β+LPS-treated DCs (possibly because those DCs were particularly effective at depleting other amino acids). In limited arginine, previous incubation with either matured or TGF-β-treated bmDCs inhibited T cell proliferation to levels obtained in medium with no arginine, which could be significantly reversed by specific inhibitors of either *arg1* or *nos2* during the preincubation (Fig. 3 *B* and *C*). Similarly, in limiting phenylalanine, histidine and valine (note that phenylalanine seems to be limiting even at 100% in Fig. 3*D*), we were able to show a reversal of T cell suppression by specific inhibition, in the bmDC cultures, of *il4i1*, *hdc*, and *bcat1*, respectively (Fig. 3 *D*–*F*). This confirmed that the multiple amino acid-consuming enzymes expressed by bmDCs were active and that significant depletion of any one of the EAAs could markedly impair T cell proliferation in a manner similar to that already known for tryptophan catabolism by IDO.

GCN2 Promotes Survival of Activated T Cells During Amino Acid Starvation. The sensing of tryptophan levels by T cells has been attributed to GCN2 (12), so we tested whether naïve or previously activated T cells from GCN2$^{-/-}$ mice could sense the lack of individual EAAs by ceasing proliferation. Surprisingly, naïve GCN2$^{-/-}$ T cells still failed to proliferate in response to a lack of any one EAA with similar, or even enhanced, sensitivity compared with wild-type mice (Fig. S2). Because this was largely reversible by adding back the missing amino acids, even after 2–3 days of starvation, it was unlikely to be entirely explained by cell death. Indeed, GCN2$^{-/-}$ and wild-type T cells starved of either single branched-chain or nonbranched chain EAAs during their first TCR-mediated activation showed a similar exponential decay in cell survival with time (Fig. 4*A*). In contrast, previously activated T cells from wild-type mice showed considerably enhanced survival when compared with similar GCN2$^{-/-}$ T cells during the first 96 h of starvation, which indicates that GCN2 and the ISR pathway may be required for survival of activated T cells during amino acid starvation, analogous to the function of GCN2 in more primitive organisms (20).

The mTOR/PI3K Pathway Is Sufficient to Control T Cell Proliferation. It seems, therefore, that the ISR pathway via GCN2 is not sufficient for T cells to sense EAA depletion and cease proliferation. Although it was possible that GCN2 was being bypassed by alternative stress response kinases (21), we found no evidence for this, because the ISR-responsive genes, CHOP (*ddit3*) and GADD34 (*myd116*), were up-regulated by EAA starvation in wild-type mice, but not GCN2$^{-/-}$ mice (Fig. S3). A known alternative nutrient sensing mechanism is via the PI3K/mTOR signaling pathway. This pathway results in phosphorylation of both 4E-BP1 and the ribosomal protein S6, which direct the ribosome to preferentially translate certain mRNAs coding for genes involved in cell cycle regulation (22, 23). Starving anti-CD3^{+}CD28-activated T cells of any single EAA (or all of them), reduced the phosphorylation of both rS6 and 4E-BP1 (Fig. S4), when compared with complete medium, suggesting that the PI3K/mTOR pathway may indeed be involved.

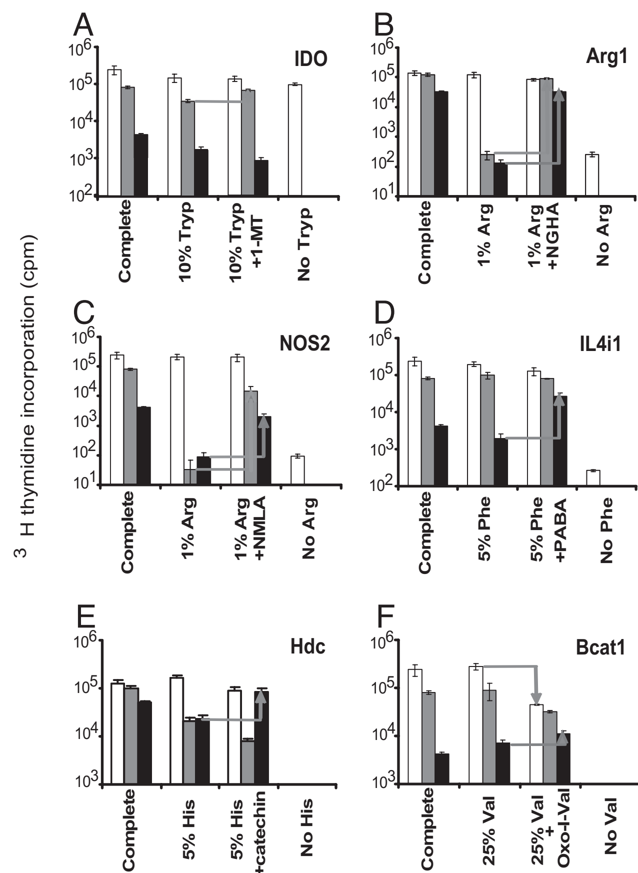

Fig. 3. DCs can suppress T cell activation through specific depletion of many different amino acids in vitro. LPS-matured (gray bars) or TGF-β + LPS-treated (black bars) bmDCs were cultured overnight with LPS at 37 °C in either complete RPMI medium 1640 or medium with a limiting concentration of the amino acid of interest, with or without a specific enzyme inhibitor (Table S1), as indicated. Control samples of each medium were similarly incubated without any cells (white bars). After removal of DCs by centrifugation and 0.2-μ filtering, fresh medium lacking the amino acid of interest was added 1:1 to each sample. Medium completely lacking the amino acid of interest was used as a negative control for T cell proliferation. Purified A1.RAG1$^{-/-}$ CD4^{+} T cells were then stimulated by anti-CD3+CD28 beads in each sample of conditioned medium and proliferation measured at 48 h by ^{3}H thymidine incorporation. The arrows indicate relief of suppression by the inhibitors (*A*–*F*) and any nonspecific toxicity of the inhibitor (*F*).

Inhibition of the mTOR/PI3K Pathway by Amino Acid Starvation Synergizes with TGF-β for Foxp3 and Treg Induction. Rapamycin, a specific inhibitor of mTOR, is an immunosuppressive agent that is thought to be permissive for transplantation tolerance and has recently been suggested to induce foxp3^{+} Tregs (15, 16). Because the PI3K/mTOR pathway is known to control cell growth in response to nutrients, including amino acids, we postulated that this pathway may be related to the mechanism of "infectious tolerance" where the tolerant Tregs, by inducing localized depletion of EAAs, may subsequently induce foxp3 and yet

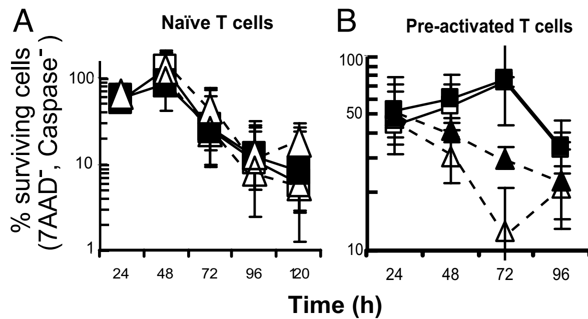

Fig. 4. GCN2 is required for the survival of activated T cells during amino acid starvation, but not the control of proliferation. $CD4^+$ T cells from C57BL/6 or C57BL/6.$GCN2^{-/-}$ mice, either naïve (*A*) or previously activated by plate bound anti-CD3 plus soluble CD28 for 48 h (*B*), were stimulated by anti-CD3+CD28 beads at 37 °C in the absence of individual EAAs. Cell samples were taken daily and analyzed by flow cytometry for the uptake or 7AAD and the fluorescent caspase product of FITC-VAD-FMK to indicate the proportion of dead (necrotic and apoptotic) cells. Plots show the geometric mean percentage of surviving cells ($7AAD^-Caspase^-$) for C57BL/6 wild type (square) compared with C57BL/6.$GCN2^{-/-}$ (triangles) T cells under conditions lacking individual branched-chain ($P < 0.05$; 2-way ANOVA), compared with nonbranched chain EAAs ($P < 0.0001$), respectively.

additional Tregs. We first confirmed that rapamycin and the PI3K inhibitor wortmannin were both able to induce foxp3 in 2 different TCR transgenic $RAG^{-/-}$ mouse models (which are devoid of any initial $foxp3^+$ Tregs) in an antigen-specific, bmDC-driven culture system and were able to synergize with suboptimal levels of TGF-β (Table S2 and Fig. 5). Decreasing the concentration of most EAAs, either singly or all together, synergized with TGF-β for foxp3 induction at both the RNA and protein level, while adding excess EAAs suppressed foxp3 (Fig. S5). By simultaneously monitoring the levels of rS6 and 4E-BP1 phoshorylation in these induced Tregs, we observed that foxp3 was most strongly induced in T cells with an intermediate level of PI3K/mTOR pathway suppression, signals from the TCR were acting to moderate the inhibitory effects of mTOR/PI3K inhibitors or amino acid starvation (Fig. 5), and foxp3 induction required PI3K/mTOR inhibition and/or TGF-β exposure within the first 24 h of activation. True synergy between mTOR inhibition by rapamycin and TGF-β was confirmed in titration experiments where the induction of foxp3 by the combination of agents was greater than the sum of the component effects, over a range of doses, during either antigen driven or APC-free anti-$CD3^+$CD28-driven T cell stimulation (Table S2).

Discussion

Immune regulation caused by EAA consumption seems to act at 2 different levels. First, there are enzymes such as *bcat1* and *tdh* that are up-regulated without the need for adaptive immunity, suggesting they may reflect an innate protective mechanism against inflammatory damage. Second, there appears to be an interplay between Tregs and APCs, leading to further up-regulation of not only IDO, but at least 4 other EAA-consuming enzymes, which all can act to limit T cell proliferation and, in addition, induce new Tregs via infectious tolerance.

We have focused on the induction of EAA-consuming enzymes within skin grafts in vivo and DCs (as APCs) in vitro, because it provides a possible molecular explanation for the linked suppression and infectious tolerance that are observed in such systems. We have not yet analyzed in detail whether there is a compartmentalization of individual enzymes to particular subsets of APCs within a tolerated tissue, although it is known that macrophages and endothelial cells, for example, can express at least some of them and are likely participating in generating an EAA-depleted microenvironment. Although the local consumption of multiple EAAs would seem to represent a redundant and therefore functionally robust system, each individual enzyme probably has additional specialized immunomodulatory properties. For example, IDO appears to be primarily expressed within APCs, requiring the appropriate tryptophan transporters to achieve extracellular depletion of tryptophan (24), whereas arginase can be secreted by neutrophils to deplete extracellular arginine (25). There are also specific functions for some of the products of amino acid consumption, such as kynurenines generated from tryptophan by IDO and NO generated by iNOS from arginine. Kynurenines have been shown in some conditions to enhance apoptosis of T cells (26) and their conversion to $foxp3^+$ Tregs during tryptophan depletion (14). Serotonin, the product of tryptophan hydoxylase activity, and histamine produced by histidine decarboxylase are generally considered as effector molecules of T helper 2 responses, but we have demonstrated here that expression of these enzymes by APCs can also deplete the amino acid substrate and cause a suppression of T cell proliferation. Other cell types expressing these enzymes, such as the mast cells that have been shown to play a role in transplantation tolerance (27, 28), might also contribute to the depletion, particularly of tryptophan and histidine. Similarly, the generation of NO by iNOS has been considered inflammatory, with arginase able to reduce this effect by competing for the substrate arginine (29), but we show here, using specific inhibitors, that both enzymes, when expressed by APCs, can have an important role in limiting arginine availability for T cell proliferation.

How amino acid levels are sensed by mammalian cells is still not entirely clear. The 2 main pathways thought to be responsible are the ISR, via GCN2, and the mTOR pathway. GCN2, which has a histidinyl-tRNA-like binding site and is thought to bind uncharged tRNAs when the relevant amino acid substrate is limiting (30), is involved in nutritional sensing of amino acid levels by the brain (31, 32), and has been implicated in the sensing of tryptophan levels during IDO-mediated immune regulation (12, 14). GCN2-

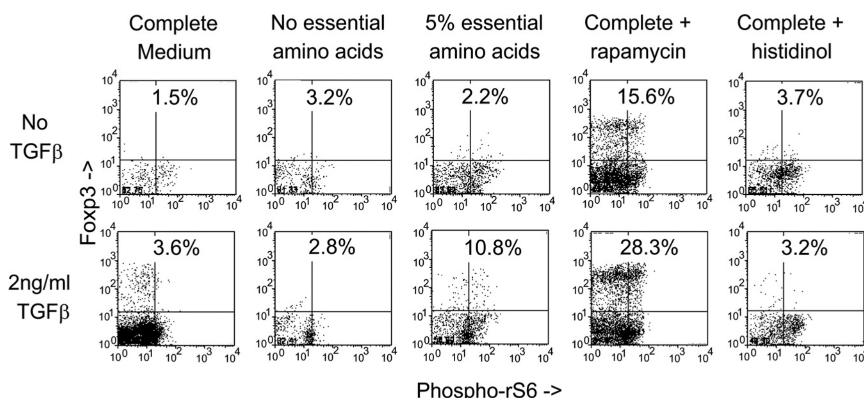

Fig. 5. Inhibition of mTOR signaling during T cell activation, either by rapamycin or amino acid depletion, induces foxp3 in synergy with TGF-β. Naive $CD4^+$ T cells from female A1.$RAG1^{-/-}$ mice were stimulated with syngeneic bmDC in the presence of 100 nM DBY peptide for 4 days at 37 °C under the conditions indicated. Cells were surface-stained for CD4 and after fixation and permeabilization for foxp3, p4E-BP1, and pS6 they were analyzed by flow cytometry. Dot plots are shown for foxp3 versus pS6 after gating on $CD4^+$ cells within the live lymphocyte forward and side scatter gates. Percentages of $foxp3^+$ for $CD4^+$ T cells are indicated. pS6 staining positively correlated with p4E-BP1 staining.

mediated activation of the ISR pathway acts, via phosphorylation of eIF2α, to inhibit translation and induce transcription factors including ATF4, which mediate changes in gene expression including the up-regulation of *chop* and *gadd34*. The mTOR/PI3K pathway, which plays an important role in many aspects of nutrient-, hormone-, and growth factor-mediated cell growth (22), has recently been found to sense amino acids via *vps34*, a class 3 PI3 kinase (33, 34), and by the Ras-related GTP-binding (RAG) proteins (17). mTOR signaling is known to control the translation of genes involved in the cell cycle, via the phosphorylation of the ribosomal protein S6 and 4E-BP1. The PI3K/mTOR pathway is often considered to be most sensitive to branched chain amino acids, particularly leucine (22), but our data suggest that it participates in detecting levels of any single EAA, even in the absence of GCN2. In wild-type T cells being activated for the first time, a lack of any single amino acid modulates both the GCN2 and PI3K/mTOR pathways, suggesting there is a potential for functional redundancy.

It has also been reported that Tregs are relatively resistant to the mTOR inhibitor rapamycin (35), which might give them a growth or survival advantage over effector cells under conditions of amino acid starvation and reduced mTOR signaling. More recently, PI3K/mTOR inhibition has been implicated in the de novo differentiation of Tregs (15, 16). We show here that reduced mTOR signaling, whether by reduced EAA levels or specific inhibitors during initial T cell activation, is indeed able to induce de novo foxp3 expression in a manner that is synergistic with TGF-β. Foxp3 expression was found to be sensitive to the entire range of EAA concentrations, such that providing excess EAAs even suppressed the induction of Tregs during exposure to normally optimal levels of TGF-β. Optimal foxp3 induction was found to depend on moderately reduced mTOR signaling (as determined by intermediate phospho S6 and 4E-BP1 levels), rather than a nonspecific response to adverse conditions, because excessive mTOR inhibition, for example by complete removal of all EAAs, was less effective for foxp3 induction. In addition, activation of the GCN2/ISR pathway by histidinol, at concentrations that inhibited proliferation, was unable to induce foxp3.

It should be noted that although Tregs were able to induce the maximal up-regulation of many of the EAA-consuming enzymes within skin grafts, both conventional inflammatory T cells and T cell-independent innate responses were also associated with the up-regulation of some enzymes, particularly *nos2*, *bcat1*, and *tdh*. This up-regulation may represent a built-in feedback system to limit pathology during the course of a normal immune response or protect tissues from attack during wound healing. Perhaps the high absolute levels of foxp3 and Tregs that are frequently associated with graft rejection (36–38) are acting to limit, but are unable to abolish, the vigorous inflammatory response.

We have shown that multiple EAA-consuming enzymes are induced in skin grafts and APCs, partly as a response to inflammation and/or wound healing, but particularly via an antigen-specific interaction with Tregs. Depletion of any 1 EAA in vitro by the expression of these enzymes on DCs leads to an inhibition of T cell activation and proliferation, combined with a synergistic induction of foxp3^{+} Tregs by TGF-β. This finding indicates that induced consumption of EAAs represents a strong candidate for a general, and possibly fundamental, component of immune regulation and infectious tolerance. The central role played by mTOR signaling may also provide a rationale for optimizing the use of mTOR pathway inhibitors in the therapeutic induction of immune tolerance in transplantation and autoimmunity.

Materials and Methods

Mice, Skin Grafting, and Tolerance Induction. CBA/Ca, CBA.RAG1$^{-/-}$, A1.RAG1$^{-/-}$, Des.RAG1$^{-/-}$, CBA.IDO$^{-/-}$ (39) (all H-2^{k}), C57BL/6, C57BL/6.IDO$^{-/-}$ (39), C57BL/6.GCN2$^{-/-}$ GCN2.KO4 (31), and Marilyn (40) (all H-2^{b}) mice were bred and maintained under specific-pathogen-free conditions in the animal facility of the Sir William Dunn School of Pathology. All procedures were conducted in accordance with the Home Office Animals (Scientific Procedures) Act of 1986. Skin grafting was performed as described (4). Cells for adoptive transfer [Dby-specific, induced Tregs (DBYT), 10^{7} per mouse] were injected into the tail vein of recipient mice the day before skin grafting. In some experiments mAbs were administered at the same time as grafting [1 mg each of anti-TGF-β (1D11), anti-IL10R (Jes5), or anti-IL25R or CD25 (PC61)].

Quantitative Real-Time RT-PCR. Total RNA was prepared from whole skin grafts by using SV Total RNA Lysis Buffer (Promega) and DNase-I treatment. cDNA was generated by using the StrataScript First Strand Synthesis System (Stratagene), with random hexamer primers. Real-time RT-PCR and analysis were performed with the ABI/PRISM 7900 sequence detector system (Applied Biosystems) and inventoried ''assay on demand'' Taqman gene expression assays (Table S1) as recommended (Applied Biosystems). Relative quantities (RQ) were calculated by the $\Delta\Delta$Ct method (41). Samples that gave no detectable signal were assigned $C_t = 40$. Data are presented as means $\pm$ SD of $\log_{10}$(RQ) with normalization to *hprt1* unless otherwise indicated.

Tissue Culture Medium. RPMI medium 1640 lacking EAAs (Invitrogen) was supplemented with antibiotics, sodium pyruvate, glutamine, 2-mercaptoethanol, and 10% (vol/vol) dialyzed FCS. EAAs were prepared as individual stock solutions and added to known concentrations relative to complete RPMI medium 1640 = 100% (for concentrations see Table S3).

T Cell Proliferation Assays. Splenic CD4^{+} and CD8^{+} T cells were prepared by using CD4 or CD8 isolation kits (Miltenyi Biotec). CD4^{+}CD25^{+} and CD4^{+}CD25^{-} populations were sorted by MoFlo using anti-CD4-PerCP and anti-CD25-PE (Becton Dickinson). A total of 5×10^{4} T cells per round-bottomed well in 200 μL were activated with equal numbers of CD3/CD28 beads (Invitrogen) at 37 °C in humidified 5% CO_2. In some experiments IL-2 and IL-7 were added at 10 units/mL and 10 ng/mL, respectively. Proliferation was measured by ^{3}H thymidine incorporation. To preactivate cells, CD4^{+} cells were stimulated for 48 h by using plate-bound anti-CD3 (1452C11, 1 μg/mL) and soluble anti-CD28 (37.51, 1 μg/mL).

DC Cultures. Splenic DCs were prepared by collagenase digestion (1 mg/mL) and selection with CD11c magnetic beads (Miltenyi) (10). Mouse CTLA4-Ig (Sigma; C4433) was used at 100 μg/mL. bmDCs were prepared as described (19, 42). DCs were harvested on day 7 and cultured at 10^{6}/mL in medium containing limiting amounts of the EAA under investigation, with or without the appropriate enzyme inhibitor (Table S1), and LPS (Sigma) was added at 1 μg/mL for 18 h. Supernatants were centrifuged, 0.2-μ-filtered, and used at a 1:1 ratio with fresh medium containing all EAAs except the one of interest, for proliferation assays with CD4^{+} T cells activated with CD3/28 beads.

Tregs. The clone Tr1D1 has been described (43). Induced Tregs (DBYT) were from naïve A1.RAG1$^{-/-}$ spleen cells cultured with bmDCs, 100 nM DBY peptide (REEALHQFRSGRKPI), and 2 ng/mL rTGF-β plus retinoic acid (1 μM). Staining indicated $\geq$75% foxp3^{+} compared with T cells activated in the absence of rTGF-β ($\leq$2%).

DC and Treg Cocultures. RNA for RT-PCR was prepared from bmDCs (10^{6}/mL) cultured either alone or with equal numbers of male-specific T cells (DBYT, DBY, or Tr1D1) and stimulated either with or without cognate DBY peptide (100 nM) for 48 h at 37 °C.

Immunofluorescence and Flow Cytometry. Cells were stained with CD4-APC, CD3-APC (BD) and for intracellular foxp3-(FITC or PE) as recommended (eBiosciences). Cells were fixed in formaldehyde and permeabilized with saponin buffer to stain with rabbit mAb conjugates Alexa488 anti-phospho-4E-BP1 (Thr-37/46) and Alexa647 anti-phospho-S6 (Ser 235/236) (Cell Signaling Technology). 7AAD (Sigma) and FITC-VAD-FMK staining was performed as recommended (Promega). Four-color immunofluorescence was measured by using a FACSCaliber and FlowJo software (Tree Star).

Statistics. Statistical differences in mRNA transcript $\log_{10}$(RQ) values were identified by using Student's T test (unpaired, 2-tailed). $P < 0.05$ was considered significant.

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Supporting Information

Cobbold et al. 10.1073/pnas.0903919106

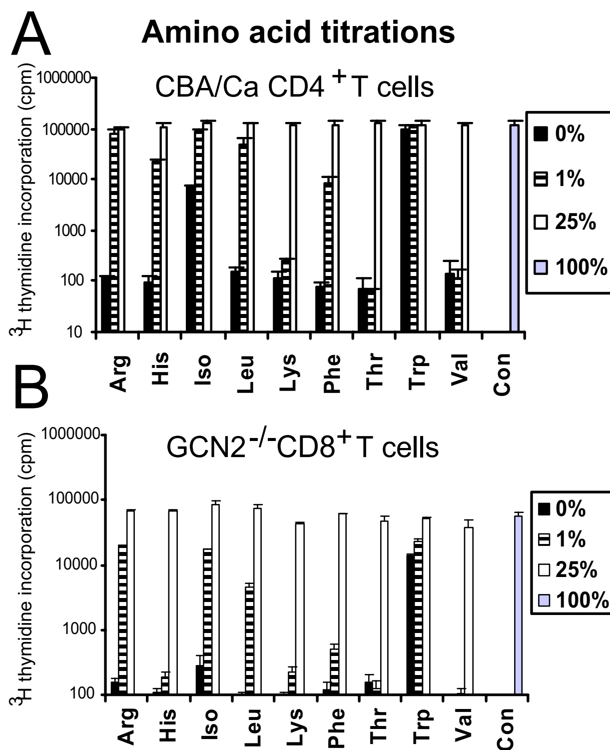

Fig. S1. The depletion of any single EAA causes a profound inhibition of naive T cell proliferation, independent of GCN2. T cells from the indicated sources, purified by magnetic negative selection, were stimulated at 37 °C for 48 h by equal numbers of anti-CD3^{+}CD28 beads in RPMI medium 1640 containing 10% (vol/vol) dialyzed FCS. Proliferation was measured by using the uptake of ^{3}H thymidine. Each amino acid of interest was titrated up to 100% of the level in complete RPMI medium 1640 (only selected concentrations are shown for clarity). Note that the apparent lack of effect of depleting tryptophan is an artifact of using dialysed FCS, as tryptophan is uniquely carried bound to serum albumin. In serum-free cultures, we confirmed that tryptophan depletion is as effective at inhibiting proliferation as for any of the other EAAs.

Fig. S2. The inhibition of proliferation caused by amino acid depletion is reversible. T cells from the indicated sources, purified by magnetic negative selection, were stimulated at 37 °C for 48 h by equal numbers of anti-CD3$^+$CD28 beads in RPMI medium 1640 containing 10% (vol/vol) dialyzed FCS. T cells were stimulated in medium lacking the indicated EAAs for 48 or 96 h before measuring proliferation (gray bars) or were then given additional, complete medium for another 48 h before measuring proliferation (slashed bars). Proliferation was measured by using the uptake of ^{3}H thymidine. Note that the apparent lack of effect of depleting tryptophan is an artifact of using dialyzed FCS (see legend to Fig. S1).

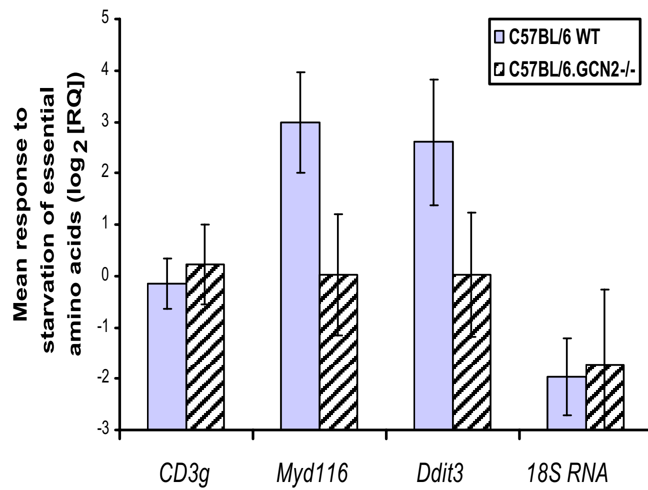

Fig. S3. Amino acid starvation does not induce the integrated stress response in GCN2$^{-/-}$ mice. CD4^{+} T cells from C57BL/6 mice (gray bars) and C57BL/6.GCN2$^{-/-}$ mice (slashed bars) were stimulated by anti-CD3^{+}CD28 beads for 48 h at 37 °C in the absence of each individual EAA. These cells were analyzed by TaqMan RT-PCR for the expression of gene transcripts downstream of the ISR, particularly *myd116* (GADD34) and *ddit3* (CHOP). The geometric means of RQ values were determined across all cells stimulated in medium lacking any single EAA (8 independent biological replicates) compared with controls stimulated in complete medium (mean of 2 biological replicates; defined as RQ = 0 for each gene tested). Data are from 1 of 2 similar experiments.

Fig. S4. The control of T cell proliferation by amino acid starvation acts via the mTOR/PI3K pathway. (*A–D*) Naïve CD4$^+$ T cells from C57BL/6 mice (*A* and *B*) or C57BL/6.GCN2$^{-/-}$ mice (*C* and *D*), were stimulated by anti-CD3$^+$CD28 beads at 37 °C for 16 h in the absence of the EAAs indicated or in the presence of rapamycin and/or wortmannin (representative examples shown for clarity). Cells were formaldehyde-fixed, permeabilized with saponin, and stained with rabbit mAbs conjugated to Alexa-647 and Alexa-488 specific for phospho-(Ser-235/Ser-236) ribosomal S6 (pS6) and phospho-(Thr-37/46) 4E-BP1 (p4E-BP1) proteins, respectively. Samples were analyzed by flow cytometry using a forward and side scatter gate appropriate to resting or activated lymphocytes. Percentage of positive staining for the same set of conditions is shown in all pS6-stained panels and median fluorescence for p4E-BP1 staining. Naïve CD4$^+$ T cells from C57BL/6 mice were stimulated by anti-CD3$^+$CD28 beads at 37 °C for 16 h in the absence of the individual essential amino acids indicated or in the presence of the mTOR inhibitor rapamycin. (*E* and *F*) Western blots for total and pS6 and β-actin (*E*) and total and p4E-BP1 (*F*) were performed as described in *Materials and Methods*. Blots shown are representative of 4 similar experiments.

Fig. S5. TGF-β and mTOR inhibition are synergistic for the induction of foxp3 message and protein. Marilyn CD4$^+$ T cells were stimulated with syngeneic bmDC plus 100 nM DBYA^b peptide, together with different concentrations of rhTGFβ1 (0, 0.2, and 2.0 ng/mL) in RPMI medium 1640 with, where indicated, various concentrations of individual or all EAAs plus 10% dialyzed FCS for 4 days at 37 °C. The inhibitors rapamycin, wortmannin, or histidinol were also added where indicated. (*A*) Cells were harvested for total RNA and quantitative RT-PCR for foxp3 transcripts normalized to the housekeeping gene *hprt1*. Each TGF-β condition was then normalized to the level of foxp3/hprt1 expressed by control cultures in complete medium to emphasize the synergy. (*B*) Cells were harvested, fixed, permeabilized, and stained with PE-conjugated monoclonal anti-foxP3 antibody before analysis by flow cytometry for percentage of foxp3-positive cells.

Table S1. Enzymes that consume essential amino acids and their inhibitors

Essential amino acid substrate	Enzyme	Gene	ABI RT-PCR ''assay on demand''	Metabolite	Inhibitor
Arginine	Arginase 1	*Arg1*	Mm00475988_m1	Orntihine	NG-hydroxy L arginine (NGHA); 0.5 mM
Arginine	iNOS	*Nos2*	Mm00440485_m1	NO	*N*-monomethyl arginine (NMLA); 10 mM
Histidine	Histidine ammonia lyase	*Hal*	Mm00456709_m1	Urocanate	3-methylhistidine; 5 mM
Histidine	Histidine decarboxylase	*Hdc*	Mm00456104_m1	Histamine	(+)-Cyanidol-3 (Catechin hydrate); 0.1 mM
Histidine	Dopa decarboxylase	*Ddc*	Mm00516688_m1	Histamine	
Leucine, isoleucine, valine	Branched chain aminotransferase (cytosolic)	*Bcat1*	Mm00500289_m1	Keto acid	2-oxoisovalerate (OXO-IVAL; competitive for valine); 0.1 mg/mL
Lysine	Aminoadipate semialdehyde synthase	*Aass*	Mm00497118_m1	Aminoadipate	
Phenylalanine	IL4i1 (L-amino acid oxidase)	*IL4i1*	Mm00515786_m1	Phenylketo acid	p-aminobenzoic acid (PABA); 10 mM
Threonine	Threonine dehydrogenase (not present in humans)	*Tdh*	Mm00451856_g1	Glycine	
Tryptophan	IDO	*Indo*	Mm00492586_m1	Kynurenines	1 methyl-DL-tryptophan (1MT); 200 mM
Tryptophan	Tryptophan dioxygenase	*Tdo2*	Mm00451266_m1	Kynurenines	1 methyl-DL-tryptophan (1MT); 200 mM
Tryptophan	Tryptophan hydoxylase	*Tph*	Mm00493794_m1	Serotonin	p-chlorophenylalanine methyl ester (PCPA); 5 mM

Table S2. Synergy between rapamycin and TGF-β for foxP3 induction

Rapamycin concentration	% Foxp3+						
	BMDC + DBY peptide stimulation				CD3+CD28 bead stimulation		
	No added TGF-β	Anti-TGF-β mAb	Plus 0.2 ng/mL TGF-β	Plus 2.0 ng/mL TGF-β	No added TGF-β	Plus 0.2 ng/mL TGF-β	Plus 2.0 ng/mL TGF-β
None	0.9	0.1	3.4	24.8	0.6	15.6	45.7
5 μM	19.0	5.7	**40.1**	41.3	nd	nd	nd
1 μM	19.3	6.3	**50.4**	**53.4**	nd	nd	nd
500 nM	20.4	4.7	**49.8**	**54.1**	nd	nd	nd
100 nM	21.6	4.2	**47.5**	**52.8**	nd	nd	nd
50 nM	21.0	3.0	**48.5**	**54.6**	9.1	**40.1**	43.7
10 nm	21.2	2.7	**42.5**	**48.4**	7.7	**44.0**	42.9
5 nM	15.7	2.5	**43.6**	**45.7**	5.9	**45.6**	43.2
1 nM	8.3	1.6	**26.6**	**38.0**	2.8	**42.9**	41.1
500 pM	4.1	1.6	**17.2**	**33.0**	4.9	**37.4**	27.8
100 pM	2.9	1.9	4.3	23.9	1.2	**36.8**	40.4
50 pM	1.9	1.4	5.8	21.6	0.9	**40.1**	**48.1**

Bold type indicates rapamycin and TGF-β synergy (ie. greater than additive effect). nd, not determined.

Table S3. RPMI medium 1640 formulation (Invitrogen catalog no. 30–2001)

Essential amino acids	mg/L
L-Arginine (free base)	200.00
L-Histidine (free base)	15.00
L-Isoleucine	50.00
L-Leucine	50.00
L-Lysine·HCl	40.00
L-Phenylalanine	15.00
L-Threonine	20.00
L-Tryptophan	5.00
L-Valine	20.00
L-Tyrosine·2Na·2HO	28.83