T Cells Encountering Myeloid Cells Programmed for Amino Acid-dependent Immunosuppression Use Rictor/mTORC2 Protein for Proliferative Checkpoint Decisions*

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Modulation of T cell proliferation and function by immuno-regulatory myeloid cells are an essential means of preventing self-reactivity and restoring tissue homeostasis. Consumption of amino acids such as arginine and tryptophan by immunoregulatory macrophages is one pathway that suppresses local T cell proliferation. Using a reduced complexity in vitro macrophage-T cell co-culture system, we show that macrophage arginase-1 is the only factor required by M2 macrophages to block T cells in G1, and this effect is mediated by l-arginine elimination rather than metabolite generation. Tracking how T cells adjust their metabolism when deprived of arginine revealed the significance of macrophage-mediated arginine deprivation to T cells. We found mTORC1 activity was unaffected in the initial G1 block. After 2 days of arginine deprivation, mTORC1 activity declined paralleling a selective down-regulation of SREBP target gene expression, whereas mRNAs involved in glycolysis, gluconeogenesis, and T cell activation were unaffected. Cell cycle arrest was reversible at any point by exogenous arginine, suggesting starved T cells remain poised awaiting nutrients. Arginine deprivation-induced cell cycle arrest was mediated in part by Rictor/mTORC2, providing evidence that this nutrient recognition pathway is a central component of how T cells measure environmental arginine.

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Modulation of T cell responses in both normal and inflamed tissue prevents immunopathology and “horror autotoxicus” self-reactivity. Among the many interlocking pathways that can suppress T cell proliferation and activity, myeloid cell-mediated amino acid deprivation is a key checkpoint in preventing immunopathology. For example, both tryptophan and arginine are essential for T cell growth and function, and their local degradation by indoleamine oxidases (IDO1 and IDO2) or arginase 1 (Arg1) and possibly arginase 2 (Arg2) expressed in myeloid cells restrains T cell proliferation, limits tissue damage, and contributes to immunologic tolerance (1–5). Amino acid starvation is also a medically important strategy to target cells autotrophic for specific amino acids. For example, asparaginase deprives leukemic cells of their exogenous supply of asparagine, and the fungal metabolite halofuginone blocks pathogenic Tfh17 responses by interfering with the glutamyl-prolyl tRNA synthase causing an amino acid stress response (6, 7). Arginine and tryptophan degradation pathways are linked to malignancy and chronic infections, where tumors and pathogens subvert the host’s T cell control pathways to suppress productive immune responses (8, 9). Amino acid metabolism is also linked to Treg development and stability at sites of infection via a process termed “infectious tolerance,” which is a self-reinforcing pathway aiding in tissue resolution and repair following inflammation (10).

The liver immune response to Schistosoma mansoni eggs is an example of how myeloid cells exert control over T cells through amino acid metabolism. In murine and human schistosomiasis, worm eggs lodged in the liver drive a Th2-mediated asynchronous granulomatous response characterized by collagen deposition and fibrosis, all of which are required to wall off the eggs, which are highly toxic (11, 12). The fibrotic granulomas protect the surrounding tissues from damage caused by the toxic eggs until they can be degraded. The Th2 response recruits inflammatory Ly6C+ monocytes from the blood to granulomas (13); there they differentiate into macrophages and become activated by IL-4 and IL-13 to the “alternatively activated” or “M2” pathway. In this context, M2 macrophages express high amounts of the arginine hydrolase Arg1 regulated via the IL-4- and IL-13-induced STAT6 pathway (14, 15). When mice lacking Arg1 specifically in macrophages were infected with schistosomes, an unregulated Th2 response occurred leading to a failure to down-regulate the pro-fibrotic response, excessive production of IL-4 and IL-13, hepatomegaly, and...
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Early lethality (16). However, when eggs are artificially introduced into the lung via intravenous administration, a T(i)2-proliferative response occurs independent of macrophage Arg1 (17). Lung tissue contains little arginase activity, whereas liver hepatocytes express high and constitutive Arg1 as part of the urea cycle, which eliminates excess nitrogen via urea. Because Arg1 catalyzes the same biochemical degradation of arginine in hepatocytes and macrophages, and because granulomas are embedded in the Arg1-rich hepatocyte parenchyma, we hypothesized that microenvironmental arginine depletion by macrophage Arg1 close to the granuloma nucleus is the key step in restricting T cell activity, thus blocking excessive immune responses. Here we developed an in vitro cellular biochemistry system to explore the mechanistic basis of microenvironmental arginine depletion sensing by T cells.

Results

T Cells Rapidly Sense Active Arginine Depletion by Arg1+ Macrophages—In the absence of macrophage Arg1, mice with schistosomiasis developed a non-resolving inflammatory T(i)2 response leading to increases in T cell number, cytokine production, fibrosis, portal hypertension, and accelerated death (16, 18). T(i)2-polarized responses induced local M2 activation of macrophages, including Arg1 expression, and liver sections from schisto-some-infected mice showed Arg1 expression was concentrated around the T(i)2-inciting stimulus, the worm egg (Fig. 1A), instead of evenly throughout the granulomatous regions. Although the mechanism of Arg1 spatial segregation within the granuloma is unclear, macrophage Arg1 is essential for suppressing excessive T cell responses in this setting even though the granulomas are embedded in the Arg1+ liver parenchyma (Fig. 1A). One explanation for the role of Arg1+ macrophages in liver granulomas is that they deplete arginine in the regions where T cells are sampling egg-derived antigens. In this context, T cells encountering Arg1+ macrophages may find themselves in a low arginine environment (19), as arginine is vital for T cell proliferation (1, 3, 20, 21). We speculated dissection of the T cell–Arg1+ macrophage interplay may give insight into how myeloid cells regulate T cells through amino acid-dependent pathways. However, the liver inflammatory microenvironment is complex and thus served as the entry point for attempts to develop simpler in vitro systems to dissect the Arg1+ macrophage–T cell interplay (Fig. 1B).

We used inflammatory peritoneal macrophages as both an antigen-presenting and immunoregulatory cell in T cell co-cultures because these cells are closely related to the type of Ly6C(â) bone marrow monocyte-derived macrophages recruited to schistosome egg granulomas (11, 13). With only antigen added, these macrophages stimulated ovalbumin (OVA)3-specific T cells to proliferate over 72 h (16). However, when macrophages were stimulated with IL-4 + IL-10 24 h preceding the addition of OVA peptide and T cells, T cell proliferation was blocked (Fig. 1C). An important element of the macrophage–T cell co-culture system common to all experiments used in this study is that all cultures were washed, and the media were replaced at time 0, regardless of any prior stimulations. The replacement of complete media included 1147 µM arginine, which is the arginine concentration in conventional RPMI 1640 medium formulations. Thus, in all experiments, time 0 conditions all begin at the same arginine concentration, unless otherwise indicated.

Using cellular biochemical approaches, we further dissected key features of the macrophage–T cell co-culture system. First, the addition of supra-physiological arginine (3 mM) alone is sufficient to rescue proliferation at any point in the culture period (Fig. 1D). T cell death in the culture was not influenced by arginine deprivation (Fig. 1E), and exogenous addition of PEGylated Arg1 was sufficient to inhibit T cell proliferation (Fig. 1F). The inhibitory effect of macrophage Arg1 was replicated by artificially manipulating the arginine concentration in the media using 20 to 0% of arginine used in normal RPMI 1640 medium (i.e. 1147 µM) where macrophages were not induced to express Arg1 (Fig. 1G). Finally, the Arg1 reaction produces two products, urea and ornithine. However, ornithine addition (Fig. 1H) or urea3 at the same molarity as the total quantity arginine, or greater, had no effect on T cell proliferation, arguing the products of the Arg1 reaction are not responsible for the blockade in T cell proliferation in the macrophage–T cell co-cultures.

IL-4- and IL-10-stimulated Macrophages Inhibit T Cell Proliferation by an Arg1-dependent Mechanism, Independent of PD-L2—IL-4 signaling is a potent inducer of the expression of PD-L2. High PD-L2 is one of the hallmarks of M2 macrophages and has previously been suggested to have a key immunoregulatory role in suppressing T cells by binding PD-1, a key negative regulatory pathway in T cells (22, 23). Indeed, IL-4 + IL-10 stimulation of macrophages caused increased and sustained expression of PD-L2 over the 72-h culture period (Fig. 2A). However, addition of blocking antibodies to PD-1, which blocks the interaction between PD-1 and its ligands PD-L1 or PD-L2, could not overcome the macrophage-mediated block in T cell proliferation (Fig. 2B). Instead, the inhibitory effect of IL-4 + IL-10-stimulated macrophages toward activated T cells was solely dependent on Arg1, as the same macrophage population isolated from mice with a pan-hematopoietic and complete deletion of Arg1 (24) failed to inhibit proliferation (Fig. 2C). These data rule out a predominant role of the mitochondrial arginase, Arg2, in the inhibitory process. Taken together with the data presented in Fig. 1, immunoregulatory macrophages programmed to express Arg1 inhibit T cell proliferation via an Arg1-dependent amino acid depletion mechanism.

Depletion of Arginase by Arg1+ Macrophages Is Efficient and Rapid—Next, we quantified the dynamics of arginine metabolism in the macrophage–T cell co-culture system using a radiolabeling approach where arginine and ornithine, the product of the Arg1 (and Arg2 reaction), were measured across time. In co-cultures where macrophages were pre-treated with IL-4 +

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3 The abbreviations used are: OVA, ovalbumin; SREBP, sterol regulatory element-binding protein; TCR, T cell receptor; mTOR, mechanistic target of rapamycin; qRT, quantitative RT; FFA, free fatty acid; iNOS, inducible nitric oxide synthase; LN, lymph node; BMDM, bone marrow-derived macrophage; CFSE, carboxyfluorescein diacetate succinimydyl ester.

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IL-10, arginine was almost undetectable, and most was converted to ornithine (Fig. 3A, 24 h). In cultures where macrophages were pre-treated with IL-4/IL-10 and supplemented with 3 mM arginine, most of the arginine was converted to ornithine within 24 h, but sufficient amounts remained for T cells to proliferate to the same extent as controls (Fig. 1D). In control cultures where macrophages were not treated with IL-4 + IL-10, arginine was also partly converted to ornithine, but this process took >48 h (Fig. 3, A–C). Arg1 and Arg2 expression in the inflammatory monocyte-derived macrophages, along with further stimulation of Arg1 expression by paracrine IL-4 and IL-13 secreted by the activated and growing CD4+ T cells, likely mediate the hydrolysis of arginine in this setting, in addition to the possibility that T cells also convert arginine to ornithine (20). These data illuminated three key points for the interpretation of T cell proliferation in the co-culture system. First, the
conversion of arginine to ornithine by Arg1+ macrophages is very efficient. Within 24 h, 1147 μM arginine was converted to ornithine, and in the case where arginine was supplemented, ~4 mM arginine was almost completely converted to ornithine in the same period. Second, T cells co-cultured with Arg1+ macrophages are exposed to a low arginine environment within a 24-h period. Therefore, the arginine “sensing” process linked to cell cycle entry occurs in the initial 24-h time period after antigen stimulation. Third, supplemental arginine, even though almost completely converted to ornithine within 24 h, was sufficient to rescue proliferation (Fig. 1D). Thus, CD4+ T cells use a decision-making process about cell cycle commitment within an ~24-h window when they estimate how much environmental arginine is available for growth.

Arg1+ Macrophages Arrest T Cells in G1, Independent of TCR Expression or Signaling—Arginine deprivation can impair TCR CD3ζ expression and therefore activation (25), but we could not find any specific effects on CD3ζ expression by intracellular staining. Instead, we measured cell surface CD3ζ, which is an essential component of the TCR, and found no loss in surface TCR expression on arginine-starved T cells at any time point across the culture period (Fig. 4A), suggesting the presence of an intact TCR throughout the assay period. Furthermore, when we profiled gene expression by CD4+ cells purified from co-
cultures with Arg1+ macrophages (Fig. 4B), we did not observe any statistically significant differences in the canonical gene expression profile activated after TCR stimulation. Taken together, these data indicate Arg1+ arginine-depleting macrophages do not affect surface TCR amounts or signaling within the time frame of the assay. Our data, however, do not exclude possible effects of low arginine on surface TCR or CD3ζ after prolonged incubation or exposure periods.

Previous work has suggested arginine-deprived T cells arrest in the G1 phase of the cell cycle (26). To confirm this observation in our co-culture system, we sorted CD4+ T cells from co-cultures at 24, 48, or 72 h, when T cells were exposed to macrophages, Arg1+ macrophages, or Arg1+ macrophages supplemented with excess exogenous arginine to rescue proliferation. We then performed immunoblotting for cyclins D2 or D3, as these are the D-type cyclins used in T cells to respond to mitogenic signaling from IL-2 and the TCR, which aids progression through the cell cycle to mitosis (27). We found cyclin D3 expression was highly induced in proliferating CD4+ T cells at 48 h, whereas arginine-starved T cells did not up-regulate cyclin D3 expression to the same extent (Fig. 4C). Addition of exogenous arginine rescued cyclin D3 expression. Expression

**FIGURE 3. Quantification of arginine depletion by thin layer chromatography.** A, macrophage-T cell co-cultures were incubated in arginine-deficient RPMI 1640 medium where [3H]arginine replaced the normal concentration of arginine. Macrophages were stimulated as in Fig. 1. At 24, 48, or 72 h, culture supernatants were isolated, precipitated, and resolved by thin layer chromatography compared with internal standards. B and C, quantification of arginine and ornithine across time in the cultures expressed as a percentage of the input concentrations. Data are representative of two experiments.
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A

CD3e CFSE

24 h 48 h 72 h

+ OVA + OVA
IL-4 + IL-10

B

+ OVA + OVA
IL-4 + IL-10

C

- + + + + + + OVA peptide
- + + + + + + IL-4 + IL-10
- + + + + + + L-arginine

Cyclin D3
Cyclin D2
GRB2

24 h 48 h 72 h

D

- + + + + + + OVA peptide
- + + + + + + IL-4 + IL-10
- + + + + + + L-arginine

pSTAT5
STAT5

24 h 48 h 72 h

E

0 hr 8 hr 24 hr 32 hr 48 hr 56 hr 72 hr

+ OVA + OVA
IL-4 + IL-10

CD4 pSTAT5

F

IL-2 24 h

Normalized expression

Normalized expression

IL-2 48 - 72 h

G

IL-2 production

- Control
- OVA
- OVA + IL-4 + IL-10
- arginine

ng/mL

Time (h)
of cyclin D2 was also regulated in a similar way, although to lower apparent amounts compared with cyclin D3 (Fig. 4C). These data are consistent with the notion that arginine-starved cells have arrested at a point in G1 preceding the increase in D-type cyclin expression.

In \textit{in vitro} T cell proliferation assays, IL-2 is the major mitogen responsible for inducing a signaling and transcription program necessary for progression through G1 and entry into mitosis (28, 29). IL-2 synthesis is induced by TCR and co-stimulatory pathways, works in an autocrine-paracrine way, and is consumed through the proliferative cycle. Therefore, we investigated the relationship between IL-2 production and signaling and arginine deprivation induced by Arg1+ macrophages. First, we measured STAT5 activation, as this is a key downstream factor required for IL-2 signaling (30). Consistent with the cyclin D3 expression data (Fig. 4C), we observed p-STAT5 amounts remained low relative to the increase at \(-48\) h observed in proliferating cells (Fig. 4D). These data were further confirmed by a finer time course using intracellular staining for p-STAT5 in CD4\(^+\)-gated T cells (Fig. 4E). Therefore, arginine deprivation affects IL-2 signaling, raising the possibility that IL-2 production was affected by Arg1+ macrophages. Interrogation of the microarray data revealed IL-2 is the highest differentially induced mRNA in CD4\(^+\) T cells exposed to Arg1+ macrophages at 48 h, which we confirmed by qRT-PCR (Fig. 4F). Furthermore, IL-2 production at the protein level was increased and sustained relative to proliferating T cells (Fig. 4G). Therefore, arginine-starved CD4\(^+\) T cells arrest in G1, but produce IL-2 mRNA and protein. However, the IL-2 signal through the IL-2R appeared blocked. We tested whether the IL-2 made in the IL-4 + IL-10 macrophage-T cell co-cultures could have been modified in some way. We added exogenous IL-2 or IL-2 superkine (a modified IL-2 with increased binding and signaling properties (31)) to Arg1+ macrophage-T cell co-cultures in excess and daily across a 72-h time window. We observed no rescue of proliferation in any case suggesting T cells respond to arginine deprivation at a point involving part IL-2 signaling but not IL-2 production. In other words, IL-2 fails to elicit progression through G1, in arginine-deprived T cells because a “downstream” component(s) of the mitogen signaling pathway is blocked in low arginine environments.

\textbf{Activation of mTORC1 in Arginine-starved T Cells Is Unaffected in G1, and Cannot Be Bypassed by Loss of TSC Inhibitors}—Activation of the mTORC1 pathway has been linked to amino acid availability, including arginine (32–37). To test whether mTORC1 was regulated in T cells exposed to Arg1+ immuno-regulatory macrophages, we took kinetic and gain-of-function approaches, noting that complete knock-out of Raptor, mTOR, or Rheb or the addition of rapamycin inhibits efficient T cell proliferation \textbf{per se}, meaning the “control” arm of the experiment (\textit{i.e.} mTORC1 loss-of-function T cells exposed to macrophages without IL-4 + IL-10) cannot be used (38–41). Kinetically, mTORC1 activity, measured by pS6 amounts, occurred equivalently in T cells exposed to control or arginine-depleting macrophages (Fig. 5A). Around the time point where the control T cells are about to divide for the first time (Fig. 5A, \textit{dashed line}), we noted pS6 amounts in arginine-starved T cells were reduced relative to controls. Nevertheless, initial mTORC1 activity occurred normally in T cells in an arginine-depleted environment. Given that the data in Fig. 3 showed arginine amounts were rapidly depleted to zero by Arg1+ macrophages within 24 h, these data suggest mTORC1 activity is not overtly affected by a low arginine extracellular milieu. The flow data were further confirmed by sorting T cells from the macrophage-T cell co-cultures at 24, 48, and 72 h. These experiments showed pS6K, p4E-BP1, and p-mTOR amounts were approximately equivalent in the first 24 h of the co-culture and declined in amount at 48 h in cultures with low arginine availability relative to proliferating controls (Fig. 5, B and C).

Previous studies have shown TSC2 is required to inhibit mTORC1 in low amino acid settings through a complex pathway that involved Rag GTPases (42). We tested a gain-of-function approach by creating \textit{Tsc1}\textsuperscript{floxed/floxed};CD4-Cre;OTII mice. In these mice both TSC1 and TSC2 are depleted, causing constitutive activation of mTORC1 as described previously (43). T cells from control or TSC1-deficient mice were both inhibited by Arg1+ macrophages in an arginine-dependent way, indicating constitutive mTORC1 activity is insufficient to bypass the arginine-depleting effects of the Arg1+ macrophages (Fig. 5D).

Taken together, our experiments to track mTORC1 activity in CD4\(^+\) T cells encountering low or zero environmental arginine activate the mTORC1 pathway following antigen stimulation, independent of whether sufficient arginine is present to provoke transition from G1 to mitosis. Our data also suggest that mTORC1 activity is responsive to low arginine and/or G1 arrest, but at least 24 h after antigen stimulation, and that promiscuous mTORC1 activation by loss of the TSC complex cannot bypass the G1 block. Therefore, arginine sensing to mTORC1 in T cells may be more complex than previously reported.

\textbf{Arginine Deprivation Causes Selective Changes in the Transcriptomes of Antigen-activated CD4\(^+\) T Cells}—To gain insights into the molecular decision-making processes in T cells exposed to Arg1+ macrophages, we first examined the tran
scriptomes of purified CD4⁺ T cells re-isolated from the co-cultures. As noted above, TCR-activated genes were unaffected (Fig. 2B), and both macrophage populations were loaded with OVA peptide prior to addition of naïve CD4⁺ T cells. The dashed line indicates the time region of the first division measured in parallel CFSE-labeled samples. B and C, immunoblotting analysis for p-mTORC1 or 4E-BP1. CD4⁺ T cells were isolated by magnetic bead separation from co-cultures at 24, 48, or 72 h. Culture conditions were no OVA peptide, OVA peptide (unstimulated macrophages), OVA peptide (IL-4 + IL-10 stimulated macrophages), and the latter with supplemental 3 mM arginine. Protein amounts were normalized by protein content and verified by total S6K amounts in C and E4BP1 in B. D, macrophage-T cell co-cultures using TSC1-deficient T cells. Shown are representative CSFE labelings where input macrophages were from C57BL/6 mice. Note that TSC1-deficient T cells have increased proliferation in +OVA controls relative to wild-type T cells on the same background.

FIGURE 5. mTORC1 activity in arginine-deprived T cells. A, pS6 expression over time in CD4⁺ cells from macrophage T cell co-cultures where macrophages were unstimulated (top) or pre-stimulated to express Arg1 (bottom), and both macrophage populations were loaded with OVA peptide prior to addition of naïve CD4⁺ T cells. The dashed line indicates the time region of the first division measured in parallel CFSE-labeled samples. 8 and C, immunoblotting analysis for p-mTORC1 or 4E-BP1. CD4⁺ T cells were isolated by magnetic bead separation from co-cultures at 24, 48, or 72 h. Culture conditions were no OVA peptide, OVA peptide (unstimulated macrophages), OVA peptide (IL-4 + IL-10 stimulated macrophages), and the latter with supplemental 3 mM arginine. Protein amounts were normalized by protein content and verified by total S6K amounts in C and E4BP1 in B. D, macrophage-T cell co-cultures using TSC1-deficient T cells. Shown are representative CSFE labelings where input macrophages were from C57BL/6 mice. Note that TSC1-deficient T cells have increased proliferation in +OVA controls relative to wild-type T cells on the same background.

The other major gene cluster down-regulated in arginine-starved T cells was a suite of mRNAs encoding proteins involved in fatty acid and cholesterol metabolism (Fig. 6A). The relative down-regulation of targets was not specific to arginine starvation as the same phenotype was also observed in T cells cultured in 1% of the normal RPMI 1640 media concentration of leucine or lysine (Fig. 6B). We confirmed these findings on selected targets using qRT-PCR (Fig. 6C). Notably, the relative decline in gene expression of the targets tested occurred after 24 h of exposure to limiting arginine. Arginine availability did not correlate with transcription of Abca1 or Abcg1, two key cholesterol transporters important for lymphocyte proliferation (44). Furthermore, expression of mRNAs encoding
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glycolysis and gluconeogenesis genes was indistinguishable between proliferating and arginine-starved and antigen-activated but cell cycle-arrested T cells (Fig. 6, D and E). Therefore, T cells respond to arginine depletion by repressing lipid biosynthesis-linked gene expression yet maintain gene expression for glucose homeostasis.

When we examined the lipid/cholesterol-associated mRNAs closely, almost every known SREBP target mRNA was repressed in arginine-starved T cells compared with proliferating or resting T cells (Fig. 6A) (45). SREBPs are membrane-anchored proteins cleaved in response to cell requirements for de novo generation of cholesterol and fatty acids. Cleavage of SREBPs releases a B-ZIP transcription factor required for activating gene expression linked to lipid biosynthesis. Two SREBP-encoding genes generate three SREBP proteins each having complex overlapping and specific functions in regulating lipid and cholesterol homeostasis (45). To determine how arginine starvation intersects with the SREBP pathway, we measured SREBP amounts by immunoblotting and found SREBP protein expression declined relative to controls at the 48-h time point (Fig. 6F). We determined the subcellular location of the active cleaved N-terminal B-ZIP SREBP proteins. In resting T cells (No OVA), SREBP-1 was localized to a membranous structure consistent with the endoplasmic reticulum/Golgi but excluded from the nucleus (Fig. 6G), whereas SREBP-1 was enriched in the nucleus in dividing T cells (OVA, Fig. 6G). By contrast, SREBP-1 was excluded from the nucleus in arginine-starved T cells but returned to the nucleus in cultures when a supraphysiological amount of arginine was supplemented, restoring proliferation (Fig. 6G). SREBP-2 is expressed at lower amounts in T cells but exhibited a similar pattern to SREBP-1.1

Free Fatty Acids Rescue Proliferation in Arginine-starved T Cells by an Indirect Mechanism That Targets Macrophage Arginine Uptake—SREBPs regulate the production of cholesterol and free fatty acids (FFAs) from acetyl-CoA at a branch point catalyzed by ACAT2 (cholesterol) or ACACA (FFAs). Because SREBP-2 were excluded from the nucleus in arginine-starved T cells (Fig. 6G), we reasoned that inhibition of cholesterol metabolites and FFAs may contribute to the growth arrest of arginine-deprived T cells. To test this, we added back downstream products of the SREBP pathway. For example, mevalonate is the product of HMG-CoA reductase, the rate-limiting step of cholesterol biosynthesis. However neither mevalonate nor cholesterol rescued growth of arginine-starved T cells.4 However, the addition of FFAs consisting of 16:0 and 18:1 saturated and unsaturated fatty acids restored growth of arginine-starved T cells (Fig. 7A). 16:0 and 18:1 are products of the endogenous FFA biosynthesis pathway. To determine whether the essential FFAs were limiting under arginine starvation, we omitted each FFA from the mixture one at a time. We found palmitic acid (16:0) was sufficient to restore growth to arginine-starved T cells (Fig. 7A). The restoration of growth in arginine-starved T cells by FFAs suggested the arginine-sensing and utilization pathway(s) and the SREBP-dependent FFA biosynthesis were linked. Growth restoration by FFAs was independent of Toll-like receptor signaling, which has been implicated in the detection of FFAs (46, 47), because the rescue of proliferation occurred in Myd88-/-;Trif-/- macropages stimulated with IL-4 + IL-10 (Fig. 7B). However, further investigation revealed the effects of FFAs were caused not by acting on T cells but instead by regulating arginine metabolism and/or uptake by macrophages in the co-culture system. This indirect mechanism was revealed in different ways. First, FFAs blocked another arginine-dependent pathway, namely the production of nitric oxide by LPS-stimulated macrophages (Fig. 7, C and D). Furthermore, FFAs caused destabilization of iNOS, whose translation, dimeric form, and stability is dependent on a threshold amount of imported intracellular arginine (Fig. 7E) (48–50). In the Arg1+ macrophage-T cell co-cultures, FFAs partly blocked arginine utilization by macrophages and therefore indirectly blocked arginine hydrolysis by Arg1 (Fig. 7F). Thus, in the Arg1+ macrophage-T cell co-cultures, FFAs partly blocked the metabolism and/or transport of arginine into macrophages, releasing sufficient arginine available to T cells to initiate growth.

Loss of Rictor in T Cells Bypasses the Growth Inhibitory Effects of Arg1+ Macrophages—In a recent study using a candidate approach in a different T cell culture system to identify T cell mutants capable of bypassing cell cycle blocks in low amino acid environments, we identified a role for Rictor/mTORC2 (51). In the absence of Rictor/mTORC2, the ability of T cells to respond to low amino acid signals and not transition through the G1 phase of the cell cycle was lost. Therefore, we wanted to understand whether the same or similar mechanisms are part of the T cell response to Arg1+ immunosuppressive macrophages. We isolated CD4+ T cells from Rictorlox/flox;CD4-Cre; DO11.10 mice and exposed them to IL-4 + IL-10-stimulated macrophages (Fig. 8, A and B). Unlike control cells, which failed to proliferate, Rictor-deficient T cells partly proliferated, even though arginine was consumed rapidly by Arg1+ macrophages (Fig. 3). This effect was dependent on arginine rather than Arg1, because macrophage-T cell co-cultures where the amounts of arginine were artificially manipulated also showed some

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**Figure 6. Amino acid-deprived T cells down-regulate SREBP protein amounts and expression of SREBP target genes.** A, microarray analysis of SREBP target expression in T cells exposed to control or low arginine environments. Each column represents an independent experiment, and shown are z-scores for selected SREBP target mRNAs. B, T cells co-cultured with macrophages under the conditions shown at top, in media containing 1% Arg, Leu, or Lys and with (OVA) or without OVA antigen. After 48 h, T cells were purified from the cultures; total RNA was isolated and subjected to microarray analysis. Shown are SREBP target mRNAs. Each column represents an individual biological replicate. One sample of 1% Arg was omitted for RNA quality control reasons. C, quantification of selected SREBP-dependent mRNAs by RT-PCR. Each dot represents a sample from independent experiments (n = 5). Data are separated into 24- or 48–72-h time periods to show that the relative down-regulation of SREBP targets occurs after 24 h. Note that the scale of relative expression differs between the 24- and 48–72-h samples. D, gene expression associated with glycolysis (top) or gluconeogenesis (bottom) was from the same data set in A. E, quantification of selected glycolysis and gluconeogenesis mRNAs by RT-PCR. Each dot represents a sample from independent experiments (n = 5). Data are separated into 24- or 48–72-h time periods as in C. F, processing of SREBP1 quantified by immunoblotting. Two different validated anti-SREBP antibodies were used. Samples are purified T cells from the same experiment shown in Fig. 5B. G, subcellular location of SREBP1 in purified T cells co-cultures.
proliferation at low arginine amounts (Fig. 8C). Therefore, Rictor controls the ability of T cells to arrest proliferation when exposed to immunoregulatory macrophages consuming arginine.

**Discussion**

Immunoregulatory macrophages play central roles in immune homeostasis and the resolution of infection and inflammation (14). To achieve suppression of potentially dam-

**FIGURE 7. Free fatty acids block arginine uptake and/or utilization by macrophages.** A, representative CFSE traces showing proliferation of T cells co-cultured with Arg1+/ macrophages rescued by a mixture of four FFAs or palmitic acid (16:0) alone in a concentration-dependent way. B, as in A but using macrophages isolated from mice lacking Trif and Myd88. FFAs were used at 100 μM. C, NO production measured using the Griess assay by LPS and IFN-γ-stimulated macrophages (x axis 0.25–2 ng/ml of IFN-γ added although LPS was 100 ng/ml in all cultures) where 1 × 10⁶ or 7 × 10⁵ bone marrow-derived macrophages (BMDMs) were incubated with FFAs (200 μM) for 24 h. D, as in C except NO production was measured across time using 1 × 10⁶ BMDMs. Note that NO production is inhibited early and is dependent on the amount of LPS + IFN-γ added. E, iNOS protein amounts were measured by immunoblotting, using normalized protein amounts verified by re-probing for STAT1 or Grb2. F, representative example of an experiment to measure the effect of FFAs on arginine utilization. The experimental conditions were the same as in Fig. 3.
aging T cell responses, immunoregulatory macrophages use multiple mechanisms, including the expression of suppressive cell surface molecules like PD-L2, secretion of cytokines like IL-10, and amino acid-metabolizing pathways that both consume amino acids and create immunoregulatory molecules such as nitric oxide from arginine and kynurenines from tryptophan (3). In TH2-dominated environments, M2 macrophages are often the predominant myeloid population and are responsible for multiple facets of immune control and resolution (11). The immune response to schistosome eggs deposited in the liver parenchyma has provided rich information about the role of immunoregulatory macrophages and the role of Arg1 (11). After egg deposition, macrophages, eosinophils, and T cells surround the egg to form granulomas. Most macrophages are recruited from the blood and intermingle with local Kupffer cells (22). After exposure to IL-4 and IL-13 made by T cells and eosinophils, egg granuloma macrophages express Arg1. We found the expression of Arg1 was highest in macrophages closest to the egg. In this context, expression of Arg1 has an obligatory role in suppressing T cell proliferation, because animals where macrophages are Arg1-deficient have large T cell-rich granulomas that fail to resolve, leading to death of infected mice by an uncontrolled inflammatory response (16). Indeed, recent work using mice lacking the IL-4 receptor, which binds both IL-4 and IL-13, has shown that IL-4-mediated expression of PD-L2 was not required, and neither were the products of the Arg1 reaction, urea and ornithine. We also excluded a role for Arg2 in this system. However, we note that in vivo, most likely many pathways elicited in immunoregulatory macrophages must work together at different times and in diverse contexts.

Our co-culture system allowed us to trace the molecular pathways involved in how T cells make decisions about proliferation when confronted with Arg1+ macrophages. First, we established that Arg1+ macrophages very efficiently deplete arginine from the culture medium. However, T cells make a decision to engage in proliferation in a narrow window of arginine availability as shown by our experiments supplementing 3 mM arginine into cultures with Arg1+ macrophages; here, arginine is almost completely depleted in 24 h but the T cells divide equivalently to controls. Thus, T cells likely have some way of estimating they have sufficient arginine to engage in the full round of proliferative cycles (i.e. 5–6 divisions in 72 h) confirming studies in human T cells (53). T cells exposed to low arginine environments had, compared with dividing controls, normal expression of the suite of TCR-activated genes, overtly normal mTORC1 activity, and normal expression of metabolic genes involved in lipid, glycolytic, and gluconeogenic pathways.
However, in arginine-deprived T cells after ~48 h, we observed a selective reduction in all SREBP-regulated genes and exclusion of mature SREBP from the cell nucleus, paralleling the shutdown of mTORC1 activity. We noted this effect was specific as gene expression of other pathways (except gene regulation involved in cell division) was identical to controls. We note that work in other cell types has determined that SREBP-dependent, glycolytic, and gluconeogenic programs are all dependent on mTORC1 to some extent (54), whereas in T cells, the same “global” system of metabolic regulation does not seem to be integrated to mTORC1. Thus, arginine-starved T cells may have a pathway that reduces resource expenditure on lipid and cholesterol biosynthesis until sufficient arginine is resupplied, as shown by the complete restoration of proliferation in growth-arrested T cells when arginine is exogenously supplied. Further work on the selective connections between SREBPs and amino acids is needed to resolve the molecular details involved.

The selective nature of Arg1+ macrophages and arginine depletion on the SREBP pathway prompted us to try and rescue T cell proliferation by supplying intermediates in the cholesterol and fatty acid biosynthesis process. Neither cholesterol nor mevalonate nor any intermediates in the pathway had any implication of our work concerns extensions to scenarios specific of the arginine-depleted cultures. Although the connections between FFA biosynthesis and arginine metabolism seemed to be a new pathway, careful experimentation revealed the effect of FFAs and palmitate was to selectively block arginine utilization in macrophages but not T cells. Thus, FFAs indirectly increased supply of arginine to T cells. Although the mechanism of FFAs on macrophage arginine metabolism or importation may or may not have biological significance, we stress that the careful design and robustness of the co-culture allowed us to draw the correct conclusions about the “rescuing” role of FFAs.

We found Rictor controls how T cells respond to an arginine-depleted environment. T cells lacking Rictor were able to proliferate in the presence of Arg1+ macrophages. By contrast, T cells lacking TSC1, and by consequence increased mTORC1 activity (43) failed to proliferate when cultured with Arg1+ macrophages. Furthermore, we were able to establish that mTORC1 activity was uncoupled from the arginine sensing event as the activity of this pathway was identical to control T cells in the period where the arginine-dependent decision to divide or not divide was made. Although we cannot exclude the possibility that Arg1+ macrophages and low arginine environments fine tune mTORC activity through one or more of the proposed upstream arginine “sensors,” the preponderance of evidence argues the initial activation (within 24 h post-TCR engagement) of mTORC1 is connected to TCR and co-stimulatory signaling, and only later in G1-arrested cells does mTORC1 activity decline. At this point, it is possible that other arginine sensors are involved, which requires verification in vivo, and in cellular biochemistry platforms that employ primary cells described herein.

How Rictor controls T cell proliferation, especially mitogen signaling, requires further understanding. However, a key implication of our work concerns extensions to scenarios where T cell activation is desired in immunosuppressive microenvironments. For example, widely used antibodies that block CTLA4 and the PD1 pathway can rescue T cell growth in immunosuppressive cancer microenvironments where Arg1+ macrophages are present, often in large numbers (1, 55). An objective of cancer therapy is the combinatorial disabling of the immunosuppressive microenvironment by targeting immune checkpoints and other pathways such as arginases and the tryptophan-metabolizing IDO proteins. Our results suggest another avenue to achieve bypass of amino acid deprivation pathways is to use molecules that specifically target Rictor/mTORC2. T cells with reduced Rictor/mTORC activity should proliferate in low amino acid environments and potentially augment other strategies to stimulate anti-tumor T cell responses.

**Mechanism of Arginase Immunosuppression**

**Experimental Procedures**

*Mice—* BALB/c (000651), C57Bl/6 (000664), DO11.10 (003303), OT-II (004194), Rictor Flox (020649), Tsc1 Flox (005680), and CD4-Cre (022071) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Arg1-deficient mice crossed to Tie2-Cre have been described previously (24). Mice lacking MyD88 and Trif were a gift from D. Green (Department of Immunology, St. Jude Children’s Research Hospital). All mice were used in accordance with the policies of the St. Jude Children’s Research Hospital Institution Animal Care and Use Committee (P. J. M.) or the National Institutes of Health (T. A. W.).

*Reagents—* Blocking antibodies to PD-1 (clone PMP1–14) were from Bio-X-cell. Cytokines (IL-2, IL-4, and IL-10) were from PeproTech. Flow antibodies were as follows: CD3ε (clone 145–2C11, Biologend); CD4 (RM4-5, Biologend); F4/80 (clone BM8, Biologend); PD-L2 (clone Ty25, Biologend); anti-phospho-S6 ribosomal protein (Ser-235/236) (clone D57.22E, Cell Signaling); and anti-phospho-STAT5 (Tyr-694) (clone D47E7, Cell Signaling). Antibodies for immunoblotting were as follows: cyclin D2 (D52F9), cyclin D3 (DCS22), phospho-mTOR (Ser-2448) (2971), 4E-BP1 (9452), iNOS (2977), phospho-p70S6 kinase (Thr-421/Ser-424) (9204), p70S6 kinase (clone 49D7), phospho-STAT5 (clone D47E7), and anti-total STAT5 (clone 3H7) were all from Cell Signaling. Antibodies to SREBP-1 (H-160 and 2A4) and STAT1 (E-23) were from Santa Cruz Biotechnology. Antibodies for immunofluorescence were SREBP-1 (clone 2121, Millipore), SREBP2 (ab28482, Abcam), and secondary antibodies from Biotium (mouse IgG (CF488A) and rabbit IgG (CF555)). Free fatty acids (16:0, 18:1, 18:2, and 20:4)) were solubilized in ethanol and used at a final concentration of 200 μM. Mevalonate (Sigma) and cholesterol (Sigma) were used at a final concentration of 10 and 200 μM and 20 μg/ml, respectively. Ornithine and urea were purchased from Sigma.

*T Cell Isolation and Culture—* Lymph nodes (LN) were isolated from all regions of the body except the mesenteric LNs. Single cell suspensions were obtained by grinding LNs through a 70-μm nylon mesh. Cells were then labeled with 2 μM carboxyfluorescein diacetate succinimidyl ester (Invitrogen) and resuspended at 2.0 x 10⁶ cells/ml.

*Macrophage Isolation and Culture—* Peritoneal inflammatory macrophages were isolated 3 days after thioglycollate injection. Peritoneal macrophages were isolated 3 days after thioglycollate injection.
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Injection and plated at $7 \times 10^5$ cells/well in RPMI 1640 medium (Corning) containing 10% fetal bovine serum (Thermo Fisher) and penicillin/streptomycin (Invitrogen) (Complete RPMI 1640 medium) in 24-well tissue culture plates. After adherence, macrophages were washed to remove non-adherent cells and stimulated overnight with IL-4 + IL-10. The following day, macrophages were washed and the media replaced containing OVA peptide at a concentration of 0.2 $\mu$g in 0.5 ml. T cells were added to the cultures in 0.5 ml. Cells were cultured for 24–72 h. Where indicated, wells were supplemented with 3 mM 1-arginine (Sigma). In PEGylated Arg1 assays, 0.1 unit/well PEGylated Arg1 or PEGylated BSA was added, and cells were assayed at 72 h or replated onto fresh macrophages for an additional 72 h. Bone marrow-derived macrophages were grown in CSF-1 as described (15).

Amino Acid Titrations—$7 \times 10^5$ peritoneal macrophages were cultured with $1 \times 10^6$ labeled T cells in SILAC RPMI 1640 medium (Sigma) supplemented with 10% dialyzed FBS (Gibco) and penicillin/streptomycin solution containing the indicated concentrations of arginine, leucine, and lysine (Sigma) plus OVA peptide. Cells were cultured for 72 h.

Flow Cytometry, Intracellular Cytokine Staining, and Gating Strategies— Cultured T cells were harvested at the indicated time points and surface-stained for 30 min at 4 °C. To assess viability, surface-stained cells were stained with Live/Dead Violet Fixable Dead Cell Stain kit (Invitrogen) according to the manufacturer’s instructions. For phospho-flow experiments, surface-stained cells were fixed in 4% paraformaldehyde for 10 min at 37 °C. Cells were washed and resuspended in ice-cold methanol and incubated on ice for a minimum of 1 h. Cells were washed and resuspended in PBS, 1% FBS containing 1:50 dilution of phospho-antibody and incubated at room temperature for 1 h. Data were acquired with a FACS CantoII BD Biosciences) and analyzed using FlowJo Version 9 (Tree Star).

RNA Isolation and Analysis—CD4+ T cells were purified from macrophage co-cultures as described (56). Purified cells were centrifuged and lysed in TRIzol (Applied Biosystems) for subsequent RNA isolation according to the manufacturer’s instructions. cDNA was synthesized using SuperScript II reverse transcriptase, random and oligo(dT) primers (Invitrogen), and analyzed by qRT-PCR. PDK1, PFKP, and TPI1 primer sets were purchased from Qiagen. SYBR Green incorporation (Applied Biosystems) was measured using an ABI Prism 7300 thermocycler (Applied Biosystems). All values were normalized to GAPDH. In-house primer pairs were IL-2 (CCTGAGCGAG-GATGGAGAATTACA and TCCAGAACATGCCGCAAG), Ldr (GCATCAGCTTGGACAAGGTGT and GGGAACAGCTAGT), Fads2 (GCCGCTGCTCAT-CACCATTGTTG), Scd1 (TGGGTTGGCTGCTTGTG and GCGTGGGCAGGATGAAG), Ldlr (GCATCAGCTTGGACAAGGTGT and GGGAACAGC- CAT) and Pmvk (CGGAAGATTGTGGAAGGCGTGTC and ACCC-AGTCAAATCGCCTTCCAAAG). Microarray Analysis—Gene expression profiles were analyzed using the Affymetrix HT MG-430 PM array. Total RNA (100 ng) from biological triplicates of each treatment condition was processed using the Affymetrix 3’IVT Express protocol. Biotinylated cRNA (6 $\mu$g) was hybridized overnight at 45 °C to an array and then stained and washed using the Affymetrix GeneTitan system. Normalized transcription measures were generated from scanned intensity files using the RMA algorithm (57). Differential expression analysis was performed by analysis of variance (Partek Genomic Suite Version 6.5), and the false discovery rate was estimated by the Benjamini-Hochberg procedure (58). $k$ means clustering of 7074 differentially expressed transcripts (>2-fold difference from the mean with false discovery rate of <0.05) was performed using TIBCO Spotfire DecisionSite Version 9.1 software. Gene lists were examined for enrichment of gene ontology and pathway terms using the DAVID bioinformatics databases (59). Data files have been deposited in the GEO database (accession GSE68804).

Immunoblotting—Lysates were prepared from purified CD4+ cells on ice in RIPA buffer containing protease inhibitors (Roche Applied Science) and phosphatase inhibitors (Calbiochem). Lysates were separated on Tris-HCl gradient gels (Bio-Rad) and transferred to nitrocellulose. Membranes were blocked in 3% nonfat milk (Bio-Rad) and probed with primary antibodies overnight at 4 °C. Membranes were washed and probed with secondary antibodies at a 1:3000 dilution and developed using chemiluminescence reagents.

Nitric Oxide Assay—Griess assay for NO production was performed as described previously (60).

$[^3]H$Arginine Chromatography—Macrophage-T cell co-cultures were grown in SILAC RPMI 1640 media with 10% FBS supplemented with 400 $\mu$M arginine (containing 40 $\mu$Ci of $[^3]H$arginine (PerkinElmer Life Sciences)). Cells were lysed with 200 $\mu$L of water and 10 $\mu$L of neat culture supernatant or cell fraction spotted on pre-activated Silica Gel G plates. The TLC plates were developed with chloroform/methanol/ammonium hydroxide/water (1:4:2:1 v/v). Standards of arginine, citrulline, and ornithine were run in parallel with samples and detected by spraying with ninhydrin. The radioactive arginine derivatives in the TLC plates were detected using a radio-TLC Imaging Scanner (Bioscan AR-2000) for 10 min per lane.

Immunofluorescence for SREBPs—Cells were fixed with 4% paraformaldehyde in PBS for 10 min, permeabilized with 0.1% Triton X-100 (Sigma) in PBS for 10 min, blocked with 1% BSA, 0.3 mM glycine in PBS for 30 min, and then incubated with primary antibodies diluted in PBS, 1% BSA buffer for 1 h at 25 °C. After washing three times with PBS, cells were incubated with conjugated secondary antibodies at 25 °C. Cells were washed three times during 10 min with PBS and finally rinsed with ultrapure water to remove excess salt. Cells were mounted using Prolong® Gold anti-fade reagent with DAPI (Molecular Probes). Confocal microscopy was conducted on a Nikon C1si inverted laser scanning confocal microscope using the 488-nm argon laser line with a ×60, 1.45 NA Apochromat oil immersion objective. Data for graphs showing SREBP-1 nuclear localization were quantified using NIH-Elements Microscope Imaging Software (Nikon Corp.). Images were assessed using the EZ-C1 3.20 Viewer (Nikon Corp.), and the figures were assembled using Adobe Photoshop CS4 software.

Statistics—Data from qRT-PCR are presented as individual samples unless otherwise indicated and analyzed in Prism 5.0 (GraphPad Software, Inc.) by one-way analysis of variance with Tukey’s multiple comparison testing or two-sided $t$ testing, where 0.05 was considered significant and a normal distribu-
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