Abstract: The metabolism of the amino acid L-arginine is emerging as a crucial mechanism for the regulation of immune responses. Here, we characterized the impact of L-arginine deprivation on T cell and macrophage (MΦ) effector functions: We show that whereas T-arginine is required unconditionally for T cell activation, MΦ can up-regulate activation markers and produce cytokines and chemokines in the absence of t-arginine. Furthermore, we show that t-arginine deprivation does not affect the capacity of activated MΦ to up-regulate t-arginine-metabolizing enzymes such as inducible NO synthase and arginase 1. Thus, our results show that to exert their effector functions, T cells and MΦ have different requirements for t-arginine. J. Leukoc. Biol. 85: 268–277; 2009.

Key Words: arginase · immune regulation · t cell hyporesponsiveness · iNOS · cytokine · chemokine

INTRODUCTION

t-arginine is classified as a semi-essential or conditionally essential amino acid, depending on the age and health of an individual. Whereas newborns are unable to produce arginine efficiently, adults can synthesize enough t-arginine so that it is not a nutritionally essential amino acid. However, in cases of physical stress such as surgery or trauma, t-arginine needs to be supplemented [1, 2]. Free arginine in the body is derived from the diet, endogenous synthesis, and protein turnover. Thus, in healthy adults, t-arginine homeostasis is mainly achieved by modulation of t-arginine intake and modulation of its catabolism [3]. There are four main enzymes that can metabolize t-arginine in macrophages (MΦ), and importantly, two of these enzymes, arginase and inducible NO synthase (iNOS), have been involved in the regulation of immune responses. Arginase 1, which is induced in myeloid cells, is up-regulated upon activation by Th2 cytokines [4], GM-CSF [5, 6], PG [7–9], and catecholamines [9]; arginase hydrolyzes t-arginine into ornithine, an amino acid that is the main intracellular source for the synthesis of polyamines. iNOS is induced in myeloid cells by proinflammatory cytokines such as TNF-α/IFN-γ and LPS [4, 10] and oxidizes t-arginine in a two-step process into NO [11]. iNOS can control arginase by the generation of the NO intermediate, hydroxy-L-arginine, which is a competitive inhibitor of arginase activity; in turn, arginase can regulate NO production via the depletion of t-arginine from the extracellular milieu [12, 13]. t-arginine is transported from the extracellular milieu into myeloid cells as a result of increased expression of the high-affinity cationic amino acid transporters [14, 15], which are coinduced with the arginine-metabolizing enzymes [16].

The metabolism of t-arginine by iNOS and arginase 1 in myeloid cells is emerging as an important mechanism of T cell regulation:

NO, which is generated by the oxidation of t-arginine by iNOS, is an important effector molecule in host defense mounted by the immune system, but it can also act as a cytotoxic agent in pathological processes and can therefore play a central role in the regulation of immune responses [17]. For example, high concentration of NO has direct, proapoptotic effects on T cells [18, 19]. NO is also known to down-regulate intracellular signaling proteins directly or indirectly [20, 21]. One of the main factors that can regulate the expression of iNOS is the availability of extracellular t-arginine [22–24].

Arginase 1 has been shown to impair T cell responses by modulating the bioavailability of t-arginine: High arginase activity expressed by myeloid cells coincides with the transport of extracellular t-arginine into the cells, thereby causing a reduction of t-arginine in the microenvironment. In turn, this decrease in t-arginine results in T cell hyporesponsiveness [25–32]. This T cell dysfunction is attributed directly to t-arginine starvation that can regulate the cell cycle and arrest the cells in the G0-G1 phase [28]. Arginase-mediated t-arginine deprivation has been shown to cause T cell hyporesponsiveness in a variety of pathological and physiological responses [8, 31, 33]. Moreover, high arginase expression has been reported in a variety of diseases such as chronic inflammation [32], asthma [34], psoriasis [35], and infectious diseases [36–39].

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During immune responses in vivo, T lymphocytes and MΦ communicate and cooperate for the execution of many effector functions. Therefore, arginine deprivation in the microenvironment could affect T cells directly or indirectly via the impairment of MΦ effector functions. Therefore, we characterized further the effects of l-arginine deprivation on T cell activation and on T cell effector functions. As MΦ can be induced by T cell-derived cytokines as well as other signals to up-regulate l-arginine-metabolizing enzymes such as arginase 1 or iNOS [40], we determined how l-arginine starvation affects MΦ biological functions.

MATERIALS AND METHODS

Mice

Female BALB/c mice (6–8 weeks old) were purchased from Charles River (UK) and were kept in individually vented cages. The animal colonies were screened regularly for mouse pathogens and tested negative consistently. Animal experiments were performed in accordance with home office and institutional guidelines.

T cell activation

Primary activation

Spleens of BALB/c mice were homogenized, and 5 × 10^6/ml cells were activated with 1 μg plate-bound anti-CD3 mAb (eBioscience, San Diego, CA, USA) and 200 ng soluble anti-CD28 mAb (eBioscience) in DMEM (400 mM l-arginine) or l-arginine-free DMEM (0 mM l-arginine), supplemented with 5% FBS, 50 IU/ml penicillin, 50 μg/ml streptomycin, and 292 μg/ml L-glutamine (Gibco, Grand Island, NY, USA). Cells were harvested after 3 days of further analysis.

Restimulation

Spleen cells from BALB/c mice were stimulated as described above. Four days later, cells were washed and rested in complete DMEM containing 400 mM l-arginine, and 3 days later, they were restimulated for 24 h in DMEM (400 μM l-arginine) or l-arginine-free DMEM (0 μM l-arginine) with 1 μg plate-bound anti-CD3 mAb and 200 ng soluble anti-CD28 mAb. Cells were harvested after 1 day for further analysis.

T cell purification

Spleens of BALB/c mice were homogenized and T cells purified using the EasySep Mouse CD90.2 (Thy-1.2) positive selection kit (Stem Cell Technologies, Canada), according to the supplier’s protocol. The purity of the CD90.2+ cells was determined by flow cytometry to be >96%. T cells (1×10^6/ml) were stimulated as described above.

Flow cytometry analysis

For the determination of IFN-γ, IL-4, and IL-10 by ELISA, 5 × 10^6/ml spleen cells were activated with 1 μg plate-bound anti-CD3 mAb (eBioscience) and 200 ng soluble anti-CD28 mAb (eBioscience). Cells were harvested after 3 days, and the levels of cytokines were determined using an ELISA kit (ELISA development kit, PeproTech, Rocky Hill, NJ, USA) following the supplier’s protocol.

BMMΦ cytokine and chemokine analyses

BMMΦ were stimulated as described above, and 48 h later, supernatants were harvested, and IL-6, IL-10, IL-12p70, and MCP-1 were detected simultaneously in each sample by the Luminex-based Multiplexed assay (Luminex 100 system). Data were analyzed using Starstation V2.0.

Determination of arginase activity

Arginase activity was measured in MΦ lysates by the conversion of l-arginine to urea as described in refs. [4, 38]. One unit of enzyme activity was defined as the amount of enzyme that catalyzes the formation of 1 μmol urea per min.

Nitrite determination

NO2− accumulation was used as an indicator of NO production and measured using the Griess reagent [44].
functions
A recent study has identified a metric parameter, the iMFI, which reflects more accurately the total functional response of activated cells [43]. iMFI is calculated by multiplying the frequency (the magnitude of the response) by the MFI (the quality of the response). As shown in Figure 1C, BrdU iMFI of TcR $\beta^+$ decreases in the media containing 100 and 20 $\mu$M, as compared with 400 $\mu$M; however, the sharpest reduction was observed in the absence of L-arginine (0 $\mu$M). These results demonstrate that the magnitude and the quality of the proliferative response are impaired greatly with decreasing levels of L-arginine.

To determine whether the absence of L-arginine impacts directly on T cell activation, T cells were purified from spleens of BALB/c mice and stimulated with anti-CD3 and anti-CD28 mAb in the presence or absence of L-arginine. As shown in Figure 1D, the absence of L-arginine clearly results in impaired T cell proliferation in response to polyclonal T cell stimulation.

To characterize further the impact of L-arginine deprivation on T cell effector functions, we assessed the ability of splenic T cells to express IFN-$\gamma$, IL-4, and IL-10 in response to anti-CD3 and anti-CD28 mAb. The strongest reduction in the iMFI of TcR $\beta^+$ IFN-$\gamma^+$, IL-4$^+$, and IL-10$^+$ was observed in the absence of L-arginine (Fig. 1E). Similar results were obtained when the levels of cytokine were determined by ELISA in the supernatant of stimulated cells (Fig. 1F). Cytokine production was also reduced when T cells, purified from spleen cell suspensions, were stimulated with anti-CD3 and anti-CD28 in the absence of L-arginine (Fig. 1G).

In agreement with the reduced capacity of T cells to proliferate and express cytokines in the absence of L-arginine, the expression of T cell activation markers such as CD25, CD28, and CD62L was also impaired (Fig. 2). Importantly, the total number of cells per well was only decreased minimally after 72 h in culture (27.1±4.6×10$^6$ in the presence of L-arginine vs. 23.8±4.1×10$^6$ in the absence of L-arginine; data not illustrated), and there was no increase in the frequency of apoptotic T cells in the absence of L-arginine, as the percentage of TcR $\beta^+$ caspase $^+$ cells was similar in the presence (400 $\mu$M) and absence of L-arginine (1.2±0.3 vs. 2.2±0.1; data not illustrated); these results confirm previously published results that show that cell death is not the cause for reduced T cell activation and function [45].

Thus, the results presented in Figures 1 and 2 clearly show that L-arginine deprivation during priming of T cells impairs their effector functions. Naive and effector T cells have different activation thresholds. Therefore, we assessed next whether restimulation of resting T cells in the absence of L-arginine also affects their effector functions. As presented in Figure 3A, L-arginine deprivation clearly impaired the capacity of restimulated T cells to proliferate, as shown by the reduced iMFI of TcR $\beta^+$ BrdU $^+$. Furthermore, there was a systematic decrease in T cell cytokine iMFI when total spleen cells (Fig. 3B) or purified T cells (Fig. 3C) were polyclonally stimulated in the absence of L-arginine.

The results presented in Figures 1–3 clearly demonstrate that activated T cells cannot exert their effector functions efficiently in the absence of L-arginine.

Arginase-induced L-arginine depletion impairs T cells functions
To assess whether L-arginine depletion by arginase-expressing MΦ can modulate T cell responses, a well-defined in vitro system was used: Arginase expression was induced in BMMΦ by stimulation with a combination of IL-4 and IL-10 [4], and purified T cells were added to the cultures. L-arginine was added daily to control cultures, and the impact of the presence...
of L-arginine-metabolizing MΦ on T cell effector functions was determined. As shown in Figure 4, addition of L-arginine clearly increases the capacity of T cells to proliferate (Fig. 4A) and to produce IFN-γ, IL-4, and IL-10 (Fig. 4B). These results demonstrate that arginase-induced L-arginine deletion by MΦ impaired T cell responses.

Effects of L-arginine deprivation on MΦ activation and effector functions

Whereas L-arginine deprivation is emerging as an important immunoregulatory mechanism for T lymphocytes, its role on other cells of the immune system is not well characterized.

Up-regulation of L-arginine-metabolizing enzymes in MΦ induces uptake of L-arginine from the microenvironment into the cells [14, 38, 46], thereby controlling the availability of this amino acid in the extracellular milieu. Therefore, in the next step, we determined whether L-arginine deprivation also affects MΦ activation and effector functions. We tested several biological functions of physiologically distinct MΦ subsets: CAMΦ and AAMΦ in the presence of L-arginine (400 μM) or in L-arginine-free DMEM (0 μM). To obtain CAMΦ, we used IFN-γ and TNF-α, which induces iNOS rather than IFN-γ and LPS, as the latter have been shown to induce iNOS and arginase 1 (refs. [4, 47]; B-S.
Choi and P. Kropf, unpublished results), and we used IL-4 for differentiation of AAMΦ. We first quantified the effects of t-arginine deprivation on phagocytic functions by determining the capacity of activated MΦ to phagocytose fluorescent particles. All groups of MΦ were capable of internalizing similar levels of fluorescent particles, even in the absence of t-arginine (data not shown).

To characterize the impact of t-arginine deprivation on MΦ activation, we analyzed the expression levels of activation markers on both MΦ subsets. As shown in Figure 5, the absence of t-arginine during the differentiation of mature MΦ into CAMΦ or AAMΦ did not significantly affect the expression of activation markers ($P > 0.05$). Of note, CD69, CD40, MHCII, CD86, and PDL1 were up-regulated in CAMΦ, whereas CD80 was down-regulated in CAMΦ, suggesting that these markers might be useful in physiologically distinguishing different types of activated MΦ; as shown previously [48], CD206 was up-regulated in AAMΦ exclusively.

In the next step, we assessed whether CAMΦ and AAMΦ require t-arginine for the production of cytokines (IL-6, IL-10, and IL-12p70) and chemokine (MCP-1) and found that in the presence or absence of t-arginine, CAMΦ produce similar levels of IL-6. Similarly, the production of MCP-1 by CAMΦ and AAMΦ was not impaired in the absence of t-arginine (data not shown). No IL-10 or IL-12p70 was detectable under those conditions (data not shown).

t-arginine deprivation also did not influence the expression of two markers of AAMΦ: YM1 (an eosinophilic chemotactic factor), and FIZZ1 (a resistin-like molecule; Fig. 6A).

Finally, we determined the expression of the two main enzymes that use t-arginine as substrate and are associated with the depletion of t-arginine from the extracellular milieu: arginase 1 and iNOS. Importantly, we show that activation of MΦ in the absence of t-arginine did not abrogate protein expression of iNOS: As shown in Figure 6A, CAMΦ did not have an impaired capacity to express iNOS when differentiated...
in the absence of L-arginine. As expected, in the absence of L-arginine as a substrate, the production of NO was reduced strongly (Fig. 6B). NO production by MΦ stimulated by IFN-γ alone in the presence of L-arginine was just above the detection limit (7.0±1.7 μMol), and no NO was detectable when MΦ were stimulated with TNF-α alone (data not illustrated).

L-arginine deprivation did not affect the capacity of AAMΦ to up-regulate arginase, as arginase protein expression and

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**Fig. 4.** L-arginine depletion by arginase-expressing MΦ impairs T cells functions. Mature BMMΦs were stimulated with IL-4/IL-10 in the presence of 100 μM L-arginine. Two days later, MΦ were washed, and purified T cells (preactivated with anti-CD3 and anti-CD28 for 24 h) were added to the MΦ. L-arginine (400 μM) was added twice/day to some of the wells (+L-arginine). The capacity of T cells to proliferate (A) and produce cytokines (B) was determined by flow cytometry. The error bars represent SD, and data show the results of one representative experiment out of three independent experiments.

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**Fig. 5.** L-arginine is not required for the expression of MΦ activation markers. Mature BMMΦ were differentiated in CAMΦ or AAMΦ or left unstimulated (nil) in the presence (400 μM, shaded bars) or absence (0 μM, open bars) of L-arginine, and the capacity to express cell-surface markers was analyzed by flow cytometry. The error bars represent SD, and data show the results of one representative experiment out of three independent experiments.
arginase activity were not impaired in the absence of L-arginine (Fig. 7). IL-10 synergizes with IL-4 and strongly enhances the expression levels of arginase [48]; therefore, we tested whether a combination of these two cytokines could still induce arginase expression in the absence of L-arginine. As shown in Figure 7, IL-4 and IL-10 induced similar expression of arginase by AAM/H9021 in the presence and absence of L-arginine. To evaluate a possible contribution of L-arginine that is present in the FBS used in the culture medium, we did similar experiments as those presented in Figures 6B and 7 with dialyzed FBS [30] and show that even in the complete absence of L-arginine in the medium, small levels of NO were produced by CAM/H9021; arginase activity expressed by AAM/H9021 remains constant under those conditions (Table 1).

The results presented here show that to exert their effector functions, T cells and M/H9021 have different requirements for L-arginine: T cells primed or restimulated in the absence of L-arginine cannot be activated, proliferate, and produce cytokines efficiently, whereas M/H9021 activation and biological functions tested here are not altered by the lack of L-arginine.

**DISCUSSION**

The metabolism of L-arginine is emerging as a crucial mechanism for local immune cell regulation [25–32]. Arginase 1-in-

**TABLE 1. Production of NO and Expression of Arginase by Activated BMMΦs in Dialyzed FBS**

|          | CAMΦ (+ L-arginine) | CAMΦ (−L-arginine) |
|----------|---------------------|--------------------|
| Dialyzed FBS | 59.6 ± 1.1          | 4.5 ± 2.9          |
| FBS       | 63.5 ± 2.4          | 2.9 ± 0.3          |

|          | AAMΦ (+ L-arginine) | AAMΦ (−L-arginine) |
|----------|----------------------|--------------------|
| Dialyzed FBS | 1657.3 ± 347.5     | 1345.4 ± 58.1      |
| FBS       | 995.2 ± 16.2        | 1034.3 ± 13.1      |

**BMMΦ** were stimulated as described in Materials and Methods for 48 h. (A) Supernatants were tested for their content in NO by the Griess reagent; the values have been corrected for the levels of NO in the medium without macrophages. (B) The activity of arginase was determined by enzymatic assay as described in Materials and Methods. Values are ± SD, and data show the results of one representative experiment out of three independent experiments.
duced t-arginine depletion has been shown to impair T cell responses by modulating the bioavailability of t-arginine. Indeed, human and mouse T cells display an impaired capacity to proliferate and express the CD3ζ chain in the absence of t-arginine in vitro; this can be reversed by addition of exogenous t-arginine. Suppression of T cell responses by t-arginine deprivation has also been demonstrated in physiological [31] and pathological [8, 27, 39] conditions. Here, we characterized further the impact of t-arginine deprivation on T cell activation and effector functions: We measured proliferation and cytokine production by T cells as functional responses. Our results show that the expression of activation markers such as CD25, CD62L, and CD28, which play crucial roles in the activation of T cells, is altered in the absence of t-arginine. Furthermore, we show that in the absence of t-arginine during priming and restimulation of T cells, the quality and the magnitude of these functional responses are impaired drastically. To determine whether t-arginine deprivation impacts directly on T cells, we also assessed the capacity of purified T cells to proliferate and produce cytokines in the absence of t-arginine. In agreement with our previous observations [32] and those of others [27], we show here that purified T cells cannot proliferate or produce cytokine efficiently in the absence of t-arginine. Of note, the decrease in proliferation and cytokine production of T cells stimulated in the absence of t-arginine was consistently lower in the cultures of total spleen cells as compared with purified T cells. This is likely to be a result of the absence of costimulation and/or bystander activation of other cells. These results demonstrate unequivocally that T cells cannot be activated efficiently and become competent effector cells in the absence of t-arginine.

Several immune dysfunctions have been associated with a change in the metabolism of t-arginine. For example, in different forms of cancer, high arginine activity in myeloid suppressor cells (MSCs) has been shown to down-regulate T cell responses; inhibition of arginase 1 alone [26, 33] or of iNOS and arginase 1 [49] has been shown to improve T cell responsiveness. These results demonstrate clearly that the metabolism of t-arginine regulates T cell responses.

Another line of evidence suggests an important immunoregulatory role for the metabolism of t-arginine. Plasma t-arginine levels in patients with pulmonary tuberculosis were significantly lower than those of negative controls; this correlated with higher levels of arginase activity and impaired T cell activation, as shown by a decreased expression of CD3ζ [39]. These studies as well as those from others [1, 30–32, 50], in combination with the results presented here, show clearly that t-arginine deprivation plays a central role in the regulation of T cell activation.

The study of the impact of t-arginine deprivation has been mainly restricted to T cells; however, its role on other cells of the immune system is not well characterized. ΨΦ not only interact with T cells, but they also can express two t-arginine-metabolizing enzymes—iNOS and arginase 1—and therefore control t-arginine availability and play an important immunoregulatory role. Indeed, the levels of t-arginine not only affect T cell activation, but they also regulate the stability of iNOS expression and NO production [22–24]. In addition, increased levels of polyamines, the metabolic products of the degradation of t-arginine by arginase, have also been shown to down-regulate ΨΦ activation [51]. Thus, in the next step, we tested the impact of t-arginine deprivation on activated ΨΦ. Interestingly, we show that the phagocytic capacity of CAMΦ and AAMΦ as well as the expression of activation markers and the production of IL-6 and MCP-1 production were not altered in the absence of t-arginine. Importantly, we also show that deprivation of t-arginine did not impair the up-regulation of iNOS and arginase; similar results were obtained with peritoneal exudate ΨΦ (data not illustrated). It has been shown recently that in the absence of t-arginine, activated T cells are unable to up-regulate cyclin D3 and cyclin-dependent kinase 4 and are therefore arrested in the G0-G1 phase of the cell cycle [28]. As activated ΨΦ do not proliferate [52], our results suggest that activated ΨΦ do not require t-arginine to the same extent as T cells for their activation and up-regulation of iNOS, arginase, and phagocytosis.

The key finding that t-arginine starvation does not abrogate biological functions of ΨΦ might explain how ΨΦ maintain their ability to express iNOS and arginase and to phagocytose, therefore regulating immune functions in critical conditions such as t-arginine deprivation. For example, in cancer, it has been demonstrated that MSCs, expressing high levels of arginase, are efficient in depleting t-arginine from their microenvironment [29, 50] and therefore, down-regulate T cell activation. Similarly, our results might also explain how myeloid cells can maintain their expression of arginase in other diseases that are associated with lower levels of t-arginine and immune dysfunctions, such as tuberculosis [39] or asthma [53]. Furthermore, this mechanism of differential t-arginine requirement might also explain the observed T cell hyporesponsiveness in diseases such as leishmaniasis and schistosomiasis, in which increased ΨΦ arginase activity has been observed [36, 38]. Indeed, high arginase expression has been associated with these pathological conditions, the subsequent depletion of t-arginine might explain impaired T cell responses, while leaving the functionality of arginase-expressing ΨΦ unaltered.

The results showing that t-arginine deprivation does not affect iNOS expression are in apparent contradiction to those presented by El-Gayar et al. [23] and Lee et al. [54], who showed that arginine availability regulates the stability of iNOS protein. Whereas both studies show that iNOS protein is clearly expressed in the absence of t-arginine, they show that the stability of the protein is altered. Although we did not test protein stability in our system, it is worth mentioning that different sources of ΨΦ have been used, as well as different stimuli. In the study by El-Gayar et al. [23], IFN-γ and LPS were used to induce iNOS, whereas we used IFN-γ and TNF-α to induce CAMΦ. As LPS also induces arginase 1 [4], it cannot be excluded that induction of arginase activity might have played a role in the down-regulation of iNOS expression. It has to be noted that whereas we used t-arginine-free DMEM, the FBS contained in the culture medium does contain t-arginine; however, even when we used dialyzed FBS, the residual levels of NO detected following stimulation without t-arginine were not significantly lower. Similarly, the expression of arginase was also not reduced when ΨΦ were stimulated in the absence of t-arginine in medium containing dialyzed FBS, as compared with nondialyzed FBS. The use of intracellular arginine pools...
represents another possibility to circumvent the consequences of t-arginine starvation in the extracellular environment. At least two pools of intracellular t-arginine have been described [55]; however, the authors suggest that these pools are not freely exchangeable with the intracellular milieu [55] and are therefore not available to be catabolized. Importantly, a complete urea cycle has been described in MΦ [56]. Therefore, it is feasible that MΦ use their own synthesized t-arginine for the induction of arginase and iNOS protein as well as the production of cytokines and chemokines and expression of activation markers.

It is important to note that during the course of diseases that are associated with lower levels of t-arginine in the plasma, the latter is still relatively high as compared with controls: 45 μM versus 94 μM (asthma; ref. [53]), 42 μM versus 77 μM (severe cerebral malaria; ref. [57]), and 88 μM versus 153 μM (tuberculosis; ref. [39]). These results, as well as those presented in our study, suggest that T cells react differently than MΦ to limiting levels of t-arginine in the extracellular milieu.

The findings presented here further our understanding about the mechanism by which t-arginine depletion regulates immune responses. Targeting the metabolism of L-arginine and the myeloid cells expressing t-arginine-metabolizing enzymes is likely to represent an important therapeutic and prophylactic strategy to treat diseases such as cancer, psoriasis, allergic asthma, and infectious diseases such as tuberculosis, leishmaniasis, and schistosomiasis.

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