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Differential impact of L-arginine deprivation on the activation and effector functions of T cells and macrophages

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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 L-arginine is required unconditionally for T cell activation, MΦ can up-regulate activation markers and produce cytokines and chemokines in the absence of L-arginine. Furthermore, we show that L-arginine deprivation does not affect the capacity of activated MΦ to up-regulate L-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 L-arginine. *J. Leukoc. Biol.* 85: 268–277; 2009.

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

INTRODUCTION

L-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 L-arginine so that it is not a nutritionally essential amino acid. However, in cases of physical stress such as surgery or trauma, L-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, L-arginine homeostasis is mainly achieved by modulation of L-arginine intake and modulation of its catabolism [3]. There are four main enzymes that can metabolize L-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 L-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 L-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 L-arginine from the extracellular milieu [12, 13]. L-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 L-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 L-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 L-arginine [22–24].

Arginase 1 has been shown to impair T cell responses by modulating the bioavailability of L-arginine: High arginase activity expressed by myeloid cells coincides with the transport of extracellular L-arginine into the cells, thereby causing a reduction of L-arginine in the microenvironment. In turn, this decrease in L-arginine results in T cell hyporesponsiveness [25–32]. This T cell dysfunction is attributed directly to L-arginine starvation that can regulate the cell cycle and arrest the cells in the G0-G1 phase [28]. Arginase-mediated L-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 M Φ 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 M Φ effector functions. Therefore, we characterized further the effects of L-arginine deprivation on T cell activation and on T cell effector functions. As M Φ 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 M Φ 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 μ g plate-bound anti-CD3 mAb (eBioscience, San Diego, CA, USA) and 200 ng soluble anti-CD28 mAb (eBioscience) in DMEM (400 μ M L-arginine) or L-arginine-free DMEM (0 μ M L-arginine), supplemented with 5% FBS, 50 IU/ml penicillin, 50 μ g/ml streptomycin, and 292 μ g/ml L-glutamine (Gibco, Grand Island, NY, USA). Cells were harvested after 3 days for 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 μ M L-arginine, and 3 days later, they were restimulated for 24 h in DMEM (400 μ M L-arginine) or L-arginine-free DMEM (0 μ M L-arginine) with 1 μ g 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

FcR blocking reagent (1 μ g; PharMingen, San Diego, CA, USA) was added to the cells to block unspecific binding before further treatment.

T cell proliferation

BrdU (0.3 μ g; Sigma Chemical Co., St. Louis, MO, USA) was added to the cells, and 18 h later, the proliferative capacity of TcR β ⁺ cells was determined by flow cytometry by measuring BrdU incorporation as described in ref. [41].

Intracellular cytokine production

The frequency of TcR β ⁺ cells expressing IL-4, IL-10, or IFN- γ was determined by flow cytometry as described in ref. [42].

Activation markers

All antibodies were used according to the suppliers' protocols [42]. T cell activation markers included anti-CD25 (PharMingen) and anti-CD62 ligand (anti-CD62L) and anti-CD28 (eBioscience); M Φ activation and differentiation

markers included anti-F4/80, anti-CD69, anti-CD40, anti-MHCII, anti-CD86, anti-CD80, and anti-programmed death ligand-1 (PDL1; eBioscience) and anti-CD206 (Serotec, UK). All flow cytometry analyses were performed on a FACSCalibur (Becton Dickinson, San Jose, CA, USA), and data were analyzed using Summit 4.0 software.

Integrated mean fluorescence intensity (iMFI)

The iMFI [43] was obtained by multiplying the percent of TcR β ⁺ cells by the value of the MFI for BrdU or by the value of the MFI for the relevant cytokine.

ELISA

For the determination of IFN- γ , IL-4, and IL-10 by ELISA, 5×10^6 /ml spleen cells were activated with 1 μ g 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.

Bone marrow M Φ (BMM Φ)

BM was obtained by flushing the femurs of naïve BALB/c mice, and precursor cells were cultured in hydrophobic Teflon bags as described in ref. [38]. After 8 days in culture, mature BMM Φ were harvested, and classically activated M Φ (CAM Φ) were obtained by stimulating 5×10^5 /ml mature M Φ with 100 U/ml IFN- γ (PeproTech) and 500 U/ml TNF- α (PeproTech); alternatively activated M Φ (AAM Φ) were obtained by stimulating 5×10^5 /ml mature M Φ with 20 U/ml IL-4 (PeproTech). IL-10 (10 U/ml; PeproTech) and 1 μ g/ml LPS (Sigma Chemical Co.) were also used. After 2 days in culture DMEM (400 μ M L-arginine) or L-arginine-free DMEM (0 μ M L-arginine) at 37°C/10% CO₂, cells were harvested for further analysis.

M Φ phagocytosis assay

The assay was adapted using the Vybrant phagocytosis assay kit (Molecular Probes, Eugene, OR, USA). Briefly, activated M Φ were incubated for 2 h with fluorescein-labeled BioParticles at 37°C with 10% CO₂; the fluorescence of any noninternalized particles was then quenched by the addition of Trypan blue. Detection of fluorescein was performed using a FACSCalibur (Becton Dickinson), and data were analyzed using Summit 4.3 software.

BMM Φ cytokine and chemokine analyses

BMM Φ 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 SStarstation V2.0.

Determination of arginase activity

Arginase activity was measured in M Φ 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 μ mol urea per min.

Nitrite determination

NO₂⁻ accumulation was used as an indicator of NO production and measured using the Griess reagent [44].

Western blot analysis

An equal amount of protein [determined by bicinchoninic acid protein assay reagent (Pierce, Rockford, IL, USA)] was resolved by the addition of reducing buffer (Invitrogen, Carlsbad, CA, USA) and sample buffer (Invitrogen) following the suppliers' recommendations before separation on SDS 4–12% gels (Invitrogen). Following electrophoresis, the protein was transferred to polyvinylidene difluoride membranes (Invitrogen) using a semi-dry transfer cell (Bio-Rad, Hercules, CA, USA) at 450 mA for 90 min. For immunodetection, membranes were blocked with 1% BSA in PBS (Sigma Chemical Co.) before the primary antibody [antiarginase 1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-NOS 2 (Santa Cruz Biotechnology), anti-Ym1 (Stem Cell Technologies), and anti-found in inflammatory zone 1 (Fizz1; PeproTech)] was added following the suppliers' recommendations, and goat anti-mouse HRP

(Santa Cruz Biotechnology) was used as the secondary antibody. Detection of bound antibodies was visualized by chemiluminescence using the Western blotting Luminol reagent kit (Santa Cruz Biotechnology).

MΦ and T cell coculture

MΦ

Mature BMMΦ were obtained as described above in BMMΦ; 5×10^5 BMMΦ were stimulated with IL-4 (20 U/ml) and IL-10 (10 U/ml) for 2 days in complete DMEM (100 μM L-arginine).

T cells

Purified T cells (1×10^6) were activated with anti-CD3 mAb (1 μg/ml) and anti-CD28 mAb (200 ng/ml) in complete DMEM (400 μM L-arginine) for 24 h.

Cocultures

MΦ were washed after 2 days of activation and resuspended in complete DMEM (100 μM L-arginine). T cells were harvested, washed, and resuspended in DMEM without L-arginine and added to the MΦ cultures. L-arginine (400 μM) was added twice/day to some of the cultures. T cells were harvested for further analysis after 2 days of coculture.

Statistical analyses

Data were evaluated using a Student's *t*-test with GraphPad PRISM, Version 2.0.

RESULTS

L-arginine deprivation impairs T cell effector functions

It has been shown previously that L-arginine deprivation induces T cell hyporesponsiveness, as defined by profound reduction of T cell proliferation and reduced CD3ζ chain expression [25, 26, 29, 31, 32, 45]. Here, we extended these results by further characterizing T cell activation and effector functions in the absence of L-arginine (L-arginine-free medium). We first assessed the capacity of T cells to undergo blast transformation and proliferate following anti-CD3 and anti-CD28 mAb stimulation. Our results show that after polyclonal stimulation of spleen cells in the presence (400 μM) of L-arginine, the large majority (>95%) of proliferating T cells (TcRβ⁺ BrdU⁺) is found in a distinct region of cells with a larger side- and forward-scatter (SSC and FSC, respectively; blasts), R1; this represents 13.2% of all gated cells (**Fig. 1A**). However, a sharp reduction in the percentage of cells in R1 was observed when cells were stimulated in L-arginine-free medium (**Fig. 1A**), as only 1.5% of all gated cells were found in R1 (88.6% reduction). In addition, L-arginine deprivation induced a clear decrease in the frequency of proliferating T cells [80.7% in the presence of L-arginine to 3.9% in the absence of L-arginine (**Fig. 1B**)] and in the MFI (14.0–9.1) of TcRβ⁺ BrdU⁺ (**Fig. 1B**). 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 TcRβ⁺ decreases in the media containing 100 and 20 μM, as compared with 400 μM; however, the sharpest reduction was observed in the absence of L-arginine (0 μM). 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-γ, IL-4, and IL-10 in response to anti-CD3 and anti-CD28 mAb. The strongest reduction in the iMFI of TcRβ⁺ IFN-γ⁺, 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 ($27.1 \pm 4.6 \times 10^6$ in the presence of L-arginine vs. $23.8 \pm 4.1 \times 10^6$ 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 TcRβ⁺ caspase⁺ cells was similar in the presence (400 μM) 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.

Naïve 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 TcRβ⁺ 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

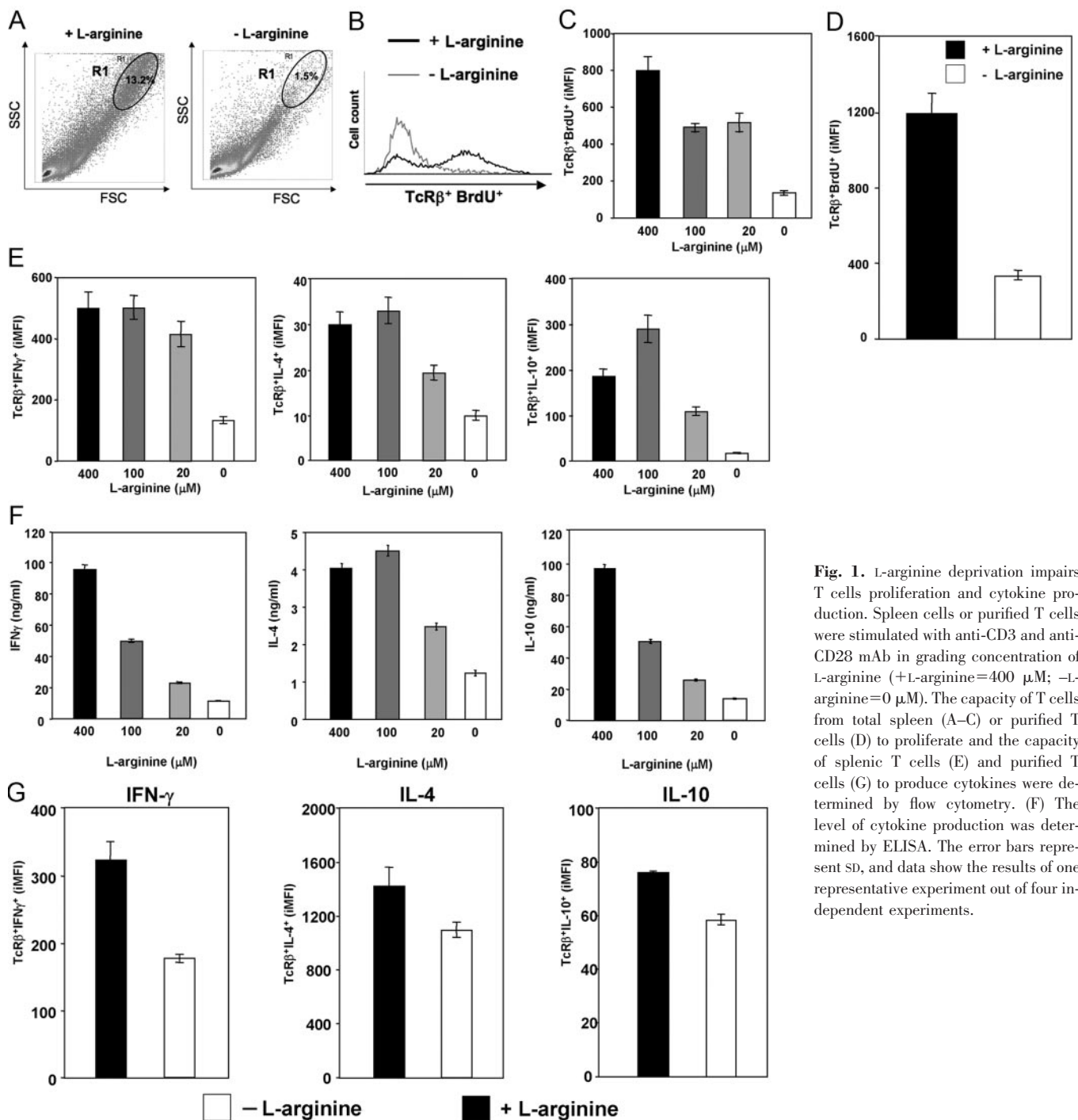


Fig. 1. L-arginine deprivation impairs T cells proliferation and cytokine production. Spleen cells or purified T cells were stimulated with anti-CD3 and anti-CD28 mAb in grading concentration of L-arginine (+L-arginine=400 μ M; -L-arginine=0 μ M). The capacity of T cells from total spleen (A-C) or purified T cells (D) to proliferate and the capacity of splenic T cells (E) and purified T cells (G) to produce cytokines were determined by flow cytometry. (F) The level of cytokine production was determined by ELISA. The error bars represent SD, and data show the results of one representative experiment out of four independent experiments.

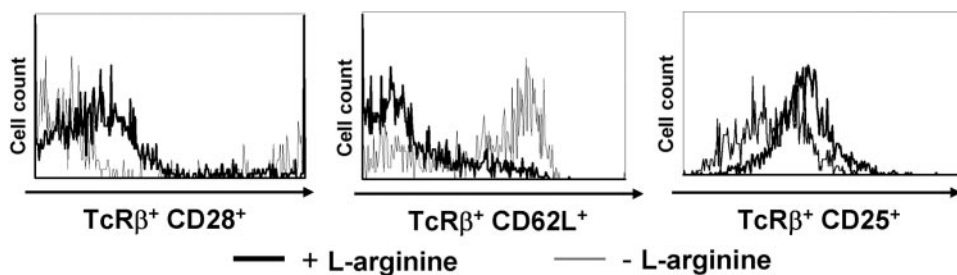
of L-arginine-metabolizing M Φ on T cell effector functions was determined. As shown in **Figure 4**, addition of L-arginine clearly increases the capacity of T cells to proliferate (Fig. 4A) and to produce IFN- γ , IL-4, and IL-10 (Fig. 4B). These results demonstrate that arginase-induced L-arginine deletion by M Φ impaired T cell responses.

Effects of L-arginine deprivation on M Φ 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 M Φ 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 M Φ activation and effector functions. We tested several biological functions of physiologically distinct M Φ subsets: CAM Φ and AAM Φ in the presence of L-arginine (400 μ M) or in L-arginine-free DMEM (0 μ M). To obtain CAM Φ , 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.

Fig. 2. L-arginine deprivation impairs expression of activation markers by T cells. Spleen cells were stimulated with anti-CD3 and anti-CD28 mAb in the presence (400 μ M, black line) or absence (0 μ M, gray line) of L-arginine, and the expression of CD28, CD62L, and CD25 on TcR β ⁺ cells was determined by flow cytometry. Data show the results of one representative experiment out of three independent experiments.



Choi and P. Kropf, unpublished results), and we used IL-4 for differentiation of AAM Φ . We first quantified the effects of L-arginine deprivation on phagocytic functions by determining the capacity of activated M Φ to phagocytose fluorescent particles. All groups of M Φ were capable of internalizing similar levels of fluorescent particles, even in the absence of L-arginine (data not shown).

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

In the next step, we assessed whether CAM Φ and AAM Φ require L-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 L-arginine, CAM Φ produce similar levels of IL-6. Similarly, the production of MCP-1 by CAM Φ and AAM Φ was not impaired in the absence of L-arginine (data not shown). No IL-10 or IL-12p70 was detectable under those conditions (data not shown).

L-arginine deprivation also did not influence the expression of two markers of AAM Φ : 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 L-arginine as substrate and are associated with the depletion of L-arginine from the extracellular milieu: arginase 1 and iNOS. Importantly, we show that activation of M Φ in the absence of L-arginine did not abrogate protein expression of iNOS: As shown in **Figure 6A**, CAM Φ did not have an impaired capacity to express iNOS when differentiated

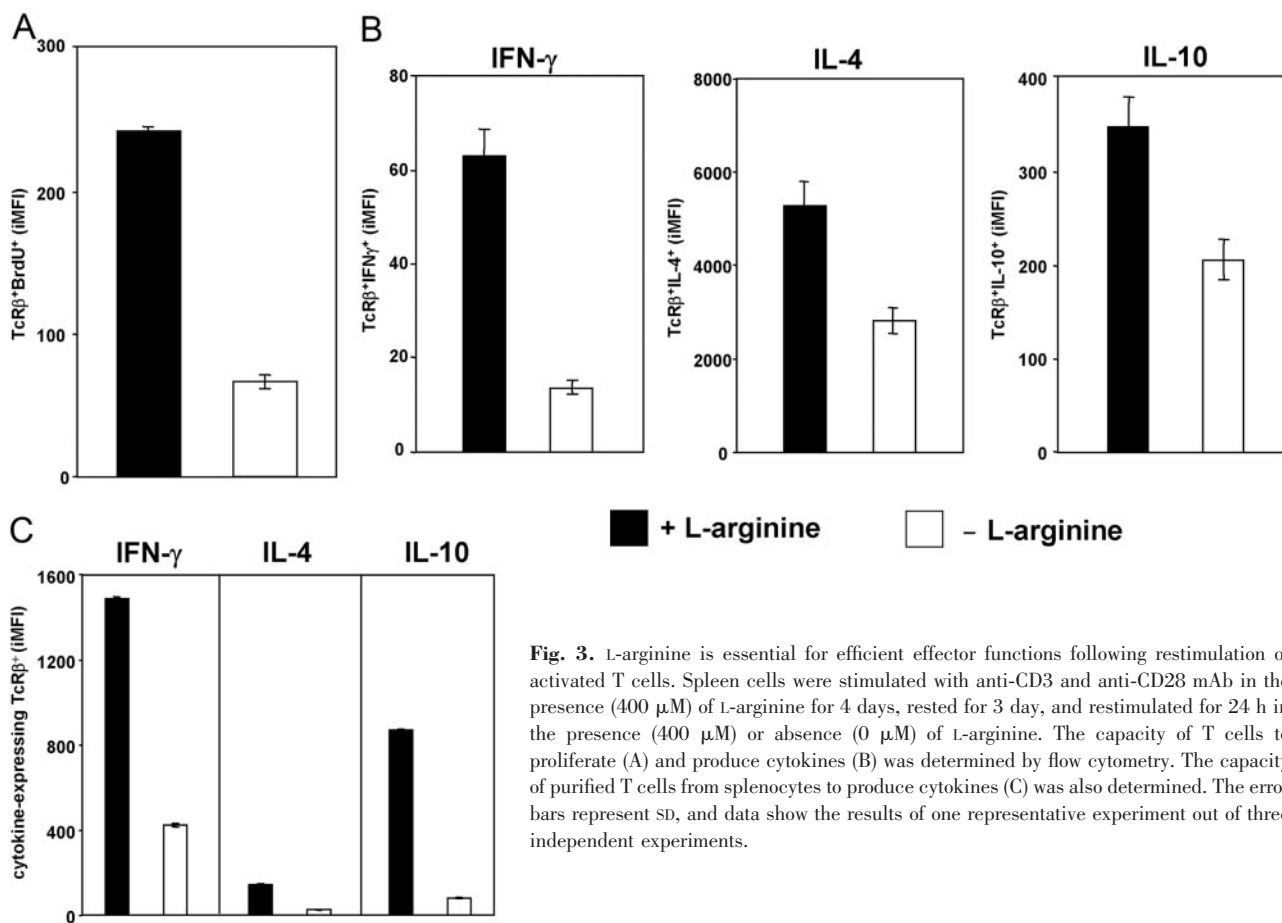


Fig. 3. L-arginine is essential for efficient effector functions following restimulation of activated T cells. Spleen cells were stimulated with anti-CD3 and anti-CD28 mAb in the presence (400 μ M) of L-arginine for 4 days, rested for 3 day, and restimulated for 24 h in the presence (400 μ M) or absence (0 μ M) of L-arginine. The capacity of T cells to proliferate (A) and produce cytokines (B) was determined by flow cytometry. The capacity of purified T cells from splenocytes to produce cytokines (C) was also determined. The error bars represent SD, and data show the results of one representative experiment out of three independent experiments.

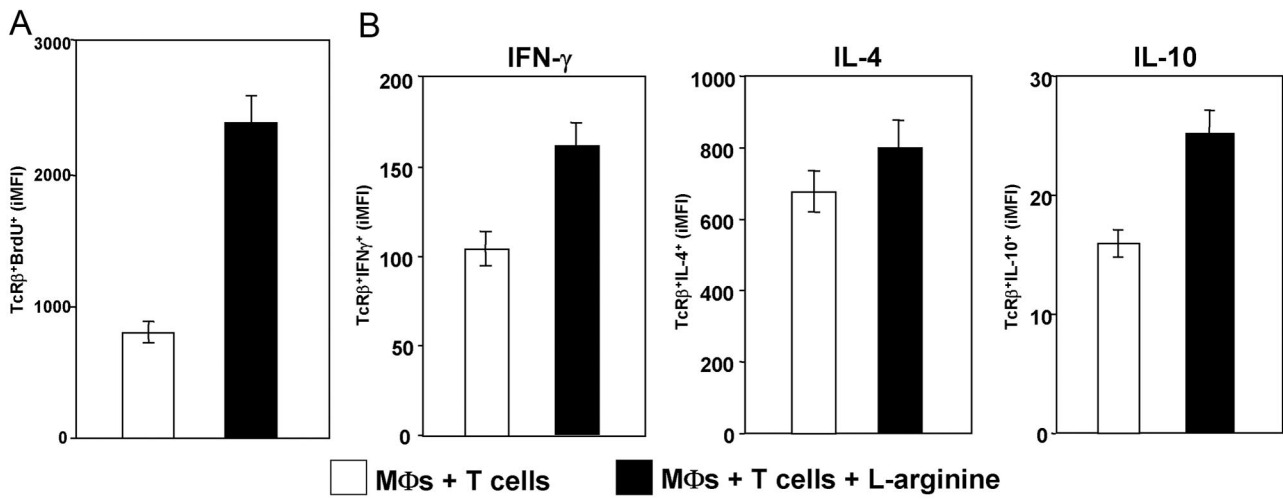


Fig. 4. L-arginine depletion by arginase-expressing MΦ impairs T cells functions. Mature BMMΦs were stimulated with IL-4/IL-10 in the presence of 100 μM L-arginine. Two days later, MΦ were washed, and purified T cells (preactivated with anti-CD3 and anti-CD28 for 24 h) were added to the MΦ. L-arginine (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.

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

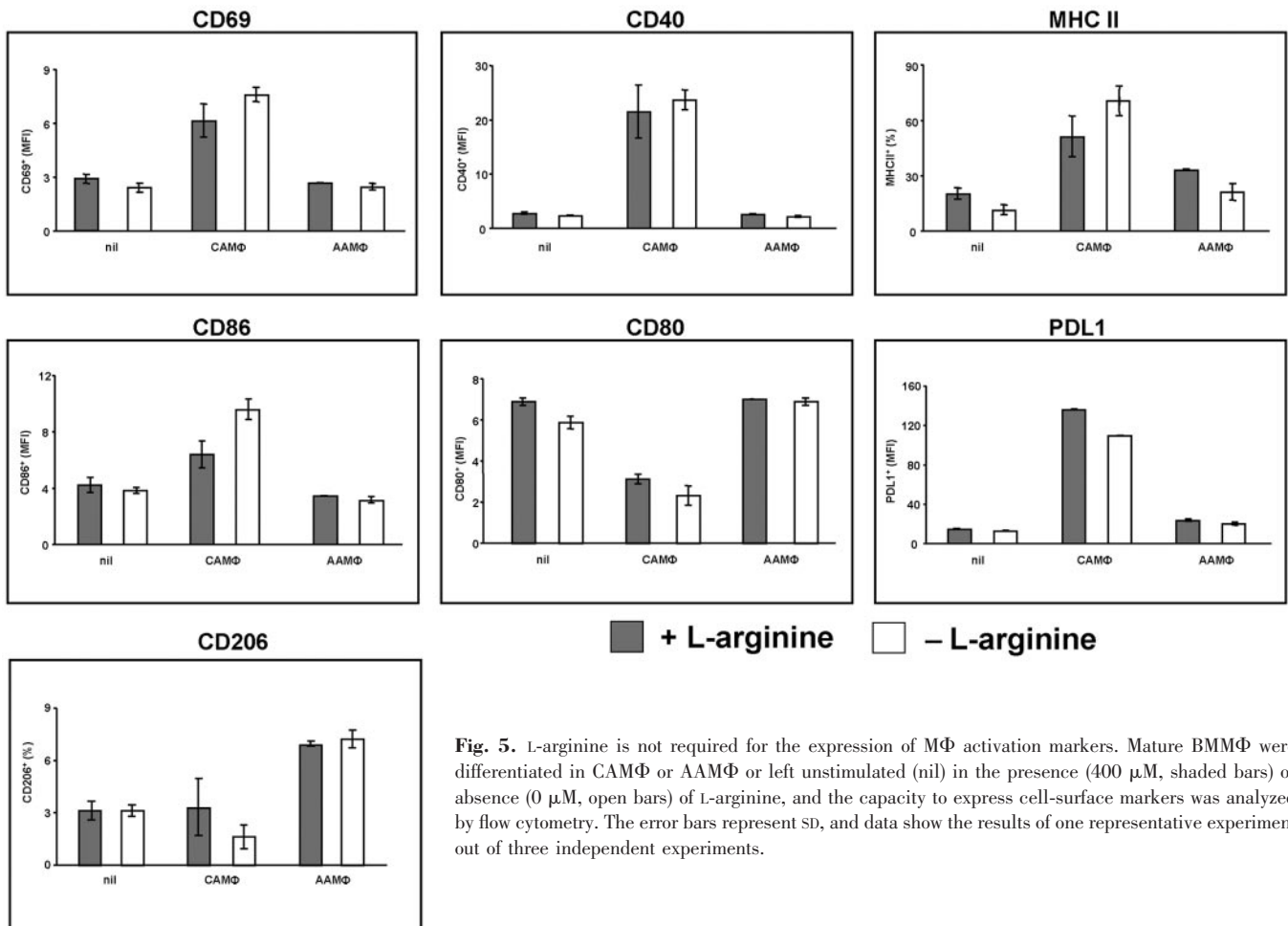


Fig. 5. L-arginine is not required for the expression of MΦ activation markers. Mature BMMΦ were differentiated in CAMΦ or AAMΦ or left unstimulated (nil) in the presence (400 μM, shaded bars) or absence (0 μM, open bars) of L-arginine, and the capacity to express cell-surface markers was analyzed by flow cytometry. The error bars represent SD, and data show the results of one representative experiment out of three independent experiments.

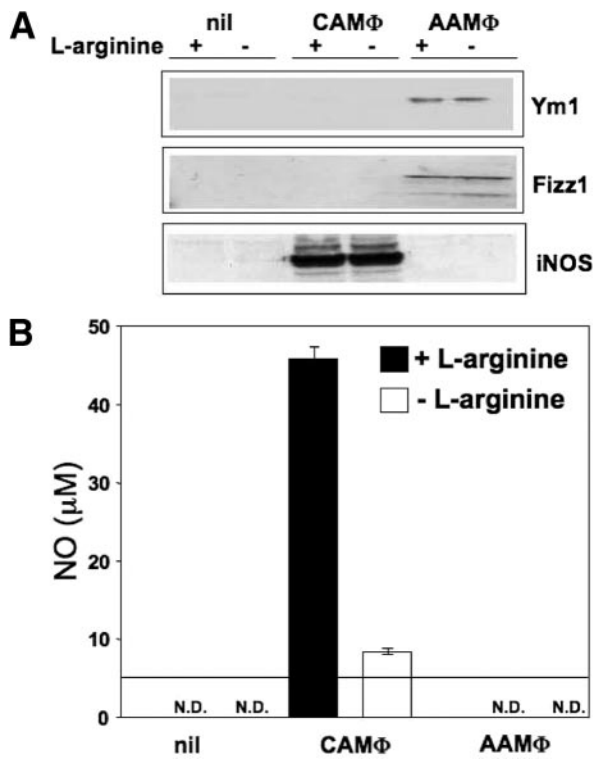


Fig. 6. L-arginine is not required for the expression of Ym1 and Fizz1, two hallmarks of AAMΦ and does not impair iNOS expression by CAMΦ. (A) Mature BMMΦ were differentiated in CAMΦ or AAMΦ or left unstimulated in the presence (400 μM) or absence (0 μM) of L-arginine, and the expression of Ym1, Fizz1, and iNOS was determined by Western blot. The same concentration of protein (14 μg) was loaded for each group. Data show the results of one representative experiment out of three independent experiments. (B) Mature BMMΦ were differentiated in CAMΦ or AAMΦ or left unstimulated in the presence (400 μM) or absence (0 μM) of L-arginine, and the production of NO was measured by the Griess reaction (B). The horizontal line represents the detection limit (5 μM). The error bars represent SD, and data show the results of one representative experiment out of five independent experiments. N.D., Not detected.

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 AAMΦ 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 CAMΦ; arginase activity expressed by AAMΦ remains constant under those conditions (**Table 1**).

The results presented here show that to exert their effector functions, T cells and MΦ 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 MΦ activation and biological functions tested here are not altered by the lack of L-arginine.

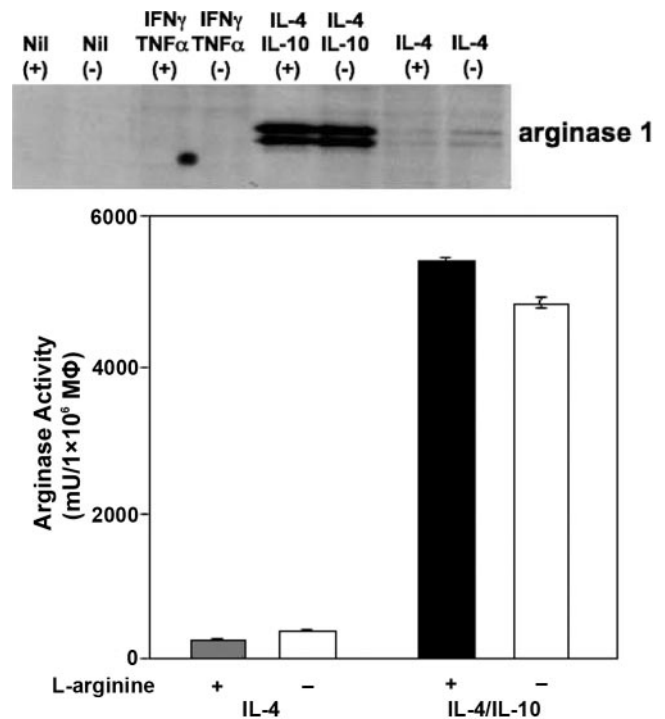


Fig. 7. L-arginine is not required for the expression and activity of arginase. Mature BMMΦ were activated with IFN-γ/TNF-α (CAMΦ), IL-4 (AAMΦ), or IL-4/IL-10 or left unstimulated in the presence (400 μM) or absence (0 μM) of L-arginine, and the expression of arginase was determined by Western blot (upper panel), and the activity of arginase was determined by enzymatic assay (lower panel). The same concentration of protein (14 μg) was loaded for each group. The error bars represent SD, and data show the results of one representative experiment out of five independent experiments.

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

A. NO (μM)		
	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
B. Arginase (mU/1 × 10 ⁶ MΦ)		
	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 L-arginine depletion has been shown to impair T cell responses by modulating the bioavailability of L-arginine. Indeed, human and mouse T cells display an impaired capacity to proliferate and express the CD3 ζ chain in the absence of L-arginine *in vitro*; this can be reversed by addition of exogenous L-arginine. Suppression of T cell responses by L-arginine deprivation has also been demonstrated in physiological [31] and pathological [8, 27, 39] conditions. Here, we characterized further the impact of L-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 L-arginine. Furthermore, we show that in the absence of L-arginine during priming and restimulation of T cells, the quality and the magnitude of these functional responses are impaired drastically. To determine whether L-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 L-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 L-arginine. Of note, the decrease in proliferation and cytokine production of T cells stimulated in the absence of L-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 L-arginine.

Several immune dysfunctions have been associated with a change in the metabolism of L-arginine. For example, in different forms of cancer, high arginase 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 L-arginine regulates T cell responses.

Another line of evidence suggests an important immunoregulatory role for the metabolism of L-arginine: Plasma L-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 CD3 ζ [39]. These studies as well as those from others [1, 30–32, 50], in combination with the results presented here, show clearly that L-arginine deprivation plays a central role in the regulation of T cell activation.

The study of the impact of L-arginine deprivation has been mainly restricted to T cells; however, its role on other cells of the immune system is not well characterized. M Φ not only interact with T cells, but they also can express two L-arginine-metabolizing enzymes—iNOS and arginase 1—and therefore control L-arginine availability and play an important immunoregulatory role. Indeed, the levels of L-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 L-arginine by arginase, have also been shown to down-regulate M Φ activation [51]. Thus, in the next step, we tested the impact of L-arginine deprivation on activated M Φ . Interestingly, we show that the phagocytic capacity of CAM Φ and AAM Φ 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 L-arginine. Importantly, we also show that deprivation of L-arginine did not impair the up-regulation of iNOS and arginase; similar results were obtained with peritoneal exudate M Φ (data not illustrated). It has been shown recently that in the absence of L-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 M Φ do not proliferate [52], our results suggest that activated M Φ do not require L-arginine to the same extent as T cells for their activation and up-regulation of iNOS, arginase, and phagocytosis.

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

The results showing that L-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 L-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 M Φ 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 CAM Φ . 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 L-arginine-free DMEM, the FBS contained in the culture medium does contain L-arginine; however, even when we used dialyzed FBS, the residual levels of NO detected following stimulation without L-arginine were not significantly lower. Similarly, the expression of arginase was also not reduced when M Φ were stimulated in the absence of L-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 L-arginine starvation in the extracellular environment. At least two pools of intracellular L-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 M Φ [56]. Therefore, it is feasible that M Φ use their own synthesized L-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 L-arginine in the plasma, the latter is still relatively high as compared with controls: 45 μ M versus 94 μ M (asthma; ref. [53]), 42 μ M versus 77 μ M (severe cerebral malaria; ref. [57]), and 88 μ M versus 153 μ M (tuberculosis; ref. [39]). These results, as well as those presented in our study, suggest that T cells react differently than M Φ to limiting levels of L-arginine in the extracellular milieu.

The findings presented here further our understanding about the mechanism by which L-arginine depletion regulates immune responses. Targeting the metabolism of L-arginine and the myeloid cells expressing L-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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