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Alternative Metabolic States in Murine Macrophages Reflected by the Nitric Oxide Synthase/Arginase Balance: Competitive Regulation by CD4$^+$ T Cells Correlates with Th1/Th2 Phenotype

Markus Munder, Klaus Eichmann, and Manuel Modolell[1]

Activated murine macrophages metabolize L-arginine via two main pathways that are catalyzed by the inducible enzymes nitric oxide synthase (iNOS) and arginase. We have previously shown that CD4$^+$ T cell-derived cytokines regulate a competitive balance in the expression of both enzymes in macrophages; Th1-type cytokines induce iNOS while they inhibit arginase, whereas the reverse is the case for Th2-type cytokines. Here we addressed the regulation of both metabolic pathways by CD4$^+$ T cells directly. Macrophages were used as APCs for established Th1 and Th2 T cell clones as well as for in vitro polarized Th1 or Th2 T cells of transgenic mice bearing an MHC class II-restricted TCR. Both systems revealed a similar dichotomy in the macrophages; Th1 T cells led to an exclusive induction of iNOS, whereas Th2 T cells up-regulated arginase without inducing iNOS. Arginase levels induced by Th2 T cells far exceeded those inducible by individual Th2 cytokines. Similarly, high arginase levels could be induced by supernatants of Th2 cells stimulated in various ways. Ab blocking experiments revealed the critical importance of IL-4 and IL-10 for arginase up-regulation. Finally, strong synergistic effects between IL-4/IL-13 and IL-10 were observed, sufficient to account for the extraordinarily high arginase activity induced by Th2 cells. Our results suggest that the iNOS/arginase balance in macrophages is competitively regulated in the context of Th1- vs Th2-driven immune reactions, most likely by cytokines without the requirement for direct cell interaction. ***The Journal of Immunology,*** **1998, 160: 5347–5354.**

The cytokine secretion pattern of mouse CD4$^+$ T cells defines two main subsets of helper cells (1–3). Th1 cells secrete IL-2, IFN-γ, and TNF-β, whereas Th2 cells secrete IL-4, IL-5, IL-6, IL-10, and IL-13. Th1 cells are mainly implicated in cell-mediated immune reactions, macrophage activation, and the production of opsonizing Abs. Th2 cells, on the other hand, are key players in humoral immunity and activate mast cells and eosinophils. The Th1/Th2 balance within an immune response is regulated by positive and negative feedback within and between, respectively, both types of cells. The importance of CD4$^+$ T cell dichotomy is underlined by the growing body of evidence that the outcome of numerous diseases critically depends on the Th1/Th2 balance in the accompanying immune responses (2, 3). Macrophages, in addition to having a role in innate immunity, participate as effector cells in adaptive immune responses. Macrophages induced in Th1-dominated immune responses secrete multiple inflammatory mediators (e.g., IL-1, IL-6, and TNF-α) and are therefore termed inflammatory macrophages. Inflammatory macrophages possess cytotoxic and antimicrobial effector functions based on their ability to produce nitric oxide (NO)[2] (4, 5). The production of NO is catalyzed by the enzyme inducible NO synthase, which oxidizes the substrate L-arginine to form NO and L-citrulline. The fundamental importance of this metabolic pathway in murine macrophages as a key defense element in various infectious diseases as well as its role in diverse settings of immunopathology is today firmly established (6, 7). In contrast, the alternative pathway of macrophages to metabolize L-arginine and the functions associated with macrophages using this pathway are less well understood. This metabolic route is catalyzed by the enzyme arginase and leads to the products L-ornithine and urea. Little information about structure, function, and regulation of murine macrophage arginase is available. The enzyme seems to act in trimeric configuration, to be located in mitochondria, and to belong to the family of extrahepatic arginases (AII), as opposed to the hepatic type (AI), which is a component of the urea cycle (for review, see Ref. 8).

We have previously demonstrated that the two enzymes are alternatively induced by Th1 and Th2 cytokines (9, 10). IFN-γ up-regulates exclusively iNOS, whereas IL-4 and IL-10 induce arginase activity. Moreover, induction of one of the enzymes is accompanied by suppression of the other, indicative of two competitive metabolic states in murine macrophages. The present study extends these findings by showing that the alternative states in macrophage metabolism are induced in cellular coculture systems resembling established or early emerging Th1- or Th2-type immune responses. The effects are mediated by cytokines endogenously produced in these cultures, with synergistic action leading to unexpectedly high arginase levels in Th2-dominated immune responses.

Materials and Methods

Medium and reagents

All cell cultures were performed in DMEM supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, 60 μM 2-ME, 1 mM sodium pyruvate, 1$\times$ nonessential amino acids, 100 U/ml penicillin, and 100 μg/ml streptomycin (Life Technologies, Paisley, U.K.).

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[2] Abbreviations used in this paper: NO, nitric oxide; iNOS, inducible nitric oxide synthase; L-NMMA, *N*-monomethyl-L-arginine; PCC, pigeon cytochrome *c*; PE, phycoerythrin; BMMϕ, bone marrow-derived macrophages.

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Conalbumin and monensin were purchased from Calbiochem (La Jolla, CA); *N*-monomethyl-L-arginine (L-NMMA) was purchased from Alexis (San Diego, CA); PMA, Con A, A23187, L-arginine, pigeon cytochrome *c* (PCC), Triton X-100, α-isonitrosopropiophenone, sulfanilamide, *N*-(1-naphthyl)ethylenediamine dihydrochloride, and saponin (lot P4170) were obtained from Sigma (Deisenhofen, Germany). Streptavidin-PE was purchased from Life Technologies (Grand Island, NY).

Cytokines and Abs

Recombinant murine IFN-γ was obtained from Genentech (South San Francisco, CA); IL-2, IL-4, IL-5, IL-12, and IL-13 were purchased from R&D Systems (Abingdon, U.K.); IL-1β, IL-6, and IL-10 were obtained from PeproTech (London, U.K.).

The following mAb were purchased from PharMingen (San Diego, CA): FITC-conjugated anti-IL-4, PE-conjugated anti-IFN-γ, PE-conjugated anti-TNF-α, anti-IL-4, anti-IL-10, isotype controls (FITC-conjugated rIgG2b, PE-conjugated rIgG1, rIgG2b, and rIgG1), biotin-conjugated anti-Vα11, PE-anti-Vβ3, FITC-conjugated anti-CD44, and biotin-conjugated anti-CD62L. Anti-CD3ϵ mAb was purified from hybridoma (145-2C11) supernatant.

Animals

Mice transgenic for the 2B4 $\alpha\beta$-TCR (Vα11, Vβ3), recognizing PCC or moth cytochrome *c* peptide$_{88-103}$ bound to I-E^k (11) were derived from a founder mouse provided by Dr. D. Mathis (Institut de Génétique et de Biologie Moleculaire et Cellulaire du Centre National de la Recherche Scientifique, Strasbourg, France). 2B4 mice (maintained as heterozygotes on a B10BR background) and AKR/N mice were obtained from the specific pathogen-free animal facilities of the Max Planck Institute and were used between 6 and 8 wk of age.

Determination of arginase activity

Arginase activity was measured in cell lysates with slight modifications as previously described (12). Briefly, cells were lysed with 100 μl of 0.1% Triton X-100. After 30 min on a shaker, 100 μl of 25 mM Tris-HCl was added. To 100 μl of this lysate, 10 μl of 10 mM $MnCl_2$ was added, and the enzyme was activated by heating for 10 min at 56°C. Arginine hydrolysis was conducted by incubating the lysate with 100 μl of 0.5 M L-arginine, pH 9.7, at 37°C for 15 to 120 min. The reaction was stopped with 800 μl of H_2SO_4 (96%)/H_3PO_4 (85%)/H_2O (1/3/7, v/v/v). The urea concentration was measured at 540 nm after addition of 40 μl of α-isonitrosopropiophenone (dissolved in 100% ethanol) followed by heating at 95°C for 30 min. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol of urea per min.

NO measurement

NO was measured as nitrite using the Griess reagent. Culture supernatant was mixed with 100 μl of 1% sulfanilamide, 0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride, and 2.5% H_3PO_4. Absorbance was measured at 540 nm in a microplate reader (Molecular Devices, Ismaning, Germany).

Generation of bone marrow-derived macrophages

Bone marrow cells were obtained by flushing the femurs of mice. Cells were cultured as previously described (13) in hydrophobic Teflon bags (Biofolie 25, Heraeus, Hanau, Germany) in DMEM containing 10% heat-inactivated FCS, 5% horse serum, and the supernatant of L929 fibroblasts at a final concentration of 15% (v/v) as a source of CSFs that drive cell proliferation toward a >95% pure population of bone marrow-derived macrophages (BMMϕ).

Cells

D10G4 is a CD4$^+$, $\alpha\beta$TCR$^+$, I-A^k-restricted Th2 T cell clone recognizing conalbumin residues 134 to 146 (14). AE7 (provided by Dr. M. Kopf, Basel Institute for Immunology, Basel, Switzerland) is a CD4$^+$, $\alpha\beta$TCR$^+$, I-E^k-restricted Th1 T cell clone recognizing the carboxyl-terminal fragment 81 to 104 of PCC (15). Both T cell clones were maintained by biweekly stimulation with 30-Gy-irradiated splenocytes (AKR/N mice) and 50 μg/ml of the appropriate Ag.

Ag presentation assays

BMMϕ-T cell coculture experiments were set up in 96-well flat-bottom plates (Costar, Cambridge, MA) in a final volume of 200 μl. Unless otherwise indicated, 5×10^4 BMMϕ were cultured together with 1×10^5 T cells in the presence of the indicated concentrations of Ag. After 48 h, supernatant was harvested for nitrite determination, and cell lysates were prepared for arginase determination.

Intracellular cytokine staining of T cells

T cells were stimulated for 5 h in six-well plates (Costar) with PMA (20 ng/ml), A23187 (300 ng/ml), and monensin (2.5 μM). After blocking FcγRII/III with culture supernatant of hybridoma 2.4G2, cells were fixed with 4% paraformaldehyde (in PBS) for 30 min on ice. After two washing steps in PBS/2% FCS, the cells were washed again in PBS/2% FCS/0.1% saponin. During the subsequent staining (each FITC- or PE-labeled Ab separately on ice for 30 min) and washing procedures, saponin was always present at a concentration of 0.1%. Finally, the cells were resuspended in PBS/2% FCS and analyzed on a FACScan (Becton Dickinson, Mountain View, CA).

In vitro differentiation of naive T cells

CD4$^+$ T cells from lymph nodes of 6- to 8-wk-old 2B4 TCR transgenic mice were isolated on a FACStar^{Plus} (Becton Dickinson), yielding purities of >98%. Sorted Vα11$^+$, Vβ3$^+$, CD62L^{high}, CD44^{low} CD4$^+$ T cells (2×10^5) were primed in 24-well plates (Costar) in a final volume of 1.5 ml with 6×10^6 irradiated (30 Gy) AKR/N spleen cells/well and 50 μg/ml PCC. To promote Th1 development, 200 U/ml IL-12 were added, whereas 25 U/ml IL-4 were added to drive T cells into a Th2 phenotype. At 72 h, the cells were diluted 10-fold in IL-2 (50 U/ml)-containing medium. On day 7, the T cells were harvested, extensively washed to remove residual cytokines, and again subjected to FACS analysis, revealing the Ag-experienced phenotype: CD4$^+$ (>98%), CD62L^{low} (>93%), CD44^{high} (>94%). In parallel, the cells were taken for intracellular cytokine staining and the Ag presentation assays with BMMϕ.

Cytokine determination

Cytokines (IL-4/IL-10/IFN-γ) in the supernatants were determined by commercially available (PharMingen) sandwich ELISA tests according to the manufacturers' protocols. The measuring ranges of the ELISA tests were the following: IFN-γ, 4 to 400 U/ml; IL-4, 0.1 to 10 U/ml; and IL-10, 0.2 to 50 U/ml.

T cell proliferation and stimulation

T cell proliferation in Ag presentation assays was assessed at 48 h by addition of 1 μCi [^{3}H]thymidine (DuPont, Boston, MA). After 16 h the cells were harvested on glass-fiber filters (GF/A, Dunn Labortechnik, Asbach, Germany), and the incorporated radioactivity was measured in an automatic beta counter (Inotech, Asbach, Germany). In T cell proliferation assays, the BMMϕ were irradiated (30 Gy) to exclude a possible contribution to the proliferative response.

To get supernatant of activated T cells, 96-well flat-bottom plates (Costar) were precoated with 50 μl of anti-CD3ϵ mAb (5 μg/ml in PBS) for 2 h at 37°C. After washing with PBS, 10^5 T cells were added to each well in a final volume of 200 μl and incubated for 48 h.

Statistical evaluation

Results were analyzed by analysis of variance without repeated measurement correction and by Dunnett's multiple comparison test.

Results

Th1-type cytokines induce iNOS and Th2-type cytokines induce arginase in AKR/N-BMMϕ

In previous experiments we had demonstrated iNOS/arginase dichotomy by cytokine stimulation of BMMϕ of (BALB/c $\times$ C57BL/6)F$_1$ (H2^d $\times$ H2^b) mice (9, 10). The T cell clones and TCR transgenic mice available for the present study were restricted for H2^k, so that we chose strain AKR/N as the donor of BMMϕ. Figure 1 shows that the previously described alternative induction of iNOS and arginase by IFN-γ and IL-4, respectively, holds true for strain AKR/N macrophages as well. Moreover, additional cytokines were tested, and cytokine stimulation was performed for 48 h in microtiter plates similar to the cellular coculture systems described below. LPS alone stimulated both pathways of macrophage arginine metabolism to moderate levels. IFN-γ was the only cytokine inducing iNOS and failed to induce arginase activity. Furthermore, IFN-γ enhanced the LPS-mediated induction of

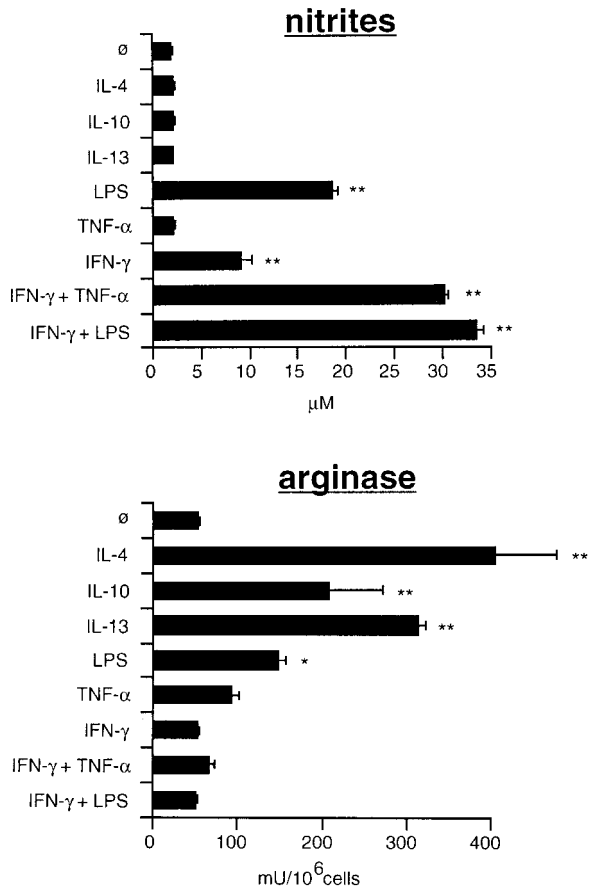

FIGURE 1. Induction of iNOS or arginase in macrophages by various cytokines. AKR/N-BMMϕ (5×10^4) were incubated in a final volume of 200 μl in 96-well flat-bottom plates with the indicated cytokines or LPS (ø, no cytokine added; IL-4, IL-10, and IL-13, 10 U/ml; TNF-α, 500 U/ml; IFN-γ, 100 U/ml; LPS, 0.1 μg/ml). After 48 h, nitrites and arginase activity were determined as described in *Materials and Methods*. The values presented are from one of four independent experiments with similar results. Data represent the mean of triplicates with the SD indicated. The significance of cytokine-mediated iNOS or arginase induction was assessed by Dunnett's multiple comparison test by comparison with the values of unstimulated BMMϕ. *, $p < 0.05$; **, $p < 0.01$.

iNOS and, at the same time, inhibited LPS-induced arginase activity (10, 16). Conversely, up-regulation of arginase activity without iNOS induction was seen with each of the typical Th2 cytokines, IL-4, IL-10, and IL-13, with IL-4 consistently being the most potent inducer. All other cytokines tested (IL1-β, IL-2, IL-5, IL-6, and IL-12) had no effect on L-arginine metabolism (data not shown).

Exclusive induction of iNOS by Th1 CD4$^+$ T cells and of arginase by Th2 CD4$^+$ T cells

In the next set of experiments, iNOS or arginase induction was determined in a cellular coculture system with AKR-BMMϕ as APCs and established Th1 (AE7) and Th2 (D10G4) CD4$^+$ T cell clones. The cytokine production patterns of both T cell clones were confirmed by intracellular cytokine staining (Fig. 2*A*). The data show that the two clones represent extremely polarized forms of Th1 and Th2 cells. BMMϕ were cocultivated with each of the T cell clones and graded amounts of the appropriate Ag. Activation of the T cells was determined by proliferation and cytokine secretion (Fig. 2, *B* and *C*), and the resulting phenotype of macrophage L-arginine metabolism was measured by nitrite production (Fig. 2*D*) or arginase activity (Fig. 2*E*). To exclude the possibility that the T cells themselves contributed to iNOS or arginase induction in our coculture system, we stimulated both T cell clones with all the above (Fig. 1) mentioned cytokines as well as with Con A (5 μg/ml), PMA (1–100 ng/ml) plus A23187 (50–1000 ng/ml), or plate-bound anti-CD3 Abs (see *Materials and Methods*); nitrites or arginase activity were never detected (data not shown). The coculture system yielded unambiguous results. In the presence of the Th1 T cell clone AE7, an Ag dose-dependent up-regulation of iNOS activity (plateauing at about 30 μM nitrites) without concurrent induction of arginase was seen. Nitrite determination truly reflected iNOS activity, as demonstrated by the addition of the iNOS inhibitor L-NMMA, which nearly abolished detectable nitrites (Fig. 2*D*) and at the same time restored the proliferative response of the T cell clone (Fig. 2*B*), confirming the known inhibitory effect of NO on T cell proliferation (17). Conversely, coculture with the Th2 T cell clone D10G4 led to an exclusive induction of arginase activity without induction of nitrites.

Unexpectedly, arginase activity induced in the coculture system reached extraordinarily high levels of about 5000 mU/10^6 cells, >10-fold greater than upon induction of BMMϕ with individual cytokines (see Fig. 1) (10). Moreover, considerable arginase activity was induced at Ag concentrations (~1 μg/ml conalbumin) that appeared too low to result in detectable production of IL-4 in the culture. This raises the question of whether cytokine secretion alone or additional mechanisms are responsible for arginase induction by Th2 cells (see also below, Fig. 3).

CD4$^+$ T cells acquire the ability to regulate the iNOS/arginase balance in Ag-presenting BMMϕ early in a developing immune response

The T cell clones D10G4 and AE7 represent the extreme endpoints of CD4$^+$ T cell polarization. The in vivo situation of a developing immune response (18) may be characterized by more moderately polarizing conditions. To mimic the latter, we chose as a model the in vitro differentiation of naive 2B4 TCR transgenic CD4$^+$ T cells in the presence of Ag/APC and either IL-4 or IL-12, without blocking endogenously produced IL-12 or IL-4, respectively. FACS-sorted naive CD62L^{high} CD4$^+$ T cells were cultured for 1 wk (as described in *Materials and Methods*), and the resulting T cell populations were analyzed by intracellular cytokine staining (Fig. 3*A*). Furthermore, they were used as responding T cells in Ag presentation assays with BMMϕ as APC (Fig. 3, *B–D*). The moderately polarizing conditions were reflected in the resulting phenotype of the T cells; the IL-12 primed population consisted of about 6% IL-4$^+$, 31% IFN-γ^+, and 54% TNF-α^+ T cells, and the IL-4-primed population consisted of about 30% IL-4$^+$, 1% IFN-γ^+, and 38% TNF-α^+ T cells (Fig. 3*A*). Furthermore, IL-10, which can be secreted by T cells or by macrophages (19), was detectable in the culture supernatant during Ag presentation assays with both types of polarized T cells (Fig. 3*B*). Thus, as expected, polarization remained incomplete in the Th1- and Th2-like cell populations. Nevertheless, their presence during Ag presentation assays resulted in macrophage phenotypes that were strongly polarized in L-arginine metabolism (Fig. 3, *C* and *D*). Depending on the Ag concentration, the IL-12-primed T cells induced high levels of nitrites in the culture (plateau at about 60 μM) without up-regulating macrophage arginase. The opposite holds true for the IL-4-primed T cells. Again, arginase levels reached a plateau at about 2000 mU/10^6 cells, a level severalfold greater than upon addition of IL-4 alone. No nitrites were detected until arginase activity had reached plateau levels. However, further increased concentrations of Ag were accompanied by iNOS induction, presumably reflecting the incomplete polarization of the IL-4-primed T cells. IFN-γ-producing

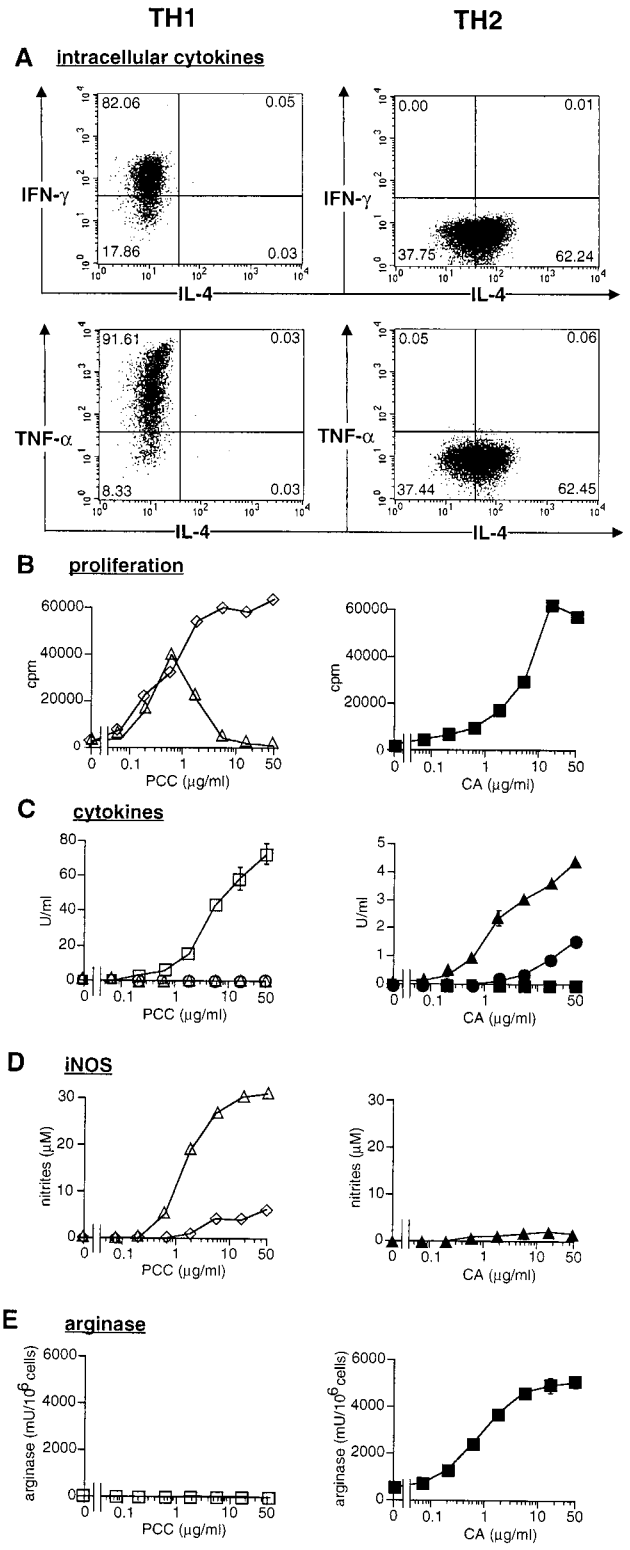

FIGURE 2. Ag-dependent specific induction of iNOS or arginase in macrophages by Th1 or Th2 T cells. *A*, Intracellular cytokine staining pattern (IFN-γ/IL-4 and TNF-α/IL-4) of the Th1 cell clone AE7 (two panels on the *left*) and the Th2 cell clone D10G4 (two panels on the *right*) after stimulation with PMA and A23187 as described in *Materials and Methods*. Quadrant gates are set according to isotype-specific control Abs. *B* through *E*, Ag presentation assays with AKR/N-BMMϕ as APC and Th1 cell clone AE7 (open symbols) or Th2 cell clone D10G4 (filled symbols) as described in *Materials and Methods*. *B*, Ag-dependent proliferation of both T cell clones with irradiated (30 Gy) BMMϕ as APC. Proliferation of the Th1 T cell clone is shown without (triangles) or with addition of L-NMMA (500 μM; diamonds). Proliferation of BMMϕ or T cells alone was always $<$450 cpm. *C*, Determination of cytokines (IFN-γ (squares), IL-4 (circles), and IL-10 (triangles)) in 48-h supernatant of Ag presentation assays as described in *Materials and Methods*. In cultures of macrophages or T cells alone, the indicated cytokines were not detectable. *D*, Determination of nitrites after 48-h coculture without (triangles) or with addition of L-NMMA (500 μM; diamonds). BMMϕ cultured without T cells showed $<$2 μM nitrites at all concentrations of Ag. Nitrites in T cell cultures alone were never detected. *E*, Arginase activity (squares) was determined in the cell lysate at the end of the culture period as described in *Materials and Methods*. BMMϕ cultured without T cells showed $<$50 mU/10^6 cells arginase activity at all concentrations of Ag. Arginase activity in T cell cultures was never detected. The data in *B* through *E* represent the mean of triplicates with the SD indicated. Similar results were obtained in a total of four independent experiments.

cells (1.35%) could clearly be detected by intracellular cytokine staining, although simultaneous cytokine determinations by ELISA (Fig. 3*B*) in the culture supernatant revealed no detectable IFN-γ. Furthermore, TNF-α produced by the IL-4-primed T cell population (38.51% TNF-α^+ cells) may synergize with low amounts of IFN-γ in iNOS induction. Essentially the same dichotomous pattern of enzyme induction was seen if peptide (moth cytochrome *c* peptide) was used as Ag instead of PCC (data not shown).

Soluble factors, most importantly IL-4 and IL-10, are responsible for arginase induction during a Th2-dominated immune response

As shown by the results in Figures 2 and 3, Th2 cell-induced arginase activity reached levels considerably greater than those inducible by the strongest soluble agonist, IL-4. Moreover, induction took place at Ag concentrations significantly lower than those required for induction of IL-4 to levels detectable by ELISA. Because interactions between macrophages and T cells include direct cell-to-cell contact (20), we considered that such direct interactions, possibly by costimulatory molecules, were involved in the induction of arginase in our coculture system. The role of soluble factors was ascertained by transfer of the 48 h supernatant of a D10G4-BMMϕ Ag presentation assay (Fig. 4*A*) onto fresh BMMϕ. The resulting levels of arginase induction in the second culture (Fig. 4, *B* and *C*) were comparable to those found in the first, even if the supernatant was used at a final concentration of only 25%. Moreover, the supernatant of plate-bound anti-CD3-stimulated D10G4 cells induced arginase in BMMϕ to levels comparable to those reached in the cellular coculture system (Fig. 4*D*).

The relative contributions of IL-4 and IL-10 were assessed by adding appropriate mAbs to our in vitro Ag presentation system. Figure 5 demonstrates that each of the two Abs alone inhibited the induction of arginase partially. Inhibition was enhanced if both Abs were added together. However, considerable arginase activity remained detectable even if both Abs were added. This may be due to the existence of other known (e.g., IL-13) or unknown arginase-inducing cytokines. Together, the experiments in Figures 4 and 5 suggest that cytokines produced by the T cells are mainly (if not exclusively) responsible for the induction of arginase, although autoregulatory cytokine secretion of the macrophages (e.g., production of IL-10) is not formally ruled out.

The cytokines IL-4 and IL-10 synergize in the Th2 cell-induced up-regulation of arginase activity in BMMϕ

We suspected synergistic mechanisms to account for the superior efficacy of Th2 cells over Th2 cytokines in the induction of arginase in macrophages. Synergistic effects in iNOS induction among

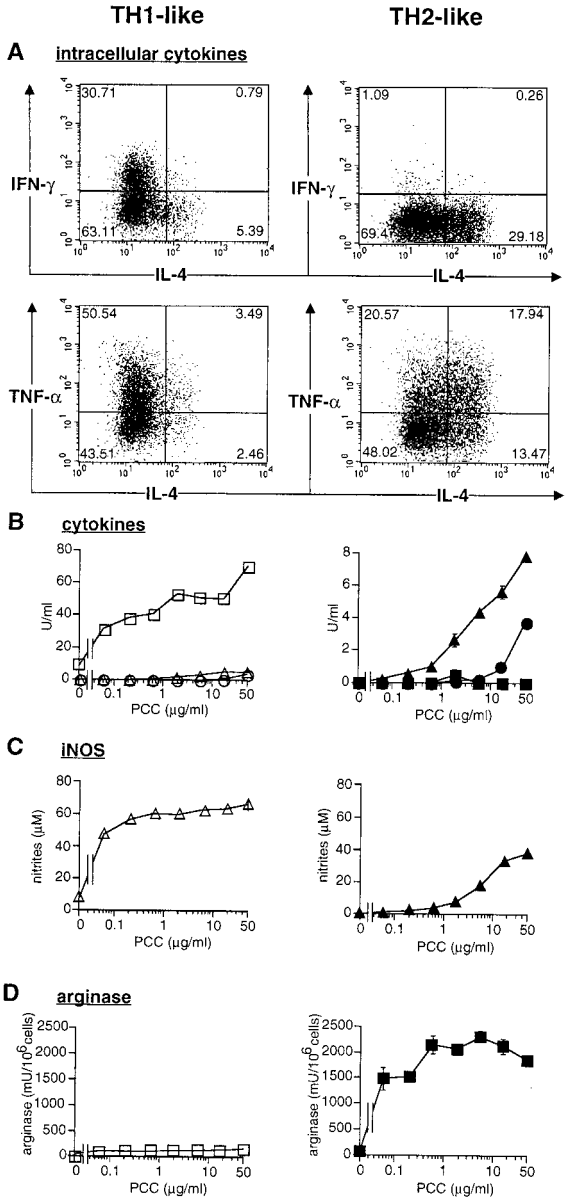

FIGURE 3. Ag-dependent specific induction of iNOS or arginase in BMMϕ by in vitro polarized CD4$^+$ T cells. *A*, Intracellular cytokine staining pattern (IFN-γ/IL-4 and TNF-α/IL-4) of in vitro polarized FACS-sorted 2B4 TCR transgenic naive (CD62L^{high}, CD44^{low}) CD4$^+$ T cells. Cells were analyzed as described in *Materials and Methods* after 1 wk of in vitro polarization under the influence of either 200 U/ml IL-12 (Th1-like, two panels on the *left*) or 25 U/ml IL-4 (Th2-like, two panels on the *right*). *B* through *D*, In vitro polarized T cells were cocultured together with AKR/N-BMMϕ as APC and various concentrations of Ag (PCC). Results with Th1-like cells (polarized under the influence of IL-12) are shown on the *left* (open symbols), whereas results with Th2-like cells (polarized under the influence of IL-4) are depicted on the *right* (closed symbols). *B*, Determination of cytokines (IFN-γ (squares), IL-4 (circles), and IL-10 (triangles)) in 48-h supernatant of the above-mentioned Ag presentation assays as described in *Materials and Methods*. BMMϕ or T cells alone produced no detectable cytokines. *C*, Determination of nitrites (triangles) in the supernatant of the above-mentioned Ag presentation assays after 48 h. Nitrites in control BMMϕ without added T cells were always <2 μM and undetectable in control T cell cultures. *D*, Determination of arginase activity (squares) in the cell lysate of the above-mentioned Ag presentation assays after 48 h. Arginase activity in control BMMϕ without added T cells was always <40 mU/10^6 cells and undetectable in T cell control cultures. Data in *B* through *D* represent the mean of triplicates with the SD indicated. Similar results were obtained in a total of three independent experiments.

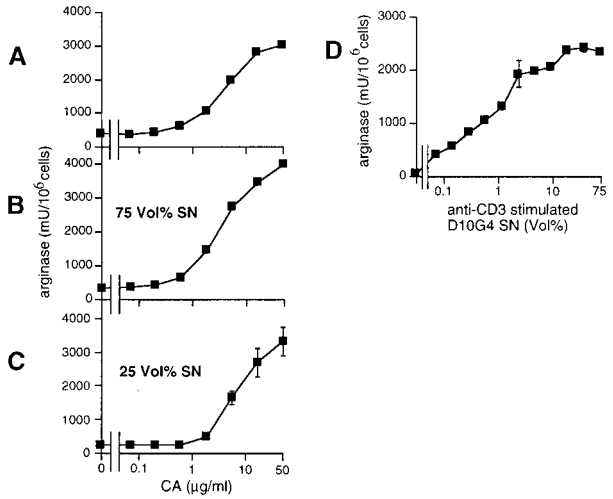

FIGURE 4. Cell membrane-bound structures are not necessary for the Th2 cell-mediated induction of arginase in BMMϕ. *A*, Arginase activity was determined after 48 h in the AKR-BMMϕ/D10G4 Ag presentation assay as described in *Materials and Methods*. *B* and *C*, In parallel, 150 μl (75 Vol%; *B*) or 50 μl (25 Vol%; *C*) supernatant (SN) of this assay was transferred after 48 h onto fresh AKR/N-BMMϕ (5×10^4; final volume, after addition of SN, 200 μl), and arginase activity was determined again after 48 h. Nitrites were always <3 μM. *D*, Supernatant of plate-bound anti-CD3-stimulated D10G4 T cells (see *Materials and Methods*) was titrated onto 5×10^4 AKR/N-BMMϕ/well (final volume, 200 μl), and arginase activity was determined after 48 h. Nitrites were always <2 μM. Data represent the mean of triplicates with the SD indicated. Similar results were obtained in a total of three independent experiments.

Th1-type cytokines have been described (21); IL-2, TNF-α, and TNF-β are unable to induce iNOS activity on their own, but increase the IFN-γ-induced iNOS activity (see also Fig. 1 for synergism between IFN-γ and TNF-α). We investigated possible additive or synergistic effects among the Th2-type cytokines, IL-4,

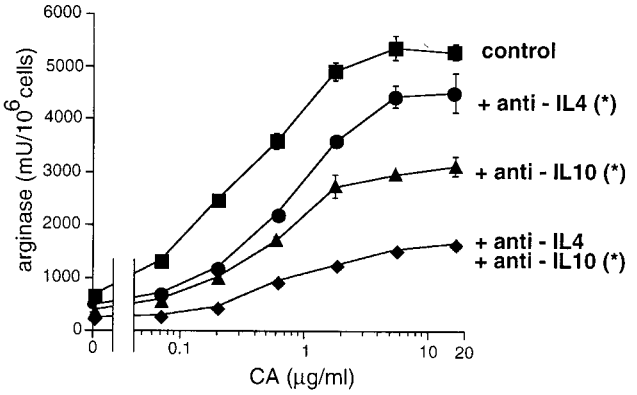

FIGURE 5. Ab-mediated inhibition of Th2 cell-induced arginase in BMMϕ. Arginase activity was determined after 48 h in an AKR/N-BMMϕ/D10G4 Ag presentation assay (control, squares). In parallel, mAbs against IL-4 (circles), IL-10 (triangles), or IL-4 and IL-10 (diamonds) were added at 5 μg/ml at the beginning of the coculture, and arginase activity was measured after 48 h. Isotype-matched control Abs (rat IgG2b and rat IgG1) had no influence on arginase induction. Data represent the mean of triplicates with the SD indicated. The values presented are from one of three independent experiments with similar results. The significance of Ab-mediated inhibition of Th2 T cell induced arginase activity was assessed by Dunnett's multiple comparison test by comparison with the values of control BMMϕ-D10G4 coculture at the same Ag concentration. *, $p < 0.01$ for all values of the corresponding curve.

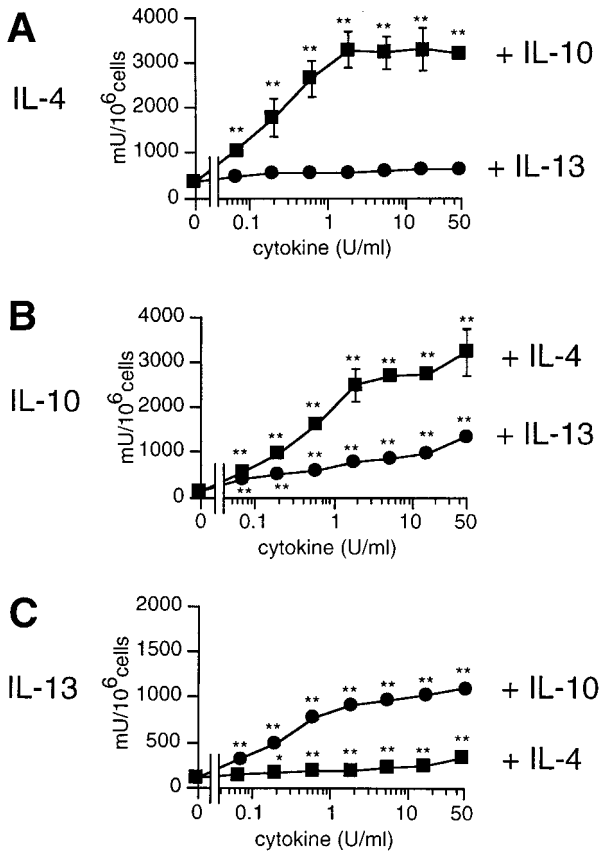

FIGURE 6. Cytokine-induced arginase in BMMϕ: synergism of inducing cytokines. AKR/N-BMMϕ (5×10^4) were incubated with a constant amount (10 U/ml) of one cytokine (indicated on the *left*; *A*, IL-4; *B*, IL-10; *C*, IL-13) and increasing concentrations of a second cytokine (mentioned at the end of each graph). After 48 h arginase activity was determined as described in *Materials and Methods*. Arginase activity of BMMϕ without cytokine stimulation was 57 mU/10^6 cells. Nitrites in the supernatants were always <2 μM. Data represent the mean of triplicate determinations with the SD indicated. The values presented are from one of three independent experiments with similar results. The significance of the enhancing effect regarding arginase induction of the added second cytokine was assessed by Dunnett's multiple comparison test by comparison with the values obtained without addition (i.e., with first cytokine alone). *, $p < 0.05$; **, $p < 0.01$.

IL-5, IL-6, IL-10, and IL-13, in arginase induction in BMMϕ. In a pilot experiment (not shown) it was determined that arginase levels induced in AKR-BMMϕ by IL-4, IL-10, and IL-13 individually plateaued at around 400, 200, and 300 mU/10^6 cells, respectively (see also Fig. 1). In subsequent experiments one cytokine was held constant (at 10 U/ml), and a second cytokine (0–50 U/ml) was titrated into the culture system. When IL-4 was held constant (Fig. 6*A*), addition of minimal concentrations of IL-10 pushed arginase levels to >1000 mU/10^6 cells, and a plateau at about 3000 mU/10^6 cells was reached. Similar plateau arginase levels were obtained in the reverse combination, although at higher concentrations of IL-4. (Fig. 6*B*), IL-13 alone was less effective in arginase induction compared with IL-4 (Fig. 1), as reflected in the lower arginase activity reached during synergism of IL-13 with IL-10, which plateaued at about 1000 mU/10^6 cells (Fig. 6, *B* and *C*). IL-4 and IL-13 showed no cooperation, not even additive effects; when arginase was already up-regulated by a fixed concentration of IL-4 (10 U/ml), IL-13 had no further influence on arginase activity. When the IL-13 concentration was fixed, the addition of IL-4 increased the response to a level similar to that reached by IL-4 alone (Fig. 6, *A* and *C*). IL-13 had no augmenting influence on IL-4- plus IL-10-induced arginase activity; IL-5 and IL-6 showed no modulating influence on Th2 cytokine-induced arginase up-regulation (data not shown). Together the data suggest an impressive synergism among Th2-type cytokines in the induction of arginase that is most pronounced between IL-4 and IL-10 and, to a lesser degree, between IL-13 and IL-10. In quantitative terms, the synergistic arginase response to combinations of IL-4 and IL-10 appears to be sufficient to account for the unexpectedly high arginase levels induced by Th2 cells.

Discussion

The powerful host-protective effects of NO are counterbalanced by the role of this compound in immunopathology (6) or endotoxic shock. A tight regulation of NO synthesis therefore appears to be crucial for the host (4). In this respect growing interest is focused on arginase (8, 22), the alternative L-arginine-metabolizing enzyme in macrophages. Both metabolic pathways have been demonstrated in LPS-stimulated murine peritoneal macrophages (23). Recently, the LPS-mediated coinduction of iNOS and arginase II in a mouse macrophage-like cell line (RAW 264.7) (16) or of iNOS and arginase I in rat peritoneal macrophages was reported (24). Following our initial findings that detoxified LPS solely up-regulates arginase without concomitant iNOS induction (9), we demonstrated the selective induction of both enzymes by $CD4^+$ T cell-derived cytokines. The Th1 cytokine IFN-γ induces specifically iNOS, and the Th2-type cytokines IL-4 and IL-10 (9, 10) as well as IL-13 (this study) up-regulate arginase. Furthermore, a mutual negative feedback between both pathways suggested a competitive regulation; IFN-γ was found to suppress arginase activity in macrophages stimulated with LPS or IL-4 (10), whereas the iNOS-suppressing activities of IL-4 and IL-10 are well established (19, 25). Interestingly, *N*-hydroxy-L-arginine, the intermediate during NO synthesis, was a strong inhibitor of liver and macrophage arginase (26). The scenario of two alternative and competitive modes of L-arginine metabolism in macrophages was further completed by our demonstration of two distinct signal transduction pathways: protein kinase A in arginase induction as opposed to protein kinase C in iNOS up-regulation (27). The competitive nature of the regulation of L-arginine metabolism in macrophages described in this paper may point toward a biologic significance of arginase or its products, similar to that of iNOS and NO. The demonstration of up-regulated iNOS within intratumor macrophages during tumor rejection as opposed to induced arginase during progressive tumor growth (28) as well as the reciprocal regulation of both enzymes during wound healing (29) further support this idea.

The purpose of the present study was to determine whether the iNOS/arginase balance in macrophages was regulated by Th1/Th2 cells in a similar fashion as by the corresponding cytokines. We chose well-known Th1 and Th2 $CD4^+$ T cell clones as representatives of completely polarized immune responses. These cells were stimulated in vitro by their specific Ag using BMMϕ as APCs. In a recent report it has been demonstrated that Th1, but not Th2, cell clones were able to induce NO production in murine macrophages (in this case in ANA-1 macrophages and thioglycolate-elicited peritoneal macrophages) (30). Conversely, we now demonstrate that a Th2, but not a Th1, cell clone up-regulated arginase. Moreover, we confirm the differential effects of Th1 vs Th2 cell clones on NO production. We could not detect nitrites or arginase activity under different modes of stimulation of the T cells used in this study. We, therefore, do not think that in our experiments T cells contributed to the production of iNOS, as described

in one earlier report for malaria-specific Th1 T cell clones (31), or arginase activity. Furthermore, our experiments with in vitro polarized naive T cells suggest that the competitive induction of the two L-arginine-metabolizing pathways may be established early in a developing immune response and is not a phenomenon restricted to highly polarized, long term cultured T cell clones. The in vitro polarized Th1 and Th2 T cells carried the same clonal TCR, excluding that the induced dichotomy in macrophage metabolism depends on factors contributed by the Ag. Furthermore, the phenomenon appears to be independent of the genetic background of the macrophages: BMMϕ of C3H, B10BR, and CBA/N mice gave essentially similar results as the AKR/N mice used here (data not shown). As an indicator of iNOS induction, we determined the accumulation of nitrite, one of NO's stable degradation products, in the supernatant at the end of the cell culture period. Arginase activity, on the other hand, was measured at the end of the culture period in the cell lysate as an end-point enzymatic assay under optimal conditions (unlimited substrate availability, optimum pH 9.5). We are aware of the fact that our results do not therefore allow for a direct quantitative comparison of the absolute fluxes of L-arginine through either of the two metabolic pathways. This indeed was not the aim of our work and should be investigated by future radioactive labeling studies. The essential finding of our study, namely the clear-cut Th1/Th2-mediated all-or-none induction of either of the two enzymes, is in our view not impaired by this restriction of quantification.

A striking feature of the cellular coculture assays was that the Th2 cell-induced arginase activity exceeded by far the levels reached after stimulation of the macrophages with individual cytokines. We considered three possible explanations for this pronounced quantitative difference: the participation of cell membrane-bound structures, the existence of as yet unknown potent arginase-inducing cytokines, and synergism among Th2-type cytokines in arginase induction. An earlier report demonstrated the participation of membrane-bound structures on T cells in iNOS induction in macrophages (32), and a recent report showed the participation of the CD40-CD40 ligand interaction during the early phase of this iNOS induction (33). We found that arginase induction was fully transferable by culture supernatants, including supernatants of anti-CD3-activated Th2 cells. Thus, direct cell-cell interactions may not be required in arginase induction by T cells. An unresolved problem is the relatively high background of arginase in the coculture assays with the established Th2 cell clone D10G4. Even without addition of Ag, a remarkable up-regulation of arginase activity in the macrophages (in the range of 400 mU/10^6 cells) was consistently found. Possible explanations include cell membrane-bound cytokines or other structures on the T cells as well as minute amounts of secreted cytokines by not completely resting T cells. Nevertheless, the conclusion that soluble factors predominate during Th2 T cell-mediated arginase induction is further strengthened by our Ab-blocking experiments (Fig. 5), demonstrating a pivotal role for IL-4 and IL-10 in Th2 cell-induced arginase activity in BMMϕ.

The excessive arginase induction by Th2 cells is fully accounted for by synergism between IL-4 and IL-10 and, to a lesser degree, between IL-13 and IL-10. This Th2 cytokine synergism also seems to be the explanation for the pronounced up-regulation of arginase by low or even undetectable individual cytokine concentrations (Figs. 2 and 3), making arginase activity a high sensitivity Th2 read-out system. The lack of synergism between IL-4 and IL-13 was no surprise, since IL-4 and IL-13 are known to partially share the same receptor and signal transduction pathways and exert similar biologic functions (34, 35). Few reports in the literature have demonstrated synergism among IL-4 and IL-10, including the inhibition of schistosomulum killing and NO production by IFN-γ-activated murine macrophages (36) or the inhibition of delayed-type hypersensitivity to *Leishmania major* in mice (37) by an unknown mechanism. These reports fit into the prevailing view on Th2 cytokines as macrophage-deactivating agents. In particular, IL-4 was demonstrated to potently inhibit NO synthesis by inflammatory murine macrophages (25, 38), whereas IL-10 preferably suppresses the macrophage release of TNF-α and reactive oxygen intermediates (39). Furthermore, IL-4 and IL-10 were recently shown to inhibit killing of *Leishmania* spp. in human macrophages by decreasing NO generation (40). In an extension of these findings, we clearly demonstrate that the down-regulating activities of IL-4, IL-10, and IL-13 are intimately associated with alternative macrophage activation. The role of Th2 cytokines as an alternative class of macrophage-activating agents is underscored by the findings that IL-4 and IL-13 potently enhance murine macrophage MHCII (35, 41) as well as mannose receptor expression (35, 42). Even for IL-10, generally considered an immunoinhibitory cytokine (19, 39), published data demonstrate immunostimulatory properties as well (43). Interestingly, TGF-β, a cytokine not attributable to either the Th1 or Th2 subset of T cells (2), was reported to suppress murine macrophage NO release (44) and to stimulate arginase activity in rats (45).

To summarize, in the present study we were able to demonstrate for the first time that arginase activity in murine macrophages is exclusively up-regulated during Th2 (as opposed to Th1)-dominated immune reactions. This process of Th2 cell-induced, Ag-dependent up-regulation of arginase activity is mediated by cytokines (especially IL-4 and IL-10). Finally, we demonstrated a previously unknown mechanism of synergy among Th2-type cytokines that fully accounts for the extraordinarily high arginase levels (as opposed to stimulation by individual Th2 cytokines) in the cellular coculture systems. Taken together with the known Th1-mediated up-regulation of iNOS, our results corroborate the hypothesis of two alternative L-arginine-metabolizing enzymatic pathways, reflecting the two alternative functional modes of the $CD4^+$ T cell compartment.

One possible function of Th2 cytokine-induced arginase might be to divert the common substrate L-arginine away from iNOS and thereby to suppress NO output (10, 23). Further speculations concern the arginase product L-ornithine, the precursor amino acid for the synthesis of polyamines (spermine, spermidine, and putrescine) that participate in the process of cell growth and are known to interact with DNA and RNA and to influence protein synthesis (46). Interestingly, spermine was shown to suppress NO production in LPS-stimulated macrophages (47) as well as to inhibit specifically the synthesis of proinflammatory cytokines in human mononuclear cells (48). The macrophage is capable of adopting extraordinarily diverse metabolic and functional phenotypes (49). The specific, high level induction of arginase in macrophages (as demonstrated here) might indeed turn out to be a key effector mechanism by which Th2 T cells regulate proinflammatory immune responses. Furthermore, it might be fruitful to investigate the role of up-regulated arginase in host-protective (50), Th2-driven immune responses as well as during allergy.

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