Th1/Th2-Regulated Expression of Arginase Isoforms in Murine Macrophages and Dendritic Cells

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Activated murine macrophages metabolize arginine by two alternative pathways involving the enzymes inducible NO synthase (iNOS) or arginase. The balance between the two enzymes is competitively regulated by Th1 and Th2 T helper cells via their secreted cytokines: Th1 cells induce iNOS, whereas Th2 cells induce arginase. Whereas the role of macrophages expressing iNOS as inflammatory cells is well established, the functional competence of macrophages expressing arginase remains a matter of speculation. Two isoforms of mammalian arginases exist, hepatic arginase I and extrahepatic arginase II. We investigated the regulation of arginase isoforms in murine bone marrow-derived macrophages (BMMφ) in the context of Th1 and Th2 stimulation. Surprisingly, in the presence of either Th2 cytokines or Th2 cells, we observe a specific induction of the hepatic isoform arginase I in BMMφ. Induction of arginase I was shown on the mRNA and protein levels and obeyed the recently demonstrated synergism among the Th2 cytokines IL-4 and IL-10. Arginase II was detectable in unstimulated BMMφ and was not significantly modulated by Th1 or Th2 stimulation. Similar to murine BMMφ, murine bone marrow-derived dendritic cells, as well as a dendritic cell line, up-regulated arginase I expression and arginase activity upon Th2 stimulation, whereas arginase II was never detected. In addition to revealing the unexpected expression of arginase I in the macrophage/monocyte lineage, these results uncover a further intriguing parallelism between iNOS and arginase: both have a constitutive and an inducible isoform, the latter regulated by the Th1/Th2 balance. The Journal of Immunology, 1999, 163: 3771–3777.

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× nonessential amino acids, 100 U/ml penicillin, and 100 μg/ml streptomycin (Life Technologies, Paisley, U.K). Conalbumin was purchased from Calbiochem (La Jolla, CA); l-arginine, pigeon cytochrome c.
(PCC), Triton X-100, α-isonitrosopropiophenone, sulfanilamide, and
N-(1-naphthyl)ethylenediamine dihydrochloride were obtained from Sigma
(Deisenhofen, Germany). LPS (from Salmonella abortus equi) was gener-
ously provided by Dr. C. Galanos (Max-Planck-Institut für Immunbiologie
Freiburg, Germany).

Cytokines and animals
Recombinant murine IFN-γ was obtained from Genentech (South
Francisco, CA), IL-10, GM-CSF, and TNF-α from Pepro-Tech (London,
U.K.), IL-4 and IL-13 were purchased from R&D Systems (Abingdon,
U.K.).

Mice of strain AKR/N, C57BL/6, and C57BL/6 in which the IL-10 gene
was deleted by homologous recombination (IL-10 KO mice) were obtained
from a modified protocol of Inaba et al. (14). Bone marrow cells (1 x 10
6 ) were cultured together with 1% sulfanilamide, 0.1% L-arginine
and arginase II sense primer, 5'-GAATGGAAGAGTCAG-3', and arginase
I antisense primer, 5'-AGAAAGTAGTTAATGGG-3'. The identities of
BMMF was investigated by RT-PCR with primers that discrimi-
nated between arginase I and II cDNA sequences. The PCR products
were resolved on a 1% (for detection of iNOS) or 12.5% (for detection of arginase) SDS-PAGE gel.

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 MnCl2 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 15–120 min. The reaction was stopped with 900
µl of 3 M H2SO4 (96%)/H3PO4 (85%)/H2O (1/3/7, v/v/v). The urea concentration
(pH 9.7) at 37°C for 5 min. And protein in BMM

was measured by the bicinchoninic acid assay (Pierce, Rockford, IL).
The samples were mixed 1:1 with sample buffer (125 mM Tris-HCl (pH 6.8), 20%
glycerol (v/v), 4% SDS, 40 mM DTT, and 0.01% bromphenol blue), boiled for
5 min, and 20 µg aliquots of protein were separated on a 7.5% (for detection of iNOS) or 12.5% (for detection of arginase) SDS-PAGE gel.

The proteins were transferred to a nitrocellulose membrane (Schleicher
& Schuell, Keene, NH), which was then blocked with 5% nonfat dry milk in
PBS at 4°C overnight. In the case of iNOS detection, the membrane was
incubated with a monoclonal anti-iNOS Ab (Transduction Laboratories,
Lexington, KY) for 1 h and subsequently with alkaline phosphatase-conju-
gated goat anti-rabbit Ig (Southern Biotechnology Associates, Birmingham,
AL). Finally, the blots were incu-

FIGURE 1. Cytokine-induced regulation of arginase I and arginase II mRNA in BMMΦ. A. AKR/N-BMMΦ (1 × 10^6) were incubated with the indicated cytokines (IFN-γ, 100 U/ml; TNF-α, 500 U/ml; IL-4 and IL-10, 10 U/ml). After 2, 8, and 24 h, RNA was extracted and cDNA prepared as described in Materials and Methods. Murine liver and kidney cDNA preparations served as internal controls. A total of 1 μl cDNA (corresponding to 50 ng RNA) was amplified by PCR with primers specific for arginase I and arginase II. To control for comparable cDNA amount, β-actin mRNA was also amplified with 0.01 μl input cDNA. One of five independent experiments, yielding comparable results, is shown. B. Control BMMΦ of strain C57BL/6 or BMMΦ of C57BL/6 mice, in which the IL-10 gene was deleted (IL-10 KO mice) were incubated with the indicated cytokines (concentrations as in Fig. 1). After 24 h, RNA was extracted, cDNA prepared, and amplified as described in Fig. 1A.

2 h of stimulation. Whereas IL-10 stimulated a far less pronounced and transient expression of arginase I mRNA, this cytokine efficiently enhanced the induction of arginase I mRNA by IL-4, confirming the previously observed synergism between these cytokines as determined by measuring arginase activity (11). Th1 cytokines did not induce arginase I mRNA until 24 h of stimulation when a moderate induction of arginase I mRNA was notable upon stimulation with IFN-γ or IFN-γ + TNF-α. Induction of arginase I mRNA by Th1 cytokines did not take place in BMMΦ of mice in which the IL-10 gene was deleted by homologous recombination (Fig. 1B), and is therefore probably a secondary effect of IL-10, secreted by the macrophages upon Th1 cytokine stimulation. On the other hand, blocking experiments with a mAb against IL-10, which completely blocked arginase induction by exogenously added IL-10, solely demonstrated a partial inhibition of arginase induction (reduction of the 48 h stimulation index of arginase induction of BMMΦ induced with IFN-γ + TNF-α from 3.1 to 2.1 upon addition of the anti-IL10 mAb, data not shown). Considering the clear-cut results with the IL-10 KO mice, endogenously produced IL-10 is probably able to signal rapidly via its receptor before it can be completely blocked by the Ab.

Arginase II mRNA was detectable in unstimulated BMMΦ, suggesting that this isoform is responsible for the background activity of arginase in resting macrophages. No significant modulation of arginase II mRNA was seen under all conditions of stimulation at all time points tested. It should be mentioned that in two of five experiments, a weak signal of arginase II mRNA was detectable by RT-PCR in unstimulated BMMΦ. Minor differences in the preactivation status of the BMMΦ may be responsible for these slightly varying results (also see below).

Induction of arginase I protein in murine BMMΦ by Th2 cytokines

Selective induction of arginase I on the protein level was investigated by Western blot analysis, employing a rabbit anti-arginase I polyclonal antiserum (18). Lysates of murine liver and kidney served as positive and negative controls, respectively (Fig. 2A). As previously shown, the polyclonal antiserum reacts with two polypeptides of murine hepatic arginase with m.w. of 35 and 38 kDa, presumably arising by alternative translation initiation (19) or by posttranscriptional modification (18, 20). Murine BMMΦ were stimulated with various cytokines for 48 h, cell lysates were separated by SDS-PAGE and analyzed for iNOS and arginase by Western Blot (Fig. 2B). Arginase activities were determined in the same cell lysates, and nitrates were determined in the supernatants of the cultures at 48 h (before lystate preparation) and are indicated below each lane. The competitive regulation of the two pathways of arginine metabolism is demonstrated by the reciprocal induction of arginase I and iNOS. Th2 cytokines did not induce iNOS activity or protein but efficiently induced arginase I activity and protein, with a pronounced synergism between IL-4 and IL-10. The Th1 cytokine IFN-γ alone failed to induce arginase or iNOS. IFN-γ in cooperation with TNF-α caused a pronounced induction of iNOS and only a marginal induction of arginase. In the experiment in Fig. 2, arginase I protein was detectable even in unstimulated BMMΦ, which was the case in two of four independent experiments. Arginase I expression in unstimulated murine BMMΦ (15–60 mU/10^6 cells) most probably reflects unintentional preactivation of the macrophages (see also above).

Induction of arginase I in BMMΦ by Th2 cells

We could recently demonstrate that the iNOS/arginase balance in macrophages is regulated by Th1 and Th2 cells via Ag-induced secretion of their corresponding cytokines (11). The isoform of arginase induced in macrophages by Th2 cells was tested using two well-defined CD4^+ T cell clones, AE7 and D10G4, which belong to the Th1 and Th2 subsets, respectively. BMMΦ of mouse strain AKR/N served as APCs and were cocultured together with the T cell clones and the corresponding Ag at several concentrations. After 48 h, cell lysates were prepared and analyzed by SDS-PAGE and Western blot analysis for the expression of arginase I and iNOS (Fig. 3). As expected, the Th1 clone induced nitrites and
iNOS protein Ag-dependently, without inducing arginase, and no induction of iNOS or nitrites by the Th2 cells was detectable. The Th2 clone D10G4 induced arginase activity, in the presence of Ag to very high levels, corresponding to a strong induction of arginase I observed by Western blotting (Fig. 3). As previously reported (11), this Th2 clone induces considerable levels of arginase activity when cocultured with the macrophages even without Ag (here 493 mU/10^6 cells). As shown in Fig. 3, this background activity corresponds to the induction of arginase I protein as well. The induction of arginase I by the Th2 clone was also confirmed at the level of RNA by RT-PCR (data not shown).

Competition regulation of the iNOS/arginase balance in murine dendritic cells by Th1 and Th2 cytokines and T cell clones

In addition to macrophages, dendritic cells represent the second major class of myeloid professional APCs (21). The iNOS/arginase balance has so far not been studied in dendritic cells. BMDC were generated and extensively analyzed phenotypically by flow cytometry (data not shown) as well as functionally in primary stimulation cultures with naive T cells (data not shown). The cells were efficient APCs and showed homogenous expression of CD11c, MAC-1, F4/80, MHCII, and CD80. However, the cells were heterogeneous (intermediate to high) in the expression of the stimulatory molecules CD86 and CD40, suggesting that they differed in their state of maturity. These BMDC, as well as the retrovirally immortalized dendritic cell line D2SC/1 (17), were an-493 mU/10^6) were stimulated in 55-mm Petriperm hydrophob plates together with 1 x 10^7 Th1 (AE7) or Th2 (D10G4) cells. The respective Ag (PCC for AE7 and conalbumin for D10G4) was added at the indicated concentrations. After 48 h, the cells were harvested, and cell lysates were subjected to SDS-PAGE (arginase: 12.5% polyacrylamide, w/v; iNOS: 7.5% polyacrylamide, w/v) and Western blot analysis. The results of parallel determinations of arginase activities (in mU/10^6 cells) in aliquots of the same cell lysates are noted below the respective lanes of the arginase blot. Nitrites were also determined in the 48 h supernatants (before harvesting the cells), and the concentrations (in µM) are shown below the respective lanes of the iNOS blot. One of two experiments with similar results is shown.

Th2 cytokine IL-4, IL-10, having no significant effect on its own, cooperated with IL-4 in arginase induction. In contrast, the Th1 cytokines up-regulated solely iNOS. A similar pattern of regulation was seen with the dendritic cell line D2SC/1 (Fig. 4), with the exception that no resting arginase activity was detectable.

To investigate the CD4^+ T cell-mediated regulation of iNOS and arginase activity in BMDC, cocultures with the T cell clones AE7 and D10G4 were performed (Fig. 5). Similar to our earlier findings with BMMΦ (11), the Th1 clone induced nitrites Ag-dependently without up-regulating arginase activity (Fig. 5). While no induction of iNOS was seen in the BMDC/Th2 cocultures, arginase activity was up-regulated Ag-dependently to very high levels (3000–3200 mU/10^6 cells). Similar to BMMΦ, the Th2 clone also caused a significant induction of arginase activity in the BMDC even without addition of Ag (here: 1750 mU/10^6 cells). As already discussed in our earlier study (11), this might be due to cell membrane-bound cytokines or to minimal amounts of cytokines secreted by not completely resting T cells. Together, the data show that the two l-arginine metabolizing enzymes in BMDC are subject to a similar competitive regulation as in BMMΦ (9, 11).

Induction of arginase I mRNA and protein in murine dendritic cells by Th2 cytokines

D2SC/1 cells were stimulated with Th1 and Th2 cytokines or LPS. Cell lysates were prepared to analyze the induction of arginase isoforms at the level of mRNA (Fig. 6A) and protein (Fig. 6B). In agreement with the data on arginase activity (Fig. 4), neither arginase I nor arginase II was detected in unstimulated D2SC/1 cells at RNA and protein level. Similar to macrophages, IL-4 and IL-13 specifically induced the hepatic isoform of arginase, while no induction of arginase II (under all conditions of stimulation) was noted.

Arginase I mRNA was induced faintly by IL-10 or by IFN-γ and more pronounced by IFN-γ + TNF-α (Fig. 6A), but neither of these modes of stimulation was reflected by protein induction detectable by Western blot (Fig. 6B). In contrast to macrophages (see Fig. 2), stimulation with LPS led to no detectable induction of
arginase activity (see also Fig. 4) or arginase protein, although a faint signal for arginase I mRNA was detectable. A prominent finding was again the strong synergism of the Th2 cytokines IL-4 and IL-10 in the induction of arginase I.

Discussion

This study describes the unexpected finding that the arginase inducible in murine macrophages in the context of a Th2 immune response corresponds to the hepatic isoform, arginase I. The extrahepatic isoform, arginase II, is constitutively expressed in macrophages. Moreover, we show that a similarly regulated iNOS/arginase balance exists in murine dendritic cells. Our study was facilitated by the availability of a specific Ab to arginase I and by the recently published cDNA sequence of murine arginase II (22). Previously, only a few reports with partially inconsistent information were available about the expression and regulation of arginase isoforms in the macrophage/monocyte lineage. A study by Louis et al. (23) on murine and rat peritoneal macrophages (PEM) reported expression of both isoforms of arginase, which were further (arginase I more pronounced than arginase II) induced upon stimulation with LPS. Hypoxia also led to an induction of arginase I, but at the same time down-regulated arginase II. Similarly, Sonoki et al. (19) reported an induction of arginase I in rat PEM by LPS. In the RAW 264.7 cell line, thought to resemble murine PEM, up-regulation of iNOS and arginase II was observed upon stimulation with LPS (24), whereas another group reported the induction of arginase I by LPS and dibutyryl-cAMP (25). Neither arginase I nor arginase II could be detected in the macrophage-like cell lines P388D1 and J774 (23), whereas another group observed the suppression of NO production via arginase in the J774 cell line (26). Similarly inconsistent results were also reported on the regulation of arginase isoforms in tissues other than macrophages. Arginase I and arginase II were both up-regulated during hyperoxic lung damage in rats (27). LPS caused an induction of arginase I in rat lung and spleen in vivo (19). Another group found the constitutive expression of arginase II in normal rat lung (alveolar + bronchial epithelium, pulmonary macrophages), whereas this expression was lost during sepsis and iNOS expression was induced (28). In a rat model of immune glomerulonephritis, arginase II was detectable in normal glomeruli, whereas nephritic glomeruli

FIGURE 4. Induction of arginase and iNOS in dendritic cells by various cytokines. AKR/N-BMDC (5 × 10⁴) were incubated in a final volume of 200 μl in 96-well flat-bottom plates with the indicated cytokines or LPS (○, no cytokine added; IL-4, IL-10, and IL-13, 10 U/ml; LPS, 0.1 μg/ml; TNF-α, 500 U/ml; IFN-γ, 100 U/ml). After 48 h, nitrites and arginase activity were determined as described in Materials and Methods. The values presented are from one of five independent experiments with similar results. Data represent mean of triplicates with SD indicated. The significance of cytokine-mediated induction of iNOS or arginase was assessed by Dunnnett's multiple comparison test by comparison with the values of unstimulated BMDC and D2SC/1. *, p < 0.05; **, p < 0.01.

FIGURE 5. Ag-dependent-specific induction of iNOS or arginase in BMDC by Th1 or Th2 cells. A total of 5 × 10⁴ BMDC was cultured together with 1 × 10⁵ T cells (Th1 cell clone AE7 or Th2 cell clone D10G4, respectively) and increasing concentrations of the respective Ag. After 48 h, nitrites in the supernatants and arginase activities in the cell lysates were determined. Arginase activity in control BMDC without added T cells was always between 220 and 245 mU/10⁶ cells for all concentrations of Ag and undetectable in T cell control cultures. Nitrites were always <2 μM in control BMDC without added T cells and undetectable in T cell control cultures. Data represent mean of triplicates with SD indicated. Similar results were obtained in a total of three independent experiments.
expressed both arginase I and arginase II. IL-4 increased urea production in nephritic glomeruli and had no effect on normal glomeruli (29).

No definitive picture so far emerges on the in vivo functions of the arginases in macrophages or other tissues, so that this issue remains for the most part at the level of speculation. Various suggestions for the extrahepatically expressed arginase II have been made (6). Our new observations corroborate our previous suggestions for a minimal function of arginase II in macrophages: the up-regulation in the context of Th2 responses points toward an anti-inflammatory role. Because arginase I and iNOS are expressed in the cytosol, arginase I can inhibit iNOS by competing for the common substrate l-arginine (9). Another antiinflammatory function of arginase might be a consequence of its synthesis of L-ornithine, the precursor amino acid for the polyamines putrescine, spermidine, and spermine. Spermine suppresses NO production in macrophages activated with LPS (30) and inhibits specifically the synthesis of proinflammatory cytokines in human mononuclear cells (31).

However, as arginase and iNOS are competitively regulated at the level of gene expression, we do not think that the function of arginase I is restricted to a negative (passive) regulation of iNOS. Rather, it is likely that arginase I plays a positive (active) role in the context of Th2-dominated immune responses. For example, arginase may participate in fibrogenic processes via the synthesis of ornithine-derived proline, an essential precursor for the production of collagen. In a murine model of granulomatous inflammation, it was shown that the tissue-destructing fibrosis of Th2 type granuloma is due to an IL-4-induced synthesis of collagen (32). Additionally, an in vivo model of liver fibrosis showed that BALB/c mice, demonstrating a Th2 cytokine profile, developed massive liver fibrosis, whereas the Th1 cytokine profile of C57BL/6 mice was accompanied by only minimal fibrosis. Neutralization of IL-4 in BALB/c mice resulted in a drastic reduction of fibrosis (33). Arginase might also play a similar role during wound healing. A reciprocal regulation of iNOS and arginase activity was already demonstrated in rats with iNOS up-regulation in the early phase of wound healing, probably creating a cytotoxic environment, and arginase up-regulation in the later, reparative phase (34). The hypoxic environment in healing wounds could be one additional inducer of arginase (23).

While an induction of iNOS in dendritic cells via stimulation with IFN-γ + LPS was already described in the literature (17, 35), we demonstrated in this study for the first time that dendritic cells express the alternative enzyme arginase. The high background level of arginase activity in the BMDC is causally unclear, but is probably induced by the cytokine GM-CSF, which is used for differentiation of the cells. In BMφ binds GM-CSF induces arginase activity in the range of 100–150 mU/10^6 cells (data not shown).

Regarding expression of arginase I, a recent paper demonstrated that the regulation in liver and RAW 264.7 macrophages clearly differs (25). Whereas arginase I is induced in the liver by glucocorticoids (36), dexamethasone led to no induction of arginase I in RAW 264.7 macrophages and even inhibited arginase I up-regulation induced by LPS (25). Dexamethasone also led to no arginase induction in our BMφ (data not shown). Besides our previous demonstration of the involvement of protein kinase A in the induction of arginase in BMφ (37), the Th2 cytokine-associated signal transduction pathways and the molecular events leading to induction of arginase in macrophages and dendritic cells are largely unknown.

Regarding the level of regulation, the induction of arginase I mRNA always seemed to account for the increase in detectable protein in the Western blot and the concomitant increase in measureable arginase activity. We recently elucidated the strong synergistic induction of arginase activity in BMφ by combinations of Th2 cytokines (most pronounced between IL-4 and IL-10) as the basis of the very efficient induction of the enzyme by Th2 cells. Now, we could demonstrate that this novel synergism is also seen on RNA and protein level. Nevertheless, further studies are needed to determine whether the Th2-mediated regulation of arginase expression is solely transcriptionally or also on translational or post-translational levels.

Our findings provide new insights into the complex regulation of this still rather unknown family of enzymes and should help to clarify the functional importance of arginase within the immune system. An intriguing observation of this study is the detection of a further strong element of similarity between the two enzymatic pathways of arginine metabolism in macrophages and dendritic cells: both pathways are mediated by two isoenzymes, one constitutively expressed and the other inducible. Most probably, this reflects different functions fulfilled by the two isoforms in different cell types and subcellular localizations under varying (patho-)physiologic circumstances.

Note added in proof. Since the submission of this manuscript a study (C. A. Louis, V. Mody, W. L. Henry, Jr., J. S. Reichner, and J. E. Albina. 1999. Regulation of arginase isoforms I and II by IL-4 in cultured murine peritoneal macrophages. Am. J. Physiol. 276: R237) was published that addressed some aspects of the work presented here and demonstrated the selective up-regulation of arginase I in murine peritoneal macrophages.
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References


