The Journal of Immunology

RESEARCH ARTICLE | NOVEMBER 01 2003

Translational Control of Inducible Nitric Oxide Synthase by IL-13 and Arginine Availability in Inflammatory Macrophages [1]

Stefan El-Gayar; ... et. al

J Immunol (2003) 171 (9): 4561–4568.
https://doi.org/10.4049/jimmunol.171.9.4561

Translational Control of Inducible Nitric Oxide Synthase by IL-13 and Arginine Availability in Inflammatory Macrophages[1]

Stefan El-Gayar,* Heike Thüring-Nahler,[2]* Josef Pfeilschifter,‡ Martin Röllinghoff,* and Christian Bogdan[3]*†

Inducible NO synthase (iNOS) and its generation of NO from L-arginine are subject to transcriptional as well as posttranscriptional control by cytokines. In this study, we describe a novel, translational mechanism of iNOS regulation by arginine availability. Using mouse inflammatory peritoneal macrophages stimulated with IFN-γ plus LPS, we demonstrate that the suppression of iNOS protein, which is observed after a 16-h (but not after a 6-h) pretreatment with IL-13, despite an unaltered iNOS mRNA level, results from arginine depletion by arginase. The addition of arginase inhibitors (in the pretreatment phase) or of arginine (in the stimulation phase) completely blocked the down-regulation of iNOS protein by IL-13. The rescuing effect of arginine supplementation was not due to a positive feedback regulation of iNOS expression via enhanced production of NO. A striking suppression of iNOS protein (but not of iNOS mRNA) was also seen, when IL-13 was replaced by purified arginase or when macrophages were stimulated with IFN-γ/LPS in arginine-free medium. Arginine deficiency specifically impaired the de novo synthesis and the stability of iNOS protein, but did not affect the production of TNF and the overall protein synthesis of the macrophages. From these results, we conclude that arginine not only functions as a substrate for iNOS, but is also critical for maintaining normal levels of iNOS protein in cytokine-stimulated macrophages. ***The Journal of Immunology*****, 2003, 171: 4561–4568.**

Inducible NO synthase (iNOS[4] or NOS2) is a dimeric enzyme that in the presence of molecular oxygen and several cofactors converts the amino acid arginine to N^{ω}-hydroxy-L-arginine (NOHA) and further to citrulline and NO. iNOS is expressed in a broad spectrum of cells, including macrophages. It is induced by cytokines (e.g., IFN-γ) and/or microbial products (e.g., LPS) and typically generates high levels of NO over a prolonged period of time (1, 2). iNOS-derived NO exerts numerous effector and immunoregulatory functions under physiological and pathophysiological conditions, such as the control or killing of infectious pathogens, the inhibition or promotion of tumor growth, the protection or damage of tissue in autoimmune diseases, and the modulation of cytokine production and Th cell development (2).

In several models of infectious or autoimmune diseases, iNOS-derived NO was shown to be both friend and foe (reviewed in Refs. 2 and 3). From this perspective, it is evident why the expression of iNOS requires tight regulation. Several mechanisms of control have been unraveled and most extensively studied in mouse macrophage cell lines or primary mouse macrophages (4, 5). In strictly resting macrophages, iNOS mRNA is undetectable. Stimulation by IFN-γ and/or LPS leads to the expression of iNOS mRNA, which is due to the transcriptional activation of the iNOS promotor and/or the stabilization of iNOS mRNA (2, 4). Experiments with radiolabeled methionine or cysteine as well as the use of proteasomal inhibitors revealed that the synthesis and the stability of iNOS protein are additional levels of regulation of iNOS by cytokines (2, 4, 6–8). It was also demonstrated that high-output generation of NO by iNOS requires extracellular arginine, even if an adequate level of intracellular arginine is already present. Thus, in macrophages, which lacked the cationic amino acid transporter CAT2 (9) or in which the activity of arginase (that degrades arginine to urea and ornithine) was up-regulated prior to the induction of iNOS (10–14), the production of NO was drastically reduced due to substrate depletion.

One of the cytokines that was shown to suppress iNOS in macrophages as well as other cells is IL-13 (15–17). IL-13 is a product of type 2 Th cells (Th2) and shares many properties with IL-4, including the ability to inhibit or alternatively activate macrophages (5, 18, 19). Previously, we observed that treatment of mouse inflammatory macrophages with IL-13 before the activation with IFN-γ and LPS inhibited the expression of iNOS protein to a similar degree as IL-4. However, unlike IL-4 (20), IL-13 did not affect the level of iNOS mRNA (21). Others reported that IL-4 as well as IL-13 induced arginase I (10, 22–24), which led to arginine consumption and reduced NO production in response to IFN-γ (± LPS) (10, 23, 24), with (24) or without (23) alteration of the level of iNOS protein.

In the present study, we investigated by which mechanism and under which circumstances IL-13 affects the expression of iNOS protein. We demonstrate that the inhibition of iNOS protein expression by IL-13 can be fully reverted by the addition of arginine

*Institute of Clinical Microbiology, Immunology and Hygiene, University of Erlangen-Nuremberg, Erlangen, Germany; †Department of Medical Microbiology and Hygiene, University of Freiburg, Freiburg, Germany; and ‡Pharmazentrum Frankfurt, Institute of Pharmacology and Toxicology, University of Frankfurt, Frankfurt, Germany

Received for publication March 28, 2003. Accepted for publication August 21, 2003.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported by the Deutsche Forschungsgemeinschaft (Grants SFB263 (project A4) and Bo996/3-1 to C.B.). This study forms part of the M.D. thesis of S.E.-G.

[2] Current address: Viramed Biotech AG, Planegg, Germany.

[3] Address correspondence and reprint requests to Dr. Christian Bogdan, Department of Medical Microbiology and Hygiene, Institute of Medical Microbiology and Hygiene, University Clinic of Freiburg, Hermann-Herder Strasse 11, D-79104 Freiburg, Germany. E-mail address: bogdancn@ukl.uni-freiburg.de

[4] Abbreviations used in this paper: iNOS (NOS2), inducible NO synthase; L-NIL, L-N^6-iminoethyl-lysine; nor-NOHA, N^{ω}-hydroxy-nor-L-arginine.

0022-1767/03/$02.00

or the presence of a arginase inhibitor. We also show that depletion of extracellular arginine mimics the effect of IL-13 and not only causes a reduced production of NO due to the lack of substrate, but in fact results in a striking down-regulation of iNOS protein due to reduced iNOS synthesis and stability with no effect on iNOS mRNA expression. These data provide evidence for a novel mode of arginine-dependent regulation of iNOS activity.

Materials and Methods

Mice and macrophages

The preparation of thioglycolate-elicited peritoneal exudate macrophages from female CD1 mice (8–12 wk old; Charles River Breeding Laboratories, Sulzfeld, Germany) was conducted as described (21, 25).

Reagents

Mouse rIFN-γ (batch M3RD48; sp. act. 5.2×10^6 U/mg) was a gift of Dr. G. Adolf (Ernst Boehringer Institut für Arzneimittelforschung, Vienna, Austria). Mouse rIL-13 (sp. act. $1.6–3.3 \times 10^5$ U/mg) was obtained from R&D Systems (Wiesbaden-Nordenstedt, Germany). Arginase (from bovine liver), L-arginine or D-arginine hydrochloride, pepstatin A, chymostatin, PMSF, leupeptin, Triton X-100, and LPS (O111:B4) were obtained from Sigma Chemie (Deisenhofen, Germany). L-cysteine, L-inositol, L-leucine, and L-methionine were from Life Technologies (Karlsruhe, Germany); L-glutamine was from Biochrom (Berlin, Germany). L-N^6-iminoethyl-lysine (L-NIL), L-N^G-monomethyl-arginine, and N^ω-hydroxy-L-arginine were obtained from Alexis (Läufelfingen, Switzerland); N^ω-hydroxy-nor-L-arginine (nor-NOHA) was from Calbiochem-Novabiochem (Bad Soden, Germany) and BACHEM Biochemica GmbH (Heidelberg, Germany).

Culture of macrophages

Macrophages were cultured in regular RPMI 1640 medium (Biochrom; supplemented with 2 mM glutamine, 10 mM HEPES, 13 mM $NaHCO_3$, 100 μg/ml penicillin, and 100 μg/ml streptomycin, and with or without 2.5% heat-inactivated FBS (Sigma)), or in amino acid-deficient RPMI 1640 medium (Life Technologies; supplemented as above and with all amino acids, except L-arginine, L-leucine, or L-cysteine, as indicated). Unless otherwise mentioned, the macrophages were seeded in 24-cm^2 tissue culture petri dishes (Nunc, Wiesbaden, Germany) in 3 ml medium at 2×10^6 macrophages/ml. After 90–120 min, nonadherent cells were washed off and the adherent macrophages were incubated with or without IL-13 (10 ng/ml), arginase (1–20 U/ml), or arginase inhibitors (5–500 μM) for 16 h, before the monolayer was stimulated with IFN-γ (20 ng/ml) plus LPS (200 ng/ml) for 24 h with or without the addition of L-arginine (10–1000 μM). In some experiments, the culture medium was replaced by fresh medium after the preincubation period before the addition of the IFN-γ/LPS stimulus.

The LPS content of the cytokine stocks and of the final culture medium was below 10 pg/ml (colorimetric *Limulus* amebocyte assay; Biowhittaker Europe, Taufkirchen, Germany).

NO production and iNOS mRNA, protein, and enzyme activity assays

The measurement of nitrite in the culture supernatants by the Griess assay, the detection of iNOS protein by 7.5% SDS-PAGE and Western blotting (with a rabbit antiserum directed against the C terminus of mouse iNOS), the determination of iNOS enzyme activity in whole cell macrophage lysates, and the analysis of iNOS mRNA expression by Northern hybridization of total RNA (with an [α-^{32}P]dCTP random primer-labeled plasmid probe specific for mouse iNOS and controlled by a [γ-^{32}P]ATP end-labeled oligoprobe specific for mouse 16S mitochondrial RNA) were performed exactly as described (21). Equal loading of the protein gels was accomplished by determination of the protein content of the macrophage lysates using the Bradford method and the protein dye reagent from Bio-Rad (Richmond, CA). All Western blots were reprobed with Abs against β-actin (sc-1616) or the 90-kDa heat-shock protein (sc-7947) (Santa Cruz Biotechnology, Heidelberg, Germany).

Analysis of iNOS protein synthesis by metabolic labeling and immunoprecipitation

Macrophage monolayers were prepared and stimulated with IFN-γ/LPS in arginine-free RPMI 1640 medium, which was supplemented with different concentrations of L-arginine (0–1000 μM) and was either serum free or contained 2.5% FBS. After stimulation, the macrophages were washed with warm PBS and the medium was replaced with 37°C serum- and L-cysteine-free RPMI 1640 pulse medium (with or without L-arginine), which was buffered with 10 mM HEPES and 23 mM $NaHCO_3$ and CO_2 equilibrated. After a 30-min starving period, 150 μCi of ^{35}S-labeled L-cysteine (>1000 mCi/mmol; Amersham Pharmacia Biotech, Freiburg, Germany) was added for 30 min before the cells were washed with PBS and lysed in 150 mM NaCl/10 mM Tris, pH 7.4, buffer containing 1% Triton X-100 and protease inhibitors, as described (21). Immunoprecipitation of equal amounts of protein with rabbit anti-mouse iNOS serum, 7.5% SDS-PAGE, and fluoroautoradiography with EN^{3}HANCE (NEN Life Science, Boston, MA) was performed as described (21). The dried gels were exposed to Kodak XAR films for various periods of time (5–12 days) to ascertain a linear relationship between image intensity and precipitated cpm.

Analysis of iNOS protein stability by pulse-chase experiments

After 18–20 h of stimulation with IFN-γ/LPS in L-arginine-containing or -free medium, the macrophage monolayers were washed and pulsed with ^{35}S-labeled L-cysteine for 30 min in L-cysteine-free medium, as mentioned above. Thereafter, chase medium (pulse medium containing 50 mg/L L-cysteine with or without 1 mM L-arginine) was added for the indicated time periods. At the conclusion of the chase, the cells were lysed, and equal amounts of protein were subjected to immunoprecipitation, 7.5% SDS-PAGE, and autoradiography, as described above.

Total protein expression and synthesis

Macrophages were stimulated with IFN-γ/LPS for 17 h in the presence of different concentrations of L-arginine (0–1000 μM). Thereafter, the cells were starved in L-cysteine-free medium (with 0–1000 μM L-arginine) for 60 min and then labeled with 50 μCi ^{35}S-labeled cysteine for 6 h. Total macrophage lysates (prepared in 1% Triton X-100 lysis buffer) were separated by 7.5% SDS-PAGE (40 μg protein/lane). The gels were fixed, stained with Coomassie brilliant blue R-250, and subjected to audioradiography.

Analysis of arginase expression and activity

Expression of arginase I (and II) protein was analyzed by 12.5% SDS-PAGE and Western blotting with rabbit antisera raised against C-terminal peptides of rat arginase I or II, which were composed of 15 aa with almost complete identity to the respective mouse sequences (26). The specificity of the obtained bands was demonstrated by their absence when preimmune sera were used and by the blocking of the staining with the peptides used for immunization of the rabbits.

Arginase enzyme activity was estimated by the measurement of the formed urea in the culture supernatants of the macrophages using the method described by Corraliza et al. (27).

Results

Replacement of culture medium or addition of arginine restores the expression of iNOS protein in macrophages pretreated with IL-13

Pretreatment of thioglycolate-elicited peritoneal macrophages with IL-13 for 16 h caused strong suppression of iNOS protein at 8–48 h of stimulation with IFN-γ/LPS without altering the expression of iNOS mRNA (21). We also noted that the degree of iNOS protein suppression was always strikingly smaller when analyzed by [^{35}S]methionine labeling as compared with steady state Western blot analysis. As the former method entailed the exchange of culture medium, we postulated that the suppression of iNOS protein by IL-13 might be due to the depletion of a medium component (21). In a first experiment, we therefore replaced the culture medium after the 16-h pretreatment period (before the stimulation with IFN-γ/LPS), which completely prevented the down-regulation of iNOS protein by IL-13 (Fig. 1*A*). The same result was obtained, when, instead of exchanging the medium, L-arginine (1 mM, which equals the concentration of L-arginine in fresh RPMI 1640 medium) was added to the cultures just before the IFN-γ/LPS stimulus (Fig. 1, *A* and *B*). The supplementation of L-arginine not only restored the expression of iNOS protein and the accumulation of nitrite, but also the iNOS enzyme activity in lysates of macrophages pretreated with IL-13 (Fig. 1*C*). As seen before (21), IL-13 was completely ineffective if added to the macrophages together with IFN-γ/LPS (cotreatment) (Fig. 1*C*).

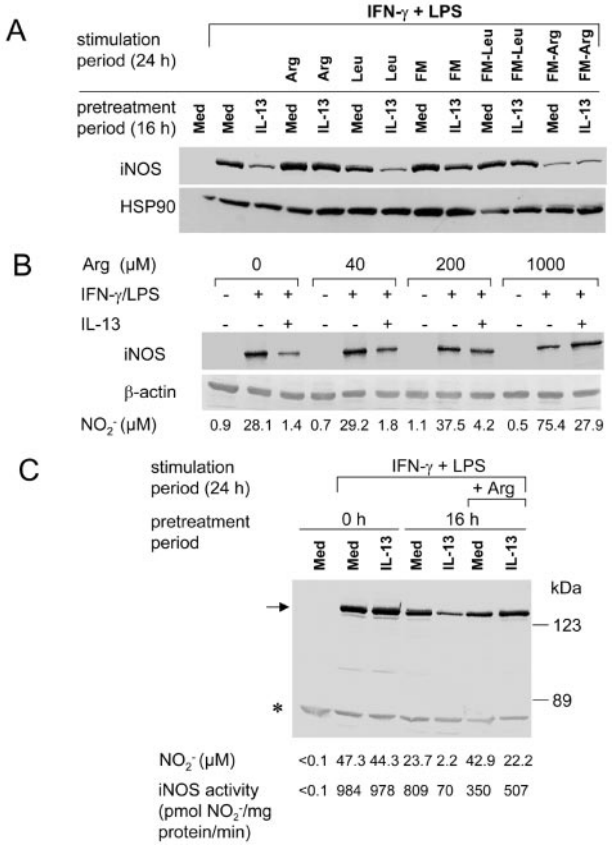

FIGURE 1. Replacement of culture medium or addition of arginine antagonizes the suppression of iNOS protein by IL-13. *A*, Macrophages were preincubated in medium with or without IL-13. After 16 h, fresh complete culture medium (FM), fresh medium without L-arginine (FM-Arg) or L-leucine (FM-Leu), or 1 mM L-arginine (Arg) or 1 mM L-leucine (Leu) were added and the cells were stimulated with IFN-γ/LPS for 24 h, when lysates for analysis by SDS-PAGE and anti-iNOS Western blotting were prepared. *B*, Macrophages were pretreated and stimulated as in *A*, but L-arginine was titrated. The concentrations of NO_2^- in the culture supernatants after 24 h of stimulation are given below the respective lanes. *C*, Macrophages were cultured in medium with or without IL-13 for 0 or 16 h before stimulation with IFN-γ/LPS. As indicated, L-arginine (1 mM) was added to some of the cultures during the stimulation period. The concentrations of NO_2^- in the culture supernatants and the iNOS enzyme activity in the lysates of the macrophages are given. →, iNOS protein (130 kDa); *, unidentified protein (≈80 kDa) demonstrating equal loading of the lanes. One of thirteen (*A*), four (*B*), and two (*C*) similar experiments.

Several controls corroborated the specificity of the effect. First, neither L-leucine (an essential amino acid like L-arginine; Fig. 1*A*) nor D-arginine (data not shown) was able to restore iNOS protein expression in IL-13-pretreated macrophages. Second, medium replacement with L-arginine-free RPMI 1640 medium was unable to revert the suppression by IL-13, whereas the addition of L-leucine-free medium was as effective as fresh complete culture medium (Fig. 1*A*). Third, pretreatment with IL-13 (with or without subsequent addition of L-arginine) did not alter the induction of TNF by IFN-γ/LPS (5.7 ± 0.9 ng/ml vs 4.6 ± 0.2 ng/ml TNF in the supernatants of IL-13-treated vs control macrophages), the expression of an unidentified protein of ~80 kDa (Fig. 1*C*, asterisk), nor the expression of arginase I (36/38-kDa doublet in Figs. 3, *A* and *B*, and 4).

These data show that supplementation of arginine specifically affects the expression of iNOS protein in IFN-γ/LPS-stimulated macrophages pretreated with IL-13.

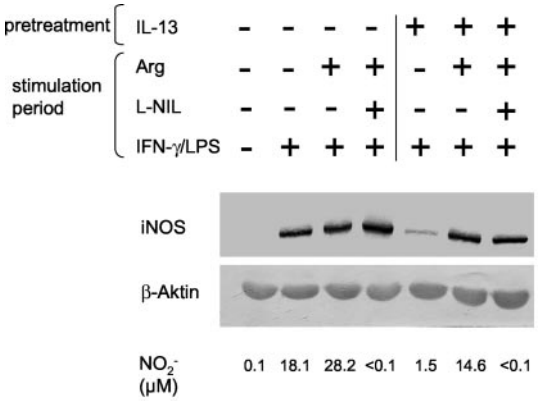

FIGURE 2. L-arginine reverts the suppression of iNOS protein by IL-13 even in the absence of NO production. Macrophages were preteated with or without IL-13 and stimulated with IFN-γ/LPS in the absence or presence of L-arginine (1 mM) and/or L-NIL (1 mM) for 12 h before analysis of macrophage lysates by SDS-PAGE and anti-iNOS Western blotting. One of three similar experiments.

Reconstitution of iNOS protein expression in IL-13-treated macrophages by L-arginine does not require production of NO

In macrophage cell lines, NO was found to exert positive or negative feedback effects on the expression of iNOS mRNA and protein (reviewed in Refs. 5, 27–29). Although in primary mouse macrophages inhibition of iNOS activity by arginine analogues (e.g., L-NIL, L-N^G-monomethyl-arginine) did not alter the levels of iNOS protein (25), we still considered the possibility that exogenous L-arginine increases the production of NO and thereby up-regulates the expression of iNOS. When macrophages pretreated with IL-13 were stimulated with IFN-γ/LPS for 6, 12, or 24 h, the addition of L-arginine restored the expression of iNOS protein at all time points even in the presence of L-NIL, a strong inhibitor of iNOS activity that completely blocked the generation of NO in the cultures (Figs. 2 (12 h) and 3*A* (24 h). Thus, L-arginine restores iNOS protein expression in IL-13-treated macrophages in a NO-independent manner.

Addition of an arginase inhibitor during the pretreatment period prevents the suppression of iNOS protein by IL-13

IL-13 up-regulates arginase activity and the expression of arginase I (but not of arginase II) in bone marrow-derived macrophages (22). Therefore, the suppression of iNOS protein by IL-13 might result from the depletion of arginine via the induction of arginase. Unlike resting peritoneal macrophages (31), bone marrow-derived macrophages (22), or macrophage cell lines (32), but similar to polypeptone-induced peritoneal macrophages (33), our inflammatory peritoneal macrophages constitutively expressed a high level of arginase I that was not altered by pretreatment with IL-13 and the stimulation with IFN-γ/LPS in the presence or absence of L-arginine or L-NIL (Fig. 3*A*, and data not shown). However, IL-13 led to a significant increase of arginase activity, as detected by the accumulation of urea in the culture supernatants at the end of the 16-h pretreatment period (Fig. 3*B*). The presence of nor-NOHA, a strong inhibitor of arginase and poor substrate of iNOS (14, 34), during the pretreatment period completely blocked the suppression of iNOS by IL-13 (Fig. 3*C*). Identical results were obtained with another arginase inhibitor, N^{ω}-hydroxy-L-arginine (34) (data not shown). Thus, the IL-13-mediated down-regulation of iNOS protein is causally related to arginase-dependent depletion of arginine.

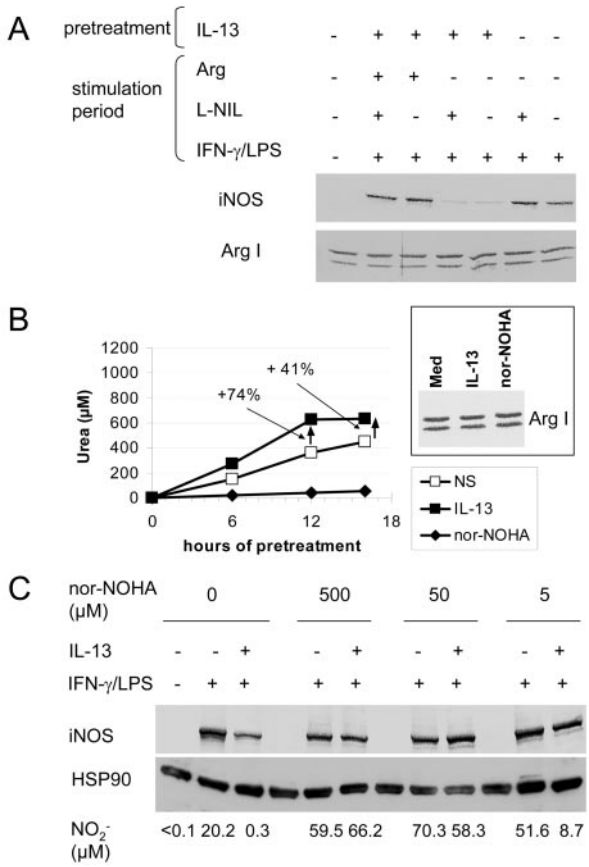

FIGURE 3. IL-13 suppresses iNOS protein expression via up-regulation of arginase activity, but without altering the expression of arginase protein. *A*, Macrophages were pretreated with or without IL-13 for 16 h and stimulated with IFN-γ/LPS in the absence or presence of L-arginine (1 mM) or L-NIL (1 mM). The macrophage lysates were analyzed for the protein expression of iNOS and arginase I. *B*, Arginase activity in macrophages pretreated with medium, IL-13, or nor-NOHA (500 μM) for 6, 12, or 16 h, as determined by anti-arginase I Western blotting and by measurement of urea in the culture supernatants (mean ± SD of triplicates; SDs were below 5% and fall within the symbols). Arrows and numbers indicate the percentage of increase in IL-13-treated vs unstimulated control macrophages. *C*, Macrophages were pretreated for 16 h with or without IL-13 in the presence or absence of nor-NOHA. iNOS protein expression was assessed by Western blotting after 24 h. One of three (*A*), two (*B*), and five (*C*) similar experiments.

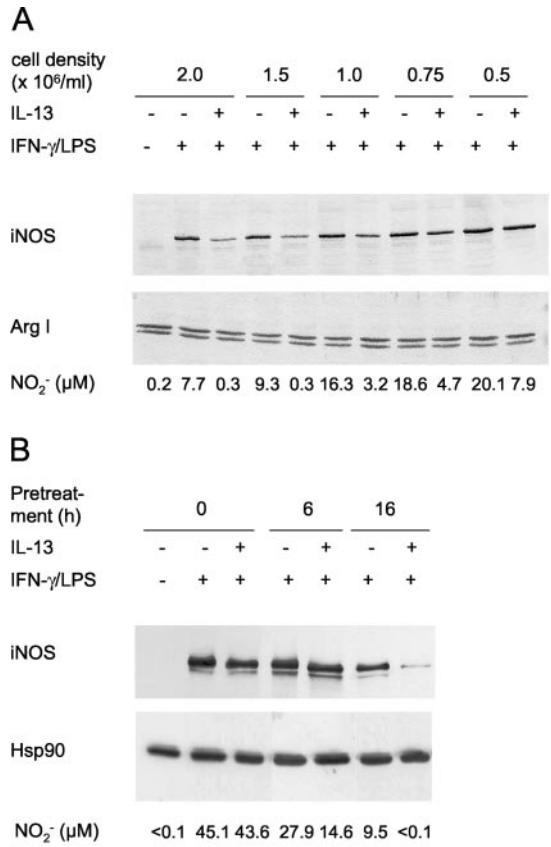

FIGURE 4. Reduction of macrophage density or shortening of the preincubation period abolishes the suppression of iNOS protein by IL-13. *A*, Macrophage cultures were set up in the same total volume (3 ml) of regular RPMI 1640 medium, but at different cell density (0.5–2 × 10^6/ml). Following preincubation with or without IL-13 for 16 h, the monolayers were stimulated with IFN-γ/LPS for 24 h and analyzed thereafter for the expression of iNOS or arginase I by Western blot. *B*, Macrophages were cultured at 2 × 10^6/ml with medium or IL-13 for 0, 6, or 16 h; stimulated with IFN-γ/LPS for 24 h; and analyzed for the expression of iNOS or heat-shock protein 90 by Western blot. *A* and *B*, One of two identical experiments.

iNOS protein suppression by IL-13 depends on the cell density of the macrophage monolayer and the length of the pretreatment period

The constitutively high arginase activity in thioglycolate-elicited peritoneal macrophages is likely to partially deplete the culture medium of arginine during the 16-h preincubation period. Indeed, macrophages stimulated with IFN-γ/LPS immediately after the 2-h adherence step produced significantly more NO than macrophages that were kept in culture overnight before stimulation (47.6 ± 2.6 μM vs 17.9 ± 2.5 μM, mean ± SEM, $n = 19$ or 33, respectively). Conversely, when nor-NOHA or L-arginine was added to the macrophages during or after the preincubation period, respectively, the IFN-γ/LPS-induced NO production was strongly enhanced compared with macrophages cultured in medium alone (see nitrite values in Figs. 1*B* and 3*C*). These observations led us to speculate that pretreatment with IL-13 further increases the consumption of L-arginine, which then might negatively affect the expression of iNOS protein, depending on the extent of arginine depletion. To test this hypothesis, we cultured macrophages in the same volume of medium (3 ml), but either at varying cell density (0.5–2.0 × 10^6/ml) (Fig. 4*A*) or for different periods of pretreatment (Fig. 4*B*).

As shown in Fig. 4*A*, IL-13 caused suppression of iNOS protein at high (2, 1.5, or 1 × 10^6/ml), but not at low cell density (0.75 or 0.5 × 10^6/ml), whereas the expression of arginase I remained unaltered. Importantly, NO_2^- accumulation after stimulation with IFN-γ/LPS increased with decreasing cell density. Down-regulation of NO production by IL-13 was also seen at the lower cell densities (i.e., without iNOS protein suppression), but clearly to a lesser extent. When macrophages were cultured at the standard density of 2 × 10^6/ml with medium alone or IL-13 for 0, 6, or 16 h before stimulation with IFN-γ/LPS, suppression of iNOS protein was only observed after 16 h of pretreatment with IL-13, whereas the production of NO was already clearly diminished with the 6-h preincubation protocol (Fig. 4*B*).

From these results, we conclude that the cell density as well as the length of the preincubation period (i.e., the level and duration of arginase activity before stimulation of the macrophages) determine whether IL-13 leads only to a reduction of NO production or to a parallel suppression of iNOS protein.

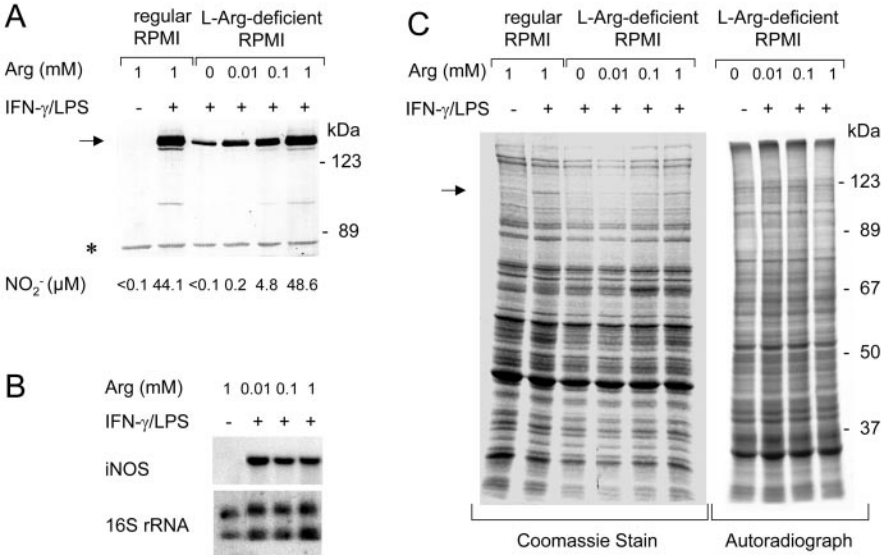

FIGURE 5. Arginine deficiency suppresses iNOS protein, but does not affect the expression of iNOS mRNA or general protein synthesis in macrophages. *A*, iNOS protein expression (Western blot); *B*, iNOS mRNA expression (Northern blot); *C*, total protein expression (Coomassie-stained 7.5% SDS-PAGE) and total protein synthesis (autoradiograph of 7.5% SDS-PAGE after ^{35}S-labeled cysteine labeling) in macrophages stimulated with IFN-γ/LPS for 24 h in regular RPMI 1640 or arginine-deficient RPMI 1640 medium supplemented with different concentrations of L-arginine. →, iNOS protein (130 kDa); ∗, unidentified 80-kDa protein demonstrating equal loading of the lanes. One of five (*A*), three (*B*), or two similar experiments (*C*).

Arginine depletion in the culture medium mimics the effect of IL-13

To directly address whether arginine availability regulates the expression of iNOS protein, we prepared and stimulated macrophage monolayers without FCS either in regular RPMI 1640 medium (contains 1 mM L-arginine) or in arginine-free RPMI 1640 medium supplemented with defined amounts of L-arginine. At 7, 24, and 48 h of stimulation, iNOS protein was strongly reduced in macrophages cultured with medium containing 0–100 μM L-arginine (Fig. 5*A*, and data not shown), whereas the expression of the unidentified 80-kDa protein (Fig. 5*A*) and of TNF (data not shown) remained unchanged. The levels of iNOS mRNA were either unaffected or slightly up-regulated, when the arginine content of the medium was reduced (Fig. 5*B*, and data not shown). Coomassie-stained protein gels of total macrophage lysates revealed a selective lack of the 130-kDa iNOS protein in the absence of L-arginine (Fig. 5*C*, arrow). ^{35}S-labeled cysteine labeling confirmed that the global protein synthesis of macrophages remained intact in the absence of L-arginine (Fig. 5*C*). Addition of arginine to macrophages stimulated with IFN-γ/LPS in arginine-free RPMI 1640 medium fully restored the expression of iNOS protein even in the presence of 1 mM L-NIL (data not shown).

When macrophages were cultured in arginine-replete medium, the addition of purified arginase 16 h before stimulation with IFN-γ/LPS caused a strong down-regulation of iNOS protein comparable to the results obtained with arginine-deficient medium. In contrast, when the preincubation period was omitted, i.e., the arginase was added to the macrophages together with the IFN-γ/LPS stimulus, the production of NO was reduced to a much lesser extent and the expression of iNOS protein was completely preserved (Fig. 6).

These results unequivocally demonstrate that the amount of arginine in the culture medium controls the expression of iNOS protein. However, they do not exclude the possibility that other IFN-γ/LPS-induced proteins that are not detectable by Coomassie-stained protein gels are also subject to regulation by arginine.

Arginine deficiency impairs iNOS protein synthesis and stability

Finally, we investigated whether the reduced steady state protein expression of iNOS is due to a reduced synthesis and/or a reduced stability of iNOS protein. To this, we performed ^{35}S-labeled cysteine pulse-labeling and pulse-chase immunoprecipitation experiments either in arginine-replete or arginine-deficient pulse medium (cysteine free) and chase medium (cysteine rich). iNOS protein synthesis was strongest at early time points of stimulation (3–10 h), in which we observed a clear down-regulation in the absence of arginine (Fig. 7*A*). We also found a reduction of iNOS protein stability in the absence of arginine (Fig. 7*B*).

Discussion

The results presented in this study have four major implications. First, our findings provide a clear explanation for the previously reported different effects of IL-13 on the IFN-γ/LPS-induced L-arginine/iNOS pathway in macrophages: the extent of arginine depletion via arginase activation appears to determine whether IL-13 reduces the production of NO without (23) or with parallel suppression of iNOS protein (21). Second, completely independent of IL-13, arginine deficiency not only transiently reduces the production of NO by iNOS due to a lack of substrate for the enzyme, but also decreases the synthesis and, to a lesser degree, the stability of iNOS. This sheds new light on the role of the extracellular arginine supply for the NO production of macrophages and suggests that arginine might be capable of regulating the expression of certain

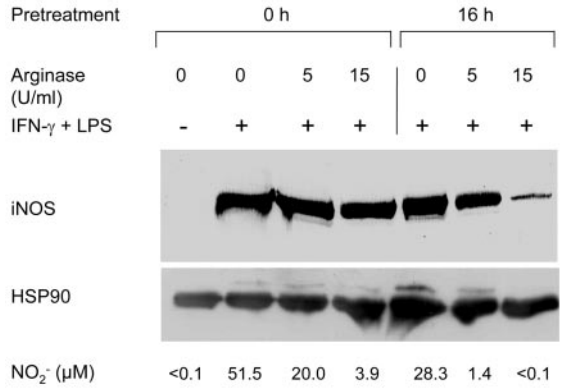

FIGURE 6. Pretreatment of macrophages with exogenous arginase down-regulates iNOS protein expression. Macrophages were cultured in regular RPMI 1640 medium (1 mM L-arginine), and arginase was added 16 h before or together with the IFN-γ/LPS stimulus. After 24 h, the expression of iNOS protein was analyzed by Western blotting. One of three similar experiments.

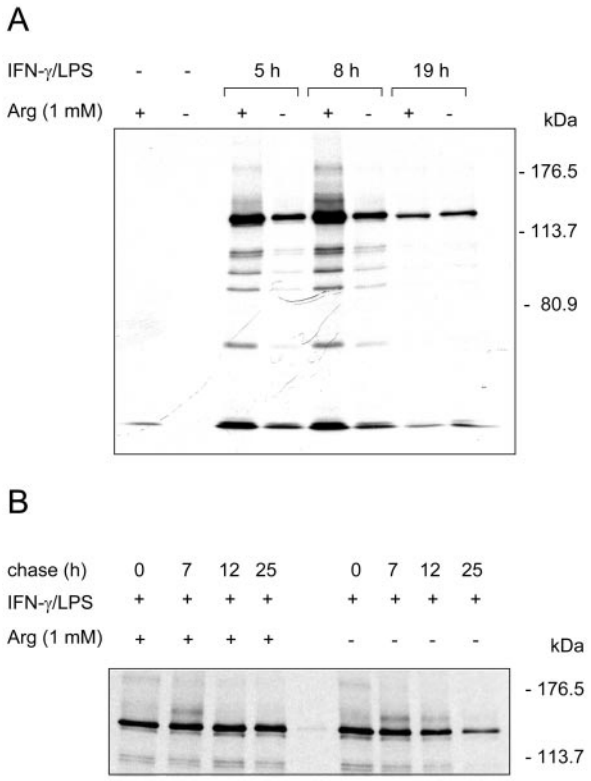

FIGURE 7. Arginine deficiency impairs iNOS protein synthesis and iNOS protein stability. After stimulation with IFN-γ/LPS in the presence or absence of L-arginine for the indicated periods of time (*A*) or for 19 h (*B*), the macrophages were pulse labeled with ^{35}S-labeled cysteine (30 min) in the respective culture medium for analysis of iNOS de novo protein synthesis (*A*) or iNOS protein stability (*B*), as described in *Materials and Methods*. One of three (*A*) and two (*B*) similar experiments.

proteins such as iNOS. Third, our data are relevant to the model of alternatively vs classically activated macrophages inasmuch as they argue for a novel interaction between these two pathways of activation. Fourth, as not only macrophages, but also bacteria, parasites, and tumor cells express arginases, our observations provide a mechanism by which infectious pathogens and tumors might locally shut down an important effector arm of the immune response.

Mechanisms of iNOS regulation by IL-13

Although IL-13 was reported to down-regulate iNOS mRNA in resident peritoneal macrophages (17) and in the J774 macrophage cell line (21), there has been consensus that in mouse bone marrow-derived macrophages and peritoneal exudate macrophages, IL-13 does not affect iNOS mRNA levels (21, 23). At the same time, however, we found that pretreatment of macrophages with IL-13 for 16 h suppressed iNOS protein (21), whereas Rutschman et al. (23) observed a reduction of the NO production by IL-13 without alteration of the iNOS protein expression using a 6-h preincubation protocol. The data of the present study strongly suggest that the suppression of iNOS protein by IL-13 only occurs from a certain cell density or length of preincubation onward, when due to the IL-13-mediated increase of arginase activity, the arginine concentration in the medium falls below the level that is required for an unimpaired expression of iNOS protein ($<$100–200 μM, see Figs. 1*B* and 5*A*, and data not shown). Of note, the increase of arginase activity by IL-13 was not accompanied by an up-regulation of arginase I protein (Fig. 3*B*). The essential role of arginase activity for the regulation of iNOS protein expression is directly demonstrated by the effects of purified arginase (Fig. 6) and of the arginase inhibitor nor-NOHA (Fig. 3*C*).

The arginine concentration in standard cell culture medium (e.g., RPMI 1640, 1 mM; MEM, 0.6 mM; DMEM, 0.4 mM) strongly exceeds the concentration of arginine in the plasma ($\sim$100 μM (35)) and at sites of inflammation (e.g., wounds, $<$50 μM (36)). Thus, the iNOS expression seen in vitro is likely to overestimate the expression of iNOS by inflammatory macrophages in vivo. Considering the a priori reduced levels of arginine in the tissue, any further up-regulation of arginase (e.g., by IL-13, IL-4, or TGF-β) (22, 31, 37, 38) will probably not only reduce the production of NO, but also impair the in situ expression of iNOS protein.

We previously showed that IL-13 inhibited the synthesis, but not the stability of iNOS protein. It is important to bear in mind that the respective assays entailed the complete exchange of culture medium, i.e., the addition of fresh labeling medium containing 1 mM arginine so that only the synthesis, but not the stability of iNOS was impeded (21). Based on the IL-13-independent iNOS synthesis and stability assays with arginine-free medium reported in this work, it is conceivable that in vivo IL-13 also affects the stability of iNOS protein when, due to the activation of arginase, the arginine levels drop below a critical limit.

Arginine pools and mechanism of regulation of iNOS protein expression by arginine

High-output generation of NO by macrophages depends on extracellular L-arginine and its transporter-mediated influx into the cells (9–14, 39). In an arginine-depleted extracellular milieu, the residual intracellular arginine pool is not accessible for iNOS in macrophages (40). To date, the only role of the extracellular arginine pool for the iNOS pathway was thought to supply substrate to the enzyme. The present data demonstrate that depletion of extracellular arginine by arginase or the use of arginine-free culture medium has a long-term, structural effect by reducing the expression of iNOS protein. The extent of suppression of iNOS protein in macrophages cultured in arginine-free RPMI 1640 medium with or without 2.5% FBS was indistinguishable (data not shown), because this amount of serum does not contain $>$2–3 μM L-arginine.

Previous in vitro studies have shown that depletion of arginine in the culture medium of macrophages does not reduce the overall protein synthesis in the resident peritoneal macrophages (36) or peritoneal macrophages activated in vivo by *Mycobacterium bovis* bacillus Calmette-Guerin and elicited by proteose-peptone (41). Our results with thioglycolate-elicited peritoneal macrophages confirm these findings, because lack of exogenous arginine did not affect: 1) the IFN-γ/LPS-induced production of TNF; 2) the level of arginase I in the macrophages; 3) the expression of an as yet unidentified 80-kDa protein; and 4) the total cellular protein synthesis, as assessed by ^{35}S-labeled cysteine incorporation.

We do not yet know how arginine deficiency affects the synthesis of iNOS protein in primary macrophages. There is evidence from various in vitro systems that the availability of certain amino acids (e.g., arginine, branched amino acids such as leucine or isoleucine) not only regulates the gene expression in mammalian cells via specific receptors/signaling pathways and amino acid response elements, but also controls mRNA translation (by modulation of elongation and initiation factors) and protein breakdown (reviewed in Ref. 42). Immunologically relevant examples as well as the molecular basis for these concepts are only emerging (42–46). When this manuscript was under review, Lee et al. (46) reported that arginine depletion (e.g., by adenovirus-mediated overexpression of arginase I) impairs cytokine-induced iNOS expression in

rat astrocytes, which was paralleled by an increased phosphorylation of the eukaryotic translation initiation factor eIF2α. In a cell line derived from adherent spleen cells and immortalized by transfection with SV40 DNA, Chiarugi et al. (45) observed that the maximal production of NO and full expression of iNOS protein after activation by IFN-γ required the presence of at least 10 μM tryptophan in the culture medium, whereas exogenous arginine was dispensable in this system.

The reduced iNOS protein stability in the absence of exogenous arginine is likely to reflect the previously demonstrated stabilizing effect of arginine on the formation of cell-free iNOS dimers. Both tetrahydrobiopterin and L-arginine promoted the dimerization of purified heme-containing iNOS monomers (47, 48). However, to date, we have been unable to directly demonstrate a higher amount of iNOS dimers compared with iNOS monomers in macrophages cultured in arginine-replete vs arginine-deficient medium, because the iNOS dimers were not sufficiently resistant against dissociation on SDS-PAGE gels even when we omitted the heat denaturation of the samples and applied low temperature conditions for the running of the gels (data not shown) (49).

iNOS, arginase, and classically vs alternatively activated macrophages

Treatment with IFN-γ ($\pm$ LPS) leads to classically activated macrophages that produce effector molecules such as NO, reactive oxygen intermediates, and TNF. In contrast, cytokines such as IL-4 and IL-13 function as alternative macrophage activators, because they cause a different phenotype characterized by the up-regulation of Fc and mannose receptors, MHC molecules, endocytic capacity, and arginase activity (19). Our previous and present data show that alternatively and classically activated macrophages do not represent entirely independent, stable, and nonconvertible functional states. First, exposure of macrophages to IL-13 did not impair the induction of iNOS mRNA by IFN-γ plus LPS (21). Second, addition of an arginase inhibitor preserved the expression of iNOS protein in response to IFN-γ/LPS, despite the continued presence of IL-13 (Fig. 3*C*). Third, depletion of arginine by IL-13 or the use of arginine-deficient medium did not lead to complete or irreversible suppression of iNOS synthesis (21) (Fig. 7*A*). Furthermore, the arginase activity in IL-13-pretreated and control macrophages became similar from 24 to 36 h of stimulation with IFN-γ/LPS onward (data not shown). Both factors are likely to account for the reported reappearance of iNOS protein at late time points of stimulation (72–100 h) (21), especially because macrophages pretreated with IL-13 never developed signs of cell death, as routinely seen in control macrophages after prolonged stimulation with IFN-γ/LPS (data not shown).

Arginase expression as a possible strategy for tumors and infectious pathogens to cause sustained suppression of the iNOS/NO effector pathway

Both tumor cells and certain infectious pathogens (e.g., *Helicobacter pylori*, *Giardia lamblia*, *Trypanosomatidae*) are known to metabolize arginine via an arginase pathway that leads to the formation of ornithine. Previous studies argued that the consumption of arginine by tumors or pathogens will support their growth and survival by promoting the synthesis of polyamines from ornithine and/or by negatively affecting the production of NO by iNOS-positive macrophages (41, 50–55). The present results suggest that a local depletion of arginine might lead to a microenvironment of iNOS-negative host cells, which will support the long-term survival of otherwise NO-sensitive infectious agents or tumor cells, despite an overall strong expression of iNOS in the host tissues.

In summary, we have shown that arginine availability is a potent regulator of iNOS protein expression. The underlying mechanism is strictly posttranscriptional and comprises a translational (iNOS protein synthesis) and a posttranslational (iNOS protein stability) component. Ongoing studies address the question as to whether in addition to IL-13 other cytokines suppress iNOS by the same mode of action and whether this mechanism of regulation of iNOS also operates in vivo during infectious dieases.

Acknowledgments

We thank Elke Lorenz and Dr. Heike Schindler for their help with some of the experiments, and Dr. Ulrike Schleicher for critical reading of the manuscript.

References

1. Nathan, C. 1992. Nitric oxide as a secretory product of mammalian cells. *FASEB J. 6:3051*.
2. Bogdan, C. 2001. Nitric oxide and the immune response. *Nat. Immun. 2:907*.
3. Kolb, H., and V. Kolb-Bachofen. 1998. Nitric oxide in autoimmune disease: cytotoxic or regulatory mediator. *Immunol. Today 19:556*.
4. MacMicking, J., Q.-w. Xie, and C. Nathan. 1997. Nitric oxide and macrophage function. *Annu. Rev. Immunol. 15:323*.
5. Bogdan, C. 2000. The function of nitric oxide in the immune system. In *Handbook of Experimental Pharmacology*, Vol. 143: *Nitric Oxide*. B. Mayer, ed. Springer, Heidelberg, p. 443.
6. Felley-Bosco, E., F. C. Bender, F. Courjault-Gautier, C. Bron, and A. F. G. Quest. 2000. Caveolin-1 down-regulates inducible nitric oxide synthase via the proteasome pathway in human colon carcinoma cells. *Proc. Natl. Acad. Sci. USA 97:14334*.
7. Musial, A., and N. T. Eissa. 2001. Inducible nitric oxide synthase is regulated by the proteasome degradation pathway. *J. Biol. Chem. 276:24268*.
8. Kolodziejski, P., A. Musial, J.-S. Koo, and N. T. Eissa. 2002. Ubiquitination of inducible nitric oxide synthase is required for its degradation. *Proc. Natl. Acad. Sci. USA 99:12315*.
9. Nicholson, B., C. K. Manner, J. Kleeman, and C. L. MacLeod. 2001. Sustained nitric oxide production in macrophages requires the arginine transporter CAT2. *J. Biol. Chem. 276:15881*.
10. Modolell, M., I. M. Corraliza, F. Link, G. Soler, and K. Eichmann. 1995. Reciprocal regulation of the nitric oxide synthase/arginase balance in mouse bone marrow-derived macrophages by Th1 and Th2 macrophages. *Eur. J. Immunol. 25:1101*.
11. Hey, C., J. L. Boucher, S. Vadon-LeGoff, G. Ketterer, I. Wessler, and K. Racke. 1997. Inhibition of arginase in rat and rabbit alveolar macrophages by Nw-hydroxy-D, L-indospicine: effects on L-arginine utilization by nitric oxide synthase. *Br. J. Pharmacol. 121:395*.
12. Sonoki, T., A. Nagasaki, T. Gotoh, M. Takiguchi, M. Takeya, H. Matsuzaki, and M. Mori. 1997. Coinduction of nitric oxide synthase and arginase I in cultured rat peritoneal macrophages and rat tissues in vivo by lipopolysaccharide. *J. Biol. Chem. 272:3689*.
13. Chang, C., J. C. Liao, and L. Kuo. 1998. Arginase modulates nitric oxide production in activated macrophages. *Am. J. Physiol. 274:H342*.
14. Tenu, J.-P., M. Lepoivre, C. Moali, M. Brollo, D. Mansuy, and J. L. Boucher. 1999. Effects of new arginase inhibitor N$^{\omega}$-hydroxy-nor-L-arginine on NO synthase activity in murine macrophages. *Nitric Oxide 3:427*.
15. Doherty, T. M., R. Kastelein, S. Menon, S. Andrade, and R. L. Coffman. 1993. Modulation of murine macrophage function by IL-13. *J. Immunol. 151:7151*.
16. Doyle, A. G., G. Herbein, L. J. Montaner, A. J. Minty, D. Caput, P. Ferrara, and S. Gordon. 1994. Interleukin 13 alters the activation state of murine macrophages in vitro: comparison with interleukin-4 and interferon-γ. *Eur. J. Immunol. 24:1441*.
17. Takeda, K., M. Kamanaka, T. Tanaka, T. Kishimoto, and S. Akira. 1996. Impaired IL-13-mediated functions of macrophages in Stat6-deficient mice. *J. Immunol. 157:3220*.
18. Bogdan, C., and C. Nathan. 1993. Modulation of macrophage function by transforming growth factor-β, interleukin 4 and interleukin 10. *Ann. NY Acad. Sci. 685:713*.
19. Gordon, S. 2003. Alternative activation of macrophages. *Nat. Rev. Immunol. 3:23*.
20. Bogdan, C., Y. Vodovotz, J. Paik, Q.-w. Xie, and C. Nathan. 1994. Mechanism of suppression of nitric oxide synthase expression by interleukin-4 in primary mouse macrophages. *J. Leukocyte Biol. 55:227*.
21. Bogdan, C., H. Thüring, M. Dlaska, M. Röllinghoff, and G. Weiss. 1997. Mechanism of suppression of macrophage nitric oxide release by IL-13. *J. Immunol. 159:4506*.
22. Munder, M., M. Eichmann, J. M. Moran, F. Centeno, G. Soler, and M. Modolell. 1999. Th1/Th2-regulated expression of arginase isoforms in murine macrophages and dendritic cells. *J. Immunol. 163:3771*.
23. Rutschman, R., R. Lang, M. Hesse, J. N. Ihle, T. A. Wynn, and P. J. Murray. 2001. Stat6-dependent substrate depletion regulates nitric oxide production. *J. Immunol. 166:2173*.
24. Chang, C.-I., B. Zoghi, J. C. Liao, and L. Kuo. 2000. The involvement of tyrosine kinases, cyclic AMP/protein kinase A, and p38 mitogen-activated protein kinase

in IL-13-mediated arginase I induction in macrophages: its implications in IL-13-inhibited nitric oxide production. *J Immunol. 165:2134*.
25. Stenger, S., H. Thüring, M. Röllinghoff, P. Manning, and C. Bogdan. 1995. L-*N*6-(1-iminoethyl)lysine potently inhibits inducible nitric oxide synthase and is superior to *NG*-monomethyl-arginine in vitro and in vivo. *Eur. J. Pharmacol. 294:703*.
26. Klasen, S., R. Hammermann, M. Fuhrmann, D. Lindemann, K.-F. Beck, J. Pfeilschifter, and K. Racke. 2001. Glucocorticoids inhibit lipopolysaccharide-induced up-regulation of arginase in rat alveolar macrophages. *Br. J. Pharmacol. 132:1349*.
27. Corraliza, I. M., M. L. Campo, G. Soler, and M. Modolell. 1994. Determination of arginase activity in macrophages: a micromethod. *J. Immunol. Methods 174:231*.
28. Sheffler, L. A., D. A. Wink, G. Melillo, and G. W. Cox. 1995. Exogenous nitric oxide regulates IFN-γ plus lipopolysaccharide-induced nitric oxide synthase expression in mouse expression. *J. Immunol. 155:886*.
29. Weisz, A., L. Cicatiello, and H. Esumi. 1996. Regulation of the mouse inducible-type nitric oxide synthase gene promotor by interferon-γ, bacterial lipopolysaccharide and N^G-monomethyl-L-arginine. *Biochem. J. 316:209*.
30. Connelly, L., M. Palacios-Callender, C. Ameixa, S. Moncada, and A. J. Hobbs. 2001. Biphasic regulation of NF-κB activity underlies the pro- and anti-inflammatory actions of nitric oxide. *J. Immunol. 166:3873*.
31. Louis, C. A., V. Mody, W. L. Henry, 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. 1999:R237*.
32. Morris, S. M., D. Kepka-Lenhart, and L.-C. Chen. 1998. Differential regulation of arginases and inducible nitric oxide synthase in murine macrophage cells. *Am. J. Physiol. 275:E740*.
33. Salimuddin, A. Nagasaki, T. Gotoh, H. Isobe, and M. Mori. 1999. Regulation of the genes for arginase isoforms and related enzymes in mouse macrophages by lipopolysaccharide. *Am. J. Physiol. 277:E110*.
34. Boucher, J. L., C. Moali, and J.-P. Tenu. 1999. Nitric oxide biosynthesis, nitric oxide synthase inhibitors and arginase competition for L-arginine utilization. *Cell. Mol. Life Sci. 55:1015*.
35. Meyer, J., N. Richter, and M. Hecker. 1997. High-performance liquid chromatographic determination of nitric oxide synthase-related arginine derivatives in vitro and in vivo. *Anal. Biochem. 247:11*.
36. Albina, J. E., M. D. Caldwell, W. L. Henry, and C. D. Mills. 1989. Regulation of macrophage functions by L-arginine. *J. Exp. Med. 169:1021*.
37. Boutard, V., R. Havouis, B. Fouqueray, C. Philippe, J.-P. Moulinoux, and L. Baud. 1995. Transforming growth factor-β stimulates arginase activity in macrophages: implications for the regulation of macrophage cytotoxicity. *J. Immunol. 155:2077*.
38. Hesse, M., M. Modolell, A. C. La Flamme, M. Schito, J. M. Fuentes, A. W. Cheever, E. J. Pearce, and T. A. Wynn. 2001. Differential regulation of nitric oxide synthase-2 and arginase-1 by type 1/type 2 cytokines in vivo: granulomatous pathology is shaped by the pattern of L-arginine metabolism. *J. Immunol. 167:6533*.
39. Granger, D. L., J. B. Hibbs, J. R. Perfect, and D. T. Durack. 1990. Metabolic fate of L-arginine in relation to microbiostatic capability of murine macrophages. *J. Clin. Invest. 85:264*.
40. Closs, E. I., J.-S. Scheld, M. Sharafi, and U. Förstermann. 2000. Substrate supply for nitric oxide synthase in macrophages and endothelial cells: role of cationic amino acid transporters. *Mol. Pharmacol. 57:68*.
41. Hibbs, J. B., Z. Vavrin, and R. R. Taintor. 1987. L-arginine is required for expression of the activated macrophage effector mechanism causing selective metabolic inhibition in target cells. *J. Immunol. 138:550*.
42. Fafournoux, P., A. Bruhat, and C. Jousse. 2000. Amino acid regulation of gene expression. *Biochem. J. 351:1*.
43. Wang, X., L. E. Campbell, C. M. Miller, and C. G. Proud. 1998. Amino acid availability regulates p70 S6 kinase and multiple translation factors. *Biochem. J. 334:261*.
44. Fehlbaum, P., M. Rao, M. Zasloff, and G. M. Anderson. 2000. An essential amino acid induces epithelial β-defensin expression. *Proc. Natl. Acad. Sci. USA 97:12723*.
45. Chiarugi, A., E. Rovida, P. D. Sbarba, and F. Moroni. 2003. Tryptophan availability selectively limits NO synthase induction in macrophages. *J. Leukocyte Biol. 73:172*.
46. Lee, J., H. Ryu, R. J. Ferrante, S. M. Morris, and R. R. Ratan. 2003. Translational control of inducible nitric oxide synthase expression by arginine can explain the arginine paradox. *Proc. Natl. Acad. Sci. USA 100:4843*.
47. Presta, A., U. Siddhanta, C. Wu, N. Sennequier, L. Huang, H. M. Abu-Soud, S. Erzurum, and D. J. Stuehr. 1998. Comparative functioning of dihydro- and tetrahydrobiopterins in supporting electron transfer, catalysis, and subunit dimerization in inducible nitric oxide synthase. *Biochemistry 37:298*.
48. Panda, K., R. J. Rosenfeld, S. Ghosh, A. L. Meade, E. D. Getzoff, and D. J. Stuehr. 2002. Distinct dimer interaction and regulation in nitric oxide synthase types I, II and III. *J. Biol. Chem. 277:31020*.
49. Klatt, P., K. Schmidt, D. Lehner, O. Glatter, H.-P. Bächinger, and B. Mayer. 1995. Structural analysis of porcine brain nitric oxide synthase reveals a role for tetrahydrobiopterin and L-arginine in the formation of an SDS-resistant dimer. *EMBO J. 14:3687*.
50. Camargo, E. P., J. A. Coelho, G. Morales, and E. N. Figueiredo. 1978. *Trypanosoma* spp., *Leishmania* spp., and *Leptospira* spp.: enzymes of ornithine-arginine metabolism. *Exp. Parasitol. 45:141*.
51. Gobert, A. P., D. J. McGee, M. Akhtar, G. L. Mendz, J. C. Newton, Y. Cheng, H. L. T. Mobley, and K. T. Wilson. 2001. *Helicobacter pylori* arginase inhibits nitric oxide production by eukaryotic cells: a strategy for bacterial survival. *Proc. Natl. Acad. Sci. USA 98:13844*.
52. Eckmann, L., F. Laurent, T. D. Langford, M. L. Hetsko, J. R. Smith, M. F. Kagnoff, and F. D. Gillin. 2000. Nitric oxide production by human intestinal epithelial cells and competition for arginine as potential determinants of host defense against the lumen-dwelling pathogen *Giardia lamblia*. *J. Immunol. 164:1478*.
53. Chang, C.-I., J. C. Liao, and L. Kuo. 2001. Macrophage arginase promotes tumor cell growth and suppresses nitric oxide mediated tumor cytotoxicity. *Cancer Res. 61:1100*.
54. Leu, S. Y., and S. R. Wang. 1992. Clinical significance of arginase in colorectal cancer. *Cancer 70:733*.
55. Singh, R., S. Pervin, A. Karimi, S. Cederbaum, and G. Chaudhuri. 2000. Arginase activity in human breast cancer cell lines: N^{ω}-hydroxy-L-arginine selectively inhibits cell proliferation and induces apoptosis in MDA-MB-468 cells. *Cancer Res. 60:3305*.