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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

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Type 2 cytokines regulate fibrotic liver pathology in mice infected with Schistosoma mansoni. Switching the immune response to a type 1-dominant reaction has proven highly effective at reducing the pathologic response. Activation of NOS-2 is critical, because type 1-deviated/NO synthase 2 (NOS-2)-deficient mice completely fail to control their response. Here, we demonstrate the differential regulation of NOS-2 and arginase 1 (Arg-1) by type 1/type 2 cytokines in vivo and for the first time show a critical role for arginase in the pathogenesis of schistosomiasis. Using cytokine-deficient mice and two granuloma models, we show that induction of Arg-1 is type 2 cytokine dependent. Schistosome eggs induce Arg-1, while Mycobacterium avium-infected mice develop a dominant NOS-2 response. IFN-γ suppresses Arg-1 activity, because type 1 polarized IL-4/IL-10-deficient, IL-4/IL-13-deficient, and egg/IL-12-sensitized animals fail to up-regulate Arg-1 following egg exposure. Notably, granuloma size decreases in these type-1-deviated/Arg-1-unresponsive mice, suggesting an important regulatory role for Arg-1 in schistosome egg-induced pathology. To test this hypothesis, we administered difluoromethylornithine to block ornithine-aminodecarboxylase, which uses the product of arginine metabolism, L-ornithine, to generate polyamines. Strikingly, granuloma size and hepatic fibrosis increased in the ornithine-aminodecarboxylase-inhibited mice. Furthermore, we show that type 2 cytokine-stimulated macrophages produce proline under strict arginase control. Together, these data reveal an important regulatory role for the arginase biosynthetic pathway in the regulation of inflammation and demonstrate that differential activation of Arg-1/NOS-2 is a critical determinant in the pathogenesis of granuloma formation. The Journal of Immunology, 2001, 167: 6533–6544.

While the immune response generated against a pathogenic micro-organism is important to control infection, a persistent inflammatory reaction can actually do more harm than the pathogen itself. To develop new treatment strategies for chronic inflammatory diseases, it is important not only to characterize the mechanisms that initiate immune responses, but also to gain a deeper understanding of the factors that maintain these reactions. Indeed, knowledge about the downstream effector cells that regulate inflammatory responses and how their functional activity is controlled by the immune response may reveal new and/or improved approaches for disease intervention in general.  

Mice infected with the helminth parasite Schistosoma mansoni develop a pathologic reaction that closely mimics the human disease. Some of the eggs laid by adult worm pairs are trapped in the liver, a process that can lead to marked inflammation, tissue eosinophilia, collagen deposition, and ultimately portal hypertension and severe hepatic fibrosis in some patients. In mice the inflammatory response is dominated by the production of type 2-associated cytokines, including IL-4, IL-5, IL-10, and IL-13. Several studies have demonstrated that these type 2 cytokines control many aspects of the egg-induced immunopathologic response (1, 2).

Previously, we showed that much of the granulomatous liver pathology is reduced in infected mice when the egg-specific response is converted from a Th2-dominant to a Th1-dominant reaction. This is accomplished by sensitizing mice to schistosome egg Ag in the presence of the Th1-inducing adjuvant, IL-12, before infection (3). Several type 1-associated cytokines were shown to be involved in the protective response (4), although recent studies suggested that NO synthase-2 (NOS-2) expression is particularly important (5). NOS-2-deficient mice sensitized with eggs/IL-12 not only fail to control their egg-induced inflammatory response, but actually display a marked exacerbation in the reaction despite developing a Th1-deviated response. A similar role for NOS-2 was recently described in Mycobacterium avium-infected mice (6).

These findings suggest that while Th1 cytokines are critical, the downstream functional activity of NO-producing cells is equally important. Thus, NOS-2 is important not only for its antimicrobial...
activities (7), but also because it can serve as a potent anti-inflammatory and antifibrotic mediator.

Murine macrophages are a dominant feature of egg-induced granulomas and represent the main cellular source of inducible NO. However, these cells possess two inducible enzymes, NOS-2 and arginase, that share L-arginine as a common substrate. Although it is clear that NOS-2 plays an important role in schistosomiasis, the specific contribution of arginase metabolism to the regulation of granulomatous pathology remains unclear. In previous in vitro studies, we showed that different combinations of cytokines induce NOS-2 and the hepatic isoform of arginase (arginase type 1 (Arg-1)). The Th1-associated cytokines IFN-γ and TNF-α activate NOS-2, whereas the Th2-type cytokines IL-4, IL-10, and IL-13 induce Arg-1 (8). Of interest, the preferential activation of these enzymes is also observed during Ag presentation by macrophages and dendritic cells to corresponding CD4+ Th1 or Th2 lymphocytes (9, 10). Thus, in a manner similar to the Th1/Th2 paradigm, the cytokine-mediated activation of one enzyme is accompanied by the active suppression of the other.

In mammals, two arginase isoforms are expressed: the cytosolic Arg-1 and the mitochondrial Arg-2 (11). The isoforms catalyze the same reaction, but are encoded by different genes and differ in their tissue distribution. Arg-1 is an essential enzyme of the urea cycle and is expressed at high levels in hepatocytes. This enzyme hydrolyzes L-arginine to urea and L-ornithine; therefore, its main function in the liver is the detoxification of ammonia. Although it is expressed at other sites, the exact role of Arg-1 in extrahepatic cells and tissues is not well understood. Because L-ornithine, a product of arginase activity, is a necessary metabolite for the production of polyamines and prolines, which control cell proliferation and collagen production, respectively, arginase activity appears to be critically linked with cell growth and connective tissue production. Notably, both of these activities are key parameters in the pathogenesis of inflammatory responses.

We hypothesized that type 1/type 2 cytokines would regulate the dominance of NOS-2 Arg-1 vs expression in vivo, which, in turn, would regulate the character and magnitude of inflammatory reactions. To test this hypothesis, we examined L-arginine metabolism in two different models of granulomatous disease. Initially, we investigated whether the expression of Arg-1 and NOS-2 was regulated in a manner similar to that described in in vitro studies. Specifically, we examined in several cytokine-deficient mouse strains whether arginase was activated following exposure to schistosome eggs, a potent Th2-inducing stimulus. In separate studies, mice treated with schistosome eggs and IL-12 or Mycobacterium avium-infected animals were used to examine the pattern of Arg-1/NOS-2 expression during type 1-dominated inflammatory responses. Finally, additional in vitro and in vivo studies were designed to determine more directly whether granulomatous pathology is influenced by the activation of arginase.

**Materials and Methods**

**Mice, parasites, bacteria, and Ag preparations**

Female 42-day-old C57BL/6, C57BL/10, C57BL/10Aki knockout (KO) IL-10, C57BL/6Ai-[KO] IL-4, and C57BL/6Ai-[KO] IL-10/LtIl4-/- mice were obtained from Taconic Farms (Germantown, NY). Wild-type (WT)-F2, IL-13-KO, and IL-4/LtIl13-KO mice (all 129Ola × C57BL/6 (F2)) were provided by Dr. A. McKenzie (Medical Research Council, London, U.K.). All mice were housed under specific pathogen-free conditions in a National Institute of Health American Association for the Accreditation of Laboratory Animal Care-approved animal facility. *Mycobacterium avium* strain 2-151 SmT was cultured on agar plates as described previously (12). Cer- cariae of a Puerto Rican (NMRI) strain of *S. mansoni* (Biomedical Research Institute) were obtained from infected Biomphalaria galbrata snails (Biomedical Research Institute). Soluble egg Ag (SEA) and soluble worm Ag preparations were obtained from homogenized *S. mansoni* eggs and adult parasites as previously described (3).

**Immunizations, infections, and difluoromethylornithine (DFMO) treatment**

*S. mansoni* eggs were extracted from the livers of infected mice (Biomedical Research Institute) and enriched for mature eggs. For the induction of pulmonary granulomas, groups of five mice were immunized with 5000 freshly isolated eggs i.p. and challenged with 5000 eggs i.v. 14 days later. All mice were sacrificed 6 days after the egg challenge. In designated experiments, C57BL/10A mice and C57BL/10Ai-[KO] IL-10 mice were also injected i.p. with 0.25 μg rIL-12 on 5 consecutive days beginning on the day of the primary egg immunization.

Infection and sensitization of mice with eggs and rIL-12 has been previously described (3). Briefly, groups of 10 C57BL/6Ai mice were injected i.p. with 5000 eggs on three occasions separated by 2-wk intervals. Animals were also injected i.p. with rIL-12 (0.25 μg/dose) on 5 consecutive days beginning on the day of each egg immunization. Naive mice and egg/rIL-12-preimmunized mice were infected 2 wk after the last egg/rIL-12 injection by percutaneous exposure of tail skin for 40 min in water containing 25 cercariae. All mice were sacrificed 8 wk after infection. We noted no mortality in any group up to the point of sacrifice. In designated experiments infected C57BL/6Ai mice were treated with 1% (42.3 mM) or 2% (84.6 mM) (D.L)-2DFMO (provided by Dr. V. Steele, National Cancer Institute) in the drinking water beginning at wk 5 postinfection. The mice were kept on DFMO-containing water until the end of the experiment.

Groups of five C57BL/6Ai mice were injected i.v. with 10⁶ CFU *M. avium* strain 2–151 SmT. Mice were sacrificed and 2 wk after infection and compared with noninfected controls. Lung tissue from individual mice was embedded in paraffin and stained with the Fite acid-fast method (American Histolabs, Histo-Path of America, Millersville, MD) to monitor bacterial infection. In addition, bacterial CFU were analyzed in lung tissue samples as previously described (12).

**Histopathology, fibrosis measurement, and immunohistochemistry**

Approximately half of the liver was fixed in Bouin-Holland solution, and half of the lung was fixed with fixative. Histologic sections were processed and stained with Giemsa (Histo-Path of America). The diameter and cell composition of granulomas (30/mouse) surrounding single eggs were measured using an ocular micrometer, and the volume of each granuloma was calculated assuming a spherical shape. The number of granulomas was counted in the same area by an experienced pathologist. Only granulomas around mature, viable eggs were measured in the liver. The collagen content of the liver samples, determined as hydroxyproline, was analyzed as described previously (1).

Formalin-fixed, paraffin-embedded tissue sections were deparaffinized and rehydrated, and the Ab epitopes unmasked by a 5-min incubation in 1% (v/v) TBS at room temperature. Endogenous peroxidase activity was quenched by a 5-min incubation in 3% H₂O₂ in H₂O. Arginase expression was detected with mouse anti-Arg-1 IgG1 (BD-Transduction Laboratories, Lexington, KY) or normal mouse IgG1 (Zymed Laboratories, South San Francisco, CA) as a negative control and using the M.O.M. kit (Vector Laboratories, Burlingame, CA) as directed by the manufacturer. The samples were developed with diaminobenzidine (Vector Laboratories) as directed by the manufacturer and counterstained with Vector Green (Vector Laboratories).

**Lymphocyte culture and cytokine detection**

Spleen and mesenteric lymph node (infection model) or lung-draining lymph node (pulmonary granuloma model) were removed aseptically, and single-cell suspensions were prepared. Cells were plated in 24-well tissue culture plates at a final concentration of 4 × 10⁶ cells/ml (lymph node) or 10⁶ cells/ml (lymph node) in RPMI 1640 supplemented with 10% FCS, 2 mM glutamine, 1 mM sodium pyruvate, 50 μM 2-ME, and antibiotics-antimycotic solution (all from Life Technologies, Gaithersburg, MD). Cultures were incubated at 37°C in a humidified atmosphere of 5% CO₂. Cells were stimulated with SEA (20 μg/ml), soluble worm Ag (50 μg/ml), Con A (1 μg/ml), or medium alone. Supernatant fluids were harvested at 72 h and assayed for cytokin production. IFN-γ and IL-5 were measured by two-site ELISA as previously described (3). Cytokine levels were calculated with standard curves constructed using recombinant murine cytokines.

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RT-PCR detection of mRNA

Relative quantities of mRNA for IFN-γ, IL-4, IL-5, IL-13, hypoxanthine phosphoribosyltransferase (HPRT), NOS-2, Arg-1, Arg-2, ornithine-aminotransferase (OAT), and ornithine-aminodecarboxylase (ODC) expressed in inflammatory tissue, were determined by RT-PCR as previously described (13). The sequences of primers and probes for HPRT, cytokines (13), and ODC have been published previously. The following primer and probes were used for arginase isoforms OAT and ODC: Arg-1 sense, 5'-CAGAAGAAGTTGAGTACG-3'; antisense, 5'-CAGATGCGGAGGTAC-3'; probe, 5'-GCGATCTGGCAGCGGAG-3'; Arg-2 sense, 5'-TGTAGTGGCAAAGGCGAG-3'; antisense, 5'-CTAGAGTGGAAATGCGTGC-3'; probe, 5'-GGGTCACCCACAGGAACACTCTG-3'; ODC sense, 5'-TGAGGCAGGCAGATACTA-3'; antisense, 5'-TGCGCTTCGTCCTCCTACTTTTCT-3'; probe, 5'-TGCCCCCCCTACGGTTGTGAC-3'. The amplified DNA was analyzed by electrophoresis, Southern blotting, and hybridization with nonradioactive specific probes. The chemiluminescent signals were quantitated using a ScanJet IIIP (Hewlett-Packard, Palo Alto, CA). The amount of PCR product was determined by comparing the ratio of molecule-specific density to that of HPRT-specific signal density for individual samples. Arbitrary densitometric units for individual samples were subsequently multiplied by 100 and compared with those for control mice (uninfected mouse tissue).

Determination of arginase activity in tissues and granulomatous liver cell isolation

The granulomatous lung or liver tissue (~100 mg) was crushed with a pestle in an Eppendorf tube (1.5 ml), resuspended in 0.5 ml 50 mM Tris-HCl buffer, pH 7.5, and homogenized with an Ultra-Turrax (IKA, Muhlheim, Germany). After addition of 0.5 ml 0.1% Triton X-100, the tubes were shaken for 10 min at 25 °C and centrifuged at 13,000 rpm. Arginase activity was determined as described previously (9), and the protein concentration was measured with the bicinchoninic acid protein assay (Pierce, Rockford, IL). One unit of arginase activity is the amount of enzyme necessary to hydrolyze 1 μmol of arginine/min.

Liver tissue samples from infected mice were passed through a sterile stainless sieve and washed twice with cold PBS. The pellet, containing sedimented granulomas, single cells, and tissue debris, was resuspended in RPMI 1640 containing 100 U/ml collagenase (D Roche, Indianapolis, IN) and 4 U/ml DNase I (Sigma) and digested for 20 min at 37 °C. The digested material was passed through a cell strainer (100 μm) to remove debris and obtain a single-cell suspension. The cells were washed twice with RPMI 1640. Erythrocytes were lysed with ACK buffer (BioWhittaker, Walkersville, MD), and cells were plated at 10^6 cells/well in 48-well culture plates. Cells were washed twice with cold PBS and lysed in 250 μl 0.1% Triton X-100, 1% protease inhibitors. Arginase activity was determined as described previously (9).

Detection of Arg-1 and NOS-2 protein expression

Equal amounts of lung tissue lysate from individual mice were pooled in each experimental group. SDS-PAGE was performed as described by Laemmli (15) in a 10% polyacrylamide gel and electrotransferred onto nitrocellulose membranes (16). Immunodetection was performed with rabbit anti-IgG, immunoreactive proteins were visualized using a ScanJet IIIP (Hewlett-Packard, Palo Alto, CA). The amount of PCR product was determined by comparing the ratio of molecule-specific density to that of HPRT-specific signal density for individual samples. Arbitrary densitometric units for individual samples were subsequently multiplied by 100 and compared with those for control mice (uninfected mouse tissue).

Analysis of macrophage proline production

Murine bone marrow-derived macrophages (BMM) were obtained by flushing the femurs of C57Bl/6 mice. Cells were cultured for 8 days in hydromorphic Teflon bags (Biofolie 25, Heraeus, Hanau, Germany) in DMEM containing 10% inactivated FCS, 5% horse serum, 1 mM sodium pyruvate, 2 mM glutamine, 60 μM 2-ME, penicillin (100 U/ml), streptomycin (100 μg/ml; Life Technologies, Paisley, Scotland) and the supernatant of L929 fibroblasts at a final concentration of 15% (v/v) of a source of M-CSF, which drives cells proliferation toward a >95% pure population of BMM.

To induce arginase activity, BMM (10^6 cells in triplicate) were pretreated with IL-4, IL-13 (both from R&D Systems, Wiesbaden, Germany), IL-10 (100 U/ml), IL-12 (10 U/ml), IFN-γ (10 U/ml; BioXCell, Rockey Hill, NJ; all 20 U/ml), or IL-1β (1 ng/ml) from Triad, Ernst-Bendt Institute, Vienna, Austria, 500 U/ml) and in designated wells with 1 mM t-hydroxyarginine (Clnalifa, Lauflingen, Switzerland) for 24 h at 37 °C and with 10% CO2 in 0.2 ml DMEM supplemented with 10% inactivated FCS, 1 mM sodium pyruvate, 2 mM glutamine, and 60 μM 2-ME. The cells were washed and placed in DMEM without arginine containing 2% FCS and pulsed with 0.2 μCi l-[1-14C]arginine (NEN, Boston, MA; sp. act., 348 mCi/mM) or 0.2, 0.4, or 0.6 μCi l-[1,14C]ornithine (NEN; sp. act., 52 mCi/mM). Cytokines and inhibitors were added at the original concentration, and after 24 h the cultures were frozen at −80 °C. The synthesized radioactive proline was isolated in a 20-μl aliquot of the culture medium by TLC in silica-coated plates (Merck, Darmstadt, Germany) using ethanoleater (3:70, v/v) as solvent. The separated compounds were detected with ninhydrine in a reference band with nonradioactive standards, and the radioactive spots with the same Rf value were placed in scintillation vials and counted.

Statistical analysis

Hepatic fibrosis on single egg decreases with increasing intensity of infection (total number hepatic eggs per tissue) in the infection experiments (19). These variables were, therefore, compared by analysis of covariance, using the log of total liver eggs as the covariate and the log of hydroxyproline per egg. All other variables were compared by Student’s t test. In all cases, results were considered significant at p < 0.05.

Results

Arg-1 expression and activity correlates with a Th2 response and maximum egg-induced pulmonary granuloma model

WT mice and mice deficient for IL-10, IL-4, and IL-10/IL-4 were sensitized i.p. with freshly isolated schistosome eggs and 2 wk later were challenged i.v. with a second bolus of eggs. On day 6 postchallenge, all animals were sacrificed, and pulmonary granuloma size and eosinophilia were assessed by microscopy. Lung tissues were also assayed for arginase activity. Large granulomas formed in WT and IL-10-deficient mice, while lesion size moderately decreased in the absence of IL-4 (Fig. 1A), which was consistent with previous observations (20, 21). In contrast to the findings in the WT and single KO animals, granuloma size (Fig. 1A) and granuloma eosinophils (Fig. 1B) were both significantly decreased in mice deficient in IL-4 and IL-10. Strikingly, lung arginase activity, examined by three different assays, closely correlated with these pathologic observations (Fig. 1, C–E). Arginase enzyme activity was induced in the WT and IL-10 KO mice after egg injection, and these mice displayed no impairment in lesion development. In marked contrast, enzyme activity was almost absent in the double IL-10/IL-4 KO mice that were highly defective for granuloma formation. Most of the arginase activity derived from the cytosolic Arg-1 isoform, as suggested by both RT-PCR (Fig. 1D) and Western blot (Fig. 1E). Consistent with the enzyme activity assays (Fig. 1C), Arg-1 mRNA (Fig. 1D) and protein expression (Fig. 1E) were almost undetectable in the double IL-10/IL-4 KO mice, while high levels were readily observed in the egg-injected WT and IL-10-deficient mice. IL-4-deficient mice displayed an intermediate phenotype in all three assays, which was again consistent with their pattern of lesion formation and tissue eosinophilia. Although it has been reported that the mitochondrial arginase (Arg-2) can also be differentially regulated in cytokine-activated macrophages (22), there was no evidence that Arg-2 mRNA expression was induced following egg injection (data not shown).

Previous in vitro studies with macrophage cultures showed that Arg-1 and NOS-2 are differentially regulated by specific type 1 and type 2 cytokines (8, 9). To determine whether similar regulatory mechanisms were operating in vivo, the expression of NOS-2 mRNA was examined in the egg-injected cytokine-deficient animals (Fig. 1F). The dominance of type 1 vs type 2 cytokine expression in the granulomatous tissues was also confirmed (20) by RT-PCR (Fig. 1, G and H). The results from these experiments showed that WT and IL-10/IL-4-deficient mice develop completely divergent type 1/type 2 cytokine and NOS-2/Arg-1 responses, while IL-10-
IL-4-deficient mice display intermediate phenotypes. IL-5 and IL-13 mRNA expression was maximally induced (Fig. 1H), while little IFN-γ mRNA was detectable in the lungs of egg-injected WT mice (Fig. 1G), which was consistent with previous findings (20, 21). There was also little NOS-2 mRNA expression in these animals (Fig. 1F). In contrast, the IL-10/IL-4-deficient mice showed high levels of IFN-γ, but almost undetectable IL-5 and IL-13 mRNA (Fig. 1H). These mice, unlike the WT group, displayed a highly significant NOS-2 mRNA response and correspondingly low levels of Arg-1 (Fig. 1, D and F). Of interest, although IFN-γ mRNA expression was markedly increased in the IL-10-deficient animals, little induction of NOS-2 was evident, and Arg-1 remained the dominant enzymatic response. The maintenance of a significant type 2 cytokine response in these animals may explain this unexpected pattern (Fig. 1H) (20). The findings from IL-4-deficient mice support this hypothesis, showing a much more impaired type 2 response and correspondingly increased expression of both IFN-γ and NOS-2 mRNA (Fig. 1, F–H).

We also examined the contribution of IL-13 to the regulation of Arg-1, because recent studies showed that IL-13 cooperates with IL-4 to generate egg-induced granulomas (21). Here, experiments conducted with IL-13-deficient and double IL-4/IL-13 mutant mice to a great extent duplicated the findings generated with IL-4- and IL-4/IL-10-deficient mice (Fig. 2). Arginase activity and egg-induced pathology were reduced, but not completely abrogated, in the IL-13-deficient mice. Strikingly, however, the double IL-4/IL-13-deficient mice displayed a much more impaired granulomatous response (Fig. 2, A and B), which correlated perfectly with an
almost complete absence of arginase activity in the tissue (Fig. 2C). Thus, in the schistosome egg-granuloma model, the expression and activation of Arg-1 in vivo directly correlates with a type 2 cytokine response and maximum egg-induced pathology, whereas peak NOS-2 expression correlates with polarized type 1 cytokine expression and reduced inflammation.

IL-12 converts the normal type 2 response to a type 1-dominant pattern, leading to decreased arginase activity and reduced granulomatous inflammation

To determine whether IL-12 and type 2 to type 1 immune deviation would affect arginase activity in the pulmonary granuloma model, we immunized WT and IL-10-deficient mice with schistosome eggs and rIL-12, challenged them with eggs i.v. 2 wk later and then sacrificed the mice on day 6. WT and IL-10-deficient mice were selected for these studies because they showed the highest arginase activity and formed large eosinophil-rich granulomas when not sensitized with eggs and IL-12 (Fig. 1). As shown in Fig. 3, granuloma size (Fig. 3A) and tissue eosinophilia (Fig. 3B) were markedly decreased in both strains when sensitized in the presence of IL-12. Arginase activity was also significantly decreased in these mice (Fig. 3C), and this correlated with a marked alteration in the type 1/type 2 cytokine response. The normal egg-specific type 2 response in WT mice was converted to an IFN-γ-dominant response by the egg/IL-12 sensitization procedure (Fig. 3D). Notably, however, although IFN-γ increased in the non-IL-12-treated IL-10 KO mice, arginase activity and granuloma formation were completely preserved in these animals. Granuloma size and arginase activity were only decreased in these mice when the animals were sensitized with eggs and IL-12. In contrast to the nonsensitized KO animals, type 2 cytokine production decreased dramatically in the sensitized mice, suggesting that type 2 cytokine expression was the most critical factor for maintaining arginase activity and granuloma formation (Fig. 3D).

NOS-2 expression dominates during M. avium infection: marginal up-regulation of Arg-1 is observed in the lung during a type 1 dominant response

C57BL/6 mice were infected with M. avium (10^8 CFU/mouse), and lung tissue was analyzed for expression and activation of Arg-1 and NOS-2 on wk 2 and 4 following infection. The local cytokine response was also examined by semiquantitative RT-PCR to show that a dominant type 1 response was induced. In addition, Fite-stained lung tissue sections were analyzed to confirm that mice were infected (data not shown). Finally, lung samples were homogenized to determine bacterial loads. The mean infection intensity on day 14 postinfection was 2.24 × 10^5 ± 1.04 × 10^5 CFU/mg tissue; by day 28 this number rose to 3.29 × 10^6 ± 8.32 × 10^5 CFU/mg. In contrast to the findings with schistosome eggs, RT-PCR analysis of M. avium-infected lungs showed a dominant type 1 cytokine mRNA response (Fig. 4A). IFN-γ was upregulated by day 14 postinfection and remained at high levels through day 28. In contrast, IL-4 was barely detectable on day 14, although a slight increase was observed by day 28 (Fig. 4A). Consistent with these observations, Arg-1 mRNA levels (Fig. 4B) and Arg-1 enzymatic activity (Fig. 4C) showed little change over background on day 14, and only a slight, but nevertheless significant, increase by day 28. Indeed, compared with the Arg-1 response observed in the lungs of schistosome egg-challenged mice, the small induction detected following M. avium infection appeared minor (Fig. 4, B and C). Western blot analysis confirmed this conclusion, because we were not able to detect Arg-1 protein expression at any time point (data not shown). In contrast to these observations, NOS-2 mRNA expression was clearly induced in the lungs of M. avium-infected mice (Fig. 4D). NOS-2 protein expression was also easily detectable by day 28 postinfection (Fig. 4E). By comparison, NOS-2 mRNA was only slightly induced in the lungs of schistosome egg-challenged mice (Fig. 4D), and no protein was detected by Western blot analysis (data not shown). Thus, Arg-1 activity does not simply result from an ongoing inflammatory reaction, but rather appears to be strictly dependent on a type 2-driven response.

Differential regulation of NOS-2/Arg-1 in the livers of infected mice

Because one of the main functions of the liver is the detoxification of ammonia, Arg-1 is highly expressed in hepatocytes, where the enzyme is essential for the urea cycle. Therefore, the liver displays
basal levels of arginase activity much higher than any other tissue. Because of its high background expression, the liver did not provide a convenient setting to investigate the differential regulation of arginase activity.

**FIGURE 3.** WT and IL-10-deficient mice sensitized with eggs and IL-12 display reduced arginase activity and granulomatous pathology following challenge. Groups of four or five WT and IL-10 KO mice were sensitized with 5000 schistosome eggs and IL-12 (0.25 μg/animal) and challenged i.v. with 5000 eggs 14 days later. On day 6 postchallenge, pulmonary granuloma size (A), tissue eosinophilia (B), and Arg-1 activity (C) were examined in individual mice. Open symbols represent data from egg/IL-12-sensitized mice; filled symbols are the non-IL-12-treated controls. The bars show group medians. The production of IL-5 and IFN-γ by SEA-restimulated lymph node cells is shown in D. The data shown are the mean cytokine concentrations ± SD. Similar results were obtained in four separate studies.

**FIGURE 4.** NOS-2 dominates during M. avium infection. Groups of five C57BL/6 mice were infected with 10⁶ CFU M. avium. Mice were sacrificed on days 0, 14, and 28 postinfection, and total lung RNA was isolated for RT-PCR analysis. IL-4, IFN-γ (A), Arg-1 (B), and NOS-2 (D) mRNA expression was analyzed in individual mice. Results shown are the group mean ± SEM. Lung homogenates were also examined for arginase activity (C). For comparison purposes, representative results from schistosome egg-challenged WT mice are shown (B–D, []). *, Group is significantly different from uninfected controls. Pulmonary expression of NOS-2 protein was also evaluated by Western blot (E).
of NOS-2/Arg-1 expression in vivo. Therefore, we initially focused on the lung, where NOS-2 and Arg-1 are both almost undetectable in naïve mice. Nevertheless, in schistosomiasis, much of the morbidity stems from the fibrotic pathology that develops in response to eggs deposited in the liver. As such, we were interested in extending our findings from the pulmonary granuloma model to the livers of infected animals. Tissue sections prepared from the liver and intestine of mice infected with cercariae from *S. mansoni* and killed 8 wk after infection were stained with anti-Arg-1 Ab. Immunohistochemical staining shows a significant accumulation of arginase-positive cells in areas surrounding the deposited eggs in both tissues (Fig. 5, A and D). Not surprisingly, high background levels were detected in the liver parenchyma of both infected and uninfected mice. Nevertheless, more intense staining was clearly visible in the granulomas. Interesting differences in the cellular localization of arginase were also noted in these sections. Specifically, dense staining for arginase was observed in cells found in the liver parenchyma, which was similar to findings in a previous report (23), while arginase-positive cells in the granuloma showed a more diffuse pattern (Fig. 5, A and B).

In additional experiments C57BL/6 mice were either sensitized with schistosome eggs and IL-12 or left untreated and subsequently infected with 25 cercariae. Similar to the immune deviation studies described above (Fig. 3), this sensitization procedure effectively deviated the normal type 2 cytokine response to a dominant type 1 phenotype (3). Unlike the findings from the pulmonary granuloma model, however, little regulation of Arg-1 activity was detected in whole liver tissue by wk 8 postinfection (Fig. 6A), consistent with previous observations (5).

**DFMO blocks the enzyme ornithine decarboxylase, resulting in increased liver fibrosis and granuloma size in *S. mansoni*-infected mice**

Hydrolysis of l-arginine by the enzyme arginase produces l-ornithine, which is a necessary metabolite for the synthesis of both polyamines and proline (Fig. 7). Generation of these products is controlled by two enzymes, ODC and OAT, respectively, both of which are constitutively expressed in normal liver and lung tissues (data not shown). ODC metabolizes l-ornithine to putrescine and is therefore the rate-limiting enzyme for polyamine synthesis. Because arginase is an essential enzyme of the urea cycle, arginase inhibitors proved toxic (M. Hesse and T. A. Wynn, unpublished observations), which made it impossible to directly examine its role in the pathogenesis of schistosomiasis. Therefore, we turned our attention to ODC, because it operates directly downstream from arginase, and its enzymatic activity is easily blocked with the inhibitor DFMO. DFMO is a selective inhibitor of ODC and is well tolerated in rodents and humans (24). The compound is commonly used as a chemotherapeutic agent for neoplasms, but, notably, has also demonstrated efficacy as curative treatment for some protozoal infections (25). Because of its documented antiparasitic activity, we initially investigated whether DFMO was toxic for schistosomes. It is important that DFMO did not affect either the viability or fecundity of adult worms cultured in vitro, even when the parasites were exposed to relatively high (31 nM) concentrations of the compound (data not shown).

To determine whether the arginase metabolic pathway was regulating the pathologic response in schistosomiasis, we administered the ODC inhibitor, DFMO, to *S. mansoni*-infected mice. Specifically, infected C57BL/6 mice were treated for 3 wk with either 1 or 2% DFMO delivered in drinking water. The treatments were initiated 5 wk postinfection, at the time when adult worms begin laying eggs. The effects on hepatic fibrosis, granuloma formation, and production of IL-4 and IL-5 were then examined at wk 8. Consistent with the in vitro experiments discussed above, DFMO had no effect on the adult worms, because similar numbers of worm pairs were seen in both the treated and nontreated animals (data not shown). Notably, however, both doses of DFMO significantly increased the development of hepatic fibrosis (Fig. 8A) as well as granuloma size (Fig. 8B). Although the granulomatous response worsened in the treated mice, no differences in the cellular make-up of their lesions were detected. Indeed, the percentages of granuloma-associated eosinophils were nearly identical in all three groups (Fig. 8C). Strikingly, the expression of IL-4 and IL-5 was totally unaffected (Fig. 8D). These findings suggest that the additional increase in fibrosis in DFMO-treated animals compared with that in infected, nontreated mice is unlikely to be explained by the enhanced production of type 2 cytokines (21, 26, 27). While induction of a type 2 cytokine response is clearly necessary to regulate pathology in infected mice (2) and to control arginase activity (Figs. 1 and 2), we hypothesize the increased bioavailability of...
L-ornithine for its competing enzyme, OAT, may be a more likely explanation for the increased fibrotic response observed in the DFMO-treated mice (Fig. 7).

Alternatively activated macrophages up-regulate proline production

Because we were unable to test the downstream function of the opposing enzyme, OAT, due to the lack of a specific inhibitor, we took a more direct in vitro approach and examined whether proline production was enhanced in macrophages that were stimulated with IL-4, IL-13, or IL-10. In these studies BMM were stimulated with the cytokines in the presence or the absence l-hydroxyarginine, a potent physiologic inhibitor of arginase produced by NOS-2 (see Fig. 7), to provide additional proof that proline production in macrophages is regulated by the activity of arginase. As shown in Fig. 9A, IL-4 and IL-13 markedly up-regulated proline production, while addition of l-hydroxyarginine to the cultures completely blocked the response. IL-10, in contrast, stimulated only very weak proline production. As described previously (8, 9), arginase activity was also significantly up-regulated in the cytokine-stimulated cultures (Fig. 9A), confirming that the BMM were developing an alternatively activated phenotype. The extent of arginase induction correlates with the amount of detected proline.

By adding radioactive labeled ornithine to cytokine-stimulated macrophages, we also investigated whether OAT-mediated proline production is regulated by substrate availability. Our results show that in the presence of ornithine, the production of proline is not dependent on cytokine-mediated activation of macrophages (Fig. 9B). Here, proline levels were only slightly affected by the addition of IL-4 and IL-13. Thus, while IL-4 stimulated a small, but significant, increase in proline in the presence of excess ornithine, the increase was only 2-fold, not nearly as dramatic as the 13-fold increase observed in arginine-enriched cultures (Fig. 9A). Moreover, even unstimulated macrophages increased proline production 10-fold (Fig. 9B) compared with control macrophages when provided the arginase substrate, arginine (Fig. 9A). Finally, incubation of macrophages with the anti-fibrotic cytokine IFN-γ (4) failed to inhibit proline production, providing additional proof that cytokines have little or no influence on OAT activity. As such, the data from these experiments demonstrate that proline production is both significant in macrophages and tightly regulated by the type 2 cytokine-dependent activation of arginase.

Discussion

Macrophages function as important effector cells during most immune responses; therefore, their activity can have a major impact on the duration, magnitude, and overall character of inflammatory reactions. There is accumulating evidence that the effector function of macrophages is controlled by specific triggering signals that
stimulate their differentiation into classically activated or alternatively activated cells (28, 29). In this regard, cytokines, glucocorticoids, and catecholamines have proven highly effective at controlling the functional diversity of macrophages (28, 30, 31). Signals such as LPS, unmethylated CpG oligodeoxynucleotides, the phagocytosis of necrotic cells, and triggering of specific toll-like receptors can also influence the effector phenotype of activated macrophages and dendritic cells (32, 33). In mice IFN-γ, IL-1, and TNF-α stimulate NO production by macrophages and therefore serve as the key signals that promote the development of classically activated macrophages (7). In contrast, recent in vitro experiments demonstrate that several Th2-associated cytokines, including IL-4, IL-13, and IL-10, are involved in the generation of alternatively activated cells, characterized by up-regulation of Arg-1, the hepatic isoform of arginase (8–10). These in vitro data suggest that the reciprocal pattern of l-arginine metabolism exhibited by activated macrophages might play an important role in regulating their effector functions during immune responses. Nevertheless, previous to this study, there was no direct evidence that these mechanisms were operating or important in vivo.

Our studies conducted in the schistosome lung granuloma model in cytokine-deficient mice demonstrate a strict requirement for type 2 cytokines in the inducible expression and activation of Arg-1 in vivo, and in agreement with in vitro data (9), IL-4 and IL-13 are the major arginase inducers (Figs. 1 and 2). The results

![FIGURE 7. NOS-2/Arg-1 expression is controlled by type 1/type 2 cytokines in vivo. The pattern of l-arginine metabolism regulates the pathological response in murine schistosomiasis. Macrophages activated in the presence of type 1 cytokines exhibit increased NOS-2 activity, which promotes l-hydroxyarginine, NO, and l-citruline production. Little arginase activity is detected in response to these cytokines. The intermediate byproduct l-hydroxyarginine also acts as a potent inhibitor of arginase. In contrast, macrophages stimulated with type 2 cytokines show increased arginase and decreased NOS-2 activity. Here, l-arginine is metabolized to urea and l-ornithine. ODC then metabolizes l-ornithine to produce polyamines, molecules that induce cell proliferation, or it can be converted by OAT to produce proline, the basic building block of collagen. DFMO effectively blocks the enzyme ODC, thereby increasing the bioavailability of l-ornithine for OAT. This situation would probably result in increased proline production and collagen deposition, particularly in situations where type 2 cytokines are the dominant response (see Figs. 8 and 9).](image)

![FIGURE 8. Blocking ODC with DFMO increases the fibrotic response in S. mansoni-infected mice. Groups of 10 C57BL/6 mice were infected with S. mansoni and treated with DFMO as described. Liver fibrosis (A) was quantified by analysis of hydroxyproline content and expressed as micromoles of hydroxyproline per 10,000 eggs. Granuloma volumes (B) and tissue eosinophilia (C) were also evaluated. Total RNA was isolated, and steady state levels of IL-4 and IL-5 mRNA were determined by semiquantitative RT-PCR (D). Results are shown for individual mice; bars denote the group median. For liver fibrosis measurements, statistical significance was determined by analysis of covariance ($p < 0.05$). All other data were analyzed by Student’s $t$ test ($p < 0.05$). Similar results were obtained in a second experiment.](image)
mycobacteria infection is low, although there might be strain differences. Interestingly, data from murine models suggest that the fibrotic response in mycobacteria infections may be attributable to an underlying type 2 cytokine response (35, 36). Regardless, these data complement the findings with schistosome eggs and demonstrate that NOS-2/Arg-1 expression is tightly regulated by type 1/type 2 cytokines. Together, these results demonstrate for the first time the differential regulation of NOS-2 and Arg-1 by type 1/type 2 cytokines at sites of pathogen-induced inflammation.

Surprisingly, although the production of IFN-γ was markedly up-regulated in schistosome egg-injected IL-10 KO mice, Arg-1 was induced to the same extent as in WT animals (Figs. 1 and 3). This was somewhat unexpected, given the established inhibitory role played by IFN-γ on Arg-1 activity (8). Nevertheless, these animals displayed no impairment of their type 2 cytokine response (IL-4/IL-13), which could explain the maintenance of both Arg-1 expression and granuloma formation (Fig. 1). To test this hypothesis, we sensitized WT and IL-10-deficient mice with eggs and IL-12 before the i.v. egg challenge. In these experiments IL-10 KO mice showed a marked reduction in Arg-1 activity and a corresponding decrease in Th2-type cytokine expression and granuloma formation (Fig. 3). These studies suggest that both the timing and the degree of exposure to Th1 and Th2 cytokines play important roles in determining the effector cell phenotype. Thus, the initial phase of an immune response is especially critical for determining the effector cell phenotype as well as the magnitude of the ensuing inflammatory response. Recent studies with rat macrophages also support this conclusion (37, 38). These findings also demonstrate that the degree of NOS-2 vs Arg-1 expression in vivo is a much better predictor of the overall magnitude of schistosome egg-induced pathology than is the relative dominance of type 1/type 2 cytokines in vivo.

Although we do not yet know the exact mechanism by which arginase regulates the granulomatous response in murine schistosomiasis or which cells besides macrophages express the enzyme, peak granuloma formation correlated with high arginase activity in every case. In contrast, NOS-2 was induced in mice sensitized with eggs and IL-12 (Fig. 6A), and the animals developed smaller and much less fibrotic granulomas when subsequently infected (5). Suppressing the type 2 response in NOS-2-deficient animals, in contrast to WT animals, completely failed to reduce egg-induced liver pathology and actually worsened the reaction (5). The inability to down-regulate pathology in the type 1-deviated NOS-2-deficient mice may be explained in part by the lack of l-hydroxyarginine, the most potent physiologic inhibitor of arginase (39, 40). It is synthesized by NOS-2 during the oxidation of arginine to NO (Fig. 7), but would be absent in NOS-2-deficient mice. Therefore, direct competition for arginase is eliminated in NOS-2-deficient mice, as is the arginase inhibitory activity of l-hydroxyarginine, which could increase the arginase-dependent production of l-ornithine and, in turn, polyamine and proline levels (Fig. 7). Indeed, this hypothesis is strongly supported by the fact that proline production by alternatively activated macrophages is blocked by l-hydroxyarginine (Fig. 9A) and is regulated exclusively by the availability of l-ornithine (Fig. 9B).

To directly test the involvement of the arginase biosynthetic pathway in the pathogenesis of schistosomiasis, we blocked the l-ornithine-dependent synthesis of polyamines with a specific inhibitor. Here, for the first time, we demonstrate that arginase activity is functionally related to the development of granulomatous pathology. When ODC was inhibited in infected mice, fibrosis and granuloma size increased significantly (Fig. 8). We hypothesize that by blocking ODC, competition between ODC and OAT for
t-ornithine is eliminated. This would favor the utilization of t-ornithine by OAT, thereby increasing the synthesis of prolines, which could explain the increase in collagen deposition (Fig. 7). Early studies of schistosomiasis pathology showed that liver fibrosis is directly influenced by the availability of proline (41, 42). We now provide a mechanistic explanation for these findings by showing a direct connection between the immune response and the arginine metabolic pathways. As our data clearly demonstrate, IL-4/IFN-γ activated macrophages are an important source of proline (Fig. 9), and by regulating the availability of this amino acid, macrophages may act as key regulators of the fibrotic reaction. Nevertheless, at this time we cannot exclude the possible contributions of other proline-expressing cells.

Type 2 cytokine-controlled arginase activity may also serve to counter-regulate the tissue-destructive potential of excessive NO production (8, 43–45). In some situations it may also promote the survival of pathogens by limiting the ability of activated macrophages to kill invading organisms (46). Macrophages pretreated with IL-4 or IL-13 become refractory to IFN-γ, which suggests that substrate competition is the primary explanation for the inability of type 2 cytokine-primed macrophages to produce NO. Strikingly, excess arginase can restore NO production and killing of Toxoplasma gondii by IFN-γ-activated IL-4-pretreated cells (45). Thus, cytokine-mediated regulation of Arg-1 expression might be an important mechanism to effect the function of classically activated macrophages and prevent NO-mediated immunopathology (47–49).

Extrahepatic arginase activity has also been observed during wound healing (50). Alternatively activated macrophages can support the healing process by providing proline (Fig. 9), which is incapable of producing NO due to substrate depletion mediated by arginase (8, 45). In these macrophages, NOS-2 expression is up-regulated normally in response to IFN-γ, which suggests that substrate competition is the primary explanation for the inability of type 2 cytokine-primed macrophages to produce NO. Strikingly, excess arginase can restore NO production and killing of Toxoplasma gondii by IFN-γ-activated IL-4-pretreated cells (45). Thus, cytokine-mediated regulation of Arg-1 expression might be an important mechanism to effect the function of classically activated macrophages and prevent NO-mediated immunopathology (47–49).

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