

Inhibition of Arginase I Activity by RNA Interference Attenuates IL-13-Induced Airways Hyperresponsiveness¹

Ming Yang,*[¶] Danny Rangasamy,* Klaus I. Matthaei,* Ailsa J. Frew,* Nives Zimmermann,[‡] Suresh Mahalingam,* Dianne C. Webb,* David J. Tremethick,* Philip J. Thompson,[§] Simon P. Hogan,* Marc E. Rothenberg,[‡] William B. Cowden,[†] and Paul S. Foster^{2,*¶}

Increased arginase I activity is associated with allergic disorders such as asthma. How arginase I contributes to and is regulated by allergic inflammatory processes remains unknown. CD4⁺ Th2 lymphocytes (Th2 cells) and IL-13 are two crucial immune regulators that use STAT6-dependent pathways to induce allergic airways inflammation and enhanced airways responsiveness to spasmogens (airways hyperresponsiveness (AHR)). This pathway is also used to activate arginase I in isolated cells and in hepatic infection with helminths. In the present study, we show that arginase I expression is also regulated in the lung in a STAT6-dependent manner by Th2-induced allergic inflammation or by IL-13 alone. IL-13-induced expression of arginase I correlated directly with increased synthesis of urea and with reduced synthesis of NO. Expression of arginase I, but not eosinophilia or mucus hypersecretion, temporally correlated with the development, persistence, and resolution of IL-13-induced AHR. Pharmacological supplementation with L-arginine or with NO donors amplified or attenuated IL-13-induced AHR, respectively. Moreover, inducing loss of function of arginase I specifically in the lung by using RNA interference abrogated the development of IL-13-induced AHR. These data suggest an important role for metabolism of L-arginine by arginase I in the modulation of IL-13-induced AHR and identify a potential pathway distal to cytokine receptor interactions for the control of IL-13-mediated bronchoconstriction in asthma. *The Journal of Immunology*, 2006, 177: 5595–5603.

Recently, we have characterized the transcriptome of two phenotypically similar models of experimental asthma induced by systemic sensitization and subsequent aeroallergen challenge with OVA or by direct inhalation of *Aspergillus fumigatus* (1). Notably, genes related to the metabolism of basic amino acids, specifically the cationic amino acid transporter 2 and arginase I and arginase II, were prominent among the asthma signature genes in both models (1). An important role for extrahepatic activity of arginase I is now recognized for the regulation of aspects of immunological processes, in particular the modulation of T cell function (e.g., T cell proliferation and expression of TCR

ζ-chain) and participation in Th2 immunity (2–4). We have also shown that arginase I activity is up-regulated in the respiratory epithelium of allergic asthmatics, in mononuclear cells with macrophage morphology, and in some granulocytes isolated in the bronchoalveolar lavage fluid (BALF)³ (1). Although we have linked increased arginase I activity with allergic inflammation of the lungs of both mice and of human asthmatics, the contribution of this pathway to disease processes remains unknown.

L-arginine is the common substrate for inducible NO synthase (iNOS) and arginase I (4, 5) and is catabolized to promote the production of NO or a range of second messengers by both of these enzymes, respectively. Recently, iNOS and arginase I activities were shown to be differentially modulated by CD4⁺ Th type 1 lymphocytes (Th1 cells) and Th2 cells and their cytokines (2, 6). Th1 cytokines (e.g., IFN-γ) activate iNOS (2), whereas Th2 cytokines (e.g., IL-13) promote arginase I activity through substrate depletion in a STAT6-dependent manner (7). Differential regulation of iNOS and arginase I activity by Th type 1 and 2 cytokines has also been observed in mice infected with *Schistosoma mansoni* and is a critical determinant of the pathogenic outcome (2). Th2 cells and cytokines also play a central role in the development and expression of allergic inflammation of the airways disorder, asthma, in a STAT6-dependent manner (8). Thus, the regulation of the metabolic fate of L-arginine by IL-13 and during Th2-dominated inflammatory responses (hepatic infection with *S. mansoni*) suggests that IL-13 and Th2 cells may also operate the arginase I pathway through STAT6 to regulate disease processes induced by allergic inflammation.

*Division of Molecular Biosciences and [†]Division of Immunology and Genetics, The John Curtin School of Medical Research, Australian National University, Canberra, Australia; [‡]Division of Allergy and Immunology, Department of Pediatrics, Children's Hospital Medical Center, University of Cincinnati College of Medicine, Cincinnati, OH 45229; [§]Centre for Asthma, Allergy and Respiratory Research, University of Western Australia, Perth, Western Australia; and [¶]Asthma, Allergy, and Inflammation Research Center, School of Biomedical Sciences, Faculty of Health, University of Newcastle and Hunter Medical Research Institute, Callaghan, New South Wales, Australia

Received for publication November 23, 2005. Accepted for publication July 21, 2006.

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 National Health Medical Research Council (NHMRC) (Australia) Program Grant 224207, a NHMRC P.C. Doherty Postdoctoral Fellowship (to S.M. and D.C.W.), an Australian International Postgraduate Research Award (to M.Y.), a University of Newcastle Postdoctoral Fellowship Scheme (to M.Y.), a Clive and Vera Ramaciotti Foundation Establishment Grant (to M.Y.), National Institutes of Health Grant R01 AI1053479 (to M.E.R.), the Human Frontier Science Program (to M.E.R. and P.S.F.), and the Commonwealths Cooperative Research Center for Asthma and Airways (to M.Y. and P.S.F.).

² Address correspondence and reprint requests to Dr. Paul S. Foster, Asthma, Allergy, and Inflammation Research Center, David Maddison Building, Royal Newcastle Hospital, School of Biomedical Sciences, Faculty of Health, University of Newcastle and Hunter Medical Research Institute, Corner of King and Watt Streets, Callaghan, New South Wales, 2300, Australia. E-mail address: Paul.Foster@newcastle.edu.au

³ Abbreviations used in this paper: BALF, bronchoalveolar lavage fluid; iNOS, inducible NO synthase; AHR, airways hyperresponsiveness; RNAi, RNA interference; WT, wild type; DETA, diethylenetriamine; RNI, reactive nitrogen intermediate; shRNA, short hairpin RNA; PDM, peritoneal-derived macrophage; Penh, enhanced pause.

Of the Th2 cytokines regulating allergic airways disease, IL-13 plays a critical role in the development of airways hyperresponsiveness (AHR) (9, 10). The mechanisms underlying the development of AHR and diminished airflow in asthma are considered to play central roles in disease pathogenesis as these underpin bronchoconstriction (8, 11, 12). The induction of AHR by IL-13 is dependent on signaling through the IL-4R α -chain (IL-4R α) and STAT6 (13, 14). There is also an important link between STAT6 expression in airway epithelial cells and the ability of IL-13 to induce AHR (15). STAT6 activation in resident pulmonary cells appears to be a critical molecular switch for the development of AHR (15). Airway epithelial cells and pulmonary macrophages are activated by IL-13 (16, 17), and we have previously shown that IL-13 can promote AHR independently of the infiltration of inflammatory cells (into the airways) in a STAT6-dependent manner (14). Collectively, these studies support the concept that IL-13 acts directly on resident pulmonary cells to promote AHR. Although IL-13 plays a critical role in the induction of AHR during allergic inflammation of the lung, the molecular processes used downstream of STAT6 to modulate airways reactivity remain unknown.

The development of AHR has been linked previously to the regulation of L-arginine catabolism by arginase I (18, 19). Diversion of L-arginine away from NO production may have important implications for the regulation of airways responsiveness to contractile agents and in the development of chronic disease. NO is a bronchodilator, which can attenuate the effect of spasmogens (20–23). Furthermore, the downstream metabolic products of L-arginine catabolism by arginase I could potentially affect cellular function in the lung that predisposes to altered airways responsiveness to spasmogens (AHR) and airways obstruction. Arginase I hydrolyzes L-arginine to urea and L-ornithine, the latter metabolite being essential for the production of proline and polyamines, which regulate collagen production and cell proliferation and differentiation, respectively (4, 24). Polyamines also modulate ion channel function and thus cell excitability (25–27). L-Arginine can also promote glutamate (and subsequently the neurotransmitter, γ -aminobutyric acid) production (28, 29).

Although arginase I activity is induced by allergen provocation of the lung in experimental models of asthma and is enhanced in the airways of allergic asthmatics, the factors up-regulating its activity and role in disease pathogenesis remain unknown.

In this investigation, we show that arginase I expression and activity in the lung are regulated by IL-13 and Th2-induced allergic inflammation in a STAT6-dependent manner. Furthermore, we dissected the specific interaction between IL-13 and arginase I for the development of AHR by delivery of this cytokine directly to the airways. The induction of arginase I expression in the lung by IL-13 directly correlated with the development, persistence, and resolution of AHR. Pharmacological manipulation of the iNOS/arginase I axis by supplementation with a NO donor or L-arginine inhibited and amplified IL-13-induced AHR, respectively. Moreover, induction of loss of function of arginase I specifically in the lung by employing RNA interference (RNAi) strategies resulted in attenuation of IL-13-induced AHR. This investigation identifies arginase I as a potential target for the treatment of IL-13-regulated bronchoconstriction in asthma.

Materials and Methods

Mice

Wild-type (WT) or STAT6-deficient (STAT6 $^{-/-}$) BALB/c mice (6–8 wk) were obtained from the Specific Pathogen Free Facility or the Gene Targeting Facility, The John Curtin School of Medical Research, Australian National University. STAT6 $^{-/-}$ mice (30) were backcrossed for 12 generations with the BALB/c strain. Mice were treated according to Australian

National University Animal Welfare guidelines and were housed in an approved containment facility.

Induction of allergic airways disease by OVA sensitization

Mice were sensitized at 6–8 wk of age by i.p. injection of 50 μ g of OVA/1 mg Alhydrogel (CSL) in 200 μ l of 0.9% sterile saline. Nonsensitized mice received 1 mg of Alhydrogel in 200 μ l of 0.9% saline. On days 12, 14, 16, and 18, all groups of mice were aeroallergen challenged with OVA, as described previously (31). Twenty-four hours after the last aeroallergen (OVA) challenge, airways responsiveness to methacholine was measured on tissues and cell samples collected for analyses.

Enumeration of eosinophils and mucus-secreting cells in the airways

Twenty-four hours after the last aeroallergen challenge, lungs were perfused with saline, removed, and fixed in 10% neutral-buffered formalin before sectioning and staining with Carbol's Chromotrope-Hematoxylin or Alcian blue/periodic acid-Schiff to identify eosinophils or mucus-secreting cells, respectively.

Induction of AHR by rIL-13

Mice were anesthetized with an i.v. injection of 100 μ l of Saffan solution (1/4 diluted with PBS) and then the trachea was intubated with a 22-gauge catheter needle, through which murine rIL-13 (provided by Dr. D. Donaldson, Wythe Genetics Institute, Cambridge, MA) (at maximally effective dose: 10 μ g dissolved in 20 μ l of vehicle: 0.1% BSA, BSA/PBS) or control vehicle (0.1% BSA/PBS) was instilled into the airways. Airways responsiveness to β -methacholine (methacholine) (doses of 3.12, 6.25, 12.5, 25, and 50 mg/ml) was measured 12 h after instillation (or as indicated in the text).

Measurement of AHR in unrestrained mice

Airways responsiveness to methacholine was assessed in conscious, unrestrained mice by barometric plethysmography, using apparatus and software supplied by Buxco. This system yields a dimensionless parameter known as enhanced pause (Penh) that reflects changes in waveform of the pressure signal from the plethysmography chamber combined with a timing comparison of early and late expiration. Measurement was performed essentially as we have described previously (14, 32). Notably, we have confirmed in the BALB/c strain that changes in Penh in response to methacholine directly correlate with changes in airway resistance to this spasmogen (our unpublished data). Thus, measuring changes in Penh reflects alterations in resistance and is indicative of enhanced airways responsiveness.

Measurement of airway resistance

Airway reactivity (resistance: Raw) was also measured using a modification of the low-frequency forced oscillation technique as described previously (33, 34).

Mice were anesthetized with 0.1 ml per 10 g of body weight with a mixture containing xylazine (2 mg/ml; Troy Laboratories) and ketamine (40 mg/ml; Parnell). Two-thirds of the dose was given to induce anesthesia, with the remainder given when the animal was attached to the ventilator (Scireq). Additional doses were given each 40 min as required. Mice were ventilated with a tidal volume of 8 ml/kg at a rate of 450 breaths/min, with a positive end-expiratory pressure of 2 cmH₂O using a custom-designed ventilator (flexivent; Scireq). Mice were then allowed to stabilize for 5 min.

Once stabilized, mice were challenged with a saline aerosol followed by increasing concentrations of β -methacholine (Sigma-Aldrich) (5, 10, 20, and 40 mg/ml). Aerosols were generated with an attached nebulizer (Scireq) and delivered to the inspiratory line between the piston and the animal. Each aerosol was delivered for 10 s during which time regular ventilation was maintained. Immediately following aerosol delivery, five measurements of airway reactivity were made at 1-min intervals and Raw determined. Changes in Raw following methacholine challenge were calculated as a percentage increase over saline control (baseline).

Role of L-arginine in IL-13-induced AHR

WT mice were injected i.p. with pharmacological concentrations of L-arginine (200 mg/kg in 100 μ l/PBS) or the same volume of PBS every 12 h for 4 consecutive days. High concentrations of L-arginine are required to modulate extrahepatic arginase I activity as the liver rapidly sequesters this amino acid from the circulation. On day 3, IL-13 (1 μ g/20 μ l of 0.1% BSA/PBS) or control vehicle (0.1% BSA/PBS) was instilled into the trachea as described above. For these experiments, IL-13 was used at 1 μ g/20

μl (50% of maximal response) instead of 10 μg (maximal effective dose). AHR was measured at 12 h to observe if L-arginase could potentiate the development of AHR.

Treatment with the NO donor 1-hydroxy-2-oxo-3,3-bis(2-aminoethyl)-1-triazene or diethylenetriamine (DETA)/NO adduct suppresses IL-13-induced AHR

DETA/NO was prepared in an identical fashion to that described by Hrabie et al. (35). Vestiges of solvent were removed in vacuo and the material stored under a nitrogen atmosphere at -20°C until use. Mice were treated with 10 μg of IL-13/20 μl of 0.1% BSA/PBS (maximal effective dose) or control vehicle (0.1% BSA/PBS) and airways responsiveness to methacholine measured 48 h later. In some experiments, IL-13-treated or saline control mice were also injected i.p. with DETA/NO (100 mg/kg in saline) 5 min before exposure to methacholine. The control mice for the DETA/NO-treated mice received acetate-buffered DETA (metabolite of DETA/NO) at a dose of 63 mg/kg. DETA/NO is degraded rapidly to liberate NO and DETA (35). These experiments were conducted 48 h after IL-13 treatment, where the effect of IL-13 on lung responsiveness to methacholine was maximal, to determine whether pharmacological supplementation with systemic NO could suppress AHR.

Measurement of arginase activity

Arginase activity was measured as described previously (36). Briefly, lungs were excised and cells lysed with 0.5 ml of 0.1% Triton X-100. After 30 min, 0.5 ml of buffer (25 mM Tris-HCl and 5 mM MnCl_2 (pH 7.4)) was added to the lysate. Arginase was then activated by heating the cell suspension for 10 min at 56°C . L-Arginine hydrolysis was conducted by incubating 25 μl of the activated lysate with 25 μl of 0.5 M L-arginine (pH 9.7) at 37°C for 60 min. The reaction was stopped with 400 μl of an acidic mixture (H_2SO_4 , H_3PO_4 , and H_2O ; 1:3:7 v/v). Urea was measured at 540 nm after addition of 25 μl of 9% α -isonitrosopropiophenone (dissolved in 100% ethanol) and heating at 100°C for 45 min to quantify arginase activity.

Assay of reactive nitrogen intermediates (RNI; nitrite and nitrate)

Levels of RNI from serum or BALF were determined by Griess reagent assay as described previously (37).

Construction of the plasmid vector for the expression short hairpin RNA (shRNA) under the control of the murine U6 promoter to silence function of arginase I

TOPO TA vector (Invitrogen Life Technologies) was used to construct the plasmid vector for the expression shRNA under the control of the murine U6 promoter (Fig. 1). The murine U6 promoter was cloned from genomic DNA, flanked with *Bam*HI at the 5' end, and cloned in front of the 19-nt sense sequence, which was separated by a short hairpin spacer (loop) from the reverse complement of the same sequence (antisense). Six thymidines (T6) were used as the termination signal. Two plasmids expressing shRNA were generated, arginase I short hairpin (AHP1) and AHP2, to specifically target arginase I expression. The oligonucleotides for AHP1 and AHP2 were synthesized chemically (Prologo) and flanked with *Eco*RI at the 3' end. The oligonucleotides were annealed and ligated downstream of the U6 promoter. All constructs were sequenced to confirm identity.

The oligonucleotide sequences are as follows: shRNA1 (AHP1), sense strand, 5'-CCAAAGTCCTTAGAGATTATTCAAGAGATAATCTCTAA GGACTTTGGTTTGGTGAATTC, and antisense strand, 5'-GAATTC AAAAAACCAAGTCCTTAGAGATTATCTCTTGAATAATCTCTAAG GACTTTGG; and shRNA2 (AHP2), sense strand, 5'-CGATTC ACCTGAGCTTTGATTCAAGAGATCAAAGCTCAGGTGAATCGT TTTTGAATTC, and antisense strand, 5'-GAATTCAAAAACGATT CACCTGAGCTTTGATCTCTTGAATCAAAGCTCAGGTGAATCG.

Isolation, culture, and in vitro transfection of macrophages with shRNA

Brewer's thioglycolate (3 ml of 4% solution) (Difco) was injected into the peritoneum of BALB/c mice, and peritoneal-derived macrophage (PDM) were then isolated 3 days later by washing the cavity with PBS (3 ml). Residual erythrocytes were eliminated with red cell lysis buffer as previously described (31) and PDM plated at 5×10^5 cells/well in 6-well plates with complete RPMI 1640 medium. After 6 h, nonadherent cells were washed away with PBS, and adherent cells were allowed to recover in fresh complete RPMI 1640 medium. Over 99% of adherent cells were macrophages as determined by FCS scanning. PDM were cultured for 4 days before being used in transfection experiments. PDM were transfected with

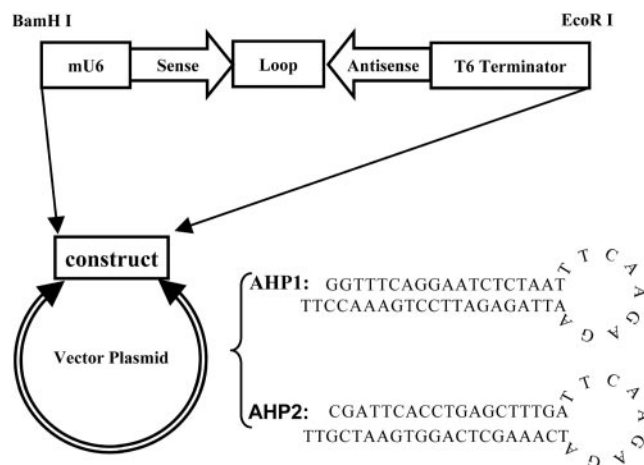


FIGURE 1. A vector-based strategy for the expression of shRNA to induce loss of function of arginase I. The murine U6 promoter was cloned and annealed in front of a 19-nt sense sequence, which was separated by a short-hairpin-spacer (loop) from the reverse complement of the same sequence (antisense sequence). Six thymidines (T6 terminator) were used as the termination signal. Two plasmids expressing shRNA were constructed, AHP1 and AHP2, to target arginase I expression. AHP2 was effective in silencing expression of arginase I, whereas AHP1 was not. Thus, AHP1 was subsequently used as a specific control for shRNA interference by AHP2. The predicted structures of shRNA are depicted.

500 ng of either plasmid containing the shRNA construct or a blank plasmid, with the transfection reagent ExGen500 (Fermentas). ExGen500/DNA complexes were administered in 5% w/v glucose in a total volume of 50 μl . IL-13 was added to the culture medium at a final concentration of 50 ng/ml 8 h after transfection. After IL-13 stimulation for 12 h, PDM were recovered and frozen at -70°C until RT-PCR and Western blot analysis was performed. We used ExGen500 in our studies because this compound belongs to an efficient new class of nonviral, nonliposomal gene delivery reagents (38, 39). ExGen500 has been shown to efficiently deliver DNA to various organs and tissues via i.v. and intratracheal administration and by direct injection into tissues (40–42). Importantly, ExGen500 has shown no or minimal toxicity in all in vivo systems tested to date (39–42).

Transfection of lung with shRNA

shRNA or control vectors (25 μg of plasmid vector) were complexed with ExGen500 and 5% w/v glucose, and a total volume of 50 μl was instilled into the trachea (and again 24 h later) through a 24-gauge catheter following the manufacturer's instructions. IL-13 was delivered to the lungs (as described above) 3 h after the last delivery of shRNA or control vectors. Lungs were then collected and frozen at -70°C for analysis of loss of function of arginase I activity.

Quantitative PCR

The method for quantitative PCR has been described in detail elsewhere (43–45). Simply, RNA was prepared from cells or tissue using the TRIzol RNA isolation buffer following the protocol suggested by the manufacturer (Invitrogen Life Technologies). cDNA was synthesized by an oligo(dT) primed reverse transcriptase reaction using 0.5 μg of RNA from each sample. Quantitative PCR was performed in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems) using murine arginase I (forward, GGTCACCTGACCTATGTGT, and reverse, ACGATGCTTTGG CAGATATGC), arginase II (forward, CTGCCATTCGAGAAGCTGG, and reverse, GGGATCATCTTGTGGGACATTAG), iNOS (forward, ACATCGACCCGTCACAGTAT, and reverse, CAGAGGGGTAGGCT TGTCTC) and GAPDH (forward, CAGGTTGCTCTCCTGGCAGTT, and reverse, CCCTGTTGCTGTAGCCGTA). SYBR-green was used to detect changes in amplicon levels with each sequential amplification cycle. The fluorescence intensity was normalized to the rhodamine derivative ROX as a passive reference label, which was present in the buffer solution. The level of mRNA, relative to GAPDH, was calculated using the formula: relative mRNA expression = $2^{-(\text{Ct of sample} - \text{Ct of GAPDH})} \times 10^6$, where Ct is the threshold cycle value. Since the levels of target mRNA were considerably lower than the level of GAPDH, all mRNA values shown were

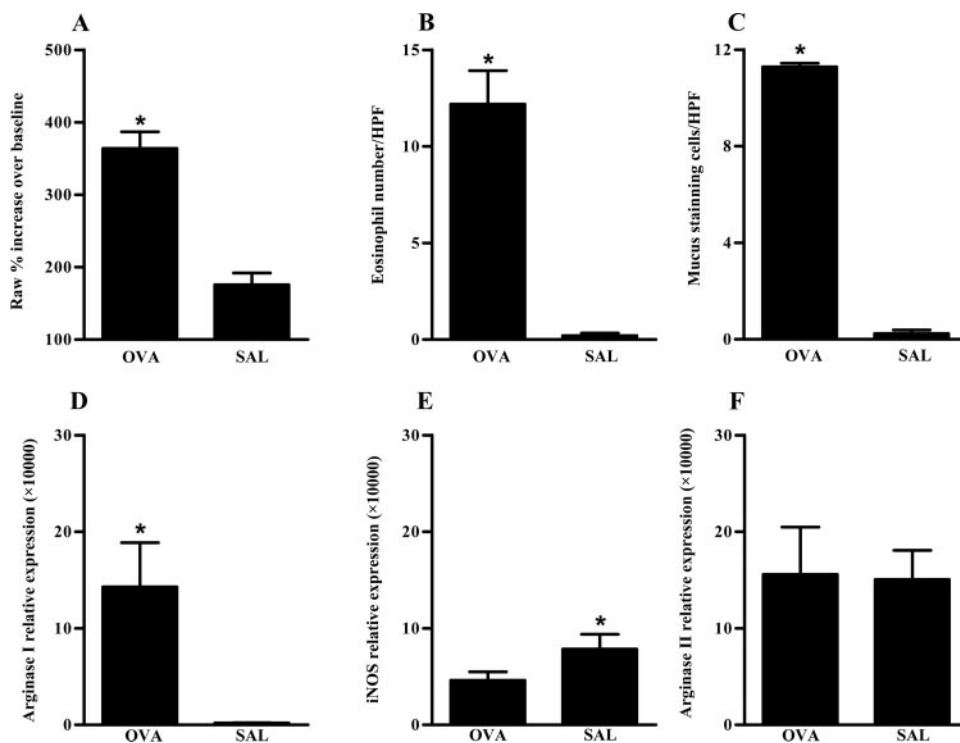


FIGURE 2. Induction of allergic airways inflammation and AHR correlates with increased expression of transcripts encoding arginase I. Levels of airways reactivity to methacholine, expression of arginase I, arginase II, and iNOS and numbers of eosinophils and mucus-staining cells in the lungs of saline (SAL)/OVA- and OVA/OVA-treated WT mice. *A*, Airways reactivity (resistance: Raw) to methacholine. Data represent the percentage increase in Raw over baseline reactivity in the absence of cholinergic stimuli. The maximal response to methacholine (40 mg/ml) is shown, but these results are representative of the full-dose-response curve (5, 10, 20, and 40 mg/ml methacholine). Numbers of peribronchial/perivascular eosinophils (*B*) and mucus-secreting cells (*C*) in the central bronchial epithelium regions. Cells were enumerated in 7–10 similar high-powered fields ($\times 40$ magnification) per mouse. Increased expression of transcripts encoding arginase I (*D*), reduced expression of transcripts encoding iNOS (*E*), and expression of transcripts encoding arginase II (*F*) in the lungs of OVA/OVA-treated mice. Gene expression levels were measured as described in *Materials and Methods*. Data are the mean $\pm$ the SEM for four to eight mice per group. *, Significant differences ($p < 0.05$) in means between OVA/OVA and SAL/OVA groups are shown.

arbitrarily multiplied by 10^6 and normalized against the background level detected in naive lung tissue.

SDS-PAGE and Western blot

Cells and lungs were harvested at the indicated time point, washed twice with cold PBS, and lysed in TNT lysis buffer (20 mM Tris, 200 mM NaCl, and 1% Triton X-100) plus protease inhibitors (mixture solution; Roche). After 20 min on ice, lysates were centrifuged at $15,000 \times g$ for 10 min to remove insoluble material. Protein concentrations were determined by us-

ing the bicinchoninic acid assay (Pierce). Twenty micrograms of lysate was separated on 12% SDS/PAGE gels and transferred to a nitrocellulose membrane (Pall) using a Multiphor Novablot semidry transfer system (Amersham Biosciences). The membrane was blocked with 2% BSA in TBST (Tris-buffered saline, 0.05% Tween 20) and probed with a 1/1000 dilution of goat anti-mouse arginase I (Santa Cruz Biotechnology), followed by an incubation with a detection Ab conjugated to HRP (DakoCytomation). Western blots were developed by using ECL (Amersham Biosciences).

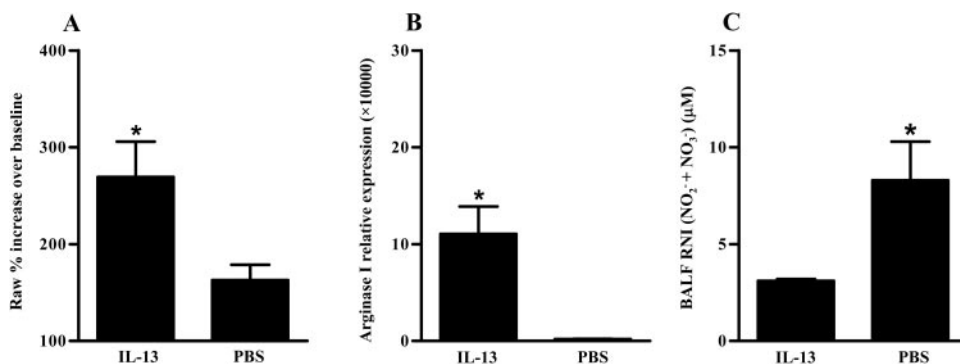


FIGURE 3. IL-13 induces AHR that correlates with arginase I expression and a reduction in the level NO metabolites in the lung. Instillation of IL-13 ($10 \mu\text{g}/20 \mu\text{l}$ 0.1% BSA/PBS per mouse) induced AHR to methacholine (*A*), increased arginase I expression in the lung (*B*), and reduced levels of NO metabolites in the BALF (*C*). Data represent the percentage increase in Raw over baseline reactivity in the absence of cholinergic stimuli. The maximal response to methacholine (40 mg/ml) is shown, but these results are representative of the full-dose-response curve (5–40 mg/ml methacholine). Gene expression and RNI levels were measured as described in *Materials and Methods*. Responses were measured 12 h after instillation of IL-13. Data are the mean $\pm$ the SEM of four to eight mice per group. *, Significant differences ($p < 0.05$) in means between IL-13- and PBS-treated groups are shown.

Statistical analysis

The significance of differences between experimental groups was analyzed using Student's unpaired *t* test. Values were reported as the mean $\pm$ SEM. Differences in mean values were considered significant if $p < 0.05$.

Results

Allergic airways inflammation induces arginase I expression in the lung

Delivery of OVA to the lung (aeroallergen challenge) of OVA-sensitized mice resulted in the induction allergic airways inflammation dominated by AHR, eosinophilia, and mucus hypersecretion (Fig. 2, A–C). Aeroallergen challenge and the development of allergic airways inflammation directly correlated with increased expression of arginase I and with a concomitant decrease in the expression of iNOS (Fig. 2, D and E). Increased expression of arginase I in response to aeroallergen challenge also correlated with increased activity (urea formed nmol/min/mg; saline (Sal) 10 ± 4 ; OVA 118 ± 20 , mean $\pm$ SEM, $n = 4$ mice/group, $p < 0.05$) indicating active catabolism of L-arginine by arginase I in the inflamed lung. Arginase II expression was not enhanced by the induction of allergic airways diseases (Fig. 2F). Although we observed a slight reduction in the relative expression of iNOS, we could not detect any measurable change in the level of RNI, nitrites and nitrates (by-products of NO production), in the BALF or serum after OVA treatment by comparison to saline-treated mice (data not shown). These data support the concept that Th2-dominated inflammatory responses shift the balance of L-arginine metabolism away from NO production by altering the expression and activity of arginase I (2).

The role of IL-13 in regulating arginase I activity in the lung

Next, we determined if IL-13 alone was sufficient for the induction of AHR and arginase I activity by delivery of this cytokine directly to the lung. IL-13-induced AHR (Fig. 3A), concomitant with promoting increased expression of arginase I activity (Fig. 3B) and enhanced production of urea (urea formed nmol/min/mg; Sal 15 ± 2.0 ; OVA 103 ± 8.4 , mean $\pm$ SEM, $n = 4$ mice/group, $p < 0.05$). IL-13 administration resulted in decreased iNOS activity as the level of RNI in the lung were significantly reduced (Fig. 3C). Notably, alterations in the expression of transcripts encoding arginase I and iNOS induced in response to allergic airways inflammation or IL-13 were also critically dependent on STAT6 (results not shown). Thus, the development of AHR and activation of arginase I activity in response to allergen provocation or IL-13 are both intimately linked via STAT6.

Development, persistence and resolution of IL-13-induced AHR directly correlate with enhanced expression of arginase I

To create an additional link between arginase I activity and the regulation of IL-13-induced AHR, we correlated expression with the development, persistence, and resolution of AHR over time. IL-13-induced AHR developed within 12 h and persisted for 96 h (data not shown) but was resolved by 8 days (Fig. 4A). Enhanced expression of arginase I, but not arginase II, directly correlated with the presence of AHR (Fig. 4, B and E). By contrast, the recruitment of eosinophils and induction of mucus production persisted after the resolution of AHR (Fig. 4, C and D). These data show a direct temporal alignment between arginase I activity and the development and resolution of AHR.

Pharmacological modulation with L-arginine or the NO donor DETA/NO modulates IL-13-induced AHR

As an additional link between arginase I and NO and the regulation of IL-13-induced AHR, we treated mice with L-arginine or the NO

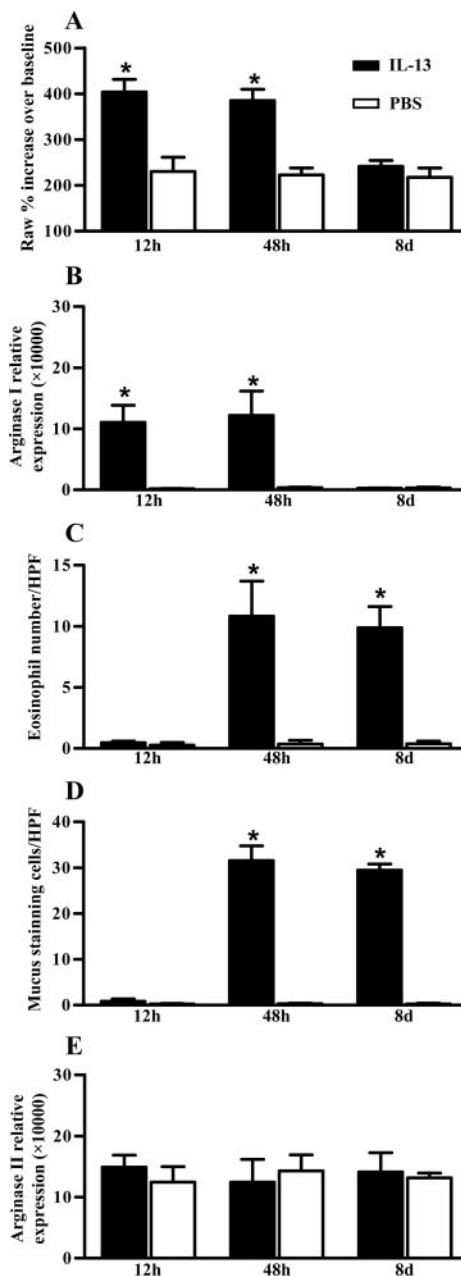


FIGURE 4. Development, persistence, and resolution of IL-13-induced AHR directly correlate with enhanced expression of transcripts encoding arginase I. IL-13 was delivered to the airways, and the effects on development of AHR (A), arginase I expression (B), eosinophil infiltration (C), mucus hypersecretion (D), and arginase II expression (E) were determined over time. AHR developed within 12 h and persisted for 96 h (data not shown). Development and persistence of AHR temporally correlated with arginase I (but not arginase II) expression. By contrast, eosinophil infiltration and increased mucus hypersecretion occurred only after 12 h (by 24 h, data not shown) and persisted for 8 days. Data represent the percentage increase in Raw over baseline reactivity in the absence of cholinergic stimuli. The maximal response to methacholine (40 mg/ml) is shown, but these results are representative of the full-dose-response curve (5–40 mg/ml methacholine). Gene expression data and eosinophil and mucus-secreting cell numbers were measured as described in *Materials and Methods*. Responses were measured at various time points after the instillation of IL-13 as indicated. Data are means $\pm$ the SEM of four to eight mice per group. *, Significant differences ($p < 0.05$) in means between IL-13- and PBS-treated groups are shown.

donor, DETA/NO, before exposure to methacholine. L-Arginine treatment amplified the development of IL-13-induced AHR (Fig. 5A). However, supplementation did not affect baseline responsiveness to methacholine, specifically linking L-arginine metabolism with the mechanism of IL-13-induced AHR to this spasmogen.

The effect of IL-13 on methacholine responsiveness was maximal 48 h after treatment (Fig. 5B). Exposure of mice to the NO donor DETA/NO significantly suppressed IL-13-induced AHR, and responses were similar to those observed in unexposed controls (PBS-treated mice) (Fig. 5B). Thus, both L-arginine and NO can pharmacologically modulate IL-13-induced AHR to methacholine, strongly implicating the iNOS/arginase I axis in the mechanism underlying enhanced airways responsiveness.

shRNAi AHP2 specifically inhibits IL-13-induced arginase I expression and protein production in isolated PDM

We generated two constructs based on the arginase I gene sequence designed to express shRNA that may result in loss of function of arginase I (AHP1 and AHP2). To examine the potential effect of AHP1 and AHP2 on arginase I expression, we initially transfected PDM with the respective vector in the presence of IL-13 (Fig. 6). As we observed with lung (Fig. 4), treatment of PDM with IL-13 induced the expression of arginase I but not arginase II. Arginase II was constitutively expressed in contrast to

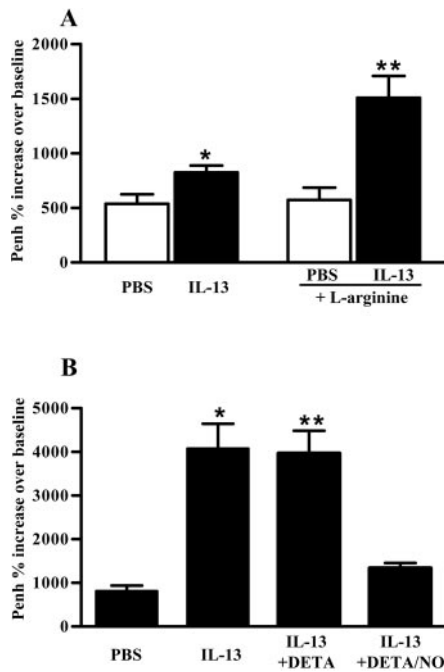


FIGURE 5. Supplementation of mice with L-arginine (200 mg/kg in 100 μ l/PBS every 12 h for 4 consecutive days) or DETA/NO (100 mg/kg in saline) modulates IL-13-induced AHR. IL-13 was instilled into the airways of mice that were pretreated with L-arginine (AHR measured 12 h after IL-13 instillation) or the NO donor DETA/NO (AHR measured 48 h after IL-13 instillation) as described in *Materials and Methods*. DETA (metabolites of DETA/NO) acts as control of DETA/NO. **A**, IL-13-induced AHR was significantly amplified by L-arginine treatment. **B**, IL-13-induced AHR was significantly attenuated by exposure to the NO donor, DETA/NO. Data are means of four to eight mice per group. **A**, **, Significant differences ($p < 0.05$) between IL-13 + L-arginine and IL-13 alone. *, Significant differences ($p < 0.05$) between IL-13 treatment and control groups. **B**, **, Significant differences ($p < 0.05$) between IL-13 + DETA/NO and IL-13 DETA. *, Significant differences ($p < 0.05$) between IL-13 alone and PBS. The IL-13 + DETA/NO group was not significantly different from the PBS group.

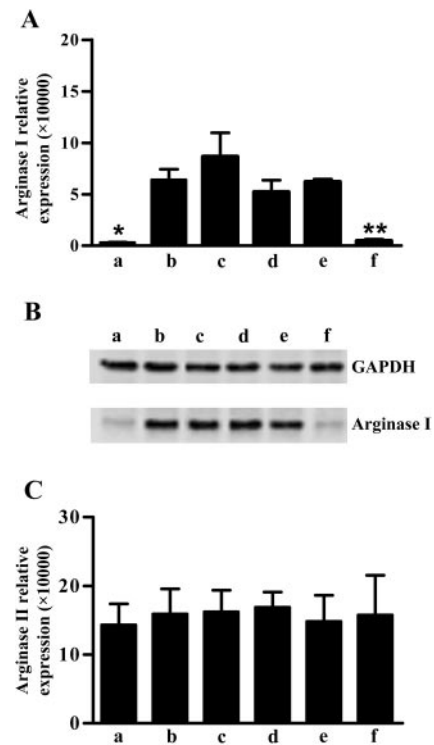


FIGURE 6. The shRNA, AHP2, induces loss of arginase I but not arginase II function in isolated PDM. The effect of shRNA (AHP1 or AHP2) interference on IL-13-induced arginase I expression (**A**) and protein production (**B**) and arginase II expression (**C**) in IL-13-treated PDM. PDM were transfected with 500 ng of AHP1 or AHP2 using ExGen 500, following the manufacturer's instructions, and then exposed to IL-13 (50 ng/ml, 8 h after transfection). Twelve hours after IL-13 stimulation, PDM were recovered and frozen at -70°C until analysis (treatments: a, PBS; b, IL-13 treated; c, IL-13 treated plus transfection control; d, IL-13 treated plus vector control; e, IL-13 treated plus AHP1; and f, IL-13 treated plus AHP2). Total RNA was extracted using RNeasy *B*, PCR was performed as described in *Materials and Methods*. To detect arginase I protein by Western blot analysis, whole cell extracts were separated on 12% SDS-PAGE. A Western blot with Ab against GAPDH was used as a control. Both PCR and Western blot analysis showed significant silencing of arginase I in response to IL-13 stimulation by AHP2 (f). AHP1 (e) and controls for the transfection reagents (c) or expression vectors (d) did not affect IL-13-induced expression of arginase I or the constitutive expression of arginase II. As a final control for specificity, we show that none of the treatments affected the expression or protein production of GAPDH. **A**, **, Significant differences ($p < 0.05$) between IL-13 + AHP2 (f) and IL-13 (b) and all other control groups (c, d, e). IL-13 + AHP2 was not significantly different from PBS (a). *, Significant differences ($p < 0.05$) between PBS and IL-13 and all other control groups (c, d, e) but not IL-13 + AHP2 (f).

arginase I. Notably, AHP2 but not AHP1 significantly inhibited the expression of transcripts for arginase I. The vectors expressing the shRNAs did not affect arginase II expression. Furthermore, controls for the transfection reagents or expression vectors did not affect IL-13-induced expression of arginase I (Fig. 6) or the constitutive expression of arginase II. Next, we determined if interference of arginase I expression by AHP2 resulted in attenuation of protein production in our IL-13-stimulated PDM. IL-13 stimulation of PDM resulted in increased protein production of arginase I, which was only inhibited by AHP2 treatment (Fig. 6B). As a final control for specificity, we showed that none of the treatments affected the expression and protein production of GAPDH.

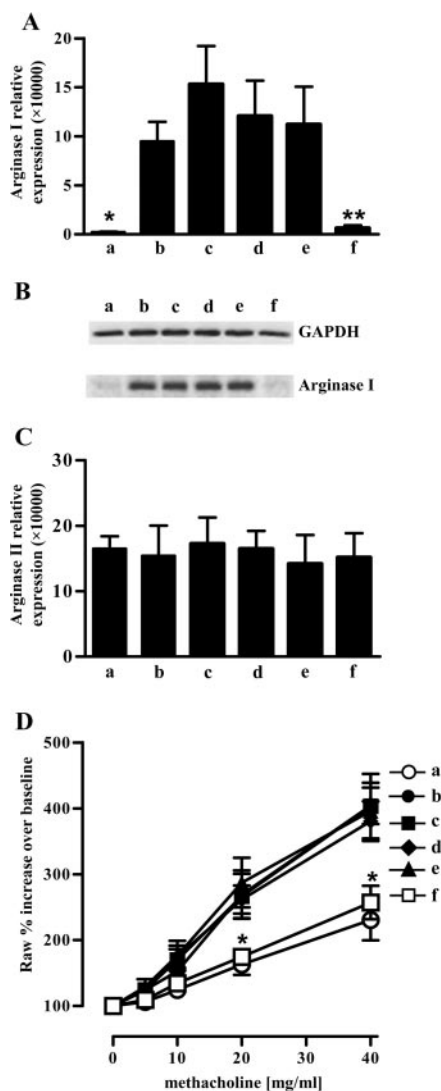


FIGURE 7. shRNA, AHP2, induces loss of arginase I but not arginase II function in the lung and inhibits IL-13-induced AHR. The effect of shRNA (AHP2) interference on IL-13-induced arginase I expression (A) and protein production (B) and arginase II expression (C) and AHR (D). BALB/c mice were transfected with 25 μ g of AHP1 (e) or AHP2 (f) (and again 24 h later by intratracheal administration) using ExGen 500, following the manufacturer's instruction. IL-13 was delivered to the lungs 3 h after the last vector treatment. Twelve hours after IL-13 delivery, airways responsiveness (Raw) to methacholine was measured. (treatments: a, PBS; b, IL-13 treated; c, IL-13 treated plus transfection control; d, IL-13 treated plus vector control; e, IL-13 treated plus AHP1; and f, IL-13 treated plus AHP2). Lungs were then collected and frozen at -70°C . AHP1 is used as a specific control for shRNA interference by AHP2. Data represent the percentage increase in Raw over baseline reactivity in the absence of cholinergic stimuli. PCR and Western blot analysis were performed as described in *Materials and Methods*. Both PCR and Western blot analysis showed that AHP2 significantly silenced arginase I function in the lung in response to IL-13 stimulation. AHP2 (or AHP1) did not alter the expression or protein production (GAPDH) of nonrelated molecules or arginase II. AHP1 and controls for the transfection reagents or expression vectors did not affect IL-13-induced expression of arginase I or the constitutive expression of arginase II. As a final control for specificity, we show that none of the treatments affected the expression or protein production of GAPDH. Significantly, loss of function of arginase I in the lung by treatment with AHP2 resulted in a marked attenuation of IL-13-induced AHR to methacholine. Data are the mean $\pm$ SEM of six to eight mice per group. A, **, Significant differences ($p < 0.05$) between IL-13 + AHP2 (f) and IL-13 (b) and all other control groups (c, d, e). IL-13 + AHP2 was not significantly different from PBS (a). *, Significant differences ($p < 0.05$)

Treatment of the airways with the shRNAi AHP2 results in loss of function of arginase I and attenuation of IL-13-induced AHR to methacholine

Next, we determined if AHP2 was effective in silencing arginase I function in the lung. Not all designed shRNA has the ability to induce loss of function, which may be due to target mRNA accessibility and individual three-dimensional structure (46, 47). We used AHP1 as a specific control for shRNAi by AHP2. AHP2 specifically inhibited IL-13-induced expression and protein synthesis of arginase I in the lung (Fig. 7, A and B, respectively), which directly correlated with the development of AHR to methacholine (Fig. 7D). AHP2 (or AHP1) did not affect the expression or protein production (GAPDH) of nonrelated molecules (Fig. 7, A and B) or of arginase II (Fig. 7C). Significantly, loss of function of arginase I in the lung by treatment with AHP2 resulted in a marked attenuation of IL-13-induced AHR to methacholine (Fig. 7D). These data in the lung show a direct and specific role for arginase I in the lung in the mechanism underlying IL-13-induced AHR to methacholine.

Discussion

The potency of IL-13 in amplifying pathways that control bronchoconstriction has identified this cytokine as a crucial regulator of airways obstruction and AHR in asthma. In this investigation, we show by specifically inducing loss of function in the lung by RNAi (AHP2) that arginase I plays a critical role in the mechanism whereby IL-13 regulates AHR. Our study also highlights the use of RNAi as a means to induce transient loss of function of target molecules in the airways for the treatment of respiratory disorders.

In our experimental model of asthma, Ag-specific Th2 cells and IL-13 are essential for the development of AHR (31, 48). In the current investigation, we extend previous observations and show that Th2 regulated allergic inflammation in the lung shifts the balance of L-arginine metabolism (iNOS/arginase I axis) away from NO production by enhancing the expression of arginase I. Increased expression of arginase I occurred concomitantly with a decrease in expression of iNOS (Fig. 2). Notably, changes in the expression of these enzymes correlated with the development of AHR (2). Increased expression of arginase I in the lung also correlated with increased activity and formation of urea, indicating active catabolism of L-arginine by arginase I in response to allergen inhalation.

To dissect the interaction between IL-13 and arginase I activation in the lung, we delivered rIL-13 protein directly to the airways (Fig. 3). IL-13-induced AHR and likewise promoted increased activity of arginase I and decreased production of RNI. Increased activation of arginase I in association with a reduction in the level of NO production in the lung suggests that iNOS activity was decreased by substrate depletion (Fig. 3). Although the in vitro kinetics of L-arginine catabolism by iNOS and arginase I appear to normally favor NO production, increased activity of arginase I in intact cells (macrophages, epithelial cells, and at sites of wound healing) can significantly limit the availability of L-arginine for NO synthesis (2, 7, 18, 49, 50). Thus, similar to observations made in the Th2-dominated granuloma models induced by *S. mansoni* egg deposition in the liver (2) and in vitro studies on isolated macrophages (7, 51, 52), IL-13 selectively modulates arginase I

between PBS and IL-13 and all other control groups (c, d, e) but not IL-13+AHP2 (f). D, *, Significant differences ($p < 0.05$) between IL-13 + AHP2 (f) and IL-13 (b) and all other control groups (c, d, e). IL-13 + AHP2 was not significantly different from PBS (a).

expression in the lung by altering the balance of L-arginine catabolism.

Although Th2 cell and IL-13-induced arginase I activity has been linked previously to the degree of pathology in granuloma models, this pathway has not been associated with other IL-13-regulated processes such as AHR. In this study, we confirm the importance of STAT6 for the development of AHR induced by allergic airways inflammation or IL-13 (data not shown). However, we extend previous observations to show that STAT6 also regulates the expression of arginase I in the lung in response to allergen inhalation or IL-13 instillation (data not shown). Furthermore, induction of arginase I expression by IL-13 temporally correlates with the development, persistence, and resolution of AHR. By contrast, other effects mediated by IL-13 such as mucus hypersecretion and eosinophil accumulation are temporally dissociated from the development and persistence of AHR (14). Bronchial hyperreactivity in asthmatics can also occur independently of the development of pulmonary inflammation (53–56). This disassociation of airways inflammation and AHR has been confirmed in animal models of allergen-induced lung disease (57–59). Eosinophilia and mucus hypersecretion (albeit reduced) are also features of allergic airways inflammation in IL-13^{-/-} mice (60, 61); however, AHR is critically dependent on this cytokine. Thus, our data on the development of AHR with rIL-13 mimic that observed in animal models of allergen-induced allergic airway diseases. Furthermore, we have linked the three critical components (Th2 cells, IL-13, and STAT6) for the development of AHR with the activation of arginase I in the lung. However, we cannot exclude the possibility that other, as yet uncharacterized pathways, may also contribute to the mechanism whereby IL-13 through STAT6 mediates AHR in the allergic lung.

However, in models of allergic lung disease STAT6 activity and AHR are intimately linked and regulated by IL-13 (15, 62). To further implicate the regulation of the arginase I in the mechanism of IL-13-induced AHR, we treated mice pharmacologically with L-arginine or the NO donor DETA/NO. By this approach, we could directly determine the effect of L-arginine supplementation on airways reactivity during increased activation and expression of arginase I by IL-13. Furthermore, we could ascertain, through NO supplementation, the potential contribution of iNOS and subsequent NO production on modulating the mechanism whereby IL-13 specifically regulates airways responsiveness. Importantly, treatment of mice with L-arginine did not have an effect on baseline airway reactivity to methacholine (Fig. 5A). However, supplementation of mice with L-arginine potentially amplified IL-13 induced AHR. These data clearly demonstrate that increased substrate availability for arginase I, only at the time of IL-13 exposure, selectively promotes AHR to methacholine, further highlighting the link between these factors for the control of bronchoconstriction. By contrast, treatment of mice with a NO donor (DETA/NO) inhibited the development of IL-13-induced AHR. Thus, NO can play an important protective role against IL-13-induced processes that promote increased airways smooth muscle reactivity. Collectively, these data strongly suggest that the metabolic fate of L-arginine plays a central role in regulating IL-13-induced AHR.

To evaluate the direct contribution of arginase I activity in the lung to IL-13-induced AHR, we induced loss of enzyme function by shRNAi. AHP2 effectively blocked IL-13-induced expression and protein production of arginase I (but did not effect arginase II) in isolated macrophages and in the lung after transfection. Notably, inhibition of arginase I function in the lung resulted in marked attenuation of IL-13-induced AHR, and methacholine responses were reduced to near baseline. A number of products of L-arginine catabolism are cell-signaling molecules that can alter smooth mus-

cle cell excitability (28, 29). Activation of arginase I in the lung may also reduce NO levels, which may promote increased airways reactivity to methacholine (5, 29, 63).

In summary, our investigation shows that Th2-regulated allergic airways inflammation and IL-13 induces alterations in L-arginine metabolism by activating arginase I in the lung in a STAT6-dependent manner. Moreover, we show that IL-13 induces arginase I expression and activity, which promote the development and persistence of AHR to methacholine, a hallmark feature of asthma. These observations may have important implications for the development of new approaches, downstream of Th2 cells and cytokines, to modulate IL-13-regulated bronchial hyperresponsiveness in asthma.

Acknowledgments

We gratefully acknowledge the generous support of Dr. D. Donaldson (Wyeth Genetics Institute, Cambridge, MA).

Disclosures

The authors have no financial conflict of interest.

References

- Zimmermann, N., N. E. King, J. Laporte, M. Yang, A. Mishra, S. M. Pope, E. E. Muntel, D. P. Witte, A. A. Pegg, P. S. Foster, et al. 2003. Dissection of experimental asthma with DNA microarray analysis identifies arginase in asthma pathogenesis. *J. Clin. Invest.* 111: 1863–1874.
- 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: 6533–6544.
- Zabaleta, J., D. J. McGee, A. H. Zea, C. P. Hernandez, P. C. Rodriguez, R. A. Sierra, P. Correa, and A. C. Ochoa. 2004. Helicobacter pylori arginase inhibits T cell proliferation and reduces the expression of the TCR ζ -chain (CD3 ζ). *J. Immunol.* 173: 586–593.
- Bronte, V., and P. Zanovello. 2005. Regulation of immune responses by L-arginine metabolism. *Nat. Rev. Immunol.* 5: 641–654.
- Wiesinger, H. 2001. Arginine metabolism and the synthesis of nitric oxide in the nervous system. *Prog. Neurobiol.* 64: 365–391.
- Munder, M., K. 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: 3771–3777.
- Rutschman, R., R. Lang, M. Hesse, J. N. Ihle, T. A. Wynn, and P. J. Murray. 2001. Cutting edge: Stat6-dependent substrate depletion regulates nitric oxide production. *J. Immunol.* 166: 2173–2177.
- Wills-Karp, M. 1999. Immunologic basis of antigen-induced airway hyperresponsiveness. *Annu. Rev. Immunol.* 17: 255–281.
- Grunig, G., M. Warnock, A. E. Wakil, R. Venkayya, F. Brombacher, D. M. Rennick, D. Sheppard, M. Mohrs, D. D. Donaldson, R. M. Locksley, and D. B. Corry. 1998. Requirement for IL-13 independently of IL-4 in experimental asthma. *Science* 282: 2261–2263.
- Wills-Karp, M., J. Luyimbazi, X. Xu, B. Schofield, T. Y. Neben, C. L. Karp, and D. D. Donaldson. 1998. Interleukin-13: central mediator of allergic asthma. *Science* 282: 2258–2261.
- Wardlaw, A. J., C. E. Brightling, R. Green, G. Woltmann, P. Bradding, and I. D. Pavord. 2002. New insights into the relationship between airway inflammation and asthma. *Clin. Sci.* 103: 201–211.
- Oga, T., K. Nishimura, M. Tsukino, T. Hajiro, S. Sato, A. Ikeda, C. Hamada, and M. Mishima. 2002. Longitudinal changes in airflow limitation and airway hyperresponsiveness in patients with stable asthma. *Ann. Allergy Asthma Immunol.* 89: 619–625.
- Tomkinson, A., C. Duez, G. Cieslewicz, J. C. Pratt, A. Joetham, M. C. Shanafelt, R. Gundel, and E. W. Gelfand. 2001. A murine IL-4 receptor antagonist that inhibits IL-4- and IL-13-induced responses prevents antigen-induced airway eosinophilia and airway hyperresponsiveness. *J. Immunol.* 166: 5792–5800.
- Yang, M., S. P. Hogan, P. J. Henry, K. I. Matthaai, A. N. McKenzie, I. G. Young, M. E. Rothenberg, and P. S. Foster. 2001. Interleukin-13 mediates airways hyperreactivity through the IL-4 receptor α -chain and STAT-6 independently of IL-5 and eotaxin. *Am. J. Respir. Cell Mol. Biol.* 25: 522–530.
- Kuperman, D. A., X. Huang, L. L. Koth, G. H. Chang, G. M. Dolganov, Z. Zhu, J. A. Elias, D. Sheppard, and D. J. Erle. 2002. Direct effects of interleukin-13 on epithelial cells cause airway hyperreactivity and mucus overproduction in asthma. *Nat. Med.* 8: 885–889.
- 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: 7151–7160.
- Matsukura, S., C. Stellato, S. N. Georas, V. Casolaro, J. R. Plitt, K. Miura, S. Kurosawa, U. Schindler, and R. P. Schleimer. 2001. Interleukin-13 upregulates eotaxin expression in airway epithelial cells by a STAT6-dependent mechanism. *Am. J. Respir. Cell Mol. Biol.* 24: 755–761.

18. Meurs, H., H. Maarsingh, and J. Zaagsma. 2003. Arginase and asthma: novel insights into nitric oxide homeostasis and airway hyperresponsiveness. *Trends Pharmacol. Sci.* 24: 450–455.
19. Mori, M., and T. Gotoh. 2004. Arginine metabolic enzymes, nitric oxide and infection. *J. Nutr.* 134(10 Suppl.): 2820S–2825S; discussion 2853S.
20. Dupuy, P. M., S. A. Shore, J. M. Drazen, C. Frostell, W. A. Hill, and W. M. Zapol. 1992. Bronchodilator action of inhaled nitric oxide in guinea pigs. *J. Clin. Invest.* 90: 421–428.
21. Hogman, M., C. Frostell, H. Arnberg, and G. Hedenstierna. 1993. Inhalation of nitric oxide modulates methacholine-induced bronchoconstriction in the rabbit. *Eur. Respir. J.* 6: 177–180.
22. Kacmarek, R. M., R. Ripple, B. A. Cockrill, K. J. Bloch, W. M. Zapol, and D. C. Johnson. 1996. Inhaled nitric oxide: a bronchodilator in mild asthmatics with methacholine-induced bronchospasm. *Am. J. Respir. Crit. Care Med.* 153: 128–135.
23. Brown, R. H., and W. Mitzner. 2003. Airway response to deep inspiration: role of nitric oxide. *Eur. Respir. J.* 22: 57–61.
24. King, N. E., M. E. Rothenberg, and N. Zimmermann. 2004. Arginine in asthma and lung inflammation. *J. Nutr.* 134(10 Suppl.): 2830S–2836S; discussion 2853S.
25. Williams, K. 1997. Interactions of polyamines with ion channels. *Biochem. J.* 325(Pt. 2): 289–297.
26. Rozov, A., Y. Zilberter, L. P. Wollmuth, and N. Burnashev. 1998. Facilitation of currents through rat Ca^{2+} -permeable AMPA receptor channels by activity-dependent relief from polyamine block. *J. Physiol.* 511(Pt. 2): 361–377.
27. Yakehiro, M., Y. Furukawa, T. Koike, E. Kimura, T. Nakajima, K. Yamaoka, and I. Seyama. 2001. Novel mechanism of blocking axonal Na^{+} channels by three macrocyclic polyamine analogues and two spider toxins. *Br. J. Pharmacol.* 132: 63–72.
28. Johnson, J. L., and E. Roberts. 1984. Arginine metabolism in mouse brain synaptosomes. *J. Neurochem.* 42: 1123–1126.
29. Wu, G., and S. M. Morris, Jr. 1998. Arginine metabolism: nitric oxide and beyond. *Biochem. J.* 336 (Pt. 1): 1–17.
30. Akimoto, T., F. Numata, M. Tamura, Y. Takata, N. Higashida, T. Takashi, K. Takeda, and S. Akira. 1998. Abrogation of bronchial eosinophilic inflammation and airway hyperactivity in signal transducers and activators of transcription (STAT)6-deficient mice. *J. Exp. Med.* 187: 1537–1542.
31. Mattes, J., M. Yang, S. Mahalingam, J. Kuehr, D. C. Webb, L. Simson, S. P. Hogan, A. Koskinen, A. N. McKenzie, L. A. Dent, et al. 2002. Intrinsic defect in T cell production of interleukin (IL)-13 in the absence of both IL-5 and eotaxin precludes the development of eosinophilia and airways hyperactivity in experimental asthma. *J. Exp. Med.* 195: 1433–1444.
32. Hamelmann, E., J. Schwarze, K. Takeda, A. Oshiba, G. L. Larsen, C. G. Irvin, and E. W. Gelfand. 1997. Noninvasive measurement of airway responsiveness in allergic mice using barometric plethysmography. *Am. J. Respir. Crit. Care Med.* 156(3 Pt. 1): 766–775.
33. Sly, P. D., M. J. Hayden, F. Petak, and Z. Hantos. 1996. Measurement of low-frequency respiratory impedance in infants. *Am. J. Respir. Crit. Care Med.* 154: 161–166.
34. Pillow, J. J., T. R. Korfhagen, M. Ikegami, and P. D. Sly. 2001. Overexpression of TGF- α increases lung tissue hysteresivity in transgenic mice. *J. Appl. Physiol.* 91: 2730–2734.
35. Hrabie, J. A., J. H. R. Klose, D. A. Wink, and L. K. Keefer. 1993. New nitric oxide-releasing zwitterions derived from polyamines. *J. Org. Chem.* 58: 1472–1476.
36. Corraliza, I. M., M. L. Campo, G. Soler, and M. Modolell. 1994. Determination of arginase activity in macrophages: a micromethod. *J. Immunol. Methods* 174: 231–235.
37. Rockett, K. A., M. M. Awburn, E. J. Rockett, W. B. Cowden, and I. A. Clark. 1994. Possible role of nitric oxide in malarial immunosuppression. *Parasite Immunol.* 16: 243–249.
38. Wiseman, J. W., C. A. Goddard, D. McLelland, and W. H. Colledge. 2003. A comparison of linear and branched polyethylenimine (PEI) with DCChol/DOPE liposomes for gene delivery to epithelial cells in vitro and in vivo. *Gene Ther.* 10: 1654–1662.
39. Li, J. Z., Q. Q. Wang, H. Yu, F. P. Shen, D. Li, and Y. Zheng. 2004. Gene transfer by novel non-viral vector polyethylenimine. *Zhejiang Da Xue Xue Bao Yi Xue Ban.* 33: 229–234.
40. Goula, D., C. Benoist, S. Mantero, G. Merlo, G. Levi, and B. A. Demeneix. 1998. Polyethylenimine-based intravenous delivery of transgenes to mouse lung. *Gene Ther.* 5: 1291–1295.
41. Ferrari, S., A. Pettenazzo, N. Garbati, F. Zaccello, J. P. Behr, and M. Scarpa. 1999. Polyethylenimine shows properties of interest for cystic fibrosis gene therapy. *Biochim. Biophys. Acta* 1447: 219–225.
42. Shi, L., G. P. Tang, S. J. Gao, Y. X. Ma, B. H. Liu, Y. Li, J. M. Zeng, Y. K. Ng, K. W. Leong, and S. Wang. 2003. Repeated intrathecal administration of plasmid DNA complexed with polyethylene glycol-grafted polyethylenimine led to prolonged transgene expression in the spinal cord. *Gene Ther.* 10: 1179–1188.
43. Fehniger, T. A., M. H. Shah, M. J. Turner, J. B. VanDeusen, S. P. Whitman, M. A. Cooper, K. Suzuki, M. Wechsler, F. Goodsaid, and M. A. Caligiuri. 1999. Differential cytokine and chemokine gene expression by human NK cells following activation with IL-18 or IL-15 in combination with IL-12: implications for the innate immune response. *J. Immunol.* 162: 4511–4520.
44. Xia, D., A. Sanders, M. Shah, A. Bickerstaff, and C. Orosz. 2001. Real-time polymerase chain reaction analysis reveals an evolution of cytokine mRNA production in allograft acceptor mice. *Transplantation* 72: 907–914.
45. Choileain, N. N., M. MacConmara, Y. Zang, T. J. Murphy, J. A. Mannick, and J. A. Lederer. 2006. Enhanced regulatory T cell activity is an element of the host response to injury. *J. Immunol.* 176: 225–236.
46. Holen, T., M. Amarzuigui, M. T. Wiiger, E. Babaie, and H. Prydz. 2002. Positional effects of short interfering RNAs targeting the human coagulation trigger tissue factor. *Nucleic Acids Res.* 30: 1757–1766.
47. Kretschmer-Kazemi Far, R., and G. Sczakiel. 2003. The activity of siRNA in mammalian cells is related to structural target accessibility: a comparison with antisense oligonucleotides. *Nucleic Acids Res.* 31: 4417–4424.
48. Zhu, Z., R. J. Homer, Z. Wang, Q. Chen, G. P. Geba, J. Wang, Y. Zhang, and J. A. Elias. 1999. Pulmonary expression of interleukin-13 causes inflammation, mucus hypersecretion, subepithelial fibrosis, physiologic abnormalities, and eotaxin production. *J. Clin. Invest.* 103: 779–788.
49. Wei, L. H., A. T. Jacobs, S. M. Morris, Jr., and L. J. Ignarro. 2000. IL-4 and IL-13 upregulate arginase I expression by cAMP and JAK/STAT6 pathways in vascular smooth muscle cells. *Am. J. Physiol.* 279: C248–C256.
50. 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: 2134–2141.
51. 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 cytokines. *Eur. J. Immunol.* 25: 1101–1104.
52. Munder, M., K. Eichmann, and M. Modolell. 1998. Alternative metabolic states in murine macrophages reflected by the nitric oxide synthase/arginase balance: competitive regulation by CD4⁺ T cells correlates with Th1/Th2 phenotype. *J. Immunol.* 160: 5347–5354.
53. Crimi, E., A. Spanevello, M. Neri, P. W. Ind, G. A. Rossi, and V. Brusasco. 1998. Dissociation between airway inflammation and airway hyperresponsiveness in allergic asthma. *Am. J. Respir. Crit. Care Med.* 157: 4–9.
54. Oddera, S., M. Silvestri, R. Penna, G. Galeazzi, E. Crimi, and G. A. Rossi. 1998. Airway eosinophilic inflammation and bronchial hyperresponsiveness after allergen inhalation challenge in asthma. *Lung* 176: 237–247.
55. Brusasco, V., E. Crimi, and R. Pellegrino. 1998. Airway hyperresponsiveness in asthma: not just a matter of airway inflammation. *Thorax* 53: 992–998.
56. Leuppi, J. D., C. M. Salome, C. R. Jenkins, H. Koskela, J. D. Brannan, S. D. Anderson, M. Andersson, H. K. Chan, and A. J. Woolcock. 2001. Markers of airway inflammation and airway hyperresponsiveness in patients with well-controlled asthma. *Eur. Respir. J.* 18: 444–450.
57. Heuer, H. O., B. Wenz, H. M. Jennewein, and K. Ulrich. 1994. Dissociation of airway responsiveness and bronchoalveolar lavage (BAL) cell composition in sensitized guinea-pigs after daily inhalation of ovalbumin. *Clin. Exp. Allergy* 24: 682–689.
58. Wilder, J. A., D. D. Collie, B. S. Wilson, D. E. Bice, C. R. Lyons, and M. F. Lipscomb. 1999. Dissociation of airway hyperresponsiveness from immunoglobulin E and airway eosinophilia in a murine model of allergic asthma. *Am. J. Respir. Cell Mol. Biol.* 20: 1326–1334.
59. Foster, P. S., Y. Ming, K. I. Matthei, I. G. Young, J. Temelkovski, and R. K. Kumar. 2000. Dissociation of inflammatory and epithelial responses in a murine model of chronic asthma. *Lab. Invest.* 80: 655–662.
60. Webb, D. C., A. N. McKenzie, A. M. Koskinen, M. Yang, J. Mattes, and P. S. Foster. 2000. Integrated signals between IL-13, IL-4, and IL-5 regulate airways hyperactivity. *J. Immunol.* 165: 108–113.
61. Mattes, J., M. Yang, A. Siqueira, K. Clark, J. MacKenzie, A. N. McKenzie, D. C. Webb, K. I. Matthei, and P. S. Foster. 2001. IL-13 induces airways hyperactivity independently of the IL-4R α chain in the allergic lung. *J. Immunol.* 167: 1683–1692.
62. Foster, P. S. 1999. STAT6: an intracellular target for the inhibition of allergic disease. *Clin. Exp. Allergy* 29: 12–16.
63. Akiho, H., P. Blennerhassett, Y. Deng, and S. M. Collins. 2002. Role of IL-4, IL-13, and STAT6 in inflammation-induced hypercontractility of murine smooth muscle cells. *Am. J. Physiol.* 282: G226–G232.