

Alterations in Nitric Oxide and Cytokine Production with Airway Inflammation in the Absence of IL-10¹

Bill T. Ameredes,^{2*†} Ruben Zamora,[‡] Jigme M. Sethi,[†] He-Liang Liu,^{*†} Lauryn K. Kohut,^{*†} Amber L. Gligonic,^{*†} Augustine M. K. Choi,[†] and William J. Calhoun^{*†}

IL-10 is an anti-inflammatory cytokine that suppresses NO synthase (NOS) and production of NO; its lack may promote NO production and alterations in cytokines modulated by NO with allergic airway inflammation (AI), such as IL-18 and IL-4. Therefore, we induced AI in IL-10 knockout ($-/-$) and IL-10-sufficient C57BL/6 (C57) mice with inhaled OVA and measured airway NO production, as exhaled NO (E_{NO}) and bronchoalveolar lavage fluid nitrite levels. E_{NO} and nitrite levels were elevated significantly in naive IL-10 $-/-$ mice as compared with C57 mice. With AI, E_{NO} and nitrite levels increased in C57 mice and decreased in IL-10 $-/-$ mice. IL-18 production fell with both AI and addition of *S*-nitroso-*N*-acetyl-D,L-penicillamine (a NO donor) but was not significantly increased by chemical NOS inhibition by L-N⁵-(1-iminoethyl)-ornithine. IL-4 AI was increased significantly (up to 10-fold greater) in the absence of IL-10 but was reduced significantly with chemical inhibition of NOS. Airway responsiveness was lower in IL-10 $-/-$ mice and was associated with alteration in production of NO and IL-4. Thus, IL-4 production was increased, and likely decreased NO production, in a way not predicted by the absence of IL-10. Inhibition of IL-4 production, with inhibition of NOS in the absence of IL-10, demonstrated the importance of a NO and IL-4 feedback mechanism regulating this interaction. *The Journal of Immunology*, 2005, 175: 1206–1213.

Interleukin-10 is a Th2 cytokine in the IL-4/IL-13 family of cytokines but differs from them in that it has anti-inflammatory and antiproliferative properties (1–3). It is produced by macrophages and other monocyte-type cells with their exposure to proinflammatory Th1 cytokines, including TNF- α (4), typically in response to allergens or proinflammatory mediators. The production of IL-10 in this manner constitutes a negative feedback loop that down-regulates TNF- α (5) and promotes resolution of the inflammatory response (6). However, IL-10 production by airway immune cells of asthmatics has been shown to be reduced (7, 8), which has led to postulation that lack of this anti-inflammatory cytokine may allow allergic proinflammatory mechanisms to dominate, which in turn, may promote development of asthma and possibly airway remodeling.

A significant aspect of these mechanisms may lie in the enhanced production of NO with a lack of IL-10. NO is a critical molecular signal in the inflammatory process, which is associated with asthma, airway inflammation (AI),³ and possibly airway remodeling (9, 10). Furthermore, inducible NO synthase (iNOS), a

key enzyme that produces NO, can be significantly up-regulated by proinflammatory cytokines and is considered a marker of AI in asthma (11). IL-10 is known to suppress NO production through suppression of iNOS activity (12), and its lack has been shown to result in greater NO production by airway immune cells from allergen-provoked mice (13); therefore, a lack of IL-10 may promote production of NO and persistence of AI.

Furthermore, cytokines known to regulate or be regulated by NO may likewise be altered in the absence of IL-10. IL-18 and IL-4 are examples of respective Th1 and Th2 cytokines that fall within the NO regulatory scheme. IL-18 is a Th1-associated cytokine, originally named “IFN- γ -inducing factor” (14), which is down-regulated by NO (15) and may be regulated as a function of differences in NOS activity in the absence of IL-10. Unlike IL-18, IL-4 is a Th2 cytokine, which promotes eosinophil survival and proliferation (16), and is known to reduce NO production by macrophages through inhibition of iNOS activity (17). This effect of IL-4 to down-regulate NO production may occur to quell the inflammation in a negative-feedback fashion as the process moves toward resolution. However, the relationship between IL-4 and NO production with AI in the absence of IL-10 has not been investigated.

Therefore, we studied the relationship between NO and IL-4 in IL-10 knockout ($-/-$) mice. Based on the known suppressive effect of IL-10 on NO production (12), we postulated that IL-10 $-/-$ mice would demonstrate enhanced AI and alterations in the production of NO and NO-associated cytokines IL-18 and IL-4. The results are consistent with this postulate, suggesting that a lack of IL-10 is an important factor in the development of AI and the modulation of NO-related mechanisms. These studies represent the first analysis of the role of the association of NO and IL-4 in noninfective allergic AI and hyperresponsiveness in the absence of IL-10.

Materials and Methods

Animals

Male mice of the C57BL/6 (C57; wild-type), IL-10 $-/-$ strain (on a C57 background) were obtained at 6 wk of age (The Jackson Laboratory) and housed under identical conditions in a specific pathogen-free/barrier animal

*Asthma, Allergy, and Airway Research Center, [†]Division of Pulmonary, Allergy, and Critical Care Medicine, and [‡]Department of Surgery, University of Pittsburgh School of Medicine Pittsburgh, PA 15261

Received for publication August 4, 2004. Accepted for publication May 3, 2005.

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 is supported by grants from the American Respiratory Alliance of West-ern Pennsylvania and National Institutes of Health Grants AI-42365 and HL-63738.

² Address correspondence and reprint requests to Dr. Bill T. Ameredes, Research Assistant Professor of Medicine, Asthma, Allergy, and Airway Research Center, Division of Pulmonary, Allergy, and Critical Care Medicine, University of Pittsburgh School of Medicine, 628NW Montefiore University Hospital, 3459 Fifth Avenue, Pittsburgh, PA 15213. E-mail address: ameredesbt@upmc.edu

³ Abbreviations used in this paper: AI, airway inflammation; iNOS, inducible NO synthase; rm, recombinant murine; E_{NO} , exhaled NO; BAL, bronchoalveolar lavage; L-NIL, L-N⁵-(1-iminoethyl)-lysine; SNAP, *S*-nitroso-*N*-acetyl-D,L-penicillamine; L-NIO, L-N⁵-(1-iminoethyl)-ornithine; AR, airway reactivity; Penh, enhanced pause; MCh, methacholine.

facility at the University of Pittsburgh. The IL-10^{-/-} mice were certified as double-knockout IL-10-null mutants (IL-10^{-/-}), originating from a strain produced by Kuhn et al. (18). All mice were allowed to age to 8–12 wk and were subjected to a series of experimental protocols described below. This maturation window allowed the mice to grow to a sufficient size (18–25g), such that airway cell recovery from each mouse was enhanced. Because of the tendency of IL-10^{-/-} mice to develop enterocolitis with age (18), throughout the study all IL-10^{-/-} mice were monitored routinely for evidence of rectal prolapse and failure to gain weight. Although housing under specific pathogen-free conditions significantly attenuates this tendency (18), any mice demonstrating these symptoms were excluded from analysis. All procedures and protocols used in these studies were approved by the University of Pittsburgh Institutional Animal Care and Use Committee, which conforms to guidelines recommended by the National Institutes of Health and the U.S. Department of Agriculture.

Airway inflammation

The inflammation protocol used to induce mild/moderate AI consisted of two OVA/alum sensitizations (0.5 ml, i.p., 50 µg/ml), on days 0 and 7, followed by three airway challenges with OVA/saline aerosol (10 min, 5 mg/ml, one on day 14 and two on day 15). The aerosol was administered to each group of unanesthetized mice by using a DeVilbiss nebulizer (droplet size < 3.5 µm) connected to an aerosol delivery chamber, specifically designed for mice (CH Technologies). Because of the potential for mild nonspecific aerosol-related airway responses, another group had OVA injected i.p., as described above, followed by aerosol with saline containing no OVA. Typically, the measurements of response to allergen were assessed in sample subsets of mice ($n = 8$ mice/subset) in each of the groups, without replacement, at time intervals of 24, 48, and 72 h after day 15 of aerosol challenge.

IL-10 administration

As previously described (19), recombinant murine (rm)IL-10 (R&D Systems) was administered to a subset of IL-10^{-/-} mice by using a subdermal implantable miniosmotic pump (ALZET) and placed in the dorsal cervicospinal area 3 days before initiation of the OVA aerosol challenge. rmIL-10 was dissolved in a normal saline and delivered at a dose of 125 µg · kg⁻¹ · day⁻¹, which had been shown previously to reduce exhaled NO (E_{NO}) levels in naive IL-10^{-/-} mice (19).

L-N^G-(1-iminoethyl)-lysine (L-NIL) administration

Inhibition of NOS in vivo was achieved by using L-NIL (3 mg/kg, i.p.), injected three days before, and on the first day of OVA aerosol challenge. This dose had been previously demonstrated to reduce E_{NO} levels in naive IL-10^{-/-} mice (19).

Bronchoalveolar lavage (BAL) supernatant and cell isolation

As previously described (13), mice were anesthetized with halothane and killed by rapid cervical dislocation, followed immediately by open-chest BAL using sterile, endotoxin-free normal saline to obtain airway cells from both lungs. The volume of saline used was 0.5 ml for the initial BAL, followed by three 1-ml volumes. The samples were immediately centrifuged (10°C, 450 × *g*), the supernatant was collected and frozen, and the cells were isolated. Cell counts were made on an aliquot of the initial BAL using Diff-Quik staining (Scientific Products), with counts made on 200–300 cells/slide. Resultant cell numbers were expressed as number of cells per body weight of mouse to normalize for size differences among mice. BAL macrophage and eosinophil numbers were used as an index of AI, whereas neutrophils were typically found to comprise ≤1% of all samples and therefore were not systematically analyzed.

BAL cell culture

Seventy-two hours after the last aerosol challenge, BAL was performed as described above. Unfractionated BAL cells were used to expedite processing and to maintain macrophage numbers and viability. This also allowed the airway cell population milieu to remain intact, providing a more physiologic assay of cell signaling and cell contact interactions, present due to the allergic treatment regime. The cells were immediately centrifuged (10°C, 450 × *g*), washed, isolated, and placed in a 96-well polystyrene culture plate with DMEM, 10% FBS, L-glutamine, and penicillin/streptomycin. Medium containing added nitrate, such as RPMI 1640 medium, was avoided to enhance the probability that measured nitrite production solely was due to cellular activity (20). Cell culture plating density was 300,000–500,000 cells/well in a total volume of 250 µl/well. The plate was then placed in a humidified incubator (5% CO₂ at 37°C) for 24 h. The conditioned medium was collected after 24 h and either snap-frozen (–80°C)

and later assayed or assayed immediately after collection. In the first set of experiments, conditioned medium from unstimulated cells and cells treated with LPS (1000 ng/ml) and IFN-γ (100 U/ml) was collected. Duplicate preparations of cells were treated with either the NO donor *S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP; Alexis) or L-N^G-(1-iminoethyl)-ornithine (L-NIO; 80 µM; Alexis), a selective iNOS inhibitor to block production of NO by these cells (21). Thus, exogenous NO was added to cells from non-OVA-treated mice through application of SNAP to approximate the NO production of cells from OVA-treated mice. The amount of SNAP added to each well was that necessary to attain a nitrite concentration of 20 µM/million cells shown previously to approximate maximal concentrations reached by BAL cells from OVA-challenged IL-10^{-/-} mice in culture (13). All samples were assayed in duplicate, with the mean of the duplicates taken as the value for that sample.

IL-18 measurement

IL-18 production by cultured airway cells was measured with a standard ELISA kit for mouse IL-18 (Quantikine M; R&D Systems), both as an index of the alteration in Th1 response to allergen (14) and because IL-18 is down-regulated by NO (15) that is, in turn, significantly down-regulated by IL-10 (12). The sensitivity limit of the assay was 8 pg/ml and was highly specific for mouse IL-18.

IL-4 measurement

IL-4 production by cultured airway cells, and within the BAL fluid, was measured by ELISA (Quantikine M; R&D Systems) as an index of induction of the Th2 response to allergen (22), and because its ability to reduce NO production by macrophages, through inhibition of iNOS activity (17). The kit relied upon a mAb reaction specific for mouse IL-4, which was evaluated using a standard curve. The sensitivity of the assay was <2 pg/ml and was highly specific for mouse IL-4, with no significant cross-reactivity with IL-10.

Nitrite measurement

Nitrite levels in the initial BAL fluid sample were measured as an index of NOS activity, using a modification of the standard Griess assay, as described previously (13, 23). The resultant colorimetric reaction was assessed using a plate reader that converted OD values to concentrations of nitrite. A standard curve was produced for known nitrite concentrations from 0.5 to 20 µM, which was linear for concentrations ≥ 1 µM and for which the lower limit of reliable detection fell between 0.5 and 1 µM. Therefore, sample values < 1 µM were considered to be beneath the limit of detectability of the assay. Final nitrite values were expressed as a function of body weight to normalize BAL nitrite production across mice of varying sizes.

E_{NO} production

E_{NO} production in unanesthetized mice was measured using a modification of a technique similar to that of Weicker et al. (24), as described previously (19). Briefly, mice were placed individually within a closed Plexiglas chamber with a low volume (~600 ml; Buxco Electronics) and allowed to breathe freely for a period of time during which the fractional CO₂ content of chamber gas attained 5–5.5%. A small sample of the chamber gas (≤85 ml) was aspirated (20 s, 250 ml/min) into a gas analyzer (Logan 2500, Logan Research Limited), which measured NO (sensitivity = 0.3 ppb, T₉₀ < 0.5 s), using photometric determination of chemiluminescence. The analyzer was calibrated with a certified gas mixture (108 ppb NO) on the morning of each trial and checked periodically with sample gases of known NO concentrations.

Airway reactivity (AR) measurement

AR was measured in unanesthetized mice as described previously (25). Briefly, mice were placed within small-volume (~600 ml) Plexiglas chambers specially designed for mice, which allowed for free movement and had transducers connected to a computer that monitored chamber pressure alterations as a function of mouse breathing patterns (Buxco Electronics). The enhanced pause (Penh) variable was calculated in the conventional way (26), in which changes in the amplitude and duration of the expiratory pressure signal determined the alterations in the Penh value, as a function of methacholine (MCh)-induced airway responses. MCh dissolved in PBS (pH 7.4) was administered as an aerosol (0–50 mg/ml) to the mice within the chambers by using a DeVilbiss ultrasonic nebulizer connected to an aerosol driver and pump apparatus (Buxco). The administration duration of each MCh concentration was 2 min, followed by a 3-min observation and data collection period. The measured response was taken as the highest Penh value achieved during the administration and observation periods.

Statistics

A one-way ANOVA was used to assess all measured variables, with the exceptions of exhaled NO and Penh, in which cases paired *t* tests and repeated measures ANOVA were used, respectively. With acquisition of a significant "F" statistic ($p < 0.05$), from the ANOVA, post hoc disreputable data analysis was performed using Student-Newman-Kuel's test, with values of $p < 0.05$ considered significant. Mean values for all other groups that were beneath the limit of nitrite assay detectability were graphically represented as either "ND" or had means shown if a value for one or more of the individual samples exceeded the detectability limit and remained within 3 SDs of the mean of their respective groups. Mean levels of BAL IL-4 were shown graphically with error bars if at least one of the values assayed was greater than the detectability limit, whereas means of groups having no detectable levels were set to one-half the detectable level for subsequent graphical display and statistical comparisons. IL-18 assay values for individual mice that were beneath the limit of detection were set at one-half the detectable limit (i.e., 4 pg/ml) for statistical comparisons.

Results

BAL cell recruitment kinetics

The total number of cells recovered in the BAL was low in all groups at 24 h post-aerosol challenge ($\sim 1 \times 10^4$ cells/g), with the exception of the OVA-challenged IL-10^{-/-} mice, in which it was significantly increased by an average of 3-fold (Fig. 1A). At 48 h post-aerosol challenge, this increase persisted in the IL-10^{-/-} mice, and the total number of cells in the C57 group was increased slightly over that of the saline-sham-challenged groups. At 72 h post-aerosol challenge, the total number of cells in the IL-10^{-/-} mice was increased four times that of the non-OVA-challenged controls and remained over twice as high as the OVA-challenged C57 mice, despite the increase by 2-fold in the C57 mice over that of the non-OVA-treated controls. These data were taken as evidence that the mild OVA sensitization and challenge protocol had provoked a differentiated allergic response between the two strains based on the presence or absence of IL-10. This inference was confirmed by administration of rmIL-10, which significantly decreased the total cell number in IL-10^{-/-} mice with AI.

The number of macrophages recovered in the BAL at 24 h post-aerosol challenge in the saline-challenged groups and that of the OVA-challenged C57 mice was low ($\sim 0.6\text{--}1 \times 10^4$ cells/g) and was the major portion of the total cells isolated ($\geq 90\%$), as observed previously (13). However, the macrophage count in the OVA-challenged IL-10^{-/-} mice at this time interval was elevated significantly by 2-fold over the others (Fig. 1B). As with the total

cell count, this increase in macrophage numbers in the IL-10^{-/-} mice persisted and increased numerically over the course of 72 h. In contrast, the macrophage count in the C57 mice displayed a small increase at 48 h that fell back to control levels by 72 h. These data were taken as further evidence that the response to allergen was different between the IL-10^{-/-} and C57 mice based on the presence or absence of IL-10. Again, addition of rmIL-10 resulted in a significant decrease in macrophage numbers in the IL-10^{-/-} mice with AI.

The number of eosinophils recovered in the BAL remained at or near 0 (<1%) over all time intervals in the saline-challenged groups (Fig. 1C). Eosinophil numbers were increased significantly in both IL-10^{-/-} and C57 mice at 24 h post-OVA aerosol challenge. The numbers rose over time to achieve levels at 72 h that were 2- to 3-fold greater than those at 24 h; however, they were not different between the IL-10^{-/-} and C57 mice, nor were they statistically different over time, suggesting that a lack of IL-10 does not differentiate the eosinophilic response to allergen in this model. Furthermore, in this case, administration of rmIL-10 resulted in no significant change in eosinophil numbers, suggesting that recruitment of eosinophils was independent of IL-10, in this model of mild AI.

IL-18 release in vitro

Average IL-18 release by isolated and cultured BAL cells of naive mice was not significantly different between C57 and IL-10^{-/-} groups (Table I). With addition of SNAP, IL-18 was significantly decreased to similarly low levels in both groups (average, ~ 30 pg/ml), suggesting a strong effect of NO on IL-18 release that was not different with a lack of IL-10. Induction of AI with OVA significantly reduced IL-18 release to ~ 300 pg/ml, consistent with allergen-driven induction of a Th2 response, but which was 10-fold higher than that observed with addition of SNAP alone. Addition of SNAP to cells from OVA-challenged mice further reduced IL-18 release to nondetectable levels in cells from the IL-10^{-/-} mice. Addition of L-NIO to cells from mice with AI increased IL-18 release as compared with the addition of SNAP in these same cells. As in the other cases, IL-18 release was not different with the lack of IL-10.

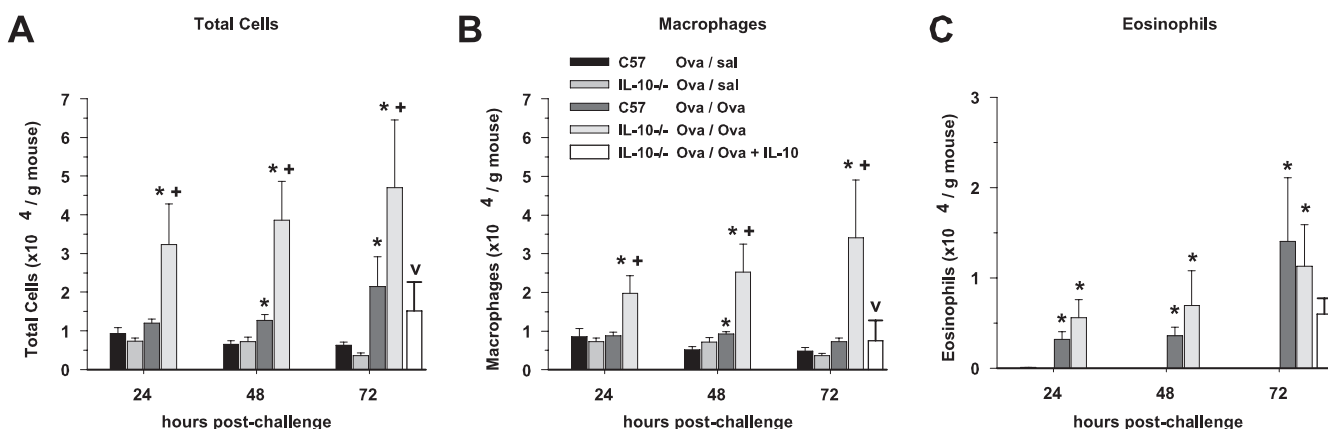


FIGURE 1. BAL cell kinetics with inflammation. **A**, Total cells recovered in BAL fluid of C57 and IL-10^{-/-} mice during post-aerosol challenge time intervals of 24, 48, and 72 h. Ova/sal = OVA sensitized/saline challenged, Ova/Ova = OVA sensitized/OVA challenged; values are means $\pm$ SE, $n = 8$ mice/bar; *, $p < 0.05$ Ova/Ova vs Ova/Sal within mouse strain, and +, $p < 0.05$ IL-10^{-/-} Ova/Ova vs C57 Ova/Ova; v, $p < 0.05$ Ova/Ova + rmIL-10 vs Ova/Ova. **B**, Macrophages recovered in BAL fluid of C57 and IL-10^{-/-} mice during post-aerosol challenge intervals. Groups, time intervals, numbers, and significance symbols as in **A**. **C**, Eosinophils recovered in BAL fluid of C57 and IL-10^{-/-} mice during post-aerosol challenge intervals. Groups, time intervals, numbers, and significance symbols as in **A**; absence of bars for Ova/Sal groups signifies eosinophils were not found.

Table I. *IL-18 alterations with AI and NO manipulation^a*

Mouse Strain	Control	Control and +SNAP	+OVA	+OVA +SNAP	+OVA +L-NIO
C57	1446 ± 225	35 ± 17*	313 ± 61*	19 ± 11* [†]	475 ± 65*
IL-10 ^{-/-}	1002 ± 237	24 ± 10*	294 ± 71*	(n.d.)* [†]	542 ± 116

^a Values are means ± SE; **p* < 0.05 vs respective C57 or IL-10^{-/-} group control (-OVA, -SNAP, -L-NIO); [†]*p* < 0.05 vs respective +OVA and +OVA+L-NIO groups; all others not significantly different, not detected (n.d.) (<8 pg/ml); -OVA (*n* = 12), -OVA+SNAP (*n* = 8), +OVA (*n* = 8), +OVA+SNAP (*n* = 6), +OVA+L-NIO (*n* = 8).

Airway IL-4 kinetics

Average levels of IL-4 in the BAL fluid were at or beneath the level of assay detectability, except in the case of the IL-10^{-/-} mice at 48 h post-OVA-aerosol challenge, at which point it was significantly elevated by an average of 2- to 4-fold as compared with the other groups (Fig. 2A). These findings suggested that the AI protocol had provoked measurable BAL IL-4 production in the absence of IL-10 and that an effect of administration of rmIL-10 on BAL IL-4 levels was not observable at 72 h after induction of AI.

Airway nitrite kinetics

Nitrite levels were elevated 48–72 h after OVA challenge in the BAL fluid of C57 mice (Fig. 2B), whereas nitrite levels in IL-10^{-/-} mice were suppressed at these same time intervals. Similar to the elevated E_{NO} levels previously observed in naive IL-10^{-/-} mice (19), BAL nitrite levels in the sham-challenged IL-10^{-/-} mice were numerically twice as great as sham-challenged C57 mice at 24 and 48 h, achieving statistical significance in the 48-h sample.

AR kinetics

AR measured as Penh remained similar in the C57 mice over all time intervals after sham-saline and mild OVA challenge (Fig. 2C). In contrast, AR fell from 24 to 48 and 72 h in OVA-challenged IL-10^{-/-} mice and was less than that of C57 mice at all time intervals studied. However, within this pattern of increasing hyporesponsiveness in the absence of IL-10, it was noted that AR was inversely related to airway nitrite levels at 24 h post-OVA aerosol challenge and that this relationship was lost in the later phases of AI as both nitrite levels and AR fell. Administration of rmIL-10 countered this pattern of hyporesponsiveness, evident as a significant increase in AR at 72 h after induction of AI.

E_{NO} in vivo

In agreement with the elevated BAL nitrite levels in the sham-saline-challenged IL-10^{-/-} mice at 24 and 48 h post-OVA challenge, average E_{NO} levels at 48 h post-OVA challenge were significantly greater in naive IL-10^{-/-} mice as compared with naive C57 mice (10.9 ± 1.1 vs 6.7 ± 0.6 (SE) ppb, *p* < 0.05) (Fig. 3A). With AI, E_{NO} levels significantly increased in C57 mice (10.3 ± 0.9 ppb, *p* < 0.05) to levels nearly identical to naive IL-10^{-/-} mice. In contrast and in agreement with decreased BAL nitrite levels, E_{NO} was significantly decreased in IL-10^{-/-} mice with AI (6.5 ± 0.9 ppb, -39% decrease, *p* < 0.05) to levels nearly identical to naive C57 mice. Administration of rmIL-10 to IL-10^{-/-} mice during induction of AI (Fig. 3B) resulted in a similar decrease in E_{NO} (-43%, NS), suggesting no significant additional effect on airway NO production. However, E_{NO} was significantly further decreased (-60%, *p* < 0.05), with administration of L-NIL to IL-10^{-/-} mice with AI (Fig. 3C), suggesting some further reduction of airway NO with NOS enzyme-specific inhibition.

AR with suppression of NO in vivo

AR in naive C57 and IL-10^{-/-} mice is shown in Fig. 4A for the full MCh concentration spectrum used and for comparison with

results obtained with at 72 h post-OVA aerosol challenge, with administration of rmIL-10 and L-NIL (Fig. 4B). Reconstitution of IL-10 resulted in a small but insignificant increase in AR; however, specific inhibition of NOS with L-NIL resulted in a significant increase in AR that produced a response profile that was not different from the C57 IL-10-sufficient case.

Nitrite production in vitro

Nitrite levels were nondetectable in conditioned medium of cells from the naive C57 mice but were numerically greater (NS) for cells from naive IL-10^{-/-} mice (Fig. 5A). Nitrite production of cells from both C57 and IL-10^{-/-} mice was increased with AI, attaining levels 2-fold higher in the absence of IL-10. Nitrite production was completely inhibited in BAL cells treated with L-NIO from both strains of mice.

IL-4 release in vitro

IL-4 release by BAL cells of naive mice was nondetectable and rose significantly in cells from both C57 and IL-10^{-/-} mice with AI, consistent with induction of an allergen-driven Th2 response (Fig. 5B). However, the magnitude of the increase in IL-4 in the absence of IL-10 was ~10-fold greater than that observed in the IL-10-sufficient case (C57 mice). Addition of L-NIO and the inhibition of NO production resulted in no significant alteration of IL-4 release in the cells from the C57 mice. However, the inhibition of NO production significantly reduced IL-4 release by cells from IL-10^{-/-} mice, down to levels similar to that released by cells from C57 mice, suggesting a difference in IL-4 modulation in the absence of IL-10.

Discussion

The main findings of this study, with AI in the absence of IL-10, were as follows: 1) significantly increased recruitment of macrophages into the airway, which was not observed when IL-10 was present; 2) decreased E_{NO} and airway nitrite levels, in contrast with increases when IL-10 was present; 3) significantly increased IL-4 release by BAL cells over that released in the IL-10-sufficient case; 4) suppression of the significant IL-4 release with inhibition of nitrite production not observed in cells sufficient for IL-10 production; 5) an early inverse association of airway NO and AR, which was diminished with continued development of AI; and 6) demonstration of additional NO-dependent increases in AR, despite a loss of the relationship between airway NO and AR, in the late stages of AI.

These results indicate that a lack of IL-10 alters modulation of NO in the setting of AI in ways that might not be predicted based on its known suppressive effect on NOS (12). Rather, the lack of IL-10 appears to allow significantly increased IL-4 production by cells within the airway that may inhibit NO production and subsequently alter physiological functions associated with its production such as AR. In addition, there appears to be a NO-associated negative feedback mechanism regulating IL-4 release from airway immune cells that may serve to limit IL-4 release in the absence of IL-10.

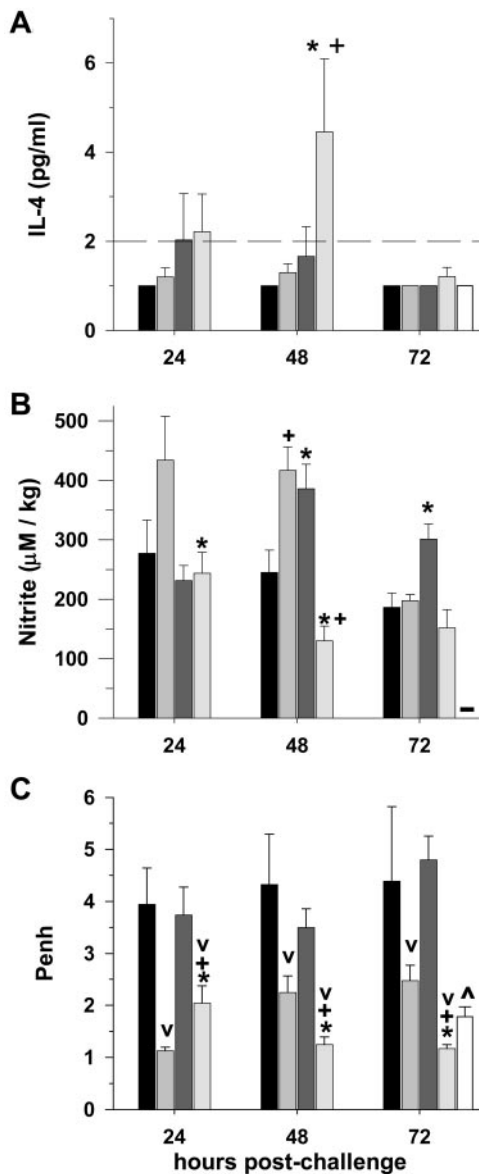


FIGURE 2. Airway IL-4, NO, and AR with AI. **A**, Concentrations of IL-4 in BAL fluid of C57 and IL-10^{-/-} mice during post-aerosol challenge time intervals of 24, 48, and 72 h. Groups, numbers, and significance symbols as in Fig. 1. Limit of detection indicated as dashed horizontal bar. **B**, Nitrite levels in BAL fluid of C57 and IL-10^{-/-} mice during post-aerosol challenge intervals of 24, 48, and 72 h. Groups, time intervals, numbers, and significance symbols as in Fig. 1. Minus symbol indicates test not done due to shortage of BAL fluid. **C**, AR (Penh) of C57 and IL-10^{-/-} mice to 50 mg/ml MCh during post-aerosol challenge intervals of 24, 48, and 72 h. Groups, time intervals, and numbers as in Fig. 1; v, $p < 0.05$ vs respective Ova/Sal or Ova/Ova strain counterpart; ^, $p < 0.05$ IL-10^{-/-} Ova/Ova + rmIL-10 vs IL-10^{-/-} Ova/Ova; other significance symbols as in Fig. 1.

BAL macrophage-dominant shifts in absence of IL-10

In the absence of IL-10, total BAL cell recruitment in response to OVA administration demonstrated an early significant increase (24 h), which was sustained at levels 2- to 4-fold higher than the C57 IL-10-sufficient mice over the 24- to 72-h time course studied. As expected, eosinophilia was demonstrated in both C57 and IL-10^{-/-} mice over this same time course. However, in the absence of IL-10, the 2- to 4-fold elevations in BAL cell numbers were due to the profound macrophage-dominant BAL cell recruitment. The present observations extend our prior work (13) and show the ki-

netic profile of AI-induced changes in the absence of IL-10. These findings are consistent with studies in humans in which increased macrophage influx occurs in allergen-challenged individuals (27) and support the notion that lack of IL-10 results in heavily skewed recruitment of macrophages to the allergen-provoked airway. Based on a prior report (28) in which administration of IL-4 to naive mice resulted in significantly increased BAL macrophages but not eosinophils, the source of this increased recruitment in the absence of IL-10 may be associated with elevated levels of IL-4, as we observed in the BAL fluid, and in stimulated cells in vitro.

IL-18 release: a Th1 cytokine profile

The profile of IL-18 release by BAL cells in these experiments is consistent with its characterization as a Th1-associated cytokine (14, 29). The presence of a similar pattern in BAL cells from both the C57 and IL-10^{-/-} mice suggests that the general profile of IL-18 production in the setting of AI is not directly affected by the absence of IL-10. IL-18 release was modifiable by NO, consistent with previous work showing suppression of IL-18 by NO (15). However, we did not observe any significant alteration in NO-associated modulation of IL-18 release in the absence of IL-10, further suggesting that a lack of IL-10 does not result in alteration of IL-18 regulation with AI.

Airway NO production

E_{NO} has been identified as a biomarker of AI associated with asthma (9, 10); however its production in vivo, in the absence of IL-10, has not been well studied. As we have reported previously, E_{NO} levels were significantly greater in naive IL-10^{-/-} mice, as compared with IL-10-sufficient C57 mice, but unlike the C57 mice, a lack of IL-10 resulted in a decrease in E_{NO} with the induction of AI with OVA. E_{NO} with AI was not statistically altered by administration of rmIL-10; however, it was further lowered with administration of L-NIL, indicating further inhibition of NOS activity using an enzyme-specific inhibitor. We interpreted these results as suggesting that the alteration in airway NO production with inflammation was not directly dependent on IL-10 but may have occurred indirectly through alterations in IL-4 production, as suggested below.

Airway IL-4, nitrite, and reactivity

IL-4 is a cytokine that is associated with allergen-mediated induction of the Th2 state and the development of AI (16, 22). IL-4 release into the airway was not detectable in sham-saline-challenged mice, suggesting no potentiation of the Th2 response without OVA. Furthermore, IL-4 remained at undetectable levels at 24 h post-OVA challenge in the absence of IL-10, whereas total cell and macrophage counts were elevated, and airway nitrite and AR demonstrated an inverse association. However, at 48 h post-OVA challenge, airway IL-4 was significantly increased in the IL-10^{-/-} mice, suggesting an OVA-driven Th2 response in the absence of IL-10, whereas both airway nitrite and AR fell; the inverse association between airway nitrite and AR was lost. This relationship continued into 72 h in the absence of IL-10. In contrast, airway nitrite levels in IL-10-sufficient C57 mice were elevated with AI at 48 and 72 h post-OVA-challenge, with no measurable change in airway IL-4 levels.

Administration of rmIL-10 resulted in a small but statistically insignificant increase in AR, suggesting a trend for an effect of reconstitution of IL-10. This trend was consistent with an AR increase reported previously (30, 31); however, its magnitude was small, perhaps due to the mild nature of our inflammatory challenge. Administration of L-NIL resulted in a significant increase in AR in the absence of IL-10 that was not different from the C57

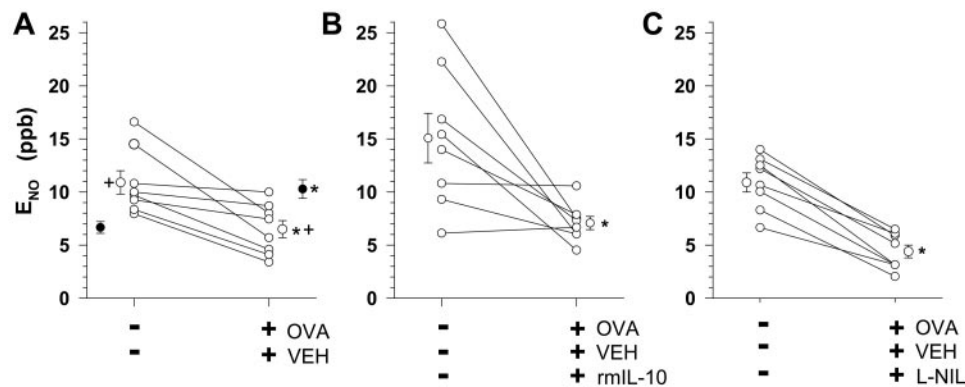


FIGURE 3. E_{NO} levels in mice. E_{NO} in individual $IL-10^{-/-}$ mice ($n = 8$ /group), connected by straight lines, before (–) and 48 h after OVA challenge (+), administered: A, sham-saline injection of L-NIL vehicle (VEH) (○); mean $\pm$ SE shown with error bars flanking individual data; +, $p < 0.05$ as compared with C57 (●) controls; *, $p < 0.05$ respective C57 and $IL-10^{-/-}$ comparisons +OVA vs –OVA; B, rmIL-10 ($125 \mu\text{g} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$), significance as in A; or C, L-NIL (3 mg/kg), significance as in A; $p < 0.05$ for average percent decrease with L-NIL (–60%) from control, as compared to average percent decrease with +OVA+VEH (–39%) in A.

IL-10-sufficient case. These findings suggest that the suppression of AR with AI in the absence of IL-10 may not be associated with loss of IL-10 modulation of NOS, per se, but may be occurring indirectly, perhaps through up-regulated IL-4 production.

Modulation of IL-4 release by airway immune cells

Unlike IL-18, IL-4 release by cultured, recruited BAL cells was negligible for both strains of naive mice and was elevated in cells from mice with OVA-induced AI, suggesting the presence of the Th2 state in our allergen-provocation model. However, the release of IL-4 in the absence of IL-10 was increased ~ 10 -fold over the IL-10-sufficient cells, suggesting altered regulation of IL-4, with a lack of IL-10. Furthermore, inhibition of NO production by cells lacking IL-10 significantly reduced IL-4 release to levels equivalent to the cells from IL-10-sufficient C57 mice, which were unchanged by this same treatment. Although the effect of IL-4 in down-regulating NO production is known (17), the present findings support an additional feedback mechanism in which NO reduces IL-4 production (32), in the absence of IL-10. Thus, these findings suggest that NO levels may play an important role in the feedback regulation of IL-4 release by immune cells recruited to the airway as part of the process of allergic inflammation. Furthermore, they suggest that the high airway NO levels present in the naive case are altered with the induction of inflammation in vivo,

perhaps through down-regulation via increasing airway IL-4 levels, with development of the Th2 state.

Association of IL-10 and airway responsiveness

The effect of IL-10 on allergen-driven AR has been reported in several previous in vivo and in vitro animal studies (30, 31, 33, 34). The evidence from those studies suggests that IL-10 influences or acts in concert with one or more inflammatory factors to increase smooth muscle activity (31), perhaps involving airway smooth muscle autocrine signaling through IL-5 (33) or regulation of surfactant protein D (35). However, there has been no further consensus. Our data suggest that the production of NO may be a factor in regulation of AR in the early stages of AI, when IL-10 is missing, but that its production may be down-regulated by increased levels of IL-4, with the progression of inflammation in the absence of IL-10. Although this idea is inconsistent with an expected linkage between eosinophilia and AR, some studies have shown dissociation between AR and eosinophilia, particularly in the C57 strain (36); therefore, their concordance does not seem to be rigid. However, given the elevated E_{NO} levels in naive $IL-10^{-/-}$ mice, the reduction of NO production with AI, and the lack of development of a typically exaggerated AI phenotype (as eosinophilia and strong airway hyperresponsiveness), the present

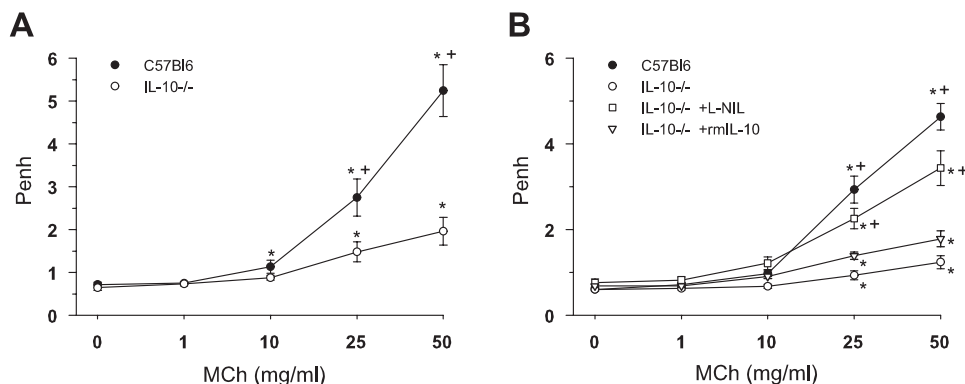


FIGURE 4. AR in mice with AI. A, AR (as Penh) in naive C57 (●, $n = 16$) and $IL-10^{-/-}$ (○, $n = 16$) mice. Values are means $\pm$ SE; *, $p < 0.05$ vs respective strain 0 mg/ml MCh and next lowest MCh concentration(s); +, $p < 0.05$ increased vs $IL-10^{-/-}$ at same MCh concentration. B, AR in C57 (●, $n = 16$), $IL-10^{-/-}$ (○, $n = 16$), $IL-10^{-/-}$ + rmIL-10 (▽, $n = 15$), and $IL-10^{-/-}$ + L-NIL (□, $n = 8$) mice, 72 h post-aerosol OVA challenge; significance symbols as in A.

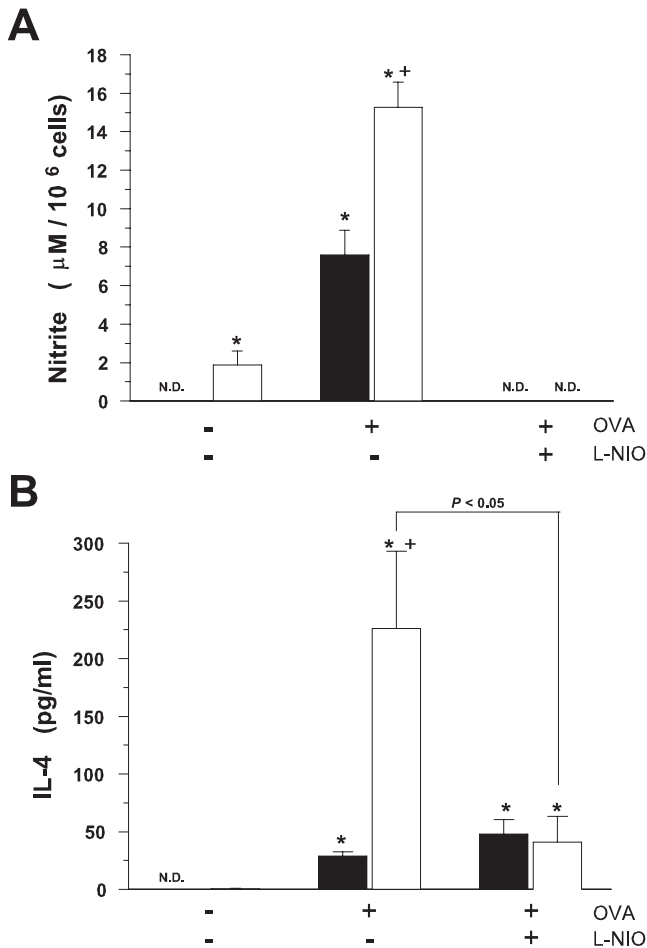


FIGURE 5. Nitrite and IL-4 production in vitro. **A**, Nitrite production by BAL cells of C57 (■) and IL-10^{-/-} (□) mice without (-OVA) and with (+OVA) AI and without or with L-NIO. Values are means $\pm$ SE; *, $p < 0.05$ vs respective C57 or IL-10^{-/-} group control (-OVA, -L-NIO); +, $p < 0.05$ IL-10^{-/-} increased vs C57 (+OVA, -L-NIO), ND = not detected ($<1 \mu\text{M}$); n same as Table I (data adapted by permission from Ref. 13). **B**, IL-4 production by BAL cells of C57 and IL-10^{-/-} mice. *, $p < 0.05$ as compared with respective strain naive (-OVA) counterpart; +, $p < 0.05$ IL-10^{-/-} +OVA increased as compared with C57 +OVA; other significance as shown for IL-10^{-/-} +OVA +L-NIO as compared with IL-10^{-/-} +OVA -L-NIO.

data would suggest that the relationship between NO and IL-4 may be important in the development of AR, in the absence of IL-10.

Critique of methods

We used the measurement of Penh as an index of AR because it allowed a rapid, repeated, noninvasive assessment of AR measurements in large numbers of unanesthetized mice over the 24–72 h postchallenge period using inhaled MCh. Earlier studies have shown that elevations in Penh are correlated with eosinophilia in BALB/c mice (37); however, this association has been reported recently to be weak or absent, and there has been some controversy as to whether Penh can accurately reflect alterations in airway responsiveness in C57 mice (38, 39). Our data suggest that alterations in AR associated with AI were consistent with expectations of airway hyporesponsiveness in IL-10^{-/-} mice, relative to their C57 IL-10-sufficient counterparts (30, 31, 33, 34). Although we acknowledge some limitations to the Penh technique, we agree with the assertion by DeLorme and Moss (40) that the choice of the Penh measurement should be linked to the study objective and

the ability to track alterations in AR previously verified by more invasive measures of lung resistance. The latter requirement in the C57 and IL-10^{-/-} strains was fulfilled by the prior study of Justice et al. (31) in which Penh was shown to display results consistent with measures of lung resistance in situ and airway smooth muscle tension development in vitro.

Although our AR and inflammation responses generally agree with those of prior studies (30, 31, 34), they are not consistent with one study in which IL-10^{-/-} mice demonstrated significantly decreased BAL macrophage counts and increased eosinophilia and AR, as compared with the C57 mice (41). Those responses were consistent with what would be expected with a lack of IL-10 as an anti-inflammatory cytokine and are in agreement with phenotypic expectations that would come from human data demonstrating a lack of IL-10 production in asthmatics (7, 8). However, it must be acknowledged that those results were obtained with a stronger OVA stimulus (five higher concentration OVA challenges), which may have profound effects on the induction of AI and the subsequent measures of cell recruitment, cytokines, NO production, and airway responsiveness (42). Even so, it is possible that fastidious precautions of sterilized germfree microisolator cages and bedding, sterile food and water provision, and sterilization of the allergen exposure chamber (41) are necessary to observe these results in this mouse model. Although we housed our mice in a barrier facility, we did not sterilize other fomites with which the mice potentially came into contact throughout the experiments, which may have led to altered AI and AR responses, in the absence of IL-10. Further study is necessary to determine whether these possibilities are germane to our understanding of systemic, cellular, and molecular responses within the airway in murine models when IL-10 is lacking.

Acknowledgments

The excellent technical assistance of Catarina Wong, Jodie Rabbitt, and Barbara Dixon-McCarthy are acknowledged.

Disclosures

The authors have no financial conflict of interest.

References

- Berkman, N., M. John, G. Roessems, P. J. Jose, P. J. Barnes, and K. F. Chung. 1995. Inhibition of macrophage inflammatory protein-1 α expression by IL-10: differential sensitivities in human blood monocytes and alveolar macrophages. *J. Immunol.* 155: 4412–4418.
- Driscoll, K. E., J. M. Carter, B. W. Howard, D. Hassenbein, M. Burdick, S. L. Kunkel, and R. M. Strieter. 1998. Interleukin-10 regulated quartz-induced pulmonary inflammation in rats. *Am. J. Physiol.* 275: L887–L894.
- Grünig, G., D. B. Corry, M. W. Leach, B. W. P. Seymour, V. P. Kurup, and D. M. Rennick. 1997. Interleukin-10 is a natural suppressor of cytokine production and inflammation in a murine model of allergic bronchopulmonary aspergillosis. *J. Exp. Med.* 185: 1089–1099.
- Wandiworun, C., and W. Strober. 1993. Predominant role of tumor necrosis factor α in human monocyte IL-10 synthesis. *J. Immunol.* 151: 6853–6861.
- de Waal Malefyt, R., J. Abrams, B. Bennett, C. G. Figdor, and J. E. de Vries. 1991. Interleukin-10 (IL-10) inhibits synthesis by human monocytes: an auto-regulatory role of IL-10 produced by monocytes. *J. Exp. Med.* 174: 1209–1220.
- Zuany-Amorim, C., S. Hailé, D. Leduc, C. Dumarey, M. Huerre, B. B. Vargaftig, and M. Pretolani. 1995. Interleukin-10 inhibits antigen-induced cellular recruitment into the airways of sensitized mice. *J. Clin. Invest.* 95: 2644–2651.
- Borish, L., A. Aarons, J. Rumbly, P. Cvietusa, J. Negri, and S. Wenzel. 1996. Interleukin-10 regulation in normal subjects and patients with asthma. *J. Allergy Clin. Immunol.* 97: 1288–1296.
- Calhoun, W. J., K. L. Hinton, J. J. Brick, A. J. Sharma, and W. Rosen. 1996. Spontaneous and stimulated IL-10 release by alveolar macrophages (AM) but not blood monocytes (BM) is reduced in allergic asthmatics (AA). *Am. J. Respir.* 153: A881.
- Hamid, Q., D. R. Springall, V. Riveros-Moreno, P. Chanez, P. Howarth, A. Redington, J. Bousquet, P. Godard, S. Holgate, and J. M. Polak. 1993. Induction of nitric oxide synthase in asthma. *Lancet* 342: 1510–1513.
- Kallio, E. A., P. K. Koskinen, E. Aavik, K. Vaali, and K. B. Lemstöm. 1997. Role of nitric oxide in experimental obliterative bronchiolitis (chronic rejection) in the rat. *J. Clin. Invest.* 100: 2984–2994.

11. Jatakanon, A., S. Lim, A. Kharitonov, K. F. Chung, and P. J. Barnes. 1998. Correlation between exhaled nitric oxide, sputum eosinophils and methacholine responsiveness in patients with mild asthma. *Thorax* 53: 91–95.
12. Cunha, F. Q., S. Moncada, and F. Y. Liew. 1992. Interleukin-10 (IL-10) inhibits the induction of nitric oxide synthase by interferon γ in murine macrophages. *Biochem. Biophys. Res. Commun.* 182: 1155–1159.
13. Ameredes, B. T., R. Zamora, K. F. Gibson, T. R. Billiar, B. Dixon-McCarthy, and W. J. Calhoun. 2001. Increased nitric oxide production by airway immune cells of sensitized and challenged IL-10 knockout mice. *J. Leukocyte Biol.* 70: 730–736.
14. Gillespie, M. T., and N. J. Norwood. 1998. Interleukin-18: perspectives on the newest interleukin. *Cytokine Growth Factor Rev.* 9: 109–116.
15. Kim, Y., R. V. Talanian, J. Li, and T. R. Billiar. 1998. Nitric oxide prevents IL-1 β and IFN- γ -inducing factor (IL-18) release from macrophages by inhibiting caspase-1 (IL-1 β -converting enzyme). *J. Immunol.* 161: 4122–4128.
16. Hamid, Q. A., and L. A. Cameron. 2000. Recruitment of T cells to the lung in response to antigen challenge. *J. Allergy Clin. Immunol.* 106(5 Suppl.): S227–S234.
17. Nemoto, Y., T. Otsuka, H. Niino, K. Izuhara, K. Yamaoka, H. Nakashima, and Y. Niho. 1999. Differential effects of interleukin-5 and interleukin-10 on nitric oxide production by murine macrophages. *Inflamm. Res.* 48: 643–650.
18. Kuhn, R., J. Lohler, D. Roanoke, K. Rajewsky, and W. Muller. 1993. Interleukin-10-deficient mice develop chronic enterocolitis. *Cell* 75: 263–274.
19. Ameredes, B. T., J. M. Sethi, H. Liu, A. M. K. Choi, and W. J. Calhoun. 2005. Enhanced nitric oxide production associated with airway hyperresponsiveness in the absence of IL-10. *Am. J. Physiol.* 288: L868–L873.
20. Weinberg, J. B. 1998. Nitric oxide production and nitric oxide synthase type 2 expression by human mononuclear phagocytes: a review. *Mol. Med.* 4: 557–591.
21. McCall, T. B., M. Feelisch, R. M. J. Palmer, and S. Moncada. 1991. Identification of N-iminoethyl-L-ornithine as an irreversible inhibitor of nitric oxide synthase. *Br. J. Pharmacol.* 102: 234–238.
22. Lederer, J. A., V. L. Perez, L. DesRoches, S. M. Kim, A. K. Abbas, and A. H. Lichtman. 1996. Cytokine transcriptional events during helper T cell subset differentiation. *J. Exp. Med.* 184: 397–406.
23. Zamora, R., K. E. Matthys, and A. G. Herman. 1997. The protective role of thiols against nitric oxide-mediated cytotoxicity in murine macrophage J774 cells. *Eur. J. Pharmacol.* 321: 87–96.
24. Weicker, S., T. A. Karachi, J. A. Scott, D. G. McCormack, and S. Mehta. 2001. Noninvasive measurement of exhaled nitric oxide in a spontaneously breathing mouse. *Am. J. Respir. Crit. Care Med.* 163: 1113–1116.
25. Ameredes, B. T., L. E. Otterbein, L. K. Kohut, A. L. Gligonic, W. J. Calhoun, and A. M. K. Choi. 2003. Low-dose carbon monoxide reduces airway hyperresponsiveness in mice. *Am. J. Physiol.* 285: L1270–L1276.
26. 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.
27. Calhoun, W. J., H. E. Reed, D. R. Moest, and C. A. Stevens. 1992. Enhanced superoxide production by alveolar macrophages and airspace cells, airway inflammation, and alveolar macrophage density changes follow segmental antigen bronchoprovocation in allergic subjects. *Am. Rev. Respir. Dis.* 145: 317–325.
28. Venkayya, R., M. Lam, M. Willkom, G. Grunig, D. B. Corry, and D. J. Erle. 2002. The Th2 lymphocyte products IL-4 and IL-13 rapidly induce airway hyperresponsiveness through direct effects on resident airway cells. *Am. J. Respir. Cell Mol. Biol.* 26: 202–208.
29. Walter, D. M., C. P. Wong, R. H. DeKruyff, G. J. Berry, S. Levy, D., and T. Umetsu. 2001. IL-18 gene transfer by adenovirus prevents the development of and reverses established allergen-induced airway hyperactivity. *J. Immunol.* 166: 6392–6398.
30. Mäkelä, M. J., A. Kanehiro, L. Borish, A. Dakhama, J. Loader, A. Joetham, Z. Xing, M. Jordana, G. L. Larsen, and E. W. Gelfand. 2000. IL-10 is necessary for the expression of airway hyperresponsiveness but not pulmonary inflammation after allergic sensitization. *Proc. Natl. Acad. Sci. USA* 97: 6007–6012.
31. Justice, J. P., S. S. Sur, J. Mustafa, M. Fan, and M. R. Van Scott. 2001. IL-10 gene knockout attenuates allergen-induced airway hyperresponsiveness in C57BL/6 mice. *Am. J. Physiol.* 280: L363–L368.
32. Fiorucci, S., A. Mencarelli, E. Distrutti, M. Baldoni, P. del Soldato, and A. Morelli. 2004. Nitric oxide regulates immune cell bioenergetic: a mechanism to understand immunomodulatory functions of nitric oxide-releasing anti-inflammatory drugs. *J. Immunol.* 173: 874–882.
33. Grunstein, M. M., H. Hakonarson, J. Leiter, M. Chen, R. Whelan, J. S. Grunstein, and S. Chuang. 2001. Autocrine signaling by IL-10 mediates altered responsiveness of atopic sensitized airway smooth muscle. *Am. J. Physiol.* 281: L1130–L1137.
34. Stämpfli, M. R., M. Cwiartka, B. U. Gajewska, D. Alvarez, S. A. Ritz, M. D. Inman, Z. Xing, and M. Jordana. 1999. Interleukin-10 gene transfer to the airway regulates allergic mucosal sensitization in mice. *Am. J. Respir. Cell Mol. Biol.* 21: 586–596.
35. Takeda, K., N. Miyahara, Y. H. Rha, C. Taube, E. S. Yang, A. Joetham, T. Kodama, A. M. Balhorn, A. Dakhama, C. Duez, et al. 2003. Surfactant protein D regulates airway function and allergic inflammation through modulation of macrophage function. *Am. J. Respir. Crit. Care Med.* 168: 783–789.
36. Wilder, J. A., D. D. S. 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.
37. Dohi, M., S. Tsukamoto, T. Nagahori, K. Shinagawa, K. Saitoh, Y. Tanaka, S. Kobayashi, R. Tanaka, Y. To, and K. Yamamoto. 1999. Noninvasive system for evaluating the allergen-specific airway response in a murine model of asthma. *Lab. Invest.* 79: 1559–1571.
38. Lundblad, L. K., C. G. Irvin, A. Adler, and J. H. Bates. 2002. A reevaluation of the validity of unrestrained plethysmography in mice. *J. Appl. Physiol.* 93: 1198–1207.
39. Adler, A., G. Cieslewicz, and C. G. Irvin. 2004. Unrestrained plethysmography is an unreliable measure of airways responsiveness in BALB/c and C57BL/6 mice. *J. Appl. Physiol.* 97: 286–292.
40. Delorme, M. P., and O. R. Moss. 2002. Pulmonary function assessment by whole-body plethysmography in restrained versus unrestrained mice. *J. Pharmacol. Toxicol. Methods* 47: 1–10.
41. Tournoy, K. G., J. C. Kips, and R. A. Pauwels. 2000. Endogenous interleukin-10 suppresses allergen-induced airway inflammation and nonspecific airway responsiveness. *Clin. Exp. Allergy* 30: 775–783.
42. Sakai, K., A. Yokoyama, N. Kohno, and K. Hiwada. 1999. Effect of different sensitizing doses of antigen in a murine model of atopic asthma. *Clin. Exp. Immunol.* 118: 9–15.