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Endogenous and Exogenous IL-6 Inhibit Aeroallergen-Induced Th2 Inflammation[1]

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Chronic Th2-dominated inflammation and exaggerated IL-6 production are characteristic features of the asthmatic airway. To understand the processes that are responsible for the chronicity of this response and the role(s) of IL-6 in the regulation of airway Th2 inflammation, we compared the responses induced by OVA in sensitized wild-type mice, IL-6 deficient (−/−) mice, and transgenic mice in which IL-6 was overexpressed in the airway (CC10-IL-6 mice). When compared with wild-type mice, IL-6$^{-/-}$ mice manifest exaggerated inflammation and eosinophilia, increased levels of IL-4, IL-5, and IL-13 protein and mRNA, exaggerated levels of eotaxin, JE/monocyte chemoattractant protein-1, macrophage inflammatory protein-1α and -2, and mRNA, increased bronchoalveolar lavage (BAL) TGF-β_1, and exaggerated airway responses to aerosolized methacholine. In contrast, CC10-IL-6 mice, on both C57BL/6 and BALB/c backgrounds, manifest diminished inflammation and eosinophilia, decreased levels of IL-4, IL-5, and IL-13 protein and mRNA, and decreased levels of bronchoalveolar lavage TGF-β_1. IL-6 also decreased the expression of endothelial VCAM-1 and airway responsiveness to methacholine in these animals. These alterations in the IL-6$^{-/-}$ and CC10-IL-6 mice were not associated with significant decreases or increases in the levels of IFN-γ, respectively. These studies demonstrate that endogenous and exogenous IL-6 inhibit aeroallergen-induced Th2 inflammation and that this inhibition is not mediated by regulatory effects of IFN-γ. IL-6 may be an important anti-inflammatory, counterregulatory, and healing cytokine in the airway. ***The Journal of Immunology,*** **2000, 165: 4051–4061.**

Asthma is a major cause of morbidity, mortality, and health care expenditure whose prevalence has increased remarkably in recent years (1, 2). Multiple lines of investigation have led to our present belief that asthma is an inflammatory disorder and that Th2-dominated inflammation plays a central role in the pathogenesis of this disease (3–5). These studies have also highlighted the impressive chronicity of this inflammatory response, which often starts in early life, can persist throughout life, and is even seen in asthma patients who are asymptomatic (6, 7). However, the mechanisms that are responsible for this chronicity are poorly understood. Specifically, it is not clear why transient responses can be mounted in vivo after exposure to some Th2-inducing stimuli, whereas chronic, often lifelong responses are seen in patients with asthma. This is due, in part, to inadequate knowledge of the mechanisms of inhibition and resolution of inflammation in the normal airway and the integrity of these responses in patients with asthma.

IL-6 is a pleiotropic cytokine that is produced during the course of a wide variety of infectious, inflammatory, and traumatic disorders (8–13). Exaggerated production of IL-6 is well documented in asthma and asthmatic tissues (10, 14–16). However, the role that IL-6 plays in asthma is not clear because equally cogent arguments can be made for pro- and anti-inflammatory effects of this important cytokine moiety. The pro-inflammatory effects include the essential role of IL-6 in IgA production (17), turpentine-induced tissue inflammation (18), Lyme arthritis (19), tumor infiltration (20), Ag-induced arthritis (21), carrageenan-induced pleural inflammation (11), and the control and/or irradication of infections caused by *Mycobacterium tuberculosis* vaccinia virus, *Listeria*, vesicular stomatitis virus, and *Candida* (13, 22, 23). In contrast, anti-inflammatory effects of IL-6 have been demonstrated in systemic and pulmonary models of endotoxin-induced injury (24, 25), immune complex lung injury (12), pneumococcal pneumonia (26), and staphylococcal enterotoxin-induced toxic shock (27). In addition, IL-6-induced pro- and anti-inflammatory effects can be tissue and stimulus specific (18, 28, 29), and IL-6 can augment (19, 30) or inhibit (13, 21, 22) Th2 tissue inflammation. Despite the well-documented dysregulation of IL-6 in asthma and the importance of chronic Th2 inflammation in asthma, the ability of IL-6 to regulate Th2 inflammation in the airway has not been adequately investigated.

To characterize the anti-inflammatory mechanisms involved in airway healing and repair and the role(s) of IL-6 in these mechanisms, we characterized the responses induced by aeroallergen in wild-type (WT)[3] mice, mice that lack the ability to make IL-6 (IL-6$^{-/-}$ mice) and mice in which IL-6 is overexpressed in an airway/lung-specific fashion (CC10-IL-6 mice). These studies demonstrate that IL-6 is a potent multifaceted inhibitor of aeroallergen-induced responses because tissue and bronchoalveolar lavage (BAL) inflammation, eosinophilia, and Th2 cytokine production are exaggerated in the IL-6$^{-/-}$ mice and inhibited the CC10-IL-6 animals.

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[3] Abbreviations used in this paper: WT, wild type; BAL, bronchoalveolar lavage; RPA, RNase protection assay; IHC, immunohistochemistry; P_{enh}, enhanced pause; MIP, macrophage inflammatory protein; MCP, monocyte chemoattractant protein.

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Materials and Methods

Genetically modified mice

Mice that were unable to make IL-6 (IL-6$^{-/-}$) and that overexpressed IL-6 in the lung/airway under the influence of the CC10 promoter (CC10-IL-6) were used in these studies. The IL-6 knockout mice were obtained on a C57BL/6 background with appropriate C57BL/6 WT controls from The Jackson Laboratory (Bar Harbor, ME). Before use their (−/−) genotype was confirmed via PCR analysis. The CC10-IL-6 mice that were used were generated in our laboratory (31). The methods that were used to generate these mice, the organ specificity of transgene expression, and the alterations induced by IL-6 have been described previously (31, 32). These mice were initially generated using CBA × C57BL/6 animals. They were subsequently bred for ≥8 generations onto C57BL/6 or BALB/c backgrounds and C57BL/6 or BALB/c transgene (−) littermates, as appropriate, were used as WT controls.

OVA sensitization and challenge

OVA sensitization and challenge were accomplished using modifications of the protocols previously described by Yang et al. (33). In brief, 6- to 8-wk-old WT mice, CC-10-IL-6 transgene (+) mice, and/or IL-6$^{-/-}$ mice received i.p. injections containing 20 μg of turkey OVA (Sigma, St. Louis, MO) complexed to alum (Resorptar; Indergen, New York, NY). This process was repeated 5 days later. After an additional 7 days, some animals were sacrificed (time = 0) and others received aerosol challenge with OVA (1% w/v) in endotoxin-free PBS or endotoxin-free PBS alone. This was accomplished in a closed 27 × 20 × 10 cm plastic aerosol chamber in which the mouse was placed for 40 min. The aerosol was generated via an Omron NE-U07 ultrasonic nebulizer (Omron Healthcare, Vernon Hills, IL). Mice were sacrificed 4, 24, 48, 72 h, or 7 days after aerosol exposure.

Bronchoalveolar lavage

BAL was performed as previously described (34). In brief, after anesthesia, a median sternotomy was performed, the trachea was dissected free from the underlying soft tissues, and a 0.6-mm tube was inserted through a small incision in the trachea. BAL was performed by perfusing the lungs in situ with 0.6 ml PBS and gently aspirating the fluid back. This was repeated three times. The samples were then pooled and centrifuged, and cell numbers and differentials were assessed. The cell-free BAL fluid was stored at −70°C until used.

Histologic analysis

Mice were anesthetized, a median sternotomy was performed, the trachea was dissected free and cannulated as described above, and the pulmonary vascular tree was perfused with calcium- and magnesium-free PBS (pH 7.40) via a catheter in the right heart. The lungs were then inflated to a 25-cm pressure with 10% formalin in PBS (pH 7.40), removed from the chest, postfixed in 10% formalin in PBS for 24 h, placed in paraffin, and sectioned. Hematoxylin and eosin and Congo red stains were performed in the Department of Pathology of Yale University School of Medicine.

Quantification of tissue inflammation

Six to eight images of each lung section were captured at ×25 final magnification on an Olympus (Tokyo, Japan) BH-2 microscope using a Sony DXC-760 MD camera attached to a Macintosh PPC8100/80 with a RasterOps 24 MxTv frame grabber board. Images were collected at 16-bit color depth, 640 × 480 pixels, final magnification 0.75 pixels per micron using the software that came with the frame grabber board. They were then analyzed in NIH Image 1.62 using a computer-generated 18 line × 13 line overlay grid. Total intersections overlying inflamed lung parenchyma, excluding vessels and airways, were counted and compared with total intersections overlying parenchyma. The percentage of total points overlying inflamed lung per animal was then determined, and an average was made over each group.

Quantification of tissue eosinophils

Tissue eosinophilia was quantitated as previously described by our laboratory (35). In brief, on each Congo red stained slide, lungs were divided into six to eight rectangular areas using a 4-mm grid. From each area, the best-defined nontangentially cut bronchovascular area was selected at ×40 final magnification using an Olympus BH-2 microscope. Eosinophils are not well visualized at that power. Eosinophils were counted at ×200 final magnification. Area was quantitated using a rectangular 10-mm square reticule grid (American Optical, Buffalo, NY) inserted into one eyepiece. The number of lower left corner grid intersections overlapping the bronchovascular bundle was used as an approximation of area. The ratio of the total number of eosinophils to total area for each mouse lung was calculated, and the mean of these results was calculated for each experimental group. In our hands this method correlates well with eosinophils quantitated using tissue digestion.

Cytokine mRNA quantification: RNase protection assays (RPAs)

The levels of mRNA encoding Th1 and Th2 cytokines and chemokines were quantitated using RPA. Briefly, mice were sensitized and challenged, and their lungs were removed as described above. They were then digested in TRI-zol reagent (Life Technologies, Gaithersburg, MD), and total RNA was obtained by processing the tissues according to the manufacturer's specifications. The levels of specific mRNA transcripts were then evaluated by RPA using Riboquant kits (PharMingen, San Diego, CA) according to the instructions provided by the manufacturer.

Cytokine quantitation

The levels of IL-4, IL-5, IL-13, IFN-γ, eotaxin, RANTES, macrophage inflammatory protein (MIP)-1α MIP-2, JE/monocyte chemottractant protein (MCP)-1, and TGF-β_1 protein were quantitated by ELISA using commercial kits according to the instructions provided by the manufacturers (R & D, Minneapolis, MN; Endogen, Cambridge, MA).

VCAM-1 immunohistochemistry (IHC)

Immunoreactive VCAM-1 was assessed by IHC as described by Yang et al. (33). Mice were sensitized, challenged with OVA, and sacrificed, then their lungs were perfused with 0.001 M periodate/0.075 M lysine/1% paraformaldehyde (PLP) and fixed overnight at 4°C followed by cryoprotection by consecutive 20-min incubations in graded cold sucrose solutions and inflated with 1 ml 40% OCT diluted in PBS. They were then embedded in 100% OCT in a cryomold and stored at −70°C until sectioning. Tissue sections were soaked in wash buffer (0.1 M phosphate buffer plus 0.01% Triton X-100) for 10 min, blocked in 3% BSA in wash buffer, and incubated with a 1:50 dilution of the primary Ab (biotinylated anti-mouse VCAM-1; PharMingen) or isotype control Ab for 1.5 h at 25°C. The slides were then washed, incubated with streptavidin-alkaline phosphatase (Zymed, San Francisco, CA) for 30 min, rewashed, developed with Fast Red, and counterstained with hematoxylin.

Physiological assessment

The baseline resistance and AHR in unrestrained, conscious animals was assessed by barometric plethysmography using whole-body plethysmography (Buxco Electronics, Troy, NY) as described by our laboratory (36) and others (37). In brief, mice were placed into whole-body plethysmographs and interfaced with computers using differential pressure transducers. Measurements were made of respiratory rate, tidal volume, and enhanced pause (P_{enh}). Airways resistance is expressed as $P_{enh} = ((t_e/0.3\ t_r) - 1) \times (2\ P_{ef}/3\ P_{if})$,where P_{enh} = enhanced pause, t_e = expiratory time (in seconds), t_r = relaxation time (in seconds), P_{ef} = peak expiratory flow (in milliliters), and P_{if} = peak inspiratory flow (in milliliters per second). Increasing doses of methacholine were administered by nebulization for 120 s, and P_{enh} was calculated over the subsequent 5 min. The P_{enh} values for each mouse are reported as the percent increase over the P_{enh} value obtained before the administration of any methacholine.

Statistical Analysis

Data are expressed as means ± SEM unless otherwise indicated. Data were assessed for significance using the Student's t test or ANOVA as appropriate.

Results

Effect of IL-6 on cellularity and eosinophilia

To determine whether endogenous IL-6 regulates aeroallergen-induced responses in the lung, we compared the effects of OVA in sensitized WT and IL-6$^{-/-}$ mice before and at intervals after aerosol challenge. Aeroallergen sensitization and challenge of WT mice caused an impressive increase in BAL cellularity (Fig. 1*A*). This increase was noted within 4 h of challenge and was maximal 48 h after Ag exposure. Before aeroallergen challenge, macrophages were the dominant cell in the BAL fluids from WT mice. However, Ag challenge caused an impressive increase in BAL eosinophils. Enhanced eosinophil recovery was noted within 4 h, peaked after 48–72 h, and was still present 7 days after Ag exposure (Fig. 1*B*). Within 48 h of Ag challenge, eosinophils were the

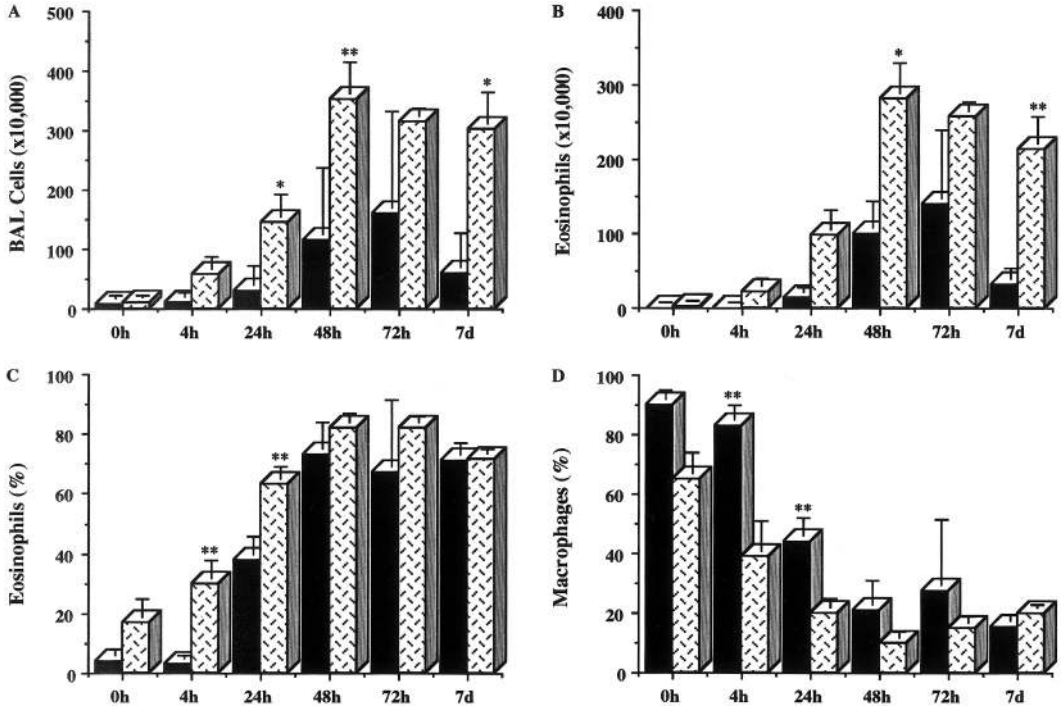

FIGURE 1. Comparison of BAL cellular profile of WT and IL-6$^{-/-}$ mice. WT (■) and IL-6$^{-/-}$ (▨) mice were sensitized and challenged with OVA as described in *Materials and Methods*. The characteristics of the BAL fluids from these animals were evaluated just before OVA challenge (0 h), or 4, 24, 48, and 72 h and 7 days (d) after OVA challenge. Total cell recovery (*A*), eosinophil recovery (*B*), the percentage of BAL cells that were eosinophils (*C*), and the percentage of BAL cells that were macrophages (*D*) are illustrated (*, $p < 0.01$; **, $p < 0.001$). The noted values represent the mean ± SEM of a minimum of seven animals at each time point.

predominant inflammatory cell in BAL fluid (Fig. 1*C*). This impressive increase in BAL eosinophils was associated with a significant decrease in the percentage of BAL cells that were macrophages (Fig. 1*D*). Comparable degrees of BAL inflammation were not seen in WT animals that were not sensitized before aeroallergen challenge or that received systemic sensitization and nebulized saline (data not shown).

When similar experiments were undertaken in the IL-6$^{-/-}$ mice, impressive differences were noted. At all time points after aerosol OVA exposure, increases in BAL cellularity were appreciated. The differences between WT and IL-6$^{-/-}$ mice were particularly prominent 24 and 48 h and 7 days after Ag challenge. At these time points, BAL fluids from IL-6$^{-/-}$ mice contained 4.7-, 3.1-, and 5-fold, respectively, more cells than BAL fluids from WT animals ($p < 0.01$ at all time points) (Fig. 1*A*). BAL eosinophil recovery was also enhanced in the IL-6$^{-/-}$ vs the WT mice at all time points after Ag challenge. Twenty four hours, 48 h, and 7 days after Ag challenge, BAL from IL-6$^{-/-}$ mice contained 6.7-, 2.8-, and 6.7-fold, respectively, greater numbers of eosinophils than BAL from WT animals ($p < 0.01$ for all time points) (Fig. 1*B*). As expected from this eosinophil influx, the percentage of BAL cells that were eosinophils was significantly greater and the percentage of BAL cells that were macrophages was significantly lower in the IL-6$^{-/-}$ than in the WT mice 4 and 24 h after OVA exposure (Fig. 1, *C* and *D*). In contrast, statistically significant differences in the percentage of BAL cells that were granulocytes or lymphocytes were not noted in comparisons of the WT and IL-6$^{-/-}$ mice at all time points (data not shown).

OVA challenge of WT mice caused a peribronchiolar and perivascular inflammatory response that contained significant tissue eosinophilia (Fig. 2*A*). This response was readily appreciated 24–48 h after OVA aerosol challenge and was not seen in animals that were not sensitized before aeroallergen challenge or that received systemic sensitization and nebulized saline (data not shown). In accord with the BAL finding, the tissue response was markedly enhanced in the IL-6$^{-/-}$ animals (Fig. 2). Compared with control WT mice, the IL-6$^{-/-}$ mice had denser pulmonary infiltrates around vessels and in the parenchyma. These infiltrates contained eosinophils and lymphocytes, the latter of which appeared more activated than in WT mice with more open chromatin and nucleoli. At 48 h after Ag challenge, the inflammatory infiltrate in the tissues was ~2.5 times greater in IL-6$^{-/-}$ mice than in WT controls. When the BAL and tissue findings are viewed in combination, they demonstrate that tissue and BAL inflammation and eosinophilia are markedly enhanced in IL-6$^{-/-}$ as vs WT mice.

Th2 cytokines in IL-6$^{-/-}$ and WT mice

Studies were next undertaken to determine whether endogenous IL-6 regulated OVA-induced Th2 cytokine production. IL-4, IL-5, and IL-13 were not appreciated in significant quantities in the BAL fluid of sensitized unchallenged mice, sensitized mice challenged with saline, or mice that were not sensitized and challenged with aerosol OVA (Fig. 3 and data not shown). IL-4, IL-5, and IL-13 proteins were readily appreciated in BAL fluids, and the mRNA moieties encoding these cytokines were readily appreciated in whole lung RNA from sensitized WT mice 24 h after OVA exposure (Figs. 3 and 4). Significant levels of IL-4, IL-5, and IL-13 protein and mRNA were not appreciated in sensitized IL-6$^{-/-}$ mice in the absence of aerosol exposure. However, aerosol exposure of sensitized IL-6-deficient mice resulted in exaggerated Th2 responses. When compared with WT controls, significantly increased levels of IL-4, IL-5, and IL-13 protein were detected in the BAL fluids from IL-6$^{-/-}$ mice. These differences were most prominent 24–48 h after Ag challenge ($p < 0.01$ for all cytokines). At 24 h, IL-6$^{-/-}$ mice produced 6.2-, 6.1-, and 14.1-fold

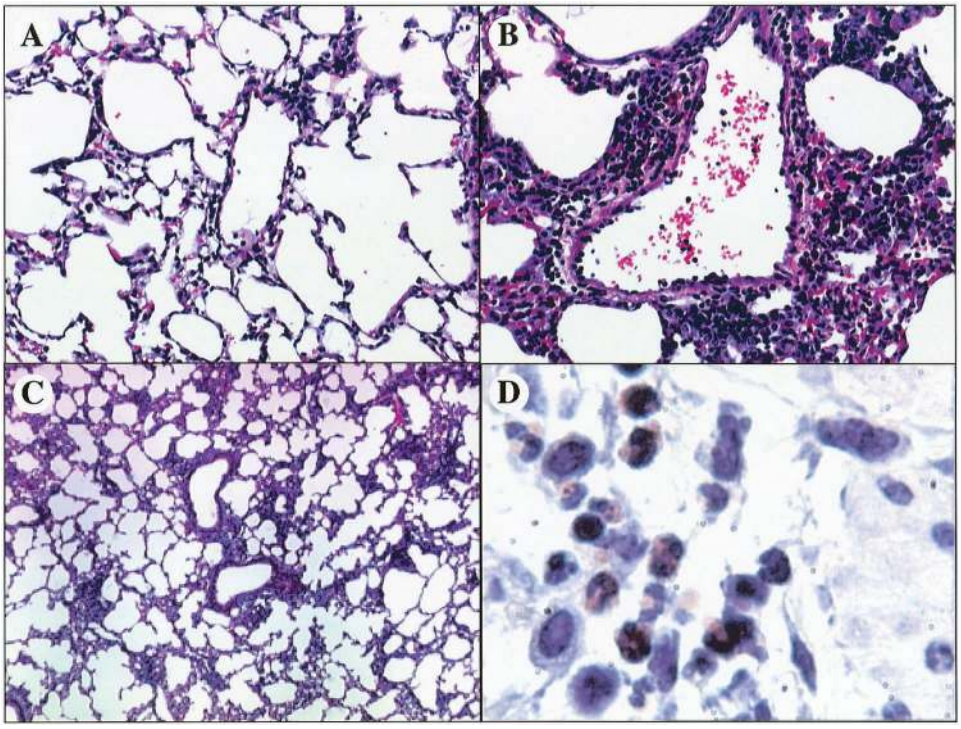

FIGURE 2. Comparison of the histology of OVA-challenged WT and IL-$6^{-/-}$ mice. WT (*A*) and IL-$6^{-/-}$ (*B*) mice were sensitized and challenged as described in *Materials and Methods*. The infiltrates in representative bronchovascular regions (hematoxylin and eosin stains) 24 h after aerosol exposure are illustrated (×50 magnification). Lower power magnification views of the lungs from IL-$6^{-/-}$ mice after OVA challenge can be seen in *C*. *D*, Peribronchial eosinophils on Congo red stain.

more IL-4, IL-5, and IL-13, respectively, than identically challenged WT controls (Fig. 3). Similar increases in the levels of IL-4, IL-5, and IL-13 mRNA were noted in lung RNA from IL-$6^{-/-}$ vs WT mice (Fig. 4). These studies demonstrate that the Th2 cytokines IL-4, IL-5, and IL-13 are produced in an exaggerated fashion in OVA-sensitized and -challenged IL-$6^{-/-}$ mice.

Th2 regulating cytokines in IL-$6^{-/-}$ and WT mice

Because IFN-γ can regulate Th2 cytokine production and effector function (5, 38), studies were undertaken to quantitate the levels of IFN-γ in lungs from sensitized and challenged IL-$6^{-/-}$ and WT animals. The levels of IFN-γ in the BAL fluids from WT mice before and after aeroallergen challenge were at or below the limits of detection of our assay. Similarly, low levels of IFN-γ were noted in the BAL fluids from the IL-$6^{-/-}$ mice before OVA challenge (Fig. 5). Four and 24 h after Ag challenge, a modest increase in the levels of BAL IFN-γ were noted in the IL-$6^{-/-}$ vs the WT mice (Fig. 5*A*). However, these increases did not reach statistical significance. Overall, the levels of IFN-γ produced by OVA-challenged IL-$6^{-/-}$ mice were greater than or equal to the levels in WT animals (data not shown). Thus, the exaggerated Th2 response in these mice is not due to decreased IFN-γ production.

TGF-β_1 has also been shown to regulate Th2 responses (39–41). Thus, studies were undertaken to determine whether endogenous IL-6 regulated the levels of TGF-β_1 induced by OVA in our modeling system. In WT mice, modest levels of immunoreactive TGF-β_1 were appreciated at baseline and these levels increased after OVA sensitization and challenge (Fig. 5*B*). Similar levels of TGF-β_1 were appreciated in IL-$6^{-/-}$ mice before Ag challenge. In contrast, significantly increased levels of TGF-β_1 were seen 4 h after OVA exposure. At this time point, BAL fluid from IL-$6^{-/-}$ mice contained 3.5-fold more TGF-β_1 than BAL from WT mice ($p < 0.05$). Thus, the exaggerated Th2 response in IL-$6^{-/-}$ mice is associated with the enhanced accumulation of TGF-β_1.

Chemokine production in IL-$6^{-/-}$ and WT mice

We next assayed the levels of protein and mRNA for a variety of chemokines that play important roles in this biologic system. In sensitized WT mice, the basal levels of BAL fluid eotaxin, MIP-1α, MIP-2, and JE/MCP-1 were at or near the limits of detection of our ELISA, and OVA challenge either did not alter or modestly increased the levels of these cytokine moieties (Fig. 6). In IL-$6^{-/-}$ mice, exaggerated levels of these chemokines were noted after OVA challenge. This effect was most prominent 4 and 24 h after OVA challenge ($p < 0.01$ for all cytokines at these time points). At the time points of maximal chemokine production by WT mice, BAL fluids from IL-$6^{-/-}$ mice contained 6.0-, 4.1-, 7.1-, and 35.2-fold more eotaxin, MIP-2, MIP-1α, and JE/MCP-1, respectively. In all cases, alterations in BAL cytokine levels were associated with comparable alterations in chemokine mRNA levels (Fig. 7). Thus, the enhanced inflammatory response in OVA-sensitized and -challenged IL-$6^{-/-}$ mice is associated with and potentially mediated by the enhanced elaboration of these eosinophil and mononuclear cell-regulating chemokines.

Exogenous IL-6 regulation of OVA-induced inflammation and eosinophilia

Because the studies noted above demonstrate exaggerated OVA-induced Th2 responses in IL-6-deficient mice, studies were undertaken to determine whether exogenous IL-6 could inhibit OVA-induced Th2 inflammation in otherwise normal animals. This was done by comparing the OVA-induced responses in WT mice and mice in which IL-6 is overexpressed in a lung/airway-specific fashion (CC10-IL-6 animals). In these experiments, the inflammatory effects of OVA sensitization and challenge of WT mice were similar to those described above. When similar experiments were undertaken with the CC10-IL-6 transgene (+) mice, a significant decrease in BAL cellularity was noted. IL-6 also caused a marked decrease in BAL eosinophil recovery and a marked decrease and increase in the percentage of BAL cells that were eosinophils and macrophages, respectively. These effects were most prominent 24 h after OVA challenge (data not shown). At this time point, BAL eosinophil recovery was 5.5% of that seen in WT animals ($p < 0.001$). Histology of the CC10-IL-6 mice 24 h after Ag challenge showed the scattered lymphoid aggregates characteristic of these animals at baseline (31). In contrast to the WT animals, no

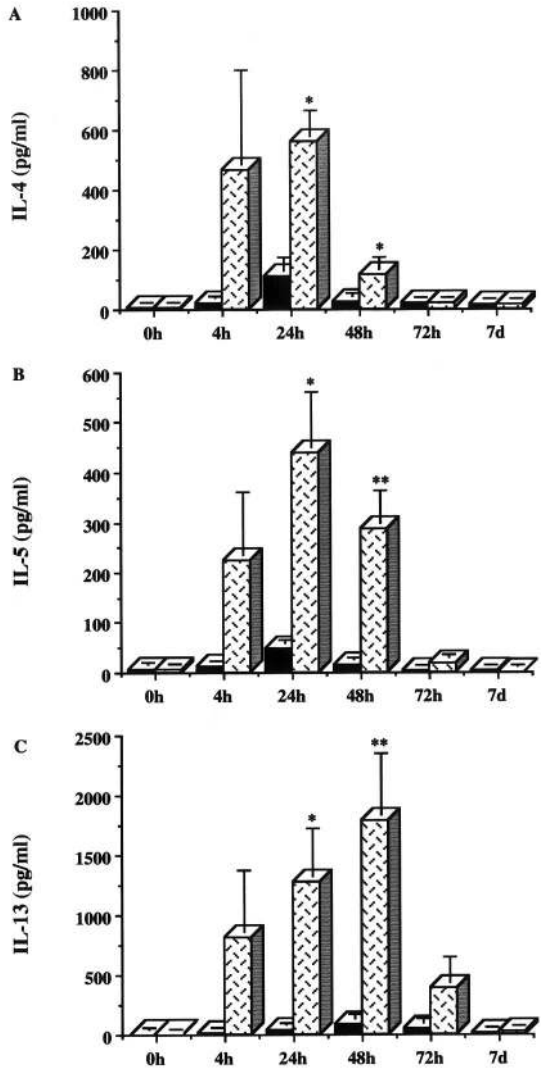

FIGURE 3. Th2 cytokine elaboration in WT and IL-6$^{-/-}$ mice. WT (■) and IL-6$^{-/-}$ mice (▨) were sensitized and challenged with OVA as described in *Materials and Methods*. At intervals before and after OVA challenge, the levels of BAL IL-4, IL-5, and IL-13 were evaluated by ELISA (*, $p < 0.01$; **, $p < 0.001$). The noted values represent the mean ± SEM of a minimum of five animals at each time point.

additional inflammation was noted (data not shown). In addition, tissue eosinophilia was virtually undetectable with the number of peribronchiolar eosinophils in the CC10-IL-6 mice being ~1% of the levels in identically manipulated WT mice ($p < 0.01$). When viewed in combination, these studies demonstrate that BAL and tissue inflammation and eosinophilia are markedly diminished in OVA-sensitized and -challenged CC10-IL-6 mice.

Th2 and Th2 regulating cytokines in CC10-IL-6 and WT mice

We next compared the production and expression of Th2 cytokines and Th2 regulating cytokines in CC10-IL-6 and WT mice. OVA sensitization and challenge caused a mild to moderate increase in IL-4, IL-5, and IL-13 protein and mRNA in WT mice (Figs. 8 and 9). IL-6 expression caused a significant decrease in the levels of these cytokines and the mRNA transcripts that encode them (Figs. 8 and 9). It also caused a significant decrease in the levels of BAL fluid TGF-β_1 (Fig. 8) and did not significantly increase the levels of IFN-γ protein or mRNA (data not shown). Thus, IL-6 inhibits OVA-induced Th2 cytokine and TGF-β_1 expression in this modeling system.

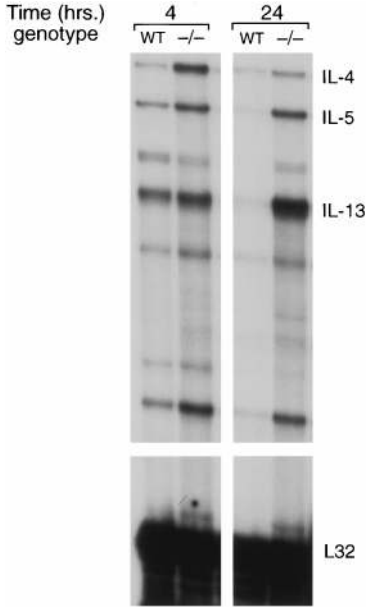

FIGURE 4. Comparison of Th2 cytokine mRNA in WT and IL-6$^{-/-}$ mice. WT and IL-6$^{-/-}$ mice were sensitized and challenged with OVA. Four or 24 h later, their lungs were harvested, mRNA was extracted, and the levels of mRNA encoding IL-4, IL-5, IL-13, and the housekeeping gene L32 were evaluated via RPA.

VCAM-1 expression in CC10-IL-6 and WT mice

Endothelial VCAM-1 staining was not detected in significant quantities in WT animals or CC10-IL-6 mice that were unchallenged, challenged with a saline aerosol, or challenged with Ag in

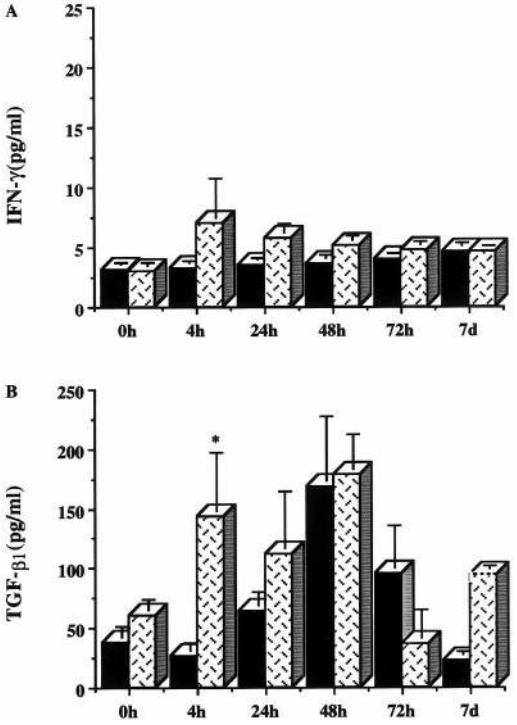

FIGURE 5. Comparison of the levels of IFN-γ and TGF-β_1 in BAL fluid from WT and IL-6$^{-/-}$ mice. WT (■) and IL-6$^{-/-}$ mice (stippled bars) were sensitized and challenged with OVA. The levels of BAL IFN-γ (*top*) and TGF-β_1 (*bottom*) were assessed at intervals after aerosol exposure. The noted values represent the mean ± SEM of a minimum of five mice at each time point. (*, $p < 0.05$)

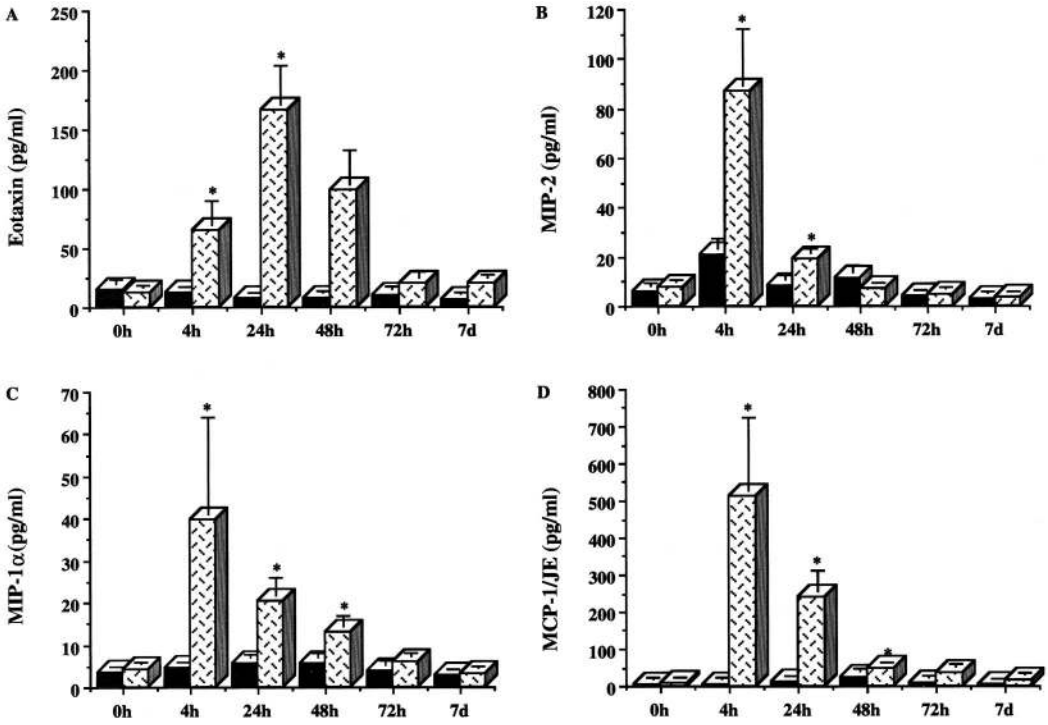

FIGURE 6. Comparison of the levels of chemokines in BAL fluid from WT and IL-6$^{-/-}$ mice. WT (■) and IL-6$^{-/-}$ (▨) mice were sensitized and challenged with OVA. At intervals after aerosol OVA exposure, the levels of BAL eotaxin (*A*), MIP-2 (*B*), MIP-1α (*C*), and MCP-1/JE (*D*) were assessed by ELISA. The noted values represent the mean ± SEM of a minimum of five animals at each time point (*, $p < 0.01$).

the absence of prior sensitization (data not shown). VCAM-1 staining of moderate intensity was noted in blood vessels and capillaries 4 and 24 h after challenge of sensitized WT animals. IL-6 markedly inhibited this inductive response because CC10-IL-6 mice had no or minimal VCAM-1 expression at both time points (Fig. 10).

Effect of IL-6 on BALb/c mice

C57BL/6 and BALB/c mice differ in their propensity to mount specific types of T cell-mediated responses with the former having a propensity toward Th1-dominated inflammation and the latter a propensity toward Th2-dominated inflammation (42). To determine whether IL-6 had similar inhibitory effects in the setting of a genetic background that is predisposed to Th2 immunity, CC10-IL-6 transgene (+) mice on a BALB/c background and appropriate littermate controls were sensitized and challenged as described above. In the transgene (−) littermate controls, a brisk response was elicited with an impressive increase in total BAL cellularity, an increase in BAL eosinophilia, and the accumulation of significant levels of BAL fluid, IL-4, IL-5, and IL-13. In the BALB/c CC10-IL-6 mice, total cellular recovery, eosinophil recovery, percent BAL eosinophilia, and the levels of BAL IL-4, IL-5, and IL-13 were all markedly diminished ($p < 0.01$ for all comparisons) (Figs. 11 and 12). Thus, IL-6 is a potent inhibitor of OVA-induced Th2 responses in BALB/c as well as C57BL/6 mice.

Effect of IL-6 on pulmonary physiology

Studies were also undertaken to determine whether the presence or absence of IL-6 altered airway responsiveness to the nonspecific agonist, methacholine, in C57BL/6 mice. This was done using barometric whole-body plethysmography with measurements of P_{enh}, a documented indicator of airway hyperresponsiveness after allergic sensitization in mice (37). In keeping with prior observations and the mild nature of our OVA challenge protocol, methacholine responsiveness was only modestly enhanced by OVA sensitization and challenge in WT mice (data not shown). In accord with prior studies with the CC10-IL-6 mice (31) and the present demonstration that IL-6 inhibits OVA-induced tissue inflammation, CC10-IL-6 mice manifest significantly decreased levels of responsiveness to methacholine at baseline and after OVA challenge when compared with WT controls (Fig. 13 and data not

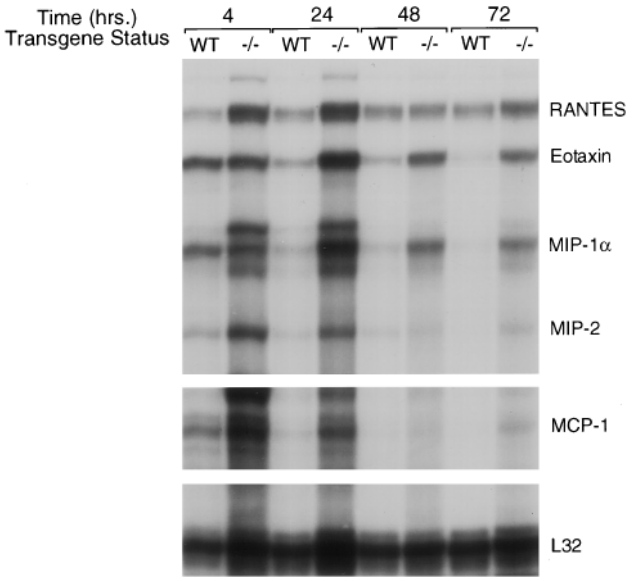

FIGURE 7. Comparison of the levels of chemokine mRNA in WT and IL-6$^{-/-}$ mice. WT and IL-6$^{-/-}$ mice were sensitized and challenged with OVA as described in *Materials and Methods*. Four, 24, 48, and 72 h after aerosol exposure, lungs were harvested, whole lung RNA was extracted, and the levels of eotaxin, MIP-1α, MIP-2, MCP-1, and L32 mRNA were evaluated via RPA.

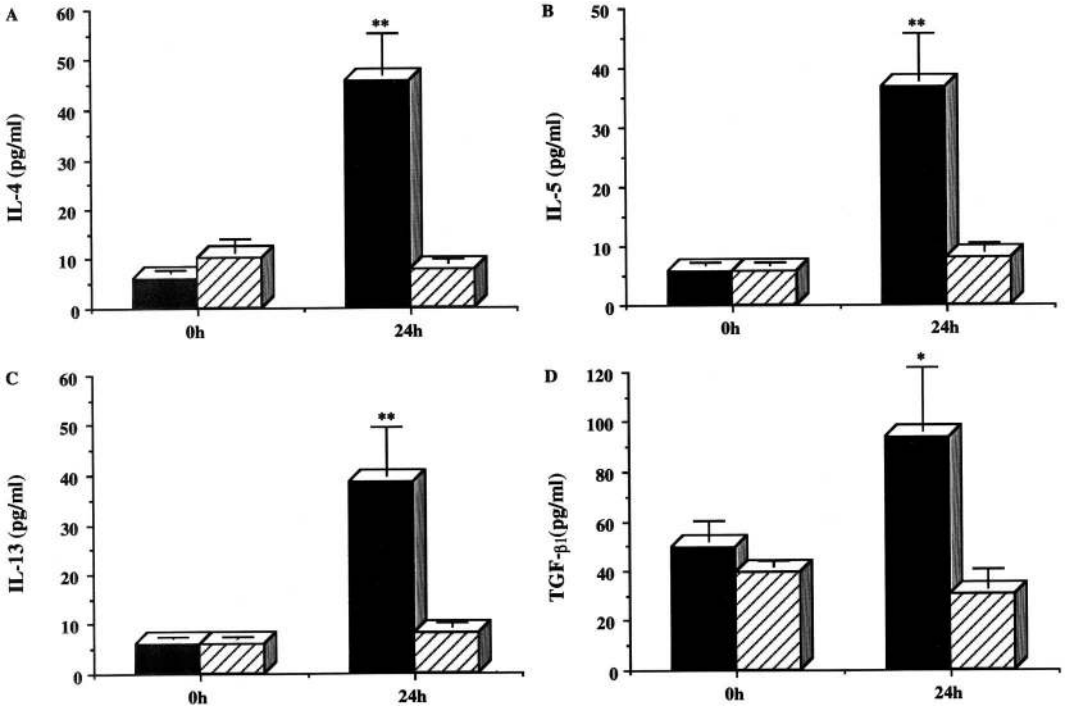

FIGURE 8. Comparison of the levels of Th2 cytokines and TGF-β_1 in BAL fluid from WT and CC10-IL-6 mice. WT (■) and CC10-IL-6 (▨) mice were sensitized and challenged with OVA. The levels of IL-4, IL-13, IL-5, and TGF-β_1 before and 24 h after aerosol challenge, were evaluated by ELISA. The noted values represent the mean ± SEM of a minimum of seven animals at each time point (*, $p < 0.01$; **, $p < 0.001$).

shown). At baseline, the IL-6$^{-/-}$ mice manifest enhanced airway responsiveness to methacholine. This approached did not achieve statistical significance. However, statistically significant increases in airway responsiveness were noted after OVA sensitization and challenge (Fig. 13). When viewed in combination, these studies demonstrate that the exaggerated expression of IL-6 diminishes, and a deficiency in IL-6 augments airway hyperresponsiveness in OVA-sensitized and -challenged C57BL/6 mice.

Discussion

The lung is exposed to airborne Ags and particulates more so than any other visceral organ. Under normal circumstances, these agents are neutralized and/or removed without overt inflammation or after a transient host response to the offending moiety. In contrast to these transient responses, patients with asthma have chronic lifelong airway inflammation, the pathogenesis of which is poorly understood. To gain insight into the mechanisms that are responsible for the transient nature of the responses in the normal lung and the systems that might be malfunctioning in chronic inflammatory disorders like asthma, we initiated studies designed to define the cytokines that inhibit and control airway inflammation. IL-6 was chosen for these studies because it can be pro-inflammatory (11, 17–21) or anti-inflammatory (12, 24–27) and can augment and/or inhibit (13, 19, 21, 22, 30) Th2 inflammation. This was done by comparing the inflammatory responses induced by aeroallergen sensitization and challenge in WT, IL-6$^{-/-}$, and CC10-IL-6 mice. These studies demonstrate, for the first time, that IL-6 deficiency results in exaggerated aeroallergen-induced Th2 inflammation, tissue and BAL eosinophilia, Th2 cytokine production, chemokine elaboration, and airway responsiveness to methacholine. They also demonstrate, for the first time, that the targeted overexpression of IL-6 in the airway decreases Th2 inflammation and eosinophilia, Th2 cytokine elaboration, endothelial VCAM-1 expression, and methacholine responsivity. When viewed in combination, these studies demonstrate that endogenous and exogenous IL-6 act to decrease Th2 inflammatory responses in the airway.

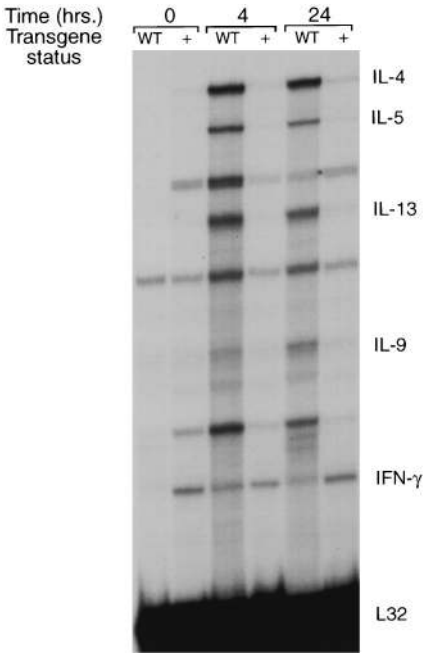

FIGURE 9. Comparison of the levels of mRNA encoding Th1 and Th2 cytokines in OVA-challenged WT and CC10-IL-6 mice. WT and CC10-IL-6 transgene (+) mice were sensitized and challenged with OVA. The levels of mRNA encoding Th2 and Th1 cytokines and the housekeeping gene L32 were evaluated before aerosol challenge (time = 0) and 4 and 24 h after OVA exposure via RPA as described in *Materials and Methods*.

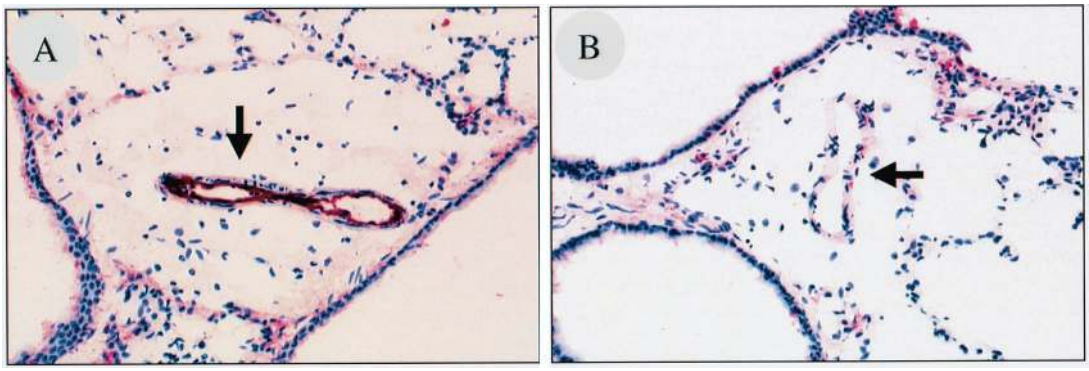

FIGURE 10. Comparison of the levels of VCAM-1 expression in WT and CC10-IL-6 mice. WT (*left*) and CC10-IL-6 (*right*) mice were sensitized and challenged with OVA, and VCAM-1 expression was assessed by IHC as described in *Materials and Methods*. The expression in representative bronchovascular regions 24 h after aerosol exposure are illustrated (×50 magnification). Vascular structures are highlighted with arrows.

Lymphocytes, eosinophils, and macrophages are characteristic of the tissue inflammatory response in asthma and models of asthmatic inflammation. An impressive volume of work has demonstrated that T cells play an important role in these responses and that Th2 cell-derived IL-4, IL-5, and IL-13 are involved in the pathogenesis of the increased IgE, eosinophilic infiltration, mucus metaplasia, and physiologic dysregulation characteristic of the disorder (4, 5, 35, 43). A substantial body of literature has also demonstrated that exaggerated IL-6 production is a feature of the asthmatic diathesis (10, 14–16). However, the contribution of IL-6 to Th2 inflammation is controversial. Some reports suggest that IL-6 can augment Th2 inflammation. Specifically, Rincon et al. reported that IL-6 polarized $CD4^+$ cells to effector Th2 cells by inducing IL-4 production (30), and Anguita demonstrated that IL-6 contributed to IL-4 production in a murine model of Lyme arthritis (19). In contrast, IL-6 has been shown to inhibit Th2 inflammation and/or cytokine production in models of Ag-induced arthritis and *M. tuberculosis* infection (21, 22), and IL-6 has been shown to oppose the Th2 promoting effects of IL-10 in a *Candida* infection model (13). Our studies address this controversy for the first time in the airway. They support the inhibitory point of view by demonstrating that IL-6 is a potent inhibitor of allergen-induced Th2 inflammation and tissue and BAL eosinophilia. In accord with these findings, endogenous and exogenous IL-6 were also potent inhibitors of aeroallergen-induced IL-4, IL-5, and IL-13 production. However, the mechanisms of these inhibitory effects are not clear. They may be the result of the ability of IL-6 to inhibit macrophage function, including the production of pro-inflammatory cytokines such as IL-1 and TNF (12, 24), induce the production of antiproteinases such as the tissue inhibitors of metalloproteinases (44), and/or stimulate the production of anti-inflammatory molecules such as IL-1 receptor antagonist (45). The ability of IL-6 to decrease endothelial cell VCAM-1 expression might also impede eosinophil entry into tissues because VCAM-1-very late antigen (VLA)4 binding appears to be crucial for this response (46). In addition, the exaggerated levels of TGF-β_1 in OVA-challenged IL-$6^{-/-}$ mice could also shift local tissue inflammation in a Th2 direction (41, 47) (see below). However, it is clear that IL-6 does not inhibit Th2 inflammation by augmenting local Th1 responses because increases and decreases in IFN-γ levels were not detected in challenged CC10-IL-6 and IL-$6^{-/-}$ mice, respectively.

Leukocyte chemotaxis is a important aspect of the pathogenesis of the complex inflammatory responses in the airway. Studies of these responses have demonstrated that multiple chemokines interact in a well-orchestrated network(s) that coordinates the recruitment and/or activation of the mononuclear cells and eosinophils seen in these disorders (48, 49). Eotaxin, JE/MCP-1, MIP-1α, and MIP-2 are well documented to contribute to these and similar tissue responses (36, 48–50). Our studies demonstrate, for the first time, that a deficiency in IL-6 results in the heightened elaboration of these chemokine moieties after OVA challenge of the airway. It is reasonable to speculate from these findings that the heightened levels of these chemokines contribute to the increased tissue and BAL inflammation seen in IL-$6^{-/-}$ mice. The well-documented ability of IL-5 and eotaxin to synergize in regulating eosinophil responses (50, 51) may also be important because both are increased in OVA-challenged IL-6-deficient animals. Recent studies from our laboratory and others have also demonstrated that Th2 cytokines, in particular IL-13, are potent inducers of chemokine elaboration by lung stromal cells (36, 52). Thus, it is likely that the increased chemokine elaboration seen in OVA-challenged IL-$6^{-/-}$ mice is due to the heightened production of Th2 cytokines in these animals. However, we cannot rule out the possibility that IL-6 has the ability to directly inhibit cellular chemokine elaboration and that this inhibiting mechanism is removed in the IL-$6^{-/-}$ mice. Thus, additional investigation will be required to define the mechanism(s) of the heightened chemokine response in IL-$6^{-/-}$ mice.

The processes that determine whether airway inflammatory responses are transient or chronic are poorly understood. Chan-Yung and Malo (7, 53) attempted to define these processes using occupational asthma as an investigational paradigm. Their studies of the natural histories of these diseases highlighted four phases: 1) the period of initial exposure; 2) the period of sensitization during which airway inflammation probably develops; 3) the period where symptoms initially are appreciated; and 4) the period of possible resolution in which some patients lose their symptoms and physiologic hyperresponsiveness, whereas others go on to persistent asthma. These studies also suggest that disease progression is associated with continuing airway inflammation (7, 53). Resolution of tissue inflammation is a complex process that can occur as a result of the removal of the offending agent, active inhibition of inflammation by cytokines such as IL-10, and the apoptosis and clearance of infiltrating leukocytes. Thus, chronic inflammation can be the result of the persistence or repeated exposure to the inciting stimulus, inadequate inhibition of tissue inflammation, heightened target tissue sensitivity and/or responsiveness to proinflammatory signals or defects in apoptosis and/or leukocyte removal. Our studies demonstrate, for the first time, that IL-6 contributes to the active inhibition of inflammation in the airway. This finding has a number of important implications. First, it suggests that abnormalities in the production and/or effector functions of IL-6 can contribute to the generation, severity, and/or chronicity of

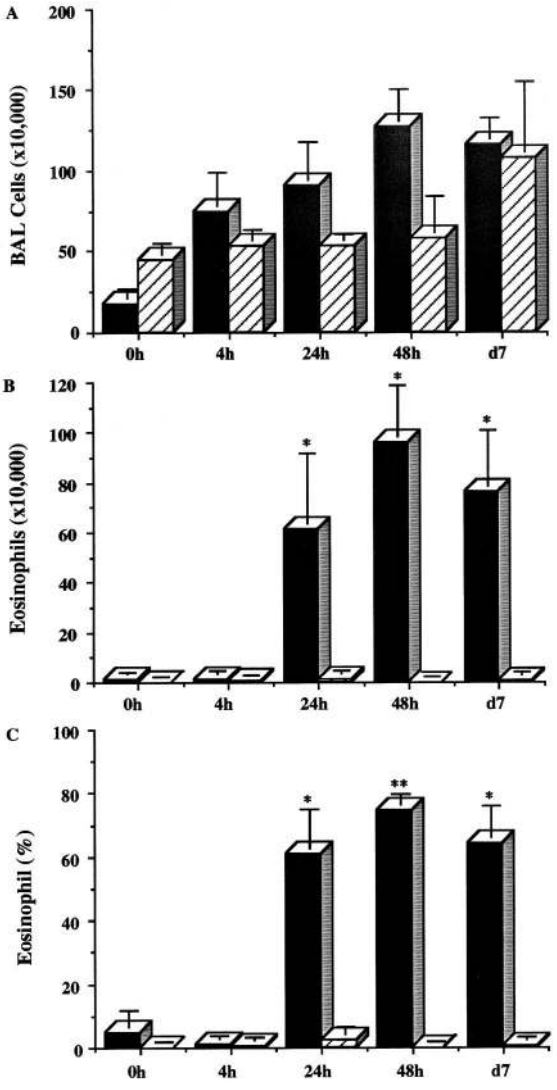

FIGURE 11. Comparison of the cellular profiles of BAL fluids from BALB/c WT and BALB/c CC10-IL-6 mice. WT and CC10-IL-6 mice on BALB/c genetic backgrounds were sensitized and challenged with OVA as described. BAL cell recovery (*top*), eosinophil recovery (*middle*), and the percentage of BAL cells that were eosinophils (*bottom*) are illustrated. The noted values represent the mean ± SEM of a minimum of four mice at each time point (*, $p < 0.01$; **, $p < 0.001$).

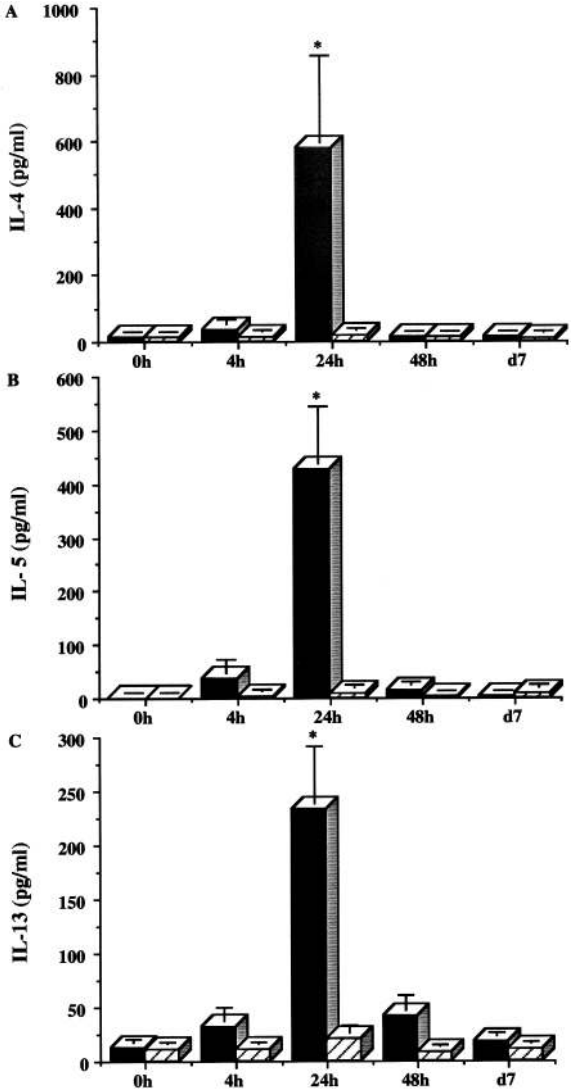

FIGURE 12. Th2 cytokines in WT and CC10-IL-6 mice on BALB/c backgrounds. WT (■) and CC10-IL-6 (▨) mice were sensitized and challenged with OVA. The levels of BAL fluid IL-4, IL-5, and IL-13 protein were evaluated by ELISA (*, $p < 0.01$). The noted values represent the mean ± SEM of a minimum of three animals at each time point.

asthma or other chronic inflammatory disorders of the airway. As a natural extension, polymorphisms of the IL-6 gene or the genes encoding the components of the IL-6 receptor and signal transduction pathway could contribute to the generation of the multiply diverse phenotypes seen in human asthma. In addition, the levels of IL-6, the soluble IL-6 receptor, and/or the presence of specific polymorphisms in the IL-6 system could serve as predictors of and biomarkers for chronic and/or severe asthmatic symptomatology.

IL-6 and TGF-β_1 interact in vivo and in vitro in a complex fashion. Studies from our laboratory and others have demonstrated that TGF-β_1 is a potent stimulator of IL-6 production and that TGF-β_1 and IL-6 can counterregulate each others' effector functions under appropriate circumstances (39, 54–56). In addition, TGF-β_1 and IL-6 can have similar (57), antagonistic (58), and additive or synergistic (59, 60) biologic effector profiles. Because TGF-β_1 is a potent anti-inflammatory cytokine, studies were undertaken to define the role of TGF-β_1 in the inflammation-regulating effects of IL-6. To our surprise, an inverse relationship was noted with increased TGF-β_1 in BAL fluid from IL-$6^{-/-}$ mice and decreased TGF-β_1 in BAL fluid from CC10-IL-6 animals. Because TGF-β_1 can shift T cell responses in a Th2 direction (40, 41, 47, 61) and stimulate chemokine production by epithelial cells (62), it is tempting to speculate that IL-6 normally inhibits TGF-β_1 elaboration and that the heightened Th2 response in IL-$6^{-/-}$ mice is due, at least in part, to the heightened accumulation of TGF-β_1 in these animals. However, this attribution of cause and effect must be viewed with caution. Eosinophils are a major source of TGF-β_1 in the asthmatic lung (63). Thus, the increased TGF-β_1 in BAL fluid from IL-$6^{-/-}$ mice could be a consequence of the increased Th2 response and increased eosinophil influx in these animals and not the cause of this exaggerated reaction. Similarly, the decrease in BAL TGF-β_1 in the CC10-IL-6 mice may reflect the decreased eosinophil influx in these animals and not an ability of IL-6 to regulate cellular TGF-β_1 elaboration. In these circumstances, eosinophil-derived TGF-β_1 may be acting to augment, or in a counterregulatory fashion, to inhibit the local Th2 inflammatory response. Regardless, these findings clearly demonstrate that the anti-inflammatory effects of IL-6 do not appear to correlate

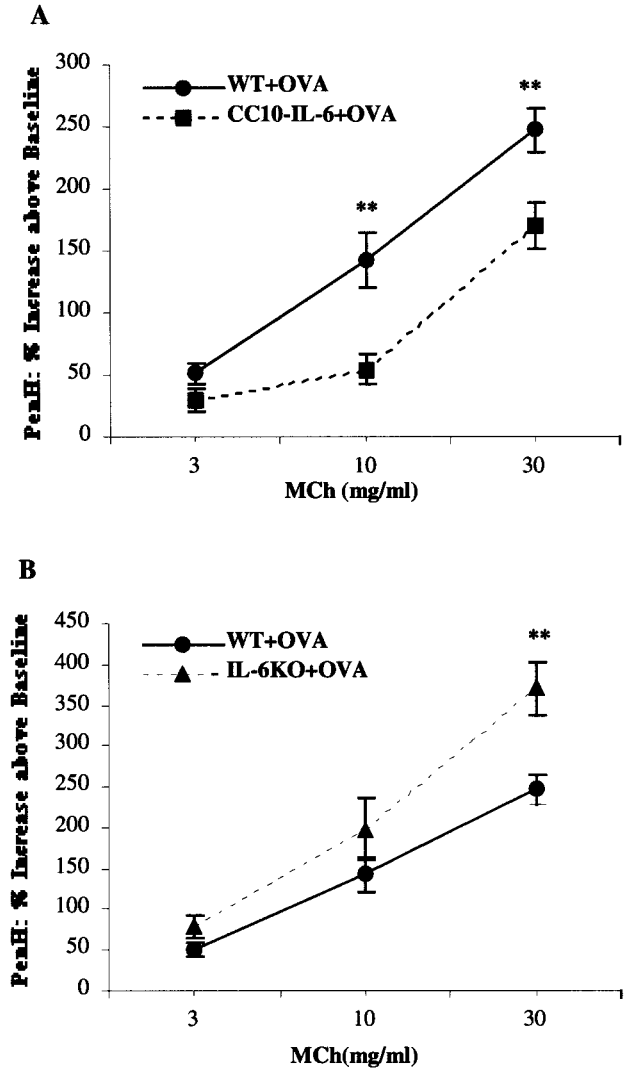

FIGURE 13. Characterization of airways hyperresponsiveness in OVA-challenged CC10-IL-6, IL-$6^{-/-}$, and WT mice. CC10-IL-6, IL-$6^{-/-}$, and WT mice were sensitized and challenged with OVA, and methacholine responsiveness was evaluated as described in *Materials and Methods*. The P_{enh} values in WT and CC10-IL-6 animals are illustrated in *A*. The methacholine responses in sensitized and challenged WT and IL-$6^{-/-}$ mice are expressed in *B* (**, $p < 0.01$).

with and are, therefore, not likely to be mediated solely by the effects of IL-6 on TGF-β_1 elaboration.

IL-6 is the prototype cytokine of the IL-6-type cytokine family. The members of this family (IL-6, IL-11, cardiotropin 1, oncostatin M, and ciliary neurotropic factor) share receptor components in their multimeric receptor complexes and have partially overlapping biologic effector profiles. These studies demonstrate that IL-6 inhibits Th2 inflammatory responses in the murine airway. Studies from our laboratory have demonstrated that IL-11 is a similarly impressive inhibitor of Th2 inflammation, eosinophilia, and cytokine elaboration in the airway (J. Wong and J. Elias, unpublished observation) and has protective effects in other injury systems (36, 64). When viewed in combination, these findings suggest that inhibition of Th2-dominated airway inflammation may be a general property of IL-6-type cytokines. They also suggest that IL-6-type cytokines and related compounds might be useful agents for the treatment of chronic Th2-dominated and other inflammatory responses in the airway.

In summary, these studies demonstrate that endogenous and exogenous IL-6 inhibit aeroallergen-induced Th2-dominated tissue inflammation. They also demonstrate that these IL-6-induced alterations in Th2 inflammation are not mediated by IFN-γ and can be appreciated in the context of Th2-prone (BALB/c) and Th1-prone (C57BL/6) murine genetic backgrounds. When these anti-inflammatory effects of IL-6 are viewed in combination with the ability of IL-6 to stimulate the acute phase response (8), antiproteinases (44), anti-inflammatory cytokines (45), and tissue fibrosis (32), it is clear that IL-6 is an important anti-inflammatory, counterregulatory, and healing cytokine in the airway.

Acknowledgments

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