Modulatory Role for Retinoid-related Orphan Receptor α in Allergen-induced Lung Inflammation

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Rationale: **Nuclear receptors play a critical role in the regulation of inflammation, thus representing attractive targets for the treatment of asthma.**

Objective: **In this study, we assess the potential regulatory function of retinoid-related orphan receptor α (RORα) in the adaptive immune response using ovalbumin (OVA)-induced airway inflammation as a model.**

Methods: **Allergen-induced inflammation was compared between wild-type (WT) and *staggerer* ($RORα^{sg/sg}$) mice, a natural mutant strain that is deficient in RORα expression.**

Measurements and Main Results: **Despite robust increases in OVA-specific IgE, $RORα^{sg/sg}$ mice developed significantly less pulmonary inflammation, mucous cell hyperplasia, and eosinophilia compared with similarly treated WT animals. Induction of Th2 cytokines, including interleukin (IL)-4, IL-5, and IL-13, was also significantly less in $RORα^{sg/sg}$ mice. Microarray analysis using lung RNA showed increased expression of many genes, previously implicated in inflammation, in OVA-treated WT mice. These include mucin Muc5b, the chloride channel calcium-activated 3 (Clca3), macrophage inflammatory protein (MIP) 1α and 1β, eotaxin-2, serum amyloid A3 (Saa3), and insulin-like growth factor 1 (Igf1). These genes were induced to a greater extent in OVA-treated WT mice relative to $RORα^{sg/sg}$ mice.**

Conclusions: **Our study demonstrates that mice deficient in RORα exhibit an attenuated allergic inflammatory response, indicating that RORα plays a critical role in the development of Th2-driven allergic lung inflammation in mice, and suggests that this nuclear receptor should be further evaluated as a potential asthma target.**

Keywords: asthma; inflammation; lung; nuclear receptor

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Nuclear receptors with antiinflammatory effects are promising pharmacological targets, and may offer novel therapeutic strategies for asthma.

What This Study Adds to the Field

Retinoid-related orphan receptor α (RORα) plays a critical role in the development of allergic lung inflammation, suggesting that this receptor should be further evaluated as a potential asthma target.

Asthma is a common, chronic inflammatory disease of the lung. Asthmatic episodes are triggered by a variety of environmental agents, and an increasing number of genetic factors are being identified that are important in the susceptibility to allergic airway disease (1, 2). Asthma is a complex genetic disorder characterized by local and systemic allergic inflammation that leads to airway hyperresponsiveness (AHR), mucosal edema, and mucus hypersecretion by goblet cells, major causes of airway obstruction (1, 3, 4). Allergen-induced inflammation involves interactions and cooperation of many cell types. Activation of $CD4^+$ lymphocytes plays a critical role in the early phase of this inflammatory cascade by releasing interleukin (IL)-4, IL-5, and IL-13. These Th2-type cytokines trigger a host of additional responses, including infiltration and activation of eosinophils, release of additional chemokines/cytokines, and induction of serum immunoglobulin E (IgE) production (5–7). Airway remodeling, defined as structural changes of the airways, is a general feature of asthma and includes increased collagen deposition, increased thickness of basement membrane, and airway smooth muscle cell hypertrophy (1, 8).

Nuclear receptors constitute a superfamily of ligand-dependent transcription factors that include receptors for steroid hormones, retinoic acid, thyroid hormone, and orphan receptors for which ligands have not yet been identified (9). Several nuclear receptors, including the glucocorticoid receptor (GR), the peroxisome proliferator-activated receptors (PPARs), and vitamin D receptor (VDR), have been reported to negatively regulate airway inflammation (10–14). VDR-deficient mice fail to develop experimental allergic asthma, thereby implicating VDR in the regulation of Th2-driven lung inflammation (14). A link between VDR and lung inflammation is further indicated by studies showing an association between VDR genetic variants and susceptibility to asthma (15, 16). Glucocorticoids, which mediate their action by binding GR, are effective antiinflammatory agents and the first-line treatment of asthma (17, 18). However, long-term treatment, particularly with oral steroids, has a number of adverse effects, including stunted growth in children and osteoporosis and high blood pressure in adults. Moreover, a subset of patients have disease that is refractory to glucocorticoids, further highlighting a need for additional therapies (18–20). Other nuclear receptors with antiinflammatory effects are promising pharmacologic targets, and may offer novel therapeutic strategies for asthma.

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The retinoid-related orphan receptor (ROR) subfamily of nuclear receptors consists of RORα, RORβ, and RORγ (named NR1F1 to −3 and RORA to −C by the Nuclear Receptor Nomenclature Committee and the Human Gene Nomenclature Committee, respectively) (21–23). Several studies have provided evidence for a role of RORα in the regulation of a number of immune functions (22, 24–29). *In vitro* stimulation of peritoneal macrophages from RORα$^{sg/sg}$ mice, a natural mutant strain with a disruption in RORα expression due to a deletion in the RORα gene, by LPS results in increased induction of IL-1α, IL-1β, and tumor necrosis factor α (TNF-α) (27). This enhanced production of cytokines may account for the greater sensitivity of RORα$^{sg/sg}$ mice to LPS-induced lung inflammation (30).

The aim of this study was to assess the role of RORα in adaptive immunity. To investigate this, we used ovalbumin (OVA)-induced airway inflammation in wild-type (WT) and RORα$^{sg/sg}$ mice as a model of allergic airway disease. We examined whether deficiency in RORα alters the induction of several well-established events in this model, including the degree of airway inflammation, mucous cell hyperplasia, AHR, and the release of several proinflammatory cytokines/chemokines. In addition, microarray analysis was performed to identify additional changes in gene expression associated with OVA-induced airway inflammation and to determine whether these changes would be affected by the lack of RORα expression. Our results demonstrate that RORα$^{sg/sg}$ mice exhibit a greatly reduced Th2-driven, airway inflammatory response, suggesting that RORα plays a regulatory role in the development of adaptive immune responses and might be a potential target for asthma therapy.

METHODS

Experimental Animals

Heterozygous C57/BL6 *staggerer* mice (RORα$^{+/sg}$) were purchased from Jackson Laboratories (Bar Harbor, ME) and bred at the National Institute of Environmental Health Sciences (NIEHS). Mice were genotyped by polymerase chain reaction (PCR) of tail DNA according to the instructions provided by Jackson Laboratories. RORα$^{sg/sg}$ mice were also easily identified by their *staggerer* phenotype (29, 31). WT littermates were used as control mice. Because RORα$^{sg/sg}$ mice weigh 20% less than WT mice, cell numbers and cytokine levels were adjusted for the differences in weight of the mice. All animal studies followed guidelines outlined by the NIH Guide for the Care and Use of Laboratory Animals, and protocols were approved by the Institutional Animal Care and Use Committee at the NIEHS and the University of North Carolina. NIH-31 feed and water were supplied *ad libitum* throughout the experiments.

OVA Sensitization and Challenge

Mice were sensitized by intraperitoneal injection with 20 μg of chicken egg OVA (grade V; Sigma, St. Louis, MO) emulsified in 200 μl of aluminum hydroxide adjuvant (Alhydrogel; Accurate Chemical and Scientific Corp., Westbury, NY) for 2 consecutive days as described (32, 33). Two weeks later, mice were challenged via the airways in a nose-only exposure chamber with an aerosol consisting of 1% OVA in saline for 5 consecutive days, 30 min/d. Control mice were primed with saline. Twenty-four hours after the last exposure, airway function was assessed and bronchoalveolar lavage (BAL) fluid and lung tissue collected for further analysis as described (30).

RESULTS

Development of OVA-induced Airway Inflammation Is Attenuated in RORα$^{sg/sg}$ Mice

To examine the role of RORα in the adaptive immune response, we compared OVA-induced airway inflammation in lungs of WT and RORα$^{sg/sg}$ mice. WT and RORα$^{sg/sg}$ mice were sensitized and challenged 2 wk later with OVA or saline as described in Methods. Mice were subsequently examined for several characteristics typically associated with OVA-induced allergic airway inflammation. We first compared the induction of histopathologic changes in the lungs of WT and RORα$^{sg/sg}$ mice. Hematoxylin and eosin–stained sections were scored using a semiquantitative histopathologic scoring system by a pathologist who was blinded to genotype and treatment. Two distinctive criteria were used: (*1*) the extent of the infiltration of inflammatory cells to the peribronchiolar/perivascular regions and (*2*) the degree of infiltration into the alveolar sacs. Lungs from saline-primed WT and RORα$^{sg/sg}$ control mice did not exhibit any significant pathologic alterations or differences. In contrast, a number of histopathologic differences were observed in lungs of both OVA-challenged WT and RORα$^{sg/sg}$ mice. The inflammatory response in the OVA-challenged WT and RORα$^{sg/sg}$ mice consisted of infiltration of lymphocytes and polymorphonuclear cells (mainly eosinophils) into the peribronchiolar and perivascular regions of the lung. These changes were less severe in the lungs of RORα$^{sg/sg}$ mice compared with those of WT mice. The peribronchiolar/perivascular infiltrates in lungs of OVA-challenged RORα$^{sg/sg}$ mice ranged from minimal to mild and scored an average of 2.1, whereas those in lungs of OVA-challenged WT mice were moderate and scored an average of 3.3 (Figure 1; $p < 0.001$). In addition to the peribronchiolar/perivascular changes, an accumulation of lymphocytes, neutrophils, eosinophils, and macrophages was observed within the alveolar sacs of lungs from both OVA-challenged WT and RORα$^{sg/sg}$ mice.

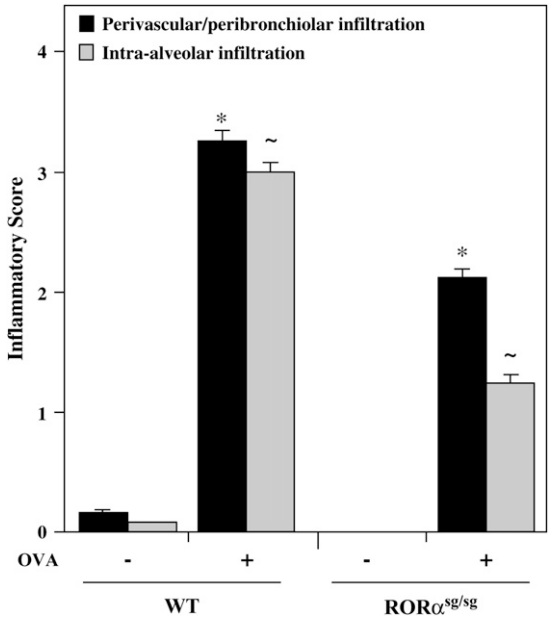

Figure 1. Comparison of inflammatory scores between wild-type (WT) and RORα$^{sg/sg}$ mice. Hematoxylin and eosin–stained sections of lungs from WT and RORα$^{sg/sg}$ mice (n = 25–28 in each group) that were challenged by either saline or ovalbumin (OVA) were scored in an unbiased manner from 0 to 4 for the extent of inflammatory cell infiltration, as described in Methods. 0–1 indicates no or little inflammation, and 4 represents severe inflammation. Perivascular/peribronchiolar infiltrates (*black bars*) and intraalveolar infiltrates (*gray bars*) were scored separately. The average histology inflammatory scores from OVA- and saline-challenged WT and RORα$^{sg/sg}$ mice were calculated and plotted. Significant differences between OVA-challenged WT and RORα$^{sg/sg}$ mice are indicated (*$p < 0.001$; ~$p < 0.001$).

The OVA-challenged WT mice contained a moderate inflammatory cell infiltration within the alveolar sacs, whereas the extent of alveolar infiltrates was consistently less in lungs from OVA-challenged RORα$^{sg/sg}$ mice and ranged from minimal to mild. The alveolar sacs in OVA-challenged WT mice also contained significant numbers of multinucleated giant cells, whereas few multinucleated giant cells were observed occasionally in the alveolar sacs from OVA-challenged RORα$^{sg/sg}$ mice. The infiltration of inflammatory cells in alveolar sacs of lungs from OVA-challenged WT mice scored an average of 3.0, whereas those from OVA-challenged RORα$^{sg/sg}$ mice averaged 1.2 (Figure 1; $p < 0.001$).

Mucous Cell Hyperplasia

Allergen-induced inflammation leads to mucous cell hyperplasia and airway obstruction. We therefore examined the extent of mucus production by periodic acid Schiff (PAS) staining of sections of lungs of OVA-challenged WT and RORα$^{sg/sg}$ mice. As shown in Figures E1A and E1C of the online supplement, very few PAS-positive cells were observed in the airways from saline-challenged WT and RORα$^{sg/sg}$ control mice. The number of PAS-positive cells significantly increased after OVA exposure (Figures E1B and E1D). However, the extent of PAS staining was significantly lower in OVA-challenged RORα$^{sg/sg}$ mice than in OVA-challenged WT mice. The average PAS score for OVA-challenged RORα$^{sg/sg}$ mice was 1.3 compared with 2.4 for the airways from OVA-challenged WT mice (Figure 2).

On the basis of these histologic observations, one may conclude that mice deficient in RORα exhibit an attenuated OVA-induced inflammatory response in the lung. These findings are in agreement with the concept that RORα plays a role in the regulation of the adaptive immune response.

Decreased Accumulation of BAL Fluid Inflammatory Cells in OVA-challenged RORα$^{sg/sg}$ Mice

The Th2-type response by $CD4^+$ lymphocytes is part of the early events in the inflammatory cascade in asthma (34, 35). The release of Th2-type cytokines, including IL-4 and IL-13, is critical in eliciting the recruitment of other inflammatory cells. We therefore compared the accumulation of inflammatory cells in BAL fluids from OVA-challenged WT and RORα$^{sg/sg}$ mice. As shown in Figure 3, the total number of inflammatory cells in BAL fluids from WT and RORα$^{sg/sg}$ control groups did not differ significantly ($p > 0.05$). OVA exposure dramatically increased the number of inflammatory cells; however, BAL fluids from OVA-challenged RORα$^{sg/sg}$ mice contained a significantly lower number of cells than those from OVA-challenged WT mice ($18.7 \times 10^5 \pm 2.7$ vs. $5.3 \times 10^5 \pm 0.4$, $p < 0.0001$). Analysis of the different types of inflammatory cells showed that the number of macrophages in BAL fluid was decreased in both OVA-challenged groups (Figure 4A); however, no significant difference was observed between WT and RORα$^{sg/sg}$ control mice. In contrast, the BAL fluids from OVA-challenged RORα$^{sg/sg}$ mice contained a significantly lower number of eosinophils and neutrophils than those from OVA-challenged WT mice (Figures 4B and 4C; $p < 0.0001$). In addition, the total number of lymphocytes in BAL fluid from OVA-challenged RORα$^{sg/sg}$ mice was much lower than that from OVA-challenged WT mice (Figure 5A; $p < 0.0001$). To examine the effect of RORα deficiency on the different subsets of lymphocytes recruited to the airway, flow cytometric analysis was performed on cells isolated from BAL fluid from OVA-challenged WT and RORα$^{sg/sg}$ mice, and the saline-challenged control groups. This analysis showed that the numbers of $CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$, and B220 cells were significantly

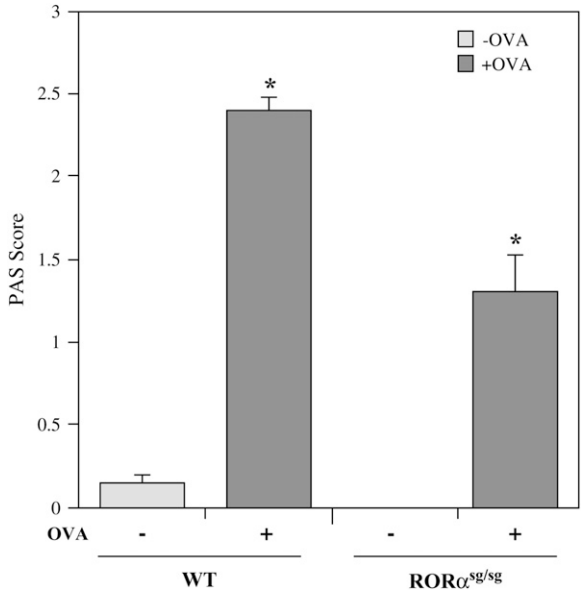

Figure 2. OVA-induced mucous cell hyperplasia in airway epithelium of WT and RORα$^{sg/sg}$ mice. Sections of airways from saline- and OVA-challenged WT and RORα$^{sg/sg}$ mice (n = 25–28 in each group) were stained by periodic acid Schiff (PAS) and then scored in an unbiased manner as described in METHODS. The average PAS scores were plotted. The difference between the average PAS score of OVA-challenged WT and RORα$^{sg/sg}$ mice was statistically significant (*$p < 0.001$).

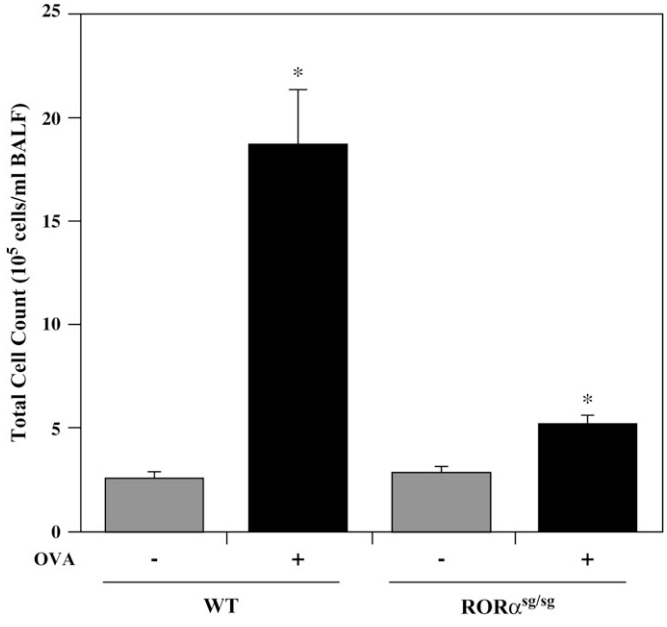

Figure 3. Decreased OVA-induced inflammation in RORα$^{sg/sg}$ mice correlates with reduced accumulation of inflammatory cells in bronchoalveolar lavage fluid (BALF). Mice (n = 25–28 in each group) were sensitized with OVA and 2 wk later challenged with either OVA or saline. Twenty-four hours after the final saline or OVA challenge, mice were killed, BALF collected, and the total number of inflammatory cells determined in a Coulter counter. A significant difference in the total number of inflammatory cells was observed between OVA-challenged WT and RORα$^{sg/sg}$ mice (*$p < 0.0001$).

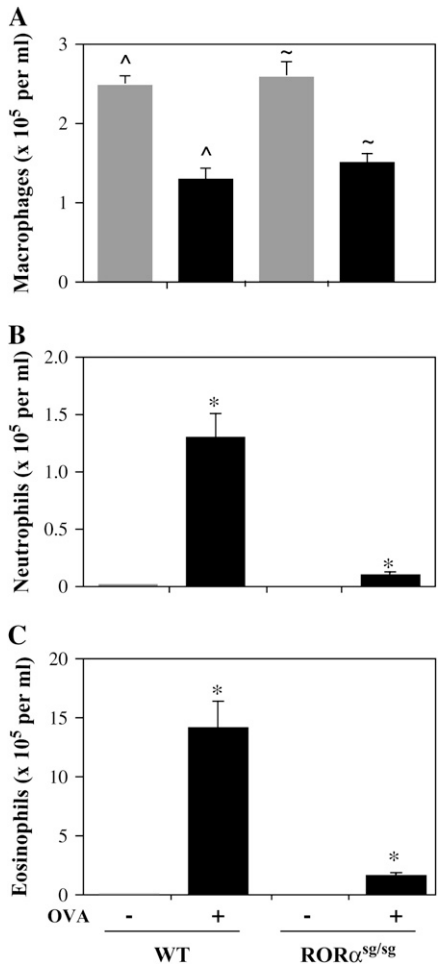

Figure 4. Reduced infiltration of eosinophils and neutrophils in BALF from OVA-challenged RORα$^{sg/sg}$ mice compared with WT mice. After saline and OVA exposure, BALF (n = 25–28 in each group) was collected, and the number of macrophages, neutrophils, and eosinophils were determined as described in METHODS. (*A*) The number of macrophages decreased in both OVA-challenged WT and RORα$^{sg/sg}$ mice (^$p < 0.001$; ~$p < 0.001$). Significant differences (*$p < 0.0001$) were observed in the number of (*B*) neutrophils and (*C*) eosinophils between OVA-challenged WT and OVA-challenged RORα$^{sg/sg}$ mice.

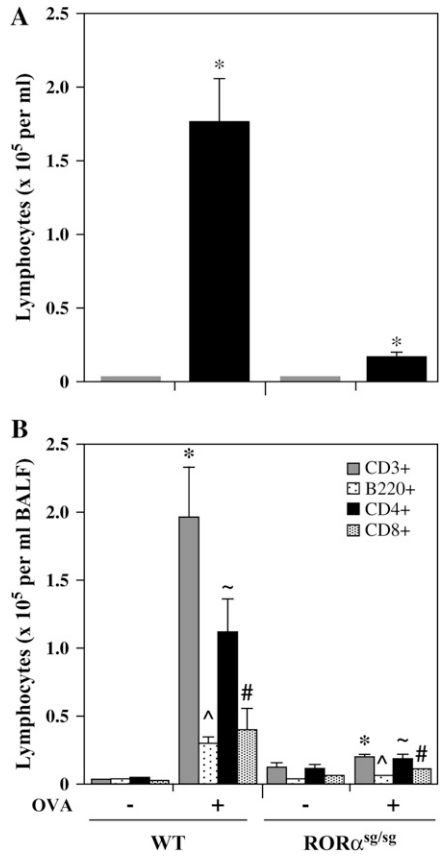

Figure 5. Reduced accumulation of various lymphocyte subpopulations in BALF from OVA-challenged RORα$^{sg/sg}$ mice compared with WT mice. BALF was collected and analyzed for the total number of lymphocytes as described in METHODS. Various lymphocyte subpopulations were examined by flow cytometry using fluorescein isothiocyanate– or phycoerythrin-conjugated CD4, CD3, CD8, and B220 antibodies. (*A*) Comparison of total number of lymphocytes. A significant difference in the total number of lymphocytes was observed between OVA-challenged WT and RORα$^{sg/sg}$ mice (*$p < 0.0001$; n = 25–28 in each group). (*B*) Comparison of different lymphocyte subpopulations (n = 9–16 in each group). Significant differences were observed in the $CD3^+$ (*$p < 0.0001$), $CD3^+CD4^+$ (~$p < 0.0001$), $CD3^+CD8^+$ (#$p < 0.0001$), and $B220^+$ (^$p < 0.0001$) lymphocytes between OVA-challenged WT mice and OVA-challenged RORα$^{sg/sg}$ mice.

($p < 0.0001$) reduced in OVA-challenged RORα$^{sg/sg}$ mice compared with OVA-challenged WT mice (Figure 5B). No significant differences were observed between the saline-challenged (control) groups. The greatly reduced recruitment of inflammatory cells observed in the lungs of OVA-challenged RORα$^{sg/sg}$ mice compared with those of WT mice is in agreement with our conclusion that RORα$^{sg/sg}$ mice are less susceptible to allergic airway inflammation and supports a modulatory role for this nuclear receptor in the pathogenesis of asthma.

Attenuated Release of Cytokines/Chemokines in OVA-challenged RORα$^{sg/sg}$ Mice

Cytokines and chemokines play a critical role in mediating many steps in the inflammatory cascade, including migration and activation of various inflammatory cells. Therefore, we evaluated whether RORα influenced the production of the cytokines IL-4, IL-5, and IL-13 and chemokines eotaxin-1 and thymus- and activation-regulated chemokine (TARC) during OVA-induced airway inflammation. IL-4, IL-5, and IL-13 levels were increased in BAL fluids from both OVA-challenged RORα$^{sg/sg}$ and WT mice; however, the induction of these cytokines in OVA-challenged RORα$^{sg/sg}$ mice was significantly less than in OVA-challenged WT mice (Figures 6A, 6D, and 6E). For example, OVA-challenged WT mice showed a 42-fold increase in levels of IL-13, whereas only a fourfold increase was observed in OVA-challenged RORα$^{sg/sg}$ mice (Figure 6A). Similarly, exposure of WT mice to OVA challenge induced eotaxin-1 in BAL fluids approximately 11-fold, whereas only a fivefold increase was observed in OVA-challenged RORα$^{sg/sg}$ mice (Figure 6B). TARC was induced approximately 60- and 10-fold, respectively (Figure 6C). No change in the level of IL-2, IL-10, IL-12, or TNF-α was observed in either OVA-challenged WT or RORα$^{sg/sg}$ mice (not shown). These observations support the conclusion that RORα plays an important role in the development of a Th2-driven airway inflammatory response in the lung.

Levels of Serum IgE Are Increased in RORα$^{sg/sg}$ Mice

Allergen-induced inflammation is associated with a significant increase in serum IgE levels. As shown in Figure 7A, the level

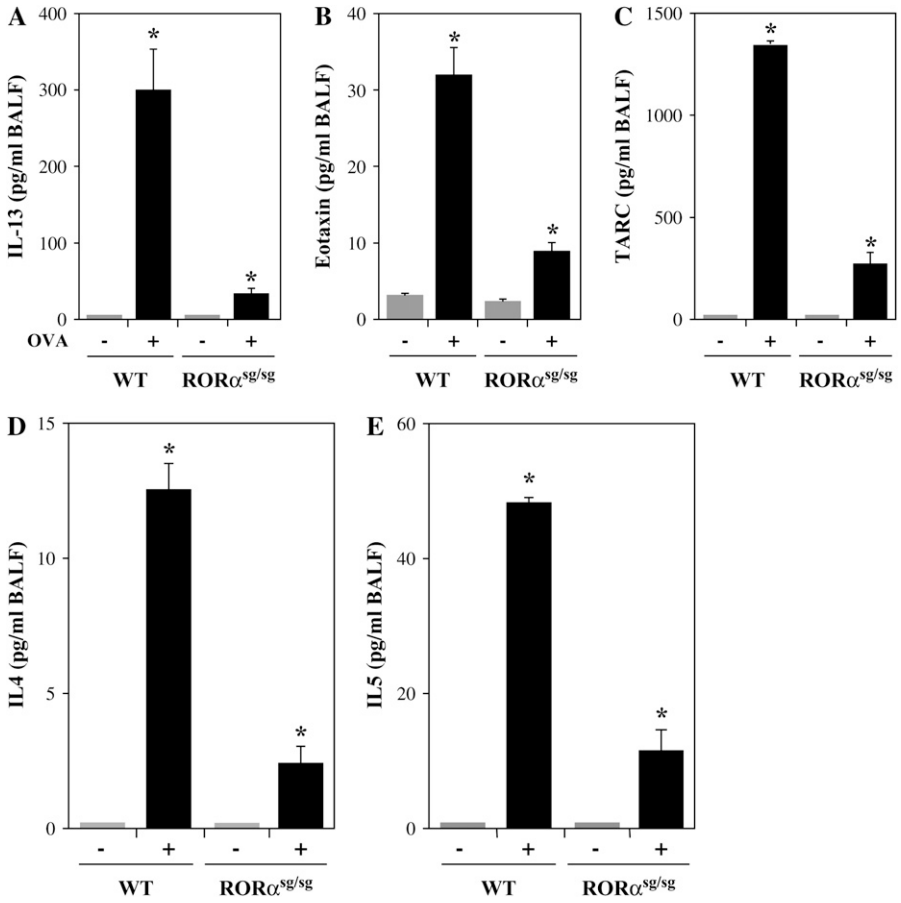

Figure 6. Reduced susceptibility of RORα$^{sg/sg}$ mice to OVA-induced inflammation correlates with decreased release of several cytokines/chemokines. BALF from saline- and OVA-challenged WT and RORα$^{sg/sg}$ mice (n = 25–28 in each group) was collected and the level of (*A*) interleukin (IL)-13, (*B*) eotaxin, (*C*) TARC, (*D*) IL-4, and (*E*) IL-5 (E) analyzed by ELISA. Significant differences (*$p < 0.0001$) were observed in the level of these cytokines/chemokines between OVA-challenged WT and OVA-challenged RORα$^{sg/sg}$ mice.

of total serum IgE was greatly elevated in unsensitized RORα$^{sg/sg}$ mice compared with WT mice. These data suggest a role for RORα signaling pathway in controlling IgE levels. OVA challenge increased OVA-specific serum IgE significantly in both WT and RORα$^{sg/sg}$ mice (Figure 7B). This increase was even more pronounced in RORα$^{sg/sg}$ than in WT mice and may involve a mechanism similar to the one responsible for the elevated levels of total IgE. These observations indicate that the elevation in total IgE or OVA-specific IgE in RORα$^{sg/sg}$ mice does not correlate with the extent of pulmonary inflammation.

AHR in RORα$^{sg/sg}$ and WT Mice after OVA Challenge

Lung resistance (RL) was evaluated in saline- and OVA-treated WT and RORα$^{sg/sg}$ mice at baseline and in response to a graded methacholine (MCh) challenge. As shown in Figure 8, baseline resistance (RL) increased after OVA sensitization/challenge in WT animals (1.3 ± 0.07 to 1.9 ± 0.15 cm $H_2O \cdot s/ml$, $p = 0.0009$). In contrast, baseline RL between RORα$^{sg/sg}$ saline and RORα$^{sg/sg}$ OVA groups was not significantly different (1.5 ± 0.05 vs. 1.7 ± 0.1 cm $H_2O \cdot s/ml$, $p = 0.12$). At lower doses of Mch, RL was similar between OVA-exposed WT and OVA-exposed

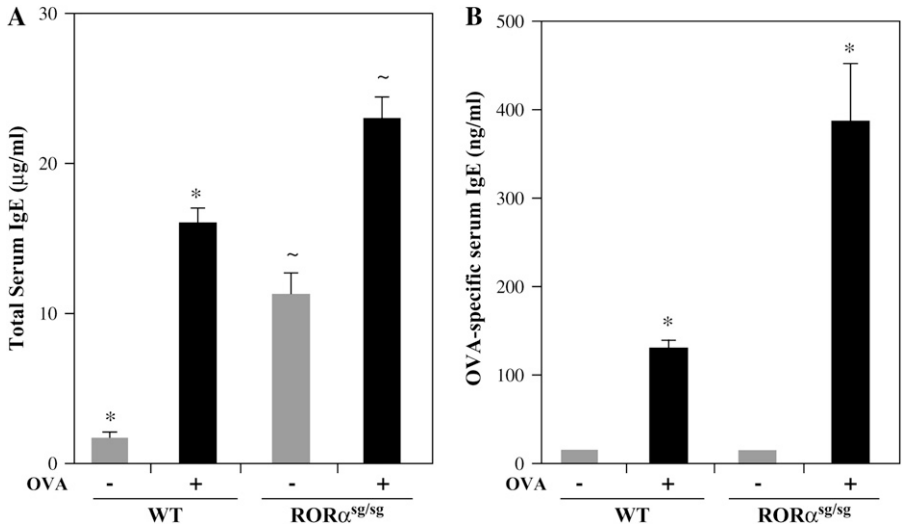

Figure 7. Comparison of the level of IgE in serum from saline- and OVA-challenged WT and RORα$^{sg/sg}$ mice. Sera from saline- and OVA-challenged WT and RORα$^{sg/sg}$ mice (n = 12–16 in each group) were collected and assayed for (*A*) total IgE and (*B*) OVA-specific IgE as described in METHODS. A significant difference (*$p < 0.0001$; ~$p < 0.001$) was observed in total serum IgE between saline-challenged RORα$^{sg/sg}$ and WT mice. A significant difference (*$p < 0.0001$) was observed in OVA-specific IgE between OVA-challenged WT and RORα$^{sg/sg}$ mice and saline-challenged mice.

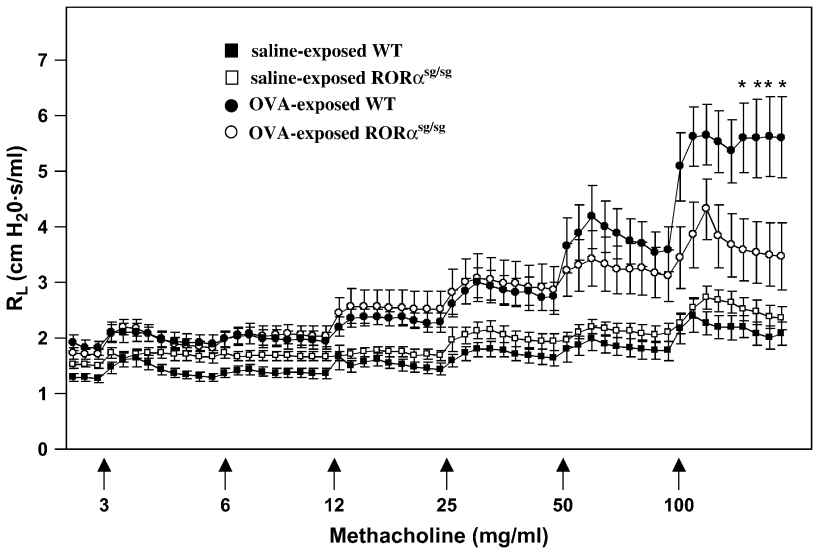

Figure 8. OVA-induced changes in resistance (R_L) and airway hyperresponsiveness in RORα$^{sg/sg}$ mice. Baseline and methacholine (MCh)-induced changes in R_L were measured in saline-exposed WT (*filled squares*; n = 16), saline-exposed RORα$^{sg/sg}$ (*open squares*; n = 16), OVA-exposed WT (*filled circles*; n = 12), and OVA-exposed RORα$^{sg/sg}$ (*open circles*; n = 12) mice. Five measurements were taken before MCh challenge, and measurements were taken every 20 s after each MCh dose. Data represent mean R_L at each interval ± SEM. *$p < 0.05$ compared with RORα$^{sg/sg}$ group.

RORα$^{sg/sg}$ groups. However, at higher doses of Mch, R_L was significantly higher in the OVA-exposed WT mice. These data suggest that airflow obstruction does not develop in OVA-treated RORα$^{sg/sg}$ mice, but that lack of RORα does not prevent the development of AHR.

Changes in Gene Expression in Lungs from OVA-challenged WT and RORα$^{sg/sg}$ Mice

To examine the effect of RORα on the expression of additional inflammatory biomarkers, we compared changes in gene expression induced during allergic inflammation in whole lungs of OVA-challenged WT and RORα$^{sg/sg}$ mice by microarray analysis using Agilent (Palo Alto, CA) oligo-chips representing approximately 20,000 genes. We first analyzed changes in gene expression in lungs from OVA-challenged WT versus saline-challenged WT mice. This analysis identified 1,545 changes in gene expression that were increased by 1.5-fold or more in lungs of OVA-challenged mice and 630 genes that were reduced by 50% or more. Table 1 provides a selective listing of several genes induced in lungs from OVA-challenged WT mice. The complete listing of all the changes in gene expression identified in the different comparisons is available at http://dir.niehs.nih.gov/microarray/jetten/home.htm. The chloride channel calcium-activated 3 (Clca3), resistin-like a (Retnla), and several chemokines, including Ccl4 (macrophage inflammatory protein 1β [MIP-1β]), Ccl3 (MIP-1α), Ccl8 (monocyte chemotactic protein 2 [MCP-2]), Ccl17 (TARC), and Ccl24 (eotaxin-2), RANTES (regulated upon activation, T-cell expressed and secreted; Ccl5), tissue inhibitor of metalloproteinase (Timp), and insulin-like growth factor 1 (Igf1), were among the genes most highly induced in lungs from OVA-challenged WT mice. Enhanced expression of many of these genes has been previously implicated in inflammation (5, 7, 36). Comparison of gene expression between saline-challenged WT and saline-challenged RORα$^{sg/sg}$ mice showed a number of moderate changes; however, the expression of most genes listed in Table 1 did not differ greatly between saline-challenged WT and RORα$^{sg/sg}$ mice (data not shown). Comparison of RNA expression between lungs of OVA-challenged WT and OVA-challenged RORα$^{sg/sg}$ mice revealed a great number of differences (for complete listing, *see* http://dir.niehs.nih.gov/microarray/jetten/home.htm). Although many of the same genes were induced or repressed in lungs of both OVA-challenged WT and OVA-challenged RORα$^{sg/sg}$, the expression of many of the RNAs were induced to a much smaller degree in OVA-challenged RORα$^{sg/sg}$ versus OVA-challenged WT mice (Table 1). The differential expression of several genes, identified by microarray analysis, was confirmed by real-time quantitative reverse transcriptase–PCR (Figure 9). Expression of Ccl17, Ccl24, Saa3, and Igf1 was induced to a much greater extent in OVA-challenged WT than in OVA-challenged RORα$^{sg/sg}$ mice. For example, Ccl17 and Ccl24 RNAs were induced, respectively, to levels 7.6- and 8.4-fold greater in OVA-challenged WT mice than in OVA-challenged RORα$^{sg/sg}$ mice. In addition to chemokines, a number of other genes were induced to a greater extent in WT than in RORα$^{sg/sg}$ mice. These include the serum amyloid proteins Saa1 and Saa3, systemic inflammation markers that are positively associated with bronchial asthma (37). MUC5b and, to a lesser degree, MUC5ac were among the genes induced in OVA-challenged WT mice, although induction of these genes did not significantly change in OVA-challenged RORα$^{sg/sg}$ mice. These results are in agreement with the observed elevated increase in the number of mucous cells in OVA-challenged WT compared with OVA-challenged RORα$^{sg/sg}$ mice. The reduced induction of Clca3, which has been implicated in the regulation of mucus production (36, 38), may at least in part be responsible for the observed reduction in mucous cell hyperplasia in RORα$^{sg/sg}$ mice. Interestingly, not all genes were affected by RORα to the same extent, suggesting that the effect of RORα on gene expression may be selective. Our microarray analysis shows increased expression of many inflammatory genes in OVA-treated WT mice. These genes are induced to a greater extent in OVA-treated WT mice relative to RORα$^{sg/sg}$ mice. These data support our conclusion that mice deficient in RORα exhibit an attenuated allergic inflammatory response.

DISCUSSION

The purpose of this study was to assess the function of the nuclear receptor RORα in the pathogenesis of allergen-induced airway inflammation. To study the potential role of this receptor, we used RORα$^{sg/sg}$ mice as a model to examine the effect of RORα deficiency on several well-known events associated with allergen-induced airway inflammation. Histologic observations show that OVA challenge induces infiltration of inflammatory cells, including eosinophils, neutrophils, and lymphocytes, into peribronchiolar and perivascular regions and within the alveolar

TABLE 1. SELECTIVE LIST OF GENES INDUCED IN LUNGS FROM OVALBUMIN-CHALLENGED WILD-TYPE VERSUS SALINE-CHALLENGED (CONTROL) WILD-TYPE OR OVALBUMIN-CHALLENGED RORα$^{sg/sg}$ MICE

			Level of mRNA (*Ratio*)	
Genbank Entry	Description	Symbol	OVA-WT/ Control-WT	OVA-WT/ OVA-RORα$^{sg/sg}$
NM_011315	Serum amyloid A 3	Saa3	15	10
NM_019577	Small inducible cytokine A24 (eotaxin-2)	Ccl24	28	8.4
NM_011332	Chemokine (C-C motif) ligand 17 (TARC)	Ccl17	20	7.6
NM_013652	Chemokine (C-C motif) ligand 4 (MIP-1β)	Ccl4	5.0	5.0
NM_008491	Lipocalin 2	Lcn2	10	4.6
NM_011337	Chemokine (C-C motif) ligand 3 (MIP-1α)	Ccl3	8.7	4.5
NM_009117	Serum amyloid A 1	Saa1	4.3	4.2
NM_007825	Cytochrome P450, 7b1	Cyp7b1	5.3	3.3
NM_013706	CD52 antigen	Cd52	5.9	3.3
NM_010512	Insulin-like growth factor 1	Igf1	7.5	3.5
NM_008605	Matrix metalloproteinase 12	Mmp12	5.0	3.5
NM_011867	Solute carrier family 26, member 4	Slc26a4	20	3.2
NM_011338	Chemokine (C-C motif) ligand 9	Ccl9	4.4	3.0
NM_007403	A disintegrin and metalloprotease domain 8	Adam8	5.0	2.9
NM_010809	Matrix metalloproteinase 3	Mmp3	3.7	2.9
NM_030712	Chemokine (C-X-C) receptor 6	Cxcr6	5.4	2.8
NM_020509	Resistin-like alpha	Retnla	68	2.8
NM_011311	S100 calcium binding protein A4	S100a4	2.9	2.7
NM_011593	Tissue inhibitor of metalloproteinase	Timp	9.5	2.7
NM_008380	Inhibin beta-A	Inhba	4.0	2.5
NM_019467	Allograft inflammatory factor 1	Aif1	4.3	2.5
NM_013653	Chemokine (C-C motif) ligand 5 (RANTES)	Ccl5	3.0	2.4
NM_010104	Endothelin 1	Edn1	3.2	2.4
AB015136	Chemokine (C-C motif) ligand 20 (MIP-3α)	Ccl20	2.2	2.3
NM_007802	Cathepsin K	Ctsk	4.2	2.2
NM_021443	Chemokine (C-C motif) ligand 8 (MCP-2)	Ccl8	3.8	2.2
NM_011331	Chemokine (C-C motif) ligand 12	Ccl12	5.5	2.1
NM_009139	Chemokine (C-C motif) ligand 6 (MRP-1)	Ccl6	2.9	2.0
NM_021281	Cathepsin S	Ctss	3.2	2.0
NM_008630	Metallothionein 2	Mt2	1.7	2.0
NM_007793	Cystatin B	Cstb	2.7	2.0
NM_028801	Mucin 5, subtype B	Muc5b	2.8	1.9
NM_008871	Proteinase inhibitor	Serpine1	2.2	1.8
NM_053113	Eosinophil-associated ribonuclease A	Ear11	2.1	1.8
NM_022325	Cathepsin Z	Ctsz	3.5	1.7
NM_008518	Lymphotoxin B	Ltb	1.8	1.7
NM_008969	Prostaglandin-endoperoxide synthase 1	Ptgs1 (Cox1)	1.7	1.6
NM_019963	Signal transducer and activator of transcription 2	Stat2	3.4	1.6
NM_011888	Small inducible cytokine A19 (MIP-3β)	Ccl19	4.4	1.6
NM_009896	Cytokine inducible SH2-containing protein 1	Clsh1	2.0	1.5
NM_017474	Chloride channel calcium-activated 3	Clca3	314	1.5
NM_016741	Scavenger receptor class B1	Scarb1	2.2	1.5
AK008656	Mucin 5 subtypes A and C	Muc5ac	1.3	1.4

Definition of abbreviations: OVA = ovalbumin; WT = wild-type.

The ratio of the levels of respective RNA is shown. A complete listing of changes in mRNA levels can be found at http://dir.niehs.nih.gov/microarray/jetten/home.htm.

sacs in lungs of both WT and RORα$^{sg/sg}$ mice. However, the degree of this infiltration was significantly less in OVA-challenged RORα$^{sg/sg}$ mice, showing that these mice develop a less severe allergic inflammatory response. The reduced numbers of eosinophils, neutrophils, and lymphocytes observed in BAL fluid from RORα$^{sg/sg}$ mice support this conclusion.

Allergic inflammatory responses are initiated by presentation of the allergen by antigen-presenting cells to CD4$^+$ lymphocytes, resulting in a Th2-type immune response (5–7, 39, 40). Induction and release of Th2-type cytokines (e.g., IL-4, IL-5, IL-9, and IL-13) by activated CD4$^+$ T cells play a pivotal role in the initiation of many events that ultimately lead to pathophysiologic abnormalities typical of asthma, namely airway obstruction and AHR. IL-4, IL-5, and IL-13 have been implicated in the regulation of several events during allergic inflammation, including eosinophilia, induction of IgE, AHR, and excessive mucus secretion. The importance of these cytokines is supported by studies showing a link between genetic polymorphisms in the IL-4 and IL-13 genes and the susceptibility to asthma (5, 41). Our data show that the induction of IL-4, IL-5, and IL-13 after OVA challenge is greatly compromised in RORα$^{sg/sg}$ mice compared with WT mice. Although IL-13 is synthesized by several cell types, it is predominantly released by Th2-type CD4$^+$ T lymphocytes. Regulation of IL-13 is complex and several inflammatory mediators have been implicated in the control of IL-13 (5, 42, 43). Our data on cytokine expression appear to suggest that lack of RORα expression affects early stages in the inflammatory cascade.

In addition to cytokines, several chemokines were induced to a significantly lesser extent in OVA-challenged RORα$^{sg/sg}$ mice than in WT mice. The levels of TARC (Ccl17) and eotaxin in BAL fluid from RORα$^{sg/sg}$ mice were considerably lower than

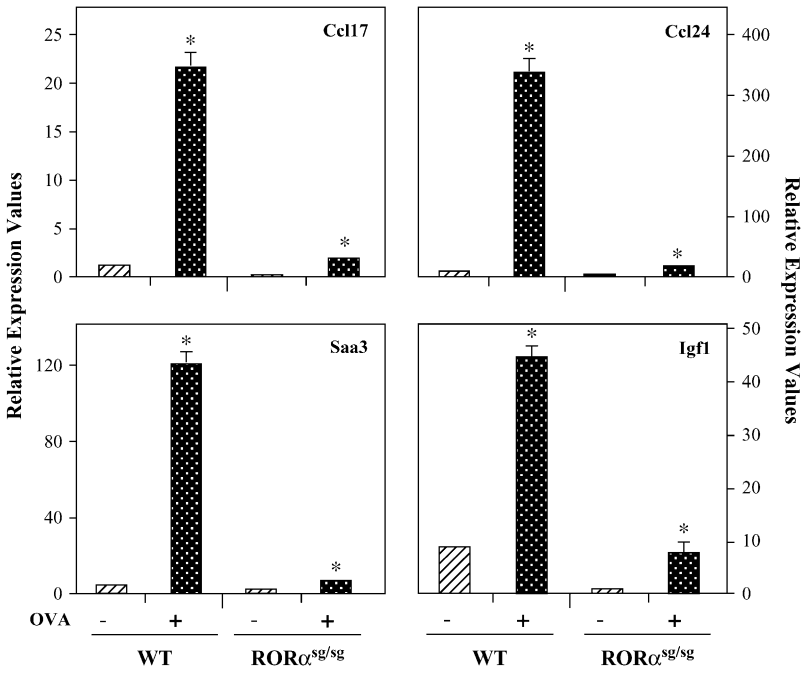

Figure 9. Differential expression of Ccl17, Ccl24, Saa3, and Igf1 mRNAs in lungs from saline- and OVA-challenged WT and RORα$^{sg/sg}$ mice. Levels of RNA were determined by real-time quantitative reverse transcriptase–polymerase chain reaction as described in METHODS.

those from WT mice. These findings are supported by our microarray analysis, which demonstrated that the expression of many chemokine genes, including TARC, eotaxin-2 (Ccl24), MCP-2 (Ccl8), and RANTES (Ccl5), was induced to a smaller degree in lungs from OVA-challenged RORα$^{sg/sg}$ mice than those from WT mice (Table 1). It is likely that the reduction in various types of inflammatory cells observed in OVA-challenged RORα$^{sg/sg}$ mice are related to these changes in chemokine expression. For example, eotaxins are potent eosinophil chemoattractants (44) and eotaxin-2 deficiency has been reported to cause a reduction particularly in luminal eosinophils, whereas peribronchial eosinophils were not affected (42). Therefore, the reduction in the number of eosinophils in BAL fluid of OVA-challenged RORα$^{sg/sg}$ mice appears to be at least in part related to the reduction in eotaxin-1 and eotaxin-2 expression. TARC has been reported to be released by a number of cell types, including macrophages, dendritic cells, natural killer cells, and bronchial epithelial cells. It has been suggested that TARC may provide a positive feedback mechanism that helps to sustain the Th2-type immune response (5, 43, 45). The reduced recruitment of CD4$^+$ lymphocytes in lungs of OVA-challenged RORα$^{sg/sg}$ mice might be due to the repression of TARC and might at least in part be responsible for the attenuated Th2-driven inflammatory response in RORα$^{sg/sg}$ mice. IL-13 has been shown to be directly or indirectly responsible for the induction of a vast array of chemokines (5, 7). The reduced level of chemokine expression in OVA-challenged RORα$^{sg/sg}$ mice might be related to the reduced induction of IL-13.

Induction of the Th2 immune response is accompanied by increased serum levels of OVA-specific IgE. Interestingly, both OVA-specific and total IgE levels were elevated in RORα$^{sg/sg}$ mice. Increased levels of total serum IgE have also been observed in VDR-null mice (14). The mechanisms underlying this elevation in total IgE in both knockout mouse models are not yet well understood and require further experimentation.

Our observation of increased RL after OVA sensitization and challenge in WT mice may reflect the development of airflow obstruction in these animals, produced collectively by the increased numbers of inflammatory cells in the airways, increased mucosal edema of the airway wall secondary to products released by these inflammatory cells, and by increased mucus released into the airway lumen. The dramatic differences in inflammatory cell numbers and goblet cell hyperplasia may explain the lack of airflow obstruction at baseline in OVA-treated RORα$^{sg/sg}$ mice. Despite the attenuated inflammatory response observed in these mice, AHR developed in OVA-exposed RORα$^{sg/sg}$ mice to a similar extent as that observed in WT animals, except at the highest MCh dose. These findings are consistent with reports showing a dissociation of AHR from airway eosinophilia, lung inflammation, and IgE levels (46, 47). At higher doses, MCh responsiveness tended to be lower in RORα$^{sg/sg}$ mice than WT animals. It has been shown previously that IL-13 is important to the development of AHR, and the lower levels of IL-13 observed in OVA-treated RORα$^{sg/sg}$ mice may, in part, explain this attenuation (48, 49).

To assess differences in gene expression in lungs from WT and RORα$^{sg/sg}$ mice after OVA challenge, we performed microarray analysis using RNA from whole lung. Gene expression analysis using whole lung versus cultured cells has both advantages and disadvantages. Clearly, regulation of specific genes can be more easily studied in cultured cells. However, allergic inflammation is a complex disease in which migration of immune cells, cell–cell interactions, and tissue remodeling play an important role. These complexities are not reflected in isolated culture systems. Furthermore, the induction of many genes, such as those encoding cytokines/chemokines, is tightly linked to the recruitment of other cell types to the lung. Despite these complications, approaches like microarray analysis using whole lung may lead to the identification of additional biomarkers to monitor inflammation in patients with inflammatory lung disease. In addition, they may lead to the identification of additional genetic determinants of susceptibility to asthma (50, 51). Our microarray analyses showed that OVA-induced airway inflammation causes a large number of changes in the pattern of gene expression in lungs of WT mice; 588 mRNAs were up-regulated more than 2.0-fold, whereas 630 mRNAs were decreased by more than 50% (*see* http://dir.niehs.nih.gov/microarray/jetten/home.htm). Many of these genes encode proteins that have been implicated in

inflammation (7, 52, 53). The chemokines MIP-1α (Ccl3) and MIP-1β (Ccl4), Retnla, Timp, metalloproteinases, endothelin 1, the acute response proteins Saa1 and Saa3, and Igf1 were among the genes most highly induced in lungs from OVA-challenged WT mice. MIP-1 proteins, which act via G-protein–coupled cell surface receptors (CCR1, CCR3, and CCR5), are expressed by lymphocytes and monocytes/macrophages and mediate migration of neutrophils (54). The reduced expression of particularly MIP-1α may be responsible for the diminished recruitment of neutrophils to lungs of RORα$^{sg/sg}$ mice. Many of the inflammatory genes identified by microarray analysis are induced to a greater extent in OVA-treated WT mice compared with OVA-treated RORα$^{sg/sg}$ mice, which therefore supports our conclusion that mice deficient in RORα exhibit an attenuated allergic inflammatory response.

It is interesting that several members of the nuclear receptor superfamily, including GR, VDR, liver X receptor (LXR), PPARs, and RORα regulate inflammation (10, 11, 14, 15, 18). Recently, the retinoid X receptor (RXR) has also been shown to play a critical role in Th2-mediated immunity (55). Although several nuclear receptors (e.g., LXR, VDR, and PPAR) form a heterodimer with RXR, the GR functions as a homodimer and the RORα as a monomer, suggesting that RXR is not a common element in the regulation of inflammation by nuclear receptors. Several of the nuclear receptors inhibit inflammation by interfering with the activation of the nuclear factor (NF)-κB signaling pathway, which plays a critical role in Th2 cell differentiation and is required for induction of allergic airway inflammation (13, 56). However, nuclear receptors inhibit the NF-κB signaling pathway by distinct mechanisms. RORα has been reported to inhibit NF-κB signaling by positively regulating the expression of IκBα (26). In contrast, other studies have demonstrated that the induction of IκBα by LPS in lung of RORα$^{sg/sg}$ mice was not impaired, suggesting that RORα is not a regulator of IκBα expression (30). In addition, induction of IκBα by LPS was unaltered in macrophage RAW 264.7 cells expressing RORα (30). These observations suggest that the attenuated Th2 response in RORα-deficient mice appears to be due to an NF-κB–independent mechanism. The inhibition of allergen-induced inflammation by PPARγ is also mediated by an NF-κB–independent mechanism. The antiinflammatory response by PPARγ agonists involves an increase in IL-10 levels (11, 57). Our results show that levels of IL-10 and of TNF-α and IL-12, two other cytokines that negatively regulate asthma (58, 59), are not significantly altered in OVA-induced inflammation in RORα$^{sg/sg}$ mice, indicating that the reduced susceptibility in these mice involves a different mechanism.

RORα is highly expressed in resting macrophages and CD4$^+$ T lymphocytes and at low levels in CD8$^+$ T lymphocytes. Although the spleen and thymus in RORα$^{sg/sg}$ mice are, respectively, 40 and 20% smaller than in WT mice (unpublished observations), the numbers of circulating lymphocytes and neutrophils in BAL fluid are not significantly different from those in WT mice (30). Although RORα$^{sg/sg}$ mice are less susceptible to allergen-induced inflammation, these mice exhibit an increased susceptibility to LPS-induced inflammation (30). It is well known that Th1 and Th2 immune responses involve different cell types and cytokines (5, 7). Because RORα can function as a repressor and activator of transcription, a simple explanation for these different responses may be that RORα differentially regulates the expression of Th1 and Th2 cytokines.

Because nuclear receptors function as ligand-dependent transcription factors, they provide excellent pharmacologic targets to interfere in (patho)physiologic processes; therefore, they may be very promising in yielding novel therapeutic strategies for human disease. Glucocorticoids, which mediate their actions by binding GR, are very effective antiinflammatory agents and are the first-line treatment of asthma (17, 18, 20, 60–62). However, long-term treatment with glucocorticoids is problematic due to long-term side effects. Moreover, a subset of patients do not respond to glucocorticoid therapy (19). Thus, additional therapeutic strategies are desirable. Ligands for PPARs, LXRs, and VDRs, which have been reported to significantly influence inflammatory responses, may be promising candidates for additional therapeutic strategies (11, 63, 64). The attenuated OVA-induced inflammatory response observed in mice deficient in RORα suggests a role for this nuclear receptor in the regulation of Th2-driven inflammation in the lung. Recent studies (65) have identified cholesterol and cholesterol sulfate as potential agonists of the RORα receptor. Activation of the RORα receptor by endogenous ligands, such as cholesterol, might be implicated in the recently reported link between obesity, hypercholesterolemia, and asthma (66). Synthetic, high-affinity antagonists could prevent these recently identified endogenous ligands from activating RORα, inhibit the activation of inflammatory genes, and have potential in the treatment of Th2-driven inflammatory diseases, such as asthma and allergy.

Conflict of Interest Statement: M.J. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. C.S. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. S.L.T. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. D.D. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. C.J.E. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. J.G.M. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. H.S.K. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. M.A. had shares in Myogen, a biotech company that focuses on cardiovascular disease and has no interest in the subject of this manuscript. He also has shares in Ambient Corp. G.L. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. J.C. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. S.G. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. A.M.J. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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