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Nuclear Receptor Nur77 Attenuates Airway Inflammation in Mice by Suppressing NF-κB Activity in Lung Epithelial Cells

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Allergic asthma is characterized by persistent chronic airway inflammation, which leads to mucus hypersecretion and airway hyperresponsiveness. Nuclear receptor Nur77 plays a pivotal role in distinct immune and inflammatory cells and is expressed in eosinophils and lung epithelium. However, the role of Nur77 in allergic airway inflammation has not been studied so far. In the present study, we determined the role of Nur77 in airway inflammation using a murine model of OVA-induced allergic airway inflammation. We found that OVA-challenged Nur77 knockout (KO) mice show significantly enhanced infiltration of inflammatory cells, including eosinophils and lymphocytes, and aggravated mucus production. The infiltration of macrophages is limited in this model and was similar in wild-type and Nur77 KO mice. Higher levels of Th2 cytokines were found in bronchoalveolar lavage fluid and draining lymph node cells of Nur77-KO mice, as well as increased serum IgG1 and IgG2a levels. Knockdown of Nur77 in human lung epithelial cells resulted in a marked increase in IκBα phosphorylation, corresponding with elevated NF-κB activity, whereas Nur77 overexpression decreased NF-κB activity. Consistently, Nur77 significantly decreased mRNA levels of inflammatory cytokines and Muc5ac expression and also attenuated mucus production in lung epithelial cells. To further corroborate these findings, we searched for association of single nucleotide polymorphisms in *Nur77* gene with asthma and with the severity of bronchial hyperresponsiveness. We identified three *Nur77* single nucleotide polymorphisms showing association with severity of bronchial hyperresponsiveness in asthma patients. Collectively, these findings support a protective role of Nur77 in OVA-induced airway inflammation and identify Nur77 as a novel therapeutic target for airway inflammation. ***The Journal of Immunology*****, 2015, 195: 000–000.**

Allergic asthma is a chronic inflammatory disorder characterized by eosinophilic airway inflammation, mucus hypersecretion, bronchial hyperreactivity, and enhanced production of Th2 cytokines and IgGs. The eventual airway obstruction in asthma varies depending on the extent of mucus hypersecretion and airway hyperresponsiveness (1, 2). The process of airway inflammation involves infiltration of various inflammatory and immune cells such as eosinophils, mast cells, neutrophils, basophils, B and T lymphocytes, and dendritic cells (3, 4). The Th2 cytokines IL-4, IL-5, and IL-13 play a major role in the development and persistence of airway inflammation, and their role in the pathogenesis of airway inflammation is well established. IL-4 is pivotal for the initiation of Th2 inflammatory responses, whereas IL-5 is essential for the growth, differentiation, recruitment and survival of eosinophils, and IL-13 is crucial for eosinophilic inflammation, mucus secretion, and airway hyperresponsiveness (5–7). Additionally, chemokines such as eotaxins and RANTES and other proinflammatory mediators play a major role in the attraction of eosinophils to the airways (8, 9). Airway smooth muscle cells are involved in the pathophysiology of asthma because excessive proliferation and contraction lead to narrowing of the airways and enhanced airway hyperresponsiveness, thereby increasing morbidity and mortality in asthma (10).

Airway epithelial cells are essential for the regulation of inflammatory, immune, and regenerative responses to several airborne allergens. Goblet cells produce mucins, which play a crucial role in maintaining epithelium homeostasis in airway disease (11). Mucus hypersecretion by these cells is, however, one of the hallmarks of allergic airway disease. Among other mucins, Muc5ac is abundantly expressed in mucus-secreting goblet cells and serves as a marker for mucus cell hyperplasia (12). NF-κB is a pleiotropic transcription factor that acts as a key regulator of immune and inflammatory genes, and activation of the NF-κB pathway has been implicated in asthma both in experimental models and in humans (13, 14). It has also been reported that proinflammatory cytokines induce Muc5ac gene expression through activation of the NF-κB pathway in lung epithelial cells (15). Despite much effort to understand the pathophysiology of allergic airway disease, the molecular events that underlie the pathogenesis of airway inflammation are not completely unraveled. To gain further mechanistic insights and design new therapeutic strategies for the treatment of airway

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Abbreviations used in this article: BALF, bronchoalveolar lavage fluid; BHR, bronchial hyperresponsiveness; DAG, Dutch Asthma Genome-Wide Association Study; KO, knockout; MBP, major basic protein; PAS, periodic acid–Schiff; qRT-PCR, quantitative RT-PCR; SNP, single nucleotide polymorphism; WT, wild-type.

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allergic disease, identification of key transcription factors responsible for allergic airway disease is essential.

Nur77 is also known as NR4A1, TR3, or NGFI-B and is a member of the NR4A subfamily of nuclear receptors that are early response genes induced by diverse extracellular signals (16–18). Nur77 plays a pivotal role in the regulation of a wide range of biological processes, including cell proliferation, differentiation, and survival. Accumulating evidence suggests that Nur77 is implicated in the regulation of genes involved in metabolism, cancer, inflammation, and immunity and vascular disease (16–19). In particular, Nur77 has been shown to have a protective function in restenosis and atherosclerosis (20, 21). In vascular endothelial cells, Nur77 reduces the proinflammatory response by inducing expression of IκBα, which is a potent inhibitor of NF-κB (22). Furthermore, Nur77 has been shown to inhibit the NF-κB pathway through direct interaction with the p65 component of NF-κB (23). Atherosclerosis may be considered a chronic inflammatory disease driven by macrophage activation in which Nur77 has an anti-inflammatory function (24, 25). Nur77, along with its family members, was also shown to be important for thymic regulatory T cell development and immune homeostasis (16). Finally, peripheral eosinophils from patients with atopic dermatitis show increased Nur77 expression (26). Limited information is available on Nur77 in the lung, but its expression has been described in lung epithelial cells, and a recent study demonstrated that Nur77 inhibits pulmonary smooth muscle cell proliferation (27, 28).

Collectively, these data suggest a specific role of Nur77 in airway inflammation and asthma, and the present study aimed to evaluate, to the best of our knowledge for the first time, the role of Nur77 in OVA-induced allergic airway inflammation. Considering the anti-inflammatory potential of Nur77, we hypothesized that Nur77 knockout (KO) mice develop enhanced allergen-induced airway inflammation. To understand underlying mechanisms involved in mediating the effect of Nur77 in lung pathology, we performed both gain and loss of function experiments in lung epithelial cells. We report in the present study that Nur77-deficient mice show significantly increased production of Th2 cytokines, eosinophil infiltrates, and histopathological changes in the lung. To substantiate the potential relevance of Nur77 in human asthma, we searched for *Nur77* single nucleotide polymorphism (SNP) association with asthma and severity of bronchial hyperresponsiveness in asthma patients.

Materials and Methods

Animals and care

Nur77 KO mice on a C57BL/6 background were provided by Prof. B. R. Binder (Vienna, Austria). All experimental protocols conducted on the mice were approved by the Committee for Animal Welfare of the Academic Medical Center, University of Amsterdam and performed in accordance with the standards established by the Dutch government.

Murine model of OVA-induced airway allergic inflammation

Eight-week-old female mice were sensitized on days 0 and 7 by i.p. injection of 10 μg OVA (Sigma-Aldrich, grade V) adsorbed in 1 mg aluminum hydroxide (Imject alum, Pierce) (n = 8/group). Control animals received aluminum hydroxide only. Subsequently, mice were challenged with aerosolized OVA (Sigma-Aldrich, grade III, 10 mg/ml) or PBS for 30 min each day for 3 consecutive days on days 14, 15, and 16. Forty-eight hours after the last challenge, mice were sacrificed for harvest of bronchoalveolar lavage fluid (BALF), lungs, lung draining lymph nodes, and serum. Two mice that did not develop airway inflammation following OVA challenge were excluded from the data analysis.

Immunohistochemistry

Lungs were inflated and fixed with 4% formaldehyde via a tracheal cannula and stored in 4% formaldehyde for fixation before histochemical processing. The whole lung was embedded in paraffin, sectioned at a 4-μm thickness, deparaffinized, and rehydrated. Sections were stained with H&E to assess inflammatory infiltrates and with periodic acid–Schiff (PAS) to detect mucin. Examining the entire slide surface, a number of inflammatory parameters were scored by experienced histopathologists in a blinded fashion, exactly as described (29). The parameters of perivascular inflammation, interstitial inflammation, peribronchial inflammation, and edema were graded in a semiquantitative fashion on a scale of 0–4 (0, absent; 1, mild; 2, moderate; 3, severe; 4, very severe). The total pathology score was expressed as the sum of the scores for all parameters. In a similar way PAS staining was quantified semiquantitatively on a scale from 0 to 3. Eosinophils were quantified in lung tissue stained by an Ab directed against major basic protein (MBP; provided by Dr. Nancy Lee and Prof. James Lee, Mayo Clinic Arizona, Scottsdale, AZ). To detect active NF-κB/RelA, we applied a Ser^{311} phospho-specific Ab (Signalway Antibody; 11260). Morphometric analyses of sections were performed using Leica QWin V3 software. To quantify the activity of p65 NF-κB, we counted the total number of nuclei of airways with a diameter <150 μm and the number of epithelial cells with nuclear staining for phosphorylated NF-κB/RelA and calculated the percentage of positive nuclei. Three sections per mouse were quantified and the percentage of positive cells is indicated (n = 6 for WT and n = 8 for Nur77 KO).

BALF and phenotyping

BALF was collected with 1 ml followed by 2× 1 ml sterile PBS containing 0.1 mM EDTA by intratracheal cannulation. The BALF was immediately centrifuged at 400 × g for 7 min at 4°C and the cell pellet was resuspended, after which flow cytometric analysis was performed as described before to assess the cellular composition of the BALF (30). Briefly, eosinophils were identified as forward $scatter^{lo}$/side $scatter^{int}$/$CD3^-$/$CD19^-$/$CCR3^+$, lymphocytes were identified as forward $scatter^{lo}$/side $scatter^{lo}$/$CD3^+$/$CD19^+$ and the distinction between B and T cells was made based on MHC class II expression by B cells. Dendritic cells were identified as $CD11c^{hi}$, MHC class II^{hi}, CD19/CD3/$CCR3^-$. Macrophages were identified as large $CD11c^{hi}$/MHC class II^{int} autofluorescent cells.

ELISA measurements of cytokines and chemokines

Cytokine levels in BALF were measured by ELISA for IL-5 and IL-13 (Ready-SET-Go!, eBioscience) and eotaxin-2 (R&D Biosystems) according to the manufacturers' instructions. TNF-α, IL-6, IFN-γ, and MCP-1 were measured in BALF and cell culture supernatants using the Cytometric Bead Array mouse inflammation kit (BD Biosciences, San Diego, CA).

Ex vivo restimulation of lung-draining lymph node cells

Mice were sacrificed and lung-draining lymph nodes were harvested 48 h after the last challenge. Cell suspensions were made from the lymph nodes and 2×10^5 cells were cultured per round-bottom well (96-well plate) and stimulated with 10 μg/ml OVA for 4 d. The supernatants from these cultures were subjected to ELISA measurements of IL-4, IL-5, and IL-13.

Serum OVA-specific Igs

Serum was analyzed to assess the level of total and OVA-specific IgG1 and IgG2a by ELISA (Opteia, BD Biosciences). We used a standard curve of murine IgG1 and IgG2a, respectively, as a semiquantitative reference.

Cell culture and lentiviral transduction

Human alveolar epithelial carcinoma (NCI-H292) cells were grown and maintained in RPMI 1640 medium containing 10% FBS and 1% penicillin/streptomycin. Recombinant lentiviral particles encoding Nur77 and short hairpin RNA targeting Nur77 were produced, concentrated, and titrated as described previously (31). The human short hairpin RNA oligonucleotide sequences of Nur77 were described earlier (20). Cells were seeded at 2.4×10^4 cells/ml and were infected with recombinant lentivirus twice with an interval of 12 h and incubated for 24 h. After 24 h, the medium was refreshed and the cells were cultured for another 24 h. Cells were serum starved for 36 h and stimulated with TNF-α (50 ng/ml) for the indicated time periods.

Quantitative RT-PCR, Western blot analysis, luciferase reporter assay, and PAS staining

Quantitative RT-PCR (qRT-PCR) and Western blot analysis were performed as described previously (32). For qRT-PCR experiments, serum-starved cells were stimulated with TNF-α for 6 h before harvesting. In some experiments, cells were pretreated overnight with BAY 11-7085 (NF-κB inhibitor) at a final concentration of 10 μM. Transduction ef-

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ficiency was determined by immunofluorescence and qRT-PCR. The following primers were used for qRT-PCR: TNF-α, forward, 5′-AGGACACCATGAGCACTGAAAG-3′, reverse, 5′-AGGAGAGGCTGAGGAACAAG-3′; RANTES, forward, 5′-CGCTGTCATCCTCATTGC-3′, reverse, 5′-CCACTGGTGTAGAAATACTCC-3′; IL-6, forward, 5′-CGCCTTCGGTCCAGTTG-3′, reverse, 5′-TCGTTCTGAAGAGGTGAGTG-3′; Muc5ac, forward, 5′-GGAACTGTGGGGACAGCTCTT-3′, reverse, 5′-GTCACATTCCTCAGCGAGGTC-3′. Abs applied in Western blot analysis were anti–p-IKBα (Cell Signaling Technology), and tubulin (Cedarlane Laboratories).

For luciferase assay, cells were transfected with an NF-κB reporter plasmid using Lipofectamine LTX plus transfection reagent (Life Technologies) according to the manufacturer's protocol, and assay was described previously (31). The construct containing the NF-κB response element of the minimal IL-6 promoter was provided by Dr. Karolien De Bosscher (Ghent University, Ghent, Belgium) and described previously (33). pRL-TK *Renilla* reporter plasmid (Promega) was cotransfected as an internal control. For PAS staining on NCI-H292 cells, the cells were infected with Nur77 lentivirus followed by serum starvation for 24 h and then cells were stimulated with TNF-α (50 ng/ml) for 48 h. After the incubation, cells were fixed with formaldehyde for 30 min and mucus glycoconjugates were visualized by PAS staining.

SNP association with asthma and severity of bronchial hyperresponsiveness

Asthma patients for single nucleotide polymorphism (SNP) analysis were recruited from the Dutch Asthma Genome-Wide Association Study (DAG) cohort (34, 35). All available SNPs in *Nur77/NR4A1* in the DAG cohort were tested for association with asthma and severity of bronchial hyperresponsiveness (BHR). In the DAG cohort all 920 asthmatics had a physician's diagnosis of asthma, asthma symptoms, and BHR to either histamine or methacholine. The 980 controls had no asthma or chronic obstructive pulmonary disease, nor any evidence of significant airway obstruction. Inclusion criteria for the association with severity of BHR were as follows: cases had a doctor diagnosis of asthma in combination with BHR with data available on smoking and inhaled corticosteroids use at the time of the test. In total, 650 asthmatics were included in this analysis. Severity of BHR was measured with a slope, which was calculated by dividing the difference between forced expiratory volume in 1 s at baseline and the dose step at which a ≥20% fall in forced expiratory volume in 1 s was reached. We divided the BHR slopes of the 30 s tidal breathing method by 4 to compare the slope of the 30 s tidal breathing method with the 2 min method. Values were log transformed to reach normal distribution. DNA of subjects was genotyped on the Illumina 317 Chip (Illumina, San Diego, CA) or with the Illumina 370 Duo Chip. Quality control was applied; subjects were removed from analysis when they were not of white descent, had a low genotyping call rate (<95%), or were discrepant or ambiguous for genetic sex. SNPs were deleted when the call rates were low (<95%), not in Hardy–Weinberg equilibrium ($p < 1 \times 10^{-4}$), or when the minor allele frequency was <0.05. After quality control, 294,932 SNPs were selected for the analysis. All statistical analyses have been performed using PLINK v1.07. Logistic regression analyses in additive models were performed to analyze the associations between *Nur77/NR4A1* SNPs and asthma. Linear regression analyses were performed in additive models to analyze the association of *Nur77/NR4A1* SNPs on slopes of the BHR test with adjustments for smoking and inhaled and/or oral corticosteroid use.

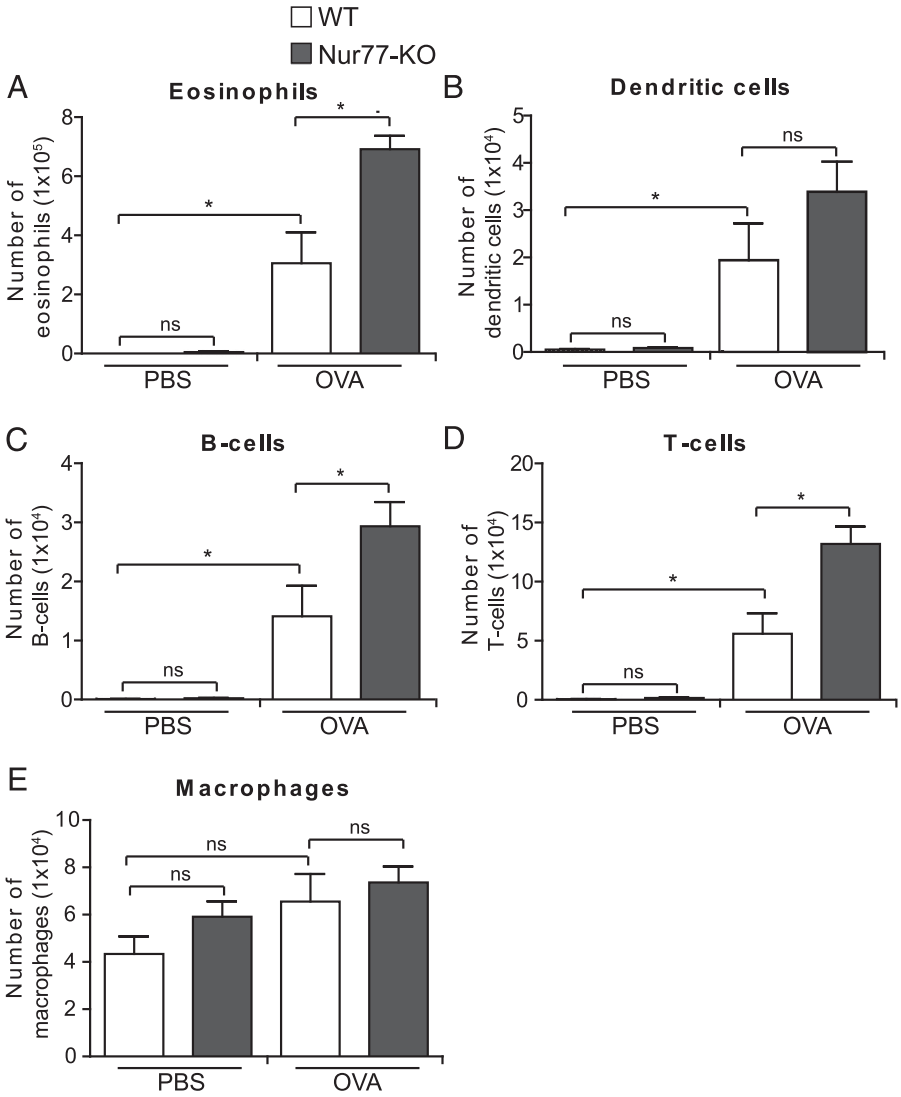

FIGURE 1. Enhanced airway inflammation in Nur77 KO mice. BALF was obtained 48 h after the last challenge from saline-treated and OVA-treated WT and Nur77 KO mice (n = 6–8/group), and differential inflammatory cell count analysis for eosinophils (**A**), dendritic cells (**B**), B cells (**C**), T cells (**D**), and macrophages (**E**) was determined. OVA challenge induced significant infiltration of inflammatory cells in WT mice. Nur77 KO mice displayed even higher numbers of eosinophils, B cells, and T cells, except dendritic cells and macrophages, compared with WT mice. Data are means ± SEM. $^*p \leq 0.05$.

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Statistical analysis

All statistical analyses and graphing were carried out with GraphPad Prism software (GraphPad Software). Comparisons between two groups were done with the Student *t* test for unpaired variables. Data are reported as means ± SEM unless otherwise specified. A p value <0.05 was considered statistically significant.

Results

OVA-challenged Nur77-deficient mice show enhanced infiltration of inflammatory cells

To define the role of Nur77 in the pathogenesis of airway inflammation, wild-type (WT) and Nur77 KO mice were sensitized and challenged with OVA. BALF was collected 48 h after the last challenge and analyzed for infiltration of inflammatory cells. OVA challenge in WT mice induced a significant increase in the number of inflammatory cells, including eosinophils, dendritic cells, and lymphocytes, except macrophages compared with non-OVA–challenged mice (Fig. 1). Among the inflammatory cell populations, eosinophils were most dominant, followed by macrophages, T cells, dendritic cells, and B cells (Fig. 1). The total number of inflammatory cells including the number of eosinophils and lymphocytes in OVA-challenged Nur77 KO mice were significantly higher than in challenged WT mice (Fig. 1). The increase in number of dendritic cells in OVA-challenged Nur77 KO mice was modest and not significant (Fig. 1B). Of note, the number of macrophages was similar for WT and Nur77 KO mice upon OVA challenge (Fig. 1E). These results demonstrate that Nur77 deficiency results in enhanced OVA-induced inflammatory cell infiltration in BALF.

Nur77 KO mice display enhanced OVA-induced allergic airway inflammation

Histological analyses revealed typical pathologic features of allergic airway inflammation in the OVA-challenged mice, as compared with the saline controls; OVA-challenged mice showed numerous inflammatory cells infiltrated around the bronchioles (Fig. 2A). OVA-challenged Nur77 KO mice showed a marked increase in inflammation compared with WT mice (Fig. 2A). Mucus cell hyperplasia is another important feature of allergic airway disease, and therefore mucus secretion was assessed by PAS staining on lung sections (11). Consistent with the enhanced inflammation, OVA-challenged Nur77 KO mice showed increased production of mucus in their lungs (Fig. 2B). Semiquantitative scoring revealed that peribronchial inflammation, interstitial inflammation, and total

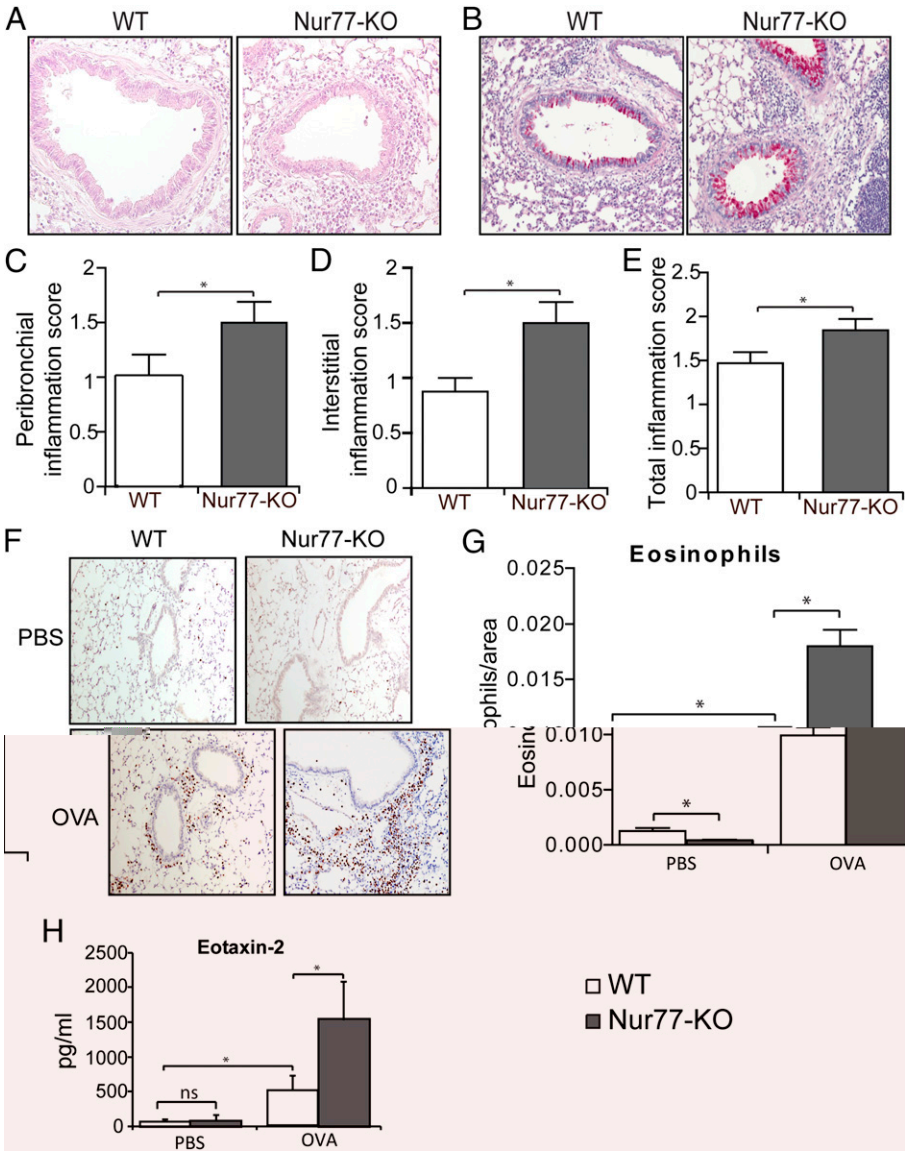

FIGURE 2. Marked infiltration of inflammatory cells and mucus production in Nur77-KO mice. (**A** and **B**) Lung sections of saline- and OVA-challenged WT and Nur77 KO mice were stained with H&E (A) or PAS (B). OVA-challenged Nur77 KO mice showed enhanced infiltration of inflammatory cells and high mucus production. (**C**–**E**) Histopathological changes in lung inflammation were scored (described in *Materials and Methods*) for peribronchial inflammation (C), interstitial inflammation (D), and total inflammation (E). (**F**) Histological analysis of lung sections stained with MBP Ab showing enhanced eosinophilic inflammation in OVA-challenged Nur77 KO mice. (**G**) Quantification of MBP^+ cells per area in WT and Nur77 KO mice. (**H**) Eotaxin-2 levels in BALF were measured by ELISA. (A, B, and F) Original magnification ×100. Data are means ± SEM ($n = 8$/group). $*p \leq 0.05$.

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inflammation were significantly enhanced in OVA-challenged Nur77 KO mice (Fig. 2C–E). Collectively, these data suggest that Nur77 plays a prominent role in regulation of allergic airway inflammation.

Enhanced eosinophil numbers in lung tissue of Nur77 KO mice

In addition to mucus hypersecretion and bronchial hyperreactivity, eosinophilic inflammation is another important hallmark of allergic airway disease. To address the impact of Nur77 deficiency on OVA-induced eosinophilic airway inflammation, we detected eosinophils by MBP staining in lung tissue sections and determined the number of eosinophils per area (Fig. 2F, 2G). Similar to the BALF data, OVA challenge caused an increase in influx of pulmonary eosinophils into lungs of WT mice compared with saline controls (Fig. 2F, 2G). OVA-challenged Nur77 KO mice displayed a significant increase in the number of eosinophils compared with WT mice, corroborating the findings in BALF (Fig. 2F, 2G).

Given that OVA-challenged Nur77 KO mice displayed an increase in recruitment of eosinophils into the lung and in BALF, we explored whether Nur77 deficiency had any impact on production of eotaxin-2, a key chemokine involved in eosinophil recruitment. Eotaxin-2 is abundantly produced 24 h after allergen challenge and plays a pivotal role in the recruitment of eosinophils into murine airways (9). Consistent with BALF and lung histopathology data of eosinophils, eotaxin-2 protein levels were highly enhanced in WT mice compared with saline control, upon OVA challenge (Fig. 2H). Furthermore, OVA-challenged Nur77 KO mice produced increased levels of eotaxin-2 compared with WT mice (Fig. 2H). Altogether, these findings suggest that Nur77 plays a crucial modulating role in pulmonary chemokine synthesis for recruitment of eosinophils after OVA challenge.

Effect of Nur77 deficiency on cytokines in BALF

BALF was examined for a variety of potentially relevant cytokines to assess whether Nur77 deficiency affects cytokine production as a mechanism by which eosinophilic and lymphocytic airway inflammation are increased in OVA-challenged Nur77 KO mice. IL-5, IL-13, TNF-α, IL-6, IFN-γ, and MCP-1 protein levels in BALF were significantly elevated upon OVA challenge in WT mice compared with saline control (Fig. 3). Levels of IL-13, TNF-α, IL-6, IFN-γ, and MCP-1 were significantly higher in OVA-challenged

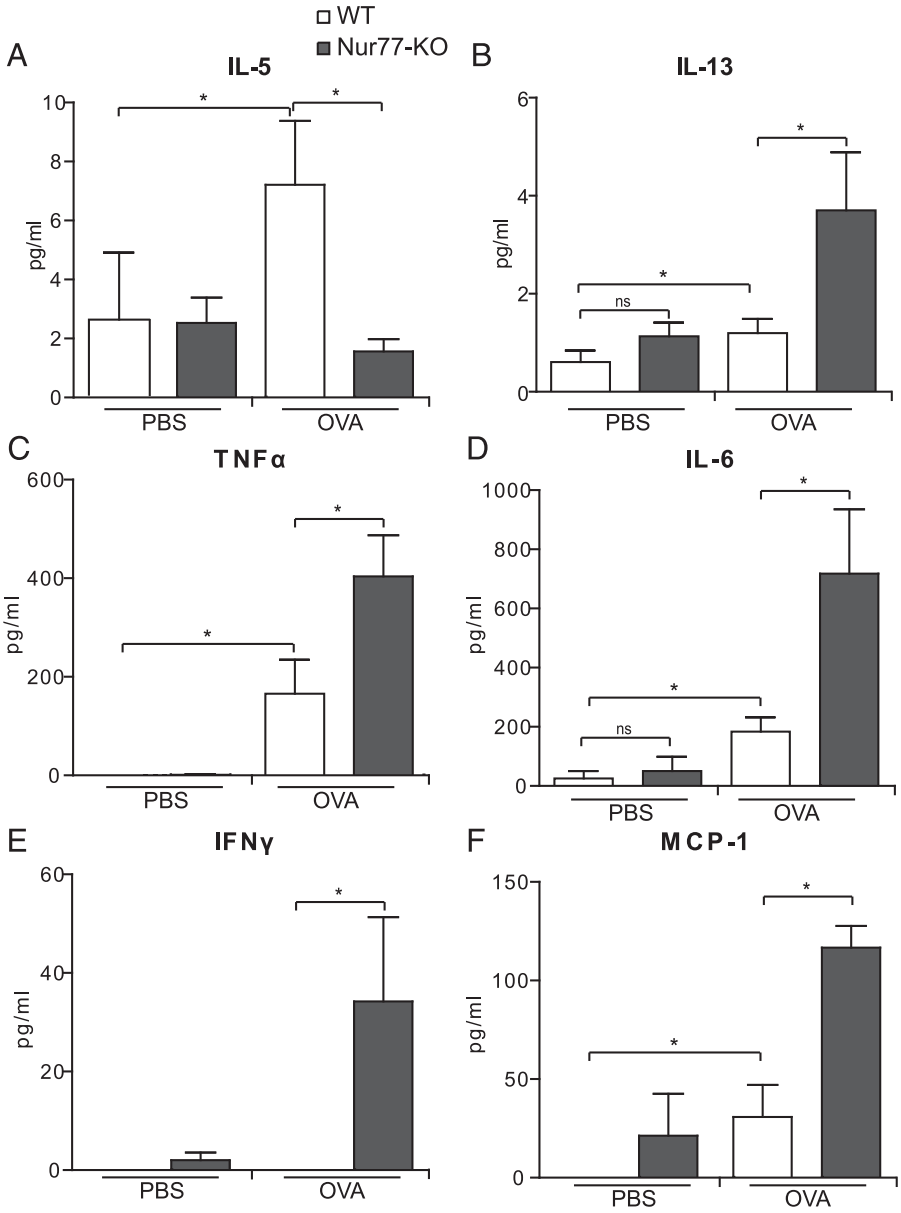

FIGURE 3. Effect of Nur77 deficiency on cytokine levels in BALF. The expression of IL-5 (**A**), IL-13 (**B**), TNF-α (**C**), IL-6 (**D**), IFN-γ (**E**), and MCP-1 (**F**) were quantified by ELISA. Data are means ± SEM (n = 8/group). *$p \leq 0.05$.

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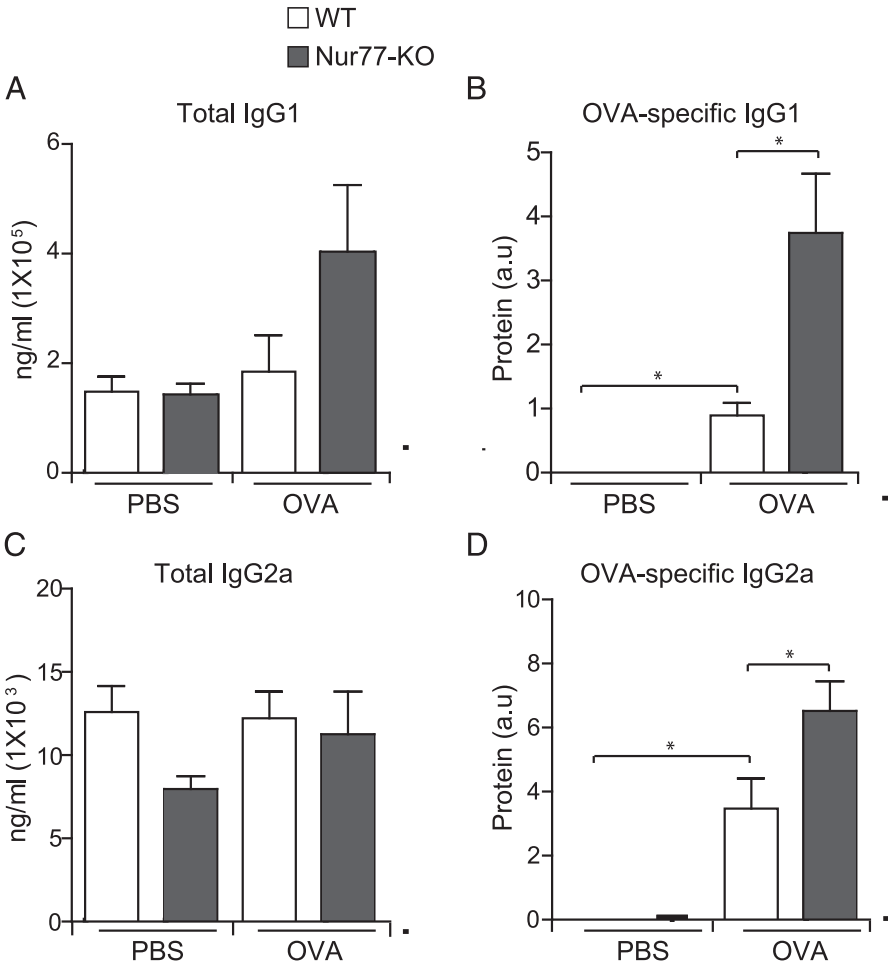

FIGURE 4. Nur77 KO mice show exacerbated production of Ag-specific IgG1 and IgG2a in serum. Total IgG1 (**A**), OVA-specific IgG1 (**B**), total IgG2a (**C**), and OVA-specific IgG2a (**D**) levels in serum obtained 48 h after saline or OVA challenge. Data are means ± SEM (n = 8/group). *$p \leq 0.05$.

Nur77 KO mice compared with challenged WT mice (Fig. 3B–F). Unexpectedly, IL-5 was significantly reduced in Nur77 KO mice compared with WT mice upon OVA challenge (Fig. 3A). Despite the decrease in IL-5 expression, our data overall showed that OVA-challenged Nur77 KO mice displayed enhanced production of cytokines compared with WT counterparts.

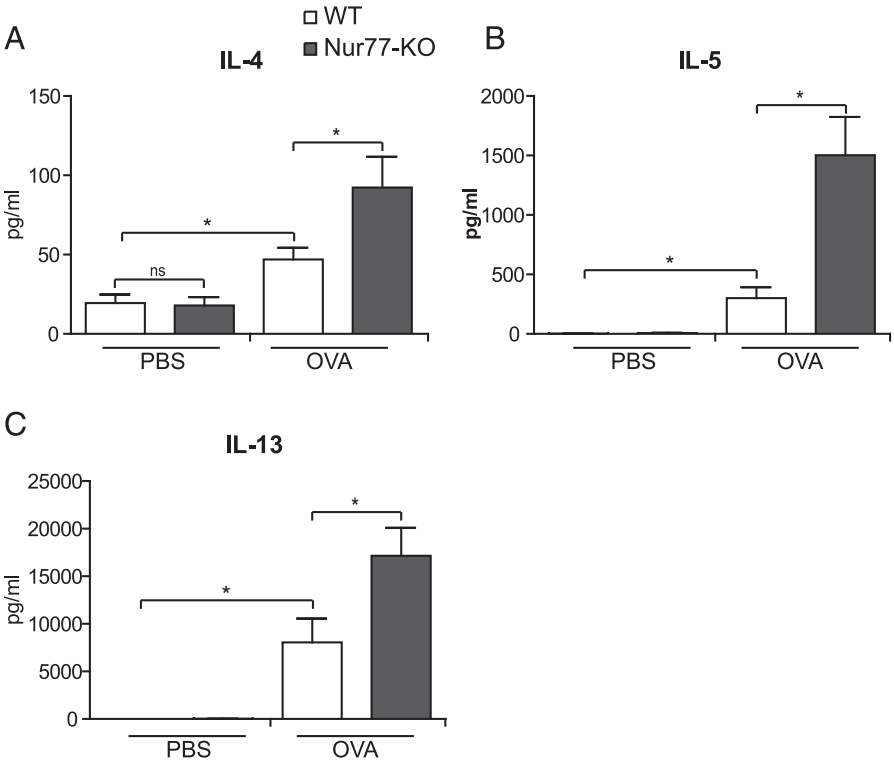

FIGURE 5. Nur77 deficiency exhibits increased levels of Th2 cytokines in the lung lymph node cells. Expression of IL-4 (**A**), IL-5 (**B**), and IL-13 (**C**) in ex vivo cultures of draining lymph node cells following restimulation with or without OVA was quantified by ELISA. Data are means ± SEM (n = 8/group). *$p \leq 0.05$.

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Nur77 KO mice show enhanced IgG1 and IgG2a responses after OVA challenge

To explore whether the augmented Th2-mediated allergic airway inflammation in Nur77 KO mice was accompanied by an increased humoral immune response, we assessed the levels of IgG1 and IgG2a in serum. WT and Nur77 KO mice showed similar levels of total IgG1 and IgG2a levels before and after OVA challenge (Fig. 4A, 4C). OVA-specific IgG1 and IgG2a levels were markedly increased in both mouse lines and higher in Nur77 KO mice compared with WT mice (Fig. 4B, 4D).

Enhanced inflammatory response of lymph node cells of Nur77 KO mice

Compelled by the findings that Nur77 deficiency led to an exacerbated inflammatory response and Th2 cytokine production, we hypothesized that this was also reflected at the level of systemic sensitization. To evaluate this, we isolated and cultured draining lymph node cells from WT and Nur77 KO mice and determined levels of IL-4, IL-5, and IL-13 in the supernatants after ex vivo stimulation with OVA. Levels of these cytokines were significantly increased in WT mice upon OVA challenge (Fig. 5). Secretion of IL-4 and IL-13, but also of IL-5, by lung lymph node cells from OVA-challenged Nur77 KO mice in response to OVA restimulation was significantly elevated compared with secretion from cells of OVA-challenged WT mice (Fig. 5A–C). Collectively, these results demonstrate that the exacerbated levels of key cytokines and chemokines may explain, at least partly, the enhanced lung inflammation in Nur77 KO mice.

Nur77 modulates inflammation and Muc5ac expression through NF-κB pathway

Several studies reported that activation of the NF-κB pathway is involved in asthma both in experimental models and in humans (13, 14). In addition, Nur77 has been shown to be expressed in activated lung epithelium and to regulate the NF-κB pathway in multiple cell systems (22, 23, 27). Therefore, we sought to explore the impact of Nur77 on p-IκBα expression in lung epithelial NCI-H292 cells. Cells were serum starved for 24 h and were stimulated with TNF-α for the indicated time points and Western blotting for p-IκBα was performed. Knockdown of Nur77 strongly enhanced phosphorylation of IκBα compared with control cells (Fig. 6A). In

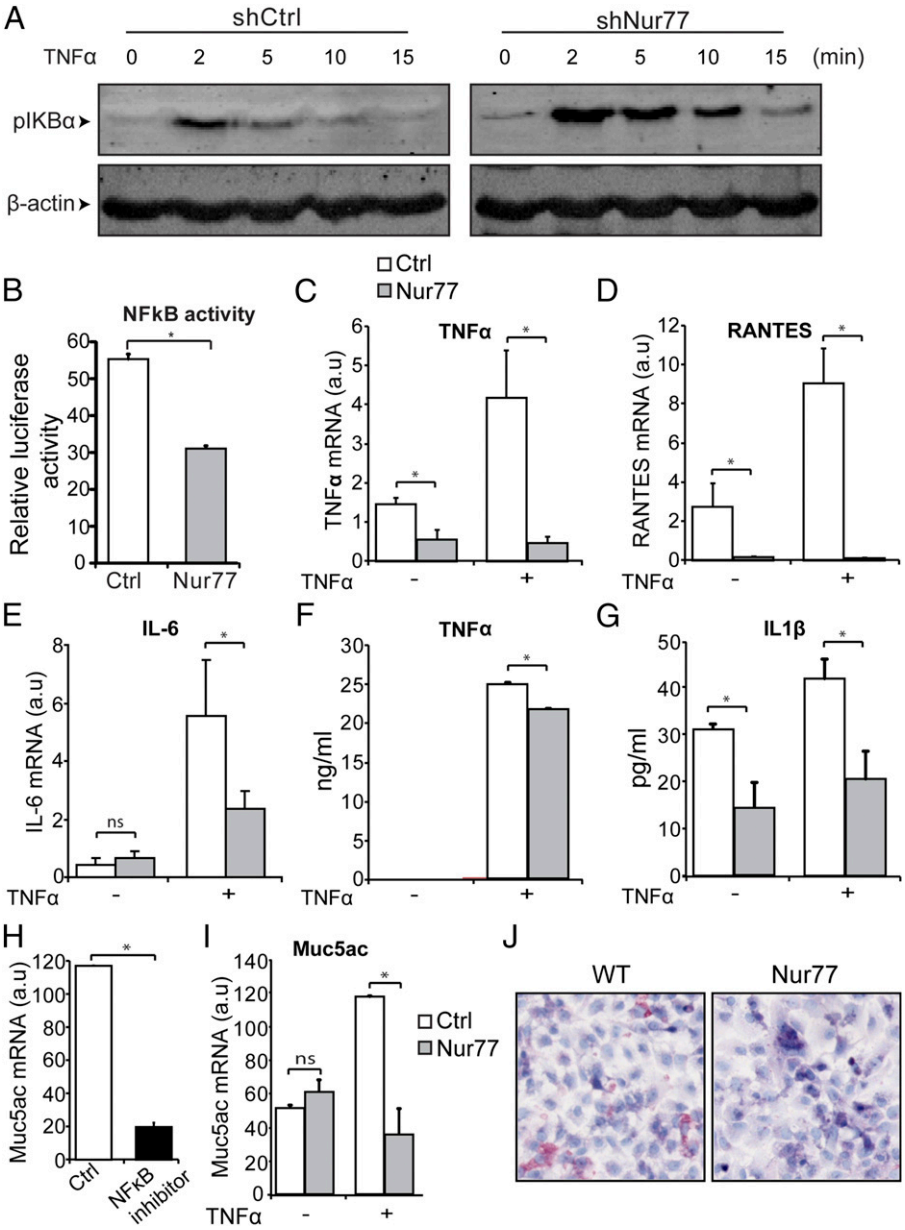

FIGURE 6. Nur77 inhibits inflammatory response and regulates Muc5ac expression through NF-κB pathway in lung epithelial cells. (**A**) Western blot analysis for p-IκBα in NCI-H292 lung epithelial cells after Nur77 knockdown and stimulation with TNF-α for the indicated time points. β-Actin served as a loading control. (**B**) Overexpression of Nur77 suppresses TNF-α–induced NF-κB activity in lung epithelial cells as measured in a luciferase reporter assay. *Renilla* is used as an internal control. (**C**–**E**) qRT-PCR analysis was performed for TNF-α (C), RANTES (D), and IL-6 (E) after overexpression of Nur77 in NCI-H292 cells. (**F** and **G**) Protein levels of TNF-α (F) and IL-1β (G) were measured in the supernatants by ELISA following overexpression of Nur77 in NCI-H292 cells. (**H**) qRT-PCR analysis was performed for Muc5ac after treatment with BAY 11-7085 (NF-κB inhibitor) and TNF-α stimulation in NCI-H292 cells. (**I**) qRT-PCR analysis was performed for Muc5ac after Nur77 overexpression and stimulation with or without TNF-α in NCI-H292 cells. (**J**) Mucus production was determined after overexpression of Nur77 and stimulation with TNF-α in NCI-H292 cells by PAS staining. Original magnification ×200. Each experiment was repeated at least three times. Data are means ± SD. $*p \leq 0.05$.

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line with this observation, overexpression of Nur77 significantly decreased NF-κB activity as determined with luciferase reporters in NCI-H292 cells (Fig. 6B).

To further substantiate these findings, we next analyzed the expression of several NF-κB–mediated proinflammatory cytokines. TNF-α stimulation enhanced the expression of cytokines in the control-infected cells and overexpression of Nur77 markedly attenuated mRNA expression of TNF-α, RANTES, and IL-6 and protein levels of TNF-α and IL-1β in lung epithelial cells (Fig. 6C–G). Collectively, these data demonstrate that Nur77 inhibits the NF-κB pathway in lung epithelial cells, corroborating the in vivo findings.

Nur77 deficiency showed enhanced mucus cell hyperplasia in vivo in airway inflammation (Fig. 2B) Therefore, we sought to assess the effect of Nur77 on Muc5ac expression, which is a key player in mucus production in airway inflammation (12). Consistent with published reports, TNF-α significantly increased Muc5ac gene expression in lung epithelial cells (Fig. 6I). Additionally, inhibition of the NF-κB pathway in lung epithelial cells significantly attenuated mRNA expression of Muc5ac (Fig. 6H). Overexpression of Nur77 had no effect on Muc5ac gene expression under basal conditions (Fig. 6I). However, Nur77 strongly decreased TNF-α–induced Muc5ac gene expression (Fig. 6I). Finally, we performed PAS staining on the lung epithelial cells after overexpression of Nur77. Consistent with decreased Muc5ac gene expression, overexpression of Nur77 inhibited TNF-α–induced mucus production (Fig. 6J). To further substantiate these in vitro observations, we analyzed NF-κB activation in lung sections of OVA-treated mice. A phosphorylation-specific Ab directed against RelA/p65 was applied and we quantified nuclear localization (Fig. 7). In line with the in vitro data, we observed increased NF-κB activation in airway epithelial cells of Nur77-deficient mice compared with WT mice. Taken together, our data indicate that Nur77 regulates Muc5ac gene expression and mucus production through NF-κB signaling.

Nur77 SNP association with asthma and severity of BHR

To gain insight on the potential involvement of Nur77 in human asthma, we searched for genetic variation in a cohort of asthma patients. In total 10 *Nur77/NR4A1* SNPs were available in the DAG cohort (34, 35) and analyzed for association with asthma and severity of BHR. No significant association was observed between *Nur77/NR4A1* and asthma (Table I). Three significant associations were found for *Nur77/NR4A1* and the severity of BHR in asthma. The minor allele of all three SNPs leads to more severe BHR in asthma (rs744691A β [CI of 95%] = 0.24 [0.02;0.45], $p = 0.03$; rs876734C β [CI of 95%] = 0.28 [0.09;0.47], $p = 0.005$; rs1283155A β [CI of 95%] = 0.25 [0.05;0.45], $p = 0.01$) (Table II). These data support functional relevance of Nur77 in human asthmatic disease.

Discussion

Allergic asthma is a chronic inflammatory disorder that involves intricate Ag interactions and immune responses, and it is characterized by bronchial hyperresponsiveness, airway inflammation highly involving eosinophils, mucus hypersecretion, and airway remodeling (1, 2). Despite many studies conducted on animals and in humans, the underlying mechanisms of asthma have not been resolved completely. In this study, we document that the nuclear receptor Nur77 plays a critical role in regulating allergic airway inflammation by using mice lacking the Nur77 gene. OVA-challenged Nur77 KO mice showed a significant increase in allergic airway inflammation as evidenced by enhanced infiltration of inflammatory cells with especially more eosinophils and lymphocytes in the airways, increased production of mucus, marked elevation of allergen-specific IgG1 and IgG2a levels, and augmented production of Th2 cytokines. These observations have in vitro relevance, as overexpression of Nur77 significantly diminished the inflammatory response in lung epithelial cells. Moreover, Nur77 decreases Muc5ac gene expression through modulation of the NF-κB pathway. Therefore, activation of Nur77 may inhibit inflammatory responses, and these findings are of considerable importance for understanding and possibly preventing airway inflammation.

Nur77 has been shown to be expressed in peripheral eosinophils and in atopic dermatitis patients. Nur77 expression in eosinophils increases with disease severity (26). In cultured eosinophils CD30 activation results in transient induction of Nur77 expression, and functional involvement has been proposed in subsequent apoptosis of the eosinophils. Of note, both other NR4A nuclear receptor subfamily members Nurr1 and especially NOR-1 are also highly expressed in (activated) eosinophils. Therefore, redundancy of activity of Nurr1 and NOR-1 in these cells cannot be excluded in Nur77 KO mice. At present, we do not know the exact function of NR4A receptors in eosinophils in allergic disease. We do know that the enhanced infiltration of eosinophils in the lung is associated with asthma severity in experimental animals and in humans, and that this process is aggravated in Nur77 KO mice,

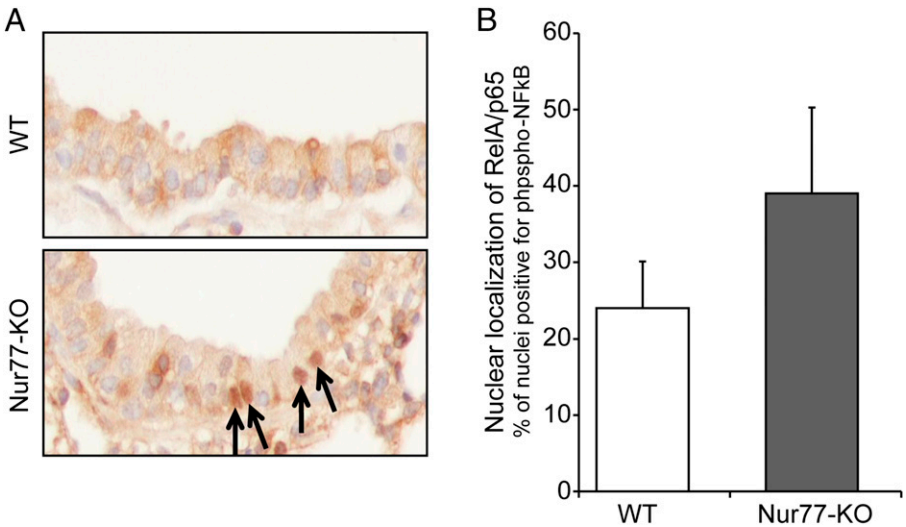

FIGURE 7. Enhanced NF-κB/RelA activation in Nur77-deficient epithelial cells after OVA challenge of mice. (**A**) Representative photomicrographs of sections from lung tissue of WT (*upper panel*) and Nur77-deficient mice (*lower panel*) after OVA challenge. The Ab is specific for active NF-κB/RelA, which is localized in the nucleus. Original magnification ×400. (**B**) Quantification of the nuclei positive for activated NF-κB/RelA in airway epithelial cells in sections of lung tissue. The arrows point at nuclei staining positive for phosphorylated NF-κB /RelA.

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Table I. Association of SNPs in Nur77/NR4A1 and asthma in the DAG cohort

CHR	BP	SNP	Allele	Odds Ratio (Fixed)	Odds Ratio (Random)	*p* (Fixed)	*p* (Random)	*Q*
12	50703829	rs10876228	T	0.92	0.92	0.26	0.26	0.60
12	50706226	rs1283176	G	0.97	0.97	0.60	0.66	0.23
12	50709301	rs879107	T	1.02	1.02	0.78	0.78	0.73
12	50710263	rs876734	C	0.95	0.95	0.53	0.53	0.90
12	50715744	rs2701129	G	1.12	1.11	0.26	0.61	0.04
12	50723758	rs2242107	A	1.08	1.08	0.31	0.32	0.31
12	50726016	rs1283155	A	0.99	0.99	0.94	0.94	0.74
12	50727817	rs744691	A	0.99	0.99	0.87	0.87	0.69
12	50729920	rs2269342	T	0.94	0.94	0.46	0.67	0.09
12	50739549	rs2603751	C	1.07	1.07	0.52	0.83	0.00

BP, base pair; CHR, chromosome.

which can be explained by increased cytokine levels in the lungs of OVA-challenged mice (5).

It is well established that also dendritic cells, lymphocytes, and smooth muscle cells all play a pivotal role in allergic airway inflammation (4, 10). Mice lacking Nur77 showed enhanced infiltration of dendritic cells in BALF following allergen challenge. Although Nur77 is expressed in dendritic cells, no change in its expression was observed after dendritic cell activation (36). Several studies showed that Nur77 is involved in T lymphocyte development and activation. However, Nur77-deficient mice do not show any obvious T cell phenotype (16). A recent study showed that Nur77 inhibits airway smooth muscle cell proliferation in vitro, but this study lacks in vivo data (28). To assess whether Nur77 similarly as in the vessel wall inhibits airway smooth muscle cell proliferation, chronic allergic experiments need to be performed. Altogether, our findings demonstrate that Nur77 is crucial in inhibiting infiltration of inflammatory cells associated with the asthmatic response and that this function is not taken over by Nurr1 and/or NOR-1.

We and others have shown that Nur77 is involved in macrophage polarization and has been shown to inhibit inflammatory response in macrophages, smooth muscle cells, and endothelial cells (20–25). The Th2 cytokines IL-4, IL-5, and IL-13, in concert with specific chemokines such as eotaxins, play a central role in the initiation and maintenance of allergic airway inflammation. Consistent with development of airway eosinophilia, OVA-challenged Nur77-deficient mice show increased levels of IL-13, IL-6, TNF-α, IFN-γ, MCP-1, and eotaxin-2 in BALF compared with WT counterparts. Similar to this, production of IL-4, IL-5, and IL-13 are significantly enhanced in lung-draining lymph node cells of OVA-challenged Nur77 KO mice. Interestingly, production of IL-5 in BALF is significantly lower in OVA-challenged Nur77 KO mice. This is in contrast to the increased influx of eosinophils into the lung and exacerbated production of IL-5 in lymph node cells in Nur77 KO mice. IL-5 is involved in growth, maturation, chemotaxis, and survival of eosinophils (5–7). These conflicting data could be due to local or systemic production of IL-5 or local consumption of IL-5. Upon OVA challenge, Nur77 deficiency resulted in increased inflammation as characterized by increased eosinophilia (associated with the increased eotaxin-2 expression), and several cell types are recruited that may consume the produced IL-5. However, this requires further investigation. In agreement with enhanced Th2 cytokine production, Nur77-deficient mice also displayed increased production of allergen-specific IgG1 and IgG2a. Because Nur77 KO mice exhibited, upon OVA challenge, enhanced upregulation of both Th1 and Th2 response, it is highly likely that Nur77 is involved in regulation of Th response in general and warrants further research.

Airway epithelial cells, among other cell types, play a key role at the onset of airway inflammation in asthma (3, 4). It has been shown that Nur77 is expressed in lung epithelial cells and causes epithelial cell apoptosis following exposure to cadmium. However, the impact of Nur77 in inflammation in epithelial cells was not investigated (37). In the present study, we found that overexpression of Nur77 in airway epithelial cells resulted in a marked reduction in expression levels of diverse proinflammatory cytokines. Several studies reported that NF-κB activity is significantly higher in asthma both in experimental models and in humans and is involved in the cytokine production from a variety of cell types, including lung epithelial cells, dendritic cells, smooth muscle cells, and lymphocytes (13, 14). Moreover, inhibition of NF-κB activity strongly decreases airway inflammation and asthma. We and others previously demonstrated that Nur77 inhibits NF-κB activity and thereby diminishes proinflammatory response in multiple cell types (20–25). Indeed, we found that knockdown of Nur77 resulted in a marked increase in IκBα phosphorylation, whereas overexpression of Nur77 decreased NF-κB activity in lung epithelial cells. As such, the enhanced production of Th2

Table II. Association of SNPs in Nur77/NR4A1 and severity of BHR in the DAG cohort

CHR	BP	SNP	Allele	β	CI of 95%	*p*
12	50703829	rs10876228	T	0.07	−0.13; 0.27	0.49
12	50706226	rs1283176	G	0.04	−0.13; 0.20	0.67
12	50709301	rs879107	T	−0.03	−0.24; 0.18	0.77
12	50710263	rs876734	C	0.28	0.09; 0.47	**0.005**
12	50715744	rs2701129	G	−0.07	−0.33; 0.19	0.58
12	50723758	rs2242107	A	0.10	−0.09; 0.30	0.30
12	50726016	rs1283155	A	0.25	0.05; 0.45	**0.01**
12	50727817	rs744691	A	0.24	0.02; 0.45	**0.03**
12	50729920	rs2269342	T	0.05	−0.17; 0.29	0.65
12	50739549	rs2603751	C	−0.03	−0.31; 0.24	0.81

Significant associations are shown in bold type.
BP, base pair; CHR, chromosome.

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cytokines in BALF and draining lymph node cells in Nur77-deficient mice upon OVA challenge may be due to enhanced activation of the NF-κB pathway in airway epithelium.

Mucus hypersecretion is another important hallmark of asthma. We found that Nur77-KO mice showed a pronounced increase in mucus production compared with control mice following allergen challenge. The NF-κB pathway has been shown to modulate Muc5ac gene expression in epithelial cells (15). In agreement with decreased NF-κB activity, Nur77 significantly decreased the mRNA expression of Muc5ac and also attenuated mucus production in lung epithelial cells. Therefore, it is likely that the exacerbated mucus production observed in Nur77-deficient mice is due to the enhanced Muc5ac expression through activation of the NF-κB pathway.

Airway hyperresponsiveness is an important characteristic feature of asthma, and measurements of airway responsiveness are useful in making a diagnosis of asthma. In the present study, we performed our experiments using OVA in C57BL/6 mice who have been shown to be less responsive to metacholine compared with other mouse strains such as BALB/c (38, 39). It was further suggested that the genetic background of mice influences several aspects of the acute allergic phenotype, including airway hyperresponsiveness (38, 39). Using higher doses of metacholine in an OVA model in C57BL/6 mice for studying airway hyperresponsiveness might be useful and requires more research. Alternatively, studying the function of Nur77 in more clinically relevant asthma models such as ozone or house dust mite may be useful to gain insight on the role of Nur77 in airway hyperresponsiveness and asthma, and future studies are warranted in this direction.

Our genome-wide association study data in the DAG cohort suggests that there is no association between genetic variation of *Nur77* with asthma. However, severity of BHR does show association with three SNPs in the *Nur77* gene. A limitation of our study is that this association was shown in only one cohort and future studies are required to confirm that our associations represent true biologically significant findings.

In conclusion, our experiments in mice and human epithelial cells demonstrate that Nur77 has a protective function in airway inflammation involving inhibition of the NF-κB pathway. Nur77 deficiency markedly enhanced OVA-induced Th2 cytokine production, pulmonary eosinophilia, serum IgG1 and IgG2a levels, and mucus hypersecretion in a model of murine airway inflammation. So far, several small-molecule agonists have been identified that enhance the activity of Nur77 (40, 41), and our findings support the possibility that Nur77 agonists provide an effective treatment for allergic airway disease.

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Disclosures

The authors have no financial conflicts of interest.

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