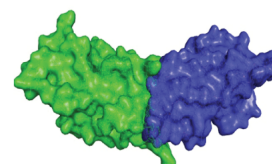




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Anti-Inflammatory Effects of the Neurotransmitter Agonist Honokiol in a Mouse Model of Allergic Asthma

Melissa E. Munroe,* Thomas R. Businga,[†] Joel N. Kline,[†] and Gail A. Bishop*,^{†,‡}

Chronic airway inflammation is a hallmark of asthma, an immune-based disease with great societal impact. Honokiol (HNK), a phenolic neurotransmitter receptor (γ -aminobutyric acid type A) agonist purified from magnolia, has anti-inflammatory properties, including stabilization of inflammation in experimentally induced arthritis. The present study tested the prediction that HNK could inhibit the chronic inflammatory component of allergic asthma. C57BL/6 mice sensitized to and challenged with OVA had increased airway hyperresponsiveness to methacholine challenge and eosinophilia compared with naive controls. HNK-treated mice showed a reduction in airway hyperresponsiveness as well as a significant decrease in lung eosinophilia. Histopathology studies revealed a marked drop in lung inflammation, goblet cell hyperplasia, and collagen deposition with HNK treatment. Ag recall responses from HNK-treated mice showed decreased proinflammatory cytokines in response to OVA, including TNF- α -, IL-6-, Th1-, and Th17-type cytokines, despite an increase in Th2-type cytokines. Regulatory cytokines IL-10 and TGF- β were also increased. Assessment of lung homogenates revealed a similar pattern of cytokines, with a noted increase in the number of FoxP3⁺ cells in the lung. HNK was able to alter B and T lymphocyte cytokine secretion in a γ -aminobutyric acid type A-dependent manner. These results indicate that symptoms and pathology of asthma can be alleviated even in the presence of increased Th2 cytokines and that neurotransmitter agonists such as HNK have promise as a novel class of anti-inflammatory agents in the treatment of chronic asthma. *The Journal of Immunology*, 2010, 185: 5586–5597.

The incidence and severity of asthma, a chronic inflammatory disease, have risen dramatically in the United States over the past 30 y, despite advances in understanding the pathogenesis and ideal approaches to this disorder. Asthma is now the most common chronic disease of children and one of the most common respiratory diseases in adults. The hallmark of asthma is chronic airway inflammation, with multiple pulmonary pathologies, including airway hyperresponsiveness (AHR) and bronchoconstriction, airway remodeling, eosinophilic infiltration, mucus hypersecretion, and collagen formation (1). The mainstay of treatment remains inhalation of corticosteroids. However, a significant proportion of patients with asthma cannot control their disease with such therapy, and only about one-third of patients benefit from the addition of leukotriene inhibitors (2). Specific immunotherapy may be beneficial in patients where known allergens contribute to asthmatic exacerbations, but its usefulness is limited by side effects, inconvenience, and disease severity (3). Severe and corticosteroid-resistant forms of asthma lead to life-threatening attacks, and the disease is associated with

a clear increase in mortality rates. There is thus an urgent need for more effective treatments for asthma with fewer undesired side effects.

Cytokines are major targets for novel allergy and asthma therapies as a result of their involvement in chronic inflammation and airway remodeling. Th2-type cytokines contribute to the initiation and pathogenesis of acute asthma (reviewed in Ref. 4). However, recent studies suggest that an array of proinflammatory mediators contribute to both the acute and chronic inflammation associated with this disease. Both Th1 (5) and Th17 cells cooperate with the Th2 response to promote pathogenic inflammation; Th17 cells are additionally believed to mediate steroid-resistant airway inflammation (6). Two additional cytokines of particular interest are TNF- α and IL-6. TNF- α , found in increased levels in the airway of asthmatic patients, plays multiple exacerbating roles in asthma, including enhancing the production of other proinflammatory cytokines, increasing levels of exhaled NO by stimulating NO synthase production, and stimulating the proliferation of sub-epithelial myofibroblasts that participate in airway remodeling (reviewed in Ref. 7). TNF- α also stimulates enhanced expression of various cellular adhesion molecules, which is important for subsequent recruitment of eosinophils, neutrophils, and lymphocytes to the airway. TNF- α can induce corticosteroid resistance, and it has been suggested that TNF- α blocking agents could be clinically useful in asthma (7). Another proinflammatory cytokine involved in various aspects of asthma pathology is IL-6, which promotes Th2 activation and allergic responses in humans, as well as inhibiting the activity of regulatory T cells. For these reasons, inhibition of IL-6 or its receptor is also proposed as a potential therapy in asthma (8).

Although cytokine-based therapies have potential benefit in asthma, clinical trials to date have achieved mixed results, underscoring the complexity of asthma pathogenesis. In addition to potential side effects, these therapies are costly, both of which might reduce compliance in this patient population. This has prompted the development and evaluation of small m.w. compounds

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Abbreviations used in this paper: ABPAS, Alcian blue and periodic acid-Schiff; AHR, airway hyperresponsiveness; BAL, bronchoalveolar lavage; GABA_A, γ -aminobutyric acid type A; GABA_AR, γ -aminobutyric acid type A receptor; HNK, Honokiol; Med/IC, medium/isotype control; N/A, not applicable; R_N, Newtonian resistance; WT, wild-type.

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capable of inhibiting inflammatory mediators. CpG-oligodeoxynucleotides have shown promise as anti-inflammatory agents in murine models of asthma, but this finding has not yet translated to human studies (9).

HNK is a small organic molecule purified from *Magnolia* species that has been well-tolerated in models of heart disease, cancer, and most recently inflammatory arthritis (10). Importantly, HNK has been used in humans without noticeable side effects for many years in traditional Asian medicine. In vitro studies in macrophages (11) and neutrophils (12) suggest an anti-inflammatory role for HNK. Our own studies suggest that HNK also has immunomodulatory effects on lymphocyte activation, both in vitro and in vivo (10). We found a prominent anti-inflammatory role for HNK, potentially in both the cognitive and effector phases of the immune response, by inhibiting cytokines that lead to chronic inflammation. HNK is a known ligand for the γ -aminobutyric acid type A (GABA_A) receptor (GABA_AR) and present on a variety of neuronal cells (13), as well as in the periphery, including lymphocytes (14, 15). The GABA_AR has recently been shown to influence asthma pathogenesis (16), and others have reported a potential anti-inflammatory role for the receptor (14).

In this paper, we present results of a study exploring the anti-inflammatory effects of HNK in a mouse model of allergic asthma (or "allergic airway disease"). In both acute and chronic models of Ag exposure, HNK treatment showed excellent potential as a new anti-inflammatory treatment for asthma, one that is already known to be well-tolerated from use in traditional medicine. Surprisingly, therapeutic effects were observed despite increased levels of Th2 cytokines, providing important new information on the complex and multiple mechanisms of inflammation pathogenesis in asthma.

Materials and Methods

Reagents and Abs

HNK was purchased from Chromadex (Irvine, CA). Bicuculline was purchased from Tocris Bioscience (Ellisville, MO). Twenty percent Intralipid was purchased from Sigma-Aldrich (St. Louis, MO). OVA (grade V) was purchased from Sigma-Aldrich, and any contaminating endotoxin was removed with an endotoxin removal column per manufacturer's instructions (Sterogene Bioseparations, Carlsbad, CA). OVA used for in vivo and ex vivo studies contained <5 ng endotoxin/mg OVA, as determined by the limulus assay (Charles River Laboratories, Wilmington, MA). Capture and detection Abs to detect human/mouse TGF- β , as well as rTGF- β , were purchased as a set from eBioscience (San Diego, CA). Streptavidin-HRP was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). ELISA tetramethylbenzidine peroxidase substrate was purchased from Kirkegaard & Perry Laboratories (Gaithersburg, MD). Biosource Multiplex buffers and Abs to mouse IL-2, TNF- α , IL-6, IL-17, IL-12 (p40/p70), IFN- γ , IL-4, IL-5, IL-13, and IL-10 were purchased from Invitrogen (Carlsbad, CA). Anti-FoxP3 (clone FJK-16s) was purified, and biotinylated anti-CD3e (clone 145-2C11), anti-CD28 (clone 37.51), and isotype control Abs were purchased from eBioscience. Anti-type IV collagen (rabbit polyclonal) and relevant rabbit IgG-negative control Abs were purchased from Abcam (Cambridge, MA). Alexa Fluor 488-labeled goat anti-rat Ab, Alexa Fluor 466-labeled streptavidin, and Alexa Fluor 633-labeled goat anti-rabbit Ab were purchased from Invitrogen.

Mice

Female C57BL/6 mice were purchased at 5–8 wk of age from the National Cancer Institute (Frederick, MD). All animal care and housing requirements of the National Institutes of Health Committee on Care and Use of Laboratory Animals were followed, and all procedures were performed as approved by the University of Iowa Animal Care and Use Committee. Animals were housed in specific pathogen-free environments and were allowed access to food and water ad libitum.

Mouse models of allergic asthma

Acute asthma model is shown in Fig. 1A. Mice remained naive or were sensitized to OVA i.p. (10 μ g OVA in 1 mg alum) on days 0 and 7,

followed by aerosol challenge with OVA (1% solution, 30 min) on days 14 and 16. Animals received either vehicle control or HNK i.p. (150 mg/kg/day HNK diluted in 20% Intralipid) on days 13–17.

Chronic asthma model is shown in Fig. 1B. Mice remained naive or were sensitized to OVA i.p. (10 μ g OVA in 1 mg alum) on days 0 and 7, followed by aerosol challenge with OVA (2.5% solution, 30 min) three times per week for 6 wk. Animals received either vehicle control or HNK i.p. (150 mg/kg/day HNK diluted in 20% Intralipid) on days 14, 16, 18, 28, 30, 32, 42, 44, and 46.

AHR to inhaled methacholine was assessed in terms of changes in central airway resistance (R_n), using the Flexivent system, as described previously (17). Mice were anesthetized with ketamine at 90 mg/kg and pentobarbital at 50 mg/kg and attached to a small-animal ventilator (Flexivent; SCIREQ, Chandler, AZ). Animals were ventilated at 150 breaths/min. Positive end-expiratory pressure was maintained between 2 and 3 cm H₂O, with the computer setting the tidal volume from the entered weight of each animal. Central airway resistance (R) was measured at baseline and after 10 s of nebulized methacholine at doses of 12.5, 25, and 50 mg/ml.

Mice were euthanized after measurement of AHR. Bronchoalveolar lavage (BAL) was prepared for cell counts, at which time lungs were removed and prepared for histopathology and cytokine determination.

Histopathology and morphometry

Lungs were excised and fixed in paraformaldehyde postmortem. Tissue blocks were embedded in paraffin, and 5 μ m sections were stained with H&E for evaluation of lung inflammation or Alcian blue and periodic acid-Schiff (ABPAS) for enumeration of mucin-positive goblet cells. Masson trichrome stain was used to evaluate collagen deposition. Lung tissue was prepared and analyzed in conjunction with the Central Microscopy Research Facility and Comparative Pathology Laboratory at the University of Iowa. Histology results were interpreted using an Olympus BX-51 Light Microscope fitted with a SPOT RT KE three-shot color camera and accompanying SPOT imaging software from Diagnostic Instruments. Histological assessment was determined by an investigator blinded to the treatment groups.

The intensity of lung inflammation/alveolitis (H&E sections) was graded on the following scale, as previously described (18): 0, normal aspect; 1, mild alteration; 2, moderate alteration; 3, strong alteration; and 4, severe alteration. Whole lung lobe sections of individual mice were observed, and inflammation scores were assigned for quantification.

The thickness of the airway epithelial layer was measured by tracing around the basement membrane and the luminal surface of epithelial cells and calculating the area between these lines, using Image Pro-Discovery software (Media Cybernetics, Bethesda, MD). The area was expressed per length (micrometers) of basement membrane to account for variation in airway diameters (19). At least 10 airway sections/animal were measured.

The extent of goblet cell hyperplasia (ABPAS sections) was determined using design-based protocols (20). The number of mucin-positive goblet cells and epithelial cells was separately enumerated, and the ratio of these cell types were calculated individually for at least eight airway sections per mouse.

Collagen deposition was quantified microscopically (Masson trichrome sections) using the point counting method, as described previously (21). Six whole lung lobe sections per mouse were digitally imaged, and each image was overlaid with a 36 $\times$ 50 (1800) point grid. Points where parenchyma and air spaces were stained green were counted as regions with collagen deposits. The percentage of collagen deposits for each mouse lung sample was calculated as the (number of collagen points in parenchyma)/(total number points in the lung parenchyma).

Immunofluorescence

Slides containing lung sections from mice in the chronic model of allergic asthma were deparaffinized and treated with Retrieval A per manufacturer's protocol (BD Biosciences, San Jose, CA). Nonspecific binding sites in the sections were blocked using 10% goat serum, 0.1 mg/ml 2.4G2, and 2.5% BSA. Endogenous biotin activity was blocked with avidin/biotin blocking solution (Vector Laboratories, Burlingame, CA). Samples were then treated with anti-FoxP3 (clone FJK-16s; eBioscience), biotinylated anti-CD3e (clone 145-2C11; eBioscience), and anti-type IV collagen (rabbit polyclonal; Abcam) at 5 μ g/ml, overnight at 4°C. After washing in PBS, the slides were subsequently incubated with Alexa Fluor 488-labeled goat anti-rat Ab for FoxP3, Alexa Fluor 466-labeled streptavidin for CD3e, and Alexa Fluor 633-labeled goat anti-rabbit Ab for type IV collagen at 10 μ g/ml for 30 min at room temperature. Slides were washed with PBS and coverslips attached in Vectashield mounting medium (Vector Laboratories). Digital images were evaluated using an Olympus BX-51 Light Microscope fitted with a SPOT RT KE three-shot color camera and accompanying SPOT imaging software from Diagnostic

Instruments. Fluorescent labeling was enumerated using Image Pro-Discovery software.

Lung homogenization

Lung lobes from individual mice used for hydroxyproline or cytokine determination were snap frozen in liquid nitrogen and then stored at -70°C until homogenization and cytokine analysis. Lung lobes were placed in Tissue Extraction Reagent (Invitrogen), according to the manufacturer's directions, then homogenized with a probe sonicator (Branson Ultrasonics, Danbury, CT). Lung homogenates were stored at -70°C .

Hydroxyproline assay

Total lung collagen was determined by analysis of hydroxyproline, as described previously (22, 23). Briefly, one part homogenate was diluted in two parts 6 N HCl for 8 h at 120°C . Five microliters of citrate/acetate buffer (5% citric acid, 7.24% sodium acetate, 3.4% sodium hydroxide, and 1.2% glacial acetic acid [pH 6.0]) and 100 μl chloramine-T solution (282 mg chloramine-T, 2 ml *n*-propanol, 2 ml H_2O , and 16 ml citrate/acetate buffer) were added to 5 μl sample, and the samples were left at room temperature for 20 min. Next, 100 μl Ehrlich's solution [4-(dimethylamino) benzaldehyde; Sigma-Aldrich] was added to each sample, and the samples were incubated for 15 min at 65°C . Samples were cooled for 10 min and read at 550 nm by a SpectraMax250 Reader (Molecular Devices, Sunnyvale, CA). Data were analyzed with SoftMax Pro software (Molecular Devices); unknowns were compared with a standard curve containing 0–100 $\mu\text{g}/\text{ml}$ hydroxyproline (Sigma-Aldrich). The coefficient of determination for the standard curve (r^2) was >0.98 .

Isotype and OVA-specific Ig ELISA

Assays for total (24) and anti-OVA (25) isotype-specific Abs in mouse sera were performed by ELISA, using isotype-specific coating Abs (total Ig assay), 5 $\mu\text{g}/\text{ml}$ OVA (OVA-specific assay), biotinylated detection Abs, isotype standards, and p-nitrophenyl phosphate substrate according to the protocol provided by Southern Biotech. The reaction was stopped with 5% EDTA. Plates were read at 405 nm by a SpectraMax²⁵⁰ Reader. Data were analyzed with SoftMax Pro software (Molecular Devices); unknowns were compared with a standard curve containing at least five to seven dilution points of the relevant isotype on each assay plate. In all cases, the coefficient of determination for the standard curve (r^2) was >0.98 . Samples were diluted 1:16,000–1:256,000 (total) and 1/1,000–1/4,000 (OVA) to fall within the standard values.

Splenocyte cell culture

Spleens were collected from female C57BL/6 mice after death on the final day of the acute or chronic asthma experimental protocol. Single-cell suspensions (4×10^6 cells/ml) were cultured in RPMI 1640 medium containing 10% FCS, 10 μM 2-ME, and antibiotics, in the presence of 100 $\mu\text{g}/\text{ml}$ OVA. OVA-specific proliferation was determined in 72-h 96-well cultures by pulsing with 1 $\mu\text{Ci}/\text{well}$ [^3H]thymidine deoxyribose (GE Healthcare, Piscataway, NJ) at 48 h, and cpm were determined by liquid scintillation 24 h later. Culture supernatants were collected at empirically derived, optimal culture times for cytokine analysis: samples for IL-2, IL-6, TNF- α , IL-10, and IL-12 (p40/p70) were collected at 48 h, and samples for IFN- γ , IL-17, IL-4, IL-5, IL-13, and TGF- β were collected at 72 h.

Cytokine ELISA/multiplex

Splenocyte culture supernatants and lung homogenates were assayed for the presence of cytokines using BioSource Multiplex system (Invitrogen Life Technologies, Carlsbad, CA) per manufacturer's protocol. Samples were analyzed using a Bio-Rad Bio-plex 200 instrument and multiplex software. TGF- β was assayed according to manufacturer's kit protocol (eBioscience), and the ELISA plate read at 450 nm by a SpectraMax 250 Reader (Molecular Devices). Data were analyzed with SoftMax Pro software (Molecular Devices). Unknowns were compared with a standard curve containing at least five to seven dilution points of the relevant recombinant cytokines on each assay plate. In all cases, the coefficient of determination for the standard curve (r^2) was 0.98. Samples were diluted to fall within the standard values.

Cell lines

The mouse B cell lines M12.4.1 and CH12.LX (10), as well as the mouse T cell line 2B4.11 (26), have been described previously. Cell lines were maintained in RPMI 1640 medium containing 10% FCS, 10 μM 2-ME, and antibiotics. Hi5 insect cells expressing mouse CD154 have been described and characterized previously (27). These cells grow at 26°C ,

rapidly die to form membrane fragments at 37°C , and therefore do not overgrow cell cultures.

Dual-luciferase reporter assays

M12.4.1 cells (1.5×10^7) were transiently transfected with 10 μg 4 $\times$ NF- κB , 40 μg 7 $\times$ AP-1, or 40 μg 4 $\times$ C/EBP β luciferase reporter plasmid (28) and 1 μg Renilla luciferase vector (pRL-null; Promega, Madison, WI) by electroporation. 2B4.11 cells were transiently transfected with 40 μg 4 $\times$ NFAT (Stratagene/Agilent Technologies, Santa Clara, CA), pSTAT4, pSTAT3, or pGATA-3 (Panomics, Fremont, CA) plasmids and 1 μg Renilla luciferase vector (pRL-null) by electroporation. Cells were rested on ice for 15 min and then stimulated (2×10^6 cells/ml) for 6 h (NF- κB) or 24 h (all others). M12.4.1 cells were stimulated with Hi5 cells (at a ratio of 1 Hi5 cell:5 B cells) expressing wild-type (WT) baculovirus or mCD154, the ligand for CD40. 2B4.11 cells were stimulated with plate-bound anti-CD3 ϵ (0.5 $\mu\text{g}/\text{ml}$) $\pm$ soluble anti-CD28 (1 $\mu\text{g}/\text{ml}$; versus medium/isotype control). After stimulation, cells were pelleted, lysed, and assayed for relative luciferase activity (NF- κB , AP-1, or C/EBP β : Renilla, M12.4.1; NFAT, STAT4, STAT3, or GATA-3: Renilla, 2B4.11) per manufacturer's protocol (Promega) using a Turner Designs 20/20 luminometer, with settings of a 2-s delay followed by a 10-s read.

Statistical analyses

All data points represent the mean $\pm$ SEM for groups of individual mice. Analyses were performed with GraphPad Instat software (San Diego, CA). A two-tailed paired Student *t* test or Mann-Whitney nonparametric test was used to determine statistical significance, where appropriate. A *p* < 0.05 was considered significant.

Results

Effect of HNK on AHR

One hallmark of asthma-associated inflammation is AHR, manifested by increased sensitivity to inhaled methacholine challenge. We investigated the effect of HNK on AHR in a mouse model of allergic airway inflammation (Fig. 1), sensitizing mice to OVA in alum i.p. on days 0 and 7, then administering a respiratory challenge of aerosolized OVA over the course of 10 d (acute allergic asthma model; Fig. 1A) or several weeks (chronic allergic asthma model; Fig. 1B). Mice were then challenged with methacholine and AHR assessed, as described in *Materials and Methods*. In both the acute (Fig. 1A) and chronic (Fig. 1B) models of allergic asthma, mice sensitized and challenged with OVA were significantly more sensitive to methacholine exposure than naive controls (*p* < 0.001) (Fig. 2).

We first asked whether HNK would have an effect on AHR in the acute model (Fig. 2A) and whether its potential anti-inflammatory effect would be beneficial if administered i.p. during the challenge phase when eosinophilic airway inflammation is seen. Untreated OVA and control mice received vehicle only. Mice receiving HNK during the challenge phase had significantly lower AHR to methacholine compared with untreated (OVA) mice at all doses tested (*p* $\leq$ 0.05), with comparable response to naive control animals at 12.5 and 25 mg/ml doses. We saw a similar significant inhibition of AHR when HNK was administered during both the sensitization and challenge phases (Supplemental Fig. 1). Given the significant effects of HNK on the acute mouse model of allergic asthma, we decided to investigate its effect in a chronic model, where airway remodeling (collagen deposition) is more pronounced. We saw a similar significant inhibition of AHR with methacholine challenge in mice exposed to OVA over a period of 6 wk and treated with HNK during the chronic challenge phase compared with the acute model (Fig. 2B), compared with untreated OVA-challenged mice.

Effect of HNK on lung inflammation

The inflammatory nature of asthma can be visualized in the lung with a preponderance of eosinophils, increased mucus production as the result of goblet cell hyperplasia, and collagen deposition in the lung as the disease progresses. We evaluated the effect of HNK

treatment in OVA-sensitized/challenged mice on overall lung inflammation histologically with H&E staining (Fig. 3A, 3B), assigning an inflammation score to multiple sections of lung from each experimental group as described in *Materials and Methods*. Untreated (vehicle only) OVA mice in both acute and chronic models of allergic asthma had significant inflammation compared with control mice, which demonstrated no inflammation (inflammation score = 0; data not shown) (Table I). In the acute model of allergic asthma (Fig. 3A), HNK treatment during the challenge phase showed significantly less inflammation than the untreated (OVA) mice ($p \leq 0.001$) (Table I). As with AHR, this was even more evident in the chronic asthma model (Fig. 3B), where HNK was also administered during the challenge phase ($p < 0.0001$) (Table I).

Lung tissue sections were also evaluated for other structural changes associated with airway remodeling. Morphometric examination of airway sections (Fig. 3A, 3B) revealed that OVA exposure significantly ($p \leq 0.001$) increased epithelial cell thickness in both the acute and chronic models of airway inflammation (Table I). Interestingly, HNK treatment during the challenge phase of either model reduced the epithelial cell thickness significantly ($p \leq 0.001$) to the level of control mice. A similar trend was seen when evaluating goblet cell hyperplasia (Fig. 3C, 3D), a source of increased mucus production in asthma. There was a significant increase in the percentage of goblet cells in the OVA mice in both the acute and chronic models of allergic asthma compared with control ($6.393 \pm 0.379\%$; $p < 0.0001$ acute model; $p = 0.01$ chronic model) (Table I). HNK treatment significantly ($p \leq 0.05$) reduced the number of stained goblet cells in both the acute and chronic models of allergic asthma, with again a greater effect seen in the chronic model.

Increased collagen deposition is a hallmark of airway remodeling due to prolonged inflammation with chronic asthma. Lung sections from mice in the chronic allergic asthma protocol were stained with Masson trichrome stain (Fig. 3E) and evaluated for collagen deposition by point counting, as described in *Materials and Methods* (Table I). In parallel with histological evaluation, hydroxyproline was assessed in lung homogenates from the same animals as an indicator of increased collagen formation (22) Table

I. Compared with control animals, animals exposed to OVA (untreated, vehicle only) had over three times the amount of collagen deposition ($p < 0.0001$) as seen by Masson trichrome staining (Fig. 3E) or hydroxyproline measurement (Table I). Mice treated with HNK during the challenge phase demonstrated half the collagen deposition compared with the untreated, OVA-immunized/challenged mice ($p < 0.0001$).

In summary, the anti-inflammatory effects of HNK treatment are seen in both acute and chronic asthma models but are more pronounced in chronic disease, with a substantially longer challenge phase (hence, course of HNK treatment) than the acute model (Fig. 1). Importantly, the effects of HNK on lung inflammation are comparable to other experimental therapies in this mouse model, including leukotriene receptor blockade (29) and CpG administration (9).

Effect of HNK on infiltrating cells of the lung

Although lung infiltration by eosinophils is considered to be a distinguishing feature of Th2-driven atopic asthma (5), other cells, including lymphocytes, macrophages, and neutrophils, also play a role in the chronic inflammation of asthma (30). The increased number and variety of cell types can be found in the lung as the adaptive immune response and its various cytokine networks signal the recruitment and activation of inflammatory cells during the effector, chronic inflammatory phase of asthma pathogenesis (31). At the end of both the acute (Fig. 1A) and chronic (Fig. 1B) experimental protocols, BAL was performed on all animals as described in *Materials and Methods*. OVA sensitization/challenge increased the total number of BAL cells, compared with naive mice in both the acute (Fig. 4A) and chronic (Fig. 4B) phases of allergic asthma ($p < 0.0001$); most of the infiltrating cells were eosinophils. Treatment with HNK in the acute model (Fig. 4A) decreased both the total cellularity and number of eosinophils found in the BAL ($p < 0.05$). A similar trend was seen in the chronic model (Fig. 4B), where HNK treatment resulted in less cellularity and fewer eosinophils in the BAL ($p < 0.05$) compared with untreated, OVA-sensitized/challenged mice.

When evaluating each BAL cell population (Fig. 4), we found that as eosinophils (black bars) were significantly inhibited with

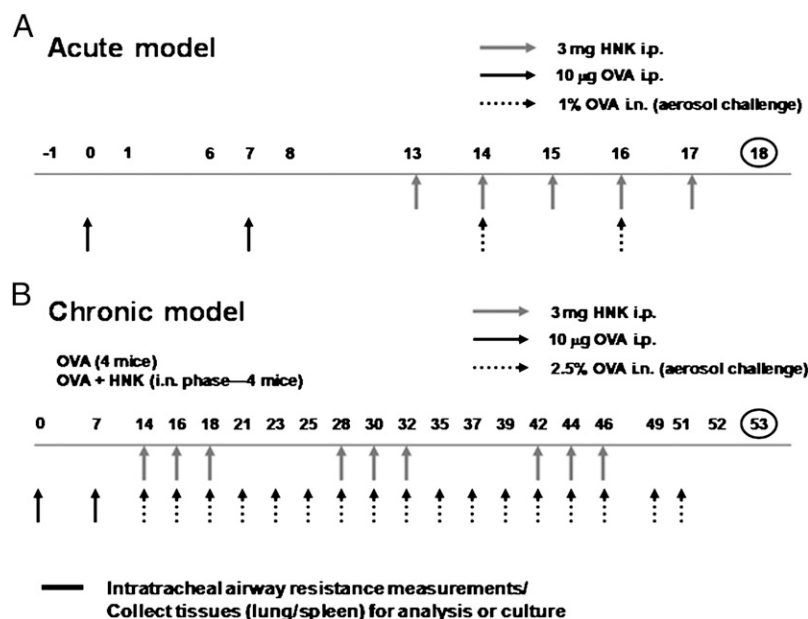


FIGURE 1. Protocols used for mouse models of allergic asthma and treatment with HNK. *A*, Acute model of allergic asthma; *B*, chronic model of allergic asthma.

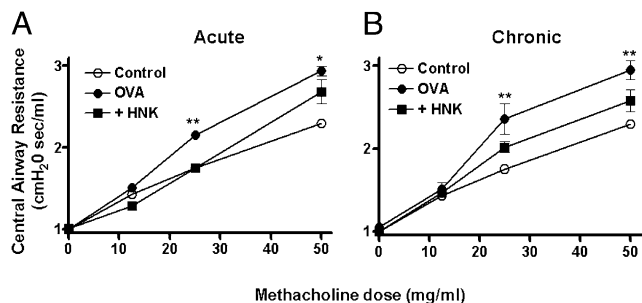


FIGURE 2. Effect of HNK on acute (A) and chronic (B) mouse models of allergic asthma. Female C57BL/6 mice remained naive (control) or were sensitized/challenged to OVA $\pm$ treatment with HNK during the challenge phase. Control and OVA-sensitized mice not receiving HNK were treated with vehicle control, as described in *Materials and Methods*. Airway hyperactivity to methacholine, as measured by an index of resistance in the major conducting airways (R_N , Newtonian resistance), was assessed 48 h after the final allergen challenge. Each bar represents the mean $\pm$ SEM of central airway resistance readings (10/methacholine dose) from individual mice. A, $n = 4$ for control; $n = 4$ for OVA; $n = 4$ for HNK (challenge). B, $n = 4$ for control, OVA, and HNK. Statistical analysis: $*p \leq 0.05$; $**p \leq 0.001$ for OVA versus control or HNK at indicated concentrations of methacholine.

HNK treatment, there was an increase in the total number and percent of macrophages (light gray bars) present in the BAL ($p < 0.05$). Neutrophils (white bars) and lymphocytes (dark gray bars) were only present in the BALs of mice that were sensitized/challenged with OVA. HNK treatment during either the acute or chronic allergic asthma model resulted in similar numbers of and lymphocytes compared with untreated OVA mice.

Effect of HNK treatment on lung cytokines

Cytokines are a driving force behind cellular infiltration, AHR, and airway remodeling in asthma (31). We thus determined the cytokine profile in lung homogenates from mice in both the acute (Fig. 5A) and chronic (Fig. 5B) protocols by evaluating lung homogenates for both pro- and anti-inflammatory/regulatory-type cytokines, as described in *Materials and Methods*. In the acute protocol (Fig. 5A), all cytokines assayed were increased in mice sensitized/challenged with OVA, compared with naive controls. HNK treatment given during the challenge phase significantly decreased proinflammatory cytokines, including IL-2, TNF- α , IL-6, IL-17, and IL-12 (IFN- γ was undetectable in lung homogenates from all mouse groups in the acute protocol). Th2 and regulatory cytokines, including IL-4, IL-13, IL-10, and TGF- β (but not IL-5), were significantly increased compared with untreated (vehicle only) OVA mice.

In the chronic protocol (Fig. 5B), a similar but distinct cytokine trend was detected in the mouse lung homogenates. As in the acute protocol, OVA-sensitized/challenged mice had significantly more cytokine production compared with naive controls (including IFN- γ). HNK treatment during the challenge phase led to significantly decreased levels of proinflammatory cytokines and increased levels of the Th2- and regulatory-type cytokines IL-13, IL-10, and TGF- β . In the chronic model, however, both IL-5 and IL-4 levels in the lung homogenates were decreased with HNK treatment.

Effect of HNK on OVA recall responses

The adaptive immune response is ignited in peripheral lymphoid organs, including the spleen, prior to immune cell recruitment to the lung during chronic inflammation, including atopic inflammation in asthma. We thus examined whether the splenic OVA-specific proliferative and cytokine responses were similar to those

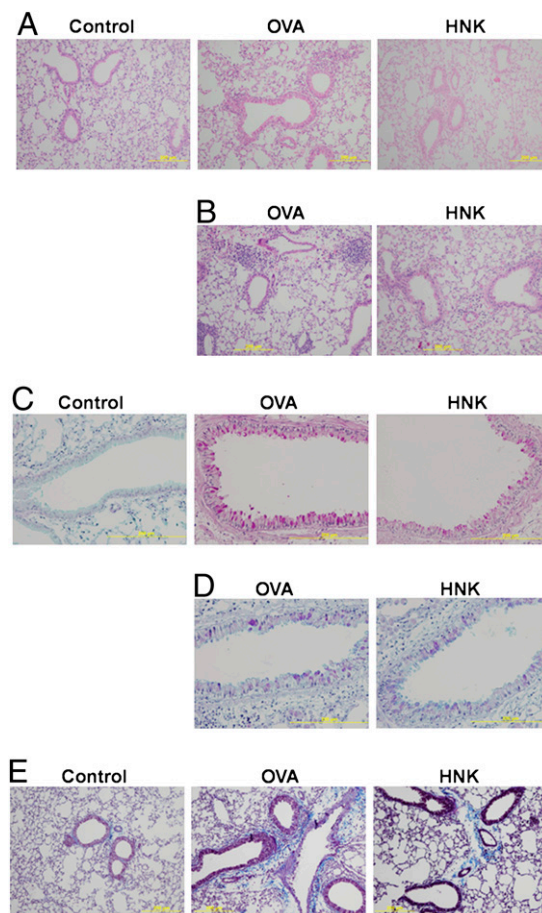


FIGURE 3. Effect of HNK on OVA-mediated lung/airway inflammation. A and B, Representative H&E-stained tissue sections of lungs from mice in acute (A) or chronic (B) models of allergic asthma. C and D, Representative ABPAS-stained tissue sections of lungs from mice in acute (C) or chronic (D) models of allergic asthma. E, Representative Masson trichrome-stained tissue sections of lungs from mice in the chronic model of allergic asthma. Original magnification $\times 10$.

detected in lung homogenates (Fig. 6). In the acute model (Fig. 6A), cells from mice sensitized/challenged with OVA had significantly more OVA-specific proliferation and cytokine production than those from naive control mice ($p \leq 0.05$ for all cytokines). Similar to lung homogenates, HNK treatment significantly decreased proinflammatory cytokines as well as the OVA-specific proliferative response in splenocytes ($p \leq 0.05$). Unlike the lung homogenates, no Th2- or regulatory-type cytokines were suppressed by HNK treatment. IL-5 and TGF- β in particular were significantly augmented when HNK was given during the challenge phase, with no negative impact on IL-4, IL-13, and IL-10.

In the chronic protocol (Fig. 6B), OVA exposure increased the OVA-specific proliferative and cytokine responses compared with naive controls. As in the acute protocol, HNK treatment during the challenge phase inhibited proinflammatory cytokine and proliferative responses. However, unlike the acute protocol, the Th2- and regulatory-type cytokines, particularly IL-5, IL-13, IL-10, and TGF- β , were significantly increased with HNK treatment.

The significant increase in IL-10 and TGF- β in both the lung (Fig. 5) and OVA-specific splenocyte response (Fig. 6), in conjunction with decreased AHR (Fig. 2) and inflammation (Fig. 3, Table I), particularly in the chronic model of allergic asthma (Fig. 1), led us to hypothesize that more FoxP3⁺ T regulatory cells might be present in the lungs of mice treated with HNK (ref).

Table I. Effect of HNK on markers of OVA-induced lung/airway inflammation

	Treatment	Acute ^a	Chronic ^a
Inflammation score ^b	OVA	1.53 ± 0.05	2.56 ± 0.09
	+ HNK	1.13 ± 0.06**	1.38 ± 0.09***
Epithelial cell thickness ^c (area [μm ²] per length of basement membrane [μm])	Control	16.20 ± 0.53	16.50 ± 0.62
	OVA	19.31 ± 0.68****	19.70 ± 1.07****
	+ HNK	16.79 ± 0.48**	15.10 ± 0.76**
Goblet cell hyperplasia ^d (% mucin + cells)	OVA	34.82 ± 2.05	44.75 ± 10.74
	+ HNK	25.44 ± 0.64***	29.56 ± 4.35*
Collagen deposition ^e (% "positive" collagen points)	Control	N/A	5.41 ± 0.66
	OVA	N/A	28.77 ± 0.89****
	+ HNK	N/A	15.76 ± 0.85***
Hydroxyproline ^f (μg/ml/g tissue)	Control	N/A	114.5 ± 0.5
	OVA	N/A	648.7 ± 9.8****
	+ HNK	N/A	369.1 ± 4.5***

^aEach value represents the mean ± SEM.
^bIntensity of lung inflammation in H&E sections from mice in the acute or chronic models of allergic asthma was graded on a scale described in *Materials and Methods*. No inflammation was seen in the control group.
^cEpithelial cell thickness in H&E-stained airway sections from mice in the acute or chronic models of allergic asthma were determined as described in *Materials and Methods*.
^dThe percentage of mucin (+) cells (number of mucin (+) cells/total number of alveolar epithelial cells) in ABPAS-stained tissue sections from mice in the acute or chronic models of allergic asthma was determined as described in *Materials and Methods*. No mucin (+) cells were seen in the control group.
^eThe amount of collagen deposition in Masson trichrome-stained tissue sections of lungs from mice in the chronic model of allergic asthma was determined by point counting as described in *Materials and Methods* (percentage of "positive" collagen points = number of collagen points in parenchyma/total number of points in lung parenchyma).
^fLung tissue was assessed for hydroxyproline as a measure of collagen deposition. *n* = 4 for control, OVA, and HNK.
^{*}*p* ≤ 0.05; ^{**}*p* ≤ 0.001; ^{***}*p* < 0.0001 OVA versus HNK; ^{****}*p* < 0.001 and ^{*****}*p* < 0.0001 control versus OVA.
N/A, not applicable.

Lung sections from mice in the chronic protocol of allergic asthma (Fig. 1) were immunofluorescently stained for FoxP3 (green), CD3 (red), and type IV collagen (blue, positive control) to determine whether HNK had an effect on the expression of FoxP3-stained cells or whether they be lung epithelial cells (32) or T regulatory cells (Supplemental Fig. 2) (33). Comparing OVA-exposed, HNK-treated mice (Supplemental Fig. 2A–D) with control (Supplemental Fig. 2E) and OVA-exposed mice (no HNK treatment; Supplemental Fig. 2F), we see a significant increase in the number of CD3⁺ T cells in the lung with OVA exposure that is not altered by HNK treatment, compared with control (Supplemental Fig. 2G). The total number of FoxP3⁺ cells is significantly decreased in the lungs of OVA-exposed mice yet increased in

HNK-treated, OVA-exposed mice (Supplemental Fig. 2H). However, the number of FoxP3⁺ T lymphocytes (T regulatory cells) increases in both the OVA-exposed groups, compared with control (Supplemental Fig. 2I), and is significantly enhanced with HNK treatment, compared with no HNK treatment. This parallels the cytokine profile observed in the lungs (Fig. 5) and splenocyte OVA recall response (Fig. 6) with HNK treatment.

Alteration of serum Ab isotype distribution by HNK treatment

B cells and the Abs they produce are indicative of Ag exposure and contribute to the pathogenesis of asthma. Certain Ig isotypes, including IgE and IgG1, have been implicated as being particularly significant (25, 34). We analyzed sera from mice in the acute (Fig. 7) and chronic (Fig. 8) models of allergic asthma to determine the isotype profile of both total and OVA-specific Abs. HNK mice had ≥30% higher levels of total and OVA-specific serum IgM

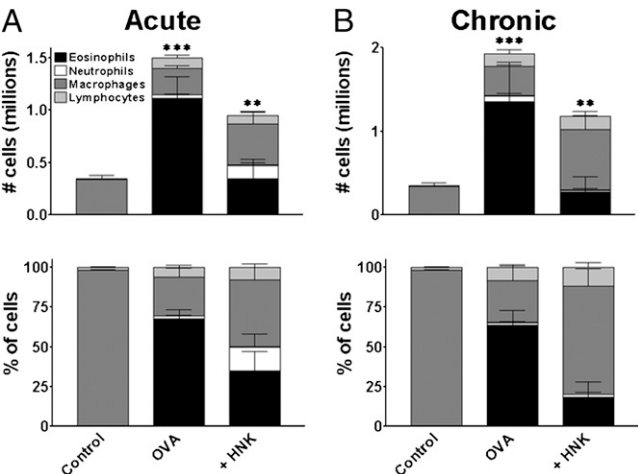


FIGURE 4. Effect of HNK on OVA-mediated cell recruitment to the lung. Postmortem, lungs from mice in the acute (A) or chronic (B) models of allergic asthma were lavaged as described in *Materials and Methods*. Total and differential cell counts were obtained. Data are presented as total number or percentage of cells in the lavage fluid. Each bar represents the mean ± SEM. A and B, *n* = 4 for control, OVA, and HNK (challenge). Statistical analysis (total number of cells): ^{**}*p* ≤ 0.001; ^{***}*p* ≤ 0.0001 for OVA versus control or HNK.

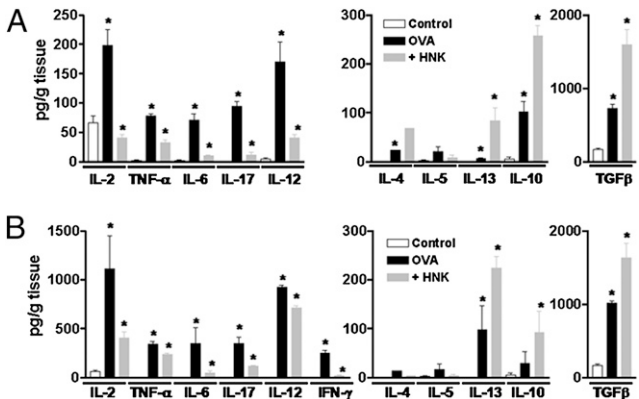


FIGURE 5. Effect of HNK treatment on lung homogenate cytokines. Lung samples from mice in acute (A) or chronic (B) asthma models were homogenized, and cytokines were assessed by multiplex ELISA as described in *Materials and Methods*. Each bar represents the mean ± SEM. IFN-γ was below the level of detection in lung homogenates from mice in the acute asthma model. A and B, *n* = 4 for control, OVA, and HNK (challenge). Statistical analysis: ^{*}*p* ≤ 0.05 for OVA versus control or HNK.

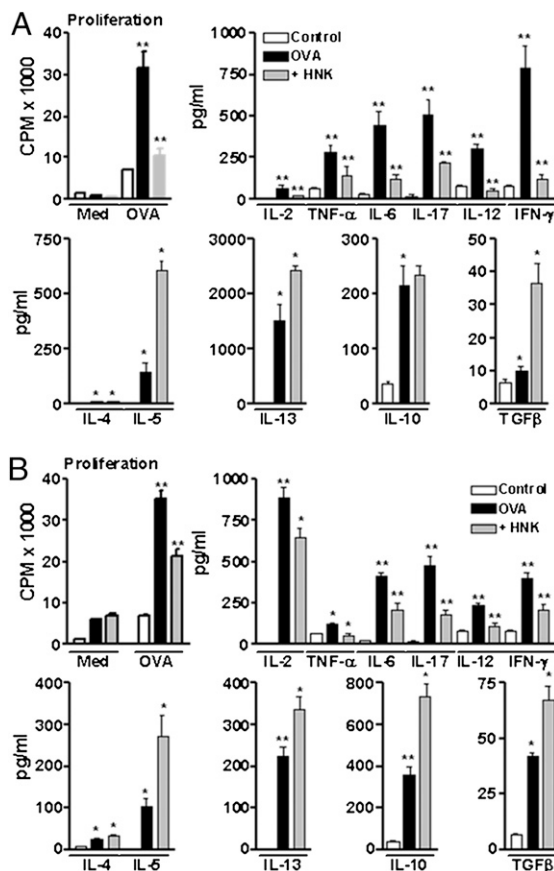


FIGURE 6. Effect of HNK treatment on OVA-specific responses in ex vivo splenocyte cultures. Single-cell suspensions of spleens from mice in acute (A) or chronic (B) asthma models were cultured with OVA and assessed for proliferation (3H incorporation, cpm) and cytokine production (by ELISA), as described in *Materials and Methods*. Each bar represents the mean $\pm$ SEM (cytokines) or the mean $\pm$ SEM of triplicate wells from individual mice (proliferation). Splenocytes cultured in medium (Med) alone induced minimal proliferative and cytokine (data not shown) responses. A and B, $n = 4$ for control, OVA, and HNK (challenge). Statistical analysis: * $p < 0.05$; ** $p \leq 0.01$ for OVA versus control or HNK.

(Figs. 7, 8A, 8B), with unchanged or slightly higher levels of serum IgG1 (Figs. 7, 8C, 8D) and IgE (Figs. 7, 8I, 8J) Abs, compared with OVA mice. However, HNK-treated mice in the chronic model had significantly decreased levels of IgG2b ([Fig. 8E, 8F]; unchanged in the acute model [Fig. 7E, 7F]), and mice from both the acute and chronic models displayed significantly lower levels of total serum IgG3 (Figs. 7, 8G, 8H) (C57BL/6 mice do not make IgG2a). When evaluating the presence of anti-OVA-specific Abs, we observed that these Abs were only present in the sera of mice sensitized/challenged with OVA. These Ab isotypes correspond to the pathogenic IgE and IgG1 Abs in human disease (34). The distribution trend (IgG2b/IgG3 versus IgG1/IgE) of Ab isotype production observed with HNK treatment parallels what was observed in the lung homogenate (Fig. 5) and, in particular, Ag recall culture responses (Fig. 6): decreased levels of proinflammatory, TNF- α , IL-6, IL-17, IL-12, and IFN- γ , with unaffected or increased levels of IL-10, IL-4, IL-5, and IL-13.

GABA_A-mediated anti-inflammatory effects HNK on CD40-mediated B cell activation

B cells contribute to the development of asthma by secreting Abs, with CD40 being required for Ab secretion and class switching (25). In addition to Ab production, proinflammatory cytokines,

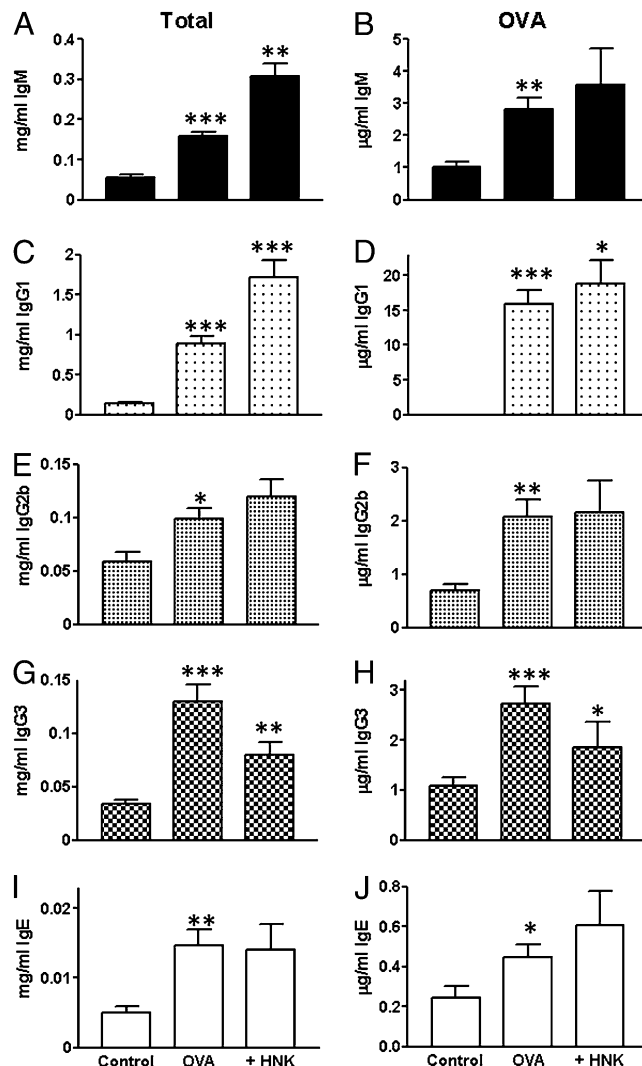


FIGURE 7. Effect of HNK treatment on total and OVA-specific serum Ig in the acute model of allergic asthma. Sera were obtained from female C57BL/6 mice on the penultimate day of the protocol, as outlined in Fig. 1. Levels of Ig isotypes were determined by ELISA, as described in *Materials and Methods*. Each bar represents the mean $\pm$ SEM of triplicate wells of sera from individual mice that were serially diluted from 1:16,000 to 1:256,000 (total, A, C, E, G, I) or 1:4,000 to 1:15,000 (OVA, B, D, F, H, J). $n = 4$ for control, OVA, and HNK (challenge). Statistical analysis: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$ for OVA versus control or HNK.

including TNF- α and IL-6, are secreted in response to CD40 (35) and are able to contribute to the pathogenesis of allergic asthma (36, 37). Given the anti-inflammatory effect of HNK on both acute and chronic models of allergic asthma (Figs. 2–4) and altered total and OVA-specific Ab response in HNK-treated mice (Figs. 7, 8), we hypothesized that HNK would also affect CD40-mediated proinflammatory cytokine production in B cells. As described earlier, HNK is a known ligand of the GABA_AR and present not only on cells in the CNS for neurotransmission but also on immune cells, including lymphocytes. We therefore examined the dependence of HNK on the GABA_AR to mediate its anti-inflammatory effects on CD40-mediated B cell activation.

To this end, we used mouse B cell lines CH12.LX and M12.4.1, which we have extensively demonstrated to mimic primary mouse B cells (38). Data in Fig. 9 demonstrate that stimulation of mouse B cell lines by CD40 results in production of elevated amounts of TNF- α (Fig. 9A) and IL-6 (Fig. 9B), compared with controls, that is

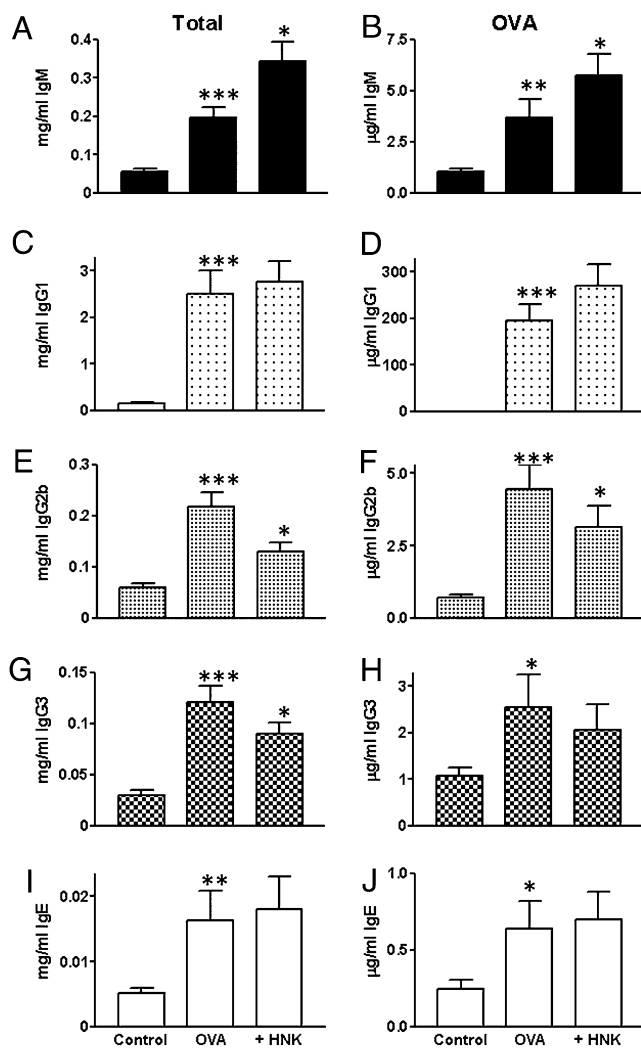


FIGURE 8. Effect of HNK treatment on total and OVA-specific serum Ig in the chronic model of allergic asthma. Sera were obtained from female C57BL/6 mice on the penultimate day of the protocol, as outlined in Fig. 1. Levels of Ig isotypes were determined by ELISA, as described in *Materials and Methods*. Each bar represents the mean + SEM of triplicate wells of sera from individual mice that were serially diluted from 1:16,000 to 1:256,000 (total, A, C, E, G, I) or 1/4,000 to 1/15,000 (OVA, B, D, F, H, J). $n = 4$ for control, OVA, and HNK (challenge). Statistical analysis: $*p \leq 0.05$; $**p \leq 0.01$; $***p \leq 0.001$ for OVA versus control or HNK.

significantly decreased ($p \leq 0.01$) with HNK treatment (25 μM was selected, based on our previous studies [10]). We have previously demonstrated that the decrease in TNF- α and IL-6 production is not due to a toxicity of HNK on cell cultures, as evidenced by IL-10 and IL-4 production (10), as well as a lack of significant apoptosis ($<5\%$ death via propidium iodide staining with HNK $\pm$ bicuculline treatment; data not shown). Using the GABA $_A$ -specific antagonist bicuculline at an empirically derived concentration (39), we see that the HNK uses the GABA $_A$ R to mediate its effect on B cell cytokine production. Bicuculline itself does not alter CD40-mediated B cell cytokine production (data not shown).

Signaling via NF- κB , AP-1, and C/EBP β are required for optimal production of TNF- α (40) and IL-6 (28). We used these cells to evaluate the GABA $_A$ -mediated effect of HNK on CD40-mediated transcriptional activation using reporter gene assays (Fig. 9C–E). CD40 was able to activate NF- κB (Fig. 9C), AP-1 (Fig. 9D), and C/EBP β (Fig. 9E), compared with controls. Again, there was GABA $_A$ -mediated decrease in transcriptional activation of TNF-

α - and IL-6-dependent regulators in the presence of HNK. These data demonstrate that HNK-mediated anti-inflammatory effects occur at the level of transcriptional activators.

Anti-inflammatory effects HNK on TCR/CD28-mediated T cell activation

Cytokines secreted by T lymphocytes, in addition to the CD40-CD154 interaction with B lymphocytes, influence the Ab isotype profile (25). We observed that HNK treatment alters OVA-specific cytokine secretion, both in the lung (Fig. 5) and the splenocyte OVA recall response (Fig. 6), that parallels the total and OVA-specific Ab isotype profile (Figs. 7, 8). Given the ability of HNK to act in a GABA $_A$ -dependent manner on B lymphocytes (Fig. 9), we hypothesized that HNK could also act directly, and in a GABA $_A$ -dependent manner, on activation of T lymphocytes (Fig. 10) (14, 39, 41). The mouse T cell line 2B4.11, shown to mimic primary mouse T cells (26), was activated with plate-bound anti-CD3 $\pm$ anti-CD28 (costimulation) in the presence of HNK $\pm$ bicuculline. Similar to what was observed in the OVA recall response (Fig. 6), HNK treatment alters both CD3- and CD3/CD28-mediated secretion of polarizing cytokines, with a significant ($p \leq 0.01$) decrease in IL-2 (Fig. 10A), IFN- γ (Th1; Fig. 10B), and IL-17 (Th17; Fig. 10C) and significant increase in IL-13 (Th2; Fig. 10D), that is reversed in the presence of the GABA $_A$ -specific antagonist bicuculline. This was not due to HNK toxicity on the cells, because of the increase in IL-13 production and lack of significant apoptosis ($<5\%$ death via propidium iodide staining with HNK $\pm$ bicuculline treatment; data not shown). Similar to B lymphocytes, we see a parallel alteration in transcriptional activation of key mediators of the cytokines tested. HNK significantly altered ($p \leq 0.01$) both CD3- and CD3/CD28-mediated activation of NFAT (IL-2 production; Fig. 10E), STAT4 (IFN- γ production; Fig. 10F), STAT3 (IL-17 production; Fig. 10G), and GATA-3 (IL-13 production; Fig. 10H) in a GABA $_A$ -dependent manner, parallel to their respective cytokines.

Discussion

There is a pressing need for safe and efficacious treatments for asthma that are cost-effective and have minimal side effects. This is especially true in corticosteroid-resistant asthmatic patients in whom IL-17 may be an important inflammatory mediator. Although inhalers can provide short-term treatment of acute attacks, alleviation of the chronic inflammatory component of the disease holds much more promise for disease modification, leading to durable control and an enhanced quality of life. Steroid agents used to treat asthma have side effects when administered in high concentrations over long periods of time, and compliance with inhalers remains an important therapeutic issue. Although biologic therapies show potential (42), they too have side effects and are costly. Data presented in this study indicate that treatment with a new type of immunomodulator, the GABA $_A$ R agonist HNK, modulated inflammation associated with asthma at a dose that was well-tolerated with multiple injections over the course of several weeks in a well-studied model of allergic airway inflammation and which falls within pharmacokinetic range (43). We have also observed that the dose of HNK administered in this study was also well-tolerated under the 7-wk, daily injection regimen of inflammatory arthritis (10).

HNK administration, whether in the acute or chronic models of asthma, resulted in decreased parameters of dysfunction, including AHR (Fig. 2) and overall lung inflammation, with reduced epithelial cell thickness, goblet cell hyperplasia, and collagen deposition (Fig. 3, Table I). The most remarkable effect of HNK treatment on asthma pathogenesis was the suppression of airway eosinophilia (Fig. 4), a hallmark of asthmatic inflammation. Cytokines are the driving

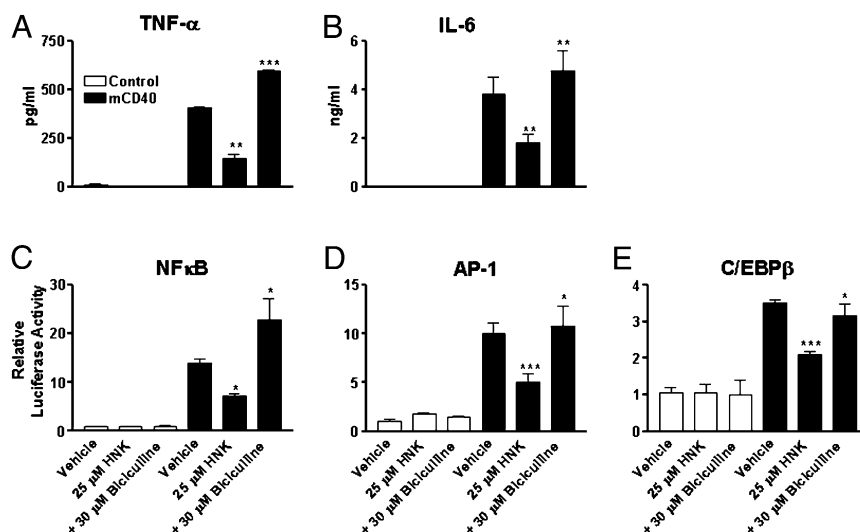


FIGURE 9. GABA_A-dependent effect of in vitro HNK treatment on CD40-mediated cytokine response and transcription factor activation in mouse B cell lines. CH12.LX (A, B) cells were cocultured with Hi-5 mCD154 (mCD40) versus Hi5-WT (control), in the absence or presence of HNK ± bicuculline, as described in *Materials and Methods*. Cytokines in culture supernatants were assessed by ELISA at 4 h (TNF-α; A) or 48 h (IL-6; B). Data points represent mean ± SEM of triplicate wells from two separate experiments. M12.4.1 cells (1.5×10^7 /ml) were transiently transfected with 10 μg 4× NF-κB (C), 40 μg 7× AP-1 (D), or 40 μg 4× C/EBPβ (E) and 1 μg *Renilla* luciferase reporter plasmids by electroporation. Cells were rested on ice, then incubated an additional 6 h (NF-κB) or 24 h (AP-1, C/EBPβ) with insect cell stimuli specific for mCD40, in the absence or presence of HNK ± bicuculline, as described in *Materials and Methods*. Relative luciferase activity of Hi-5 mCD154 (mCD40) versus Hi5-WT (control) was calculated as the mean ± SEM of duplicate samples from two independent experiments. No cell death was seen in the cultures. Statistical analysis: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$ for HNK versus vehicle or bicuculline.

force behind asthma pathogenesis and eosinophilia (31); HNK treatment led to distinct alterations in the cytokine profile both within the lung (Fig. 5) and those detected in ex vivo Ag-specific responses (Fig. 6) that could explain its influence on the asthmatic response. Throughout we see a greater benefit of HNK in the chronic versus the acute phase of disease, consistent with substantial reduction in Th1 cytokines and increase in regulatory cytokines, despite no reduction in

Th2-type cytokine (Figs. 5, 6) or total/OVA-specific Ig (Figs. 7, 8) responses. The lack of reduction in total and OVA-specific IgG1 and IgE responses is indicative of the robustness of the OVA-specific immune response, even with HNK treatment (25).

Although asthma was originally thought to be a Th2-mediated disease (4), proinflammatory cytokines also play significant and distinct roles in asthma pathogenesis. TNF-α and IL-6, as well as

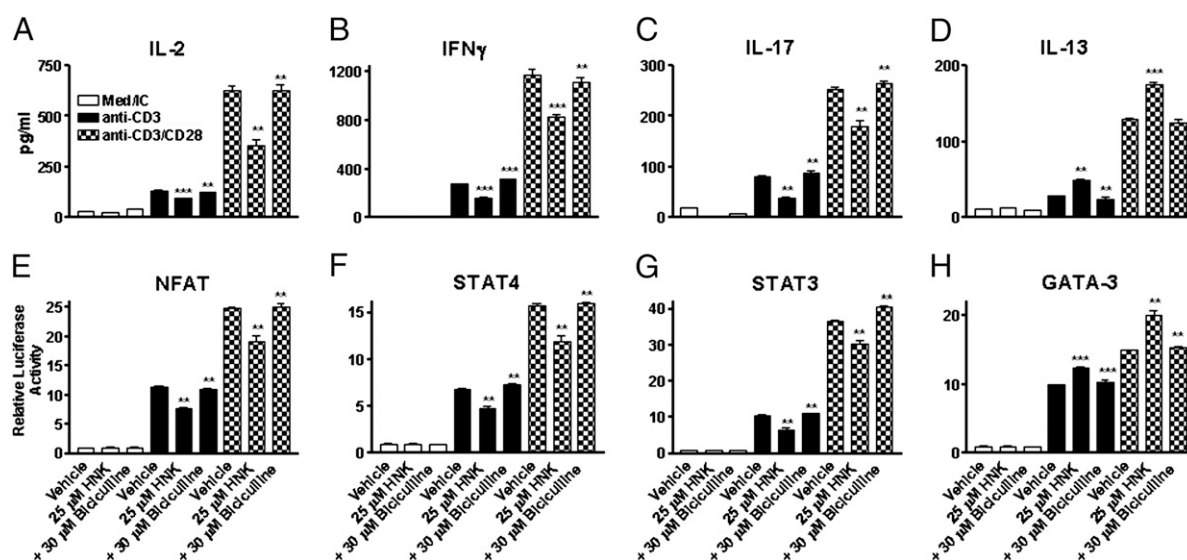


FIGURE 10. GABA_A-dependent effect of in vitro HNK treatment on CD3- or CD3/CD28-mediated cytokine response and transcription factor activation in 2B4.11 cells. 2B4.11 cells (1×10^6 /ml) were cultured in the presence of plate-bound anti-CD3ε (CD3) ± soluble anti-CD28 (CD3/CD28) versus medium/isotype control (Med/IC), in the absence or presence of HNK ± bicuculline, as described in *Materials and Methods*. Cytokines in culture supernatants were assessed by ELISA at 48 h (A, IL-2) or 72 h (B, IFN-γ; C, IL-17; and D, IL-13). Data points represent mean ± SEM of triplicate wells from two separate experiments. Alternatively, 2B4.11 cells (1.5×10^7 /ml) were transiently transfected with 40 μg 4× NFAT (E), pSTAT4 (F), pSTAT1 (G), or pGATA-3 (H) and 1 μg *Renilla* luciferase reporter plasmids by electroporation. Cells were rested on ice and then incubated an additional 24 h with insect cell stimuli specific for CD3 ± CD28, in the absence or presence of HNK ± bicuculline, as described in *Materials and Methods*. Relative luciferase activity of CD3 or CD3/CD28 versus Med/IC (control) was calculated as the mean ± SEM of duplicate samples from two independent experiments. No cell death was seen in the cultures. Statistical analysis: ** $p \leq 0.01$; *** $p \leq 0.001$ for HNK versus vehicle or bicuculline.

Th1- and Th17-type cytokines, were consistently inhibited by HNK treatment in both the lung (Fig. 5) and in ex vivo Ag recall cultures (Fig. 6), irrespective of the model used. This is consistent with the anti-inflammatory nature of HNK treatment in a mouse model of rheumatoid arthritis, collagen-induced arthritis (10). TNF- α and its receptors play a role in asthma, contributing to constriction of the airway in late-phase AHR and the recruitment of eosinophils to the lung (7). TNF- α is increased in both adult and pediatric patients, particularly those with corticosteroid-dependent and refractory disease (44). Its blockade with Etanercept contributes to improved lung function and quality of life scores in asthma patients (44) and decreased BAL eosinophils, similar to our findings with HNK treatment (Figs. 2, 4). IL-6 acts in coordination with membrane and soluble IL-6Rs to affect the asthmatic response. Blockade of the soluble receptor, found to be increased in asthmatic patients (45), reduces the number of Th2 cells in the lung. Complete blockade of IL-6 results in decreased fibrosis and collagen deposition, with little alteration of the AHR secondary to an increase in IL-13 (46).

Not only do Th1 proinflammatory cells fail to counterbalance the effect of Th2-mediated inflammation in asthma, they actually contribute to severe airway inflammation, including increased AHR, eosinophilia, and pulmonary fibrosis with increased collagen deposition (5). Th17 cells also contribute to the chronic inflammation associated with asthma. IL-17 is increased in the airway of asthmatic patients and enhances fibroblast activity, leading to increased collagen deposition (47). In addition, Th17 cells enhance Th2-mediated eosinophilia, goblet cell hyperplasia with increased mucin gene expression (48), and AHR (49). The consistent inhibition of these cytokines by HNK treatment in both models of asthma in parallel with altered AHR, goblet cell hyperplasia, collagen deposition, and eosinophilia suggests that inhibition of proinflammatory cytokines by HNK may contribute to its alleviating effects on asthma pathogenesis.

Interestingly, although HNK consistently blocked proinflammatory cytokines, this did not require inhibition of Th2-type cytokines. IL-13 was increased in both models of asthma with HNK treatment, both in the lung (Fig. 5) and in ex vivo Ag-specific recall cultures (Fig. 6). Unlike IL-13, IL-4 (chronic) and IL-5 (acute and chronic) were differentially decreased in the lung (Fig. 5) yet increased in the Ag recall response in splenocytes (Fig. 6). The relative decrease in lung IL-4 and IL-5 may be due to a decrease in TNF- α (50) and/or the consistent increase in IL-10 levels (51) and may contribute to decreased AHR and eosinophilia (4). IL-13 also promotes the activation of macrophages, whose cell numbers and percentage were increased as the number and proportion of eosinophils decreased with HNK treatment (Fig. 4). This may be partly due to the presence of IL-13 and partly due to the lack of eosinophils, a significant source of macrophage inhibitory factor (30).

Both IL-10 and TGF- β , consistently increased in the lung (Fig. 5) and splenic Ag recall response (Fig. 6), can act both as modulators and activators of asthma pathogenesis (52). IL-10 has the ability to alleviate airway inflammation and reduce Th2 cytokines and eosinophilia yet can increase AHR, particularly at the onset of asthma (53). When CD4 helper cells are the source of IL-10 and TGF- β , they become regulatory in nature. Regulatory cells, whether TGF- β -producing Th3 cells, IL-10-producing regulatory T cells, or TGF- β - and IL-10-producing CD4⁺CD25⁺ FoxP3⁺ regulatory T cells, are able to dampen asthma pathogenesis (54). TGF- β -producing Th3 cells (55) and IL-10-producing TR cells (56) are able to decrease inflammation and AHR. Regulatory T cells have less effect on AHR than on eosinophilic lung inflammation, mucus production, collagen deposition, and lung

Th2 cytokine levels (57). HNK treatment promoted Th2 cytokines while diminishing hallmarks of asthma pathogenesis, particularly when administered during the challenge phase. Thus, it seems likely that HNK is promoting regulatory, as well as anti-inflammatory, immune function. This is further demonstrated by the increase in the number of FoxP3⁺ T cells in the lungs of HNK-treated mice (Supplemental Fig. 2). Interestingly, there was also an increase in non-T cell FoxP3 expression with HNK treatment. FoxP3 (32), as well as other forkhead (Fox) transcription factors (58), have been shown to be widely expressed in the lung and play significant roles in lung morphometry and function, including lung repair after injury (59).

One possible common mechanism to explain the effect of HNK on these varied mediators of inflammation is its binding to peripheral GABA_ARs (60). Peripheral GABA_ARs are present on immune cells and have been shown to inhibit lymphocyte activation and lymphocyte-mediated inflammatory disease (14). In addition to its systemic anti-inflammatory effects in vivo, we find that HNK directly alters cytokine production in a GABA_A-dependent manner in both B cells (Fig. 9) and T cells (Fig. 10) at the level of transcription. That the in vitro findings mimic what is seen in/ex vivo suggests that the cytokine response in allergic asthma contributes to the ability of HNK to affect both acute and chronic models of the disease. In addition to the immune response, HNK may also have GABA_AR-mediated effects directly in the airway. Another ligand of the GABA_AR muscimol has been shown to reverse AHR in a guinea pig model of allergic asthma (16), as well as directly contribute to relaxation of airway smooth muscle (61). Furthermore, engagement of GABA_ARs potentiates the effects of β -agonists that are a mainstay of allergic asthma treatment (62), supporting the notion that HNK would be effective in conjunction with currently prescribed asthma therapy.

HNK has considerable promise as a clinical therapeutic agent. It has been long used in traditional Asian medicine and appears to be safe and effective in mice for models of asthma (this study), inflammatory arthritis (10), and as an antiangiogenic agent in cancer (63). HNK is potentially advantageous in its ability to alleviate the inflammatory processes contributing to asthma in a manner that does not require decreasing Th2-type cytokines. This would be particularly helpful in chronic asthma, where inflammation is persistent and particularly difficult to ameliorate. Results presented in this paper also highlight that a simple "Th1/Th2" dichotomy is often insufficient to explain complex immune responses.

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Disclosures

The authors have no financial conflicts of interest.

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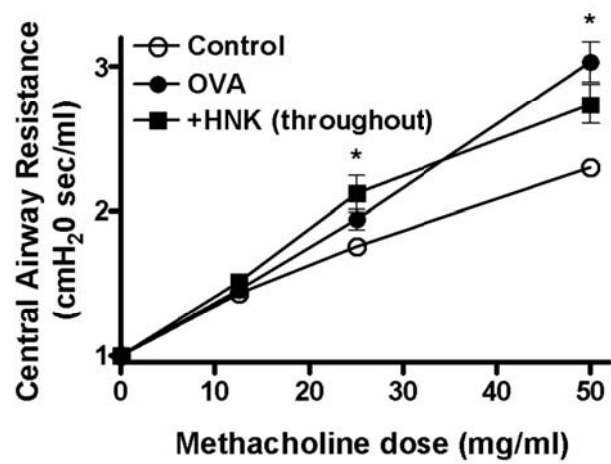
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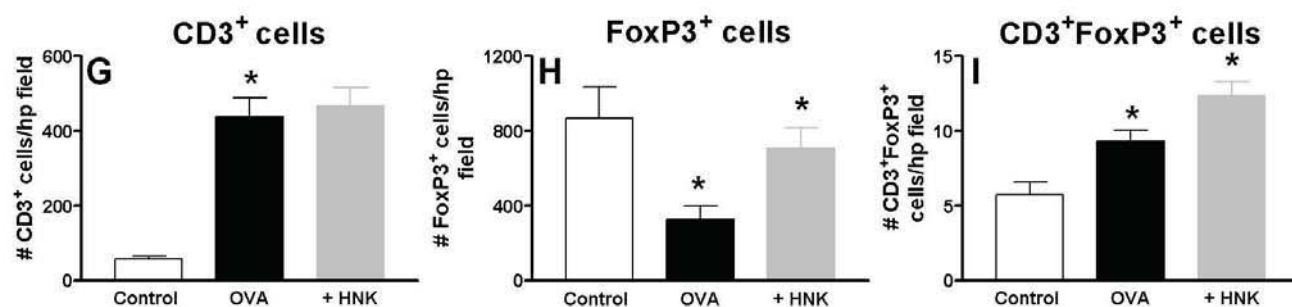
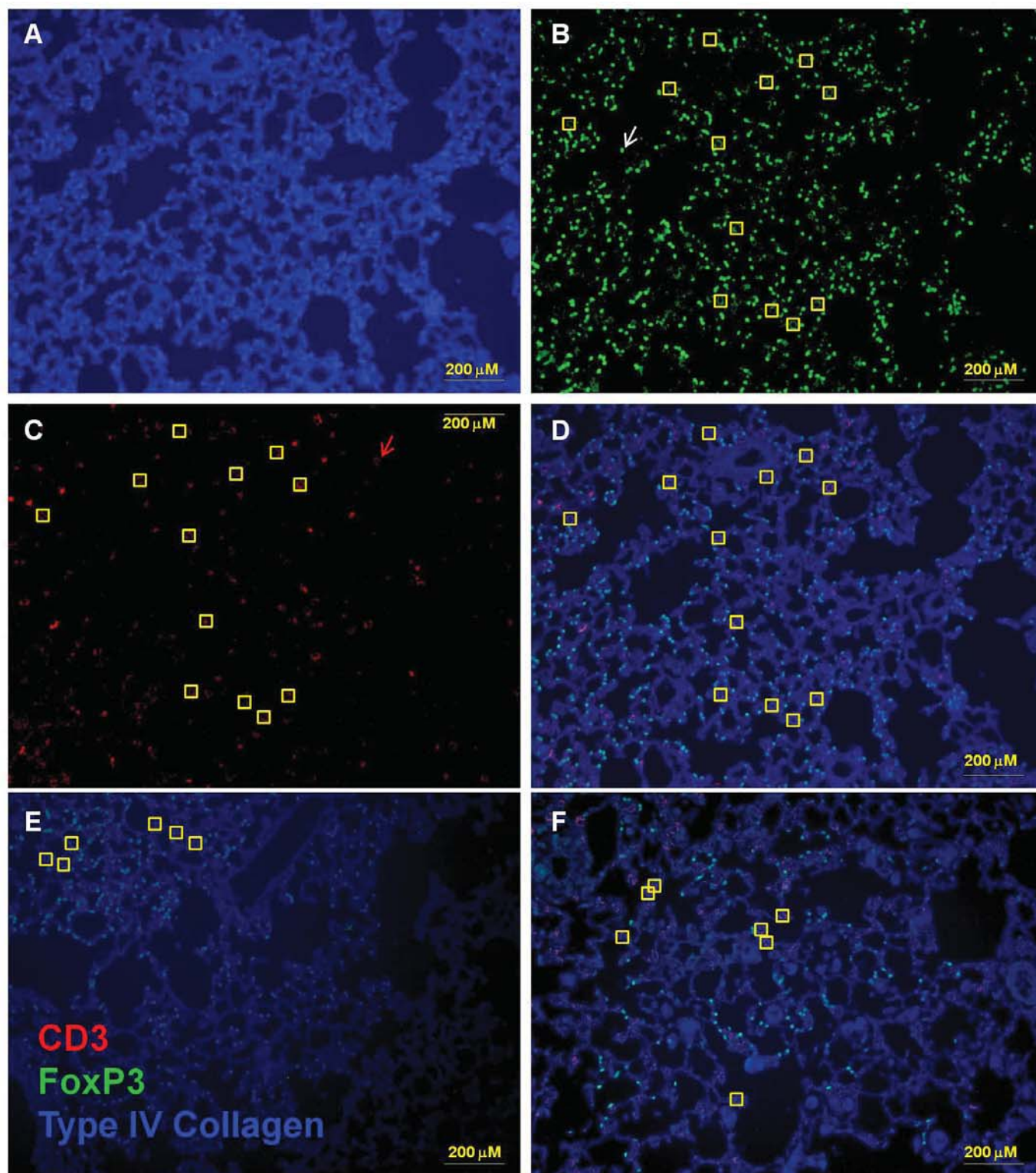
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Supplemental Figure 1. Effect of administering HNK throughout acute model of allergic asthma. Female C57Bl/6 mice remained naïve (Control) or were sensitized/challenged to OVA $\pm$ treatment with HNK throughout the protocol. Control and OVA-sensitized mice not receiving HNK were treated with vehicle control. Airway hyperactivity to methacholine, as measured by an index of resistance in the major conducting airways (R_N , Newtonian resistance), was assessed 48 h after the final allergen challenge. Each bar represents the mean $\pm$ SEM of central airway resistance readings (10 per methacholine dose) from individual mice. $n = 4$ for Control; $n = 8$ for OVA; $n = 8$ for HNK (throughout). Statistical analysis: $p \leq 0.05$ (*) for OVA vs. Control or HNK at indicated concentrations of methacholine.

Supplemental Figure 2. Effect of administering HNK during the challenge phase of the chronic model of allergic asthma on FoxP3 expression. Lung sections of mice in the chronic model of allergic asthma (Control [E], OVA [F], or HNK [challenge; A-D]) were stained for CD3 ϵ (red), FoxP3 (green), and Type IV collagen (blue). Representative slides are shown for each fluorochrome (A-C), as well as merged fields for each group (D-F). Representative single-stained cells are marked with an arrow (B, C), while dual CD3 ϵ^+ FoxP3 $^+$ cells are marked with yellow boxes (D-F). The number of CD3 ϵ^+ (G), FoxP3 $^+$ (H), and CD3 ϵ^+ FoxP3 $^+$ (I) cells/high power field was determined using Pro Plus 5.1 software (MediaCybernetics). Each data point represents the mean $\pm$ SEM of 10 high power field analyses/animal. $n = 4$ for Control, OVA, and HNK (challenge). Statistical analysis: $p \leq 0.05$ (*) for OVA vs. Control or HNK.



Supplemental Figure 1



Supplemental Figure 2