

Inhibition of allergic inflammation by supplementation with 5-hydroxytryptophan

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Abdala-Valencia H, Berdnikovs S, McCary CA, Urick D, Mahadevia R, Marchese ME, Swartz K, Wright L, Mutlu GM, Cook-Mills JM. Inhibition of allergic inflammation by supplementation with 5-hydroxytryptophan. *Am J Physiol Lung Cell Mol Physiol* 303: L642–L660, 2012. First published July 27, 2012; doi:10.1152/ajplung.00406.2011.—Clinical reports indicate that patients with allergy/asthma commonly have associated symptoms of anxiety/depression. Anxiety/depression can be reduced by 5-hydroxytryptophan (5-HTP) supplementation. However, it is not known whether 5-HTP reduces allergic inflammation. Therefore, we determined whether 5-HTP supplementation reduces allergic inflammation. We also determined whether 5-HTP decreases passage of leukocytes through the endothelial barrier by regulating endothelial cell function. For these studies, C57BL/6 mice were supplemented with 5-HTP, treated with ovalbumin fraction V (OVA), house dust mite (HDM) extract, or IL-4, and examined for allergic lung inflammation and OVA-induced airway responsiveness. To determine whether 5-HTP reduces leukocyte or eosinophil transendothelial migration, endothelial cells were pretreated with 5-HTP, washed and then used in an in vitro transendothelial migration assay under laminar flow. Interestingly, 5-HTP reduced allergic lung inflammation by 70–90% and reduced antigen-induced airway responsiveness without affecting body weight, blood eosinophils, cytokines, or chemokines. 5-HTP reduced allergen-induced transglutaminase 2 (TG2) expression and serotonylation (serotonin conjugation to proteins) in lung endothelial cells. Consistent with the regulation of endothelial serotonylation in vivo, in vitro pretreatment of endothelial cells with 5-HTP reduced TNF- α -induced endothelial cell serotonylation and reduced leukocyte transendothelial migration. Furthermore, eosinophil and leukocyte transendothelial migration was reduced by inhibitors of transglutaminase and by inhibition of endothelial cell serotonin synthesis, suggesting that endothelial cell serotonylation is key for leukocyte transendothelial migration. In summary, 5-HTP supplementation inhibits endothelial serotonylation, leukocyte recruitment, and allergic inflammation. These data identify novel potential targets for intervention in allergy/asthma.

5-hydroxytryptophan metabolites; serotonylation; endothelial cell; leukocyte recruitment; lung

TISSUE-DERIVED MEDIATORS stimulate endothelium to express adhesion molecules and secrete cytokines that then regulate leukocyte recruitment. Leukocyte binding to endothelial cell adhesion molecules activates intracellular signaling in the endothelial cells that then induces localized endothelial cell retraction. This retraction opens narrow passageways through which the leukocytes migrate. Inhibition of signals in endothelial cells blocks recruitment of leukocytes to tissues (2, 9, 25, 94).

Activated endothelium also secretes cytokines, chemokines, and neurotransmitters that regulate immune responses. Endothelial cells synthesize the neurotransmitter serotonin (33, 39, 81, 87) and express stimulatory and inhibitory serotonin receptors (33, 62, 78–80). The serotonin receptors are also expressed by leukocytes, and it is reported that serotonin regulates leukocyte chemotaxis, cytokine production, and dendritic cell activation of T cells (3, 4, 13, 23, 66, 72, 73, 85, 88, 115, 122). Serotonin also regulates cell signaling by covalent linkage of serotonin to proteins through transamidation of glutamines (serotonylation) (30, 65, 75, 93, 119). Therefore, changes in localized levels of metabolites in the serotonin pathway would alter signaling through serotonylation and inhibitory/stimulatory serotonin receptors.

A reduction in serotonin synthesis in vivo results in anxiety/depression (116). Symptoms of anxiety/depression are commonly associated with allergy/asthma in humans and mice (21, 27, 36, 100, 106). Moreover, 46% of patients who have allergies and anxiety/depression do not receive or recognize the need for psychopharmacological treatment (28). The mechanisms for the association of anxiety in individuals with allergy/asthma are not known. Anxiety/depression, which correlates with serotonin deficiencies, are associated with polymorphisms in tryptophan hydroxylase 1 or 2 (Fig. 1A), the rate-limiting enzymes that catalyze the formation of 5-hydroxytryptophan (5-HTP) (44). 5-HTP is further metabolized to serotonin by decarboxylases (Fig. 1A).

Anxiety/depression has been treated with antidepressant medication, such as selective serotonin reuptake inhibitors (SSRIs), atypical antipsychotics, and monoamine oxidase inhibitors (114). Little research has addressed the impact of antidepressants on asthma (17, 112). In one clinical study of asthmatics, treatment with the antidepressant bupropion is associated with improvements in depression and lung function (15). SSRIs, which are used as antidepressants, block the reuptake of serotonin and thus regulate the function of cells that express selective serotonin reuptake receptors, including neurons, platelets, and smooth muscle cells. However, an undesired effect of SSRIs such as citalopram and fluoxetine is that they increase vasoconstriction of the pulmonary arteries and the aorta (32, 54, 117). In contrast to SSRIs that block uptake of serotonin, the antidepressant Tianeptine stimulates uptake of free serotonin by neurons and platelets, resulting in reduced free serotonin in the plasma (41, 91, 92). Because symptomatic patients with asthma can have elevated free serotonin in plasma and this free serotonin associates with asthma symptoms (71), Tianeptine has been used in clinical studies of asthma. It is reported that Tianeptine reduces free serotonin in plasma and that this associates with reduced asthma symptoms in children (69, 70, 112).

Supplementation with the serotonin precursor 5-HTP is an alternative approach for anxiety/depression and anxiety in

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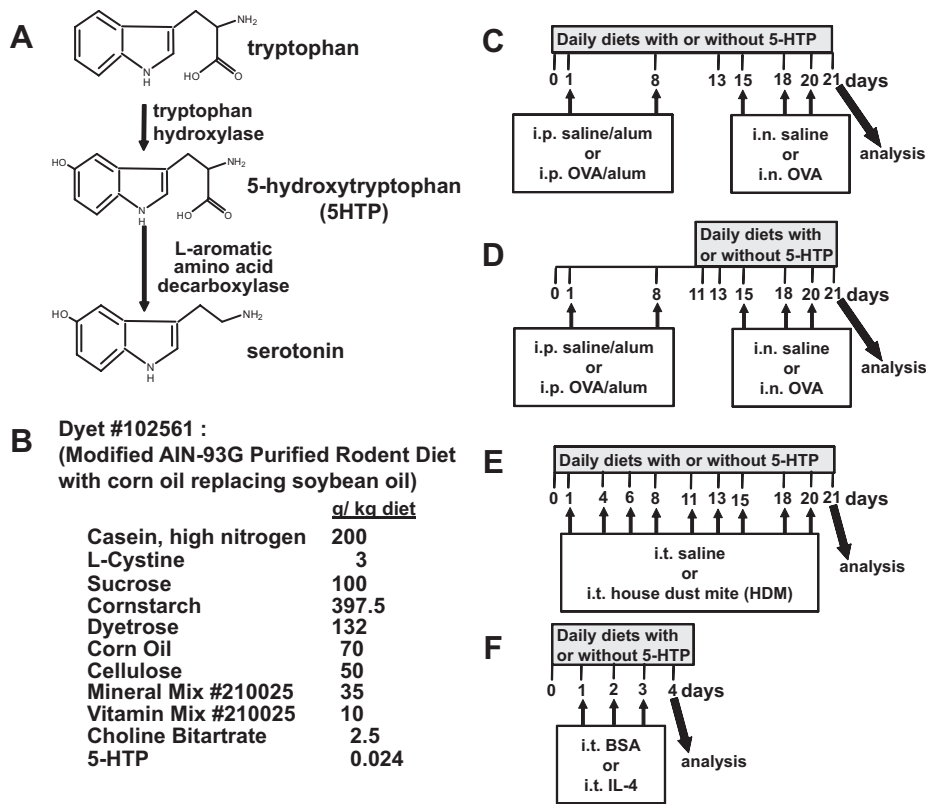


Fig. 1. 5-Hydroxytryptophan (5-HTP) supplementation. **A:** serotonin synthesis. **B:** purified 5-HTP from Sigma was used to supplement the diets by Dyets. Consumption of this diet (0.024 g 5-HTP/kg diet) at 4 g diet/day/20 g mouse is equivalent to 200 mg 5-HTP/100 pound person/day. This calculation is as follows: $200 \text{ mg } 5\text{-HTP}/100 \text{ pound person/day} = \{(0.024 \text{ g } 5\text{-HTP} \times 1,000 \text{ mg/g})/(1,000 \text{ g diet}) \times 4 \text{ g diet/mouse/day}\}/[20 \text{ g mouse} \times (1 \text{ pound}/453 \text{ g})]$. The control diet was the same except without 5-HTP. We confirmed the concentration of 5-HTP in the diet as 0.024 g 5-HTP/kg diet by HPLC/electrochemical detector (ECD). **C:** timeline for 5-HTP supplementation during ovalbumin fraction V (OVA) sensitization and OVA challenge treatments. **D:** timeline for 5-HTP supplementation after OVA sensitization. **E:** timeline for 5-HTP supplementation during treatments with house dust mite (HDM) extract from *Dermatophagoides pteronyssius*. **F:** timeline for 5-HTP supplementation during intratracheal IL-4 treatments. On day of analysis in C–F, the bronchoalveolar lavage (BAL), perfused lungs, blood, brains, and intestines were collected. i.p., intraperitoneal. i.n., intranasal. i.t., intratracheal. $n = 6\text{--}10$ mice/group.

panic disorder (12, 101), albeit perhaps 5-HTP is used less frequently than pharmacological agents. Interestingly, 5-HTP and its metabolite serotonin have been reported to have opposing outcomes on some physiological responses. For example, when administered systemically, 5-HTP decreases blood pressure, whereas serotonin can increase vasoconstriction in humans and animal models (37, 39, 81, 105, 109, 118). In another report, 5-HTP did not affect heart rate or mean arterial pressure (11). The opposing functions of 5-HTP and its metabolite serotonin on vasoconstriction may occur as an outcome of changes in local microenvironment concentrations of 5-HTP and its metabolites that would generate a change in the microenvironment balance of serotonylation and the stimulation of the multiple inhibitory serotonin receptors and the multiple stimulatory serotonin receptors (5-HTRs) that are differentially expressed by cells. Because leukocytes and endothelial cells express inhibitory and stimulatory serotonin receptors (5HTRs), recruitment of leukocytes during inflammation may be regulated by a localized balance of the 5-HTP/serotonin pathway in the microenvironment of tissues. Clinical studies with inhibitors of specific serotonin receptors such as 5-HT_{1A} and 5-HT_{2A} have had only marginal benefit for asthma as summarized in a review by Cazzola et al. (17). In contrast to targeting inhibition of individual serotonin receptors during allergies/asthma, we propose that a balance of serotonylation and serotonin inhibitory/stimulatory receptors may be achieved by supplementation with 5-HTP such that 5-HTP is metabolized in local microenvironments.

For these studies, we hypothesized that 5-HTP supplementation reduces allergic inflammation because 5-HTP reduces symptoms of anxiety/depression, anxiety/depression is increased with allergy/asthma, and because 5-HTP administra-

tion has regulatory functions that can be opposite to serotonin. Moreover, we focused the studies in this report on 5-HTP rather than SSRIs or individual serotonin receptors because many cells that regulate immune responses do not have selective serotonin reuptake receptors that are the target of SSRIs and because previous studies targeting individual receptors have only marginal effects on asthma (17).

We report that supplementation with 5-HTP reduced allergic inflammation induced by ovalbumin fraction V (OVA), house dust mite (HDM) or IL-4 administration. In addition, 5-HTP supplementation decreased leukocyte recruitment, at least, through inhibition of transglutaminase (TG) expression and serotonylation in endothelial cells. Because 5-HTP is also reported to reduce anxiety/depression, these data have important implications for coordinated regulation of anxiety/depression and allergic inflammation.

MATERIALS AND METHODS

Animals. C57BL/6J mice (female, 6–8 wk old) were from Jackson Laboratory (Bar Harbor, ME) and the NJ.1638 IL-5 transgenic mice were from James J Lee (Mayo Clinic, AZ). The studies are approved by the Northwestern University Institutional Review Committee for animals.

Cells. The murine endothelial cell line mHEVa was cultured as previously described (1, 84). Spleen cells were prepared from male BALB/c or 4-mo-old NJ1638 mice (84), and the spleen red blood cells were lysed by hypotonic shock (84). These cells from BALB/c mice were >90% lymphocytes as previously described (110). The NJ1638 spleen cells were >85% eosinophils as determined by cyto-spin.

5-HTP and control rodent diets. The diet with 5-HTP (diet no. 102561) and the control diet without 5-HTP (diet no. 101591) were from Dyets; the 5-HTP (catalog no. 9772) added to the diet was from Sigma (St. Louis, MO). C57BL/6J female mice were maintained on

the diets with or without 5-HTP at 0.024 g 5-HTP/kg diet (Fig. 1B). Corn oil, which was commonly used in rodent diet in the past, was used in this diet instead of the more recent formulas with soybean oil because we wanted to avoid the proinflammatory contribution of high γ -tocopherol in soybean oil as we recently reported (9, 26). Diets were started on the days indicated in Fig. 1, C–F. The diets were replaced every 3 to 4 days.

OVA, HDM, or IL-4 administration in vivo. Many mouse strains have polymorphisms in the genes in the serotonin system, and this is thought to contribute to their survival (16, 47, 59, 83, 90, 104, 108). C57BL/6 mice were used because they are commonly used to examine regulation of the serotonin pathway in vivo (16, 47, 59, 83, 90, 104, 108). For the OVA models (Fig. 1, C and D), mice (8–10 mice/group) received intraperitoneal (i.p.) injections (200 μ l) of chicken egg OVA (catalog no. 5503, Sigma) (10 μ g)/alum or saline/alum on days 1 and 8 (2). OVA grade V was used for sensitization because it contains low endotoxin levels that are required for adequate OVA sensitization (40); in contrast, high levels of endotoxin suppress the OVA response (40). On days 15, 18, and 20, mice received intranasal endotoxin-free OVA fraction VI (catalog no. A2512, Sigma) (150 μ g) in

saline or saline alone. For the HDM (*Dermatophagoides pteronyssius* extract containing DerP1) was from Greer Laboratories, Lenoir, NC) model (Fig. 1E), mice (8–10 mice/group) received an intratracheal 10 μ g HDM/50 μ l saline three times per week for 3 wk. For the IL-4 model (Fig. 1F), mice (4 mice/group) received intratracheal administration of endotoxin-free murine IL-4 (catalog no. 200–18; Shenandoah Biotechnology, Warwick, PA) (4 μ g IL-4/mouse/day) or the protein control bovine serum albumin grade VI (BSA, catalog no. A7030, Sigma) on days 1, 2, and 3. For these models, 24 h after the last treatment, tissues were collected. Blood eosinophils were stained and counted (2). Bronchoalveolar lavage (BAL) cells were counted and cytopun for differential counts (2). For platelet-containing plasma, blood was collected in EDTA tubes and centrifuged for 15 min at 1,500 g. Lungs were perfused free of blood, and right lung lobes were collected and weighed. The plasma and lungs were frozen at -80°C until analyzed for neurotransmitters by HPLC/electrochemical detector (ECD). Left lung lobes were collected and frozen in optimal cutting temperature compound for tissue sections.

OVA-induced airway responsiveness. The mice (8–10 mice/group) were sensitized by intraperitoneal (i.p.) injection (200 μ l) of OVA

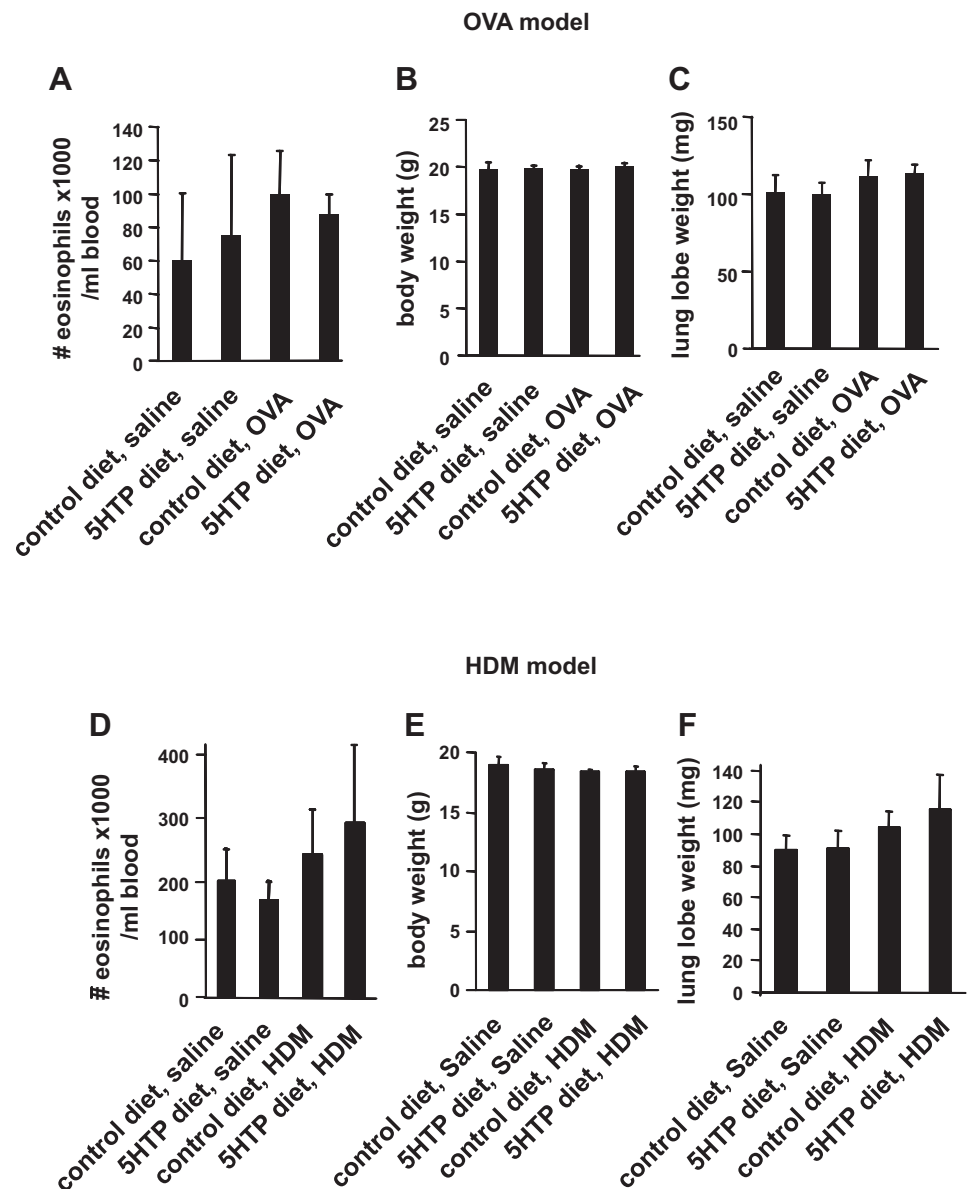


Fig. 2. 5-HTP supplementation did not alter blood eosinophils, body weight, or lung weight. Mice were supplemented with 5-HTP and received the antigens OVA (A–C) or HDM (D–F) as in the timeline Fig. 1, C and E. The mice were weighed, and blood eosinophils were counted. A and D: blood eosinophils. B and E: mouse body weight. C and F: mouse lung lobe weight. $n = 8$ –10 mice/group. The groups are not statistically different.

grade V (10 μ g)/alum or saline/alum on days 1 and 8 (2). On days 10 and 12, the mice received intranasal endotoxin-free OVA fraction VI (150 μ g) in saline or saline alone. On day 13, the mice were anesthetized, tracheostomized, and mechanically ventilated. Exposure to mechanical ventilation and measurements of lung mechanics were performed using a flexiVent mouse ventilator (Scireq, Montreal, Quebec, Canada) according to Scireq (113). A standard ventilation history for each mouse was obtained with two total lung capacity maneuvers before the mice were treated retro-orbitally with 500 μ g OVA VI in 50 μ l sterile saline to induce a response to antigen (29). The forced oscillation and quasistatic pressure volume curve protocols were used to calculate airway resistance. The data are presented as percentage of baseline lung airway resistance.

In vitro leukocyte association and migration assays in Transwells or under laminar flow. For Transwell migration assays, spleen leukocytes (>90% lymphocytes) (110) from control and 5-HTP-supplemented mice were added overnight to TNF- α -stimulated confluent monolayers of endothelial cells grown on 8- μ m pore Transwell inserts, and the number of migrated leukocytes was determined as we previously described (84). For transendothelial migration under laminar flow, the endothelial cells were stimulated overnight with TNF- α (catalog no. 300-01A; PeproTech, Rocky Hill, NJ) (5 ng TNF- α /ml) in the presence or absence of 5-HTP (catalog no. 9772, Sigma Aldrich) in PBS or the inhibitors 3-hydroxybenzylhydrazine dihydrochloride (NSD1015, catalog no. 54880, Sigma), dansylcadaverine (MDC, catalog no. 30432, Sigma), cystamine dihydrochloride (catalog no. 30050, Sigma), 1-methyl-L-tryptophan (catalog no. 447439, Sigma), and 1-methyl-D-tryptophan (catalog no. sc200313; Santa Cruz

Biotechnology; Santa Cruz, CA). The treatments had no effect on cell viability as determined by Trypan blue exclusion and were not cytotoxic to the cells as determined by the Vibrant Cytotoxicity Assay Kit (catalog no. V-23111; Molecular Probes, Eugene, OR). At 18 h after treatment, the endothelial cells were washed, and spleen leukocyte transendothelial migration was examined using a parallel plate flow chamber under conditions of laminar flow of 2 dynes/cm², as previously described (2, 31). This migration assay is dependent on endothelial cell expression of VCAM-1 and the chemokine monocyte chemoattractant protein (MCP)-1 (84, 96). Spleen leukocytes (>90% lymphocytes) (110) or NJ.1638 spleen eosinophils (>85% eosinophils) were added to the endothelial cell monolayers, and leukocyte migration was examined at 15 min by phase contrast microscopy, whereas initial spleen cell contact with the endothelial cells is determined after 2 min of laminar flow (2, 31).

HPLC measurement of amino acid metabolites. This method measures free unconjugated serotonin. Dopamine (DA, catalog no. H60255), 5-hydroxyindoleacetic acid (5-HIAA, catalog no. H2255), homovanillic acid (HVA, catalog no. H1252), 5-hydroxytryptamine (5-HT, catalog no. H9523), and the 3,4-dihydroxybenzylamine internal standard, zinc sulfate, sodium hydroxide and the HPLC grade chemicals for HPLC mobile phase including sodium acetate, citric acid, disodium-EDTA (Na²⁺EDTA), dibutylamine, sodium octyl sulfate (SOS), and methanol were purchased from Sigma. Lung and plasma 5-HTP and dopamine metabolites were analyzed by HPLC according to standard methods (20). Briefly, the diet, frozen plasma (100 μ l) or lung tissue (0.1 mg) were thawed and diluted with an equal amount of HPLC-grade water containing 8 ng/ml of the internal standard 3,4-dihydroxy-

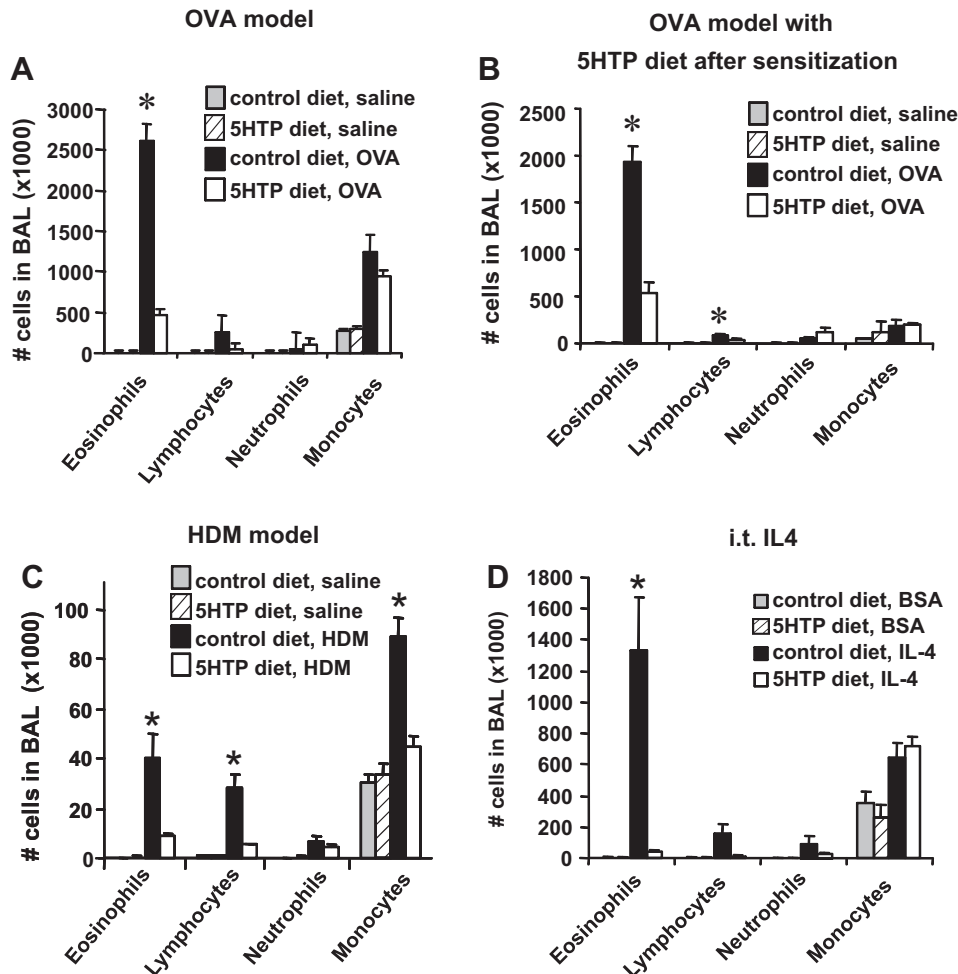
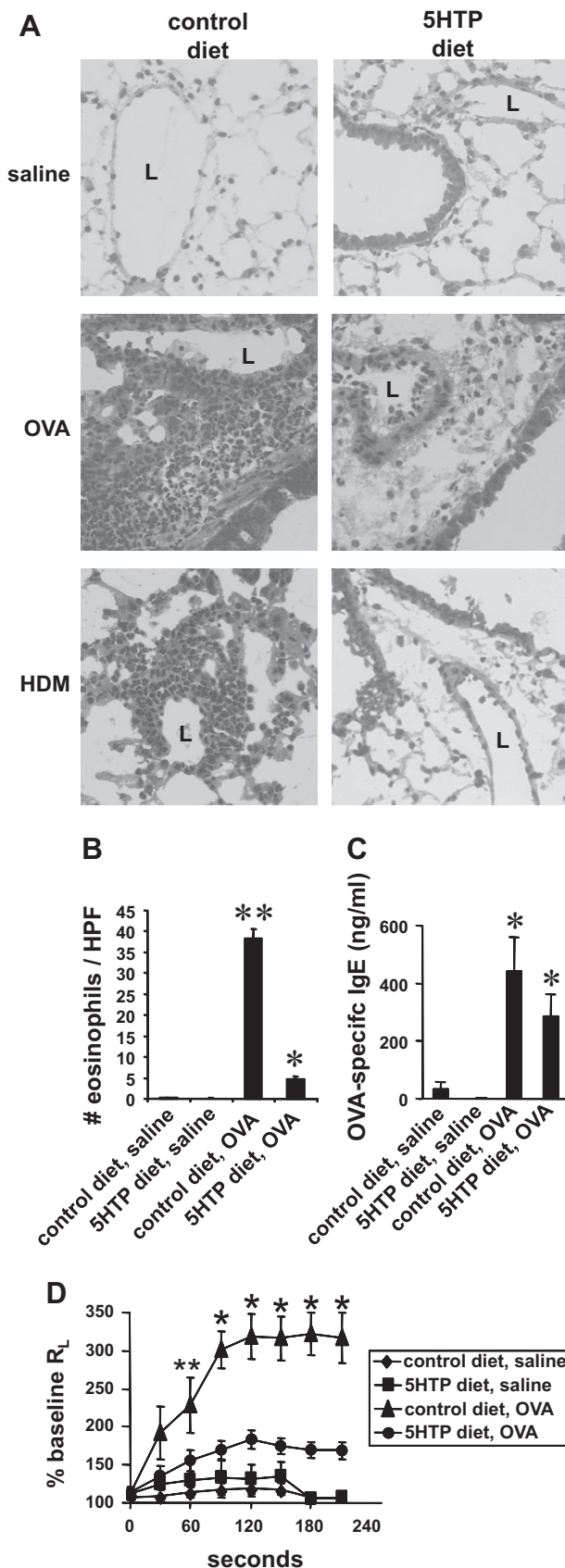


Fig. 3. 5-HTP supplementation reduced the number of leukocytes in the BAL. A: mice were supplemented with 5-HTP and received OVA as in the timeline Fig. 1C. $n = 8-10$ mice/group. B: mice were supplemented with 5-HTP after OVA sensitization as in the timeline in Fig. 1D. $n = 6-8$ mice/group. C: mice were supplemented with 5-HTP and received HDM as in the timeline Fig. 1E. $n = 8-10$ mice/group. D: mice received intratracheal (i.t.) administration of 4 μ g IL-4/mouse/day or the protein control BSA grade VI as in the timeline in Fig. 1F. $n = 6$ mice/group. The BAL cells were collected and cytospun and then neutrophils, eosinophils, monocytes, and lymphocytes were counted by standard morphological criteria. * $P < 0.05$ compared with the other groups.



benzylamine. To the diluted samples was added 0.2 ml of 20% $ZnSO_4$. The samples were vortexed and then centrifuged for 10 min at 4°C. The supernatant was collected, treated with 1 μ l of 10% NaOH, vortexed, and centrifuged as above. The supernatant from the second centrifugation was filtered through a 0.2- μ m syringe filter, and 1–20 μ l was injected into the HPLC for separation and quantification. The HPLC Breeze system (Waters, Milford, MA) was equipped with an ECD. The ECD system is comprised of a glassy carbon working electrode, an auxiliary electrode, and an Ag/AgCl reference electrode. Chromatographic separations were performed using a 3.9×150 mm stainless steel resolve column packed with octadecylsilane C18 on microparticulate (5 μ m) spherical silica gel. The mobile phase contained sodium acetate 0.1 M, citric acid 0.1 M, SOS 0.75 mM, sodium EDTA 0.15 mM, dibutylamine 1.0 mM, and methanol 15%. The solution was adjusted to pH 4.0 and was filtered through a 0.2- μ m filter. All separations were performed isocratically at a flow rate of 0.5 ml/min and at a potential of +0.60 V. Standard curves were prepared with mixtures of standard solutions containing various concentrations of 5-HTP, DA, 5-HIAA, HVA, and 5-HT. In analysis of mouse tissues, metabolite concentrations are expressed as ng/ml plasma, ng/mg tissue, or ng/g tissue ($n = 8$ –10 mice/group).

Immunolabeling of tissue sections and cultured endothelial cells. Lung tissue sections and cultured endothelial cell monolayers were fixed in -20°C methanol for 15 min and rehydrated with PBS for 1 h, which removes free serotonin. The samples were blocked with goat serum in PBS-0.3% BSA, incubated with rabbit antisero-tonin antibodies (catalog no. 8250-0004; AbD Serotec, Dusseldorf, Germany) (2 μ g Ab/200 μ l PBS-0.3% BSA), rabbit anti-TG2 antibodies (catalog no. NB120-421; Novus Biologicals, Littleton, CO) (2 μ g Ab/200 μ l PBS-0.3% BSA), rat anti-mouse major basic protein (MBP) (kind gift from James J. Lee, Mayo Clinic) (2 μ g Ab/200 μ l PBS-0.3% BSA) or isotype control antibodies for 1 h at room temperature, washed, then labeled with FITC-conjugated goat anti-rabbit IgG antibodies (catalog no. 554020; BD Pharmingen, San Diego, CA) (2 μ g Ab/200 μ l PBS-0.3% BSA) or peroxidase-conjugated goat anti-rat IgG antibodies (catalog no. 112-035-003, Jackson ImmunoResearch Laboratory, Bar Harbor, ME) (2 μ g Ab/200 μ l PBS-0.3% BSA), washed, and analyzed by fluorescence microscopy with Slidebook software. Data are presented as the sum of the pixel intensities per μm^2 of vessel endothelium, vessel lumen, airway epithelium, or lung tissue. Data for all cell types within three sections from each mouse were collected and averaged to obtain an average for each mouse. Then, the data in the graphs are the means $\pm$ SE for 8 mice/group. Immunohistochemistry for MBP was done as previously reported (2).

Cytokines, chemokines, and adhesion molecules. The BAL supernatants were tested for protein levels of cytokines using the Luminex 20plex or 6plex cytokine kits (catalog no. LMC0006 or LMC0002; Invitrogen, Carlsbad, CA). The expression of those cytokines and chemokines that are not in the Luminex kit were examined by qPCR. Briefly, total RNA was isolated from 10–15 mg of lung tissue or 1 million endothelial cells using the Qiagen RNeasy Mini Kit. cDNA

Fig. 4. 5-HTP supplementation reduced allergen-induced lung tissue inflammation and lung responsiveness. **A:** frozen lung tissue sections from the mice in Fig. 3, A and C, were stained with hematoxylin and eosin. Shown are representative micrographs of perivascular regions in lung tissue. $n = 8$ –10 mice/group. L, vessel lumen. **B:** number of antimajor basic protein (MBP)-labeled lung perivascular eosinophils per high-powered field (HPF) from mice in Fig. 3A. **C:** 5-HTP diets did not alter plasma OVA-specific IgE from mice in Fig. 3A as measured by ELISA. $n = 8$ –10 mice/group. **D:** to examine OVA-induced lung resistance, OVA-treated mice were retro-orbitally challenged with 500 μ g OVA VI in 50 μ l saline or saline alone (29). Exposure to mechanical ventilation and measurements of lung mechanics were performed using a flexiVent mouse ventilator. Presented are the percentages of baseline lung resistance (R_L). ** $P < 0.05$ compared with saline groups. * $P < 0.05$ compared with all other groups.

was prepared from 500 ng of mRNA/reaction using qScript cDNA Synthesis Kit (catalog no. 95047–100, Quanta BioSciences, Gaithersburg, MD) and analyzed by real-time PCR on an 7500 Real Time PCR System (Applied Biosystems, Foster City, CA) using Taqman probes and Taqman Universal Master Mix (Applied Biosystems) or using PrimeTime probes and PrimeTime qPCR assays (Integrated DNA Technologies). VCAM-1 and ICAM-1 expression by mHEVα cells was also examined by immunolabeling with antibodies from BD PharMingen (FITC-conjugated hamster anti-mouse ICAM-1, catalog no. 01544D; rat anti-mouse VCAM-1 clone MVCAMα, catalog no. 550547; FITC-conjugated goat anti-rat IgG antibodies, catalog no. 554020; FITC-conjugated control antibody, catalog no. 554679; and

FITC-conjugated goat anti-rat IgG, catalog no. 554016). Analysis was by flow cytometry.

In vitro chemotaxis and chemokinesis assays. Spleen leukocytes isolated from mice supplemented with 5-HTP after OVA sensitization and before OVA challenge (Fig. 1D) were used in Transwell chemotaxis assays with eotaxin (CCL11). Both lymphocytes and eosinophils express the CCL11 receptor CCR3 and respond to CCL11. For chemotaxis, 1×10^6 cells/100 μ l were added to the upper chamber of 5.0- μ m pore Transwell inserts (Corning COSTAR no. 3421; Corning, NY). The lower chamber contained 600 μ l of media with 0.05 ng/ μ l recombinant murine CCL11 (PeproTech no. 250–01). After 3 or 6 h at 37°C, cells in the lower chamber were counted using a hemocytometer.

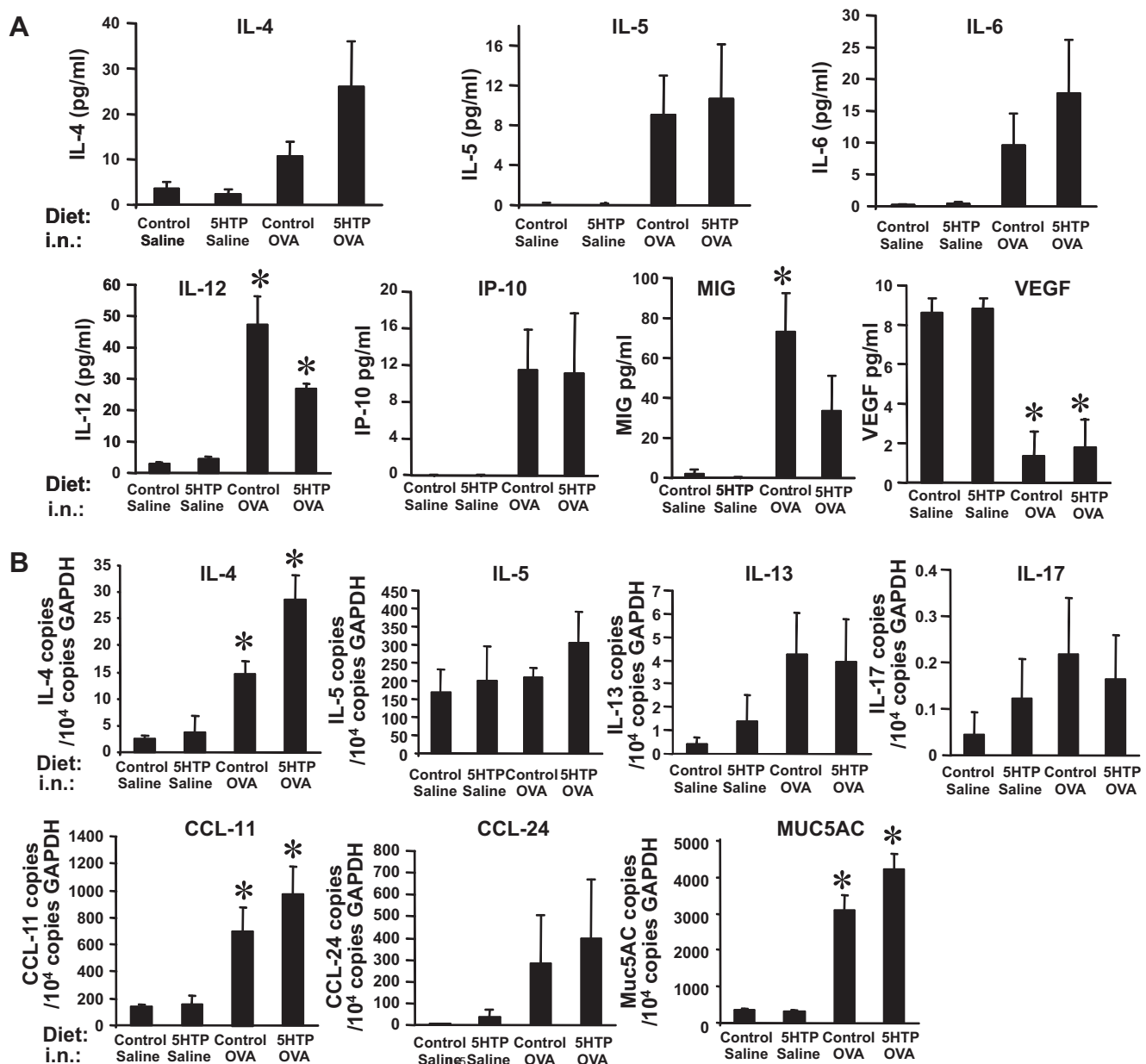


Fig. 5. For OVA-challenged mice, 5-HTP supplementation did not alter expression of lung cytokines, chemokines, growth factors, or mucin. The lungs and BAL were collected from the OVA-challenged mice in Fig. 3A. A: BAL supernatants from lungs in Fig. 3A were tested for levels of cytokines using the Luminex 20plex cytokine kit. Not detected were IFN- γ , IL-1 α , IL-1 β , IL-2, KC, monocyte chemoattractant protein (MCP)-1, and TNF- α ; IL-17 was very low (data not shown). B: lung tissue from mice in Fig. 3A was placed in RNeasy lysis buffer and then examined by quantitative PCR for IL-4, IL-5, IL-13, IL-17, CCL11, CCL24, and MUC5AC expression. $n = 8-10$ mice/group. * $P < 0.05$ compared with the saline groups. There is not a significant effect of 5-HTP supplementation of OVA-treated mice compared with control diet OVA-treated mice.

tometer. For chemokinesis, 0.05 ng/ μ l recombinant murine CCL11 was added to the upper well of the Transwells ($n = 6$ animals/group).

Statistics. Data were analyzed by a one-way ANOVA followed by Tukey's or Dunnett's multiple-comparisons test (SigmaStat; Jandel Scientific, San Ramon, CA). $P < 0.05$ is considered significant. Presented are the means $\pm$ SE.

RESULTS

5-HTP reduces lung inflammation in two models of allergy/asthma. We determined whether dietary supplementation with 5-HTP reduces inflammation in mice challenged with OVA (Fig. 1C) or HDM extract (Fig. 1E). We also determined whether inflammation was reduced by supplementation with

5-HTP during the OVA challenge phase (after OVA sensitization) (Fig. 1D). The level of 5-HTP in the rodent diets (Fig. 1B) was calculated to be equivalent to that for human consumption of 200 mg 5-HTP/100 pound person/day because clinical studies with 5-HTP supplementation use 100–300 mg 5-HTP/person/day (12, 45, 50, 60, 76, 101, 103). We confirmed the 5-HTP concentration in the diet by HPLC/ECD (Fig. 1B). The 5-HTP in this diet is about fivefold lower than the 5-HTP concentrations reported to increase systemic serotonin, increases serotonin-mediated intestinal peristalsis, and induces diarrhea in mice (8, 14, 19, 58, 99). 5-HTP supplementation as in Fig. 1, C and E, did not affect the number of blood eosinophils, body

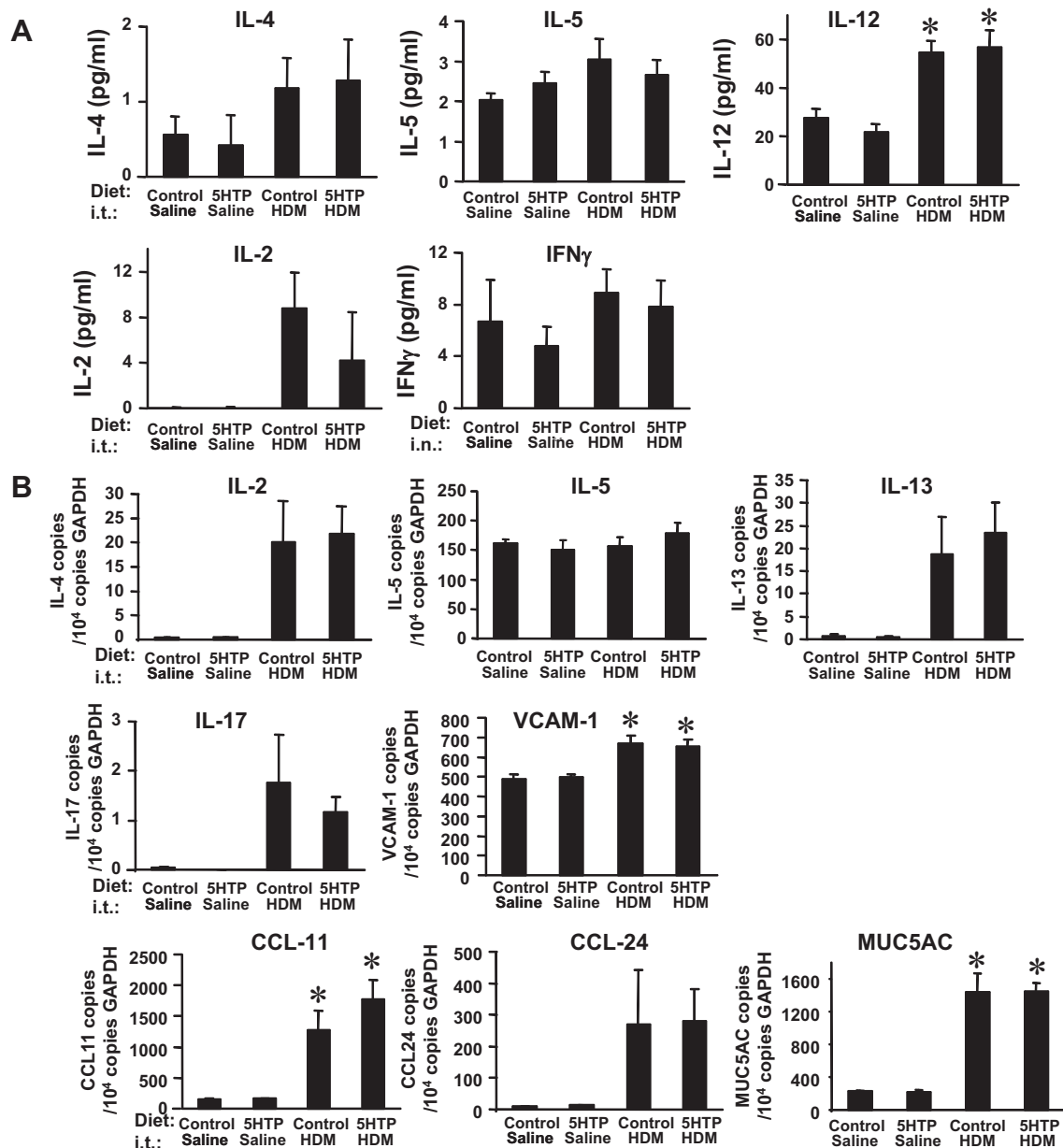


Fig. 6. For the HDM-challenged mice, 5-HTP supplementation did not alter lung cytokines, chemokines, mucin, or adhesion molecules. The lungs and BAL were collected from the HDM-challenged mice in Fig. 3C. A: BAL supernatants were tested for levels of cytokines using the Luminex 6plex cytokine kit. B: lung tissue was placed in RNeasy lysis buffer and then examined by quantitative PCR for IL-4, IL-5, IL-13, IL-17, VCAM-1, CCL11, CCL24, and MUC5AC. $n = 8$ –10 mice/group. * $P < 0.05$ compared with the saline groups. There is not a significant effect of 5-HTP supplementation of HDM-treated mice compared with control diet HDM-treated mice.

weight, or lung lobe weight of the mice (Fig. 2). 5-HTP supplementation reduced the number of eosinophils in the BAL of OVA-challenged mice and reduced the number of eosinophils, lymphocytes, and monocytes of HDM-challenged mice (Fig. 3, A–C). 5-HTP supplementation also reduced OVA- and HDM-induced lung tissue inflammation (Fig. 4A), OVA-in-

duced lung tissue eosinophils (Fig. 4B), HDM-induced lung tissue eosinophils (data not shown), and OVA-induced lung responsiveness (Fig. 4D), but not OVA-specific IgE levels (Fig. 4C). Because 5-HTP did not alter antigen sensitization (OVA-specific IgE), we determined whether 5-HTP reduced leukocyte recruitment when antigen presentation was bypassed

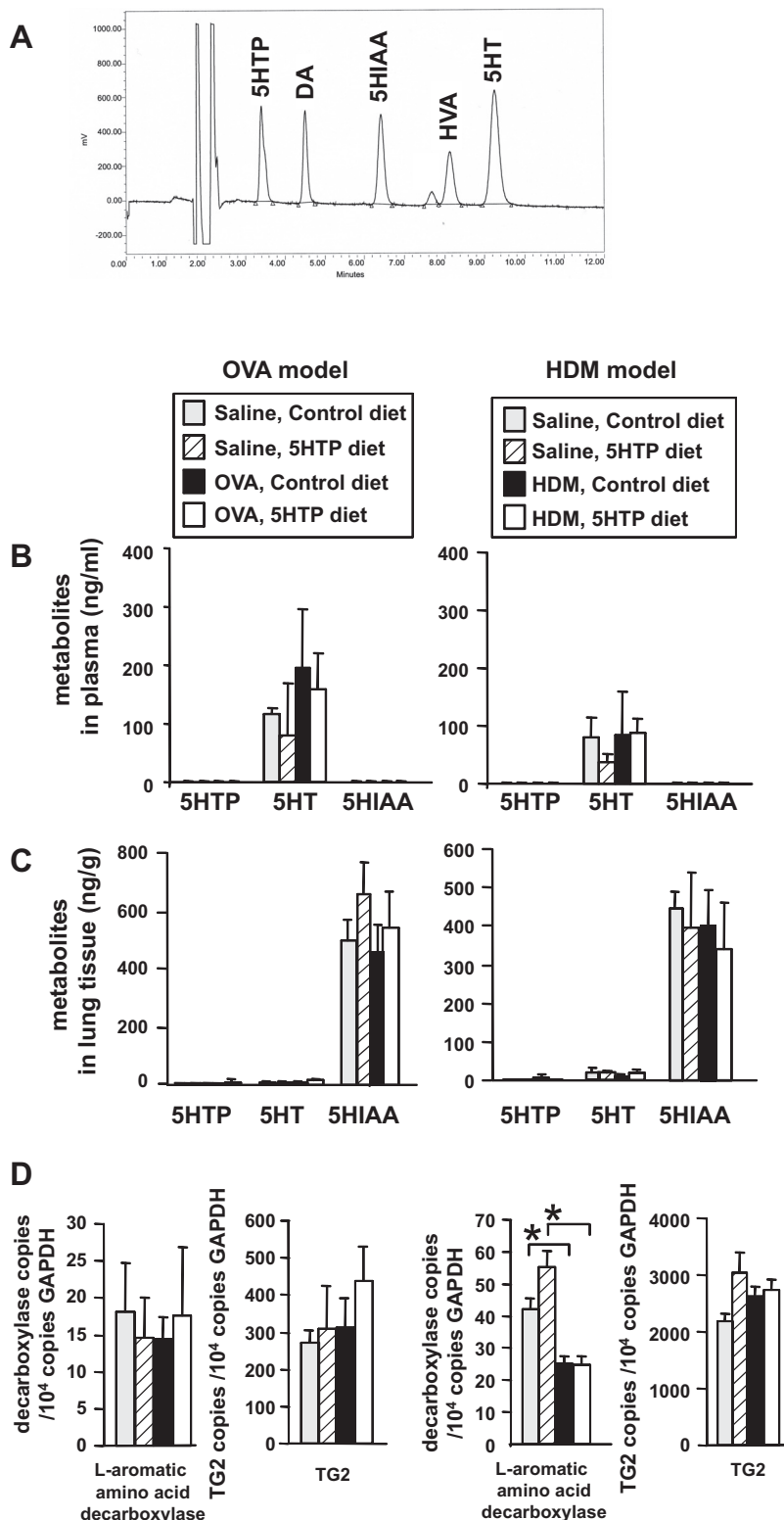


Fig. 7. 5-HTP supplementation did not alter 5-HTP or its metabolites in plasma or perfused whole lung tissue. A: HPLC/ECD profile of standards demonstrating separation of 5-HTP and its metabolites serotonin (5-HT) and 5-hydroxyindoleacetic acid (HIAA) as well as dopamine (DA) and its metabolite homovanillic acid (HVA). The solvent peaks come off the column before the metabolites (unlabeled peaks). Peaks are labeled with the metabolite. From mice in Fig. 3, A and C, plasma (B) and lungs (C), which had been perfused free of blood, were examined for 5-HTP, 5-HT, and HIAA by HPLC with electrochemical detection. D: lung tissue was placed in RNAlater and then examined by quantitative PCR for L-aromatic amino acid decarboxylase and transglutaminase (TG)2 in total lung tissue. $n = 8-10$ mice/group. $*P < 0.05$ for the indicated groups.

by administration of intratracheal IL-4. 5-HTP reduced IL-4-induced recruitment of BAL eosinophils (Fig. 3D). Thus 5-HTP supplementation reduced allergic inflammation and allergen-induced lung hyperresponsiveness without altering body weight, OVA-specific IgE, or blood eosinophils.

5-HTP supplementation does not alter expression of several cytokines, chemokines, or adhesion molecules. We determined whether 5-HTP supplementation alters lung cytokines, chemokines, or endothelial cell adhesion molecules that regulate allergic inflammation. We measured mRNA expression of cytokines, chemokines, and adhesion molecules in whole lung tissue and examined cytokines and chemokines in BAL. MUC5AC was examined because it is a quantitative measure of goblet cell metaplasia. For the mice from the OVA model and HDM model (Fig. 3, A and C), 5-HTP supplementation of OVA-treated mice did not change BAL or lung tissue protein expression (Figs. 5A and 6A) or mRNA expression (Figs. 5B and 6B) for the cytokines IL-4, IL-5, IL-6, IL-12, IL-13, and IL-17, the chemokines CCL11, CCL24, MIG, and IP-10, or the adhesion molecule VCAM-1 compared with OVA-treated mice on control diet. Consistent with no change in IL-13 mRNA in the lung tissue, there was no change in MUC5AC mRNA. 5-HTP supplementation after OVA sensitization (mice in Fig. 3B) also did not alter lung tissue cytokines (data not shown). The 5-HTP supplementation did not induce a switch of the Th2 cell response to a Th1 response because there was no effect of 5-HTP diets on expression of the Th1 cytokines IFN γ and IL-2 (Fig. 6A and data not shown for OVA model). The antigen-treated lungs and BAL had no significant induction of IL-1 α , IL-1 β , IL-2, IL-10, KC, MCP-1, macrophage inflammatory protein-1 α , TNF- α , granulocyte-macrophage colony-stimulating factor, and fibroblast growth factor basic; this was not altered by 5-HTP diets (data not shown).

5-HTP diets did not alter total tissue levels of 5-HTP and its metabolites. It is reported that low levels of 5-HTP supplementation (≤ 10 mg 5-HTP/kg body wt/day) does not alter 5-HTP metabolites in blood and tissues (8, 58). Consistent with this, 5-HTP supplementation for mice in Fig. 3, A and C, at the low levels used in our studies (about 5 mg 5-HTP/kg body wt/day), did not alter systemic levels of 5-HTP, free unconjugated serotonin, or the metabolite of serotonin HIAA as determined by HPLC/ECD analysis of lung, plasma, intestine, or brain when tissues were collected midday (Figs. 7 and 8). Mice are nocturnal, and it is reported that plasma serotonin levels are greatest within a couple of hours of feeding (5, 55). Therefore, we also examined 5-HTP metabolites in lung, plasma, intestine, and brain at 2 h after feeding the diets because this is the time of maximal increases in 5-HTP metabolites as determined in a time course in our studies (data not shown) and in a previous report with oral gavage of 5-HTP (77). At 2 h after feeding, there was a fourfold increase in 5-HTP and its metabolites compared with values in Figs. 8 and 9, but there were no differences among the treatment groups for these tissues (data not shown).

It is reported that high levels of 5-HTP reduce serum dopamine and that serotonin counterregulates dopamine (35, 43). It is also reported that dopamine regulates Th17 and mast cell responses (67, 68, 89). Therefore, we determined whether low-level 5-HTP supplementation reduced dopamine concentrations in the mice from Fig. 3, A and C. The 5-HTP supplementation did not alter dopamine or its metabolite HVA as

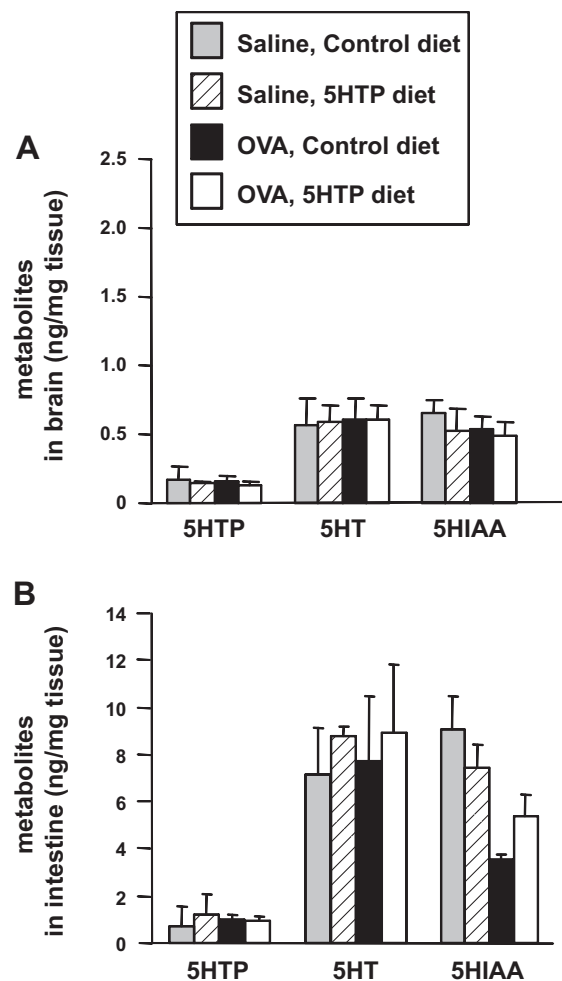


Fig. 8. 5-HTP supplementation did not alter 5-HTP or its metabolites in brain or intestine. Brain and intestines were collected from OVA-challenged mice from Fig. 3A and frozen. Brains (A) and intestines (B) were examined for 5-HTP, 5-HT, and HIAA by HPLC/ECD. $n = 8-10$ mice/group. There is not a significant difference among the treatment groups.

determined by HPLC/ECD analysis of lung, plasma, or brain (Fig. 9). Therefore, the low level of 5-HTP supplementation in these studies did not alter systemic serotonin or dopamine and their metabolites.

5-HTP diets reduced endothelial cell TG2 expression and serotonylation in the lung. TG is increased in allergic disease, TG catalyzes localized serotonin transamidation of glutamines (serotonylation), and serotonylation regulates cell functions (30, 65, 75, 93, 119). Therefore, we determined whether 5-HTP diets altered localized TG2 expression and serotonylation by immunolabeling lung tissue sections from mice in Fig. 3A with anti-TG2 antibodies and antiserotonin antibodies, respectively. Lung endothelial cell TG2 protein expression was increased after OVA challenge (Fig. 10, A and B). The endothelial TG2 expression in saline and OVA-treated mice was reduced with 5-HTP (Fig. 10, A and B), suggesting a negative feedback regulation of TG2 expression with 5-HTP supplementation. In contrast, total lung expression of TG2 and decarboxylase were not altered (Fig. 7D). To examine serotonylation, lung tissue sections from the OVA model (Fig. 3A) were fixed, which removes free serotonin, and labeled with antiserotonin antibody-

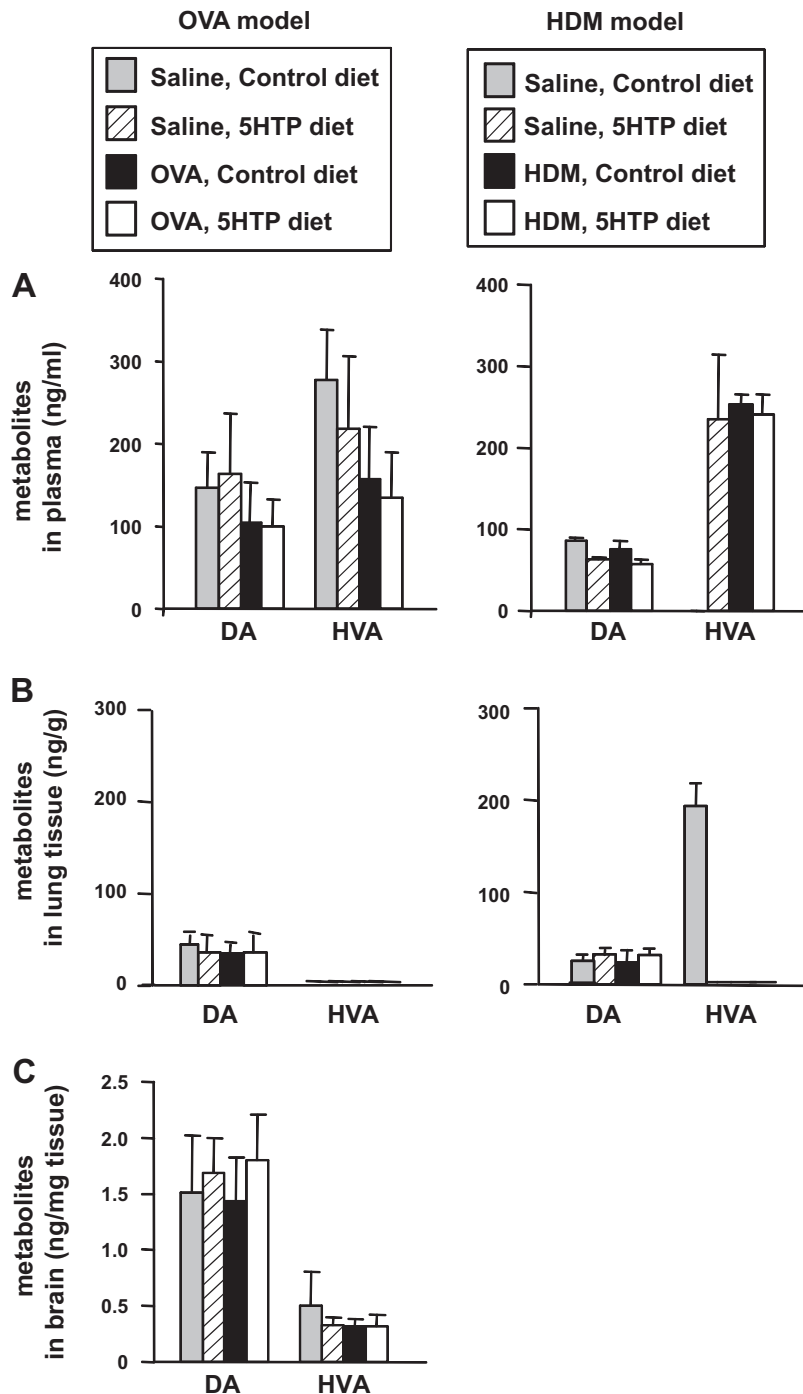


Fig. 9. 5-HTP supplementation did not alter dopamine or its metabolite HVA in plasma, perfused whole lung tissue, or brain. From mice in Fig. 3, A and C, plasma (A), lungs that had been perfused free of blood (B), and brains (C) were examined for DA and HVA by HPLC/ECD. DA and HVA were not detected in intestines (data not shown). $n = 8-10$ mice/group. There is not a significant difference among the treatment groups.

ies to detect bound serotonin (serotonylation). OVA induced a significant increase in lung endothelial cell serotonylation (Fig. 10, C and D), and this was completely blocked in the group with 5-HTP supplementation (Fig. 10, C and D). The airway epithelium was serotonylated, but this was not regulated by OVA challenge or by 5-HTP supplementation (Fig. 10D).

5-HTP reduced endothelial serotonylation in vitro. Because 5-HTP regulated serotonylation in vivo in lung endothelium, we also examined 5-HTP regulation of endothelial cell serotonylation in vitro. In vitro TNF- α treatment of endothelial cells increased endothelial cell serotonylation, and this seroto-

nylation was blocked by treatment with 5-HTP or the TG inhibitors cystamine or dansylcadaverine (MDC) (Fig. 10, E and F); the treatments were not cytotoxic to the endothelial cells as determined by the G6PDH release assay (Fig. 14). Thus 5-HTP reduces endothelial cell serotonylation in vivo and in vitro.

5-HTP supplementation in vivo does not alter leukocyte expression of chemokines or adhesion molecules or leukocyte chemotaxis, chemokinesis, or transendothelial migration. Because 5-HTP reduced leukocyte recruitment in vivo, we examined whether pretreatment of leukocytes or endothelial cells

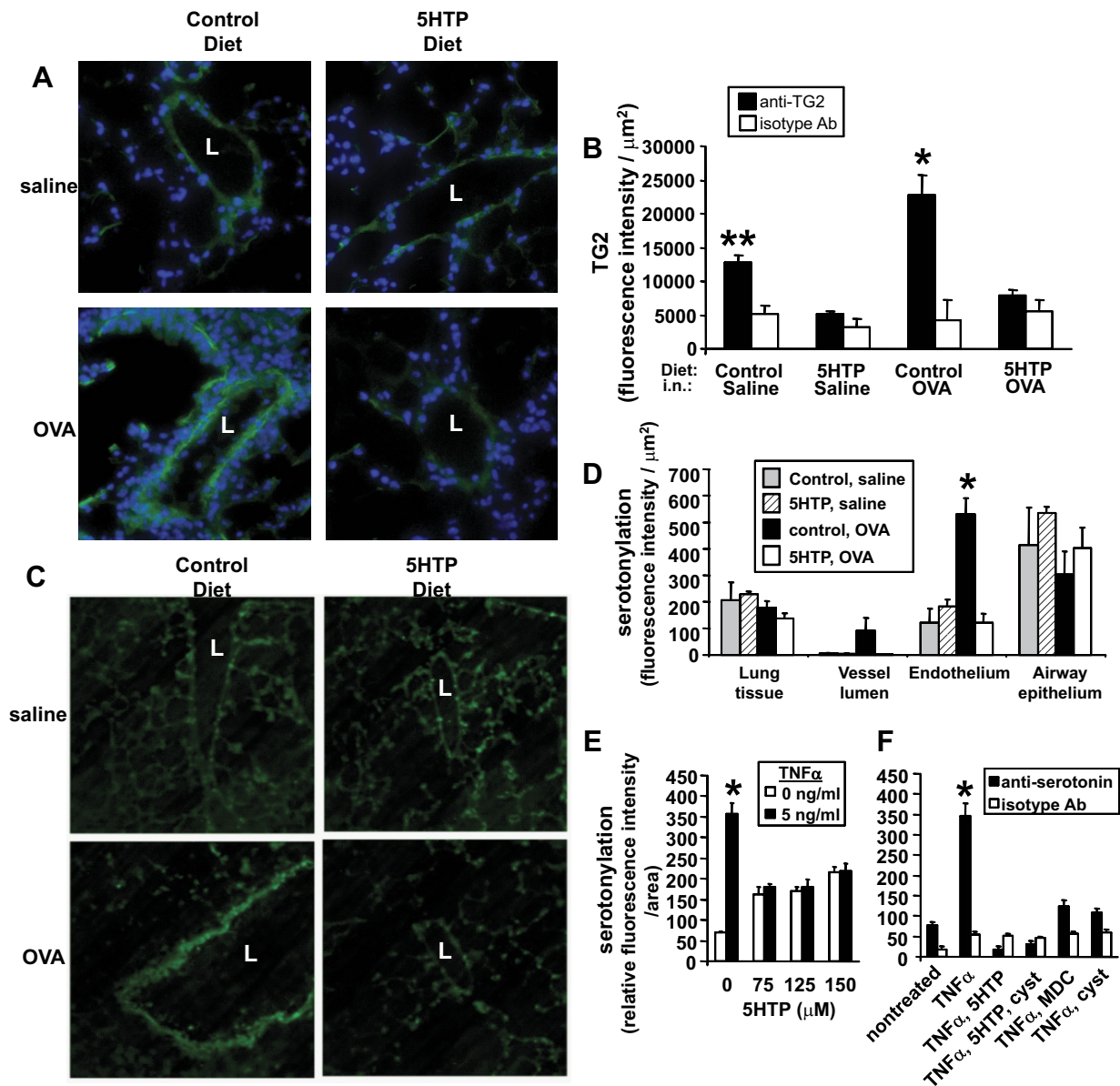


Fig. 10. 5-HTP supplementation reduced OVA-induced lung vascular TG2 expression and serotonylation in vivo and reduced TNF- α -stimulated endothelial cell serotonylation in vitro. *A–D*: tissue sections from perfused lungs of mice from Fig. 3A were labeled with anti-TG2 or antiseroitonin antibodies and examined by fluorescence microscopy. Labeling with isotype control antibodies was negative such that the fluorescence was lower than the intensity in the vessel lumen of the saline control groups (data not shown). *A*: anti-TG2 immunofluorescence labeling (green) and DAPI (blue). Representative micrographs with vessels. *B*: fluorescence intensity of TG2/ μm^2 of vessel endothelium. *C*: antiseroitonin immunofluorescence labeling. Representative micrographs with vessels. *D*: fluorescence intensity of serotonylation/ μm^2 of vessel endothelium, vessel lumen, airway epithelium, or lung tissue. *B* and *D*: for each cell type, data from 3 sections per mouse were collected and averaged to obtain an average for each mouse. Then the data in the graphs are from the average $\pm$ the SE of 8 mice/group. *E–F*: in vitro, monolayers of endothelial cells were stimulated with TNF- α in the presence or absence of 125 μM 5-HTP, and/or the TG inhibitors cystamine (Cyst, 100 μM) or dansylcadaverine (MDC, 200 μM). $n = 3–5$ for in vitro serotonylation. The inhibitors did not affect cell viability (shown in Fig. 14). L, vessel lumen. * $P < 0.05$ compared with the other treatment groups. ** $P < 0.05$ compared with the other treatment groups.

with 5-HTP regulates leukocyte transendothelial migration, which is regulated by chemokine receptors and adhesion molecules. Leukocytes from the 5-HTP- and OVA-treated mice in Fig. 3B were used to examine adhesion molecule expression, chemokine receptor expression, and transendothelial migration across a monolayer of endothelial cells in vitro. Leukocytes from 5-HTP-supplemented mice did not have altered mRNA expression of the chemokine receptor CCR3 or the VCAM-1 ligand $\alpha 4$ -integrin (Fig. 11, A and B). As in previous reports (107), chemotaxis to eotaxin (CCL11) induced a decrease in

mRNA expression of $\alpha 4$ -integrin after 6 h (Fig. 11A). Leukocytes from 5-HTP-supplemented mice did not have altered leukocyte chemotaxis or leukocyte chemokinesis in a 6-h response to CCL11 in vitro (Fig. 11, C and D). In addition, spleen leukocytes from 5-HTP-supplemented mice did not have altered transmigration across microvascular endothelial cells in vitro (Fig. 11E). Therefore, leukocytes from 5-HTP-supplemented mice did not have altered adhesion molecule expression, chemokine receptor expression, chemotaxis, chemokinesis, or transendothelial migration in vitro.

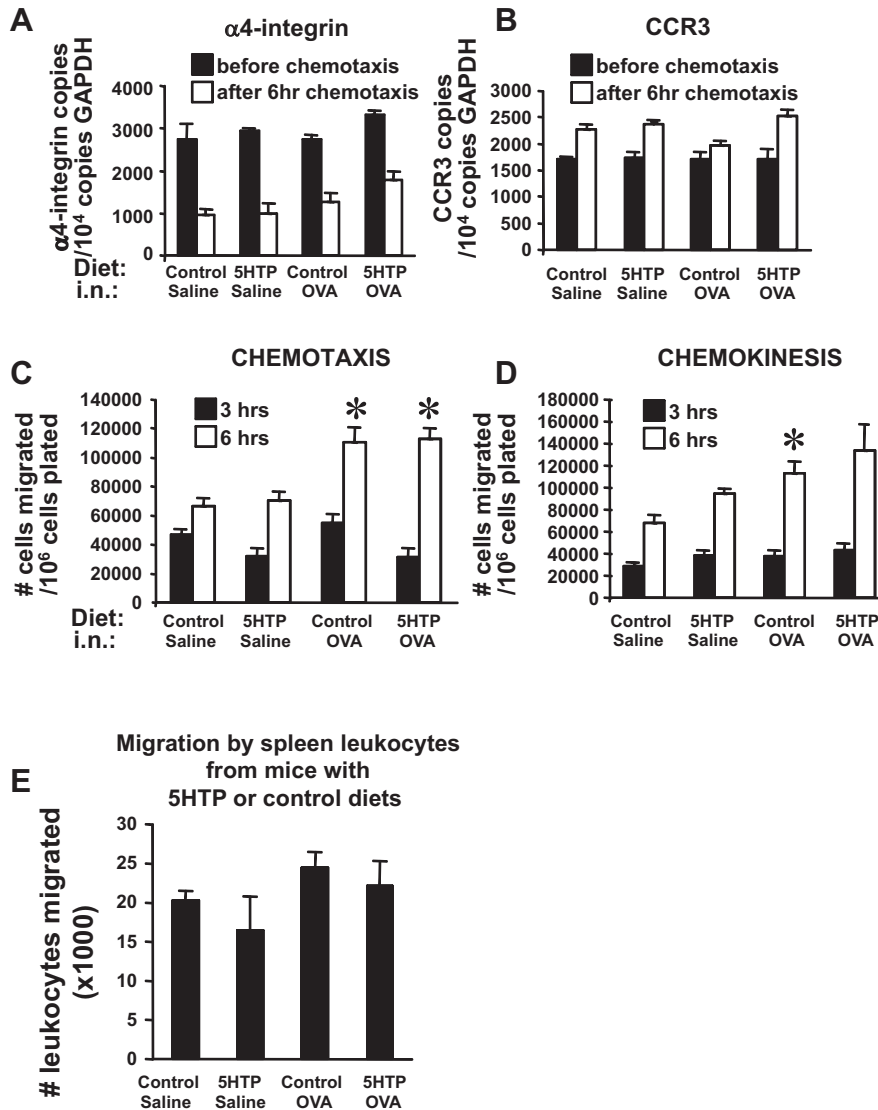


Fig. 11. For OVA-challenged mice, 5-HTP supplementation of leukocytes did not alter expression of leukocyte $\alpha 4$ -integrin, the chemokine receptor CCR3, chemotaxis, chemokinesis, or transendothelial migration. *A* and *B*: spleen leukocytes from mice in Fig. 3*B* were placed in RNAlater before and after the chemotaxis in *C*. These cells were examined by quantitative PCR for $\alpha 4$ -integrin (*A*) and CCR3 expression (*B*). *C* and *D*: chemotaxis and chemokinesis of spleen cells isolated from mice in Fig. 3*B*. The assays were performed with 50 ng CCL-11/ml for 3 or 6 h. *E*: transendothelial migration by spleen leukocytes from mice supplemented with 5-HTP as in Fig. 3*B*. Leukocytes were added to confluent monolayers of endothelial cells in 8- μ m-pore Transwells overnight. The migrated leukocytes in the bottom chamber were counted with a hemocytometer. i.n., intranasal. $n = 8$ –10 mice/group. * $P < 0.05$ compared with the saline groups. There is not a significant effect of 5-HTP supplementation of OVA-treated mice compared with control diet OVA-treated mice.

5-HTP pretreatment of endothelial cells blocks endothelial cell TG2 support of leukocyte transendothelial migration *in vitro*. Endothelial cells express TG2, enzymes for synthesis of serotonin, and enzymes for metabolism of serotonin (33, 62, 78–80). Endothelial cells also regulate the migration of leukocytes into the tissue (25). To examine 5-HTP regulation of endothelial function during leukocyte migration, endothelial cells (1, 31) were treated with 5-HTP at doses reported to regulate other functions of endothelial cells and other cell types *in vitro* (51, 123). The endothelial cells were pretreated overnight with TNF- α (5 ng/ml) and with 5-HTP (0–125 μ M) or with the TG2 inhibitors cystamine or dansylcadaverine (Fig. 12*A*), washed, and placed in the leukocyte transendothelial migration assay under laminar flow at 2 dynes/cm² (1, 31). In this assay, leukocytes migrate on VCAM-1 and respond to the endothelial cell-derived chemokine MCP-1 (84, 96). Leukocyte and eosinophil transendothelial migration was blocked by pretreatment of TNF- α -stimulated endothelial cells with 5-HTP, cystamine, or dansylcadaverine (Fig. 12, *B*, *C*, and *G*), suggesting that endothelial cell transglutamination is important for migration. There was no effect on leukocyte or eosinophil

association with endothelial cells (Fig. 12, *D* and *H*), VCAM-1 expression (Fig. 13, *A* and *C*), MCP-1 expression (Fig. 13*B*), endothelial cell viability (data not shown), or cell cytotoxicity as determined by the G6PDH release assay (Fig. 14*A*). To determine whether 5-HTP metabolism to serotonin in endothelial cells is required for leukocyte transendothelial migration, endothelial cells were pretreated with the aromatic amino acid decarboxylase inhibitor NSD1015 (Fig. 12*A*). 5-HTP inhibition of leukocyte and eosinophil transendothelial migration was blocked by NSD1015 (Fig. 12, *E* and *I*), whereas NSD1015 did not alter leukocyte or eosinophil association with the endothelial cells (Fig. 12, *F* and *J*). NSD1015 was not cytotoxic (Fig. 14*A*).

There is one report that suggests that 5-HTP may be metabolized to 5-hydroxykynurenine; intestines treated *ex vivo* with 5-HTP generate 5-hydroxykynurenine (46) (Fig. 12*A*). Therefore, we determined whether 5-HTP inhibition of leukocyte transendothelial migration is blocked by treatment with the indoleamine 2,3-dioxygenase inhibitor 1-methyl-DL-tryptophan (1MT). 1MT did not alter 5-HTP-induced inhibition of leukocyte or eosinophil transendothelial migration (Fig. 12, *E*

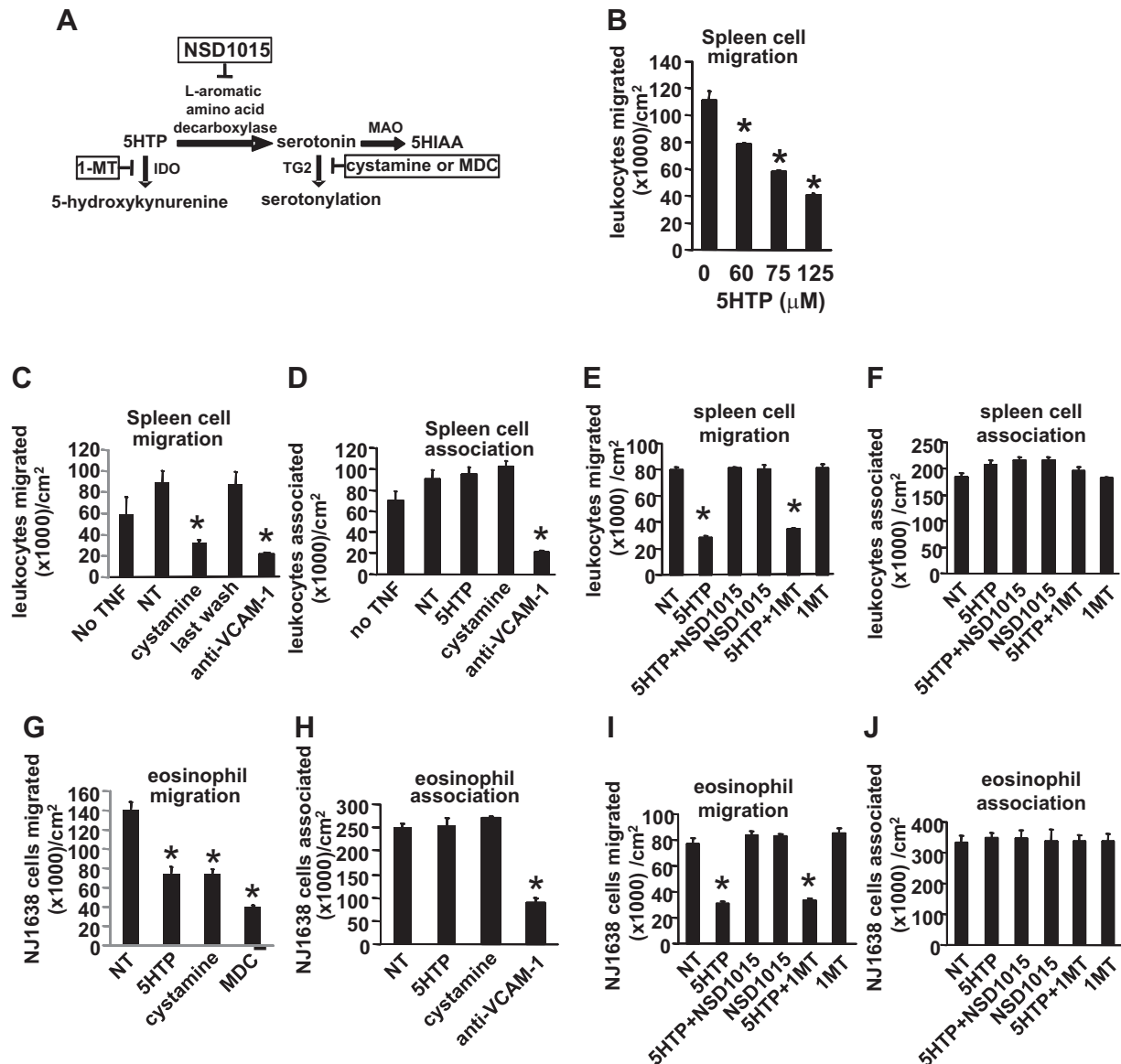


Fig. 12. 5-HTP pretreatment of endothelial cells in vitro reduced leukocyte transendothelial migration. A: diagram of 5-HTP metabolism to HIAA or serotonylation. Also indicated is the potential 5-HTP metabolism to 5-hydroxykynurenine; it is reported that 5-HTP application to intestines ex vivo generates 5-hydroxykynurenine (46). In boxes are the inhibitors. B–J: endothelial cell monolayers were pretreated with 5 ng/ml TNF- α overnight except where indicated as “no TNF”. TNF- α -stimulated endothelial cell monolayers on slide flasks were pretreated overnight as indicated with 5-HTP (75–125 μ M), the TG2 inhibitors cystamine (100 μ M) and dansylcadaverine (MDC, 200 μ M), the aromatic amino acid decarboxylase inhibitor NSD1015 (30 μ M), or the indolamine dioxygenase (IDO) inhibitor 1-MT (100 μ M 1-L-methyl-tryptophan + 100 μ M 1-D-methyl-tryptophan) and then washed before addition of BALB/c mouse spleen leukocytes (>90% lymphocytes) (B–F) or NJ1638 mouse eosinophils (>85% eosinophils) (G–J) for the migration and adhesion assays in a parallel plate flow chamber. The above inhibitors did not affect endothelial cell viability (data not shown) or cell cytotoxicity (Fig. 14A). Where indicated, anti-VCAM-1 antibodies (54 μ g Ab/800 μ l/slide flask) were added to the endothelial cells 15 min before addition of leukocytes. B–J: comparisons can only be made within an experiment because total migration can vary somewhat among experiments. In C, the “last wash” designates cells that were treated with the 5th wash from cystamine-pretreated endothelial cells, indicating that the cells were sufficiently washed to remove effective concentrations of the inhibitor. NT, nontreated. MAO, monoamine oxidase. $n = 3$ –5. * $P < 0.05$ compared with nontreated controls.

and D). 1MT also did not alter leukocyte or eosinophil association with the endothelial cells (Fig. 12, F and J).

The 5-HTP-treated endothelial cells were examined by HPLC with electrochemical detection as in Fig. 7A. Fresh culture medium with 20% heat-inactivated fetal calf serum did not contain detectable levels of 5-HTP, serotonin, or HIAA as determined by HPLC/ECD (data not shown). 5-HTP pretreatment of endothelial cells yielded an increase in 5-HTP and the metabolite HIAA (Fig. 14B). The L-aromatic amino acid de-

carboxylase inhibitor NSD1015 (6, 34, 52, 120) was used to determine whether it blocked catabolism to HIAA. The increase in HIAA in the 5-HTP-treated cells was blocked by NSD1015 (Fig. 14B) without inducing cell cytotoxicity (Fig. 14A). This suggests that the endothelial cells metabolized 5-HTP to serotonin and then HIAA. Although NSD1015 also blocks L-dihydroxyphenylalanine conversion to dopamine, we did not observe detectable levels of dopamine or its metabolite HVA in the endothelial cell lines with or without 5-HTP

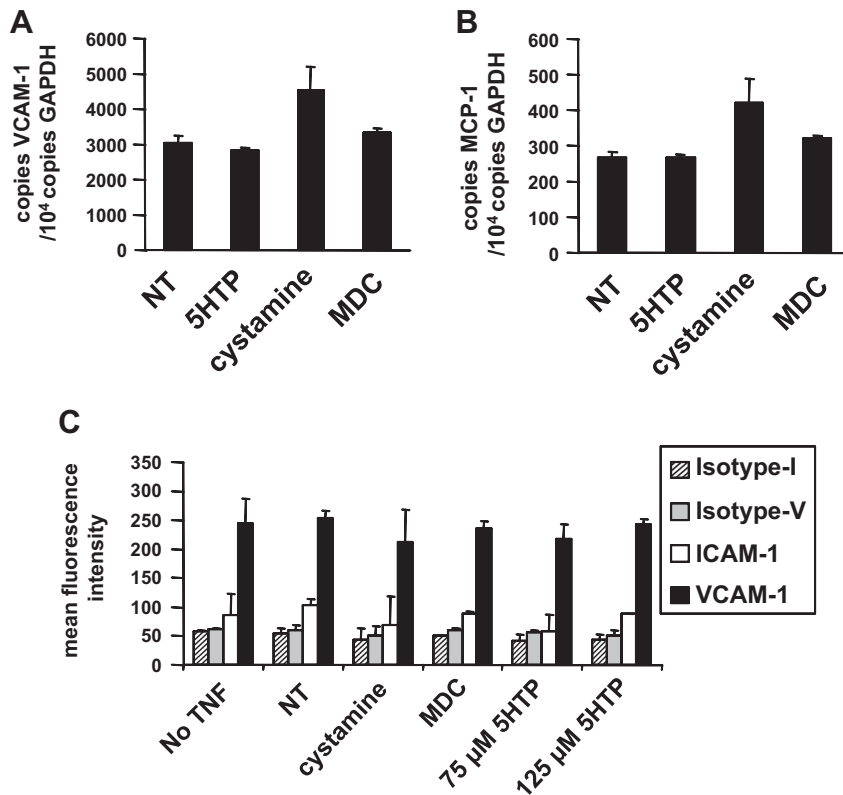


Fig. 13. 5-HTP pretreatment of endothelial cells did not alter endothelial cell expression of VCAM-1 or MCP-1. *A* and *B*: TNF- α -treated endothelial cells were incubated overnight with 125 μ M 5-HTP, 100 μ M cystamine, or 200 μ M MDC as in Fig. 12, and the cells were placed in RNAlater; the samples were then examined by quantitative PCR for expression of the adhesion molecule VCAM-1 (*A*) or the chemokine MCP-1 (*B*). *C*: endothelial cells were treated as indicated, immunolabeled with anti-ICAM-1 or an isotype antibody control for the ICAM-1 antibody (isotype-I) or immunolabeled with anti-VCAM-1 or an isotype antibody control for the VCAM-1 antibody (isotype-V), and examined by flow cytometry. The endothelial cell lines expressed VCAM-1 but not ICAM-1 as previously reported (24, 25). $n = 2$ experiments.

treatment (data not shown). Free serotonin was not detected in the 5-HTP-treated endothelial cells or in the culture supernatants from the endothelial cells as determined by HPLC/ECD (Fig. 14*B*), but serotonylation in the endothelial cells was detected (Fig. 10, *E* and *F*), indicating that serotonin is generated, used for serotonylation, as well as rapidly metabolized to HIAA. To detect 5-HTP catabolism to serotonin, we blocked serotonin metabolism to HIAA by using the inhibitor clorgyline to block monoamine oxygenase-A (MAO-A) and pargyline to block monoamine oxygenase-B (MAO-B) during 5-HTP treatment. MAO-A is expressed in peripheral cells including endothelial cells. In contrast, MAO-B is expressed by neurons (38) but not the endothelial cells (data not shown). Consistent with this, clorgyline but not the control pargyline increased serotonin in 5-HTP-treated endothelial cells (Fig. 14*B*). Together these data indicate that 5-HTP is metabolized to serotonin and then HIAA in the endothelial cells. In summary (overview in Fig. 14), 5-HTP supplementation reduced endothelial cell TG2 expression (Fig. 10*B*), endothelial cell serotonylation (Fig. 10, *D–F*) and leukocyte recruitment in vivo and in vitro (Figs. 3, 4, and 12).

DISCUSSION

We report that 5-HTP supplementation blocks allergen-induced airway responsiveness and blocks allergic lung inflammation induced by OVA, HDM, or intratracheal administration of IL-4. This occurred without reducing lung tissue mRNA and/or protein expression of mucins, cytokines, chemokines, or adhesion molecules and without altering leukocyte adhesion molecules, chemokine receptors, chemotaxis, or chemokinesis. A mechanism for 5-HTP inhibition of inflammation was, at least in part, by inhibition of endothelial cell function during

leukocyte recruitment. 5-HTP inhibited allergen-induced TG2 expression in lung endothelial cells and allergen-induced serotonylation in lung endothelial cells without altering epithelial serotonylation. Thus in vivo there were selective microenvironment changes with 5-HTP supplementation. In vitro, 5-HTP inhibited cytokine-induced serotonylation in endothelial cells and inhibited leukocyte and eosinophil transendothelial migration. Treatment of endothelial cells with TG inhibitors reduced leukocyte and eosinophil transendothelial migration. These data suggest that, during inflammation, endothelial cell serotonylation functions to support leukocyte recruitment and that this is inhibited by 5-HTP supplementation (Fig. 15).

It is reported that TG expression can be elevated in human asthmatic airways (56). In the mouse, daily intraperitoneal administration of a TG2 peptide inhibitor (KVLGQDP) is reported to block OVA-induced lung inflammation (64, 65). In these studies, the cellular targets of the TG peptide inhibitor are not known. We found that, in the lung, serotonylation is present in epithelium and endothelial, but, interestingly, OVA challenge induced an increase in TG2 and serotonylation in lung endothelial cells but not in lung epithelial cells. Moreover, 5-HTP supplementation blocked the increase in TG2 expression and serotonylation in lung endothelial cells. 5-HTP did not alter total lung TG2 mRNA expression in the mixed cell population of the whole lung, but 5-HTP did reduce protein expression of TG2 in lung endothelial cells, suggesting that 5-HTP supplementation mediates a negative feedback regulation of the serotonin pathway for serotonylation in endothelial cells. There is precedence for negative feedback regulation of gene expression in the serotonin pathway. For example, treatment of mice with clorgyline to block breakdown of serotonin results in

Fig. 14. Endothelial cell metabolism of 5-HTP to serotonin or HIAA was blocked by the inhibitors without inducing endothelial cell cytotoxicity. **A:** inhibitors in Figs. 10, 12, and 13 did not induce cell cytotoxicity. The endothelial cell monolayers were treated for 15 min with the following inhibitors used in Figs. 10, 12, and 13: 5-HTP (75 or 125 μ M), the aromatic amino acid decarboxylase inhibitor NSD1015 (NSD, 30 μ M), the monoamine oxygenase inhibitors clorgyline (Clorgyl, 1 μ M) or pargyline (Pargyl, 100 μ M), the TG inhibitor dansylcadaverine (MDC, 100 μ M), the IDO inhibitor 1-MT (100 μ M 1-L-methyl-tryptophan + 100 μ M 1-D-methyl-tryptophan), or the solvent control 0.01% DMSO. Then the cells were stimulated with 5 ng/ml TNF- α . After overnight culture, the endothelial cells were examined for cell cytotoxicity with the Vybrant Cytotoxicity assay. A set of cells were lysed as a positive control for cytotoxicity. **B:** endothelial cells were pretreated with NSD1015 (NSD, 30 μ M), 5-HTP (75 or 125 μ M), clorgyline (1 μ M), or pargyline (Pargyl, 100 μ M) where indicated and then stimulated with 5 ng/ml TNF- α . After overnight culture, the endothelial cells were examined for 5-HTP, serotonin (5-HT), and HIAA by HPLC/ECD. $n = 3$. * $P < 0.05$ indicates a significant increase compared with the nontreated (NT) group.

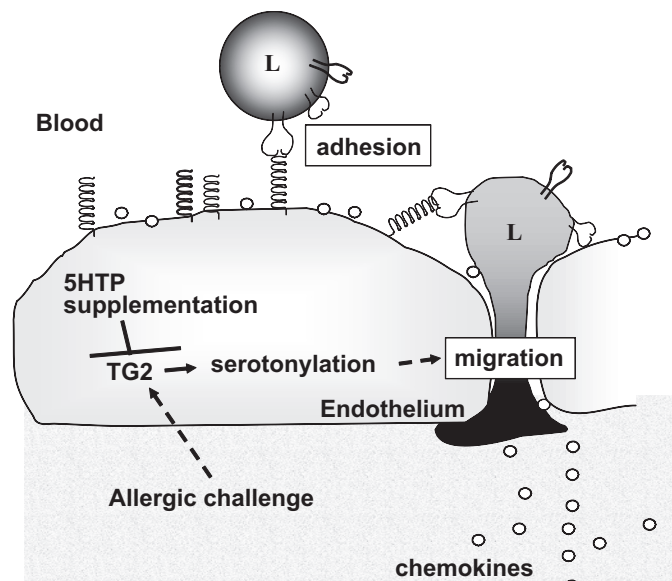
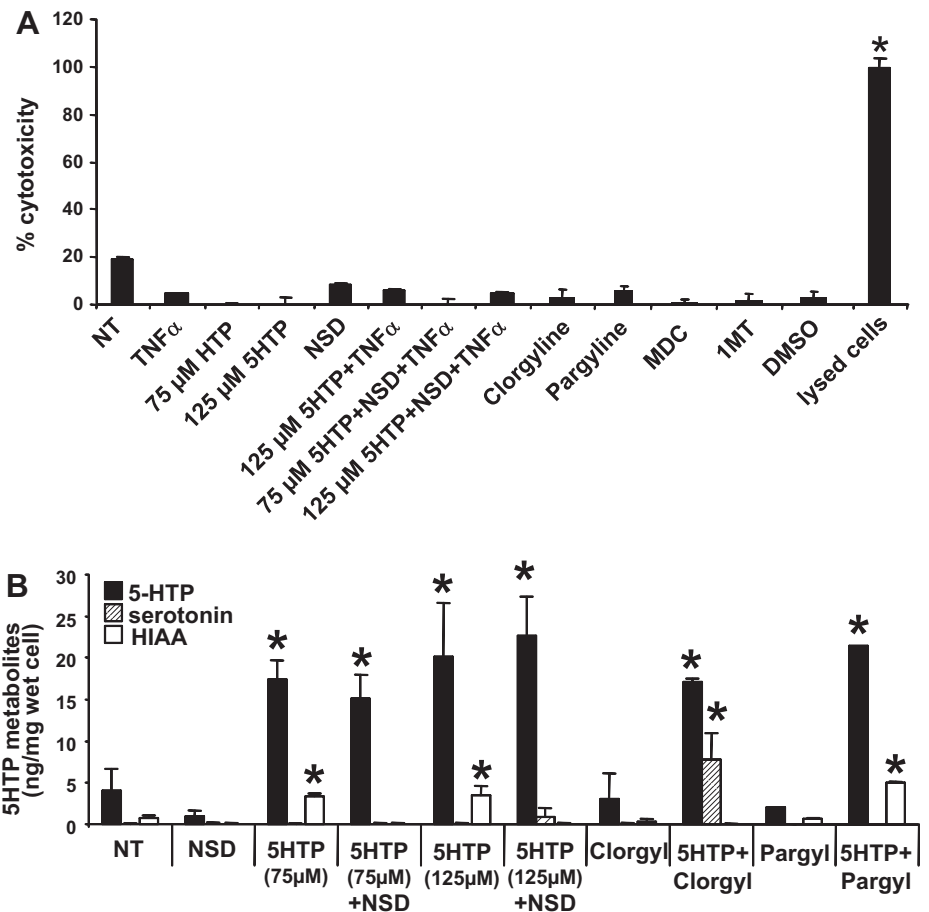


Fig. 15. Overview of 5-HTP inhibition of endothelial cell function during leukocyte transendothelial migration. During allergic inflammation, tissue-derived cytokines activate endothelial cells to increase expression of TG2, adhesion molecules, and chemokines. Furthermore, endothelial cell TG2-mediated serotonylation increases leukocyte transendothelial migration in response to chemokines. 5-HTP supplementation blocks allergen-induced increases in TG2 expression, TG2-mediated serotonylation, and leukocyte transendothelial migration. L, leukocyte.

reduced brain expression of L-aromatic amino acid hydroxylase, the enzyme for 5-HTP conversion to serotonin (22).

Endothelial cells express intracellular and cell surface TG2 (7, 10, 42). TG2-mediated serotonylation is important because serotonylation regulates the function of signaling proteins (30, 65, 75, 93, 119), and it is reported that adhesion molecule signaling in endothelium is required for eosinophil recruitment in allergic lung inflammation (2, 9, 25, 63, 94). It is also reported that antibody blockade of TG on the endothelial cell surface blocks CD8+ T cell transendothelial migration in vitro, but anti-TG treatment of CD8+ cells does not have an effect (86). In our studies, pretreatment of the endothelial cells in vitro with 5-HTP blocked endothelial cell serotonylation and blocked leukocyte transendothelial migration, suggesting that 5-HTP supplementation induces a negative feedback to down-regulate endothelial serotonylation and leukocyte transendothelial migration.

A TG2 peptide inhibitor (KVLGQDP) administered by daily intraperitoneal injection is reported to block OVA-induced lung expression of TG2, VCAM-1, IL-4, IL-5, and IL-13 (64, 65). In contrast to these studies with a peptide and unknown cellular targets of the peptide, we report that supplementation with 5-HTP did not alter OVA-induced lung tissue mRNA expression of adhesion molecules, cytokines, chemokines, or chemokine receptors, including lung tissue expression for the Th2 cell cytokines IL-4 and IL-5. In addition, lung lavage cytokines were not altered. Th2 cytokines are produced by many cells in the lung. Our finding that 5-HTP inhibits

allergic inflammation, OVA-induced airway responsiveness, and endothelial cell serotonylation without affecting cytokines or chemokines is consistent with previous reports, where inhibition of signals in endothelial cells blocks allergic inflammation and airway responsiveness without altering expression of cytokines and chemokines (2, 9, 25, 63, 94). This is also consistent with the active function of endothelial cells in the opening of endothelial passageways through which leukocytes migrate. Our in vitro studies demonstrate that cytokine-induced endothelial cell serotonylation functions to support leukocyte transendothelial migration and that this is blocked by treatment with 5-HTP (Fig. 12).

Very high doses of 5-HTP (1 mg/mouse, twice per day) are reported to be proinflammatory in a mouse model of colitis (49). However, these high doses of 5-HTP increase diarrhea (14, 19, 99) and thus would negatively impact colitis inflammation. It is also reported that high doses of 5-HTP (25–100 mg 5-HTP/kg body wt) increases blood or brain serotonin, whereas at 10 mg 5-HTP/kg body wt, there is no increase in 5-HTP metabolites (8, 48, 58, 95). In addition, it is reported that elevating systemic 5-HTP decreases blood pressure, whereas elevating systemic serotonin can increase vasoconstriction in humans and animal models (37, 39, 81, 105, 109, 118). Consistent with these previous reports that 10 mg 5-HTP/kg body wt did not increase 5-HTP metabolites, in our studies with lower doses of 5-HTP (0.1 mg 5-HTP/mouse/day = 5 mg 5-HTP/kg body wt/day), there was no change in systemic serotonin in plasma, lung, intestine, or brain. Nevertheless, we demonstrated that this level of 5-HTP supplementation decreased endothelial cell TG2 expression, endothelial cell serotonylation, and allergic inflammation.

Allergic inflammation is also reported to be regulated by the precursor to 5-HTP, tryptophan. Tryptophan can be converted to kynurenines by indolamine 2,3-dioxygenase and kynurenines are produced by the intestine and by dendritic cells. It is reported that kynurenines reduce OVA-induced allergic inflammation (121). In contrast to the studies with kynurenines, in our studies, 5-HTP did not alter tissue or BAL cytokine or chemokine production in vivo. In addition, when we used intratracheal IL-4 administration to bypass antigen presentation and kynurenine regulation of antigen presentation, 5-HTP blocked the IL-4-induced eosinophil inflammation in the lung. In vitro, 5-HTP inhibition of migration was blocked by pretreatment of endothelial cells with inhibitors of aromatic amino acid decarboxylase, which converts 5-HTP to serotonin in endothelial cells (33, 62, 78–80).

In summary, 5-HTP administration reduced allergic inflammation in vivo and leukocyte transendothelial migration in vitro, at least in part, by endothelial cell metabolism of 5-HTP for inhibition of TG2 (Fig. 15). Thus 5-HTP, which is reported to reduce hypertension (37) and decrease anxiety/depression (18, 53, 57, 61, 74, 82, 97, 98, 102, 111), has profound anti-inflammatory effects on allergic inflammation. This occurs without systemic increases in serotonin, which is an advantage because increases in systemic serotonin can cause vasoconstriction. These data identify 5-HTP as a potential target for intervention in allergy/asthma and the commonly asthma-associated clinical symptoms of anxiety/depression (21, 27, 36, 100, 106).

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: H.A.-V., S.B., C.A.M., D.U., R.M., M.E.M., K.S., L.W., and J.M.C.-M. performed experiments; H.A.-V., S.B., C.A.M., D.U., R.M., M.E.M., K.S., L.W., and J.M.C.-M. analyzed data; H.A.-V., S.B., C.A.M., M.E.M., G.M.M., and J.M.C.-M. interpreted results of experiments; H.A.-V., S.B., D.U., G.M.M., and J.M.C.-M. edited and revised manuscript; H.A.-V., S.B., C.A.M., D.U., R.M., M.E.M., K.S., L.W., G.M.M., and J.M.C.-M. approved final version of manuscript; R.M., G.M.M., and J.M.C.-M. conception and design of research; J.M.C.-M. prepared figures; J.M.C.-M. drafted manuscript.

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