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Serotonin 5-HT_2 receptor activation prevents allergic asthma in a mouse model

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ABSTRACT

Asthma is an inflammatory disease of the lung characterized by airways hyperresponsiveness, inflammation, and mucus hyperproduction. Current mainstream therapies include bronchodilators that relieve bronchoconstriction, and inhaled glucocorticoids to reduce inflammation. The small molecule hormone and neurotransmitter serotonin has long been known to be involved in inflammatory processes; however, its precise role in asthma is unknown. We have previously established that activation of serotonin 5-HT_{2A} receptors has potent anti-inflammatory activity in primary cultures of vascular tissues, and in the whole animal in vasculature and gut tissues. The 5-HT_{2A} receptor agonist, (*R*)-2,5-Dimethoxy-4-iodoamphetamine ((*R*)-DOI) is especially potent. In this work, we have examined the effect of (*R*)-DOI in an established mouse model of allergic asthma. In the ovalbumin mouse model of allergic inflammation, we demonstrate that inhalation of (*R*)-DOI prevents the development of many key features of allergic asthma including airways hyperresponsiveness, mucus hyperproduction, airways inflammation, and pulmonary eosinophil recruitment. Our results highlight a likely role of the 5-HT_2 receptors in allergic airways disease, and suggest that 5-HT_2 receptor agonists may represent an effective and novel small-molecule based therapy for asthma.

Keywords: Serotonin, Inflammation, 5-HT_2 receptor, 5-HT_{2A} receptor, Asthma, DOI

INTRODUCTION

Serotonin (5-hydroxytryptophan, 5-HT) is a ubiquitous small hormone molecule present in nearly all eukaryotes that mediates a wide spectrum of physiological processes. In mammals it exerts its action through 14 different receptor subtypes that comprise seven distinct families (5-HT_{1-7}) (34). All but one family, the ligand-gated 5-HT_3 receptor ion channel, are G-protein coupled receptors (34). The 5-HT_{2A} receptor is primarily known for its role in mediating complex cognitive behaviors within the central nervous system, and for mediating physiological processes such as vasoconstriction in the periphery (32, 34). Interestingly, the 5-HT_{2A} receptor is the primary target of classic hallucinogenic drugs like lysergic acid diethylamide (LSD), which produces intoxicating effects. Although 5-HT_{2A} receptor mRNA is expressed at higher levels in immune related tissues such as spleen, thymus, and peripheral circulating lymphocytes compared to other serotonin receptor subtypes (i.e. 5-HT_{1A}, 5-HT_{1D}, 5-HT_{2C}, 5-HT_4, 5-HT_{5A}, and 5-HT_{5B}) (42), its precise role in inflammatory processes is not well defined. With regard to of the potential role of serotonin in asthma, 5-HT_{2A} receptors are functionally expressed in activated $CD4^+$ T cells, alveolar macrophages, eosinophils, and lung epithelial and smooth muscle cells, (8, 20, 21, 23, 30). In fact, migration of eosinophils in allergic asthma has been recently shown to be dependent on 5-HT_{2A} receptor activation (21), and 5-HT_2 receptors have been implicated in platelet function relevant to allergic asthma (13).

We recently reported that 5-HT_{2A} receptor agonists potently inhibit inflammation *in vitro* (53). The anti-inflammatory effects of one particular 5-HT_{2A} receptor agonist, (*R*)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane [(*R*)-DOI], is extremely potent, with an

EC_{50} of ~15 picomolar. Through activation of the 5-HT_{2A} receptor, *(R)*-DOI blocks the expression and activation of proinflammatory markers including expression of chemokines (e.g. *MCP-1*), cellular adhesion molecules (*ICAM1* and *VCAM1*), cytokines (e.g. *IL-6*), nitric oxide synthase, and activation/nuclear translocation of NF-kB in a variety of cell types, including primary aortic smooth muscle cells (53). We have translated these *in vitro* findings to a whole animal mouse model of inflammation by demonstrating that (*R*)-DOI, also through 5-HT_{2A} receptor activation, has potent anti-inflammatory effects when administered systemically prior to systemically administered TNF-α. These effects are most pronounced in the vasculature and the gut, where preadministration of (*R*)-DOI blocks TNF-α induced increases in proinflammatory gene and protein expression, including circulating IL-6 (33).

In an effort to extend our findings to the potential use of (*R*)-DOI as a therapeutic in inflammatory airways disease, herein we examine the ability of (*R*)-DOI to block the key features of allergic asthma in the well-established mouse model of ovalbumin (OVA) induced allergic asthma. In this model, mice are sensitized and challenged with inhaled chicken OVA peptide to induce a phenotype resembling human asthma, including airways hyperresponsiveness in response to methacholine (MeCh), mucus hyperproduction, and pulmonary inflammation characterized by eosinophilia (5). We show here that inhaled (*R*)-DOI blocks airways hyper-responsiveness, recruitment of eosinophils to the lung, mucus hyperproduction, and inflammatory airway remodeling. We speculate that, 5-HT_2 receptor agonism may represent a novel therapeutic strategy for asthma.

MATERIALS AND METHODS

Drugs and Reagents. (*R*)-DOI was generously provided by Dr. David E. Nichols (Purdue University, IN) and was dissolved in sterile physiological saline prior to use. Ovalbumin (OVA) and methacholine were purchased from Sigma-Aldrich (St. Louis, MO).

Animals. For the inhalation/asthma experiments, specific pathogen free wild type BALB/c mice were purchased from Harlan Laboratories (Indianapolis, IN). Mice were maintained in the animal care facility at LSUHSC in ventilated cages housed in a pathogen-free animal facility with free access to food and water. Animal protocols were prepared in accordance with the Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee at Louisiana State University Health Sciences Center.

Induction of allergic inflammatory airways disease (i.e. the OVA mouse model of asthma). Mice (male; 6-8 weeks old) were sensitized and challenged with chicken ovalbumin grade V (OVA; Sigma, St. Louis, MO) as previously described (4). Briefly, mice were sensitized by an intraperitoneal injection (100 ul) of 20 μg OVA emulsified in 2 ml Imject Alum (Al [OH]3/Mg [OH]2; Pierce Rockford, IL) on days 0 and 14. Mice were subsequently challenged with an OVA aerosol generated using an ultrasonic nebulizer (PariNeb Pro Nebulizer) using a 1% (wt/vol) OVA solution in saline for 20 min on days 24, 25 and 26. Thirty minutes prior to each OVA challenge each mouse was treated with

one of two different concentrations of (*R*)-DOI (nose-only inhalation of 0.01 mg/kg or 1.0 mg/kg) or vehicle control using an ultrasonic nebulizer (Aerogen, Galway, Ireland).

Measurement of airway inflammation, pulmonary mechanics, and BALF cellularity. Pulmonary function testing, BAL and tissue harvests were performed on day 28 (when mice were 10 to 12 weeks of age). For the forced oscillation method, pulmonary resistance was measured as previously described (11). In brief, anesthetized animals were mechanically ventilated with a tidal volume of 10 ml/kg and a frequency of 2.5 Hz using a computer-controlled piston ventilator (Flexivent, SCIREQ; Montreal, Canada). Bronchial tone was determined in response to increasing concentrations of the aerosolized bronchoconstrictor methacholine (MeCh, at 0, 6.25, 12.5, 25 and 50 mg/ml in isotonic saline). The single compartment model was used to calculate airway resistance values, and peak values obtained after each MeCh challenge were plotted (17). On protocol day 28, bronchoalveolar lavage fluid (BALF) was harvested after pulmonary function testing and analyzed for cellularity as previously described (3). Differential cell counts were performed by two blinded observers using standard morphological criteria to classify individual leukocyte populations. All mice from each group were used for these analyses, and over 200 cells were counted per animal. For the whole body method, airway hyperresponsiveness to MeCh (0, 6.25, 12.5, 25, 50, and 100 mg/ml in isotonic saline) was measured using whole body plethysmography (Buxco Electronics, Troy, NY and EMKA Technologies, Falls Church, VA) and performed as described previously (51). Mice were exposed to aerosolized MeCh for 1 minute at each dose and peak enhanced pause (PenH) response was recorded for 3

minutes. The max PenH was averaged for each dose and data were plotted as percent change from vehicle controls.

Lung histopathology. Lungs were isolated and prepared as previously described (52). Sections (4 μm) were cut from paraffin embedded lungs and stained with Periodic acid-Schiff (PAS) staining to visualize mucus and imaged as previously described (52). Adjacent sections were stained with hematoxylin and eosin to visualize airway morphology and cellular inflammation.

Measurement of Total Protein in BALF. Total protein was measured from BALF isolated on Day 28 using the Pierce BCA Protein Assay kit following manufacturer's directions (Thermo Scientific # 23228, Rockford, IL).

Measurement of Total IgE and OVA-Specific IgE. Whole blood was taken via cardiac puncture by a 23 gauge needle on protocol Day 28. Whole blood was placed into plasma separator tubes coated in lithium heparin (Becton, Dickenson and Company, Franklin Lakes, NJ). Plasma was isolated from whole blood following manufacturer's protocols. Total Mouse IgE in the isolated plasma was determined using the ELISA MAX Deluxe kit (Cat. No. 432404) and Mouse OVA Specific IgE Legend MAX kit (Cat. No. 439807) purchased from BioLegend, INC (San Diego, CA).

Cytokine and chemokine analysis by qRT-PCR. Lungs were harvested 48 hours after the final OVA exposure, and expression levels of cytokines were determined using reverse transcription and quantitative, realtime PCR (qRT-PCR). For all lung tissues, RNA was extracted with TRIzol Reagent purchased from Life Technologies (Carlsbad, CA) following manufacturer's instructions. RNA was processed into first strand cDNA using the ImProm-II cDNA synthesis kit (Promega, Madison, WI) following the manufacturer's instructions. The input cDNA for each reaction was 500 ng total RNA. Cytokine and chemokine mRNA expression examined by probe-based QPCR included: *Il-4*, *Il-5*, *Il-6*, *Il-10*, *Il-13*, *Tnfα, Mcp-1*, *and Gm-csf*. Primers were designed and synthesized by Integrated DNA Technologies, INC (Coralville, IA). Primer sequences used in this study are as listed: *Il-4* forward 5'-catcggcattttgaacgag-3' and reverse 5'-cgagctcactctctgtggtg-3'; *Il-5* forward 5'-acattgaccgccaaaaagag-3' and reverse 5'-caccatggagcagctcag-3'; *Il-6* forward 5'-tctaattcatatcttcaaccaagagg-3' and reverse 5'-tggtccttagccactccttc-3'; *Il-10* forward 5'-cagagccacatgctcctaga-3' and reverse 5'-tgtccagctggtcctttgtt-3'; *Il-13 forward 5'*-cctctgacccttaaggagcttat-3' and reverse 5'-cgttgcacaggggagtct-3'; *Tnfα* forward 5'-tcttctcattcctgcttgtgg-3' and reverse 5'-ggtctgggccatagaactga-3'; *Mcp-1* forward 5'-tcactgaagccagctctctct-3' and reverse 5'-gatcatcttgctggtgaatgagt-3'; *Gm-csf* forward 5'-gcatgtagaggccatcaaaga-3' and reverse 5'-cgggtctgcacacatgtta-3'. Probes used were from the Universal Probe Library (Roche, Indianapolis, IN) and are listed with the universal probe number: U2, U97, U78, U41, U17, U49, U22, and U79, for *Il-4, Il-5, Il-6, Il-10, Il-13, Tnfα, Mcp-1, and Gm-csf* respectively. Quantification of gene expression shown in **Figure 6** was performed on a Roche LightCycler 480II LC (Roche, Indianapolis, IN). Gene expression levels were

calculated using the $\Delta\Delta C_T$ method and normalized to internal *Gapdh* expression as determined using the Mouse *Gapdh* Gene Assay (Roche Diagnostics, Cat. no. 05046211001) in multiplex format.

Statistics. All statistical analysis was performed using GraphPad Prism (La Jolla, CA). In **Figure 1**, n=5-9 animals per treatment group , * $p < 0.05$ OVA vs Naive; # $p < 0.05$ OVA vs (*R*)-DOI (1.0 mg/kg); ^ $p < 0.05$ OVA vs (*R*)-DOI (0.01 mg/kg); error bars represent ± SEM; the statistical analysis used was 2-way ANOVA with Bonferroni post hoc test. In **Figure 3**, n=3 for all groups, all airways were scored blinded, and both lungs were represented in the sections, * $p<0.0001$ vs Naive; # $p<0.0001$ vs OVA; ^ $p<0.001$ vs Naive; error bars represent ± SEM; the statistical analysis used was an ANOVA with Tukey post hoc test. In **Figure 4,** n=5-6 animals per treatment group, * represents Naive mice are significantly different ($p<0.001$) from OVA-only mice; # represents (*R*)-DOI treated mice are significantly different ($p<0.01$) from OVA-only mice; error bars represent ± SEM; the statistical analysis used was 2-way ANOVA with Bonferroni post hoc test. In **Figure 5,** n=7-17 animals per treatment group, *** $p<0.001$ vs OVA; n.s. stands for no significance vs OVA; error bars represent ± SEM, ANOVA with Tukey post hoc test. In **Figure 6**, n=4 animals for the Naïve group, n=10 animals for the OVA and DOI+OVA treatment groups; *** $p<0.0001$; ** $p<0.01$, *$p<0.05$; n.s. stands for no significance, error bars represent ± SEM, ANOVA with Tukey post hoc test

RESULTS

Pulmonary administration of (*R*)-DOI is effective in preventing airways hyperresponsiveness (AHR) in a mouse model of allergic asthma.

Following OVA sensitization and challenge, we measured airways resistance by two different methods in separate groups of mice. For the first method we used the forced oscillation technique, and in the second method whole body plethysmography in awake freely moving mice. As expected, mice receiving only OVA develop significant AHR in both methods (**Figure 1**). Mice pretreated with inhaled (*R*)-DOI at either 0.01 (Figure 1A and 1B) or 1.0 mg/kg (Figure 1A and 1C) prior to OVA challenge display airways responsiveness not significantly different from naïve as measured by either method.

(*R*)-DOI prevents pulmonary inflammation and mucus hyperproduction.

Histopathological analysis of lung sections from the different treatment groups demonstrated that, as expected, OVA mice develop significant pulmonary inflammation and mucus. Animals treated with (*R*)-DOI (1.0 mg/kg) prior to OVA exposure exhibit very little peribronchial inflammation or mucus. Mice treated with two orders of magnitude less (*R*)-DOI (0.01 mg/kg) demonstrate significantly reduced inflammation and mucus production compared to the OVA only exposed lungs (**Figures 2** and **3**).

(*R*)-DOI reduces pulmonary inflammation and BALF eosinophilia.

Pulmonary inflammation is a common feature of asthma and is partly responsible for increased AHR (15). To associate (*R*)-DOI treatment and decreased AHR as well as normal appearing histological results with lack of inflammation, we performed cell differential counts on BALF cell populations for each mouse in each group. As

expected, OVA induced a significant increase in the total number of cells recovered in the BALF when compared to naïve and (*R*)-DOI treated animals. A large fraction of the BALF cellularity was due to elevated numbers of eosinophils (**Figure 4**). Total BALF cell numbers and eosinophil numbers for naïve, 0.01 mg/kg DOI + OVA and 1.0 mg/kg DOI + OVA were significantly lower than the OVA-only mice (**Figure 4**). Although the eosinophil numbers for the (*R*)-DOI treated mice were greater than those of naïve mice they were not significantly different. There is a trend for a decrease in the neutrophil numbers in (*R*)-DOI treated mice compared to AVA treated mice, however the difference was not significant.

(*R*)-DOI does not alter lung leak or plasma IgE levels.

Increased protein content of the BALF is a hallmark of asthma and the OVA model (46). Analysis of BALF total protein by BCA assay from different treatment groups revealed a significant increase between naïve and OVA groups but showed no difference between mice treated with (*R*)-DOI + OVA and those animals that were treated with OVA only (**Figure 5).** The OVA model characteristically produces increased serum levels of IgE and OVA-specific IgE (19, 28, 56), therefore we tested the effects of (*R*)-DOI on total IgE and OVA specific IgE. In both cases, we measured a significant increase between naïve and OVA treated groups. (*R*)-DOI treatment, however, had no effect on either total IgE or OVA-specific IgE as induced by OVA (**Figure 5)**.

(*R*)-DOI suppresses expression of genes involved in the T-cell and innate immune cell response.

A panel of cytokines and chemokines typically involved in asthma and the OVA model (*Il-4, Il-5, Il-6, Il-10, Il-13, Tnfα, Mcp-1, and Gm-csf*) was examined in the lungs by QPCR (6, 10, 17, 29, 36, 45, 49). There were, as anticipated, significant increases in mRNA for *Il-4*, *Il-5*, *Il-10, Il-13, Mcp-1* and *Gm-csf* with OVA treatment compared to naïve mice. There was a trend that did not reach significance for *Il-6* and *Tnfα* expression. (*R*)-DOI had no effect on the increased expression levels of OVA induced *Il-4, or Il-10.* Interestingly, (*R*)-DOI treatment significantly repressed the OVA-induced increases in mRNA expression for *Mcp-1*, *Il-13*, *Il-5*, and completely blocked the increase in *Gm-csf* (**Figure 5**). Although *Il-6* expression was not significantly up-regulated in the OVA group compared to vehicle control, (*R*)-DOI did significantly reduce *Il-6* expression levels in OVA treated mice, as expected from our previous studies in different inflammatory models (**Figure 6**).

DISCUSSION:

To determine if serotonin $5HT_2$ receptor activation with (*R*)-DOI is an effective mechanism to treat a pathological inflammatory disease, we investigated the effects of the highly selective 5-HT_2 receptor agonist (*R*)-DOI in a mouse model of allergic asthma. Building upon our earlier *in vitro* and *in vivo* studies, we demonstrate here that inhaled (*R*)-DOI has potent anti-inflammatory effects and blocks the development of allergic asthma in the OVA mouse model. Importantly, we have already established that the anti-inflammatory effects of (*R*)-DOI *in vitro* and *in vivo* are mediated through activation of the serotonin 5-HT_{2A} receptor subtype (33, 53). Here, we tested two different doses of (*R*)-DOI. The 1.0 mg/kg dose is in the range of that used in typical

behavioral experiments (41). The very low dose of 0.01 mg/kg was chosen to test the super potency of (*R*)-DOI predicted by our previous cellular studies (53). Anti-inflammatory effects of this very low dose were also observed in our recent *in vivo* study examining the ability of (*R*)-DOI to block the effects of systemic administration of TNF-α (33). Because activation of the 5-HT_{2A} receptor subtype, and not the 5-HT_{2C} receptor subtype, was found to be necessary for the anti-inflammatory effects of (*R*)-DOI in our previous studies, we hypothesized that the effects of (*R*)-DOI against allergic asthma were also mediated through 5-HT_{2A} receptor activation. Although we were not able to validate this here, we have confirmed the presence of 5-HT_{2A} receptor mRNA on whole lung tissue (33). Furthermore, the expression of 5-HT_{2A} receptors has been reported in airway smooth muscle cells (2) and alveolar macrophages (30), and although naïve T cells do not express high levels of the 5-HT_{2A} receptor, activated T cells do express high levels of 5-HT_{2A} receptor mRNA (23). We suggest that (*R*)-DOI's site of therapeutic action in this model is the pulmonary tissues, including resident activated T-cell populations and/or innate immune cells.

The major components of allergic asthma in humans include AHR, pulmonary inflammation, and mucus hyperproduction (7). In addition, eosinophils, which release cytotoxic mediators and leukotrienes are recruited in large numbers to the lungs of asthmatic individuals (37). Eosinophil production, chemotaxis, and survival are controlled by RANTES (CCL5), macrophage inflammatory protein 1*α*, eotaxins, IL-5, and GM-CSF (18, 25, 35, 43, 47). IL-5 and GM-CSF are derived from activated pulmonary epithelial cells, eosinophils themselves, and activated T-lymphocytes (1, 27, 50). IL-5 and GM-CSF are molecules important in the development of asthma, and are

295 increased in serum and BALF of asthmatics in the clinic (12, 44). Significantly, our data
296 show that both genes are suppressed by administration of (*R*)-DOI in the OVA mouse
297 model.

298 The role of eosinophils in asthma is both direct, causing bronchoconstriction and
299 destruction to airways, and indirect by provoking degranulation of mast cells and
300 basophils (7). We demonstrate here that (*R*)-DOI blocks recruitment of eosinophils to
301 the lung, prevents mucus cell mucus hyperproduction, blocks AHR, and represses Th_2
302 and innate immune cell gene expression (e.g. *Il-5, and Mcp-1*). We directly delivered
303 (*R*)-DOI to the lung using inhalation techniques in these experiments, and it remains to
304 be determined if systemically injected (*R*)-DOI has the same or similar effects on the
305 development of asthma. Importantly, effective levels of (*R*)-DOI administered by this
306 route (inhalation) are orders of magnitude less than those necessary to produce either
307 behavioral intoxication, as indicated by the classical head twitch response (9), or
308 airways constriction in mice (>10 mg/kg inhaled; data not shown).

309 Although the presence of 5-HT_{2A} receptor mRNA has been demonstrated in
310 pulmonary tissues by our lab and others, the role of this receptor in the lung has
311 remained largely undefined. A few reports have suggested that the 5-HT_{2A} receptor
312 mediates AHR in allergic asthma (14, 40, 54). However, these studies used the
313 antagonist ketanserin, which is non-selective in rodents for 5-HT_2 receptors and also
314 has high affinity for histamine H1 and α-adrenergic receptors, to block the effects of
315 serotonin. This makes it difficult to interpret results using ketanserin. In any case, these
316 reports indicated that serotonin activation of 5-HT_{2A} receptors contribute to AHR, not
317 prevent it. Serotonin itself has been implicated in airways inflammation in allergic

asthma by acting as a critical factor to recruit inflammatory cells and prime Th_2 responses by activation of bone marrow derived dendritic cells, although the receptor(s) mediating these effects remain unknown (13). Conversely, blockade of serotonin receptors with a non-selective antagonist for multiple subtypes has demonstrated anti-asthma effects in the OVA model (24, 40). Why then, if serotonin appears to have a pro-inflammatory effect in the lung, does activation of 5-HT_2 receptors with (*R*)-DOI have an anti-inflammatory effect? One possibility is that selective activation of 5-HT_2 receptors with (*R*)-DOI avoids activation of other serotonin receptor types responsible for the inflammatory response. A more likely explanation is that (*R*)-DOI, which has a much higher affinity for the 5-HT_2 receptors than serotonin, is acting as a functionally selective ligand and recruiting anti-inflammatory effector pathways that serotonin itself does not (26, 31). Significantly, DOI has already been demonstrated to activate different signaling pathways than serotonin at the 5-HT_{2A} receptor *in vivo* to modulate biological responses (38, 39).

It is unlikely that the therapeutic mechanistic site of action of (*R*)-DOI is on the B-cell or the antigen presenting cell (APC) as (*R*)-DOI has no effect on OVA-induced *Il-4* gene expression. Recent reports indicate that IgE-dependent mast cell activation is involved in the development of AHR (28). That (*R*)-DOI has no measureable effect on humoral IgE production, yet prevents AHR, suggests that (*R*)-DOI is acting on activated rather than naïve T cells to block AHR through non-mast cell dependent mechanisms. Because (*R*)-DOI blocks *Mcp*-1 and *Gm-csf* mRNA production, the therapeutic target may also include innate immune cells. There is also the possibility that (*R*)-DOI may be acting on the naïve $CD4^+$ population, however, naïve T cells do not express high levels

of 5-HT_{2A} receptor mRNA until activated. Our data demonstrate that (*R*)-DOI treatment significantly inhibits the OVA-induced expression of Th_2 related genes that include *Il-13*, *Il-5*, and *Gm-csf* in the lung. Interestingly, vascular (or more likely epithelial) permeability is not improved with (*R*)-DOI, as total protein in the BALF is not reduced compared to OVA alone.

We propose a model, shown in **Figure 7,** where the pool of 5-HT_{2A} receptors activated by (*R*)-DOI that responds with anti-inflammatory properties could reside on activated Th_2 cells and/or innate immune cells. In this proposed model, 5-HT_{2A} receptor activation would lead to reduced *IL-5*, *GM-CSF,* and *MCP-1* secretion, in turn decreasing eosinophil recruitment, Th_2 polarization, and Il-13 production (16, 22, 48, 55). Overall, these effects would combine to reduce inflammation and AHR. The precise cellular signaling pathways, however, remain to be elucidated.

In conclusion, we have identified an important and new functional role of 5-HT_2 receptors in the lung. (*R*)-DOI activation of serotonin 5-HT_2 receptors potently prevents the development of a clinically relevant mouse model of allergic asthma at drug levels far below those necessary to invoke adverse cardiovascular or behavioral effects. Based on our previous *in vitro* and *in vivo* studies, we predict that it is the 5-HT_{2A} receptor that is the therapeutic target of (*R*)-DOI in our model. Our results demonstrate that activation of 5-HT_2 receptors differentially regulates Th_2 signaling, innate cytokine responses, and other relevant inflammatory effector pathways, and that selective activation with (*R*)-DOI, or perhaps other 5-HT_{2A} agonists in its class, represent a novel small molecule based therapeutic strategy for the treatment of asthma.

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FIGURE LEGENDS

Figure 1. (*R*)-DOI prevents the development of airways hyperresponsiveness. **A)** In forced oscillation resistance measurements (FlexiVent), naive mice and those treated nose-only (NO) with 0.01 and 1.0 mg/kg (*R*)-DOI during the sensitization process exhibited significantly different resistances from the OVA-only treated group at 50 mg/mL MeCh, and were not significantly different from naive saline treated mice. (Resistance = the average of the fractional difference of the value measured vs the individual baseline values). **B** and **C**: Results from whole body plethysmography experiments in awake freely moving mice are consistent with the forced oscillation results: Pretreatment with **(B)** 0.01 mg/kg (*R*)-DOI NO, and **(C)** 1.0 mg/kg (*R*)-DOI NO significantly reduced the development of airways resistance. (* $p < 0.05$ OVA vs Naive; # $p < 0.05$ OVA vs (*R*)-DOI (1.0 mg/kg); ^ $p < 0.05$ OVA vs (*R*)-DOI (0.01 mg/kg); n=5-9 animals per treatment group; error bars represent ± SEM; 2-way ANOVA with Bonferroni post hoc test) (PenH Max values represent baseline normalized values).

Figure 2. OVA-induced lung inflammation and mucus hyperproduction is inhibited by nose-only (*R*)-DOI. Representative sections of airways (4 μm) stained with the PAS technique are shown in this figure to highlight mucus (bright pink color). Saline treated animals have normal airway morphology and no mucus or inflammation (**A**, **E**). OVA-alone treated animals have thickened airways with a significant amount of mucus present (**B**), as well as peribronchial inflammation (**F**, arrows indicate inflammatory cells). Animals pre-treated with (*R*)-DOI (1.0 mg/kg and 0.01 mg/kg nose only)

587 demonstrate normal airway morphology, with little to no detectable mucus or
588 inflammation (**C**, **G; D, H**). Panels A - D = 40x obj, and panels E - H = 10x obj.

589 **Figure 3.** Inhaled (*R*)-DOI reduces mucus cell hyperplasia and mucus production in the
590 airway. The fraction of airway cells containing mucus, as determined by PAS staining (see
591 ***Figure 2***), was determined for two doses of (*R*)-DOI in two separate experiments. Results
592 are presented as normalized to OVA = 100%. Naive airways did not contain PAS
593 positive cells, OVA sensitization dramatically increases mucus production (OVA). **A)**
594 The number of airway cells containing mucus is significantly reduced by administration of
595 aerosolized (*R*)-DOI prior to OVA challenge (0.01 mg/kg DOI+OVA). **B)** The number of
596 airway cells containing mucus is nearly abolished by inhaled (*R*)-DOI treatment at 1.0
597 mg/kg (DOI+OVA). (* $p<0.0001$ vs Naive; # $p<0.0001$ vs OVA; ^ $p<0.001$ vs Naive; error
598 bars represent ± SEM; ANOVA with Tukey post hoc test; All airways per section, which
599 included both lungs, were scored by an unbiased observer for each of three animals per
600 treatment (n=3)).

601

602 **Figure 4.** BAL fluid cellularity is altered in mice exposed to OVA but not in mice exposed
603 to OVA and treated with (*R*)-DOI. BAL cellularities and differentials are expressed as the
604 product of the total number of cells recovered and the percentages of each cell type derived
605 from differentials. The total number of cells is nearly double in the OVA alone treated mice
606 compared to naïve (Total). (*R*)-DOI treatment prior to OVA exposure reduced the total
607 cellularity back to naïve levels. This difference was primarily due to a significant reduction in

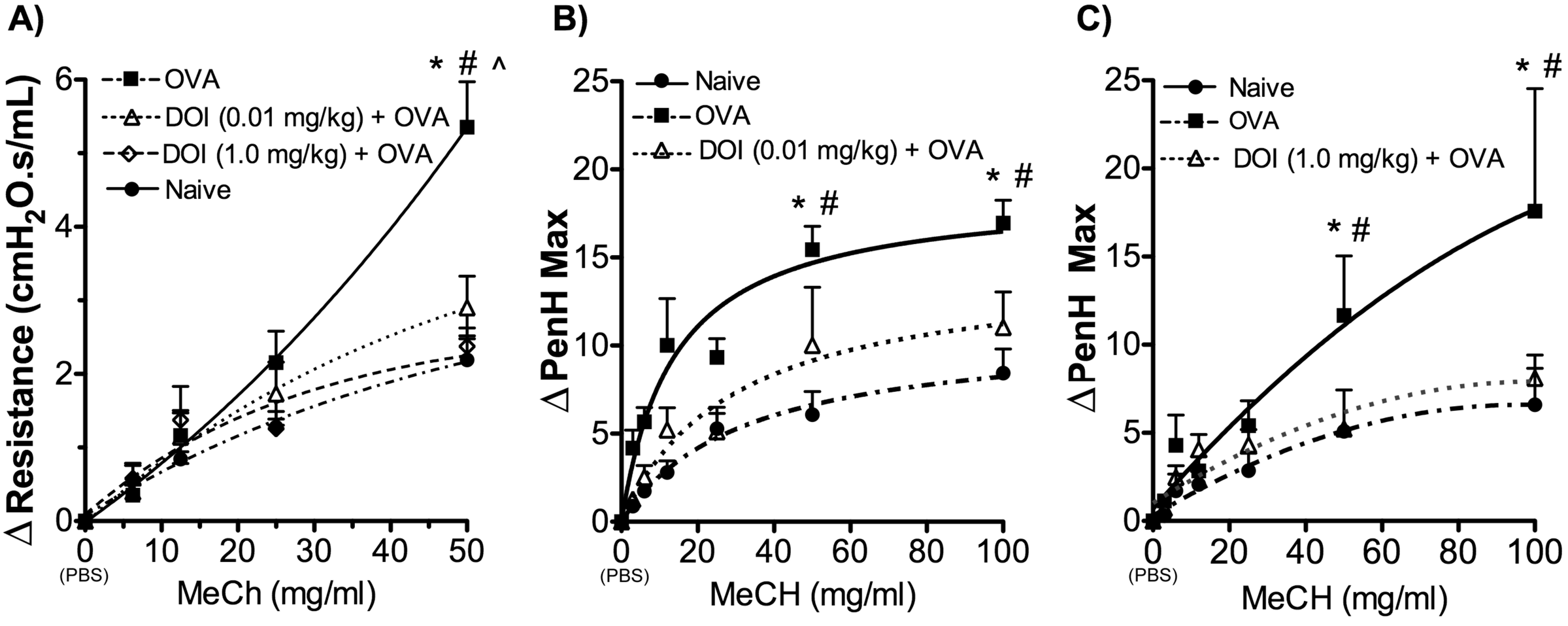
eosinophils (Eos) in the mice treated with (*R*)-DOI. No significant differences in the numbers of macrophages (Macs), lymphocytes (Lymphs), or neutrophils (Neutros) were observed between treatment groups. (* = OVA-only vs Naive ($p<0.001$); # = OVA-only vs (*R*)-DOI + OVA ($p<0.01$); n=5-6 animals per treatment group; error bars represent ± SEM; 2-way ANOVA with Bonferroni post hoc test).

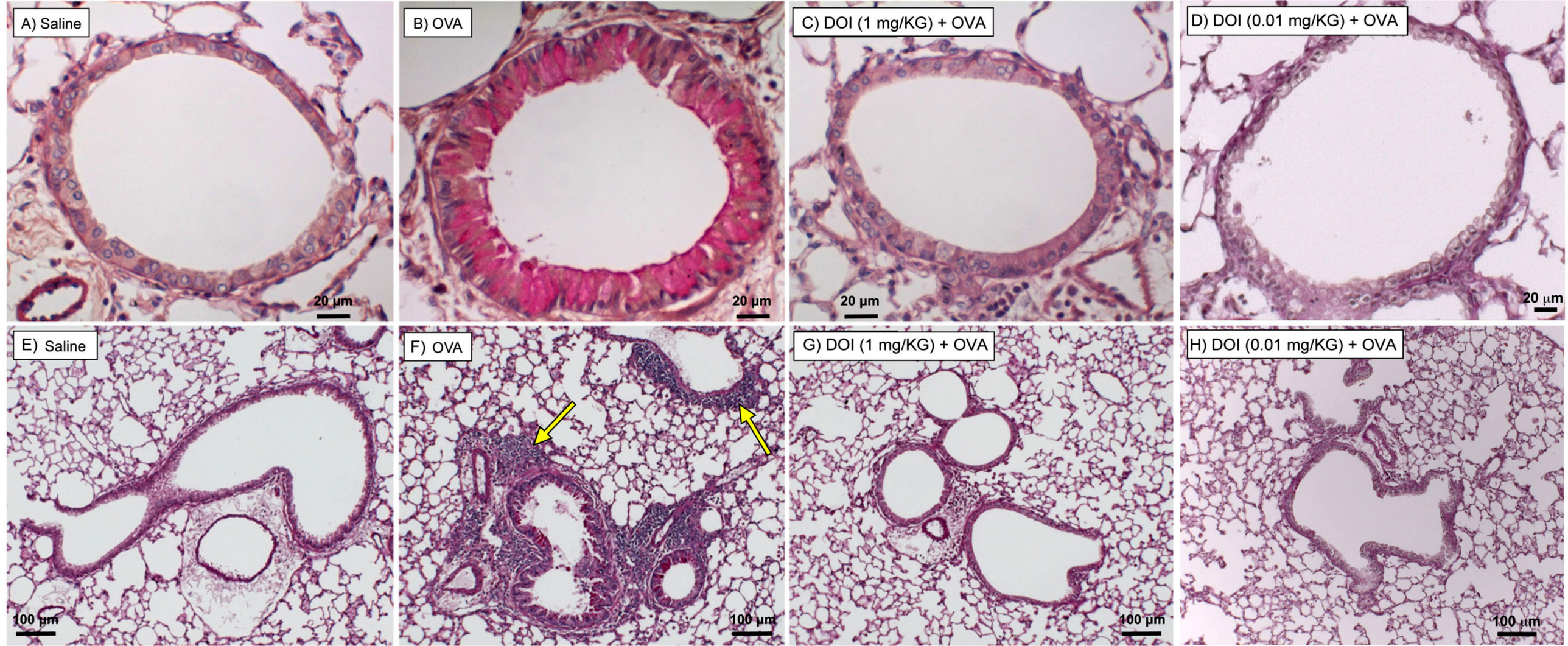
Figure 5. Total protein and IgE levels are not affected by (*R*)-DOI (1.0 mg/ml, NO). **A)** The total of protein content in the BALF as measured by BCA assay is significantly increased in the OVA-only treated lungs compared to naïve. (*R*)-DOI does not alter total BALF protein induced by OVA. **B)** Total plasma IgE as measured by ELISA is significantly increased by OVA treatment. (*R*)-DOI administered prior to OVA challenge has no effect on total plasma IgE. **C)** OVA specific plasma IgE as measured by ELISA is significantly increased by OVA treatment. (*R*)-DOI administered prior to OVA challenge has no effect on OVA specific plasma IgE (*** $p<0.001$ vs OVA; n.s. = no significance vs OVA, n = 7-17 animals per treatment group, error bars represent ± SEM, ANOVA with Tukey post hoc test).

Figure 6: Inhaled (*R*)-DOI (1.0 mg/kg) inhibits pro-inflammatory gene expression in the whole lung. qRT-PCR measurement of mRNA expression levels of several inflammatory markers are shown. OVA produces a significant increase in the mRNA levels of *Il-4* **(A)**, *Il-10* **(B)**, *Mcp-1* **(E)**, *Il-13* **(F)**, *Il-5* **(G)**, and *Gm-csf* **(H)** compared to naïve. No significant effect of OVA was observed on *Tnfα* **(C)** *or Il-6* **(D)** expression. (*R*)-DOI produces

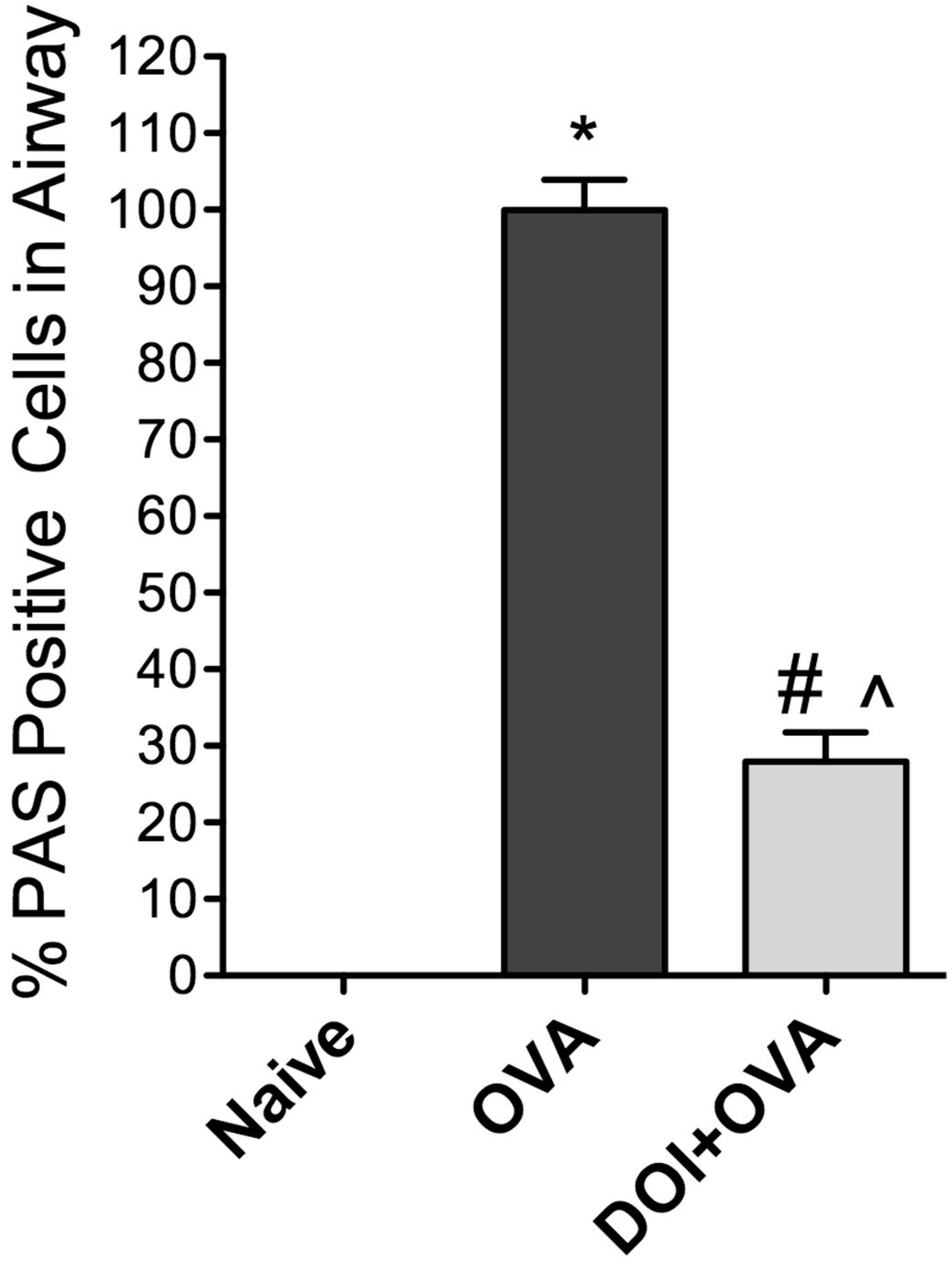
significant inhibition of the OVA-induced increases in the mRNA expression of *Mcp-1, Il-13, Il-5, and Gm-csf*. Although *Il-6* levels were not statistically different between naïve and OVA groups, (*R*)-DOI elicited a significant decrease in *Il-6* expression levels when administered prior to OVA exposure compared to OVA alone. (*** $p<0.0001$; ** $p<0.01$, *$p<0.05$; n.s. stands for no significance, n=4 animals for the Naïve group, n=10 animals for the OVA and DOI+OVA treatment groups, error bars represent ± SEM, ANOVA with Tukey post hoc test.

Figure 7: A proposed therapeutic mechanism of (*R*)-DOI. The presented data shows that (*R*)-DOI has no effect on *Il-4* gene expression, as well as no effect on humoral IgE production. These data provide evidence that the therapeutic action of (*R*)-DOI is not on the B-cell, the Antigen Presenting Cell (APC), and/or the Naïve $CD4^+$ population. Importantly, we show that (*R*)-DOI treatment significantly inhibits expression of Th_2 related genes including ***Mcp-1***, ***Il-13***, ***Il-5***, and ***Gm-csf*** when compared to asthmatic animals. Taken together, we suggest that (*R*)-DOI exerts its therapeutic action in the OVA asthma model by activating anti-inflammatory signaling pathways through the 5-HT_{2A} receptors on T cells and/or innate immune cells, leading to a decrease in secretion of Il-13, resulting in a decrease in mucus production, a decrease in *Il-5* and *Gm-csf* secretion, leading to a decrease in eosinophilia recruitment, and a decrease in Mcp-1 production leading to a decrease in Th_2 polarization. These changes contribute to a general decrease in both inflammation and AHR.

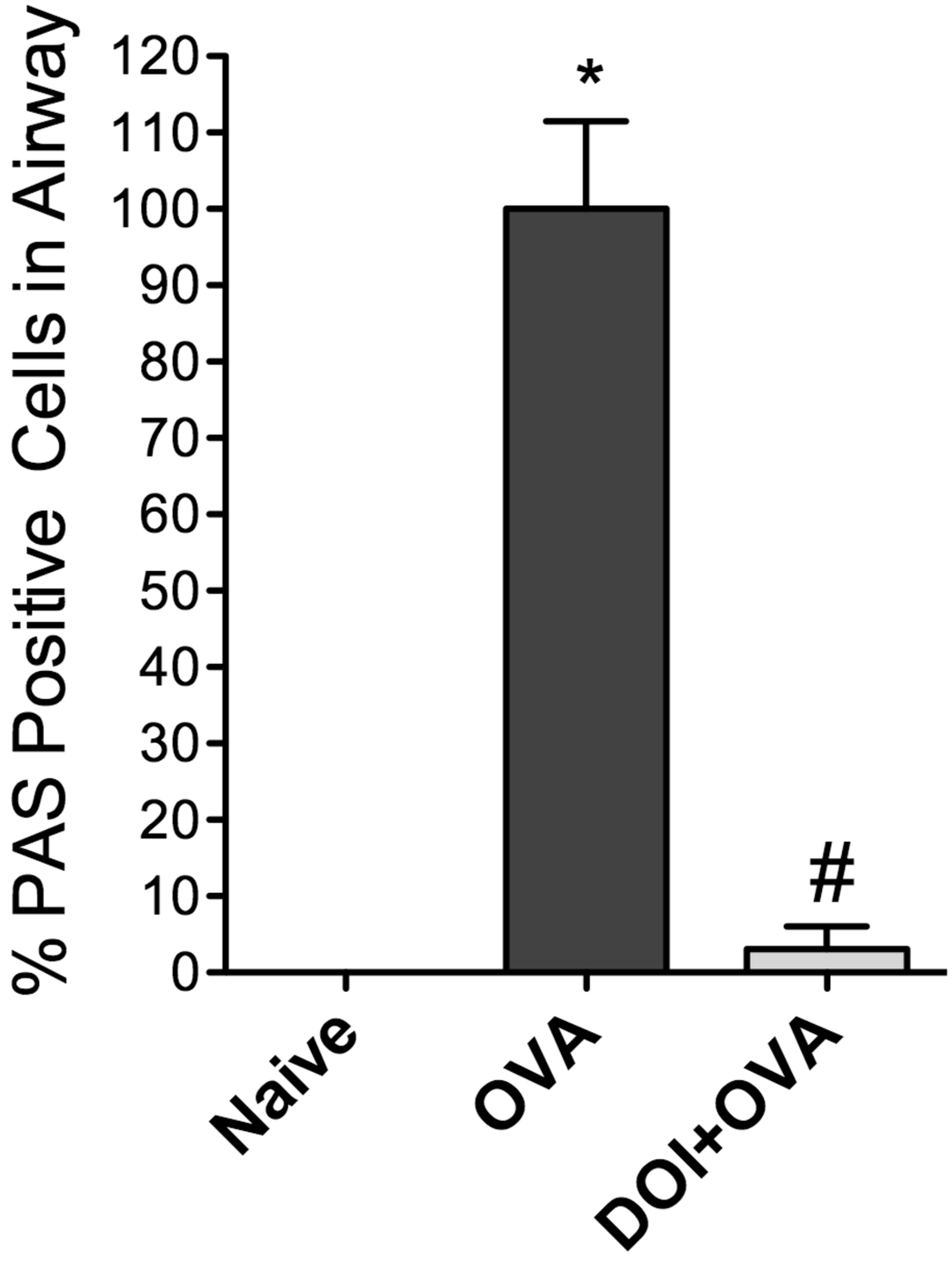

A)
B)
C)
OVA
DOI (0.01 mg/kg) + OVA
DOI (1.0 mg/kg) + OVA
Naive
Δ Resistance (cmH2O.s/mL)
ΔPenH Max
MeCh (mg/ml)
MeCH (mg/ml)
(PBS)
* # ^
* #

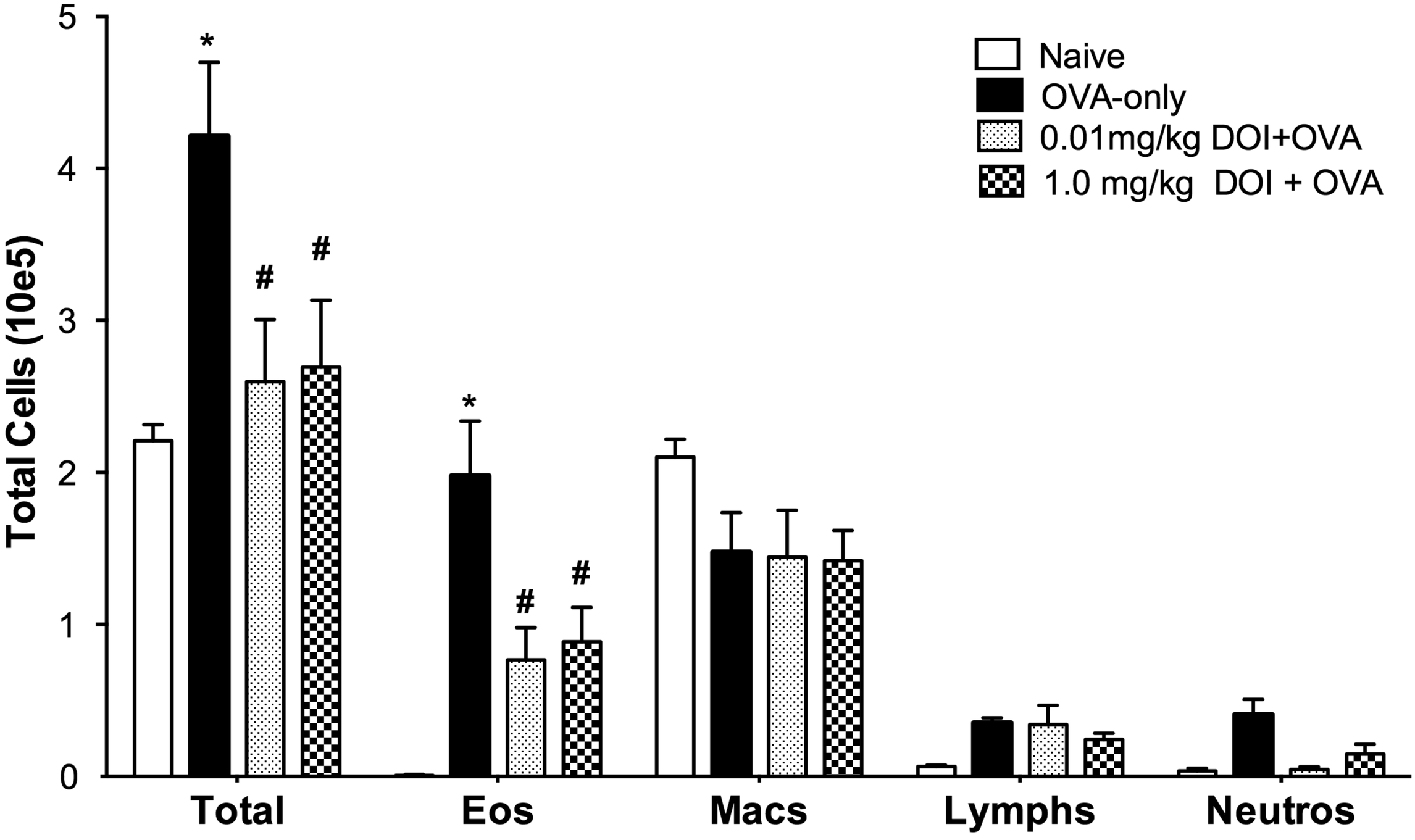

A) Saline
20 µm
B) OVA
20 µm
C) DOI (1 mg/KG) + OVA
20 µm
D) DOI (0.01 mg/KG) + OVA
20 µm
E) Saline
100 µm
F) OVA
100 µm
G) DOI (1 mg/KG) + OVA
100 µm
H) DOI (0.01 mg/KG) + OVA
100 µm

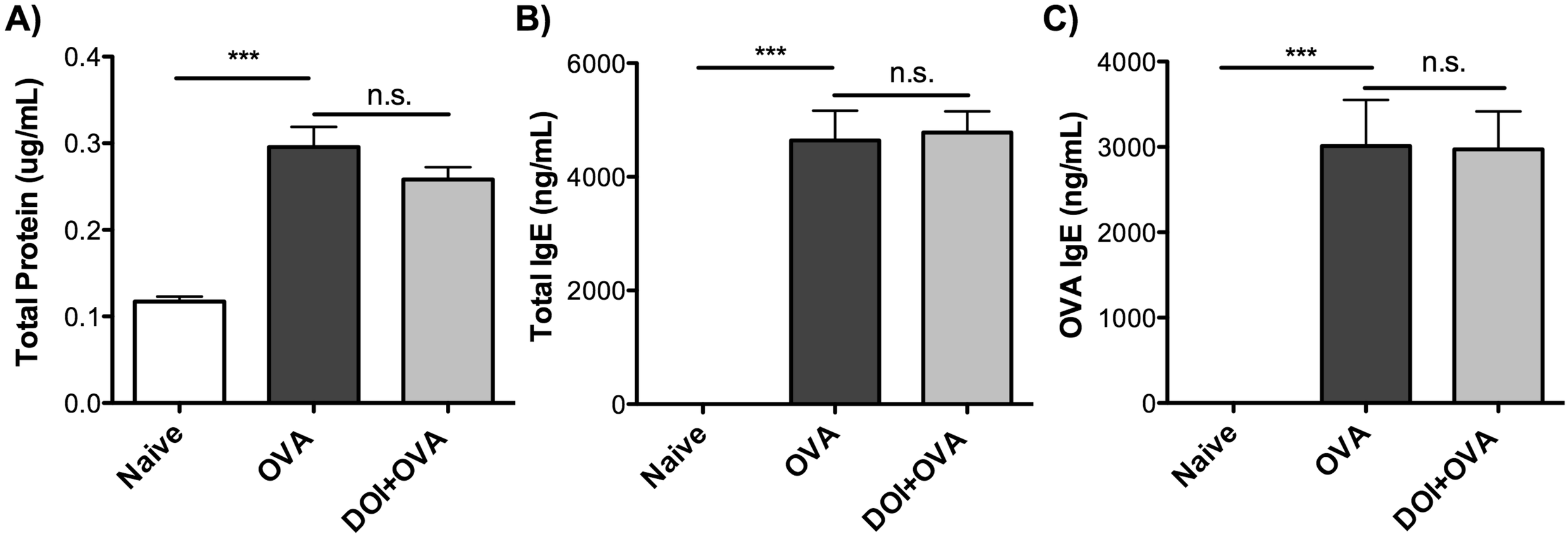
A)

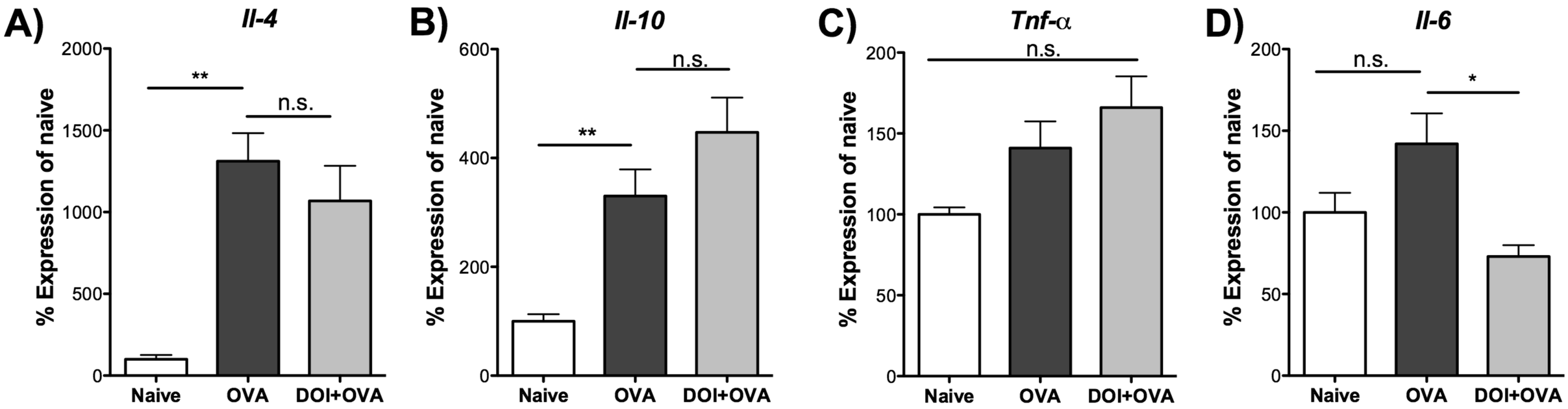

% PAS Positive Cells in Airway
120
110
100
90
80
70
60
50
40
30
20
10
0
*
^
Naive
OVA
DOI+OVA

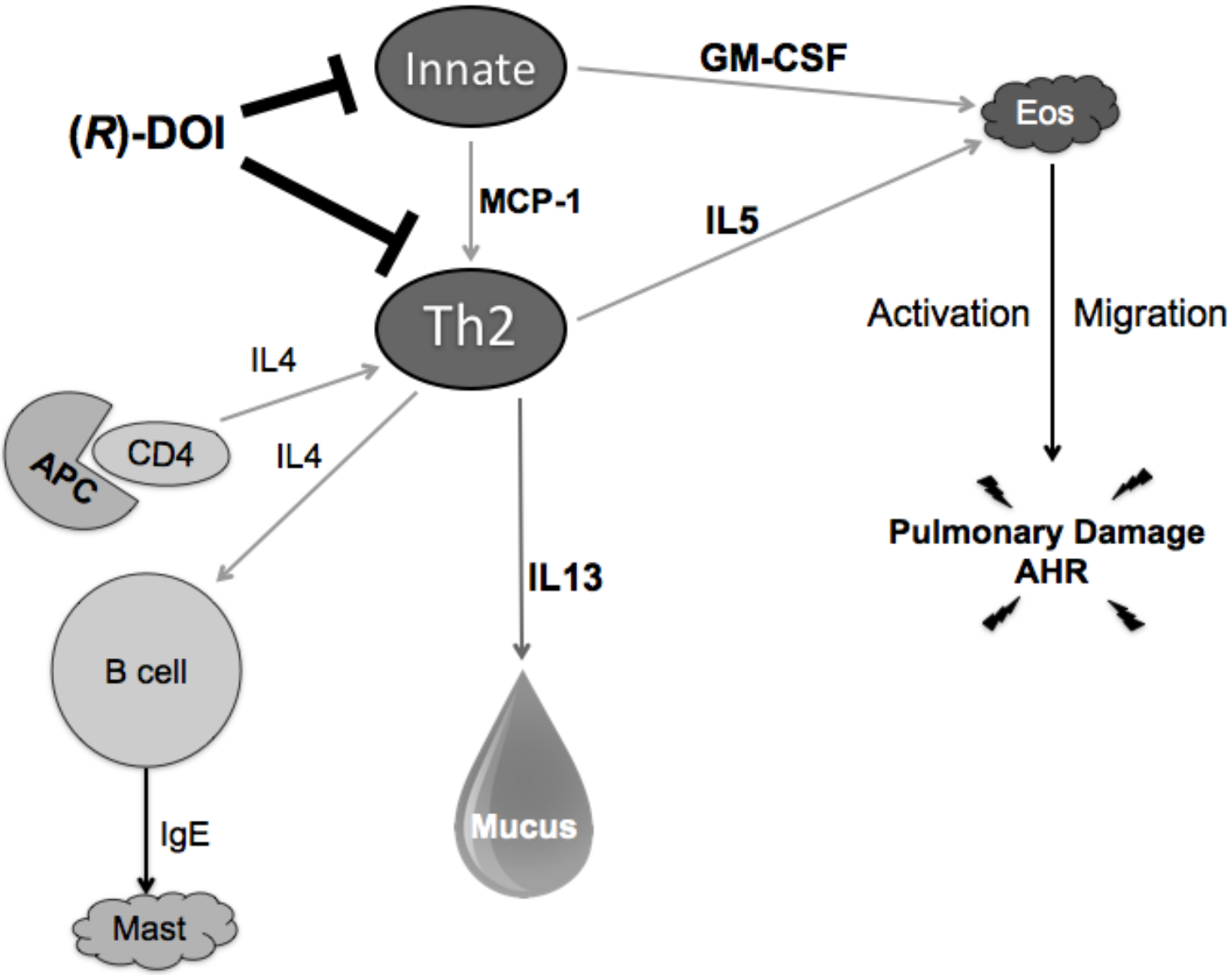
B)


% PAS Positive Cells in Airway
120
110
100
90
80
70
60
50
40
30
20
10
0
*
#
Naive
OVA
DOI+OVA


Total Cells (10e5)
0
1
2
3
4
5
Naive
OVA-only
0.01mg/kg DOI+OVA
1.0 mg/kg DOI + OVA
*
#
#
*
#
#
Total
Eos
Macs
Lymphs
Neutros


A)
Total Protein (ug/mL)
0.4
0.3
0.2
0.1
0.0
***
n.s.
Naive
OVA
DOI+OVA
B)
Total IgE (ng/mL)
6000
4000
2000
0
***
n.s.
Naive
OVA
DOI+OVA
C)
OVA IgE (ng/mL)
4000
3000
2000
1000
0
***
n.s.
Naive
OVA
DOI+OVA


A)
Il-4
% Expression of naive
2000
1500
1000
500
0
**
n.s.
Naive
OVA
DOI+OVA
B)
Il-10
% Expression of naive
600
400
200
0
**
n.s.
Naive
OVA
DOI+OVA
C)
Tnf-α
% Expression of naive
200
150
100
50
0
n.s.
Naive
OVA
DOI+OVA
D)
Il-6
% Expression of naive
200
150
100
50
0
n.s.
*
Naive
OVA
DOI+OVA
E)
Mcp-1
% Expression of naive
600
400
200
0
**
*
Naive
OVA
DOI+OVA
F)
Il-13
% Expression of naive
25000
20000
15000
10000
5000
0
**
*
Naive
OVA
DOI+OVA
G)
Il-5
% Expression of naive
1000
800
600
400
200
0
***
*
Naive
OVA
DOI+OVA
H)
Gm-csf
% Expression of naive
800
600
400
200
0
***
***
Naive
OVA
DOI+OVA


Innate
GM-CSF
(R)-DOI
Eos
MCP-1
IL5
Th2
Activation
Migration
IL4
APC
CD4
IL4
Pulmonary Damage
AHR
IL13
B cell
Mucus
IgE
Mast