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The absence of the VPAC₂ receptor does not protect mice from *Aspergillus* induced allergic asthma

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Abstract

Allergic asthma is a T_H2-mediated disease marked by airway inflammation, increased mucus production, and elevated serum IgE in response to allergen provocation. Among its ascribed functions, the neuropeptide Vasoactive Intestinal Peptide (VIP) is believed to promote a T_H2 phenotype when signaling through its VPAC₂ receptor. In this study, we assessed the requirement for the VIP/VPAC₂ axis in initiating the allergic pulmonary phenotype in a murine model of fungal allergic asthma. C57BL/6 wild-type (WT) and VPAC₂ knock-out (KO) mice were sensitized with *Aspergillus fumigatus* antigen and challenged with an aerosol of live conidia to induce allergic airways disease. WT and KO mice exhibited similar peribronchovascular inflammation, increased number of goblet cells, and elevated serum IgE. However, the absence of VPAC₂ receptor resulted in a marked enhancement of *MUC5AC* mRNA with an associated increase in goblet cells and a reduction in eosinophils in the airway lumen at day 3 when *VIP* mRNA was undetectable in the KO lung. Sustained elevation of serum IgE was noted in KO mice at day 14, while the level in WT mice declined at this time point. These data suggest that the absence of VPAC₂ does not protect mice from developing the signs and symptoms of allergic asthma.

Keywords

Vasoactive intestinal peptide; Matrix metalloproteinase-2; Eosinophil; Vipr2; Allergy; Asthma

1. Introduction

Allergic asthma is a pulmonary syndrome characterized by bronchial inflammation, hyperresponsiveness, excessive mucus production, remodeling, and increased serum IgE in response to allergen provocation. Exposure to the triggering allergen results in the release of chemotactic agents to recruit eosinophils [14] and T_H2-type lymphocytes [39], the hallmark leukocytes associated with allergic asthma.

Vasoactive intestinal peptide (VIP) is a ubiquitous, pleiotropic neuropeptide with cytokine properties, produced by cells of the immune system such as macrophages, eosinophils,

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neutrophils, T and B lymphocytes [32]. Known effects of VIP include, broncho- and vasodilation [29], inhibition of smooth muscle cell proliferation [23], and regulation of leukocyte migration [2]. VIP is abundant in the lung, and immune cells contribute to the amounts produced by the non-adrenergic non-cholinergic innervation [22]. The observations that VIP availability is decreased in patients with asthma [4,25], and that increased airway inflammation and hyperresponsiveness occur in the absence of VIP [33], have led to the recognition of VIP as an “anti-asthma” factor.

VIP can function via two G protein-coupled receptors VPAC₁ and VPAC₂. Most cells of the immune system express both VPAC₁ and VPAC₂, and macrophages and T cells change their receptor ratio in favor of VPAC₂ upon activation [10]. However, the cell type that produces and responds to VIP, as well as the temporal and spatial expression of its receptors, dictate the outcome of the immune response.

Transgenic mice with constitutively upregulated T cell expression of human VPAC₂ have been shown to have increased serum IgE and eosinophils [37], supporting the role of the VIP/VPAC₂ axis in skewing the immune balance in favor of T_H2. In contrast, VPAC₂ null mice have increased delayed-type hypersensitivity marked by decreased serum IgE and eosinophilia [15] indicating that VPAC₂ may be VIP’s “pro-allergy” receptor.

In the current study, we hypothesized that in the absence of VPAC₂, mice would show the amelioration of the hallmark signs and symptoms of allergic asthma in an *Aspergillus fumigatus*-induced murine model system. Although VPAC₂ null mice had a delay and reduction in eosinophils, the absence of VPAC₂ did not protect these mice from developing the characteristics of allergic asthma marked by increased serum IgE and goblet cell metaplasia. To our knowledge, this is the first report to determine the outcome of VPAC₂ KO animals subjected to a pulmonary allergy model. These findings contribute to our understanding of VIP and its receptors in allergic asthma, emphasizing that the absence of a single VIP receptor, VPAC₂, does not result in the amelioration of asthma symptoms.

2. Materials and Methods

2.1. Animals

Heterozygous VPAC₂ null (KO) mice on C57BL/6 background were a kind gift from Dr. Anthony Harmor, University of Edinburgh, UK. These were crossed to achieve homozygous animals which were used for the study. C57BL/6 mice were purchased from Jackson Laboratories, Bar Harbor, ME, USA. Animals were housed on Alpha-dri™ paper bedding (Shepherd Specialty Papers, Watertown, TN, USA) in microfilter-topped cages (Anicare, Bellmore, NY, USA) in a specific pathogen-free facility with ad libitum access to food and water. The study described was performed in accordance with IACUC guidelines of North Dakota State University.

2.2. Fungal antigen and *Aspergillus fumigatus* culture and aerosol inoculation system

Soluble *Aspergillus fumigatus* antigen was purchased from Greer Laboratories (Lenoir, NC, USA) and fungal culture stock (strain NIH 5233) was purchased from American Type Culture Collection (Manassas, VA, USA). All experimental procedures utilizing *A. fumigatus* were conducted with prior approval of the Institutional Biological Safety Office of North Dakota State University. A single lyophilized *A. fumigatus* culture was reconstituted per ATCC recommendations, and 60- μ l aliquots of the suspension were stored at 4°C until use.

An aerosol inoculation chamber with nose ports for three mice was fitted with an inline 25-cm² culture flask in which a mature (8-day-old), *A. fumigatus* culture had been grown on Sabouraud Dextrose Agar. Airflow through the culture flask was delivered at 2 psi to liberate

the hydrophobic spores and allow their delivery through the chamber, which was housed in a Class II biosafety cabinet for inhalation experiments. The ports were plugged and the chamber was coated with airborne conidia for 10-min prior to animal challenge. The fungal culture was changed with each new set of mice.

2.3. Allergen sensitization and allergen challenge by aerosol delivery

Animals were sensitized with a modification of Hogaboam's published protocol [18]. Briefly, 10 µg of *A. fumigatus* antigen (Greer Laboratories) in 0.1 ml normal saline (NS) mixed with 0.1 ml of Imject[®] Alum (Pierce, Rockford, IL, USA) was injected subcutaneously (0.1 ml) and intraperitoneally (0.1 ml). Two weeks later, mice received the first of three weekly intranasal sensitizations with 20 µg of *A. fumigatus* antigen in 20 µl of NS.

One week after the last sensitization, mice were anesthetized with a cocktail of Ketamine (75 mg/kg) and Xylazine (25 mg/kg) and challenged with a 10-min, nose-only, aerosol exposure to live *A. fumigatus* spores. Each anesthetized animal was placed supine with its nose in an inoculation port inhaling the fungal spores for 10 min, constituting the allergen challenge. Two weeks following the first allergen challenge, mice were subjected to a second 10-min aerosol fungal challenge. Naïve animals were neither sensitized nor challenged. After the second allergen exposure, sensitized and challenged mice were separated into groups of five for analysis at days 3, 7, and 14.

2.4. Serum sample collection and bronchoalveolar lavage

Approximately 500 µl of blood was removed from each mouse via ocular bleed and centrifuged at 13,000 ×g for 10 min to yield sera which was stored at -20°C until use. Bronchoalveolar lavage (BAL) was performed through a tracheostomy tube on each mouse with 1.0 ml of sterile NS. BAL contents were centrifuged at 3000 ×g for 10 min to separate the cells from the fluid. The BAL fluid was stored at -20°C until use, and cells were used for morphometric analysis (see below). Left lungs were harvested and fixed in 10% neutral buffered formalin for histological analysis. Right lungs were snap frozen in liquid N₂ and stored at -20°C for RNA extraction and analysis.

2.5. Morphometric and histological analysis

BAL cells were resuspended in 0.2 – 1.0 ml of NS and cytopun (Shandon Scientific, Runcorn, UK) onto microscope slides and differentially stained (Quick-Dip stain, Mercedes Medical, Sarasota, FL, USA). Cells from five randomly chosen high-powered fields (HPFs) were counted to determine the mean number of each cell type in the airway lumen of each mouse.

Formalin-fixed, paraffin-embedded lungs were cut longitudinally across the coronal plane in 5-µm sections and stained with hematoxylin and eosin (H&E) to assess inflammation or with periodic acid Schiff's (PAS) stain for goblet cells. Peribronchovascular eosinophils (identified by eosin stained cytoplasm) were counted in 50 randomly selected HPFs by an investigator blind to the treatments and sample groups. Results were reported as the mean per 50 HPFs.

2.6. Quantification of serum IgE and matrix metalloproteinase (MMP)-2 in BAL fluid

The amount of total IgE in sera (BD OptEIA, San Diego, CA, USA) and MMP-2 in BAL fluid (Quantikine kit for MMP-2, R&D Systems, MN, USA) were quantified via specific ELISA according to manufacturers' guidelines. Serum samples were diluted 1/100 and BAL fluid samples were diluted 1/10.

2.7. Real-time qPCR analysis of mean fold change in gene expression

RNA was extracted from lung tissue with Trizol[®] reagent (Invitrogen, Carlsbad, CA, USA), and 2 µg of DNase treated RNA was used for cDNA synthesis. All necessary reagents for DNase treatment and cDNA synthesis (DNase, random primers, M-MLV reverse transcriptase, dNTPs) were purchased from Promega (Madison, WI, USA). RNA-specific QuantiTect primer assays for *MUC5AC*, *VIP*, *VPAC₁*, *VPAC₂* and for the internal control, *HPRT-1*, (Qiagen, Valencia, CA, USA) were used in a Sybr-Green Fast PCR master mix (Qiagen) reaction and run on an ABI-7500 real-time PCR machine (Applied Biosystems, Carlsbad, CA, USA). The $2^{-\Delta\Delta CT}$ method was used to calculate the relative fold change in gene expression of all samples standardized to naïve controls.

2.8. Statistical analysis

Allergic WT and KO mice were compared against their respective naïve controls and against one another at each time point. An unpaired, Student's two tailed *t*-test with Welch's correction was used to determine statistical significance with Prism GraphPad software (San Diego, CA, USA). All results are expressed as mean ± SEM. *p* < 0.05 was considered statistically significant.

3. Results

The predominant differences between the WT and *VPAC₂* KO mice occurred three days after the fungal challenge. At day 3, the WT had a 4.5-fold induction of *VIP* mRNA in the lung, approximately 400 eosinophils/HPF in the airway lumen, and a 300-fold induction of *MUC5AC* mRNA. In comparison, the *VPAC₂* KO mice had no detectable *VIP* mRNA expression in the lung, about 160 eosinophils/HPF in the airways, and a 750-fold increase in *MUC5AC* mRNA expression.

3.1. The absence of *VPAC₂* altered the dynamics of leukocyte recruitment and egression

In order to determine whether the absence of *VPAC₂* played a role in leukocyte recruitment into the sensitized and challenged lungs, we differentially stained and counted the cells extracted via bronchoalveolar lavage (BAL) from WT and *VPAC₂* KO mice at each time point. The naïve *VPAC₂* KO mice had approximately the same BAL cell profile as the naïve WT mice (Fig 1). All four types of leukocytes analyzed (monocytes/macrophages, neutrophils, eosinophils, lymphocytes) were significantly increased in the airways as a result of allergen exposure in both WT and KO mice at all time points (Fig 1). Fewer monocytes/macrophages were found in the airways of KO mice at each time point compared to WT (Fig 1A). Neutrophils were the least abundant in allergic airways (~2–5/HPF) in allergen exposed mice of both groups (Fig 1B). The peak in lymphocytes occurred in the both groups at day 7 at which point the number of lymphocytes in the airways of KO was 1.75-fold higher than that of the WT (Fig 1C).

Eosinophils are the hallmark leukocyte of allergic asthma. We counted the number of eosinophils in the lungs and in the BAL contents based on the eosin staining of the cytoplasmic granules. *VPAC₂* KO mice appeared to have a delay in eosinophil recruitment into the lung, and a delay and reduction in eosinophil migration into the airway lumen (both peaking at day 7) compared to WT mice which had rapid eosinophil recruitment and egression (Fig 1D and E). MMP-2 advocates leukocyte migration into the airway lumen [5]. Therefore, we measured the amount of MMP-2 available in the BAL fluid in WT and *VPAC₂* KO mice. The peak in MMP-2 in the WT occurred at day 3 (Fig 1F), corresponding to the leukocyte peaks in the BAL (Fig 1). MMP-2 peaked in the BAL of KO mice at day 7 (Fig 1F) at which time monocytes/macrophages, lymphocytes and eosinophils also peaked in the airway lumen (Fig 1A, C, and E).

3.2. The absence of VPAC₂ did not result in a difference in lung inflammation compared to WT mice

We examined the amount of peribronchovascular inflammation in the lung tissue of allergen exposed animals in comparison to naïve controls with H&E stained tissue sections. Naïve animals of both the WT and VPAC₂ KO genotypes had no signs of airway inflammation (Fig 2A and B). Both groups of mice showed massive influx of leukocytes at all time points analyzed (Fig 2C-H). Eosinophils were marginated at the basal ends of the airway epithelia in both groups at all time points although decreasing in number with time (insets, Fig 2C-H). Both WT and KO mice had smooth muscle cell hyperplasia around the blood vessels as a result of allergen sensitization and challenge. However, this characteristic was more evident in the KO mice (Fig 2F). The latest time point (day 14) was still marked by considerable inflammation in both the WT and KO lungs (Fig 2G and H).

3.3. Serum IgE was increased and sustained in high amounts in the VPAC₂ KO

Serum IgE is a hallmark of allergic asthma. We measured the increase in total IgE in response to allergen exposure in the sera of WT and KO mice to determine whether the absence of the VPAC₂ receptor would decrease IgE production (Fig 3). All allergen exposed animals at each time point had significantly elevated IgE in the sera compared to naïve controls of both genotypes (Fig 3). Both WT and KO mice at days 3 and 7 produced equivalent amounts of IgE (Fig 3). However, KO mice sustained elevated levels of IgE even at day 14 which was in stark contrast to that of the WT which showed a decreasing trend by this time point (Fig 3).

3.4. The absence of VPAC₂ receptor resulted in enhanced goblet cell metaplasia

Goblet cell (GC) metaplasia and associated increase in mucus production can lead to airway obstruction in allergic asthma. We measured this phenomenon by counting the number of GCs interspersed in the airway columnar epithelium as well as by the induction of *MUC5AC* in the lung. Naïve animals of both groups did not have PAS stained mucus producing GCs while allergen sensitized and challenged animals had marked GC metaplasia marked by PAS staining of mucus globules (Fig 4). Allergen exposed WT and KO mice had increased number of GCs in the airways at all time points analyzed, although KO mice had significantly more GCs at early time points compared to the WT (Fig 4I). This trend was also observed in the fold induction of *MUC5AC* (Fig 4J). The mRNA for *MUC5AC* was approximately 2.5-fold higher in the KO at day 3 compared to their WT counterparts (Fig 4J).

3.5. Allergen sensitization and challenge resulted in altered VIP and VPAC gene expression

We measured the mRNA of *VIP* and *VPAC* in allergen sensitized and challenged WT and KO in comparison to their respective naïve controls to determine whether the changes in allergic phenotype could be due to altered expression of *VIP* and *VPAC*. While WT animals had a 4.5-fold upregulation of *VIP* mRNA (Fig 5A), KO mice had undetectable levels of *VIP* mRNA at day 3 (Fig 5B), which was the only time point at which *VIP* mRNA expression was significantly different between the WT and KO. The trend in the induction of *VPAC₁* between the allergen exposed WT and KO mice was similar, marked by an upregulation at day 7 and a downregulation at day 14 (Fig 5A and B). Interestingly, allergen exposed WT mice had significantly downregulated expression of *VPAC₂* at all time points analyzed (Fig 5A).

4. Discussion

The hallmark characteristics of allergic asthma such as airway inflammation, hyperresponsiveness, excessive mucus production and increased serum IgE were represented in mice subjected to the *A. fumigatus* aeroallergen. *VIP* is a neuropeptide with a broad spectrum of functions which vary based on the immune cell and its expression of receptors. Contrary to

previous reports in the literature showing that the VPAC₂ knock-out animals have decreased immediate-type hypersensitivity reactions [15], we found that the VPAC₂ KO mice were susceptible to the development of allergic asthma exhibiting airway inflammation, goblet cell metaplasia, and elevated serum IgE.

Numerous reports indicate VIP's ability to impact leukocyte migration [2,11,13], inhibit inflammation [33], inhibit airway hyperresponsiveness [33] and smooth muscle cell proliferation [23], and promote a T_H2 phenotype [38]. Asthmatics have more eosinophils in the inner airways than the outer airways; however, those in the outer airways are more highly activated [17]. Therefore, the migration of eosinophils into the airway lumen is important in minimizing the damage that can be induced by activated eosinophils in peribronchial areas. Although eosinophils are recognized as producers of VIP [1,24], VIP's direct impact on eosinophil migration is limited to one report by Dunzendorfer et al., emphasizing its importance in promoting the migration of eosinophils via VPAC₁ [11]. In the absence of *VIP* (day 3), luminal eosinophils are markedly reduced in the VPAC₂ KO mice. The restoration of *VIP* mRNA to WT-equivalent levels by day 7 in our model also restored eosinophil migration into the airway lumen in the KO, indicating that it is not the absence of the VPAC₂ receptor, rather the absence of its ligand (*VIP*) that causes a delay and reduction in eosinophil migration into the airway lumen. Consequently, the VIP/VPAC₁ axis may mediate eosinophil migration to the airway lumen in this model system. Furthermore, VIP has been proposed to be an endogenous modulator of nuclear factor of activated T cells (NFAT) in the lungs [28]. Mice deficient in NFAT produce more IL-4 and IL-5 and have increased eosinophils in the BAL, indicating that the absence of NFAT promotes a T_H2 phenotype [36]. In addition, the translocation of NFAT on T cells is inhibited by VIP/VPAC₂ signaling [8]. Therefore, the reduction in BAL eosinophils in the KO may result from the absence of the VIP/VPAC₂ modulation of NFAT in T cells.

Matrix metalloproteinase-2 (MMP-2) is also an important mediator of leukocyte migration. The retention of leukocytes in the lung tissue can lead to asphyxiation, and has been shown to be a cause of death in patients with asthma [35]. Animals lacking MMP-2 subjected to an asthma model died of asphyxiation resulting from inhibited leukocyte (predominantly eosinophils) egression into the airway lumen [5]. Naïve mice with a targeted deletion in *VIP* have increased MMP-2 in the BAL [33], and *VIP* has been shown to downregulate MMP-2 in a model of rheumatoid arthritis [7]. However, in our model, WT and KO mice showed no difference in MMP-2 availability in the BAL fluid or its mRNA expression in the lung (data not shown). Hence, the absence of VPAC₂ did not alter MMP-2 expression in the lung in this model of allergic asthma.

Pulmonary inflammation between the WT and VPAC₂ KO animals was similar. The accumulation of leukocytes in the lungs of both WT and KO groups in this model implies that, in the absence of VPAC₂, *VIP* may not provide an anti-inflammatory protection mechanism in allergic asthma. While both WT and KO mice exhibited vascular smooth muscle hyperplasia, this feature was more apparent in the VPAC₂ KO. *VIP*-deleted mice have vascular smooth muscle cell proliferation [30]. Together, this indicates that *VIP* is important in vascular integrity and that this function may be mediated through the VPAC₂ receptor.

Constitutive production of mucins by GCs maintains the mucus layer for clearance of inhaled particles through ciliary movement which is promoted by *VIP* [31]. Goblet cell metaplasia can be induced by T_H2 cytokines [27], and the VIP/VPAC₂ axis promotes a T_H2 response as reported in the VPAC₂ transgenics [37]. Of the 20 mucin genes expressed in the lung, *MUC5AC* predominates in the airway mucus [34] and can be used as a marker of GC metaplasia. *VIP* is directly linked to the increase in *MUC2* expression [19] and increases the expression of mucin genes in cultured colon epithelial cells through VPAC₁ [20]. However,

to our knowledge, its effect on *MUC5AC* has not been previously investigated or reported for pulmonary allergy. When *VIP* mRNA was undetectable in the *VPAC₂* KO lungs, there were more GCs in the airways, accompanied by a 2.5-fold greater induction of *MUC5AC* mRNA compared to WT, decreasing the likelihood that the *VIP/VPAC₁* axis mediates *MUC5AC* induction in the lung.

Elevated serum IgE is an important hallmark of allergic asthma that is used to diagnose the disease in humans. Contrary to previous reports that indicate the reduction of serum IgE in the *VPAC₂* KO mouse [15], we have shown that these mice respond to allergen challenge with the same vigor in IgE production as their WT counterparts and elevated levels were sustained longer than in the WT. The differences in our data from that of Goetzl et al. may be due to the disparity in the model systems employed [15]. This indicates that the *VIP/VPAC₂* axis is not important in mediating IgE production, in fact the absence of *VPAC₂* may have contributed to the maintenance of serum IgE possibly by initiating the survival of activated *T_H2* cells [9] to induce B cell activation and class switching. Since *VIP* has been shown to be important in these mechanisms, perhaps its function is predominantly through *VPAC₁* rather than *VPAC₂*.

The predominant differences between the WT and *VPAC₂* KO animals occurred at a time in which *VIP* mRNA was not detected in the KO. This emphasizes the importance of the ligand (*VIP*) in altering the immune response in pulmonary allergy. *VIP*'s anti-inflammatory [6,12,22,26] and anti-asthmatic [16,21,23,29,33] properties have been clearly demonstrated in the literature. Since multiple receptors (*VPAC₁*, *VPAC₂*, *PAC₁* [3]) through which *VIP* can function are available in the lung, one cannot dismiss the possibility that the absence of one receptor may be compensated by the presence of another.

It is apparent that *VIP*'s role in immunoregulation is complicated and dependent on the disease state, tissue, and environmental factors. In this allergic asthma model system, mice lacking the *VPAC₂* receptor responded with pathology that is similar to that of WT mice including increased serum IgE, eosinophilic inflammation, and GC metaplasia with associated *MUC5AC* induction. However, KO mice had a delay and reduction in eosinophil egression into the airway lumen and a dramatic upregulation of *MUC5AC* when *VIP* mRNA was undetectable in the lung. This indicates that *VPAC₂* is necessary for *VIP* to promote eosinophil migration and downregulate GC metaplasia thereby promoting lung homeostasis by ridding the tissue of a potentially harmful leukocyte and maintaining the integrity of the airway columnar epithelium in this murine model system of allergic asthma. In summary, the absence of the *VIP/VPAC₂* axis does not protect mice from developing an allergic phenotype.

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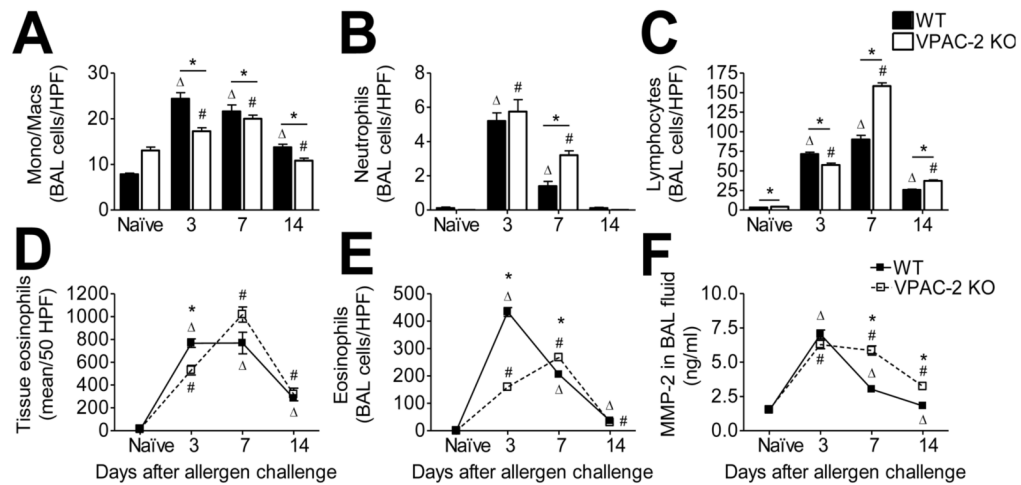


Figure 1.

Leukocyte infiltration was increased as a result of allergen challenge in both WT and KO groups. Monocytes/macrophages (A), neutrophils (B), lymphocytes (C), and eosinophils (E) in cytospun bronchoalveolar lavage (BAL) contents were differentially stained and counted. Peribronchovascular eosinophils were counted in 50 random HPFs in H&E stained sections (D). Total MMP-2 in the BAL was quantified via ELISA (F). All data are represented as the mean \pm SEM, n=5 mice per group. Δ denotes p<0.05 of allergen exposed WT animals compared to their naïve controls, while # denotes p<0.05 of allergen exposed KO animals compared to their naïve controls. * indicates p<0.05 between WT and KO.

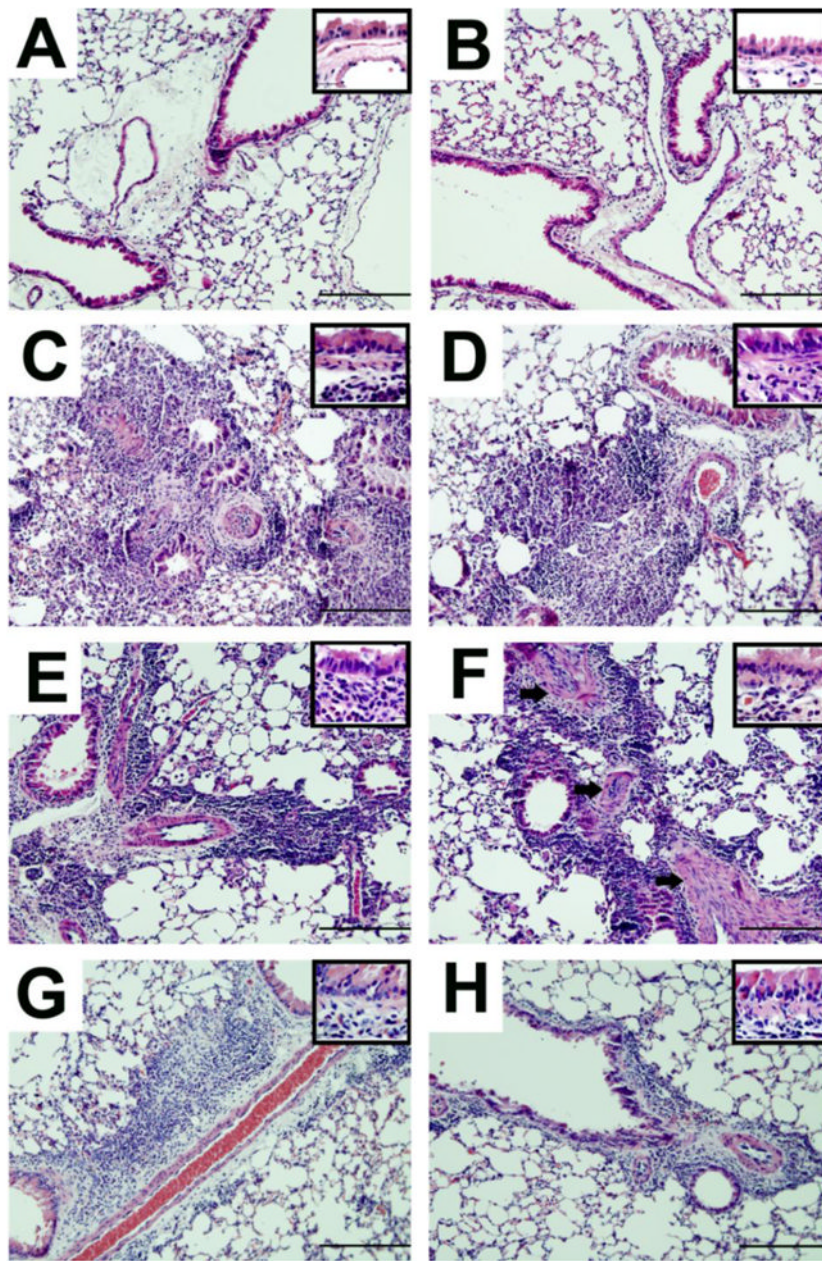


Figure 2.

Representative photomicrographs of H&E-stained whole lung sections of naïve and allergen-challenged WT (left column) and VPAC₂ KO (right column) mice. Naïve mice in both groups did not show inflammation (WT, A; KO, B). Peribronchovascular accumulation of leukocytes was prominent in both groups at day 3 (WT, C; KO, D). Inflammatory cell recruitment was still substantial at day 7 in both groups (WT, E; KO, F), and although slightly subsided continued well into day 14 (WT, G; KO, H). Eosinophils were absent in naïve lungs, but evident in allergic lungs seen marginated at the basolateral ends of the columnar airway epithelia in both groups (insets, C-H). Thickening of the blood vessel walls was observed in both groups, although this was more prominent in the KO (arrows, F). Scale bars = 200 μm, insets = 50 μm

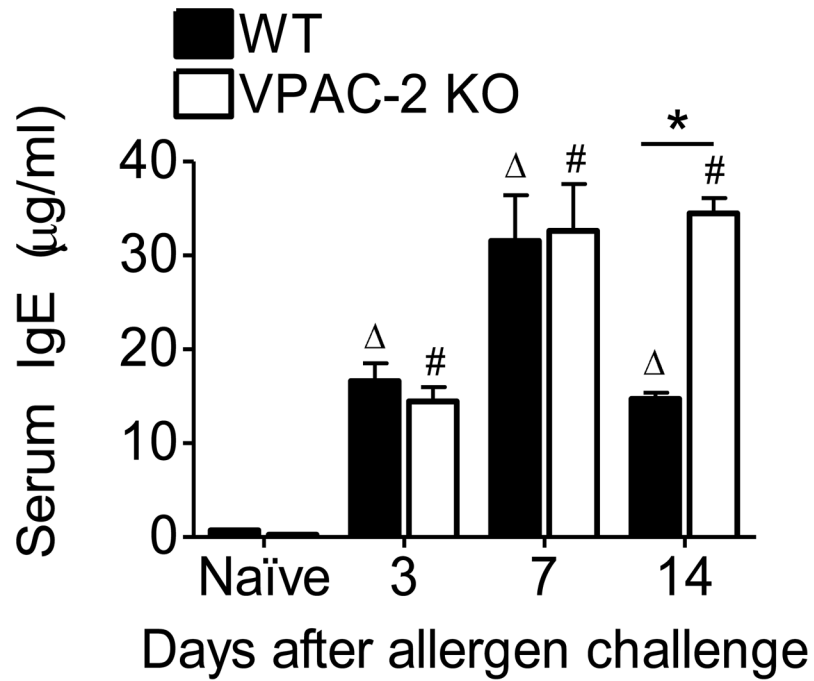


Figure 3.

Elevated IgE was evident in the sera of WT and VPAC₂ KO mice after allergen exposure. All data are represented as the mean \pm SEM, n=5 mice per group. Δ denotes p<0.05 of allergen exposed WT animals compared to their naïve controls, while # denotes p<0.05 of allergen exposed KO animals compared to their naïve controls. * indicates p<0.05 between WT and KO.

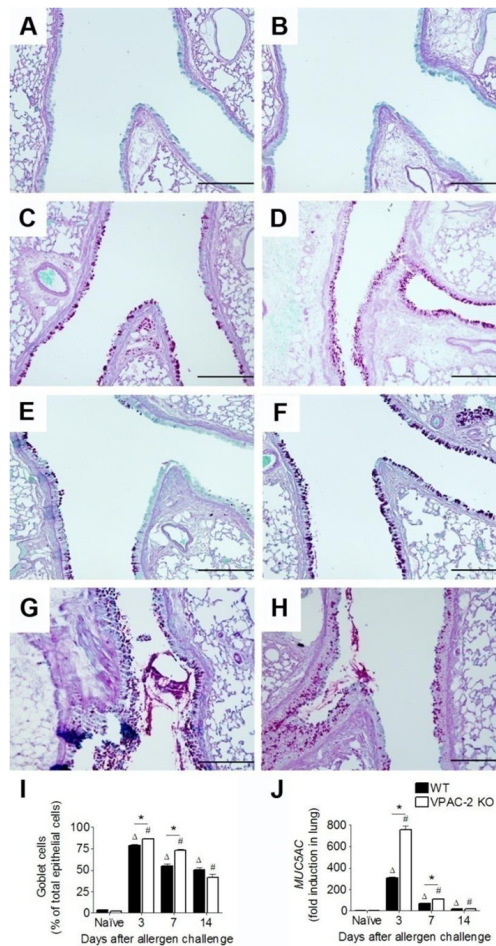


Figure 4.

Representative photomicrographs of PAS-stained whole lung sections of naïve and allergen exposed WT (left panel) and VPAC₂ KO (right panel) mice. Goblet cells (GCs) were scarce in naïve animals of WT (A) and KO (B) groups. GCs and mucus were evident in the airways of both groups at days 3 (C & D), 7 (E & F), and 14 (G & H). GCs were counted and reported as the percentage of total epithelial cells that lined the airways (I). The induction of *MUC5AC* mRNA in lung tissue was measured via real-time qPCR and reported standardized to naïve controls using the $2^{-\Delta\Delta C_t}$ method. While both groups had *MUC5AC* induction in the allergic state, a marked difference between WT and KO levels occurred at day 3 (J). All data are represented as the mean \pm SEM, n=3–5 mice per group. Δ denotes p<0.05 of allergen exposed WT animals compared to their naïve controls, while # denotes p<0.05 of allergen exposed KO animals compared to their naïve controls. * indicates p<0.05 between WT and KO. Scale bars = 200 μ m

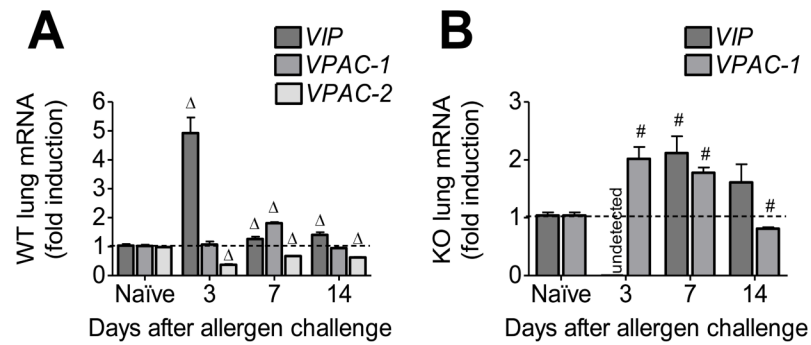


Figure 5.

Vasoactive intestinal peptide (*VIP*) and *VPAC* receptor mRNA in whole lung of WT (A) and *VPAC*₂ KO (B) standardized to naïve controls using the $2^{-\Delta\Delta C_t}$ method. *VIP* was markedly upregulated in WT at day 3 (A) while it was undetected in KO (B). Both groups had a similar expression pattern for *VPAC*₁ (A & B), and the WT had a marked downregulation of *VPAC*₂ at all time points analyzed (A). All data are represented as the mean \pm SEM, n=3 mice per group. Δ denotes p<0.05 of allergen exposed WT animals compared to their naïve controls, while # denotes p<0.05 of allergen exposed KO animals compared to their naïve controls.