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The absence of $VPAC_2$ leads to aberrant antibody production in *Aspergillus fumigatus* sensitized and challenged mice

Amali E. Samarasinghe[a], **Scott A. Hoselton**[a], and **Jane M. Schuh**[a]

Amali E. Samarasinghe: a.samarasinghe@ndsu.edu; Scott A. Hoselton: Scott.Hoselton@ndsu.edu; Jane M. Schuh: Jane.Schuh@ndsu.edu

[a] North Dakota State University, Department of Veterinary and Microbiological Sciences, PO Box 6050-Dept 7690, Fargo, ND 58108

Abstract

Vasoactive intestinal peptide (VIP) facilitates a “pro-allergy” phenotype when signaling through its G protein-coupled receptor, $VPAC_2$. We have shown that $VPAC_2$ knock-out (KO) mice developed an allergic phenotype marked by eosinophilia and elevated serum IgE. Therefore, we hypothesized that the humoral response to allergen challenge in these mice was T_H2 dominant similar to wild-type (WT) C57BL/6 mice. Antibody responses in WT and KO mice were measured after *Aspergillus fumigatus* conidia inhalation. In contrast to previous reports, basal levels of serum IgG_{2a} and IgA were significantly higher in naïve $VPAC_2$ KO animals. Antibody availability in the serum as well as the bronchoalveolar lavage fluid after fungal challenge was dominated by the pro-inflammatory isotype IgG_{2a} and the mucosal isotype, IgA. IgA localizing cells dominated in the peribronchovascular areas of allergic KO mice while IgE immune complexes were found in WT allergic lungs. This research shows for the first time that $VPAC_2$ has a significant effect on antibody regulation, in the context of allergy.

Keywords

VIP; IgG_{2a}; IgA; Asthma

1. Introduction

Allergic asthma is a debilitating T_H2 mediated pulmonary syndrome marked by acute exacerbations of reversible airway constriction and inflammation triggered by the inhalation of a sensitizing agent, which may result in permanent airflow dysfunction due to chronic airway wall remodeling. A distinct neurological component, including the attendant inflammation [35], cough [22], airway hyperresponsiveness [24,25,29], and mucus production [26], is associated with asthma symptoms. Indeed, dysfunction of pulmonary

Correspondence. Amali E. Samarasinghe, North Dakota State University, Department of Veterinary and Microbiological Sciences, P. O. Box 6050 – Dept 7690, Fargo, ND 58108-6050, Phone: (701) 231-7696, Fax: (701) 231- 9692.

Disclosures
The authors declare no conflict of interest.

Author contributions
A.E.S. and J.M.S. designed the study; A.E.S. and S.A.H. performed the research; A.E.S. analyzed the data; A.E.S. wrote the paper; A.E.S., S.A.H., and J.M.S. edited the paper.

nervous responses has been implicated as a causative or an aggravating factor in the immunomodulation of allergic asthma [32,37]. Neuropeptides, such as vasoactive intestinal peptide (VIP), are supplied to peripheral organs like the lungs by an abundant supply of synapsing nerves [2]. Additionally, macrophages and lymphocytes contribute to the local production of "neuropeptides" in lymphoid and other organs and respond to these mediators in an autocrine/paracrine manner. While the availability of neuropeptides in the lung is indisputable, their role in the communication between the nervous and immune networks is complicated and has not been fully elucidated.

Originally identified in 1970 [33], VIP has emerged as an important neuropeptide for its anti-inflammatory effects on immune cells. Acting through at least two G-protein coupled receptors in the lung, $VPAC_1$ [36] and $VPAC_2$ [28], VIP regulates leukocytes by hindering pro-inflammatory mediator release. VIP augments the T_H2 phenotype by: (i) acting directly and indirectly on antigen presenting cells to preferentially recruit T_H2 cells, (ii) promoting the generation of T_H2 cells, and (iii) enhancing the survival and proliferation of existing T_H2 cells [31]. The VIP/$VPAC_2$ axis on $CD4^+$ T cells promotes T_H2 cytokine production inhibiting the production of classic T_H1 cytokines [38]. The strength and duration of the T_H2 immune phenotype is enhanced by the auto-regulatory function of VIP produced by T_H2 cells [8]. While $VPAC_2$ transgenic mice exhibit increased IgE, eosinophilia, and decreased delayed-type hypersensititvity (DTH) [38]; $VPAC_2$ KO mice have an inflammatory phenotype with increased DTH and decreased immediate-type hypersensitivity (ITH) responses [15].

VIP is a potent bronchodilator and chemically modified VIP has been actively pursued as a potential therapeutic for use in asthma, yet the physiologic role of VIP and its receptors in the development and maintenance of allergic asthma has not been fully explored. Our recent studies using $VPAC_2$ KO mice showed that the absence of this receptor was not protective in the development of fungal allergy and that IgE was produced in the absence of $VPAC_2$ signaling [34]. This was interesting, since other investigators have reported that ITH is significantly decreased in $VPAC_2$ KO mice [15]. In this study, using an *Aspergillus fumigatus* induced murine model system of fungal asthma, we show that $VPAC_2$ KO mice have aberrant humoral responses to systemic and local allergen provocation.

2. Materials and Methods

2.1. Model of allergic airways disease

C57BL/6 mice (WT, Jackson Laboratories, Bar Harbor, ME) and $VPAC_2$ KO mice on a C57BL/6 background (heterozygous animals, a kind gift from Dr. Anthony Harmar, University of Edinburgh, UK, were bred to produce homozygous KO) were used for the study. All animals were housed in a specific pathogen-free facility in microisolator cages throughout the study and bedded on paper bedding, which harbors extremely low microbial contamination. As part of the IACUC-approved animal care protocol, sentinel animals that share bedding with the WT and KO animals were routinely tested for viral and bacterial profiles. None were detected. The study described was performed in accordance with IACUC and Institutional Biosafety Committee guidelines of North Dakota State University.

Animals were sensitized as previously described [34]. Briefly, 10 μg of *A. fumigatus* antigen (Greer Laboratories, Inc., Lenoir, N.C.) adsorbed in 0.2 ml of Alum 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 normal saline. Sensitized mice were challenged as previously described [34]. Briefly, one week after the final sensitization, anesthetized mice were subjected to two 10-min, nose-only exposure to live fungal conidia two weeks apart. Naïve animals were neither sensitized

nor challenged. Mice were separated into groups of five and euthanized at predetermined time points (days 3, 7, 14, and 28 after the second allergen exposure). Figure 1 provides a schematic representation of the allergen sensitization and challenge protocol.

2.2. Sample collection and determination of antibody concentration in sera and BAL fluid by ELISA

Approximately 500 μl of blood was collected by ocular bleed, and a bronchoalveolar lavage (BAL) was performed on each mouse with 1 ml of sterile saline. Left lungs were harvested and fixed in formalin for histological analysis. Blood harvested from each mouse was centrifuged at 15,000 × *g* for 10 min to obtain sera which was frozen at −20°C until use. BAL contents were centrifuged at 600 × *g* for 10 min to separate the cell ular component from the fluid. The BAL fluid was stored at −20°C until used.

To assess the affect of pro-inflammatory (T_H1-type) skewing on Ab production in the absence of VIP's "pro-allergy" receptor $VPAC_2$, and to determine baseline levels for allergic sensitization, we measured the concentrations of IgG_{2a} (pro-inflammatory), and IgA (mucosal), and IgE (pro-allergy) in sera and BAL fluid. IgE, IgG_{2a} (BD OptEIA, San Diego, CA), and IgA (Bethyl Labs, Montgomery, TX) were quantified with ELISA per manufacturer's protocols.

2.3. Immunohistochemical analysis of VIP, IgE, and IgA

Left lungs were inflated with 10% neutral buffered formalin and longitudinal 5-μm sections were cut across the coronal plane and used for immunohistochemical (IHC) analyses. Goat anti-rabbit VIP antibody (Abcam, Cambridge, MA) diluted at 1:20 was used with the anti-rabbit HRP-AEC tissue staining kit from R&D Systems (Minneapolis, MN) for VIP immunostaining. Goat anti-mouse IgA antibody and goat anti-mouse IgE antibody (Southern Biotech, Birmingham, AL) at 1:50 dilution with the anti-goat HRP-AEC tissue staining kit from R&D stained IgA and IgE red against the Gill III hematoxylin (Surgipath, Richmond, IL) blue counterstain.

2.4. Statistical analysis of data

All results are expressed as mean ± S.E.M. WT and KO mice at each time point were compared with Prism GraphPad software (San Diego, CA) using an unpaired Student's two tailed *t*-test with Welch's correction to determine statistical significance at predetermined time points after allergen challenge. $p < 0.05$ was considered statistically significant.

3. Results

Both groups of mice were demonstrated to develop an allergic phenotype against *A. fumigatus* conidia previously [34]. Based on this evidence, we hypothesized that $VPAC_2$ null mice would have similar humoral responses against allergen challenge compared to WT controls. In contrast to previous reports that demonstrated $VPAC_2$ null mice to have "normal basic immune characteristics" [15], we show that these mice have aberrant antibody responses in naïve and allergic states.

3.1. VIP localization in the naïve and allergic lungs was similar in WT and KO mice

Since VIP can serve as a regulator of leukocyte migration [4,10,17], and we have shown differences in VIP mRNA expression after allergen challenge between WT and KO lungs [34], we used immunohistochemistry to identify the cellular localization of VIP in the naïve and allergic WT and KO lungs. Naïve mice of both groups exhibited a similar pattern of VIP localization with the predominant site being the columnar epithelia (Fig 2A & C). While naïve WT columnar epithelia showed homogenous VIP localization (Fig 2A), the naïve

$VPAC_2$ KO lungs had prominent VIP localization in the apical ends of the columnar epithelia (Fig 2C). After allergen challenge, there was a reduction in VIP in the columnar epithelia of both groups (Fig 2B & D).

3.2. $VPAC_2$ KO mice had elevated IgG_{2a} and IgA in naïve and disease states

We have previously demonstrated that $VPAC_2$ KO mice produced IgE equivalent to that of WT animals after allergen challenge and that they sustained elevated levels of IgE in the sera for longer than their WT counterparts [34]. Naïve KO mice demonstrated significant overexpression of IgG_{2a} in serum when compared to WT mice (Fig 3A) which was in stark contrast with a previous report [15]. Serum levels of IgG_{2a} in naïve KO mice were approximately 175× that of naïve WT mice (Fig 3A). A continual increase in IgG_{2a} occurred in the KO mice after allergen challenge, while a reduction was noticed in allergic WT mice (Fig 3A). Serum IgG_{2a} was maintained at a much higher level in the KO at all time points after the final conidia inhalation (Fig 3A).

The IgA content in the sera in the naïve KO and WT groups were equivalent (Fig 3B). Although serum IgA levels were significantly different between WT and KO after allergen challenge, there was no difference in IgA availability in each group compared to their respective naïve controls (Fig 3B). While WT IgA in the sera decreased by day 28, KO levels remained elevated (Fig 3B).

3.3. $VPAC_2$ KO mice had an aberrant local antibody response to fungal challenge

Since fungal allergen challenge predominantly impacts mucosal immunity in the lung, we assessed the local availability of immunoglobulins in the BAL fluid of the lung. While both groups of mice increased the availability of IgE in the lung in response to fungal challenge, KO mice had significantly more IgE at days 7 and 14 compared to that in WT mice (Fig 4A). Both groups maintained IgE in the BAL fluid at day 28 reiterating their allergic phenotype (Fig 4A). However, IgE was the least abundant of the isotypes in the BAL after allergen challenge (Fig 4).

In WT mice, IgG_{2a} was not a major Ab component of the naïve BAL, nor was it induced in the lung after allergen challenge (Fig 4B). After allergen challenge, IgG_{2a} concentration was increased in the BAL fluid of $VPAC_2$ KO animals and remained significantly elevated through the day-28 time point as compared to naïve levels and that produced in allergic WT animals (Fig 4B). IgA was observed at very low levels in naïve WT and KO (Fig 4C). In contrast to the pattern seen in the serum, IgA was induced in the BAL fluid following allergen challenge in both groups (Fig 4C). While WT mice maintained these same level of antibodies at all subsequent time points, KO mice had significantly more of IgA at later time points (Fig 4C). The trend in IgA in the BAL followed that of IgE for both groups (Fig 4).

3.4. Immune complexes with IgE were prominent in WT allergic lungs while allergic $VPAC_2$ KO lungs had more IgA localizing cells in the peribronchovascular areas

IgE is important in the allergic response because of its ability to cross link antigen to elicit mast cell degranulation and activation of the T_H2 immune cascade. After allergen challenge, we observed that serum and BAL IgE was significantly increased in both WT and KO animals. The source of the BAL IgE was not readily apparent, since either increased serum leak into the airways or increased local production of IgE would result in similar findings. We analyzed IgE's local production by immunohistochemistry in the allergic lungs 7 days after allergen challenge, a time point at which both WT and KO animals had peak production of this immunoglobulin. Immunohistochemical analysis showed that naïve controls of WT (Fig 5A) and KO (Fig 5C) groups had very few IgE-producing cells. While IgE staining was observed in the peribronchovascular areas in the WT lungs at day 7 (Fig

5B), IgE-stained cells were localized to the perivascular areas in the KO allergic lung (Fig 5D). Contrasting with the ELISA data, IgE staining was much more prominent in WT lungs (Fig 5B) than in KO lungs (Fig 5D), an observation that also supports the extrapulmonary production of the majority of IgE in the KO animals.

Mucosal protection is largely mediated by IgA that is actively pumped across the mucus membrane. While naïve lungs of both the WT (Fig 5E) and KO (Fig 5G) groups showed little evidence of active IgA production, WT allergic lungs contained IgA-producing cells in the peribronchovascular areas (Fig 5F). IgA-positive cells which were obvious in peribronchovascular areas in $VPAC_2$ KO allergic lungs, were more abundant than in the WT lung (Fig 5H). These cells may contribute to the local IgA in the BAL fluid quantified by ELISA.

4. Discussion

VIP is a potent anti-inflammatory cytokine and neuropeptide that has been targeted as a potential bronchodilator for therapeutic use in asthma. However, its production by leukocytes in addition to the nervous system contribution, its ability to signal through multiple receptors, and the widespread availability of these receptors on leukocytes and structural cells of the lung makes VIP a complex peptide to study in health and disease. Using a mouse strain deficient in $VPAC_2$, we show for the first time that $VPAC_2$'s regulation significantly impacts antibody production by B cells. In this study, $VPAC_2$ regulation restrained systemic inflammatory antibody production in the naïve animal, a fact that has not been recognized before. This is in contrast to other reports that failed to identify significant differences in the immune regulation between WT and KO animals [15].

The immunoregulatory role played by VIP is complex and involves cells of the innate and adaptive branches. T lymphocyte differentiation into various subsets is dependent on the signals conveyed by antigen presenting cells. The type and load of antigen, the activation state of the antigen presenting cell and the genetic background of the host with the cytokine microenvironment available, all mediate the T cell bias toward a T_H1/T_H2 phenotype [8]. Macrophages are capable of producing various cytokines depending on the stimulus. Macrophages express VPAC receptors and those that mature in the presence of VIP reduce IL-12 production [6] and increase IL-10 [5]. Functions of dendritic cells (DCs) depend on the mediators in the environment that they are in at the time of antigen uptake. DCs that mature in the presence of VIP, downregulate CD80 and CXCL10, while upregulating the production of CCL22, thereby preferentially attracting T_H2 cells [4]. The decreased VIP in the $VPAC_2$ knock-out lung immediately after fungal challenge [34] removes VIP's effect on dendritic cells that transport antigen for presentation to T cells in the lymph nodes, thereby limiting the interaction between T_H2 cells and allowing T_H1 polarization instead. Since $VPAC_2$ elevates the migration of T_H2 cells [9] T_H2 migration toward antigen presenting dendritic cells may be hindered in the absence of $VPAC_2$ further promoting dendritic cell interaction with T_H1 cells (Fig 6).

Analysis in naïve $VPAC_2$ mice has led to the conclusion that there was no immunological difference in these mice compared to their WT counterparts [15]. However, we have shown that naïve $VPAC_2$ KO mice had far greater abundance of serum IgG_{2a} and IgA compared to WT, marking this as the major differentiating characteristic between the two groups. We did not find evidence of enlarged spleens or differences in splenic $CD4^+$ T cells and $CD19^+$ B cells in naïve KO mice (data not shown), confirming the findings by by Goetzl and colleagues [15]. Therefore, the increased production of systemic antibodies in the KO could not result from increased lymphocytes. Naïve B cells require antigen stimulus, CD40 signaling, and cytokines in order to activate switch enzymes that dictate class-switch

recombination [39]. Of the various cytokines, IFNγ, IL-4, and TGFβ are the main cytokines known to induce class-switching to IgG, IgE, and IgA respectively [12]. The generation of each isotype analyzed is dependent on T cell help via CD40L and cytokines. T cell migration is promoted by VIP [23]. However, in our model, the equal expression of *CD40L* mRNA (data not shown) between WT and KO indicates that the massive induction in *VIP* mRNA in the WT at day 3 did not result in an equally greater T cell recruitment [34]. However, the possibility that it may regulate the number of CD40L expressed on each T cell will need to be investigated in the future.

In the absence of $VPAC_2$, VIP may act as a secretogogue through $VPAC_1$ on T_H2 cells (activated independent of VIP) to enhance cytokine secretion. The VIP/$VPAC_2$ axis acts on T cells to reduce IL-2 [14] and IFNγ [15], increase IL-4 [15], and acts on macrophages to decrease IL-6 and IL-12 [7] all of which hinders a T_H1 response. The diminished regulatory effect of VIP may skew the T_H1/T_H2 balance in favor of T_H1 cells thereby promoting B cell switch recombination toward IgG_{2a} and IgA in the $VPAC_2$ KO mice (Fig 6).

Antibodies induced in the allergic lungs marked by their presence in the BAL fluid may aid in the clearance of the inhaled fungal conidia. Allergic asthma is characterized by increased mediators such as histamine and leukotrienes which result in blood vessel permeability [20]. Therefore, circulating antibodies from vascular leakage probably adds to the antibodies produced in situ in the allergic lungs by recruited and resident B cells. Although IgE is not known to perform a mucosal defense function like IgA, it has been found in the BAL fluid of asthmatics [30] and allergen challenged mice contain immune complexes with IgE in the BAL fluid [40]. Based on our staining pattern, IgE containing immune complexes surrounding the peribronchovascular areas of allergic lungs may serve as a method of fungal clearance in the WT lungs. In the $VPAC_2$ null mice, the IgE-staining was modest although the amount of IgE in the BAL fluid was significantly greater than that of WT suggesting that these immune complexes with IgE may largely be in the airway lumen of KO lungs. The systemic and local availability of IgE in this "T_H1-skewed" mouse suggests that $VPAC_2$ is not sufficient to control the generation of T_H2 cells and IL-4 production in allergic asthma.

By performing numerous functions such as neutralization, immune exclusion, and antigen clearance in the lumen, IgA is believed to be vital for mucosal defense. B cells produce VIP and express $VPAC_1$ [16], and VIP has been shown to induce class switching in CD40-activated B cells [13]. VIP induces B cell class switching to IgA directly [13,27] or indirectly through regulation of IgA-inducing protein expression by DCs [11]. Peyer's patches contain the most amount of IgA producing cells [3], and the gut-associated lymphoid organs are the predominant site of IgA production [21]. Synapsing of VIPergic nerves at these locations perfectly positions VIP to regulate T cells, DCs, and B cells to regulate IgA production. Similarly, we propose based on the close proximity of IgA stained cells and VIP localizing columnar epithelia, that IgA induction and secretion in the lung occurs in situ as part of mucosal defense which may be hindered in the presence of $VPAC_2$. The decrease in $VPAC_2$ compared to an increase in $VPAC_1$ in the WT allergic lungs [34] indicates that $VPAC_1$ may be the predominant VIP receptor in allergic asthma. However, this does not speak to individual cell expression of VIP receptors since we looked at VIP receptor expression in the whole lung rather than cellular subsets. The immediate reduction in VIP in the KO may allow unhindered production of IL-6 by activated macrophages promoting class-switching to IgA [1] (Fig 6).

Decades have passed since the discovery of VIP by Said and Mutt [33]. While our understanding of VIP has expanded, its effects on the respiratory and immune systems in allergic asthma are still unclear. The $VPAC_2$ null mouse has shown that the VIP/$VPAC_2$ axis neither impedes organ development [18,19] nor immune system development [15].

While Goetzl and colleagues have shown that the $VPAC_2$ null mice have decreased ITH and increased DTH responses [15], we have shown that these mice are capable of developing allergic responses against *A. fumigatus* allergens [34]. Furthermore, in this report, we show for the first time, that the VIP/$VPAC_2$ axis plays a significant role in the regulation of immunoglobulin production by B cells. The absence of the regulatory receptor $VPAC_2$ promotes the T_H1 skewing of the C57BL/6 mouse strain further or fails to shift the balance in favor of T_H2. Nonetheless, $VPAC_2$ knock-out mice are still susceptible to a T_H2 mediated disease such as allergic asthma indicating the fine balance between T_H1 and T_H2 immune phenotypes in disease pathogenesis. Taken as a whole, this research supports the increasing body of knowledge that VIP regulates inflammatory responses by signaling through $VPAC_2$ receptors on leukocytes. Future studies analyzing various cell types such as T and B lymphocytes, and antigen presenting cells from allergic WT and $VPAC_2$ KO mice will shed light on the intricate cellular interactions that occur between these cells with/without the $VPAC_2$ receptor.

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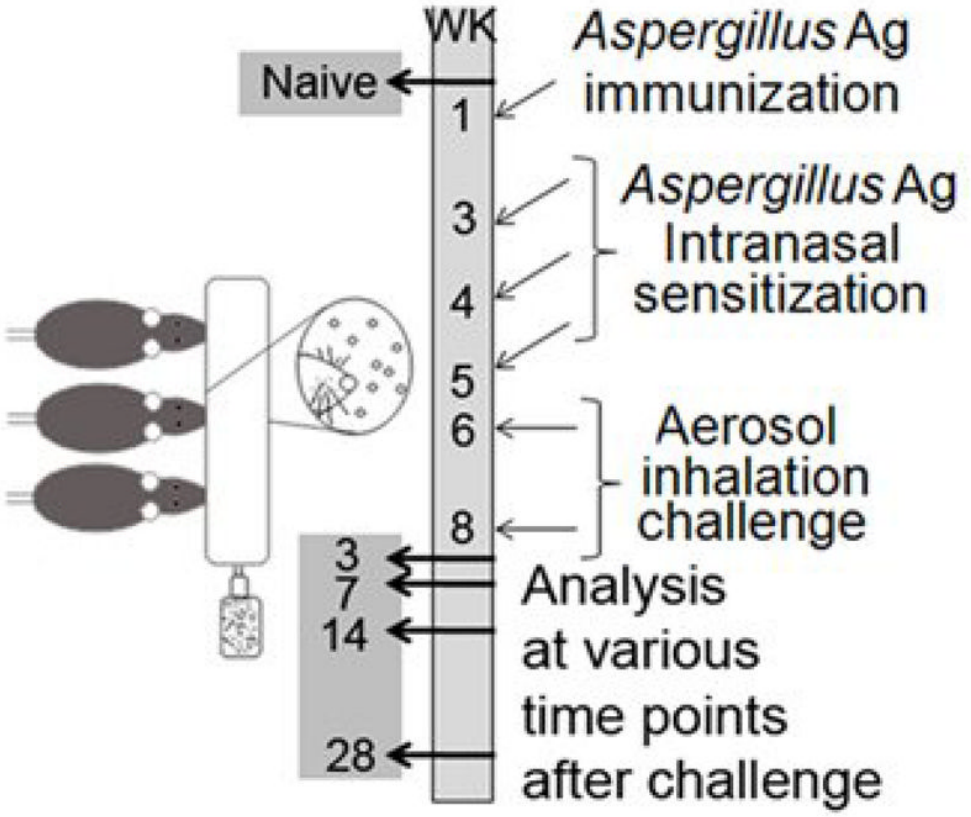

Figure 1.
Schematic representation of the *Aspergillus fumigatus* induced murine model system of allergic asthma. Mice were sensitized against antigen from *A. fumigatus* over the course of five weeks. Sensitized mice were exposed to un-manipulated conidia via normal inhalation for 10 minutes two weeks apart and analyzed at days 3, 7, 14, and 28 after the second inhalation challenge.

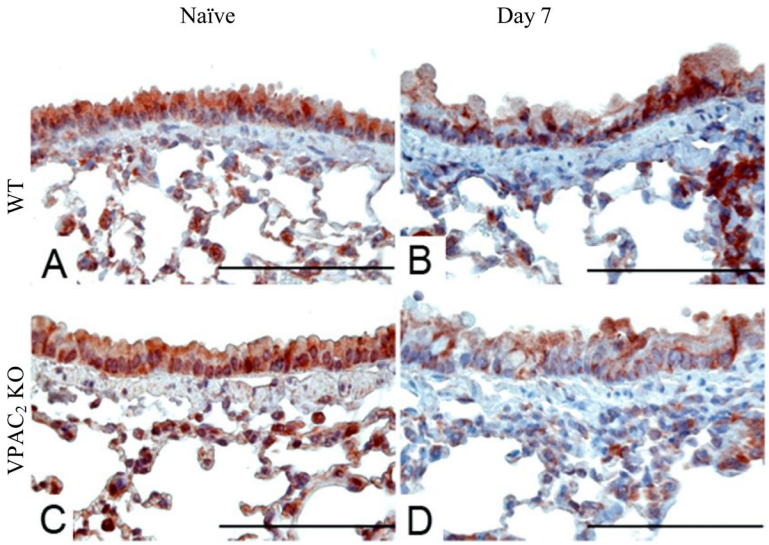

Figure 2.

Immunohistochemical staining for VIP localization in naïve and day 7 wild-type (WT) and $VPAC_2$ knock-out (KO) lungs. The columnar epithelia and Type II pneumocytes were the predominant areas of VIP localization in both the WT (A) and KO (C) naïve lungs. VIP localization was decreased in these areas after allergen challenge, but equivalent localization occurred in WT (B) and KO (D) day 7 lungs. Scale bars = 100 μm

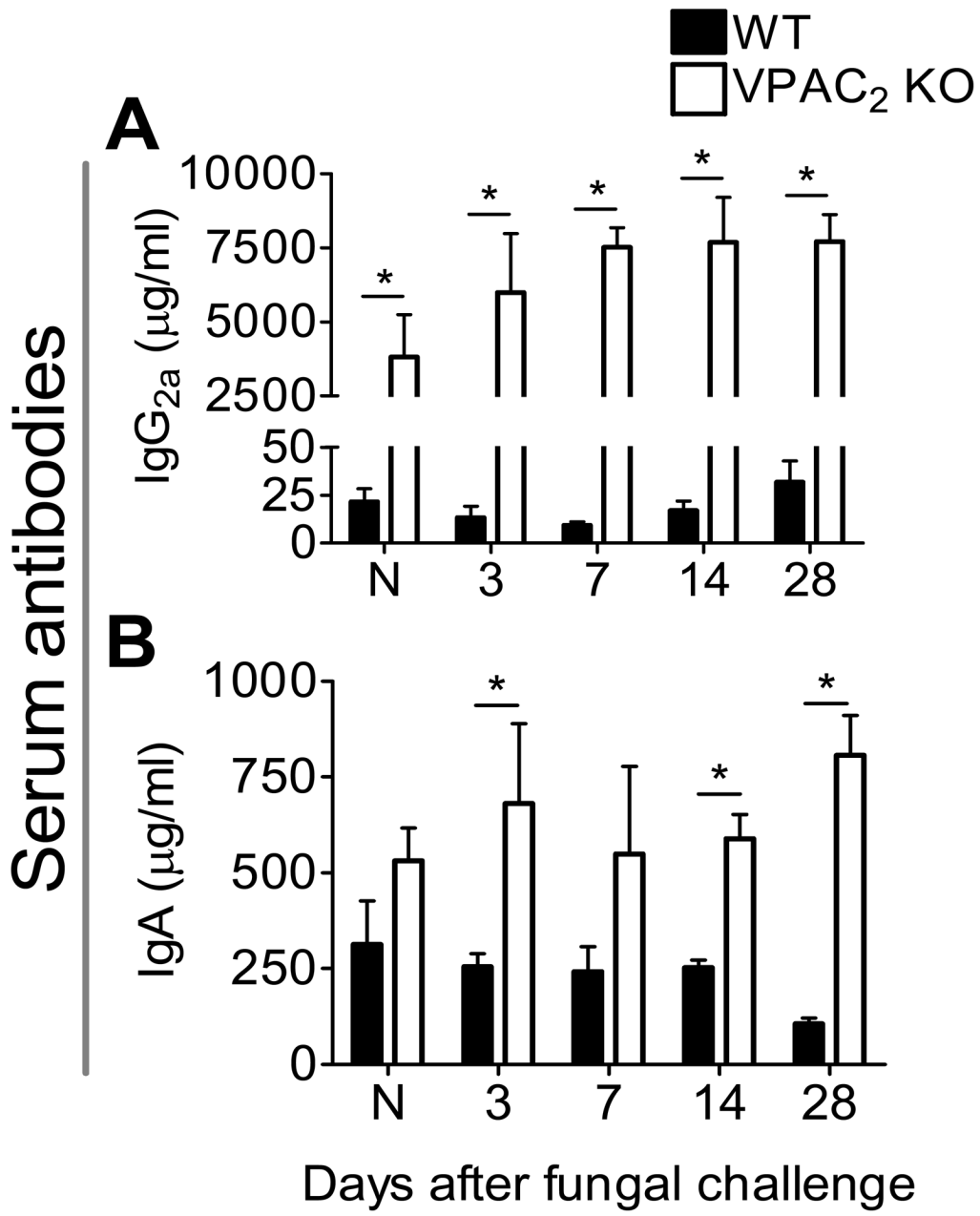

Figure 3.

Antibodies quantified in sera of naïve and allergic wild-type (WT) and $VPAC_2$ knockout (KO) mice via ELISA. Naïve KO mice contained more IgG_{2a} (A) and IgA (B) in the sera compared to naïve WT mice. Allergen challenge led to a decreasing trend in serum IgG_{2a} in the WT, while KO mice had increased IgG_{2a} production as a result of fungal inhalation (A). The amount of IgA in the sera did not change in either group during the early time points after allergen challenge (B). However, at day 28, WT IgA availability decreased while KO levels remained elevated (B). All values expressed as the mean ± S.E.M. n=5 mice/group, *$p<0.05$

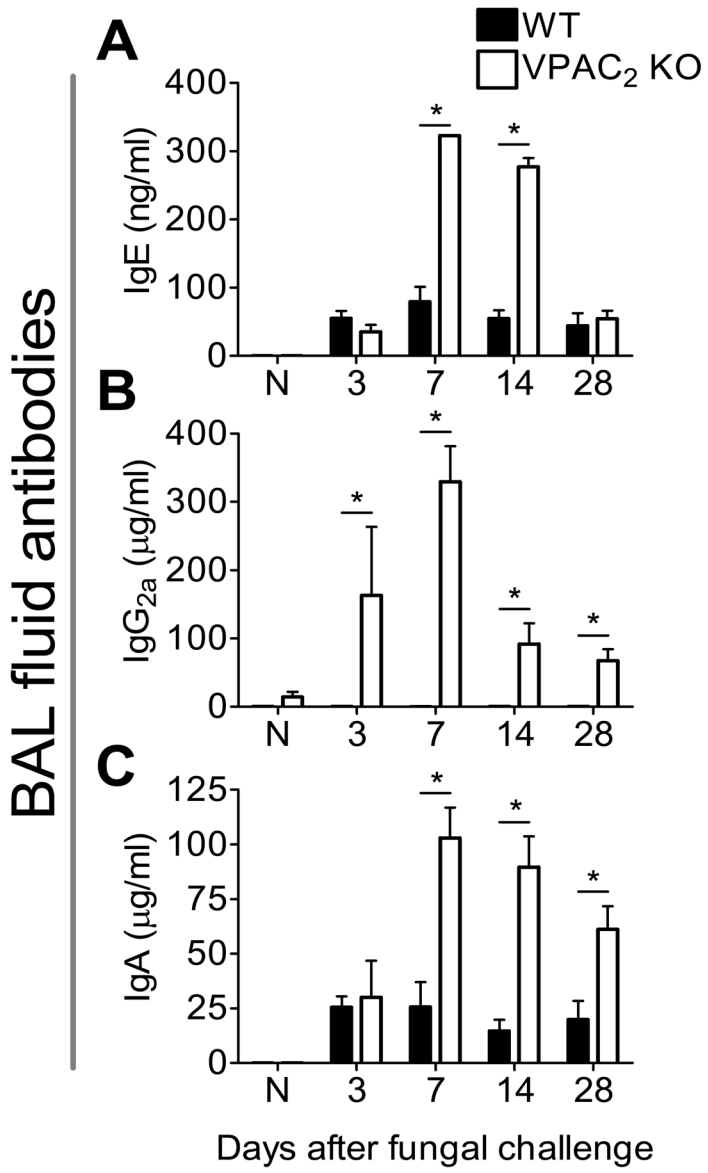

Figure 4.

Antibodies quantified in the bronchoalveolar lavage (BAL) fluid of naïve and allergic wild-type (WT) and $VPAC_2$ knock-out (KO) mice. Naïve animals in both groups had minimal antibody production in the lungs (A-C). IgE in the BAL fluid increased after allergen challenge in both groups although KO mice had a marked increase in local IgE production at days 7 and 14 compared to WT controls (A). WT mice had negligible levels of IgG2a in the BAL fluid after allergen challenge while a massive increase in IgG2a was observed in the KO BAL fluid (B). The mucosal antibody, IgA, was increased as a result of fungal inhalation in both groups, although KO had far more IgA after day 3 compared to WT (C). All values expressed as the mean ± S.E.M. n=5 mice/group, *$p<0.05$

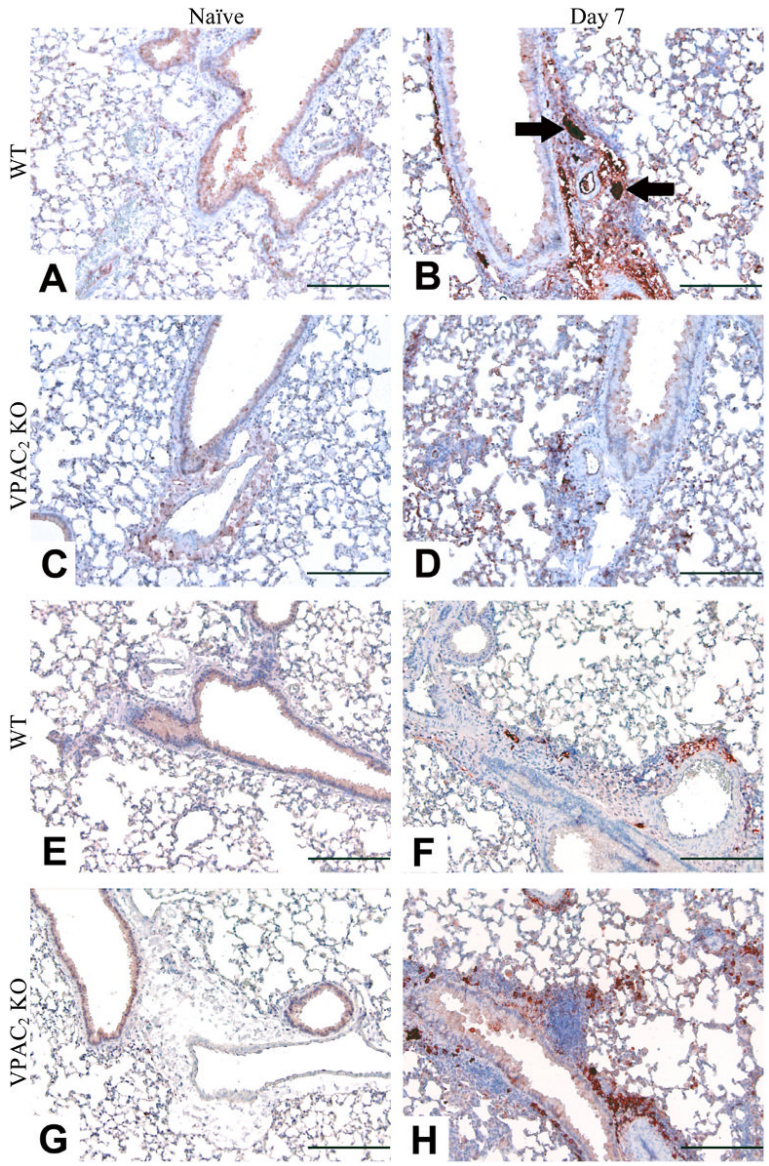

Figure 5.

Immunohistochemical staining for IgE (A-D) and IgA (E-H) localization in naïve and day 7 wild-type (WT) and $VPAC_2$ knock-out (KO) lungs. Cells that localized IgE in naïve WT (A) and KO (C) mice were scarce. The pattern of IgE staining in the WT at day 7 showed the presence of immune complexes (arrows, B) rather than cellular localization. In the KO lungs at day 7, a few IgE localizing cells were found around the perivascular areas (D). Naïve lungs in both groups had very few cells that localized IgA (E & G). Cells that localized IgA was identified in the peribronchovascular areas in the WT lungs at day 7 (F). Compared to WT, the day 7 KO lungs contained substantial numbers of IgA localizing cells around the airways and blood vessels (H). Scale bars = 200 μm

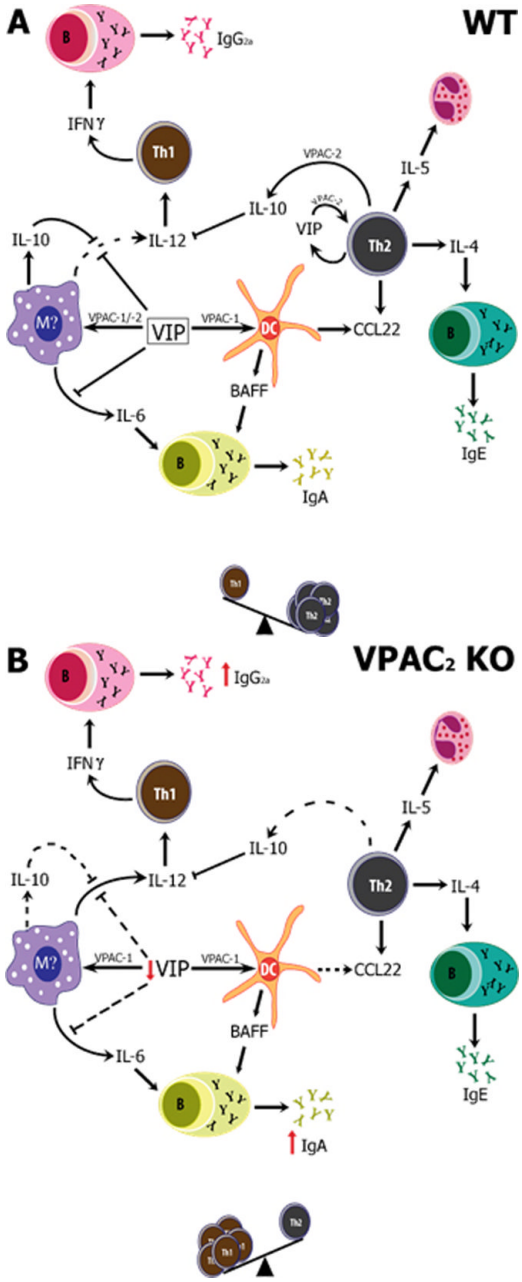

Figure 6.

Hypothetical model for VIP's regulation of antibody production in the presence and absence of the $VPAC_2$ receptor. In the presence of $VPAC_2$, VIP's effect on dendritic cells (DCs) would promote the preferential recruitment and activation of T_H2 cells to produce IL-10 that suppresses macrophage (Mφs) production of IL-12 to negatively impact T_H1 activation (A). In the absence of $VPAC_2$, VIP's effect on the T_H2 population would be reduced thereby leading to a reduction in pro-inflammatory Mφ function. The promotion and activation of the T_H1 population will therefore promote preferential class switching to the IgG_{2a} and IgA in the $VPAC_2$ KO mice (B). IL = interleukin; CCL = CC chemokines ligand; IFN = interferon; BAFF = B cell activating factor of the TNF family