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Spatio-temporal localization of vasoactive intestinal peptide and neutral endopeptidase in allergic murine lungs

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Abstract

Vasoactive intestinal peptide (VIP) is a neuropeptide with cytokine properties that is abundant in the lung. *VIP* null mice exhibit spontaneous airway inflammation and hyperresponsiveness emphasizing VIP's "anti-asthma" potential. Although VIP's impending protective role in the lung has been demonstrated, its localization in the naïve and allergic murine lungs has not. To this aim, we analyzed the availability of VIP and its protease, neutral peptidase (NEP), in naïve and *Aspergillus*-sensitized and challenged murine lungs after 3, 7, and 14 days. Both VIP and NEP were predominantly localized to the columnar epithelia of the airways in naïve lungs. A marked decrease in VIP occurred in these cells 3 days after allergen challenge. NEP localization in the columnar epithelia decreased after allergen challenge. At day 14, VIP localization in the columnar epithelia and arteriolar smooth muscle increased while NEP localization at these sites remained low. This study provides new insights into the local regulation of VIP in the columnar epithelia of the allergic lung. Its altered availability in the context of allergy provides fresh evidence for the modulation of pulmonary inflammation by VIP.

Keywords

VIP; NEP; Asthma; Columnar epithelia; Smooth muscle; Immunohistochemistry

1. Introduction

Vasoactive intestinal peptide (VIP) is a neuropeptide originally isolated from porcine intestines by Drs. Said and Mutt in 1970 [1]. A series of post transcriptional modifications of prepro-VIP yields the final 28-amino acid peptide that is now known to be an important regulator of functions in various organ systems including the respiratory and immune systems [2]. VIPergic nerves associated with smooth muscle of the trachea, bronchi, and pulmonary vessels supply the lung with VIP [3,4] wherein it facilitates vaso- and broncho-dilation [5,6]. In addition, mast cells [7], eosinophils [8], and T_H2 lymphocytes [9] produce VIP in the allergic lung. VIP regulates the immune response in an anti-inflammatory manner [10], and VIP's effect on T cell differentiation and maturation has resulted in its consideration as a T_H2-type cytokine [9,11].

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Allergic asthma is a T_H2 mediated disease of the airways characterized by airway hyperresponsiveness, eosinophilic inflammation, and elevated serum IgE. VIP is decreased in the lungs of asthmatics [12,13] and VIP null mice have spontaneous airway inflammation and hyperresponsiveness [14] highlighting the importance of VIP in allergic asthma. Although numerous studies have demonstrated the localization of VIP receptors in cells of the respiratory system [15–18], to our knowledge, the *in vivo* localization of VIP in murine allergic lungs has not been fully elucidated. We investigated the spatio-temporal localization of VIP and its predominant protease neutral endopeptidase (NEP, also known as Neprilysin) in an *Aspergillus*-induced murine model of allergic asthma. We found that the columnar epithelium of the airways and the smooth muscle surrounding pulmonary arterioles to be the predominant sites of VIP localization in the lungs of allergic mice, while NEP was localized mainly to the alveolar lining. The results of this study provide new insights into the local regulation of VIP in the columnar epithelia of the allergic lung.

2. Materials and Methods

2.1. Animals

C57BL/6 mice were purchased from Jackson Laboratories, Bar Harbor, ME, USA. Animals were housed on Alpha-driTM paper bedding (Shepherd Specialty Papers, Watertown, TN, USA) in microfilter-topped cages (Ancare, 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 in-line 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 per Hogaboam's published protocol using alum in place of incomplete Freund's adjuvant [19]. Briefly, 10 μ g 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 μ g of *A. fumigatus* antigen in 20 μ l 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.

Sensitized and challenged mice were separated into groups of five for analysis at days 3, 7, and 14. Naïve animals were neither sensitized nor challenged.

2.4. Airway response measurement and sample collection

Mice were anesthetized with sodium pentobarbital (Butler, Columbus OH; 0.1 mg/10 g of mouse body weight), intubated, and ventilated with a Harvard pump ventilator (Harvard Apparatus, Reno, NV) to assess allergic airway responses. Restrained plethysmography (Buxco, Troy, NY) was used to assess airway response as described previously [19]. Baseline airway resistance was measured for each animal before an intravenous injection of acetyl β methacholine (420 μ g/kg) to determine AHR at days 3, 7, and 14 after *A. fumigatus* challenge. This dose had been previously determined to double baseline airway response in a naïve mouse.

Approximately 500 μ l 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, inflated with 10% neutral buffered formalin, fixed in formalin for 16 h, and paraffin embedded for immunohistochemical analysis.

2.5. Determination of allergic airways disease

Serum IgE level was used to assess allergy. Blood harvested from each mouse was centrifuged at $13,000 \times g$ for 10 min to obtain sera. IgE (BD OptEIA, San Diego, CA) concentration in the sera was quantified by ELISA per manufacturer's guidelines.

BAL cell counts and histology were used to assess eosinophil recruitment into the airways. Individual BAL samples were centrifuged at $3000 \times g$ for 10 min. BAL cells were resuspended and cytospun (Shandon Scientific, Runcorn, UK) onto microscope slides for differential staining (Quick-Dip stain, Mercedes Medical, Sarasota, FL) and counting. Eosinophils from five randomly chosen high-powered fields (HPFs) were counted to determine the mean number of eosinophils in the airway lumen of each mouse.

2.6. Immunohistochemical analysis of VIP and NEP in the lung

Immunohistochemistry (IHC) was carried out using longitudinal 5- μ m sections across the coronal plane of the left lungs. Sections affixed to glass slides were submerged in 10-mM citric acid at pH 6.0 and cooked in a pressure cooker for 10 min for antigen retrieval. Staining was performed using the HRP-AEC cell and tissue staining kits by R&D Systems (Minneapolis, MN, USA) following the manufacturer's recommended protocol. Endogenous peroxidase, serum, avidin, and biotin blocking steps were carried out at room temperature as specified using the appropriate blocking reagents provided in the staining kits. Sections were incubated in rabbit polyclonal antibody against full-length VIP (Abcam, Cambridge, MA, USA) or goat anti-mouse Neprilysin (R&D Systems) primary antibodies at 1:20 and 1:10 dilutions respectively, overnight at 4°C. Control sections were not incubated in primary antibody. Incubation in secondary antibodies was carried out at room temperature for one hour. A 30 min incubation in horseradish peroxidase conjugate followed by a 10 min incubation in 3-amino-9-ethylcarbazole resulted in the formation of a red precipitate in areas of antigen localization. Sections were counterstained in Gill's III hematoxylin (Surgipath, Richmond, IL, USA) for 10 sec.

The staining intensity of each section was rated independently using the plus (+) minus (−) system. A − score was used when no staining was observed. A + score was used when the staining was low, ++ when the staining was of medium intensity, and +++ when there was highly intense staining. Parentheses were used to denote scores that were in-between intensities.

2.7. Statistical analysis

Allergic mice were compared against their respective naïve controls 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 $\pm$ Standard Error of the Mean (S.E.M). $p < 0.05$ was considered statistically significant.

3. Results

Allergen challenge resulted in a marked increase in airway hyperresponsiveness, serum IgE, and airway eosinophilia. VIP and NEP localization were altered in the lung as a result of allergen challenge. The columnar epithelium, arteriolar smooth muscle, and mononuclear cells in the alveolar spaces were the predominant sites of VIP localization in the naïve lung, and an early decrease in production in these cells marked the allergic state. NEP localization was limited to the columnar and alveolar epithelia in naïve lungs with a down-modulation in these same areas after allergen challenge. The photomicrographs of immunostaining for VIP and NEP show the same locations in serial lung sections for comparison when possible.

3.1. Allergen challenge induced hallmark characteristics of allergic asthma in mice

Airway hyperresponsiveness (AHR), airway eosinophilia, and serum IgE were measured to determine the allergic state of mice used for the study. Allergen challenge led to increased AHR compared to naïve controls peaking at day 3 (Fig 1A). Total serum IgE was also increased over naïve as a result of allergen exposure. The peak in serum IgE occurred at day 14 (Fig 1B). Eosinophils are the hallmark leukocyte of allergy; we quantified the average number of eosinophils in the airway lumen and found that eosinophils peaked in the allergic lungs at day 3 (Fig 1C). Although eosinophils persisted in the allergic lungs, they decreased over time in the model (Fig 1C). These characteristics indicated the allergic state of the animals used to determine the spatio-temporal localization of VIP and NEP in the lung.

3.2. VIP localization decreased in the lung as a result of allergen challenge

The spatio-temporal availability of VIP in the lung was determined by IHC. Table 1 provides a summary of the distribution pattern of VIP in the naïve and allergic lungs based on staining intensities of sections from each mouse. The majority of VIP localization in the lung occurred in the columnar epithelium, arteriolar smooth muscle, and mononuclear cells within the alveolar spaces. Endothelial cells that lined the arteries and veins also stained for VIP. The major differences between the naïve and allergic lungs occurred in the columnar epithelia and the smooth muscle around the arteries wherein a massive decrease in VIP localization was observed after allergen exposure.

VIP was predominantly localized to the columnar epithelium of the airways (Fig 2A) and the arteriolar smooth muscle (Fig 2B) in the naïve lung. Lung myocytes located at the basal end of the columnar epithelium did not stain for VIP in naïve (Fig 2A) or at days 3 and 7 (Fig 2D, G), while very diffuse light staining was observed in this cell layer at day 14 (Fig 2J). The controls consistently did not stain for VIP. At day 3, there was a marked decrease in VIP staining in the major areas of localization, the columnar epithelium (Fig 2D) and the arteriolar smooth muscle (Fig 2E). Distal blood vessels (BVs) were occluded in the allergic lungs (Fig 2F, I, L) but not in naïve lungs (Fig 2C). The smooth muscle layer as well as the recruited inflammatory cells that surrounded these occluded BVs at day 3 did not stain for VIP (Fig 2F). There was an increase in VIP in the lung at day 7, marked by increased localization in the columnar epithelium (Fig 2G) and arteriolar smooth muscle (Fig 2H). Recruited cells that surrounded the occluded distal BVs also stained for VIP at this time point (Fig 2I). VIP localization increased further in the columnar epithelium (Fig 2J) and

arteriolar smooth muscle (Fig 2K) 14 days after allergen challenge. Recruited cells at this time point also contributed to the local VIP in the lung (Fig 2L).

3.3. NEP localization decreased in the lung after allergen challenge

The spatio-temporal availability of NEP in the lung was determined by IHC. Table 2 summarizes the distribution pattern of NEP in the naïve and allergic lungs based on staining intensities of lung sections. The majority of NEP localization in the lung occurred in the columnar epithelium, the alveolar lining (types I and II pneumocytes), and mononuclear cells in the alveolar spaces. Contrary to VIP, the arteriolar smooth muscle only stained lightly for NEP. The major differences between the naïve and allergic lungs occurred in the columnar epithelia wherein allergen exposure resulted in a massive decrease in NEP localization.

The columnar epithelia that lined the airways of naïve animals localized NEP albeit at a lower staining intensity than that of VIP (Fig 3A) while the arteriolar smooth muscle had very little NEP staining (Fig 3B). The distal BVs in the naïve lungs were not occluded and the smooth muscle had very diffuse NEP staining (Fig 3C). Types I and II pneumocytes were the predominant sites of NEP localization. Basal myocytes did not have NEP localization in the naïve (Fig 3A) or allergic states (Fig 3D, G, J). The control sections were void of any red precipitate.

Allergic animals at day 3 showed a marked decrease in NEP localization in the columnar epithelia (Fig 3D) and still showed no change in NEP availability in the arteriolar smooth muscle (Fig 3E). The occluded distal BVs also did not stain for NEP, nor did the recruited cells at this time point (Fig 3F). At day 7, the columnar epithelium showed a slight increase in NEP (Fig 5G). Similar to the arterial smooth muscle (Fig 3H) the distal occluded BV smooth muscle did not stain for NEP (Fig 3I); recruited cells that surrounded these BVs stained lightly for NEP (Fig 3I). Allergic lungs at day 14 showed a slight increase in NEP in the columnar epithelium (Fig 3J) and endothelium (Fig 3K), and recruited cells also localized NEP (Fig 3L) although the smooth muscle around the BVs did not (Fig 3K, L). NEP localization in the endothelia of blood vessels increased at day 14 (Fig 3K, L).

4. Discussion

Vasoactive intestinal peptide is an important regulator of functions in the pulmonary and immune systems. In the lung, VIP has been shown to have bronchodilatory effects [20] and inhibit smooth muscle cell proliferation [21]. A downregulation in VIP content has been reported in human asthmatics [12,13] and VIP null mice have spontaneous airway hyperresponsiveness and inflammation [14] highlighting the protective roles played by VIP in the lung. While the availability of VIP and its receptors in the lung are documented [2], the exact localization of VIP in the lung in steady- and diseased-states has not been fully characterized. With the use of immunohistochemistry, we identified the cells that localize VIP and its protease NEP, in naïve and *Aspergillus*-induced allergic murine lungs.

The columnar epithelia of the airways play a vital role in protecting the lung by serving as a barrier that shields the lung from inhaled agents which may be innocuous or offensive. The ciliated columnar cells also perform a vital role in clearance of inhaled material via the mucociliary escalator. VIP predominantly localized to the columnar epithelia of the airways in naïve lungs which may further establish its roles in enhancing ciliary beat [22] and wound healing [23]. Its availability in the columnar epithelium may enable its secretagogue effect on mucus secreting cells [24,25] that intersperse this cell layer thereby leading to an increase in mucus production. Its decreased localization in these cells in the allergic state may be due to increased goblet cells in the airways as a result of allergen challenge (data not shown).

Therefore, in the allergic state, although the amount of mucus increases in the lung, decreased VIP in the ciliated cells may contribute to a decrease in mucociliary clearance which could cause mucus accumulation which is characteristic of asthma.

Prominent VIP localization in the columnar epithelia may function as a potential chemotactic gradient wherein cells expressing the VIP receptors may be drawn toward the peribronchial area. A decrease in VIP localization in these structural cells therefore, may be an anti-inflammatory mechanism to decrease the influx of inflammatory cells. Alternatively, the decrease in VIP localization in these cells may promote the asthma phenotype by decreasing a potential “anti-asthma” factor from the lung. Dendritic cells (DCs) are important mediators of the immune response. VIP promotes the chemotaxis and CD86 expression in immature DCs while hindering that in mature DCs [26–28]. The trends in VIP localization of VIP in the columnar epithelium may have an impact on the maturation and functions of DCs. Furthermore, VIP increases the expression of E-cadherin on epithelial cells which is vital to airway homeostasis [23]. The immediate decrease in VIP localization in the columnar epithelium after allergen challenge may lead to a reduction in E-cadherin which would trigger epithelial shedding that is a characteristic of asthma [29].

The importance of VIP in maintaining normal lung function is emphasized since its absence results in spontaneous AHR and inflammation in mice [14]. Increased AHR and luminal eosinophils were characteristic at time points when VIP was decreased in the lung, which reiterates VIP’s protective role in allergic asthma. Smooth muscle cell proliferation is inhibited by VIP [21]. While VIP did not localize in basal myocytes in naïve and allergic lungs, the arteriolar smooth muscle was a predominant site of VIP localization in the naïve lungs which decreased significantly immediately after allergen challenge. Since VIP’s inhibitory effect on smooth muscle proliferation *in vitro* was most pronounced at early days [21], the reduction in VIP at early time points in our model may contribute to smooth muscle cell hyperplasia around blood vessels like that observed in the distal lung.

As an immune regulator, VIP performs an anti-inflammatory role by downregulating macrophages; inhibiting respiratory burst, nitric oxide production, chemotaxis, pro-inflammatory cytokine production, and phagocytosis [30–33]. The mononuclear cells within the alveolar spaces that showed VIP localization are likely alveolar macrophages. Since VIP has been demonstrated to hinder the phagocytic and chemotactic ability of alveolar macrophages *in vitro* [30], its localization in these cells may further reiterate VIP’s anti-inflammatory function in the lung.

Proteases involved with VIP modulation are important in maintaining homeostasis. Neutral endopeptidase regulates VIP under steady-state, while mast cell proteases are known regulators of VIP in inflammation [34–36]. However, since mast cells in mouse lungs are rare [37], and we have only been able to locate very few in our model, we investigated the distribution pattern of NEP. While NEP is produced by various cells in the body, its local production in the lung is mainly by airway epithelia, smooth muscle, lung parenchymal tissue, and the Vagus nerve [38]. Functions of NEP include regulating the growth and maturation of the fetal lung and lymphoid progenitors [39], inhibiting macromolecule secretion [40], and modulating the effects of tachykinins [38]. Airway epithelial cells are known producers of NEP [41] in which NEP is active and its inhibition promotes VIP-induced relaxation of airways [2]. Substance P is a tachykinin that plays a role in smooth muscle cell contraction and inflammation [42]. The reduction in NEP after allergen challenge may therefore promote inflammation, AHR, and mucus hypersecretion by mediating these neuropeptides. Although NEP has been shown to cleave various substrates *in vitro*, its proteolytic function *in vivo* has been hypothesized to be dependent on its expression and tissue distribution in relation to the distribution of its substrates [43]. The co-

localization of NEP and VIP in the columnar epithelium and other cells in the naïve lung indicates that the juxtaposition of NEP in respect to VIP is important in this regulation process.

A decrease in NEP in the lung is associated with inflammation by promoting the adhesion and margination of leukocytes [44]. Neutrophil produced NEP cleaves f-Met thereby modulating neutrophil migration [45]. In our study, we observed that recruited inflammatory cells largely localized NEP, which may indicate that NEP plays similar roles in allergic asthma. Contrary to reports in other species [46,47], NEP did not localize to the vascular smooth muscle in the naïve or allergic mouse lungs. However, the absence of NEP in the vascular smooth muscle may facilitate VIP's vasodilatory properties in the lung. Other substrates of NEP in the lung such as substance P, IL-1 β , and neurokinins [48] all of which are known mediators of inflammation.

The identification of the *in vivo* localization of VIP in naïve and allergic lungs may be important in elucidating the exact role played by VIP in the lung in healthy and disease states. We have shown that VIP is mainly localized in the columnar epithelia and arteriolar smooth muscle in the naïve lung which decreases after allergen challenge, and that NEP also co-localizes in the columnar epithelia and decreases in these cells after allergen challenge. Future studies aim to identify whether VIP and NEP functions are interrelated or independent with the use of NEP inhibitors. We have shown that the localization of VIP after allergen challenge is dynamic and that a marked decrease occurs immediately after allergen challenge, which may explain why not all reports indicate a decrease in VIP production in allergic airways. Understanding the local distribution of VIP and the regulation of this peptide in allergic airways disease provides new insights into possible means of VIP regulation at the cellular level in the lung.

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Figure 1.

Characterization of allergic asthma in allergen sensitized and challenged mice. Baseline responsiveness prior to methacholine administration as an average for all groups is indicated by the dotted line (1.35 ± 0.10 cm H₂O/ml/sec). Airway hyperresponsiveness (AHR) was increased following allergen challenge at days 3 and 7 compared to naïve controls (A). Total serum IgE was quantified by ELISA and found to be elevated after allergen exposure (B). Eosinophils in the cellular component of the bronchoalveolar lavage (BAL) were counted and found to be markedly increased after allergen challenge (C). All data are represented as the mean $\pm$ S.E.M., n=4–5 mice per group. * denotes p<0.05 of allergen challenged mice compared to naïve controls.

Figure 2.

Immunohistochemical localization of vasoactive intestinal peptide in naïve and allergic lungs. VIP localization in various cell types were scored based on staining intensity and summarized in Table 1 for each group. Representative photomicrographs of lungs from each group were taken to highlight the differences between naïve and allergic lungs. The columnar epithelium (A) and arteriolar smooth muscle (B) were the predominant cells of VIP localization in the naïve lungs. Distal blood vessels were not occluded in naïve lungs and the smooth muscle stained lightly for VIP (C). A marked decrease in VIP occurred immediately after allergen challenge (day 3) in the columnar epithelium (D) and the arteriolar smooth muscle (E). Distal blood vessels were occluded in the allergic state and the surrounding smooth muscle did not stain for VIP (F, I, L). A gradual increase in VIP in the columnar epithelium occurred at days 7 (G) and 14 (J), as did the VIP in the arteriolar smooth muscle at these time points (H, K). Cells around the distal occluded BVs at days 7 (I) and 14 (L) stained for VIP. Scale bars = 50 μ m

Figure 3.

Immunohistochemical localization of neutral endopeptidase in naïve and allergic lungs. NEP localization in various cell types were scored based on staining intensity and summarized in Table 2 for each group. Representative photomicrographs of lungs from each group were taken to highlight the differences between naïve and allergic lungs. The predominant cells of NEP localization were the columnar epithelium (A) and the alveolar lining. NEP localization in the arteriolar smooth muscle was very weak in naïve (B) and allergic states (E, H, K). The smooth muscle surrounding distal blood vessels showed minimal NEP localization in naïve mice (C). A marked decrease in NEP localized to the columnar epithelium occurred at days 3 (D), 7 (G), and 14 (J). Distal blood vessels were occluded after allergen challenge and the surrounding smooth muscle did not stain for NEP (F, I, L). Perivascular recruited cells did not stain for NEP at day 3 (F) but showed localization of NEP at days 7 (I) and 14 (L). Scale bars = 50 μ m

Intensity of VIP localization within naïve and allergic lungs based on immunohistochemical staining

Table 1

Time-point	Columnar epithelium	Basal myocytes	Type II pneumocytes	Type I pneumocytes	Arteriolar smooth muscle	Venular smooth muscle	Endothelium	Alveolar mononuclear cells
Naïve	+++	-	++	+	+++	-	++	+++
D 3	(+)/+	-	+	-	(+)/+	-	++	+++
D 7	+(+)	-	(+)	-	+	-	++	+++
D 14	++	-/(+)	+(+)	+	++	-	++	+++

Regen. Pept. Author manuscript; available in PMC 2011 September 24.

Intensity of NEP localization within naïve and allergic lungs based on immunohistochemical staining

Table 2

Time-point	Columnar epithelium	Basal myocytes	Type II pneumocytes	Type I pneumocytes	Arteriolar smooth muscle	Venular smooth muscle	Endothelium	Alveolar mononuclear cells
Naïve	++	-	+++	++	(+)	-	(+)	++
D 3	(+)	-	+++	+	(+)	-	(+)	+++
D 7	+	-	+++	+	(+)	-	(+)	+++
D 14	+(+)	-	+++	++	-	-	+	+++

Regen. Pept. Author manuscript; available in PMC 2011 September 24.