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Eosinophils Increase Transient Receptor Potential V1 and Substance P Expression in Dorsal Root Ganglia *In Vivo*

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ABSTRACT

Background: Asthma is characterized by excessive bronchoconstriction and cough. Airway nerves control these reflexes. In asthma, an abundance of airway eosinophils cause airway nerve dysfunction by altering neurotransmitter content and neuronal receptor expression.

Objective: To characterize the effects of eosinophils on Transient Receptor Potential V1 (TRPV1) and substance P (SP) expression in dorsal root ganglia sensory neurons.

Methods: Dorsal root ganglia were isolated from wild-type C57BL/6 mice, IL-5 transgenic mice with airway eosinophilia driven by high IL-5 (IL5tg, NJ.1726 lineage), and transgenic eosinophil-deficient (PHIL) mice. Ganglia were immersed for 24 hours at 4 degrees in Zamboni's fixative, immunolabeled with antibodies against TRPV1 and SP, and imaged on a Zeiss laser scanning confocal microscope (LSM780). Neuronal TRPV1 and SP intensity were measured using ImageJ.

Results: In wild-type mice, 24% of dorsal root ganglia neurons expressed TRPV1 and 26% expressed SP. In contrast, in IL5tg mice, 99% of neurons expressed TRPV1 and 97% expressed SP. TRPV1 expression in eosinophil-deficient PHIL mice was similar to wild-type control with 20% of neurons expressing TRPV1. However, SP expression in eosinophil-deficient PHIL mice was increased compared to wild-type, with 73% of neurons expressing SP. In total, 39% of TRPV1-positive neurons also expressed SP in wild-type mice whereas 97% of TRPV1-positive neurons expressed SP in IL5tg mice and 90% of TRPV1-positive neurons expressed SP in PHIL mice.

Conclusions: Eosinophils increase TRPV1 and SP expression in dorsal root sensory neurons. These changes may underlie increased cough and bronchoconstriction in asthma.

INTRODUCTION

Asthma is a chronic respiratory disease characterized by inflammation and narrowing of the airways. Patients with asthma experience recurrent wheezing and shortness of breath. Twenty-four million Americans suffer from asthma, with a disproportionate burden falling on minorities and the poor. Despite advances in asthma treatment, many patients continue to struggle with severe asthma symptoms and in some cases, the condition can be fatal.

Bronchoconstriction (narrowing of the airways) is controlled by airway nerves. Specifically, airway sensory nerves detect irritants including cigarette smoke, changes in pH or temperature, allergens, and pollutants, and relay signals to efferent parasympathetic cholinergic nerves via synapses in the central nervous system (11, 12, 13, 14, 15; **Figure 1**). Parasympathetic nerves then provoke cough and bronchoconstriction via axons that project from the brain to the airways (16). Sensory afferent nerve cell bodies reside in clusters known as ganglia, which include the vagal ganglia located at the base of the skull and dorsal root ganglia located along the thoracic spine (20). Nerve cell bodies in the dorsal root ganglia also provide sensory input from the skin.

In asthma, increased bronchoconstriction occurs due in part to the development of airway nerve dysfunction. Eosinophils, which are a white blood cell subtype often found in asthmatic airways, cause nerve dysfunction (7-8) by altering neurotransmitter content and receptor expression. However, the full range of eosinophils' effects on sensory afferents are not fully known.

Transient receptor potential vanilloid 1 (TRPV1) is expressed by airway sensory nerves (1) and is highly relevant for asthma given that TRPV1 activation triggers cough and since asthmatics have

exaggerated responses (2-5) to TRPV1 agonists capsaicin and citric acid. Previously, studies found that severe asthmatics have increased TRPV1 expression on airway epithelial cells (6). Evidence that TRPV1 mediates cough and bronchoconstriction via activation of sensory nerves and findings that asthmatics have increased responses to inhaled TRPV1 agonists suggest that similar changes in TRPV1 expression may contribute to neuronal dysfunction in asthma (2, 3).

In asthma, neurogenic inflammation occurs when sensory nerve axons (10) release inflammatory mediators such as substance P (SP) (9). SP belongs to the tachykinin family (17) of neuropeptides—a family of neuronal signaling molecules expressed by primary sensory neurons in the respiratory tract (18) that cause airway edema and inflammatory cell recruitment. SP binds to neurokinin receptors (NK1 and NK2). In patients with asthma, airway NK-1R expression is increased and responses to exogenous SP are increased (17), suggesting that SP contributes to airway pathology in asthma as well.

In this study, we evaluated TRPV1 and substance P expression in dorsal root ganglia sensory neurons using immunofluorescence and confocal microscopy in mouse models of asthma. Specifically, we utilized IL5 transgenic mice (IL5tg) with airway eosinophilia driven by interleukin-5 (IL5) over-expression in lung epithelium. In these mice, airway expression of IL5 leads to peribronchial accumulation of eosinophils and pathologic changes seen in human asthma, including goblet cell hyperplasia and airway hyperreactivity (26). We compared TRPV1 and SP expression in IL5tg mice to eosinophil-deficient mice (PHIL) that constitutively express an eosinophil-specific diphtheria toxin that congenitally ablates eosinophils, and to C57BL/6 wild-type mice (WT).

METHODS

Animals

Wild-type C57BL/6 (Jackson Laboratories), IL5tg mice with airway eosinophilia driven by high IL5 (NJ.1726 lineage) and transgenic eosinophil-deficient PHILmice (gift from Dr. James J Lee, Mayo Clinic, Scottsdale, AZ) were handled in accordance with the U.S. Animal Welfare Act. Protocols were approved by the Institutional Animal Care and Use Committee.

Fixation

Mice were sedated with isoflurane and euthanized by subcutaneously injection with 200 μ L of 30 mg/mL pentobarbital. Lungs were perfused with 20 mL of phosphate-buffered saline (PBS) and airways were lavaged with 1 mL of Zamboni's fixative. Dorsal root ganglia from thoracic vertebrae (T3) were dissected from mice and fixed in Zamboni's overnight at 4°C.

Immunohistochemistry

Ganglia were washed in Tris-buffered saline (TBS; pH 7.4) for 1 hour x 5 and blocked in 4% normal goat serum (NGS) with 5% concentrated milk powder in TBS and 1% Triton X-100 overnight at 4°C. Tissues were then incubated in rat polyclonal antibody against SP (1:500 concentration; **Table 1**) and rabbit polyclonal antibody against TRPV1 (1:100) overnight at 4°C. Next, ganglia were washed in TBS 5 x 1 hour and overnight at 4°C. Following washes, tissues were immersed in goat anti-rat 555 (1:1000) F(ab')₂-conjugated secondary antibody and goat anti-rabbit 647 (1:1000) F(ab')₂-conjugated secondary antibody overnight at 4°C. Control slides were not treated with primary antibodies (no primary control); all slides were stored flat at 4°C prior to imaging.

Microscopy

Ganglia were imaged on an LSM780 laser scanning confocal microscope (Zeiss, 63x/1.40 Oil) provided by the OHSU Advanced Light Microscopy Core (Portland, OR). One overview image was taken of each dorsal root ganglia and a tile function was used to stitch sections together.

Image J/ FIJI software

ImageJ software (freeware, NIH, Bethesda, MD) was used to measure fluorescent intensity and to determine the percent co-localization of TRPV1 and SP within each dorsal root ganglia. All identifiable neurons (approximately 140 per DRG) were manually traced and fluorescent intensity of TRPV1 and SP was measured within the defined area. In all images, background fluorescence was determined by measuring fluorescence in a 10 μm x 10 μm area of the slide without any tissue, and then subtracted from total intensity prior to measuring fluorescent intensities of the stained tissues.

Statistics

SP and TRPV1 expression were analyzed using a one-way analysis of variance with a Bonferroni's multiple comparison post-hoc test (GraphPad Software, La Jolla, CA). A *p* value less than 0.05 was considered significant. Data is presented as mean \pm standard error of the mean (SEM).

RESULTS

Eosinophils induce TRPV1 expression in dorsal root ganglia sensory neurons

A total of twelve dorsal root ganglia were analyzed from WT, IL5tg and PHIL mice (n=4/group). In wild-type mice, 24% \pm 5% of dorsal root ganglia neurons expressed TRPV1 while in IL5tg mice, 99% \pm 0.2% of neurons expressed TRPV1 (***p* < 0.0001). Eosinophil-deficient PHIL mice were similar to wild-type, with 20% \pm 13% of neurons expressing TRPV1 (**Figure 2**).

SP expression within dorsal root ganglia sensory neurons is increased in mice with airway eosinophilia and in eosinophil-deficient mice

In WT mice, 26% \pm 5% of dorsal root ganglia neurons expressed SP. In contrast, 97% \pm 3% of neurons expressed SP in IL5tg mice and 73% \pm 4% of neurons expressed SP in eosinophil-deficient PHIL mice (***p* < 0.005; **Figure 3**).

Co-expression of TRPV1 and SP in sensory neurons of mice is increased by eosinophilia

The percentage of TRPV1-positive neurons that also express SP was calculated within dorsal root ganglia of WT mice, IL5tg mice with eosinophilia, and eosinophil-deficient PHIL mice. In wild-type mice, 39% \pm 7% of TRPV1-positive neurons also expressed SP. In IL5tg mice, 97% \pm 3% of TRPV1-positive neurons expressed SP while 90% \pm 5% of TRPV1-positive neurons also expressed SP in PHIL mice (***p* < 0.0001; **Figure 4**).

A majority of dorsal root ganglia neurons express both TRPV1 and SP in mice with eosinophilia.

The percentage of total neurons that expressed both TRPV1 and SP was calculated within dorsal root ganglia of WT mice, IL5tg with eosinophilia, and eosinophil-deficient (PHIL) mice. In WT mice, TRPV1 and SP co-localization occurred in $10\% \pm 2\%$ of all WT neurons. In contrast, in IL5tg mice, TRPV1 and SP co-localization occurred in $97\% \pm 3\%$ of all IL5tg neurons and in PHIL mice, TRPV1 and SP co-localization occurred in $17\% \pm 12\%$ of all neurons (** $p < 0.0001$). **Figure 5).**

DISCUSSION

Asthma is a chronic respiratory disease where airway inflammation causes excessive bronchoconstriction and cough. These reflexes are controlled by airway nerves whose function is modulated by eosinophils. In asthma, eosinophils invoke dysfunction of airway nerves via changes in neurotransmitter content and expression of neuronal receptors. Airway sensory nerves express TRPV1 (1) and release inflammatory mediators like SP (10). For these reasons, the neuropeptide SP and neuronal receptor TRPV1 were of interest in this study. We hypothesized that eosinophils increase TRPV1 and SP expression in dorsal root ganglia sensory neurons. We found eosinophils induce TRPV1 expression in sensory neurons (**Figure 2**). In contrast, regulation of SP expression was more complex, showing upregulation in both the presence of eosinophilia and when eosinophils were congenitally absent.

TRPV1 upregulation likely contributes to asthmatics' heightened cough responses (2-5) to TRPV1 agonists capsaicin and citric acid. Furthermore, since stimulating sensory C fiber afferents with irritants like capsaicin among other noxious stimuli (22) induces a central neuronal reflex that results in bronchoconstriction, increased TRPV1 expression may also contribute to excessive bronchoconstriction in asthma. Our data and other studies suggest that altered TRPV1 expression may be an integral factor leading to dysfunction of airway nerves in asthma (2, 3). In contrast to previous studies that described eosinophils and TRPV1 independently of each other, we examined eosinophils' specific role in regulating TRPV1 expression. Our findings further underscore the importance of studying eosinophils' effects on sensory neuronal receptors.

We also found SP expression was significantly increased in dorsal root ganglia sensory neurons of IL5tg mice (**Figure 3**). Surprisingly, in the absence of eosinophils, SP expression in dorsal root neurons was also increased. These seemingly contradictory findings suggest that eosinophils have an important homeostatic role in regulating neuronal phenotype at baseline. Therefore, both an increase and absence of eosinophils produces alterations in neuronal SP.

SP expression is relevant in asthma because SP-expressing nerves are increased in the submucosa of patients with severe or fatal asthma (19) and asthmatics are hyperresponsive to SP (21). In addition, SP leads to many of the common features of asthmatic inflammation in the airway. For example, SP promotes eosinophil activation and degranulation, mucus cell hypersecretion, and smooth muscle contraction. The neuropeptide SP amplifies local neurogenic inflammation by inducing pro-inflammatory cytokine secretion from inflammatory and epithelial cells in the airway (17). As a result, therapies that limit the degree of neurogenic inflammation may prove effective in asthma. For example, neutral endopeptidase (NEP) cleaves and inactivates SP. Thus, promoting NEP-mediated breakdown of SP may reduce the extent of neurogenic inflammation in asthma (23). Future therapeutic trials may consider this target and others in treatment of clinical asthma.

The percent of TRPV1-positive neurons that also express SP was greatest in IL5tg mice (**Figure 4-5**). Previous studies have suggested TRPV1-expressing neurons release SP (24). Hence, the production of neuropeptides by activated TRPV1 receptors may be an important contributor in the development of asthma by enhancing immune reaction (25). Our study of co-localization has

revealed that populations of neuropeptide and TRPV1-expressing neurons within dorsal root ganglia are substantially impacted by eosinophils.

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Figure 1. Overview of Sensory and Autonomic Innervation

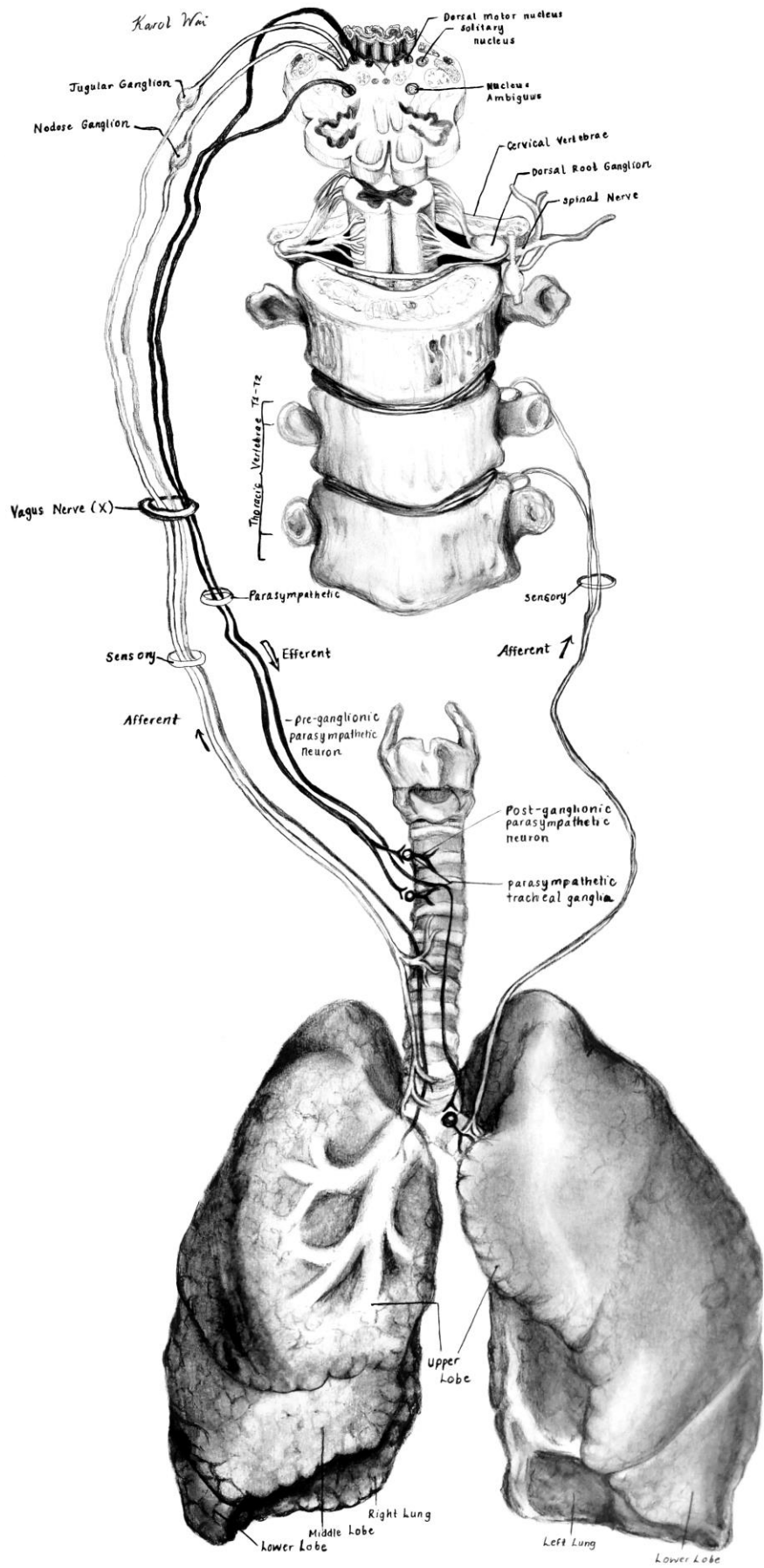


Figure 2

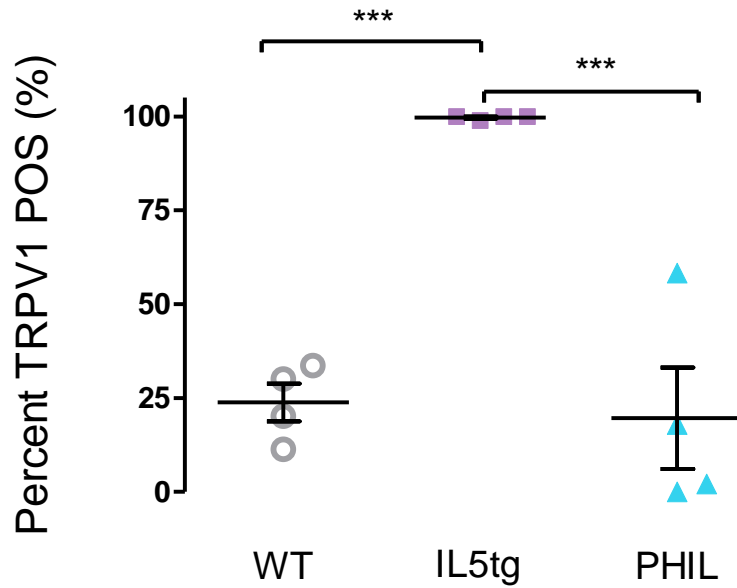


Figure 2. IL5tg mice contain more TRPV1-positive sensory neurons than wild-type and eosinophil-deficient PHIL mice. TRPV1 expression was quantified within dorsal root ganglia sensory neurons in C57BL/6 wild-type mice, IL5 transgenic mice with airway eosinophilia driven by high IL-5 (IL5tg), and transgenic eosinophil-deficient (PHIL) mice. TRPV1 expression was significantly increased in IL5tg mice compared to WT and PHIL mice ($***p < 0.0001$). Data are expressed as the mean \pm SEM.

Figure 3

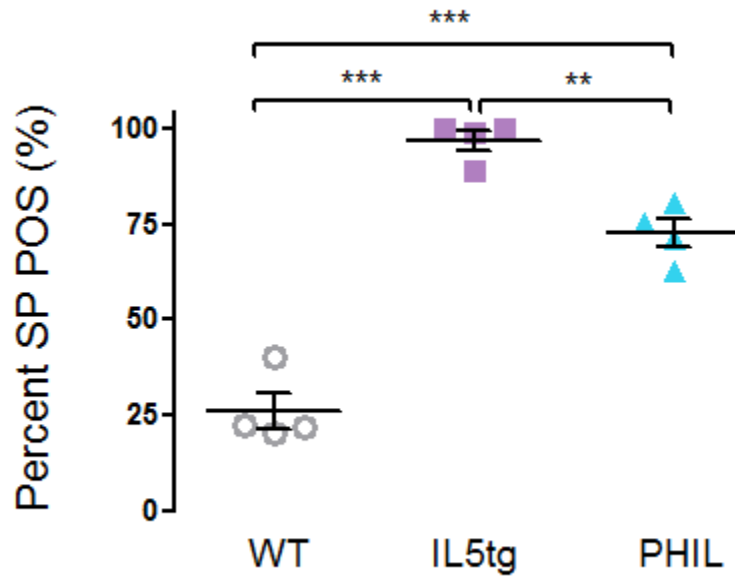


Figure 3. IL5tg mice contain more SP-positive neurons than wild-type and eosinophil-deficient PHIL mice. Substance P (SP) expression was quantified in dorsal root ganglia sensory neurons in C57BL/6 wild-type mice, IL5 transgenic mice with airway eosinophilia driven by high IL-5 (IL5tg), and transgenic eosinophil-deficient (PHIL) mice. SP expression was increased in both IL5tg mice and in PHIL mice compared to WT controls. Data are expressed as the mean \pm SEM. (** $p < 0.005$ and *** $p < 0.0001$).

Figure 4

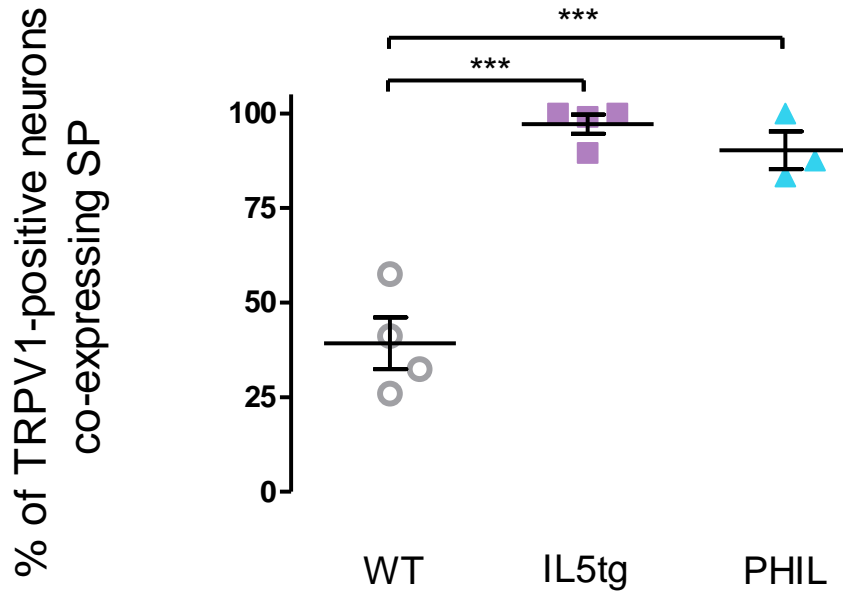


Figure 4. SP expression displayed as a percent of all TRPV1-positive neurons in dorsal root ganglia sensory neurons in C57BL/6 wild-type mice, IL5 transgenic mice with airway eosinophilia driven by high IL-5 (IL5tg), and transgenic eosinophil-deficient (PHIL) mice ($***p < 0.0001$). Data are expressed as the mean \pm SEM.

Figure 5

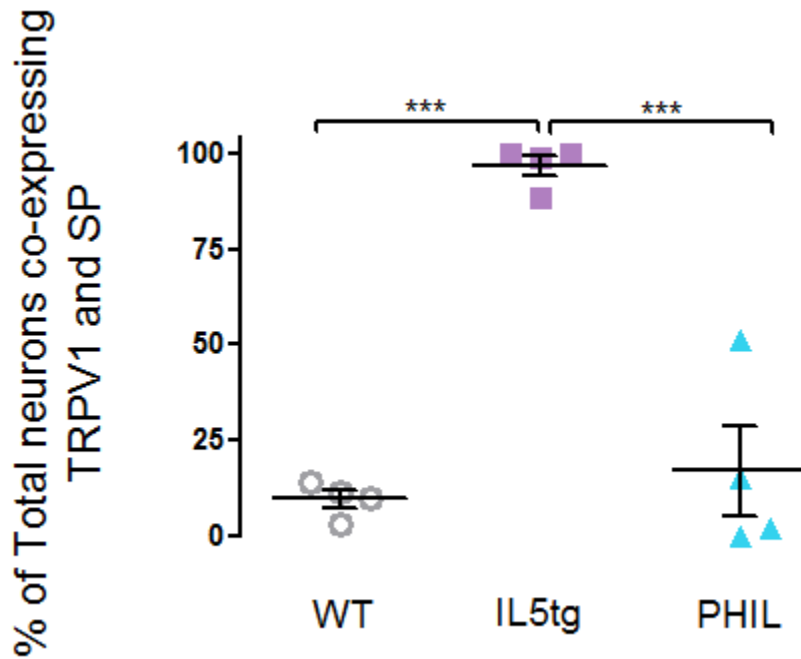


Figure 5. SP and TRPV1 co-localization is increased in IL5tg compared to eosinophil-deficient PHIL mice and wild-type mice. TRPV1 and SP co-expression displayed as the percent of all sensory neurons in dorsal root ganglia of wild-type mice, transgenic mice with airway eosinophilia driven by high IL-5 (IL5tg), and transgenic eosinophil-deficient (PHIL) mice (** $p < 0.0001$). Data are expressed as the mean \pm SEM.

Table 1: Immunohistochemistry Antibodies

Antibody	Concentration (ab: block)	Details
Rat polyclonal antibody against Substance P	1:500	BD Pharmigen #556312 lot 4311672
Goat anti-rat 555 F(ab') ₂ - conjugated secondary antibody	1:1000	Lifotech Alexa Fluor
Rabbit polyclonal antibody against TRPV1	1:100	Alomone labs ACC-030, lot ACC030AN1902
Goat anti-rabbit 647 F(ab') ₂ - conjugated secondary antibody	1:1000	Lifotech Alexa Fluor