Sensory neurons regulate stimulus-dependent humoral immunity.

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Abstract

Sensory neurons sense pathogenic infiltration, serving to inform immune coordination of host defense. However, sensory neuron-immune interactions have been predominantly shown to drive innate immune responses. Humoral memory, whether protective or destructive, is acquired early in life - as demonstrated by both early exposure to streptococci and allergic disease onset. Our study further defines the role of sensory neuron influence on humoral immunity in the lung. Using a murine model of *Streptococcus pneumonia* pre-exposure and infection and a model of allergic asthma, we show that sensory neurons are required for B-cell and plasma cell recruitment and antibody production. In response to *S. pneumoniae*, sensory neuron depletion resulted in a larger bacterial burden, reduced B-cell populations, IgG release and neutrophil stimulation. Conversely, sensory neuron depletion reduced B-cell populations, IgE and asthmatic characteristics during allergen-induced airway inflammation. The sensory neuron neuropeptide released within each model differed. With bacterial infection, vasoactive intestinal polypeptide (VIP) was preferentially released, whereas substance P was released in response to asthma. Administration of VIP into sensory neuron-depleted mice suppressed bacterial burden and increased IgG levels, while VIP1R deficiency increased susceptibility to bacterial infection. Sensory neuron-depleted mice treated with substance P increased IgE and asthma, while substance P genetic ablation resulted in blunted IgE, similar to sensory neuron-depleted asthmatic mice. These data demonstrate that the immunogen differentially stimulates sensory neurons to release specific neuropeptides which specifically target B-cells. Targeting sensory neurons may provide an alternate treatment pathway for diseases involved with insufficient and/or aggravated humoral immunity.

Abstract word count: 246

Keywords: sensory neurons, vagus, B-cell, plasma cell, substance P, vasoactive intestinal polypeptide, humoral immunity, immunoglobulin, *Streptococcus pneumoniae, Alternaria alternata*

Introduction

With each breath, the lung risks exposure to atmospheric infectious agents and allergens. Sensory neurons work alongside immune cells and influence host-defense against infection and injury[1–7], which can exacerbate allergy[8–12]. Stimulation of sensory neurons by cytokines[4,5,8,12,13], proteases[11,14–17], PAMPs[1,1–3] and immunoglobulins[9,18–20] lead to neuronal depolarization. The release of neuropeptides by sensory neurons is a necessary cofactor in immune coordination[21,22]. The unique interplay between neurons and immune cells is pathology-dependent. A growing body of work demonstrates immune stimulus-dependent neuropeptide release and, therefore, differential coordination of varying cell types[1–3,7,23–26]. Such differences speak to the dichotomous neuro-immune response, which can elicit a pro- or anti-inflammatory state[21,27].

Most evidence involving sensory neuron and immune cell crosstalk involves cellular-mediated immunity. For example, sensory neurons inhibit bacterial clearance and fungal infection by suppressing Th1 and Th17-mediated cytokine release and, therefore, suppress neutrophil-mediated clearance of infection in response to acute infection in naïve mice[2,7]. Given that many infectious diseases are recurring, prolonged, or stem from commensal microbes becoming invasive[28,29], further inquiry into the neural influence on humoral immunity in response to infection is warranted.

Conversely, during allergy and asthma, vasoactive intestinal polypeptide (VIP)[8] and substance P[11] release activate ILC2s[8] and dendritic cells[11] to increase eosinophilic infiltration[8] and mast cell degranulation[11]. However, the catalytic event that drives allergic symptoms[30] is a dysregulated increase of allergen-specific IgE and its downstream effects. Given the very high prevalence of allergic disorders, there is a great need to further understand the role of sensory neurons in regulating humoral immunity that may also lead to future therapeutic targets.

B-cells are responsible for immune memory by class switch recombination, a process of VDJ recombination where heavy chains are cleaved from IgM to produce, IgG, IgA, IgE, or IgD depending on the immune effector response required[31]. Early investigations have shown that B-cells respond to exogenous neuropeptides *in vitro* and can steer the predominant release of specific immunoglobulin[32–36]. For example, B-cells express neurotransmitter receptors for, substance P[32], vasoactive intestinal peptide[37,38], noradrenaline, and neuropeptide Y[39,40]. *In vitro*, some of these neurotransmitters appear to increase immunoglobulin release[32–36]. Sensory neurons also mediate B-cell development in the mucosa[37,41] and promote B-cell class switching in the spleen[42] and lung[10]. It was shown that sensory neuron ablation reduced IgE release subsequent to a reduction of B220+ cells in the lung, in response to house dust mite asthma and calcipitrol skin allergy[10]. Substance P was demonstrated to be responsible for IgE class switch recombination *in vitro*; however, whether this was the case *in vivo* is unresolved. Our study investigates whether sensory neuron neuropeptide release can be a global effector for infection and whether the interplay between sensory neurons and B-cells is antigen-dependent.

We analyzed how sensory neurons traffic and stimulate B-cells in response to *Streptococcus pneumoniae* pulmonary infection and *Alternaria alternata*-induced airway inflammation. We show that the release of sensory neuropeptides differs between our model of *S. pnemoniae* pre-exposure and infection and in an *A. alternata*-induced asthma model. Sensory neuron depletion suppressed select sensory neuropeptides in the lungs of each model and, as a result, reduced B-cell and plasma cell recruitment and immunoglobulin release. Neuronal depletion also

exacerbated infection but improved asthma, proving the necessity of sensory neurons to regulate humoral immunity. Supplementation of neuropeptides back into sensory neuron-depleted mice in each model either improved bacterial clearance or worsened asthma features due to a restoration of immunoglobulin production and release- thus supporting the sufficiency of sensory neurons to regulate humoral immunity. In summary, sensory neurons modulate B-cell production of immunoglobulins by variations in neurotransmitter release. The release of neuropeptides is altered by the immune stimulus, which appears to provide a way to steer the humoral response.

Results

TRPV1+ neurons mediate survival and bacterial clearance in pneumonia. Transient receptor potential vanilloid 1+ (TRPV1) neurons have been demonstrated to influence T-cell mediated coordination of immunity by the release of specific neuropepties[2,3,8]. The TRPV1 ion channel responds to capsaicin, protons, prostaglandins, lipids and heat stimuli[43]. TRPV1+ neurons have been found to coordinate cellular immunity and influence T-cell stimulation of neutrophils in acute *Staphylococcus aureus* infection[2] and eosinophils and mast cells in allergic asthma[8]. Thus, we tested how TRPV1+ sensory neurons play a role in humoral immunity.

Using an established model of sensory neuron chemical ablation with resiniferatoxin (RTX)[2,44,45], we suppressed TRPV1 containing neurons similarly in the vagal (nodose/jugular) ganglion by >85% compared to mice injected with vehicle as assessed by qPCR and TRPV1 immunohistochemistry (**Supplementary Figure 1**). RTX, administered by subcutaneous injection, induces global ablation of TRPV1+ neurons, thus blocking sensory neuron vesicle emission of neuropeptides and rendering them ineffective/atrophied[2,44,45]. Although there are indications that dorsal root ganglia (distal site, primarily innervate skin and visceral organs[46]) provide some innervation of the lungs[47], recent tracer studies have demonstrated sparse innervation with an overwhelming majority of lung sensory neuron innervation provided by the vagal ganglia[48].

To investigate humoral immunity in response to *S. pneumoniae* lung infection, we reasoned that replicating the colonization and infection observed in humans, as mice are not naturally colonized with *S. pneumoniae*[49,50]. We established a model of *S. pneumoniae* infection, which involved an initial exposure of mice with a low inoculum on Day 0, and a subsequent exposure on Day 9 to a high infectious dose of bacteria (**Figure 1A, B**). Compared to naïve mice, our *S. pneumoniae* model showed an initially increased bacterial burden. However, sensory neuron ablation with RTX did not suppress the bacterial burden 16hrs after infection (**Figure 1B**). Forty-eight hours after the last infectious dose of *S. pneumoniae*, sensory neuron intact mice almost completely cleared their bacterial burden, while RTX treated mice exhibited an elevated bacterial burden in comparison to sensory intact mice and naïve mice (**Figure 1B**). Importantly, RTX treatment alone did not affect bacterial burden in comparison to vehicle (**Figure 1B**). Additionally, in response to a single high dose of *S. pneumoniae,* in both sensory neuron intact and RTX ablated mice, bacterial burden and IgG remained unchanged (**Supplementary Figure 2**); similar to previous investigations[2]. Furthermore, at 48hrs, bacterial clearance between RTX-treated and targeted vagal ganglion TRPV1 ablation with direct diphtheria toxin (DTX) injection into the vagal ganglia of TRPV1-DT receptor (DTR) mice[51] was similar (**Supplementary Figure 3**). Consequently, our findings suggest that vagal-specific sensory neurons are required to control *S. pneumoniae* during the course of lung infection in our “recall” model.

Next, we determined whether nociceptors regulated pro-inflammatory-cytokine production before clearance of infection in the lung Post-infection, RTX-treated mice showed reduced inflammatory cytokine levels, including IL-1β, IL-6 and TNFα (**Figure 1C, Supplementary Figure 4**). Furthermore, cytokines involved in B-cell stimulation (BAFF, TNFSF13, IL-2 and IL-5) and chemokines involved in B-cell attraction (CXCL13, CCL5 and CCL7) were reduced in RTX mice compared to control mice (**Figure 1C, Supplementary Figure 4**).

TRPV1+ neurons regulate B-cells.

Given that cytokines and chemokines for B-cell recruitment (**Figure 1C**) were reduced, we assessed immunoglobulin levels in lung homogenates. Immunoglobulins were reduced in RTX mice compared to sensory neuron intact mice after 16hrs of infection (**Figure 2A**). *S. pneumoniae* serotype 19F specific IgG was also reduced in RTX as the IC50 calculated from lung homogenate dilutions was higher in RTX compared to Veh (**Figure 2A**). The reduction in immunoglobulins was directly due to a reduction in isotype switched memory, resident memory B-cells, plasma cells and plasmablasts at 16h (**Figure 2B-G, Supplementary Figure 5-7**). In contrast, T-cells recruitment was unaffected by sensory neuron ablation (**Supplementary Figure 8**). The reduction in B-cell populations remained significant at 48h (**Figure 2B-G, Supplementary Figure 6**), in direct accord with an augmented bacterial burden in RTX mice (**Figure 1B**).

To confirm that the bacterial clearance in response to pre-exposure and infection was due to intact B-cells, we compared bacterial burden from our sensory neuron intact and depleted mice to mice lacking mature B-cells (µMT mice). Like RTX mice, µMT mice had a significantly increased bacterial burden at 48hrs after our recall model of *S. pneumoniae* (**Figure 2H**),

Neutrophil depletion in sensory neuron intact mice significantly increased the bacterial burden to a level of RTX mice (**Figure 2I, Supplementary Figure 9**). Neutrophil depletion did not further augment bacterial burden in RTX mice, showing that the interaction between B-cells and neutrophils may be an important mechanism of bacterial clearance in our recall model (**Figure 2I, Supplementary Figure 9**). Therefore, the main effect of sensory neurons in response to infection is to coordinate B-cell homing and immunoglobulin production to enhance neutrophil-mediated *S. pneumoniae* clearance.

TRPV1+ neuron neuropeptide release of VIP stimulates B-cell immunoglobulin release. B-cells express select neuropeptide receptors[52]. Therefore, we reasoned that neurons must attract B-cells within their proximity. Using a sensory neuron reporter mouse (Vglut-TdTomato[48]) we showed that B220+ cells were localized within <20um of sensory neurons in the lungs 48h after the final infectious dose of *S. pneumoniae* (**Figure 3A**). In comparison, infected mice treated with RTX, showed a more dispersed distribution of B220+ cells, extending further from sensory neurons. Of note, whereas RTX depletes TRPV1 positive neurons, sensory neurons have a wide array of genetic composition[48,53], and therefore, some remain following TRPV1 depletion (**Figure 3A**). Thus, it was likely that sensory neuropeptides released by TRPV1+ neurons were suppressed with RTX. Therefore, we tested, known sensory neurotransmitters with noted immunological effects (CGRP[2], Substance P[10,11,54,54–56], VIP[8,38,57,58], NPY[33,40,59]). We show pre-exposure and infection increased NPY and VIP (**Figure 3B,C**). In comparison to sensory neuron intact mice, we demonstrate that RTX suppresses NPY, VIP and CGRP (**Figure 3D,E**). Therefore, we hypothesized that NPY or VIP increases B-cell immunoglobulin release, stimulating neutrophil-mediated bacterial clearance. Neuropeptides bind to specific receptors.

We show that VIP1R and PNY1R are both present on B cells and plasma cells, demonstrating the likelihood of stimulation by sensory neuropeptides (**Supplementary Figure 10**).

We isolated splenic B-cells and cultured them with NPY or VIP. VIP stimulation increased IgG secretion and surface-bound levels in CD19^{mid}/CD138+ plasma cells (**Figure 3F,H**). However, neurotransmitter stimulation did not affect proliferation of B-cells and plasma cells, assessed by Ki67+ intracellular staining (**Figure 3G**).

To test a functional outcome of VIP release in response to *S. pneumoniae*, we supplemented sensory neuron intact and RTX sensory depleted mice with VIP (**Figure 4A**). VIP supplementation rescued RTX mice by suppressing their bacterial burden at 48h (**Figure 4I**). Furthermore, B memory, plasma cells, plasmablasts and lung IgG were increased with VIP supplementation in RTX mice compared to RTX mice supplemented with PBS (**Figure 4B-G**). Finally, *Vip1r*$^{-/-}$ had increased bacterial burden in comparison to WT littermates in response to pre-exposure and infection (**Figure 4H, J**).

Neural stimulation of immunoglobulins exacerbate asthma features. Given that immunoglobulins and humoral immunity are an important mechanism for bacterial clearance, we then asked whether an overproduction of immunoglobulins as a result of neural stimulation could result in immune dysfunction. We hypothesized that this would be neuropeptide driven but, likely mediated by an alternate peptide as previous data supports[10,56]. *A. alternata* causes a swift and distinct rise of IgE in response to four inoculations (**Figure 5A**) as demonstrated previously[60]. *A. alternata*-induced asthma increased airway hyper-responsiveness and goblet cell metaplasia, which are reduced in sensory neuron depleted mice (**Supplementary Figure 11**). Specific B-cell recruitment cytokines are suppressed with RTX sensory neuron ablation (**Supplementary Figure 12**). RTX also suppressed B-cells, immunoglobulins and mast cells in asthmatic mice (**Figure 5A-G, Supplementary Figure 13**). *A. alternata* specific IgE was also reduced in RTX as the IC50 calculated from lung homogenate dilutions was higher in RTX compared to Veh (**Figure 5A**). We reasoned however, that a different neuropeptide mechanism might be at play considering the major difference in immunogen delivery between *S. pneumoniae and A. alternata*. NPY and Substance P are increased during *A. alternata*-induced asthma, in comparison to naïve mice, while decreased with RTX (**Supplementary Figure 14A-D**), VIP was not increased in response to A. alternata induced lung inflammation (**Supplementary Figure 14D**). Substance P receptors (Neurokinin 1 and 2) were not present in our isolated naïve B-cells (**Supplementary Figure 10**). However, substance P also binds to mas related g protein coupled receptor[11] which we show is present on naïve B-cells (**Supplementary Figure 10**). NPY and Substance P increased bound and released IgE in naïve splenic B-cells (**Supplementary Figure 14E-G**). To test whether substance P was able to increase B-cells, we supplemented Substance P into *A. alternata* mice (**Figure 6A**). We show that substance P supplementation increased B-cells in lungs of RTX sensory neuron depleted mice treated with *A. alternata* (**Figure 6B-F**). Further, when *A. alternata* RTX mice were supplemented with substance P, IgE was increased (**Figure 6G**). This was in line with the removal of substance P, as substance P knockout mice showed reduced levels of IgE in response to *A. alternata* induced lung inflammation compared to WT mice with *A. alternata* induced lung inflammation (**Figure 6H**). These data demonstrate that substance P exacerbates asthma features in mice by increasing B-cell production of IgE.

Discussion

Models of repeated immune stimulation show that sensory neurons may stimulate humoral immunity[10]. In contrast, sensory neurons appear to suppress innate responses to select single-dose models of bacterial and fungal infection[1–7]. Recent data show that sensory neurons modulate B-cell populations in the lungs[10] and contribute to B-cell maturation in the mucosa[37,41]. Select neuropeptides, released by sensory neurons, can induce IgE, IgG and IgA release *in vitro*[32–36]. Therefore, we reasoned that sensory neurons could play critical roles in humoral immunity[10,61–63]. The differential roles of neurons with innate and adaptive immunity may provide an additional explanation why sensory neurons can produce divergent results depending on the underlying immune stimulus, which is likely dependent on the timing and type of neuropeptide released.

While humans are colonized with pneumococcal strains at birth, mouse models require pre-exposure prior to infection in order to engage humoral memory and mimic the human condition[28,29,49,50]. Depletion of sensory neurons reduced B-cell populations, IgG, and neutrophil activity, which subsequently augmented bacterial burden in our recall model. This humoral regulation was due to the release of VIP from sensory neurons and the resulting stimulation of plasma cells via the VIP1 receptor. We demonstrate that B-cells have VIP1 receptors, which stimulate PKCs that are critical to the development and release of immunoglobulins[64,65].

Our results are consistent with early *in vitro* data which show that VIP and substance P can stimulate B-cell immunoglobulin release[32–36]. In the context of bacterial lung infection, our results contrast with the finding that sensory neurons suppress immunity in response to acute infection[2]. Specifically, sensory neuron release of CGRP suppressed bacterial clearance of *Staphylococcus aureus* by dampening γδT cell-neutrophil stimulation[2]. When we used a single dose of *S. pneumoniae*, we see that sensory neuron depletion had similar bacterial burden and IgG as sensory neuron intact mice 48 hours following the single challenge. In response to pre-exposure and infection, CGRP was not significantly increased. We suggest that CGRP may be necessary for early innate neuroimmune interaction, which is superseded by VIP upon re-infection.

B-cells can become dysregulated and exacerbate diseases. In the context of allergen-induced lung inflammation, we show that sensory neurons become hypersensitized and stimulate B-cells. This is in direct accord with many investigations demonstrating that hypersensitization of sensory neurons leads to augmented cytokine release and subseqeunt eosinophil trafficking by T-cells or resident innate lymphocytes[8,9,26]. Our study extends these findings by showing that sensory neuron depletion reduces B-cell-attracting cytokines and as a result reduces B-cell recruitment. Mast cells were also reduced in sensory neuron depleted mice. It is likely that the combined reduction in B-cells and IgE release also reduced mast cell stimulation. Conversely, mast cells also harbor neuropeptide receptors[66] and sensory neuron ablation could result in a direct reduction of mast cell stimulation.

Substance P released by sensory neurons regulates B-cell stimulation in the asthma model. We and others show the ability of Substance P to induce class switch recombination[10]. We further demonstrate that, instead of tachykinin receptors, B-cells express Mas related gene protein-coupled receptors that are stimulated by substance P[11]. Our data significantly extend previous data by showing that the chemical ablation of neurons reduced B-cell populations and IgE levels. Further, when substance P was supplemented in asthmatic sensory neuron depleted mice, IgE was once again restored. Finally, genetic ablation of the ability to produce Substance P completely prevented IgE production in response to *A. alternata.*

While the direct effects of neuropeptides on B-cells have been demonstrated, recent findings show that the lymph node innervation by sensory neurons is restricted to the macrophage-rich medulla, not the lymphocyte-rich cortex[61,67]. Therefore, sensory neurons or neural circuits are unlikely to recruit B-cells from lymph nodes or secondary lymphoid tissues. In line with this reasoning, we show no changes in B-cells in the spleens or bone marrow in response to sensory neuron depletion. Our data demonstrate that the effects of sensory neuropeptides are confined to the lungs and produce these effects locally. Tissue resident B-cells establish themselves after primary infection or colonization[68], and are poised to release antibodies[69,70] upon antigen encounter with a challenge infection. Our data demonstrate that B-cells cluster around neurons and that the changes to B-cell numbers were only in the lungs. Further, CD62L negative B resident cells were prominent in our model and reduced with sensory neuron ablation, demonstrating that tissue-resident B-cells were employed upon infection after pre-exposure. In contrast to the lengthy time course required for germinal center-mediated B-cell maturation, our rapid antibody responses were consistent with tissue-resident extra-follicular B-cell antibody production[71,72]. We suggest that neuropeptides act to assist in the maturation and development of tissue-resident B-cells.

Our data provides a new role of sensory neuropeptides acting as B-cell stimulants and highlights the humoral influence of sensory neurons. This study demonstrates that sensory neurons are critical in regulating pulmonary humoral immunity and the outcomes of bacterial lung infections and asthma. Targeting neuro-immunological communication through VIP and Substance P or other potentially uncovered molecular mechanisms may be an effective approach to enhance host protection or reduce allergic responses by influencing immunoglobulin production through accessory pathways.

METHODS

Mice. All animal experiments were approved by The Lundquist Institute at Harbor UCLA Institutional Animal Care and Use Committee protocol # 32183. Mice were housed in a specific-pathogen free animal facility at The Lundquist Institute. C57BL/6J, Tac-/- (B6.Cg-Tac1tm1Bbm/J) and, µMT (B6.129S2-Ighmtm1Cgn/J) mice were purchased from Jackson Laboratories. Vglut-TdTomato mice were provided by X. Sun (UCSD- crossed from Vglut2cre- and RosaTdTomato originally attained from JAX B6J.129S6(FVB)-Slc17a6tm2(cre)Lowl/MwarJ and B6.Cg-Gt(ROSA)26Sortm14(CAG-tdTomato)Hze/J). TRPV1-DTR mice were obtained from Dr. Isaac Chiu, with MTA provided by M Hoon (NIH). VIP1R-/- were kindly donated by Joseph Pisegna, Patrizia Germano and, James Waschek (UCLA, MGI: 177616). At the start of experiments micer were 5-8-weeks old. Age-matched male and female mice were used for experiments.

***Streptococcus pneumoniae* cultures**. *S. pneumoniae* strain 49619 serotype 19F was purchased from ATCC. Cultures were originally grown on Tryptic Soy Agar (BD Biosciences 236950) supplemented with 5% defibrinated sheep's blood (Remel R54012) and cultured for 24h, then re-cultured for an additional 24h in 5%CO2 at 37°C. Colonies were then picked for shape and hemolytic activity and cultured in Todd Hewitt Broth supplemented with 2% Yeast Extract (Fisher 50489152) or Brain Heart infusion media (BD DF0037178) in 5%CO2 at 37°C. Cultures were adjusted to OD600 1.0 and resuspended in PBS prior to inoculation.

Infection model. Colonies were checked for OD, then spun at 4000 rpm, 10 min, washed in PBS. On day 0, CFUs were diluted to obtain 10^4 CFU/ml. Mice were then infected with 50 µl of

10^4 CFU/ml in PBS intranasally. Then on day 9, mice were infected with 50 µl of 10^8 CFU/ml in PBS intranasally, experiments took place 16 or 48 hours later. Control animals were intranasally infused with 50µl PBS only.

Allergy model. *A. alternata* extract was purchase from CiteQ Laboratories (09.01.26) and dissolved to yield a final concentration of 25ug/ml. Then mice were inoculated intranasally with 50ul on days 0, 3, 6, 9 where experiments took place 16 or 48 hours later[73]. All control mice received PBS in the same volume intranasally.

Sensory neural ablation. For chemical ablation of TRPV1+ neurons, mice 5 weeks of age were treated with RTX (Adipogen 502053716) as previously described[2,4,7]. Mice were injected subcutaneously in the dorsal flank on consecutive days with three increasing doses of RTX (30, 70, and 100 µg/kg) dissolved in 2% DMSO with 0.15% Tween 80 in PBS. Control mice received vehicle.

For targeted TRPV1+ neuron depletion, we injected 20 ng DT in 200 nl PBS containing 0.05% Retrobeads (LumaFluor INC) into nodose/jugular/petrosal VG with a nanoinjector (Drummond Scientific Company)[74]. Mice were anesthetized with 87.5mg/kg ketamine and 12.5mg/kg xylazine. The vagal ganglion was exposed after a midline incision in the neck (~1.5 cm in length). DT was gently injected at a rate of 5nl/s. Once completed, the injection needle remained in the ganglia for 2-3 minutes in order to reduce spillover of DT. This process was repeated for the vagal ganglion on the other side of the body.

Neurotransmitter dosing. Vasoactive intestinal peptide (200ng in 50 µl PBS) was delivered intranasally at 48, 24, 0h before and 24h after (for 48h harvest only) final infection with 10^8 CFU of *S. pneumoniae*. Control mice received PBS at the same timepoints. When mice received bacteria on the same day, VIP was combined with the bacterial inoculum.

Substance P (1000ng in 50uL PBS) was delivered intranasally on Days 5,6,7,8,9 of the *A. alternata* model. On days where *A. alternata* was delivered, Substance P was mixed with *A. alternata.*

Neutrophil depletion. For neutrophil depletion, we followed an established protocol[75]. Mice were injected i.p. with 150 µg of anti-A18 (clone 1A8, BioXCell BE-0075-1) (in 200 µl) per mouse 48, 24, 0h before lung infection and 24h after lung infection (48h analysis only). Control mice received 150 µg of rat IgG isotype control (2A3 BioXCell).

Bacterial burden determination. Lungs were weighed and then homogenized in 2ml sterile PBS with a tissue homogenizer. Homogenates were serially diluted on tryptic soy agar plates with 5% sheep's blood and 10ug/ml neomycin (Fisher Biochemicals BP2669-5). The bacterial CFU were enumerated after overnight incubation at 37°C and 5% CO2.

Quantitative real-time PCR. RNA was isolated from tissues or cells using the inTron Easy spin Total RNA extraction Kit (Boca Scientific 17221), which was reverse transcribed to cDNA with an Tetro cDNA Synthesis Kit (Tetro biosystems NC1352749). Relative gene expression was determined by quantitative real-time PCR on a QuantStudio 3 System (Thermo Fisher Scientific) with TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific 4444557) with the Taqman probe sets (ThermoScientific Applied Biosystems). Expression values relative to HPRT were calculated by Δ ΔCTT method. All taqman probes utilized were purchased from Thermofisher (Vip1r Mm00449214_m1; Vip2r Mm01238618_g1; Tac1r Mm00436892_m1; Tac2r

Mm01175997_m1; NPY1r Mm00650798_g1; MRGPRA Mm01984314_s1; TRPV1 Mm01246300_m1; HPRT Mm03024075_m1).

Flexivent lung function. Mice anesthetized with ketamine/xylaxine (87.5/12.5mg/kg), paralyzed with pancuronium bromide (MP Biomedicals ICN15605350) and subject to methacholine challenge with 200µl of doubling doses of methacholine (MP Biomedicals ICN19023110) dissolved in PBS for 20 breaths. Single and double compartment model Respiratory resistance was measured with the Flexivent system (SCIREQ) as previous[74]. Both absolute and percentage change values from PBS were calculated.

Tissue collection. For flow cytometry, mice were euthanized by Isoflurane inhalation. The lungs were then dissected and flushed with PBS, coarse dissected and incubated at 37°C for 45min in 1.75mg/ml collagenase IV (C4-22-1g Sigma) in PBS; then washed, macerated through a 21 gauge needle and filtered through a 70 µm mesh filter, then treated for flow cytometry. Spleens were macerated through a 70 µm mesh filter, then treated for flow cytometry. Bone marrow was flushed with RPMI media supplemented with 2mM EDTA and 10% FBS, filtered through a 70 µm mesh filter, then treated for flow cytometry.

For cytokine analysis, lungs were dried, weighed, then macerated in 2ml PBS supplemented with 25ul protease inhibitors (consisting of AEBSF HCl (100 mM), Aprotinin (80 µM), Bestatin (5 mM), E-64 (1.5 mM), Leupeptin (2 mM) and Pepstatin (1 mM) Tocris 5500), spun at 8000g 4 min 4C and then the supernatant was snap frozen and stored t -80°C until analysis.

Cytokine, neurotransmitter and, immunoglobulin analysis. Cytokine and immunoglobulin levels in lung homogenates were measured through Luminex multiplex assay using R&D discovery or Thermo kits; analytes were measured with the Luminex 200 system. Enzyme-linked immunosorbent assay (ELISA) kits were used according to the manufacturer's instructions (R&D systems, Raybiotech or Thermofisher) and analyzed with a BioTek Synergy H1 plate analyzer. Luminex and ELISA kits are all commercially available and listed in supplementary materials list.

Specific immunoglobulin analysis. For specific IgG determination, crude S. pneumoniae (12.53µg/ml ATCC 49619) extract was incubated in 96 well plates overnight at 4°C, then blocked with goat serum for 1h at 4°C. Then log dilutions of lung homogenates (from groups above) were incubated at RT for 2 hours. Goat anti-mouse IgG HRP conjugated secondary antibody (Abcam ab205719, 1:1000) was then added and incubated for 2 hours RT. The ELISA was developed with 0.5% TMB solution (Fisher AAJ61325AP) and stopped with 2N H2SO4 (Fisher 828016). For specific IgE determination, *A. alternata* (100µg/ml CiteQ 09.01.26) was incubated as above and developed as above except Goat anti mouse IgE HRP secondary (Thermofisher PA1-84764, 1:1000) was used. OD450 for each dilution was used with a 4 point sigmoidal curve to determine IC50 for each sample, in each group.

Flow cytometry. Red blood cells were lysed with ACK lysing buffer (Gibco A10492-01), treated with Fc Block (Biolegend Trustain 101320), and resuspended in FACS buffer (HBSS-Gibco 10010-023 with 2% FBS Gibco. Incubations with antibody cocktails were conducted at 4°C for 60 min, and samples were subjected to two washes and resuspension in FACS buffer (HBSS +2% FBS Sigma F8192). For intracellular staining, cells were fixed/permeabilized with BD cytofix/cytoperm kit (554714), washed and stained overnight at 4°C. Flow cytometry was conducted on a Symphony A5 flow cytometer (BD). Data were collected with BD DIVA software, and files were analyzed with FlowJo (Treestar, version 10.0.8r1). A live-cell stain (eFluor 780,

ebioscience) was used to exclude dead cells. Gating strategies are provided in **Supplementary Figure 5.** Positive staining and gates for each fluorescent marker was defined by comparing full stain sets with fluorescence minus one (FMO) control stain sets. Antibodies used: Biolegend- CD11c (Clone N418), MHCII (Clone M5/114.15.2), CD64 (Clone X54-5/7.1), Ly6g (Clone 1A8), CD11b (Clone M1/70). CD24 (Clone M1/69), CD117 (Clone 2B8), CD4 (Clone GK1.5), CD8 (Clone SK1), T-bet (Clone 4B10), GATA3 (Clone 16E10A23), B220 (Clone RA3-6B2), CD19 (Clone 6D5), CD62L (Clone MEL-14), CD44 (Clone IM7), CD138 (Clone 281-2), IgG1 (Clone RMG1-1), IgE (Clone RME-1) CD45 (Clone 30-F11), MRGPRX (Clone K125H4); BDbiosciences- IgM (Clone IL/41), IgD (Clone 11-26C.2a), RorγT (Clone Q31378; Invitrogen- Siglec F (Clone 1RNM44N), TCR (Clone GL-3), CD38 (Clone 90), Ki67 (Clon SolA15); AlomoneLabs- VIP1R (Clone AB_2341081).

Immunofluorescence, histology and microscopy. For immunostaining, lungs were perfused with PBS followed by 4% paraformaldehyde (PFA) in PBS. Lungs were dissected and postfixed overnight in 4% PFA/PBS at 4 °C, incubated at 4 °C with 30% sucrose/PBS for 2 days and stored in 0.1% sodium azide in PBS until cutting. Lungs were embedded in optimal cutting temperature compound (OCT, Tissue-Tek, PA) and 50-µm cryosections were cut at -20°C and then blocked for 4 h in PBS with 10% donkey serum, 2% bovine serum albumin (BSA), and 0.8% Triton X-100. Sections were immunostained with the following antibodies: B220 (Biolegend 103225; 1:100 FITC).

Vagal (nodose/jugular) and dorsal root ganglia were dissected, fixed in 4%PFA for 1 hour, cryopresereved in 30% sucrose overnight (all 4C), then placed in OCT and 14µm sections were cut and placed on gelatin coated slides. Sections were stained with TRPV1 (Alomone labs Clone- AB_2313819 1:200) followed by incubation with secondary antibody and secondary (Goat Anti Rabbit IgG H&L, Abcam). All sections were mounted in Prolong diamond antifade compound (Thermofisher P36961).

For histology, lungs were embedded in paraffin and cut on a microtome (Leica) 5µm and stained with hematoxylin (Epredia 6765001) and eosin (Epredia 6766007), gram stain (Sigma HT90T-1KT) or periodic acid, schiffs reagent (Sigma395B-1kit). Blood smears were stained with Giemsa solution (AB150670). Additionally, to confirm neutrophil counts in select experiments, Giemsa stain (Abcam AB150670) was used on lysed blood smears. Histological slides were mounted with permount toluene solution.

Sections were either imaged with a Leica SP8 confocal microscope or Leica Thunder system. Data were imaged and analyzed with Leica LASx software. For quantification of the proximity of B-cells to nerves, LASx online calipers were used to measure the distance from cell-to-nerve in 3D from the z-stack.

B-cell culture. Spleens from 3 mice were pooled to yield one sample. Spleens were macerated through a 70µm mesh filter and cells were separated using the EasySep Mouse B-cell separation kit (StemCell Technologies 19854A). Cells were then treated in B-cell culture media made using 438 mL RPMI 1640 (Gibco 11835-030), 2.5mL 1 M HEPES buffer (Sigma H3662), 5 mL GlutaMAX (Thermo Fisher Scientific 35050061), 5 mL penicillin/streptomycin stock (Sigma P0781), 30 mL heat-inactivated fetal bovine serum stock (Gibco A38400-01), and 454 µL β-mercaptoethanol (Gibco 21985-023) and vacuum filter sterilized (0.22 µm filter)[76]. Cells were then treated with either media + IL4 (20ng/ml, Sigma Aldrich I1020-5UG) + LPS (10µg/ml, L6529-1MG Sigma Aldrich), media+IL4+LPS+NPY (50ng/ml, Tocris 1153), media+IL4+LPS+VIP

(50ng/ml, Tocris 1911) or, media+IL4+LPS+Substance P (50ng/ml, 1156 Tocris). After 96 hours media was stored at -80°C until immunoglobulin analysis. Cells were stained and analyzed with a Symphony A5 flow cytometer and data were processed with FlowJo (Treestar 10.881).

Sample size and statistical analysis. For bacterial burden studies, we used animal numbers between 5 and 12 mice per experimental group/genotype. For measurement of cytokine, neuropeptide and immunoglobulins 5-8 mice per group/genotype were used. For flow cytometry 5-10 mice per group/genotype were used. For in vitro experiments, at least 3 animals were pooled into one sample and 5-7 replicates were used. Bacterial burden data were analyzed with the two-way RM ANOVA (time x group) with either Tukey or Holm-Sidak post-hoc test; FACS and cytokines were compared with ANOVA with Tukey or Holm-Sidak post-hoc tests, two-tailed unpaired t tests for parametric analyses, or Mann–Whitney test for nonparametric analyses. Data were plotted in Prism (GraphPad).

Acknowledgements

NJ is supported by NIH grant R21AI159221, UCLA CTSI UL1TR001881-01 and T32IP4707 of the Regents of the University of California Tobacco-Related Diseases Research Program. M.S. is supported by NIH grant, R01DE031382. T.A.D. is supported by NIH AI171795 and Veterans Affairs BLR&D BX005073.N.M. and D.A. are supported by a California Institute for Regenerative Medicine Stem Cell Biology Training Grant EDUC4-12837. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. We are grateful to Dr. Isaac Chiu (Harvard) for providing TPV1-DTR mice as per the direction of Dr. Mark Hoon (NIH). We are grateful to Dr. Xin Sun for providing Vglut-Tdtomato mice. Figures 1A, 2H,I, 4A, 6A and Supplementary Figures 2A, 11A were made using Biorender.

Author contributions

D.A. and F.Z. contributed to experimental design, conducted experiments and analyzed data. A.M. and N.M. contributed to experimental execution and data analysis. P.G. J.P., T. A.D assisted with experimental design and analysis. M.S contributed to study design and manuscript preparation. N.J. designed the study, conducted experiments, analyzed data, prepared the manuscript and figures. All authors agree on the manuscript.

Conflict of interest statement

D.A, M.S. and N.J. declare the following competing interest. U.S. Patent Application Serial Number 63/492,846. Methods of using sensory neuron neurotransmitters to enhance humoral immunity. The remaining authors do not declare competing interests.

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Figure 1.

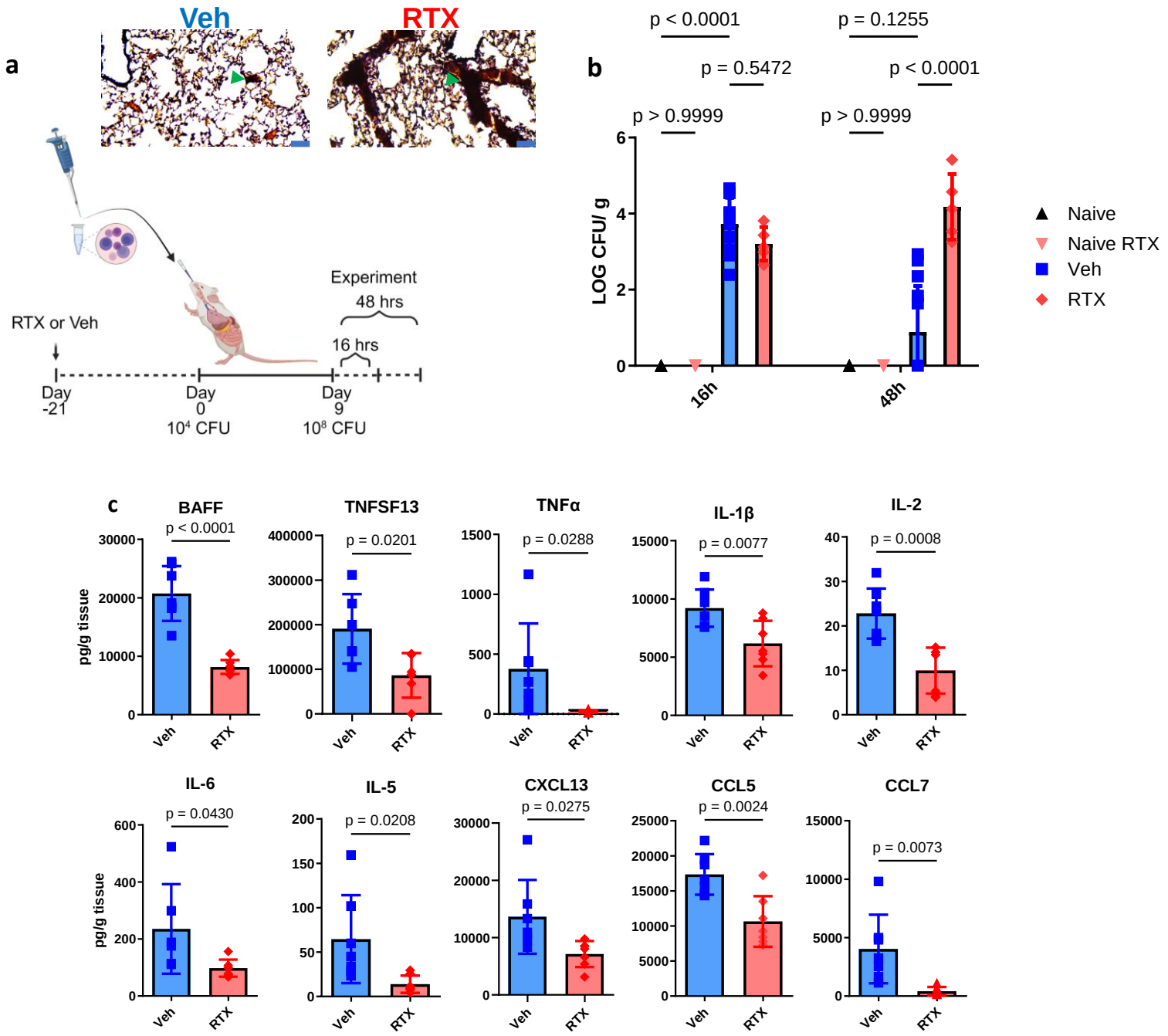

Figure 1. Sensory neurons are required for successful clearance of *S pneumoniae* following pre-exposure and infection. a) Model of *S pneumoniae* pre-exposure and infection. Mice received escalating doses of RTX or Veh starting 21 days prior to the beginning of inoculation. 10^4 CFU of *S pneumoniae (Serotype 19F, ATCC49619)* was delivered on day 0 to expose mice, then 10^8 CFU was delivered on Day 9. Experiments took place 16 or 48h later. Gram stain shows *S pneumoniae* infection (purple, green arrows point to example of gram positive space) was greater in RTX compared to Veh. Scale bar=20µm.

b) Bacterial recovery from lungs in untreated sensory neuron intact mice (Naïve), untreated sensory neuron ablated mice (Naive RTX), pre-exposed and infected sensory neuron intact (Veh) or pre-exposed and infected sensory neuron ablated mice (RTX). 16h: Naïve n=6, Naïve RTX: n=5, Veh: n=13, RTX: n=5. 48h: Naïve n=4, Naïve RTX: n=5, Veh: n=13, RTX: n=5. Two-way ANOVA with Holm-Sidak post hoc test. Data were pooled from three independent experiments. **c)** Quantification of B cell activating factor (BAFF), APRIL13 (TNFSF13), TNFα, IL-1β, IL-2, IL-6, IL-5, CXCL13, CCL5, CCL7 in sensory neuron intact (Veh n=7) and sensory neuron ablated (RTX, n=6) mice 16h following the final 10^9 CFU dose of *S pneumoniae*. Samples were analyzed with Luminex bead assay. IL-6 was re-run with ELISA. Two-sided t-test. Data were pooled from 2 independent experiments.

Figure 2.

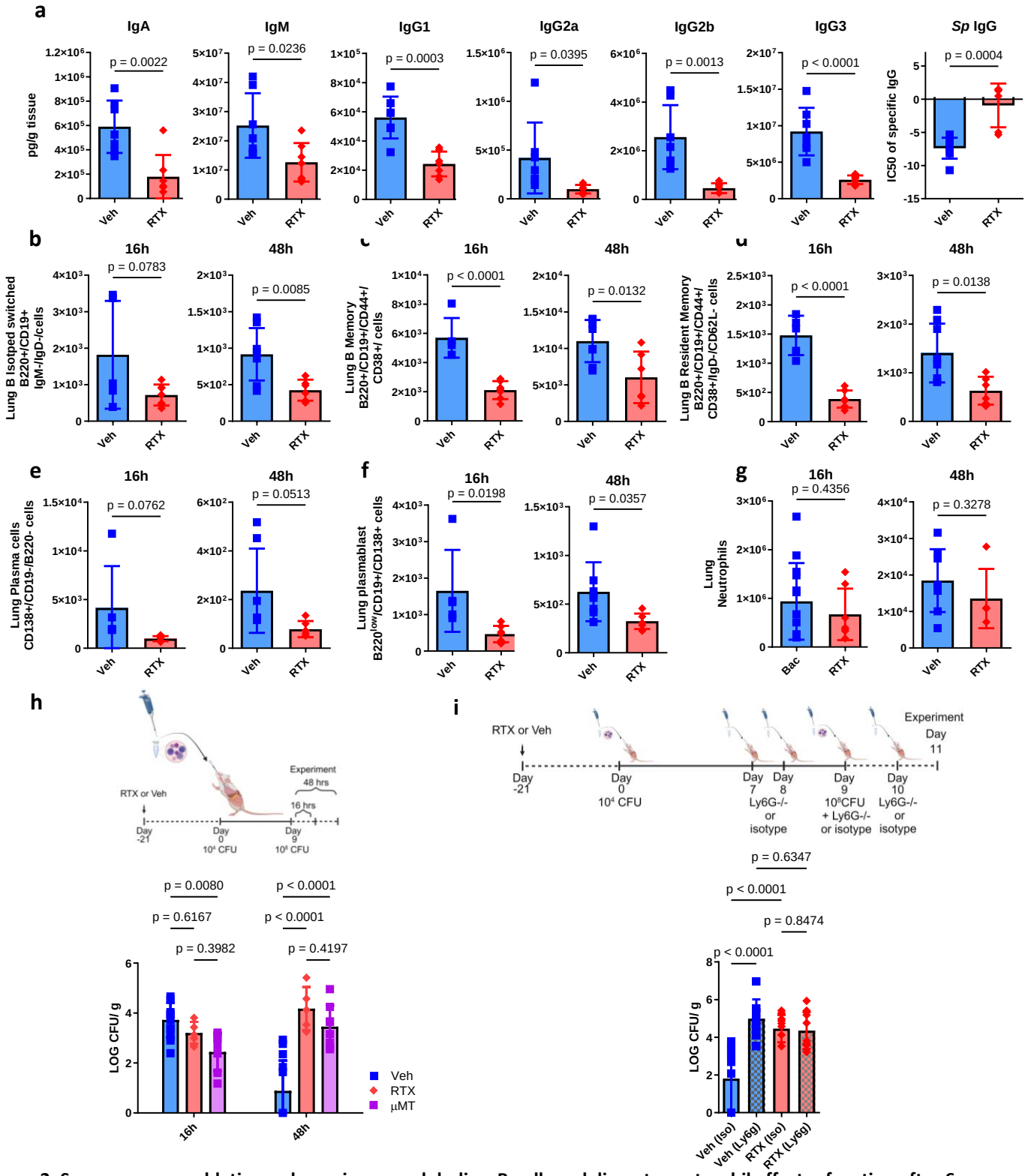

Figure 2. Sensory neuron ablation reduces, immunoglobulins, B-cells and disrupts neutrophil effector function after *S pneumoniae*. a) IgA, IgM, IgG1, IgG2a, IgG2b, IgG3 were reduced with sensory neuron ablation after final infection. Veh: n=7, RTX: n=7. Isotyping Luminex was used to analyze lung homogenates. Two-sided T-test. IgG specific to *S. pneumoniae* was tested with crude *Sp* extract. Serial dilutions were used to model the IC50 with a 4 point log model to determine differences between Veh (n=8) and RTX (n=6). Two-sided T-test. Data were collected from two independent experiments. **b**) Isotype switched B220+/CD19+/IgM-/IgD-, **c**) B-memory B220+/CD19+/CD44+/CD38+, **d**) B resident memory B220+/CD19+/CD44+/CD38+/IgD-/CD62l-, **e**) Plasma cells CD138+ and, **f**) Plasmablasts CD138+/B220-/CD19- cells were downregulated by sensory neuron ablation at 16 and 48h after infection. **g)** neutrophils remained similar between sensory neuron intact and ablated conditions. Cells were gated on live/CD45+ cells and counting bead calculation determined total cell population. Red gate is the measured cell population. Veh: n=5-14, RTX: n=6-7. Two-sided t-test. Data from 3 independent experiments. **h)** µMT mice had reduced burden at 16h in comparison to sensory neuron intact mice (Veh). Sensory neuron ablated (RTX) mice demonstrated a bacterial burden similar to mice with immature B cells (µMT) 48h after infection. Sensory neuron intact mice (Veh) reduced their bacterial burden at 48h. Veh n=13, RTX n=5, uMT infected n=7. Data from four independent experiments. Two-way ANOVA with Holm-Sidak tests. **i**) Ly6G A18 neutrophil depletion antibody or isotype control was delivered before, during and after the final infection dose of *S pneumoniae.* Bacterial burden (Log CFU/ g tissue mass) from lung homogenates was greater with neutrophil depletion in sensory neuron intact infected mice compared to isotype control. Sensory neuron depleted mice (RTX) did not further increase bacterial burden with neutrophil depletion demonstrating that B-cell neutrophil interaction was disrupted. One-way ANOVA with Holm-Sidak post-hoc test. Veh(Iso): n=12, Veh(Ly6g): n=8, RTX(iso): n=10, RTX(Ly6g):n=9. Data were pooled from two independent experiments.

Figure 3.

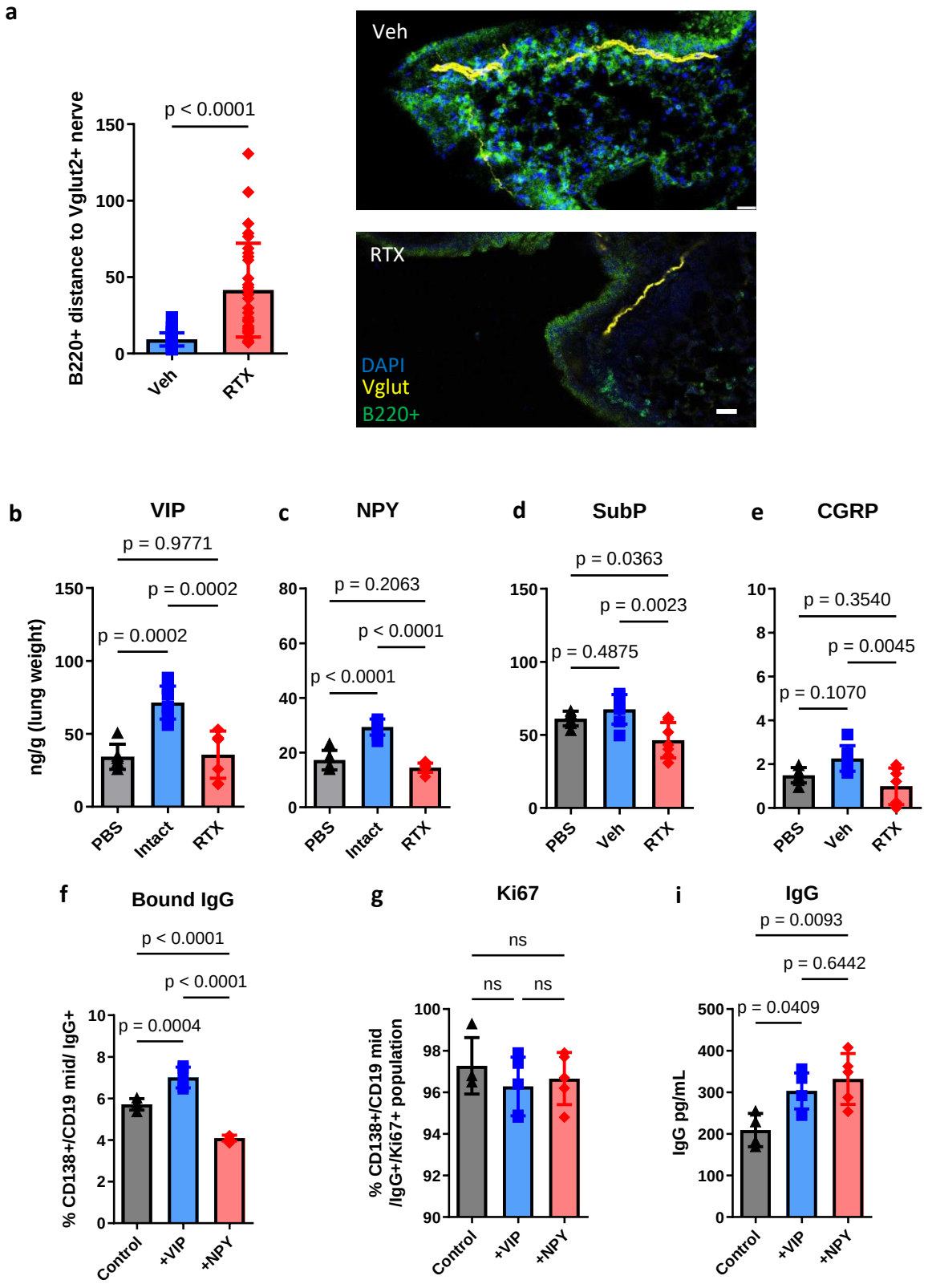

Figure 3. Vasoactive intestinal peptide attracts B-cells and regulates immunoglobulin production and release. a) B220+ cells cluster around sensory nerves from lungs of sensory neuron intact mice (Veh). In RTX sensory neuron depleted mice, B220+ cells are further dispersed. 30 cells (10 cells per sample) from n=3 mice for Veh & RTX. Two-sided t-test. **b-e)** Prominent sensory neuron neuropeptides were increased in lung homogenates 16 hours after pre-exposure and infection with *S. pneumoniae*. **b**) VIP was increased with infection and suppressed with sensory neuron depletion. **c**) NPY was increased with infection and suppressed with sensory neuron depletion. **d**) Substance P did not significantly increase following infection with *S pneumoniae.* **e**) CGRP did not significantly increase following infection with *S pneumoniae.* Lung homogenates analyzed with ELISA. PBS: n=6, Veh: n=7, RTX: n=7. One-way ANOVA with Tukey's post hoc test. Data from 2 independent experiments. **f-h)** B cells were isolated from spleens of naïve mice and cultured in media with IL4+LPS, and supplemented with VIP or NPY. Cells and media were sampled after 96 hours of incubation. **f**) VIP increased bound IgG in CD138+/CD19+ plasma cells. **g**) Proliferation as assessed by intracellular Ki67 staining was not affected by neuropeptides (from CD138+/CD19+ gate). **h**) Secreted IgG was increased by VIP and NPY. IL4+LPS: n=4, +VIP: n=5, +NPY: n=5. One-way ANOVA and Tukey's post-hoc test. Data from 2 independent experiments.

Figure 4.

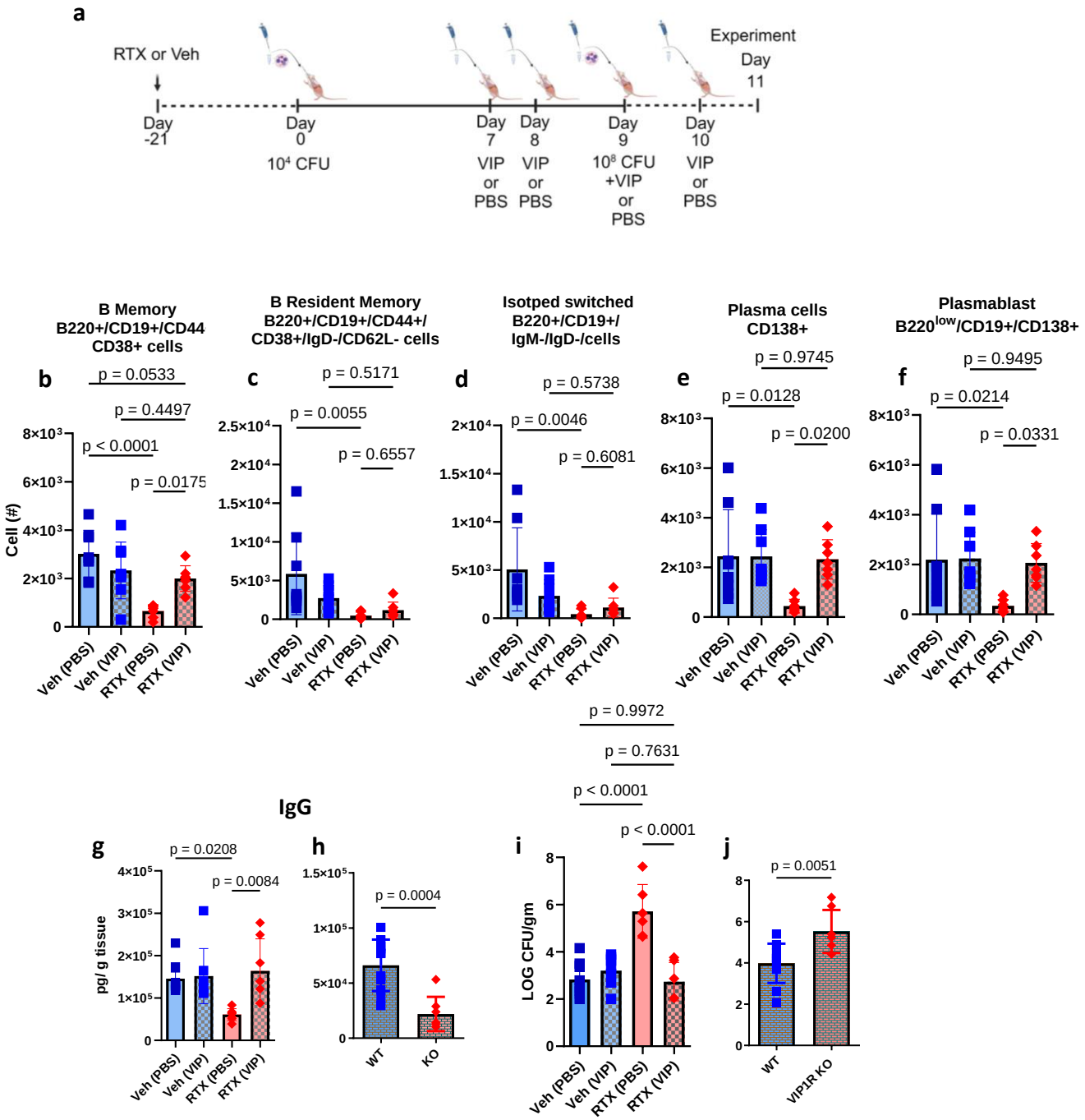

Figure 4. Vasoactive intestinal peptide supplementation improves bacterial clearance, memory B cells, plasma cells and IgG in response to pre-exposure and infection with *Streptococcus pneumoniae*. a) Vasoactive intestinal peptide (10ng/µl) or vehicle (PBS) was delivered as per the schematic in addition to the model of *Streptococcus pneumoniae* pre-exposure and infection. Cells were gated on Live/CD45+ cells and total populations assessed with counting beads. **b**) B memory cells, **c**) B resident memory, **d**) Isotype switched, **e**) Plasma cells, **f**) plasmablasts were reduced with sensory neuron ablation. Supplementation with VIP increased B-cell populations. VIP supplementation did not further increase B-cell populations in sensory neuron intact mice. **g**) IgG was reduced with RTX as previous but, increased with the supplementation of VIP to RTX mice. **h**) VIP1R knockout mice had reduced IgG following colonization and infection compared to WT littermates. **j**) VIP supplementation suppressed bacterial burden in RTX mice. No effect of VIP supplementation was observed in Veh mice. **j**) VIP 1 receptor knockout mice (VIP1RKO) have an increased bacterial burden following pre-exposure and infection with *S pneumoniae* compared to WT littermates. Veh (PBS): n=8, Veh (VIP): n=7-8, RTX(PBS): n=6-7, RTX(VIP): n=6-7. One-way ANOVA with Holm sidak post hoc test. Data from 3 independent experiments. WT n=11, VIP1RKO n=7. Two-sided t-test. Data from 3 independent experiments.

Figure 5.

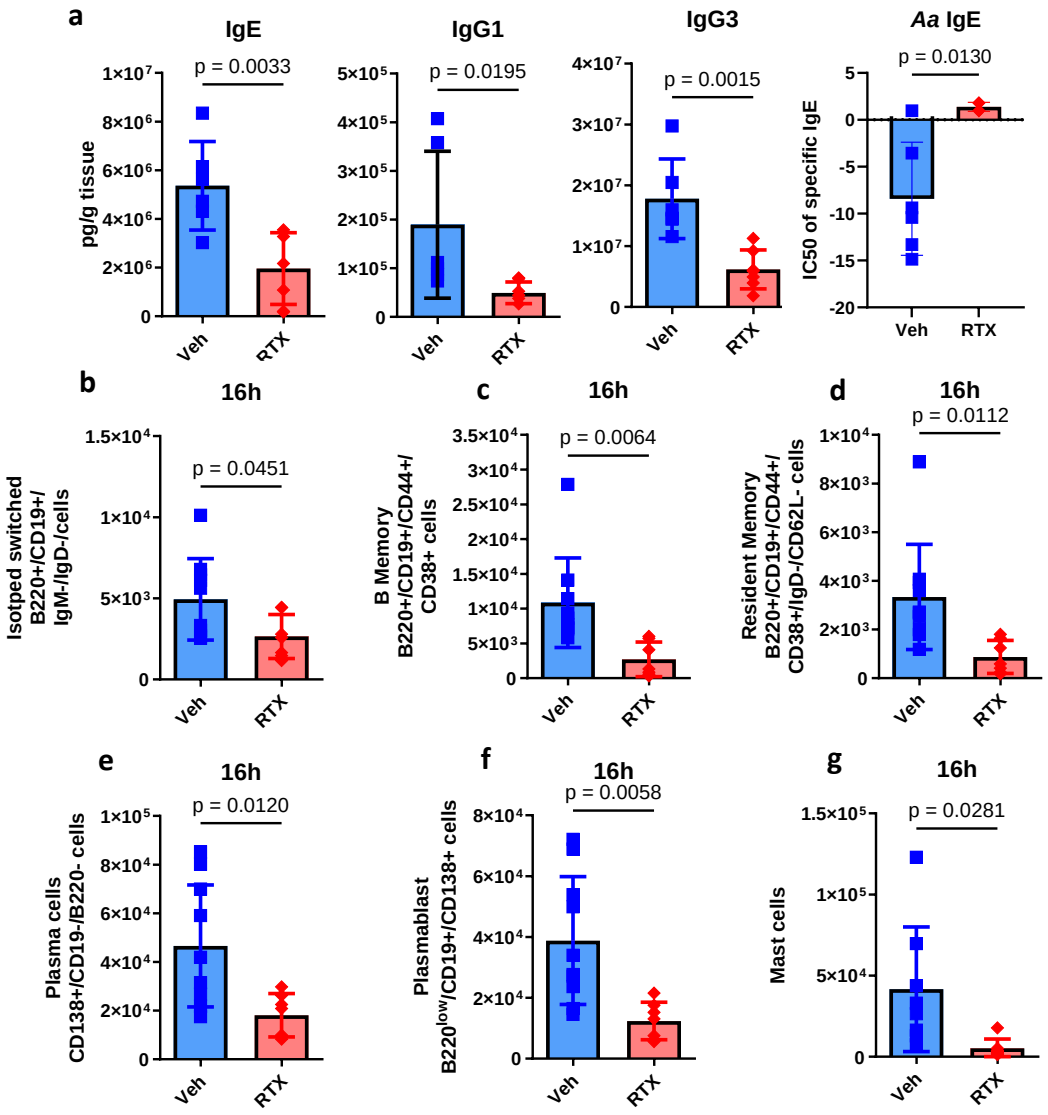

Figure 5. Sensory neuron ablation reduces B-lymphocytes, immunoglobulins and, mast cells in *A alternata* treated mice. a) IgE, IgG1, IgG3 (n=6, RTX: n=7) were reduced with sensory neuron ablation after final *A alternata dose*. Isotyping Luminex was used to analyze lung homogenates. *A. alternata* extract with lung homogenates and IC50 modeling was used to determine IgE specific to *A. alternata* (Veh n=6, RTX n=4) Two-sided T-test. Data were collected from two independent experiments. **b**) Isotype switched B220+/CD19+/IgM-/IgD-, **c**) B-Memory B220+/CD19+/CD44+/CD38+, **d**) B resident memory B220+/CD19+/CD44+/CD38+/IgD-/CD62l-, **e**) Plasma cells CD138+ and, **f**) Plasmablasts CD138+/B220-/CD19- cells and **g**) mast cells CD117+ were downregulated by sensory neuron ablation 16h after *A alternata* asthma induction. Cells were gated on live/CD45+ cells and counting bead calculation determined total cell population. Red gate is the measured cell population. Veh: n=10, RTX: n=7. Two-sided t-test. Data from 3 independent experiments.

Figure 6.

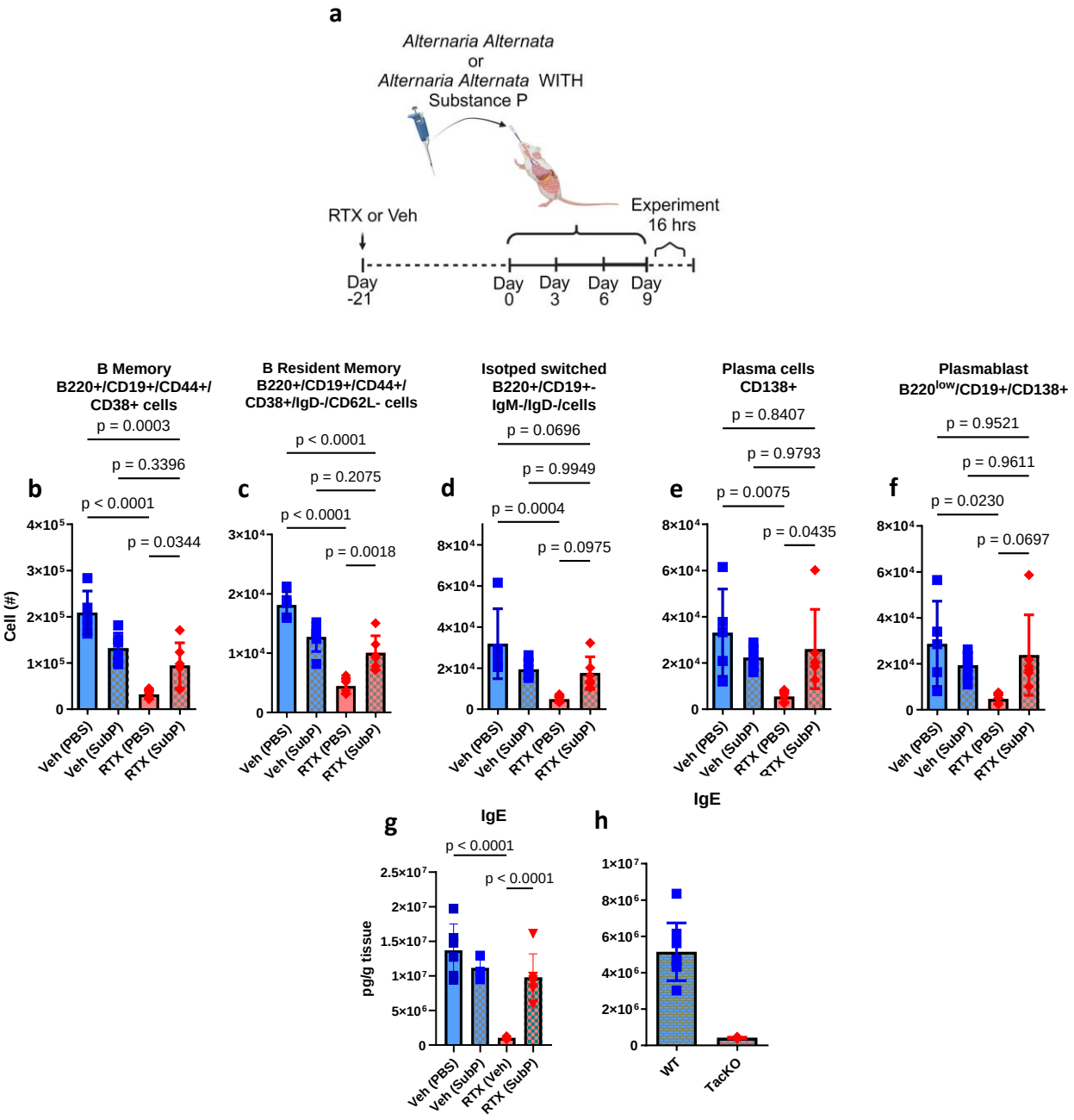

Figure 6. Substance P supplementation increases B-cells and IgE in mice treated with *A. alternata*. a) Substance P (100ng/µl) or vehicle (PBS) was delivered in conjunction with *A. alternata* extract as per the schematic, in accordance with our *A. alternata* model of fungal asthma. Cells were gated on Live/CD45+ cells and total populations assessed with counting beads. **b**) B memory cells, **c**) B resident memory, **d**) Isotype switched, **e**) Plasma cells, **f**) plasmablasts were reduced with sensory neuron ablation. Supplementation with Substance P increased B-cell populations. Substance P supplementation did not further increase B-cell populations in sensory neuron intact mice. . Veh (PBS): n=5, Veh (Sub P): n=6, RTX(PBS): n=6, RTX(SubP): n=6. One-way ANOVA with Holm sidak post hoc test. Data from 3 independent experiments. **g**) Supplementation with substance P increased IgE in *A. alternata* mice treated with RTX to a level consistent with Veh. Substance P did not further increase IgE in Veh mice (All groups n=6). One-way ANOVA with Holm sidak post hoc test. Data from 2 independent experiments. **h**) Substance P knockout mice (TacKO, n=7) had reduced IgE compared to WT *A. alternata* mice (n=8). Unpaired t-test. Data from 2 independent experiments.