# The CGRP-RAMP1 signaling enhances anti-*Aspergillus fumigatus* immune response

by

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B.Sc., University of Nigeria, 2017

A THESIS

submitted in partial fulfillment of the requirements for the degree

MASTER OF SCIENCE

Division of Biology
College of Arts and Sciences

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2023

Approved by:

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# Abstract

*Aspergillus fumigatus* is the single most common cause of fungal pneumonia and is listed as the World Health Organization's fungal priority pathogen. *Aspergillus fumigatus* opportunistic infections are on the rise due to an increasing proportion of immunocompromised and chronic lung disease patients. These individuals are more likely to acquire Invasive Pulmonary Aspergillosis (IPA) and lethal pneumonia caused by *A. fumigatus*. IPA and fungal pneumonia lead to dysregulated inflammation, rapid destruction of airway barrier permeability, and immune dysfunction. Innate immune cells, macrophages, neutrophils, and monocytes are critical for rapid fungal clearance and host protection against *A. fumigatus*. The lungs are heavily innervated by nociceptive sensory neurons which help to maintain homeostasis and immune cell functions, and they also secrete the neuropeptide calcitonin gene-related peptide (CGRP) to mediate pain sensations. Although *A. fumigatus*-specific therapeutics exist, they fail to provide absolute treatment to IPA and come with huge clinical limitations. Therefore, this study aims to understand the crosstalk between lung-innervating nociceptive neurons and the innate immune system to identify novel anti-*A. fumigatus* therapeutic target(s) through the signaling of these two systems. To understand the role of CGRP signaling in anti-fungal innate immune response, we employed immunohistochemistry, intracellular conidia killing assay, and flow cytometry analyses. We found an increase in the level of phagocytosis and intracellular killing of conidia by Bone Marrow (BM)-derived Macrophages and BM monocytes in the presence of CGRP. Also, flow cytometry and total cellular ROS analyses indicate significant increases in phagocytosis and effector function within neutrophils in the presence of CGRP. These effects were abolished when immune cells that lack the RAMP1 (receptor for CGRP) were employed. Altogether, our

findings support the notion that the CGRP-RAMP1 signaling increases anti-*A. fumigatus* innate immune response during *A. fumigatus* infection. This highlights the relevance of this interaction to *A. fumigatus* infections and points to it as a potential therapeutic option for the treatment/management of this infection. Further studies are required to understand the translation of these findings into *in vivo* and/or clinical settings.

# Table of Contents

# List of Figures

# Acknowledgments

My deepest gratitude and appreciation go to my advisor, Dr. Pankaj Baral for believing in me and giving me the shoulder to stand upon. I am immensely grateful for the mentorship, opportunities and time put into my mentorship during the period of this study.

I am thankful to my committee members: Dr. Kristin Michel, Dr. Ari Jumpponen and Dr. Stephanie Shames for their insightful comments and advise all through my study.

I acknowledge and appreciate the members of Baral Lab – Prabhu Joshi, Sandeep Adhikari, Abigail Judd and Camille Carrier for all their support and insights during the period of this study. I specially acknowledge and thank Michael Bartkoski of Baral Lab for his unwavering support and dedication toward this study.

Finally, I recognize and deeply appreciate my family and amazing friends who in numerous ways encouraged me to always be at my best and let me fly as high as I can reach. Thank you all.

# Dedication

This work is dedicated to my parents Marcellus Ndubisi Onah, Cordelia Onah and Felicia Ugwu for laying the foundation on which my achievements today are built upon.

# Chapter 1 - Introduction

## 1.1 *Aspergillus fumigatus* and Aspergillosis

*Aspergillus fumigatus* is the single most common cause of mold pneumonia and is currently recognized by the World Health Organization as a priority fungal pathogen (World Health Organization, 2022). It is a ubiquitous, environmental fungus in the phylum Ascomycota. It is widely distributed in air, soil, decaying vegetation, and compost heaps (Sugui et al., 2015) where they decompose organic matter because of their saprophytic lifestyle (Kwon-Chung & Sugui, 2013). This suggests their natural role in carbon and nitrogen recycling. Multiple features ensure efficient dispersal and adaptation of *A. fumigatus* in the environment. They thrive in a range of temperatures (25°C to above 37°C); they produce asexual spores (conidia) with characteristic hydrophobic nature and 2-3 µm in size. These characteristics allow *A. fumigatus* to build up to large numbers and thus the estimated daily inhalation dose of 100 – 1000 conidia by humans. Such characteristics also make it easy for these conidia to reach the lower respiratory tract and airways (Abad et al., 2010; Latgé, 1999; van de Veerdonk et al., 2017).

*Aspergillus fumigatus* is not a critical concern for individuals with competent immune systems whose ciliated airway epithelial cells, resident alveolar macrophages, monocytes, and neutrophils act to clear the spores upon inhalation. However, in immunocompromised individuals, the human physiological temperature is favorable for *A. fumigatus* survival. Hence, conidia germinate into tissue invasive hyphae after 4 – 8 hours of inhalation in high-risk human populations (McDonagh et al., 2008; van de Veerdonk et al., 2017), in particular patients with hematologic malignancies, bone marrow (BM) transplant recipients, HIV-AIDS patients, and more recently, patients with severe influenza or COVID-19. *A. fumigatus* causes a range of conditions in these individuals, including but not limited to invasive pulmonary aspergillosis

(IPA), allergic bronchopulmonary aspergillosis (ABPA), chronic pulmonary aspergillosis (CPA) or chronic necrotizing pulmonary aspergillosis (CNPA), and aspergilloma (Lionakis et al., 2023; Sugui et al., 2015; van de Veerdonk et al., 2017).

Risk factors for these situations include immunodeficiency, chronic lung diseases (such as cystic fibrosis or chronic obstructive pulmonary disease (COPD)), organ and hematopoietic stem cell transplantations (HSCTs), pre-existing lung injury, extensive hospitalization and severe influenza or COVID-19 pneumonia (Hoenigl et al., 2022; Latgé & Chamilos, 2019; Lionakis et al., 2023; Salazar et al., 2021; van de Veerdonk et al., 2017). Secondary immunodeficiency is the major risk factor for IPA and occurs in various forms: chemotherapy that eliminates both cancer cells and neutrophils during cancer treatments (Gargiulo et al., 2021; Kasi & Grothey, 2018; Shitara et al., 2011) or prevents the generation of new hematopoietic stem cells during HSCTs (Bhatia, 2011; Wingard et al., 2002), or immunosuppressive drug regimen administered during HSCTs and solid organ transplantations (SOTs) (Chinen & Buckley, 2010). HSCTs and SOTs have risen in recent years due to developments in medical standards and as a consequence, an increased proportion of the human population are subjected to immunosuppressive regimen in clinical settings, leading to an increased number of high-risk individuals and increased incidence of *A. fumigatus*-related diseases (van de Veerdonk et al., 2017).

Even with the existence of prophylactic measures and targeted antifungal therapy ( broadly reviewed in Patterson et al., 2016), *A. fumigatus* infections have risen and remain a major public health challenge. There is no approved vaccine against *A. fumigatus*. In addition to these outlined conditions, antifungal resistance is also on the rise as increasing levels of chemicals are used in agricultural settings as fungicides (Berger et al., 2017; Fisher et al., 2022; World Health Organization, 2022). *A. fumigatus* is responsible for respective 3,000,000 and

300,000 chronic and invasive clinical cases annually with mortality rates ranging from 30-95% in immunocompromised patients while 100% mortality has been reported in certain studies involving azole-resistant *A. fumigatus*-infected individuals (Bongomin et al., 2017; Brown et al., 2012; World Health Organization, 2022).

*Aspergillus fumigatus* diagnosis involves simultaneous histopathologic/cytologic and culture examination of tissue and fluid specimens (serum and Bronchioalveolar Lavage (BAL)) for galactomannan (GM), which serves as an accurate marker for the diagnosis of IPA in adult and pediatric patients; chest radiograph and computed tomographic (CT) scan is also used for IPA diagnosis (Patterson et al., 2016; van de Veerdonk et al., 2017). While triazoles rank as the primary choice for treatment among the current antifungal agents against aspergillosis including triazoles, amphotericin B, and echinocandins, combination therapy is also possible with these antifungal drug families (Patterson et al., 2016; van de Veerdonk et al., 2017). An alternative effective host-based immunotherapy against *A. fumigatus* is much needed to combat this infection.

## 1.2 Immune Response against *Aspergillus fumigatus*

The lung innate immune system is critical for rapid fungal clearance and to mediate protective immunity after inhalation of airborne *A. fumigatus* conidia (Lionakis et al., 2023; Lionakis & Levitz, 2018). In humans, genetic or pharmacologic defects in neutrophil, macrophage or monocyte function or numbers underlie susceptibility to IPA, while injury to lymphoid cells is largely dispensable for immunity in the lung (Lionakis et al., 2023).

The outer layer of the conidia masks the pathogen-associated molecular patterns (PAMPs) necessary for immune cell recognition of conidia presence. However, *A. fumigatus* PAMPs including β-1,3-glucan are exposed upon the germination of the conidia. These PAMPs

are recognized by the pattern recognition receptors (PRRs) (such as dectin-1) on host innate immune cells, leading to their phagocytosis, induction of inflammatory response and conidia clearance (Becker et al., 2015; Gresnigt et al., 2012). Conidia clearance occurs mainly through phagocytosis and killing via the mechanism that involves engagement with PRRs (such as dectin-1, -2, -3) or with non-PRR molecules. Activation of PRRs also trigger the production of cytokines (TNF-α, IL-6, IL-1β, IL-10, IL-17) and chemotactic mediators (CXCL1/2/5, CCL2, G-CSF, CXCL9/10) that are involved in immune cell recruitment and anti-aspergillus effector functions (van de Veerdonk et al., 2017).

The hallmark of innate effector mechanism against *A. fumigatus* involves the Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-mediated production of Reactive Oxygen Species (ROS) in phagocytes that culminates in phagocytic conidial killing for the fungal clearance (Akoumianaki et al., 2016; de Luca et al., 2014; Garlanda et al., 2002; Kyrmizi et al., 2013). Intercellular crosstalk that occurs within the airway and alveolar environment is a starting point for anti-*A. fumigatus* immunity. This regulates the induction of proinflammatory responses against the fungus and the recruitment of other myeloid immune cells for fungal clearance. Altered or weakened immune activation, fungal virulence, and defective conidial phagocytic clearance are some determinants of *A. fumigatus* infection severity.

When *A. fumigatus* conidia resist the host mucociliary defense mechanism in the upper airways including the mucous layer that presents surfactant proteins SPA and SPD, the pentraxin-3-ficolin complexes, and the β-defensins (Alekseeva et al., 2009; Ma et al., 2013; Madan et al., 2010; Okamoto et al., 2004), they reach the alveolar compartment where they are recognized by sentinel cells (such as alveolar macrophages) for their clearance. The tissue-resident alveolar macrophages internalize the conidia by phagocytosis followed by their killing

through ROS-mediated mechanism. Conidia expose their PAMPs and increase their cell-wall permeability to phagocyte toxic molecules when they swell, germinate, and shed their rodlet layer in the phagolysosome. A further consequence of these morphological changes is the induction of cytokine and chemokine production that coordinates the recruitment of myeloid cells such as neutrophils and monocytes into the alveolar space (Sugui et al., 2015).

In addition to the crucial role of monocytes in phagocytosis and conidial clearance during *A. fumigatus* infections, recruited $CCR2^{+}$ monocytes differentiate into monocyte-derived dendritic cells (DCs) that enhance overall fungicidal activity and initiate adaptive immune responses when they take up and convey the conidia to the draining lymph nodes (Espinosa et al., 2014; Hohl et al., 2009). Like alveolar macrophages, neutrophils are key myeloid phagocytic cells, and neutrophil-mediated elimination of *A. fumigatus* relies on two mechanisms. First, intracellular killing of conidia by neutrophils involves phagocytosis and fusion of the phagosomes with toxic granule-filled vesicles (lysosomes) that include ROS. For the hyphae, neutrophils secrete antifungal compounds including ROS to initiate fungal killing. Second, they use NADPH oxidase-induced NETosis – the formation of Neutrophil Extracellular Traps (NETs) (Röhm et al., 2014) as a mechanism to damage both conidia and hyphae. NETs are made up of released neutrophil DNA that contain antimicrobial/antifungal peptides and proteases. These structures arrest and destroy fungal conidia and hyphae (Branzk et al., 2014; McCormick et al., 2010; Urban et al., 2006).

However, in neutropenic patients, *A. fumigatus* conidia get established in lung tissues and they readily germinate into tissue invasive hyphae within 6-8 hours after the exposure (McDonagh et al., 2008). Some level of unfavorable growth conditions also present themselves in this tissue environment, but their complex gene expression system enhance their adaptation to

nutrient deprivation, iron limitation, hypoxia, and other unfavorable host conditions (McDonagh et al., 2008). This allows their excessive growth and dissemination, increasing the risk of and eventually causing the condition of invasive aspergillosis (Dagenais & Keller, 2009; Hohl & Feldmesser, 2007; Sugui et al., 2015).

## 1.3 Neuroimmune Interactions in Homeostasis and Diseases

The peripheral arm of the nervous system comprises the somatosensory and motor domain. The somatosensory domain includes nociceptors and proprioceptors which integrate sensory cues such as pain, touch, noxious and itch-inducing stimuli at barrier surfaces (Blake et al., 2019a). The motor domain contains the autonomic and somatic nervous system, with populations of sympathetic, parasympathetic, and enteric neurons making up the autonomic arm (Blake et al., 2019a). The immune system detects and neutralizes pathogenic materials and drives tissue repair after injury. These are achieved through a clear correspondence and concerted actions of the innate and adaptive aspects of the immune system.

In his encyclopedia contribution 2 millenniums ago, Aulus Cornelius Celsus pointed out what is today regarded as the cardinal signs of inflammation to include rubor (redness), tumor (swelling), calor (heat), and dolor (pain), and are all driven by neurons in the form of neurogenic inflammation (Chiu et al., 2012). This has been the initial premise on which the logic of neuroimmune communication is built upon. For instance, immune activation releases cytokines that act on neurons to cause pain production. Similarly, neurons secrete neuropeptides and neurotransmitters that act on immune cells to modulate their functions. This supports the idea of an ongoing neuroimmune crosstalk between these two systems that is bi-directional in nature.

Neuroimmunology research has noticed increasing amount of efforts to uncover the mechanisms involved in the signaling and what they mean to organisms under homeostatic and

disease conditions. Immune cells express receptors for the neuropeptides and neurotransmitters released by neurons. Like immune cells, neurons express the PRRs and cytokine receptors. With this, the nervous system is able to directly sense microbial and antigenic components and help monitor mucosal and tissue environment. Compared to the nervous system (seconds to minutes), the immune system is slower in action (minutes to hours) during infection. Therefore, via the cytokine and PRR expression in response to microbes, neurons rapidly react to danger conditions and coordinate the immune system to pre-act for host defense.

The conditions that dictate neuroimmune interactions during infections is complicated. Previous studies demonstrate the pathogen-specific and site-specific differences in neuro-immune crosstalk. Several studies show that the type and nature of tissue, neurons and their mediators, immune cells and their cytokines, and pathogens or antigens involved during infections greatly impact the outcomes of these neuroimmune interactions. For instance, they are tissue dependent in some cases as the role of a given neuronal or immune mediator have varying effects in different tissues. Again, a neuropeptide released in a particular tissue can have diverse influence on the outcome of infections and depending on the kind of pathogen involved, and can initiate a positive or negative response in that specific disease condition. Additionally, neuroimmune interactions do not necessarily imply host protective or detrimental outcome of infections because of the conditions mentioned above.

In recent years, studies are increasingly uncovering the rules of engagement and the endpoint of neuroimmune interactions in different disease states. To fully comprehend the range of peripheral neuroimmunology, it is important to specifically examine recent and ongoing studies that aim to uncover the intricate relationship between the peripheral nervous system and the immune system during pathogenic infections involving the major mucosal organs – gut, skin

and lungs. There are currently more discoveries in the skin and gut neuroimmunology as compared to discoveries in the lungs. Some of the discoveries in these organs will be highlighted in the following sections to point out how neuroimmune interactions work at barrier tissues. Each section starts with an overview of the neuroanatomy of the respective organ followed by an outline of how the neuroimmune crosstalk in them has progressed over the years.

### 1.3.1 Neuroimmune Contribution to Gut Antimicrobial Immunity

The subdivisions of the gastrointestinal tract (GIT) - mesentery, serosa, muscularis, submucosa, lamina propria and epithelium, are innervated by different neuron populations near resident immune cells, that lead to crosstalk under different conditions. The gut-brain axis has two major neuron units - the Intrinsic Enteric Nervous System (ENS) which has cell bodies within the GIT and the Extrinsic Enteric Nervous System with ganglia, spinal cord and brainstem-associated cell bodies (somatosensory and autonomic neurons). The sympathetic and parasympathetic autonomic neurons, respectively traced to the spinal cord and brainstem, facilitate the brain-gut communication and at the myenteric plexus create local neural circuits by networking with the ENS to further mediate the relay of signals.

Arising from the vagal ganglia and dorsal root ganglia, the extrinsic gut-innervating sensory neurons perceive stimuli including cytokines, food nutrients, mechanical and luminal disturbances in the gut and relay this information to the brainstem and spinal cord, respectively. In addition, these neurons secrete neuropeptides and neurotransmitters through their nerve endings in the intestine to influence both the immune and stromal cells of the GIT. The extrinsic celiac, and superior and inferior mesenteric ganglia originate from the sympathetic chain of the spinal cord to innervate the stomach, small intestine, and proximal colon whereas the sympathetic pelvic nerves innervate the distal colon. In contrast, the parasympathetic neurons

arise from the brainstem to supply the intestine through the efferent vagus nerve. The enteric neurons situate and emerge from the myenteric and submucosal plexus to innervate other sections of the gut. In addition to maintaining gut peristalsis and secretions, these neuron subsets incorporate sensory, parasympathetic, and sympathetic cues to modulate intestinal physiology.

The gut neuroimmune interactions are crucial for maintaining the mucosal barrier during homeostasis and infections. Recent findings suggest that specific neuronal populations secrete and respond to neurotransmitters and neuropeptides to mediate critical functions in infections and homeostasis. As an example, IL-18 expression by the intrinsic subset of the ENS is critical for survival during intestinal salmonellosis, leading to activation and production of antimicrobial peptides by goblet cells, which regulates the mucosal barrier immunity protecting against *Salmonella* Typhimurium (S. t.) infection and the spread to peripheral tissues **(Figure 1.1a)** (Jarret et al., 2020). Also, immune cells release neuropeptides and neurotransmitters to mediate pathogen clearance. During *Citrobacter rodentium* infection, sensory nociceptive neurons and CXCL13 recruit T cells to the infection site (Ramirez et al., 2019), which serve as non-neuronal sources of acetylcholine to lower bacterial burden, modulate inflammation, and enhance mucosal defense. Nociceptive neurons also maintain mucosal adhesin and IL-22 levels critical for host protection and T cell recruitment. These neurons may release other neuropeptides apart from CGRP to exert further host protective outcomes (Ramirez et al., 2020).

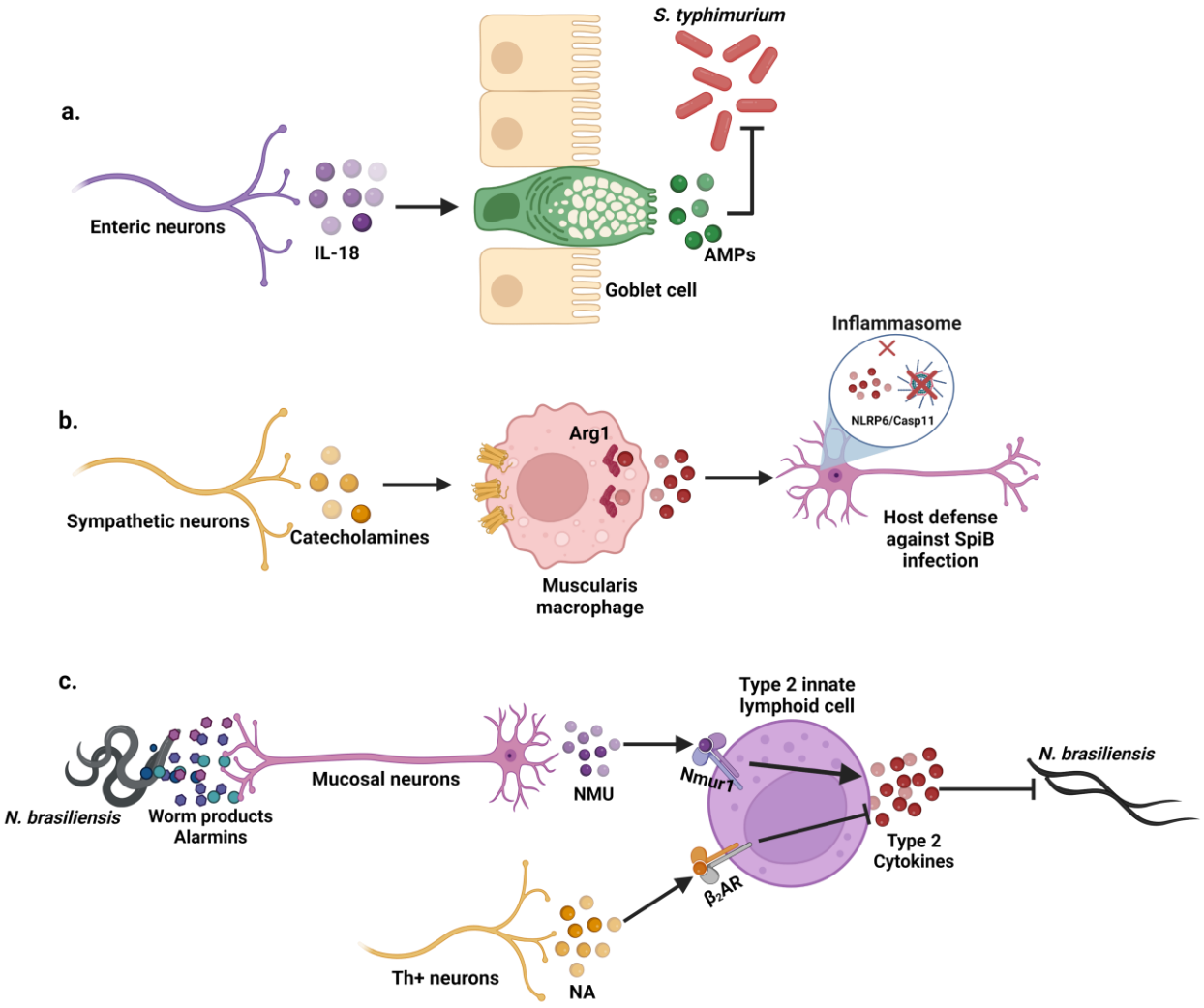


**Figure 1.1: Neuroimmune Contribution to Gut Antimicrobial Immunity.**
**(a).** Epithelial and immune cell-derived IL-18 is dispensable for combating *S. typhimurium* gut infections. Instead, Enteric neuron-derived IL-18 drives goblet cell production of antimicrobial peptides to potentiate the elimination of enteric *S. typhimurium*. **(b.)** Caspase-11 and NLRP6 induce irreversible loss of intestinal EANs (iEANs) in the ileum and colon while muscularis macrophages (MMs) act via the MM-AR-Arg1-polyamine axis to upregulate Arginase1 upon infection. Arginase1 mediates the production of spermine to repress the activation of the NLRP6 inflammasome. Induction of catecholamine release during pre-infection acts in concert with MM β2-AR signals to alleviate post-spiB infection-induced neuronal loss. **(c.)** The Neuromedin U (NMU) expressing cholinergic and Tyrosine hydroxylase (TH) expressing adrenergic neurons coexist with NMU Receptor 1 (NMUR1) and β2-adrenergic receptor expressing ILC2s in the intestine. These neurons directly sense alarmin and worm products to secrete NMU which activates ILC2s to promote their proliferation, and expression of the type 2 cytokines IL-5 and IL-13. $NMU^{+}$ neurons respond during *Nippostrongylus brasiliensis* infection by secreting NMU to cause ILC2s-mediated expulsion of the nematode through a combination of antimicrobial, inflammatory, and tissue-protective type 2 responses. Noradrenaline signaling via β2AR on ILC2s controls the inflammation by limiting the level of type 2 cytokines secretion by ILC2s. (Figures were made with BioRender).

The mesenteric layer of the gut has Enteric Associated Neurons (EANs) that regulate the gut in healthy and diseased states. In post-infectious Irritable Bowel Syndrome (IBS), caspase-11

and NLRP6 induce irreversible loss of excitatory VGLUT2$^{+}$ intestinal EANs **(Figure 1.1b)** (Matheis et al., 2020). Moreover, muscularis macrophages (MMs) signal via β2-adrenergic receptors (ARs) to limit inflammation and loss of iEANs. Similarly, induction of catecholamine release during pre-infection acts with MM β2-AR signals to alleviate post-infection neuronal loss (Matheis et al., 2020). Enteric *C. rodentium* infection causes mucosal enlargement, decreased serotonin and somatostatin (SST) expressing cell numbers, reduced SERT immunoreactivity, elevated iNOS levels, but does not damage the enteric nervous system. (O'Hara et al., 2006). In homeostasis, neuronal signaling drives gut resident macrophage programming, with lamina propria macrophages (LpMs) and muscularis macrophages (MMs) exhibiting pro-inflammatory and tissue-protective phenotypes, respectively. Also, infection models show that norepinephrine signaling induces tissue-protective phenotypes in MMs (Gabanyi et al., 2016), while substance P (SP) secretion by Ileal sensory neurons and activated LpMs increases TNF-alpha secretion in *Clostridium difficile* toxin A-induced enteritis (Castagliuolo et al., 1997). Vagus nerve signaling via α4/β2 nicotinic acetylcholine receptors upregulates phagocytosis and enhances transport of luminal *Enterococcus faecium* bacteria, priming LpMs towards an anti-inflammatory state (van der Zanden et al., 2009).

Hematopoietic cell fates are indirectly influenced by neuronal signaling and this also helps to maintain the intestinal microbiota. Postganglionic norepinephrine-activated dendritic cells act through β2-ARs on naïve T cells to cause their differentiation into CD4$^{+}$ChAT$^{+}$ T cells, which communicate with gut epithelia to increase their expression of antimicrobial peptides (Dhawan et al., 2016; Satoh-Takayama et al., 2008; Yang et al., 2008).

The ability to discern between noxious and innocuous stimuli in the gut is critical to maintaining normal functions of the gut especially during feeding when several foreign

molecules/substances are present. Therefore, it is necessary to regulate the assimilation of these molecules while maintaining gut barrier integrity and pathogen clearance. Neuroimmune interactions during feeding lead to a compromise between barrier immunity and nutrient uptake. This involves the activation of VIPergic neurons upon food consumption to signal via VIPR2 expressed by $CCR6^+$ ILC3. This causes a reduction in IL-22 mediated secretion of antimicrobial peptides by intestinal epithelial cells and increased lipid uptake (Talbot et al., 2020). Vasoactive intestinal peptide secreting ($VIP^+$) and $CGRP^+$ enteric neurons lie near ILC2s and ILC3s that express high levels of VIP receptors, suggesting potential crosstalk between them. Rapid VIP secretion upon neuronal activation can induce an anticipatory response of ILCs for pathogens that may be present in food (Pascal et al., 2022).

The extrinsic arm of the enteric nervous system secretes CGRP to regulate gut function. Moreover, Peyer's patch M cells were found to play a critical role in gut barrier function and regulation of gut microbiota. CGRP suppresses M cell population during *Salmonella enterica* serovar Typhimurium infection to elevate segmented filamentous bacteria levels that leads to enhanced host defense (Lai et al., 2020). Substance P receptor expression is increased during infection, correlating with early induction of IL-12 and IFNγ mRNA expression to strengthen immunity against enteric salmonella infection (Kincy-Cain & Bost, 1996). In *Caenorhabditis elegans* model, *Staphylococcus aureus* infection causes the release of acetylcholine, which upregulates Wnt and its receptor to increase host defense gene transcription (Labed et al., 2018). Lamina propria ILC3s express RET and colocalize with neurotrophic-factor-expressing glial cells, indicating a potential to moderate gut defense and inflammation. Glial cells release neurotrophic factors that induce downstream secretion of IL-22 by ILC3 upon RET activation.

*Ret*Δ mice exhibited decreased frequency of IL-22-producing ILC3, reduced gut epithelial reactivity/repair genes, and lower survival in *C. rodentium* infection (Ibiza et al., 2016).

6-Hydroxydopamine (6-OHDA)-mediated sympathectomy in mice can increase immune response to *Listeria monocytogenes* by reducing bacterial load in the spleen, mediated by increased neutrophil recruitment (Rice et al., 2001). Neuromedin U (NMU) expressing cholinergic neurons coexist with NMU Receptor 1 (NMUR1) expressing ILC2s in the intestine, suggesting a crosstalk between these cell units. ILC2s can be activated by NMU to promote proliferation and expression of type 2 cytokines IL-5 and IL-13 **(Figure 1.1c)** (Cardoso et al., 2017; Klose et al., 2017). In *Nippostrongylus brasiliensis* model of helminth infection, $NMU^{+}$ neurons communicate with neighboring ILC2s to control *N. brasiliensis* infection, and β2AR signaling regulates the effect of NMU during the infection (Moriyama et al., 2018).

### 1.3.2 Skin Neuroimmune Crosstalk and Implication to Immunity

The skin being the largest organ in the human body is constantly exposed to the external environmental hazards including microbes and their products. This implies that the skin anatomy and physiology are critical not just for protection against pathogens but in maintaining barrier functions and homeostasis. Skin neuroimmune interactions are key orchestrators in maintaining homeostasis and host defense. The skin comprises the epidermal and dermal layers made up of various cell populations including keratinocytes, fibroblasts, immune, endothelial, and Schwann cells (Blake et al., 2019a; Peters et al., 2012). These cell populations are also closely associated with the neurons that innervate the skin. The sensory nerve fibers are the greatest neuron subset that innervates the skin and oversee signals of touch, itch, pain as well as thermal and mechanical sensations. These sensory nerve fibers have their origins either in the DRG in the spinal cord that supply the thoracic and abdominal skin, or the trigeminal ganglia that innervate the head and

face. The sensory projections of the DRG connect with the dorsal horn of the spinal cord to convey cues to the brainstem and thalamus (Blake et al., 2019a; Laverdet et al., 2015; Schwendinger-Schreck et al., 2015). A proportion of the sympathetic arm of the autonomic nervous system also supplies the hair follicles, blood vessels, lymphatic vessels, apocrine and eccrine glands, and erector pili muscles in the dermal layers of the skin (Blake et al., 2019a; Laverdet et al., 2015; Vetrugno et al., 2003).

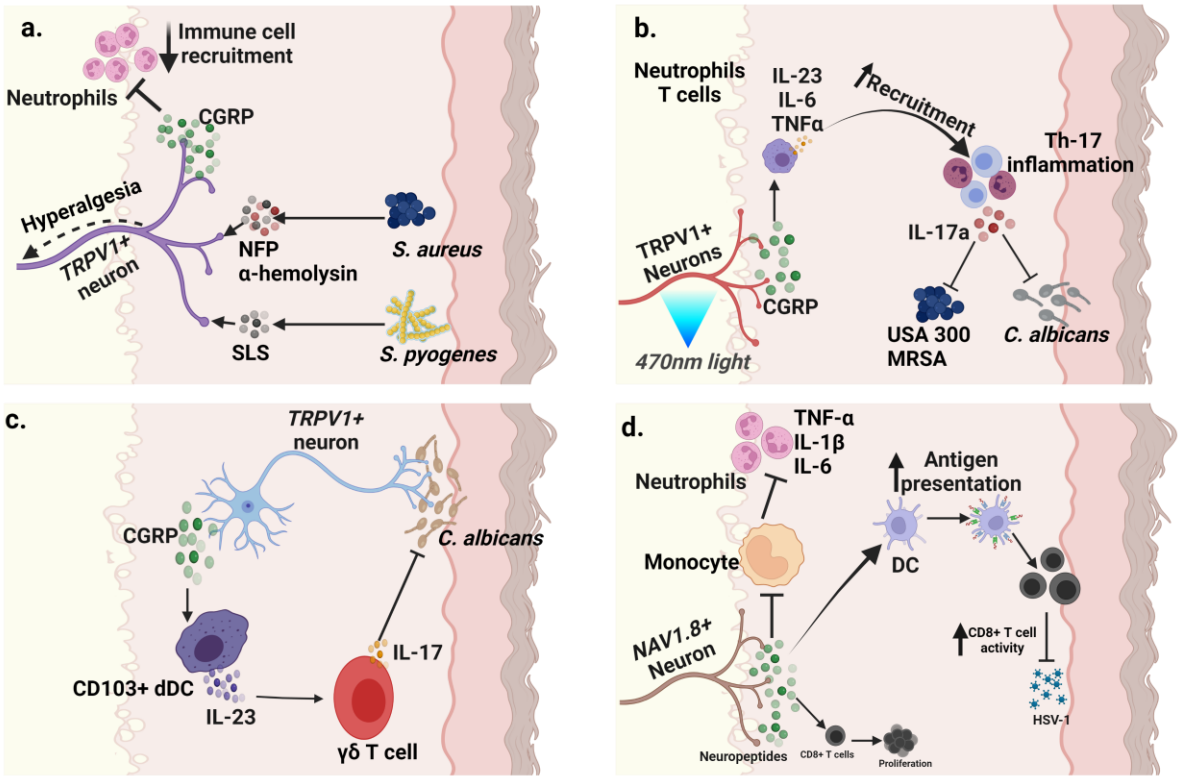


**Figure 1.2: Skin Neuroimmune Crosstalk and Implication to Immunity.**
**(a).** *S. aureus* N-formylated peptides and α-hemolysin directly stimulate skin nociceptors to cause pain sensation. In necrotizing fasciitis, *S. pyrogens* streptolysin S causes acute pain, heat, and mechanical hyperalgesia through the activation of TRPV1$^+$ neurons. CGRP release due to pain in these two conditions dampens the recruitment and activity of recruited immune cells. **(b.)** Optogenetic stimulation of TRPV1$^+$ neurons increases CGRP levels. CGRP signaling initiates local skin inflammation by increasing IL-23, IL-6, TNF-α, and type 17 inflammatory cytokines. This causes T cell and neutrophil recruitment that augment IL-17A and type 17 inflammation. This significantly reduce *C. albicans* and USA300 MRSA during epicutaneous infections. **(c.)** TRPV1$^+$ nociceptors directly sense *C. albicans* to release CGRP that directs IL-23 secretion by CD301b$^+$ dDCs. IL-23 increases γδT cell proliferation that secretes more IL-17 to limit the *C. albicans* burden. **(d.)** Nav1.8$^+$ sensory neurons balance inflammation by controlling inflammatory cytokine production by monocytes, and neutrophil infiltration to HSV-1-infected skin. They influence CD8 T cell function and proliferation during HSV-1 infection and increase their immune response by enhancing efficient antigen presentation by dendritic cells. (Figures were made with BioRender).

Several factors are associated with neuroimmune regulation of antimicrobial immunity in the skin. The outcome of these interactions depends on the skin microenvironment, neuron subsets involved, the immune cells resident in the skin local tissue environment, the nature of invading pathogen, and the nature of molecules secreted by these interacting cells. Neurons can directly sense microbial products and initiate sensations that lead to pathogen clearance or sensitization of the host system.

For instance, *S. aureus* N-formylated peptides and α-hemolysin directly stimulate skin nociceptors and cause pain sensation that is due to bacterial load and not immune cells. Whereas N-formylated peptides act through the Formyl Peptide Receptor 1 (FPR1) to induce calcium flux and mechanical hyperalgesia, α-hemolysin mediates heat hyperalgesia by causing ion infiltration into the nociceptor neurons and their further depolarization when they form pores on these nociceptor neuron membranes (Blake et al., 2018; Chiu et al., 2013). Pain may be a mechanism of immune system evasion by these bacteria since their induction of pain causes the release of neuropeptides such as CGRP, galanin and somatostatin that dampen the activity of recruited immune cells (Chiu et al., 2013). Similarly, in *Streptococcus pyogenes* model of necrotizing fasciitis, the bacterial toxin streptolysin S (SLS) drives acute pain, heat and mechanical hyperalgesia through the activation of $TRPV1^+$ neurons arising from the DRG (Pinho-Ribeiro et al., 2018). *S. pyogenes* SLS initiates a pain sensation that promotes its invasion by directly stimulating the $TRPV1^+$ neurons. CGRP released from these neurons modulates the host immunity in *S. pyogenes* infection (**Figure 1.2a**). CGRP prevents the recruitment of neutrophils and reduces the bactericidal actions required for the clearance of the pathogen by inhibiting myeloperoxidase activity in these neutrophils. However, local treatment with botulinum neurotoxin A (BoNT/A) which prevents neuronal vesicle release or CGRP receptor antagonist

resolves the infection and increases host survival. This suggests that blocking CGRP signaling can serve as a therapeutic support to treating necrotizing fasciitis (Pinho-Ribeiro et al., 2018).

In another study, optogenetic stimulation of $TRPV1^+$ neurons results in CGRP mediated induction of local skin inflammation by elevating IL-23, IL-6, and TNF-α type 17 inflammatory cytokine levels. This initiates the recruitment of TCRγδ T cells, $CD4^+$TCRαβ T cells, and neutrophils that potentiate an increased IL-17A and type 17 inflammation. Because of this, photostimulation of $TRPV1^+$ neurons during epicutaneous infection with *C. albicans* and USA300 strain of methicillin-resistant *S. aureus* (MRSA) significantly reduces pathogen burden (**Figure 1.2b**). Furthermore, prior optogenetic or *C. albicans* stimulation of $TRPV1^+$ neurons stimulate adjacent regions via a nerve reflex arc, hence complementing host defense and anticipatory immunity in the neighboring tissue regions (Cohen et al., 2019). In another mouse model of cutaneous candidiasis, $TRPV1^+$ nociceptors directly sense *C. albicans* to release CGRP. Different dendritic cell (DC) subsets in different tissue compartments express neuropeptide receptors and specifically, dermal DCs (dDCs) express the CALCRL and RAMP3 receptors for CGRP (Kashem et al., 2015; Li et al., 2014; Miller et al., 2012). CGRP signals to $CD301b^+$ dDCs to induce IL-23 secretion during dermal *C. albicans* infection. This increases γδT cell proliferation and their secretion of IL-17 that plays a host protective role and limits the fungal burden (**Figure 1.2c**) (Kashem et al., 2015).

Along with their assistance in the regulation of skin homeostasis, there is a possibility that resident immune cells indirectly control the phenotypic state of other immune cell populations by contributing to the maintenance of specific neuron populations. Long-term absence of Langerhans cells (LC) resulted in the loss of $MrgprD^+$ nonpeptidergic neurons in the skin. Thus, at homeostasis, glutamate release by these neuron subset acts to mediate the

neuroimmune crosstalk that regulates cutaneous mast cell activity. MrgprD$^+$ nonpeptidergic neuron ablation reprograms mast cell gene expression to upregulate Mrgprb2 (the activating receptor that enhances mast cell activity) causing their degranulation that propagates skin inflammation (Zhang et al., 2021). Ablation of LC or MgbrD$^+$ neurons during localized dermonecrotic *S. aureus* USA300 infection enhances host defense which is manifested by smaller skin lesions that contained 10-fold fewer pathogens on day 10 post-infection (Zhang et al., 2021).

Nav1.8$^+$ neurons depletion exaggerated skin inflammatory lesions with Herpes Simplex Virus 1 (HSV-1) infection. This is due to elevated levels of proinflammatory cytokines (TNF-α, IL-1β and IL-6) from activated monocytes, and chemokines that cause excessive neutrophil infiltration in the skin which persists even after virus clearance. Also, the depletion of these neurons impairs viral antigen presentation by the DCs and their activation of the CD8$^+$ T cells (**Figure 1.2c**) (Filtjens et al., 2021).

### 1.3.3 Lung Neuroimmune Crosstalk in Infection

As in other barrier sites, neuroimmune interactions during lung infections show both positive and negative impacts on the host. Neuropeptides and neurotransmitters may act to ensure that the level of inflammation occurring during infection is limited to prevent excessive organ damage. This action backfires in some occasions because certain pathogens require very high inflammatory immune status for their clearance. For instance, vagal TRPV1$^+$ nociceptive neurons release of CGRP during MRSA-induced pneumonia suppresses lung immunity. Although these neurons are important in the maintenance of lung epithelia and barrier functions, their CGRP secretion reduces neutrophil recruitment and lung-resident γδ T cell activity, hence increasing mortality, bacterial load, and dysregulated core body temperature. However, CGRP

antagonist treatment post-infection rescued MRSA-infected mice from the infection (**Figure 1.3a**) (Baral et al., 2018a). A similar effect is noticed with the lung cholinergic system which greatly impacts immunomodulation and disease progression during pulmonary tuberculosis (TB) in favor of the disease. *Mycobacterium tuberculosis* (Mtb) infection induces increased lung epithelial cells and macrophage acetylcholine levels with elevated α7 nAChR expression. This persists in lung lymphocytes and macrophages later in the infection. The release of acetylcholine consequently antagonizes the protective Th1 and Th17 immune response against Mtb to increase disease pathology, unrestrained inflammation, and bacterial burden. Also, nAChR antagonists are bactericidal and co-treatment with second-line antibiotics eliminated the lung Mtb burden (Islas-Weinstein et al., 2021). In a rat model of early-life *Pseudomonas aeruginosa* infection, $TRPV1^+$ neuron stimulation specifically increases both intravascular and intervascular pulmonary permeability in young rats which is associated with elevated levels of nerve growth factor (NGF). This increases the escape of polymorphonuclear neutrophils (PMNs) into the alveolar space and a change in metabolic status expressed as increased weight loss. These outcomes are partly due to NGF-induced proinflammatory activities. However, the phenotypes are rescued by NGF blockade, thus pointing to the role of the NGF pathway targeting for the management of *P. aeruginosa*-related chronic respiratory infections (Cardenas et al., 2010).

Two neuropeptides may either work in concert or oppose the actions of each other under disease conditions. CGRP and NMU play directly opposing roles during *N. brasiliensis* lung infection in which they propagate different inflammatory profiles in ILC2s. scRNA-seq identifies a subset of ILC2s to constitutively express CGRP and its coreceptors Receptor Activity-Modifying Protein 1 (RAMP1) and CALCR1. In addition, *in vitro* analysis of infected ILC2s identifies NMU to improve ILC2 proliferation and their type 2 immune response (IL-13 and IL-5

secretion) whereas CGRP antagonizes this by inhibiting IL-13 secretion. The *in vivo* significance is seen in CGRP reduction of ILC2 response to alarmins and *N. brasiliensis* infection by limiting their level of inflammation which further reduces inflammatory cell influx and worm expulsion from the lungs of infected mice (Nagashima et al., 2019).

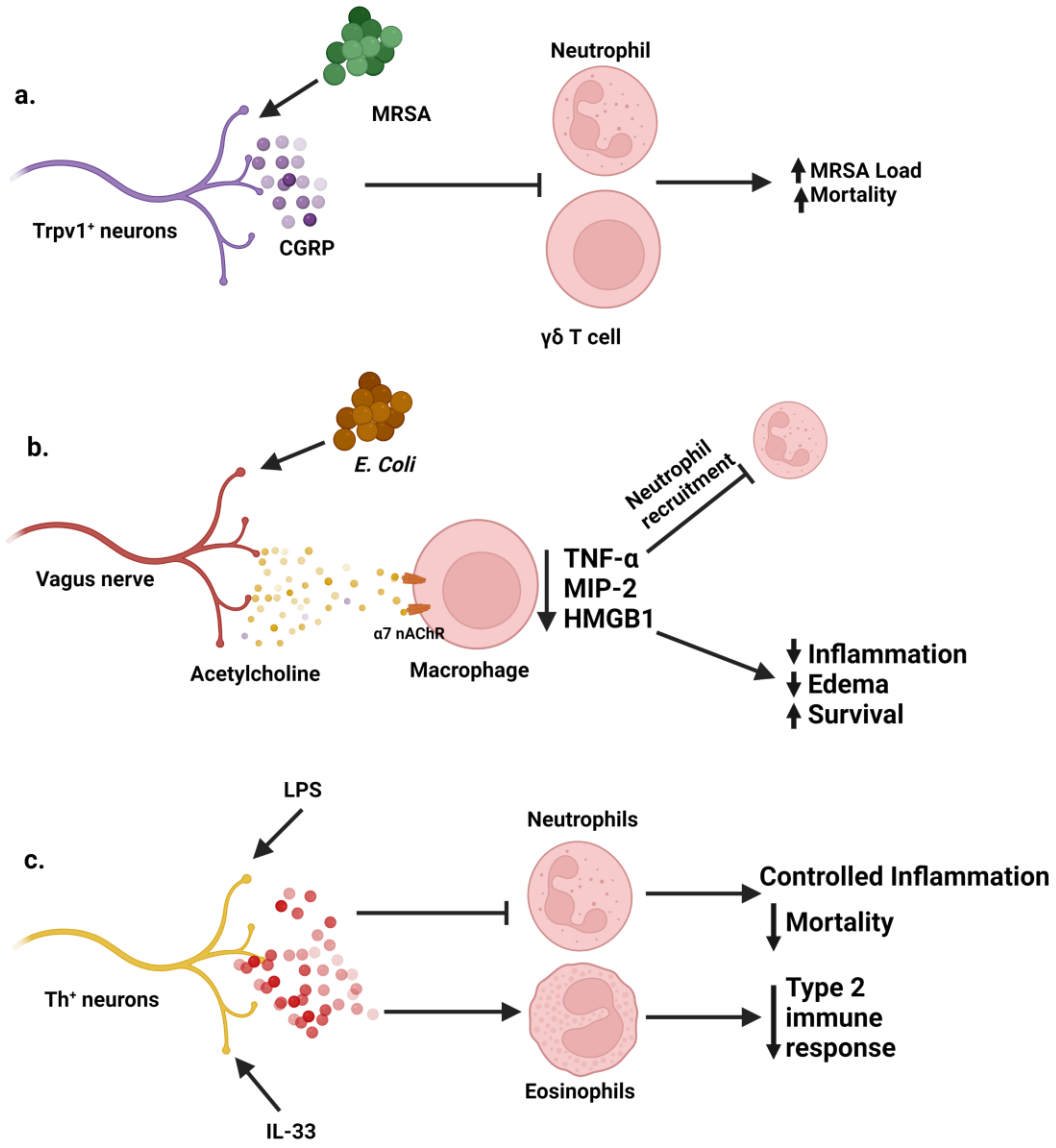


**Figure 1.3: Lung Neuroimmune Crosstalk in Infection.**
**(a.)** CGRP from vagal TRPV1$^+$ nociceptive neurons suppresses inflammation during MRSA-induced pneumonia by inhibiting neutrophil recruitment into the lungs and resident γδ T cells activity. This aggravates MRSA load and mortality. **(b.)** In LPS and *E. coli*–induced ALI, α7 nAChR signaling reduces lung inflammation through the reduction of TNF-α, MIP-2, and HMGB1 levels, and reduces lung vascular and epithelial permeability to limit neutrophil infiltration. This reduced ALI and increased survival. **c.** Lung sympathetic neurons signal via norepinephrine to balance immune cell recruitment and survival during LPS and IL-33-induced inflammation. Lung sympathetic neurons reduce neutrophil recruitment and decrease type 2 innate immune response in this condition. (Figures were made with BioRender).

Additional findings also highlight the multifactorial dependency of neuronal influence on disease outcome and the fact that some neuronal secretions act to stabilize the inflammatory states of immune cells. In a study involving vagus nerve-originating acetylcholine, this neurotransmitter signals via the α7 nicotinic acetylcholine receptor (α7 nAChR) pathway to mediate the outcome of LPS and *E. coli*–induced Acute Lung Injury (ALI) in mice. Acetylcholine interacts with α7 nAChR$^+$ alveolar macrophages and recruited neutrophils. Mechanistically, α7 nAChR signaling reduces lung inflammation through the reduction of TNF-α, MIP-2 and HMGB1, and reduces lung vascular and epithelial permeability which limits neutrophil infiltration. This interaction significantly ameliorated ALI and increased the survival outcome of mice in this condition (**Figure 1.3b**) (Su et al., 2010). Although this occurs through a different route, the lung sympathetic innervations greatly impact the outcome of Influenza A Virus (IAV) infection in mice via noradrenaline secretion. This exacerbates the pulmonary inflammatory response to the virus by increasing IL1-β and IL-6 levels with downstream stimulation of neutrophil, inflammatory monocyte, and NK cell recruitment, hence increasing pathology and mortality (Grebe et al., 2010). In a similar finding, β-adrenergic receptor abundance on DCs enables the sympathetic nervous system influence on CD8$^+$ T cell responses to IAV. In this manner, norepinephrine-β2 AR interaction modifies these antigen presenting cells (APCs) by reducing their inherent stimulation of CD8$^+$ T cells. However, 6-OHDA sympathectomy or β2 antagonists yield robust activation of these T cells against IAV (Grebe et al., 2009).

In LPS and IL-33 induced inflammation, lung sympathetic neurons balance immune cell recruitment and survival. Lung sympathetic neuron ablated mice experience dysregulated

neutrophil recruitment and decreased type 2 innate immune response (**Figure 1.3c**). These neurons control lung inflammation through their norepinephrine signaling. This points out a potential for the immunomodulatory role of sympathetic neurons in the control of bacteria-induced inflammation and their elimination during infection (Liu et al., 2020). LPS also stimulates nerve injury-associated gene expression in lung innervating sensory neurons originating from jugular-nodose complex and makes them more sensitive to peptides and to readily sense pain signals (Kaelberer et al., 2020).

Although neuron-derived neurotrophic factors arise from neurons and associated cells, an *ex vivo* study of lung influenza virus infection induces neurturin (NRTN) and interferon β (IFN-β) expression by epithelial cells and alveolar macrophages respectively. While these macrophages constitutively express GNFRα, their released IFN-β autocrinally induces RET expression. NRTN binding to GNFRα and RET coreceptors elevates macrophage MMP2 secretion. Under this condition, MMP2 alters normal macrophage anti-viral response by reducing their inflammatory potentials as seen by lowered IL-6 and IL12p40 levels, but not TNF-α and IL-10. This illustrates the possibility of driving the immune response toward controlled inflammation and lowered tissue pathology (Connolly et al., 2020).

## 1.4 Scope of Study

The bronchioles and airways are heavily innervated by nociceptive neurons – a subset of the sensory portion of the peripheral nervous system (Blake et al., 2019b; Godinho-Silva et al., 2019a; Hiroki et al., 2021). The role of neuroimmune interactions in the lungs has not been integrated in a model of antifungal immunity. Recent findings during bacterial and viral infections suggest that these neurons play critical roles in the maintenance of homeostasis and immune cell functions. Also, there is evidence that the immune and nervous systems mutually

influence each other and their responses are not self-regulated (Dantzer, 2018). The nervous system for instance impacts the immune system to determine the outcome of infections, and this depends on a host of factors including tissue type, the neuronal mediator involved, pathogen type, and immune cell type. While it is well established that these neurons and the immune system interact to modulate tissue homeostasis during infections (Godinho-Silva et al., 2019b; McMahon et al., 2015), it is unknown how these interactions regulate *A. fumigatus* infection and antifungal immunity. This study aims to understand the crosstalk between lung-innervating nociceptive neurons and the immune system to identify novel mechanism of anti-fungal therapeutic target(s) through the signaling of these two systems.

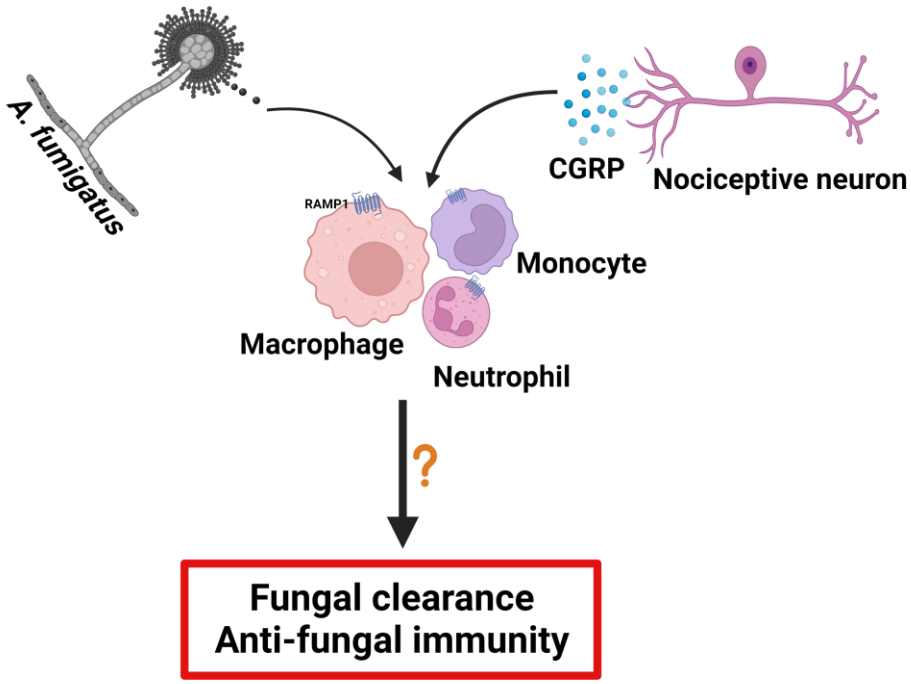


**Figure 1.4: Schematic of project aim.**
To understand the role of CGRP signaling in anti-*A. fumigatus* innate immune response in the major innate immune cells involved in the clearance of *A. fumigatus* conidia during pulmonary infections, and to understand the crosstalk between lung-innervating nociceptive neurons and the immune system to identify a novel anti-fungal therapeutic target(s). CGRP - Calcitonin Gene-Related Peptide. (Figures were made with BioRender).

# Chapter 2 - Materials and Methods

## *Aspergillus fumigatus* Strain, Culture, and Harvest

*Aspergillus fumigatus* (AF293) was grown on 1.5% malt extract agar slants for 7 days at 37°C without $CO_2$. Conidia were harvested by adding 0.02% Tween 20 in PBS (PBS-T) on top of the fungus. The conidia suspension was sieved into a 50 mL centrifuge tube using a 40 µm cell strainer to remove any residual mycelium and washed by centrifuging at 4000 rpm and 4°C for 5 minutes. Conidia were resuspended in 20 mL PBS and washing was repeated twice. The supernatant was removed and the conidia resuspended in 1mL of sterile PBS. Conidia concentration was determined using a hemocytometer.

## Bone Marrow-Derived Macrophage (BMDM) Derivation

Bone marrow (BM) cells were isolated from the femur and tibia of 8-12 weeks old C57BL/6J and *Ramp1*$^{-/-}$ mice by crushing them in a mortar and filtering through a 70 µm cell strainer. Red blood cells were lysed using RBC lysis buffer (Thermofisher) and washed 3X with PBS containing 2% FBS. Bone marrow cells were resuspended in complete DMEM containing 30% L929 conditioned media, 10% fetal bovine serum (FBS), and 1% antibiotics/antimycotics (containing penicillin, streptomycin and amphotericin B) (cDMEM). The BM cells were cultured in cDMEM for 7 days followed by isolation of the BMDMs using 2.5 mM ice-cold EDTA solution, and counted using a hemocytometer.

## BMDM Stimulation for Cytokine Analysis in the Presence of Neuropeptides

$1.5x10^5$ BMDMs were seeded overnight in a 96-well plate and stimulated with *A. fumigatus* conidia swollen at 37°C for 6 hours or at 37°C for 7 hours with shaking at 250 rpm.

The BMDMs were stimulated with swollen conidia at MOI of 2 and 10 in the presence or absence of 100 nM and 1 µM CGRP, Substance P, VIP, and Somatostatin for 16 hours.

## Cytokine Analysis

TNF-α, IL-6, and MCP-1 cytokine levels from stimulated BMDMs and BALF were measured using enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions (BioLegend).

## BMDM Phagocytosis and Killing Experiments

$1.5 \times 10^5$ BMDMs were seeded in 96-well plates and incubated overnight in 5% $CO_2$, 37˚C, and humidity. *A. fumigatus* conidia were added afterward in the presence or absence of 1 µM CGRP, Substance P, VIP, and Somatostatin at MOI of 10 followed by brief centrifugation at 200 g for 5 minutes at 4˚C. The plates were incubated for 6 hours and each well was washed 3 times with PBS. Experiments that involved forskolin, rolipram, and protein kinase inhibitor (PKAi) also followed the same steps as described above, but with co-incubation with these molecules in their respective parameters **(**as in **figure 3.8)**. This BMDM-conidia coculture was incubated again with nystatin (20 µg/mL) in cDMEM for 4 hours (for phagocytosis) or 10 hours (for killing activity). The monolayers were washed twice with PBS-T and lysed by incubating them on ice with 0.25% Triton X-100 (IBI Scientific) for 10 minutes. The lysate was diluted, plated onto SDA plates, and incubated at 37˚C for 24 h, followed by colony count to determine the total intracellular conidia.

## Confocal Microscopy and Analysis of BMDM Phagocytosis

*Aspergillus fumigatus* conidia were labeled with Alexa Fluor™ 488 NHS Ester (Succinimidyl Ester) (Thermofisher) by adding $25x10^6$ conidia in 1 mL of 0.1 M sodium

bicarbonate buffer followed by the addition of 1 mL of 0.1mg/mL Alexa Fluor™ 488 dye. The suspension was incubated at 37˚C for 2 hours and washed twice with 1X PBS at 4000 rpm for 5 mins. $3x10^5$ BMDMs were cultured overnight in a 4-well chambered slide (Cell treat) at 37˚C with 5% $CO_2$ and stimulated with Alexa Fluor 488-labeled *A. fumigatus* conidia at a MOI of 2 for 4 hours at 37˚C. Wells were washed 3 times with PBS-T and fixed with 4% paraformaldehyde solution for 15 min at 4˚C. Blocking was performed by adding Normal Goat Serum (Sigma) in PBS containing Triton X-100 for 30 mins at room temperature. This was followed by F4/80 primary antibody (Abcam, 1:500) staining for 2 hours at room temperature. After 3 washing steps with PBS-T, Alexa Fluor 594 (Thermofisher, 1:500) secondary antibody was added and incubated for 1 hour at room temperature, washed 3 times again before adding DAPI-containing mounting media and preparing the slides for microscopy. BMDM phagocytosis was imaged using Carl Zeiss LSM 880 confocal microscope and analyzed by counting the number of *A. fumigatus* conidia engulfed per image field.

## Monocyte Phagocytosis and Killing Experiments

C57BL/6J and *Ramp1*$^{-/-}$ mice monocytes were isolated using the EasySep™ mouse monocyte isolation kit (STEMCELL - 19861) following the manufacturer's guidelines. $1.5 \times 10^5$ monocytes were seeded in 96-well plates and incubated overnight in 5% $CO_2$, 37˚C, and humidity. *Aspergillus fumigatus* conidia were added afterward in the presence or absence of 1 µM CGRP at MOI of 10 followed by brief centrifugation at 200 g for 5 minutes at 4˚C. The plates were incubated for 6 hours and each well was washed 3 times with PBS. The monocyte-conidia coculture was incubated with nystatin (20 µg/ml) in cDMEM for 3 hours (for phagocytosis) or 6 hours (for killing activity). The monolayers were washed twice with PBS-T and lysed by incubating in 0.25% Triton X-100 for 10 minutes on ice. The lysates were diluted,

plated onto SDA plates, and incubated at 37°C for 24 h, followed by colony count to determine the total intracellular conidia.

## Neutrophil Phagocytosis of *A. fumigatus* Conidia using Flow Cytometry

FITC solution (0.1 mM) was prepared by dissolving FITC powder in sterile 0.1 M $Na_2CO3$ (dissolved in PBS). $10^8$ conidia were resuspended in 5 mL of FITC solution in a 50 mL tube and incubated for 20 minutes at 37°C in a rotator at 250 rpm. For the negative control, conidia were resuspended in 0.1 M $Na_2CO3$ (without FITC) in a 50 mL tube and incubated for 20 minutes at 37°C in a rotator. At the end of the rotation, the stained conidia were washed by adding 10 mL of PBS-T to the suspension and centrifuging at 4000 rpm, 4°C for 5 minutes. The supernatant was removed and the washing step was repeated twice with 10 mL of PBS. C57BL/6J and *Ramp1*$^{-/-}$ mouse bone marrow cells were harvested and their bone marrow neutrophils were isolated with an EasySep™ mouse neutrophil enrichment kit (StemCell Technologies - 19762) according to the manufacturer's instructions and the purity was confirmed using flow cytometry. For the neutrophil phagocytosis assay, 0.5 x $10^6$ neutrophils were incubated with $10^6$ FITC-labeled conidia (MOI = 2) in 500 µL of RPMI + 10% FBS in a 48-well cell culture plate. Cells only (no conidia) and cells + unlabeled conidia were also included as controls. The cells were incubated in a humidified $CO_2$ incubator at 37°C for 4 hours and were harvested into a 1.5 mL tube after incubation. The suspension was centrifuged for 5 minutes at 1500 rpm and 4°C before resuspension in 2 mM FACS wash buffer.

## Antibody Staining

Antibody cocktail was prepared (100 µL per well) in FACS staining buffer using Alexa Fluor 700 anti-Ly-6G, APC-Cy7 anti-CD45, and APC anti-FITC antibodies. A live-cell stain

was used to exclude dead cells. 100 µL of each sample was added into respective wells of a 96-well V-bottom plate followed by 100 µL of FACS wash buffer. The plate was secured from direct light and centrifuged at 1500 rpm and 4°C for 5 minutes. The supernatant was discarded and cells were resuspended in 100 µL of antibody cocktail and incubated in darkness and on ice for 25 minutes. 100 µL of FACS staining buffer was added to each well and washed by centrifuging at 1500 rpm and 4°C for 5 minutes. Cells were resuspended in 200 µL of FACS fixative buffer, fixed in ice, and transferred into a round bottom FACS tube. Flow cytometry was carried out using an LSR Fortessa X-20 flow cytometer (Becton Dickinson) and data was acquired with BD DIVA software. The acquired data were analyzed with FlowJo (FlowJo™ v10.9). To record sample data, the FACSDiva acquisition software was set to display FSC and SSC in the acquisition software and gates set around neutrophils. Based on the neutrophil gate, a dot plot was displayed for SSC/Ly6G and conidia were separated from $Ly6G^+$ cells. Neutrophils were displayed in a dot plot anti-FITC/FITC and quadrants for anti-FITC and FITC signals set. At least 50000 events were recorded in the neutrophil gate.

## Animal Handling

C57BL/6J, *B6.Trpv-1-cre*$^{+/+}$ and *B6.Dta*$^{+/+}$ mice were purchased from Jackson Laboratories. All mice used were duly approved by the Kansas State University Institutional Animal Care and Use Committee (IACUC). Mice were bred and harbored in a pathogen-free animal facility at the Kansas State University Division of Biology Animal facility and infected in the College of Veterinary Medicine Animal Facility. *TRPV1-cre*$^{+/-}$ mice were bred with B6.*Dta*$^{+/+}$ mice to generate *TRPV1-cre*$^{+/-}$; *Dta*$^{+/-}$ mice and control littermates (*TRPV1-cre*$^{-/-}$; *Dta*$^{+/-}$). Age-matched 8- to 12-week-old male and female mice were used for experiments.

## Chemical Ablation of TRPV1$^+$ Nociceptors

To ablate TRPV1$^+$ neurons in both the vagal ganglia (VG) and the dorsal root ganglia (DRG) in adult mice, the mice were treated with resiniferatoxin (RTX, pharmacologic approach), following an already established protocol (Baral et al., 2018a; Kashem et al., 2015; Riol-Blanco et al., 2014). RTX is a high-affinity agonist for the ion channel TRPV1. RTX treatment leads to calcium overload in neurons, denervation, and a specific loss of TRPV1$^+$ neurons, including those that innervate the respiratory tract. Four-week-old C57BL/6 mice were subcutaneously treated with RTX as previously described (Baral et al., 2018a; Kashem et al., 2015; Riol-Blanco et al., 2014). RTX was dissolved in 2% DMSO with 0.15% Tween 80 in PBS and subcutaneously injected in the flank of these mice for three consecutive days at an increasing dose (30, 70, and 100 μg/kg respectively). The control mouse group received the vehicle alone. In past studies, RTX neither affects splenic or lymph node immune cell subsets at homeostasis nor has off-target effects on lung function and overall mouse physiology.

## Lung Fungal Infections

Age-matched 8- to-12-week-old male and female C57BL/6J, TRPV1$^{DTA}$ mice and control littermates were anesthetized by isoflurane inhalation followed by the inoculation of $10^8$ *A. fumigatus* conidia resuspended in PBS. 17.5 μL of the conidia suspension was administered into each nostril of the mouse through intranasal route.

## Bronchoalveolar Lavage Fluid (BALF) Analysis

Mice were euthanized by $CO_2$ inhalation, and the trachea was exposed and cannulated with a 20-gauge catheter (BD Insyte Autoguard). BALF was collected two times by instilling 0.8 ml of cold PBS containing heparin and dextrose (BALF buffer). The collected BALF was

centrifuged at 1500 rpm and 4°C for 5 minutes, and the cell pellet was separated from the supernatant. Total BALF leukocytes were counted after red-blood-cell lysis (RBC lysis buffer, Thermofisher). Cell-free BALF supernatant was mixed with a protease/phosphatase-inhibitor cocktail, then kept at −80°C for BALF total protein and cytokine analyses.

## BALF Fungal-load measurements

BALF was serially diluted in PBS and plated on SDA plates. The fungal CFU were enumerated after 24 hours incubation of the SDA plates at 37°C.

## CGRP ELISA

CGRP levels from uninfected TRPV1$^{+}$ neuron-ablated and control mice were measured using mouse CGRP1 ELISA Kit (Novus Biologicals: NBP3-00522) according to the manufacturer's instructions.

## Reproducibility and Statistical Analysis

To enhance experimental rigor, investigators were blinded to animal group and/or experimental condition during data collection and analysis, whenever possible. Phagocytosis and killing assays, FACS, and cytokines were compared with one-way ANOVA, two-tailed unpaired t tests for parametric analyses, or Mann–Whitney test for nonparametric analyses. Statistical significance was accepted at $p \leq 0.05$. Data were plotted in Prism (GraphPad, Boston, MA; Version 9.3.0 (463)). For *in vivo* experiments, we included both male and female mice.

# Chapter 3 - Results

## 3.1 CGRP Suppresses Macrophage Inflammatory Cytokine Response during *A. fumigatus* Infection

Recognition of *A. fumigatus* β-1,3-glucan by the macrophage dectin-1 induces a chain of proinflammatory responses (Hohl et al., 2005; Lionakis et al., 2023). The initial responses aim to recruit phagocytic neutrophils and monocytes into the alveolar space for pathogen clearance. The airway epithelia and lung resident immune cells (alveolar macrophages and dendritic cells) secrete cytokines and chemokines including TNF-α, IL-1α/β that induces the epithelial cell, and autocrine secretion of IL-6, MCP-1, CXCL1, and CXCL5 necessary for neutrophil and monocyte recruitment (Caffrey et al., 2015; Jhingran et al., 2015; Sugui et al., 2015). With the idea that these resident immune cells are colocalized with lung nociceptive neurons, we reasoned that these neurons can secrete neuropeptides to influence the response of these immune cells to *A. fumigatus* infection. We aimed to screen these neuropeptides (CGRP, SP, SST and VIP) to understand how they regulate inflammation. Therefore, we stimulated Bone Marrow-Derived Macrophages (BMDMs) with swollen *A. fumigatus* conidia (swollen for 6 hours at 37˚C without shaking) to determine how these neuropeptides control the secretion of the key inflammatory cytokine TNF-α after 16 hours of stimulation. The TNF-α levels were determined using Enzyme-linked Immunoassay (ELISA). CGRP suppresses BMDM secretion of the proinflammatory cytokine TNF-α in a dose-dependent manner and at both conidia MOI of 2 and 10 **(Figure 3.1a-b)**. Similar effects were observed for other neuropeptides - substance P, somatostatin and VIP at MOI of 2 but not 10 **(Figure 3.1c-d & Figure 3.2a-d)**. To further understand the reproducibility of this result, RAW 264.7 cells, a macrophage cell line used as a model for macrophages (Taciak et al., 2018) was stimulated using the same procedure as described above. The response was

similar to the observation for CGRP that showed dose-dependent decrease in TNF-α secretion with CGRP co-treatment **(Figure 3.3)**. The level of BMDM inflammatory response is dependent on the time and conditions during conidia swelling (Hohl et al., 2005). In attempt to induce more inflammation, we also swelled the conidia longer for 7 hours at 37°C with shaking at 250 rpm throughout the swelling time. With this, the level of TNF-α secretion was elevated to about 7-fold **(Figure 3.4a)**. We also observed an intense suppression of TNF-α secretion with 1μM CGRP concentration but not with SP at MOI of 10 **(Figure 3.4a-b)**. However, the difference in TNF-α release disappeared when RAMP1$^{-/-}$ BMDMs were applied for this study **(Figure 3.4a)**. These observations are important and relevant because TNF-α is one of the earliest cytokines secreted during infections and therefore serves to understand how these neuropeptides may influence inflammation during *A. fumigatus* infections. CGRP is particularly important as it has been well cited to play varying roles during some bacterial and specifically *Candida albicans* infections (Baral et al., 2018a; Kashem et al., 2015) but not for *A. fumigatus*. Our subsequent experiments focus on CGRP as it showed more consistency and can be replicated with another macrophage model.

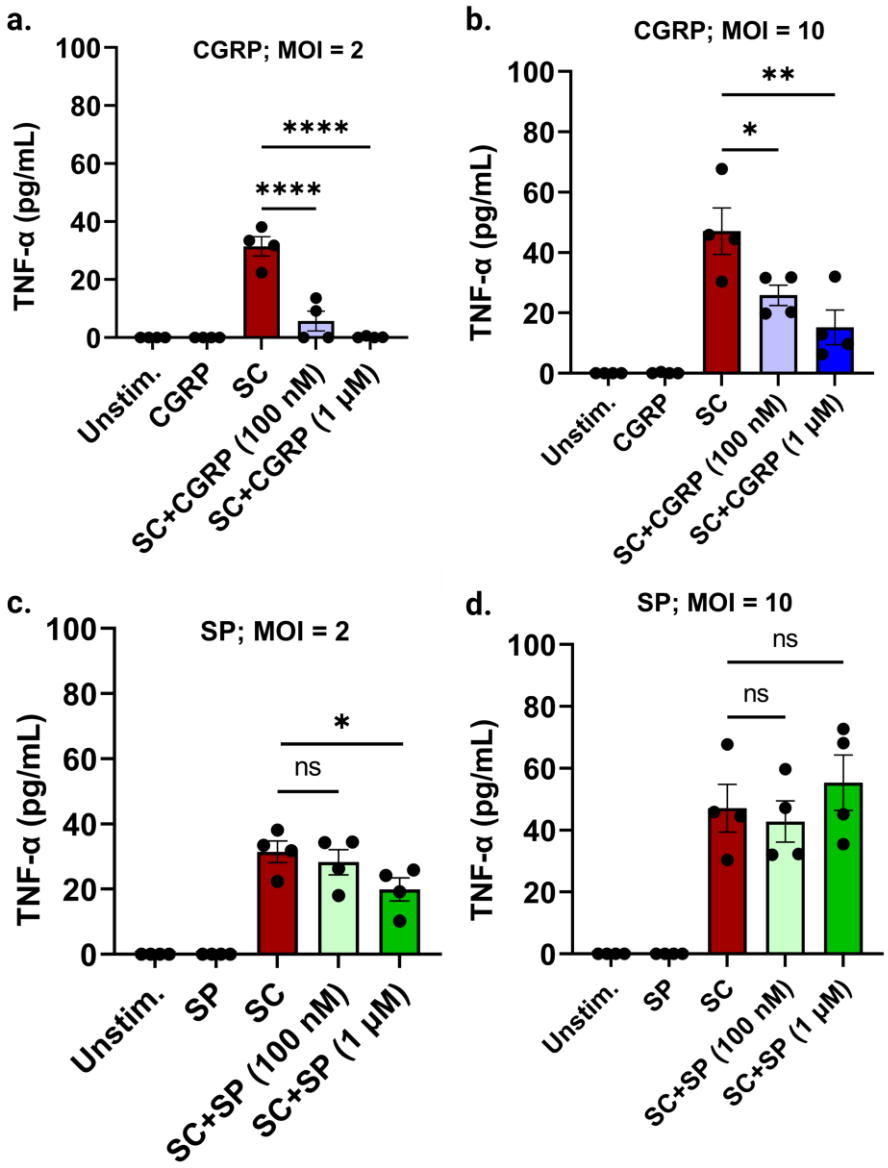


**Figure 3.1: CGRP reduces BMDM TNF-α secretion during *A. fumigatus* stimulation.**
BMDMs were stimulated with *Aspergillus fumigatus* at MOI of 2 and 10 in the presence and absence of CGRP **(a & b)** and SP **(c & d)** at concentrations of 100 nM and 1 μM. This was followed by ELISA analysis of TNF-α secretion in the supernatant after 16 hours of stimulation. CGRP = Calcitonin Gene-Related Peptide; SP = substance P; SC = swollen conidia. Statistical analysis: Ordinary one-way ANOVA (Tukey's multiple comparisons test), *=p<0.05, **=p<0.01, and ****=p<0.0001.

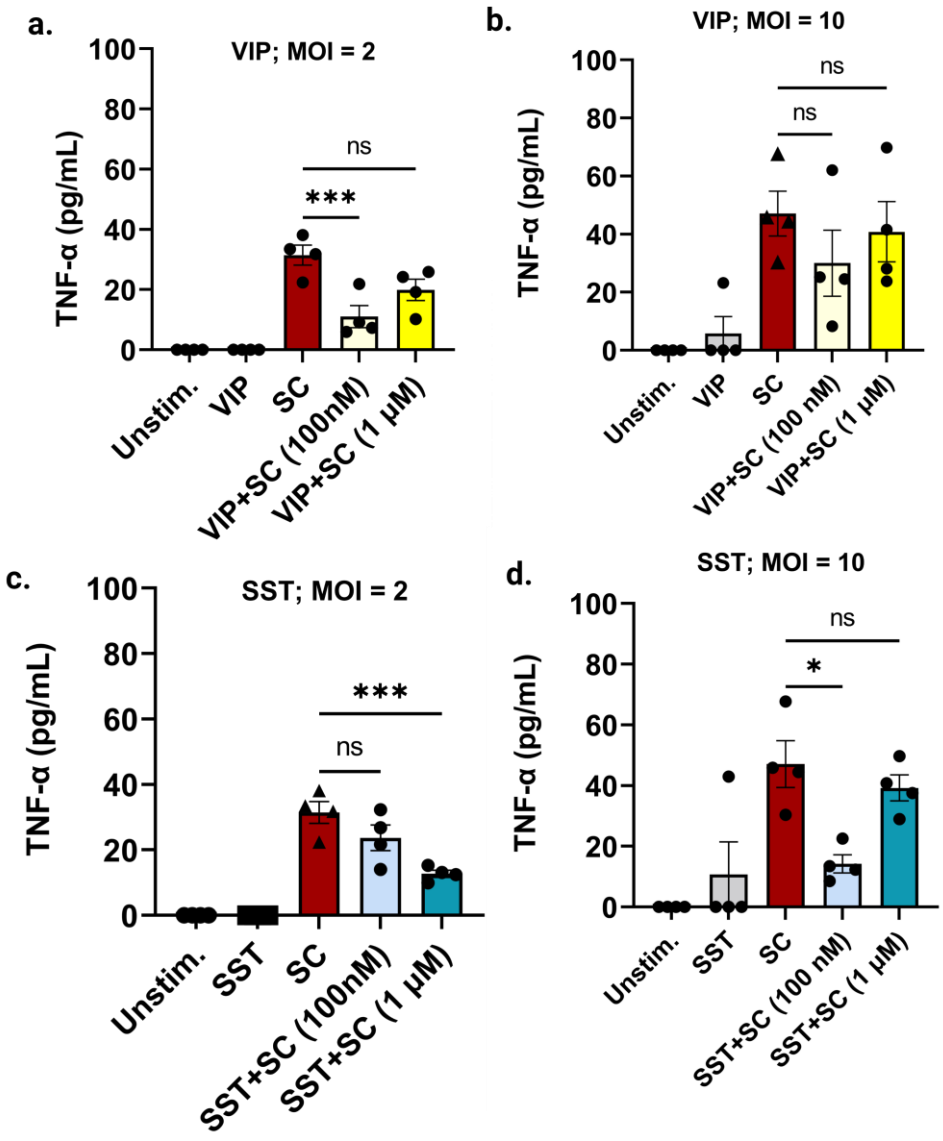


**Figure 3.2: VIP and SST influence BMDM TNF-α response during *A. fumigatus* stimulation.**

BMDMs were stimulated with *Aspergillus fumigatus* at MOI of 2 and 10 in the presence and absence of VIP **(a & b)** and SST **(c & d)** at concentrations of 100 nM and 1 µM. This was followed by ELISA analysis of TNF-α secretion in the supernatant after 16 hours of stimulation. VIP = Vasoactive Intestinal Peptide; SST = Somatostatin; SC = swollen conidia. Statistical analysis: Ordinary one-way ANOVA (Tukey's multiple comparisons test), *=$p<0.05$, and ***=$p<0.001$.

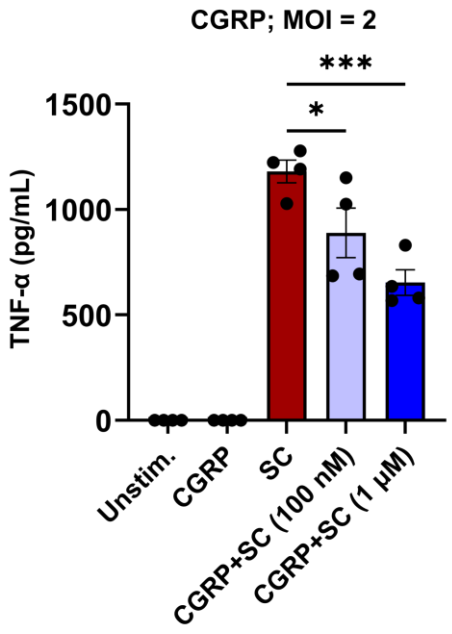


**Figure 3.3: CGRP reduces TNF-α secretion in RAW 264.7 cells during *A. fumigatus* stimulation.**

RAW 264.7 cells were stimulated with *Aspergillus fumigatus* at MOI of 2 in the presence and absence of CGRP and at concentrations of 100 nM and 1 µM. This was followed by ELISA analysis of TNF-α secretion in the supernatant after 16 hours of stimulation. CGRP = Calcitonin Gene-Related Peptide; SC = swollen conidia. Statistical analysis: Ordinary one-way ANOVA (Tukey's multiple comparisons test), *=p<0.05, and ***=p<0.001.

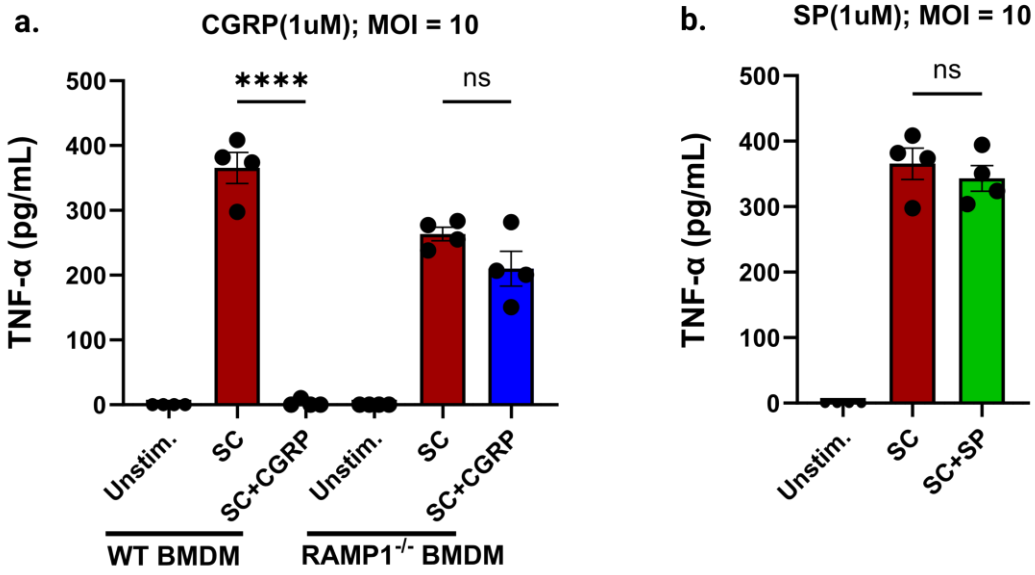


**Figure 3.4: The effect of CGRP on BMDM TNF-α secretion during *A. fumigatus* stimulation is rescued with RAMP1$^{-/-}$ BMDM.**

C57BL/6 and RAMP1$^{-/-}$ BMDMs were stimulated with *Aspergillus fumigatus* at MOI of 10 in the presence and absence of 1 µM CGRP **(a)** and SP **(b)**. This was followed by ELISA analysis of TNF-α secretion in the supernatant after 16 hours of stimulation.

CGRP = Calcitonin Gene-Related Peptide; SP = substance P; SC = swollen conidia. Statistical analysis: **a** - Ordinary one-way ANOVA (Tukey's multiple comparisons test) and **b** – Unpaired t test, *=p<0.05, **=p<0.01, and ****=p<0.0001

## 3.2 CGRP increases Macrophage and Monocyte Phagocytosis and Killing Capacity of *A. fumigatus* Conidia

Lung-resident alveolar macrophages are among the first point of contact during *A. fumigatus* invasion. Downstream signaling after fungal interactions with the resident cells extends to the recruitment of $Ly6C^{hi}$ monocytes and monocyte-derived DCs which are also important phagocytic mediators. Alveolar macrophages and recruited monocytes engage *A. fumigatus* mainly through their uptake and killing of these conidia. We investigated the effect of CGRP, SP, VIP and SST on the phagocytosis and killing ability of macrophages and monocytes. First, we employed nystatin protection assay to analyze the killing ability of BMDMs in the presence of these neuropeptides and found that whereas CGRP increases the BMDM killing ability, VIP suppresses this activity, but SP and SST exerted no effect on this activity **(Figure 3.5a & Figure A.4.2)**. We went further to study more of this phenomenon starting from the effect of CGRP on the phagocytosis and killing activity of BMDMs and noticed that phagocytosis and killing activity are increased in these immune cells in the presence of CGRP. This was confirmed by using BMDMs isolated from C57BL/6 (B6) and *Ramp1*$^{-/-}$ (KO) mice in which we noticed that the effect of CGRP treatment was abrogated in $RAMP1^{-/-}$ BMDMs (**Figure 3.5 a-b**). Alternatively, we utilized immunocytochemistry and confocal microscopy techniques in which we labeled the *A. fumigatus* conidia with Alexa Fluor™ 488 NHS Ester (Succinimidyl Ester), BMDMs with F4/80 antibody and nucleus with DAPI and used confocal microscopy to analyze BMDM phagocytosis after conidia-CGRP coinfection. Again, CGRP resulted in increased phagocytosis of these cells while its effect was annulled in the absence of

the RAMP1 co-receptor **(Figure 3.6 a-b)**. Nystatin assay was also performed with both monocytes isolated from B6 and KO mice, and CGRP was observed to play the same role in these cells during phagocytosis and fungal killing **(Figure 3.7 a-b)**.

Cyclic AMP (cAMP) acts as a second messenger during intracellular signaling that involves GPCRs (Sunahara et al., 1996). Following its activation by cAMP, Protein Kinase A (PKA) mediates a number of intracellular responses (Di Benedetto et al., 2008) including the response to CGRP. Phosphodiesterase 4 specifically degrades cAMP to decrease the magnitude of its signaling (Cedervall et al., 2015). PKA inhibitors (PKAi) also decrease cAMP-dependent signaling. Rolipram is widely used as a phosphodiesterase 4 (PDE4) inhibitor (Eschenhagen, 2013) whereas Rp-8-CPT-Cyclic AMP (sodium salt) serves as a site-selective inhibitor of PKA (Dostmann et al., 1990).

Cyclic AMP (cAMP) and Protein Kinase A (PKA) are both involved in CGRP signaling, hence the effect noticed with CGRP treatment is dependent on these molecules. Also, changing the cellular levels of cAMP and PKA in BMDMs would affect the fungicidal activity of these cells. Therefore, we explored the effect of stabilizing cAMP levels, and the effect of inhibiting the activity of PKA by using rolipram and Rp-8-CPT-Cyclic AMP (sodium salt). cAMP stabilization increases the conidial killing activity of BMDMs whereas PKA inhibition reduced this effect. PKAi addition in the presence of CGRP also eliminated the fungicidal activity noticed earlier with CGRP treatment **(Figure 3.8 a-b)**.

This indicates that the increased fungicidal activity of BMDMs noticed is due to CGRP signaling. Altogether, CGRP signals through its RAMP1 receptor to increase the phagocytic and fungicidal activity of BMDMs and monocytes.

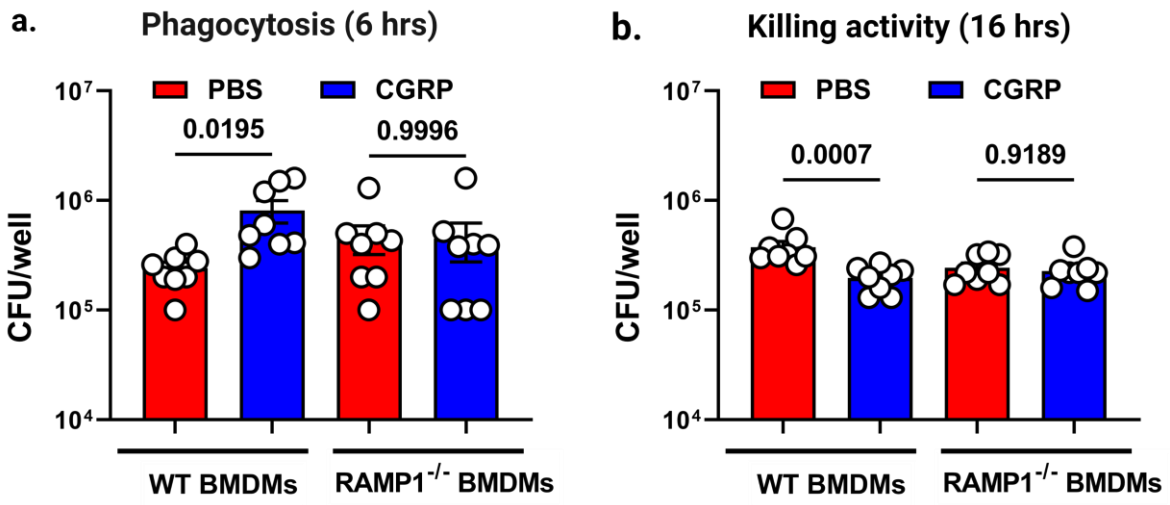


**Figure 3.5: CGRP increases macrophage phagocytosis and killing of *A. fumigatus* conidia.**
BMDMs from C57BL/6 and *Ramp1*$^{-/-}$ mice were challenged with *A. fumigatus* conidia in the presence or absence of 1 μM CGRP at MOI of 10 for 6 hours (phagocytosis, **a**) or incubated again afterward with nystatin (20 μg/mL) for another 10 hours (killing activity, **b**), lysed with Triton X-100, diluted, plated onto SDA plates, and followed by colony count after 24 hours. Statistical analysis: Ordinary one-way ANOVA (Sidak's multiple comparisons test).

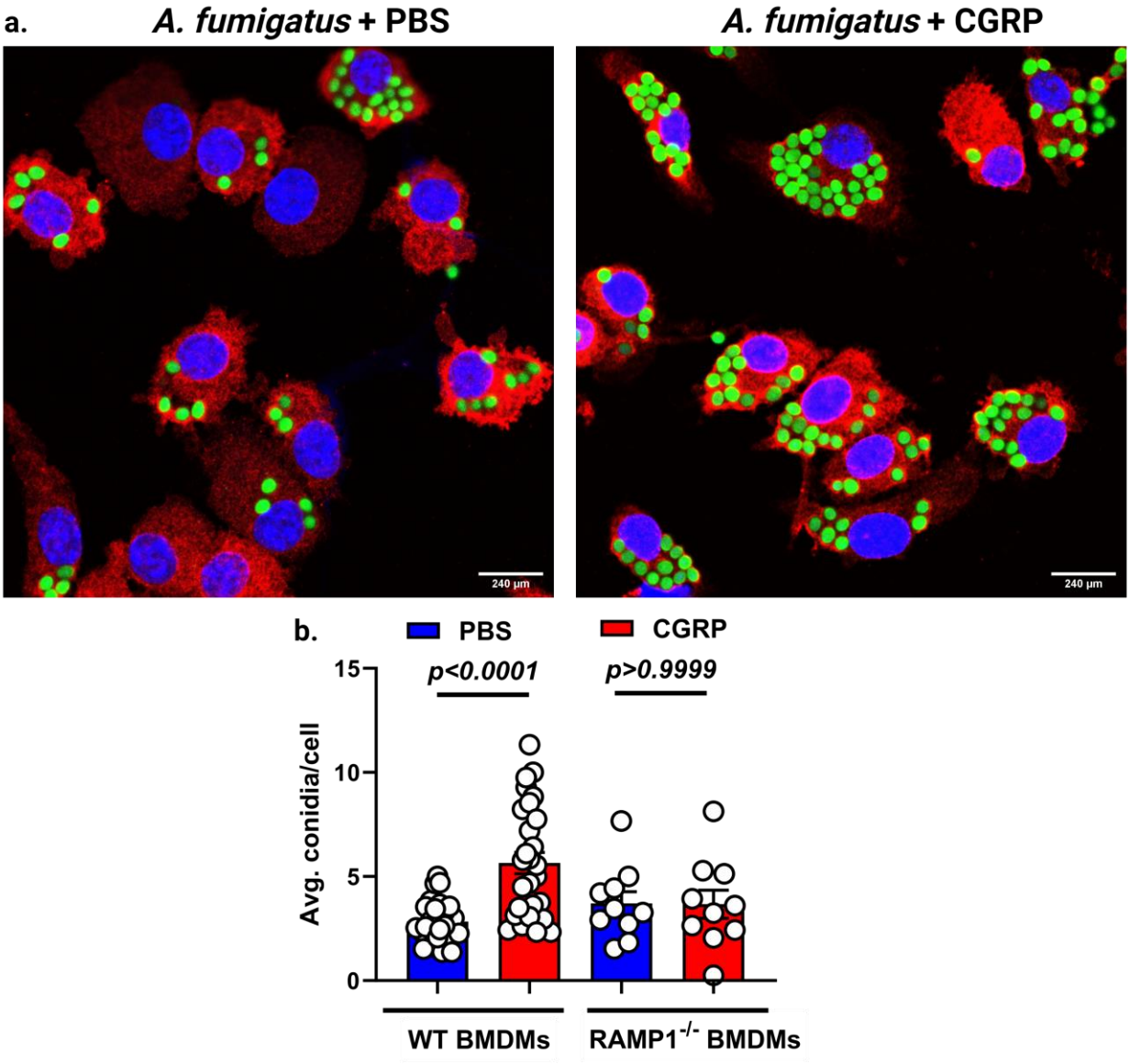


**Figure 3.6: Confocal images and plotted data of BMDMs infected with *A. fumigatus* conidia.**

**(a)** BMDMs were co-incubated with *A. fumigatus* conidia (MOI = 2) and 1µM CGRP for 4 hours followed by fixation and immunocytochemistry. F4/80 was stained with Alexa Fluor 594 (red), Nucleus with DAPI (blue), and *A. fumigatus* conidia labeled with Alexa Fluor 488 (green). **(b)** Analysis of the level of phagocytosis displayed in **a**; Each data point represents average conidia engulfed per cell in each image field. Each experiment was performed in quadruplicates per condition. Statistical analysis: Ordinary one-way ANOVA (Tukey's multiple comparisons test).

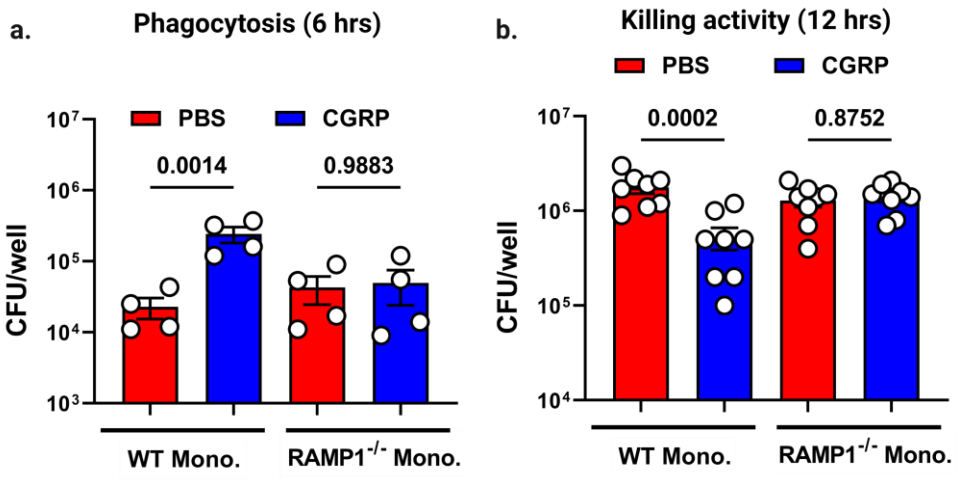


**Figure 3.7: CGRP increases monocyte phagocytosis and killing of *A. fumigatus* conidia.**
**(a-b)** Monocytes from C57BL/6 and *Ramp1*$^{-/-}$ mice were challenged with *A. fumigatus* conidia in the presence or absence of 1 µM CGRP at MOI of 10 for 6 hours (phagocytosis, **a**) or 12 hours (killing activity, **b**), lysed with Triton X-100, diluted, plated onto SDA plates, and followed by colony count after 24 hours. Statistical analysis: Ordinary one-way ANOVA (Sidak's multiple comparisons test).

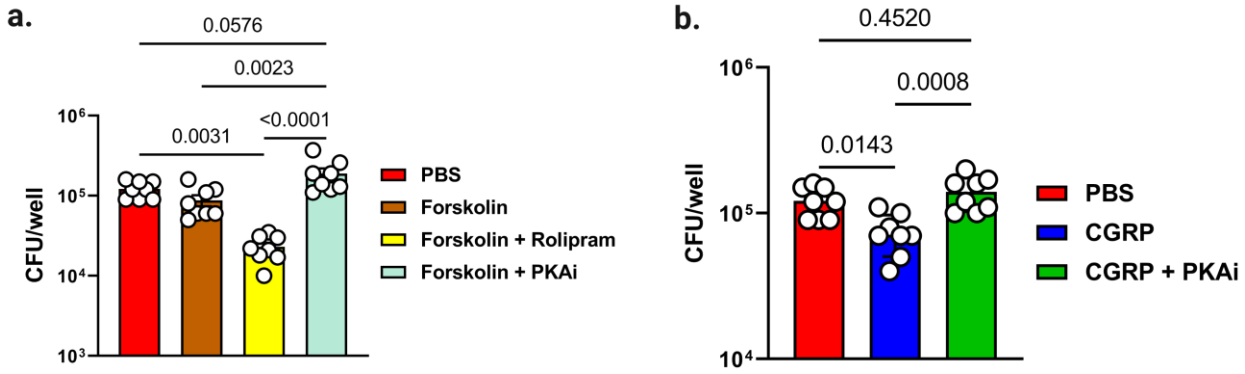


**Figure 3.8: Increased fungicidal activity of BMDMs relies on CGRP-RAMP1 signaling.**
**(a-b)** BMDMs from C57BL/6 mice were challenged with *A. fumigatus* conidia according to the following parameters: forskolin only, forskolin + rolipram, and forskolin + PKAi **(a)**; CGRP, and CGRP + PKAi **(b)** at MOI of 10 for 6 hours, washed and followed by incubation with nystatin (20 µg/mL) for another 10 hours, lysed with Triton X-100, diluted, plated onto SDA plates, and followed by colony count after 24 hours. Statistical analysis: Ordinary one-way ANOVA (Tukey's multiple comparisons test).

## 3.3 CGRP increases the Phagocytic and Fungicidal Activity of Neutrophils

Recruited neutrophils are critical for the elimination of *A. fumigatus* during infections. They mediate fungal clearance through phagocytosis, ROS production and Neutrophil Extracellular Traps (NETs) to damage and degrade conidia. Here, we employed flow cytometry-

based techniques to analyze how CGRP affects antifungal activity of neutrophils. This technique allowed us to determine the proportion of internalized FITC-labelled *A. fumigatus* conidia and the uninternalized/extracellular antibody-bound conidia. We stained the neutrophils with CD45, Ly6G and anti-FITC antibodies, and distinguished internalized conidia (R2) from conidia that are attached on the surface of neutrophils (R1). We also utilized cytochalasin D (CytD) as a negative control to block the internalization of conidia by the neutrophils (**Figure 3.10a).** We observed no difference in conidia phagocytosis at 2 hours post-stimulation of neutrophils between CGRP-treated and untreated conditions **(Figure 3.10 a-b)**. However, CGRP-treated neutrophils phagocytosed higher proportion of conidia after 4 hours compared to the untreated control cells, and using neutrophils isolated from *Ramp1*$^{-/-}$ mice abolished the difference in phagocytosis noticed with CGRP treatment of B6 mice-isolated neutrophils **(Figure 3.10 a & c)**.

*Aspergillus fumigatus* phagocytosis by neutrophils is followed by the step-wise deployment of NADPH-oxidase-derived ROS into the phagolysosome to further arrest conidial germination into its hyphal forms. DCFDA / $H_2$DCFDA - Cellular ROS Assay was employed to understand the influence of CGRP on neutrophil ROS production. In this case, a higher induction of ROS indicates an increased conidial killing and vice versa. As shown in Figure 3.11a, neutrophils not co-incubated with conidia exhibited <0.05% $ROS^+$ cells. In contrast, PMA stimulation, used as positive control, robustly increased the proportion of $ROS^+$ cells. CGRP-treated neutrophils induced higher ROS production than the untreated controls **(Figure 3.11 & Figure 3.12 a-b)**. These findings point out the role of CGRP in enhancing neutrophil-mediated elimination of *A. fumigatus* upon their recruitment into the airways during infections.

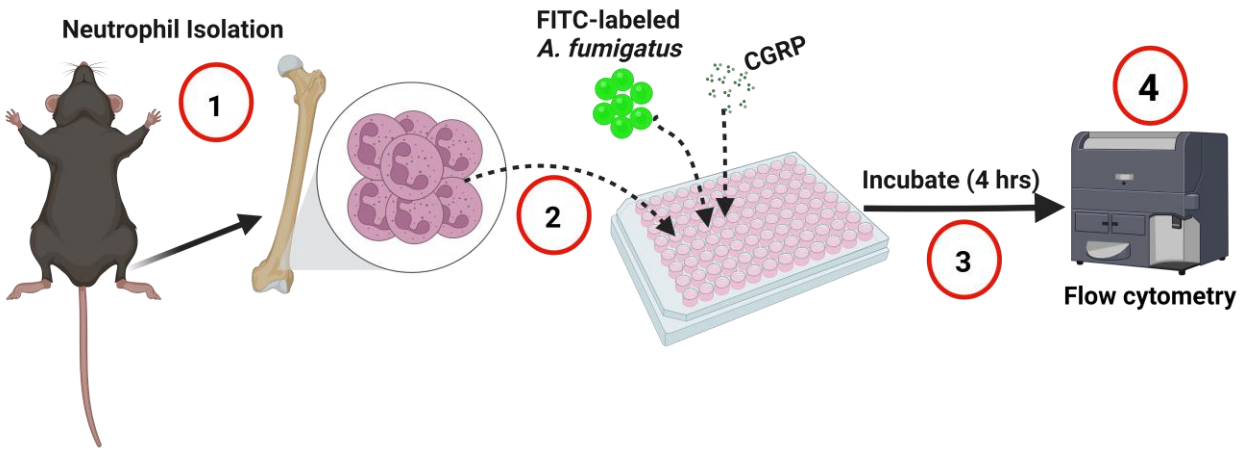


**Figure 3.9: Schematic of the procedure for neutrophil phagocytosis of *A. fumigatus* conidia.**
C57BL/6 and RAMP1$^{-/-}$ Bone marrow neutrophils were isolated using STEMCell neutrophil isolation kit and stimulated with FITC-labelled *A. fumigatus* conidia for 2 and 4 hours followed by antibody staining and processing for flow cytometry. (Figures were made with BioRender).

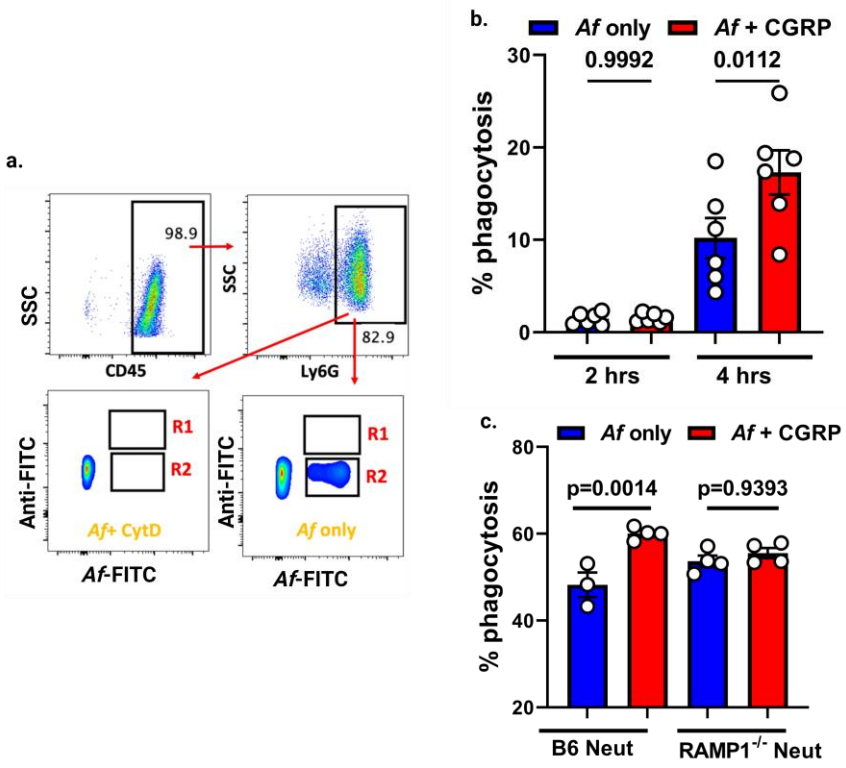


**Figure 3.10: CGRP increases neutrophil phagocytosis of *A. fumigatus* conidia.**
Bone marrow isolated neutrophils were stimulated with FITC-labelled *A. fumigatus* conidia (MOI 2) for 2 and 4 hours before flow cytometric determination of phagocytosis. **(a)** Gating strategy. R1 gate represents the proportion of cells with conidia attached on the surface of neutrophils. R2 gate represents the proportion of cells with internalized conidia. **(b)** Quantification of % phagocytosis. **(c)** Quantification of % phagocytosis in neutrophils isolated from C57BL/6 and *Ramp1*$^{-/-}$ mice. Each experiment was performed in quadruplicates per condition. Cytochalasin D (CytD) was used as negative control. Statistical analysis: Ordinary one-way ANOVA (Sidak's multiple comparisons test), Mean±SEM.

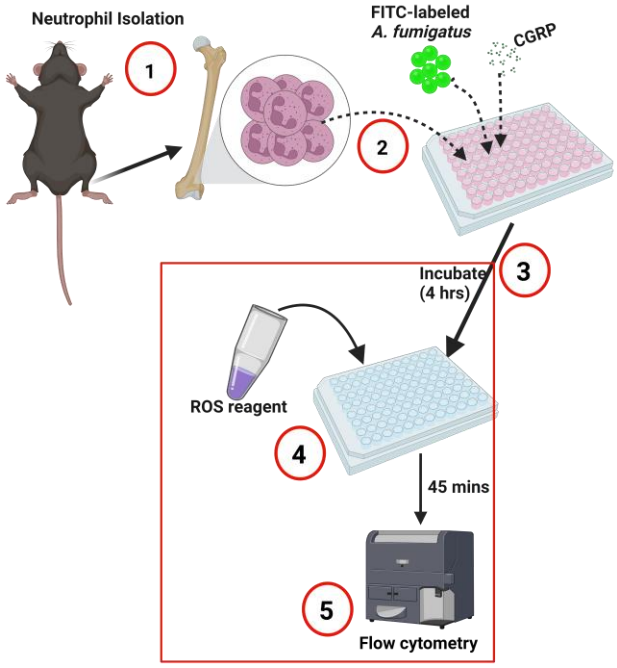


**Figure 3.11: Schematic of the procedure to measure neutrophil ROS production in response to *A. fumigatus* challenge.**

C57BL/6 and RAMP1$^{-/-}$ Bone marrow neutrophils were isolated using STEMCell neutrophil isolation kit and stimulated with *A. fumigatus* conidia for 4 hours followed by treatment with ROS detection reagent (DCFDA dye) and antibody staining, and the level of total ROS production analyzed by flow cytometry. (Figures were made with BioRender).

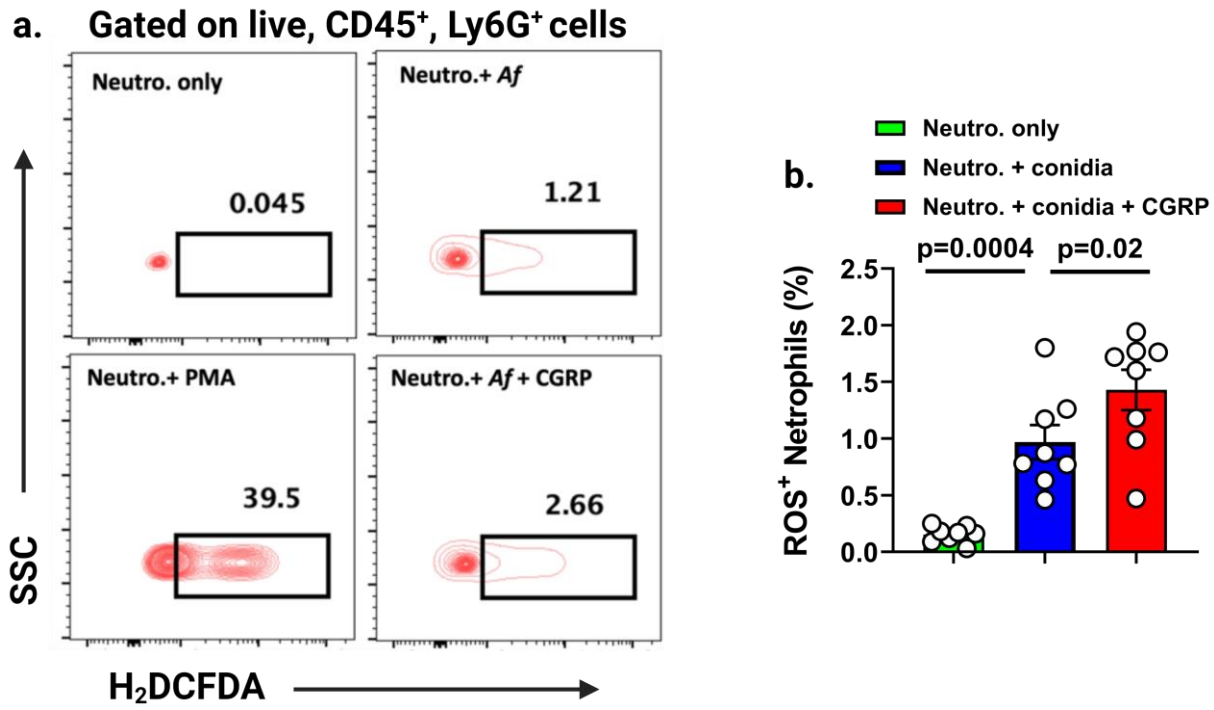


**Figure 3.12: CGRP increases neutrophil ROS production during *A. fumigatus* challenge.**
**(a)** Flow cytometry plots of Ly6G$^+$ROS$^+$ neutrophils. **(b)** Quantification of % ROS$^+$ neutrophils 4 hours post stimulation (n=8). Statistical analysis: Ordinary one-way ANOVA (Two-stage linear step-up procedure of Benjamini, Krieger and Yekutieli).

## 3.4: Nociceptive Neurons Control Fungal Clearance and Inflammatory Cytokine Levels during *A. fumigatus* Infection

We hypothesized that lung-innervating nociceptors would initiate and drive robust immune response during airway challenge with *A. fumigatus*. We reasoned that transient receptor potential vanilloid 1 expressing (TRPV1$^+$) neurons might affect pulmonary host defense. TRPV1 is a member of the transient receptor potential channel (TRP) superfamily that is selectively expressed in a subpopulation of pain-sensing primary afferent neurons. This neuronal subset expresses CGRP and other neuropeptides that have been implicated in neurogenic inflammation and to influence the response of other cellular subsets (Baral et al., 2018b; Mishra et al., 2011; Pogorzala et al., 2013). We studied the role of nociceptive neurons during *A. fumigatus* pulmonary infection by using Resiniferatoxin (RTX) as a chemical approach to ablate the

nociceptive neurons. RTX is a capsaicin analog that binds to and activates TRPV1 leading to the desensitization of the sensory neurons. We intranasally inoculated RTX- and vehicle-treated mice with $10^8$ *A. fumigatus* conidia. At 24 hours post-infection, RTX-treated mice, compared with vehicle-treated controls exhibited a trend towards higher inflammation and lung tissue leakage as judged by their secretion of higher inflammatory TNF-α, IL-6, and MCP-1 cytokine and chemokine, and higher protein levels in their Bronchioalveolar lavage (BAL) fluid respectively **(Figure 3.14 a-d)**. Alternatively, we employed a genetic approach as a second strategy to ablate $TRPV1^+$ neurons. $TRPV1^{DTA}$ mice express diphtheria-toxin A (DTA) under the control of mouse TRPV1 regulatory sequences (Mishra et al., 2011) **(Figure 3.15 a)**. Mouse cells are normally resistant to diphtheria toxin (DT)-induced apoptosis because they lack the receptor, but are rendered susceptible by their expression of DTA (Baral et al., 2018). We confirmed the success of this approach by determining the concentration of CGRP in the BALF of uninfected $TRPV1^+$ neuron-ablated and control mice. These mice showed a significant reduction in CGRP concentration in comparison with the control group **(Figure 3.15 b)**. Analysis of fungal load after $TRPV1^{DTA}$ mice infection with *A. fumigatus* indicates that compared with control littermates, $TRPV1^+$ neuron-ablated mice exhibited higher fungal burden recovered from BALF at 24 hours post-infection **(Figure 3.15 c)**. In agreement with our RTX data, *A. fumigatus*-infected $TRPV1^+$ neuron-ablated mice exhibited higher TNF-α, IL-6 and MCP-1 in their BALF compared to control littermates **(Figure 3.15 d-f)**.

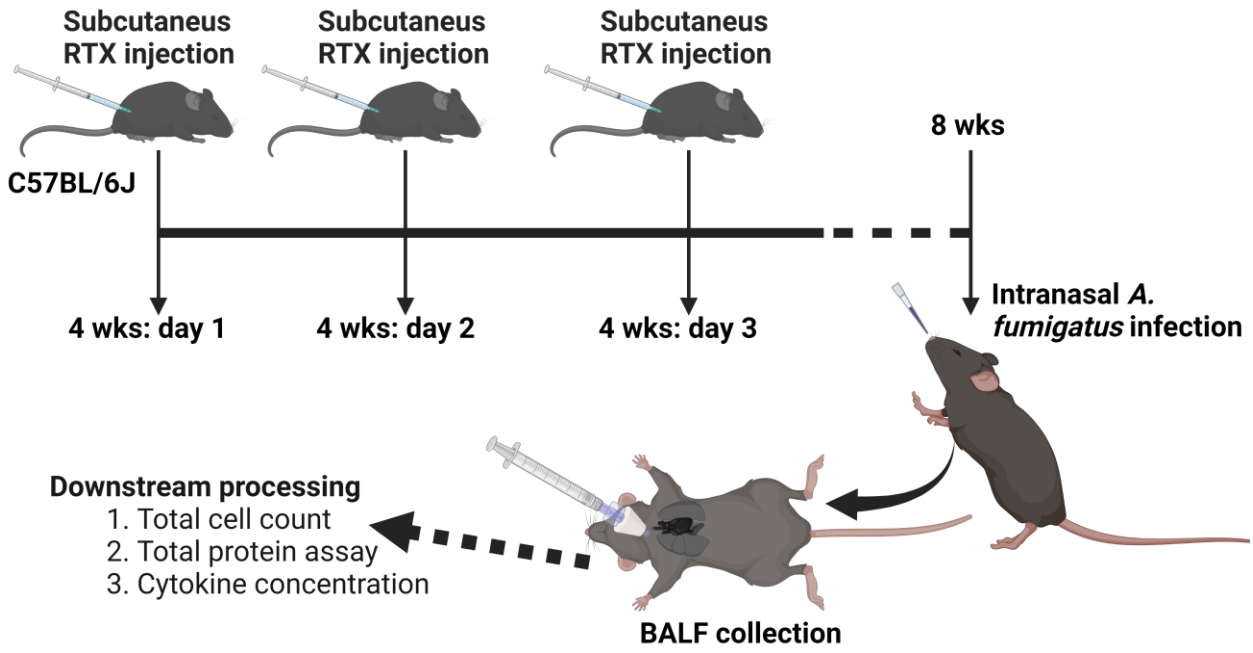


**Figure 3.13: Schematic for chemical ablation of TRPV1$^+$ nociceptive neurons and their infection with *A. fumigatus* conidia.**

TRPV1$^+$ nociceptive neurons in 4-week-old C57BL/6 mice were ablated by subcutaneously injecting their flanks with resiniferatoxin for three consecutive days at an increasing dose of 30, 70, and 100 μg/kg respectively. Mice were intranasally infected with $10^8$ conidia at 8 weeks of age. (Figures were made with BioRender).

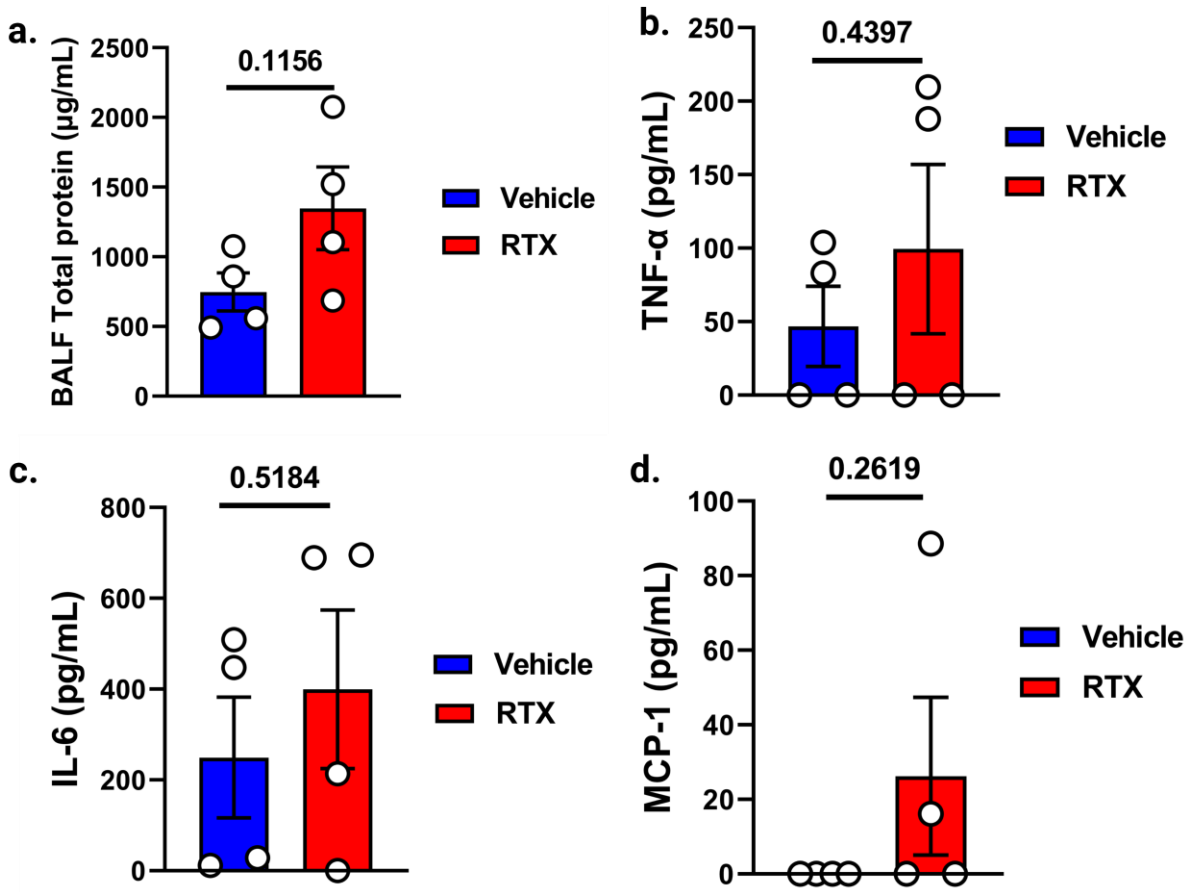


**Figure 3.14: Nociceptive neurons control inflammatory cytokine levels during *A. fumigatus* infection.**

Bronchoalveolar Lavage Fluid (BALF) was collected from *A. fumigatus*-infected nociceptive neuron ablated and control littermates after 24 hours of infection followed by analysis for total protein **(a)**, TNF-α **(b)**, IL-6 **(c)**, and MCP-1 **(d)**; n = 4. Statistical analysis: Unpaired t test.

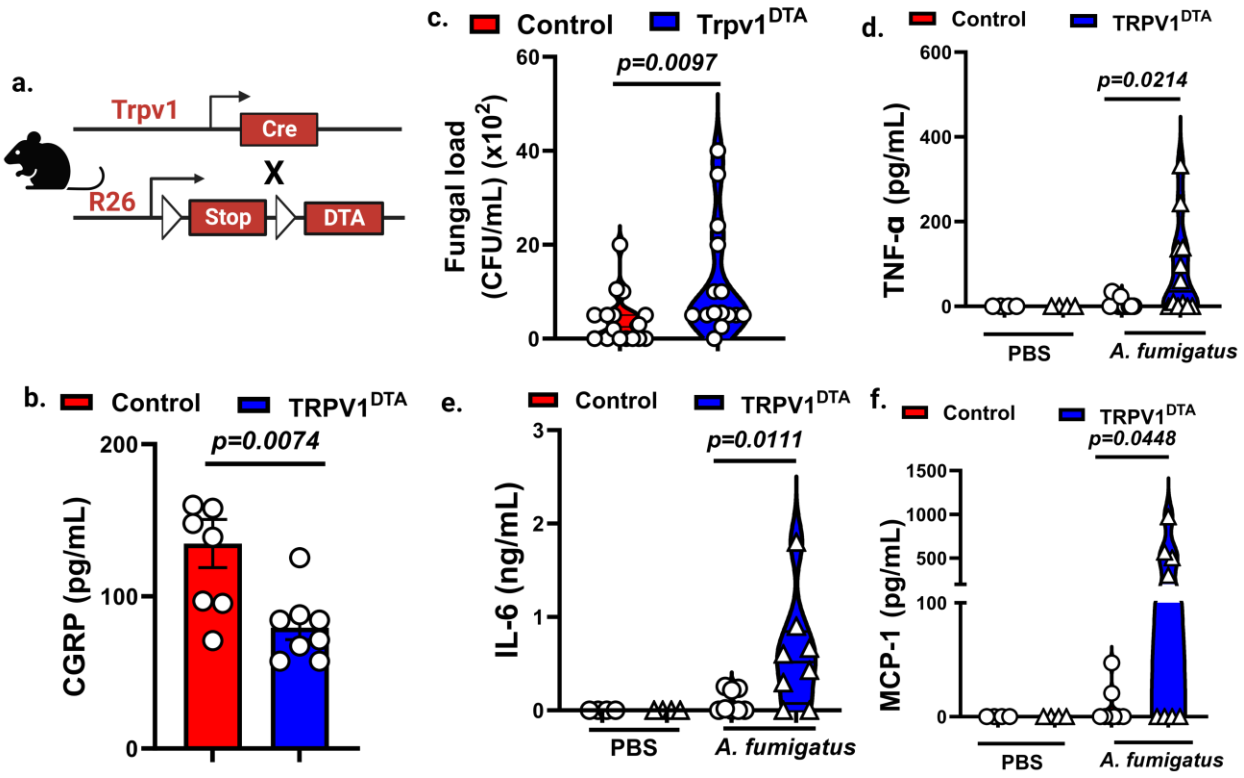


**Figure 3.15: Nociceptive neurons control fungal clearance and inflammatory cytokine levels during *A. fumigatus* infection.**

**(a)** Schematic for the genetic ablation of TRPV1$^+$ nociceptive neurons. **(b)** Concentration of CGRP in BALF of uninfected TRPV1$^+$ neuron-ablated and control mice. **(c-f)** (Bronchoalveolar Lavage Fluid (BALF) was collected from *A. fumigatus*-infected nociceptive neuron ablated and control littermates after 24 hours of infection followed by fungal load enumeration **(c)**, TNF-α **(d)**, IL-6 **(e)**, and MCP-1 **(f)**; n = 4 - 15. Statistical analysis: Unpaired t test **(a)**, Mann-Whitney test **(c)**, Ordinary one-way ANOVA: Sidak's multiple comparisons test **(d & f)**, Two-stage linear set-up procedure of Benjamin, Krieger and Yekutieli **(e)**.

# Chapter 4 - Discussion and Conclusion

In this study, we discovered CGRP involvement in the clearance of *A. fumigatus* - it influences the action of the major innate immune cells that determine the outcome of *A. fumigatus* infection. The *in vitro* results from this study are in line with the *in vivo* system using the nociceptive neuron-ablated mice. CGRP treatment lowers the level of inflammatory TNF-α released by the BMDMs but increases their ability to engulf and kill these conidia. Consistent with our *in vitro* data, we noticed a trend towards rising levels of TNF-α, IL-6, MCP-1, and total protein levels during *A. fumigatus* infection in RTX-treated mice that lack nociceptive neurons (the sources of CGRP). This was further substantiated by the use of an alternative genetic approach to ablate the $TRPV1^{+}$ neurons. Here, these mice released significantly higher TNF-α, IL-6, MCP-1, and showed reduced ability to kill the *A. fumigatus* conidia as they contained higher fungal load in their BALF compared with the control mice. This means that CGRP may act to balance the inflammatory responses initiated during *A. fumigatus* infection, and still enhance the clearance of the spores from the airways. Further studies are needed to identify the relationship between this result, immune cell recruitment, and lung fungal burden. We also observed CGRP-mediated increase in phagocytosis and killing of conidia by monocytes and neutrophils which are mostly recruited to the airways during infections. To underscore the impact of CGRP in these phenomena, we utilized immune cells that lack the RAMP1 co-receptor for CGRP. We proved that these phenotypes were due to CGRP signaling as the effects noticed in the presence of CGRP were eliminated in the absence of its co-receptor - they took up and killed similar quantities of conidia and produced similar levels of ROS in the presence of CGRP.

The calcitonin receptor-like receptor (CLR), a G-protein-coupled receptor (GPCR) requires the RAMP1 as a vital molecule to become a functionally active receptor and to initiate

CGRP signaling (Walker et al., 2010). Upon CGRP binding, Gα protein activates adenylate cyclase to increase cyclic AMP (cAMP) levels which activates protein kinase A (PKA) (Hay et al., 2003) to drive further actions of CGRP. Blockade or elevation of CGRP-dependent cAMP and PKA accumulation would have a significant impact on the effects of CGRP signaling on immune cells. This also means that Gαs activation during *in vitro A. fumigatus* stimulation of immune cells will result in similar effects as CGRP. Our findings that cAMP stabilization/accumulation increases fungal killing by BMDMs and that PKA inhibition reverses their killing activity further lays credence to our findings about the immunomodulatory effects of CGRP during *A. fumigatus* infection.

Dectin-1 is a transmembrane signaling receptor and a C-type lectin expressed by myeloid cells including macrophages, neutrophils, and dendritic cells. It binds to and recognizes fungal PAMPs like the β-glucan to initiate an intracellular signaling cascade that potentiates fungal binding, phagocytosis, and killing. It also mediates the induction of cytokine and chemokine signaling for fungal clearance (Brown, 2006). We reason that the phenotypes noticed here with CGRP may involve either – 1) the upregulation of dectin-1 gene transcription to increase dectin-1 availability for fungal binding after CGRP-RAMP1 downstream signaling, or 2) the direct coupling of the CGRP-RAMP1 pathway with dectin-1 to increase its response to *A. fumigatus* interaction **(Figure 4.1)**. Future approaches to this study will focus on understanding the mechanisms involved in CGRP-RAMP1 signaling-mediated anti-*A. fumigatus* immune response.

Overall, our work shows that the CGRP-RAMP1 signaling pathway increases the anti-fungal innate immune response during *A. fumigatus* infection. This highlights the importance of this signaling pathway during *A. fumigatus* infections and points to it as a potential avenue in the

therapeutic treatment of these infections. Further studies are required to understand the translation of these findings into *in vivo* and/or clinical settings.

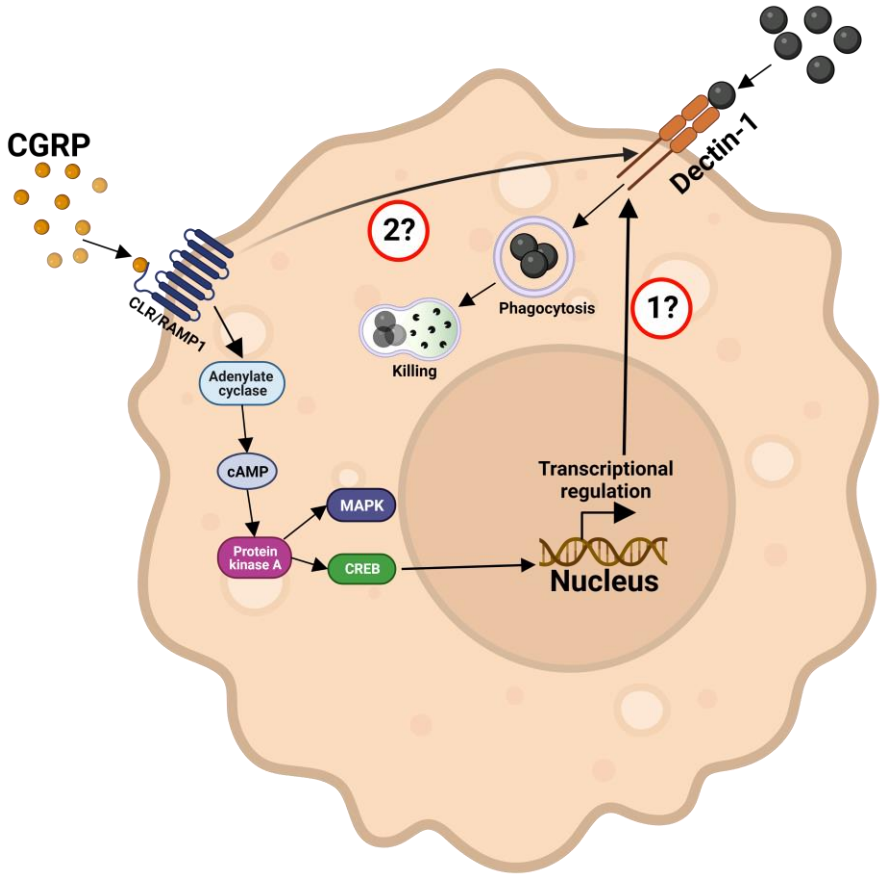


**Figure 4.1: Potential mechanism of CGRP-RAMP1 signaling in anti-*A. fumigatus* immune response.**
CGRP binding to CALCRL/RAMP1 receptor activates several signaling pathways including the activation of adenylate cyclase that increases cyclic AMP (cAMP) levels that further activates protein kinase A (PKA). This enables the phosphorylation of multiple target molecules to cause increased dectin-1gene transcription **(1).** The CGRP-RAMP1 signaling can directly couple with dectin-1 to increase its response to *A. fumigatus* interaction **(2)**. (Figures were made with BioRender).

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# Appendix A - Effects of SP, VIP and SST on BMDM Phagocytosis of *A. fumigatus*

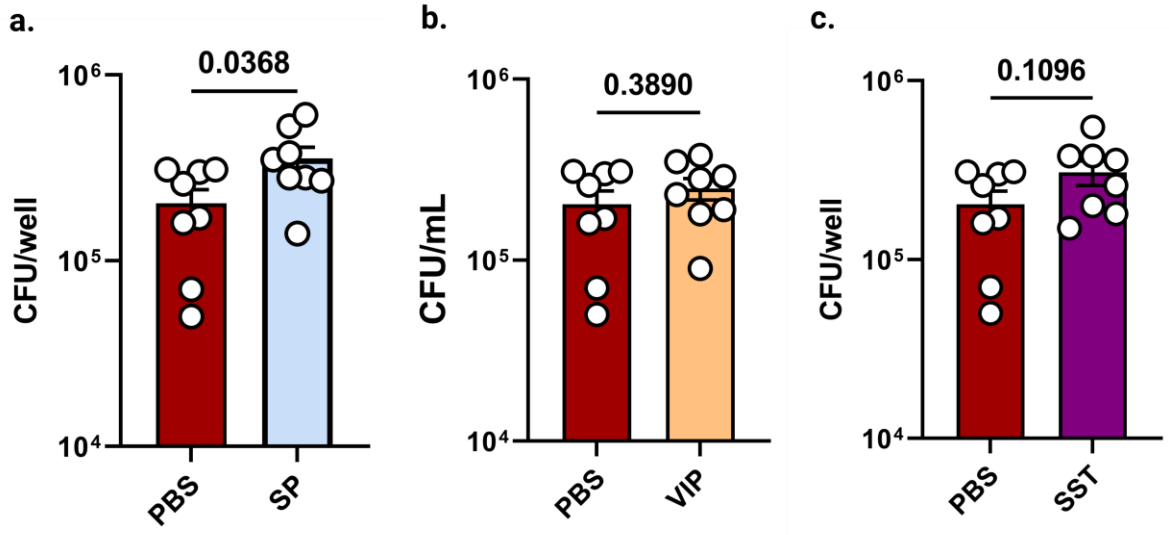


**Figure A.4.2: Effects of SP, VIP and SST on BMDM Phagocytosis of *A. fumigatus*.**
**(a–c)** BMDMs from B6 mice were challenged with *A. fumigatus* conidia in the presence or absence of 1 µM Substance P, VIP, and Somatostatin at MOI of 10 for 6 hours, washed and followed by incubation with nystatin (20 µg/mL) for another 10 hours, lysed with Triton X-100, diluted, plated onto SDA plates, and followed by colony count after 24 hours. Statistical analysis, Unpaired students t-test.