Is repurposing of CGRP antagonists for asthma a strategy worth pursuing?

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Abstract

Asthma is a disease that affects 262 million people worldwide. The symptoms result from an inflammatory reaction to irritants that would not provoke the same response in healthy individuals. Severe cases of asthma are often characterized by airway remodeling, which includes an increase in sensory neurons. These neurons produce CGRP, which is also elevated in fatal asthma. Some severe cases of asthma are unresponsive to current treatments, and CGRP antagonists may represent a potential new treatment option. We utilized cell databases, mast cell gene expression studies, and a literature review to investigate this possibility. The primary source of CGRP in asthma was identified as pulmonary endocrine cells, which are also elevated in asthma. Most CGRP receptors were found on the endothelial cells of blood vessels, aligning with the mechanism of CGRP antagonists in migraines, where they inhibit vasodilation. CGRP is thought to increase mast cell activity, though this has not yet been confirmed by laboratory results, as qRT-PCR analysis showed no significant difference after CGRP stimulation of mast cells. The literature review concluded that CGRP's anti-inflammatory effects mainly occur through eosinophils, mast cells, ILC2 cells, and Th2 cells. Additionally, CGRP exerts tissue-protective effects by increasing IL-10, promoting regulatory T-cells, and inhibiting dendritic cell migration. In conclusion, repurposing CGRP antagonists appears to be a promising strategy, given their effect on eosinophils and mast cells, two key players in asthma pathology and possible vasodilatory effects.

Introduction

According to the World Health Organization, asthma effects approximately 262 million people worldwide and it is also the most common chronic disease in children (1). Asthma patients experience recurrent attacks with symptoms such as dyspnoea, wheezing, chest tightness and sometimes coughing (2) These symptoms result from an inflammatory reaction to irritants that healthy individuals would not typically react to. The exposure to these stimuli causes inflammation, bronchial hyperreactivity, and reversible airway obstruction (3).

The severity of disease is classified into four stages: intermittent, mild, moderate and severe. Treatment and management strategies vary across these stages (4). Treatment typically includes a controller regiment, often consisting of an inhaled corticosteroid, which can be combined with a long-acting beta-agonist (LABA). The dosage of these medications increases with disease severity. Exacerbations are managed with a short-acting beta-agonist (SABA) (5).

Due to its heterogeneous nature, asthma remains difficult to treat (2). Severe asthma is often unresponsive to standard treatments (6). In the United States alone, asthma attacks still result in over 3,000 deaths annually (7). For severe asthma, alternative therapies such as tiotropium, anti-IgE, or anti-IL5 may be considered (5). However, severe asthma is characterized by airway remodelling (6), a condition not effectively addressed by the therapies currently available, leading to uncontrolled asthma ((2).

Airway remodelling

Airway remodelling can affect both the small and large airways (6) leading to thickening of the airway cell walls and obstruction of airflow (8) It is most likely caused by inflammation involving eosinophilic granulocytes and CD4+ Th cells, resulting in a prolonged inflammatory process. Airway remodelling affects epithelial cells (leading to shedding, goblet cell hyperplasia, and thickening of the basal membrane), peribronchial tissue (causing subepithelial fibrosis), and leads to hyperplasia and hypertrophy of airway smooth muscle cells. Additionally, it impacts bronchial vasculature and results in an increase in nerve tissue (9). The topic of increased neuronal tissue remains underexplored and therefore warrants further investigation.

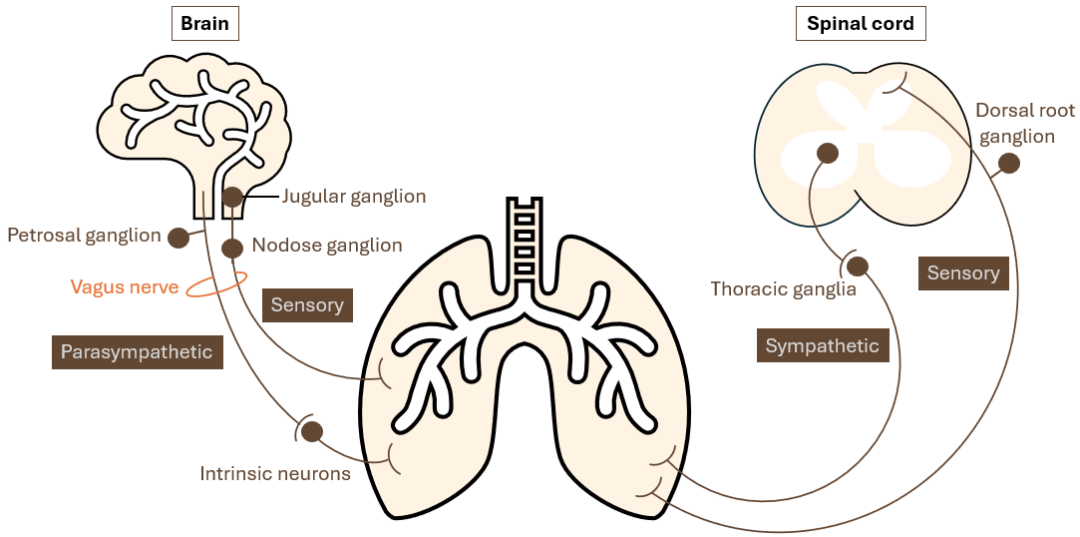

Figure 1 Human neuronal anatomy of the lung.

As shown in Figure 1, there are two primary routes of neuronal regulation in the lungs. Sympathetic innervation, originating from the spinal cord, provides noradrenergic input to bronchial blood vessels and submucosal glands. Additionally, some sensory nerves arise from the dorsal root ganglia (10). However, the bronchial tone—and, consequently, airway hyperresponsiveness—are primarily controlled by the parasympathetic nervous system (11). The parasympathetic nervous system, originating from the vagus nerve, also supplies most of the sensory nerves present in the lungs (10).

Stimulation of the parasympathetic nervous system, mediated by muscarinic M3 receptors, contributes to asthma symptoms as it induces bronchoconstriction, mucus secretion and bronchial vasodilation. Furthermore, macrophages in the lung are found to express M2 and M3 receptors. This indicates that parasympathetic acetylcholine release could directly influence inflammation by influencing the activity of macrophages (12)

Sensory nerves primarily regulate airway hyperreactivity and immune responses (12). These sensory nerve fibres originate from the jugular and nodose ganglia (Figure 1) and extend to the trachea, as well as the primary and secondary bronchi (12,13). The remaining portions of the lower respiratory tract are innervated by smaller branches of neurons, which terminate in the airway epithelium, smooth muscle, glands, autonomic ganglia, and around the capillary beds in the alveoli (13).

Sensory nerve fibers in the lung can be further subdivided based on their ability to detect mechanical or chemical stimuli. Mechanical sensory fibers are sensitive to touch and are typically myelinated Aδ fibers (13) Chemoreceptors, which are unmyelinated C-fibers, are usually dormant in the lung but can detect endogenous irritants (13). These chemoreceptors (also known as nociceptors) contain neuropeptides, which play a role in their function (12).

Neuroplasticity

In healthy individuals, sensory neurons are involved in normal physiological activities, including bronchoconstriction, breathing, and the removal of exogenous materials through the induction of cough (11). However, recent studies have shown that in asthma, the density of sensory nerves in the lungs is increased. This conclusion is based on confocal microscopy images from patient biopsies (13). And presence of pan-neuronal marker PGP9.5 in fatal asthma. These structural changes in nerves are classified as neuroplasticity, which involves alterations in nerve structure, increased neuropeptide release, and overactivation of nerve terminals (14). Furthermore, asthma patients with poorer lung function tend to exhibit a greater increase in nerve density. This observation supports the hypothesis that nerve density contributes to asthma severity (13).

Cholinergic neuroplasticity was also confirmed using bronchial biopsies, with sections stained using vesicular acetylcholine transporter (VAChT) as a marker (11). The increase in cholinergic tone contributes to reversible airway obstruction by controlling bronchoconstriction and mucus secretion, which leads to dyspnoea. This airway obstruction can be reversed with anticholinergic treatment, which exerts a broncho-dilatory effect. Neuroplasticity induces neurogenesis, phenotypic switching, increased peptide synthesis, enhanced neurotransmission and excitability, and a decreased threshold for activation. These changes contribute to or exacerbate asthma symptoms, such as coughing, wheezing, mucus secretion, airway hyperresponsiveness, airway inflammation, and remodelling (15).

Neuroimmune reactions

Notably, a study investigating neuroplasticity in fatal asthma found an increased presence of inflammatory cells, such as eosinophils, mast cells, and cell adhesion molecule 1 positive (CADM-1+) cells. These cells were observed near or even inside nerve bundles, with others in contact with the epineurium (14). The increase in eosinophils was also noted in other studies, where it was explained by the expression of eotaxin-1 in nerve cells. Eotaxin-1 binds to the CCR3 receptor on eosinophils, promoting chemotaxis toward the nerve bundles. When eosinophils are exposed to irritants, they disrupt the parasympathetic nerve function. In combination with other inflammatory cells, this leads to sensory nerve activation, alterations in nerve phenotypes, increased nerve density, and the release of neuron stimulants such as prostaglandins, histamine, thromboxane's, and tachykinins. These findings suggest that eosinophils play a crucial role in neuroplasticity in asthma (13).

Additionally, evidence from biopsies of patients who succumbed to fatal asthma indicated that neuroimmune reactions play a significant role in the severity of the disease. These reactions involve both the immune cells affecting neurons, as previously described with eosinophils, and neurons influencing immune cells. For instance, sensory neurons affect mast cells. A study using human pluripotent stem cell (hPSC)-derived sensory neurons co-cultured with mast cells found that mast cell differentiation is upregulated. Sensory neurons induce the upregulation of several genes, including PTGS2, C-kit, GATA2, HDC, CPA3, ATXN1, VCAM1, and CADM-4, which promotes further mast cell differentiation and increases nerve adhesion. This process is believed to play a crucial role in fatal asthma exacerbations in individuals with asthma (14).

Neurotrophins

Neuronal activity and sensory nerve development in the lungs are regulated by neurotrophins (13). Additionally, neurotrophins control neuronal survival, differentiation and function (11). Neurotrophins are growth factors, including brain-derived neurotrophic factors (BDNF) and nerve growth factors (NGF), which respectively bind to tropomyosin receptor kinase A and B. These neurotrophins are primarily synthesized in the central nervous system or autonomic nervous system. However, they can also be synthesized by bronchial epithelial cells, airway smooth muscle cells, and immune cells such as lymphocytes and eosinophils (16).

The tropomyosin receptor kinase B (TrkB) is expressed in the nerve endings of the airways. In mice, it has been shown that, TrkB signalling is crucial for the development of increased total and cholinergic nerve density. In humans, BDNF expression in sputum and bronchial samples has been found to correlate with asthma severity (11) Both BDNF and NGF play an essential role in neuronal airway remodeling (16). Additionally, they increase TRPV1 expression and activity, thereby sensitizing the cough response and inducing bronchoconstriction. NGF also increases the activity of the TRPA1 channel, which stimulates neuropeptide expression in sensory neurons (13).

Neuropeptides

Neuropeptides are small, biologically active proteins that, once released from the sensory neurons, modify the nerve function, stimulate the production of neuronal growth factors, and recruit inflammatory cells to the airways. Among the various neuropeptides, substance P is the most extensively studied. It generates bronchoconstriction and induces cough (13). Substance P is stored in the nerve endings of sensory neuron C fibres in the airways, alongside calcitonin gene-related peptide (CGRP) (16). CGRP expression has been found to be upregulated in patients with fatal asthma (14). This neuropeptide exists in two isoforms: αCGRP, which is present in both the central and peripheral nervous system, and βCGRP, which is found in the enteric nervous system and immune cells (16).

The primary CGRP receptor is CALCRL (14), which forms a heterodimeric complex with calcitonin-like receptor (CLR) (17) and receptor activity-modifying protein 1 (RAMP1) (16). Additionally, CGRP receptors may also contain RAMP2, RAMP2-AS1, and RAMP3 (14). The cellular effects of CGRP are summarized in Figure 2.

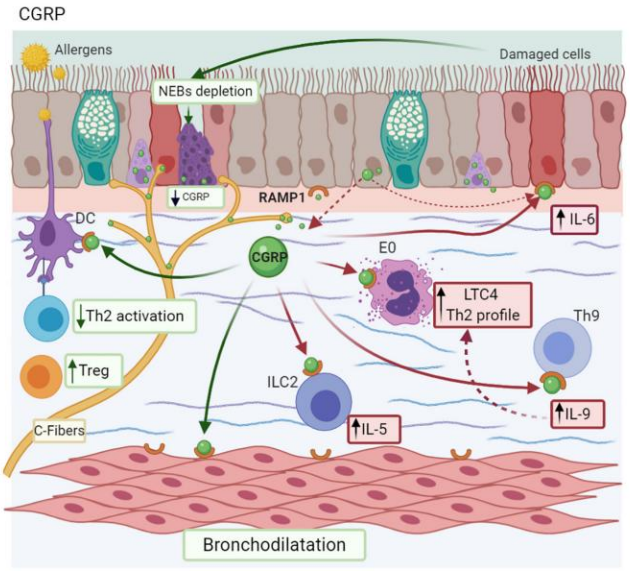

Figure 2 The effects of Calcitonin gene-related peptide (CGRP) (16).

CGRP induces the differentiation of innate lymphoid type 2 (ILC2) cells, which in turn increase the secretion of IL-5. Correlations between CGRP levels and eosinophil counts have been observed in late-phase allergen reactions (14). Furthermore, CGRP promotes the synthesis of leukotriene C4 in eosinophils, triggering the Th2 response. In the airway epithelium, it stimulates the production and presentation of IL-6, an inflammatory interleukin. CGRP also enhances the Th9 response by upregulating GATA3 and PU.1, which are transcription factors for Th9 cells, and increases the production of IL-9, further exacerbating the airway inflammation (16).

On its own, αCGRP induces bronchodilation by increasing cellular levels of cAMP. However, other studies suggest that CGRP has a bronchoconstrictor effect (18). In mice, CGRP has been shown to inhibit dendritic cell maturation, reduce antigen-specific T cell activation (specifically Th2 cells), increase Treg cells, and decrease eosinophil levels. These findings suggest that CGRP could potentially be a therapeutic target for asthma (16). However, studies on fatal asthma patients provide a contrasting perspective, linking CGRP to allergy development, inflammation modulation, and oedema (14)

At this point, it remains unclear whether CGRP is ultimately harmful or beneficial. To move closer to an answer, the cell types in the lung that express CGRP and its receptors are being investigated. Additionally, to give more insight, the effects of CGRP on mast cell gene expression in vitro are tested. CGRP antagonists have already been approved for the treatment of migraines (17), raising the question: Is repurposing CGRP antagonists a strategy worth pursuing? To explore this, a literature review is conducted.

Methods

The idiopathic pulmonary fibrosis (IPF) cell atlas

To identify which cells express CGRP and its receptors, the IPF cell atlas is used. This database includes single-cell RNA sequencing to investigate tissues at a cell-level resolution. The Cell Atlas contains multiple studies, and for this research, the Kaminski/Rosas study was chosen, because it included the largest number of cells (19). The cells were collected from the lungs of IPF patients, as well as from smokers, non-smokers and COPD patients (20).

Chan Zuckerberg (CZ) CELLxGENE Discover

As a supplement to the IPF Cell Atlas, the CZ CELLxGENE Discover data platform is used. This community-contributed database contains data from over 93 million cells and employs a standardized schema for gene expression, cell assays, and donor metadata. It is a comprehensive collection of single-cell transcriptomic data. The CZ CELLxGENE platform was chosen to provide a broader view of cells not included in the IPF Cell Atlas. Unlike the IPF cell atlas, the CZ CELLxGENE database includes a wide range of cells from the entire lung, rather than focusing solely on deep lung tissue (21) The gene expression tool was used, organism homo sapiens and tissue lung.

Effect of CGRP on mast cells

LUVA culturing protocol

The mast cells are procured by adding human mast cells (LUVA) to a 50/50 maturation/LUVA medium for 5 days. Afterwards the cells were harvested using 0.5 mM EDTA and centrifuged at 300 xg for 10 minutes. The supernatant was removed, and the cells were frozen (14).

CGRP stimulation of mast cells

After LUVA cell harvesting, the cells were washed with LUVA (StemPro-34 SFM) medium, then seeded into wells at a density of 200,000 LUVA cells per well. The following day, CGRP was added at the following concentrations: 0 nM, 0.001 nM, 0.01 nM, 0.1 nM, and 1 nM. After 24 hours, the medium was refreshed to maintain the neuropeptide concentration (22).

On the next day, the mast cells were stimulated by adding IgE (1 µg/mL) to all wells except the control wells. After 24 hours, anti-IgE (0.1 µg/mL) was added to the same wells. The cells were then counted using a cell counter, centrifuged at 170 rcf for 5 minutes at 4°C, and the pellets were frozen for RNA analysis (22).

RNA extraction and cDNA synthesis

For RNA isolation, Trizol was added to the cell pellets, which were then kept on ice before being frozen at -80°C. After freezing, the samples were thawed on ice and centrifuged at 4°C for 15 minutes at 12,000 g. The transparent top layer, containing the RNA, was transferred to a new tube. To wash the RNA, isopropanol was added, and the samples were incubated at 4°C for 10 minutes. Following incubation, the samples were centrifuged at 4°C for 10 minutes at 12,000 g. The supernatant was discarded, and the pellets were resuspended in 75% ethanol. After vortexing, the samples were centrifuged at 4°C for 5 minutes at 7,500 g. The supernatant was removed, and the pellets were left to dry. After adding RNase-free water, RNA concentrations were measured using the Nanodrop machine. All samples were adjusted to an RNA concentration of 75 ng/µL using RNase-free water (22).

Next, a master mix containing a random primer and dNTPs was added to each sample. To break secondary structures, the samples were heated to 70°C for 5 minutes, then immediately cooled on ice. The second master mix (containing M-MLV 5x reaction buffer, RNasin ribonuclease inhibitor, and M-MLV reverse transcriptase in a 10:1:1 ratio) was added to each sample to reach a final volume of 20 µL, and the samples were vortexed. The cDNA synthesis program was then run as follows:

- 10 minutes at 25°C
- 50 minutes at 37°C
- 15 minutes at 70°C
- 99 hours at 4°C (storage)
- 99 hours at 4°C (storage)

Afterwards, the samples were diluted to 10 ng/µL for RT-qPCR and stored at -20°C (22).

RT-qPCR

For the RT-qPCR analysis, special 384-well plates from ERIBA were used. Seven genes were investigated: PTSG2, C-Kit, GATA2, HDC, CPA3, ALOX15, and ALOX5, along with two housekeeping genes, SDHA and B2M, as controls. For each gene, a master mix was prepared containing forward primer, reverse primer, RNase-free water, and SYBR Green in a 1:1:38:50 ratio, with a final volume of 9 µL. To each well, 1 µL of cDNA was added, resulting in a cDNA concentration of 10 ng. To avoid contamination, the plate was centrifuged at 1000 rpm for 1 minute. The master mix was then added to the wells, and the plate was sealed and centrifuged again at 1000 rpm for 1 minute (22). RT-qPCR analysis was carried out using QuantStudio software, and the data was analysed using the double delta Ct method in Excel. Statistical analysis was performed using one-way ANOVA in SPSS.

Literature search method

Articles were obtained from the scientific database PubMed. The following search terms were used:

- “CGRP and Asthma’’
- “CGRP and Antagonist’’
- “Neuroimmune and Asthma’’
- “Calcitonin-gene related peptide’’
- “Neuropeptide CGRP’’

The relevance of each article was initially assessed by reading the title and abstract. Full articles were then reviewed to gather more detailed information. Key aspects, including the methods, results, and conclusions of the studies, were used to evaluate whether repurposing CGRP antagonists is a viable strategy.

Results

Identification of cell types that express CGRP and its receptors

CGRP

Using the Idiopathic Pulmonary Fibrosis (IPF) Cell Atlas (23), the expression of the CGRP gene (CALCA) was investigated within the Kaminski/Rosas dataset. The results are presented in Figure 3.

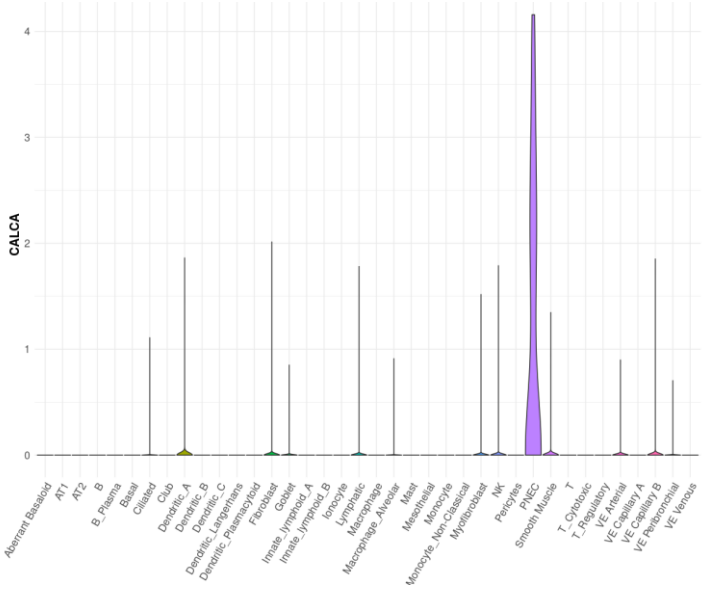

Figure 3 CGRP gene expression in healthy individuals of the Kaminski/Rosas dataset (23).

The data indicates that CGRP is moderately expressed in dendritic cells, lymphatic cells, natural killer (NK) cells, smooth muscle cells, venous endothelial cells, and venous capillary B cells. Notably, CGRP expression was high in pulmonary neuroendocrine (PNEC) cells.

To further validate and complement the findings from the IPF cell atlas, data was retrieved from a single-cell data platform (CZ Celgene). This additional data supported the conclusions of the IPF database

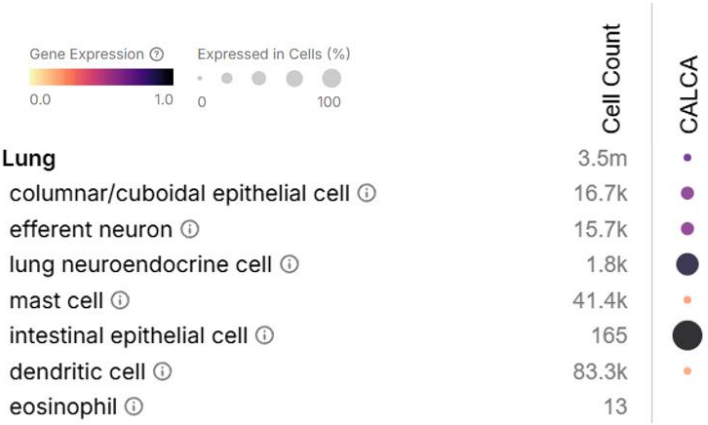

Figure 4 CGRP gene expression in healthy individuals of the CZ CELLxGENE dataset (24).

Additionally, as shown in Figure 3, the database indicates that efferent neurons and lung neuroendocrine cells are the primary producers of CGRP. The expression in intestinal epithelial cells present in the mouth and the columnar/cuboidal epithelial cells is also noted.

CGRP receptor

Using the Idiopathic Pulmonary Fibrosis (IPF) Cell Atlas (23), the expression of the CGRP receptor gene (CALCRL) was investigated within the Kaminski/Rosas dataset. The results are presented in Figure 5.

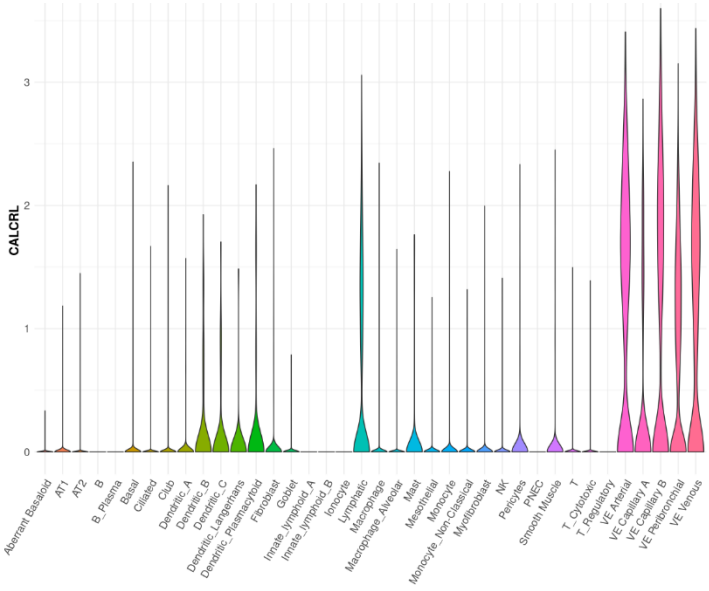

Figure 5 CGRP receptor gene expression in healthy individuals of the Kaminski/Rosas dataset.

The CGRP receptor is predominantly found in blood vessel endothelial cells. In the context of asthma, it is noteworthy that the receptor is present on various immune cells, including dendritic cells, macrophages, mast cells, monocytes, and NK cells. It is also expressed on smooth muscle and lymphatic cells. However, there is no available information regarding the presence of the CGRP receptor on eosinophils.

To further validate and complement the findings from the IPF cell atlas (24), data was retrieved from a single-cell data platform (CZ GENExCELL database). This additional data supported the conclusions of the IPF database.

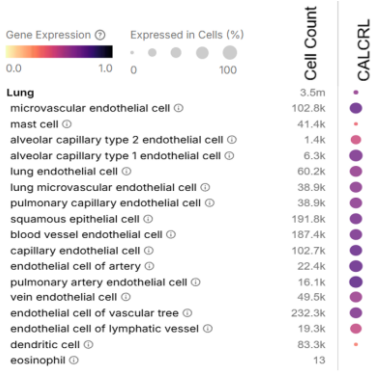

Figure 6 CGRP receptor gene (CALCRL) expression in healthy individuals of the CZ CELLxGENE dataset (24).

The CELLxGENE dataset revealed that the CGRP receptor is highly expressed in microvascular endothelial cells, lung endothelial cells, and other cells involved in the blood supply of the lung as well as the lymph vessels. Additionally, the receptor appears to be mildly expressed in mast cells and dendritic cells. However, no information was available regarding eosinophils.

RAMP1 receptor

Using the Idiopathic Pulmonary Fibrosis (IPF) Cell Atlas (23), the expression of the RAMP1 receptor gene (CRAMP1) was investigated within the Kaminski/Rosas dataset. The results are presented in Figure 7.

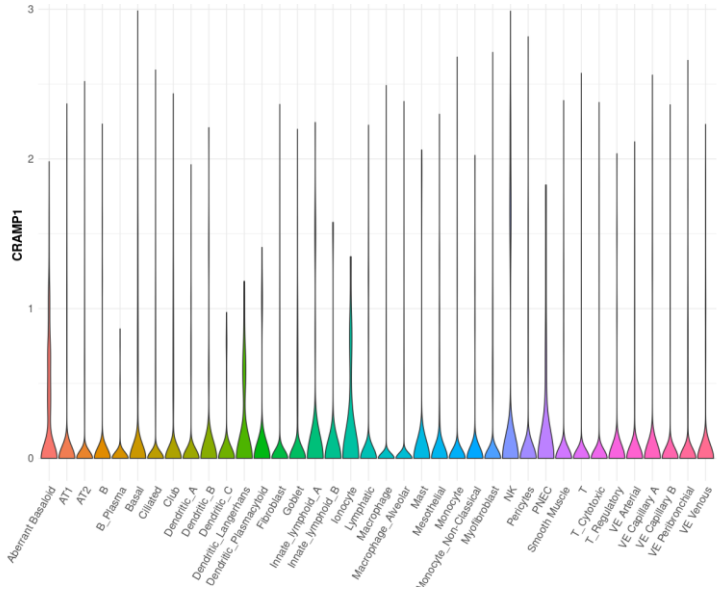

Figure 7 CRAMP1 receptor gene expression in healthy individuals of the Kaminski/Rosas dataset.

The RAMP1 Gene is present in all cells, but is predominantly expressed in aberrant basaloid cells, dendritic Langerhans cells, innate lymphoid cells, ionocytes, mast cells, NK cells, and PNEC cells.

To further validate and complement the findings from the IPF cell atlas (24), data was retrieved from a single-cell data platform (CZ GENExCELL database). This additional data supported the conclusions of the IPF database.

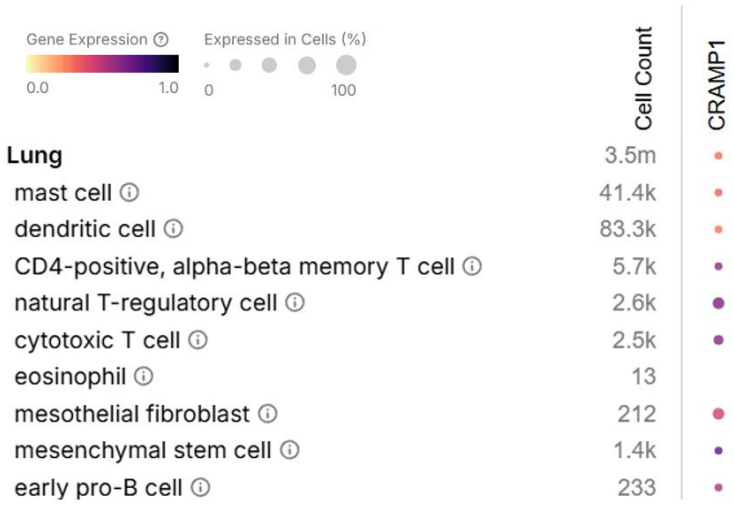

Figure 8 RAMP1 receptor gene (CRAMP1) expression in healthy individuals of the CZ CELLxGENE dataset (24).

Additionally, this database indicated particularly high expression in natural regulatory T-cells, CD4-positve αβ memory T cells, cytotoxic T cells, mesothelial fibroblasts, mesenchymal stem cells, and early pro-B cells.

An investigation into the effects of CGRP on mast cell gene expression in vitro.

The experiment outlined in the methods was conducted six times for the genes ALOX5, ALOX15, and HDC. Using this data, a one-way ANOVA was performed to determine whether there is a statistically significant difference in gene expression across the various CGRP concentrations.

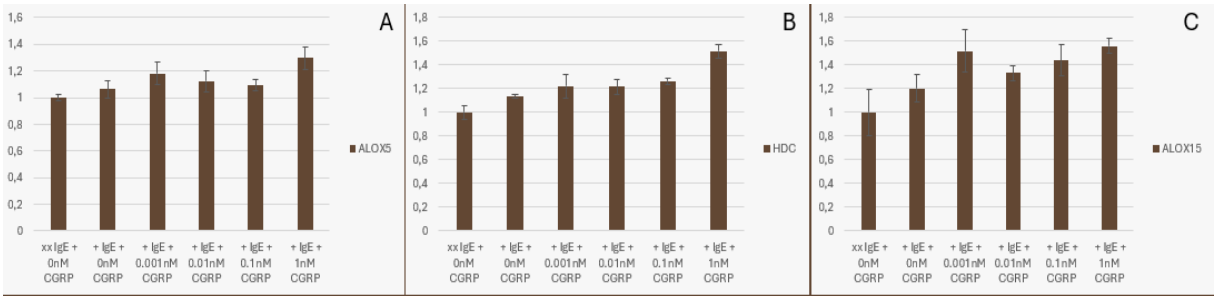

Figure 9 RT-qPCR results of ALOX5, HDC and ALOX15.

In Figure 9a, the results for the ALOX5 gene, which is expressed in human mast cells as a lipid mediator biosynthetic enzyme (25), show a slight increase at the highest CGRP concentration (1 nM). However, a one-way ANOVA (Appendix 1) indicated no significant difference between the groups. Figure 9b presents the results for the HDC gene, which is essential for histamine synthesis (26). This gene also shows a slight increase, but again, no significant difference is observed. Lastly, Figure 9c shows the results for the ALOX15 gene, which promotes the infiltration of mast cells into airway tissues (27). A slight increase is observed after CGRP addition, but, as with the previous genes, no significant difference is found. The lack of significant difference at these concentrations suggests that either the concentration of CGRP is too low to produce an effect. Or CGRP does not have a promotional effect on mast cells.

The sixth time the experiment was conducted more genes were included to provide a clearer understanding of the effect on mast cells. However, this experiment was only performed twice, so no statistical analysis could be conducted on the data.

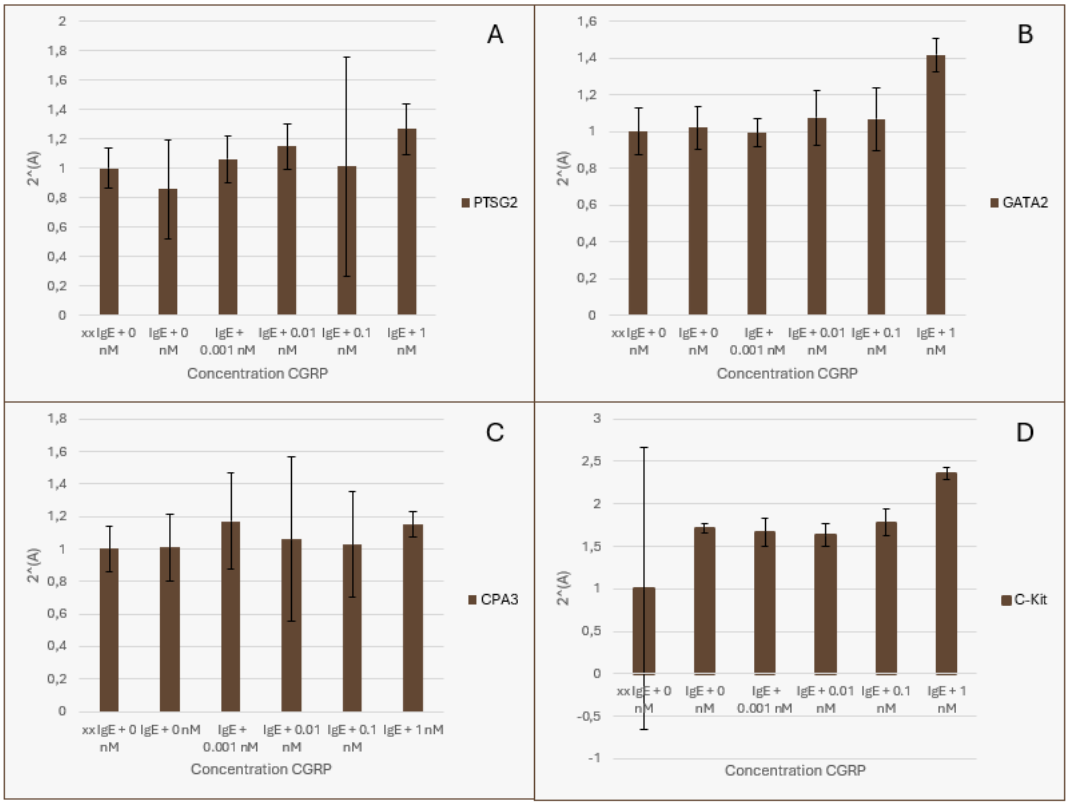

Figure 10 RT-qPCR results of PTSG2, GATA2, CPA3 and C-Kit.

Figure 10a shows the RT-qPCR results for PTSG2, a gene involved in prostaglandin production (28). The gene expression of PTSG2 shows a slight increase, however, the standard deviation at the 0.1 nM CGRP concentration is high. Additionally, GATA2 gene expression is slightly increased at the highest CGRP concentration (Figure 10b). The GATA2 gene is associated with blood cell production (29). The CPA3 gene shows a very small increase in expression (Figure 10c). The CPA3 gene encodes a mast cell protease (30). The gene that shows the most significant increase in the experiment is the C-Kit gene, as shown in Figure 10d. The C-kit gene is important for proliferation, survival, and differentiation of mast cells (31). This result seems promising, and the experiment should be repeated to determine if the increase is statistically significant.

Is repurposing CGRP-antagonists a strategy worth pursuing?

Possible advantages of anti-GCRP treatment

For anti-CGRP treatment to be effective, CGRP must be pro-inflammatory or capable of stimulating the inflammatory process. A study in Brown-Norway rats indicated that CGRP contributed to hypersensitivity reactions, and additionally mucus production by glands and goblet cells (32). Assuming this is true and CGRP does aid hypersensitivity reactions in asthma, the next question would be how.

At first glance, the effect of CGRP is believed to be pro-inflammatory, as CGRP levels are elevated in patients with asthma. An in vitro culture model using hPSC-derived sensory neuron/mast cell co-culture showed upregulation in mast cell activation and differentiation markers, including CADM4, PTGS2, C-KIT, GATA2, HDC, CPA3, ATXN1, and VCAM1 (14). This suggests that CGRP activates mast cells, which play a significant role in asthma. Additionally, another study indicated that activation of the CGRP receptor causes mast cell degranulation (33).

Not only has a link been established between mast cells and CGRP, but there is also evidence for a potential connection between CGRP and eosinophils. A study using BALB/c mice sensitized with ovalbumin (OVA) provided evidence for this, observing a potential link between CGRP and increased eosinophil cell counts, as well as recruitment factors like IL-5 (18). Another central contributor to the pathogenesis of asthma is Th2 cells (34). CGRP is believed to induce the release of CCL17 and CCL22, while simultaneously inhibiting CXCL9 and CCL10, thereby enhancing Th2 cell recruitment and responses (33).

CGRP's effect on mast cells, eosinophils, and Th2 cells suggests that it may play a role in the inflammatory response. When the effects of CGRP on asthma were investigated using RAMP1-deficient mice the pro-inflammatory response was confirmed. Before any tests, the mice were found to be viable and appeared normal. They were then sensitized with ovalbumin to stimulate asthma. The absence of RAMP1 resulted in reduced airway resistance and inflammation. Similair effects were observed with a 50% reduction of CLR. However, when CLR was lost from the smooth muscle cells in the lung, no alteration in airway resistance was observed (35).

It is worth noting that the RAMP1 receptor is not only present in the CGRP receptor. The CALCRL receptor was also tested, and the results are presented in the following figure:

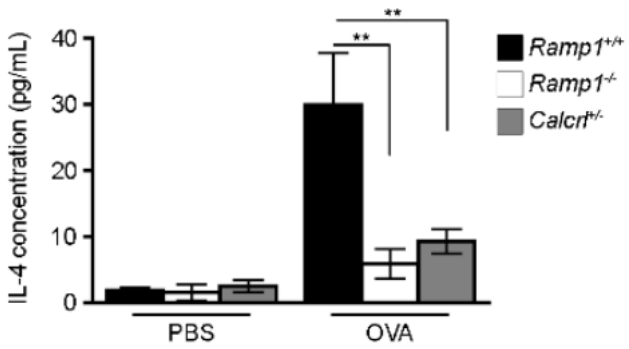

Figure 11 IL-4 levels from bronchial lavage fluid in RAMP1- and CALCRL-deficient mice compared to wild-type mice (35).

Compared to wild-type mice, the RAMP1-deficient, but most notably the CALCRL-deficient, mice showed reduced IL-4 concentrations. This strengthens the link between CGRP and Th2 cells, as IL-4 activates Th2 cells and is a potent pro-inflammatory cytokine (35).

CGRP is expressed by sensory neurons, but pulmonary neuroendocrine cells (PNECs) have also been shown to play an essential role in amplifying allergic asthma responses. Analysis using qRT-PCR on whole lung samples from OVA-treated mice indicated that CGRP expression is elevated (36).

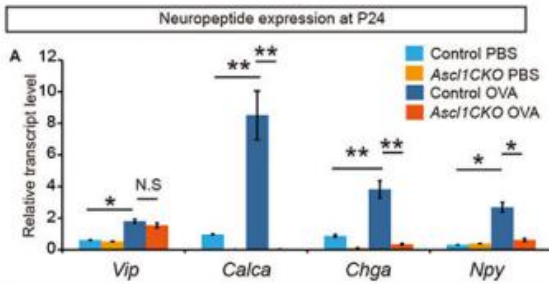

Figure 12 Neuropeptide expression in OVA-treated mice (36).

This increased CGRP expression is likely generated by PNECs. The localization of PNECs was also investigated using histology, as shown in Figure 13 (36).

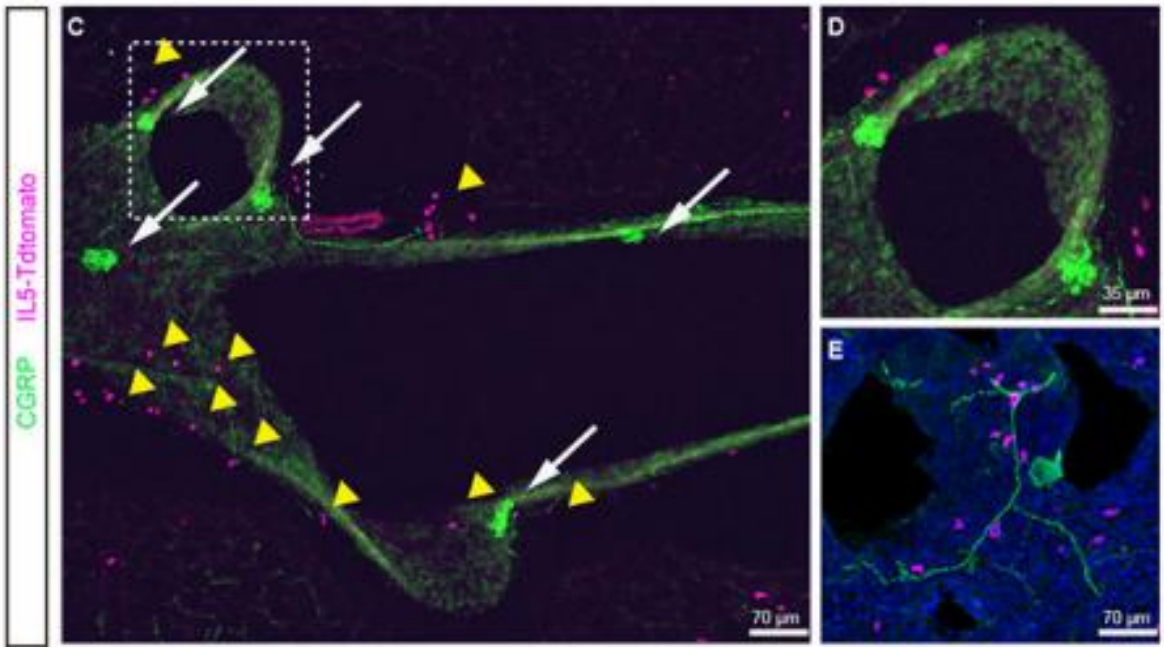

Figure 13 Localization of PNECs in OVA-induced mice (36).

In Figure 13, anti-CGRP was used to label CGRP in green, which is expressed by sensory neurons, as shown in Figure 13e. Figure 13c displays clusters of CGRP, with the boxed area magnified in Figure 13d. These clusters were identified as pulmonary neuroendocrine cells (PNEC). The pink highlights the ILC2 cells in close proximity. This study concluded that PNECs use CGRP to stimulate ILC2 cells, which in turn produce IL-5. And an additional ELISA test showed an increased IL-5 secretion. And IL-5 would increase eosinophil cell count and activity. The study also confirmed PNECs to be elevated in human asthma patients (36).

The study of a CGRP antagonist in an asthmatic mouse model strengthened the hypothesis that CGRP is a pro-inflammatory neuropeptide. According to the study, Rimegepant targeted ILC2 cells, and treatment led to a decrease in airway inflammation and goblet cell hyperplasia. This was evidenced by a reduction in the total number of eosinophils, lymphocytes and PAS-stained cells (goblet cells). A possible explanation for the reduction in these cells is the decrease in cytokine levels of IL-5 and IL-13, which were significantly reduced when measured using the ELISA technique. This reduction in cytokine levels further supports the anti-inflammatory effects of Rimegepant, particularly in relation to the Th2 cytokines. Furthermore, the number of KLRG+ ILC2 cells was reduced, indicating a decrease in the overall ILC2 population and their ability to produce Th2 cytokines (37).

These results suggest that CGRP may be pro-inflammatory by increasing cytokine levels and attracting and activating mast cells, eosinophils, Th2 cells, and ILC2 cells, thereby promoting the inflammatory response in asthma. CGRP also increased mucus production and is a potent vasodilator (17). This could also be a mechanism for the supply of immune cells into the lungs.

Possible disadvantages of anti-GCRP treatment

There are studies that conclude that CGRP is an anti-inflammatory neuropeptide and suggest that a CGRP agonist should be administered to patients as asthma treatment. One such study investigated the effect of CGRP on smooth muscle contraction in BALB/c OVA-sensitized mice. The study concluded that exogenous CGRP helped normalize lung function and demonstrated that endogenous CGRP does not exacerbate airway hyperresponsiveness through smooth muscle contraction. Stimulation with methacholine was used to assess smooth muscle contraction (18). This finding was supported by other studies using knockout mice (33). The suggested mechanism is that CGRP may exert a bronchodilator effect through cAMP signalling (18). Thus, on smooth muscle cells CGRP supposedly has a bronchodilator effect. However, based solely on its effects on smooth muscle, it cannot be concluded that CGRP normalizes lung function.

The function of CGRP in asthma most probably involves the immune system. The neuropeptide has an effect on dendritic cells, mast cells, eosinophils, Th2 cells and ILC2 cells. However, whether the effect is pro-, or anti-inflammatory remains a debate. The following experiment was conducted in vitro, some of the results are depicted in the figure below (38).

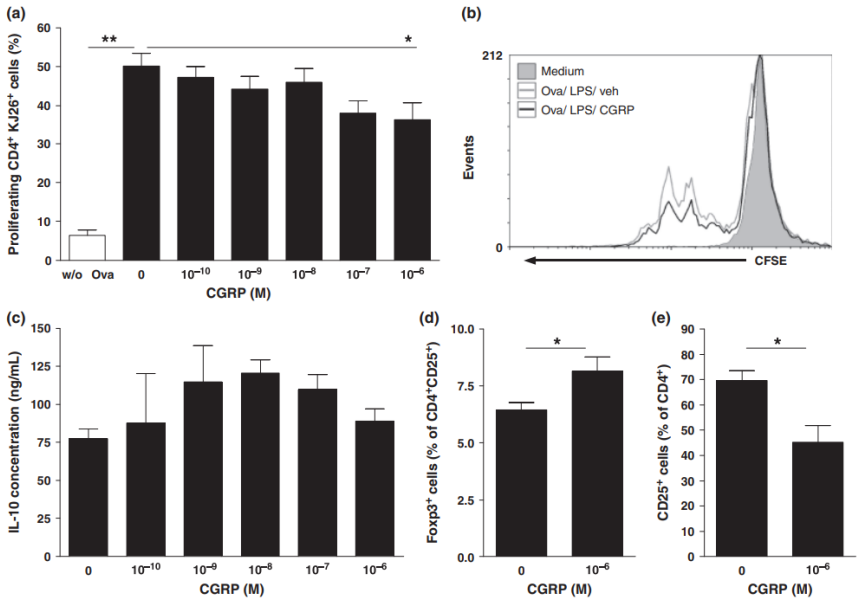

Figure 14 The effect of CGRP on LPS-induced expression of costimulatory molecules by bone marrow dendritic cells and the subsequent modulation of DC-induced T cell responses (38).

According to the study, inhibition of OVA-specific T cells (CD4+ KJ26+ cells) was observed, with this reduction illustrated in Figure 14a. The study also reported a significant 28% reduction in the proliferation of OVA-specific T-cells. Additionally, the increase in IL-10 was discussed as an anti-inflammatory effect of CGRP, as shown in Figure 14c (38). IL-10, an anti-inflammatory cytokine, limits the immune system's response to pathogens, preventing damage and maintaining tissue homeostasis (39). This could represent an anti-inflammatory effect induced by CGRP.

However, the study also indicates an increase in regulatory T-cells (Figure 14d) (38). These cells act as guardians of the immune system, and their increase contributes to the anti-inflammatory effects (40). This effect contrasts with previous reports, but it involves different cell types. These findings suggest that CGRP could elicit both anti-inflammatory and pro-inflammatory responses.

To further investigate the effect of CGRP on inflammation, a study examining lung lymphocytes during helminth infection in vivo using RNA sequencing found that ILC2 cells stimulated by CGRP reversed pro-inflammatory mediators, such as NMU, IL-33, and IL-25. This, in turn, decreased IL-13 production and ILC2 proliferation, effectively suppressing type 2 immunity by inhibiting mast cell degranulation, ILC2 proliferation, IL-13 secretion, and dendritic cell migration. These findings again suggest an anti-inflammatory effect. However, CGRP was also shown to selectively promote IL-5, a cytokine with pleiotropic effects on eosinophils (33). These findings were further supported by another study, both in vivo and in vitro (41). The results of these studies are summarized in the figure below.

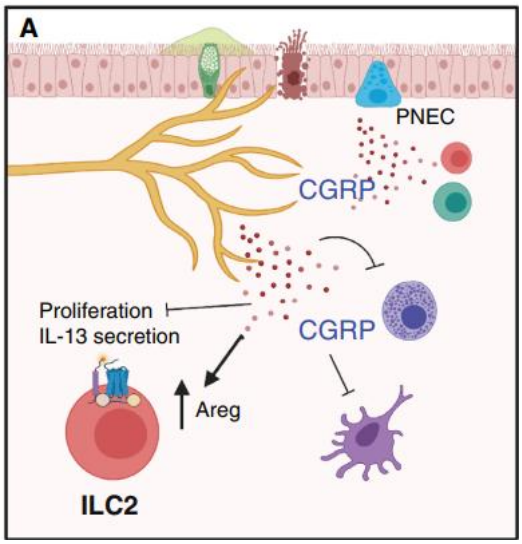

Figure 15 the anti-inflammatory effects of CGRP (42)

Discussion

Identification of cell types that express CGRP and its receptors

Literature has indicated that CGRP is expressed by nociceptive neurons, a finding confirmed by the CZ CELLxGENE database. However, both the IPF Cell Atlas and the CZ CELLxGENE database revealed another cell type with high CGRP expression: the pulmonary neuroendocrine cell (PNEC). According to literature, pulmonary neuroendocrine cells are a rare type of epithelial cell, which are disproportionately elevated in asthma. These cells are closely innervated by neurons and have been found in proximity to airway ILC2 cells, where they are reported to play a role in driving inflammation (42).

The CGRP receptor (CALCRL) is predominantly expressed in cells involved in the blood supply of the lung and lymph vessels. This is not surprising, as counteracting vasodilation is the mechanism behind the use of CGRP antagonists in migraines (17). The receptor is also mildly expressed on certain immune cells, such as mast cells. However, no information is available regarding the expression of the receptor on eosinophils. For this reason, the expression of RAMP1 was also investigated. According to the IPF Cell Atlas, it is present in all of the cells examined. It is important to note that RAMP1 is not specific to CGRP (16), which makes the CGRP receptor data more valuable. Data from the CZ CELLxGENE database showed that RAMP1 is also expressed on immune cells, but again, no data was found regarding eosinophils. The expression of RAMP1 does not necessarily correlate with the expression of the CGRP receptor.

An investigation into the effects of CGRP on mast cell gene expression in vitro.

The graph of ALOX5, HDC, and ALOX15 gene expression showed an increasing trend, but none of these increases were statistically significant. Therefore, it cannot be concluded that CGRP has a pro-inflammatory effect on mast cells, though the opposite cannot be concluded either. Then, when the results show no pro-inflammatory effect, it is possible that an anti-inflammatory effect could be present. Further testing of additional genes is necessary to determine whether there is a pro-inflammatory effect. In added gene cases, an increase in gene expression was observed at the highest CGRP concentrations, particularly for C-kit. A recommendation for future research would be to include genes with anti-inflammatory effects, as mast cell production of IL-10 can suppress inflammation (43)

Is repurposing CGRP-antagonists a strategy worth pursuing?

Results have shown that CGRP exhibits both anti-inflammatory and pro-inflammatory effects. The main anti-inflammatory effects include increased IL-10 levels, reduced dendritic cell migration, and an increase in regulatory T-cells. Studies conducted in mice have found an increase in ILC2 cells, while in vitro studies showed a reduction in ILC2 cell numbers. It is possible that a combination of activating factors is necessary to increase ILC2 numbers, such as CGRP and GABA. Studies have shown that the absence of PNECs results in a reduction in GABA concentrations (36) This combination could also be the mechanism that links CGRP to increased regulatory T-cells (44). Additionally, ILC2 cells in vivo have been shown to be closely located next to PNECs. Therefore, distance should not be considered the primary issue. Both in vivo and in vitro studies indicated an increase in IL-5 levels. Therefore, the pro-inflammatory effect of CGRP is strongly associated with eosinophils, which play a key role in asthma pathogenesis. Mast cells, on the other hand, show conflicting results. Literature reports an increase in mast cell gene expression, but this has not been confirmed in the lab experiments conducted.

The CGRP receptor was found to be primarily expressed on the endothelial cells of the lung's blood supply. CGRP can induce vasodilation directly by activating the receptor in the vascular smooth muscle (protein kinase A mediated) or indirectly with nitric oxide from endothelial cells (45). Clusters of PNECs, also called neuroepithelial bodies (NEBs), are predominantly found at the airway bifurcations and in intraepithelial nerve fibers in the lungs (46). This proximity to the epithelium suggests that vasodilation in the lung may be mediated indirectly. CGRP may exert its pro-inflammatory effect via vasodilation, leading to an increased influx of immune cells. The vasodilatory effect of CGRP in vivo could be tested by measuring vasoreactivity using nitric oxide (47) to assess its impact on the lungs. The effect of CGRP on eosinophils could be tested using the same method as with the mast cells, employing bone-marrow-derived eosinophils (48) to determine whether CGRP has a direct effect on eosinophils. Alternatively, the impact of CGRP on ILC2 cells or Th2 cells could be tested in combination with measuring IL-5 concentrations.

Conclusion

When revisiting the question of whether repurposing CGRP antagonists is a strategy worth pursuing, the answer is yes. Although mast cells have not yet shown promising results, literature suggests that the main effect of CGRP in asthma patients is likely due to eosinophils. The location of the CGRP receptors and the use of CGRP antagonists indicate that the vasodilation effect may play a significant role as well. CGRP antagonists have already shown promise in studies conducted. Given CGRP's dual nature, it should be noted that it could act as a pro-inflammatory neuropeptide while exhibiting tissue-protective effects through stimulation of IL-10, regulatory T-cells, and inhibition of dendritic cell migration.

When revisiting the question of whether CGRP antagonists should be repurposed, the answer, based solely on literature research, might lean towards yes. Given CGRP's dual nature and its likely involvement in inflammation in asthma through eosinophils or vasodilation, it remains an interesting neuropeptide with potential. While it is unlikely that a CGRP agonist would be beneficial for treatment, CGRP antagonists show promise in studies already conducted.

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Appendix 1

The ALOX5 gene

A one-way ANOVA was conducted on the qRT-PCR results for the ALOX5 gene in mast cells, a gene expressed in human mast cells as a lipid mediator biosynthetic enzymes (25). The results of the ANOVA test are shown in the figure below.

ANOVA[a]

Expression

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	,319	5	,064	1,561	,201
Within Groups	1,225	30	,041		
Total	1,544	35			

a. Genes = ALOX5

Figure 16 One-way ANOVA results of the ALOX5 gene of 6 experiments using qRT-PCR.

The p-value is greater than 0.05, indicating no statistically significant difference between the groups. Thus, there does not appear to be an significant increase in ALOX5 expression following CGRP stimulation.

The ALOX15 gene

A one-way ANOVA was conducted on the qRT-PCR results for the ALOX15 gene in mast cells, a gene promoting the infiltration of mast cells into airway tissues (27). The results of the ANOVA test are shown in the figure below.

ANOVA[a]

expression

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	1,001	5	,200	1,195	,349
Within Groups	3,182	19	,167		
Total	4,183	24			

a. gene = ALOX15

Figure 17 One-way ANOVA results of the ALOX15 gene of 6 experiments using qRT-PCR.

The p-value is greater than 0.05, indicating no statistically significant difference between the groups. Thus, there does not appear to be an significant increase in ALOX15 expression following CGRP stimulation.

The HDC gene

A one-way ANOVA was conducted on the qRT-PCR results for the HDC gene in mast cells, a gene essential for histamine synthesis (26) The results of the ANOVA test are shown in the figure below.

ANOVA[a]

Expression

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	,870	5	,174	1,954	,115
Within Groups	2,671	30	,089		
Total	3,540	35			

a. Genes = HDC

Figure 18 One-way ANOVA results of the HDC gene of 6 experiments using qRT-PCR.

The p-value is greater than 0.05, indicating no statistically significant difference between the groups. Thus, there does not appear to be an significant increase in HDC expression following CGRP stimulation.