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# CGRP inhibits SARS-CoV-2 infection of bronchial epithelial cells, and its pulmonary levels correlate with viral clearance in critical COVID-19 patients

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**ABSTRACT** Upon infection with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), patients with critical coronavirus disease 2019 (COVID-19) present with life-threatening respiratory distress, pulmonary damage, and cytokine storm. One unexplored component in COVID-19 is the neuropeptide calcitonin gene-related peptide (CGRP), which is highly abundant in the airways and could converge in multiple aspects of COVID-19-related pulmonary pathophysiology. Whether CGRP affects SARS-CoV-2 infection directly remains elusive. We show that in critical COVID-19 patients, CGRP is increased in both plasma and lungs. Importantly, CGRP pulmonary levels are elevated in early SARS-CoV-2-positive patients and restored to baseline upon subsequent viral clearance in SARS-CoV-2-negative patients. We further show that CGRP and its stable analog SAX directly inhibit infection of bronchial Calu-3 epithelial cells with SARS-CoV-2 Omicron and Alpha variants in a dose-dependent manner. Both pre- and post-infection treatments with CGRP and/or SAX are enough to block SARS-CoV-2 productive infection of Calu-3 cells. CGRP-mediated inhibition occurs via activation of the CGRP receptor and involves down-regulation of both SARS-CoV-2 entry receptors at the surface of Calu-3 cells. Together, we propose that increased pulmonary CGRP mediates beneficial viral clearance in critical COVID-19 patients by directly inhibiting SARS-CoV-2 propagation. Hence, CGRP-based interventions could be harnessed for management of COVID-19.

**IMPORTANCE** The neuropeptide CGRP is highly abundant in the airways. Due to its immunomodulatory, vasodilatory, and anti-viral functions, CGRP could affect multiple aspects of COVID-19-related pulmonary pathophysiology. Yet, the interplay between CGRP and SARS-CoV-2 during COVID-19 remains elusive. Herein, we show that pulmonary levels of CGRP are increased in critical COVID-19 patients, at an early stage of their disease when patients are SARS-CoV-2-positive. Upon subsequent viral clearance, CGRP levels are restored to baseline in SARS-CoV-2-negative patients. We further show that pre- and post-infection treatments with CGRP directly inhibit infection of Calu-3 bronchial epithelial cells with SARS-CoV-2, via activation of the CGRP receptor leading to decreased expression of both SARS-CoV-2 entry receptors. Together, we propose that increased pulmonary CGRP is beneficial in COVID-19, as CGRP-mediated inhibition of SARS-CoV-2 infection could contribute to viral clearance in critical COVID-19 patients. Accordingly, CGRP-based formulations could be useful for COVID-19 management.

**KEYWORDS** Calu-3, CGRP, COVID-19, SARS-CoV-2, SAX

COVID-19 is characterized by pulmonary symptoms ranging from mild/moderate upper-airway disease to severe/critical acute respiratory distress syndrome (ARDS)

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and lung damage (1). Life-threatening critical COVID-19 develops in patients with pre-disposing risk factors, including the age of >65 y, being male, and presenting with co-morbidities, such as hypertension, obesity, and diabetes (2). These patients develop a cytokine storm characterized by elevated levels of pro-inflammatory cytokines such as interleukin 6 (IL-6) and tumor necrosis factor alpha (TNF $\alpha$ ) (3) but impaired type I interferon responses (4–6), promoting altogether viral sepsis (7). Airway epithelial cells, which are key elements in the host innate immune response against respiratory infections (8), are a major cellular source for secretion of these pro-inflammatory cytokines (9, 10) upon SARS-CoV-2 sensing (11). In turn, SARS-CoV-2 replicates in epithelial cells throughout the airways (12), with a decreasing infection gradient from upper bronchial/bronchiolar to lower alveolar epithelial cells (13).

The 37 amino acids neuropeptide CGRP (14) is one unexplored component in COVID-19. CGRP is highly abundant in the airways, where it is principally secreted by sensory nerve fibers (15) that innervate the lungs (16) and by rare bronchial epithelial cells with specialized sensory function termed pulmonary neuroendocrine cells (PNECs) (17). Due to its immunomodulatory and vasodilatory functions, CGRP might intersect with multiple COVID-19 pulmonary symptoms, which could result in opposite consequences. Accordingly, CGRP is a key regulator of inflammatory processes and affects the release of pro-inflammatory cytokines. While CGRP increases IL-6 secretion from bronchial epithelial cells (18), which was recently confirmed in a mouse model of SARS-CoV-2 infection (19), it also decreases TNF $\alpha$  levels in murine models of bacterial sepsis in which CGRP levels are elevated (20). In addition, CGRP is a potent vasodilator (21) believed to be secreted during local hypoxia, with beneficial actions in cardiovascular diseases including hypertension (22, 23). Hence, CGRP levels/effects vary in pathologies that characterize and/or represent risk factors for the development of critical COVID-19. CGRP further promotes lung tissue repair and regeneration (24) by inducing bronchial epithelial cell migration (25) and wound healing (26). These studies indicate that CGRP plays both harmful and protective roles in airways physiopathology.

Besides its immunomodulatory and vasodilator roles, CGRP also exerts anti-viral functions, as we previously discovered (27–31). Indeed, CGRP strongly inhibits infection of antigen-presenting Langerhans cells (LCs) with HIV type 1 (HIV-1) and herpes simplex virus types 1 and 2 (HSV-1/2) (27–31). One shared CGRP-mediated anti-viral mechanism involves modulation of surface expression of different viral attachment and entry receptors. In particular, CGRP: (i) enhances surface expression of atypical double-trimers of the LC-specific pathogen-recognition lectin langerin, i.e., the receptor of both HIV-1 and HSV-2, resulting in reduced viral capture (31); (ii) decreases surface expression of the HIV-1 entry co-receptor CCR5 (28), and the HSV-1 entry receptor 3-O sulfated heparan sulfate (31).

Herein, we explored the interplay between CGRP and COVID-19 *in vivo* and investigated whether CGRP exerts direct anti-SARS-CoV-2 effects when acting on pulmonary epithelial cells.

## RESULTS

### CGRP levels are increased in SARS-CoV-2-positive critical COVID-19 patients

To explore the involvement of CGRP in COVID-19, we used some of our previously described plasma samples (4), obtained from COVID-19 patients with different disease severities, namely mild/moderate, severe, and critical. Control samples included plasma from patients with non-COVID-19-related pathologies as we described (32), as well as from otherwise healthy individuals at the time of sampling that we also described (33). As shown in Table 1, all cases had mean age of >65 y, were mostly males, and were matched for both age and sex. When compared to all other groups, critical COVID-19 patients had a significantly higher incidence of smokers (69%,  $P = 0.0473$ ). These patients also had a higher incidence of hypertension yet not reaching statistical significance (62%,  $P = 0.0790$ ). On multivariate analysis in this cohort, smoking was associated with critical COVID-19 [odds ratio (OR) 6.8; 95% confidence intervals (CIs) 1.3–42.8;  $P = 0.0259$ ]. We

TABLE 1 Clinical characteristics of the study patients

Characteristics	Plasma					<i>P</i>	Bronchoalveolar lavage			
	Healthy controls <i>N</i> = 5	Non COVID-19 <i>N</i> = 13	Mild/moderate COVID-19 <i>N</i> = 5	Severe COVID-19 <i>N</i> = 13	Critical COVID-19 <i>N</i> = 13		Non COVID-19 <i>N</i> = 7	Critical COVID-19 positive <i>N</i> = 4	Critical COVID-19 negative <i>N</i> = 4	<i>P</i>
Age mean (IQR) <sup>ac</sup>	66 (60–77)	67 (61–77)	77 (70–84)	65 (53–74)	67 (62–75)	0.3397	57 (45–72)	60 (48–66)	63 (48–72)	0.8575
Biological sex male, <i>N</i> (%) <sup>b</sup>	5 (100)	9 (69)	2 (40)	11 (85)	11 (85)	0.1877	5 (71)	4 (100)	2 (50)	0.2751
Co-morbidities <i>N</i> (%) <sup>b</sup>										
Smoking	0 (0)	4 (31)	1 (20)	4 (31)	<b>9 (69)<sup>d</sup></b>	<b>0.0473</b>	NA	NA	NA	
Hypertension	0 (0)	3 (23)	2 (40)	7 (54)	<b>8 (62)</b>	<b>0.0790</b>				
Cardiovascular disease							1 (14)	0 (0)	1 (25)	0.5793
Obesity	0 (0)	0 (0)	0 (0)	2 (15)	2 (15)	0.4263	1 (14)	1 (25)	<b>4 (100)</b>	<b>0.0157</b>
Diabetes	0 (0)	3 (23)	1 (20)	5 (38)	6 (46)	0.3224	0 (0)	0 (0)	1 (25)	0.2292
Additional pathologies <i>N</i> (%) <sup>b</sup>										
ARDS	NA	NA	NA	NA	NA		1 (14)	0 (0)	<b>3 (75)</b>	<b>0.0337</b>
Lung disease	0 (0)	3 (23)	1 (20)	1 (8)	0 (0)	0.2863	NA	NA	NA	
Cancer	0 (0)	4 (31)	0 (0)	4 (31)	1 (8)	0.2063	3 (43)	1 (25)	0 (0)	0.3014
Infectious disease	0 (0)	2 (15)	0 (0)	0 (0)	0 (0)	0.2167	1 (14)	2 (50)	0 (0)	0.1833
Kidney disease	0 (0)	1 (8)	0 (0)	0 (0)	2 (15)	0.4785	0 (0)	0 (0)	0 (0)	
Coronary disease	0 (0)	1 (8)	1 (20)	2 (15)	3 (23)	0.6878	0 (0)	0 (0)	0 (0)	
Others	0 (0)	3 (23)	0 (0)	0 (0)	1 (8)	0.2063	2 (29)	2 (50)	1 (25)	0.7059

<sup>a</sup>Comparison of all groups with Kruskal-Wallis.

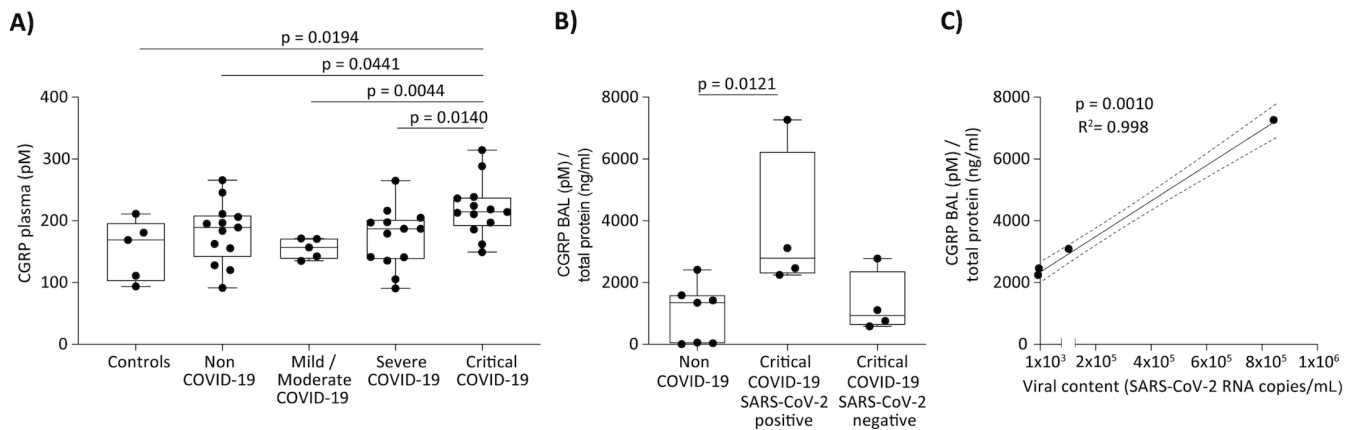
<sup>b</sup>Comparison of all groups with  $\chi^2$  tests.

<sup>c</sup>IQR, interquartile range.

<sup>d</sup>The values in bold are statistically (or almost) significant.

measured CGRP in these plasma samples by an enzyme immunoassay (EIA) and found that critical COVID-19 patients had significantly elevated CGRP levels when compared to the other groups of COVID-19 patients, as well as to non-COVID-19 patients and healthy controls (Fig. 1A).

SARS-CoV-2 detection and isolation from non-respiratory specimens, including blood, are unsuccessful in most studies (34). Therefore, we also measured CGRP in bronchoalveolar lavages (BALs) of additional critical COVID-19 and non-COVID-19 patients (i.e., serving as controls), which were included in our previous study (35). We previously quantified the viral content in these BAL samples and reported that SARS-CoV-2 presence in BALs (i.e., SARS-CoV-2-positive patients) corresponds to an early phase of viral replication, whereas SARS-CoV-2 absence in BALs (i.e., SARS-CoV-2-negative patients) corresponds to a later phase of the infection when the virus has been cleared from the lungs (35). These cases were matched in terms of age and sex (see Table 1) and were not statistically different from the cases from which we obtained plasma samples in terms of both age ( $P = 0.2477$ ) and sex ( $P = 0.2197$ ). When compared to the SARS-CoV-2-positive critical COVID-19 and non-COVID-19 patients, SARS-CoV-2-negative critical COVID-19 patients had significantly higher incidence of obesity (100%,  $P = 0.0157$ ) and ARDS (75%,  $P = 0.0337$ ). We measured CGRP by EIA in these BAL samples as above and normalized CGRP levels to that of total BAL protein (i.e., to account for potential differences in protein content upon BAL collection). When compared to the non-COVID-19 patients, normalized CGRP levels were significantly higher in BALs of SARS-CoV-2-positive critical COVID-19 patients (Fig. 1B). Moreover, these levels significantly and positively correlated with SARS-CoV-2 viral content in these samples (Fig. 1C). Interestingly, in BALs obtained from SARS-CoV-2-negative critical COVID-19



**FIG 1** CGRP levels are increased in critical COVID-19 patients. (A and B) CGRP levels were determined using an EIA in plasma of COVID-19 patients with different disease severities, non-COVID-19 patients and healthy controls (A), and in BALs of SARS-CoV-2-positive or negative critical COVID-19 and non-COVID-19 patients (B). In (B), CGRP levels were normalized to that of BAL total protein content. Shown are box and whisker plots with exact  $P$  values calculated with the Mann-Whitney test. (C) Correlation between SARS-CoV-2 viral content expressed as RNA copies per mL and normalized CGRP levels, in BALs of SARS-CoV-2-positive critical COVID-19 patients. The solid line denotes a linear correlation with numbers representing the corresponding  $P$  and  $R^2$  values, and broken lines denote 95% CIs.

patients that correspond to a late stage of the disease, normalized CGRP levels were restored to baseline and were not different from that in the control group (Fig. 1B).

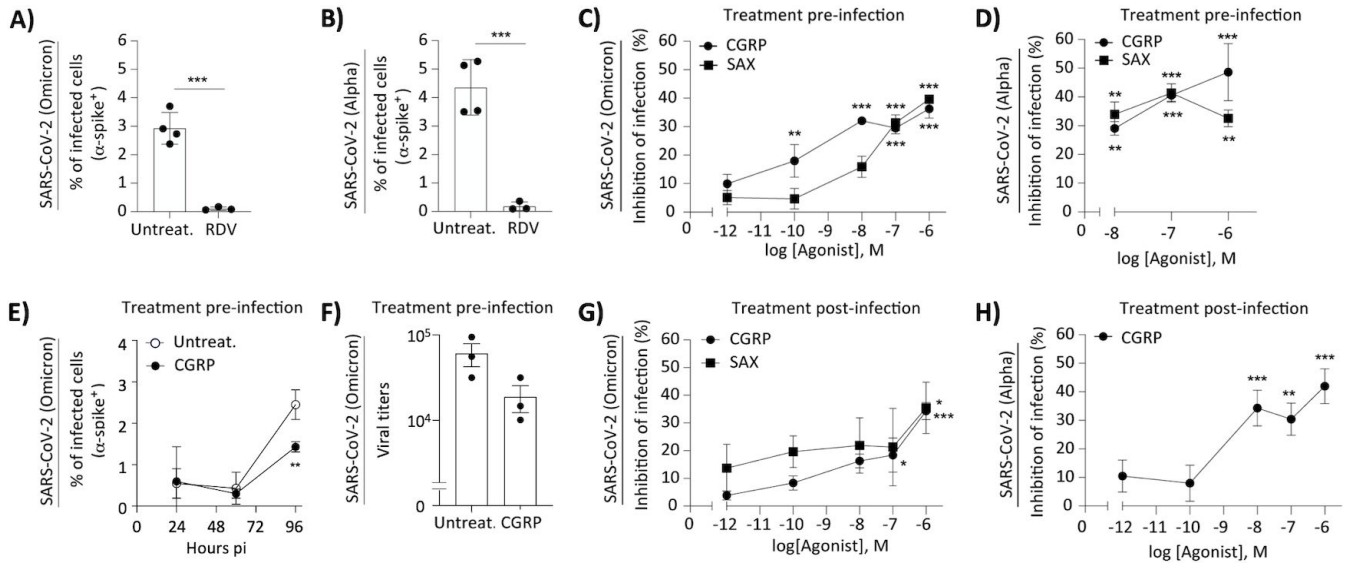
These results show that CGRP levels increase in critical COVID-19 patients and correlate with their pulmonary viral content.

### CGRP and its analog SAX inhibit SARS-CoV-2 infection of Calu-3 cells

SARS-CoV-2 content in BALs (i.e., reflecting pulmonary viral content) is transitory, suggesting that CGRP might interfere with viral replication. Thus, we next explored the direct effects of CGRP on SARS-CoV-2 infection, using the well-characterized bronchial epithelial cell line Calu-3 (36) that is permissive to infection with different SARS-CoV-2 variants (37). We first confirmed that Calu-3 cells were productively infected by pulsing confluent Calu-3 monolayers with SARS-CoV-2 for 4 h, alone or following pre-incubation with the antiviral drug remdesivir (RDV). Four days later, viral replication was evaluated by intracellular expression of the SARS-CoV-2 spike protein using flow cytometry. These experiments showed that Calu-3 cells were infected with both Omicron (Fig. 2A; Fig. S1A) and Alpha (Fig. 2B) variants. As expected, infection was productive and was completely inhibited by RDV for both SARS-CoV-2 variants (Fig. 2A and B; Fig. S1A).

Next, Calu-3 cells were pre-treated for 24 h at 37°C with CGRP within its concentration range that inhibits LCs-mediated HIV-1 and HSV-1/2 infection, as we reported (30, 31). The cells were also pre-treated with the CGRP metabolically stable analog SAX (serinyl-CGRP<sub>2-37</sub>-amide with an albumin binding fatty acid moiety in the N-terminus) that, like CGRP, exerts protective cardiovascular effects (38–41) and anti-viral functions (30, 31), as we reported. The cells were then washed, pulsed with SARS-CoV-2 variants for 4 h in the absence of CGRP/SAX, further incubated for 4 days, and infection was determined by flow cytometry as above. CGRP and SAX significantly inhibited infection of Calu-3 cells with both Omicron (Fig. 2C; Fig. S1A) and Alpha (Fig. 2D) variants, in a dose-dependent manner. The potency of SAX was lower than that of CGRP for the Omicron variant (Fig. 2C;  $P = 0.0268$ ), which is in accordance with steric interference by its bulk moiety (38, 40).

The inhibitory effect following pre-treatment for 24 h at 37°C with  $10^{-6}$ M CGRP (i.e., the highest effective concentration we tested), against infection of Calu-3 cells with the Omicron variant, was also time-dependent (Fig. 2E). To further evaluate SARS-CoV-2 titers at these settings, the culture supernatants were collected at 4 days post-infection (pi), centrifuged, serially diluted, and added to replicate wells of non-infected Calu-3



**FIG 2** CGRP and SAX inhibit SARS-CoV-2 infection of Calu-3 cells. (A and B) Calu-3 cells were left untreated or pre-incubated for 24 h at 37°C with RDV, washed, pulsed with the SARS-CoV-2 Omicron (A) or Alpha (B) variants for 4 h at 37°C, washed again, and further incubated for 4 days. Infection was then determined by intracellular staining using an anti-spike Ab and flow cytometry. Graphs represent mean ± SEM (*n* = 4 independent experiments) percentages of spike-positive infected cells. (C and D) Calu-3 cells were left untreated or pre-treated for 24 h at 37°C with the indicated molar concentrations of CGRP or SAX. The cells were then washed, pulsed with SARS-CoV-2 Omicron (C) or Alpha (D) variants in the absence of CGRP/SAX and examined for infection by flow cytometry as above. Graphs represent mean ± SEM (*n* = 3–7 independent experiments) percentages of SARS-CoV2 infection inhibition, normalized against untreated cells serving as the 100% set point (i.e., no inhibition). (E) Calu-3 cells were left untreated or pre-treated for 24 h at 37°C with 10<sup>-6</sup>M CGRP. The cells were then washed, pulsed with SARS-CoV-2 Omicron, and examined by flow cytometry as above at different time points pi. The graph represents mean ± SEM (*n* = 3 independent experiments) percentages of spike-positive infected cells. (F) Calu-3 cells were left untreated or pre-treated for 24 h at 37°C with 10<sup>-6</sup>M CGRP, washed, and pulsed with SARS-CoV-2 Omicron. At 4 days pi, the culture supernatants were collected and viral titers were determined. The graph represents mean ± SEM (*n* = 3 independent experiments) TCID<sub>50</sub>/mL. (G and H) Untreated Calu-3 cells were pulsed with SARS-CoV-2 variants as above, washed, and post-treated with the indicated molar concentrations of CGRP or SAX that were kept throughout the 4 days of culture. Results (*n* = 4–9 independent experiments) are shown as in (C and D). In all experiments, statistical significance was evaluated by the Student's *t* test.

monolayers, and the 50% tissue culture infectious doses (TCID<sub>50</sub>)/mL were calculated. In agreement with the results above, CGRP also reduced viral titers (Fig. 2F; *P* = 0.0761, Student's *t* test). Calu-3 cells were similarly pre-treated for 24 h at 37°C with 10<sup>-6</sup>M CGRP, washed, and pulsed with the SARS-CoV-2 Alpha variant for 4 h, and viral production was evaluated 24 h later by quantifying viral genome copies in the culture supernatants. CGRP reduced viral production from Calu-3 cells, with mean ± SEM (derived of *n* = 4 different experiments) relative SARS-CoV-2 RNA folds of 1.0 ± 0.13 for untreated cells vs 0.61 ± 0.08 for CGRP-treated cells (*P* = 0.0432, Student's *t* test).

To next evaluate the mechanisms of action of CGRP and SAX, Calu-3 cells were first pulsed with the SARS-CoV-2 variants, washed, and CGRP or SAX were added after the viral pulse and maintained throughout the 4 days culture period. Such post-infection treatment significantly inhibited infection of Calu-3 cells with both SARS-CoV-2 variants, in a dose-dependent manner (Fig. 2G and H; Fig. S1B). Moreover, the maximal extent of inhibition mediated by treatment pre- (Fig. 2C and D) and post-infection (Fig. 2G and H) was similar.

These results indicate that CGRP directly inhibits SARS-CoV-2 replication in bronchial epithelial cells.

### CGRP-mediated inhibition of SARS-CoV-2 infection involves activation of the CGRP receptor in Calu-3 cells

The heteromeric CGRP receptor complex is composed of calcitonin receptor-like receptor (CLR) and receptor activity modifying protein 1 (RAMP1; important for surface trafficking

of the receptor complex), as well as the intracellular receptor component protein (important for signaling of this receptor) (42). Primary airway epithelial cells were reported to express the CGRP receptor (43), but data are missing for Calu-3 cells. Hence, we stained Calu-3 cells with commercially available antibodies (Abs) that are validated for flow cytometry and directed against extracellular epitopes of human CLR and RAMP1. Approximately 10% of Calu-3 cells co-expressed CLR and RAMP1 on their surface (Fig. 3A), indicating the presence of a potentially functional CGRP receptor.

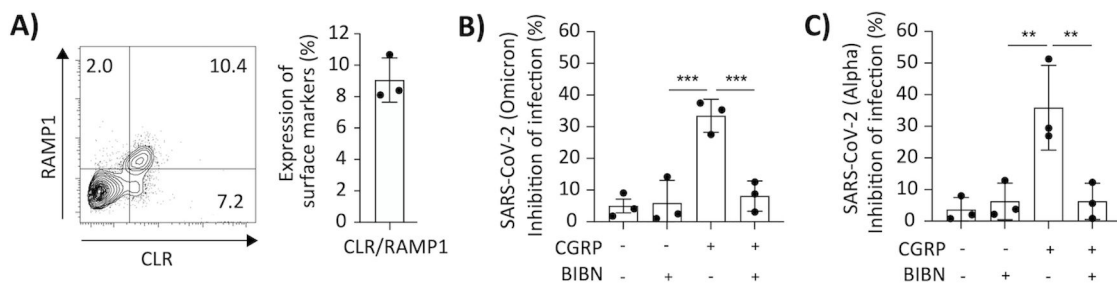
To determine the implication of the CGRP receptor in the anti-viral activity of CGRP, Calu-3 cells were first incubated for 15 min with the CGRP receptor antagonist BIBN4096 (BIBN), followed by pre-treatment with  $10^{-6}$ M CGRP for 24 h. The cells were then washed and pulsed with SARS-CoV-2 for 4 h, and infection was evaluated 4 days later by flow cytometry as above. BIBN alone had no significant effect but completely abrogated CGRP-mediated inhibition of infection with both Omicron and Alpha variants (Fig. 3B and C; Fig. S2).

These results indicate that the anti-SARS-CoV-2 activity of CGRP is mediated via activation of its cognate receptor expressed at the surface of Calu-3 cells.

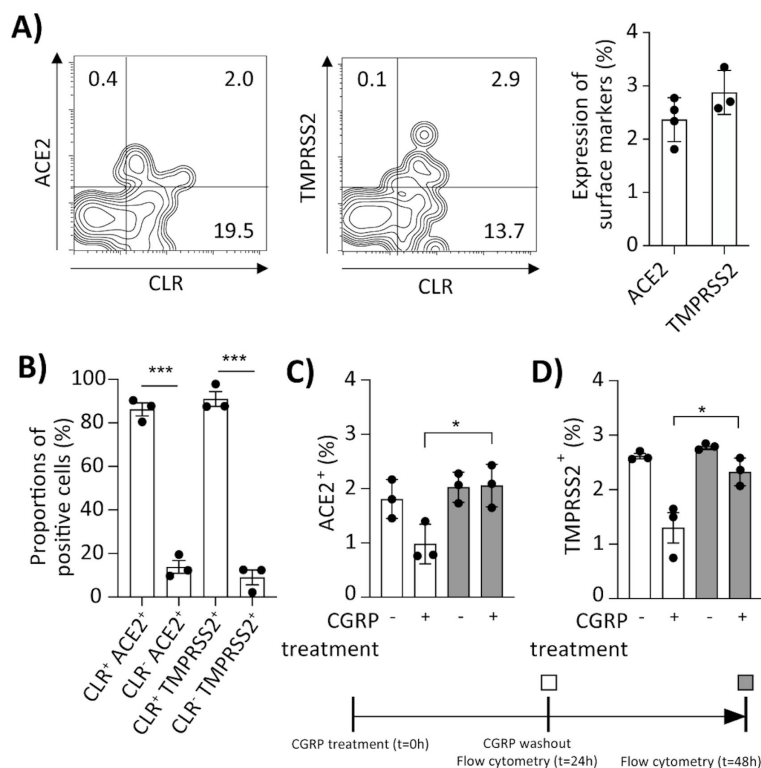
### CGRP reduces the expression of SARS-CoV-2 entry receptors in Calu-3 cells

We next determined by flow cytometry the surface expression in Calu-3 cells of SARS-CoV-2 entry receptors, namely angiotensin-converting enzyme 2 (ACE2) and transmembrane protease serine 2 (TMPRSS2), as well as the CGRP receptor component CLR. These experiments showed that Calu-3 cells expressed both ACE2 and TMPRSS2 (Fig. 4A and B), confirming previous observations (44). Moreover, Calu-3 cells expressing ACE2 or TMPRSS2 represented almost exclusively sub-populations of cells expressing CLR (Fig. 4A and B). We then investigated whether CGRP affects the surface levels of ACE2 or TMPRSS2, like its capacity to modulate the expression of HIV-1 and HSV-1/2 entry receptors mentioned above. Calu-3 cells were left untreated or treated with  $10^{-6}$ M CGRP for 24 h. Surface expression of ACE2/TMPRSS2 was then determined either after CGRP treatment or following washing and chasing of the cells in the absence of CGRP for an additional 24 h. These experiments showed that surface expression of either ACE2 (Fig. 4C) or TMPRSS2 (Fig. 4D) was significantly decreased by approximately half following CGRP treatment (white bars in Fig. 4C and D). Surface expression of both receptors was restored to their normal levels the next day, when the cells were further cultured in a medium without CGRP (gray bars in Fig. 4C and D).

These results suggest that CGRP acts directly on ACE2/TMPRSS2-expressing Calu-3 cells. By reducing SARS-CoV-2 entry receptor expression, which in turn decreases SARS-CoV-2 infection in infected patients, CGRP could limit viral propagation and accordingly play important roles in the resolution of infection.



**FIG 3** CGRP inhibits SARS-CoV-2 infection of Calu-3 cells by activating its receptor. (A) Representative flow cytometry contour plot showing surface expression of CLR and RAMP1 in Calu-3 cells, with a graph showing mean  $\pm$  SEM ( $n = 3$  independent experiments) percentages of double positive cells. (B and C) Calu-3 cells were left untreated or pre-treated for 24 h at 37°C with  $10^{-6}$ M CGRP. When indicated, the CGRP receptor antagonist BIBN was added at  $10^{-6}$ M 15 min before the addition of CGRP. Cells were then washed, pulsed with the SARS-CoV-2 Omicron (B) or Alpha (C) variants for 4 h at 37°C, washed again, further incubated for 4 days, and infection was determined by flow cytometry. Graphs represent mean  $\pm$  SEM ( $n = 3$  independent experiments) percentages of SARS-CoV-2 infection inhibition, and statistical significance was evaluated by the Student's *t* test.



**FIG 4** CGRP decreases ACE2 and TMPRSS2 surface expression in Calu-3 cells. (A and B) Representative flow cytometry contour plots showing surface expression of CLR and either ACE2 or TMPRSS2 in untreated Calu-3 cells. In (A), the graph shows mean  $\pm$  SEM ( $n = 3-4$  independent experiments) percentages of ACE2 or TMPRSS2 positive cells. In (B), the graph shows mean  $\pm$  SEM ( $n = 3$  independent experiments) proportions of CLR/ACE2 or CLR/TMPRSS2 double-positive vs single-positive cells expressing ACE2 or TMPRSS2 but not CLR. (C and D) Calu-3 cells were left untreated or treated for 24 h at 37°C with  $10^{-6}$ M CGRP, followed by washout of CGRP and further incubation for 24 h. Surface expression of ACE2 or TMPRSS2 was determined immediately after CGRP treatment (white bars) or the next day following CGRP washout (gray bars). Graphs represent mean  $\pm$  SEM ( $n = 3$  independent experiments) percentages of positive cells. In all graphs, statistical significance was evaluated by the Student's *t* test.

## DISCUSSION

The rapid development of vaccines against SARS-CoV-2 and direct-acting antivirals was crucial for the management of the COVID-19 pandemic. Although these clinical interventions prevent severe disease, vaccine-induced protection weakens in months and SARS-CoV-2 variants escape vaccine-acquired immunity (2). As these strategies fail to fully block viral transmission, identifying endogenous pulmonary factors limiting SARS-CoV-2 infection could offer novel preventive and therapeutic approaches complementing vaccination.

We show that CGRP levels are increased in the plasma of critical COVID-19 patients. Moreover, we tested for the first time CGRP levels in BALs of COVID-19 patients, namely samples representing the tissue site of SARS-CoV-2 replication, and observed similar CGRP elevation. Importantly, CGRP increases only when BALs from critical COVID-19 patients contain SARS-CoV-2. This suggests that CGRP increases when virus replicates in the lungs of these patients, helping to control pulmonary SARS-CoV-2 replication. As plasma CGRP is considered spillover from its tissue production sites, the changes in CGRP levels we observed in the plasma of critical COVID-19 patients correspond to different stages of their disease. Future studies should now quantify CGRP levels in BALs of non-critical COVID-19 patients, which we speculate will not contain elevated CGRP.



Interestingly, the overall outcome we observe is the elevation of CGRP in both plasma and SARS-CoV-2-positive BALs, although the samples tested herein were obtained from critical COVID-19 patients with several co-morbidities that have opposite effects on CGRP. Specifically, our samples were obtained from patients with a high incidence of smoking/hypertension (for plasma) or obesity (for SARS-CoV-2-negative BALs), which associates with either increased [i.e., smoking (45) and obesity (46)] or decreased [i.e., hypertension (22)] CGRP levels. When the plasma samples of critical COVID-19 patients were stratified according to smoking status, CGRP levels were higher in smokers compared to non-smokers (i.e., mean  $\pm$  SEM of  $229.3.0 \pm 16.6$  vs  $197.0 \pm 12.0$ , respectively) yet not reaching statistical significance ( $P = 0.1986$ , Mann-Whitney test). These results suggest that modulation of CGRP levels in COVID-19 patients cannot be attributed to a single clinical parameter.

Of note, age and sex are additional factors that should be considered, as plasma CGRP levels decrease with age (46) and are lower in males (47, 48). These observations could explain why a previous study concluded that plasma CGRP levels were lower in COVID-19 patients, as the control group in that study included healthy volunteers that were significantly younger than the COVID-19 patients tested. Nevertheless, although not reaching statistical significance, CGRP levels were higher in COVID-19 patients admitted to the intensive care unit (ICU) compared to patients in the general ward or asymptomatic (49). A second study reported that plasma CGRP levels were higher in a cohort of COVID-19 patients that excluded ICU patients. Yet, cases were younger and included males and females at equal proportions (50), differing from our cohort which includes principally elderly males. Finally, a third study evaluated plasma CGRP in a cohort of moderate-to-severe COVID-19 patients but without comparison to controls. This study included elderly and mostly male patients and reported that CGRP levels were higher in patients with negative disease evolution, including eventual ICU admission (51). Collectively, and together with our results described herein, we conclude that elevated CGRP is a hallmark of early critical COVID-19.

The control of SARS-CoV-2 replication by CGRP, which we observed *in vitro*, suggests the induction of beneficial CGRP-mediated protective mechanisms. Indeed, in other lung pathologies such as asthma and cystic fibrosis, elevated CGRP does not cause damage but is rather a compensatory protective response to ameliorate the damage (23). Similarly, increase in plasma CGRP levels in sepsis is considered a compensatory mechanism by which septic shock is attenuated (52). Our results indicate that this compensatory response probably also occurs in COVID-19, as we find that CGRP directly inhibits SARS-CoV-2 replication in bronchial epithelial cells. Such inhibition involves activation of the CGRP receptor and leads to down-regulation of SARS-CoV-2 entry receptors. Importantly, reduced infection following CGRP pre-treatment correlates with the decrease in ACE2/TMPRSS2 surface expression at the time of the viral pulse. In CGRP post-infection treatment, we speculate that the maintenance of CGRP throughout the infection period provides a sufficient time frame, permitting CGRP to exert a similar ACE2/TMPRSS2 decreasing effect, which is similarly accompanied by reduced infection. CGRP post-infection treatment could also impair viral propagation by reducing ACE2/TMPRSS2 surface expression on cells not yet infected after SARS-CoV-2 pulse, helping to resolve the infection. We, therefore, propose that future CGRP-based formulations might be useful for COVID-19 prevention. Such formulations might also be useful as COVID-19 treatments, at an early time point when the virus is still present in the airways. Our results also extend the anti-viral function of CGRP to pulmonary viruses and cells, in addition to its activity exerted by acting on immune cells that are targeted by sexually transmitted viruses, as we previously discovered (27–31).

Different cell types secrete CGRP in the airways, principally sensory nerves and PNECs but also non-neuronal cells, including macrophages (53) and epithelial cells (43, 54). Previous studies reported that SARS-CoV-2 preferentially targets ciliated lung epithelial cells (55). These results are in line with the SARS-CoV-2 permissiveness of Calu-3 cells, which give rise to ciliated cells upon their differentiation in submerged and air-liquid

culture conditions (56). Interestingly, while bronchial epithelial cells secrete pro-inflammatory cytokines upon sensing of SARS-CoV-2, we did not observe induction of CGRP secretion from SARS-CoV-2-infected Calu-3 cells (data not shown). Hence, it would be instrumental to determine precisely CGRP cellular sources during SARS-CoV-2 infection. In this context, a recent study reported that the SARS-CoV-2 papain-like protease directly activates lung-innervating sensory nerves and induces their secretion of CGRP (57). These findings strongly suggest that the elevated levels of CGRP we observed herein in BALs of SARS-CoV-2-positive patients result from direct activation of lung-innervating sensory nerves by the virus.

The use of CGRP receptor antagonists was proposed, at the early stages of the pandemic, as a potential therapeutic approach against COVID-19 (58), mainly due to the induction by CGRP of IL-6 secretion that was recently confirmed (19). Yet, concerns have been raised that CGRP blockade might counteract its anti-hypertensive and protective cardiopulmonary functions, leading to adverse effects (59). Two recent studies reported that CGRP neutralizing Abs [nAbs; used clinically in migraine patients, in which CGRP is elevated and detrimental (23)] present no association with increased risk of COVID-19 infection and outcomes (60, 61). Importantly, both cohorts included mainly younger individuals (46–47 years old) presenting with non-critical COVID-19. Based on our results, we speculate that CGRP levels in these cases would be normal, explaining why CGRP blockade had no effects in these studies. In contrast, we argue that CGRP elevation would exert protective effects in elderly patients with early critical COVID-19.

Collectively, we reveal a novel anti-viral function of CGRP and suggest that its direct anti-SARS-CoV-2 function mediates beneficial pulmonary viral clearance in critical COVID-19, hence representing a protective compensatory mechanism. Caution should be taken when considering the use of CGRP receptor antagonists and nAbs in critical COVID-19, which would counteract these direct protective effects of CGRP against SARS-CoV-2 infection.

## MATERIALS AND METHODS

### Plasma and BAL levels of CGRP

Plasma samples were obtained between November 2020 to January 2021 from different groups of COVID-19 patients, which were hospitalized in either the internal medicine unit (IMU) or ICU at the Cochin Hospital (Paris). These patients were described in our previous study (4) and presented with different COVID-19 disease severities at the sampling date. In the current study, we used samples from  $n = 5$  mild/moderate patients at the IMU,  $n = 13$  severe patients at the IMU and  $n = 13$  critical patients at the ICU. As a control, we used plasma samples from  $n = 13$  patients admitted due to various non-COVID-19-related pathologies that we described in another previous study (32), as well as from  $n = 5$  otherwise healthy individuals that we recently described (33).

BAL samples were obtained between March to June 2020 from additional critical COVID-19 patients hospitalized at the ICU of the Cochin (Paris) or Ambroise Paré (Boulogne-Billancourt) Hospitals, which we previously described (35). Herein, we included  $n = 4$  SARS-CoV-2-positive and  $n = 4$  SARS-CoV-2-negative critical COVID-19 patients, as well as  $n = 7$  patients admitted due to non-COVID-19-related pathologies.

Plasma and BAL CGRP levels were determined using a competitive CGRP EIA kit (Phoenix Pharmaceuticals), and total BAL protein content was determined using the Pierce bicinchoninic acid assay kit (Thermo Scientific), both according to the manufacturer's instructions.

### Cells and viruses

The human bronchial Calu-3 epithelial cell line was obtained from the American Type Culture Collection. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Gibco) supplemented with 10% fetal calf serum (Eurobio), 2 mM L-glutamine, 1%

non-essential amino acids, 100 U/mL penicillin, and 100 µg/mL streptomycin (Gibco). Cells were incubated at 37°C and 5% CO<sub>2</sub> and were detached using 0.05% trypsin (Gibco).

SARS-CoV-2 included the Alpha and Omicron variants we described previously (62), and viral stocks were prepared and titrated using Vero-E6 cells as before (63).

### Calu-3 infection

Calu-3 cells were plated in 48-wells plate ( $2.5 \times 10^5$  cells/well), incubated overnight at 37°C, and pre-treated for 24 h with the indicated molar concentrations of CGRP (Sigma) or its stable analog SAX (41), as we previously described (30, 40). Of note, at their effective concentrations of  $10^{-8}$ – $10^{-6}$ M, CGRP and SAX had no effect on the viability of Calu-3 cells, which remained around 90%–95% (Fig. S3). When indicated, the CGRP receptor antagonist BIBN4096 (Sigma) was added 15 min before the addition of agonists, and the antiviral drug RDV (MedChemExpress) was added at 10 µM overnight before infection. Cells were next washed, pulsed for 4 h with SARS-CoV-2 variants at multiplicities of infection of 0.05 (for Alpha) or 0.0015 (for Omicron), and further incubated for 4 days. In some experiments, CGRP and SAX were added after the pulse period and kept until the end of the culture. Next, cells were detached with trypsin, transferred to 96 round-bottom wells plate, and fixed/permeabilized with the Cytofix/Cytoperm kit (BD Bioscience). Cells were then stained for 30 min at room temperature with Alexa-Fluor-488-conjugated mouse monoclonal Ab directed against the SARS-CoV-2 spike protein [R&D systems; clone #1035206, diluted 1:50 in Perm/Wash buffer (BD)]. Infection was determined by flow cytometry, using a Guava easyCyte Flow Cytometer (Merck-Millipore) and analyzed by FlowJo software (FlowJo LLC).

In some experiments with the Alpha variant, the culture supernatants were collected 24 h following the viral pulse, and infectious viral particle production was quantified by reverse transcription qPCR, as we previously described (64). In other experiments with the Omicron variant, the culture supernatants were collected 4 days following the viral pulse, and viral titers were determined via a standard TCID<sub>50</sub> assay. Titers were calculated based on the Reed-Muench method using a freely available TCID<sub>50</sub> calculator and expressed as TCID<sub>50</sub>/mL (<https://www.virosin.org/tcid50/TCID50.html>).

### Surface expression of SARS-CoV-2 entry receptors

Calu-3 cells ( $5 \times 10^4$  cells/well, in 96 round-bottom wells plate) were surface stained with unconjugated rabbit-anti-human RAMP1 (Proteintech; 1:100), ACE2 (Biorbyt; 1:25), or TMPRSS2 (Invitrogen; 1:10), followed by cyanine-5-conjugated donkey-anti-rabbit-IgG secondary Ab (Jackson ImmunoResearch; 1:200). Cells were next surface stained with fluorescein-isothiocyanate-conjugated rabbit-anti-human CLR (Alomone labs; 1:100). All Abs were diluted in phosphate-buffered saline, and each staining step was performed with 30 µL/well for 30 min on ice. Cells were analyzed by flow cytometry, as above.

### Statistical analyses

Statistical significance was analyzed using Prism 9 software (GraphPad). For clinical data of COVID-19 patients and controls (Table 1), the non-parametric Kruskal-Wallis or  $\chi^2$  tests were used to compare continuous or categorical parameters across groups, respectively. A multivariate logistic regression model was applied to calculate ORs and 95% CIs, and the non-parametric unpaired Mann-Whitney test was used for pairwise comparisons of CGRP levels. Liner correlation was calculated for the association between SARS-CoV-2 content and CGRP levels in BAL samples. For experimental data using Calu-3 cells, the Student's *t* test was used. CGRP and SAX dose-response curves were compared using the [log(inhibitor) vs normalized response – variable slope] non-linear regression model. All tests were two-sided, and differences were considered significant when the *P* values were <0.05, with asterisks denoting the degree of significance (\**P* < 0.05; \*\**P* < 0.005; \*\*\**P* < 0.0005).

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## DATA AVAILABILITY

All research data associated with the paper can be obtained upon request from Y.G.

## ADDITIONAL FILES

The following material is available [online](#).

## Supplemental Material

Supplemental material (JV100128-24-s0001.pdf). Fig. S1 to S3.

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