Role of C3a as a novel regulator of 25(OH)D$_3$ to 1,25(OH)$_2$D$_3$ metabolism in upper airway epithelial cells

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Abstract

In patients with chronic rhinosinusitis with nasal polyps (CRSwNP), primary human sinonasal epithelial cell (HSNEC) 1α-hydroxylase levels are reduced, as is their ability to metabolize 25(OH)D$_3$ to its active metabolite, 1,25(OH)$_2$D$_3$. Here we sought to identify the factor responsible for the regulation of HSNEC metabolism of 25(OH)D$_3$, focusing on C3 and C3a. Multiple inhaled irritants trigger the release of complement components, C3 and C3a, leading to suppression of 1α-hydroxylase levels in HSNEC. Recombinant C3a was able to decrease 1α-hydroxylase and impair 25(OH)D$_3$ to 1,25(OH)$_2$D$_3$ metabolism, while addition of a C3a receptor antagonist (C3aRA) restored conversion. Conversely, 1,25(OH)$_2$D$_3$ suppressed *Aspergillus fumigatus* (Af)-induced C3 and C3a levels in HSNEC supernatant. Given 1,25(OH)$_2$D$_3$'s ability to modulate LL37 in other cell types, we examined its regulation in HSNEC and relationship to C3a. 1,25(OH)$_2$D$_3$ stimulated the secretion of LL37, whereas Af and C3a suppressed it. Conversely, LL37 reduced the release of C3/C3a by HSNEC. Lastly, oral steroid use, and in vitro dexamethasone application, both failed to increase 1α-hydroxylase or reduce C3a levels. In summary, here we describe for the first time a novel relationship between complement activation and local vitamin D metabolism in airway epithelial cells. The presence of elevated C3/C3a in patients with asthma and/or CRSwNP may account for their impaired HSNEC 25(OH)D$_3$ to 1,25(OH)$_2$D$_3$ metabolism and explain why they receive limited therapeutic benefit from oral vitamin D3 supplementation.

Keywords

vitamin D; 1α-hydroxylase; cathelicidin; nasal mucus; sinusitis; epithelial cell; nasal polyp; complement; C3a; *Aspergillus fumigatus*

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INTRODUCTION

Chronic rhinosinusitis (CRS) affects up to 16% of the United States population with direct costs of nearly $22 billion per year.(1) CRS represents one disease that is composed of a wide variety of clinical phenotypes. CRS with nasal polyps (CRSwNP) is the most difficult form of the disease to treat and will be the focus of these studies. CRSwNP is characterized by a polarized type 2 microenvironment.(2) Further complicating the treatment of CRSwNP is that nearly 50% of patients also have a diagnosis of asthma.(3)

One anti-inflammatory agent that may have beneficial effects in the treatment of inflammatory airway disorders is vitamin D3 (VD3). VD3 is a secosteroid hormone whose synthesis begins in the skin where pro-vitamin D3 is metabolized to pre-vitamin D3. Following binding to vitamin D binding protein, it is transported to the liver and metabolized to 25-hydroxycholecalciferol [$25(OH)D_3$]. In the final step of metabolism, 1α-hydroxylase converts $25(OH)D_3$ to its active form, $1\alpha,25$-dihydroxyvitamin D3 [$1,25(OH)_2D_3$]. $1,25(OH)_2D_3$ mediates its actions through the vitamin D receptor (VDR) which is expressed on nearly all cells in the body. It was originally thought that the metabolism of $25(OH)D_3$ to $1,25(OH)_2D_3$ occurred solely in the kidneys, however, studies have now shown it can occur throughout the body including the upper and lower airways.(4–6) Still little is known regarding the role and significance of these extra-renal sites of VD3 metabolism in health and disease, nor how this process is regulated at the tissue level.

Previously we have shown that sinonasal epithelial cells from patients with chronic rhinosinusitis with nasal polyps (CRSwNP), including those with asthma, have a suppressed ability to metabolism 25(OH)D3 to 1,25(OH)D3. What remains unclear, and the focus of these studies, is the mechanism by which sinonasal epithelial cell $25(OH)D_3$ to $1,25(OH)_2D_3$ metabolism is regulated. Here we investigate the role of the central complement component C3 and its cleavage fragment, C3a, as a novel regulator of HSNEC $25(OH)D_3$ metabolism. Extrarenal metabolism has been studied in the context of immune cells, particularly monocytes and macrophages.(7–9) However, the regulation of $25(OH)D_3$ metabolism in epithelial cells appears to be unique from immune cells.(10) Furthermore, while it has been hypothesized that antimicrobial peptides such as LL37 (the human cathelicidin peptide) and complement may collaborate in regulating functions in the mucosal environment,(11) it was unclear in human tissues if a mutual relationship exists. Here we describe that HSNEC production of $1,25(OH)_2D_3$ serves as regulator of the interactions between the complement system and LL37. Lastly, we show that commonly utilized pharmacotherapeutics used in the treatment of CRSwNP and/or asthma, such as oral steroid administration, fail to improve HSNEC 1α-hydroxylase expression or reduce nasal mucus C3a levels in patients with CRSwNP.

METHODS

Patient enrollment, inclusion, and exclusion criteria

Sinus tissue was collected at the time of endoscopic sinus surgery. Inclusion criteria included CRSwNP patients that met the diagnostic criteria outlined by the European Position Paper on Rhinosinusitis and Nasal Polyps 2020.(12) Control sinus tissue was collected from

the uncinate process of subjects who were undergoing surgery for repair of cerebrospinal fluid leak repair or removal of non-hormone secreting pituitary tumor. Exclusion criteria included CRS without nasal polyps, active smokers, immunomodulatory agents within the preceding 30 days, other immunologic, renal, gastrointestinal, endocrine, skeletal disorders, or pregnancy. A subset of patients actively taking oral steroids preoperatively was used to determine its impact on sinonasal 1α-hydroxylase and nasal mucus C3a levels. In all other experiments, samples from patients using oral steroids in the preceding 30 days were excluded.

Human sinonasal epithelial cell line establishment and treatments

Sinus mucosa was processed and used to establish human sinonasal epithelial cell (HSNEC) lines or single cell suspensions (SCS), as previously described.[13, 14] HSNECs were cultured and treated in *serum-free media* and used at passage two. The use of serum free media assures that the complement present is produced by the HSNEC and is not from exogenous sources.

Aspergillus fumigatus (Af) was obtained from Greer Laboratories (Lenoir, NC) and was a combination of mycelial extract and culture filtrate. We have previously established an optimal dose of 5 μg/ml (range 0.1 – 20 μg/ml) and have determined that this dose does not induce cell death, as determined by lactate dehydrogenase (LDH) assay (ThermoFisher Scientific, Waltham, MA). Additional HSNEC treatments included 24-hour incubations with 5 μg/ml *Alternaria alternata* (Alt)(Greer Laboratories), 10 μg/ml house dust mite antigen (HDM) (Greer Laboratories), 10 μg/ml *Pseudomonas*-derived LPS (Sigma-Aldrich, St. Louis, MO) and 10 μg/ml Staphylococcal enterotoxin B (SEB) from *Staphylococcus aureus* (Sigma-Aldrich). Cigarette smoke extract (CSE) was used at a concentration of 10% and was generated as previously described.[5, 15] In separate studies, cells were pre-treated with 100 mmol/L dexamethasone for 1 hour prior to Af treatment as well as concurrent to Af treatment. Af and Alt were determined to be endotoxin free by Limulus Amebocyte Lysate assay (ThermoFisher Scientific). C3a was obtained from Complement Technology (Tyler, Texas) and used at the indicated doses. Recombinant TNF-α, IFN-γ and IL-4 for each of these mediators were used at doses that have previously been reported to modulate 1α-hydroxylase in immune cells.[16–19] Montelukast (Sigma-Aldrich) was used at 100 nM as previously described [20]. Neutralizing antibody to the IL-4 receptor (R&D Systems, Minneapolis, MN) was used at the ND_{50} dose recommended by the manufacture. The $1,25(OH)_2D_3$ was administered at a dose of 100 pg/ml, which is similar to the levels found in circulation and the same as we have previously reported *in vitro*.[5] C3a receptor antagonist (C3aRA) (Calbiochem/EMD Millipore, Burlington, MA) was used at a dose of 50 μM and given one hour prior to stimulation or $25(OH)D_3$ treatment. Recombinant LL37 (Novus Biologicals) doses ranged from 1–10 μg/ml to treat HSNEC and are similar to prior reports from *in vitro* models of the lower airway having described physiological doses of LL37 as ranging from 1–20 μg/mL.[21]

Measurements of $25(OH)D_3$ to $1,25(OH)_2D_3$ metabolism, C3 and C3a

Cells were treated with 1×10^{-6} M $25(OH)D_3$ for 24 hours as we previously described.[5] After this time $1,25(OH)_2D_3$ was assayed immediately by enzyme immunoassay

(Immunodiagnostic Systems, Fountain Hills AZ) according to the manufacturer's instructions. C3 (Genway, San Diego, CA) and C3a (BD Biosciences, San Jose, CA) levels were measured by ELISA according to the manufacturer's instructions.

Immunoblot analysis.

Cells were collected by centrifugation then lysed in cell lysis buffer. Supernatants from the lysed cells were collected and stored at −80. Immunoblot analysis was performed in samples that separated in reduced or nonreduced 4%–12% SDS-PAGE buffer, transferred to PVDF membranes, and probed with goat anti-C3 (A213, Complement Technologies, 1:5000), rabbit anti-human C3a (A218, Complement Technologies, 1:4000) or C3aR (PA5–50364, Invitrogen, 1:1000). Primary antibodies were visualized by HRP-conjugated secondary antibodies using SuperSignal West Pico PLUS (Thermo, Rockford, IL). Antibody binding was imaged on an In-vitrogen iBright 1500 Imager (Thermo, USA). Mouse β-actin was used as a loading control (12262, Cell signaling, 1:5000). The C3 content of the cell lysates was analyzed by immunoblot followed by densitometric scanning (Fiji, USA).

Flow cytometric analysis of 1α-hydroxylase, C3, C3a, and C3aR.

HSNEC and sinonasal tissue explants levels of 1α-hydroxylase were determined by flow cytometric analysis as previously described.(22–24) Intracellular expression of C3, C3a and C3aR was conducted as we and others have previously described.(13, 25) Samples were assayed immediately following staining using a Guava 8HT flow and analyzed with FCS Express 6.0. Dead cells were determined by Zombie viability dye uptake (eBioscience) and were excluded from the final data analysis. Marker gates were set using matched isotype controls or exclusion of primary antibody when a secondary antibody was used.

Statistical Analysis & Data Presentation

Statistical analysis was conducted using GraphPad Prism 9.0 software (La Jolla, CA). A D'Agostino & Pearson omnibus test was used to determine if data sets were normally distrusted. For data in which all groups were normally distributed, a one-way ANOVA with post-hoc, *t*-test was used. For analysis where all data sets were not normally distributed, a Kruskal-Wallis test followed by Dunns multiple comparisons was used. A Pearson correlation analysis was used to determine if a significant correlation existed between 1α-hydroxylase and C3 or C3a levels, as well as between $25(OH)D_3$ and C3 or C3a levels. The results in all bar graphs are mean ± SD and are 24 hours after the initial treatment. All experiments were performed in duplicate wells with the average of the wells being combined from multiple patients (except for correlation analysis). Each data represents results from a different individual. Asterisks were generated using GraphPad Prism's threshold for significances level and are associated with the following p values; NS p 0.05, * p = 0.01 to 0.05, **p= 0.001 to 0.01, *** 0.0001 to 0.001 and **** p< 0.0001.

RESULTS

Multiple types of inflammatory stimuli trigger HSNEC release of C3 and its cleavage fragment, C3a leading to the suppression of 1α-hydroxylase

Previously we have shown that patients with CRSwNP have lower HSNEC levels of 1α-hydroxylase, resulting in reduced sinonasal levels of $1,25(OH)_2D_3$ which is associated with more severe disease.(5, 23) Mice with Af-induced CRS also showed down regulation in sinonasal 1α-hydroxylase and $1,25(OH)_2D_3$ levels.(22) Furthermore, tobacco smoke exposure, either from active smoking or exposure to environmental tobacco smoke, also suppressed HSNEC 1α-hydroxylase expression *in vitro* and *in vivo*.(5) Given that tobacco smoke is unlikely to be the sole contributor to the suppression of 1α-hydroxylase in CRSwNP patients, we examined if other factors commonly found within the upper airway, and associated with CRS, could suppress local $25(OH)D_3$ metabolism.

As shown in Figure 1A, numerous inhaled stimuli were capable of suppressing control HSNEC 1α-hydroxylase including Alt, HDM, CSE and Af. While SEB has been hypothesized to play a role in the pathogenesis of CRSwNP,(26) we did not find that it impacted 1α-hydroxylase expression. Due to LPS' well described role in regulating extrarenal $25(OH)D_3$ metabolism in immune cells,(27, 28) we examined its ability to modulate 1α-hydroxylase. LPS exposure had no significant effect on 1α-hydroxylase levels. As IFN-γ, IL-4 and TNF-α have been previously described to regulate vitamin D metabolism, again in immune cells, they were also examined herein. However, we found that none of the tested cytokines altered HSNEC $25(OH)D_3$ to $1,25(OH)_2D_3$ metabolism (Supplemental Fig. 1).

Our recent proteomic and ELISA studies have demonstrated that local sinonasal, but not systemic, complement 3 (C3) levels correlate with CRSwNP subjective disease severity. (13) Given these findings we sort to examine whether C3/C3a played a role HSNEC 1α-hydroxylase suppression. As shown in Figure 1B & C, HDM, CSE, Alt and Af treatment of control HSNEC all resulted in increased supernatant levels of C3 and C3a, while SEB and LPS had no impact. This response closely mirrored the modulation of 1α-hydroxylase by each of these stimuli. Since C3 elicits many of its pro-inflammatory effects through its cleavage fragments, we next examined the ability of C3a to modulate HSNEC 1α-hydroxylase. Addition of recombinant C3a to control HSNEC resulted in a significant reduction in 1α-hydroxylase expression (Fig. 1D).

Given the ubiquitous presence of Af, our prior findings that mice with Af-induced CRS display suppressed sinonasal levels of 1α-hydroxylase and $1,25(OH)_2D_3$, similar to that seen in humans with CRSwNP(22), and Af's ability to trigger the release of C3/C3a *in vitro* HSNEC(22) and *in vivo*(13) (and Fig. 1A), we elected to use Af as a pathogenically significant stimulus for the following series of experiments. Next, we examined if inhibition of C3a signaling, through antagonism of the receptor with C3a Receptor antagonist (C3aRA), could block the Af-induced reduction of 1α-hydroxylase and subsequent $25(OH)D_3$ to $1,25(OH)_2D_3$ conversion we have demonstrated in control HSNEC. Treatment with C3aRA blocked Af-induced suppression of 1α-hydroxylase (Fig. 2A), and further, treatment blocked the ability of Af to suppress $25(OH)D_3$ to $1,25(OH)_2D_3$

metabolism (Fig. 2B). These data support the existence of C3a-C3aR axis in the control of HSNEC 1α-hydroxylase expression. To determine whether exposure of control HSNEC to Af leads to changes in C3aR expression that could influence our results we performed flow cytometry and western blot analysis. Neither Af exposure nor $1,25(OH)_2D_3$, treatment significantly impacted C3aR expression (Fig. 2C & D). Together, these data demonstrate that C3a reduces the expression of HSNEC 1α-hydroxylase, and subsequently impairs the metabolism of $25(OH)D_3$ to $1,25(OH)_2D_3$.

Exogenous $1,25(OH)_2D_3$ can reduce C3 secretion, resulting in reduced C3a levels following Af stimulation

We next examined if $1,25(OH)_2D_3$ could reduce Af-induced complement secretion in CRSwNP HSNEC. In cells treated with $1,25(OH)_2D_3$ alone, there was a significant reduction in C3 release compared to control (Fig. 3A). Additionally, $1,25(OH)_2D_3$ was able to reduce supernatant levels of C3 present following Af exposure. For C3a, $1,25(OH)_2D_3$ alone, had no significant impact on supernatant levels of C3a between control and $1,25(OH)_2D_3$ treated cells (Fig. 3B), albeit levels were extremely low. However, in HSNECs treated with Af plus $1,25(OH)_2D_3$, $1,25(OH)_2D_3$ was able to significantly reduce the levels of C3 and C3a found in the culture supernatants. Taken together these data suggest that $1,25(OH)_2D_3$'s ability to reduce C3a levels, may be the result of a reduced secretion of C3, resulting in less product available for cleavage by Af, ultimately resulting in diminished supernatant C3a levels. To further explores this, we performed C3 western blot analysis of HSNEC lysates to determine the levels, and to access the different C3 forms, present within cells in all treatment groups. Immunoblotting for C3 revealed the presence of pro-C3 and native C3 in all groups, a finding that is in keeping with that seen in respiratory epithelial cells of the lower airway (Fig. 3C) (29). Densitometry quantification of C3 showed a small but significant increase of native C3 in Af plus $1,25(OH)_2D_3$ treated cells as compared to Af alone, perhaps suggesting that $1,25(OH)_2D_3$ leads to a retention of intracellular C3 stores (Fig. 3D). We could demonstrate no intracellular C3a stores in control HSNECs by immunoblotting supporting our hypothesis that C3 is cleaved extracellularly in response to exposure to Af.

LL37 and C3a reciprocally regulate each other's expression in HSNEC

One of the most well described immunological roles of vitamin D is the stimulation of cells to make cathelicidin, of which LL37 is the only human form. LL37 is an antimicrobial peptide that can kill gram positive and gram-negative bacteria, viruses, fungi, and protozoa though its ability to form holes in cell membranes. Furthermore, it has previously been described in non-human tissues that an association between complement and cathelicidin exists, though whether this relationship exists in human remains unclear.(11) Therefore, we sought to determine if such a relationship existed in HSNEC. In keeping with other epithelial and immune cell types, HSNEC increased their section of LL37 in response to $1,25(OH)_2D_3$ treatment (Fig. 4). Whereas treatment with Af or C3a, significantly reduced LL37 secretion.

After observing these results, we next examined in control and CRSwNP-patient derived HSNEC the impact of LL37 treatment on C3 and C3a secretion. Similar to our prior reports,

(13) basal rates of C3 and C3a secretion were higher in CRSwNP vs control HSNEC (Figs. 5A & 5B, respectively). Treatment with LL37 resulted in a dose dependent decrease in C3 release in both CRSwNP and controls (Figs. 5A & B). C3a levels were also significantly reduced with LL37 treatment (Figs. 5C & D). Collectively, these findings suggest that in HSNEC a reciprocal relationship exists between C3a and LL37.

C3aR inhibition restores disease associated suppression in CRSwNP HSNEC, while commonly used therapies including steroids do not

Given our previous reports that CRSwNP HSNEC had reduced *CYP27B1* gene expression, 1α-hydroxylase protein levels and an impaired $25(OH)D_3$ metabolism, (5, 23) we next examined in an *ex vivo* analysis of sinonasal tissue if there was any association between 1α-hydroxylase and intracellular stores of C3 or C3a. In single cell suspensions of sinonasal tissue explants of CRSwNP patients, we found a strong correlation between intracellular C3 and 1α-hydroxylase (Fig. 6A). Intracellular levels of C3a were also examined with no correlation between C3a with 1α-hydroxylase being observed (Fig. 6B). These results were similar to our *in vitro* studies in which $1,25(OH)_2D_3$ secretion impacted the release of C3, but not C3a (Fig. 3A & B). After observing the association between C3 and 1α-hydroxylase, we next examined *in vitro* the ability of C3aRA to reverse the impaired metabolism of $25(OH)D_3$ to $1,25(OH)_2D_3$ in diseased, CRSwNP HSNECs. As shown in Figure 6C, CRSwNP HSNECs have impaired basal production of $1,25(OH)_2D_3$ compared to control HSNECs. C3aRA treatment increased the production of $1,25(OH)_2D_3$ by CRSwNP HSNEC to levels equivalent to control subject derived HSNEC. Together, these data implicate C3a as a novel regulator of 1α-hydroxylase, and increased complement activation leads to impaired $25(OH)D_3$ to $1,25(OH)_2D_3$ metabolism in patients with CRSwNP.

Since steroids are one of the most widely used therapies to treat CRSwNP, we tested if they had any impact of 1α-hydroxylase or nasal mucus levels of C3a. As shown in Figure 7A, sinonasal tissue levels of 1α-hydroxylase were no different between CRSwNP patients taking oral steroids prior to surgery and those that were not. Consistent with prior reports that corticosteroids have little to no impact on the production of complement,(30, 31) we observed oral steroid use was not associated with any changes in nasal mucus C3a in CRSwNP patients (Fig. 7B). To model this *in vivo* finding we pre-treated HSNECs with dexamethasone, prior to exposing them to Af. In keeping with our *in vivo* data pre-treatment with dexamethasone failed to block Af's suppression of 1α-hydroxylase (Figure 7C). To expand the scope of our findings we further investigated the impact of other therapies utilized for CRS/asthma treatment. Using our in vitro model, we pre-treated HSNECs with either monteleukast or IL4 receptor alpha inhibitors and again determined the expression of 1α-hydroxylase. In keeping with our steroid studies treatment with neither therapeutic blocked Af's suppression of 1α-hydroxylase (Supplement Fig. 2).

In conclusion and highlighted in Figure 8, environmental insults such as Af trigger the release of HSNEC intracellular stores of C3. Upon interaction with environmental factors, C3 is cleaved to C3a, which in an autocrine fashion suppresses the local production of $1,25(OH)_2D_3$. Conversely, the presence of $1,25(OH)_2D_3$ blocks the release of C3 which reduces the available product that can be converted to C3a. The reduction in locally

produced $1,25(OH)_2D_3$ prevents the HSNEC from being induced to release LL37 and could potentially account for the for increases seen in C3/C3a. Finally, The presence of elevated C3/C3a in patients with asthma and/or CRSwNP may account for their impaired HSNEC $25(OH)D_3$ to $1,25(OH)_2D_3$ metabolism and explain why they receive limited therapeutic benefit from oral vitamin D3 supplementation.

DISCUSSION

To our knowledge, these studies are the first to demonstrate that any member of the complement family of proteins can modulate $25(OH)D_3$ to $1,25(OH)_2D_3$ metabolism. These data also provide a link between two prior, independent studies that found reductions in 1α-hydroxylase and elevations in sinonasal C3 were both associated with worse subjective disease severity.(5, 23) Similar to 1α-hydroxylase, HSNEC are also the predominate sinonasal source of C3.(13) In our current study, we have expanded upon these findings and now have identified a closely coordinated collaboration between complement C3/C3a and $1,25(OH)_2D_3$. Here we demonstrate that $1,25(OH)_2D_3$ reduces the secretion of C3, both at rest and in response antigen stimulation. Given that CSE, HDM, Alt and Af have all been shown to cleave C3 into C3a,(32–34) by suppressing the secretion of C3, $1,25(OH)_2D_3$ prevents its subsequent cleavage by inhaled antigens to form the anaphylatoxin, C3a.

The etiology of CRSwNP is unknown and likely complicated by the fact that no singular pathogen or gene has been identified as causing CRSwNP.(35) As such we initially screened a broad range of inhaled and microbial factors associated with CRSwNP to determine their potential to suppress 1α-hydroxylase. While prior studies have hypothesized that SEB contributes to the pathogenesis of CRSwNP,(36) our data suggest that SEB is not responsible for the release C3 and C3a by HSNEC. LPS was tested given prior reports of its ability to modulate immune cell 1α-hydroxylase.(27) However, similar to reports utilizing colon epithelial cells,(10) we found that LPS had no significant impact on 1α-hydroxylase expression. In addition to the examining stimuli responsible for the suppression of 1α-hydroxylase, we also examined if one of the most used treatments for CRS, dexamethasone, could reverse Af-induced suppression. Both oral steroids and *in vitro* dexamethasone treatment failed to improve 1α-hydroxylase suppression. This is likely because corticosteroids have little to no impact on numerous innate immune mediators,(31) including as we have shown sinonasal C3a. We expanded the scope of these studies to include other pharmacotherapeutics used in the treatment of CRS. Interestingly, neither IL-4 nor leukotriene inhibitors impacted 1α-hydroxylase levels. Perhaps the most compelling data that a link between C3a and 1α-hydroxylase suppression occurs is that exogenous addition of C3a suppresses 1α-hydroxylase, and C3aR antagonism restores 1α-hydroxylase to near normal levels. Taken together these data implicate a C3a-C3aR axis. To conform that this axis was not associated with changes in C3aR expression we showed that C3aR levels were not significantly different by both flow cytometry and western blot analysis.

While $25(OH)D_3$ metabolism in epithelial cells has previously been shown to be unique from immune cells,(10) here our studies add the complement component C3a as additional regulator. Similar to prior reports in normal human bronchial epithelial cells (NHBE),(37) we did not see a change in 1α-hydroxylase when treating HSNEC with TNF-α. TGF-β has

been shown to stimulate an increase in *CYP27B1* expression in 16HBE cells.(38) Unlike patients with asthma, CRSwNP is characterized by a decrease in TGF-β as compared to control subjects,(39) and thus it is unlikely to be responsible for the observed suppression. Beyond cytokines, swollen conidia from *A. fumigatus* have been previously reported to increase *CYP27B1* gene expression in 16HBE cells, which differs from the results described herein.(40) This could reflect differences in the vitamin D metabolism pathways between the upper and lower airways.(41) Alternatively, the studies presented herein utilized a combination of mycelial extract and culture filtrate, which could have different roles in the regulation of *CYP27B1*/1α-hydroxylase as compared to conidia. However, given the broad range of environmental factors that we observed here capable of triggering the release of C3, and its cleavage to C3a, it is highly unlikely that the suppression of 1α-hydroxylase in patients with CRSwNP is caused by a singular pathogen. Additionally, we did not investigate if C3a had any impact on VDR expression. However, given that patients with CRSwNP have locally elevated C3a and no alterations in VDR expression, it is unlikely that changes in VDR expression are caused by C3a.(5)

Vitamin D, complement and the cathelicidin family represent three of our oldest and most evolutionarily conserved innate immune systems. While regulation of each other's functions has been described in other species,(11) here, we describe for the first time the interplay of these three systems in the modulation of airway epithelial cell functions. While LL37 has been shown to be expressed in the sinonasal environment, reports are inconsistent if levels are altered in diseased tissue versus healthy controls. This is likely confounded by a number of variables including if patients were colonized with *Staphylococcus aureus*.(42–45) Collectively, the data presented here suggest that if local levels of $1,25(OH)_2D_3$ could be increased, that it may have the ability to reduce the inflammatory effects of C3a while simultaneously improving anti-microbial immunity by increasing LL37 production.

While a number of reports have linked $25(OH)D_3$ deficiency to worsened clinical outcomes in asthma and CRSwNP, the use of oral VD3 supplementation has yielded mixed results for the treatment of airway diseases, primarily asthma.(41) Most notably the VIDA trial (*Vitamin D Add-on Therapy Enhances Corticosteroid Responsiveness in Asthma*) demonstrated that oral VD3 supplementation did not improve patient outcomes(46) or improve sinonasal symptoms in asthmatics with CRS.(47) This is consistent with our own unpublished studies where we observed that oral vitamin D supplementation failed to improve disease severity in CRSwNP patients (NCT01185808). The mechanisms associated with the failure of oral supplementation to improve patient outcomes are unclear, especially given that these strategies restored systemic $25(OH)D_3$ to sufficient levels [30 ng/ml] in both the VIDA trial.(46, 47) Similar to patients with CRSwNP, numerous reports have previously described that asthmatics have increased levels of C3 and C3a in circulation and in respiratory tissues.(48) Collectively these studies demonstrate that C3a, which is not suppressed by steroids, can inhibit HSNECs from making $1,25(OH)_2D_3$ and may provide mechanistic incites as to why oral vitamin D supplementation has not been successful clinically in the treatment of asthma or CRSwNP.

Here we have demonstrated a novel interaction of C3 with sinonasal epithelial cell vitamin D metabolism. A limitation of the current study, and area for future investigation,

is to determine the precise mechanisms by which $1,25(OH)_2D_3$ induces alterations in C3 responses. Exogenous treatment of several different immune and parenchymal cell types has shown that 1,25(OH)2Vit D3 can reduce cytokine secretion (49–52), reduce pro-inflammatory gene transcription (53, 54), and impact membrane and junction protein functions leading to alterations in peptide trafficking (55, 56). In all these studies, the mechanisms by which 1,25(OH)2Vit D3 acts was either undetermined or varied by cell type and organ system. For example, 1,25(OH)2Vit D3 suppression of IL-17A expression was found to be transcriptional repression, mediated by the VDR (53). Whereas studies demonstrating 1,25(OH)2Vit D3 downregulation of cytokine secretion largely did not directly address the mechanism (49–52). Based on these published studies and the data presented herein future studies are needed to determine the mechanism nature of 1,25(OH)2Vit D3 ability to alter C3 responses.

In conclusion, environmental insults such as Af trigger the release of HSNEC intracellular stores of C3. Upon interaction with environmental factors, C3 is cleaved to C3a, which in an autocrine fashion suppresses the local production of $1,25(OH)_2D_3$. Conversely, the presence of $1,25(OH)_2D_3$ reduces the secretion of C3 which reduces the available product that can be converted to C3a. The reduction in locally produced $1,25(OH)_2D_3$ prevents the HSNEC from being induced to release LL37 and could potentially account for the for increases seen in C3/C3a.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

FUNDING:

ABBREVIATIONS

$1,25(OH)_2D_3$	1α,25-dihydroxyvitamin D3
$25(OH)D_3$	25-hydroxycholecalciferol
Af	*Aspergillus fumigatus*
Alt	*Alternaria alternata*
BEGM	Basal epithelial cell growth medium
C3	Complement 3
C3a	Complement 3a
C3aRA	C3a receptor antagonist

CRS	Chronic rhinosinusitis
CRSsNP	Chronic rhinosinusitis without nasal polyps
CRSwNP	Chronic rhinosinusitis with nasal polyps
CS	Cigarette smoke
CSE	Cigarette smoke extract
ELISA	Enzyme-linked immuno sorbent assay
HDM	House dust mite antigen
HSNEC	Human sinonasal epithelial cells
LPS	Lipopolysaccharide
MFI	Mean fluorescent intensity
SEB	*Staphylococcal* enterotoxin B
VD3	Vitamin D3
VDR	Vitamin D receptor

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KEY POINTS

1. Complement component C3 suppresses 1α-hydroxylase in sinonasal epithelial cells

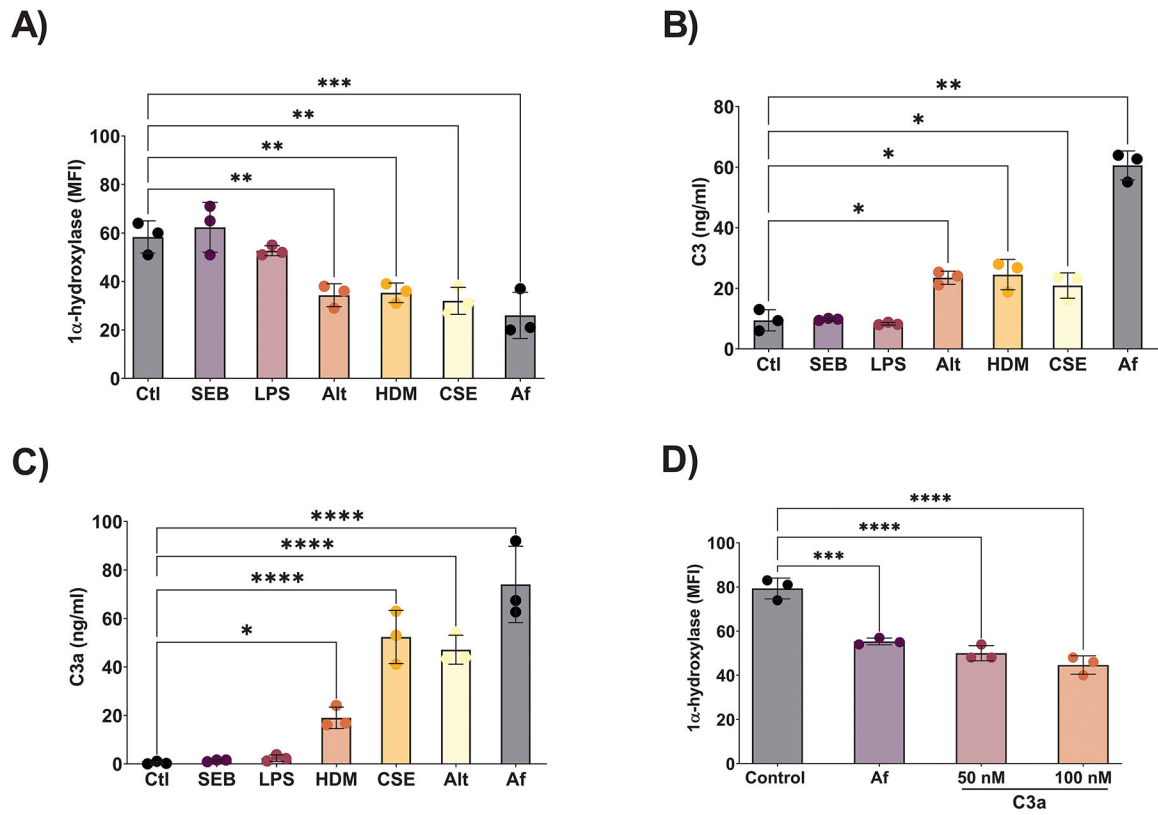2. The active metabolite $1,25(OH)_2D_3$ suppresses sinonasal epithelial cell release of C3

Figure 1: Numerous CRS-associated inflammatory stimuli reduce control HSNEC 1α-hydroxylase expression and trigger the release of C3 and C3a.

Numerous CRS associated stimuli suppress trigger the release of **A)** C3 and **B)** C3a. **C)** The same factors the triggered the release of C3 and C3a, also suppressed HSNEC 1α-hydroxylase expression. **D)** Addition of exogenous C3a reduced HSNEC 1α-hydroxylase similar to Af.
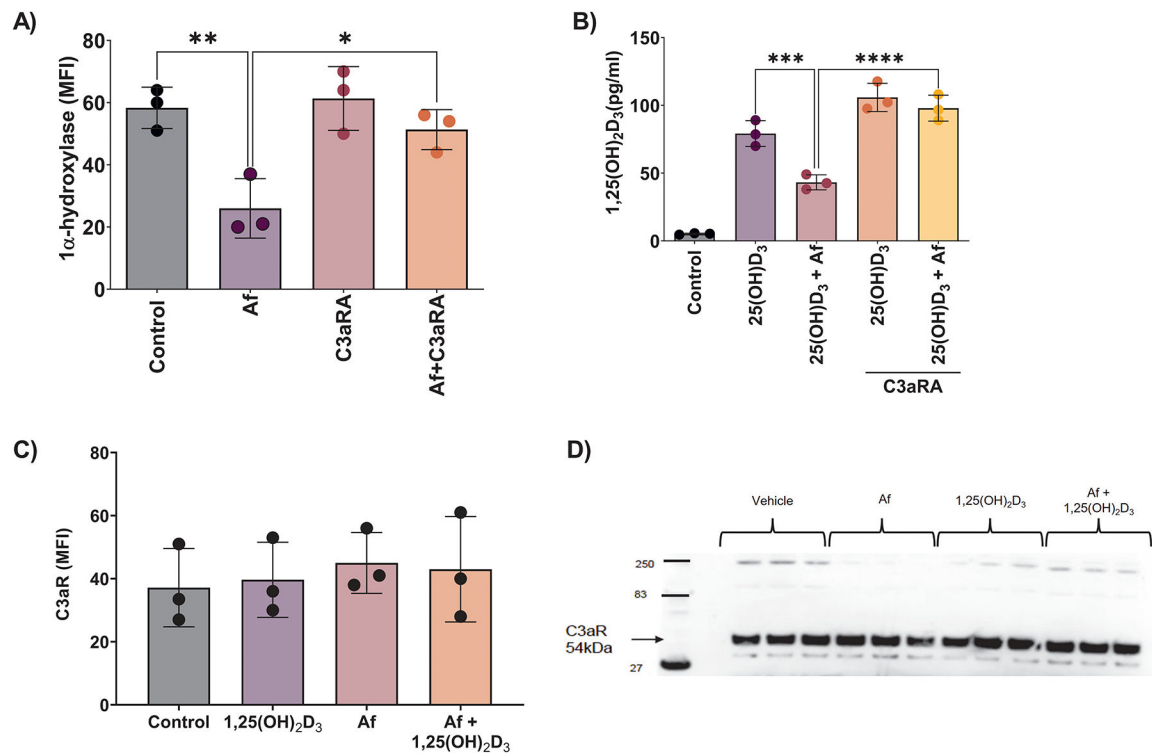
Figure 2. Inhibition of C3a signaling blocks Af induced suppression of 1α-hydroxylase and 25(OH)D$_3$ to 1,25(OH)$_2$D$_3$ metabolism.
A) Control HSNEC 1α-hydroxylase and **B)** 25(OH)D$_3$ to 1,25(OH)$_2$D$_3$ metabolism is protected from Af induced suppression by C3a receptor antagonist (C3aRA) treatment. C3aR expression was not impacted by Af or 1,25(OH)$_2$D$_3$ treatment as determined by flow cytometry **C)** and western blotting **D)**.
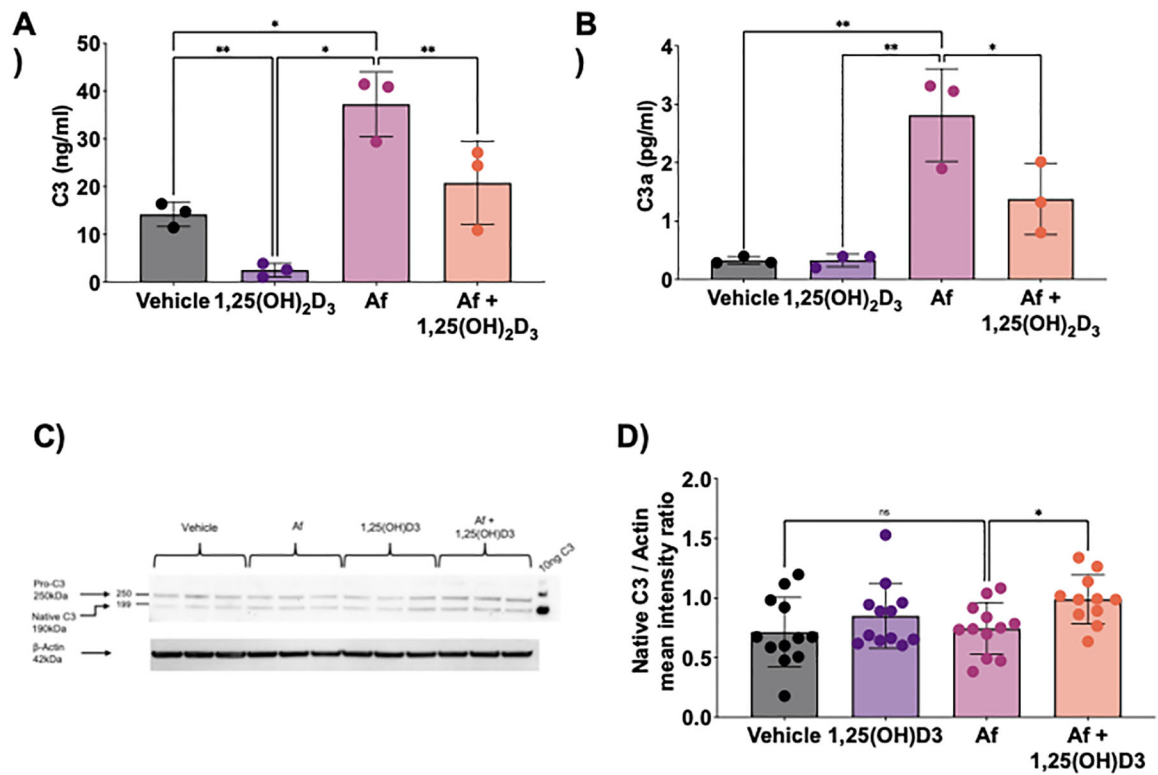
Figure 3: Endogenous C3 is blocked by addition of 1,25(OH)$_2$D$_3$.
Control HSNEC were treated with 100 pg/ml of 1,25(OH)$_2$D$_3$ for 24 hours with and without
Af. 24 hours after treatment, conditioned media was assessed by ELISA for **A)** C3, **B)**
C3a, and **C)** and **D)** HSNEC lysate assessed for C3 conformations. **A)** and **B)**. Note that
Af treatment induces a significant increase in C3 and C3a release that is inhibited in
1,25(OH)$_2$D$_3$. **C)**. Investigation of HSNEC lysates demonstrates the presence of two distinct
intracellular forms of C3, pro-C3 and native C3. **D)**. Densitometry analysis of native C3
shows a small but significant retention of C3 in Af plus 1,25(OH)$_2$D$_3$ treated HSNECs.
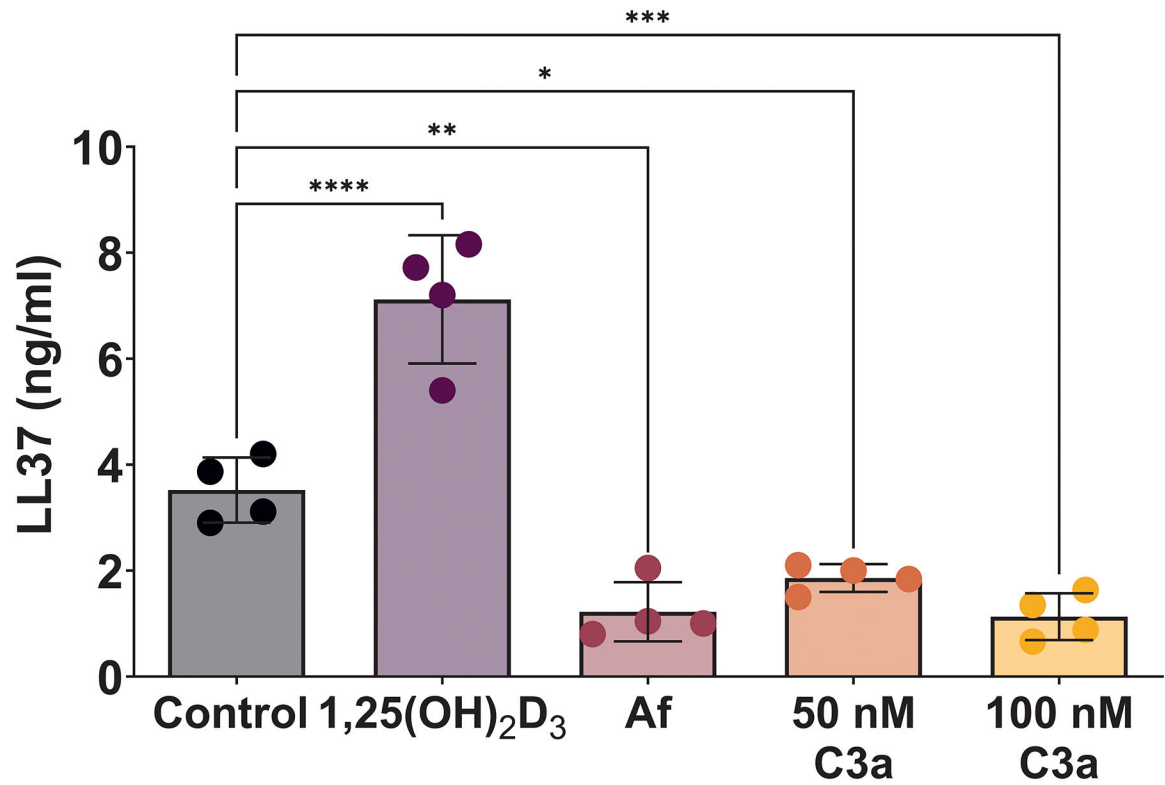
Figure 4: HSNEC increase secretion of LL37 in response to 1,25(OH)$_2$D$_3$, while Af and recombinant C3a reduce its secretion.

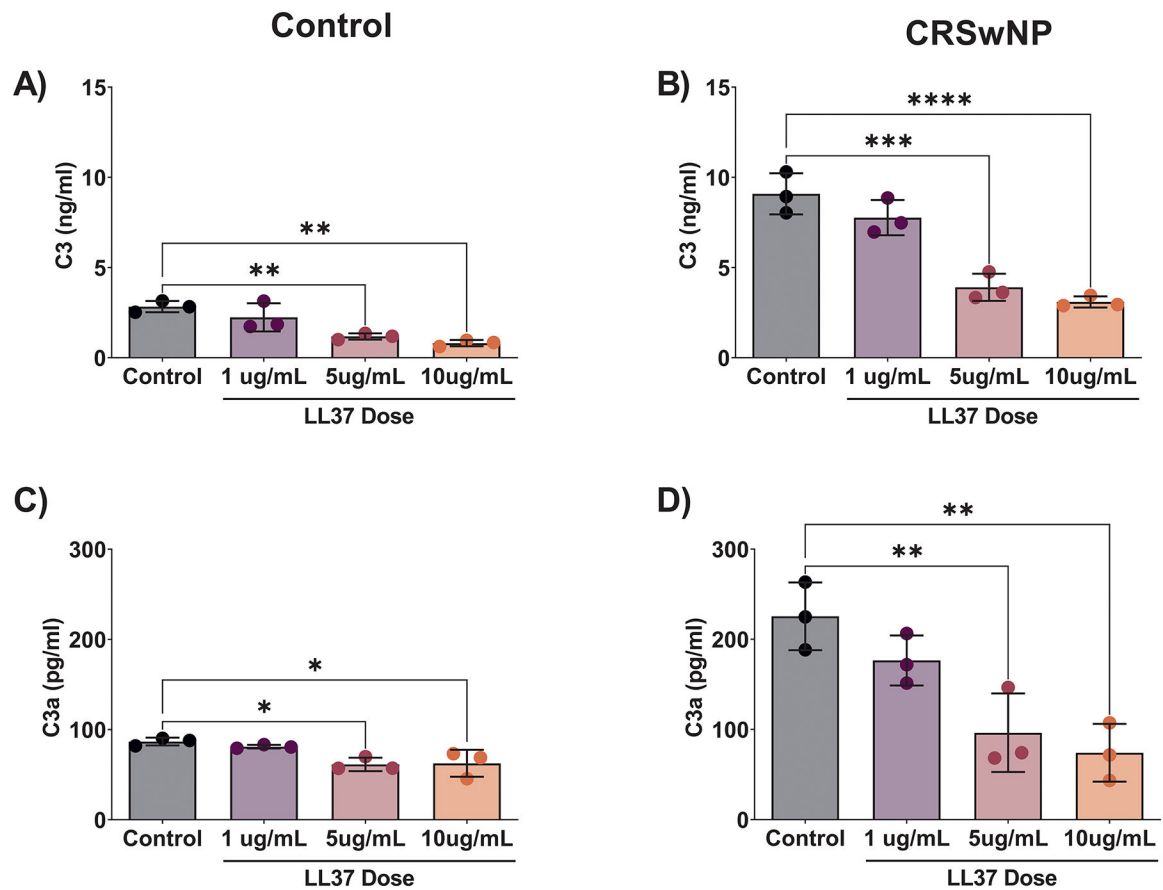Control HSNEC secrete LL37 in response treatment with 1,25(OH)D3, while treatment with Af or C3a suppress its release.

Figure 5: LL37 suppresses HSNEC secretion of C3 and C3a.

C3 and C3a secretion by control HSNEC **(A & C)** and by CRSwNP HSNEC **(B & D)** C3a were measured in supernatants from following treatment with LL37. Note that baseline C3 and C3a was elevated in CRSwNP HSNEC.
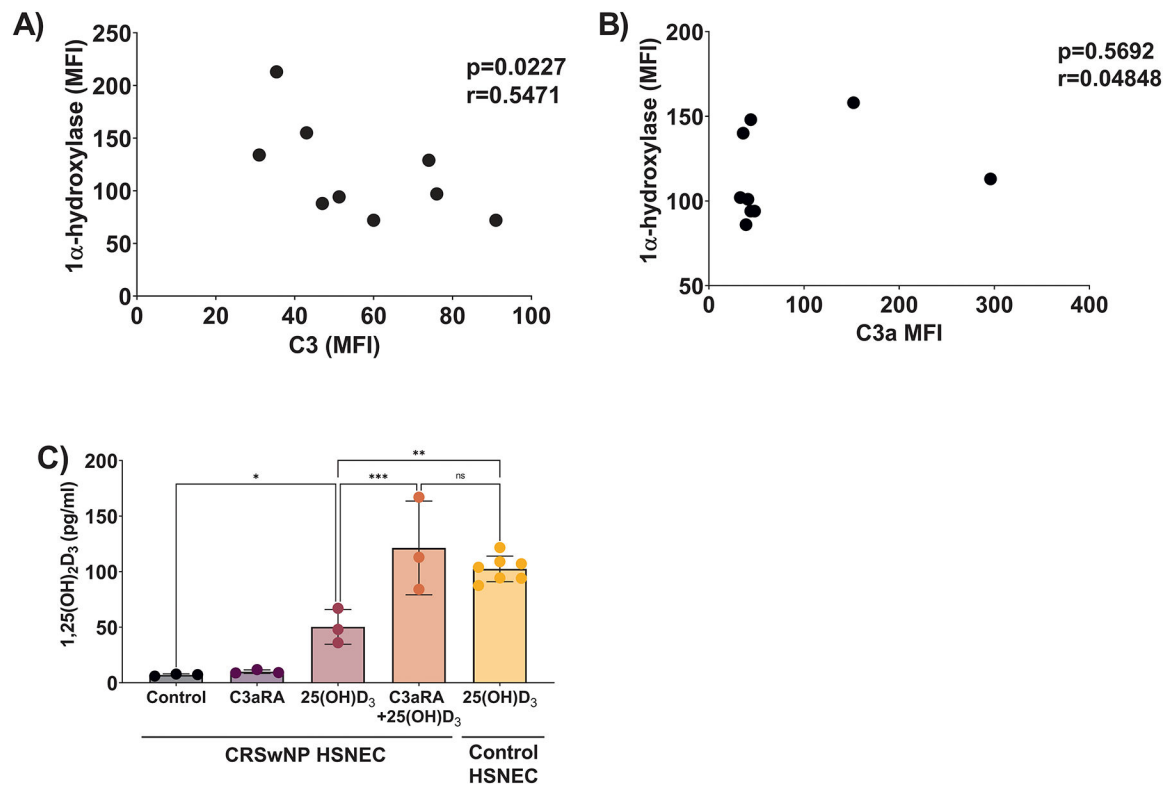
Figure 6: Inhibition of C3a signaling restores CRSwNP patient derived-HSNEC ability to metabolize 25(OH)D$_3$ to 1,25(OH)$_2$D$_3$.

Single cell suspensions of sinonasal tissues were examined for 1α-hydroxylase and either **A)** C3 or **B)** C3a. **C)** 25(OH)D$_3$ metabolism in CRSwNP samples is restored to levels similar to control-subject derived cells with C3aRA.
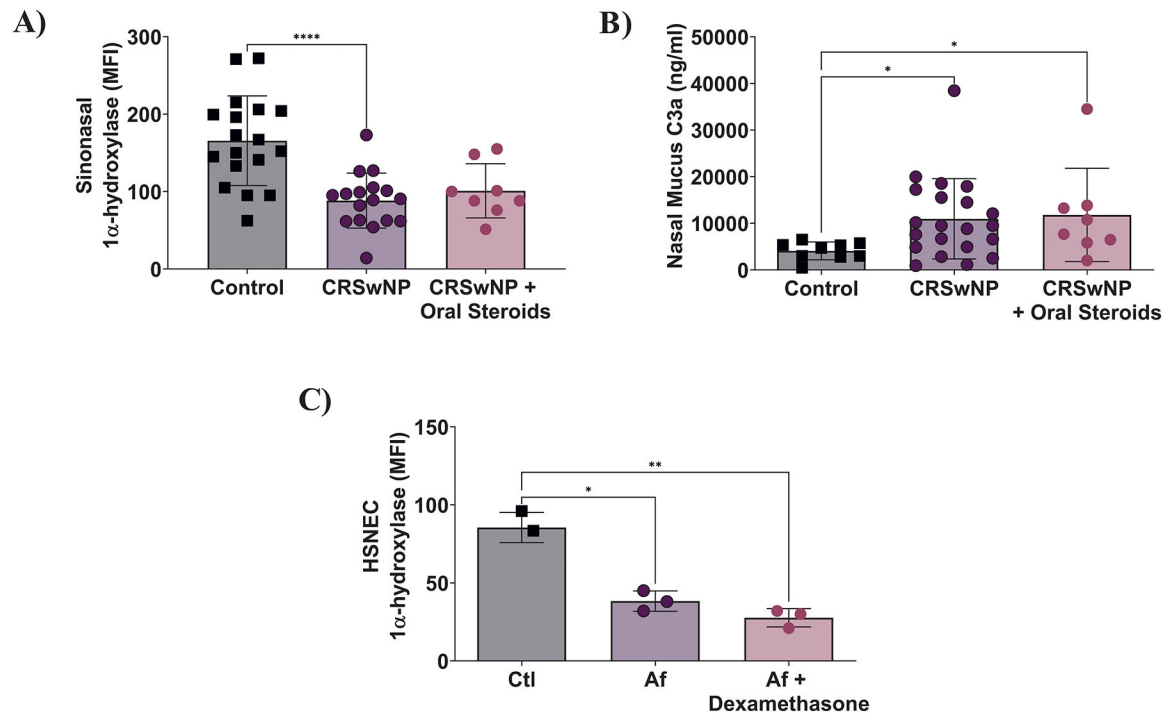
Figure 7: Steroids have no impact on 1α-hydroxylase expression or nasal mucus C3a.
A) Oral steroids had no impact on sinonasal tissue 1α-hydroxylase expression. **B)** Steroids have no impact on nasal mucus C3a levels. **C)** HSNEC treatment with dexamethasone does not block Af-induced suppression of 1α-hydroxylase.
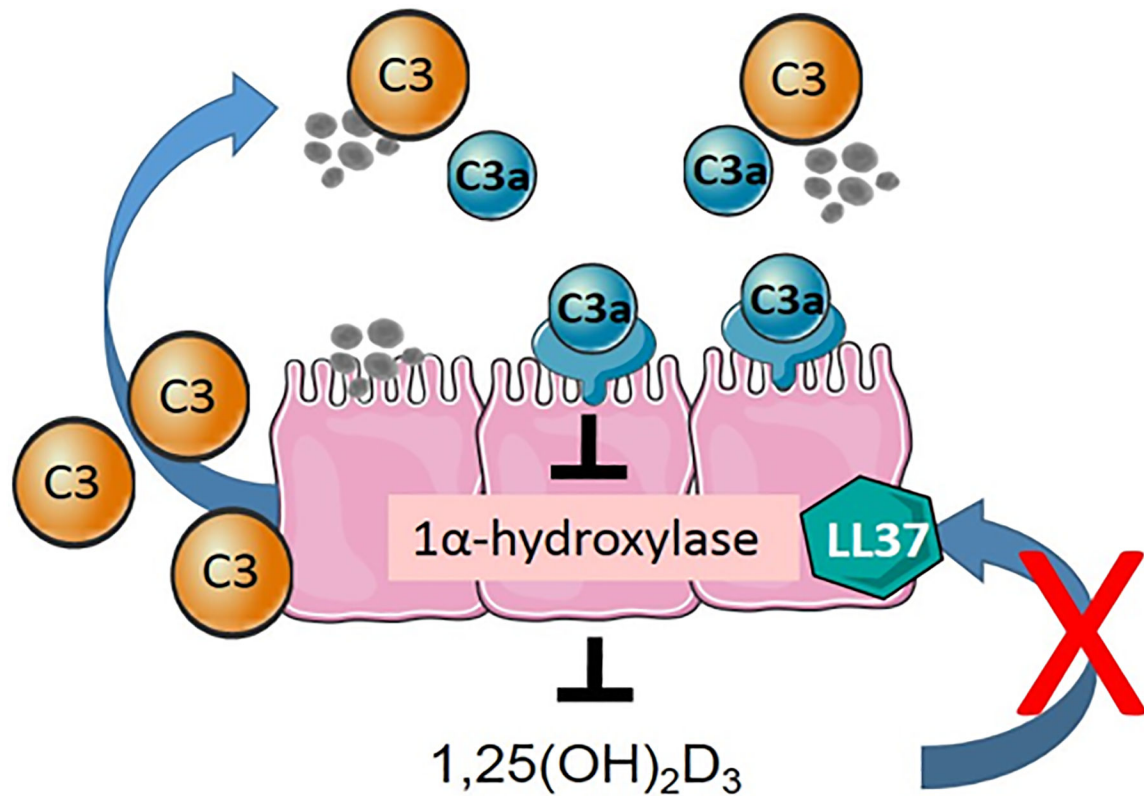
Figure 8: Results Summary.
Upon epithelial exposure to exogenous stimuli, such as *Aspergillus fumigatus* (Af), complement is released into the extracellular space where it is cleaved by inhaled antigens to form C3a. C3a in an autocrine manner binds to the C3aR on HSNEC, which causes the suppression of 1α-hydroxylase and subsequent metabolism of $25(OH)D_3$ to $1,25(OH)_2D_3$. Given prior reports that **1)** Af suppresses HSNEC LL37 section and **2)** complement regulates LL37 expression, we hypothesize that $1,25(OH)_2D_3$ may be responsible for the reciprocal interactions between antimicrobial peptide and complement expression.