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Super Resolution Microscopy Analysis Reveals Increased Orai1 Activity in Asthma and Cystic Fibrosis Lungs

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Abstract

Question: In diseases such as asthma and cystic fibrosis (CF), the immune response is dysregulated and the lung is chronically inflamed. Orai1 activation is required for the initiation and persistence of inflammation. However, Orai1 expression in the lung is poorly understood. We therefore tested the hypothesis that Orai1 expression was upregulated in asthmatic and CF lungs.

Materials and methods: We used LungMAP to analyze single-cell RNAseq data of Orai1 and stromal interaction molecule 1 (STIM1) expression in normal human lungs. We then performed RNAscope analysis and immunostaining on lung sections from normal, asthma, and CF donors. We imaged sections by confocal and super resolution microscopy, and analyzed Orai1 and STIM1 expression in different pulmonary cell types.

Results: Orai1 was broadly-expressed, but expression was greatest in immune cells. At mRNA and protein levels, there were no consistent trends in expression levels between the three phenotypes. Orai1 must interact with STIM1 in order to activate and conduct Ca^{2+}. We therefore used STIM1/Orai1 co-localization as a marker of Orai1 activity. Using this approach, we found significantly increased co-localization between these proteins in epithelia, interstitial and luminal immune cells, but not alveoli, from asthma and CF lungs. Orai1 also aggregates as part of its interaction process. Using super resolution microscopy, we also found significantly increased Orai1 aggregation in immune cells from asthmatic and CF lungs.

Conclusion: We found evidence that Orai1 was more active in asthma and CF than normal lungs. These data suggest that Orai1 is a relevant target for reducing pulmonary inflammation.

Summary:

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Author Contributions:

A.S. Goriounova, R.C. Gilmore and J.A. Wrennall performed experiments. A.S. Goriounova and R. Tarran analyzed data. A.S. Goriounova and R. Tarran designed experiments and wrote the manuscript. All other authors edited and approved the manuscript.

Disclosures

RT is a founder of Eldec Pharmaceuticals, has equity in this company and serves as its President and Chief Scientific Officer. RT is also listed as an inventor on intellectual property related to Orai1 inhibition. The other authors have nothing to disclose.

Orai1 is a key regulator of inflammation in multiple cell types. Using super resolution microscopy, we found that Orai1 was activated in asthma and cystic fibrosis lungs. We propose that Orai1 is a clinically useful biomarker of inflammation.

Keywords

Cystic fibrosis; asthma; inflammation; Orai1/STIM1; RNAscope; super resolution microscopy

1. Introduction

Orai1 is a plasma membrane Ca^{2+} channel that is involved in store operated calcium entry (SOCE) [1]: Depletion of Ca^{2+} from the endoplasmic reticulum (ER) activates SOCE by causing STIM1 to move to ER/plasma membrane junctions and form oligomers. These oligomers interact with Orai1 causing it to also oligomerize, form puncta, and activate, permitting SOCE and an increase in cytosolic Ca^{2+} that serves to initiate cellular events [1]. Orai1/SOCE are activated by G_q-linked G-protein-coupled receptors. In pulmonary cells, SOCE regulates gene expression and stimulates cytokine, mucin, and protease secretion [2, 3]. Activation of Orai1 results in the recruitment of neutrophils to the lungs [4]. Orai1 activation is also essential for T cell activation, irrespective of the stimuli, and is upstream of transcription factors like Nuclear Factor of Activated T cells (NFAT), which facilitate the onset of inflammation [5]. In airway epithelia, SOCE can regulate ciliary beating and Cl^- secretion [6]. The importance of Orai1 to the immune system is further illustrated by patients with global loss-of-function Orai1 mutations. These mutations (e.g. $Orai1^{R91W}$), are immunosuppressive and cause severe combined immune deficiency syndrome, which is characterized by recurring pneumonia and sepsis [7]. Thus, appropriate regulation of SOCE is important for maintaining a robust, but not excessive, immune response.

Cystic fibrosis (CF) is an autosomal recessive genetic disease which affects approximately 70,000 people worldwide [8]. CF is characterized by mutations in the CF transmembrane conductance regulator (CFTR) anion channel [9, 10]. Disease-causing CFTR mutations lead to persistent lung infections and neutrophil-dominated pulmonary inflammation that destroy the lung [10]. SOCE is upregulated in immortalized CF cell lines and leads to increased secretion of the neutrophil chemoattractant IL-8 [11]. Asthma is another chronic lung disease characterized by persistent inflammation that affects ~260 million people worldwide [12]. In asthma, Type-2 helper T lymphocytes initiate the release of pro-inflammatory cytokines, such as IL-13, which results in inflammatory immune cell (e.g. eosinophils and neutrophils) infiltration into the lung [12]. The excessive immune cell infiltration leads to abnormal airway smooth muscle function and causes progressive lung damage [12, 13].

Orai1 has been proposed as a therapeutic target to treat inflammatory diseases [14]. Indeed, inhibition of Orai1 either with shRNA, antibodies, or inhibitory peptides, decreased pulmonary inflammation in mice [15–17]. Orai1 has recently been clinically validated, and a small molecule Orai1 antagonist was successfully used to treat severe COVID19 patients [16]. Despite the importance of Orai1 to inflammation and the immune response, information on Orai1 expression in the lung is sparse and is limited to studies of individual

cell types [18–20]. Orai1 is a validated drug target and understanding its expression in the lung is important to inform drug development processes. We therefore performed a full analysis of Orai1 expression in the lung. Since Orai1 is predicted to be upstream of both Th-1 and Th-2 inflammation, we also compared Orai1 expression in two lung diseases with different inflammatory stimuli (asthma and CF).

2. Materials and Methods

2.1 Lung tissue sections

All sections were acquired from UNC Marsico Lung Institute's Tissue Procurement Core which collects tissue samples under an approved IRB (99–0615). All lung tissue sections were from post-mortem or post-transplant donors that had been de-identified and biobanked. An equal number of male/female sections (4M/4F) were used for each group (normal, asthma, and CF; supplementary table S2). We selected regions from asthmatic and CF lungs that exhibited significant lung luminal immune cell infiltration as a marker of local inflammation. Based on this criterion, asthma and CF tissues had substantial amounts of neutrophilic inflammation, whereas normal tissues lacked neutrophils and/or significant luminal immune cells.

2.2 RNA *in situ* hybridization

In situ RNA detection was performed using the RNAscope® Multiplex Fluorescent Reagent Kit and a HybEZ oven (Advanced Cell Diagnostics Inc. Hayward, CA) per manufacturer's instructions.

2.3 Immunostaining

Paraffin-embedded sections were immunostained as described [17] and imaged using a Leica SP8 confocal microscope either in normal or super resolution mode using the Leica LAS-AF software package with Lightning deconvolution enabled.

2.4 Peripheral blood neutrophil collection and isolation

Blood was drawn from antecubital veins of volunteers (5 normal/5 CF; supplementary table S3) and collected in a K2-EDTA vial under an approved IRB (UNC-20–1765). Neutrophils were isolated using the EasySep Direct Human Neutrophil Isolation Kit (Stem Cell Technologies; 19666) with immunomagnetic negative isolation of neutrophils from whole blood per the manufacturer's protocol.

2.5 Statistical Analysis

Statistical analysis was performed using Graph Pad Prism software. For comparison between multiple groups, one-way ANOVA with Tukey's multiple comparisons post-test, Kruskal-Wallis tests with Dunn's multiple comparisons post-test, or repeated measures two-way ANOVA were used as appropriate. For comparison between 2 groups, the Mann-Whitney test was used. For statistical comparison, no outliers were removed. However, data were visually compared with and without outliers to assess their impact on the means. Data were represented as mean ± standard deviation and $p<0.05$ was considered significant.

3. Results

3.1 Single cell RNAseq expression of Orai1 and STIM1 RNA in normal human lungs

Little is known about Orai1 expression in the lung. To address this deficiency, we studied Orai1 and STIM1 expression in normal human lungs by mining single-cell RNA sequencing (scRNA-seq) data deposited in LungMAP (http://app.lungmap.net/app/shinycell-lung-cell-cards-human-mnn; figure 1a). This dataset used transcriptomes from whole lung digests [21]. 259,565 cells contributed to the dataset and both Orai1 and STIM1 were expressed in at least one cell of each cell type present in the lung (figures 1b, c; supplementary table 1). Overall, STIM1 was expressed in more total lung cells than Orai1 (19.58% vs 9.43%, supplementary figure S2a, b). Orai1 was highly expressed in mucous cells (36.83% of cells analyzed), secretory cells (30.45%), serous cells (28.99%), ionocytes (26.24%) and goblet cells (25.01%) and to a lesser extent in ciliated cells and immune cells (figure 1d and supplementary figure S1a). STIM1 was also expressed in these cell types, but tended to be more highly expressed in immune cells than in specialized airway cells (figure 1e and supplementary figure S1b). Of the cells that expressed Orai1, 64.79% also expressed STIM1, and the greatest degree of co-expression was observed in immune cells (figure 1f; supplementary table 1).

3.2 Orai1 and STIM1 mRNA expression in normal, asthma, and CF lungs

To study Orai1 expression in patients with inflammatory diseases, we compared Orai1 and STIM1 gene expression in proximal and distal lung sections (proximal and distal lung sections from each donor were mounted on separate slides) from normal, asthma, and CF donors using RNAscope *in situ* hybridization. Since the scRNA-seq analysis indicated that STIM1 and Orai1 were expressed in multiple regions of the lung (figure 1), we focused our analysis on immune cells, epithelia, interstitia and alveoli. Based on gross visual appearance and α-tubulin staining for epithelial cells, we selected four regions of interest to be analyzed per donor that contained (i) interstitial, (ii) alveolar, (iii) epithelial and (iv) luminal immune cells. To validate our approach, we used the manufacturer's positive and negative controls (See Online Methods). The positive control probe was detected in our samples and the negative control probe was not detected (supplementary figure S2a). As per the scRNA-seq data, Orai1 mRNA was detected in all regions of the lung (figure 2a). STIM1 was more highly expressed than Orai1 in epithelial, interstitial, and alveolar cells. Epithelial cells had higher STIM1 and Orai1 mRNA expression than interstitial or alveolar cells. When normalized to area of analysis, immune cells had significantly more Orai1 and STIM1 mRNA expression than other cell type (supplementary figure S3). Orai1 was upregulated in asthma-derived epithelia (figure 2b), but not in any other region from asthmatic lungs, or in CF lungs (figures 2c–e). STIM1 mRNA was not upregulated in either asthma or CF epithelia (figure 2f). However, STIM1 mRNA was upregulated in both asthma and CF interstitial cells (figure 2g), and was upregulated in asthma-derived alveolar and immune cells (figures 2h–i).

3.3 Orai1 and STIM1 protein expression in normal, asthma, and CF lungs

Since Orai1 and STIM1 mRNA expression were similar between normal, asthma, and CF lungs, we next compared Orai1 and STIM1 protein expression in the same lung sections (figure 3a). Both STIM1 and Orai1 could be readily detected in all samples, while

STIM1 and Orai1 isotype controls did not show any expression (supplementary figure S2b), suggesting that our antibody staining was specific. Unlike Orai1 mRNA, Orai1 protein was downregulated in asthma epithelia compared to normal or CF epithelia (figure 3b). Orai1 protein expression was also downregulated in asthma interstitial cells (figure 3c), while Orai1 protein expression was at similar levels to normal for both diseases in alveolar cells (figure 3d). In contrast, Orai1 expression was greater in asthma-derived pulmonary immune cells than in CF cells (figure 3e and supplementary figure S3). STIM1 expression was downregulated in both asthma and CF epithelia (figure 3f) and in CF alveoli (Fig 3h), but was not altered in interstitial or immune cells (figures 3g, i).

3.4 Orai1-STIM1 colocalization is increased in asthma, and CF airways

Orai1 expression was inconsistently changed at protein and mRNA levels (figures 2 and 3). However, STIM1 must directly interact with Orai1 in order to activate SOCE [22]. Therefore, we next looked for Orai1/STIM1 colocalization as a marker of Orai1 activation. In epithelial, interstitial, and immune cells, Orai1/STIM1 colocalization was significantly greater in asthma and CF than normal samples (figures 4b, c, and e), while Orai1/STIM1 colocalization was not significantly different between groups in alveolar cells (figure 4d). The greatest degree of colocalization was seen in immune cells (supplementary figure S5). As a control, we used Orai1/α-tubulin (cilia) colocalization and saw minimal colocalization for all lung sections analyzed (figure 4f).

3.5 Orai1/STIM1 puncta are larger in asthma and CF airways and immune cells

STIM1 and Orai1 molecules aggregate into puncta as a prerequisite for Orai1 activation [22, 23]. Since we observed the greatest Orai1/STIM1 colocalization in asthma and CF lung immune cells, we evaluated whether Orai1 puncta formation was also increased. To validate that increased puncta size equates with activation, we first treated isolated blood neutrophils from normal and CF patients with thapsigargin, a Sarcoplasmic/Endoplasmic Reticulum Calcium ATPase (SERCA) pump inhibitor that stimulates puncta formation and activation [23]. Neutrophils were stained for Orai1 and imaged using super resolution microscopy (~30 nm resolution; figure 5a, supplementary figure S6). Interestingly, CF neutrophils had significantly larger puncta than normal neutrophils at baseline, which suggested that CF neutrophil Ca^{2+} signaling was dysregulated (figure 5b). Irrespective of genotype, thapsigargin-treated neutrophils had significantly larger Orai1 particles, indicating puncta formation and Orai1 activation (figure 5b).

We then used super resolution microscopy to image lung immune cells (figure 5c). DAPI was used to stain nuclei and identify macrophages/monocytes and neutrophils. We initially focused on macrophages because they were the only immune cell types found abundantly in all three phenotypes. Both Orai1 (figure 5d) and STIM1 (figure 5e) puncta were significantly larger in macrophages derived from asthma and CF patients than those from normal subjects. While neutrophils were sparce in normal lungs, CF neutrophils were abundant. Orai1 puncta in CF lung neutrophils appeared larger than normal neutrophils ($p = 0.069$, Mann-Whitney), however insufficient NL neutrophils were identified for a robust analysis (figure 5f).

4. Discussion

We have previously demonstrated that both STIM1 and Orai1 were expressed in non-polarized primary human bronchial epithelia and that knockdown of either protein abolished SOCE [24]. Orai1 was also expressed in airway smooth muscle from humans and mice [18, 25]. These data are consistent with earlier studies which demonstrated the presence of SOCE in pulmonary cells [2, 3]. However, Orai1 expression has not systematically been evaluated in the lung. We therefore evaluated Orai1 expression using LungMAP scRNAseq data. Cytosolic Ca^{2+} triggers secretion of mucins, cytokines and anions [26, 27]. Consistent with these findings, we found that Orai1 was highly expressed in pulmonary cells associated with either protein and/or salt secretion (figure 1, table S1). Surprisingly, despite good evidence for STIM1 and Orai1 expression in airway smooth muscle [18, 25], Orai1 was expressed at very low levels in these cells at the scRNAseq level (figure 1). scRNAseq is a new technique that relies upon cell isolation before analysis and this procedure may not be fully optimized for every cell type. However, STIM1 and Orai1 showed a similar pattern of expression (figure 1f), suggesting that key components of the machinery required for functional SOCE are expressed in most pulmonary cell types, as well as in immune cells.

We then looked for STIM1 and Orai1 mRNA expression using the RNAscope technique, which allows for *in situ* hybridization of RNA in paraffin-embedded sections. Using this approach, we again found that STIM1 and Orai1 were broadly-expressed in the lungs (figure 2). With RNAscope, we were able to take advantage of an existing library of asthma and CF lungs. Asthma and CF are two very different pulmonary diseases that are characterized by Th2 allergic inflammation and Th1-type inflammation respectively [17, 28]. However, Orai1 activation is predicted to be applicable to both diseases [29]. In general, we found that STIM1, but not Orai1, expression was different between groups (figure 2). In all three phenotypes, both Orai1 and STIM1 mRNA were most highly expressed in immune cells (supplementary figure S3). This is partially contrasted by the scRNA-seq data from LungMAP in which Orai1 was more highly expressed in ciliated and basal cells than macrophages. However, the scRNA-seq data aligns with the STIM1 RNAscope data, with macrophages having the second-highest STIM1 expression, behind only dendritic cells. Therefore, it is likely that RNAscope is more reliable when studying individual subjects, but lacks the comprehensiveness and high through-put capability of scRNA-seq.

Next, we evaluated Orai1 and STIM1 protein expression by immunohistochemistry. We detected robust expression of both STIM1 and Orai1 (figure 3) but not the isotype controls, indicating antibody specificity (supplementary figure S2). Unlike the mRNA expression where STIM1 had more differences between groups than Orai1, protein expression between groups varied more with Orai1 than STIM1 (figure 3). Overall, Orai1 expression was highest in immune cells, but STIM1 was equally expressed in epithelia and immune cells for all three phenotypes. We found that any potential outliers trended in the positive direction and the removal of outliers from data analysis had little effect on significance (supplementary figure S4). Further, we speculate that these points of higher expression may be hot spots of greater inflammation and an increased sample size in future studies will provide more insight into the range of Orai1 expression levels in asthma and CF.

We did not detect consistent trends in Orai1 expression levels between diseases. However, in order for SOCE to occur, STIM1 must relocate to the ER-plasma membrane junction where it directly interacts with and stimulates Orai1. Indeed, colocalization between Orai1 and STIM1 has been shown to increase during SOCE [1] and when STIM1 is knocked down, SOCE no longer occurs [24]. Preformed Orai1-STIM1 complexes in unstimulated T-cells have been reported [30]. However, these complexes were active and enabled local calcium entry in the unstimulated cells. Thus, the assumption is that the vast majority of Orai1-STIM complexes are activated and can conduct Ca^{2+}. We next looked for co-localization between STIM1 and Orai1. Using Mander's overlap coefficient, we found that STIM1/Orai1 co-localization was significantly greater in asthmatic and CF epithelia, interstitial and immune cells as compared to normal controls (figure 4a–c, f). There were only 3 potential outliers for colocalization comparison and they had no effect on significance (supplementary figure S5). Moreover, the degree of co-localization was not different between the asthma and CF cells, suggesting similar levels of Orai1 activation. Asthma and CF are airway rather than alveolar diseases, and consistent with this, we saw no difference in STIM1/Orai1 co-localization in alveolar cells (figure 4d).

Super resolution imaging exceeds the resolution limits imposed by the wavelengths of light (typically ~250 nm) and with our system, yields ~30 nm resolution (supplementary figure S6). This technique has previously been used to study STIM1/Orai1 puncta in HeLa and T cells, suggesting that the approach is valid [30, 31]. We confirmed that Orai1/SOCE activation correlated with increased puncta size in peripheral blood neutrophils from normal and CF subjects (figure 5). There is evidence that peripheral blood neutrophil phenotype reflects pulmonary inflammation in CF patients [32–34]. The Vertex CFTR modulator/corrector combination therapy Trikafta increases CFTR function in CF patients [35]. All CF subjects that we studied were undergoing Trikafta treatment, but under baseline conditions, there were significantly more puncta in CF neutrophils than in normal neutrophils, suggesting that these patients continued to have significant inflammation despite Trikafta treatment. Indeed, the mean percent predicted FEV_1 in our cohort was 55±22% (supplementary table S3), indicating that these patients had persistent lung disease.

We then used super resolution microscopy to look at our lung sections. Immune cells were readily identifiable in the lung lumens of all three phenotypes. As expected, based on visual analysis, macrophages and/or monocytes were prevalent in normal epithelia, while neutrophils were common in CF lung lumens and neutrophils/eosinophils were found in asthma lungs. Indeed the exclusion criterion for normal patients was that they had no inflammatory disease, and the inclusion criterion for asthma and CF patients was that they had a severe presentation of their respective disease. Due to commonality, we compared macrophages from each group and found that Orai1 and STIM1 puncta were significantly larger in asthma and CF macrophages (figure 5d, e). Robust comparison of lung neutrophils between phenotypes was not possible due the sparce presence of neutrophils in NL lungs and difficulties delineating asmatic neutrophils and eosinophils, Orai1 particle size in CF neutrophils trended towards a larger size than those observed in NL lungs ($p = 0.069$; Mann-Whitney) (figure 5f). Orai1/SOCE activation is directly upstream of the pro-inflammatory transcription factors such as NFAT [5]. Thus, based on the co-localization and super resolution data, we conclude that Orai1 is more active in asthmatic and CF than normal

lungs. However, for the asthma and CF patients, we cannot rule out the possible contribution of other inflammatory comorbidities to the increased Orai1 activation.

The CF lung is subject to exuberant and poorly-resolving inflammatory responses, typified by the over recruitment of neutrophils and high levels of proteases and pro-inflammatory cytokines [10]. Immune cell recruitment and cytokine release are disproportionate to bacterial load, indicating dysregulation of the inflammatory response [36]. The mechanisms underlying the hyper-inflammatory state of the CF lung are not fully understood. However, dysregulated Ca^{2+} homeostasis may play a role: CF airway epithelia display elevated Ca^{2+} levels and increased secretion of IL-8, a pro-inflammatory cytokine central to the recruitment of neutrophilia [11]. CF T cells display exaggerated Ca^{2+} signaling upon activation, which promotes cytokine release via NFAT [37]. Ca^{2+} homeostasis was also elevated in neutrophils from CF mice, which was associated with impaired bacterial killing. Here, the increase in intracellular Ca^{2+} correlated to significantly decreased NADPH oxidase response, which impaired neutrophil extracellular trap formation [38]. Thus, we posit that the STIM1/Orai1/SOCE axis is an important, but under-appreciated, player in the CF inflammatory response that is convergent for multiple inflammatory pathways and which may contribute to the hyperinflammation seen in CF patients [11].

Orai1 regulates airway smooth muscle proliferation and contraction [18, 25]. However, beyond this, little is known about STIM1/Orai1 expression levels in asthmatic lungs. STIM1/Orai1 have the potential to drive aberrant/pro-inflammatory cell signaling in asthmatic immune cells, airway smooth muscle and epithelia. While we did not specifically stain for airway smooth muscle, we analyzed broad sections of the interstitial region, which included airway smooth muscle cells. Future experiments would benefit from staining with markers specific to airway smooth muscle, and/or different immune cell types. Indeed, our data indicate that while Orai1 expression may or may not be upregulated in asthma airways, Orai1 activation (i.e. STIM1/Orai1 co-localization and puncta formation) is increased. As with CF airways, these increases as predicted to lead to more cytokine production (albeit Th2-mediated) and greater airway smooth muscle proliferation/contraction. Ca^{2+} regulatory genes including Orai1 were upregulated in peripheral blood monocytes from both allergic and non-allergic pediatric asthma patients [39], suggesting that as with CF (figure 5a, b) altered Ca^{2+} signaling extends beyond the pulmonary system in asthma patients. Allergens have been shown to stimulate SOCE and cytokine production in airway epithelia [40], and the 5-fold increased Orai1-STIM1 co-localization in asthmatic airways would suggest an exaggerated epithelial inflammatory response to allergens.

Orai1 puncta formation, which we propose is an upstream biomarker of inflammation, is increased in asthma and CF patients' lungs. However, additional studies should be performed in order to replicate these data and look for correlations between Orai1 puncta formation and disease severity, medication status, lung function, race, gender etc. The super resolution microscopy technique is not particularly time consuming, so performing a larger study is feasible. In addition to regulating inflammation, Orai1 also controls cell proliferation, and Orai1 expression in lungs inversely correlates with non-small cell lung cancer survival rates. Moreover, *ex vivo*, Orai1 inhibition reduces proliferation of non-small cell lung cancer cells [41]. Thus, beyond asthma and CF, using STIM1/Orai1 co-localization

or Orai1 puncta formation to better understand Orai1 activation may lead to improved diagnostics and predictors of survival for this cancer. A limitation of our study is the lack of functional data on actual Ca^{2+} influx (i.e. SOCE) in asthmatic and CF airways. However, since Orai1 has a unique mechanism of activation that requires changes in its cellular localization, it is highly likely that Orai1 is indeed more active in asthmatic and CF lungs. While functional studies can only be performed on freshly-isolated cells, more studies should be performed to better correlate Ca^{2+} influx with our immunohistochemical data.

Inhibition of Orai1 has been proposed therapeutically for a number of inflammatory diseases [17, 42]. Inhibition of Orai1 with a novel antibody has recently been shown to reduce mast cell degranulation and prevent T cell activation in mice [15]. Moreover, we have recently developed a novel, inhaled Orai1-inhibitory peptide that inhibits SOCE in a number of relevant human immune cell types including neutrophils, alveolar macrophages and mast cells, and reduces inflammation in house dust mite-extract-sensitized mice [17]. Legitimate concerns exist regarding Orai1 inhibition as a therapy due to the powerful effects that Orai1 can exert on the immune system. However, Auxora (CM4620), a small molecule injectable Orai1 antagonist was recently shown to be beneficial in patients with severe COVID-19, providing clinical evidence that Orai1 inhibition is beneficial against severe pulmonary inflammation [16]. Importantly, our data suggest that (i) Orai1 is broadly-expressed in the lungs, and (ii) since Orai1 is more active (i.e. increased STIM1-Orai1 co-localization) in epithelia, interstitial and immune cells that inhibition of Orai1 and/or STIM1 are valid targets for treating inflammatory lung diseases.

In conclusion, this is the first comprehensive analysis of Orai1 and STIM1 gene and protein expression in lungs from normal, asthma, and CF donors. Using super resolution microscopy, we have shown that Orai1 activation is upregulated in asthma and CF donors. This novel application of super resolution microscopy has the potential to be used in clinical settings for the analysis of *ex vivo* patient samples and an evaluation of a patient's inflammation. Although traditional biomarkers of inflammation, such as serum cytokine levels, are broadly useful for rapid detection of systemic inflammation, our technique allows for the localization of upstream inflammatory signaling at the cellular level. This would facilitate research into the pathogenesis of inflammatory diseases and potentially allow for the development of personalized immunomodulatory therapies via the identification of key target cell populations. These data can also be used to inform Orai1 drug development by providing critical information regarding Orai1 expression in inflammatory diseases. Additionally, super resolution microscopy of lung immune cells can also be used to compare Orai1 activation before and after treatment with a drug candidate. Therefore, we propose that these data serve as proof of principle regarding the applicability and feasibility of using Orai1 puncta formation and super resolution microscopy to identify inflammation in human lungs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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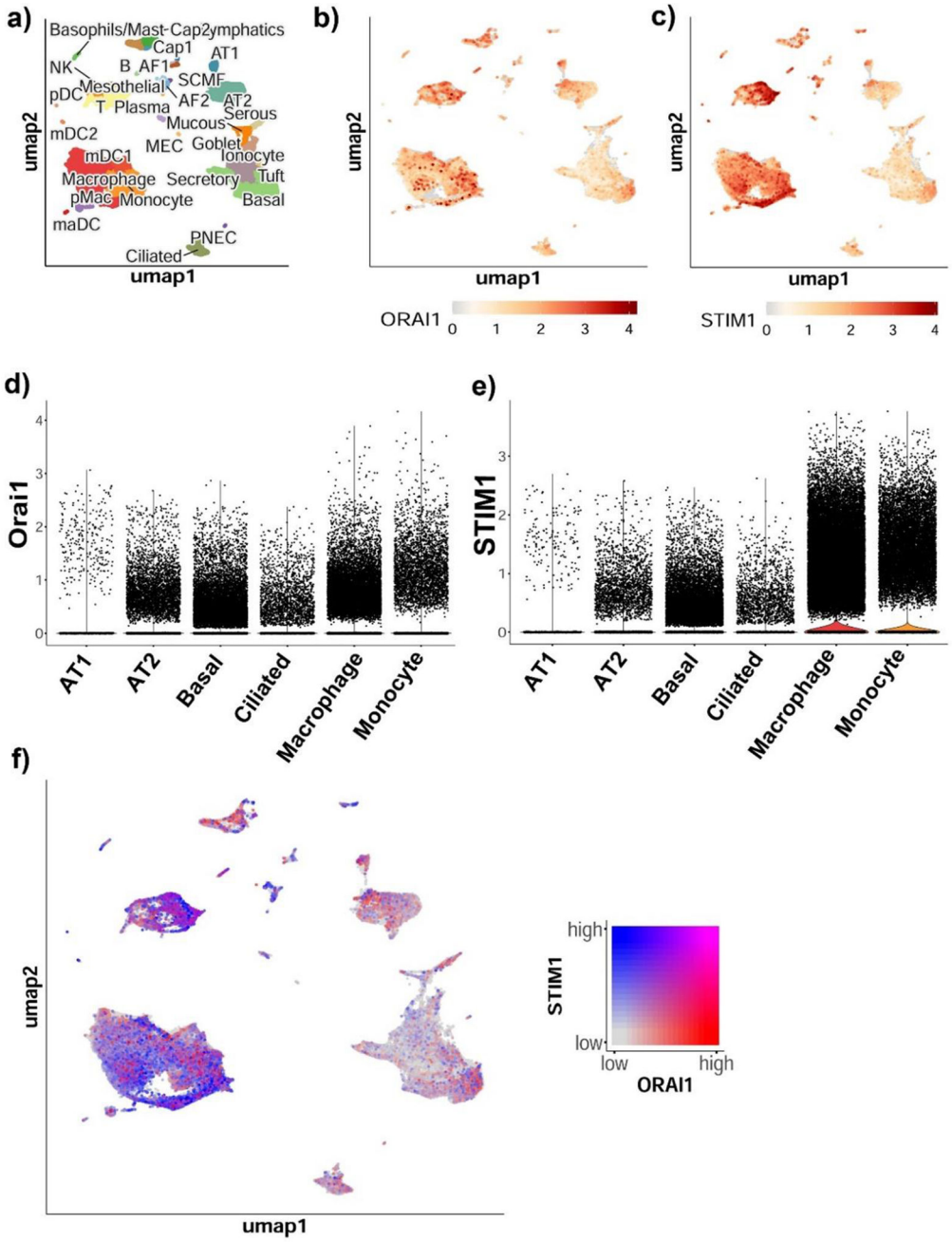

Figure 1: Single cell RNAseq analysis indicates that Orai1 and STIM1 are broadly expressed in the lung.

(a) Reference UMAP (uniform manifold approximation and projection) depicting labeled cell type clusters. UMAP of **(b)** Orai1 and **(c)** STIM1 showing lung cell mRNA distribution. Violin plots of **(d)** Orai1 and **(e)** STIM1 mRNA frequency in select cell types. **(f)** UMAP showing Orai1/STIM1 co-expression in lung cell type clusters.

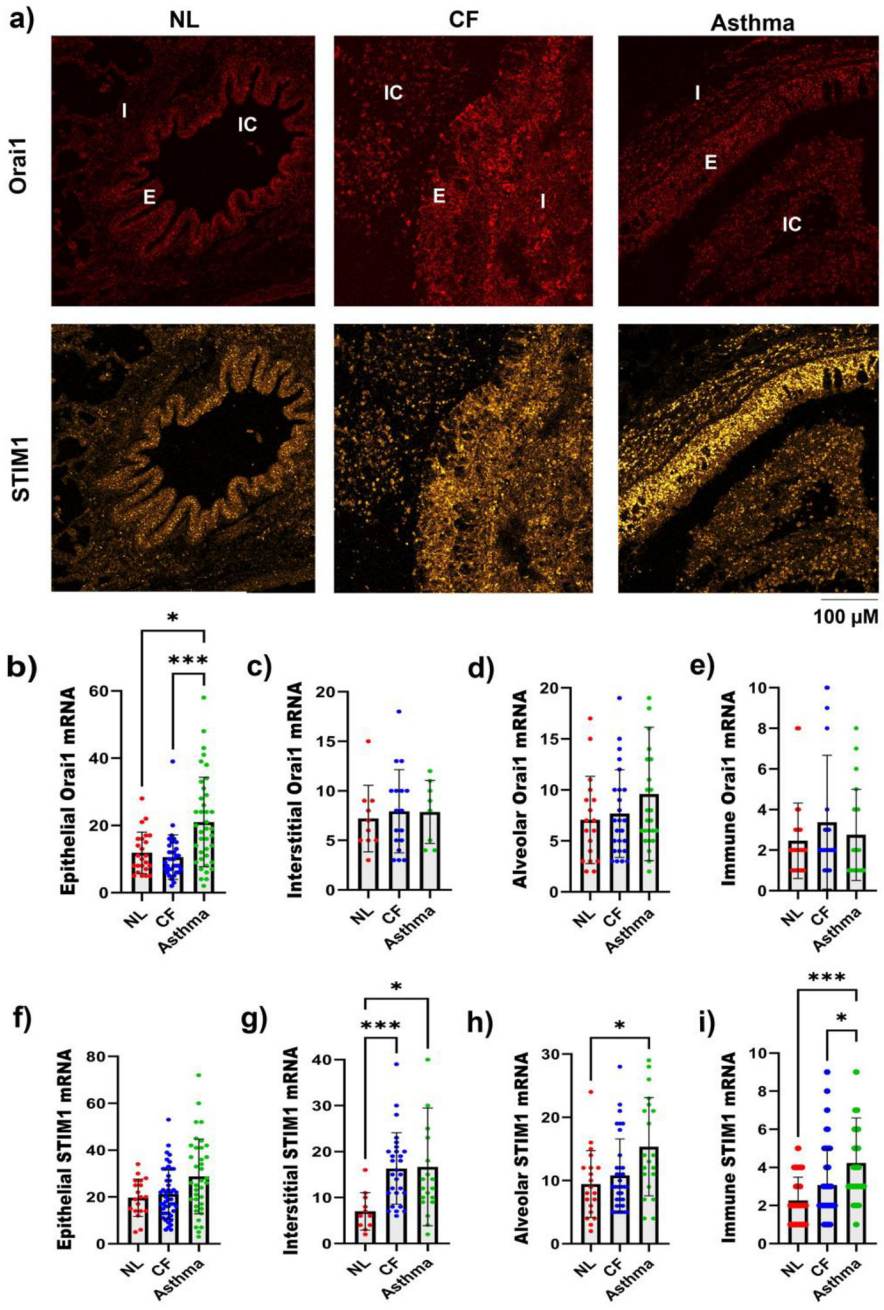

Figure 2: Orai1 and STIM1 mRNA expression is upregulated in some regions of asthma and CF lung.

(a) Representative images of Orai1 (red) and STIM1 (yellow) mRNA from normal (NL), CF, and asthma lungs. I, interstitial; E, epithelia; IC, immune cell. Orai1 mRNA count in **(b)** epithelial, **(c)** interstitial, **(d)** alveolar, and **(e)** immune cells. STIM1 mRNA count in **(f)** epithelial, **(g)** interstitial, **(h)** alveolar, and **(i)** immune cells. *=p<0.05, ***=p<0.001, ****=p<0.0001. Each data point represents an analyzed region. Data shown as mean ± SD. Data were analyzed using Kruskal-Wallis ANOVA. N=8 subjects per group.

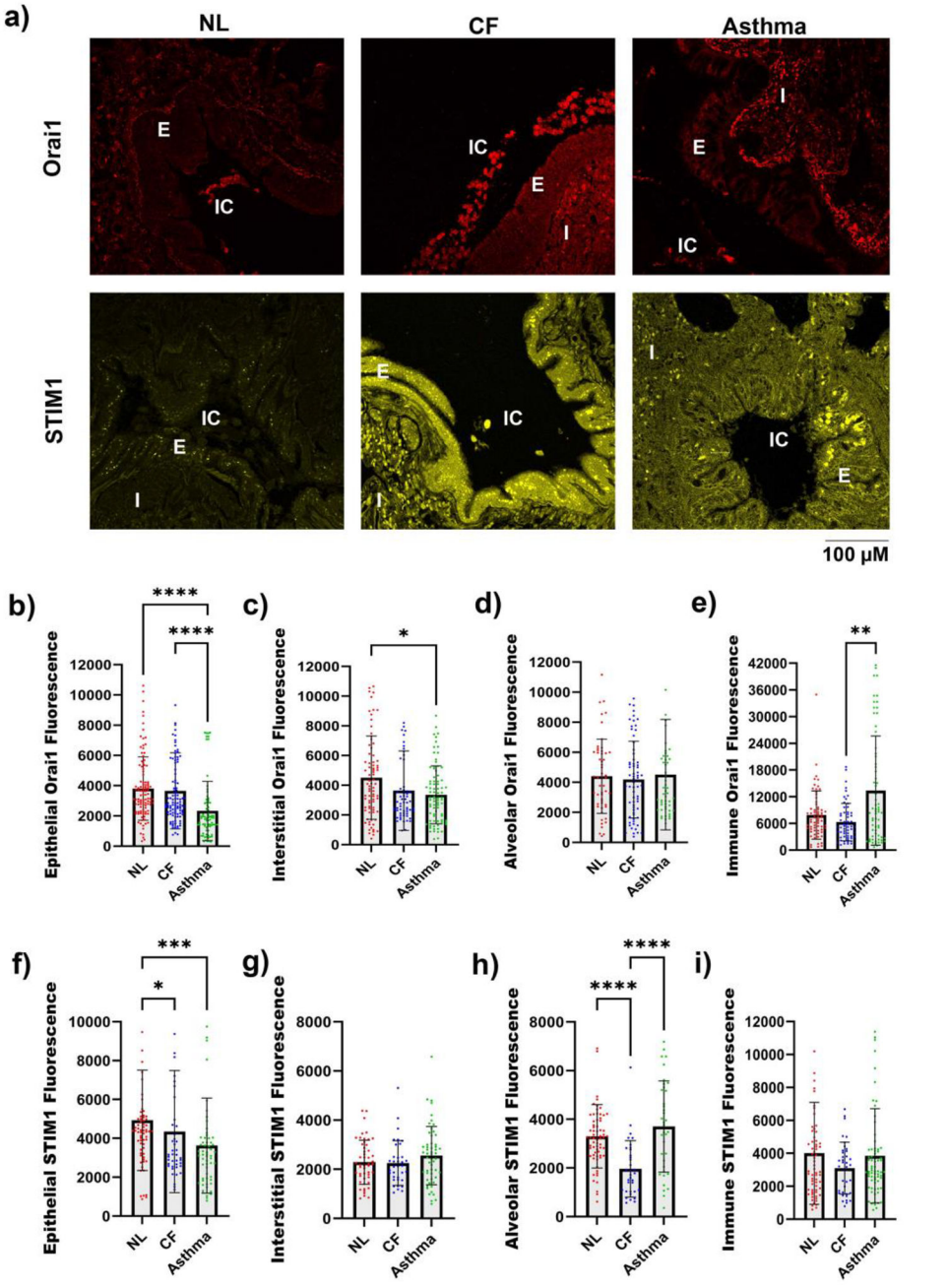

Figure 3: Orai1 and STIM1 protein expression are not overly different between groups.

(a) Representative images of Orai1 (red) and STIM1 (yellow) protein expression in normal, asthma and CF lungs. I, interstitial; E, epithelia; IC, immune cell. Orai1 fluorescence intensity in **(b)** epithelial, **(c)** interstitial, **(d)** alveolar, and **(e)** immune cells. STIM1 fluorescence intensity in **(f)** epithelial, **(g)** interstitial, **(h)** alveolar, and **(i)** immune cells. *=p<0.05, ***=p<0.001, ****=p<0.0001. Each data point represents an analyzed region. Data shown as mean ± SD. Data were analyzed using Kruskal-Wallis ANOVA. N=8 subjects per group.

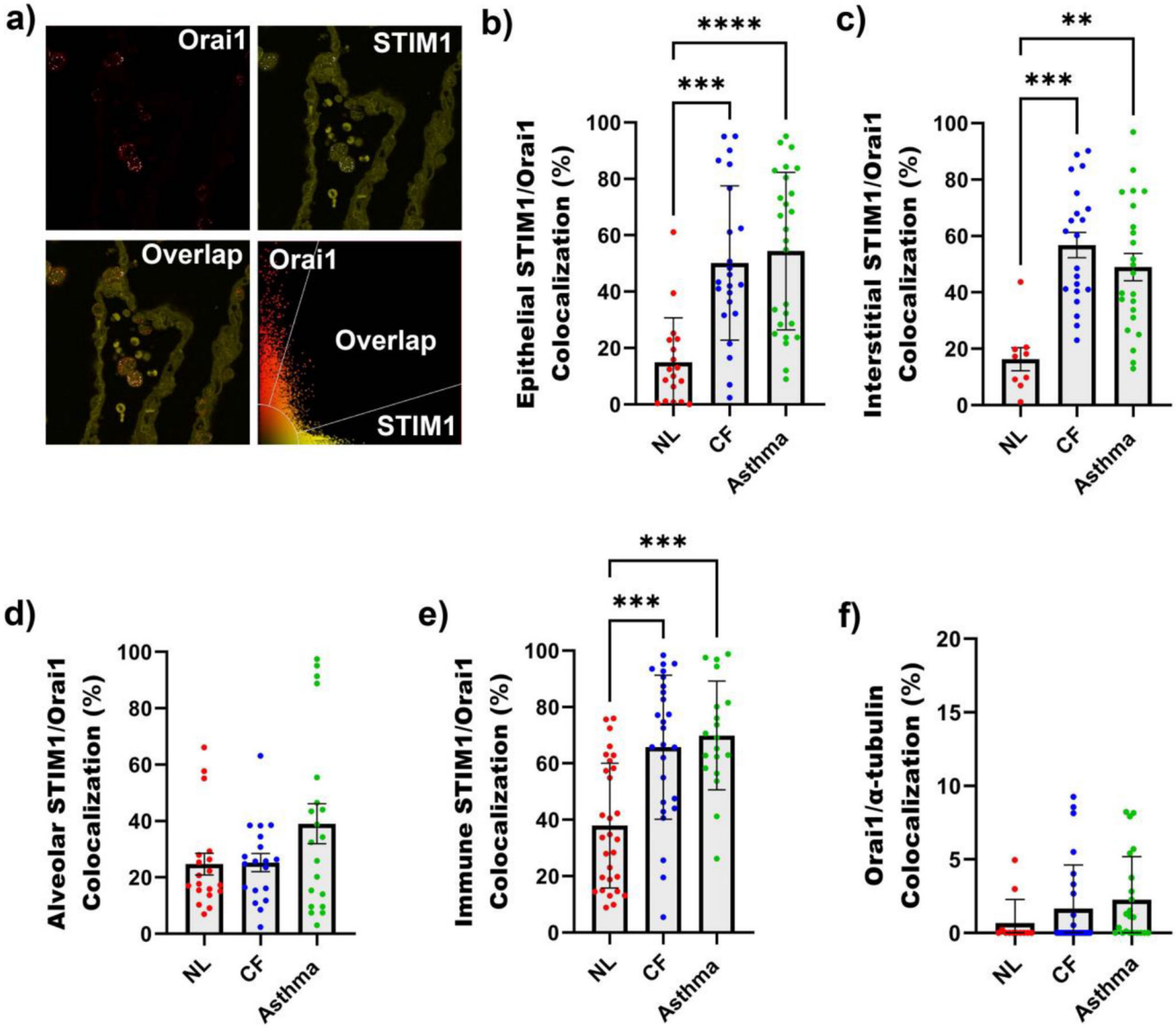

Figure 4: Orai1/STIM1 colocalization is significantly increased in asthma and CF lungs.

(a) Representative image of the Leica SP8 colocalization software process. Orai1/STIM1 colocalization in **(b)** epithelial, **(c)** interstitial, **(d)** alveolar, and **(e)** immune cells. **(f)** Orai1/α-tubulin colocalization in ciliated cells. **=p<0.01, ***=p<0.001, ****=p<0.0001. Each data point represents an analyzed region. Data shown as mean ± SD. Data were analyzed using Kruskal-Wallis ANOVA. N=8 subjects per group.

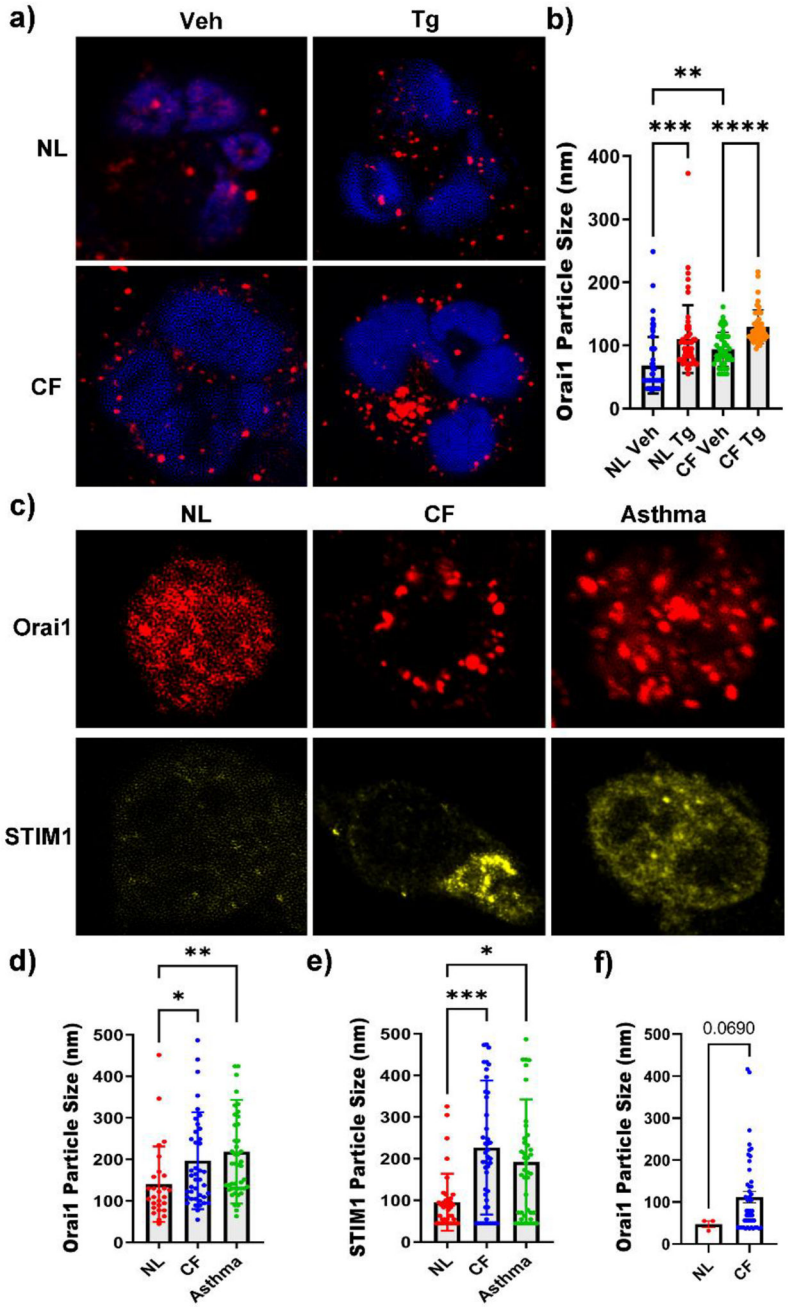

Figure 5: Orai1 puncta are significantly larger in CF and asthma immune cells, indicating increased Orai1 activation.

(a) Representative images of Orai1 puncta formation in vehicle-treated and thapsigargin-treated isolated human neutrophils from normal and CF blood. Blue, DAPI; red, Orai1. **(b)** Orai1 particle size (i.e. puncta) in isolated normal and CF blood neutrophils ± thapsigargin. N=5 subjects per group. **(c)** Representative images of Orai1 and STIM1 puncta formation in normal, asthma, and CF lung macrophages. **(d)** Orai1 and **(e)** STIM1 particle size in normal, asthma, and CF lung macrophages. **(f)** Orai1 particle size in NL and CF neutrophils. N=8 subjects per group. *=p<0.05, **=p<0.01, ***=p<0.001, ****=p <0.0001. Each data

point represents an individual cell. Data shown as mean ± SD. Data were analyzed using the Kruskal-Wallis ANOVA or a Mann Whitney test.