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# Club Cell TRPV4 Serves as a Damage Sensor Driving Lung Allergic Inflammation

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AUTHOR CONTRIBUTIONS

D.L.W. conceived, designed and performed experiments, and drafted the manuscript. S.J.E. and N.N.J. prepared bronchial biopsies. M.N. and N.P.K. assisted with design and execution of experiments related to *Aspergillus* culture supernates. R.A.B.-S. and J.E.G. provided ciliated cells grown at air-liquid interface. M.B.F. and J.M.V. prepared club cells. D.J.J., M.D.E., and C.O. collected data from COAST patients, analyzed the data, and assisted in writing the manuscript. T.W. analyzed histology of bronchial tissue biopsies. R.M.M. and G.C.K. assisted with analysis of *TRPV4* genotype and SNP analysis. B.S.K assisted with study concept, design, and execution and helped write the manuscript.

DELCARATION OF INTERESTS

M.B.F. is an employee of Vertex Pharmaceuticals and may own stock in that company. All other authors declare no competing interests.

SUPPLEMENTAL INFORMATION

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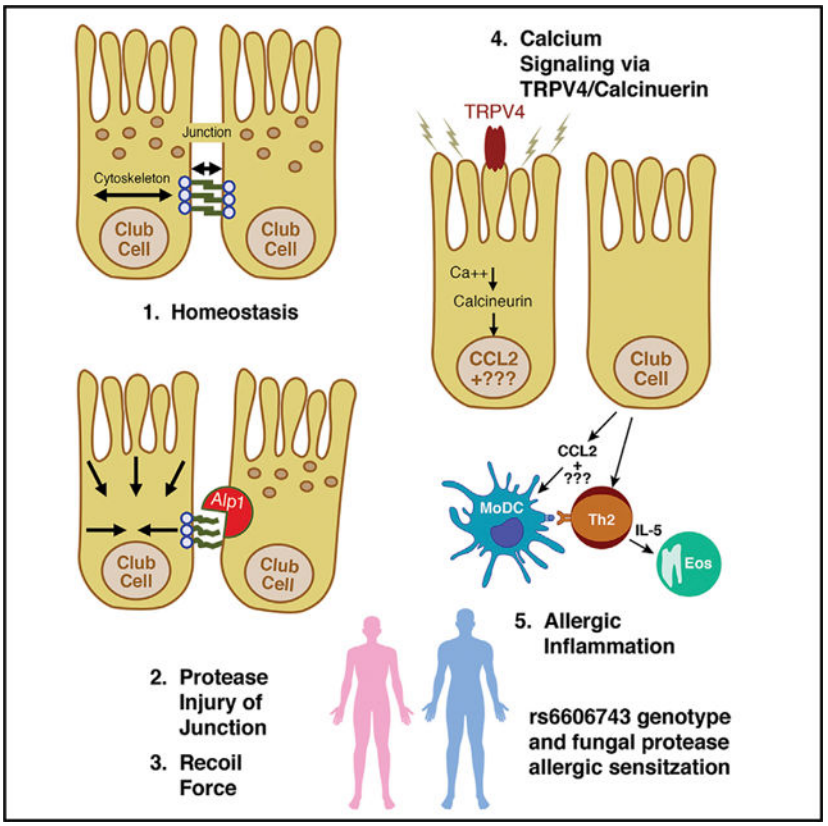
## SUMMARY

Airway epithelium is the first body surface to contact inhaled irritants and report danger. Here, we report how epithelial cells recognize and respond to aeroallergen alkaline protease 1 (Alp1) of *Aspergillus* sp., because proteases are critical components of many allergens that provoke asthma. In a murine model, Alp1 elicits helper T (Th) cell-dependent lung eosinophilia that is initiated by the rapid response of bronchiolar club cells to Alp1. Alp1 damages bronchiolar cell junctions, which triggers a calcium flux signaled through calcineurin within club cells of the bronchioles, inciting inflammation. In two human cohorts, we link fungal sensitization and/or asthma with SNP/protein expression of the mechanosensitive calcium channel, TRPV4. TRPV4 is also necessary and sufficient for club cells to sensitize mice to Alp1. Thus, club cells detect junction damage as mechanical stress, which signals danger via TRPV4, calcium, and calcineurin to initiate allergic sensitization.

## In Brief

Wiesner et al. show a secreted fungal protease allergen of humans induces inflammation in mice with hallmarks of allergic asthma. The protease damages junctions of bronchiolar epithelial club cells, which the mechanosensor and gated calcium channel TRPV4 detects. Calcineurin mediates the calcium signaling and cellular alarms initiating lung allergic inflammation.

## Graphical Abstract




## INTRODUCTION

Asthma is often triggered by inhalation of environmental allergens, many produced by household molds (Denning et al., 2006; Knutsen et al., 2012). *Aspergillus* is a major source of allergens (Simon-Nobbe et al., 2008), and alkaline protease 1 (Alp1) is the most abundant secreted protein by this mold (Sriranganadane et al., 2010; Wartenberg et al., 2011). Alp1 is a clinically important human allergen (Asp f 13), and the presence of Alp1 in the lungs is associated with severe asthma (Basu et al., 2018). Alp1 reportedly interrupts the interactions between smooth muscle cells and matrix components in the lung. Although these events impact airway hyperreactivity, the effect of Alp1 inhalation on allergic sensitization at the lung mucosa is poorly understood.

The immune consequences of allergen exposure are well known. Briefly, type-2 helper T (Th2) cells drive IgE antibody class-switching by B cells (Lambrecht and Hammad, 2015). Th2 cells, in collaboration with innate lymphoid cells (ILCs), also produce cytokines that propel granulocyte recruitment, mucous production, and bronchiolar constriction (McKenzie, 2014). In contrast, the earliest events that prime this allergic cascade are just beginning to be appreciated (von Moltke and Pepper, 2018). The lung epithelium interfaces with the host and allergen and functions as both a mechanical barrier and dynamic responder (Wiesner and Klein, 2017). Upon allergen exposure, lung epithelial cells rapidly release signals that lead to type-2 leukocyte accumulation in the lungs (Roy et al., 2012; Van Dyken et al., 2014). However, the lung epithelium is not a uniform tissue, and a lack of appreciation

for the heterogeneity in the epithelium has impeded our understanding of how epithelial cells recognize and respond to allergens (Wiesner and Klein, 2017).

Type-2 immune responses, besides promoting allergies, have a beneficial role in wound repair (Gause et al., 2013). Many allergens are proteases, which suggests that allergic diseases may arise when proteolytic damage to the airway is followed by dysregulated wound healing (Holgate, 2007). In fact, airway injury and loss of barrier function are correlates of allergic disease in humans (Bousquet et al., 2000). However, the mechanisms by which epithelial cell barrier damage leads to Th cell sensitization represents a gap in our knowledge. Airway integrity is maintained by junction proteins that mechanically link adjoining epithelial cells, and intercellular tension is balanced by intracellular forces exerted through the cytoskeleton (Ng et al., 2014). These forces are tightly regulated, and mechanosensing at the junction governs epithelial morphogenesis and cytokinesis (Pinheiro and Bellaïche, 2018). We explored the possibility that protease damage to the junction causes the epithelium to experience a mechanical recoil force that initiates proinflammatory signaling.

Transient receptor potential (TRP) channels are a family of proteins that sense varied stimuli, including chemicals, cold, pain, light, and pressure (Venkatachalam and Montell, 2007). To understand how the epithelium may sense mechanical strain, we investigated a particular TRP channel (i.e., TRPV4) that has osmosensory (Liedtke et al., 2000; Strotmann et al., 2000) and mechanosensory functions in various tissues (Lyons et al., 2017; Mendoza et al., 2010; O'Conor et al., 2014; Suzuki et al., 2003; Yin and Kuebler, 2010). TRPV4 is a gated calcium channel, and calcium ion currents are rapid signaling events that can occur when a cell is perturbed (Shannon et al., 2017; Zhao et al., 2006). This signaling pathway is best studied in leukocytes where calcineurin functions as a calcium-responsive phosphatase that regulates the activity of nuclear factor of activated T cells (Crabtree and Olson, 2002), which in turn transcribes various proinflammatory genes.

Here, we exploited Alp1 to test the hypothesis that allergen protease is initially sensed as damage and this signal is translated as mechanical recoil detected via TRPV4 in epithelial cells. We report an epithelial cell subset in the bronchioles—club cells—that responds to junction damage and elicits calcineurin-dependent inflammation. We show that TRPV4 is linked to fungal sensitization and asthma in humans and is highly expressed in the bronchioles of human asthmatic patients. Finally, we establish a causal relationship between club cell TRPV4 and allergic inflammation in our fungal asthma murine model. Collectively, our data indicate that mechanical stress due to protease erosion of the epithelial cell junction creates a danger signal sensed by TRPV4 on bronchiolar club cells, which initiates a rapid calcium flux that evokes calcineurin-driven allergic sensitization.

## RESULTS

### Inhalation of *Aspergillus* Alkaline Protease 1 Induces Helper T Cell-Dependent Eosinophilia

*Aspergillus fumigatus* is a ubiquitous household mold that abundantly secretes a human aeroallergen, Alp1 (*Asp* f13) (Namvar et al., 2015). We used *Pichia pastoris* to express

recombinant Alp1. A ~32-kDa protein corresponding to the size of mature Alp1 was present in the culture supernate of the Alp1-expressing strain of *Pichia* and not in the untransformed control (Figure S1A). Strong proteolytic activity was detected in supernate collected from the recombinant strain and absent when the sample was heat inactivated or in the supernate of the untransformed control (Figure S1B).

We established a murine model to investigate the immunologic events that follow inhalation of Alp1 (Figure 1A). In contrast to heat-inactive Alp1, repeated instillation of enzymatically active protease caused allergic pathology in the bronchiolar region of the lung by day 15, including leukocyte infiltration (H&E) and goblet cell metaplasia and hyperplasia (PAS) (Figure 1B). We quantified the lung cellular immune response to protease by flow cytometry, excluding cells in the blood vasculature from analysis (Anderson et al., 2014). Cell suspensions from lung digests were stained with myeloid and lymphoid antibody panels with overlapping fluorophores, which allowed simultaneous quantification of 15 leukocyte and functional subsets that accounted for >90% of the total CD45+ hematopoietic cells in the lungs (Figures S2A–S2C). Eosinophils increased ~500-fold (relative to heat-inactivated protease) and represented the dominant leukocyte subset by 15 days post-inoculation (dpi) (Figure 1C; Figure S2C).

Eosinophil differentiation is regulated by Interleukin (IL)-5, which is produced mainly by lymphocytes in non-lymphoid tissues and transcriptionally regulated by GATA3 (Lambrecht and Hammad, 2015; Van Dyken et al., 2016). We analyzed the kinetics of GATA3 and IL-5 expression in CD4+ Th cells and ILCs in the lungs of protease-challenged mice to determine which of these lymphocytes is involved in the eosinophilia. Alp1 exposure resulted in expansion of $GATA3^{+}$ Th cells and ILCs in the lungs (Figure 1D). Pulmonary leukocytes from unchallenged and challenged mice were stimulated *ex vivo* with titrations of heat-inactive protease to assess the capacity of Th cells and ILCs to produce IL-5. Th cells from challenged mice produced IL-5 in an antigen dose-dependent manner, whereas ILCs were less responsive to Alp1 (Figure 1E). IL-5 neutralization abrogated the eosinophilic response to protease, indicating IL-5 is a requirement for eosinophilia (Figure 1F). Complete lymphocyte deficiency in IL-2Rg/$Rag1^{-/-}$ mice resulted in a failure to produce IL-5 or recruit eosinophils (Figures 1G and 1H). Elimination of Th cells (with CD4 antibody or Rag deficiency) reduced both IL-5 and eosinophil accumulation to naive levels (Figures 1G and 1H).

We also adoptively transferred CD90.1+ ovalbumin-specific T (OTII) cells into mice to track the anatomical location of antigen-specific cells in the lungs. A higher proportion of OTII cells expressed GATA3 compared to the endogenous Th cell population (Figure 1I), indicating OTII cells differentiate into Th2 cells. OTII cells also accumulated in the bronchiolar region of the lung when both ovalbumin and protease were present in the inoculum (Figures 1J and 1K). Thus, in this model, allergen-specific, GATA3+ Th cells that accumulate around the bronchioles produce IL-5 to drive a hallmark feature of allergic asthma, eosinophilia, and this allergic response requires enzymatically active Alp1.

We related the protease used in our studies to that used in similar, well-established fungal proteinase-driven allergic inflammation models (Drouin et al., 2002; Millien et al., 2013).

We compared the amount and enzymatic activity of proteinase acquired from Sigma-Aldrich (product of *Aspergillus melleus* used in the previous studies) to our recombinant Alp1 (Figures S1C and S1D). A band of similar size to Alp1 is apparent in the Sigma-Aldrich proteinase, suggesting that Alp1 could be present in that proteinase. Sigma-Aldrich proteinase and recombinant Alp1 elicited similar magnitudes of allergic response when the two protease concentrations were adjusted to have equal enzymatic activity (Figure S1E). Thus, our model is comparable to established fungal proteinase-based allergic inflammation models. We also inoculated mice with culture filtrates collected from two strains of *Aspergillus fumigatus* (Af293 or CEA10) grown in glucose minimal media or DMEM+10% fetal bovine serum (FBS). Each *Aspergillus* culture filtrate elicited allergic responses in mice (Figure S1F). Thus, secreted products, which include Alp1, from several *Aspergillus* strains and growth conditions cause allergic inflammation similar to purified Alp1.

## Bronchiolar Club Cells Recruit Monocyte-Derived Dendritic Cells to the Lungs and Sensitize Allergic Response to Alp1

We sought to identify an antigen-presenting cell population in the lungs that directs Th cell responses to Alp1. MHCII+ CD11c+CD11b+ CD64+ monocyte-derived dendritic cells (Mo-DCs) rapidly accumulated in the lungs within 3 dpi (Figure 2A). Using pulmonary instillation of an epitope-tagged fluorescent protein (EαRFP) as a surrogate for Alp1, we noted that more than a third of the Mo-DCs captured Ea-RFP and presented Ea peptide on MHCII at 3 dpi (Figure 2B). C-C chemokine receptor 2 (CCR2) is critical for monocyte recruitment from the bone marrow and Mo-DC formation in the lung (León et al., 2005). To test the requirement of Mo-DCs for Th cell responses to Alp1, we transferred OTII cells into wild-type and $CCR2^{-/-}$ mice and treated the mice with ovalbumin alone or together with protease. ~20to 50-fold more OTII cells accumulated in the lungs of wild-type mice inoculated with protease and ovalbumin than wild-type mice treated with ovalbumin alone or $CCR2^{-/-}$ mice treated with protease and ovalbumin (Figure 2C). Thus, Mo-DCs arrive in the lung early in response to protease, capturing and presenting antigen leading to pulmonary accumulation of Th cells.

Alarimins are signals rapidly released upon tissue injury. Thymic stromal lymphopoietin (TSLP), IL-25, IL-33, and CCL2 are recognized as important allarmin signals during allergic lung inflammation (Lambrecht and Hammad, 2014). We analyzed these factors in lung lysates at 0, 8, and 24 h after Alp1 exposure. TLSP did not increase from baseline, whereas IL-25 and IL-33 rose significantly at 24 h post-inoculation (Figure 3A). CCL2 rose significantly within 8 h and remained elevated 24 h after Alp1 inhalation (Figure 3A). Because we were interested in early recognition events of protease, we pursued a mechanism involving CCL2 that underpins allergic responses to Alp1.

C-C chemokine ligand (CCL) 2 is one ligand for CCR2 (Deshmane et al., 2009). CCL2-deficiency had a modest effect on Mo-DC recruitment, Th2 cell accumulation, and eosinophilia after Alp1 exposure than did CCR2-deficiency (Figures S3A and S3B), but these responses were still significantly reduced in $CCL2^{-/-}$ mice versus wild-type controls (Figure S3B). Because CCL2 significantly affects Mo-DC accumulation in the lungs, we investigated the initial source of CCL2 in the lungs of Alp1-inoculated mice. Three types of

cells could produce CCL2 in response to the protease: resident phagocytes, resident lymphocytes, and stromal tissues. To see which population is required, we ablated phagocytes with clodrosomes (Figure S3C), genetically eliminated lymphocytes (IL-2Rg/Rag$^{-/-}$), or did both. Within 8 h of Alp1 inhalation, CCL2 levels were several fold higher in wild-type mice than naive controls (Figure 3B). The CCL2 response to protease remained at or above wild-type mouse levels even in the absence of phagocytes and/or lymphocytes, indicating that stromal tissues are an important early source of CCL2 in this model (Figure 3B).

Epithelial cells release CCL2 in response to sterile lung injury and inhaled chitin (Mercer et al., 2009; Roy et al., 2012). However, the lung epithelium is not a homogeneous tissue and is comprised of at least seven distinct subsets with varying functions (Wiesner and Klein, 2017). We used confocal microscopy to identify if/which epithelial cell subset produces CCL2. Within 8 h of Alp1 exposure, CCL2 expression was confined to secretoglobin (Scgb1a1)+ club cells in the bronchiolar regions (Figure 3C; Figure S3D). In mice with Scgb1a1-estrogen receptor cre and cre-inducible *Diptheria* toxin, tamoxifen administration eliminated club cells, which were replaced by Forkhead box J1 (Foxj1)+ ciliated epithelial cells (Figure 3D). This system allowed us to test the requirement of club cells in response to protease. In club-cell-depleted mice, the numbers of Mo-DCs and OTII cells in the lung returned toward baseline, and eosinophil recruitment fell sharply by 15 dpi (Figure 3E). Thus, an individual epithelial cell subset (i.e., club cells) drives allergic inflammation. Interestingly, club cells are a major constituent of the bronchiolar epithelium, which coincides with the focal areas of inflammation in Figures 1B and 1K.

## Proposed Models that Fail to Explain Alp1-Instigated Allergic Inflammation

To understand how protease is recognized by the epithelium, we tested several models. Investigators have proposed that fungal proteases cleave host proteins, coopting physiologic pathways to cause atopic disease (Cayrol et al., 2018; Drouin et al., 2002; Kauffman et al., 2000; Millien et al., 2013). We tested the roles of (1) protease-activated receptors (PARs) 1 or 2, (2) complement C3, (3) fibrinogen, and (4) IL-33 as sensors of Alp1. First, Alp1 failed to cleave at the precise site of PAR1 or PAR2 required for tetheredligand signaling (Figures S4A and S4B). The allergic response to Alp1 in whole genome PAR1-or PAR2-knockout mice also was not different from wild-type animals (Figures S4C and S4D). Second, we asked if Alp1 cleaves complement C3 to generate C3a anaphylatoxin, which could bind C3a receptors on club cells (Drouin et al., 2002). We found that club cells display C3a receptors, and that Alp1 excises a fragment from C3 similar in size to C3a (Figures S5A S5B). However, MALDITOF analysis revealed that the resultant fragment was not identical in sequence to C3a, and the allergic response to Alp1 was also unaffected in C3$^{-/-}$ mice (Figures S5C and S5D).

We next tested whether Alp1 releases fibrinopeptides from fibrinogen to signal inflammation. We found that the largest amount of inhaled thrombin—the natural enzyme for fibrinogen—that mice could survive (2,000 units) had only a subtle effect on inflammation and was not additive or synergistic with Alp1 (Figure S6A). Likewise, hirudin, a thrombin inhibitor, failed to suppress inflammation 3 dpi when administered together with

Alp1 (Figure S6B). Alp1 neither induced clot formation when incubated with human fibrinogen, nor released fibrinopeptides A or B into the reaction mixture (Figures S6C and S6D). Furthermore, the reaction mixture was only able to induce inflammation if the sample was not heat inactivated to remove protease activity (Figure S6E). Lastly, we found that the presumptive receptor for fibrinogen cleavage products, toll-like receptors (Millien et al., 2013), were dispensable for the allergic response to Alp1 (Figure S6F). MyD88, the downstream signaling intermediate for toll-like receptors and the IL-33 receptor, also was not required in this model of allergic inflammation (Figure S6F). Of note, ILC2 numbers were reduced to naive levels in protease-treated MyD88$^{-/-}$ mice, suggesting that the inflammatory effects of IL-33 involve ILC2 cells more than Th2 cells.

## Alp1 Uniquely Interacts with Club Cells to Incite Allergic Inflammation

We evaluated if club cells sense and respond to a variety of proteases or if this behavior is unique to Alp1. We normalized concentrations of the protease allergens produced by plants (papain) and bacteria (subtilase) to that of Alp1 based on enzymatic activity and administered the proteases to mice. All three proteases elicited qualitatively (Mo-DCs, GATA3+ Th2 cells, and eosinophils) and quantitatively similar responses (Figure S6G). However, allergic responses to papain and subtilase were unaffected by club depletion in Scgb1a1-creERT2 × LSLDTA mice (Figures S6H and S6I). Thus, Alp1 uniquely interacts with club cells to instigate allergic inflammation.

## Protease Damage to Epithelial Junctions in the Bronchioles Instigates Inflammation

Alp1 lacks an exosite that would render it specific for other mammalian proteins beyond those mentioned in the preceding paragraphs. Therefore, we modified our hypothesis and tested if Alp1 broadly disrupts the epithelial barrier. Inhalation of biotin in the presence or absence of Alp1, followed 1 h later by lung processing for microscopy, revealed that the protease caused biotin to leak from the bronchiolar regions of the lung, indicating loss of airway integrity (Figure 4A).

The mechanical barrier of the airway is maintained by solute impermeable tight junctions (TJs) at the apical reach of the epithelia, whereas adherens junctions (AJs) are situated just beneath the TJ. Whereas TJ proteins are heterogeneous in identity and expression, the AJ is formed through highly conserved, homotypic interactions of E-cadherin between adjacent epithelial cells (Hartsock and Nelson, 2008; Perez-Moreno and Fuchs, 2006). We posited that measuring the effect of Alp1 on E-cadherin is a direct way to test whether protease disrupts the epithelial junction. Commercial antibody to mouse E-cadherin (clone DECMA-1) recognizes the cytosolic portion of E-cadherin, which appeared unaffected by Alp1 in the bronchioles of mice despite the hyperplastic appearance of the airway cells (Figure 4B).

Some additional monoclonal antibodies recognize individual extracellular (EC) domains of human E-cadherin (Shiraishi et al., 2005). To test if Alp1 degrades E-cadherin in the absence of confounding host enzymes (Zemans et al., 2011), we incubated recombinant E-cadherin with titrations of Alp1 for 1 h. Western blots showed that even low concentrations of Alp1 destroyed the epitopes of E-cadherin recognized by antibodies against EC1 and EC2 (Figure

4C). Next, we grew human bronchiolar epithelial cells at an air-liquid interface and tested whether Alp1 penetrates the TJ and damages E-cadherin *in situ*. After 1 h, Alp1 compromised barrier integrity (Figure 4D), as measured by fluorescein isothiocyanate (FITC) leakage and reduced transepithelial electrical resistance (TEER), and also degraded the EC1 domain of E-cadherin (Figure 4E).

Cationic polypeptides such as poly-L arginine interrupt epithelial junctions (Shahana et al., 2002). When this compound was administered into the lungs of mice, direct junction damage (without the intrinsic ability to cleave host proteins) was sufficient to drive Mo-DC accumulation 3 dpi (Figure 4F). Collectively, these data establish that Alp1 disrupts epithelial junctions and that damage to junctions between conducting airway cells signals inflammation.

## Allergic Response to Alp1 Requires Calcineurin Signaling in Club Cells

We investigated the signaling events in club cells that initiate inflammation upon protease injury to the epithelial junction. The AJ is at the center of a critical signaling pathway involved in spatial patterning during lung development, cancer metastasis, and epithelial wound healing (Perez-Moreno and Fuchs, 2006). Many of these processes feed into the Wnt pathway, where β-catenin dissociates from E-cadherin at the AJ and migrates to the nucleus (Komiya and Habas, 2008). Although active β-catenin was expressed at the lateral membrane of club cells, active β-catenin did not concentrate in the nucleus of club cells in protease-damaged regions of the bronchioles (Figure 5A). Nuclear factor κ light chain of B cells (NF-κB) is another major signaling pathway implicated in epithelial cell responses to allergen (Lambrecht and Hammad, 2014; Whitsett and Alenghat, 2015). However, protease-challenged mice with pan-lung epithelial cell deficiency in the positive regulator of NF-κB, inhibitor of nuclear factor kappa-B kinase subunit beta (IKK2) (PerezNazario et al., 2013), did not elicit GATA3+ Th cell response differently from wild-type littermates (Figure 5B). Thus, unexpectedly, neither Wnt nor NF-κB signaling is responsible for club-cell-dependent inflammation.

The findings above suggested that an atypical pathway in club cells drives inflammation to inhaled protease. Calcium signaling is another canonical inflammatory pathway in leukocytes, and so we investigated calcium signaling in club cells based on what is known in leukocytes. In transgenic mice that express a calcium indicator protein, GCamp6s, specifically in club cells, these cells fluxed calcium within 15 min of exposure to inhaled protease (Figure 5C). To assess the downstream consequences of calcium signaling, we used transgenic mice with tamoxifeninducible calcineurin-or IKK2-deficient club cells. Mice with IKK2-deficient club cells maintained unaffected allergic responses to protease (Figure 5D), consistent with Figure 5B. Conversely, mice with calcineurin-deficient club cells demonstrated blunted allergic responses of Mo-DCs, GATA3+ Th cells, eosinophils, and CCL2 levels in responses to protease (Figures 5D–5F). We also investigated mice lacking calcineurin or IKK2 in other epithelial cell subsets—Sftpc+ Type-2 alveolar cells, Foxj1+ ciliated cells, or Ascl1+ pulmonary neuroendocrine cells—and found no effect of these pathways on inflammation (Figure S7A and S7B).

We used Poly L-arginine to disrupt lung epithelial cell junctions, as we did in Figure 4F, to determine if junction damage alone is sufficient to drive T cell sensitization and if T cell priming is dependent on calcineurin in club cells. Here, we adoptively transferred OTII T cells into the mice, included ovalbumin in the inoculum, and harvested the lungs at 15 dpi. Targeting the junction directly with Poly L-arginie caused a large expansion of OTII cells in wild-type mice, and this response was reduced to control levels with an induced deletion of calcineurin in club cells (Figure 5G). Thus, junction damage alone can sensitize T cells, and similar to protease, this response signals via calcineurin in club cells.

Finally, we asked if calcium signaling in club cells is sufficient to instigate rapid inflammation. Scgb1a1-creERT2 mice were crossed with hM3Dq (i.e., DREADD) mice and treated with clozapine n-oxide (CNO) to induce G-protein-mediated calcium flux in club cells. Mice receiving both CNO and tamoxifen recruited a larger population of Mo-DCs to the lungs compared to mice treated only with CNO (Figure 5H). In sum, protease damage to the epithelial junction elicits a calcium flux in club cells that signals through calcineurin to mediate allergic inflammation.

## Apical Bronchiolar Epithelium Expression of TRPV4 Is Associated with Fungal Asthma

We searched for a receptor/channel that could connect protease injury at the bronchiolar junction with calcium entry into club cells. We posited that TRPV4 could exert this function, because it acts as a mechanosensative gated-calcium channel. Genetic variation at the *TRPV4* locus has been associated with lung pathology in prior studies in humans. In particular, the G allele or AG+GG genotypes at rs6606743 were associated with osmotic airway hyperresponsiveness (Naumov et al., 2016) and increased risk of chronic obstructive pulmonary disease (Zhu et al., 2009). We therefore tested for the association of genotype at rs6606743 with fungal sensitization and asthma in 211 participants in the Childhood Origins of Asthma (COAST) study. We identified significant associations with both phenotypes (Figures 6A and 6B). This SNP resides 496 kb upstream of the transcription start site of the *TRPV4* gene, within an intron of a long non-coding RNA (lncRNA), XR_945333.1. This variant is also in linkage disequilibrium ($r^2 > 0.8$ in Northern Europeans from Utah, i.e. CEU) with three SNPs spanning about 2.5 kbp, located within the lncRNA gene and overlapping an enhancer site annotated in multiple cell types, including keratinocytes (NHEK) and lung epithelial cells (A549) in the Roadmap Epigenomics project (Roadmap Epigenomics Consortium, 2015). To directly examine whether rs6606743, or SNPs in linkage disequilibrium with it, are associated with expression of the *TRPV4* gene, we used expression quantitative locus (eQTL) data from epithelial tissues, esophagus and skin, available in the GTEx database (GTEx Consortium et al., 2017). Indeed, the G allele at rs6606743 is associated with increased expression of *TRPV4* in the epithelium of esophagus ($p = 4.5e-5$) and skin ($p = 2.4e-7$). Collectively, these data suggest that increased expression of TRVP4 is associated with a risk of sensitization to fungal allergen and asthma.

In a separate group of individuals, we analyzed the location and level of TRPV4 expression in human bronchiolar cells and biopsies. Cell lysates of club/goblet cells differentiated from primary human lung basal cells (Feldman et al., 2019) were enriched for membrane proteins (Sokabe et al., 2010). The membrane proteins were immunoprecipitated with anti-β-catenin

antibody to determine if TRPV4 physically interacts with β-catenin at the AJ, as reported with keratinocytes (Sokabe et al., 2010). TRPV4 was more abundant in the unbound fraction than the lysate, indicating TRPV4 concentrates in the cell membrane (Figure 6C). However, TRPV4 was absent in the bound fraction, indicating TRPV4 does not form an avid interaction with β-catenin at the AJ in human club/goblet cells.

We alsoimmunologically stained bronchialbiopsiesforTRPV4in five healthy donors and three patients with asthma. TRPV4 was most apparent in the apical tip of the bronchiolar epithelium (Figures 6D and 6G). TRPV4 expression was scored by a sampleblinded pathologist, and, when compared with healthy individuals, TRPV4 expression trended higher in asthmatics (Figure 6E). Five mild-asthmatic patients were subjected to segmental challenge with ragweed, as previously described (Kelly et al., 2017). TRPV4 expression scores did not significantly increase with allergen challenge (Figure 6F), suggesting that baseline differences in TRPV4 expression between asthmatics and healthy donors is not due to recent antigen encounter. Taken together, TRPV4 is highly expressed in the apicalmembrane of bronchiolar cells of asthma patients.

### Club Cell TRPV4 Signals Allergic Inflammation

To determine if a directional relationship exists between TRPV4, club cells, and Th cell sensitization, we exploited our murine model. First, we used a functional assay to assess TRPV4 expression by club cells. Cell suspensions from club-cell-reporter mice were loaded with a ratiometric calcium indicator dye and analyzed by flow cytometry. Club cells rapidly shifted fluorescence in response to a TRPV4 agonist (GSK1016790A), indicating the cells express a functional TRPV4 calcium channel (Figure 7A). Second, we tested the requirement of club cell TRPV4 in protease-driven allergic inflammation by using Scgb1a1-creERT2 mice with a floxed *TRPV4* allele. Tamoxifen administration to delete *TRPV4* in club cells resulted in significantly decreased CCL2 production within 8 h of protease inhalation and reductions in OTII cell, GATA3+ Th cell, Mo-DCs, and eosinophil accumulation 15 dpi (Figures 7B–7D). Lastly, we modeled the human condition of enhanced TRPV4 expression by overexpressing TRPV4 *in vivo* with adeno-associated virus (AAV) 6. We found that AAV6 expressed mCherry efficiently in club cells (Figure 7E). We thus infected Scgb1a1-creERT2 mice with an AAV6-doublefloxed inverse orf-TRPV4 virus that overexpresses TRPV4 in cells with a functional cre recombinase (Wheeler et al., 2016). When viral gene expression was activated by tamoxifen, Scgb1a1-creERT2 mice inoculated with protease accumulated significantly more OTII cells, Mo-DCs, and eosinophils than wild phenotype mice not receiving tamoxifen (Figure 7F). Remarkably, the enhanced OTII cell response due to TRPV4 overexpression was absent in mice with club cell calcineurin deficiency, indicating that TRPV4 and calcinueurin are in epistasis (Figure 7G). In sum, club cell TRPV4 is required for CD4+ T cell sensitization to Alp1 and that recapitulating the features of human asthma by overexpressing TRPV4 enhances sensitivity to Alp1.

## DISCUSSION

Aeroallergens, including proteases, are irritants (Holgate, 2007), and the airway epithelium is the first tissue assaulted by inhaled substances. We investigated how the lung epithelium

alerts immune cells to protease allergen exposure thereby sensitizing CD4+ T cells. We report an advance in understanding the pathogenesis of asthma, revealing that the mechanical force exerted on epithelial cells that suffer junction damage is a danger signal detected by TRPV4. We define a linear pathway connecting epithelial junction damage with calcium flux via TRPV4, calcineurin signaling, and Th cell sensitization.

Patients with asthma are often allergic to environmental molds. Many fungi, including *Aspergillus*, are saprophytes, and secreted proteases facilitate nutrient acquisition in the environment (St Leger et al., 1997). However, these proteases are not virulence factors (Bergmann et al., 2009) and have not undergone host selection to evolve exosites that mammalian elastases, convertases, or thrombin require for specific interaction with their substrate (Overall and Blobel, 2007). Organisms spanning every reach of the biologic kingdom produce proteases that elicit allergic responses in humans (Reed and Kita, 2004). The mammalian host has evolved multiple layers to detect potentially injurious proteases, including PARs, sensor cytokines (e.g., IL-25 and IL-33), and junction damage (Cayrol et al., 2018; Florsheim et al., 2015; Goswami et al., 2009). How the host responds to a protease is likely dependent on enzyme substrate specificity and host redundancy for those targets. For example, papain and subtilase are reported to cleave PAR2, which could explain why neither of these proteases required club cells to mount an allergic response. Conversely, Alp1 does not require *PAR2* or *MyD88* (i.e., IL-33), yet club cells are instrumental in priming an allergic response.

We tested several models of protease-induced allergic inflammation. The most striking finding upon protease inhalation was damage to the bronchiolar epithelial junction. We propose a model of asthma pathogenesis in which protease injury to epithelial cell junctions elicits an unconventional “damage response” sensed as recoil by a mechanoreceptor TRPV4. This mechanism of sensing damage runs counter to classic damage receptorligand interactions. The most widely recognized mechanism by which cells report injury is via damage-associated molecular patterns (DAMPs). In general, DAMPs are proteins (e.g., IL-33, highmobility group box 1, F-actin), nucleic acids, or metabolites (e.g., ATP) that are sequestered within cells during steady state. These molecules spill from cells when they rupture during necrosis (Kaczmarek et al., 2013). DAMPs thereby alert a healing response in neighboring cells bearing DAMP receptors. Although protease injury induced hyperplasia of the bronchiolar epithelium, we found little necrosis in the affected regions (data not shown). These observations indicate that junction disruption, independent of cell lysis/death, likely signals damage. We posit that mechanical perturbations exerted on club cells as they detach from one another are sensed by TRPV4, prompting cellular calcium flux and calcineurin-dependant inflammatory signaling.

TRP proteins perform various sensory functions, and several TRP channels have been implicated in asthma. TRPV4 expressed by smooth muscle cells responds to osmotic stimuli and contributes to hypotonic airway contractility (Jia et al., 2004). SNP rs6606743 of TRPV4 has been associated with osmotic hyperresponsiveness in patients with bronchial asthma (Naumov et al., 2016). Mice with a whole genome deletion of TRPV4 are also protected from airway remodeling after exposure to house dust mite antigens (Gombedza et al., 2017). Our work extends previous studies of airway hyperreactivity and fibrosis by

defining a role for epithelial cell TRPV4 in sensing damage and driving allergic inflammation. The pain-sensing channel, TRPV1, and cold sensing channel, TRPM8, also expressed by lung epithelia, promote inflammatory responses when stimulated and have been associated with asthma (Cantero-Recasens et al., 2010; Choi et al., 2018; McGarvey et al., 2014; Sabnis et al., 2008). TRPV1 and TRPM8 are calcium channels, and their involvement in epithelial responses and asthma also could be mediated by calcium signaling through calcineurin, as we found with TRPV4. Thus, calcium signaling and calcineurin might be a central pathway in the pathogenesis of allergic asthma, perhaps amenable to therapeutic targeting.

In contrast to the widely held belief that NF-κB is the master regulator of inflammatory signaling within the epithelium (Janssen-Heininger et al., 2009), we found that NF-κB was not required for the epithelial response to protease. Our model is distinct in two ways that could potentially explain this discrepancy. First, we avoided lipopolysaccharide (LPS) contamination in our allergen preparations by producing Alp1 with a yeast expression system. Many experimental allergens, like those from dust mites, are crude extracts containing unknown quantities of LPS and other undefined contaminants (Cates et al., 2004). The presence of toll-like receptor ligands in these allergens could prompt NF-κB-dependent inflammation in epithelial cells. Second, mice only received allergen intranasally in our model, whereas other approaches, including ovalbumin/alum, require systemic sensitization followed by intranasal challenge. With intranasal sensitization, the epithelium is intimately involved in the early innate cell activation and T cell priming events, whereas challenge after systemic sensitization prompts epithelial cells to recruit activated T cells from the periphery to lung. These disparate situations may rely on a different set of signals in the epithelium.

The bronchiolar epithelium is a central feature in our model of protease-driven allergic inflammation: (1) protease damage was concentrated in the bronchioles, (2) bronchiolar club cells had a requisite role in driving a large part of the response to protease, (3) the inflammation was confined to bronchiolar regions of the lung, and (4) TRPV4 was highly expressed in the bronchiolar epithelium of humans. Mice and humans have similar epithelial cell subsets, and these cells retain comparable functions across species. However, the proportions of epithelial cell subsets that comprise the conducting airways (i.e., bronchioles) differ between mice and humans (Iwasaki et al., 2017). Although the bronchioles are densely populated by club cells with a few intermittent ciliated cells in mice, club cells and ciliated cells are present in more balanced proportions in similar regions of human lungs. In our murine model, club cells had a potent effect, and our manipulations of the epithelium were targeted to club cells. When we depleted club cells, Foxj1+ ciliated cells repopulated the bronchioles, and the inflammatory response to protease was reduced. Knockout of IKK or calcineurin in Foxj1+ cells did not reveal a strong phenotype in our model, as well. Collectively, these observations suggest that ciliated cells are not sufficient or required for the response to protease. However, Foxj1+ cells expressed high levels of Ki67 after club-cell depletion (data not shown), and the proliferative state of these cells could prevent them from producing proinflammatory cytokines. Also, the bronchiolar epithelium remains broadly intact when genes are disrupted in ciliated cells in mice, and club cells could perform redundant functions masking any effect that ciliated cells have in the response. Thus, the

mechanisms we detailed in mouse club cells may not be restricted to only club cells in humans and could involve ciliated cells as well.

Immune responses function like electronic circuits. Cells in a network cooperate with one another to amplify a small signal into a highly ordered response. Evolution has built complexity and redundancy into the circuit, making identification of causal relationships difficult. Vaccinologists define correlates of immunity to elucidate complex biology (Plotkin, 2010). We propose that CCL2, Mo-DCs, and eosinophils are correlates of immunity and not an epistatic pathway feeding into and out of Th cell sensitization. We do not claim there is a stoichiometric relationship between these allergic parameters. For example, we showed that neutralizing CCL2 only modestly affected allergic inflammation, even under idealized conditions. Likewise, elimination of Mo-DCs did not abrogate Th cell sensitization. Other lung resident DC populations could have redundant functions, as seen in house dust mite allergy models in which CD11b+ DCs overlap with Mo-DCs depending on allergen dose (Plantinga et al., 2013). It is also likely that many signals emanate from the epithelium and instruct Mo-DCs (e.g., CCL7, CCL8, CCL12, CCL13) or CD11b+ DCs (e.g., colony stimulating factor-1) (Moon et al., 2018). Finally, whether Mo-DCs, CD11b+ DCs, or another DC subset traffics from the lung to the draining lymph to initiate T cell priming remains unresolved. Based on our *in vivo* EαRFP assays, we do know that Mo-DCs are capable of capturing and presenting antigen. We also know from our ex vivo restimulation experiments that a lung antigen-presenting cell is involved in regulating IL-5 production by Alp1-specific Th cells, because MHCII-restricted Th cells produce IL-5 when Alp1 antigen is added to the *ex vivo* culture. In summary, our goal was to understand how the epithelium recognizes and responds to protease allergen to drive Th cell sensitization. Th cell accumulation in the lungs was the primary read out in all experiments in which it was technically and biologically feasible. This criterion let us uncover a linear pathway within club cells, linking epithelial junction damage, calcium flux through TRPV4, calcineurin signaling, and Th cell sensitization.

Most cases of asthma begin in early childhood, and allergic sensitization in the first 2–3 years of life is a major risk factor for persistent childhood asthma. In our model, calcium signaling in club cells is an important mediator of Th cell priming, and elevated TRPV4 expression exacerbated the inflammatory response to protease. We also show that children with an SNP in *TRPV4* are a risk group for fungal sensitization and asthma. Genotyping at the *TRPV4* locus could identify children at increased risk for sensitization to protease-containing allergens such as fungi. Our findings also suggest that targeting bronchiolar epithelial cells directly with calcineurin inhibitors, perhaps through the inhaled route to minimize systemic effects, could inhibit the onset of allergic sensitization.

## STAR★METHODS

### LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources and reagents should be directed to and fulfilled by the lead contact, Bruce S. Klein (bsklein@wisc.edu). This study did not generate new unique reagents.

## EXPERIMENTAL MODEL AND SUBJECT DETAILS

**Mice—**All mice were C56BL/6 genetic background and were used for experimentation between the ages of 8 and 16 weeks. Males and females were used throughout the study. Sex and age had no obvious confounding effect in this model, but animals were age and sex matched for scientific rigor, nonetheless. Mice were bred in house and maintained in specific pathogen free conditions. All procedures were approved by the Institutional Care and Use Committee. See Key Resources Table for an inventory of the transgenic and knockout animals used in this study (Kerschen et al., 2007; Moore et al., 2013; Pasparakis et al., 2002; Perez-Nazario et al., 2013; Tabeta et al., 2006).

**Human Subjects:** Paraffin-embedded bronchial biopsies were obtained by bronchoscopy from subjects with asthma and from normal subjects as previously described (Nakamura et al., 2004). The study was approved by the University of Wisconsin-Madison Human Subjects Committee. Human lung epithelial cells were obtained from the tracheas of donor lungs and were differentiated at an air liquid interface (ALI) as described (Ashraf et al., 2015). Human adult airway basal stem cells were also recovered from discarded lung tissue obtained from the New England Organ Bank from donors under IRB approved protocols. These cells were isolated and differentiated into club and goblet cells as described (Feldman et al., 2019; Mou et al., 2016)

Participants of the COAST birth cohort study (N = 289) were recruited in Madison, Wisconsin and surrounding areas from November 1998 to May 2000 (Lemanske, 2002). The study was approved by the University of Wisconsin-Madison Human Subjects Committee, and all families provided informed consent before enrollment. When the children reached 7 years of age, they themselves provided assent. Asthma was defined by parental report of physician diagnosis and/or use of asthma medications as previously described (Jackson et al., 2008). All COAST children were genotyped with the Illumina Infinium Exome Core array, and untyped SNPs (including rs6606743) were imputed using 1000Genome reference panel (CEU) as the reference.

## METHOD DETAILS

**Allergic Inflammation Model—**Mice were sedated with isoflurane. 25 μg of *Aspergillus* rAlp1 or heat-inactivated (95°C, 10 min) control protease in 25 μL of PBS was placed into the mouse pharynx. 6.25 μg of Sigma-Aldrich fungal proteinase, 12.5 μg of subtilase, 50 μg of papain, or 25 μL of *Aspergillus* culture filtrate were also used in some of these studies, where indicated. The tongue of the mouse was retracted with a forceps until the solution was completely aspirated into the lungs. This procedure was repeated on days 1, 2, 7, and 14 post-initiation. Lungs were harvested 24 h after the last dose and processed for histology or flow cytometry. The protocol was also modified to track antigen-specific T cells. $10^6$ splenocytes from CD90.1+ OTII mice were injected into the tail vein 24 h prior to the initial protease treatment. 25 μg of ovalbumin was added to the protease inoculum, and all other aspects of the treatment remained unchanged.

**Histology—**Animals were euthanized by $CO_2$ asphyxiation. The lungs were inflated with 10% paraformaldehyde via the trachea, the trachea was sutured shut, and the lungs were

removed and placed in 10% paraformaldehyde for 1 h. For histological dyes, the fixed lungs were rinsed with PBS, embedded in paraffin, cut into 10 μm thick sections, and stained with periodic acid-Schiff or hematoxylin and eosin dyes. For immunofluorescence, the lungs were transferred to cryoprotectant (30% sucrose) and kept at 4°C for ~4 days or until the lungs fell to the bottom of tube (i.e., fully saturated). The preserved lungs were washed with PBS, embedded in optimal cutting temperature (OCT) compound, cut into 8 μm sections, and stored at −80°C. The slides were retrieved from −80°C storage, warmed to room temperature, soaked in PBS to remove OCT, and air-dried for 15 min. Slides were incubated with Animal-free Block and Diluent with anti-mouse Fab fragment antibodies for 1 h at room temperature in a humidified chamber. Slides were washed three times with PBS before adding primary antibodies at the indicated concentrations (see Key Resources Table). The slides were stored overnight at 4°C. The next day, the slides were washed three times with PBS, and the respective fluorophore-conjugated secondary antibody was added to the slides for 1 h at room temperature in a humidified chamber. The slides were washed once with PBS, and 1 μg/mL of DAPI was added to the slide for 10 min at room temperature. The slides were washed three times and allowed to air dry before adding hardset antifade reagent and coverslips. ~24 h later, a Nikon A1R confocal microscope was used to capture images of the slides. Images were processed with Fiji (Schindelin et al., 2012). For immunocytochemistry, we followed the manufacturer's instructions without modification. TRPV4 staining intensity in the bronchial biopsies was scored by a blinded, surgical pathologist on a scale from 0 (weak) to 3 (strong).

**Cellular Analysis—**3 μg of anti-CD45 APC-e780 antibodies were injected into the tail veins of mice to mark cells circulating in the blood vasculature. After 3 min, mice were euthanized by cervical dislocation. The lungs were harvested and minced with a gentleMACS (Miltenyi, San Diego, CA, USA) in digest solution, containing: RPMI-1640, 5% fetal bovine serum, 1500 U/mL type-1 collagenase, 1 mM $CaCl_2$, 1 mM $MgCl_2$. The samples were agitated at 37°C for 1 h and further disrupted by gentleMACS. The cell solution was passed through a 70 μm filter, pelleted, and resuspended in 40% Percoll-RPMI medium. A Percoll density gradient was created (40% top, 67% bottom), and the samples were centrifuged for 20 min at 650 x g. The leukocytes at the interface were removed, washed twice with PBS + 0.1% bovine serum albumin (i.e., FACS buffer). ~1/4 of each sample was placed in a 96 well plate. 1:500 LIVE/DEAD Fixable Dye and Fc block (1:100) was added to the samples for 10 min. The sample was washed and incubated for 1 h at 4°C with fluorophore-conjugated primary antibodies (see Key Resources Table). The samples were washed, resuspended in FACS buffer, and processed by flow cytometry (LSRFortessa, BD Bioscience, San Diego, CA, USA). 50,000 count beads were added to the sample immediately prior to data collection. The data were analyzed with Flowjo X. See Figure S2 for gating strategy.

**Mouse Treatments—**For IL-5 blockade and CD4+ T cell depletions, mice were injected into the peritoneal cavity with 1 mg of antibody on days −1, 6, and 13 post-inoculation with protease. For phagocyte depletion, mice were given 3 intranasal doses of clodronate liposomes, followed by a 24 h rest period before protease inoculation. To induce cre activity in Scb1a1-creERT2 mice, mice intraperitoneal injections of 2mg of tamoxifen on days −7 to

−4, −1, 6, and 13 days post protease inoculation. For AAV6 *in vivo* gene expression experiments, mice were infected with $10^{11}$ AAV6 weekly for 3 consecutive weeks before commencing protease treatment. For junction targeting experiments, 25 μg of poly L-arginine or poly L-glutamic acid were aspirated by mice on the same schedule as protease treatments. For thrombin inhibition experiments, mice were given 5U hirudin in the normal protease solution.

**In Vivo Antigen Capture/Presentation Assay—**100 μg of Eα-RFP (McLachlan et al., 2009) or RFP was instilled into the lungs of sedated mice. 6 h later, the mice were euthanized. The lungs were processed for flow cytometry, as explained above.

**Calcium Flux Flow Cytometry—**After euthanasia, 1ml of dispase was injected into the lungs of tamoxifen-treated Scgb1a1-creERT2 × LSL-Cas9 GFP mice. The lungs were removed, placed into collagenase solution, minced with a gentleMACS, and incubated with agitation for 30 min at 37°C. The lung cell suspensions were loaded with calcium dye (1 μM Indo-1, 1mM $MgCl_2$, 0.1% pluronic acid, 4mM probenecid) for 30 min at 37C. The samples were washed, stained with fluorescent antibodies, and finally suspended in calcium (1μM $CaCl_2$) containing running buffer. 15 s of events were collected on the cytometer before retrieving the sample, adding 100 μM final concentration of TRPV4 agonist, and replacing the sample in the cytometer for additional data collection.

**Cytokine—**Lungs from mice 0, 8H, 24H, 3d or 15d post-inoculation with Alp1 were excised, snap frozen in liquid nitrogen, and homogenized in 2 mL of Tissue Protein Extraction Reagent with Complete Protease Inhibitor Cocktail. The lung homogenate was pelleted, and the supernatant was collected and stored at 80°C until analysis. CCL2, IL-5, IL-25, IL-33, and TSLP were measured in lung lysates by ELISA.

**Co-immunoprecipitation—**Club and goblet cells were generated as previously described (Feldman et al., 2019). ~$2\times10^7$ cells collected from 2 T75 flasks were flash frozen in liquid nitrogen. Membrane fractions were obtained (Sokabe et al., 2010), and the immunopreciptation was performed according to the manufacturer's instructions.

**Ex Vivo Restimulation—**Lung leukocyte suspensions were incubated for 2 h with 100 μg of heat-inactivated protease in restimulation buffer (RPMI, 10% fetal bovine serum, 1X pen/strep, 1 μM CaCl2, Beta-mercaptoethanol). 1X Brefeldin A was added to the well for the remaining 4 h of incubation. At the conclusion, the cells were washed, stained with live/dead dye and surface antibodies, fixed and permeabilized with Foxp3 transcription buffer set, and incubated over night with antibodies to intracellular targets (i.e., Foxp3, IL-5, and GATA3).

**Epithelium Permeability—**125 μg of Sulfo-NHS-Biotin in 25 uL of PBS + 1mM $CaCl_2$ with protease or heat-inactive protease was aspirated into the lungs of sedated mice. After 1 h, the lungs were processed for histology, as described above. Barrier integrity of primary human epithelial cells grown *ex vivo* was also evaluated with two methods. 2 mg/mL of 4kDa FITC-dextran with serial dilution of protease were added to the insert of the ALI device and left overnight in a tissue culture incubator. 100 μL of solution from the lower chamber of the ALI device was collected and 485/530 nm fluorescence was measured with a

fluorimeter. Lastly, transepithelial electrical resistance was measured with a voltohmeter after 1 h of incubation with varying concentrations of Alp1.

**Enzyme Assays—**All enzymatic reactions were performed at room temperature in 25mM Tris-base + 15mM NaCl + 10mM $CaCl_2$, pH7.2. To assess cleavage activity, 2-fold serial dilutions of protease were incubated with 2.5mg/mL FITC-in an opaque 96-well plate. 485/530nm fluorescence intensity was detected after 15 min. 100 μg human E-cadherin or 5 μg C3 was incubated with 500ng of Alp1 for 1 h, and western blots were performed with the reactions. 10mM of protease activated receptor peptides or 20mg/mL of human fibrinogen was incubated with 1mg/mL Alp1, 500U thrombin, or 10mg/mL trypsin for one h. The solutions were analyzed by MALDI-TOF.

**Culture Filtrates—**100 million spores of *Aspergillus fumigatus* strains CEA10 and Af293 were grown in 100mL of DMEM+ 10% FBS for 2 days or glucose minimal medium (GMM) for 5 days at 37C with agitation. The culture supernates were sterile filtered and concentrated 20-fold with 10kD centrifugal filter.

### QUANTIFICATION AND STATISTICAL ANALYSIS

In the COAST patient cohort, associations between genotype and rates of allergic sensitization and asthma were assessed with an additive genetic model using the chi-square test for trend in proportions. All other statistical tests were Mann-Whitney U test with Bonferoni adjustment for multiple comparisons when > 2 groups were compared. $p < 0.05$ was deemed statistically significant.

### DATA AND CODE AVAILABILITY

This study did not generate/analyze any large-scale datasets or computer code.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Abbreviations

| | |
|---|---|
| **AAV** | Adeno-Associated Virus |
| **ALI** | Air-Liquid Interface |

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|---|---|
| **Alp1** | Alkaline protease 1 |
| **Arg** | Arginine |
| **CCL** | Chemokine (C-C motif) Ligand |
| **CCR** | Chemokine (C-C motif) Receptor |
| **Cdh1** | E-cadherin |
| **CNB1** | Calcineurin |
| **CNO** | Clozapine N-Oxide |
| **DAMP** | Damage-Associated Molecular Patterns |
| **dpi** | days post-inoculation |
| **Dex** | Dextran |
| **EC** | Extracellular |
| **Eos** | Eosinophils |
| **Glu** | Glutamate |
| **H&E** | Hematoxylin/Eosin |
| **HBEC** | Human Bronchiolar Epithelial Cells |
| **IL** | Interleukin |
| **ILC** | Innate Lymphoid Cell |
| **IKK** | Inhibitor of Nuclear Factor kappa-B Kinase Subunit beta |
| **lncRNA** | long non-coding RNA |
| **LSL-GFP** | Lox-Stop-Lox Green Fluorescent Protein |
| **MHC** | Major Histocompatibility Class |
| **MFI** | Mean Fluorescence Intensity |
| **Mo-DC** | Monocyte-derived Dendritic Cells |
| **NF-κB** | Nuclear Factor kappa-light-chain-enhancer of Activated B cells |
| **Ova** | Ovalbumin |
| **PAS** | Periodic Acid-Schiff |
| **PAR** | Protease Activated Receptor |
| **RFP** | Red Fluorescent Protein |
| **Rag** | Recombination activating gene |

| | |
|---|---|
| **Scgb1a1** | Secretoglobin |
| **TEER** | Electrical Resistance |
| **THC** | T helper cell |
| **TSLP** | Thymic Stromal Lymphopoietin |
| **TRPV4** | Transient Receptor Potential cation channel subfamily V member 4 |

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## Highlights

- An inhaled fungal protease allergen damages the junctions of bronchiolar club cells
- The mechanosensor TRPV4 senses the junction injury, triggering allergic inflammation
- TRPV4 is sufficient for inflammation in mice and linked with fungal asthma in humans
- Calcineurin mediates TRPV4-dependent calcium signalling within bronchiolar club cells

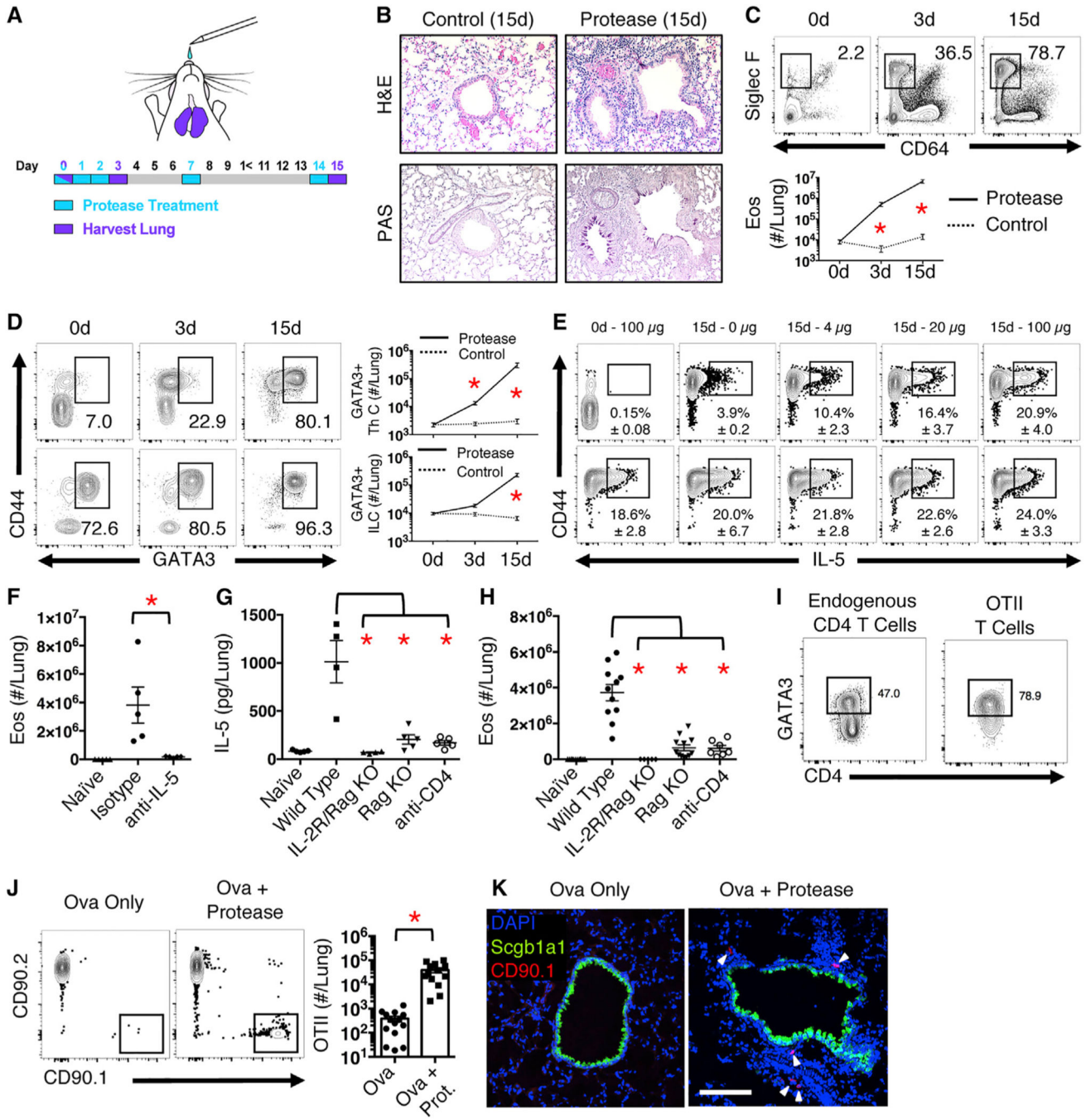


**Figure 1. Inhalation of *Aspergillus* Alkaline Protease 1 Induces Helper T Cell-Dependent Eosinophilia**

(A) Protease treatment schedule.

(B) Lung sections indicating leukocyte infiltration (H&E) and mucous production (PAS) of conducting airways of mice 15 dpi with protease or control (heat-inactiveprotease).

(C) Siglec F+ CD64 eosinophil accumulation in the lungs of protease-treated mice.

(D) ThC (top) and ILC (bottom) expression of GATA3 in response to protease.

(E) IL-5 production by ThC (top) or ILC (bottom) upon ex vivo restimulation with indicated quantities of heat-inactivated protease.

(F) Eosinophils in the lungs of mice 15 dpi (or naive) after treatment with anti-IL-5 antibody (or isotype control).
(G and H) (G) IL-5 or (H) eosinophil quantification in lungs of protease-treated (or heat-inactive control), wild type, and knockout animals at 15 dpi.
(I) Flow plot with GATA3 expression of CD90.1+ OTII cells (left) and CD90.2 CD4+ endogenous cells (right).
(J and K) (J) Flow plots or (K) immunofluorescence of CD90.1 OTII T cells (arrow heads) in lungs of mice 15 dpi. Scale bars: 100 nm.
Statistics: unpaired, Mann-Whitney U test with Bonferroni correction; *$p < 0.05$. Error bars are standard error of the mean. (B) and (K) are representative images of at least two independent experiments. Other panels provide compiled data of two or more independent experiments.

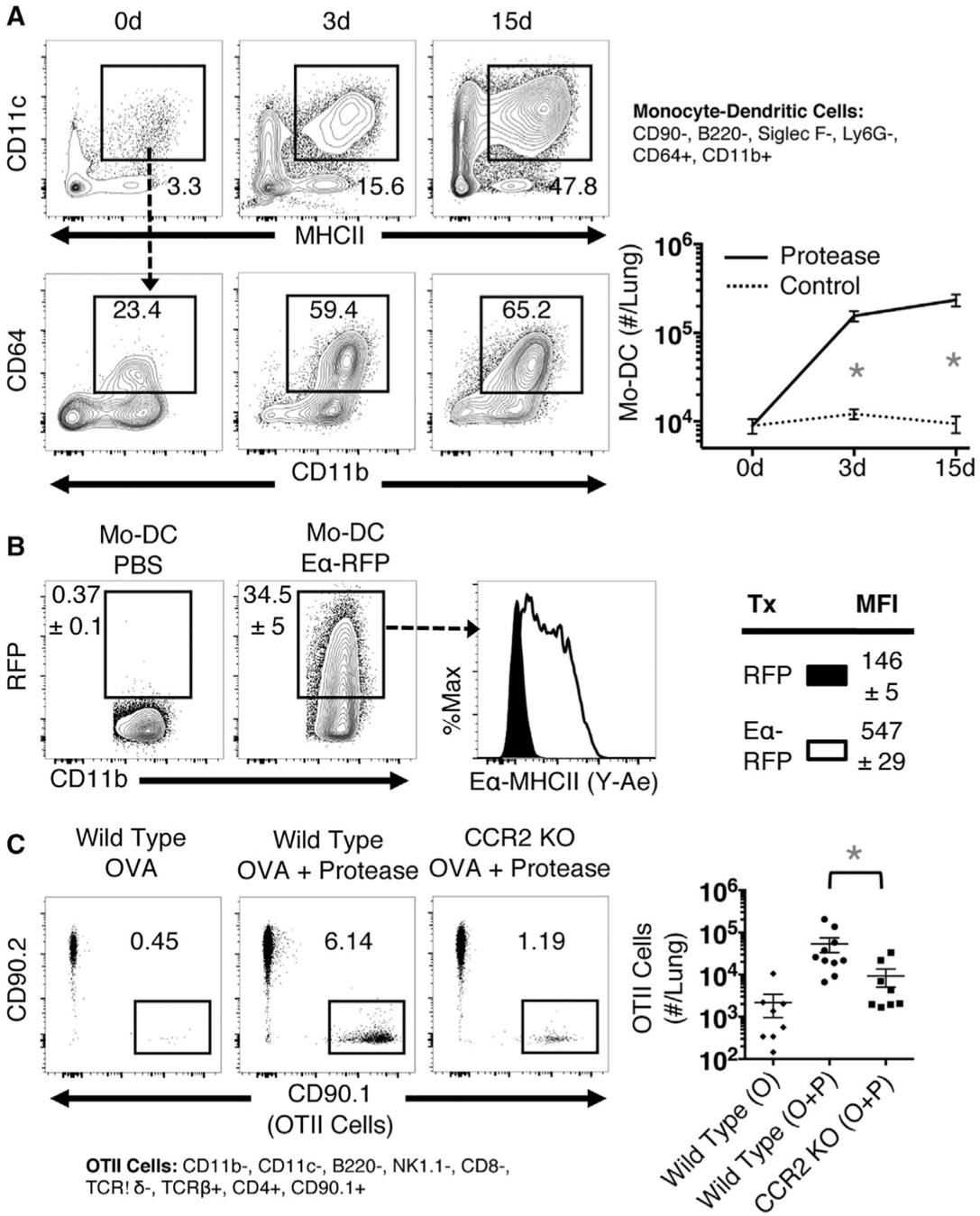


**Figure 2. Monocyte-Derived Dendritic Cells Capture and Present Antigen and Promote Accumulation of Pulmonary Helper T Cells in Response to Fungal Protease**

(A) Kinetic response of CD11c+ MHCII+ CD64+ CD11b+ Mo-DC after protease challenge.

(B) Mo-DC antigen uptake (top) and presentation on MHCII (bottom) 6 h post-inhalation of PBS, RFP, or EαRFP.

(C) CD90.2 endogenous and CD90.1 OTII helperT cells from lungs of wild type and CCR2$^{-/-}$ mice 15 days post-treatment with indicated antigens.

Statistics: unpaired, Mann-Whitney U test with Bonferroni correction; *p < 0.05. Error bars are standard error of the mean. (B) and (J) are representative images of at least two

independent experiments. All panels provide compiled data of two or more independent experiments.

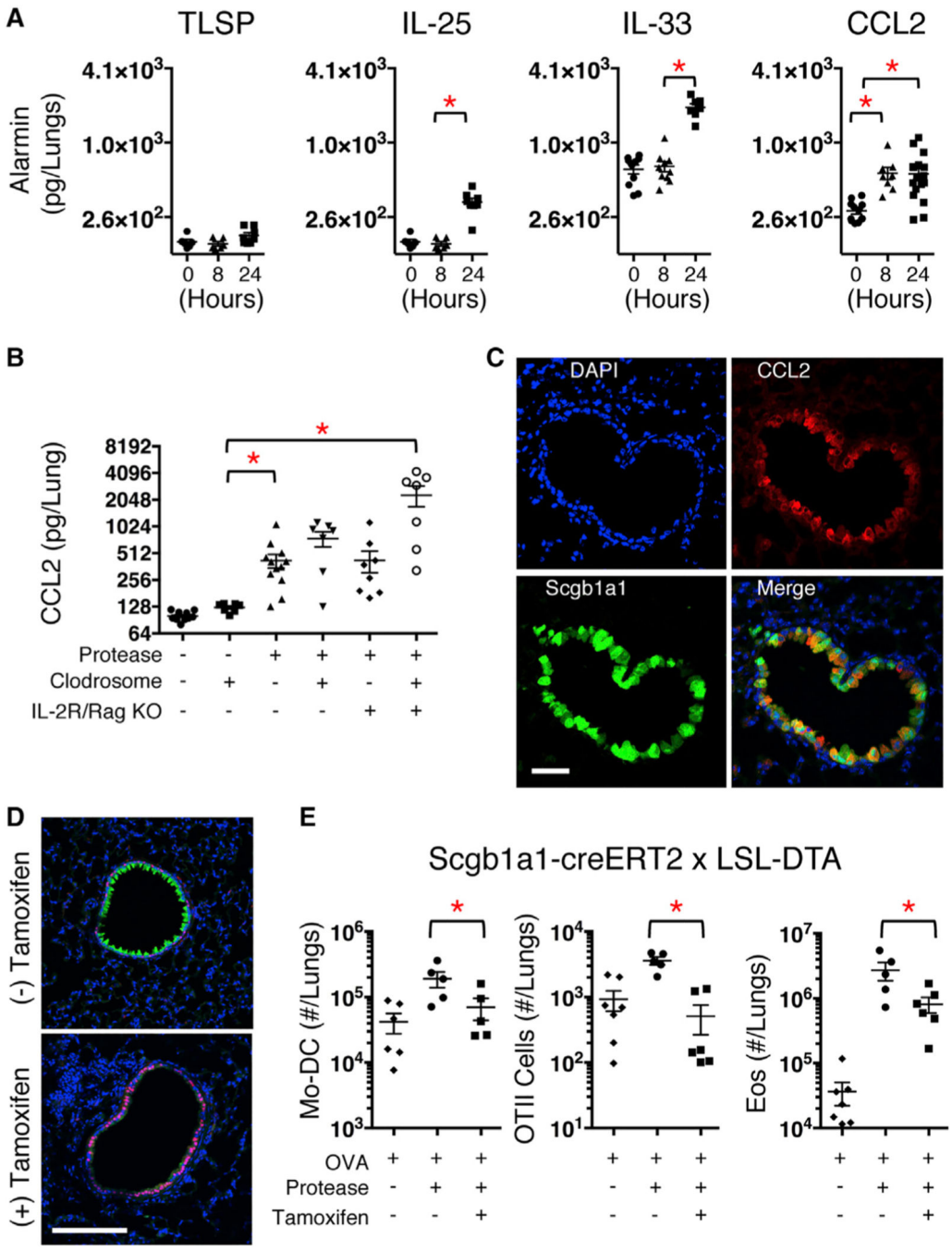


**Figure 3. Bronchiolar Club Cells Recruit Monocyte-Derived Dendritic Cells to the Lungs and Sensitize Allergic Response to Fungal Protease**

(A) Alarmins measured in lung lysates 0, 8, and 24 h post inoculation with Alp1.

(B) CCL2 in lung homogenates of protease-and control-treated wild-type or lymphocyte-deficienct mice. A subgroup of these mice received clodrosomes to deplete phagocytes in the lungs.

(C) Immunofluorescence images of club cells (Scgb1a1+) and CCL2 from mice treated with protease 6 h prior to harvest.

(D) Scgb1a1-creERT2 × LSL *Diptheria* toxin mice ± tamoxifen every day for 4 days. Green = Scgb1a1, Red = Foxj1, Blue = DAPI.
(E) Enumeration of Mo-DCs, OTII T cells, and eosinophils from lungs of club-cell-depleted mice and phenotypically wild mice. Scale bars: (C) 20 nm, (D) 50 nm.
Statistics: unpaired, Mann-Whitney U test with Bonferroni correction; *$p < 0.05$. Error bars are standard error of the mean. (C) and (D) are representative images of at least two independent experiments. Other panels provide compiled data of two or more independent experiments.

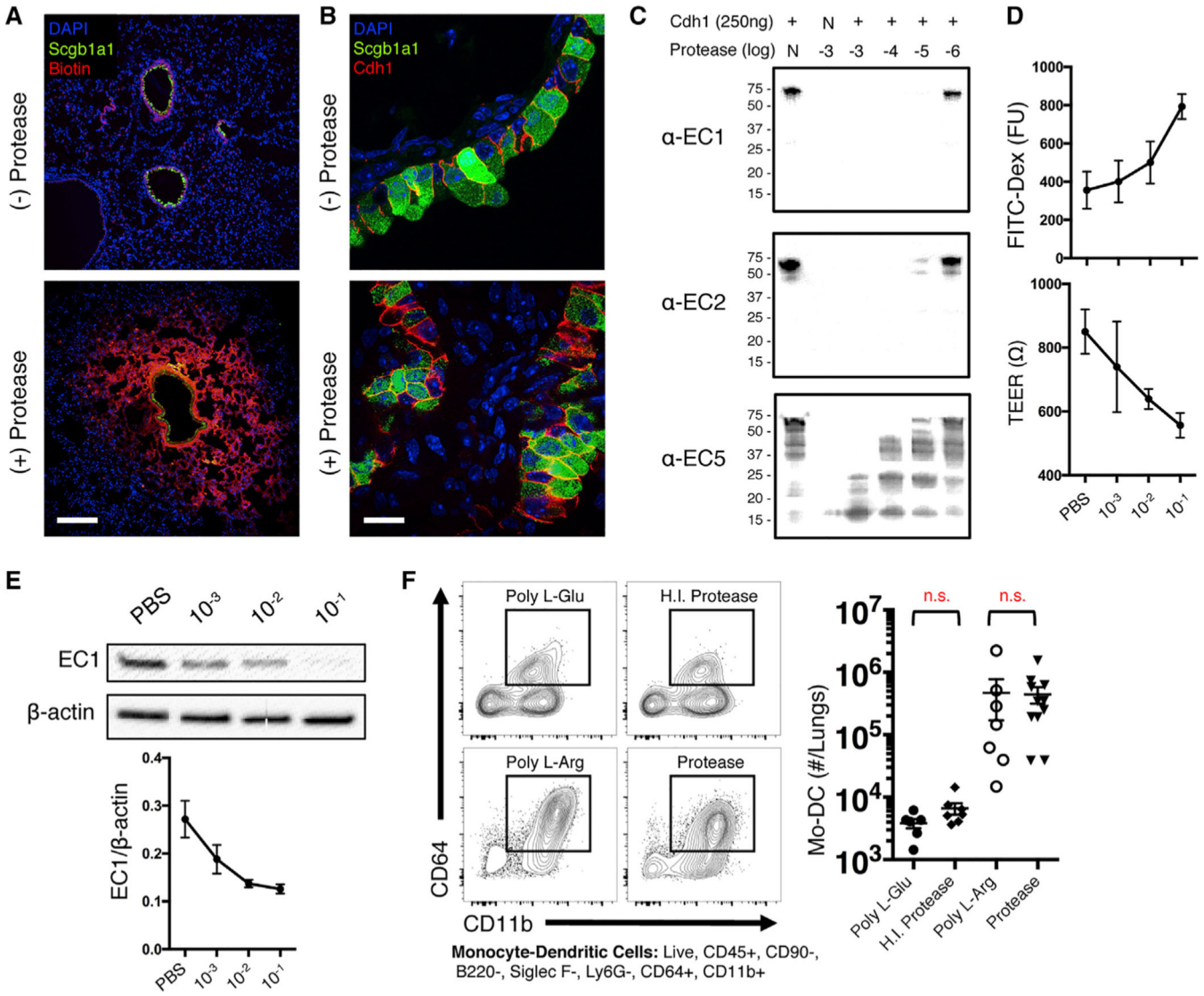


**Figure 4. Protease Damage to Epithelial Junctions in the Bronchioles Instigates Inflammation**

(A) Biotin leakage from the airways with (bottom) or without (top) Alp1 1 h post-treatment.

(B) Immunofluorescence of lungs 3 dpi.

(C) Purified Cdh1 incubated with log-fold dilutions of protease and blots probed with monoclonal antibodies targeting Cdh1 extracellular domains.

(D) Primary human lung epithelial cells grown in an air-liquid interface incubated with log dilution of protease. Barrier integrity was quantified by FITC-dextran leakage or TEER.

(E) Lysates from (D) were blotted for EC1.

(F) Mice received 25 μg of either Poly-L-Glu, heat-inactive protease, Poly-L-Arg, or protease for 3 consecutive days. Lungs were processed for flow cytometry, and the Mo-DC response quantified. Scale bars: (A) 100 nm, (B) 20 nm.

Statistics: unpaired, Mann-Whitney U test with Bonferroni correction; *$p < 0.05$. Error bars are standard error of the mean. (A), (B), and (C) are representative images of at least two independent experiments. Other panels provide compiled data of two or more independent experiments.

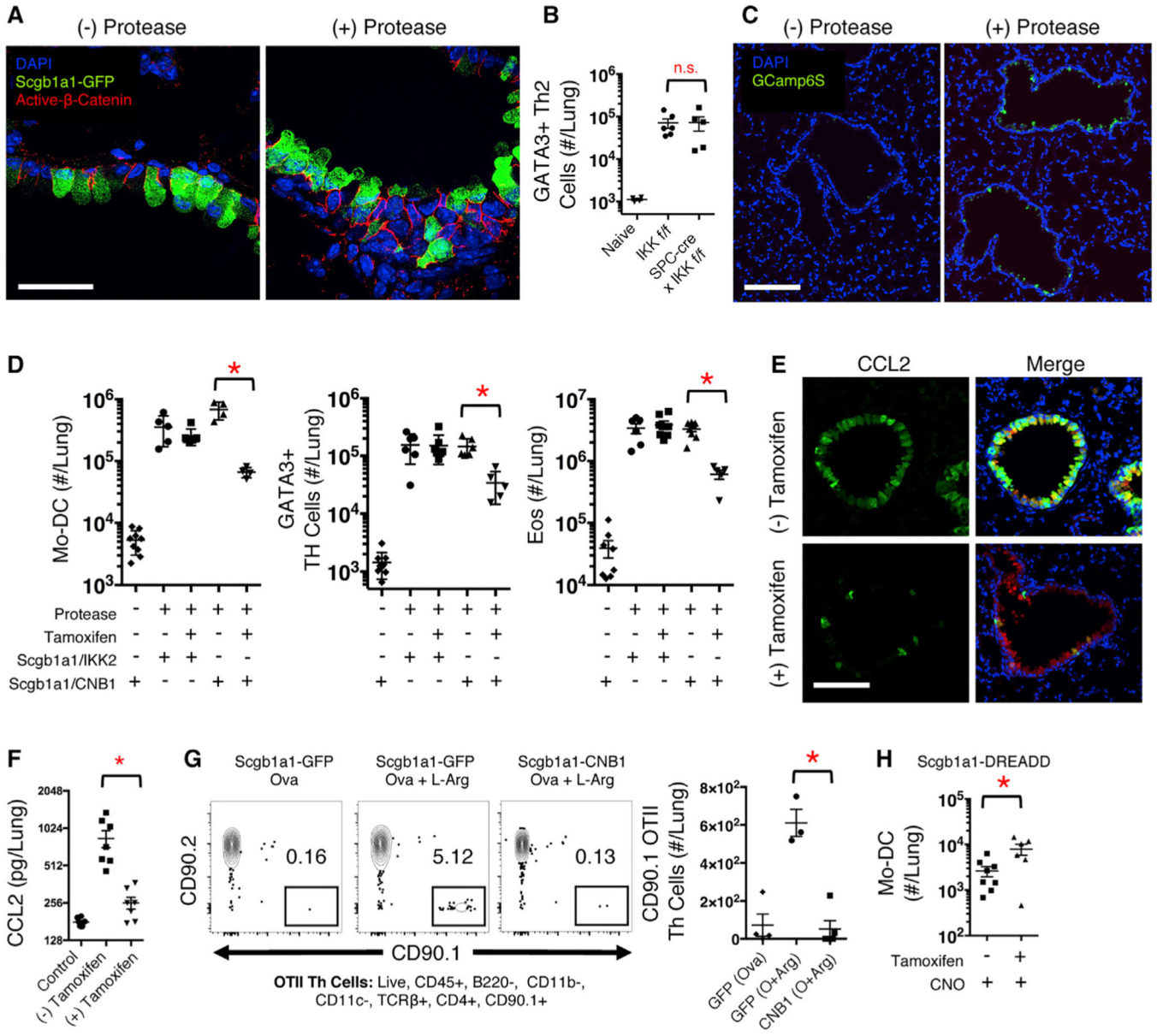


**Figure 5. Allergic Response to Alp1 Requires Club Cell Calcineurin**

(A) Immunofluorescence images of lungs 3 dpi.

(B) GATA3+ CD4+ T cells from lungs of mice with NF-κB deficiency in entire lung epithelium 15 dpi protease exposure.

(C) Scgb1a1-creERT2 × GCamp6S (calcium indicator protein) mice treated with control (left) or protease (right). Green = Calcium flux. Blue = Nuclei.

(D) Cellular response to protease 15 dpi in mice with club cell deficiencies in NF-κB (IKK2) or nuclear factor of activated T cells (CNB1) pathways.

(E) Immunofluorescence images collected from Scgb1a1-CreERT2 × CNB1-floxed mice treated with or without tamoxifen 3 dpi with protease. Green = CCL2, Red = Scgb1a1, Blue = DAPI.

(F) CCL2 quantified from lung lysates of Scgb1a1-CreERT2 × CNB1-floxed mice treated with or without tamoxifen, analyzed 3 dpi with protease.
(G) OTII response 15 dpi with Ova, with or without Poly-L-Arg in wild phenotype mice or mice with calcineurin-deficiency in club cells.
(H) Mo-DCs from Scgb1a1-CreERT2 × DREADD (hM3Dq) mice 3 dpi treatment with agonist (CNO). Scale bars: (A) 20 nm, (C) 100nm, (E) 50 nm.
Statistics: unpaired, Mann-Whitney U test with Bonferroni correction; *$p < 0.05$. Error bars are standard error of the mean. (A), (C), and (E) are representative images of at least two independent experiments. Other panels provide compiled data of two or more independent experiments.

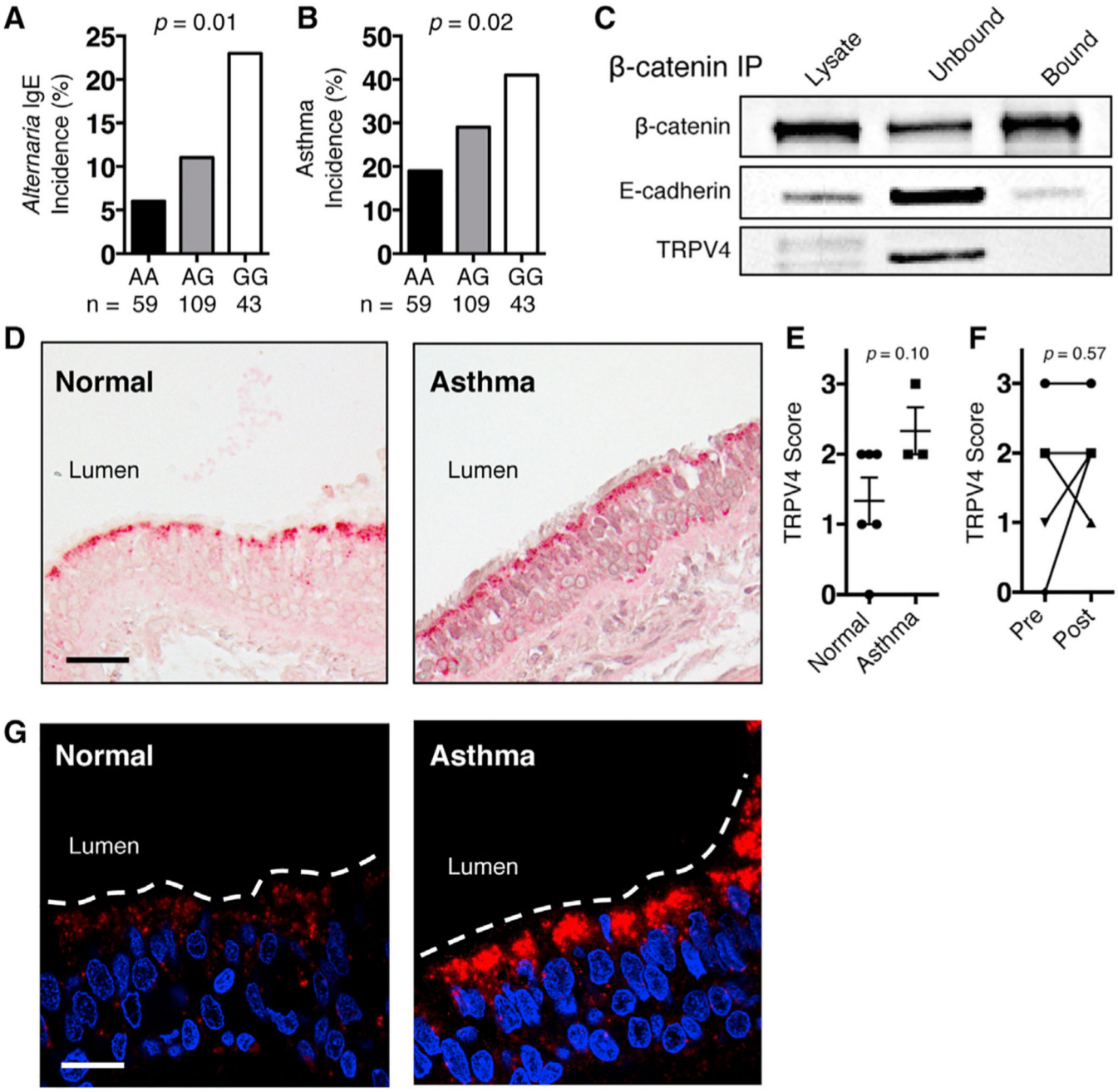


**Figure 6. Apical Membrane Expression of TRPV4 Is Associated with Fungal Sensitization and Asthma in Humans**

(A and B) Frequency and Chi-square test of the onset of (A) fungal-specific IgE at 3 years and (B) asthma at 6 years associated with SNP at position rs6606743 of *TRPV4*. N = 211 children.

(C) Western blots of lysates or membrane-enriched samples from human club and goblet cells subjected to immunoprecipitation with anti-β-catenin antibody.

(D–G) (D) Immmunocytochemistry or (G) immunofluorescence of bronchial biopsies from healthy donors (N = 5, Age = 23 ± 1.7 years, Sex = 3F, 2M) and patients with asthma (N =

3, Age = 25.3 ± 11 years, Sex = 3M). TRPV4 is indicated by red chromogen and red fluorescence. TRPV4 expression at (E) baseline or (F) pre-and post-segmental challenge with ragweed. Error bars are standard error of the mean.
Scale bars: 10nm. Unpaired, non-parametric t test.
(C) is a representative image of at least two independent experiments.

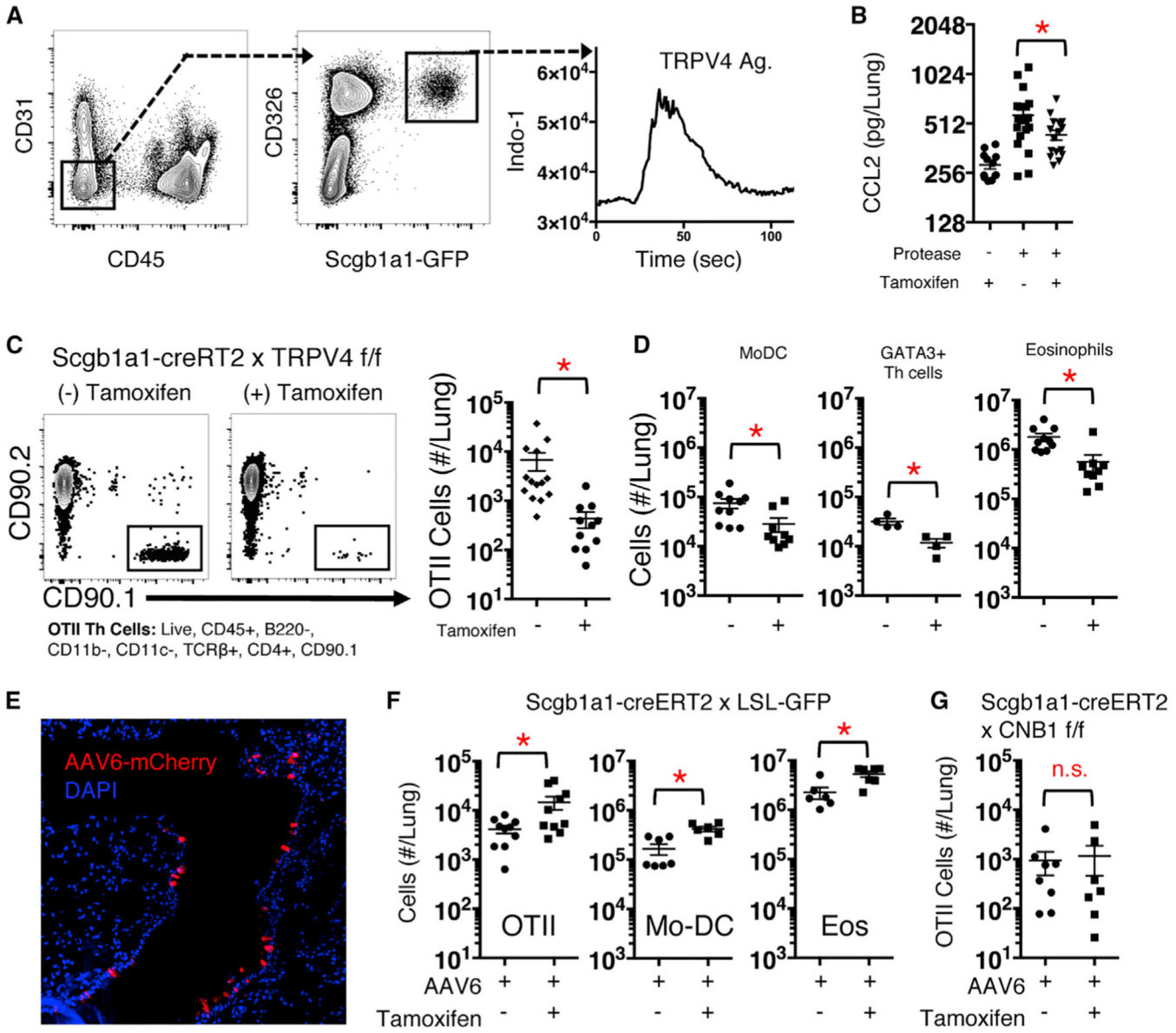


**Figure 7. Club Cell TRPV4 Signals Allergic Inflammation**

(A) Flow plots of lung cells from Scgb1a1-GFP mice loaded with a calcium indicator dye, Indo-1, and stimulated with a TRPV4 agonist GSK1016790A.

(B–D) (B) CCL2 (8 h), (C) CD90.1+ OTII cell, (D) GATA3+ Th cells, Mo-DCs, and eosinophils (15 dpi) response to protease in Scgb1a1-creERT2 × TRPV4-floxed mice.

(E–G) (E) Immunofluorescent images of lung sections from mice intranasally infected with AAV6-mCherry 3 weeks prior. CD90.1 OTII T cell, Mo-DC, and eosinophil response to protease 15 dpi in (F) Scgb1a1-creERT2 × LSL GFP or (G) OTII cell response in Scgb1a1-creERT2 × $CNB1^{fl/fl}$ mice. Scale bar: 50 nm.

Statistics: unpaired, Mann-Whitney U test with Bonferroni adjustment for multiple comparisons; *p < 0.05. Error bars are standard error of the mean. (A) and (D) are

representative data of multiple independent experiments. Other panels provide compiled data of two or more independent experiments.

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Antibodies | | |
| Active B-Catenin | EMD Milipore | 105–665 |
| Anti-Eα Biotin | Thermo | 13–5741-82 |
| B-catenin | BD | 610153 |
| B-catenin | Abcam | ab16051 |
| B220 AF700 | Biolegend | 103231 |
| Sav-BUV395 | BD | 564176 |
| C3aR | Hycult Biotech | HM1123–20UG |
| CC10 | Seven Hills Bioreagents | WRAB-3950 |
| CCL2 | Bio X cell | BE0185 |
| CCR6 AF647 | BD | 557976 |
| CD103 eFluor 450 | eBioscience | 48–1031-82 |
| CD11b BV650 | Biolegend | 301335 |
| CD11b AF700 | Biolegend | 301355 |
| CD11c PE-Cy7 | Biolegend | 117317 |
| CD11c AF700 | Biolegend | 117319 |
| CD19 PE-Dazzle | Biolegend | 115553 |
| CD28 | Biolegend | 102101 |
| CD31 PerCP-Cy5.5 | Biolegend | 102522 |
| CD326 (EpCAM) BV421 | Biolegend | 118225 |
| CD4 BUV395 | BD | 563790 |
| CD4 | Bio X cell | BP0003–1 |
| CD44 AF488 | Biolegend | 103015 |
| CD45 PE-Cy7 | Biolegend | 103113 |
| CD45 BUV395 | BD | 564279 |
| CD64 PE | Biolegend | 139303 |
| CD8 BV650 | Biolegend | 100741 |
| CD90.1 AF488 | Biolegend | 202505 |
| CD90.2 BV785 | Biolegend | 105331 |
| E-cadherin | Biolegend | 147301 |
| E-cadherin | Kerafast | EG1010 |
| E-cadherin (EC1) | Thermo | 13–5700 |
| E-cadherin (EC2) | Thermo | 13–1700 |
| E-cadherin (EC5) | Abcam | ab40772 |
| Foxj1 | ebioscience | 14–9965-80 |
| Foxp3 eFluor 450 | eBioscience | 48–5773-82 |
| GATA3 AF657 | BD | 560068 |
| GATA3 AF657 | Biolegend | 653809 |
| GATA3 eFluor 660 | eBioscience | 50–9966-42 |
| γδ TCR PerCP-Cy5.5 | Biolegend | 118117 |

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Goat Ig Cy3 | Jackson ImmunoRes | 705–165-147 |
| Goat Ig AF488 | Jackson ImmunoRes | 705–545-147 |
| Hamster Ig AF488 | Jackson ImmunoRes | 127–545-099 |
| hC3a | CompTech | A218 |
| IL-25R PE | eBioscience | 12–7361-82 |
| IL-33Ra PE | Biolegend | 146607 |
| IL-5 PE | Biolegend | 504303 |
| IL-5 | Bio X cell | BE0198 |
| Ly6C BV510 | Biolegend | 128033 |
| MHCII AF700 | Biolegend | 107621 |
| Mouse Ig Cy3 | Jackson ImmunoRes | 715–165-151 |
| Mouse Ig AF488 | Jackson ImmunoRes | 715–545-151 |
| NK1.1 AF700 | Biolegend | 108729 |
| NK1.1 eFluor 450 | Biolegend | 108731 |
| Non-phospho B-catenin | Cell Signaling | 8814S |
| Rabbit Ig Cy3 | Jackson ImmunoRes | 111–165-144 |
| Rabbit Ig AF488 | Jackson ImmunoRes | 111–545-144 |
| Rat Ig Cy3 | Jackson ImmunoRes | 712–165-153 |
| Rat Ig AF488 | Jackson ImmunoRes | 712–545-153 |
| Siglec F PerCP-Cy5.5 | BD | 565526 |
| TCRβ PE-Cy7 | Biolegend | 109221 |
| TER-119 AF700 | Biolegend | 116220 |
| TRPV4 | Alome Labs | ACC-034 |
| TruStain | Biolegend | 101319 |
| TSLPR BV421 | Biolegend | 151807 |
| Bacterial and Virus Strains | | |
| pAAV-CMV-DIO-TRPV4-p2A-ferritin-sNRPpA | Addgene | 74306 |
| AAV6 mCherry | Signagen | SL101273 |
| Biological Samples | | |
| *Pichia pastoris* Alp1 | This Manuscript | N/A |
| *E. coli* Eα RFP | Marc Jenkins (U of Minnesota) | (McLachlan et al., 2009) |
| *E. coli* RFP | This Manuscript | N/A |
| Chemicals, Peptides, and Recombinant Proteins | | |
| 4–15% polyacrylamide gel | Bio-rad | 4561084 |
| *Aspergillus melleus* proteinase | Sigma | P4032 |
| Brefeldin A | Sigma | B6542–5MG |
| Clodrosome | Clodrosome | Standard Mac Depletion Kit |
| Clozapin n-oxide hydrochloride | Tocris | 6422 |
| cOmplete, Mini Protease Inhibitor Cocktail tablets (25) | Sigma | 11836153001 |
| Counting Beads | Invitrogen | C36950 |

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| DAPI | Thermo Fisher | D1306 |
| Dispase | Corning | 354235 |
| Fibropeptide A TDTEDKGEFLSEGGGVR | Genscript | N/A |
| Fibropeptide B p-EGVNDNEEGFFSAR | Genscript | N/A |
| FITC-Casein | Fisher | 23267 |
| FITC-Dextran | Sigma | 46944–100MG-F |
| Foxp3 Staining Buffer Set | eBioscience | 00–5523-00 |
| GSK1016790A | Cayman Chemical | 17289 – 5mg |
| HBSS | Sigma | 55021C-1000ML |
| Hirudin (Thrombin Inhib) | Sigma | H7016–100UN |
| huC3 | CompTech | A113 |
| huC3a | CompTech | A118 |
| huE-cadherin (*E. coli*) | Sigma | 5085–100UG |
| Human Fibrinogen | Sigma | F3879–250MG |
| Indo-1 | eBioscience | 65–0857-78 |
| Live Dead (APC-780, Near IR) | Invitrogen | L-34975 (80 Assays) |
| Ova Albumin Grade 5 | Sigma | A5503 |
| Papain | Sigma | P4762 |
| PAR1 Cleavage Peptide RTDATVNPRSFFLRNPSENTFELVPLGDEE | Genscript | N/A |
| PAR2 Cleavage Peptide PGRNNSKGRSLIGRLETQPPITGKGVPVEP | Genscript | N/A |
| Percoll | Sigma | P1644–1L |
| Pluronic F-127 (20% in DMSO) | Fisher | P3000MP |
| Poly L Arginine | Sigma | P4663–50MG |
| Poly L Glutamic Acid | Sigma | P4636–100MG |
| Probenecid 25mM (10X) | AAT Bioquest | 20062 |
| Protease Assay | Fisher | 23266 |
| RTU Animal-free Blocking Reagent | Vector Labs | SP5035 |
| Streptavidin-BUV395 | BD Bioscience | 564176 |
| Stretavidin-AF555 | Thermo Fisher | S21381 |
| Sulfo-NHS-Biotin | Thermo Fisher | 21217 |
| Subtilase | Sigma | P5380 |
| Tamoxifen | Sigma | T5648–1G |
| Thrombin | Milipore | 605195–1000U |
| Tissue-Protein Extraction Reagent | Invitrogen | 78510 |
| Type 1 Collagenase | Worthington | LS004194 |
| Vectashield hardset | Vector Labs | H-1400 |
| Western ECL substrate | Bio-rad | 1705060S |
| Critical Commercial Assays | | |
| CCL2 ELISA | Thermo Fisher | 88–7391-88 |
| IL-5 ELISA | Thermo Fisher | 88–7054-86 |

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| IL-25 ELISA | Biolegend | 447107 |
| IL-33 ELISA | Invitrogen | 88–7333-22 |
| TSLP ELISA | Biolegend | 434107 |
| Co-immunoprecipitation Kit | Thermo Fisher | 26149 |
| VECTASTAIN Immunocytochemistry Kit | Vector Labs | D1306 |
| Experimental Models: Organisms/Strains | | |
| Mouse: GCamp6S$^{LSL}$: B6J.Cg-Gt(ROSA) 26Sortm96(CAG-GCaMP6s)Hze/MwarJ | Jackson Labs | 028866 |
| Mouse: C3$^{-/-}$: B6;129S4-C3tm1Crr/J | Jackson Labs | 003641 |
| Mouse: Cas9-GFP$^{LSL}$: B6;129-Gt(ROSA)26Sortm1 (CAG-cas9*,-EGFP)Fezh/J | Jackson Labs | 024857 |
| Mouse: CCL2$^{-/-}$: B6.129S4-Ccl2tm1Rol/J | Jackson Labs | 004434 |
| Mouse: CCR2$^{-/-}$: B6.129S4-Ccr2tm1Ifc/J | Jackson Labs | 004999 |
| Mouse: CD90.1: B6.PL-Thy1a/CyJ | Jackson Labs | 000406 |
| Mouse: Calcineurin$^{fl/fl}$: B6;129S-Ppp3r1tm2Grc/J | Jackson Labs | 017692 |
| Mouse: DTA$^{LSL}$: B6.129P2-Gt(ROSA)26Sortm1(DTA)Lky/J | Jackson Labs | 009669 |
| Mouse: hMR3$^{LSL}$: B6N;129-Tg(CAG-CHRM3*,-mCitrine) 1Ute/J | Jackson Labs | 026220 |
| Mouse: IKK2$^{fl/fl}$: B6 | Manolis Pasparakis (CECAD) | (Pasparakis et al., 2002) |
| Mouse: IL-2R/Rag2$^{-/-}$: B10;B6-Rag2tm1Fwa Il2rgtm1Wjl | Taconic | 4111 |
| Mouse: MyD88$^{-/-}$: B6.129P2(SJL)-Myd88tm1.1Defr/J | Jackson Labs | 009088 |
| Mouse: OTII: B6.Cg-Tg(TcraTcrb)425Cbn/J | Jackson Labs | 004194 |
| Mouse: PAR1$^{-/-}$: B6 | Harmut Weiler (Medical College of Wisconsin) | (Kerschen et al., 2007) |
| Mouse: PAR2$^{-/-}$: B6.Cg-F2rl1tm1Mslb/J | Jackson Labs | 004993 |
| Mouse: Rag1$^{-/-}$: B6.129S7-Rag1tm1Mom/J | Jackson Labs | 002216 |
| Mouse: Scgb1a1$^{creERT2}$: B6N.129S6(Cg)-Scgb1a1tm1(cre/ERT)Blh/J | Jackson Labs | 016225 |
| Mouse: SPC-cre x IKK2 floxed: B6 | Terry Wright (U of Rochester) | (Perez-Nazario et al., 2013) |
| Mouse: TLR23479$^{/}$: B6 | In house | (Tabeta et al., 2006) |
| Mouse: TRPV4$^{fl/fl}$: B6 | Wolfgang von Liedtke (Duke) | (Moore et al., 2013) |
| Software and Algorithms | | |
| FlowJo X | Treestar | FlowJo 10.6.1 |
| Fiji | ImageJ | https://fiji.sc |