Epigenetic regulation of epithelial dectin-1 through an IL-33-STAT3 axis in allergic disease

IL-33-STAT3 epigenetically represses dectin-1

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Abstract

Allergic diseases arise in susceptible individuals in part because of decrements in protective pathways. The mechanism by which these anti-inflammatory molecules become repressed remains unclear. We have previously reported that epithelial dectin-1 prevents aberrant type 2 responses and is downregulated in the epithelium of allergic patients. Here we report that dectin-1 is constitutively expressed by the respiratory epithelium in humans, and that IL-33 specifically acts as a repressor of dectin-1. Mechanistically, this occurs via IL-33-dependent STAT3 activation and the subsequent repression of the dectin-1 gene, CLEC7A. We have identified a novel enhancer region upstream of the proximal promoter of CLEC7A that is only accessible in epithelial cells, but not in hematopoietic cells. Epigenetic repression of CLEC7A through this newly identified locus, downstream of an aberrant IL-33-STAT3 axis, occurs in the epithelium of allergic individuals. Collectively, our data identify a mechanism of epigenetic fine-tuning of dectin-1 expression in epithelial cells that may participate in allergenicity.

Keywords: allergy, allergic diseases, IL-33, dectin-1, epithelium

Introduction

Allergic diseases such as asthma, allergic rhinitis, chronic rhinosinusitis with nasal-polyps (CRSwNP) are driven by dysregulated type 2 responses. There are several pathways that prevent overzealous activation of these responses and confer protection to the development of allergy. In susceptible individuals, it is thought that a insufficiency in these protective mechanisms can drive disease upon exposure to sources of allergens. Several molecular pathways, including pattern recognition receptors like CLEC10A, TLR2, TLR7/8, and TLR9, as well as well-known anti-inflammatory intracellular mediators like A20, have been shown to be protective in asthma[1-4]. Some of these pathways are epithellial-expressed and their activity can be decreased in allergic patients[2,5]. Consistent with this, we observed lower levels of dectin-1 in epithelial cells from individuals with asthma and CRSwNP and found that it protects against the development of allergic asthma by recognizing invertebrate tropomyosin in house dust mite (HDM) extract and by preventing the aberrant release of IL-33, an epithelial-derived type 2-promoting cytokine[6].

It is thought that in susceptible individuals, the combination of genetic and epigenetic mechanisms contributes to the decrease in protective pathways. Single nucleotide polymorphisms (SNP) have been associated with the regulation of asthma-protective genes. For example, a coding SNP in the A20 gene TNFAIP3 causes insufficiencies in protein function and increases the propensity to the development of asthma[2]. Further, we found an intronic SNP in the *CLEC7A* locus associated with lower expression of *CLEC7A* in children with asthma[6]. However, in addition to genetic mechanisms of gene regulation, several epigenetic modifications in pro-inflammatory and anti-inflammatory genes have been identified in patients with allergic diseases[7-10]. These epigenetic mechanisms can, in combination with SNPs, control gene expression in disease, and have also been shown to regulate these transcripts in the asthmatic epithelium[10-12]. Epigenetic pathways involve not only methylation and non-coding RNAs, but also histone modifications, which work in concert with repressors, enhancers and transcription factors to regulate gene expression. The importance of these mechanisms in allergy is especially evident in studies done in T cells. For example, enhancer regions in the genes coding for type 2 cytokines in T cells are controlled by transcription factors like STAT6 and GATA3 and confer susceptibility to allergic diseases[13,14]. Also, a recent report shows differentially regulated enhancer regions are associated with upregulated and downregulated genes in epithelial cells from asthmatics[12]. Despite this, little remains known about the epigenetic regulation of gene expression in the epithelium and the identity of external mediators that can influence this biology.

Based on this, we set out to determine whether there are epigenetic mechanisms that repress epithelial dectin-1 expression in allergy. Understanding how these restorative mechanisms become dysregulated is an essential aspect in expanding our knowledge of allergic disease pathogenesis.

Materials and Methods

<u>Mice</u>

BALB/c mice were obtained from Jackson Labs (Bar Harbor, Maine) and bred in our facility. *Il33*$^{cit/cit}$ mice on the BALB/c background (gift from Dr. Andrew McKenzie) were bred to BALB/c to generate heterozygous animals. *Il33*$^{cit/+}$ mice were used to generate homozygous progeny that was used for experiments. Mice were housed in a specific pathogen free animal facility in micro-isolator cages. Mice were provided autoclaved food (Lab diet 5010) and water ad libitum. All procedures were approved by the Animal Care and Use Committee of Johns Hopkins University.

HDM *in vivo* administration

Il33+/+ and *Il33cit/cit* mice were given PBS (40 ul) or HDM (40ul/50 ug) intratracheally on days 0 and 14. Mice were sacrificed on day 17 for analysis of dectin-1 expression.

Flow cytometry

Mouse lung cells were obtained by digestion of lung tissue with 0.05 mg/ml Liberase TL (Roche) and 0.5 mg/ml DNaseI (Sigma) for 45 min at 37°C in 5% CO_2. Digested tissue was filtered through a 70-um nylon mesh (BD Biosciences) and centrifuged. Pellet was resuspended in red blood cell lysis buffer (ACK lysis buffer). Recovered cells were counted (trypan blue exclusion), plated at 4-5x10^6 cells/ml. Cells were washed with PBS and labeled with Zombie Aqua live/dead dye (Biolegend) for 10 min at RT, and blocked with 10 ug/ml anti-CD16/32 (BioLegend or BioXCell) for an additional 20 min at RT. Cells were stained with fluorochrome-labeled antibodies as detailed in Table 1. Human sinonasal tissue was processed similarly to mouse lungs. Cells were labeled with live/dead dye (Zombie Aqua) for 10 min at RT and blocked with human TruStain FcX (BioLegend) for 20 min at RT. See Table 1 for a list of antibodies used. Data was acquired on an LSRII flow cytometer (BD Biosciences) and gated to exclude debris and to select single cells (SSC-W/SSC-A). Data was analyzed using FlowJo (BD Biosciences).

Human bronchial epithelial cell line culture

16HBE14o- (Sigma) and BEAS-2B (ATCC) were maintained in DMEM media (10% FBS). For transfections, 16HBE were seeded at 10,000 cells/well of a 96-well flat-bottom dish and transfected using FuGENE HD (Promega).

SNEC and NHBE cell culture

Human nasal epithelial cells and sinonasal mucosa were obtained from nasal brushings and tissue removed during endoscopic sinus surgery from control and patients with or without nasal polyps as previously described. The research protocol was approved through the Johns Hopkins Institutional Review process, and all subjects gave signed informed consent. Inclusion criteria included continuous symptoms of rhinosinusitis as defined by the AAO-HNS Chronic Rhinosinusitis Task Force for greater than 12 weeks, computed tomography of the sinuses revealing isolated or diffuse sinus mucosal thickening or air-fluid levels, and nasal polyps visible on diagnostic endoscopy. None of the subjects had a history of tobacco use, cystic fibrosis, ciliary dyskinesia, systemic inflammatory or autoimmune disease, or immunodeficiency. Characteristics of the subjects are summarized in Table 2. Isolated SNECs cells were grown in Bronchial Epithelial cell Growth Media (BEGM bullet kit, Lonza). At 80% confluency, cells were lifted using trypsin-EDTA (0.05%, Gibco) and seeded into a 96-well culture plate pre-coated with fibronectin-collagen. NHBE (Lonza) were cultured similarly to SNECs. Upon reaching 80-90% confluency, cells were starved overnight (BEGM media without the bovine pituitary extract-BEGMnoBP). Cells were treated with HDM (100 ug/ml) for 2h and cells were harvested for RNA. Where indicated, cells were pretreated for 1h with 5 ug/ml isotype or neutralizing monoclonal anti-hIL-33 antibodies (R&D Systems, MAB36254), followed by stimulation with HDM (100 ug/ml) for 2h. RNA was extracted (TRIzol), and reverse-transcribed to analyze *CLEC7A* expression.

Transfection of SNECs and NHBE

Transfection was done in BEGM media in 96-well dishes (65-85% confluent) with 0.098 ug *CLEC7A*.luc and 0.002 ug pGL4.PGK control vector per well (Promega) using Trans-IT X2 (Mirus). The following day, cells were starved in BEGMnoBP overnight. Media was removed, cells were rinsed once in PBS, and 150 ul/well BEGMnoBP was added, after 4-6h, 25 ul was collected for determination of secreted Nano-Glo, and cells lysed (25 ul/well, Passive Lysis Buffer, Promega) for detection of the firefly control (pGL4-PGK).

Dendritic cell culture

Human normal dendritic cells (Lonza) were cultured in 50 ng/ml GM-CSF and 50 ng/ml IL-4 for 4-5 days, after which they were stimulated with HDM (100 ug/ml) in combination with 10 ug/ml isotype control or neutralizing anti-IL-33 antibodies for 4h.

Plasmids

To generate *CLEC7A*.luc reporter vectors, a 3.5kb fragment of the human *CLEC7A* promoter region (chr12:10129421-10132905) was synthesized (GenScript) and cloned into the secreted Nano-Glo luciferase vector pNL1.3 (Promega). Mutation of the STAT3 binding site (wt: TTTCCAAGCAC, mut: TGGAAGGGCAC) in *CENSER* was performed commercially (GenScript). CENSER and proximal deletion constructs were done using the QuikChange mutagenesis kit (Agilent). Deletion were verified by gel electrophoresis.

Chromatin immunoprecipitation and ChIP-PCR.

0.5-1.0x10^6 epithelial cells (BEAS-2B) were trypisinized and fixed in 1% PFA (8 min) followed by quenching with 1 M glycine (10 min). Cells were washed twice in ice-cold PBS before storage at -80°C. Cells were lysed in 150 uL lysis buffer (1% SDS, 50 mM Tris-Cl (pH 8.1), 10 mM EDTA (pH 8.0)) and sonicated in TPX microtubes (Diagenode) in a 4°C-cooled water bath sonicator (Diagenode Bioruptor, HI setting, 30 sec ON/30 sec OFF) for 25-30 cycles to properly shear the DNA. Lysates were cleared by centrifugation (10 min, 18,000xg) and pre-cleared for non-specific binding with 40 ul BSA-blocked protein A/G magnetic Dynabeads (Invitrogen) for 4h at 4°C. Lysates were diluted 10-fold (1.1% Triton X-100, 0.01% SDS, 167 mM NaCl, 16.7 mM Tris-Cl pH 8.1, 1.2 mM EDTA) and incubated with 3.5 ug anti-STAT3 (clone ST3-5G7, Invitrogen) antibody overnight at 4°C. Antibodies were captured with 40 ul BSA-blocked protein A/G magnetic Dynabeads on a rocking platform for 4h at 4°C. Beads were washed in 1 mL low salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris pH 8.1, 150 mM NaCl), then, high salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris pH 8.1, 500 mM NaCl), lithium chloride buffer (0.25 M LiCl, 1% Nonidet P-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris pH 8.1), followed by 2 washes in Tris-EDTA (pH 8.1). Chromatin was eluted from the beads using 200 ul elution buffer for 15 min at RT (1% SDS, 0.1 M $NaHCO_3$), followed by de-crosslinking in 0.3 M NaCl at 65°C overnight. Chromatin was RNase-digested (30 min at 37°C), followed by proteinase K (1h at 56°C). Chromatin was purified using PCR purification columns (Qiagen). PCR was performed using various primers in the *CLEC7A* locus. All data was normalized to input and presented as enrichment over the *IGX1A* negative control region (primers from Qiagen).

Results

Respiratory epithelial cells express dectin-1

While the expression, regulation and function of prototypical PRRs such as TLRs have been extensively studied in the allergic epithelium, our understanding of the role of C-type Lectin Receptors (CLRs) in allergic diseases is mostly unclear and has majorly been derived from their expression on hematopoietic cells (macrophages, dendritic cells, etc). Exploration of c-type lectin receptors in human tracheal and bronchial epithelial cells reveals that several CLRs are constitutively expressed, including CLEC7A, the gene coding for dectin-1, suggesting that they may play an important role in regulating epithelial function (Fig.1a). The majority of these CLRs are poorly understood, and many have unknown function, especially on the epithelium. Dectin-1 is probably the best studied CLR and its function is relatively well understood in myeloid cells in context of fungal infections. However, we[6] and others[15-20] have reported expression of dectin-1 in epithelial cells, and found that it has a key role in preventing aberrant IL-33 release in

response to dust mite allergen. Further analysis of the CLEC7A locus using ENCODE data shows the transcribed exons across the human respiratory epithelium in nasal, tracheal, bronchial and airway epithelial cells (Fig.1b). These data support the notion of epithelial cells as important non-hematopoietic dectin-1-expressing cells. In humans and mice, respiratory epithelial cells can be divided into subsets that can be deconvoluted based on EpCAM and CD104 or CD49f expression[21-24]. Where $EpCAM^{+}CD104^{-}$ are luminal cells, $EpCAM^{+}CD014^{+}$ are luminal progenitors and $EpCAM^{lo}CD104^{+}$ represent basal cells. We sought to characterize the expression of dectin-1 in these subsets. To this end, we used human sinonasal tissue, and we found that luminal progenitor and basal epithelial cells have the highest levels of dectin-1-expressing cells, with marginal expression in luminal epithelial cells (Fig. 1c). Our findings demonstrate that the epithelium maintains tonic levels of dectin-1 across the respiratory system.

Allergen-induced IL-33 represses epithelial dectin-1

Our previous work has shown that dectin-1 is downregulated in the allergic epithelium, and that it functions to inhibits allergen-induced IL-33[6]. Based on this, we postulated that dectin-1 is repressed by an autocrine pathway. As IL-33 is enriched in allergic airway tissue, we hypothesized that epithelial-released IL-33 may counter dectin-1 expression. To begin to explore this, we treated cultured human sinonasal epithelial cells (SNECs) from nasal polyp patients with media or HDM and treated with isotype control or blocking anti-IL-33 mAbs for 24h. Interestingly, we observed that HDM reduced *CLEC7A* expression, and this repression was restored when IL-33 was neutralized (Fig. 2a). Interestingly, these culture conditions had no effect on expression of *CLEC7A* in human primary dendritic cells (Fig. 2b), suggesting a cell-type specific regulation of dectin-1. Consistent with this, we found that isolated mouse tracheal epithelial cells from IL-33 deficient ($Il33^{cit/cit}$) mice express more *Clec7a* than wildtype cells (Fig. 2c).

IL-33 represses epithelial dectin-1 *in vivo*

To further examine the regulation by IL-33 of dectin-1 *in vivo*, we exposed $Il33^{cit/cit}$ and $Il33^{+/+}$ animals to PBS or HDM. We find that lack of IL-33 results in an increase in the percentage of dectin-1^{+} basal epithelial cells in mice treated with HDM, when compared to control (Fig. 3a,b). Similarly, we find that the frequency of dectin-1^{+} luminal progenitor epithelial cells increases significantly in *Il33*-deficient animals after HDM exposure, as compared to IL33-sufficient mice (Fig. 3c,d). Surprisingly, we show that, while dectin-1 expression is abundant in myeloid cells, IL-33 does not appear to regulate it. Various myeloid populations were identified (see gating approach, SupFig. 1), and we found that the frequency of dectin-1^{+} alveolar macrophages (Fig. 3e), interstitial macrophages (Fig. 3f), and $CD11b^{+}$ dendritic cells (Fig. 3g) remains unaffected in $Il33^{cit/cit}$ animals. Overall, our data suggest that IL-33 represses the expression of dectin-1 specifically in epithelial cells and not hematopoietic cells, implying the involvement of cell-type specific epigenetic regulation of dectin-1. We did not observe that HDM caused a decrease in the frequency of mouse dectin-1^{+} epithelial cells, as it does in human nasal polyp-derived epithelial cells, suggesting that HDM may elicit non-epithelial factors that could promote survival or proliferation of lung epithelia. Nonetheless, these data overall demonstrate that allergen-induced IL-33 specifically represses epithelial dectin-1 in mouse and humans.

Identification of a functional epithelial-specific enhancer region in the *CLEC7A* loci

To understand the epithelial-specific regulation of dectin-1, we investigated its regulatory landscape using ENCODE data where we analyzed DNaseI hypersensitive sites (DHS-seq) from primary human epithelial cells from various tissues, as well as from hematopoietic cells such as $CD14^{+}$ monocytes and $CD1c^{+}$ dendritic cells. This analysis revealed a previously reported proximal promoter[25], which is active in both cell types, as demonstrated by an open chromatin configuration (Fig. 4a). In addition, we found a second, previously unreported, distal

site of open chromatin, seen only in epithelial cells, we refer to this region as the ClEc7a Nonhematopoietic-SpEcific Region-CENSER. This suggests that CENSER acts as a unique regulatory region, differentially regulating dectin-1 expression between non-hematopoietic and hematopoietic cells. To evaluate the function of these regulatory elements in controlling CLEC7A expression, we generated a 3.5kb promoter construct containing both the proximal and CENSER region upstream of secreted NanoLuc. We observed significant activity of this CLEC7A reporter construct in transiently transfected primary human sinonasal epithelial cells (Fig. 4b). Deletion of the proximal region attenuated *CLEC7A* promoter activity in primary human nasal epithelial cells, consistent with the important role of proximal promoters in regulating gene function (Figure 4b). Notably, removal of CENSER also reduced *CLEC7A* promoter activity in SNECs (Fig. 4b). Similarly, deletion of the CENSER region also leads to a decrease in *CLEC7A* promoter activity in human bronchial epithelial (NHBE) cells (Fig. 4c). Overall our data suggests a role for this CENSER region as an enhancer regulating dectin-1activity in epithelial cells.

In order to elucidate the mechanism through which CENSER region controls dectin-1 promoter activity we analyzed ENCODE ChIP-seq data, and this revealed this region to be a target of the transcription factors TFAP2A, TFAP2C, MYC, FOS, BACH1 and STAT3 (SupFig 2). We next assessed the levels of these CENSER-binding transcription factors (TFs) in cultured SNECs from control and CRSwNP patients (Fig. 4d-i). We found STAT3 mRNA to be significantly higher in the allergic epithelium (Fig. 4i). Consistently, exposure to HDM has been shown to activate STAT3 in mouse lung epithelial cells, leading to allergen-induced experimental asthma[26,27]. These data suggest a pro-allergic function for STAT3 where it could act as repressor of dectin-1 during aberrant Th2 responses.

STAT3 represses CLEC7A through CENSER.

In order to test the direct role of STAT3-CENSER interaction in controlling dectin-1 expression, we performed site-directed mutagenesis of the CENSER STAT3 binding site. We found that disrupting the STAT3 binding site significantly enhanced *CLEC7A* promoter activity in bronchial epithelial cell line (Fig. 5a), establishing the repressive function of STAT3 binding at the *CLEC7A* locus. Analysis of NCBI GEO data (GDS3106) also demonstrates that levels of *Clec7a* are higher in *Stat3*$^{-/-}$ cultured mouse lung epithelial cells, as compared to controls (Fig. 5b). We then determined whether pharmacological inhibition of STAT3 could upregulate CLEC7A in cultured CRSwNP SNECs. We found that HDM-mediated repression of *CLEC7A* in the allergic epithelium was completely restored by a STAT3 inhibitor (Fig. 5c). In total, these findings demonstrate that STAT3 is an inhibitor of dectin-1. Several reports have demonstrated that STAT3 is activated by IL-33[28-30], and based on that, we hypothesized that an IL-33-STAT3 axis acts to repress CLEC7A transcription. Next, we performed ChIP-PCR and, in line with our findings that IL-33-mediated activation of STAT3 represses the *CLEC7A* locus, we find that treating a bronchial epithelial cell line with IL-33 led to increased recruitment of STAT3, specifically to the CENSER and not the proximal region (Fig. 5d). Taken together, these findings reveal that an aberrant IL-33-STAT3 axis represses *CLEC7A* through its interaction with a newly described enhancer region. These data uncover a potential novel epigenetic mechanism underlying the regulation of CLEC7A in the respiratory epithelium.

Discussion

In susceptible individuals, allergic airway diseases like asthma and CRS are thought to develop due to the aberrant expression of pathogenic pathways. However, we now know that insufficiencies in protective mechanisms also contribute to disease. Recent reports have uncovered molecular pathways, including PRRs like dectin-1 and CLEC10A, that mediate

protection against aberrant type 2 responses[1-3,6] and where decrement in these pathways, many of which in the epithelium[2,6], result in a greater propensity to develop allergy. Thus, understanding the function and regulation of these protective epithelial sensors will be important to explain how individuals develop susceptibility to allergy.

Dectin-1, while traditionally associated with expression on macrophages and dendritic cells, is as we found, constitutively expressed by human respiratory epithelial cells, and it is also expressed by epithelial cells of other barrier surfaces like the skin, eyes, and gut[15-17,19,20,31-33]. Nevertheless, little remains known about the regulation and function of epithelial dectin-1. Reports have shown that stimulation of human corneal epithelial cells with heat-killed *Candida albicans* (HKCA) results in the upregulation of dectin-1[34], and that it is required for the secretion of anti-fungal peptidoglycan recognition proteins (PGLYRP). Consistent with this, live, but not heat- killed, *Aspergillus fumigatus* conidia upregulated dectin-1 in primary human bronchial epithelial cells (HBE) in a TLR2-dependent manner[35]. Furthermore, exposure of HBE to *A. fumigatus* resulted in dectin-1-dependent cytokine release[35]. These findings are in line with the well-documented role of dectin-1 as a receptor for fungal β-glucan moieties, where this interaction triggers anti-fungal responses.

In addition to epithelial dectin-1 expression being triggered by fungus, some have shown that it can be induced by TLR2 ligand-containing bacteria. Specifically, while *Mycobacterium tuberculosis* (Mtb) and *Staphylococcus aureus* induced dectin-1 expression in the human alveolar epithelial A549 cell line[31], TLR4-ligand containing bacteria, like *Escherichia coli,* could not activate dectin-1[31]. Functionally, siRNA-mediated knockdown of dectin-1 resulted in impaired cytokine response to Mtb and elevated Mtb colony forming units (CFUs)[31]. Others have shown that A549 cells overexpressing dectin-1 and infected with non-typable *Haemophilus influenzae* are driven to produce cytokines but this is abolished in cells expressing a non-functional dectin-1[20]. In the context of allergy, we have previously shown that in addition to these microbial ligands, the HDM protein allergen *Der p 10*, also known as invertebrate tropomyosin, is recognized by dectin-1 and this interaction prevent aberrant release of IL-33 by the epithelium[6]. Taken together, these data suggest that intact levels of epithelial dectin-1 promote anti-microbial responses and protect against allergic manifestations.

While some microbial interactions appear to drive dectin-1 expression in epithelial cells, the molecular mechanisms that regulate levels of epithelial dectin-1 are not understood. Although we have previously reported that an intronic SNP in *CLEC7A* is associated with lower gene expression in children with asthma[6], epigenetic influences in response to inflammatory mediators also significantly contribute to gene expression. This supports the longstanding view that gene-environment interactions, especially in the epithelium, are regulators of susceptibility to allergy.

We found that exposure to dust-mite allergen led to a decrease in CLEC7A mRNA in epithelial cells from patients with CRS, which was restored by neutralizing IL-33. Moreover, mice lacking IL-33 also display significantly more dectin-1^{+} basal epithelial cells and dectin-1^{+} luminal progenitors. Consistent with this, IL-33 and other type 2-promoting innate epithelial cytokines like TSLP and IL-25 are known to downregulate protective molecules in human keratinocytes including filaggrin and claudin-1 [28,29,36] and decrement in these pathways is thought to play a role in driving skin allergy. Thus, IL-33, IL-25 and TLSP may promote disease by decreasing protective mucosal mechanisms in addition to directly driving type 2 inflammation.

We now know that many PRRs are also epithelial cell-expressed, and reports have shown that they may function differently in the epithelium than they would on myeloid cells. For example,

key studies have reported that epithelial TLR4 is a key driver of type 2 responses to HDM[37,38], whereas its expression on myeloid cells promotes neutrophilic responses to dust mite[38]. In addition to cell-type functional difference, PRR expression may also be differentially regulated in hematopoietic and non-hematopoietic compartments. Here we find that while IL-33 represses epithelial dectin-1, blockade of IL-33 had no impact on haematopoietically derived dectin-1.

We found that the cell-type specific (dys)regulation of *CLEC7A* in the human epithelium originated in *CENSER,* an upstream regulatory region of the *CLEC7A* locus that is in an open chromatin configuration only in epithelial cells, and not in macrophages or DCs. Regulatory regions that are designed to control gene expression in immune versus non-immune cells have been reported before, for example, an epithelial-specific repressor in the *TLR4* locus has been found to maintain low levels of TLR4 in gut epithelial cells, while having no effect in monocytes[39]. Perhaps there is an evolutionary advantage to epigenetically regulate the expression of PRRs differentially in mucosal epithelial cells versus immune cells. For example, maintainance expression of protective PRRs, like dectin-1, while keeping pro-Th2 PRRs like TLR4 at low levels at the epithelial interface would favor homeostatic responses to environmental exposures.

Specifically, we found that *CENSER*, acting as an enhancer, is bound by several experimentally verified transcription factors. However, it remains unclear which TF acts as a driver of CLEC7A transcription through this region. We found STAT3 to be one of those CENSER-binding TFs, and as we find that STAT3 is significantly upregulated in nasal polyp epithelial cells as compared to controls, suggesting that STAT3 may promote type 2 responses. This is supported by other reports showing that epithelial STAT3 promotes type 2 inflammation in the lungs in response to HDM in mice[26], and that pharmacological blockade of STAT3 inhibits both IL-13 and IL-17A responses to HDM[27] . As STAT3 has been reported to act as a transcriptional repressor[40-42], in addition to several reports demonstrating that IL-33 activates STAT3[29,30,43], we postulated a role for an IL-33-STAT3 axis in inhibiting dectin-1 expression in epithelial cells. Our data shows that impairing STAT3 function enhances dectin-1 gene expression, and thus establishes STAT3 as repressor of dectin-1. Moreover, treating an epithelial cell line with IL-33 leads to the accumulation of STAT3 binding at CENSER, but not at the proximal promoter region. This is reminiscent of repression of the protective molecules filaggrin and claudin-1 in atopic dermatitis being thought to also be driven by IL-33-mediated STAT3 activation in human keratinocytes and human skin equivalent models[28,29]. This supports the notion that dysregulation of STAT3 in the allergic epithelium is a mechanism that disrupts homeostatic levels of protective molecules like dectin-1.

Taken together, our findings suggest the existence of a pathophysiologically relevant pathway that leads to diminishing of protective epithelial dectin-1 expression. We propose that aberrant production of IL-33 by respiratory epithelial cells from allergic individuals results in an autocrine-mediated increase in STAT3 activation. A dysregulated IL-33-STAT3 axis then targets the *CENSER* enhancer, only accessible in epithelial cells, to repress dectin-1 gene expression. Our results provide new insights into the mechanisms by which dysregulated innate epithelial cytokines may drive allergy through the reduction of protective pathways.

Author contributions

HMY and NG performed the experiments with help from DS and SMK. APL provided samples from control and CRS patients. NG and SL designed the experiments and performed data analysis. SL performed the *in silico* (ChIP-seq, DHS-seq, RNA-seq) analysis and wrote the manuscript.

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Table 1. Summary of CRS patient characteristics used

Table 1

Controls		
Age	**Sex**	**Nasal Polyps**
69	M	No
63	F	No
59	F	No
24	M	No
50	M	No
20	M	No
69	M	No
43	F	No
46	F	No
67	F	No
50	M	No
CRS		
Age	**Sex**	**Nasal Polyps**
46	M	Yes
63	M	Yes
68	F	Yes
49	M	Yes
31	M	Yes
47	M	Yes
54	M	Yes
56	F	Yes
57	M	Yes

Table 2. List of flow antibodies

Table 2

Antibody	Reactivity	Clone	Dilution	Company
CD45-APC-Cy7	mouse	30-F11	1/800	BioLegend
CD45-Alexa 700	mouse	30-F11	1/800	BioLegend
CD31-APC-Cy7	mouse	390	1/300	BioLegend
CD90-APC-Cy7	mouse	30-H12	1/200	BioLegend
EpCAM-PE-Cy7	mouse	G8.8	1/600	BioLegend
CD104-APC	mouse	346-11A	1/400	BioLegend
CD49f-FITC	mouse	GoH3	1/400	BioLegend
Dectin-1-PE	mouse	RH1	1/100	BioLegend
CD11c-BV786	mouse	N418	1/400	BioLegend
CD11b-PE-Cy7	mouse	M1/70	1/2500	BioLegend
Siglec-F-PerCP-Cy5.5	mouse	E50-2440	1/400	BioLegend
CD64-BV421	mouse	X54-5/7.1	1/150	BioLegend
MerTK-APC	mouse	2B10C42	1/150	BioLegend
IA-IE-Alexa 488	mouse	M5/114.15.2	1/3000	BioLegend
CD45-Alexa 700	human	2D1/HI30	1/500	BioLegend
CD31-APC-Cy7	human	WM59	1/500	BioLegend
CD90-APC-Cy7	human	5E10	1/500	BioLegend
EpCAM-PerCP-Cy5.5	human	9C4	1/250	BioLegend
EpCAM-APC	human	9C4	1/250	BioLegend
CD104-BV786	human	439 9B	1/250	BD
Dectin-1-PE	human	15E2	1/100	BioLegend
Zombie Aqua viability dye	all		1/1000	Biolegend

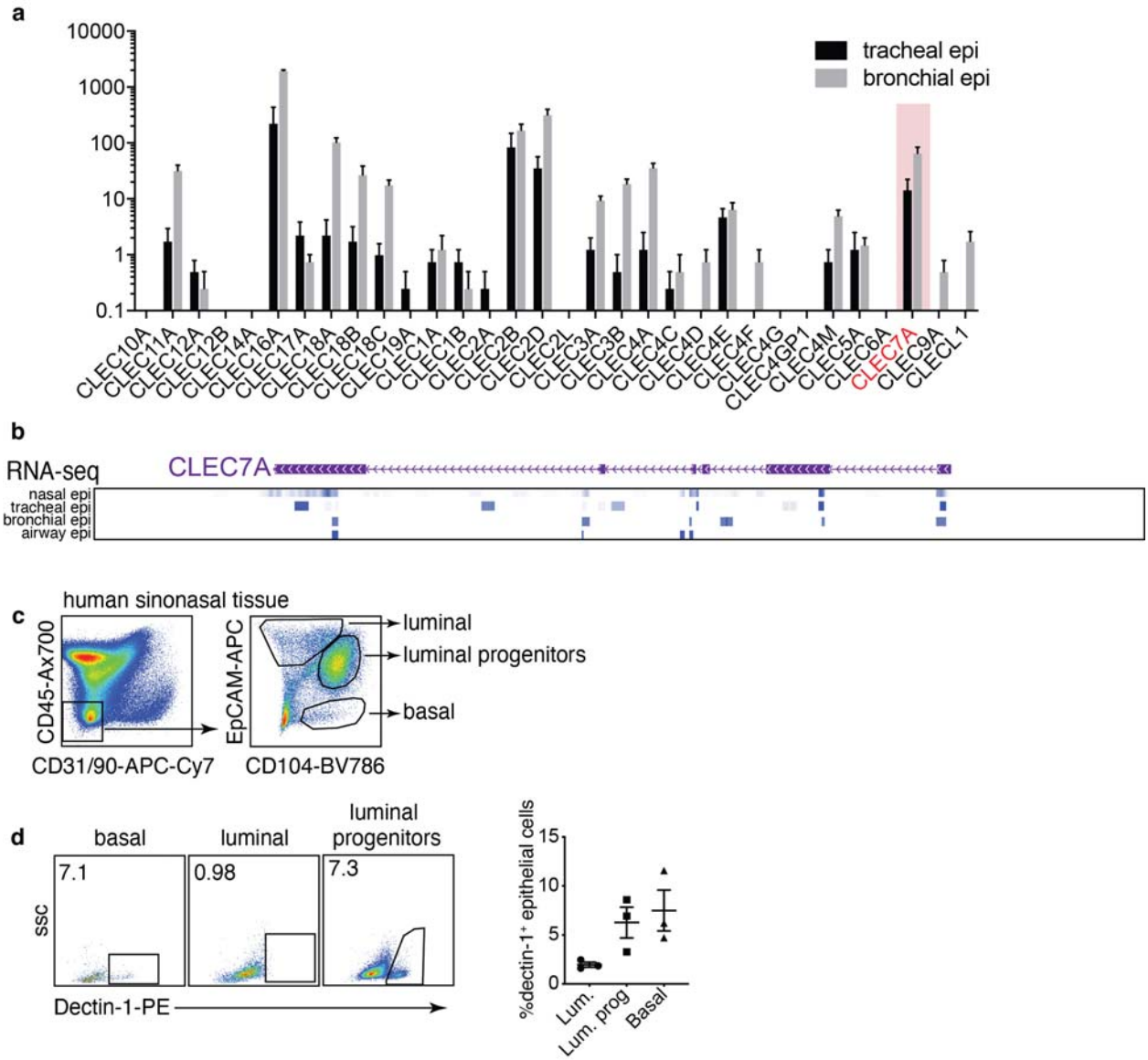


Figure 1 Respiratory epithelial cells express dectin-1. (a) Levels of c-type lectin mRNAs in human bronchial and tracheal epithelial cells from healthy donors. (b) RNA-seq from ENCODE highlighting CLEC7A exon transcription in nasal, tracheal, bronchial and airway epithelial cells. (c) gating scheme identifying various lung epithelial subsets by flow cytometry and (d) expression of dectin-1 in these populations. Data is means+SEM and pooled from 4 (a) or 3 (d) donors.

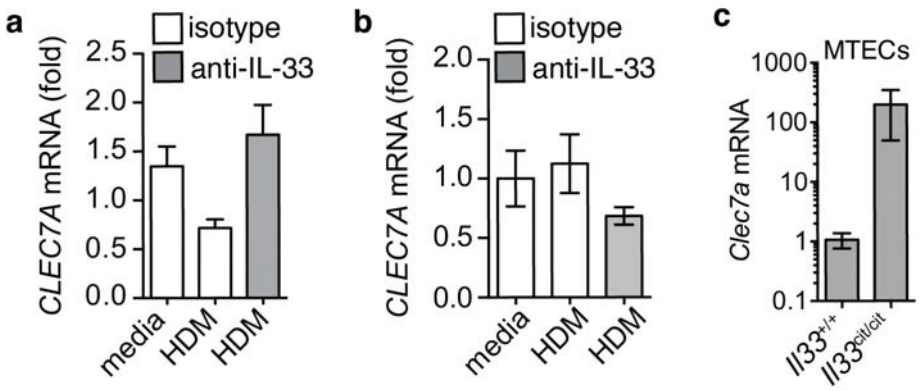


Figure 2. Allergen-induced IL-33 represses epithelial dectin-1. CLEC7A mRNA levels in (a) CRSwNP SNECs or (b) dendritic cells cultured in the presence of media, HDM (100 ug/ml) in combination with isotype or blocking anti-IL-33 antibodies (5 ug/ml) for 2h. Clec7a mRNA in cultured mouse tracheal epithelial cells from wildtype or *Il33*$^{cit/cit}$ mice. Data is means+SEM and represents representative data from 3 donors or 3 independent experiments.

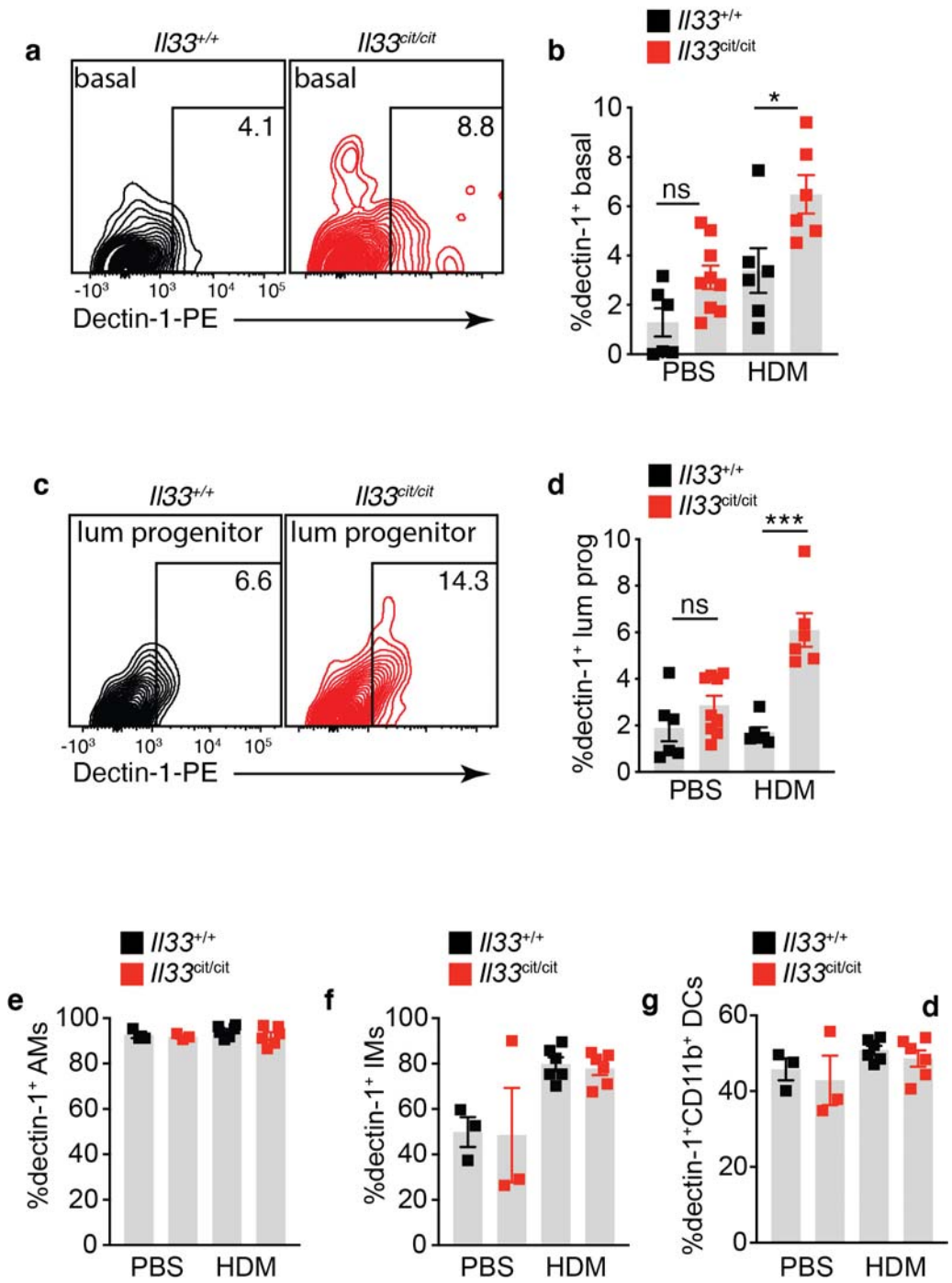


Figure 3. IL-33 represses epithelial dectin-1 *in vivo*. Il33$^{+/+}$ and Il33$^{cit/cit}$ were treated with PBS or HDM and lung dectin-1^{+} cells were analyzed. Dectin-1^{+} basal epithelial cells in (a) representative flow plots and (b) frequencies. Dectin-1^{+} luminal progenitors in (c) representative flow plots and (d) frequencies. Dectin-1+ (e) alveolar macrophages (f) interstitial macrophages and (g) CD11b^{+} dendritic cells. Data is means+SEM and representative of at least 2 independent experiments. Data was analyzed by one-way ANOVA followed by post-hoc test. *p<0.05; ***p<0.001.

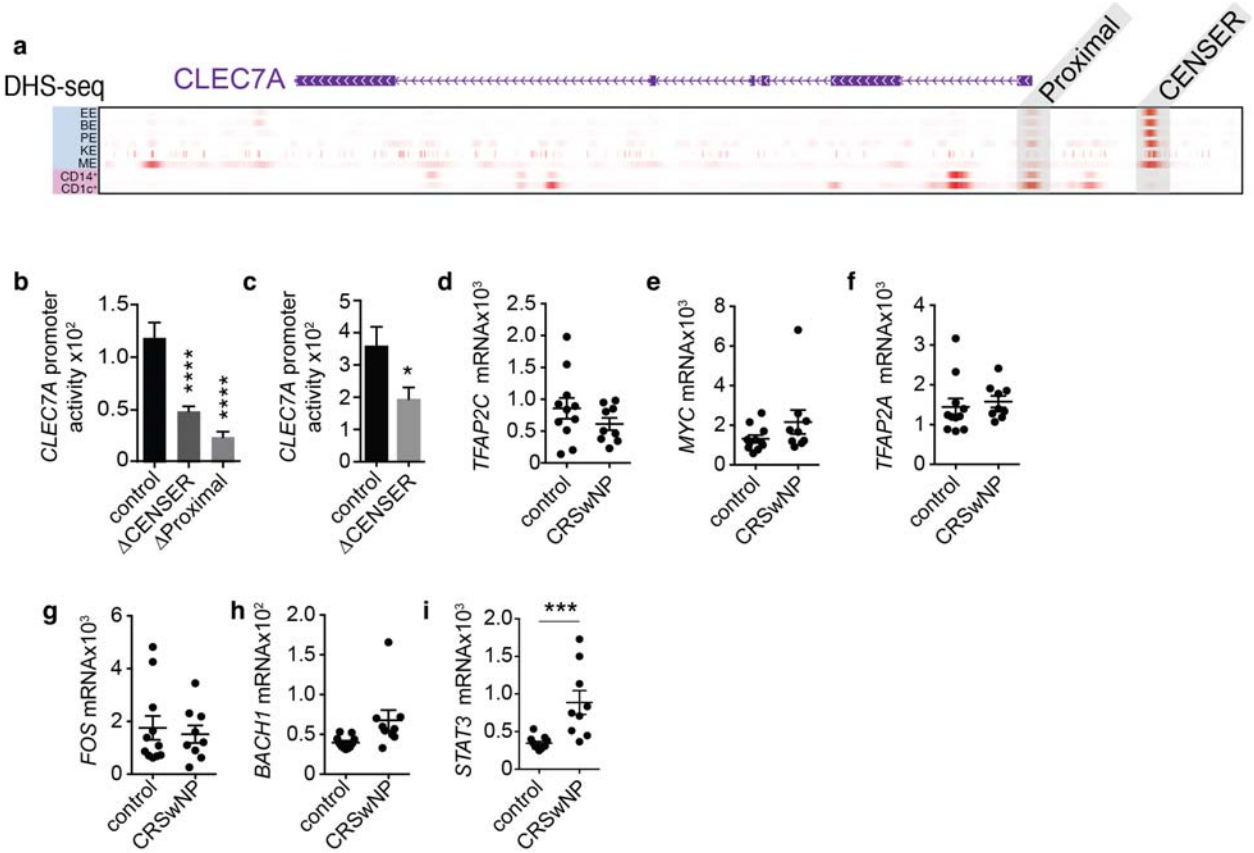


Figure 4. Identification of a functional epithelial-specific enhancer region in the *CLEC7A* loci. (a) DHS-seq data from ENCODE for the CLEC7A locus for primary esophageal (EE), bronchial (BE), prostate (PE), mammary epithelial (ME) cells, keratinocytes (KE), $CD14^+$ monocytes and $CD1c^+$ dendritic cells. CLEC7A promoter activity in (b) SNECs and (c) NHBE transfected with full-length or truncated promoter constructs. Levels of (d) TFAP2C, (e) MYC, (f) TFAP2A, (g) FOS, (h) BACH1, and (i) STAT3 mRNA in control and CRSwNP SNECs. Data is means+SEM and representative of 2-3 independent experiments. Data was analyzed by one-way ANOVA, Student's T test, or Mann Whitney test (control vs. CRSwNP). *$p<0.05$, ***$p<0.001$, ****$p<0.0001$.

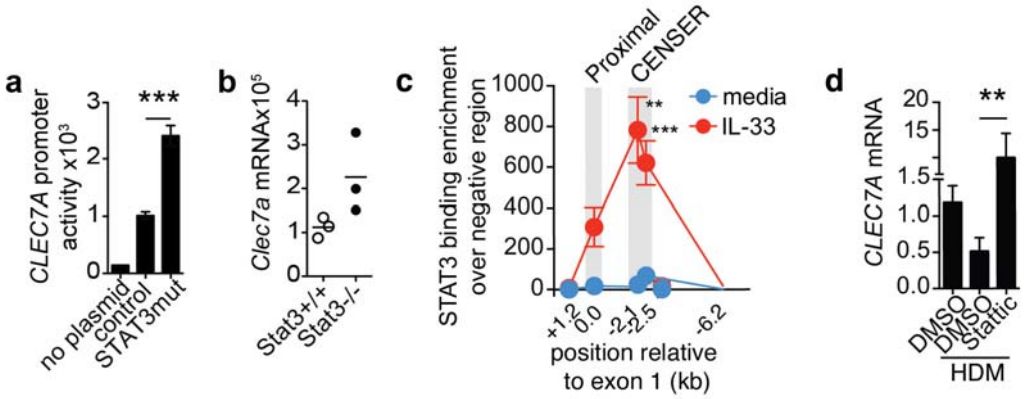
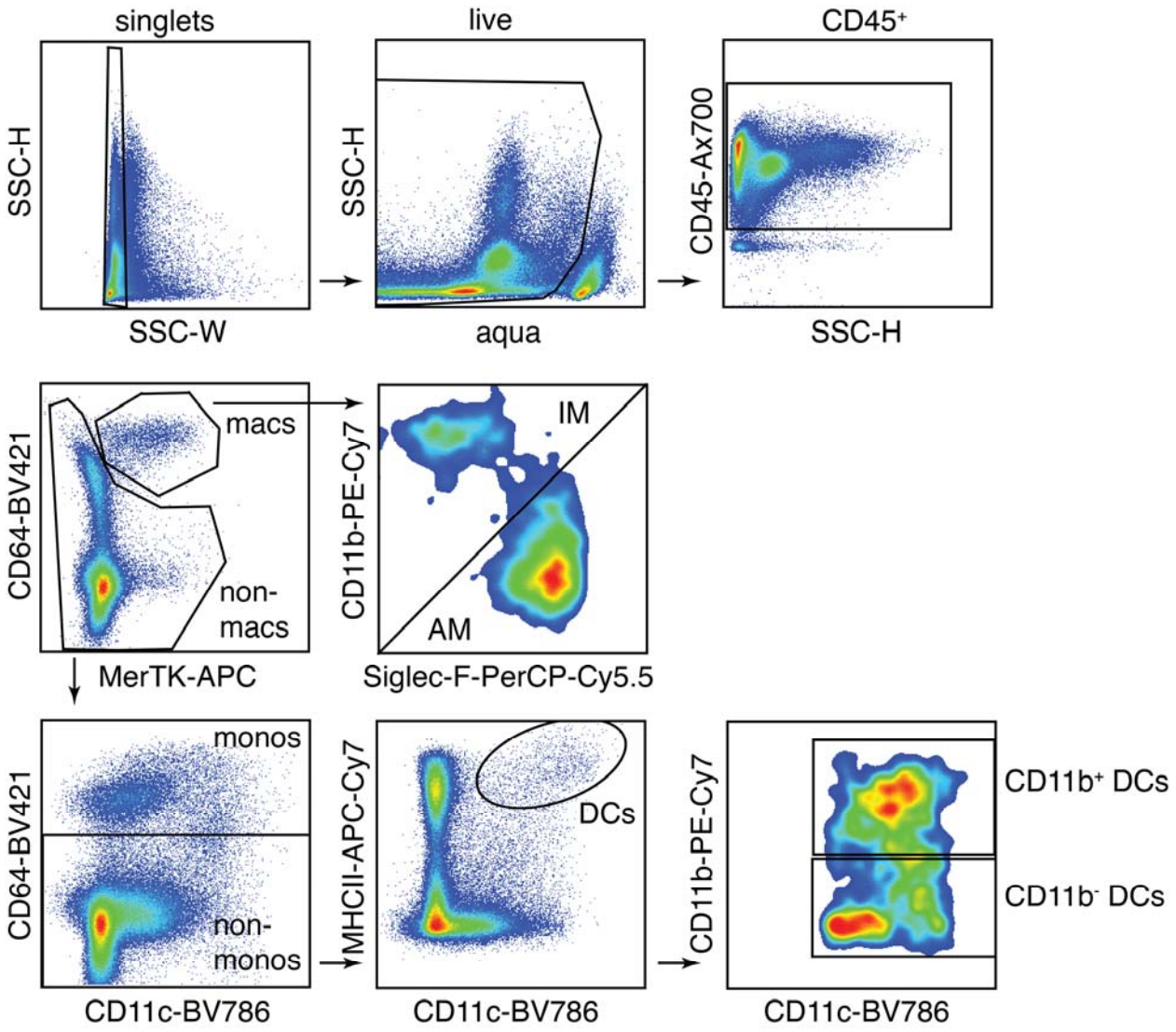


Figure 5. STAT3 represses CLEC7A through CENSER. (a) CLEC7A promoter activity in bronchial epithelial cell line transfected with full-length or STAT3-mutated constructs. (b) Clec7a mRNA in isolated lung epithelial cells from *Stat3+/+* and *Stat3-/-* mice. (c) STAT3 binding at the CLEC7A locus in bronchial epithelial cell line treated with media or IL-33 (10 ng/ml) for 2h. (d) CLEC7A mRNA in CRSwNP SNECs treated with HDM (100 ug/ml) and vehicle or 5 uM STAT3 inhibitor, stattic for 2h. Data is means+SEM and representative of 2-3 independent experiments (a, c) or of 2 patients (d). Data was analyzed by one-way ANOVA followed by post-hoc test. **p<0.01; ***p<0.001.



Supplementary Figure 1. Gating scheme for lung immune cells.

Supplementary Figure 2. ENCODE DHS-seq and ChIP-seq data for the human CLEC7A locus.

Author contributions

HMY and NG performed the experiments with help from DS and SMK. APL provided samples from control and CRS patients. NG and SL designed the experiments and performed data analysis. SL performed the *in silico* (ChIP-seq, DHS-seq, RNA-seq) analysis and wrote the manuscript.

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