Clara Cells Attenuate the Inflammatory Response through Regulation of Macrophage Behavior

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Chronic lung diseases are marked by excessive inflammation and epithelial remodeling. Reduced Clara cell secretory function and corresponding decreases in the abundance of the major Clara cell secretory protein (CCSP) are characteristically seen in these disease states. We sought to define the impact of Clara cell and CCSP depletion on regulation of the lung inflammatory response. We used chemical and genetic mouse models of Clara cell and CCSP deficiency (CCSP^{-/-}) coupled with *Pseudomonas aeruginosa* LPS elicited inflammation. Exposure of Clara cell-depleted or CCSP^{-/-} mice to LPS resulted in augmented inflammation as assessed by polymorphonuclear leukocyte recruitment to the airspace. Gene expression analysis and pathway modeling of the CCSP^{-/-} inflammatory response implicated increased TNF- α signaling. Consistent with this model was the demonstration of significantly elevated TNF- α in airway fluid of LPS-stimulated CCSP^{-/-} mice compared with similarly exposed wild-type mice. Increased LPS-elicited TNF- α production was also observed in cultured lung macrophages from CCSP^{-/-} mice compared with wild-type mice. We demonstrate that macrophages from Clara cell-depleted and CCSP^{-/-} mice displayed increased Toll-like receptor 4 surface expression. Our results provide evidence that Clara cells can attenuate inflammation through regulation of macrophage behavior, and suggest that epithelial remodeling leading to reduced Clara cell secretory function is an important factor that increases the intensity of lung inflammation in chronic lung disease.

Keywords: Clara cell; Clara cell secretory protein; inflammation; LPS; macrophage

Chronic lung diseases (CLD), such as chronic obstructive pulmonary disease (COPD) and asthma are a global health problem, with COPD expected to be the third leading cause of mortality by 2020, and asthma currently affecting 300 million people worldwide (1, 2). Pathological remodeling of the lung in CLD includes changes in the abundance and differentiated properties of the airway epithelium, subepithelial fibrosis, increased basement membrane thickness, and smooth muscle hypertrophy. These pathological alterations are accompanied by excessive inflammation contributing significantly to declining lung function, morbidity, and, ultimately, mortality (3, 4). The abundance of low molecular weight nonmucinous secretory proteins in airway lining fluid and serum have been directly correlated with severity of CLD (5). Levels of Clara cell secretory protein (CCSP;

CLINICAL RELEVANCE

This study defines a new role for Clara cell secretions in regulation of innate immunity. This finding is of clinical importance, as Clara cell abundance and secretory capacity are attenuated in the setting of chronic lung diseases.

Scgb1a1) are dramatically reduced in either serum or airway lining fluid of patients with COPD, asthma, bronchopulmonary dysplasia, silicosis, and post-transplant rejection (6–11). Even though these studies associate epithelial remodeling with progression of CLD, it is not clear if secretory cell dysfunction directly contributes to the disease process.

CCSP is the principal secreted product of the nonciliated bronchiolar Clara cell, and the founding member of the secretoglobulin family of secretory proteins (12, 13). Two different lines of CCSP^{-/-} mice have been developed that differ in their steady-state phenotypes, but each show evidence for alterations in their susceptibility to environmental agents and injury-related induction of tissue inflammation (14–17). CCSP^{-/-} mice mount an increased inflammatory response to live *Pseudomonas aeruginosa* administered intratracheally, as measured by increased total inflammatory cell exudates in bronchoalveolar lavage (BAL), as well as secretion of proinflammatory cytokines, implicating a role for CCSP regulation of innate immunity (18). However, these studies failed to define molecular mechanisms by which CCSP regulates lung inflammatory responses. The recent demonstration that CCSP deficiency leads to altered post-translational modification (PTM) of annexin A1 (ANXA1) within ciliated cells and macrophages suggests that epithelial and inflammatory cell function may be influenced by Clara cell secretions through a paracrine signaling mechanism (19).

The present study was designed to test the hypothesis that Clara cells regulate the lung response to inflammatory stimuli. We demonstrate that Clara cell depletion and CCSP deficiency enhance the LPS-elicited inflammatory response *in vivo*. Furthermore, we establish that macrophages from CCSP^{-/-} mice show increased LPS-stimulated TNF- α production and cell surface Toll-like receptor (TLR)-4 relative to macrophages from wild-type (WT) mice. These data suggest that Clara cells can attenuate the inflammatory response through regulation of macrophage behavior, and that reduced Clara cell secretory function seen in patients with CLD may directly enhance lung inflammation.

MATERIALS AND METHODS

Animal Husbandry

Inbred CCSP^{-/-} 129J, CCSP^{-/-} C57Bl6/J congenic, and WT FVB/N mice were maintained as in-house breeding colonies under specific pathogen-free conditions in an Association for Assessment and Accreditation of Laboratory Animals accredited vivarium. WT strain

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129Sv/Ev and C57Bl/6J mice were purchased directly from the supplier (Taconic, Germantown, NY and Jackson Laboratory, Bar Harbor, ME), and housed for a minimum of 2 weeks before experimentation. Mice were maintained on a 12 hours light/dark cycle and provided food and water *ad libitum*. Sentinel screening was conducted on a quarterly basis to determine pathogen status. For *in vivo* exposures, male 129Sv/ev mice aged 6–10 weeks were used. All *in vitro* experiments were conducted with male and female C57Bl/6 WT and CCSP^{-/-} mice aged 6–12 weeks. Animal procedures and cell isolations were approved by Duke University Medical Center and the University of Pittsburgh's Institutional Animal Care and Use Committee.

Naphthalene and LPS Dual Exposure

WT adult male C57Bl/6 mice and FVB/N were exposed to 250 mg/kg and 275 mg/kg naphthalene, respectively. Naphthalene was dissolved in Mazola corn oil (ACH Food Companies, Memphis, TN) and injected into the peritoneal cavity, as described previously (20). Groups that received a dual exposure were aerosol challenged with a 10-ng deposited dose of LPS 24 hours after naphthalene injection. Mice were recovered for 2 or 7 days after naphthalene treatment.

LPS Exposure

WT and CCSP^{-/-} 129Sv/ev mice (6–10 wk of age, male) were coexposed to *P. aeruginosa* 10 LPS (L8643; Sigma, St. Louis, MO), as previously described, resulting in an estimated deposited dose of 10 ng (21). LPS (5 mg) from *P. aeruginosa* 10 (L8643; Sigma) were reconstituted in 4 ml of sterile and pyrogen-free 0.9% NaCl (Hospira, Lake Forest, IL). Immediately before exposures, aliquots of 1.25 mg/ml LPS were diluted to 62.5 µg/ml in sterile 0.9% NaCl; 5 ml of the 62.5 µg/ml LPS solution were loaded into a jet nebulizer (DeVilbiss, Somerset, PA) and nebulized with breathing-quality air (Valley Natural Gases, Wheeling, WV) at 15 psi for 20 minutes. Animals were killed by administering general anesthesia to achieve a surgical plane followed by exsanguination.

Analysis of Polymorphonuclear Leukocyte Recruitment, Apoptosis, and Necrosis

Inflammatory cells were sampled by total lung lavage. Briefly, animals were cannulated with a 20-GA BD Insyte Autoguard intravenous Catheter. Each animal was lavaged a total of eight times with 1 ml of sterile PBS. Cells were collected by centrifugation at 4°C and resuspended in 1.0 ml PBS. Total cell counts were determined in technical triplicates using a Z1 Coulter Particle Counter (BeckmanCoulter, Fullerton, CA). Particles greater than 7 µm were counted to exclude red blood cells and cellular debris. Polymorphonuclear leukocyte (PMN) apoptosis was measured using a BD FACS CANTO Flow Cytometer (BD BioSciences, San Jose, CA). PMNs were identified by staining with Ms-Ly6G/Ly-6C (Gr-1) directly conjugated Allophycocyanin (APC)-Cy7 (cat. no. 557,661; BD Pharmingen, San Jose, CA) for 15 minutes (22). Cells were also stained for annexin V-FITC and propidium iodide (PI) as a measure of apoptosis and necrosis, respectively (Biovision, Mountain View, CA). Appropriate unstained and single-stained controls were analyzed to set compensation voltages. Apoptotic and necrotic PMNs were defined as Gr-1⁺/annexin V⁺/PI⁻ and Gr-1⁺/annexin V⁺/PI⁺ populations, respectively. Unstained total BAL cells were used for microscopic classification of cell types using a differential staining kit, according to the manufacturer's recommendation (Fisher Scientific, Pittsburgh, PA).

Gene Expression Analysis

RNA was isolated from left lung according to an established protocol (23). CodeLink UniSet Mouse 20K I Bioarrays (GE Healthcare BioSciences Corp., Piscataway, NJ) were used for gene expression profiling of isolated total lung RNA. Determination of RNA integrity, generation of biotin-labeled cRNA, hybridization to the array, array scanning, and data collection were conducted at the University of Pittsburgh Cancer Institute Clinical Genomics Facility.

Bioinformatic Analysis

Data analysis was conducted in three steps, as previously described (24, 25). This included: (1) generation of complete data tables, including full

annotations; (2) normalization of raw data and statistical analysis to determine significant changes in gene expression; (3) identification of clusters of similarly expressed genes; and (4) pathway modeling. Raw data were annotated using the freeware program, *Source* (Stanford University, Stanford, CA). Data were next imported into the *ScoreGenes* software package (Jerusalem, Israel) for normalization, statistical analysis, and clustering (26). Raw data were log2 transformed and normalized to the average across all groups. Significant gene expression changes were assessed by fulfilling two criteria: a significant Student's *t* test *P* value, and a threshold number of misclassifications (TNOM) equal to 0, according to previously established data-mining methods (27). LPS-responsive and CCSP-independent genes were revealed by identifying all genes in WT and CCSP^{-/-} data sets that were differentially expressed compared with control (TNOM = 0; *t* test *P* < 0.1) and not dependent upon genotype (TNOM > 0 in a WT versus CCSP^{-/-} comparison). LPS-responsive and CCSP-dependent genes were determined by first identifying all LPS-responsive genes. These genes were further evaluated for genotype-dependent expression differences by pair-wise comparisons of WT and knockout data sets (TNOM = 0, and *t* test *P* value < 0.1). Independent clustering of the LPS-responsive/CCSP-independent and LPS-responsive/CCSP-dependent genes was conducted with the *ScoreGenes* package using the "pcluster" command. Data were visualized using *Treeview* software (Hebrew University, Jerusalem, Israel), as previously described (28). Finally, PathwayArchitect (Stratagene, La Jolla, CA) was used as a means to create biological pathways. This software package allowed for transformation of gene lists into biological pathways based on known functional characteristics of the selected genes. The ontological definitions are based upon the composite of human curated databases and analysis of published abstracts through language processing algorithms. Microarray analysis was conducted according to MIAME (minimum information about a microarray experiment) parameters and gene expression data deposited into the Gene Expression Omnibus microarray repository (accession number GSE16409).

Protein Characterization of Bronchoalveolar Lavage

WT and CCSP^{-/-} mice were lavaged twice with 1 ml of sterile PBS and centrifuged at 300 × *g* for 5 minutes to remove cells and debris. Protein-enriched supernatants were assayed for cytokine and chemokine protein abundance using a Luminex system (Luminex Corp., Austin, TX) with either Mouse-18-plex or Mouse-6-plex chemokine/cytokine detection kits (Bio-Rad, Hercules, CA). CCSP was measured in BAL with a CCSP ELISA that we designed using CCSP anti-serum from goat and rabbit.

Mouse Tracheal Epithelial Cultures

Primary airway epithelial cultures were established and maintained at the air-liquid interface using the method of You and colleagues (29). Cultures prepared from WT and CCSP^{-/-} mice were exposed to LPS on Day 10 of air-liquid culture, as maximal expression of the secretory cell marker, CCSP, occurs at this time. Epithelial cells were exposed on the basolateral surface to 1 µg/ml *P. aeruginosa* LPS (Sigma) in normal growth medium. A basolateral stimulus was conducted, as an apical stimulus can have detrimental effects on epithelial integrity (30). Apical surface fluid was harvested 0, 6, or 24 hours after LPS exposure by washing the apical surface of cultures with 500 µl sterile PBS, and assayed for cytokine/chemokine protein expression using a Mouse-6-plex assay as outlined above. RNA was extracted from epithelial cells using an SV Total RNA Isolation System (Promega, Madison, WI) according to manufacturer's instructions. Air-liquid interface experiments were repeated with three independent epithelial cell isolations.

Ex Vivo LPS Exposure of WT and CCSP^{-/-} Macrophages

Lung macrophages were isolated from naive WT and CCSP^{-/-} C57Bl/6 (non-sex/age-matched) mice, as previously described by Schurr and colleagues (31), and exposed to *P. aeruginosa* LPS (Sigma). Three WT and three CCSP^{-/-} animals were lavaged eight times with 1 ml PBS. Cells were recovered by centrifugation at 300 × *g* for 10 minutes, and resuspended in media composed of Dulbecco's modified Eagle's medium (Cambrex, East Rutherford, NJ), 10% FBS (ThermoFisher, Waltham, MA), penicillin-streptomycin (50 IU/ml), and 2 mM

L-Glutamine (Chemicon, Millipore, Billerica, MA). Cells were incubated for 2 hours to allow for adherence to the tissue culture plate. Media were replaced with LPS-spiked media at concentrations indicated in the figure legends. Cells were incubated with LPS containing media for 6 hours, and the supernatant collected for further analysis. TNF- α protein concentration was determined using a mouse TNF- α cytosol sandwich ELISA, according to manufacturer's recommendations (Biosource/Invitrogen, Carlsbad, CA). The *ex vivo* macrophage experiments were repeated with two independent macrophage isolations.

Macrophage Gene Expression Analysis

Alveolar macrophages were isolated from naive WT and CCSP^{-/-} mice for analysis of TLR4 and CD14 mRNA abundance. WT and CCSP^{-/-} mice were lavaged, and cells recovered by centrifugation. Three mice were pooled per group for a total of three groups per genotype ($n = 3$). RNA was extracted using an SV total RNA Isolation System. cDNA synthesis was performed as described previously here. Real-time PCR was conducted to assay for TLR4 mRNA abundance as described subsequently.

TLR4 Surface Distribution on Lung Macrophages

Lung macrophages were isolated from WT and CCSP^{-/-} lung, and TLR4 distribution analyzed as previously described (32). Briefly, whole-lung lavage and centrifugation was performed to obtain lung macrophages. Flow cytometry analysis was performed using a Becton Dickinson FACSCalibur (BD BioSciences) and analyzed with CELLQuest software (BD BioSciences). A laser scanning confocal microscope (LSM510 UV mounted on an Axio Observer microscope; Carl Zeiss, Inc., Gottingen, Germany) was used to obtain the fluorescence and transmitted images. Zeiss LSM510 v4.2 and LSM Image Examiner v3.2 software were used for image acquisition and analysis, respectively.

Real-Time PCR

Taqman real-time PCR was conducted on an Applied Biosystems 7000 Real-Time PCR System (Applied Biosystems, Foster City, CA). RNA was analyzed for quantity and quality using a Nanodrop 1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE). Equal amounts

of RNA were used to generate cDNA using the First strand cDNA synthesis kit according to manufacturer's recommendations (Invitrogen). Taqman real-time PCR was conducted using validated Taqman probe and primer combinations (Applied Biosystems). Relative mRNA abundance was determined by the $\Delta\Delta C_T$ method for real-time PCR (33).

Statistical Analysis

Statistical analysis was conducted in Minitab 15 for Windows. Pair-wise comparisons were tested for significance using the Student's *t* test or a Mann Whitney U test. The general linear model and Tukey's *post hoc* analysis was used to test multiple pair-wise comparisons.

RESULTS

Clara Cell Depletion Exacerbates the LPS-Induced Inflammatory Response

To address the contribution of the airway epithelium to the inflammatory response, we used a dual-exposure mouse model in which Clara cells were specifically ablated by naphthalene exposure before LPS challenge. A critical component of this experiment was to first identify the time at which naphthalene-induced injury resulted in maximal Clara cell ablation and diminished secretory function. We exposed WT FVB/N mice to naphthalene and recovered for 1, 2.5, 4, 8, or 12 hours and 1, 3, 5, 10, or 30 days. Protein abundance of CCSP was measured in the cell-free fraction of BAL as an indicator of both Clara cell ablation and secretory capacity. CCSP protein in BAL increased 1.0 hour after injury, indicative of Clara cell degranulation/hypersecretion, returned to normal levels by 2.5 hours, and reached minima from 8 hours to 5 days, suggesting that Clara cell ablation and/or diminished secretory capacity had occurred by 24 hours (Figure 1A). This finding parallels other endpoints of Clara cell depletion, including depletion of CCSP mRNA within total lung RNA, and the loss of CCSP-immunoreactive cells within lung tissue by immunocytochemistry (34).

Figure 1. The airway epithelium attenuates the inflammatory response. Wild-type (WT) adult mice were exposed to naphthalene, LPS, or naphthalene and LPS. The abundance of (A) Clara cell secretory protein (CCSP) in bronchoalveolar lavage (BAL) from 0, 1, 2.5, 4, 8, and 12 hours (solid bars indicate measurements in hours; $n = 4$ /group) and 1, 2, 3, 5, 10, and 30 days (open bars indicate measurements in days; $n = 5$ /group) was measured by an ELISA. (B) Recruited polymorphonuclear leukocytes (PMNs) were analyzed in BAL from control ($n = 3$), naphthalene- ($n = 3$), LPS- ($n = 3$), and naphthalene plus LPS-exposed (open bars, dual exposure; $n = 4$) mice. (C and D) Toll-like receptor (TLR)-4 protein expression was measured on alveolar macrophages at steady state (solid bars, control) and 1 day after naphthalene (open bars, Day 1) through cytometric analysis, and

presented as the geometric mean of TLR4-positive macrophages and percentage of TLR4^{high}-positive macrophages (C and D, respectively). Data are presented as means ($\pm$ SEM). * $P < 0.05$ for pair-wise comparison of exposure group or comparison to control, respectively; Student's *t* test and general linear model/Tukey's *post hoc* analysis was used in A and B, respectively; $n = 8$ for naphthalene.

To test the physiological impact of Clara cell ablation on the inflammatory response, mice were exposed to naphthalene, LPS, or naphthalene and LPS. The 24-hour recovery time point after naphthalene exposure was selected for further LPS challenge, as it represented the interval showing maximal depletion of Clara cells and their major secretory product, CCSP (Figure 1A). Naphthalene treatment alone resulted in only a subtle increase in lavageable PMNs (Figure 1B). In contrast, 30% of lavageable cells were classified as PMNs 1 day after treatment with LPS alone (Figure 1B). Naphthalene and LPS exposure (dual exposure) resulted in a synergistic increase in PMN recruitment, as evidenced by a PMN contribution of 73% of BAL cells (Figure 1B). As the Clara cell and macrophage signaling axis has been shown to be an important regulator of the LPS-induced inflammatory response, we investigated how Clara cell ablation affects macrophage behavior (35). Increased abundance of TLR4 on the surface of lung macrophages has previously been correlated with an exacerbated LPS-elicited inflammatory response (32). Therefore, macrophages from control and naphthalene-exposed mice were isolated from BAL and stained for the macrophage marker, F4/80, and TLR4. TLR4 surface expression was evaluated on F4/80-positive macrophages through flow cytometry, and demonstrated a robust increase on macrophages isolated from naphthalene-exposed mice relative to steady-state controls (Figures 1C and 1D). Although these data do not rule out indirect effects of injury on modulation of the LPS response, they suggest that Clara cells function in an anti-inflammatory capacity within airways by modulating macrophage behavior.

Increased LPS-Induced Recruitment of PMNs to Lungs of CCSP-Deficient Mice

Based upon these data, we sought to establish a mechanism whereby Clara cells may moderate the inflammatory response. Clara cells may attenuate the inflammatory response through a variety of mechanisms, including secretion of paracrine factors. We have previously shown that CCSP^{-/-} mice display increased lung inflammation relative to WT mice after exposure to inhaled oxidant pollutants (15, 16). Additionally we have established that a signaling axis exists between Clara cells and macrophages, as revealed by changes in ANXA1 PTM in macrophages of CCSP^{-/-} mice (19). Therefore, we sought to test the hypothesis that Clara cell attenuation of the inflammatory response is CCSP dependent. WT and CCSP^{-/-} mice were coexposed to 10 ng deposited dose of LPS and recovered for 3 or 24 hours. Analysis of cell types recovered from BAL revealed the expected shift in inflammatory cell profile, from greater than 99.5% macrophages in BAL of unexposed mice, to predominantly recruited PMNs in BAL from both WT and CCSP^{-/-} mice either 3 or 24 hours after LPS exposure (Figure 2A). Although a similar overall response was observed, differences were apparent in the magnitude of PMN recruitment. PMNs represented 44.9 and 74.4% of WT and CCSP^{-/-} isolated BAL cells 3 hours after LPS exposure, indicating a statistically significant 65.7% increase in PMNs in airways of CCSP-deficient mice (Figure 2A). Furthermore, PMNs represented 70.9 and 81.3% of WT and CCSP^{-/-} BAL 24 hours after LPS exposure, demonstrating a more subtle, but 14.6%, increase in PMN contribution to CCSP^{-/-} BAL. Corresponding decreases were observed in the fractional contribution of macrophages to BAL cells recovered from airways of WT and CCSP^{-/-} mice after LPS exposure (data not shown).

Changes in total cell numbers were also measured to better define CCSP-dependent differences in inflammatory cell recruitment. Total BAL cell counts were increased by 68 and 43% in BAL of CCSP^{-/-} mice compared with WT mice at 3 and

Figure 2. CCSP deficiency augments the LPS-induced inflammatory response. WT (solid bars) and CCSP^{-/-} mice (open bars) were simultaneously challenged with LPS and recovered for 3 or 24 hours. Control ($n = 4$) mice were unexposed to LPS (0). (A) Cellular infiltrates were analyzed using a differential cellular stain to determine PMN recruitment. (B) Total cells were determined in WT and CCSP^{-/-} BAL using a Coulter Particle Counter (left). To reveal cell type-specific changes to the total cellular infiltrate, total macrophages (center) and PMNs (right) were calculated by multiplication of total cells by the percentage of macrophage or PMN in BAL, as determined in differential cell analysis. Data are presented as means ($\pm$ SEM). * $\dagger$ Tukey's *post hoc* $P < 0.05$ in pair-wise comparisons of WT and CCSP^{-/-} or comparison to control, respectively. Solid bars, WT; open bars, CCSP^{-/-}.

24 hours after LPS exposure, respectively (Figure 2B). No changes were observed in total macrophages recovered in BAL from WT and CCSP^{-/-} mice (Figure 1B). Increased cellular recruitment after LPS exposure could be accounted for by recruitment of PMNs. Consistent with differential cell type analysis in BAL, PMNs were recruited to significantly higher levels in BAL of LPS-exposed CCSP^{-/-} mice compared with similarly exposed WT mice. PMNs in BAL of LPS-exposed CCSP^{-/-} mice showed 2.79-fold and 1.66-fold increases over WT control mice at the 3- and 24-hour after exposure time points, respectively (Figure 2B).

Instantaneous measurements of PMN abundance represent the sum of recruitment and apoptotic clearance. To determine whether CCSP deficiency was associated with changes in either of these parameters, the abundance of apoptotic and necrotic PMNs was determined within BAL of WT and CCSP^{-/-} mice by FACS analysis 24 hours after LPS exposure. Isolated BAL cells were stained with Gr-1 (22), and the fraction of apoptotic cells determined by staining for annexin V-FITC and PI. As was the case with cytological identification of cell types, FACS analysis revealed greater than 50% contribution of PMNs to the total cell fraction of BAL from LPS-exposed WT and CCSP^{-/-} mice (Figures 3A and 3B). The abundance of cells showing cell surface staining for annexin V and either exclusion or staining with PI indicated no statistically significant differences in apoptotic and necrotic PMNs, respectively, in BAL of CCSP^{-/-} mice compared with WT mice (Figures 3C–3G). These data suggest that CCSP deficiency modifies the initial response to

proinflammatory stimuli, leading to increased PMN recruitment, but has no impact on PMN turnover.

Microarray Analysis Reveals CCSP-Dependent and CCSP-Independent Affects to the LPS Response

To better understand mechanisms by which CCSP impacts LPS-induced lung inflammation, we conducted a genome-wide expression screen of WT and CCSP^{-/-} lung RNA from mice at 0, 1.5, 6, and 12 hours after exposure to an estimated deposited dose of 10 ng LPS. A total of 1,815 genes were identified that demonstrated a similar LPS response in WT and CCSP^{-/-} lung. This gene list was designated as CCSP independent and LPS responsive, relevant portions of which are represented as a clustered heat map in Figure 4A. A representative gene list and fold change relative to WT or CCSP^{-/-} controls is presented in Table E1 in the online supplement. A second gene set of 267 transcripts was comprised of genes that were differentially LPS responsive in a WT and CCSP^{-/-} pair-wise comparison. This gene list was designated as CCSP dependent and LPS responsive, relevant portions of which are represented as a clustered heat map in Figure 4B. The representative gene list and fold change relative to WT is presented in Table E2. To validate the gene expression signatures displayed in Figure 4, mRNA abundance for genes from each cluster was determined by Taqman real-time PCR (data not shown; validated genes indicated in Tables E1 and E2).

Pathway Analysis Identifies TNF- α and IL-6 as Key Cytokines that Are either Directly or Indirectly Impacted by CCSP Deficiency

PathwayArchitect was used to organize the gene set *in silico* based upon transcriptional regulators and gene product interactions (36–38). Genes within the LPS-responsive CCSP-dependent gene cluster were organized into an interactive pathway to provide further insights into regulatory mechanisms accounting for CCSP-dependent regulation of the inflammatory response. In addition to these genes, LPS, CCSP, and two known regulated downstream target genes, SCGB3A2 and ANXA1, were included within the gene set to identify evidence of involvement in this pathway. Generation of the *in silico* generated pathway recapitulated the previously established model of LPS-elicited inflammation, including LPS recognition by CD14 and TLR4. Included within this pathway are key nodes of regulation implicated in the CCSP-dependent modulation of LPS-elicited inflammation. Two key signaling molecules implicated in the CCSP-dependent component of the inflammatory response were TNF- α and IL-6, as demonstrated by the high connectivity between genes the mRNAs of which were significantly altered in a CCSP-dependent, LPS-dependent fashion (Figure 5A, red asterisk). Importantly, neither TNF- α nor TLR4 mRNA abundance were found to be CCSP dependent, as indicated by similar responses to LPS treatment between WT and CCSP^{-/-} mice and their absence from the

Figure 3. Turnover of PMNs is not impacted by CCSP deficiency. BAL cells from WT (solid bars) and CCSP^{-/-} lung (open bars) were isolated 24 hours after LPS exposure ($n = 4$) for determination of PMN apoptosis and necrosis. Appropriate one-color controls were used to set compensation voltages (data not shown). (A) Unexposed controls were used to determine the Gr-1 (–) fraction of inflammatory cells. (B–D) Isolated cells were stained with Gr1-APC-Cy7, annexin V (AnxV)-FITC, and PI to determine PMN specific apoptosis and necrosis. (E) Scatterplot of PI versus AnxV-FITC Gr-1⁺ cells to determine necrotic and apoptotic PMNs. Quadrants 2 (AnxV⁺/PI⁺) and 4 (AnxV⁺/PI[–]) represent necrotic and apoptotic PMNs respectively. Quantitative analysis of the percentage of (F) apoptotic and (G) necrotic PMN (no significance was reached using a two-sample Student's *t* test). Data are presented as means ($\pm$ SEM).

Figure 4. Hierarchical clustering of genome-wide expression analysis to identify CCSP-dependent and -independent components of the LPS-induced inflammatory response. WT and CCSP^{-/-} mice were coexposed to LPS aerosol for 20 minutes and recovered 0, 1.5, 3, 6, 9, and 12 hours ($n = 4$ /group). RNA from 0, 1.5, 6, and 12 hours was screened for total message expression using Codelink 20K Bioarrays ($n = 3$ /group). Hierarchical clustering of gene expression is represented in *heat maps*, where *columns* are experiments and *rows* are genes (yellow and magenta indicate decreased and increased expression, respectively, with respect to the average across all time points). The *heat map* represents a subset of the clusters from (A) CCSP-independent/LPS-responsive and (B) CCSP-dependent/LPS-responsive gene signatures. The rows (genes) are annotated in ascending numerical order. For complete annotations, including accession number, gene name, and ontology, refer to Tables E1 and E2.

corresponding gene list used for pathway analysis. Rather, PathwayArchitect's computational algorithm added TNF- α and TLR4 to the pathway, as they were both predicted to be key upstream regulators of genes represented within the CCSP-dependent and LPS-dependent gene list. This observation suggests that increased signaling by TNF- α and IL-6 may contribute to enhanced LPS-elicited inflammatory response observed in CCSP^{-/-} mice relative to coexposed WT mice.

We next sought to determine whether the *in silico* prediction of increased IL-6 and TNF- α signaling could be validated *in vivo*. Protein abundance in BAL fluid was determined by cytokine/chemokine multiplex analysis at 0, 1.5, 3, 6, 9, and 12 hours after LPS stimulation of WT or CCSP^{-/-} mice. Statistically significant 3.2- and 5.8-fold increases in TNF- α protein abundance were observed in BAL fluid isolated from CCSP^{-/-} mice compared with similarly exposed WT at either 1.5 or 3 hours after LPS exposure times (Figure 5B). This was accompanied by a trend toward increased IL-6 protein in CCSP^{-/-} BAL fluid 3 hours after LPS exposure compared with WT (Figure 5C). These data support the prediction generated from microarray data of CCSP-dependent changes in LPS-elicited TNF- α and IL-6 production *in vivo*.

Airway Epithelium and Lung Macrophages Are Intrinsically Hyperresponsive to LPS Stimulation

Our previous studies have revealed alterations to airway epithelial cells and macrophages that result from CCSP deficiency (19, 39). Because each of these cellular compartments plays a central role in mediating the lung response to inhaled LPS, we sought to determine their intrinsic responsiveness to LPS *in vitro*. Differentiated airway epithelial cell cultures were prepared from WT and CCSP^{-/-} mice. Expression of cell type-specific marker genes was used to verify differentiation status of cultured epithelia and, similarly, of cultures prepared from WT and CCSP^{-/-} mice. Steady-state analysis of gene expression indicated that *in vitro* cultures of WT and CCSP^{-/-} epithelium

were similarly differentiated before and throughout the exposure as indicated by equivalent expression of the ciliated and secretory cell lineage markers FoxJ1 and Cyp2f2, respectively (Figures 6A and 6B). The expected difference in CCSP gene expression was observed between WT and CCSP^{-/-} cultures, with the latter showing no detectable CCSP mRNA (Figure 6C). Interestingly, CCSP mRNA abundance significantly decreased after 24 hours of LPS exposure (Figure 6C). This observation correlates with a study by Harrod and Jaramillo (40), demonstrating that CCSP promoter activity is attenuated by *P. aeruginosa* exposure both *in vitro* and *in vivo*.

To determine whether LPS had direct effects on epithelial cytokine production, apical fluid was recovered from cultures and the abundance of proinflammatory cytokines and chemokines measured by multiplex immunoassay. LPS stimulated epithelial secretion of IL-6 into apical fluid of cultures from WT and CCSP^{-/-} mice. A trend toward differences between WT and CCSP^{-/-} cultures was observed. Levels of IL-6 present within apical fluid of epithelium from CCSP^{-/-} mice was 6.18- and 3.5-fold higher at 6 and 24 hours than that of similarly exposed cultures from WT mice (Figure 6D). These differences did not reach significance due to variability at the 24-hour time point and stringency of the general linear model. However, a pair-wise comparison using a Student's unpaired *t* test yielded a statistically significant *P* value of 0.001 at the 6-hour time point. Additionally, we also saw a similar trend in the abundance of keratinocyte-derived chemokine (KC) and regulated upon activation, normal T-cell expressed and secreted (data not shown). Together, these data indicate that the CCSP^{-/-} epithelium is intrinsically hyperresponsive to LPS. However, these changes are only weakly associated with the CCSP-dependent component of the LPS response. Therefore, we investigated levels of TNF- α secreted into the media, as this is the most robust difference between genotypes after LPS exposure *in vivo*, as identified by pathway analysis and subsequent protein validation. TNF- α was below the limit of detection in control and

Figure 5. Pathway analysis of the CCSP-dependent and LPS-responsive gene signature identifies regulatory networks that can be biologically validated. Genes that were identified to be LPS-responsive and CCSP-dependent (Figure 4B, Table E2) were used to construct an interaction network, as discussed in MATERIALS AND METHODS. Gene products (ellipses) are linked functionally by connecting lines in accordance with functional annotation (refer to legend). Note the high connectivity of TNF- α and IL-6 in the proposed pathway. Red and black asterisks denote molecules/processes that were added to the pathway in either an unsupervised (added by the pathway analysis software) or supervised (manually by the user) fashion, as discussed in the text. (B and C) WT and CCSP^{-/-} mice were simultaneously aerosol exposed to *Pseudomonas aeruginosa* and killed 1.5, 3, 6, 9, and 12 hours after exposure ($n = 4$ per group). Control mice were unexposed to LPS (0). Airway surface fluid was collected by lavage and characterized using a multiplex immunoassay. (B) TNF- α (pg/ml) and (C) IL-6 protein levels are depicted. Data are presented as means ($\pm$ SEM). * $\dagger$ Tukey's *post hoc* $P < 0.05$ in comparison of WT and CCSP^{-/-} or comparison to control, respectively. Solid bars, WT; open bars, CCSP^{-/-}.

LPS-exposed WT and CCSP^{-/-} epithelial cultures (data not shown), confirming that the cell types mediating synthesis and secretion of TNF- α are absent within epithelial cultures.

In light of our previous finding of biochemical changes to macrophages of CCSP^{-/-} mice, we sought to determine if there were functional alterations to macrophages of CCSP^{-/-} mice that might account for the observed phenotype (19). WT and CCSP^{-/-} macrophages were isolated by lavage of steady-state lung and exposed to 0.00, 0.39, 0.78, 1.56, 3.13, 6.25, 12.5, and 25.00 ng/ml LPS, and media harvested for analysis of TNF- α release after 6 hours. Lung macrophages recovered

from CCSP^{-/-} mice displayed a robust increase in LPS-elicited TNF- α release compared with similarly exposed macrophages from WT mice (Figure 7A). Levels of TNF- α released into medium of macrophages cultured from CCSP^{-/-} mice were increased over that present in macrophages from WT mice by 1.75- to 2.6-fold when stimulated by LPS concentrations of 3.1–12.5 ng/ml (Figure 7A). These results indicate that CCSP deficiency may moderate the macrophage-elicited LPS response.

We reasoned that CCSP deficiency caused alterations to the LPS recognition machinery on lung macrophages in parallel to

Figure 6. CCSP^{-/-} airway epithelial cells are hyper-responsive to LPS. Primary cultures of WT (solid bars) and CCSP^{-/-} epithelium (open bars) were generated and maintained at the air-liquid interface, according to established protocols as described in MATERIALS AND METHODS ($n = 3$ /genotype/treatment group). The control group was unexposed to LPS (0). RNA was isolated from each culture for Taqman real-time PCR assessment of (A) FoxJ1, (B) Cyp450-2f2, and (C) CCSP expression to determine differentiation status of the epithelium. (D) IL-6 protein secreted into the apical surface fluid was measured using a multiplex immunoassay. Data are presented as means ($\pm$ SEM). * $\dagger$ Tukey's *post hoc* $P < 0.05$ in comparison of WT and CCSP^{-/-} or comparison to control, respectively.

our findings after naphthalene-induced Clara cell depletion (Figure 1). Determination of mRNA abundance of TLR4 and CD14 in WT and CCSP^{-/-} alveolar macrophages demonstrated no significant difference between genotypes (Figures 7B and 7C, respectively). As naphthalene-induced Clara cell depletion resulted in increased surface expression of TLR4 on lung macrophages, we reasoned that macrophages isolated from steady-state CCSP^{-/-} mice may have a similar alteration in TLR4 distribution. Cells were stained with macrophage marker F4/80 and TLR4 to determine if trafficking had been affected. Confocal microscopy of F4/80⁺ WT and CCSP^{-/-} macrophages (Figures 7D and 7F, respectively) revealed increased TLR4 expression in F480⁺ CCSP^{-/-} macrophages (Figure 7G) compared with WT macrophages (Figure 7E). To quantify the observed increase in TLR4 expression on CCSP^{-/-} macrophages, we also performed flow cytometric analysis of WT and CCSP^{-/-} macrophages. Lavaged WT and CCSP^{-/-} cells were stained for F4/80 and TLR4. TLR4 expression was evaluated on F4/80⁺ macrophages, and demonstrated that CCSP^{-/-} macrophages have a robust increase in surface TLR4 surface expression represented by the geometric mean of the fluorescence intensity and percent of macrophages with high TLR4 surface expression (Figures 7F and 7G, respectively). These results confirm that macrophages from CCSP^{-/-} mice are hyperresponsive to LPS, and that the increased TLR4 surface expression on macrophages was most likely responsible for the observed increase in TNF- α production *in vivo* after delivery of aerosolized LPS.

We next sought to determine if CCSP directly regulates macrophage responsiveness to LPS in cultured macrophages. Fractionated BAL was prepared from WT and CCSP^{-/-} mice according to a previously published protocol (41), and used to test the inhibitory effects of CCSP on macrophage responsiveness. No differences in LPS responsiveness were observed after either preincubation of LPS or pretreatment of macrophages before LPS treatment with BAL preparations from WT or CCSP^{-/-} mice (Figure E1). These data suggest that neither CCSP nor other changes to the acellular fraction of airway lining fluid from CCSP^{-/-} mice directly regulate macrophage

responsiveness to LPS. Rather, our data support a model in which attenuated Clara cell function results in intrinsic functional differences in the lung macrophage population.

DISCUSSION

In this study, we provide evidence for anti-inflammatory roles of the airway epithelium and elucidate a mechanism whereby Clara cells may regulate this process. Injured airway epithelium and mice deficient in expression of CCSP respond more robustly to inhaled *P. aeruginosa* LPS, leading to increased recruitment of PMNs. Using microarray analysis, pathway modeling, and *in vivo* validation, we demonstrated that TNF- α signaling was robustly increased in the CCSP^{-/-} inflammatory response. Functional analysis of macrophages isolated from WT and CCSP^{-/-} lungs revealed a robust increase in LPS responsiveness in the CCSP^{-/-} isolated macrophage compared with WT macrophage, and was paralleled by increased TLR4 surface abundance on CCSP^{-/-} macrophages at steady state. The response of cultured macrophages could not be altered *in vitro* with fractionated CCSP-enriched BAL, indicating that CCSP does not directly regulate macrophage responsiveness. Instead, our data argue that CCSP deficiency causes an intrinsic alteration to lung macrophage behavior.

Recent reports have suggested that the airway epithelium provides a proinflammatory role in the innate immune response through modulation of epithelial NF- κ B signaling and subsequent secretion of inflammatory cytokines and chemokines (42–45). However, the present study defines normal Clara cell secretory function, and suggests that the production of CCSP is a key mediator of the innate pulmonary response, providing evidence that the airway epithelium confers anti-inflammatory properties in the lung. Of particular interest is the apparent discrepancy between our findings presented herein and those of Elizur and colleagues (44), describing the effects of acute Clara cell injury on the inflammatory response. In their study, Elizur and colleagues concluded that Clara cells are proinflammatory, based upon their observation of attenuated TNF- α and KC production in LPS-challenged, naphthalene-injured lung. We

Figure 7. CCSP^{-/-} augments the macrophage elicited inflammatory response. (A) Primary lung macrophages from WT (solid bars) and CCSP^{-/-} mice (open bars) were exposed to 0.00, 0.39, 0.78, 1.56, 3.13, 6.25, 12.5, and 25.00 ng/ml *P. aeruginosa*-derived LPS-spiked media for 6 hours (*n* = 4 per group). TNF-α (pg/ml) was determined in media by a mouse TNF-α sandwich ELISA. *†Tukey's *post hoc* *P* < 0.05 in comparison of WT and CCSP^{-/-} or comparison to control, respectively; solid bars, WT; open bars, CCSP^{-/-}. (B and C) Real-time PCR analysis of TLR4 and CD14 mRNA abundance in isolated alveolar macrophages indicates no difference (*t* test *P* > 0.05) between WT and CCSP^{-/-} in expression of key components of the LPS signaling machinery (*n* = 3/genotype). BAL cells from WT (D and E) and CCSP^{-/-} (F and G) lungs were stained for the macrophage marker F4/80 (D and F), TLR4 (E and G), and analyzed by confocal microscopy. Flow cytometric analysis of BAL cells from WT (*n* = 5) and CCSP^{-/-} (*n* = 5) lungs were stained for F4/80 and TLR4. TLR4 expression on F4/80-positive macrophages was quantified and presented as geometric mean fluorescent intensity (H) (**t* test *P* < 0.001) and percentage of macrophages with high TLR4 expression (I) (**t* test *P* < 0.001). Data are presented as means (±SEM).

demonstrate that Clara cells are anti-inflammatory, based upon our observation that PMN recruitment is synergistically increased in LPS-challenged, naphthalene-injured lung. We speculate that this may be attributed to differences in the time at which injured mice are challenged with LPS. Elizur and colleagues challenged with LPS 2.5 hours after injury, which was preceded 1.5 hours earlier by a supramaximal level of Clara cell secretions in the airway, as measured by CCSP protein abundance in BAL (Figure 1A). We speculate that an overabundance of such proteins delivered into the airway before LPS stimulus may diminish the inflammatory response. In contrast, we demonstrate that protracted loss of Clara cell secretory function results in alteration in lung macrophage behavior and an exacerbation of the response to proinflammatory stimuli.

These data have important implications regarding regulation of the inflammatory response in normal and diseased airway. In the normal adult airway, Clara cells function to moderate responses to inhaled inflammatory agents, a function that involves paracrine influences of Clara cell secretions on lung macrophages (19). Our data suggest that Clara cell secretory dysfunction that accompanies airway remodeling results in loss of the intrinsic immunosuppressive properties provided by the epithelium, leading to exacerbation of the inflammatory response. Furthermore, we show that exposure of epithelial cells from WT mice to LPS results in a dramatic decrease in expression of CCSP. This finding is consistent with previous reports demonstrating that *in vivo* exposure of mice to live *P. aeruginosa* results in decreased expression of CCSP. However, in contrast to the finding of Harrod and Jaramillo (40), we demonstrate that decreased CCSP mRNA abundance can occur independent of TNF- α . When put in the context of these findings from other laboratories, our data argue that airway inflammation resulting from acute exposure of normal airways to proinflammatory bacterial products is actively suppressed, but that chronic infection leads to decreased Clara cell secretory function and elevated immune responsiveness that is typical of individuals with CLD.

The postulated paracrine/endocrine function of CCSP implies recognition by a cell surface receptor. A candidate receptor system has previously been described in the proximal tubule of the kidney, in which receptor-mediated endocytosis occurs through a megalin/cubilin-dependent mechanism. Receptor-mediated uptake of CCSP within the proximal convoluted tubule results in its catabolism, which has the potential to release bound ligands (46). However, this receptor-ligand interaction was shown to be specific to the kidney, with no evidence for its activity within structural cells or macrophages of the lung. Other candidate receptors have been described previously, leading to speculation that CCSP may function as a cytokine (47, 48). Recently, another candidate receptor has been identified that mediates interaction with other structurally related members of the secretoglobin family. SCGB3A2 was shown in an expression cloning screen to interact with the macrophage receptor with collagenous structure (49). Moreover, SCGB3A2 was shown to compete with LPS for interaction with macrophage receptor with collagenous structure, suggesting an alternative mechanism by which secretoglobin family members may alter the macrophage response to LPS. However, our data suggest that a similar mechanism does not operate in the CCSP-dependent moderation of LPS-induced lung inflammation, as the CCSP-containing fraction of BAL fails to attenuate LPS-elicited TNF- α release in cultured macrophages.

The inability of fractionated BAL to modulate macrophage responsiveness to LPS suggests that CCSP does not directly regulate this process. Interestingly, the demonstration in the present study that macrophage function is altered in the setting of CCSP deficiency is consistent with our previous finding of

changes in ANXA1 PTM within macrophages of CCSP^{-/-} mice (19). A reasonable explanation is that the macrophage population in the Clara cell-depleted and CCSP-deficient lung is intrinsically altered compared with the steady-state and WT populations, respectively. Another possible explanation is that CCSP^{-/-} results in altered steady-state monocyte/macrophage populations within the lung. Recently, a subpopulation of monocytes has been identified, termed "inflammatory monocytes." These cells can be distinguished based upon the expression of several genetic markers, including ANXA1, and their proinflammatory gene signature (50). Moreover, these cells are required for a robust neutrophilic response to LPS (51). More recently, Kim and colleagues (52) demonstrated alterations to macrophage subsets in the setting of CLD. In particular, they identified an increased abundance of alternatively activated macrophages in CLD, in both mouse models and in patients. These cells contributed directly to the progression of mucus cell metaplasia and epithelial remodeling that is typical of CLD. Additional studies will be necessary to determine if altered Clara cell secretory function impacts the monocyte/macrophage populations within the lungs of CCSP^{-/-} mice.

CLD and associated inflammatory exacerbations are a result of complex interactions between the host and environmental stimuli (53). We reveal here a mechanism of epithelial-macrophage cross-talk, which, when perturbed, as in the setting of CLD, results in an augmented innate inflammatory response to inhaled environmental agents. The precise molecular mechanism by which CCSP or other unidentified Clara cell-secreted proteins mediate these immunomodulatory effects will be the subject of future studies.

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