

Gob-5 Contributes to Goblet Cell Hyperplasia and Modulates Pulmonary Tissue Inflammation

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Gob-5 is a member of the calcium-activated chloride channel family and has been associated with allergic response in mouse models of pulmonary inflammation. Gene expression of Gob-5 has been shown to be induced in allergic airways and has been strongly associated with mucin gene regulation and goblet cell hyperplasia. We investigated the physiologic role of Gob-5 in murine models of pulmonary inflammation using mice deficient in Gob-5. After sensitization and aerosol challenge with ovalbumin (OVA), Gob-5 knockout mice exhibit significantly increased bronchoalveolar lavage (BAL) inflammation as compared with wild-type controls. The augmented inflammation in BAL consisted predominantly of neutrophils. Examination of perivascular inflammation revealed that tissue inflammation was decreased in OVA-challenged Gob-5^{-/-} mice. OVA-challenged Gob-5 knockout mice also had decreased goblet cell hyperplasia as well as decreased mucus production. These mice also had decreased airway hypersensitivity after cholinergic provocation with methacholine. Gob-5 knockout mice were also challenged via intranasal LPS, a TLR-4 agonist. Gob-5^{-/-} mice responded with increased neutrophilic BAL inflammation and decreased perivascular tissue inflammation as compared with wild-type controls. There was little effect on goblet cell hyperplasia and mucus production after LPS challenge. These observations reinforce findings that associate Gob-5 with goblet cell hyperplasia and mucus production in the allergic immune response, but also implicate Gob-5 in the regulation of tissue inflammation in the innate immune response.

Keywords: Gob-5; goblet cell hyperplasia; inflammation; neutrophils

Gob-5 is a member of the calcium-activated chloride channel family of proteins, which has been associated with the asthmatic phenotype in animal models of allergic inflammation (1–6). The association of Gob-5 with asthma models was observed in antigen-challenged mice that were intratracheally administered adenoviral antisense oligonucleotides directed against Gob-5 (1). After antigen challenge, blockade of Gob-5 resulted in decreased mucus production as measured by periodic acid–Schiff (PAS)-stained lung sections. Airway hyperresponsiveness in response to cholinergic stimulation was decreased as well. *In vitro* experiments showed that overexpression of Gob-5 in NCI-H292 cells resulted in increased expression of the mRNA for the mucin gene MUC5AC as well as an increase in PAS staining of cell monolayers (1).

Protein expression of Gob-5 surrounds mucin granules in respiratory, uterine, and intestinal goblet cells (7). In some respiratory goblet cells, the staining pattern showed capping at the

luminal surface. Gob-5 was not present in salivary glands or in the intestinal crypts (7). Because of its association with mucin granules and MUC5AC gene expression, Gob-5 has been used as a marker of mucus production and goblet cell hyperplasia. Model systems in which mucus hypersecretion is predominant have been used to study regulation of the expression of Gob-5 mRNA. Respiratory syncytial virus (RSV), the leading cause of bronchiolitis in infants (8), was shown in mouse models of allergic inflammation to strongly induce both MUC5AC and Gob-5 mRNA. Administration of RSV to ovalbumin (OVA)-challenged mice augmented Gob-5 and MUC5AC gene induction (5). This response correlated with induction of IL-17 mRNA. Induction of MUC5AC and Gob-5 mRNA was negatively modulated with the addition of antibodies to the chemokine receptor CXCR2 (3), a receptor for α chemokines (9). Mice deficient in CXCR2 show a diminished response to RSV infection in relation to MUC5AC and Gob-5 gene expression (3).

Antibodies to Gob-5 have been used to isolate mucin granules by immunoprecipitation confirming the association of this protein with granules (10). The mucin granules were isolated to study the role of the MARCKS, a molecule critical for the organization and fusion of mucin granules to plasma membranes (10). Although Gob-5 antibodies were used to isolate the granules, it is unclear if there is any mechanistic link between Gob-5 and MARCKS (11).

The regulation of Gob-5 gene expression in the lung has been studied in relation to various cellular stimuli. It has been demonstrated that Gob-5 mRNA was induced by overexpression of the cytokines IL-9, IL-10, and IL-13 in the lungs of transgenic mice (4, 12, 13). Expression of Gob-5 mRNA was also induced when IL-9, IL-10, IL-13, and IL-4 were administered to mice intratracheally (13). These data show a strong association of Gob-5 gene expression with Th2 T cell cytokine signals.

In the present study, the effect of Gob-5 gene disruption was evaluated in mouse models of pulmonary inflammation. The absence of Gob-5 resulted in increased bronchoalveolar lavage (BAL) inflammation and a decrease in tissue inflammation in response to multiple stimuli. There was also a decrease in mucin production and goblet cell hyperplasia. The results of this study confirm the role of Gob-5 in mucus production and indicate that Gob-5 may also play a regulatory role in neutrophilic pulmonary inflammation.

MATERIALS AND METHODS

Generation of Gob-5 Knockout and Conditional Knockout Mice

Gob-5 knockout (KO) mice were developed by gene-targeted mutagenesis in 129SvEvBrd ES cells and Cre-mediated recombination. Mice carrying the conditional knockout (CKO) allele of Gob-5 were created by Lexicon Genetics, Inc. (The Woodlands, TX). Gene-targeted ES cell clones were identified by southern blot RFLP analysis of BglII-digested ES cell DNA using a 3' probe external to the targeting construct in which the wild-type (wt) band is 11 kb and the targeted band is 9 kb. The CKO allele contains LoxP sites flanking exon 7. The Gob5

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KO allele was derived from the CKO allele via germline Cre-mediated deletion by crossing Protamine-Cre transgenic mice (129SvEvBrd) with Gob-5-CKO mice.

Genotyping of Gob-5 KO and CKO Mice

Mice were genotyped by PCR analysis of proteinase K lysate of tail biopsies. Wild-type and Gob-5-CKO alleles were distinguished using PCR of an amplicon spanning the 5' LoxP insertion site with a forward primer of the sequence (5'-AGACCTGGGATGACGCCTGTCT-3') and a reverse primer of the sequence (5'-AGTCTCTGCACTCC CAGGCTTG-3'), yielding a 321-bp wt product and a 392 CKO product. The Gob-5-KO (deletion) allele was distinguished from the wt allele by PCR of an amplicon spanning the deletion junction using the forward primer (5'-AGACCTGGGATGACGCCTGTCT-3') and a reverse primer of the sequence (5'-GAGCCACAGGGATTGCTGTC-3'), yielding an 871-bp wt product and a 341-bp product for the Gob-5-KO allele.

Mouse Model of Allergic Pulmonary Inflammation

All animal studies were performed with Institutional Animal Care and Use Committee approved protocols in accordance with the Animal Welfare Act. Age-matched 4- to 6-wk-old female 129SvEvBrd mice (Lexicon Genetics) and Gob-5 null mice were sensitized intraperitoneally with 0.15 mg/ml OVA (Sigma Aldrich, St. Louis, MO) with a combination aluminum hydroxide and magnesium hydroxide (ImjectAlum; Pierce, Rockford, IL) as an adjuvant. Sensitization occurred on Days 0 and 14. Starting on Day 26, mice were subjected to either aerosolized PBS or aerosolized 5% OVA for 30 min. This was repeated for 3 d. The mice were allowed to rest for a day, and on Day 30 animals were killed and analyzed.

LPS Induction of Pulmonary Inflammation

Female age-matched wt and Gob-5^{-/-} mice were intranasally administered PBS or LPS (Sigma Aldrich) at a dose of 1 mg/kg in a volume of 50 μ l. Mice were killed 24 h later and analyzed.

Measurement of Airway Function

To assess increases in airway resistance to aerosolized methacholine, mice were anesthetized, tracheostomized, and placed into PLY 3111 whole-body plethysmographs (Buxco, Wilmington, NC), each with a manifold built into the head plate of the chamber with ports to connect to the trachea, to the inspiration and expiration ports of a ventilator, and to a pressure transducer that monitors the tracheal pressure. A pneumotachograph in the wall of each plethysmograph monitored the airflow into and out of the chamber due to the thoracic movement of the ventilated animal. Animals were ventilated at a rate of 150 breaths/min and a tidal volume of 150 μ l. A MAX II preamplifier (Buxco) was used to interface with BioSystems XA software (Buxco). Resistance computations were derived from the tracheal pressure and airflow signals, using an algorithm of covariance. Aerosolization of methacholine was performed by a nebulizer in-line with the inspiratory tubing from the ventilator. Increases in airway resistance were analyzed over a 3-min period.

Peptide Polyclonal Antibody Generation

New Zealand white rabbits (age 3–9 mo) were immunized with keyhole limpet hemocyanin (KLH) peptide (*see below*) emulsified in Freund's adjuvant by subcutaneous injections at three dorsal sites (0.1 mg peptide per immunization). Animals were bled from the articular artery. Serum was collected and titer was determined via free peptide enzyme-linked immunosorbent assay (ELISA). The peptide used corresponded to the N-terminus of Gob-5, corresponding to amino acids 124–140 (RSTWEVIQESDFK). Antibodies were then purified using affinity chromatography. Pre-immune sera was tested in Western blots to account for nonspecific signal (data not shown).

Western Blot Analysis

Equal volumes of BAL fluid (BALF) (20 μ l) were resolved using SDS-PAGE on 4–20% Tris-Glycine precast gels (Invitrogen, Carlsbad, CA). Protein was transferred to nitrocellulose (Invitrogen), blocked with 5% nonfat dry milk, and blotted for 1 h with 2.5 μ g/ml of anti-Gob-5

peptide polyclonal antibody. Blots were washed and then incubated with 1:10,000 anti-rabbit Ig-horseradish peroxidase (Southern Biotechnology Associates, Birmingham, AL). Blots were developed using ECL reagent (Amersham Bioscience, Piscataway, NJ) and developed on Kodak film (Kodak, New Haven, CT).

Generation of Lung Sections

Lungs from mice were harvested immediately after BALF collection and placed in a 10% neutral buffered formalin solution (VWR International, Bridgeport, NJ). After tissues were fixed, lungs were trimmed of extraneous tissue and dehydrated with 70% ethanol, then embedded in paraffin. Embedded tissue was sectioned in 5- μ m-thick sections onto glass slides. Sections were stained with either hematoxylin and eosin (H&E) or PASchiff stain for mucus.

Measurement of BAL Inflammation

BALF was harvested from killed mice by placing a 20-gauge Luer stub adapter as a cannula into the trachea. The lungs were then lavaged three times with 0.7 ml PBS. The first of the three lavages was collected and saved for future analysis. Cells from all three lavages were combined and counted on a Cell-Dyn 35 hematology analyzer (Abbott Labs, Abbott Park, IL) for total cell counts. Cytospins were made for each sample and stained with Hema-3 stain (Biochemical Sciences, Swedesboro, NJ). Individual cell types were counted manually for determination of differential cell counts with total counts of 200 cells per each animal.

Measurement of Perivascular Inflammation

Tissue inflammation was measured by using H&E-stained lung sections and Bioquant Image analysis software (Bioquant Image Analysis Corporation, Nashville, TN). The area of the entire $\times 40$ magnified field was measured. The area of the vascular space was then measured and subtracted from the area of the entire field. The number of infiltrating leukocytes in the entire field was counted manually. The number of cells counted was then divided by the area of the lung for the final measurement of cells per unit area.

Determination of Epithelial Thickening and Mucus Production

Morphometric analysis was performed using a Nikon Eclipse E800 microscope and Bioquant software (Bioquant Image Analysis Corporation, Nashville, TN) for the measurement of epithelial area. To do this, the basement membrane length was measured and then the area of the corresponding epithelium was determined by tracing around the luminal edge of the epithelium. The data were represented as epithelial area per unit basement membrane length. Bioquant software then calculated the area of the epithelial layer, and the values were normalized to the basement membrane length. Mucus content was quantitated by measuring the basement membrane length and then counting the PAS-positive cells in the corresponding epithelium. The number of PAS-positive cells were then normalized per length of basement membrane.

Chemokine and Cytokine Measurement

Measurements of KC, MIP-2, and IL-17 were measured using ELISA (Quantikine M ELISA kits; R&D Systems, Minneapolis, MN). ELISA tests were performed as per the manufacturer's instructions.

Statistical Analysis

Differences between groups were analyzed using an ANOVA. *Post hoc* analysis was performed using Fisher's protected least significant difference test. This analysis was performed using StatView 5 statistical analysis software (SAS Institute, Inc., Cary, NC). Data was presented as mean values $\pm$ SEM.

RESULTS

Generation of Gob-5-KO Mice

A schematic diagram of the generation of Gob-5-CKOs is shown (Figure 1). Southern blot analysis was used to confirm targeted deletion in the CKO yielding a 9-kb band as compared with an 11-kb band in the wild type (Figure 1, *lower left*). Genotyping of wt and Gob-5-CKO alleles was distinguished using PCR of

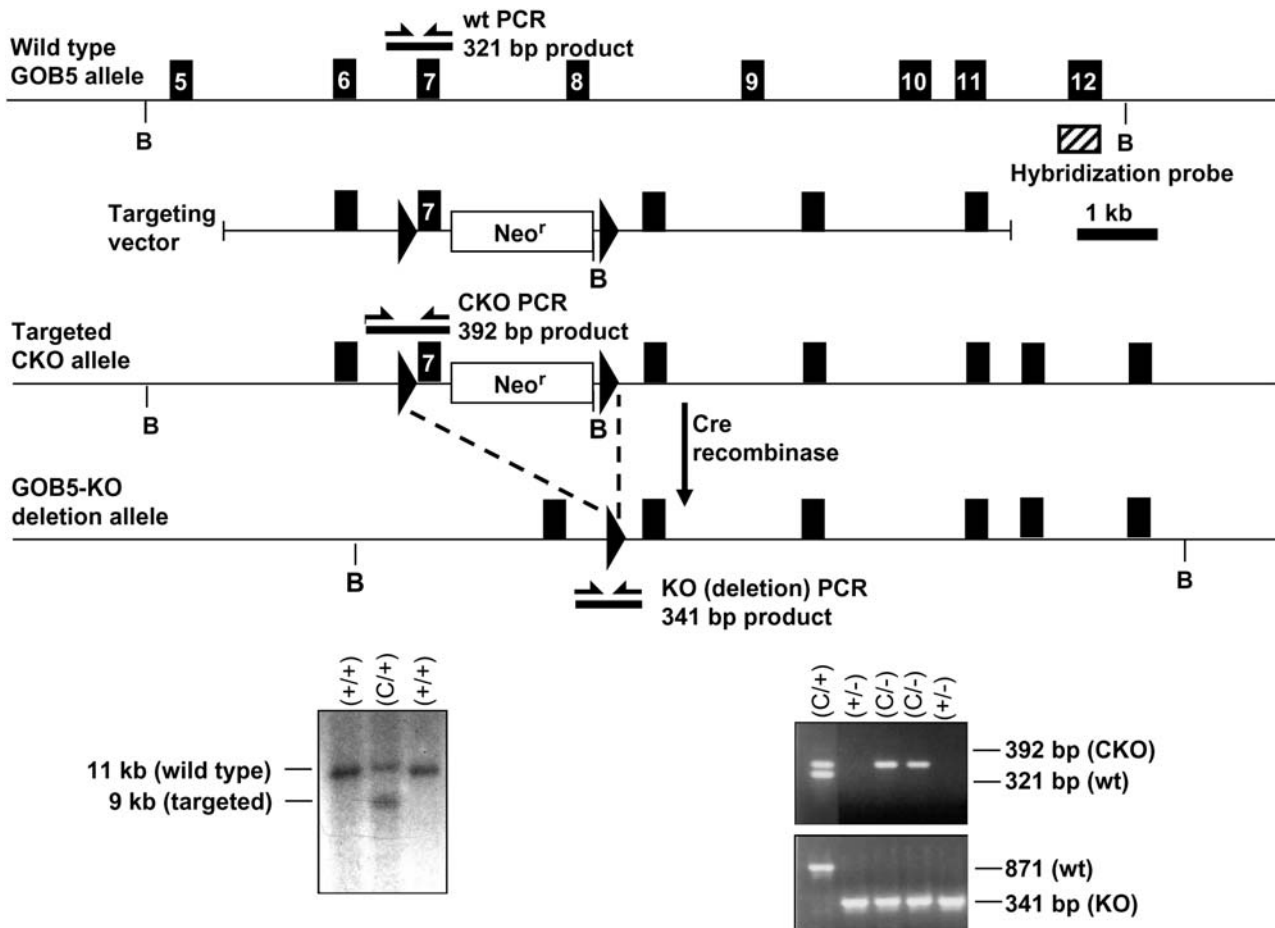


Figure 1. The Gob-5-CKO construct is composed of an 8-kb fragment of the Gob-5 gene with a 3-kb short arm and a 5-kb long arm flanking exon 7 and a neomycin resistance selectable marker gene (Neo^r). Exon 7 and Neo^r are flanked with LoxP sites indicated by *solid triangles*. Position of the BglII restriction sites are indicated by B. Also indicated are positions of PCR primers, amplicons, and product sizes used for genotyping. Southern blot analysis showing wt (+/+) and targeted (C/+) ES cell clones (*lower left*). PCR genotyping results (*lower right*) from the same set of tail biopsies distinguishing wt (+) and conditional alleles (top) and wt (+) and KO (–) alleles (*bottom*).

an amplicon spanning the 5' LoxP insertion site, yielding a 321-bp wt product and a 392-bp CKO product (Figure 1, *lower right, top panel*). The Gob-5-KO (deletion) allele was distinguished from the wt allele by PCR of an amplicon spanning the deletion junction, yielding an 871-bp wt product and a 341-bp product for the Gob-5-KO allele (Figure 1 *lower right, bottom panel*).

Inflammatory Response to Antigen

Wild-type or Gob-5-deficient animals were appropriately sensitized to OVA and challenged with aerosolized saline or antigen (5% OVA). After BALF harvest, total inflammatory infiltrates were counted and cell differential counts analyzed. Wild-type animals as well as Gob-5^{–/–} mice responded to antigen with a robust total inflammation that was significantly higher than PBS-challenged animals ($*P < 0.01$; Figure 2A). Gob-5^{–/–} mice had significantly higher total BAL cell inflammation than wt mice in response to antigen ($*P < 0.01$; Figure 2A). There was also a statistically significant increase in neutrophils ($*P < 0.01$) in Gob-5^{–/–} mice as compared with wt mice (Figure 2A). Monocytes and eosinophils increased in the KO mice after OVA challenge, but the difference in these cell types failed to reach statistical significance compared with the wt response (Figure 2A). Lymphocytes did not change significantly between OVA-challenged wt and Gob-5^{–/–} mice (Figure 2A).

Measurement of Perivascular Inflammation in OVA-Challenged Mice

Perivascular tissue inflammation was measured and normalized to lung area (Figure 2B). There was a significant increase in perivascular inflammation response to OVA challenge in both the wt ($*P < 0.01$) and the Gob-5^{–/–} mice ($*P < 0.01$). Compared with the wt animals, Gob-5^{–/–} mice exhibited a slight but significant decrease ($*P = 0.03$) in the amount of perivascular tissue inflammation (Figure 2B).

Western Analysis of BALF of OVA-Challenged Mice

Based on previous reports that showed that Gob-5 gene expression was induced in the lung of antigen-challenged mice (1, 2, 5, 13), as well as studies which demonstrated that Gob-5 is secreted and present in the BALF of challenged mice (14), we tested by Western blot for the presence of Gob-5 in the BALF from both wt mice and Gob-5^{–/–} mice challenged with either PBS (Figure 2C) or OVA (Figure 2C). Each *lane* represents an individual animal. An 85-kD Gob-5-immunoreactive band was detected after antigen challenge in the BALF of OVA-challenged wt mice (Figure 2C), but was not present in the PBS-challenged wt mice (Figure 2C). That same immunoreactive band was absent in Gob-5^{–/–} mice challenged with either PBS or OVA (Figure 2C), consistent with the KO phenotype.

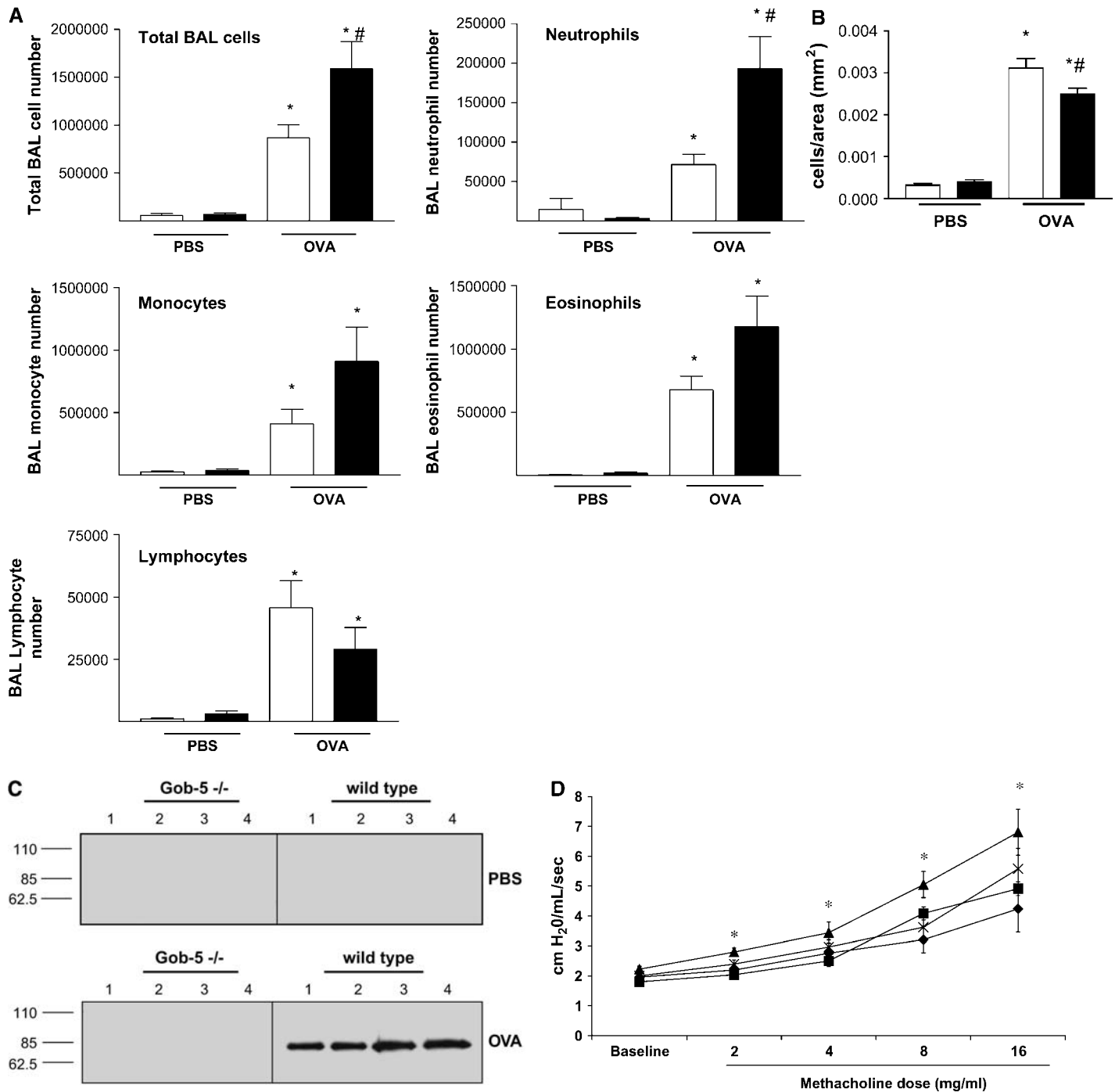


Figure 2. Total BAL inflammation was measured in (A) wt mice (open bars) or Gob-5^{-/-} mice (filled bars) in response to PBS or OVA challenge (as marked). Neutrophils, monocytes, eosinophils, and lymphocytes were determined by counting Wright-Geimsa stained cytopsin slides from BALF. Each group represents 20 mice. Statistically, groups were compared using ANOVA (* $P < 0.01$ for OVA versus PBS groups, # $P < 0.05$ for OVA-challenged Gob-5^{-/-} versus OVA-challenged wt groups). Data are presented as mean values $\pm$ SEM. OVA-challenged Gob-5^{-/-} mice exhibited a significant increase in BAL inflammation as compared with OVA-challenged wt mice (* $P < 0.05$) (B) Perivascular tissue inflammation was measured in wt mice (open bars) or Gob-5^{-/-} mice (filled bars) in response to PBS or OVA challenge (as marked). Wild-type mice and Gob-5^{-/-} mice respond to antigen with a significant increase in total inflammation compared with PBS-treated wt (* $P < 0.01$) and PBS-treated Gob-5^{-/-} (* $P < 0.01$). Gob-5^{-/-} mice exhibited a significant decrease in tissue inflammation as compared with wild type (* $P = 0.03$). Data are presented as mean values $\pm$ SEM. BALF from PBS-challenged and OVA-challenged wt (open bars) and Gob-5^{-/-} mice (filled bars). (C) Gob-5 was induced in the BALF in wt mice challenged with OVA, but was undetectable in Gob-5^{-/-} mice. Each lane represents a separate animal. Maximum airway resistance was measured (D) at baseline and in response to increasing concentrations (as indicated) of methacholine in PBS-challenged wt mice (diamonds), PBS-challenged Gob-5^{-/-} mice (squares), OVA-challenged wt mice (triangles), and OVA-challenged Gob-5^{-/-} mice (X symbols).

Airway Resistance Measurements

To measure changes in lung function in response to antigen challenge, we measured airway resistance and dynamic compliance in wt and Gob-5-null mice challenged with either aerosolized PBS or OVA. There was no difference in the baseline resistance between wt and Gob-5^{-/-} mice (Figure 2D). Wild-type mice challenged with OVA had an increase in maximum airway resistance after methacholine provocation as compared with PBS-treated animals (Figure 2D). OVA-challenged Gob-5-null mice exhibited a decrease of maximum airway resistance as compared with the OVA-challenged wt controls (Figure 2D). This difference was statistically significant at every dose of methacholine tested ($*P < 0.05$).

Measurement of Epithelial Cell Thickening in OVA-Challenged Mice

Epithelial thickening and goblet cell hyperplasia are characteristic features in the airway of mice after exposure to antigen (15). To address the role of Gob-5 in the induction of goblet cell hyperplasia, we measured the epithelial cell area in lung sections from both wt and Gob-5^{-/-} OVA-challenged mice (Figure 3A). There was no difference between wt and KO mice in the epithelial area after PBS aerosol challenge (Figure 3A). There was a 2-fold increase in epithelial area ($**P < 0.01$) in OVA-challenged wt mice as compared with PBS challenge. The epithelial area in the OVA-challenged Gob-5-null mice was also increased as compared with PBS-challenged animals ($*P < 0.01$), but was significantly reduced ($\#P < 0.01$) compared with wt OVA-challenged controls (Figure 3A).

Histology sections stained with H&E show the increased thickness of the epithelium. The epithelia in the airway of OVA-challenged wt animals (Figure 3A, *left panel*) have increased epithelial thickness that consists of multiple layers as compared with the airway epithelia in the OVA-challenged Gob-5^{-/-} mice (Figure 3A, *right panel*). The increase in epithelial thickening in OVA-challenged wt mice was associated with goblet cell hyperplasia (see below).

Mucus Production in OVA-Challenged Mice

Lungs from both wt and Gob-5^{-/-} mice challenged with either PBS or OVA were imbedded in paraffin, sectioned, and stained with PAS stain. To quantitate mucus metaplasia, the number of PAS-positive cells were counted and normalized to the length of basement membrane (Figure 3B). Minimal PAS staining was present in the PBS-treated wt and Gob-5^{-/-} mice (Figure 3B). In the OVA-treated groups, PAS staining was highly induced in the wt animals ($P < 0.01$) (Figure 3B). OVA-challenged Gob-5^{-/-} mice exhibited a significant increase ($*P < 0.01$) in PAS-positive staining as compared with PBS-treated animals but showed a significant decrease in the amount of PAS-positive cells ($\#P < 0.01$) as compared with OVA-challenged wt controls (Figure 3B). Representative histologic sections stained with PAS (Figure 3B) showing an induction of mucin-containing cells in the lungs of OVA-treated wt mice (Figure 3B, *left panel*) and a reduction PAS staining in the Gob-5^{-/-} mice (Figure 3B, *right panel*).

LPS Induction of Pulmonary Inflammation

To address whether the increase in pulmonary inflammation in response to antigen was a Th2 T cell-specific phenomenon, we tested the response of wt and Gob-5^{-/-} mice to LPS, a TLR4 ligand. Mice were intranasally administered either PBS or 1 mg/kg LPS. Twenty-four hours after LPS challenge, wt mice exhibited a robust total BAL cell inflammation (Figure 4A) ($*P < 0.01$). Gob-5^{-/-} mice had a significant increase ($*P <$

0.05) in the total BAL inflammation (Figure 4A) after challenge with LPS. The inflammatory response was predominantly neutrophilic (Figure 4A). LPS-challenged wt mice exhibited a significant increase in neutrophils as compared with PBS-challenged wt mice ($*P < 0.01$). Gob-5^{-/-} mice responded to LPS challenge with an even greater neutrophilic inflammation ($**P < 0.05$) than LPS-challenged wt mice (Figure 4A). Monocyte numbers were not influenced by PBS or LPS challenge in either the wt or Gob-5^{-/-} mice. Eosinophils or lymphocytes were undetectable in the BALF from PBS- or LPS-challenged wt or Gob-5^{-/-} mice (data not shown).

Perivascular Tissue Inflammation in Response to LPS

Perivascular tissue inflammation was measured as with OVA challenge. A similar finding was observed in LPS-challenged mice (Figure 4B). The perivascular tissue inflammation was significantly increased in both LPS-challenged wt ($*P < 0.01$) and LPS-challenged Gob-5^{-/-} mice ($\#P < 0.01$) compared with PBS-challenged animals. LPS-challenged Gob-5^{-/-} mice had significantly less perivascular inflammation than LPS-challenged wt animals ($**P = 0.02$) (Figure 4B).

Western Analysis of BALF of LPS-Challenged Mice

To test whether Gob-5 levels were induced in the lungs of LPS-challenged animals, BALF from wt and Gob-5-KO mice were tested by Western blot for Gob-5 protein expression. Gob-5 was not present in the BALF of PBS-challenged wt or Gob-5^{-/-} mice with the exception of one wt animal tested (Figure 4B). Gob-5 was induced in the BALF of three out of four wt animals tested (Figure 4B), while there was no Gob-5 induction in the BALF from Gob-5^{-/-} mice after intranasal LPS challenge (Figure 4B).

Measurement of Epithelial Thickening in LPS-Challenged Mice

Epithelial thickening was measured as before in the OVA-challenged mice. When the wt or Gob-5-deficient mice were challenged with LPS, a Th1-like stimulus, there was an increase in epithelial area in the wt mice as compared with PBS-treated animals (Figure 5) ($*P < 0.01$). In the Gob-5^{-/-} mice, there was no significant increase compared with PBS-treated mice. There was a complete reduction in epithelial area as compared with the LPS-challenged wt animals ($**P = 0.012$) (Figure 5).

Mucus Production in LPS-Challenged Mice

When challenged intranasally with LPS, neither wt mice nor Gob-5^{-/-} mice showed any increase in mucus production as measured by measuring PAS positively stained cells in lung sections (Figure 5). None of the PBS-challenged groups or the LPS-challenged groups exhibited any significant change in PAS-positive cells when compared with each other. The magnitude of the effect was similar to the PBS groups in the OVA-challenged animals (Figure 5).

Chemokine Production in LPS-Challenged Mice

Based on the increase in neutrophilia in both the antigen- and LPS-challenged mice, we tested for the presence of the neutrophil-specific α chemokines, KC and MIP-2 (Figure 6). In the BALF of LPS-challenged wt mice, mouse KC levels were significantly increased ($*P < 0.01$) compared with PBS-challenged wt mice (Figure 6). KC levels in the LPS-challenged Gob-5^{-/-} mice were significantly increased compared with PBS-challenged Gob-5^{-/-} mice ($*P < 0.01$) (Figure 6) and significantly greater than LPS-challenged wt mice ($**P = 0.05$). Levels of another α chemokine, MIP-2, was measured in the BALF from PBS- and LPS-challenged mice (Figure 6). MIP-2 levels were significantly

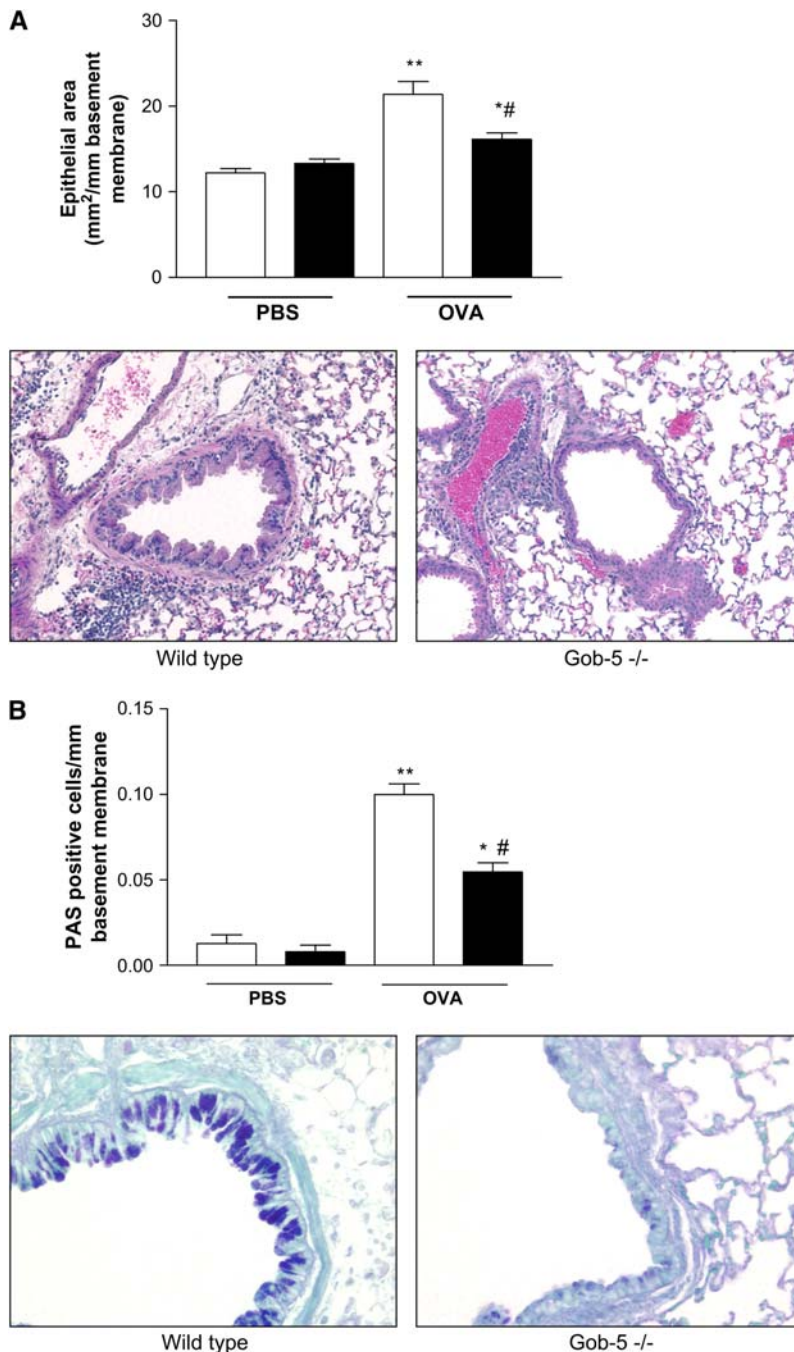


Figure 3. Epithelial thickening is represented as epithelial area as a function of basement membrane length (A). Measurements were made using Bioquant software at $\times 40$ magnification on H&E-stained sections of wt mice (*left panel*) and Gob-5^{-/-} mice (*right panel*). Each group represents 30 measurements from six animals each. OVA challenge in wt mice induces significant epithelial thickening ($**P < 0.01$). Gob-5^{-/-} mice (*filled bars*) had significant increase in area over baseline ($*P < 0.01$) but significantly less than wt (*open bars*) ($#P < 0.01$). Representative lung sections from OVA-challenged wt mice (*left panel*) and OVA-challenged Gob-5^{-/-} mice (*right panel*) stained with H&E are shown. The number of PAS-stained cells were counted and the resulting number normalized to length of basement membrane (B). OVA challenge in wt mice induces significant numbers of PAS-positive cells ($**P < 0.01$). Gob-5^{-/-} mice had significant increase in number of PAS-positive cells compared with baseline ($*P < 0.01$) but significantly less than wt ($#P < 0.01$). Representative lung sections from OVA-challenged wt mice (B, *left panel*) and OVA-challenged Gob-5^{-/-} mice (B, *right panel*) stained with PAS are shown. Data are presented as mean values $\pm$ SEM.

increased in LPS-challenged wt and Gob-5^{-/-} mice compared with the PBS-challenged cohorts ($*P < 0.01$). LPS-challenged wt and Gob-5^{-/-} did not differ in the MIP-2 levels in the BALF (Figure 6). Given the association of Gob-5 induction with IL-17 expression (5), as well as the ability of IL-17 to induce neutrophilic chemokines (16), levels of IL-17 in the BALF were measured (Figure 6). IL-17 is increased in wt and Gob-5^{-/-} mice challenged with LPS ($*P < 0.01$, $#P < 0.01$) compared with PBS-challenged animals. Levels of IL-17 were not significantly changed in LPS-challenged Gob-5^{-/-} mice as compared with LPS-challenged wt controls (Figure 6).

DISCUSSION

In the present study, Gob-5-deficient mice were characterized in different preclinical models of pulmonary inflammation. The

phenotype of the Gob-5^{-/-} mice in response to various stimuli implicate Gob-5 as an important molecule involved in the allergic response by regulating epithelial responses as well as the inflammatory partitioning in the lung.

When challenged in the lung with antigen after sensitization with OVA, a strong Th2-like stimulus, Gob-5-deficient mice exhibited an increase in total BAL inflammation that was 1.5 times the normal inflammatory response. A similar increase in total inflammation was seen when the mice were challenged with LPS. In response to both types of stimuli, the augmented BAL inflammation consisted mainly of neutrophils. In contrast to the BAL inflammation, the amount of perivascular tissue inflammation was decreased, suggesting that the increased inflammation in the BALF was due, in part, to increased trafficking from the tissue to the alveolar space. This observation suggests expression

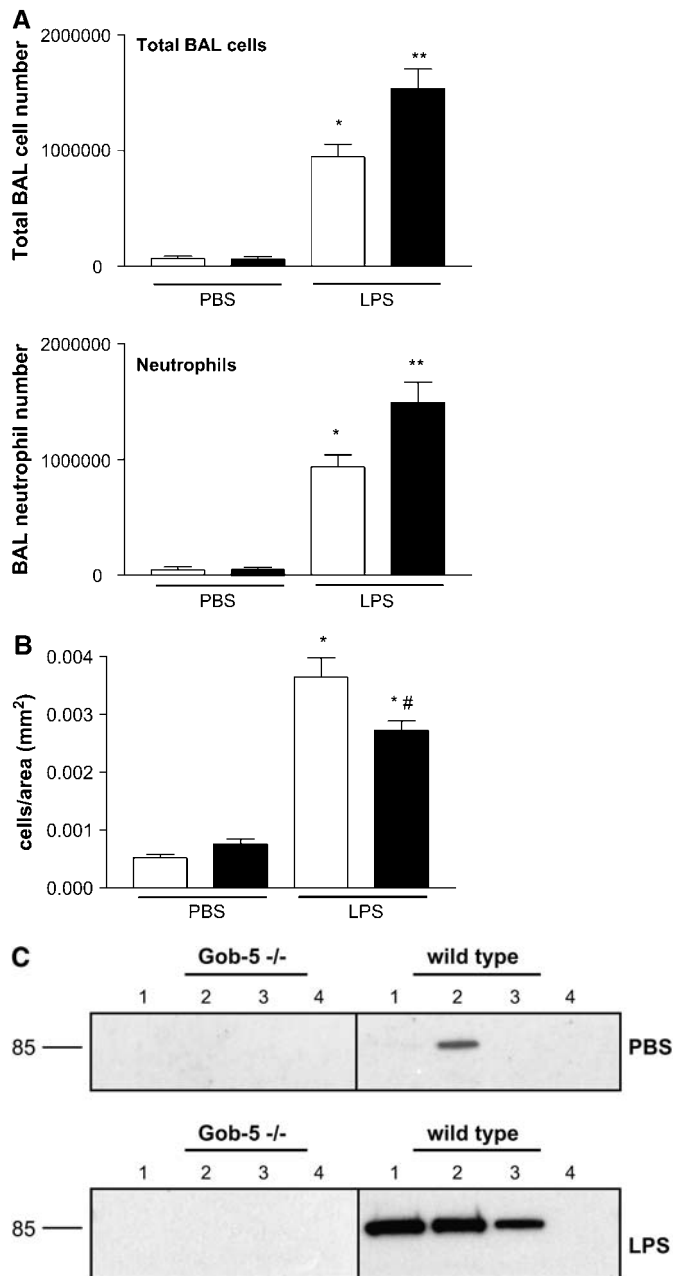


Figure 4. Total BAL cell inflammation in wt (open bars) and Gob-5^{-/-} mice (filled bars) in response to intranasal LPS. (A) There is an increase in pulmonary inflammation in wt and Gob-5^{-/-} mice compared with PBS-challenged controls (* $P < 0.001$) and a significant increase in LPS-challenged Gob-5^{-/-} mice compared with LPS-challenged wt mice (** $P < 0.05$). Neutrophils were also increased in LPS-challenged wt mice compared with PBS-challenged controls (* $P < 0.01$) as well as being increased in LPS-challenged Gob-5^{-/-} mice as compared with LPS-challenged wt mice (** $P = 0.012$). Perivascular tissue inflammation was measured in wt mice (open bars) or Gob-5^{-/-} mice (filled bars) in response to PBS or LPS challenge (as marked) (B). Wild-type mice and Gob-5^{-/-} mice respond to LPS with a significant increase in perivascular inflammation compared with PBS-treated wt (* $P < 0.01$). LPS-challenged Gob-5^{-/-} mice exhibited a significant decrease in tissue inflammation compared with LPS-challenged wt animals (* $P = 0.03$). Data are presented as mean values $\pm$ SEM. (C) Western analysis showing that Gob-5 was induced in the BALF in wt mice challenged with LPS but was undetectable in LPS-challenged Gob-5^{-/-} mice. Each lane represents a separate animal.

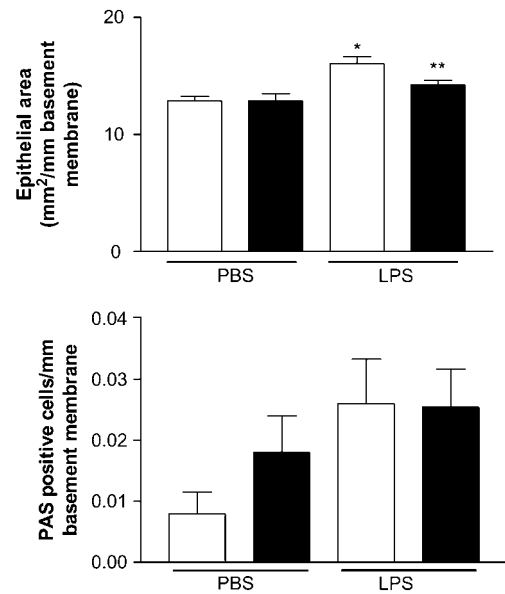


Figure 5. Epithelial thickening was measured in wt mice (open bars) or Gob-5^{-/-} mice (filled bars) in response to PBS or LPS challenge (as marked). Wild-type mice exhibited a slight but significant increase in epithelial thickening (* $P < 0.01$). LPS-challenged Gob-5^{-/-} mice exhibited a decrease in epithelial thickening compared with LPS-challenged wt mice (** $P < 0.01$). Mucus production was measured by counting PAS-positive cells. LPS challenge had no effect on any of the groups tested. Data are presented as mean values $\pm$ SEM.

of Gob-5 contributes to maintaining the localization of the inflammatory response in the tissue. It is unclear whether this phenomenon is the result of a direct or indirect effect of Gob-5, although the chemokine KC was significantly increased in BAL samples, suggesting that Gob-5 may affect the regulation of chemotactic signals. The increased concentration of KC in the alveolar space may be responsible for increased neutrophil trafficking into the BALF and the resulting decrease in the amount of tissue inflammation. MIP-2, another α chemokine that is chemotactic for neutrophils was not changed in the BALF of KO mice. The BALF was tested for levels of IL-17 based on previous studies which had shown that IL-17 induced neutrophilic chemokines (16) and was associated with Gob-5 gene expression after infection with respiratory syncytial virus (5). However, levels of IL-17 were unchanged in the BAL of LPS-treated mice. Gob-5, therefore, does not appear to regulate the induction of KC through an IL-17-dependent mechanism. The concentrations of IL-17, KC, and MIP-2 in the BALF from OVA-challenged mice were below the level of detection.

Gob-5 was necessary for some level of goblet cell hyperplasia and the development of mucus production in response to antigen challenge. The defect in Gob-5-KO mice appears to be in mucus-producing cells rather than a defect in mucus secretion, because in the absence of Gob-5 there was less goblet cell hyperplasia. The decrease in goblet cell hyperplasia and mucin production are not complete, suggesting that, in addition to Gob-5, some other pathway, or pathways are involved in the initiation of goblet cell hyperplasia. This does not seem to be the case when wt and Gob-5^{-/-} mice were challenged with LPS. The magnitude of the LPS effect on epithelial thickening in wt mice was less pronounced as compared with OVA-challenged mice, consistent with what has been shown previously (17). In wt mice, there was slight, but significant epithelial thickening essentially in the

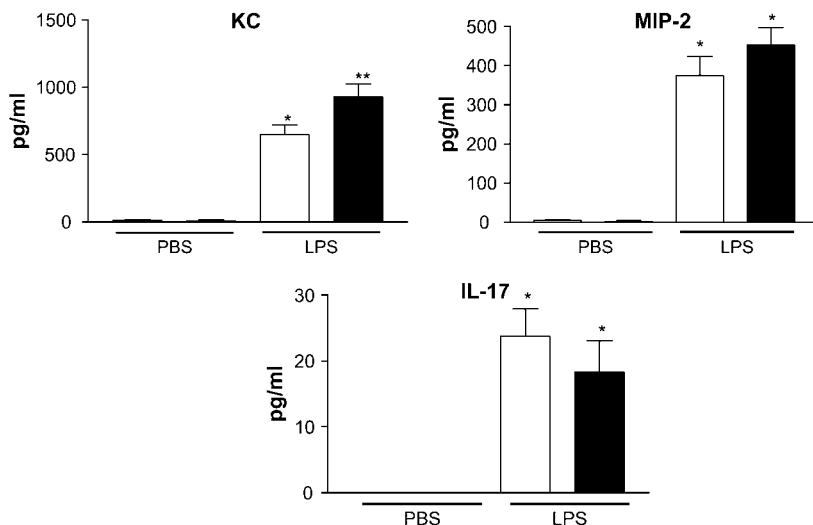


Figure 6. BALF from PBS-challenged or LPS-challenged wt mice (open bars) or Gob-5^{-/-} mice (filled bars) was used to measure mouse KC, MIP-2, and IL-17. All proteins tested were significantly increased in LPS-treated mice as compared with PBS-treated mice (* $P < 0.01$). Mouse KC was significantly enhanced in LPS-challenged Gob-5^{-/-} mice as compared with LPS-treated wt mice (** $P = 0.05$). Data are presented as mean values $\pm$ SEM.

absence of mucus. In the absence of Gob-5, however, the effect was completely ablated. This would suggest that Gob-5 is required for the epithelial cell hyperplasia in response to LPS but that other signals are required in a Th2 cell-driven response. The reduction in PAS staining in the Gob-5^{-/-} epithelium is consistent with a decrease in mucus production; however, we cannot exclude the possibility that the decreased staining is due to increased secretion from the epithelium.

When taken together, the change in the inflammatory compartmentalization and the decrease in goblet cell hyperplasia suggest that Gob-5 plays an important pathophysiologic role in allergic inflammation of the lung. In addition, the observation that Gob-5 is involved in the response to LPS challenge suggest that this pathway may also be important in regulation of innate immune responses in the lung. Upon challenge with a noxious stimulus, it would be important for the lung to be able to increase its epithelial cell layer as a physical barrier to invading pathogens while maintaining a regulated inflammatory response. In asthma, this type of response is exaggerated and the pathology of the disease is a consequence. It is clear, however, that to stimulate an increase in mucus, Gob-5 alone is not sufficient, given that LPS stimulation in the absence of additional an Th2 signal(s) did not enhance the production of mucus.

In a transgenic mouse model, overexpression of IL-10 in the mouse lung resulted in mucus metaplasia, changes in epithelial thickening, and a redistribution of inflammatory cells with an increase in tissue inflammation (4). This type of response would be predicted if Gob-5 were overexpressed in a similar fashion, given that we have shown that the opposite pattern of changes occurs in the absence of the protein. Consistent with this prediction, IL-10 overexpression induced expression of Gob-5 mRNA in the lungs of IL-10 transgenic mice (4). When the IL-10 transgenics were crossed with IL-13-KO mice or STAT-6-KO mice, the induction of Gob-5 mRNA, as well as the effects on tissue inflammation, mucus metaplasia, and remodeling, were ablated (4). These data indicate Gob-5 as a downstream mediator of these effects of IL-13 and STAT-6. The data presented in this report extend these findings and indicate that Gob-5 may be contribute to the inflammatory effects seen in the IL-10 transgenic mouse in addition to the mucus metaplasia.

Another report has described Gob-5-null mice and their response to allergic stimulus to the lung (18). The elimination of Gob-5 in these mice did not affect goblet cell hyperplasia, mucus formation, or cellular inflammation. These studies were very

similar to the present study in design, but with seemingly conflicting results. There are a number of differences between these two studies. First is a strain difference. Our studies were performed using 129SvEvBrd mice while the previous study had been conducted using Gob-5-KO mice that had been backcrossed onto a C57BL/6 background. It has been shown that allergen-induced airway disease is mouse strain dependent (19). 129SvJ mice have been shown to have a greater inflammatory response to antigen than C57BL/6 (19) and may explain the differences in response. Levels of Th2 cytokines are considerably higher in 129SvJ mice compared with C57BL/6 mice as well.

The results of the present study reinforce the association between Gob-5 and goblet cell hyperplasia. It also appears that Gob-5 is involved in regulating the inflammatory response, more specifically, the neutrophilic component of airways inflammation. These observations provide additional insight into the role of Gob-5, and by extension hCLCA1, in the asthmatic response. Gob-5 contributes to a decrease in lung function by increasing the amount of mucus obstruction. Lung function is a key measure of disease severity in human asthma and mucus accumulation has been implicated in decline in disease progression (20).

The regulation and function of Gob-5 has been thought to be regulated predominantly by Th2 cell cytokines. The results of the studies presented here show that the induction of goblet cell hyperplasia via Gob-5 was driven predominantly by Th2 cell cytokines. However, the changes seen in lung inflammation in the Gob-5-KO mice were not restricted to Th2 cell stimuli. The Gob-5-KO animals responded similarly with regards to the partitioning of the inflammation in response to either LPS or OVA. This is the first demonstration of a function of Gob-5 that is not restricted to Th2 cell-like stimuli.

It remains unclear whether the pleiotropic effects of Gob-5 are the result of a common signaling pathway or whether they are the result of separate, distinct molecular and cellular processes. The observations in this report that show the absence of Gob-5 resulted in an altered pattern of inflammatory cell trafficking, increased levels of a specific chemokine (KC), and decreased epithelial cell hyperplasia and goblet cell formation have identified specific cellular responses that should help enable the identification of molecular targets associated with Gob-5 activity.

Conflict of Interest Statement: All the authors are employees of Wyeth Research and declare that they have no other competing financial interests.

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References

1. Nakanishi A, Morita S, Iwashita H, Sagiya Y, Ashida Y, Shirafuji H, Fujisawa Y, Nishimura O, Fujino M. Role of gob-5 in mucus overproduction and airway hyperresponsiveness in asthma. *Proc Natl Acad Sci USA* 2001;98:5175–5180.
2. Kuperman DA, Lewis CC, Woodruff PG, Rodriguez MW, Yang YH, Dolganov GM, Fahy JV, Erle DJ. Dissecting asthma using focused transgenic modeling and functional genomics. *J Allergy Clin Immunol* 2005;116:305–311.
3. Miller AL, Strieter RM, Gruber AD, Ho SB, Lukacs NW. CXCR2 regulates respiratory syncytial virus-induced airway hyperreactivity and mucus overproduction. *J Immunol* 2003;170:3348–3356.
4. Lee CG, Homer RJ, Cohn L, Link H, Jung S, Craft JE, Graham BS, Johnson TR, Elias JA. Transgenic overexpression of interleukin (IL)-10 in the lung causes mucus metaplasia, tissue inflammation, and airway remodeling via IL-13-dependent and -independent pathways. *J Biol Chem* 2002;277:35466–35474.
5. Hashimoto K, Graham BS, Ho SB, Adler KB, Collins RD, Olson SJ, Zhou W, Suzutani T, Jones PW, Goleniewska K, *et al.* Respiratory syncytial virus in allergic lung inflammation increases Muc5ac and Gob-5. *Am J Respir Crit Care Med* 2004;170:306–312.
6. Dahesia M, Iian NT, Connolly T, Drawid A, Wu Q, Bienvenu J, Cavallo J, Jupp R, De Sanctis GT, Minnich A. Molecular characterization of antigen-induced lung inflammation in a murine model of asthma. *Ann N Y Acad Sci* 2002;975:148–159.
7. Leverkoehne I, Gruber AD. The murine mCLCA3 (alias gob-5) protein is located in the mucin granule membranes of intestinal, respiratory, and uterine goblet cells. *J Histochem Cytochem* 2002;50:829–838.
8. Busse W, Banks-Schlegel S, Wenzel S. Pathophysiology of severe asthma. *J Allergy Clin Immunol* 2000;106:1033–1042.
9. Lee J, Cacalano G, Camerato T, Toy K, Moore M, Wood W. Chemokine binding and activities mediated by the mouse IL-8 receptor. *J Immunol* 1995;155:2158–2164.
10. Singer M, Martin L, Vargaftig B, Park J, Gruber A, Li Y, Adler K. A MARCKS-related peptide blocks mucus hypersecretion in a mouse model of asthma. *Nat Med* 2004;10:193–196.
11. Fahy JV. Goblet cell and mucin gene abnormalities in asthma. *Chest* 2002;122:320–326.
12. Chen Q, Rabach L, Noble P, Zheng T, Lee CG, Homer RJ, Elias JA. IL-11 receptor α in the pathogenesis of IL-13-induced inflammation and remodeling. *J Immunol* 2005;174:2305–2313.
13. Zhou Y, Dong Q, Louahed J, Dragwa C, Savio D, Huang M, Weiss C, Tomer Y, McLane MP, Nicolaides NC, *et al.* Characterization of a calcium-activated chloride channel as a shared target of Th2 cytokine pathways and its potential involvement in asthma. *Am J Respir Cell Mol Biol* 2001;25:486–491.
14. Gibson A, Lewis AP, Affleck K, Aitken AJ, Meldrum E, Thompson N. hCLCA1 and mCLCA3 are secreted non-integral membrane proteins and therefore are not ion channels. *J Biol Chem* 2005;280:27205–27212.
15. Blyth D, Pedrick M, Savage T, Hessel E, Fattah D. Lung inflammation and epithelial changes in a murine model of atopic asthma. *Am J Respir Cell Mol Biol* 1996;14:425–438.
16. Linden A, Laan M, Anderson GP. Neutrophils, interleukin-17A and lung disease. *Eur Respir J* 2005;25:159–172.
17. Blyth DI, Pedrick MS, Savage TJ, Bright H, Beesley JE, Sanjar S. Induction, duration, and resolution of airway goblet cell hyperplasia in a murine model of atopic asthma: effect of concurrent infection with respiratory syncytial virus and response to dexamethasone. *Am J Respir Cell Mol Biol* 1998;19:38–54.
18. Robichaud A, Tuck SA, Kargman S, Tam J, Wong E, Abramovitz M, Mortimer J, Burston HE, Masson P, Slipetz D, Kennedy B, *et al.* Gob-5 is not essential for mucus overproduction in preclinical murine models of allergic asthma. *Am J Respir Cell Mol Biol* 2005;33:303–314.
19. Whitehead GS, Walker JKL, Berman KG, Foster WM, Schwartz DA. Allergen-induced airway disease is mouse strain dependent. *Am J Physiol Lung Cell Mol Physiol* 2003;285:L32–L42.
20. Lange P, Parner J, Vestbo J, Schnohr P, Jensen G. A 15-year follow-up study of ventilatory function in adults with asthma. *N Engl J Med* 1998;339:1194–1200.