Lymphotoxin beta receptor signaling directly controls airway smooth muscle deregulation and asthmatic lung dysfunction

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Background: Dysregulation of airway smooth muscle cells (ASM) is central to the severity of asthma. Which molecules dominantly control ASM in asthma is unclear. High levels of the cytokine LIGHT (aka TNFSF14) have been linked to asthma severity and lower baseline predicted FEV_1 percentage, implying that signals through its receptors might directly control ASM dysfunction.

Objective: Our study sought to determine whether signaling via lymphotoxin beta receptor (LTβR) or herpesvirus entry mediator from LIGHT dominantly drives ASM hyperreactivity induced by allergen.

Methods: Conditional knockout mice deficient for LTβR or herpesvirus entry mediator in smooth muscle cells were used to determine their role in ASM deregulation and airway hyperresponsiveness (AHR) *in vivo*. Human ASM were used to study signals induced by LTβR.

Results: LTβR was strongly expressed in ASM from normal and asthmatic subjects compared to several other receptors implicated in smooth muscle deregulation. Correspondingly, conditional deletion of LTβR only in smooth muscle cells in $smMHC^{Cre}LT\beta R^{fl/fl}$ mice minimized changes in their numbers and mass as well as AHR induced by house dust mite allergen in a model of severe asthma. Intratracheal LIGHT administration independently induced ASM hypertrophy and AHR *in vivo* dependent on direct LTβR signals to ASM. LIGHT promoted contractility, hypertrophy, and hyperplasia of human ASM *in vitro*. Distinguishing LTβR from the receptors for IL-13, TNF, and IL-17, which have also been implicated in smooth muscle dysregulation, LIGHT promoted NF-κB–inducing kinase-dependent noncanonical nuclear factor kappa–light-chain enhancer of activated B cells in ASM *in vitro*, leading to sustained accumulation of F-actin, phosphorylation of myosin light chain kinase, and contractile activity.

Conclusions: LTβR signals directly and dominantly drive airway smooth muscle hyperresponsiveness relevant for pathogenesis of airway remodeling in severe asthma. (J Allergy Clin Immunol 2023;151:976-90.)

Key words: *LTβR, asthma, airway smooth muscle, AHR, contractility, noncanonical NF-κB, TNF superfamily, LIGHT, TNFSF14*

The pathologic features of asthma are chronic airway inflammation associated with an aberrant airway constriction response to allergen, termed airway hypersensitivity or hyperresponsiveness (AHR).[1] Deregulation of airway smooth muscle cells (ASM), which play a pivotal role in constriction and dilation of the airways, may be key to AHR.[2] ASM mass is greater in patients with severe asthma compared to those with moderate asthma.[3] Increased ASM mass is also observed in children with severe asthma despite their having a relatively short duration of asthma, suggesting that this change is directly linked to asthma severity.[4] Moreover, ASM isolated from asthmatic subjects also exhibit enhanced contractile activity and enhanced proliferation.[2,5] However, what drives these changes is still not fully appreciated.

Specifically, it remains unclear whether immune-mediated inflammatory signals into ASM are directly responsible for lung dysfunction, and if so, which signals may be essential. Several cytokines linked to asthma, notably IL-13, TNF, and IL-17, have been shown to promote contractility or other responses in ASM *in vitro* and can contribute to AHR *in vivo* in mouse models.[6-11] Nevertheless, no data have yet shown that their direct actions on smooth muscle cells *in vivo* are essential for ASM changes or for AHR in response to allergen. In particular, studies of the receptor for IL-13, which has gained strong prominence in asthma pathogenesis from clinical trials of the IL-4Rα–targeting antibody dupilumab,[12,13] failed to find a defect in AHR and smooth muscle deregulation to allergen when IL-4Rα was conditionally deleted in mice only in smooth muscle cells.[14,15] Thus, the key molecules directly and dominantly controlling asthma-related changes in ASM activity that are relevant for AHR are still not clear.

We previously linked the cytokine LIGHT (homologous to lymphotoxin, exhibits inducible expression, and competes with herpes simplex virus glycoprotein D for binding to herpesvirus

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Abbreviations used
AHR: Airway hyperresponsiveness
ASM: Airway smooth muscle cells
αSMA: Alpha smooth muscle actin
BAL: Bronchoalveolar lavage
BrdU: Bromodeoxyuridine
EpCAM: Epithelial cell adhesion molecule
HDM: House dust mite
HVEM: Herpesvirus entry mediator
LIGHT: TNF superfamily 14, aka TNFSF14
LTαβ: Lymphotoxin alpha beta
LTβR: Lymphotoxin beta receptor
MCAM: Melanoma cell adhesion molecule
MLC: Myosin light chain
MLCK: MLC kinase
MYPT: Myosin phosphatase targeting protein
NF-κB: Nuclear factor kappa–light-chain enhancer of activated B cells
NIK: NF-κB–inducing kinase
PAK1: p21-activated kinase 1
PAS: Periodic acid–Schiff
PDGFRα: Platelet-derived growth factor receptor A
RNA-Seq: RNA sequencing
siRNA: Small interfering RNA
smMHC: Smooth muscle myosin heavy chain

entry mediator [HVEM], a receptor expressed on T lymphocytes), also known as TNF superfamily member 14, or TNFSF14, to the severity of lung inflammation and to changes in ASM mass with studies of LIGHT-deficient mice.[16] LIGHT can be expressed by several immune cell types, including activated T cells, dendritic cells, and neutrophils, that are found in asthmatic lungs, and higher levels of soluble LIGHT or cell-associated LIGHT in the sputum of asthmatic patients are associated with severe disease.[17-19] Structural cells of the lung such as epithelial cells and fibroblasts express the receptors for LIGHT, namely HVEM (TNFRSF14) and lymphotoxin beta receptor (LTβR) (LTBR/TNFRSF3), and LIGHT can promote inflammatory activity in these cells.[20-24] However, whether LIGHT and its receptors act directly on ASM *in vivo* to promote exaggerated responsiveness and play a direct role in AHR has not been demonstrated.

In this study, we now show that LTβR is constitutively expressed on mouse and human ASM. With conditional deletion of LTβR in smooth muscle cells *in vivo,* we demonstrate that LTβR activity in these cells is essential for smooth muscle remodeling, lung dysfunction, and AHR driven by inhaled allergen. This is explained by LTβR activating sustained signaling pathways involving nuclear factor kappa–light-chain enhancer of activated B cells (NF-κB)-inducing kinase (NIK) and activation of noncanonical NF-κB—pathways not triggered by the other ASM-associated cytokines IL-13, TNF, and IL-17.

METHODS

Mice

C57BL/6J and smooth muscle myosin heavy chain (smMHC) *(Myh11)*/Cre/eGFP transgenic mice (B6.Cg-Tg(Myh11-cre,-EGFP)2Mik/J)[25] were purchased from The Jackson Laboratory (Bar Harbor, Me). HVEM-floxed mice were generated in house as previously described.[23,26] LTβR-floxed mice were generated by Alexei Tumanov as previously described.[27] HVEM flox/flox or LTβR flox/flox mice crossed to smMHC^{Cre} transgenic mice were bred in house on a C57BL/6 background. Animal experiments were performed with 6- to 8-week-old female mice. All animals used in this study were maintained in specific-pathogen-free conditions. All experiments were performed in compliance with the regulations of the La Jolla Institute for Immunology Animal Care Committee in accordance with guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care.

Mouse models of airway remodeling and acute airway inflammation

For allergen airway remodeling experiments, mice were intranasally administered house dust mite (HDM), *Dermatophagoides pteronyssinus,* extract (Greer, Lenoir, NC): 200 μg on day 0 and 100 μg on days 7 and 14, followed by 50 μg of HDM provided twice a week for 4 weeks. For LIGHT experiments, mice were injected intratracheally with 10 μg of recombinant LIGHT (R&D Systems, Minneapolis, Minn) or PBS on days 1 and 2 and analyzed on day 3. For acute airway inflammation, mice were sensitized by administration of 20 μg HDM protein in 2 mg alum provided intraperitoneally on day 0, and challenged with 10 μg HDM protein intranasally on days 10 to 13.

Confocal microscopy of lung tissue

Lungs were intubated, filled, and embedded in the Cryomold with OCT compound, and frozen. The fresh frozen lung tissues were postfixed in 4% paraformaldehyde, and lung sections were cut (30 μm) and stained with rabbit polyclonal antibody to α-smooth muscle actin (αSMA; Abcam, Cambridge, United Kingdom), phalloidin–Alexa Fluor 568 (Thermo Fisher Scientific, Waltham, Mass), and Hoechst 33342 (BD Biosciences, Franklin Lakes, NJ). All 3-D high-resolution tertiary bronchi image stacks were acquired with an inverted Zeiss 780 or 880 Airyscan laser scanning confocal microscope using a 40× (1.4na) objective and the 32-channel GaAsP-PMT area detector (Carl Zeiss, Jena, Germany). Image stacks through 15 to 20 μm of lung tissue, on average 35 slices, were acquired with Nyquist resolution parameters using a 0.421 μm step size and optimal frame size of 2048 × 2048.

Lung tissues were further processed in Imaris software (Imaris, Concord, Mass) using the isosurface module (Bitplane) to outline and quantify the volume and area of αSMA or phalloidin around the mouse bronchioles. Phalloidin (F-actin) was more consistent in labeling the smooth muscle, based on the density of the signal and circular localization pattern in addition to the co-localization with αSMA. The actin labeling in all other nonmuscle cells was omitted from analysis by masking only the bronchioles using Imaris software and then thresholding the F-actin signal to omit very low to sparsely labeled F-actin in epithelial and other cells beyond the smooth muscle layer. A total of 10 to 15 tertiary bronchi per 4 or 5 mouse lungs per condition and mouse model were analyzed.

Airway hyperresponsiveness

Airway resistance in response to different doses of methacholine was measured using the FlexiVent system (Scireq, Montreal, Quebec, Canada) as previously described.[16] Peak airway resistance was analyzed by Scireq flexiWare v8 software (Scireq).

Flow cytometry

Lungs were dissociated with the gentleMACS Dissociator and a Lung Dissociation Kit (Miltenyi Biotec, San Diego, Calif). Single cells were stained with monoclonal antibodies to mouse CD45 (clone 30-F11), CD11b (clone M1/70), CD11c (clone HL3), and SiglecF (clone E50-2440) from BD Biosciences; epithelial cell adhesion molecule (EpCAM; clone G8.8), CD31 (clone 390), platelet-derived growth factor receptor A (PDGFRα; clone 1A4/asm-1), and melanoma cell adhesion molecule (MCAM; clone ME-9F1) from BioLegend (San Diego, Calif); vimentin (clone 280618) from R&D Systems; and αSMA (clone 1A4/asm-1) from

Novus Biologicals (Littleton, Colo). Live/dead cells were stained with Fixable Aqua Dead Cell Staining Kit (Thermo Fisher Scientific). For intracellular staining, Foxp3 Transcription Factor Staining Buffer Set (eBioscience, San Diego, Calif) was used for fixation and permeabilization. Flow analysis was performed on a Fortessa device (BD Biosciences), and data were analyzed by FlowJo v10 software (Treestar, Ashland, Ore). Live$^+$CD45$^+$ lung immune cells were gated for neutrophils, eosinophils, alveolar macrophages, CD4$^+$ T cells, and CD8$^+$ T cells following the gating strategy shown in Fig E2 in the Online Repository available at www.jacionline.org. Live$^+$CD45$^-$ lung structural cells were gated into alternative populations by surface staining for EpCAM, CD31, PDGFRα, Mcam, and intracellular staining for vimentin and αSMA.

Histology

Whole lung lobes were fixed with 10% formalin and embedded in paraffin. Sections were stained with hematoxylin and eosin or periodic acid–Schiff (PAS). Mucus production was assessed by measuring the percentage of PAS-positive cells airway epithelial cells in the bronchioles. More than 5 bronchi per section were randomly selected and used for quantification.

Bronchoalveolar lavage cytokines

Cytokines in bronchoalveolar lavage (BAL) fluid were assayed by sandwich ELISA with paired antibody sets according to the manufacturers' instructions. Mouse IL-4, IL-5, and IL-13 kits were purchased from R&D Systems.

ASM culture and analysis

Healthy donor human ASM or asthmatic diseased donor ASM were purchased from ScienCell (catalog 3400; Carlsbad, Calif) or Lonza (00194850; Walkersville, Md). Cells from several donors were used for reproducibility. Other asthmatic donor ASM were isolated from postmortem lungs provided by Richard Kurten from the Arkansas Regional Organ Recovery Agency as previously described[28] (Hl129: asthma, 7 years old, male, White, nonsmoker, cause of death cerebrovascular accident/stroke; Hl227: asthma, 21 years old, male, White, nonsmoker, cause of death head trauma). For flow analyses, cells were stained with monoclonal antibodies to human LTβR (clone 31G4D8) or HVEM (clone 122), purchased from BioLegend. Cells were maintained in Smooth Muscle Cell Media (ScienCell) with supplied supplements and FBS added. Cells were used between passages 2 and 4 and were cultured in smooth muscle basal media for 16 hours or 7 days before stimulation with recombinant LIGHT (100 ng/mL). In some experiments, inhibitors of NIK/noncanonical NF-κB (NIK-SMI, 25 nmol; MedChemExpress, Monmouth, NJ), canonical NF-κB (BAY11-7082, 1 nmol, MedChemExpress), or Rac1 (NSC 23766, 10 nmol; Tocris Bioscience, Bristol, United Kingdom) were added for 1 hour before stimulation with rLIGHT. For small interfering RNA (siRNA) knockdown, ON-TARGETplus siRNA to human LTβR and NIK as well as a nontargeting control were purchased from Dharmacon (Pittsburg, Pa). siRNA Oligo Duplex siRNA to human HVEM was purchased from OriGene Technologies (Rockville, Md). A total of 50 nmol of siRNA was transfected into ASM using HiPerFect transfection reagent (Qiagen, Hilden, Germany) as described previously[21] and according to the manufacturer's instructions.

ASM gel contraction assay

We performed 3-D gel contraction assays using collagen contraction assay kits purchased from Cell Biolabs (San Diego, Calif) according to the manufacturer's instructions. In brief, suspensions of ASM were mixed with collagen solution at a density of 0.5-1 $\times$ 10^6 cells per well. After confirming the polymerization of collagen gels, smooth muscle basal medium was added and incubated for 16 hours. Collagen gels were then stimulated with rLIGHT (100 ng/mL), rLTαβ (100 ng/mL), or PBS and analyzed for gel contraction at different time points (24, 48, and 72 hours). Images were obtained with Image-Lab (Bio-Rad, Hercules, Calif), and the area of collagen gel matrix was measured by ImageJ software (imagej.nih.gov/ij/).

ASM intracellular protein analysis

For analyzing actin polymerization and focal adhesions, ASM were incubated in smooth muscle basal media without serum for 16 hours and seeded as monolayers at 70% to 80% confluency on coverslips coated with Collagen Type I solution (Sigma-Aldrich, St Louis, Mo). Cells were stimulated with 100 ng/mL of rLIGHT or PBS with or without NIK-SMI or Rac inhibitors. After 6 to 12 hours, cells were washed and fixed with 4% formalin and permeabilized with 0.1% Triton X-100 (Thermo Fisher Scientific). Cells were stained with phalloidin–Alexa Fluor 568 (Thermo Fisher Scientific), anti-vinculin antibody (Abcam), and Hoechst 33342 (BD Biosciences) and analyzed by confocal microscopy. Single ASM images were processed in Imaris software using the isosurface module (Bitplane) to outline and quantify the volume of intracellular vinculin clusters and F-actin expression (phalloidin). All 3-D high-resolution single ASM cell images were acquired with an inverted 880 Airyscan laser scanning confocal microscope using a 63$\times$ (1.4na) objective and the 32-channel GaAsP-PMT area detector (Zeiss). Image stacks through 3 to 5 μm of spreading ASM, on average 10 to 15 slices, were acquired with Nyquist resolution parameters using a 0.3 μm step size and optimal frame size of 4164 $\times$ 4164. A total of 300 to 400 individual ASM, in 2 replicate experiments for the various conditions, were analyzed.

ASM migration/wound assay

ASM were seeded in monolayers on coverslips coated with Collagen Type I solution (Sigma-Aldrich) and cultured in smooth muscle media until reaching 90% confluency. Cells were incubated in smooth muscle basal media for 16 hours before making a scratch wound in a straight line, followed by washing out of any debris. Smooth muscle basal media containing 100 ng/mL of rLIGHT or PBS was then added and incubated for 12 hours. Cells were washed and fixed with 4% formalin and permeabilized with 0.1% Triton X-100 (Thermo Fisher Scientific). Cells were stained with phalloidin–Alexa Fluor 568 (Thermo Fisher Scientific) and Hoechst 33342 (BD Biosciences) and analyzed by confocal microscopy. All 3-D multistitched image panels were acquired on an inverted Zeiss LSM 780 confocal laser-scanning microscope using a 20 $\times$ 0.8 NA objective, a z-step size of 0.85 μm, and the automated tiling function of Zen software with a 10% overlap between tiles. Images were stitched, and 3-D stacks were maximum intensity projected using Zen software, then imported into Image Pro Premier 10 (IPP10; Media Cybernetics, Rockville, Md) for further processing. Briefly, by using the phalloidin–Alexa Fluor 568 signal to define the cells along the wound edge and the thresholding tool in IPP10 to define areas devoid of cells (set at a dynamic range of 0-50 for 8-bit images), the autocount tool automatically outlined and quantified the area along the wound front, allowing quantification of the wound area and the extent of ASM migration into the wound.

ASM proliferation

ASM were cultured in the presence of 100 ng/mL rLIGHT or PBS. After 48 hours, cells were collected, and the proliferating cells were detected by bromodeoxyuridine (BrdU) incorporation using a fluorescein isothiocyanate BrdU Flow Kit (BD Biosciences) according to the manufacturer's protocol.

Western blot analysis

Cells were lysed in radioimmunoprecipitation assay buffer containing protease inhibitor (Roche, Basel, Switzerland). Whole-cell lysates were run in WedgeWell 8-16% Trys-Glysin gradient gels (Novex, Richmond, British Columbia, Canada) and transferred to nitrocellulose membrane (Bio-Rad). Membranes were incubated with 5% BSA (Sigma-Aldrich) or nonfat dry milk (R&D Systems) in Tris-buffered saline and polysorbate 20 for blocking, then incubated with primary antibodies to pNF-κB p65 (3033, 1:1000), NF-κB p65

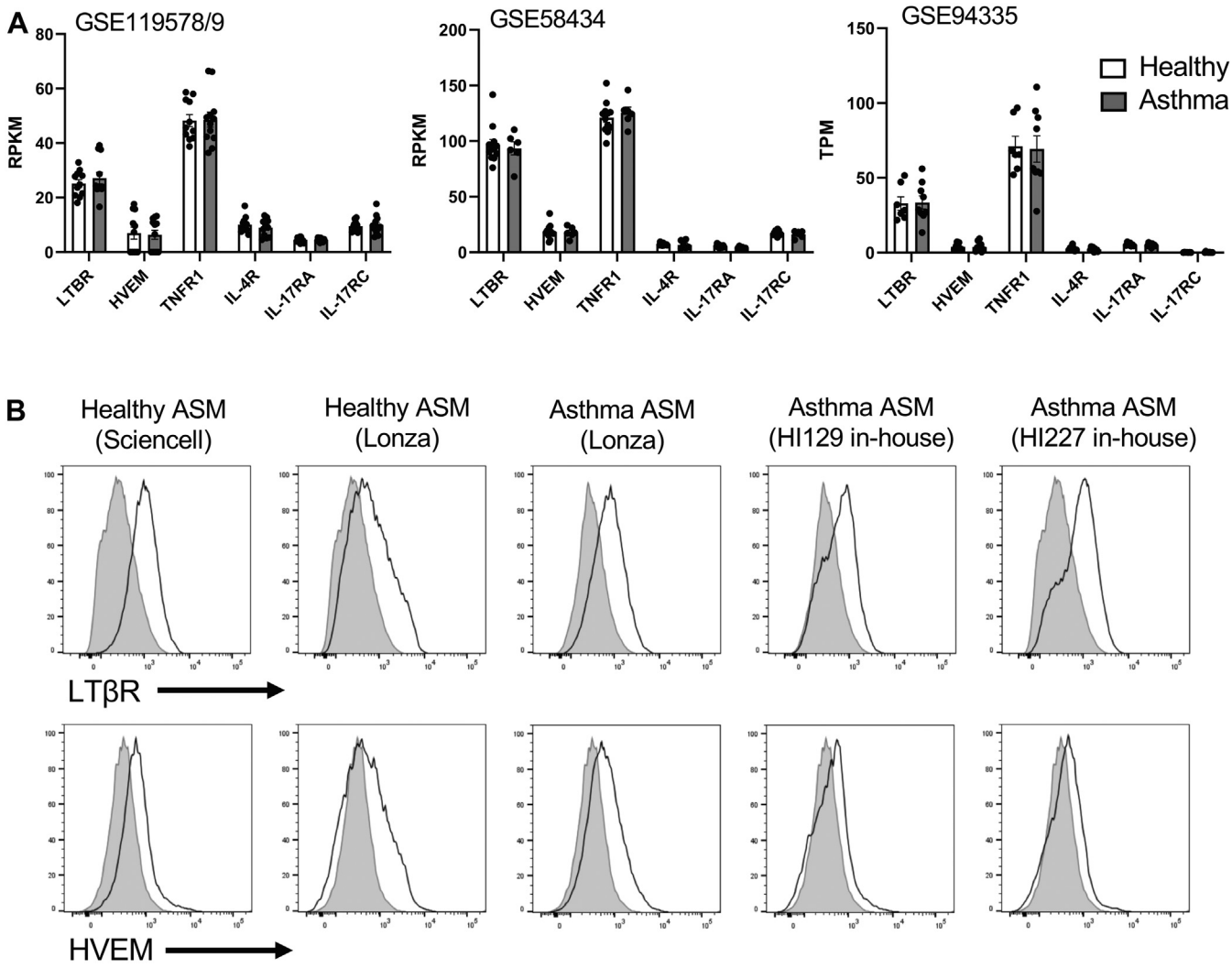

FIG 1. LTβR and HVEM are expressed by human healthy and asthmatic ASM. **(A)** Expression of transcripts of the receptors for LIGHT, TNF, IL-13/IL-4, and IL-17 from published RNA-Seq data of human healthy and asthmatic ASM (GSE119578/9, GSE58434, GSE94335). **(B)** Flow analysis of LTβR and HVEM expression on healthy and asthmatic donor–derived ASM.

(8242, 1:1000), NF-κB p100/52 (3017, 1:1000), PAK1 (2602,1:1000), pMYPT (Thr696) (5163, 1:1000), pMLC2 (3672, 1:1000), and MLC2 (8505, 1:500) from Cell Signaling Technology (Danvers, Mass); to pPAK1 (135755, 1:200) and MYPT (514261, 1:100) from Santa Cruz Biotechnology (Santa Cruz, Calif); followed by horseradish peroxidase–conjugated secondary antibodies to mouse IgG (516102, Santa Cruz Biotechnology) or rabbit IgG (2357, Santa Cruz Biotechnology). Chemiluminescence was enhanced with Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare, Waukesha, Wis) and visualized after exposure to Gel Doc (Bio-Rad). Stripping and reblotting were performed with Restore Western Blot Stripping Buffer (Thermo Fisher Scientific). Immunoblotting for GAPDH (Santa Cruz Biotechnology) was used as loading control. For Rac1 detection, whole cell lysates were used for immunoprecipitation using the Active Rac1 detection kit (Cell Signaling Technology) according to the manufacturer's instructions.

Real-time reverse transcriptase PCR

Total RNA from ASM was isolated with the RNeasy kit (Qiagen). cDNA was prepared using a SuperScript IV reverse transcriptase kit (Thermo Fisher Scientific). Real-time PCR was performed with a LightCycler (Roche) using PowerUp SYBR Green master mixes (Applied Biosystems; Thermo Fisher Scientific). Data were normalized with housekeeping gene *GAPDH* and are presented as relative expressions or relative quantifications to control samples, which were derived from the difference in cycle threshold (C_t) between the gene of interest and the housekeeping genes using the equation RQ = $2 - \Delta\Delta C_t$. Designed primers for human MAP3K14 were purchased from Bio-Rad. The oligonucleotide primer sequences were as follows: GAPDH, sense AGCCAAAAGGGTCATCATCTCT, antisense AGGGGCCATCCACAGTCTT; HVEM, sense AGCAGCTCCCACTGGGTATG, antisense GATTAGGCCAACTGTGGAGCA; and LTβR, sense CCGACACAACCTGCAAAAAT, antisense GAGCAGAAAGAAGGCCAGTG.

Analysis of published RNA sequencing data

Bulk RNA sequencing (RNA-Seq) data of healthy and asthma ASM were analyzed for transcripts of cytokine receptors.[29-31] Fong et al[29] used ASM from 11 asthmatic and 12 nonasthmatic donors over 2 GEO (Gene Expression Omnibus, www.ncbi.nlm.nih.gov/geo) submissions: GSE119578 (GSE119578_GeneCount_rpkm_by_gene_RDB.txt.gz) and GSE119579 (GSE119579_GeneCount_rpkm_by_gene_SW.txt.gz). Himes et al[30] used ASM from 6 donors with fatal asthma and 12 control donors. Only baseline (no treatment) measurements from GSE58434 were analyzed

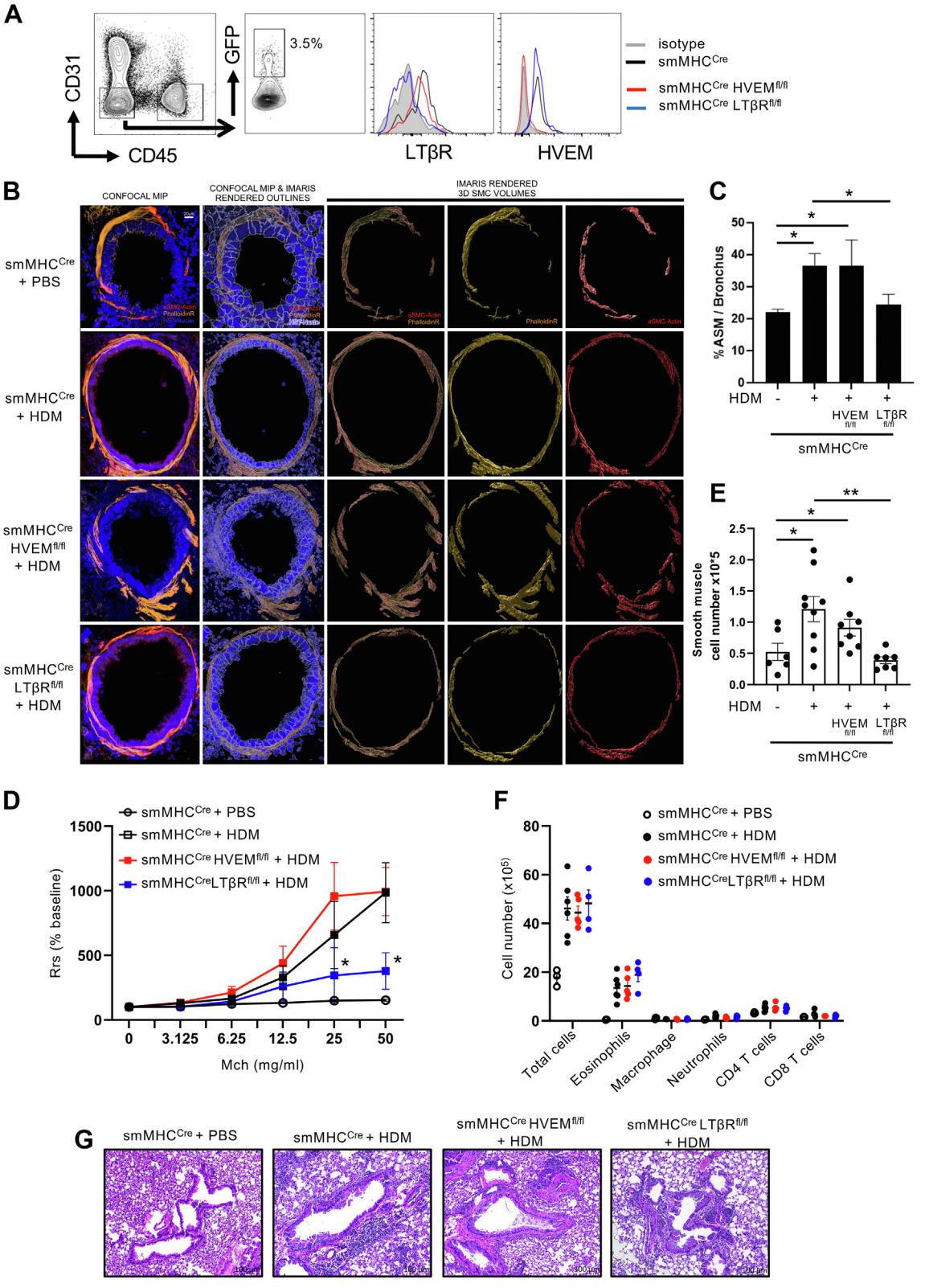

FIG 2. ASM-specific LTβR-deficient mice exhibit reduced ASM mass and AHR in allergen-induced experimental severe asthma. **(A)** HVEM and LTβR expression on ASM (GFP^+) from $smMHC^{Cre}$ (eGFP), $smMHC^{Cre}HVEM^{fl/fl}$, or $smMHC^{Cre}LT\beta R^{fl/fl}$ mice. Data from 3 experiments. **(B-G)** $smMHC^{Cre}$, $smMHC^{Cre}HVEM^{fl/fl}$, or $smMHC^{Cre}LT\beta R^{fl/fl}$ mice were provided intranasal challenges of HDM extract over 6 weeks (n = 4-6 per group). *(B)* Confocal analysis of lung bronchi (scale, 20 μm). F-actin (phalloidin, *yellow*), αSMA *(red),* 4′,6-diamidino-2-phenylindole (DAPI) *(blue).* Data are representative of 3 experiments. *(C)*

(GSE58434_All_Sample_FPKM_Matrix.txt.gz). Kan et al[31] used ASM from 9 fatal asthma and 8 nonasthma donors. Only vehicle control samples from GSE94335 were analyzed (GSE94335_PostQC_all_sample_TPM.txt.gz).

Statistical analysis

All results are presented as means ± SEMs. Statistical significance was analyzed by Mann-Whitney test for comparison of means between 2 independent groups or by 1-way ANOVA followed by Tukey *post hoc* multiple comparison test for differences of means between multiple groups by GraphPad Prism 8 software (GraphPad Software, La Jolla, Calif). $P < .05$ was considered statistically significant (*$P < .05$, **$P < .01$, ***$P < .001$, ****$P < .0001$).

RESULTS

LTβR and HVEM are expressed on ASM from healthy and asthmatic individuals

We first studied the expression of the receptors for LIGHT, LTβR, and HVEM, in published RNA-Seq data from ASM isolated from healthy and asthmatic individuals.[29-31] Three separate data sets revealed strong expression of transcripts for LTβR and lower levels of transcripts for HVEM (Fig 1, *A*). Interestingly, compared to the receptors for the 3 primary asthma-related cytokines linked to activities in ASM, namely TNF-R1, IL-4R, and IL-17RA and IL-17RC, only TNF-R1 transcripts were as, or more, abundant compared to LTβR transcripts. No differences in transcript levels were observed for any of the receptors between healthy and asthmatic ASM (Fig 1, *A*). Flow cytometric analyses of 2 preparations of ASM from healthy individuals obtained from commercial sources confirmed that LTβR was more strongly expressed on the cell surface than HVEM. Analysis of ASM from asthmatic subjects from a commercial source and from postmortem lungs of people with asthma isolated in house furthermore showed equivalent expression of both molecules compared to healthy donor ASM, with LTβR being the most highly expressed (Fig 1, *B*).

ASM-specific LTβR-deficient mice display reduced ASM mass and AHR in allergen-induced experimental asthma

To assess the importance of LTβR or HVEM on ASM *in vivo,* we used smMHC^{Cre} *(Myh11)*-eGFP transgenic mice.[25] Similar to human cells, each receptor was expressed constitutively on ASM in mouse lungs (Fig 2, *A*). We next generated smooth muscle cell–specific receptor knockout mice by crossing smMHC^{Cre} mice to HVEM$^{fl/fl}$ or LTβR$^{fl/fl}$ mice, and confirmed deletion of each receptor in ASM after gating on lung GFP-smMHC–positive cells (Fig 2, *A*). Previous studies using single cell RNA sequencing have revealed several markers to identify structural cell subsets in the lung.[32,33] However, no established gating strategy yet exists to identify ASM by conventional flow cytometric methods without a marker tag. We found that among the CD45$^-$EpCAM$^-$PDGFRα$^-$ and Vimentin^{low} population, which excludes epithelial and endothelial cells and fibroblasts, there are 2 distinct αSMA-positive populations (see Fig E1, *A,* in the Online Repository available at www.jacionline.org). MCAM is known to be highly expressed on pericytes and lower in smMHC-positive mature smooth muscle cells.[34] By analyzing smMHC^{Cre} conditional knockout mice, we found that both HVEM and LTβR were deficient in the αSMA$^+$MCAM$^-$ population, but not in the αSMA$^+$MCAM$^+$ population or in fibroblasts or epithelial cells. This further showed the specificity of the conditional deletion of the 2 receptors and revealed that ASM can be distinguished effectively as CD45$^-$EpCAM$^-$PDGFRα$^-$Vimentin^{low}αSMA$^+$MCAM$^-$ cells in the lung (Fig E1, *B*).

The conditional knockout mice and control animals (smMHC^{Cre}) were then exposed to chronic repetitive intranasal challenges with HDM allergen extract over 6 weeks in a model of asthma that results in airway remodeling. Similar to what we previously reported,[16] the allergen drove an eosinophilic inflammatory response in the lungs of control animals, accompanied by a strong increase in bronchial ASM mass, as visualized and quantified by high-resolution 3-D confocal microscopy of the immunofluorescent staining of αSMA and phalloidin (filamentous actin). These changes were associated with profound AHR, reflected by increased lung resistance to methacholine when administered intranasally (Fig 2, *B-G*). Strikingly, we found that no significant increase in bronchial ASM mass was observed in ASM-specific, LTβR-deficient mice compared to the approximate doubling in mass seen in control animals when quantified by histologic analysis (Fig 2, *B* and *C*). In contrast, a normal smooth muscle response to allergen was found in mice specifically lacking HVEM in ASM. Importantly, AHR was strongly reduced in LTβR conditional knockout mice challenged with allergen, whereas AHR was induced in HVEM conditional knockout mice comparable to control wild-type mice (Fig 2, *D*).

Flow cytometric analysis of dissociated lung tissue cells furthermore revealed another specific requirement for LTβR in controlling ASM. While the total number of smooth muscle cells was enhanced in the lungs of allergen-exposed smMHC^{Cre} and ASM-specific, HVEM-deficient mice, no increase was found in LTβR-deficient mice (Fig 2, *E*). Moreover, demonstrating that this was not a bystander effect of a poor inflammatory response, the number of inflammatory cells, including eosinophils, that accumulated in the lung tissue, and the overall infiltrates around the bronchioles, as well as T_H2 cytokine production in BAL fluid, were not different between the control and LTβR conditional knockout mice challenged with allergen (Fig 2, *F* and *G,* and see Fig E2, *A* and *B,* in the Online Repository available at www.jacionline.org). Mucus production determined by PAS staining of bronchial epithelium was also not different between

Quantitation of ASM volume based on phalloidin staining around the bronchi from *(B)* (n = 6-9 per group, 10-15 tertiary bronchi per mouse). Data are representative of 3 experiments. *(D)* Airway resistance (AHR) after methacholine challenge, measured by FlexiVent (n = 5-8 per group). Data are representative of 2 experiments. *$P < .05$, smMHC^{Cre} versus LTβR$^{fl/fl}$. *(E)* Flow quantitation of total lung tissue smooth muscle cells (ASM). Data points indicate individual mice. *(F)* Flow quantitation of total lung inflammatory/immune cells. Data points indicate individual mice. *(G)* Representative hematoxylin and eosin–stained lung sections (scale, 100 μm); n = 4-6 mice per group. Data are presented as means ± SEMs and are representative of 3 experiments. *$P < .05$, **$P < .01$.

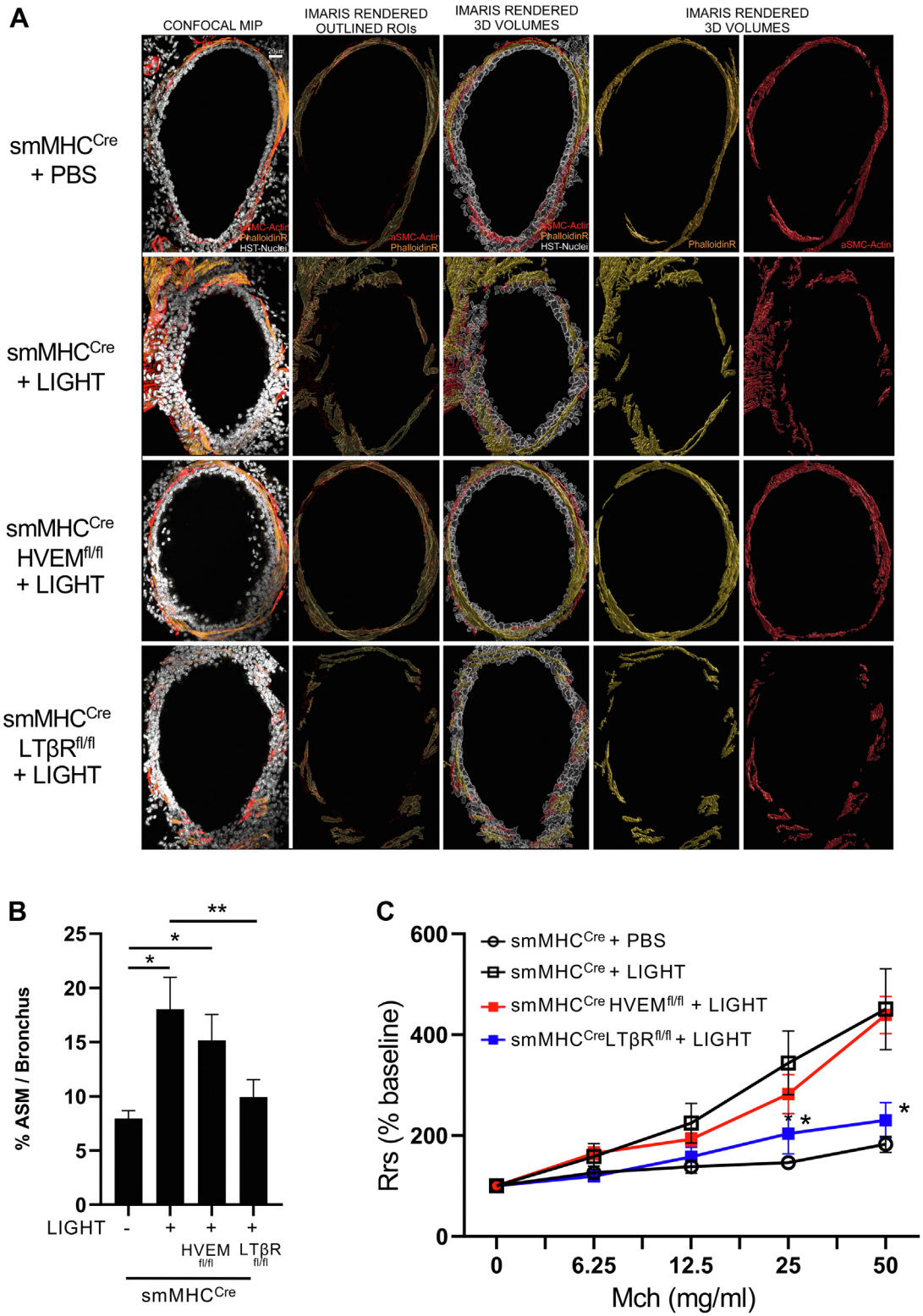

FIG 3. LIGHT-LTβR signals increase airway smooth muscle mass and induce AHR in mice. Mice received intratracheal recombinant LIGHT or PBS over 3 days. (**A** and **B**) Confocal microscopy of lung bronchial sections for expression of F-actin (phalloidin, *yellow*) and αSMA *(red). (A)* Representative images of bronchi (scale, 20 μm). *(B)* Imaris 3-D imaging analysis quantification of ASM volume (phalloidin) around individual bronchi (n = 4-5 mice per group, 10-15 tertiary bronchi per mouse). Data are presented as means ± SEMs and are representative of 3 experiments. **P* < .05, ***P* < .01. **(C)** Airway resistance (AHR) after methacholine challenge, measured by FlexiVent (n = 4 mice per group). **P* < .05, smMHC^{Cre} versus LTβR$^{fl/fl}$. Data are presented as means ± SEMs and are representative of 2 experiments.

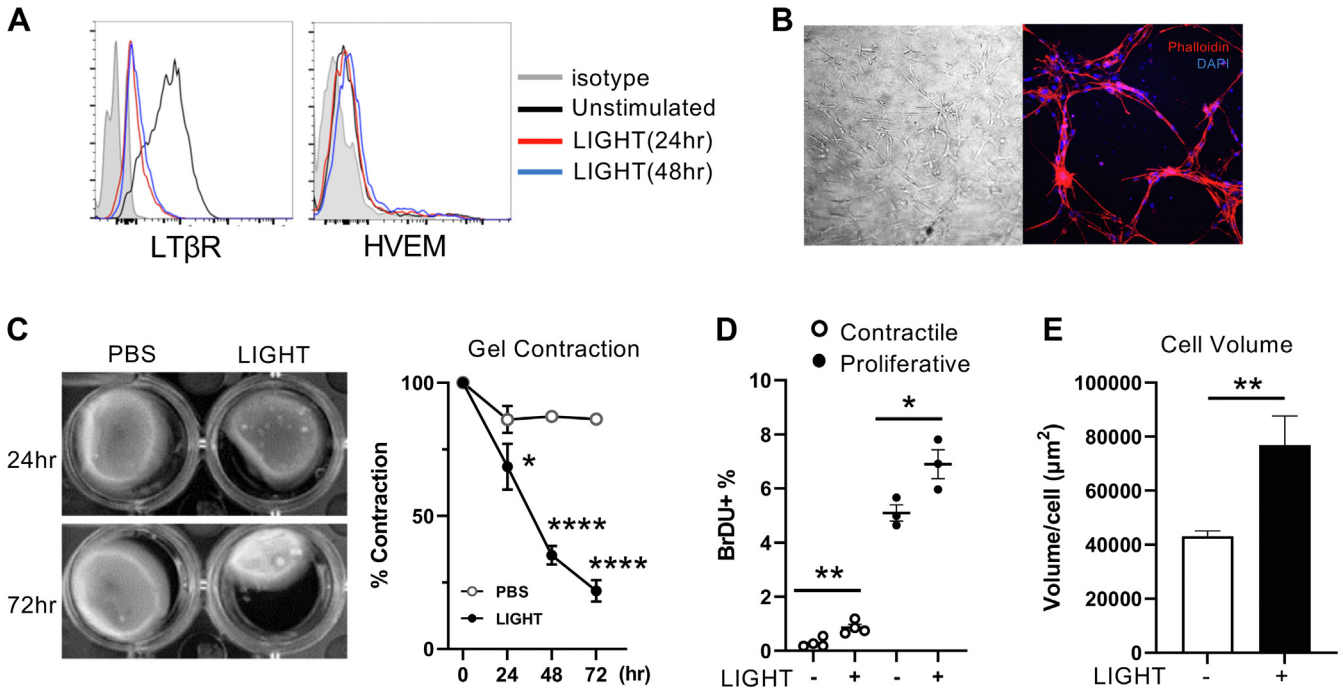

FIG 4. LIGHT induces human ASM proliferation and contractile activity. **(A)** HVEM and LTβR expression on human ASM assessed by flow after stimulation with LIGHT for 24 or 48 hours. Similar data in 3 experiments. **(B)** Images of ASM in collagen 3-D gels. *Left,* Bright field; *right,* phalloidin *(red)* and 4′,6-diamidino-2-phenylindole (DAPI) *(blue).* **(C)** ASM collagen gel contraction at 24-72 hours after stimulation with LIGHT. Data are combined means from triplicates from 3 experiments. **(D)** Percentages of BrdU-positive ASM stimulated with LIGHT for 48 hours. Cells were either first incubated in serum-free media for 16 hours for proliferative ASM or for 7 days for contractile ASM. Data are means of triplicates and are representative of 3 experiments. **(E)** ASM volume after stimulation with LIGHT, measured by confocal microscopy; 300-400 ASM, in 2 replicate experiments, were analyzed. Data are presented as means ± SEMs. **P* < .05, ***P* < .01, ****P* < .001, *****P* < .0001.

the control and LTβR conditional knockout mice challenged with allergen (Fig E2, *C*).

Thus, LTβR controlled hyperplasia and hypertrophy of ASM in the context of chronic repetitive airway allergen exposure, and this strongly contributed to the development of AHR and lung dysfunction. Importantly, the latter was dissociated from generalized inflammation, demonstrating the requirement for direct signals to smooth muscle cells.

We also assessed if LTβR played a role in AHR in an acute asthma model without chronic repetitive allergen exposure, where airway remodeling does not occur to any extent. In this case, only a weak AHR response resulted in control mice challenged with HDM. However, this was reduced in LTβR conditional knockout mice to the level seen in naive unchallenged mice (Fig E2, *D*). All this suggests that the primary effect of LTβR on AHR is explained by its action on driving ASM remodeling, but that LTβR might also control ASM contractility in the absence of significant remodeling.

LIGHT-LTβR signals increase ASM mass and AHR *in vivo*

The above findings in allergen-induced airway remodeling correlated with the reduction in airway smooth muscle mass we found in LIGHT-deficient mice.[16] To directly relate the results to the activity of LIGHT as a cytokine, we then injected rLIGHT at a high dose intratracheally into unimmunized control and conditional knockout mice over 3 days. The smooth muscle layer was again quantified by high-resolution 3-D confocal microscopy (see Fig E3, *A,* in the Online Repository available at www.jacionline.org). Replicating our observations from 2 previous studies,[16,24] rLIGHT administration to the lungs induced a significant increase in smooth muscle mass surrounding the bronchi in control mice (Fig 3, *A* and *B*). Importantly, this increased ASM mass was not observed in mice with conditional deletion of LTβR in ASM, whereas ASM-specific deletion of HVEM did not diminish the LIGHT-driven response compared to that in smMHC^{Cre} control mice (Fig 3, *A* and *B*).

To extend this data, we quantified the number of total lung smooth muscle cells by flow cytometry as before. In this case, intratracheal rLIGHT administration did not alter the number of ASM; nor did it have an effect on promoting increased numbers of total cells and eosinophils in the lung tissue (Fig E3, *B*). Thus, the primary action of recombinant LIGHT in this setting *in vivo* was to promote ASM hypertrophy, suggesting that the LTβR-driven hyperplasia seen with repetitive allergen exposure (Fig 2, *E*) may have been dependent on other inflammatory factors elicited by the allergen acting together with LIGHT. However, regardless of the latter, rLIGHT injection still significantly induced AHR, albeit less than that observed with repetitive allergen (Fig 3, *C;* cf Fig 2, *D*). Most importantly, ASM-specific, LTβR-deficient animals displayed strongly reduced

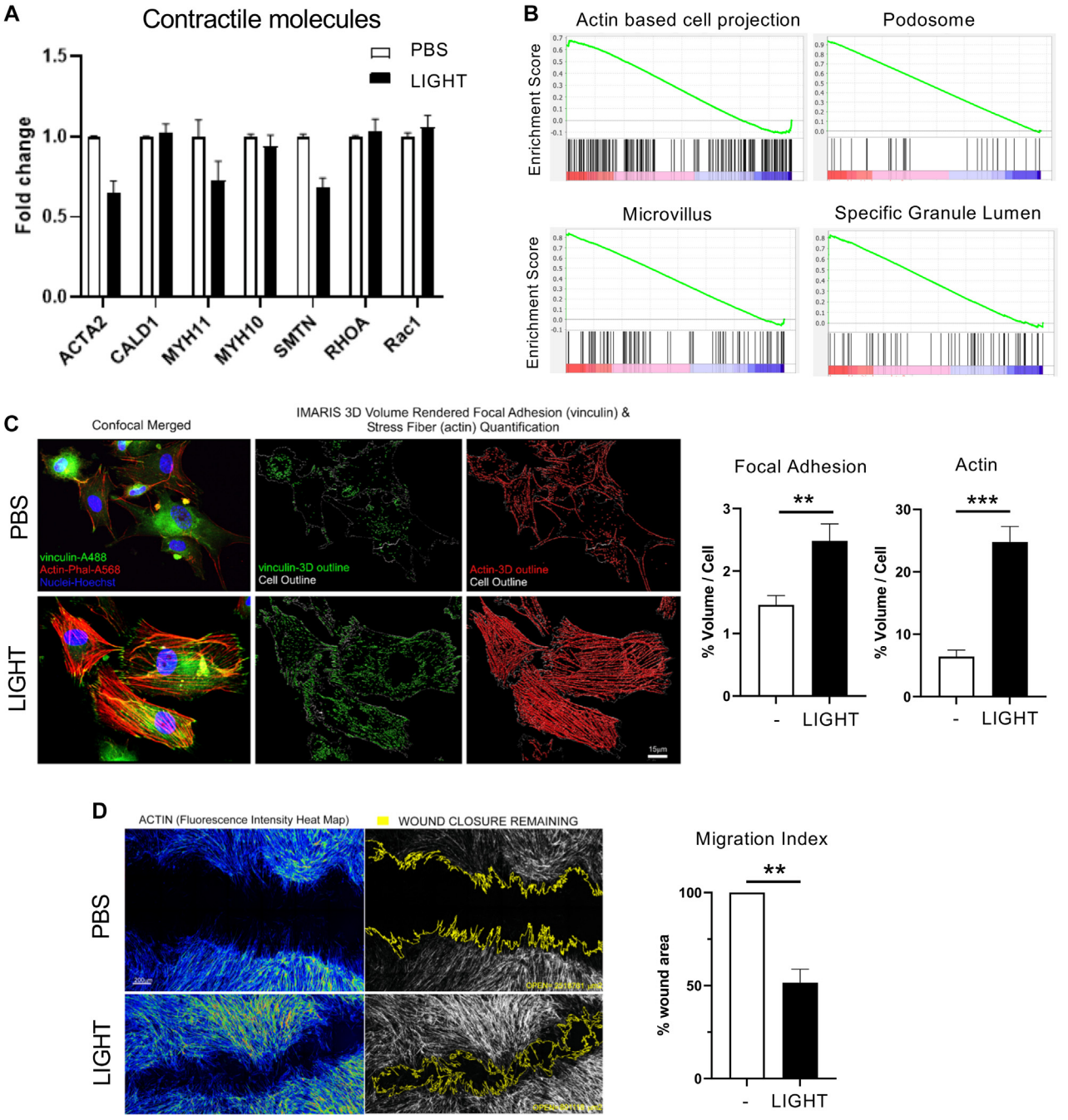

FIG 5. LIGHT induces actin polymerization and migratory ability in human ASM. **(A)** RNA-Seq analysis of contractile molecules in human ASM treated with LIGHT. Log_2 fold change after 4 hours compared to PBS-treated cells. **(B)** Gene set enrichment analysis of cell structure–related genes from RNA-Seq of ASM stimulated with LIGHT compared to PBS for 4 hours. **(C)** Confocal images of ASM treated with LIGHT for 6 hours and stained with phalloidin *(red),* vinculin *(green),* and 4′,6-diamidino-2-phenylindole (DAPI) *(blue).* Indicated are volume of F-actin (phalloidin) and focal adhesions (vinculin) normalized to individual cell size (scale, 15 μm); 300-400 ASM, in 2 replicate experiments, were analyzed. **(D)** Images of wounded monolayers of ASM stimulated with LIGHT for 12 hours. *Left,* Fluorescence intensity of F-actin (*yellow* to *red*); *right,* remaining wound highlighted in *yellow outline.* Percentage of wound area remaining after LIGHT stimulation compared to PBS (scale, 200 μm). Data are combined from 3 independent experiments and are presented as means ± SEMs. $*P < .05$, $**P < .01$, $***P < .001$.

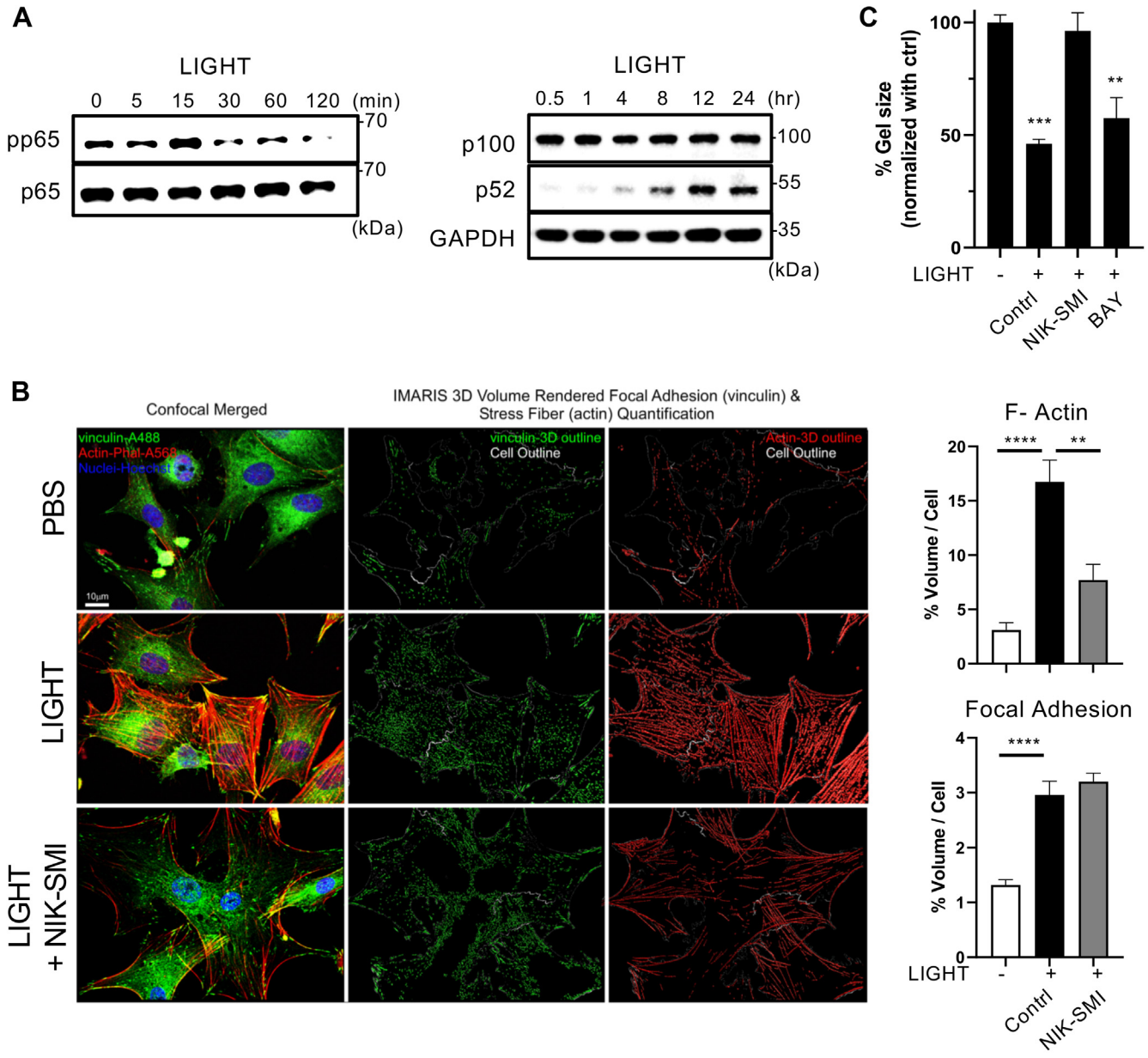

FIG 6. LIGHT-dependent noncanonical NF-κB signaling induces actin polymerization and ASM contractility. **(A)** Activation of canonical NF-κB (pp65) and noncanonical NF-κB (processing of p100 to p52) assessed in human ASM stimulated with LIGHT over 120 minutes or 24 hours. Data are representative of 3 experiments. **(B)** Confocal analysis of ASM treated with LIGHT with or without NIK-SMI, an inhibitor of noncanonical NF-κB. Volume of phalloidin and vinculin normalized to cell size from triplicates (scale, 10 μm); 300-400 ASM, in 2 replicate experiments, were analyzed. **(C)** Gel contraction assay of LIGHT-stimulated ASM with or without inhibitors of canonical (BAY11-7082) and noncanonical (NIK-SMI) NF-κB. Percentage gel contraction compared to PBS in triplicate. Data are representative of 2 experiments and are presented as means ± SEMs. $^{**}P < .01$, $^{***}P < .001$, $^{****}P < .0001$.

AHR, unlike HVEM-deficient animals, which showed similar AHR compared to the control mice treated with rLIGHT (Fig 3, *C*). These results collectively demonstrate that LIGHT can signal through LTβR on ASM in the lungs and directly drive smooth muscle hypertrophy and lung dysfunction (Fig 3), and in the context of a response to allergen, the LTβR signal is also critical and dominant for smooth muscle hyperplasia, leading to even greater lung dysfunction associated with airway remodeling (Fig 2).

LIGHT-LTβR interactions promote contractile activity, hypertrophy, and hyperplasia in human ASM

To further investigate the direct actions of LIGHT-LTβR signals on ASM, we analyzed normal human primary bronchial smooth muscle cells. In an indication that LTβR was active, it was downregulated after culture with LIGHT, a phenomenon previously associated with initiation of signaling through this

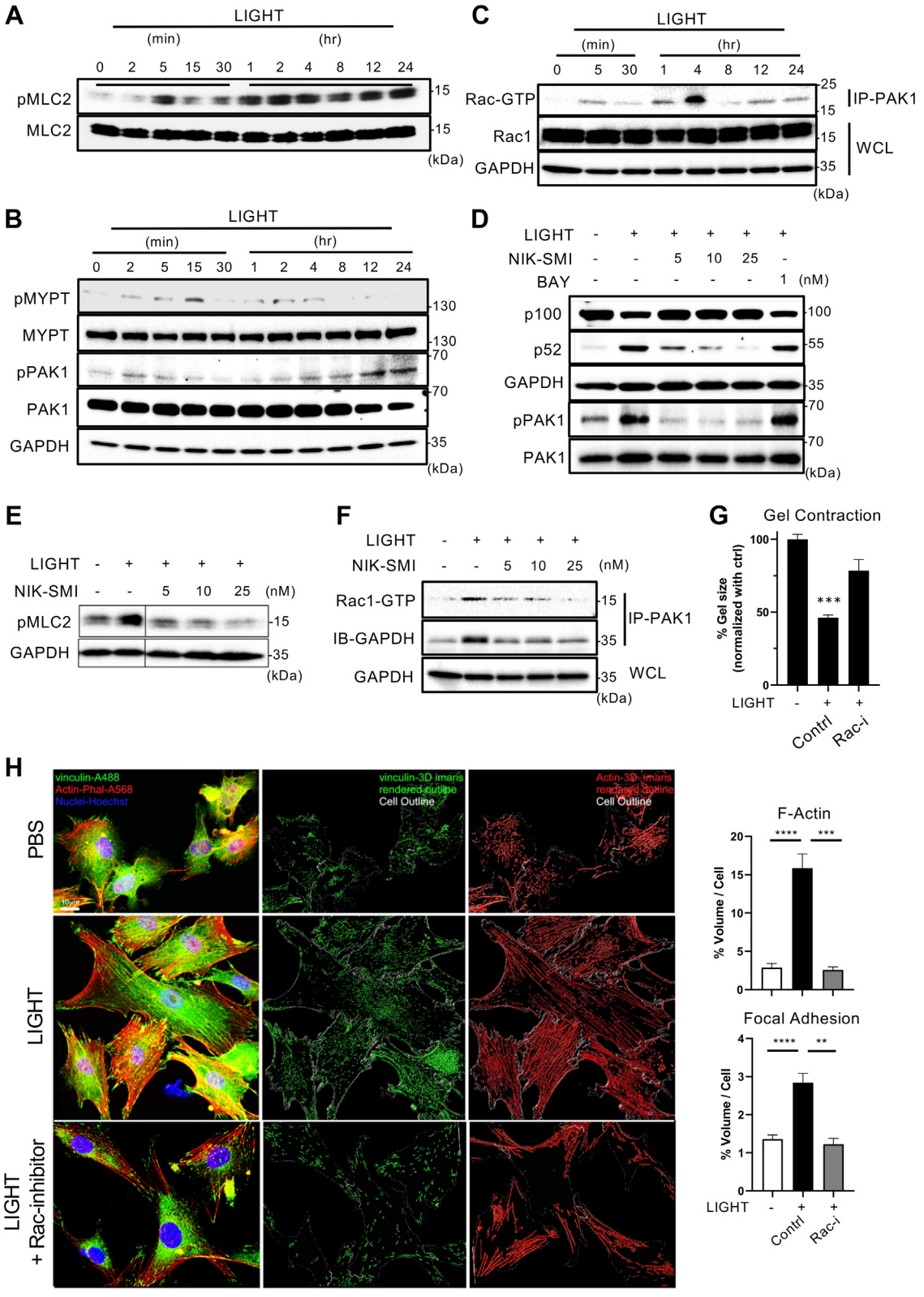

FIG 7. LIGHT induces noncanonical NF-κB–dependent activation of Rac1/PAK1 and phosphorylation of MLC in ASM. **(A-C)** pMLC2 *(A)*, pMYPT and pPAK1 *(B)*, and active Rac1-GTP immunoprecipitated with PAK1 *(C)* in human ASM stimulated with LIGHT for the indicated times. **(D** and **E)** Processing of p52, and pPAK1 *(D)* and pMLC2 *(E)* in ASM stimulated with LIGHT for 12 hours, with or without inhibitors of canonical (BAY) and noncanonical NF-κB (NIK-SMI). **(F)** Active Rac1-GTP immunoprecipitated with PAK1 in ASM stimulated with LIGHT for 4 hours with or without NIK-SMI. All data in *(A-F)* are representative of 3 experiments. **(G)**

receptor (Fig 4, *A*). The human ASM were then cultured in 3-D collagen gels to make a net structure (Fig 4, *B*) and stimulated with LIGHT to assess contractile activity. Significantly, LIGHT induced strong activity in this assay, as shown by the collagen gel's shrinking, largely seen from 24 to 48 hours (Fig 4, *C*). In line with the notion that this was due to LTβR signaling, knockdown of this receptor with siRNA, but not knockdown of HVEM, prevented LIGHT-induced contractile activity (see Fig E4, *A* and *B,* in the Online Repository available at www.jacionline.org). The membrane version of lymphotoxin, lymphotoxin alpha beta (LTαβ), is also a ligand of LTβR. Recombinant LTαβ induced identical gel contraction compared to LIGHT (Fig E4, *C*), further supporting the dominant role of LTβR in driving smooth muscle cell activity.

We next assessed whether LIGHT-LTβR interactions can promote proliferation of ASM. Functional changes in cultured ASM have been studied previously, with both contractile and synthetic/proliferative phenotypes observed to be dependent on culture conditions. Synthetic/proliferative cells can mature into contractile cells by prolonged serum deprivation.[2] We thus compared early passage human ASM with a proliferative phenotype to those with a contractile phenotype cultured without serum for 7 days. By assessing BrdU incorporation, proliferative ASM exhibited a high background rate of BrdU uptake (Fig 4, *D*), and this was significantly enhanced by LIGHT stimulation. Contractile ASM weakly incorporated BrdU, which was less strongly, albeit significantly, affected by LIGHT (Fig 4, *D*). Finally, human ASM hypertrophy was assessed *in vitro,* and LIGHT significantly increased the volume of individual cells (Fig 4, *E*), again corresponding to the *in vivo* phenotype observed in the mouse (Figs 2 and 3).

LIGHT increases actin polymerization in ASM

Contractile force in smooth muscle cells is generated by ATP-dependent interaction between F-actin and myosin, regulated in part by phosphorylation of myosin light chain by myosin light chain (MLC) kinase (MLCK). In asthmatic patients, in addition to increased numbers of the contractile actin/myosin-expressing ASM subset, upregulation of MLCK and other proteins associated with MLCK activity has been reported.[5] Furthermore, cell hypertrophy is also directly related to several factors associated with contractility, including actin polymerization, as is migration of ASM, another process thought to be relevant to ASM mass changes in asthma.[35]

We therefore first assessed by RNA-Seq whether LIGHT might promote responsiveness in ASM by regulating the expression of contractile molecules and signaling molecules associated with contraction. We did not detect any upregulation of transcripts for such molecules, including αSMA, contractile myosin heavy chain 11 isoform, and RhoA (Fig 5, *A*). In contrast, by gene set enrichment analysis, we found significant enrichment by LIGHT of transcriptional machinery associated with the cell membrane and actin rearrangement (Fig 5, *B*). This led us to focus on the quantitative analysis of intracellular F-actin and vinculin expression, which reflect cytoskeletal macromolecular changes associated with mechanical force, migration, and hypertrophy. By confocal imaging analysis, human ASM stimulated with LIGHT were found to significantly increase the volume of vinculin rich focal adhesions and polymerized F-actin–rich stress fibers compared to unstimulated cells within 6 to 12 hours (Fig 5, *C*). Further in line with this, migration of ASM was enhanced by LIGHT stimulation in migration/wound assays of monolayers analyzed at 12 hours, correlating with increased expression of actin filaments (Fig 5, *D*). Thus, overall, these results suggested that actin polymerization might have been central to many or all of the activities of LTβR on driving ASM dysregulation.

LIGHT-induced noncanonical NF-κB signaling induces actin polymerization in ASM

Because LTβR signals are known to induce both early-onset canonical NF-κB activation and late-onset noncanonical NF-κB activation in other cell types, we asked if one or both of these pathways were activated by LIGHT in human ASM. Indeed, LIGHT stimulation induced a small increase in phosphorylation of p65 (canonical NF-κB) after 15 minutes. However, most interestingly, LIGHT resulted in processing of p100 to p52 (the signature of noncanonical NF-κB pathway activation) starting about 4 hours after stimulation and extending to 24 hours (Fig 6, *A*). We then assessed whether LIGHT-induced noncanonical NF-κB signaling was required for actin rearrangement and contractile activity using NIK-SMI, an inhibitor of NF-κB–inducing kinase (NIK), the kinase that is central to p100 processing into p52.[36] Treatment with NIK-SMI significantly reduced LIGHT-induced actin polymerization, although it did not block the increase in focal adhesions (Fig 6, *B*). Moreover, NIK-SMI also effectively prevented LIGHT-induced contractile activity visualized in 3-D collagen gels, whereas in contrast, BAY11-7082, a canonical NF-κB inhibitor, had no effect on LIGHT-induced gel contraction (Fig 6, *C*). Knockdown of NIK (MAP3K14) with siRNA further supported the inhibitor studies and prevented LIGHT-driven gel contraction (Fig E4, *A* and *B*). Notably, noncanonical signaling, initiated from 4 hours and sustained at least up to 24 hours (Fig 6, *A*), preceded the initiation of contraction at 24 hours (Fig 4, *C,* and Fig E4), and preceded or coincided with changes in actin polymerization and migration related to actin polymerization (Fig 5, *C* and *D*), thus reinforcing our conclusion regarding the relevance of this pathway from the NIK inhibition assays. Thus, LIGHT-LTβR–dependent noncanonical NF-κB signaling is central to ASM dysregulation. Moreover, distinguishing LIGHT from other cytokines reported to be capable of acting on ASM, namely IL-13, TNF, and IL-17, none of these cytokines induced noncanonical NF-κB activation in ASM (p52 processing), even though canonical NF-κB (pp65) was induced, particularly by TNF, as previously reported (see Fig E5 Online Repository available at www.jacionline.org).

Gel contraction of ASM stimulated with LIGHT, with or without an inhibitor of Rac1. Percentage gel contraction was compared to PBS in triplicate. **(H)** Confocal analysis of ASM treated with LIGHT with or without a Rac1 inhibitor. Volume of phalloidin and vinculin normalized to cell size from triplicates (scale, 10 μm); 300-400 ASM, in 2 replicate experiments, were analyzed. Data are representative of 2 experiments and are presented as means ± SEMs. $^{**}P < .01$, $^{***}P < .001$, $^{****}P < .0001$.

LIGHT induces actin polymerization and sustained phosphorylation of MLC via noncanonical NF-κB activation of Rac1/PAK1

To further understand the role of noncanonical NF-κB signaling, we examined other changes associated with activity in ASM. Phosphorylation of myosin light chain (MLC) is regulated directly by MLC kinase and MLC phosphatase, and a Rho-dependent pathway inhibits MLC phosphatase whereas calcium dependent calmodulin signaling activates MLC kinase.[5,37] LIGHT promoted phosphorylation of MLC in human ASM within 5 minutes, but importantly, it sustained MLC phosphorylation over 24 hours (Fig 7, *A*). We also detected late and sustained phosphorylation of the serine threonine kinase p21-activated kinase 1 (PAK1) over 24 hours; it is a target of the GTP binding protein Rac1 and has been implicated in cytoskeleton/actin reorganization, and it is also upstream of MLC kinase (Fig 7, *B*). Consistent with this, the active (GTP) form of Rac1, bound to PAK1, was detected between 1 and 4 hours after LIGHT stimulation (Fig 7, *C*). We also assessed phosphorylation of myosin phosphatase targeting protein (MYPT, a subunit of MLC phosphatase) downstream of the Rho-dependent pathway,[38] but in this case, LIGHT induced only early, transient, and weak phosphorylation of MYPT at 15 minutes (Fig 7, *B*).

Because PAK1 has been reported to interact with NIK,[39] we hypothesized that the late phosphorylation of MLC and activation of Rac1/PAK1 was also related to noncanonical NF-κB signaling. Indeed, LIGHT-induced phosphorylation of PAK1 (Fig 7, *D*) and sustained phosphorylation of MLC (Fig 7, *E*) was reduced by NIK inhibition in a dose-dependent manner. Correspondingly, inhibiting NIK also prevented LIGHT from promoting the binding of active Rac1 to PAK1 (Fig 7, *F*). Last, to further consolidate the significance of this signaling pathway to ASM dysfunction, we found that a Rac1 inhibitor blocked LIGHT-induced contraction in 3-D gels (Fig 7, *G*). Correspondingly, treatment with the Rac1 inhibitor reduced actin polymerization, and in this case also blocked the increase in focal adhesions driven by LIGHT (Fig 7, *H*).

Together with our *in vivo* data, these results indicate that LTβR-induced noncanonical NF-κB signaling is a primary and dominant driver of ASM hyperresponsiveness.

DISCUSSION

ASM have long been considered to be central for lung dysfunction in asthma as a result of their ability to narrow the airways. However, it remains unclear whether there are dominant receptors on ASM that explain the ASM remodeling observed in severe asthma and that explain AHR. Our present study now reveals the novel finding that LTβR is essential for allergen-driven ASM remodeling and the resultant increased AHR that is associated with remodeling. Importantly, to our knowledge, out of the primary cytokine receptors that have been associated with asthma and ASM activity, namely IL-13, TNF, and IL-17, this is the first study to show that deletion of a single cytokine receptor only on smooth muscle cells can strongly affect ASM deregulation and AHR in response to allergen *in vivo.* This correlates with the unique ability of LTβR to drive noncanonical NF-κB signaling, an activity not shared by these other cytokine receptors. The results have major implications for our understanding of the control of airway remodeling and hyperresponsiveness in asthma.

We favor the notion that LIGHT, rather than LTαβ—the other ligand for LTβR—is the primary factor relevant for smooth muscle remodeling. Higher expression of LIGHT has been reported in the sputum of severe asthmatic patients,[17-19] and in previous studies of LIGHT-deficient mice, we showed that this cytokine participated in remodeling of the ASM mass that was induced by repetitive exposure to allergen.[16] LIGHT-deficient mice were protected from ASM remodeling at a similar level as wild-type mice treated with LTβR.Ig that blocks both LIGHT and LTαβ,[16] further implying that at least in our mouse model systems, LIGHT is the primary ligand. However, it is possible that LTαβ could be important in human asthmatic subjects because it is capable of inducing the same signals as LIGHT through the LTβR, and we thus cannot definitively rule out a contribution of LTαβ.

Increased ASM mass has previously been related to the severity and baseline lung function in patients with asthma,[3] with ASM hyperplasia and an increased number of bronchial ASM being well established as features of asthmatic airways.[35] Although ASM hypertrophy in asthma is less discussed,[35] asthmatic bronchial biopsy samples have been found to contain smooth muscle cells of larger diameter compared to control subjects, and severe asthmatic subjects presented with the highest smooth muscle cell size.[40] Our data here show that LIGHT-LTβR promoted both ASM hyperplasia and hypertrophy *in vivo* and *in vitro.* LIGHT drove only moderate proliferation in cell culture, but LTβR signals to ASM were essential for the increased number of lung smooth muscle cells seen *in vivo* in response to allergen. In addition, LIGHT directly promoted an increase in ASM size *in vitro,* accompanied by increased actin polymerization, and *in vivo,* recombinant LIGHT administration without other inflammatory signals increased peribronchial ASM mass without affecting the total number of lung ASM. Thus, these observations strongly imply that LTβR signals could be central to both smooth muscle cell hyperplasia and hypertrophy seen in asthma.

The finding that LTβR activates the noncanonical NF-κB pathway in ASM and drives sustained changes in actin polymerization and rearrangement distinguishes it from the other primary cytokine receptors that have been described to also induce ASM responses *in vitro,* namely those for IL-13, TNF, and IL-17.[41] Noncanonical NF-κB signaling is a late-onset and more continual inflammatory signal reliant on recruitment of the NF-κB–inducing kinase, NIK. TNF via canonical NF-κB was found to induce activation of RhoA in ASM leading to inhibition of MLC phosphatase.[9,42] IL-13 was shown to upregulate the expression of RhoA or enhance Ca^{2+} signaling dependent on signal transducer and activator of transcription 6 (aka STAT6), mitogen-activated protein kinase, and phosphatidylinositol 3-kinase (aka PI3K) activity.[7,43-46] Moreover, IL-17 was also found to drive migration, contraction, or other activities in ASM, dependent on several mitogen-activated protein kinase pathways, canonical NF-κB, and protein kinase C.[47-49] Thus, although these cytokine receptors might then promote other signaling pathways compared to LTβR—or, in the case of the receptors for TNF and IL-17, affect canonical NF-κB activation similar to LTβR—they have not been described to activate NIK, and we show they do not drive p100 processing to p52 in ASM. Therefore, they are more likely to trigger early and transient activities compared to LTβR.

Collectively, this suggests that LTβR signaling can differ from the actions of these other cytokine receptors in engendering sustained increases in ASM mass and a contractile phenotype. This

could explain the dominant effect of deleting the LTβR in smooth muscle cells that we observed *in vivo.* Our data, however, do not rule out important contributions for these other cytokine receptors on ASM in asthma, simply that LTβR signaling in the context of allergen is essential. In fact, recent results with mice with conditional deletion of IL-4Rα in both epithelial cells and smooth muscle cells supports a role for IL-13 and IL-4R in control of ASM, albeit dependent on epithelial cell activity.[50] We suggest that LIGHT might act on ASM in concert with these other cytokines, dependent on their production and availability within the lung, given that IL-13, IL-17, and TNF are also products of T cells and other inflammatory cells that can make LIGHT. We previously reported that LIGHT stimulated IL-13 production from eosinophils in experimental asthma models[16] and that injection of LIGHT into the naive lungs also upregulated IL-13, potentially from innate lymphoid cells, coincident with a partial requirement for IL-4Rα in promoting ASM mass[24]—both observations that are in line with LIGHT working together with IL-13 to amplify smooth muscle responsiveness. Whether LIGHT cooperates with TNF or IL-17 to regulate ASM activities has not yet been shown, but it will be interesting in future studies to fully understand the extent of cross talk and synergy between these cytokines on ASM.

In summary, our study reveals direct evidence that LTβR, most likely activated by LIGHT, can drive ASM hypertrophy, hyperplasia, and contractile activity, and that it is essential for airway hyperreactivity that results after chronic exposure to airway allergen. The data suggest that signaling through LTβR is critically required for smooth muscle changes and AHR regardless of the potential for other cytokines to be active, as shown by the almost complete lack of ASM hypertrophy, hyperplasia, and AHR in allergen-challenged mice where LTβR was specifically deleted in smooth muscle cells. Existing asthma treatments largely target inflammatory cells such as eosinophils or mast cells, and airway constriction is only transiently controlled by β-agonist inhalers. Blocking LIGHT-LTβR interactions, and possibly LTαβ-LTβR interactions, or disrupting noncanonical NF-κB signaling in smooth muscle cells, could represent important therapeutic approaches for persistent and chronic AHR in severe asthmatic patients or patients with similar lung dysfunction.

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Key messages

- **LTβR interactions on smooth muscle cells in vivo control airway smooth muscle mass and AHR driven by inhaled allergen.**
- **LIGHT-LTβR noncanonical NF-κB signaling promotes contractile activity, hyperplasia, and hypertrophy in ASM.**

REFERENCES

1. Busse WW, Lemanske RF Jr. Asthma. N Engl J Med 2001;344:350-62.
2. Zuyderduyn S, Sukkar MB, Fust A, Dhaliwal S, Burgess JK. Treating asthma means treating airway smooth muscle cells. Eur Respir J 2008;32:265-74.
3. Pepe C, Foley S, Shannon J, Lemiere C, Olivenstein R, Ernst P, et al. Differences in airway remodeling between subjects with severe and moderate asthma. J Allergy Clin Immunol 2005;116:544-9.
4. Kaminska M, Foley S, Maghni K, Storness-Bliss C, Coxson H, Ghezzo H, et al. Airway remodeling in subjects with severe asthma with or without chronic persistent airflow obstruction. J Allergy Clin Immunol 2009;124:45-51.e1-4.
5. Mahn K, Ojo OO, Chadwick G, Aaronson PI, Ward JP, Lee TH. Ca^{2+} homeostasis and structural and functional remodelling of airway smooth muscle in asthma. Thorax 2010;65:547-52.
6. Lee JH, Kaminski N, Dolganov G, Grunig G, Koth L, Solomon C, et al. Interleukin-13 induces dramatically different transcriptional programs in three human airway cell types. Am J Respir Cell Mol Biol 2001;25:474-85.
7. Tliba O, Deshpande D, Chen H, Van Besien C, Kannan M, Panettieri RA Jr, et al. IL-13 enhances agonist-evoked calcium signals and contractile responses in airway smooth muscle. Br J Pharmacol 2003;140:1159-62.
8. Amrani Y, Panettieri RA Jr, Frossard N, Bronner C. Activation of the TNF alpha-p55 receptor induces myocyte proliferation and modulates agonist-evoked calcium transients in cultured human tracheal smooth muscle cells. Am J Respir Cell Mol Biol 1996;15:55-63.
9. Hunter I. Tumor necrosis factor-alpha–induced activation of RhoA in airway smooth muscle cells: role in the Ca^{2+} sensitization of myosin light chain20 phosphorylation. Mol Pharmacol 2003;63:714-21.
10. Chang Y, Al-Alwan L, Risse PA, Roussel L, Rousseau S, Halayko AJ, et al. T_H17 cytokines induce human airway smooth muscle cell migration. J Allergy Clin Immunol 2011;127:1046-53, e1-2.
11. Kudo M, Melton AC, Chen C, Engler MB, Huang KE, Ren X, et al. IL-17A produced by alphabeta T cells drives airway hyper-responsiveness in mice and enhances mouse and human airway smooth muscle contraction. Nat Med 2012;18:547-54.
12. Wechsler ME, Ford LB, Maspero JF, Pavord ID, Papi A, Bourdin A, et al. Long-term safety and efficacy of dupilumab in patients with moderate-to-severe asthma (TRAVERSE): an open-label extension study. Lancet Respir Med 2022;10:11-25.
13. Wenzel S, Ford L, Pearlman D, Spector S, Sher L, Skobieranda F, et al. Dupilumab in persistent asthma with elevated eosinophil levels. N Engl J Med 2013;368:2455-66.
14. Kirstein F, Horsnell WG, Kuperman DA, Huang X, Erle DJ, Lopata AL, et al. Expression of IL-4 receptor alpha on smooth muscle cells is not necessary for development of experimental allergic asthma. J Allergy Clin Immunol 2010;126:347-54.
15. Perkins C, Yanase N, Smulian G, Gildea L, Orekov T, Potter C, et al. Selective stimulation of IL-4 receptor on smooth muscle induces airway hyperresponsiveness in mice. J Exp Med 2011;208:853-67.
16. Doherty TA, Soroosh P, Khorram N, Fukuyama S, Rosenthal P, Cho JY, et al. The tumor necrosis factor family member LIGHT is a target for asthmatic airway remodeling. Nat Med 2011;17:596-603.
17. Hastie AT, Moore WC, Meyers DA, Vestal PL, Li H, Peters SP, et al. Analyses of asthma severity phenotypes and inflammatory proteins in subjects stratified by sputum granulocytes. J Allergy Clin Immunol 2010;125:1028-36.
18. Frossing L, Silberbrandt A, Von Bulow A, Kjaersgaard Klein D, Ross Christensen M, Backer V, et al. Airway gene expression identifies subtypes of type 2 inflammation in severe asthma. Clin Exp Allergy 2021;52:59-69.
19. Hirano T, Matsunaga K, Oishi K, Doi K, Harada M, Suizu J, et al. Abundant TNF-LIGHT expression in the airways of patients with asthma with persistent airflow limitation: association with nitrative and inflammatory profiles. Respir Investig 2021;59:651-60.
20. da Silva Antunes R, Madge L, Soroosh P, Tocker J, Croft M. The TNF family molecules LIGHT and lymphotoxin alphabeta induce a distinct steroid-resistant inflammatory phenotype in human lung epithelial cells. J Immunol 2015;195:2429-41.
21. da Silva Antunes R, Mehta AK, Madge L, Tocker J, Croft M. TNFSF14 (LIGHT) exhibits inflammatory activities in lung fibroblasts complementary to IL-13 and TGF-beta. Front Immunol 2018;9:576.
22. Manresa MC, Chiang AWT, Kurten RC, Dohil R, Brickner H, Dohil L, et al. Increased production of LIGHT by T cells in eosinophilic esophagitis promotes differentiation of esophageal fibroblasts toward an inflammatory phenotype. Gastroenterology 2020;159:1778-92.e13.
23. Herro R, Shui JW, Zahner S, Sidler D, Kawakami Y, Kawakami T, et al. LIGHT-HVEM signaling in keratinocytes controls development of dermatitis. J Exp Med 2018;215:415-22.
24. Herro R, Da Silva Antunes R, Aguilera AR, Tamada K, Croft M. Tumor necrosis factor superfamily 14 (LIGHT) controls thymic stromal lymphopoietin to drive pulmonary fibrosis. J Allergy Clin Immunol 2015;136:757-68.

25. Xin HB, Deng KY, Rishniw M, Ji G, Kotlikoff MI. Smooth muscle expression of Cre recombinase and eGFP in transgenic mice. Physiol Genomics 2002;10:211-5.
26. Seo GY, Shui JW, Takahashi D, Song C, Wang Q, Kim K, et al. LIGHT-HVEM signaling in innate lymphoid cell subsets protects against enteric bacterial infection. Cell Host Microbe 2018;24:249-60.e4.
27. Wang Y, Koroleva EP, Kruglov AA, Kuprash DV, Nedospasov SA, Fu YX, et al. Lymphotoxin beta receptor signaling in intestinal epithelial cells orchestrates innate immune responses against mucosal bacterial infection. Immunity 2010;32:403-13.
28. Pham AK, Miller M, Rosenthal P, Das S, Weng N, Jang S, et al. ORMDL3 expression in ASM regulates hypertrophy, hyperplasia via TPM1 and TPM4, and contractility. JCI Insight 2021;6:e136911.
29. Fong V, Hsu A, Wu E, Looney AP, Ganesan P, Ren X, et al. Arhgef12 drives IL17A-induced airway contractility and airway hyperresponsiveness in mice. JCI Insight 2018;3L:e123578.
30. Himes BE, Koziol-White C, Johnson M, Nikolos C, Jester W, Klanderman B, et al. Vitamin D modulates expression of the airway smooth muscle transcriptome in fatal asthma. PLoS One 2015;10:e0134057.
31. Kan M, Koziol-White C, Shumyatcher M, Johnson M, Jester W, Panettieri RA, et al. Airway Smooth Muscle-Specific Transcriptomic Signatures of Glucocorticoid Exposure. Am J Respir Cell Mol Biol 2019;1:110-20.
32. Tsukui T, Sun KH, Wetter JB, Wilson-Kanamori JR, Hazelwood LA, Henderson NC, et al. Collagen-producing lung cell atlas identifies multiple subsets with distinct localization and relevance to fibrosis. Nat Commun 2020;11:1920.
33. Zepp JA, Zacharias WJ, Frank DB, Cavanaugh CA, Zhou S, Morley MP, et al. Distinct mesenchymal lineages and niches promote epithelial self-renewal and myofibrogenesis in the lung. Cell 2017;170:1134-48.e10.
34. Kumar A, D'Souza SS, Moskvin OV, Toh H, Wang B, Zhang J, et al. Specification and diversification of pericytes and smooth muscle cells from mesenchymoangioblasts. Cell Rep 2017;19:1902-16.
35. Bara I, Ozier A, Tunon de Lara JM, Marthan R, Berger P. Pathophysiology of bronchial smooth muscle remodelling in asthma. Eur Respir J 2010;36:1174-84.
36. Brightbill HD, Suto E, Blaquiere N, Ramamoorthi N, Sujatha-Bhaskar S, Gogol EB, et al. NF-kappaB inducing kinase is a therapeutic target for systemic lupus erythematosus. Nat Commun 2018;9:179.
37. Sakai H, Suto W, Kai Y, Chiba Y. Mechanisms underlying the pathogenesis of hyper-contractility of bronchial smooth muscle in allergic asthma. J Smooth Muscle Res 2017;53:37-47.
38. Andre-Gregoire G, Dilasser F, Chesne J, Braza F, Magnan A, Loirand G, et al. Targeting of Rac1 prevents bronchoconstriction and airway hyperresponsiveness. J Allergy Clin Immunol 2018;142:824-33.e3.
39. Tong L, Tergaonkar V. Rho protein GTPases and their interactions with NFkappaB: crossroads of inflammation and matrix biology. Biosci Rep 2014;34:e00115.
40. Benayoun L, Druilhe A, Dombret MC, Aubier M, Pretolani M. Airway structural alterations selectively associated with severe asthma. Am J Respir Crit Care Med 2003;167:1360-8.
41. Lauzon AM, Martin JG. Airway hyperresponsiveness;smooth muscle as the principal actor. F1000Res 2016;5:5:F1000 Faculty Rev-306.
42. Chiba Y, Ueno A, Shinozaki K, Takeyama H, Nakazawa S, Sakai H, et al. Involvement of RhoA-mediated Ca^{2+} sensitization in antigen-induced bronchial smooth muscle hyperresponsiveness in mice. Respir Res 2005;6:4.
43. Eum SY, Maghni K, Tolloczko B, Eidelman DH, Martin JG. IL-13 may mediate allergen-induced hyperresponsiveness independently of IL-5 or eotaxin by effects on airway smooth muscle. Am J Physiol Lung Cell Mol Physiol 2005;288:L576-84.
44. Moynihan B, Tolloczko B, Michoud MC, Tamaoka M, Ferraro P, Martin JG. MAP kinases mediate interleukin-13 effects on calcium signaling in human airway smooth muscle cells. Am J Physiol Lung Cell Mol Physiol 2008;295:L171-7.
45. Farghaly HS, Blagbrough IS, Medina-Tato DA, Watson ML. Interleukin 13 increases contractility of murine tracheal smooth muscle by a phosphoinositide 3-kinase p110delta-dependent mechanism. Mol Pharmacol 2008;73:1530-7.
46. Chiba Y, Nakazawa S, Todoroki M, Shinozaki K, Sakai H, Misawa M. Interleukin-13 augments bronchial smooth muscle contractility with an up-regulation of RhoA protein. Am J Respir Cell Mol Biol 2009;40:159-67.
47. Chang Y, Al-Alwan L, Risse PA, Halayko AJ, Martin JG, Baglole CJ, et al. Th17-associated cytokines promote human airway smooth muscle cell proliferation. FASEB J 2012;26:5152-60.
48. Nakajima M, Kawaguchi M, Ota K, Fujita J, Matsukura S, Huang SK, et al. IL-17F induces IL-6 via TAK1-NFκB pathway in airway smooth muscle cells. Immun Inflamm Dis 2017;5:124-31.
49. Bulek K, Chen X, Parron V, Sundaram A, Herjan T, Ouyang S, et al. IL-17A recruits Rab35 to IL-17R to mediate PKCalpha-dependent stress fiber formation and airway smooth muscle contractility. J Immunol 2019;202:1540-8.
50. McKnight CG, Potter C, Finkelman FD. IL-4Ralpha expression by airway epithelium and smooth muscle accounts for nearly all airway hyperresponsiveness in murine allergic airway disease. Mucosal Immunol 2020;13:283-92.

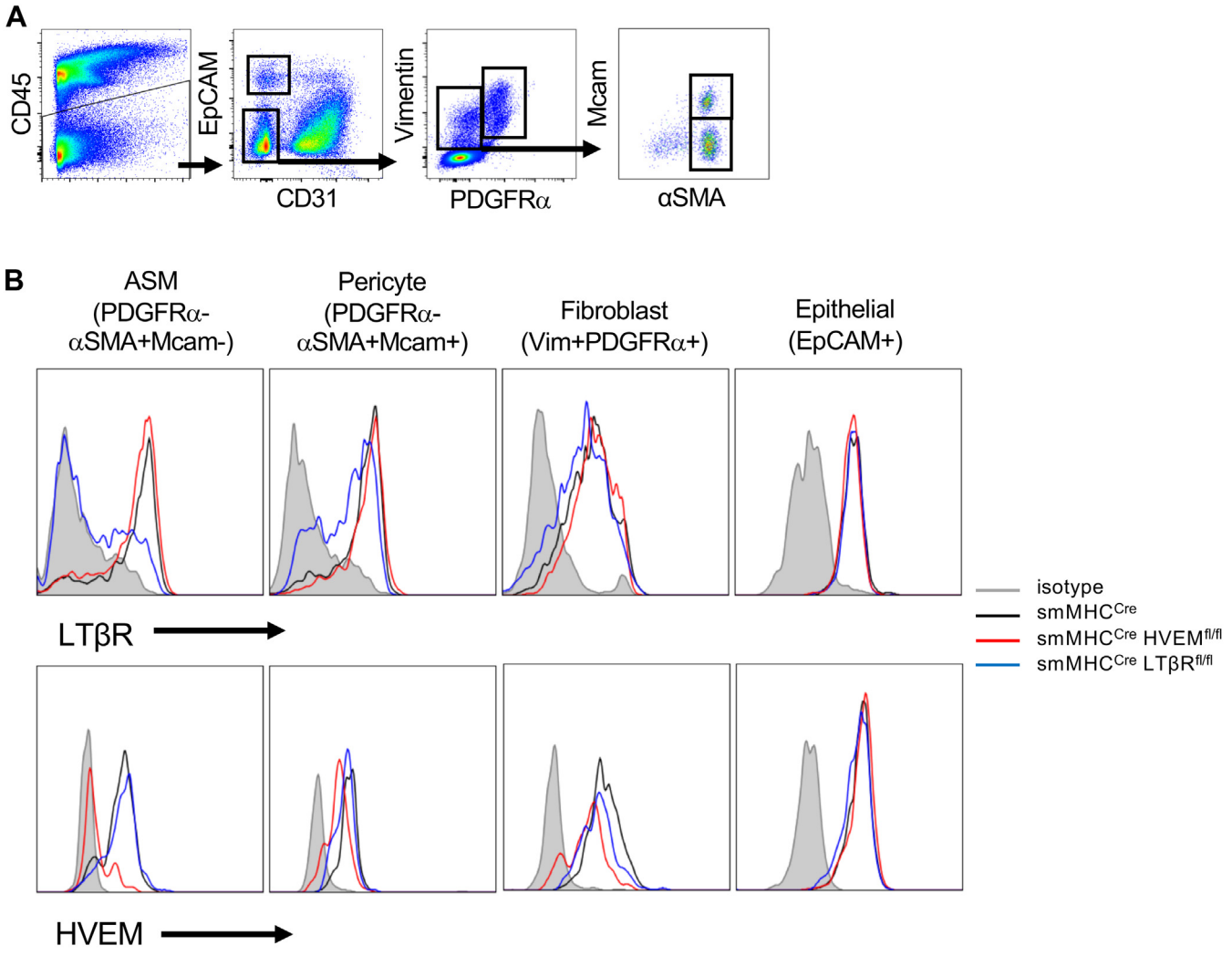

FIG E1. Flow cytometric analysis of lung structural cells. **(A)** Flow gating of epithelial cells ($CD45^-EpCAM^+CD31^-$), fibroblasts ($CD45^-EpCAM^-CD31^-Vimentin^{hi}PDGFR\alpha^+$), pericytes ($CD45^-EpCAM^-CD31^-Vimentin^{med}PDGFR\alpha^-\alpha SMA^+Mcam^+$), and ASM ($CD45^-EpCAM^-CD31^-Vimentin^{med}PDGFR\alpha^-\alpha SMA^+Mcam^-$) in mouse lung. **(B)** Expression of HVEM and LTβR on gated lung structural cells from smMHC^{Cre}, smMHC^{Cre}HVEM$^{fl/fl}$, or smMHC^{Cre}LTβR$^{fl/fl}$ mice.

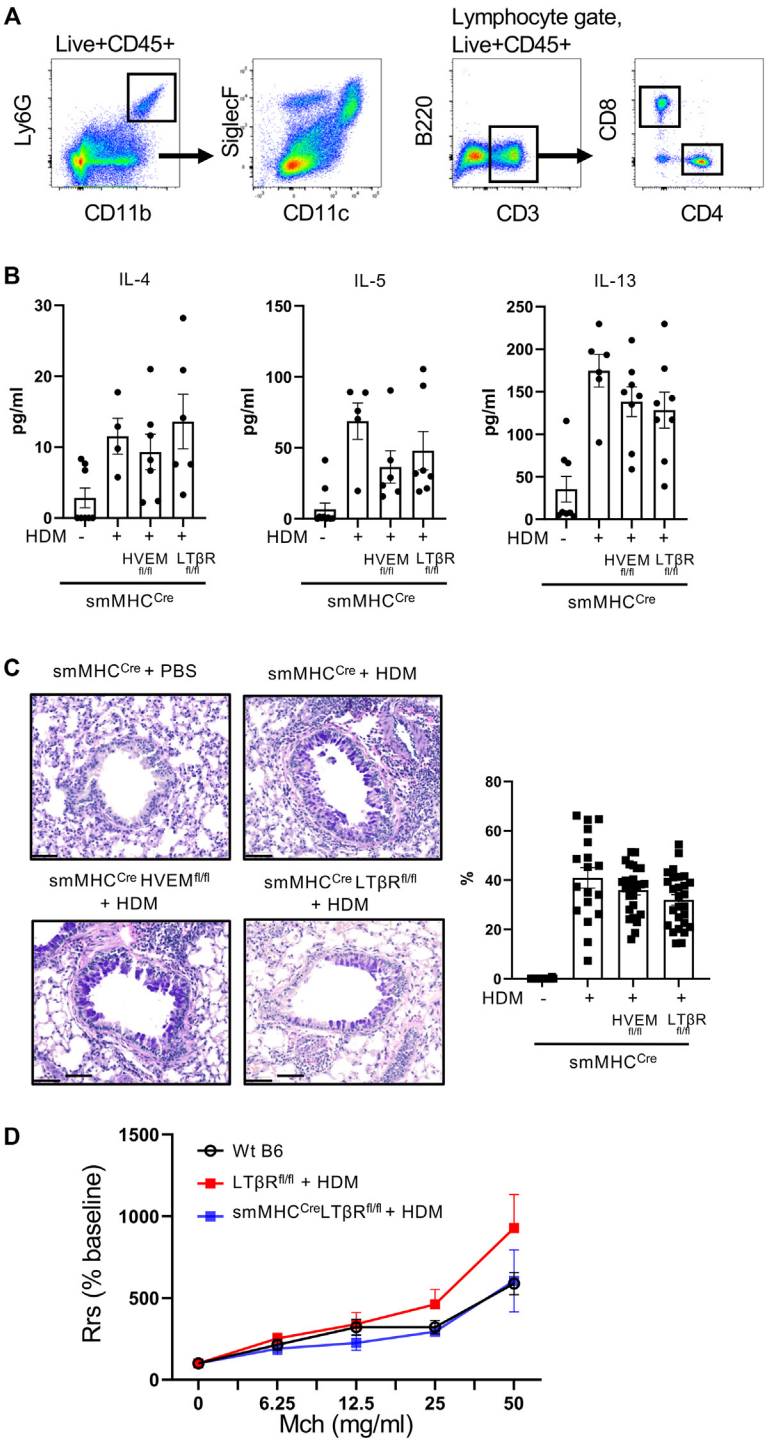

FIG E2. Analysis of inflammatory features in allergen-induced lungs, and AHR in acute lung inflammation. **(A)** Flow gating of $CD45^+$ lung immune cells: neutrophils ($CD11b^+Ly6G^+$), eosinophils ($CD11b^-Ly6G^-CD11c^{lo}SiglecF^+$), alveolar macrophages ($CD11b^-Ly6G^-CD11c^+SiglecF^+$), $CD4^+$ T cells ($CD3^+CD4^+CD8^-$), and $CD8^+$ T cells ($CD3^+CD4^-CD8^+$). **(B)** IL-4, IL-5, and IL-13 production in BAL fluid of $smMHC^{Cre}$, $smMHC^{Cre}HVEM^{fl/fl}$, or $smMHC^{Cre}LT\beta R^{fl/fl}$ mice challenged chronically with HDM over 6 weeks (n = 6-8 per group). **(C)** Representative PAS-stained lung sections (scale, 100 μm) and percentages of PAS-positive bronchial epithelial cells; n = 4-5 mice per group, 5 bronchi per mouse. Data are representative of 3 experiments. **(D)** Airway resistance (AHR) after methacholine challenge, measured by FlexiVent (n = 4-5 mice per group), in mice acutely challenged with HDM over 14 days. Data are presented as means ± SEMs.

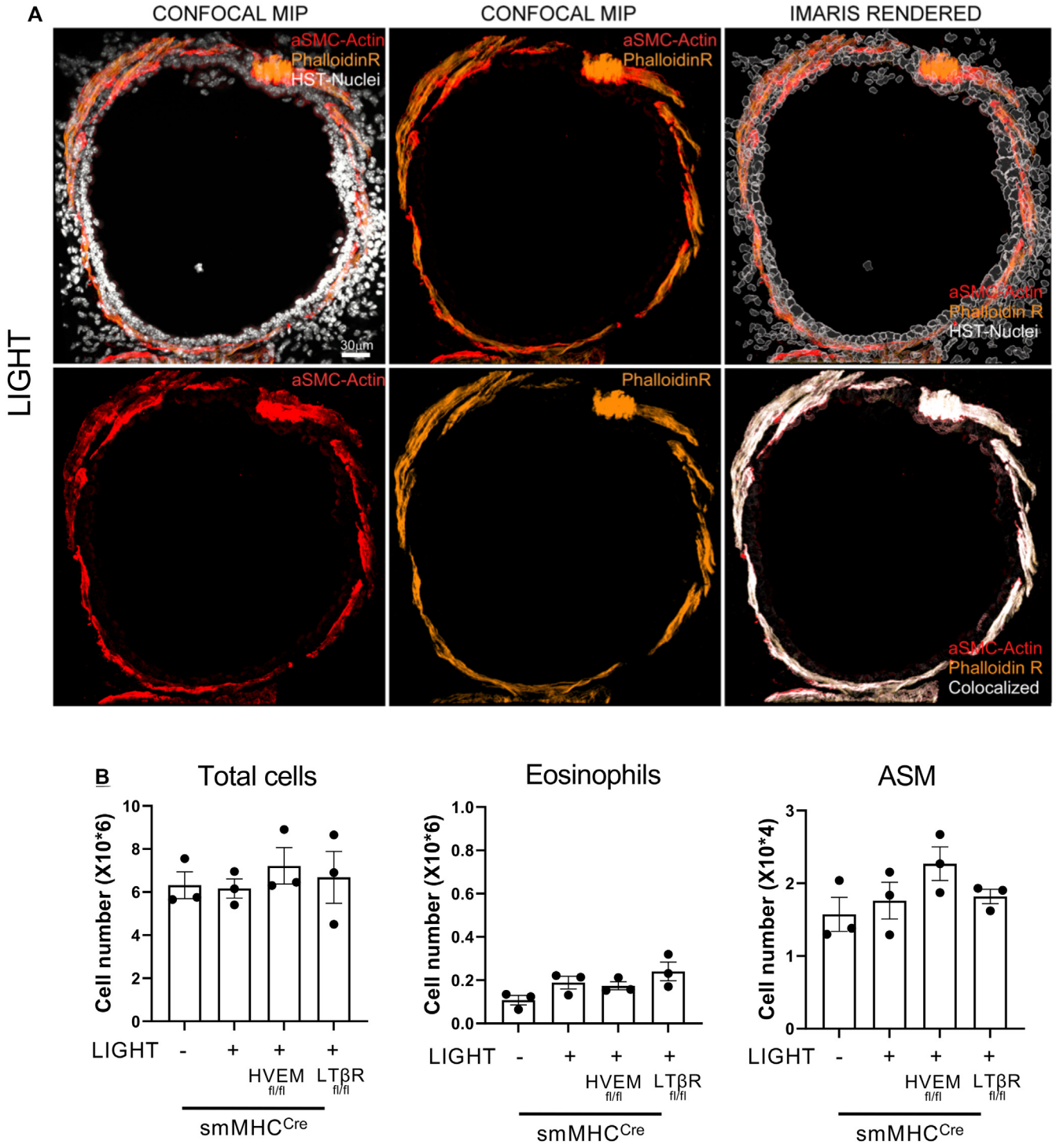

FIG E3. Bronchial smooth muscle and other cellular changes in mice injected with LIGHT. **(A)** Confocal images of lung bronchi from mice treated with LIGHT and stained with phalloidin *(orange)* and αSMA *(red)* (scale, 30 μm). **(B)** smMHC^{Cre}, smMHC^{Cre}HVEM$^{fl/fl}$, or smMHC^{Cre}LTβR$^{fl/fl}$ mice were treated intratracheally with LIGHT or PBS, and numbers of total cells, eosinophils, or ASM in lung tissues were analyzed by flow cytometry.

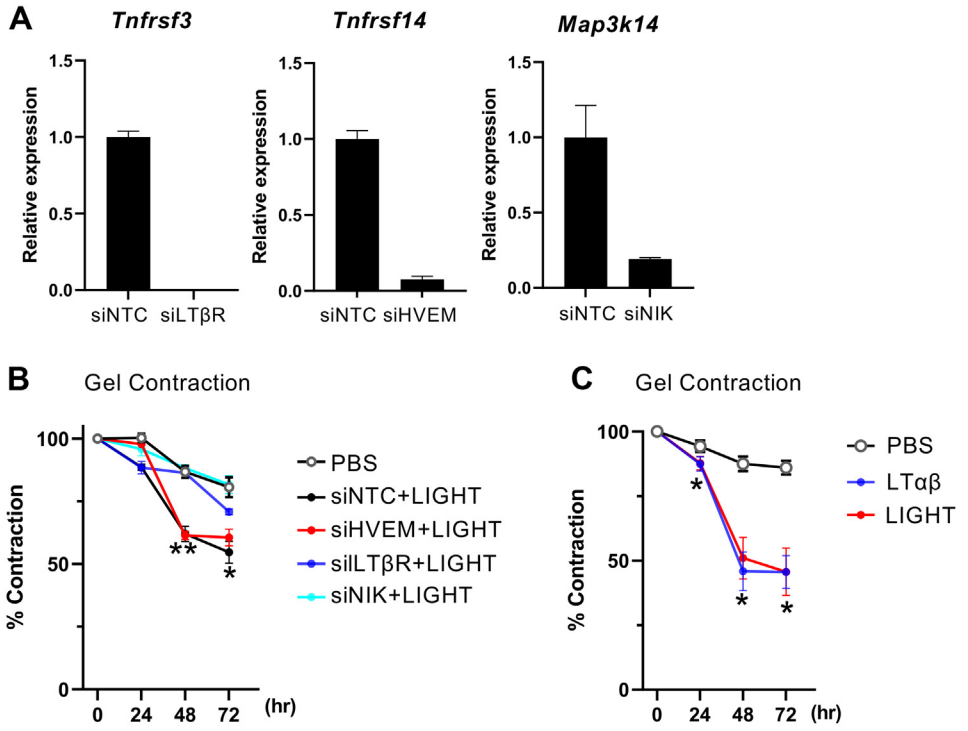

FIG E4. Involvement of LTβR and NIK in ASM contractility. **(A)** mRNA expression of TNFRSF3 (LTβR), TNFRSF14 (HVEM), and Map3k14 (NIK) in ASM after siRNA targeting. **(B)** Gel contraction of ASM with siRNA knockdown after stimulation with LIGHT. **(C)** Gel contraction of ASM stimulated with LIGHT or LTαβ. Means ± SEMs are from triplicate cultures. Data are presented as means ± SEMs and are representative of 2 experiments. **P* < .05, ***P* < .01.

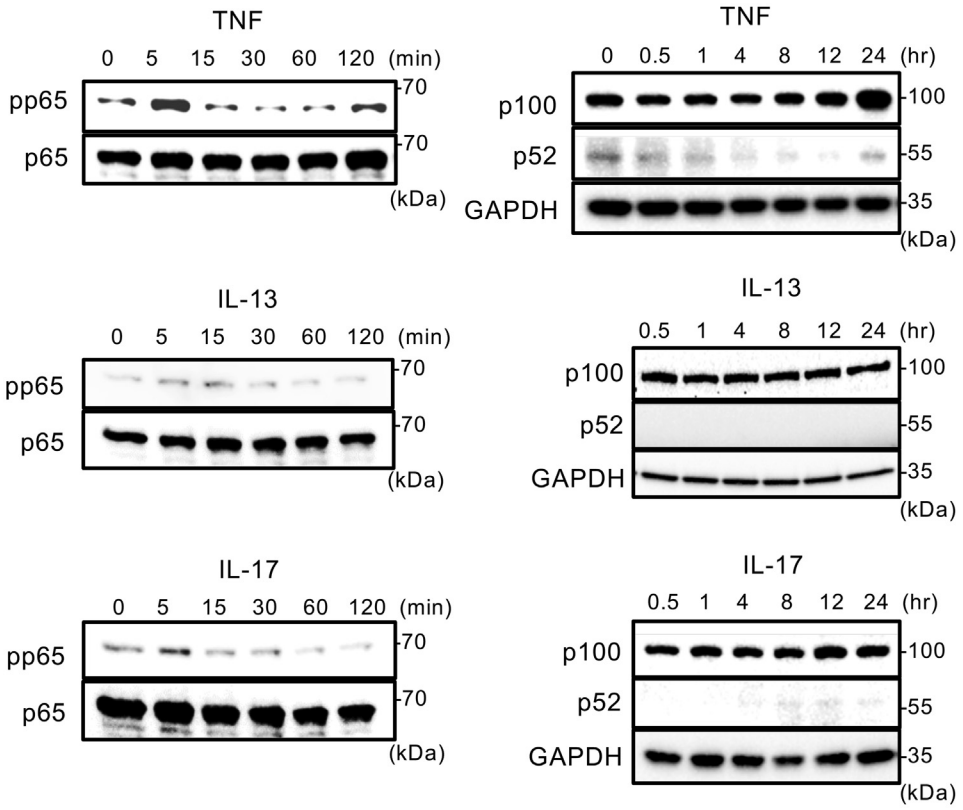

FIG E5. TNF, IL-13, and IL-17 activation of canonical or noncanonical NF-κB in ASM. Activation of canonical NF-κB (pp65, *left*) and noncanonical NF-κB (processing of p100 to p52, *right*) assessed in human ASM stimulated for various times with TNF, IL-13, or IL-17.