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Conflict of interest statement: M.C has patents related to LIGHT and lung inflammation.

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ABSTRACT

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

Objective: To determine whether signaling via LTβR or HVEM from LIGHT dominantly drives ASM hyperreactivity induced by allergen.

Methods: Conditional knockout mice deficient for LTβR or HVEM 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, and 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 that have also been implicated in smooth muscle dysregulation, LIGHT promoted NIK-dependent non-canonical NF-κB in ASM in vitro, leading to sustained accumulation of F-actin, phosphorylation of myosin light chain kinase, and contractile activity.

Conclusion: LTβR signals directly and dominantly drive airway smooth muscle hyperresponsiveness relevant for pathogenesis of airway remodeling in severe asthma.

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Key Messages:

- LTβR interactions on smooth muscle cells in vivo control airway smooth muscle mass and airway hyperresponsiveness driven by inhaled allergen
- LIGHT-LTβR non-canonical NF-κB signaling promotes contractile activity, hyperplasia and hypertrophy in airway smooth muscle cells

Capsule Summary:

Deletion of LTβR in smooth muscle cells limits allergen-driven airway smooth muscle remodeling and airway hyperresponsiveness, implying that LTβR may control excessive bronchial smooth muscle activity related to remodeling and lung function impairment in asthma.

Keywords

LTβR, Asthma, Airway Smooth Muscle, AHR, contractility, non-canonical NF-κB, TNF superfamily, LIGHT, TNFSF14

Abbreviations

HDM, house dust mite; ASM, airway smooth muscle cells; AHR, airway hyperresponsiveness; LIGHT, homologous to lymphotoxin, exhibits inducible expression and competes with HSV glycoprotein D for binding to HVEM, a receptor expressed on T lymphocytes; HVEM, herpes virus entry mediator; LTβR, lymphotoxin beta receptor; LTαβ, lymphotoxin alpha beta

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INTRODUCTION

The pathological features of asthma are chronic airway inflammation, associated with an aberrant airway constriction response to allergen that is 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 having a relatively short duration of asthma, suggesting that this change is directly linked with asthma severity [4]. Moreover, ASM isolated from asthmatics also exhibit enhanced contractile activity and enhanced proliferation [2, 5]. However, what drives these changes is still not fully appreciated.

Specifically, it has not been clear 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 has 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 HSV glycoprotein D for binding to HVEM, a receptor expressed on T lymphocytes), also known as TNF superfamily member 14 (TNFSF14), to the severity of lung inflammation and to changes in ASM mass with studies of LIGHT-deficient mice [16]. LIGHT can be

92 expressed by several immune cell types including activated T cells, dendritic cells, and neutrophils,
93 that are found in asthmatic lungs, and higher levels of soluble LIGHT or cell-associated LIGHT in
94 the sputum of asthmatic patients are associated with severe disease [17-19]. Structural cells of the
95 lung such as epithelial cells and fibroblasts express the receptors for LIGHT, namely HVEM
96 (TNFRSF14) and LTβR (LTBR/TNFRSF3), and LIGHT can promote inflammatory activity in these
97 cells [20-24]. However, whether LIGHT and its receptors act directly on airway smooth muscle cells
98 in vivo to promote exaggerated responsiveness and play a direct role in AHR has not been
99 demonstrated. In this study, we now show that LTβR is constitutively expressed on mouse and
100 human airway smooth muscle cells. With conditional deletion of LTβR in smooth muscle cells in
101 vivo, we demonstrate that LTβR activity in these cells is essential for smooth muscle remodeling,
102 lung dysfunction, and airway hyperresponsiveness driven by inhaled allergen. This is explained
103 by LTβR activating sustained signaling pathways involving NFκB-inducing kinase (NIK) and
104 activation of non-canonical NF-κB, pathways not triggered by the other ASM-associated cytokines
105 IL-13, TNF, and IL-17.
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METHODS

Mice

C57BL/6J and smMHC (*Myh11*)/Cre/eGFP transgenic mice (B6.Cg-Tg(Myh11-cre,-EGFP)2Mik/J) [25] were purchased from Jackson Laboratory. 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 the C57BL/6 background. Animal experiments were performed with 6–8-wk-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 given intranasal administrations of HDM, *Dermatophagoides pteronyssinus,* extract (Greer Laboratories, Lenoir, NC, USA): 200 µg on day 0 and 100 µg on days 7 and 14, followed by 50 µg of HDM given twice per week for 4 weeks. For LIGHT experiments, mice were injected intratracheally with 10 µg of recombinant LIGHT (R&D, Mineapolis, MN, USA) or PBS on day 1 and 2 and analyzed on day 3. For acute airway inflammation, mice were sensitized by administration of 20 µg HDM protein in 2mg alum given intraperitoneally on day 0, and challenged with 10 µg HDM protein intranasally on days 10-13.

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 post-fixed in 4% PFA and lung sections were cut (30 µm) and stained with rabbit polyclonal antibody to αSMA (alpha smooth muscle actin: Abcam, Cambridge,

UK), Phalloidin-AlexaFluor 568 (Thermo Fisher Scientific, Hampton, NJ, USA), and Hoechst33342 (BD Bioscience, San Jose, CA, USA). All 3D high resolution tertiary bronchi image stacks were acquired with an inverted Zeiss 780 or 880 Airyscan laser scanning confocal microscope (LSCM) using a 40x (1.4na) objective and the 32-channel GaAsP-PMT area detector (Zeiss Microscopy LLC, White Plains, NY, USA). Image stacks through 15-20 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 2048x2048.

Lung tissue were further processed in imaris software using the isosurface module (Bitplane) to outline and quantify the volume and area of α-smooth muscle actin 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 colocalization with αSMA. The actin labeling in all other non-muscle 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. 10-15 tertiary bronchi per 4-5 mouse lungs per condition and mouse model were analyzed.

Airway hyperresponsiveness

Airway resistance in response to different doses of Methacholine were measured using the FlexiVent system (SCIREQ Inc, Montreal, Canada) as previously described [16]. Peak airway resistance was analyzed by Scireq flexiWare software V8.

Flow cytometry

Lungs were dissociated using gentleMACS Dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany) with Lung Dissociation Kit (Miltenyi Biotec). Single cells were stained with monoclonal antibodies to mouse CD45 (clone:30-F11), CD11b (clone:M1/70), CD11c (clone:HL3),

SiglecF (clone: E50-2440) from BD Biosciences; EpCAM (clone:G8.8), CD31 (clone:390), PDGFRα (clone:1A4/asm-1), Mcam (clone:ME-9F1) from BioLegend; vimentin (clone:280618) from R&D; and αSMA (clone:1A4/asm-1) from Novus. Live/Dead cells were stained with Fixable Aqua Dead Cell Staining Kit (Thermo Fisher). For intracellular staining, Foxp3 Transcription Factor Staining Buffer Set (eBioscience, San Diego, CA, USA) were used for fixation and permeabilization. Flow analysis was performed on a Fortessa (BD Biosciences) and data were analyzed using FlowJo Software (version 10, FlowJo, LLC, Ashland, OR). $Live^{+}CD45^{+}$ lung immune cells were gated for neutrophils, eosinophils, alveolar macrophages, $CD4^{+}$ T cells and $CD8^{+}$ T cells following the gating strategy shown in the supplemental figure. $Live^{+}CD45^{-}$ lung structural cells were gated as described in the figures 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.

BAL cytokines

Cytokines in 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 from R&D.

ASM culture and analysis

Healthy donor human ASM or asthmatic diseased donor ASM were purchased from ScienCell (cat:3400; Carlsbad, CA, USA) or Lonza (cat: 00194850; Walkersville, MD, USA). 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, Caucasian, Non-smoker, COD CVA/Stroke; Hl227: Asthma, 21 years old, Male, Caucasian, Non-smoker, COD Head trauma. For flow analyses, cells were stained with monoclonal antibodies to human LTβR (clone:31G4D8), or HVEM (clone:122) from BioLegend. Cells were maintained in Smooth Muscle Cell Media (ScienCell) with supplied supplements and FBS added. Cells were used between passages 2–4 and 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/non-canonical NF-κB (NIK-SMI, 25nM, MedChemExpress, Monmouth, NJ, USA), canonical NF-κB (BAY11-7082, 1nM, MedChemExpress), or Rac1 (NSC 23766, 10nM, Tocris, Birstol, UK) were added for 1 hour before stimulation with rLIGHT. For siRNA knockdown, ON-TARGETplus siRNA to human LTβR and NIK, and an NTC control, were purchased from Dharmacon (Pittsburg, PA, USA). siRNA Oligo Duplex siRNA to human HVEM was purchased from Origene（Rockville, MD, USA). 50 nM of siRNA was transfected into ASM using HiPerFect transfection reagent (Qiagen, Venlo, Netherlands) as described previously[21] and according to the manufacturer's instructions.

ASM gel contraction assay

3D Gel contraction assays were performed using collagen contraction assay kits purchased from Cell Biolabs (San Diego, CA, USA) according to the manufacturer's instructions. In brief, suspensions of ASM were mixed with collagen solution at a density of 0.5-1x10^6 cells/well. After confirming the polymerization of collagen gels, smooth muscle basal media 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 timepoints (24, 48, 72 hours). Images were obtained with ImageLab (Bio-Rad Laboratories, Hercules, CA) and the area of collagen gel matrix measured using ImageJ software.

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 70-80% confluency on coverslips coated with Collagen Type I solution (Sigma-Aldrich, St. Louis, MO, USA). Cells were stimulated with 100 ng/ml of rLIGHT or PBS with or without NIK-SMI or Rac inhibitors. After 6-12 hours, cells were washed and fixed with 4% Formalin and permeabilized with 0.1% TritonX-100 (Thermo Fisher Scientific). Cells were stained with Phalloidin-Alexa Fluor 568 (Thermo Fisher Scientific), anti-Vinculin antibody (Abcam) and Hoechst 33342 (BD Bioscience) 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 3D high resolution single ASM cell images were acquired with an inverted 880 Airyscan laser scanning confocal microscope (LSCM) using a 63x (1.4na) objective and the 32-channel GaAsP-PMT area detector (Zeiss Microscopy LLC). Image stacks through 3-5 µm of spreading ASM, on average 10-15 slices, were acquired with Nyquist resolution parameters using a 0.3 µm step size and optimal frame size of 4164x4164. 300-400 individual ASM, in two 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% TritonX-100 (Thermo Fisher Scientific). Cells were stained with Phalloidin-Alexa Fluor 568 (Thermo Fisher Scientific) and Hoechst 33342 (BD

Bioscience) and analyzed by confocal microscopy. All 3D multi-stitched image panels were acquired on an inverted Zeiss LSM 780 LCSM using a 20x 0.8 NA objective, a z-step size of 0.85 microns and by using the automated tiling function of the Zen software with a 10% overlap between tiles. Images were stitched and 3D stacks were maximum intensity projected using the Zen software then imported into Image Pro Premier 10 (IPP10) Media Cybernetics) for further processing. Briefly, by using the phalloidin-AF555 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 auto count tool automatically outlined and quantified the area all 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 harvested and the proliferating cells were detected by BrdU incorporation using a FITC BrdU Flow Kit (BD Bioscience) according to the manufacturer's protocol.

Western Blotting

Cells were lysed in RIPA Buffer containing protease inhibitor (Roche, Mannheim, Germany) Whole cell lysates were run in WedgeWell 8-16% Trys-Glysin gradient gels (Novex) and transferred to Nitrocellurose membrane (Bio-Rad). Membranes were incubated with 5% BSA (Sigma-Aldrich) or non-fat dry milk (R&D) in TBS-T for blocking, then incubated with primary antibodies to p-NFκB p65 (cat:3033, 1:1000), NFκB p65 (cat:8242, 1:1000), NFκB p100/52 (cat:3017, 1:1000), PAK1 (cat:2602,1:1000), p-MYPT(Thr696) (cat:5163, 1:1000), p-MLC2 (cat:3672, 1:1000), MLC2 (cat:8505, 1:500) from Cell Signaling Technology; to p-PAK1 (cat:135755, 1:200) and MYPT (cat:514261, 1:100) from Santa Cruz; followed by HRP-conjugated secondary antibodies to mouse IgG (cat:516102, Santa Cruz) or rabbit IgG (cat:2357,

Santa Cruz). Chemiluminescence was enhanced with Amersham ECL Prime Western Blotting Detection Reagent (GE Health Care, Madison, WI, USA) 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, Dallas, TX, USA) was used as loading control. For Rac1 detection, whole cell lysates were used for immunoprecipitation using Active Rac1 detection kit (Cell Signaling Technology, Danvers, MA, USA) according to the manufacturer's instruction.

RT-PCR

Total RNA from ASM was isolated using RNeasy kit (Qiagen). cDNA was prepared using a SuperScript IV reverse transcriptase kit (Thermo Fisher Scientific). Real-time PCR was performed with LightCycler (Roche, Indianapolis, Ind) using PowerUp SYBR Green master mixes (Applied Biosystems, Foster City, CA). Data were normalized with housekeeping gene GAPDH and presented as relative expression or relative quantification to control samples which were derived from the difference in cycle threshold (Ct) between the gene of interest and the housekeeping genes using the equation RQ = 2−ΔΔct. Designed primers for human MAP3K14 were purchased from Bio-Rad (Hercules, CA, USA). The oligonucleotide primer sequences were: GAPDH, sense AGCCAAAAGGGTCATCATCTCT, anti-sense AGGGGCCATCCACAGTCTT; HVEM, sense AGCAGCTCCCACTGGGTATG, anti-sense GATTAGGCCAACTGTGGAGCA; LTβR, sense CCGACACAACCTGCAAAAAT, anti-sense GAGCAGAAAGAAGGCCAGTG.

Analysis of published RNA-seq data

Bulk RNA-seq of healthy and asthma ASM were analyzed for transcripts of cytokine receptors. Fong et al [29-31] used ASM from 11 asthmatic and 12 non-asthmatic donors over two GEO submissions: GSE119578 (GSE119578_GeneCount_rpkm_by_gene_RDB.txt.gz) and GSE119579 (GSE119579_GeneCount_rpkm_by_gene_SW.txt.gz). Himes et al [29-31] used ASM

from 6 donors with fatal asthma and 12 control donors. Only baseline (no treatment) measurements from GSE58434 were analyzed (GSE58434_All_Sample_FPKM_Matrix.txt.gz). Kan et al (Kan et al., 2019) used ASM from 9 fatal asthma and 8 non-asthma donors. Only vehicle control samples from GSE94335 were analyzed (GSE94335_PostQC_all_sample_TPM.txt.gz).

Statistical analysis

All results are presented as mean ±SEM. Statistical significance was analyzed by Mann-Whitney test for comparison of means between 2 independent groups or by 1-way ANOVA followed by Tukey's post hoc multiple comparison test for differences of means between multiple groups using Prism 8 software (GraphPad Prism, San Diego, CA). P value <0.05 was considered statistically significant. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

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RESULTS

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

We first studied expression of the receptors for LIGHT, LTβR and HVEM, in published RNA-seq data from airway smooth muscle cells (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. 1A**). Interestingly, compared to the receptors for the three primary asthma-related cytokines linked to activities in airway smooth muscle cells, namely TNFR1, IL4R, and IL17RA and IL17RC, only TNFR1 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. 1A**). Flow cytometric analyses of two 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 post-mortem lungs of asthmatics isolated in-house, furthermore showed equivalent expression of both molecules compared to healthy donor ASM, with LTβR being most highly expressed (**Fig. 1B**).

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

To then assess the importance of LTβR or HVEM on ASM in vivo, we used smooth muscle Myosin Heavy Chain (smMHC)^{Cre} (*Myh11*)-eGFP transgenic mice [25]. Similar to human cells, each receptor was expressed constitutively on ASM in the mouse lungs **(Fig. 2A)**. 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. 2A).** Previous studies using scRNAseq have revealed several markers to identify structural cell subsets in the lung [32, 33], however there is still a lack of an established gating

strategy to identify ASM by conventional flow cytometric methods without a marker tag. We found that among the CD45$^-$ EpCAM$^-$ PDGFRα$^-$ and Vimentin^{low} population, that excludes epithelial and endothelial cells and fibroblasts, there are two distinct αSMA (alpha smooth muscle actin) positive populations subdivided based on MCAM (CD146) expression (**Fig. E1A**). MCAM is known to be highly expressed on pericytes and lower in smMHC$^+$ mature smooth muscle cells [34]. By analyzing the smMHC^{Cre} conditional knockout mice, we found that both HVEM and LTβR were deficient in the αSMA$^+$ MCAM$^-$ population, but not the αSMA$^+$ MCAM$^+$ population or in fibroblasts or epithelial cells. This further showed the specificity of the conditional deletion of the two receptors and revealed that ASM can be distinguished effectively as CD45$^-$ EpCAM$^-$ PDGFRα$^-$ Vimentin^{low} αSMA$^+$ MCAM$^-$ cells in the lungs (**Fig. E1B**).

The conditional knockout mice and control animals (smMHC^{Cre}) were then exposed to chronic repetitive intranasal challenges with house dust mite 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-dimensional confocal microscopy of the immunofluorescent staining of αSMA and phalloidin (Filamentous actin). These changes were associated with profound airway hyperresponsiveness, reflected by increased lung resistance to methacholine when administered intranasally (**Fig. 2B-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 histological analysis (**Fig. 2B-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. 2D**).

Flow cytometry 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. 2E**). 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 Th2 cytokine production in BAL fluid, were not different between the control and LTβR conditional knockout mice challenged with allergen (**Fig. 2F-G, Fig. E2A-B**). Mucus production determined by PAS staining of bronchial epithelium was also not different between the control and LTβR conditional knockout mice challenged with allergen (**Fig. E2C**).

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 naïve unchallenged mice (**Fig. E2D**). Together, 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-dimensional confocal microscopy (**Fig. E3A**). Replicating our observations from two previous studies [16, 24], rLIGHT administration to the lungs induced a significant increase in smooth muscle mass surrounding the bronchi in control mice **(Fig. 3A-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. 3A-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, as well as having no effect on promoting increased numbers of total cells and eosinophils in the lung tissue (**Fig. E3B**). 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. 2E) 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 see with repetitive allergen (**Fig. 3C**, compare to Fig. 2D). Most importantly, ASM-specific LTβR-deficient animals displayed strongly reduced AHR, unlike HVEM-deficient animals that showed similar AHR compared to the control mice treated with rLIGHT **(Fig. 3C)**. 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**).

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

To further investigate the direct actions of LIGHT-LTβR signals on airway smooth muscle cells, we analyzed normal human primary bronchial smooth muscle cells. Indicating that LTβR was active, it was downregulated after culture with LIGHT, a phenomenon previously associated with initiation of signaling through this receptor **(Fig. 4A**). The human ASM were then cultured in 3D collagen gels to make a net structure (**Fig. 4B**) and stimulated with LIGHT to assess contractile activity. Significantly, LIGHT induced strong activity in this assay shown by shrinking of the collagen gel, largely seen from 24-48 hours (**Fig. 4C)**. 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 (**Fig. E4A-B**). The membrane version of lymphotoxin, LTαβ, is also a ligand of LTβR. Recombinant LTαβ induced identical gel contraction compared to LIGHT (**Fig. E4C**), 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 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. 4D**), and this was enhanced significantly by LIGHT stimulation. Contractile ASM weakly incorporated BrdU, which was less strongly, albeit significantly, affected by LIGHT (**Fig. 4D**). Lastly, human ASM hypertrophy was assessed in vitro, and LIGHT significantly increased the volume of individual cells (**Fig. 4E**), 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 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 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 alpha smooth muscle actin, contractile myosin heavy chain 11 isoform, and RhoA (**Fig. 5A**). In contrast, by GSEA, we found significant enrichment by LIGHT of transcriptional machinery associated with the cell membrane and actin rearrangement (**Fig. 5B**). 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 6-12 hr (**Fig. 5C**). Further in line with this, migration of ASM was enhanced by LIGHT stimulation in migration/wound assays of monolayers assayed at 12hr, correlating with increased expression of actin filaments (**Fig. 5D**). 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 non-canonical NF-κB signaling induces actin polymerization in ASM

As LTβR signals are known to induce both early onset canonical NF-κB activation, as well as late onset non-canonical 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 mins. However, most interestingly, LIGHT resulted in processing of p100 to p52 (the signature of non-canonical NF-κB pathway activation) starting ~4 hrs after stimulation and extending to 24 hrs (**Fig. 6A**). We then assessed whether LIGHT-induced non-canonical 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. 6B**). Moreover, NIK-SMI also effectively prevented LIGHT-induced contractile activity visualized in 3D collagen gels, whereas in contrast, BAY11-7082, a canonical NF-κB inhibitor, had no effect on LIGHT-induced gel contraction (**Fig. 6C**). Knockdown of NIK (Map3k14) with siRNA further supported the inhibitor studies and prevented LIGHT-driven gel contraction (**Fig. E4A-B**). Notably, non-canonical signaling, initiated from 4hr and sustained at least up to 24hr (Fig. 6A), preceded the initiation of contraction at 24hr (Fig. 4C and S4), and preceded or coincided with changes in actin polymerization and migration related to actin polymerization (Fig. 5C and D), reinforcing the conclusion regarding the relevance of this pathway from the NIK inhibition assays. Thus, LIGHT-LTβR dependent non-canonical NF-κB signaling is central to dysregulation of ASM. 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 non-canonical NFκB activation in ASM (p52 processing), even though canonical NF-κB (pp65) was induced, particularly by TNF, as previously reported (**Fig. E5)**.

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

To further understand the role of non-canonical 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 hrs (**Fig. 7A**). We also detected late and sustained phosphorylation of the serine threonine kinase PAK1 (p21-activated kinase 1) over 24 hrs, that is a target of the GTP binding protein Rac1, and has been implicated in cytoskeleton/actin reorganization as well as being upstream of MLC kinase (**Fig. 7B**). Consistent with this, the active (GTP) form of Rac1, bound to PAK1, was detected between 1-4 hours after LIGHT stimulation (**Fig. 7C**). We also assessed phosphorylation of 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 mins (**Fig. 7B**).

Since 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 non-canonical NF-κB signaling. Indeed, LIGHT-induced phosphorylation of PAK1 (**Fig. 7D**) and sustained phosphorylation of MLC (**Fig. 7E**) 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. 7F**). Lastly, to further consolidate the significance of this signaling pathway to ASM dysfunction, we found that a Rac1 inhibitor blocked LIGHT-induced contraction in 3D gels (**Fig. 7G**). Correspondingly treatment with the Rac1 inhibitor reduced actin polymerization, and in this case also blocked the increase in focal adhesions driven by LIGHT (**Fig. 7H).**

Together with our in vivo data, these results indicate that LTβR-induced non-canonical NF-κB signaling is a primary and dominant driver of airway smooth muscle cell hyperresponsiveness.

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DISCUSSION

Airway smooth muscle cells have long been considered to be central for lung dysfunction in asthma due to their ability to narrow the airways. However, whether there are dominant receptors on ASM that explain ASM remodeling that is seen in severe asthma and explain airway hyperresponsiveness has not been clear. Our present study now reveals the novel finding that LTβR is essential for allergen-driven ASM remodeling and the resultant increased airway hyperresponsiveness 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 those for 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 impact ASM deregulation and AHR in response to allergen in vivo. This correlates with the unique ability of LTβR to drive non-canonical 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 WT 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 asthmatics as it is capable of inducing the same signals as LIGHT through the LTβR, and thus we 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 biopsies have been found to contain smooth muscle cells of larger diameter compared to control subjects, and severe asthmatics 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 non-canonical 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]. Non-canonical 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 Ca2+ signaling dependent on STAT6, MAPK, and PI3K activity [7, 43-46]. Moreover, IL-17 was also found to drive migration, contraction, or other activities in ASM, dependent on several MAPK pathways, canonical NF-κB, and PKC [47-49]. Thus, while these cytokine receptors might then promote other signaling pathways compared to LTβR, or in the case of the receptors for TNF and IL-17 impact 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 ILC, 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 co-operates with TNF or IL-17 to regulate ASM activities has not been shown yet, 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 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 airway hyperresponsiveness in allergen-challenged mice where

575 LTβR was specifically deleted in smooth muscle cells. Existing asthma treatments largely target
576 inflammatory cells such as eosinophils or mast cells, and airway constriction is only transiently
577 controlled by beta agonist inhalers. Blocking LIGHT-LTβR interactions, and possibly LTαβ-LTβR
578 interactions, or disrupting non-canonical NF-κB signaling in smooth muscle cells, could represent
579 important therapeutic approaches for persistent and chronic airway hyperresponsiveness in
580 severe asthmatic patients or patients with similar lung dysfunction.
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Author Contributions

H.M. designed and conducted experiments, and wrote the paper. W.B.K designed and conducted experiments. M.M, M.C.M, R.H, R.D.S.A, R.K.G and G.S.S assisted with assays or experiments. K.F and A.S performed bioinformatics analysis. P.D and F.A analyzed published RNA-seq data. K.D. assisted histology studies. M.M and D.H.B provided human ASM and assisted with assay design. A.V.T provided mice. M.C directed the study, designed experiments, and wrote the paper.

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Figure Legends

Figure 1. LTβR and HVEM are expressed by human healthy and asthmatic airway smooth muscle cells. (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 asthma donor-derived ASM.

Figure 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 given intranasal challenges of HDM extract over 6 weeks (n = 4-6/group). (B) Confocal analysis of lung bronchi (scale 20 μm). F-actin (Phalloidin, yellow), alpha smooth muscle actin (red), DAPI (blue). Data representative of 3 experiments. (C) Quantitation of ASM volume based on phalloidin staining around the bronchi from (B) (n = 6-9/group, 10-15 tertiary bronchi per mouse). Data representative of 3 experiments. (D) Airway resistance (AHR) after methacholine challenge, measured by Flexivent (n = 5-8/group). Data representative of two experiments. *P < 0.05, $smMHC^{Cre}$ vs. $LT\beta R^{fl/fl}$. (E) Flow quantitation of total lung tissue smooth muscle cells (ASM). Data points, individual mice. (F) Flow quantitation of total lung inflammatory/immune cells. Data points, individual mice. (G) Representative H&E stained lung sections (scale 100 μm). n =4-6 mice/group. Data representative of 3 experiments. Means ± SEM. *P < 0.05, **P < 0.01.

Figure 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-B) Confocal microscopy of lung bronchial sections for expression of F-actin (Phalloidin, yellow) and α-smooth

768 muscle actin (red). (A) Representative images of bronchi (scale 20 μm). (B) Imaris 3D imaging
769 analysis quantification of ASM volume (phalloidin) around individual bronchi (n = 4-5 mice/group,
770 10-15 tertiary bronchi per mouse). Data means ± SEM and representative of 3 experiments. *P <
771 0.05, **P < 0.01. (C) Airway resistance (AHR) after methacholine challenge, measured by
772 Flexivent (n = 4 mice/group). *P < 0.05, $smMHC^{Cre}$ vs. $LT\beta R^{fl/fl}$. Data means ± SEM and
773 representative of 2 experiments.
774
775 **Figure 4. LIGHT induces human ASM proliferation and contractile activity.** (A) HVEM and
776 LTβR expression on human ASM assessed by flow after stimulation with LIGHT for 24 or 48hr.
777 Similar data in 3 experiments. (B) Images of ASM in collagen 3D gels. Left: Bright field, Right:
778 Phalloidin (red), DAPI (blue). (C) ASM collagen gel contraction at 24-72hr after stimulation with
779 LIGHT. Data combined means from triplicates from 3 experiments. (D) Percentages of $BrdU^+$
780 ASM stimulated with LIGHT for 48hr. Cells either first incubated in serum free media for 16 hours
781 for proliferative ASM, or for 7 days for contractile ASM. Data means of triplicates and
782 representative of 3 experiments. (E) ASM volume after stimulation with LIGHT, measured by
783 confocal microscopy. 300-400 ASM, in two replicate experiments were analyzed. Data means ±
784 SEM. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.
785
786 **Figure 5. LIGHT induces actin polymerization and migratory ability in human ASM.** (A) RNA-
787 seq analysis of contractile molecules in human ASM treated with LIGHT. Log2 fold change after
788 4hr compared to PBS treated cells. (B) GSEA of cell structure-related genes from RNA-seq of
789 ASM stimulated with LIGHT compared to PBS for 4hr. (C) Confocal images of ASM treated with
790 LIGHT for 6hr and stained with Phalloidin (red), Vinculin (green), DAPI (blue). Graphs show
791 volume of F-actin (phalloidin) and focal adhesions (vinculin) normalized to individual cell sizes
792 (scale 15 μm). 300-400 ASM, in two replicate experiments were analyzed. (D) Images of wounded
793 monolayers of ASM stimulated with LIGHT for 12hr; left, fluorescence intensity of F-actin shown

(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 combined from 3 independent experiments. Data means ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 6. LIGHT-dependent non-canonical NF-κB signaling induces actin polymerization and ASM contractility. (A) Activation of canonical NF-κB (pp65) and non-canonical NF-κB (processing of p100 to p52) assessed in human ASM stimulated with LIGHT over 120 mins or 24hr. Data representative of 3 experiments. (B) Confocal analysis of ASM treated with LIGHT with or without NIK-SMI, an inhibitor of non-canonical NF-κB. Volume of phalloidin and vinculin normalized to cell size from triplicates (scale 10 μm). 300-400 ASM, in two replicate experiments were analyzed. (C) Gel contraction assay of LIGHT-stimulated ASM with or without inhibitors of canonical (BAY11-7082) and non-canonical (NIK-SMI) NF-κB. Percentage gel contraction compared to PBS in triplicates. Data representative of 2 experiments. Data means ± SEM. **P < 0.01, ***P < 0.001, ****P < 0.0001.

Figure 7. LIGHT induces non-canonical 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-E) Processing of p52, and pPAK1 (D) and pMLC2 (E) in ASM stimulated with LIGHT for 12hr, with or without inhibitors of canonical (BAY) and non-canonical NF-κB (NIK-SMI). (F) Active Rac1-GTP immunoprecipitated with PAK1 in ASM stimulated with LIGHT for 4hr with or without NIK-SMI. All data in A-F representative of 3 experiments. (G) Gel contraction of ASM stimulated with LIGHT, with or without an inhibitor of Rac1. Percentage gel contraction compared to PBS in triplicates. (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 two replicate experiments were analyzed. Data representative of 2 experiments. Data means ± SEM. **P < 0.01, ***P < 0.001, ****P < 0.0001.

Journal Pre-proof

Figure 1

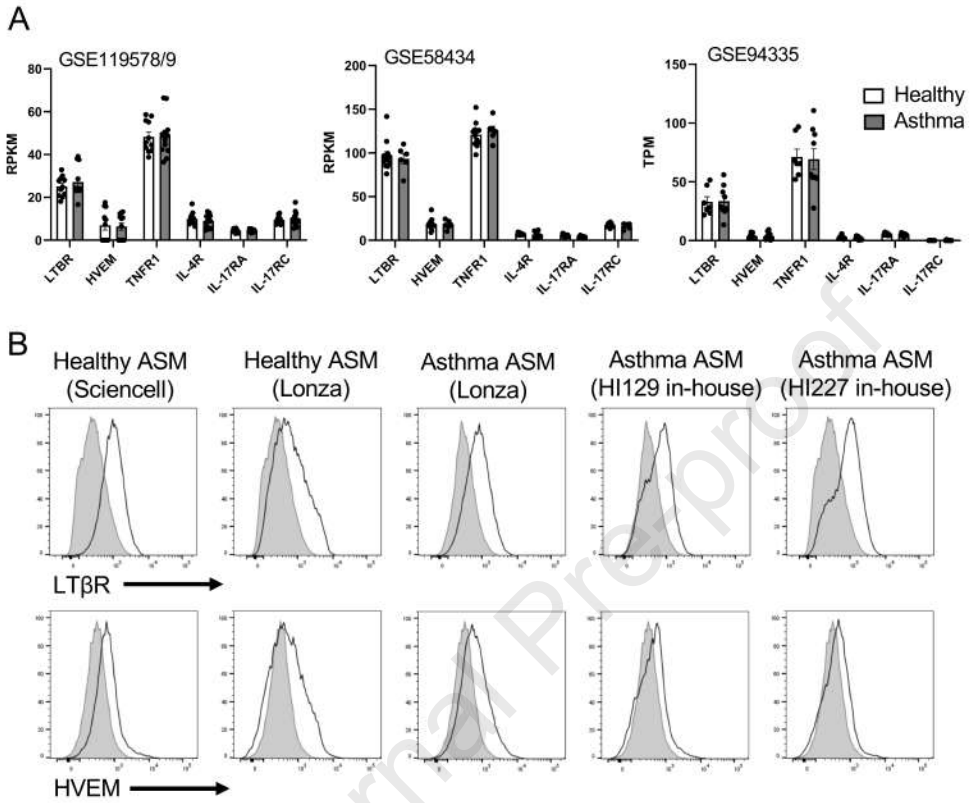

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Figure 2

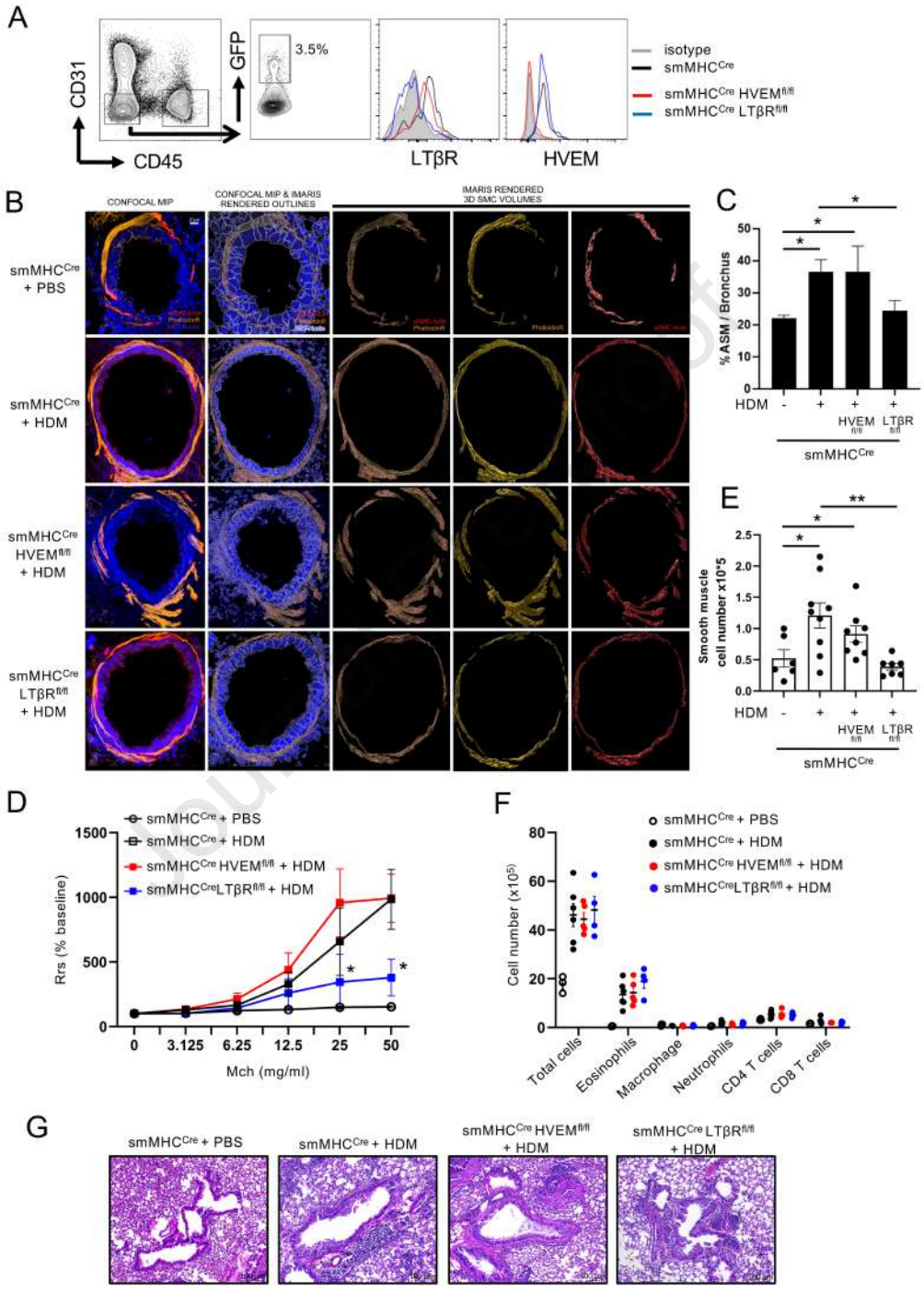
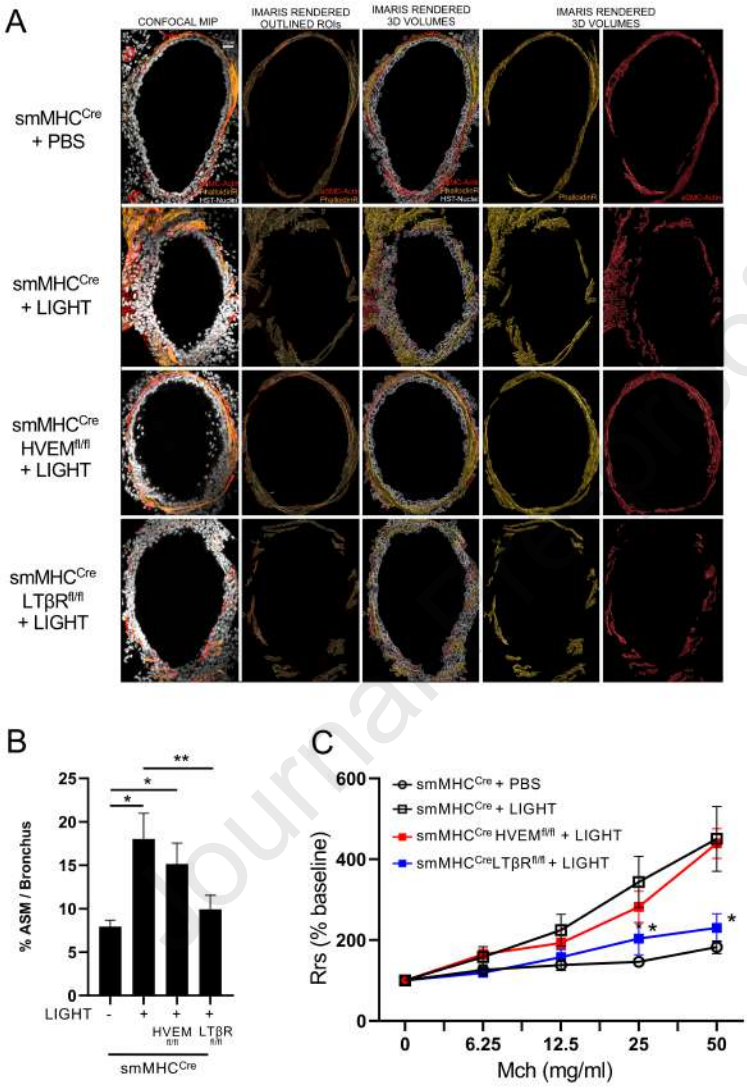

A
CD31
CD45
GFP
3.5%
LTβR
HVEM
isotype
smMHC^{Cre}
smMHC^{Cre} HVEM$^{fl/fl}$
smMHC^{Cre} LTβR$^{fl/fl}$
B
CONFOCAL MIP
CONFOCAL MIP & IMARIS RENDERED OUTLINES
IMARIS RENDERED 3D SMC VOLUMES
smMHC^{Cre} + PBS
smMHC^{Cre} + HDM
smMHC^{Cre} HVEM$^{fl/fl}$ + HDM
smMHC^{Cre} LTβR$^{fl/fl}$ + HDM
C
% ASM / Bronchus
HDM
HVEM$^{fl/fl}$
LTβR$^{fl/fl}$
smMHC^{Cre}
D
smMHC^{Cre} + PBS
smMHC^{Cre} + HDM
smMHC^{Cre} HVEM$^{fl/fl}$ + HDM
smMHC^{Cre}LTβR$^{fl/fl}$ + HDM
Rrs (% baseline)
Mch (mg/ml)
E
Smooth muscle cell number x10^5
F
Cell number (x10^5)
Total cells
Eosinophils
Macrophage
Neutrophils
CD4 T cells
CD8 T cells
G
smMHC^{Cre} + PBS
smMHC^{Cre} + HDM
smMHC^{Cre} HVEM$^{fl/fl}$ + HDM
smMHC^{Cre} LTβR$^{fl/fl}$ + HDM

Figure 3



Journal Pre-proof

Figure 4

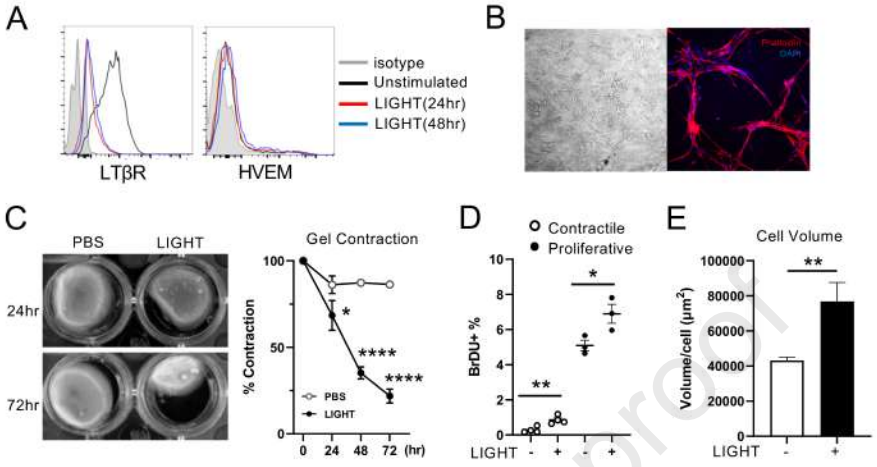


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Figure 5

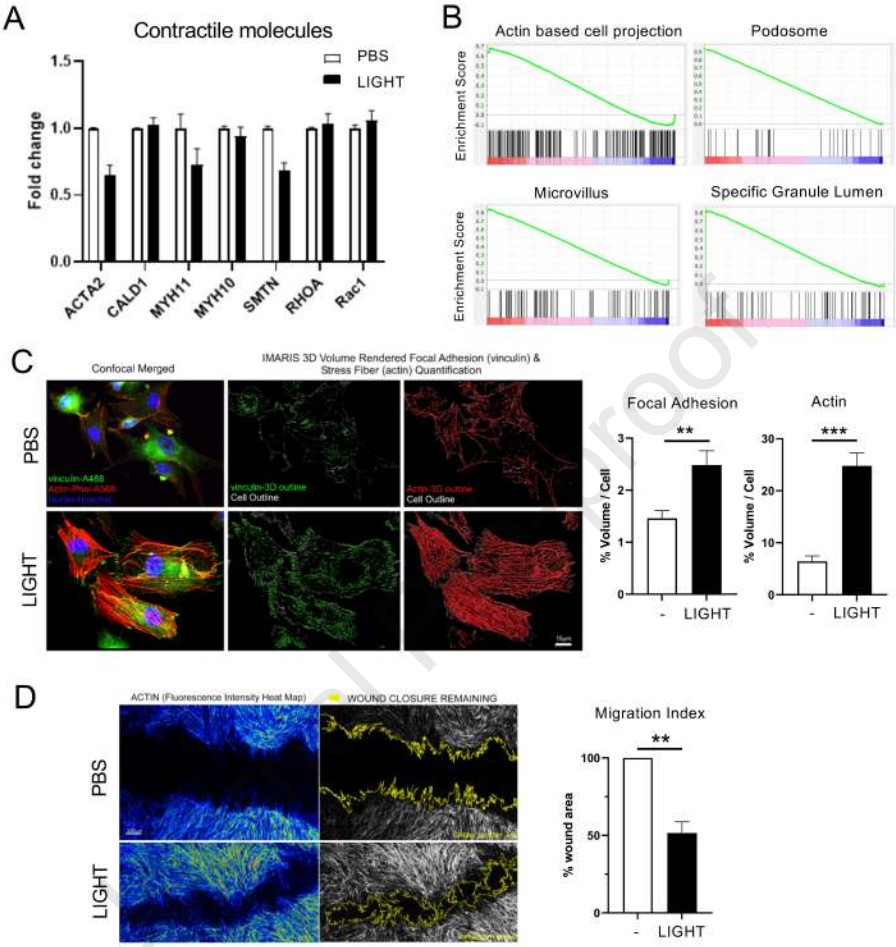
A
Contractile molecules
PBS
LIGHT
Fold change
1.5
1.0
0.5
0.0
ACTA2
CALD1
MYH11
MYH10
SMTN
RHOA
Rac1
B
Actin based cell projection
Podosome
Microvillus
Specific Granule Lumen
Enrichment Score
C
Confocal Merged
IMARIS 3D Volume Rendered Focal Adhesion (vinculin) &
Stress Fiber (actin) Quantification
PBS
LIGHT
Cell Outline
Focal Adhesion
Actin
% Volume / Cell
**
***
-
LIGHT
D
ACTIN (Fluorescence Intensity Heat Map)
WOUND CLOSURE REMAINING
PBS
LIGHT
Migration Index
% wound area
100
50
0

Figure 6

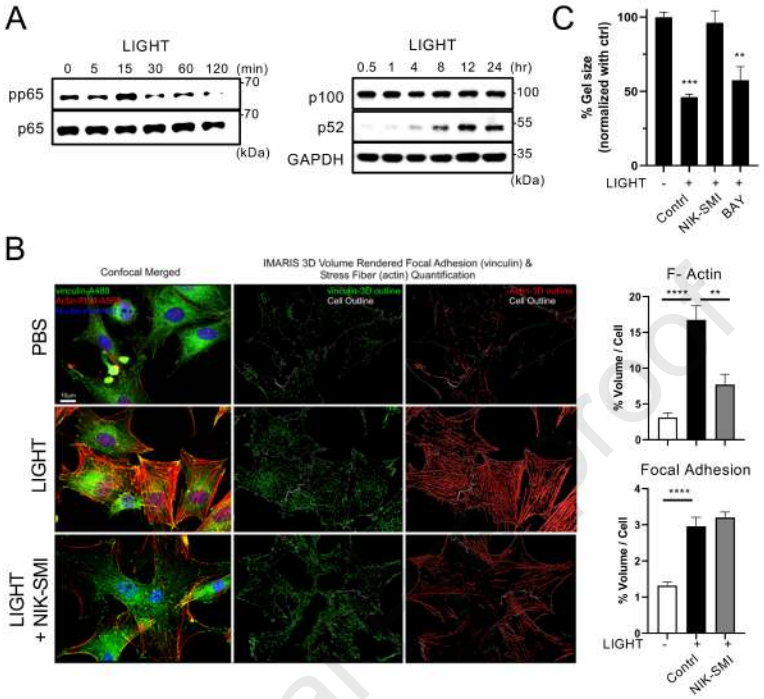

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Figure 7

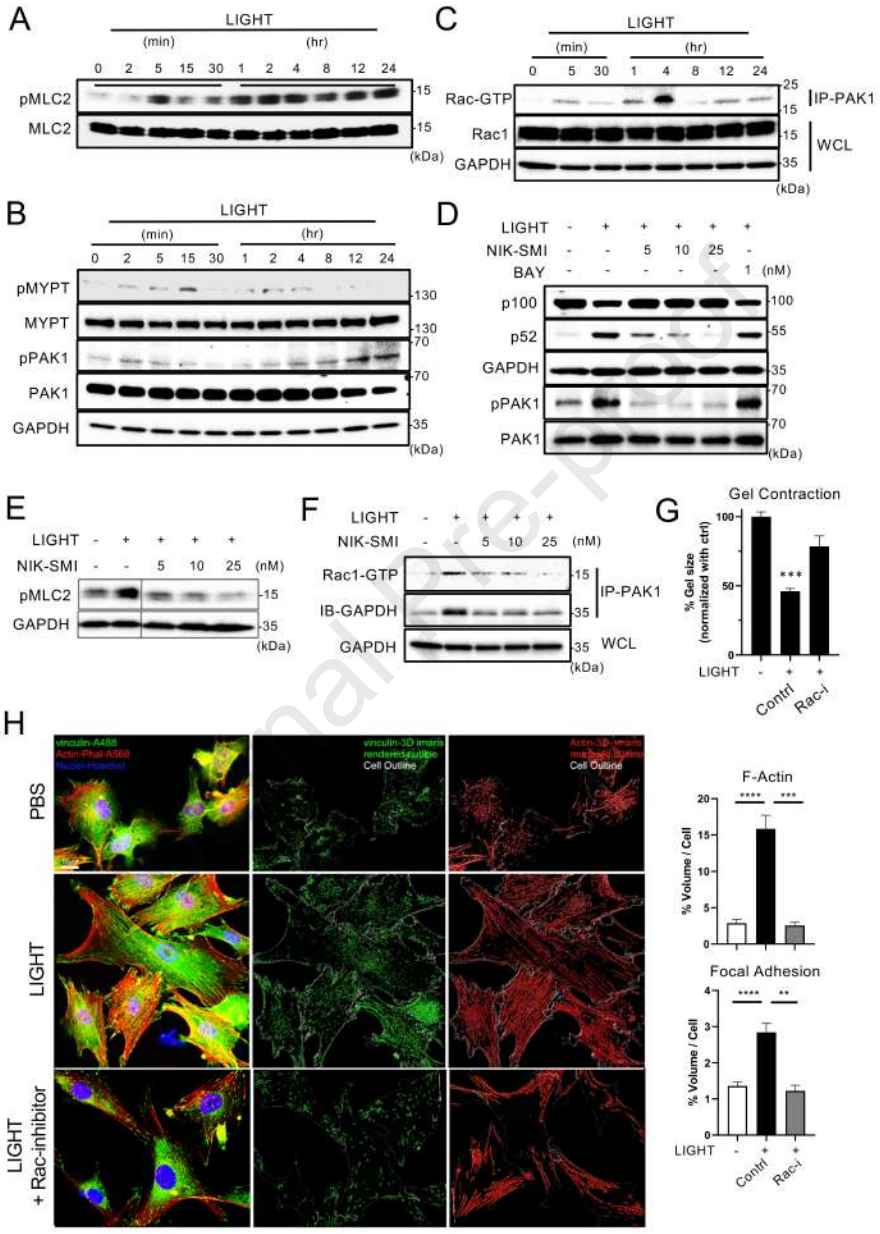
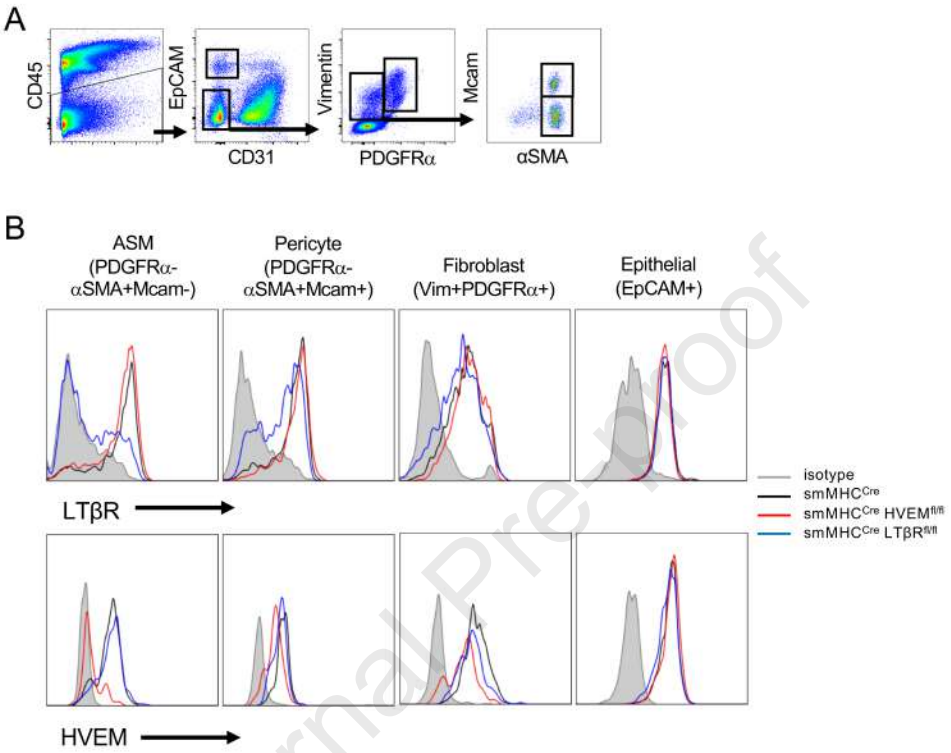

A
LIGHT
(min)
(hr)
0 2 5 15 30 1 2 4 8 12 24
pMLC2
MLC2
(kDa)
B
LIGHT
(min)
(hr)
0 2 5 15 30 1 2 4 8 12 24
pMYPT
MYPT
pPAK1
PAK1
GAPDH
(kDa)
C
LIGHT
(min)
(hr)
0 5 30 1 4 8 12 24
Rac-GTP
Rac1
GAPDH
IP-PAK1
WCL
(kDa)
D
LIGHT
NIK-SMI
BAY
(nM)
p100
p52
GAPDH
pPAK1
PAK1
(kDa)
E
LIGHT
NIK-SMI
5 10 25 (nM)
pMLC2
GAPDH
(kDa)
F
LIGHT
NIK-SMI
5 10 25 (nM)
Rac1-GTP
IB-GAPDH
GAPDH
IP-PAK1
WCL
(kDa)
G
Gel Contraction
% Gel size (normalized with ctrl)
LIGHT
Contrl
Rac-i
H
PBS
LIGHT
LIGHT + Rac-inhibitor
Cell Outline
F-Actin
% Volume / Cell
Focal Adhesion
% Volume / Cell
LIGHT
Contrl
Rac-i

Figure E1



Journal Pre-proof

Figure E2

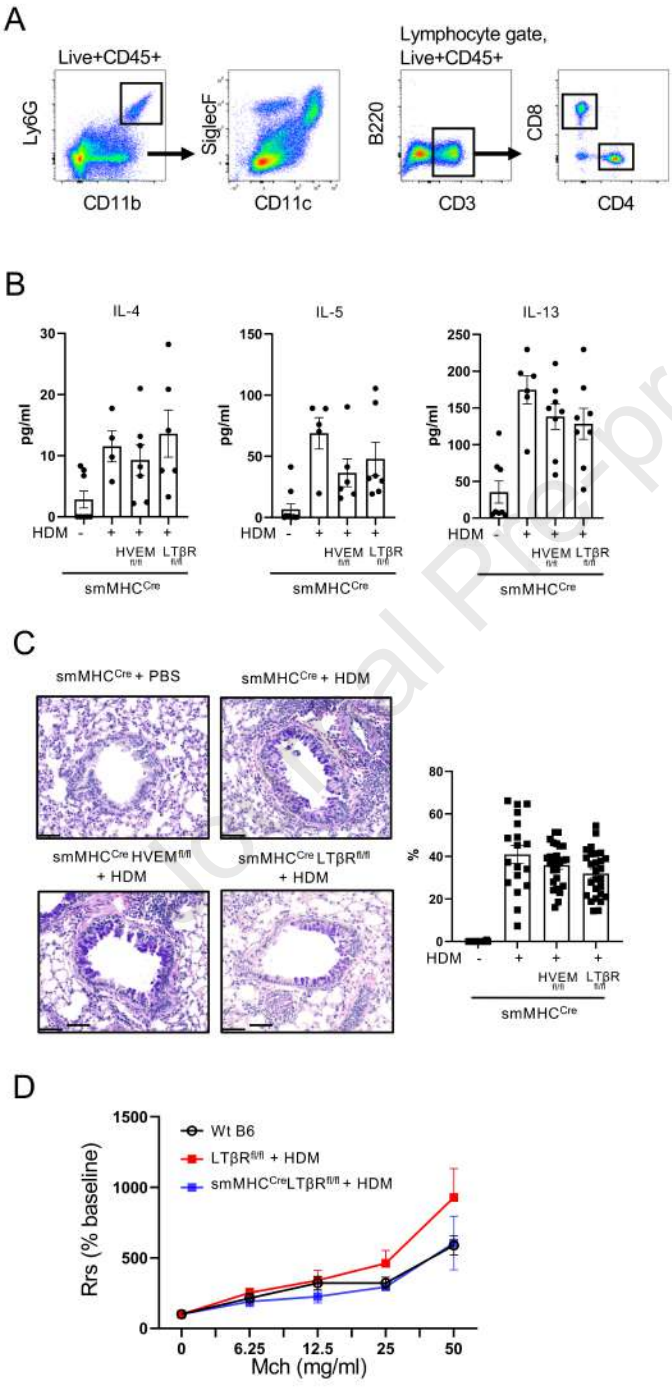
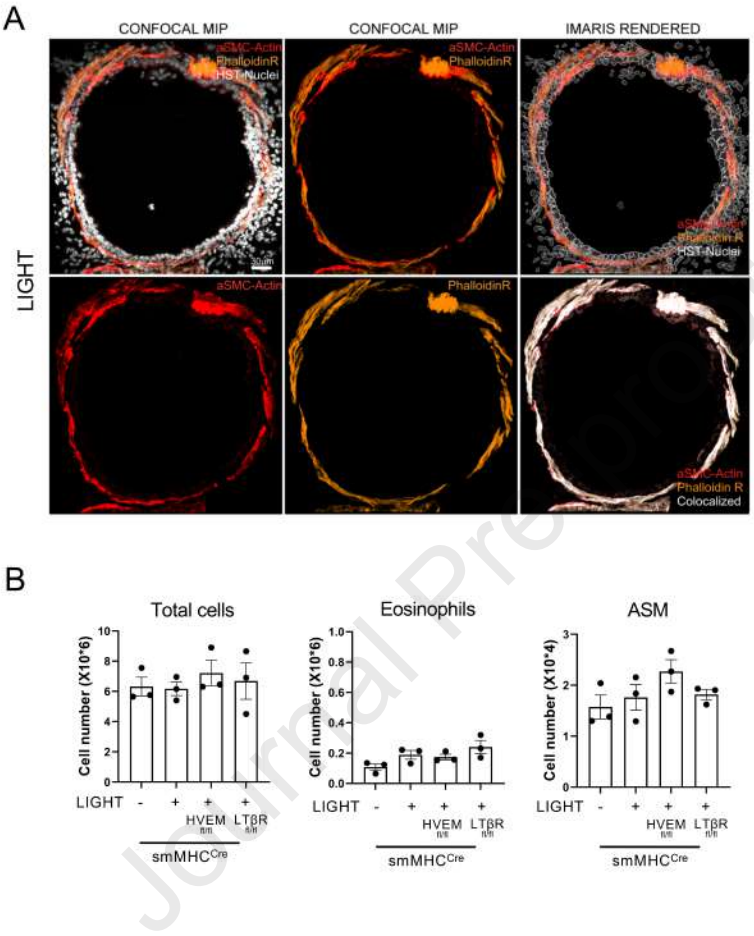
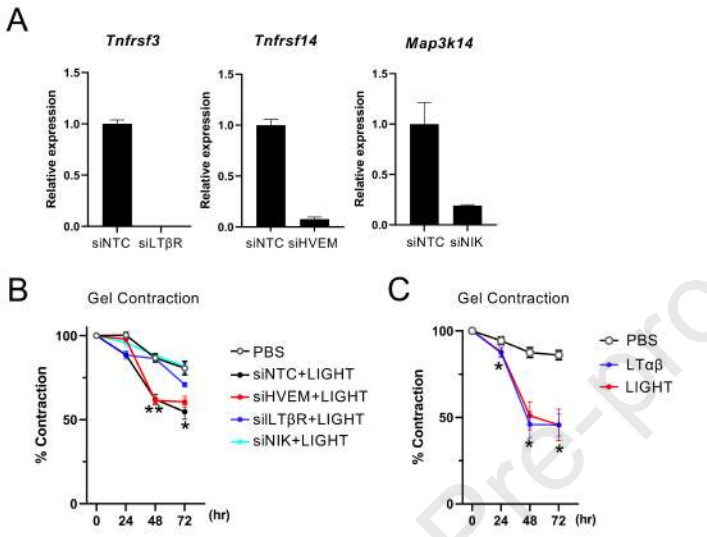

A
Live+CD45+
Ly6G
CD11b
SiglecF
CD11c
Lymphocyte gate,
Live+CD45+
B220
CD3
CD8
CD4
B
IL-4
IL-5
IL-13
pg/ml
HDM
HVEM fl/fl
LTβR fl/fl
smMHC Cre
C
smMHC Cre + PBS
smMHC Cre + HDM
smMHC Cre HVEM fl/fl + HDM
smMHC Cre LTβR fl/fl + HDM
%
D
Wt B6
LTβR fl/fl + HDM
smMHC Cre LTβR fl/fl + HDM
Rrs (% baseline)
Mch (mg/ml)

Figure E3



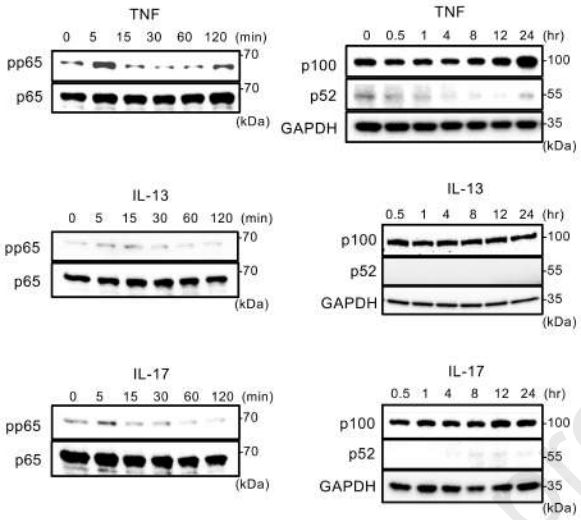
Journal Pre-proof

Figure E4



Journal Pre-proof

Figure E5



Journal Pre-proof

Supplementary Figure Legends

Figure E1. Flow cytometry 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 lungs. (B) Expression of HVEM and LTβR on gated lung structural cells from $smMHC^{Cre}$, $smMHC^{Cre}HVEM^{fl/fl}$, or $smMHC^{Cre}LT\beta R^{fl/fl}$ mice.

Figure 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/group). (C) Representative PAS stained lung sections (scale 100 μm) and percentages of PAS-positive bronchial epithelial cells. n =4-5 mice/group, 5 bronchi per mouse. Data representative of 3 experiments. (D) Airway resistance (AHR) after methacholine challenge, measured by Flexivent (n = 4-5 mice/group), in mice acutely challenged with HDM over 14 days. Data means ± SEM.

Figure 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 α-smooth muscle actin (red), scale 30 μm. (B) $smMHC^{Cre}$, $smMHC^{Cre}HVEM^{fl/fl}$, or $smMHC^{Cre}LT\beta R^{fl/fl}$ mice were treated intratracheally with LIGHT or PBS, and numbers of total cells, eosinophils, or ASM in lung tissues were analyzed by flow cytometry.

Figure 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 ± SEM from triplicate cultures. Data means ± SEM and representative of 2 experiments. *P < 0.05, **P < 0.01.

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

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