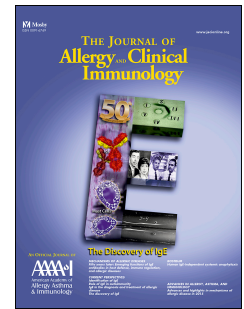


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LRP-1 Attenuates House Dust Mite-induced Eosinophilic Airway Inflammation by Suppressing Dendritic Cell-mediated Adaptive Immune Responses

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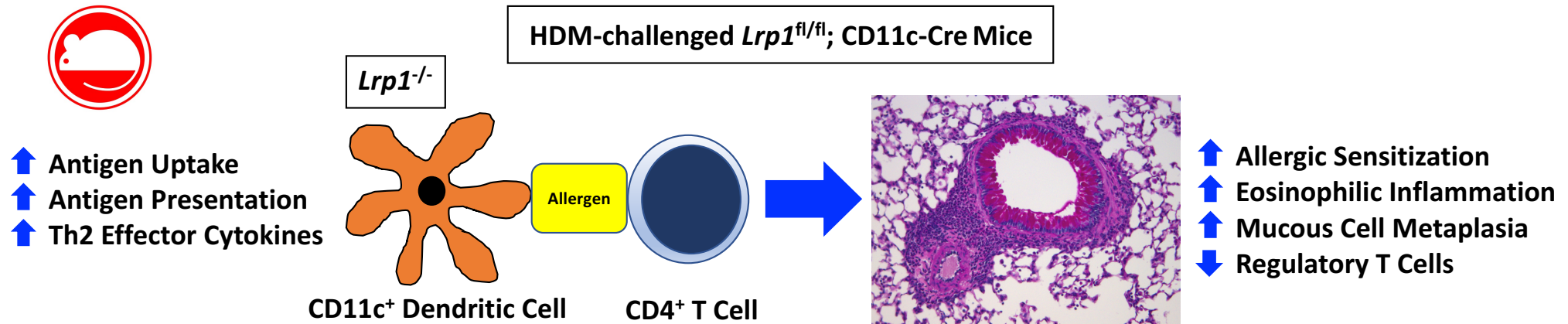
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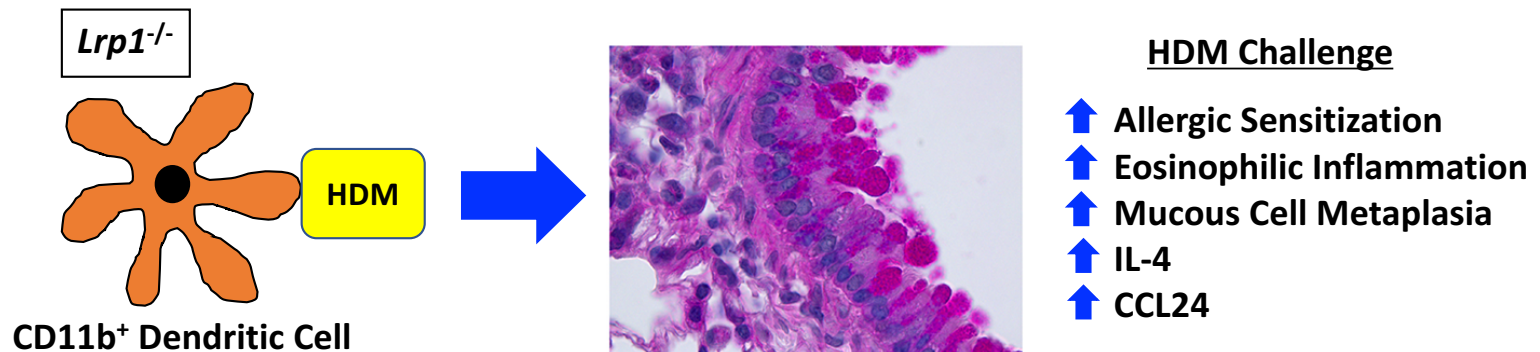
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Lrp-1 is a Negative Regulator of Dendritic Cell-mediated Adaptive Immune Responses in Allergic Airways Disease



Adoptive Transfer of HDM-pulsed CD11b⁺ Dendritic Cells from *Lrp1^{fl/fl}*; CD11c-Cre Mice to Naïve Wild Type Mice



Abbreviations: Lrp-1, LDL-receptor related protein 1; HDM, house dust mite

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Capsule summary: LRP-1 expression by myeloid dendritic cells is reduced in eosinophilic asthmatics and negatively regulates house dust mite-induced eosinophilic airway inflammation, Th2-mediated adaptive immune responses, and allergic sensitization in mice by modulating antigen uptake and presentation.

Key words: LDL-receptor related protein-1 (LRP-1); dendritic cells; eosinophilic airway inflammation; house dust mite; type 2-high asthma.

Abbreviations used: DCs (dendritic cells); MDC (myeloid dendritic cells); BMDC (bone marrow-derived dendritic cells); cDCs (conventional dendritic cells); moDCs (monocyte-derived dendritic cells); HDM (house dust mite); OVA (ovalbumin); BAL (bronchoalveolar lavage); MLN (mediastinal lymph node); AHR (airway hyperresponsiveness); TLR (Toll-like

receptor); Tregs (regulatory T cells); Foxp3 (Forkhead box protein 3); AF (Alexa Fluor); WT (wild-type)

Key messages:

- LRP-1 expression by circulating myeloid DC subsets from eosinophil-high asthmatics is reduced as compared to healthy, non-asthmatic subjects. In addition, LRP-1 expression by human peripheral blood myeloid DC subsets is negatively correlated with peripheral blood eosinophil counts in asthmatics.
- LRP-1 functions as a negative regulator of dendritic cell-mediated adaptive immune responses in house dust mite-induced eosinophilic airway inflammation in mice by modulating antigen uptake and presentation.

Abstract

Background: The LDL-receptor related protein 1 (LRP-1) is a scavenger receptor that regulates adaptive immunity and inflammation. LRP-1 is not known to modulate the pathogenesis of allergic asthma.

Objective: To assess whether LRP-1 expression by dendritic cells (DCs) modulates adaptive immune responses in house dust mite (HDM)-induced airways disease.

Methods: LRP-1 expression on peripheral blood DCs was quantified by flow cytometry. The role of LRP-1 in modulating HDM-induced airways disease was assessed in mice with a deletion of LRP-1 in CD11c⁺ cells (*Lrp1^{fl/fl}*; CD11c-Cre) and by the adoptive transfer of HDM-pulsed CD11b⁺ DCs from *Lrp1^{fl/fl}*; CD11c-Cre mice to wild-type mice.

Results: Human peripheral blood myeloid DC subsets from eosinophilic asthmatics have lower LRP-1 expression than cells from healthy, non-asthmatic subjects. Similarly, LRP-1 expression by CD11b⁺ lung DCs was significantly reduced in HDM-challenged wild-type mice. HDM-challenged *LRP-1^{fl/fl}*; CD11c-Cre mice have a phenotype of increased eosinophilic airway inflammation, allergic sensitization, Th2 cytokine production, and mucous cell metaplasia. The adoptive transfer of HDM-pulsed LRP-1-deficient CD11b⁺ DCs into wild-type mice generated a similar phenotype of enhanced eosinophilic inflammation and allergic sensitization. Furthermore, CD11b⁺ DCs in the lungs of *Lrp1^{fl/fl}*; CD11c-Cre mice have an increased ability to take up HDM antigen, whereas bone marrow-derived DCs display enhanced antigen presentation capabilities.

Conclusion: This identifies a novel role for LRP-1 as a negative regulator of DC-mediated adaptive immune responses in HDM-induced eosinophilic airway inflammation. Furthermore, the reduced LRP-1 expression by circulating myeloid DCs from eosinophilic asthmatics suggests a possible role for LRP-1 in modulating type 2-high asthma.

Introduction

The low-density lipoprotein receptor-related protein-1 (LRP-1), is a ubiquitously expressed, multifunctional, scavenger-type endocytic receptor that binds a broad spectrum of more than 40 structurally unrelated ligands, including lipoproteins (e.g., apolipoprotein E-containing chylomicrons and VLDL remnants), proteases (e.g., urokinase plasminogen activator (uPA) and tissue-type plasminogen activator (tPA)), protease-inhibitor complexes (i.e., uPA and tPA complexes with plasminogen activator inhibitor (PAI-1) and activated α -2-macroglobulin), extracellular matrix proteins (e.g., thrombospondins-1 and -2), growth factors (i.e., platelet-derived growth factor and transforming growth factor- β), bacterial toxins (e.g., *Pseudomonas* exotoxin A), viruses (e.g., Rhinovirus), and amyloid β (A β) peptides¹⁻⁷. LRP-1, is one of the largest members of the low-density lipoprotein (LDL)- receptor gene family that also includes the apolipoprotein E (ApoE) receptor 2, the very low-density lipoprotein (VLDL) receptor, and megalin. LRP-1 is derived from a 600-kDa precursor protein to yield a 515 kDa α -chain and an 85 kDa β -chain that are associated in a non-covalent fashion on the cell surface. Furthermore, LRP-1 is expressed by multiple cell types, including dendritic cells, macrophages, vascular endothelial cells, neurons, Schwann cells, hepatocytes, and fibroblasts^{6, 8}. This broad spectrum of cellular expression allows LRP-1 to participate in multiple cellular processes, such as tissue homeostasis, intracellular signaling, cell survival, trafficking, proliferation and (trans)-differentiation⁹⁻¹².

Besides its canonical role in endocytosis, LRP-1 also participates in the regulation of inflammation and adaptive immune responses¹³⁻¹⁵. For example, the macrophage-specific deletion of LRP-1 decreases VLDL uptake and thereby promotes atherogenesis in hypercholesterolemic mice by increasing matrix metalloproteinase 9 activity and the production of pro-inflammatory mediators, such as TNF- α and CCL2 (MCP-1)¹³. *Lrp1*^{-/-} mice display a phenotype of enhanced ligand-specific inflammatory responses that are regulated by NF- κ B and miR-155 in macrophages⁷. Furthermore, the mechanism by which LRP-1 suppresses NF- κ B signaling may involve the down-regulation of signaling by TNF receptor-1¹⁶. Consistent with an anti-inflammatory function in macrophages, LRP-1 has been demonstrated to promote a M2 phenotype¹⁷. LRP-1 has also recently been shown to attenuate neuroinflammation and

microglial immune responses by suppressing signaling via c-Jun N-terminal kinase and NF- κ B pathways¹⁸.

LRP-1 modulates host defense and anti-tumor immune responses by facilitating antigen uptake and presentation. For example, an important function of LRP-1 is the binding, internalization, and clearance of apoptotic cells via the formation of a multimeric protein complex comprised of the scaffolding protein, RAN-binding protein 9 (RANBP9), and the receptor tyrosine kinase, AXL¹⁹. This multimeric protein complex facilitates efferocytosis by dendritic cells (DCs) and the subsequent cross-presentation of viral antigens expressed by apoptotic cells to CD8⁺ T cells, which promotes host defense against viral infections. Similarly, binding of bacterial or tumor cell-derived heat shock proteins to LRP-1, which is also known as CD91, facilitates the high efficiency uptake of exogenous antigenic peptides by antigen presenting cells for chaperone-based presentation that primes effector CD4⁺ and CD8⁺ T cells in the setting of infection or malignancy²⁰⁻²². Interestingly, LRP-1 also modulates pro-inflammatory cytokine production and the initiation of inflammatory responses in LPS-induced acute lung injury by a calreticulin axis that binds surfactant proteins, SP-A and SP-D, to activate downstream p38 and NF- κ B signaling pathways²³.

Previously, we identified an endogenous apoE-LDL receptor pathway in the lung that regulates airway hyperreactivity and mucous cell metaplasia in house dust mite (HDM)-induced allergic asthma²⁴. Moreover, we demonstrated that the VLDL receptor on dendritic cells (DCs) acts as a negative regulator and thereby attenuates HDM-induced airway inflammation and adaptive immune responses²⁵. Here, we hypothesized that another LDL receptor family member, LRP-1, might similarly regulate DC function and thereby modify adaptive immune responses to HDM, which is a common and clinically relevant aeroallergen. We show that the cell-specific deletion of LRP-1 on CD11c⁺ cells enhances HDM-induced allergic sensitization, adaptive Th2 immune responses, and eosinophilic airway inflammation via a mechanism that involves increased antigen uptake and presentation by DCs. In addition, we demonstrate that circulating myeloid DC subsets from human subjects with eosinophilic asthma have decreased LRP-1 expression as compared to healthy, non-asthmatic subjects. We also show that LRP-1 expression by circulating myeloid DC subsets is negatively correlated with peripheral blood eosinophil counts in asthmatics, which suggests a possible role for LRP-1 in modulating adaptive immune responses in humans. Therefore, we propose

156 that LRP-1 functions as a negative regulator of DC-mediated adaptive immune responses in
157 HDM-induced allergic eosinophilic airway inflammation.

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Methods

Quantification of LRP-1 expression on Peripheral Blood DCs. Informed consent was obtained from 38 subjects who participated in protocol 99-H-0076, which was approved by the Institutional Review Board of the National Heart, Lung, and Blood Institute (NHLBI). All asthmatics had evidence of either a positive response to an inhaled β 2-agonist or a positive methacholine bronchoprovocation challenge test, whereas all healthy, non-asthmatic subjects had a negative methacholine bronchoprovocation challenge test. The presence or absence of allergy was determined by skin testing, which was performed either at NHLBI or by their local physician, or by patient history. Allergy skin testing at NHLBI was performed using a Multi-Test II applicator, which was a generous gift from Lincoln Diagnostics (Decatur, IL) with glycerin as a negative control and histamine as a positive control (Holister-Stier, Spokane, WA). Allergy was defined by the presence of skin test reactivity to at least one of six common aeroallergens, which included house dust mite (*Dermatophagoides farinae*), cockroaches (*Periplaneta americana* and *Battella germanica*), cat hair, *Aspergillus fumigatus*, Ragweed (giant and short) and grasses (Kentucky Bluegrass, Orchard Redtop, Timothy Sweet Vernal grass, Meadow Fescue, and Perennial Ryegrass).

Peripheral blood was collected in 10-ml sodium heparinized Vacutainers (Becton Dickinson, Franklin Lakes, NJ) and red blood cells (RBCs) were lysed using ACK lysing buffer. Peripheral blood cells were reacted with the following antibodies for flow cytometry: anti-human CD11c-BV785 (clone 3.9); anti-human CD1c-PE/Dazzle 594 (clone L161); anti-human CD141-PECy7 (clone M80); anti-human CD16-PerCP/Cy5.5 (clone 3G8); anti-human CD123-BV650 (clone 6H6); anti-human HLA-DR- APC Cy7 (clone W6/32); anti-human Lin 1-FITC all from BioLegend (San Diego, CA) and AF647-conjugated rabbit monoclonal antibody to LRP-1 (ab92544) from Abcam (Cambridge, UK). Cells were incubated for 15 mins with human Fc γ R-binding inhibitor (ThermoFisher Scientific, Waltham, MA), reacted with antibodies, and washed with PBS. The fixable viability dye was then added, followed by fixation and permeabilization. Cellular debris and doublets were excluded using physical scatter properties of RBC-lysed whole blood cells and dead cells were excluded using fixable live-dead exclusion dye. Myeloid-DCs type I (MDC1) were identified as viable/Lin-1⁻ cells expressing CD11c⁺/CD1c⁺/CD141⁻

/HLA-DR⁺, whereas myeloid-DCs type II (MDC2) were CD11c⁺/CD1c⁻/CD141⁺/HLA-DR⁺, while CD16⁺ DCs were identified as CD11c⁺/CD16⁺ cells and plasmacytoid DCs were CD11c⁻/CD123⁺ using Fluorescence Minus One (FMO) as a control²⁶.

Mice. Experimental protocols were approved by the Animal Care and Use Committee of the National Heart, Lung, and Blood Institute, Bethesda, MD. *Lrp1*^{fl/fl} (B6;129S7-*Lrp1*^{tm2Her}/J) and CD11c-Cre (C57BL/6J-Tg(ltgax-cre,-EGFP)4097Ach/J) mice were purchased from The Jackson Laboratories (Bar Harbor, ME), and crossed to generate homozygous *Lrp1*^{fl/fl}; CD11c (Cre) mice. The cell-specific deletion of *Lrp1* from CD11c⁺ dendritic cells was confirmed by PCR of genomic DNA and Western blotting of cellular proteins. PCR was performed on genomic DNA from SSC^{lo}/SiglecF⁻/CD11c⁺/CD11b⁺/MHCII^{hi} mouse lung DCs and SSC^{lo}/SiglecF⁻/CD11c⁻/CD11b⁻/EpCAM^{hi}/MHCII^{lo} lung epithelial cells (LECs), as well as SSC^{lo}/CD11c⁺/CD11b⁺/MHCII^{hi} splenic DCs and CD4⁺ T cells, which had been purified by flow cytometry, using the following PCR primers that detected the recombined *Lrp1* floxed allele: primer rec1, 5'-GGT GTG ACA TAG AGT TTT AAA GAG G-3'; primer rec2, 5'-GCA AGC TCT CCT GCT CAG ACC TGG A-3'. Bone marrow-derived DCs (BMDCs) and murine lungs were lysed in RIPA buffer and 100 µg of protein was separated by SDS-PAGE using 4-20% Tris-Glycine gels (Invitrogen) and transferred to nitrocellulose membranes (GE healthcare Life sciences, PA). Membranes were reacted with anti-LRP-1 (1:1000) and β-actin antibodies (Abcam). Blots were stripped using Restore Western Blot Stripping Buffer (Thermo Scientific).

HDM sensitization and challenge models. *Lrp1*^{fl/fl} and *Lrp1*^{fl/fl}; CD11c (Cre) mice received two intraperitoneal injections of HDM (100 µg) (*Dermatophagoides pteronyssinus* extract, Greer Laboratories, Lenoir, NC) with aluminum hydroxide (40 mg ml⁻¹, Invivogen, San Diego, CA) on day 4 and day 8 followed by intranasal administration of HDM (50 µg) on days 8, 10, 12, 15 before harvest on day 17. For the adoptive transfer model, bone marrow cells from *Lrp1*^{fl/fl} and *Lrp1*^{fl/fl}; CD11c (Cre) mice were cultured at a density of 2 x 10⁶ cells ml⁻¹ in Iscove's Modified Dulbecco's medium (Gibco/Life Technologies, Carlsbad, CA) supplemented with 10% heat-inactivated FCS, penicillin (100 U ml⁻¹), streptomycin (100 µg ml⁻¹), L-glutamine (2 mM), 2-mercaptoethanol (50 µM), and recombinant mouse GM-CSF (20 ng ml⁻¹; BioLegend, San Diego, CA). An equal volume of medium was added on day 3 and 50% of the medium was

replaced on day 6. On day 8, floating non-adherent BMDCs were pulsed with HDM (100 $\mu\text{g ml}^{-1}$) or sham-pulsed with saline and flow sorted, viable CD11c⁺/CD11b⁺/MHCII^{hi} BMDCs (0.05 $\times 10^6$) were adoptively transferred in 20 μl of PBS via intranasal administration on day 0 to recipient naïve C57BL/6 wild-type mice. All recipient mice received daily intranasal HDM challenges (50 μg) on day 9, 11, 13, 15 and endpoints were analyzed on day 15. Two independent experiments were performed for each model.

BAL and Lung Histopathology. Bronchoalveolar lavage (BAL) was performed three times with 0.5 ml PBS and red blood cells were lysed with ACK buffer for 2 min at 4°C, as previously described²⁷. BALF cells were re-suspended in 0.3 ml RPMI-1640 containing 10% FBS and cell counts were performed using a hemocytometer, while differential cell counts were performed on Diff-Quik-stained cytospin slides (Siemens, Deerfield, Illinois). Lungs were inflated with 10% formalin to a pressure of 25 cm H₂O, fixed in 10% formalin for 24 h, dehydrated through gradient ethanol, and embedded in paraffin. Sagittal sections were cut at a thickness of 5 μm and stained with hematoxylin and eosin and periodic acid Schiff (PAS). Quantification of mucous cell metaplasia was performed as previously described. All the airways present (large (conducting), medium (central), and small (distal)) within a representative lung section were inspected. The number of airways that contained PAS-positive cells was counted and mucous cell metaplasia is reported as the percentage of airways that contained PAS-positive cells, as previously described²⁴.

Quantification of HDM-specific IgE and IgG1. Levels of HDM-specific serum IgE and IgG1 were quantified, as previously described²⁷. 96 well plates were coated overnight with 0.01% HDM in PBS and blocked with 1% BSA in PBS. Serum samples were added to the plates for 1 hr, washed 6X with PBS with 0.05% Tween-20 prior to incubation with biotinylated anti-mouse IgE or IgG1 (Pharmingen, San Jose, CA) for 1 hr. Plates were washed 6 additional times and streptavidin-HRP (R&D Systems, Minneapolis, MN) was added for 30 min and the amount of bound HDM-specific antibody was determined by the addition of TMB substrate.

Measurement of Airway Hyperresponsiveness. Mice were anesthetized and airway resistance to different doses of methacholine was measured using an Elan RC Fine Pointe

system (Buxco, Wilmington, NC), as previously described²⁴. Mice were mechanically ventilated following tracheal cannulation with a 19-gauge beveled metal catheter, with a constant inspiratory flow before nebulization with increasing doses of methacholine (0-10 mg/ml). Airway resistance was recorded at 10-seconds interval for 3 mins and average values are represented as cm-H₂O/ml/s.

House dust mite re-stimulation of mediastinal lymph node cells. Single cell suspensions from both right and left mediastinal lymph nodes (MLN) of mice were generated. MLN cells (0.2×10^6 /ml) were cultured in round-bottom 96-well plates and re-stimulated with HDM (100 µg/ml) for 72 hours at 37°C in RPMI medium with 10 % FBS. The concentrations of IL-4, IL-5, IL-13, or IFN-γ in the supernatants were measured using sandwich ELISA kits with limits of sensitivity of 15.6 pg/ml for IL-4, 31.25 pg/ml for IL-5 and IFN-γ, and 62.5 pg/ml for IL-13 (R & D Systems, Minneapolis, MN).

Lung dendritic cell migration assay. HDM was labeled with AF-647 protein labeling kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Mice received intratracheal administration of AF647-HDM (50 µg per administration of sensitization) dissolved in 80 µl of PBS using a 24-gauge sterile polyurethane catheter connected to the outlet of a micropipette, as previously described²⁷. At 72 hours after injection, migrating DCs were quantified in digested lungs and MLNs by flow cytometry as CD11c⁺/MHCII^{hi}/AF-647-HDM⁺ cells.

Flow cytometry. Lungs cells were isolated by enzymatic digestion with liberase (100 µg/ml, Roche, Indianapolis, IN) and DNase I (0.2 mg/ml; DN25, Sigma, St. Louis, MO) (total volume 1-2 ml/lung) and incubated at 37°C for 25 min with agitation. Cells from digested lungs were incubated with Fc BlockTM (BD Biosciences, San Jose, CA) to react with Fcγ III/II receptors before surface staining. Mouse DC subsets were identified and characterized by using anti-mouse Siglec F-PE-CF594, CD11c-APC Cy7, MHCII-PE Cy7, CD11b-PerCP Cy5.5, CD103-APC, PDCA1-AF488, Mar-1-eFluor450, CD64-PE, TLR-4-APC, Dectin-1-PE, CD205-PerCPCy5.5, and CD206-BV785 antibodies, all from BioLegend (San Diego, CA); CD80-PECy5, CD86-605NC, and DC-SIGN-eFluor660 all from ThermoFisher Scientific, Waltham,

MA); Dectin-2-AF647 (BioRad, Hercules, CA); and TLR 5-PE (Novus, Littleton, CO). CD4⁺ regulatory T cells (Treg) were identified using APC-conjugated anti-CD3, BV650-conjugated anti-CD4, and PE-Cy7-conjugated anti-CD25, all from ThermoFisher Scientific. For intracellular Foxp3 staining, cells were fixed and permeabilized with Foxp3 staining buffer (ThermoFisher Scientific), then reacted with a PE-conjugated anti-mouse Foxp3 antibody (0.5 µg per 10⁶ cells) (Clone NRRF-30, ThermoFisher Scientific). Data were acquired on a LSRII (BD Biosciences, USA) equipped with 407, 488, 532, and 633 LASER lines using DIVA 8 software and analyzed with the Flow Jo software version 9.9.5 (Treestar, San Carlos, CA). Cellular debris was excluded using a forward light scatter/side scatter plot.

OT-II co-culture. The ability of DCs to induce *ex vivo* antigen-specific T-cell proliferation was assessed using CFSE-labeled splenic CD4 T cells from naïve (DO11.10 TCR (C.Cg-Tg(DO11.10)10Dlo/J) transgenic mice (Jackson Laboratories, Bar Harbor, ME) that express a transgenic MHCII-restricted TCR that recognizes the OVA peptide antigen²⁷. BMDCs from *Lrp1^{fl/fl}* and *Lrp1^{fl/fl}*; CD11c (Cre) mice were pulsed overnight with 1 µg ml⁻¹ of OVA 323-339 peptide (AnaSpec, Fremont, CA, USA) or PBS as a control and viable CD11c⁺ cells were isolated by flow sorting using an anti-mouse CD11c-APC-Cy7 antibody (BD Biosciences). Naïve CD4⁺ T cells were purified from the spleens of DO11.10 TCR mice by flow sorting using an anti-mouse CD4-BV 650 antibody (ThermoFisher Scientific) and were labelled with 5 µM CFDA-SE (carboxyfluoresceine diacetate succinimidyl ester; Cayman Chemical, MI), for 15 min. 1 x 10⁵ OVA peptide-specific CD4⁺ OTII cells were co-cultured with 2 x 10⁴ CD11c⁺ BMDCs in 96-well plates for 4 days and T cell proliferation was quantified by flow cytometry using CFSE dye dilution. CD4⁺-gated cells were analyzed using proliferation profile, percent divided, and division index as calculated by the FlowJo Proliferation Platform²⁸.

Statistics. Data were analyzed using Graph Pad Prism version 7.0b using either an unpaired t test for normally distributed data or a Mann-Whitney test for non-normally distributed data. Multiple comparisons were analyzed using a one-way ANOVA with a Sidak's multiple comparison test or a two-way ANOVA. Correlation analyses between LRP-1 expression and peripheral blood eosinophil counts were performed by Spearman correlation. Data are presented as mean ± SEM. A *P* value < 0.05 was considered significant.

Results

LRP-1 Expression is Decreased on Peripheral Blood Myeloid Dendritic Cells from Eosinophilic Asthmatics

Elevated peripheral blood eosinophils are a biomarker of type 2-high asthma²⁹⁻³². Here, we hypothesized that LRP-1 expression by peripheral blood DCs might be modified in asthmatics with high peripheral blood eosinophils counts (> 260 cells/ μ l) as compared to asthmatics with low peripheral blood eosinophils counts (< 260 cells/ μ l) or healthy, non-asthmatics who also had low peripheral blood eosinophil counts (< 260 cells/ μ l) (Figure 1A)³³⁻³⁵. Demographic information regarding the high and low eosinophil groups are presented in Repository eTable 1. LRP-1 expression was quantified by flow cytometry on four specific peripheral blood DCs subsets; Lin⁻/HLA-DR⁺/CD11c⁺/CD1c⁺/CD141⁻ myeloid DC type I (MDC1), Lin⁻/HLA-DR⁺/CD11c⁺/CD1c⁻/CD141⁺ myeloid DC type II (MDC2), Lin⁻/HLA-DR⁺/CD16⁺/CD11c⁺ (CD16⁺ DCs) or Lin⁻/HLA-DR⁺/CD11c⁺/CD123⁺ (plasmacytoid DCs, pDCs) (Repository eFigure 1). As shown in Figure 1B, LRP-1 expression by the MDC1, MDC2, and CD16⁺ myeloid DC subsets was significantly decreased in the eosinophil-high asthmatic group as compared to the healthy, non-asthmatic group, whereas there was no difference between the groups regarding LRP-1 expression by plasmacytoid DCs. Furthermore, when all eosinophil-high and eosinophil-low asthmatic subjects were combined into a single cohort, there were significant negative correlations between peripheral blood eosinophil counts and LRP-1 expression by the MDC1 (Spearman $\rho = -0.46$, $p = 0.019$), MDC2 (Spearman $\rho = -0.51$, $p = 0.008$), and CD16⁺ (Spearman $\rho = -0.39$, $p = 0.047$) myeloid DC subsets, whereas there was a trend for a negative correlation among plasmacytoid DCs (Spearman $\rho = -0.37$, $p = 0.06$). Collectively, these results show that myeloid DC subsets from eosinophil-high asthmatics have lower levels of LRP-1 expression than healthy, non-asthmatic subjects and that a negative correlation exists between LRP-1 expression by myeloid DC subsets and peripheral blood eosinophil counts in asthmatics.

Next, we used a murine model to assess whether sensitization and challenge with HDM modifies the cell surface expression of LRP-1 on CD11c⁺/CD11b⁺ lung DCs. As shown in

Figure 1C, LRP-1 is expressed on both CD11b⁺/CD11c⁺/Siglec F⁻/MHC-II^{hi}/Mar-1⁻/CD64⁻ conventional myeloid DCs (cDC) and CD11b⁺/CD11c⁺/Siglec F⁻/MHC-II^{hi}/Mar-1⁺/CD64⁺ monocyte-derived DCs (moDC) in the lungs of saline-challenged mice³⁶. Furthermore, following sensitization and challenge with HDM, the percentage of both LRP-1⁺ cDCs and moDCs in the lung were significantly reduced.

The CD11c-specific deletion of LRP-1 Augments HDM-induced Eosinophilic Airway Inflammation

Having shown that both human and murine DCs express LRP-1, experiments were next conducted to assess the role of LRP-1 on CD11c⁺ cells in initiating HDM-induced allergic sensitization, Th2 immune responses, and eosinophilic airway inflammation. To address this question, we created a *Lrp1*^{fl/fl}; *CD11c*-Cre mouse, in which LRP-1 is specifically deleted in CD11c⁺ cells, which include both DCs and alveolar macrophages (Repository eFigure 2). *Lrp1*^{fl/fl}; *CD11c*-Cre mice were sensitized by intraperitoneal injection of HDM plus aluminum hydroxide, followed by multiple intranasal HDM challenges (Figure 2A). As shown in Figure 2B, the total number of BALF inflammatory cells recovered from HDM-challenged *Lrp1*^{fl/fl}; *CD11c*-Cre mice were significantly increased as compared to control *Lrp1*^{fl/fl} mice, which represented increases in eosinophils, alveolar macrophages, and lymphocytes. Similarly, lung histopathology showed an increase in the extent of peribronchial inflammatory cell infiltrates (Figure 2C) and mucous cell metaplasia (Figure 2C and 2D) in HDM-challenged *Lrp1*^{fl/fl}; *CD11c*-Cre mice as compared to *Lrp1*^{fl/fl} mice. In contrast, there was no significant difference in airway hyperresponsiveness between HDM-exposed *Lrp1*^{fl/fl}; *CD11c*-Cre and *Lrp1*^{fl/fl} mice (Figure 3A). Re-stimulation of mediastinal lymph node (MLN) cells from *Lrp1*^{fl/fl}; *CD11c*-Cre mice with HDM showed significant increases in the production of the Th2 cytokines, IL-4, IL-5 and IL-13, whereas there was no change in interferon- γ (Figure 3B). Flow cytometry demonstrated a significant reduction in the percentage of CD3⁺/CD4⁺/CD25⁺/Foxp3⁺ regulatory T cells (Tregs) in the lungs of HDM-challenged *Lrp1*^{fl/fl}; *CD11c*-Cre mice as compared to *Lrp1*^{fl/fl} mice (Figure 3C). Lastly, HDM-challenged *Lrp1*^{fl/fl}; *CD11c*-Cre mice had increases in HDM-specific serum IgE and IgG1 levels (Figure 3D) in as compared with *Lrp1*^{fl/fl} mice, which is indicative of augmented allergic sensitization. Collectively, these data demonstrate that HDM-

challenged *Lrp1^{fl/fl}*; *CD11c*-Cre mice have a phenotype of increased allergic sensitization, Th2-mediated adaptive immune responses, mucous cell metaplasia, and eosinophilic airway inflammation in response to HDM that was associated with a reduction in lung Tregs.

To confirm that the phenotype in HDM-challenged *Lrp1^{fl/fl}*; *CD11c*-Cre mice of augmented eosinophilic inflammation in the absence of modified AHR was not specifically related to the use of aluminum hydroxide as an adjuvant during the sensitization phase, we assessed if similar results would be obtained if mice were sensitized and challenged by intranasal administration of HDM (25 µg) alone, on a daily basis, five days per week for five weeks (Repository eFigure 3A). Consistent with the results obtained using the HDM plus adjuvant model, the total number of BALF inflammatory cells recovered from *Lrp1^{fl/fl}*; *CD11c*-Cre mice that had been sensitized and challenged with HDM alone were significantly increased as compared to control *Lrp1^{fl/fl}* mice, which again represented increases in eosinophils, alveolar macrophages, and lymphocytes (Repository eFigure 3B). Again, there was no significant difference in airway hyperresponsiveness between HDM-challenged *Lrp1^{fl/fl}*; *CD11c*-Cre and *Lrp1^{fl/fl}* mice (Repository eFigure 3C), while flow cytometry demonstrated that there was a significant reduction in the number of CD3⁺/CD4⁺/CD25⁺/Foxp3⁺ regulatory T cells (Tregs) in the lungs of HDM-challenged *Lrp1^{fl/fl}*; *CD11c*-Cre mice as compared to *Lrp1^{fl/fl}* mice (Repository eFigure 3D). BALF levels of CCL24 were also significantly increased in HDM-challenged *Lrp1^{fl/fl}*; *CD11c*-Cre mice as compared to *Lrp1^{fl/fl}* mice (Repository eFigure 3E). Thus, a similar phenotype of enhanced eosinophilic airway inflammation that was dissociated from airway hyperreactivity was present when *Lrp1^{fl/fl}*; *CD11c*-Cre mice were sensitized and challenged by intranasal administration of HDM without aluminum hydroxide as an adjuvant.

The Adoptive Transfer of HDM-pulsed CD11b⁺ DCs from *Lrp1^{fl/fl}*; *CD11c*-Cre mice Augments Eosinophilic Airway Inflammation in HDM-challenged recipient mice

Next, adoptive transfer experiments were performed to confirm that the augmented HDM-induced eosinophilic airway inflammatory responses in *Lrp1^{fl/fl}*; *CD11c*-Cre mice were specifically mediated by DCs. CD11b⁺ BMDCs from *Lrp1^{fl/fl}*; *CD11c*-Cre and *Lrp1^{fl/fl}* mice were pulsed with HDM or saline and adoptively transferred to naïve wild-type (WT) mice that

subsequently received multiple intranasal HDM challenges to induce allergic airway inflammation (Figure 4A). As shown in Figure 4B, the number of total BALF inflammatory cells, which were specifically comprised of eosinophils and alveolar macrophages, were significantly increased in WT recipients of CD11b⁺ DCs from *Lrp1^{fl/fl}; CD11c-Cre* donor mice as compared to WT recipients of CD11b⁺ DCs from *Lrp1^{fl/fl}* donor mice. Similarly, lung histology showed increases in peribronchial inflammatory cell infiltrates (Figure 4C) and mucous cell metaplasia (Figure 4D) in WT recipients of CD11b⁺ DCs from *Lrp1^{fl/fl}; CD11c-Cre* donor mice, as compared to WT recipients of CD11b⁺ DCs from *Lrp1^{fl/fl}* donor mice. Serum levels of HDM-specific IgE and IgG1 (Figure 4E) and BALF levels of the C-C chemokine, CCL24 (Figure 4F), were also significantly increased in the WT recipients of CD11b⁺ DCs from *Lrp1^{fl/fl}; CD11c-Cre* donor mice as compared to WT recipients of CD11b⁺ DCs from *Lrp1^{fl/fl}* donor mice. In addition, the production of IL-4, but not IL-5, IL-13, or IFN- γ , was increased following HDM re-stimulation of MLN cells isolated from WT recipient mice that received the adoptive transfer of CD11b⁺ DCs from *Lrp1^{fl/fl}; CD11c-Cre* donor mice as compared to WT recipient mice that received the adoptive transfer of CD11b⁺ DCs from *Lrp1^{fl/fl}* donor mice (Figure 4G). Collectively, these experiments demonstrate that CD11b⁺ DCs from *Lrp1^{fl/fl}; CD11c-Cre* donor mice significantly up-regulated eosinophilic airway inflammatory responses in WT recipient mice upon subsequent HDM challenge, as compared to WT recipient mice that received the adoptive transfer of HDM-pulsed CD11b⁺ BMDCs from *Lrp1^{fl/fl}* donor mice. Thus, the adoptive transfer of HDM-pulsed LRP-1-deficient CD11b⁺ DCs into WT mice recapitulated the phenotype of enhanced allergic sensitization and eosinophilic inflammation that was observed in HDM-challenged *Lrp1^{fl/fl}; CD11c-Cre* mice.

CD11b⁺ Lung DCs from HDM-challenged *Lrp1^{fl/fl}; CD11c-Cre* mice have Increased Expression of CD86 and TLR5

Next, we considered whether the specific deletion of LRP-1 in CD11c⁺ cells would modify the number of lung DC subsets or the surface expression of co-stimulatory molecules in response to HDM challenge in mice that had been sensitized with HDM plus aluminum hydroxide. As shown in Figure 5A, there were no significant differences in the numbers of total lung CD11c⁺/Siglec F^{hi}/MHC II^{hi} DCs in HDM-challenged *Lrp1^{fl/fl}; CD11c-Cre* mice as compared to

HDM-challenged *Lrp1^{fl/fl}* mice. In addition, there were no differences in the number of CD11b⁺ cDCs and moDCs or CD11b⁺ CD103⁺ DCs and pDCs in the lungs of HDM-challenged *Lrp1^{fl/fl}*; *CD11c*-Cre mice and *Lrp1^{fl/fl}* mice (Figure 5B). Therefore, the phenotype of enhanced eosinophilic airway inflammation in HDM-challenged *Lrp1^{fl/fl}*; *CD11c*-Cre mice was not mediated by changes in the number of DC subsets in the lung. Similarly, there were no differences in the numbers of cDCs, moDCs, CD103⁺ DCs and pDCs in the lungs of *Lrp1^{fl/fl}*; *CD11c*-Cre mice and *Lrp1^{fl/fl}* mice that had been sensitized and challenged by administration of intranasal HDM without adjuvant (Supplemental Repository eFigure 4).

Since, CD11b⁺ cDCs and moDCs have been shown to be the primary DC subset in the lung that mediates antigen presentation and Th2 responses^{27, 36, 37}, we characterized these DC subsets further in *Lrp1^{fl/fl}*; *CD11c*-Cre mice. We found that cell surface expression of the co-stimulatory molecule CD86 was increased on CD11b⁺ DCs from the lungs of HDM-challenged *Lrp1^{fl/fl}*; *CD11c*-Cre mice, whereas there was no difference in the expression of CD80 (Figure 5C). We also considered whether cell surface expression of C-type lectin receptors (CLRs) and toll-like receptors (TLRs) by CD11b⁺ lung DCs might be modified in *Lrp1^{fl/fl}*; *CD11c*-Cre mice as a possible mechanism by which allergic inflammation is augmented³⁸⁻⁴¹. As shown in Figures 5D and 5E, there were no differences in the mean fluorescence intensity (MFI) of the CLRs, Dectin 1, Dectin 2, DC-SIGN, and CD206, or TLR4 by CD11b⁺ lung DCs from HDM-challenged *Lrp1^{fl/fl}*; *CD11c*-Cre mice as compared to *Lrp1^{fl/fl}* mice, whereas the expression of CD205 was decreased. This suggests that the phenotype of HDM-challenged *Lrp1^{fl/fl}*; *CD11c*-Cre mice was not mediated at the level of CLRs. In contrast, TLR5 expression by lung CD11b⁺ DCs from HDM-challenged *Lrp1^{fl/fl}*; *CD11c*-Cre mice was significantly increased. These data suggest that the up-regulated expression of CD86 and TLR5 by CD11b⁺ myeloid lung DCs from HDM-challenged *Lrp1^{fl/fl}*; *CD11c*-Cre mice might in part contribute to the phenotype of enhanced eosinophilic inflammation and Th2 responses.

LRP-1-deficient DCs have Increased Antigen Uptake and Presentation

We next hypothesized that antigen uptake and presentation functions of LRP-1-deficient DCs might be enhanced as a mechanism by which HDM-induced eosinophilic airway inflammation

is augmented in *Lrp1^{fl/fl}*; *CD11c*-Cre mice. AF-647-labelled HDM was administered to the lungs of *Lrp1^{fl/fl}*; *CD11c*-Cre and *Lrp1^{fl/fl}* mice and antigen uptake was assessed by flow cytometry. As shown in Figure 6A, there was a significant increase in HDM uptake by CD11b⁺ lung DCs from *Lrp1^{fl/fl}*; *CD11c*-Cre mice as compared to *Lrp1^{fl/fl}* mice (14.28 % $\pm$ 2.6 vs. 2.46 % $\pm$ 0.31), whereas there was no difference in the uptake of HDM antigen by lung CD11b⁻ DCs. In addition, there was no difference in the frequency of AF-647-HDM⁺ CD11b⁺ or CD11b⁻ DCs from *Lrp1^{fl/fl}*; *CD11c*-Cre and *Lrp1^{fl/fl}* mice that migrated to MLNs following uptake of HDM antigen in the lung (Figure 6B). Collectively, these experiments show that lung DCs from *Lrp1^{fl/fl}*; *CD11c*-Cre mice have an enhanced antigen uptake capability, whereas subsequent migration to MLNs was not modified.

Next, CD11c⁺ BMDCs from *Lrp1^{fl/fl}*; *CD11c*-Cre and *Lrp1^{fl/fl}* mice were used to investigate whether LRP-1 expression modifies antigen-specific T cell proliferation and Th2 cytokine production. Co-culture experiments of splenic CD4⁺ T cells from mice expressing the major histocompatibility complex II (MHC II)-restricted DO11.10 T-cell receptor that recognizes the OVA 323-339 peptide showed that CD11c⁺ BMDCs from *Lrp1^{fl/fl}*; *CD11c*-Cre mice mediate significant increases in both T cell proliferation (Figure 6C) and Th2 cytokine production (Figure 6D) as compared with CD11c⁺ BMDCs from *Lrp1^{fl/fl}* mice following *ex vivo* stimulation with the OVA 323-339 peptide. Collectively, these experiments show that LRP-1-deficient CD11c⁺ DCs have an enhanced ability to present antigen to CD4⁺ T cells and thereby induce effector Th2 inflammatory responses.

Discussion

CD11b⁺ cDCs play key roles in initiating Th2 cell differentiation and effector cytokine production that mediate allergic airway inflammation⁴²⁻⁴⁴. We have previously shown that the VLDL receptor functions as a negative regulator of DC function in experimental HDM-induced asthma. However, limited data exists regarding the function of another LDL receptor family member, LRP-1, in mediating the ability of DCs to induce allergen-mediated adaptive immune responses in asthma^{21, 45-47}. For example, *LRP1* polymorphisms have been associated with food allergen sensitization in asthmatic children who participated in the Mexico City Childhood Asthma Study⁴⁸. In other settings, LRP-1 expression by DCs has been shown to mediate antigen-specific immune responses. LRP-1 on DCs can facilitate the receptor-mediated uptake of a wide variety of antigens, including soluble tetanus toxin C fragment (TTC) or activated α 2-macroglobulin-TTC complexes, that elicit T cell proliferation and enhance primary antigen-specific immune responses^{45, 49, 50}. Furthermore, LRP-1 has been shown to bind and internalize immunogenic heat shock proteins (HSPs), such as HSP70, HSP90, gp96, and calreticulin^{22, 46, 51}, which are processed and presented by MHC class I or MHC class II molecules to provide the appropriate signal for T cell priming²¹. LRP-1 also mediates DC responses in the setting of host defense against viral infections and in cancer^{19, 51, 52}.

Peripheral blood eosinophil counts can be used as a biomarker to identify eosinophilic asthmatics and are also correlated with asthma severity^{29, 53, 54}. Here, we show that eosinophil-high asthmatics have decreased LRP-1 expression on circulating MDC1, MDC2, and CD16⁺ myeloid DC subsets as compared to healthy, non-asthmatic subjects. Furthermore, we show LRP-1 expression by MDC1, MDC2, and CD16⁺ myeloid DC subsets is negatively correlated with peripheral blood eosinophil counts in asthmatics. Interestingly, LRP-1⁺/Lin⁻/CD11c⁺ DCs, but not CD14⁺ monocytes are the primary antigen presenting cells (APCs) in circulating blood that facilitate receptor-mediated antigen uptake and presentation⁴⁵. This is clinically relevant as these DC subsets also express the high-affinity IgE receptor (Fc ϵ R1), which contributes to the pathogenesis of allergic asthma⁵⁵. Similarly, we show that HDM suppresses LRP-1 expression by CD11b⁺ cDCs and moDCs in the murine lung. This is consistent with prior reports demonstrating down-regulation of cell surface LRP-1 expression

on myeloid cells in response to bacterial LPS and cigarette smoke^{56, 57}. Collectively, these findings support the possibility that reduced LRP-1 expression by circulating myeloid DCs in eosinophilic asthmatics, as well as by CD11b⁺ cDCs and moDCs lung DCs from HDM-challenged mice, modify the severity of eosinophilic asthma.

We used two murine models to investigate whether LRP-1 expression by DCs modulates the pathogenesis of allergic sensitization to HDM and the subsequent induction of eosinophilic airway inflammation and Th2 adaptive immune responses in the lung. First, we created a mouse with a specific deletion of LRP-1 in CD11c⁺ cells, such as DCs and alveolar macrophages, to characterize the role of LRP-1 expression in HDM-induced airways disease. We show that HDM-challenged *Lrp1^{fl/fl}; CD11c-Cre* mice display a phenotype of augmented allergic sensitization and enhanced Th2 immune responses to HDM that were associated with increased eosinophilic airway inflammation and mucous cell metaplasia. HDM re-stimulation of draining mediastinal lymph node cells from *Lrp1^{fl/fl}; CD11c-Cre* mice showed increased production of the Th2 cytokines, IL-4, IL-5 and IL-13, which are key regulators of eosinophilic airway inflammation and mucous cell metaplasia⁵⁸⁻⁶⁰. Furthermore, there was a reduction CD4⁺ Tregs, which function to suppress eosinophilic airway inflammation and Th2 immune responses in the lungs of HDM-challenged *Lrp1^{fl/fl}; CD11c-Cre* mice. This suggests that the decrease in lung Tregs in HDM-challenged *Lrp1^{fl/fl}; CD11c-Cre* mice might foster a permissive immunologic environment that augments eosinophilic airway inflammation^{61, 62}. In addition, CD11b⁺ lung DCs from HDM-challenged *Lrp1^{fl/fl}; CD11c-Cre* mice had increased expression of CD86 (B7-2), which mediates co-stimulatory signaling upon interacting with CD28 on CD4⁺ T cells to promote Th2 immune responses^{63, 64}. CD11b⁺ lung DCs from HDM-challenged *Lrp1^{fl/fl}; CD11c-Cre* mice also had increased expression of TLR5, which promotes allergic sensitization to flagellin-containing aeroallergens⁶⁵. Thus, the concomitant decrease in Tregs, and increased expression of CD86 and TLR5 on CD11b⁺ lung DCs, may in part contribute to the phenotype of enhanced allergic sensitization and eosinophilic airway inflammation in HDM-challenged *Lrp1^{fl/fl}; CD11c-Cre* mice.

Interestingly, the phenotype of augmented eosinophilic airway inflammation in HDM-challenged *Lrp1^{fl/fl}; CD11c-Cre* mice that were sensitized with HDM plus adjuvant (i.e.,

aluminum hydroxide) was not associated with concomitant modifications in AHR. This finding was confirmed when HDM alone was used for both allergic sensitization and challenge, which shows that the dissociation between AHR and eosinophilic airway inflammation was not merely a consequence of a sensitization model that utilized aluminum hydroxide as an adjuvant. A dissociation between eosinophilic airway inflammation and AHR has been previously reported both in murine models, as well as in human studies, which suggests that distinct pathogenic pathways can regulate the development of these cardinal manifestations of asthma⁶⁶⁻⁷². Consistent with this concept, our results suggest that LRP-1 regulates HDM-induced eosinophilic airway inflammation, but not AHR, in *Lrp1^{fl/fl}*; *CD11c*-Cre mice.

CD11b⁺ cDCs that reside in the airway continuously sample the local environment and take up inhaled aeroallergens, such as HDM, after which they migrate to nearby draining lymph nodes to present antigens to T cells⁷³. As such, CD11b⁺ cDCs are the major lung DC subset that mediates the priming and activation of CD4⁺ effector T cells in allergen-mediated airway inflammation^{27, 74, 75}. Furthermore, following sensitization and challenge with HDM, migratory CD11b⁺ cDCs have been identified as the primary DC subset that induces Th2 immune responses in lymph nodes, while the function of CD11b⁺ moDCs is to produce pro-inflammatory cytokines and facilitate allergen presentation and allergen-mediated eosinophilic inflammation in the lung³⁶. Here, we show that the adoptive transfer of HDM-pulsed CD11b⁺ DCs from *Lrp1^{fl/fl}*; *CD11c*-Cre mice have an increased ability to induce allergic sensitization, as indicated by elevated serum levels of HDM-specific IgE and IgG₁, as well as eosinophilic airway inflammation. In addition, BALF levels of the C-C chemokine, CCL24 (eotaxin-2), which has chemotactic activity towards eosinophils, were increased⁷⁶. Collectively, these results confirm the conclusion that the phenotype of increased allergic sensitization and eosinophilic airway inflammation that we observed in HDM-challenged *Lrp1^{fl/fl}*; *CD11c*-Cre mice was specifically mediated by CD11b⁺ DCs.

Uptake of HDM antigen in the lung is almost exclusively mediated by the CD11b⁺ DC subset, whereas CD11b⁻ DCs can also mediate HDM uptake at high doses^{36, 38}. Following antigen uptake in the lung, DCs are transported to draining MLNs by a CCR7-dependent process, where they induce the expansion of naïve T cells to mount a Th2 immune response⁷⁷. By sub-

dividing the lung and MLN DC populations, we show that CD11b⁺ DCs from the lungs of naïve *Lrp1^{fl/fl}*; *CD11c*-Cre mice have an increased ability to take up HDM antigen, whereas there was no difference in the migration of HDM⁺ CD11b⁺ DCs to the draining MLNs. In addition, we show that OVA-peptide pulsed CD11c⁺ BMDCs *Lrp1^{fl/fl}*; *CD11c*-Cre mice have enhanced antigen presentation capabilities as evidenced by their ability to increase the proliferation of naïve DO11.10 T cells, as well as up-regulate the production of IL-5 and IL-13 Th2 effector cytokines. Thus, increased uptake of HDM antigen, as well as enhanced antigen presentation and induction of Th2 immunity, may contribute to the phenotype of augmented eosinophilic airway inflammation in HDM-challenged *Lrp1^{fl/fl}*; *CD11c*-Cre mice.

In conclusion, the present study demonstrates, for what we believe is the first time, a role for LRP-1 in modulating dendritic cell function in the pathogenesis of Th2 immune responses to HDM in experimental allergen-induced asthma. Our data are consistent with the conclusion that LRP-1 functions as a negative regulator of CD11b⁺ DCs, which thereby attenuates HDM-induced allergic sensitization and eosinophilic airway inflammation. We propose that the mechanism by which LRP-1 negatively regulates DC function involves attenuated antigen uptake and presentation capabilities, which reduces allergic sensitization and Th2 immune responses in the lung. Furthermore, we demonstrate that LRP-1 expression is reduced on circulating myeloid DC subsets from eosinophilic asthmatics, which also suggests a possible role for LRP-1 in modulating the type 2-high asthma endotype in humans. Therefore, these findings support a novel role for the LDL receptor family member, LRP-1, as a negative regulator of DC function during the pathogenesis of allergic asthma.

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Figure Legends

Figure 1. Cell surface LRP-1 expression is reduced on myeloid dendritic cell subsets from eosinophilic asthmatics. **A)** Asthmatics (n = 14) and healthy, non-asthmatics (n = 12) with low peripheral blood eosinophil counts (≤ 260 cells/ μ l), or asthmatics with high (> 260 cells/ μ l) peripheral blood eosinophil counts (n = 12) (* P< 0.0001, one-way ANOVA with Sidak's multiple comparison test). **B)** Mean fluorescence intensity (MFI) of LRP-1 expression was assessed by flow cytometry on viable/ Lin⁻/ HLA-DR⁺ peripheral blood dendritic cell subsets that express CD11c⁺/CD1c⁺/CD141⁻ (MDC1), CD11c⁺/CD1c⁻/CD141⁺ (MDC2), CD11c⁺/CD16⁺ (CD16⁺ DCs), and CD11c⁻/CD123⁺ (pDCs). Data shown as means $\pm$ SEM (*P< 0.05, one-way ANOVA with Sidak's multiple comparison test). **C)** The percentage of LRP-1⁺ CD11c⁺/CD11b⁺/Siglec F⁻/MHC II^{hi}/Mar-1⁻/CD64⁻ conventional myeloid DCs (cDCs) and CD11c⁺/CD11b⁺/Siglec F⁻/MHC II^{hi}/Mar-1⁺/CD64⁺ monocyte-derived DCs (moDCs) from saline- and house dust mite (HDM)-challenged wild-type C57BL6 mice were quantified (n = 9 mice, * P< 0.0001, saline- versus HDM-challenged, Mann-Whitney test).

Figure 2. Eosinophilic airway inflammation and mucous cell metaplasia are increased in HDM-challenged *Lrp1*^{fl/fl}; *CD11c*-Cre mice. **A)** *Lrp1*^{fl/fl} and *Lrp1*^{fl/fl}; *CD11c*-Cre mice were sensitized with two *intraperitoneal* injections of 100 μ g HDM (with alum 40 mg ml⁻¹) on day 0 and day 4 and challenged on days 8, 10, 12 and 15 by intranasal administration of HDM (50 μ g) before harvest and endpoint analysis on day 17. **B)** The number of total BALF inflammatory cells and inflammatory cell types (eosinophils (Eos), alveolar macrophages (AM), neutrophils (PMN) and lymphocytes (Lymph)) from saline- and HDM-challenged *Lrp1*^{fl/fl}; *CD11c*-Cre and *Lrp1*^{fl/fl} mice (n = 7 - 16 mice per group, * P< 0.01, HDM-challenged *Lrp1*^{fl/fl}; *CD11c*-Cre versus *Lrp1*^{fl/fl}, one-way ANOVA with Sidak's multiple comparison test). **C)** Representative lung histology sections stained with hematoxylin and eosin (H&E) or Periodic acid-schiff (PAS). Scale bars 100 μ m for the 200x images and 20 μ m for the 1,000x images. **D)** Quantification of mucous cell metaplasia as assessed by the percentage of airways that contained PAS⁺ cells. Data are mean $\pm$ SEM (n = 5 - 7 mice per group, * P < 0.05, HDM-

challenged *Lrp1^{fl/fl}*; *CD11c-Cre* versus *Lrp1^{fl/fl}* mice, Mann-Whitney test). Results shown are pooled data from two independent experiments.

Figure 3. Th2 cytokine production and allergic sensitization are increased in HDM-challenged *Lrp1^{fl/fl}*; *CD11c-Cre* mice. **A)** Airway resistance (cm H₂O per ml s⁻¹) to inhaled methacholine from HDM-challenged *Lrp1^{fl/fl}*; *CD11c-Cre* and *Lrp1^{fl/fl}* mice (n = 4 - 6 mice per group). (P = NS, two-way ANOVA). **B)** Cytokine secretion by ex vivo cultures of MLN cells that had been re-stimulated with saline or HDM (100 µg ml⁻¹, n = 6 mice per group, * P < 0.05, HDM-challenged *Lrp1^{fl/fl}*; *CD11c-Cre* versus *Lrp1^{fl/fl}* mice, one-way ANOVA with Sidak's multiple comparison test). **C)** The percentage of CD4⁺/CD25⁺/FoxP3⁺ Tregs in the lungs of HDM-challenged *Lrp1^{fl/fl}*; *CD11c-Cre* mice and *Lrp1^{fl/fl}* mice (n = 5 mice per group, *P < 0.008, Mann-Whitney test). **D)** Serum levels of HDM-specific IgE and IgG₁ (n = 9 - 10 mice per group), * P < 0.05, HDM-challenged *Lrp1^{fl/fl}*; *CD11c-Cre* versus *Lrp1^{fl/fl}* mice, one-way ANOVA with Sidak's multiple comparison test).

Figure 4. The adoptive transfer of CD11b⁺ DCs from *Lrp1^{fl/fl}*; *CD11c-Cre* mice increases HDM-mediated eosinophilic airway inflammation. **A)** BMDCs from *Lrp1^{fl/fl}*; *CD11c-Cre* and *Lrp1^{fl/fl}* donor mice were pulsed ex vivo with saline or HDM (100 µg ml⁻¹) for 16 hours. 2.5 x 10⁴ CD11c⁺/CD11b⁺/MHC II^{hi} DCs were adoptively transferred to wild-type (WT) C57BL6 recipient mice by intranasal administration on day 0 and intranasal HDM challenges (50 µg) were administered on alternate days, from day 9 through day 15, to all recipient mice, prior to endpoint analysis on day 16. **B)** The number of total BALF inflammatory cells and inflammatory cell subtypes in recipient WT mice (n = 6 - 10 mice per group, *P < 0.05, HDM-pulsed *Lrp1^{fl/fl}*; *CD11c-Cre* DCs versus HDM-pulsed *Lrp1^{fl/fl}* DCs, one-way ANOVA with Sidak's multiple comparison test). **C)** Representative histologic lung sections stained with H&E and PAS. Scale bars indicate 100 µm for the 200x images and 20 µm for the 1,000x images. **D)** Quantification of mucous cell metaplasia as assessed by the percentage of airways that contained PAS⁺ cells. (n = 5 mice per group, * P < 0.008, HDM-pulsed *Lrp1^{fl/fl}*; *CD11c-Cre* DCs versus HDM-pulsed *Lrp1^{fl/fl}* DCs, Mann-Whitney test). **E)** Serum levels HDM-specific IgE and IgG₁ (n = 9 - 10 mice per group, *P < 0.01, Mann-Whitney test, HDM-pulsed *Lrp1^{fl/fl}*; *CD11c-Cre* DCs versus HDM-pulsed *Lrp1^{fl/fl}* DCs). **F)** BALF levels of CCL24 from WT recipient mice that received

HDM-pulsed *Lrp1^{fl/fl}*; *CD11c*-Cre DCs or HDM-pulsed *Lrp1^{fl/fl}* DCs (n = 6 - 10 mice per group, *P < 0.05, unpaired t test). **G)** Cytokine secretion by ex vivo cultures of MLN cells that had been re-stimulated with saline or HDM (100 µg ml⁻¹, n = 6 - 10 mice per group, * P < 0.01, HDM-pulsed *Lrp1^{fl/fl}*; *CD11c*-Cre DCs versus HDM-pulsed *Lrp1^{fl/fl}* DCs, one-way ANOVA with Sidak's multiple comparison test). Results shown are pooled data from two independent experiments.

Figure 5. Increased expression of CD86 and TLR5 by CD11c⁺/CD11b⁺ DCs from HDM-challenged *Lrp1^{fl/fl}*; *CD11c*-Cre mice. **A)** The percentage of CD11c⁺/Siglec F⁻/MHC II^{hi} total lung DCs and **B)** the percentage of lung DC monocyte-derived dendritic cells (moDCs), conventional myeloid dendritic cells (cDCs), CD103⁺ DCs, and plasmacytoid dendritic cells (pDCs) subsets as quantified by flow cytometry (P = NS, HDM-challenged *Lrp1^{fl/fl}*; *CD11c*-Cre versus *Lrp1^{fl/fl}* mice, n= 9 mice per group, unpaired t test). **C)** Cell surface expression of CD80 and CD86 by lung CD11c⁺/CD11b⁺/SSC^{lo}/SiglecF⁻/MHC II^{hi} DCs from HDM-challenged *Lrp1^{fl/fl}*; *CD11c*-Cre versus *Lrp1^{fl/fl}* mice (n = 8 - 9 mice per group, * P < 0.01, Mann-Whitney test). **D)** Cell surface expression of Dectin1, Dectin 2, CD205, CD206, and DC-SIGN by lung CD11c⁺/CD11b⁺/SSC^{lo}/SiglecF⁻/MHC II^{hi} DCs from HDM-challenged *Lrp1^{fl/fl}*; *CD11c*-Cre versus *Lrp1^{fl/fl}* mice (n = 4 - 9 mice per group, * P = 0.029, Mann-Whitney test). **E)** Cell surface expression of TLR4 and TLR5 by lung CD11c⁺/CD11b⁺/SSC^{lo}/SiglecF⁻/MHC II^{hi} DCs from HDM-challenged *Lrp1^{fl/fl}*; *CD11c*-Cre versus *Lrp1^{fl/fl}* mice (n= 4 - 9 mice per group, *P < 0.05, unpaired t test).

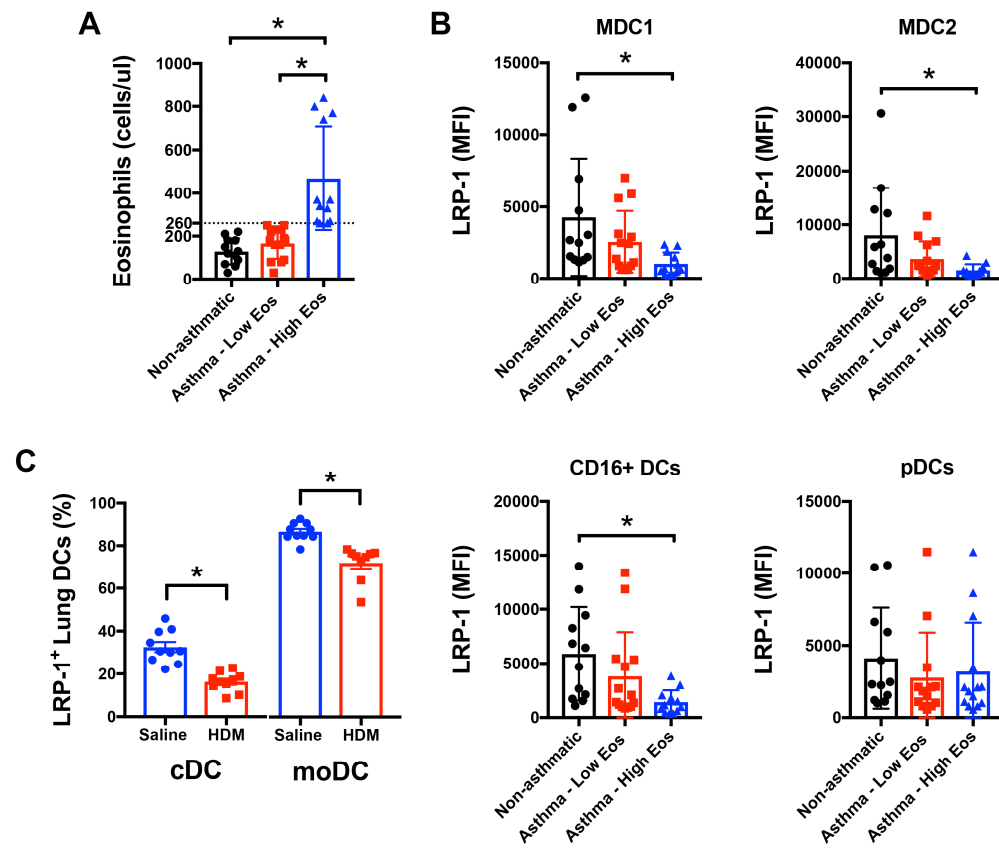
Figure 6: *Lrp1*-deficient DCs have augmented antigen uptake and presentation capabilities. **A and B)** Uptake of Alexa Fluor 647-labeled HDM (50 µg) by CD11c⁺/SSC^{lo}/SiglecF⁻/MHCII^{hi} CD11b⁺ or CD11b⁻ DCs in the lungs (A) and MLNs (B) of *Lrp1^{fl/fl}*; *CD11c*-Cre and *Lrp1^{fl/fl}* mice 72 hours after intranasal administration (n= 10 mice per group, * P < 0.05, unpaired t test). **C)** CD11c⁺ BMDCs from *Lrp1^{fl/fl}* and *Lrp1^{fl/fl}*; *CD11c*-Cre mice were pulsed with the OVA 323-339 peptide and incubated at a 1:5 ratio with CFSE-labelled splenic CD4⁺ DO11.10 T cells for 4 days. OVA-specific proliferation is presented as % divided OT-II cells (n = 12 – 15 per group, * P < 0.0001, one-way ANOVA with Sidak's multiple comparison test). **D)** Th2 cytokines released by co-cultures of OVA 323-339 peptide-pulsed

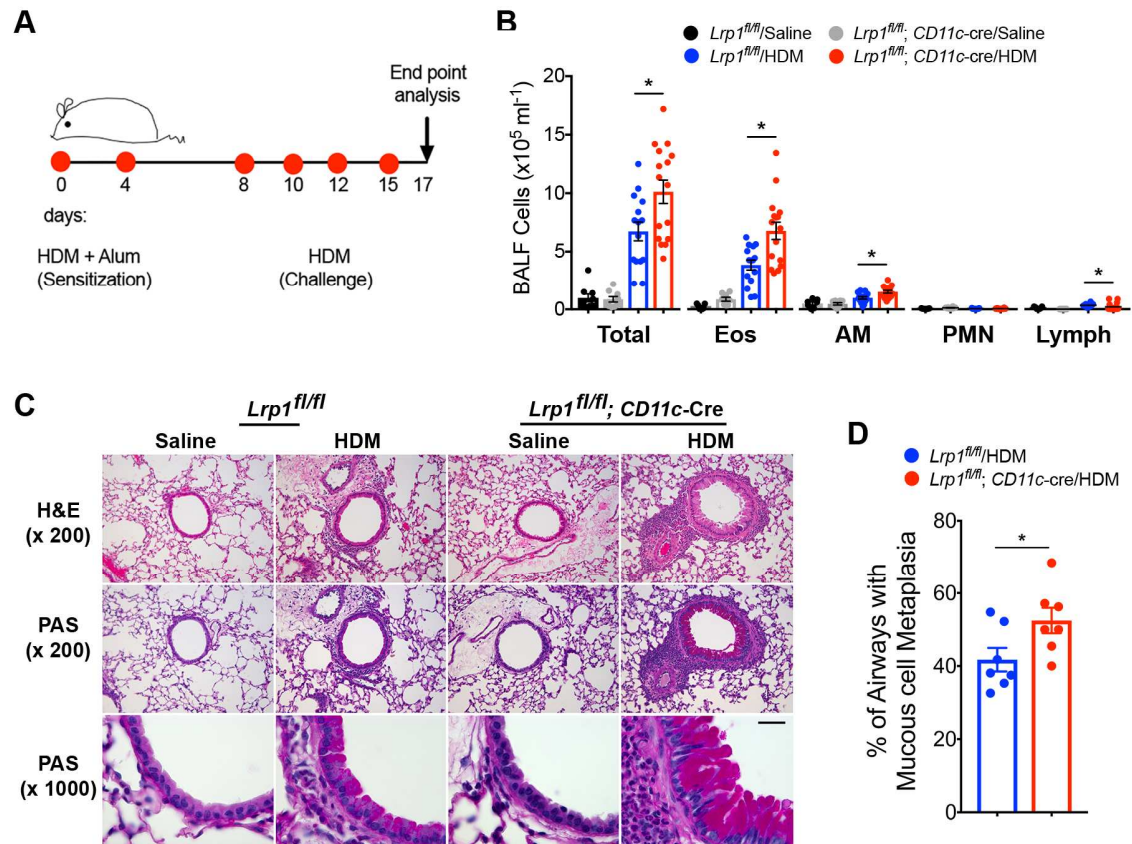
934 BMDCs and CFSE-labelled CD4⁺ DO11.10 T cells (n= 10 per group, * P < 0.05, *Lrp1*^{fl/fl};
935 *CD11c*-Cre versus *Lrp1*^{fl/fl}, unpaired t test). Results shown are pooled data from two
936 independent experiments.

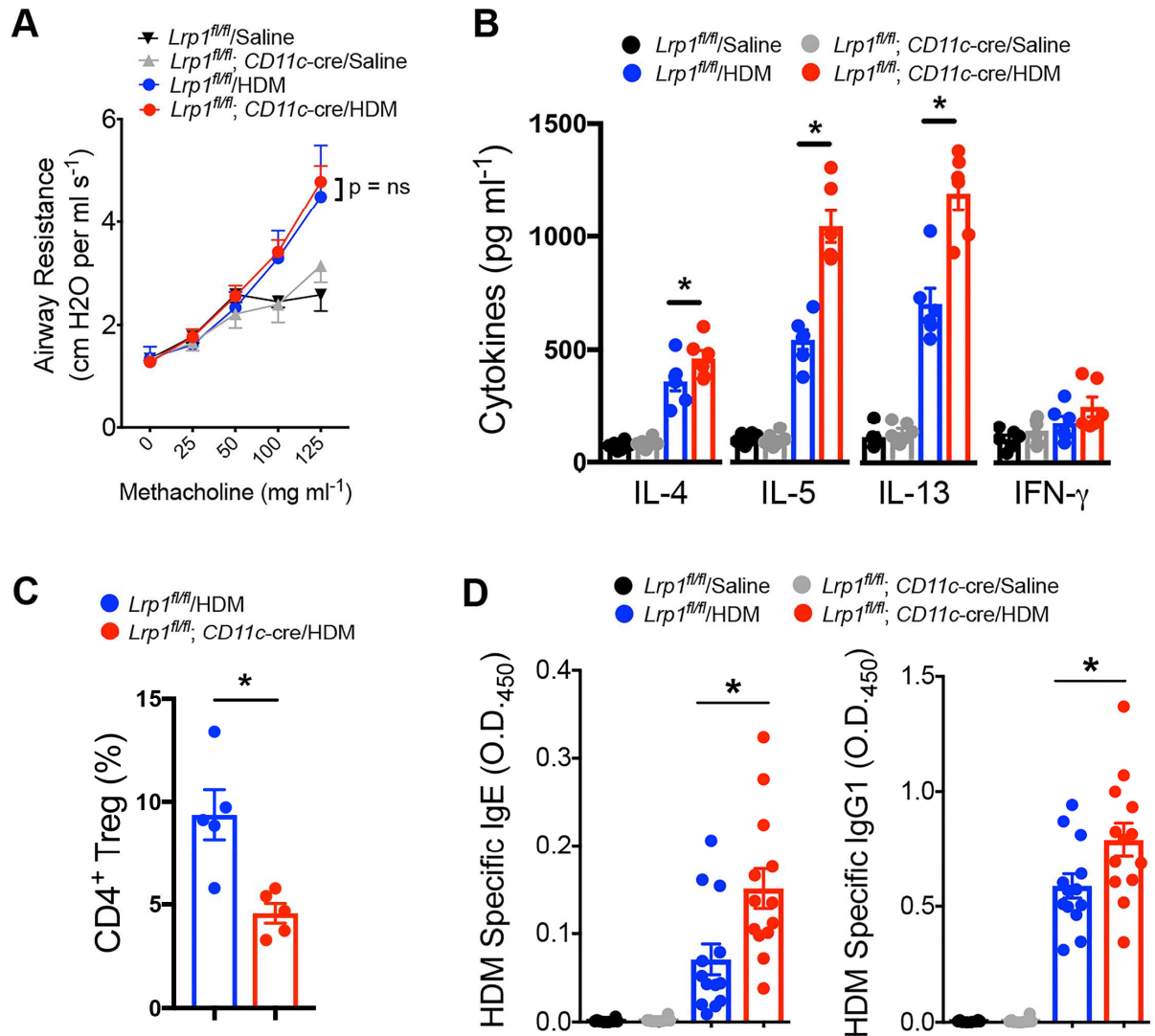
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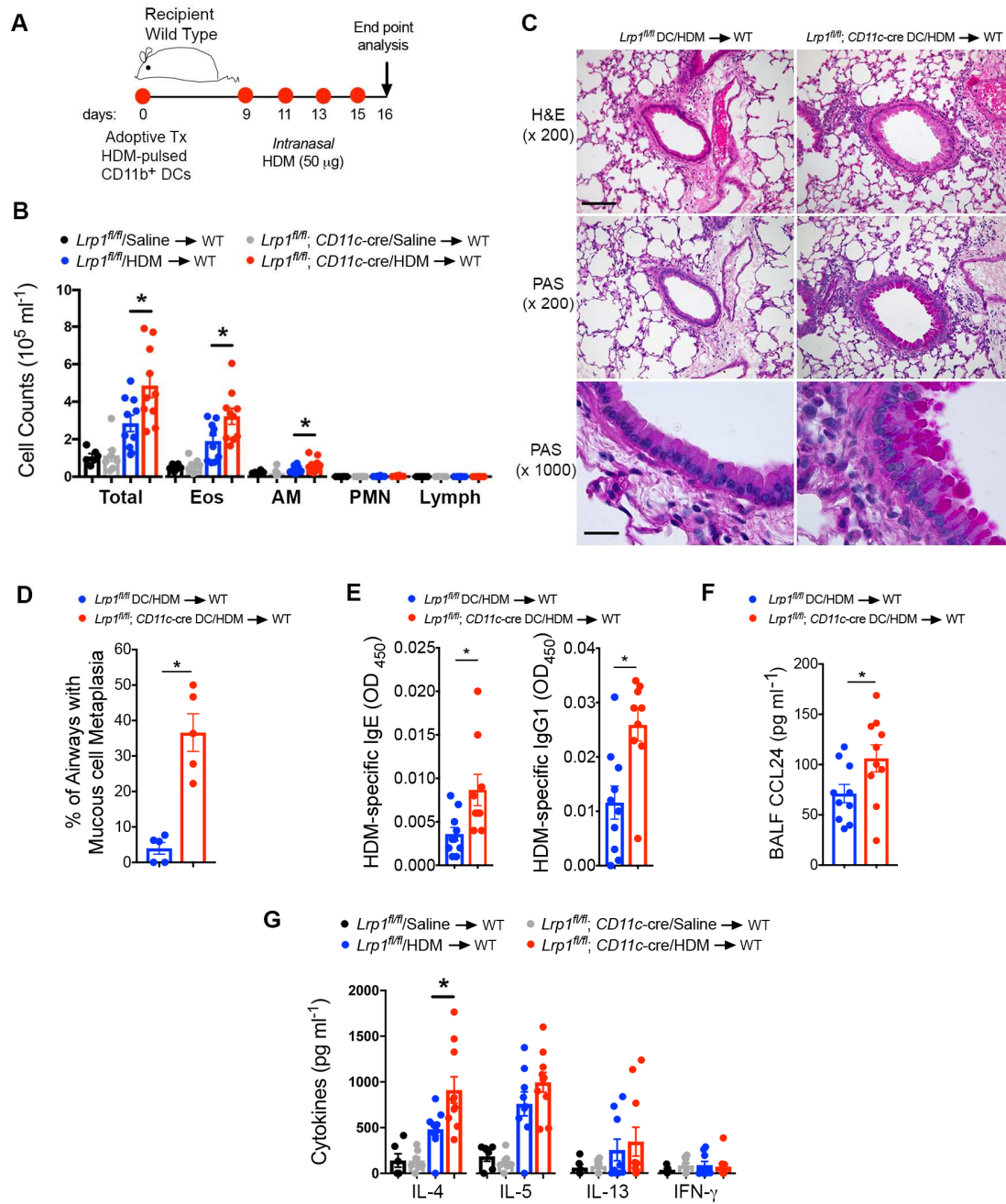
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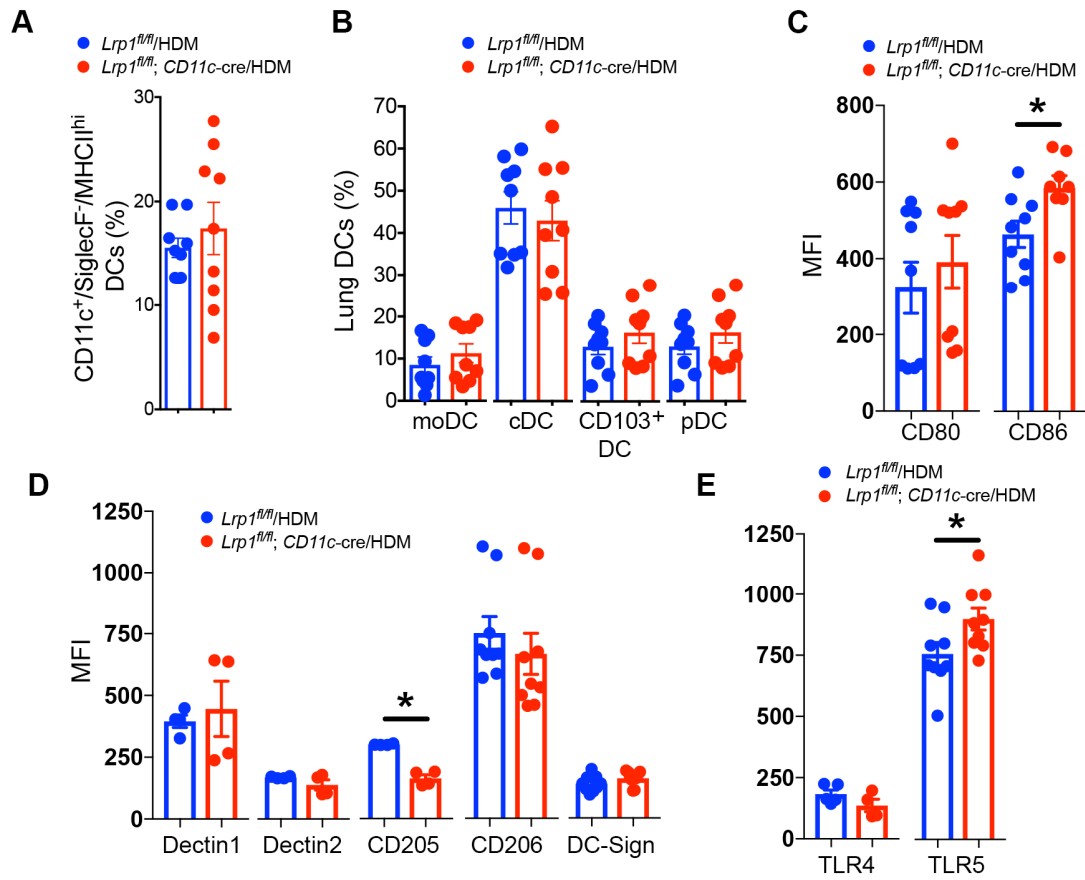
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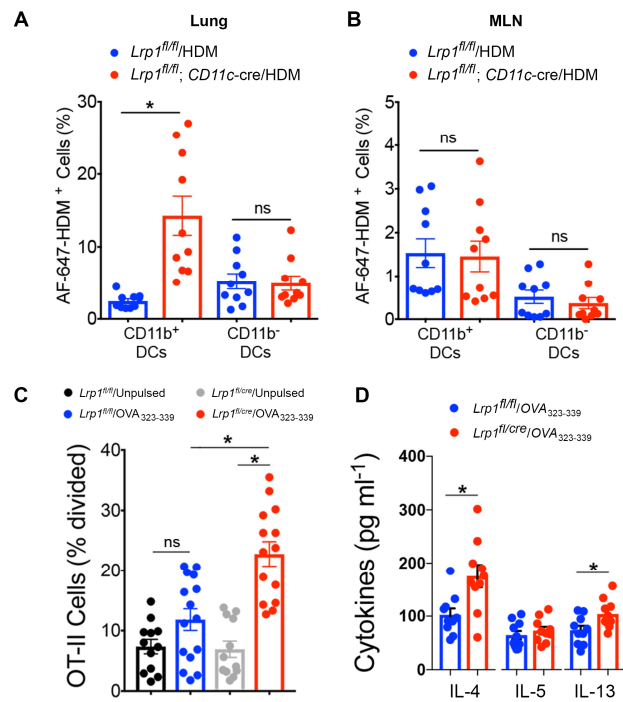












Repository eTable 1. Demographics of subjects who participated in the study.

	Healthy, non-asthmatics, low eosinophils (<260 cells/ μ l)	Asthmatics, low eosinophils (<260 cells/ μ l)	Asthmatics, high eosinophils (>260 cells/ μ l)
# of subjects	12	14	12
Age (years)	53 ± 8	42 ± 15	49 ± 13
Gender (M/F)	5/7	7/7	5/7
Severe Asthma	n/a	2	3
Inhaled Steroids	0	8	10
Oral Steroids	0	1	0
Allergy	6	14	12
FEV ₁ (liters)	3.32 ± 0.7	2.89 ± 0.8	$2.46 \pm 0.7^*$
FEV ₁ (% predicted)	103 ± 9	$85 \pm 14^*$	$81 \pm 19^*$
FeNO (ppb)	19 ± 10	29 ± 24^a	33 ± 21^b
IgE (IU/ml)	73 ± 93	150 ± 194	150 ± 150
Blood eosinophils (cells/ μ l)	129 ± 61	166 ± 71	$468 \pm 240^{*, **}$
Blood eosinophils (%)	2.4 ± 1.4	2.9 ± 1.8	$6.9 \pm 3^{*, **}$

Data are presented as mean $\pm$ standard deviation.

* $P < 0.05$ versus Healthy, non-asthmatics.

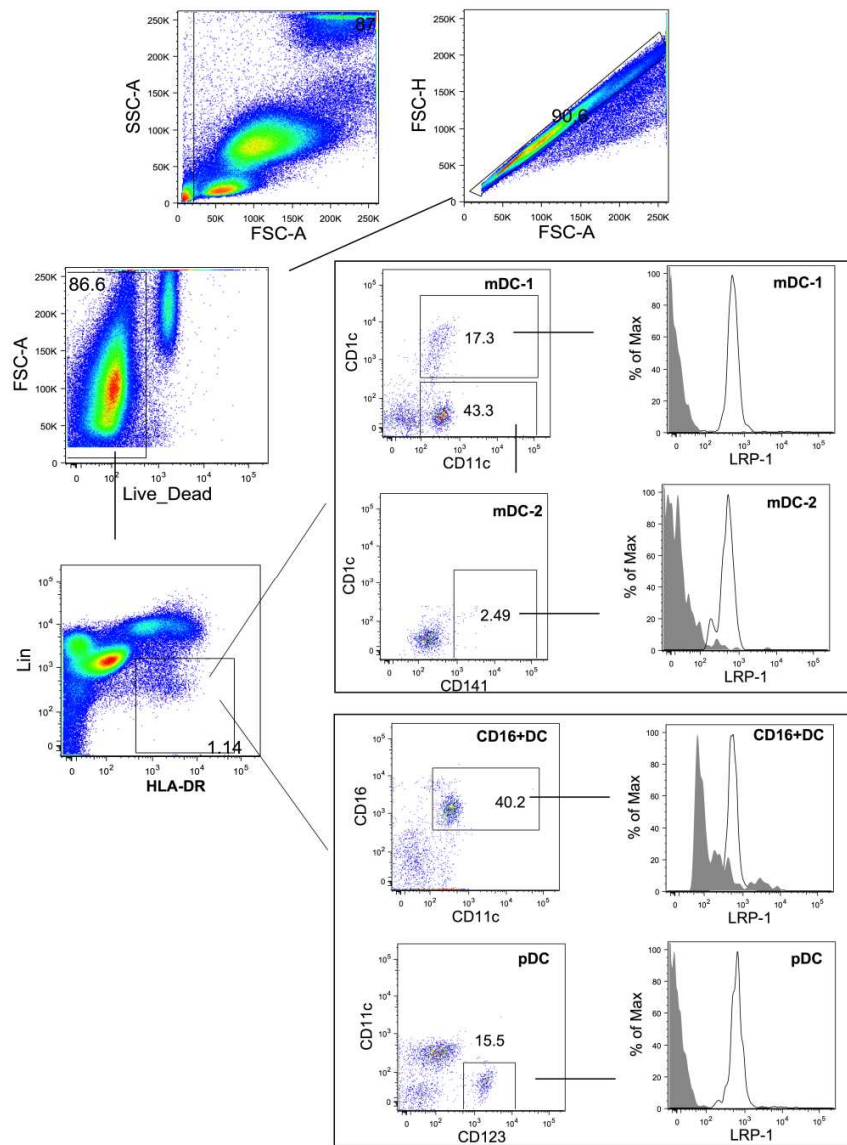
** $P < 0.05$ versus Asthmatics, low-eosinophils.

One-way ANOVA with Sidak's multiple comparison test.

^a Data available for 13 subjects.

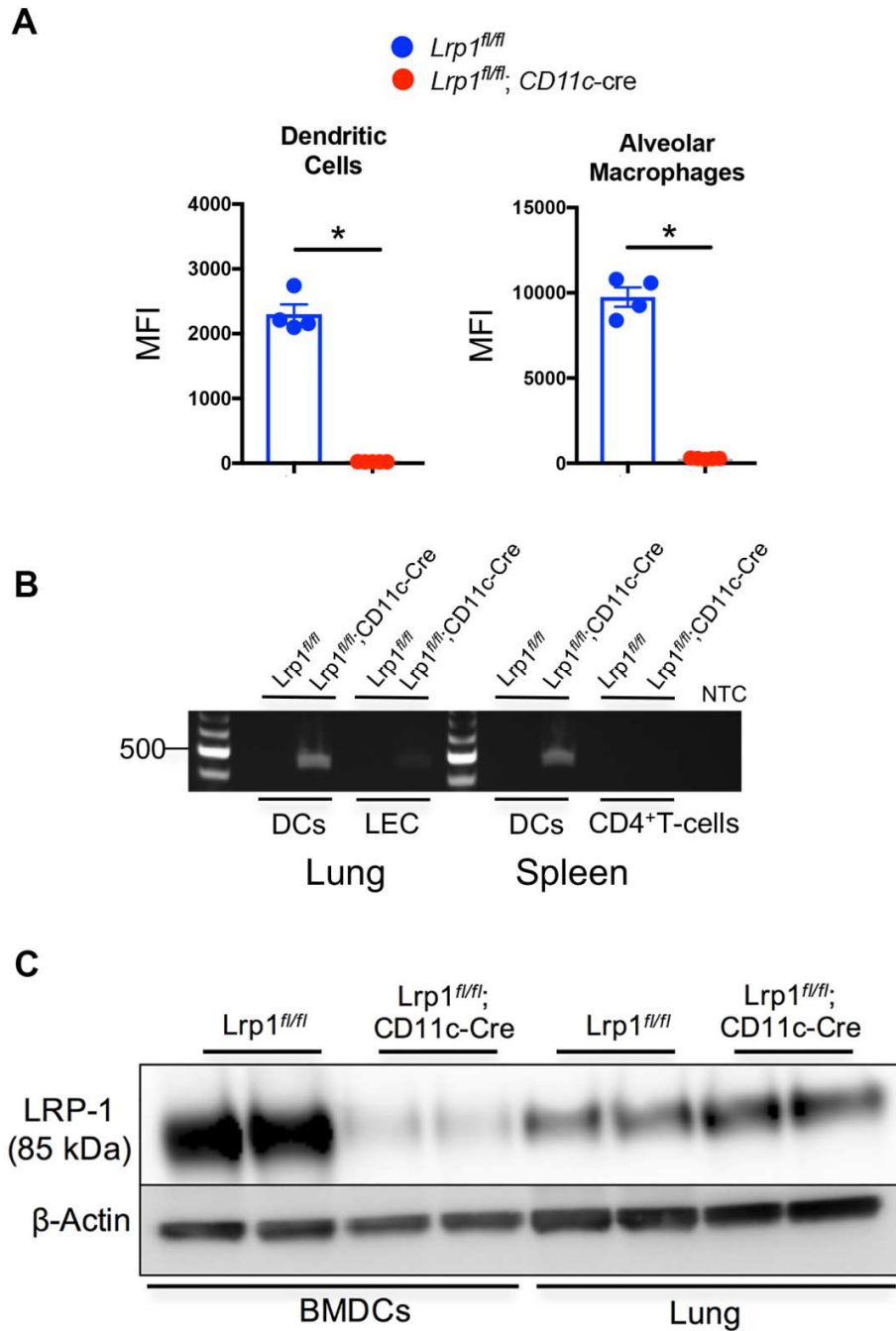
^b Data available for 11 subjects.

Repository eFigure 1.



Representative flow cytometric analysis of viable/Lin⁻/HLA-DR⁺ dendritic cells in peripheral blood of asthmatic subjects. Cellular debris and doublets were excluded using physical scatter properties of whole blood cells. Dead cells were excluded using a fixable live-dead exclusion dye. Cell surface expression of LRP-1 was assessed on DCs subset that express CD11c⁺/CD1c⁺/CD141⁻ (MDC1), CD11c⁺/CD1c⁻/CD141⁺ (MDC2), CD11c⁺/CD16⁺ (CD16⁺ DCs), and CD11c⁻/CD123⁺ (pDCs).

Repository eFigure 2.



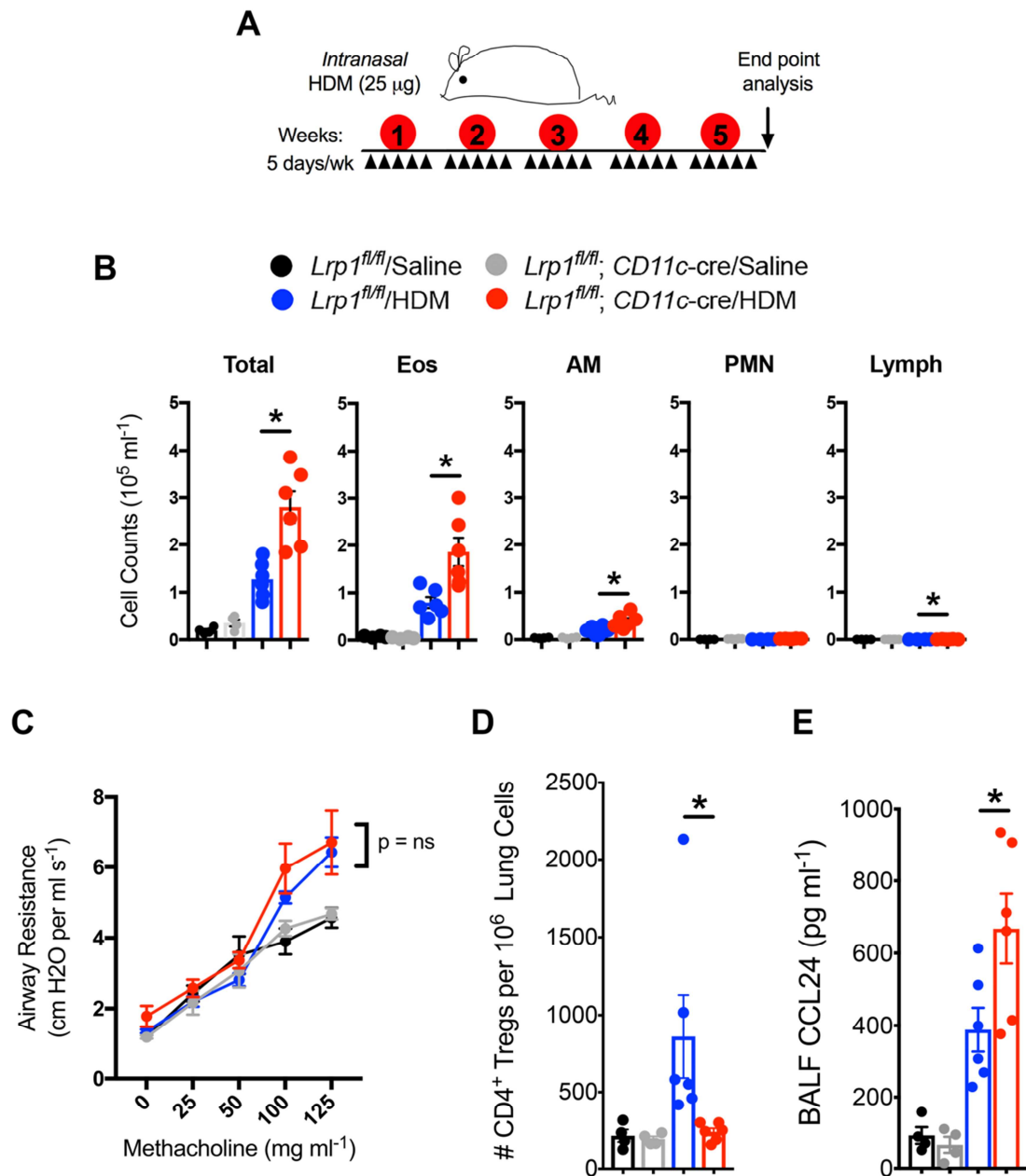
Selective recombination of LRP-1 in CD11c⁺ cells from *Lrp1^{fl/fl}*; *CD11c-Cre* mice.

A) LRP-1 expression by SSC^{lo}/CD11c⁺/Siglec F⁻/F4/80⁻/MHCII^{hi} lung DCs and SSC^{hi}/CD11c⁺/Siglec F⁺/F4/80⁺/MHCII^{lo} alveolar macrophages from *Lrp1^{fl/fl}*; *CD11c-Cre* mice were assessed by flow cytometry. LRP-1 expression is markedly reduced by both CD11c⁺ lung DCs and alveolar macrophages isolated from *Lrp1^{fl/fl}*; *CD11c-Cre* mice as compared to *Lrp1^{fl/fl}* mice ($P < 0.02$, *Lrp1^{fl/fl}*; *CD11c-Cre* versus *Lrp1^{fl/fl}* mice, $n = 4 - 5$ mice per group, one-way ANOVA with Sidak's multiple comparison test). B) Genomic DNA from lung DCs, lung epithelial cells (LECs), splenic DCs, and CD4⁺ T cells was prepared from *Lrp1^{fl/fl}* and *Lrp1^{fl/fl}*; *CD11c-Cre* mice. SSC^{lo}/Siglec F⁻/CD11c⁺/CD11b⁻/MHC II^{hi} DCs were isolated from lungs and spleens by flow sorting, which was also utilized to isolate SSC^{lo}/Siglec F⁻/CD11c⁻/CD11b⁻/EpCAM^{hi}/MHCII^{lo} lung epithelial cells and CD3⁺/CD4⁺ splenic T cells as previously described¹. PCR analysis with primers specific for the recombined allele was performed. A PCR product indicative of deletion of the floxed *Lrp1* gene is detected in CD11c⁺ dendritic cells from lungs and spleen, but not from LECs or CD4⁺ T cells. C) Bone-marrow derived dendritic cells and whole lung cell suspensions were prepared from *Lrp1^{fl/fl}* and *Lrp1^{fl/fl}*; *CD11c-Cre* mice and proteins were subjected to SDS-polyacrylamide gel electrophoresis and Western. LRP-1 protein levels are reduced in the BMDCs, but not lung tissue, from *Lrp1^{fl/fl}*; *CD11c-Cre* mice as compared to *Lrp1^{fl/fl}* mice, which is consistent with the CD11c-specific deletion of LRP-1.

References

¹ Mishra A, Brown AL, Yao XL, Yang ST, Park SJ, Liu CY, et al. Dendritic cells induce Th2-mediated airway inflammatory responses to house dust mite via DNA-dependent protein kinase. *Nature Communications* 2015; 6:6224.

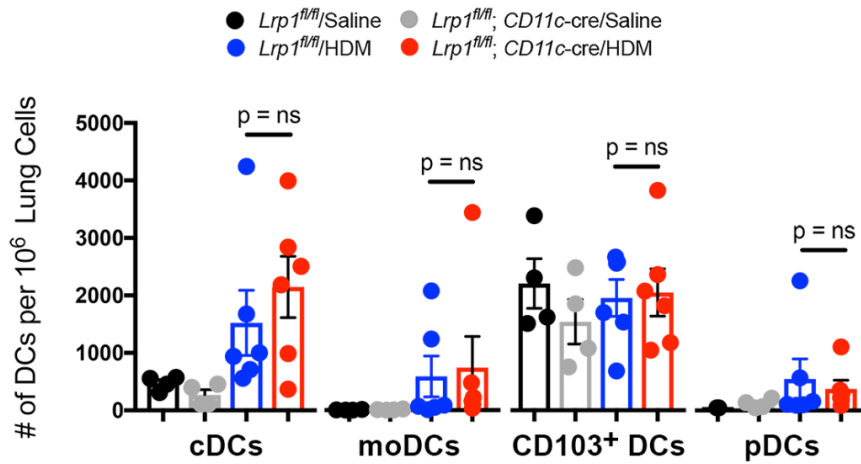
Repository eFigure 3.



Eosinophilic airway inflammation is dissociated from airway hyperreactivity in *Lrp1^{fl/fl}; CD11c-Cre* mice that were sensitized and challenged by intranasal administration of house dust mite without adjuvant. **A)** *Lrp1^{fl/fl}* and *Lrp1^{fl/fl}; CD11c-Cre* mice were sensitized and challenged by intranasal administration of 25 µg HDM without adjuvant, in 10 µl of saline, 5 days per week, for 5 weeks. **B)** The number of total BALF inflammatory cells and inflammatory cell types (eosinophils (Eos), alveolar

macrophages (AM), neutrophils (PMN) and lymphocytes (Lymph)) from saline- and HDM-challenged *Lrp1^{fl/fl}*, *CD11c-Cre* and *Lrp1^{fl/fl}* mice (n = 4 - 6 mice per group, * P < 0.05, HDM-challenged *Lrp1^{fl/fl}*, *CD11c-Cre* versus *Lrp1^{fl/fl}*, one-way ANOVA with Sidak's multiple comparison test). **C)** Airway resistance (cm H₂O per ml s⁻¹) to inhaled methacholine from HDM-challenged *Lrp1^{fl/fl}*, *CD11c-Cre* and *Lrp1^{fl/fl}* mice (n = 4 - 6 mice per group). (P = NS, two-way ANOVA). **D)** The number of CD4⁺/CD25⁺/FoxP3⁺ Tregs per 10⁶ total lung cells in HDM-challenged *Lrp1^{fl/fl}*, *CD11c-Cre* mice and *Lrp1^{fl/fl}* mice (n = 4 - 6 mice per group, *P < 0.05, one-way ANOVA with Sidak's multiple comparison test). **E)** BALF CCL24 levels in HDM-challenged *Lrp1^{fl/fl}*, *CD11c-Cre* mice and *Lrp1^{fl/fl}* mice. (n = 4 - 6 mice per group, *P < 0.01, one-way ANOVA with Sidak's multiple comparison test).

Repository eFigure 4.



The number of lung dendritic cells are not modified in *Lrp1^{fl/fl}; CD11c-Cre* mice that were sensitized and challenged by intranasal administration of house dust mite without adjuvant. *Lrp1^{fl/fl}* and *Lrp1^{fl/fl}; CD11c-Cre* mice were sensitized and challenged by intranasal administration of 25 μ g HDM without adjuvant, in 10 μ l of saline, 5 days per week, for 5 weeks. The percentage of lung DC monocyte-derived dendritic cells (moDCs), conventional myeloid dendritic cells (cDCs), CD103⁺ DCs, and plasmacytoid dendritic cells (pDCs) subsets were quantified by flow cytometry. Data are shown as number of each DC subset per 10⁶ total lung cells (P = NS, HDM-challenged *Lrp1^{fl/fl}; CD11c-Cre* versus *Lrp1^{fl/fl}* mice, n= 4 - 6 mice per group, one-way ANOVA with Sidak's multiple comparison test).