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CD36 and Platelet-Activating Factor Receptor Promote House Dust Mite Allergy Development

Preeyam S. Patel¹ and John F. Kearney

Over 89% of asthmatic children in underdeveloped countries demonstrate sensitivity to house dust mites (HDMs). The allergic response to HDMs is partially mediated by epithelial cell–derived cytokines that activate group 2 innate lymphoid cells, induce migration and activation of dendritic cells, and promote effector differentiation of HDM-specific TH2 cells. However, the contribution of innate receptor engagement on epithelial or dendritic cells by HDMs that ultimately mediates said innate and adaptive allergic responses is poorly understood. We and other investigators have demonstrated that HDMs express phosphorylcholine (PC) moieties. The major PC receptors involved in immune responses include CD36 and platelet-activating factor receptor (PAFR). Because CD36 and PAFR are expressed by epithelial cells and dendritic cells, and expression of these receptors is higher in human asthmatics, we determined whether engagement of CD36 or PAFR on epithelial or dendritic cells contributes to HDM allergy development. Testing bone marrow chimeric mice revealed that CD36 engagement on radioresistant cells and PAFR engagement on radioresistant and radiosensitive cells in the lung promote allergic responses to HDMs. Additionally, passive anti-PC IgM Abs administered intratracheally with HDMs decreased allergen uptake by epithelial cells and APCs in the lungs of C57BL/6 mice but not CD36^{−/−} or PAFR^{−/−} mice. These results show that CD36 and PAFR are important mediators of HDM allergy development and that inhibiting HDM engagement with PC receptors in the lung protects against allergic airway disease. *The Journal of Immunology*, 2017, 199: 000–000.

In the United States, asthma affects 8% of adults and 10% of children (1). Among these individuals, 40% of adults with allergies (1) and 89% of children with asthma are sensitive to house dust mite (HDM) allergen (2, 3). Innate and adaptive immunological cascades initiating and maintaining allergic responses to HDMs have been well characterized in mice. In response to HDMs, epithelial cells release cytokines, such as thymic stromal lymphopoietin (TSLP) and IL-33, which activate tissue-resident group 2 innate lymphoid cells (ILC2s) (4) and induce activation and migration of dendritic cells (DCs) (5, 6). Meanwhile, pulmonary CD103⁺ DCs prime HDM-specific TH2 cells in the draining lymph node (7). Once in the lungs, TH2 cell effector differentiation is driven by TSLP and IL-33 (8). TH2 cells produce cytokines that, along with ILC2s, drive eosinophilia, IgE production, and airway hyperresponsiveness (AHR) (4). Although epithelial cells and DCs appear to function separately to activate ILC2s and prime TH2 cells, respectively, extensive cross-talk occurs between DCs and epithelial cells (9–11).

However, the mechanisms by which HDMs engage these cells and initiate the innate and adaptive allergic responses are poorly understood. HDM allergen is complex, containing a major allergy-inducing protein, Der p 1, which is targeted by TH2 cells and IgE in HDM-allergic individuals (12–14). HDM particulates also contain many pathogen-associated molecular patterns, such as β -glucan, LPS, and chitin, which can engage innate immune receptors, such as dectin-1, dectin-2, TLR2, TLR4, and mannose receptor (15–17). Many studies have demonstrated that these and other innate receptors are involved in HDM allergy development; however, very few studies have clearly demonstrated to what extent engagement of these receptors on epithelial cells and/or DCs contributes to allergic disease. These few studies detail how dectin-2 on DCs, but not on epithelial cells (18), and TLR-4 on epithelial cells, but not on DCs (19), are involved in HDM allergy development.

Although the contributions of glycan-binding receptors, such as TLR-2 and the mannose receptor, to HDM allergy have already been investigated (15), phospholipid-binding receptor involvement in allergic responses is unclear. HDMs contain phosphorylcholine (PC) moieties (20–22). Two well-characterized PC receptors include CD36 and platelet-activating factor receptor (PAFR). CD36 (scavenger receptor) and PAFR (G protein–coupled receptor) differ in their expression patterns, signaling cascades, and functions (23, 24). Individually, these receptors are immunomodulatory in diseases involving PC-expressing bacteria, helminths, apoptotic cells, and oxidized lipids (23–30). Additionally, some evidence indicates that CD36 and PAFR synergistically promote the removal of apoptotic cells (28).

CD36 and PAFR are expressed on epithelial cells and DCs (23, 24, 31), and expression of both receptors is increased in asthmatics (32, 33). Based on these observations, we investigated whether HDM allergy development depended on CD36 or PAFR and whether engagement of these receptors on epithelial cells or DCs in the lung was involved in HDM-induced allergic disease. Induced or passively administered anti-PC IgM Abs are protective in disease models involving PC-expressing bacteria, parasites,

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Abbreviations used in this article: AHR, airway hyperresponsiveness; BALF, bronchoalveolar lavage fluid; DC, dendritic cell; HDM, house dust mite; ILC2, group 2 innate lymphoid cell; i.t., intratracheal(ly); medLN, mediastinal lymph node; PAFR, platelet-activating factor receptor; PC, phosphorylcholine; Rrs, respiratory system resistance; TGP, timothy grass pollen; TSLP, thymic stromal lymphopoietin.

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apoptotic cells, and metabolic products (34–39). Similarly, we have shown that increased anti-PC IgM levels in serum and lungs are associated with suppressed allergy development (22).

We demonstrated previously that HDM particulates contain PC epitopes (22). In this article, we demonstrate that HDM feces coexpress PC and Der p 1. In these studies, we show that, in mice lacking CD36 and/or PAFR, the uptake of HDM by pulmonary epithelial cells and DCs is impaired, and these mice exhibit suppressed development of allergic disease compared with C57BL/6 mice. Using bone marrow chimeric mice, we also demonstrate that CD36 engagement on radioresistant cells in the lung and PAFR engagement on radioresistant and radiosensitive cells in the lung contribute to priming of Der p 1-specific T cells and HDM allergy. We have previously shown that anti-PC IgM Abs decrease the uptake of HDMs by APCs in the lung (22). In this article, we demonstrate that anti-PC IgM Abs do not decrease HDM uptake in CD36- or PAFR-deficient mice, indicating that this effect depends on PC receptor expression in the lung.

Materials and Methods

Animals

C57BL/6 mouse breeders were originally obtained from The Jackson Laboratory. CD36^{-/-} mice were donated by Dr. M. Febbraio (University of Alberta) (27). PAFR^{-/-} mice, originally generated by Dr. T. Shimizu (University of Tokyo) (40), were obtained from Dr. T. Jilling (The University of Alabama at Birmingham). CD36^{-/-} and PAFR^{-/-} mice were crossed to generate CD36^{-/-} × PAFR^{-/-} double-knockout mice. All mice were maintained under specific pathogen-free conditions using approved animal protocols from the Institutional Animal Care and Use Committee at The University of Alabama at Birmingham.

Bone marrow chimera generation

Six- to eight-week-old C57BL/6, CD36^{-/-}, and PAFR^{-/-} mice were sublethally irradiated with 950 rad, delivered in two doses. On the same day, irradiated mice were reconstituted with 2×10^6 bone marrow cells from C57BL/6, CD36^{-/-} or PAFR^{-/-} mice and were rested for 12–15 wk.

HDM and timothy grass pollen allergen

HDM particulates for allergen challenge were prepared as previously described (22). HDMs, along with timothy grass pollen (TGP; *Phleum pratense*; Greer Laboratories), were labeled with Alexa Fluor 647 (Life Technologies), according to the manufacturer's instructions. Intact HDM fecal pellets were isolated by passing whole unmilled HDMs (Greer) through a 40-μm filter.

In vivo intratracheal challenge and the HDM allergy model

For intratracheal (i.t.) challenge, adult mice were anesthetized with 3–5% isoflurane and then immobilized on a vertical board. Suture string was looped around the upper incisors. To facilitate liquid pipetting into the oral cavity, the tongue was extended using blunt-end forceps, and the nares were manually plugged to promote inhalation of the liquid suspension. Adult mice were challenged i.t. with 5 μg of HDMs resuspended in 50 μl of PBS. Mice were rested for 7 d before i.t. challenge for five consecutive days with 5 μg of HDMs in 50 μl of PBS. Following the last HDM allergen challenge, mice were rested for 2 d before euthanization. Where indicated, the above procedure was also conducted using Alexa Fluor 488- or Alexa Fluor 647-labeled HDMs for sensitizations and subsequent challenges in the stated experimental groups.

For single-challenge experiments, mice were administered 20 μg of Alexa Fluor 647-labeled HDMs or Alexa Fluor 647-labeled TGP and were euthanized 2, 24, or 48 h later. When stated, mice were administered Alexa Fluor 647-labeled HDMs or TGP with PBS, 50 μg of anti-PC IgM Ab, or 50 μg of an irrelevant isotype-matched control Ab i.t. and were euthanized 24 h later.

Bronchoalveolar lavage fluid, lung, and mediastinal lymph node collection

A 5-ml lavage via trachea cannulation was used to extract cellular infiltrates from the bronchoalveolar space. Lungs were minced and treated with 1 mg/ml collagenase (Sigma) in 5 ml HBSS and 10 U/ml DNase I (Bovine Pancreas) for 40 min at 37°C. One third of these cells were used

for epithelial cell analysis, and the remaining cells were subjected to lymphocyte separation (CellGro). Mediastinal lymph node (medLN) cells were collected after mechanical disruption. All cells were manually enumerated using a hemocytometer.

Ab reagents, flow cytometry, and cell identification

Maintenance of hybridomas and plasmacytomas, Ab purification, and conjugation of these reagents to Alexa Fluor dyes were described previously (22). Whole, intact HDM fecal samples were stained with purified or Alexa Fluor-labeled monoclonal anti-PC IgM (BH8), anti-PC IgA (S107), anti-Der p 1 IgG1 (5H8; Indoor Biotechnologies), or isotype-control Abs. Staining with purified Abs was followed by staining with fluorescently conjugated secondary goat anti-mouse against the respective isotypes (SouthernBiotech, Life Technologies).

Bronchoalveolar lavage fluid (BALF), lung, and medLN cells were stained for flow cytometry with propidium iodide for excluding dead cells and with fluorochrome-conjugated Abs specific for the following molecules: CD3 (145-2C11), CD4 (GK1.5), CD11c (HL3), CD11b (M1/70), CD19 (1D3), CD36 (MZ1), CD44 (IM7), CD45 (30-F11), CD90.2 (53-2.1), CD103 (M290), CD117 (2B8), CD127 (SB/199), EpCAM (G8.8), Foxp3 (MF23), GATA3 (L50-823), B220 (RA3-6B2), IgE (R35-72), Siglec-F (E50-2440), Ly6G (IA8), Ly6C (AL-21), PAFR (ab104162), Gr-1 (RB6-8C5), KLRG1 (2F1), ST2 (RMST2-2), TER-119, CD49b (DX5), TSLP (eBio28F12), and IL-33 (396118). All Abs were purchased from BD, with the exception of EpCAM and TSLP (eBioscience), PAFR (Abcam), IL-33 (R&D Systems), and MZ1 (41). medLN T cells were also stained with allophycocyanin-conjugated I-A(b) tetramers (National Institutes of Health Tetramer Core Facility) containing human CLIP (PVSKMRMATPLLMQA) or Der p 1 (CQIYPPNVNKI) peptides (42, 43). To detect epithelial expression of TSLP or IL-33, as well as CD4 T cell expression of Foxp3 or GATA3, cells were fixed and permeabilized with an eBioscience Transcription Factor Staining Buffer Set (00-5523-00), using the manufacturer's instructions. Cells were also stained to detect CD36 or PAFR expression (as shown in Supplemental Fig. 1). Cells of interest were identified as described in Fig. 2B and Supplemental Fig. 2. All flow cytometry analyses were performed on a FACSCalibur or LSR II (both from BD Biosciences) and analyzed using FlowJo software (TreeStar).

ELISA

Anti-Der p 1-IgE was detected by coating plates with 2 μg/ml natural Der p 1 (Indoor Biotechnologies), and serum Ab was detected using alkaline phosphatase-conjugated goat anti-mouse IgE (SouthernBiotech). Total IgE levels were determined by coating plates with 2 μg/ml rat anti-mouse IgE, and standard curves were prepared with known concentrations of IgE Abs (all from Southern Biotechnology). For all ELISA assays, p-nitrophenyl phosphate substrate (Sigma) was added, and color development was detected with a SPECTROstar Omega reader (BMG LABTECH) at 405 nm.

Single-frequency forced oscillation technique for mechanical ventilation

Mice were anesthetized with ketamine xylazine (100 mg/kg) and pancuronium bromide (0.8 mg/kg). Tracheas was cannulated with an 18-G tube connected to the respiratory and expiratory ports of a flexiVent ventilator (SCIREQ), from which each mouse was ventilated at a rate of 160 breaths per minute. After acquiring baseline resistance measurements without challenge, increasing concentrations (10–50 mg/ml) of methacholine (Sigma) were vaporized, and total respiratory system resistance (Rrs) was recorded every 12 s continuously for up to 3 min. Averages from each methacholine dose were calculated from four to six mice per group to determine AHR.

Histology and fluorescence staining

Whole HDM fecal pellets were cytocentrifuged (Shandon Elliott) onto glass slides at 1000 rpm for 5 min and stained with Alexa Fluor 647-conjugated anti-PC IgA Ab (S107) or anti-Der p 1 IgG1 Ab (Indoor Biotechnologies), followed by Alexa Fluor 488-conjugated anti-mouse IgG1 Ab. Replicate slides were also stained with Alexa Fluor 647-conjugated isotype-control Ab (J558) or purified mouse IgG1 isotype-control Ab (MOPC-21; BD), followed by Alexa Fluor 488-conjugated anti-mouse IgG1 Ab. Lungs were embedded in paraffin, as described previously (22). Six-micron-thick paraffin-embedded lung sections were cut (Leica RM2235), rehydrated, and stained with H&E (Sigma), according to the manufacturer's instructions, before being dehydrated and mounted in a xylene-based mounting medium (Poly-mount xylene). Sections and cells were imaged with a Leica/Leitz DMRB fluorescence microscope equipped with appropriate filter cubes.

Sample size and statistics

Data are the mean $\pm$ SEM from three independent experiments with 4–10 mice per group. Statistical calculations were performed with Prism 4.0 software (GraphPad). Comparison of three or more groups was performed using a one-way ANOVA test, followed by Tukey post hoc analysis. Only statistically significant differences between C57BL/6 mice and reconstituted or receptor-deficient mice are shown. Data between two groups were analyzed using a two-tailed unpaired *t* test to determine statistical significance. In the figures, statistically significant differences are indicated by **p* < 0.05, ***p* < 0.01, and ****p* < 0.001.

Results

Der p 1 and PC are coexpressed on HDM feces

Separate studies have demonstrated that HDM feces contains Der p 1 (44), and HDMs expresses PC moieties (20, 22). Abs against Der p 1 (5GH) (Fig. 1A), and IgM (BH8) and IgA (S107) Abs to PC (Fig. 1B) individually react with HDM fecal pellets. Flow cytometry and fluorescence microscopy revealed that most of the HDM fecal particles coexpress Der p 1 and PC (Fig. 1C–G).

HDM uptake by pulmonary APCs and epithelial cells is impaired in mice lacking CD36 and/or PAFR

To determine whether CD36 and/or PAFR mediate HDM uptake, we challenged C57BL/6, CD36^{-/-}, PAFR^{-/-}, and CD36^{-/-} $\times$ PAFR^{-/-} mice i.t. with fluorescently labeled HDMs and then calculated the number of APCs or epithelial cells containing HDMs. We assessed

HDM uptake by alveolar macrophages, epithelial cells, and four subtypes of DCs at 2, 24, or 48 h postchallenge, respectively, to reflect the time point for optimal phagocytosis of each cell type (7, 45–47) (Fig. 2A). Pulmonary APC subsets were identified as previously described (48) (Fig. 2B). Following HDM challenge, lower numbers of alveolar macrophages (Fig. 2C), epithelial cells (Fig. 2D), CD11b⁺CD11c⁺ DCs (Fig. 2E), CD11c⁺CD103⁻ DCs (Fig. 2F), CD11c⁺CD103⁺ DCs (Fig. 2G), and CD11b⁺Ly6C⁺ DCs (Fig. 2H) from mice lacking CD36 and/or PAFR had taken up HDMs compared with C57BL/6 mice. When we analyzed HDM uptake by alveolar macrophages, epithelial cells, and CD103⁺ DCs from CD36^{-/-}, PAFR^{-/-} and CD36^{-/-} $\times$ PAFR^{-/-} mice earlier or later than the frame time described, HDM uptake was also impaired (Supplemental Fig. 3). Thus, APCs and epithelial cells from CD36^{-/-}, PAFR^{-/-}, and CD36^{-/-} $\times$ PAFR^{-/-} mice are defective in their ability to take up HDMs in the lung. Of the DC subtypes that had taken up HDMs in C57BL/6 mice, most (67%) were CD11c⁺CD103⁺ DCs (Fig. 2I). Therefore, the only DC subset analyzed in the rest of our studies was the CD11c⁺CD103⁺ phenotype.

CD36 and PAFR are essential for induction of HDM allergy

To determine whether CD36 or PAFR expression is necessary for the development of HDM-induced allergy, C57BL/6, CD36^{-/-}, PAFR^{-/-}, and CD36^{-/-} $\times$ PAFR^{-/-} mice were repeatedly sensitized and challenged with HDMs (Fig. 3A). As controls, groups

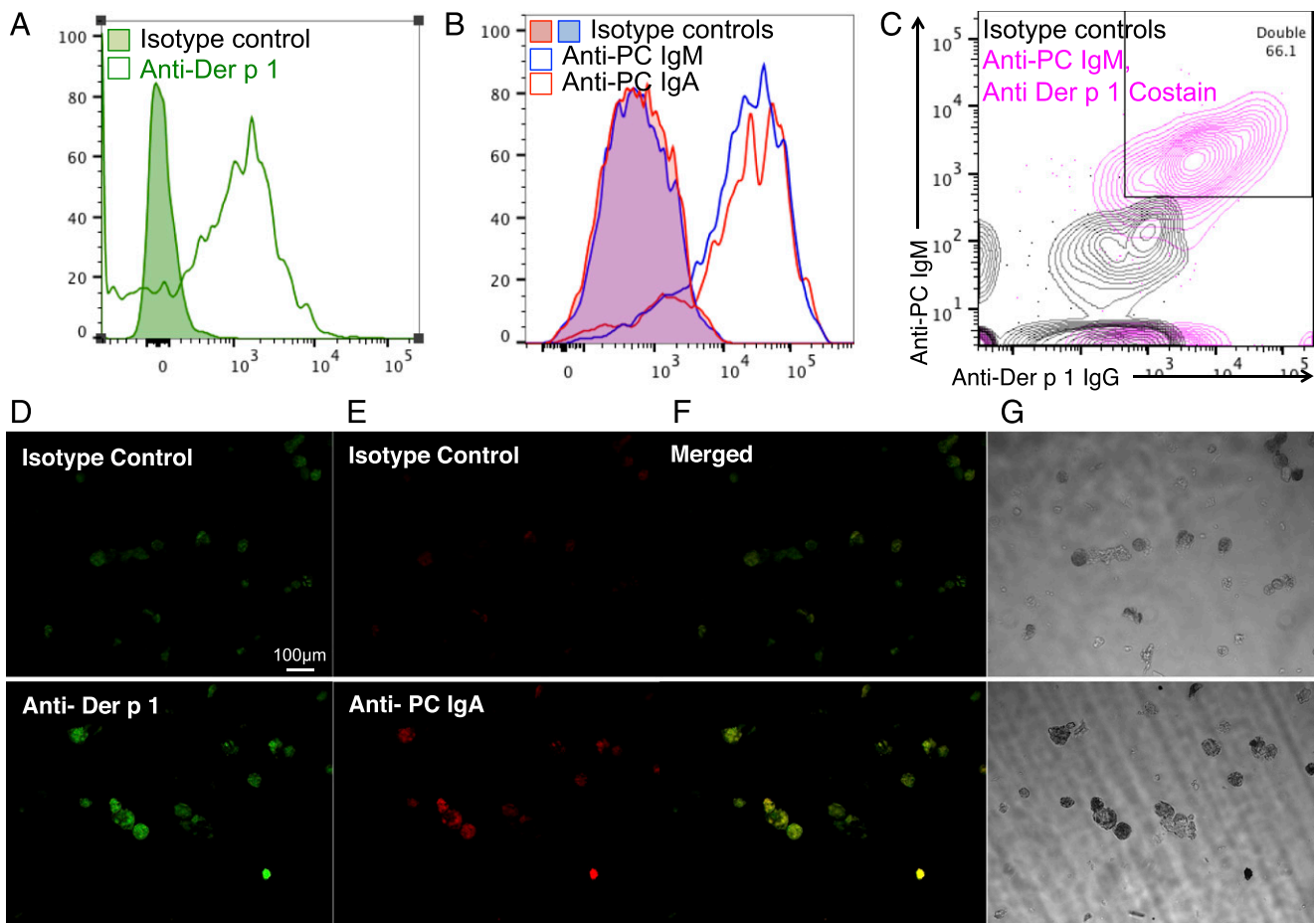


FIGURE 1. HDM feces coexpress Der p 1 and PC. HDM fecal pellets were stained with purified monoclonal anti-Der p 1 IgG1 (5H8) (A), anti-PC IgM (BH8) (B), anti-PC IgA (S107), anti-Der p 1 IgG1 and anti-PC IgM (C), or isotype-matched control Abs (A–C). Ab binding was detected after incubation with fluorescently conjugated goat anti-mouse secondary Abs against the respective isotypes for analysis by flow cytometry. Suspensions of whole HDM fecal pellets were cytocentrifuged (Shandon Elliott) onto glass slides and stained with anti-Der p 1 IgG1 Ab (D) and Alexa Fluor 647-conjugated anti-PC IgA Ab (E), followed by Alexa Fluor 488-conjugated anti-mouse IgG1 Ab. Replicate slides were stained with isotype-control Abs (D and E), and Ab costaining was determined by fluorescence microscopy (D–F). (G) Phase-contrast view of (D)–(F).

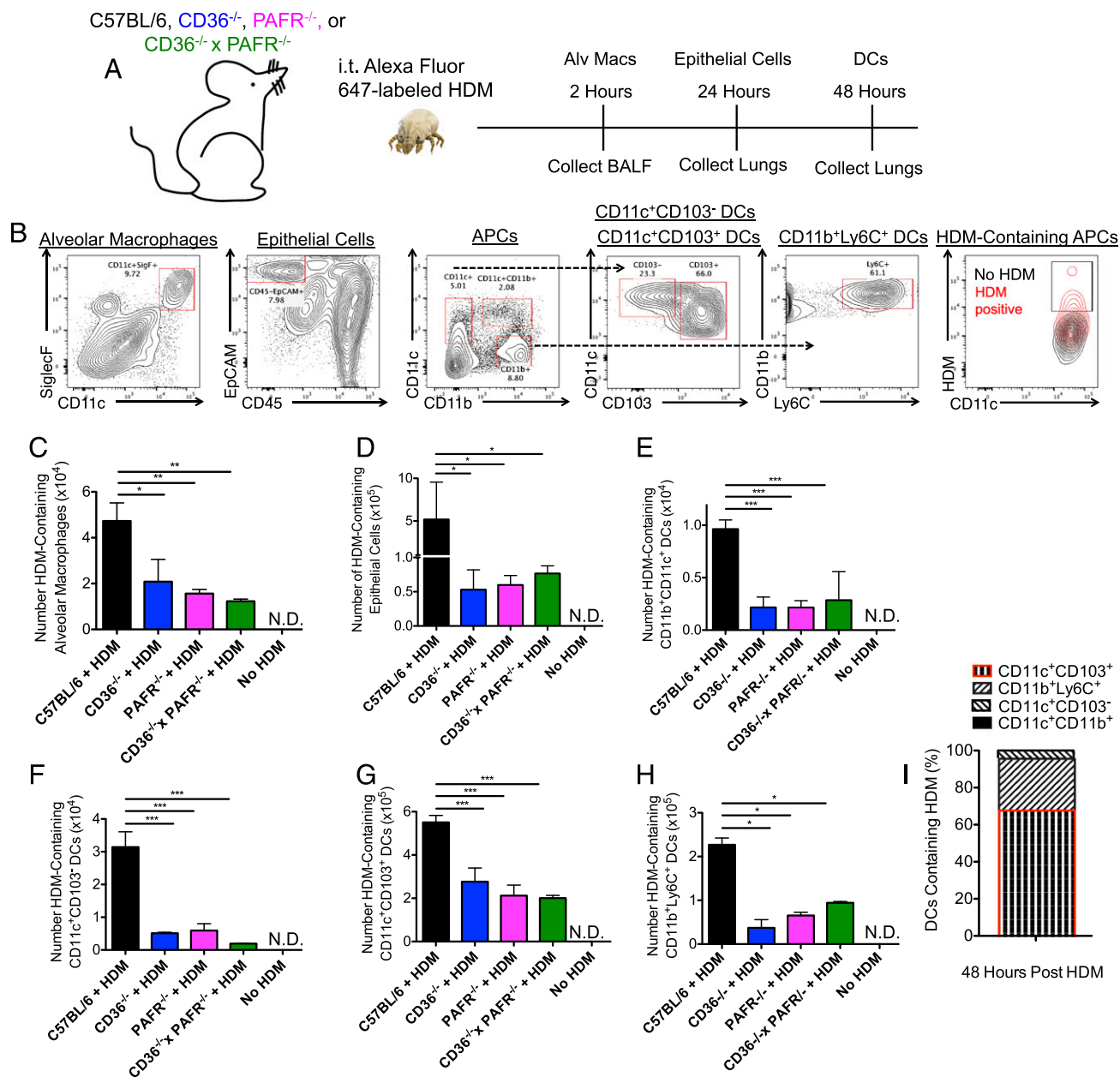


FIGURE 2. Pulmonary alveolar macrophages, epithelial cells, and CD103⁺ DCs from CD36^{-/-} and/or PAFR^{-/-} mice are defective in HDM particle uptake in vivo. **(A)** Six- to eight-week-old C57BL/6, CD36^{-/-}, PAFR^{-/-}, and CD36^{-/-} × PAFR^{-/-} mice were challenged i.t. with 20 µg of fluorescently labeled HDMs. Alveolar macrophages, epithelial cells, and DCs were isolated 2, 24, and 48 h following challenge, respectively. **(B)** Representative gating scheme for alveolar macrophages (SiglecF⁺CD11c⁺), epithelial cells (CD45⁺EpCAM⁺), CD11c⁺CD11b⁺ DCs, CD11c⁺CD103⁺ DCs, CD11b⁺Ly6C⁺ DCs, and HDM-containing APCs. Total frequencies of pulmonary alveolar macrophages **(C)**, epithelial cells **(D)**, CD11b⁺CD11c⁺ DCs **(E)**, CD11c⁺CD103⁻ DCs **(F)**, CD11c⁺CD103⁺ DCs **(G)**, and CD11b⁺Ly6C⁺ DCs **(H)** containing HDMs from C57BL/6, CD36^{-/-}, PAFR^{-/-}, and CD36^{-/-} × PAFR^{-/-} mice were determined by flow cytometry. **(I)** Percentages of pulmonary DCs containing HDMs for four subsets from C57BL/6 mice. Values represent the mean ± SEM from three independent experiments with 5–10 mice per group. Data were analyzed by a two-tailed unpaired *t* test comparing CD36^{-/-}, PAFR^{-/-}, and CD36^{-/-} × PAFR^{-/-} mice receiving HDMs to C57BL/6 + HDM mice. Values lower than the limits of detection are noted as not detectable (N.D.). Statistically significant differences are indicated as **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

of C57BL/6, CD36^{-/-}, PAFR^{-/-}, and CD36^{-/-} × PAFR^{-/-} mice that were not challenged with HDMs were also analyzed. Compared with C57BL/6 mice, the number of pulmonary alveolar macrophages and lung and lymph node CD11c⁺CD103⁺ DCs from CD36^{-/-}, PAFR^{-/-}, and CD36^{-/-} × PAFR^{-/-} mice that had taken up HDMs is significantly lower (Fig. 3B, 3C). Following HDM challenge, CD36^{-/-}, PAFR^{-/-}, and CD36^{-/-} × PAFR^{-/-} mice also have decreased numbers of CD4⁺ T cells, eosinophils, and neutrophils in their BALF and lungs (Fig. 3D, 3G), as well as CD11c⁺CD103⁺ DCs, ILC2s, basophils, and mast cells in their

lungs (Fig. 3H), compared with C57BL/6 mice. Additionally, compared with C57BL/6 mice, CD36^{-/-}, PAFR^{-/-}, and CD36^{-/-} × PAFR^{-/-} mice develop less AHR (Fig. 3E), have fewer numbers of TSLP- or IL-33-producing epithelial cells in their lungs (Fig. 3F), and express lower levels of serum Der p 1-specific IgE (Fig. 3I). CD36^{-/-}, PAFR^{-/-}, and CD36^{-/-} × PAFR^{-/-} mice also exhibit smaller medLNs (Fig. 3J, 3K) that are composed of fewer B cells, DCs, and CD4⁺ T cells that are GATA3⁺ or Foxp3⁺ compared with C57BL/6 mice (Fig. 3L, 3M). Therefore, CD36 and PAFR play a major role in HDM-induced allergy development.

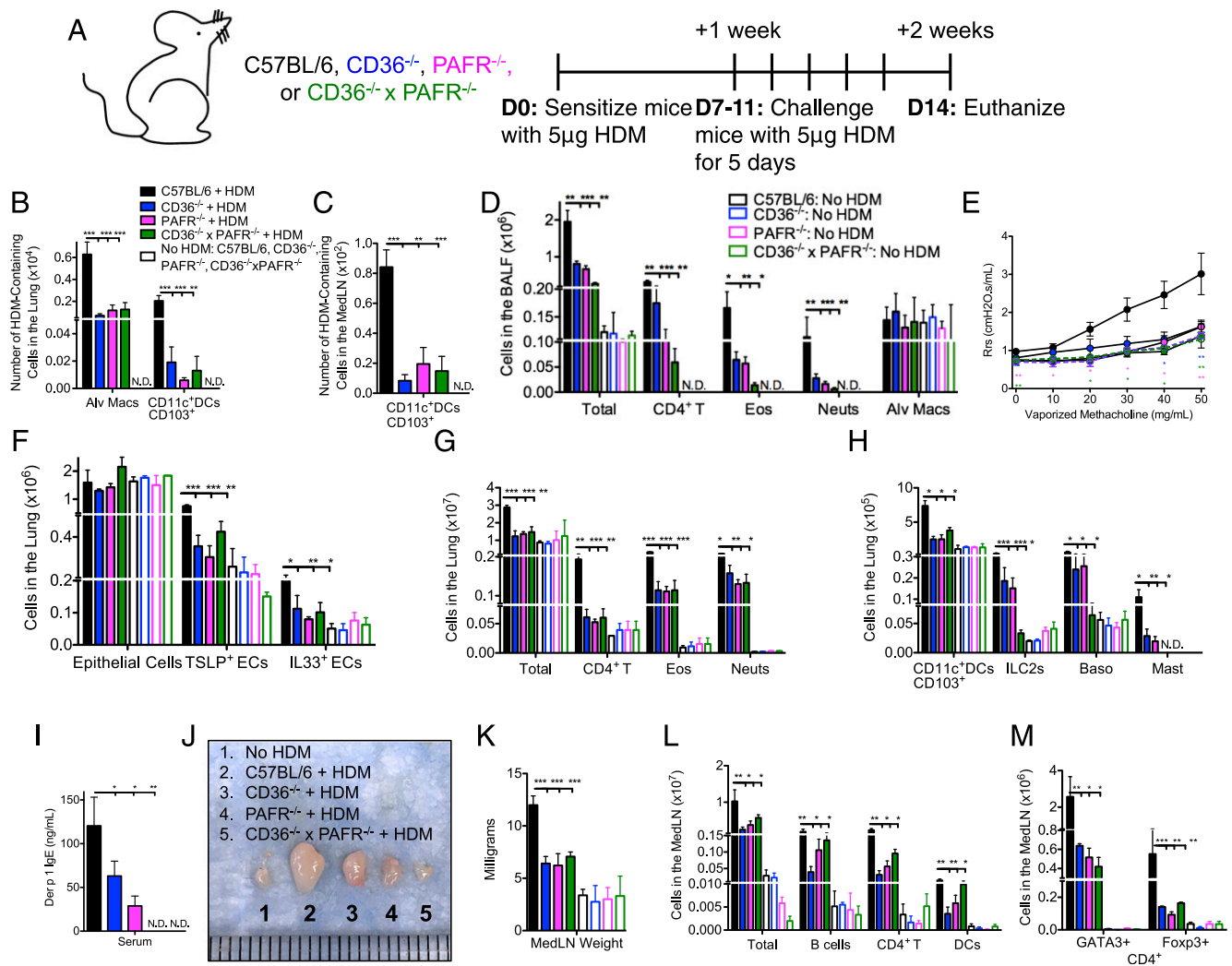


FIGURE 3. CD36^{-/-}, PAFR^{-/-}, and CD36^{-/-} × PAFR^{-/-} mice exhibit decreased allergic responses to HDMs compared with C57BL/6 mice. **(A)** Six- to eight-week-old C57BL/6, CD36^{-/-}, PAFR^{-/-}, and CD36^{-/-} × PAFR^{-/-} mice were sensitized and challenged i.t. with HDMs. As a control, littermate C57BL/6, CD36^{-/-}, PAFR^{-/-}, and CD36^{-/-} × PAFR^{-/-} mice were not administered HDMs. Numbers of HDM-containing APCs from the lung **(B)** and medLN **(C)**. **(D)** Numbers of cells in BALF. **(E)** AHR determined by Rrs following challenge with the indicated concentration of vaporized methacholine. Number of cytokine-producing epithelial cells **(F)** and lymphocytes **(G and H)** in the lung. **(I)** Levels of serum anti-Der p 1-IgE measured by ELISA. Representative image **(J)**, weight **(K)**, and cellular composition **(L and M)** of medLNs. Cells were identified by flow cytometry, as documented in Supplemental Fig. 2. Values represent the mean ± SEM from three independent experiments with four to eight mice per group. Data were analyzed by a two-tailed unpaired *t* test comparing CD36^{-/-}, PAFR^{-/-}, and CD36^{-/-} × PAFR^{-/-} mice receiving HDMs to C57BL/6 + HDM mice. Values lower than the limits of detection are noted as not detectable (N.D.). Statistically significant differences are indicated as **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

CD36 engagement on radioresistant airway structural cells is necessary for HDM allergy development

To assess whether CD36 expression on airway structural cells or hematopoietic cells contributes to HDM allergic responses, we generated chimeric mice by irradiating C57BL/6 and CD36^{-/-} mice and reconstituting them with C57BL/6 or CD36^{-/-} bone marrow to generate donor → recipient mice (Fig. 4A). As expected, CD36 is not detected on the epithelial cells of CD36^{-/-} → CD36^{-/-} or C57BL/6 → CD36^{-/-} mice or on the CD11c⁺ CD103⁺ DCs of CD36^{-/-} → CD36^{-/-} or CD36^{-/-} → C57BL/6 mice (Fig. 4B, 4C).

Following reconstitution, chimeric mice were sensitized i.t. and challenged with HDMs, and the allergic response to HDMs was assessed. Compared with C57BL/6 → C57BL/6 mice, CD36^{-/-} → CD36^{-/-} and C57BL/6 → CD36^{-/-} mice have lower frequencies of epithelial cells expressing TSLP or IL-33 (Fig. 4D) and ILC2s (Fig. 4E). Additionally, CD36^{-/-} → CD36^{-/-} and C57BL/6 → CD36^{-/-} mice exhibit less HDM uptake by pulmonary alveolar

macrophages, as well as lung and medLN CD11c⁺CD103⁺ DCs, compared with C57BL/6 → C57BL/6 mice (Fig. 4F, 4G).

CD36^{-/-} → CD36^{-/-} and C57BL/6 → CD36^{-/-} mice have lower frequencies of CD4⁺ T cells, eosinophils, and neutrophils in their BALF and lungs (Fig. 5A, 5B), lower levels of IgE in the BALF (Fig. 5C), reduced cellular infiltrates, as detectable by H&E staining (Fig. 5D–H), and decreased AHR (Fig. 5I) compared with C57BL/6 → C57BL/6 mice following HDM challenge. Additionally, medLNs of CD36^{-/-} → CD36^{-/-} and C57BL/6 → CD36^{-/-} mice are reduced in size and weight (Fig. 6A, 6B), contain fewer B cells, CD4⁺ T cells, and DCs, and have lower frequencies of CD44^{high}, Der p 1-specific, GATA3⁺, and Foxp3⁺ CD4⁺ T cells (Fig. 6D–F). CD36^{-/-} → CD36^{-/-} and C57BL/6 → CD36^{-/-} mice also have lower levels of serum Der p 1-specific IgE compared with C57BL/6 → C57BL/6 mice (Fig. 6C). Therefore, CD36 engagement on radioresistant cells in the lung, such as epithelial cells, is involved in the allergic response to HDMs.

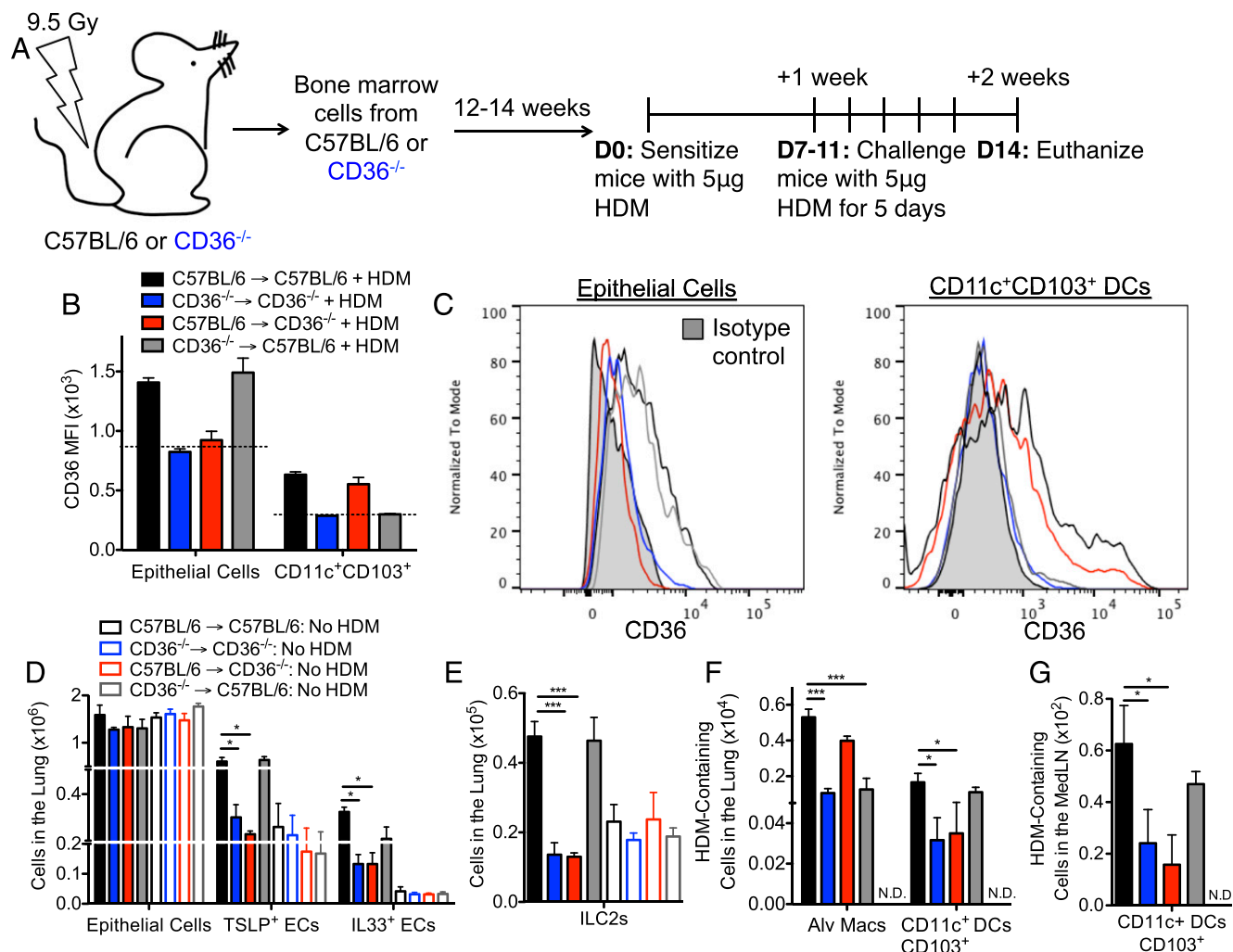


FIGURE 4. CD36 engagement on radioresistant airway structural cells is associated with epithelial cytokine production, ILC2 proliferation, and HDM uptake by pulmonary APCs. (A) C57BL/6 and $CD36^{-/-}$ mice were irradiated (950 rad) and reconstituted with C57BL/6 or $CD36^{-/-}$ bone marrow to generate the following donor → recipient bone marrow chimeric mice: C57BL/6 → C57BL/6, $CD36^{-/-}$ → $CD36^{-/-}$, C57BL/6 → $CD36^{-/-}$, and $CD36^{-/-}$ → C57BL/6. Chimeric mice were rested for 12–15 wk, sensitized, and challenged with HDM allergen. (B and C) CD36 expression on pulmonary epithelial cells or CD11c⁺CD103⁺ DCs was determined from chimeric mice by flow cytometry. The dotted horizontal line in (B) and the shaded graph in (C) correspond to isotype-control Ab for anti-CD36. Numbers of epithelial cells expressing TSLP or IL-33 (D) and ILC2s (E) in the lung. Numbers of alveolar macrophages (F) and CD11c⁺CD103⁺ DCs (G) containing HDMs from the lung and medLNs. Validation of CD36 staining is shown in Supplemental Fig. 1. Values represent the mean ± SEM from three independent experiments with four to eight mice per group. Values lower than the limits of detection are noted as not detectable (N.D.). Data were analyzed by a two-tailed unpaired *t* test comparing all bone marrow chimeric mice to C57BL/6 → C57BL/6 mice. Statistically significant differences are indicated as **p* < 0.05, ****p* < 0.001.

PAFR engagement on radioresistant and radiosensitive cells in the lung is involved in HDM allergy development

Having determined that CD36 expression on radioresistant airway structural cells is necessary for HDM allergy, we next asked whether PAFR expression on pulmonary structural cells or hematopoietic-derived cells contributes to HDM-induced allergic responses. We generated chimeric mice by irradiating C57BL/6 and PAFR^{-/-} mice and reconstituting them with C57BL/6 or PAFR^{-/-} bone marrow (Fig. 7A). As expected, PAFR is not detectable on the epithelial cells of PAFR^{-/-} → PAFR^{-/-} or C57BL/6 → PAFR^{-/-} mice or on the DCs or macrophages of PAFR^{-/-} → PAFR^{-/-} or PAFR^{-/-} → C57BL/6 mice (Fig. 7B, 7C).

Following HDM challenge, only PAFR^{-/-} → PAFR^{-/-} mice have fewer TSLP- or IL-33-producing epithelial cells (Fig. 7D), ILC2s (Fig. 7E), and HDM-containing pulmonary alveolar macrophages and CD11c⁺CD103⁺ DCs from the lung and medLN (Fig. 7F, 7G). PAFR^{-/-} → PAFR^{-/-} mice also have decreased frequencies of CD4⁺ T cells, eosinophils, and neutrophils in their

BALF and lungs (Fig. 8A, 8B), reduced levels of IgE in their BALF (Fig. 8C), decreased numbers of cellular infiltrates detected by H&E staining (Fig. 8D–H), and decreased AHR (Fig. 8I) compared with C57BL/6 → C57BL/6 mice.

Additionally, only PAFR^{-/-} → PAFR^{-/-} mice have medLNs that are reduced in size and weight (Fig. 9A, 9B) and contain fewer B cells, CD4⁺ T cells, and DCs, along with fewer CD44^{high}, Der p 1-specific, GATA3⁺, and Foxp3⁺ CD4⁺ T cells (Fig. 9D–F). PAFR^{-/-} → PAFR^{-/-} mice also have lower levels of serum Der p 1-specific IgE compared with C57BL/6 → C57BL/6 mice (Fig. 9C). Therefore, PAFR expression on radioresistant and hematopoietic cells in the lung, such as epithelial cells and DCs, respectively, is required for the allergic response to HDMs.

In the absence of CD36 or PAFR, anti-PC IgM Abs no longer decrease HDM uptake by epithelial cells or APCs in the lung

The data above demonstrate that CD36 and PAFR are major contributors to HDM-induced allergic disease. Having previously

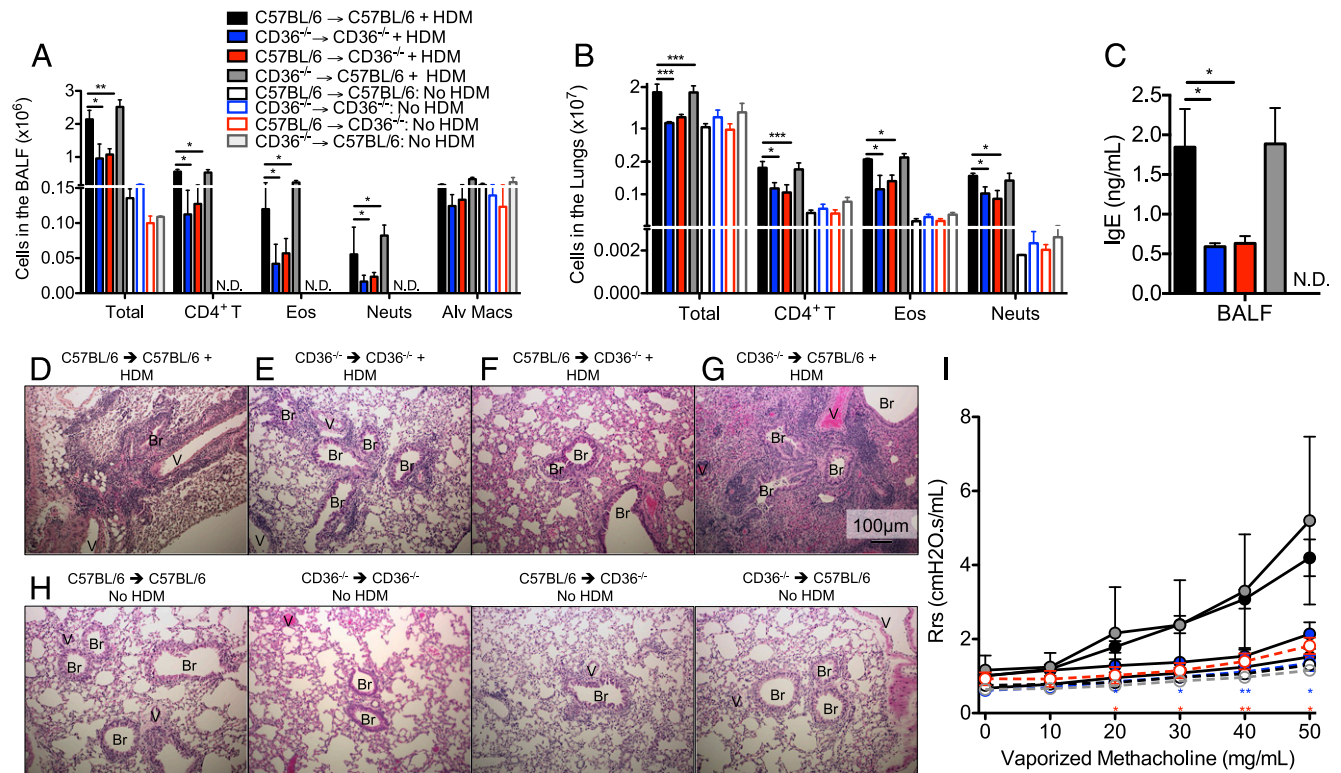


FIGURE 5. CD36 engagement on radioresistant airway structural cells is involved in the pulmonary allergic disease in response to HDMs. (**A** and **B**) Cells were enumerated from BALF and lungs. (**C**) Levels of IgE in BALF were determined by ELISA. (**D–H**) Paraffin-embedded lungs were stained with H&E. Br, bronchus; V, vessel. (**I**) AHR was determined by Rrs following challenge with the indicated concentration of vaporized methacholine. Values are the mean $\pm$ SEM from three independent experiments with four to eight mice per group. Values lower than the limits of detection are noted as not detectable (N.D.). Data were analyzed by a two-tailed unpaired *t* test comparing all bone marrow chimeric mice to C57BL/6 $\rightarrow$ C57BL/6 mice. Statistically significant differences are indicated as **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

demonstrated that anti-PC IgM decreases the uptake of HDM particles by APCs in the lung (22), we determined whether this effect was mediated by inhibited engagement of CD36 or PAFR.

Labeled HDMs were administered i.t. to C57BL/6, CD36^{-/-}, and PAFR^{-/-} mice in the presence of PBS alone, an isotype-control Ab, or anti-PC IgM Ab (Fig. 10A). The number of alveolar

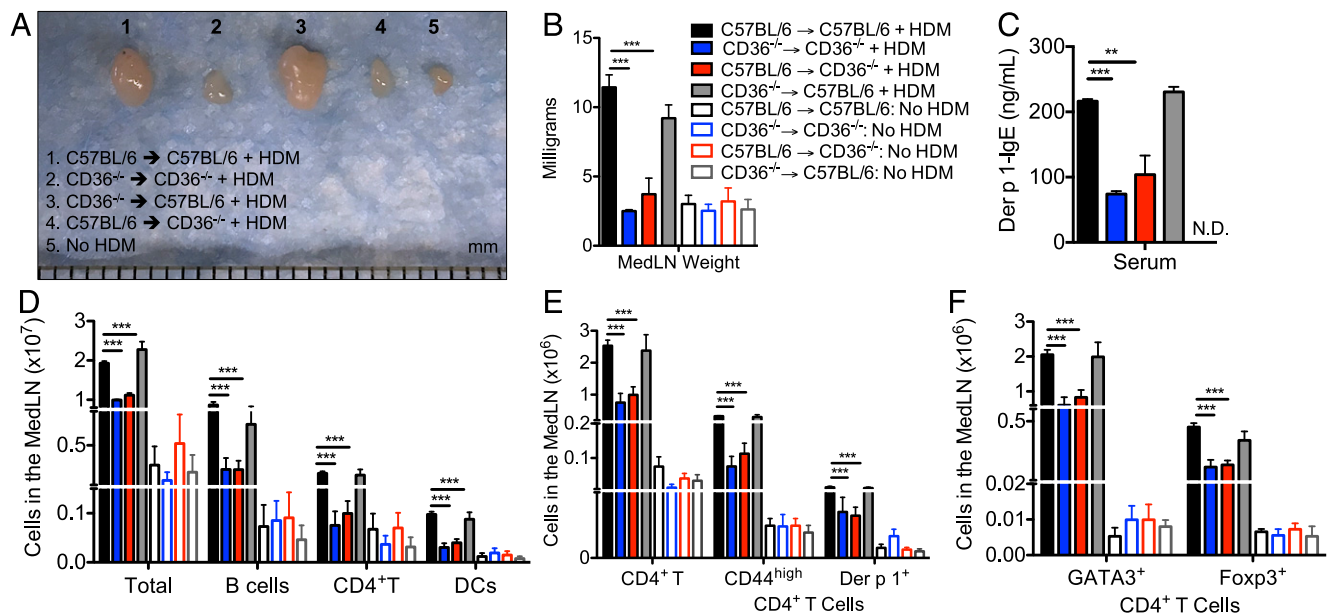


FIGURE 6. CD36 engagement on radioresistant airway structural cells is involved in Der p 1-specific TH2 cell priming in the medLN. Representative image (**A**), weight (**B**), and cellular composition (**D**) of medLNs. (**C**) Serum Der p 1-IgE, as determined by ELISA. Number of CD4⁺ T cells from the medLN that are CD44^{high}, Der p 1 specific (**E**) or GATA3⁺ or Foxp3⁺ (**F**), as determined by flow cytometry. Identification of tetramer-specific or Foxp3⁺ CD4⁺ T cells is documented in Supplemental Fig. 2. Values are the mean $\pm$ SEM from three independent experiments with four to eight mice per group. Values lower than the limits of detection are noted as not detectable (N.D.). Data were analyzed by a two-tailed unpaired *t* test comparing all bone marrow chimeric mice to C57BL/6 $\rightarrow$ C57BL/6 mice. Statistically significant differences are indicated as ***p* < 0.01, ****p* < 0.001.

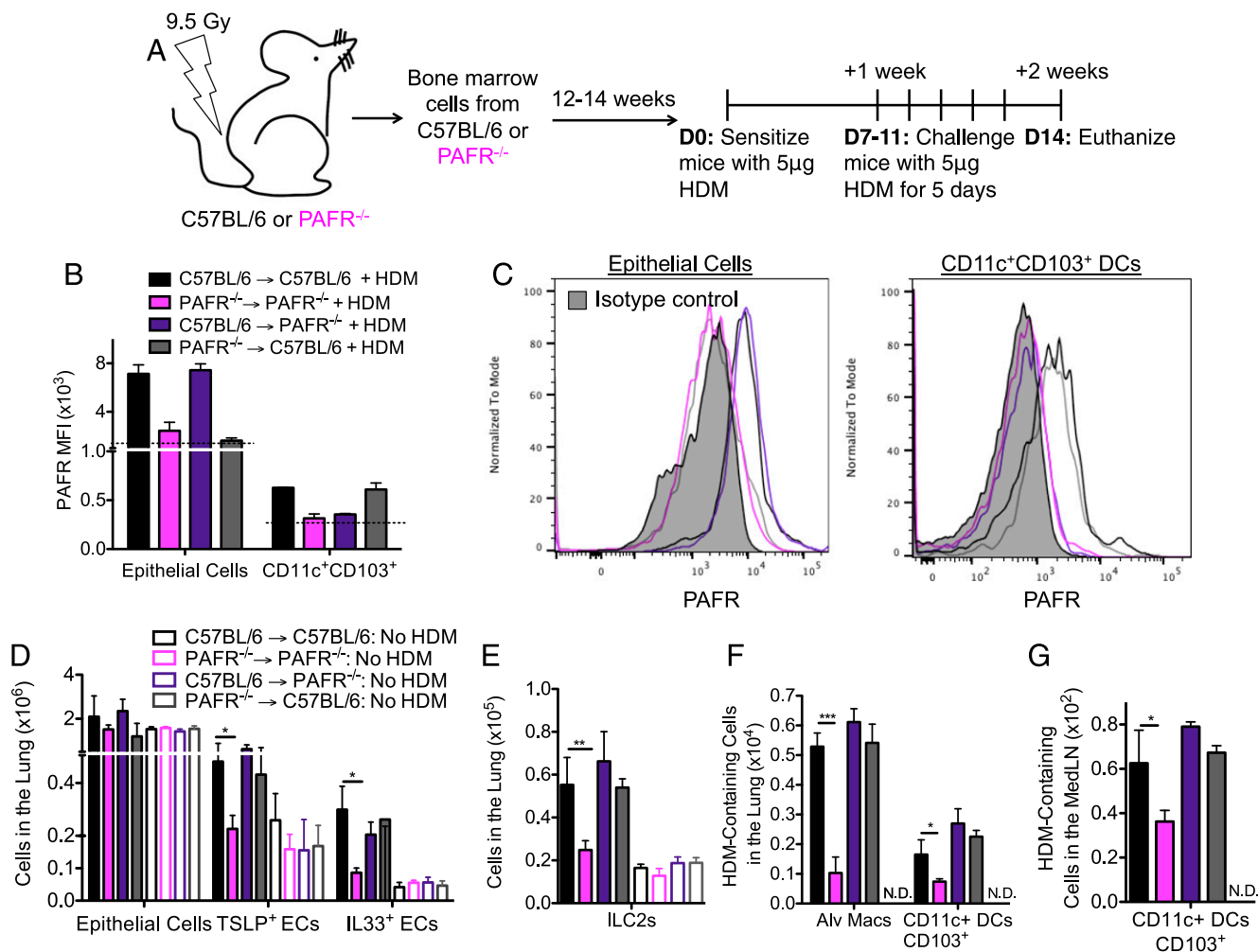


FIGURE 7. PAFR engagement on radioresistant airway structural cells and radiosensitive hematopoietic-derived cells is involved in epithelial cytokine production, ILC2 proliferation, and HDM uptake by pulmonary APCs. (A) C57BL/6 or PAFR^{-/-} mice were irradiated (950 rad) and reconstituted with C57BL/6 or PAFR^{-/-} bone marrow to generate the following bone marrow chimera mice: C57BL/6 → C57BL/6, PAFR^{-/-} → PAFR^{-/-}, C57BL/6 → PAFR^{-/-}, and PAFR^{-/-} → C57BL/6. Mice were rested for 12–15 wk, sensitized, and challenged with HDM allergen. (B and C) PAFR expression on pulmonary epithelial cells, DCs, and macrophages was determined from chimeric mice (dotted line and shaded graph = isotype control). Numbers of epithelial cells making TSLP or IL-33 (D) and ILC2s (E) in the lung. Numbers of alveolar macrophages (F) and CD11c⁺CD103⁺ DCs (G) containing HDMs from the lung and medLN. Validation of PAFR staining is shown in Supplemental Fig. 1. Values represent the mean ± SEM from three independent experiments with four to eight mice per group. Values lower than the limits of detection are noted as not detectable (N.D.). Data were analyzed by a two-tailed unpaired *t* test comparing all bone marrow chimeric mice to C57BL/6 → C57BL/6 mice. Statistically significant differences are indicated as **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

macrophages, epithelial cells, or CD103⁺ DCs that had taken up HDMs was determined 24 h later. As a control, we administered labeled TGP, which we confirmed did not express PC, in place of HDMs (Fig. 10A). As expected, HDM uptake by APCs and epithelial cells is defective in CD36- and PAFR-deficient mice (Fig. 10B–D) compared with C57BL/6 mice, consistent with our findings in Fig. 2. Although anti-PC IgM Ab decreases HDM uptake by alveolar macrophages (Fig. 10B), epithelial cells (Fig. 10C), and CD103⁺ DCs (Fig. 10D) in the lungs of C57BL/6 mice, there is no further decrease in CD36^{-/-} or PAFR^{-/-} mice (Fig. 10B–D). Additionally, TGP uptake is not altered in mice lacking CD36 or PAFR, and anti-PC IgM Ab did not decrease TGP uptake by lung DCs (Fig. 10E). Therefore, anti-PC IgM-mediated inhibition of HDM uptake largely depends on CD36 and PAFR expression.

Discussion

In these studies, we demonstrate that HDM particles express PC in addition to Der p 1, the target of pathogenic TH2 cells and IgE Abs

in HDM-allergic humans and mice (12–14). In the absence of a PC receptor, CD36 or PAFR, HDM uptake in the lung by alveolar macrophages, epithelial cells, and CD103⁺ DCs is impaired. Following HDM sensitization, mice ubiquitously lacking CD36 and/or PAFR exhibit a defect in HDM uptake by pulmonary CD103⁺ DCs and epithelial production of TSLP and IL-33. These mice also present with decreased frequencies of Der p 1-specific TH2 cells in the medLNs and ILC2s in the lung, as well as suppressed allergic disease development. Thus, CD36 and PAFR play a major role in promoting the development of HDM allergy.

Additionally, we generated bone marrow chimeric mice selectively expressing CD36 or PAFR on radioresistant cells in the lung, including epithelial cells, or radiosensitive cells in the lung, such as DCs, to determine the mechanism by which cellular engagement of these receptors drives HDM allergy. Loss of CD36 expression on radioresistant epithelial cells in the lung results in decreased production of epithelial cell cytokines, uptake of HDM by CD11c⁺CD103⁺ DCs in the lung, and accumulation of TH2 cells and ILC2s. However, CD36 expression on radiosensitive cells in the

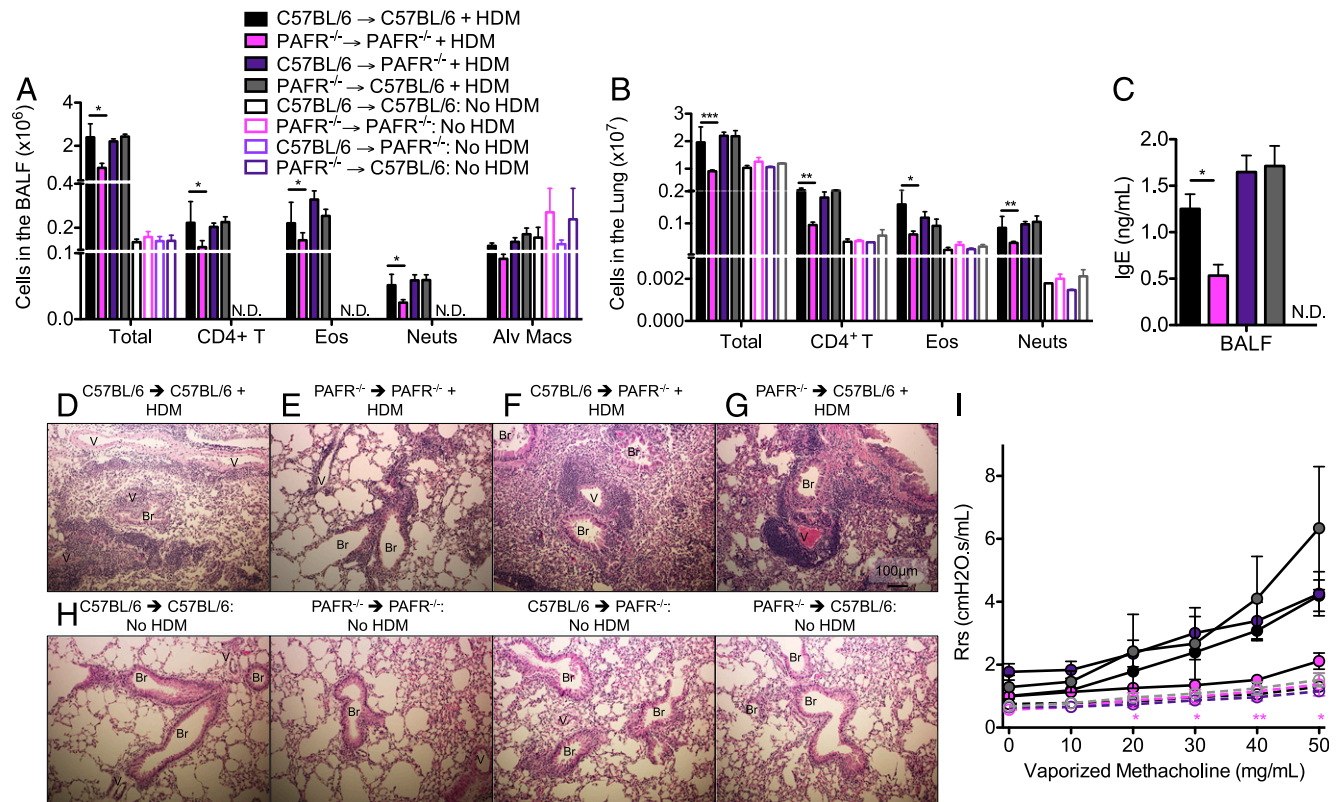


FIGURE 8. PAFR engagement on radioresistant airway structural cells and radiosensitive hematopoietic-derived cells is involved in the pulmonary allergic response to HDM. (**A** and **B**) Cells in BALF and lungs were enumerated. (**C**) Level of IgE from BALF was determined by ELISA. (**D–H**) Paraffin-embedded lungs were stained with H&E. Br, bronchus; V, vessel. (**I**) AHR determined by Rrs following challenge with the indicated concentration of vaporized methacholine. Values are the mean $\pm$ SEM from three independent experiments with four to eight mice per group. Values lower than the limits of detection are noted as not detectable (N.D.). Data were analyzed by a two-tailed unpaired *t* test comparing all bone marrow chimeric mice to C57BL/6 $\rightarrow$ C57BL/6 mice. Statistically significant differences are indicated as **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

lung, such as DCs, is dispensable for priming Der p 1-specific CD4⁺ T cells and HDM allergy development. Additionally, the loss of only PAFR expression on radioresistant and radiosensitive cells in the lung, such as DCs, resulted in diminished HDM-induced allergic responses. Therefore, CD36 expression on radioresistant cells and PAFR expression on radioresistant and radiosensitive cells are necessary to promote HDM allergy.

These results are not surprising considering that epithelial cell cytokines drive DC activation and migration, ILC2 proliferation, and TH2 cell effector differentiation in the lung (4, 8, 9). However, it raises the question of why PAFR expression on radioresistant cells in the lung alone does not appear to be involved in HDM-induced allergic disease. Although CD36 and PAFR bind PC, they have varied receptor functions and initiate different signaling cascades (23, 24). Compared with CD36, PAFR is associated with less-extensive signaling pathways linked to cytokine secretion (24, 49). Instead, PAFR has the unique ability to translocate materials, such as bacterial components, across cell surfaces (50, 51). It is possible that PAFR-mediated translocation of HDM components between epithelial and APCs contributes to the priming of TH2 cells and promotes HDM allergy development. Such a PAFR-dependent cross-talk between DCs and epithelial cells may be involved in promoting communication between these cells (9).

One study demonstrated that CD36 and PAFR synergize to stimulate clearance of apoptotic cells (28). In our studies, when multiple allergic disease parameters were assessed, we observed that CD36^{-/-} $\times$ PAFR^{-/-} double-knockout mice exhibited less allergic activity in some categories compared with CD36^{-/-} or PAFR^{-/-} mice. These allergy-associated parameters included

decreased levels of serum Der p 1-IgE, as well as lower frequencies of CD4⁺ T cells, eosinophils, and neutrophils in the BALF; ILC2s, basophils, and mast cells in the lung; and CD4⁺ GATA3⁺ T cells in the medLNs (Fig. 3). Therefore, some pathways of HDM-induced allergic inflammation, such as GATA3-dependent IL-4 production, may require cooperative engagement of both PC receptors.

Compared with nonasthmatics, asthmatic humans have higher PAFR expression in their lungs (32) and increased CD36 expression on circulating monocytes (33), which may result from engagement of CD36 or PAFR and upregulated expression of these receptors (24, 52). However, an increased basal expression of these receptors could also indicate increased susceptibility to allergic disease. CD36 and PAFR expression in the lungs of humans relative to age and allergen exposure has not been investigated. CD36 and PAFR are also involved in the clearance of oxidized lipids, apoptotic cells, and pneumococci from the host (24, 53). Therefore, direct blocking of these receptors systemically or in the airways with an Ab or an agonist is likely to have undesirable side effects. Instead, shielding PC epitopes on HDM particles to prevent their engagement with receptors on DCs in the lung may be a more clinically feasible approach. In the current and previous studies, we also demonstrate that passive or induced anti-PC IgM Abs in the lung prevent uptake of HDM particles by epithelial and multiple APC subsets in C57BL/6 mice and reduce the allergic response (22). This effect is not observed in CD36^{-/-} or PAFR^{-/-} mice, suggesting that anti-PC IgM Abs function in PC receptor-expressing C57BL/6 mice to prevent specific engagement of HDMs with CD36 or PAFR in the lung. Therefore,

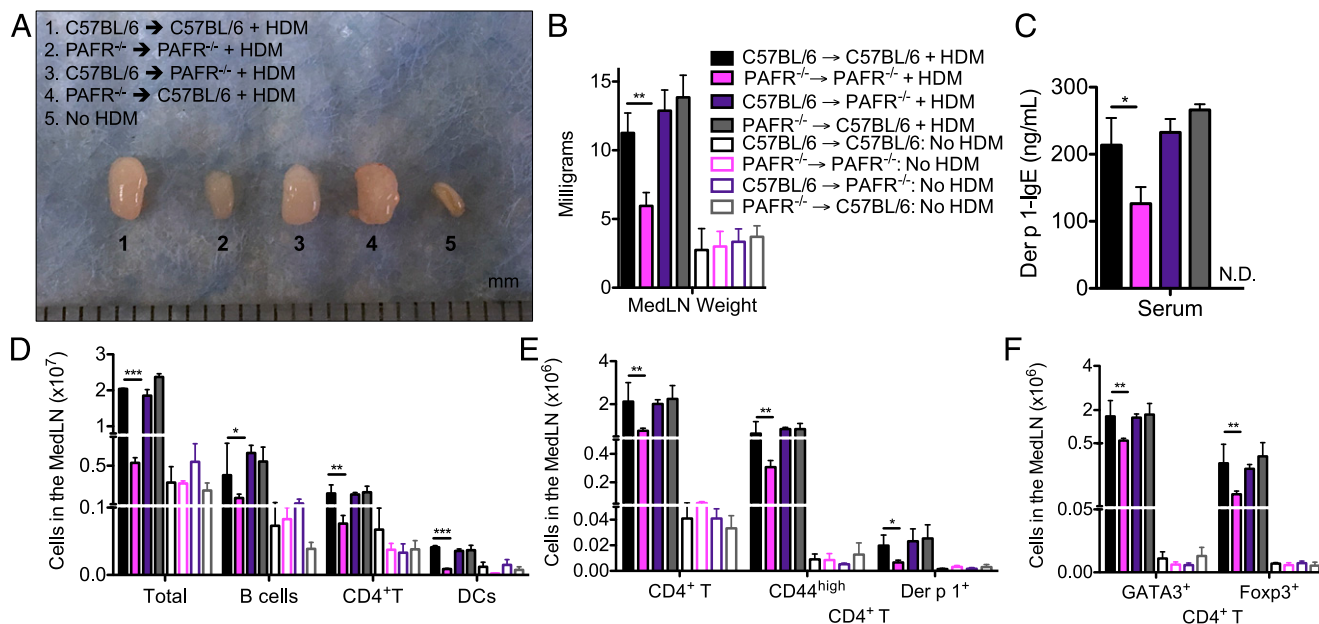


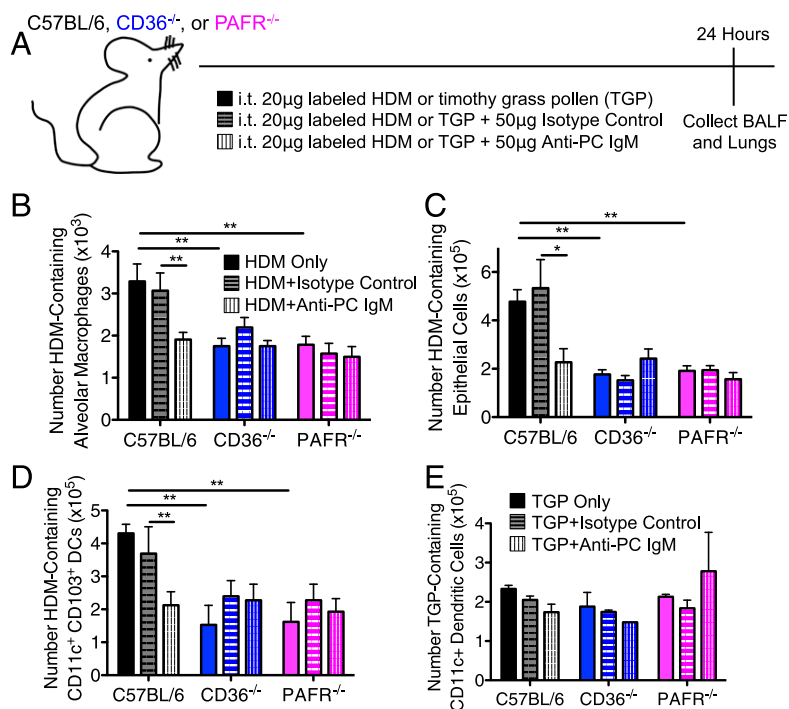
FIGURE 9. PAFR engagement on radioresistant airway structural cells and radiosensitive hematopoietic-derived cells is involved in Der p 1-specific TH2 cell priming in the medLN. Representative image (A), weight (B), and cellular composition (D) of medLN. (C) Serum Der p 1-IgE was determined by ELISA. Number of CD4⁺ T cells from the medLN that are CD44^{high}, Der p 1 specific (E) or GATA3⁺ or Foxp3⁺ (F), as determined by flow cytometry. Values are the mean ± SEM from three independent experiments with four to eight mice per group. Values lower than the limits of detection are noted as not detectable (N.D.). Data were analyzed by a two-tailed unpaired *t* test comparing all bone marrow chimeric mice to C57BL/6 → C57BL/6 mice. Statistically significant differences are indicated as **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

low levels of anti-PC IgM Ab in human lungs could predict susceptibility to asthma development. Modulating the levels of these Igs could be a potential therapeutic for preventing and treating HDM- and fungal-induced allergic disease.

PC is expressed by several fungal allergens (21); therefore, CD36 and PAFR may also be involved in orchestrating other allergic diseases. When CD36^{-/-} and PAFR^{-/-} mice were challenged with labeled PC-negative TGP, there was no defect in the allergen uptake by epithelial cells and APCs in the lung. Thus, CD36- and PAFR-mediated recognition of PC-expressing HDM

particles promotes HDM allergy in a ligand-specific fashion. In addition to phospholipids, it is clear that aeroallergens, including fungi and pollens, are associated with allergic proteins (15–17) and are composed of multiple structural components (e.g., glycan and phospholipid epitopes) that can engage innate receptors, such as TLR2, TLR4, dectin-1, dectin-2, mannose receptor, CD36, and PAFR (15–17). Engagement of these innate receptors on epithelial cells and DCs provides access to the allergenic protein cargo, which can then induce proliferation of ILC2s and priming of allergen-specific TH2 cells, respectively (4, 54, 55). Mouse

FIGURE 10. Decreased HDM particle uptake in C57BL/7 mouse lungs by anti-PC IgM Abs is not observed in the absence of CD36 and PAFR. (A–E) C57BL/6, CD36^{-/-}, and PAFR^{-/-} mice were sensitized i.t. with 20 µg of labeled HDMs or labeled HDMs in the presence of 50 µg of isotype control Ab or 50 µg of anti-PC IgM Ab. BALF and lungs were collected 24 h later, and the numbers of alveolar macrophages (B), epithelial cells (C), and CD103⁺ DCs (D) containing HDMs were determined by flow cytometry. (E) C57BL/6, CD36^{-/-}, and PAFR^{-/-} mice were sensitized i.t. with 20 µg of labeled TGP in the presence of 50 µg of isotype control Ab or 50 µg of anti-PC IgM Ab. (E) Lungs were collected 24 h later, and the numbers of DCs that contained HDMs were calculated. Gating strategy for APCs and epithelial cells is documented in Supplemental Fig. 2. Data were analyzed by a two-tailed unpaired *t* test comparing CD36^{-/-} or PAFR^{-/-} mice to C57BL/6 mice. Values lower than the limits of detection are noted as not detectable (N.D.). Statistically significant differences are indicated as **p* < 0.05, ***p* < 0.01.



models of allergic disease that use recombinant or modified proteins derived from allergens, such as HDM, or portions of the allergen that are soluble, LPS depleted, or manipulated to be “clean” (56–60) may not recapitulate the mechanisms involved in natural sensitization to environmental allergens. Because innate immune recognition of these particles is an initiating factor in disease pathogenesis, these potential ligands for multiple innate receptors may have complex downstream effects on disease outcome. Using mouse models in which innate immune receptors have been genetically deleted has provided information about how allergic disease is initiated; however, this information has not led to viable therapeutics for treating or preventing asthma. We suggest instead that pulmonary Abs targeting these conserved Ag-linked glycans, such as β -1,3-glucan or GlcNAc, or those targeting pathogen-associated molecular patterns, such as *Bartonella*-derived LPS in the case of HDM, may more effectively neutralize allergenic particles in the lung (22, 61, 62). The levels of these and other Abs in the lungs of children may also be predictive for asthma development. Therapeutic manipulation of these levels, through a vaccine or probiotic, may be sufficient to suppress allergic disease development.

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Disclosures

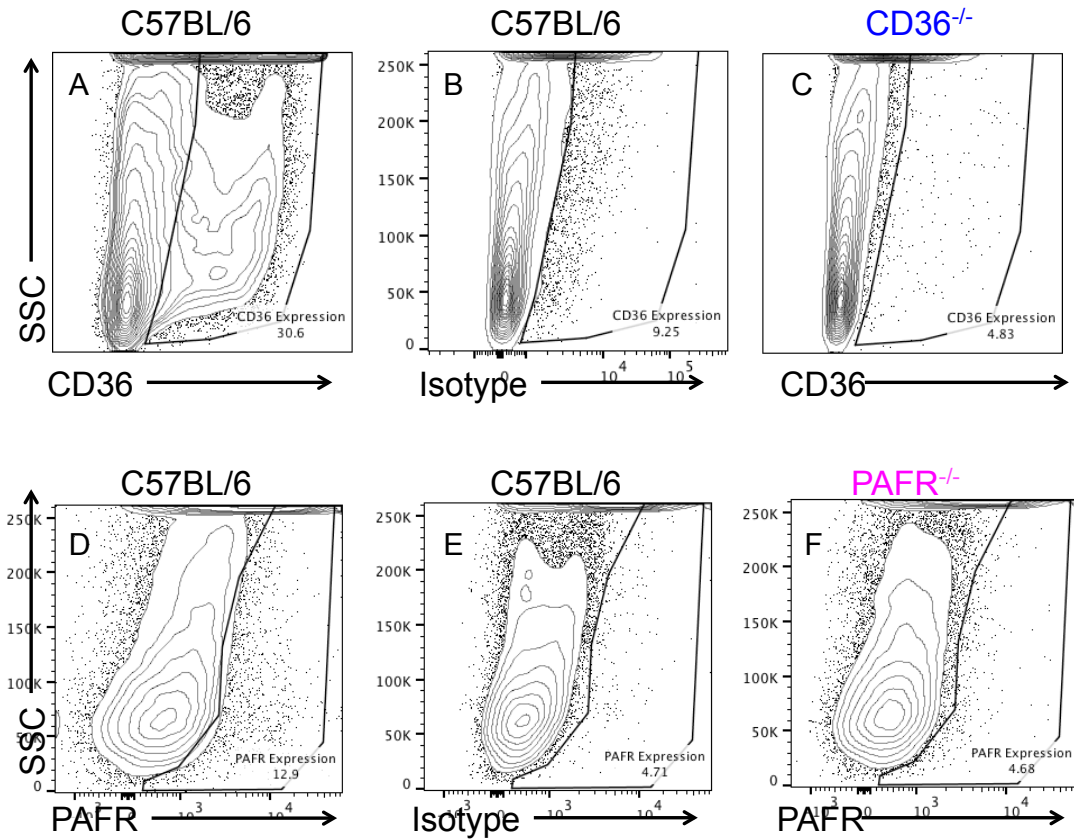
The authors have no financial conflicts of interest.

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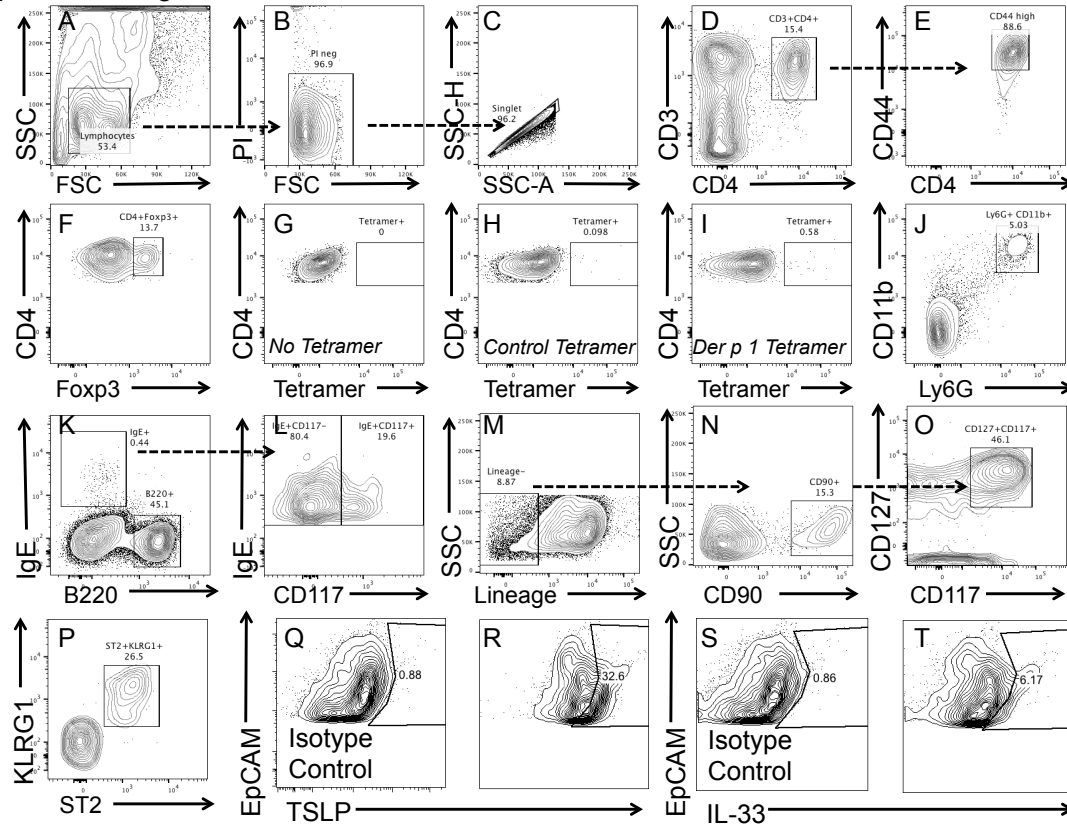
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Supplemental Figure 1



Supplemental Figure 1: Flow cytometric detection of cells expressing CD36 or PAFR in the lung. Enzymatically digested lung cells from (A-B, D-E) C57BL/6, (C) CD36^{-/-} or (F) PAFR^{-/-} mice were stained for flow cytometry using antibodies specific for (A, C) CD36 or (D, F) PAFR or (B, E) isotype control antibodies.

Supplemental Figure 2



Supplemental Figure 2: Representative gating scheme for cells detected by flow cytometry. Cells from suspensions of BALF, enzymatically digested lungs, and mediastinal lymph nodes were analyzed by flow cytometry as described below: (A) representative forward scatter (FSC) and side scatter (SSC) of cells in the lungs, (B) live cells (PI⁻), (C) single cells, (D) CD4⁺ T cells, (E) CD44^{high} CD4⁺ T cells, (F) Foxp3⁺ CD4⁺ T Cells, (G-I) CD4⁺ T cells from the medLN were incubated with (G) no tetramer, (H) APC-conjugated I-A(b) human CLIP tetramer (control) or (I) APC-conjugated I-A(b) Der p 1 tetramer, (J) neutrophils (CD11b⁺ Ly6G⁺), (K) B cells (B220⁺), (L) basophils (B220⁻ IgE⁺ CD117⁺) and mast cells (B220⁻ IgE⁺ CD117⁺), (M-P) ILC2s were identified by excluding (M) lineage⁺ cells (CD3, CD4, CD8, CD19, B220, CD11c, F4/80, Gr-1, TER119, and CD49b) and gating on cells that expressed (N) CD90.2, (O) CD127 and CD117, (P) ST2 and KLRG1. Epithelial cells (CD45⁻ EpCAM⁺) cells were assessed for intracellular production of (Q, R) TSLP or (S, T) IL-33.

Supplemental Figure 3

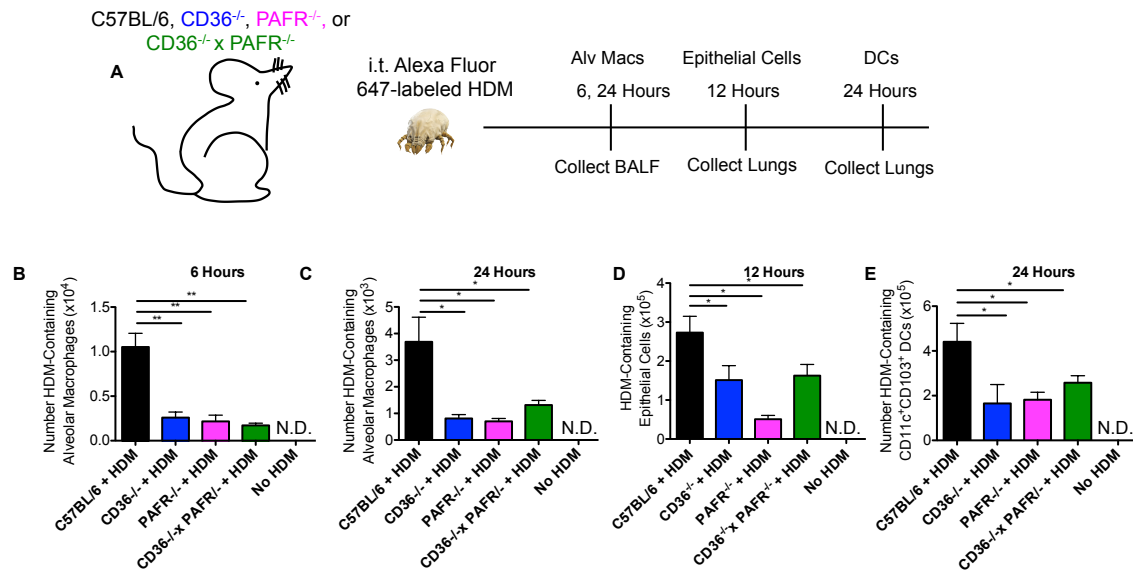


Figure 3: Pulmonary alveolar macrophages, epithelial cells, and CD103⁺ DC from mice lacking CD36^{-/-} and/or PAFR^{-/-} mice are defective in their ability to take up HDM particles *in vivo* at different time points.

(A) Six- to eight-week-old C57BL/6, CD36^{-/-}, PAFR^{-/-}, and CD36^{-/-} x PAFR^{-/-} mice were challenged i.t. with 20μg of fluorescently labeled HDM. (B, C) Alveolar macrophages were isolated 6 and 24 hours following challenge, (D) epithelial cells were isolated 12 hours following challenge, and (E) pulmonary CD103⁺ DCs were isolated 24 hours following challenge. (B-E) Total numbers of these cells containing HDM was determined by flow cytometry. Values represent the mean ± SEM from 3 independent experiments with 5-10 mice per group. Data were analyzed by a two-tailed unpaired *t* test comparing CD36^{-/-}, PAFR^{-/-} mice and CD36^{-/-} x PAFR^{-/-} to C57BL/6 mice. Values below the limits of detection are noted as not detectable “N.D.” Statistically significant differences are indicated as **p*<0.05, ***p*<0.01, and ****p*<0.001.