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CD36 and Platelet Activating Factor Receptor (PAFR) Promote House Dust Mite Allergy Development[1]

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Abstract

Over 89% of asthmatic children in underdeveloped countries demonstrate sensitivity to house dust mites (HDM). The allergic response to HDM is partially mediated by epithelial cell-derived cytokines that activate group-2 innate lymphoid cells (ILC2s), induce migration and activation of dendritic cells (DCs), and promote effector differentiation of HDM-specific TH2 cells. However, the contribution of innate receptor engagement on epithelial or dendritic cells by HDM that ultimately mediates said innate and adaptive allergic responses is poorly understood. We and others have demonstrated that HDM express phosphorylcholine (PC) moieties. The major PC receptors involved in immune responses include CD36 and platelet activating factor receptor (PAFR). Because both CD36 and PAFR are expressed by epithelial cells and DCs, 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 both radioresistant and radiosensitive cells in the lung promote allergic responses to HDM. Additionally, passive anti-PC IgM antibodies administered intratracheally with HDM decreased allergen uptake by epithelial 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 inhibiting HDM engagement with PC receptors in the lung protects against allergic airway disease.

Introduction

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 HDM have been well-characterized in mice. In response to HDM, 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 type-2 helper (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

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cells produce cytokines that, along with ILC2s, drive eosinophilia, IgE production, and airway hyperresponsiveness (4). Although epithelial and dendritic cells appear to function separately to activate ILC2s and prime TH2s, respectively, extensive crosstalk occurs between dendritic and epithelial cells (9–11).

However, the mechanisms by which HDM engage these cells and initiate both the innate and adaptive allergic response 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 (PAMPs) such as β-glucan, LPS, and chitin, which can engage innate immune receptors such as Dectin-1, Dectin-2, TLR-2, TLR-4, 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 and/or dendritic cells contributes to allergic disease. These few studies detail how Dectin-2 on dendritic, but not epithelial, cells (18) and TLR-4 on epithelial, but not dendritic, cells (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. HDM contains phosphorylcholine moieties (PC) (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 or dendritic cells in the lung is involved in HDM-induced allergic disease. Induced or passively administered anti-PC IgM antibodies are protective in disease models involving PC-expressing bacteria, parasites, 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 previously demonstrated that HDM particulates contain PC epitopes (22). Here, 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 and dendritic cells is impaired and these mice exhibit suppressed development of allergic disease compared to C57BL/6 mice. Using bone marrow chimeric mice, we also demonstrate that CD36 engagement on radioresistant cells in the lung and PAFR engagement on both 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 antibodies decrease the uptake of HDM by APCs in the lung (22). Here, we demonstrate that anti-PC IgM

antibodies do not decrease HDM uptake in CD36 or PAFR deficient mice, indicating that this effect depends on PC receptor expression in the lung.

Methods

Animals

C57BL/6 mouse breeders were originally obtained from Jackson Laboratory. $CD36^{-/-}$ mice were donated by Dr Maria Febbraio (University of Alberta) (27). $PAFR^{-/-}$ mice, originally generated by Dr Takao Shimizu (University of Tokyo) (40), were obtained from Dr Tamas Jilling (University of Alabama at Birmingham). $CD36^{-/-}$ and $PAFR^{-/-}$ mice were crossed to generate $CD36^{-/-} \times PAFR^{-/-}$ 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 rads, delivered in 2 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 weeks.

House dust mite (HDM) and Timothy grass pollen (TGP) allergen

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

In vivo intratracheal challenge, and the HDM allergy model

For intratracheal challenge, adult mice were anesthetized with 3–5% isoflurane, 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 intratracheally (i.t.) with 5μg HDM resuspended in 50μL PBS. Mice were rested for 7 days before daily i.t. challenge for 5 consecutive days with 5μg HDM in 50μL PBS. Following the last HDM allergen challenge, mice were rested for 2 days before euthanization. Where indicated, the above procedure was also conducted using Alexa Fluor 488- or 647-labeled HDM for sensitizations and subsequent challenges in the stated experimental groups.

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

Bronchoalveolar lavage fluid (BALF), lung, and mediastinal lymph node (medLN) collection

A 5mL 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 5mL HBSS and 10U/mL DNAse I (Bovine Pancreas) for 40 minutes at 37°C. A third of these cells were taken for epithelial cell analysis and the remaining cells were subjected to lymphocyte separation (Cellgro). MedLN cells were collected after mechanical disruption. All cells were manually enumerated using a hemocytometer.

Antibody reagents, flow cytometry, and cell identification

Maintenance of hybridomas and plasmacytomas, antibody purification, and conjugation of these reagents to Alexa fluor dyes have been previously described (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 antibodies. Staining with purified antibodies was followed by staining with fluorescently conjugated secondary goat anti-mouse against the respective isotypes (Southern Biotechnology, Life Technologies).

BALF, lung, and medLN cells were stained for flow cytometry with propidium iodide (PI) for excluding dead cells and with fluorochrome-conjugated antibodies 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 antibodies were purchased from BD, except EpCAM and TSLP (Ebiosciences), PAFR (Abcam), IL-33 (R&D Systems), and MZ1 (41). MedLN T cells were also stained with APC-conjugated I-A(b) tetramers (NIH Tetramer Core Facility) containing human CLIP (PVSKMRMATPLLMQA) or Der p 1 (CQIYPPNVNKI) peptides (42, 43). To detect epithelial expression of TSLP or IL-33 and CD4 T cell expression of Foxp3 or GATA3, cells were fixed and permeabilized with eBioscience Transcription Factor Staining Buffer Set (00–5523-00) using manufacturer's instructions. Cells were also stained to detect CD36 or PAFR expression (as shown in Supplemental Figure 1). Cells of interest were identified as described in Figure 2B and Supplemental Figure 2. All flow cytometry analyses were performed on a FACSCalibur (BD Biosciences) or LSR II (BD Biosciences) and analyzed using FlowJo software (Tree Star).

Enzyme-Linked Immunosorbent Assay (ELISA)

Anti-Der p 1-IgE was detected by coating plates with 2μg/mL natural Der p 1 (Indoor Biotechnologies), and serum antibody was detected using alkaline phosphatase-conjugated goat anti-mouse IgE (Southern Biotechnology Associates, AL). Total IgE levels were determined by coating plates with 2μg/mL rat anti-mouse IgE (Southern Biotechnology), and standard curves were prepared with known concentrations of IgE antibodies (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 405nm.

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 resistance (Rrs) was recorded every 12 seconds continuously for up to 3 minutes. Averages from each methacholine dose were calculated from 4–6 mice per group to determine airway hyperreactivity.

Histology and fluorescence staining

Whole HDM fecal pellets were cytocentrifuged (Shandon Elliott) onto glass slides at 1,000 rpm for 5 minutes and stained with Alexa fluor 647-conjugated anti-PC IgA antibody (S107) or anti-Der p 1 IgG1 antibody (Indoor Biotechnologies) followed by 488-conjugated anti-mouse IgG1 antibody. Replicate slides were also stained with Alexa fluor 647-conjugated isotype control antibody (J558) or purified mouse IgG1 isotype control antibody (MOPC-21; BD) followed by 488-conjugated anti-mouse IgG1 antibody. 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 manufacturer's instructions before being dehydrated and mounted in a xylene-based mounting media (Poly-mount xylene). Sections and cells were imaged with a Leica/Leitz DMRB fluorescence microscope equipped with appropriate filter cubes.

Sample size and Statistics

Values are the mean ± SEM from 3 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 by a one-way ANOVA test followed by Tukey's post-hoc analysis. Only statistically significant differences between C57BL/6 mice and reconstituted or receptor deficient mice are displayed. Data between two groups were analyzed by a two-tailed unpaired *t* test to determine statistical significance. In the figures, statistically significant differences are indicated as *p*-values of *<0.05, **<0.01, and ***<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 HDM expresses PC moieties (20, 22). Antibodies against Der p 1 (5GH) (Figure 1A), and IgM (BH8) and IgA (S107) antibodies to PC (Figure 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. (Figure 1C–G).

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

To determine if CD36 and/or PAFR mediate HDM uptake, we challenged C57BL/6, $CD36^{-/-}$, $PAFR^{-/-}$, and $CD36^{-/-} \times PAFR^{-/-}$ mice intratracheally with fluorescently labeled HDM, then calculated the number of antigen-presenting or epithelial cells containing HDM. We assessed HDM uptake by alveolar macrophages, epithelial cells, and 4 subtypes of DCs at 2, 24, or 48 hours post challenge, respectively, to reflect the time point for optimal phagocytosis of each cell type (7, 45–47) (Figure 2A). Pulmonary APC subsets were identified as previously described (48) (Figure 2B). Following HDM challenge, lower numbers of alveolar macrophages (Figure 2C), epithelial cells (Figure 2D), $CD11b^{+}CD11c^{+}$ DCs (Figure 2E), $CD11c^{+}CD103^{-}$ DCs (Figure 2F), $CD11c^{+}CD103^{+}$ DCs (Figure 2G), and $CD11b^{+}Ly6C^{+}$ DCs (Figure 2H) from mice lacking CD36 and/or PAFR take up HDM compared to C57BL/6 mice. When we analyzed HDM uptake by alveolar macrophages, epithelial cells, and $CD103^{+}$ DCs from $CD36^{-/-}$, $PAFR^{-/-}$ and $CD36^{-/-} \times PAFR^{-/-}$ mice at earlier or later than the frame time described, HDM uptake is also impaired (Supplemental Figure 3). Thus, both antigen-presenting and epithelial cells from $CD36^{-/-}$, $PAFR^{-/-}$ and $CD36^{-/-} \times PAFR^{-/-}$ mice are defective in their ability to take-up HDM in the lung. Of the DC subtypes that had taken-up HDM in C57BL/6 mice, most (67%) of the HDM-containing cells were $CD11c^{+}CD103^{+}$ DCs (Figure 2I). Therefore, the only DC subset analyzed in the rest of our studies is the $CD11c^{+}CD103^{+}$ phenotype.

CD36 and PAFR are essential for induction of HDM allergy

To determine if CD36 or PAFR expression is necessary for development of HDM induced allergy, C57BL/6, $CD36^{-/-}$, $PAFR^{-/-}$ and $CD36^{-/-} \times PAFR^{-/-}$ mice were repeatedly sensitized and challenged with HDM (Figure 3A). As controls, groups of C57BL/6, $CD36^{-/-}$, $PAFR^{-/-}$ and $CD36^{-/-} \times PAFR^{-/-}$ mice that were not challenged with HDM were also analyzed. Compared to C57BL/6 mice, the number of pulmonary alveolar macrophages, lung and lymph node $CD11c^{+}CD103^{+}$ DCs from $CD36^{-/-}$, $PAFR^{-/-}$ and $CD36^{-/-} \times PAFR^{-/-}$ that had taken-up HDM is significantly lower (Figure 3B, C). Following HDM challenge, $CD36^{-/-}$, $PAFR^{-/-}$ and $CD36^{-/-} \times PAFR^{-/-}$ also have decreased numbers of $CD4^{+}$ T cells, eosinophils, and neutrophils in their BALF and lungs (Figure 3D, G), and $CD11c^{+}CD103^{+}$ DCs, ILC2s, basophils, and mast cells in their lungs (Figure 3H) compared to C57BL/6 mice. Additionally, compared to C57BL/6 mice, $CD36^{-/-}$, $PAFR^{-/-}$, and $CD36^{-/-} \times PAFR^{-/-}$ mice developed less airway hyperresponsiveness (Figure 3E), have fewer numbers of TSLP or IL-33 producing epithelial cells in their lungs (Figure 3F), and express lower levels of serum Der p 1-specific IgE (Figure 3I). $CD36^{-/-}$, $PAFR^{-/-}$ and $CD36^{-/-} \times PAFR^{-/-}$ mice also exhibit smaller medLNs (Figure 3J, K) that are composed of fewer B cells, DCs, and $CD4^{+}$ T cells that are $GATA3^{+}$ or $Foxp3^{+}$ compared to C57BL/6 mice (Figure 3L, M). Therefore, CD36 and PAFR play a major role in HDM induced allergy development.

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 either C57BL/6 or $CD36^{-/-}$ bone marrow to generate donor→ recipient mice (Figure 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 (Figure 4B, C).

Following reconstitution, chimeric mice were intratracheally sensitized and challenged with HDM, and the allergic response to HDM was assessed. Compared to C57BL/6→ C57BL/6 mice, both $CD36^{-/-}$→ $CD36^{-/-}$ and C57BL/6→ $CD36^{-/-}$ mice have lower frequencies of epithelial cells expressing TSLP or IL-33 (Figure 4D) and ILC2s (Figure 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 to C57BL/6→ C57BL/6 mice (Figure 4F, G).

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

PAFR engagement on both 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 (Figure 7A). As expected, PAFR is neither detectable on the epithelial cells of $PAFR^{-/-}$→ $PAFR^{-/-}$ nor C57BL/6→ $PAFR^{-/-}$ mice nor the dendritic cells and macrophages of $PAFR^{-/-}$→ $PAFR^{-/-}$ or $PAFR^{-/-}$→C57BL/6 mice (Figure 7B, C).

Following HDM challenge, only $PAFR^{-/-}$→ $PAFR^{-/-}$ mice have a decreased number of TSLP or IL-33-producing epithelial cells (Figure 7D), ILC2s (Figure 7E), and HDM-containing pulmonary alveolar macrophages and $CD11c^{+}CD103^{+}$ DCs from the lung and medLN (Figure 7F–G). $PAFR^{-/-}$→ $PAFR^{-/-}$ mice also have decreased frequencies of $CD4^{+}$ T cells, eosinophils, and neutrophils in their BALF and lungs (Figure 8A, B), reduced levels

of IgE in their BALF (Figure 8C), numbers of cellular infiltrates detected by H&E staining (Figure 8D–H), and decreased airway hyperreactivity (Figure 8I) compared to C57BL/6→ C57BL/6 mice.

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

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

The data above demonstrate that both CD36 and PAFR are major contributors to HDM-induced allergic disease. Having previously demonstrated that anti-PC IgM decreases uptake of HDM particles by APCs in the lung (22), we determined if this effect was mediated by inhibited engagement of CD36 or PAFR. Labeled HDM was administered i.t. to C57BL/6, CD36$^{-/-}$ and PAFR$^{-/-}$ mice in the presence of either PBS alone, an isotype control antibody, or anti-PC IgM antibody (Figure 10A). The number of alveolar macrophages, epithelial cells, or CD103^{+} DCs that had taken up HDM was determined 24 hours later. As a control, we administered labeled timothy grass pollen (TGP), which we confirmed did not express PC, in place of HDM (Figure 10A). As expected, HDM uptake by antigen-presenting and epithelial cells is defective in CD36 and PAFR deficient mice (Figure 10B–D) compared to C57BL/6 mice, consistent with our findings in Figure 2. Although anti-PC IgM antibody decreases HDM uptake by alveolar macrophages (Figure 10B), epithelial cells (Figure 10C), and CD103^{+} DCs (Figure 10D), in the lungs of C57BL/6 mice, there is no further decrease in CD36$^{-/-}$ or PAFR$^{-/-}$ mice (Figures 10B–D). Additionally, TGP uptake is not altered in mice lacking CD36 or PAFR, and anti-PC IgM antibody did not decrease TGP uptake by lung DCs (Figure 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 coexpress PC in addition to Der p 1, the target of pathogenic TH2 cells and IgE antibodies in HDM-allergic humans and mice (12–14). In the absence of PC receptors, 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 medLN, ILC2s in the lung, and suppressed allergic disease development. Thus, both 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 either 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 TH2s and ILC2s. However, CD36 expression on radiosensitive cells in the lung, such as DCs, is dispensable for priming Der p 1-specific $CD4^{+}$ T cells and HDM allergy development. Additionally, only loss of PAFR expression on both radioresistant and radiosensitive cells in the lung, such as DCs, resulted in lessening of HDM induced allergic responses. Therefore, CD36 expression on radioresistant cells and PAFR expression on both radioresistant and radiosensitive cells is 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 both CD36 and PAFR bind PC, they have varied receptor functions and initiate different signaling cascades (23, 24). Compared to 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 antigen-presenting cells contributes to the priming of TH2 cells and promotes HDM allergy development. Such a PAFR-dependent mechanism of crosstalk 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 to $CD36^{-/-}$ or $PAFR^{-/-}$ mice. These allergy-associated parameters included: lower frequencies of $CD4^{+}$ T cells, eosinophils, and neutrophils in the BALF, ILC2s, basophils, and mast cells in the lung, $CD4^{+}$ $GATA3^{+}$ T cells in the medLN, and levels of serum Der p 1-IgE (Figure 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 to non-asthmatics, asthmatic humans have higher PAFR expression in their lungs (32) and CD36 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. To date, 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 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 antibody 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 antibodies 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 antibodies function in PC receptor-expressing C57BL/6 mice to prevent specific engagement of HDM with CD36 or PAFR in the lung. Therefore, low levels of anti-PC IgM antibody levels in human lungs could predict susceptibility to asthma development. Modulating the levels of these immunoglobulins 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 timothy grass pollen, there was no defect in the allergen uptake by epithelial 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 comprised of multiple structural components such as glycan and phospholipid epitopes, which 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 and dendritic cells 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 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 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 yet lead to viable therapeutics for treating or preventing asthma. We suggest instead that pulmonary antibodies targeting these conserved antigen-linked glycans such as β-1,3-glucan or GlcNAc, or those targeting PAMPs such as Bartonella-derived LPS in the case of HDM may more effectively neutralize allergenic particles in the lung (22, 61, 62). Levels of these, and other, antibodies 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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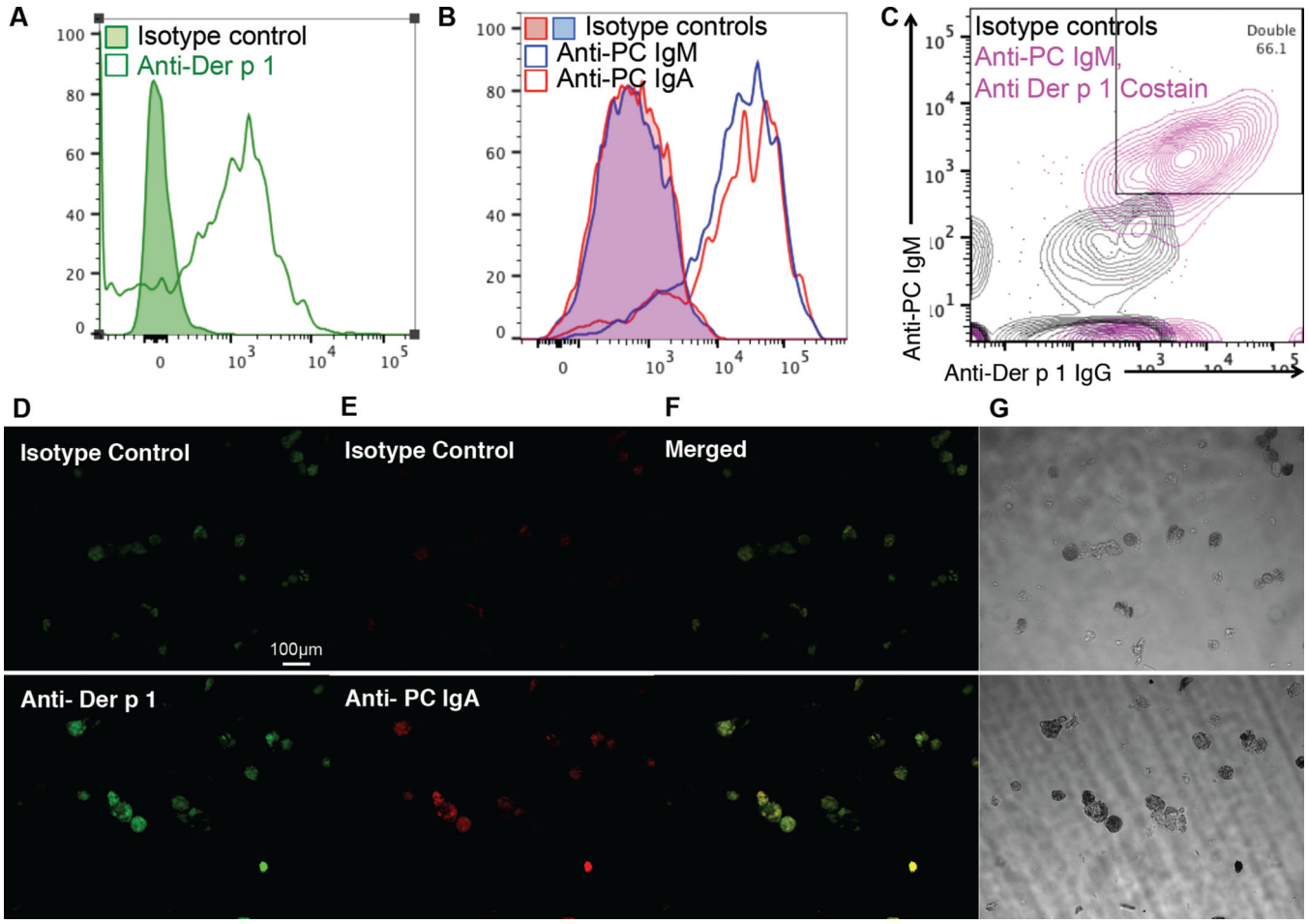

Figure 1. HDM feces coexpress Der p 1 and PC

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

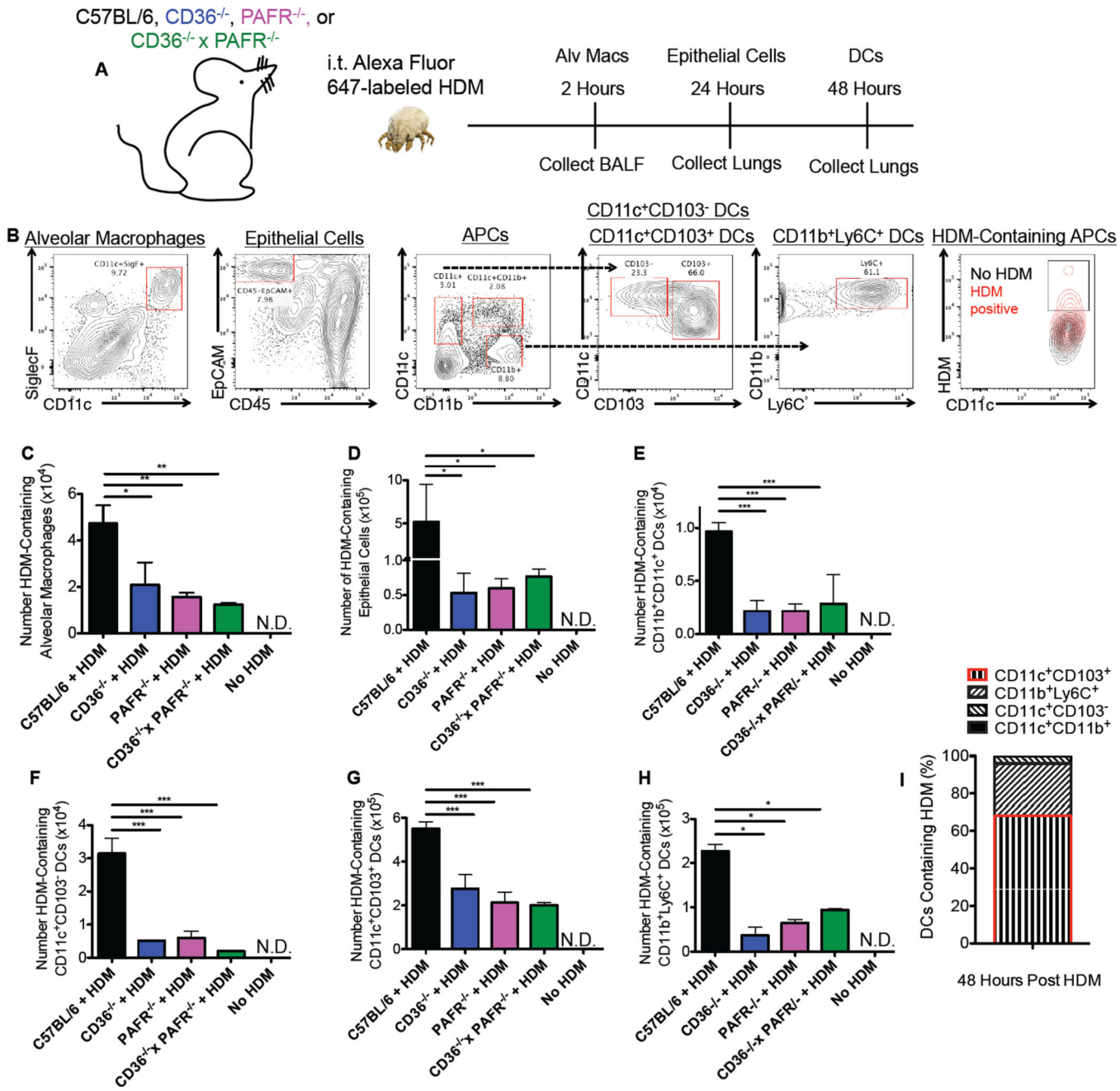

Figure 2. Pulmonary alveolar macrophages, epithelial cells, and CD103$^+$ DC from mice lacking CD36$^{-/-}$ and/or PAFR$^{-/-}$ mice are defective in HDM particle uptake *in vivo*

(A) Six- to eight-week-old C57BL/6, CD36$^{-/-}$, PAFR$^{-/-}$, and CD36$^{-/-}$ × PAFR$^{-/-}$ mice were challenged i.t. with 20μg fluorescently labeled HDM. Alveolar macrophages, epithelial cells, and DCs were isolated 2, 24, and 48 hours following challenge, respectively. (B) Representative gating scheme for alveolar macrophages (Siglec-F$^+$CD11c$^+$), epithelial cells (CD45$^-$EpCAM$^+$), CD11c$^+$CD11b$^+$ DCs, CD11c$^+$CD103$^-$ DCs, CD11c$^+$CD103$^+$ DCs, CD11b$^+$Ly6C$^+$ DCs, and HDM-containing APCs. (C-H) Total frequencies of pulmonary (C) alveolar macrophages, (D) epithelial cells, (E) CD11b$^+$CD11c$^+$, (F) CD11c$^+$CD103$^-$, (G)

$CD11c^{+}CD103^{+}$, and (H) $CD11b^{+}Ly6C^{+}$ DCs containing HDM from C57BL/6, $CD36^{-/-}$, $PAFR^{-/-}$, and $CD36^{-/-} \times PAFR^{-/-}$ mice were determined by flow cytometry. (I) Percentage of pulmonary DCs containing HDM for four subsets from C57BL/6 mice was determined. 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 $CD36^{-/-}$, $PAFR^{-/-}$ and $CD36^{-/-} \times PAFR^{-/-}$ mice receiving HDM 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 $*p<0.05$, $**p<0.01$, and $***p<0.001$.

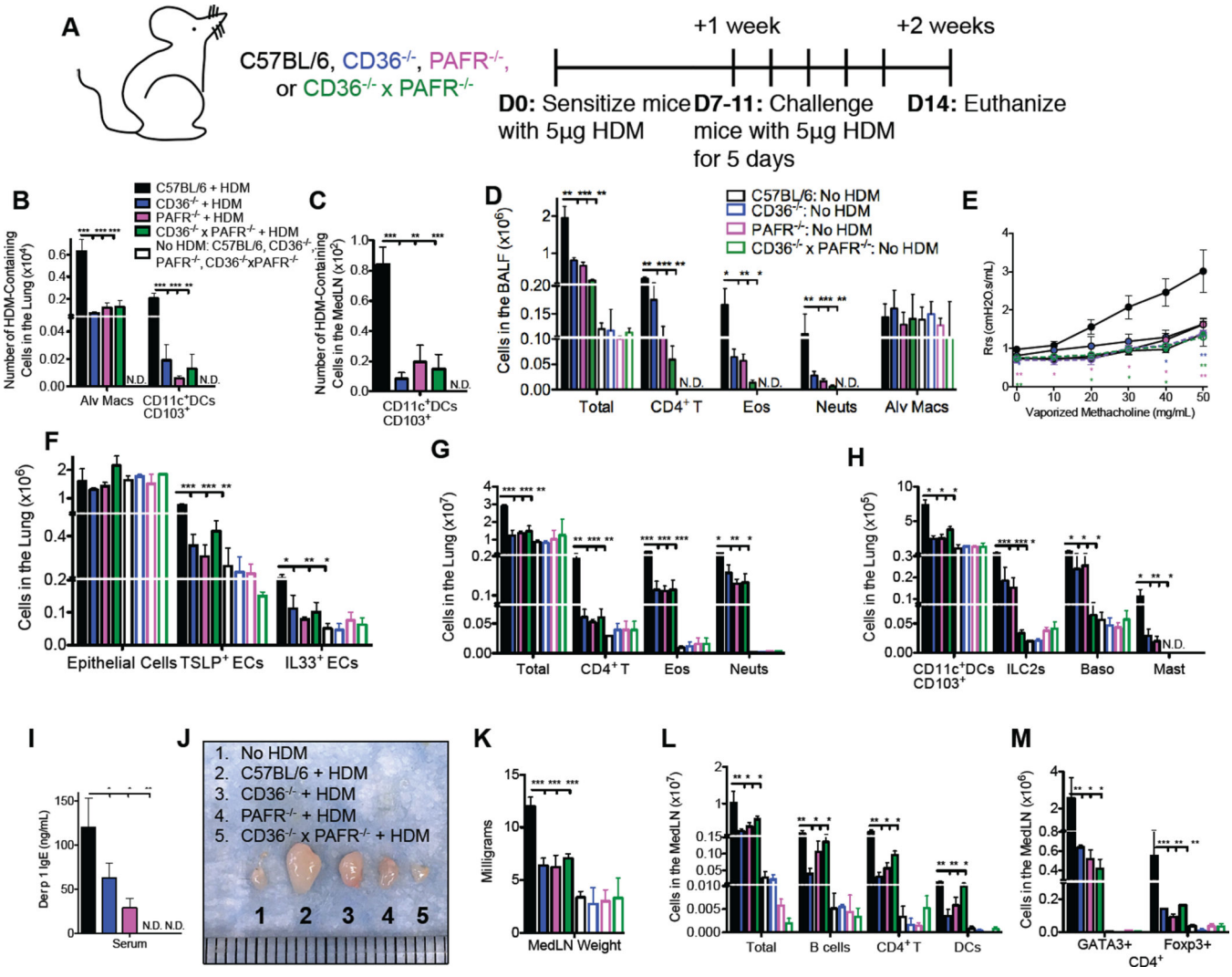

Figure 3. CD36$^{-/-}$, PAFR$^{-/-}$, and CD36$^{-/-}$ × PAFR$^{-/-}$ mice exhibit decreased allergic responses to HDM compared to C57BL/6 mice

(A) Six- to eight-week-old C57BL/6, CD36$^{-/-}$, PAFR$^{-/-}$, and CD36$^{-/-}$ × PAFR$^{-/-}$ mice were sensitized and challenged i.t. with HDM. As a control, littermate C57BL/6, CD36$^{-/-}$, PAFR$^{-/-}$, and CD36$^{-/-}$ × PAFR$^{-/-}$ mice were not administered HDM. Number of HDM-containing APCs from the (B) lung and (C) medLN. (D) Numbers of cells in the BALF. (E) Airway hyperresponsiveness determined by respiratory system resistance (Rrs) following challenge with the indicated concentration of vaporized methacholine. (F) Number of cytokine-producing epithelial cells (G, H) and lymphocytes in the lung. (I) Levels of serum anti-Der p 1-IgE measured by ELISA. (J) Representative images, (K) weight, and (L, M) the cellular composition of medLNs. Cells were identified by flow cytometry as documented in Supplemental Figure 2. Values lower than the limits of detection are noted as not detectable "N.D.". Values represent the mean ± SEM from 3 independent experiments with 4–8 mice per group. Data were analyzed by a two-tailed unpaired *t* test comparing CD36$^{-/-}$, PAFR$^{-/-}$ and CD36$^{-/-}$ × PAFR$^{-/-}$ mice receiving HDM to C57BL/6 + HDM mice. Statistically significant differences are indicated as *p<0.05, **p<0.01, and ***p<0.001.

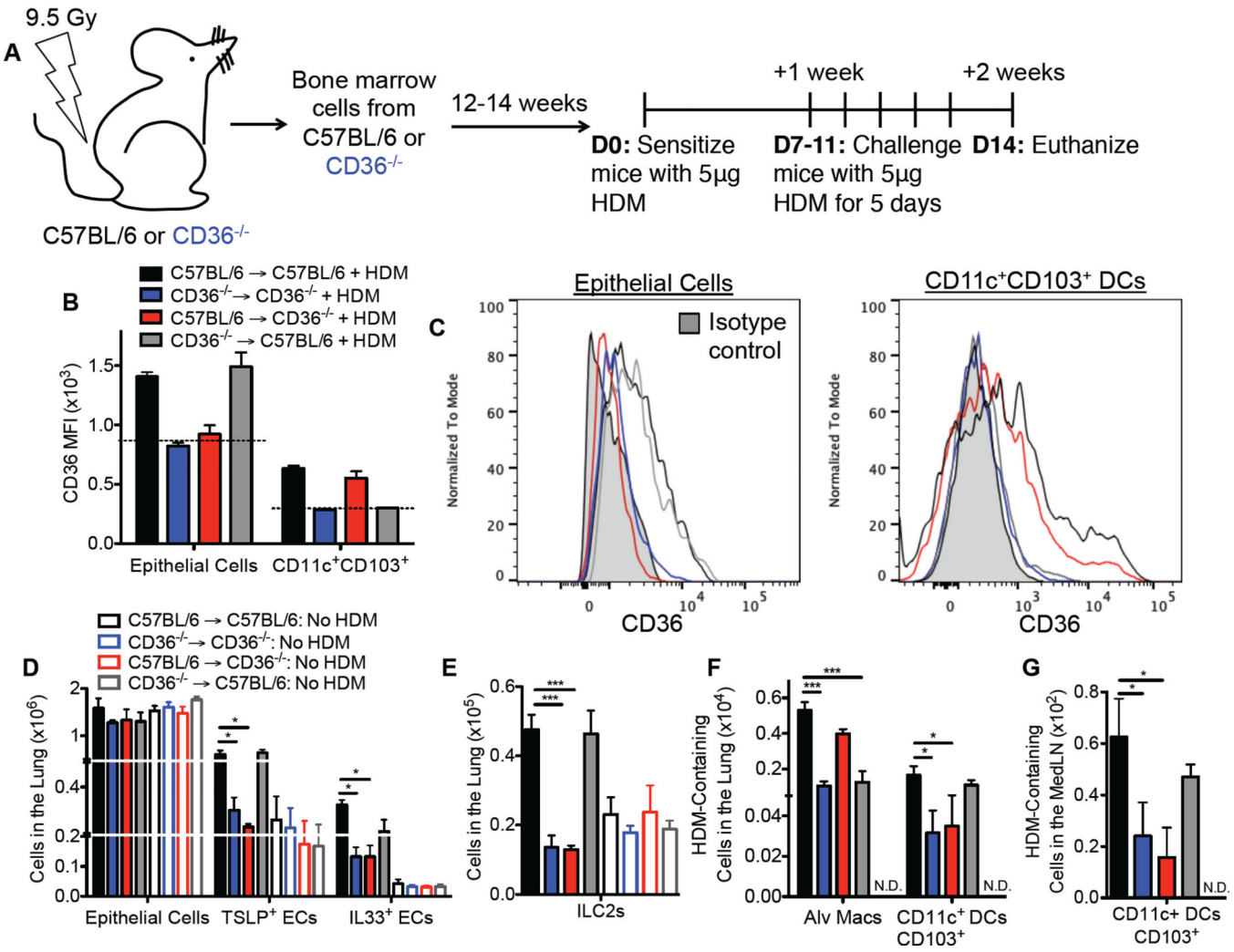

Figure 4. CD36 engagement on radioresistant airway structural cells is associated with epithelial cytokine production, ILC2 proliferation, and HDM uptake by pulmonary APCs

(A) C57BL/6 and CD36$^{-/-}$ mice were irradiated (950 rads) 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 weeks, then sensitized and challenged with HDM allergen. (B, C) CD36 expression on pulmonary epithelial cells or CD11c^{+}CD103^{+} DCs was determined from chimeric mice by flow cytometry. (B) Dotted horizontal line and (C) filled-in grey histogram correspond to isotype control antibody for anti-CD36. (D) Numbers of epithelial cells expressing TSLP or IL-33 and (E) ILC2s in the lung. (G) Numbers of alveolar macrophages and CD11c^{+}CD103^{+} DCs containing HDM from the lung and medLN. Validation of CD36 staining is shown in Supplemental Figure 1. Values lower than the limits of detection are noted as not detectable “N.D.” Values represent the mean ± SEM from 3 independent experiments with 4–8 mice per group. 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, and ***p<0.001.

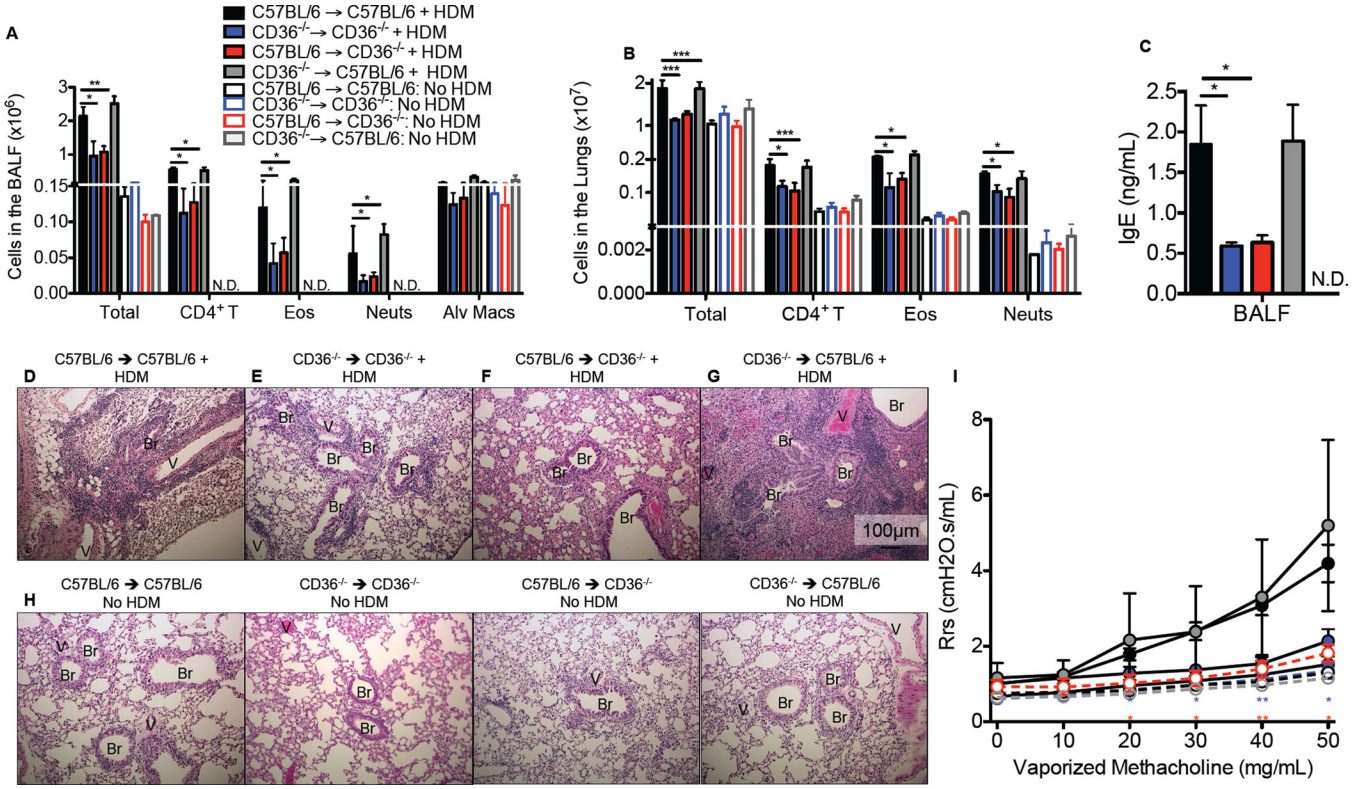

Figure 5. CD36 engagement on radioresistant airway structural cells is involved in the pulmonary allergic disease in response to HDM

(A, B) Cells were enumerated from the BALF and lungs. (C) Levels of IgE from the BALF were determined by ELISA. (D–H) Paraffin-embedded lungs were stained with Hematoxylin and Eosin. (I) Airway hyperresponsiveness was determined by Rrs following challenge with the indicated concentration of vaporized methacholine. Values lower than the limits of detection are noted as not detectable “N.D.”. Values are the mean ± SEM from 3 independent experiments with 4–8 mice per group. Data were analyzed by a two-tailed unpaired *t* test comparing all bone marrow chimera mice to C57BL/6→ C57BL/6 mice. Statistically significant differences are indicated as *p<0.05, **p<0.01, and ***p<0.001.

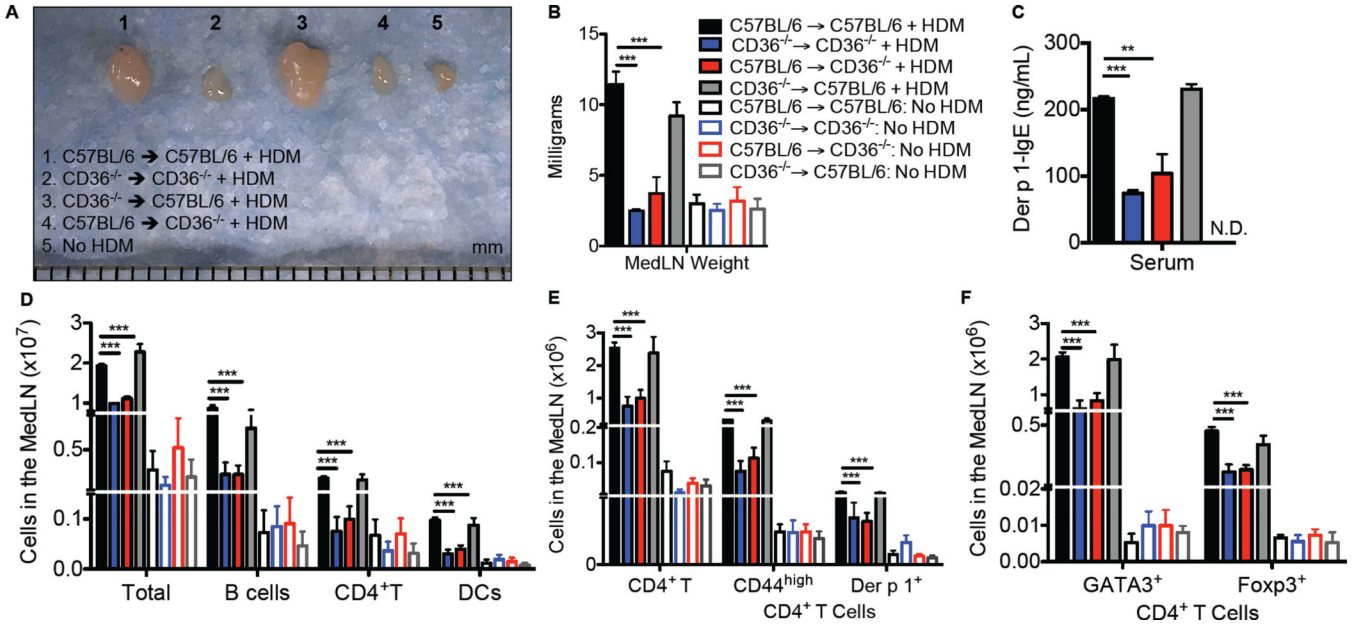

Figure 6. CD36 engagement on radioresistant airway structural cells is involved in Der p 1-specific TH2 cell priming in the medLN

(A) Representative image, (B) weight, and (D) cellular composition of medLN. (C) Serum Der p 1-IgE determined by ELISA. (E, F) Number of $CD4^+$ T cells that are (E) $CD44^{high}$, Der p 1-specific, (F) $GATA3^+$, or $Foxp3^+$ from the medLN determined by flow cytometry. Identification of tetramer-specific or $Foxp3^+$ $CD4^+$ T cells is documented in Supplemental Figure 2. Values are the mean ± SEM from 3 independent experiments with 4–8 mice per group. Data were analyzed by a two-tailed unpaired *t* test comparing all bone marrow chimera mice to C57BL/6→ C57BL/6 mice. Statistically significant differences are indicated as *p<0.05, **p<0.01, and ***p<0.001.

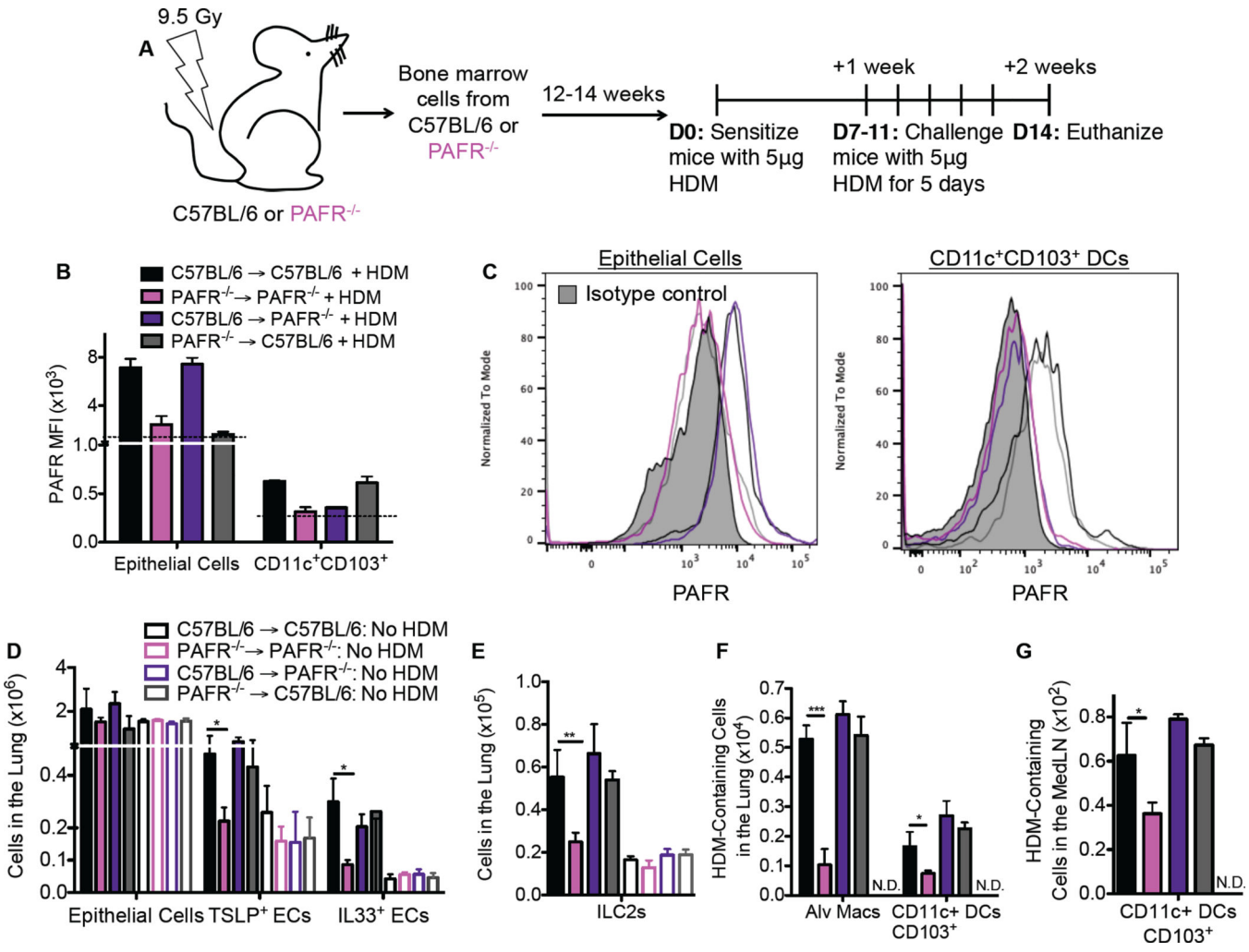

Figure 7. PAFR engagement on both radioresistant airway structural cells and radiosensitive hematopoietic-derived cells is involved in epithelial cytokine production, ILC2 proliferation, and HDM uptake by pulmonary APCs

(A) C57BL/6 or PAFR$^{-/-}$ mice were irradiated (950 rads) 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 weeks, then sensitized and challenged with HDM allergen. (B, C) PAFR expression on pulmonary epithelial cells, dendritic cells and macrophages was determined from chimeric mice (dotted line and shaded histogram = isotype control). (D) Numbers of epithelial cells making TSLP or IL-33 and (E) ILC2s in the lung. (G) Numbers of alveolar macrophages and CD11c^{+}CD103^{+} DCs containing HDM from the lung and medLN. Validation of PAFR staining is shown in Supplemental Figure 1. Values represent the mean ± SEM from 3 independent experiments with 4–8 mice per group. Data were analyzed by a two-tailed unpaired *t* test comparing all bone marrow chimera mice to C57BL/6→ C57BL/6 mice. Statistically significant differences are indicated as *p<0.05, **p<0.01, and ***p<0.001.

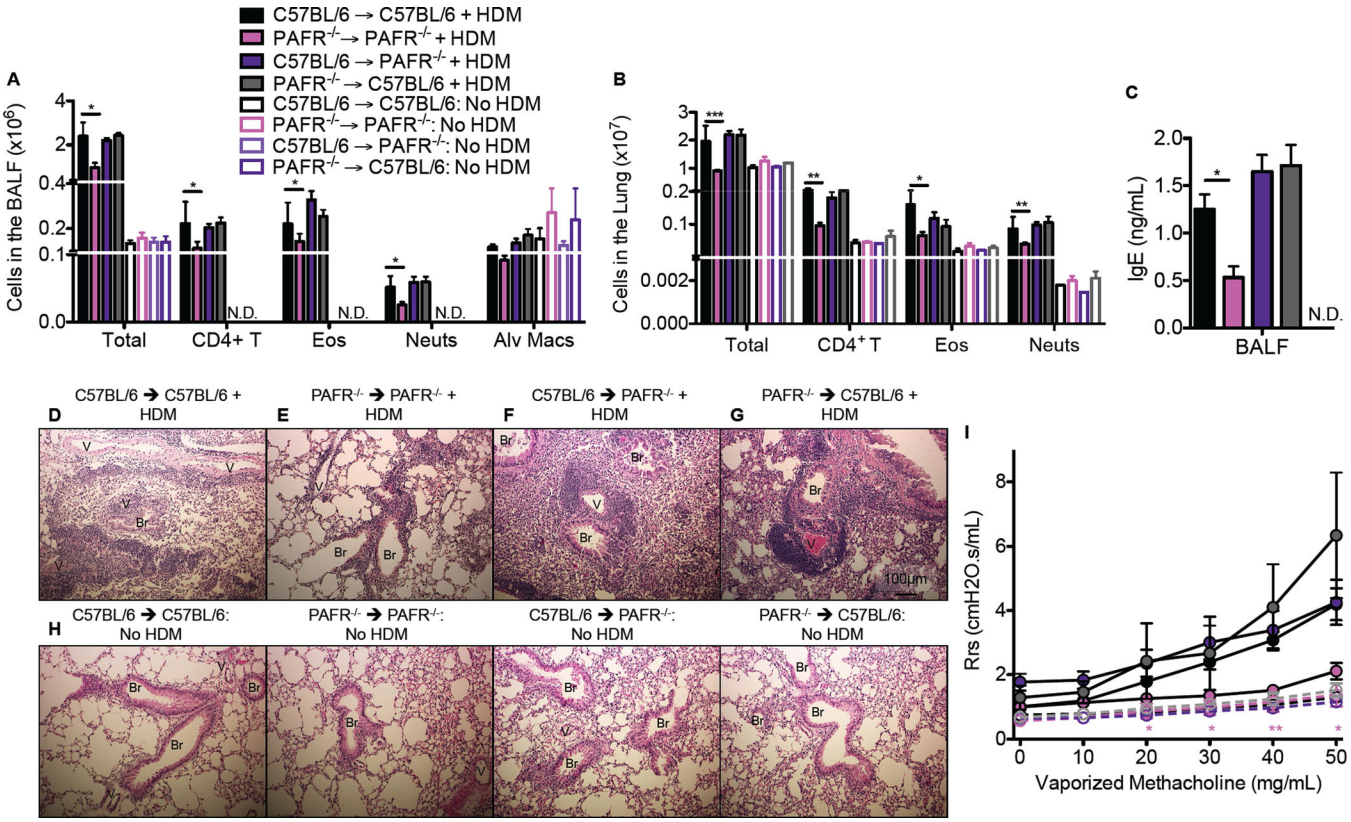

Figure 8. PAFR engagement on both radioresistant airway structural cells and radiosensitive hematopoietic-derived cells is involved in the pulmonary allergic response to HDM

(A, 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 hematoxylin and eosin. (I) Airway hyperresponsiveness determined by respiratory system resistance (Rrs) following challenge with the indicated concentration of vaporized methacholine. Values lower than the limits of detection are noted as not detectable "N.D.". Values are the mean ± SEM from 3 independent experiments with 4–8 mice per group. Data were analyzed by a two-tailed unpaired *t* test comparing all bone marrow chimera mice to C57BL/6→ C57BL/6 mice. Statistically significant differences are indicated as *p<0.05, **p<0.01, and ***p<0.001.

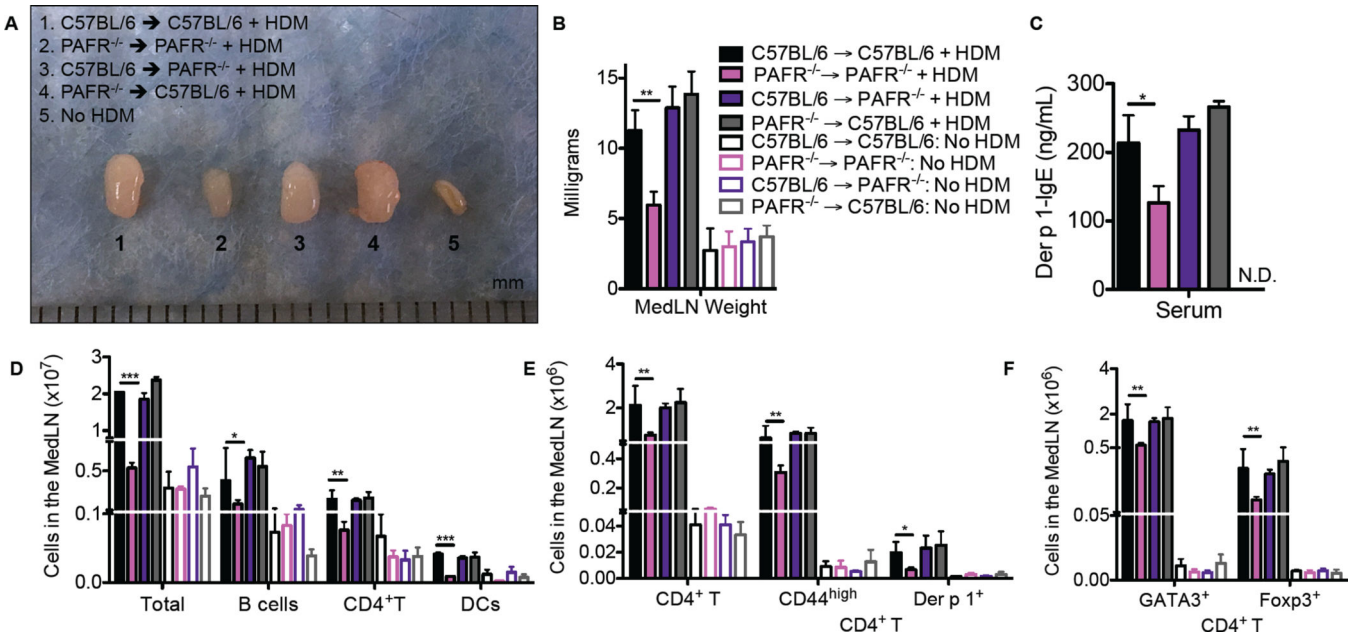

Figure 9. PAFR engagement on both radioresistant airway structural cells and radiosensitive hematopoietic-derived cells is involved in Der p 1-specific TH2 cell priming in the medLN

(A) Representative image, (B) weight, and (D) cellular composition of medLN. (C) Serum Der p 1-IgE was determined by ELISA. (E, F) Number of $CD4^+$ T cells that are (E) $CD44^{high}$, Der p 1-specific, (F) $GATA3^+$, or $Foxp3^+$ from the medLN determined by flow cytometry. Values are the mean ± SEM from 3 independent experiments with 4–8 mice per group. Data were analyzed by a two-tailed unpaired *t* test comparing all bone marrow chimera mice to C57BL/6→ C57BL/6 mice. Statistically significant differences are indicated as *p<0.05, **p<0.01, and ***p<0.001.

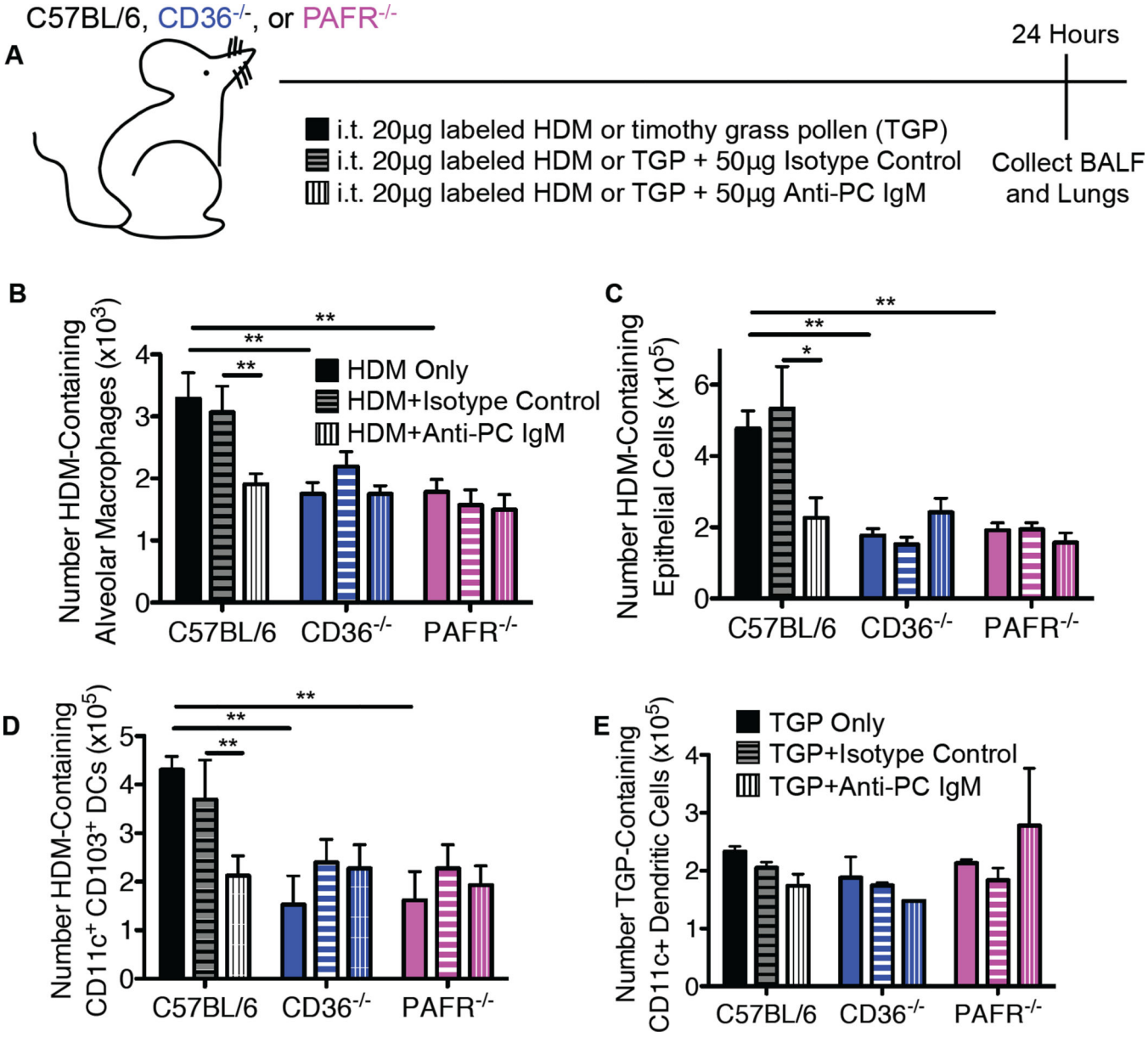

Figure 10. Decreased HDM particle uptake in the of C57BL/7 mouse lungs by anti-PC IgM antibodies is not observed in the absence of CD36 and PAFR

(A–E) C57BL/6, CD36$^{-/-}$ and PAFR$^{-/-}$ mice were sensitized i.t. with 20µg labeled HDM or labeled HDM in the presence of 50µg isotype control antibody or 50µg anti-PC IgM antibody. BALF and lungs were collected 24 hours later, and the number of (B) alveolar macrophages, (C) epithelial cells and (D) CD103^{+} DCs containing HDM was determined by flow cytometry. (E) C57BL/6, CD36$^{-/-}$ and PAFR$^{-/-}$ mice were. sensitized i.t. with 20µg labeled timothy grass pollen (TGP) in the presence of 50µg isotype control antibody or 50µg anti-PC IgM antibody. Lungs were collected 24 hours later, and the numbers of (E) DCs that contained HDM was calculated. Gating strategy for antigen-presenting and epithelial cells is documented in Supplemental Figure 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 “N.D.” Statistically significant differences are indicated as *p<0.05, **p<0.01, and ***p<0.001.