



Look beyond the status flow  
The Attune™ NXT Flow Cytometer

Find out more

**ThermoFisher**  
SCIENTIFIC



## **PGI<sub>2</sub> Controls Pulmonary NK Cells That Prevent Airway Sensitization to House Dust Mite Allergen**

This information is current as of December 12, 2016.

Bryan Simons, Maria E. Ferrini, Sophia Carvalho, David J. P. Bassett, Zeina Jaffar and Kevan Roberts

*J Immunol* published online 28 November 2016  
<http://www.jimmunol.org/content/early/2016/11/26/jimmunol.1600275>

- 
- Supplementary Material** <http://www.jimmunol.org/content/suppl/2016/11/26/jimmunol.1600275.DCSupplemental.html>
- Subscriptions** Information about subscribing to *The Journal of Immunology* is online at: <http://jimmunol.org/subscriptions>
- Permissions** Submit copyright permission requests at: <http://www.aai.org/ji/copyright.html>
- Email Alerts** Receive free email-alerts when new articles cite this article. Sign up at: <http://jimmunol.org/cgi/alerts/etoc>



# PGI<sub>2</sub> Controls Pulmonary NK Cells That Prevent Airway Sensitization to House Dust Mite Allergen

Bryan Simons,\* Maria E. Ferrini,\* Sophia Carvalho,\* David J. P. Bassett,<sup>†</sup>  
Zeina Jaffar,\*<sup>1</sup> and Kevan Roberts\*,<sup>1</sup>

**In allergic asthma, inhalation of airborne allergens such as the house dust mite (HDM) effectively activates both innate and adaptive immunity in the lung mucosa. To determine the role of the eicosanoid PGI<sub>2</sub> and its receptor IP during allergic airway sensitization, HDM responses in mice lacking a functional IP receptor (i.e., PGI<sub>2</sub> IP receptor-deficient [IP<sup>-/-</sup>]) were compared with wild type (WT) mice. Surprisingly, IP<sup>-/-</sup> mice had increased numbers of pulmonary CD3<sup>-</sup>NK1.1<sup>+</sup>Ly49b<sup>+</sup> NK cells producing IFN- $\gamma$  that was inversely associated with the number of type 2 innate lymphoid cells (ILC2s) expressing IL-33R $\alpha$  and IL-13 compared with WT animals. This phenomenon was associated with elevated CX<sub>3</sub>CL1 levels in the airways of IP<sup>-/-</sup> mice and treatment with a neutralizing Ab to CX<sub>3</sub>CL1 reduced IFN- $\gamma$  production by the lung NK cells. Remarkably, IP<sup>-/-</sup> mice were less responsive to HDM challenge than WT counterparts because intranasal instillation of the allergen induced markedly reduced levels of airway eosinophils, CD4<sup>+</sup> lymphocyte infiltration, and mucus production, as well as depressed levels of CCL2 chemokine and Th2 cytokines. NK cells were responsible for such attenuated responses because depletion of NK1.1<sup>+</sup> cells in IP<sup>-/-</sup> mice restored both the HDM-induced lung inflammation and ILC2 numbers, whereas transfer of CD3<sup>-</sup>NK1.1<sup>+</sup> NK cells into the airways of WT hosts suppressed the inflammatory response. Collectively, these data demonstrate a hitherto unknown role for PGI<sub>2</sub> in regulating the number and properties of NK cells resident in lung tissue and reveal a role for NK cells in limiting lung tissue ILC2s and preventing allergic inflammatory responses to inhaled HDM allergen. *The Journal of Immunology*, 2017, 198: 000–000.**

**A**sthma is largely associated with atopy, typified by an IgE response to known allergens. Exacerbations of this disease are coincident with Th2-mediated airway inflammation characterized by the infiltration of eosinophils, goblet cell hyperplasia, and airway remodeling resulting in AHR and luminal narrowing. In highly populated areas of North and South America, almost 85% of asthmatics are allergic to house dust mites (HDMs) (1, 2). It seems likely that an improved understanding of the cellular and molecular events that underpin bronchial sensitization to allergens will prove a prerequisite for the development of novel intervention strategies. The interaction of HDM allergens with innate cells in the respiratory tract is a critical event that precedes adaptive immunity. In this context, HDMs entering the mouse

airway have been shown to interact with formyl peptide receptors on eosinophils (3), protease-activated receptor-2 on epithelial cells (4), TLR4 on stromal cells (5), and Dectin-2 on dendritic cells (DC) (6) and alveolar macrophages (7).

NK cells are effector cells of innate immunity classically recognized for their ability to identify and kill tumors and virally infected cells (8). Such reactivity is tightly regulated by an expansive system of activating (e.g., NKp46 and NKG2D) and inhibiting (e.g., NKG2A and NKG2B) receptors expressed on the surface of NK cells (9, 10). Recent advances in the understanding of NK cell interaction with other innate and adaptive immune cell populations has generated interest in the immunoregulatory role of NK cells beyond their capacity as killers. NK cells residing in the lung mucosal tissue have been described previously, however, their relationship to conventional circulating NK cells remains unclear (11–13). Previous reports have investigated the effect of NK cells on allergic lung inflammation with contradictory findings, with some instances inhibiting allergic lung inflammation (14, 15) and others promoting eosinophilic inflammation (16, 17). Importantly, NK cells are responsive to a range of endogenously produced eicosanoids, which include Resolvin-E1 (18), Lipoxin-A4 (19), PGD<sub>2</sub> (20), PGE<sub>2</sub> (21), and leukotrienes (22). Eicosanoids generated in the lung likely regulate the inflammatory response and provide a means to integrate the opposing needs of maintaining the normal barrier function of both endothelial and epithelial surfaces yet facilitate immune responses to airborne pathogens and allergens. The early events elicited by the interaction of allergens with the innate immune system include the biosynthesis of cysteinyl leukotrienes and PGs (23–25). PGI<sub>2</sub> (also known as prostacyclin) is a metabolite of arachidonic acid and forms a prominent component of the COX-2 response (reviewed in Ref. 26). This mediator plays a critical role in pain perception and inflammation and is a potent vasodilator and inhibitor of platelet aggregation (27).

\*Center for Environmental Health Sciences, Biomedical and Pharmaceutical Sciences, University of Montana, Missoula, MT 59812; and <sup>†</sup>Department of Family Medicine and Public Health Sciences, School of Medicine, Wayne State University, Detroit, MI 48201

<sup>1</sup>K.R. and Z.J. contributed equally to this work.

Received for publication February 16, 2016. Accepted for publication October 31, 2016.

This work was supported by National Institutes of Health Grants R01-HL079189 (to K.R.) and R15-HL112073 (to Z.J.) and the Center for Environmental Health Sciences Core Facilities (Fluorescence Cytometry, Histology and Inhalation) supported by National Institute of General Medical Sciences, National Institutes of Health Grant P30GM103338.

Address correspondence and reprint requests to Dr. Kevan Roberts and Zeina Jaffar, Center for Environmental Health Sciences, Biomedical and Pharmaceutical Sciences, Skaggs Building, The University of Montana, Missoula, MT 59812. E-mail addresses: kevan.roberts@umontana.edu (K.R.) and zeina.jaffar@umontana.edu (Z.J.)

The online version of this article contains supplemental material.

Abbreviations used in this article: BALF, bronchoalveolar lavage fluid; DC, dendritic cell; EPO, eosinophil peroxidase; HDM, house dust mite; ILC2, type 2 innate lymphoid cell; IP<sup>-/-</sup>, PGI<sub>2</sub> IP receptor-deficient; LMC, lung mononuclear cell; moDC, monocyte-derived dendritic cell; PAS, periodic acid-Schiff; pNK cell, pulmonary NK cell; WT, wild type.

Copyright © 2016 by The American Association of Immunologists, Inc. 0022-1767/16/\$30.00

To resolve the contribution of PGI<sub>2</sub> and its IP receptor during sensitization of the airways to the common airborne allergen HDM, lung mucosal immune responses were examined in PGI<sub>2</sub> IP receptor-deficient (IP<sup>-/-</sup>) mice and compared with wild-type (WT) counterparts. In mice lacking IP, the size of the NK cell pool in the lung tissue was increased 2-fold and these cells produced high levels of IFN- $\gamma$ . Remarkably, IP<sup>-/-</sup> mice were less responsive to HDM allergen sensitization than WT counterparts because intranasal instillation of the allergen induced markedly reduced levels of eosinophils, CD4<sup>+</sup> lymphocyte infiltration, and mucus production in the airways. The reduced allergic inflammation was associated with a decrease in the number of type 2 innate lymphoid cells (ILC2s) in the lungs of HDM-challenged IP<sup>-/-</sup> mice. NK cells appeared linked to the reduced ILC2s numbers because depletion of NK cells in IP<sup>-/-</sup> mice restored ILC2 numbers. These findings reveal a previously unknown capacity for PGI<sub>2</sub> in regulating pulmonary NK (pNK) cells and reveal a role for NK cells in preventing airway sensitization to inhaled allergen and subsequent development of HDM-induced allergic lung inflammation.

## Materials and Methods

### Mice

C57BL/6 (The Jackson Laboratory, Bar Harbor, ME) and IP<sup>-/-</sup> mice (10–16 wk old) were used throughout this study. IP<sup>-/-</sup> mice were originally developed and kindly provided by Dr. Garret A. FitzGerald's Laboratory, Institute for Translational Medicine and Therapeutics, Philadelphia, PA. All mice were bred under pathogen-free conditions in a barrier facility. Experimental animals were maintained in microisolator cages and treated in accordance with National Institutes of Health guidelines and the American Association of Laboratory Animal Care regulations. Animal experiments were approved by the University of Montana, Institutional Animal Care and Use Committee according to National Institutes of Health guidelines.

### Intranasal administration of house dust mite allergen

To elicit allergic lung inflammation, mice were sensitized to HDM on day 0 by the intranasal administration of 100  $\mu$ g of HDM allergen (*Dermaphagoides pteronyssinus*; Greer Laboratories, Lenoir, NC) in 30  $\mu$ l of PBS and then on days 7 and 14 by intranasal treatment with 50  $\mu$ g of HDM (30  $\mu$ l total volume). HDM allergen preparations used throughout this study contained minimal levels of LPS. Control groups comprised mice receiving 30  $\mu$ l of PBS on days 0, 7, and 14. To determine the level of mucosal inflammation, bronchoalveolar lavage fluid (BALF) and lung tissue were harvested 48 h after the last challenge on day 16.

### Determining the level of pulmonary inflammation

Bronchoalveolar lavage was performed to collect BALF for analysis. Eosinophil peroxidase (EPO) levels in the lavage cells were determined by colorimetric analysis. Cell differential percentages were determined by light microscopic evaluation of Hema3-stained cytospin preparations and expressed as absolute cell numbers. Lung tissue was dispersed by collagenase (Type IV; Sigma-Aldrich, St Louis, MO), and lung mononuclear cells (LMC) were isolated by Percoll (Sigma-Aldrich) density gradient for functional analysis.

### CX<sub>3</sub>CL1 immunochemical detection and neutralization in vivo

Paraffin-embedded lung tissues were deparaffinized and treated with 0.3% H<sub>2</sub>O<sub>2</sub>, followed by blocking non-specific binding and the sections were then incubated with 5  $\mu$ g/ml goat anti-mouse CX<sub>3</sub>CL1 polyclonal Ab (R&D Systems, Minneapolis, MN) at 4°C overnight. After washing, they were incubated with horse anti-goat IgG peroxidase (Vector Labs, Burlingame, CA) for 1 h at room temperature. Peroxidase binding sites were amplified with avidin-biotin complex reagent (ABC reagent; Vector Labs) for 1 h, and peroxidase activity was visualized using diaminobenzidine. Sections were counterstained with hematoxylin and visualized using Nikon Eclipse 800 light microscope equipped with Olympus DP71 camera and cellSens software.

In certain experiments, CX<sub>3</sub>CL1 activity was inhibited by the administration of a neutralizing mAb to CX<sub>3</sub>CL1 (rat IgG<sub>2A</sub>; R&D Systems) or control IgG<sub>2A</sub> isotype mAb by oropharyngeal instillation (25  $\mu$ g in 30  $\mu$ l PBS over a 6-d period on days 0 and 3) into the airways of naive mice prior to isolating lung tissues for generating LMC and enumeration of pNK cell numbers (on day 6).

### Measurement of cytokines, chemokines, and eicosanoids

To examine cytokine production in vitro, dispersed LMC or spleen cells were stimulated with immobilized anti-CD3 (2  $\mu$ g/ml, 2C11; ATCC), anti-NKp46 (10  $\mu$ g/ml, 29A1.4; BioLegend, San Diego, CA) or anti-NK1.1 Ab (PK136, 20  $\mu$ g/ml) in the absence or presence of 10 ng/ml mouse IL-2 (R&D Systems). After culture for 24 h, supernatants were harvested and cytokines (IL-4, IL-5, IL-13, TNF- $\alpha$ , IFN- $\gamma$ , IL-12, IL-18 and IL-15) measured by ELISA (R&D Systems).

BALF chemokine or cytokine levels were determined using ELISA for measurement of CCL2, CX<sub>3</sub>CL1 (DuoSet; R&D Systems), and IL-13 (Quantikine; R&D Systems), or using the sensitive Mouse V-Plex Pro-Inflammatory Panel 1 assay and Meso Quickplex 120 reader (MesoScale Discovery, MD) for measurement of other cytokines (IL-4, IL-5, TNF- $\alpha$ , IFN- $\gamma$ , and IL-12p70). PG and cysteinyl leukotriene levels in the BALF were measured using PG screening and cysteinyl leukotriene ELISA kits (Cayman Chemical, Ann Arbor, MI).

### Histological determination of peribronchial inflammation and goblet cell hyperplasia

Lung tissue was fixed in 4% paraformaldehyde and embedded in paraffin using a Leica ASP 300 tissue processor (Leica, Bannockburn, IL). Microtome sections were cut at 5- $\mu$ m thickness and stained with H&E using a Shandon Varistain 24-4 (Thermo Fisher Scientific). Alternatively, sections were stained using periodic acid-Schiff (PAS) reagent. The level of peribronchial inflammation (H&E stained) or mucus production (PAS stained) was analyzed by microscopy and the transmitted light images were collected on a Nikon Eclipse 800 microscope equipped with an Olympus DP 71 camera and cellSens software (Version 1.9).

### In vivo depletion of NK1.1<sup>+</sup> NK cells

To determine the in vivo effects of NK cells on allergic lung inflammation, NK1.1<sup>+</sup> cells were depleted using anti-NK1.1 Ab. The anti-NK1.1 MoAb (PK136 obtained from the American Type Culture Collection (ATCC), Manassas, VA) was purified by protein-A affinity chromatography (28). Briefly, C57BL/6 WT and IP<sup>-/-</sup> mice were injected i.p with 250  $\mu$ g anti-NK1.1 Ab or control IgG (mouse IgG2a) 24 h prior to the start of HDM challenge and then twice weekly for a period of 2 wk (on days -1, 3, 6, 10 and 13). Mice were challenged with PBS (control) or HDM allergen on days 0, 7 and 14 with the allergic inflammatory response characterized on day 16. In certain experiments, naive IP<sup>-/-</sup> or WT mice were similarly treated with the anti-NK1.1 or control mAb, twice weekly over a period of 2 wk. After treatment, the number of NK cells remaining in the lungs and spleen was determined by enumerating CD3<sup>+</sup>NK1.1<sup>+</sup>NKp46<sup>+</sup> cells.

### Purification of splenic NK cells and transfer to the airways

NK cells were purified by magnetic cell sorting (MagCelect; R&D Systems) of spleen cells prepared from WT or IP<sup>-/-</sup> mice. Sorted cells were selected on the basis of being CD3<sup>+</sup>NK1.1<sup>+</sup> and purity was checked by measuring the proportion of CD3<sup>+</sup>NK1.1<sup>+</sup> cells (87–92% over three experiments). Purified splenic NK cells (5  $\times$  10<sup>5</sup>) were suspended in RPMI 1640 (lacking FBS) and instilled directly into the airways of WT mice by the oropharyngeal route in a 30  $\mu$ l volume 24 h after the start of HDM allergen sensitization period as detailed above. Purified NK cells were pretreated with 10 ng/ml of IL-2 prior to transfer into hosts to maintain cell viability and function. Sham control mice received an equivalent suspension media alone by the oropharyngeal route. Mice were subsequently challenged with 50  $\mu$ g of HDM allergen on days 7 and 14 and the airway inflammatory characterized on day 16. Examination of the eosinophilic infiltration into the airways was determined by cell differential counts and measuring cell-associated EPO activity.

### Flow cytometry

Cells (LMC, BALF, or splenic) were Fc $\gamma$ R blocked using 2.4G2 Ab (ATCC) and stained with combinations of the following mouse-conjugated mAb (all purchased from BioLegend): allophycocyanin or FITC anti-CD3, allophycocyanin/Cy7 anti-CD4, PE anti-CD8a, allophycocyanin-Cy7 anti-CD19, allophycocyanin or PE anti-CD49b DX5 (pan-NK cells) or Ly49a, FITC or allophycocyanin anti-NK1.1 (PK136), PE or allophycocyanin anti-CD335 (NKp46), PE or allophycocyanin CD27, PE anti-CD94 (NKG2), FITC or PE anti-NKG2D (CD314), allophycocyanin or PE anti-CD11c, PE or allophycocyanin/Cy7 anti-I-A/I-E, allophycocyanin/Cy7 anti-Ly6G, allophycocyanin or allophycocyanin/Cy7 anti-Ly6C, allophycocyanin/Cy7 anti-Ly-6G/Ly6C (Gr1), PE, FITC or Brilliant Violet 421 anti-CD11b, allophycocyanin or PE anti-F4/80. In addition, PE anti-Siglec-F (BD Biosciences, to stain eosinophils), Alexa Fluor 647 anti-Dectin-2 (AbD Serotec), and PE or allophycocyanin anti-CX3CR1 (R&D Systems) mAb

were used. For intracellular staining, FITC anti-granzyme B, allophycocyanin anti-granzyme A, and allophycocyanin anti-IFN- $\gamma$ , (all Abs from BioLegend) were used and cells stained intracellularly as previously (29). Flow cytometric acquisition was performed on a FACSaria II (BD Biosciences, San Jose, CA) by four-color analysis using FACSDiVa software and FlowJo, and a minimum of 50,000 live, single-cell events were collected. Lung ILC2s were lineage negative (Lin $^{-}$ ) cells that were stained with Thyl.2/CD90.2 (AlexaFluor 405), CD45 (CLA) (allophycocyanin/Cy7), and IL-33R $\alpha$ /ST2 (allophycocyanin) mAb (all from BioLegend). Lin $^{-}$  cell determination was attributed to cells that were CD3 $^{-}$ , CD4 $^{-}$ , CD8 $\alpha$  $^{-}$ , CD5 $^{-}$ , CD11c $^{-}$ , CD19 $^{-}$ , B220 $^{-}$ , TCR $\beta$  $^{-}$ ,  $\gamma\delta$ -TCR $^{-}$ , Gr1 $^{-}$ , NK1.1 $^{-}$ , TER-119 $^{-}$  (all FITC mAb from BioLegend), and Siglec-F $^{-}$  (using PE mAb; BD Biosciences). Additionally, eFluor 660 anti-IL-13 (Affymetrix eBioscience, San Diego, CA) was used for intracellular staining. Flow cytometric acquisition was performed on a FACSaria II (BD Biosciences) by six-color analysis using FACSDiVa software or Attune NxT flow cytometer (Thermo Fisher Scientific, Waltham, MA).

### Statistical analyses

Data were analyzed using GraphPad Prism 5.0 (GraphPad, La Jolla, CA). Results involving two variables were analyzed by two-way ANOVA with a Bonferroni post test. Data comparing two groups were analyzed using an unpaired *t* test. Figures show combined data from multiple studies or independent repeats (two or more). Data shown are mean  $\pm$  SEM. A *p* value  $< 0.05$  was considered statistically significant.

## Results

*The lungs of mice lacking the PGI $_2$  receptor, IP $^{-/-}$ , contain increased numbers of NK cells that express elevated levels of NKp46*

pNK were characterized in IP $^{-/-}$  mice following dispersion of lung tissue using collagenase and the resulting LMC examined by multicolor flow cytometry. The NK1.1 alloantigen (a member of

the Mkrp1c gene family) was used as a specific marker for NK cells (30). CD3 $^{-}$ CD19 $^{-}$ NK1.1 $^{+}$  pNK cells from IP $^{-/-}$  mice were enumerated and compared with C57BL/6 WT counterparts. Unexpectedly, a 2-fold increase in the number of pNK cells was found in the lungs of IP $^{-/-}$  compared with WT mice ( $2.3 \times 10^5$  versus  $1.2 \times 10^5$  NK cells per mouse respectively, Fig. 1A), although the total number of leukocytes present in the lungs of these mice was similar. Given the elevated numbers of IP $^{-/-}$  pNK cells it was important to establish their relationship to circulating NK cells. To this end, the expression of known NK differentiation markers by CD3 $^{-}$ CD19 $^{-}$ NK1.1 $^{+}$  cells was evaluated. IP $^{-/-}$  pNK cells expressed over 4-fold elevated levels of NKp46 (CD335 or natural cytotoxicity triggering receptor-1) compared with WT NK cells (Fig. 1B). In marked contrast to the elevated pNK cell numbers, the number of CD3 $^{-}$ CD19 $^{-}$ NK1.1 $^{+}$  NK cells present in spleen, bone marrow, axillary, and mesenteric lymph nodes, and Peyer's Patches of IP $^{-/-}$  mice were not significantly different from that observed in WT mice. However, NKp46 expression by NK cells in IP $^{-/-}$  mice in all such tissue sites examined was elevated and comparable to pNK cells (Supplemental Fig. 1). The IP $^{-/-}$  pNK cells were indistinguishable from circulating NK cells because  $>95\%$  of CD3 $^{-}$ CD19 $^{-}$ NK1.1 $^{+}$  pNK cells were DX5 $^{+}$  (the  $\alpha 2$  integrin CD49b), a late NK maturation marker (Fig. 1B). Subsets of pNK cells in IP $^{-/-}$  mice expressed CD27 and Ly49A ( $\sim 19.9\%$  and  $14.7\%$ , respectively) but no appreciable difference to that observed in WT mice was evident. Intracellular staining of the pulmonary cells revealed that the majority ( $>95\%$ ) of the IP $^{-/-}$  pNK cells expressed granzyme A (and granzyme B, data not shown). Innate lymphoid cells have also been reported to express

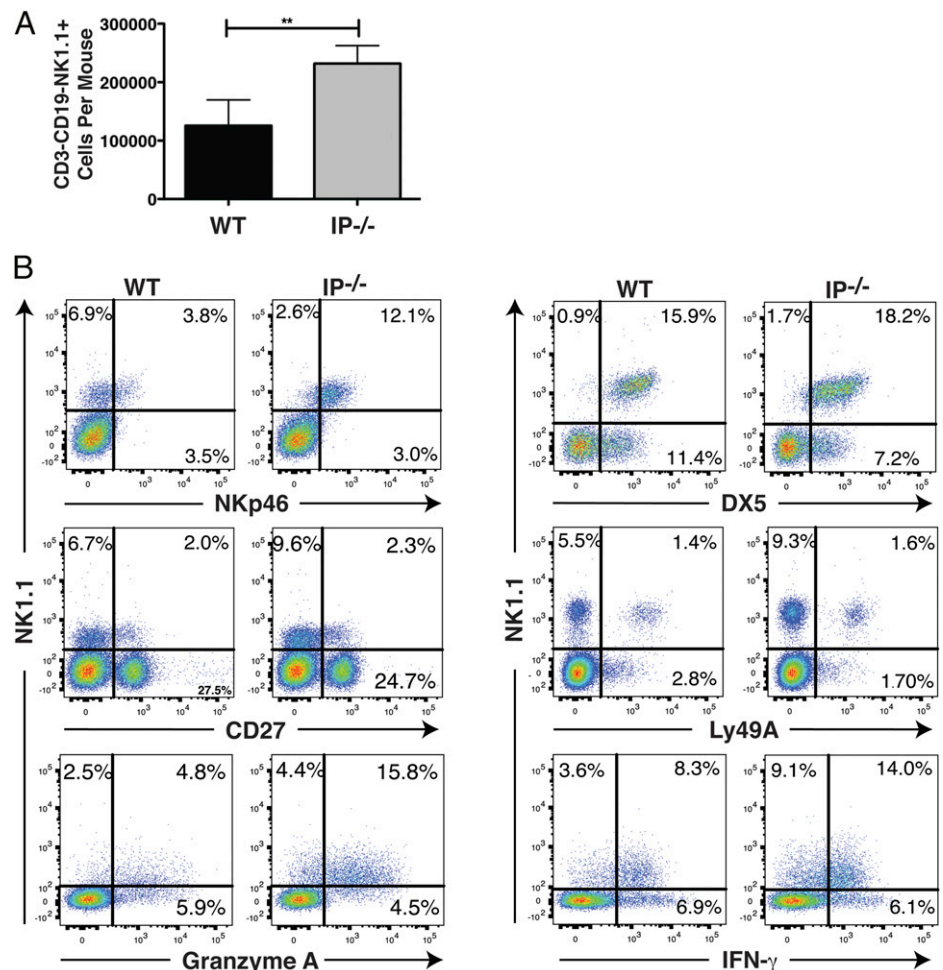


**FIGURE 1.** IP $^{-/-}$  mice exhibit increased numbers of pulmonary NK cells that express elevated levels of NKp46. Lung tissue from C57BL/6 WT and IP $^{-/-}$  mice (six per group) was dispersed using collagenase to prepare lung mononuclear cells (LMC) and the characteristics of CD3 $^{-}$ CD19 $^{-}$ NK1.1 $^{+}$  NK cells was determined by four-color flow cytometry. (A) After gating on CD3 $^{-}$ CD19 $^{-}$  cells, the total number of NK1.1 $^{+}$  NK cells was assessed, and (B) the level of NKp46, DX5, CD27, and Ly49A expression as well as intracellular Granzyme A and IFN- $\gamma$  expression by the NK1.1 $^{+}$  NK cells was determined. Data are mean  $\pm$  SEM and expressed as total number of lung CD3 $^{-}$ CD19 $^{-}$ NK1.1 $^{+}$  NK cells per mouse ( $n = 6$ ). Results are representative of three independent experiments,  $**p < 0.01$ .

NKp46 in some instances but these cells typically coexpress CD127 (31). CD127 was universally absent from the CD3<sup>+</sup>CD19<sup>+</sup>NK1.1<sup>+</sup> pNK cells in both IP<sup>-/-</sup> and WT mice (data not shown). Collectively, these results demonstrate that IP<sup>-/-</sup> mice have elevated numbers of NK cells expressing NKp46, DX5, and granzymes A and B, and revealed that the pNK cells are indistinguishable from mature NK cells.

#### Increased production of IFN- $\gamma$ by pNK cells in IP<sup>-/-</sup> mice

Although largely recognized by their ability to mediate cytolytic activity, NK cells are also an important source of proinflammatory cytokines that include IFN- $\gamma$  and TNF- $\alpha$ . Given the elevated numbers of NK1.1<sup>+</sup> NK cells present in IP<sup>-/-</sup> lungs, we next examined the levels of IFN- $\gamma$  and TNF- $\alpha$  produced by these pNK cells. Intracellular staining of IFN- $\gamma$  revealed that the majority of pNK in WT and IP<sup>-/-</sup> mice expressed this cytokine (Fig. 1B). Because NK1.1 and NKp46 are activating receptors, an effective way to determine cytokine production by NK cells was to determine LMC response to immobilized anti-NK1.1 or anti-NKp46 Abs. Cytokine release from LMC was determined using Ab-coated plates in the absence or presence of exogenous IL-2 as detailed previously (32). A significant elevation in IFN- $\gamma$  production in response to anti-NKp46 or anti-NK1.1 stimulation (in the presence of IL-2) was observed in LMC from IP<sup>-/-</sup> mice (2-fold or more) compared with WT counterparts (Fig. 2). These responses are consistent with a 2-fold increase in the number of NK1.1<sup>+</sup> cells present in the lungs and elevated NKp46 expression by NK cells. Interestingly, a slight elevation in IFN- $\gamma$  production by LMC from IP<sup>-/-</sup> mice was also observed in the presence of exogenous IL-2 alone or spontaneously, possibly due to elevated myeloid cells in these mice (Fig. 2). In both IP<sup>-/-</sup> and WT LMC the amount of production of TNF- $\alpha$  by pNK was negligible and <40 pg/ml. Because NK cells are responsive to the cytokines IL-12, IL-18, and IL-15, we examined whether any of them were elevated in the airways or lung tissue of IP<sup>-/-</sup> mice. Although none of these cytokines were detected in the BALF of naive IP<sup>-/-</sup> mice (data not shown), their contribution to the increased numbers of pNK cells cannot be excluded.

#### Elevated CX<sub>3</sub>CL1 levels in the airways of IP<sup>-/-</sup> mice regulate cytokine production by pNK cells

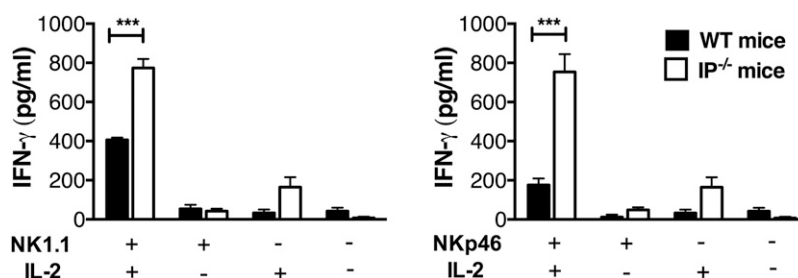
CX<sub>3</sub>CL1/fractalkine and its receptor CX<sub>3</sub>CR1 promote the chemotaxis of macrophages (33) and cytolytic lymphoid cells including NK cells, CD8<sup>+</sup> cells, and  $\gamma\delta$  T cells (34). CX<sub>3</sub>CL1 is unusual insofar as it exists as a membrane-associated form that is proteolytically released from the cells. Given the elevated numbers of pNK cells in IP<sup>-/-</sup> mice, we examined whether the chemokine CX<sub>3</sub>CL1 was present in the airways of these mice. Interestingly, 2-fold higher levels of CX<sub>3</sub>CL1 were found in the BALF of naive IP<sup>-/-</sup> mice compared with WT animals (Fig. 3A). Because expression of this chemokine is reported to be predominantly by epithelial and endothelial cells (35), we investigated its expression in lung tissue of both WT and IP<sup>-/-</sup> mice by immunohistochemistry. The majority

of CX<sub>3</sub>CL1 expression in the lungs of IP<sup>-/-</sup> mice was found to be associated with the airway epithelium, with lower levels of expression observed in the lung tissue of WT mice (Supplemental Fig. 2). To confirm that NK cells in both IP<sup>-/-</sup> and WT mice potentially respond to this chemokine, the expression of CX<sub>3</sub>CR1 receptor by NK cells was examined. Flow cytometric analysis revealed that CD3<sup>+</sup>NK1.1<sup>+</sup> NK cells in both naive IP<sup>-/-</sup> and WT mice expressed CX<sub>3</sub>CR1 (Fig. 3B). To examine whether CX<sub>3</sub>CL1 was responsible for influencing cytokine production by pNK cells or promoting the homeostatic recruitment of these cells to the lungs of IP<sup>-/-</sup> mice, a neutralizing Ab to CX<sub>3</sub>CL1 or control IgG<sub>2A</sub> isotype Ab (25  $\mu$ g in 30  $\mu$ l PBS over a 6 d period on days 0 and 3) was administered by oropharyngeal instillation into the airways of naive IP<sup>-/-</sup> or WT mice. Lung tissues were harvested (on day 6) and cytokine production or the frequency of CD3<sup>+</sup>CD19<sup>+</sup>NK1.1<sup>+</sup> NK cells in LMC was determined. Interestingly, in vivo administration of anti-CX<sub>3</sub>CL1 mAb to naive WT or IP<sup>-/-</sup> mice resulted in a significant reduction in the production of IFN- $\gamma$  by pNK from IP<sup>-/-</sup> mice (but not WT) in response to IL-15 + IL-18 or NKp46 stimulation (Fig. 3C). In contrast, the number of CD3<sup>+</sup>CD19<sup>+</sup>NK1.1<sup>+</sup> NK cells present in the lungs of both WT or IP<sup>-/-</sup> mice was unchanged after treatment with anti-CX<sub>3</sub>CL1 Ab (Fig. 3D). These findings suggest that CX<sub>3</sub>CL1 plays a role in the maintenance of cytokine production by pNK cells rather than the recruitment of NK cells to the lungs.

#### HDM-induced allergic lung inflammation and Th2 cytokine production are attenuated in IP<sup>-/-</sup> mice

Given the increase in pNK cell numbers in IP<sup>-/-</sup> mice, it was important to resolve whether these cells influenced the development of adaptive immunity in the lung. NK cells have been shown to be capable of augmenting or suppressing the development of allergic lung inflammation (15, 17, 36). We thus evaluated whether the presence of increased numbers of pNK cells evident in the IP<sup>-/-</sup> mice impacts lung mucosal immune responses in these animals. To this end, acute allergic sensitization and challenge was elicited by repeated intranasal administration of HDM allergen over a 2-wk period. The level of HDM-induced pulmonary inflammation in IP<sup>-/-</sup> mice was compared with WT counterparts. HDM inhalation evoked a pronounced lung inflammation in C57BL/6 WT mice, characterized by the influx of eosinophils and lymphocytes into the airways and an increase in cell-associated EPO levels in the BALF (Fig. 4A). In marked contrast, the number of eosinophils and lymphocytes present in BALF of HDM-challenged IP<sup>-/-</sup> mice was markedly reduced compared with WT mice (by ~50%, Fig. 4A). IP<sup>-/-</sup> and WT mice that were repeatedly exposed to intranasal PBS (control) did not exhibit any evidence of eosinophil infiltration into the airway. Lung histological analysis (using H&E staining and PAS staining) revealed that HDM-exposed WT mice developed a pronounced peribronchial and perivascular inflammation and an increase in mucus production or goblet cell hyperplasia compared with control animals exposed to PBS (Fig. 4B). Both the peribronchial inflammation and mucus

**FIGURE 2.** Increased IFN- $\gamma$  production by pulmonary NK cells in IP<sup>-/-</sup> mice. The production of IFN- $\gamma$  by NK cells was determined by stimulating LMC, generated by collagenase dispersion of lung tissue, from naive WT or IP<sup>-/-</sup> mice (four per group) with either immobilized anti-NK1.1 or anti-NKp46 Ab in the presence or absence of IL-2. After culture for 24 h, IFN- $\gamma$  production was determined using ELISA (in triplicate). Data represent mean  $\pm$  SEM of three independent experiments, \*\*\* $p$  < 0.001.



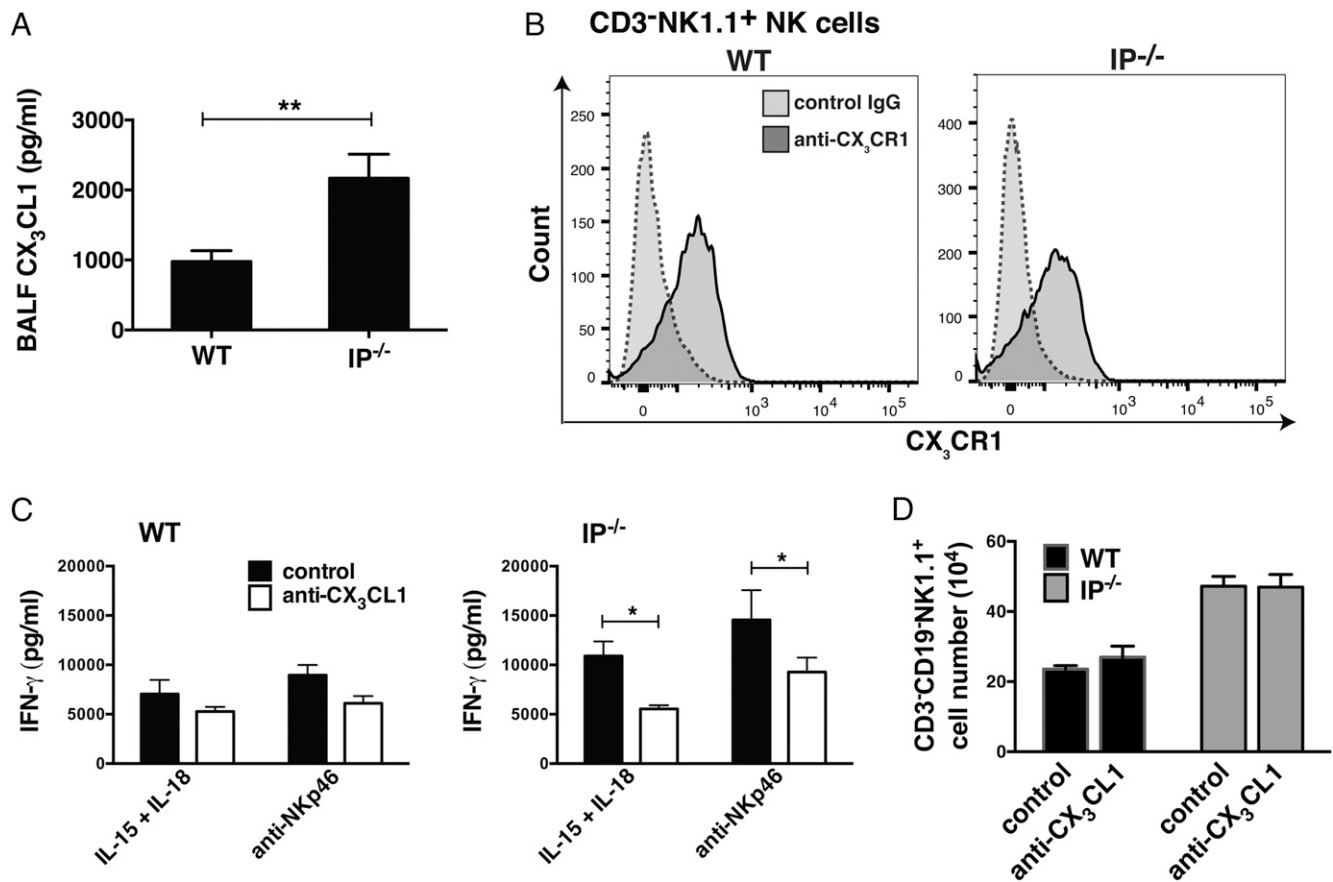


**FIGURE 3.** Elevated CX<sub>3</sub>CL1 levels in the airways of IP<sup>-/-</sup> mice regulate cytokine production by pNK cells. CX<sub>3</sub>CL1 chemokine levels in BALF from naive WT and IP<sup>-/-</sup> mice (six per group) were determined in conjunction with CX<sub>3</sub>CR1 receptor expression by NK1.1<sup>+</sup> NK cells. **(A)** CX<sub>3</sub>CL1 levels present in the BALF from naive IP<sup>-/-</sup> or WT mice measured by ELISA. Data are mean  $\pm$  SEM ( $n = 6$ ),  $^{**}p < 0.01$ . **(B)** Receptor CX<sub>3</sub>CR1 expression by purified splenic CD3<sup>+</sup>NK1.1<sup>+</sup> NK cells from WT or IP<sup>-/-</sup> mice determined by flow cytometry. The contribution of CX<sub>3</sub>CL1 in influencing cytokine production by pNK cells in IP<sup>-/-</sup> or WT mice was examined in vivo using a neutralizing Ab to CX<sub>3</sub>CL1. Anti-CX<sub>3</sub>CL1 or isotype control Ab was administered by oropharyngeal instillation (25  $\mu$ g in 30  $\mu$ l PBS over a 6-d period on days 0 and 3) into the airways of naive IP<sup>-/-</sup> or WT mice (six per group). Lung tissue was collected and dispersed in collagenase to prepare LMC. **(C)** IFN- $\gamma$  production by pNK cells was determined using ELISA (in triplicate) following stimulation of LMC with either IL-15 + IL-18 or anti-NKp46 Ab for 24 h. Data are mean  $\pm$  SEM,  $^{*}p < 0.05$ . **(D)** The effect of anti-CX<sub>3</sub>CL1 treatment on the frequency of pulmonary CD3<sup>+</sup>CD19<sup>+</sup>NK1.1<sup>+</sup> NK cells was determined by flow cytometry. Results are representative of three independent experiments.

secretion were markedly reduced in HDM-challenged IP<sup>-/-</sup> mice (Fig. 4B), with limited evidence for eosinophil infiltration. The infiltration of eosinophils into the airway was further demonstrated by flow cytometry where the number of Gr1<sup>+</sup>CD11b<sup>+</sup> Siglec-F<sup>+</sup> eosinophils present in the BALF of HDM-treated IP<sup>-/-</sup> mice was clearly reduced compared with WT counterparts (Fig. 4C). To determine if the reduced inflammatory response was associated with decreased numbers of APCs present in the lungs, we enumerated the number of monocyte-derived DC (moDC) (CD11c<sup>+</sup>MHC-II<sup>bright</sup>CD11b<sup>+</sup>) and epithelial-associated DC (CD11c<sup>+</sup>MHC-II<sup>bright</sup>CD103<sup>+</sup>) in the LMC. Following the onset of HDM-induced allergic inflammation, the number of CD11c<sup>+</sup>MHC-II<sup>bright</sup> DC in WT mice was markedly increased (0.64% in PBS controls versus 4.52% in HDM challenged) and these were predominantly CD11b<sup>+</sup> moDC rather than CD103<sup>+</sup> conventional DC (Fig. 4D). In marked contrast, the number of CD11c<sup>+</sup>MHC-II<sup>bright</sup>CD11b<sup>+</sup> DC in IP<sup>-/-</sup> mice was not significantly increased. Coincident with the onset of allergic inflammation in HDM-challenged WT mice was the presence of high levels of IL-13 in the BALF and increased concentration of IL-4, IL-5, and TNF- $\alpha$  (Fig. 5A). The production of all three cytokines was markedly depressed in BALF from IP<sup>-/-</sup> mice treated with HDM allergen. However, levels of IFN- $\gamma$  and IL-12 in the

BALF of HDM-exposed IP<sup>-/-</sup> or WT mice were undetectable (Fig. 5A), suggesting that the unresponsiveness in IP<sup>-/-</sup> mice was not a consequence of immune deviation of the HDM response toward a Th1 phenotype. CCL2 has been implicated in the recruitment of moDC during allergic inflammation (37) and is essential for Th2 polarization (38). Coincident with the onset of allergic inflammation in HDM-challenged WT mice, high levels of CCL2 were produced in the airways of these animals. However, consistent with the depressed Th2 cytokine levels, there was a marked reduction in CCL2 production in the BALF of HDM-exposed IP<sup>-/-</sup> compared with WT mice (Fig. 5B). To confirm that the observed effects in IP<sup>-/-</sup> mice did not arise as a consequence of altered eicosanoid biosynthesis, the levels of PGs and cysteinyl leukotrienes present in BALF of HDM-challenged or control IP<sup>-/-</sup> mice was compared with WT littermates. However, no significant differences in de novo levels of eicosanoids were observed in the airways of WT and IP<sup>-/-</sup> mice, although production in both strains tended to increase after allergen inhalation (Supplemental Fig. 3). The early induction of allergic airway inflammation has been reported to involve HDM interaction with alveolar macrophages via a Dectin-2-dependent mechanism (7). Given that the airways of IP<sup>-/-</sup> animals had elevated levels of CX<sub>3</sub>CL1, we examined whether the reduced responsiveness of IP<sup>-/-</sup> mice to allergen sensitization was due to changes in

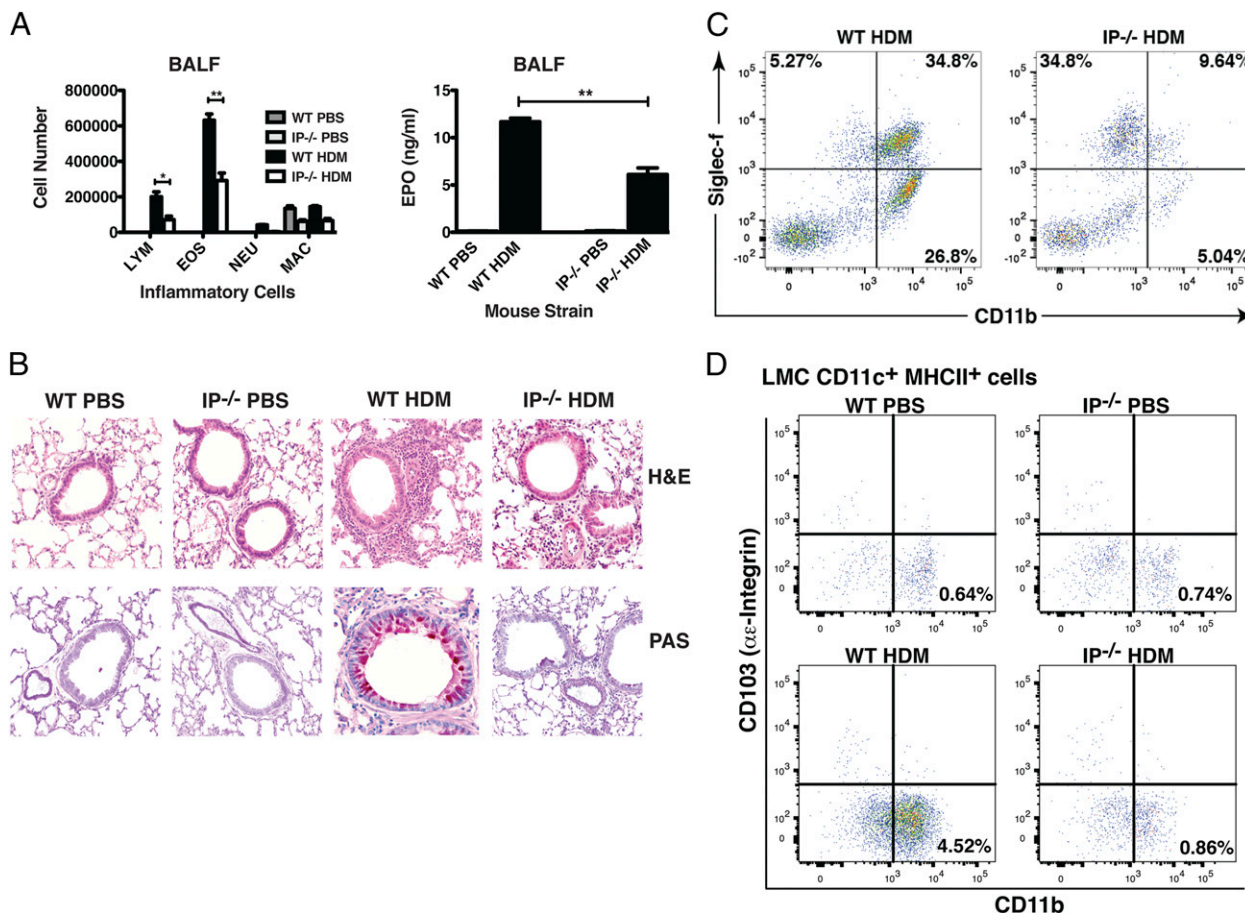


**FIGURE 4.** HDM-induced allergic lung inflammation and mucus production is attenuated in IP<sup>-/-</sup> mice. WT or IP<sup>-/-</sup> mice (six per group) were subjected to acute allergen sensitization and challenge by repeated intranasal administration of HDM allergen or PBS (control) on days 0, 7, and 14. BALF and lung tissue were harvested (on day 16) to determine the level of allergic inflammation. **(A)** The inflammatory cells present in the BALF were determined by cell differential counts and expressed as absolute cell numbers (per mouse) of lymphocytes (Lym), macrophages (Mac), eosinophils (Eos), and neutrophils (Neu). Eosinophil infiltration was also determined by measuring cell-associated eosinophil peroxidase (EPO) by colorimetric analysis. Data are mean  $\pm$  SEM ( $n = 6$ ), \* $p < 0.05$ , \*\* $p < 0.01$ . **(B)** Peribronchial and perivascular inflammation (H&E) and mucus production (PAS staining) was determined using histological analysis (original magnification  $\times 20$ ). **(C)** The number of Gr1<sup>+</sup>CD11b<sup>+</sup>Siglec-F<sup>+</sup> eosinophils present in BALF was determined using four-color flow cytometry. **(D)** The presence of moDC in LMC (prepared by collagenase dispersion of lung tissue) from WT and IP<sup>-/-</sup> mice following HDM challenge was examined by four-color flow cytometry. After gating on CD11c<sup>+</sup>MHC-II<sup>bright</sup> cells, the expression of  $\alpha$ E integrin (CD103) and CD11b was determined. Data are representative of three or four independent experiments.

phenotype and early allergen responses of CX<sub>3</sub>CR1<sup>+</sup> myeloid cells present in the lung tissue and airways. Flow cytometric analysis revealed that naive IP<sup>-/-</sup> mice had augmented numbers of CD11c<sup>+</sup>CX<sub>3</sub>CR1<sup>+</sup> cells in the BALF that exhibited markedly increased levels of MHC-II<sup>int</sup> expression but reduced levels of Dectin-2 compared with WT counterparts (Supplemental Fig. 4).

#### Depletion of NK cells restores allergic lung inflammation in IP<sup>-/-</sup> mice

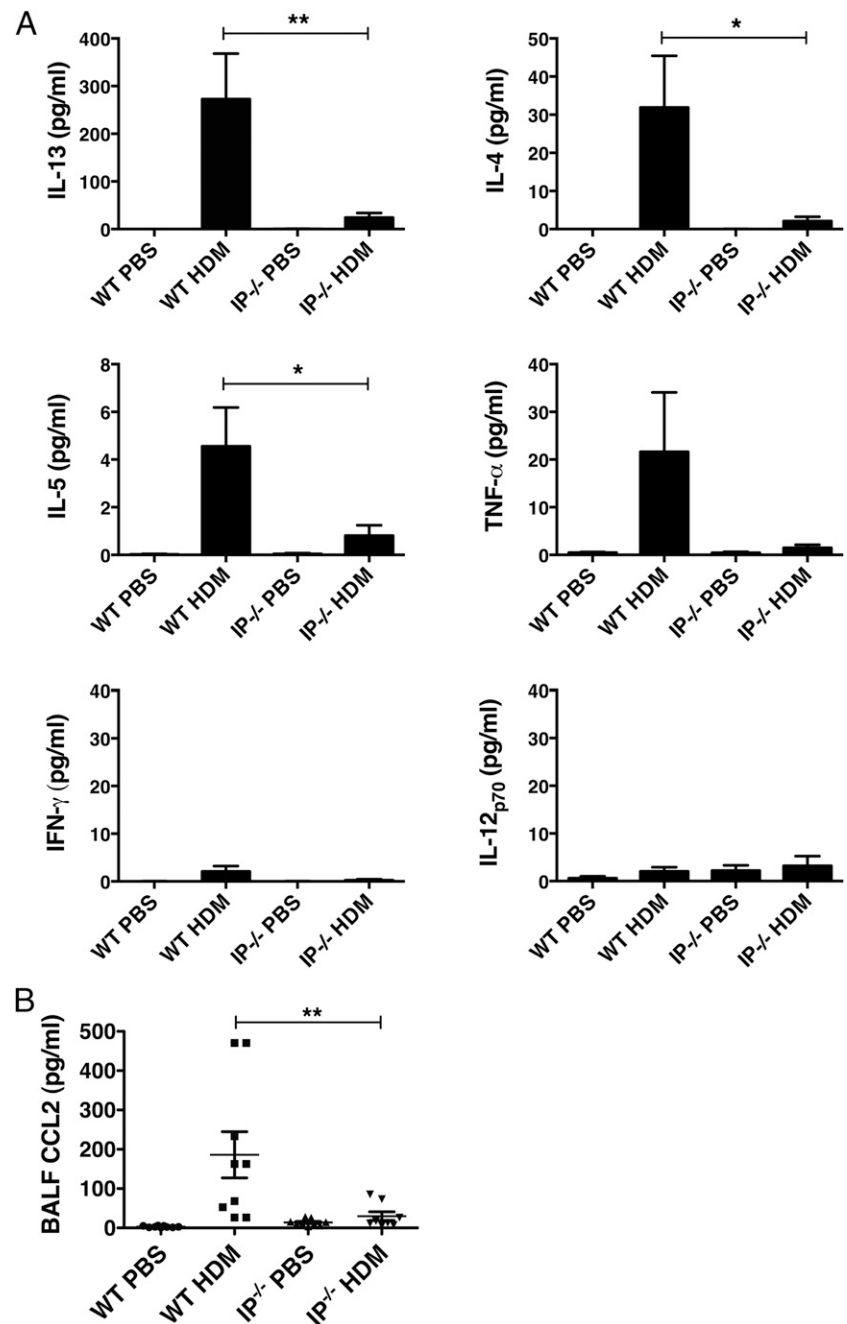
Because naive IP<sup>-/-</sup> mice had elevated numbers of CD3<sup>+</sup>CD19<sup>-</sup>NK1.1<sup>+</sup> NK cells in the lungs compared with WT animals, we examined the effect of depleting NK cells on the allergic inflammatory response. To deplete NK cells *in vivo*, IP<sup>-/-</sup> and WT mice were treated twice weekly with either the anti-NK1.1 Ab, PK136 (250  $\mu$ g), or control IgG during the acute allergen challenge period. Treatment with PK136 resulted in  $>98\%$  depletion of NK1.1<sup>+</sup> cells when evaluated at day 16 (data not shown). Interestingly, depletion of NK1.1<sup>+</sup> cells in HDM-exposed IP<sup>-/-</sup> mice restored the airway eosinophilia and cell-associated EPO levels to a similar amount to that observed in WT mice and this effect was not evident in mice receiving control IgG (Fig. 6A). The restoration of eosinophilic inflammation in IP<sup>-/-</sup> mice by depletion of NK1.1<sup>+</sup> cells, strongly implies that NK cells are

responsible for preventing the development of allergic lung inflammation in response to HDM. Treatment of WT mice with anti-NK1.1 also resulted in a slight increase in the number of eosinophils, possibly due the presence of fewer NK cells in these mice. As observed previously, production of the Th2 cytokine IL-13 in the airways of mice after allergen challenge was clearly reduced in IP<sup>-/-</sup> mice. However, treatment of HDM-exposed IP<sup>-/-</sup> mice with anti-NK1.1 Ab restored airway IL-13 production to a level similar to that found in WT mice (Fig. 6B). Moreover, lung histological analysis revealed that HDM-exposed IP<sup>-/-</sup> mice had reduced peribronchial inflammation and mucus production compared with allergen-challenged WT mice. Consistently, depletion of NK1.1 cells markedly increased the inflammatory pockets and mucus production in the lungs of HDM-challenged IP<sup>-/-</sup> mice (Fig. 6C). Dectin-2 expression by myeloid cells in the lungs of IP<sup>-/-</sup> mice was not restored to normal levels following NK cell depletion (data not shown) even though lung inflammatory responses were.

#### Adoptive transfer of NK cells into the airways suppresses the development of allergic lung inflammation

The depletion of NK cells using the anti-NK1.1 mAb (PK136) strongly implied that NK1.1<sup>+</sup> cells in the IP<sup>-/-</sup> mice are responsible for preventing the development of HDM-induced allergic airway

**FIGURE 5.** The attenuated allergic inflammation in  $IP^{-/-}$  mice is associated with a reduction in Th2 cytokine and CCL2 chemokine production. WT or  $IP^{-/-}$  mice (8–10 per group) were subjected to acute allergen challenge by repeated intranasal administration of HDM allergen or PBS (control) on days 0, 7 and 14. **(A)** Cytokine production in the BALF was assessed using ELISA or V-Plex assay, and **(B)** CCL2 chemokine levels were determined using ELISA. Results represent mean  $\pm$  SEM of three independent experiments, \* $p < 0.05$ , \*\* $p < 0.01$ .



inflammation. However, a limitation encountered when treating mice with the PK136 Ab is that both invariant NK-T cells and NK cells are depleted. To resolve that the anti-inflammatory effects were mediated by NK cells, we examined whether purified  $IP^{-/-}$  and WT splenic NK cells ( $5 \times 10^5$ ) instilled directly into the airways of WT mice via the oropharyngeal route (24 h after the start of allergen sensitization period) were able to inhibit HDM-induced lung eosinophilic inflammation. Remarkably, adoptive transfer of either  $IP^{-/-}$  or WT NK cells into the lungs of WT hosts significantly reduced the level of HDM-induced eosinophilic inflammation (Fig. 7A) and cell-associated EPO levels (Fig. 7B) compared with the sham group (no cell transfer). Interestingly, on a per-cell comparison WT and  $IP^{-/-}$  NK cells were equally effective at suppressing allergic pulmonary inflammation, suggesting that the major contributing factor to the attenuated responses evident in  $IP^{-/-}$  mice likely arose from the preponderance of NK cells in their lungs.

*The elevated number of pNK cells in  $IP^{-/-}$  mice is associated with depressed lung ILC2 numbers*

Although the elevated numbers of pNK cells in  $IP^{-/-}$  mice appeared responsible for reduced HDM-induced inflammatory responses, how they mediate immune suppression was unclear. To examine if a defect in pulmonary ILC2 function was responsible for the attenuated HDM responses, we characterized the number and properties of ILCs present in the lungs of  $IP^{-/-}$  mice by enumerating  $Lin^{-}$  cells that coexpressed CD45 (CLA) and CD90.2 using flow cytometry. The ILC2 phenotype was confirmed by intracellular staining for IL-13 and surface expression of IL-33R $\alpha$ /ST2, as detailed previously (39). This approach revealed that lung tissues from  $IP^{-/-}$  mice had markedly reduced numbers of  $Lin^{-}CD45^{+}CD90.2^{+}$  ILC2s expressing IL-33R $\alpha$  (4.90% in WT versus 0.65% in  $IP^{-/-}$  mice) and IL-13 (4.74% in WT versus 2.53% in  $IP^{-/-}$  mice) compared with WT mice (Fig. 8A). As

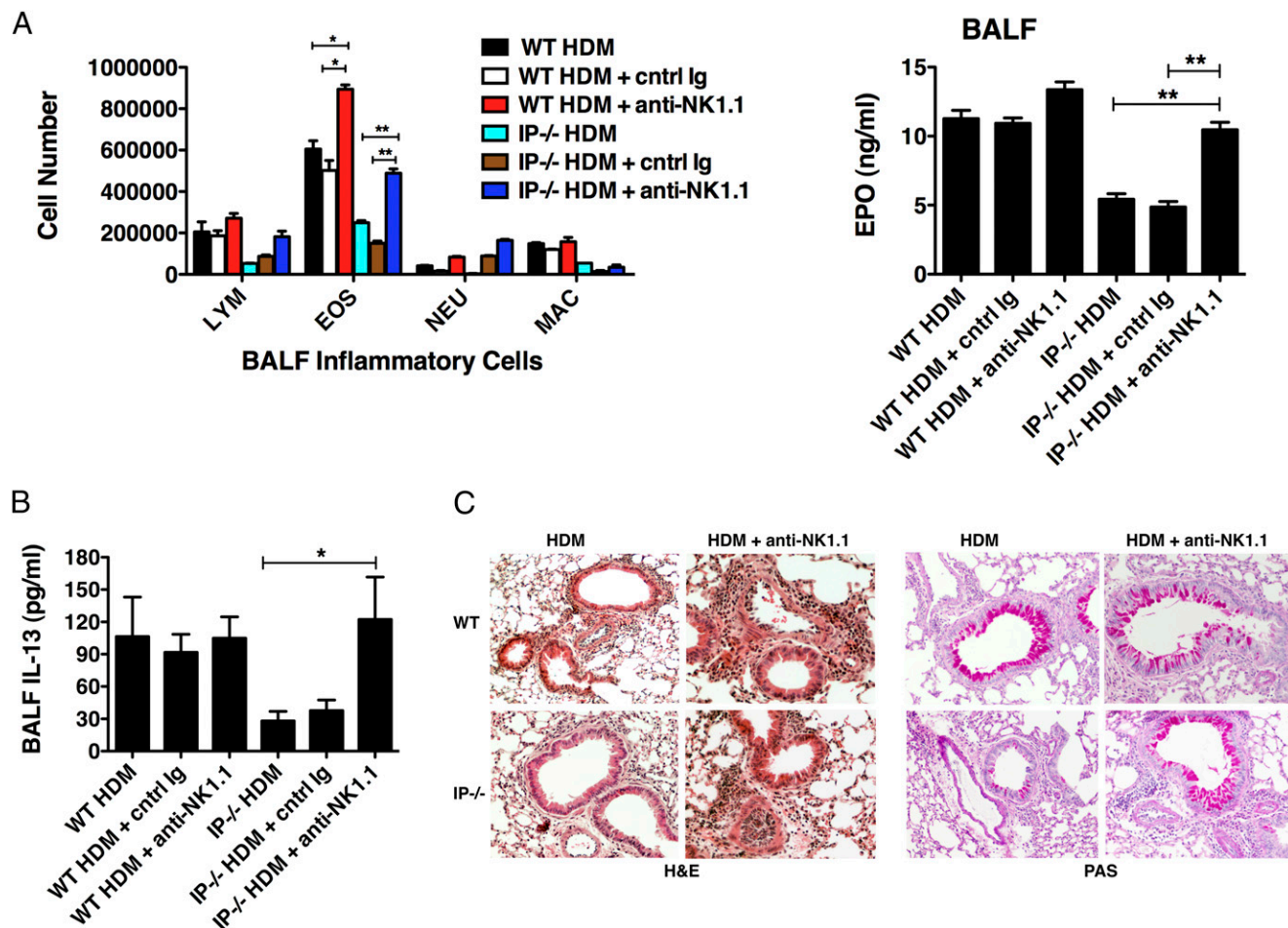


**FIGURE 6.** Depletion of NK1.1<sup>+</sup> cells in IP<sup>-/-</sup> mice restores allergic lung inflammation. To deplete NK1.1<sup>+</sup> cells in vivo, WT and IP<sup>-/-</sup> mice (six per group) were injected with anti-NK1.1 Ab (250  $\mu$ g i.p.) or control IgG twice weekly over a period of 2 wk during the acute allergen sensitization and challenge period. Following intranasal administration of HDM or control PBS (on days 0, 7 and 14), BALF and lung tissue were harvested (on day 16) to examine the level of allergic airway inflammation and IL-13 production. **(A)** Cell differential counts in the BALF were determined and expressed as absolute numbers (per mouse) of lymphocytes (Lym), eosinophils (Eos), neutrophils (Neu) and macrophages (Mac). Eosinophil infiltration was also assessed by measuring cell-associated eosinophil peroxidase (EPO) using colorimetric analysis. Results are mean  $\pm$  SEM ( $n = 6$ ),  $*p < 0.05$ ,  $**p < 0.01$ . **(B)** IL-13 levels in BALF were measured by ELISA. Results are mean  $\pm$  SEM ( $n = 6$ ),  $*p < 0.05$ . **(C)** Peribronchial inflammation (H&E staining) and mucus production (PAS staining) was determined by histological analysis of lung tissue (original magnification  $\times 20$ ). Data are representative of three or four independent experiments.

evidenced by the previous observations, the reduced number of ILC2s in IP<sup>-/-</sup> mice was coincident with an increased total number of pNK cells (6.84% in WT versus 12.24% in IP<sup>-/-</sup>, Fig. 8B). To examine the possibility that NK1.1<sup>+</sup> NK cells were directly linked to and/or responsible for the reduced numbers of ILC2s, NK cells were depleted by administration of anti-NK1.1 Ab to naive WT and IP<sup>-/-</sup> mice. The effect of treating mice with anti-NK1.1 mAb or control Ig (twice weekly over a period of 2 wk) on the total number of lung ILC2s was determined by flow cytometry. Depletion of NK cells was associated with a significant increase in the number of Lin<sup>-</sup>CD45<sup>+</sup>CD90.2<sup>+</sup> IL-33R $\alpha$  ILC2s in the lungs of IP<sup>-/-</sup> mice to a level similar to WT counterparts (Fig. 8C). These data are consistent with the possibility that the decreased HDM responses in IP<sup>-/-</sup> mice arises because of reduced lung ILC2 numbers caused by NK cell-mediated regulation.

## Discussion

HDM is a clinically relevant allergen that is capable of conferring immunity following inhalation without the need to resort to the use of immunological adjuvants to prime the response. In mice, repeated inhalation of airborne HDM allergen results in the activation

of adaptive immunity characterized by a pronounced CD4<sup>+</sup> Th2 response, generation of specific IgE, and infiltration of eosinophils into the airway (40). The mucosal allergic response in the airway is typified by increased mucus production and AHR, analogous to inflammatory processes observed in atopic asthma. The surprising finding in this study was that the inflammatory response elicited by inhaled HDM allergen was compromised in mice lacking IP receptors. To explain the observed defect in allergic sensitization in IP<sup>-/-</sup> mice, deficiencies in the resident lung mucosal lymphoid and myeloid cells were sought. The most notable immunological characteristic of IP<sup>-/-</sup> mice was that their lung tissues contained 2-fold more NK cells when compared with WT littermates and that these pNK cells produced IFN- $\gamma$ . The increased size of the NK cell pool in IP<sup>-/-</sup> mice was only observed in the lungs because NK cell numbers present in lymphoid sites and other tissues, such as liver, remained normal. The elevated number of pNK cells in IP<sup>-/-</sup> mice could conceivably arise from expansion of these cells in the lung tissue and/or increased recruitment of NK cells to this site. NK cells in IP<sup>-/-</sup> mice expressed high levels of NKp46 compared with WT NK cells. NKp46 is a 46-kDa protein type I glycoprotein natural cytotoxicity receptor that associates with the

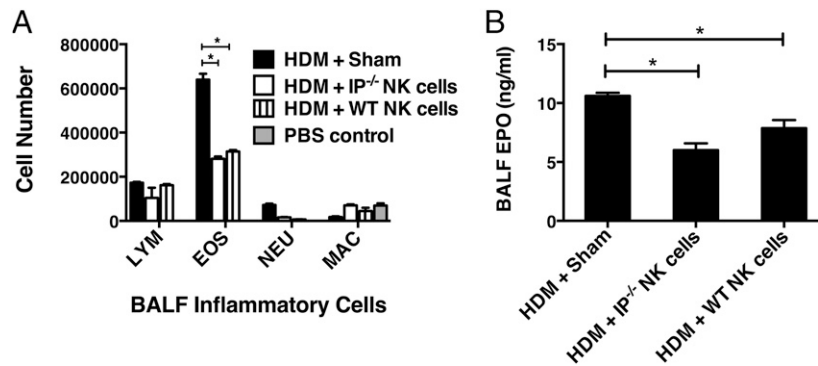


**FIGURE 7.** Adoptive transfer of NK cells suppresses allergic lung inflammation. NK cells were purified from spleens of IP<sup>-/-</sup> and WT mice (using MagCelect protocol) and the NK cells or PBS alone (sham group) were adoptively transferred into the airways of WT mice (six per group) by oropharyngeal instillation ( $5 \times 10^5$  cells per mouse). Following acute allergic sensitization and challenge with intranasal HDM or control PBS (on days 0, 7, and 14), the level of allergic lung inflammation was determined by examining the inflammatory cells present in the BALF. **(A)** Cell differential counts in the BALF were determined and expressed as absolute numbers (per mouse) of lymphocytes (Lym), eosinophils (Eos), neutrophils (Neu) and macrophages (Mac). **(B)** Eosinophil infiltration was assessed by measuring cell-associated eosinophil peroxidase (EPO) using colorimetric analysis. Results represent mean  $\pm$  SEM ( $n = 6$ ) of three independent experiments,  $*p < 0.05$ .

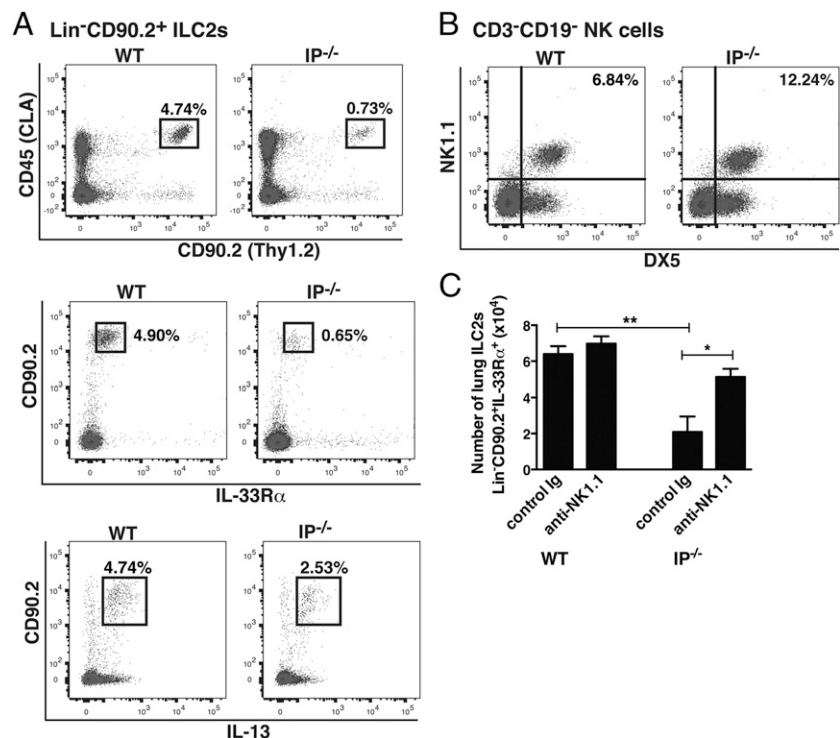
CD3 $\zeta$  and Fc $\epsilon$ RI $\gamma$  (41). It serves as a receptor for several ligands, which include vimentin (42) tumor Ags (43) or influenza viral hemagglutinin (44). Of these ligands, only vimentin is ubiquitous and its expression appeared normal in null mice, raising the possibility that elevated Nkp46 expression arises from an increased frequency of mature NK cells (45).

PGI<sub>2</sub> biosynthesis is promoted during allergic lung inflammation (46) and this mediator is known to have several immunoregulatory properties (29, 46, 47). That IP null mice have an immunological defect where NK cells are selectively affected is noteworthy. NK cells are responsive to a range of endogenously produced eicosanoids including Resolvin-E1 (18), Lipoxin-A4 (19), PGD<sub>2</sub> (20), PGE<sub>2</sub> (21), and leukotrienes (22). In the current study, no gross changes in eicosanoid levels in BALF of IP<sup>-/-</sup> mice was observed, suggesting that the altered phenotype in these animals was linked with lack of IP expression. A notable characteristic of the null mice was the augmented production of

CX<sub>3</sub>CL1 in the BALF that likely contributed to the altered phenotype of IP<sup>-/-</sup> mice. Our study confirms previous work that CX<sub>3</sub>CL1 is highly expressed by airway epithelial cells (35). Unusually CX<sub>3</sub>CL1 exists as both trans-membrane and soluble forms, the latter being generated following cleavage from the cell surface by the action of the proteases ADAM10, ADAM17 or  $\gamma$ -secretase (48). CX<sub>3</sub>CL1 appeared not to be responsible for the increased size of the pNK pool observed in IP<sup>-/-</sup> mice (49). However, administration of blocking Abs to the chemokine reduced IFN- $\gamma$  production by pNK cells in IP<sup>-/-</sup> mice, suggesting that CX<sub>3</sub>CL1 promotes cytokine production by pNK cells rather than their recruitment. In this context, CX<sub>3</sub>CL1 has been shown to augment both NK cell cytolytic activity (50, 51) and the release of IFN- $\gamma$  (51, 52). The relative contribution of cell-associated and soluble forms of CX<sub>3</sub>CL1 in augmenting pNK cell function remains unclear.

The elevated number of NK cells present in the lungs of IP<sup>-/-</sup> mice prompted speculation that they may be responsible for the

**FIGURE 8.** The elevated number of pNK cells in IP<sup>-/-</sup> mice is associated with depressed lung ILC2 numbers. Lung tissue was collected from WT and IP<sup>-/-</sup> mice (six per group) and LMC prepared by dispersion of lung tissue. **(A)** ILC2 numbers in the LMC were determined by enumerating Lin<sup>-</sup>CD45CLA<sup>+</sup>CD90.2<sup>+</sup> cells (after removing Lin<sup>-</sup> cells from the analysis), which expressed either IL-33R $\alpha$  or IL-13 (intracellular staining) using five-color flow cytometry. **(B)** The number of pNK cells was determined by enumerating NK1.1<sup>+</sup>DX5<sup>+</sup> cells in CD3<sup>-</sup>CD19<sup>-</sup> gated LMC. **(C)** The effect of depleting NK1.1<sup>+</sup> NK cells by administration of anti-NK1.1 mAb or control Ig to naive WT or IP<sup>-/-</sup> mice (twice weekly over a period of 2 wk) on the number and phenotype of lung ILC2s was determined by enumerating Lin<sup>-</sup>CD90.2<sup>+</sup>IL-33R $\alpha$ <sup>+</sup> cells. Results are expressed as cell number per mouse and are mean  $\pm$  SEM ( $n = 6$ ). Data are representative of three independent experiments,  $*p < 0.05$ ,  $**p < 0.01$ .



reduced allergic lung inflammation elicited by HDM in these animals. Critically, the unresponsiveness of IP<sup>-/-</sup> mice to inhaled HDM was strictly dependent on the presence of NK cells because anti-NK1.1 Ab treatment restored the lung inflammatory response. Moreover, the suppression of allergic inflammation was demonstrable by the oropharyngeal transfer of purified IP<sup>-/-</sup> NK cells into WT mice. Interestingly, both WT and IP<sup>-/-</sup> NK cells were equally effective at suppressing the development of allergic pulmonary inflammation, suggesting that the NK-mediated regulation evident in IP<sup>-/-</sup> mice was primarily a consequence of the preponderance of pNK cells in these animals. These observations reveal that NK cells in IP<sup>-/-</sup> mice play a pivotal role in preventing the onset of inflammation elicited by an inhaled allergen. Previous reports studying the impact of NK cells on the development of allergic lung inflammation have yielded contradictory findings. In certain instances, NK cells inhibited allergic lung inflammation (14, 15) and in others they promoted eosinophilic inflammation (16, 53), reviewed in Ref. 54. In some studies, the Ag sensitization phase was more susceptible to NK cell-mediated regulation compared with the subsequent lung inflammatory response elicited by OVA inhalation (55). It is noteworthy that these reports did not use HDM to elicit lung inflammation and the effect of endogenous eicosanoids was not considered. Certainly, NK cells have been shown to prevent the development of T cell immunity by multifarious mechanisms that typically fall into either affecting the APC or the responding T cells (56).

IP<sup>-/-</sup> mice are not overtly immune compromised, however, in studies utilizing protein Ags such as OVA, inflammatory responses are typically elevated (57, 58), often after systemic immunization using adjuvants (29). An important aspect of the current study is that it examines the responses elicited by the clinically relevant HDM allergen instilled directly into the airways, without the use of adjuvants (47). A notable difference between OVA and HDM responses in the lung is that ILC2s contribute to the lung inflammatory responses to allergens, such as papain (59, 60) and HDM (61–64), but have a less prominent role in OVA responses following systemic immunization (61, 64). ILC2s are a potent source of innate type 2 cytokines IL-5 and IL-13 in allergic lung inflammation (64, 65). They are present in human respiratory tissue and studies from mouse models of asthma demonstrate a role for these innate cells in promoting allergic inflammation (66, 67). ILC2s secrete IL-5 constitutively and are induced to express IL-13 during inflammatory processes and promote eosinophil homeostasis (68). Indeed, the raised frequency of NK cells in IP<sup>-/-</sup> mice was coincident with a reduction in the number of ILC2s present in the lung tissues, and NK cell depletion resulted in a rise in ILC2 numbers. These findings are consistent with the possibility that tissue NK cells, either directly or indirectly, limit ILC2 responses and subsequent allergic inflammation. Importantly, ILC2 function is susceptible to regulation by the action of IFN- $\gamma$  and IFN- $\alpha/\beta$  (69–71). This form of regulation could arise from either the direct effect of NK cells on ILC2 cells (likely by the action of IFN- $\gamma$ ) or, alternatively, by inhibiting IL-33 release in the lung mucosa. Such regulation likely would impact the development of T cell-mediated immunity because ILC2s promote the development of CD4<sup>+</sup> Th2 responses (72, 73).

In summary, our data demonstrate a hitherto unknown role for PGI<sub>2</sub> in regulating the number and properties of NK cells resident in lung tissue and reveal a role for NK cells in preventing allergic inflammation by limiting the number of HDM-responsive ILC2s in the lung. In recent years, development of novel therapeutic strategies that selectively target the Th2 response has proven difficult. We propose that the improved understanding of the role of eicosanoids, such as PGI<sub>2</sub>, in promoting NK cell maturation and

expansion in the lung is critical not only for potentiating innate immunity in the mucosal site, but also for preventing sensitization to airborne allergens. Certainly, approaches that seek to prevent allergic inflammation by specifically promoting mucosal NK cell response would form a novel immunotherapeutic approach.

## Acknowledgments

We thank Britt Postma, Pam Shaw (FACS Core), Lou Herritt and Diane Brooks (Histology Core) for valuable technical assistance.

## Disclosures

The authors have no financial conflicts of interest.

## References

1. Thomas, W. R., B. J. Hales, and W. A. Smith. 2010. House dust mite allergens in asthma and allergy. *Trends Mol. Med.* 16: 321–328.
2. Platts-Mills, T. 1989. Dust mite allergens and asthma - a worldwide problem. *J. Allergy Clin. Immunol.* 83: 416–427.
3. Svensson, L., E. Redvall, C. Björn, J. Karlsson, A. M. Bergin, M. J. Rabiet, C. Dahlgren, and C. Wennerås. 2007. House dust mite allergen activates human eosinophils via formyl peptide receptor and formyl peptide receptor-like 1. *Eur. J. Immunol.* 37: 1966–1977.
4. Asokanathan, N., P. T. Graham, D. J. Stewart, A. J. Bakker, K. A. Eidne, P. J. Thompson, and G. A. Stewart. 2002. House dust mite allergens induce proinflammatory cytokines from respiratory epithelial cells: the cysteine protease allergen, Der p 1, activates protease-activated receptor (PAR)-2 and inactivates PAR-1. *J. Immunol.* 169: 4572–4578.
5. Hammad, H., M. Chieppa, F. Perros, M. A. Willart, R. N. Germain, and B. N. Lambrecht. 2009. House dust mite allergen induces asthma via Toll-like receptor 4 triggering of airway structural cells. *Nat. Med.* 15: 410–416.
6. Barrett, N. A., A. Maekawa, O. M. Rahman, K. F. Austen, and Y. Kanaoka. 2009. Dectin-2 recognition of house dust mite triggers cysteinyl leukotriene generation by dendritic cells. *J. Immunol.* 182: 1119–1128.
7. Clarke, D. L., N. H. Davis, C. L. Campion, M. L. Foster, S. C. Heasman, A. R. Lewis, I. K. Anderson, D. J. Corkill, M. A. Sleeman, R. D. May, and M. J. Robinson. 2014. Dectin-2 sensing of house dust mite is critical for the initiation of airway inflammation. *Mucosal Immunol.* 7: 558–567.
8. Yokoyama, W. M. 2013. Natural killer cells. In *Fundamental Immunology*, 7th Ed. Lippincott Williams & Wilkins, Philadelphia, New York, p. 395–431.
9. Yokoyama, W. M. 1995. Natural killer cell receptors. *Curr. Opin. Immunol.* 7: 110–120.
10. Kärre, K. 2002. NK cells, MHC class I molecules and the missing self. *Scand. J. Immunol.* 55: 221–228.
11. Takeda, K., M. Nakayama, M. Sakaki, Y. Hayakawa, M. Imawari, K. Ogasawara, K. Okumura, and M. J. Smyth. 2011. IFN- $\gamma$  production by lung NK cells is critical for the natural resistance to pulmonary metastasis of B16 melanoma in mice. *J. Leukoc. Biol.* 90: 777–785.
12. Sojka, D. K., B. Plougastel-Douglas, L. Yang, M. A. Pak-Wittel, M. N. Artymov, Y. Ivanova, C. Zhong, J. M. Chase, P. B. Rothman, J. Yu, et al. 2014. Tissue-resident natural killer (NK) cells are cell lineages distinct from thymic and conventional splenic NK cells. *eLife* 3: e01659.
13. Sojka, D. K., Z. Tian, and W. M. Yokoyama. 2014. Tissue-resident natural killer cells and their potential diversity. *Semin. Immunol.* 26: 127–131.
14. Han, X., Y. Fan, S. Wang, L. Jiao, H. Qiu, and X. Yang. 2008. NK cells contribute to intracellular bacterial infection-mediated inhibition of allergic responses. *J. Immunol.* 180: 4621–4628.
15. Haworth, O., M. Cernadas, and B. D. Levy. 2011. NK cells are effectors for resolvin E1 in the timely resolution of allergic airway inflammation. *J. Immunol.* 186: 6129–6135.
16. Walker, C., J. Checkel, S. Cammisuli, P. J. Leibson, and G. J. Gleich. 1998. IL-5 production by NK cells contributes to eosinophil infiltration in a mouse model of allergic inflammation. *J. Immunol.* 161: 1962–1969.
17. Farhadi, N., L. Lambert, C. Triulzi, P. J. Openshaw, N. Guerra, and F. J. Culley. 2014. Natural killer cell NKG2D and granzyme B are critical for allergic pulmonary inflammation. *J. Allergy Clin. Immunol.* 133: 827–835.
18. Haworth, O., M. Cernadas, R. Yang, C. N. Serhan, and B. D. Levy. 2008. Resolvin E1 regulates interleukin 23, interferon-gamma and lipoxin A4 to promote the resolution of allergic airway inflammation. *Nat. Immunol.* 9: 873–879.
19. Barnig, C., M. Cernadas, S. Dutilleul, X. Liu, M. A. Perrella, S. Kazani, M. E. Wechsler, E. Israel, and B. D. Levy. 2013. Lipoxin A4 regulates natural killer cell and type 2 innate lymphoid cell activation in asthma. *Sci. Transl. Med.* 5: 174ra26.
20. Chen, Y., B. Perussia, and K. S. Campbell. 2007. Prostaglandin D2 suppresses human NK cell function via signaling through D prostanoid receptor. *J. Immunol.* 179: 2766–2773.
21. Zielinski, C. C., C. Gisinger, C. Binder, J. W. Mannhalter, and M. M. Eibl. 1984. Regulation of NK cell activity by prostaglandin E2: the role of T cells. *Cell. Immunol.* 87: 65–72.
22. Bray, R. A., and Z. Brahami. 1986. Role of lipooxygenation in human natural killer cell activation. *J. Immunol.* 136: 1783–1790.

23. Liu, T., T. M. Laidlaw, C. Feng, W. Xing, S. Shen, G. L. Milne, and J. A. Boyce. 2012. Prostaglandin E2 deficiency uncovers a dominant role for thromboxane A2 in house dust mite-induced allergic pulmonary inflammation. *Proc. Natl. Acad. Sci. USA* 109: 12692–12697.
24. Stebbins, K. J., A. R. Broadhead, L. D. Correa, J. M. Scott, Y. P. Truong, B. A. Stearns, J. H. Hutchinson, P. Prasit, J. F. Evans, and D. S. Lorrain. 2010. Therapeutic efficacy of AM156, a novel prostanoid DP2 receptor antagonist, in murine models of allergic rhinitis and house dust mite-induced pulmonary inflammation. *Eur. J. Pharmacol.* 638: 142–149.
25. Herrerias, A., R. Torres, M. Serra, A. Marco, L. Pujols, C. Picado, and F. de Mora. 2009. Activity of the cyclooxygenase 2-prostaglandin-E prostanoid receptor pathway in mice exposed to house dust mite aeroallergens, and impact of exogenous prostaglandin E2. *J. Inflamm. (Lond.)* 6: 30.
26. Catella-Lawson, F., and L. J. Crofford. 2001. Cyclooxygenase inhibition and thrombogenicity. *Am. J. Med.* 110(Suppl. 3A): 28S–32S.
27. Murata, T., F. Ushikubi, T. Matsuo, M. Hirata, A. Yamasaki, Y. Sugimoto, A. Ichikawa, Y. Aze, T. Tanaka, N. Yoshida, et al. 1997. Altered pain perception and inflammatory response in mice lacking prostacyclin receptor. *Nature* 388: 678–682.
28. Koo, G. C., and J. R. Peppard. 1984. Establishment of monoclonal anti-Nk-1.1 antibody. *Hybridoma* 3: 301–303.
29. Jaffar, Z., M. E. Ferrini, P. K. Shaw, G. A. FitzGerald, and K. Roberts. 2011. Prostaglandin I<sub>2</sub> promotes the development of IL-17-producing  $\gamma\delta$  T cells that associate with the epithelium during allergic lung inflammation. *J. Immunol.* 187: 5380–5391.
30. Kim, S., K. Iizuka, H. L. Aguila, I. L. Weissman, and W. M. Yokoyama. 2000. In vivo natural killer cell activities revealed by natural killer cell-deficient mice. *Proc. Natl. Acad. Sci. USA* 97: 2731–2736.
31. Monticelli, L. A., G. F. Sonnenberg, M. C. Abt, T. Alenghat, C. G. Ziegler, T. A. Doering, J. M. Angelosanto, B. J. Laidlaw, C. Y. Yang, T. Sathiyawala, et al. 2011. Innate lymphoid cells promote lung-tissue homeostasis after infection with influenza virus. *Nat. Immunol.* 12: 1045–1054.
32. Arase, H., N. Arase, and T. Saito. 1996. Interferon gamma production by natural killer (NK) cells and NK1.1+ T cells upon NKR-P1 cross-linking. *J. Exp. Med.* 183: 2391–2396.
33. Gevrey, J. C., B. M. Isaac, and D. Cox. 2005. Syk is required for monocyte/macrophage chemotaxis to CX3CL1 (Fractalkine). *J. Immunol.* 175: 3737–3745.
34. Jones, B. A., M. Beamer, and S. Ahmed. 2010. Fractalkine/CX3CL1: a potential new target for inflammatory diseases. *Mol. Interv.* 10: 263–270.
35. Kim, K. W., A. Vallon-Eberhard, E. Zigmund, J. Farache, E. Shezen, G. Shakh, A. Ludwig, S. A. Lira, and S. Jung. 2011. In vivo structure/function and expression analysis of the CX3C chemokine fractalkine. *Blood* 118: e156–e167.
36. Korsgren, M. 2002. NK cells and asthma. *Curr. Pharm. Des.* 8: 1871–1876.
37. Chen, K., M. Liu, Y. Liu, C. Wang, T. Yoshimura, W. Gong, Y. Le, L. Tassarollo, and J. M. Wang. 2013. Signal relay by CC chemokine receptor 2 (CCR2) and formylpeptide receptor 2 (Fpr2) in the recruitment of monocyte-derived dendritic cells in allergic airway inflammation. *J. Biol. Chem.* 288: 16262–16273.
38. Gu, L., S. Tseng, R. M. Horner, C. Tam, M. Loda, and B. J. Rollins. 2000. Control of TH2 polarization by the chemokine monocyte chemoattractant protein-1. *Nature* 404: 407–411.
39. Klein Wolterink, R. G., and R. W. Hendriks. 2013. Type 2 innate lymphocytes in allergic airway inflammation. *Curr. Allergy Asthma Rep.* 13: 271–280.
40. Wills-Karp, M., A. Nathan, K. Page, and C. L. Karp. 2010. New insights into innate immune mechanisms underlying allergenicity. *Mucosal Immunol.* 3: 104–110.
41. Westgaard, I. H., S. F. Berg, J. T. Vaage, L. L. Wang, W. M. Yokoyama, E. Dissen, and S. Fossum. 2004. Rat Nkp46 activates natural killer cell cytotoxicity and is associated with Fc $\epsilon$ s1R1gamma and CD3zeta. *J. Leukoc. Biol.* 76: 1200–1206.
42. Harris, D. T., R. Kapur, C. Frye, A. Acevedo, T. Camenisch, L. Jaso-Friedmann, and D. L. Evans. 1992. A species-conserved NK cell antigen receptor is a novel vimentin-like molecule. *Dev. Comp. Immunol.* 16: 395–403.
43. Halftack, G. G., M. Elboim, C. Gur, H. Achdout, H. Ghadially, and O. Mandelboim. 2009. Enhanced in vivo growth of lymphoma tumors in the absence of the NK-activating receptor Nkp46/NCR1. *J. Immunol.* 182: 2221–2230.
44. Mandelboim, O., and A. Porgador. 2001. Nkp46. *Int. J. Biochem. Cell Biol.* 33: 1147–1150.
45. Fathman, J. W., D. Bhattacharya, M. A. Inlay, J. Seita, H. Karsunky, and I. L. Weissman. 2011. Identification of the earliest natural killer cell-committed progenitor in murine bone marrow. *Blood* 118: 5439–5447.
46. Jaffar, Z., K. S. Wan, and K. Roberts. 2002. A key role for prostaglandin I<sub>2</sub> in limiting lung mucosal Th2, but not Th1, responses to inhaled allergen. *J. Immunol.* 169: 5997–6004.
47. Jaffar, Z., M. E. Ferrini, M. C. Buford, G. A. FitzGerald, and K. Roberts. 2007. Prostaglandin I<sub>2</sub>-IP signaling blocks allergic pulmonary inflammation by preventing recruitment of CD4+ Th2 cells into the airways in a mouse model of asthma. *J. Immunol.* 179: 6193–6203.
48. Garton, K. J., P. J. Gough, C. P. Blobel, G. Murphy, D. R. Greaves, P. J. Dempsey, and E. W. Raines. 2001. Tumor necrosis factor- $\alpha$ -converting enzyme (ADAM17) mediates the cleavage and shedding of fractalkine (CX3CL1). *J. Biol. Chem.* 276: 37993–38001.
49. Inngjerd, M., B. Rolstad, and J. C. Ryan. 2003. Activating and inhibitory Ly49 receptors modulate NK cell chemotaxis to CXCL chemokine ligand (CXCL10) and CXCL12. *J. Immunol.* 171: 2889–2895.
50. Robinson, L. A., C. Nataraj, D. W. Thomas, J. M. Cosby, R. Griffiths, V. L. Bautch, D. D. Patel, and T. M. Coffman. 2003. The chemokine CX3CL1 regulates NK cell activity in vivo. *Cell. Immunol.* 225: 122–130.
51. Yoneda, O., T. Imai, M. Nishimura, M. Miyaji, T. Mimori, T. Okazaki, N. Domae, H. Fujimoto, Y. Minami, T. Kono, et al. 2003. Membrane-bound form of fractalkine induces IFN- $\gamma$  production by NK cells. *Eur. J. Immunol.* 33: 53–58.
52. Fraticelli, P., M. Sironi, G. Bianchi, D. D'Ambrosio, C. Albanesi, A. Stoppacciaro, M. Chieppa, P. Allavena, L. Ruco, G. Girolomoni, et al. 2001. Fractalkine (CX3CL1) as an amplification circuit of polarized Th1 responses. *J. Clin. Invest.* 107: 1173–1181.
53. Mathias, C. B., L. A. Guernsey, D. Zammit, C. Brammer, C. A. Wu, R. S. Thrall, and H. L. Aguila. 2014. Pro-inflammatory role of natural killer cells in the development of allergic airway disease. *Clin. Exp. Allergy* 44: 589–601.
54. Karimi, K., and P. Forsythe. 2013. Natural killer cells in asthma. *Front. Immunol.* 4: 159.
55. Ple, C., M. Barrier, L. Amniai, P. Marquillies, J. Bertout, A. Tsicopoulos, T. Walzer, P. Lassalle, and C. Duez. 2010. Natural killer cells accumulate in lung-draining lymph nodes and regulate airway eosinophilia in a murine model of asthma. *Scand. J. Immunol.* 72: 118–127.
56. Crome, S. Q., P. A. Lang, K. S. Lang, and P. S. Ohashi. 2013. Natural killer cells regulate diverse T cell responses. *Trends Immunol.* 34: 342–349.
57. Nagao, K., H. Tanaka, M. Komai, T. Masuda, S. Narumiya, and H. Nagai. 2003. Role of prostaglandin I<sub>2</sub> in airway remodeling induced by repeated allergen challenge in mice. *Am. J. Respir. Cell Mol. Biol.* 29: 314–320.
58. Takahashi, Y., S. Tokunaga, T. Masuda, Y. Hirano, M. Nagao, H. Tanaka, N. Inagaki, S. Narumiya, and H. Nagai. 2002. Augmentation of allergic inflammation in prostaglandin IP receptor deficient mice. *Br. J. Pharmacol.* 137: 315–322.
59. Halim, T. Y. 2016. Group 2 innate lymphoid cells in disease. *Int. Immunol.* 28: 13–22.
60. van Rij, L., H. von Richthofen, and R. van Ree. 2016. Type 2 innate lymphoid cells: at the cross-roads in allergic asthma. *Semin. Immunopathol.* 38: 483–496.
61. Gold, M. J., F. Antignano, T. Y. Halim, J. A. Hirota, M. R. Blanchet, C. Zaph, F. Takei, and K. M. McNagny. 2014. Group 2 innate lymphoid cells facilitate sensitization to local, but not systemic, TH2-inducing allergen exposures. *J. Allergy Clin. Immunol.* 133: 1142–1148.
62. Scanlon, S. T., and A. N. McKenzie. 2012. Type 2 innate lymphoid cells: new players in asthma and allergy. *Curr. Opin. Immunol.* 24: 707–712.
63. Everaere, L., S. Ait-Yahia, O. Molendi-Coste, H. Vong, S. Quemener, P. LeVu, S. Fleury, E. Bouchaert, Y. Fan, C. Duez, et al. 2016. Innate lymphoid cells contribute to allergic airway disease exacerbation by obesity. *J. Allergy Clin. Immunol.*
64. Christianson, C. A., N. P. Goplen, I. Zafar, C. Irvin, J. T. Good, Jr., D. R. Rollins, B. Goren, W. Liu, M. M. Gorska, H. Chu, et al. 2015. Persistence of asthma requires multiple feedback circuits involving type 2 innate lymphoid cells and IL-33. *J. Allergy Clin. Immunol.* 136: 59–68 e14.
65. Barlow, J. L., S. Peel, J. Fox, V. Panova, C. S. Hardman, A. Camelo, C. Bucks, X. Wu, C. M. Kane, D. R. Neill, et al. 2013. IL-33 is more potent than IL-25 in provoking IL-13-producing nuocytes (type 2 innate lymphoid cells) and airway contraction. *J. Allergy Clin. Immunol.* 132: 933–941.
66. Neill, D. R., S. H. Wong, A. Bellosi, R. J. Flynn, M. Daly, T. K. Langford, C. Bucks, C. M. Kane, P. G. Fallon, R. Pannell, et al. 2010. Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity. *Nature* 464: 1367–1370.
67. Spits, H., D. Artis, M. Colonna, A. Diefenbach, J. P. Di Santo, G. Eberl, S. Koyasu, R. M. Locksley, A. N. McKenzie, R. E. Mebius, et al. 2013. Innate lymphoid cells - a proposal for uniform nomenclature. *Nat. Rev. Immunol.* 13: 145–149.
68. Nussbaum, J. C., S. J. Van Dyken, J. von Moltke, L. E. Cheng, A. Mohapatra, A. B. Molofsky, E. E. Thornton, M. F. Krummel, A. Chawla, H. E. Liang, and R. M. Locksley. 2013. Type 2 innate lymphoid cells control eosinophil homeostasis. *Nature* 502: 245–248.
69. Molofsky, A. B., F. Van Gool, H. E. Liang, S. J. Van Dyken, J. C. Nussbaum, J. Lee, J. A. Bluestone, and R. M. Locksley. 2015. Interleukin-33 and Interferon- $\gamma$  counter-regulate group 2 innate lymphoid cell activation during immune perturbation. *Immunity* 43: 161–174.
70. Duerr, C. U., C. D. McCarthy, B. C. Mindt, M. Rubio, A. P. Meli, J. Pothlichet, M. E. Eva, J. F. Gauchat, S. T. Qureshi, B. D. Mazer, et al. 2016. Type 1 interferon restricts type 2 immunopathology through the regulation of group 2 innate lymphoid cells. *Nat. Immunol.* 17: 65–75.
71. Stehle, C., P. Saikali, and C. Romagnani. 2016. Putting the brakes on ILC2 cells. *Nat. Immunol.* 17: 43–44.
72. Mirchandani, A. S., A. G. Besnard, E. Yip, C. Scott, C. C. Bain, V. Cerovic, R. J. Salmond, and F. Y. Liew. 2014. Type 2 innate lymphoid cells drive CD4+ Th2 cell responses. *J. Immunol.* 192: 2442–2448.
73. Liu, B., J. B. Lee, C. Y. Chen, G. K. Hershey, and Y. H. Wang. 2015. Collaborative interactions between type 2 innate lymphoid cells and antigen-specific CD4+ Th2 cells exacerbate murine allergic airway diseases with prominent eosinophilia. *J. Immunol.* 194: 3583–3593.

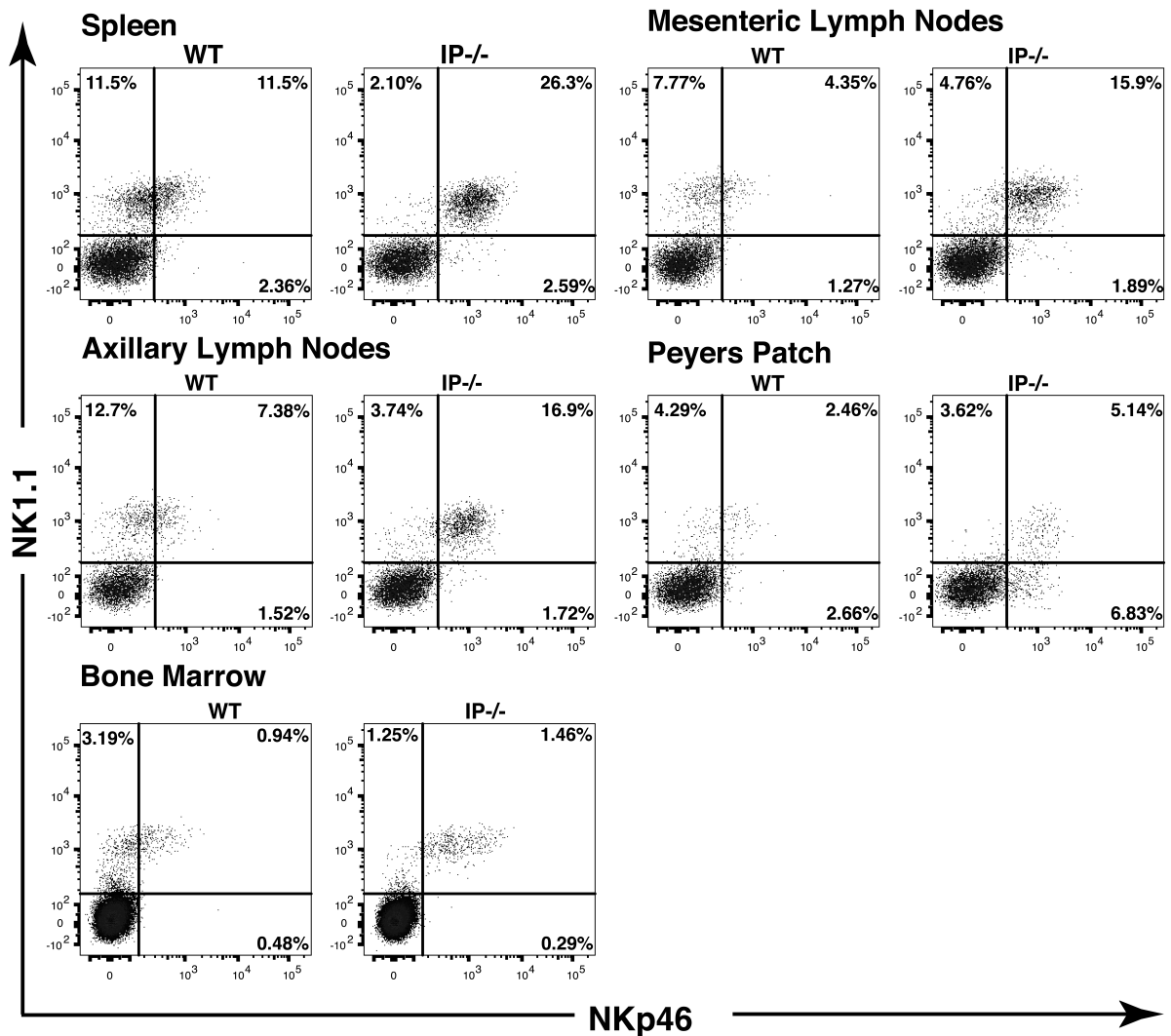


**Figure S1.** NKp46 expression is elevated in all lymphoid tissues in naïve IP<sup>-/-</sup> mice. The number of CD3<sup>-</sup>CD19<sup>-</sup>NK1.1<sup>+</sup> NK cells and expression levels of NKp46 present in the bone marrow, axillary lymph nodes, mesenteric lymph nodes, Peyers Patch and spleens (from 4 mice) was determined by 4-color flow cytometry. Data are representative of 2 independent experiments.

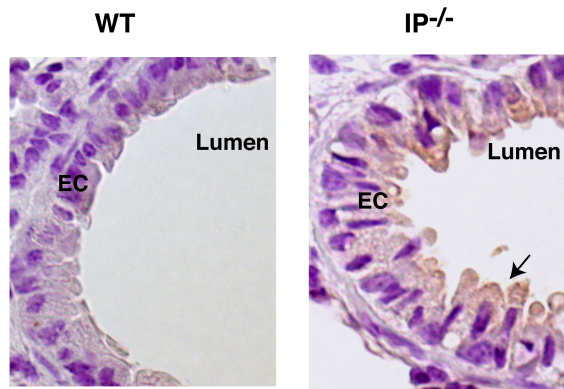


**Figure S2.** CX<sub>3</sub>CL1 expression in the lungs of IP<sup>-/-</sup> mice is predominantly by airway epithelial cells. Expression of CX<sub>3</sub>CL1 by lung tissue sections from IP<sup>-/-</sup> and WT mice (4 per group) was examined by immunohistochemical staining using anti-CX<sub>3</sub>CL1 Ab (5 µg/ml) and visualized by light microscopy (x40). Data are representative of 2 independent experiments.

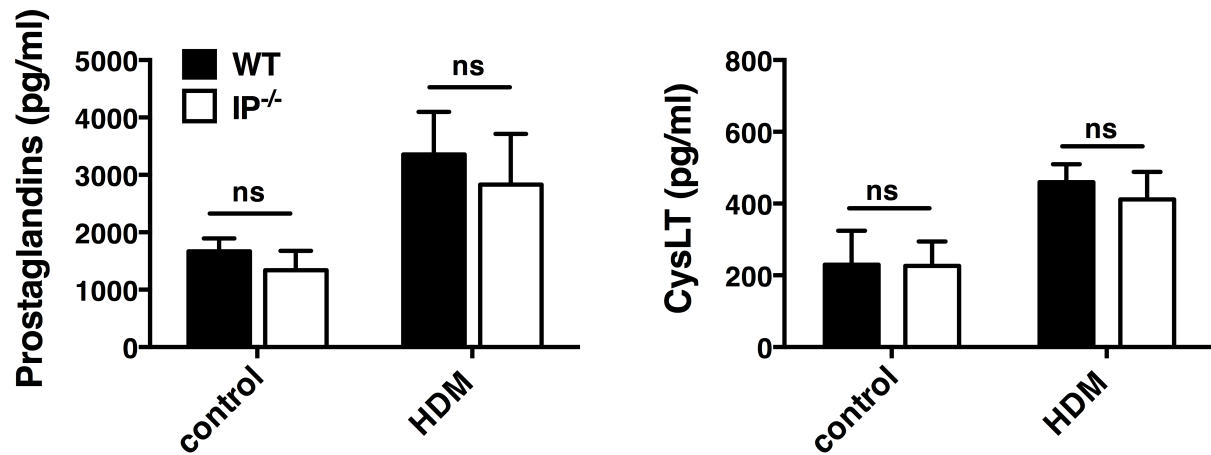


**Figure S3.** The levels of endogenous eicosanoid biosynthesis in IP<sup>-/-</sup> and WT mice. WT or IP<sup>-/-</sup> mice (6 per group) were subjected to acute allergen sensitization and challenge by repeated intranasal administration of HDM allergen or PBS (control). Prostaglandin and cysteinyl leukotriene (cysLT) levels in the BALF were determined using ELISA. Results are mean  $\pm$  SEM of 3 independent experiments, ns = not significant.

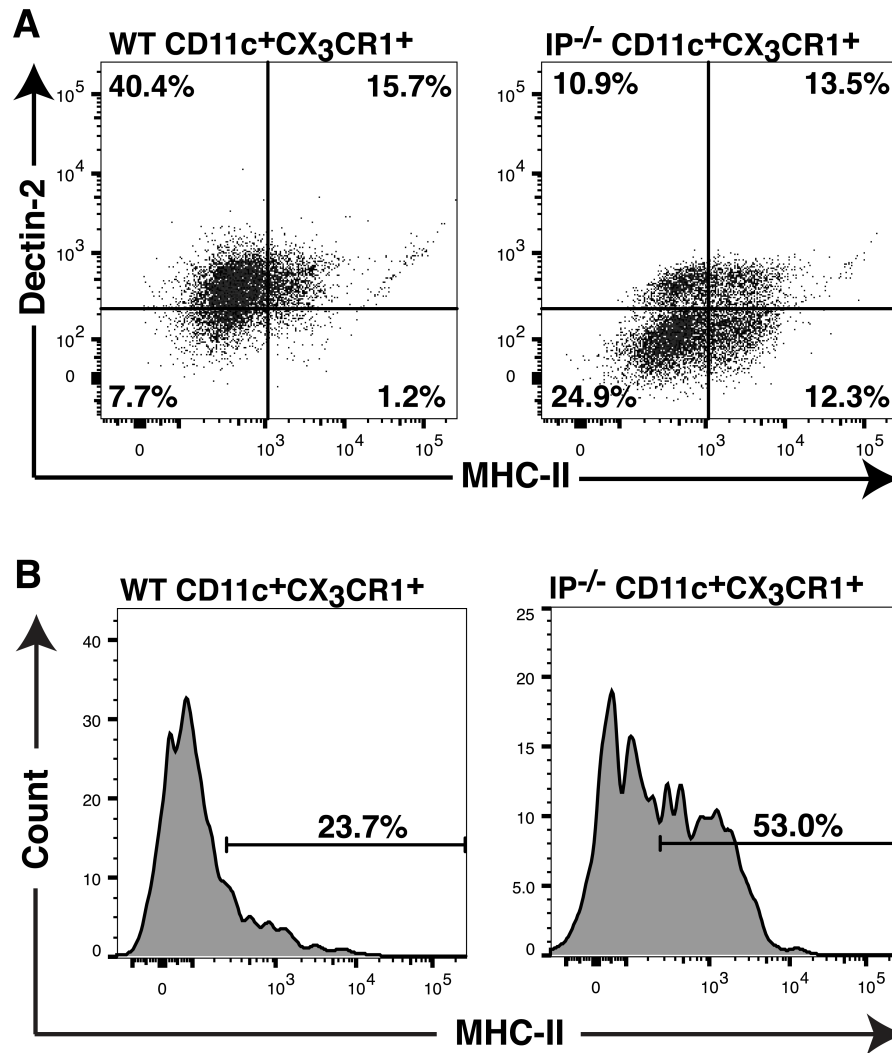


**Figure S4.** CD11c<sup>+</sup>CX<sub>3</sub>CR1<sup>+</sup> alveolar macrophages in the lungs of IP<sup>-/-</sup> mice have reduced levels of Dectin-2 expression. Myeloid cells present in the BALF and LMC of naïve WT or IP<sup>-/-</sup> mice (4 per group) were examined by 4-color flow cytometry. (A) After gating on CD11c<sup>+</sup>CX<sub>3</sub>CR1<sup>+</sup> cells, the expression of MHC-II and Dectin-2 by myeloid cells present in the BALF was determined. (B) Similarly, the number and expression of MHC-II by CD11c<sup>+</sup>CX<sub>3</sub>CR1<sup>+</sup> cells in the LMC was assessed. Results are representative of 3 independent experiments