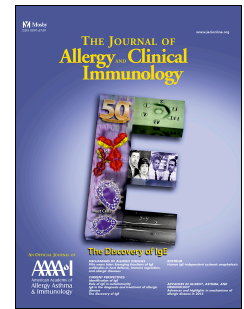


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PKC theta controls ILC2 and Th2 responses to house dust mite allergen

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## Original article

**PKC theta controls ILC2 and Th2 responses to house dust mite allergen**

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**Author contributions**

DT and BR conceived the study; FM, PC, LF, MG, TM, NR, AL, QM and DT performed experiments and analyzed the results; CB, VL and LM provide human material and data, VQ, FE, FT, LA and MLB provided materials, critically read and comment the paper; DT and BR wrote the paper.

**Additional information**

This article contains **online supporting information**

**Conflict of interest:** D. Togbe, L. Fauconnier, T. Marchiol, A. Ledru, N. Rouxel and F. Trovero are employees at ArtImmune. L. Mascarell, V. Lombardi and C. Beuraud are employees at Stallergenes Greer. All the authors have no conflict of interest related to this study.

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**ABSTRACT: 250 words**

Background: Protein Kinase C theta (PKC- $\theta$ ), a serine/threonine kinase is involved in T helper 2 (Th2) cell activation and proliferation. Innate lymphoid cells 2 (ILC2) resemble Th2 cells; produce the Th2 cytokines IL-5 and IL-13, but lack antigen specific receptors. The mechanism by which PKC- $\theta$  drives innate immune cells to instruct Th2 responses in allergic lung inflammation remains unknown.

Objectives: We hypothesized that PKC- $\theta$  contributes to ILC2 activation and may be necessary for ILC2 to instruct Th2 response.

Methods: PKC- $\theta$  **gene expression was assessed in ILC subsets purified from human peripheral blood mononuclear cells (PBMC) and mouse lung ILC2.** ILC2 activation and eosinophil recruitment, Th2 related cytokine and chemokine production, lung histopathology, IRF4 mRNA and NFAT1 protein expression, were determined. Adoptive transfer of ILC2 from WT mice was performed in WT and PKC- $\theta^{-/-}$  mice.

Results: Here we report that PKC- $\theta$  is expressed in both human and mouse ILC2. Mice lacking PKC- $\theta$  had reduced ILC2, Th2 cell numbers and activation, airway hyperresponsiveness (AHR) as well as the expression of transcription factors IRF4 and NFAT1. Importantly, adoptive transfer of ILC2 restored eosinophil influx, IL-4, IL-5 and IL-13 production in lung tissue as well as Th2 cell activation. The pharmacological PKC- $\theta$  inhibitor (compound 20) given during allergen challenge reduced ILC2 numbers and activation as well as airway inflammation, IRF4 and NFAT1 expression.

Conclusions: Therefore, our finding identifies PKC- $\theta$  as a critical factor for ILC2 activation and contributes to Th2 cell differentiation which is associated to IRF4 and NFAT1 expression in allergic lung inflammation.

## Clinical Implications

Pharmacological inhibition of established asthma suggests that PKC- $\theta$  represents a potent therapeutic target of the allergic response.

## Capsule Summary

Our finding shows that PKC- $\theta$  expression in both human and mouse ILC2 contributes to the effector functions of Th2 cells with activated transcription factors IRF4 and NFAT1 in the HDM induced airway inflammation model.

## Key words

House dust mite, allergic asthma, eosinophils, protein kinase C theta, innate lymphoid cells (ILC), nuclear factor of activated T-cells (NFAT), interferon regulating factor 4 (IRF4).

## Abbreviations used

AHR: Airway hyperresponsiveness

BALF: Bronchoalveolar lavage fluid

BMDC: Bone marrow derived dendritic cells

CCL: C-C motif ligand

HDM: house dust mite

EPO: eosinophil peroxidase

IL- : Interleukin

ILC: Innate lymphoid cells

IRF4: Interferon regulating factor 4

NFAT1: Nuclear factor of activated T-cells

- 88 PAS: Periodic acid–Schiff
- 89 PKC- $\theta$ : Protein Kinase C theta
- 90 qRT-PCR: Quantitative real-time PCR
- 91 Th: T helper

## INTRODUCTION

Allergic asthma is an inflammatory pulmonary disease associated with aberrant innate and adaptive immune responses to allergens. The key clinical features of the disease are underpinned by mucus hypersecretion, inflammatory infiltrates and airway hyperreactivity. Asthma is typically characterized by Th2 responses and their related secreted cytokines, namely, IL-4, IL-5 and IL-13<sup>1</sup>. Asthma in humans is induced by several allergens such as innocuous antigens including birch pollen (BP) and protease antigens such as papain, *Alternaria alternata* or house dust mite (HDM). Mouse models of allergic asthma are induced either by protease allergens but also ovalbumin (OVA) after systemic priming.

HDM contains several antigens with protease activity and plays an important role in initiating as well as sustaining inflammation through the induction of Th2 and IgE responses<sup>2-4</sup>. HDM causes epithelial barrier dysfunction with disruption of tight junctions, the production of pro-inflammatory cytokines and modulates function of various cell types including Th2 response<sup>3, 5-10</sup>.

The interactions between naive T lymphocytes and antigen presenting cells (APC) mediated by the contact between peptides-major histocompatibility complex (MHCII) and T cell receptor (TCR) as well as by costimulatory molecules such as CD28<sup>11</sup> allow the formation of a immunological synapse which comprises Protein Kinase C Theta (PKC- $\theta$ )<sup>12</sup>. PKC- $\theta$  recruitment into the immunological synapse is required for full effector T lymphocyte activation<sup>13</sup>. PKC- $\theta$  regulates T cell activation in several inflammatory disease models such as allergic asthma, multiple sclerosis, arthritis and inflammatory bowel disease (IBD)<sup>11, 14</sup>. PKC- $\theta$  is critical for the activation of several transcription factors such as NF- $\kappa$ B, Nuclear factor of activated T-cells (NFAT), C-Jun, C-Fos and AP-1<sup>15</sup> and also promotes the

development of Treg cells by enhancing Foxp3 expression via activation of the calcineurin/NFAT pathway<sup>16</sup>.

More recently innate lymphoid cells 2 (ILC2) have been identified as critical source for the production of an early Th2 type cytokine including IL-5 and IL-13<sup>17-18</sup>. While the role of PKC- $\theta$  has been explored in Th2 cells, its contribution to ILC2 activation remains unclear.

Herein, we demonstrate that PKC- $\theta$  is critical for ILC2 activation and contributes to the acquisition of Th2 cell effector functions. We demonstrate that PKC- $\theta$  is expressed in both human and mouse ILC2s and its deletion decreases the emergence and activation of ILC2 followed by reduced Th2 cell response which is associated with reduced NFAT1 and IRF4 expression. Importantly, adoptive transfer of ILC2 restores eosinophil influx and Th2 cytokines IL-4, IL-5 and IL-13 production in lung. Pharmacological inhibition of PKC- $\theta$  by a specific PKC- $\theta$  inhibitor (C20) markedly reduced the number and activation of ILC2 and cardinal features of allergic lung inflammation induced by HDM.

## METHODS

### *Animals*

PKC- $\theta$ -deficient mice on C57BL/6 background (7–8 weeks old) were generated by Littman D. laboratory<sup>19</sup> and obtained from Manfred Kopf (Molecular Biomedicine, ETH Zürich, Switzerland), Rag2<sup>-/-</sup>Ly5.1 were obtained from Benedita Rocha (INEM, Necker Institute, Inserm U1151-CNRS UMR 8253, Paris, France)<sup>20</sup>. Both PKC- $\theta$ -deficient and littermate WT controls on C57BL/6 background were bred in the Transgenose Institute animal facility (TAAM, UPS44 CNRS, Orleans). All animals were maintained according to FELASA regulations and complied with the French Government's ethical and animal experiment regulations. Mice were maintained in a temperature-controlled (23°C) facility with a strict 12 h light/dark cycle with food and water provided *ad libitum*. Experiments were performed with gender-matched mice aged 8-10 weeks. All protocols were approved by the CNRS institutional animal research ethical committee under the number (CLE CCO 2013-1004).

### *Human PBMCs and sorting of ILC subsets and subsequent gene expression analysis*

Blood samples from healthy volunteers were collected by the French blood bank (French Blood institute; EFS, Rungis, France), after approval of the study protocol by an ethical committee (N° #12/R/129). Human PBMCs were isolated by density gradient centrifugation (Ficoll, Eurobio Les Ulis, France) from buffy coats. ILC subsets were isolated as described in supplemental methods.

### *HDM induced allergic airway inflammation induction*

To induce HDM-driven eosinophilic airway inflammation, isoflurane anesthetized mice were administered intranasally twice at days 0 and 7 with 25  $\mu$ g HDM ((*Dermatophagoides*

*pteronysinus*) extracts (ALK-Abello, Denmark) without adjuvant, control mice were challenged with saline alone and were challenged on days 14-16 with 5 µg of HDM extracts. Twenty four hours after the last challenge, airway hyperreactivity was measured and mice were sacrificed; bronchoalveolar lavage (BAL) and lung were collected for EPO enzyme, cytokines, chemokines, histology, qRT-PCR, cytokines and chemokines, and Western blot analysis.

#### ***Inhibition of PKC $\theta$ in vivo with compound 20 (C20)***

To inhibit PKC- $\theta$  *in vivo*, mice received during challenge from day 14-16 by oral route, 10 mg/kg of PKC- $\theta$  selective inhibitor Compound 20, (C20/BIX02656, Boehringer Ingelheim, USA) as described previously<sup>13</sup>.

#### ***Bronchoalveolar lavage (BAL)***

BAL was performed by washing the lungs four times with 0.5 ml of saline solution at room temperature. After centrifugation at 400 x g for 10 min at 4°C, the supernatant (cell-free BAL fluid) was stored at -20°C for cytokine analysis. FACS analysis on BAL and lung mononuclear cells was performed.

#### ***Pulmonary eosinophil peroxidase (EPO) activity***

EPO activity was determined in order to estimate the recruitment of eosinophils to the lung parenchyma as described<sup>21</sup>.

#### ***Determination of bronchial hyperresponsiveness (AHR)***

For invasive measurement of dynamic resistance, mice were anesthetized with intra-peritoneal injection of solution containing ketamine (100 mg/kg, Merial) and xylazine (10

mg/kg, Bayer), paralyzed using D-tubocurarine (0.125%, Sigma), and intubated with an 18-gauge catheter. Respiratory frequency was set at 140 breaths per min with a tidal volume of 0.2 ml and a positive end-expiratory pressure of 2 ml H<sub>2</sub>O. Increasing concentrations of aerosolized methacholine (9.375, 18.75, 37.5, 75 and 150 mg/ml) were administered. Resistance was recorded using an invasive plethysmograph (Buxco, London, UK). Baseline resistance was restored before administering the subsequent doses of methacholine.

### ***ILC2 expansion, isolation and adoptive cell transfer***

Mice were anesthetized with isoflurane (3%) and treated with recombinant IL-33 (1 µg/mouse, Ser109-Ile266, obtained from Jean Philippe Girard laboratory, CNRS-Institute of Pharmacology and Structural Biology IPBS, 31077 Toulouse, France) daily for 5 days, as described previously<sup>22</sup>.

Briefly, lungs were harvested after perfusion on day 6 and digested in 125 mg/ml Liberase TL and 1 mg/ml DNase 1 (Roche Diagnostics). Nonadherent cells were stained for ST2-FITC (clone DJ8, MD Biosciences), lineage markers (B220, FcεRI, CD11b, CD3ε and Siglec F) labeled with PE, ICOS-PECy7 (clone 7E-17G9, eBioscience), CD45-APC (clone 30-F11, eBioscience) and ILC2 were sorted with a MoFlo (Beckman coulter, Fort Collins, Co, USA).

IL-5, IL-13 and IRF4 expression were detected by intracellular/intranuclear staining with anti IL-5-PE (clone TRFK5, BD Biosciences) or anti IL-13-APC (clone ebio13A, eBioscience), APC anti IRF4 (clone REA201, Miltenyi Biotec), after activation with phorbol 12-myristate 13-acetate (PMA) (50 ng/ml) and ionomycin (750 ng/ml) in the presence of Brefeldin A (1 µl/1x10<sup>6</sup> cells, BD Biosciences) and cell permeabilization (BD Cytofix/Cytoperm, BD Biosciences).

Sorted lung ILC2 (10<sup>4</sup> cells/well) were expanded *in vitro* in 96 wells round bottom plate in 10 % (vol/vol) FBS RPMI complete medium containing IL-2 (10 ng/ml) and IL-33 (10 ng/ml)

for 5 days as described previously<sup>23</sup>. The medium was removed and fresh medium or recombinant mouse IL-33 (100 ng/ml) was added for 4 hours followed by RNA extraction. For ILC2s transfer,  $1 \times 10^5$  ILC2 in 50  $\mu$ l of PBS were inoculated intra-tracheally at day 0 followed by HDM (25  $\mu$ g/mouse) challenge at days 1 and 5-7 to WT and PKC- $\theta$  mice under isoflurane (3%) anesthetized. Mice were killed at day 8, BAL was performed in some experiments to assess lung inflammation and FACS analysis. In some experiments, lung mononuclear cells were isolated in the whole lungs. ILC2 proliferation was detected with Ki-67 staining (BV421 labeled anti mouse Ki-67, clone16A8, Biolegend).

#### ***Lung mononuclear cell isolation and stimulation***

Lung mononuclear cells were isolated from WT and PKC- $\theta$ <sup>-/-</sup> mice 24h after the last challenge as described previously<sup>24</sup>. Briefly the aorta and the inferior vena cava were sectioned and the lungs were perfused with saline. The lobes of the lungs were sliced into small cubes and then incubated for 45 min in 2 ml RPMI 1640 solution and digested in 125 mg/ml of Liberase TL and 1 mg/ml DNase 1 (Roche Diagnostics). Isolated lung mononuclear single cells were plated in round bottom 96-well plates ( $2 \times 10^6$ /ml) and restimulated 4h in vitro with phorbol 12-myristate 13-acetate (PMA) (50 ng/ml) and ionomycin (750 ng/ml; both from Sigma-Aldrich) in the presence of Brefeldin A ( $1 \mu$ l/ $1 \times 10^6$  cells, BD Biosciences) for intracellular flow cytometry analysis. Lung mononuclear cell ( $1 \times 10^6$  cells) were restimulated either with anti CD3e/CD28 (3  $\mu$ g/ml respectively) or HDM (100  $\mu$ g/ml) in a 96 well plate for 3 days. Supernatants were analyzed for the presence of cytokines using Multiplex immunoassay (MagPix, BioRad).

#### ***Flow cytometry analysis on BAL cells***

Single cell suspensions were prepared from BAL, after blockade of Fc receptors with CD16/32 blocking antibody, cells were stained with antibodies in FACS buffer (D-PBS, containing 3% heat-inactivated FCS and 0.005% NaN<sub>3</sub>) for 30 minutes with APC-conjugated anti-I-Ad/I-Ed (macrophages/DCs), PE-conjugated anti-SiglecF (clone E50-2440) (eosinophils), FITC-conjugated anti-CD3 (clone 145-2C11) and anti-CD45R/B220 (clone RA5-6B2) (lymphocytes), efluor450-conjugated anti F4/80 (clone BM8) and biotin-conjugated anti-CD11c (clone HL3) (macrophages/DCs). Differential cell counts were analyzed by flow cytometry, and cytopins as previously described<sup>25</sup>. Data were acquired using FACS Canto II flow cytometer and analyzed using Diva and FlowJo software.

### ***Lung histology***

Lungs were fixed in 4% buffered formaldehyde and 3 µm sections were stained with periodic acid Schiff reagent (PAS) and examined with a Leica DM2500 microscope (x40 magnification). Peribronchial infiltrates and mucus hypersecretion were assessed by a semi-quantitative score (0-5) by two observers independently as described before<sup>21</sup>.

### ***Cytokines and chemokines measurement by ELISA***

IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-13, IL-33, CXCL1 (KC), CCL11 (Eotaxin), CCL17 (TARC), CCL20, CCL22 (MDC) and CCL24 (Eotaxin2) concentrations in cell culture supernatant from restimulated lung mononuclear cells and lung homogenate were determined by enzyme-linked immunosorbent assay (ELISA), using commercial kits from R&D (Abingdon, UK) according to the manufacturer's recommendations or by multiplex immunoassay using a MagPix reader (Biorad).

252

**253 Immunoblotting of NFAT1**

254 Lung tissues were homogenized in Tris-HCl 0,05M pH8 with Polytron PT 2100 (Kinematica  
255 AG). Protein concentration was determined using the Bradford method (Bio-Rad). Equal  
256 amounts of protein (30 µg/sample) were denaturated by boiling (95°C, 5min), separated by  
257 SDS/PAGE and transferred to nitrocellulose membranes (GE Healthcare). The membrane was  
258 blocked in Tris-buffer saline (TBS), tween 20, containing 5% lipid free dry milk. Antibodies  
259 used for detection were diluted in Tris-buffer saline, tween 20, containing 5% (TBST) lipid  
260 free dry milk and were from Sigma unless otherwise stated.

261 The control  $\beta$ -actin was detected with primary mouse anti  $\beta$ -actin antibody followed by  
262 biotinylated secondary antibody HRP conjugated goat anti-mouse Ig. NFAT1 (NFATc2) were  
263 detected with primary polyclonal anti-mouse NFAT1 (Cell Signaling) followed by a  
264 secondary antibody HRP conjugated goat anti-rabbit Ig (Cell Signaling). Immuno-reactive  
265 bands were visualized by enhanced chemiluminescence kit (GE Healthcare).

266

**267 RNA isolation and quantitative RT-PCR**

Lung lobes were transferred in RNA later solution for 24 h at 4°C and snap-frozen in liquid nitrogen. Total RNA was isolated from homogenized mouse lung or ILC2 sorted cells using Tri Reagent (Sigma) and quantified by NanoDrop (Nd-1000). Reverse transcription was performed with SuperScript III Kit according to manufacturers' instructions (Invitrogen). cDNA was subjected to quantitative PCR using primers for mouse *Muc5ac* (Forward 5'CAGCCGAGAGGAGGGTTTGATCT and reverse 5'AGTCTCTCTCCGCTCCTCTCA; Sigma), *Gata-3* (QT00170828), *Il-4* (QT00160678), *Il-5* (QT00099715), *Il-13* (QT00099554), *Irf4* (QT00109984), *Rn18s* (QT02448075) and *Il1rl1*(QT01063062) from Qiagen. Relative transcript expression of a gene is given as  $2^{-\Delta\Delta C_t}$ . For ILC2 analysis, messenger RNA expression was assessed using  $\beta$ -actin or RN18s for human and mouse samples respectively as endogenous controls. The relative amount of target genes in each sample was calculated in comparison with a calibrator sample (ILC1s) for human samples and naïve primary mouse tracheal epithelial cells (mTEC) or naïve lymph node cells (mLN) for mouse samples.

### Statistical analysis

A Mann-Whitney U analysis and non parametric Kruskal-Wallis followed with Dunns post test were used to determine significant differences between groups using GraphPad Prism software version 5.00. Data are expressed as mean  $\pm$  SEM. Statistically significant differences were defined as \*P<0.05, \*\*P<0.01 and \*\*\*P<0.001.

## RESULTS

### ***Increased PKC- $\theta$ gene expression in human and mouse ILC2s***

To determine whether PKC- $\theta$  has a role in ILC2, we assessed PKC- $\theta$  expression in human ILCs subsets isolated from human PBMCs and mouse ILC2s populations purified from lung. We observed significant expression of *Pkc- $\theta$*  and *Gata-3* genes in ILC2s compared with ILC1 and ILC3 subsets (Fig 1, A). Purified mouse lung ILC2 from WT mice showed a significant induction of *Pkc- $\theta$*  transcript (Fig 1, B) compared with primary mouse tracheal epithelial cells (mTEC) which express low levels of *Pkc- $\theta$* . IL-33 restimulation did not further increase *Pkc- $\theta$*  gene levels. To investigate whether isolated ILC2s are functional, we assessed Th2 associated genes expression on ILC2 (*IL-4*, *IL-5*, *IL-13*, *Il1rl1* (ST2), *Gata-3* and *Irf4*) by quantitative RT-PCR analysis. Lung ILC2s had upregulated the expression of numerous Th2 associated genes such as *IL-4*, *IL-5*, *IL-13*, *Il1rl1* (ST2) and *Gata-3* in comparison with naive lymph node cells (Fig 1, C). IL-33 restimulation did not further increase *IL-4*, *IL-5*, *IL-13*, *Il1rl1* (ST2) and *Gata-3* gene levels. However, *Irf4* was down-regulated in ILC2s when compared with naive LN cells (Fig 1, D).

Therefore, PKC- $\theta$  gene is expressed in both human and mouse functional ILC2 populations.

### ***Reduced innate lymphoid cell activation in PKC- $\theta$ <sup>-/-</sup> mice***

ILC2 accumulate in lungs shortly after allergen exposure and produce IL-5 and IL-13<sup>26</sup>. To determine the relative contribution of PKC- $\theta$  signaling on lung ILC2 activation, PKC- $\theta$  deficient mice (PKC- $\theta$ <sup>-/-</sup>) or littermate wild type (WT) control were immunized with house dust mite (HDM, 25  $\mu$ g by intranasal route at day 0 and 7), followed by three intranasal challenges (5  $\mu$ g HDM on days 14-16) (Fig 2, A). Twenty four hours after the last HDM challenge, Total ILC2, IL-5 and IL-13 producing ILC2 were analyzed. The frequency and

total cell counts of ILC2<sup>+</sup>, ILC2<sup>+</sup>IL-5<sup>+</sup>, ILC2<sup>+</sup>IL-13<sup>+</sup> and ILC2<sup>+</sup>IRF4<sup>+</sup> were significantly reduced in PKC- $\theta$ <sup>-/-</sup> mice as compared with HDM stimulated WT mice (Fig 2, B-F). This data indicate that the PKC- $\theta$  signalling is required for ILC2 activation and the production of IL-5 and IL-13. We then asked whether the inhibitory effect correlated with a diminished adaptive Th2 response.

### ***PKC- $\theta$ <sup>-/-</sup> mice develop reduced airway eosinophilia and Th2-driven airway inflammation***

Th2 cells drive the adaptive immune response in allergic airway inflammation and PKC- $\theta$  has been shown to be important for the response to ovalbumin<sup>11, 27</sup>. To determine whether PKC- $\theta$  is also involved in airway inflammation induced by the complex and relevant allergens such as HDM extracts, WT or PKC- $\theta$ <sup>-/-</sup> mice were treated as reported in Fig 2, A, bronchoalveolar lavage (BAL) was performed for inflammatory cell counts. Saline sensitized and challenged WT and PKC- $\theta$ <sup>-/-</sup> mice were used as a negative control and did not exhibit any inflammatory response, as evidenced by the negligible number of leukocytes in the BAL fluid. However, WT mice which were HDM sensitized and challenged had a dramatic increase in the total leukocyte number in the BAL fluid (Fig 3, A). The predominant cell infiltrate in BALF consisted of eosinophils and EPO activity in the lung (Fig 3, B-C), with lower lymphocyte counts (Fig 3, D). In striking contrast, HDM-primed PKC- $\theta$  deficient mice (PKC- $\theta$ <sup>-/-</sup> HDM) exhibited a 3 fold lower total leukocyte counts in the BAL fluid compared with their wild type counterparts (Fig 3, A). In particular, there was a significant decrease in eosinophilia (3-fold reduction) (Fig 3, B-C), as well as a lower lymphocyte counts (2-fold reduction compared with WT-HDM) (Fig 3, D).

A hallmark of allergic lung inflammation is airway resistance, which was measured by invasive plethysmography in WT and PKC- $\theta$ <sup>-/-</sup> mice. Of note, PKC- $\theta$ <sup>-/-</sup> mice treated with HDM had reduced airway resistance compared with WT mice (Fig 3, E).

Histological analysis revealed a prominent lung inflammatory response characterized by perivascular and peribronchiolar infiltration of eosinophils and mononuclear cells in WT-HDM mice (Fig 3, F). By contrast, a significant reduction of inflammatory cell infiltration was found in PKC- $\theta^{-/-}$  mice, consistent with reduced cells in the BAL fluid (Fig 3, G). Additionally, the extensive goblet cell hyperplasia and mucus production observed in primed and challenged WT mice was reduced in PKC- $\theta^{-/-}$  mice, since approximately 80% of goblet cells stained strongly with PAS in WT mice versus only approximately 10%–20% in PKC- $\theta^{-/-}$  mice (Fig 3, H). The reduced mucus overproduction was confirmed at the mRNA expression level for Muc5ac in lung (Fig 3, I).

Moreover, pro-inflammatory and Th2 cytokines such as IL-1 $\alpha$ , IL-1 $\beta$ , IL-4, IL-5, IL-13 were significantly reduced (2-3 fold reduction) in lung of PKC- $\theta^{-/-}$  HDM compared with WT-HDM mice (Fig 4, A-B, D-F). By contrast, IL-33 levels were unchanged in the absence of PKC- $\theta$  (Fig 4, C).

We further investigated the Th2 cell numbers in the lung in HDM challenged mice and found an increase of TCR $\beta^+$  cells expressing IL-4 (Fig E2, A-C) and IL-5 (Fig E2, D-F). In the absence of PKC- $\theta$  the number of TCR $\beta^+$ IL-4 $^+$  and TCR $\beta^+$ IL-5 $^+$  cells were significantly reduced concomitant with reduced IL-4 level in the lung.

In addition, in the absence of PKC- $\theta$  significant lower levels of neutrophils/monocytes-attracting chemokines CXCL1 (2 fold reduction), basophils and eosinophil-attracting chemokines CCL11 and CCL24 (2 fold reduction) as well as Th2 cell-attracting chemokines CCL17 and CCL20 (approximately 2 fold reduction) were found in the lungs as compared with WT-HDM mice (Fig 4, G-K).

Taking together, these data indicate an important role of PKC- $\theta$  in the initiation of airway allergic inflammation to HDM.

***PKC- $\theta$  deletion reduces IRF4 and NFAT1 expression and determines lung Th2 cell numbers and activation***

The transcription factors IRF4 and NFAT1 are known to promote the expression of Th2 cell differentiation and function including IL-4 and IL-5 expression, and contribute to IL-10 production<sup>28-30</sup>. To analyze the transcription factors involved in the PKC- $\theta$  phenotype upon HDM exposure we investigated the expression of IRF4 by qPCR (Fig 4, L) and NFAT1 (NFATC2) by Western Blot analysis in lung tissues (Fig 4, M). We found a distinct reduction of these transcription factors in PKC- $\theta$  deficient lungs. Therefore the data suggest that lack of PKC- $\theta$  is associated with decreased expression of NFAT1 and IRF4.

We investigated whether the number and activation of CD45<sup>+</sup>CD3 $\epsilon$ <sup>+</sup> T cells are different in the lung from PKC- $\theta$ <sup>-/-</sup> versus WT mice upon HDM challenge (Fig E3, A, C). Consistent with the reduced allergic response total CD4<sup>+</sup> T cell recruitment within the CD45<sup>+</sup> CD3 $\epsilon$ <sup>+</sup> population was reduced, and T cells have a reduced expression of the activation marker CD69 (Fig E3, B, D). Since regulatory T cells may contribute to the overall response to the allergen HDM, we determined the pulmonary Treg cell numbers and found a significant reduction of FoxP3 cells in the absence of PKC- $\theta$  (Fig E3, E-G).

PKC- $\theta$  is expressed in NK cells and was found to be important for tumor growth control, IFN $\gamma$  and TNF $\alpha$  production and the function of NKT cells<sup>31-33</sup>. To determine whether PKC- $\theta$  is involved in NKp46 cell counts, we isolated mononuclear cells from the lung of PKC- $\theta$ <sup>-/-</sup> versus WT mice after HDM challenge. Similar CD45<sup>+</sup>NKp46<sup>+</sup> cell counts were found in PKC- $\theta$ <sup>-/-</sup> and WT control mice (Fig E4, A-C).

Collectively, these data demonstrate that PKC- $\theta$  is important for the activation of Th2 responses and FoxP3<sup>+</sup> Treg cell recruitment.

***PKC- $\theta$  deficient APCs process the allergen, but cannot activate T cells in vitro***

To exclude any effect on antigen uptake and processing we investigated these parameters in bone-marrow derived DCs (BMDCs) using OVA as antigen. The uptake and processing in PKC- $\theta^{-/-}$  BMDCs was not different from WT-BMDCs (Fig E5, A-B). However, PKC- $\theta^{-/-}$  BMDCs showed reduced MHC II expression (Fig E5, C). We further analyzed the ability of the BMDCs to produce pro-inflammatory cytokines upon HDM activation and found reduced IL-1 $\alpha$  production (Fig E5, D) while IL-1 $\beta$  and IL-10 were not affected (Fig E5, E, F).

Co-culture of WT-BMDCs primed with HDM with naïve CD4<sup>+</sup> T cells induced a robust cell proliferation of CFSE labeled T cells and IL-2 production, which was dramatically reduced when co-cultured with PKC- $\theta^{-/-}$  CD4<sup>+</sup> T cells (Fig E5, G, H). Therefore, PKC- $\theta^{-/-}$  CD4<sup>+</sup> T cells are unable to proliferate to HDM *in vitro*. The lack of the response in PKC- $\theta^{-/-}$  mice may be at level of the T cells and of DCs due to reduced IRF4 expression, since DC activation relies on IRF4 expression<sup>34</sup>. Consistent with published data PKC- $\theta^{-/-}$  T cells do not respond to TCR activation by CD3 $\epsilon$  antibody (Fig E5, I). The data reveal diminished T cell and DC activation as reported before.

***Adoptive transfer of ILC2 in PKC- $\theta^{-/-}$  restores lung inflammation and Th2 cytokine production.***

IL-33 is known to be an important cytokine for the promotion of ILC2 expansion<sup>17, 35</sup>. To address whether PKC- $\theta$  signalling affected the fate of ILC2, we treated WT and PKC- $\theta^{-/-}$  deficient mice with recombinant IL-33 for 5 days. We observed a significant increase of ILC2 in WT mice. In contrast, the percentage and number of ILC2 were greatly reduced in PKC- $\theta^{-/-}$  (Fig 5, A-B). Thus, PKC- $\theta$  plays an important role in ILC2 induction.

To test whether the role of ILC2s in HDM induced airway inflammation is dependent on PKC- $\theta$  signaling, we adoptively transferred purified ILC2s from either WT or Rag2<sup>-/-</sup>Ly5.1 mice into PKC- $\theta$ <sup>-/-</sup> and congenic WT (Ly5.2) mice (Fig. 5 and Fig. E6). Mice were challenged four times at days 1 and 5-7 with HDM extract and analyzed 24h after the last HDM challenge. The ILC2 transfer and HDM challenge led to increased lung inflammation compared with ILC2 transfer alone in WT mice (3 fold increase) (Fig 5, C-E and Fig E6, B). To determine whether the transferred ILC2 have the ability to proliferate, we used Ki-67 as a surrogate marker of proliferation and found high ILC2<sup>+</sup>Ki-67<sup>+</sup> cell population in lung after HDM administration in WT mice. However, ILC2 proliferation was reduced in PKC- $\theta$ <sup>-/-</sup> compared with WT mice. In addition, the transferred ILC2<sup>+</sup> (ILC2<sup>+</sup> CD45.1<sup>+</sup>) proliferated and restored the low proliferation seen in PKC- $\theta$ <sup>-/-</sup> mice (Fig E6, F, G; Fig E7 G-M). The transferred ILC2 also produced both IL-5 and IL-13 (Fig E7 N and data not shown). We investigated Th2 cells within the lung after ILC2 adoptive transfer and found an increased number of TCR $\beta$ <sup>+</sup>CD4<sup>+</sup> cells expressing IL-5 (Fig E6, H) and IL-13 (Fig E6, I) in WT mice after HDM administration. In the absence of PKC- $\theta$  the numbers of TCR $\beta$ <sup>+</sup>CD4<sup>+</sup> IL-5<sup>+</sup> and TCR $\beta$ <sup>+</sup>CD4<sup>+</sup> IL-13<sup>+</sup> cells were significantly reduced in the lung. ILC2 transfer together with HDM administration restored the IL-5 and IL-13 producing TCR $\beta$ <sup>+</sup>CD4<sup>+</sup> cell populations. These changes were accompanied by increased Th2 cytokines and chemokines, IL-4, IL-5, IL-13, CCL17 and CCL24 production in cell culture supernatant from lung mononuclear cells after HDM restimulation in WT mice (Fig 5, F-H and Fig E6, C). Interestingly, ILC2 transfer and HDM challenge into PKC- $\theta$ <sup>-/-</sup> mice restored lung inflammation including total leukocyte, eosinophil and lymphocyte counts in BALF (Fig 5, D-F) and IL-4, IL-5, IL-13, CCL17 and CCL24 production in culture supernatant from lung mononuclear cells (Fig 5, G-K and Fig E6, C).

Therefore PKC- $\theta$  signaling is critical for ILC2 expansion and activation and potentially mediated Th2 driven allergic airway inflammation.

#### ***PKC inhibitor (C20) reduces HDM induced ongoing allergic asthma***

To determine the mechanism that may account for the suppressed allergic phenotype in response to PKC- $\theta$  signaling during the effector phase in the allergic response to HDM, we investigated the effect of the specific inhibitor C20 of PKC- $\theta$ , compound 20 (C20, BIX02656), which was shown to inhibit PKC- $\theta$  in vitro<sup>13</sup>. Sensitized-WT mice were treated orally with PKC- $\theta$  inhibitor at doses of 10 mg/kg for 3 days during HDM challenge on day 14-16 as described in Figure 6A. At day 14, before starting C20 treatment, we have checked whether lung inflammation is already established in WT mice. At day 14 after two HDM immunization, WT mice exhibited a strong lung inflammation as evidenced by the increased eosinophils and lymphocytes recruitment in the bronchoalveolar space (BALF), *Muc5ac* gene expression in lung tissue as well as IL-13, IL-33, CCL17 and CCL24 production in lung homogenate after anti CD3 $\epsilon$  or HDM restimulation (Fig E8, A-I). C20 treatment yielded a significant inhibition (2-3 fold reduction) of eosinophils and lymphocytes in the airways (Fig 6, B-E), with a significant reduction of HDM induced increase in airway resistance (Fig 6, F), reduced ILC2 numbers, ILC2 expressing IL-5, IL-13 and IRF4, airway inflammation and mucus overproduction as evidenced by histological analysis and mRNA expression level for *Muc5ac* (Fig 6, G-K) as well as Th2 cytokine/chemokine production (IL-1 $\alpha$ , IL-1 $\beta$ , IL-4, IL-5, IL-13, CCL11, CCL17 and CCL24 (Fig 6, L-M; O-R). Moreover, IRF4 expression from pulmonary tissue was reduced, as well as NFAT1 production in lung (Fig 6, S-T). Importantly, pulmonary cells produced similar amounts of IL-33 in response to HDM immunization either in the presence or absence of PKC- $\theta$  blockade (Fig 6, N).

457 Therefore disruption of the PKC- $\theta$  signalling pathways with the PKC- $\theta$  inhibitor C20 inhibits  
458 ongoing allergic inflammation and reduces IRF4 and NFAT1 expression.

**DISCUSSION**

Recent evidence has implicated ILC2 cells in Th2 responses<sup>17, 36</sup>. We demonstrate for the first time that PKC- $\theta$  is expressed by both human and mouse ILC2 populations and likely required for efficient Th2 cell mediated allergic lung inflammation upon challenge with the protease allergen HDM. The reduced ILC2 and Th2 cell counts and activation is linked to an inability to mount a strong allergic lung inflammation with impaired cardinal features of asthma including airway hyperresponsiveness and Th2 cytokines and chemokines release in PKC- $\theta$  deficient mice. This is the first report suggesting that ILC2 activation is dependent on PKC- $\theta$  signalling pathway. ILC2 can expand and be activated after local protease allergens administration including papain, *Alternaria alternata* and house dust mite<sup>17, 26, 37</sup> and we show that the emergence of ILC2 is inhibited in the absence of PKC- $\theta$ . This finding is novel and PKC- $\theta$  is required for ILC2 activation. ILC2 precedes the Th2 differentiation, which is drastically reduced in the absence of PKC- $\theta$ . We questioned whether IL-33 known to be involved in ILC2 activation resulting in IL-5 and IL-13 production is responsible for the lack of response, but we did not find any difference of IL-33 production in lung tissue in PKC- $\theta$  - deficient mice.

PKC- $\theta$  is involved in the T cell receptor synapse and inhibition of PKC- $\theta$  reduces the Th2 mediated response<sup>12</sup>. While previous studies reported PKC- $\theta$  dependent OVA-induced lung inflammation, we show here that all features of HDM-induced allergic asthma are dramatically reduced in the absence of PKC- $\theta$ <sup>11, 14</sup>.

IRF-4 functions either as a transcriptional activator or repressor depending on DNA-binding sequences and/or protein-interacting partners. IRF-4 interacts with its family member PU.1 or NFAT1 to synergistically enhance transcriptional activity of a variety of genes including IL-4 in T cells and Th2 cell response<sup>34</sup>. Our study reveals that the decreased *IRF4*/NFAT1

expression is associated with decreased allergic inflammation to HDM in PKC- $\theta$  -deficient mice

Furthermore we identified that dendritic cell functions are PKC- $\theta$  dependent. Specifically MHC class II expression is reduced and associated with the inability of HDM primed PKC- $\theta$  deficient T cell to mediate T cell proliferation and induce the production of IL-2. Thus we confirm that PKC- $\theta$  deficient T cells are unable to respond to TCR stimulation<sup>12</sup>. We report a role of PKC- $\theta$  on DC activation and suggest that the defect might be at the class II molecule dependent antigen platform which may be related to reduced *IRF4* expression as previously by the study showing that IRF4 signalling in DC cells controls Th2 responses<sup>34</sup>. Importantly it was shown that PKC- $\theta$  during CD8<sup>+</sup> T cell antiviral response may be overcome by activated DC<sup>38</sup>. Therefore, NFAT1 and *IRF4* expression in DC and T cells are likely PKC- $\theta$  dependent and contribute to allergic response.

Since the net inflammatory response depends on other factors we thought that augmented FoxP3 regulatory cells could contribute to reduced allergic response. However we found reduced FoxP3 cell counts in PKC- $\theta$  deficient lungs. Therefore Treg cells are unlikely to be involved in our model.

While we demonstrate that PKC- $\theta$  is required for the allergic response, it was unknown whether PKC- $\theta$  blockade could interrupt an ongoing inflammatory response and ILC2 activation of sensitized mice. To address this question we used the PKC- $\theta$  inhibitor and found that C20 had a potent therapeutic effect on ongoing HDM induced allergic inflammatory responses with a drastic inhibition of the immune response, lung inflammation and airway hyperreactivity. This is an important finding and our data points to an interesting therapeutic strategy by using the inhibitor to decrease allergic responses.

506 In conclusion our data suggest that PKC- $\theta$  contributes to ILC2 activation and T cell  
507 proliferation both contributing to Th2 response in allergic lung inflammation in response to  
508 protease allergens. This response is associated with IRF4 and NFAT1 transcription factor  
509 expression. Together with the potent inhibition of asthma by the PKC- $\theta$  inhibitor C20 during  
510 allergen challenge, our data suggest that PKC- $\theta$  may be a novel therapeutic target in asthma.

511

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**Figure legends**

**Figure 1.** *Prkcq* (*Pkc-θ*) is expressed in human circulating ILC subsets and mouse lung ILC2s.

(A) *Prkcq* (*Pkc-θ*) and *Gata-3* mRNA expression in human ILC1s, ILC2s and ILC3s (n=8). (B, C) *Prkcq* (*Pkc-θ*), *IL-4*, *IL-5*, *IL-13*, *Irf4*, *Gata-3* and *Il-1rl1* (*ST2*) mRNA expression in mouse lungs ILC2 (n=3). Data are expressed as means of gene expression fold changes relative to  $\beta$ -actin or RN18s. Values are the mean  $\pm$  s.e.m. (\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ).

**Figure 2.** *PKC-θ* is required for ILC2 activation.

(A), Scheme of HDM model protocol in *PKC-θ*<sup>-/-</sup> and wild type (WT) mice. (B-F), Total ILC2s, *IL-5*<sup>+</sup>*ILC2*<sup>+</sup>, *IL-13*<sup>+</sup>*ILC2*<sup>+</sup> and *IRF4*<sup>+</sup>*ILC2*<sup>+</sup> frequency and absolute number in the lungs. Representative data of two independent experiments are given (n=4 for saline control and 8 mice for HDM group). Values are the mean  $\pm$  s.e.m. \*\* $P \leq 0.01$ ; \*\*\*  $P \leq 0.001$ .

**Figure 3.** Impaired *Th2*-mediated lung inflammation in *PKC-θ*<sup>-/-</sup> mice

(A-E) Total cells, eosinophils and lymphocytes recruitment in BALF, eosinophil peroxidase activity (EPO) in the lungs and airway resistance. (F-I) PAS staining (scale bars: 100  $\mu$ m), semi-quantitative histological score and *Muc5ac* gene expression. Data are derived from four for saline and eight mice for HDM group of three independent experiments. Values are the mean  $\pm$  s.e.m. n.s., not significant, \* $P \leq 0.05$ ; \*\* $P \leq 0.01$ ; \*\*\* $P \leq 0.001$ .

**Figure 4.** *Th2* associated cytokines and chemokines analysis in lung homogenates of *PKC-θ*<sup>-/-</sup> mice.

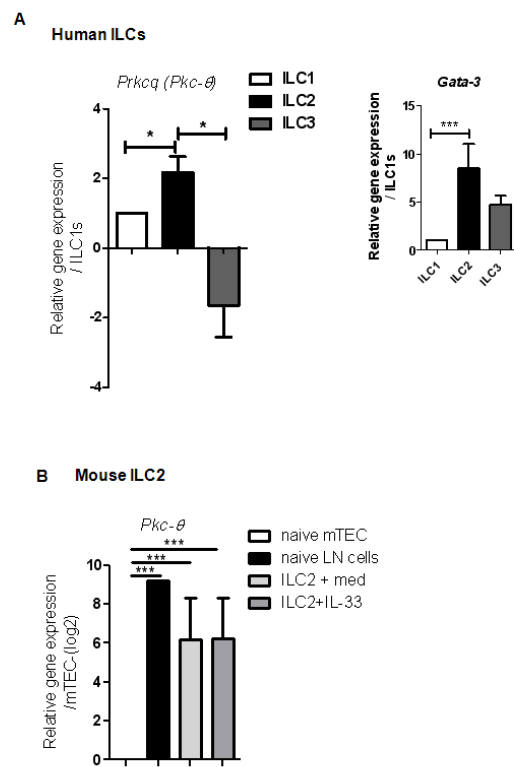
(A-K) IL-1 $\alpha$ , IL-1 $\beta$ , IL-4, IL-5, IL-13, IL-33, CXCL1 (KC), CCL11, CCL17 (TARC), CCL20 (MIP-3 $\alpha$ ), and CCL24 (Eotaxin-2) levels in lung homogenates. (L-M) *IRF4* gene expression and NFAT1 (NFATc2) protein expression in lung homogenates. These experiments were performed two times (n=4 for saline and 8 mice for HDM group). Values are the mean  $\pm$  s.e.m. \*P $\leq$ 0.05; \*\*P $\leq$ 0.01.

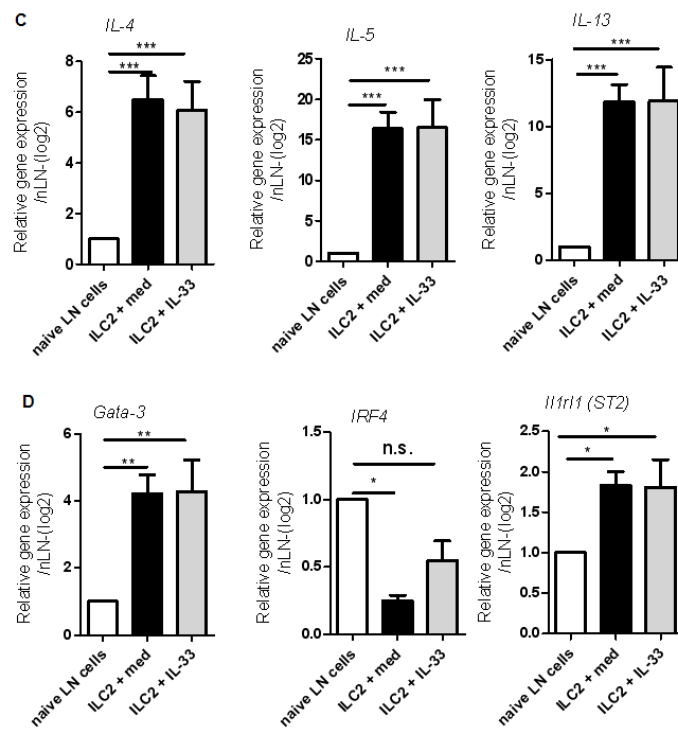
**Figure 5.** *ILC2 cell transfer from WT mice restores eosinophilia and Th2 cytokines.*

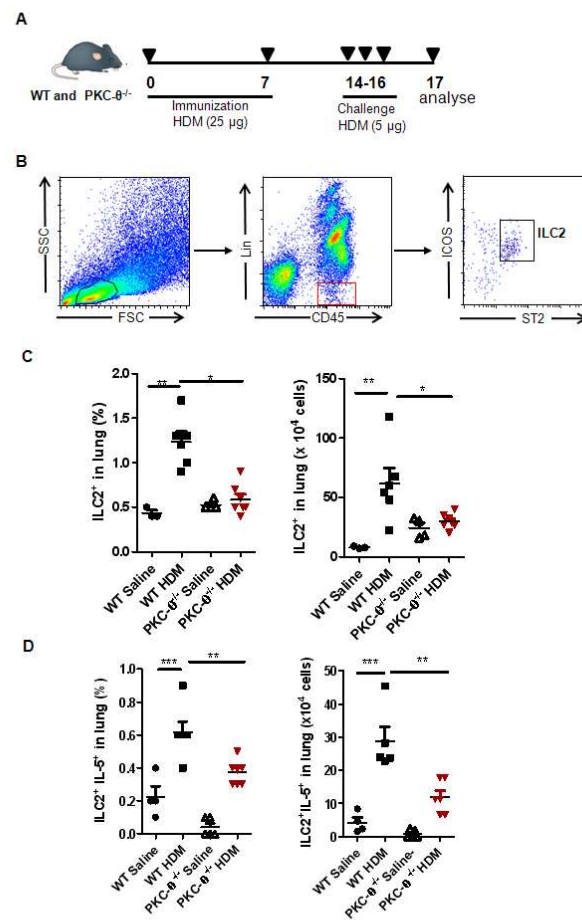
(A) Recombinant IL-33 was administered by intranasal route to WT and PKC- $\theta^{-/-}$  mice for 5 days. (B) The frequency and total number of ILC2s in lungs are shown. (C-E) Total cells, eosinophils and lymphocytes recruitment in BALF were determined after adoptive transfer of purified ILC2 from WT mice to WT and PKC- $\theta^{-/-}$  mice. (F-J) IL-4, IL-5, IL-13, CCL17 (TARC), and CCL24 (Eotaxin-2) levels after lung mononuclear cell restimulation with anti CD3 $\epsilon$ /CD28 for 72h. Values are the mean  $\pm$  s.e.m. \*P $\leq$ 0.05; \*\*P $\leq$ 0.01; \*\*\*P $\leq$ 0.001.

**Figure 6.** *Oral administration of PKC- $\theta$  inhibitor (C20) reduces HDM-driven inflammation, AHR and IRF4 gene expression*

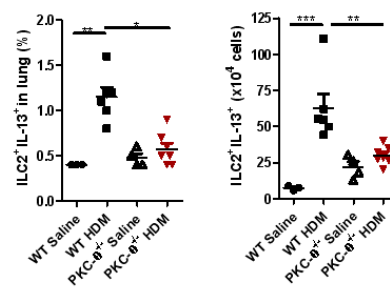
(A) Schematic diagram of PKC- $\theta$  inhibitor treatment (C20, 10 mg/kg, po). (B-G) Total cells, eosinophils and lymphocytes counts in BALF, EPO activity in the lung, airway resistance, ILC2 numbers, ILC2 expressing IL-5, IL-13 and IRF4 are shown. (H-K) Cell infiltration and mucus production using PAS staining (scale bars: 100  $\mu$ m and 200  $\mu$ m) and a semi-quantitative histological score of cell infiltration and mucus hypersecretion and *Muc5ac* gene expression. (L-R) IL-1 $\alpha$ , IL-1 $\beta$ , IL-4, IL-5, IL-13, CCL11, CCL17 (TARC), and CCL24 (Eotaxin-2) levels in lung homogenates after HDM restimulation were shown. (S-T) *IRF4* gene expression and NFAT1 (NFATc2) protein levels in lung tissue. Values are the mean  $\pm$  s.e.m. n.s., not significant, \*



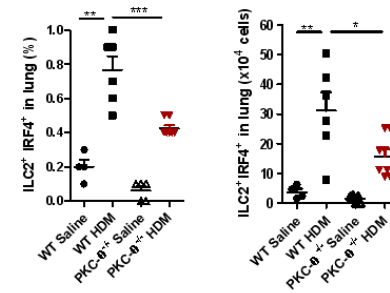


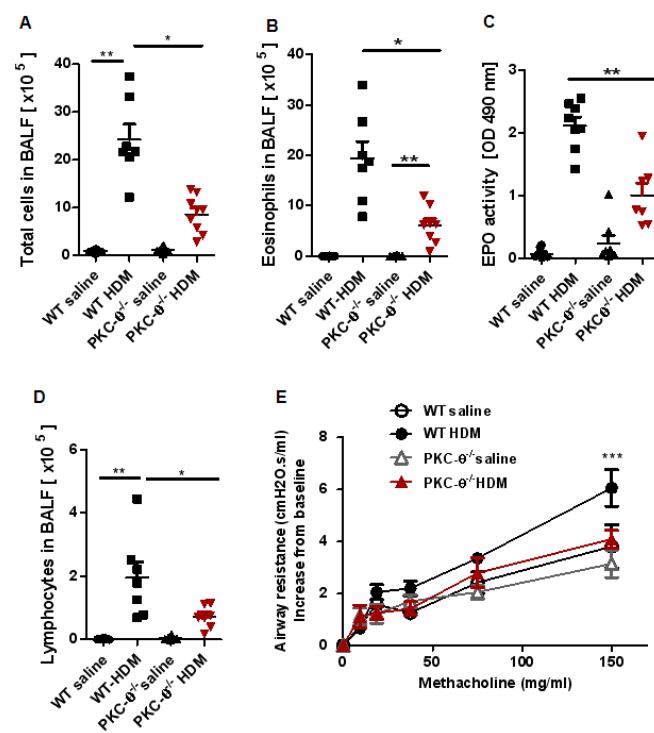


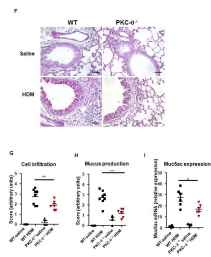
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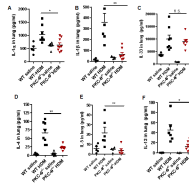


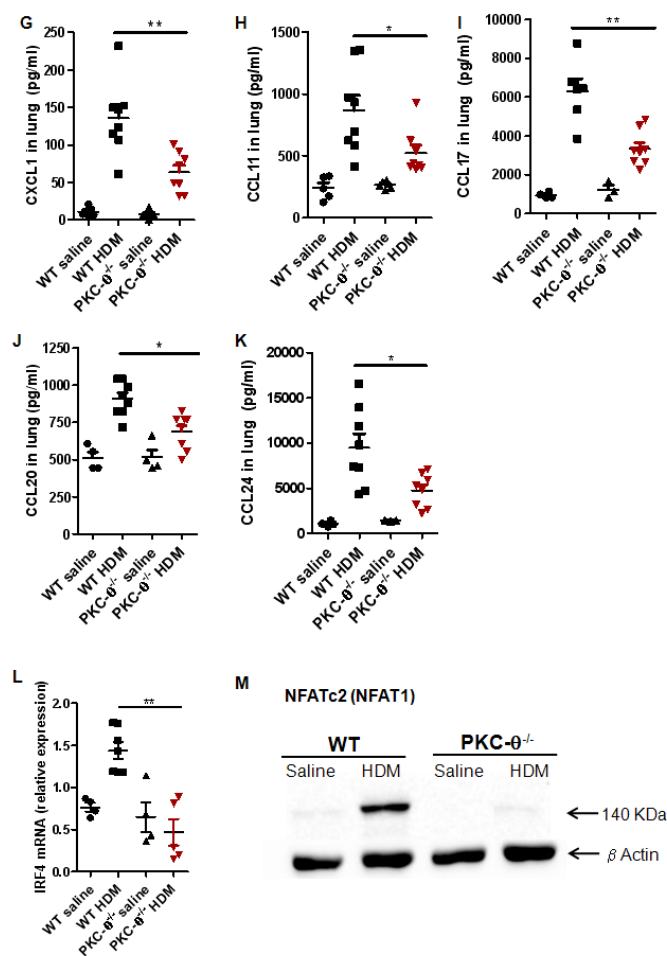
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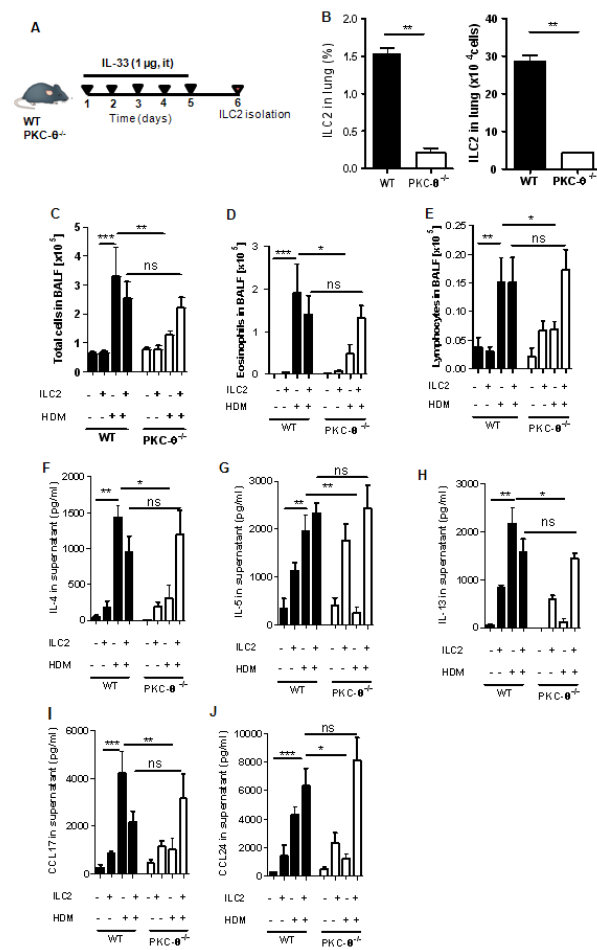


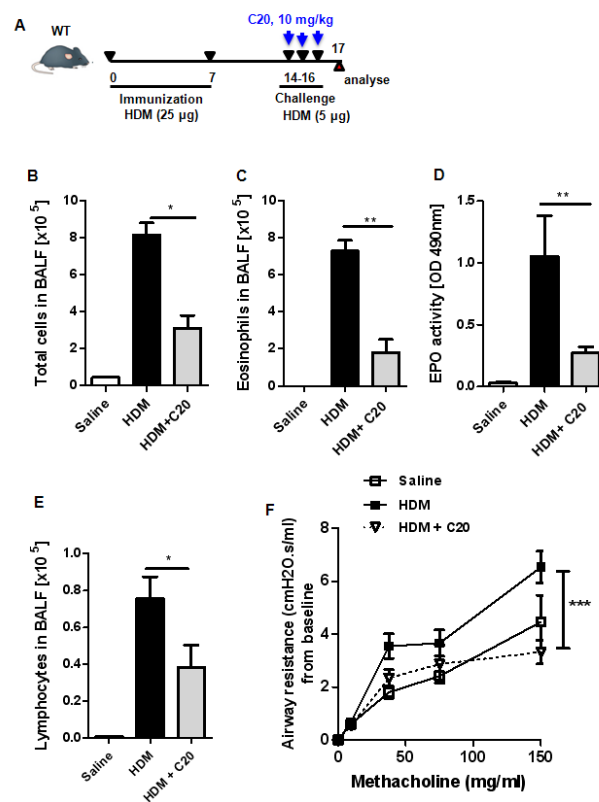




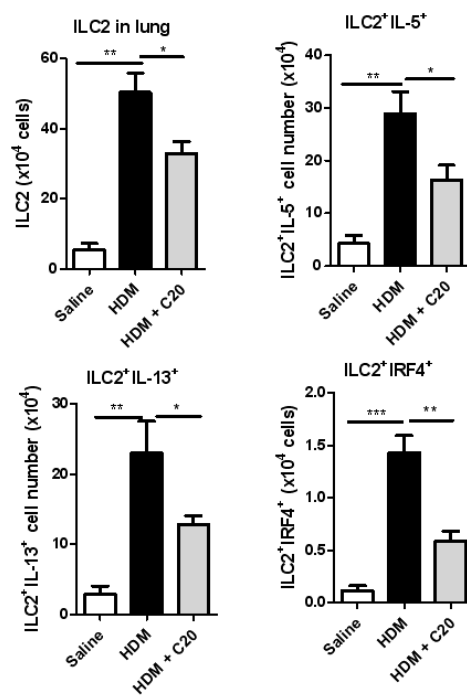


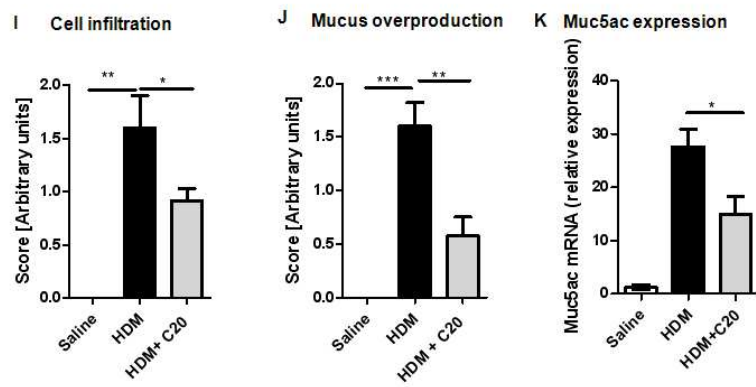
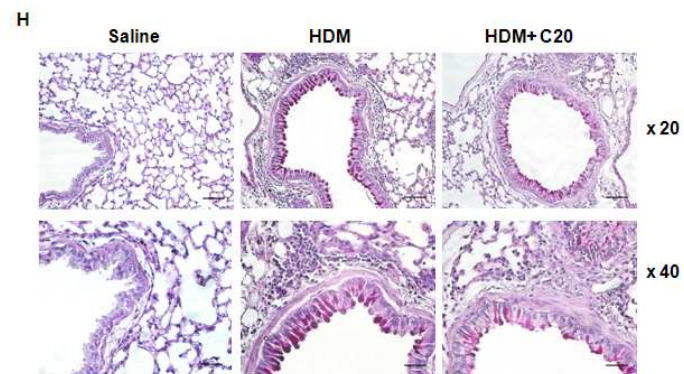


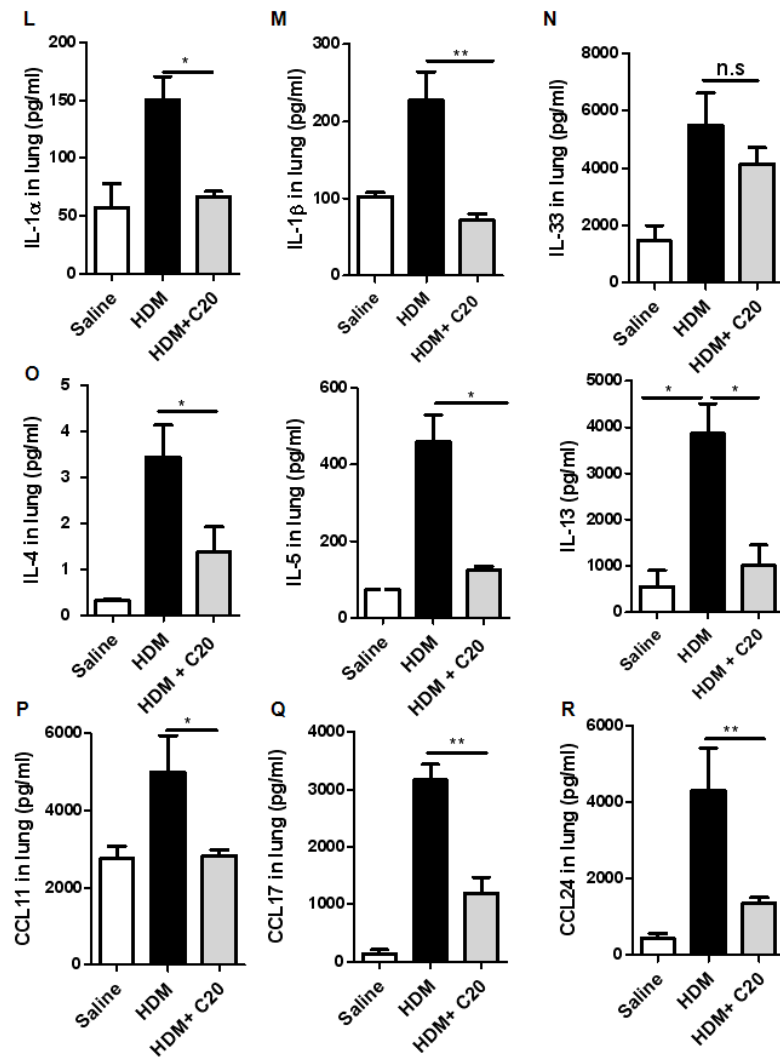


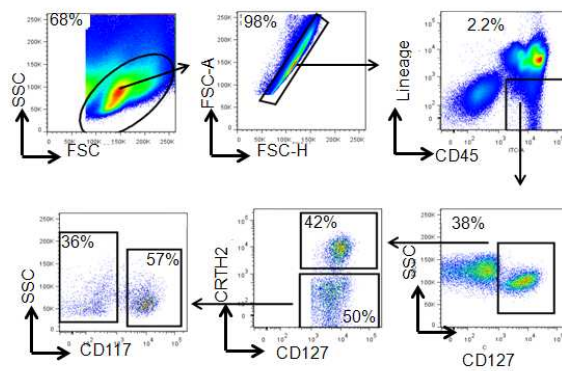
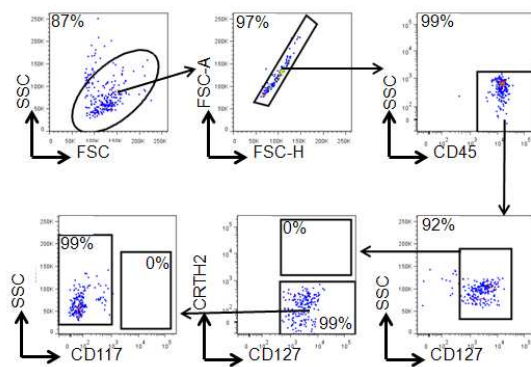


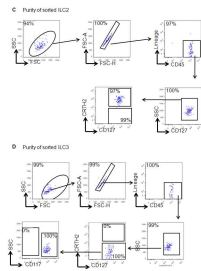
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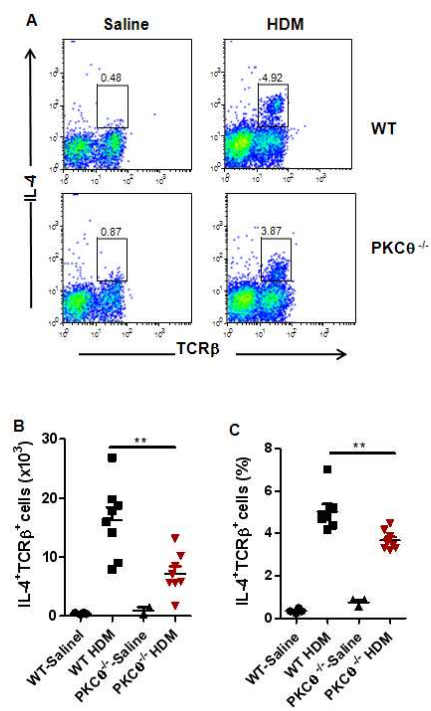


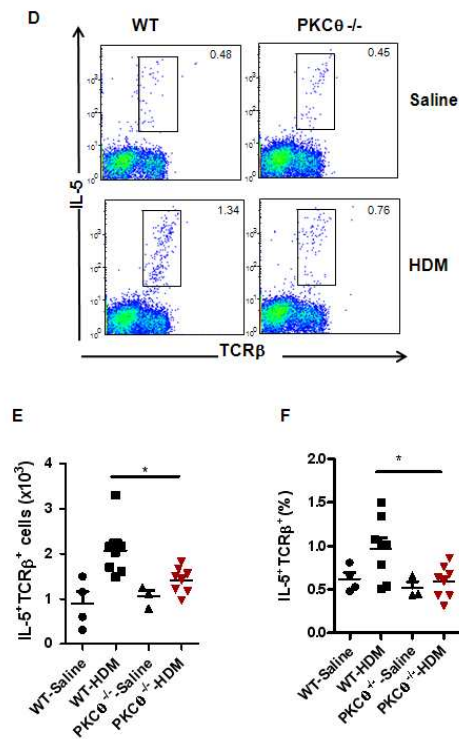


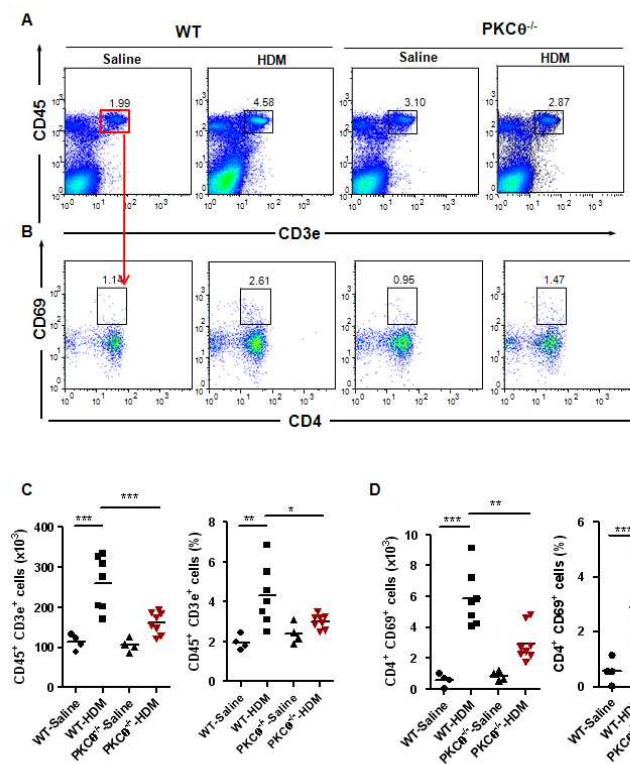


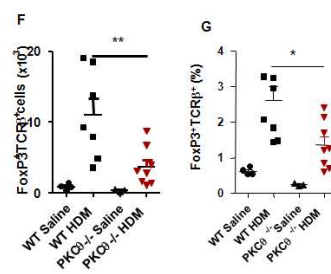
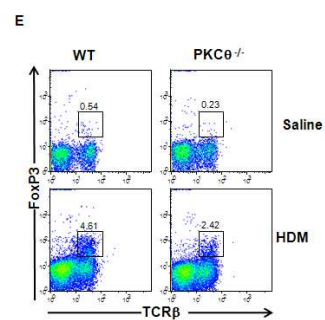
**A** Sorting strategy**B** Purity of sorted ILC1

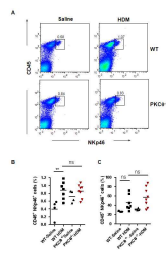


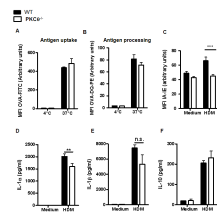


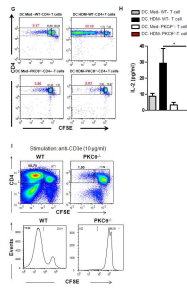




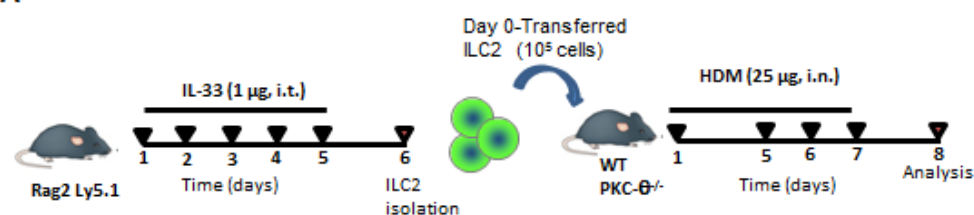




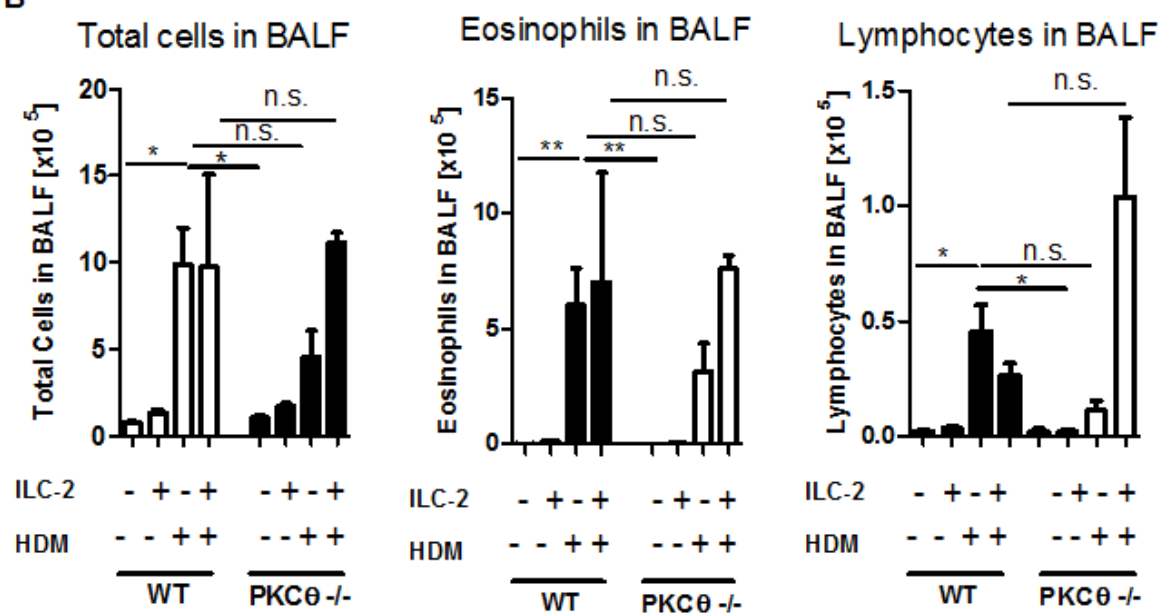




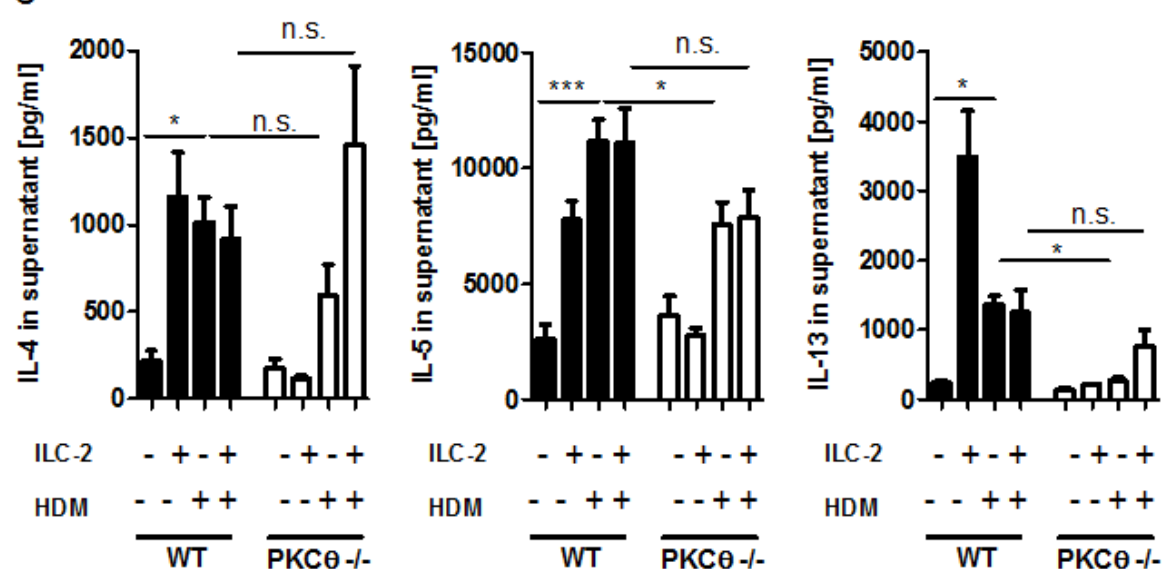
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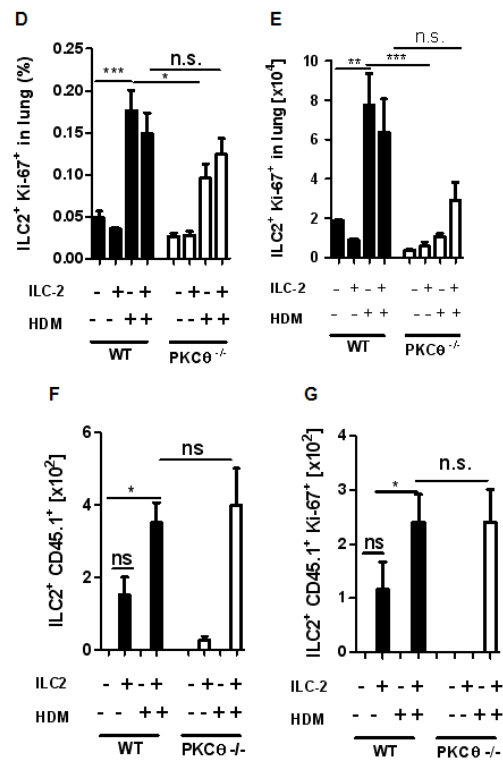


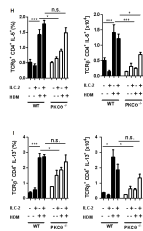
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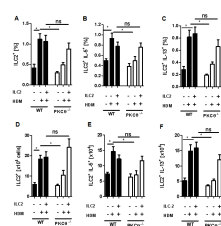


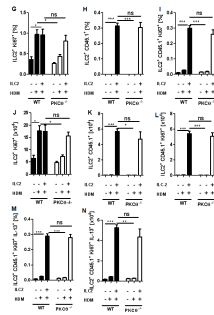
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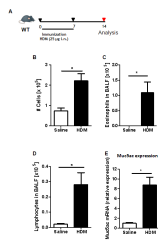


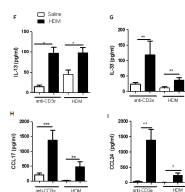












**PKC theta controls ILC2 and Th2 responses to house dust mite allergen**

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**SUPPLEMENTAL MATERIAL**

## SUPPLEMENTAL METHODS

### Sorting of ILC subsets and subsequent gene expression analysis

Human PBMCs were isolated by density gradient centrifugation (Ficoll, Eurobio Les Ulis, France) from buffy coats obtained from healthy volunteers (Etablissement Français du Sang, Rungis, France). Most of lineage positive cells were depleted using the Lineage Cell Depletion kit (Containing CD2, CD3, CD11b, CD14, CD15, CD16, CD19, CD56, CD123, and CD235a antibodies, Miltenyi Biotec, Bergisch Gladbach, Germany) and an autoMACS Pro Separator device following the manufacturer's instructions. To isolate ILC subsets, lineage positive cells-depleted PBMCs were stained with Fluorescein isothiocyanate (FITC)-conjugated anti-CD45 (HI30, BD Biosciences, San Diego, CA), Brilliant Violet 421-conjugated anti-CD117 (YB5.B8, Biolegend, San Diego, CA), Phycoerythrin-cyanine 7 (PE-Cy7)-conjugated anti-CD127 (M15-18C9, Miltenyi Biotec), PerCP-Cy5.5-conjugated anti-CD294 (CRTH2, BM16, BD Biosciences) and a lineage cocktail conjugated with Viogreen or Brilliant Violet 510 comprising anti-CD3 (BW264/56), anti-CD14 (TÜK4), anti-CD16 (VEP13), anti-CD20 (LT20), anti-CD34 (561) antibodies (all from Miltenyi Biotec), anti-CD5 (UCHT2), anti-CD11c (B-ly6), anti-CD56 (NCAM16.2), anti-CD123 (9F5) and anti-TCR $\alpha/\beta$  (T10B9.1A-31) antibodies (from BD Biosciences). ILC1s (Lineage<sup>-</sup>, CD45<sup>+</sup>, CD127<sup>+</sup>, CRTH2<sup>-</sup>, CD117<sup>-</sup>), ILC2s (Lineage<sup>-</sup>, CD45<sup>+</sup>, CD127<sup>+</sup>, CRTH2<sup>+</sup>) and ILC3s (Lineage<sup>-</sup>, CD127<sup>+</sup>, CD45<sup>+</sup>, CRTH2<sup>-</sup>, CD117<sup>+</sup>) were sorted using a FACS Aria III (BD Biosciences) apparatus and data were analyzed with the FlowJo software (TreesStar, Ashland, OR). Total RNA was extracted from sorted ILC subsets using the RNeasy Micro kit (Qiagen, Courtaboeuf, France), and cDNAs were synthesized using the Super Script VILO master mix (Applied Biosystems, Les Ulis, France) according to the manufacturer's instructions. Messenger RNA expression was evaluated by quantitative PCR using a 7900HT Real-time PCR system (Applied Biosystems) with predesigned TaqMan gene expression assays and

reagents, according to the manufacturer's instructions. Expression of the following genes was assessed in ILC subsets: GATA3 (Hs00231122\_m1) and PRKCQ (Hs00989370\_m1). Data were interpreted for each target gene in comparison with the endogenous control  $\beta$ -actin (Hs99999003\_m1). The relative amount of target genes in each sample was calculated in comparison with a calibrator sample (ILC1s) using the  $\Delta\Delta C_t$  method<sup>1</sup>.

#### **Antigen uptake and processing assays.**

BMDCs ( $10^6$  cells) were incubated for 2 h at 4 °C or 37 °C with 50  $\mu$ g of ovalbumin coupled to fluorescein isothiocyanate alone or OVA-DQ (Molecular probes, Invitrogen). After incubation, cells were collected, thoroughly washed and analyzed by flow cytometry (Canto II, BD Bioscience).

#### **Proliferation assay.**

A single-cell suspension was isolated from lymph nodes, CD4<sup>+</sup> T cells were purified by magnetic separation (Dyna, Invitrogen) according to the manufacturer's instructions, and proliferation was determined as described<sup>2</sup>.

Briefly purified CD4<sup>+</sup> T cells ( $10^5$  cells) from WT and PKC- $\theta^{-/-}$  mice were co-cultured with  $10^4$  WT dendritic cells preloaded with HDM (10  $\mu$ g/ ml, 2h). T cell proliferation was assessed by CFSE staining (0.5  $\mu$ M; Molecular probes, Invitrogen).

#### ***Flow cytometry analysis on lung mononuclear cells***

Lung mononuclear cells were stained with PerCP-conjugated anti-CD45 (clone 30F11), FITC-conjugated anti-CD3 $\epsilon$  (clone 145-2C11), v450-conjugated anti-CD4 (clone RM4-5), APC-Cy7 -conjugated anti-CD8 $\alpha$  (clone 53-6.7), PE-Cy7-conjugated anti-CD69 (clone H1.2F2), eFluor 660-conjugated anti-NKp46 (clone 29A1, 4). After washing, cells were

permeabilized for 20 min with cytofix/cytoperm kit (BD Biosciences, France) and stained with PE-conjugated IL-4 (clone 11B11), APC-conjugated anti-IL-5 (clone TRFK5), APC-conjugated anti-IL-13 (clone eBio13A, eBioscience) or APC-conjugated anti-FoxP3 (clone FJK-16s, eBioscience). All antibodies used in this were from BD Biosciences, unless otherwise specified. Data were acquired using FACS Canto II flow cytometer and analyzed using Diva and FlowJo software.

### Supplementary figure legends

#### **Figure E1:** *Flow cytometry sorting of human ILC subsets*

PBMCs depleted from lineage positive cells were stained with anti-CD45, anti-CD117, anti-CD127, anti-CRTH2 antibodies and a lineage cocktail comprising anti-CD3, anti-CD11c, anti-CD14, anti-CD16, anti-CD20, anti-CD34, anti-CD123 and anti-TCR $\alpha\beta$  antibodies. ILC subtypes, i.e. ILC1s (Lineage<sup>-</sup>, CD45<sup>+</sup>, CD127<sup>+</sup>, CRTH2<sup>-</sup>, CD117<sup>-</sup>), ILC2s (Lineage<sup>-</sup>, CD45<sup>+</sup>, CD127<sup>+</sup>, CRTH2<sup>+</sup>) and ILC3s (Lineage<sup>-</sup>, CD45<sup>+</sup>, CD127<sup>+</sup>, CRTH2<sup>-</sup>, CD117<sup>+</sup>) were isolated using a cell sorter. All gates were set up based on reactivity with relevant isotype controls. (A) Gating strategy, (B-D) purity of ILC1, ILC2 and ILC3 sorted populations was controlled after each experiment. Representative dot plots are shown.

#### **Figure E2:** *Impaired IL-4<sup>+</sup>TCR $\beta$ <sup>+</sup> cells and IL-5<sup>+</sup>TCR $\beta$ <sup>+</sup> cells in absence of PKC- $\theta$ .*

HDM sensitized WT and PKC- $\theta$ <sup>-/-</sup> mice were challenged three times with HDM extract. Twenty hours after the third HDM challenge at day 17, lung mononuclear cells from WT and PKC- $\theta$ <sup>-/-</sup> mice were isolated and restimulated for 4 h with PMA (50 ng/ml) and ionomycin (750 ng/ml) in presence of Brefeldin A followed by extracellular staining of CD45, TCR $\beta$ , CD4 and CD8 $\alpha$  and intracellular staining of IL-4 (A-C) or IL-5 (D-F). (A-F) Representative dot plots and scatter plot are shown. Values are the mean  $\pm$  .s.e.m. of 4 mice for saline group and 8 mice per group for HDM group. \*P $\leq$  0.05; \*\*P $\leq$ 0.01. n.s., not significant.

#### **Figure E3.** *Reduced activated T cells and Treg cells recruitment in the absence of PKC- $\theta$ .*

CD45, CD3, TCR $\beta$  $\square\square$ , CD4, CD69 and FoxP3 expression on lung mononuclear cells were shown. (A-G) Representative dot plots, the absolute numbers and the frequency of

CD45<sup>+</sup>CD3e<sup>+</sup> (C), CD4<sup>+</sup>CD69<sup>+</sup> (D) and TCRβ<sup>+</sup>FoxP3<sup>+</sup> cells (F,G). These experiments were performed twice. Values are the mean ± s.e.m. of 4 mice for saline group and 8 mice per group for HDM group. \*P ≤ 0.05; \*\* P ≤ 0.01.

**Figure E4:** Similar number of CD45<sup>+</sup>NKp46<sup>+</sup> cells in lung in absence of PKC-θ.

HDM sensitized WT and PKC-θ<sup>-/-</sup> mice were challenged three times with HDM extracts. (A-C) Twenty hours after the third HDM challenge at day 17, lung mononuclear cells from WT and PKC-θ<sup>-/-</sup> mice were isolated followed by extracellular staining of CD45, NKp46. Representative dot plots and scatter plot are shown. Values are the mean ± s.e.m. of 4 mice for saline group and 8 mice per group for HDM group. n.s. not significant, \*\*P ≤ 0.01.

**Figure E5.** Antigen uptake and processing and HDM activated DCs promote T-cells proliferation of naive CD4<sup>+</sup> T cells from PKC-θ.

(A-C) *In vitro* antigen uptake and processing on BMDCs from WT and PKC-θ<sup>-/-</sup> mice MFI of OVA-FITC (a), OVA-DQ (b) and MHCII<sup>+</sup> DCs (c) were shown. (D-F) IL-1α, IL-1β and IL-10 levels in BMDC culture supernatant. (G) CFSE labeled CD4<sup>+</sup> T cells from naive WT or PKC-θ mice were cocultured with untreated or HDM-treated WT-DCs at a ratio of 1:10 (T cells:DCs) for 3 days. (H) IL-2 levels in the supernatant. Data are representative of two independent experiments (n = 4 mice per group). Data are presented as mean ± s.e.m. ; n.s., not significant, \* P ≤ 0.05; \*\* P ≤ 0.01; \*\*\* P ≤ 0.001.

**Figure E6.** ILC2 cells transfer from Rag2<sup>-/-</sup>Ly5.1 mice restored eosinophilia and Th2 cytokines in PKC-θ<sup>-/-</sup> mice.

(A) Recombinant IL-33 was administered by intranasal route to Rag2<sup>-/-</sup>Ly5.1 mice for 5 days. Twenty four hours after the last IL-33 administration, lung mononuclear cell were isolated

and stained with lineage markers and ILC2 were sorted. **(B)** Purified ILC2s from *Rag2<sup>-/-</sup>Ly5.1* mice were adoptively transferred into WT or PKC- $\theta^{-/-}$  followed by four HDM challenges. Broncho-alveolar lavage (BAL) was performed and total cells, eosinophils and lymphocytes recruitment in BALF were determined. **(C)** IL-4, IL-5 and IL-13 levels in cell culture supernatant of lung mononuclear cells after anti-CD3 $\epsilon$ /CD28 restimulation were shown. **(D, G)** Proliferation defined as Ki-67 expression on total ILC2, transferred ILC2 (CD45.1<sup>+</sup> ILC2<sup>+</sup>) were shown. **(H, I)** IL-5 and IL-13 producing TCR $\beta^+$  CD4<sup>+</sup> cells in the lung were assessed. Data are representative of one experiment (n = 4-8 mice per group). Data are presented as mean  $\pm$  s.e.m. ; n.s., not significant, \*  $P \leq 0.05$ ; \*\*  $P \leq 0.01$ ; \*\*\*  $P \leq 0.001$ .

**Figure E7.** Adoptively transferred wild type ILC2 cells proliferated in PKC- $\theta^{-/-}$  mice.

Recombinant IL-33 was administered by intranasal route to *Rag2<sup>-/-</sup>Ly5.1* mice for 5 days. Twenty four hours after the last IL-33 administration, lung mononuclear cell were isolated and stained with lineage markers and ILC2 were sorted. Purified ILC2 from *Rag2<sup>-/-</sup>Ly5.1* mice were adoptively transferred into WT or PKC- $\theta^{-/-}$  mice followed by four HDM challenges. **(A, F)** Frequency and absolute cell counts of ILC2<sup>+</sup>, ILC2<sup>+</sup>IL-5<sup>+</sup> and ILC2<sup>+</sup>IL-13<sup>+</sup> populations are given. **(G, N)** Frequency and absolute cell counts of ILC2<sup>+</sup>Ki-67, ILC2<sup>+</sup>CD45.1<sup>+</sup> and ILC2<sup>+</sup>CD45.1<sup>+</sup>Ki-67<sup>+</sup>IL-13<sup>+</sup> populations were shown. Data are representative of one experiment (n = 4-8 mice per group). Data are presented as mean  $\pm$  s.e.m.; n.s., not significant, \*  $P \leq 0.05$ ; \*\*  $P \leq 0.01$ ; \*\*\*  $P \leq 0.001$ . Data are representative of one experiment (n = 4-8 mice per group). Data are presented as mean  $\pm$  s.e.m. ; n.s., not significant, \*  $P \leq 0.05$ ; \*\*  $P \leq 0.01$ ; \*\*\*  $P \leq 0.001$ .

**Figure E8.** Lung inflammation is established at day 14 after two HDM immunizations

**(A)** WT mice were immunized twice (days 0 and 7) with HDM (25  $\mu$ g per mouse) by intranasal route under Isoflurane anesthesia (3%). Control mice received saline alone. On day 14, BAL and differential cell counts were performed. **(B-I)** Total cells, eosinophils and lymphocytes recruitment in BALF, *Muc5ac* gene expression levels, and IL-13, IL-33, CCL17

and CCL24 productions in lung mononuclear cells culture supernatant after anti-CD3 or HDM restimulation were shown. Data are presented as mean  $\pm$  s.e.m. of 4 mice for saline group and 8 mice per group; n.s., not significant, \*  $P \leq 0.05$ ; \*\*  $P \leq 0.01$ ; \*\*\*  $P \leq 0.001$ .

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1. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc 2008; 3:1101-8.
2. Besnard A, Togbe D, Guillou N, Erard F, Quesniaux V, Ryffel B. IL-33-activated dendritic cells are critical for allergic airway inflammation. European Journal of Immunology 2011; 41:1675-86.