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Fibrinogen-cleavage products and TLR4 promote the generation of programmed cell death 1 ligand 2(PD-L2)+ dendritic cells in allergic asthma

Minkyong Cho, PhD, Jeong-Eun Lee, BS, Hoyong Lim, PhD, Hyun-Woo Shin, MD, Roza Khalmuratova, PhD, Garam Choi, MS, Hyuk Soon Kim, PhD, Wahn Soo Choi, PhD, Young-Jun Park, PhD, Inbo Shim, BS, Byung-Seok Kim, PhD, Chang-Yuil Kang, PhD, Jae-Ouk Kim, PhD, Shinya Tanaka, PhD, Masato Kubo, PhD, Yeonseok Chung, PhD



PII: S0091-6749(17)31582-8

DOI: [10.1016/j.jaci.2017.09.019](https://doi.org/10.1016/j.jaci.2017.09.019)

Reference: YMAI 13047

To appear in: *Journal of Allergy and Clinical Immunology*

Received Date: 7 March 2017

Revised Date: 23 August 2017

Accepted Date: 22 September 2017

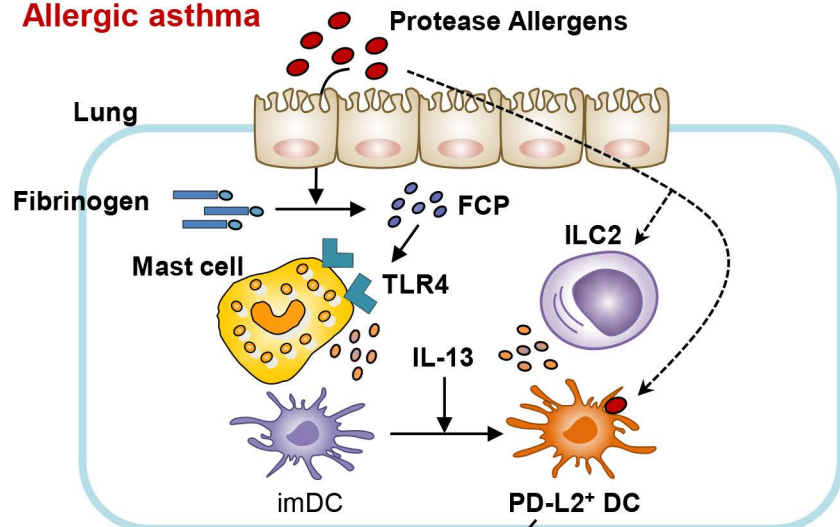
Please cite this article as: Cho M, Lee J-E, Lim H, Shin H-W, Khalmuratova R, Choi G, Kim HS, Choi WS, Park Y-J, Shim I, Kim B-S, Kang C-Y, Kim J-O, Tanaka S, Kubo M, Chung Y, Fibrinogen-cleavage products and TLR4 promote the generation of programmed cell death 1 ligand 2(PD-L2)+ dendritic cells in allergic asthma, *Journal of Allergy and Clinical Immunology* (2017), doi: 10.1016/j.jaci.2017.09.019.

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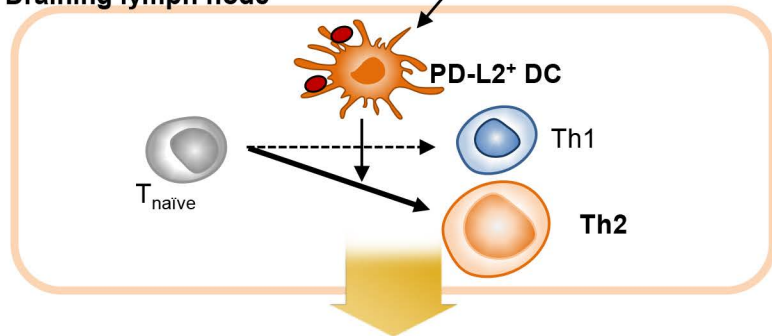


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Allergic asthma

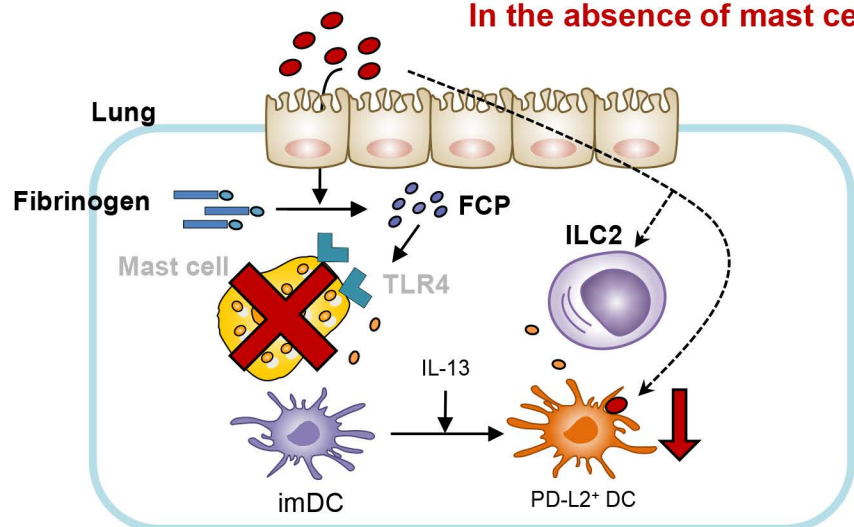


Draining lymph node

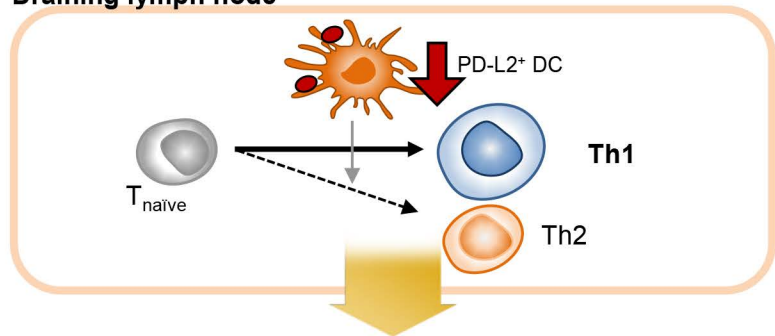


Th2-dominant allergic lung inflammation

In the absence of mast cell



Draining lymph node



Relief of allergic lung inflammation

DC, dendritic cell; FCP, fibrinogen cleavage products; ILC2, type 2 innate lymphoid cell; PD-L2, programmed cell death 1 ligand 2; TLR, Toll-like receptor

**Fibrinogen-cleavage products and TLR4 promote the generation of programmed cell death
1 ligand 2(PD-L2)+ dendritic cells in allergic asthma**

Minkyung Cho, PhD,^a Jeong-Eun Lee, BS^a, Hoyong Lim, PhD,^a Hyun-Woo Shin, MD^{b,c}, Roza Khalmuratova, PhD^b, Garam Choi, MS^a, Hyuk Soon Kim, PhD^d, Wahn Soo Choi, PhD^d, Young-Jun Park, PhD^a, Inbo Shim, BS^a, Byung-Seok Kim, PhD^a, Chang-Yuil Kang, PhD^a, Jae-Ouk Kim, PhD^e, Shinya Tanaka, PhD^f, Masato Kubo, PhD^f, Yeonseok Chung, PhD^a

^aLaboratory of Immune Regulation, Research Institute of Pharmaceutical Sciences, College of Pharmacy, Seoul National University, Seoul 08826. ^bDepartment of Pharmacology, College of Medicine, Seoul National University, Seoul, 08826. ^cDepartment of Biomedical Sciences and Otorhinolaryngology-Head and Neck Surgery, Seoul National University Hospital, Seoul 03080. ^dSchool of Medicine, Konkuk University, Chungju 27478. ^eLaboratory Science Division, International Vaccine Institute, Seoul 08826, Republic of Korea. ^fResearch Institute for Biomedical Science, Tokyo University of Science, Chiba, 278-0022 and the RIKEN Center for Integrative Medical Sciences (IMS), Yokohama, Kanagawa, 230-0045, Japan

Correspondence: Yeonseok Chung, PhD, College of Pharmacy, Seoul National University, Seoul, 08826, Republic of Korea. Tel: +82-2-880-7874. Fax: +82-2-872-1795. E-mail: yeonseok@snu.ac.kr

Funding: The work is supported by research grants 2017R1A2B3007392 (YC) from the National Research Foundation of Korea (NRF) grant funded by the Korea government (MEST) and

HI14C2282 (YC) from the Korean Health Technology R&D Project, Ministry of Health & Welfare, Republic of Korea.

ABSTRACT

Background: Inhaled protease allergens preferentially trigger Th2-mediated inflammation in allergic asthma. The role of dendritic cells (DCs) on the induction of Th2 cell responses in allergic asthma has been well documented; however, the mechanism by which protease allergens induce Th2-favorable DCs in the airway remains unclear.

Objective: We sought to determine a subset of DCs responsible for Th2 cell responses in allergic asthma and the mechanism by which protease allergens induce the DC subset in the airway.

Methods: Mice were intranasally challenged with protease allergens or fibrinogen-cleavage product (FCP) to induce allergic airway inflammation. DCs isolated from the mediastinal lymph nodes were analyzed for surface phenotype and T cell stimulatory function. Anti-Thy1.2 and Mas-TRECK mice were used to deplete innate lymphoid cells and mast cells, respectively. Adoptive cell transfer, bone marrow DC culture, anti-IL-13, and TLR4-deficient mice were used for further mechanistic studies.

Results: Protease allergens induced a remarkable accumulation of Th2-favorable PD-L2⁺ DCs in the mediastinal lymph nodes, which was significantly abolished in mice depleted of mast cells and, to a lesser extent, innate lymphoid cells. Mechanistically, FCP generated by protease allergens triggered IL-13 production from wild-type mast cells but not from TLR4-deficient mast cells, which resulted in the increase of PD-L2⁺ DCs. Intranasal administration of FCP induced an increase of PD-L2⁺ DCs in the airway, which was significantly abolished in TLR4- and mast cell-deficient mice. Injection of IL-13 restored PD-L2⁺ DC population in mice lacking mast cells.

Conclusion: Our findings unveil the ‘protease-FCP-TLR4-mast cell-IL-13’ axis as a molecular mechanism for the generation of Th2-favorable PD-L2⁺ DCs in allergic asthma, and suggest that

targeting PD-L2⁺ DC pathway might be effective in suppressing allergic T cell responses in the airway.

Key messages:

Protease allergens induce PD-L2⁺ DCs in the airway that favorably stimulate Th2 cells while suppressing Th1 cells.

Mast cell-derived IL-13 induces an increase of PD-L2⁺ DCs in a TLR4-dependent manner.

Protease allergens generate FCPs that signal through TLR4 to stimulate IL-13 production from mast cells.

Capsule summary:

FCPs, generated by proteolytic activity of allergens, stimulate IL-13 production from mast cells via TLR4 to induce PD-L2⁺ DCs that preferentially trigger Th2 cell responses during allergic asthma.

Keywords: protease allergen, PD-L2⁺ DC, fibrinogen cleavage product, mast cell, TLR4

Abbreviations used:

BAL: Bronchoalveolar lavage

BMDC: bone marrow derived DC

CM: conditioned media

- 44 DC: dendritic cell
- 45 DT: diphtheria toxin
- 46 FCP: fibrinogen cleavage products
- 47 ILC: innate lymphoid cell
- 48 mLN: mediastinal lymph node
- 49 PAO: protease from *Aspergillus orizae*
- 50 TLR: Toll-like receptor

INTRODUCTION

Type 2 airway inflammation including allergic asthma, allergic rhinitis and chronic rhinosinusitis is one of the common causes of chronic immune disorders in humans¹. The critical contribution of Th2 cells to the pathology of allergic airway inflammation is well-established; however, it is unclear how allergens trigger the differentiation of native CD4⁺ T cells into Th2 cell responses *in vivo*. In this regard, it is noteworthy that a number of allergens are proteases, such as Der p and Der f proteins from house dust mite, papain from *Carica papaya*, and aspergillopepsin from *Aspergillus*^{2, 3}, since proteolytic activity of allergens has been suggested to exert Th2 adjuvant effects during the induction of allergic asthma. Suggested Th2-promoting effects of protease allergens include release of proinflammatory cytokines from innate immune cells, and release of IL-33, IL-25 and TSLP from mucosal epithelial cells²⁻⁴, although the molecular and cellular events driving Th2 cell responses remain incompletely understood.

Several studies have suggested that basophils could act as Th2-inducing antigen-presenting cells⁴⁻⁶. In addition, mast cells have been proposed to be required for the allergen-driven Th2 cell responses^{7, 8}. On the other hand, a number of studies have demonstrated that DCs and alveolar macrophages are necessary for the differentiation of allergen-specific Th2 cells in asthma^{9, 10}, even in the absence of basophils¹¹. Among diverse subsets of DCs in the periphery, recent studies have identified PD-L2 and CD301b as reliable markers of dermal DCs that efficiently mount Th2 cell responses to subcutaneously administered protease allergens and helminth infection in mice^{12, 13}. Nevertheless, it has been unclear whether these innate immune cells are cooperatively or separately involved in initiating Th2 cell responses, especially in case of

allergic asthma.

In the present study, we found that that mast cells and innate lymphoid cells play a crucial role for the initiation of Th2 cell responses to intranasal protease allergens by inducing Th2-favorable PD-L2⁺ DCs in the mediastinal lymph nodes in a TLR4-dependent manner. Mechanistically, protease allergens generate fibrinogen cleavage products (FCPs) which stimulate mast cells to produce IL-13 via TLR4. Thus, protease allergens preferentially promote Th2 cell responses in the airway by inducing PD-L2⁺ DCs through FCP-mediated TLR4 signaling pathway in mast cells.

METHODS

Ethics statements

All animal experiments were approved by the Seoul National University Institutional Animal Care and Use Committee (protocol #: SNU-140602-2 and SNU-141203-1), and were performed in accordance with guidelines for the care and use of laboratory animals of Seoul National University.

Mice

Rag1^{-/-} mice, *Kit*^{W-sh/W-sh}, Mgl2-DTR and C57BL/6 mice were purchased from Jackson laboratory (Bar Harbor, ME, USA) or Orient Bio (Gyeonggi, South Korea). Mas-TRECK and Bas-TRECK mice were generated as previously described¹⁴. *Rag1*^{-/-} Mas-TRECK mice were generated by crossing *Rag1*^{-/-} mice with Mas-TRECK mice. TLR4-deficient mice were provided by Drs. Shizhuo Akira (Osaka University) and Seung-Yong Sung (Seoul National University). All mice were bred and maintained in the specific pathogen-free vivarium of the Seoul National University.

Depletion and analysis of immune cell population

To deplete basophils and/or mast cells, Bas-TRECK and Mas-TRECK mice were i.p. injected with diphtheria toxin (DT; 12 ng/gram body weights), as described previously¹⁴. To deplete innate lymphoid cells (ILCs), wild-type and Mas-TRECK mice were i.p. injected with anti-Thy1.2 antibody or IgG2b antibody as isotype control (all from BioXcell, NH, USA). In some experiments, Mgl2-DTR mice were i.p. injected with DT (50 ng/gram body weights) one day before antigen challenges. Peritoneal cells, bone marrow cells and single lymphoid cells of the

lungs and the mLN were harvested to examine the depletion of mast cells, basophil and ILCs, respectively. To analyze DC population in the mLNs, B220⁺F4/80⁺CD11c⁺MHCII⁺ cells were gated and the expression of CD80, CD86, OX40L, PD-L1, PD-L2, MHCII and CD103 was examined. To examine ILC population, lymphoid cells from the lung and the mLNs were obtained, and CD90.2⁺Lineage⁻ cells among CD45.2⁺ cells were analyzed. To determine CD4⁺ T helper cell subsets, lymphoid cells were stimulated with PMA and Ionomycin in the presence of Golgistop for four hours, and the intracellular expression of IFN γ (Th1), IL-4, IL-5 or IL-13 (Th2) or IL-17A (Th17) in the CD45.2⁺CD4⁺ T cells was analyzed (see Fig. E1 in the Online Repository at www.jacionline.org).

Induction of allergic airway inflammation

Allergic airway inflammation was induced as described previously¹⁵. In brief, mice were intranasally challenged with 7 μ g *Aspergillus* protease combined with 20 μ g ovalbumin (PAO/Ova) (all from Sigma, MO, USA) every other day for total 5 times. In some experiments, 1 μ g of recombinant IL-13 (Peprotech, NJ, USA) was injected with PAO/Ova. Fibrinogen-cleavage products (FCP) were generated as previously described¹⁶. In brief, pre-warmed 5 mg of fibrinogen (Haematologic technologies Inc., Essex Junction, VT, USA) was incubated with 6 μ g of PAO in 1 ml PBS at 37 °C for 30 minutes. In some experiments, mice were intranasally challenged with 40 μ g FCPs alone (1 time) or together with 20 μ g ovalbumin every other day (4 times). Eighteen hours after the last challenge, mice were euthanized and analyzed as previously described¹⁷. In some experiments, 1×10^7 of bone marrow cultured mast cells were i.v. injected into *Kit*^{W-sh/W-sh} mice, and after 5 weeks, allergic airway inflammation was induced in the recipient

mice by intranasal challenges with PAO/Ova. The endotoxin units (EU) of PAO, Ova and fibrinogen are 1.7×10^{-3} EU/ μ g, 1.8×10^{-3} EU/ μ g and 1.8×10^{-5} EU/ μ g, respectively.

DC functional studies

To isolate DCs, the mLN of allergen-challenged mice were obtained and treated with collagenase IV (1 mg/ml) (Gibco, Grand Island, NY, USA) and DNase I (10 μ g/ml) (Bio basic Inc., Amherst, NY, USA) in Ca^{2+} -free HBSS containing 5 mM EDTA (All from Gibco, Grand Island, NY, USA) at 37 °C for 30 minutes. CD11c⁺ cells were enriched with anti-CD11c-microbeads (Miltenyi Biotec., Bergisch Gladbach, Germany). PD-L2⁻ or PD-L2⁺ DCs among B220⁻F4/80⁻CD11c⁺MHCII⁺ cells were sorted by FACS Aria III (Becton Dickinson, San Jose, CA). For *in vitro* functional study, the FACS sorted PD-L2⁺ or PDL2⁻ DCs (2×10^4 /well)s were co-cultured with CD62L^{lo}CD44^{hi}CD25⁻ effector/memory CD4⁺ T cells (1×10^5 / well) in the presence of anti-CD3 ϵ (0.5 μ g/ml) antibody for 72 hours in round-bottom 96 well-plates. In some experiments, anti-PD-L2 blocking antibodies were added (20 μ g/ml). The levels of indicated cytokines in the supernatant were measured by ELISA.

For *in vivo* functional study, naïve C57BL/6 mice were i.v. injected with FACS-sorted PD-L2⁻ or PD-L2⁺ DCs (1×10^5 cells/injection) that had been incubated with 10 μ M Ova₃₂₃₋₃₃₉ for 2 hours at 37 °C. Mice injected with PBS were used as control. One week after DC transfer, the recipient mice were intranasally challenged with 100 μ g ovalbumin (Grade VII, Sigma, Louis, MO, USA) for three consecutive days, and the CD4⁺ T cells in the lungs and in the mLN were analyzed.

DC stimulation with mast cell-conditioned media

Bone marrow-derived mast cells were generated with 10 ng/ml of IL-3 and SCF (Peprotech, NJ, USA). Mast cells were stimulated with native or heat-inactivated PAO, fibrinogen and FCP for 8 hours before being washed and cultured for additional 24 hours. The culture supernatants were harvested and used as 'conditioned media (CM)'. Bone marrow-derived DCs (BMDCs) were prepared by culturing bone marrow cells with 10 ng/ml of GM-CSF (Peprotech, NJ, USA). BMDCs were stimulated by CM or 10 ng/ml of IL-13 with or without anti-mouse IL-13 antibody (Abcam, San Francisco, USA) for 48 hours.

Measurement of cytokines

To prepare lung homogenates, lungs of allergen-challenged mice were homogenized in 1 ml of PBS including protease cocktail (GenDEPOT, TX, USA) using tissue homogenizer (PRO Scientific Inc., Connecticut, USA). Supernatants from the lung homogenates were filtered through 100 μ m-pore nylon mesh (Small Parts Inc., FL, USA). The levels of cytokines in the lung extracts, CM, and in the supernatant of T cells cocultured with DCs were measured by ELISA according the manufacturer's instructions (eBioscience).

Real-time RT-PCR

Total RNA from FACS-sorted PD-L2⁻ or PD-L2⁺ DCs of mice was isolated by TRIzol reagent (Ambion, CA, USA) and cDNA was synthesized with a cDNA Synthesis kit (Thermo Fisher Scientific Inc., NY, USA). Quantitative real-time PCR was conducted using SYBR Green (Bio-Rad, PA, USA) on an ABI 7500 Fast Real-Time PCR Systems (Applied Biosystems, MA, USA). Target genes were normalized to the β -actin level in each sample. Primer sets for genes were

synthesized at Cosmogenetech (Seoul, South Korea): *Nos2* (sense, 5'-CGAAACGCTTCACTTCCAA-3', antisense, 5'-TGAGCCTATATTGCTGTGGCT-3'), *Il12a* (sense, 5'-CCACCCTTGCCCTCCTAAAC-3', antisense, 5'-GGCAGCTCCCTCTTGTTGTG-3'), *Arg1* (sense, 5'-CTTGCGAGACGTAGACC CTG-3', antisense, 5'-TCCATCACCTTGCCAATCCC-3'), *Pdcd1lg2* (sense, 5'-CTGCTGCCG ATACTGAACCT-3', antisense, 5'-CTGCGGTCAAAATCGCACTC-3'), *Il13ra1* (sense, 5'-GGCCATCCTGCAAAATAGTG-3', antisense, 5'-ACAGCGTCGGCAAGAACA-3'), *Il4ra* (sense, 5'-CACCTGGAGTGAGTGGAGTC-3', antisense, 5'-AGGCAAAACAACGGGATG-3'), β -actin (sense, 5'-TGGAATCCTGTGGCATCCATGAAAC-3', antisense, 5'-TAAAACGCAGCT CAGTAACAGTCCG-3').

Statistical Analysis

All experiments were performed two to four times. Statistical analysis was conducted with mean $\pm$ SEM by unpaired two-tailed Student's *t* test with Prism Graphpad 5.0 (GraphPad Software Inc., USA).

RESULTS

Protease allergens induce the accumulation of PD-L2⁺ DCs in the airway

As a first step to investigate the mechanism by which protease allergens induce allergic Th2 cell responses *in vivo*, we sought to determine if a certain subset of dendritic cells (DCs) is induced by protease allergens in the airway. Although DCs expressing PD-L2 have been recently identified as critical mediators of protease allergen-specific Th2 cell responses in animal models of skin sensitization^{13, 18, 19}, their role in the generation of Th2 cell responses in the airway remains incompletely understood. To examine if PD-L2⁺ DC is involved in this process, we analyzed DC population in the mediastinal lymph nodes (mLN) after intranasal challenges with allergens. We used protease allergen from *Aspergillus oryzae* (PAO) in combination with ovalbumin (PAO/Ova), since intranasal challenge with PAO/Ova is known to trigger Th2 cell dominant inflammation in the airway^{15, 20, 21}. As depicted in Figure 1A, we observed a significant increase in the frequency of PD-L2⁺ DCs among B220⁺F4/80⁺CD11c⁺ DC population in the mLN of the allergen-challenged mice in comparison to those of unchallenged ones. Kinetic analysis revealed that the frequency of PD-L2⁺ DCs gradually increased upon intranasal PAO/Ova (Fig. 1B). Intranasal PAO alone induced the accumulation of PD-L2⁺ DCs in the mLN as efficiently as PAO/Ova, while heat-inactivated PAO failed to do so (Fig. 1C). In addition, the number of PD-L2⁺ DCs appeared to be positively correlated with the number of IL-4⁺ and IL-13⁺ Th2 cells as well as with that of eosinophils in the bronchoalveolar lavage (BAL) fluid in the allergen-challenged mice (Fig. 1D).

PD-L2⁺ DCs promote Th2 cell responses in allergic airway inflammation

To define the phenotype and function of the PD-L2⁺ DCs induced by intranasal protease allergens, we compared the expression of costimulatory molecules between PD-L2⁺ and PD-L2⁻ DCs obtained from the mLN of PAO/Ova challenged mice. Compared with PD-L2⁻ counterparts, PD-L2⁺ DCs exhibited increased levels of CD80, CD86, PD-L1 and MHCII (Fig. 2A). The levels of OX40L and CD103 were also higher in the PD-L2⁺ DCs than PD-L2⁻ DCs. To examine if PD-L2 is expressed in a specific DC subset, we determined the expression of PD-L2 on CD11b⁺ and CD103⁺ DC subsets. The level of PD-L2 expression was higher on CD11b⁺ and CD103⁺ DC subsets than their respective counterparts (see Fig. E2A in the Online Repository at www.jacionline.org). The majority of PD-L2⁺ DCs co-expressed CD11b. In addition, PD-L2⁺ DCs expressed significantly lower levels of *Nos2* and *Il12a*, but higher levels of *Arg1* (encoding Arginase-1), *Pdcd1lg2* (encoding PD-L2), *Il13ra1* and *Il4ra* than PD-L2⁻ DCs (Fig. 2B). To compare the helper T cell stimulatory function of the two DC subsets, each DC population was FACS-sorted from the mLN of PAO/Ova challenged mice, and was co-cultured with CD62L^{lo}CD44^{hi}CD25⁻CD4⁺ T cells in the presence of soluble anti-CD3. CD4⁺ T cells stimulated with PD-L2⁺ DCs produced significantly higher levels of IL-4, IL-5, IL-13 as well as IL-10 than those stimulated with PD-L2⁻ DCs (Fig. 2C). In addition, the amount of IL-17 was increased while that of IFN γ was slightly but significantly reduced in the former group. Addition of anti-PD-L2 blocking antibody did not significantly affect the production of IL-5 and IL-13, indicating little to no role of PD-L2 in inducing the Th2 cytokines from effector/memory CD4⁺ T cells by PD-L2⁺ DC subset (see Fig. E2B in the Online Repository at www.jacionline.org).

To determine if PD-L2⁺ DCs induced by intranasal protease allergens can trigger Th2 cell responses *in vivo*, we purely isolated PD-L2⁻ and PD-L2⁺ DCs, loaded with Ova₃₂₃₋₃₃₉ peptide, and transferred them into naïve syngeneic mice. One week later, the recipients were intranasally challenged with Ova for three consecutive days. As shown in Fig. 2D, the frequencies of IL-4, IL-5, or IL-13-expressing Th2 cells in the lung were significantly higher in the recipients of PD-L2⁺ DCs than those of PD-L2⁻ DCs, while the reverse was true for the frequencies of Th17 and Th1 cells. These results demonstrate that PD-L2⁺ DCs were capable of inducing Th2 cell responses *in vivo*. To further determine the Th2-inducing capacity of PD-L2⁺ DCs *in vivo*, we employed Mgl2-DTR mice in which injection with diphtheria toxin (DT) depleted about 50 % of PD-L2⁺ DCs due to the expression of CD301b on the majority of PD-L2⁺ DCs (see Fig. E3A in the Online Repository at www.jacionline.org). Compared with DT-treated wild-type mice, DT-treated Mgl2-DTR mice exhibited significantly attenuated Th2 cell responses in the lungs and in the mLN (see Fig. E3B/C in the Online Repository at www.jacionline.org). Collectively, these results demonstrate that PD-L2⁺ DCs were induced in the airway by protease allergens, and that they represented a specialized DC subset promoting Th2 cell responses *in vivo*.

A critical role of mast cells and innate lymphoid cells in inducing PD-L2⁺ DCs in the airway

We next aimed to determine the cellular and molecular requirements for the generation of PD-L2⁺ DCs in the airway by protease allergens. To this end, we examined the involvement of innate lymphoid cell (ILC), basophil and mast cell since they have been shown to be associated with Th2 cell responses in allergic asthma^{6, 22, 23}. To deplete basophils and/or mast cells, we employed two lines of knock-in mice, Bas-TRECK and Mas-TRECK, in which DT treatment depletes

solely basophils or both basophils and mast cells, respectively¹⁴ (see Fig. E4A and B in the Online Repository at www.jacionline.org). To deplete ILCs *in vivo*, we utilized anti-Thy1.2 antibody. DT-treated Mas-TRECK mice showed a moderate reduction in the number of ILCs in the lung when compared to wild-type mice (Fig. E4C and D in the Online Repository at www.jacionline.org). Of note, we observed that the frequency of PD-L2⁺ DCs in the mLNs after PAO challenges was profoundly decreased in the DT-treated Mas-TRECK mice and, to a lesser extent, in the anti-Thy1.2-treated mice (Fig. 3A). Since DT treatment depleted both mast cells and basophils, we next asked which cell type is responsible for the induction of PD-L2⁺ DCs by comparing the PD-L2⁺ DCs in Bas-TRECK and Mas-TRECK mice. As shown in Figure 3B, we observed that the frequency and absolute cell number of PD-L2⁺ DCs were profoundly reduced in the mLNs of DT-treated Mas-TRECK, but not in the DT-treated Bas-TRECK, indicating that mast cells rather than basophils were required for the induction of PD-L2⁺ DCs in the airway. DT-treatment did not directly deplete PD-L2⁺ DCs *in vitro* (see Fig. E5 in the Online Repository at www.jacionline.org). The decrease of PD-L2⁺ DCs in the Mas-TRECK mice was evident as early as 24 hours after PAO/Ova intranasal challenge (see Fig. E5B in the Online Repository at www.jacionline.org). Similarly, we observed that the accumulation of PD-L2⁺ DCs in the mLNs of *Rag1*^{-/-} Mas-TRECK mice was significantly attenuated by anti-Thy1.2 and DT-treatment, indicating that the induction of PD-L2⁺ DCs by ILCs and mast cells was independent of adaptive immunity (see Fig. E5C and D in the Online Repository at www.jacionline.org). In parallel with these findings, we observed a significant reduction in the frequency and number of PD-L2⁺ DCs in mast cell-deficient *Kit*^{W-sh/W-sh} mice compared with those of wild-type mice upon PAO/Ova challenges, which was fully restored by adoptive transfer of bone marrow-derived mast cells (Fig.

3C).

To further determine the role of mast cells in inducing Th2 cell responses and allergic inflammation in the airway, we intranasally challenged wild-type, Bas-TRECK, and Mas-TRECK mice with PAO/Ova every other days for 5 times. All mice were treated with DT prior to allergenic challenges to deplete respective cell population and to normalize the effect of the toxin treatment. Differential cell analysis of BAL fluid showed a profound reduction in the numbers of eosinophil and, to less extent, neutrophil in Mas-TRECK mice compared with those of wild-type and Bas-TRECK mice (see Fig. E6A in the Online Repository at www.jacionline.org). Similarly, the lungs of Mas-TRECK mice showed reduced infiltration of inflammatory cells, goblet cell hyperplasia, and mucus production compared with the other two groups. Consistent with reduced allergic phenotypes in the airway, CD4⁺ T cells from the BAL fluid of Mas-TRECK mice showed significantly lower frequencies of IL-5⁺ and IL-13⁺ cells, and significantly higher frequencies of IL-17⁺ or IFN γ ⁺ cells than the other two groups, leading to the decrease in the ratios of Th2 to Th1 or to Th17 cells (see Fig. E6B/C/D in the Online Repository at www.jacionline.org). These results together demonstrate that mast cells play a crucial role in inducing the accumulation of PD-L2⁺ DCs and Th2 cell-mediated allergic inflammation in the airway in response to protease allergens.

Protease allergen-induced IL-13 from mast cells generates PD-L2⁺ DCs

To investigate the mechanism by which mast cells promote the induction of PD-L2⁺ DC, we asked if the increase of PD-L2⁺ DCs is mediated by soluble factor(s) from mast cells. To answer

this question, we prepared ‘mast cell-conditioned media (CM)’ by obtaining culture supernatant
 of mast cells that had been previously stimulated with PAO, as illustrated in Fig. 4A. Of note,
 addition of the CM prepared with native PAO, but not with heat-inactivated PAO, significantly
 increased the frequency of PD-L2⁺ population among MHCII⁺CD11c⁺ bone marrow-derived DCs
 (Fig. 4B). To identify the soluble factor(s) responsible for the increase of PD-L2⁺ DC in the
 conditioned media, we measured the levels of innate cytokines and found that PAO stimulation
 remarkably triggered the production of IL-13 and, to a lesser extent, IL-25 and TSLP from mast
 cells (Fig. 4C), while the level of IL-4 was undetectable (<2 pg/ml). Importantly, neutralizing
 antibodies to IL-13 almost completely abolished the increase of PD-L2⁺ DCs induced by the CM
 (Fig. 4D). Moreover, recombinant IL-13 was sufficient to induce PD-L2⁺ population, indicating
 that mast cell-derived IL-13 is necessary and sufficient to induce PD-L2⁺ DCs by PAO in this *in*
vitro experimental setting.

The levels of IL-13 in the lung increased as early as 12 hours after a single intranasal challenge
 with PAO (Fig. 4E). To determine if mast cells play any role in the early production of IL-13 after
 intranasal protease allergens *in vivo*, we intranasally challenged PAO into wild-type and mast
 cell-deficient mice. We found that the induction of IL-13 by protease allergens was significantly
 diminished in the DT-treated Mas-TRECK mice as well as in the *Kit*<sup>W^{-sh}/W^{-sh} mice (Fig. 4F and G),
 indicating that mast cells were required for the induction of IL-13 in the lung upon protease
 allergens *in vivo*. These results demonstrate that protease allergens stimulated mast cells to
 produce IL-13 which, in turn, induced the accumulation of PD-L2⁺ DC in the airway *in vivo*.</sup>

Fibrinogen-cleavage products generated by protease allergens trigger IL-13 from mast cells via TLR4

We next sought to explore the molecular mechanism by which protease allergens activate mast cells to produce IL-13. Mast cells are located in the subepithelial region in steady state²⁴, and thus unlikely have a direct access to exogenous protease allergens during the primary exposure. Hence we hypothesized that a soluble factor generated by protease allergens at epithelium reaches mast cells in the subepithelial region and stimulates the release of IL-13 during the primary exposure to the allergens. As shown in Figure 5A, the production of IL-13 from mast cells upon PAO stimulation was significantly decreased when TLR4-deficient mast cells were used (Fig. 5A). Moreover, the accumulation of PD-L2⁺ DCs in the mLN after intranasal PAO/Ova was significantly attenuated in TLR4^{-/-} mice (Fig. 5B), indicating a role for TLR4 in inducing IL-13 production from mast cells and in inducing PD-L2⁺ DCs PAO. In this regard, a recent report revealed that fibrinogen cleavage product (FCP) generated by proteases can act as an endogenous TLR4 ligand on airway epithelial cells and pulmonary macrophages¹⁶. We therefore, hypothesized that FCP generated from fibrinogen by enzymatic activity of PAO might act as an endogenous TLR4 ligand that mediates the production of IL-13 from mast cells and subsequent accumulation of PD-L2⁺ DCs. Of note, FCPs stimulated mast cells to produce IL-13 *in vitro*, which was significantly abolished when TLR4-deficient mast cells were used (Fig. 5C). In addition, conditioned media prepared by stimulating mast cells with FCPs remarkably increased PD-L2⁺ DCs whereas conditioned media prepared with TLR4-deficient mast cells failed to do so (Fig. 5D). Conditioned media prepared by stimulation with native fibrinogen also failed to induce PD-L2⁺ DCs, indicating that proteolytic cleavage of fibrinogen by protease allergen was required

for the PD-L2⁺-inducing capacity of FCPs (Fig. 5D).

To examine the role of FCPs *in vivo*, we intranasally administered FCPs into C57BL/6 mice and found a remarkable increase in the level of IL-13 in the lungs as well as in the number of PD-L2⁺ DCs in the mLN, which was significantly blunted in TLR4-deficient mice (Fig. 5E and F). It is noteworthy that the induction of IL-13 and PD-L2⁺ DCs in the TLR4-deficient mice was significantly but not completely abolished, suggesting that a pathway other than TLR4 might be involved in the sensing of FCPs by mast cells. To determine if FCP can promote Th2 cell responses *in vivo*, we intranasally challenged C57BL/6 mice with FCP/Ova or Ova alone, and found that mice challenged with FCP/Ova exhibited higher numbers of inflammatory cells in the BAL fluid as well as higher frequencies of Th2 cells, but not Th1 and Th17 cells, in the lungs than those challenged with Ova alone (see Fig. E6E and F in the Online Repository at www.jacionline.org). To confirm the role of mast cells in the FCP-mediated increase of IL-13 and PD-L2⁺ DCs *in vivo*, we intranasally injected FCPs into DT-treated Mas-TRECK or wild-type mice. Compared to wild-type mice, the amount of IL-13 in the lungs as well as the number of PD-L2⁺ DCs in the mLNs was profoundly attenuated in the DT-treated Mas-TRECK (Fig. 5G and H), indicating that mast cells played a dominant role for the FCP-mediated increase of IL-13 and PD-L2⁺ DCs *in vivo*. Collectively, these results demonstrate that FCPs generated by protease allergens stimulate mast cells to produce IL-13 through, at least in part, TLR4 to induce PD-L2⁺ DCs in the airway.

Recombinant IL-13 restores PD-L2⁺ DC population and subsequent Th2 cell responses in

the absence of mast cells *in vivo*

To directly determine if the decreased IL-13 in the mast cell-deficient mice accounts for the defective PD-L2⁺ population and Th2 cell responses *in vivo*, we intranasally administered recombinant IL-13 or saline into the DT-treated Mas-TRECK mice at the time of PAO/Ova challenges and examined PD-L2⁺ population and Th2 cell responses. As depicted in Figure 6A, intranasal IL-13 profoundly increased the frequency and number of PD-L2⁺ DCs in the mLNs of the DT-treated Mas-TRECK. In addition, IL-13 significantly increased the number of eosinophils in BALF, leading to the increase of total leukocytes in the airway (Fig. 6B). Moreover, IL-13 significantly increased the frequency of Th2 cells while decreasing that of Th1 cells in the DT-treated Mas-TRECK mice, leading to Th2 cell-dominant responses in the airway over Th1 cell and Th17 cell (Fig. 6C and D). Taken together, these results demonstrate that IL-13 treatment restores the induction of PD-L2⁺ DCs and subsequent Th2 cell-dominant responses in the airway in mice lacking mast cells *in vivo*.

DISCUSSION

Proteolytic activity of allergens has been thought to be necessary for inducing Th2 cell-mediated inflammation in the airway; however, the molecular mechanism governing the initiation of Th2 cell responses by protease allergens has been incompletely understood. We demonstrate that protease allergens induced an increase of PD-L2⁺ DC population in the mLN in an IL-13-dependent manner through stimulating mast cells, and that this DC subset was specialized for inducing Th2 cell responses. We also demonstrate that FCPs generated by protease allergens can stimulate mast cells to produce IL-13 in a TLR4-dependent fashion. Intranasal administration of FCPs resulted in the increase of PD-L2⁺ DCs in the mLN. Moreover, transfer of antigen-loaded PD-L2⁺ DCs efficiently induced Th2 cell responses *in vivo*. Hence, our findings unveil ‘protease allergens→FCP→TLR4→mast cell→IL-13→PD-L2⁺ DC’ axis as a fundamental molecular basis that contributes to Th2 cell dominant responses in the airway in response to protease allergens.

The role of basophils and mast cells in the initiation of Th2 cell immunity has been controversial. In the present study, all indicators of type 2 inflammation were profoundly diminished in the absence of mast cells. Notably, PD-L2⁺ DCs were accumulated in the mLN upon intranasal protease allergens, which was profoundly abolished in mast cell-deficient mice, and reconstitution of mast cells almost completely restored PD-L2⁺ DC population in the airway in allergen-challenged mice. By contrast, we observed little changes in PD-L2⁺ DC population in DT-treated Bas-TRECK mice, indicating little, if any, role of basophils in inducing the DC subset *in vivo*. Hence, mast cells rather than basophils are required for the induction of Th2-favorable PD-L2⁺ DCs in our experimental setting. On the other hand, basophils have been shown to be

activated to produce IL-4 in an animal model of papain-induced airway inflammation²⁵. Thus it is likely that both mast cells and basophils are involved in sensing protease allergens, probably depending on the type of allergens. It will be interesting to investigate if basophils also trigger the accumulation of PD-L2⁺ DCs. cKit⁺ DCs have been proposed to mediate Th2 cell responses in allergic asthma^{26, 27}; however, we observed that PD-L2⁺ DCs did not express cKit (data not shown). A recent study by Lewkowich *et al.* has shown that anti-PD-L2 did not affect Th2 cytokine production in an HDM-induced asthma model, although such treatment attenuated airway hyperresponsiveness²⁸. In parallel with this study, we observed that anti-PD-L2 had little effect on Th2 cytokine production from effector/memory T cells stimulated with PD-L2⁺ DCs. Thus, it is likely that PD-L2 does not exert critical role in Th2 cell responses, but is a reliable marker for Th2-favorable DCs. On the other hand, the surface phenotypes and Th2 cell-stimulatory function of PD-L2⁺ DC induced by protease allergens in the airway appeared to be similar to those found in the skin draining lymph nodes induced by subcutaneous injection of protease allergens¹³. Hence, it is possible that the induction of PD-L2⁺ DC is a common mechanism for the generation of Th2 cell responses by protease allergens. Further studies will be needed to identify the molecular mechanism by which PD-L2⁺ DCs induce and/or maintain Th2 cell responses in the airway. Mast cells reside close to the blood vessel in dermis and act as critical mediators of Th2 cell responses via IgE-mediated degranulation of inflammatory molecules^{29, 30}. It would be intriguing to study if mast cells also have a role in inducing PD-L2⁺ DCs in the skin-associated lymphoid tissues.

Protease allergens are known to induce cytokine release from airway epithelial cells, mast cells and basophils^{4, 31, 32}. Although house dust mite extracts have been widely used to induce allergic asthma in mice, we utilized protease from *Aspergillus oryzae* as a model protease allergen since it contains little endotoxin (1.7×10^{-3} endotoxin unit (EU)/ μg)¹⁶ and intranasal injection of PAO with ovalbumin has been used to trigger Th2 dominant allergic asthma in mice^{15, 20, 21}. We found that mast cell-conditioned media, prepared by protease allergens, increased the frequency of PD-L2⁺ DC in an IL-13 dependent fashion. This observation raised the possibility that protease allergens directly stimulate mast cells to produce IL-13 to trigger PD-L2⁺ DCs. However, mast cells are typically located in the sub-epithelial region of the airway and thus not immediately accessible to allergens at the beginning of allergens exposure⁷. A recent study showed that protease allergens yield FCPs which can stimulate airway epithelial cells and macrophages through TLR4 to induce the expression of *IL13Ra1* and *Muc5AC*¹⁶. Fibrinogen is abundant in circulation and also present in fetal bovine serum, although not as much as unclotted plasma¹⁶. Interestingly, we found that FCPs induced IL-13 production from mast cells *in vitro*, and the accumulation of PD-L2⁺ DCs in the mLN *in vivo*. These results strongly suggest that protease allergens stimulate mast cells indirectly by generating FCP to produce IL-13 in a TLR4-dependent manner. By contrast, since we observed that the production of IL-13 by PAO was not completely abolished in TLR4-deficient mast cells, it is possible to surmise that PAO activates mast cells independently of TLR4 and FCP. Further studies will be needed to clarify the mechanism for TLR4-independent activation of mast cells by protease allergens, the molecular characteristics of FCP and downstream signaling pathways leading to the release of IL-13.

Several studies demonstrated that ILC2s have a crucial role in Th2 cell immunity in animal models of helminth infection as well as allergic asthma^{22, 33, 34}. By contrast, a recent study showed that the induction of Th2 cell responses was relatively normal in the absence of ILC2s³⁵. We found that the size of ILC population in the lung after challenges with intranasal allergens was diminished in DT-treated Mas-TRECK mice, suggesting a role for mast cells in expanding ILCs in the airway. Depletion of ILCs by anti-Thy1.2 slightly reduced PD-L2⁺ DC population *in vivo*, although the magnitude of reduction was greater in mice lacking mast cells. Hence, we propose that mast cells play two distinct roles in the inductive phase of type 2 inflammation; (i) by producing IL-13 through recognition of FCP via TLR4, and (ii) by expanding ILC2 cells that also contribute to IL-13 production, although it is not clear how mast cells regulate the expansion of ILCs. Collectively, the present study indicates that mast cells and ILCs cooperatively promote PD-L2⁺ DC in the airway.

In summary, the present study unveils the essential role of ‘protease-FCP-mast cell-IL-13-PDL2⁺ DC’ pathway as a fundamental molecular basis for the initiation of Th2 cell responses during protease allergen-induced airway inflammation. Targeting this pathway might pave the development of therapeutic strategies for the treatment of allergic asthma.

Acknowledgments

We thank Dr. Gustavo Martinez (Rosalind Franklin University) for critical comments and suggestions on the manuscript and Drs. Shizhuo Akira (Osaka University) and Seung-Yong Sung (Seoul National University) for providing TLR4-deficient mice.

Author Contributions

M.C., J.E.L., G.C., Y.J.P., I.S., B.S.K., J.O.K. H.W.S., and R.K. performed the animal studies and *in vitro* experiments; H.S.K., W.S.C., C.Y.K., S.T., and M.K. provided experimental materials and advised the experiments; M.C., J.E.L. and Y.C. designed the experiments and wrote the manuscript.

465 **Competing Financial Interest**

466 The authors declare no competing financial interest.

FIGURE LEGENDS

Figure 1. Induction of PD-L2⁺ DCs in the mLN in response to intranasal protease allergens.

(A) The frequencies of PD-L2⁺ DCs in the mLNs of mice challenged with intranasal PAO/Ova every other days for five times (n=5). The mice were analyzed one day after the last challenge. **(B)** The frequencies of PD-L2⁺ DCs in the mLNs after single intranasal challenge with PAO/Ova (n=5). **(C)** The frequencies of PD-L2⁺ DCs in the mLNs at 24 hours after single challenge of Ova, heat-inactivated PAO (HI-PAO), PAO, or PAO/Ova (n=4). **(D)** Linear regression analysis between PD-L2⁺ DCs in the lung and IL-4 or IL-13-producing CD4⁺ T cells in the lung, or eosinophils in the BAL fluid. Data are mean $\pm$ SEM and represent two or three independent experiments. **, $p < 0.01$; *** $p < 0.001$ vs 0 hour (B), or between two groups.

Figure 2. Phenotypic and functional characterization of PD-L2⁺ DCs induced by intranasal protease allergens.

(A-C) Wild-type mice were intranasally challenged with PAO/Ova every other day for five times and the profile of PD-L2⁻ or PD-L2⁺ DCs in the mLN were analyzed at 18 hours after the last challenge. **(A)** The histogram and geometric MFI of indicated molecules on PD-L2⁻ (shaded grey) or PD-L2⁺ DCs (solid black line) (n=3). **(B)** The expression of *Nos2*, *Il12a*, *Arg1*, *Pdcd1lg2*, *Il13ra1* and *Il4ra* transcripts in the PD-L2⁻ DCs and the PD-L2⁺ DCs (triplicated). **(C)** Production of the indicated cytokines by effector/memory CD4⁺ T cells after stimulation with PD-L2⁻ DCs or PD-L2⁺ DCs for 3 days in the presence of anti-CD3 antibody (triplicated). **(D)** The frequencies of the indicated cytokine-producing CD4⁺ T cells in the lung of wild-type recipients of Ova-loaded PD-L2⁻, PD-L2⁺ DCs or PBS (control) (n=4). Data are mean

± SEM and represent at least two independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Figure 3. Mast cells and ILCs play a crucial role for the increase of PD-L2⁺ DCs in allergen

challenged mice. (A) The frequency of PD-L2⁺ DCs in the mLNs of wild-type and Mas-TRECK mice after single intranasal challenge with PAO or PBS (control) (n=3-4). All mice were treated with DT, and additionally given anti-Thy1.2 or control IgG prior to the intranasal challenge. **(B, C)** The frequency and number of PD-L2⁺ DCs among B220⁺F4/80⁺CD11c⁺MHCII⁺ DCs in the mLN of DT-treated wild-type, Bas-TRECK (BT) and Mas-TRECK (MT) mice (n=4-5) **(B)** or in the wild-type, mast cell-deficient mice *Kit*^(w-sh/w-sh) and mast cell-reconstituted *Kit*^(w-sh/w-sh) mice after intranasal PAO/Ova every other day for five times (n=3) **(C)**. Data are mean ± SEM and represent at least two independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Figure 4. Protease-stimulated mast cells induce PD-L2⁺ DCs in the bone marrow-derived

dendritic cell via IL-13. (A) The scheme of bone marrow-derived mast cell (BMMC) stimulation by protease and the generation of BMMC-conditioned medium (CM). **(B)** The frequencies of PD-L2⁺ DCs after culturing bone marrow-derived dendritic cells with CM prepared by stimulating mast cells with PAO, heat-inactivated PAO (HI-PAO), or PBS (control). **(C)** The amounts of IL-13, IL-25 and TSLP in the CM after stimulation with the indicated reagents (triplicated). **(D)** The frequency of PD-L2⁺ DCs among bone marrow-derived DCs after stimulation with CM in the presence or absence of anti-IL-13 (5 µg/ml), or with recombinant murine IL-13 (rmIL-13, 1 µg/ml). **(E)** Kinetics of IL-13 in the lung homogenates after a single

dose of intranasal PAO/Ova (n=5). **(F-G)** The quantity of IL-13 in the lung of wild-type and Mas-TRECK mice (F) or wild-type and *Kit*^(w-sh/w-sh) mice (G) at 18 hour after allergen challenge (n=5). Data are mean $\pm$ SEM and represent at least two independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ between two groups (A-D, F-G), or vs 0 hour (E).

Figure 5. Fibrinogen cleavage products generated by protease allergens induce IL-13 and PD-L2⁺ DCs in a TLR4 and mast cell-dependent manner. **(A)** The production of IL-13 from wild-type (WT) or TLR4-deficient (TLR4 KO) BMMC after stimulation with PAO (filled bar) or HI-PAO (open bar) (triplicated). **(B)** The frequency of PD-L2⁺ DCs in the mLN of wild-type or TLR4 KO mice after single intranasal challenge with PAO (n=3). **(C)** The production of IL-13 from wild-type or TLR4 KO mast cells after stimulation with FCP (filled bar) or heat-inactivated FCP (open bar) (triplicated). **(D)** The frequency of PD-L2⁺ BMDCs after culturing bone marrow-derived DCs with CM from WT (open circle) or TLR4 KO (closed circle) mast cells stimulated with fibrinogen or FCP (triplicated). **(E)** The amount of IL-13 in the lung extracts of wild-type or TLR4 KO mice at 24 hour after single intranasal injection of FCP or PBS (control) (n=4). **(F)** The frequency and number of PD-L2⁺ DCs in the mLN of wild-type or TLR4 KO mice at 24 hour after single intranasal injection of FCP (n=3-4). **(G-H)** The amount of IL-13 in the lung extracts of wild-type or Mas-TRECK mice (G), and the frequency and number of PD-L2⁺ DCs in the mLN of wild-type or Mas-TRECK mice (H) at 24 hour after single intranasal injection of FCP (n=4). Data are mean $\pm$ SEM and represent at least two independent experiments. **, $p < 0.01$; ***, $p < 0.001$.

Figure 6. Administration of IL-13 drives Th2 cell responses in the absence of mast cells. (A)

The frequencies and numbers of PD-L2⁺ DCs in the mLN of DT-treated wild-type or Mas-TRECK mice at 24 hours after single intranasal PAO/Ova with or without recombinant IL-13 (n=3). **(B-D)** DT-treated Mas-TRECK mice were intranasally injected with PAO/Ova with recombinant IL-13 (rmIL-13) or PBS every other day for five times. Differential counts of leukocytes in BAL fluid **(B)**, the frequencies of the indicated cytokine-producers among CD4⁺ T cells in the lung **(C)**, and the ratios of Th2 cell to Th17 cell or to Th1 cell **(D)** were analyzed at 18 hour after the last challenge (n=4-5). Data are mean $\pm$ SEM and represent two independent experiments. *, p < 0.05; **, p < 0.01; ***, p < 0.001.

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EXTENDED FIGURE LEGENDS

FIG E1. Gating strategy for DC, ILC and CD4⁺ T cell analysis. Cells were first gated on size and singularity followed by FSC and SSC. **(A)** The cells were further gated on B220⁻ and F4/80⁻ to exclude B cells and macrophages, and CD11c⁺ and MHCII⁺ cells were defined as DCs. **(B-C)** For ILC or CD4⁺ T cell analysis, CD90.2⁺Lineage⁻ cells or CD4⁺ cells were gated from CD45.2⁺ cells. Among CD4⁺ T cells, IL-4- and/or IL-5-producers (Th2), IL-17A-producers (Th17), and IFN γ -producers (Th1) were further analyzed by intracellular staining.

FIG E2. Expression of PD-L2 on CD11b⁺ and CD103⁺ DCs. **(A)** PD-L2 expression on CD11b⁺ and CD103⁺ DCs from the mLN of mice in mice given 5 times challenges of PAO/Ova every other day (triplicated). **(B)** FACS sorted PD-L2⁻ and PD-L2⁺ DCs were co-cultured with CD62L^{lo}CD44^{hi}CD25⁻ effector/memory CD4⁺ T cells for 3 days after incubation with 20 μ g of anti-PDL2 antibody for 2 hours. The level of IL-5 and IL-13 in culture supernatant was evaluated by ELISA (triplicated). Data are mean $\pm$ SEM and represent one experiment of two independent experiments. **, p<0.01; ***, p<0.001.

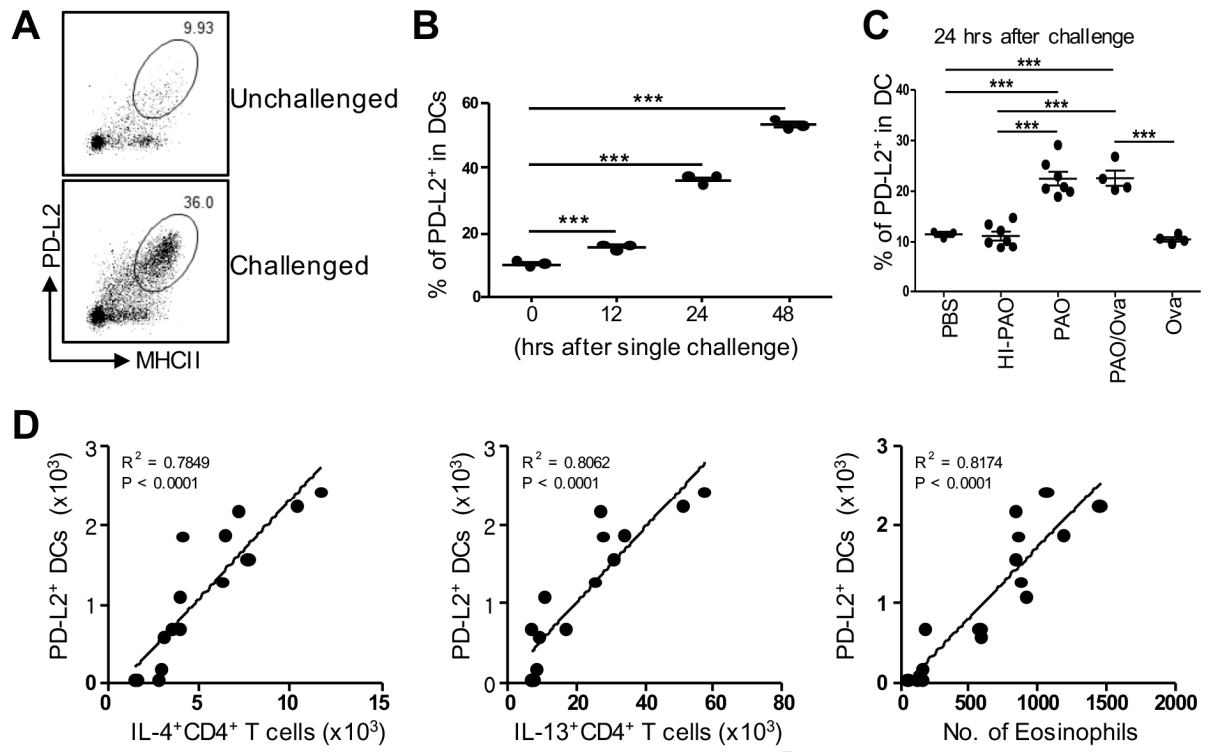
FIG E3. Depletion of CD301b⁺ DCs attenuated Th2 cell responses in the airway. **(A)** The frequency of PD-L2⁺ DCs in the mLNs of DT-treated wild-type or Mgl2-DTR mice after intranasal challenges with PAO/Ova (n=3-4). **(B)** The frequencies of IL-4-, IL-5-, IL-13-, IL-17A and IFN γ -producing CD4⁺ T cells in the lung. **(C)** The amounts of Th2 cytokines after stimulation of lymphoid cells from the mLN with Ova. Data are mean $\pm$ SEM and represent three independent experiment. *, p < 0.05; **, p < 0.01; ***, p < 0.001.

FIG E4. Depletion of mast cells, basophils and ILC. (A and B) Wild-type, Bas-TRECK or Mas-TRECK mice were i.p. injected with diphtheria toxin (DT) every day for 3 days. One day after the last injection, bone marrow cells and peritoneal cavity cells were analyzed for the presence of basophil (CD49b⁺FcεRI⁺) (A) and mast cell (c-kit⁺FcεRI⁺) (B), respectively. **(C-D)** Wild-type and Mas-TRECK mice were i.p. injected with anti-Thy1.2 antibody or isotype antibody (IgG2b). 24 hours after single PAO/Ova challenge, the frequency (C) and the number (D) of ILCs (CD90.2⁺Lineage⁻ gated from CD45.2⁺ cells) in lungs were analyzed. Data are mean ± SEM and represent two independent experiment. ***, $p < 0.001$.

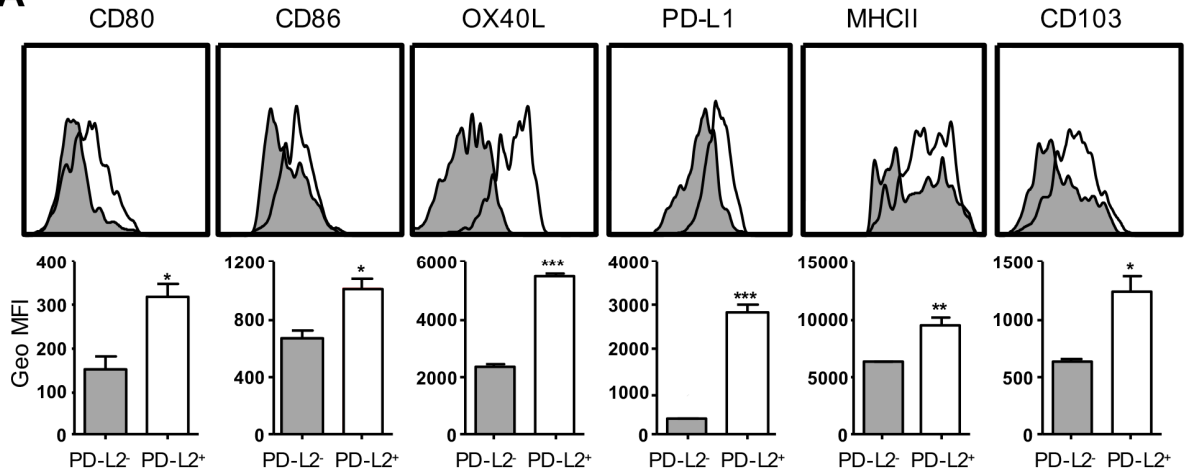
FIG E5. Defective accumulation of PD-L2⁺ DCs in the mLNs of mast cell-depleted mice after intranasal protease allergens. (A) BMDCs of wild-type or Mas-TRECK mouse were treated with various doses of DT. After 24 hour, BMDCs were analyzed for the PD-L2⁺ expression (triplicated). **(B-CD)** The frequency of PD-L2⁺ DCs in the mLNs of Mas-TRECK (B), *Rag1*^{-/-} Mas-TRECK (C) or anti-Thy1.2-treated *Rag1*^{-/-} Mas-TRECK (D) mice (n=3-4). Mas; Mas-TRECK. Data are mean ± SEM and represent two independent experiments. **, $p < 0.01$; ***, $p < 0.001$.

FIG E6. Development of allergic airway inflammation by PAO/Ova or FCP/Ova. (A-D) DT-treated wild-type, Bas-TRECK and Mas-TRECK mice were intranasal injected with PAO/Ova in total of 5 times every other day (n=4-6). **(A)** Differential count of leukocytes in the BAL fluid. **(B)** Histochemical analysis of the lung of the indicated mice. **(C)** The frequencies of the indicated

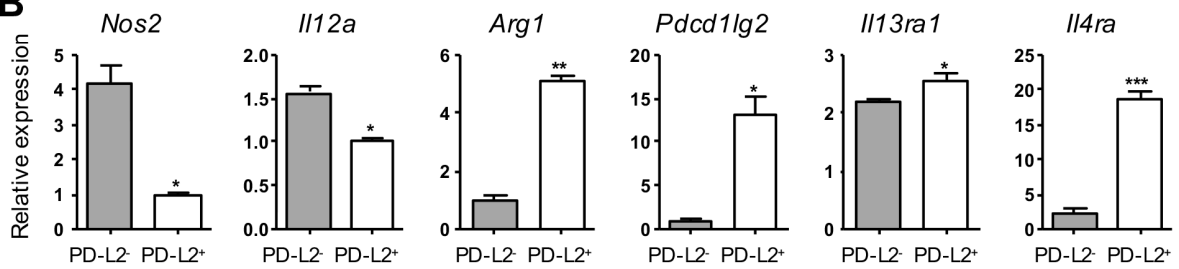
676 cytokine producers among CD4⁺ T cells in the BAL fluid. **(D)** The ratios of Th2 cell to Th17 cell
677 or Th2 cell to Th1 cell. BT; Bas-TRECK, MT; Mas-TRECK. **(E-F)** Differential cell count in the
678 BAL fluid **(E)** and the frequencies of the indicated cytokine-producers among CD4⁺ T cells in the
679 BAL fluid **(F)** in wild-type mice given four times of intranasal Ova with or without FCP (n=4).
680 Data are mean $\pm$ SEM and represent at least two independent experiments. *, $p < 0.05$; **, $p <$
681 0.01 ; ***, $p < 0.001$.



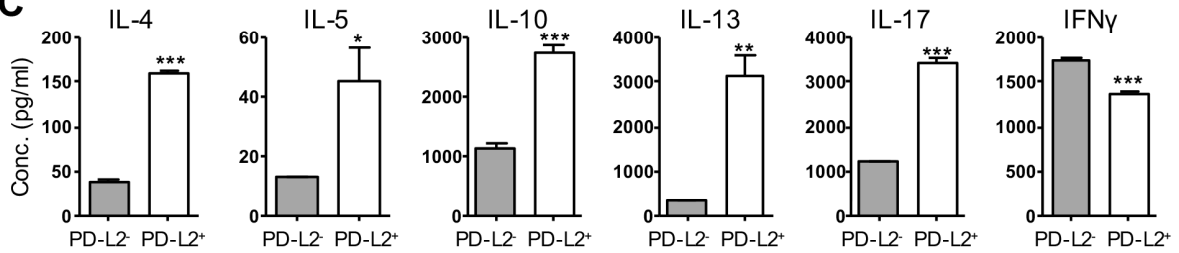
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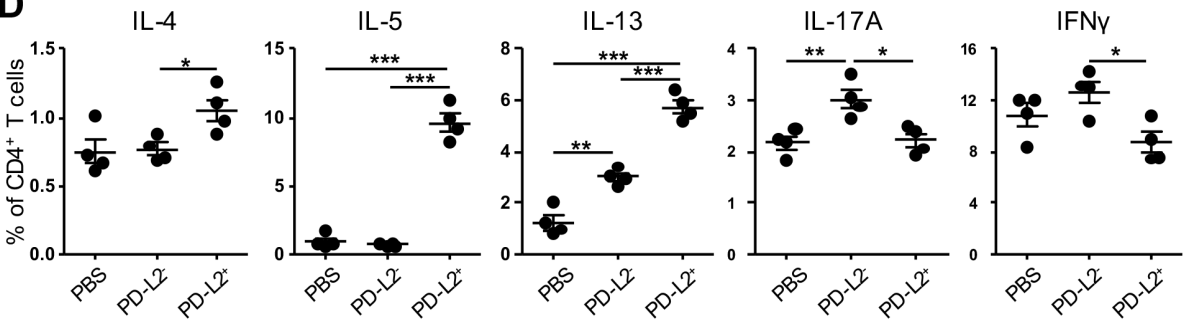
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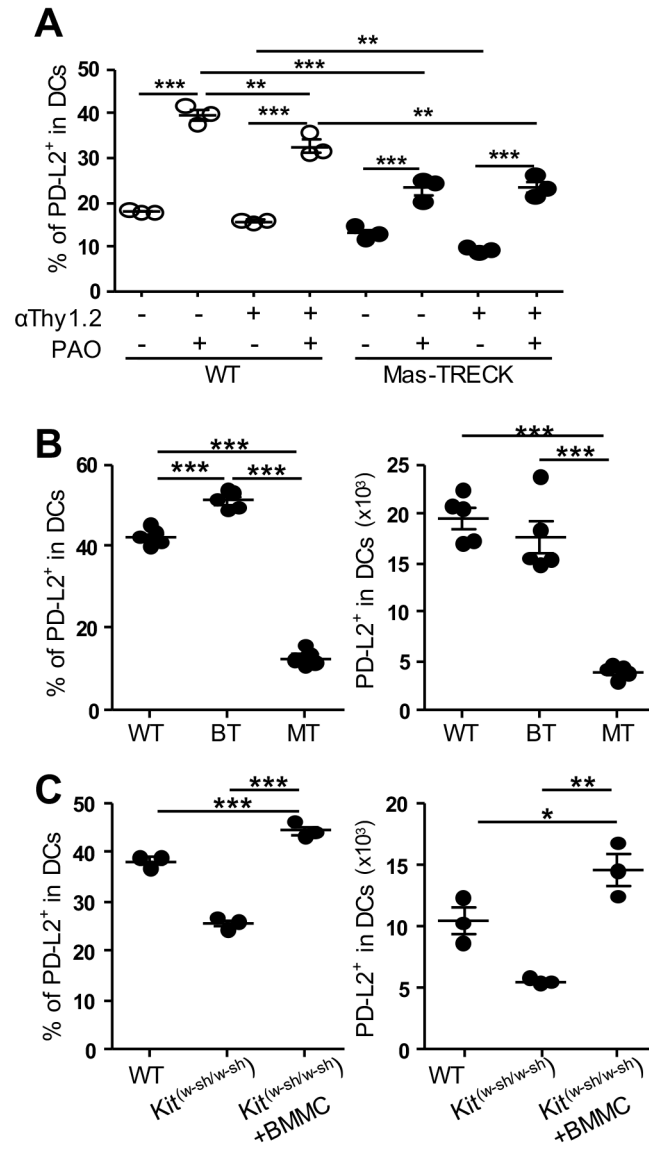


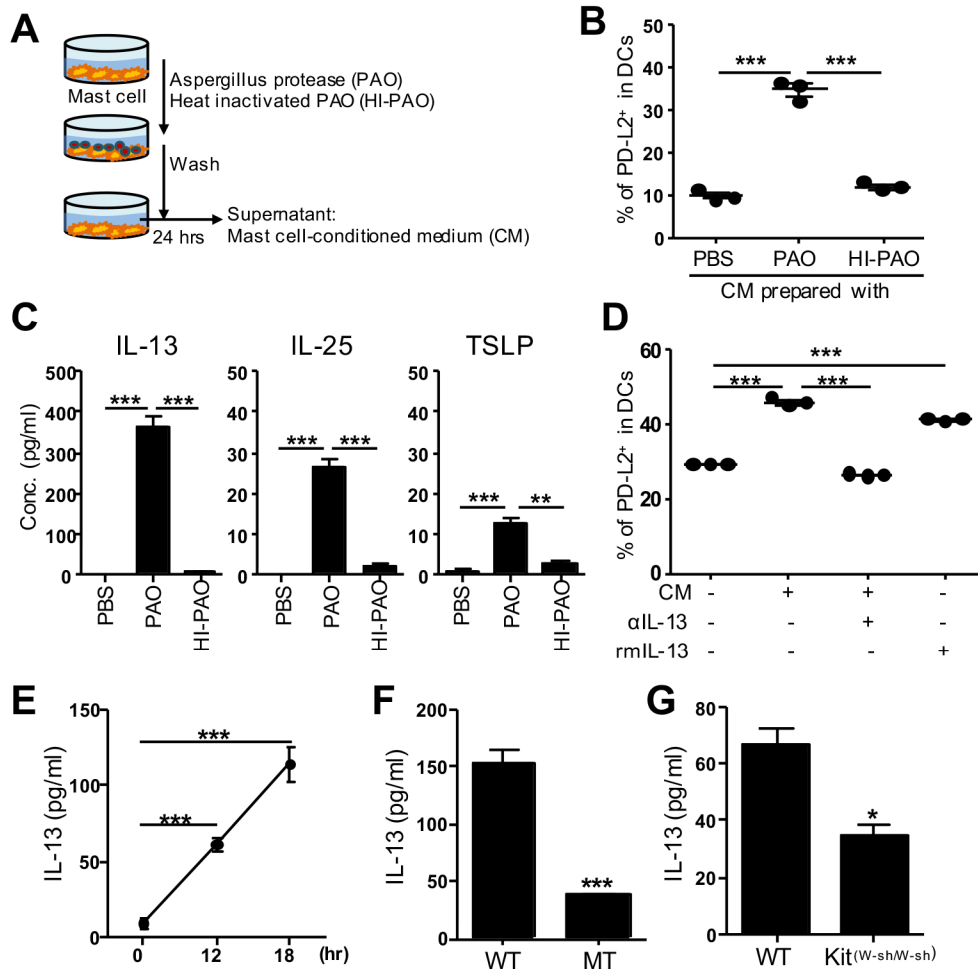
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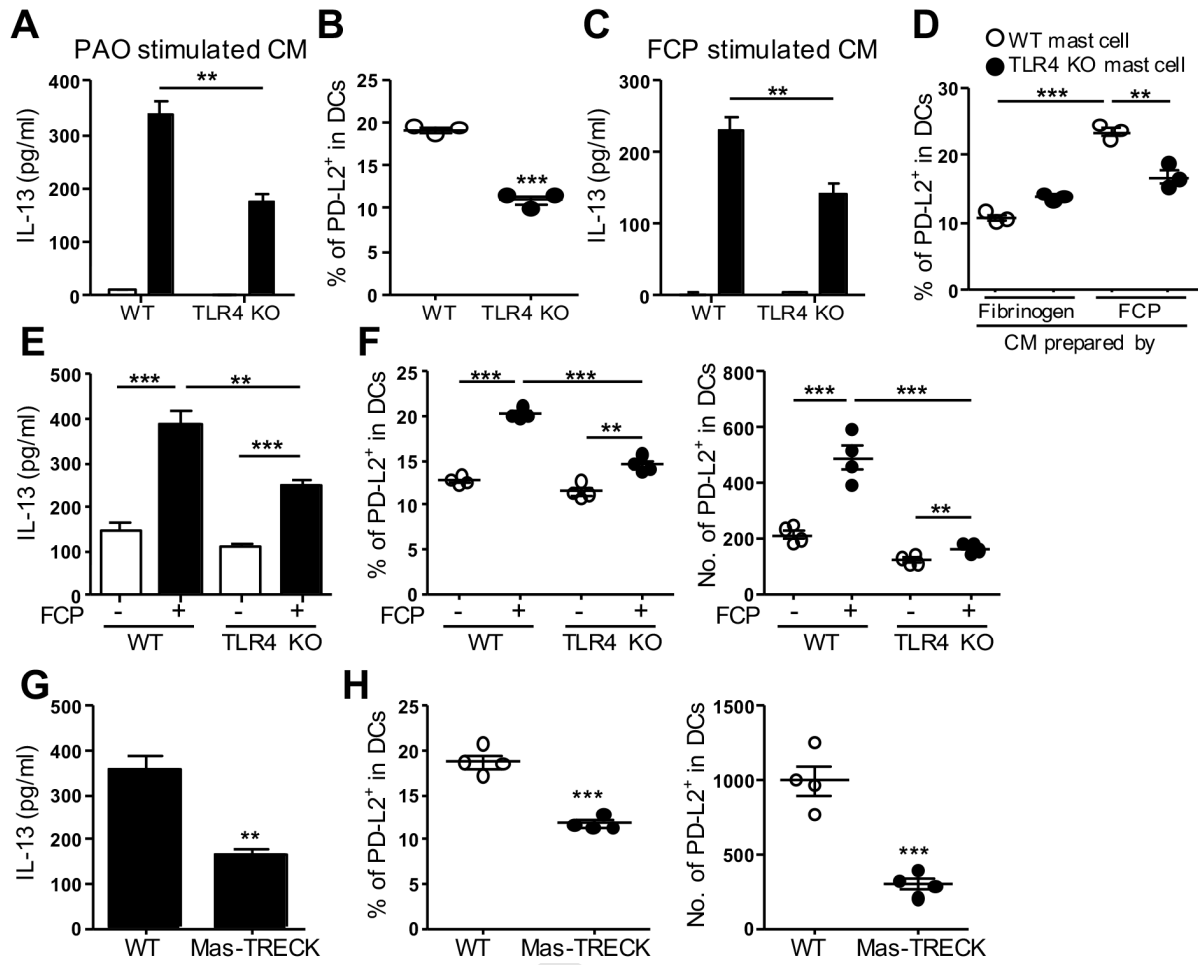


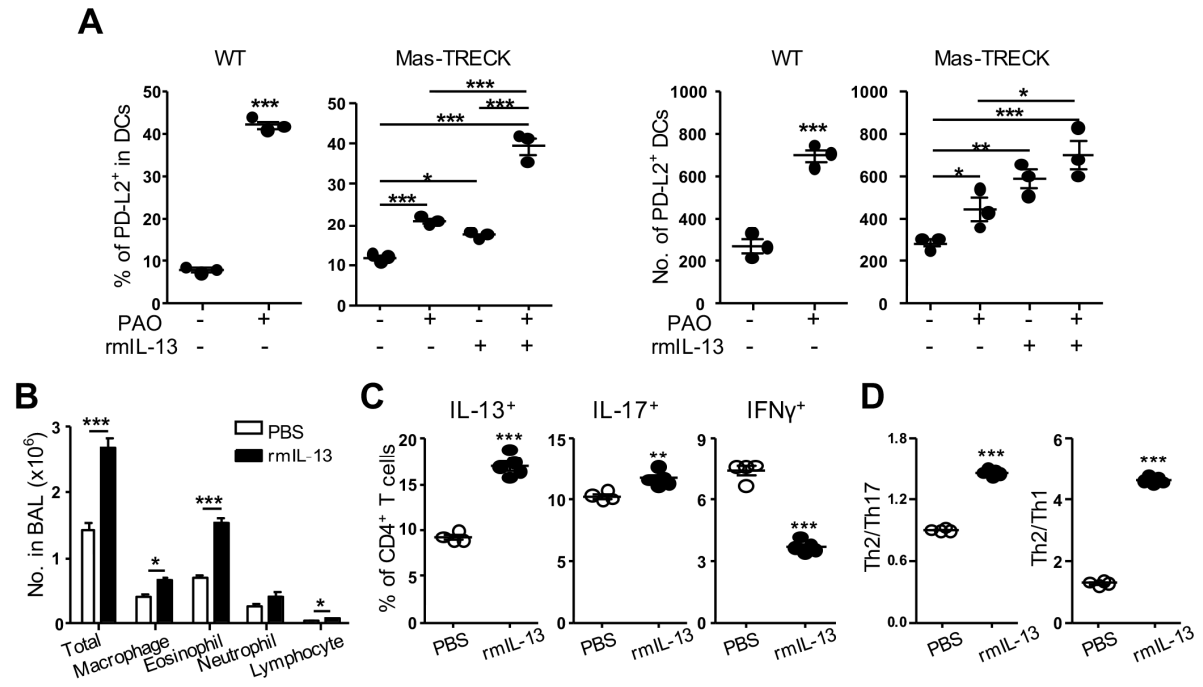
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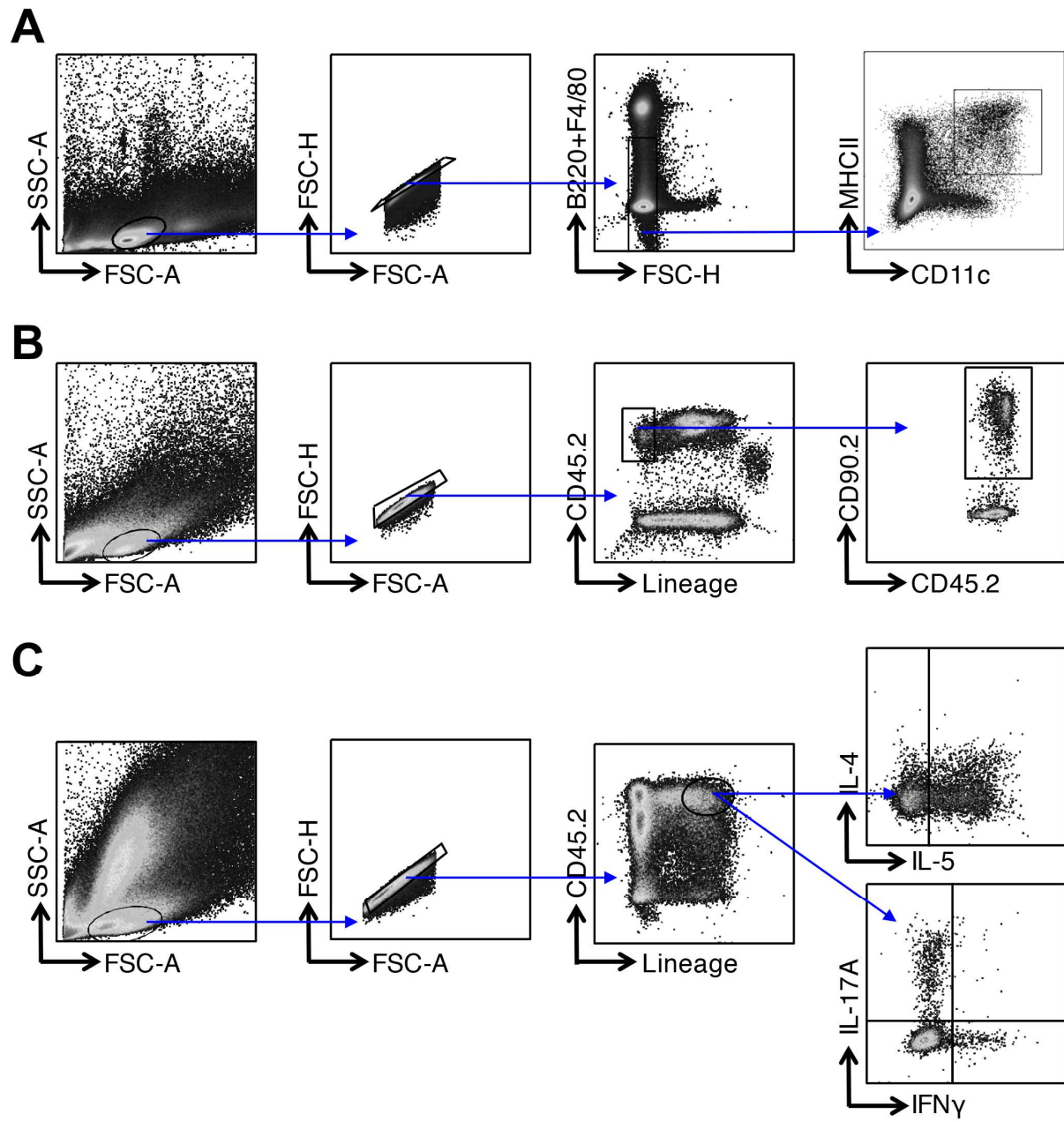


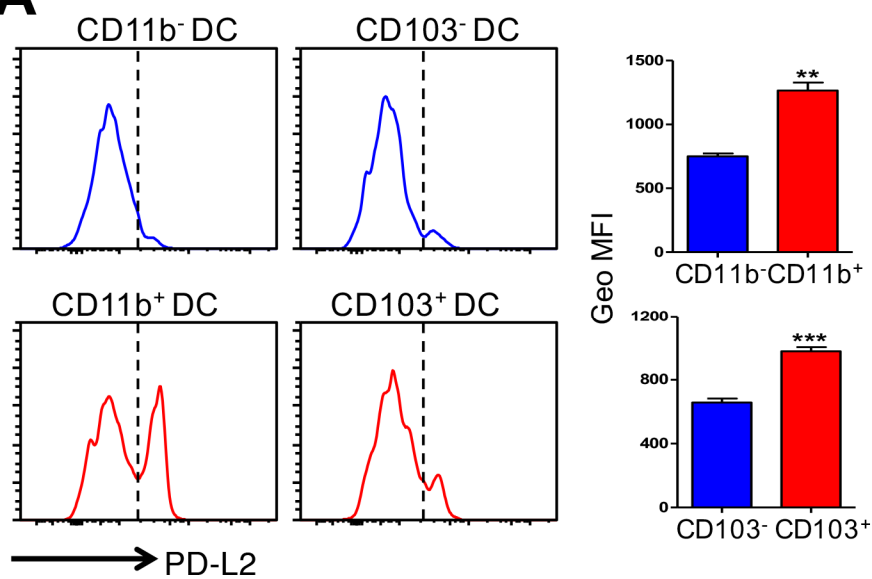










A**B**