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DOCK8 deficiency causes a skewing to type 2 immunity in the gut with expansion of group 2 innate lymphoid cells

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ABSTRACT

Dedicator of cytokinesis 8 (DOCK8) is a guanine nucleotide exchange factor (GEF) for Cdc42. In humans, homozygous or compound heterozygous deletions in *DOCK8* cause a combined immunodeficiency characterized by various allergic diseases including food allergies. Although group 2 innate lymphoid cells (ILC2s) contribute to the development of allergic inflammation by producing interleukin (IL)-5 and IL-13, the role of ILC2s in DOCK8 deficiency has not been fully explored. With the use of cytometry by time-of-flight (CyTOF), we performed high-dimensional phenotyping of intestinal immune cells and found that DOCK8-deficient (*Dock8*$^{-/-}$) mice exhibited expansion of ILC2s and other leukocytes associated with type 2 immunity in the small intestine. Moreover, IL-5− and IL-13−producing cells markedly increased in *Dock8*$^{-/-}$ mice, and the majority of them were lineage-negative cells, most likely ILC2s. Intestinal ILC2s expanded when DOCK8 expression was selectively deleted in hematopoietic cells. Importantly, intestinal ILC2 expansion was also observed in *Dock8*^{VAGR} mice having mutations in the catalytic center of DOCK8, thereby failing to activate Cdc42. Our findings indicate that DOCK8 is a negative regulator of intestinal ILC2s to inhibit their expansion via Cdc42 activation, and that deletion of DOCK8 causes a skewing to type 2 immunity in the gut.

1. Introduction

Innate lymphoid cells (ILCs) represent a novel family of hematopoietic cells that serve protective roles in innate immune responses to infectious microorganisms [1,2]. ILCs consist of three distinct groups: group 1 ILCs (ILC1s), group 2 ILCs (ILC2s) and group 3 ILCs (ILC3s) [1,2]. ILC1s express the transcription factor T-bet and produce interferon-γ (IFN-γ), and ILC3s require RORγt and produce interleukin (IL)-22 [1,2]. On the other hand, ILC2s are characterized by the expression of GATA3 and surface markers, such as T1/ST2 (IL33R), Sca-1, CD25 (IL-2R), and Thy1.2 (CD90.2) [3,4]. It is known that ILC2s produce a large amount of type 2 cytokines, including IL-5 and IL-13, in response to epithelial-derived cytokines (IL-25, IL-33, and TSLP) [3–5]. IL-5 secreted by tissue-resident ILC2s is particularly important for the development, recruitment and activation of eosinophils [6]. In addition, IL-13 has been reported to induce goblet cell hyperplasia, mucus overproduction, mast cell proliferation, and immunoglobulin E (IgE) secretion from B cells [7,8]. Several studies also showed that ILC2s play an important role in the development of atopic dermatitis, asthma and food allergy in mice and humans [9–11].

DOCK8 is a member of the evolutionarily conserved DOCK family proteins that function as guanine nucleotide exchange factors (GEFs) for Rho family of GTPases [12,13]. Although DOCK8 does not contain Dbl homology (DH) domain typically found in GEFs, DOCK8 binds to Cdc42 and mediates the GTP-GDP exchange reaction through the DOCK homology region 2 (DHR2) domain [12,13]. So far, much attention has been paid to the signaling and functions of DOCK8, because the bi-allelic loss-of-function mutations in *DOCK8* cause a combined immunodeficiency in humans [14]. DOCK8 immunodeficiency syndrome is characterized by early-

Abbreviations: CyTOF, Cytometry by time-of-flight; DH, Dbl homology; DHR2, DOCK homology region 2; DOCK8, Dedicator of cytokinesis 8; *Dock8*$^{-/-}$, DOCK8-deficient; GEF, Guanine nucleotide exchange factor; IFN-γ, Interferon-γ; IgE, Immunoglobulin E; IL, Interleukin; ILCs, Innate lymphoid cells; T_H2, Type 2 CD4$^+$ helper T; *t*-SNE, t-distributed stochastic neighbor embedding; viSNE, Visualization of t-distributed stochastic neighbor embedding.

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onset malignancy and persistent or recurrent skin, mucosal and respiratory infections, while DOCK8-deficienct patients also suffer from allergic diseases, such as atopic dermatitis, asthma and eosinophilic esophagitis with elevated serum IgE and eosinophilia [14–16]. Moreover, multiple food allergies are especially severe in DOCK8-deficient patients, often resulting in life-threatening food-induced anaphylaxis [14–16]. However, the underlying mechanisms of allergic diseases in DOCK8 deficiency has not been thoroughly investigated. In this study, we provide evidence that DOCK8 deficiency causes a skewing to type 2 immunity with expansion of ILC2s in the gut.

2. Materials and methods

2.1. Mice

DOCK8-deficient (*Dock8*$^{-/-}$) mice have been previously described [17], and *Dock8*$^{-/-}$ mice were backcrossed onto a C57BL/6J background for more than 9 generations prior to analyses. *Dock8*^{VAGR} mice, *Dock8*$^{flox/flox}$ mice, *Vav1-Cre* Tg mice, *Rorc-Cre* Tg mice, *Lyz2-Cre* KI mice, *Cd4-Cre* Tg mice, *Cd19-Cre* KI mice, *Itgax-Cre* Tg mice, and *Vil1-Cre* Tg mice have been previously described [18–26]. Male and female mice were used at 7–12 weeks of age. Age- and sex-matched littermate mice were used as controls. Mice were maintained in specific pathogen-free conditions in the animal facility of Kyushu University. The protocol of animal experiments was approved by the committee of Ethics on Animal Experiments, Kyushu University.

2.2. Mass cytometry (CyTOF)

CyTOF analysis of immune cells isolated from the lamina propria of small intestine was performed as described previously [27,28]. The used antibodies are listed in Supplemental Table 1. Detailed methods can be found in the Supplemental Methods.

2.3. Flow cytometry

Intracellular cytokine staining and flow cytometric analysis of intestinal ILC2s were performed as described previously [18]. The used antibodies are listed in Supplemental Table 2. ILC2s were identified as Lineage (CD11b, CD11c, Gr-1, CD3ε, CD4, CD8α, B220, FcεR1α, NK1.1)$^{-}$Sca-1^{+}T1/ST2^{+} cells. Detailed methods can be found in the Supplemental Methods.

2.4. Gene expression analysis

Intestinal lamina propria cells were isolated from the small intestine of *Dock8*$^{+/-}$ or *Dock8*$^{-/-}$ mice, and total RNA extraction, reverse transcription and real-time PCR analysis were performed as described previously [18]. The expressions of target genes were normalized with *Hprt* expression for each sample. The following PCR primers were used: *Hprt*, 5′–CTGGTGAAAAGGACCTCTCG–3′ and 5′–TGAAGTACTCATTATAG

TCAAGGGCA–3’; *Il5*, 5′–AAGAGAAGTGTGGCGAGGAGA–3′ and 5′– CACCAAGGAA

CTCTTGCAGGTAA–3’; *Il13*, 5′–CGCAAGGCCCCCACTAC–3′ and 5′–TGGCGAAACA

GTTGCTTTGT–3’.

2.5. Immunoblotting

Intestinal epithelial cells were isolated from the small intestine and immunoblotting was performed as described previously [19]. Detailed methods can be found in the Supplemental Methods.

2.6. Statistical analysis

Statistical analyses were performed using Prism 7.0 (GraphPad Software, San Diego, CA, USA). The data were initially tested with a Kolmogorov-Smirnov test for normal distribution. Parametric data were analyzed using a two-tailed unpaired Student’s *t*-test and nonparametric data were analyzed with a two-tailed Mann-Whitney test when two groups were compared. P-values less than 0.05 were considered significant.

3. Results

3.1. DOCK8-deficient mice exhibit expansion of immune cells associated with type 2 immunity in the small intestine

To comprehensively understand the immune profiles in the gut from *Dock8*$^{-/-}$ mice, we performed high-dimensional phenotyping of intestinal immune cells by using CyTOF [29]. In this study, after isolation of the intestinal lamina propria cells from steady-state *Dock8*$^{+/-}$ or *Dock8*$^{-/-}$ mice, the samples were stained with 29 metal-conjugated antibodies. The antibody panel was designed to encompass the major innate and adaptive immune cell populations (Supplemental Table. 1). We also used a DNA-intercalator containing two iridium isotopes (191Ir and 193Ir) and platinum-based reagent cisplatin (194 Pt) to distinguish singlets from doublets, and live cells from dead cells, respectively (Supplemental Fig. 1A). CyTOF data were processed using manual gating and visualization of t-distributed stochastic neighbor embedding (viSNE) algorithms for the purpose of simultaneously resolving the many distinct immune populations (Supplemental Fig. 1B).

After gating on singlets, live CD45^{+} cells in intestinal lamina propria were subdivided into 16 populations. Cell populations on the viSNE plot were identified by differential expression of individual lineage markers (Fig. 1A and B, and Supplemental Fig.2A). These data revealed significant differences in the distribution of immune cells between *Dock8*$^{+/-}$ and *Dock8*$^{-/-}$ mice (Fig. 1C and Supplemental Fig. 2B–K). Consistent with our previous study [18], the percentage of ILC3s decreased in *Dock8*$^{-/-}$ mice compared to *Dock8*$^{+/-}$ mice (Fig. 1D). By contrast, *Dock8*$^{-/-}$ mice exhibited expansion of immune cells associated with type 2 immunity. For example, the percentages of eosinophils, mast cells, T_H2 cells, and ILC2s significantly increased in *Dock8*$^{-/-}$ mice compared to *Dock8*$^{+/-}$ mice (Fig. 1E–H). These results suggest that DOCK8 deficiency causes a skewing to type 2 immunity in the gut even at steady state.

3.2. IL-5– and IL-13–producing lineage-negative cells increase in DOCK8-deficient mice

IL-5 and IL-13 are involved in the induction of type 2 immune responses and allergic inflammation [4,5]. It has been reported that IL-5 and IL-13 promote tissue eosinophilia and goblet cell hyperplasia, respectively [6–8,30]. In light of the importance of these type 2 cytokines, we performed real-time PCR for the gene expression of *Il5* and *Il13* in the small intestine. Real-time PCR analysis revealed that total lamina propria cells in *Dock8*$^{-/-}$ mice exhibited notably enhanced expression of *Il5* and *Il13*, as compared with *Dock8*$^{+/-}$ mice under steady-state conditions (Fig. 2A). IL-5 and IL-13 are known to be produced by ILC2s and T_H2 cells [3–5,31]. To examine what cell type produces these type 2 cytokines, we analyzed IL-5– and IL-13–producing cells in the intestinal lamina propria by intracellular cytokine staining. The percentages of IL-5– and IL-13–producing cells markedly increased in *Dock8*$^{-/-}$ mice, and the majority of IL-5– and IL-13–producing cells were lineage-negative cells, most likely ILC2s (Fig. 2B). In addition, among lineage^{-} cells, about half of IL-13–producing cells

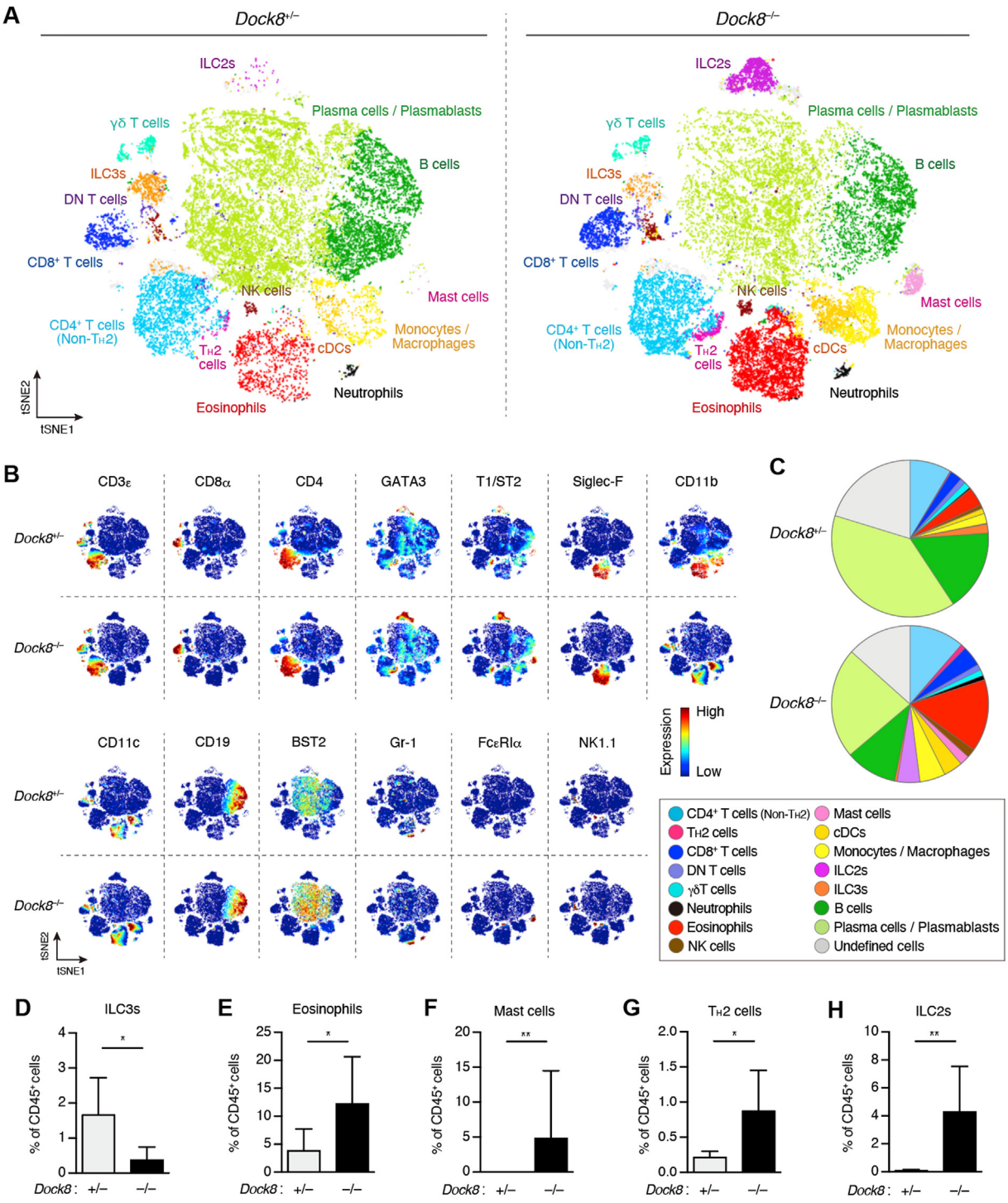

Fig. 1. DOCK8-deficient mice exhibit expansion of cells associated with type 2 immunity in the small intestine. **(A)** FlowSOM analyses of immune cells isolated from the intestinal lamina propria of steady-state *Dock8*$^{+/-}$ or *Dock8*$^{-/-}$ mice. After gating on singlets, live CD45^{+} cells were concatenated from 6 mice per group with equal sampling (23,419 total cells per group), and clustered using the viSNE on the expression of the following parameters: CD3ε, CD8α, CD4, GATA3, T1/ST2, Siglec-F, CD11b, CD11c, CD19, Gr-1, FcεR1α, NK1.1, CD90.2 (Thy1.2), TCRγδ. The distinct metaclusters are shown in different colors. **(B)** t-SNE plots overlaid with the expression heatmaps of individual markers (red and blue indicate high and low expression, respectively). **(C)** Small intestine composition as the mean percentage of each population per group. Colors indicate cell subsets as shown in **(A)**. **(D–H)** Frequency of intestinal immune cell populations as percentage of live CD45^{+} cells from the viSNE clustering outlined in **(A)**. Data (n = 6) are expressed as mean ± SD. *P < 0.05; **P < 0.01; ns, not significant by two-tailed unpaired Student's t-test **(D, G, H)** and two-tailed Mann-Whiteney test **(E, F)**.

co-produced IL-5 in *Dock8*$^{-/-}$ mice (Supplemental Fig. 3A). Importantly, the percentages of IL-5− and IL-13−co-producing cells to the total lineage^{-} cells were much higher than to the total lineage^{+} cells (Supplemental Fig. 3B). These results indicate that ILC2s rather than T_H2 cells have the potential to produce both IL-5 and IL-13 in the small intestine of DOCK8-deficient mice.

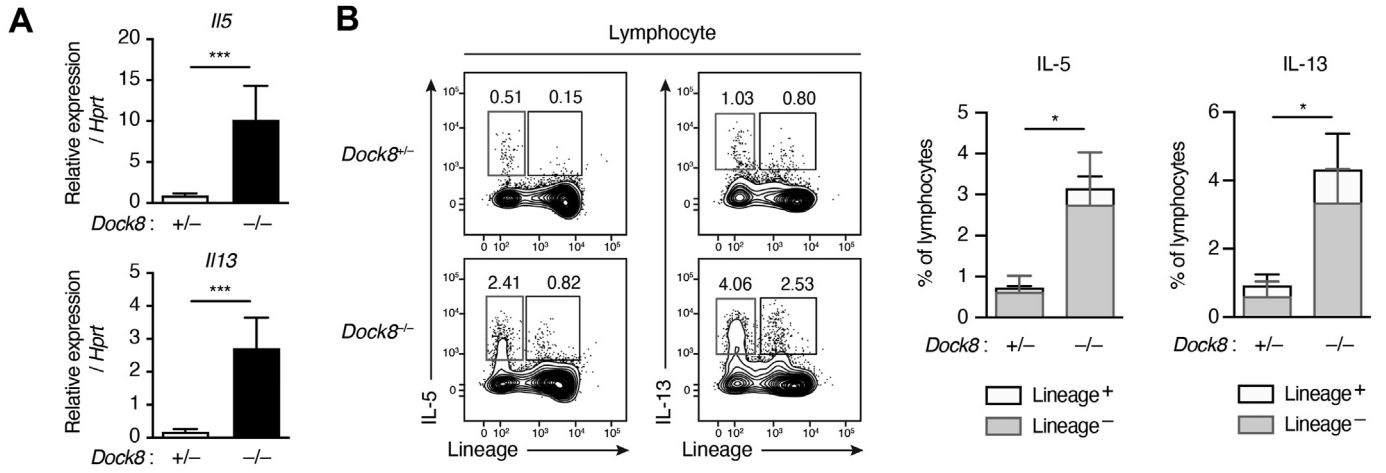

Fig. 2. IL-5− and IL-13−producing cells increase in DOCK8-deficient mice. (A) Real-time PCR analyses for *Il5* and *Il13* gene expressions in total lamina propria cells isolated from *Dock8*$^{+/-}$ or *Dock8*$^{-/-}$ mice. Each expression was normalized with *Hprt* expression, and data (n = 6) are expressed as mean ± SD. **(B)** Flow cytometric analyses for IL-5− and IL-13−producing cells in the intestinal lamina propria. The percentages of IL-5− and IL-13−producing cells to the total lymphocytes were compared between them. The following were used as lineage markers: CD11b, CD11c, Gr-1, CD3ε, CD4, CD8α, B220, FcεR1α, NK1.1. Data (n = 4) are expressed as mean ± SD. *P < 0.05; ***P < 0.001 by two-tailed unpaired Student's t-test **(A)** and two-tailed Mann-Whiteney test **(B)**.

3.3. DOCK8 deficiency in hematopoietic cells increases intestinal ILC2s

Having found that more IL-5− and IL-13−producing lineage-negative cells exist in *Dock8*$^{-/-}$ mice than *Dock8*$^{+/-}$ mice, we confirmed the frequency and the number of ILC2s in the intestinal lamina propria by flow cytometric analysis. Consistent with the CyTOF data, the percentages of Lineage^{-}Sca-1^{+}T1/ST2^{+} ILC2s in total lineage^{-} populations and their absolute numbers significantly increased in *Dock8*$^{-/-}$ mice at steady state (Fig. 3A). DOCK8 is expressed not only in hematopoietic cells but also in non-hematopoietic cells including intestinal epithelial cells [13,19]. To identify the specific cell types that require DOCK8 expression for ILC2 expansion, we used the gene-targeted mice harboring *loxP*-flanked exon3 of *Dock8* allele (*Dock8*$^{flox/flox}$) [19]. Since ILC2s are known to proliferate in response to epithelial-derived cytokines, we compared intestinal ILC2s between Villin (*Vil1*)-*Cre Dock8*$^{flox/flox}$ mice and control mice. Although DOCK8 expression was deleted in intestinal epithelial cells isolated from *Vil1-Cre Dock8*$^{flox/flox}$ mice (Supplemental Fig. 4A), the frequency and the number of intestinal ILC2s were comparable between *Vil1-Cre Dock8*$^{flox/flox}$ mice and control mice (Fig. 3B).

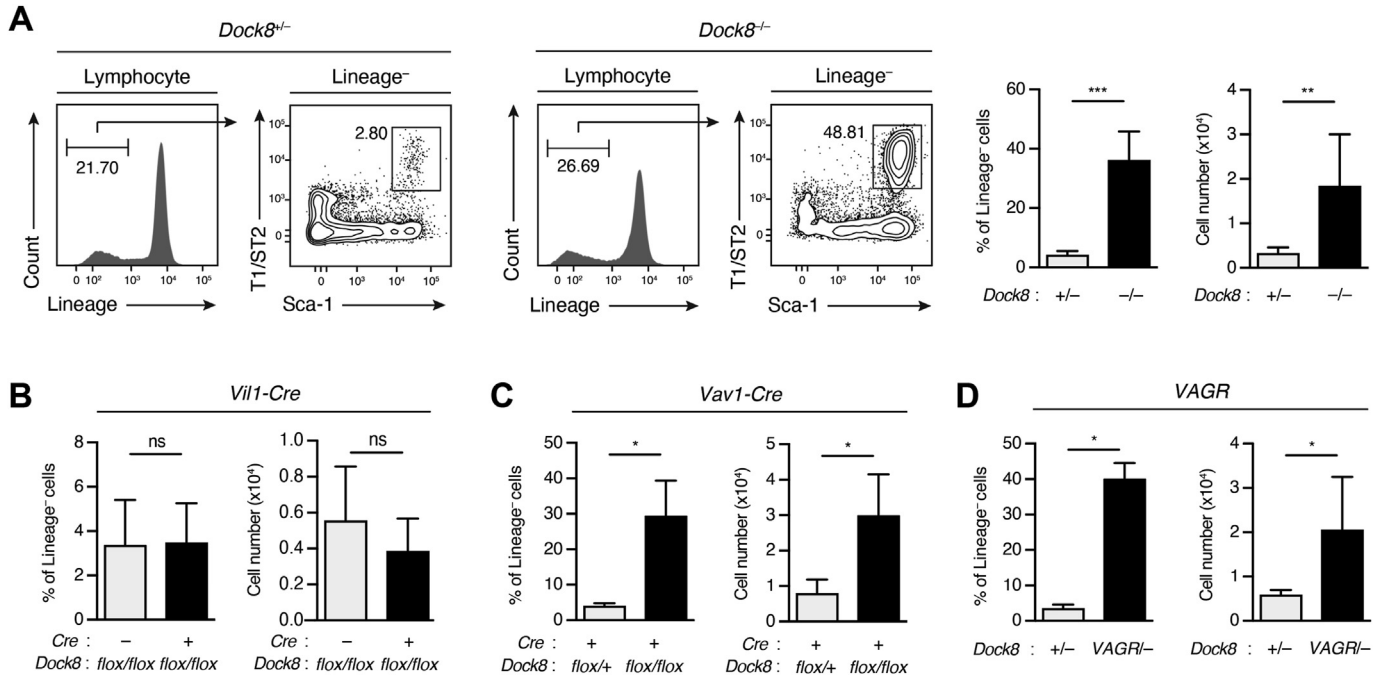

Fig. 3. DOCK8 deficiency increases intestinal ILC2s in a leukocyte-dependent manner. (A–E) Flow cytometric analyses for ILC2s (Lineage^{-}Sca-1^{+}T1/ST2^{+}) in the intestinal lamina propria. The percentage of ILC2s to the total lineage^{-} cells and their absolute numbers were compared between *Dock8*$^{+/-}$ and *Dock8*$^{-/-}$ mice **(A)**, *Dock8*$^{flox/flox}$ and *Vil1-Cre Dock8*$^{flox/flox}$ mice **(B)**, *Vav1-Cre Dock8*$^{flox/+}$ and *Vav1-Cre Dock8*$^{flox/flox}$ mice **(C)**, *Dock8*$^{+/-}$ and *Dock8*$^{VAGR/-}$ mice **(D)**. Data (**A**, n = 6; **B–D**, n = 4) are expressed as mean ± SD. *P < 0.05; **P < 0.01; ***P < 0.001; ns, not significant by two-tailed unpaired Student's t-test **(A)** and two-tailed Mann-Whiteney test **(B–D)**.

Vav1 protein is a DH type GEF for Rho family GTPases and specifically expressed in hematopoietic cells [20]. To examine whether the lack of DOCK8 expression in hematopoietic cells is required for ILC2 expansion, we analyzed Vav1 (*Vav1*)-*Cre Dock8*$^{flox/flox}$ mice and found that the frequency and the number of ILC2s increased in those mice (Fig. 3C). In contrast, genetic deletion of DOCK8 in RORγt$^+$ cells, LysM$^+$ cells, CD11c$^+$ cells, CD4$^+$ cells, or CD19$^+$ cells alone did not affect intestinal ILC2 expansion (Supplemental Fig. 4B–F). These data suggest that DOCK8 protein negatively regulates ILC2 expansion in a hematopoietic cell-dependent manner.

3.4. DOCK8-Cdc42 signaling controls the expansion of intestinal ILC2s

Our previous studies demonstrated that DOCK8 regulates immune responses through GEF activity-dependent or -independent mechanisms [13,32]. Therefore, we finally examined whether intestinal ILC2 expansion in DOCK8-deficient mice requires the GEF activity for Cdc42, by analyzing *Dock8*^{VAGR} mice. *Dock8*^{VAGR} mice have point mutations (valine-to-alanine and glycine-to-arginine mutations) in the catalytic center of DOCK8 DHR-2 domain and fail to activate Cdc42 [18]. We found that the percentages of ILC2s in total lineage$^-$ populations and their absolute numbers markedly increased in the intestinal lamina propria of *Dock8*$^{VAGR/-}$ mice (Fig. 3D). These results indicate that DOCK8 inhibits intestinal ILC2 expansion through Cdc42 activation.

4. Discussion

DOCK8-deficienct patients suffer from severe allergic diseases [14–16]. Although it is assumed that excessive and chronic activation of the type 2 immune responses exacerbates allergy symptoms in DOCK8 deficiency, the underlying mechanisms remain unknown. Recently, Tangye et al. have reported that DOCK8-deficient CD4$^+$ T cells are biased to a T$_H$2 effector fate at the expense of T$_H$1 and T$_H$17 cells [33]. However, considering that ILC2s are associated with the initiation and orchestration of allergic inflammation [5,6], we presumed that tissue-resident ILC2s also promote the development of allergic diseases in tissues or organs of DOCK8-deficient patients. To investigate comprehensively, we compared the immune profiles of lamina propria cells in the small intestine between *Dock8*$^{+/-}$ and *Dock8*$^{-/-}$ mice by performing CyTOF analyses. Our data revealed that various immune cells associated with type 2 immunity, such as eosinophils, mast cells, T$_H$2 cells, and ILC2s, markedly increased in *Dock8*$^{-/-}$ mice, compared with those in *Dock8*$^{+/-}$ mice. Moreover, we found that the majority of IL-5− and IL-13−producing cells was lineage-negative cells, most likely ILC2s. These results indicate that tissue-resident ILC2s would play a pivotal role in the initiation of type 2 immunity in DOCK8 deficiency through IL-5 and IL-13 secretion.

The epithelial cytokines IL-25, IL-33 and TSLP are powerful ILC2 activating ligands [3–5]. Based on the fact that intestinal epithelial cells express DOCK8 proteins [19], we crossed *Dock8*$^{flox/flox}$ mice with *Vil1-Cre* mice and analyzed the population of ILC2s in the small intestine. However, there was no difference between the population of intestinal ILC2s in *Vil1-Cre Dock8*$^{flox/flox}$ mice and those in control mice, indicating that DOCK8 deficiency in epithelial cells are irrelevant to ILC2 expansion under steady-state conditions. In contrast, intestinal ILC2s were expanded when DOCK8 expression was selectively deleted in Vav1$^+$ hematopoietic cells. Thus, DOCK8 expression in hematopoietic cells is required for inhibiting ILC2 expansion in the gut. Since intestinal ILC2 expansion was also observed in *Dock8*^{VAGR} mice, it is clear that this inhibitory activity is mediated through Cdc42 activation. It is known that Cdc42 binds to multiple effector molecules and acts as a master regulator of cell polarity in eukaryotic organisms ranging from yeasts to humans [34,35]. Moreover, recent evidence indicates that Cdc42 is involved in cell survival, proliferation, differentiation, and migration in mice [36–38]. Although the precise mechanisms remain to be elucidated, DOCK8-Cdc42 axis may be a potential target for controlling allergic responses induced by tissue-resident ILC2s.

More recently, Eken et al. have reported that DOCK8-deficient patients exhibited the reduction of ILC2s in the peripheral blood; however, they have not described the absolute numbers or frequency of ILC2s in tissues or organs, including skin, lungs and intestines [39]. Therefore, whether and how DOCK8 deficiency affects tissue-resident ILC2s in humans is important issue that should be investigated in future studies.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2021.04.094.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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