

Intrinsic functional defects of type 2 innate lymphoid cells impair innate allergic inflammation in promyelocytic leukemia zinc finger (PLZF)-deficient mice

Philip A. Verhoef, MD, PhD,^{a,b,c} Michael G. Constantinides, PhD,^a Benjamin D. McDonald, PhD,^a

Joseph F. Urban, Jr, PhD,^d Anne I. Sperling, PhD,^{a,b} and Albert Bendelac, MD, PhD^{a,e}

Chicago, Ill, and Beltsville, Md

Background: The transcription factor promyelocytic leukemia zinc finger (PLZF) is transiently expressed during development of type 2 innate lymphoid cells (ILC2s) but is not present at the mature stage. We hypothesized that PLZF-deficient ILC2s have functional defects in the innate allergic response and represent a tool for studying innate immunity in a mouse with a functional adaptive immune response.

Objective: We determined the consequences of PLZF deficiency on ILC2 function in response to innate and adaptive immune stimuli by using PLZF^{-/-} mice and mixed wild-type:PLZF^{-/-} bone marrow chimeras.

Methods: PLZF^{-/-} mice, wild-type littermates, or mixed bone marrow chimeras were treated with the protease allergen papain or the cytokines IL-25 and IL-33 or infected with the helminth *Nippostrongylus brasiliensis* to induce innate type 2 allergic responses. Mice were sensitized with intraperitoneal ovalbumin-alum, followed by intranasal challenge with ovalbumin alone, to induce adaptive Th2 responses. Lungs were analyzed for immune cell subsets, and alveolar lavage fluid was analyzed for ILC2-derived cytokines. In addition, ILC2s were stimulated *ex vivo* for their capacity to release type 2 cytokines. **Results:** PLZF-deficient lung ILC2s exhibit a cell-intrinsic defect in the secretion of IL-5 and IL-13 in response to innate stimuli, resulting in defective recruitment of eosinophils and goblet cell hyperplasia. In contrast, the adaptive allergic inflammatory response to ovalbumin and alum was unimpaired. **Conclusions:** PLZF expression at the innate lymphoid cell precursor stage has a long-range effect on the functional

properties of mature ILC2s and highlights the importance of these cells for innate allergic responses in otherwise immunocompetent mice. (J Allergy Clin Immunol 2015;■■■:■■■-■■■.)

Key words: Allergic mechanisms, innate lymphoid cells, mouse models

In recent years, an important role has emerged for lymphoid lineage cells with innate properties, such as innate lymphoid cells (ILCs), innate-like $\gamma\delta$ T cells, and invariant natural killer T (iNKT) cells.¹⁻⁵ ILCs develop from the common lymphoid progenitor (CLP) through an ILC precursor in the bone marrow, whereas iNKT cells and $\gamma\delta$ T cells are derived from the CLP but require thymic selection, although with a limited T-cell receptor (TCR) repertoire. Lymphoid-derived innate cells serve to exert and promote early defense to pathogens and allergens, as well as repair and regeneration at mucosal barriers. They also provide a link to adaptive responses in the setting of infection, allergies, and autoimmune disease.

In studying the development of innate-like iNKT cells, we identified the transcription factor promyelocytic leukemia zinc finger protein (PLZF) as a master regulator of this lineage and other distinct innate-like cells. Mice lacking the gene for PLZF have markedly reduced numbers of iNKT cells, and the few remaining iNKT cells have a naive rather than effector phenotype, whereas forced expression of PLZF in T cells induced an effector phenotype.^{6,7} Using a transgenic mouse that expresses a green fluorescent protein-Cre fusion protein under control of the endogenous PLZF gene, we observed that PLZF was also expressed by a newly identified common precursor to ILCs, although expression was subsequently downregulated in mature type 1 (ILC1), type 2 (ILC2), and type 3 (ILC3) ILCs.⁸ Although the frequency of ILCs was not altered in PLZF^{-/-} mice, it was markedly decreased in mixed bone marrow chimeras, where the mutant cells competed with the wild-type (WT) cells. Thus the current study was aimed at evaluating potential functional defects in ILC2s from PLZF^{-/-} mice.

We focused on the pulmonary ILC2-dependent innate type 2 inflammatory response in mice lacking PLZF. Pulmonary ILC2s respond to cytokines (thymic stromal lymphopoietin, IL-25, or IL-33) that are generated in the setting of helminth infection, viral infection, or inhalation of protease allergens, such as papain.⁹⁻¹¹ On stimulation, ILC2s can release large amounts of the type 2 cytokines IL-5 and IL-13, which in turn promote recruitment of eosinophils to the lung, airway hyperreactivity, mucous secretion, and airway smooth muscle thickening.¹²⁻¹⁴ Most published reports have used recombination-activating gene (Rag) 1-deficient mice depleted of ILCs or Rag2^{-/-}IL2rg^{-/-}

From ^athe Committee on Immunology; ^bthe Department of Medicine, Section of Pulmonary and Critical Care; ^cthe Department of Pediatrics, Section of Pediatric Critical Care; and ^dthe Department of Pathology, University of Chicago, and ^ethe Diet, Genomics, and Immunology Laboratory, Beltsville Human Nutrition Research Center, Agricultural Research Service, US Department of Agriculture, Beltsville.

Supported by National Institutes of Health grants T32 HL007605 (to P.A.V.) and R01HL118092, R01AI038339, and AI108643 (A.B.) and the University of Chicago Digestive Research Core Center Grant P30DK42086.

Disclosure of potential conflict of interest: P. A. Verhoef and A. Bendelac have received research support from the National Institutes of Health (NIH; R01 HL118092, R01 AI038339, and P30DK42086) and are employed by the University of Chicago. M. G. Constantinides has received research support from the NIH (R01 HL118092, R01 AI038339, and R01AI108643). B. D. McDonald has received research support from the NIH (R01 HL118092, R01 AI038339, and P30DK42086). The rest of the authors declare that they have no relevant conflicts of interest.

Received for publication April 30, 2015; revised July 19, 2015; accepted for publication July 31, 2015.

Corresponding author: Albert Bendelac, MD, PhD, Committee on Immunology, Department of Pathology, University of Chicago, 929 E 57th St, Gordon Center for Integrative Science W506, Chicago, IL 60637. E-mail: abendela@bsd.uchicago.edu. 0091-6749/\$36.00

© 2015 American Academy of Allergy, Asthma & Immunology
<http://dx.doi.org/10.1016/j.jaci.2015.07.050>

Abbreviations used

CLP:	Common lymphoid progenitor
DAPI:	4',6-Diamidino-2-phenylindole
ICOS:	Inducible costimulator
ILC:	Innate lymphoid cell
ILC2:	Type 2 innate lymphoid cell
IL-7R α :	IL-7 receptor α
iNKT:	Invariant natural killer T
KO:	Knockout
PLZF:	Promyelocytic leukemia zinc finger
PMA:	Phorbol 12-myristate 13-acetate
Rag:	Recombination-activating gene
TCR:	T-cell receptor
WT:	Wild-type

mice reconstituted with ILCs, and the exact role of ILC2s in nonimmunocompromised mice has not been well studied.^{9,11} We found that PLZF^{-/-} mice manifested a markedly impaired response to various innate type 2 inflammatory stimuli because of cell-intrinsic defects in ILC2 function. However, adaptive T_H2 responses remained intact. These data indicate that PLZF specifically controls the effector phenotype of ILC2s and suggest that the PLZF^{-/-} mouse is the first of its kind to have a functionally impaired innate lymphoid response but maintain an adaptive lymphocyte response.

METHODS

Mice

C57BL/6J, CD1d^{-/-}, and CD45.1 congenic mice (B6.SJL-Ptprca Pep3b/BoyJ) on the C57BL/6 background were purchased from The Jackson Laboratory (Bar Harbor, Me). *Plzf*^{-/-} mice were a gift from P. P. Pandolfi and were backcrossed to C57BL/6J for at least 10 generations. Animals were 4 to 10 weeks of age when analyzed and were compared with WT littermate control mice. Mice were housed in a specific pathogen-free environment at the University of Chicago, and experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee.

Induction of type 2 innate immune responses

Either papain (Calbiochem, San Diego, Calif), IL-25 (eBioscience, San Diego, Calif), or IL-33 (eBioscience) in PBS with carrier protein was administered intranasally on days 1, 2, and 3 to the anesthetized mice to induce type 2 immune responses, followed by harvest and analysis on day 4. As controls, PBS or heat-inactivated papain was used. For infection with *Nippostrongylus brasiliensis*, 500 L3 larvae were resuspended in 200 μ L of sterile PBS according to published protocols and injected intradermally into 6- to 8-week-old mice.¹⁵ On day 5 after infection, the mice were killed for analysis.

Bronchoalveolar lavage

Mice were anesthetized with ketamine/xylazine and immobilized. The trachea was cannulated with a 20-gauge blunt-end catheter, and 800 μ L of cold PBS was slowly infused into the lungs and withdrawn. This was repeated a total of 3 times, yielding approximately 2 mL of recovered saline per mouse. These samples were immediately centrifuged at 400g for 5 minutes to pellet alveolar cells. The supernatants were removed and frozen at -20°C for subsequent cytokine analysis, whereas the cells were resuspended in HBSS containing 0.25% BSA and 0.65 mg $\cdot$ L⁻¹ sodium azide for subsequent flow cytometric analysis.

Preparation of cell suspensions

For the isolation of lung leukocytes, mice were anesthetized with ketamine/xylazine, and approximately 1 mL of PBS (Sigma-Aldrich, St Louis, Mo) was injected into the right ventricle to perfuse the lung tissue. Pairs of lungs were diced and incubated in 5 mL of prewarmed RPMI 1640 (Cellgro; Mediatech, Manassas, Va) containing 0.01% DNase I (Roche, Mannheim, Germany) and 650 U/mL collagenase I (Worthington Biochemical, Lakewood, NJ) in a 37°C shaking incubator for 30 minutes. The digested tissue was passed through a 70- μ m filter, washed with 25 mL of RPMI/10% FCS, and centrifuged at 400g for 5 minutes. Cells were resuspended in 5 mL of 44% Percoll underlaid with 3 mL of 66% Percoll and centrifuged at 800g for 20 minutes with no brake. Lymphocytes were isolated from the interface, washed, and resuspended in HBSS containing 0.25% BSA and 0.65 mg $\cdot$ L⁻¹ sodium azide for subsequent flow cytometry.

Microscopy

For histology, lungs were perfused through a needle inserted in the right ventricle with cold PBS *in situ* before removal and fixation in 4% paraformaldehyde (histological grade; Thermo Fischer Scientific, Waltham, Mass) under a vacuum overnight and then transferred to PBS for 24 hours at 4°C. Lobes were sectioned sagittally, embedded in paraffin, and cut into 5- μ m sections before staining with periodic acid-Schiff. Histologic micrographs were taken with the FSX-100 microscope camera system (Olympus, Center Valley, Pa). Data were analyzed with ImageJ software (National Institutes of Health, Bethesda, Md).

Flow cytometry

Cell suspensions were incubated with purified anti-CD16/32 (clone 93) for 10 minutes on ice to block Fc receptors. Fluorochrome or biotin-labeled mAbs (clones denoted in parentheses) against B220 (RA3-6B2), CD3e (17A2), CD8 α (53-6.7), CD11b (M1/70), CD11c (N418), CD25 (PC61), Gr-1 (RB6-8C5), inducible costimulator (ICOS; C398.4A), IL-7 receptor α (IL-7R α)/CD127 (A7R34), NK1.1 (PK136), Sca-1 (D7), T1/ST2 (D1H9), and TCR β (H57-597) were purchased from BioLegend (San Diego, Calif); against CD4 (RM4-5 or GK1.5), CD19 (6D5), CD45.1 (A20), CD45.2 (104), Siglec-F (E50-2440), Thy1.2/CD90.2 (52-2.1), and IL-5 (TRFK5) were purchased from BD Biosciences (San Jose, Calif); and against IL-13 (JES-105A2) was purchased from eBioscience (San Diego, Calif). CD1d-PBS57 tetramer was from the National Institutes of Health tetramer facility. 4',6-Diamidino-2-phenylindole (DAPI; Molecular Probes, Eugene, Ore) was added to all live samples to exclude dead cells. Cells were run on an LSRII (BD Biosciences) or sorted with a FACSAria II (BD Biosciences) and analyzed with FlowJo software (TreeStar, Ashland, Ore). Collected events were gated on DAPI⁻CD45⁺ leukocytes, and doublets were excluded.

Lung ILC2s were identified as lineage⁻ (B220, CD3e, CD8 α , CD11b, CD11c, CD19, Gr-1, NK1.1, and TCR β) and positive for IL-7R α , Thy1.2, ICOS, Sca-1, and CD25. Eosinophils were gated as Siglec-F⁺CD11b⁺CD11c⁻ side scatter high. Alveolar macrophages were identified as Siglec-F⁺, CD11c⁺, autofluorescent on the fluorescent isothiocyanate channel, CD11b^{low}, forward scatter high, and side scatter high. CD4⁺ T cells were identified as TCR β ⁺, CD4⁺, CD8⁻, and B220⁻. CD8 T cells were identified as TCR β ⁺, CD4⁻, CD8⁺, and B220⁻. B cells were identified as B220⁺ and TCR β ⁻. iNKT cells were identified as TCR β ⁺, tetramer⁺, CD8⁻, and B220⁻.

For isolation of lung ILC2s, lung leukocytes were stained with allophycocyanin-conjugated anti-CD25 antibody, bound to anti-APC microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), and subjected to double-column enrichment on an autoMACS (Miltenyi Biotec). The CD25⁺ fraction was then sorted by using the strategy described above for identifying lung ILC2s.

For intracellular cytokine staining, lung leukocytes were isolated and incubated with phorbol 12-myristate 13-acetate (PMA; 50 ng/mL) and ionomycin (1 mmol/L) for 3 hours at 37°C in the presence of 1 mmol/L Brefeldin A (BD Biosciences). Nonadherent cells were then stained for identification of lung ILC2s, as noted above, followed by fixation and intracellular staining with the BD Cytofix/Cytoperm Kit. As a control, unlabeled anti-IL-5 or anti-IL-13 antibody was preincubated with the cells at a 25-fold excess to allow setting for positive and negative gates.

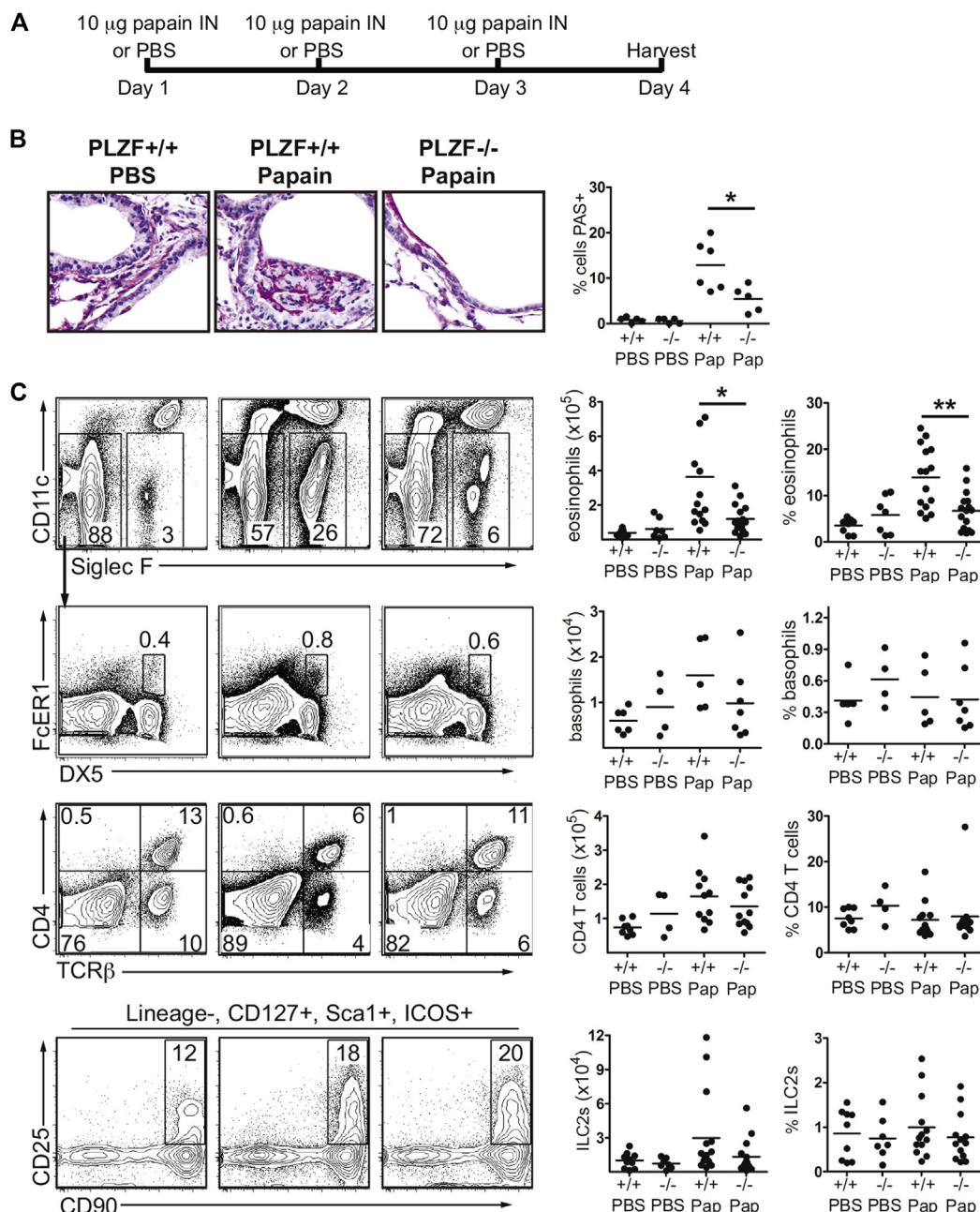


FIG 1. PLZF^{-/-} mice have reduced type 2 inflammation in response to papain (Pap) exposure. **A**, Schematic for intranasal papain treatment of PLZF^{+/+} versus PLZF^{-/-} littermate mice. **B**, Histology at ×50 magnification of lungs treated as indicated, with counts of the percentage of periodic acid-Schiff (PAS)⁺ epithelial cells. **C**, Flow cytometric plots showing cell percentages within each plot, quantified cell counts, and quantified cell percentages in lung digests from mice treated as indicated for eosinophils (Siglec-F⁺ and CD11c⁺), basophils (Siglec-F⁺, CD11c⁺, FcεR1⁺, and DX5⁺), ILC2s (lineage⁻, CD90⁺, ICOS⁺, Sca1⁺, CD25⁺, and CD127⁺), and CD4⁺ T cells (CD19⁺, TCRβ⁺, and CD4⁺). Experiments are pooled data from at least 2 separate experiments. *P ≤ .05 and **P ≤ .01.

For measurement of cytokine concentrations in alveolar lavage fluid, the BD Cytometric Bead Array was used, analyzing levels of IL-4, IL-5, IL-13, and IFN-γ. Samples were analyzed in duplicate.

Cytokine production assay

Flow cytometry-purified lung ILC2s from PLZF^{-/-} or WT littermates were cultured in 200 mL of RPMI-1640 media containing 10% FBS, penicillin and streptomycin, and 50 mmol/L 2-mercaptoethanol at 37°C. Cells were

stimulated with IL-7 (10 ng/mL) and either IL-25 (10 ng/mL) or IL-33 (10 ng/mL) for 3 days. Extracellular media were then collected and analyzed for IL-5 and IL-13 by means of ELISA in triplicate (eBioscience).

Generation of mixed bone marrow chimeras

Recipient CD45.1/CD45.2⁺ mice were lethally irradiated with 1000 Rads from a Gammacell 40 Exactor irradiator with a cesium source (Best Theratronics, Ottawa, Ontario, Canada). Irradiated mice were injected intravenously

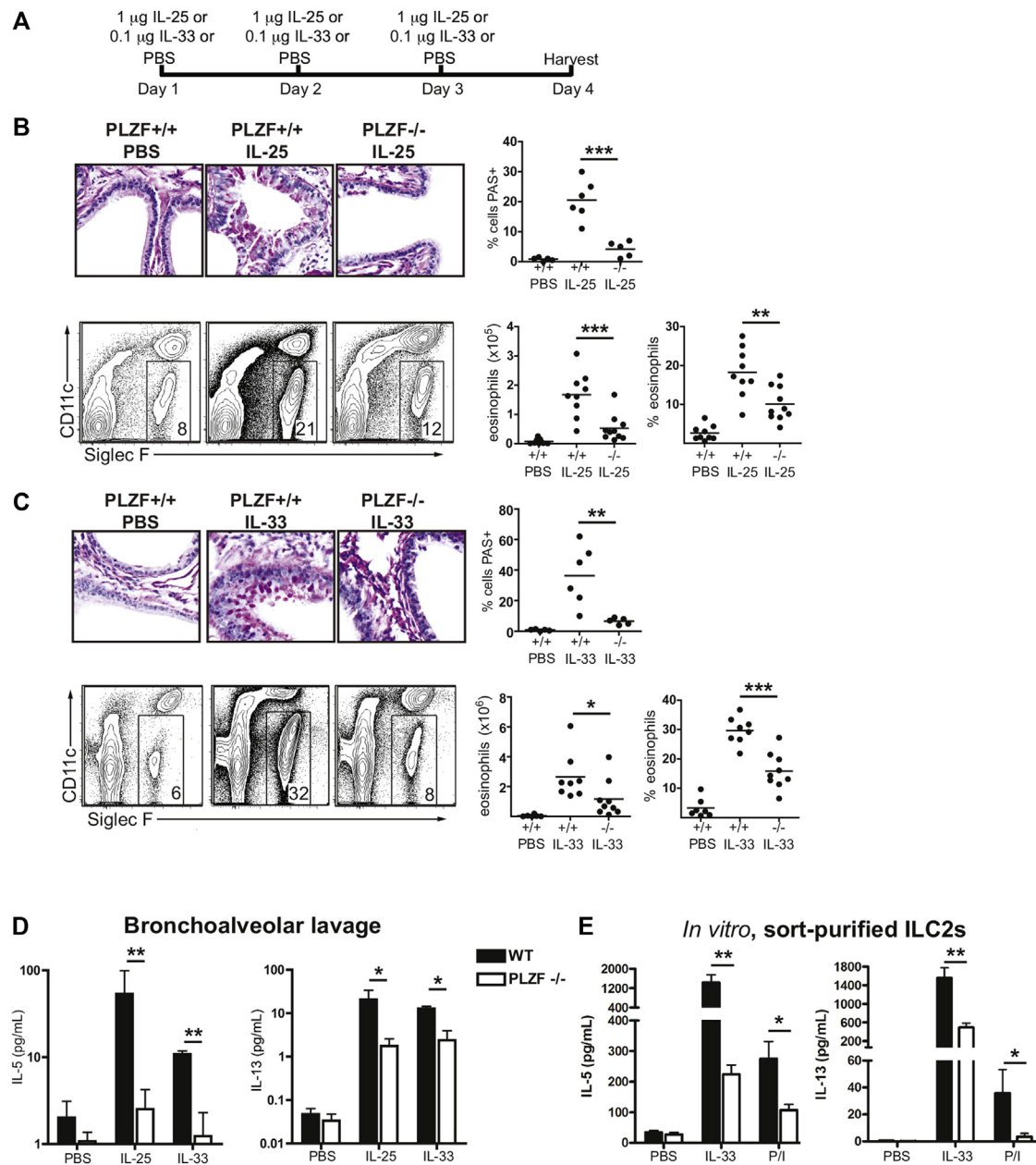
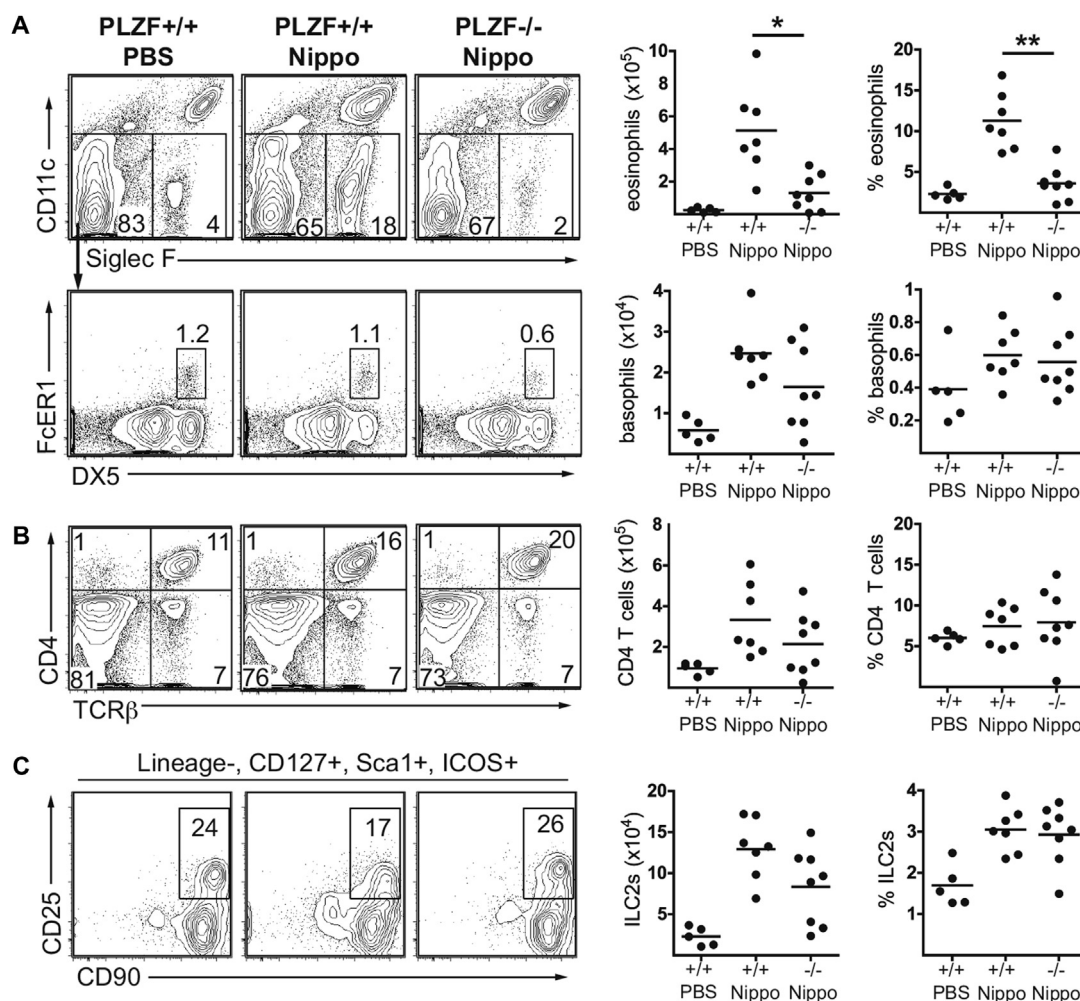


FIG 2. PLZF^{-/-} mice have reduced type 2 inflammation and ILC2 activation in response to IL-25 or IL-33 inhalation. **A**, Schematic for intranasal IL-25 or IL-33 treatment of PLZF^{+/+} versus PLZF^{-/-} mice. **B** and **C**, Histology at $\times 50$ magnification of lungs treated with IL-25 (Fig 2, B) or IL-33 (Fig 2, C) with counts of periodic acid-Schiff (PAS)⁺ epithelial cells and flow cytometric plots and cell percentages of eosinophils (Siglec-F⁺ and CD11c⁻) in lung digests from mice treated as indicated. **D**, Bronchoalveolar lavage fluid from mice treated with the indicated stimuli for 3 days was tested for either IL-5 or IL-13 by using a cytometric bead array. Samples were tested in duplicate, with each experiment using at least 3 mice and repeated 2 to 3 times. **E**, Sort-purified ILC2s were treated for 3 days in media containing IL-7 and either PBS, IL-33, or PMA/ionomycin (P/I). Media were then collected and tested for levels of IL-5 and IL-13 by means of ELISA. Results represent pooled results from 4 different samples repeated twice. * $P \leq .05$, ** $P \leq .01$, and *** $P \leq .001$.

with a mixture of bone marrow cells isolated from CD45.2⁺PLZF^{-/-} mice and CD45.1 WT congenic mice at a ratio of 2:1, favoring the PLZF^{-/-} bone marrow. This ratio was chosen because of our observation that bone marrow from the PLZF^{-/-} mice has fewer ILC2s, thus potentially biasing mixed chimera results in favor of the WT (unpublished observations). Cell numbers were then normalized to splenic CD45.2/CD45.1 B-cell ratios for each mouse, reflecting chimeric reconstitution. Mice were analyzed at least 6 weeks after reconstitution.

Sensitization and challenge with ovalbumin

PLZF^{-/-} mice, WT littermates, and mixed bone marrow chimeras were treated with a 23-day model of sensitization and challenge for C57BL/6 mice, as previously described.¹⁶ Briefly, 50 μ g of ovalbumin (Sigma) was mixed with 50 μ L of Inject Alum (Thermo Scientific) to a final volume of 100 μ L and injected into each mouse intraperitoneally on days 0, 7, and 14. This was followed by a challenge phase, in which 100 μ g of ovalbumin was



delivered intranasally to anesthetized mice on days 20, 21, and 22, with death on day 23.

Statistical analysis

The 2-tailed Student *t* test was performed with Prism software (GraphPad Software, La Jolla, Calif) to determine whether data differed from the expected value. If the groups that were compared had significantly different variances (*P* < .05, *F* test), Welch correction was applied.

RESULTS

PLZF^{-/-} mice treated with papain have a reduced type 2 inflammatory response

We treated PLZF^{-/-} mice and PLZF^{+/+} WT littermates with papain intranasally for 3 days before analysis of alveolar lavage and lung digests (Fig 1, A). As shown in Fig 1, B, PLZF^{-/-} mice exhibited significantly less periodic acid-Schiff-positive goblet cell hyperplasia compared with PLZF^{+/+} WT mice,

indicating an impairment in innate type 2 inflammation. Furthermore, PLZF^{-/-} mice exhibited a significantly decreased percentage of eosinophils in lung digests (Fig 1, C) in response to papain treatment. Importantly, recruitment of other type 2 inflammatory cell types (basophils, CD4 T cells, and ILC2s) was preserved in the PLZF^{-/-} mice.

Type 2 inflammation is reduced in PLZF^{-/-} mice treated with either inhaled IL-25 or IL-33 because of defective ILC2 function

Because papain administration activates ILC2s through the stimulation of IL-25 and IL-33 release from epithelial cells and macrophages, we studied type 2 pulmonary inflammation after intranasal administration of IL-25 or IL-33 (Fig 2, A). As indicated in Fig 2, B and C, PLZF^{-/-} mice treated with 1 μg of IL-25 intranasally or 0.1 μg of IL-33 intranasally for 3 days showed markedly less eosinophilia and goblet cell hyperplasia

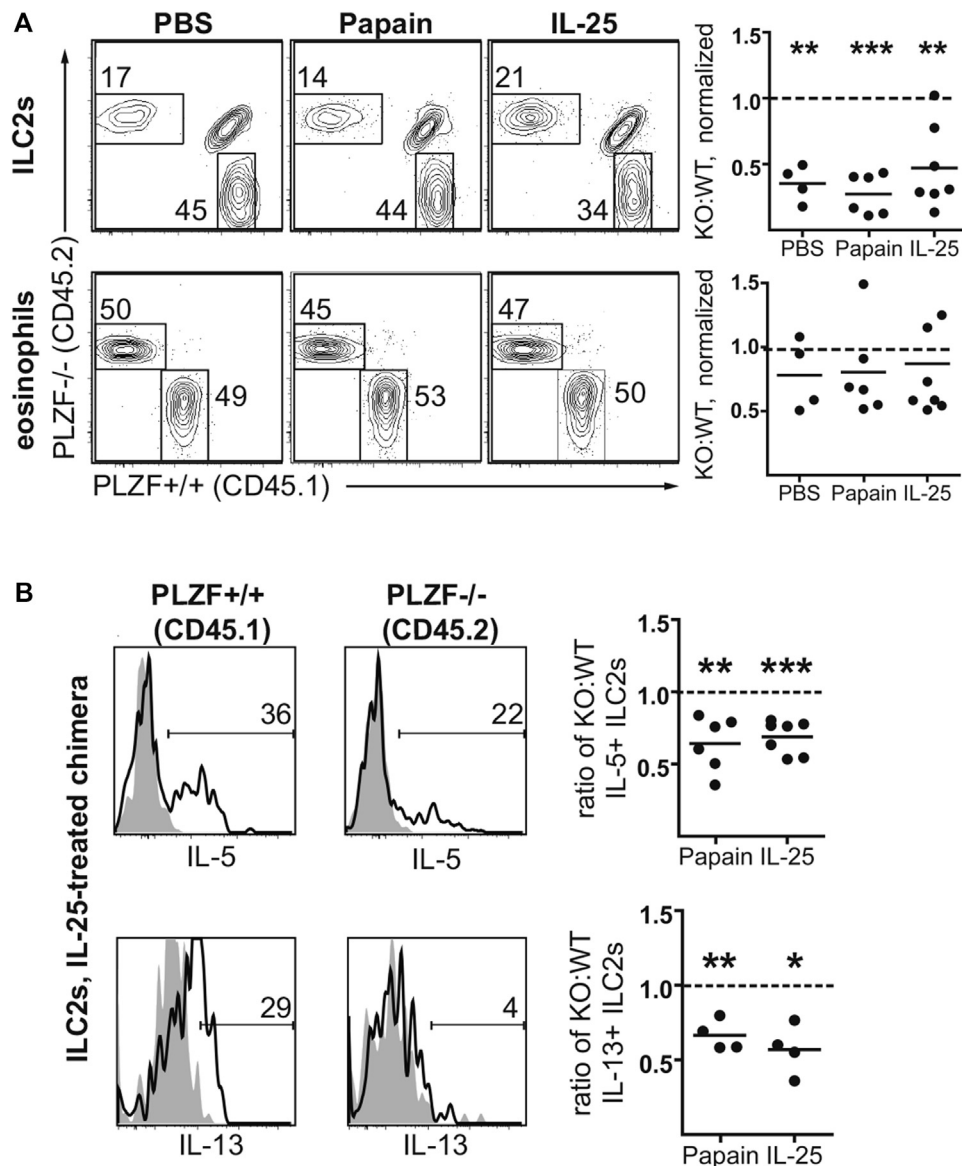


FIG 4. Mixed bone marrow chimeras reveal cell-intrinsic reconstitution and activation defects in ILC2s from PLZF^{-/-} mice. Mixed bone marrow chimeras were generated by using bone marrow from CD45.1 PLZF^{+/+} (WT) and CD45.2 PLZF^{-/-} (KO) mice, and 6 weeks after reconstitution, they were treated for 3 days with papain, IL-25, or PBS intranasally, followed by analysis of lung digests. **A**, Flow cytometric plots and KO/WT ratios (normalized to splenic B cells) for ILC2s (CD25⁺, CD127⁺, Thy1⁺, ICOS⁺, and lineage⁻) and eosinophils (Siglec-F⁺ and CD11c⁻). *Dashed line* indicates predicted reconstitution ratio if no reconstitution defect were present. **B**, Flow cytometric histograms and quantitation of ILC2s from a representative IL-25-treated mixed chimera mouse comparing the percentage of CD45.2⁺ ILC2s from PLZF^{-/-} mice, which are IL-5⁺ or IL-13⁺ (*solid line*), with CD45.1⁺ ILC2s from PLZF^{+/+} mice. The negative control (*gray shading*) was derived by preincubating ILC2s with excess unlabeled anti-cytokine antibody before staining with the indicated anti-cytokine antibody. Quantitation plots represent the ratio of CD45.2⁺, cytokine-positive ILC2s from PLZF^{-/-} mice to CD45.1⁺, and cytokine-positive ILC2s from PLZF^{+/+} mice for either papain or IL-25 treatment. *Dashed line* indicates predicted ratio if no activation defect were present. Experiments were repeated 2 to 3 times with 4 to 6 mice per condition. Statistical analysis was done with the Student *t* test for variation from a predicted ratio of 1. **P* ≤ .05, ***P* ≤ .01, and ****P* ≤ .001.

compared with their WT counterparts. We measured the IL-5 and IL-13 levels in the alveolar lavage fluid and found that PLZF^{-/-} mice had lower amounts of IL-5 and IL-13 in response to either IL-25 or IL-33, reflecting the reduced type 2 inflammation in the lung (Fig 2, D). Of note, with such a short stimulation in the absence of a specific adaptive response to a protein allergen,

T_H2 lymphocytes were not a significant source of IL-5 or IL-13, as assessed by means of intracellular cytokine staining (data not shown). To track the defect in cytokine secretion specifically to ILC2s, we sort purified lung ILC2s and cultured them *in vitro* for 3 days with IL-7 and IL-33 or with PMA/ionomycin, as described previously.⁹ IL-33 induced robust release of both

IL-5 and IL-13 from WT ILC2s, whereas PLZF^{-/-} ILC2s released significantly less IL-5 and IL-13 (Fig 2, E). In addition, the response to ionomycin and PMA was markedly decreased in PLZF^{-/-} ILC2s. Given the robust nature of the defect we observed to both IL-33 and PMA/ionomycin, we elected not to test responses to other cytokine combinations previously used to stimulate ILC2s *in vitro*.^{9,17}

Impaired responses of the PLZF^{-/-} mice to several innate type 2 stimuli indicated a global defect in ILC2 function, which might be sufficient to explain the decreased allergic airway inflammation induced by papain. However, the PLZF^{-/-} mouse also lacks iNKT cells. Therefore we tested the response to innate type 2 stimuli in CD1d^{-/-} mice that lack iNKT cells but do not have defects in any other hematopoietic lineages, such as ILC2s. CD1d^{-/-} mice did not demonstrate a defect in eosinophilia in response to intranasal IL-25 or papain treatment compared with their WT littermates, indicating that, in contrast with ILC2s, iNKT cells are dispensable for the pulmonary innate response to these stimuli (see Fig E1 in this article's Online Repository at www.jacionline.org).

PLZF^{-/-} mice infected with the helminth *N brasiliensis* have reduced pulmonary eosinophilia

ILC2s play a critical role during the pulmonary phase of infection with the helminth *N brasiliensis*, leading to eosinophilia and helminth clearance.^{10,18,19} Therefore we infected PLZF^{-/-} mice or WT littermates with 500 L3 *N brasiliensis*, followed by examination 5 days later to assess pulmonary eosinophilia. As shown in Fig 3, A, we observed a significant decrease in both the percentage and total number of eosinophils recruited to the lungs in PLZF^{-/-} mice, suggesting a defect in the acute response to helminth infection. Importantly, the percentages of basophils (Fig 3, A), CD4⁺ T lymphocytes (Fig 3, B), and ILC2s (Fig 3, C) were not significantly altered in PLZF^{-/-} mice. These results are consistent with defective eosinophil recruitment by functionally impaired ILC2s similar to that seen with papain and inhaled IL-25 and IL-33.

Mixed bone marrow chimeras reveal cell-intrinsic reconstitution and activation defects in PLZF^{-/-} mouse-derived ILC2s

To prove further that the defects in the responses of PLZF^{-/-} mice were cell intrinsic to ILC2s, we generated mixed bone marrow chimeras using congenically labeled WT CD45.1 bone marrow mixed with PLZF^{-/-} CD45.2 bone marrow into an irradiated CD45.1/CD45.2 recipient. This approach allows us to establish whether ILC2s from PLZF^{-/-} mice are equally capable of reconstituting the ILC2 compartment in the lungs compared with those of their PLZF^{+/+} counterparts and to examine the specific activation of ILC2s from PLZF^{-/-} mice relative to ILC2s from PLZF^{+/+} mice in response to stimulation. At 6 weeks after reconstitution, these chimeras were treated for 3 days with PBS, papain, or IL-25 to induce type 2 inflammation, as described above. Consistent with prior results from our laboratory, reconstitution of ILC2s from PLZF^{-/-} mice in the lungs was substantially impaired at baseline compared with that of WT ILC2s, as shown in Fig 4, A.⁸ Furthermore, these cell-intrinsic defects were not corrected after inflammation induced by IL-25

or papain treatment. Importantly, eosinophil reconstitution, recruitment, and expansion were similar in the PLZF-deficient and sufficient compartments.

We then compared intracellular IL-5 and IL-13 staining between ILC2s from WT mice and ILC2s from PLZF^{-/-} mice in these chimeras. Histograms in Fig 4, B, show a representative sample of ILC2s from the lungs of a mixed chimera mouse treated with IL-25 for 3 days and stained for either IL-5 or IL-13. Of note, radiation-resistant, CD45.1/CD45.2 double-positive ILC2s were excluded from this analysis. A smaller percentage of CD45.2⁺ ILC2s from PLZF^{-/-} mice stained positive for IL-5 or IL-13 compared with CD45.1⁺ ILC2s from PLZF^{+/+} mice. To account for the ILC2 reconstitution defect noted in Fig 4, A, the ratio of cytokine-positive knockout (KO)/WT ILC2s was expressed relative to the total ratio of KO/WT ILC2s. If there were no defect in activation of IL-5 or IL-13 in PLZF^{-/-} mouse-derived ILC2s, the ratio of ILC2s from PLZF^{-/-} mice to ILC2s from WT mice in the cytokine-positive population would have been identical to that of the total ILC2 population or 1. However, the quantitation plots in Fig 4, B, reveal a significant activation defect in ILC2s from PLZF^{-/-} mice in response to either papain or IL-25 treatment for both IL-5 and IL-13 expression. Thus the data establish the cell-intrinsic nature of the ILC2 functional defects and suggest that defective eosinophilia in papain- or IL-25-treated PLZF^{-/-} mice is secondary to impaired ILC2 effector function.

T cells from PLZF^{-/-} mice are capable of mediating T_H2 responses to ovalbumin in both PLZF^{-/-} mice and mixed bone marrow chimeras

Whether ILC2s can also control T_H2 responses triggered by injection of antigen with alum has not been investigated. WT or PLZF^{-/-} mice were sensitized with ovalbumin plus alum intraperitoneally on days 0, 7, and 14, followed by ovalbumin inhalation on days 20, 21, and 22 and death on day 23.¹⁶ As shown in Fig 5, A, there was no significant difference in the degree of eosinophilia or in recruitment of CD4⁺ T cells to the lungs of PLZF^{-/-} mice compared with WT mice, indicating that this T_H2 response is independent of ILC2s.

To prove that PLZF-derived CD4⁺ T cells function comparably to WT cells in a competitive environment independent of ILC2 function, we examined the T_H2 response in mixed CD45.1⁺, WT:CD45.2⁺, PLZF^{-/-} bone marrow chimeras, as described above. Ovalbumin sensitization and challenge led to a marked allergic inflammatory response with significant pulmonary eosinophilia (Fig 5, B). Analysis of the normalized ratios of PLZF^{-/-}-derived to WT-derived eosinophils again revealed no cell-intrinsic defect in reconstitution or expansion, whereas ILC2s from PLZF^{-/-} mice demonstrated a marked defect in reconstitution and expansion. Furthermore, analysis of PLZF^{-/-} mouse-derived CD4⁺ T cells revealed no defect in reconstitution or expansion.

PLZF is not expressed in T or B cells (with the exception of iNKT cells), but to confirm that T cells derived from PLZF^{-/-} bone marrow retain functionality in a mixed chimera, we stained lung digest lymphocytes for IL-5 and IL-13.⁸ Comparison of the normalized ratio of cytokine-positive CD4⁺ T cells from PLZF^{-/-} mice/WT mice to total CD4⁺ T cells from

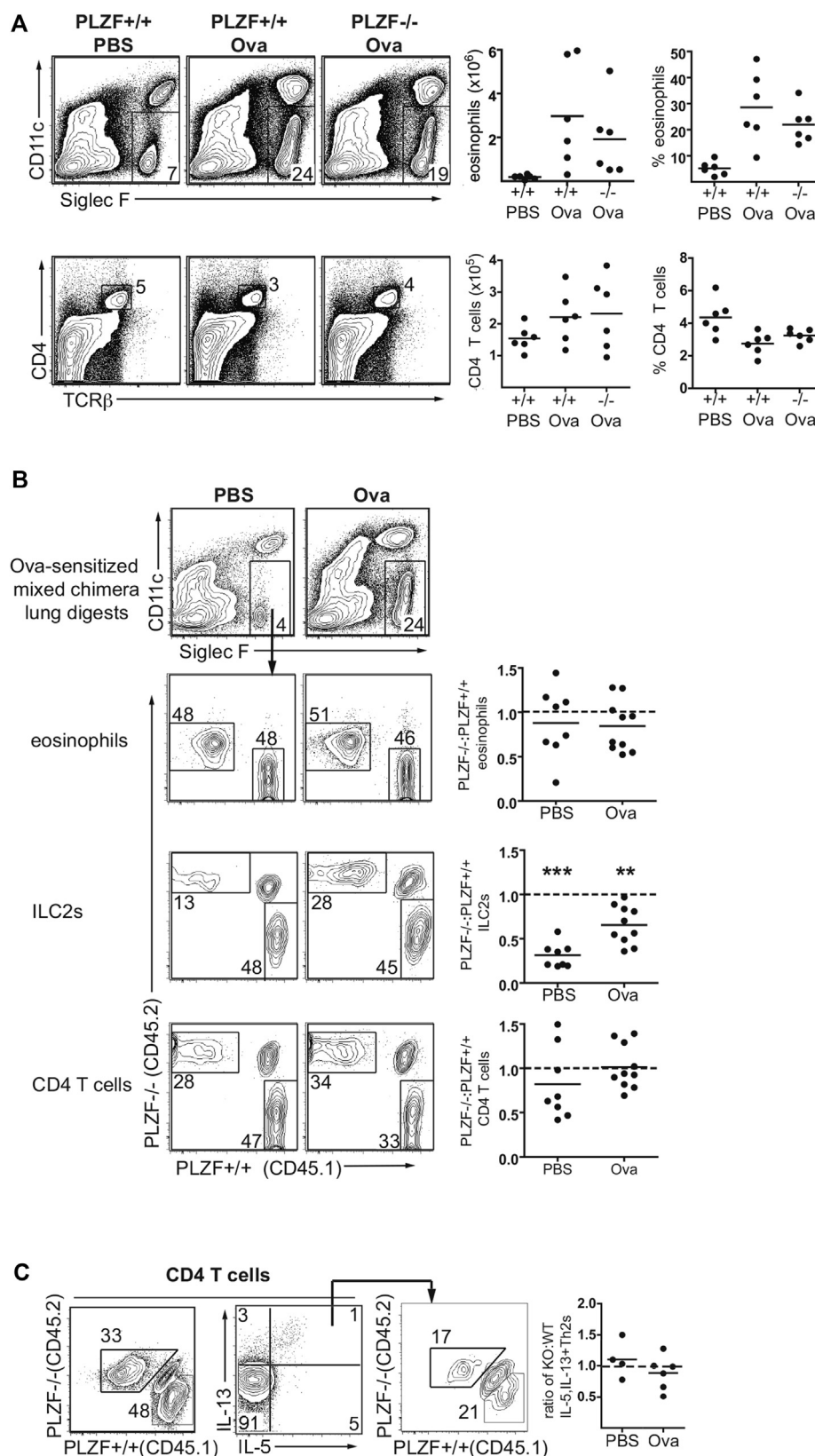


FIG 5. PLZF^{-/-} mice generate normal adaptive T_H2 responses to ovalbumin. PLZF^{-/-} or PLZF^{+/+} littermate mice were sensitized and challenged with ovalbumin (Ova). **A**, Flow cytometric plots and percentages of lung digests for eosinophils and CD4⁺ T cells, as indicated. **B**, Mixed bone marrow chimeras were generated by using bone marrow from CD45.1 PLZF^{+/+} mice and CD45.2 PLZF^{-/-} mice and sensitized to ovalbumin, as described

PLZF^{-/-} mice/WT mice showed no defect in upregulation of cytokine expression among CD4⁺ T cells derived from PLZF^{-/-} bone marrow, indicating a functionally intact adaptive immune response (Fig 5, C).

DISCUSSION

An important limitation of previously published research on ILC2 function has been the need to use systems in which the endogenous immune system is significantly altered. Adoptive transfer of purified ILC2s into *Rag2*^{-/-}*IL2rg*^{-/-} mice proves that ILC2s can mediate innate responses, but these mice lack endogenous lymphoid immune cells and lymph nodes.⁹ Functional studies with antibody-based depletion of ILC2s in *Rag1*^{-/-} mice are also hindered by the lack of a functional adaptive immune response.^{9,11} Reconstitution of lethally irradiated mice with bone marrow from *Rora*^{sg/-sg} mice (possessing a mutant form of retinoic acid receptor–related orphan nuclear receptor α) partly circumvents this limitation.^{13,20} The resulting chimeras do not have a significant ILC2 compartment but do have T cells. However, this approach is limited by the potential presence of radiation-resistant cells (including ILC2s, which we have observed) coupled with concerns about the function of the reconstituted immune system compared with that of the WT unirradiated mouse. As we have demonstrated in this work, the PLZF^{-/-} mouse model does not depend on irradiation or reconstitution of immune-deficient mice and therefore complements the published research on ILC2 function. In addition, the PLZF^{-/-} mouse model is a novel tool for dissecting the role that lymphoid-derived innate cells play in an otherwise immunocompetent system.

This work also reveals a unique role for PLZF in establishing effector function of lymphoid lineage innate cells. ILCs develop from the CLP in the bone marrow through an ILC precursor and subsequently migrate to the periphery.⁸ In contrast, iNKT cells undergo thymic selection before emigration and maturation in the periphery.⁶ In spite of these disparate developmental pathways, PLZF acts as a global master transcription factor in conferring an effector phenotype on both lymphoid-derived innate cell types. Our previous work suggested that the PLZF^{-/-} mouse has a reduced number of ILC precursors in the bone marrow, and we confirmed this defect using the bone marrow mixed chimera. However, the PLZF^{-/-} mouse has a nearly normal number of ILC2s in the lung, indicating that PLZF expression is not required for development, which is in contrast to its effect on ILC2 function. This is consistent with our observation that residual immature ILC2s in PLZF^{-/-} mice express high levels of

CD62 ligand and lower levels of ICOS compared with their WT counterparts, which will likely affect tissue targeting and recirculation. The precise transcriptional targets of PLZF expression are currently under investigation but likely include effector cytokines and other tissue-targeting integrins. Furthermore, we are evaluating epigenetic changes induced by PLZF that define the unique capacity of PLZF to pattern these effector phenotypes. Ultimately, ILC2s lacking PLZF have a profound functional defect, even in the face of a relatively preserved cell frequency.

Our findings are supported by several recent publications on ILC2 function. Maazi et al²¹ recently reported that mice lacking ICOS have impaired type 2 inflammation in response to inhaled IL-33. Given that we previously demonstrated reduced ICOS expression on ILC2s from PLZF^{-/-} mice, this might explain in part the reduced functionality of PLZF^{-/-} ILC2s.⁸ Huang et al²² have postulated the existence of distinct ILC2 subsets, termed inflammatory ILC2 (responsive to IL-25) and natural ILC2 (responsive to IL-33). The diminished responsiveness observed in ILC2s from PLZF^{-/-} mice to both stimuli is consistent with PLZF expression at an early common progenitor stage for both inflammatory ILC2s and natural ILC2s, rendering both subsets hypofunctional. Christianson et al²³ recently reported a feed-forward mechanism for type 2 inflammation in mouse lungs, in which IL-13 secretion from stimulated ILC2s induces additional IL-33 secretion from epithelial cells to augment the allergic inflammatory response. The decreased secretion of IL-13 from ILC2s from PLZF^{-/-} mice would lead to a diminished feed-forward signaling cascade and presumably render PLZF^{-/-} mice less susceptible to induction of allergic airways disease. Papain-induced ILC2 activation also depends on IL-4 secretion from basophils; our *in vitro* and mixed chimera experiments confirm that the ILC2 defect is cell intrinsic and not due to dysfunctional basophil function.²⁴ However, ILC2s from PLZF^{-/-} mice might be hyporesponsive to basophil-derived IL-4, thereby further decreasing IL-5 and IL-13 expression.

Although our data unambiguously demonstrate an important role for PLZF in determining the effector function of ILC2s, the molecular mechanism remains to be investigated. Nevertheless, this study reinforces the importance of PLZF expression during development of lymphoid-derived innate cells by defining a previously unrecognized functional role for PLZF and suggests that PLZF represents a unique tool for dissecting the function of these innate cells in disease models.

We thank the past and present members of the Bendelac laboratory for productive discussion and David Leclerc and Ryan Duggan for help with cell sorting.

above. Eosinophils, ILC2s, and CD4⁺ T cells were normalized to splenic B-cell numbers and expressed as a ratio of CD45.2 PLZF^{-/-} to CD45.1 PLZF^{+/+} to determine whether there were cell-intrinsic defects in cells participating in lung inflammation in response to ovalbumin. *Dashed lines* indicate the predicted PLZF^{-/-}/PLZF^{+/+} ratio if no cell-intrinsic defect were present. **C**, Flow cytometric plots of CD4⁺ T cells (CD4⁺, TCR β ⁺, CD8⁻, and CD19⁻) from ovalbumin-sensitized mixed chimera mice challenged with ovalbumin and gated as either CD45.2 PLZF^{-/-} versus CD45.1 PLZF^{+/+} or as IL-5⁺ versus IL-13⁺. Cytokine-positive cells are then gated for CD45.2 PLZF^{-/-} versus CD45.1 PLZF^{+/+}. The quantitation plot shows the ratio of KO/WT IL-5⁺/IL-13⁺ CD4⁺ T cells to total CD4⁺ T cells, and the *dashed line* indicates the predicted ratio of KO/WT IL-5⁺/IL-13⁺ CD4⁺ T cells if no activation defect were present. Experiments were repeated 2 to 3 times with 4 to 6 mice per condition. Statistical analysis was done with the Student *t* test for variation from a ratio of 1. ***P* < .01 and ****P* < .001.

Key messages

- ILC2s from mice lacking the transcription factor PLZF have functional defects in response to various type 2 immune stimuli.
- ILC2s are not required for alum-OVA adjuvanted T_H2 responses in the lungs.
- PLZF^{-/-} mice are a novel tool for studying innate immune responses in an otherwise immunocompetent mouse.

REFERENCES

- Brennan PJ, Brigl M, Brenner MB. Invariant natural killer T cells: an innate activation scheme linked to diverse effector functions. *Nat Rev Immunol* 2013; 13:101-17.
- Bendelac A, Savage PB, Teyton L. The biology of NKT cells. *Annu Rev Immunol* 2007;25:297-336.
- Fuchs A, Colonna M. Innate lymphoid cells in homeostasis, infection, chronic inflammation and tumors of the gastrointestinal tract. *Curr Opin Gastroenterol* 2013;29:581-7.
- Vantourout P, Hayday A. Six-of-the-best: unique contributions of gammadelta T cells to immunology. *Nat Rev Immunol* 2013;13:88-100.
- Zeissig S, Blumberg RS. Commensal microbiota and NKT cells in the control of inflammatory diseases at mucosal surfaces. *Curr Opin Immunol* 2013;25:690-6.
- Constantinides MG, Bendelac A. Transcriptional regulation of the NKT cell lineage. *Curr Opin Immunol* 2013;25:161-7.
- Savage AK, Constantinides MG, Han J, Picard D, Martin E, Li B, et al. The transcription factor PLZF directs the effector program of the NKT cell lineage. *Immunity* 2008;29:391-403.
- Constantinides MG, McDonald BD, Verhoef PA, Bendelac A. A committed precursor to innate lymphoid cells. *Nature* 2014;508:397-401.
- Halim TY, Krauss RH, Sun AC, Takei F. Lung natural helper cells are a critical source of Th2 cell-type cytokines in protease allergen-induced airway inflammation. *Immunity* 2012;36:451-63.
- Price AE, Liang HE, Sullivan BM, Reinhardt RL, Eislely CJ, Erle DJ, et al. Systemically dispersed innate IL-13-expressing cells in type 2 immunity. *Proc Natl Acad Sci U S A* 2010;107:11489-94.
- Monticelli LA, Sonnenberg GF, Abt MC, Alenghat T, Ziegler CG, Doering TA, et al. Innate lymphoid cells promote lung-tissue homeostasis after infection with influenza virus. *Nat Immunol* 2011;12:1045-54.
- Scanlon ST, McKenzie AN. Type 2 innate lymphoid cells: new players in asthma and allergy. *Curr Opin Immunol* 2012;24:707-12.
- Halim TY, Steer CA, Matha L, Gold MJ, Martinez-Gonzalez I, McNagny KM, et al. Group 2 innate lymphoid cells are critical for the initiation of adaptive T helper 2 cell-mediated allergic lung inflammation. *Immunity* 2014;40:425-35.
- Drake LY, Iijima K, Kita H. Group 2 innate lymphoid cells and CD4+ T cells cooperate to mediate type 2 immune response in mice. *Allergy* 2014;69: 1300-7.
- Camberis M, Le Gros G, Urban J Jr. Animal model of *Nippostrongylus brasiliensis* and *Heligmosomoides polygyrus*. *Curr Protoc Immunol* 2003;55:chapter 19: 19.1-19.27.
- Caceres AI, Brackmann M, Elia MD, Bessac BF, del Camino D, D'Amours M, et al. A sensory neuronal ion channel essential for airway inflammation and hyperreactivity in asthma. *Proc Natl Acad Sci U S A* 2009;106:9099-104.
- Bartemes KR, Kephart GM, Fox SJ, Kita H. Enhanced innate type 2 immune response in peripheral blood from patients with asthma. *J Allergy Clin Immunol* 2014;134:671-8.e4.
- Turner JE, Morrison PJ, Wilhelm C, Wilson M, Ahlfors H, Renaud JC, et al. IL-9-mediated survival of type 2 innate lymphoid cells promotes damage control in helminth-induced lung inflammation. *J Exp Med* 2013;210:2951-65.
- Hung LY, Lewkowich IP, Dawson LA, Downey J, Yang Y, Smith DE, et al. IL-33 drives biphasic IL-13 production for noncanonical Type 2 immunity against hookworms. *Proc Natl Acad Sci U S A* 2013;110:282-7.
- Halim TY, Maclaren A, Romanish MT, Gold MJ, McNagny KM, Takei F. Retinoic-acid-receptor-related orphan nuclear receptor alpha is required for natural helper cell development and allergic inflammation. *Immunity* 2012;37:463-74.
- Maazi H, Patel N, Sankaranarayanan I, Suzuki Y, Rigas D, Soroosh P, et al. ICOS: ICOS-ligand interaction is required for type 2 innate lymphoid cell function, homeostasis, and induction of airway hyperreactivity. *Immunity* 2015; 42:538-51.
- Huang Y, Guo L, Qiu J, Chen X, Hu-Li J, Siebenlist U, et al. IL-25-responsive, lineage-negative KLRG1(hi) cells are multipotential 'inflammatory' type 2 innate lymphoid cells. *Nat Immunol* 2015;16:161-9.
- Christianson CA, Goplen NP, Zafar I, Irvin C, Good JT Jr, Rollins DR, et al. Persistence of asthma requires multiple feedback circuits involving type 2 innate lymphoid cells and IL-33. *J Allergy Clin Immunol* 2015;136:59-68.e14.
- Motomura Y, Morita H, Moro K, Nakae S, Artis D, Endo TA, et al. Basophil-derived interleukin-4 controls the function of natural helper cells, a member of ILC2s, in lung inflammation. *Immunity* 2014;40:758-71.

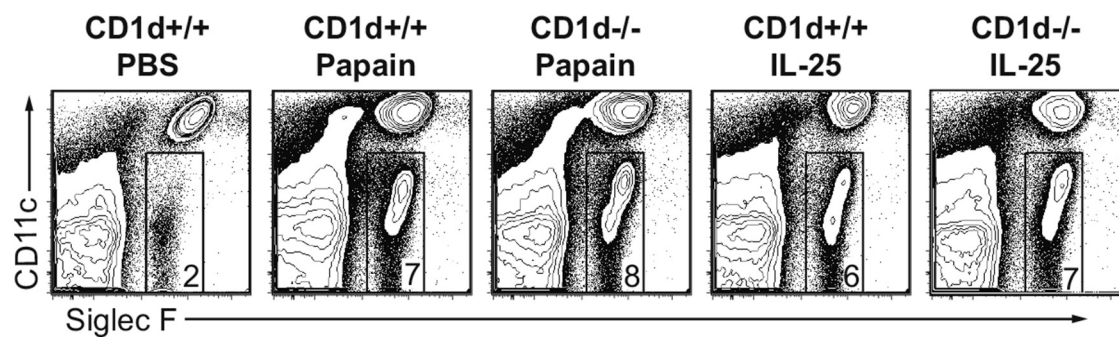


FIG E1. CD1d^{-/-} mice do not have reduced eosinophilia in response to papain or IL-25 inhalation. Flow cytometric plots of eosinophils (Siglec-F⁺ and CD11c⁻) from lung digests of CD1d^{+/+} or littermate CD1d^{-/-} mice treated for 3 days with PBS, papain, or IL-25.