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

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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 T_H2 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 γδ 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 γδ 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*$^{-/-}$

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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-7Rα: 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 μL 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 μL 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 400*g* 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 · 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-μm filter, washed with 25 mL of RPMI/10% FCS, and centrifuged at 400*g* for 5 minutes. Cells were resuspended in 5 mL of 44% Percoll underlaid with 3 mL of 66% Percoll and centrifuged at 800*g* 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 · 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-μm 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), CD3ε (17A2), CD8α (53-6.7), CD11b (M1/70), CD11c (N418), CD25 (PC61), Gr-1 (RB6-8C5), inducible costimulator (ICOS; C398.4A), IL-7 receptor α (IL-7Rα)/CD127 (A7R34), NK1.1 (PK136), Sca-1 (D7), T1/ST2 (D1H9), and TCRβ (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, CD3ε, CD8α, CD11b, CD11c, CD19, Gr-1, NK1.1, and TCRβ) and positive for IL-7Rα, 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 TCRβ$^+$, CD4$^+$, CD8$^-$, and B220$^-$. CD8 T cells were identified as TCRβ$^+$, CD4$^-$, CD8$^+$, and B220$^-$. B cells were identified as B220$^+$ and TCRβ$^-$. iNKT cells were identified as TCRβ$^+$, 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.