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Cutting Edge: Natural Helper Cells Derive from Lymphoid Progenitors

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Natural helper (NH) cells are recently discovered innate immune cells that confer protective type 2 immunity during helminth infection and mediate influenza-induced airway hypersensitivity. Little is known about the ontogeny of NH cells. We report in this study that NH cells derive from bone marrow lymphoid progenitors. Using RAG-1Cre/ROSA26<sup>YFP</sup> mice, we show that most NH cells are marked with a history of RAG-1 expression, implying lymphoid developmental origin. The development of NH cells depends on the cytokine receptor Flt3, which is required for the efficient generation of bone marrow lymphoid progenitors. Finally, we demonstrate that lymphoid progenitors, but not myeloid–erythroid progenitors, give rise to NH cells in vivo. This work therefore expands the lymphocyte family, currently comprising T, B, and NK cells, to include NH cells as another type of innate lymphocyte that derives from bone marrow lymphoid progenitors. *Journal of Immunology, 2011, 187: 000–000.

The Th2 cytokines IL-4, IL-5, and IL-13 play an important role in the pathophysiology of allergic diseases and in protective immunity against helminth infection (1). The innate immune pathways responsible for the induction, maintenance, and amplification of Th2 cytokine responses during allergic diseases and parasite infection remain poorly understood. Recent studies have discovered novel populations of innate immune cells that promote Th2 cytokine responses (2–6). MPP<sup>type2</sup> cells produce IL-4 in response to IL-25 and give rise to myeloid cells and mast cells in vitro (6). Other innate effector populations termed natural helper (NH) cells, nuocytes, and innate type 2 helper cells appear to be terminally differentiated cell types that produce IL-5 and IL-13 in response to IL-25 or IL-33 (2–5). These novel innate immune cells promote type 2 immunity during helminth infection and mediate influenza-induced airway hypersensitivity (2–6).

We have investigated the developmental origin of type 2 innate effector cells. We found that more than half of NH cells were marked with a history of RAG-1 expression, suggesting lymphoid origin. Development of NH cells relies on the cytokine receptor Flt3 that is required for the efficient generation of lymphoid progenitors. Finally, adoptive transfer of purified hematopoietic progenitors established that NH cells are generated by lymphoid but not myeloid–erythroid progenitors. Taken together, these results indicate that NH cells predominantly originate from bone marrow (BM) lymphocyte progenitors.

Materials and Methods

**Mice**

B6-Ly5.2 (CD45.1), RAG-2<sup>−/−</sup>, and nu/nu mice were purchased from the National Institutes of Health or The Jackson Laboratory. RAG-1Cre/ROSA26<sup>YFP</sup> mice (7) and Flt3<sup>−/−</sup> mice (8) were bred in accordance with the Institutional Animal Care and Use Committee policies at the University of Pennsylvania.

**Isolation of hematopoietic cells from the lung**

Mice were exsanguinated and lungs were perfused by injecting 10 ml PBS into the right ventricle of the heart. Lungs were carefully cut into small fragments and digested in HBSS containing 0.025 mg/ml Liberase D (Roche Diagnostics) and 10 U/ml DNase 1 (Roche Diagnostics). Cells were filtered using a cell strainer.

**Flow cytometry**

All Abs used in this study were purchased from eBioscience unless specified otherwise. Abs in the lineage (Lin) mixture included anti-FcεR (MAR-1), anti-B220 (RA3-6B2), anti-CD19 (1D3), anti–Mac-1 (M1/70), anti–Gr-1 (RC6), anti-CD11c (HL3), anti–NK1.1 (PK136), anti–Ter-119 (Ter-119), anti-CD3 (2C11), anti–CD8α (53.6-7), anti-CD8β (53-5.8), anti–TCR β (H57), and anti–γδTCR (GL-3). Additional Abs used included anti-CD45.2 (104), anti-CD45.1 (A20), anti–Kit (2B8), anti–Sca1 (D7), anti–CD4 (GK1.5), anti–Flt3 (A2F10), anti–IL-7Rα (A7R34), anti–CD25 (PC61.5), anti–Thy1.2 (53-2.1), and anti–T1/ST2 (D78; BD Biosciences).

**Bone marrow transplantation and intrathymic injections**

For BM transplantation, BM cells were depleted of Thy1<sup>high</sup> cells by magnetic beads. Thy1-depleted donor cells or sorted progenitors (CD45.2) were mixed with 2 × 10<sup>5</sup> competitor BM cells (CD45.1), and together they were injected i.v. into lethally irradiated (9.5 Gy) recipient mice (CD45.1) through retro-orbital injection. Recipient mice were examined at different time points posttransplant.

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The online version of this article contains supplemental material. Abbreviations used in this article: BM, bone marrow; CLP, common lymphoid progenitor; Lin, lineage; LMPF, lymphoid-primed multipotent progenitor; NH, natural helper; WT, wild-type.

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For intrathymic transfer, 5000 sorted NH cells or control BM Flt3high-Lin2 Kit+ cells (CD45.2) were transferred intrathymically into sublethally irradiated (6.5 Gy) mice (CD45.1). Mice were examined 12 d and again 21 d after injection.

Cell culture

Sorted NH cells were cultured in MEM-a medium with 20% FCS containing 10 ng/ml IL-7, IL-2, or IL-33 for 7 d. Cytokine production was determined by intracellular staining using a Cytofix/Cytoperm fixation/permeabilization solution kit (BD Biosciences).

Statistics

Intergroup comparisons were performed using a Student’s t test. The differences were considered significant with a p value of <0.05.

Results and Discussion

Characterization of NH cells in lung

Lung resident NH cells mediate influenza-induced airway hypersensitivity (2). The lung NH cells in C57BL/6 mice are negative for Lin markers and express T1/ST2 (9) and Thy1 (Fig. 1A). They also express the stem cell Ag Sca-1 and the cytokine receptors IL-7Rα, CD25, and Kit, but not Flt3 (Supplemental Fig. 1A). Similar to the previously characterized NH cells in fat-associated lymphoid clusters (3), lung NH cells express a high level of GATA-3, a transcription factor essential for Th2 cell differentiation (Supplemental Fig. 1B). These cells are present in RAG2−/− mice and athymic nude mice, but they are diminished in IL-7Rα−/− mice (Supplemental Fig. 1C). Lung NH cells expand in the presence of IL-2 and IL-7, and they produce type 2 cytokines IL-5 and IL-13 in response to IL-33 stimulation (Supplemental Fig. 1D, 1E). Unlike the T1/ST2+ NH cells, the lung resident T1/ST2−Lin−Thy1high cells do not expand in vitro with the same cytokines that support NH cell growth and activation, distinguishing them from functional NH cells (Supplemental Fig. 1E). Thus, the phenotype of Lin−Thy1highT1/ST2− was used to identify NH cells. Together, lung resident NH cells display the same phenotype, gene expression, and type 2 cytokine-producing activity of previously described NH cells in the fat-associated lymphoid clusters (3).

MPPtype2 cells are another newly described tissue-resident type 2 innate immune cells (6).
myeloid cells and mast cells in vitro. Lung NH cells, however, did not expand or differentiate in myeloid differentiation conditions, which distinguished NH cells from MPPtype2 cells (Supplemental Fig. 2A). NH cells also phenotypically resemble thymic DN2 cells or circulating T cell progenitors (10). However, they did not differentiate into T cells when cocultured with OP9-DL1 stromal cells (Supplemental Fig. 2B) or after intrathymic transfer (Supplemental Fig. 2C), thus excluding the possibility that NH cells are extrathymic T cell progenitors.

Defective NH cell development upon ablation of lymphocyte progenitors

To investigate the ontogeny of NH cells, we used the RAG-1Cre/ROSA26YFP mice in which the YFP+ cells either express RAG-1 or derive from RAG-1–expressing progenitors. NH cells themselves do not express RAG-1 (Fig. 1B). However, more than half of the NH cells express YFP, indicating a history of RAG-1 expression (Fig. 1C, 1D). These results suggest that NH cells develop from BM lymphoid progenitors in which RAG expression has initiated. Such progenitors include common lymphoid progenitors (CLPs) and developmentally more primitive lymphoid-primed multipotent progenitors (LMPPs), which are efficient progenitors of lymphocytes although they retain a degree of myeloid potential (11, 12).

To understand whether lymphoid progenitors are required for the generation of NH cells, we asked whether NH cell development is dependent on the cytokine receptor Flt3. Previous studies have established that Flt3 is expressed at high levels on BM lymphoid progenitors and is necessary for the efficient generation of CLPs and LMPPs (13–17). Using mixed BM chimeras, we first confirmed that Flt3 signaling was important for the generation of the lymphoid progenitors LMPPs and CLPs (Fig. 2A, 2B), which was previously suggested in Flt3 ligand-deficient mice (13, 15–17). Because we could not use Flt3 itself to define lymphoid progenitors derived from the Flt3<sup>−/−</sup> progenitors, we instead used L-selectin (CD62L). L-selectin has been suggested to distinguish lymphoid progenitors from myeloid progenitors within the BM (18), and in this study we show that L-selectin is a valid marker to replace Flt3 in defining CLPs (Supplemental Fig. 2D). More than 90% of BM Lin<sup>−</sup>Sca-1<sup>hi</sup>Kit<sup>lo</sup>IL-7R<sup>a</sup>L-selectin<sup>hi</sup> cells expressed a high level of Flt3 consistent with the phenotype of CLPs, whereas L-selectin<sup>−</sup> cells were almost entirely negative for Flt3 (Supplemental Fig. 2D). Conversely, L-selectin<sup>hi</sup> cells constituted most (>90%) total CLPs (Lin<sup>−</sup>Sca-1<sup>−</sup>Kit<sup>lo</sup>IL-7R<sup>a</sup>L-selectin<sup>hi</sup>) (data not shown). Thus, we were able to identify CLPs from Flt3<sup>−/−</sup> donor progenitors as Lin<sup>−</sup>Sca-1<sup>−</sup>Kit<sup>lo</sup>IL-7R<sup>a</sup>L-selectin<sup>hi</sup> cells. Flt3<sup>−/−</sup> BM cells gave rise to a greatly reduced number of CLPs in competition with wild-type (WT) BM cells (Fig. 2A, 2B), confirming the
requirement of Flt3 signaling for the efficient generation of these lymphoid progenitors (13, 15–17). The reconstitution of Mac-1+Gr-1+ granulocytes remained intact despite the absence of Flt3, indicating that Flt3 signaling is dispensable for myeloid development (Fig. 2A, 2B). Similar to T cells and NK cells, NH cell development was also dependent on Flt3 (Fig. 2A, 2B). Frequencies of donor-derived NH cells were reduced >10-fold in the absence of Flt3. We cultured NH cells in vitro and confirmed that Flt3 knockout progenitors gave rise to ~10-fold fewer IL-5– and IL-13–producing functional NH cells (Fig. 2C). These results indicate that Flt3 signaling is important for the development of NH cells. NH cells themselves do not express Flt3, and their proliferation and activation are unaffected by Flt3 ligand (Supplemental Fig. 1A and data not shown); thus, Flt3 signaling is required at earlier stages of NH cell development. We reason that NH cells, similar to T cells and NK cells, likely derive from Flt3/Likt3 ligand-dependent hematopoietic progenitors, most likely LMPPs and CLPs (13, 15–17).

NH cells derive from lymphoid progenitors

We next directly tested the capability of purified populations of hematopoietic progenitors to generate NH cells in vivo. Sorted BM hematopoietic stem cells (Lin− Sca-1+Kit+Flt3hi), LMPPs (Lin− Sca-1+Kit+Flt3lo), CLPs (Lin− Sca-1+Kit+IL-7R−Flt3hi), and myeloid–erythroid progenitors (Lin− Sca-1−Kit+) were competed with a radioprotective dose of CD45.1 host-type competitor WT BM cells. At 7 d posttransplant, none of these progenitors gave rise to NH cells (data not shown). At 14 d posttransplant, however, NH cells developed from hematopoietic stem cells and FLt3芝 LMPPs and CLPs, a finding consistent with the Flt3 dependency of NH cell development (Fig. 3A, 3B). No detectable NH cells were generated by myeloid–erythroid progenitors (Fig. 3A, 3B). Lymphoid progenitors gave rise to functional NH cells, which produced IL-5– and IL-13–producing NH cells, whereas myeloid–erythroid progenitors did not give rise to type 2 cytokine-producing functional NH cells (Fig. 3C). Lymphoid progenitor-derived NH cells expressed a high level of GATA3, a major Th2-associated transcription factor (Fig. 3D). They also expressed mRNA for IL-4, another type 2 cytokine, but did not produce IL-4 protein (data not shown). Expression of CD3ε was not detected in the lymphoid progenitor-derived NH cells, distinguishing them from T cells (Fig. 3D). RAG-2−/− CLPs and LMPPs also efficiently gave rise to NH cells, confirming that NH cells in these assays are innate lymphocytes (Fig. 3E). Because the myeloid–erythroid progenitors are ~10-fold more abundant in BM (19), we repeated these experiments using 20 times as many myeloid–erythroid progenitors as LMPPs and CLPs. Again, donor-derived NH cells were not detected from myeloid–erythroid progenitors but were easily detected from LMPPs and CLPs (Fig. 3E). Interestingly, LMPPs were more efficient than CLPs in generating NH cells (Fig. 3E, 3C, 3E), a difference that is similar to the previously noted difference in the efficiency with which these progenitor populations generate T cells in vivo (15, 20). It remains to be determined whether NH cells can directly derive from LMPPs, as has been suggested for T cells (15), or whether LMPPs are more efficient progenitors of NH cells because they efficiently generate CLPs (20). However, results from this experiment indicate that lymphocyte progenitor populations of LMPPs and CLPs, but not myeloid–erythroid progenitor populations, possess the potential to efficiently develop into NH cells in vivo.

In summary, we have established that NH cells are a new type of innate lymphocyte that derives from lymphoid progenitors. We demonstrate that the development of NH cells depends on Flt3 signaling, which is required for the efficient generation of lymphoid progenitors. Most NH cells are marked with a history of RAG-1 expression and derive from BM lymphoid but not myeloid–erythroid progenitors in vivo. Although we cannot exclude the possibility that a rare subset of myeloid–erythroid progenitors might possess the potential to give rise to NH cells, our results from multiple lines of evidence suggest a predominantly lymphoid origin of NH cells. The possibly overlapping yet distinct molecular pathways that orchestrate development of NH cells and other innate and adaptive lymphocytes are an important area for future research.

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Disclosures

The authors have no financial interests of interest.

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