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SHORT COMMUNICATION

Retinoic acid receptor activity is required for the maintenance of type 1 innate lymphoid cells

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Abstract

Group 1 innate lymphoid cells (G1-ILCs) are innate immune effectors critical for the response to intracellular pathogens and tumors. G1-ILCs comprise circulating natural killer (NK) cells and tissue-resident type 1 ILCs (ILC1s). ILC1s mainly reside in barrier tissues and provide the initial sources of interferon-γ (IFN-γ) to prime the protecting responses against infections, which are followed by the response of recruited NK cells. Despite such distribution differences, whether local environmental factors influence the behavior of NK cells and ILC1s is unclear. Here, we show that the signaling of retinoic acid (RA), active metabolites of vitamin A, is essential for the maintenance of ILC1s in the periphery. Mice expressing RARα403, a truncated form of retinoic acid receptor α (RARα) that exerts dominant negative activity, in a lymphoid cell- or G1-ILC-specific manner showed remarkable reductions of peripheral ILC1s while NK cells were unaffected. Lymphoid cell-specific inhibition of RAR activity resulted in the reduction of PD-1$^+$ ILC progenitors (ILCPs), but not of common lymphoid progenitors (CLPs), suggesting the impaired commitment and differentiation of ILC1s. Transcriptome analysis revealed that RARα403-expressing ILC1s exhibited impaired proliferative states and declined expression of effector molecules. Thus, our findings demonstrate that cell-intrinsic RA signaling is required for the homeostasis and the functionality of ILC1s, which may present RA as critical environmental cue targeting local type 1 immunity against infection and cancer.

Keywords: ILC1, NK cell, vitamin A

Introduction

Group 1 innate lymphoid cells (G1-ILCs) play vital roles in response to intracellular infections and tumors (1, 2). G1-ILCs contain circulating natural killer (NK) cells and tissue-resident type 1 ILCs (ILC1s), with similar features such as the expression of surface NK1.1 and NKp46, transcription factor T-bet, and production of abundant interferon-γ (IFN-γ) (1, 3, 4). In contrast, murine ILC1s are generally distinguished from NK cells by expression of CD49a as well as a lack of CD49b and transcription factor Eomes (5–7). The developmental trajectory of ILC1s also clearly differs from NK cells, as evidenced by the fact that common lymphoid progenitors (CLPs) can generate both NK cells and ILC1s while PLZF$^+$PD-1$^+$ ILC progenitors (ILCPs), a progeny of CLPs, are committed to ILC1s, ILC2s and ILC3s but not to NK cells (8–10). Recent studies have also shed light on the unique ILC1 roles as the early IFN-γ producers and the local mediators of type 1 immune responses during virus infection and liver injury (11–13). Another important feature of ILC1s is heterogeneity among tissues; NK cells are observed as relatively uniform populations through the body whereas ILC1s represent highly diverse surface molecule expression and transcription factor requirements across organs (7, 14, 15), suggesting that tissue-specific cues play key roles in ILC1 regulation. However, how local environmental factors differentially regulate NK cells and ILC1s to maintain their pool size and the proper functionality is unclear.

Retinoic acid (RA), highly bioactive metabolites of vitamin A, is essential for various biological processes such as embryonic development, epithelial integrity and immune function (16, 17). Vitamin A deficiency is associated with increases in various infectious diseases such as diarrhea, respiratory infections including measles and human immunodeficiency

virus (HIV) infection (18, 19). Mechanistically, RA locally produced by retinaldehyde dehydrogenases (RALDH)-expressing cells binds to target cell retinoic acid receptors (RARα, β and γ), that form heterodimers with retinoid X receptors (RXRs). RAR/RXR complexes bind to RA response elements (RAREs) to directly modulate expression of vast target genes including immune-related genes such as *Nfatc1* (20), *Il22* (21), *Il9* (22) and *Rorc* (23). RA thereby regulates T cell function, localization and effector differentiation in the intestine (17, 24). RA is also involved in ILC regulation: promoting the migration and development of ILC3s and inhibiting ILC2 development in the intestine (23, 25–27). However, despite the well-known relevance between RA and infection prevention, how RA influences NK cells and ILC1s still remains unknown.

To address this question, we examined the lymphoid cell- and G1-ILC-intrinsic requirements for RAR activity *in vivo* by using mice carrying RARα403 transgenes, a truncated form of RARα that can bind to ligands but no longer modulates transcription (28), in the Rosa26 locus (Rosa26-RARα403/+ mice) (29). Mice in which RA signaling is specifically inhibited in lymphoid cells (Il7r-Cre Rosa26-RARα403/+ mice) lacked ILC1s, in contrast to unaffected NK cells, in the liver and mesenteric lymph nodes (mLNs). In addition, ILCPs were decreased in Il7r-Cre Rosa26-RARα403/+ mice, suggesting that commitment and differentiation of ILCs depend on RAR activity. Moreover, we demonstrated that ILC1s depended on RA signaling in a cell-intrinsic manner by using Ncr1-Cre Rosa26-RARα403/+ mice, in which RAR activity is ablated specifically in G1-ILCs. Transcriptome analysis showed the impaired proliferative and functional signatures in ILC1s of Ncr1-Cre Rosa26-RARα403/+ mice. Thus, our findings reveal that RA signaling is critical for the homeostasis and functionality of peripheral ILC1s.

Methods

Mice

C57BL/6 mice were purchased from Japan SLC (Hamamatsu, Japan). Mice with an age of about 6- to 12-weeks and on a C57BL/6 background were analyzed. Rosa26-RARα403/+ mice (29) were kindly provided by Dr Cathy L. Mendelsohn (Columbia University, USA) and crossed with either Ncr1-Cre transgenic (Tg) mice (30) provided by Dr Veronika Sexl or Il7r-Cre knock-in (KI) mice (31) provided by Dr Hans-Reimer Rodewald. All mice were maintained under specific pathogen-free conditions in the Experimental Research Center for Infectious Diseases in the Institute for Life and Medical Sciences, Kyoto University. All procedures were carried out under sevoflurane or isoflurane anesthesia to minimize animal suffering. All mouse protocols were approved by the Animal Experimentation Committee of the Institute for Life and Medical Sciences, Kyoto University.

Cell preparation and isolation

Liver and mLNs were dissociated mechanically and passed through 70-μm cell strainers (Greiner Bio-One, Milan, Italy). Adult liver leukocytes were then separated by centrifugation through 40% Percoll. Bone marrow (BM) cells were obtained by flushing out the marrow fraction of femurs and tibias using a syringe with a 27 G needle (Terumo Corporation, Tokyo, Japan).

Flow cytometry and cell sorting

Flow cytometry and cell sorting were performed on BD FACSVerse or BD LSRFortessa X-20 flow cytometers (BD Biosciences) and BD FACS Aria II or Aria III cell sorters (BD Biosciences), respectively. Data were analyzed on FlowJo software (FlowJo, Ashland, OR, USA). Debris and dead cells were excluded from analysis by forward and side scatter and propidium iodide (PI) gating. In figures, values in quadrants, gated areas and interval gates indicate percentages in each population. For antibody staining, the following fluorescent dye- or biotin-conjugated antibodies were used: CD3ε (145-2C11), NK1.1 (PK136), NKp46 (29A1.4), CD49a (HMα1), CD49b (DX5), IL-7Rα (A7R34), F4/80 (BM8), Gr-1 (RB6-8C5), CD19 (6D5), B220 (RA3-6B2), TCRβ(H57 – 597), FcεRI (MAR-1), PD-1 (29F.1A12), α4β7 (DATK32), Flt3 (A2F10), CD25 (PC61), Ki-67 (SolA15), Bcl-2 (BCL/10C4) and RORγt (Q31-378) (BioLegend, San Diego, CA, USA; Thermo Fisher Scientific, Waltham, MA, USA; BD Bioscience, San Jose, CA, USA; TONBO Biosciences, San Diego, CA, USA). Early apoptosis of NK cells and ILC1s was detected using a MEBCYTO Apoptosis Kit (MBL Life Science, Nagoya, Japan), and live annexin V$^+$ cells were termed as "Annexin V$^+$ cells". Biotinylated monoclonal antibodies were detected with Brilliant Violet 421-conjugated streptavidin (Thermo Fisher Scientific).

RNA sequencing (RNA-seq) and data analysis

NK cells (CD49a$^-$CD49b$^+$) and ILC1s (CD49a$^+$CD49b$^-$IL-7R$^+$) were freshly sorted (2×10^2 cells) from control or Ncr1-Cre Rosa26-RARα403 mice, lysed with Buffer RLT (Qiagen, Hilden, Germany), and purified with RNAClean XP (Beckman Coulter, Brea, CA, USA). Double strand cDNA was synthesized, and sequencing libraries were constructed using a SMART-seq HT Plus kit (Takara Bio, Otsu, Japan). Sequencing was performed with 150 bp paired-end reads on the Illumina HiSeq X (Illumina, San Diego, CA, USA) sequencer. fastp (32) was used to assess sequencing quality and to exclude low-quality reads and adaptor contaminations. Reads were mapped on the mouse reference genome (mm10) using HiSat2. The read counts were determined at the gene level with featureCounts. Normalization of gene expression levels and differential gene expression analysis were performed using DESeq2. Genes were considered as differentially expressed genes (DEG) when they had an adjusted p (p_{adj}) value < 0.3. Metascape (33) was used for enrichment analysis.

Statistical analysis

Statistical differences were evaluated by the two-tailed unpaired Student's *t*-test and one-way analysis of variance (ANOVA) using GraphPad Prism 8 (GraphPad Software, San Diego, California, USA). Asterisks in all figures indicate as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$.

Data availability

The accession number for RNA-seq data of G1-ILCs in control or Ncr1-Cre Rosa26-RARα403 mice generated in this study is deposited in Gene Expression Omnibus (GEO): GSE205895.

Results and discussion

Lymphoid cell-specific inhibition of RAR activity depletes ILC1s

Vitamin A and RA promote ILC3 development whereas they suppress the population sizes of ILC2s (23, 26, 27). Although the overall cellularity of NK1.1$^+$ G1-ILCs is unaffected by RA signaling inhibition (26, 27), how RA impacts NK cells and ILC1s individually remains unclear. To address this question, we generated Il7r-Cre Rosa26-RARα403/+ mice (Il7r-Cre^{RARα403} mice) (29), in which RAR activity was inhibited in all lymphoid lineage cells (lymphoid lineage precursors such as CLPs, conventional and unconventional T cell and B cell lineages, and all ILCs and ILCPs) (31) in a dominant negative manner. In flow cytometry (FCM) analysis, NK cells and ILC1s were identified as CD49a$^-$CD49b$^+$ and CD49a$^+$CD49b$^-$ populations, respectively, within CD3$^-$NK1.1$^+$NKp46$^+$ G1-ILCs in the liver and the mLNs (Fig. 1(A and B)). In both Il7r-Cre Rosa26-+/+ (Il7r-Cre^{WT}) control mice and Il7r-Cre^{RARα403} mice, CD3$^+$ T cells and CD19$^+$ B cells were normally developed (Fig. 1(C and D)). NK cells were also unchanged in Il7r-Cre^{RARα403} mice (Fig. 1(E)). Surprisingly, Il7r-Cre^{RARα403} mice mostly lacked ILC1s in the liver and the mLNs (Fig. 1(F)). In addition, CD3$^+$NK1.1$^+$ NKT cells were also reduced in Il7r-Cre^{RARα403} mice (Fig. 1(G)). These results show that RAR activity impacts the homeostasis of innate-like lymphocytes in a subset-dependent manner and is especially critical for the presence of ILC1s.

RAR activity is involved in the ILC commitment and differentiation

To address the effects of RAR activity in ILC development, we examined whether RARα403 expression impacts the precursor fractions of ILCs, such as CLPs (Lin$^-$IL-7R$^+$FLT3$^+$) and ILCPs (Lin$^-$IL-7R$^+$FLT3$^-$α4α7$^+$PD-1$^+$) (34) (Fig. 2(A)). The frequency of ILCPs decreased in BM of Il7r-Cre^{RARα403} mice compared to controls, whereas the frequency of CLPs was slightly increased ($p = 0.064$) (Fig. 2(B)). We next analyzed the ILCP progenies other than ILC1s. Consistent with previous studies (23, 26), the cell number of ILC3s (Lin$^-$IL-7R$^{+ROR\gamma t+}$) were decreased in mLNs of Il7r-Cre^{RARα403} mice (Fig. 2(C)). In addition, ILC2s (Lin$^-$IL-7R$^+$FLT3$^-$CD25$^+$) were also reduced in BM and mLNs of Il7r-Cre^{RARα403} mice (Fig. 2(D)), suggesting that the effect of systemic vitamin A deficiency and treatment of RA antagonists examined so far, that suppress the ILC2 number (27, 35), may differ from that of cell-intrinsic inhibition of RA signaling. It is also possible that occupancy of RXRs by RARα403 might exert unexpected effects on the function of other RXR partners, such as VDR and PPARs (36, 37), although minimal levels of expression of these genes were detected at least in NK cells and ILC1s (data not shown). Collectively, these results show that RAR activity is required for ILCPs but not CLPs, suggesting that RA promotes commitment and differentiation of ILCs but not overall development of lymphocytes.

ILC1s require RA signaling for their homeostasis in a cell-intrinsic manner

To address the G1-ILC-intrinsic requirement for RAR activity, we analyzed Ncr1-Cre Rosa26-RARα403/+ mice (Ncr1-Cre^{RARα403} mice) compared with Ncr1-Cre Rosa26-+/+ (Ncr1-Cre^{WT}) controls. As expected, T cells and NKT cells were unchanged in Ncr1-Cre^{RARα403} mice (Fig. 3(A and B)), consistent with specific G1-ILC targeting of Ncr1-Cre mice (30). Notably, ILC1s, but not NK cells, were significantly reduced in the liver of Ncr1-Cre^{RARα403} mice (Fig. 3(C and D)). Similar trends were observed in the small intestine and the spleen (data not shown). These results demonstrate the cell-intrinsic requirement for RAR activity in ILC1s across organs.

RA signaling maintains the proliferative state and functionality of ILC1s

An ILC1-restricted reduction in Ncr1-Cre^{RARα403} mice indicates that RA signaling directly modulates the cellular state of mature ILC1s. To address the RA signaling-mediated effects in detail, we conducted bulk (RNA-seq) experiments on liver NK cells, liver ILC1s, and spleen ILC1s freshly sorted from control or Ncr1-Cre^{RARα403} mice. In RNA-seq experiments, liver and spleen ILC1s were identified as CD49a$^+$CD49b$^-$IL-7R$^+$ G1-ILCs to achieve further purification of ILC1s (9) (Supplementary Fig. S1(A)). Genes significantly downregulated in Ncr1-Cre^{RARα403} mice compared to controls (down-DEGs) were detected and calculated in each G1-ILC population: 8 genes for NK cells, 56 genes for liver ILC1s and 42 genes for splenic ILC1s (Fig. 4(A) and Supplementary Figure S1(B)), confirming the larger impacts of RAR activity against ILC1s than NK cells. Enrichment analysis using Metascape (33) revealed that both down-DEGs of liver and splenic ILC1s were enriched with the pathways related to proliferation and cell cycle (Fig. 4(B, C, and D)), whereas down-DEGs of NK cells had no enriched pathway. Consistent with this, *Mki67* gene and Ki-67 protein expression were reduced in liver ILC1s in Ncr1-Cre^{RARα403} mice (Fig. 4(E and F)). We found no change in their Bcl-2 expression and the frequency of Annexin V$^+$ cells (Fig. 4(G and H)), suggesting that the ILC1 survival is intact in Ncr1-Cre^{RARα403} mice. These data suggest that RA activity supports the proliferation of ILC1s.

Interestingly, down-DEGs of liver ILC1s contained several chemokines (*Ccl3*, *Ccl4* and *Xcl1*) (Fig. 4(I)), suggesting impaired functionality. Since ILC1s localize in the frontline of infection and play a critical role in the priming of type 1 immune responses (11), it is possible that ILC1s are involved in leukocyte recruiting or directly suppress viruses via these chemokines (38). In addition, five genes were identified as the intersection of down-DEGs in liver and splenic ILC1s (Fig. 4(J)), in which *Egr1* was the most variant one. RARs directly bind to RARE at the 5'-proximal region of *Egr1* and promote its expression (39–41). EGR1 has been reported to enhance chemokine expression including CCL3 and CCL4 (42–44). Thus, these results suggest the possibility that RAR activity promotes chemokine-mediated ILC1 functions via direct induction of EGR1.

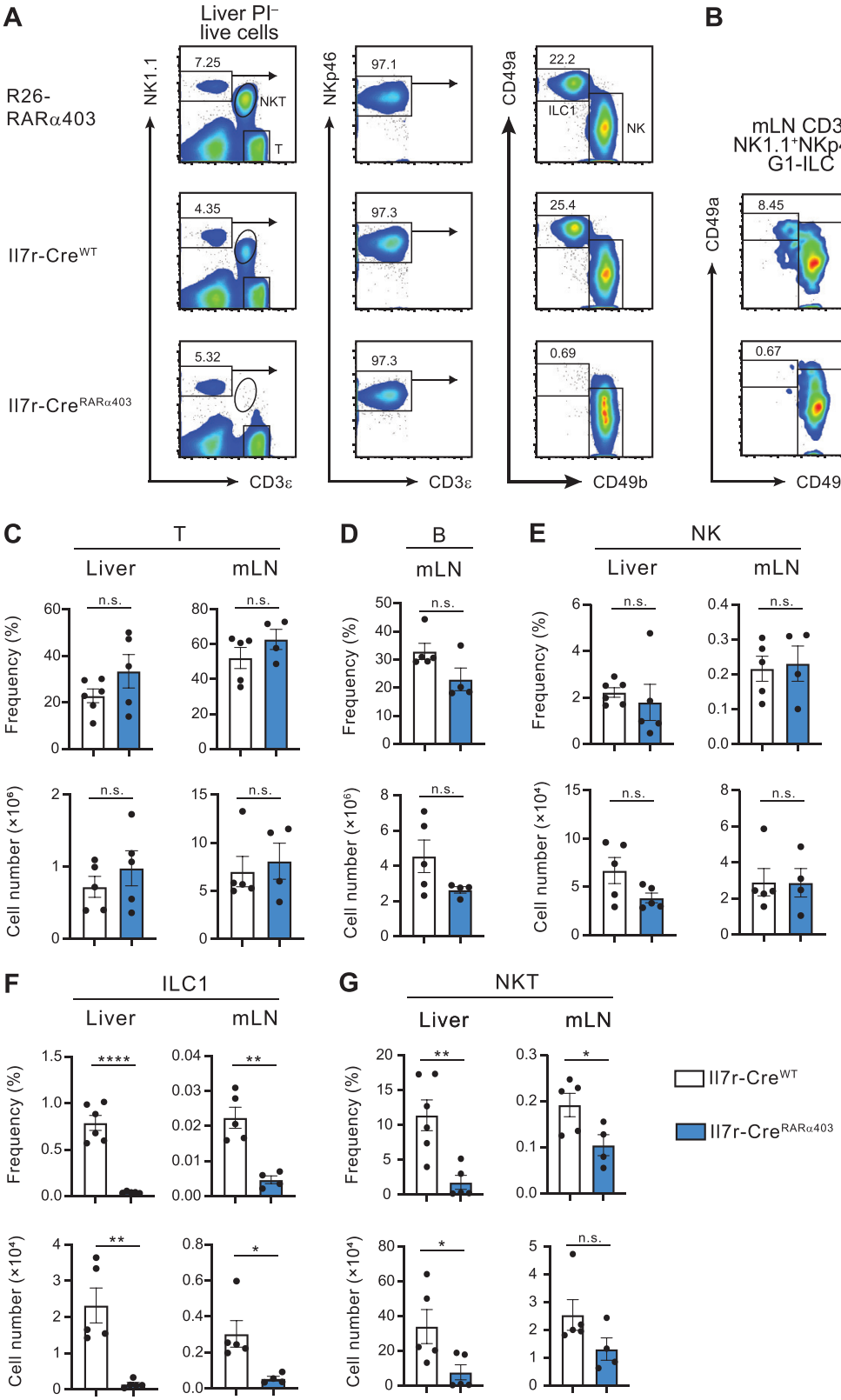

Fig. 1. Lymphoid cell-specific inhibition of RAR activity depletes ILC1s but not NK cells. (A–G) Flow cytometric (FCM) analysis of liver and mLN lymphocytes in Rosa26-RARα403/+ mice, Il7r-Cre^{WT} mice or Il7r-Cre^{RARα403} mice. Representative FCM profiles in the liver (A) and mLNs (B), the percentages (*upper*) and the cell numbers (*lower*) of T cells (C), B cells (D), NK cells (E), ILC1s (F) and NKT cells (G) in indicated tissues are shown. Data represent two to three independent experiments ($n = 4–6$). Data are presented as mean ± SEM. *$p < 0.05$, **$p < 0.01$ and ****$p < 0.0001$.

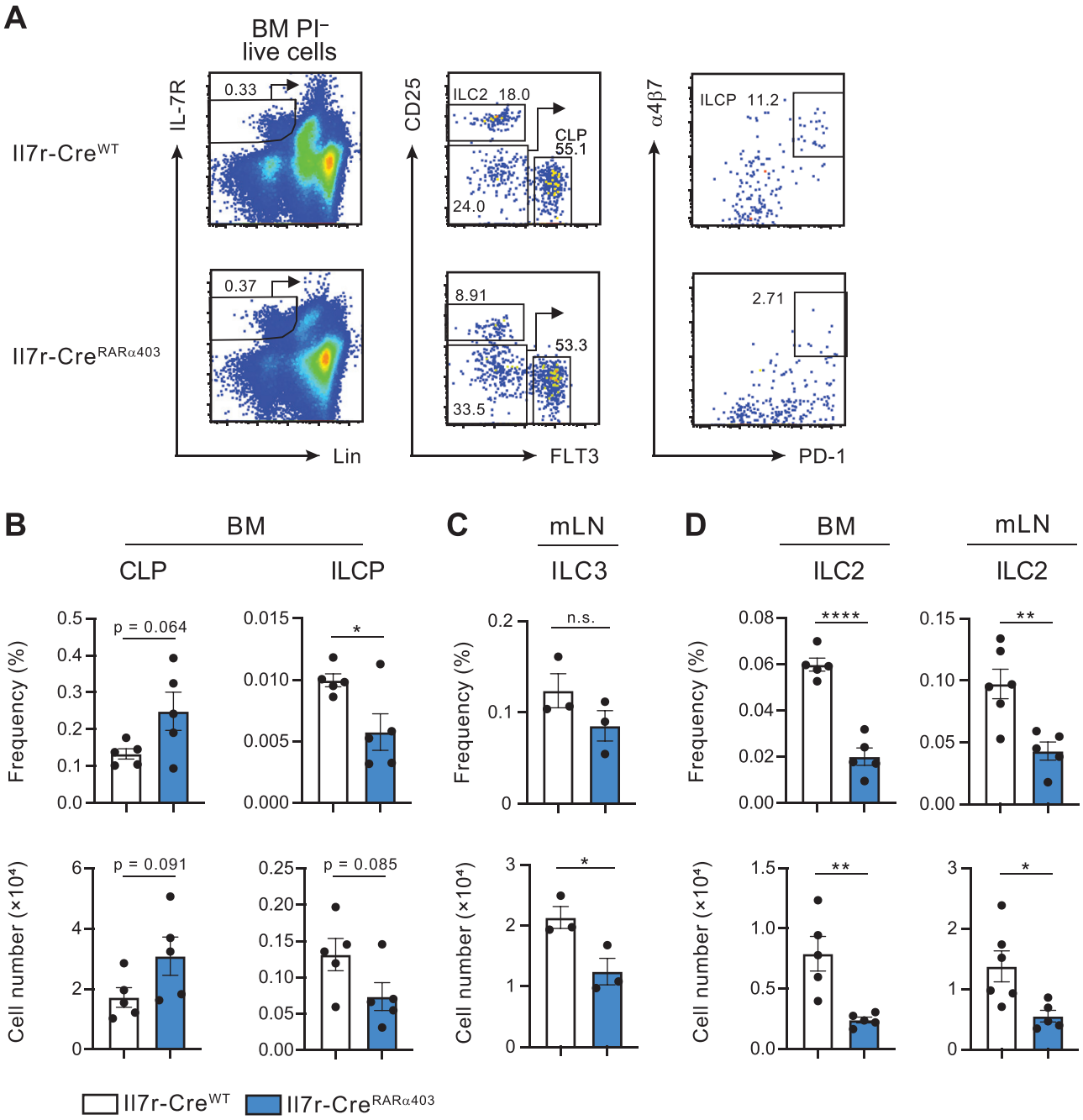

Fig. 2. RA signaling is required for the development of ILCPs. (A–D) FCM analysis of CLPs, ILCPs, ILC3s and ILC2s in Il7r-Cre^{WT} (control) or Il7r-Cre^{RARα403} mice. Representative FCM profiles in BM (A) and the percentages (*upper*) and the cell numbers (*lower*) of CLPs and ILCPs (B), ILC3s (C) and ILC2s (D) in indicated tissues are shown. Data represent two to three independent experiments (n = 3–6). Data are presented as mean ± SEM. *$p < 0.05$, **$p < 0.01$ and ****$p < 0.0001$.

Accumulating evidence has shown that RA is a critical niche factor regulating development, maintenance and function of immune cells such as intestinal induced Tregs (iTregs) (45) and peritoneal macrophages (46). Given that, it is possible that RA-producing cells across multiple tissues provide niches for ILC1s, which locally maintain the ILC1 pool size and proper functionality to immediately respond to infection. Therefore, it would be increasingly critical to determine the precise RA source for ILC1s. In the liver, RALDH expression is detected in hepatic stellate cells (47), liver sinusoidal endothelial cells (LSECs) (48) and hepatocytes (49), although their roles *in vivo* have not been determined. Future investigations addressing the actual RA source for ILC1s will lead to further understanding of the mechanism of local immune regulation. Taken together, our findings have presented RA as a possible key regulator for the local type 1 immunity and provide mechanistic insights into the critical roles of vitamin A in infection prevention.

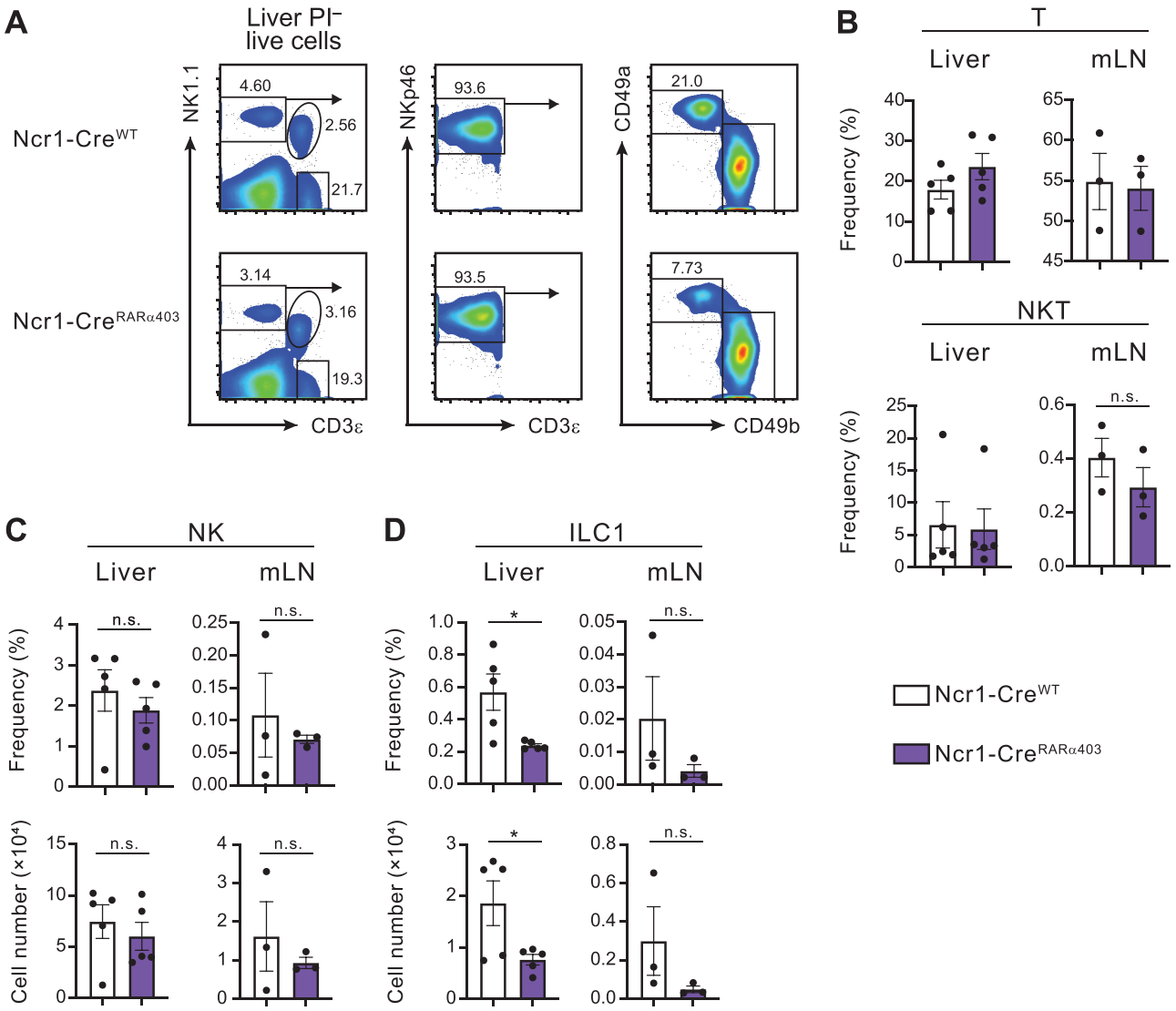

Fig. 3. Cell-intrinsic RAR activity is required for the maintenance of peripheral ILC1s. (A–D) FCM analysis of liver and mLN lymphocytes in Ncr1-Cre^{WT} or Ncr1-Cre^{RARα403} mice. Representative FCM profiles in the liver (A), the percentages of T cells and NKT cells (B) and the percentages (*upper*) and the cell numbers (*lower*) of NK cells (C) and ILC1s (D) in the liver and the mLNs are shown. Data represent two to three independent experiments (n = 3–5). Data are presented as mean ± SEM. *$p < 0.05$.

Supplementary data

Supplementary data are available at *International Immunology* Online.

Supplementary Fig. S1. Transcriptome analysis of G1-ILCs in Ncr1-Cre^{RARα403} mice. (A) IL-7R expression of the liver (*upper*) and spleen (*lower*) CD49a^{+}CD49b^{-} G1-ILCs in Ncr1-Cre^{WT} or Ncr1-Cre^{RARα403} mice. (B) Volcano plots showing gene expression of each G1-ILC population in Ncr1-Cre^{WT} (control) mice relative to that in Ncr1-^{CreRARα403} mice. Genes significantly downregulated (red; down-DEGs) or upregulated (blue; up-DEGs) in each cell population of Ncr1-Cre^{RARα403} mice are highlighted. p_{adj}, adjusted p-value. FC, fold change. Data represent three (liver) and one (spleen) experiments (A) or are from RNA-seq experiments with three biological replicates (B).

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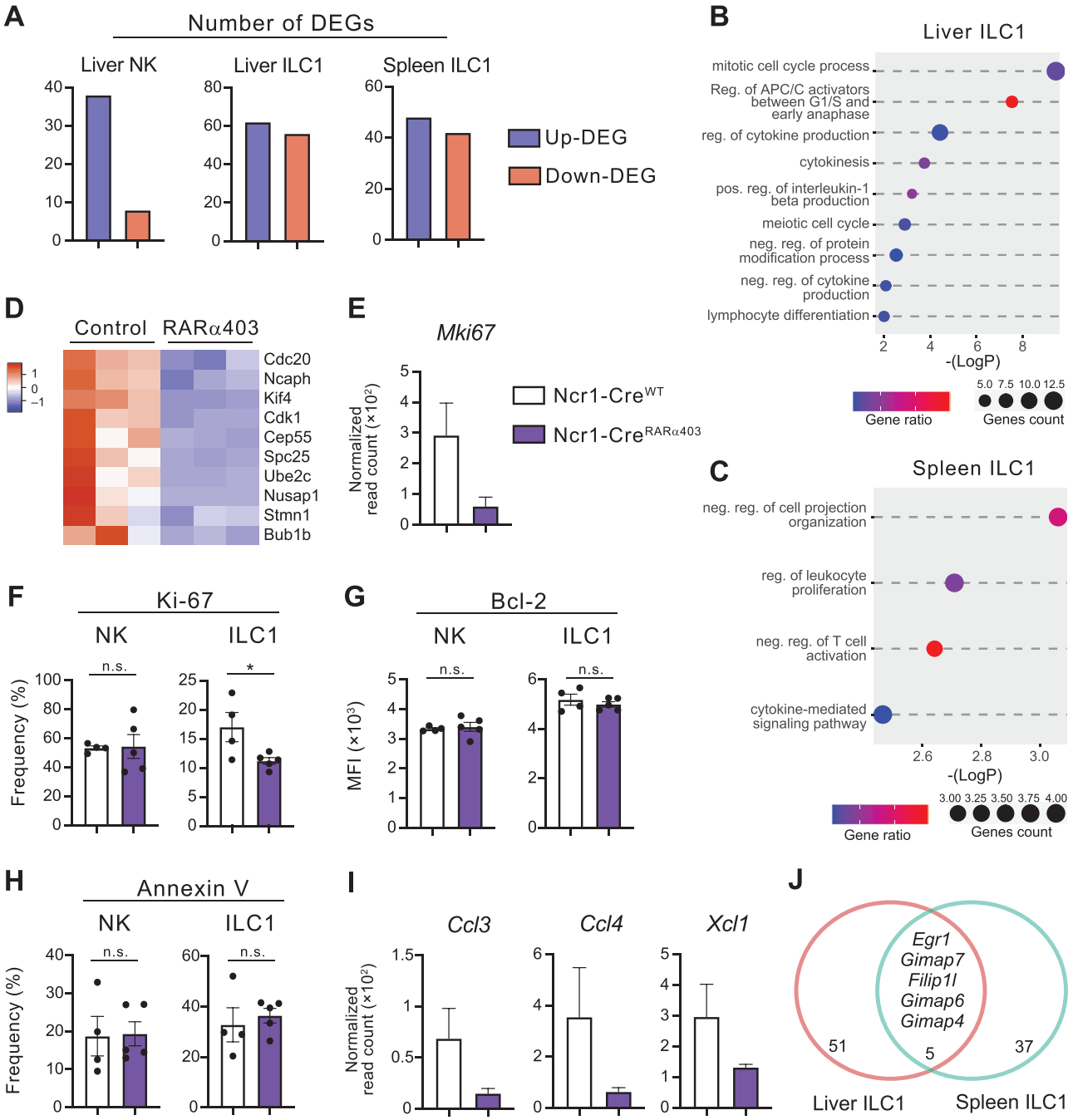

Fig. 4. RA signaling supports the proliferative and functional statuses in ILC1s. (A) The number of genes significantly upregulated (blue; up-DEGs) and downregulated (red; down-DEGs) in each cell population of Ncr1-Cre$^{RAR\alpha403}$ mice compared to Ncr1-Cre^{WT} mice are shown. (B and C) Dot plots showing the enriched pathways on down-DEGs of the liver (B) and spleen (C) ILC1s. Genes count indicates the number of DEGs included in the pathway. Gene ratio is the ratio of genes counts to the total gene number in the pathway. (D) Heatmap representing normalized expression levels of the genes related to the cell cycle in liver ILC1s from Ncr1-Cre^{WT} (control) or Ncr1-Cre$^{RAR\alpha403}$ mice followed by range scaling. (E) Normalized read counts of *Mki67* (*padj* = 0.254) expressed in liver ILC1s from control or Ncr1-Cre$^{RAR\alpha403}$ mice. (F–H) FCM analysis of proliferation and survival marker expression in liver NK cells and ILC1s of Ncr1-Cre^{WT} or Ncr1-Cre$^{RAR\alpha403}$ mice. The percentages of Ki-67$^+$ cells (F), MFI levels of Bcl-2 (G) and the percentages of Annexin V$^+$ cells (H) are shown. (I) Normalized read counts of *Ccl3* (*padj* = 0.013), *Ccl4* (*padj* = 0.203) and *Xcl1* (*padj* = 0.078) expressed on liver ILC1s from control or Ncr1-Cre$^{RAR\alpha403}$ mice. (J) Venn diagram showing the overlap between down-DEGs of liver and spleen ILC1s. Data represent two independent experiments (F–H; n = 4–5) or are from RNA-seq experiments with three biological replicates (A–E, I and J). Data are presented as mean ± SEM. *$p < 0.05$.

Conflicts of interest statement: the authors declared no conflicts of interest.

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