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Original Article

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# Bone Marrow Progenitors and IL-2 Signaling Contribute to the Strain Differences of Kidney Innate Lymphoid Cells

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**Conflict of Interest**
The authors declare no potential conflicts of interest.

**Abbreviations**
BM, bone marrow; ChILP, common helper innate lymphoid cell progenitor; CLP, common lymphoid progenitor; DEG, differentially expressed gene; GO, Gene Ontology;

## ABSTRACT

Innate lymphoid cells (ILCs) are critical immune-response mediators. Although they largely reside in mucosal tissues, the kidney also bears substantial numbers. Nevertheless, kidney ILC biology is poorly understood. BALB/c and C57BL/6 mice are known to display type-2 and type-1 skewed immune responses, respectively, but it is unclear whether this extends to ILCs. We show here that indeed, BALB/c mice have higher total ILCs in the kidney than C57BL/6 mice. This difference was particularly pronounced for ILC2s. We then showed that three factors contributed to the higher ILC2s in the BALB/c kidney. First, BALB/c mice demonstrated higher numbers of ILC precursors in the bone marrow. Second, transcriptome analysis showed that compared to C57BL/6 kidneys, the BALB/c kidneys associated with significantly higher IL-2 responses. Quantitative RT-PCR also showed that compared to C57BL/6 kidneys, the BALB/c kidneys expressed higher levels of IL-2 and other cytokines known to promote ILC2 proliferation and/or survival (IL-7, IL-33, and thymic stromal lymphopoietin). Third, the BALB/c kidney ILC2s may be more sensitive to the environmental signals than C57BL/6 kidney ILC2s since they expressed their transcription factor GATA-3 and the IL-2, IL-7, and IL-25 receptors at higher levels. Indeed, they also demonstrated greater responsiveness to IL-2 than C57BL/6 kidney ILC2s, as shown by their greater STAT5 phosphorylation levels after culture with IL-2. Thus, this study demonstrates previously unknown properties of kidney ILC2s. It also shows the impact of mouse strain background on ILC2 behavior, which should be considered when conducting research on immune diseases with experimental mouse models.

**Keywords:** Innate immunity; Innate lymphoid cells; Inbred strains; Kidney

## INTRODUCTION

Innate lymphoid cells (ILCs) are the innate counterparts of T cells. While they do not express antigen-specific receptors, they are exquisitely sensitive to cytokine signals and thus are able to respond rapidly to a dynamically changing microenvironment. This response is largely mediated by their production of immunomodulatory cytokines, which allows them

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ICOS, inducible co-stimulator; IL-2C, IL-2 complexes; ILC, innate lymphoid cell; ILC2P, innate lymphoid cell 2 progenitor; ILCP, innate lymphoid cell progenitor; KEGG, Kyoto Encyclopedia of Genes and Genomes; KLRG1, killer cell lectin-like receptor G1; MFI, mean fluorescence intensity; ns, not significant; PCA, principal component analysis; RBC, red blood cell; RNA-seq, RNA sequencing; TLSP, thymic stromal lymphopoietin; αLP, α-lymphoid progenitor.

**Author Contributions**

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to communicate with and direct various immune and non-immune cells (1). There are three ILC subsets designated group 1, 2, and 3 ILCs (ILC1s, ILC2s, and ILC3s, respectively), which share the cytokine and master transcription factor profiles as Th1, Th2, and Th17/22 cells, respectively. However, despite these similarities between ILCs and Th cells, many studies with T-cell depleted mice (*Rag1* or *Rag2* knock-out mice) show that ILCs are essential for protection against infection and other immune activities rather than being redundant to T cells. In addition, there is increasing evidence that ILCs can themselves play important pathogenic and protective roles in various diseases (2).

While ILCs were once thought to mainly reside in mucosal tissues, it is now clear that they also occupy and act in non-mucosal tissues, including fat (3), the brain (4), muscles (5), and the kidneys (6). This variation in anatomical location is significant because different locations are likely to bear disparate microenvironments and the biological functions of ILCs are highly dependent on their local context (7). For example, in the lung, ILC2s help exacerbate allergic asthma by producing type-2 cytokines in response to the alarmins (IL-33, IL-25, and thymic stromal lymphopoietin [TLSP]) that are released by the damaged tissue (8-10). Conversely, ILC2s in the lean adipose tissues may protect against metabolic diseases such as diabetes and obesity by secreting enkephalin, which induces the beiging of white adipocytes, and/or by producing IL-13, which promotes M2 macrophage polarization (11,12). With regard to ILCs in the kidney, a recent review suggests that they may suppress acute and chronic kidney diseases (13). However, at present, the underlying mechanisms are not unclear.

It is well-known that the inbred laboratory mouse strains that are used worldwide for immunological studies can differ in their responses to the same trigger (14). For example, BALB/c and C57BL/6, the two most commonly used strains, respectively exhibit type-2 and type-1 skewed immune responses. As a result, the former is favored for studying allergic diseases (15-17) and the latter for infectious diseases (18). These biases have been attributed in part to differences in preferential Th1/Th2-cell and M1/M2-macrophage polarization (18,19). However, the role of ILCs in these strain differences has to date not been extensively addressed: to our knowledge, only two comparative studies on lung ILC2s have been conducted (20,21).

During our initial explorations on the roles of ILCs in the homeostatic immunological differences between murine strains, we noted that while BALB/c mice indeed had higher ILC2 frequencies in their lung than C57BL/6 mice, this strain-related disparity was much more pronounced in the kidney. Since little is known about kidney ILCs, we explored the strain differences of these cells further. We showed that the higher ILC2 frequencies in BALB/c kidneys may reflect multiple factors, namely, greater production of ILC2s from their progenitors in the bone marrow (BM), a kidney microenvironment that is enriched in IL-2 signaling and may thereby promote ILC2 proliferation or survival, and a higher sensitivity of kidney ILC2s to the kidney microenvironmental cues.

## MATERIALS AND METHODS

### Mice

BALB/c and C57BL/6 mice (6–8 wk old, female) were purchased from Koatech (Pyeongtaek, Korea). All mice were maintained in the Seoul National University Hospital Biomedical Research Institute animal facility under specific pathogen-free conditions. All experiments

were approved by the Seoul National University Hospital Institutional Animal Care and Use Committee (approval No. 21-0278).

### Immune cell preparation

Kidney and lung tissues were minced into small pieces and digested with RPMI1640 containing 1 mg/ml of collagen IV (Worthington Biochemical, Lakewood, NJ, USA) and 50 ug/ml of DNase I (Sigma-Aldrich, St. Louis, MO, USA) for 30–60 min at 37°C. After digestion, cell suspensions were passed through 40-um strainers. The leukocytes in the kidney cells were further enriched with 40%–80% Percoll (GE Healthcare, Uppsala, Sweden) density gradient centrifugation (300 x*g*, 25 min). Red blood cells (RBCs) were lysed with RBC lysis buffer (BioLegend, San Diego, CA, USA), and the cells were washed with FACS buffer (2% bovine calf serum in PBS) for further analysis.

### Flow cytometry

To exclude dead cells from the analysis, Zombie-Aqua dye (BioLegend) was applied according to the manufacturer's instructions. Single-cell suspensions were incubated with anti-mouse CD16/32 antibody (TruStain FcX Plus; BioLegend) for 10 min. Cells were stained for 30 min with fluorochrome- or biotin-conjugated antibodies as follows. All were from BioLegend unless otherwise indicated: anti-CD45 (30-F11), anti-CD127 (A7R34), anti-CD90.2 (30-H12), anti-Flt3 (A2F10), anti-α4β7 (DATK32), anti-CD25 (PC61), anti-IL-18Rα (A17071D), anti-ST2 (RMST2-33; Invitrogen, Waltham, MA, USA), anti-IL-17RB (9B10), anti-inducible co-stimulator (ICOS, C398.4A), anti-killer cell lectin-like receptor G1 (KLRG1, 2F1/KLRG1), anti-CD117 (ACK2), Sca-1 (D7), and the Lineage antibody cocktail containing anti-CD3e (145-2C11), anti-CD19 (1D3/CD19), anti-CD11b (M1/70; BD Biosciences, Franklin Lakes, NJ, USA), anti-CD11c (HL3, BD Biosciences), anti-F4/80 (BM8), CD49b (DX5), and anti-FcεRIα (MAR-1). For intracellular transcription factor staining, cells were fixed and permeabilized with Foxp3/Transcription factor staining kit (Invitrogen). For intracellular cytokine staining, cells were restimulated with PMA (100 ng/ml; Sigma-Aldrich) and ionomycin (1 ug/ml; Sigma-Aldrich) for 3 h at 37°C, and fixed and permeabilized with BD fixation/permeabilization solution kit (BD Biosciences) for 20 min at 4°C. The permeabilized cells were incubated for 1 h with fluorochrome-conjugated antibodies as follows all from BD Biosciences unless otherwise indicated: anti-T-bet (4B10; BioLegend), GATA-3 (L50/823), ROR-γt (Q31-378), TCF-1 (S33-966), IL-5 (TRFK5; BioLegend), and IL-13 (eBio13A; Invitrogen). All samples were acquired using LSRFortessa X-20 (BD Biosciences). Data were analyzed with FlowJo v10.8.1 software (BD Biosciences).

### IL-2 induced intracellular STAT5 phospho-protein analysis

Kidney cells were stained with Zombi-Aqua dye and surface antibodies as described above. Following a washing step, the cells were initially cultured for 30 min in serum-free RPMI1640 medium at 37°C. The same volume of IL-2-containing RPMI 1640 medium was then added to the wells and the cells were further incubated for another 30 min. After washing, cells were fixed for 15 min in the pre-warmed fixation buffer (BioLegend) at 37°C and then suspended in the pre-chilled True-Phos Perm buffer (BioLegend) for an 1 h at −20°C. The samples were finally stained with pSTAT5 antibody (47/Stat5(pY694); BD Biosciences) for 30 min.

### RNA sequencing (RNA-seq) analysis

Total RNA from kidney tissue was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. RNA quality was assessed with Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). A cDNA library was generated using Lexogen

QuantSeq 3' mRNA-Seq Library Prep Kit (Vienna, Austria). Samples were sequenced with NextSeq500 system (Illumina, San Diego, CA, USA) with single-end 75 bp mode. Raw reads were trimmed with BBDuk to set Q20. Filtered reads were mapped to the reference genome (mm10) using Bowtie2 and calculated for reading counts using Bedtools. Normalization and gene expression comparison was performed using DESeq2 (version 1.32.0) in R (version 4.1.0) (22). The differentially expressed genes (DEGs) were identified as p-value <0.05 and log2 fold-change >1. A hierarchical clustering plot was generated using the pheatmap (version 1.0.12). A volcano plot was generated using the EnhancedVolcano (version 1.13.2). Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis were performed with clusterProfiler (version 4.0.5) (23). Raw data files are deposited in the National Center for Biotechnology Information Gene Expression Omnibus database under accession number GSE208404.

## Quantitative PCR analysis

RNA was extracted as prepared for RNA-seq above and reverse transcribed with a SensiFAST cDNA synthesis kit (Bioline, London, UK). Genes of interest were amplified using the SensiFAST SYBR Lo-Rox kit (Bioline). Sequences for primers (Integrated DNA Technologies, IA, USA) were as follows: *Il2*, forward 5′-GCA GGA TGG AGA ATT ACA GGA A-3′ and reverse 5′-GCA GAG GTC CAA GTT CAT CTT C-3′; *Il7*, forward 5′-CCG CAG ACC ATG TTC CAT-3′ and reverse 5′-GTC TTT AAT GTG GCA CTC AGA TG-3′; *Il25*, forward 5′-CGA TTC AAG TCC CTG TCC AA-3′ and reverse 5′-AAG TGG AGC TCT GCA TCT G-3′; *Tslp*, forward 5′-TCT CAA TCC TAT CCC TGG CT-3′ and reverse 5′-TTG TGC CAT TTC CTG AGT ACC-3′; *Il33*, forward 5′-GTG CTA CTA CGC TAC TAT GAG TC-3′ and reverse 5′-TCA TGT TCA CCA TCA GCT TCT-3′; *Gapdh*, forward 5′-AAT GGT GAA GGT CGG TGT G-3′ and reverse 5′-GTG GAG TCA TAC TGG AAC ATG TAG-3′. Gene expression was normalized to the housekeeping gene *Gapdh* according to the $2^{-\Delta Ct}$ method.

## IL-2 complex treatment

Recombinant IL-2 (1 ug; BioLegend) and anti-IL-2 (10 ug; BioLegend) were preincubated at 37°C for 30 min to form IL-2 complexes (IL-2C). Mice were injected intraperitoneally with IL-2C, daily for 2 consecutive days. Seven days after the first IL-2C injection, the frequencies of ILC2 progenitors in the BM and ILC2s in the kidney were measured by FACS analysis. In the second set of experiments, IL-2C treated mice were injected intravenously with a BV650-labeled anti-CD45 antibody (BioLegend) 5 min before sacrifice on Day 7.

## Statistical analysis

Statistical analyses were performed using Prism 9 software (GraphPad Software, San Diego, CA, USA). Data were expressed as mean ± SEM. Shapiro-Wilk test was performed to decide the normality of the data. Parametric Student's unpaired 2-tailed *t*-tests or nonparametric Mann-Whitney tests were used for 2-group comparisons. One-way ANOVA with Turkey's multiple comparisons tests or nonparametric Kruskal-Wallis tests with Dunn's multiple comparisons tests were used for 3-group comparisons. A p-value less than 0.05 was considered statistically significant.

## RESULTS

### BALB/c mice have higher ILC2 frequencies in the lung and especially the kidney than C57BL/6 mice

The ILCs in the lung have been extensively investigated, particularly with regard to their crucial pathogenic roles in asthma (1), and one study has shown that BALB/c mice bear significantly more ILC2s in the homeostatic lung than C57BL/6 mice (20). To explore this further, we first compared BALB/c and C57BL/6 mice in terms of the frequencies of total ILCs in the $CD45^+$ leukocyte population of the lung (**Fig. 1A**). As expected (20), the lungs of BALB/c mice bore 2.45-fold higher frequencies of total ILCs than the lungs of C57BL/6 mice. More interestingly, when we conducted the same analysis with the kidneys, we found the strain-related difference in total ILCs was much more pronounced: the BALB/c kidneys had 4.46-fold higher total ILC frequencies than the C57BL/6 kidneys (**Fig. 1B**). Although the pattern of higher absolute numbers in BALB/c was consistent across organs, there were significantly more ILCs in the lung regardless of strain (**Supplementary Fig. 1A**).

Therefore, we next determined the frequencies of the ILC1s, ILC2s, and ILC3s in the lungs and kidneys of the 2 mouse strains by FACS staining for the master transcription factors of these ILC types, namely, T-bet, GATA-3, and ROR-γt, respectively. The GATA-$3^+$ ILC2s were particularly prevalent in both organs and are higher in BALB/c than in C57BL/6 (kidney: 56.13% vs. 31.45%; lung: 63.84% vs. 53.36%) (**Fig. 1C**, **Supplementary Fig. 1B and C**). T-$bet^+$ ILC1s were rare in the lung (up to 5% in both strains) but prominent in the kidney (33.25% in the C57BL/6 vs. 49.56% in the BALB/c). ROR-$γt^+$ ILC3s were infrequent in the kidney regardless of strain (up to 5%); however, while the BALB/c lungs also had low ILC3 frequencies (2.17%), this ILC subset was moderately frequent in the C57BL/6 lung (19.66%) (**Fig. 1C**). Thus, the two strains showed marked differences in the ILC frequency profiles of their lungs and kidneys.

### BALB/c mice have higher ILC progenitor (ILCP) frequencies in the BM than C57BL/6 mice

We then asked whether these disparate strain-specific ILC distributions reflect fundamental differences in ILC development in the BM (**Fig. 2A**). All lymphoid lineages, including T cells, B cells, and ILCs, differentiate from the common lymphoid progenitor (CLP) in the BM; these cells are defined as $Lineage^-$ $Flt3^+$ $CD127^+$ (24,25). However, the two strains did not differ in CLP frequency (**Fig. 2B**).

CLP differentiates into either T-cell progenitor, B-cell progenitor, or the α-lymphoid progenitor (αLP), which is defined as $Lineage^-$ $α4β7^+$ $CD127^+$ and gives rise to all innate lymphocytes (**Fig. 2A**) (24,25). The BALB/c mice had significantly higher αLP frequencies and numbers in the BM than the C57BL/6 mice (**Fig. 2B**, **Supplementary Fig. 2A**). αLP differentiates into the common helper innate lymphoid cell progenitor (ChILP) and then the ILCP. Both still have multi-lineage potential but it is restricted to ILC1, ILC2, and ILC3. ChILP and ILCP are both $Lineage^-$ $α4β7^-$ $CD127^+$ cells but ILCP also expresses CD90.2 (Thy1.2). ILCP then differentiates into the ILC2 progenitor (ILC2Ps) (as well as the ILC1 and ILC3 progenitors): ILC2P bears the same surface marker profile as ILCP except that it also expresses CD25 (**Fig. 2A**) (26). The BALB/c mice had significantly higher frequencies and numbers of ChILP, ILCP, and ILC2P in their BM than the C57BL/6 mice (**Fig. 2B**, **Supplementary Fig. 2A**).


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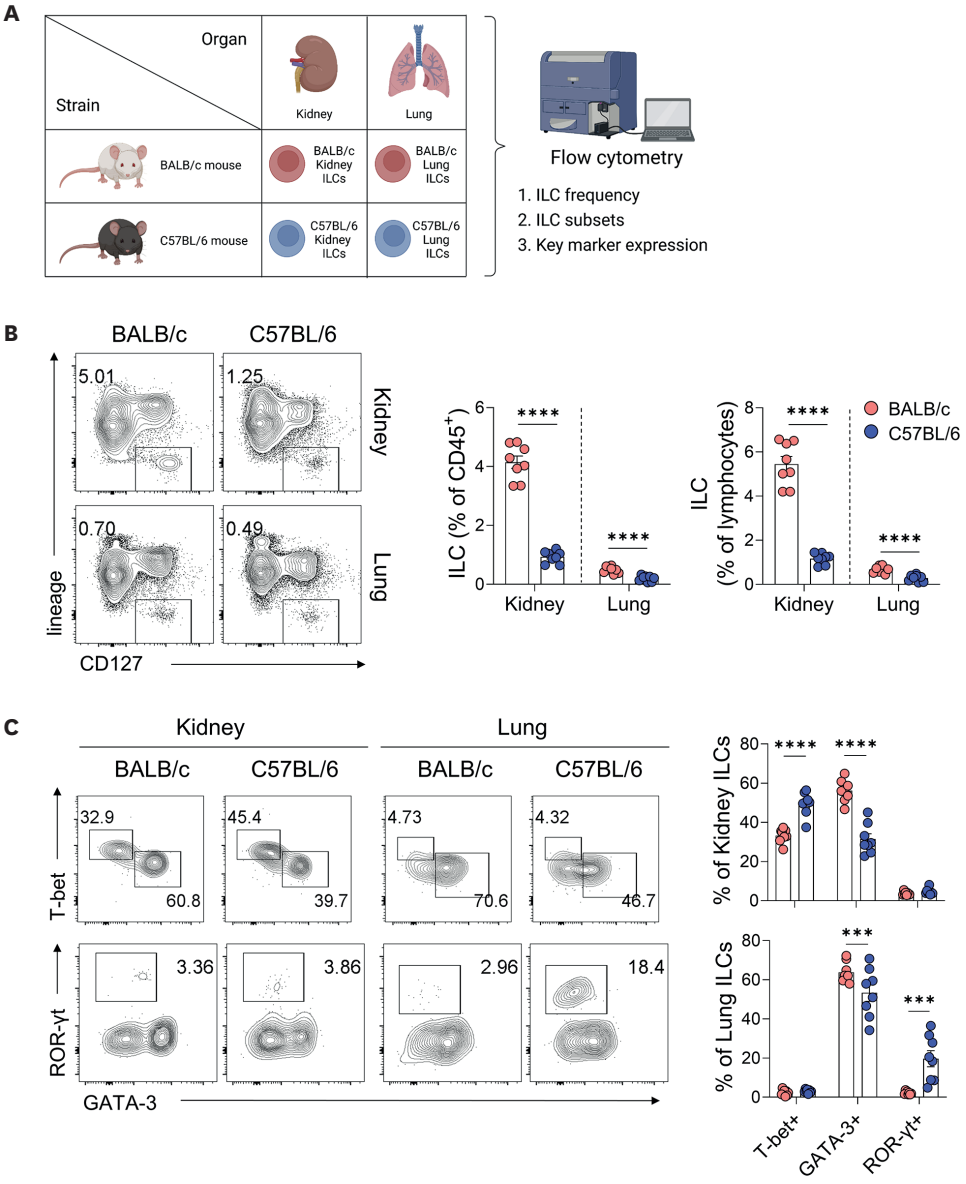


**Figure 1.** Kidney ILC2s are increased in BALB/c mice. (A) Experimental scheme. (B) Kidney and lung ILCs from naïve adult mice identified as Lineage$^-$ CD127$^+$. (C) ILC1s, ILC2s, and ILC3s gated as positive for T-bet, GATA-3, and ROR-γt, respectively. All results are shown as mean ± SEM and statistical analysis was performed using a 2-tailed Student's t-test.
***p<0.001; ****p<0.0001; n = 8–10 mice in each group.

Recent studies reported that the lungs of adult mice contain tissue ILCPs that express TCF-1 and IL-18Rα, but not ST2 (IL-33R), and that can differentiate into multiple ILC lineages *in vivo* and *in vitro* (27-29). To determine the contribution of such tissue-residing progenitors to the strain-dependent ILC frequency profiles, we compared the BALB/c and C57BL/6 mice in terms of the TCF-1$^+$ IL-18Rα$^+$ ILCP frequencies in the lungs and kidneys. The kidney had higher frequencies of these precursors than the lung (**Fig. 2C**), which is consistent with its higher frequency of total ILCs (**Fig. 1B**). The absolute number of tissue ILCPs is higher in BALB/c kidneys but their frequency is comparable between strains (**Fig. 2C**, **Supplementary Fig. 2B**).


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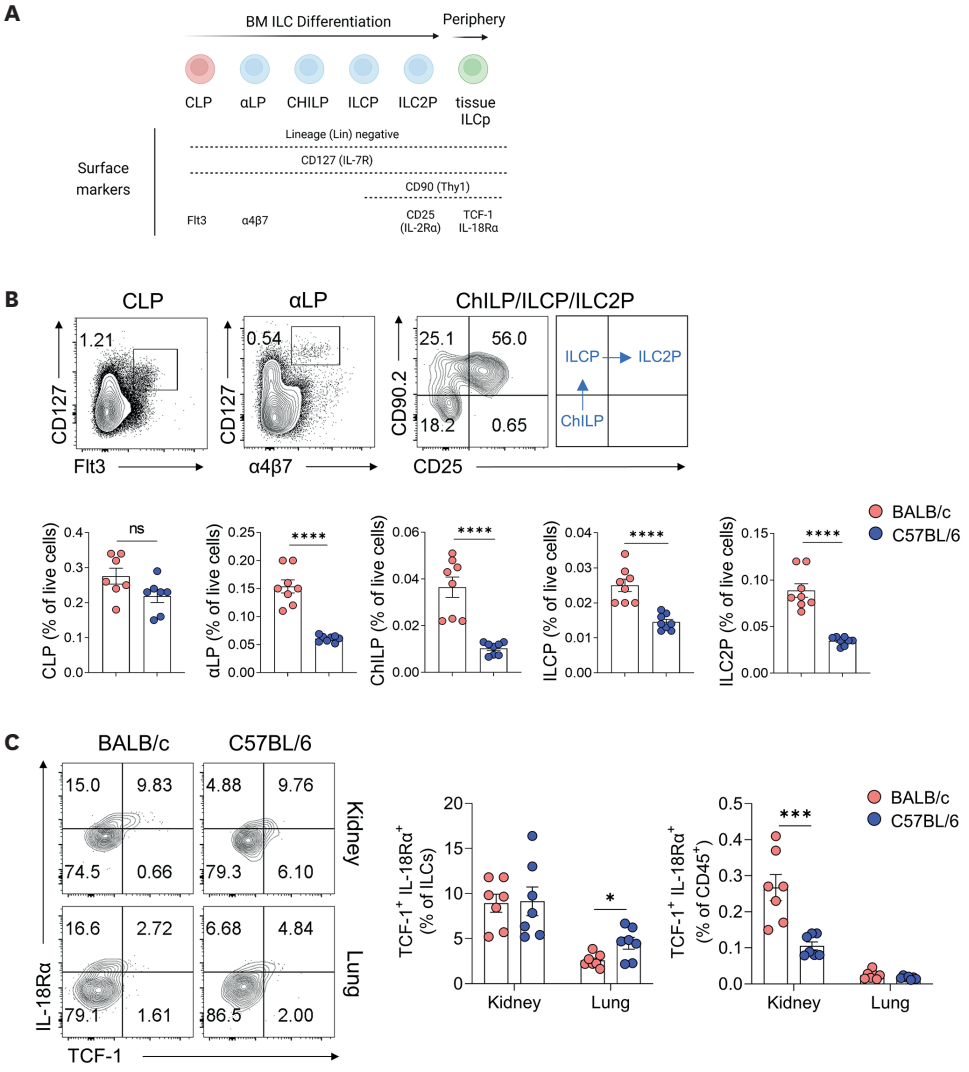


**Figure 2.** Bone marrow from BALB/c mice harbors more ILCPs. (A) Mouse BM ILC differentiation process from CLPs to ILC2ps with the key surface marker expression. (B) Comparison of BM CLP (Lineage$^-$ Flt3$^+$ CD127$^+$), αLP (Lineage$^-$ α4β7$^+$ CD127$^+$), ChILP (Lineage$^-$ CD127$^+$ CD90.2$^-$), ILCp (Lineage$^-$ CD127$^+$ CD90.2$^+$ CD25$^-$), and ILC2p (Lineage$^-$ CD127$^+$ CD90.2$^+$ CD25$^+$). (C) Tissue resident ILCPs from kidney and lung tissues identified as Lineage$^-$ CD127$^+$ IL-18Rα$^+$ TCF-1$^+$. All results are shown as mean ± SEM and statistical analysis was performed using two-tailed Student's *t*-test (B) and Mann-Whitney test (C). *p<0.05; ***p<0.001; ****p<0.0001; n=7 mice in each group.

Thus, the greater frequency of ILC2s in both the kidney and lung of BALB/c mice may be due to higher frequencies of the bone-marrow ILCP - ILC2P and its ChILP, ILCP, and αLP predecessors. Whether the differences in ChILP, ILCP, and αLP frequencies also dictate the higher ILC1 and ILC3 frequencies in the C57BL/6 kidney and lung, respectively remains to be determined. By contrast, disparate tissue ILCP frequencies do not seem to be responsible for the strain differences in ILC distributions.

## BALB/c kidneys associate with greater IL-2 responses compared to C57BL/6 kidneys, as determined by transcriptome analysis

Another, not mutually exclusive, explanation for the higher ILC2 frequencies in the BALB/c lung and kidney is that the local environment promotes the proliferation and/or survival of ILC2s. Since the kidney showed a particularly profound disparity between the mouse strains in ILC2s (**Fig. 1B**), we subjected the BALB/c and C57BL/6 kidneys to whole transcriptome sequencing


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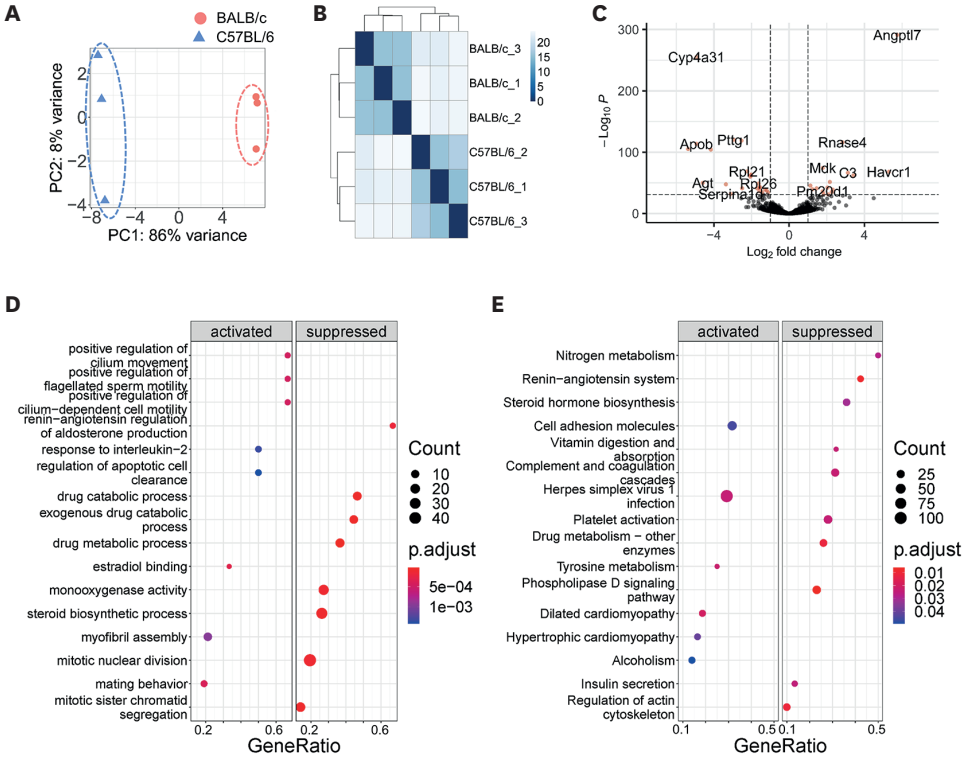


**Figure 3.** IL-2 response genes are upregulated in the BALB/c kidney. (A) PCA plot of transcriptomes of kidney cells from BALB/c and C57BL/6 (n=3 each). (B) Heat map of gene expression profiles using hierarchical clustering analysis. (C) Volcano plots with log2 fold change and −log10 p-value for the DEGs in BALB/c kidney. GO pathway (D) and KEGG analysis (E) showing the top 16 pathways (activated [upregulated] or suppressed [downregulated]) in BALB/c kidney, identified with the ClusterProfiler R package.

(RNA-seq) in triplicate followed by principal component analysis (PCA) and hierarchical clustering analysis (**Fig. 3A and B**). The PCA showed a clear separation of the 2 strains (**Fig. 3A**), as did the heatmap combined with hierarchical clustering analysis (**Fig. 3B**). Thus, the kidney transcriptomes of the strains differed significantly. The RNA-seq analyses showed that the BALB/c transcriptome contained 227 upregulated DEGs and 235 downregulated DEGs compared to the C57BL/6 kidney transcriptome (**Fig. 3C**). To further interpret these transcriptome differences, we conducted gene set enrichment analysis for GO terms and KEGG pathways using the clusterProfiler implemented in R (**Fig. 3D and E**). The GO analyses indicated that the DEGs that were upregulated in the BALB/c kidney associate with responses to IL-2 and the regulation of apoptotic cell clearance (**Fig. 3D**). Moreover, the KEGG analysis showed that the BALB/c kidneys express significantly higher levels of cell adhesion molecule-related gene sets (**Fig. 3E**). Thus, the higher frequencies of ILC2s in the BALB/c kidney may reflect greater IL-2 signaling and cell adhesion molecule expression in the microenvironment.

## BALB/c kidneys express higher levels of innate type-2 signals than C57BL/6 kidneys

IL-2 was shown to promote ILC2 proliferation and survival (30). This together with our findings above led us to speculate that the BALB/c kidneys bear higher ILC2 frequencies because of higher homeostatic IL-2 signaling in the kidney microenvironment. To test this, we examined the BALB/c and C57BL/6 kidney expression of innate cytokines that are known to contribute to ILC maintenance and activation, namely, *Il2* and *Il7* for all ILC subsets, and *Il33*, *Il25,* and *Tslp* for ILC2s (30-32). The BALB/c kidney expressed more *Il2*, *Il7, Il33*, and *Tslp*


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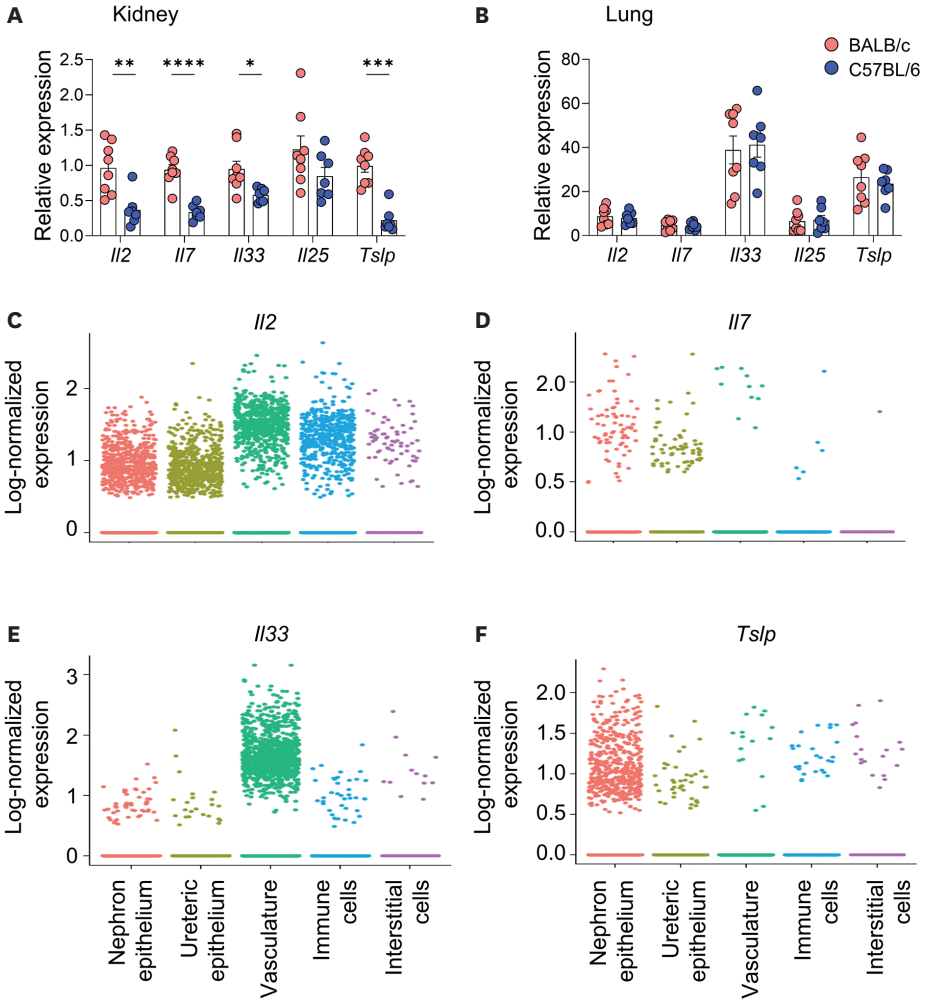


**Figure 4.** BALB/c kidney shows increased innate cytokine production for ILC2 maintenance. (A-B) Relative mRNA expression of *Il2*, *Il7*, *Il33*, *Il25*, and *Tslp* in (A) kidney and (B) lung tissues compared between BALB/c and C57BL/6 strains. (C) *Il2*, (D) *Il7*, (E) *Il33*, and (F) *Tslp* expression in the compartments of mouse kidney tissue was analyzed using a web-based public single-cell RNA-seq data platform, KidneyCellExplorer (https://cello.shinyapps.io/kidneycellexplorer/). Results of (A and B) are shown as mean ± SEM and statistical analysis was performed using a two-tailed Student's *t*-test or Mann-Whitney test (TSLP in A).
*p<0.05; **p<0.01; ***p<0.001; ****p<0.0001; n=7–8 mice in each group.

but not *Il25,* than the C57BL/6 kidney (**Fig. 4A**). Interestingly, when we conducted the same experiment with the lungs, we did not observe these strain differences (**Fig. 4B**).

Next, we checked the expression of innate cytokines in various kidney compartments by using KidneyCellExplorer, which is a web-based public dataset of mouse kidney single-cell RNA sequences (https://cello.shinyapps.io/kidneycellexplorer) (33). Several kidney compartments, namely, the nephron epithelium, the ureteric epithelium, the renal vasculature, and immune cells, frequently demonstrated strong *Il2* expression (**Fig. 4C**). By contrast, all compartments showed relatively low *Il7* expression (**Fig. 4D**), and *Il25* was not detected at all (data not shown). Notably, the vasculature and nephron epithelium respectively expressed *Il33* and *Tslp* at high levels, much higher than the other kidney compartments (**Fig. 4E and F**). Thus, these findings together suggest not only that the BALB/c and C57BL/6 kidney milieus differ, but also that the greater innate type-2 cytokine levels in the BALB/c kidney microenvironment are responsible for its higher ILC2 frequencies.


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## BALB/c kidney ILC2s demonstrate altered transcription factor, cytokine receptor, and functional regulator expression compared to C57BL/6 kidney ILC2s

Another explanation for the higher ILC2 frequencies in BALB/c kidneys is that the BALB/c kidney ILC2s are more sensitive to microenvironmental signals that promote their proliferation and/or survival. Such greater sensitivity could be induced by early imprinting events that shape the transcriptional responsiveness of the cells and their expression of key cytokine receptors and self-regulatory molecules (**Fig. 5A**). To test this, we compared the ILC2s from BALB/c and C57BL/6 kidneys in terms of their expression of 1) GATA-3, a master ILC2 transcription factor, 2) the receptors for IL-2 (CD25), IL-7 (CD127), IL-25 (IL-17RB), IL-33 (ST3), and TSLR (TSLP-R), and 3) ICOS and KLRG1, which regulate ILC2 proliferation/

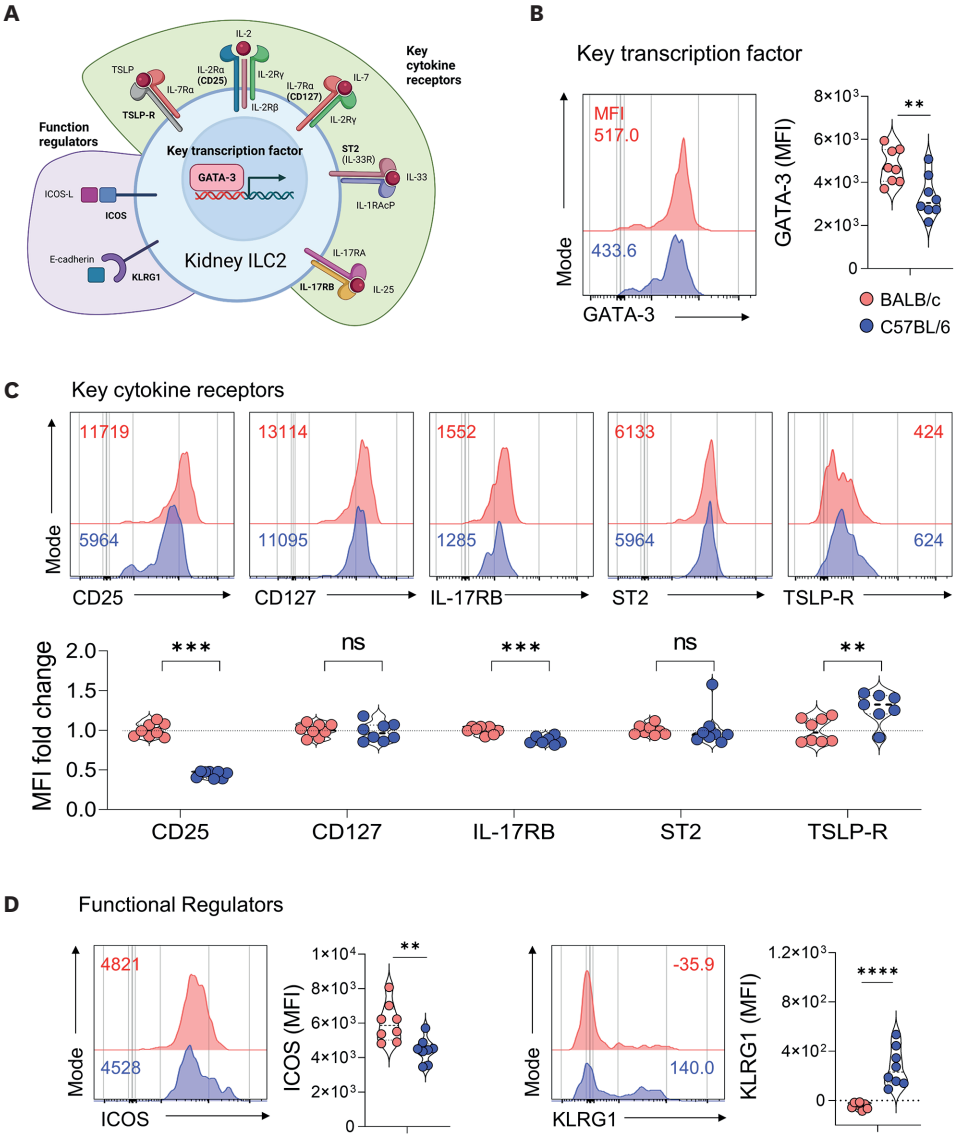


**Figure 5.** BALB/c kidney ILC2s have increased expression levels of a key transcription factor, cytokine receptors, and function regulators. (A) Illustration showing the relevance of transcription factors and surface markers in ILC2s. (B) Expression of GATA-3 (MFI), a key transcription factor of ILC2s. (C) Expression of CD25, CD127, IL-17RB, ST2, and TSLP-R (fold change of MFI). (D) Expression of ICOS and KLRG1 (MFI), functional regulators of ILC2s. All results are shown as mean ± SEM and statistical analysis was performed using a 2-tailed Student's *t*-test or Mann-Whitney test (CD25, ST2, and TSLP-R in C).
**p<0.01; ***p<0.001; ****p<0.0001; n=7–8 mice in each group.


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survival (34). Indeed, compared to the C57BL/6 kidney ILC2s, the BALB/c ILC2s expressed higher levels of GATA-3 (**Fig. 5B**), CD25, IL-17RB, and ICOS; and lower levels of the TSLP receptor and KLRG1 (**Fig. 5C and D**). However, the BALB/c and C57BL/6 ILC2s did not differ in CD127 and ST2 expression (**Fig. 5C**). Thus, ILC2s from BALB/c mice may in part sustain their higher numbers in the kidney by expressing transcription factors, function-related cytokine receptors, and stimulatory signals at higher levels.

### Homeostatic BALB/c kidney ILC2s are more receptive to IL-2 signaling than C57BL/6 kidney ILC2s

Given the higher IL-2 response-related gene expression (**Fig. 3D**) and microenvironmental IL-2 levels (**Fig. 4A**) in the BALB/c kidney, and the greater IL-2 receptor expression by BALB/c kidney ILC2s relative to C57BL/6 kidney ILC2s (**Fig. 5C**), we asked whether the two cell groups differed in terms of responsiveness to IL-2.

Referring to the result that IL-2 and IL-2 monoclonal antibody complex (IL-2C) increase CD25-dependent IL-2 signaling to immune cells (35), we adopted the IL-2C treatment regimen to compare the IL-2 reactivity from two strains. In both strains, IL-2C increased kidney ILC2s. However, the frequency of BALB/c ILC2s is higher after IL-2C treatment (**Fig. 6A**). Although IL-2C increased ILC precursors, including αLP, ChILP, and ILC2P, only in C57BL/6 (**Supplementary Fig. 3A-C**), the BALB/c-dominant alteration in kidney ILC frequency demonstrates that BM precursors do not significantly contribute to kidney ILCs. To examine the hypothesis, we examined the origin of kidney ILCs by intravascular staining of CD45 by the systemic administration of IL-2C (**Fig. 6B**). Most of the ILC2 after IL-2C treatment was CD45 negative population, suggesting that the increase in the ILC2s by IL-2C treatment was due to the proliferation of kidney resident ILC2 rather than an exogenous influx (**Fig. 6B**). Moreover, IL-2C treatment specifically increases the $ST2^{+}CD25^{+}$ ILC2 subset and type 2 cytokine secretion in both stains (**Fig. 6C**). It is well known that IL-2 signaling plays a crucial role in the type 2 cytokine expression in ILC2s and Th2 cells through activation of STAT5 (30,36-38). Therefore, STAT5 phosphorylation was further evaluated to validate the significance of IL-2 signaling on ILC2s. Phosphorylated STAT5 increased in an IL-2 dose-dependent manner only in BALB/c kidney ILC2s (**Fig. 6D**). Collectively, these results suggest that BALB/c kidney ILC2s may respond more robustly to IL-2, leading to differences from C57BL/6 ILC2s.

## DISCUSSION

Although kidney ILCs may contribute to inflammatory kidney diseases (13), this cell population remains poorly characterized. Here, we showed that two mouse strains possess different frequencies of ILCs in the homeostatic kidney, especially ILC2s, and that the higher ILC2 frequencies in BALB/c kidneys may reflect 1) greater production of ILC2 progenitors in the BM, 2) a kidney microenvironment that is rich in IL-2 signaling, which may promote ILC2 proliferation and/or survival, and 3) homeostatic imprinting of the kidney ILC2s that makes them more sensitive to the microenvironmental signals.

Several studies show that mouse strains differ in terms of susceptibility to kidney inflammation (39-41). In particular, C57BL/6 mice demonstrate more severe glomerular injury in the nephrotoxic serum-induced glomerulonephritis model than BALB/c mice (39). Moreover, BALB/c mice and six other strains are resistant to immune-mediated nephritis in

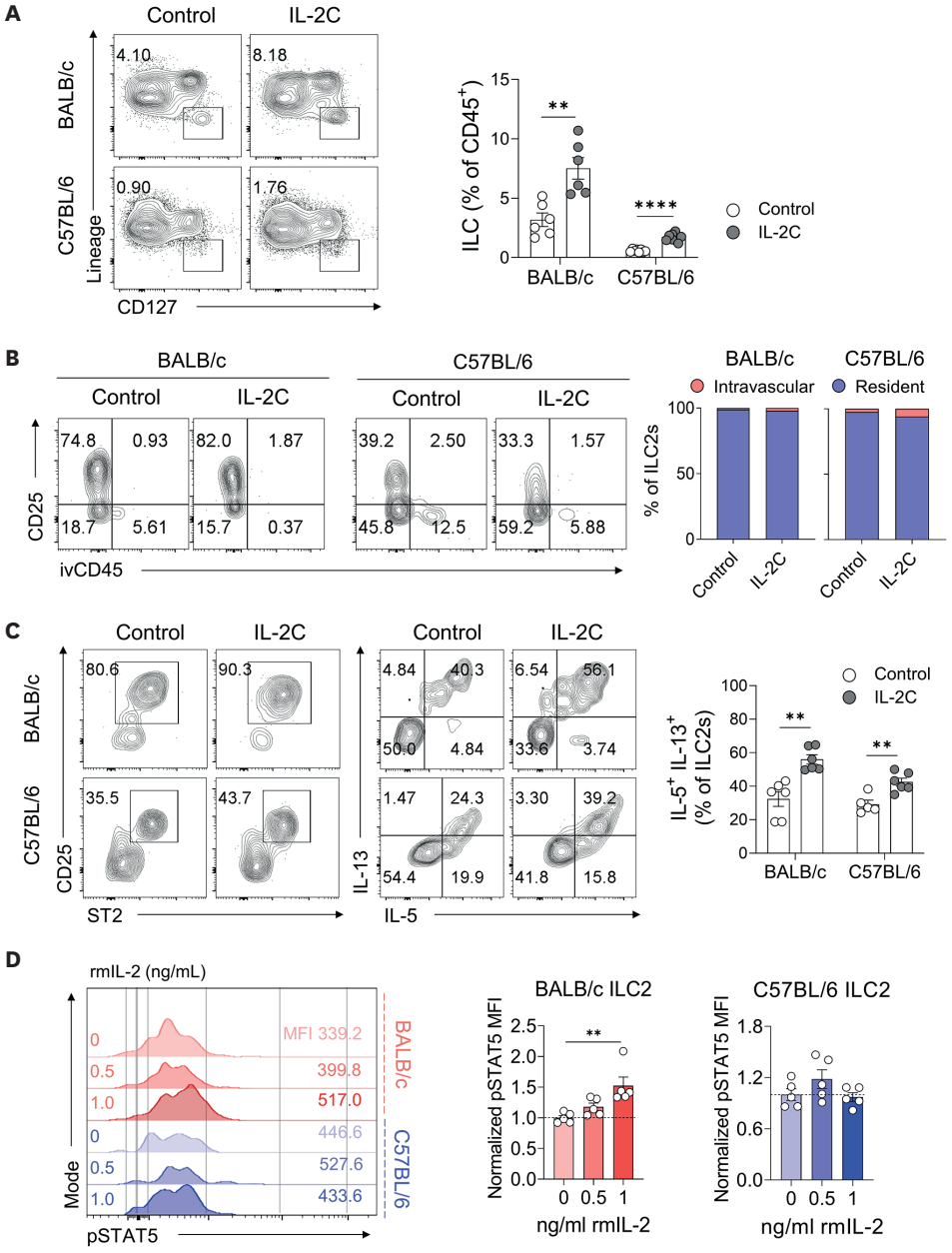


**Figure 6.** BALB/c kidney ILC2s have an intrinsically higher response to IL-2 via STAT5 signaling. IL-2C was systemically treated in BALB/c and C57BL/6 adult mice. (A) Kidney ILCs were analyzed after IL-2C treatment. (B) Resident and intravascular distribution of ILC2s studied using intravascular labeling with monoclonal anti-CD45 fluorescent-labeled antibodies. (C) Kidney ILC2s were identified as Lineage$^-$ ST2$^+$ CD25$^+$. IL-5 and IL-13 production from ILC2s challenged by IL-2C. (D) IL-2-induced STAT5 phosphorylation in kidney ILC2s. All results are shown as mean ± SEM and statistical analysis was performed using a 2-tailed Student's *t*-test (A, C), one-way ANOVA (D, left graph), or Kruskal-Wallis test (D, right graph).
**p<0.01; ****p<0.0001; n=6 mice in each group (A-C), n=5 tests in each group (D).

the anti-glomerular basement membrane-induced glomerulonephritis model whereas the C58, CBA, NZW, 129/svJ, BUB/BnJ, and DBA/1J strains develop the severe disease (C57BL/6 mice were not examined) (41). Interestingly, *de novo* whole-genome sequencing of 16 mouse strains showed that 2,567 regions displayed considerable sequence diversity and that these regions were enriched for immunity-related gene sets (42). These and our findings suggest that the outcomes of immunological studies on human diseases that employ murine models can be significantly impacted by the choice of mouse strain.


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While the mechanisms by which the different strain-specific gene profiles favor kidney or lung disease remain to be fully elucidated, our finding that BALB/c mice have higher frequencies of ILC2s (the innate equivalent of Th2 cells) in the kidney and lung than C57BL/6 mice is consistent with the well-established notion that C57BL/6 and BALB/c mice are prototypes of type-1 and type-2 inflammation, respectively (43,44). Our study extends this notion by showing that the larger ILC2 (type 2) population in the kidney of BALB/c mice may be due in part to greater baseline production of ILC2s from progenitors in the BM.

In addition, our study showed that the type-1/-2 skewing of C57BL/6 and BALB/c mice may also reflect fundamentally different microenvironments: specifically, we observed that IL-2 signaling and *Il2* expression were enriched in BALB/c kidneys compared to in C57BL/6 kidneys. It may promote ILC2 maintenance since these cells express CD25, and IL-2 was shown recently to promote ILC2 proliferation/survival (30). We also noted that compared to the C57BL/6 kidney, the BALB/c kidney expressed higher levels of cytokines that drive type-2 responses, including ILC2 responses, namely, IL-33, IL-25, and TSLP (45). Moreover, the BALB/c kidney displayed higher *Il7* transcription: IL-7 is thought to be critical for the survival and maintenance of tissue ILC2s (46). Thus, the cytokine milieu of each strain may also contribute to the strain differences in ILC2 frequencies. Notably, we did not observe strain differences in *Il2*, *Il7*, *Il33*, *Il25*, or *Tslp* expression in the lung, even though this organ also bore higher ILC2 frequencies in BALB/c mice compared to C57BL/6 mice. Thus, the factors that shape ILC2 predominance in mouse strains may vary from organ to organ.

The higher ILC2 frequencies in BALB/c kidneys may also be due to the putative greater sensitivity of these cells to the local environmental signals, as shown by their higher expression of their master transcription factor GATA-3, IL-2 and IL-25 receptors, and ICOS, which promotes ILC2 survival (34). They also expressed less KLRG1: upregulation of this molecule is known to associate with inhibition of ILC2 proliferation (47). Moreover, BALB/c kidney ILC2s were more responsive to IL-2 than C57BL/6 kidney ILC2s, as shown by their higher STAT5 phosphorylation levels. Notably, the possibility that IL-2 signaling is a major driver of the ILC2 predominance in BALB/c kidneys is supported by a previous study showing that naïve splenic $CD4^+$ T cells from BALB/c mice express much more IL-4 than the equivalent cells in C57BL/6 mice and that this difference is due to greater intrinsic sensitivity to STAT5 and IL-2 signaling (48). Thus, the latter study shows that not only ILCs but also T cells can be intrinsically hyperresponsive to IL-2 in a strain-dependent manner. Despite the intrinsic difference in response to IL-2, the proliferation capacity of kidney ILC2s from both stains are comparable when we administrated the same dose of IL-2C. Thus, it is more likely that the key limiting factor determining the properties of kidney ILC2s in the homeostatic condition is the tissue level of IL-2 rather than the difference in responsiveness to IL-2 between the two strains. Thus, lower CD25 expression in ILC2s from C57BL/6 than BALB/c kidneys may be responsible for less STAT5 phosphorylation in response to IL-2. However, high doses of IL-2C overcame these defects seen in C57BL/6 ILC2s. Also, the possibility that differences in innate cytokines other than IL-2 and their receptor expression may contribute to ILC2 differences between the strains should not be excluded.

Our finding that BALB/c kidney ILC2s expressed high levels of CD25, IL-17RB, and ICOS while C57BL/6 kidney ILC2s showed increased expression of the TSLP receptor and KLRG1 is consistent with the studies showing ILC2 phenotypic heterogeneity. It has recently been recognized that the ILC2s are not a uniform population: different subpopulations express different markers and respond to different stimuli (49). For example, a study on the lung

ILCs that promote worm expulsion showed that while the uninfected lung is populated by ST2$^+$ IL-33-responsive ILC2s, the worm infection initially generates an inflammatory ILC2 that expresses KLRG1 but not ST2 and that responds to IL-25 but not IL-33 (50,51). Moreover, a single-cell RNA-seq analysis suggests that there may be up to 15 different subcategories of pulmonary ILC2s. These findings suggest that the tissue-specific cytokine milieu of each strain also contributes to the strain-derived difference of ILC2s.

The current study has important implications for research on both ILC biology and kidney immunity. First, we showed that two different strains displayed quite disparate innate cytokine milieus, in particular, IL-2 signaling and that this could shape the kidney ILC2 profile. Therefore, it is critical to select the murine strains for kidney ILC studies with care because not accounting for strain background may overestimate or underestimate the role of these cells. Second, we found significant differences between strains in the frequencies of ILCPs in the BM, which could affect the distribution of ILCs in all organs. These findings together suggest that care should also be taken when studying ILCs in general. Given the link between ILCs and inflammatory kidney diseases, there is a growing interest in ILCs in the field of nephrology (13). However, current studies on ILCs in kidney diseases mainly focus on their potential as cell therapeutics, and little is known about kidney ILCs in terms of how they interact with other immune and non-immune cells during kidney homeostasis and inflammation. Further studies of this nature will help further our understanding of these cells.

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## SUPPLEMENTARY MATERIALS

### Supplementary Figure 1

The number of ILC2s in BALB/c kidneys is higher than in C57BL/6 kidneys. (A) Numbers of total ILCs, subsets of ILCs in the (B) kidney and (C) lung between BALB/c and C57BL/6 strains. Results are shown as mean ± SEM and statistical analysis was performed using a 2-tailed Student's *t*-test or Mann-Whitney test.

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### Supplementary Figure 2

BALB/c harbor the higher number of progenitors of ILCs. (A) Comparison of the number of CLPs, αLPs, ChILPs, ILCPs, and ILC2Ps between BALB/c and C57BL/6 strains. (B) Comparison of tissue resident ILCP, IL-18Rα$^+$ TCF-1$^+$ cells, between strains. All results are shown as mean ± SEM and statistical analysis was performed using 2-tailed Student's *t*-test or Mann-Whitney test.

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### Supplementary Figure 3

Treatment with IL-2C promotes the expansion of ILCPs in the bone marrow of C57BL/6 mice.

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