

# Suppression of Akt Signaling by the Phosphatase SH-PTEN Inhibits Cell Proliferation and Promotes Apoptosis in Human Prostate Cancer Cells

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## ABSTRACT:

helper 2 cell responses in lung (8). Intestinal CD103<sup>+</sup>CD11b<sup>-</sup> DCs, but not CD103<sup>+</sup>CD11b<sup>+</sup> DCs, restrain colitis by triggering a reversible early anti-inflammatory response in epithelial cells *via* proteins induced by IFN- $\gamma$ , creating an anti-inflammatory environment (9). These studies were generally performed with flow cytometry or immunofluorescence, which cannot provide enough targets for characterizing the heterogeneous and functional diversity of DCs.

In the steady state and in inflammatory diseases, DC subsets express distinct levels of function-related molecules and thus display a series of functional properties (10), including those related to maturation (11), migration (12), oxidized LDL (oxLDL) engulfment (13), adhesion (14), and T-cell stimulation (15). In the steady state, the DCs in nonlymphoid tissues display an immature phenotype with low levels of the costimulatory molecules CD40, CD80, and CD86 (16). After up-regulation of chemokine receptors, such as CCR7, DCs migrate from nonlymphoid tissues *via* the afferent lymph to the draining lymph nodes where they up-regulate expressions of costimulatory molecules and display mature phenotypes (17). DCs can uptake oxLDL *via* scavenger receptors (SRs), such as LOX-1, CD36, and CD205, and this process may lead to aggravation of vascular inflammation (18). The TLR adapter MyD88 of DCs has been reported to be required for the T-cell response in inflammatory bowel disease (19). All of these studies suggest that it is quite necessary to identify the heterogeneity of DCs based on a series of functional markers. Most recently, single-cell analyses (20) have been used to identify heterogeneous populations, and massively parallel RNA single-cell sequencing has provided a new way to explore DC phenotypic heterogeneity (21). However, the important functional changes of DCs were ignored in the massive information of RNA-seq. Compared with single-cell sequencing, single-cell quantitative PCR (sc-qPCR) has advantages of DC classification based on not only phenotypic but also functional markers and shows potential value in clinical application at an economical price with more-easily commercialized target genes.

To demonstrate the functional diversity of DCs, 48 genes were selected in the present study, illustrating the classic phenotype as well as 8 groups of important functional markers of DCs. By a combination of fluorescence-activated cell sorting (FACS) and sc-qPCR, the expression profiles of 48 genes in single DCs from the mouse liver and spleen were detected and then analyzed *via* bioinformatic methods. Furthermore, the functional heterogeneity of single DCs was also compared between a healthy and an atherosclerotic mouse model. The present analyses may provide a new method for functionally discriminating of DC heterogeneity and may expand the understanding of the phenotypic and functional divergence of DCs in different tissues and different diseases.

## MATERIALS AND METHODS

### Mice

C57BL/6, C57BL/6J and apolipoprotein E-knockout (*ApoE*<sup>-/-</sup>) mice were obtained from Vital River Laboratory (Beijing, China). All mice were male. The mice were maintained under specific

pathogen-free conditions and were fed with a normal diet in the Animal Research Committee facility of Shanghai Jiao Tong University. To establish the atherosclerotic model and the control group, *ApoE*<sup>-/-</sup> mice and C57BL/6J mice were fed a high-fat diet or normal chow, respectively, for 12 wk beginning at 8 wk old. Oil-red O (Sangon Biotech, Shanghai, China) staining of the mouse aortae was used to validate the atherosclerosis. All studies were performed in accordance with the principles of the Basel Declaration and recommendations of the *Guide for the Care and Use of Laboratory Animals* (National Institutes of Health, Bethesda, MD, USA) (22). The protocol was approved by the Animal Research Committee of Shanghai Jiao Tong University.

### Preparation of the single-cell suspensions

The spleens and livers were isolated from the mice. The tissues were flushed with PBS and excised into small pieces. The mouse spleens were ground and passed through a cell strainer. Next, ACK buffer (ammonium-chloride-potassium; Beyotime, Shanghai, China) was used to remove the red blood cells from the spleen suspensions. The liver cells were prepared following a previously published method (23). The mouse livers were incubated with 770 U/mg collagenase type IV in HBSS containing 10% fetal bovine serum for 1 h at 37°C with gentle shaking. The liver suspensions were then run through a Ficoll 1.077 (GE Healthcare, Waukesha, WI, USA) centrifugation at room temperature to enrich the hematopoietic cells. The spleen suspensions and enriched liver suspensions were kept on ice for the flow cytometry analyses.

### Flow cytometry and single-cell sorting

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well of a 96-well plate was supplemented with 0.2  $\mu$ l SuperScript III RT-PCR System with Platinum Taq Mix (Thermo Fisher Scientific), 2.5  $\mu$ l of the mixture of the 48 pooled primers, and 1.3  $\mu$ l of nuclease-free water (Thermo Fisher Scientific). The plates were then placed on a PCR machine (Bio-Rad Laboratories, Hercules, CA, USA) and incubated for 90 s at 70°C to denature the single cells. The mRNA of each cell lysate was reverse transcribed into cDNA (50°C for 15 min). Then, the reverse transcription was inactivated, and Taq polymerase was activated (95°C for 2 min). Subsequently, preamplification of the specific target was performed in the same tube for 22 PCR cycles (each cycle: 95°C for 15 s and 60°C for 4 min). Finally, a cleanup step was used to remove the unincorporated primers with exonuclease I (New England BioLabs, Ipswich, MA, USA).

### High-throughput real-time PCR on a real-time PCR reader

The amplified cDNA from each single cell was diluted 1:5 with nuclease-free water. The diluted samples for the 48 assays were loaded into a M48 chip (Fluidigm, South San Francisco, CA, USA) with an IFC Controller HX (Fluidigm) with EvaGreen DNA Binding Dye (Bio-Rad Laboratories). Then, the chip was transferred to a Biomark real-time PCR reader (Fluidigm). All steps were performed in strict accordance with the manufacturer's protocols and instructions.

### Computational analysis of the single-cell data

The single-cell data were analyzed and displayed with the Singular Analysis Toolset (Bell Laboratories, Madison, WI, USA) of the R software (R Foundation for Statistical Computing, Vienna, Austria). The background value was set to 28. Because DCs were commonly defined as lineage (LIN) (CD3, CD19, NK1.1)<sup>-</sup>CD45<sup>+</sup>CD11c<sup>+</sup>MHCII<sup>+</sup> cells (24), the sorted single CD11c<sup>+</sup>MHCII<sup>+</sup> DCs by flow cytometry were further quantified with sc-qPCR *in a* series of genes, including the lineage-restricted marker genes (*CD3*, *CD19*, and *KLRB1C*), leukocyte common marker gene *PTPRC* (CD45), and the commonly used DCs marker genes *ITGAX* (CD11c) and *H2-AA* (MHCII). T, B, and NK cells were recognized by the expression of *CD3*, *CD19*, and *KLRB1C* (NK1.1) and were removed from the data set. The cells with no expression of *CD45*, *CD11c*, and

*MHCII* were identified as non-DCs and excluded from the data set. The cells with no or low housekeeping gene expression levels were also removed from the analysis. Hierarchical clustering (HC) analysis was used to connect the data points to form clusters based on distance and to help build a binary tree in the form of a heat map. Forty-eight genes were clustered by the Pearson method, and the single DCs from the different mouse tissues were clustered by the Euclidean method (25). Principal component analysis (PCA), a dimension-reduction analysis, was used to simplify the multidimensional data sets from the sc-qPCR experiment for plotting purposes. Forty-eight genes were transformed into 2 principal components. A radar map was drawn to provide an overall concept of the expression differences. The mean values of each of the genes were separately calculated for the liver and spleen DCs and then converted into log<sub>2</sub>-fold changes. Violin plots were used to display the gene characteristics of burst size and frequency. Some of the genes had unimodal distributions, and others had bimodal distributions.

### Immunofluorescence staining

The spleens, livers, and hearts from healthy mice and atherosclerotic model mice were flushed with PBS and fixed with 4% paraformaldehyde. Frozen sections of the mouse livers, spleens, and aorta roots were cut at a thickness of 6  $\mu$ m using a cryosection technique. The slides were permeabilized with 0.3% Triton X-100 (MilliporeSigma, Burlington, MA, USA). CD11c and CCR7 were stained with biotin-labeled anti-CD11c (clone N418; BioLegend, San Diego, CA, USA) and anti-CCR7 antibody (clone Y59; Abcam, Cambridge, MA, USA), respectively, and detected with an Alexa Fluor 555 Tyramide SuperBoost kit (Thermo Fisher Scientific) and an Alexa-Fluor-488 Anti-Rabbit IgG Kit (Cell Signaling Technology, Danvers, MA, USA), respectively. The nuclei were stained using DAPI (Cell Signaling Technology). Images of the stained sections were collected with a TCS SP8 STED  $\times$ 3 confocal microscope (Leica, Wetzlar, Germany).

## RESULTS

### Genes for phenotypic and functional classification of single DCs

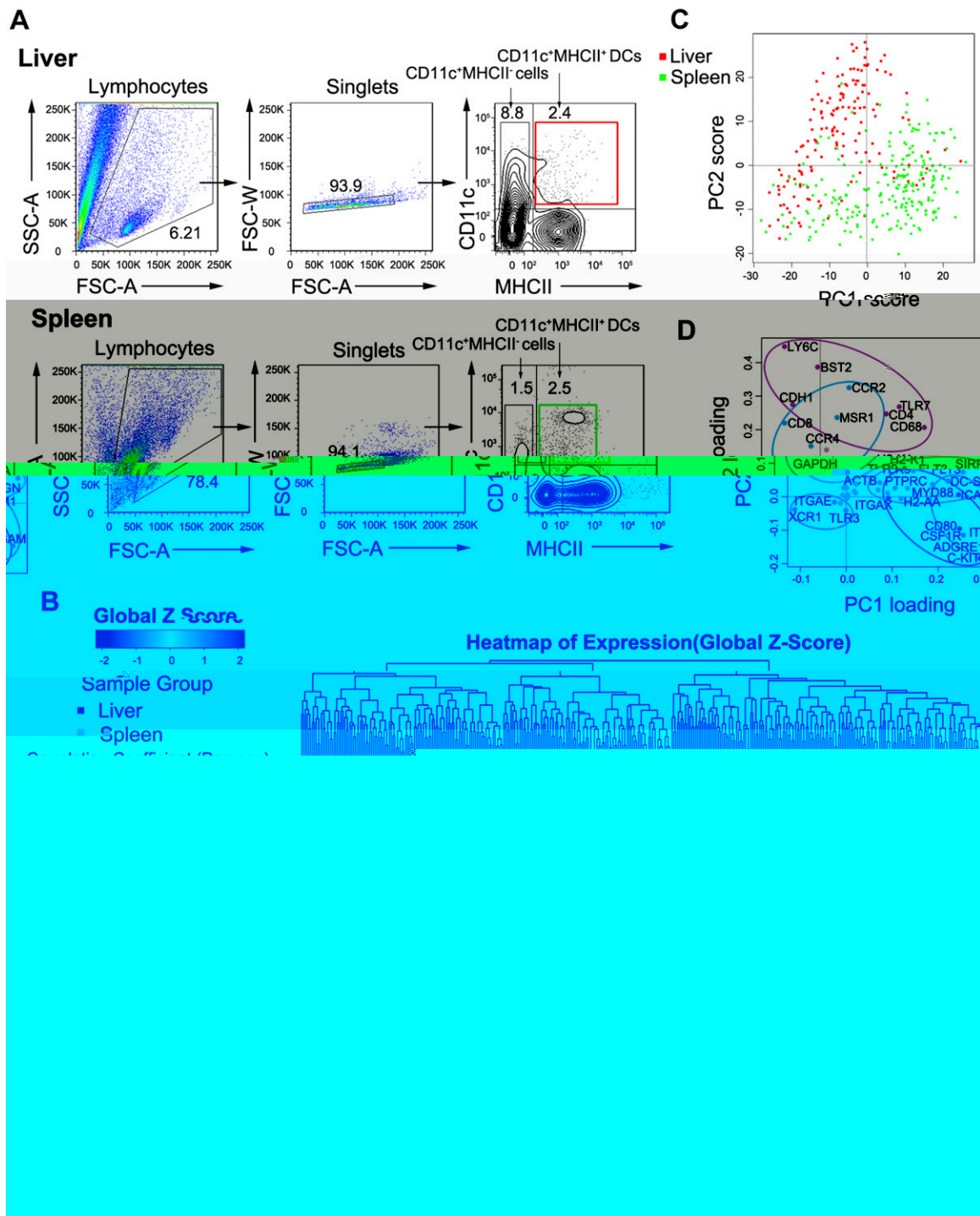
To identify the phenotypic and functional classification of single DCs, 48 target genes, divided into the following 4

TABLE 1. Forty eight Genes for phenotypic and functional classification of single DCs

Group	Class	Gene
Housekeeping genes DC definition-related markers	Housekeeping genes	<i>GAPDH</i> , <i>ACTB</i>
	Lineage restricted genes	<i>CD3</i> , <i>CD19</i> , <i>KLRB1C</i> , <i>PTPRC</i>
Phenotypic markers	Makers of DCs	<i>ITGAX</i> , <i>H2-AA</i> , <i>H2-K1</i>
	Makers of macrophages	<i>FCGR1</i> , <i>MERTK</i> , <i>CD68</i> , <i>ADGRE1</i>
	Ontogeny markers of DCs	<i>C-KIT</i> , <i>FLT3</i> , <i>CSF1R</i>
	Reported phenotype genes	<i>LY6C</i> , <i>BST2</i> , <i>XCRI</i> , <i>SIRPA</i> , <i>CD4</i> , <i>CD8</i> , <i>ITGAE</i> , <i>ITGAM</i> , <i>DC-SIGN</i> , <i>CD207</i>
Functional markers	Costimulatory molecules	<i>CD40</i> , <i>CD80</i> , <i>CD86</i>
	Adhesion molecules	<i>ICAM1</i> , <i>ICAM2</i> , <i>CDH1</i>
	TLR	<i>TLR3</i> , <i>TLR7</i> , <i>TLR9</i>
	TLR adaptor molecule	<i>MYD88</i>
	Scavenger receptors	<i>OLR1</i> , <i>MSR1</i> , <i>LY75</i>
	Mannose receptor	<i>MRC1</i>
	Chemokine receptors	<i>CCR1</i> , <i>CCR2</i> , <i>CCR4</i> , <i>CCR7</i> , <i>CCL17</i> , <i>CX3CR1</i>
	Cytokines	<i>IL-10</i> , <i>IL-12</i>

groups, were selected (**Table 1** and Supplemental Table S1): 1) housekeeping genes, 2) DC definition-related, 3) phenotypic, and 4) functional markers.

*GAPDH* and *ACTB* were selected as the housekeeping genes. The DC definition-related markers included lineage-restricted marker genes *CD3*, *CD19*, and *KLRB1C* to exclude T, B, and NK cells; *PTPRC* (CD45) as a leukocyte common marker (24); *FCGR1* and *MERTK* to distinguish macrophages from DCs (6); the commonly used DCs markers *ITGAX* (CD11c) and *f156 313.3dT/32*



**Figure 2.** Single-cell gene expression analysis to distinguish the profiles of the DCs from the mouse liver and spleen. *A*) Gating strategy for sorting the single DCs from the mouse liver and spleen. First, live lymphocytes were segregated on FSC-A and SSC-A plots, and singlets from live lymphocytes were further gated by FSC-A and FSC-W. Then, liver CD11c<sup>+</sup>MHCII<sup>+</sup> DCs (red box) and spleen CD11c<sup>+</sup>MHCII<sup>+</sup> DCs (green box) were segregated from singlets on CD11c and MHCII plots, respectively. The percentages of CD11c<sup>+</sup>MHCII<sup>+</sup> DCs in liver and spleen suspensions were calculated with the percentages of the 3 parts. *B*) The heat map revealed the expression levels of the 48 target genes from 151 single liver DCs (red square) and 229 single spleen DCs (green square). Each square in the heat map indicates the expression of the specific gene in the corresponding column from a specific single cell in the corresponding row. The genes were grouped into 6 gene clusters (correlation coefficient >0.15), which are marked with different colors (pink, brown, green, blue, orange, and purple). *C*) PCA of single DCs from the liver (red square) and spleen (green square). *D*) Gene loading for the PCA analysis; the genes are colored and circled according to the 6 clusters that were identified in the HC analysis depicted in *B*. The gray dots are unclustered genes.

CD11c<sup>+</sup>MHCII<sup>+</sup> DCs in the liver was 0.12–0.16%. In the spleen cell suspensions, CD11c<sup>+</sup>MHCII<sup>+</sup> DCs accounted for 1.58–1.89%.

The 336 single liver CD11c<sup>+</sup>MHCII<sup>+</sup> DCs and the 304 single spleen CD11c<sup>+</sup>MHCII<sup>+</sup> DCs from 6 individual mice were initially sorted by FACS. Then, the expression levels of the 48 target genes in each individual cell were quantified in parallel using 48.48 Dynamic Array chips with the Biomark system (Fluidigm). After genetic confirmation as LIN(CD3, CD19, NK1.1)<sup>-</sup>CD45<sup>+</sup>CD11c<sup>+</sup>MHCII<sup>+</sup> cells, 154 liver DCs and 229 spleen DCs were identified and entered into the bioinformatic analysis.

### Liver DCs and spleen DCs show different single-cell expression patterns

Based on the expression profiles of the 48 genes in the single DCs from liver and spleen, HC was applied (Fig. 2B). *ITGAM* was enriched in the spleen DCs, which suggested an accumulation of CD11b<sup>+</sup> DCs. *LY6C* and *BST2* exhibited higher expression levels in the liver DCs, which revealed a higher percentage of pDCs.

PCA was also performed to reduce the dimensionality of the 48 gene expression profiles of single DCs. Using the first and second principal components (PC1 and PC2), the DCs from the liver and spleen were separated (Fig. 2C). Additionally, the contributions of each of the genes to PC1 and PC2 (termed the PC projections of the 48 genes) were identified. The genes were grouped into 6 gene clusters according to the HC analysis (Fig. 2D). For example, *CSF1R*, *ADGRE1*, *ITGAM*, and *DC-SIGN* were clustered together and primarily contributed to PC1, whereas genes such as *LY6C*, *BST2*, and *CDH1* were clustered together and provided a major contribution to PC2.

This classification of the single DCs using HC and PCA reflects the divergence of the DCs in lymphoid and non-lymphoid tissues, which can be identified by changes in both phenotypic and functional markers.

### Functional classification reveals functional heterogeneity of liver and spleen DCs

To illustrate the different expression patterns of the single DCs from the liver and spleen, a radar map was drawn to display the log<sub>2</sub>-fold changes of the genes with significant *P* values (*P* < 0.05) (Fig. 3A and Supplemental Table S2). The results revealed that *LY6C*, *BST2*, *CDH1*, *CCR2*, and *MSR1* were remarkably up-regulated in the liver DCs, whereas genes *C-KIT*, *ADGRE1*, *CD80*, *ICAM1*, and *MYD88* were significantly enriched in the spleen DCs.

The single DCs were firstly subdivided using the classic phenotypic markers: conventional type 1 DCs (cDC1s), which are characterized by *XCR1*, *ITGAE* (CD103), and *CD8*; conventional type 2 DCs (cDC2s), which are identified by *SIRPA* (CD172), *ITGAM* (CD11b), *CD4*, and *DC-SIGN*; and pDCs, which can be recognized by *LY6C* and *BST2* (31) (Fig. 3B). Violin plots revealed increased ratios of *XCR1*<sup>+</sup>, *ITGAE*<sup>+</sup>, and *CD8*<sup>+</sup> DCs in the liver and up-regulated ratios of *SIRPA*<sup>+</sup>, *ITGAM*<sup>+</sup>, *CD4*<sup>+</sup>, and *DC-SIGN*<sup>+</sup>

DCs in the spleen, with similar expression levels of positive groups. The ratios of *LY6C*<sup>+</sup> and *BST2*<sup>+</sup> DCs were increased in the liver as well as up-regulating expression levels. As a marker gene of epidermal LCs (32), *CD207* (Langerin) was negatively expressed in the liver and spleen DCs.

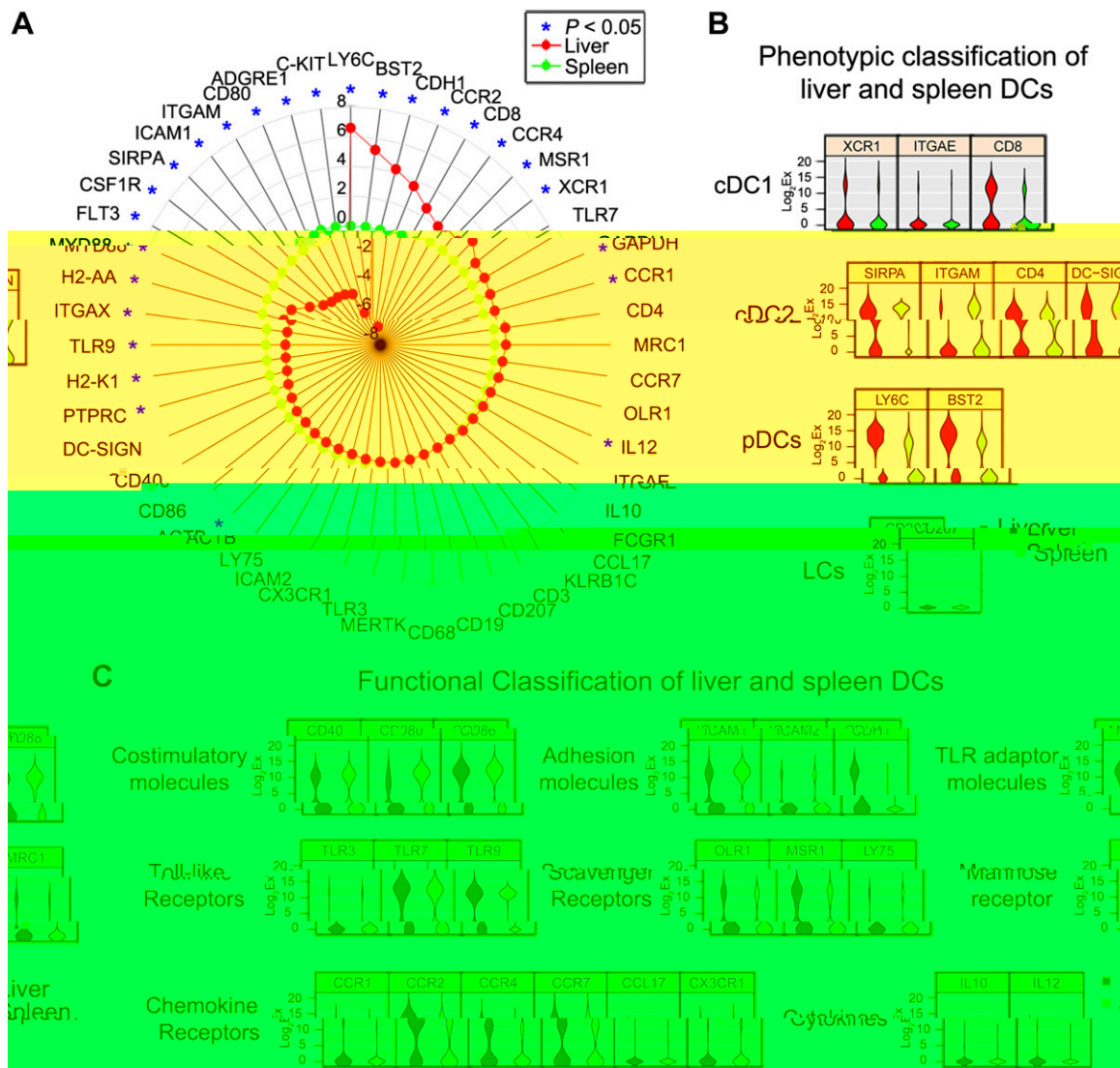
In addition to classic phenotypic classification, our results also revealed a deep divergence of the single DCs based on a series of function-related markers (Fig. 3C). The expressions of 10 molecules in 7 functional groups was changed remarkably between the liver and the spleen DCs. *CD80* was significantly up-regulated in the spleen DCs, suggesting a state of greater maturation in the spleen DCs. *MYD88* was increased in the spleen DCs, which revealed an increased ability of the DCs in the spleen to activate CD4<sup>+</sup> T cells. *TLR9* was elevated in the spleen DCs, which indicated increased pathogen recognition. *MSR1* was enriched in the liver DCs, revealing greater lipid-uptake ability by the DCs from the liver. The remarkable accumulations of *CCR1*, *CCR2*, and *CCR4* in the liver DCs may indicate enhanced migration properties of the DCs in the nonlymphoid tissues compared with the lymphoid tissues. Interestingly, *CDH1* up-regulated in liver DCs, whereas *ICAM1* was enriched in spleen DCs, which suggests that their adhesion ability may depend mainly on different adhesion molecules.

Moreover, the expression patterns of the DC definition-related markers provided further examples (Supplemental Fig. S2). The spleen DCs expressed greater levels of DC markers *ITGAX* (CD11c) and *H2-AA* (MHCII), which also indicated maturation of DCs (11). The increased level of *C-KIT* in the spleen DCs indicated that more DCs in the lymphoid organ expressed progenitor markers. We also analyzed several markers of macrophages: *FCGR1* (CD64) and *MERTK* were rarely expressed in DCs and can thus be used as specific markers of macrophages. Although *CD68* and *ADGRE1* (F4/80) have also been reported to be markers of macrophages (33), our present research indicated that those 2 molecules were not specific and were also found on DCs.

These results suggested that single DCs reveal different phenotypic and functional classification in lymphoid and nonlymphoid tissue. Hence, this method was further examined in atherosclerosis, an immune-related disease.

### Quantitatively increase of DCs in the atherosclerotic mouse model

Suspensions of mouse livers and spleens from healthy and atherosclerotic mice were subjected to flow cytometry (Fig. 4A, B), and the quantities and percentages of DCs, defined as CD11c<sup>+</sup>MHCII<sup>+</sup>, were analyzed (Fig. 4C, D). Compared with healthy liver DCs, the percentages of *ApoE*<sup>-/-</sup> liver DCs were significantly increased from 0.14–0.26% to 0.42–0.50%. The CD11c<sup>+</sup>MHCII<sup>+</sup> DCs in the healthy spleen accounted for 1.98–2.32% of the cells, and that value increased to 3.45–4.07% in the *ApoE*<sup>-/-</sup> spleen. The remarkable recruitments of CD11c<sup>+</sup>MHCII<sup>+</sup> DCs revealed the increased inflammatory responses in



**Figure 3.** Phenotypic and functional classification of mouse liver and spleen DCs. *A*) The 48 genes are ordered in a clockwise manner with increasing  $\log_2$ -fold change. Each data point is the average expression level for a certain gene in the liver DCs (red points) and the spleen DCs (green points). \* $P < 0.05$  by Student's *t* test. *B*) Violin plots showing the classifications of the liver DCs (red plots) and spleen DCs (green plots) with the reported phenotypic markers for cDC1, cDC2, pDC, and LCs. *C*) Classifications of the DCs from the liver (red plots) and spleen (green plots) based on 8 groups of function-related markers. See also Supplemental Fig. S2 and Supplemental Table S2.

both the lymphoid and nonlymphoid tissues during atherosclerosis.

### DCs show distinct functional classification in the atherosclerotic mouse model

We proceeded to study the functional variations of the DCs between the healthy and *ApoE*<sup>-/-</sup> mouse model. Single DCs from healthy mouse livers ( $n = 212$ ), single DCs from *ApoE*<sup>-/-</sup> mouse livers ( $n = 206$ ), single DCs from healthy mouse spleens ( $n = 224$ ), and single DCs from *ApoE*<sup>-/-</sup> mouse spleens ( $n = 231$ ) were included in the analyses.

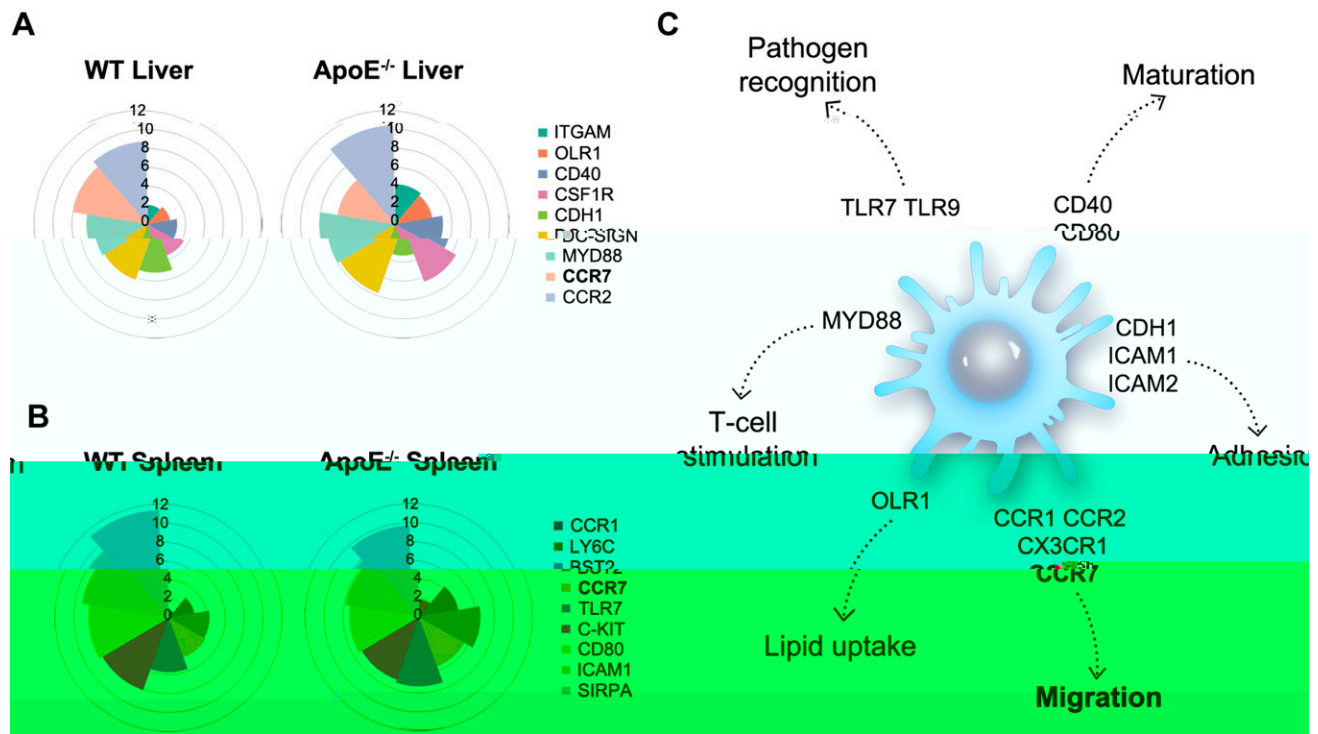
To probe the divergence of the expression patterns of the single DCs in the healthy and atherosclerotic mouse models, *P* values were calculated for the 48 target genes.

Supplemental Figure S3A lists all genes that changed significantly between the healthy and *ApoE*<sup>-/-</sup> liver DCs, including *CSF1R*, *ITGAM*, *CCR2*, and *CCR7*, and Supplemental Fig. S3B lists the genes that were markedly different between the healthy and *ApoE*<sup>-/-</sup> spleen DCs, such as *BST-2*, *CCR1*, *SIRPA*, and *ICAM1*.

Comparisons of the expression patterns of single DCs in liver and spleen from the healthy and *ApoE*<sup>-/-</sup> mice revealed variations in both phenotypic genes and functional genes (Fig. 5A, B and Supplemental Tables S3, 4). Regarding the phenotypic markers, *ITGAM* and *DC-SIGN* were enriched in the *ApoE*<sup>-/-</sup> liver DCs, which indicated increased rates of cDC2. *LY6C* and *BST2* accumulation in the *ApoE*<sup>-/-</sup> spleen DCs suggested an increased proportion of pDCs. Differently expressed functional markers and their potential roles were also demonstrated (Fig. 5C). The expression of the TLR genes *TLR7* and *TLR9* was

significantly increased in the *ApoE*<sup>-/-</sup> spleen DCs, which indicated elevated innate responses. *MYD88* was markedly up-regulated in the *ApoE*<sup>-/-</sup> liver DCs, which indicated increased T-cell stimulation. *OLR1* was significantly increased in the *ApoE*<sup>-/-</sup> liver DCs, and that increase might lead to enhanced oxLDL-induced DC activation. Regarding the chemokine receptors, *CCR2* and *CX3CR1* were elevated in the *ApoE*<sup>-/-</sup> liver DCs, and *CCR1* was up-regulated in the *ApoE*<sup>-/-</sup> spleen DCs, which may be suggestive of increased migration and T-cell-recruitment abilities. Interestingly, *CCR7* was down-regulated in the *ApoE*<sup>-/-</sup> liver DCs. Regarding adhesion genes, the expression of the *CDH1* and *ICAM1* genes was decreased in the *ApoE*<sup>-/-</sup> liver and





**Figure 5.** DCs in the healthy and *ApoE*<sup>-/-</sup> mice showed different gene expression patterns. *A, B*) Wind rose maps show the different expression patterns of the DCs in the liver (*A*) and spleen (*B*) from the wild-type (WT) and *ApoE*<sup>-/-</sup> mice with the average Log<sub>2</sub>Ex values. The genes that exhibited large differences in the Log<sub>2</sub>Ex values (>1) are displayed. *C*) Diagram of differentially expressed genes of the DCs and their potential roles in atherosclerosis. Each figure represents 6 experiments. *CCR7*, which is marked in bold, was the migration-related molecule used in the subsequent portion of the study. See also Supplemental Fig. S3 and Supplemental Tables S3, S4.

*CCR7* was slightly up-regulated in the *ApoE*<sup>-/-</sup> spleen DCs, and the DCs (CD11c<sup>+</sup>) in atherosclerotic livers exhibited a down-regulation of *CCR7* at the single-cell level.

Next, the distributions of DCs (CD11c<sup>+</sup>) and the expression of *CCR7* in healthy and atherosclerotic aorta were compared (Fig. 6*E, F*). We observed a subendothelial location of DCs in the healthy aorta, and the DCs dramatically accumulated in atherosclerotic plaques. Additionally, there was an up-regulation of *CCR7* in the DCs of atherosclerotic aorta, which was thought to mediate DC egress during lesion regression (34).

## DISCUSSION

The rarity of DCs in tissues has made it rather difficult to explore their heterogeneity. *In vivo* study by culturing DCs cannot reflect the real biology (35), and bulk cell analysis may mask DC heterogeneity (36). Limited by detection methods, prior studies used only a few phenotypic markers to divide DCs at 1 time (24). Recently, mass cytometry, based on 14 markers, was used to analyze DC populations across tissues and species (2). In the present analysis, we simultaneously analyzed 46 phenotypic and functional markers, which revealed a similar DC heterogeneity to previous flow cytometry results (2). For example, *XCR1*, the marker of cDC1, accounted for 21.4% in liver DCs and 13.1% in spleen DCs, whereas the ratio of *SIRPA*, the marker of cDC2, was 61.7% in liver DCs and 86.0% in spleen DCs.

Furthermore, our work also contributes to an understanding of functional DC heterogeneity, which has never been used for DC classification before. We showed distinct expression patterns of functional markers in lymphoid and nonlymphoid DCs, suggesting their potential roles in migration, maturation, and T-cell stimulation. Chemokine receptors *CCR1*, *CCR2*, and *CCR4* were up-regulated in liver DCs, suggesting enhanced migration ability. Previous studies were mainly focused on *CCR7* (37), which may not be sufficient to understand DC migration in nonlymphoid tissues. Spleen DCs exhibited a more-mature state with an enriched costimulatory molecule *CD80* (16), which corresponded to our immunofluorescence showing more dendrites. DCs were close to the T-cell zone in the spleen. Up-regulation of the TLR adaptor *MYD88* in spleen DCs indicated enhanced T-cell activation (15), which may be related with DC-T cell interactions.

Previous research revealed that DCs accumulate in atherosclerotic lesions (38) and are predisposed to atherosclerosis, which is a well-known, chronic inflammatory disease of the large and medium arteries (39). We then concentrated on the migration ability of DCs in *ApoE*<sup>-/-</sup> mice and demonstrated an unexpected change in DCs, which could be hidden by traditional bulk-cell analysis. Our results showed an increase in *CCR7* in spleen DCs in the *ApoE*<sup>-/-</sup> mice. Interestingly, *CCR7* was decreased in single DCs from the *ApoE*<sup>-/-</sup> liver. *CCR7* was reported to participate in the egress of DCs. In the atherosclerosis regression model, blockade of *CCR7* ligands *CCL19* and *CCL21* preserved CD11c<sup>+</sup> cells in atherosclerotic plaques,

suggesting a CCR7-dependent emigration for DCs (34). Decreased expression of *CCR7* in *ApoE*<sup>-/-</sup> liver DCs may indicate impaired migration under sustained hyperlipidemia conditions, which remains to be explained.

Additionally, the present phenotypic and functional classifications have been focused on commonly defined DCs, that is, CD11c<sup>+</sup>MHCII<sup>+</sup>, whereas flow cytometry also identified another subgroup of cells, characterized as CD11c<sup>+</sup>MHCII<sup>-</sup>, which are considered to be DC precursors in mice (40, 41). The percentages of CD11c<sup>+</sup>MHCII<sup>-</sup> cells were significantly up-regulated from 0.42–1.17% in the healthy liver to 1.08–1.30% in the *ApoE*<sup>-/-</sup> liver, and the ratios were increased from 1.58–1.95% in the healthy spleen to 1.96–2.60% in the

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