

Supporting Information

Comprehensive Characterization of Age-Related Changes in Cell Subpopulations and Tissue Structural Properties in Secondary Lymphoid Organs

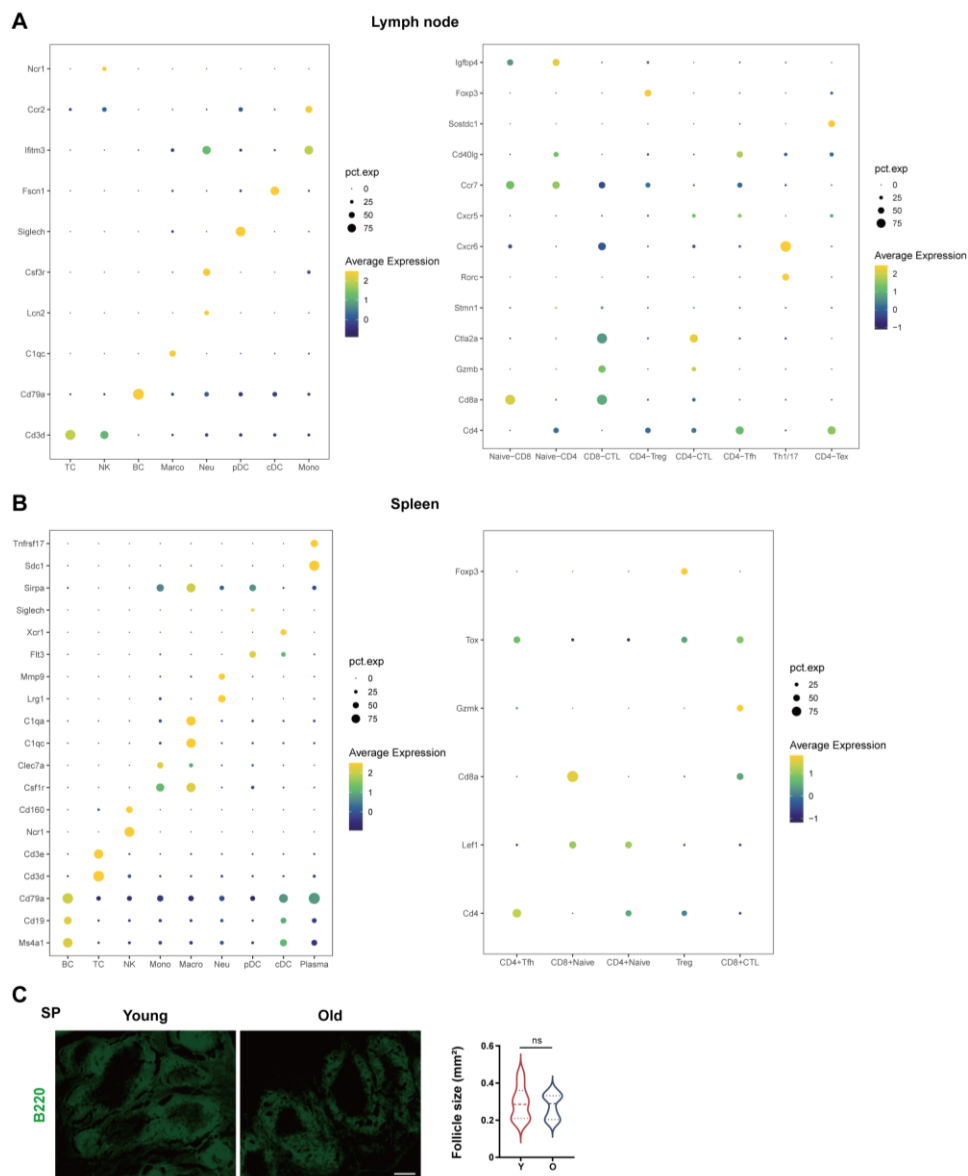
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The PDF file includes:

Fig. S1 to 6

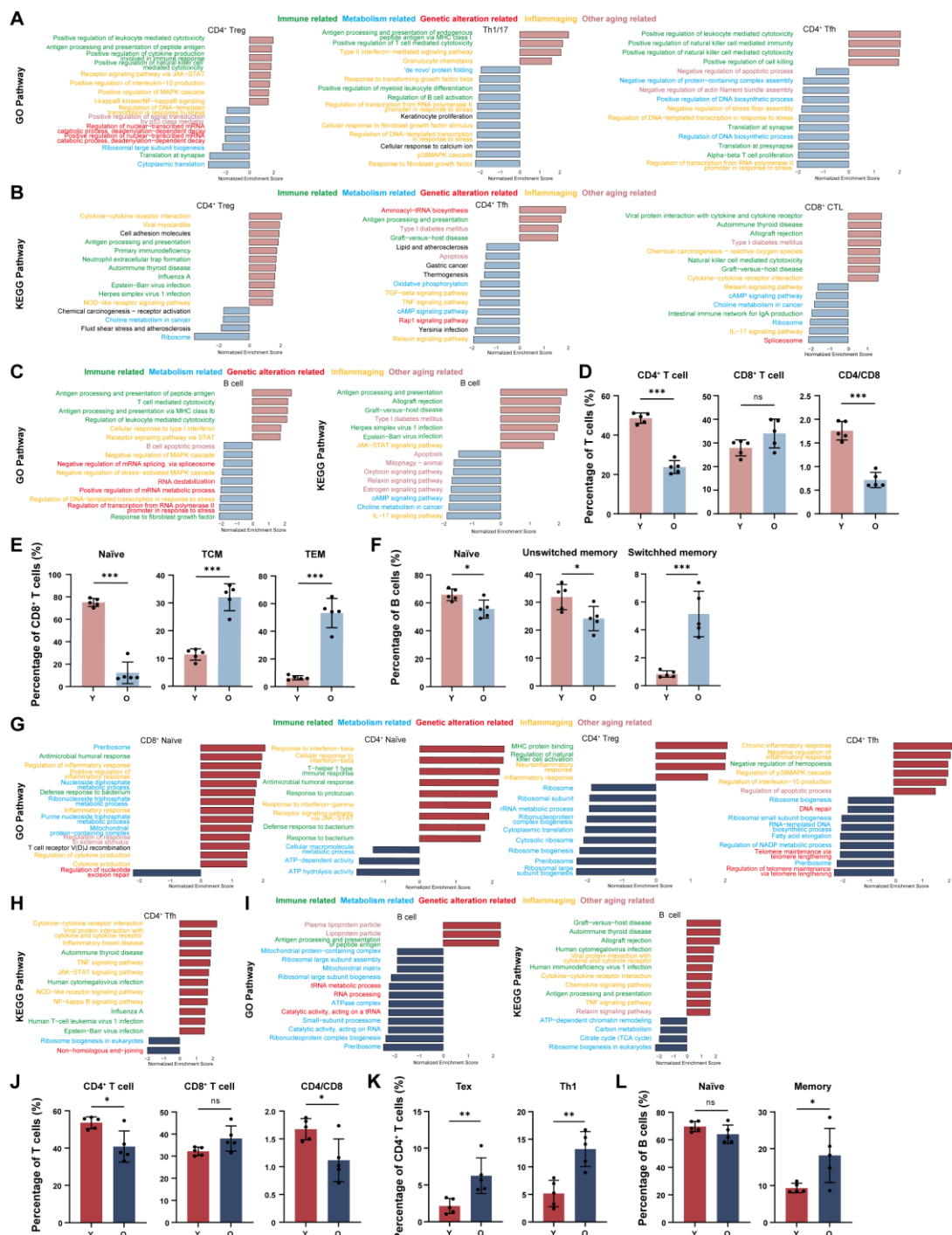
Table S1

Fig. S1



(A) The heatmap illustrating the average expression levels of key genes across different cell clusters and T cell subsets in young and old LNs. (B) The heatmap illustrating the average expression levels of key genes across different cell clusters and T cell subsets in young and old spleen. (C) Histological sections of spleen with immunostaining for B cells (B220, green) and measurement of the area of B220⁺ B cell follicles in young and old mice. Scale bars represent 200 μ m ($n = 5$ mice/group). P -values were calculated between two groups using an unpaired t -test. ns, not significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

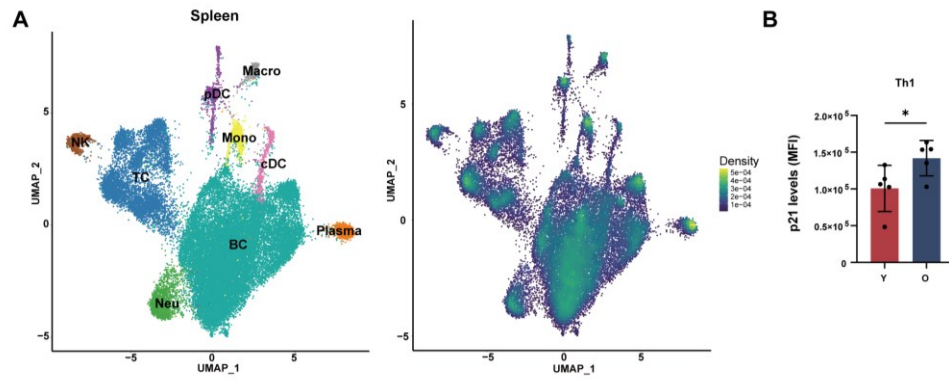
Fig. S2



(A-B) NES of enrichment for GO biological process terms and KEGG terms with an age-related change. Only top 15 GO or KEGG terms with P -value < 0.05 were listed for all T subsets in young and old LNs. (C) NES of enrichment for GO biological process terms and KEGG terms with an age-related change. Only top 15 GO or KEGG terms with P -value < 0.05 were listed for B cells in young and old LNs. (D) Flow cytometric analysis of CD4⁺ T cells and CD8⁺ T cells was performed. The

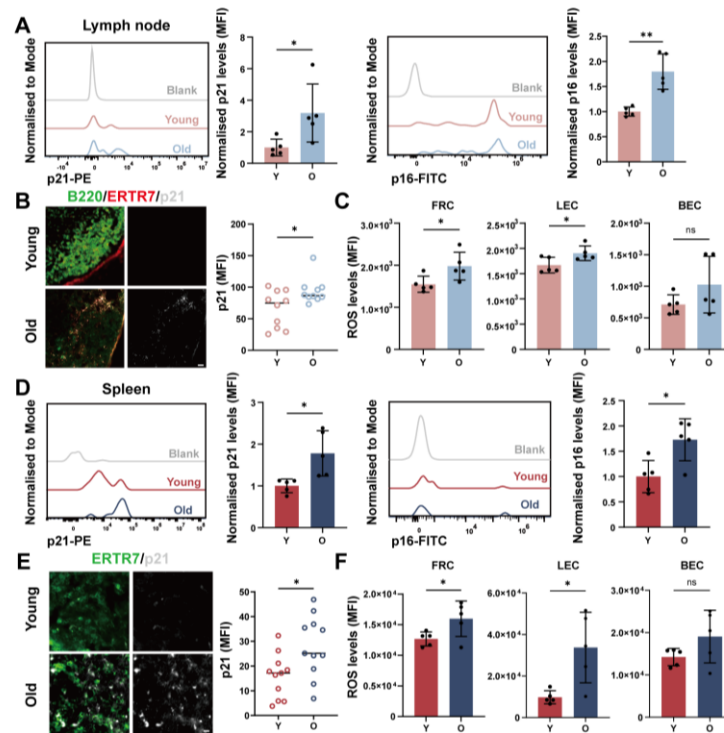
percentage representation of the populations was shown in young and old LNs (n = 5 mice/group). (E) Flow cytometric analysis of CD8⁺ naïve T cells, TCM cells and TEM cells was performed. The percentage of the populations was shown in young and old LNs (n = 5 mice/group). (F) Flow cytometric analysis of naïve B cells (CD19⁺, IgD⁺, CD27⁻), unswitched memory cell (CD19⁺, IgD⁺, CD27⁺) and switched memory cell (CD19⁺, IgD⁻, CD27⁺) was performed. The percentage representation of the populations was shown in young and old LNs (n = 5 mice/group). (G-H) NES of enrichment for GO biological process terms and KEGG terms with an age-related change. Only top 15 GO or KEGG terms with *P*-value < 0.05 were listed for each major T cell subset in young and old spleen. (I) NES of enrichment for GO biological process terms and KEGG terms with an age-related change. Only top 15 GO or KEGG terms with *P*-value < 0.05 were listed for B cells in young and old spleen. (J) Flow cytometric analysis of CD4⁺ T cells and CD8⁺ T cells was performed. The percentage of the populations was shown in young and old spleen (n = 5 mice/group). (K) Flow cytometric analysis of CD4⁺ Tex cells and CD4⁺ Th1 cells was performed. The percentage of the populations was shown in young and old spleen (n = 5 mice/group). (L) Flow cytometric analysis of naïve B cells and memory B cells was performed. The percentage of the populations was shown in young and old spleen (n = 5 mice/group). *P*-values were calculated between two groups using an unpaired *t*-test. ns, not significant; **P*<0.05; ***P*<0.01; ****P*<0.001.

Fig. S3



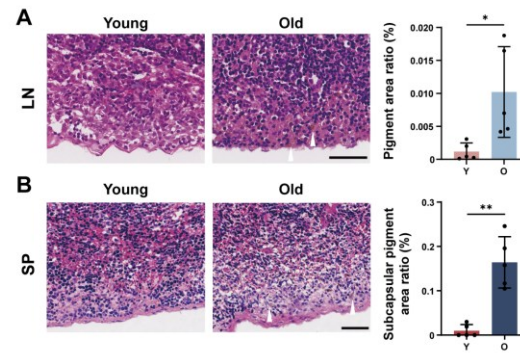
(A) *Left Panel*: UMAP plot illustrating subclusters of cell subsets in young and old spleen; *Right Panel*: Density distribution plot of aging scores. (B) Flow cytometric analysis of p21 levels in Th1 cells populations in young and old spleen (n = 5 mice/group). *P*-values were calculated between two groups using an unpaired *t*-test. ns, not significant; **P*<0.05; ***P*<0.01; ****P*<0.001.

Fig. S4



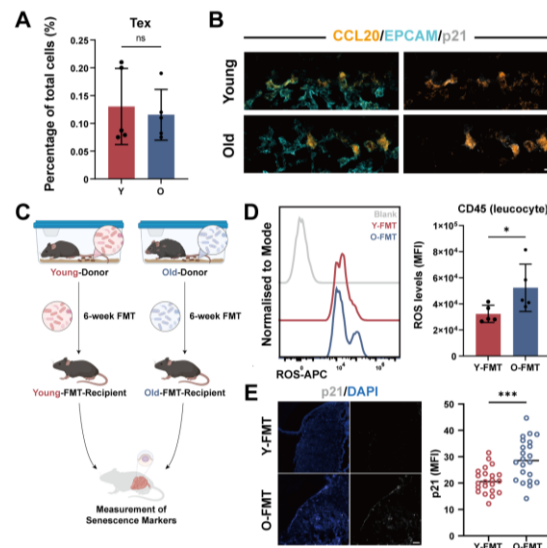
(A) Flow cytometric analysis of p21 and p16 levels in stromal cells (CD45⁻, PDPN⁺) in young and old LNs (n = 5 mice/group). (B) Histological sections of LNs with immunostaining for stromal cells (ERTR7, red) and measurement of p21 MFI of ERTR7⁺ stromal cell area in young and old LNs. Scale bars represent 50 μ m (n = 5 mice/group). (C) Flow cytometric analysis of ROS levels in FRCs (PDPN⁺, CD31⁻), LECs (PDPN⁺, CD31⁺) and BECs (PDPN⁻, CD31⁺) in young and old LNs (n = 5 mice/group). (D) Flow cytometric analysis of p16 and p21 levels in stromal cells (CD45⁻PDPN⁺) in young and old spleen (n = 5 mice/group). (E) Histological sections of spleen with immunostaining for stromal cells (ERTR7, green) and measurement of p21 MFI of ERTR7⁺ stromal cell area in young and old spleen. Scale bars represent 10 μ m (n = 5 mice/group). (F) Flow cytometric analysis of ROS levels in FRCs (PDPN⁺, CD31⁻), LECs (PDPN⁺, CD31⁺) and BECs (PDPN⁻, CD31⁺) in young and old spleen (n = 5 mice/group). *P*-values were calculated between two groups using an unpaired *t*-test. ns, not significant; **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

Fig. S5



(A) Representative images of LN tissue sections after HE staining and measurement of pigment area within the visual field in young and old mice. Scale bars represent 50 μm ($n = 5$ mice/group). (B) Representative images of spleen tissue sections after HE staining and measurement of subcapsular pigment area within the visual field in young and old mice. Scale bars represent 50 μm ($n = 5$ mice/group). P -values were calculated between two groups using an unpaired t -test. ns, not significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Fig. S6



(A) Flow cytometric analysis of CD4⁺ Tex cells was performed. The percentage of the populations was shown in young and old PPs (n = 5 mice/group). (B) Histological sections of intestinal PPs with immunostaining for mature M cells (CCL20, bright orange) and epithelial cells (EPCAM, soft cyan). Scale bars represent 50 μ m (n = 5 mice/group). (C) Illustration of faecal microbiota transplantation (FMT) experiment. (D) Flow cytometric analysis of ROS levels in CD45⁺ leucocyte populations in PPs from young-FMT recipients and old-FMT recipients (n = 5 mice/group). (E) Immunostaining of PP histological sections and measurement of p21 MFI from young-FMT recipients and old-FMT recipients. Scale bars represent 50 μ m (n = 5 mice/group). *P*-values were calculated between two groups using an unpaired *t*-test. ns, not significant; **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

Table S1. Key Resources

ANTIBODIES	SOURCE	IDENTIFIER
Waf1/Cip1/CDKN1A p21 antibody (F-5)	Santa Cruz Biotechnology	Cat# sc-6246; RRID: AB_628073
CDKN2A/p16 antibody (F-12)	Santa Cruz Biotechnology	Cat# sc-1661; RRID: AB_628067
LYVE-1 antibody (E9VA4)	Santa Cruz Biotechnology	Cat# sc-65647; RRID: AB_1123635
podoplanin antibody (F-3)	Santa Cruz Biotechnology	Cat# sc-376962
MAdCAM-1 antibody (H-3)	Santa Cruz Biotechnology	Cat# sc-365934; RRID: AB_10916705
Fibroblast Marker antibody (ER-TR7)	Santa Cruz Biotechnology	Cat# sc-73355; RRID: AB_1122890
APC anti-mouse CD21/CD35 (CR2/CR1) Antibody	BioLegend	Cat# 123411; RRID: AB_940395
FITC anti-mouse CD45 Antibody	BioLegend	Cat# 103107; RRID: AB_312972
PerCP/Cyanine5.5 anti-mouse CD3 Antibody	BioLegend	Cat# 100218; RRID: AB_1595492
Brilliant Violet 785™ anti-mouse CD19 Antibody	BioLegend	Cat# 115543; RRID: AB_11218994
PE anti-mouse CD4 Antibody	BioLegend	Cat# 100407; RRID: AB_312692
APC anti-mouse/human CD44 Antibody	BioLegend	Cat# 103011; RRID: AB_312962
PE anti-mouse CD169 (Siglec-1) Antibody	BioLegend	Cat# 142404; RRID: AB_10915697
PE anti-mouse CD31 Antibody	BioLegend	Cat# 102407; RRID: AB_312902
Brilliant Violet 421™ anti-mouse F4/80 Antibody	BioLegend	Cat# 123131; RRID: AB_10901171
Brilliant Violet 785™ anti-human/mouse Granzyme B Recombinant Antibody	BioLegend	Cat# 396437; RRID: AB_3106140
Alexa Fluor® 647 anti-mouse CD326 (Ep-CAM) Antibody	BioLegend	Cat# 118211; RRID: AB_1134104
CCL20/MIP-3 alpha Polyclonal antibody	Proteintech	Cat# 26527-1-AP; RRID: AB_2880543
CD3e Monoclonal Antibody (145-2C11) APC	Thermo Fisher Scientific	Cat# 17-0031-82; RRID: AB_469315
CD19 Monoclonal Antibody (eBio1D3 (1D3)) APC	Thermo Fisher Scientific	Cat# 17-0193-82; RRID: AB_1659676
CD8a Monoclonal Antibody (53-6.7) Alexa Fluor™ 700	Thermo Fisher Scientific	Cat# 56-0081-82; RRID: AB_494005
CD62L (L-Selectin) Monoclonal Antibody (MEL-14) eFluor™ 450	Thermo Fisher Scientific	Cat# 48-0621-82; RRID: AB_1963590
CD27 Monoclonal Antibody (LG.7F9) PE-Cyanine7	Thermo Fisher Scientific	Cat# 25-0271-82; RRID: AB_1724035
IgD Monoclonal Antibody (11-26c (11-26)) eFluor™ 450	Thermo Fisher Scientific	Cat# 48-5993-82; RRID: AB_1272202
Podoplanin Monoclonal Antibody (eBio8.1.1(8.1.1)), Super Bright™ 436	Thermo Fisher Scientific	Cat# 62-5381-82; RRID: AB_2744800
NK1.1 Monoclonal Antibody (PK136), FITC	Thermo Fisher Scientific	Cat# 11-5941-82; RRID: AB_465318
FITC Rat Anti-Mouse CD45R/B220	BD Bioscience	Cat# 553087; RRID: AB_394617
BV510 Rat Anti-Mouse CD4	BD Bioscience	Cat# 740105; RRID: AB_2739863
R718 Hamster Anti-Mouse CD183 (CXCR3)	BD Bioscience	Cat# 752155; RRID: AB_2917263
Alexa Fluor™ 647 Rat Anti-Mouse CD366 (TIM-3)	BD Bioscience	Cat# 568797
PE-Cy™7 Rat Anti-Mouse CD185 (CXCR5)	BD Bioscience	Cat# 560617; RRID: AB_1727521
BV650 Hamster Anti-Mouse CD154	BD Bioscience	Cat# 740480; RRID: AB_2740205
Ms CD279 (PD-1) BV421 RMP1	BD Bioscience	Cat# 748268; RRID: AB_2872696
BV421 Rat Anti-Mouse Foxp3	BD Bioscience	Cat# 562996; RRID: AB_2737940
Anti-GP2 (Glycoprotein 2) (Mouse) mAb	MBL Life Sciences	Cat# D278-3