

# PNAS

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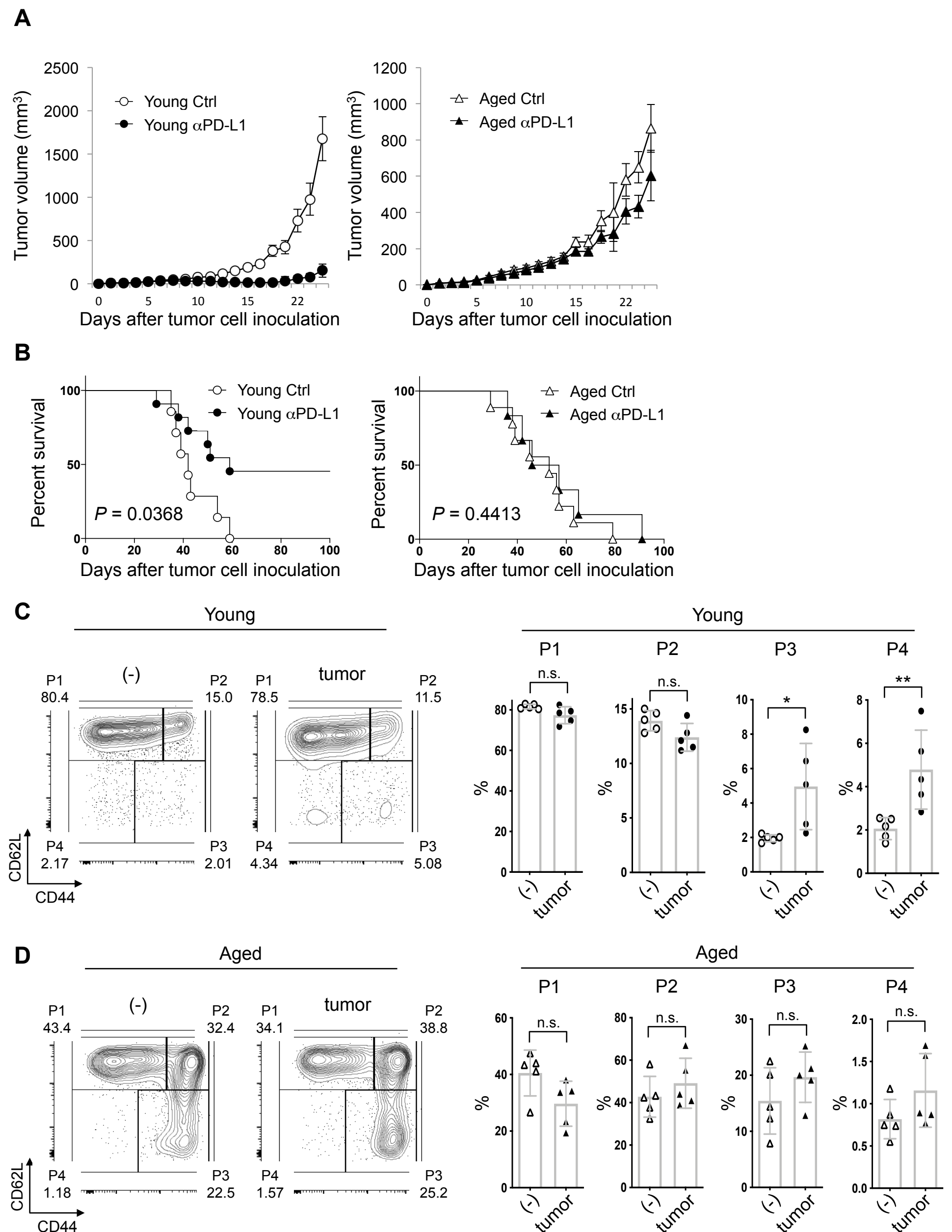
**Supporting Information for  
Critical role of the CD44<sup>low</sup>CD62L<sup>low</sup> CD8<sup>+</sup> T cell subset in  
restoring antitumor immunity in aged mice**

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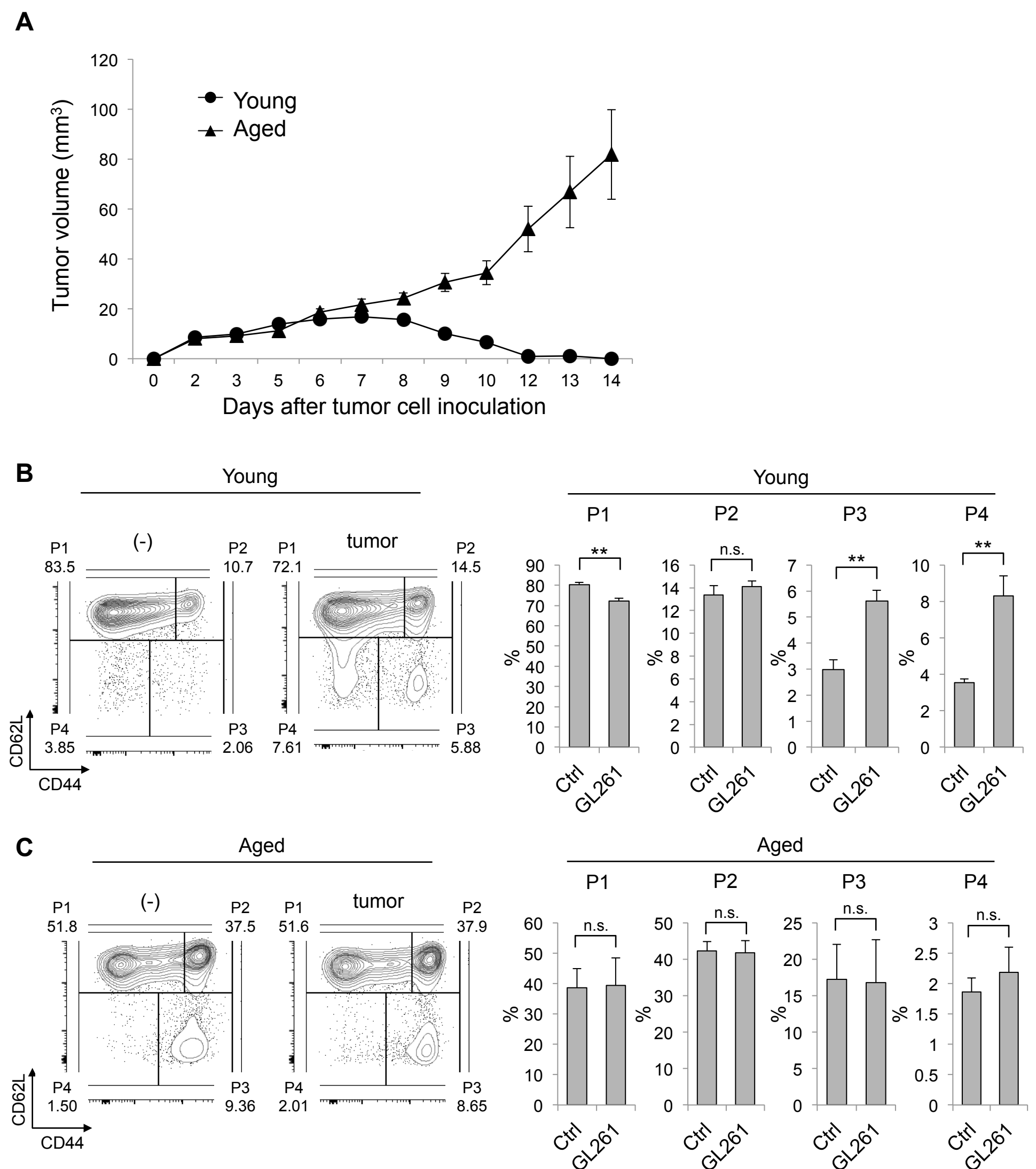
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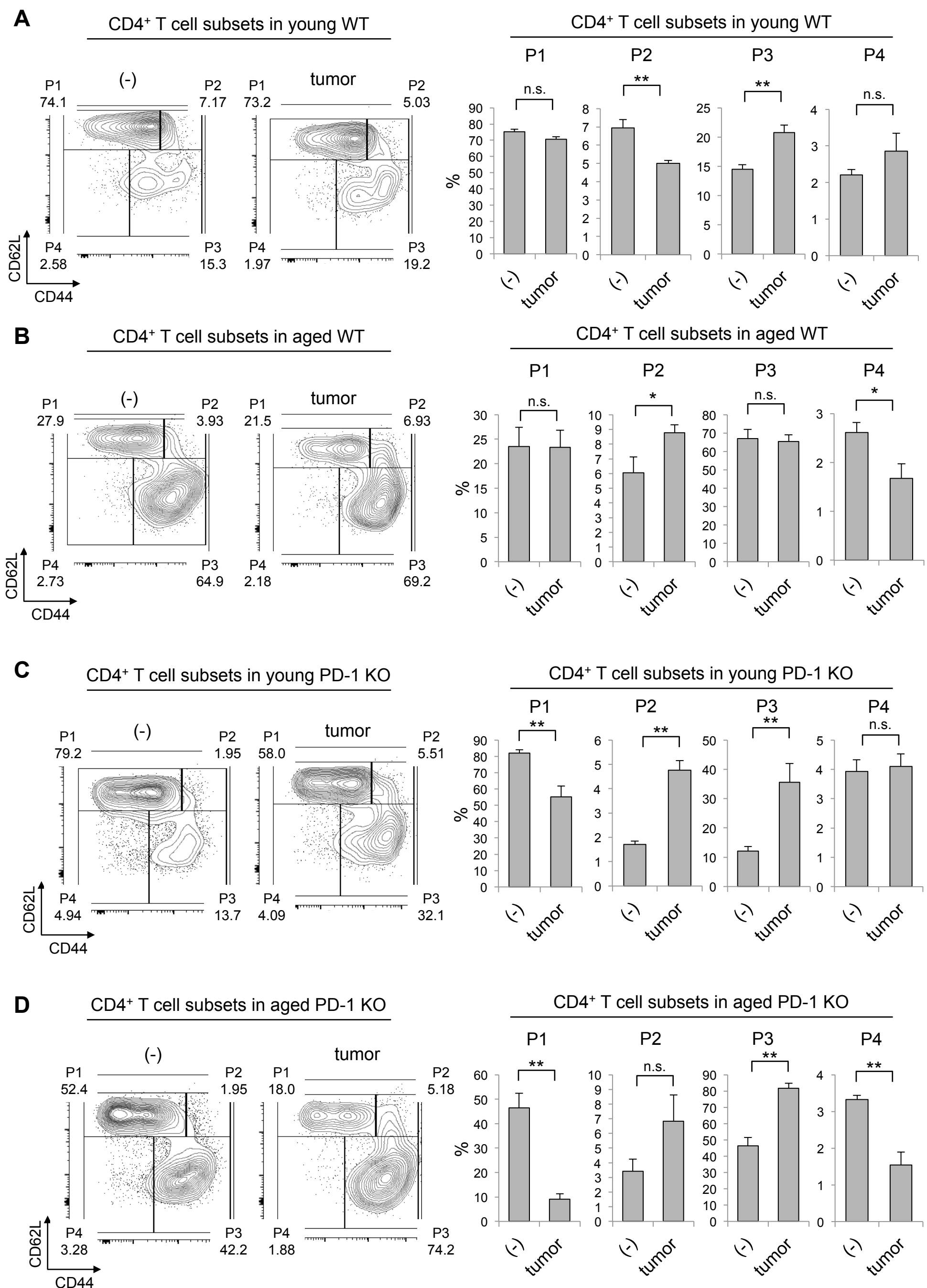
Figures S1 to S10



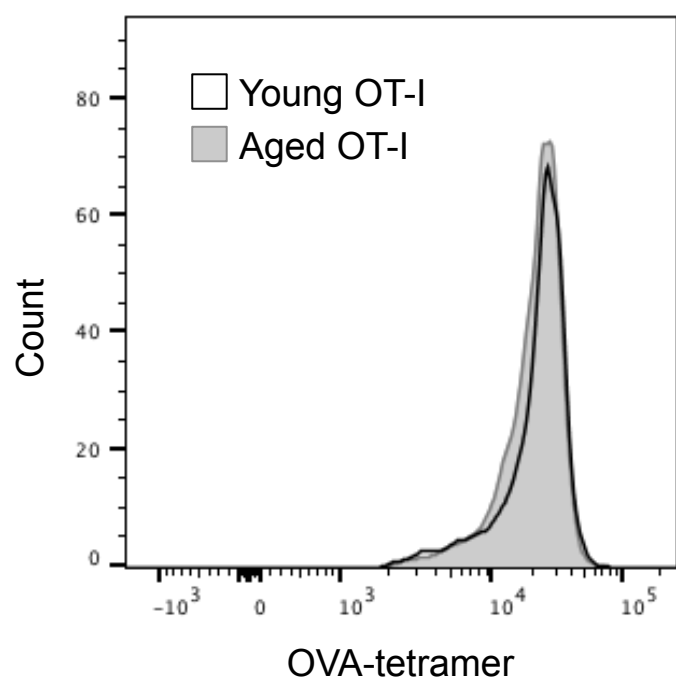
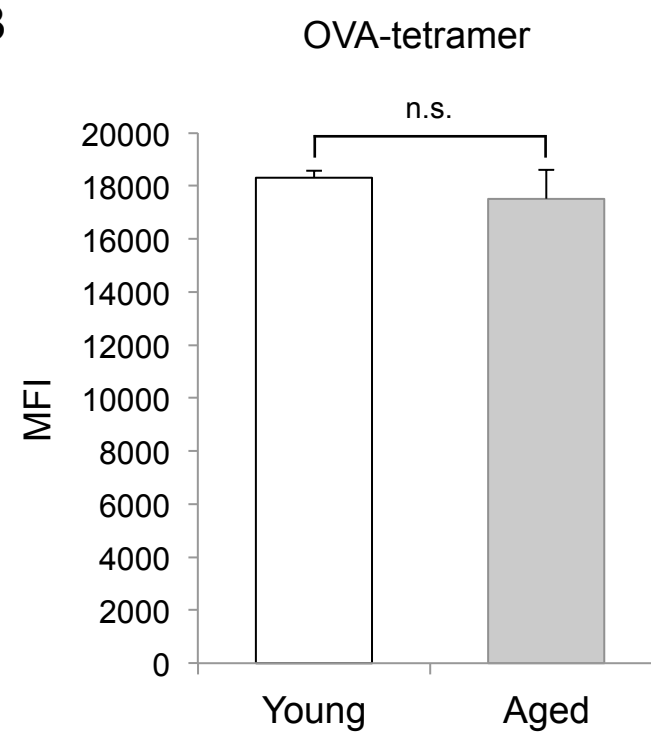
**Fig. S1: Loss of antitumor activity and reduced P4 cell induction in aged WT mice with MC38 tumors. (A and B)** MC38 cells were i.d. inoculated into young (2 mo old) and aged (14 mo old) C57BL/6 WT mice. Then, those mice were injected with anti-PD-L1 monoclonal antibody (mAb) on day 5, 11, and 17. **(A)** MC38-tumor sizes in young and aged WT mice. **(B)** Percent survival of MC38-tumor-bearing WT mice. *P* values were calculated by log-rank test. **(C and D)** Analysis of CD8<sup>+</sup> T cell subsets in young (**C**, 2 mo old) or aged (**D**, 15–19 mo old) WT mice with or without injection of MC38 cells. Stained PLN and DLN cells on day 9. Representative plots showing CD44 and CD62L expression gated on CD3<sup>+</sup>CD8<sup>+</sup> T cells and percentages of CD8<sup>+</sup> T cell subsets; CD44<sup>low</sup>/CD62L<sup>high</sup> (naive; P1), CD44<sup>high</sup>/CD62L<sup>high</sup> (central memory; P2), CD44<sup>high</sup>/CD62L<sup>low</sup> (effector/memory; P3), CD44<sup>low</sup>/CD62L<sup>low</sup> (P4). \**p* < 0.05; \*\**p* < 0.01; n.s., not significant (two-tailed unpaired Student's *t* test). Data are presented as the mean ± SEM (*n* = 5 for A, C, & D, *n* = 9–11 for B).



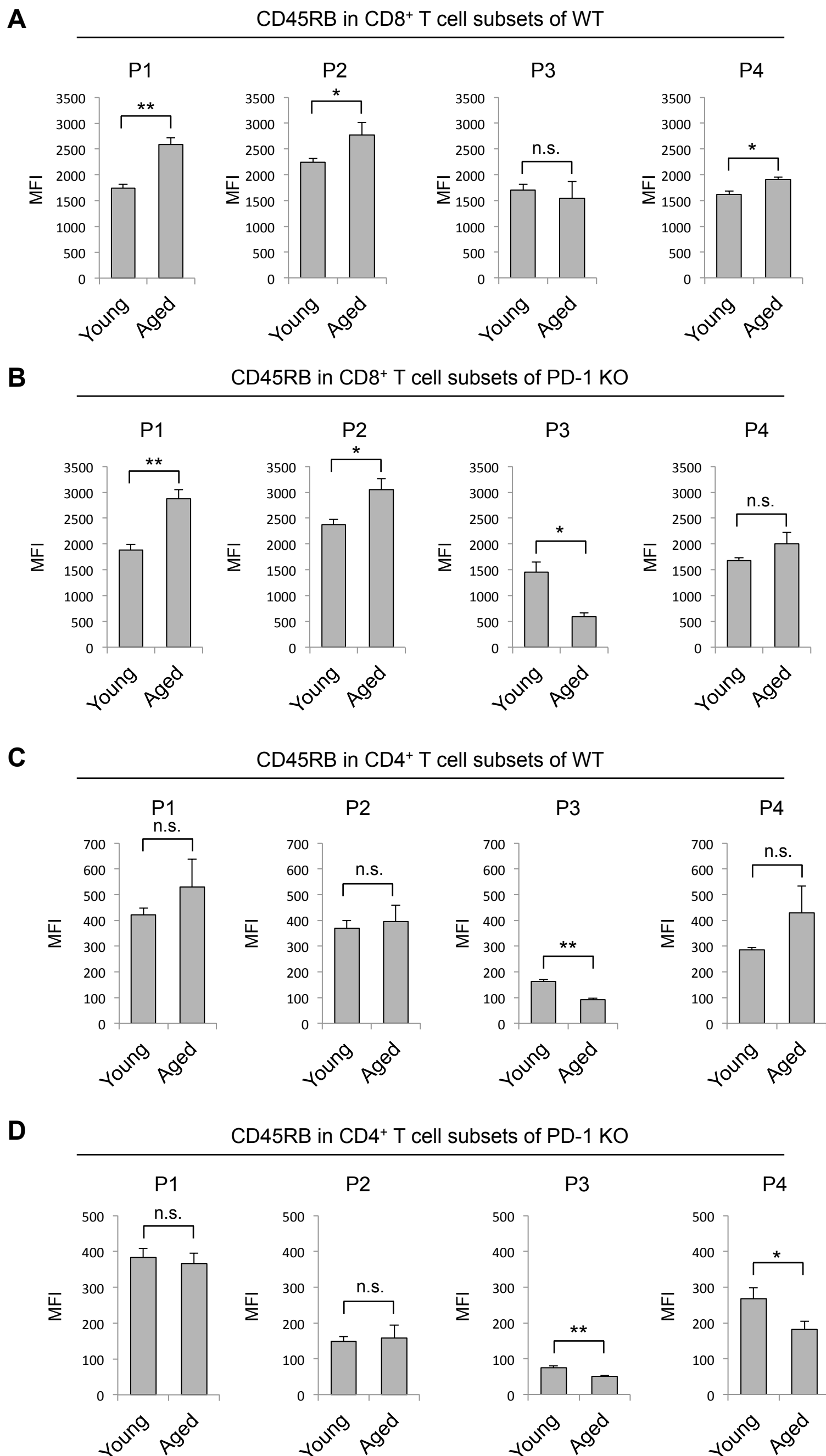
**Fig. S2: Loss of antitumor activity and reduced P4 cell induction in aged PD-1 KO mice with GL261 tumors. (A)** GL261-tumor sizes in young and aged PD-1 KO mice. GL261 cells were i.d. inoculated into young (3 mo old) and aged (15–19 mo old) PD-1KO mice. **(B and C)** Analysis of CD8<sup>+</sup> T cell subsets in young (1 mo old) or aged (13–15 mo old) PD-1 KO mice with or without injection of GL261 cells. Stained PLN and DLN cells on day 9. Representative plots showing CD44 and CD62L expression gated on CD3<sup>+</sup>CD8<sup>+</sup> T cells and percentages of CD8<sup>+</sup> T cell subsets. \* $p < 0.05$ ; \*\* $p < 0.01$ ; n.s., not significant (two-tailed unpaired Student's  $t$  test). Data are presented as the mean  $\pm$  SEM ( $n = 6$ ).



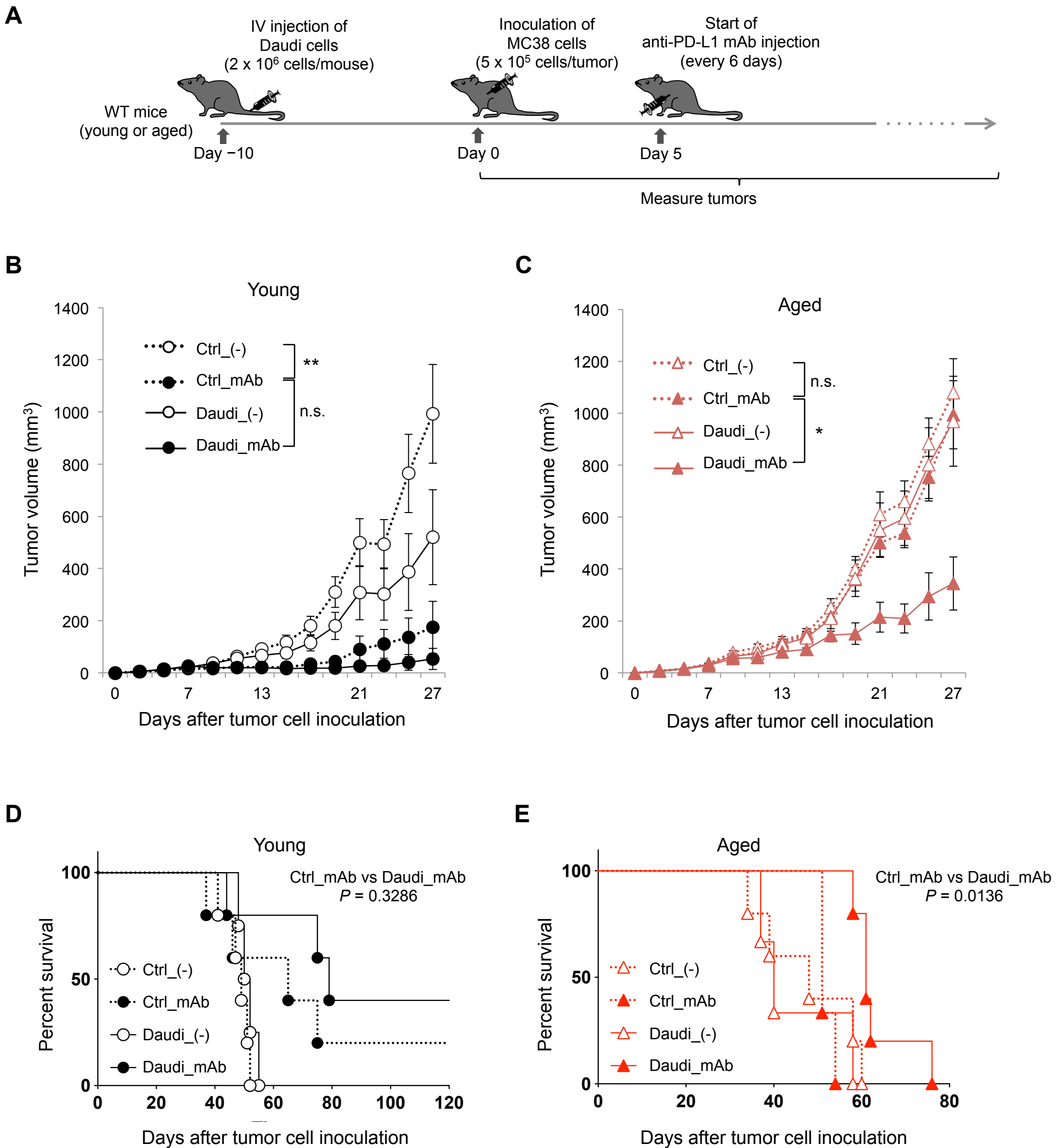
**Fig. S3: Effects of aging on CD4<sup>+</sup> T cell subsets of WT and PD-1 KO mice.** Analysis of CD4<sup>+</sup> T cell subsets in young WT (**A**, 2–3 mo old), aged WT (**B**, 14–17 mo old), young PD-1 KO (**C**, 2–3 mo old), or aged PD-1 KO (**D**, 13–17 mo old) mice with or without MC38 tumors. Stained PLN and DLN cells on day 9. Representative plots showing CD44 and CD62L expression gated on CD3<sup>+</sup>CD4<sup>+</sup> T cells and percentages of CD4<sup>+</sup> T-cell subsets; CD44<sup>low</sup>/CD62L<sup>high</sup> (naive; P1), CD44<sup>high</sup>/CD62L<sup>high</sup> (central memory; P2), CD44<sup>high</sup>/CD62L<sup>low</sup> (effector/memory; P3), CD44<sup>low</sup>/CD62L<sup>low</sup> (P4). \**p* < 0.05; \*\**p* < 0.01; n.s., not significant (two-tailed unpaired Student's *t* test). Data are presented as the mean ± SEM (*n* = 4–8).

**A****B**

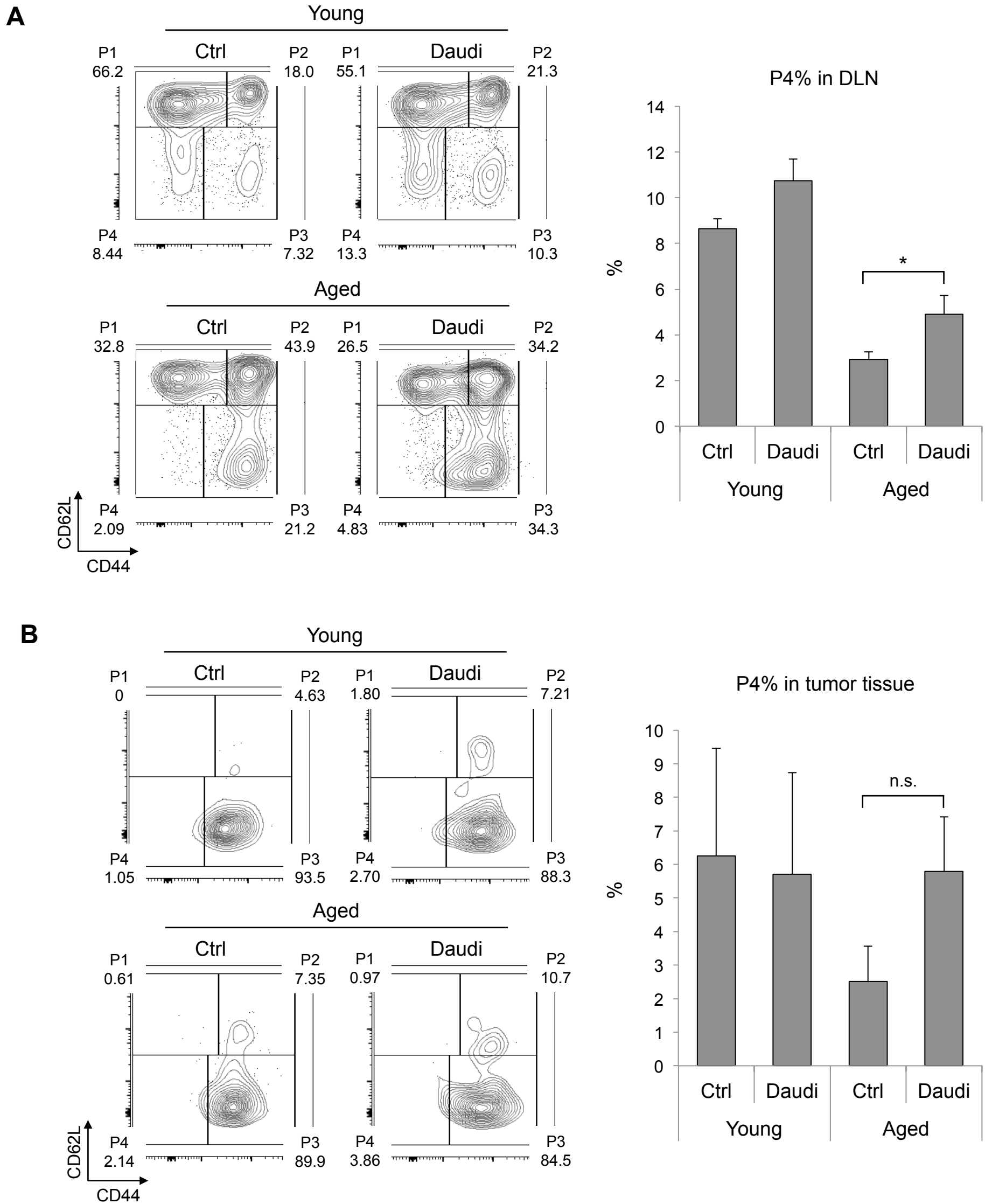
**Fig. S4: Similar TCR avidity to OVA antigen between young and aged OT-I CD8<sup>+</sup> T cells.** Mean fluorescence intensity (MFI) of OVA-specific MHC tetramer in young and aged OT-1 mice is shown by representative plot (**A**) and graph (**B**). Data are presented as the mean  $\pm$  SEM ( $n = 4-6$ ), n.s., not significant (two-tailed unpaired Student's  $t$  test).



**Fig. S5: Enhanced CD45RB expression in CD8<sup>+</sup> and CD4<sup>+</sup> T cells from aged WT and PD-1 KO mice.** (**A** and **B**) CD45RB expression levels in CD8<sup>+</sup> T cell subsets from PLNs of Young (2 to 3 mo old) and aged (14 to 17 mo old) WT (**A**) or PD-1 KO (**B**) mice. (**C** and **D**) CD45RB expression levels in CD4<sup>+</sup> T cell subsets from PLNs of young (2 to 3 mo old) and aged (13 to 17 mo old) WT (**C**) or PD-1 KO (**D**) mice. Data are presented as the mean  $\pm$  SEM of three to six mice, \* $p$  < 0.05; \*\* $p$  < 0.01; n.s., not significant (two-tailed unpaired Student's  $t$  test).

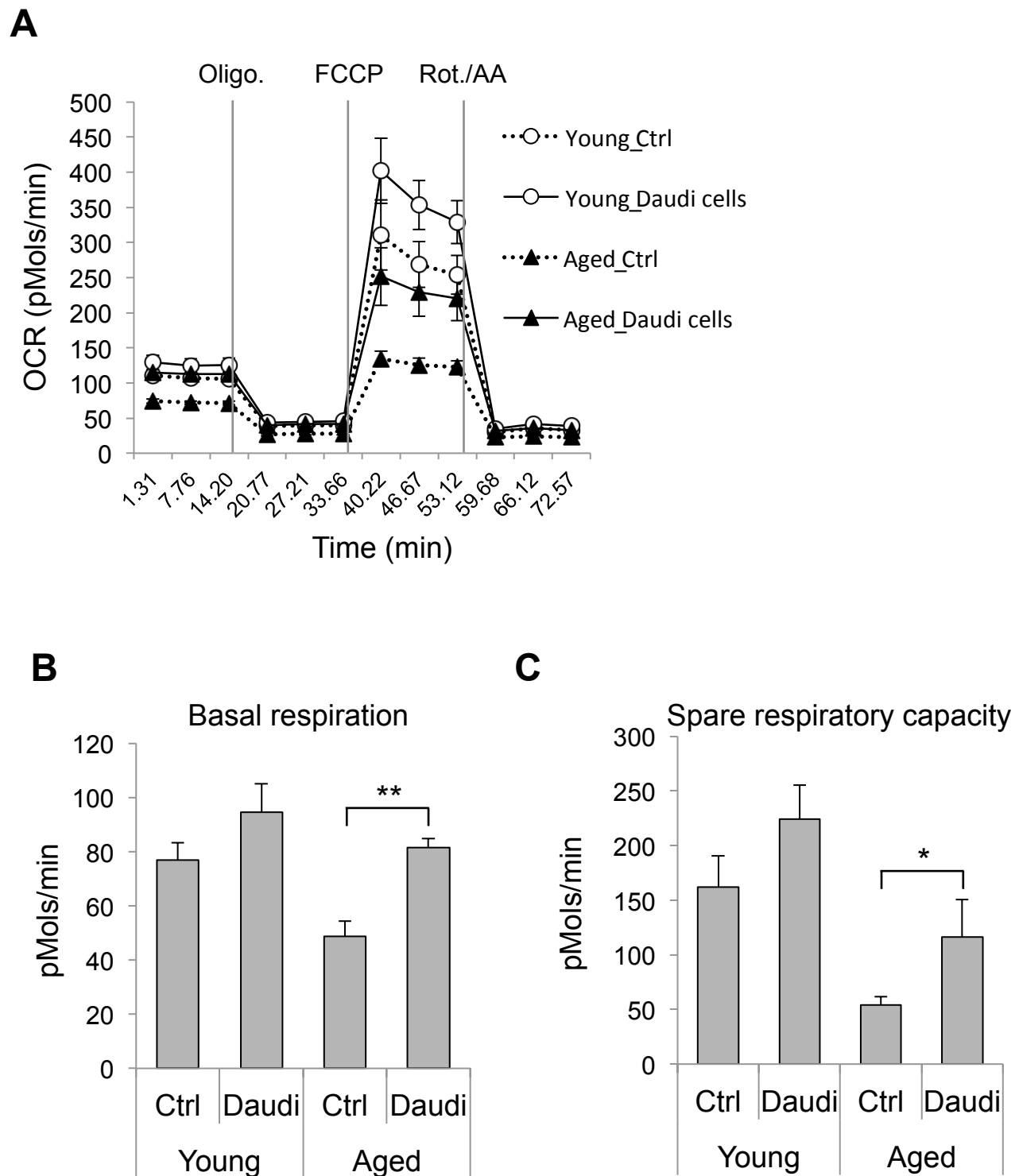


**Fig. S6: Amelioration of age-related resistance to PD-1 blockade therapy in WT mice by xenogeneic cell injection.** Young (1 mo old) and aged (17–19 mo old) C57BL/6 WT mice were i.v. injected with or without (Ctrl) Daudi cells. Ten days after the transfer, the mice were i.d. inoculated with MC38 cells. The treatment of anti-PD-L1 mAb was started on day 5, and repeated 3 times every 6 days. **(A)** Schematic diagram of the experimental schedule. **(B and C)** MC38-tumor sizes in young **(B)** and aged **(C)** WT mice. **(D and E)** Percent survival of MC38-tumor-bearing young **(D)** and aged **(E)** WT mice. Data are presented as the mean  $\pm$  SEM of five to six mice, \* $p < 0.05$ ; \*\* $p < 0.01$ ; n.s., not significant (one-way ANOVA followed by Tukey's test or log-rank test).

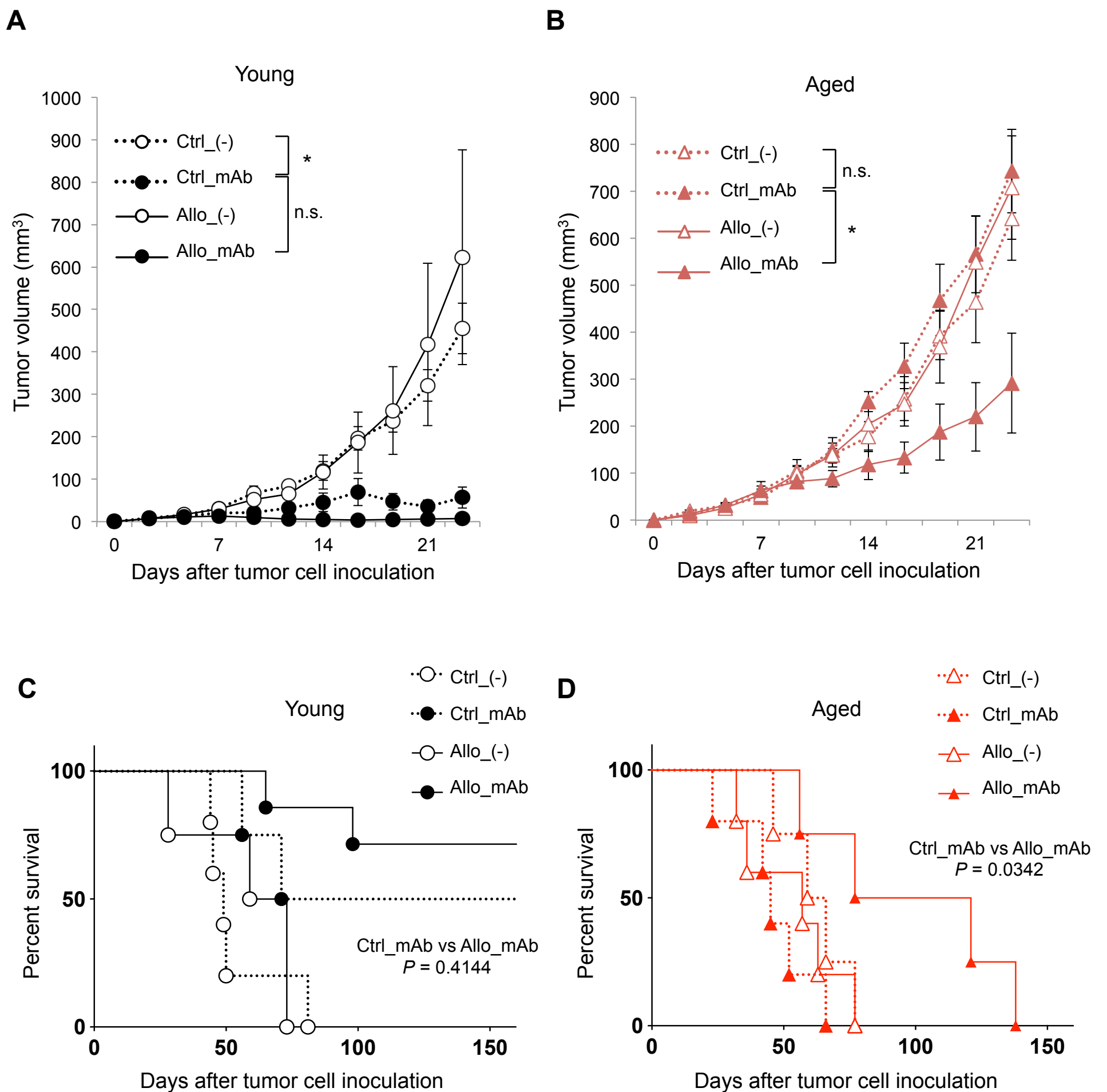


**Fig. S7: Effects of Daudi cell injection on CD8<sup>+</sup> T cell subsets in DLNs and tumors of PD-1 KO mice with MC38 tumors.** Analysis of CD8<sup>+</sup> T cell subsets in DLNs (**A**) and tumors (**B**) of young (3–4 mo old) and aged (16–18 mo old) PD-1 KO mice with the injection of MC38 cells (day 6). Representative plots showing CD44 and CD62L expression gated on CD3<sup>+</sup>CD8<sup>+</sup> T cells and percentages of P4 cells. \* $p < 0.05$ ; n.s., not significant (two-tailed unpaired Student's  $t$  test). Data are presented as the mean  $\pm$  SEM ( $n = 5-6$ ).

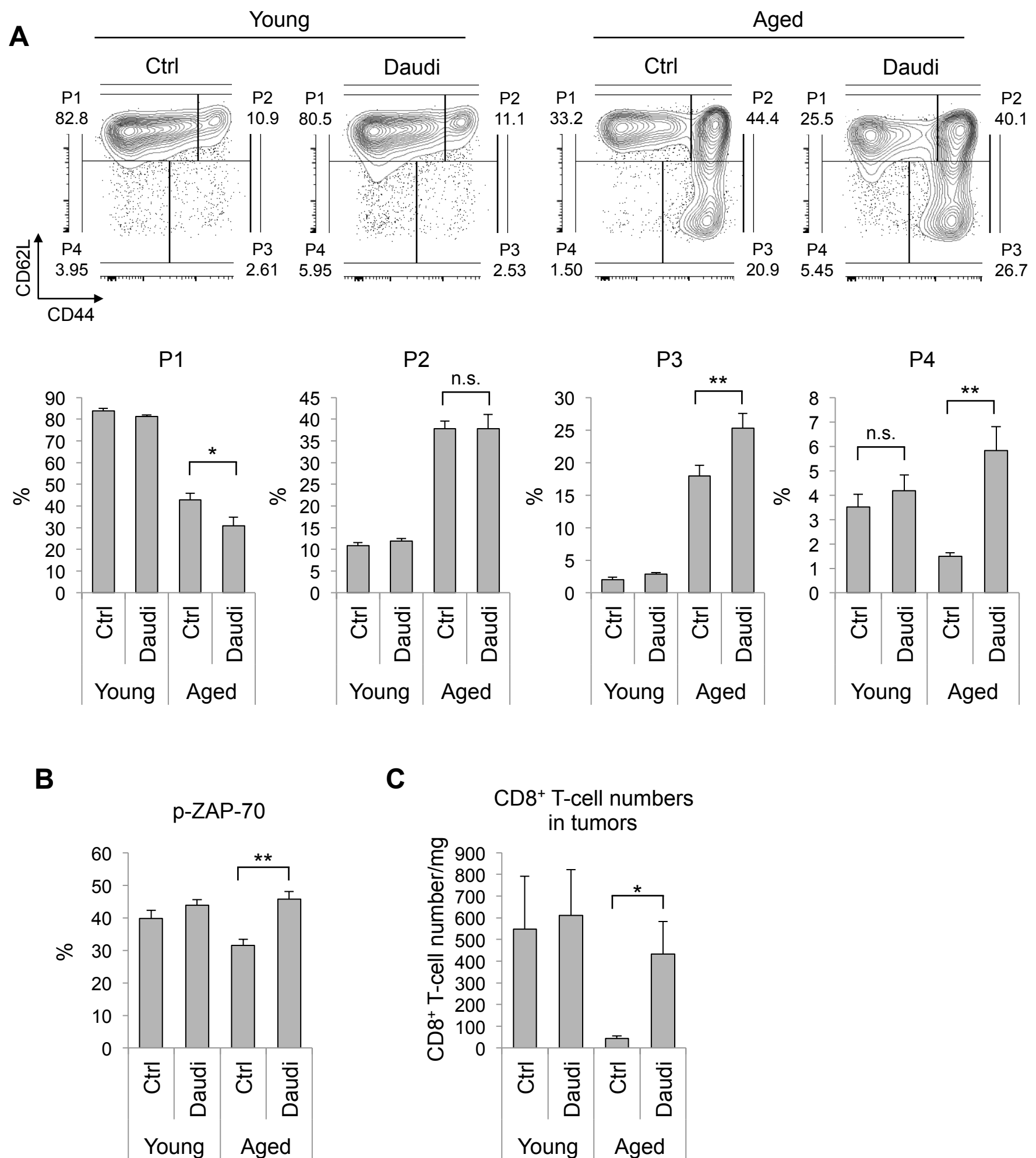




**Fig. S8: Recovery of age-induced mitochondrial disorder by Daudi cell injection.** MC38-OVA cells were i.d. inoculated into young (3–4 mo old) and aged (17–18 mo old) PD-1 KO mice 10 days after PBS (Ctrl) or Daudi cell injection. Six days after the inoculation, OCR of CD8<sup>+</sup> T cells isolated from DLNs of the indicated mice was measured using a Seahorse XFe96 analyzer. OCR trace (**A**), and basal respiration (**B**) and spare respiratory capacity (**C**). Data are presented as the mean  $\pm$  SEM ( $n = 3$ ), \* $p < 0.05$ ; \*\* $p < 0.01$  (one-way ANOVA followed by Tukey's test).



**Fig. S9: Rescue of age-induced resistance to PD-1 blockade therapy by allogeneic cell injection.** Mitomycin C treated splenocytes from C57BL/6 (Ctrl) or Balb/c (Allo) mice were i.v. injected into young (1 mo old) and aged (14–18 mo old) C57BL/6 WT mice. Following experimental condition was same as Figure S6A. MC38-tumor sizes in young (**A**) and aged (**B**) C57BL/6 WT mice. Data are presented as the mean  $\pm$  SEM of five to six mice, \* $p < 0.05$ ; n.s., not significant (one-way ANOVA followed by Tukey's test). (**C** and **D**) Percent survival of MC38-tumor-bearing young (**C**) and aged C57BL/6 WT (**D**) mice.  $P$  values were calculated by log-rank test ( $n = 5-6$ ).



**Fig. S10: Amelioration of age-related resistance to PD-1 blockade therapy in WT mice with established MC38 tumors by xenogeneic cell injection.** MC38 cells were i.d. inoculated into young (2–3 mo old) and aged (17 mo old) C57BL/6 WT mice. Five days after the inoculation, the mice were treated with anti-PD-L1 mAb and were i.v. injected with or without (Ctrl) Daudi cells. Two days after the administration, the mice were used for analysis. **(A)** The percentages of P1–P4 subsets relative to all CD8<sup>+</sup> T cells in DLNs. **(B)** The percentages of p-ZAP-70<sup>+</sup> cells relative to all CD8<sup>+</sup> T cells from DLNs on day 7. **(C)** The number of CD8<sup>+</sup> T cells in tumor tissues. Data are presented as the mean ± SEM of five to six mice, \* $p < 0.05$ ; \*\* $p < 0.01$ ; n.s., not significant (one-way ANOVA followed by Tukey's test or two-tailed unpaired Student's  $t$  test).