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Supplementary Materials for

Tissue-resident CD8$^+$ T cells drive age-associated chronic lung sequelae following viral pneumonia

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The PDF file includes:

Other Supplementary Material for this manuscript includes the following:

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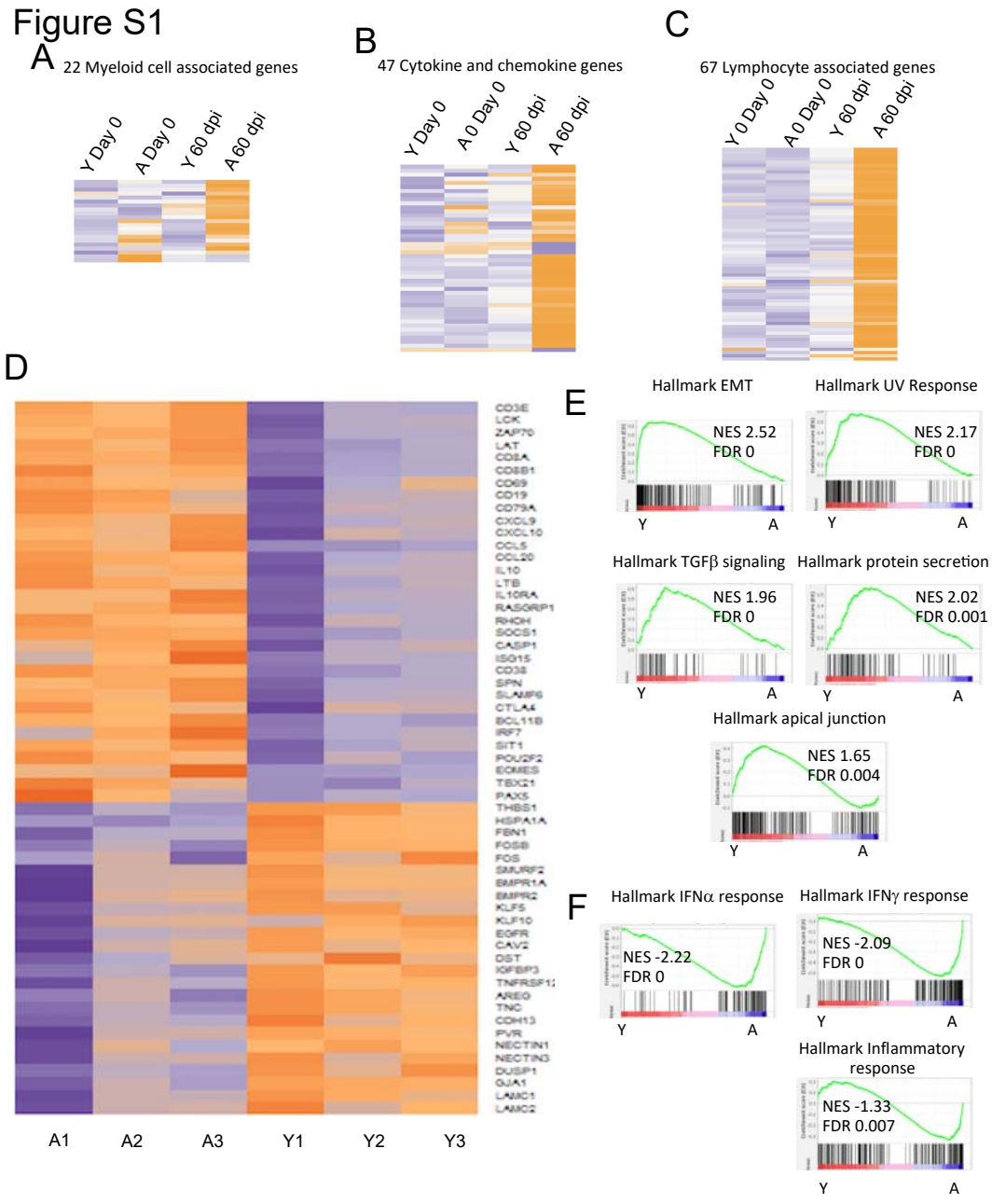

Fig. S1. Aged lungs exhibit persistent inflammatory responses after influenza infection.

A-C. Young (Y) or aged (A) C57BL/6 mice were infected (day 60) or not (day 0) with PR8. 560 immune-associated genes in the lungs were analyzed by Nanostring (at least 3 pooled samples/group). Myeloid **(A)**, cytokine and chemokine (**B)**, or lymphocyte (**C)** associated DEGs that were at least 1.5 fold expression level changes from Aged to young infected samples. **(D)** RNAseq heatmap showing DEGs in young or aged infected lungs 60 d.p.i. **E, F.** GSEA plots of RNAseq data showing genes enriched in young infected lungs **(E)** or aged infected lungs (**F)** with associated normalized enrichment scores (NES) and false-discovery rates (FDR).Nanostring

data were representative of 2 experiments with pooled samples. RNAseq data were from a single replicate with samples in triplicate.

Figure S2

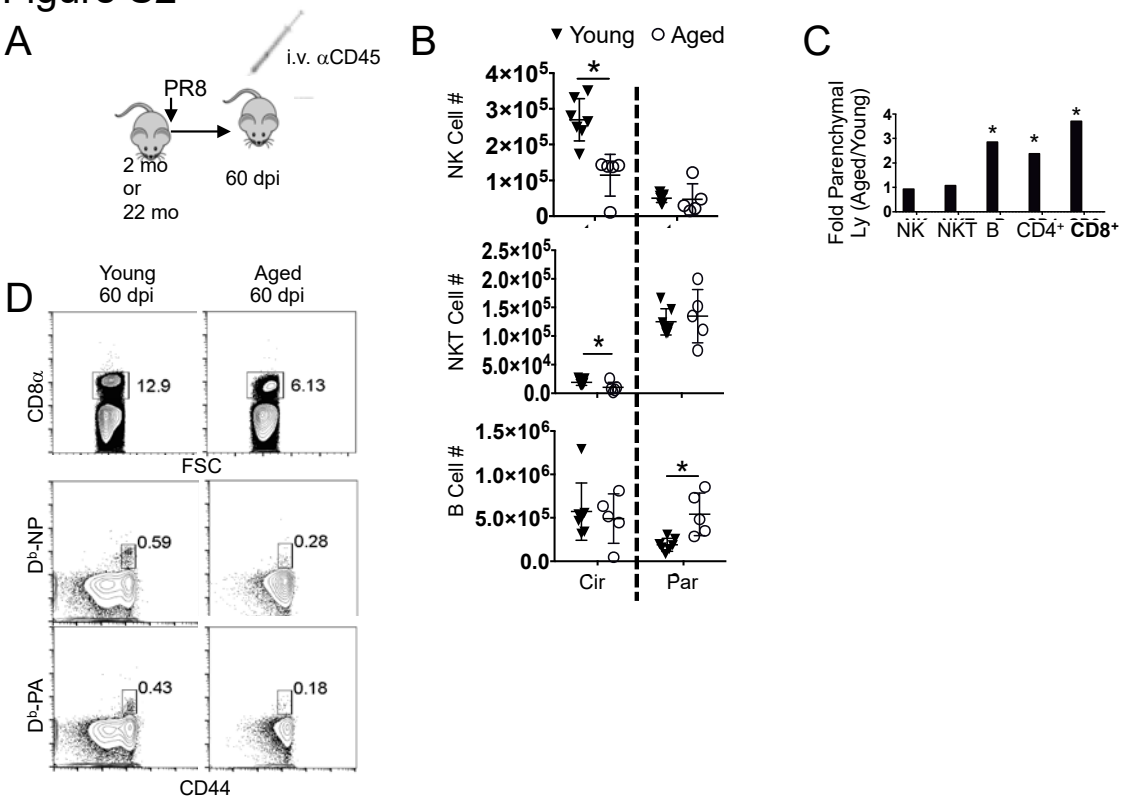

Fig. S2. Enhanced presence of adaptive immune cells in aged lung parenchyma. Young or aged C57BL/6 mice were infected with PR8 and injected intravenously (i.v.) with anti-CD45 to label circulating white blood cells prior to sacrifice. **A.** Schematics of experimental procedure. **B.** NK, NKT, and B cells were enumerated in the lung vasculature (Cir) and parenchyma (Par) at 60 d.p.i. **C.** Fold change of aged/young cells in parenchyma with indicated lymphocyte population at 60 d.p.i. **D.** Representative flow cytometry gating of $CD8^+$ $CD44^{Hi}$ D^b-tetramer$^+$ NP and PA specific memory T cells in the spleen at 60 d.p.i. **B-D** were representatives of 3 experiments. * $p<0.05$ Student's two-tailed t-test with unequal variance.

Figure S3

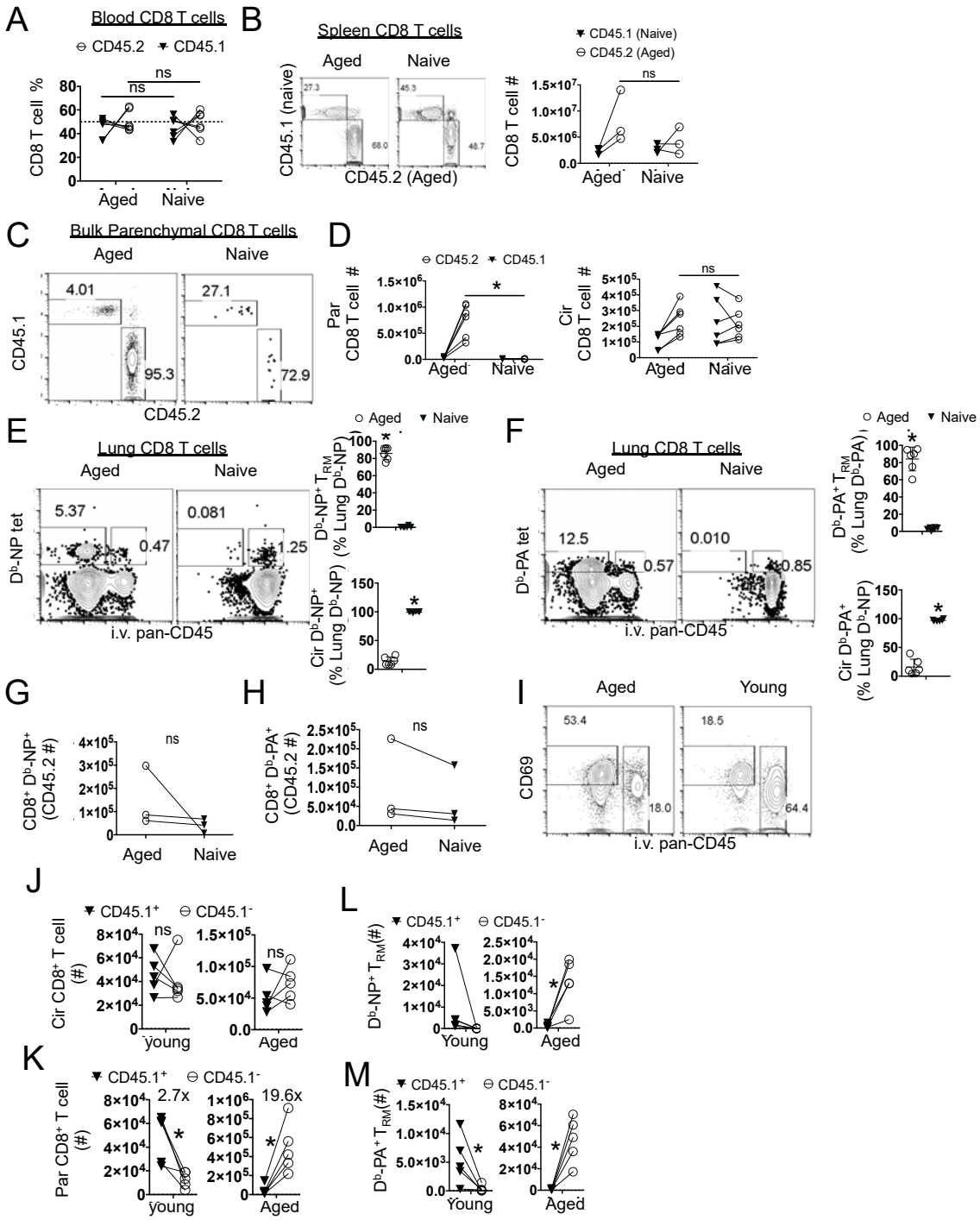
A
Blood CD8 T cells
CD45.2
CD45.1
ns
CD8 T cell %
Aged
Naive
B
Spleen CD8 T cells
Aged
Naive
CD45.1 (naive)
CD45.2 (Aged)
CD45.1 (Naive)
CD45.2 (Aged)
CD8 T cell #
C
Bulk Parenchymal CD8 T cells
4.01
95.3
27.1
72.9
CD45.1
CD45.2
D
Par CD8 T cell #
Cir CD8 T cell #
E
Lung CD8 T cells
5.37
0.47
0.081
1.25
Db-NP tet
i.v. pan-CD45
Db-NP+ TRM (% Lung Db-NP)
Cir Db-NP+ (% Lung Db-NP)
F
Lung CD8 T cells
12.5
0.57
0.010
0.85
Db-PA tet
i.v. pan-CD45
Db-PA+ TRM (% Lung Db-PA)
Cir Db-PA+ (% Lung Db-NP)
G
CD8+ Db-NP+ (CD45.2 #)
H
CD8+ Db-PA+ (CD45.2 #)
I
Aged
Young
53.4
18.0
18.5
64.4
CD69
i.v. pan-CD45
J
CD45.1+
CD45.1-
Cir CD8+ T cell (#)
young
K
Par CD8+ T cell (#)
2.7x
19.6x
L
Db-NP+ TRM(#)
M
Db-PA+ TRM(#)

Fig. S3. Aged parenchymal CD8$^+$ CD69$^+$ memory T cells are tissue resident. Aged mice (CD45.2$^+$) were infected with PR8 and parabiosed with young naive (CD45.1$^+$) mice at 5 weeks after infection. **A.** Percent of host or donor CD8$^+$ T cells in aged or naive hosts where each line represents total CD8$^+$ T cells in blood from one mouse. **B.** Representative flow plot of total splenic CD8 T cells (left) and enumeration (right) of their host of origin. **C.** Representative flow pot of bulk parenchymal CD8$^+$ T cells. **D.** Enumeration of source of lung parenchymal (Par; CD69$^+$CD8$^+$ T cells protected from i.v. CD45 labeling) or circulating (Cir) total CD8 T cells. **E.** Representative flow plot showing tetramer staining of D^b-NP$^+$ cells (Left) and percentages of total lung D^b-NP$^+$ cells found in the parenchyma (top right panels) or circulation (bottom right panels) of each mouse. **F.** Representative flow plot showing tetramer staining of D^b-PA$^+$ cells (Left) and percentages of total lung D^b-PA$^+$ cells found in the parenchyma (top right panels) or circulation (bottom right panels) of each mouse. **G-H.** Quantitation of tetramer positive D^b-NP **(G)** or D^b-PA **(H)** CD44^{Hi} CD8$^+$ memory T cells from the spleen. **I-M.** Young (CD45.1$^+$) or aged (CD45.2$^+$) mice were infected with PR8. At 5 weeks p.i., infected young or aged mice were parabiosed for 4 weeks. **I.** representative flow cytometry plot showing intravenous labeling with CD45 antibody and CD69 expression on lung CD8$^+$ T cells. **J, K.** Enumeration of circulating (**J**.) or CD69$^+$ parenchymal CD8 T cells (**K.)** from young or aged origin in each parabionts. **L, M.** Enumeration of D^b-NP (**L**) or D^b-PA (**M**) CD69$^+$ parenchymal CD8$^+$ T cells from young or aged origin in each parabionts. **A-F** was repeated twice with 3 pairs each and data was pooled. **I-M** was repeated twice with a total of 5 pairs and pooled. * $p<0.05$ or not significant (ns) by Student's two-tailed t-test with unequal variance.

Figure S4

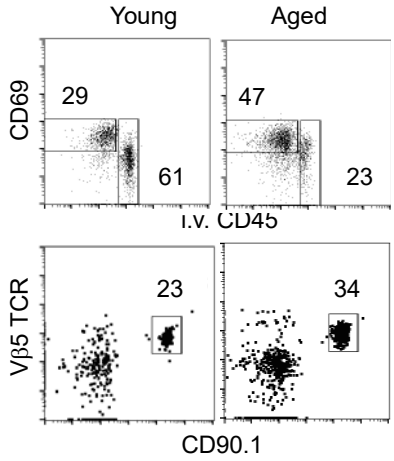

Fig. S4. The accumulation of CD8⁺ T_{RM} in aged lungs is dependent on TGF-βR signaling.
Wild type (Wt, CD45.1⁺) or *TGFbR2$^{fl/fl}$ dLck-Cre* OT-I cells (KO, CD45.1⁺) were adoptively transferred from young donors into separate young or aged hosts (CD45.2⁺) one day prior to PR8-OVA infection**.** Representative flow cytometry plots in lungs at 50 d.p.i., showing the gating strategy for resident OT-I T cells (CD8⁺CD69⁺ i.v.CD45⁻ Vβ5⁺CD90.1⁺).

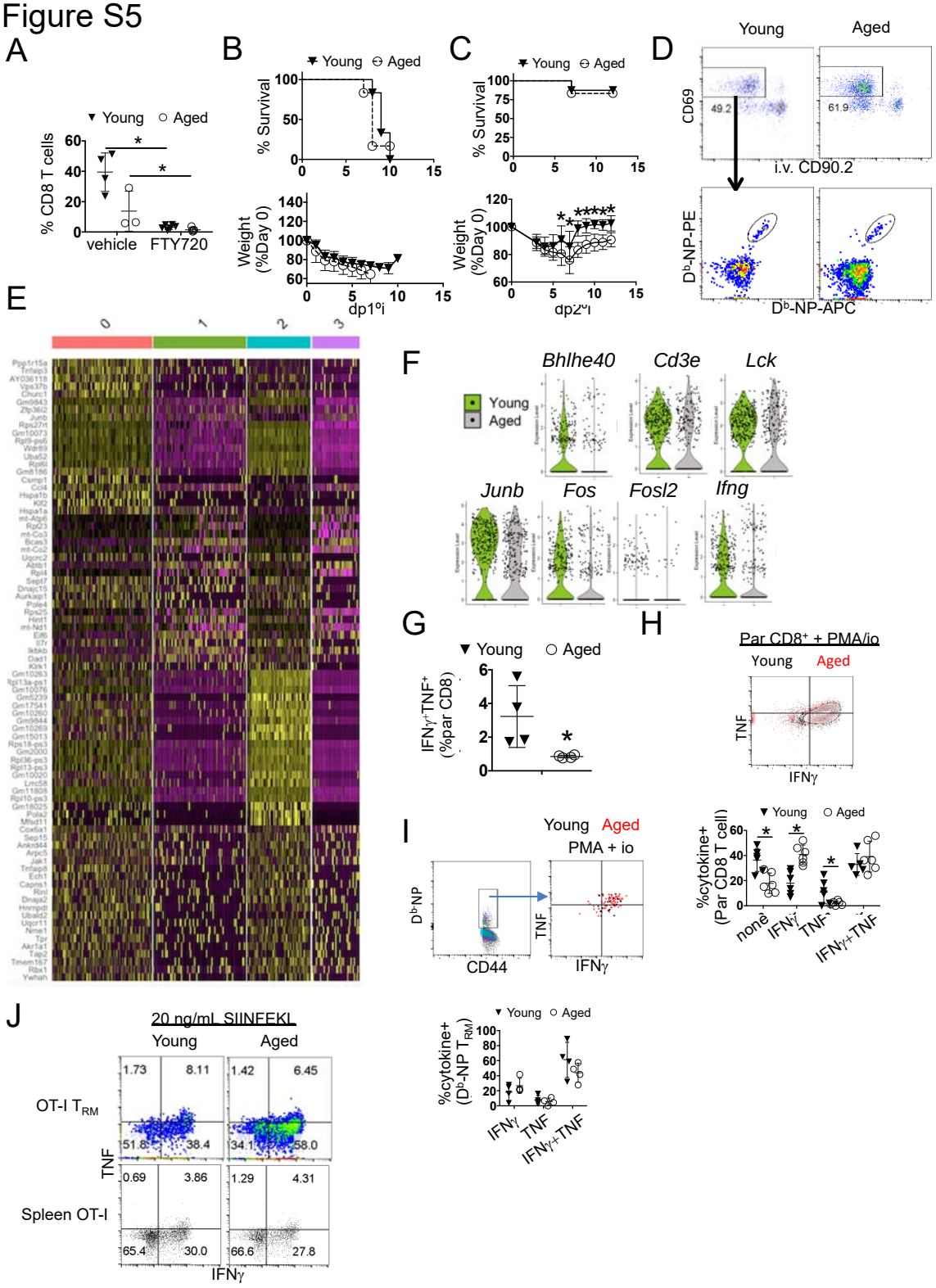

Fig. S5. scRNA-seq on Dᵇ-NP T_{RM} from young or aged lungs.
A. Percent of peripheral blood CD8 T cells in CD45⁺ white blood cells were evaluated 48 hours following FTY720 or vehicle treatment in young and aged memory mice. **B-C.** Survival (top) and weight loss (bottom) following lethal X31 infection of naive young or aged mice in the presence (B) or absence (C) of FTY720. **D.** Sorting scheme for Dᵇ-NP specific T_{RM} cells. Following CD8 enrichment (Miltenyi

kit), $CD44^{Hi}CD69^{+}$i.v.$CD90.2^{-}D^{b}$-NP-PE^{+}/ D^{b}-NP-APC^{+} cells were sorted from young (n=18) or aged (n=11) mice at 60 d.p.i. for scRNAseq. **E.** Heat map of top 20 DEGs by cluster for scRNA seq data. **F.** Violin plots of *Bhlhe40, Cd3e, Lck, Junb, Fos, Fosl2, and Ifng* expression in young or aged D^{b}-NP T_{RM} cells. **G.** Young or aged mice were infected with PR8. At 60 d.p.i., young lung cells were labeled with cell-proliferation dye and then mixed 1:1 with aged lung cells prior to stimulating for 5 hours with NP peptide. IFN-$\gamma^{+}TNF^{+}$ production by parenchymal $CD8^{+}$ T cells derived from young or aged lungs was measured. **H.** Representative plots (top) or percent (bottom) of IFN-γ^{+} TNF^{+} lung-resident $CD8^{+}$ T cells following stimulation with PMA/Ionomycin at 60 d.p.i. **I** Representative plots (left) or percent of IFN-γ^{+} TNF^{+} lung-resident $CD8^{+}$ D^{b}-NP^{+} T cells following stimulation with PMA/Ionomycin at 60 d.p.i. **J.** OT-I T cells ($CD90.1^{+}$) from young donors were adoptively transferred into young or aged C57BL/6 mice 1 day prior to infection with PR8-OVA virus. Percent of IFN-γ^{+} TNF^{+} lung-resident (top) or splenic (bottom) OT-I cells following stimulation with increasing amounts of SIINFEKL peptide at 50 d.p.i. **A & B** were single replicates. scRNAseq data (**D-F**) is from a sorted pool of 18 young or 11 aged mouse lungs. **G-J** were representative data from 2-3 replicates each. * $p<0.05$ or not significant (ns) by Student's two-tailed t-test with unequal variance or Log-rank (Mantel-Cox) test for survival curve comparison (**B & C).**

Figure S6

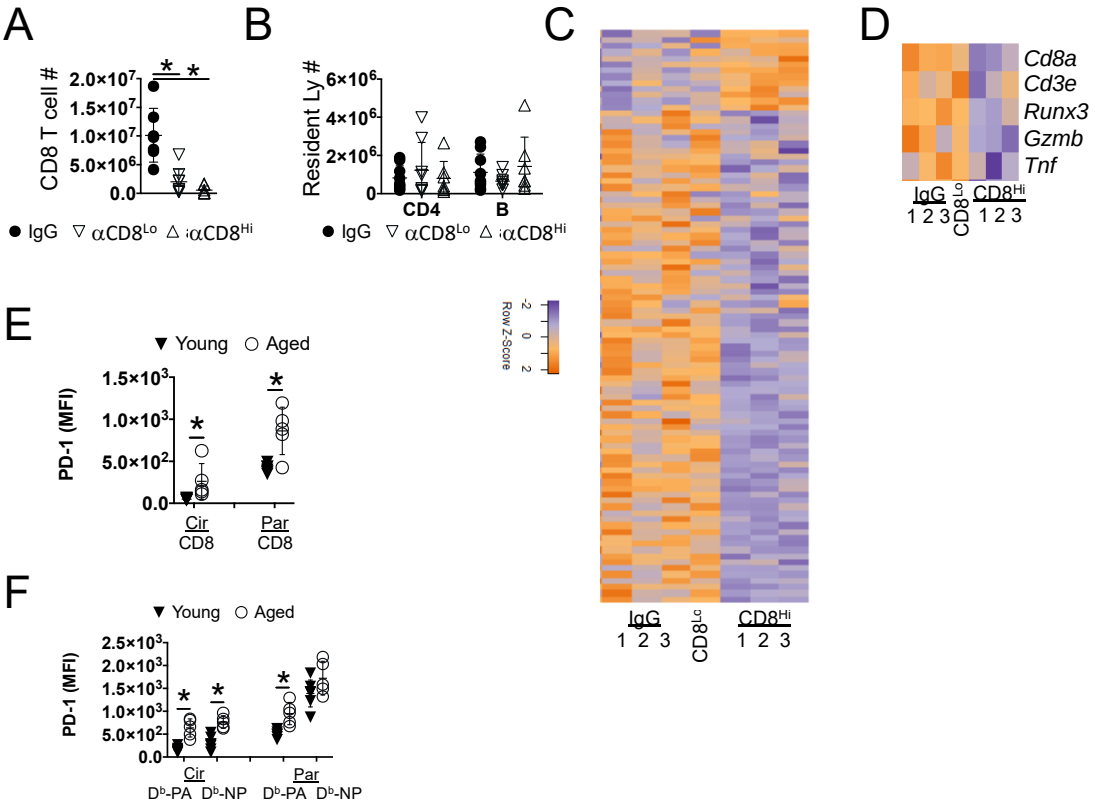

Fig. S6. High dose of CD8 Ab treatment diminishes lung inflammatory responses.

Aged or young C57BL/6 mice were infected with PR8. Mice received high or low dose of anti-CD8 treatment starting at day 21 as indicated. **A.** Splenic CD8⁺ T cell numbers in aged mice following CD8 Ab treatment. **B.** Resident CD4 and B cells were quantitated in the lungs. **C.** Complete set of DEGs (1.5 fold) from Nanostring data. **D.** Select nanostring DEGs showing CD8-related gene depletion after high, but not low dose Ab treatment in aged mice at 60 d.p.i. **E-F.** PD-1 expression (MFI) was measured in young or aged lung circulating (Cir) or parenchymal (Par) CD8⁺ T cells (**E**), or D^b-NP and D^b-PA influenza-specific CD8⁺ T cells at 60 d.p.i. (**F**). **A & B** were 2 pooled experiments, **C & D** were a single experiment in triplicate or pooled from 3 mice ($aCD8^{Lo}$). **E & F** are representatives of 2 independent experiments. * $p<0.05$ ANOVA with correction for multiple tests.