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Supplemental information

Vaccine-induced ICOS^{+}CD38^{+} circulating Tfh are sensitive biosensors of age-related changes in inflammatory pathways

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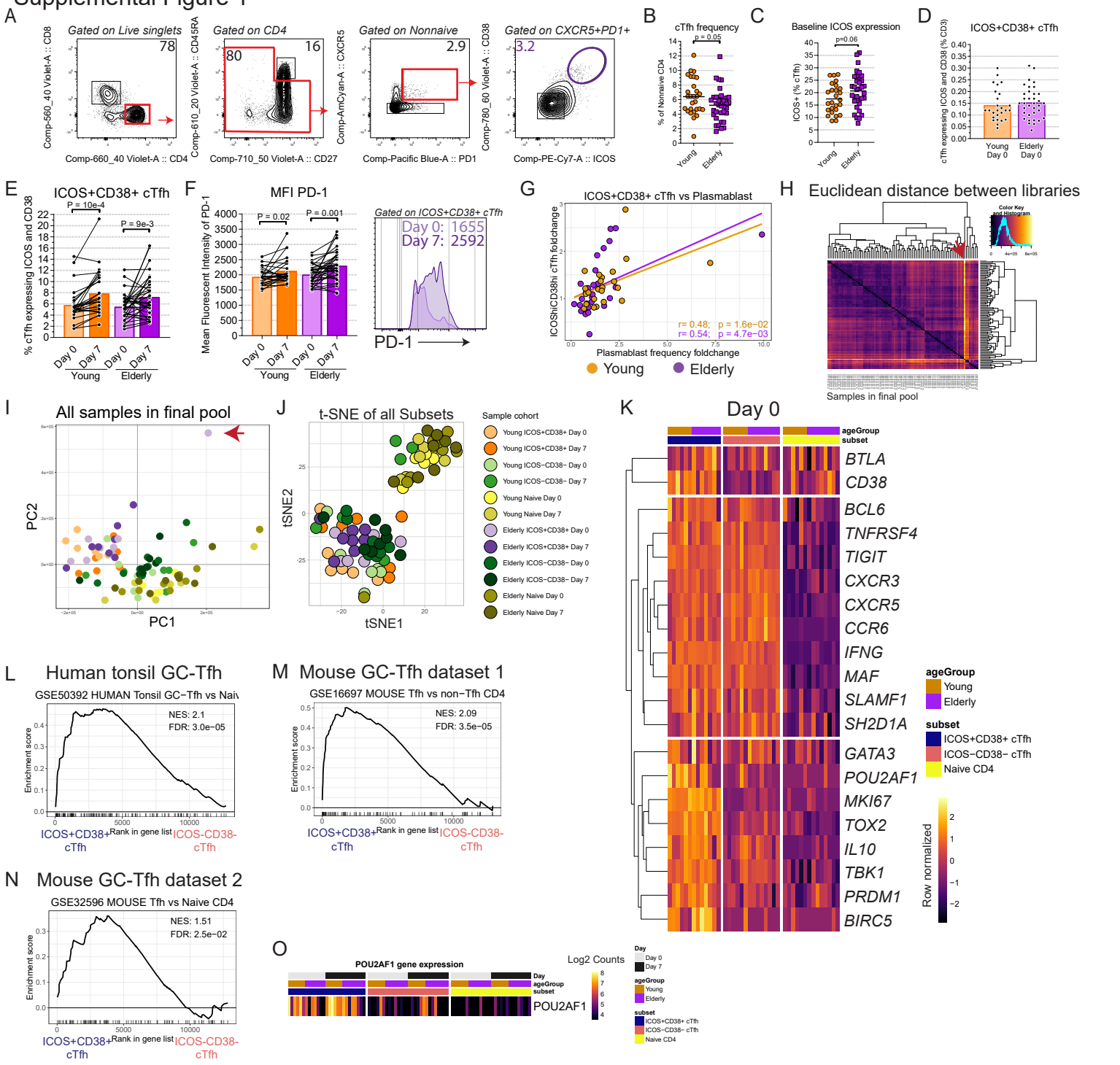
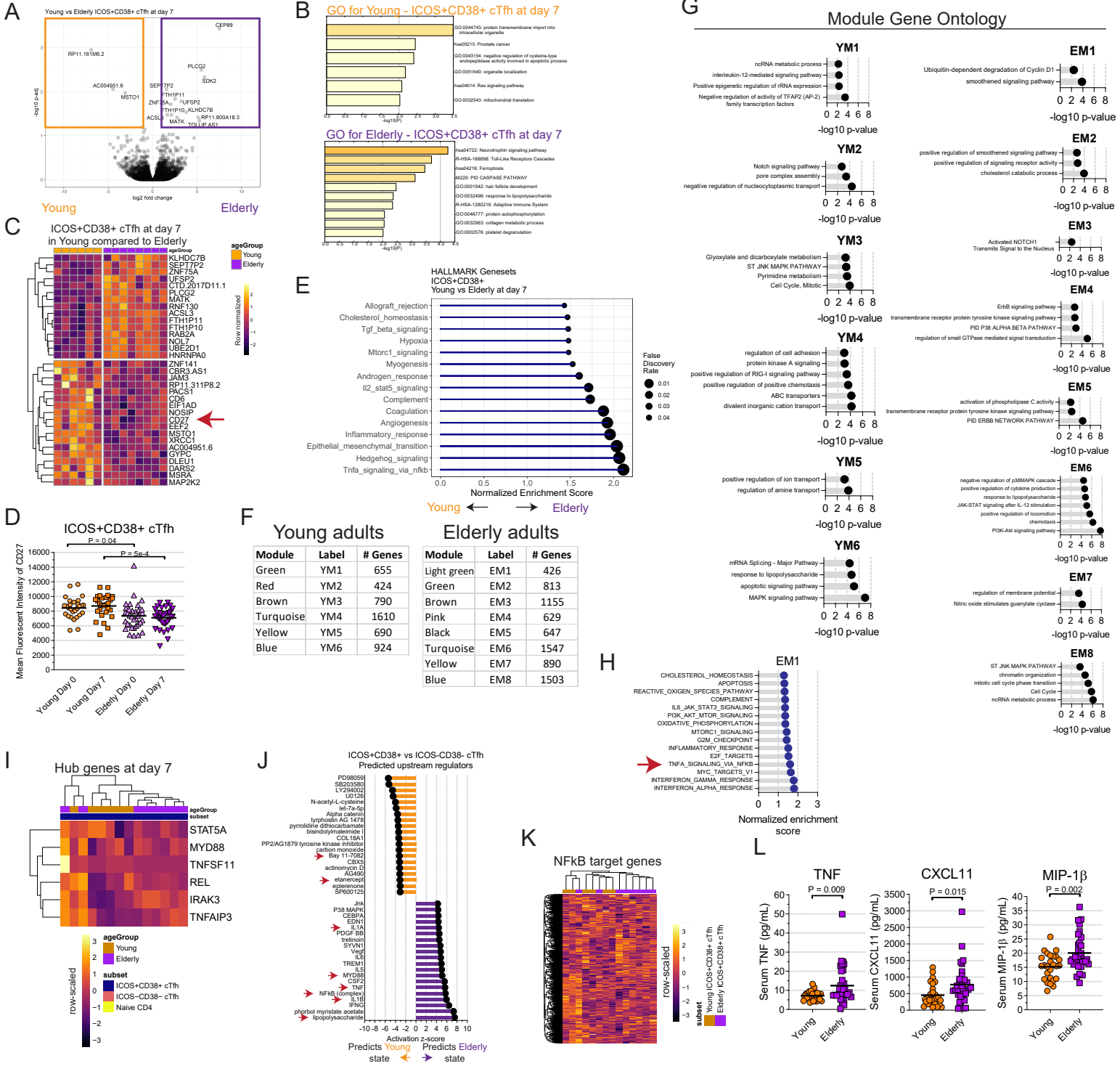


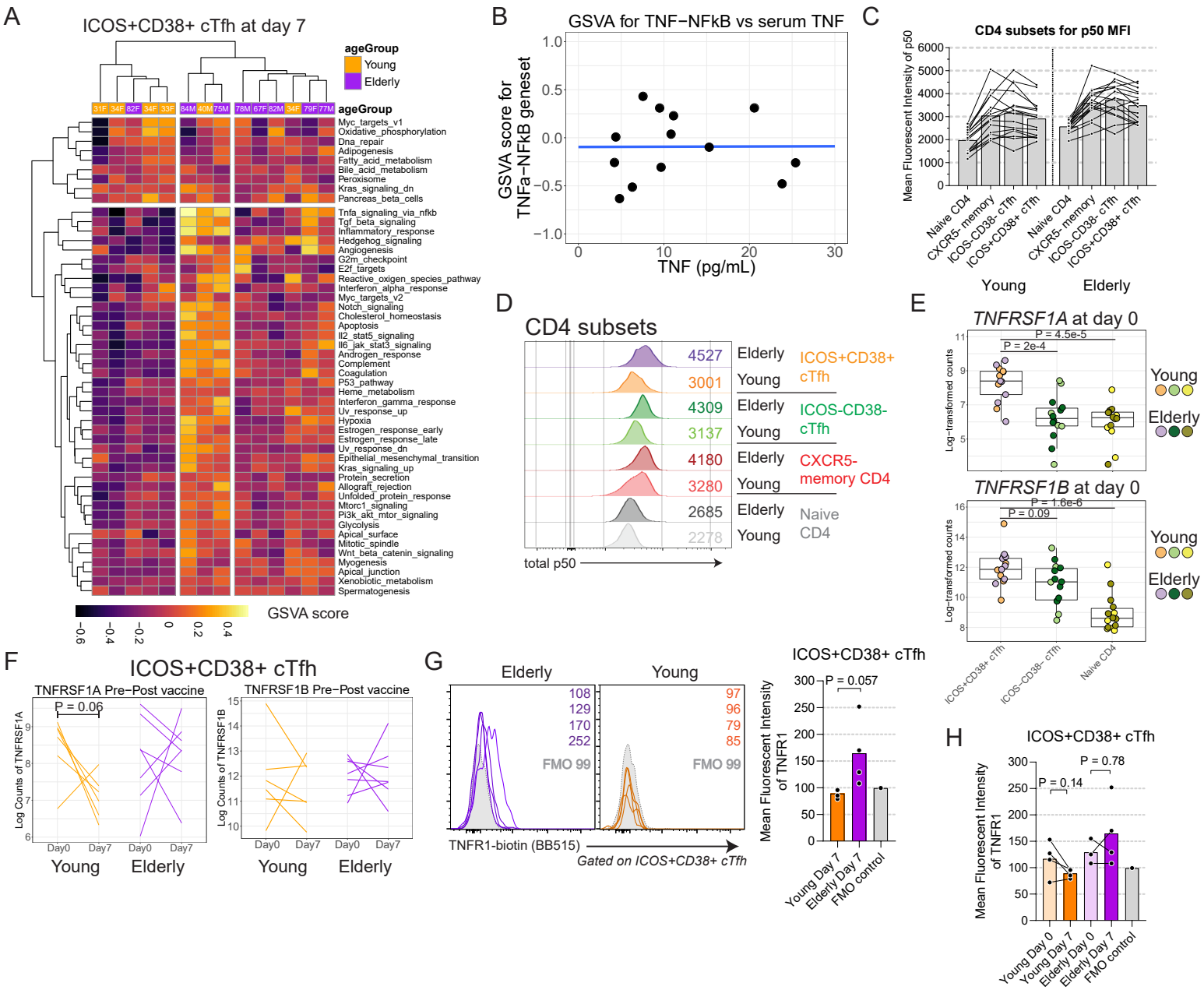
Supplemental Figure 1. Transcriptional profiling of circulating CD4 subsets. Related to Figure 1. **A**. Flow cytometry gating scheme shown for identifying cTfh subsets from peripheral blood. **B**. cTfh frequency shown as a proportion of nonnaive CD4 cells (P=0.05, t-test, n=28 young and n=35 elderly). **C**. Proportion of cTfh expressing ICOS (P=0.06, t-test, n=26 young and n=35 elderly). **D**. ICOS+CD38+ cTfh shown for young and elderly at baseline as a proportion of all CD3+ cells. **E**. Summary plots shown for frequency of cTfh co-expressing ICOS and CD38 for young (orange, $P=10^{-3}$, paired t-test, n=27) and elderly (purple, $P=8.6x10^{-3}$, paired t-test, n=35) at days 0 and 7 after vaccination. **F**. Summary plots shown for Mean Fluorescence Intensity of PD-1 (CD279) in ICOS+CD38+ cTfh for young (orange, P=0.015, paired t-test; n=27) and elderly (purple, $P=10^{-3}$; paired t-test; n=35) at days 0 and 7 after vaccination. Example PD-1 stain shown for one subject at day 0 (light purple) and 7 (dark purple) after influenza vaccination. Plot gated on ICOS+CD38+ cTfh. Geometric mean fluorescent intensity shown. **G**. Pearson correlation shown for the ICOS+CD38+ cTfh frequency fold-change from day 7 compared to day 0, vs the plasmablast response fold-change in frequency from day 7 compared to day 0. Shown is the full dataset with all outliers included for young (orange) and elderly (purple) subjects. **H**. Euclidean distance matrix calculated for all samples in the final pool for RNAseq after raw data processing. One outlier was identified by the red arrow and removed from further analysis. **I-J**. Principal component analysis (**I**) on all samples in the final pool for RNAseq after raw data processing. The outlier (same as in H) is indicated by arrow and was excluded from all subsequent analyses. t-Stochastic neighbor embedding (t-SNE) plot (**J**) showing the remaining 83 samples. **K**. Log-transformed transcriptional profiling data was queried for selected genes from the literature for young (orange bars) and elderly (purple bars) for CD4 subsets at day 0 after vaccination. Each column represents one unique subject. Heatmap is row-normalized. Row gaps and column gaps are based on hierarchical clustering and CD4 subsets, respectively. **L-N**. Normalized Enrichment Score (NES) and False Discovery Rate (FDR) q-value are shown for the pre-ranked gene set enrichment analysis (GSEA) comparison of ICOS+CD38+ cTfh to ICOS-CD38- cTfh at day 0 for all subjects combined, for GSE50392 (human tonsillar Tfh vs naïve CD4) (**L**), GSE16697 (mouse Tfh vs non-Tfh CD4) (**M**), and GSE32596 (mouse Tfh vs naïve CD4) (**N**). **O**. Log counts data shown for POU2AF1 for all samples in the final dataset.

Supplemental Figure 2



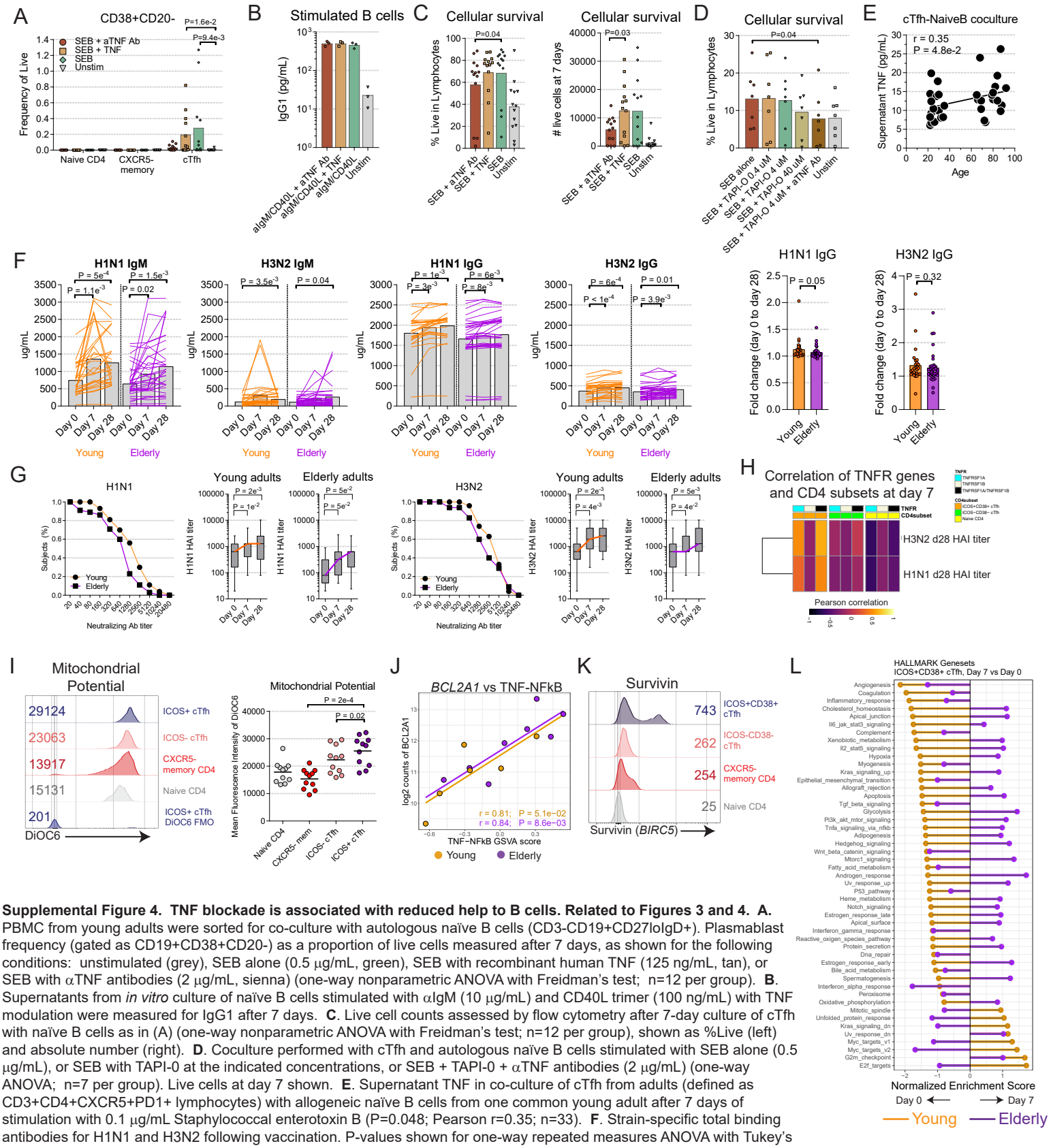
Supplemental Figure 2. Aging is associated with increased TNF-NFkB signaling in elderly. Related to Figure 2. **A**. Volcano plot for the differential expression analysis of ICOS+CD38+ cTfh from young adults and elderly adults at day 7 after vaccination. **B**. Gene ontology was performed on the 100 most differentially-expressed genes for ICOS+CD38+ cTfh at day 7 for young (middle) or elderly (bottom) subjects. **C**. Log-transformed transcriptional profiling data (left) was queried for differentially-expressed genes for the ICOS+CD38+ cTfh at day 7 from young (orange) and elderly (purple). Each column represents one unique subject. Heatmap is row-normalized and gaps are based on hierarchical clustering. **D**. Geometric mean fluorescence intensity of CD27 (right) is shown for the ICOS+CD38+ cTfh at days 0 and 7 (one-way ANOVA with Tukey's post-test; n=28 for young, n=35 for elderly). **E**. Pre-ranked GSEA was used to compare ICOS+CD38+ cTfh at day 7 from young vs elderly subjects. Pathways with FDR <0.05 shown. Positive NES indicate greater enrichment in the elderly than young. **F**. Weighted gene correlation network analysis was performed for ICOS+CD38+ cTfh at day 7 after vaccination for young adults and elderly adults separately. The six young adult modules and eight elderly adult modules were relabeled as indicated. **G**. Gene ontology by Metascape was performed for all genes in each module with module membership >0.80. **H**. Pre-ranked GSEA was performed on Elderly module EM1 using the Hallmark gene sets from MSigDB. TNF-NFkB gene set is indicated by the red arrow. **I**. Log_2-transformed gene counts as a row-normalized heatmap for ICOS+CD38+ cTfh at day 7 for the hub genes identified by network analysis. **J**. Ingenuity Pathway Analysis was used to assess predicted upstream regulators in the comparison of ICOS+CD38+ cTfh in young and elderly subjects at day 7 after vaccination. Top 20 highest and 20 lowest-scoring terms by activation z-score shown. Arrows indicate terms relevant to NFkB signaling. **K**. Gene targets of NFkB shown for ICOS+CD38+ cTfh at day 7 for young (orange) or elderly (purple) adults. Heatmap shows row-normalized log-transformed expression data for genes with nonzero variance. **L**. Plasma was profiled for inflammatory cytokines TNF, CXCL11, and MIP1β prior to vaccination.

Supplemental Figure 3



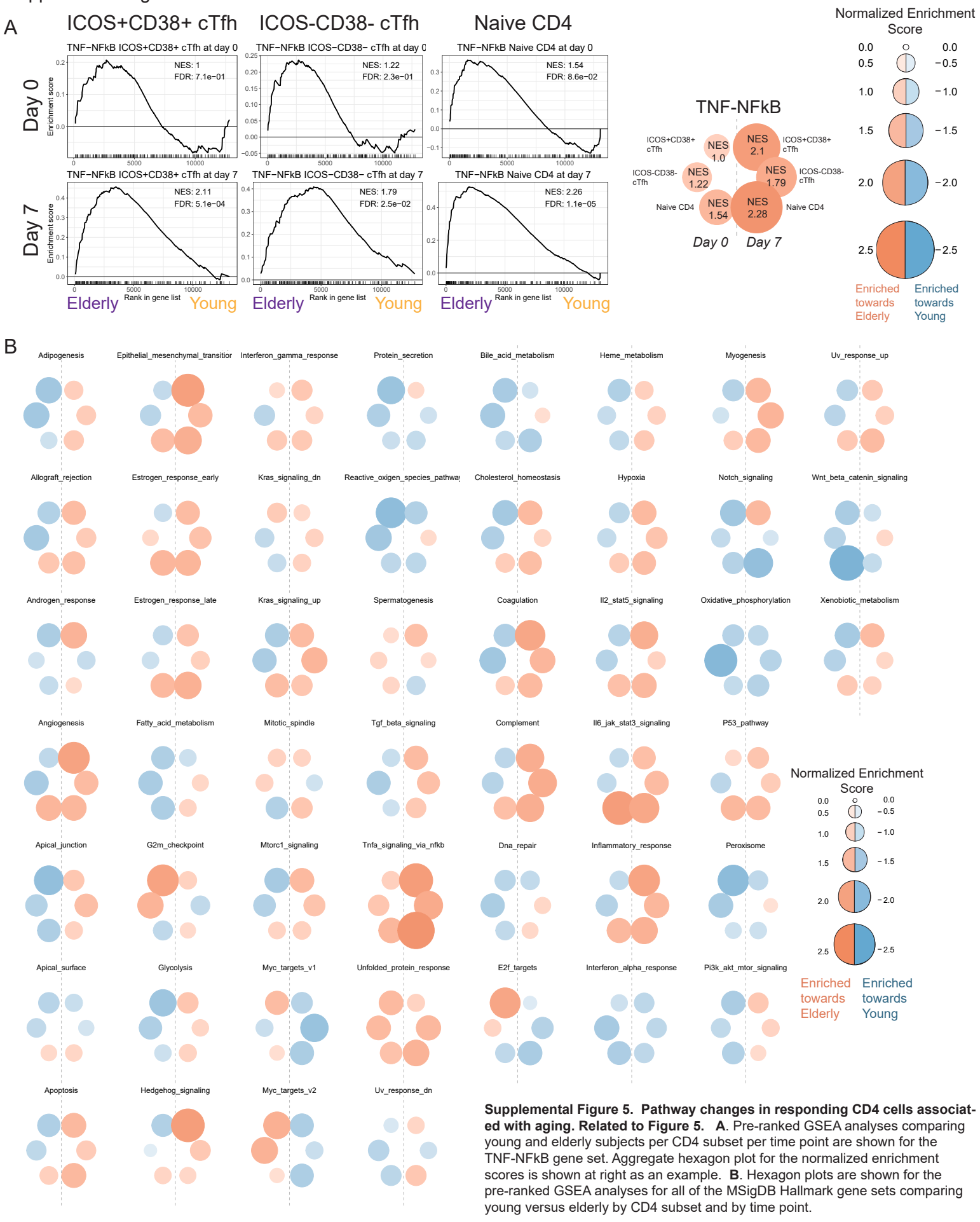
Supplemental Figure 3. Increased TNFR expression in cTfh. Related to Figure 2. A. Gene set variation analysis (GSVA) was performed for ICOS+CD38+ cTfh at day 7 from all 14 subjects for the Hallmark collection in MSigDB. Subject age and sex are shown. **B**. Scatter-plot shows GSVA score per subject compared to the plasma TNF concentration at baseline. **C-D**. PBMC were assayed by flow cytometry for total NFkB p50 protein expression. Numbers on histogram indicate mean fluorescence intensity for p50. Summary data and example plot shown for one young and one elderly subject for p50 protein in the different CD4 subsets. **E**. Log_2-transformed mRNA counts shown for gene expression for *TNFRSF1A* (left) or *TNFRSF1B* (right) in CD4 subsets for young and elderly subjects (one-way ANOVA with Tukey's, n=6 or 8). **f**. Gene expression of *TNFRSF1A* (left) and *TNFRSF1B* (right) were assessed from RNA-seq data, shown as before-and-after plots for normalized mRNA counts. Each line indicates one unique subject. **G**. TNFR1 protein was assessed by flow cytometry 7 days after influenza vaccination in an independent, randomly-selected subset of young and elderly adults from the same cohort. FMO control is shown in gray histogram on each plot. Summary plot is shown (P=0.057, t-test, n=3 for young and n=4 for elderly). **H**. Summary data for TNFR1 protein by flow cytometry is shown before and 7 days after vaccination for young (P=0.14, paired t-test, n=3) and elderly (P=0.78, paired t-test, n=3). Connected dots represent paired observations.

Supplemental Figure 4



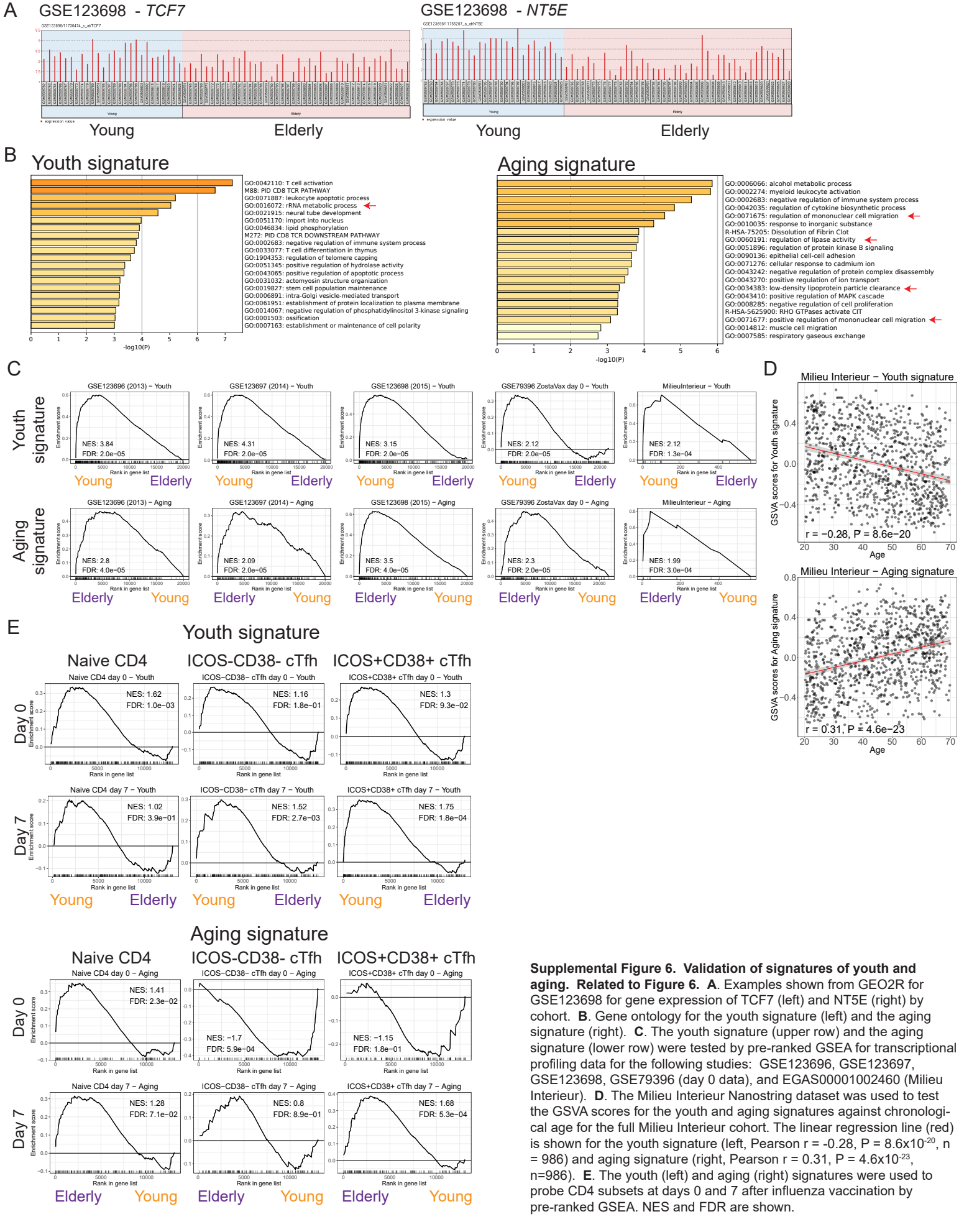
Supplemental Figure 4. TNF blockade is associated with reduced help to B cells. Related to Figures 3 and 4. A. PBMC from young adults were sorted for co-culture with autologous naïve B cells (CD3-CD19+CD27loIgD+). Plasmablast frequency (gated as CD19+CD38+CD20-) as a proportion of live cells measured after 7 days, as shown for the following conditions: unstimulated (grey), SEB alone (0.5 μg/mL, green), SEB with recombinant human TNF (125 ng/mL, tan), or SEB with αTNF antibodies (2 μg/mL, sienna) (one-way nonparametric ANOVA with Freidman's test; n=12 per group). **B.** Supernatants from *in vitro* culture of naïve B cells stimulated with αIgM (10 μg/mL) and CD40L trimer (100 ng/mL) with TNF modulation were measured for IgG1 after 7 days. **C.** Live cell counts assessed by flow cytometry after 7-day culture of cTfh with naïve B cells as in (A) (one-way nonparametric ANOVA with Freidman's test; n=12 per group), shown as %Live (left) and absolute number (right). **D.** Coculture performed with cTfh and autologous naïve B cells stimulated with SEB alone (0.5 μg/mL), or SEB with TAPI-0 at the indicated concentrations, or SEB + TAPI-0 + αTNF antibodies (2 μg/mL) (one-way ANOVA; n=7 per group). Live cells at day 7 shown. **E.** Supernatant TNF in co-culture of cTfh from adults (defined as CD3+CD4+CXCR5+PD1+ lymphocytes) with allogeneic naïve B cells from one common young adult after 7 days of stimulation with 0.1 μg/mL Staphylococcal enterotoxin B (P=0.048; Pearson r=0.35; n=33). **F.** Strain-specific total binding antibodies for H1N1 and H3N2 following vaccination. P-values shown for one-way repeated measures ANOVA with Tukey's post-test within each cohort. P-values for fold-change plots were calculated using Mann-Whitney U-test. **G.** Day 28 strain-specific neutralizing antibody titers as a cumulative distribution for H1N1 and H3N2 viral strains. Per-visit titers shown, with whiskers representing range and boxes representing the 25-75th percentiles. Line connects medians of each visit. P values calculated using repeated-measures ANOVA with Tukey's post-test. **H.** Pearson correlation for $\log_2$-transformed gene counts for TNFR genes as a heatmap for CD4 subsets against the neutralizing antibody response against each viral strain. **I.** Mitochondrial potential assessed with DiOC6. Example plot and summary data shown (one-way repeated-measures ANOVA with Tukey's; n=11 per group). **J.** Gene expression of *BCL2A1* from transcriptional profiling of ICOS+CD38+ cTfh at day 7 correlated against the GSVA scores for TNF-NFkB for young (orange, P=5.1x10^{-2}, Pearson r=0.81, n=6) and elderly (purple, P=8.6x10^{-3}, Pearson r=0.84, n=8). **K.** Flow cytometry for one young subject for Survivin protein. MFI shown. **L.** Aggregated GSEA results for the complete Hallmark gene sets collection for the comparison of day 7 vs day 0 for ICOS+CD38+ cTfh in young (orange) and elderly (purple). Positive enrichment scores indicate enrichment at day 7.

Supplemental Figure 5



Supplemental Figure 5. Pathway changes in responding CD4 cells associated with aging. Related to Figure 5. **A**. Pre-ranked GSEA analyses comparing young and elderly subjects per CD4 subset per time point are shown for the TNF-NFkB gene set. Aggregate hexagon plot for the normalized enrichment scores is shown at right as an example. **B**. Hexagon plots are shown for the pre-ranked GSEA analyses for all of the MSigDB Hallmark gene sets comparing young versus elderly by CD4 subset and by time point.

Supplemental Figure 6



Supplemental Figure 6. Validation of signatures of youth and aging. Related to Figure 6. A. Examples shown from GEO2R for GSE123698 for gene expression of TCF7 (left) and NT5E (right) by cohort. **B**. Gene ontology for the youth signature (left) and the aging signature (right). **C**. The youth signature (upper row) and the aging signature (lower row) were tested by pre-ranked GSEA for transcriptional profiling data for the following studies: GSE123696, GSE123697, GSE123698, GSE79396 (day 0 data), and EGAS00001002460 (Milieu Interieur). **D**. The Milieu Interieur Nanostring dataset was used to test the GSVA scores for the youth and aging signatures against chronological age for the full Milieu Interieur cohort. The linear regression line (red) is shown for the youth signature (left, Pearson r = -0.28, P = 8.6×10^{-20}, n = 986) and aging signature (right, Pearson r = 0.31, P = 4.6×10^{-23}, n=986). **E**. The youth (left) and aging (right) signatures were used to probe CD4 subsets at days 0 and 7 after influenza vaccination by pre-ranked GSEA. NES and FDR are shown.

Supplementary Table 1. Clinical characteristics. Related to Figure 1

Cohort	Young (n=28)	Elderly (n=35)
Age		
Median	34	79
Mean (Standard deviation)	34.1 (2.59)	77.7 (4.93)
Range	30-40	66-85
Sex		
Male (%)	8 (29)	14 (40)
Race		
White	25 (89)	32 (91)
Black or African American	2 (7.1)	2 (5.7)
Other	1 (3.6)	1 (2.9)

Data are presented as number (% total) unless otherwise indicated.

Supplemental table 6: Whole-blood transcriptional profiling studies. Related to Figure 6.

StudyIdentifier	Young		Elderly		PMID Reference(s)	Weblink
	n	**range**	**n**	**range**		
SDY739	27	30-40	34	65-85	30186359, 27588486,	https://www.immport.org/shared/study/SDY739
	n	**range**	**n**	**range**		
GSE79396	33	25-40	44	60-79	28502771	https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE79396
GSE123696	17	23-34	49	66-96	30842675	https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE123696
GSE123697	25	23-32	35	67-97	30842675	https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE123697
GSE123698	26	24-36	42	68-97	30842675	https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE123698
Milieu Interieur	395	20-39	76	66-69	29282317, 25562703	https://www.ebi.ac.uk/ega/studies/EGAS00001002460
SDY622	27	30-40	35	65-88	30186359, 27588486,	https://www.immport.org/shared/study/SDY622
SDY648	28	30-40	33	65-87	30186359, 27588486,	https://www.immport.org/shared/study/SDY648
SDY819	15	30-40	30	65-89	30186359, 27588486,	https://www.immport.org/shared/study/SDY819