

Supplemental Figure 1:

Flow Cytometry gating strategy of dissociated tumors. A) Scatter, singlet, live dead, and CD45⁺ leukocyte example gates for tumor infiltrating leukocyte immunophenotyping. B) Myeloid cell gating strategy for CD11b⁺F480⁺ macrophages, M2 like macrophage phenotype Arg⁺, M1-like macrophage phenotype being MHCII⁺iNOS⁺, and dendritic cells CD11b⁻CD11c⁺MHCII⁺. These gates compare representative replicates of Py8119 and Py117 tumors from figure 2. C) Gating strategy of infiltrating T cells and example of CD4⁺FoxP3⁺ cells (left) and FMO (right).

Supplemental Figure 2:

Flow cytometry gating strategy of tumor draining lymph nodes. A) Scatter, singlet, live dead and CD45⁺ leukocyte gate of dissociated tumor draining lymph nodes. B) Gating strategy of dendritic cells, to exclude lymphocytes by scatter, MHCII⁺TCRb⁻ gating, then CD11c⁺MHCI⁺ gating to avoid lymphocytes that bled in. C) Middle and right panels show subsets of CD4⁺, CD8⁺, and CD69⁺ cells. Representative samples of Py8119 + 20 Gy and Py8119 + 20 Gy + CD40 Ag are shown to highlight differences in dendritic cells and T cell activation.

Supplemental Figure 3:

Tumor response in immunodeficient mice and immune cell populations in mixed tumors. A) Py5050 mixed cells and Py117 cells were implanted into athymic nude mice and irradiated with 12 Gy 10 days after implantation. Tumors from Py8119/Py117 mixed experiments in Figure 1 in immunocompetent mice were harvested dissociated, stained, and analyzed by flow cytometry. B) The CD45⁺ cells as a proportion of live cells. C) CD8⁺ and CD4⁺ T cells as a proportion of live cells in treated tumors. D) Immature myeloid cells (CD45⁺CD11b⁺GR-1^{hi}) are few in these tumors as a proportion of CD45⁺ cells and thus. N=5 mice each group

Supplemental Figure 4:

Mixed tumor immune cell infiltrate 3 days after irradiation. Mice implanted with Py8119 or Py5050 mix 50% of Py8119 and Py117 tumor cells were treated with or without 20 Gy RT and harvested 3 days. A) CD4⁺ and CD8⁺ T cells infiltrated in the tumor as a proportion of live cells was not significantly different. B) Dendritic cells (CD11b⁻CD11c⁺MHCII⁺) were also not significantly different 3 days after treatment. N=5 mice each.

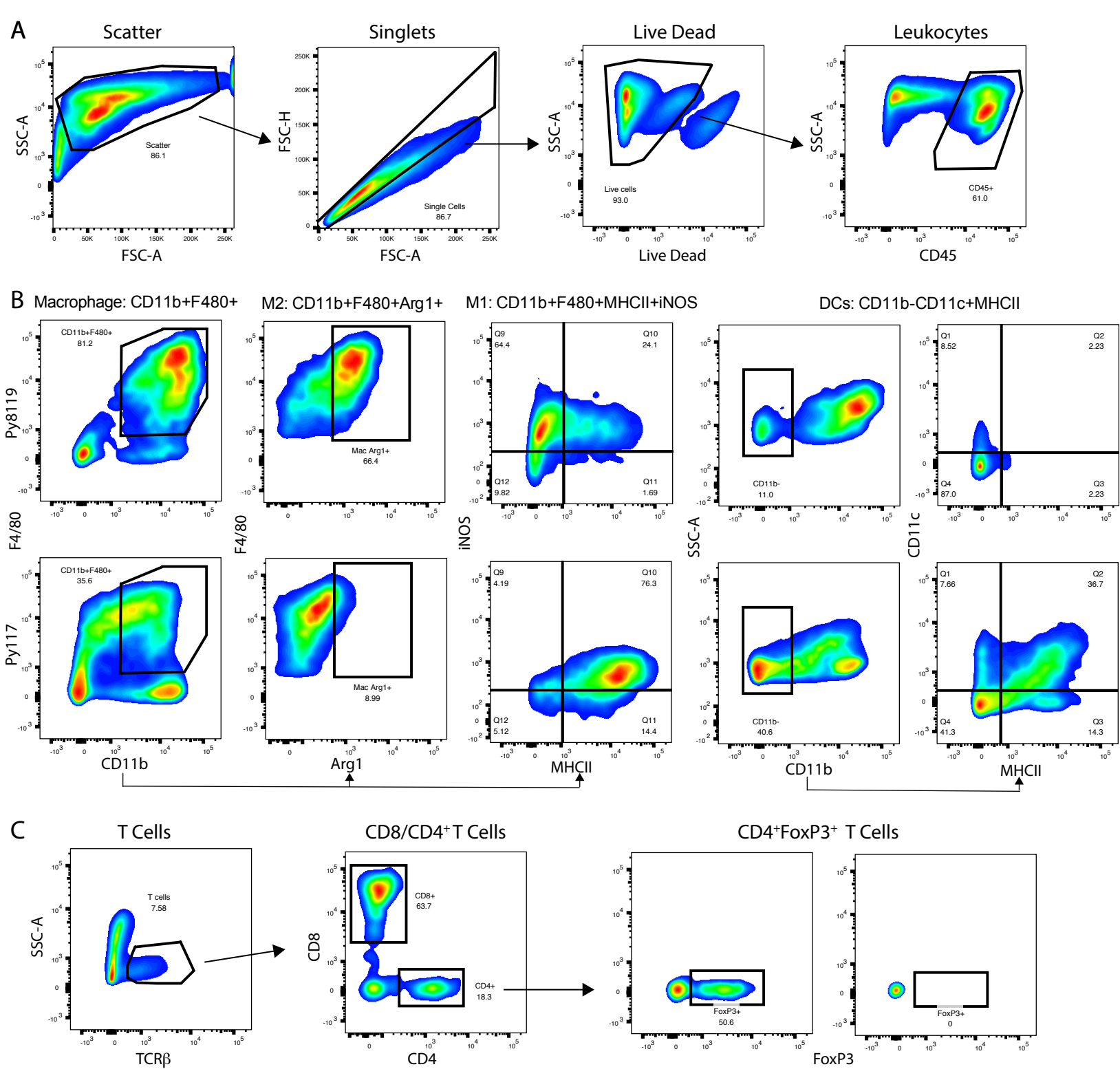
Supplemental Figure 5:

Immune cell infiltration in the tumor microenvironment determined by RNAseq deconvolution of the TCGA data sets using the TIMER algorithm. Immune infiltration analysis of key CD40 pathway genes CD40LG, CD40, and MAP3K14 from the TCGA from A) melanoma and B) breast cancer reveal correlations.

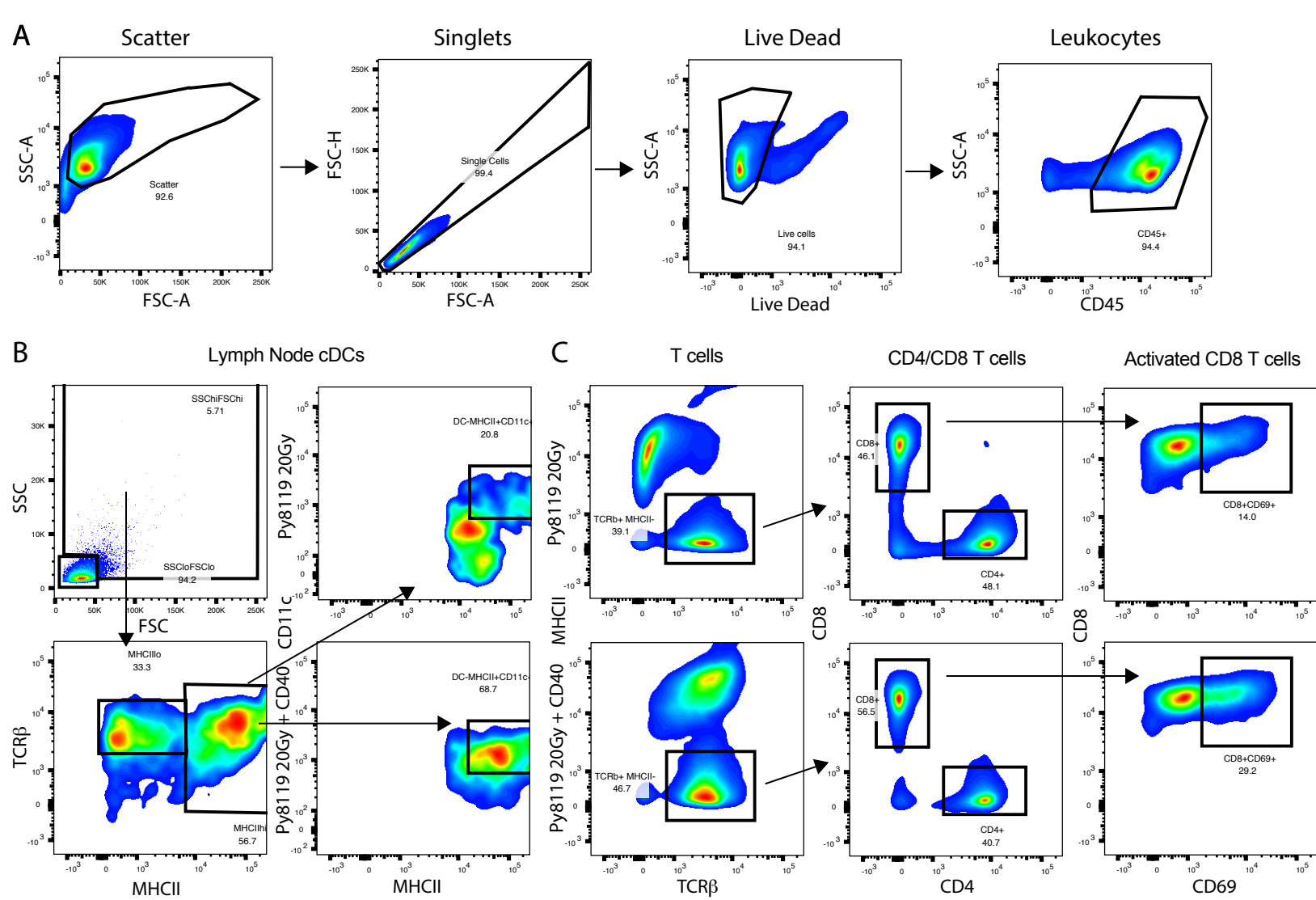
Supplemental Figure 6:

CD40 pathway correlates with outcomes and response is associated with on treatment biopsies of patients that received immune checkpoint therapy. A) GSEA of TCGA breast cancer data reveals enrichment of the CD40 pathway and B) multivariate cox model using Oncolnc revealed significance associations of CD40LG and survival of breast and melanoma with high gene rank in the top 10% for the same tumors. C) CD40LG was associated with survival of the TCGA data sets by log rank when you slice groups by a broad range in melanoma and breast cancer. D)

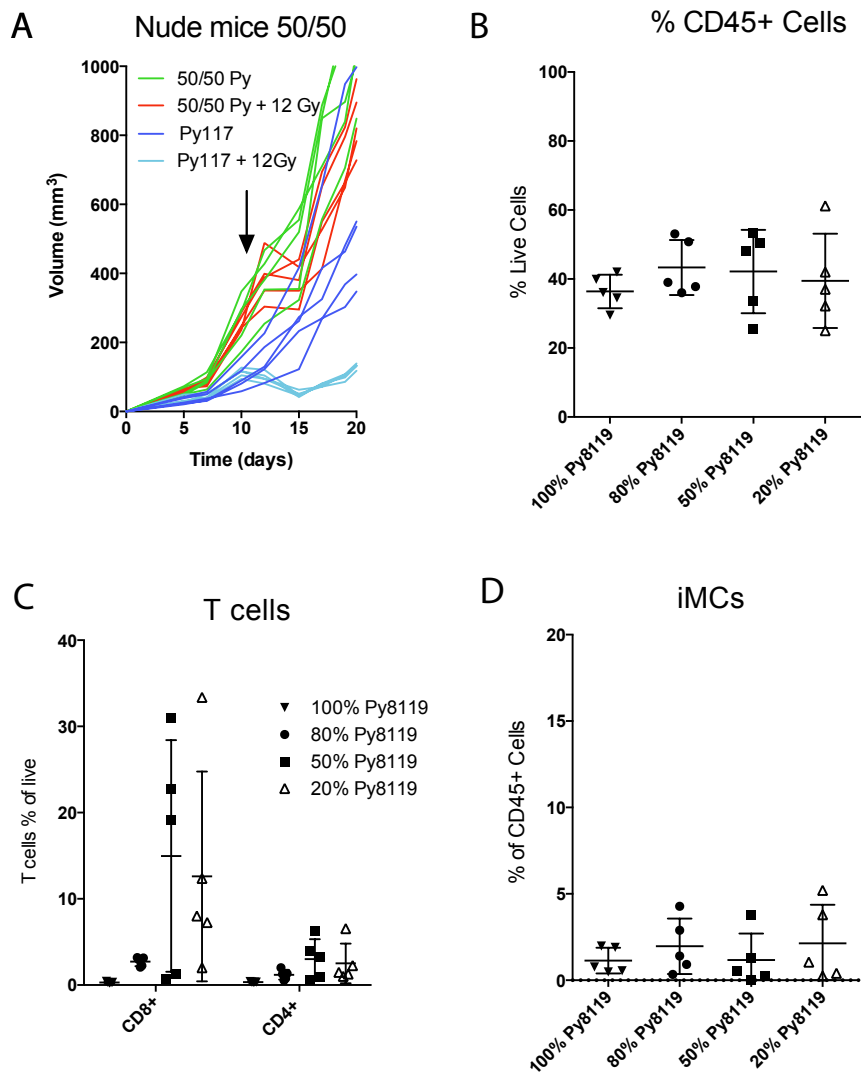
Kaplan-Meier curve of breast, melanoma, and pancreatic cancer CD40LG expression level cutoff at the median and p-value showing significance difference breast and melanoma. E) CD40LG/CD40 ratio reveals a greater ratio in immunotherapy responders that was significant during on treatment biopsies from Chen et al. F) CD40LG/CD40 normalized RNAseq expression value ratio of all PD-1 treatment biopsies from Riaz et al. showing no correlation between CR/PR/SD compared to PD as opposed to what was observed from on treatment biopsies.



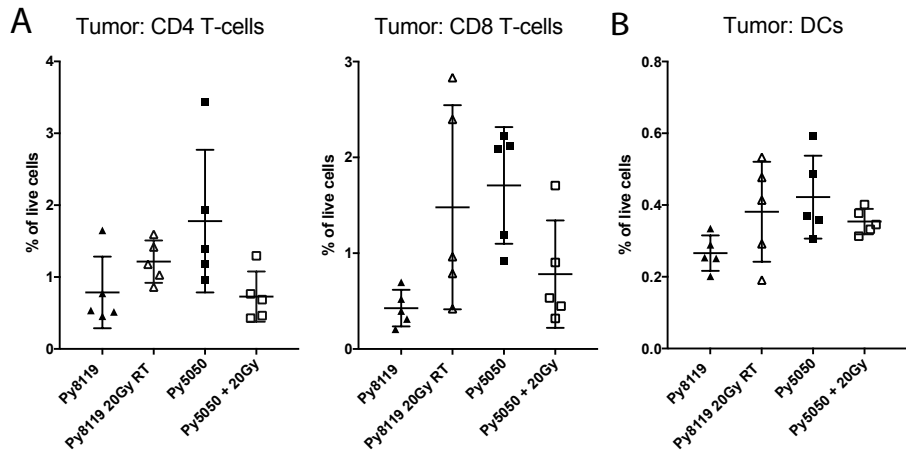
Supplemental Figure 1



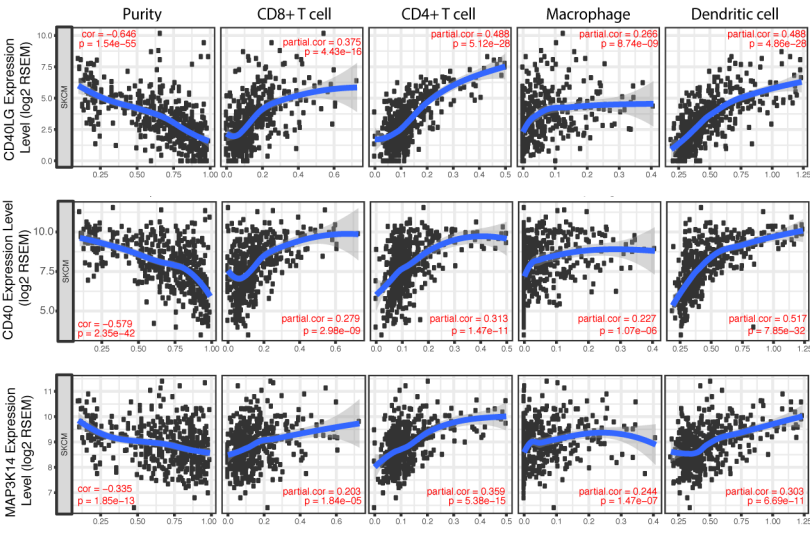
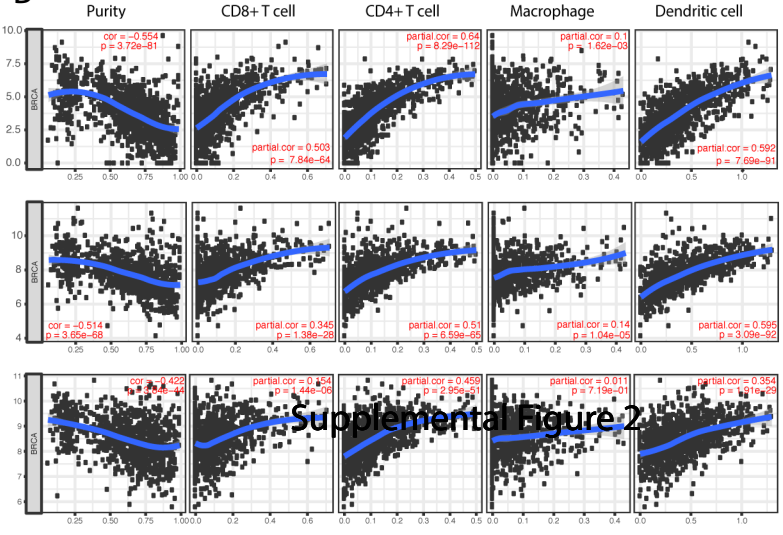
Supplemental Figure 2



Supplemental Figure 3



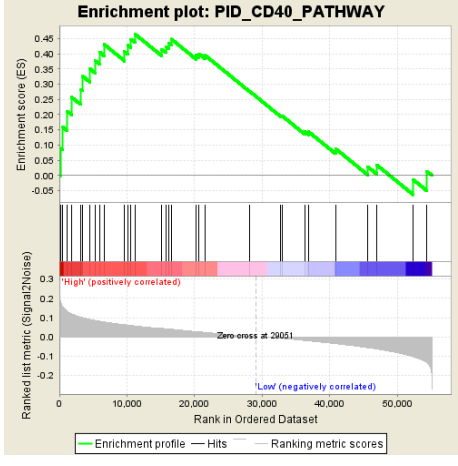
Supplemental Figure 4

A**TCGA: Melanoma****B****TCGA: Breast Cancer**

Supplemental Figure 2

Supplemental Figure 5

A Breast TCGA GSEA: Survival <3 yr (66 pts) vs >3 yr (429 pts)

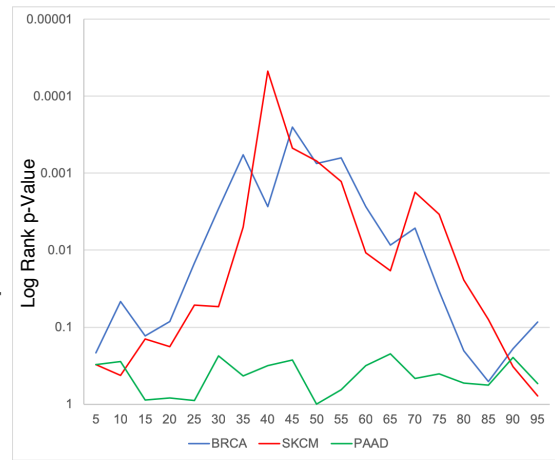


Breast				
Cancer TCGA				
GSEA CD40				
Pathway	0.465	0.014	0.0257	

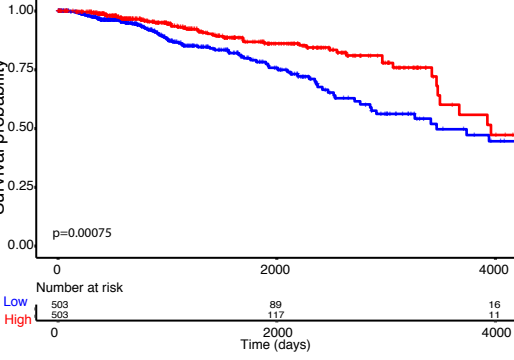
B CD40LG: survival and expression rank

Cancer	Cox Coefficient	P-Value	FDR Corrected	Rank	Number of genes
BRCA	-0.215	1.50E-02	2.57E-01	938	16,602
SKCM	-0.171	1.00E-02	6.25E-02	2538	16,030
PAAD	-0.085	4.20E-01	6.25E-01	11490	17,177

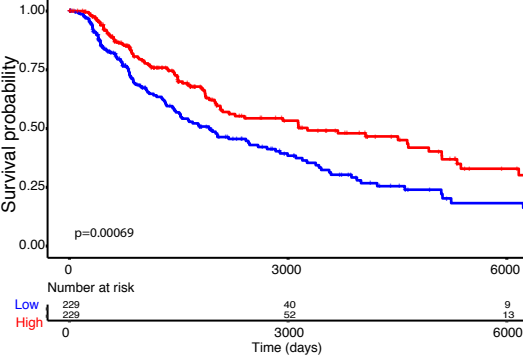
C CD40LG and Survival: Slice by expression value



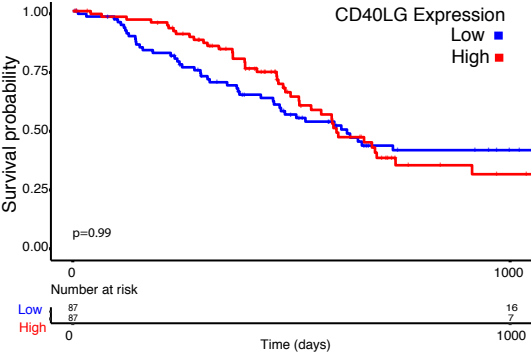
D TCGA: Breast Cancer



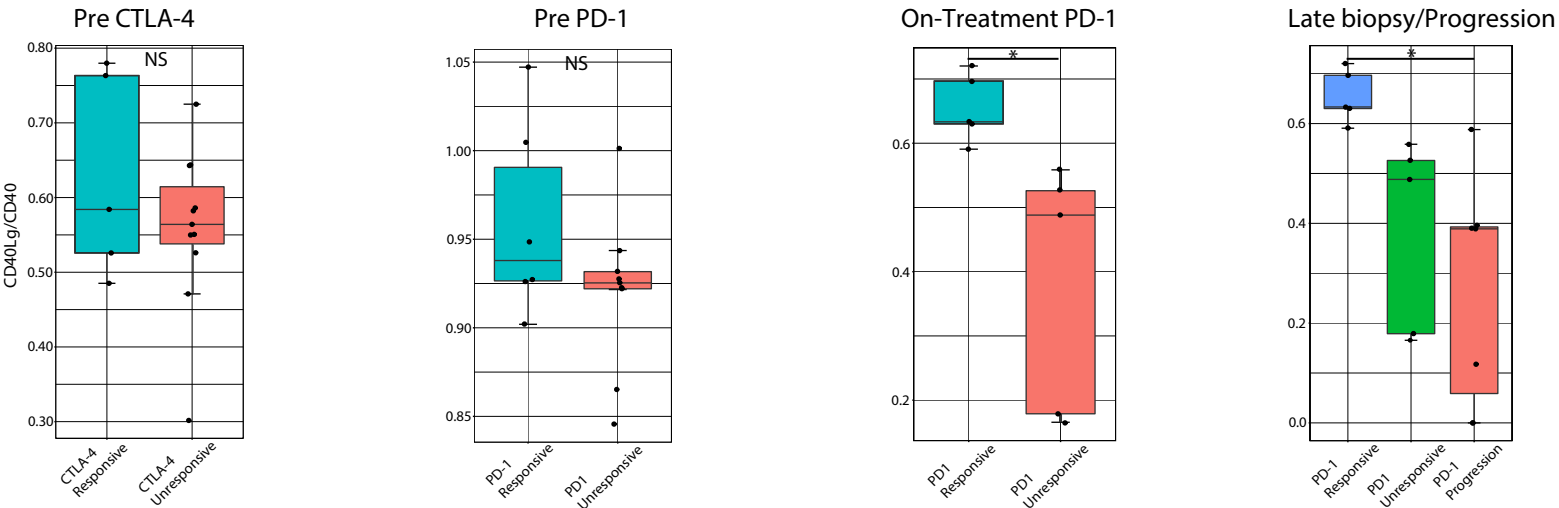
TCGA: Melanoma



TCGA: Pancreatic Cancer



E Chen et al: Melanoma serial biopsies during immunotherapy



F Riaz et al: Melanoma biopsies Pre PD-1 Treatment

