Figure S3

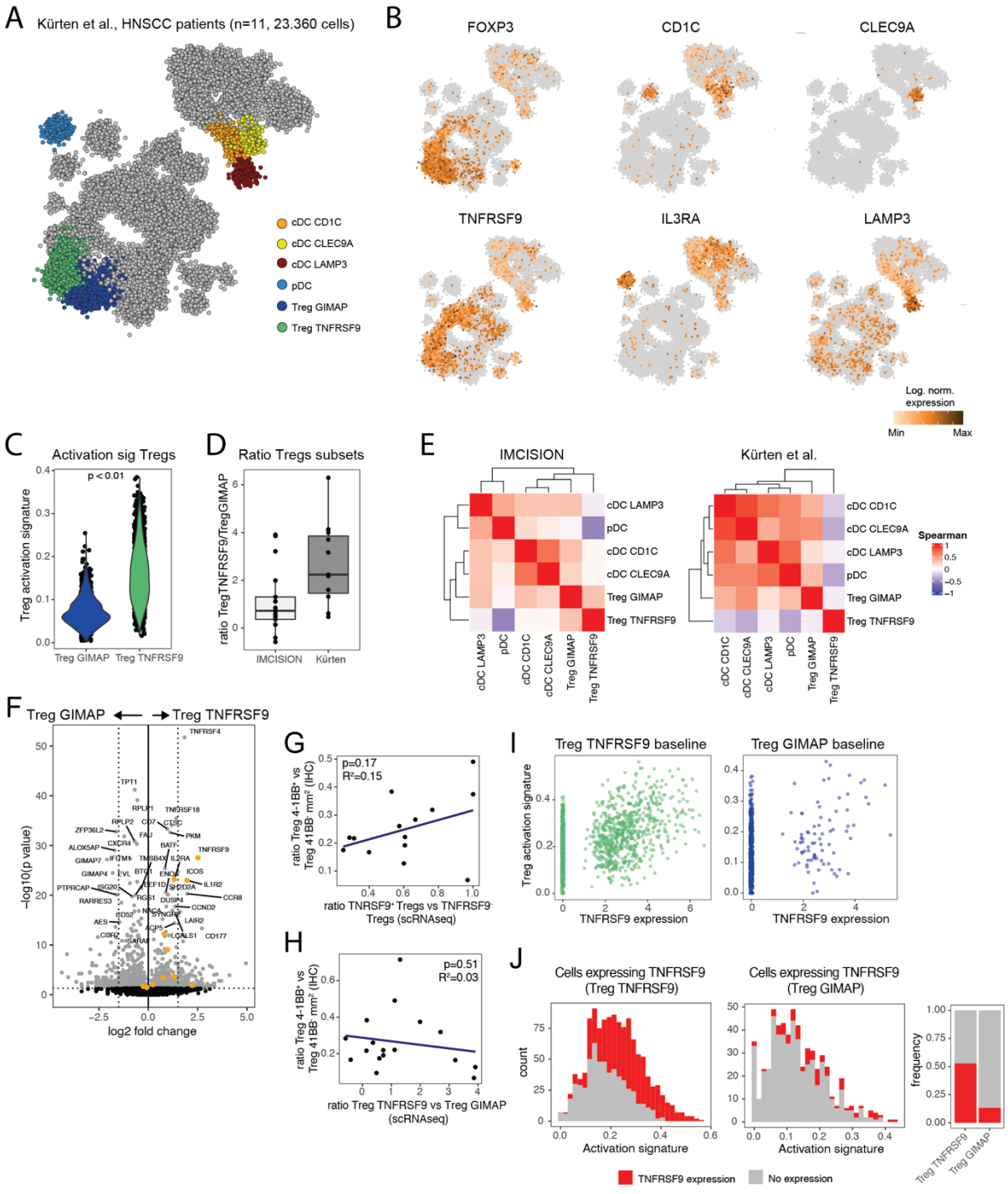

Figure S3. Validation of the differential abundance of Treg subpopulations at baseline using orthogonal strategies and an independent HNSCC dataset

A) 2D projection of all immune cells from all 11 HNSCC patients in the cohort of Kürten et al.[31]. Cell populations that were found to be differentially abundant between responding and non-responding

patients at baseline (Figure 2A) are highlighted on the 2D projection and colored by cell state. B) Expression of marker genes that characterize Tregs (*FOXP3*), activated T cells (*TNFRSF9*), CD1C-expressing cDCs (*CD1C, LAMP3* and *CLEC9A)* and pDCs (*IL3A*) across all metacells, is projected on the 2D map shown in A. Color scale bar represents log normalized expression, scaled to the minimum and maximum expression levels for each gene. C) Expression of the Treg activation signature[32] used in Figure 2E in $Treg_{TNFRSF9}$ and $Treg_{GIMAP}$ cells across all patients from the Kürten et al. cohort[31]. Log normalized expression of activation-related transcripts are depicted and a two-tailed Mann–Whitney U test was performed. D) Pre-treatment ratio of $Treg_{TNFRSF9}$ over $Treg_{GIMAP}$ for all patients (responders and non-responders, excluding IMC-04) from our cohort and the Kürten et al. cohort[31], depicted as the log fold ratio in fraction. Boxes show the median and 25th and 75th percentiles. Whiskers depict 1.5 × IQR and data points represent individual patients. E) Correlation plot of the abundance between differentially present cell states in our cohort (baseline samples) and the cohort of Kürten et al.[31], calculated with a Spearman correlation. Color scale bar represent Spearman's rank correlation coefficient. F) Differential gene expression between the $Treg_{GIMAP}$ and $Treg_{TNFRSF9}$ subsets, shown as log fold change. Highly significantly differentially expressed genes ($P < 1 \times 10^{-15}$) are marked in yellow. Mann Whitney U test with false detection rate (FDR) correction was used to determine p-values. G) Correlation between the ratio of 4-1BB^{+}FOXP3^{+} / 4-1BB^{-}FOXP3^{+} cells as scored by immunohistochemistry and *TNFRSF9*$^{+}$ / *TNFRSF9*$^{-}$ Tregs as identified by single cell RNA sequencing (i.e. cells classified as Treg, in which the *TNFRSF9* gene is or is not detected). Each dot represents data from a single patient. Blue line represents the linear regression, correlation coefficient was calculated by a Pearson correlation. H) Correlation between the ratio of 4-1BB^{+}FOXP3^{+}/4-1BB^{-}FOXP3^{+} cells as scored by immunohistochemistry and the ratio of $Treg_{TNFRSF9}$/$Treg_{GIMAP}$ cells as observed in single cell RNAseq data. Each dot represents data from a single patient. Blue line represents the linear regression, correlation coefficient was calculated by a Pearson correlation. I) Scatter plot of TNFRSF9 expression values versus the signature score of the Treg activation signature[32] within individual cells, within $Treg_{TNFRSF9}$ (left) and $Treg_{GIMAP}$ cells (right) only. J) Left and middle: Stacked histograms of Treg

activation signature scores in $Treg_{TNFRSF9}$ (left) and $Treg_{GIMAP}$ cells (right). Cells in which *TNFRSF9* gene expression was detected are depicted in red. Right: Bar plot showing the fraction of cells in which TNFRSF9 transcripts were detected (depicted in red), within each Treg subset.