Supplementary materials

Title:

Neoadjuvant intratumoral flu vaccine treatment in patients with proficient mismatch repair colorectal cancer leads to increased tumor infiltration of CD8+ T-cells and upregulation of PD-L1: A phase 1/2 clinical trial

Running title:

Intratumoral flu vaccine in pMMR colorectal cancer

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Supplementary methods

Staining of slides for immunohistochemistry analyses

Staining was performed using anti-Cytokeratin clone BS5 (Nordic Biosite Aps, Denmark, # BSH-7124-1), anti-CD3 clone LN10 (Leica/Triolab AS, Denmark, # NCL-L-CD3-565), and anti-CD8 clone C8/144B (Agilent/Dako, Denmark, # GA623). Double-labelling staining was performed on the automated instrument Omnis (Agilent/Dako, Denmark). Briefly, antigen retrieval was accomplished using EnVision™ FLEX Target Retrieval Solution, High pH (Agilent/Dako, Denmark, # GV804) and slides were subsequently incubated with primary antibodies, CD3 (1:50) or CD8 (Ready-to-Use) for 30 minutes at 32°C. Reactions were detected using the standard polymer technique EnVision™ FLEX /HRP Detection Reagent (Agilent/Dako, Denmark, # GV800/GV821) and visualized using EnVision™ Flex DAB+ Chromogen system (Agilent/Dako, Denmark, # GV825) following instructions given by the manufacturer. In the second sequence, slides were incubated with anti-Cytokeratin (1:800) applying the same protocol settings as described above except for visualizing the reactions with EnVision™ Flex Magenta Chromogen system (Agilent/Dako, Denmark, # GV900). Finally, sections were counterstained with hematoxylin and mounted with pertex mounting media (Pertex™/Histolab, Sweden, #00801-EX).

RNA isolation and panel preparation for nCounter analysis of mRNA expression

RNA isolation and panel preparation: Total RNA was extracted from FFPE slides (10 µm sections) using the High Pure FFPET RNA Isolation Kit (Roche Life Science, Germany) according to the manufacturer's instructions. Total RNA was quantified using spectrophotometry (NanoDrop, Thermo Scientific, USA), and RNA quality assessment was done with the Bioanalyzer (Agilent, Denmark). Approximately 300 ng total RNA was used to determine gene expression levels for each sample adequately. We performed RNA hybridization overnight using two distinct panels: for gene expression analysis, we used the nCounter® IO360 panel of 750 endogenous human transcripts; for T-cell receptor (TCR) expression analysis – the nCounter® TCR Diversity panel of 119 T-cell receptor variable and constant regions and lymphocyte

transcripts (NanoString, USA). Sample acquisition was done by using the nCounter® system and following the manufacturer's instructions.

Preparation of plots related to nCounter analysis of mRNA expression:

PCA was performed using the top 400 most variable genes and results were plotted using the functions “pca” and “biplot” of the PCAtools package (v.2.5.3) in R, respectively[1]. Hierarchical clustering was performed based on sample Euclidean distances and farthest neighbor linkage method. A heatmap of the full gene panel (n = 750) was generated using “Heatmap” function from ComplexHeatmap package (v.2.8.0) in R[2]. DE results were visualized with “EnhancedVolcano” package (v.1.11.1) in R[3]. Enrichment analysis was performed to test for functional enrichment in samples before and after vaccination based on pair-wise down-regulated (higher expression in samples before) and up-regulated (higher expression in samples after) DE genes. Gene set annotations were downloaded from the Molecular Signatures Database v7.4 and comprised gene sets from the Hallmark gene sets[4], and “biological processes” from Gene Ontology (GO) database[5]. Additionally, NanoString specific gene sets were retrieved from Danaher *et* al[6]. We used enrichment functions from “clusterProfiler” (v.4.0.2)[7] specific for each gene set annotation, e.g. “enrichGO” for GO database. Genes were included in enrichment analysis if they met threshold requirements of adjusted p value < 0.1 and log2 fold change (logFC) ≥ 0.5.

Gene expression analysis:

Shortly, quality control was performed by evaluating technical sample quality, generating principal component analysis (PCA) plot, Euclidean distance heat map, and gene count histogram. Raw gene counts were normalized by first running upper quartile normalization, followed by variance stabilizing transformation. Estimated unwanted variation was removed using the “removeBatchEffects” function from limma package (v.3.48.1). After iterative QC and normalization, we removed n = 5 vectors of unwanted variation from the data set.

TCR expression analysis

The same iterative QC process was performed for raw TCR data as described for gene expression data. Normalization was performed as described in technical note provided by Nanostring[8]. Shortly, we used "procedure 2", which included three steps: (1) panel standard normalization, (2) creation of housekeeping normalization factors for each sample, and (3) housekeeping normalization. From further analysis we removed n = 1 sample, as normalization could not overcome technical sample artifacts. TCR scores were calculated to quantify the diversity of TCR alpha, beta, delta and gamma variable regions within a sample. The score is based on the Shannon diversity index calculations, which was calculated using base R.

GeoMX data collection

For QC, a FOV detection of >75, binding density of 0.1-2.25, minimum nuclei count of 20, minimum surface area of 0.016 mm, and a positive control normalization factor not between 0.3–3.0 was chosen.

References

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6. Danaher, P. *et al.* Gene expression markers of Tumor Infiltrating Leukocytes. *J. Immunother. Cancer* **5**, 18 (2017).
7. Yu, G., Wang, L.-G., Han, Y. & He, Q.-Y. clusterProfiler: an R package for comparing biological themes among gene clusters. *OMICS* **16**, 284–287 (2012).
8. TECH NOTE: Panel Standard and Calibration Sample Usage. 8 https://nanostring.com/wp-content/uploads/TN_MK3415_Panel-Standard_r9.pdf (2021).

Figure S1 – Flow chart of patient inclusion

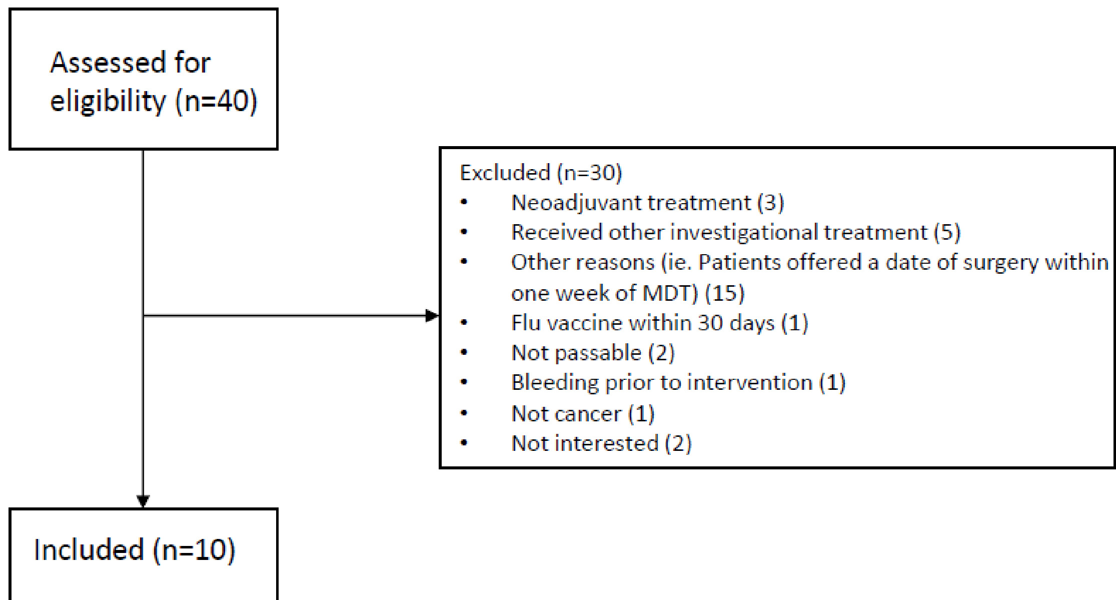

Figure S2 – Overview of distribution and correlation plots after K=5 normalization

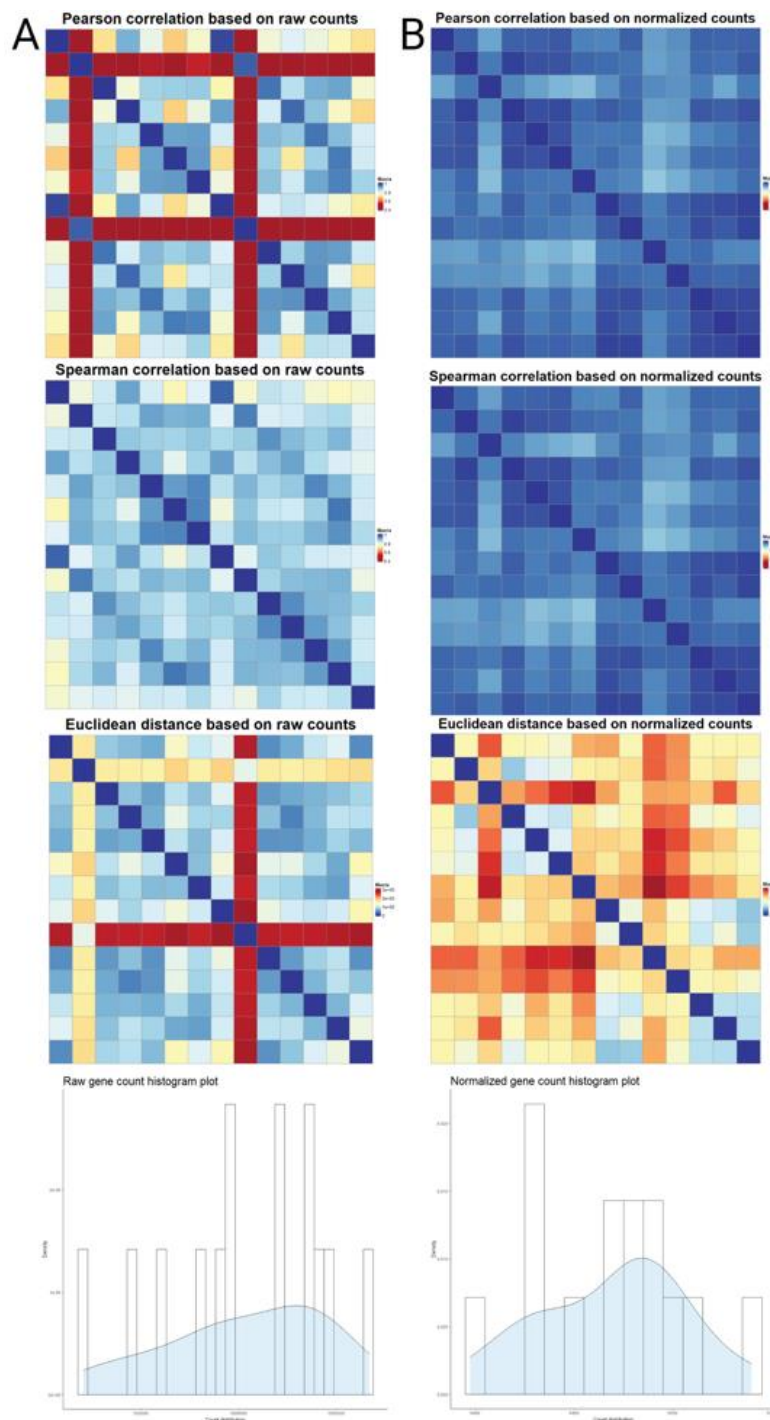

Figure S2 - Overview of distribution and correlation plots before vs. after K=5 normalization. A: Pearson, Spearman, Euclidean distance correlation plots, and data distribution plot before normalization of tumor mRNA gene expression data. B: Pearson, Spearman, Euclidean distance correlation plots, and data distribution plot after RUVseq K=5 normalization of tumor mRNA gene expression data.

Figure S3 – Overview of mRNA gene expression analysis after vs. before IT-flu vaccination

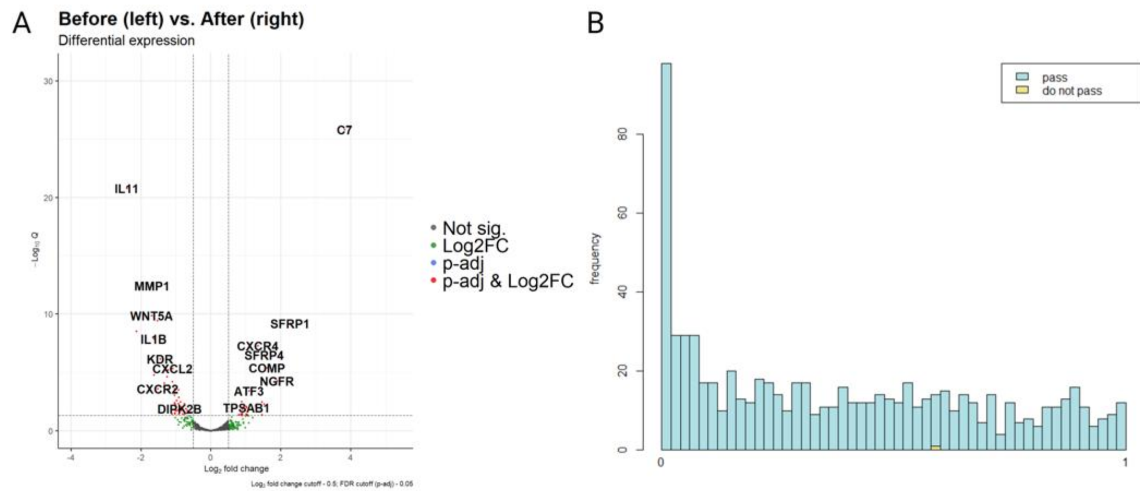

Figure S3 – Overview of mRNA gene expression comparison in tumor samples before vs. after vaccination. A: Volcano plot, B: P-value distribution plot.

Figure S4 – Overview of upregulated genes in mRNA gene expression analysis

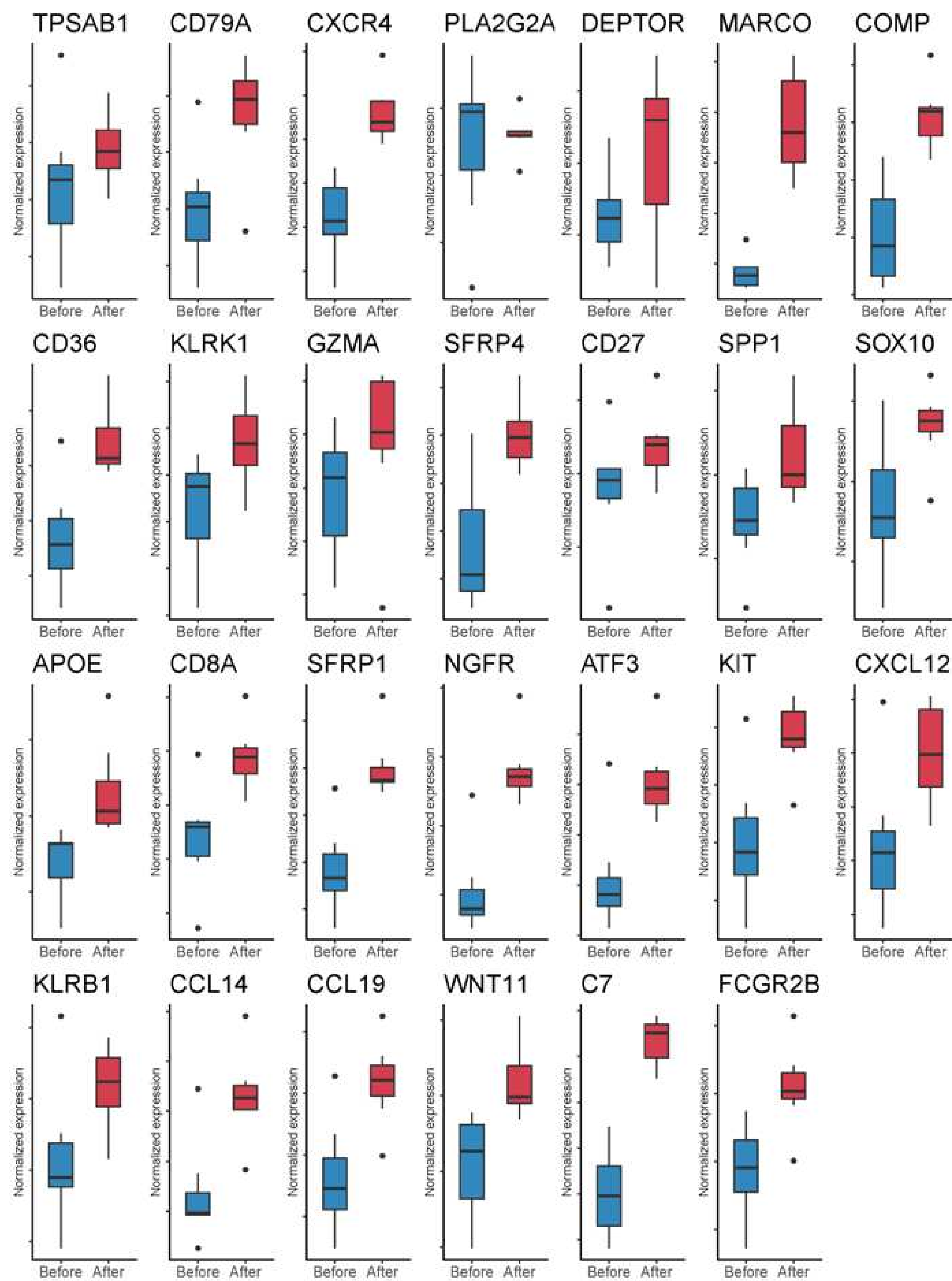

Figure S4 - All upregulated genes after vaccination

Figure S5 – Overview of downregulated genes in mRNA gene expression analysis

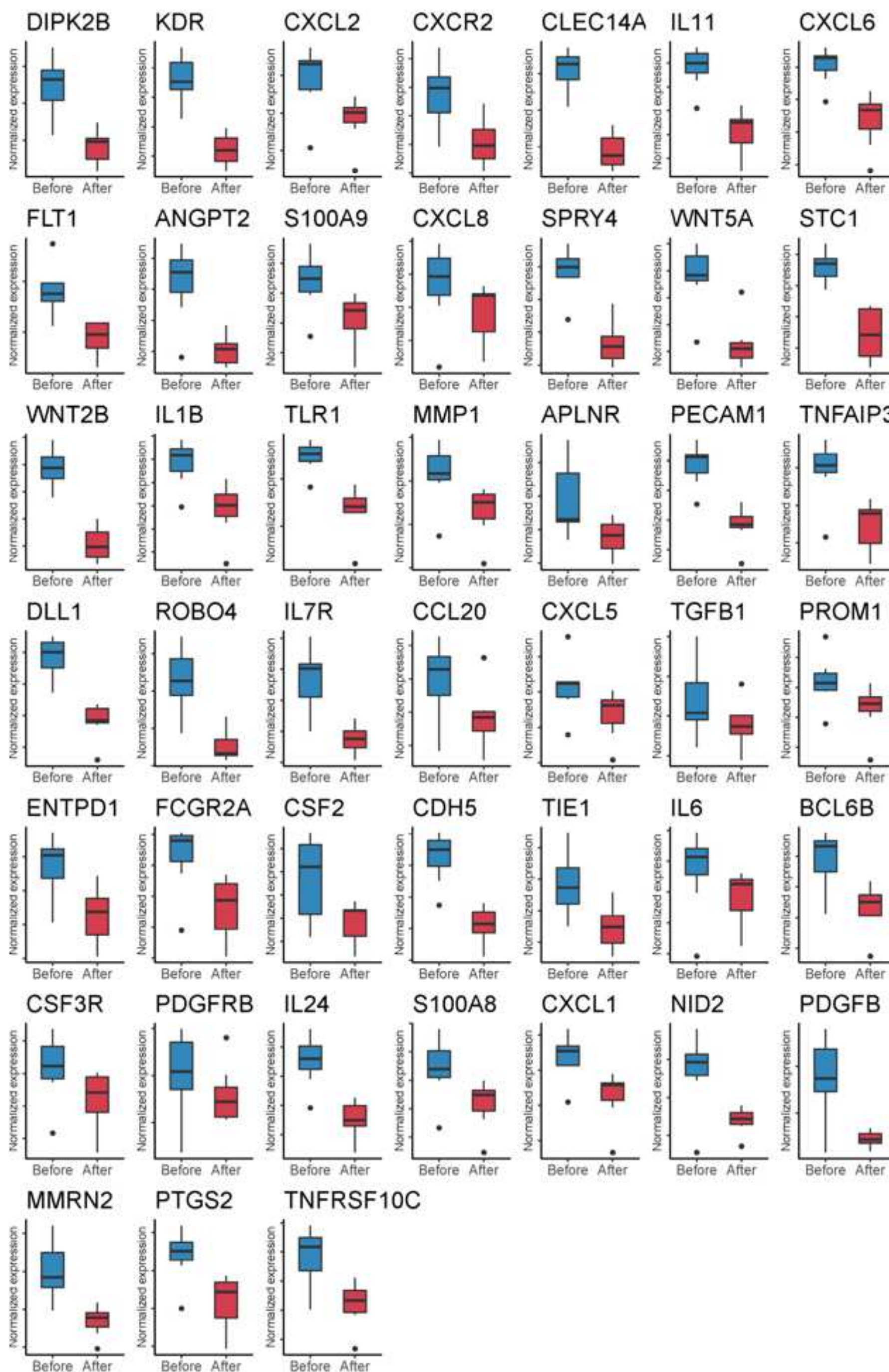

Figure S5 - All downregulated genes at after vaccination.

Figure S6 – Representative ROI's of normal tissues regions before vs. after IT-flu vaccination

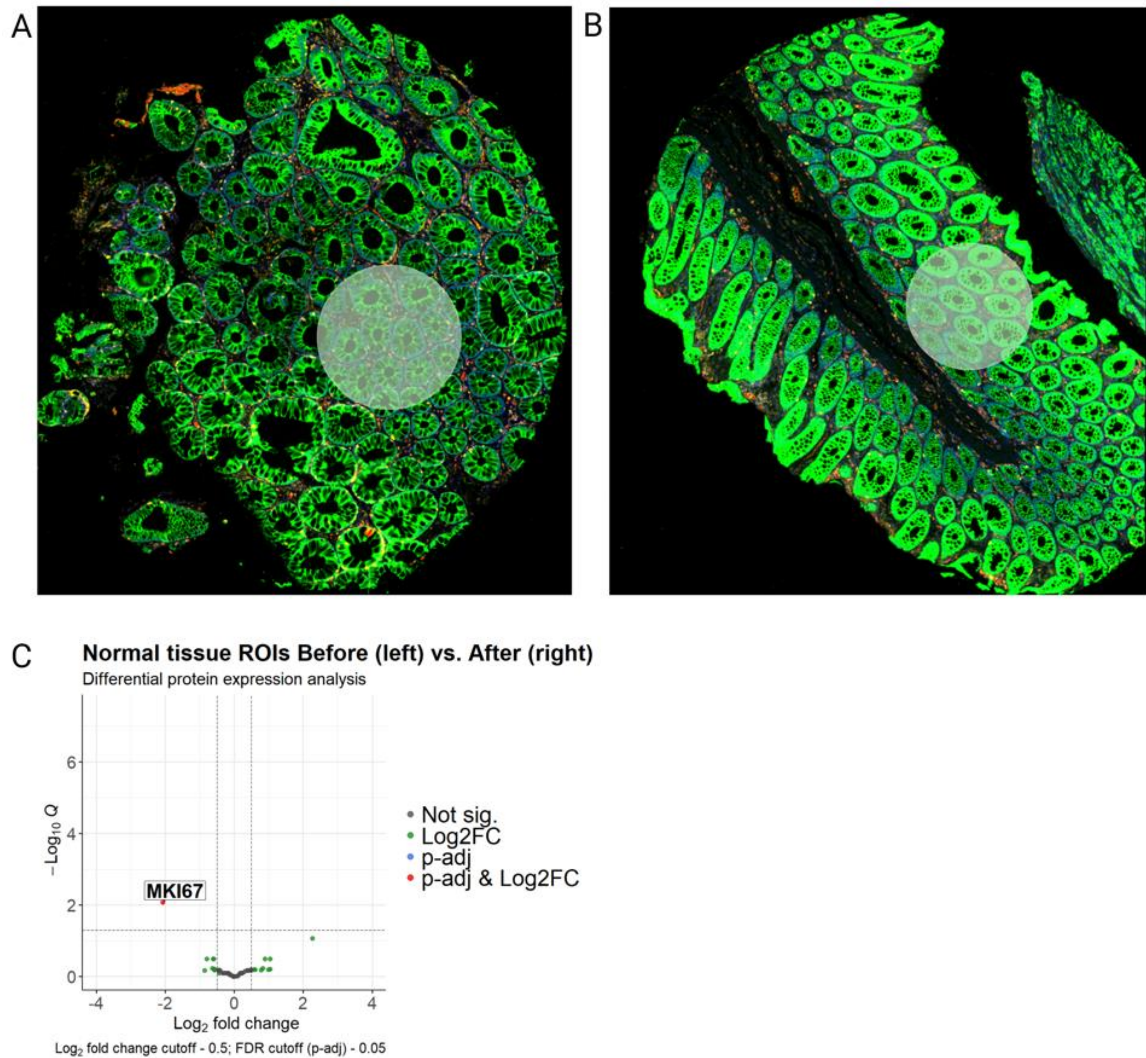

Figure S6 - Representative ROI's of normal tissue regions before vs. after IT-flu vaccination. A: Normal tissue region before vaccination (baseline). B: Normal tissue region after vaccination. C: Volcano plot of differentially expressed proteins at different time points.