Legends for Supplemental Material

Supplemental Fig. 1 PSA responses**. a** Percent change from baseline to lowest on-study PSA prior to prostatectomy. **b** Individual PSA responses (percent change from baseline) during vaccination course.

Supplemental Fig. 2 Percent change in ADC with vaccination. Percent change in apparent diffusion coefficient (ADC) with vaccination. Results are shown for each of 42 lesions in 22 patients with measurements at baseline and post-vaccination. Each lesion number is in the format 'subject #.evaluable lesion #', and each bar color indicates lesions from a single patient. Lower ADC values indicate increasing hypercellularity associated with malignancy.

Supplemental Fig. 3 T-cell densities in pre-vaccination biopsies (Pre) and in radical prostatectomy sections post-vaccination (Post) with PROSTVAC. Paired results connected by lines are shown for compartmental and noncompartmental analysis as indicated.

Supplemental Fig. 4 Compartmental distribution of CD4 and CD8 T cells post-vaccination (PROSTVAC) in intraprostatic tissue compared to a non-treated cohort (CONTROL). CD4 and CD8 T-cell infiltrate average densities were quantified in each compartment. CD4 and CD8 immune-cell density medians were compared by the Mann Whitney test. Median ± IQR (interquartile range) shown with horizontal lines.

Supplemental Fig. 5 Average density ratios (Post/Pre) of IC infiltrates (CD4 and CD8) compared between patients with a change in PD-L status (PD-L1+, n=3), and patients with no change in their PD-L1 status (PD-L1-, n= 23). A) Data representing the mean of density ratios of IC in compartmental and non-compartmental analysis. B) Representative case with change in PD-L1 status post PROSTVAC. Paired path views (generated from multispectral images) of markers CK, PD-L1, CD4, and CD8 are shown.

Supplemental Fig. 6 Polyfunctional PSA, Brachyury, and MUC1-specific T-cell Responses. (A) Frequency of polyfunctional TAA responses ($CD4^+$ or $CD8^+$ T cells expressing 2 or more of the following: IFNγ, TNFα, IL-2, or CD107a) developed after (vs before) vaccination in 25 patients. (B) Representative plots from Patient #22 developing multifunctional MUC1-specific

$CD4^+$ T cells (producing both IFNγ and TNFα) post (vs pre) vaccination. Signal in negative control (HLA) and positive control (CEFT II) is shown.

Supplemental Table 1 Grade ≥ 2 toxicities

Supplemental Table 2 MRI responses

Supplemental Table 3 TIME IC response summary tables
Table 3A Number and percent of cases with >2-fold change from baseline in CD4 and CD8 infiltrates in NCA
Table 3B Number and percent of cases with >2-fold change from baseline in CD4 and CD8 infiltrates in CA

Table 3C Number and percent of cases with >2-fold change from baseline in Tregs and activated (Ki67+) CD8 infiltrates in NCA

Table 3D Number and percent of cases with >2-fold change from baseline in Tregs and activated (Ki67+) CD8 infiltrates in NCA

Table 3E Number of cases with ≥2-fold change from baseline in CD4 and CD8 infiltrates in any of 3 compartments (tIR) in association with peripheral immune response (pIR) to any of 3 target antigens tested

Table 3F Case-by-case data for post/pre CD4 and CD8 T-cell densities by compartment in parallel with peripheral immune response to 3 target antigens tested (pIR).

Supplemental Table 4 PSA, Brachyury, and MUC1-specific intracellular cytokine staining responses. Immune responses reported in this table are calculated by comparing the absolute # of $CD4^+$ or $CD8^+$ T-cells producing cytokine (IFNγ, IL-2, TNFα) or positive for CD107a per $1x10^6$ PBMCs plated at the start of the *in vitro* stimulation at the specified time point post (vs pre) vaccine. Background (obtained with the negative control peptide pool, HLA) and any response prior to vaccine are subtracted: [TAA post vaccine – HLA post vaccine] – [TAA pre vaccine – HLA pre vaccine]. Positive immune responses are defined as >250 (highlighted).

Supplemental Table 5 Recurrence predictions and outcomes, and association with peripheral immune responses. Predicted pre- and post-radical prostatectomy progression-free probabilities calculated using Memorial Sloan Kettering predictive nomograms. The 4 subjects highlighted in red experienced biochemical recurrence after radical prostatectomy, and none developed antigen-specific responses. 12 of the 20 recurrence-free patients that had samples analyzed (60%) generated peripheral antigen-specific immune responses.

Supplemental Methods

1. Staining protocol Opal multiplexing is a serial immunofluorescence method that relies on tyramide signal amplification, which creates an amplification of signal that then covalently binds to the epitope in a specific manner. Primary and secondary antibody complexes are subsequently removed for serial immunofluorescence, while the covalent fluorescent signal remains. Single controls and an unstained slide were stained with each group of slides.

2. Data analysis Cell segmentation was adjusted based on minimum DAPI signal and membranous staining to accurately locate all cells and avoid hyper- and/or hyposegmentation. CD4, CD8, and CK stains were used to create four phenotypes: CD4 cells, CD8 cells, tumor or epithelial cells, and other (not fitting any previously mentioned phenotypes). Thresholds were set by a research pathologist matching adequate signals to reflect true morphology of any particular stain. FOXP3, Ki67, and PD-L1 stains were used to obtain thresholds.

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