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B Cells and Tertiary Lymphoid Structures Promote Immunotherapy Response

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Intro paragraph:

Treatment with immune checkpoint blockade (ICB) has revolutionized cancer therapy, and efforts to better understand therapeutic responses are ongoing. To date, predictive biomarkers[1-10] and strategies to augment clinical response have largely focused on the T-cell compartment. However other immune subsets (including B-cells and tertiary lymphoid structures, TLS) may also contribute to anti-tumor immunity[11-15], though these are not well-studied in ICB[16]. We conducted a neoadjuvant ICB trial in melanoma patients and demonstrated that B-cell signatures were enriched in tumors of responders (R) versus non-responders (NR) via targeted expression profiling[17].To build on this, we performed bulk RNA sequencing on these tumor specimens and demonstrated that markers associated with B-cell development and function were the most differentially expressed genes in R versus NR. Findings were corroborated using a computational method to estimate immune and stromal composition of tumor samples (MCP counter[18]), and were corroborated in another melanoma cohort and in a cohort of renal cell carcinoma (RCC) patients on ICB. Histologic evaluation was performed in these cohorts, highlighting localization of B-cells within TLS. Potential functional contributions of B-cells were assessed via bulk and single-cell RNAseq analysis, demonstrating clonal expansion of B-cells in responders to ICB and unique transcriptional states associated with response. Mass cytometry (CyTOF) was performed in tumor and blood samples from our cohort demonstrating an enrichment of switched memory B-cells and decreased naïve B-cells in tumors of R versus NR to ICB, suggesting that these intra-tumoral B-cells may actively contribute to the anti-tumor response following ICB. Together, these data provide novel insight into the potential role of B-cells and TLS in the response to ICB with implications for the development of biomarkers and potentially therapeutic targets. Further studies to fully elucidate their role are critically needed and are currently underway.

Main:

Immunotherapy has afforded patients with melanoma and other cancers the potential for long term survival, and we are beginning to gain insight into the mechanisms of therapeutic response as well as biomarkers of response and resistance. Significant progress has been made in this regard, with the identification of several validated biomarkers, particularly for immune checkpoint blockade (ICB)[1-10]. It is clear that cytotoxic T lymphocytes play a dominant role in response to ICB and other forms of immunotherapy; however there is a growing appreciation of other components of the tumor microenvironment that may influence therapeutic response – including myeloid cells and other immune cell subsets[11].

Tumor infiltrating B lymphocytes have been identified, yet their overall functional role in cancer is incompletely understood[14,15,19-24] – with some studies suggesting that they are tumor-promoting while others show a positive association with improved cancer outcomes, particularly when they are found in association with organized lymphoid aggregates known as tertiary lymphoid structures (TLS)[12,13,16,25-27].

TLS have been identified within a wide range of human cancers at all stages of disease, though their presence is highly variable between cancer types as well as between patients[12,16]. Significant heterogeneity also exists with regards to the cellular constituents of TLS and their location within tumor, and this may influence the overall impact on anti-tumor immunity and outcome[12-14,16]. These TLS structures are not just a surrogate marker of a brisk immune response; rather, it is thought they actively modulate anti-tumor immune activity. In this regard, the benefit of a high $CD8^+$ T-cell density within a tumor is abrogated in the absence of TLS-associated DCs[28]. Mature TLS exhibit evidence for germinal center formation[29,30], and oligoclonal B-cell responses have been identified in cutaneous melanoma and metastases[31,32], further suggesting an active humoral anti-tumor response within the TLS and one driven by B-cells. Notably, the role of B-cells and, in particular, TLS in response to ICB remains unclear.

We recently conducted a phase 2 clinical trial of neoadjuvant treatment with ICB in patients with high-risk resectable (clinical stage III or oligometastatic stage IV) melanoma to assess the safety

and feasibility of this treatment in this patient population (NCT02519322)[17]. Importantly, longitudinal tumor samples were taken in the context of therapy, and molecular and immune profiling was performed to gain insight into mechanisms of therapeutic response and resistance. In these studies, known and novel biomarkers of response were identified, and targeted protein expression profiling (via Nanostring Digital Spatial Profiling) revealed significantly higher expression of B-cell markers in baseline and on-treatment samples of responders to ICB[17].

To gain a deeper understanding of potential mechanisms of therapeutic response to ICB in this cohort, we performed RNA sequencing (RNAseq) in longitudinal tumor samples from this patient cohort. In these studies, significantly higher expression of B-cell related genes such as *MZB1*, *JCHAIN*, and *IGLL5* were observed in responders versus non-responders to ICB at baseline (p<0.001) with over-representation of these genes compared to T-cell and other immune markers (with evaluable tumors from 7R and 9NR) (**Figure 1 a-b, Extended Data Tables 1 ; Supplementary Table 1**). Additional genes that are expected to alter B-cell function were also significantly enriched in R vs NR, such as *FCRL5*, *ID01, IFN- γ, and BTLA.* Low tumor purity was observed in some samples, particularly in the context of an effective therapeutic response, limiting conventional analysis of RNAseq data. To address this, we next performed a more focused interrogation of the tumor immune microenvironment using the MCP-counter method[18] on RNAseq data in baseline and on-treatment tumor samples - focusing more specifically on immune-related genes (**Supplementary Table 2**), allowing inclusion of samples with low tumor purity (10 R and 11 NR at baseline, 9 R and 11 NR on-treatment). In these analyses, we again observed enrichment of a B-cell signature in R versus NR at baseline and early on-treatment (p=0.036 and 0.038, respectively). Notably, these analyses included samples from patients with nodal and extra-nodal disease with no obvious contribution based on site of disease (**Fig. 1c, Extended Data Fig. 1a-b and 2a, Supplementary Table 3 and 7**), suggesting that B-cell signatures were not merely related to the presence of these tumors within lymph nodes. Importantly, findings of high B-cell lineage scores in responders were replicated in samples from an additional cohort of melanoma patients treated with neoadjuvant versus adjuvant checkpoint

blockade (NCT02437279, OpACIN trial (n=12 R and 6 NR)[33] (**Extended Data Fig. 1d and 2c**, **Extended Data Table 2, Supplementary Tables 4 and 7**). B-cell signatures alone were predictive of response in univariable analyses (OR 2.6, p=0.02 for our trial, and OR 2.9, p=0.03 for combined melanoma cohorts), but not in multivariable analyses when considering other components of the immune cell infiltrate, suggesting that B-cells are likely acting in concert with other immune subsets and not acting in isolation; however these analyses were limited due to the low sample size **(Extended Data Table 4 and 5).**

To evaluate the validity of these findings across additional cancer types, we next assessed the expression of these immune cell gene expression signatures in a pre-surgical ICB trial for patients with metastatic renal cell carcinoma (RCC) (NCT02210117, PD-1 blockade monotherapy versus combined CTLA-4 and PD-1 blockade versus combined PD-1 blockade and bevacizumab) **(Extended Data Table 3)**. Gene expression profiling by microarray and subsequent MCP-counter analysis of baseline tumor samples was performed, demonstrating significantly higher expression of B-cell related genes in R vs NR to therapy (p=0.0011, n=17R and 11 NR) (**Fig. 1d, Extended Data Fig. 1c, 2b, and 3, Supplementary Table 5-7**). As in the case of melanoma, B-cell signatures were predictive of response in univariable analysis in the RCC cohort (OR 61.2, p=0.05) but not multivariable analysis, again suggesting cooperative function with other immune subsets; however, sample size was again quite limited (**Extended Data Table 6)**.

Based on these data along with existing data regarding a potential prognostic role for TLS in melanoma and other cancer types outside the context of treatment with ICB[18,34,35], we next assessed the expression of these immune related genes in cutaneous melanoma from The Cancer Genome Atlas platform (TCGA-SKCM, n=136)[36]. To do this, we applied the MCP-counter algorithm to available RNAseq data from a subset of patients with non-recurrent Stage III disease (regional lymph node or regional subcutaneous metastases), as these were most comparable to our clinical cohort. In these studies, we identified 3 distinct melanoma immune clusters (MICs), with significantly higher expression of B-cells in cluster C versus cluster A

(p<0.0001) and cluster B (p<0.0001) (**Extended Data Fig. 4a, Supplementary Table 8-10).** Notably, there was no clear association of MIC with known genomic subtypes of melanoma (BRAF, NRAS, NF1, triple WT)[36] or disease site (nodal versus non-nodal) (**Extended Data Fig. 4a**, **Supplementary Table 10**). Importantly, survival analyses revealed that cases in cluster C had significantly improved overall survival (OS) compared to cluster A (p=0.0068) (**Extended Data Fig. 4b**). To assess the association with B-cell signatures specifically, we next compared OS between B-cell lineage high versus low demonstrating prolonged survival in patients with B-cell lineage high versus to B-cell lineage low tumors (p=0.053) (**Extended Data Fig. 4c**). Furthermore, univariable Cox Proportional Hazards modeling demonstrated that tumors with low B-cell infiltrate had significantly increased risk of death (HR is 1.7 for B-cell low, p=0.05) in comparison to B-cell high group (**Extended Data Table** 7). Similar analyses were performed to assess the expression of immune-related genes in clear cell RCC from the TCGA (TCGA-KIRC, n=526)[37]. In these studies, similar immune clusters were observed; however, immune infiltrate was not associated with survival in these patients (p=0.24) (**Extended Data Fig. 5, Supplementary Tables 11-13**), possibly owing to the heterogeneous nature of this disease and other driving mechanisms of patient outcomes.

Based on these insights from gene expression profiling data, we next assessed tumor samples histologically to gain insight into the density and distribution of B-cells as well as their relationship to TLS in patients treated with neoadjuvant ICB. The density of $CD20^+$ B-cells, TLS, and ratio of TLS to tumor area was higher in R versus NR in our neoadjuvant melanoma cohort, particularly in early on-treatment samples (p=0.0008, p=0.001, p=0.002 respectively), though statistical significance was not reached for all of these markers in baseline samples (p=0.132, p=0.078, p=0.037, respectively) (**Fig. 2a**), which is in line with our prior published work suggesting that assessment of early on-treatment immune infiltrate is far more predictive of response to ICB than assessment of pre-treatment samples[1]. Findings between gene expression profiling and IHC were complementary, and had modest correlation as described by others[18] (**Extended Data Fig 9**). Intriguingly, we also demonstrate increased abundance of B-cell related

exosomes ($CD20^+$ exosome-coated beads) in the peripheral blood of Rs as compared to NRs at early on-treatment timepoints (**Extended Data Fig. 6**).

Importantly, architectural analysis identified that CD20+ B-cells were localized in TLS within tumors of Rs with co-localization of $CD20^+$ B-cells with $CD4^+$, $CD8^+$, and $FoxP3^+$ T lymphocytes. Co-localization with $CD21^+$ follicular dendritic cells and $MECA79^+$ high endothelial venules (HEV) was also demonstrated (**Fig. 2d-f, Extended Data Fig. 7a and 8a**). The vast majority of evaluated TLS in these patients represented mature secondary-follicle like TLS, as indicated by the presence of both CD21+ follicular dendritic cells and $CD23^+$ germinal center B-cells[29] (**Fig. 2d-f, Extended Data Fig. 7a and 8a**). We identify similar mature TLS in patients with extra-nodal metastases (**Extended Data Fig. 8b**), suggesting that TLS may develop in non-nodal sites and are associated with response to ICB. Analogous immunohistochemical findings were observed in our cohort of RCC patients treated with pre-surgical ICB with increased CD20+ cell infiltration and TLS density associated with response (**Extended Data Fig. 10a-c**); these TLS are morphologically similar to those found in melanoma (**Extended Data Fig. 7b and 10d-f**). We also assessed the potential functional role of B-cells and TLS in promoting T-cell responses in our cohort via additional spatial profiling analyses, noting increased markers of activation on T-cells within as compared to those outside these TLS **(Extended Data Fig 11)**.

Next we performed several more in-depth analyses to gain insight into the phenotype and function of the infiltrating B-cells, and how they might be contributing to responses to ICB. Reasoning that differences in clonotypes of B-cell receptors (BCRs) between Rs and NRs would be indicative of an antitumor B-cell response, we probed our RNAseq data for BCR sequences using the modified TRUST algorithm. In these studies, we identified significantly increased clonal counts for both immunoglobulin heavy chain (IgH) and immunoglobulin light chain (IgL) ($p=0.001$ and $p=0.004$, respectively) and increased BCR diversity in Rs as compared to NRs ($p=0.002$ and $p=0.0008$) suggesting an active role for B-cells in anti-tumor immunity **(Fig. 3a, Extended Data Fig. 12-13)**. To complement these analyses, we next analyzed single-cell RNA sequencing data from baseline and on-treatment samples from an independent cohort of metastatic melanoma

patients treated with ICB (n=48 tumor samples--1760 B-cells from 32 patients treated with PD-1 blockade monotherapy, CTLA-4 blockade monotherapy, or combined PD-1 and CTLA-4 blockade, including samples from some patients in our neoadjuvant ICB cohort[38]) (**Extended Data Tables 8 and 14**). Similar to observations made in our clinical trial cohort, we demonstrated that B-cells were significantly enriched in R versus NR tumors and were predictive of response (OR 1.05, p=0.02), whereas interestingly T-cells overall (as opposed to differential T-cell states) were not predictive of response in this cohort (**Fig. 3b and Extended Data Fig. 14a, Extended Data Table 9**). Unbiased analysis for B-cell markers (using all expressed genes in the $CD45^{+}CD19^{+}$ population only) associated with clinical outcome demonstrated 46 markers to be significantly enriched in R and 147 markers significantly enriched in NR lesions (**Extended Data Fig. 14b, Supplementary Tables 14-15**)**.** Pathways upregulated in Rs as compared to NRs include those consistent with increased immune activity including CXCR4 signaling, cytokine receptor interaction and chemokine signaling pathways (**Extended Data Fig. 15a and Extended Data Table 10**). Unsupervised clustering of B-cells using k-means clustering, after testing for the robustness of each solution, identified 4 distinct B-cell clusters, G1 (B-cells, switched, activated IgD- cells), G2 (plasma cells), G3 (B-cells unswitched IgD^{+}) and G4 (B-cells, switched, activated IgD- cells, with unique markers relative to G1), each of which is associated with different functional state (**Fig. 3c, Extended Data Fig. 14c and 15b and Extended Data Table 11**). No significant differences were identified when testing for associations of each individual cluster (G1-G4) with the clinical outcome, likely owing to limited sample size. Pathway analysis was also performed on bulk RNA sequencing data from our clinical trial cohort, revealing increased immune signaling pathways in Rs as compared to NRs including TCR signaling, MHC-mediated antigen processing and presentation, Th1- and Th2-cell differentiation, and co-stimulatory signaling associated with T-cell signaling **(Extended Data Fig. 16 and Extended Data Table 12 and 13).**

To gain additional insight into the potential functional role of B-cells in response to ICB, we performed mass cytometry (CyTOF) in evaluable tumor and peripheral blood (PB) samples (n =

7 R and n = 3 NR for tumor and n = 4 R and n = 4 NR for PB from our neoadjuvant ICB trial). Sample size was somewhat limited due to the amount of tumor available given prioritization for other studies as well as tumor viability. Notably, these analyses include patients with nodal and non-nodal metastases (**Extended Data Fig 17a and Extended Data Table 15**). We first assessed differences between intra-tumoral B-cells and those in the peripheral blood of patients. In these studies, unique clusters of $CD45^{+}$ $CD19^{+}$ (B-cell) populations including naïve ($CD19^{+}$, $CD27^{-}$, IgD^{+}), transitional (CD19+, $CD24^{++}$, $CD38^{++}$, $CD10^{+}$, $CD27^{-}$, IgD^{+}), unswitched and switched memory ($CD19^{+}$, $CD27^{+}$, $IgD^{+/-}$), double-negative ($CD19^{+}$, $CD27^{-}$, IgD^{-}), and plasma(like) cell ($CD19^{+}$, $CD20^{-}$, $CD22^{-}$, $CD38^{++}$, $CD27^{++}$) populations were demonstrated in peripheral blood and tumor samples, with distinct profiles in the tumor compared to peripheral blood samples (**Fig. 3e, Extended Data Fig. 17 and 18**). Intratumoral B-cells had reduced expression of CD21, CD23, CD79b, and CXCR5, pointing to distinct functional and migratory profiles compared to similar B-cell populations in the peripheral blood (**Extended Data Fig 19a)**. We next compared the phenotypes of B-cells in Rs as compared to NRs to ICB in both tumor and peripheral blood. Though B-cell subsets (naïve, memory and transitional B-cells and plasma cells) in the peripheral blood had a similar distribution in R and NR (**Extended Data Fig. 17b**), significant differences were noted in B-cell subsets in tumors of R vs NR to ICB (**Extended Data Fig. 17b**). Specifically, tumors from R had a significantly higher frequency of memory B-cells, whereas NR had a significantly higher frequency of naïve B-cells ($p=0.033$ for naïve and $p=0.033$ for memory) (**Fig. 3f-g, Extended Data Fig. 17**). Other notable differences included an increase in plasma cells in R vs NR; however this did not reach significance and was largely driven by data from one patient ($p=0.3$). An increased percentage of $CXCR3^{+}$ and $CD86^{+}$ B-cells were identified in Rs as compared to NRs, which are markers of memory B-cells and germinal center B-cells **(Extended Data Fig. 19c)**.

In summary, we present multi-omic data supporting a role for B-cells within TLS in the response to ICB in patients with melanoma and RCC. While the distinct mechanisms through which B-cells contribute are incompletely understood, our data suggests that the same properties of memory

B-cells and plasma cells desirable for acquired immune responses may also be contributing to an effective T-cell response following ICB. Importantly, these B-cells are likely acting in concert with other key immune constituents of the TLS by altering T-cell activation and function as well as through other mechanisms. Memory B-cells may be acting as antigen-presenting cells, driving the expansion of both memory and naive tumor-associated T-cell responses. B-cells can also secrete an array of cytokines, including TNF-α, IL-2, IL-6 and IFNγ, through which they activate and recruit other immune effector cells, including T-cells. The observation of switched memory B-cells (that can differentiate into plasma cells) in responders suggests that they could be potentially contributing to the anti-tumor response by producing antibodies against tumor antigens, though we did not have adequate samples to study this in our cohort. Though findings in these cohorts are provocative, further studies need to be performed in additional cohort and pre-clinical models to better understand the mechanisms through which B-cells and TLS may favorably impact response to immunotherapy. Nonetheless, this represents an important insight into therapeutic responses to ICB and will likely stimulate further research in this area.

Materials and Methods:

Patient Cohort(s) and Sample Collection

For the melanoma neoadjuvant cohort (NCT02519322)[17] , 23 patients enrolled in a phase II clinical trial of neoadjuvant ICB. Twelve patients received nivolumab monotherapy with 3 mg/kg every 2 weeks for up to 4 doses, and 11 patients received ipilimumab 3 mg/kg with nivolumab 1 mg/kg every 3 weeks for up to 3 doses followed by surgical resection. These patients were treated at the University of Texas MD Anderson Cancer Center and had tumor samples collected and analyzed under Institutional Review Board (IRB)-approved protocols (2015-0041, 2012-0846). Of note, these studies were conducted in accordance with the Declaration of Helsinski and approved by the UT MD Anderson Cancer Center IRB. Response was defined as achieving a complete or partial radiographic response by RECIST 1.1 between pre-treatment imaging and post-neoadjuvant treatment imaging prior to surgical resection. Tumor samples were collected at several time-points for correlative studies including baseline and on-treatment (weeks 3 and 5 for nivolumab monotherapy, weeks 4 and 7 for combination ipilimumab with nivolumab). Tumor samples were obtained as core, punch or excisional biopsies performed by treating clinicians or an interventional radiologist. Samples were immediately formalin fixed and paraffin-embedded (FFPE), snap frozen, or digested following tissue collection.

Additional patients off-protocol included 5 patients with widely metastatic melanoma who were treated at the University of Texas MD Anderson Cancer Center and had tumor samples collected and analyzed under Institutional Review Board (IRB)-approved protocols (LAB00-063 and PA17 – 0261). Samples were immediately formalin fixed and paraffin-embedded (FFPE) following tissue collection.

For the validation melanoma cohort, we used samples of 18 patients enrolled in the OpACIN trial (NCT02437279). In the phase 1b OpACIN trial 20 patients with palpable stage III melanoma were randomized 1:1 to receive ipilimumab 3 mg/kg and nivolumab 1 mg/kg, either four courses after surgery (adjuvant arm), or 2 courses prior to surgery and two courses post-surgery

(neoadjuvant arm). Co-primary endpoints were safety/feasibility and tumor specific T-cell expansion. For this current correlative study, response was defined as not having disease relapse. These patients were treated at the Netherlands Cancer Institute (Amsterdam, the Netherlands). The study was conducted in accordance with the Declaration of Helsinki and approved by the medical ethics committee of the Netherlands Cancer Institute. All subjects provided informed consent prior to their participation in the study. Patients underwent a pre-treatment tumor biopsy (1x formalin fixed and paraffin-embedded (FFPE) and 2x fresh frozen) obtained as a core biopsy performed by a radiologist. RNA was extracted from one frozen biopsy for RNA-Sequencing analysis. We only included 18 patients in our analysis because the tumor purity in the frozen pretreatment biopsy of two patients was too low, therefore no RNA could be isolated and these patients could not be included in this analysis. The clinical responses of this cohort has been previously described[33].

The renal cell carcinoma (RCC) trial was an open-label, randomized, pre-surgical/pre-biopsy trial (NCT02210117) whereby adults with metastatic RCC without prior immune checkpoint therapy and anti-VEGF therapy were enrolled and randomized 2:3:2 to receive nivolumab (3mg/kg q2wks x3 doses), nivolumab+bevacizumab (3mg/kg q2wks x3 +10mg/kg x3) or nivolumab+ipilimumab (3mg/kg q2wks x3 1mg/kg x2), followed by surgery (cytoreductive nephrectomy or metastasectomy), or biopsy at week 8-10, and subsequent nivo maintenance therapy up to 2 years. Response was assessed at 8 weeks and then at ≥12 weeks by RECIST 1.1 criteria. Clinical response data collection is still ongoing at this time. For this current correlative study, response was defined as achieving a complete or partial response. Pre- and post-treatment blood and tumors were obtained for correlative studies by IRB-approved lab protocol PA13-0291. Tumor samples were obtained as core biopsies or surgical resection performed by interventional radiologists or surgeons. Samples were immediately formalin fixed and paraffin-embedded (FFPE) or snap frozen following tissue collection.

The single-cell RNA sequencing B-cell analysis used a dataset from 32 metastatic melanoma patients (n=48 samples) treated with anti-PD1 (n=37), anti-CTLA4 (n=2), or anti-PD1/CTLA4

(n=9)[38]. Patient response was determined by RECIST criteria: CR and PR for R or SD and PD for NR. For the analysis we focused on individual lesions and classified them into two categories: Responder (R; n=17) including CR and PR samples; Non-responder (NR; n=31) including SD and PD samples, based on radiologic tumor evaluations. Samples were collected after patients provided a written consent for research and genomic profiling of collected tissue as approved by the Dana-Farber/Harvard Cancer Center Institutional Review Board (DF/HCC protocol 11-181) and UT MD Anderson Cancer Center (LAB00-063 and 2012-0846).

Gene Expression Profiling and Analysis

RNA extraction for neoadjuvant melanoma ICB cohort. Total RNA was extracted from snap-frozen tumor specimens using the AllPrep DNA/RNA/miRNA Universal Kit (Qiagen) following assessment of tumor content by a pathologist, and macrodissection of tumor bed if required. RNA quality was assessed on an Agilent 2100 Bioanalyzer using the Agilent RNA 6000 Nano Chip with smear analysis to determine DV200 and original RNA concentration. Based on RNA quality, 40-80ng of total RNA from each sample then underwent library preparation using the Illumina TruSeq RNA Access Library Prep kit according to the manufacturer's protocol. Barcoded libraries were pooled to produce final 10-12 plex pools prior to sequencing on an Illumina NextSeq sequencer using one high-output run per pool of 76bp paired-end reads, generating 8 fastq files (4 lanes, paired reads) per sample.

RNA-seq data processing and quality check. RNA-seq FASTQ files were first processed through FastQC (v0.11.5)[39], a quality control tool to evaluate the quality of sequencing reads at both the base and read levels. The reads that had ≥15 contiguous low-quality bases (phred score <20) were removed from the FASTQ files. STAR 2-pass alignment (v2.5.3)[40] was then performed on the filtered FASTQ files with default parameters to generate RNA-seq BAM file for each sequencing event. After that, RNA-SeQC (v1.1.8)[41] was run on the aligned BAM files to generate a series of RNA-seq related quality control metrics including read counts, coverage, and correlation. A matrix of Spearman correlation coefficients was subsequently generated by RNA-SeQC among all sequencing events. The correlation matrix was carefully reviewed and the

sequencing event generated from one library pool that showed poor correlation with other library pools from the same RNA sample were removed before sample-level merging of BAM files.

Gene expression quantification and normalization. HTSeq-count (v0.9.1)[42] tool was applied to aligned RNA-seq BAM files to count for each gene how many aligned reads overlap with its exons. The raw read counts generated from HTSeq-count (v0.9.1)[42] were normalized into fragments per kilobase of transcript per million mapped reads (FPKM) using the RNA-seq quantification approach suggested by the bioinformatics team of NCI Genomic Data Commons (GDC)[43]. Briefly, FPKM normalizes read count by dividing it by the gene length and the total number of reads mapped to protein-coding genes using a calculation described below:

$$FPKM = \frac{RC_g * 10^9}{RC_{pc} * L}$$

RC_g, number of reads mapped to the gene; RC_{pc}: number of reads mapped to all protein-coding genes; L, length of the gene in base pairs (calculated as the sum of all exons in a gene). The FPKM values were then log_2-transformed for further downstream processes.

RNA Sequencing and Analysis for OpACIN trial. RNA sequencing and data analysis were performed as previously described[33].

Affymetrix Microarray for RCC. The Affymetrix microarray data were created using the Affymetrix Clariom™ D Assay (Human). There are 28 available pre-treatment samples from 3 arms: Nivolumab (n= 6), Nivolumab+Bevacizumab (n= 14) and Nivolumab+Ipilimumab (n= 8). The raw CEL files were normalized using the built-in SST-RMA method of the Affymetrix Transcriptome Analysis Console (TAC, v4.0) software. The cell lineage scores were calculated using the R package MCP-counter algorithm (v. 1.1.0). The Limma R software package[44] was used to identify DEGs from normalized microarray data for the RCC cohort.

TCGA SKCM and KIRC data downloading and patient selection. The normalized RNA-seq expression data of TCGA skin cutaneous melanoma (TCGA-SKCM) and Kidney Renal Clear

Cell Carcinoma (TCGA-KIRC) was downloaded from NCI Genomic Data Commons (GDC, https://portal.gdc.cancer.gov) and the relevant clinical data was downloaded from recent TCGA PanCancer clinical data study[45]. The information of SKCM genomic subtypes was obtained from the TCGA-SKCM study[36].To achieve a uniform cohort of patients with Stage III (non-recurrent) melanoma for analysis, we applied an appropriate set of sequential filters: The TCGA-SKCM cohort was filtered to include patients with biospecimen tissue sites that included regional lymph node or regional subcutaneous metastases. We excluded patients presenting with Stage IV disease. Then, to exclude patients with *recurrent* Stage III disease, we excluded all patients for whom the number of days from the diagnosis of the primary to the accession date was > 90 days. Additionally, for a patient to be included, their tumor must also have had a defined melanoma driver type. Finally, we eliminated those lacking sufficient gene expression data, yielding a final Stage III TCGA-SKCM cohort of n=136. Survival data missing for 9 or 136 samples, so n=127 for overall survival analyses. For TCGA-KIRC, the cases without available expression data were excluded and a total of 526 cases were taken into subsequent analysis.

Identification of differentially expressed genes. The HTSeq normalized read count data for all expressed coding transcripts was processed by Deseq2 (v3.6)[46] software to identify differentially expressed genes (DEGs) between two response (R versus NR) groups. A cut-off of gene expression fold change of ≥2 or ≤0.5 and a FDR q-value of ≤0.05 was applied to select the most differentially expressed genes. The Limma R software package[44] was used to identify DEGs from normalized microarray data for the RCC cohort.

Deconvolution of the cellular composition with MCP-counter. The R package software MCP-counter[18] was applied to the normalized $\log_2$-transformed FPKM expression matrix to produce the absolute abundance scores for 8 major immune cell types ($CD3^+$ T-cells, $CD8^+$ T-cells, cytotoxic lymphocytes, NK cells, B lymphocytes, monocytic lineage cells, myeloid dendritic cells, and neutrophils), endothelial cells, and fibroblasts. The deconvolution profiles were then hierarchically clustered and compared across response and treatment groups.

Pathway Enrichment Analyses. The network based pathway enrichment analysis was performed using differentially expressed genes across responder and non-responder groups in the bulk-tissue RNA sequencing data from melanoma neoadjuvant cohort and single-cell RNA sequencing data from metastatic melanoma cohort. In the bulk-tissue, the differentially expressed genes which had a q-value <0.05 and log2foldchange >1.5 were & < -1.5 were selected as input for network based pathway enrichment analysis using ReactomeFiViz[47] application in Cytoscape[48,49]. In single-cell, the differentially expressed genes with q-value<0.1 were selected as input for pathway enrichment analysis. Pathway enrichment was calculated using several biological databases (KEGG, NCBI, Reactome, Biocarta, and Panther) with hypergeometric test false discovery rate (FDR) <0.01.

Survival analyses. In TCGA cohort, survival data was not available for 9 samples and these were excluded from survival analysis. As described previously[36], the survival time for each patient was "Curated TCGA survival (i.e., from time of TCGA biospecimen procurement). The time to event was defined as the time interval from date of accession for each sample to date of death or censoring from any cause (curated value CURATED_TCGA_days_to_death_or_last_follow-up; aka TCGA post-accession survival). The survival analysis was performed using Cox Proportional Hazards model and survival curves were plotted using Kaplan-Meier method. The statistical comparison of the survival curves was done using the log rank test. The analysis was done using R package *survival* [50].

Statistical analyses. The statistical comparison between responder and non-responder groups for a given continuous variable was performed using two-sided Mann-Whitney U test. The association between two continuous variables was assessed using Spearman's rank correlation coefficient. To control for multiple comparisons, we applied the Benjamini-Hochberg method[51] and calculated adjusted P-values. Univariable and multivariable analysis predicting response to ICB was performed using logistic regression modeling.

Single Immunohistochemistry

Hematoxylin (H&E) and immunohistochemistry (IHC) staining were performed on FFPE tumor tissue sections. The tumor tissues were fixed in 10% formalin, embedded in paraffin, and transversely sectioned. 4 µm sections were used for the histo-pathological study.

Sections were stained with mouse or rabbit anti-human monoclonal antibodies against CD20 (Dako, cat# M0755, 1:1400), CD21 (Novocastra, NCL-L-CD21-2G9, 1:10 or Leica, CD21-2G9; 1:20), CD23 (Leica, CD23-1B12, 1:15), CD4 (Novocastra, CD4-368-L-A, 1:80) CD8 (Thermo Scientific, MS-457-S, 1:25), FoxP3 (Biolegend, Cat# 320102, 1:50). All sections were counterstained with hematoxylin, dehydrated, and mounted. All sections were processed with peroxidase-conjugated avidin/biotin and 3'-3-diaminobenzidine (DAB) substrate (Leica Microsystem) and slides were scanned and digitalized using the scanscope system from Scanscope XT, Aperio/Leica Technologies.

Quantitative analysis of IHC staining was conducted using the image analysis software ImageScope-Aperio/Leica. Five random areas (1 mm^2 each) were selected using a customized algorithm for each marker in order to determine the number of positive cells at high power field (HPF). The data is expressed as a density (total number of positive cells/mm^2 area). IHC staining was interpreted in conjunction with H&E stained sections.

Tertiary lymphoid structure quantification

Tertiary lymphoid structures (TLS) were qualified and quantified using both H&E and CD20 IHC staining. Structures were identified as aggregates of lymphocytes having histologic features with analogous structures to that of lymphoid tissue with follicles, appearing in the tumor area[52-55]. For the current study, criteria used for the quantification of TLS includes: 1) the total number of structures identified either within the tumoral area or in direct contact with the tumoral cells on the margin of the tumors (numbers of TLS / mm^2 area); and 2) a normalization of the total area occupied by the TLNs in relation of the total area of the tumor analyzed (ratio: area of TLS / area tumor + TLNs).

Multiplex immunofluorescence assay and analysis

For Images shown in Figure 2, Extended Data Fig. 7 and 10. For IF multiplex staining, we followed the staining method for the following markers: CD20 (Dako, cat# M0755, 1:500) with subsequent visualization using fluorescein Cy3 (1:50); CD21 (Novocastra, NCL-L-CD21-2G9, 1:10) with subsequent visualization using fluorescein Cy5 (1:50); CD4 (CM153BK, Biocare, 1:25) with subsequent visualization using fluorescein Cy5.5 (1:50); CD8 (1:200, M7103, Dako) with subsequent visualization using fluorescein Cy3.5 (1:50); FoxP3 (Biolegend, Cat# 320102, 1:50) with subsequent visualization using fluorescein FITC (1:50) and nuclei visualized with DAPI (1:2000). All of the sections were cover-slipped using Vectashield Hardset 895 mounting media.

The slides were scanned using the Vectra slide scanner (PerkinElmer). For each marker, the mean fluorescent intensity per case was then determined as a base point from which positive calls could be established. For multispectral analysis, each of the individually stained sections was utilized to establish the spectral library of the fluorophores. Five random areas on each sample were analyzed blindly by a pathologist at 20X magnification.

For additional multiplex images shown in Extended Data Figure 8. For additional multiplex staining, we followed similar methods to the above for the following markers: MECA79-Dy550 (Novus, MECA-79, 1:100); CD20-Dy594 (Novus, IGEL/773; 1:100); CD4-AF647 (abcam, ERP6855, 1:100,); and nuclei visualized with Syto13 at 500 nM. The slides were scanned with the GeoMx DSP machine as described below.

GeoMx Digital Spatial Profiling

Microscope and fluidics system overview. For immune profiling of T cells located within and outside TLS structures in patient samples, the GeoMx Digital Spatial Profiler (NanoString, Seattle WA), a custom-built high-speed automated system and integrated instrument software, was utilized. A multiplexed cocktail of primary antibodies with UV photocleavable indexing oligos and 4 fluorescent markers was applied to a slide-mounted FFPE tissue section. For the fluorescent markers, we utilized Syto13 at 500uM for nuclei visualization; CD20-Dy594 (Novus,

IGEL/773; 1:100); CD3-AF647 (Novus, C3e/1308; 1:100); and PMEL-Dy550 (Novus, HMB45; 1:100) with S100B-Dy550 (Novus, 15F4NB; 1:100). 20x images were assembled to yield a high-resolution image of the tissue area of interest. The specific regions of interest (ROIs) for molecular profiling were then selected based on location (TLS or non-TLS areas of tumor) and CD3-positive staining and sequentially processed by the microscope automation. ROIs was selectively illuminated with UV light to release the indexing oligos by coupling UV LED light with a double digital mirror device (DDMD) module. Following each UV illumination cycle, the eluent was collected from the local region via microcapillary aspiration and transferred to an individual well of a microtiter plate. Once all ROIs were processed, pools of released indexing oligos were hybridized to NanoString optical barcodes for digital counting and subsequently analyzed with an nCounter Analysis System.

nCounter hybridization assay for photocleaved oligo counting. Hybridization of cleaved indexing oligos to fluorescent barcodes was performed using the nCounter Protein PlexSet reagents based on manufacturer's directions. Hybridizations were performed at 65°C overnight in a thermocycler. After hybridization, samples were processed using the nCounter Prep Station and Digital Analyzer as per manufacturer instructions.

B-Cell Clonotype Analyses

The modified TRUST algorithm[56] was applied to extract the B-cell immunoglobin hypervariable regions from the bulk RNA-seq data and assembly the complementarity-determining region 3 (CDR3) sequences of the B-cell heavy chain (IgH) and light chain (IgL). BCR clonotypes were identified and the clonal fraction was automatically calculated by TRUST. The output of TRUST was parsed by the R package tcR (version 3.4.1)[57] for downstream analyses. Only in-frame productive clonotypes were taken into subsequent analysis. The total number of BCR clonotypes detected per sample was normalized by the corresponding sequencing depth of each individual sample and calculated as per 100 million mapped reads. The top 5 clonotypes were selected by

their clonal expression abundance. The BCR repertoire diversity was calculated by entropy from the tcR package[57].

Single-cell sequencing and analysis of CD45+ B-cells

Fresh isolated tumor samples were dissociated using the human tumor dissociation kit (Miltenyi Biotec; 130-095-929), sorted into 96 well plates containing 10µl of TCL buffer (Qiagen) with 1% β-mercaptoethanol, using the following anti-human antibodies: FcX (Biolegend, 422302), CD45-PE (Biolegend, 304008), CD3-APC (Biolegend, 300412), CD235a-APC/Cy7 (Biolegend, 349116) and HLA-A,B,C-FITC (Biolegend, 311426). Sorting of viable cells was performed using the live/ dead dye Zombie Violet (Biolegend, 77477). Single-cell libraries were generated using a modified version of the full length Smart-seq2 protocol a previously described[58], and were sequenced on a NextSeq 500 sequencer (Illumina), resulting in a median of ~1.4 million paired-end reads and a median of 2588 genes detected per cell. A cutoff of $\log_2(\text{TPM}+1) \geq 2$ was used to define a gene as expressed in each single cell. For each sample we computed the fraction of B-cells using pre-defined markers (*CD19* and/or *MS4A1*). Notably, this is a is a plate based protocol; thus, for each patient, we collected and sequenced the same number of cells (n=384 CD45+ cells per plate). Thus, the number of cells per patient is equal, and the frequency reflects patients with either high or low B-cell infiltrate.

Unsupervised clustering of immune cells. To cluster all cells that passed QC we applied the *k*-means algorithm with a correlation distance metric, testing $k = 3, \ldots, 15$. The algorithm was applied using all genes with variance >6, yielding ~4000 genes. This value was selected based on the relation between the variance and the fraction of cells expressing each gene. To determine the optimal number of clusters we applied the following steps: (1) We first examined how much of the complexity each cluster captures by applying the elbow method. This was done by computing the Pearson correlation matrix R and the distance matrix D as $(1 - R)$. We then computed the sum of pair-wise distances between all cells in different clusters $Dis_b = \sum_{l=1}^{k} (\sum_{i \in C_l, j \notin C_l} D(i,j))$ and the total distance $Dis_t = \sum_{i,j} D(i,j)$. The ratio

between these two measures $V = Dis_b/Dis_t$ was used to estimate the variance explained by a given solution, such that in the extreme case where all cells are clustered together or the case where each cell is a single cluster, this ratio would be 0 and 1, respectively. Exploring this ratio, we then select the solutions that are near plateau ($k = 10, \ldots, 15$). (2) We then performed differential expression analysis (see below) to search for gene markers that are significantly more highly expressed in a specific cluster as compared to all other clusters. Then, in order to avoid complex solutions, we excluded solutions with clusters that have too few marker genes (<20) distinguishing between them and the rest of the cells. (3) Finally, we performed a robustness analysis and selected the clustering solution with the highest median robustness score. Specifically, to determine the robustness of each clustering solution, we performed 100 iterations in which we randomly removed 10% of the cells, and re-ran the *k*-means algorithm and checked the stability of the clustering solution. We quantified the agreement of a given solution with the original one as the number of pairs of cells that were either clustered together, or not clustered together, in both solutions, divided by the total number pairs shared between the runs. This process yielded a median robustness measure of 0.96 for the selected $k = 11$.

Differential expression analysis. In all cases, differential expression analysis was applied to all genes that had an average expression level $\log_2$(TPM+1) > 2 in either tested groups, G_1 and G_2. Then, for each gene i, we count the number of cells in G_1 and G_2 that express it with an expression level $\log_2$(TPM+1) > 2 or $\leq$ 2. We then apply Fisher's Exact test for the corresponding 2x2 table. To identify significant differences, we considered genes with a Bonferroni-corrected q-value $\leq$ 0.05 and $\log_2$(fold-change) > 0.5.

Details for Mass Cytometry (CyTOF)

Antibody conjugation. In-depth characterization of R and NR B-cells was performed using metal-tagged antibodies. Metal conjugated antibodies were purchased from Fluidigm or conjugated to unlabeled antibodies in-house. All unlabeled antibodies were purchased in carrier-

free form and conjugated with the corresponding metal tag using Maxpar X8 polymer per manufacturer's instructions (Fluidigm). Metal isotopes were acquired from Fluidigm and indium (III) chloride was acquired from Sigma-Aldrich. Antibody concentration was determined by measuring the amount of A280 protein using Nanodrop 2000 (Thermo Fisher Scientific). Conjugated antibodies were diluted using PBS-based antibody stabilizer supplemented with 0.05% sodium azide (Sigma-Aldrich) to a final concentration of 0.5 mg/ml. The list of antibodies with the corresponding metal tag isotopes is shown in table below.

TARGET	Clone	ISOTOPE	Source
CD45	HI30	^{89}Y	Fluidigm
CD80	2D10	^{115}In	Biolegend
CD138	MI15	^{141}Pr	BD Biosciences
CD19	HIB19	^{142}Nd	Fluidigm
CD5	UCHT2	^{143}Nd	Fluidigm
HLA-ABC	EMR8-5	^{144}Nd	BD Biosciences
CD178	NOK-1	^{145}Nd	Biolegend
IgD	IA6-2	^{146}Nd	Biolegend
CD20	2H7	^{147}Sm	Fluidigm
PDL-1	29E.2A3	^{148}Nd	Fluidigm
HLA-DR	L243	^{149}Sm	Biolegend
CD25	2A3	^{150}Nd	BD Biosciences
IGM	MHM-88	^{151}Eu	Biolegend
CD95	DX2	^{152}Sm	BD Biosciences
CXCR5	RF8B2	^{153}Eu	Fluidigm
CD86	IT2.2	^{154}Sm	BD Biosciences
CD27	L128	^{155}Gd	Fluidigm
CXCR3	G025H7	^{156}Gd	Biolegend
CD10	HI10a	^{158}Gd	Fluidigm
PDL-2	24F.10C12	^{159}Tb	Biolegend
CD39	A1	^{160}Gd	Fluidigm
BAFF-R	11C1	^{161}Dy	Biolegend

CD79b	CB3.1	^{162}Dy	Fluidigm
CD1d	51.1	^{163}Dy	Biolegend
CD23	EBVCS-5	^{164}Dy	Fluidigm
CD40	5C3	^{165}Ho	Biolegend
CD24	ML5	^{166}Er	BD Biosciences
CD38	HIT2	^{167}Er	BD Bioscience
CD21	Bu32	^{168}Er	Biolegend
ICOS	C398.4A	^{169}Tb	Biolegend
CTLA-4	14D3	^{170}Er	Fluidigm
CD9	HI9a	^{171}Yb	Biolegend
CD11c	Bu15	^{172}Yb	Biolegend
CD14	HCD14	^{173}Yb	Biolegend
PD1	PD1.3.1.3	^{174}Yb	Miltenyi
CXCR4	12G5	^{175}Lu	Biolegend
CD22	HIB22	^{176}Yb	Biolegend
CD3	UCHT-1	^{194}Pt	Biolegend
Cisplatin		^{198}Pt	Fluidigm
CD16	3G8	^{209}Bi	Fluidigm

Sample preparation and acquisition. Peripheral blood mononuclear cells (PBMCs) and tumor cells were harvested and washed twice with wash buffer (0.5% bovine serum albumin (BSA) in PBS). For tumor, this included 9 R and 9 NR, and for PBMCs, 8 R and 8 NR. To determine the live population, cells were stained with cisplatin 1μM for 3 minutes. The reaction was stopped with FACS buffer (2% Fetal Bovine Serum (FBS) in PBS), and the cells were washed once with wash buffer. Cells were then incubated with 5 μl of Fc receptor blocking buffer reagent (Miltenyi) for 10 minutes at room temperature. Cells were incubated with surface antibodies at room temperature for 60 minutes, washed twice with wash buffer and stored overnight in 1ml of 1.6% paraformaldehyde (EMD Biosciences) in PBS with 125 nM iridium nucleic acid intercalator (Fluidigm). The next day, samples were washed twice with cell staining buffer, re-suspended in 1

ml of MilliQ dH2O, filtered through a 35 μm nylon mesh (cell strainer cap tubes, BD, San Jose, CA) and counted. Before analysis, samples were resuspended in MilliQ dH_2O supplemented with EQ™ four element calibration beads at a concentration of $0.5x10^5$/ml. Samples were acquired at 300 events/second on a Helios instrument (Fluidigm) using the Helios 6.5.358 acquisition software (Fluidigm).

Data analysis. Mass cytometry data were normalized based on EQ™ four element signal shift over time using Fluidigm normalization software 2. Initial data processing was performed using Flowjo version 10.2. Mass cytometry data were normalized based on EQTM four element signal shift over time using Fluidigm normalization software 2. Initially, all R and NR normalized FCS files were either concatenated or separately exported for downstream analyses. Data were processed and analyzed using Cytobank; $CD19^+$ sample 'clean-up' was performed by gating on intact ($^{191}Ir^+$ DNA stain), no beads ($^{140}Ce^-$), live ($^{198}Pt^-$), no T-cells $CD3^-$(^{194}Pt), no monocytes $CD14^-$(^{173}Yb) and $CD45^+$(^{89}Y), no NK Cells $CD16^-$(^{209}Bi), $CD19^+$ B-cells. Mass cytometry complex data were analyzed using viSNE, in combination with heat map, to identify distinct subpopulations using the following parameters: CD19(^{142}Nd), CD20(^{147}Sm), CD5(^{143}Nd), HLA-ABC(^{144}Nd), IgD(^{146}Nd), PDL-1(^{148}Nd), HLA-DR(^{149}Sm), CD25(^{150}Nd), IgM(^{151}Eu), CD95(^{152}Sm), CXCR5(^{153}Eu), CD86(^{154}Sm), CD27(^{155}Gd), CXCR3(^{156}Gd), CD10(^{158}Gd), CD39(^{160}Gd), BAFFR(^{161}Dy), CD79b(^{162}Dy), CD1d(^{163}Dy), CD23(^{164}Dy), CD40(^{165}Ho), CD24(^{166}Er), CD38(^{167}Er), CD9(^{171}Yb), CD11c(^{172}Yb), CXCR4(^{175}Lu), and CD22(^{176}Yb). Samples with less than 200 $CD45^+CD19^+$ B-cells were not utilized for downstream analyses. Percentages of different sub-populations of B-cells were measured in aggregated R and NR PBMC and tumor samples for each run; statistical analyses performed via unpaired Student's t-test.

Analysis of Peripheral Blood Exosomes

Isolation of exosomes from human plasma. Approximately 1ml of plasma per patient sample contained in a cryovial was thawed rapidly in a 37ºC water bath. The plasma was transferred into a 1.5ml Eppendorf tube and centrifuged at room temperature (RT) for 5 min at 800*g* and 10

min at 2,000*g*. The supernatant was filtered with a 0.22 µm filter (cat. #6789-1302) directly into an ultracentrifuge tube (Lot #Z80615SCA, ref. #331372). A distinct filter was used for each 500µl of plasma filtered, and each filter was subsequently cleared with 2 x 1 ml phosphate buffer saline (PBS), all of which was collected into the ultracentrifuge tube. Additional PBS was added to the ultracentrifuge tube to reach 11ml. The tubes were the ultracentrifuged at 4°C for 15 to 16 hours at 100,000*g* using a Beckman Optima XE-90 ultracentrifuge. The pellet was resuspended in 200 to 300 µl of PBS by pipetting up and down. The exosomes contained in this resuspension were stored at -80°C until further use.

Flow cytometry analyses of exosomes. Exosomes were thawed on ice. Concentration was determined using the NanoSight NS300 nanoparticle tracking analyzer according to the manufacturer's directions, and 15 µl of exosomes (which was equivalent to approximately 4 x 10^9 particles on average) were mixed with 30µl of pre-washed anti-human CD63-coated Dynabeads® (Invitrogen, 10606D). For one sample, the Nanosight measurement was erroneous and was excluded. All samples were included in the flow cytometric analyses. Round bottom 2 ml tubes were used. All pre-wash and washes thereafter were performed using 0.22 µm filtered 0.1% Bovine Serum albumin (BSA) in PBS (0.1% BSA/PBS) and the samples were mixed well by pipetting up and down at each wash steps. 100µl of 0.1% BSA/PBS was added to beads + exosomes mixture for a final volume of 145 µl (15 µl of exosomes + 30 µl of Dynabeads® + 100 µl of 0.1% BSA/PBS). The samples were mixed by pipetting up and down and allowed to incubate for 4 to 16 hours at RT on a benchtop rotator. 300 µl 0.1% BSA/PBS was added to the samples and the samples were placed on a magnet (1 min incubation minimum). The supernatant was discarded and the beads (and bound exosomes) were washed once with 400 µl 0.1% BSA/PBS.

The beads (with bound exosomes) were resuspended in 400 µl of 0.1% BSA/PBS and subsequently split into 4 distinct round bottom 2 ml tubes, each containing 100 µl. To each of

these tubes, either antibodies or isotype control were added. These include: PE/Cy7 anti-human CD20 (Biolegend, cat.# 302312, clone 2H7) or isotype control PE/Cy7 mouse IgG2b (Biolegend, cat.# 400326, clone MCP-11); APC/Cy7 anti-human CD27 (Biolegend, cat.# 356424, clone M-T271) or isotype control APC/Cy7 mouse IgG1 (Biolegend, cat.# 400128, clone MOPC-21); PE/Cy7 anti-human CD9 (Biolegend, cat.# 312116, clone HI9a) or isotype control PE/Cy7 mouse IgG1 (Biolegend, cat.# 400126, clone MOPC-21); and Alexa Fluor 647 anti-human CD63 (Biolegend, cat.# 353016, clone H5C6) or isotype control Alexa Fluor 647 mouse IgG1 (Biolegend, cat.# 400130, clone MOPC-21). For each antibodies or isotype control, 0.4 µg per tube was added to each tube. The samples were allowed to incubate at RT for 1 to 3 hours, in the dark. 300µl 0.1% BSA/PBS was added to the samples and the samples were placed on a magnet (1min incubation). The supernatant was discarded and the beads (and bound exosomes) were washed once with 400 µl 0.1% BSA/PBS. The beads were visible on the magnet at each step of the procedure described above. The supernatant was discarded and the beads were resuspended in 200 µl of 0.1% BSA/PBS and transferred into flow cytometry (FC) tubes for FC analysis. The FC data were captured within 24 hours of completing the staining of the beads-exosomes samples. If not read immediately after completing the staining, the FC tubes were stored at 4°C in the dark. The data was subsequently analyzed using FlowJo. Responder vs. non-responder status was blinded until FC data capture and FlowJo analyses were completed.

For GPC1 staining, 3 tubes of beads with exosomes were processed parallelly. One tube did not receive any antibody (exosomes alone), one tube received primary antibody (1 hr) followed by secondary antibody (1 hr), and one tube received secondary antibody only (1 hr). All three tubes were processed similarly, including for a wash step after one hour (post primary antibody incubation, 300µl 0.1% BSA/PBS was added to the samples and the samples were placed on a magnet for 1min incubation, and then resuspended into 100µl of 0.1% BSA/PBS), and again another hour later (after the secondary antibody incubation), before transferred into a FC tube. All incubations were carried out at RT and covered from light, and beads were visible at each

step when placed on the magnet. Rabbit anti-human GPC1 antibody was used (Sigma, SAB2700282, 3µl per tube), and Alexa Fluor 488 conjugated goat anti-rabbit IgG (Invitrogen, A-11008, 2µl per tube) were used. The samples were analyzed by FC and positive signal was gated on the secondary only-paired sample.

Nanoimager analyses. Beads with exosomes stained for FC analysis for CD63 (Alexa Fluor 647 anti-human CD63) or isotype control described above (Flow cytometry analyses of exosomes) were evaluated by using the on the Nanoimager S Mark I from ONI (Oxford Nanoimaging) with the lasers 405nm/150mW, 488nm/200mW, 561nm/300mW, 640nm/1W and dual emission channels split at 560nm. Data was processed on NimOS (Version [1.25]) from ONI. Briefly, 25 µl of sample was spotted onto a slide (Fisher Scientific, 12-550-15), covered with a 1.5H coverslip (Zeiss, 474030-9000), and immediately placed on the stage. All images were captured using HILO mode (highly inclined and laminated optical sheet) at an illumination angle of 35.0 degrees with a 10.0 ms exposure setting for 200 frames. To minimize photobleaching, the focal plane of the beads was found under the 405 nm laser at 37% power, then switched to the 640 nm laser at 25% power for image acquisition.

Electron microscopy analyses. Bead only and beads with exosomes were prepared as described above (Flow cytometry analyses of exosomes). The samples were magnetized and resuspended in 50µl of 1% glutaraldehyde/PBS at 4ºC, or in 30µl of 0.1% BSA/PBS, and mixed with 30µl of warm (60ºC) 1% agarose in distilled water. The agarose-bead mixture was allowed to cool on ice, and the gels were cut into ~1mm^3 pieces and placed in 1% glutaraldehyde/PBS at 4ºC. Fixed samples were washed in 0.1 M sodium cacodylate buffer and treated with 0.1% Millipore-filtered cacodylate buffered tannic acid, postfixed with 1% buffered osmium, and stained en bloc with 1% Millipore-filtered uranyl acetate. The samples were dehydrated in increasing concentrations of ethanol, infiltrated, and embedded in LX-112 medium. The samples were polymerized in a 60ºC oven for approximately 3 days. Ultrathin sections were cut in a Leica

Ultracut microtome (Leica, Deerfield, IL), stained with uranyl acetate and lead citrate in a Leica EM Stainer, and examined in a JEM 1010 transmission electron microscope (JEOL, USA, Inc., Peabody, MA) at an accelerating voltage of 80 kV. Digital images were obtained using AMT Imaging System (Advanced Microscopy Techniques Corp, Danvers, MA).

Statistical analyses

Once unblinded and plotted into GraphPad prism, the samples were evaluated for statistically significant differences. The Kruskall-Wallis test or a two-sided Mann-Whitney test was used to determine significance as defined in the figure legend.

Data Availability:

The authors declare that the data supporting the findings of this study are available within the paper and its supplementary information files.

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