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Immune profiling of human tumors identifies CD73 as a combinatorial target in glioblastoma

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Authors Contributions

Supervision and Study Oversight by P.S. Funding Acquisition, P.S.; Experiments were performed by S.G., S.A., M.O., L.Y.K. Writing, Review & Editing, P.S., S.G., T.W., A.E.C., S.B.; Data analysis and interpretation were performed by P.S., S.G., T.W., A.E.C., S.B., S.A., I.F., L.V., J.B., H.Z., D.P., S.S., J.A.P. Patient samples were provided by A.B.H., J.d.G., B.S., M.O., S.K., P.S.

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Competing Interests Statement

P.S. has ownership in Jounce, Neon, Constellation, Oncolytics, BioAtla, Forty-Seven, Apricity, Polaris, Marker Therapeutics, Codiak, ImaginAb, Dragonfly, Lytx and Hummingbird. P.S. serves as a consultant for Constellation, Jounce, Neon, BioAtla, Pieris Pharmaceuticals, Oncolytics Biotech, Forty-Seven, Polaris, Apricity, Marker Therapeutics, Codiak, ImaginAb, Dragonfly, Lytx and Hummingbird.

Data Availability Statement:

CytoTOF data (Figure. 1, 2A–B, 3C–G and 4C–D) are deposited to FlowRepository and the Repository ID is FR-FCM-Z2B3. Single cell RNAseq data (Figure. 2C–E, 3A) are deposited in the Sequence Read Archive (SRA) with accession number PRJNA588461. All requests for data and materials are available from the corresponding author, following verification of any intellectual property or confidentiality obligations.

Further information on research design is available in the [Nature Research Reporting Summary](#) linked to this article

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Abstract

Immune checkpoint therapy (ICT) with anti-CTLA-4 and anti-PD-1/PD-L1 has revolutionized the treatment of many solid tumors. However, the clinical efficacy of ICT is limited to a subset of patients with specific tumor types^{1,2}. Multiple clinical trials with combinatorial immune checkpoint strategies are ongoing, however, the mechanistic rationale for tumor specific targeting of immune checkpoints remains elusive. To garner insight into tumor specific immunomodulatory targets, we analyzed tumors (N=94) representing 5 different cancer types, including those that respond relatively well to ICT and those that do not, such as glioblastoma (GBM), prostate cancer (PCa) and colorectal cancer (CRC). Through mass cytometry and single cell RNA-sequencing, we identified a unique population of CD73^{hi} macrophages in GBM that persists after anti-PD-1 treatment. To test if targeting CD73 would be important for a successful combination strategy in GBM, we performed reverse translational studies using CD73^{-/-} mice. We found that the absence of CD73 improved survival in a murine model of GBM treated with anti-CTLA-4 and anti-PD-1. Our data identified CD73 as a specific immunotherapeutic target to improve anti-tumor immune responses to ICT in GBM, and demonstrate that comprehensive human and reverse translational studies can be used for rational design of combinatorial immune checkpoint strategies.

ICT provides durable anti-tumor response to a subset of patients with specific tumor type³⁻⁹. Independent studies have recently provided in-depth single-cell analyses of tumor infiltrating leukocytes (TILs) from individual tumors namely renal cell carcinoma (RCC), hepatocellular carcinoma (HCC), Non-Small Cell Lung Carcinoma (NSCLC) and melanoma¹⁰⁻¹³. These studies bring new insights and validate prior findings on the immune infiltrates of different cancers, but the non-uniformity of response amongst cancer types may be a result of tumor type-specific immune checkpoint expression patterns and demands a comprehensive comparison of the TIL phenotypes across multiple tumors. To address this need, we applied mass cytometry (CyTOF) to profile immune cell subsets in 85 patients with 5 different tumor types: NSCLC (n=15), RCC (n=25), MSI stable Colorectal Cancer (CRC) (n=11), Prostate Cancer (PCa) (n=21) as well as Glioblastoma Multiforme (GBM) (n=13) (Supplementary Table 1). This is the first CyTOF dataset evaluating immune cell subsets across different human tumor types.

We first compared the major immune infiltrates present in each tumor type (Extended Data Fig. 1). We observed that NSCLC, RCC and CRC tumors were CD3⁺ T cell rich with CD4⁺FoxP3⁺ cells being most frequent in CRC (Figure 1A). While both PCa and GBM were poorly infiltrated by CD3⁺ T cells, GBM had higher abundance of CD68⁺ myeloid cells (Figure 1A). To identify shared phenotypes across the different tumor types, we performed PhenoGraph clustering of CD45⁺ cells that identified 18 meta-clusters (L1–18), with 8 CD3⁺ T cell meta-clusters and 10 CD3⁻ meta-clusters, including 6 CD68⁺ myeloid

clusters and 1 NK cell meta-cluster (Figure 1B and Extended Data Fig. 2A–B). We identified a group of 6 immune meta-clusters which were present in all 5 tumor types. These clusters displayed a high Shannon entropy which is a measure of higher uniformity in their distribution across tumor types. We also identified 8 immune meta-clusters that displayed low Shannon entropy values, indicating tumor specific distribution (Figure 1C).

Analysis of the frequency of different T cell clusters identified $CD3^+ CD4^+ PD-1^{hi}$ and $CD3^+ CD8^+ PD-1^{hi}$ meta-clusters (**L3** & **L6** respectively) in NSCLC, RCC and CRC (Figure 1D & Extended Data Fig. 2 C–D). Upon analysis of PBMC samples from the RCC cohort, we identified T cell subsets (**P33** and **P24**) which correlated with **L3** and **L6** clusters respectively. Interestingly, **P33** and **P24** clusters were found to be expanded in responders compared to non-responders to ICT (Extended Data Fig. 3A–C). We also noted higher abundance of $CD4^+ FoxP3^{hi}$ regulatory T cells (**L12**) and $CD8^+ VISTA^+$ (**L14**) cells in CRC and PCa respectively (Figure 1D, Extended Data Fig. 2D), which could be contributing to the lack of response to ICT^{14,15}. PhenoGraph clustering of all CD3-gated cells from 30 samples across 3 T cell infiltrated tumor types (NSCLC, RCC and CRC) revealed 17 meta-clusters (Extended Data Fig. 4A–B). We performed hierarchical clustering of all of these 30 patient samples based on their T cell meta-cluster frequencies and identified 3 primary sub-groups (**I**, **II**, and **III**) (Figure 1E). A higher frequency of T cell meta-clusters **T1** ($PD-1^{hi} ICOS^+ CD4^+$ T cell like **L3**) and **T4** ($PD-1^{hi} CD8^+$ T cell like **L6**) were observed in sub-group **II**, which predominantly comprised NSCLC and RCC, two tumor types that respond favorably to ICT (Figure 1F). Sub-group **III** included higher frequencies of meta-clusters **T2** ($CD4^+$ T cell) and **T3** ($CD8^+$ T cell), which were low in checkpoint-receptor expression, while sub-group **I** showed intermediate frequencies of different T cell subsets with both high expression and low expression of immune checkpoints (Extended Data Fig. 4C).

Next, we performed in-depth analysis of the $CD3^- CD68^+$ myeloid clusters identified from the PhenoGraph clustering of $CD45^+$ cells across the different tumor types. We observed 2 $PD-L1^-$ subset (**L5** and **L17**) and 2 $PD-L1^+$ subsets (**L1** and **L8**) across tumor types (Figure 2A & Extended Data Fig. 5A). **L5** was identified as a $VISTA^+$ subset and was present at a higher frequency in CRC as compared to NSCLC and PCa. **L17** was also identified as a $VISTA^+$ subset but was only found in CRC. **L1** was identified as myeloid subset shared by all tumor types.

Meta-cluster **L8** was a unique subset found only in GBM (this was further validated by manual gating) (Figure 2A & Extended Data Fig. 5A–C). **L8** expressed high levels of CD73 in addition to other co-inhibitory molecules such as VISTA and PD-1 (Extended Data Fig. 5D). IHC and IF studies further revealed that human GBM tumors have high density of $CD68^+$ macrophages that co-express CD73 (Extended Data Fig. 5E–H). To demonstrate the validity of our findings on leukocyte infiltration in GBM we analyzed macrophage and T cell infiltration by CyTOF in an independent cohort of 9 GBM patients (Extended Data Fig. 6). As compared to our first GBM cohort, we found similar high frequencies of $CD73^{hi}$ macrophages and low T cell numbers.

CD73 is an ectonucleotidase which works with its upstream signaling molecule CD39 to convert extracellular ATP to adenosine¹⁶. CD73 has been shown to promote tumor progression and induce immune suppression in GBM^{16–20}. Further, it was recently shown that kynurenine produced by murine GBM cells can upregulate CD39 in macrophages¹⁹. To obtain a deeper understanding of genes that may define CD73^{hi} myeloid cells we performed single cell RNA sequencing (sc-RNA seq) on 4 additional GBM tumors (Supplementary Table 1). This analysis revealed 17 clusters, of which 4 were CD3⁺ T cell clusters and 10 were CD3[−]CD68⁺ myeloid cell clusters. Of the 10 myeloid clusters, 4 were CD73^{hi} (R7, R14, R3 and R17) (Figure 2B, indicated by arrows). We found that CD73^{hi} myeloid clusters had high expression of genes suggestive of a blood derived macrophage signature as opposed to microglial signature²¹ (Figure 2C). CD73^{hi} macrophages were also found to express *CCR5*, *CCR2*, *ITGAV/ITGB5* and *CSF1R* suggesting that CD73^{hi} macrophages are probably recruited to the GBM tumor microenvironment by these factors^{22–26} (Figure 2D). We also evaluated the CD73^{hi} myeloid cells for expression of immunostimulatory genes or immunosuppressive genes and found that CD73^{hi} myeloid cells had high expression of immunosuppressive and hypoxia related genes (Figure 2E).

Next, we derived a gene signature specific for CD73^{hi} macrophages using 4 CD73^{hi} clusters (R7, R14, R3 and R17) (Figure 3; see methods). *MARCO*, *TGFB* and several *SIGLECs* were found to be expressed in the CD73^{hi} cells (Figure 3A). To understand the significance of the gene signature, we evaluated the CD73^{hi} gene signature for potential correlation with survival. To perform this analysis we used the TCGA-GBM cohort (N=525). We found a significant negative correlation between overall survival (OS) and high expression of the CD73^{hi} gene signature (Figure 3B, $p=0.013$, $HR=1.268$) in TCGA-GBM cohort. Based on the potential immune-suppressive function of CD73^{hi} myeloid cells, we evaluated GBM samples from patients treated with anti-PD-1 to determine whether prevalence of these cells may correlate with lack of response to therapy. We used a cohort of 5 patients with GBM who were enrolled on a phase II study assessing the effect of pembrolizumab in patients with recurrent GBM ([NCT02337686](#), Methods). PhenoGraph clustering of 7 untreated tumors and the cohort of 5 patients with GBM treated with pembrolizumab revealed 17 clusters, consisting of 12 subsets that were characterized as CD3[−]CD68⁺ myeloid subsets, 2 CD3⁺ T cell subsets and 1 NK cell CD3[−]CD56⁺ subset (Figure 3C–D; Extended Data Fig 7). Out of the 12 CD68⁺ myeloid subsets, there were 3 CD73^{hi} myeloid clusters (Figure 3D; **G2**, **G8**, **G11** indicated by red arrows). Upon comparison of untreated with anti-PD-1 treated GBM samples, we found that these 3 CD73^{hi} myeloid clusters persisted despite treatment with ICT (Figure 3E). Evaluation of the remaining myeloid-like clusters that were CD73 low or CD73 negative also persisted despite treatment with ICT, which is consistent with results from a previous study in which there was no change in the myeloid cell markers following anti-PD-1 treatment²⁷. Of note, 2 T cell clusters were identified (Figure 3D; **G3**, **G6**, indicated by blue arrow), representing CD4 and CD8 respectively, which did not demonstrate any significant difference between untreated and anti-PD-1 treated GBM tumors (Figure 3F). GSEA analysis from the untreated and anti-PD-1 treated tumors revealed higher expression of IFN- γ responsive genes in anti-PD-1 treated patients (Figure 3G), in accordance with a recent study which suggested moderate clinical benefit of anti-PD-1 treatment in a neoadjuvant setting²⁸. Our findings suggest that anti-PD-1, despite

possibly inducing modest immunological responses in TIL, does not profoundly change the GBM TME, which is characterized by its high content of CD73^{hi} myeloid cells. It is possible that the prevalence of the CD73^{hi} myeloid cells contributed to the lack of T cell infiltration thereby leading to poor clinical outcome.

To test our hypothesis that targeting CD73 would be important for a successful combination strategy in GBM, we performed reverse translational studies using wild-type (WT) and CD73^{-/-} mice orthotopically inoculated with GL-261 GBM tumor cells. In the absence of CD73, intracranial tumor growth was impeded (Extended Data Fig. 8A) and mice exhibited improved survival, confirming the immunosuppressive role of CD73 in GBM (p=0.01) (Extended Data Fig. 8B). To understand the effect of CD73 in the tumor microenvironment, we performed comparative immune profiling of the tumor microenvironment and assessed the differences in immune infiltrates between the WT and CD73^{-/-} mice using CyTOF (Extended Data Fig. 8C). Although, the absence of CD73 has been shown to increase intra-tumoral T cell abundance in murine tumor models such as B16-F10 melanoma and MC-38 colon cancer²⁹, clustering of CD45⁺ gated cells did not reveal significant changes in the T cell subsets between WT and CD73^{-/-} GBM tumor bearing mice. In our GBM model, we noted differences in the myeloid (CD11b⁺F4/80⁺) subsets, including a decrease in the immunosuppressive CD206⁺Arg1⁺VISTA⁺PD-1⁺CD115⁺ myeloid cluster (**Gmm20**, p=0.0079) in the CD73^{-/-} mice as compared to WT mice (Extended Data Fig. 8D). Interestingly, we also observed a concomitant increase in iNOS⁺ myeloid clusters (**Gmm13**, p=0.0159) in the CD73^{-/-} mice (Extended Data Fig. 8D–E) as compared to the WT mice. This data support the role of CD73 in macrophage polarization. Overall, our data indicate that absence of CD73 in the murine GBM tumor model improves survival by modulating the intra-tumoral myeloid subsets.

Next, we assessed if CD73-mediated changes in macrophage phenotype could impact the efficacy of ICT. We treated GBM-tumor bearing mice with anti-PD-1 antibody or with a combination of anti-PD-1 and anti-CTLA-4 antibodies. Figure 4A shows representative MRI images of the GBM tumors from untreated and ICT treated mice. Significant improvement in survival was noted in WT and CD73^{-/-} mice treated with a combination of anti-PD-1 plus anti-CTLA-4 compared to untreated controls (p<0.0001) (Figure 4B). Importantly, following treatment with combination of anti-PD-1 and anti-CTLA-4, CD73^{-/-} mice had improved survival as compared to WT GBM tumor bearing mice (p=0.03, Figure 4B). We did not find any significant survival benefit from anti-PD-1 treatment in WT and CD73^{-/-} mice. (Figure 4B). We noted that the ratio of iNOS⁺ immune stimulatory macrophages to CD206⁺ immune suppressive macrophages was significantly higher in CD73^{-/-} mice compared to WT mice. This was more evident in tumor bearing mice treated with combination therapy. Similarly, the ratio of the granzymeB⁺ CD8 T cells to the CD206⁺ immune suppressive macrophages was significantly higher in the CD73^{-/-} mice compared to WT, and is further pronounced following combination therapy (Figure 4 C–D). Our data thus suggest that increased T cell infiltration using combination ICT, coupled with polarization of macrophages to an immune stimulatory phenotype in CD73^{-/-} mice, play a critical part in determining response to ICT.

Multiple immune checkpoints exist^{30–32}, however, our data suggest dynamic interaction of immune checkpoints in the tumor microenvironment is specific to each tumor type. Clinical trials with combination immunotherapy are ongoing at an unprecedented rate; however, a comprehensive understanding of the tumor-immune interactions are still limited, to design rational combination therapy in a tumor-specific manner. Our study coupled in-depth human tumor analyses with murine reverse translational studies to generate a combination strategy for a future clinical trial in GBM. Overall, our study highlights that reverse translational studies are critical to test the relevant hypotheses generated from human datasets for precision immunotherapy.

In our study, we provided immune profiling data from 1) multiple different human tumors and 2) an anti-PD-1 clinical trial in patients with GBM. We identified CD73^{hi} myeloid population to be specifically present in GBM that persisted even after treatment with anti-PD-1 therapy. Further, we derived a gene signature from the CD73^{hi} myeloid cell clusters that negatively correlated with OS in TCGA-GBM cohort. scRNA sequencing showed that CD73^{hi} myeloid cells are enriched in immune-suppressive genes and have a signature distinct from the resident microglial signature. CD73^{hi} myeloid cells are further characterized by higher expression of chemokines/chemokine receptors such as *CCR5*, *CCR2*, *ITGAV/ITGB5* and *CSF1R*. Although several clinical trials are testing the utility of targeting these individual chemokine receptors in patients with advanced solid tumors including GBM, cumulative expression of these receptors in CD73^{hi} myeloid cells suggest that CD73 itself is a more relevant target as it is highly expressed on the majority of cells expressing all of these receptors. For example, clinical trials targeting CSF1R have demonstrated limited clinical efficacy^{33,34}, which may be due to ongoing presence of myeloid populations expressing other immunosuppressive markers.

Our data demonstrate the persistence of an immunosuppressive CD73^{hi} myeloid subsets in patients with GBM who received anti-PD-1 therapy and the therapeutic benefit of immune checkpoint inhibitors in a CD73 deficient mouse model. Based on our data and earlier studies, we propose a combination therapy strategy to target CD73 plus dual blockade of PD-1 and CTLA-4. Anti-CD73 antibody has yielded promising results in preclinical as well as early clinical studies^{35,36}, therefore our data have clinical applications with rapid translation of combination therapy for GBM with currently available anti-CD73 antibodies.

Methods:

Patients and surgical samples

Patients' samples were collected after appropriate informed consent was obtained on MD Anderson IRB-approved protocol PA13–0291. Patients with relapsed glioblastoma multiforme were treated with pembrolizumab every 3 weeks on MDACC clinical protocol 2014–0820 (NCT02337686) and consented for PA13–0291. Clinical characteristics of individual patients are indicated in Supplementary Table 1.

Mice

C57Bl/6 (5–7 weeks) mice were purchased from the National Cancer Institute (Frederick, MD) and CD73 knockout (CD73^{-/-}) mice in the C57BL/6 background (stock no. 018986, 5–7 weeks) were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were kept in specific pathogen-free conditions in the Animal Resource Center at The University of Texas MD Anderson Cancer Center. Animal protocols were approved by the Institutional Animal Care and Use Committee of The University of Texas MD Anderson Cancer Center.

Cell lines and tumor model

Murine Glioblastoma cancer cell line (GL-261) were obtained from the National Cancer Institute (Rockville, MD, USA). Cells were collected in the logarithmic phase and washed twice with PBS just before tumor injections. 50,000 cells were injected intracerebrally in the mice (5 or 10 mice per group) as described previously³⁷. Anti-CTLA-4 (clone 9H10) and anti-PD-1 (RMP1–14) antibodies were purchased from BioXcell (West Lebanon, NH). Mice were injected intraperitoneally with anti-PD-1 and combination of anti-PD-1 plus anti-CTLA-4 on day 7 (200 µg/mouse), day 10 (100 µg/mouse) and day 13 (100 µg/mouse) post tumor inoculation.

Mass cytometry (CyTOF)

Patient PBMC were isolated from blood by density gradient centrifugation, resuspended in 90% AB serum and 10% DMSO and stored in liquid nitrogen until the analysis. Fresh tumor tissue was dissociated with GentleMACS system (Miltenyi Biotec; Bergisch Gladbach, Germany) as per the manufacturer's instructions and cultured overnight in a 96 well plate with RPMI 1640 medium; supplemented with 10% human AB Serum, 10 mM Hepes, 50 µM β-ME, penicillin/streptomycin/l-glutamine. For mouse experiments, freshly collected tumors were dissociated with Liberase/DNAse solution, incubated for 30 minutes at 37°C prior to single cell being made. Cells were stained with up to 36 antibodies. Antibodies were either purchased pre-conjugated from Fluidigm or purchased purified and conjugated in house using MaxPar X8 Polymer kits (Fluidigm) according to the manufacturer's instructions (See Supplementary Table 4). Briefly, samples were stained with cell-surface antibodies in phosphate-buffered saline (PBS) containing 5% goat serum and 30% BSA for 30 minutes at 4°C. Optimal antibody concentrations were determined by serial dilution stainings of human PBMCs. After viability staining with 5µM cisplatin (Fluidigm) in PBS containing 30% BSA, samples were washed in PBS containing 30% BSA, fixed and permeabilized according to manufacturers' instructions using the FoxP3 staining buffer set (eBioscience) before being incubated with intracellular antibodies in permeabilization buffer for 30min at 4°C. Samples were washed and incubated in Ir intercalator (Fluidigm) and stored at 4°C until acquisition, generally within 12 hours. Right before acquisition samples were washed and re-suspended in water containing EQ 4 element beads (Fluidigm). Samples were acquired on a Helios mass cytometer (Fluidigm).

Mass Cytometry Analysis

Four separate cohorts of human patient samples were analyzed using CyTOF (after removing samples with too few cells for analysis, as explained for different data sets

individually below): 1) 66 samples from TILs extracted from 5 different tumor types; 2) 5 additional GBM TIL samples extracted from tumors resected from patients after treatment with Pembrolizumab; 3) A validation cohort of 9 additional immune checkpoint therapy naïve GBM TIL samples; and 4) 14 Matched PBMC samples from both before and after two and/or four cycles of treatment with combination Ipilimumab and Nivolumab treatment from 14 separate RCC patients. The panels used for the multi-tumor and GBM cohorts were identical (besides one difference as to which channel was used for HLA-DR in some samples, explained below), though a separate panel was used for the RCC PBMC cohort, which was analyzed entirely separately (Supplementary Table 1). For the most part the various analyses using these different cohorts proceeded in a similar if not identical fashion; where they differed will be referenced explicitly below.

First, files (fcs) were uploaded into Cytobank and normalized using a bead-based normalization software for mass Cytometry data (R package premissa, Parker Institute for Cancer Immunotherapy)³⁸. As the RCC PBMC samples (Cohort 3 above) were labeled using mass-tag cell barcoding for each sample from a given patient, they were additionally demultiplexed using the strategy outlined in Zunder et al., 2015³⁹, prior to bead-based normalization between patients. For the initial TIL (Cohort 1) and additional post-treatment GBM (Cohort 2) samples we merged signals for the 174Yb and 209Bi isotopes into a single channel for HLA-DR, as we used an antibody to HLA-DR that was conjugated to either 174Yb or 209Bi for different samples.

Samples were then manually gated in FlowJo by event length, live/dead discrimination, and for populations of interest using lineage markers (CD45 and CD3) for separate analyses. Data were then exported into Matlab or R as fcs files for downstream analysis, and arcsinh transformed using a coefficient of 5 ($x_{\text{transformed}} = \text{arsinh}(x/5)$). Samples with less than 600 events in the final gate (e.g. CD45⁺ cells or CD3⁺ cells) were excluded due to insufficient cells for clustering, dimension reduction, and other analyses. In the case of the GBM-specific TIL analysis, 4300 cells (chosen as it was the smallest number of viable, post-gating cells from all but one of the samples) were randomly selected from each of 11 samples; as file 1814 contained 1170 cells it was not subsampled and all 1170 cells were included in analysis.

To visualize the high-dimensional data in two dimensions, the t-Distributed Stochastic Neighbor Embedding (t-SNE) dimension reduction algorithm⁴⁰ was applied to the analyses of the multi-tumor TIL samples and separately to the analysis of the 12 total GBM samples (including also 5 post-treatment samples along with the 7 initial samples). For the multi-tumor samples, 10,000 cells were randomly selected from each tumor type, using all markers besides CD326 (EPCAM) and those used to manually gate the population of interest (e.g. CD45 and CD3). For the GBM TIL analysis, subsampling was done as explained above. All t-SNE maps were produced using the Barnes-Hut implementation of the algorithm in the R package Rtsne, and data was displayed using the ggplot2 R package (). For t-SNE plots overlaid with expression of individual markers, the arcsinh transformed signal intensity for all values was divided by the 99th percentile of intensity for that channel, leading to signal intensities ranging between 0 and 1 for each channel.

For the murine CyTOF samples, both pre-processing and normalization was done identically (though with an entirely separate murine panel). Clustering and other downstream analysis was done in a different manner, explained below.

Mass cytometry clustering

Clustering analysis was performed using the MATLAB implementation of the PhenoGraph clustering algorithm⁴¹. For the clustering analysis of the multi-tumor samples (Cohort 1), to reduce noise from batch and other effects as well as compress marker redundancies, data from each individual patient were projected onto principal components accounting for 90% of observed variance prior to clustering, using all markers besides CD326 (EPCAM) and those used to manually gate the population of interest (CD45 and CD3, respectively, as well as CD68 for separate T Cell analysis as it was used as a negative gate). This approach was employed to avoid capturing physiologically irrelevant populations as well as reduce residual noise not accounted for by bead normalization. Clusters were identified using PhenoGraph on a per sample basis in the space formed by these principal components, with the parameter k for the number of nearest neighbors selected uniquely for each sample using the formula $k = \text{minimum}(0.002 * \text{number of cells}, 10)$. For each individual sample, pan-positive (expressing high levels of all markers, i.e. likely doublets) and pan-negative (expressing no markers) clusters were excluded from downstream meta-clustering and frequency analyses due to being likely artifacts; they accounted for less than 0.4% of each parent population.

For the murine CyTOF data, normalized data was clustered using the FlowSOM clustering method via Cytobank⁴².

To compare phenotypes across samples while accounting for batch effects, clusters from each sample were represented by their centroid across all non-discarded channels and merged into a single matrix, of size 794 clusters (across 45 samples) by 34 markers for the CD45⁺ TIL analysis, and 486 clusters (across 30 samples) by 32 markers for the CD3⁺ TIL analysis. PhenoGraph was run with parameter $k = 10$ on both of these matrices individually, resulting in 18 meta-clusters in the CD45⁺ analysis, and 17 meta-clusters in the CD3⁺ analysis.

To find tumor type agnostic immune landscape across tumor types, the frequency of cells belonging to each meta-cluster was calculated for each sample and each tumor type in the multi-tumor TIL analysis. Samples were hierarchically clustered by their meta-cluster frequencies using hierarchical clustering with Ward's method and visualized with dendrograms.

In the case of the RCC PBMC analysis (**Figure S3**), barcoding reduced the need for an initial sample-specific clustering step followed by meta-clustering; consequently, all cells from all pre and post-treatment samples (without subsampling, resulting in over 1 million total cells) were clustered together. In the case of the clustering analysis of the 12 pre or post-treatment GBM samples (Figure 3A), clustering was also performed on cells (subsampling identically as in the tSNE section above, with 4300 cells from each samples besides 1170 cells from sample 1814) from all samples together, as the number of clusters

obtained from each individual patient in this smaller set (~200 total) did not allow for stable and robust downstream meta-clustering. This may lead to mildly increased batch effects in this particular analysis, which should be accordingly taken into account in interpretation. In this analysis one small pan-positive cluster of 147 cells (0.3% of the total) was also excluded from downstream analysis. All 9 samples in the GBM validation cohort (Cohort 3) were also clustered together. In all of these analyses PCA pre-processing was done as above.

For heatmap displays of marker expression by either cluster or meta-cluster, depending on the analysis, expression was normalized via dividing by the maximum mean cluster value for each parameter and displayed in R with a custom-made script using the `geom_tile` function in the `ggplot2` package. In all box plots, depicted boxes indicate interquartile range with central bar indicating median and whiskers range.

Statistical analysis

Metacluster and subset frequencies were compared in a two-step approach. First, Kruskal-Wallis tests were performed for the 14 metaclusters from the multi-tumor CyTOF analysis and corrected for multiple comparisons using the Benjamini and Hochberg method. L2, L4, L15 and L18 as well as T12, T13 were removed from multiple comparison corrections as they were either not expressed in the analyzed dataset, expressed by only one patient or of undefined lineage and thus not amenable for comparison. Q-values were calculated using the `p.adjust()` function (R studio Version 1.0.153) and $q < 0.05$ was considered statistically significant. Second, pairwise comparisons were only performed for metaclusters/subsets with statistically significant variation across tumor types using Mann-Whitney tests and corrected for multiple comparisons within the respective clusters using the Benjamini and Hochberg method with a $q < 0.05$ considered as statistically significant.

For calculation of ratios of the cell cluster frequencies in the murine experiments (Figure 4D), 3 CD8 T cell clusters expressing granzymeB were identified (clusters 19, 26 and 27) and their cell frequencies were added. Similarly, 4 iNOS expressing myeloid clusters (clusters 1, 2, 6 and 7) were identified and cell frequencies added. Only 1 CD206 expressing myeloid cluster was observed (cluster 5) and hence was taken individually. The cumulative frequencies of granzyme B⁺ CD8 T cell clusters and the cumulative frequencies of iNOS⁺ myeloid clusters were divided by the frequency of CD206⁺ myeloid cluster respectively and the ratios were plotted in GraphPad Prism 7 to obtain statistics. Summary of the statistical methods used for these analyses are included in Supplementary Table 2.

Cluster mixing

Mixing of the 18 CD45⁺ immune meta-clusters across the 6 tumor types (including mCRC) was estimated using a bootstrapping technique to correct for the different sizes of clusters, which ranged from just over 1,800 cells to just over 180,000 cells. Shannon entropy was computed for the empirical distribution of tumor types across 1000 cells, sampled uniformly from each cluster with replacement. This sampling procedure was repeated 1000 times per cluster in order to bootstrap cluster size-corrected standard errors of entropy. Figure 2C shows boxplots of entropy values in each cluster, ordered by mean entropy.

Immunohistochemistry

For IHC analyses, GBM tumor tissues were fixed in 10% formalin, embedded in paraffin, and transversely sectioned. Sections of 4 μm were stained with hematoxylin and eosin (H&E). IHC analyses were conducted on paraffin-embedded tissue sections. Primary antibody was used to detect CD3 (Dako, Cat#A0452), CD8 (Thermo Scientific, Cat# MS-457-S), CD68 (Dako, Cat# M0876). Antibodies were detected with secondary antibodies, followed by peroxidase-conjugated avidin/biotin and 3,3'-diaminobenzidine (DAB) substrate (Leica Microsystem). All IHC slides were scanned and digitalized using the scanscope system from Scanscope XT, Aperio/Leica Technologies. Quantitative analyses of IHC staining were conducted using the image analysis software provided (ImageScope-Aperio/Leica). Five random areas (at least 1 mm² each) were selected using a customized algorithm for each specific marker for analysis of density of positive cells (numbers of positive cells/mm²).

Multiplex immunofluorescence assay and multispectral analysis

For multiplex staining, we followed the Opal protocol staining method³⁶ for the following markers: CD73 (1:200, Abcam, ab91086) with subsequent visualization using fluorescein Cy3 (1:50); CD163 (1:25, Leica Biosystems, NCL-L-CD163) with visualization accomplished using Cy5 (1:50); and CD68 (1:100, Dako, M0876) with visualization using Cy5.5 (1:50). Nuclei were subsequently visualized with DAPI (1:2000). All of the sections were cover-slipped using Vectashield H-1400 mounting media. For multispectral analysis, a detailed methodology was followed as described previously (Stack et al., 2014). Each of the individually stained sections was utilized to establish the spectral library of fluorophores required for multispectral analysis. The slides were scanned using the Vectra slide scanner (PerkinElmer) under fluorescent conditions. For each marker, the mean fluorescent intensity per case was then determined as a base point from which positive cells could be established. Finally, the co-localization algorithm was used to determine percent of CD68, CD163 and CD73 staining.

Single-cell RNA sequencing

Single-cell RNA sequencing (sc-RNA seq) was performed using the 10x genomics chromium single cell controller. Briefly, tumor cell single cell suspensions were prepared as indicated above. Cells were resuspended in freezing media containing 90% AB serum and 10% DMSO and stored in liquid nitrogen until analysis. For sc-RNA seq analysis cells were thawed, washed and sorted for viable CD45⁺ cells using the BD FACSaria. Next, cells were droplet separated using ChromiumTM Single Cell 3' v2 Reagent Kit with the 10x genomics microfluidic system creating cDNA library with individual barcodes for individual cells. Barcoded cDNA transcripts from GBM patients were pooled and sequenced using the Illumina HiSeq 4000 Sequencing System.

Single cell RNA sequencing clustering and statistical analysis

For each of the 4 GBM sc-RNA seq samples Illumina fastq files were preprocessed and converted into count matrices using the Sequence Quality Control (SEQC) package. Briefly, SEQC takes as input Illumina barcode and genomic sequence fastq or bcl files; merges them

into a single fastq file containing alignable sequence and metadata; filters reads for common errors including barcode substitution errors and low-complexity errors; aligns reads using STAR; resolves multiple alignment reads; and groups the error-reduced and filtered reads by cell, molecule, and gene annotation into count matrices. It also outputs a series of QC metrics by which to evaluate the library quality. The pipeline is described in full detail in Azizi, et al. 2018⁴³.

These four separate count matrices were then merged into one large count matrix, of size 13,263 cells (ranging from 2,763 to 3666 cells per patient) by 19,187 genes. The data was first preprocessed in three sequential ways: first, it was normalized according to the median library- size for each cell, as is the standard practice for sc-RNA seq data; next, it was log-transformed; and finally, principal component analysis was applied to further decrease noise and maximize signal robustness while taking advantage of the redundancy inherent to gene expression (so- called “intrinsic dimensionality”), with principal components accounting for 90% of the variance retained.

Next, the median number of unique molecules (UMI) per cell was low across the four samples (1170, 1210, 1468, and 1592, respectively), resulting in a sparse data matrix, as is common to sc-RNA seq data. Thus, we used the imputation algorithm Markov affinity-based graph imputation of cells (MAGIC) to denoise the count matrix and correct for data sparsity and gene dropout. MAGIC exploits shared information across similar (“neighboring”) cells, via data diffusion, to both de-noise the count matrix and, crucially, fill in missing transcripts that are likely present but have been lost to sampling error (“dropout” or false negatives). This is particularly important in the case of interrogating gene-gene relationships, such as in the case of co-expression patterns in important cell populations. Of minor note, MAGIC also performs PCA as a pre-processing step but returns a full (non-dimension reduced) imputed count matrix; for downstream analysis (clustering, etc.) PCA pre-processing as described above was applied to this imputed count matrix. A full, detailed description of the intuition, biological and mathematical theory, and algorithmic procedure of MAGIC is provided in van Dijk et al., 2018⁴⁴. For this analysis, the R implementation of MAGIC was used, with the following parameter settings: all genes; k (number of nearest neighbors) of 10; alpha of 15; and the automatic (“t = auto”) value for the power by which the diffusion operator is powered, such that t was selected according to the Procrustes disparity of the diffused data (the value of t chosen in this manner was 8).

t-SNE visualization of the sc-RNA seq data was again performed using the reduced PCA space, applied to all cells from all four patients, using the Barnes-Hut implementation of the algorithm and signal intensities relative to maximum imputed expression of either the individual markers or the mean expression of multi-gene signatures.

Clustering of the sc-RNA seq data was performed using PhenoGraph in the reduced PCA space on all cells, with k again set to $0.002 * \text{number (cells)} = 38$. One cluster of cells, totaling less than one-half of one percent of the total, was identified that did not express any canonical immune typing markers at an appreciable frequency (CD45, CD3, CD8, CD4, CD14, CD68, etc.) It did, however, express high levels of several markers associated with neurons. Thus, we conclude that it is likely a rare contaminant that was erroneously missed

by the CD45-based sorting process, and was removed from all analyses and it was outside the scope of the immune populations that are the object of investigation in this study.

Hypoxia, anti-inflammatory (“immunosuppressive”), and pro-inflammatory (“immunostimulatory”) gene signatures were taken from Azizi, et al. 2018⁴³, while the microglial versus bone-marrow derived signatures were taken from Muller et al., 2017²¹. In all cases the intensity of expression of the signature in question was computed as the mean expression of the genes included in the signature.

In order to define a gene signature representative of the CD73⁺ macrophage populations of special interest in this study, the four sc-RNA seq PhenoGraph clusters expressing high levels of CD73 and varying combinations of other immuno-suppressive factors (R3, R7, R14, and R17) were grouped into one (with all cells from the four clusters merged), and their differential expression compared to all cells not in one of those four clusters (i.e. belonging to any of the 13 other clusters, including T cell, myeloid, and NK cell populations). Together there were 3453 cells in one of these three clusters. Though traditional bulk RNA-seq methods for differential expression rely on mean expression and fold-change between samples/cell populations, a crucial aspect of single cell data is the ability to utilize the full distribution (with respect to multi-dimensional gene expression) of cells in a population of cells (i.e. a distribution as opposed to a point representation). A method for assessing differential expression between populations that maximally exploits these full distributions, and has been increasingly used in recent studies, is the Earth Mover’s Distance (EMD). In physical terms, the EMD quantifies the minimum “cost” of converting one pile of some material (e.g. dirt) into another, defined as the amount of material moved multiplied by the distance by which it is moved. In probability theory, it thus measures the distance between two distributions (again, as opposed to the distance between simply e.g. their means). For one-dimensional distributions (in our case for the distribution of expression of a single gene in a group of cells) it can be conveniently and efficiently computed as the L1 norm of the cumulative density functions for two distributions. Thus, we calculated the EMD using this method for each gene, between the two distributions of interest (cells belonging to the 4 CD73⁺ clusters and cells belonging to all other clusters), and ranked all of the over 19,000 genes by their EMD (with the top genes being differentially highly expressed in the CD73⁺ clusters, and the bottom genes the inverse). The EMD values, and associated z-scores across all genes, are provided for all genes in Supplementary Table 3. All genes with a z-score above 2.0 are shown in Figure 3A.

Nanostring gene expression analysis

RNA were isolated from formalin fixed paraffin embedded (FFPE) tumor sections by de-waxing using deparaffinization solution (Qiagen, Valencia, CA), and total RNA was extracted using the RecoverALL™ Total Nucleic Acid Isolation kit (Ambion, Austin, TX) according to the manufacturer’s instructions. The RNA purity was assessed on the ND-Nanodrop1000 spectrometer (Thermo Scientific, Wilmington, MA, USA). For the NanoString platform, 100 ng of RNA was used to detect immune gene expression using nCounter PanCancer Immune Profiling panel along with custom CodeSet. Counts of the reporter probes were tabulated for each sample by the nCounter Digital Analyzer and raw

data output was imported into nSolver (<http://www.nanosttring.com/products/nSolver>). nSolver data analysis package was used for normalization and hierarchical clustering heatmap analysis were performed with Qlucore Omics Explorer version 3.5 software (Qlucore, NY, USA).

MRI image quantification

The MRI images were quantified using ImageJ Software version 1.52a. First, the images were imported and the Brightness/Contrast was adjusted. The images slices were then scanned to identify tumor sections. A gate was drawn around the tumor in each section and the area was measured. The image geometry indicated the slice thickness to be 0.75mm and the distance between two sections to be 1 mm. Tumor area in each section was multiplied by 0.75 and the average between the tumor area in 2 sections was taken and multiplied by (1–0.75) 0.25 (this gave the value for depth).The volume for each tumor was obtained by multiplying the tumor area and depth from section containing tumor. All the values were added to determine the volume of tumor in cubic mm.

Survival Analysis

A gene expression signature using this method was defined by taking the top 44 genes, with a z-score above 3.0. The gene expression data based on microarray panel were downloaded from cBioportal (<http://www.cbioportal.org/datasets>, Glioblastoma Multiforme (TCGA, Provisional), as of Nov. 7, 2018). In our analysis, we used 525 patients with primary tumors whose clinical data are available. In our provisional dataset we utilized data from 201 patients published in Nature 2008, and we utilized data from 151 patients published in Cell 2013; 35 of the 44 signature genes were used, because 9 genes were not found in the U133 microarray data. The patients were sorted by the average z-score values of the signature genes and then split into a group with high expression (n=263) and a group with low expression (n=262). A log-rank test showed a significant negative association of the survival with the expression level of the signature genes (p=0.013) (Figure 3B).

Statistical analyses for murine experiments:

All data are representative of at least two to three independent experiments with 5–10 mice in each in vivo experiment. The data are expressed as mean $\pm$ standard error of the mean (SEM) and were analyzed using Prism 7.0 statistical analysis software (GraphPad Software, La Jolla, CA). Student t-tests (two tailed), ANOVA, and Bonferroni multiple comparison tests were used to identify significant differences (p<0.05) between treatment groups. The log-rank test was used to analyze data from the survival experiments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Extended Data

Extended Data Fig. 1. Gating strategy for identification of immune cell subsets by manual gating
 Contour plots indicating the gating strategy used to define manually gated CD3, CD4, CD8
 and FoxP3 positive populations in Figure 1A.

Extended Data Fig. 2. Heterogeneity of Tumor Infiltrating Leukocytes

A) Scatter plot indicating the absolute number of CD45⁺ live singlets of mass cytometry samples used for the multi-tumor comparison. Dashed line depicting the 600-cell threshold for sample inclusion. **(B)** Stacked bars (left) depicting the distribution of the identified meta-cluster frequencies within different tumor types in the color code indicated below. t-SNE map of 10,000 randomly selected cells per tumor type colored by tumor type with color legend indicated on the right (right, top panel), or by meta-cluster (right, bottom panel) with color legend indicated in the left panel. **(C)** Box-plots indicating CD45⁺ immune meta-cluster frequencies across tumor types from the PhenoGraph-based clustering approach in Figure 1. (Number of patients, GBM =7, NSCLC=11, RCC=11, CRC=11, and PCa=5). In all the box plots depicted, boxes indicate interquartile range with central bar indicating median and whiskers indicating the range. Individual patients are represented with dots. **(D)**

Histograms depicting expression of immune markers on the respective meta-clusters indicated on the left related to Figure 1D.

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T cell phenotypes in PBMC suspensions from renal cell carcinoma (RCC) patients undergoing combined ipilimumab and nivolumab ICT were analyzed by mass cytometry and identified using the PhenoGraph algorithm on viable CD45⁺ cells (n=14). **(A)** Heatmap indicating normalized expression of selected markers on CD45⁺ meta-clusters identified by PhenoGraph. **(B)** CD4⁺ T cell cluster P33 and CD8⁺ T cell cluster P24 frequencies at pre-treatment (T0) and after two cycles (T2) or four cycles (T4) of combination ICT in responders (n=7) and non-responders (n=7). P values were calculated using Mann-Whitney U tests (two-sided). Q values were calculated with the output p values. **(C)** Heat map displaying correlation matrix of clusters from PBMC samples and TIL. The Pearson correlation coefficient between each RCC PBMC cluster (above the threshold described in

the Methods) and each TIL clusters was computed using z-scored values (for RCC PBMC and TIL clusters, respectively, to account for their separate normalization) across all 29 channels shared between each experiment.

Extended Data Fig. 4. Distribution of T cell phenotypes across tumor types

T cell phenotypes in single cell suspensions of tumors from immune-checkpoint naïve patients were analyzed by mass cytometry and identified using the PhenoGraph algorithm on viable CD45⁺CD3⁺ cells. **(A)** Scatter plot indicating the absolute number of CD45⁺CD3⁺ live singlets in tumor single cell suspensions from immune checkpoint therapy naïve patients (n=37). Dashed line depicting the 600-cell threshold for sample inclusion (see Methods). **(B)** Box-plots indicating frequencies of selected T cell meta-clusters across tumor types (number of patients: NSCLC n=10, RCC n=11, CRC n=9). Samples are identical to samples used in Figures 2, 3, 5. **(C)** Histograms depicting expression of immune markers on the respective CD4 and CD8 T cell meta-clusters indicated on the left. Related to Figure 1F.

Extended Data Fig. 5. Characterization of Myeloid metaclusters

(A) Histograms below depicting the expression of immune markers on the respective metaclusters indicated on the left and in Figure 2A. (B) Contour plots indicating the gating strategy used to manually define myeloid cells phenotypically similar to L8 metacluster identified by PhenoGraph. All cells were gated on CD45⁺ live cells according to the gating strategy outlined in Figure S1. (C) Box plots indicating manually gated L8 subset frequencies as percentage of CD45⁺ live cells. (Number of patients, GBM=7, NSCLC=11, RCC=11, pCRC=7, mCRC=4, PCa=5). For pairwise comparisons, p values were computed by Mann-Whitney tests. Q values were calculated with the output p values. (D) Histogram overlay of CD73 expression of CD68⁺ cells in normal donor PBMCs (blue) and GBM-TILs

(red) by CyTOF. **(E)** Representative IHC images of GBM patient samples **(F)** Box-plot indicating density of CD3⁺, CD8⁺ and CD68⁺ cells/mm² in IHC sections of GBM patients samples (n=7) **(G)** Representative images of multicolor IF in GBM tumor samples (n=6). **(H)** Box-plot indicating percentage of CD68⁺ cells and CD68⁺CD73⁺ cells in total nucleated cells (n=6).

Extended Data Fig. 6. Similarities of tumor infiltrating leukocyte phenotypes between first and second cohort of untreated GBM patients

Leukocyte phenotypes in single cell suspensions of tumors from immune-checkpoint naïve patients were analyzed by mass cytometry and identified using the PhenoGraph algorithm on viable CD45⁺ cells. **(A)** Grouped box-plots indicating frequencies of CD45⁺ cells as indicated in single cell suspension of tumors from untreated cohort 1 patients (n=7) and untreated cohort 2 patients (n=9). Boxes indicate interquartile range with central bar indicating median and whiskers indicating the range. Individual patients are represented with dots. **(B)** Heatmap indicating normalized expression of selected markers on CD45⁺ meta-clusters identified by PhenoGraph from cohort 2 patients.

Extended Data Fig. 7. Distribution of tumor infiltrating leukocyte phenotypes in pembrolizumab treated and untreated GBM patients

Leukocyte phenotypes in single cell suspensions of tumors from immune-checkpoint naïve patients (untreated) and Pembrolizumab treated patients (pembro) were analyzed by masscytometry and identified using the PhenoGraph algorithm on viable CD45⁺ cells. (A) Scatter plot indicating the absolute number of CD45⁺ live singlets in single cell suspension of tumors from untreated patients (n=8) and pembro treated patients (n=5). (B) Grouped box-plots indicating CD45⁺ immune meta-cluster frequencies identified by PhenoGraph in untreated (n=7) and pembrolizumab treated tumors (n=5).

Extended Data Fig. 8. Distribution of tumor infiltrating leukocyte phenotypes from orthotopically injected GL-261 gliomas in untreated wild type and CD73^{-/-} mice
 CD73^{-/-} and WT mice were inoculated with GL-261 gliomas intracranially. **(A)** Left: Box plots indicating tumor sizes as determined by MRI in WT (blue) and CD73^{-/-} mice (red). Data is representative of two independent experiments, n=5 mice per group. Student *t* test (two-sided) was used to evaluate statistical significance. Data in the box plots are means $\pm$ SEM. Right: Indicated are representative MRI images on day 14 of tumor cell inoculation. Arrows indicate the tumor bulks. **(B)** Kaplan-Meier plot showing overall survival of untreated wild-type or CD73^{-/-} (n=10) orthotopically injected with GL-261 gliomas, P values were calculated using a logrank test (two sided). Data shown are representative of two

experiments. **(C)** Representative heatmap indicating intra-tumoral CD11b⁺ immune populations in both WT and CD73^{-/-} mice bearing GBM tumors by FlowSOM analysis. **(D)** The clusters on the right indicate clusters that show significant changes. P-values were calculated using Mann-Whitney U tests (two sided). Data is representative of two independent experiments, n=5 mice per group. **(E)** Bar graphs depicting CD45⁺ immune cluster frequencies identified by heatmap (n=5 mice per group). Boxes indicate interquartile range with central bar indicating median and whiskers indicating the range. Individual mice are represented with dots.

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Figure 1. Identification of Tumor infiltrating leukocyte phenotypes TILs were analyzed by CyTOF and identified using the PhenoGraph algorithm on viable CD45⁺ cells.

(A) Box-plots indicating frequency of CD3, CD4, CD8 or CD68 positive cells and CD4⁺FoxP3⁺ cells from live singlets obtained by manual gating of mass cytometry data (n=66). In all the box plots depicted, boxes indicate interquartile range with central bar indicating median and whiskers indicating the range. Individual patients are represented with dots. p values were computed by Mann-Whitney tests (two sided). Q values were calculated using the p.adjust function. $q < 0.05$ was considered statistically significant. (B) Heatmap depicting normalized expression of different immune markers by our PhenoGraph-based

clustering approach on CD45⁺ cells obtained from NSCLC (n=11), RCC (n=11), CRC (n=11), PCa (n=5) and GBM (n=7) patients. The color bar on the right indicates the leukocyte lineage of the respective meta-cluster (Myeloid: CD3⁻CD68⁺; T cell: CD3⁺; NK cell: CD3⁻CD56⁺). Bar graphs on the right indicate the relative frequency of the respective meta-clusters. **(C)** Box-plots indicating Shannon entropy of the distribution of tumor types in immune meta-clusters. Shannon entropy was computed for an empirical distribution of tumor across 1000 cells. This procedure was repeated 1000 times per cluster in order to bootstrap cluster size-corrected standard errors of entropy (n=1000). Boxplots of entropy values in each cluster, ordered by mean entropy. Dashed line indicating the expected entropy value, if in-cluster tumor type distribution matches tumor type distribution of all cells in the dataset. **(D)** Box-plots indicating frequencies of the respective CD4 and CD8 T cell meta-clusters across tumor types. (Number of patients: GBM =7, NSCLC=11, RCC=11, CRC=11 PCa=5). Kruskal- Wallis tests were performed for the 14 metaclusters and corrected for multiple comparisons using the Benjamini and Hochberg (BH) method. **(E)** Stacked bar graph visualizing meta-clusters frequencies in individual patients using the color-code indicated on the right. Dendrogram on the left indicating hierarchical clustering of patient meta-cluster frequencies. Black frames highlighting patient subgroups identified by this clustering approach. Color bar on the left indicating tumor types of the individual patients using the color code indicated below. **(F)** Box-plots indicating T cell meta-cluster frequencies across the patient subgroups identified in (E). Group I =11, group II =8, group III=9. In all the box plots depicted, boxes indicate interquartile range with central bar indicating median and whiskers indicating the range. Individual patients are represented with dots. Kruskal-Wallis tests were used to compare across the subgroups. Mann-Whitney tests were used for pairwise comparisons. Significant pairwise comparisons are indicated (FDR=5%).

Figure 2. CD73^{hi} macrophages are specifically present in GBM Myeloid cells (CD3⁻CD68⁺) were analyzed by CyTOF in patients of multiple tumor types and further characterized by sc-RNA seq in GBM.

(A) Box-plots indicating frequencies of L1, L5, L8 and L17 meta-cluster across tumor types (number of patients: NSCLC=11, RCC=11, CRC=11, PCa=5, GBM=7). Q-values were calculated using Kruskal-Wallis tests (across different tumor types) and the Benjamini & Hochberg method. Pairwise comparisons were performed using Mann-Whitney U tests within and corrected for multiple comparisons using the Benjamini & Hochberg. Significant pairwise comparisons are indicated (FDR=5%). (B) TILs from untreated GBM tumors (n=4) were analyzed by sc-RNA seq and identified using the MAGIC algorithm. Heatmap

indicating normalized expression of selected markers in leukocyte clusters identified by MAGIC. Black arrows indicate the CD73^{hi} myeloid cell clusters. **(C)** Upper top panel: t-SNE maps depicting cluster phenotypes and relative expression levels of CD73 on a single cell level with color legend on the right. Oval area highlighting CD73^{hi} macrophage clusters (R3, R7, R14 and R17). Lower bottom panel: t-SNE maps indicating relative expression levels of a blood-derived macrophage gene signature and microglial gene signature at a single cell level with color legend on the right (n=4). **(D)** Heatmap indicating normalized expression of chemokine receptors on CD73^{hi} macrophage clusters identified by MAGIC. Black arrows indicate the CD73^{hi} myeloid cell clusters. **(E)** Upper panel: t-SNE maps indicating relative expression levels of immunosuppressive and immunostimulatory gene signature at a single cell level. Lower bottom panel: t-SNE maps indicating relative expression levels of a hypoxia-induced gene signature, (n=4).

Figure 3. CD73^{hi} myeloid cells persist after anti-PD-1 therapy and correlates with reduced overall survival in TCGA-GBM cohort
(A) CD73^{hi} macrophage gene signature of differentially expressed genes ($z > 3.0$, 45 genes) (Supplementary Table 3). Heatmap indicating normalized expression of top differentially expressed genes in CD73^{hi} macrophages (z score > 2.0) identified by MAGIC. **(B)** Kaplan-Meier plot showing overall survival of GBM patients from the TCGA database with above (blue= high expression, number of patients: $n=263$) or below (red=low expression, number of patients: $n=262$) median expression of 45 genes signature derived in (A). Log rank p value (two-sided) and hazard ratio (HR) displayed. Leukocyte phenotypes in single cell suspensions of tumors from immune-checkpoint naïve patients (untreated) and

Pembrolizumab treated patients (pembro) were analyzed by mass cytometry and identified using the PhenoGraph algorithm on viable CD45⁺ cells. **(C)** t-SNE map depicting degree of phenotypic similarity of GBM infiltrating leukocytes in pembrolizumab-treated (n=5) or untreated patients (n=7) at a single cell level. **(D)** TILs from GBM tumors after treatment with pembrolizumab (n=5) or ICT naïve GBM patients (n=7) were analyzed by mass cytometry and identified using the PhenoGraph algorithm on viable CD45⁺ cells. Heatmap indicating normalized expression of selected markers on CD45⁺ meta-clusters identified by PhenoGraph. **(E-F)** Stacked-bars indicating frequencies of CD73^{hi} myeloid meta-clusters and T cell clusters in pembrolizumab treated and untreated GBM patients. **(G)** Representative heat map of transcriptome profiling using GSEA of tumor specimens from untreated (n=6) and anti-PD-1 treated (n=4) patients using customized 739- gene Nanostring panel.

Figure 4. Absence of CD73 enhances efficacy of ICT in murine model of GBM

A) Representative MRI images on day 14 of inoculation of GL-261 tumor line orthotopically into $CD73^{-/-}$ and wild-type mice with and without ICT treatment. Figures are representative of three independent experiments. **(B)** Kaplan-Meier plot showing overall survival of wild-type and $CD73^{-/-}$ ($n \sim 10$ mice) treated with anti-PD-1 alone, anti-PD-1 and anti-CTLA-4 or untreated mice orthotopically injected with GL-261 gliomas, p values were calculated using a logrank test (two sided). Please refer to Supplementary Table 2 for more details. **(C)** Heatmap indicating intra-tumoral $CD45^{+}$ immune populations as determined by FlowSOM in both WT and $CD73^{-/-}$ mice bearing GBM tumors. Color code on the upper right indicates z-scored expression values. Legend on the lower right indicates cell types for each colored cluster. **(D)** Box-plots indicating abundance ratio of leukocyte subsets ($n=5$ mice per group). Data representative of 2 independent experiments. Data in the box plots are means $\pm$ SEM. P-values were calculated using Mann-Whitney U tests (two sided) for pairwise comparisons.