

1 **Multiplex spatial systems analysis of local nanodose drug responses predicts effective**
2 **treatment combinations of immunotherapies and targeted agents in mammary carcinoma**

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18 **SUMMARY**

19 Better methods are needed to identify effective combinations of immunotherapies with
20 chemotherapies and targeted anti-cancer agents. Here we present a Multiplex Implantable
21 Microdevice Assay (MIMA) system for rapid *in vivo* assessment of the effects of multiple,
22 spatially separate anticancer drugs directly in the complex tumor microenvironment. In
23 prototypic experiments, olaparib, lenvatinib, palbociclib, venetoclax, panobinostat, doxorubicin,
24 and paclitaxel and combinations thereof were administered simultaneously to murine mammary
25 tumor models. Quantitative multiplex immunohistochemistry and spatial systems analyses of
26 each local drug response defined cellular relations of fibroblasts, endothelial cells, immune
27 lineages, immunogenic cell death, tumor proliferation and/or cancer stem cells that were used to
28 predict effective drug combinations. A predicted combination of panobinostat, venetoclax and
29 anti-CD40 showed long-term anti-tumor efficacy in multiple mouse models with no observable

30 toxicity when administered systemically. Future MIMA use promises to design effective drug
31 combinations for tumor cell control and immune activation on a personalized basis.

32

33 **KEYWORDS**

34 personalized combination treatments, tumor microenvironment, implantable microdevice, spatial
35 systems analyses

36

37 **INTRODUCTION**

38 Modern cancer therapies increasingly seek to effect tumor control by simultaneously attacking
39 tumor intrinsic vulnerabilities, enhancing anti-tumor immune activity and/or mitigating stromal
40 mediators of resistance. Targeted drugs typically are designed to attack genetic or transcriptional
41 vulnerabilities on which tumor cells depend for survival but non-malignant cells do not.

42 Genomic screening approaches have supported such tumor-intrinsic aspects of precision
43 medicine, leading to matching of genomic aberrations with specific targeted agents (Li et al.,
44 2021). In breast cancer, treatments targeting tumors that depend on estrogen receptor (ER)
45 signaling, aberrant signaling resulting from human epidermal growth factor receptor 2 (HER2)
46 amplification and/or over expression, CDK4/6 signaling and defects in DNA repair in triple
47 negative breast cancer (TNBC) have been particularly effective (Bettaieb et al., 2017; Hanker et
48 al., 2020; Harbeck et al., 2021; Lord and Ashworth, 2017). Unfortunately, these treatments are
49 not uniformly effective even in primary tumors carrying the target and are usually only
50 transiently effective in metastatic disease (Brady et al., 2017; Janiszewska et al., 2021; Jeselsohn
51 et al., 2017). This may be due in part to drug modulation of aspects of the tumor
52 microenvironment (TME) including immune function that negatively influence cancer control.
53 This suggests that treatment efficacy can be increased by combing these drugs with agents that
54 increase immunogenicity and/or counter microenvironment-mediated resistance, a hypothesis
55 that we address in this paper.

56 The concept of enhancing cancer treatment efficacy by combining chemotherapies and targeted
57 drugs with agents that enhance immune-mediated cancer cell killing is increasingly well
58 established. The clearest example is the use of immunotherapies, including immune checkpoint
59 blocking (ICB) antibodies as complements to tumor targeted therapies in various liquid and solid

60 malignancies (Dummer et al., 2020; Robert, 2020). However, many cancers do not benefit from
61 ICB including in breast cancer where efficacy has been limited to a subset of TNBC patients
62 (Adams et al., 2019; Force et al., 2019). This lack of efficacy is attributed in part to two
63 mechanisms: i) Low antigenicity through decreased expression of major histocompatibility
64 complex class I (MHC-I)—observed mainly in luminal ER+ BC (Brady et al., 2017; Lee et al.,
65 2016) and HER2+ BCs (Inoue et al., 2012; Janiszewska et al., 2021); and ii) a naturally
66 immunosuppressive TME associated mainly with TNBC and HER2+ BC (Denardo et al., 2011;
67 Gil Del Alcazar et al., 2017; Guerrouahen et al., 2020). Both of these mechanisms may limit the
68 CD8+ T cell-mediated anti-tumor response, which then cannot be leveraged to improve efficacy
69 of ICB therapies (Palucka and Coussens, 2016). Combinations of conventional chemotherapies
70 and/or targeted anticancer drugs that increase immunogenic cell kill promise significant
71 improvements in overall outcome (Galluzzi et al., 2018). However, further understanding of
72 drug-immune system interactions is required to design effective and safe immune modulating
73 combinatorial regimens.

74 A variety of experimental approaches have been deployed to elucidate the effects of drug
75 combinations on the tumor and stromal components and to identify biomarkers that inform on
76 the efficacy of treatment combination decisions (Letai, 2017). Biomarkers typically are identified
77 by establishing associations between tumor features and outcomes in population-based clinical
78 studies (Hellmann et al., 2018; Hugo et al., 2016) such as those supported by The Cancer
79 Genome Atlas (Hutter and Zenklusen, 2018) and Human Tumor Atlas Network (Rozenblatt-
80 Rosen et al., 2020) programs. However, these association-based approaches need to be tested for
81 causality in systems that faithfully recreate the interactions of the various components of the
82 TME. Common model systems include tumors that arise in immune competent mice and short-
83 or long-term ex vivo cultures comprised of tumor and stromal components using miniscule
84 scaffolds and active fluidics to closely model specific aspects of the TME (Deng et al., 2017;
85 Jenkins et al., 2017; Tatárová et al., 2016). However, these studies in mice typically are slow,
86 expensive and labor-intensive, and comprehensive modeling of tumor-microenvironment
87 interactions in ex vivo systems remains a major challenge (Yuki et al., 2020).

88 We report now on a high-throughput in vivo approach to rapidly, safely and efficiently assess the
89 effects of multiple drug combinations on TME composition and architecture in living mouse
90 models that is also applicable to humans (Dominas et al., 2021). Our study focuses on mouse

91 mammary cancers and our approach integrates two recently introduced high-throughput and
92 high-content techniques: a minimally invasive implantable microdevice (IMD) (Jonas et al.,
93 2016, 2015; Watson et al., 2018) for intratumor delivery of nanoliter doses (nanodoses) of
94 multiple drugs or drug combinations into spatially separate regions, and multiplexed
95 immunohistochemistry (mIHC) (Lin et al., 2015; Tsujikawa et al., 2017) to assess the in-situ
96 responses of the tumor-microenvironment milieu near each drug delivery site. Computational
97 analyses of serial mIHC staining and imaging of 30+ proteins allow comprehensive
98 categorization of standard immune and non-immune stromal cell types and states as well as
99 complementary characterization of tumor proliferation, apoptosis, differentiation and/or
100 immunogenicity. Assessment of the composition and spatial distribution of the functionally
101 different cell types in each drug delivery area facilitates identification of drug-mediated
102 mechanisms of response and resistance that rapidly reveal new therapeutic intervention
103 strategies. We refer to the overall approach as the Multiplex Implantable Microdevice Assay
104 (MIMA).

105 We used the MIMA to evaluate the effects of FDA approved drugs olaparib, palbociclib,
106 doxorubicin, venetoclax, panobinostat, lenvatinib and paclitaxel and combinations thereof on the
107 tumor and TME, in the immunocompetent MMTV-PyMT (mouse mammary tumor virus-
108 polyoma middle tumor-antigen) mouse model. This commonly used genetically engineered
109 mouse model for breast cancer research mirrors many aspects of human breast cancer
110 progression and heterogeneity (Attalla et al., 2021; Guy et al., 1992; Lin et al., 2003). Out of
111 eight treatments tested, five showed unique, significantly enriched histopathological signature
112 that we used to predict synergistic TME modulating combination treatments that subsequently
113 were validated in traditional systemic dosing studies. Among these, the combination of
114 panobinostat, venetoclax and agonist anti-CD40 monoclonal antibodies (mAB) provided the
115 strongest response for long-term disease control in multiple models of mammary carcinoma and
116 was well-tolerated.

117

118 **RESULTS**

119 **MIMA components and design to identify effective TME modulating combination** 120 **treatments**

121 The IMD used for drug delivery in the MIMA system was a 5.5 mm long, 0.75 mm diameter
122 biocompatible resin cylinder capable of delivering multiple drugs or drug combinations at up to
123 18 spatially separate regions inside a living tissue per device (Figure 1A). IMDs were loaded
124 with drugs in pegylated semi-solid form within the wells of the cylinder so that drugs were
125 released upon implantation via passive diffusion (Jonas et al., 2015). Local concentrations of
126 drugs in the IMD were tuned to produce drug levels at each site in the tissue to match those
127 achieved during systemic treatment in clinical practice (Figure S1A and Table S1) (Jonas et al.,
128 2016). Importantly, the miniscule nanodoses of drugs delivered via IMDs do not generate the
129 toxicities typically associated with systemic treatments (Jonas et al., 2015).

130 After treatment for 1 to 8 days, tumors were harvested with the IMD in place, prepared as
131 formalin-fixed, paraffin-embedded (FFPE) samples and serial tissue sections were cut orthogonal
132 to the axis of the IMD (Figure 1A). Sections through each drug delivery well in the IMD were
133 analyzed implementing mIHC (Figure S1B) according to published procedures (Chang et al.,
134 2017; Tsujikawa et al., 2017) to assess drug effects using a range of markers with specificity
135 being cross-validated using cyclic immunofluorescence (cycIF) (Lin et al., 2015) (Figure S1C-
136 F). mIHC generated multiprotein images of each tissue section through a process of serial
137 immunostaining, imaging and stripping of each FFPE section (Figure 1B, Figure S1B) using
138 antibodies to proteins that define cell types and/or functions *in situ* (Figure 1C, D and S2A). In
139 our studies, 30+ proteins were interrogated for each section. Each mIHC image was analyzed by
140 segmenting individual cells and calculating cell positivity for each segmented cell (Figure 1C,
141 S2B-D). The mIHC antibody panel (Figure 1D, Table S2 and S3) was specifically designed to
142 capture a broad range of TME states and to identify actionable phenotypes with preferential
143 detection of early and local responses (Table S4).

144 We implemented a binary gating strategy (Figure 1E, S2E) using measurements of 13 proteins
145 (Epcam, CD45, CD31, α SMA, CD3, CD4, CD8, CD11b, F4/80, CSF1R, CD11c, Ly6G, MHC-
146 II; Figure 1D, S2A; baseline discovery panel) to define 17 major tumor cell types or states with
147 focus mostly on immune and non-immune stromal cells. We refer to these cells as “standard cell
148 types” hereafter. They included, for example, T cells (Epcam-CD45+CD3+), antigen presenting
149 macrophages (Epcam-CD45+F4/80+CD11c-MHC-II+), immature myeloid cells (Epcam-
150 CD45+F4/80-CD11c-Ly6G-CD11b+MHC-II-) and endothelial cells (Epcam-CD45- α SMA-

151 CD31+). For the full list see [Figure 1F](#). We measured the abundances and spatial organizations
152 of these standard cell types for all test conditions to narrow down the target stromal phenotypes
153 in a controlled and unbiased manner. Then, we interrogated additional proteins to refine the
154 standard cell types and/or to report on other aspects of tumor and stromal cell biology including
155 on basic drug sensitivity (CC3, Ki67), immunogenic cell death and/or processes typically
156 associated with resistance or breast cancer progression such as cancer stem cells (Epcam+CD45-
157 PyMT+Ki67-Sox9+), invasion (Keratin-14, K14); or immune suppression (arginase-1, arg-1)
158 ([Figure 1D](#); extended readout).

159

160 **Quantification of single cell events at local delivery sites reveal unique drug specific** 161 **histopathological signatures of TME response**

162 We used the MIMA system to perform a small-scale screening study in which we quantitatively
163 assessed the responses to five FDA approved targeted therapies and two chemotherapeutic agents
164 with distinct modes of action. The targeted drug were the poly (adenosine diphosphate [ADP])
165 ribose polymerase (PARP) inhibitor, olaparib (Lord and Ashworth, 2017); the multi-kinase
166 vascular endothelial growth factor receptor (VEGFR)-1/2/3 inhibitor, lenvatinib (Kato et al.,
167 2019); the cyclin dependent kinase (CDK)-4/6 inhibitor, palbociclib (Harbeck et al., 2021); the
168 B-cell lymphoma (BCL)-2 inhibitor, venetoclax (Montero and Letai, 2018); and the pan- histone-
169 deacetylase (HDAC) inhibitor, panobinostat (Atadja, 2009). The chemotherapeutic drugs were
170 the DNA-intercalating agent, doxorubicin and the microtubule poison, paclitaxel, which are often
171 used in first line therapy for breast cancer (Cardoso et al., 2018; Kumar et al., 2018). We
172 assessed responses in late stage MMTV-PyMT mice with spontaneously growing tumors that
173 mirror the morphology and aspects of progression of human breast cancers (Guy et al., 1992).
174 These tumors initially express ER strongly but expression decreases as they progress to late-
175 stage carcinoma (Lin et al., 2003). Gene expression profiling reveal that tumors cluster with the
176 luminal B subtype at the stage used herein (Herschkowitz et al., 2007; Lin et al., 2003). We
177 chose a spontaneous rather than transplanted tumor model to better account for all stages of
178 immune-biology associated with de novo tumor progression (Hanahan and Coussens, 2012),
179 including editing (Dunn et al., 2004).

180 IMDs loaded with individual agents were implanted in tumors for three days since our previous
181 work indicated that TME responses were apparent by this time (Watson et al., 2018). Our
182 analyses of harvested tumors focused on the cell and molecular compositions and organizations
183 that were significantly enriched in regions close to the drug deliver sites compared to remote
184 intratumoral controls (Figure 1G). Quantification of the 17 standard cell types following seven
185 candidate drug exposures revealed unique drug-specific histopathological signatures of TME
186 response that are summarized in Figure 1H with Figures 1I-L showing computed images of the
187 most prominent response cell types after treatment. Lenvatinib and paclitaxel produced no
188 detectable local TME changes; olaparib was associated with a modest increase in macrophage,
189 neutrophil and fibroblast density, whereas doxorubicin induced a significant increase in
190 vasculature (Figure 1H). Palbociclib, venetoclax, and panobinostat produced significant
191 ecosystem changes in both the immune and non-immune stromal states and thus we studied and
192 described these targeted anti-cancer agents in more detail in the following sections. Predicted
193 effective combinations derived from these studies (Table S4) were then validated in whole
194 animals implementing commonly used murine mammary cancer cell lines (E0771 and/or EMT6
195 models (Herschkowitz et al., 2007)) allografted into syngeneic immunocompetent hosts.

196

197 **Palbociclib induces enrichment of CSF1R⁺ macrophages associated with pericyte** 198 **branching and de novo tumor proliferation**

199 Intratumoral treatment with the CDK4/6 inhibitor, palbociclib, induced a significant
200 accumulation of several stromal cell types into the assay area including leukocytes, endothelial
201 cells, pericytes and mesenchymal cells (Figure 1H, J, Figure 2A, Figure S3A, B). Among
202 leukocytes, colony-stimulating factor 1 receptor (CSF1R)-positive macrophages were the most
203 enriched subset (Figure 1H, 2A) but only 9% were positive for class II major histocompatibility
204 complex (MHC-II) (Figure S3C) indicating these macrophages are not professional antigen
205 presenting cells (APCs) and may be protumorigenic (Kowal et al., 2019; Reis E Sousa, 2006).
206 CSF1R⁺ cells, in general, were uniquely and significantly enriched by palbociclib (Figure 2A, B
207 and S3A, D), however, the marker was not solely expressed on macrophages as defined by the
208 standard cell type classification (Figure 1F). Extended mIHC analyses revealed that CSF1R also
209 was expressed on epithelial cells, fibroblasts and endothelial cells (Figure 2C). The majority
210 (46%) of CSF1R⁺ cells were positive for the F4/80 macrophage and CD11b pan-myeloid

211 markers, however, the CD45 leukocyte marker was under the detection limit of IHC (Epcam-
212 CD45-CD31- α SMA-F4/80+CD11b+CSF1R+; [Figure 2C](#), [S3B](#), [D](#)) indicating they might be less
213 differentiated protumorigenic macrophages (Deszo et al., 2001; Norazmi et al., 1989; Sophie
214 Mokas et al., 2009). Spatial analyses showed that while the CD45+ macrophages were localized
215 to regions immediately proximal to the drug delivery well, CD45- less-differentiated
216 macrophages were localized both proximally and more distally and in some regions were
217 associated with the contractile pericytes (Epcam-CD45- α SMA-CD31+) (Bergers and Song,
218 2005) ([Figure 2B-D](#)). This spatial distribution is apparent in a profile plot ([Figures 2D](#), [S3B](#))
219 which shows the relative abundance of cells at increasing distances from the drug delivery well.
220 We also assessed the propensity of specific cell types to cluster together by mapping the
221 locations where 10 or more cells of a defined phenotype occurred together in regions 30, 50 or
222 75 μ m in diameter ([Figure 2E](#) and [S3E](#)). The selection of cluster sizes was based on the highest
223 variance between palbociclib treated and PEG control regions excluding strategies that showed
224 treatment unspecific clusters in untreated regions (e.g. clusters of as few as 5 cells or distances of
225 ≥ 100 μ m; [Figure S3F](#)). These analyses showed that the CSF1R+ macrophages and CD31+
226 endothelial cell/pericyte structures were organized together in response to palbociclib drug
227 stimulus and did not appear in PEG control tissues. The patterns for the CD31+ cell aggregates
228 were branch-like with pericytes integrated within endothelial structures suggestive of
229 neovascularization and blood pressure/flow control (Bergers et al., 2003) ([Figure 2E](#), [S3E](#)). The
230 profile plot and distance-based cluster analyses also showed clusters of Ki67-positive neoplastic
231 cells (Epcam-CD45+PyMT+Ki67+) distal to the drug delivery site and proximal to the
232 macrophage-pericyte networks ([Figure 2D](#), [E](#) and [S3B](#), [D](#) and [E](#)) indicating that the macrophage-
233 pericyte structures may be linked to the increased tumor cell proliferation in local microculture
234 as summarized schematically in [Figure 2F](#). The high density of CSF1R on multiple cell types and
235 the associated increase in Ki67+ tumor cells suggested to us that compounds targeting the
236 CSF1/CSF1R axis might enhance palbociclib efficacy by countering these CSF1R-mediated
237 processes ([Table S4](#)).

238 We tested this concept in a systemic study of the EMT6 breast cancer model, by treating mice
239 bearing orthotopically implanted tumors into the mammary fat pads of immunocompetent
240 syngeneic mice with intraperitoneal injections of palbociclib, an anti-CSF1R antibody and a

241 combination of the two. Whereas the individual drugs did not impact the rate of tumor growth,
242 the combination treatment significantly reduced tumor growth rate (Figure 2G). Thus, the
243 efficacy of palbociclib/anti-CSF1R revealed by analyses of responses to intratumoral treatments
244 was affirmed in whole animal experiments.

245

246 **Venetoclax recruits phenotypically distinct clusters of dendritic cells, immature myeloid cells** 247 **and endothelial cells**

248 Intratumor treatment with the BCL-2 inhibitor venetoclax, did not produce significant cell death
249 as indicated by IHC staining for cleaved caspase 3 (CC3) (see Figure 4C). However, it resulted
250 in significant recruitment of leukocytes including dendritic cells (DCs), immature myeloid cells;
251 and endothelial cells to the region proximal to the drug release well (Figures 1H, K and 3A and
252 S4). Unlike palbociclib, the endothelial cells were not associated with α SMA (Figure 3B)
253 suggesting they form small blood vessels (Bergers et al., 2003) that are not supported by
254 pericytes. The recruitment of dendritic cells is noteworthy since they play important roles in
255 regulating the balance between immune tolerance and activity (Domogalla et al., 2017).
256 Interestingly, CD11c⁺ cells (primarily a dendritic cell marker) aggregated into clusters in regions
257 near venetoclax delivery, but not in random intratumoral regions far from the drug releasing
258 reservoir (Figure 3C). The clusters were phenotypically distinct as defined by their morphology
259 and positivity for Epcam, CD45, MHC-II and CD11b (Figure 3D, E). The dendritic cell clusters
260 located nearest to the drug delivery well were intermixed with neoplastic cells, exhibited brighter
261 and smaller nuclei, (Figure 3D; 1a and 1b) and greater than 60% of this population
262 Epcam⁺CD45⁻ (Figure 3E, S4C) suggesting these cells might be phagocytic DCs (Goodridge et
263 al., 2011). Dendritic cells further from the reservoir were aligned and intermixed with endothelial
264 cells (Figure 3D; 3), possibly resulting from migration from/to the TME and they were the only
265 population expressing MHC-II (Figure 3B and E) (Kedl et al., 2017). Thus, only a small subset
266 of the DCs recruited to the venetoclax well were antigen presenting cells. DCs distal from the
267 reservoir were mostly present in tumor cleared areas (Figure 1K and 3D; top right). These cells
268 were CD11b and CD45 positive (Figure 3E) and all three stains CD45, CD11b and CD11c
269 showed a “bulls-eye” pattern around an unstained cytoplasmic area centered on the nucleus

270 (Figure 3D; 4). The surface CD45 expression and the “bulls-eye morphology” suggest these cells
271 were unstimulated DCs (Goodridge et al., 2011).

272 It is thus far unknown whether the spatially separated and phenotypically distinct DC clusters are
273 functionally related, or whether they were induced by venetoclax as separate entities (Figure 3F).
274 However, the literature suggests that MHC-II negative DCs have limited antigen presenting
275 potential and limited capacity to prime T cells (Reis E Sousa, 2006). Additionally, unstimulated
276 DCs as well as immature myeloid cells, which also were significantly enriched near the
277 venetoclax well (Figure 1H, K, 3A, and S4A, C), have been reported to induce immunological
278 tolerance (Domogalla et al., 2017; Griffiths et al., 2016; Sotomayor et al., 1999) raising the
279 possible therapeutic utility of agents that could mitigate the tolerogenic potential of these cells
280 (Table S4). Agonist monoclonal anti-CD40 antibodies act on DCs and immature myeloid cells
281 by increasing their antigen presenting capacity, maturation and activation potential (called
282 licensing) (Sotomayor et al., 1999). Licensed DCs have the capacity to shift the balance from
283 tolerance to anti-tumor immunity (Griffiths et al., 2016; Hoves et al., 2018; Kowal et al., 2019).
284 We reasoned that anti-CD40 immunotherapy could be used to enhance the anti-tumor immune
285 capacity of DCs and immature myeloid cells that were recruited by venetoclax treatment (Figure
286 3F).

287 We tested this concept in E0771 breast cancer cells grown orthotopically in the mammary fat
288 pads of immunocompetent C57BL/6 mice by treating systemically with intraperitoneal injections
289 of venetoclax, an anti-CD40 agonist antibody and a combination of the two. Neither agent was
290 effective as a single drug but the combination of the two significantly reduced the tumor growth
291 rate and increased overall survival with 60% of mice surviving for >180 days (Figure 3G). For
292 comparison, the combination of venetoclax with a programmed death ligand-1 (PD-1) inhibitory
293 antibody did not significantly affect tumor growth rate or survival (Figure 3G). Again, a
294 therapeutic strategy suggested by the MIMA proved to be effective in whole animal experiments.

295

296 **Panobinostat induces immunogenic cell death associated with recruitment of antigen** 297 **presenting neutrophils and macrophages**

298 Intratumor delivery of the pan-HDAC inhibitor, panobinostat, led to significant recruitment of
299 several immune cell populations including dendritic cells, antigen presenting macrophages and

300 (antigen presenting) neutrophils (Figure 1H, L, 4A and S5A, B). The cells located *immediately* at
301 the drug releasing well were MHC-II positive antigen presenting macrophages (Figure 1L, 4B,
302 5A and S5B-C). The presence of such macrophages inside tumor nests has been associated with
303 better clinical responses, improved survival and improved efficacy of PD-1/programmed death
304 ligand-1 receptor (PD-L1) therapies (Guerriero et al., 2017; Kawai et al., 2008). Depletion of
305 these macrophages using a CSF1R inhibitor significantly decreased panobinostat-induced cell
306 death (Figure 4C) implying this immune subset has a functional, anti-tumor role. This result is in
307 line with previous studies showing that concurrent depletion of macrophages abrogates the anti-
308 tumor efficacy of HDAC inhibitors (Guerriero et al., 2017).

309 Neutrophils were the most abundant immune population recruited to the panobinostat well
310 (Figure 1H, 4A) and these were organized in a band slightly farther from the well than
311 macrophages (Figure 1L, 4B, E and S5B, C). Neutrophils are considered to be rapid responders
312 in the first line of defense against pathogens and classically are not categorized as professional
313 antigen presenting cells as compared to DCs, B-cells, monocytes and macrophages which have
314 superior ability to prime naïve T cells in some settings (Lin and Loré, 2017; Reis E Sousa, 2006).
315 However, 13% of neutrophils were MHC-II-positive (Figure 4F) suggesting they have undergone
316 strong phenotypic maturation (Garg et al., 2017). MHC-II-positive neutrophils have recently
317 been linked to immunogenic cell death (ICD) during which they phagocytose dying tumor cells
318 and mediate respiratory-burst-dependent cytotoxicity against residual cells (Garg et al., 2017).
319 Interestingly, panobinostat was the only one of the seven drugs tested that induced substantial
320 cell kill near the drug delivery site as indicated by significantly increased CC3 expression
321 (Figure 4C, D). Based on our observation of a significant enrichment of MHC-II+ antigen
322 presenting neutrophils at the panobinostat well, we hypothesized that panobinostat-mediated cell
323 death would be immunogenic and the efficacy of this targeted therapy would be enhanced by
324 PD-1 blockade.

325 We confirmed that panobinostat-induced tumor killing was immunogenic by performing a whole
326 animal vaccination study (Ma et al., 2013; Md Sakib Hossain et al., 2018). Specifically, aliquots
327 of E0771 and EMT6 tumor cells treated with panobinostat *in vitro* or killed by freeze thawing
328 (negative control for non-immunogenic cell death) were injected subcutaneously into syngeneic
329 immunocompetent mice, and then mice were then re-challenged with live tumor cells of the
330 same type after seven days. We observed significantly increased tumor-free survival in mice

331 immunized with panobinostat-treated tumor cells as compared to mice inoculated with untreated
332 freeze-thawed dead cells (P value <.0001 and 0.0027 for E0771 and EMT6, respectively; [Figure](#)
333 [4G](#)). Consistent with this, systemic treatment of mice with panobinostat significantly increased
334 the proportion of intratumoral CD8⁺ T cells as compared to stromal parenchyma ([Figure 4H](#) and
335 [Figure S5D](#)).

336 Next, we tested the utility of combining panobinostat with an anti-PD-1 antibody by systemically
337 treating mice carrying syngeneic mammary tumors with these drugs administered singly and in
338 combination. Panobinostat alone significantly decreased the rate of tumor growth in early-stage
339 tumors ([Figure S5E](#)) but later stage tumors did not respond to the single agent ([Figure 4I](#), [S5F](#)).
340 However, combining panobinostat with anti-PD-1 immunotherapy, as suggested by our MIMA
341 studies, significantly decreased tumor growth rate and increased survival in both EMT6 and
342 E0771 models ([Figure 4I](#), [6H](#)) thus indicating effective induction of antitumor immunity.

343

344 **Biomarkers of response and mechanisms of resistance help to identify early intratumor** 345 **signatures of panobinostat-induced anti-tumor immunity in mammary carcinoma**

346 Because our local and systemic studies indicated panobinostat to be increasing immunogenicity
347 of mammary carcinoma ([Figure 4](#), [S5](#)), we further evaluated potential mechanisms of response
348 and resistance associated with this targeted therapy. We extended the mIHC readout to enable
349 assessment of functional biomarkers related to induced anti-tumor immunity by adding
350 antibodies to probe expression of calreticulin which facilitates folding of the MHC-I complex in
351 the endoplasmic reticulum (Raghavan et al., 2013) and dictates immunogenicity of cell death
352 (Obeid et al., 2007), intercellular adhesion molecule 1 (ICAM1) and the β -galactoside-binding
353 lectin, galectin-3; which play roles in recruitment of neutrophils to tissues (Gittens et al., 2018;
354 Yang et al., 2005) and licensing of myeloid cells (Radsak et al., 2000; Vonderheide, 2018),
355 myeloperoxidase (MPO) to identify cytotoxic neutrophils (Patnaik et al., 2017), and MHC-I and
356 neuropilin-1 to report on proficient antigen presentation capacity (Chawla et al., 2016; Kerros et
357 al., 2017; Luo et al., 2018). These early in situ markers as well as PD-L1 have been directly or
358 indirectly associated with immunogenic cell death, increased tumor CD8⁺ T cell infiltrate and/or
359 immune checkpoint blockade efficacy (Aguilera et al., 2016; Guerriero et al., 2017; Hu and
360 McArthur, 2018; Luo et al., 2018; Obeid et al., 2007; Patnaik et al., 2017). We measured the

361 expression of these proteins at the panobinostat reservoir and refer to them in ensemble as
362 “biomarkers of immunogenic cell death” (Figure 1D, in yellow). We also added antibodies to
363 probe resistance mechanisms associated with breast cancer progression; specifically, the cancer
364 stem cell (CSC) marker, Sox9 (Guo et al., 2012); the immune suppression marker arginase-1
365 (Geiger et al., 2016; Rodriguez et al., 2017); Keratin-14 as a marker of the tumor invasive front
366 (Cheung et al., 2016, 2013); and matrix metalloproteinases (MMPs) and collagens as markers of
367 extracellular matrix (ECM) processing and deposition (Sahai et al., 2020) (Figure 1D, in blue).
368 We assessed the locations of the 17 standard cell types and the expression of the various
369 biomarkers of response and resistance thereon and found that they were organized in distinct
370 layers (zones) at increasing distances from the panobinostat delivery well. These zones were
371 designated as *immediate*, *proximal*, *border*, *distal* and *remote* as illustrated in Figure 5B.

372 Antigen presenting macrophages were located in the *immediate* region at the panobinostat well
373 such as described above (Figure 5A, B). The *proximal* region was populated predominantly by
374 neutrophils. These and other cells expressed MPO, ICAM1 and neuropilin-1 (Figure 5A-C,
375 Figure S5C, SA, B). More than half (65%) of Ly6G⁺ neutrophils were positive for MPO (Figure
376 S6C) suggesting they have cytotoxic capacity. Co-treatment with panobinostat and an anti-Ly6G
377 antibody decreased panobinostat mediated cell death implying that these neutrophils have anti-
378 tumor function as a result of the drug’s mechanism of action (Figure S6D). These results indicate
379 that recruitment/induction of cytotoxic neutrophils is an important mechanism by which
380 panobinostat causes cell death. We expected ICAM-1 to be expressed in the perivascular space
381 (Patnaik et al., 2017). However, it was mostly expressed on neutrophils (44%), tumor cells
382 (37%) and DCs (10%, Figure 5E). The expression of ICAM-1 by myeloid cells including
383 neutrophils suggests that they might be activated and capable of priming T cells to induce anti-
384 tumor immunity (Banchereau and Steinman, 1998; Radsak et al., 2000). Neuropilin-1 is a
385 molecule with pleiotropic function and is mostly pro-tumorigenic in other cancers (Graziani and
386 Lacal, 2015; Matkar et al., 2016; Overacre-Delgoffe et al., 2017); however, in breast cancer, it
387 was recently reported to improve class I antigen presentation machinery and cross-presentation
388 (Chawla et al., 2016; Kerros et al., 2017). The majority (up to 88%) of neuropilin-1 positive cells
389 proximal to the panobinostat well were cytotoxic neutrophils (Figure 5A, C and S5C, S6E, F).
390 The high phagocytic and tumor-killing potential, high expression of ICAM-1 and mutually
391 exclusive expression of the immune suppressive molecule arginase-1 on the panobinostat

392 induced neutrophil population (Figure S5C, S6A) indicate these are likely anti-tumor (reported
393 also as N1) rather than protumor (N2) neutrophils (Fridlender et al., 2009; Shaul et al., 2016).
394 These results raise the possibility that neutrophils induce increased MHC-I expression on
395 neoplastic cells and suggest that neuropilin-1 may be a novel biomarker of anti-tumor
396 neutrophils in BC – hypotheses that remain to be functionally tested.

397 The *distal* region was populated predominantly by tumor cells expressing high levels of galectin-
398 3, MHC-I, calreticulin and PD-L1 (Figure 5A-E; S5C and S6); the latter being expressed
399 >500um from the well at the outer border of a galectin-3 rich region (Figure 5D, S6A). Relative
400 increase of MHC-I and calreticulin expression on the surface of cells was present in a gradient
401 pattern that was highest in proximal cell death/neutrophil regions and decreased with distance
402 from the panobinostat well (Figure 5A and S6A). We observed a similar spatial cell organization
403 in a different genetically engineered BC mouse model – ErbB2 Δ Ex16 (Turpin et al., 2016)
404 (Figure S7A, B) highlighting the generality of this phenomenon.

405 Two apparent cellular barrier transition zones co-evolved with the biomarkers of immunogenic
406 cell death: (i) first at the outer *border* of the proximal neutrophil rich region composed of cancer
407 stem cells associated with DCs limiting propagation of cell death; (ii) second at the outer border
408 of galectin-3, MHC-I and calreticulin-rich region *remotely* from the well (Figure 5A-C).

409 The *border* region was populated by CSCs defined as Epcam+CD45-PyMT+ cells with nuclear
410 expression of Sox9 (Guo et al., 2012) which were intermixed with CC3+ dying cells and cell
411 debris (Figure 1L, S5B, C). CSCs have been reported to have self-renewal and tumor-initiating
412 capacity and often exhibit resistance to therapy (Jeselsohn et al., 2017; Xue et al., 2019).

413 Importantly, cellular expression of CC3 and Sox9 staining was mutually exclusive (Figure 5F
414 and Figure S5C) providing direct *in vivo* evidence that the CSCs were resistant to the most potent
415 tumor killing therapy in our screen. Inversely, galectin-3 and Sox9 were co-expressed in many
416 areas in the border region (Figure 5F) and we found that 22% of galectin-3+ cells were CSCs
417 (Figure 5E). This indicates galectin-3 might be another biomarker enriching CSCs in breast
418 cancer. Finally, macroscopic profile plots of relative cell abundance (Figure 5A), distance-based
419 cluster analyses (Figure 5D) and pairwise proximity measurements in Sox9 microcultures
420 (Figure 5G, H and S6G, H) showed that, among immune cells, CD11c+ dendritic cells were

421 preferentially located in close proximity to the CSCs suggesting functional interactions between
422 the two cell types.

423 Spindle-shaped α SMA⁺ cells – likely activated fibroblasts – consistently appeared in the *remote*
424 region (Figure 1L, 5C, S6F). We speculate that these cells may act as a barrier to physically
425 restrict cellular and molecular movements since they form a sharp boundary that appears to limit
426 the galectin-3 signal propagation (Figure 5C, top right). This α SMA barrier became increasingly
427 prominent at day 8 and more phenotypes emerged at that time that have been associated with
428 mechanisms of resistance; including K14⁺ cells comprising a tumor invasive front, immune
429 suppressive expression via arginase-1, strong deposition of Collagen VI, Ki67+Ly6G⁺ (likely
430 neutrophil extracellular trap (NET) formation (Albregues et al., 2018)), and accumulation of
431 Sox9 cancer stem cells in close proximity to both dendritic cells and fibroblasts (Figure S7C).
432 The transition of galectin-3 from diffuse spreading across the distal zone (Figure 5C, D, S5C and
433 S6A, F, G), into a sharp barrier (Figure S7C) suggests it may be a resistance marker and critical
434 component spatially connecting fibroblasts to the CSCs/DCs niche.

435 Pro-tumorigenic macrophages with low expression of CSF1R (Figure S7C) and sparse single
436 cytotoxic B cells expressing CD45R, granzyme B, galectin-3 and calreticulin (Figure S7D)
437 appeared at the outer edge of the resistant zone remotely from the well at day 8 of panobinostat
438 exposure. Expression and spatial association of galectin-3 with functionally distinct – both
439 response and resistance – phenotypes (calreticulin, antigen presentation, cytotoxicity, PD-L1,
440 stem cells, immune suppression) suggest a broad and possibly time-dependent function of this
441 protein indicating that targeting galectin-3 during immunogenic cell death should be carefully
442 considered (Figure S6D). While quantitative evaluation of the spatial cell composition of
443 immunogenic cell death across multiple time points is outside the scope of this study, the
444 appearance of spatially segregated phenotypes after three and eight days shows that spatial
445 assessment of cellular responses with increasing distance from the reservoir provides insight into
446 the sequence of emerging cellular events that follow treatment (Figure S7A) and ultimately may
447 be reverse engineered to devise effective treatment schedules (Figure 5I, Figure S8). The cause-
448 consequence spatial cell associations describing the early in situ events of induced anti-tumor
449 immunity in BC are summarized in Figure 5I.

450

451 **Combination of panobinostat, venetoclax and anti-CD40 immunotherapy maximizes tumor**
452 **killing and immune surveillance in mammary carcinoma**

453 We used information from additional MIMA analyses to identify drugs that would enhance
454 panobinostat mediated tumor killing and immune-modulation. In one analysis, we assessed
455 effects by combining panobinostat with other drugs in individual MIMA reservoirs. We
456 decreased the baseline concentration of panobinostat to approximately 25% of the original
457 concentration in order to facilitate identification of highly synergistic combinations. We then
458 measured the relative increase of cell death (CC3) and leukocyte density (CD45) compared to
459 control and panobinostat treatments after 3 days. Combinations of panobinostat with venetoclax,
460 doxorubicin and palbociclib were highly effective at increasing cell death and leukocyte density.
461 Combining panobinostat with venetoclax significantly potentiated immune modulation, while its
462 combination with doxorubicin significantly increased tumor kill (Figure 6A). In a second
463 analysis, we delivered single drugs in adjacent wells and assessed the effects in regions of drug
464 overlap between the wells (Figure 6B) over the course of eight days. These studies were carried
465 out in orthotopic MMTV-PyMT derived mammary tumors. Orthotopic models exhibit a lower
466 level of non-immune stroma at baseline (Dunn et al., 2004; Yang et al., 2017) and the eight day
467 exposure was chosen to provide sufficient time for the drugs to diffuse to create a zone of drug
468 overlap. We focused on the combinations of panobinostat with venetoclax or palbociclib in
469 subsequent experiments as panobinostat/doxorubicin combination treatment efficacy had been
470 evaluated previously (Budman et al., 2012).

471 Remarkably, the combination of panobinostat and venetoclax resulted in complete clearance of
472 tumor cells at the intersection of the two drugs (Figure 6B, Figure S9A, B). The observed TME
473 responses included significant enrichment of macrophages, dendritic cells, immature myeloid
474 cells and CD4+ T cells (Figure 1H, 6C, D and Figure S9C). A cluster of MHC-II positive DCs
475 co-expressing Ki67+ CD4 and CD8 appeared near stroma and assay region (Figure 6E and S9A)
476 suggesting the drug combination induced intra-tumoral T cell infiltration and stimulation (Broz
477 et al., 2014). However, this event was rare, and the vast majority of the panobinostat/venetoclax
478 assay area was dominated by myeloid cells (Figure 1H, 6C and S9C) implying that a myeloid
479 targeting agent rather than ICB could optimally exploit the panobinostat/venetoclax induced
480 tumor microenvironmental state.

481 Since CSF1R positive protumorigenic macrophages were not significantly enriched at the
482 intersection of wells (Figure S9A-C) and the anti-CD40 agonist antibody can positively modulate
483 DCs, immature myeloid cells (Hegde et al., 2020; Sotomayor et al., 1999) as well as resting and
484 proinflammatory macrophages (Verreck et al., 2006), we tested the possibility that anti-CD40
485 immunotherapy would further increase the efficacy of the panobinostat/venetoclax combination
486 by licensing the accumulated myeloid cell subsets as described above. We tested this by
487 systemically treating mice bearing EMT6 and E0771 orthotopic tumors and compared the
488 responses to those obtained using a panobinostat/venetoclax/anti-PD-1 combination. Treatment
489 with panobinostat/venetoclax/anti-PD-1 significantly reduced the tumor burden as compared to
490 dual panobinostat/venetoclax and panobinostat/anti-PD-1 (Figure 6G, H) treatments with
491 survival rates of 40% in mice bearing EMT6 tumors (Figure 6H). The triple combination of
492 panobinostat/venetoclax/anti-CD40, however, was superior and eliminated visible tumors in
493 100% of EMT6 tumors and 85% of E0771 tumors, respectively (Figure 6G, H). The antitumor
494 effect of panobinostat/venetoclax/anti-CD40 against spontaneous tumors arising in the MMTV-
495 PyMT model, inhibited tumor progression and doubled the overall survival (Figure 6I).

496 We note that none of the combination treatments in whole animal studies were associated with
497 adverse events, likely because we used lower concentrations of drugs than published previously
498 (Table S5). We measured underconditioned body score (level 2; as established by (Morton and
499 Griffiths, 1985)) associated with palbociclib monotherapy treatment; and two out of eight mice
500 died in the anti-CD40 monotherapy group. Lethal toxicity of anti-CD40 used as a single agent
501 was previously reported due to a shock-like syndrome (Van Mierlo et al., 2002) and our data also
502 suggest this immunotherapy is tolerable only with prior administration of anti-cancer agent(s).
503 Overall, these results suggest the triple combination of panobinostat, venetoclax and anti-CD40
504 immunotherapy as a highly synergistic therapeutic strategy for long term breast cancer control.

505

506 **DISCUSSION**

507 Much research is now underway to develop synergistic multi-drug cancer treatment strategies
508 that directly target neoplastic cells, enhance anti-tumor immune activation, normalize tumor
509 vasculature and/or favorably alter aspects of the tumor microenvironment to improve tumor
510 control. Successful strategies increasingly use combinations of drugs, each of which impacts one

511 or more components of the TME. We developed the MIMA system to efficiently develop
512 effective combination regimens by assessing tumor cell control and drug-induced changes in
513 immune and stromal composition, architecture and function that correlate with overall antitumor
514 efficacy. MIMA attributes include an IMD for delivery of nanoliter doses of multiple drugs, each
515 delivered into a spatially separate region of a single living tumor growing in an immunologically
516 intact host; a 30+ multiplex IHC analysis platform that provides a comprehensive description of
517 tumor and stromal responses to each localized drug treatment and the functional status of
518 selected immune and other tumor and stromal cells. The approach provides a highly precise,
519 multiplexed platform to systematically identify candidate biomarkers of response and quantify
520 cell interactions and to inform on treatment sequencing (Figure S8).

521 While majority of our studies were performed using a three day long microdevice implantation,
522 the in-dwelling time of the IMD can be varied from a few hours to many days in order to capture
523 a range of tissue responses and TME reorganization. Importantly, the approach is minimally
524 toxic even when testing multiple therapies in a single tumor. The local drug doses match
525 concentrations that are achieved during systemic treatments but are systemically insignificant
526 such that drug toxicity is negligible. The focal drug delivery begins at the time of implantation,
527 and follows a characteristic diffusion gradient controlled by the PEG polymer formulation in a
528 more-or-less radial direction away from each drug delivery well (Jonas et al., 2015). The focal
529 delivery can then be treated as a spatial and temporal perturbation. Analyses of the responses
530 produced by devices left in place across a range of time points provide data about drug induced
531 changes in cellular densities, molecular phenotypes, cell motility and functional interactions. In
532 addition, analyses of the regions between drug delivery wells where drugs are allowed to overlap
533 via diffusion serves as a measurement of the effect of the combination of those drugs. Since
534 distances from the drug delivery wells reflect recruitment events, computational modeling of
535 these patterns can provide actionable information to guide the development of effective drug
536 doses and schedules.

537 Although not pursued explicitly in this study, the timing of combination immunotherapies in
538 whole animals was estimated based on immune component responses at increasing distances
539 from the drug delivery wells (Figure S8). Many of these effects are difficult or impossible to
540 study in animal models treated systemically, due to heterogeneous and indeterminate drug

541 distribution that can vary greatly over different regions of a tumor. Importantly, we validate the
542 significance of the locally observed response phenotypes from MIMA studies by reverse-
543 engineering combination treatments involving targeted and immuno-therapies (Table S4) that
544 demonstrate synergistic anti-tumor efficacy in systemic studies. This is particularly important
545 since it enables systems level studies of the effects of multiple drugs in a single organism, and
546 leads to accurate predictions of responses to systemic treatments in animal models with intact
547 immune systems. Furthermore, recent work by Jonas et al has demonstrated that IMD
548 applications are safe and feasible in patients across multiple cancer indications including breast
549 cancer, prostate cancer, T cell lymphoma and glioblastoma (Dominas et al., 2021). It may
550 become feasible then to use the MIMA assay in patients to measure a range of combination
551 regimens in each patient to guide rational treatment design on a personalized basis.

552 Although intended as proof-of-concept that local nanodose drug phenotypes can effectively
553 guide systemic treatment strategies, we have already identified specific therapeutic strategies that
554 warrant clinical consideration. One finding is that the CDK4/6 inhibitor, palbociclib, recruits a
555 significant number of CSF1R+, MHC II- protumorigenic macrophages that appear to induce
556 formation of CD31+ endothelial/pericyte networks that contribute to neovascularization and
557 provide nutrients to support tumor cell proliferation. These results provide direct evidence of
558 how specific changes in tumor microenvironmental states induced by monotherapy can mediate
559 acquired resistance. We hypothesized that this protumorigenic resistance phenomenon may be
560 overcome by combining palbociclib with anti-CSF1R antibody. Our test of this concept in the
561 EMT6 model demonstrated that systemic treatment with this drug combination significantly
562 reduced tumor growth.

563 Our studies of venetoclax demonstrated that this BCL-2 inhibitor induced formation of
564 phenotypically distinct clusters of CD11c+ dendritic cells associated with immature myeloid cell
565 and endothelial cell enrichment. Many of the dendritic cells were Epcam+, CD45- suggesting
566 that they were phagocytic, while others shared morphological features of unstimulated myeloid
567 cells. However, only a small fraction of these cells were MHC-II positive and thus were likely
568 limited in their ability to respond to available tumor antigens. This finding led us to add an anti-
569 CD40 immunotherapy to increase antigen presentation, maturation and activation (aka licensing)
570 in a population that was already poised to have antitumor activity. Our test of this hypothesis in

571 the E0771 model showed that systemic treatment with a combination of venetoclax and an anti-
572 CD40 agonist indeed reduced tumor growth rate and increased overall survival as predicted
573 (Figure 3G).

574 Our demonstration that the pan HDAC inhibitor, panobinostat, increased immunogenicity (by
575 induction of immunogenic cell death and antigenicity; (Yatim et al., 2017)) of mammary
576 carcinomas when administered locally or systemically in four different animal models of breast
577 cancer corroborates previous studies showing the importance of HDAC inhibitors as pleiotropic
578 effectors of many immune surveillance processes (Conte et al., 2018). This immune component
579 is important in breast cancer since many patients do not benefit from treatments including
580 immune checkpoint blockade (Force et al., 2019). However, the exact mechanisms by which
581 HDAC inhibitors influence immune surveillance are still being elucidated (Conte et al., 2018)
582 and likely vary between inhibitors. Treatment of EMT6 and E0771 model tumors with
583 panobinostat plus anti-PD-1 increased survival duration and reduced tumor growth rate relative
584 to treatment controls or to treatment with panobinostat alone (Figure 4H, 6H). However, these
585 treatments did not achieve long term tumor control and in vaccination studies – not all mice in
586 either EMT6 and E0771 model rejected the tumor post re-challenge (Figure 4G). These studies
587 suggest that resistance mechanisms exist that might counter the full potential panobinostat
588 mediated antitumor immunity and thus we explored this treatment condition in more detail.

589 Our studies indicate that panobinostat enhances anti-tumor immunity by recruitment of antigen
590 presenting macrophages, cytotoxic and antigen presenting antitumor neutrophils and ICAM-1
591 positive myeloid cells leading to upregulation of MHC-I and calreticulin expression on tumor
592 cells. However, this is counterbalanced by the emergence of multiple potential resistance
593 mechanisms including preferential clustering of dendritic cells with cancer stem cells, the
594 formation of fibroblast/ECM barriers to treatment and antigen presentation, recruitment of
595 immune suppressive cells (arg-1), development of highly metastatic tumor cells expressing K14,
596 NETs and CSF1R+ cells. Recruitment of CSF1R+ cells at a late time point indicatings sequential
597 or alternating administration of HDAC inhibitors with CSF1/CSF1R targeting agents may be
598 efficacious while simultaneous dosing is not (Guerriero et al., 2017)) (Figure 4C and S8). Our
599 studies also identified neuropilin-1 and galectin-3 as candidate biomarkers that may inform on
600 panobinostat mechanism of action. Neuropilin-1 appears to mark anti-tumor (N1) neutrophils

601 that may potentiate antigen presentation in mammary carcinoma (Chawla et al., 2016; Kerros et
602 al., 2017) while galectin-3 expression is associated with several anti-tumor endpoints including
603 increased tumor MHC-I, calreticulin; cytotoxic granzyme B positive B cells as well as pro-tumor
604 phenotypes (cancer stem cells, immune suppression). The validated biomarker of early induced
605 antitumor immunity may allow early stratification of breast cancer patients to immune
606 checkpoint blockade. This concept may be further investigated in window of opportunity
607 treatment clinical trials.

608 Analyses of the tumor and its microenvironments in regions of panobinostat and venetoclax
609 intersection revealed almost complete tumor clearance. These two targeted anticancer agents
610 worked together to increase total intratumoral immune cell counts – panobinostat by inducing
611 neutrophils and macrophages and venetoclax by inducing recruitment of DCs and immature
612 myeloid cells. Importantly, all these myeloid cells can be positively modulated by anti-CD40
613 immunotherapy. DCs are generally thought to be the main antigen presenting cells which can
614 activate naïve T cells to become effectors (Mempel et al., 2013). However, CD40 ligation also
615 upregulates antigen presenting molecules and adhesion molecules such as ICAM-1 and increases
616 the type-1 proinflammatory state to support immunogenic processes in neutrophils, resting and
617 proinflammatory macrophages and immature myeloid cells (Oehler et al., 1998; Radsak et al.,
618 2000; Sotomayor et al., 1999; Verreck et al., 2006) (Figure 6J). Additionally, in pancreatic
619 cancer, this immunotherapy modulates the TME to degrade fibrosis (Long et al., 2016). It
620 remains to be determined if such matrix remodeling of the formed fibrotic barriers could also
621 occur in breast cancer (Figure 6J). Also, the spatial association of DCs and CSCs might be of
622 particular importance in the panobinostat/venetoclax/anti-CD40 mechanism of action. We
623 hypothesize that panobinostat induces anti-tumor immunity to bulk tumor while cancer stem
624 cells remain resistant in the TME. Venetoclax induces recruitment of DCs which we have
625 revealed to localize specifically to the CSC niche. We propose that if CD40 ligation induces
626 licensing of DCs which already captured and processed CSC antigen, this might result in
627 activation of CSC-specific anti-tumor immunity and eventually to complete tumor clearance
628 (Figure 6J). Thus, we suggest that panobinostat induces antitumor immunity on the level of bulk
629 tumor, while venetoclax/anti-CD40 induces anti-tumor immunity on the level of resistant, tumor
630 initiating cancer stem cells. This model of response is so far hypothetical as antigen specific T
631 cell responses remain to be critically evaluated. Nevertheless, our observations suggest that the

632 combination of lower dose panobinostat/venetoclax/anti-CD40, and the anti-CD40
633 immunotherapy in general, should be considered clinically for treatment of breast cancer.

634 In sum, the MIMA platform described here provides a strategy to design effective combination
635 regimens based on intratumor nanodose exposure to a range of agents, coupled with highly
636 multiplexed phenotyping and integrated spatial analysis of tumor response to each therapy. By
637 testing multiple therapeutic strategies in the same tumor, we can for the first time perform
638 systems level analysis using multiple parallel pharmacological perturbations in the same
639 organism. The low drug toxicity of intratumor nanodosing further supports clinical use of
640 MIMA, which is currently being investigated in multiple human studies. Thus, MIMA represents
641 a new approach to identification of effective combination regimens for individual patients on a
642 personalized basis. Extended use of MIMA will also open new opportunities in *in silico*
643 modeling to model dynamic drug-tumor-stromal interactions.

644

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654

655 **AUTHOR CONTRIBUTIONS**

656 Conceptualization, ZT, OJ, JWG; Methodology, ZT, OJ, JWG; Software, ZT, DCB;
657 Investigation, ZT, DCB; Data Analysis and Interpretation, ZT, OJ, JWG; Writing – original and
658 final draft, ZT, OJ, JWG; Writing – review & editing, JEK, LMH, JLM, PJS, SWA, GBM,
659 LMC; Resources, JLM, LMC, OJ, JWG; Funding, OJ, JWG; Supervision, OJ, JWG.

660

661 **DECLARATION OF INTERESTS**

662 J.E.K. is a cofounder and stock holder of Convergent Genomics.

663 GBM has licensed technologies to Myriad Genetics and Nanostring; is on the SAB or is a
664 consultant to Amphista, AstraZeneca, Chrysallis Biotechnology, GSK, ImmunoMET, Ionis,
665 Lilly, PDX Pharmaceuticals, Signalchem Lifesciences, Symphogen, Tarveda, Turbine, and
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673 and Cell Signaling Technologies; and is a member of the Scientific Advisory Boards of Syndax
674 Pharmaceuticals, Carisma Therapeutics, Zymeworks, Inc, Verseau Therapeutics, Cytomix
675 Therapeutics, Inc., and Kineta Inc., HiberCell, Inc., Cell Signaling Technologies, Alkermes, Inc.

676 O.J. is a consultant to Kibur Medical. Dr. Jonas's interests were reviewed and are managed by
677 BWH and Mass General Brigham in accordance with their conflict of interest policies.

678 JWG has licensed technologies to Abbott Diagnostics, PDX Pharmaceuticals and Zorro Bio; has
679 ownership positions in Convergent Genomics, Health Technology Innovations, Zorro Bio and
680 PDX Pharmaceuticals; serves as a paid consultant to New Leaf Ventures; has received research
681 support from Thermo Fisher Scientific (formerly FEI), Zeiss, Miltenyi Biotech, Quantitative
682 Imaging, Health Technology Innovations and Micron Technologies; and owns stock in Abbott
683 Diagnostics, AbbVie, Alphabet, Amazon, AMD, Amgen, Apple, Berkshire, Cisco systems,
684 Clorox, Colgate Palmolive, Crown Castle Int., Humana, Keysight, Linde, Proctor and Gamble,
685 Qualcomm, Unilever, Gilead, Intel, Johnson & Johnson, Microsoft, Nvidia, Taiwan
686 Semiconductor, and Zimmer Biomet.

687 The other authors declare no competing interests.

688

689 **FIGURE LEGENDS**

690 Figure 1. MIMA components and testing of locally induced drug effects on TME

691 (A) Schematic of IMDs implanted into a multifocal mouse model of mammary carcinoma (i).

692 Treatments are released from device reservoirs into spatially separated regions of tumors through
693 passive diffusion (ii). Following incubation, the tumor is extracted with the device in place and is
694 formalin fixed paraffin embedded (FFPE). Each condition is assayed individually (iii) using 30+
695 color staining (B, D).

696 (B) Schematic of the mIHC technique composed of iterative histological staining on a single
697 FFPE slide which is alternated with digital scanning microscopy to detect the target marker.

698 (C) Acquired images are co-registered with nuclear staining and the mean intensity of antibody
699 staining within a mask is calculated for each cell to count marker positive cells in an intact tissue.

700 (D) Antibody list used to interrogate a broad range of tumor intrinsic and tumor-
701 microenvironmental states as categorized and color-coded in the table. DST; drug sensitivity
702 testing.

703 (E) Multidimensionality reduction in hierarchical gating to classify standard cell types.

704 (F) List of probe combinations from the baseline discovery panel (D, top half) identifying
705 standard cell types.

706 (G) Macroscopic view of the hematoxylin-stained tumor tissue implanted with IMD.

707 Experimental condition (red box, assay area) was compared to control, untreated intra-tumor,
708 regions distant from the drug-releasing site (blue boxes). To obtain greater control over
709 cofounding variables, paired sample one tailed t-tests were used to determine enrichment of
710 induced TME states.

711 (H) Heatmap of mean percentage of positive cells (left) and level of significance (right) at
712 depicted targeted agents and chemotherapies (y-axis). Polyethylene glycol (PEG) served as
713 negative control. Total cell counts to define percentage of positivity were between 3000 to 5000
714 cells per assay area and were matched ± 300 total cells for paired samples (experimental vs
715 control region). Minimum population proportion within 5% margin of error and 95% confidence
716 level was set to 0.75% (represents 12 cells) to discriminate noise from specific signal. n=3 wells
717 from 3 tumors from 2-3 mice per treatment. MMTV-PyMT mice with late stage spontaneously

718 growing tumors were implanted for three days. *For panobinostat/venetoclax (pano/veneto)
719 condition details see Figure 6C.

720 (I-L) Presentation of selected standard cell types in XY space. [0,0] coordinate is the drug
721 releasing site; and direction of release is upward.

722

723 Figure 2. Local TME changes induced by targeted therapy palbociclib and whole animal studies
724 testing the combination efficacy with predicted anti-CSF1R immunotherapy.

725 (A) Quantification of single cell events induced by palbociclib using individual markers and
726 standard cell type classification. Bars are mean \pm s.e.; n=3 palbociclib reservoirs in three tumors
727 from three different mice. Significance was calculated by paired sample one tailed t-test. Only
728 significantly enriched cells are presented. For quantification of all TME lineages, see [Figure](#)
729 [S3A](#).

730 (B) Sample composite image of key response markers. Arrow indicates the source and direction
731 of palbociclib drug release. Dashed lines define the magnified area. Scale bar is 100 μ m (left);
732 and 25 μ m (right).

733 (C) Percentage of top five cell types expressing CSF1R stratified by zones in the palbociclib
734 assay area. Immediate pool zone analyzed is visualized by the dashed line in [Figure S3D](#). The
735 number of cells (n) quantified is presented at the top of the figure.

736 (D) Line profile of relative cell abundance as a function of distance from well (left to right).
737 Assay zones are color-coded in the legend.

738 (E) Distance-based clustering of depicted cell types as a set of XY coordinates. Coordinate [0,0]
739 identifies the drug source. The direction of the drug release is upward. Clusters were identified
740 by a minimum 10 cells within maximum distances of 50 μ m, 75 μ m and 30 μ m for CSF1R+
741 protumorigenic macrophages, endothelial/pericyte network and proliferating tumor cells,
742 respectively. Each cluster is depicted with a randomized color; clusters were merged and share
743 one color if present within the maximum distance range. Individual (non-clustering) cells are
744 shown as smaller light gray points.

745 (F) Palbociclib model of response presented as line diagram and site of intervention using
746 immunotherapy depicted in red.

747 (G) Tumor burden measurement of mice bearing EMT6 tumors after systemic treatment using
748 drugs as color-coded in the graph. Shown is mean \pm s.e.; n=8 to 10 tumors per group.
749 Significance was calculated using an independent two-sample two-tailed t-test with equal
750 variance.

751
752 Figure 3. Local TME changes induced by Venetoclax and whole animal studies testing the
753 combination treatment efficacy with the predicted anti-CD40 immunotherapy.

754 (A) Quantification of single cell events using individual markers and standard cell types. Bars
755 are mean \pm s.e.; n=3 venetoclax reservoirs in two tumors from two different mice. Significance
756 was calculated by paired sample one tailed t-test. Only significantly enriched cells are presented.
757 For quantification of all cells, see [Figure S4A](#).

758 (B) Marker co-expression in XY coordinates in the palbociclib (left graph) and venetoclax (mid
759 and right graph) assay area. Each color-coded dot represents a marker positive cell. Coordinate
760 [0,0] identifies the drug source. The direction of the drug release is upward.

761 (C) Distance-based cluster analysis of CD11c positive cells as a set of XY coordinates in random
762 intratumoral (left) and venetoclax assay (right) regions. Clusters are displayed in randomized
763 colors if at least 10 cells are present within maximum distance range 50 μ m; individual (non-
764 clustering) cells are shown as light gray points.

765 (D) Sample composite image of key response markers as color-coded on the left. The arrow
766 indicates the source and direction of the venetoclax drug release. DHashed boxes define the
767 magnified area on the right. Here individual markers are overlaid on DNA signal (in white).
768 Scale bar 100 μ m; and 30 μ m for the magnified image.

769 (E) Percentages of Epcam and CD45 (top) and CD11b and MHC-II (bottom) positive cells
770 within morphologically different CD11c + cells presented as a stack bar graph. The number of
771 cells (n) in each analysis is presented above the bar. Two to three ROIs from two venetoclax
772 samples were analyzed and summed per each zone.

773 (F) A model of venetoclax response presented as an influence diagram with sites of intervention
774 using immunotherapy depicted in red. The relation of morphologically distinct and spatially
775 separate CD11c DC clusters remains unclear (gray dashed arrows).

776 (G) Survival rates (left) and tumor burden measurements (right) of mice bearing E0771 tumors
777 after systemic treatment using drugs as color-coded in the line graphs. Shown is mean \pm s.e.;
778 n=7-8 mice per group. Significance was calculated by log-rank (Mantel-Cox) and by an unpaired
779 two-tailed t-test with equal variance for survival and tumor burden rate, respectively. The
780 treatment dose and schedule are presented schematically. For single treatment effects of anti-PD-
781 1 and anti-CD40 see [Figure 6H](#).

782

783 Figure 4. Local effects of panobinostat and whole animal studies testing induction of anti-tumor
784 immunity in mouse mammary carcinoma

785 (A) Quantification of single cell events using individual markers and standard cell types. Bars
786 are mean \pm s.e.; n=3 panobinostat reservoirs in two tumors from two different mice. Significance
787 was calculated by paired sample one tailed t-test. Only significantly enriched cells are presented
788 here; for quantification of all cell, see [Figure S5A](#).

789 (B) Composite image of the most prominent markers appearing at the panobinostat reservoir as
790 color-coded on the left. An arrow indicates the source and direction of the drug release. A dashed
791 box defines the magnified area (right), which shows F4/80 staining in red and DNA signal and
792 DNA-derived mask (white). Ly6G, MHC-II, aSMA or F4/80 expression was not enriched in
793 random, drug-remote, intratumoral regions (far right). Scale bar, 100 μ m.

794 (C) Quantification of PEG normalized average mean CC3 intensity (px value) in the assay
795 region. The graph shows mean \pm s.e signal intensity; n=3 wells from 3 tumors from 2-3 mice per
796 treatment; significance was calculated using an independent two-sample t-test with equal
797 variance. CSF1R inhibitor (PLX3397 in mouse chow at average 40mg/kg dose) was
798 administered for seven days before the three-day-long IMD application.

799 (D) CC3 IHC image of a sectioned tissue surrounding the IMD at depicted targeted agents and
800 chemotherapies. Three replicates are presented for the most potent death-inducing drug,
801 panobinostat. A computationally processed CC3 signal for the 20% panobinostat image is shown
802 in the lower right as a binary image.

803 (E) Marker co-expression in XY coordinates. Each dot represents a marker positive cell as color
804 coded on the bottom. Coordinate [0,0] identifies the drug source. The direction of the drug
805 release is upwards.

806 (F) Percentage of MHC-II+ neutrophils. Shown is mean \pm s.e.; n=3 panobinostat reservoirs.

807 (G) Induction of anti-tumor immunity measured in a vaccination study using panobinostat treated
808 cells and negative control (cells killed by three freeze/thaw cycles). Line graphs show
809 percentages of mice free from palpable tumors. The P-value was calculated by log-rank (Mantel-
810 Cox) test. n=7 per each group for E0771 model; and n=4 (control) and n=5 (experimental) for
811 EMT6 model, respectively.

812 (H) Quantification of intratumoral CD8+ T cell infiltration into ErbB2 Δ Ex16 spontaneously
813 growing tumors 7 days after systemic panobinostat treatment. The central mark indicates median
814 and the bottom and top edges of the box indicate 25th and 75th percentiles, respectively. Green
815 diamonds show the means. n=8 randomly selected ROIs using four tumors from two to three
816 mice per group. A two-color composite image of Epcam and CD8 staining (top right) and a
817 processed image showing intraepithelial CD8+ cells depicted in white (bottom right). Scale bar,
818 100 μ m.

819 (I) Survival rates (left; 100% to 0%) and tumor burden measurements (right) of E0771 tumor
820 bearing mice treated with depicted treatments as color-coded in the line graphs. Mean \pm s.e. per
821 timepoint are presented; n=8 to 12 mice per group. Significance was calculated by log-rank
822 (Mantel-Cox) and by unpaired two-tailed t-test with equal variance for survival and tumor
823 burden rate, respectively. The treatment dose and schedules are presented.

824

825 Figure 5. Spatial cell analyses of immunogenic cell death biomarkers and associated resistance
826 mechanisms

827 (A) Profile plot of the relative abundance of standard cell types and individual biomarkers with
828 distance from the well and overlay with the assay zones (colored vertical lines).

829 (B) A schematic presentation of cell phenotype separation into zones with distance from the
830 panobinostat reservoir.

831 (C) 3D composite image showing biomarkers associated with immunogenic cell death and
832 α SMA barrier limiting the propagation of these biomarkers presented in green. The white arrow
833 indicates the source and direction of the panobinostat release. A profile plot of relative cell
834 abundance at the depicted area (dashed) is presented top right.

835 (D) Distance-based clustering of Sox9, CD45, CD11c and galectin-3 positive cells (top) and PD-
836 L1 and galectin-3 positive cells (bottom) in XY coordinates with overlay (black line) on Sox9
837 and PD-L1 cluster border, respectively. Individual clusters were identified by a minimum 10
838 cells within a maximum 50 μ m distance for all but PD-L1 marker which clustered with a
839 maximum distance set to 150 μ m.

840 (E) Percentages of cells expressing biomarkers of ICD on standard cell types presented in form
841 of a stack bar graph. The number of cells quantified is presented above the bar.

842 (F) A composite image showing mutually exclusive staining of Sox9 and CC3; and co-
843 expression of Sox9 with galectin-3 (bottom left image). Scale bar; 100 μ m.

844 (G, H) Number of Sox9+ pairwise distances with other marker positive cells presented in form of
845 a histogram (G) and bar graph showing average proportion of Sox9 pairwise distances which
846 were less than 50 μ m (H). n=4 ROIs of 175 μ m diameter in the border assay zone. Significance
847 was determined by paired two tailed t test.

848 (I) Line diagram of a proposed panobinostat mechanism of action determined by MIMA and
849 sites of intervention (depicted in red). Phenotypes that remain to be tested/validated are presented
850 in gray color. All experiments were performed using the MMTV-PyMT mice with late stage
851 spontaneously growing tumors and a three-day device implant.

852

853 Figure 6. Efficacy of the triple combination of panobinostat, venetoclax and anti-CD40
854 immunotherapy in mammary carcinoma and rationale for the combination.

855 (A) Effects of drug combinations on CC3 and CD45 expression as measured by IHC at three
856 days of exposure. Graph shows mean \pm s.e normalized signal intensity. n=3-10 wells from at
857 least 3 tumors from 2-3 MMTV-PyMT mice with spontaneous tumors per treatment; significance
858 was calculated by independent two-sample t-test with equal variance.

859 (B) Macroscopic view of a hematoxylin-stained tumor tissue section showing the intersection
860 between two drugs. Drug release sites are shown by black arrows. The device was implanted for
861 eight days in MMTV-PyMT tumor induced by orthotopic implant into mammary fat pad of
862 syngeneic mice. Note the tumor cleared region lacking nucleated cells at the intersection of
863 panobinostat and venetoclax.

864 (C) Quantification of single cell events using individual markers and marker combinations for
865 standard cell types. Bars are mean \pm s.e.; n=2 reservoirs at the intersection of panobinsostat and
866 venetoclax. Significance was calculated by paired sample one tailed t-test. Only significantly
867 enriched cells are presented. For quantification of all cells, see [Figure S9C](#).
868 (D) A five-color 3D composite image showing key response markers induced at the intersection
869 of panobinostat and venetoclax. White arrows indicate the source and direction of the drug
870 release. CD11c dendritic cell marker is presented in high view. Scale bar; shown.
871 (E, F) Limited infiltration of CD4+, CD8+ T cells and CD45R+ B cells localized to MHC-II
872 antigen presenting CD11c+ dendritic cells lacking CD11b pan-myeloid marker expression (E)
873 and quantification of their Ki67 proliferative and Foxp3 regulatory potential (F).
874 (G, H, I) Survival rate (left and bottom; 100% to 0%) and tumor burden measurements (right and
875 top) over time in E0771 (G), EMT6 (H) orthotopically induced tumor bearing mice and MMTV-
876 PyMT mice with spontaneously growing tumors (I). C, control; P, panobinostat, PV,
877 panobinostat-venetoclax combination. Treatment schedules and doses match those in [Figure 3F](#)
878 and [4H](#) except the doses for panobinostat and venetoclax were decreased to 11.5mg/kg and
879 18mg/kg, respectively, when drugs were combined. For survival rate, P-value was calculated by
880 log-rank (Mantel-Cox). For tumor burden, line graphs are mean \pm s.e. per timepoint; n= 8-12
881 mice, and 6-12 tumors and 6-8 mice per group in (G), (H) and (I), respectively. Significance was
882 calculated by unpaired two-tailed t-test with equal variance.
883 (J) Hypothetical model of response for panobinostat/venetoclax/anti-CD40 triple combination
884 treatment efficacy in breast cancers. Briefly, the tumor is composed of bulk tumor and cancer
885 stem cells (i). Panobinostat induces immunogenic cell death of the bulk tumor while cancer stem
886 cells remain resistant in the tumor microenvironment (ii). Venetoclax induces recruitment of
887 dendritic cells in close proximity to cancer stem cells (iii). We hypothesize that if CD40 ligation
888 induces licensing of dendritic cells which captured and processed antigen from neighboring
889 CSCs, the triple combination potentiates CSC-specific anti-tumor immunity leading to complete
890 tumor rejection (iv).

891

892 **STAR METHODS**

893 **CONTACT FOR REAGENT AND RESOURCE SHARING**

894 Further information and requests for resources and reagents should be directed to and will be
895 fulfilled by the lead contact Joe W. Gray (grayjo@ohsu.edu).

896

897 **EXPERIMENTAL MODELS AND SUBJECT DETAILS**

898 **Murine Models**

899 Mice were purchased from The Jackson Laboratory. All animal studies were conducted in
900 accordance with protocols approved by Institutional Animal Care and Use Committee (IACUC)
901 at OHSU (protocol number: IP00000956). All mice were bred and housed under specific
902 pathogen free conditions under a standard 12h light / 12h dark cycle. C57LB/6, BALB/c, and
903 FVB/N mice were purchased from the Jackson Laboratory. MMTV-PyMT were from Dr. Lisa
904 Coussens and purchased from the Jackson Laboratory. Virgin female mice of 8-24 weeks of age
905 were used for all experiments.

906

907 **Cell lines**

908 EMT6 (mouse breast cancer) cells were purchased from American Type Culture Collection and
909 were maintained in Waymouth's medium with 10% FBS, and 2mM L-glutamine. E0771 (mouse
910 breast cancer) cells were purchased from CH3 BioSystems® and were cultured in RPMI-1640
911 with 10% FBS and 10mM HEPES. Both cell lines were pathogen tested and were grown at 5%
912 CO₂ and 37C.

913

914 **METHOD DETAILS**

915 **Experimental design**

916 The objective of the studies in figures is to show how intact tumor microenvironment responds to
917 local stimulus of drug release and to test whether this response was significantly different from
918 the baseline tumor microenvironmental state in tumor region distant from the drug site. The
919 number of independent biological replicates of each experiment (n) performed are given in the
920 figure legends. Spatial systems analyses were designed to quantitatively define directional spatial
921 cell dependencies and cause consequence association with distance from the reservoir translating
922 to models of drug response. Within these models we aimed to identify therapeutic vulnerabilities
923 to predict rational immune or TME modulating treatment combinations and their optimal
924 schedule/sequencing.

925

926 **Microdevice implantation studies and sample collection**

927 Nanodose drug delivery devices were manufactured and implanted as described previously in
928 (Jonas et al., 2015). Briefly, cylindrical microdevices 5.5mm in length and 750 μ m in diameter
929 were manufactured from medical-grade Delrin acetyl resin blocks (DuPont) by micromachining
930 (CNC Micromachining Center) with 18 reservoirs 200 μ m (diameter) x 250 μ m (depth) on the
931 outer surface. Reservoirs were packed with drugs mixed with Polyethylene glycol (PEG, MW
932 1450, Polysciences) polymer at the concentrations indicated in Table S1. Recommended
933 systemic dose in cancer patients was derived from the <https://rxlist.com> web page to June 2017.
934 Systemic doses ranging between 0-1mg/kg, 1-2mg/kg, 2-4mg/kg, >4mg/kg translate to 20%,
935 25%, 30% and 40% of drug concentration in PEG, respectively, when released from the
936 nanowell. The calibration was determined previously using mass spectrometry measurements
937 (Jonas et al., 2015). Pure PEG was used in control conditions. Implanting multiple devices per
938 tumor and/or multifocal animal model can increase the throughput up to 50-70 times as
939 compared to conventional systemic treatment studies. When two drugs were loaded into one
940 reservoir, they were at approximately 1:1 ratio. The combination partner was loaded on the
941 bottom of the well; panobinostat was released first. Microdevices were implanted for three days
942 in MMTV-PyMT and ErbB2 Δ Ex16 mice with late stage spontaneously growing tumors in all
943 experiments but those presented in [Figure 6](#) and [S9](#). Tumor size was between 1.2 - 1.5cm in the
944 longest dimension at the time of implant. Tumors were excised at three days after device
945 implantation unless otherwise stated, fixed for 48h in 10% formalin or 4% paraformaldehyde,
946 then perfused with paraffin. Specimen were sectioned using a standard microtome and 5 μ m
947 tissue sections were collected from each reservoir. Dry FFPE tissues were baked in a 65`C oven
948 for 30mins. Following deparaffinization with xylene and rehydration in serially graded alcohol to
949 distilled water, slides were subjected to endogenous peroxidase blocking in fresh 3% H₂O₂ for 10
950 minutes at RT. Sections were then stained by multiplex immunohistochemistry and/or cyclic
951 immunofluorescence (see also [Figure S1B, C](#)).

952

953 **Cyclic Immunofluorescence**

954 Before iterative cycles of (i) staining, (ii) whole slide scanning and (iii) fluorophore bleaching,
955 the slides were subjected to heat-mediated antigen retrieval immersed in citrate buffer (pH 5.5,

956 HK0809K, BioGenex Laboratories Citra Plus Antigen Retrieval), then in Tris/EDTA buffer (pH
957 9.0, S2368, Dako Target Retrieval Solution) using Cuisinart Electric Pressure Cooker (CPC-
958 600N1) for total of 35 to 40 minutes. Protein blocking was performed for 30 minutes RT with
959 10% normal goat serum (S-1000, Vector Lab) and 1% bovine serum albumin (BP1600-100) in
960 1xPBS. (i) Slides were incubated with primary antibody (concentrations defined in Table S2) for
961 2 hours at RT while being protected from light in a dark humid chamber. All washing steps were
962 performed for 3 x 2-5 minutes in 1xPBS while agitating. Slides were mounted with SlowFade
963 Gold antifade mountant with DAPI (S36938) using a Corning Cover Glass (2980-245). (ii)
964 Images were acquired using Zeiss Axio Scan.Z1 Digital Slide Scanner (Carl Zeiss Microscopy)
965 at 20x magnification after which the coverslips were gently removed in 1xPBS while agitating.
966 (iii) Fluorophores were chemically inactivated using a 3% H₂O₂ and 20mM NaOH in 1xPBS for
967 30 minutes at RT while being continuously illuminated. The fluorophore inactivation was
968 repeated twice with a short, 10-minute, 1xPBS wash in between. Efficacy of bleaching was
969 imaged before antibody incubation (baseline autofluorescence) and every third to fourth cycle in
970 average. After protein blocking, samples were subjected to the next round of staining. Single cell
971 feature extraction was not applied to evaluate sections stained by cyclic immunofluorescence.

972

973 **Multiplex Immunohistochemistry**

974 Before iterative cycles of (i) staining, (ii) whole slide scanning and (iii) and heat and chemical
975 stripping of antibodies and chromogen, the slides were subjected to staining with F4/80 and
976 CSF1R antibodies (cycle zero, no antigen retrieval, [Table S3](#)) and hematoxylin staining (S3301,
977 Dako) for 1-5mins followed by whole slide scanning. Slides were then subjected to the first heat-
978 mediated antigen retrieval in 1x pH 5.5-6 citrate buffer (Biogenex Laboratories, HK0809K) for
979 90 seconds in a low power microwave and 16 minutes in a steamer followed by protein blocking
980 with 10% normal goat serum (S-1000, Vector Lab) and 1% bovine serum albumin (BP1600-100)
981 in 1xPBS for 30 minutes RT. (i) Slides were incubated with primary antibodies (concentrations
982 defined in [Table S3](#)) for 1 hour at RT or 16-17 hours at 4 degrees Celsius while being protected
983 from light in a dark humid chamber. Signal was visualized with either anti-rabbit or anti-rat
984 Histofine Simple Stain MAX PO horseradish peroxidase (HRP) conjugated polymer (Nichirei
985 Biosciences) followed by peroxidase detection with 3-amino-9-ethylcarbazole (AEC). Two or
986 three drops of HRP polymer were used for up to nickel-size or whole slide tissue sample,

987 respectively. Timing of AEC development was determined by visual inspection of positive
988 control tissue (Figure S1 D-F) for each antibody. All washing steps were performed for 3 x 5-10
989 minutes in 1xPBS while agitating. Slides were mounted with a filtered 1xPBS with 0.075%
990 Tween20 (BP337100) using a Signature Series Cover Glass cover glass (Thermo Scientific,
991 12460S). (ii) Images were acquired using the Aperio ImageScope AT (Leica Biosystems) at 20x
992 magnification after which the coverslips were gently removed in 1xPBS while agitating. (iii)
993 Within one cycle, removal of AEC and HRP inactivation was accomplished by incubating the
994 slides in 0.6% fresh H₂O₂ in methanol for 15 minutes; AEC removal and stripping of antibodies
995 was accomplished by Ethanol gradient incubation and heat-mediated antigen retrieval such as
996 described above between cycles (see also Figure S1B) (Banik et al., 2020; Tsujikawa et al.,
997 2017). After washing and protein blocking, samples were subjected to the next round of staining.
998

999 **Image processing and feature extraction of mIHC images**

1000 The iteratively digitized images were co-registered using Matlab (The MathWorks, Inc., Natic,
1001 MA, version 2019b) utilizing the detectSURFFeatures algorithm from the Computer Vision
1002 Toolbox. The imperfectly registered images were additionally processed using the Linear Stack
1003 Alignment with SIFT plugin (Fiji) so that cell features overlap down to a single pixel level.
1004 Hematoxylin-stained images were color deconvoluted for single cell nuclear segmentation to
1005 generate a binary mask using watershed function and standard image processing steps (noise
1006 removal, erosion, dilation; Fiji) (Schneider et al., 2012). AEC chromogenic signal was extracted
1007 using the NIH plugin RGB_to_CMYK to separate AEC signal into the yellow channel for
1008 improved sensitivity of IHC evaluation (Banik et al., 2020; Pham et al., 2007). Gray scale images
1009 of all proteins and the binary mask were imported to CellProfiler (version 3.1.8, Broad Institute)
1010 (Carpenter et al., 2006) to quantify single cell signal mean intensity as defined by mask which
1011 was scaled to a range 0-1. IdentifyPrimaryObjects module was used to identify nuclei from
1012 mask; MeasureObjectIntensity module measured mean intensity for each object for each protein.
1013 The mean signal intensity per cell output was imported to FCS Express 6 and 7 Image Cytometry
1014 Software (DeNovo Software) to perform multidimensionality reduction to classify “cell
1015 standards”. Gating strategies and hierarchical cell classification is presented in Figure 1E and
1016 Figures S2E and F. Polygonal gates moving around central vertex without changing the polygon
1017 shapes was used to obtain quantitatively reproducible multiplex data, batch to batch, independent

1018 of the condition measured. Positive control tissues were used to help to define single parameter
1019 threshold for positivity by manual gating. Total of 3000-5000 cells were analyzed for feature
1020 extraction in the assay area located above the drug releasing site with ± 300 total cells for paired,
1021 experimental vs control, region. Minimum population proportion within 5% margin of error and
1022 95% confidence level was set to 0.75% (represents 12 cells) to discriminate noise from specific
1023 cell enrichment induced by e.g. increased protein expression or cell recruitment into the assay
1024 region. Experimental condition of the assay area was compared to random control intratumoral
1025 region located perpendicular and/or far from the drug-releasing reservoir. To obtain greater
1026 control over confounding variables, paired sample one tailed t-tests were used to determine
1027 enrichment of induced TME states. Percentage of positivity and significance was presented in
1028 form of a heatmap or bar graphs. Quality of the single cell data was ensured by excluding
1029 deformed (folded), lost or unevenly stained tissue (border effects). The assay area was
1030 determined by the first 3000-5000 cells above the well excluding these deformed regions. Single
1031 cell data from FCS Express was extracted in data grid to Matlab for downstream spatial systems
1032 analyses. In computed images, neutrophils are presented independent of the Epcam \pm status.

1033

1034 **Spatial Systems Analyses**

1035 Distance based cluster function finds clusters in a set of spatial points expressed in XY space
1036 (adapted and modified from Yann Marcon; Matlab October 2019). The clustering is based on
1037 Euclidean distance between the points (cells). The function does not require the number of
1038 clusters to be known beforehand. Each cell clusters with the closest neighboring cell if distance
1039 between the two cells is shorter than the defined threshold. Minimal number of cells per cluster
1040 are defined by user. The function outputs non-clustering cells in gray color while each cluster
1041 meeting the defined parameters (minimal number of cells within maximum distance range) are
1042 presented in randomized colors. Clusters within the maximum defined distance merge and share
1043 one color. Number of clusters and total coverage in the assay area was calculated using distinct
1044 cluster sizes (defined by minimal number of cells within maximum distance range, [Figure S3F](#))
1045 for control PEG and palbociclib which identified that cells cluster in response to treatment if
1046 minimum 10 cells are present within maximum distance range 30-75 μ m. Cluster parametrization
1047 using as few as 5 cells and distances as large as 100 μ m resulted in treatment non-specific cluster
1048 formation in PEG negative control. Treatment specific cluster formation with cluster definition

1049 of minimum 10 cells within 50 μ m distance was generalizable to all marker and standard cell
1050 types which was confirmed in panobinostat condition by comparing assay area and distal region
1051 side by side in one field of view (Figure S6G). This treatment specific cluster parametrization
1052 was applied in downstream analytics to identify hotspots/zones of interest (e.g. *proximal*, *border*,
1053 *distal*, network adjacent, CD11c+ DC clusters) in an objective, biology driven, manner.

1054 For the relative abundance profile plot, marker positive cells and the standard cell types were
1055 extracted to XY coordinate space, signal was blurred using Gaussian Blur filter and relative
1056 abundance of positive cells was displayed with distance from the well in a profile plot as outlines
1057 in Figures S3B and S6A. A moving average filter with 50 μ m; and 100 μ m window size
1058 (movmean function; Matlab) was additionally applied to smoothen the feature signal for
1059 palbociclib and panobinostat condition, respectively. Signal in the profile plots was not scaled.

1060 Inside the hotspot, spatial (geographical) interactions between marker positive cells were
1061 determined by proximity measurements in local microculture by using the pdist2 function in
1062 Matlab (MathWorks, Inc., Natic, MA, version 2019b) which returns the distance of each pair of
1063 observations (positive cells) in X and Y using metric specified by Euclidean distance. Random
1064 circular regions of 175 μ m diameter (defined by Figure S6H) were selected in the border, cancer
1065 stem cell, zone of the panobinostat assay area and Euclidean distance was measured between
1066 Sox9+ and other marker positive cells. The number of distances was presented in form of a
1067 histogram. To quantify spatially interrelated phenomenon, proportions of distances lower than
1068 50 μ m (as defined by distance-based cluster analyses) was compared between different cell pairs
1069 (e.g. Sox9+/Ly6G+ vs Sox9+/CD11c+).

1070 Extended hierarchical cell classification was applied to characterize the significantly enriched
1071 cell phenotypes forming zones of interest which were outside the standard cell type classification
1072 (e.g less differentiated macrophages or phagocytic DCs). Probe combination, number of cells
1073 analyzed within number of clusters are defined in the figures and figure legends.

1074 2D composite and 3D composite images were presented by using Fiji (Schneider et al., 2012)
1075 and QiTissue Quantitative Imaging System (<http://www.qi-tissue.com>).

1076 The spatial systems analyses were used to identify drug models of response (presented as line
1077 diagrams) and the identified therapeutic vulnerabilities were tested in whole animal studies.

1078

1079 **Whole animal treatment studies**

1080 MMTV-PyMT transgenic mice that were 80 days old were randomized and included in the study
1081 when their total tumor burden was between 150-550mm³ (treatment initiation). For the
1082 orthotopically induced tumor models of mammary carcinoma, EMT6 (0.5 x 10⁶ in 1xPBS per
1083 site), E0771 (0.5 x 10⁶ in Corning matrigel per site) and primary tumor derived LPA3 (0.8 x 10⁵
1084 in Corning matrigel per site) cells were injected into the #4 mammary fat pad of female virgin
1085 C57LB/6, BALB/c, and FVB/N mice, respectively. One tumor was induced in the E0771, LPA3
1086 models and two tumors were induced in the EMT6 model. Caliper measurements were used to
1087 calculate the tumor volumes using formula length x width² / 2. Treatments were initiated when
1088 total tumor burden was between 60-150mm³ or as defined in the figure legend (Figure S5E, F).
1089 For all models, the endpoint was determined by tumor volume above 2000mm³ in two
1090 consecutive measurements or one measurement above 2200mm³. Treatments were administered
1091 by intraperitoneal injection. Dose, schedule and duration are indicated in the respective figures
1092 and figure legends. Treatment schedule was estimated depending on the location of the targetable
1093 cell phenotype in proximity to the well or more distal from the drug source. E.g. cells in the
1094 *immediate proximity* to the drug well at 3 days of exposure were likely recruited first to the drug
1095 assay area thus early targeting (pre-treatment) of these cells is preferred. Inversely, cells located
1096 in *distal* regions at late timepoints (e.g. day 8) should be targeted by posttreatment approach. See
1097 also Figure S8. Diluent and IgG2a isotype control (BioXCell) concentrations were equivalent to
1098 the highest dose of the respective drug used in each experiment.

1099 The mice were monitored daily to determine any possible effects on the general condition of the
1100 animals using parameters as established by (Morton and Griffiths, 1985). The guidelines for
1101 pain, discomfort and distress recognition were used to evaluate weight loss, appearance,
1102 spontaneous behavior, behavior in response to manipulation and vital signs. Specifically, general
1103 appearance (dehydration, missing anatomy, abnormal posture, swelling, tissue masses, prolapse)
1104 skin and fur appearance (discoloration, urine stain, pallor, redness, cyanosis, icterus, wound,
1105 sore, abscess, ulcer, alopecia, ruffled fur), eyes (exophthalmos, microphthalmia, ptosis, reddened
1106 eye, lacrimation, discharge, opacity), feces (discoloration, blood in the feces, softness/diarrhea),
1107 locomotor (hyperactivity, coma, ataxia, circling) were monitored to determine loss of body
1108 condition (BC) score, namely: BC 1 (emaciated) score applied when skeletal structure was

1109 extremely prominent with little or no flesh/muscle mass and vertebrae was distinctly segmented;
1110 BC 2 (under-conditioned) score applied when segmentation of vertebrate column was evident,
1111 dorsal pelvic bones were readily palpable and muscle mass was reduced; BC 3 (well-
1112 conditioned) applies when vertebrae and dorsal pelvis were not prominent/visible, and were
1113 palpable with slight pressure. Loss of BC was also considered when anorexia (lack or loss of
1114 appetite) or failure to drink; debilitating diarrhea, dehydration/reduced skin turgor; edema,
1115 sizable abdominal enlargement or ascites, progressive dermatitis, rough hair coat/unkept
1116 appearance, hunched posture, lethargy, loss of righting reflex, neurological signs or bleeding
1117 from any orifice appeared in treated mice. Majority of treated groups were well-conditioned (BC
1118 score 3); less than 20% of mice in each group experienced mild diarrhea for up to 2 days once
1119 during the course of treatment (typically post first or second therapy administration). Mice
1120 receiving palbociclib monotherapy were under-conditioned (BC score 2) starting from day 3 till
1121 the end of the treatment. Two out of eight mice in the MMTV-PyMT model died within 1-3 days
1122 after first injection of α CD40 immunotherapy when administered as single agent. Surviving mice
1123 receiving Venetoclax/ α CD40 combination experienced fur graying to different degree starting
1124 approximately four weeks post treatment. No signs of pain, discomfort or distress were observed
1125 in the surviving mice. Emaciated (BC score 1), over-conditioned (BC score 4) nor obese (BC
1126 score 5) were observed in our studies. LPA-3 mice become obese with tumor development but
1127 this sign was independent of administered treatment (treatment naïve).

1128 To measure CD8⁺ T cell infiltration inside the tumor bed, ErbB2 Δ Ex16 mice with spontaneously
1129 growing tumors were intraperitoneally injected with panobinostat (15mg/mg) on day 0, 2 and 4.
1130 Tumors were extracted at day 7, were FFPE processed and were stained for Epcam and CD8 to
1131 compare rate of intratumoral (Epcam⁺) vs stromal (Epcam⁻) CD8⁺ T cells in panobinostat
1132 treated vs control (diluent) treated tumors.

1133

1134 **Vaccination study**

1135 EMT6 and E0771 cells in tissue culture were treated with a soluble drug panobinostat at 5 μ M
1136 concentration when they would reach 60-70% confluency. After two days the cells (80-90%
1137 death rate) were harvested and were injected subcutaneously (total 2-3 x 10⁶ cells) into lower left
1138 flank of BALB/c and C57Bl6 mice, respectively. Cells freeze-thawed three times served as

1139 negative control for non-immunogenic form of cell death. After 7-8 days, the mice were re-
1140 inoculated by injecting living cells orthotopically into one #4 mammary fat pad (total 0.5×10^6
1141 cells) and tumor appearance was monitored by minimal tumor size approximately 5mm and
1142 3.5mm in the longest dimension for E0771 and EMT6 model, respectively (palpable tumors). We
1143 note the E0771 tumors after re-challenge appeared at the primary subcutaneous site and no
1144 tumors were developed in the orthotopic site.

1145

1146 **Statistical analysis**

1147 All data are combined from two to three independent experiments, unless specifically noted. To
1148 accomplish randomization for systemic mouse experiments, animals were sorted by a blinded
1149 investigator and then groups were assigned. Each group was checked post-hoc to verify no
1150 statistical significance in average starting tumor size. There was no sample-size estimation of in
1151 standard drug treatment experiments. Data are shown as mean \pm SEM, unless otherwise noted.
1152 For tumor growth rate, significance was calculated by unpaired two-tailed t-test with equal
1153 variance. For survival and tumor free analyses, Kaplan-Meier curves were generated to
1154 demonstrate time to event and log-rank (Mantel-Cox) test was used to evaluate statistical
1155 significance.

1156

1157 **SUPPLEMENTAL INFORMATION**

1158 **SUPPLEMENTARY TABLE LEGENDS**

1159 Table S1. Drug list and drug concentration calibration used in the MIMA system; Related to
1160 Figure 1.

1161 Table S2. Antibody order, catalog and concentration used in mouse multiplex IHC; Related to
1162 Figure 1.

1163 Table S3. Antibody order, catalog and concentration used in mouse cycIF; Related to Figure 1.

1164 Table S4. Rationale to select effective immunotherapy or non-immune stroma modulating
1165 combination partner based on the TME signature induced by primary treatment.

1166 Table S5. Comparison of systemic drug dosing in our and the previously reported studies.

1167

1168 SUPPLEMENTARY FIGURE LEGENDS

1169 Figure S1. Components of the MIMA system and mIHC/cycIF anti-mouse antibody validation;
1170 Related to Figure 1.

1171 (A) Dosing for individual drugs was calibrated using mass spectrometry measurements
1172 comparing concentration of the same drug in situ after systemic treatment versus after local
1173 delivery (adapted and modified from (Jonas et al., 2015)). Sample images of intratumoral
1174 doxorubicin distribution at 6 hours after systemic treatment (top left image) and PEG-formulated
1175 doxorubicin transport from device at 20 hours after release (bottom left image). Signal mean
1176 intensity was extracted (n=3 each) and plotted using a moving average window filter to
1177 smoothen the signal (right). For detailed information on the pharmacokinetics of intratumoral
1178 drug release from the IMD see (Jonas et al., 2015).

1179 (B,C) Schematic overview of multiplex immunohistochemistry (mIHC; B) and cyclic
1180 immunofluorescence (cycIF, C). (C) mIHC utilizes indirect staining, iterative deposition of
1181 chromogen/enzyme pairs and brightfield microscopy to image the target signal. The chromogen
1182 used in this study is called 3-amino-9-ethylcarbazol (AEC) and it produces a red precipitate
1183 when visualized with polymer-based peroxidase conjugated to a secondary antibody (anti-rabbit
1184 or anti-rat; Histofine® Simple Stain). AEC is susceptible to organic solvents which is used to
1185 remove the red signal and detect two target proteins in one cycle. Primary antibody mixture is
1186 stripped in heated low pH citrate buffer is every cycle after scanning in order to further multiplex
1187 the staining on a single FFPE slide. Antibodies raised in rabbit and rat hosts alternate to prevent
1188 crosstalk between cycles. Hematoxylin counterstains nuclei to allow cell count and downstream
1189 image analysis (Figure S2). (C) cycIF utilizes fluorophores as reporters via direct labeling. Four
1190 to five non-overlapping fluorescent signals can be detected in a single cycle against dark
1191 background. DAPI signal is used to visualize nuclei for cell count. Progressive staining is
1192 enabled by inactivating the fluorophore using base hydrogen peroxide mixture and heat.
1193 Antibody specificity is cross-validated by performing chromogenic mIHC on the adjacent tissue
1194 section.

1195 (D) List of biomarkers (left column) and positive control tissues used for antibody validation and
1196 signal thresholding.

1197 (E) Hematoxylin staining of an FFPE section containing all positive control organs from an adult
1198 wild type FVB/N female mouse: thymus (Th), heart (H), lungs (Lu), liver (Li), mammary gland

1199 (MG), lymph node (LN), spleen (Sp), pancreas (P), adrenal gland (A), kidney (K), fat (F), uterine
1200 horn (UH). Tumor (T) with implanted device and attached skin (Sk) was embedded into the same
1201 FFPE block.

1202 (F) Representative images of individual biomarkers using mIHC (red signal in bright field
1203 images) or cycIF (magenta signal in fluorescent images unless otherwise stated). Biomarker
1204 name is located on the top left; while the name of the organ is located on the top right side of
1205 each image, respectively. Some positive signal can be detected in a macroscopic view (Ki67,
1206 CD31, CD4, CD45, NF-KB, desmin, arginase-1, ICAM-1). Section stained without primary
1207 antibody served as negative control in the mIHC procedure (last image). Green fluorescent
1208 channel served to detect autofluorescence and to separate background from specific staining in
1209 the cycIF procedure. Only antibodies with very strong specific staining such as aSMA (marked
1210 with a star *), were used in conjugation with Alexa fluor-488. Scale bars; shown.

1211

1212 Figure S2. Analytical design to quantify single cell events in MIMA; Related to Figure 1.

1213 (A) The baseline discovery readout panel for MIMA is composed of a total of 13 probes we
1214 identified to be the minimal and satisfactory requirement to capture the major TME states to
1215 predict drug-induced changes which are actionable (see also [Table S4](#)).

1216 (B) Tissue section of an early MMTV-PyMT mammary carcinoma and adjacent lymph node
1217 (brightfield image of PyMT, CD45, α SMA, CD31 shown) was used to establish hierarchical
1218 gating strategies in image cytometry (in E) to define “standard cell types”. This for two reasons:
1219 presence of a lymph node in the same section offers the possibility to utilize mutual exclusivity
1220 (top) for reproducible signal thresholding. Second, early tumors provide with the opportunity to
1221 evaluate relatively broader range of phenotypically distinct cell types as compared to late-stage
1222 tumors (quantification, bottom). Number of cells analyzed is shown; data is derived from one
1223 and two tumors for early and late tumor sample, respectively.

1224 (C) Image processing for image cytometry analysis is composed of the following steps, briefly:
1225 hematoxylin staining (1) is color-deconvoluted and the signal is segmented using ImageJ
1226 watershed function (Schneider et al., 2012) to generate mask (2). Red AEC signal (3) mean
1227 intensity in a selection as defined by mask (4) is calculated for each cell (5).

1228 (D) Pixel intensity measurements and shape size measurements are used to gate cells for positive
1229 marker expression (CD45 in this case). FCS Express 6 and 7 Image Cytometry Software (De

1230 Novo Software), was used to obtain accurate thresholding using the cell population shape and
1231 dimensions. Correct gating is also monitored through visual inspection (second column).
1232 (E) Density plot of dimensionality reduction in hierarchical clustering to define “standard cell
1233 types” (middle column). The shape of the gates was designed to obtain quantitatively
1234 reproducible multiplex data, batch to batch, independent of the condition measured: early tumor
1235 and lymph node (top row), mammary gland and lymph node (middle row) and panobinostat
1236 implanted tumor sample two days post exposure (bottom row) are shown for comparison. For
1237 probes other than “standard cell types” (pleiotropic/undefined biology), threshold for positivity
1238 was determined manually using FCS Express 6 and 7 Image Cytometry software and positive
1239 control tissue (Figure S1D-F) (right). Sample pictures for marker positive cells; left.

1240

1241 Figure S3. Locally induced tumor-TME changes at the palbociclib delivery sites; Related to
1242 Figure 2

1243 (A) Quantification of single cell events using individual markers and marker combinations
1244 (including standard cell types). Bars are mean \pm s.e.; n=3 palbociclib reservoirs in three tumors
1245 from three different MMTV-PyMT mice with late stage (d93-d100) spontaneously growing
1246 tumors implanted with IMD for three days. Significance was calculated by paired sample one
1247 tailed *t*-test.

1248 (B) Presentation of key response cell types (biomarker combination displayed) in XY space.
1249 Black arrow shows the drug releasing site; direction of the release is from the bottom to the top.
1250 The black line depicts region analyzed to quantify relative abundance of cell types with distance
1251 from the well in Figure 2D.

1252 (C) Percentage of MHC-II+ protumorigenic (M2) macrophages as defined by standard cell types.
1253 Stack bars are mean \pm s.e.; n=3 palbociclib reservoirs in three tumors from three different mice.

1254 (D) Gray scale single channel images of depicted markers at the palbociclib reservoir (left) and
1255 merge composite images with or without overlay on the nuclei defined mask (top right and
1256 bottom right, respectively). Dashed line stratifies the “immediate pool” zone for Figure 2C. Scale
1257 bar, 100 μ m.

1258 (E) Distance based clustering of CSF1R, CD31 and Ki67 positive cells as a set of XY
1259 coordinates. Coordinate [0,0] identifies the drug source. Direction of the drug release is always
1260 from bottom to the top. Individual clusters were identified by minimum 10 cells within

1261 maximum distance 50 μ m, 75 μ m and 30 μ m for CSF1R+, CD31+ and Ki67+, respectively.
1262 Clusters were merged together if present within the maximum distance range. Each cluster is
1263 depicted with a different randomized color; individual (non-clustering) cells are shown as light
1264 gray points. Function was adapted and modified from Yann Marcon (Matlab, Oct 2019). Note
1265 larger cluster formation when analyzing individual markers as compared to standard cell types
1266 (Figure 2E) suggesting other than standard cell types express the CSF1R and CD31 marker
1267 (potential cell trans-differentiation).
1268 (F) Systematic testing of endothelial cell, endothelial and pericyte (union) cell and proliferating
1269 tumor cell cluster formation at palbociclib and control PEG reservoir based on cluster size
1270 presented in form of a heatmap. Cluster size was defined by minimal number of cells (x axis)
1271 within maximum distance range (y axis). Total coverage, number of clusters in the assay area
1272 variance between palbociclib and PEG in these two parameters was evaluated. Yellow rectangle
1273 defines cluster sizes that form specifically at the palbociclib stimulus site and have maximal
1274 variance (PEG vs palbociclib). Treatment specific cluster formation appears when minimum 10-
1275 20 cells are present with 50-75 μ m and 30-50 μ m for endothelial/pericyte cells and proliferating
1276 tumor cells, respectively.
1277 (G) Three-dimensional composite image of another palbociclib tumor tissue section. F4/80
1278 macrophage marker is presented in high-view. Triangle arrow, which shows the localization and
1279 direction of the drug release, is shifted slightly to the right so that both normal tissue and
1280 Palbociclib affected region can be seen at once. Note slightly different extent of the TME
1281 response as compared to replicate number 1 (Figure 2B), while the shape and the order of the cell
1282 response with distance remains the same: CSF1R+, F4/80+ macrophages located in close
1283 proximity to the well; CD31 α SMA pericyte form network outside this region and Ki67
1284 proliferating cells appear de novo (in the local microculture) around network.

1285

1286 Figure S4. Locally induced tumor-TME changes at the venetoclax delivery sites; Related to
1287 Figure 3.

1288 (A) Quantification of single cell events at the venetoclax delivery site in spontaneous MMTV-
1289 PyMT tumors at three days of exposure by using individual markers and marker combinations
1290 (including standard cell types). Total cell counts to define percentage of positivity were between
1291 3000 to 5000 cells per assay area and were matched \pm 300 total cells for paired samples

1292 (experimental vs control region). Minimum population proportion within 5% margin of error and
1293 95% confidence level was set to 0.75% (represents 12 cells) to discriminate noise from specific
1294 cell enrichment. Bars are mean \pm s.e.; n=3 venetoclax reservoirs in two tumors from two
1295 different mice. Significance was calculated by paired sample one tailed t-test.

1296 (B) Gray scale single channel images of depicted markers at the Venetoclax reservoir.
1297 Macroscopic view is on the left; magnified view of stromal regions and venetoclax proximal
1298 region are in the middle and right, respectively. Composite of depicted markers is in the colored
1299 image. Scale bar, 500 μ m and 50 μ m for macroscopic and zoomed view, respectively.

1300 (C) Presentation of key response cell types/states (biomarker combination displayed) in XY
1301 space. [0,0] coordinate is the drug releasing site; direction of the release is from the bottom to the
1302 top.

1303

1304 Figure S5. Local and systemic effects of the pan-HDAC inhibitor, panobinostat, in different
1305 mouse models of breast cancer. Related to Figure 4.

1306 (A) Quantification of single cell events at the panobinostat delivery site in spontaneous MMTV-
1307 PyMT tumors at three days of exposure by using individual markers and marker combinations
1308 including standard cell types. Total cell counts to define percentage of positivity were between
1309 3000 to 5000 cells per assay area and were matched \pm 300 total cells for paired samples
1310 (experimental vs control region). Minimum population proportion within 5% margin of error and
1311 95% confidence level was set to 0.75% (represents 12 cells) to discriminate noise from specific
1312 cell enrichment. Bars are mean \pm s.e.; n=3 panobinostat reservoirs in two tumors from two
1313 different mice. Significance was calculated by paired sample one tailed t-test.

1314 (B) Presentation of the most prominent response cell types (biomarker combination displayed) in
1315 XY space. Black arrow marks source and direction of drug release.

1316 (C) Gray-scale single channel images of depicted markers at the panobinostat reservoir (replicate
1317 1). Dashed line in the Sox9 image marks the border of the device. Scale bar, 100 μ m. Dashed
1318 yellow box in the Ly6G image marks magnified view of a region at the intersection of dying
1319 cells (by CC3) and surrounding TME. Scale bar, 50 μ m.

1320 (D) ErbB2 Δ Ex16 mice with spontaneously growing tumors were treated with diluent (control)
1321 and panobinostat systemically for 4 days, after which the tumors were extracted at day 7 and

1322 formalin fixed paraffin processed (FFPE). Images show tumor tissue sections stained with anti-
1323 mouse CD8 antibody (red AEC signal) and hematoxylin (blue). Note the gradient of high CD8
1324 infiltration closer to stroma with decreasing tendency towards the tumor center (arrows)
1325 suggesting CD8 T cells are recruited from stroma regions. Scale bar, 100 μ m
1326 (E, F) Tumor growth rate in syngeneic LPA3 mice in which tumors were induced by orthotopic
1327 injection of primary tumor cells into mammary fat pad. The mice were treated systemically by
1328 depicted treatments as shown in the graph. Early (<20mm³; D) and more advanced (>100mm³,
1329 E) tumors were tested as for the treatment start (day 0). Mice were treated intraperitoneally with
1330 dose and schedule as defined in [Figure 3G](#) and [4H](#). Line graphs show mean \pm s.e., n=5 tumors in
1331 five mice per group. Significance was calculated by two sample two-tailed t-test with equal
1332 variance.

1333

1334 **Figure S6. Biomarkers of immunogenic cell death and associated mechanisms of resistance**
1335 **induced by local panobinostat drug stimulus; Related to Figure 5.**

1336 (A) Large field of view three-color composite images showing biomarkers of immunogenic cell
1337 death induced by panobinostat (replicate 2) reservoir at three days of exposure. Calreticulin and
1338 PD-L1 IHC (red AEC signal) overlaid on hematoxylin nuclei (in blue) are presented in bright
1339 field zoomed image on the left.

1340 (B) Quantification of single cells positive cells for depicted biomarker with distance from the
1341 reservoir; total cell counts (left) and rate of positive cells (right) are presented in form of a 3D
1342 bar graph. 2-3 ROIs are presented per assay zone (proximal, distal, distal, remote, control).

1343 (C) Expression rate of CD45, MPO and arginase-1 on Ly6G⁺ cells in the panobinostat assay area
1344 to stratify phagocytic, cytotoxic and immune suppressive neutrophils, respectively. Number (n)
1345 of analyzed cells is presented.

1346 (D) Panobinostat reservoirs were co-loaded with anti-Ly6G (clone 1A8) and galectin-3 (clone
1347 M3/38) antibodies at 5:1 to 10:1 ratio and CC3 IHC signal was quantified at the drug releasing
1348 site. n=2 for experimental and 1 for control conditions, respectively. All results were obtained
1349 from a single IMD in one tumor which was implanted for two instead of typical three days to
1350 account for antibody half-live.

1351 (E) Image cytometry measuring neuropilin-1 expression on cytotoxic neutrophils. For
1352 comparison, population distribution of all cells is presented on the bottom left.

1353 (F) Bright field large field of view images of CD11b, neuropilin-1, galectin-3 and α SMA IHC at
1354 the panobinostat reservoir (replicate 3) at 3 days of exposure. Zoomed images show color-coded
1355 extracted signal overlaid on the true signal of the depicted biomarkers (white).
1356 (G) Distance based cluster analysis testing different cluster size strategies to identify treatment
1357 specific cluster formation. The function implements a user defined cluster sizes set by minimal
1358 number of cells (first number in the top right legend) within maximum distance range (second
1359 number in the top right legend) to display cluster formation in randomized color while individual
1360 cells not meeting the clustering criteria are presented in gray. Drug source is shifted to the left to
1361 stratify cluster formation in assay area (proximity to the well) vs side/random regions. Clustering
1362 strategies using low cell number (e.g. 5 cells, first two columns) and high distances (e.g. 100 μ m,
1363 right column) show clusters forming unspecifically outside the assay area; Clustering strategy
1364 using minimal 10 cells within maximum distance range 50 μ m (10/50 column) shows cluster
1365 formation specifically above the drug site for all presented markers (F4/80, Galectin-3, Ly6G,
1366 Sox9). Magnified Sox9 cluster formation; bottom.
1367 (H) Frequency of Sox9 cluster sizes. Cluster size around 175 μ m in diameter, which were the
1368 most prominent, were used for downstream analysis of pairwise proximity measurements of
1369 Sox9 with other markers (Figure 5G, H).

1370

1371 Figure S7. Local panobinostat efficacy in other mouse model and at a later (day 8) timepoint;
1372 Related to Figure 5

1373 (A) A schematic presentation of cell phenotype separation into zones with distance from the
1374 panobinostat reservoir at day three and day eight timepoint. Shared phenotypes between the two
1375 timepoints suggest order of the cell transition with distance from the well defines the sequence of
1376 cellular events (earliest to latest) and is as follows: 1) MHC-II antigen presenting cells and F4/80
1377 macrophages are recruited first to the drug delivery well as they are located *immediately* at the
1378 drug well at early timepoint; 2) MPO cytotoxic, ICAM1 adhesive/activated, neuropilin-1 positive
1379 N1 neutrophils are recruited second (*proximal* zone) and this phenotype is halt by the *border*
1380 barrier composed of 3) CD11c dendritic cells, Sox9 cancer stem cells and galectin-3. Relative
1381 increase of MHC-I and calreticulin on the cell surface starts to form in this border zone and
1382 propagates to (4) *distal* region with decreasing gradient profile. 5) galectin-3 expression is
1383 associated with PD-L1 and is halt by α SMA fibroblast *remotely* from the well. Over time, the last

1384 three zones (3-5) merge into a single border zone composed of CD11c dendritic cells, arg-1
1385 immune suppressive cells, Sox9 cancer stem cells, K14 cells of invasive front and ECM
1386 deposition/processing components (collagen VI and MMP2). The immediate macrophage zone
1387 is missing at the day eight timepoint; instead, two new cell types appear at the new border zone:
1388 (6) CSF1R+ cells (C) and (7) granzyme B cytotoxic CD45R B cells (D).
1389 (B) ErbB2 Δ Ex16 mice with spontaneously growing tumors were implanted with IMD loaded
1390 with panobinostat and the tumor with the device in place was extracted at three days and was
1391 formalin fixed and paraffin processed. Picture shows a five-color composite image of biomarkers
1392 associated with immunogenic cell death. While the absolute extent of the phenotype is larger as
1393 compared to those observation in MMTV-PyMT model; the order of the phenotypes (spatial cell
1394 pattern with distance from the drug source) remains identical with Ly6G+ leukocytes and CC3
1395 apoptosis present at the proximal, CD11c dendritic cells present at border and galectin-3 present
1396 at the distal regions. We note that the ErbB2 Δ Ex16 model of breast cancer express both basal
1397 and luminal cytokeratin markers (Turpin et al., 2016). The different extent of the signal might be
1398 associated with the tumor subtype difference or the compactness/"fluidity" of the tumor tissue.
1399 (C) Sectioned tissue surrounding the implantable microdevice containing panobinostat for eight
1400 days was stained with cycIF using panel of mouse specific antibodies (Table S3) to display cellular
1401 phenotypes supporting the panobinostat model of response describe in (A).
1402 (D) Adjacent section as described in (C) was stained by multiplex immunohistochemistry using
1403 mouse specific antibodies (Table S2) and displays presence of cytotoxic granzyme B+ CD45R+
1404 B cells expressing calreticulin and galectin-3. No other marker was expressed on these cells (or
1405 under the IHC sensitivity limit; not shown). Also, no other cells expressed the granzyme B
1406 cytotoxicity marker intratumorally; inside the tumor bed. Panobinostat drug release site is
1407 marked by triangle in all images. Direction of the drug release is always from the bottom to the
1408 top. Scale bars; shown.

1409

1410 Figure S8. Illustration of complex tumor tissue response to targeted therapies and rational for
1411 immune modulatory combinations.

1412 Schematic presentation of the spatial cell associations induced by palbociclib, venetoclax and
1413 panobinostat at three days of local drug exposure; and panobinostat at eight days of drug
1414 exposure (bottom right). Palbociclib efficacy is associated with a "tree" or "delta-like" cell

1415 organization with targetable protumorigenic macrophages being localized in immediate
1416 proximity to the reservoir leading to endothelial/pericyte network formation and proliferation of
1417 tumor cells in proximity to this network. The optimal schedule of immunotherapy application
1418 can be estimated based on the location of the targetable phenotype from the drug well. Early
1419 (pre-treatment) modulation of protumorigenic macrophages using CSF1/CSF1R targeted
1420 immunotherapy was used to potentiate the efficacy of palbociclib. Venetoclax induces small
1421 vessel formation and recruitment of dendritic cells which appeared as “split clusters” at the drug
1422 well and it is yet unknown whether and how the subsets are functionally related. Anti-CD40
1423 mediated licensing of these DCs can shift the balance from immune tolerance to T cell priming
1424 and this immunotherapy was applied at three days of venetoclax treatment since DCs were
1425 present in all regions of the assay area. Panobinostat induces immunogenic cell death associated
1426 with infiltration of antigen presenting neutrophils and macrophages but the propagation of this
1427 positive response is limited by enrichment of cancer stem cells and recruitment of dendritic cells
1428 and fibroblasts which merge into a resistant barrier over time and subsequently induce
1429 recruitment of protumorigenic macrophages and cytotoxic B cells. The cellular pattern of
1430 response has a “layering” or bay-like” structure implying more intense cell interaction might be
1431 involved at the cell layer interfaces. Anti-PD-1 immunotherapy was identified as the rational
1432 combination partner for panobinostat due to induction of immunogenic cell death and increased
1433 antigenicity. While early (pretreatment) administration of CSF1/CSF1R targeted immunotherapy
1434 can negatively affect antitumor function of macrophages (tested, [Figure 4C](#)); late administration
1435 of this immunotherapy might be beneficial to deplete/polarize the protumor macrophage which
1436 are recruited at the later timepoint (hypothetical).

1437

1438 Figure S9. Local and systemic effects of the pan-HDAC inhibitor, panobinostat, in different
1439 mouse models of breast cancer.

1440 (A) Quantification of single cell events at the intersection of venetoclax and panobinostat
1441 delivery sites in orthotopic MMTV-PyMT tumors at eight days of exposure by using individual
1442 markers and marker combinations. Total cell counts to define percentage of positivity were
1443 between 3000 to 5000 cells per assay area and were matched ± 300 total cells for paired samples
1444 (experimental vs control region). Minimum population proportion within 5% margin of error and
1445 95% confidence level was set to 0.75% (represents 12 cells) to discriminate noise from specific

1446 cell enrichment. Bars are mean \pm s.e.; n=2 wells from one tumor in one mouse. Significance was
1447 calculated by paired sample one tailed t-test.

1448 (B) Macroscopic view of marker positive cells at the panobinostat/venetoclax drug intersection.
1449 Histograms show mean expression intensity of individual biomarkers. Red line defines threshold
1450 for positivity. Marker positive cells as defined by threshold are presented as blue dots in XY
1451 space. Black arrow is pointing on the tumor stroma region remote from the reservoir. Yellow
1452 triangle arrows indicate the source and direction of the drug release. Yellow box shows the
1453 approximation of the assay area.

1454 (C) Five-color composite images showing most prominent biomarkers induced at the intersection
1455 of panobinostat and venetoclax. White arrows indicate the source and direction of the drug
1456 release. Scale bar; 200 μ m. Macroscopic view of tumor tissue sections stained with anti-mouse
1457 F4/80 (red AEC signal) and hematoxylin (blue). Tumor cleared tissue is shown by lack of
1458 hematoxylin staining in the center suggesting lack of nucleated cells (bottom left image).
1459 Magnified view of intratumoral T cell infiltration to professional antigen presenting DC region
1460 near panobinostat/venetoclax assay area. The device was implanted for eight days in MMTV-
1461 PyMT tumor induced by orthotopic implant into mammary fat pad of syngeneic mice.

1462

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Figure 2

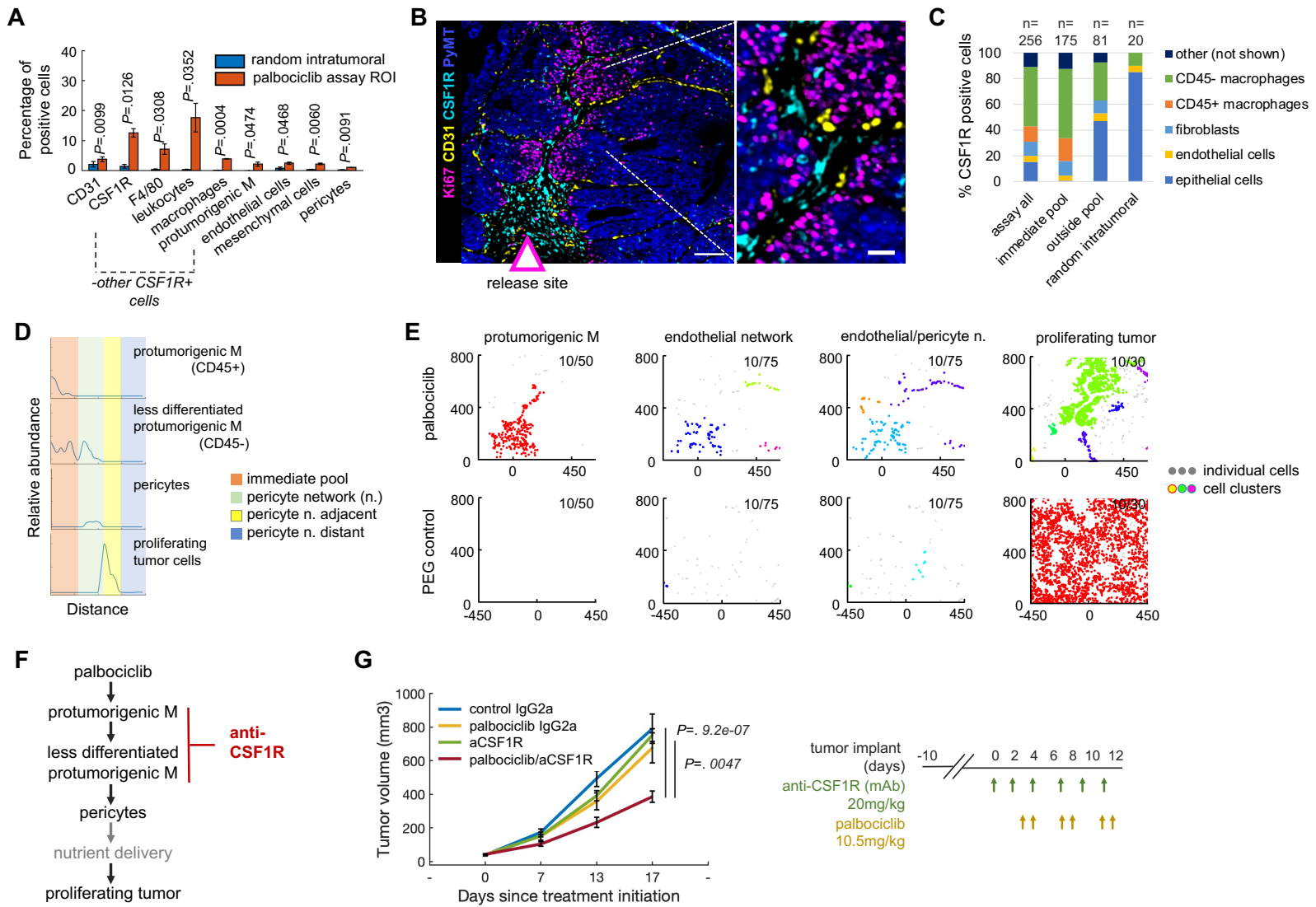


Figure 3

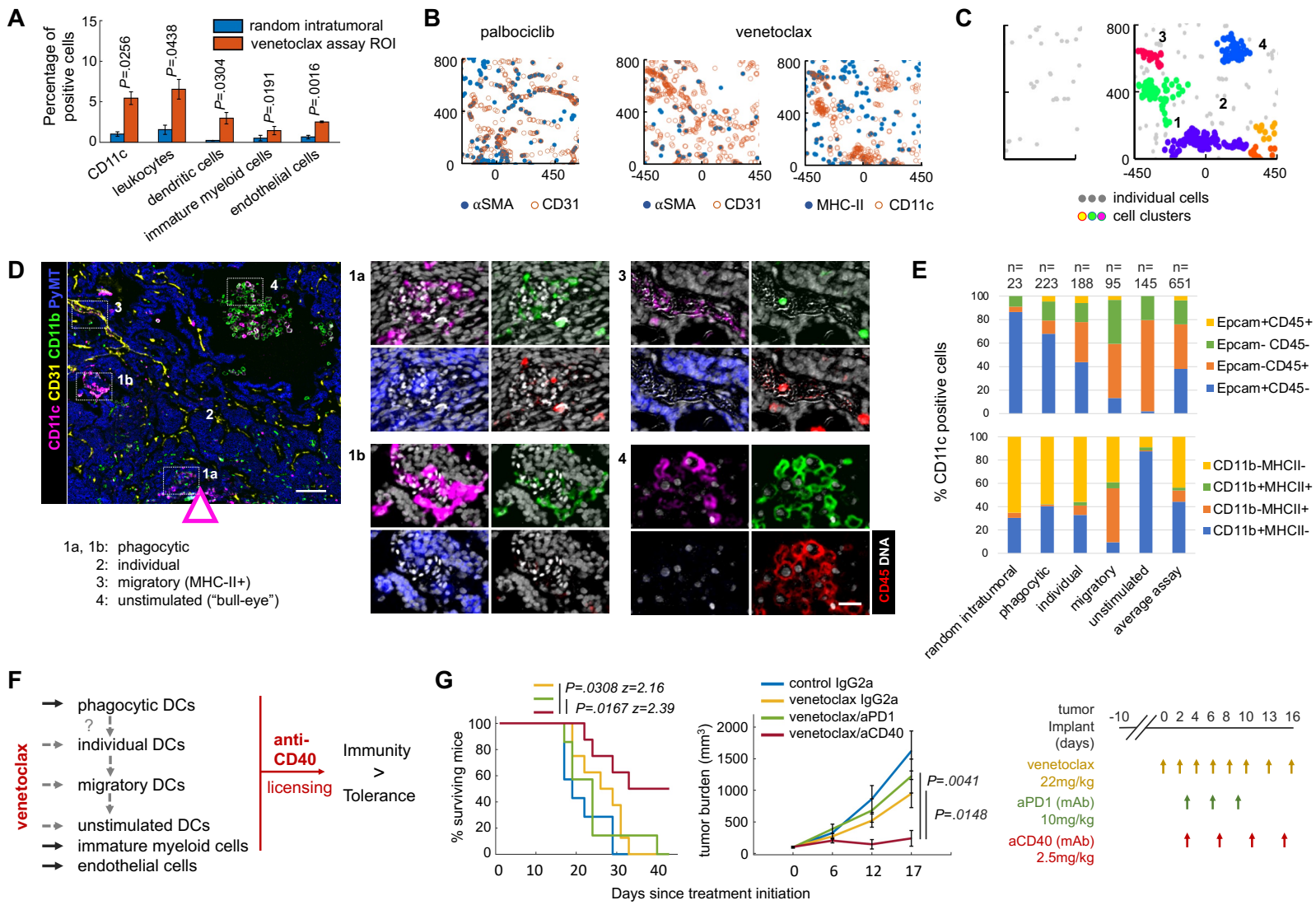


Figure 4

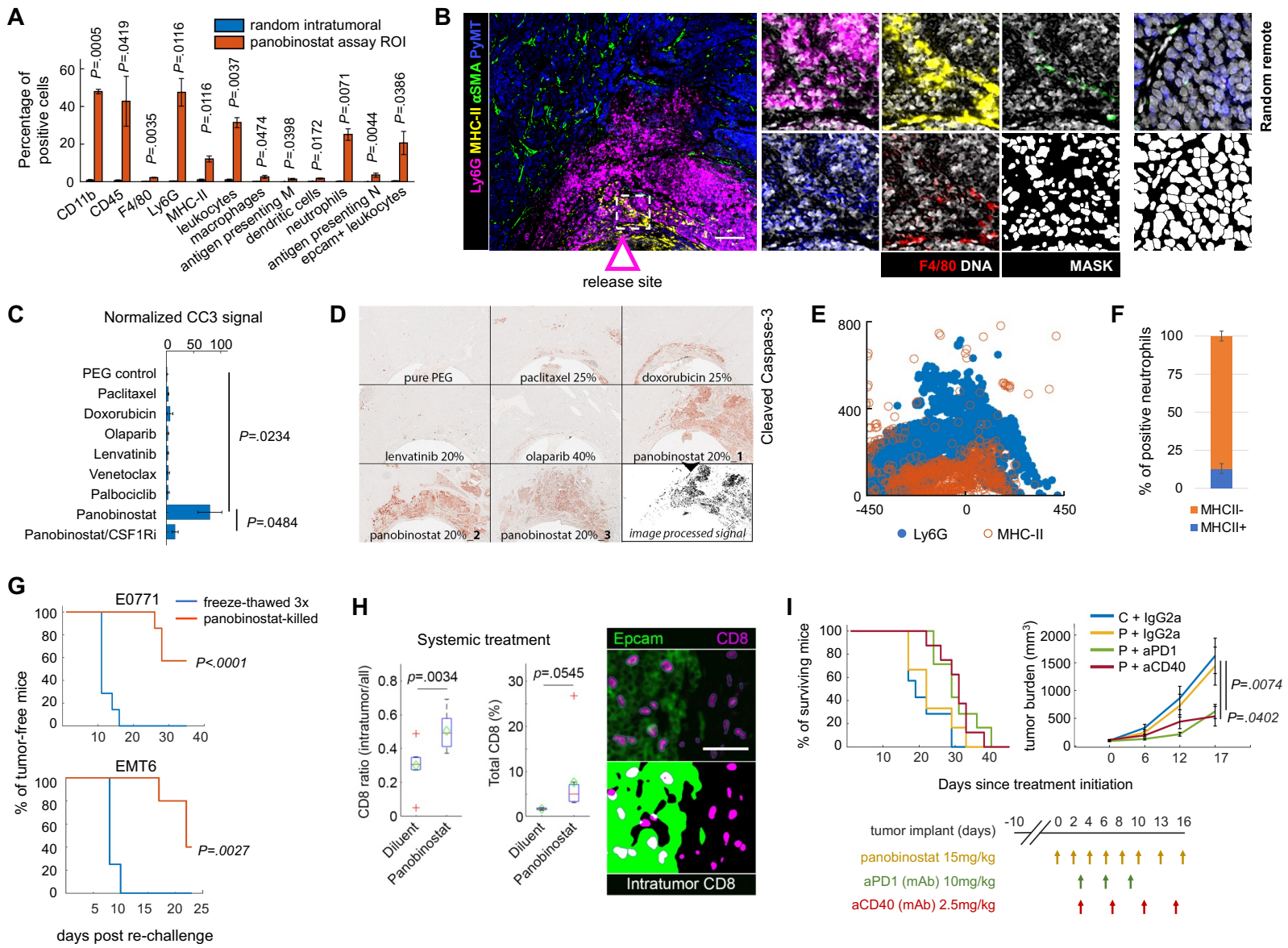


Figure 5

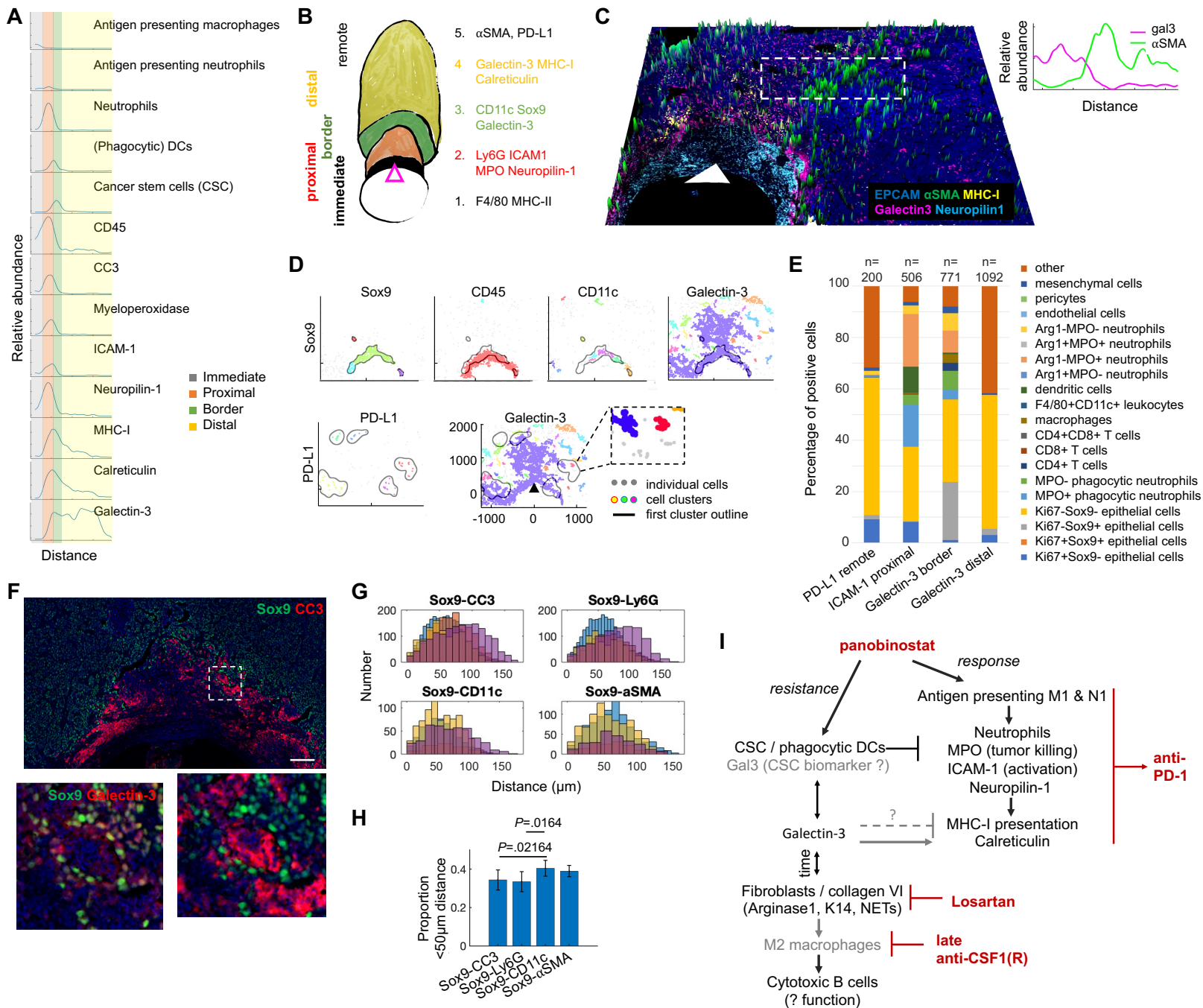
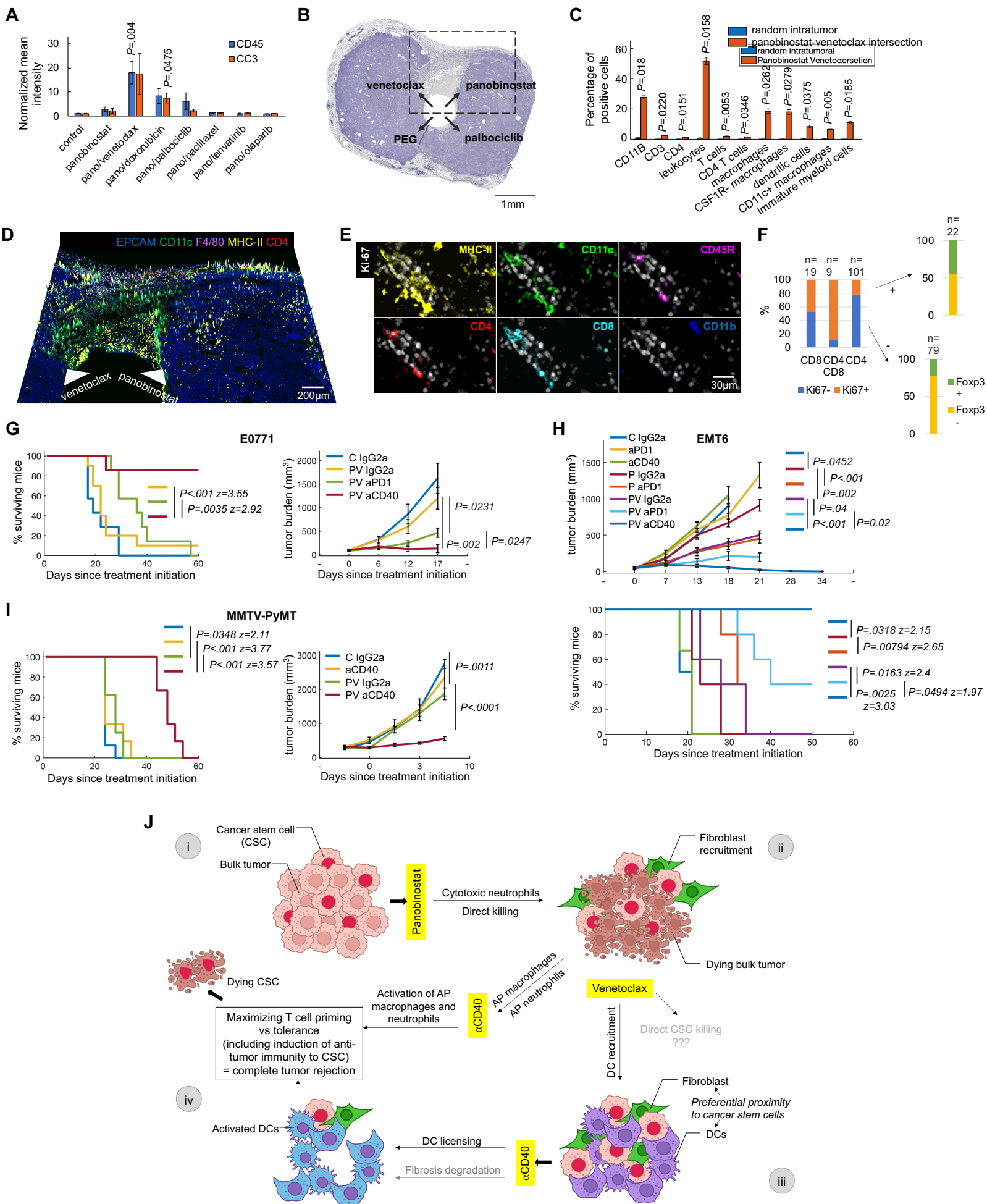
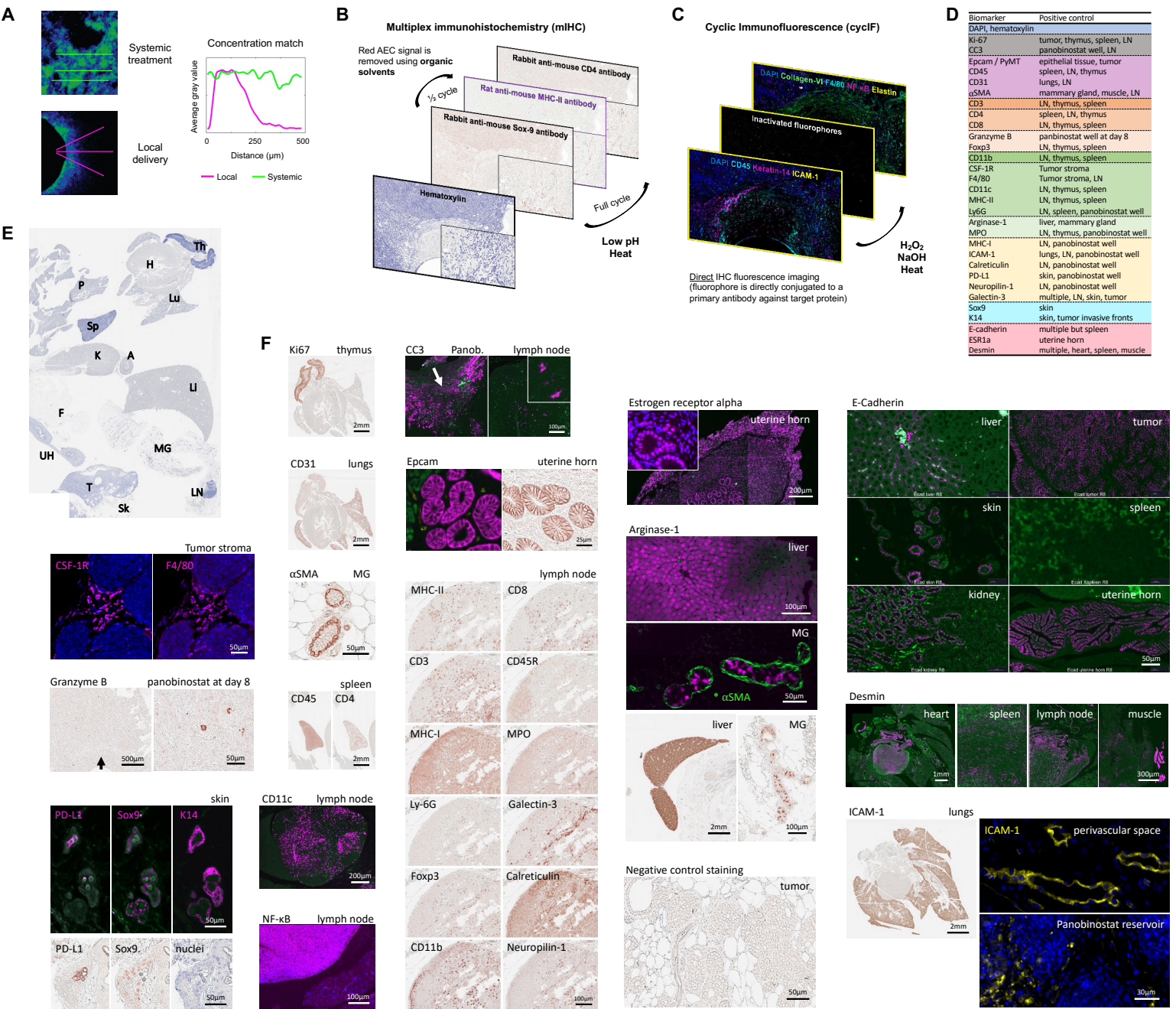
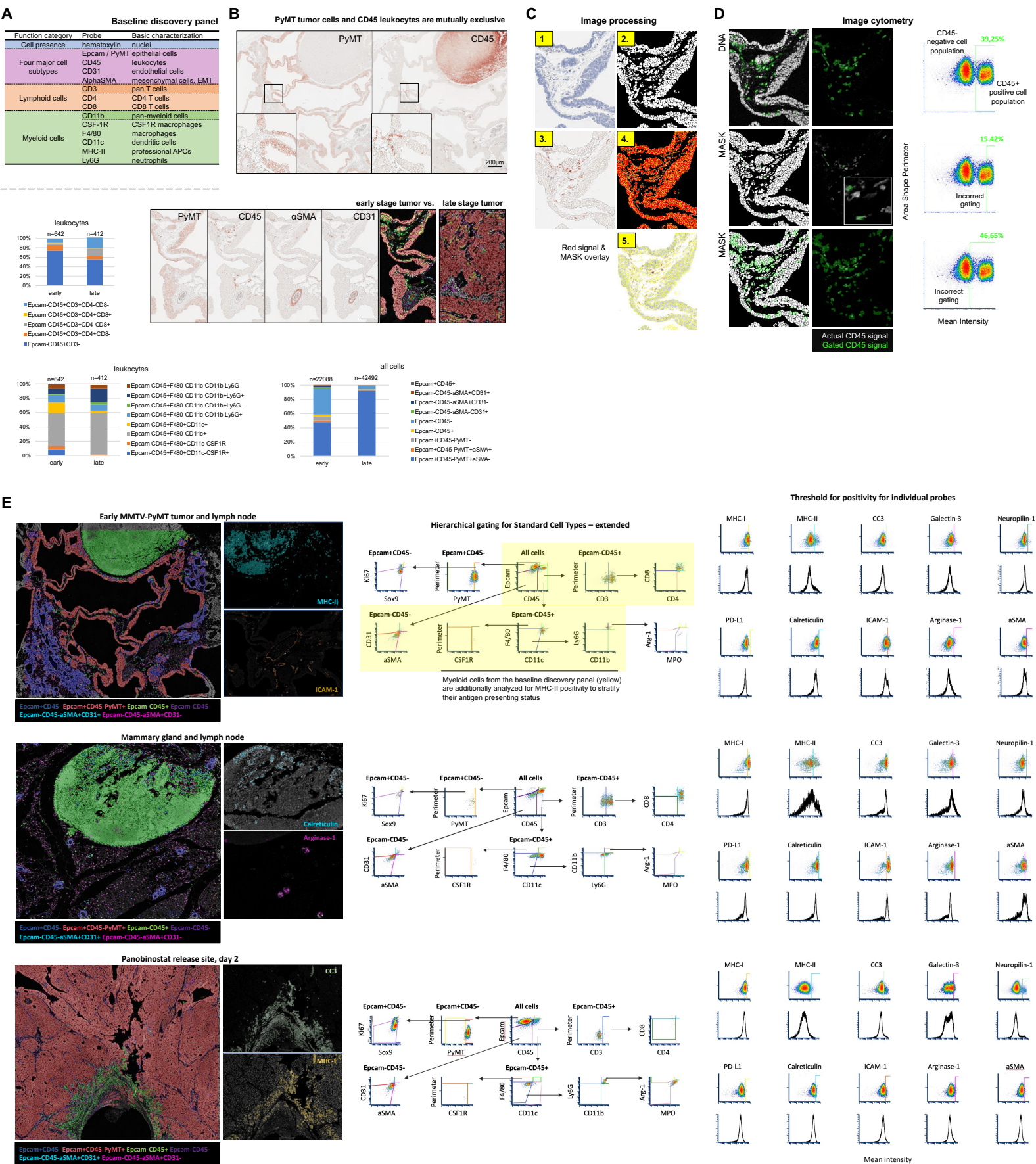


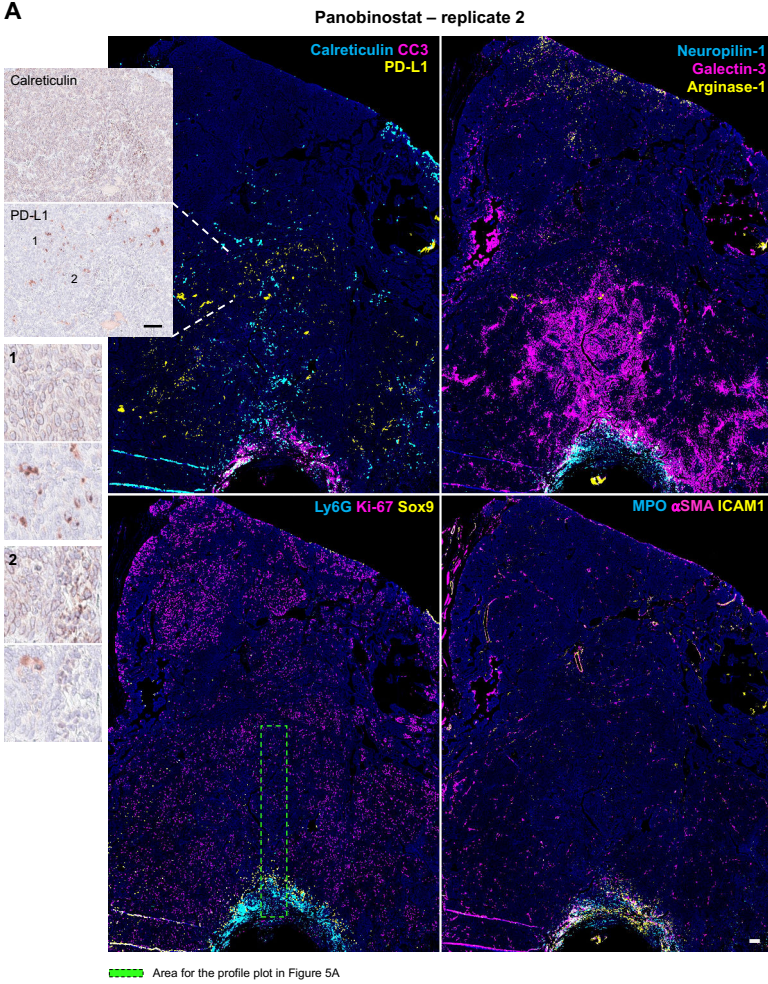
Figure 6



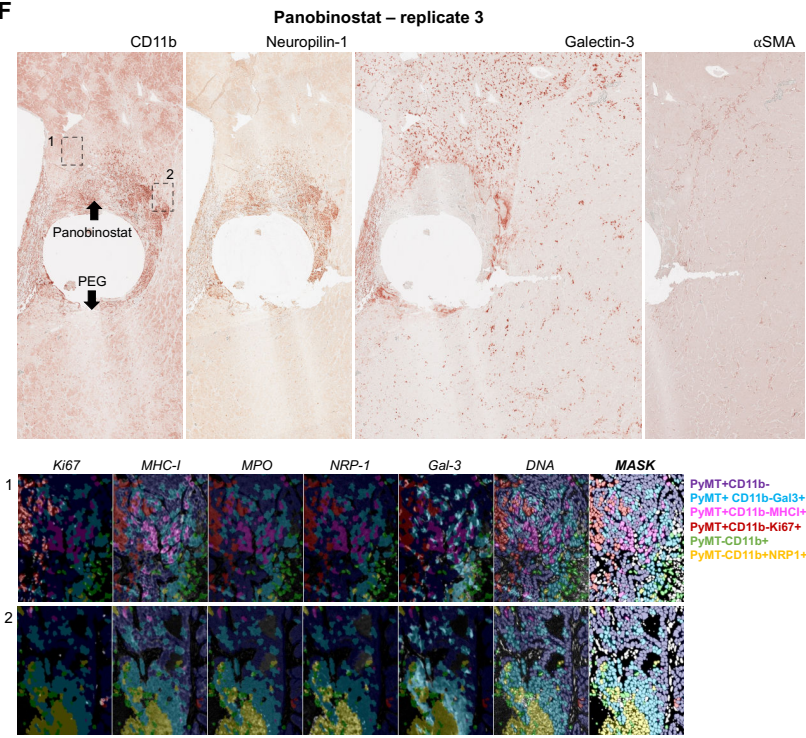




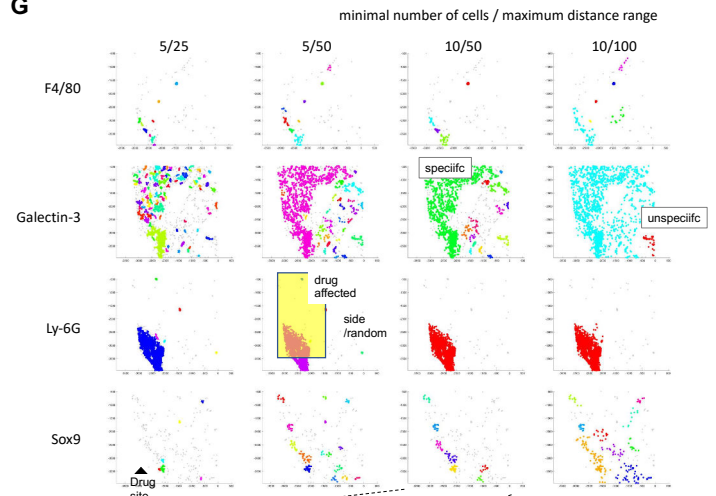
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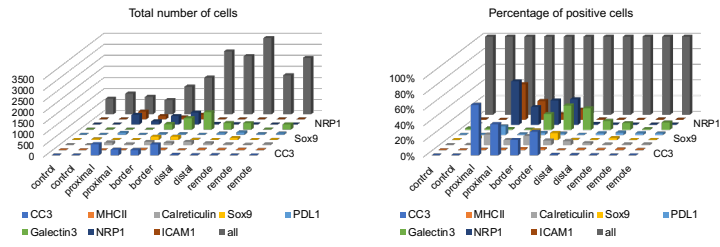
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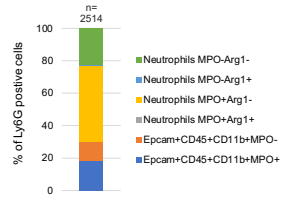
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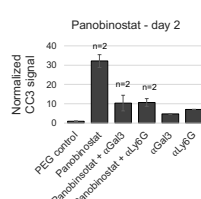
B



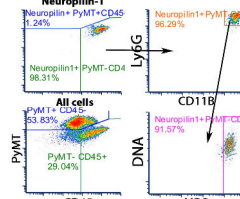
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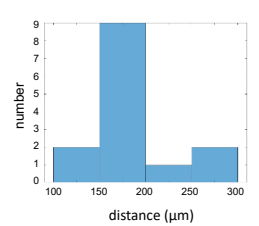
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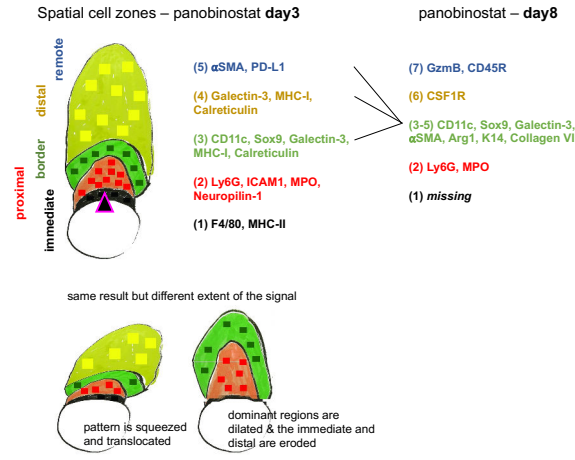
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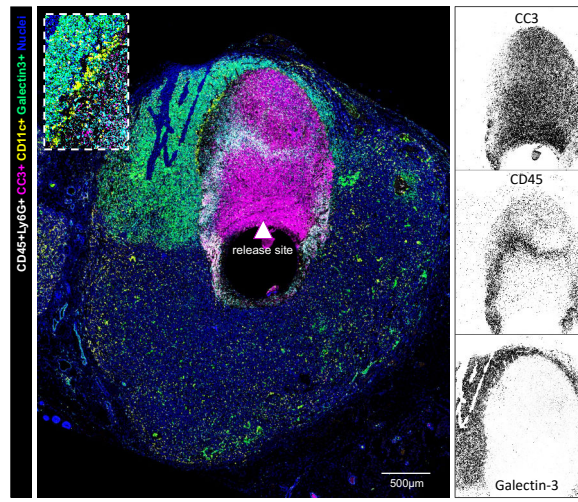
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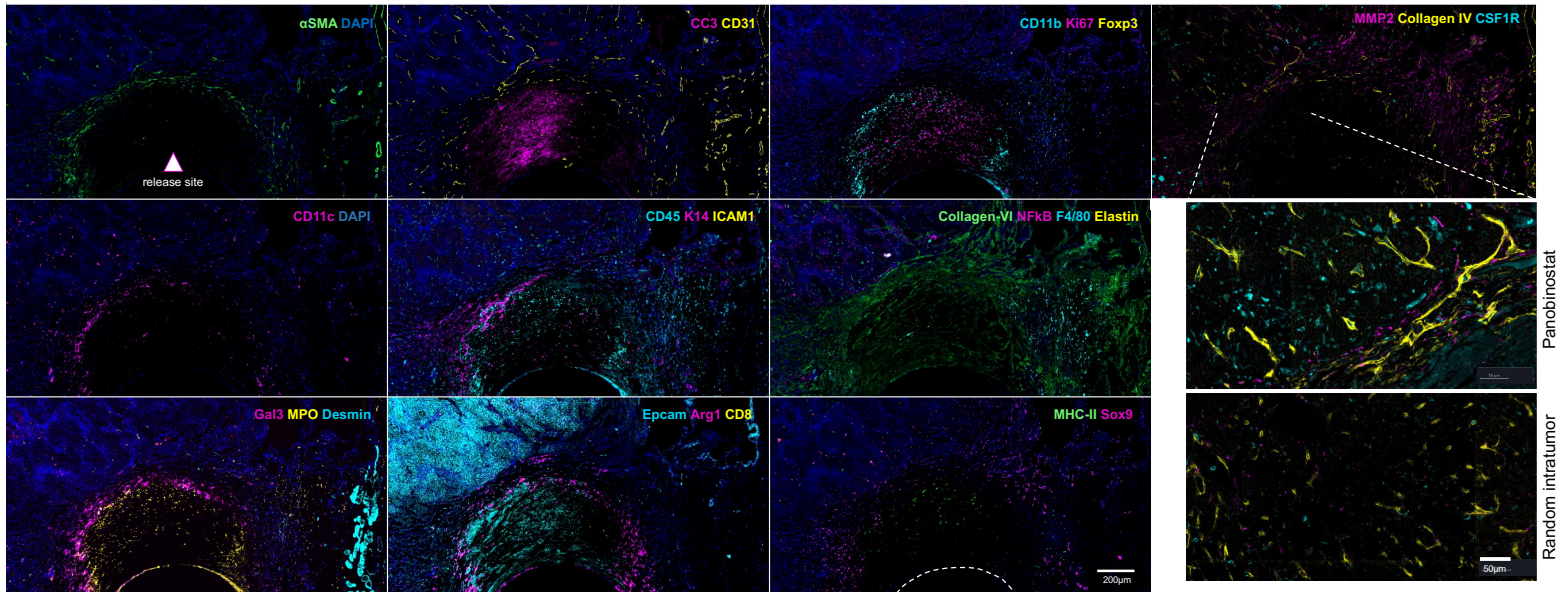
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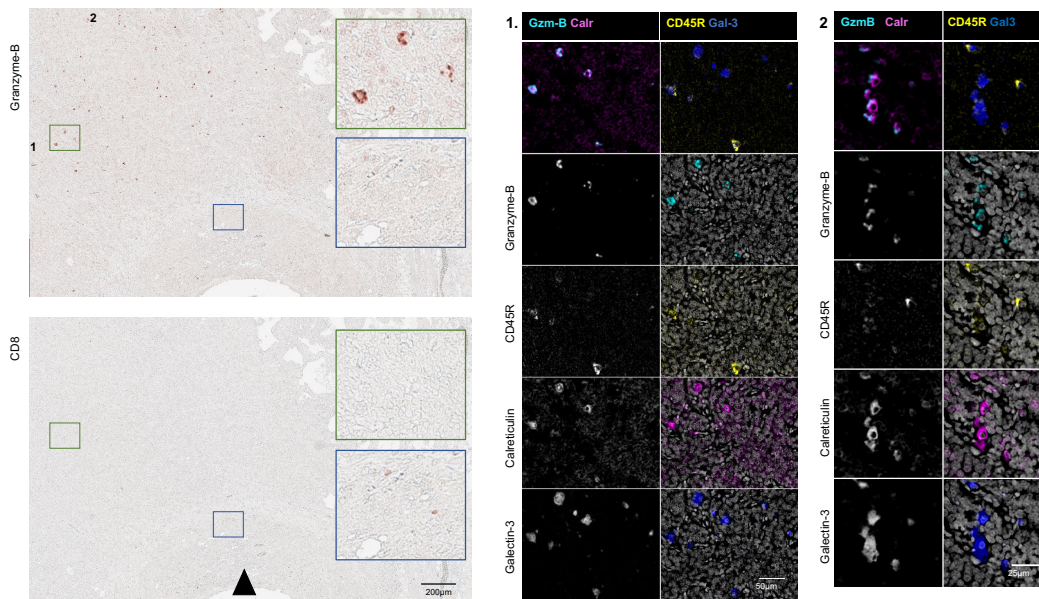
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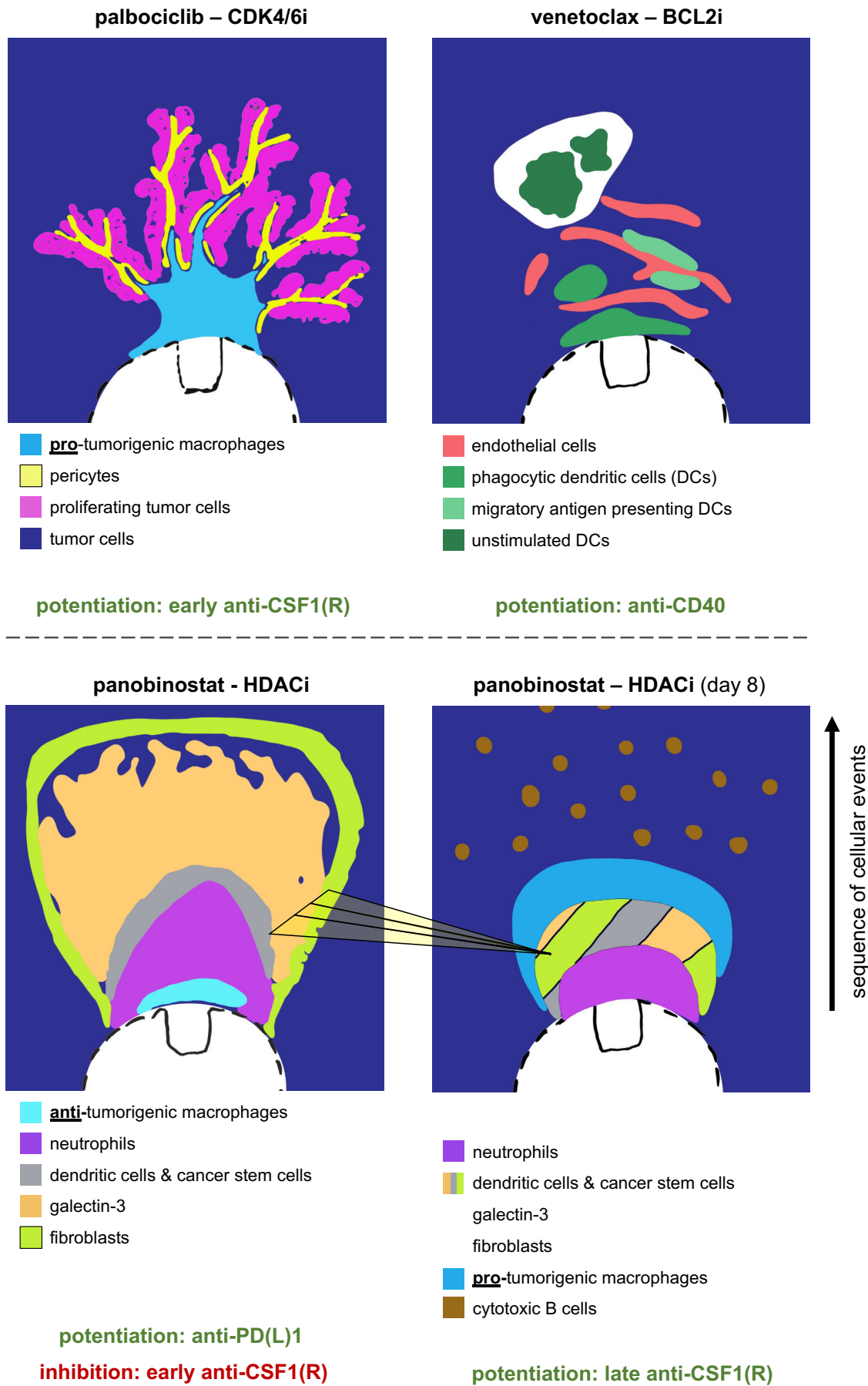


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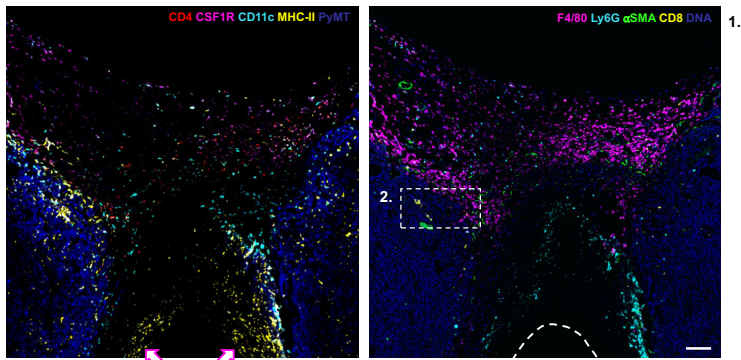
D



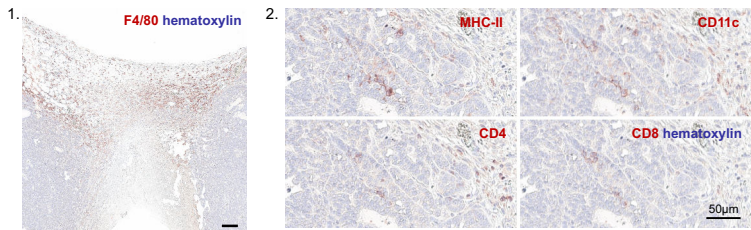


A

venetoclax panobinostat intersection day 8 – replicate 2

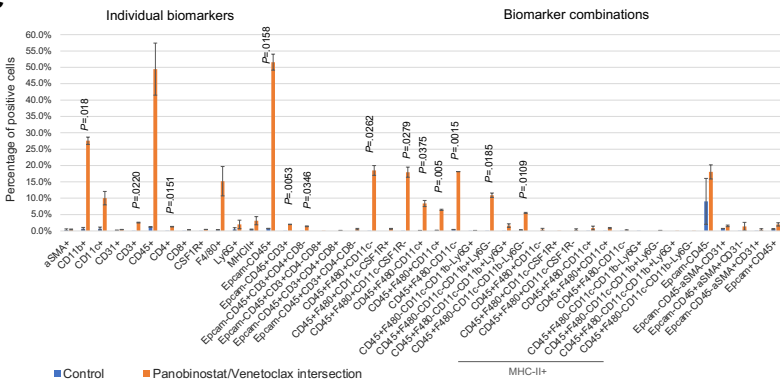


Veneto Pano



Cleared tumor tissue without nuclei staining

C



B

