Cells in the Polyaneuploid Cancer Cell State are Pro-Metastatic

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58

Ethics Statement:

60 All animal experiments were reviewed and approved by the Johns Hopkins
61 Animal Care and Use Committee. Human elements of this study were approved by the
62 corresponding institutional review boards and were conducted in accordance with
63 ethical principles founded in the Declaration of Helsinki. All patients gave written
64 informed consent.
65

Data Availability Statement:

83 Nearly all data generated in this study are available within the article and its
84 supplementary data files. Detailed data regarding the 247-panel cytokine array are
85 available upon request from the corresponding author.
86

Abstract:

88 There remains a large need for a greater understanding of the metastatic
89 process within the prostate cancer field. Our research aims to understand the adaptive
90 – ergo potentially metastatic – responses of cancer to changing microenvironments.
91 Emerging evidence has implicated a role of the Polyaneuploid Cancer Cell (PACC)
92 state in metastasis, positing the PACC state as capable of conferring metastatic

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competency. Mounting *in vitro* evidence supports increased metastatic potential of cells in the PACC state. Additionally, our recent retrospective study of prostate cancer patients revealed that PACC presence in the prostate at the time of radical prostatectomy was predictive of future metastatic progression. To test for a causative relationship between PACC state biology and metastasis, we leveraged a novel method designed for flow-cytometric detection of circulating tumor cells (CTCs) and disseminated tumor cells (DTCs) in subcutaneous, caudal artery, and intracardiac mouse models of metastasis. This approach provides both quantitative and qualitative information about the number and PACC-status of recovered CTCs and DTCs. Collating data from all models, we found that 74% of recovered CTCs and DTCs were in the PACC state. *In vivo* colonization assays proved PACC populations can regain proliferative capacity at metastatic sites following dormancy. Additional direct and indirect mechanistic *in vitro* analyses revealed a PACC-specific partial Epithelial-to-Mesenchymal-Transition phenotype and a pro-metastatic secretory profile, together providing preliminary evidence that PACCs are mechanistically linked to metastasis.

Statement of Significance:

We provide the first evidence that cells in the polyaneuploid cancer cell state contribute to increased metastatic competency *in vivo*.

Keywords:

Polyaneuploid Cancer Cell (PACC), Polyploid Giant Cancer Cell (PGCC), Metastasis, Metastatic Competency, Circulating Tumor Cell (CTC), Disseminated Tumor Cell (DTC), partial-Epithelial-to-Mesenchymal-Transition (pEMT), *in vivo* Metastatic Models

I. Introduction:

Though early detection of prostate cancer favors diagnosis of eradicable localized disease, metastatic prostate cancer remains lethal and incurable. Metastatic disease arises when metastatically-competent cells in the primary tumor i) invade local tissue, ii) intravasate into the vasculature, iii) survive circulatory transit, iv) extravasate into a distant organ, and v) colonize that organ (1). In 2024, it is projected that over 35,000 men in the US will die from metastatic prostate cancer (2). Clearly, there remains a large need for a greater understanding of the metastatic process within the prostate cancer field. One approach relies on understanding how tumor microenvironmental stressors constantly influence the adaptive potential of cancer cells, potentially driving phenotypes with increased metastatic potential.

Emerging evidence has highlighted the role of the Polyaneuploid Cancer Cell (or PACC) state as a phenotype of metastatically competent cells (3-7). Cells in the PACC state (also termed Polyploid Giant Cancer Cells, Multinucleate Giant Cells, and Pleomorphic Cells) exhibit a transient and adaptive cellular response to genotoxic stress. Most notably, the PACC state is characterized by an increase in genomic content coincident with an indefinite pause in cell division (8). Canonically, it is understood that genotoxic stress results in a G2/M cell cycle checkpoint stall that allows for an attempt at genomic repair, and in the event of failure, promotes apoptosis. Cells

139 in the PACC-state adopt an interphase-restricted cell cycle following an expected
140 stress-induced G2/M pause (9-11). This alternative cell cycle pattern, termed an
141 endocycle, consists of subsequent cycles of G1, S, and G2 phases without any
142 intervening M phases (12). In addition to explaining the increase in genomic content and
143 lack of cell division, an endocycle also explains the vast nuclear and cytoplasmic
144 enlargement typical of cells in the PACC state, which together create a distinct
145 morphological phenotype useful in identifying PACCs in cell culture as well as
146 histopathologic contexts. Indeed, these morphological features have been frequently
147 used as markers of the PACC state phenotype, which has been observed to arise in
148 various cell lines (prostate, breast, ovarian, brain, and melanoma, among others) in
149 response to multiple classes of anti-cancer stressors (13-18).
150 We and others have published data supporting that cells in the PACC state have
151 increased metastatic potential. Functionally, Xuan et. al. has reported that breast cancer
152 MDA-MB-231 PACCs exhibit a persistent migratory phenotype driven by an enriched
153 Vimentin filament network (19, 20). More recently, we have shown that prostate cancer
154 PC3 PACCs demonstrate an identical motility phenotype to that observed by Xuan et al.
155 that can be influenced by presence of a chemotactic gradient (21). In mice, Zhang et. al.
156 showed that serial metastatic passage of PC3 cells increased not only the cells'
157 metastatic rates but also their percentage PACC makeup with each cycle of selection
158 (22).
159 Clinical research has also indicated a potential role for PACCs in contributing to
160 metastasis. Stromal-invasive PACCs identified by histology were more frequently
161 identified in patients with metastatic (vs. nonmetastatic) ovarian cancer: PACCs were
162 found in 18/21 high-grade primary tumors from patients with metastases, but only 6/26
163 low-grade primary tumors from patients without metastasis (23). Similar trends have
164 been reported in prostate cancer; of 27 patients with PACC-positive cases of prostate
165 cancer, all 27 had Gleason-scores of 9 or 10, indicating that presence of cells in the
166 PACC state is linked to more aggressive disease (24). An independent study published
167 nearly identical findings: of 30 patients presenting with PACC-positive cases of prostate
168 cancer, all 30 had Gleason-scores of 9 or 10, and 11 patients were dead at a median of
169 8 months after diagnosis (25). The Michigan Legacy Tissue program identified PACCs
170 in all 16 osseous and non-osseous metastatic sites of 5 randomly selected cases of
171 prostate cancer, and most PACCs identified were concentrated around tumor hotspots
172 (26). Most recently, we reported that presence of PACCs in the primary tumor at the
173 time of radical prostatectomy was predictive of future metastatic progression in men
174 with prostate cancer (27). These studies reveal a correlation between PACC state
175 biology and metastasis.
176 To test for a causative relationship between PACC state biology and metastasis,
177 we leveraged our recently published flow cytometry method designed for the detection
178 of rare circulating tumor cells (CTCs) and disseminated tumor cells (DTCs) in metastatic
179 mouse models (28). This approach is powerful because it provides both quantitative and
180 qualitative information about the number and PACC-status of metastasizing
181 CTCs/DTCs recovered from animal tissues. We used various *in vivo* models to test
182 distinct steps of the metastatic cascade. Measurement of spontaneous metastasis of
183 blood CTCs and distant organ DTCs from subcutaneous tumors tested invasion,
184 intravasation, and circulation survival (for CTCs), as well as extravasation (for DTCs).

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185 Measurement of DTCs from caudal artery and tail vein injections specifically tested
186 extravasation. Evaluation of primary tumor growth in subcutaneous models tested
187 dormancy and colonization. Lastly, measurement of metastatic lesion outgrowth
188 following intracardiac injection tested circulation survival, extravasation, dormancy, and
189 colonization.
190   Across the various models, 75% of recovered CTCs and 72% of recovered DTCs
191 were in the PACC state, as defined by a DNA content greater than 4N (>4N). Two *in*
192 *vivo* colonization assays proved that PACC populations can regain proliferative capacity
193 following long periods of dormancy, a phenomenon frequently observed and yet
194 inadequately understood in the clinic. *In vitro* studies revealed a PACC-specific partial-
195 Epithelial-to-Mesenchymal-Transition (pEMT) as a likely mechanism of increased
196 metastatic behavior in PACCs. Notably, PACCs identified in the blood of human
197 prostate cancer patients also demonstrated a pEMT phenotype characterized by co-
198 expression of EpCAM and Vimentin. Additionally, an analysis of PACC-conditioned
199 media and its effects on nonPACC cells indicated that a PACC-specific pro-metastatic
200 secretory phenotype may increase metastatic potential of nonPACCs. Together, our
201 results provide strong evidence that the clinically observed links between PACC
202 presence and risk of metastasis move beyond mere correlation. Our data point to a
203 combination of direct and indirect mechanisms that together support a causative
204 relationship between PACCs and increased metastatic risk.
205
206 **II. Results:**
207
208 **The majority of circulating tumor cells are in the PACC state**
209
210   We used size-matched and time-matched subcutaneous murine metastasis
211 models to measure the differential metastatic potential of PACCs through evaluation of
212 CTCs recovered from the blood. Mice were injected subcutaneously with either parental
213 cells or PACC-enriched cells confirmed to have increased ploidy at the population level
214 (Figure 1A, 2A).
215   In a time-matched model, blood from each mouse was collected and analyzed 6
216 weeks following tumor cell injection. At experimental endpoint, parental-injected mice
217 produced larger tumors than PACC-injected mice (Figure 1B) probably (?) due to the
218 transiently nonproliferative phenotype of PACCs abundant in the PACC-enriched
219 population. At experimental endpoint, the proportion of PACCs to nonPACCs in each
220 tumor equilibrated to similar levels (Figure 1C, appreciable by comparison of cell size).
221 In total, 3 CTCs were recovered, 2 of which were in the PACC state (66%) (Figure 1D,
222 Supplemental Figure S1).
223   To increase the number of recoverable CTCs, we repeated the experiment using
224 a tumor size-matched model, in which the experimental endpoint of each mouse was
225 independently determined. Blood was collected and analyzed when tumors reached
226 approximately 350 mm^3 (Figure 2B). Again, at experimental endpoint, the proportion of
227 PACCs to nonPACCs in each tumor equilibrated to similar levels (Figure 2C,
228 appreciable by comparison of cell size). In total, 33 CTCs were recovered, 25 of which
229 were in the PACC state (75%) (Figure D, Supplemental Figure S3). Altogether, these

230 data confirm that i) PACCs can survive as CTCs in the context of spontaneous
231 metastasis, and ii) the majority of CTCs recovered in this context are in the PACC state.
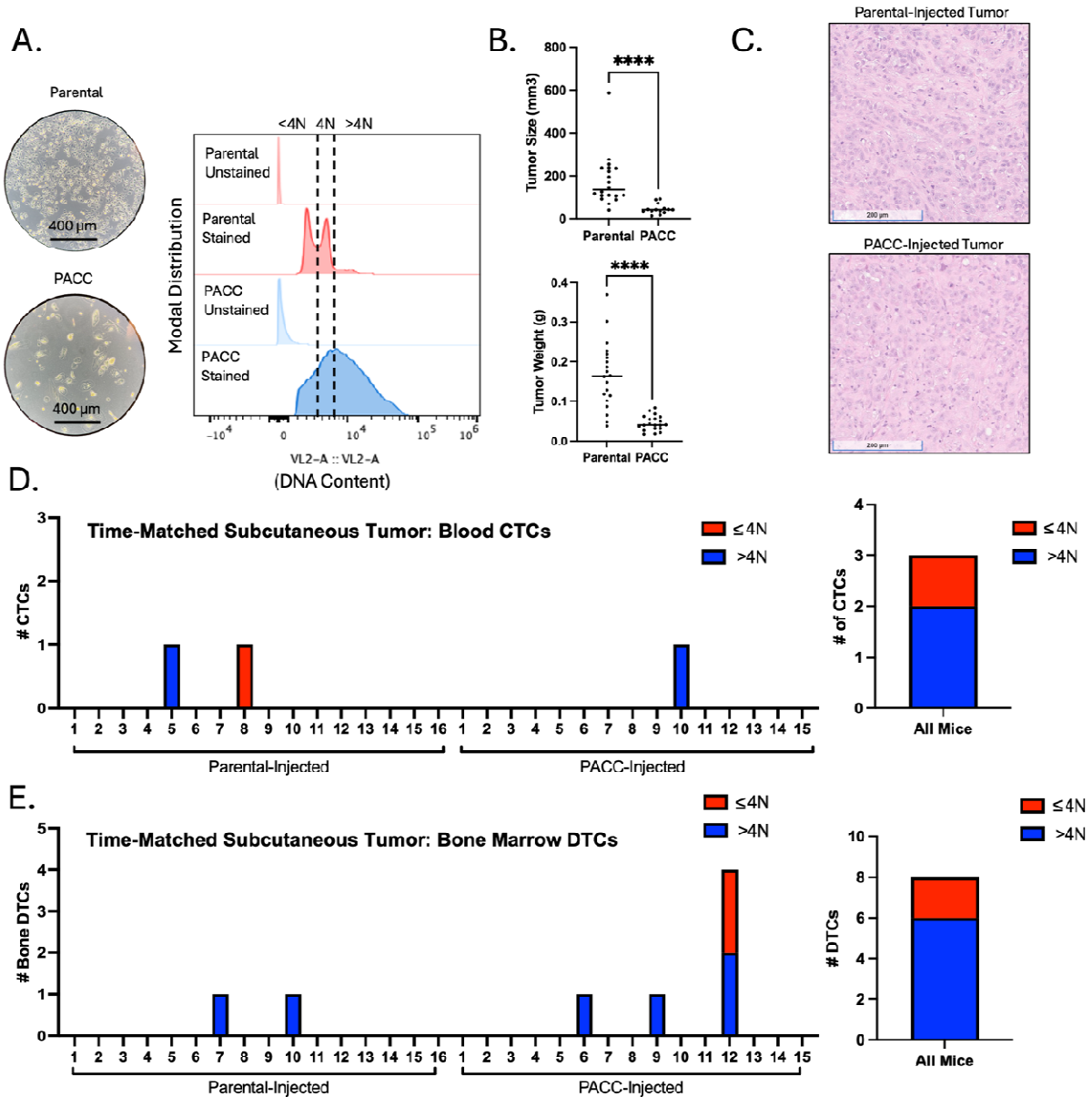
232
Figure 1:
Time-matched, subcutaneous injection of PC3-GFP-Luc parental population vs. PACC-enriched population: A) Light microscopy photos and flow-cytometric ploidy analysis of injected cells per injection group. B) Tumor volume and tumor weight measurements per injection group at experimental endpoint. C) Representative H&E photos of primary tumors per injection group. D) Enumeration of CTCs sourced from the blood of each animal and quantification of % >4N CTCs vs. ≤ 4N CTCs. E) Enumeration

240 of DTCs sourced from the bone marrow of each animal and quantification of % >4N
241 DTCs vs. ≤ 4N DTCs.

Supplemental Figure S1:
Time-matched, subcutaneous injection of PC3-GFP-Luc parental population vs. PACC-enriched population: A) Raw cytometric data reporting GFP+ signal across all blood samples. B) Raw cytometric data reporting DNA content of GFP+ cells across all blood samples.
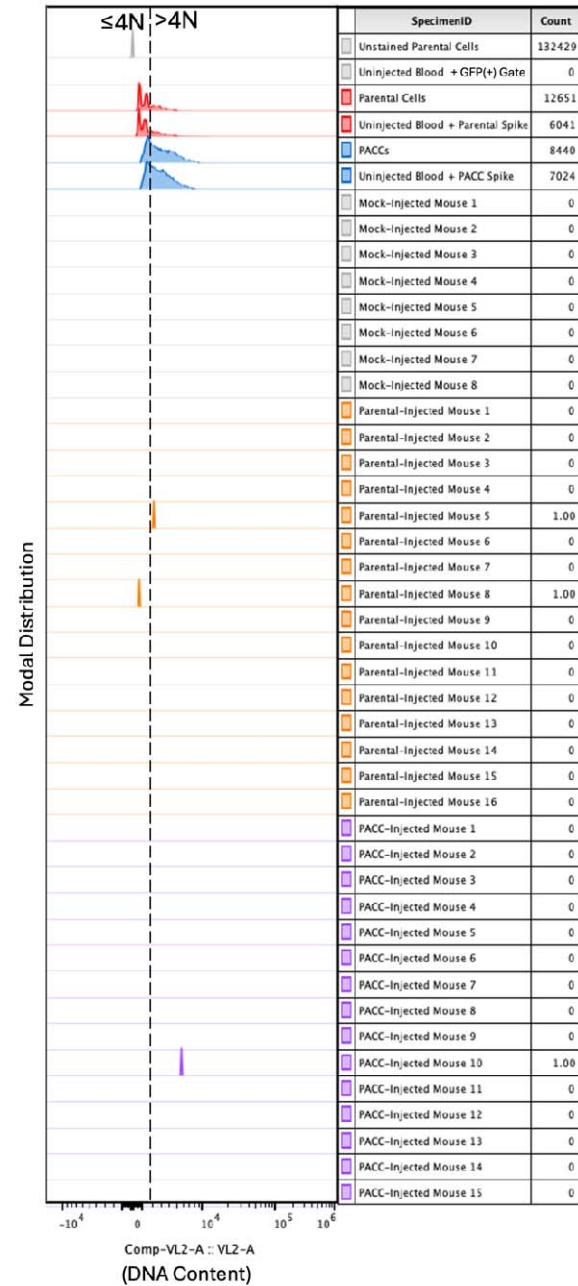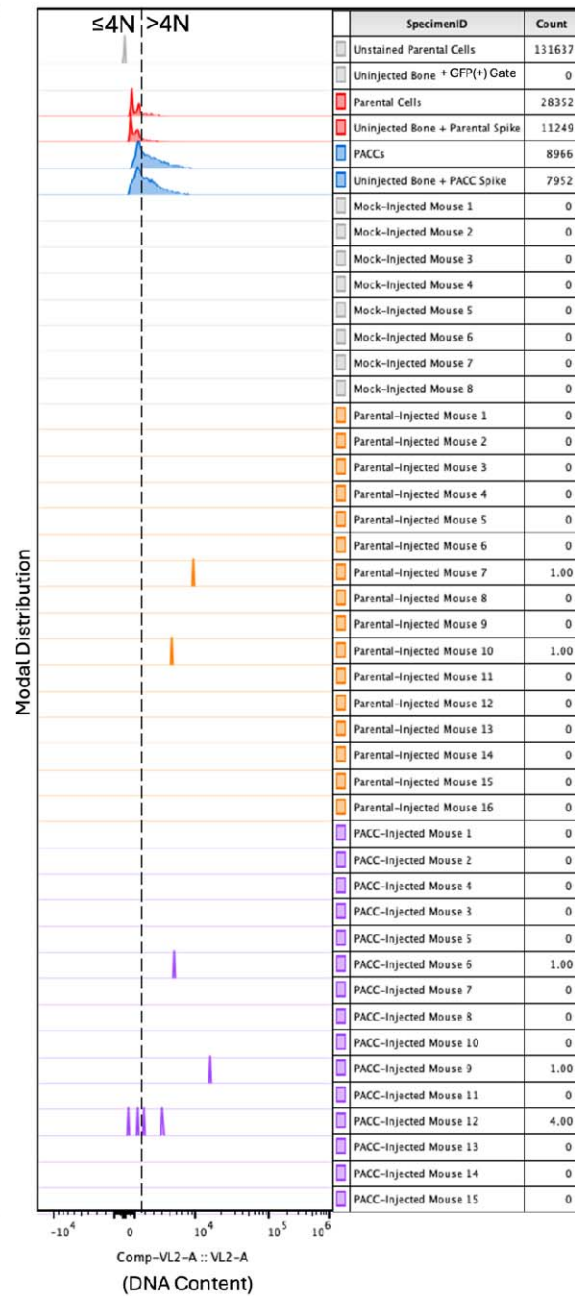
Supplemental Figure S2:
Time-matched, subcutaneous injection of PC3-GFP-Luc parental population vs. PACC-enriched population: A) Raw cytometric data reporting GFP+ signal across all bone marrow samples. B) Raw cytometric data reporting DNA content of GFP+ cells across all bone marrow samples.
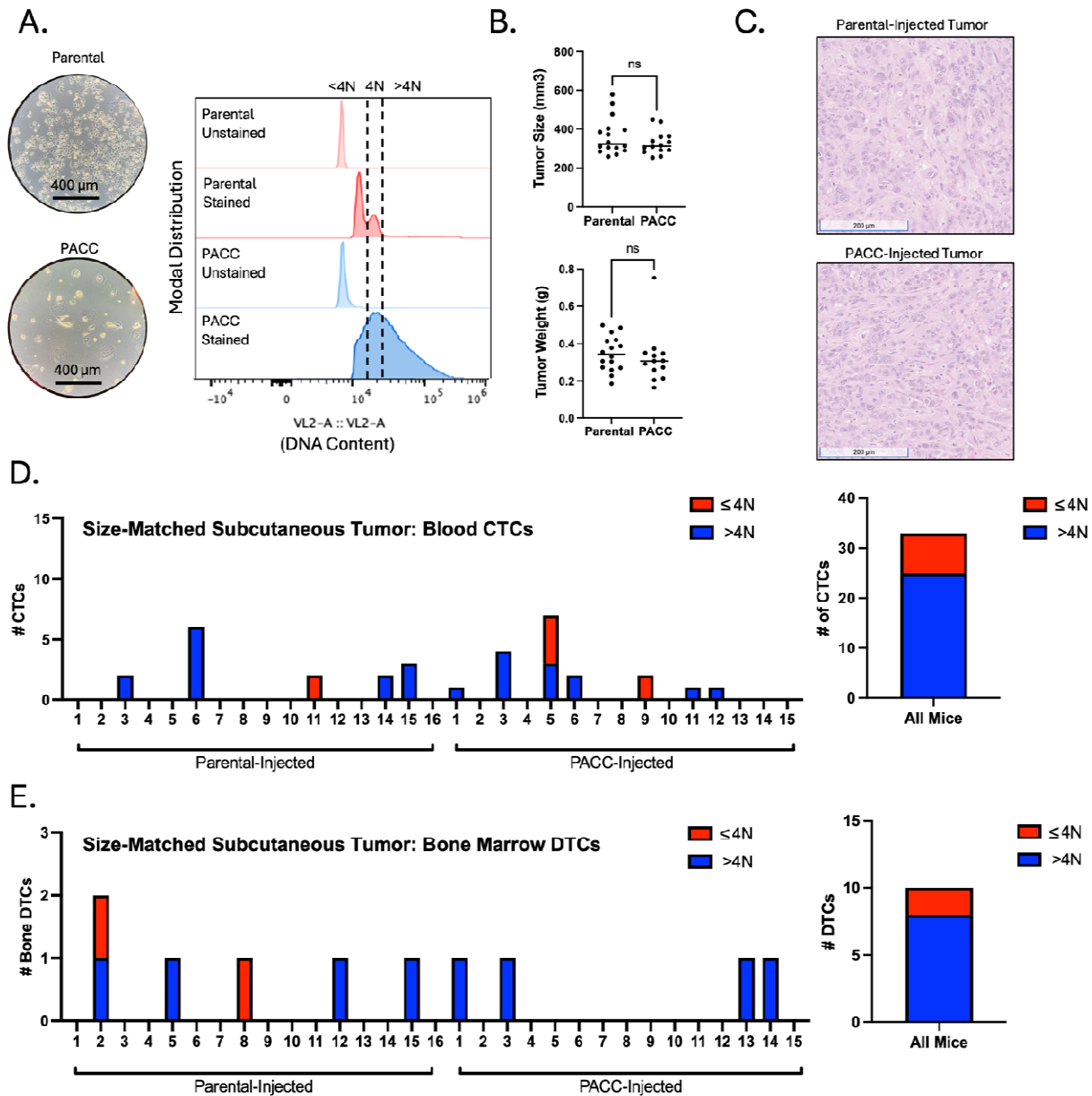
254
The majority of disseminated tumor cells are in the PACC state
256

257 The same two subcutaneous models used to measure blood CTCs were also
258 used to quantify and characterize DTCs recovered from hind-limb bone marrow. Across
259 both models, a majority of the DTCs recovered contained >4N DNA content, indicating
260 they are in the PACC state. In the time-matched model, 8 bone marrow DTCs were
261 recovered, 6 of which were in the PACC state (75%) (Figure 1E, Supplemental Figure
262 S2). In the size-matched model, 10 DTCs were recovered, 8 of which were in the PACC
263 state (80%) (Figure 2E, Supplemental Figure S4).
264 Subcutaneous tumor models are useful for investigating multiple steps of the
265 metastatic cascade (i.e. invasion, intravasation, survival in the circulation, and
266 extravasation) within one animal. However, they limit the ability to specifically query
267 differential extravasation capacity between cell phenotypes owing to potential upstream
268 bottlenecks that may differentially skew the numbers of each cell type surviving in the
269 circulation. To directly measure the differential extravasation potential of PACCs, we
270 used a caudal-artery injection model. Mice were injected with either parental cells or
271 PACC-enriched cells confirmed to have increased ploidy at the population level (Figure
272 3A). Bone marrow and lung tissue were collected and analyzed 3 days after injection.
273 Caudal artery injection introduces cells directly into the vasculature and directs them to
274 the hind limb bone marrow capillaries and lung capillaries, wherein they become lodged
275 due to size. After 72 hours, lodged cells have either been cleared from the vasculature
276 or, more rarely, have extravasated into surrounding tissue (Figure 3B). After 72 hours,
277 81 DTCs were recovered from the bone marrow, 59 of which were in the PACC state
278 (73%) (Figure 3C, Supplemental Figure S5). 132 DTCs were recovered from the lung,
279 111 of which were in the PACC state (84%) (Figure 3D, Supplemental Figure S6). This
280 data suggests that cells in the PACC state have increased extravasation potential
281 compared to their nonPACC counterparts. As such, not only are PACCs uniquely adept
282 at reaching and surviving within the circulatory system as CTCs, but they are also adept
283 at extravasating into secondary site tissues.

Figure 2:

Size-matched, subcutaneous injection of PC3-GFP-Luc parental population vs. PACC-enriched population: A) Light microscopy photos and flow-cytometric ploidy analysis of injected cells per injection group. B) Tumor volume and tumor weight measurements per injection group at experimental endpoint. C) Representative H&E photos of primary tumors per injection group. D) Enumeration of CTCs sourced from the blood of each animal and quantification of % >4N CTCs vs. ≤ 4N CTCs. E) Enumeration of DTCs sourced from the bone marrow of each animal and quantification of % >4N DTCs vs. ≤ 4N DTCs.

Supplemental Figure S3:
Size-matched, subcutaneous injection of PC3-GFP-Luc parental population vs. PACC-enriched population: A) Raw cytometric data reporting GFP+ signal across all blood samples. B) Raw cytometric data reporting DNA content of GFP+ cells across all blood samples.

Supplemental Figure S4:
Size-matched, subcutaneous injection of PC3-GFP-Luc parental population vs. PACC-enriched population: A) Raw cytometric data reporting GFP+ signal across all bone marrow samples. B) Raw cytometric data reporting DNA content of GFP+ cells across all bone marrow samples.

Figure 3:

Caudal Artery injection of PC3-GFP-Luc parental population vs. PACC-enriched population: A) Light microscopy photos and flow-cytometric ploidy analysis of injected cells per injection group. B) Representative BLI images capturing the cellular distribution and signal intensity immediately following caudal artery injection and 72 hours following caudal artery injection. C) Enumeration of DTCs sourced from the bone marrow of each animal and quantification of % >4N DTCs vs. ≤ 4N DTCs. E) Enumeration of DTCs sourced from the lung tissue of each animal and quantification of % >4N DTCs vs. ≤ 4N DTCs.

A. Caudal Artery Injections: Bone Marrow DTCs

B. Caudal Artery Injections: Bone Marrow DTCs

Supplemental Figure S5:

Caudal Artery injection of PC3-GFP-Luc parental population vs. PACC-enriched population: A) Raw cytometric data reporting GFP+ signal across all bone marrow samples. B) Raw cytometric data reporting DNA content of GFP+ cells across all bone marrow samples.
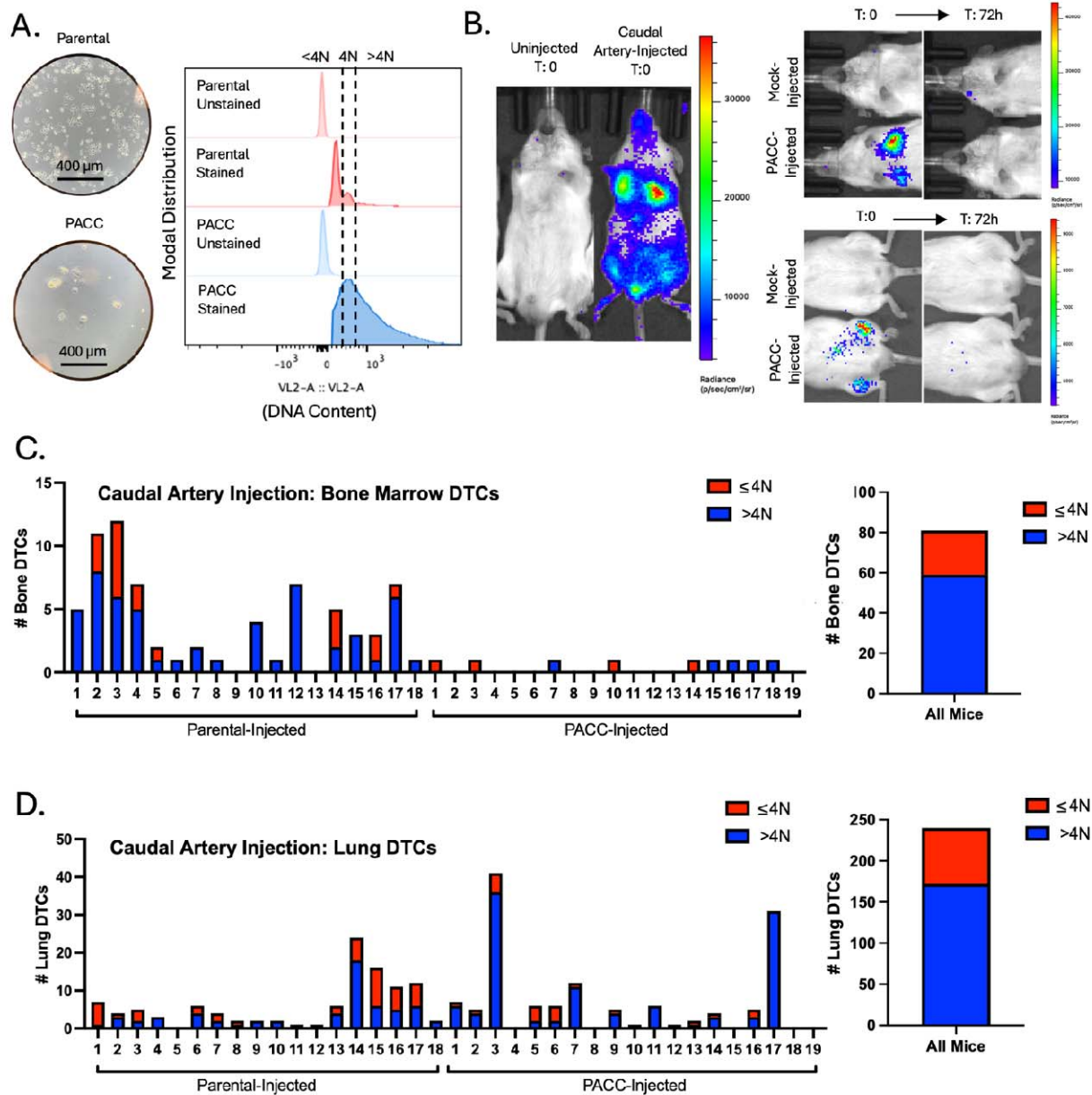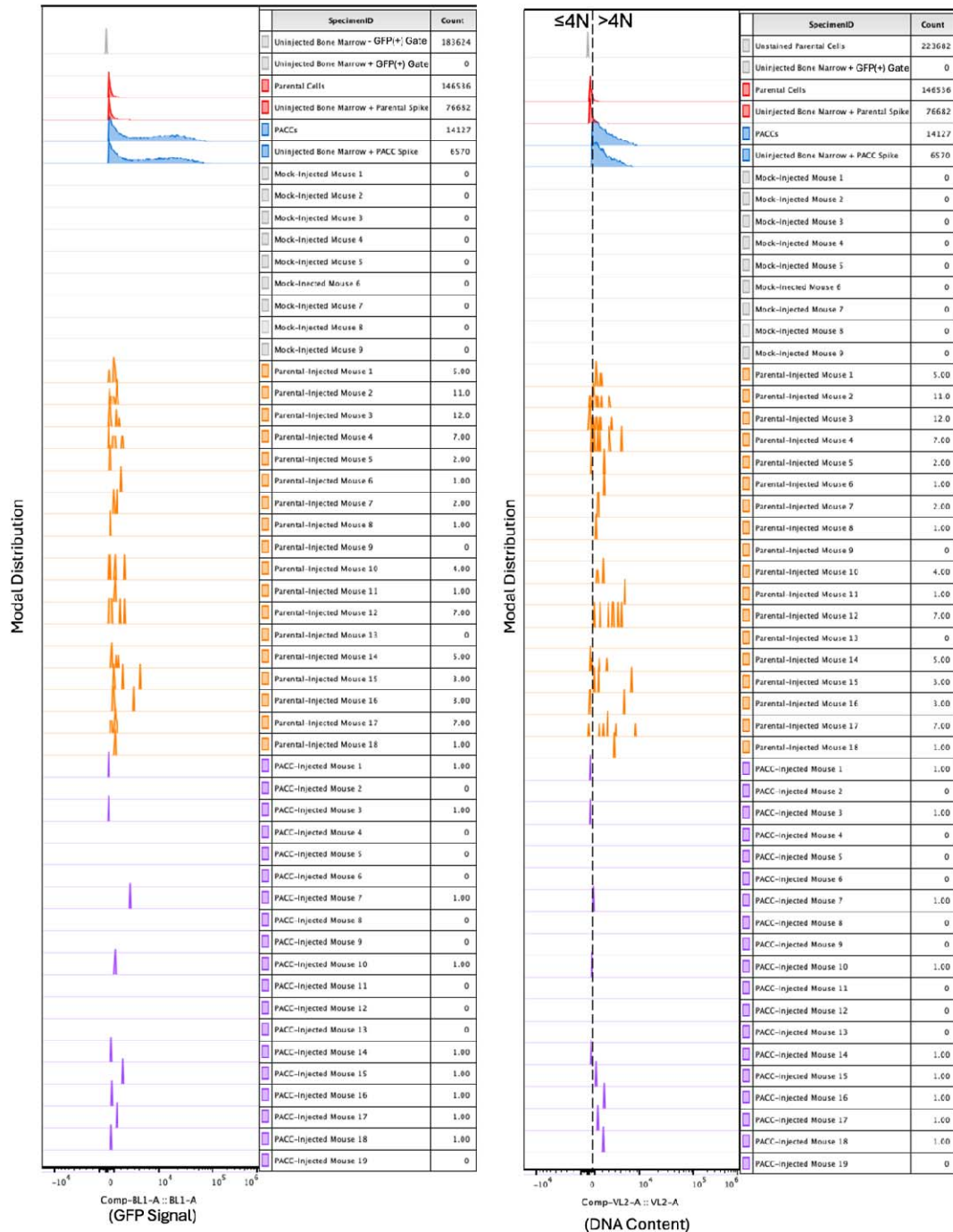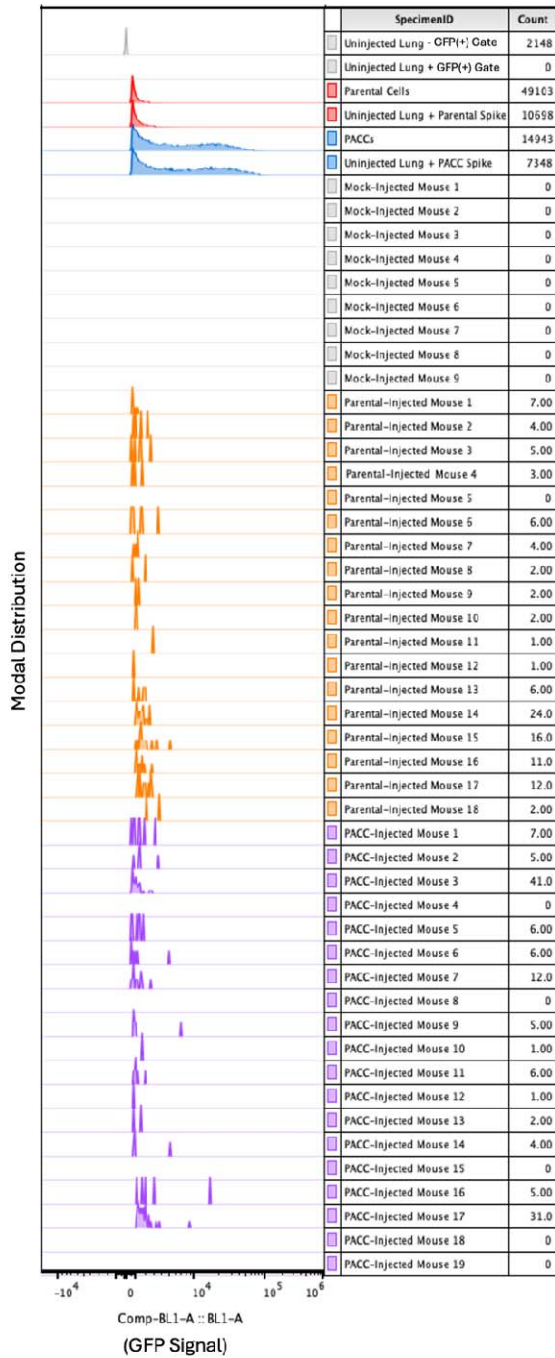
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A. Caudal Artery Injections: Lung DTCs

SpecimenID	Count
Uninjected Lung - GFP(+) Gate	2148
Uninjected Lung + GFP(+) Gate	0
Parental Cells	49103
Uninjected Lung + Parental Spike	10698
PACCs	14943
Uninjected Lung + PACC Spike	7348
Mock-Injected Mouse 1	0
Mock-Injected Mouse 2	0
Mock-Injected Mouse 3	0
Mock-Injected Mouse 4	0
Mock-Injected Mouse 5	0
Mock-Injected Mouse 6	0
Mock-Injected Mouse 7	0
Mock-Injected Mouse 8	0
Mock-Injected Mouse 9	0
Parental-Injected Mouse 1	7.00
Parental-Injected Mouse 2	4.00
Parental-Injected Mouse 3	5.00
Parental-Injected Mouse 4	3.00
Parental-Injected Mouse 5	0
Parental-Injected Mouse 6	6.00
Parental-Injected Mouse 7	4.00
Parental-Injected Mouse 8	2.00
Parental-Injected Mouse 9	2.00
Parental-Injected Mouse 10	2.00
Parental-Injected Mouse 11	1.00
Parental-Injected Mouse 12	1.00
Parental-Injected Mouse 13	6.00
Parental-Injected Mouse 14	24.0
Parental-Injected Mouse 15	16.0
Parental-Injected Mouse 16	11.0
Parental-Injected Mouse 17	12.0
Parental-Injected Mouse 18	2.00
PACC-Injected Mouse 1	7.00
PACC-Injected Mouse 2	5.00
PACC-Injected Mouse 3	41.0
PACC-Injected Mouse 4	0
PACC-Injected Mouse 5	6.00
PACC-Injected Mouse 6	6.00
PACC-Injected Mouse 7	12.0
PACC-Injected Mouse 8	0
PACC-Injected Mouse 9	5.00
PACC-Injected Mouse 10	1.00
PACC-Injected Mouse 11	6.00
PACC-Injected Mouse 12	1.00
PACC-Injected Mouse 13	2.00
PACC-Injected Mouse 14	4.00
PACC-Injected Mouse 15	0
PACC-Injected Mouse 16	5.00
PACC-Injected Mouse 17	31.0
PACC-Injected Mouse 18	0
PACC-Injected Mouse 19	0

Modal Distribution

-10^4 0 10^4 10^5 10^6

Comp-BL1-A :: BL1-A

(GFP Signal)

B. Caudal Artery Injections: Lung DTCs

≤4N | >4N

SpecimenID	Count
Unstained Parental Cells	223682
Uninjected Lung + GFP(+) Gate	0
Parental Cells	49103
Uninjected Lung + Parental Spike	10698
PACCs	8243
Uninjected Lung + PACC Spike	6361
Mock-Injected Mouse 1	0
Mock-Injected Mouse 2	0
Mock-Injected Mouse 3	0
Mock-Injected Mouse 4	0
Mock-Injected Mouse 5	0
Mock-Injected Mouse 6	0
Mock-Injected Mouse 7	0
Mock-Injected Mouse 8	0
Mock-Injected Mouse 9	0
Parental-Injected Mouse 1	7.00
Parental-Injected Mouse 2	4.00
Parental-Injected Mouse 3	5.00
Parental-Injected Mouse 4	3.00
Parental-Injected Mouse 5	0
Parental-Injected Mouse 6	6.00
Parental-Injected Mouse 7	4.00
Parental-Injected Mouse 8	2.00
Parental-Injected Mouse 9	2.00
Parental-Injected Mouse 10	2.00
Parental-Injected Mouse 11	1.00
Parental-Injected Mouse 12	1.00
Parental-Injected Mouse 13	6.00
Parental-Injected Mouse 14	24.0
Parental-Injected Mouse 15	16.0
Parental-Injected Mouse 16	11.0
Parental-Injected Mouse 17	12.0
Parental-Injected Mouse 18	2.00
PACC-Injected Mouse 1	7.00
PACC-Injected Mouse 2	5.00
PACC-Injected Mouse 3	41.0
PACC-Injected Mouse 4	0
PACC-Injected Mouse 5	6.00
PACC-Injected Mouse 6	6.00
PACC-injected Mouse 7	12.0
PACC-Injected Mouse 8	0
PACC-Injected Mouse 9	5.00
PACC-Injected Mouse 10	1.00
PACC-Injected Mouse 11	6.00
PACC-Injected Mouse 12	1.00
PACC-Injected Mouse 13	2.00
PACC-Injected Mouse 14	4.00
PACC-Injected Mouse 15	0
PACC-Injected Mouse 16	5.00
PACC-Injected Mouse 17	31.0
PACC-Injected Mouse 18	0
PACC-Injected Mouse 19	0

Modal Distribution

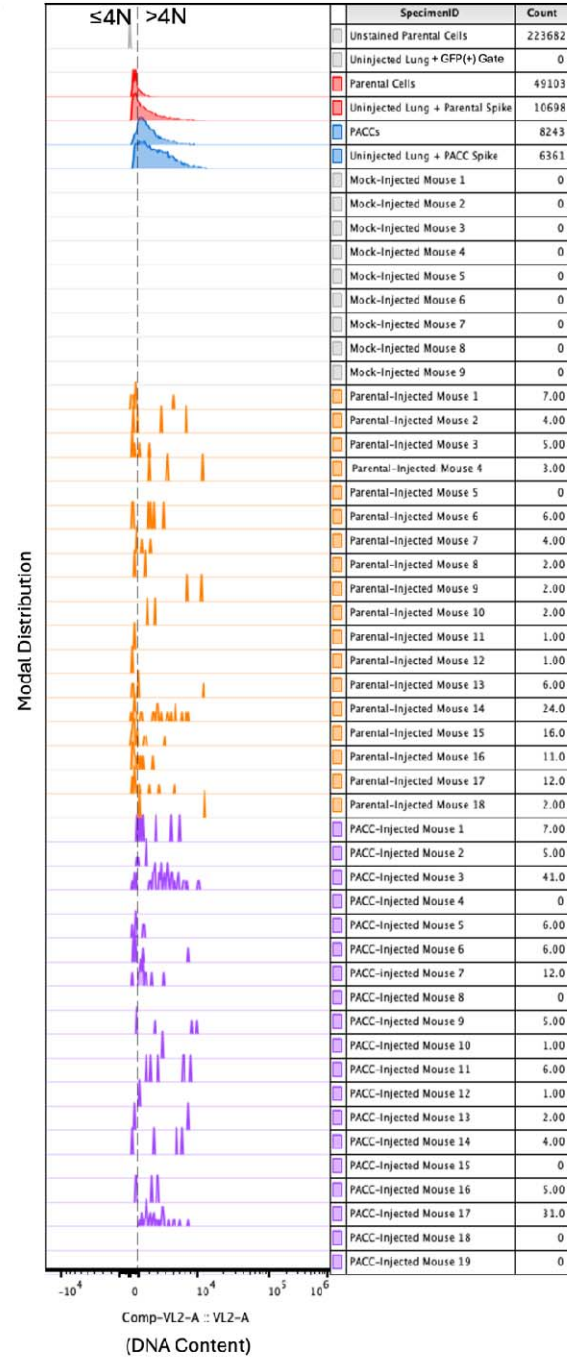-10^4 0 10^4 10^5 10^6

Comp-VL2-A :: VL2-A

(DNA Content)

Supplemental Figure S6:

Caudal Artery injection of PC3-GFP-Luc parental population vs. PACC-enriched population: A) Raw cytometric data reporting GFP+ signal across all lung tissue samples. B) Raw cytometric data reporting DNA content of GFP+ cells across all lung tissue samples.

15

325   PACCs can be found at a low baseline level (5%) in parental populations of the
326   PC3 cell line and are generally thought to reflect the stress inherent to cell culturing.
327   Therefore, it is possible that PACC-state DTCs recovered from parental-injected mice
328   result from selection of pre-existing PACCs in the parental population and reflect the
329   increased metastatic risk of cells in the PACC state. Alternatively, it is possible that
330   stress experienced during the metastatic process induced nonPACCs in the parental
331   population to access the PACC state *in vivo*. To investigate the source of the PACC-
332   state DTCs recovered from parental-injected mice, we performed a tail vein experiment
333   comparing a population of parental cells (inherently containing a small percentage of
334   PACCs) to a population of nonPACC enriched cells generated by size-filtering a
335   parental population through a 10-micron filter to remove PACCs. Mice were injected
336   with either unfiltered (parental) cells or filtered (nonPACC-enriched) cells confirmed to
337   contain fewer cells of increased ploidy (Supplemental Figure S7A). After 72 hours, lung
338   tissues were collected and analyzed for DTCs. Across all mice, 566 DTCs were
339   recovered, 328 of which were in the PACC state (58%). This is consistent with our
340   previous findings (Supplemental Figure S7B). When comparing the proportion of lung
341   DTCs in the PACC state between the two injection groups, we found no difference
342   (unfiltered: 58% vs. filtered: 57%) (Supplemental Figure S7C). These data show that a
343   reduction in the percentage of baseline PACCs present in the parental population does
344   not change the percentage of PACCs present among recovered DTCs, suggesting a
345   possibility that nonPACCs in the parental population may access the PACC state *in*
346   *vivo.*
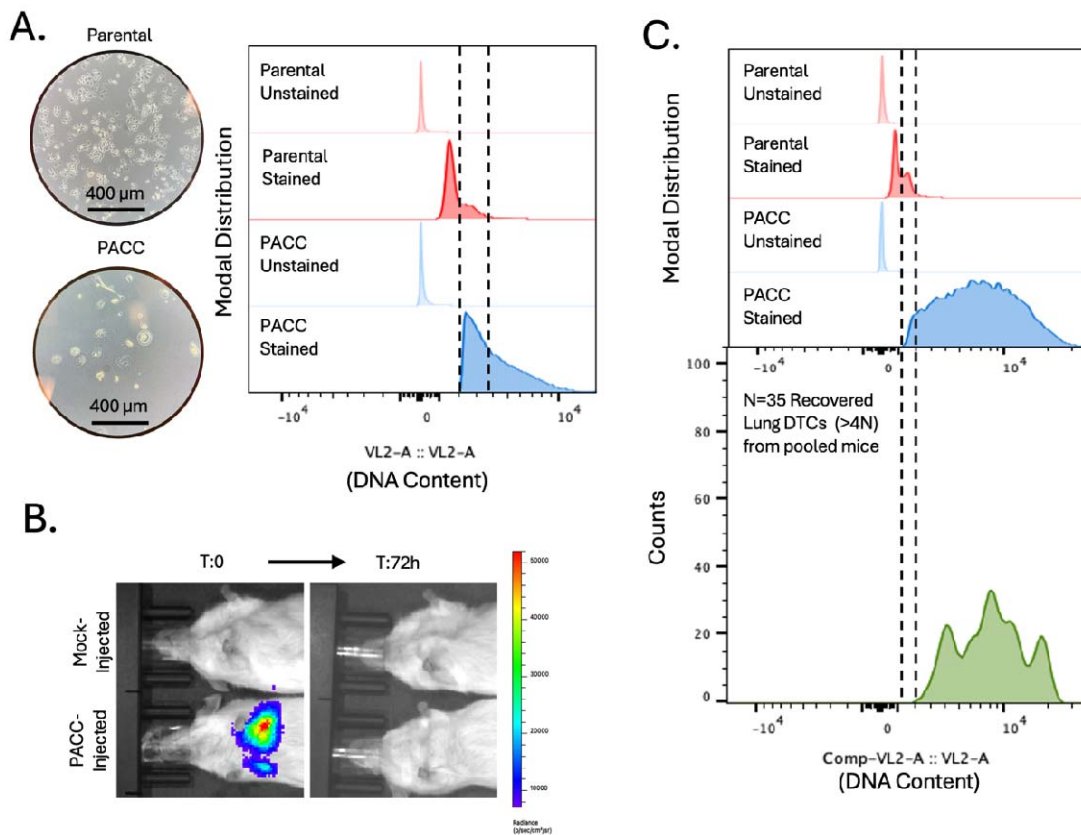
347 **Supplemental Figure S7:**
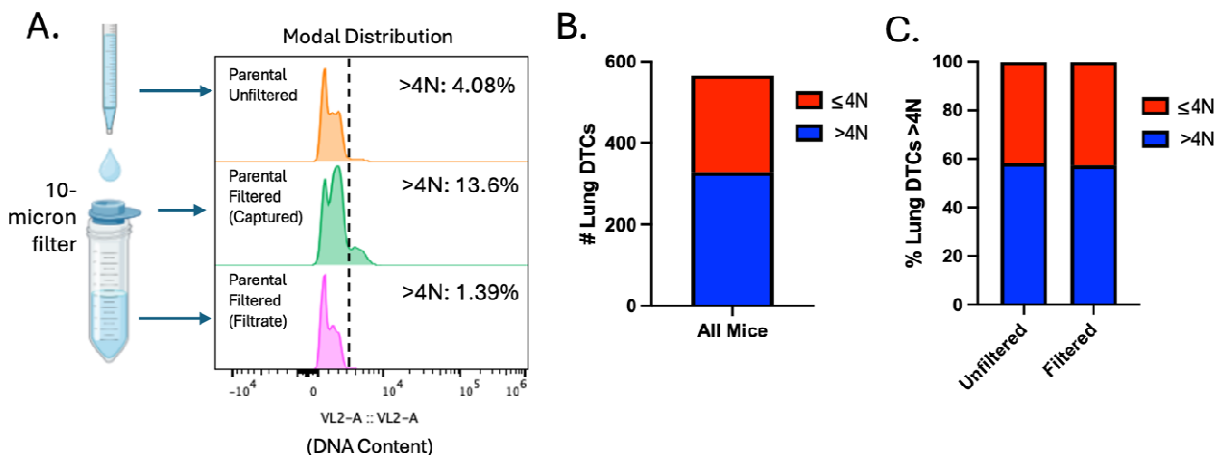
348   Tail Vein injection of PC3-GFP-Luc parental population vs. PACC-depleted
349   parental population: A) Flow-cytometric ploidy analysis of injected cells per injection
350   group. B) Proportion of lung DTCs with >4N DNA content across all experimental mice.
351   C) Comparative proportions of lung DTCs with >4N DNA content between two injection
352   groups.
353
354 **PACCs are capable of colonization following a period of dormancy:**

16

355   Clinically relevant metastatic potential requires colonization capacity. *In vitro*,
356   cells induced to enter the PACC state become transiently nonproliferative, existing in a
357   nondividing state. It has been observed that PACCs can survive in this state for months,
358   a phenomenon compatible with that of metastatic tumor cell dormancy. Following a
359   period of nonproliferative dormancy, some cells within the *in vitro* PACC-induced
360   population return to a proliferative mitotic cell cycle, consistent with latent metastatic
361   outgrowth frequently observed in human patients. To initially test the short-term survival
362   status of PACCs when introduced *in vivo*, PACC-enriched cells were injected into the
363   tail-vein of mice (Supplemental Figure S8A). The lungs were analyzed for surviving
364   DTCs in the PACC state after 21 days (Supplemental Figure S8B). After pooling mice
365   together, 35 DTCs in the PACC state were found still surviving in lung tissue
366   (Supplemental Figure S8C).
367

368 **Supplemental Figure S8:**
369   Tail Vein injection of PC3-GFP-Luc parental population vs. PACC-enriched
370 population: A) Light microscopy photos and flow-cytometric ploidy analysis of injected
371 cells per injection group. B) Representative BLI images capturing the cellular distribution
372 and signal intensity immediately following tail vein injection and 3 days following tail vein
373 injection. C) Raw cytometric data reporting the presence of >4N Lung DTCs in a pooled
374 sample 21 days following PACC injection.

Satisfied that PACCs do not immediately re-enter a proliferative cell cycle nor immediately die when introduced *in vivo*, we sought to understand the long-term survival and growth kinetics of PACCs *in vivo*. Mice were subcutaneously injected with either a population of parental cells (serving as a positive control) or a population of size filtered PACCs and monitored for 12 weeks using bioluminescent imaging (BLI). The size filtered PACC population was confirmed to contain only cells of at least 4N or greater ploidy. (Figure 4A). Within 4 weeks, 6/6 positive control mice developed appreciable tumors and showed the expected marked increase in BLI flux. At that time, 0/12 PACC-injected mice presented with palpable tumors, but BLI showed evidence of tumor cell survival at injection site (Figure 4B). Positive control mice reached ethical tumor burden and were euthanized 6 weeks post-injection. PACC-injected mice were monitored for an additional 6 weeks before they were euthanized. At experimental-endpoint, all PACC-injected mice showed greater levels of BLI flux than negative controls. 7/12 PACC-injected mice showed BLI evidence of nonproliferative tumor cell survival localized to the injection site, and an additional 2/12 PACC-injected mice had developed slow-growing palpable subcutaneous tumors following a latent dormancy phase (Figure 4C, 4D).


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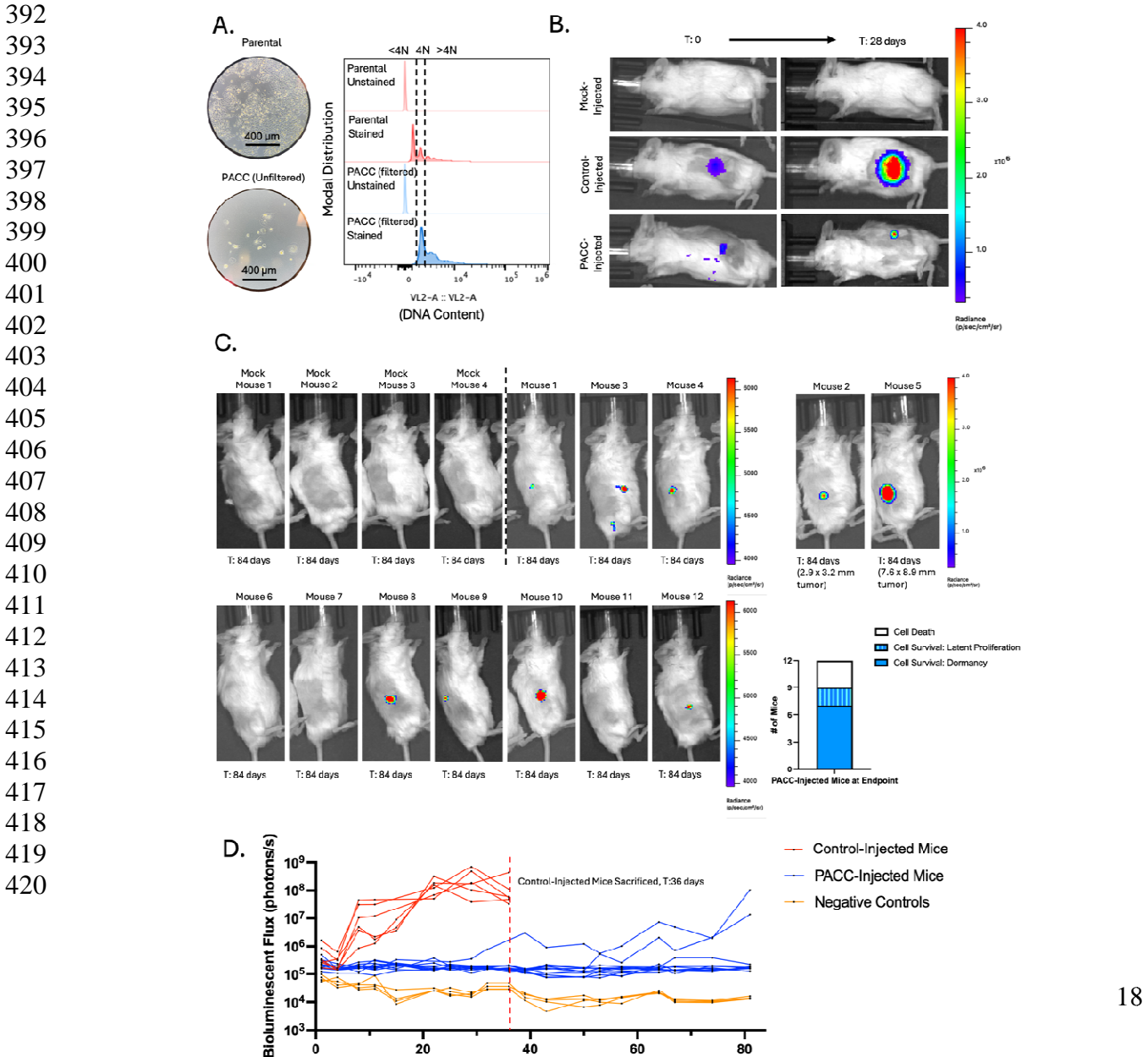
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441 **Figure 4:**
442 Subcutaneous injection of PC3-Luc parental population vs. size filtered PACC
443 population: A) Light microscopy photos and flow-cytometric ploidy analysis of injected
444 cells per injection group. B) Representative BLI images capturing the cellular distribution
445 and signal intensity immediately following subcutaneous injection and 28 days following
446 subcutaneous injection. C) BLI images of 4 mock-injected and 12 PACC-injected mice
447 82 days following subcutaneous injection. Note the different scale for Mouse 2 and
448 Mouse 5. Quantification of the status of injected cells across all 12 PACC-injected mice
449 at experimental endpoint. D) Weekly BLI flux of all experimental mice over 84 days.
450
451 We next used an intracardiac injection model to test the dormancy and
452 colonization kinetics of PACCs in a metastasis-relevant context (i.e. following survival in
453 the circulation and subsequent extravasation). Mice were injected directly into the left
454 ventricle with either a population of parental cells (serving as a positive control) or a
455 population of PACC-enriched cells confirmed to have increased ploidy at the population
456 level (Figure 5A). Within 6 weeks, 6/7 positive control mice had evidence of liver and/or
457 bone metastases by BLI. At that time, 0/7 PACC-injected mice showed any BLI positive
458 lesions (Figure 5B). Positive control mice reached ethical tumor burden and were
459 euthanized between 6 and 14 weeks. PACC-injected mice were monitored for an
460 additional 5 weeks. By experimental endpoint, 4/7 PACC-injected mice had showed
461 evidence of liver metastases by BLI, but none of these metastases reached a BLI flux
462 threshold indicative of rapidly progressive disease (Figure 5C, 5D). Taken together,
463 these data indicate that PACCs are capable of both i) long-term *in vivo* survival in a
464 nonproliferative state and ii) return to a proliferative phenotype able to seed metastatic
465 colonization following a period of dormancy.

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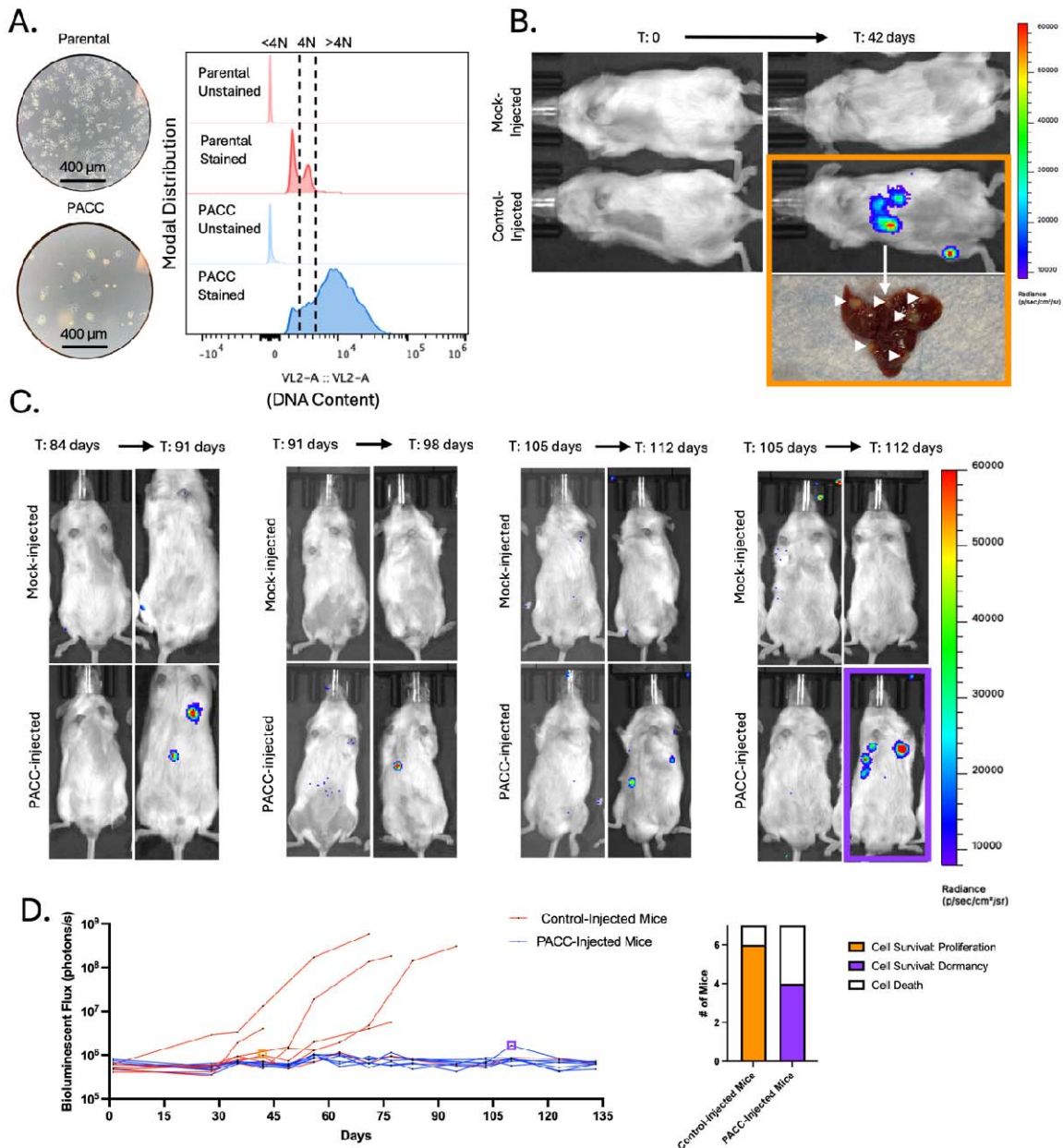
Figure 5:
Intracardiac injection of PC3-GFP-Luc parental population vs. PACC-enriched population: A) Light microscopy photos and flow-cytometric ploidy analysis of injected cells per injection group. B) Representative BLI images capturing the cellular distribution and signal intensity immediately following intracardiac injection and 42 days following intracardiac injection. C) BLI images of 4 mock-injected and 4/7 PACC-injected mice that showed evidence of colonization following dormancy between 91- and 112-days post intracardiac injection. D) Weekly BLI flux of all experimental mice over 135 days.

20

474   Quantification of the status of injected cells across all 7 PACC-injected mice at
475   experimental endpoint.

PACCs display a partial EMT phenotype:

477   *In vitro* studies show that cells in the PACC state display enhanced metastatic
478   phenotypes including motility, chemotaxis, and invasion (20, 21). The present work
479   shows that most CTCs and DTCs recovered across various metastatic models are in
480   the PACC state, supporting the hypothesis that PACCs promote a tumor's metastatic
481   potential through cell-intrinsic metastatic competency. In the past decade, it has been
482   increasingly reported that metastatic competency relies heavily on a partial-Epithelial-to-
483   Mesenchymal-Transition (pEMT) phenotype. pEMT (also called hybrid-EMT and mixed-
484   EMT, among other similar names) is a nonbinarized variant of the canonically mutually
485   exclusive epithelial vs. mesenchymal phenotypes that is characterized by co-expression
486   of proteins typically associated with only one or the other. Using the PC3 prostate
487   cancer cell line, we found that at the RNA level, PACCs show an increase in *ZEB1*,
488   *VIM*, and *CLDN1* expression and no difference in *SNAI1, SNAI2, TWIST1, CDH2,*
489   *CDH1,* or *EPCAM* expression (Figure 6A). At the protein level, PACCs show an
490   increase in VIM, CDH2, CLDN1, and CDH1 expression, a decrease in SNAI1, SNAI2,
491   and EPCAM expression, and no change in low-level TWIST1 expression (Figure 6B).
492   High co-expression of VIM (a classic mesenchymal marker) and CLDN1 (a known
493   epithelial marker) in PACCs indicate a pEMT phenotype. Absence of inverse expression
494   between PACC CDH2 (a classic mesenchymal marker) and PACC CDH1 (a classic
495   epithelial marker) also supports a PACC-specific pEMT phenotype (Figure 6C).
496   To evaluate the pEMT status of metastatic PACCs in a clinical context, we
497   stained cancer cells in the bone marrow isolated from prostate cancer patients with a
498   DNA content dye, a cocktail of epithelial-specific markers, and Vimentin. Cells with 4x-
499   greater-than-average nuclear area were deemed PACCs. Single-cell copy number
500   analysis confirmed that the PACCs were tumor-derived (Figure 6D). 34 of 44 patients
501   were found to have PACC cancer cells. Of those 34, 6 patients were found to have
502   pEMT PACC cancer cells positive for both pan-Epithelial (including EPCAM) and VIM
503   stains (Representative images: Figure 6E). These data support our *in vitro* observations
504   of PACC-specific pEMT in metastatic prostate cancer patients and provide a plausible
505   mechanism for increased metastatic competency among cells in the PACC state.
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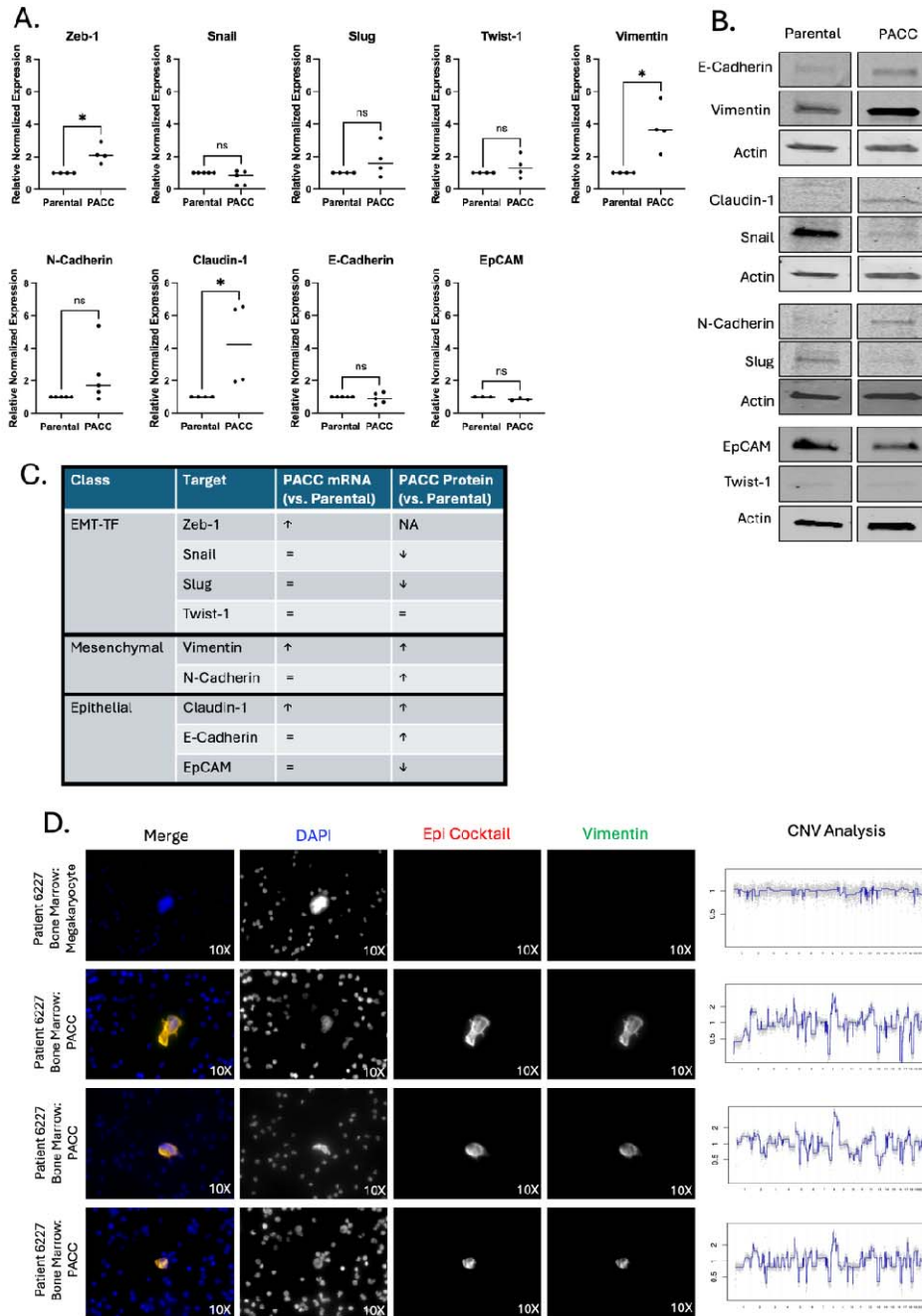
Figure 6:

PACCs have a pEMT phenotype: A) RNA expression of a panel of EMT markers by RTqPCR in a PC3-Luc parental population vs. size filtered PACC population. B) Protein expression a panel of EMT markers by Western blot in a PC3-Luc parental population vs. size filtered PACC population, where each biological replicate reported is an average of three technical replicates. C) Summary table of RNA and protein expression. D) Representative immunofluorescent mages of PACCs identified as DTCs in the bone marrow of a metastatic prostate cancer patient, stained for DAPI, an Epithelial-origin cocktail, and VIM. CNV analysis of the corresponding single cell.

PACCs have a pro-metastatic secretory profile:

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552   In addition to the hypothesis that PACCs promote a tumor's metastatic potential
553 because PACCs themselves are more metastatic, PACCs might otherwise promote a
554 primary tumor's metastatic potential by increasing the metastatic phenotype of
555 surrounding tumor cells. To test this alternative model, we evaluated the motility
556 phenotype of nonPACC cells cultured in PACC-conditioned media compared to control
557 parental cell-conditioned media. We found that PACC-conditioned media increases
558 nonPACC motility by two common motility assays (Figure 7A, Supplemental Figure
559 S9A), which was not accompanied by an increased transcriptional EMT phenotype
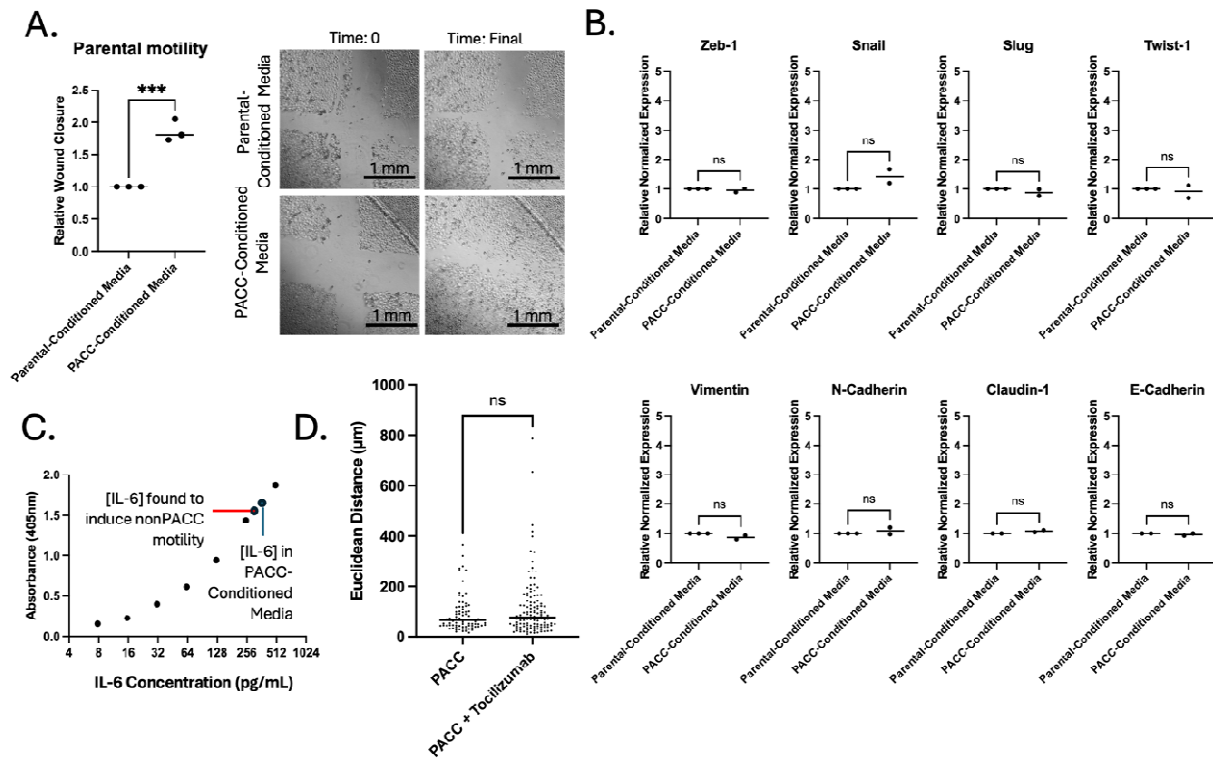560 (Supplemental Figure S9B).
561   We performed a cytokine array on parental cell-conditioned media and PACC-
562 conditioned media to identify abundant cytokines differentially secreted by PACCs. IL6
563 was the strongest candidate for further study (Figure 7B). We measured the
564 concentration of IL6 in PACC-Conditioned Media (Supplemental Figure S9C) and found
565 that a similar concentration was sufficient to produce increased motility in nonPACCs
566 (Figure 7C). This increased motility phenotype was abrogated by inhibition of the IL6
567 Receptor (IL6R) via addition of Tocilizumab (Figure 7D). We and others have previously
568 published the PACCs are more motile than nonPACCs (20, 21). To rule out the
569 possibility that increased PACC motility is the result of autocrine-acting IL6, we applied
570 Tocilizumab to PACC samples and observed no change in PACC motility
571 (Supplemental Figure S9D). These data suggest that in addition to cell-autonomous
572 pro-metastatic effects, PACCs contribute to a tumor microenvironment that promotes
573 pro-metastatic phenotype in nonPACC tumor cells.
574
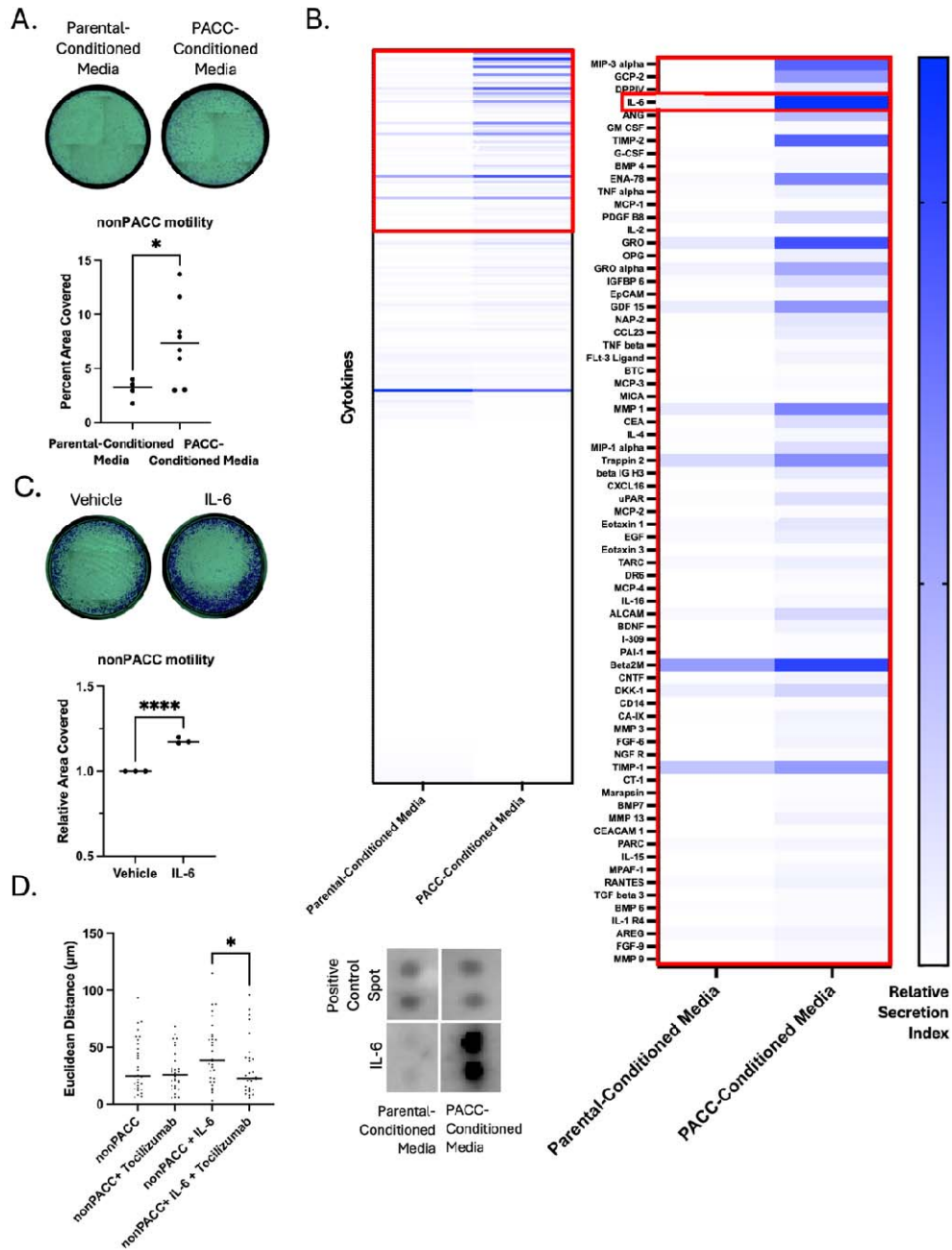
Figure 7:

PACCs have a pro-metastatic secretory profile: A) Transswell assay to measure the differential motility of a PC3-Luc population exposed to parental-conditioned media vs. PACC-conditioned media, where each reported biological replicate is an average of two technical replicates. B) Cytokine array to measure the relative abundance of 274 cytokines of interest in parental-conditioned media vs. PACC-conditioned media. C) Transswell assay to measure the effect of addition of recombinant IL6 on the motility of a PC3-Luc population. D) Single cell tracking to measure the effects of addition of recombinant IL6 and/or Tocilizumab on the motility of a PC3-Luc population.

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Supplemental Figure S9:

PACCs have a pro-metastatic secretory profile: A) Wound-healing assay to measure the differential motility of a PC3-Luc population exposed to parental-conditioned media vs. PACC-conditioned media, where each reported biological replicate is an average of two technical replicates. B) RNA expression of a panel of EMT markers by RTqPCR in a PC3-Luc parental population exposed to parental-conditioned media vs. PACC-conditioned media, were each biological replicate reported is an average of three technical replicates. C) Elisa assay to measure the concentration of IL6 in PACC conditioned media. D) Single cell tracking to measure the effects of addition of Tocilizumab on the motility of a PACCs.

III. Discussion:

Our recent study demonstrating the clinical relevance of cells in the PACC state as reliable predictors of the risk of prostate cancer recurrence (27) raised important questions about the roles that PACCs may play in metastatic progression. For example, accession of the PACC state may increase intrinsic pro-metastatic features, making PACCs more metastatically competent than their nonPACC counterparts. Alternatively, PACCs may increase the metastatic competency of nearby nonPACCs via a paracrine-functioning pro-metastatic phenotype. Or perhaps PACCs are merely uninvolved "third-party" cells incidentally produced by an unknown stimulus that itself is driving metastatic risk via an otherwise unrelated mechanism. Here, we sought to test for a direct causative link between PACC presence and metastatic propensity. We tested for the presence of PACCs throughout various steps – and therefore locations – of the metastatic cascade using distinct metastatic mouse models.

When testing the blood for metastatic cells capable of invading, intravasating, and then surviving in the circulation, we found that 75% of recovered CTCs were in the PACC state (27/36 CTCs). When testing the bone marrow for metastatic cells capable of invading, intravasating, surviving, and then extravasating, we found that 77% of recovered DTCs were in the PACC state (14/18 DTCs). It was pertinent to test both time-matched and size-matched subcutaneous models to account for any potential effects that the differing growth kinetics of the two injections groups might have had on the initiation of the angiogenic switch. Appropriately, an increased number of total CTCs and DTCs were recovered in the size-based experiment that at endpoint had larger primary tumors than the time-based experiment. We did not consistently find that PACC-injected mice produced a larger number of metastasizing cells. Analysis of the proportion of PACCs within the tumors of each injection group at experimental endpoint offered a potential explanation: the initial difference between the proportions of PACCs within each injected population (less than 5% PACCs among parental populations, but over 50% among PACC-enriched populations) equilibrated within 6 weeks. A decrease in PACC proportion over time in the PACC-enriched tumors could be caused by a delayed return to mitotic cell cycle among PACCs, or a presence of a small number of mitotic nonPACCs in the injection population. An increase in PACC proportion over time in the parental population could be caused by accumulation of tumor microenvironmental stressors *in vivo* that initiate PACC state entry as has been previously reported (18, 29, 30).

The similarity between the proportions of PACCs found among the recovered CTCs and the recovered DTCs (75% vs 77%) in the subcutaneous tumor models does not adequately support a conclusion of increased extravasation potential among cells in the PACC state. To specifically test for increased extravasation potential among PACCs, we used a caudal artery injection model that allowed for analysis of bone marrow, the most common site of prostate metastasis in patients. When testing the bone marrow for metastatic cells capable of extravasation, we found that 73% of recovered DTCs were in the PACC state (59/81 DTCs). When testing the lung for metastatic cells capable of extravasation, we found that 84% of recovered DTCs were in the PACC state (111/132 DTCs). This data indicates that cells in the PACC state have increased extravasation potential compared to nonPACCs. We and others have

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653   previously published that PACCs demonstrate both persistent chemotactic-driven
654   motility and functional deformability, phenotypes which may explain their increased
655   ability to extravasate (20, 21). An alternative hypothesis supposes that extravasated
656   nonPACCs entered the PACC state after entering secondary organ tissue owing to the
657   inherent stressors of that organ site. When comparing the portion of PACCs found
658   among total DTCs in the lungs of animals injected with parental populations with low-
659   level baseline PACCs vs. a PACC-depleted population, we found that reduction of
660   baseline PACCs present does not change the percentage of PACCs present among
661   recovered DTCs. This data suggests that it is possible that stress experienced during
662   the metastatic process may induce nonPACCs to access the PACC state *in vivo,* but
663   further study is necessary.

664   Interestingly, in the caudal-artery model, most of the bone marrow DTCs were
665   sourced from parental-injected mice (though the majority of those DTCs were in the
666   PACC state), but the majority of lung DTCs were sourced from PACC-injected mice.
667   This difference may represent biologically interesting differences in tissue-specific
668   extravasation-barriers. For example, potential size or biophysical rigidity restrictions in
669   the bone marrow may select for cells in the PACC state on the smaller end of what is
670   known to be a heterogenous spectrum: the number of endocycles is inherently linked to
671   the size of a PACC's nucleus and cell body. Notably, baseline PACCs found in parental
672   populations are frequently smaller than PACCs found in chemotherapy induced
673   populations. It is possible, therefore, that bone-specific environmental pressure selects
674   for extravasation of small PACCs, which are disproportionately found in parental
675   populations.

676   Presence of CTCs and DTCs alone is not sufficient to claim complete metastatic
677   competency; evidence of functional colonization is also needed. Multiple *in vitro* studies
678   have reported on the latent depolyploidization (also called ploidy reversal or ploidy
679   reduction) of cells in the PACC state following long stints of survival without cell division
680   (31-35). The progeny of PACCs appear to be of typical cancer cell size and genomic
681   content and display a typical mitotic cell cycle. We used two models to specifically test
682   the dormancy and colonization potential of PACCs *in vivo.* In both settings, the PACC
683   state life cycle closely followed what has been observed *in vitro.* 12 weeks following
684   subcutaneous injection of size filtered PACCs (to remove any infiltrating mitotic
685   nonPACC cells), 7/12 mice showed survival of nonproliferative PACCs at the injection
686   site, and 2/12 mice showed delayed tumor establishment followed by slow proliferation,
687   indicative of an *in vivo* depolyploidization event. By 19 weeks following intracardiac
688   injection of PACCs, 4/7 mice had established, presumably proliferative, metastatic
689   lesions.

690   Taken together, our *in vivo* data provides strong evidence of a causative
691   relationship between PACCs and metastasis. Next, we sought to establish a preliminary
692   mechanism underlying this pro-metastatic phentoype. In the past decade, there has
693   been a notable shift away from a binary and mutually exclusive Epithelial vs.
694   Mesenchymal phenotype. Instead, researchers have begun to appreciate the existence
695   – and importance – of pEMT phenotypes. In fact, many groups have reported that cells
696   with pEMT expression profiles display increased metastatic competency (36-39),
697   perhaps owning to the needs of successfully metastatic cells to have motility programs
698   that mesenchymal cells provide and the proliferative programs that epithelial cells

699 provide. Our RNA and protein analysis of cells in the PACC state revealed a clear
700 pEMT pattern, most strongly supported by simultaneous expression of *CDH1* and *VIM*.
701 Notably, others have also reported pEMT phenotypes in PACCs (40). Analysis of bone
702 marrow DTCs from patients with prostate cancer supported our *in vitro* findings: patient
703 bone marrow contained PACCs co-expressing pan-Epithelial markers (including
704 EPCAM) and VIM at the protein level.
705   To test for a potentially indirect mechanistic link, we turned to the literature citing
706 the similarities between what we have termed cells in the PACC state and what others
707 have termed therapy-induced senescent cells (41-45). The therapy-induced senescent
708 cell literature defines this cell type as one arising in response to treatment and
709 characterized, among other features, by a transient pause in cell cycle. Though therapy-
710 induced senescent cells depart from classically terminal senescent cells in several
711 ways, they have been reported to share the senescence-associated secretory
712 phenotype (SASP). Most notably, the SASP has been reported to contribute to a more
713 pro-metastatic tumor microenvironment (46, 47). Accordingly, we thought it possible
714 PACCs might promote the metastatic phenotype of surrounding nonPACC tumor cells
715 by contributing to a pro-metastatic tumor microenvironment. We found that PACCs
716 produce a SASP-like secretory profile rich in MIP3-alpha, GCP-2, DPPIV, IL-6, GM-
717 CSF, G-CSF, ENA-78, TNF alpha, MCP-1, IL-2, and GRO, among many others (For
718 clarity, cytokine annotations used here are consistent with those on the product sheet).
719 Furthermore, this co-culture with PACC conditioned media increased the motility of
720 nonPACC cells. A validation of the most differentially secreted cytokine, IL6, showed
721 that it was sufficient to induce motility induction in a PACC-to-nonPACC paracrine, but
722 not a PACC-to-PACC autocrine, setting. We are not the first to find an IL6 rich PACC-
723 secretome (48), and of note, the motility-inducing nature of IL6 has been previously
724 published in other contexts (49).
725   The PACC state represents an emerging area of cancer research committed to
726 understanding the adaptive phenotypic potential of cancer cells. It has been well
727 established that treatment of cancer with chemotherapeutic agents inevitably leads to
728 the eventual rise of treatment-resistant disease. Though outside the scope of this
729 manuscript, it has been repeatedly shown that cells in the PACC state are broadly
730 resistant to a wide swath of anti-cancer agents (8, 10, 13, 43, 48, 50, 51). In addition to
731 chemotherapy-induced resistance, evidence has begun to suggest that cancers treated
732 with chemotherapeutics are prone to become more metastatic. For example, emerging
733 evidence show that chemotherapy induces cancer-cell intrinsic changes such as
734 upregulation of anti-apoptotic genes and increased migration (52). When tested in mice
735 using spontaneously metastatic orthotopic breast models, it was found that paclitaxel
736 increased metastasis despite decreases in tumor burden (52). Karagiannis et. al,
737 demonstrated that this observation holds true in the patient setting: clinically validated
738 prognostic markers of metastasis in breast cancer patients were increased in patients
739 who received neoadjuvant paclitaxel after doxorubicin plus cyclophosphamide (53).
740 Considering that nearly all anti-cancer agents tested have shown to induce cells into the
741 PACC state, our data demonstrating the increased metastatic competency of PACCs
742 positions the PACC state as an important unforeseen ramification of neoadjuvant
743 regimens that may help to explain clinical correlations between chemotherapy and
744 metastatic progression.

IV. Materials and Methodology:

Cell Culture:

Experiments were performed using either the PC3-Luc prostate cancer cell line or the PC3-GFP-Luc prostate cancer cell line generated as previously described (28). All cells were cultured with RPMI 1640 media containing L-glutamine and phenol red additives (Gibco) and further supplemented with 10% Premium Grade Fetal Bovine Serum (Avantor Seradigm) and 1% 5000U/mL Penicillin-Streptomycin antibiotic (Gibco) at 37 degrees Celsius and in 5% CO_2. Cells were routinely lifted using TrypIE (Gibco) following a single PBS wash (Gibco). Cells were STR-profile authenticated and tested for *mycoplasma* contamination biannually (Genetica).

PACC induction:

Cells were induced to enter the PACC state as previously described (11, 21). Briefly, 625,000 PC3 cells/T75 flask (scaled for appropriately for larger tissue culture vessels) were treated with 6 µM Cisplatin (Millipore Sigma) resuspended in sterile PBS supplemented with 140mM NaCl (Sigma-Aldrich) for 72 hours. Where indicated, PC3-GFP-Luc cells were treated with 12 µM Cisplatin for 72 hours. After 72 hours, Cisplatin-treated media was removed and replaced with fresh complete media. Cells were injected (or assayed, where appropriate) 10 days after Cisplatin-treatment removal, unless indicated otherwise. When indicted, cells were filtered through a 10-micron cell strainer (PluriSelect) to either isolate or remove PACCs, which are larger in size, from the other cells in the population, as previously described (21).

Animal Models:

All murine protocols were approved by the Johns Hopkins Animal Care and Use Committee. Most experiments were performed using 8–12-week-old male Nod-Scid-Gamma (NSG) mice (Jackson Laboratories). Intracardiac injections were performed on 6-week-old mice. Subcutaneously injected mice received a 100 uL injection of a 1:1 ratio of 10 mg/mL Matrigel (Corning) to 200,000 PC3-GFP-Luc cells suspended in complete media (or a mock suspension of media alone). Caudal artery-injected mice received a 100 uL injection of 200,000 cells suspended in sterile PBS. Tail vein-injected mice received a 100 uL injection of 200,000 PC3-GFP-Luc cells suspended in sterile PBS. Intracardiac-injected mice received a 100 uL injection of 50,000 PC3-GFP-Luc cells suspended in sterile PBS into the left ventricle. Tumor progression of subcutaneously injected mice was monitored via weekly caliper measurements and tumor volume was calculated using the following formula: $V = 0.5 * L * W^2$. Progression of caudal artery-injected, tail vein-injected, and intracardiac-injected mice was monitored via BLI, wherein mice were injected with 100 µL of 30 mg/mL luciferin (Regis) and imaged within 15 minutes using the IVIS Spectrum BLI imager (Revity). At experimental endpoint, subcutaneous tumors were dissection, fixed in 10% Neutral Buffered Formalin (Sigma-Aldrich) for 24 hours, washed 3x 5 minutes in PBS, and embedded into paraffin blocks. 4-micron thick sections were mounted on slides, stained with Hematoxylin and Eosin, and imaged using a 40X objective.

CTC and DTC Analysis:

791   CTC and DTC detection and analysis was performed as previously described
792   (28). CTCs were defined as GFP-positive cells identified in mouse blood by flow
793   cytometry. 500 µL of blood was collected via terminal tail bleed. Blood samples were
794   individually transferred to 5 mL Eppendorf tubes and supplemented with ACK lysis
795   buffer (Quality Biological) at a 1:4 blood:lysis buffer ratio. This solution was incubated
796   on an end-over-end turner for 10 minutes. Following incubation, all samples were
797   centrifuged at 1500 xg for 10 minutes at 4 degrees C. Cell pellets were resuspended in
798   1 mL complete media and stained with 1 µL of Vybrant DyeCycle Violet (Thermo Fisher
799   Scientific).
800   DTCs were defined as GFP-positive cells identified in mouse hind-limb blood
801   marrow or homogenized lung tissue by flow cytometry. Bone marrow was collected
802   from the hind limb bones of freshly euthanized mice using a standard centrifugation
803   protocol. Briefly, the right and left femurs and tibias of each mouse were dissected. The
804   distal femoral epiphysial plate from each femur and the proximal tibial epiphysial plate
805   from each tibia were removed to ensure access to red bone marrow. Bones were
806   placed marrow-exposed side down in a 0.5 mL tube punctured with a small hole which
807   was then nested into a 1.5 mL tube. Tubes were centrifuged at maximum speed for 30
808   seconds to collect bone marrow into the 1.5 mL tube. All samples were resuspended in
809   200 µL PBS and then supplemented with 800 µL of ACK lysis buffer. This solution was
810   incubated on an end-over-end turner for 10 minutes. Following incubation, all samples
811   were centrifuged at 1500 xg for 10 minutes at 4 degrees C. Cell pellets were
812   resuspended in 3 mL complete media and stained with 15 µL of Vybrant DyeCycle
813   Violet.
814   To collect lung tissue, all 5 lobes of the lungs were dissected from freshly
815   euthanized mice. Each sample was transferred to a petri dish and minced into small
816   pieces with a fresh, straight-edged razor blade. Each sample was transferred to a 14
817   mL round-bottom tube and suspended in 5 mL of a solution containing 250 µL
818   Collagenase/Hyaluronidase (Stem Cell Technologies), 375 µL DNase I (Stem Cell
819   Technologies) at 1 mg/mL, and 1.875 mL complete media. The samples were incubated
820   at 37 degrees C for 20 minutes with shaking, before being pushed through a fresh 70-
821   micron strainer placed over a 50 mL conical tube using the rubber end of a fresh 5 mL
822   syringe plunger. 45 mL of complete media was used per sample to facilitate straining.
823   Following straining, samples were centrifuged at 300 xg for 10 minutes at room
824   temperature. The cell pellets were resuspended in 2 mL of ACK lysis buffer and
825   incubated at room temperature for 3 minutes before adding 47 mL of complete media.
826   Samples were then centrifuged at 300 xg for 10 minutes at room temperature, using a
827   slow deceleration setting. Cell pellets were resuspended in 2 mL of complete media and
828   stained with 10 µL of Vybrant DyeCycle Violet.
829   The entire volume of all stained samples was run on the Attune NxT Acoustic
830   Focusing Cytometer (Thermo Fisher Scientific) at a flow rate of 1000 µL per minute. As
831   previously described, it was critical to implement the following four modifications to our
832   cytometer to accommodate analysis of cells in the PACC state: 1) The largest
833   commercially available blocker bar was installed. 2) An alternative optical configuration
834   was used. 3). Thresholding was performed using SSC rather than the standard FSC. 4)
835   Area scaling factors of 0.6 were used for all lasers, rather than the standard area
836   scaling factors. With these modifications, data was collected in the SSC, VL1-A, VL2-A,

837 and BL1-A channels. All data were analyzed using FlowJo (BD), following our
838 previously published analysis protocol (28).
839
840 **Patient bone marrow samples:**
841   Bone marrow aspirate (BM) samples were collected from castrate-resistant
842 prostate cancer patients as previously described (54). Briefly, liquid biopsy samples
843 were taken from participants at baseline and immediately started treatment on trial
844 NCT01505868 which evaluated cabazitaxel with or without carboplatin. Samples were
845 collected at MD Anderson at baseline prior to clinical trial treatment administration. The
846 study was approved by the corresponding institutional review boards and was
847 conducted in accordance with ethical principles founded in the Declaration of Helsinki.
848 All patients gave written informed consent.
849
850 **Patient sample processing**:
851   Samples were processed as previously described (54). In short, bone marrow
852 samples were delivered overnight (7.5 mL) in Cell-Free DNA Blood Collection Tubing
853 (Streck) at the clinical sites and sent to the University of Southern California for
854 processing. Erythrocytes were lysed via ammonium chloride and the entire nucleated
855 cell population was plated onto a specialized cell adhesion glass slide (Marienfield) at a
856 density of 2-3 million cells per slide. Slides were stored at -80°C until use.
857
858 **Immunofluorescent staining**:
859   Slides were fixed with 2% PFA for 20 minutes. Slides were then blocked with 2%
860 BSA in PBS. Slides were then incubated overnight at 4°C with an antibody cocktail
861 consisting of mouse IgG1/Ig2a anti-human cytokeratins (CK) 1, 4, 5, 6, 8, 10, 13, 18,
862 and 19 (clones: C-11, PCK-26, CY-90, KS-1A3, M20, A53-B/A2, C2562, Sigma, St.
863 Louis, MO, USA), mouse IgG1 anti-human CK 19 (clone: RCK108, GA61561-2, Dako,
864 Carpinteria, CA, USA), mouse EpCAM (14-9326-82, Thermo), and rabbit IgG antihuman
865 vimentin (VIM): Alexa Fluor 488 (clone: D21H3, 9854BC, Cell Signaling Technology).
866 Slides were then washed with PBS and incubated at room temperature for two hours
867 with Alexa Fluor® 555 goat anti-mouse IgG1 antibody (A21127, Invitrogen, Carlsbad,
868 CA, USA), and counter-stained with 4′,6-diamidino-2-phenylindole (DAPI; D1306,
869 Thermo Fisher Scientific, Waltham, MA, USA).
870
871 **High content imaging and analysis**:
872   Slides were imaged as previously reported (55). Briefly, an automated high
873 throughput microscope equipped with a 10x optical lens was used to collect 2304
874 images across the slide. An image analysis tool, available at
875 https://github.com/aminnaghdloo/if_utils was used to identify PACCs. Briefly, each
876 fluorescent channel was segmented individually using adaptive thresholding and
877 merged into one cell mask. PACCs were identified as having a nuclear diameter two-
878 times larger than the rare cell population (four-times the area).
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882 **Single cell picking:**

883 AN Eppendorf TransferMan NK2 micromanipulator was used to collect the cell of
884 interest in a 100 μM micropipette. The cell was transferred into a sterile solution of 1x
885 PBS. Single cells were stored at -80°C.
886
887 **Copy number profiling:**
888 Copy number profiling from low pass whole genome sequencing samples was
889 conducted as previously described (56, 57). Briefly, the commercially available WGA4
890 kit (Sigma) was used for single cell whole genome amplification. Sequencing libraries
891 were prepared with the NEB Ultra FS II with 50 ng of starting material. Cells were
892 sequenced at a depth of 1-2 million reads on an Illumina HiSeq 4000 (Fulgent, Inc.).
893 Raw sequencing reads were aligned with BWA-MEM to the hg19 reference. Count data
894 was segmented via the R package DNACopy (version 1.70.0) and median values were
895 reported for copy number ratio data.
896
897 **Western Blot:**
898 PC3 parental cells or PC3 PACCs were lysed with an appropriate amount of
899 RIPA Lysis and Extraction Buffer (Thermo Scientific) with Halt Protease and
900 Phosphatase Inhibitor Cocktail (Thermo Scientific) for 30 minutes, rotating in 4 degrees
901 Celsius. Lysates were spun at 21,000 xg for 15 minutes in 4 degrees Celsius and the
902 proteinaceous supernatant was stored at -80 degrees Celsius. 50 ng of protein
903 (measured by Pierce BCA Protein Assay, following manufacturer's protocol) (Thermo
904 Scientific) was added to a 1:4 mixture of Laemmli Sample Buffer (BioRad) and 2-
905 Mercaptoethanol (BioRad) and ran through a 4-20% Mini-ProTEAN TGX gel (BioRad).
906 The gel was transferred via Trans-Blot SD Semi-Dry Transfer Cell (BioRad) onto a 0.2-
907 micron Nitrocellulose Trans-Blot Turbo Transfer Pack using the 7-minute, 2.5A, 25V
908 protocol designed for Mixed Molecular Weights. The blot was blocked in Casein
909 Blocking Buffer (Sigma-Aldrich) for 1 hour at room temperature with shaking, and then
910 transferred to primary antibody diluted in casein and incubated overnight at 4 degrees
911 Celsius with shaking. The blot was then washed 3 times for 5 minutes each with pH 7.4
912 Tris-Buffered Saline (Quality Biological) with 0.1% Tween 20 (Sigma) (TBST) and
913 incubated in secondary antibody diluted 1:20,000 in Casein for 1 hour at room
914 temperature. The blot was then washed 3 times for 5 minutes with TBST and imaged
915 using the Odyssey Western Blot Imager (Li-Cor). See Table 1 below for antibodies
916 used.
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927 **Table 1: Western Blot Antibodies**
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Target (Alias)	Antibody	Concentration	Secondary
SNAI1 (Snail)	Rabbit IgG Monoclonal: C15D3 (Cell Signaling Technologies 3879)	1:1000	IRDye 800CW Goat anti Rabbit IgG (Licor)
SNAI2 (Slug)	Rabbit IgG Monoclonal: C19G7 (Cell Signaling Technologies 9585)	1:1000	IRDye 800CW Goat anti Rabbit IgG (Licor)
TWIST1 (Twist-1)	Rabbit IgG Monoclonal: E7E2G (Cell Signaling Technologies 31174)	1:500	IRDye 800CW Goat anti Rabbit IgG (Licor)
VIM (Vimentin)	Rabbit IgG Monoclonal: D21H3 (Cell Signaling Technologies 5741)	1:5000	IRDye 800CW Goat anti Rabbit IgG (Licor)
CDH2 (N-Cadherin)	Rabbit IgG Monoclonal: D4R1H (Cell Signaling Technologies 13116)	1:500	IRDye 800CW Goat anti Rabbit IgG (Licor)
CLDN1 (Claudin-1)	Rabbit IgG Monoclonal: D5H1D (Cell Signaling Technologies 13255)	1:1000	IRDye 800CW Goat anti Rabbit IgG (Licor)
CDH1 (E-Cadherin)	Rabbit IgG Monoclonal: 24E10 (Cell Signaling Technologies 3195)	1:1000	IRDye 800CW Goat anti Rabbit IgG (Licor)
EPCAM (EpCAM)	Rabbit IgG Monoclonal: E678Y (Cell Signaling Technologies 93790)	1:1000	IRDye 800CW Goat anti Rabbit IgG (Licor)
ACTB (Beta-Actin)	Mouse IgG2B Monoclonal: 8H10D10 (Cell Signaling Technologies 3700)	1:5000	IRDye 680RD Goat anti Mouse IgG (Licor)

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941 **RT-qPCR:**
942 PC3 parental cells or PC3 PACCs were lysed using the QIAshredder Kit
943 (Qiagen), following the manufacturer's protocol. Where indicated, PC3 parental cells or
944 PC3 PACCs were incubated in parental-conditioned media or PACC-conditioned media
945 for 24 hours prior to lysis. RNA was extracted from lysates using an RNeasy Mini Kit

946   (Qiagen) following the manufacturer's protocol. RNA was converted to cDNA (1 ug RNA
947   per reaction) using the iScript cDNA Synthesis Kit (Bio-Rad) following the
948   manufacturer's protocol. RT-qPCR reactions were performed using SsoFast EvaGreen
949   Supermix (Bio-Rad), following the manufacturer's protocols, and assorted primers
950   (Integrated DNA Technologies) at a concentration of 10 uM. See Table 2 below for
951   primers used. Data was collected using the CFX96 Real-Time PCR Detection System
952   (Bio-Rad) with a standard cycle protocol. Gene expression was normalized to the
953   housekeeping gene Beta-Actin and calculated using the delta-delta Ct method. Each
954   biological replicate reported is an average of three technical replicates.
955
956   **Table 2: RT-qPCR Primers**
957

Target (Alias)	Forward Primer	Reverse Primer
ZEB-1 (Zeb-1)	5-AGCAGTGAAAGAGAAGGGAATGC-3	5-GGTCCTCTTCAGGTGCCTCAG-3
SNAI1 (Snail)	5-ACTGCAACAAGGAATACCTCAG-3	5-GCACTGGTACTTCTTGACATCTG-3
SNAI2 (Slug)	5-CTCTGTTGCAGTGAGGGCAAG-3	5-AATATGTGAGCCTGGGCGC-3
TWIST1 (Twist-1)	5-GTCCGCAGTCTTACGAGGAG-3	5-GCTTGAGGGTCTGAATCTTGCT-3
VIM (Vimentin)	5-TGCCGTTGAAGCTGCTAACTA-3	5-CCAGAGGGAGTGAATCCAGATTA-3
CDH2 (N-Cadherin)	5-TGTGGGAATCCGACGAATG-3	5-GTCATATGGTGGAGCTGTGGG-3
CLDN1 (Claudin-1)	5-TCTGGCTATTTTAGTTGCCACAG-3	5-AGAGAGCCTGACCAAATTCGT-3
CDH1 (E-Cadherin)	5-ATTTTTCCCTCGACACCCGAT-3	5-TCCCAGGCGTAGACCAAGA-3
EPCAM (EpCAM)	5-CGCAGCTCAGGAAGAATGTG-3	5-TGAAGTACACTGGCATTGACGA-3
ACTB (Beta-Actin)	5-ACGTGGACATCCGCAAAGAC-3	5-CAAGAAAGGGTGTAACGCAACTA-3

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963   **Conditioned-Media Generation:**

964 To generate PACC-conditioned media, PC3 PACCs were generated using our
965 standard induction-approach as described above, in a T150 tissue-culture flask. On day
966 10 of treatment-removal, exactly 20 mL of fresh complete media were added. Exactly 24
967 hours later, on day 11 of treatment-removal, all 20 mL of PACC-conditioned media was
968 collected. Media was centrifuged at 1000 xg for 5 minutes to remove any debris, and
969 then filtered through a 0.45-micron PES filter into 1 mL aliquots (to prevent freeze-
970 thawing) that were stored at -80 degrees Celsius.
971 To generate parental-conditioned media, 1,250,000 PC3 parental cells were
972 seeded in a T150 tissue-culture flask. After 12 hours, exactly 20 mL of fresh complete
973 media were added. Exactly 24 hours later, all 20 mL of parental-conditioned media was
974 collected. Media was centrifuged at 1000 xg for 5 minutes to remove any debris, and
975 then filtered through a 0.45-micron PES filter into 1 mL aliquots (to prevent freeze-
976 thawing) that were stored at -80 degrees Celsius.
977
978
979 **Motility Assays:**
980 Transwell assays were performed using 6.5-millimeter, 8-micron, PES
981 membranes. PC3 parental cells were seeded on the membrane at 100,000 cells/mL in
982 250 µL of complete media. After 12 hours, 500 µL of testing-condition media was placed
983 in the top and bottom wells for 24 hours. Where indicated, recombinant IL6 was added
984 to complete media at a concentration of 0.5 ng/mL. After 24 hours, membranes were
985 washed in PBS and nonmigrated cells were removed from the top of the membrane with
986 a cotton-tipped applicator. Membranes were fixed in 100% ice cold methanol for 10
987 minutes, and then stained in 0.5% crystal violet resuspended in 20% methanol for 10
988 minutes. Membranes were washed with deionized water to remove excess stain and
989 tiled imaging of the entire membrane was acquired with an EVOS FL Digital Inverted
990 Fluorescence Microscope (Thermo Fisher Scientific) using a 4X objective. Image
991 analysis to calculate percent area coverage was performed using the Phansalkar auto-
992 local threshold method in ImageJ. Each reported biological replicate is an average of
993 two technical replicates.
994 Wound healing assays were performed in 24-well tissue culture plates. PC3
995 parental cells were seeded at 100,000 cells/mL in 2.5 mL of complete media. After 12
996 hours, a P1000 pipette tip was used to create a scratch wound. After 3 washes with
997 PBS, the scratch wounds were imaged on an EVOS FL Digital Inverted Fluorescence
998 Microscope using a 4X phase objective. 1mL of testing-conditioned media was added
999 for 24 hours, after which the wounds were re-imaged. Image analysis to calculate
1000 percent wound closure was performed using the Wound Healing Size Tool Updated
1001 plug-in in ImageJ. Each reported biological replicate is an average of two technical
1002 replicates.
1003 Single-cell tracking assays were performed using live-cell, time-lapse microscopy
1004 in 24-well tissue culture plates. PC3 parental cells were seeded at 25,000 cells/mL in 2
1005 mL of complete media. PC3 PACCs were seeded 5,000 cells/mL in 2 mL of complete
1006 media. After 12 hours, testing-condition media was added for 24 hours. Where
1007 indicated, Tocilizumab (Selleck Chemicals) was added to complete media at a
1008 concentration of 2.5 µg/mL. Where indicated, Recombinant IL6 was added to complete
1009 media at a concentration of 0.5 ng/mL. An EVOS FL Digital Inverted Fluorescence

1010 Microscope was used to take 10X phase images every 30 minutes for the 24-hour
1011 testing-condition incubation. An on-stage environment chamber was used to maintain
1012 cell conditions at 37 degrees Celsius, 5% CO_2, and 20% O_2. Images were analyzed
1013 using the Manual Tracking and Chemotaxis Tool plug-ins in ImageJ image analysis
1014 software. All cells analyzed were randomly selected. Cells that underwent division,
1015 apoptosis, or moved out of frame were excluded from analysis.
1016
1017 **Cytokine Array:**
1018 Cytokine analysis was performed using a 274-target chemiluminescent human
1019 cytokine antibody array, following manufacturer's protocol. (Abcam ab198496).
1020 Nondiluted PACC-conditioned media and Parental-conditioned media were tested,
1021 using nonconditioned media as a background control. Membranes were imaged using
1022 the ChemiDoc XRS+ imaging system (Biorad) and densitometry data was obtained
1023 using Image Lab Software (Biorad) with signal-specific automatic background
1024 thresholding enabled. Background subtraction, positive control normalization differential
1025 secretion calculations were performed following manufacturer's protocol.
1026
1027 **Elisa:**
1028 Quantification of IL6 present in PACC-Conditioned media was performed using
1029 an IL6 Elisa, following the manufacturer's protocol (BioLegend 430504), with the
1030 following alteration: sample incubation time was lengthened from 2 hours to 3 hours.
1031 PACC-Conditioned media was diluted 1:4 in protocol buffer Assay A prior to analysis.
1032 Data was collected using FLUOStar Omega Plate Reader (BMG LabTech) at 405 nm.
1033
1034 **Statistics:**
1035 Nonparametric T-Tests (Mann-Whitney) were performed to generate reported P
1036 values. Power calculations were performed to determine appropriate sample size for *in*
1037 *vivo* experiments, wherein an alpha value of 0.05 and a beta value of 0.8 were used.
1038
1039 NS = nonsignificant = $P > 0.05$.
1040 * = $P < 0.05$
1041 ** = $P < 0.01$
1042 *** = $P < 0.001$
1043 **** = $P < 0.0001$
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