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## **Netrin-1 blockade inhibits tumor associated Myeloid-derived suppressor cells, cancer stemness and alleviates resistance to chemotherapy and immune checkpoint inhibitor**

Benjamin Ducarouge, Anna-Rita Redavid, Camille Victoor, Ruxanda Chira, Aurélien Fonseca, Maëva Hervieu, Roméo Bergé, Justine Lengrand, Pauline Vieugué, David Neves, et al.

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1           **Netrin-1 blockade inhibits tumor associated Myeloid-**  
2           **derived suppressor cells, cancer stemness and alleviates**  
3           **resistance to chemotherapy and immune checkpoint**  
4           **inhibitor**

5 Benjamin Ducarouge<sup>1,2\*</sup>, Anna-Rita Redavid<sup>1\*</sup>, Camille Victoor<sup>1,2</sup>, Ruxanda Chira<sup>1,2</sup>  
6 Aurélien Fonseca<sup>3</sup>, Maëva Hervieu<sup>1</sup>, Roméo Bergé<sup>1,2</sup>, Justine Lengrand<sup>1,2</sup>, Pauline  
7 Vieugué<sup>1</sup>, David Neves<sup>2</sup>, Isabelle Goddard<sup>1</sup>, Mathieu Richaud<sup>1</sup>, Pierre-Alexandre  
8 Laval<sup>1</sup>, Nicolas Rama<sup>1</sup>, David Goldschneider<sup>2</sup>, Andrea Paradisi<sup>1</sup>, Nicolas Gourdin<sup>4</sup>,  
9 Sylvie Chabaud<sup>5</sup>, Isabelle Treilleux<sup>5</sup>, Nicolas Gadot<sup>6</sup>, Isabelle Ray-Coquard<sup>6</sup>, Stéphane  
10 Depil<sup>2</sup>, Didier Decaudin<sup>7</sup>, Fariba Némati<sup>7</sup>, Elisabetta Marangoni<sup>7</sup>, Eliane Mery-  
11 Lamarche<sup>8</sup>, Catherine Génestie<sup>9</sup>, Séverine Tabone-Eglinger<sup>6</sup>, Mojgan Devouassoux-  
12 Shisheboran<sup>10</sup>, Kathryn J Moore<sup>11</sup>, Benjamin Gibert<sup>1§£</sup>, Patrick Mehlen<sup>1,2§£</sup> and Agnes  
13 Bernet<sup>1,2£</sup>

14  
15 <sup>1</sup>Apoptosis, Cancer and Development Laboratory- Equipe labellisée 'La Ligue', Labex  
16 DEVweCAN, Institut Convergence PLAsCAN, Centre de Cancérologie de Lyon,  
17 INSERM U1052-CNRS UMR5286, Université de Lyon, Centre Léon Bérard, 69008  
18 Lyon, France. <sup>2</sup>Netris Pharma, Centre Léon Bérard, 69008 Lyon, France. <sup>3</sup>Research  
19 Pathology Department, Centre Léon Bérard, Lyon, France. <sup>4</sup>Targeting of the tumor and  
20 its immune environment, Centre de Cancérologie de Lyon, INSERM U1052-CNRS  
21 UMR5286, Université de Lyon, Centre Léon Bérard, 69008 Lyon, France. <sup>5</sup>UBET,  
22 Centre Léon Bérard, Lyon, France. <sup>6</sup>Pathology Department, Centre Léon Bérard, Lyon,  
23 France. <sup>7</sup>Laboratory of Preclinical Investigations, Translational Research Department,  
24 Institut Curie, Université Paris-Sciences-et-Lettres, 75005 Paris, France. <sup>8</sup>Department  
25 of Pathology, IUCT Oncopole, Toulouse, France. <sup>9</sup>Department of Pathology, Gustave  
26 Roussy, Villejuif, France. <sup>10</sup>Hospices Civils de Lyon, Department of Pathology, 69002  
27 Lyon, France <sup>11</sup>Department of Medicine, Leon H. Charney Division of Cardiology, New  
28 York University School of Medicine, New York, New York, USA.

29 <sup>§</sup>Corresponding authors: P. Mehlen; e-mail: [patrick.mehlen@lyon.unicancer.fr](mailto:patrick.mehlen@lyon.unicancer.fr) , A.  
30 Bernet; e-mail: [agnes.bernet@lyon.unicancer.fr](mailto:agnes.bernet@lyon.unicancer.fr) and B. Gibert; e-mail:  
31 [benjamin.gibert@lyon.unicancer.fr](mailto:benjamin.gibert@lyon.unicancer.fr)

32 <sup>£</sup>Co-senior authors

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34 **Abstract:**

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Drug resistance and cancer relapse represent significant therapeutic challenges after chemotherapy or immunotherapy, and a major limiting factor for long-term cancer survival. Netrin-1 was initially identified as a neuronal navigation cue but has more recently emerged as an interesting target for cancer therapy, which is currently clinically investigated. We show here that netrin-1 is an independent prognostic marker for clinical progression of breast and ovary cancers. Cancer stem cells (CSCs)/Tumor initiating cells (TICs) are hypothesized to be involved in clinical progression, tumor relapse and resistance. We found a significant correlation between netrin-1 expression and cancer stem cell (CSC) markers levels. We also show in different mice models of resistance to chemotherapies that netrin-1 interference using a therapeutic netrin-1 blocking antibody alleviate resistance to chemotherapy and triggers an efficient delay in tumor relapse and that this effect is associated with CSCs loss. We also demonstrate that netrin-1 interference limits tumor resistance to immune checkpoint inhibitor and provide evidence linking this enhanced anti-tumor efficacy to the blockage of recruitment of a subtype of myeloid-derived suppressor cells (MDSCs) called PMN-MDSCs. We have functionally demonstrated that these immune cells promote CSCs and, consequently, resistance to anti-cancer treatments. Together, these data support the view of both a direct and indirect contribution of netrin-1 to cancer stemness and we propose that this may lead to therapeutic opportunities by combining conventional chemotherapies and immunotherapies with netrin-1 interfering drugs.

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61 **Introduction:**

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63 Netrin-1 is a multifunctional, secreted, laminin-related glycoprotein that plays  
64 key roles in neuronal navigation, angiogenesis, and cell survival<sup>1-3</sup>. Netrin-1 is also  
65 implicated in numerous pathologies including type 2 diabetes, cardiovascular disease,  
66 and cancer<sup>4-9</sup>. Its activity has been shown to principally occur through the regulation of  
67 the signaling pathways transduced by its main receptors, Deleted in Colorectal  
68 Carcinoma (DCC) and UNC5-Homolog (UNC5H- *i.e.*, UNC5A, UNC5B, UNC5C,  
69 UNC5D)<sup>10,11</sup>. Notably, netrin-1 has been shown to be up-regulated in many tumor  
70 types, and this up-regulation has been proposed to act as a selective mechanism that  
71 blocks apoptosis induced by the dependence receptors DCC and UNC5B<sup>4,12</sup>.

72 Efforts to develop drugs that inhibit the interaction of netrin-1 with its receptors  
73 have therefore been initiated. Several pre-clinical proof-of-concept studies have  
74 highlighted that candidate drugs interfering with netrin-1-receptor interactions  
75 markedly inhibit tumor growth and metastasis<sup>13-16</sup>. NP137, a netrin-1 targeted  
76 monoclonal antibody strongly accumulate within tumors and was recently evaluated in  
77 a phase I clinical trial among patients with advanced solid cancers<sup>17,18</sup>. Interim results  
78 included both excellent safety profiles and encouraging signs of clinical activity,  
79 particularly for gynecological indications<sup>19</sup>. Benefits were seen in patients despite  
80 highly advanced disease resistant to standard of care, including chemotherapies and  
81 immune checkpoint inhibitors<sup>20,21</sup>.

82 A common view is that resistance to these anti-proliferative drugs is intrinsically  
83 linked to the presence of Cancer Stem Cells (CSCs) or Tumor-Initiating Cells (TICs)  
84 within the tumors. The notion is that CSCs and TICs rely on the existence of a distinct  
85 subset of tumor cells that possesses the capacity to sustain tumor growth<sup>22</sup>.  
86 CSCs/TICs are thought to express specific markers at the cell surface and are  
87 identified functionally based on their ability to propagate tumors when serially  
88 transplanted into recipient mice<sup>23</sup>. Whether CSCs are a distinct subset of cancer cells,  
89 or whether they represent a functional state of some cancer cells, remains a matter of  
90 debate. To date, the general consensus is that CSCs are key to clinical progression  
91 due to their ability to self-renew and resist chemotherapies or more recently to immune  
92 checkpoint inhibitors<sup>24-28</sup>, thereby facilitating tumor relapse<sup>29</sup>.

93 In the present study, we thus investigated netrin-1 implication in breast and  
94 ovary and its putative link with cancer stemness and resistance to



95 chemotherapy/immunotherapy. We show that netrin-1 expressed by cancer stem cells  
96 promotes resistance to anticancer drugs. We also associate netrin-1 expression with  
97 clinical progression and demonstrate that in various animal models, netrin-1  
98 interference selectively impacts CSCs/TICs, thus potentiating sensitivity to  
99 chemotherapy and anti-CTLA-4 monoclonal Antibody (mAb) immunotherapy. Finally,  
100 we describe the integration of netrin-1 within the tumoral microenvironment and show  
101 that the anti-netrin-1 inhibits tumor recruitment of a subtype of myeloid-derived  
102 suppressor cells (MDSCs) called PMN-MDSCs. We provide functional evidence that  
103 these immune cells promote the CSC phenotype, and consequent resistance to anti-  
104 cancer treatments.

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106

107 **Results:**

108

109 **Netrin-1 expression is associated with clinical progression and cancer stemness**  
110 **in ovary and breast tumors.**

111 Previous studies described high-level netrin-1 expression in a sizeable fraction  
112 of human breast and ovarian cancers<sup>13,17,30</sup>. Moreover, patients with metastasis at  
113 diagnosis harbored primary tumors with greater netrin-1 expression than patients who  
114 were metastasis-free<sup>13</sup>. To evaluate the impact of netrin-1 on prognosis, we analyzed  
115 tumor expression of netrin-1 by quantitative-RT-PCR (q-RT-PCR) across a panel of 89  
116 breast cancer patients treated with conventional chemotherapies, correlating netrin-1  
117 expression data with clinical data. Clinical characteristics of the patients are presented  
118 in **Supplementary Table 1**. Patients with tumors with elevated netrin-1 expression had  
119 a significantly worse prognosis than patients with tumors with low netrin-1 expression.  
120 The median survival of breast cancer patient was 9.8 years in the high netrin-1 group.  
121 By contrast, the median survival was not yet reached at 15 years in the low netrin-1  
122 group (**Fig. 1A**). Importantly, netrin-1 expression emerged as an independent  
123 prognostic factor in multivariate analysis, together with the presence of metastasis at  
124 diagnosis (**Supp. Table 2**). To better evaluate the association between netrin-1  
125 expression and the risk of tumor relapse or progression, we evaluated the prognostic  
126 value of netrin-1 tumor expression in the 67 patients with a localized disease, using  
127 treatment-free survival as an endpoint in this retrospective analysis. As shown in  
128 **Figure 1B**, median treatment-free survival was 9.9 years for patients with high netrin-  
129 1 tumor and was not reached for low netrin-1 group.

130

131 CSCs/TICs are hypothesized to play a major role in clinical progression and  
132 tumor recurrence<sup>22,31,32</sup>. As shown in **Figure 1C**, netrin-1 expression was significantly  
133 elevated in CSC marker CD44 high tumors vs CD44 low tumors (n=89). Similar data  
134 were obtained with other CSC markers (**Supp. Fig. 1A**). In parallel, using fresh tumoral  
135 tissues from patients, we generated mammospheres to enrich stem-like  
136 subpopulations. We observed that netrin-1 expression is lower in the overall bulk tumor  
137 as compared to the paired mammospheres (**Fig. 1D**), indicating an enrichment of  
138 netrin-1 in the subpopulation of cancer cells capable of regenerating spheres.

139 To analyze whether the results obtained in breast cancer could be extended to  
140 other pathologies, we have investigated by immunohistochemistry (IHC) analysis the  
141 expression of netrin-1 in ovarian carcinomas (**Supp. Fig.1B**). IHCs were performed on  
142 biological samples taken at diagnosis in a cohort of 51 patients with high-grade serous  
143 ovarian carcinomas without neoadjuvant therapy. Clinical characteristics of the  
144 patients are presented in **Supplementary Table 3**. As shown in **Figure 1E**, patients  
145 had a significantly poorer prognosis with increasing netrin-1 expression. Median  
146 survivals were respectively 9.9, 5.7 and 3.3 years in the Negative, Low and High netrin-  
147 1 groups respectively. As shown in **Figure 1F**, median treatment-free survivals were  
148 respectively 5.6, 3.1 and 1.8 years for patients with Negative, Low and High expression  
149 of netrin-1 within the tumor. Similarly, netrin-1 was elevated in patients whose tumors  
150 were resistant to platinum therapy (**Fig. 1G**) and increasing netrin-1 was concordant  
151 with the CA-125, a circulating tumor marker known to correlate with tumor relapse  
152 (**Supplementary Fig. 1C**). To determine the association of netrin-1 expression with  
153 the CD44 stem marker, we performed q-RT-PCR analysis on a cohort of 22 patients  
154 with high-grade serous ovarian carcinoma. As shown in **Figure 1H**, netrin-1 expression  
155 was significantly higher in High CD44 tumors than in Low CD44 tumors. Taken  
156 together, these expression data suggest that the level of netrin-1 is associated with  
157 relapse and progression of breast and ovarian tumors and that netrin-1 is specifically  
158 upregulated in CSCs/TICs compared to the bulk tumor.

159 The expression of netrin-1 in patient's tumors and its association with  
160 CSCs/TICs led us to investigate the link between netrin-1 expression and stem  
161 properties in a series of breast and ovarian cancer cell lines. With the idea that  
162 chemoresistance is associated with stemness, we first identified Hs578t and SKBR7  
163 as partially chemo-sensitive to Adriamycin (*i.e.*, ~20% of cells are still alive after 48h  
164 of Adriamycin (**Supp. Fig. 2A**)) relative to other netrin-1 positive human cell lines  
165 screened. Interestingly, following Adriamycin treatment, the surviving Hs578t and  
166 SKBR7 cells were enriched for stem cell markers such as CD44 or CD49f, as well as  
167 netrin-1 (**Fig. 2A; Supp. Fig. 2B-C**). We next investigated the ability of cells to form  
168 spheres in breast and ovarian cell lines as a functional test to identify the existence of  
169 a stem cell population. BT474, Hs578t, and OV90 form spheres (**Supp. Fig. 2D**) and  
170 we observed a concordant elevation of stem cell markers and netrin-1, when compared  
171 to the parental 2D cultures (**Fig. 2B; Supp. Fig. 2E**). To avoid the potential bias of

172 analyzing CSCs enrichment after chemotherapy or sphere formation, we also  
173 performed an unbiased examination of netrin-1 expression in cells after sorting for  
174 stem cell markers positivity. Hs578t, SKBR7 breast cancer cells and OV90 ovary  
175 cancer cells have been sorted for respectively CD49f, ALDH or CD44 markers  
176 positivity associated with a clear increase of netrin-1 (**Fig. 2C**; **Supp. Fig. 2F-G**). This  
177 result highlights the association between netrin-1 and functional CSC markers in  
178 different tumor types.

179 In order to evaluate the involvement of netrin-1 in CSCs/TICs-mediated  
180 treatment resistance, we first took advantage of a patient-derived xenograft (PDX)  
181 immunocompromised mouse model in which a human triple negative breast tumor  
182 sample *-i.e.*, HBC146- is grafted. This model was shown to be sensitive to a single  
183 injection of chemotherapeutic agents, such as adriamycin and cyclophosphamide<sup>33</sup>.  
184 Human residual disease pathology includes a remission phase during which the tumor  
185 is virtually undetectable, followed by a relapse phase in ~80% of the mice (**Fig. 2D**).  
186 Netrin-1 expression was relatively low in the tumor bulk during the tumor growth phase  
187 but was markedly elevated during the regression phase, returning to the basal level at  
188 the time of relapse (**Fig. 2E**). Of interest, netrin-1 level appears fully correlated with the  
189 presence of CD44 marker during all these stages, indicating a strong association of  
190 netrin-1 with the stem character of the tumor cells (**Fig. 2E**). In order to link these higher  
191 expression levels of netrin-1 and CD44 with an enrichment of CSCs during the tumor  
192 regression phase more specifically, we dissected out untreated tumors or  
193 chemotherapy-treated tumors prior to complete regression and performed CD44  
194 analysis by flow cytometry or mammospheres forming assays. CD44<sup>high</sup> cells were  
195 enriched during the regression phase (**Fig. 2F**). Consistent with reports of CSC  
196 resistance to chemotherapy, mammospheres formation occurred more frequently  
197 when the tumors cells used were derived immediately following chemotherapy (**Fig.**  
198 **2G**). We then proceeded to the enrichment of tumor-initiating cells from the untreated  
199 tumor, either by CD44<sup>high</sup> sorting or by mammospheres' cultures. Netrin-1 expression  
200 was enriched in CD44<sup>high</sup> cells (Supp. **Fig. 2H**). Paralleling our results in human breast  
201 cancer samples, netrin-1 was less expressed in bulk tumors than in mammospheres  
202 generated from these same tumors (**Fig. 2H**). Taken together, these data support the  
203 view that in this breast cancer PDX model, netrin-1 is only weakly expressed in bulk

204 tumors but is more strongly expressed in a population of tumors cells resistant to the  
205 chemotherapeutic treatment that display elevated CSC markers.

206

### 207 **Netrin-1 blockade induces cell death and delays relapse.**

208 We and others groups have shown that netrin-1 up-regulation is a mechanism  
209 of escape from netrin-1 dependence receptor-induced apoptosis<sup>13-16,34</sup>. In order to  
210 investigate the potential survival role of netrin-1 in CSC, we first knocked down netrin-  
211 1 expression by siRNA in different cell lines. Silencing of netrin-1 is associated with  
212 cell death induction in Hs578t as measured by a SubG1 assay (**Fig. 2I**). Moreover, this  
213 effect is increased when Adriamycin is combined as measured by cell viability (**Fig.**  
214 **2J**). Along the same line, netrin-1 silencing impairs sphere formation ability of both  
215 Hs578t and OV90 cells (**Fig. 2K**).

216 In order to achieve a pre-clinical and clinical development of targeting netrin-1,  
217 a specific blocking monoclonal antibody (mAb) has been developed<sup>19,35</sup>. When we  
218 examined the effect of this antibody (mAb) in the previously described HBC146-PDX  
219 model, we found that systemic delivery of the anti-netrin-1 mAb did not reveal any  
220 significant tumor growth-inhibiting effect (**Supp. Fig. 3A**). This was consistent with the  
221 low level of netrin-1 expressed in this tumor. However, evaluating this together with  
222 combined chemotherapy, we observed a marked decrease of tumor regrowth at the  
223 expected time of relapse observed in the chemotherapy-treated mice (**Fig. 3A**). While  
224 the relapse-free median time in chemotherapy-treated animals was 56 days, this was  
225 significantly delayed (*i.e.*, beyond the 75 days indicating the end of the experiment) in  
226 the anti-netrin1 mAb-treated group (**Fig. 3B**). Consistent with a selective effect of  
227 netrin-1 blockade on CSCs/TICs, the anti-netrin1 mAb treatment was associated with  
228 an increase of cell death associated with a decrease in the number of cells able to form  
229 spheres (**Fig. 3C**). This result was confirmed by immunohistochemical staining of  
230 CD44, where anti-netrin-1 mAb-treated tumors showed a decrease of this stemness  
231 marker in residual cancer cells (**Fig. 3D**).

232 To more definitively test the ability of netrin-1 blockade to impact mammary TICs  
233 in this model, we next performed serial transplant assays. HBC146 breast tumors were  
234 initially grafted in mice and treated twice weekly with either anti-netrin-1 mAb or a  
235 control IgG1. After 32 days, tumors were dissociated and regrafted into new recipient  
236 mice to evaluate tumor take in the absence of further treatment. As shown in **Figure**

237 **3E**, anti-netrin-1 treatment is associated with a significant reduced tumor take in  
238 HBC146 serial transplantation assay. Importantly, similar results were obtained in a  
239 second breast cancer PDX model (BRE-012) where anti-netrin-1 treatment similarly  
240 failed to inhibit tumor growth (**Supp. Fig. 3B**) but was associated with a dramatic  
241 suppression of tumor take in serial transplantation assay (**Fig. 3F**).

242 To determine whether the results could be extended to other type of tumors, we  
243 next performed a combination treatment using anti-netrin-1 with the standard of care  
244 Carboplatin/Paclitaxel chemotherapy in the ovarian OV21 PDX model. Again, anti-  
245 netrin-1 mAb had no activity as a single agent (**Supp. Fig. 3C**) but combining anti-  
246 netrin-1 mAb with chemotherapeutic treatment triggered a better significant efficacy  
247 than chemotherapy alone (**Fig.3G**). Similar effects were obtained with ovarian OV90  
248 cells xenografts (**Supp. Fig. 3D**). This observation was associated with inhibition of  
249 tumor take in a serial transplantation with anti-netrin-1 alone (**Supp. Fig. 3E**). This  
250 was confirmed in mice engrafted with in high-grade serous ovarian carcinoma cell lines  
251 OV90 and OVCAR3, where no inhibition of initial tumor growth was seen with anti-  
252 netrin-1 mAb (**Supp. Fig.3F-G**) but where strong inhibition of tumor take in a serial  
253 transplantation assay was observed (**Fig. 3H-I**). Because in cancer settings the netrin-  
254 1 receptor involved appears to be mainly UNC5B, we have here investigated whether  
255 the impact on CSCs was dependent on UNC5B. We used CRISPR interference to  
256 invalidate UNC5B in HCC1937 breast cell line and grafted in mice and observed that  
257 the absence of UNC5B completely altered the ability to initiate tumors (**Supp. Fig. 3H**).  
258 Together, these data strongly support the idea that netrin-1/UNC5B blockade has a  
259 specific impact on CSCs/TICs and prevents recurrence/resistance of breast and ovary  
260 tumors after chemotherapy.

261

### 262 **Netrin-1 blockade alleviates resistance to immune checkpoint inhibitor therapy.**

263 Based on the impact on CSCs/TICs described here and the existing literature  
264 on cancer stemness, plasticity and resistance to immunotherapy<sup>24,36</sup>, we next  
265 investigated whether netrin-1 blockade might also attenuate tumor resistance to  
266 immune checkpoint inhibitor (ICPI). For this we used the mouse mammary tumor cell  
267 line EMT6, which expresses netrin-1(**Supp. Fig. 4A**) and has some susceptibility to  
268 anti-CTLA-4<sup>37,38</sup>. This model demonstrates a ~35% of tumor growth inhibition when  
269 treated with anti-CTLA-4 (**Supp. Fig. 4B**), though spaghetti curves indicate that it acts

270 as an on/off model where mice may respond or not. About 60% of mice are non-  
271 responders (**Supp. Fig. 4C**). This heterogeneity is highlighted by individual animal-  
272 specific cytokine secretion profiles, which reveals "cold" and "hot" animals in terms of  
273 immune response (**Supp. Table 4**).

274 Consistent with anti-CTLA4 activity, the level of regulatory T (T-reg) cells<sup>39</sup>, was  
275 downregulated in all mice, but less than 40% of mice demonstrated modulation of  
276 tumor growth (**Supp. Fig.4D**). Anti-netrin-1 mAb was unable to significantly inhibit  
277 tumor growth at all, when used as a single agent (**Supp. Fig. 4E**). However, the  
278 combination of anti-netrin-1 with anti-CTLA-4 dramatically blocked tumor growth, and  
279 strongly enhanced the long-term survival of mice bearing EMT6 tumors (**Fig. 4A-B**).  
280 This increased response to the anti-CTLA-4 mAb induced by netrin-1 blockade was  
281 associated with increased secretion of pro-inflammatory cytokines such as IFN $\gamma$   
282 (**Supp. Fig. 4F**), suggesting a robust immune response<sup>24,40</sup>.

283 In parallel studies, we analyzed the tumor immune infiltrate using both lymphoid  
284 and myeloid panels by flow cytometry. We noticed an increase of CD8<sup>+</sup> T lymphocytes  
285 within tumors of mice treated with the combination of both antibodies, supporting the  
286 notion that immune cell populations promote the enhanced anti-tumor effect observed  
287 (**Fig. 4C-D**). In addition to T cell response, we analyzed the regulation of innate  
288 immunity. A decrease in tumor associated macrophages was observed within tumors  
289 of mice treated with the combination anti-netrin-1/anti-CTLA-4, supporting the view that  
290 these immune cell populations promote the enhanced anti-tumor effect observed (**Fig.**  
291 **4E**).

292

### 293 **PMN-MDSCs participate to netrin-1 mediated cancer stem cell enrichment and** 294 **resistance to immune checkpoint inhibitors.**

295 To explore the underlying mechanisms linking immune infiltration and its  
296 possible link with the anti-netrin-1 response, we next analyzed immune subpopulations  
297 known to be involved in resistance to immune checkpoint inhibitors. We identified, in  
298 the EMT6 engrafted model, a specific decrease in PMN-MDSCs<sup>41</sup>, a subtype of  
299 myeloid-derived suppressor cells (MDSCs) derived from the  
300 neutrophil/polymorphonuclear compartment (CD11b<sup>+</sup> Ly6G<sup>+</sup> Ly6C<sup>Interm</sup>) within tumors  
301 treated with anti-netrin-1 (**Fig.5A**). The same population was found to be down-  
302 regulated upon anti-netrin-1 mAb treatment in a spontaneous genetic breast cancer

303 model MMTV/NeuT. These mice spontaneously develop mammary tumors closed to  
304 human pathology<sup>42</sup> (**Fig.5B**).

305 MDSCs have been reported to be able to control and protect cancer stem cell  
306 populations<sup>43</sup>. PMN-MDSCs are immature cells capable of inhibiting the functions of T  
307 cells. They can accumulate during tumor growth and contribute to cancer  
308 development<sup>41</sup>. We thus hypothesized that these PMN-MDSCs could link the effect of  
309 netrin-1 interference on the tumor microenvironment and the resistance to treatments  
310 mediated by CSCs/TICs. To analyze *in vivo* the MDSC function, we differentiate  
311 immature myeloid cells into PMN-MDSCs. These immature myeloid cells were purified  
312 from bone marrow of wild type mice and differentiated into PMN-MDSCs by treatment  
313 with IL-6 and GM-CSF, that we reinjected into EMT6 tumor, treated with anti-netrin-1  
314 and anti-CTLA-4. As a result, injection of PMN-MDSCs inhibit the effect of the combo-  
315 therapy highlighting the immuno-suppressive role of these cells (**Fig. 5C**). To analyze  
316 if PMN-MDSCs can directly impact on CSCs, we evaluated their impact on CD49<sup>f<sup>High</sup></sup>  
317 populations in a coculture model with EMT6 cells in which netrin-1 had been silenced.  
318 As shown in **Figure 5D**, we observed that PMN-MDSCs sustained the CD49<sup>f<sup>+</sup></sup> stem  
319 EMT6 cell population, an effect that was lost when netrin-1 was silenced. *In vitro*, PMN-  
320 MDSCs inhibit CD3<sup>+</sup> T-cell proliferation and IFN $\gamma$  production concomitant with the  
321 activation of cytotoxic CD8<sup>+</sup> lymphocytes, which clearly demonstrates their  
322 immunosuppressive roles in this context (**Supp. Fig.5A-B**).

323 To determine whether the potentiation of the anti-CTLA-4 response by netrin-1  
324 blockade was related to the impact of netrin-1 interference on CSCs/TICs, we analyzed  
325 stemness markers in EMT6 tumors treated either with anti-netrin-1 mAb alone or in  
326 combination with anti-CTLA-4. By selecting CD45<sup>-</sup> non-immune cells that were either  
327 CD49<sup>f<sup>+</sup></sup> or CD326<sup>-</sup> (murine stemness markers<sup>26</sup>), we found that systemic anti-CTLA-4  
328 treatment was associated with an enhancement of the stem cell populations (**Fig. 5E**;  
329 **Supp. Fig. 5C**). This suggests that the anti-CTLA-4 antibody principally affects bulk  
330 tumor cells, thus leaving residual tumors enriched for CSCs/TICs responsible for tumor  
331 recurrence. Moreover, we quantified at the same time point PMN-MDSCs cells by  
332 immunohistochemistry analysis (flow cytometry analysis was not possible due to a high  
333 rate of necrosis linked to CTLA-4 cytotoxic activity). We show that Ly6G<sup>+</sup> cells are  
334 decreased both in the monotherapy with anti-netrin-1 mAb and in the combo with anti-  
335 CTLA-4, arguing in a protective role of these cell for CSCs (**Fig. 5F**).



336 We also assessed cancer stem cell function by performing a serial  
337 transplantation assay comparing anti-netrin-1, anti-CTLA-4, or the combination. As  
338 shown in **Figure 5G**, while treatment with anti-netrin-1 or its combination with anti-  
339 CTLA-4 completely abolished tumor take in serially transplanted mice, treatment with  
340 anti-CTLA-4 only modestly inhibited tumor take. These data extend a literature which  
341 notes that ICPIs only partially impact cancer stem cell populations<sup>24</sup>. Of major interest,  
342 the combination of anti-CTLA-4 and anti-netrin-1 completely inhibits the enrichment in  
343 these stem cell populations associated with anti-CTLA-4 antibody, making it a  
344 promising strategy.

345 To further link our observations on cell subpopulations expressing stem cell  
346 markers and CSC/TIC function, we sorted cells using CD49f and CD326 markers from  
347 untreated EMT6 transplanted tumors and then transplanted them serially. As might be  
348 predicted, we found that CD49f<sup>+</sup> or CD326<sup>-</sup> EMT6 cells were more prone to form tumors  
349 than their CD49f<sup>-</sup> and CD326<sup>+</sup> counterparts (**Supp. Fig. 5D-E**). Moreover, CD49f<sup>+</sup> cells  
350 sorted from tumors treated with anti-netrin-1 mAb are losing their enhanced ability to  
351 form new tumors in serial transplantation assays (**Fig. 5H**). To unravel the mechanism  
352 behind, this effect we observed that while the *In vitro* treatment of EMT6 cells with anti-  
353 netrin-1 mAb leads to the decrease of CD49f<sup>+</sup> cells (**Supp. Fig. 5F**), this decreased is  
354 reverted when the general caspase inhibitor BOC was added (**Supp. Fig. 5F**),  
355 supporting further the view that netrin-1 blockade impacts on CSCs by affecting cell  
356 death, In order to demonstrate the role of the receptor in these experiments, we  
357 invalidate UNC5B by shRNA in EMT6 and identified that the knock-down of UNC5B  
358 altered the ability to initiate tumors in mice (**Supp. Fig. 5G**). Collectively, our data  
359 support the view that the combination of ICPI and anti-netrin-1 is sensitizing grafted  
360 tumors to ICPI by the impact of the anti-netrin-1 mAb on cancer stem cell populations  
361 supported by the tumoral microenvironment.

362

363

## 364 Discussion

365

366 The present data identifies, for the first-time, netrin-1 as an independent  
367 progression factor in breast and high grade serous ovarian cancers, associated with a  
368 higher expression in CSCs/TICs. We show that netrin-1 interference may not only drive  
369 tumor growth inhibition but may also be important in the prevention of tumor relapse  
370 as well as in resistance to chemotherapy or immunotherapy. In support of this notion,  
371 we identified that targeting netrin-1 in association with standard of care  
372 chemotherapies induced a significant extension of the tumor-free survival (associated  
373 with a delay in tumor relapse) in animal models of both breast and high grade ovarian  
374 serous carcinoma. Similarly, we have shown that resistance to the ICPI anti-CTLA-4  
375 could be alleviated by the association with the anti-netrin-1 mAb.

376 A challenging question remains; to understand the molecular mechanisms  
377 inhibited or unleashed upon netrin-1 blockade that confer relapse delay and/or alleviate  
378 resistance. We propose here that netrin-1 may play a specific role in CSCs/TICs since  
379 (i) netrin-1 is often specifically more expressed in CSCs/TICs compared to bulk tumor  
380 cells and (ii) netrin-1 blockade appears to reduce CSCs/TICs *-i.e.*, monitored using  
381 both functional assays or markers. Although netrin-1 was initially considered as a  
382 guidance cue, several recent published works seem to describe a role of netrin-1 in  
383 induced pluripotent stem cells, embryonic stem cells and naïve pluripotency<sup>44-46</sup>. The  
384 link between normal and embryonic stemness has not been extended to cancer  
385 “stemness,” a prior *in vitro* study reported a possible role of netrin-1 in glioblastoma  
386 CSCs<sup>8</sup>. Based on an initial work from our lab, netrin-1 has been described as a survival  
387 factor in iPSC and ES, and acts in part by blocking the death induced by the  
388 dependence receptor UNC5B<sup>44</sup>. More recent work suggests that netrin-1 also links the  
389 Wnt and MAPK pathways<sup>45</sup>. Whether the biology behind “normal” stemness and  
390 CSCs/TICs relies on similar mechanisms should now be examined. Similarly, whether  
391 the reduction of CSCs/TICs associated with netrin-1 blockade seen in this manuscript  
392 is related to increased cell death or “lineage” commitment remains to be investigated.  
393 Nonetheless, our work, for the first time, links netrin-1 to breast and ovarian cancer  
394 progression and suggests it to be a *bona fide* clinical target. Based on these studies,  
395 a phase 2 clinical trial (<https://clinicaltrials.gov/ct2/show/NCT04652076>) was started in

396 late 2020 combining anti-netrin-1 and standard of care chemotherapy as well as  
397 immunotherapy.

398 The data obtained with the combination of ICPI with the anti-netrin1 mAb are of  
399 specific importance taken into account the currently limited clinical activity of ICPI in  
400 breast cancer<sup>37,38</sup>. The data presented here support the view that the suppression of  
401 ICPI resistance by netrin-1 blockade is associated with its impact on CSCs/TICs.  
402 However, we cannot yet exclude others additional mechanisms. Moreover, previous  
403 works have described the role of netrin-1 in the migration or the survival of immune  
404 cells such as macrophage<sup>4-7</sup> or CD4<sup>+</sup> cells<sup>47</sup> during inflammatory arthritis or obesity.  
405 MDSCs have already been reported to modulate the response to ICPI drugs<sup>41,48</sup>.

406 PMN-MDSCs have already been shown to be capable of sustaining tumor  
407 progression<sup>41</sup>. In our investigations, we identify that netrin-1 blockade silences these  
408 cells. We propose a novel pro-tumoral effect of immune cells, sustain CSCs/TICs and  
409 thus may indirectly affect resistance to treatment. This proposed mechanism is  
410 consistent with a mechanism in which the potentiation of ICPI by netrin-1 blockade  
411 does not necessarily occur through direct reinforcement of immune effector cells but  
412 rather through an indirect effect on CSCs/TICs. We thus propose that netrin-1  
413 produced by cancer cells sustains PMN-MDSC which in turn sustains CSCs/TICs that  
414 contribute to both recurrence and to resistance to chemotherapies and ICPIs.

415 However it is fair to say it probably includes additional players of the tumor  
416 microenvironment more generally impacting on tumoral plasticity such as CAFs<sup>49</sup>,  
417 additional immune cells, endothelial cells, and extracellular matrix but also more  
418 generally the cellular stress reported in tumoral and stromal cells<sup>25</sup>. Whether and how  
419 netrin-1 might interact with these additional players remains an open question.

420

421 **Materials and Methods:**

422

423 **Ethics, Human tumors samples and biological annotations:**

424 The usage of all patient tumor samples was supported according to French regulations  
425 on the protection of biomedical investigation subjects. The collection of human  
426 samples was realized with primary female breast tumors from patients treated at the  
427 Centre Léon Bérard, conserved in the Centre des Ressources Biologiques (CRB) of  
428 the Centre Léon Bérard. Informed consent was obtained from all patients, and the  
429 study use samples which were approved by the ethics committee of the institutions.  
430 Fresh samples were addressed for mammosphere formation experiments, frozen  
431 tissues for q-RT-PCR analysis and fixed tissues to immunohistochemistry.

432

433 **Cell culture**

434 All the cell lines were obtained from ATCC. Murine mammary carcinoma EMT-6 cells  
435 were cultured in Eagle's Minimum Essential Medium (EMEM, ATCC) complemented  
436 with 10% of Serum Foetal Bovine (FBS, Gibco) and antibiotics (Streptomycin and  
437 Penicillin). They were cultured in Dulbecco's Modified Eagle's Medium (DMEM) for  
438 SKBR7 and Hs578t or RPMI for BT474, T47D, MDA-MB468, OV90 and OVCAR3,  
439 complemented with 10% FBS (Gibco) and antibiotics. HCC1937 cell line was cultured  
440 in RPMI complemented with 10% FBS (Gibco), 2% Na Bicarbonate, 1% Na Pyruvate,  
441 1% HEPES and antibiotics. Cells were maintained in culture at 37°C under humidified  
442 atmosphere consisting of 20% O<sub>2</sub> and 5% CO<sub>2</sub>. For the CRISPRi mediated silencing  
443 of hUNC5B in HCC1937 cell line, a small guide RNA (sgRNA) targeting hUNC5B  
444 promoter was designed using the sgRNA designer tool  
445 (<https://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design-crisprai>). A  
446 control sgRNA targeting Firefly luciferase was also designed. Sequences are as  
447 follows: sgUNC5B: 5'- GCGCGCCTCTCGGAGCCCCG -3' and sgLUC: 5'-  
448 CGCCCACGGCCCTTCCCGGG-3'. Forward and reverse sgRNA-coding oligos with  
449 Esp3I linkers were ordered from Eurofins Genomics and then annealed by incubation

450 for 3 min at 90°C and 15 min at 37°C and ligated in pLV hU6-sgRNA hUbC-dCas9-  
451 KRAB-T2a-Puro<sup>50</sup> Insertion of sgRNA was confirmed by sequencing. The use of pLV  
452 hU6-sgRNA hUbC-dCas9-KRAB-T2a-Puro allows for the simultaneous expression of  
453 dCas9-KRAB and sgRNA and for selection for puromycin resistance. pLV hU6-sgRNA  
454 hUbC-dCas9-KRAB-T2a-Puro was a gift from Charles Gersbach (Addgene plasmid #  
455 71236; <http://n2t.net/addgene:71236> ; RRID:Addgene\_71236). For the shRNA  
456 mediated KO of mUNC5B in EMT6 cell line, a pLKO.1-puro vector encoding shRNA  
457 targeting mUNC5B (Clone ID: TRCN0000072079, shRNA sequence: 5'-  
458 CGCCTACATCGTAAAGAACAA-3') was purchased from Sigma-Aldrich. Production of  
459 VSV-g pseudotyped lentivirus was made as described previously.

460

#### 461 ***In vivo* mice models and materials:**

462 Human anti-netrin-1 monoclonal antibodies (anti-netrin1) were supplied by Netris  
463 Pharma (Lyon, France). Female Swiss nude mice, 6-weeks old, were purchased from  
464 Charles River laboratories (Les Oncins, France) and maintained in specific pathogen-  
465 free conditions (Anican, Lyon, France) and stored in sterilized filter-topped cages. Their  
466 care and housing were in accordance with institutional European guidelines as put  
467 forth by the CECCAP local Ethical Committee (C2EA-15, CLB\_2014\_001;  
468 CLB\_2014\_012; CECCAPP\_CLB\_2016\_017). The human human triple negative  
469 breast cancer patient derived xenograft PDX HBC146 was established and  
470 characterized for tumor recurrence by Marangoni *et al*<sup>63</sup>. The human triple negative  
471 breast cancer patient derived xenograft BRE-012 was established by the IMODI  
472 consortium(<https://imodi-cancer.org/fr/>). PDX-OV21 human high grade serous ovarian  
473 carcinoma model has been established by Curie Institute and previously published<sup>51</sup>.  
474 For PDX experiments, mice were grafted as described by Marangoni *et al*, 2007<sup>24</sup>.  
475 Tumor growth was evaluated by measurement of two perpendicular diameters of  
476 tumors with a caliper twice per week. Individual tumor volumes were calculated as  $V =$   
477  $(\text{length} \times \text{width}^2) / 2$ , a being the largest diameter, b the smallest. When tumors reached  
478 a volume of 300mm<sup>3</sup>, the mice were randomly separated in two groups and submitted

479 anti-netrin-1 or Control alone, or in combination to standard or care chemotherapy:  
480 Adriamycin, 2 mg/kg (Doxorubicin, Teva Pharmaceuticals), and cyclophosphamide,  
481 100 mg/kg (Endoxan, Baxter) were inoculated with a single i.p. injection or Carboplatin  
482 66 mg/Kg (generic provided by Hospira) and Paclitaxel 30 mg/Kg (generic provided by  
483 Hospira), i. p. injection once a week Anti-netrin-1 mAb or IgG1 control were i.p. injected  
484 with 10mg/kg twice per week for the rest of the experiment. Animal work has been  
485 done by Netris pharma, Institut Curie or the Laboratoire des Modèles Tumoraux (LMT).  
486  $5 \times 10^6$  OV90 and OVCAR3 cells were subcutaneously inoculated with Matrigel  
487 (Corning) in the dorsal flank of 8-week-old female Swiss/Nude mice. The mice were  
488 housed under specific pathogen free (SPOF) conditions (Anican, Lyon, France). Tumor  
489 volumes were evaluated approximately every 3 days after initial detection using  
490 calliper<sup>52</sup>

491 For experiments of immune checkpoints inhibitors, eight-week-old (20-22 g body  
492 weight) female BalbC/J mice were obtained from Janvier Labs.  $1 \times 10^6$  EMT-6 cells  
493 were subcutaneously inoculated in the dorsal flank of 8-week-old female BALB/cJ wild-  
494 type mice. The mice were housed under specific pathogen free (SPF) conditions as  
495 previously described (Anican, Lyon, France)<sup>53</sup>. Tumor volumes were evaluated  
496 approximately every 2 days after initial detection using calliper<sup>52</sup>. Based on initial intra-  
497 group homogeneity of tumor size, four different treatments have been administrated:  
498 IgG1 control isotype (Netris Pharma), anti-netrin-1 mAb (NP137, Netris Pharma)  
499 combination of anti-CTLA-4 mAb (BioXCell, Clone 9H10) with IgG1 and combination  
500 of anti-CTLA-4 with anti-netrin-1 mAb. The treatments were ip. administered respecting  
501 the following dose: 20 mg/kg anti-CTLA-4, 10 mg/kg anti-netrin-1 and 10 mg/kg mice  
502 IgG1 on days 8, 11 and 14 after the implant. Mice were sacrificed when individual  
503 tumours reached a predefined endpoint (1000-2000 mm<sup>3</sup>).

504 For the injection of MDSCs cells, 500 000 EMT6 breast cancer cells were mixed with  
505 50 000 PMN-MDSCs (ratio 10:1) produced *in vitro* and were inoculated in the dorsal  
506 flank of 8-week-old female BALB/cJ wild-type mice. Subsequently mice were  
507 intratumorally inoculated with PMN-MDSC at day 8 and 13.

508

509 **Serial transplantation, tumor-initiating cell sorting and mammosphere culture:**

510 Tumors were dissociated with a collagenase (Sigma Aldrich) / DNase (Life  
511 Technologies) for 45 minutes at 37°C, submitted to red blood cell lysis (Miltenyi  
512 Biotech) and passed through a 30 µm filter to obtain a single cell suspension. Cell  
513 viability has been evaluated by trypan blue count under the microscope to ensure the  
514 engraftment of the same number of cells with a similar viability in the different  
515 conditions of treatment, residual cells were after analyzed and sorted by FACS or  
516 submitted to mammosphere formation. FACS sorting was performed on a BD  
517 FACSDiVa. Anti-CD44-PE (BD system) was used to count and sort CD44<sup>high</sup> cells and  
518 dead cells were counted with propidium iodine staining. ALDEFLUOR (Stemcell  
519 Technologies) was used to count and sort ALDH<sup>high</sup> cells and dead cells were counted  
520 with propidium iodine staining. Mammosphere enrichments were obtained by culturing  
521 the cells on 6 wells ultra-low attachment dishes in mammosphere medium (Stem cell  
522 technologies). Cells were allowed to form clonal mammospheres for 7 to 10 days of  
523 culture. For mammosphere forming assays, viable singlets were sorted by FACS and  
524 directly plated on 96 wells ultra-low attachment dishes at the density of 1000 cells per  
525 well in mammosphere medium. Mammospheres were counted after 7 days of culture. To  
526 analyze spheres passages, 7 days spheres were dissociated and 1000 cells per well  
527 were again plated in the conditions mentioned above and counted after 7 days of  
528 culture.

529

530 **Immunohistochemistry:**

531 Immunohistochemistry analysis was performed on 4-µm-thick sections of paraffin  
532 embedded mouse tissues stained with anti-CD44 (156-3C11, Thermo Fisher) or anti-  
533 netrin-1 (CPA2389, Cohesion Biosciences) or anti-Ly-6G (E6Z1T- Rabbit mAb #87048  
534 Cell signaling) dilution 1/500. Expressions are revealed by brown DAB staining. Nuclei  
535 are counterstained by hematoxylin (blue).

536

537 **Flow cytometry analysis:**

538 EMT6 subcutaneous tumors were mechanically disaggregated in RPMI-1640 medium  
539 5% FBS and enzymatically digested with 1 mg/ml of collagenase I and 40 U/ml of  
540 DNase I (Sigma Aldrich) for 30 min at 37 °C. Cells were passed through a 70 µm-cell  
541 strainer and filtered. After RBC lysis, cells were passed through a 30 µm-cell strainer  
542 and filtered. Cell pellets were resuspended in HBSS (Gibco) before counting.  $2 \times 10^6$   
543 cells were stained for the viability with Zombie Aqua (Biolegend) for 30 minutes at 4°C.  
544 Cells were washed and incubated with Fc Block purified anti-mouse CD16/CD32  
545 (Clone 93, Biolegend), followed by staining with mouse anti-CD4 (RM4-5, Brilliant  
546 Violet 785), anti-CD19 (6D5, Brilliant Violet 605), anti-NKp46 (29A1.4, PE), anti-CD45  
547 (30-F11, Alexa Fluor 700), anti-CD25 (PC61, APC), anti-CD69 (H1.2F3, FITC), anti-  
548 IA-IE (M5/114.15.2, BV421), anti-Ly6C (HK1.4, APC-Cy7) anti-Ly6G (1A8, FITC),  
549 CD11b (M1/70, BV785) and F4/80 (BM8, APC) from Biolegend and anti-CD3 (clone  
550 500A2, PerCP-eFluor 710, anti-CD8b (eBioH35-17.2, eFluor 450), anti-CD44 (IM7,  
551 PerCP-Cy5.5), anti-CD326 (G8.8, PE-Dazzle 594) from eBioscience, anti-CD49f  
552 (GoH3, eFluor 450) from Life Technologies, for 45 min at 4 °C. Cells were fixed and  
553 permeabilized with the Foxp3 transcription factor staining buffer set (eBiosciences),  
554 and overnight stained for 30 min at 4 °C for IFN  $\gamma$  (XMG1.2, PE, Biolegend) and with  
555 anti-Foxp3 (FJK-16s, PE-Cyanine7, eBioscience). Analyses were performed with  
556 Fortessa HTS flow cytometer (BD Biosciences). Data were analyzed with FlowLogic  
557 Version 6 (Miltenyi Biotec, Germany). All gates were defined using fluorescence minus  
558 one control (FMO).

559

560 **Cell isolation**

561 CD3<sup>+</sup> T-cells were isolated from the Lymph nodes of 7-week-old BALB/cJ wild-type  
562 mice. The separation was performed by using Pan T Cell Isolation Kit (Miltenyi Biotec)  
563 and MACS column. Purity of cell population were evaluated by flow cytometry. CD3<sup>+</sup>  
564 T-cells were cultivated *in-vitro* for 3-days with Anti-CD3 and Anti-CD28 (Dynabeads,  
565 Gibco) and IL-2 (10 ng/ml). Immature myeloid cells were purified from bone marrow of



566 7-week-old BALB/cJ wild-type mice. Red blood cells (RBC) were lysate with ACK  
567 (Ammonium-Chloride-Potassium) Lysing Buffer, cells were filtered through a 70 µm-  
568 cell strainer. Immature myeloid cells were cultivated *in-vitro* for 4-days with GM-CSF  
569 and IL-6 (40ng/ml) to differentiate them. T-cells and PMN-MDSCs were cultivated in  
570 RPMI complemented with 10% FBS (Gibco), 1% Na Bicarbonate, 1% Na Pyruvate,  
571 1% L-glutamine (Gibco), 1% HEPES, 0.05 mM 2-mercaptoethanol (Gibco) and  
572 antibiotics.

573

#### 574 **Proliferation viability and cell death assays**

575 CD3<sup>+</sup> T-cells were labeled with cell trace violet (CTV, Invitrogen) as follows: 10<sup>6</sup> cells  
576 were incubated with 1 µM of CTV in PBS for 20 min at 37°C, 5% CO<sub>2</sub>. 5-volume of  
577 culture medium were added, and cells were left at 37°C 5% CO<sub>2</sub> in culture medium for  
578 additional 5 min. Cells were analyzed by flow cytometry for the proliferation baseline  
579 or co-cultured with PMN-MDSCs. WST1 assay was used according to the  
580 manufacturer (Cell proliferation Reagent, Roche) to measure cell viability. Cells are  
581 fixed in 70% Ethanol and stained with Propidium iodide (PI). SubG1 population is  
582 further identified by flow cytometry.

583

#### 584 **IFN-γ Production**

585 Activated CD3<sup>+</sup> T-cells were stimulated with phorbol myristate acetate (PMA),  
586 Ionomycin, Brefeldin (Biolegend) and Monensin (Biolegend) for 4 hours at 37°C, 5%  
587 CO<sub>2</sub> in complete culture medium. IFN γ released by CD8<sup>+</sup> T- cells was evaluated by  
588 flow cytometry.

589

#### 590 **Co-culture**

591 To test the immunosuppressive activity of PMN-MDSCs were co-cultured with purified  
592 CD3<sup>+</sup> T-cells for 3 days at different ratio (MDSCs: CD3<sup>+</sup> T-cells, 1:1, 1:4) in 96-well  
593 plate. To analyze the effect PMN-MDSCs on stemness were co-cultured with EMT6-  
594 control vector EMT6-Sh netrin-1 in a ratio 1:10 (MDSCs: Tumoral cells) for 24 hours in

595 24 well plate. CD49<sup>high</sup> cells were analyzed by flow cytometry.

596

597 **Cytokine analysis:**

598 To measure the systemic release of IFN $\gamma$ , fresh stabilized whole blood was drawn from  
599 BALB/cJ wild-type mice 16h hours after 1<sup>st</sup>, 2<sup>nd</sup> and 3<sup>rd</sup> treatments. Plasma was  
600 separated and used to measure the protein levels of IFN  $\gamma$  with the Mouse IFN-gamma  
601 Quantikine ELISA Kit (R&D System, Minnesota, USA) according to the manufacturer's  
602 instructions.

603 For cytokines array analysis, 31 cytokines and chemokines were analyzed 18 hours  
604 after the 2<sup>nd</sup> CTLA-4 treatment, using the multiplex assays manufactured by Meso  
605 Scale Discovery (MSD; Gaithersburg, MD). EMT6 subcutaneous tumors were  
606 mechanically disaggregated in 500 $\mu$ l of RPMI-1640 medium (in absence of FBS) for  
607 500 mg of tumor. The cytokines and chemokines analyzed accordingly to the  
608 manufacturer recommandations and were IL-1 $\beta$ , IL-2, IL-4, IL-5, IL-6, IL-9, IL-10, IL-  
609 12p70, IL-15, IL-16, IL-17A, IL-17A/F, IL-17C, IL-17E/IL-25, IL-21, IL-22, IL-23,  
610 IL27p28/IL30, IL-31, IL-33, IFN  $\gamma$ , MIP-1 $\alpha$ , MIP-2, MIP-3 $\alpha$ , IP-10, MCP-1, KC/GRO,  
611 TNF- $\alpha$ , TGF-  $\beta$ 2, TGF- $\beta$ 3, TGF- $\beta$ 1. Analyses were performed using the software  
612 Discovery workbench 4.0.

613

614 **Immunoblots:**

615 Confluent cells were washed with cold PBS and scrapped in lysis buffer (Tris 10 mM  
616 pH 7.6; SDS 5; Glycerol 10%; Triton X-100 1%, DTT 100mM). After sonication proteins  
617 were dosed using Pierce 660nm Protein Assay Reagent (Thermo Fisher Scientific) and  
618 after loading on 4%-15% SDS-polyacrylamide gels (Bio-Rad) transferred to  
619 nitrocellulose membranes using Trans-Blot Turbo Transfer (Bio-Rad). Membranes  
620 were blocked for 1 hour at room temperature with 5% of nonfat dried milk for the Netrin-  
621 1 and with 5% of BSA and 1% of nonfat dried milk for UNC5B. Staining was performed  
622 overnight with primary antibody: anti-netrin1 antibody (Ab126729, Abcam) and anti-  
623 UNC5B (D9M7Z, Cell signaling technology). After washing, membranes were

624 incubated with secondary antibody, goat anti-rabbit coupled with HRP for 1 hour at  
625 room temperature. West Dura Chemiluminescence System (Pierce) was used to  
626 intensify the signal. Imaging was performed using Chemidoch Touch (Bio-Rad). Full  
627 membrane westernblots were included in the supplementary material.

628

#### 629 **RNA expression analysis:**

630 Total mRNAs were extracted from tissues or cells using Nucleospin RNAII kit  
631 (Macherey-Nagel) and 1 µg was reverse transcribed using the iScript cDNA Synthesis  
632 kit (Bio-Rad). Real-time quantitative RT-PCR was performed on a LightCycler 2.0  
633 apparatus (Roche, Meylan, France) using the Light Cycler Master Taqman kit (Roche,  
634 Meylan, France). Oligonucleotide sequences are available upon request.

635

#### 636 **Statistical analysis:**

637 To test netrin-1 expression, cohorts were dichotomized based on Netrin-1 median  
638 expression. Survival curves were produced by the Kaplan-Meier method on Prism  
639 (GraphPad Software, San Diego, California). Data were analyzed using a Mantel-Cox  
640 test. n defines the total number of replicates. All statistical tests were two-sided. The  
641 multivariate analysis was realized with COX model in the R software. Kaplan-Meier  
642 curves of disease-free survival of overall survival were analyzed with a Gehan-  
643 Breslow-Wilcoxon Test in the GraphPad Prism software.

644 For mice experiments, statistical methods were not used to predetermine necessary  
645 sample size, but sample size was chosen based on pilot experiments which applying  
646 appropriate statistical test could return significant results. Tumor growth with time was  
647 analyzed by two-way ANOVA with Bonferroni correction. Survival curves were  
648 analyzed using the Log-rank (Mantel-Cox) test. Flow cytometry data are reported as  
649 mean±s.e.m. in case of representative experiment; the pool of different experiments is  
650 expressed as a *ratio* of the control group of the single experiment. Statistical analysis  
651 was performed by Mann-Whitney test (GraphPad).

652

653

654

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662  
663 **Conflict of interest:**

664 BD, CV, JL, DN, DG, SD, AB and PM declare to have a conflict of interest as  
665 respectively employees (BD, DN, DG, CV, JL and SD) and shareholders (AB and PM)  
666 of NETRIS Pharma.

667  
668 **Author contributions:**

669 BD, ARR, CV, RC, RB, AP and JL performed the cell experiments and *in vitro* data;  
670 BD, ARR, MH, PV, DN, MR, IG, FN and PAL performed mice experiments;  
671 CV, RB, AP and DG produced the lentiviruses and CRISPR and Sh cell lines;  
672 BD, AF, IT and NG made anatomopathological analysis;  
673 NR performed bioinformatical analysis;  
674 NG, IT, IRC, EML, CG, STE and MDS provided human samples and cell lines;  
675 BD, ARR and SC performed statistical analysis;  
676 BD, SD, DD, EM, KJM, BG, PM and AB have participated to the research design and  
677 to write the paper.

678  
679 **Data Availability statement:**

680 Data sharing not applicable to this article as no datasets were generated or analysed during the  
681 current study.

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## 837 **Figure legends**

838

839 **Figure 1: Netrin-1 expression is a poor prognosis marker and correlates with**  
840 **stem cells marker in human breast and ovary cancers.**

841 **A-B.** Kaplan-Meier curves representing the percentage of overall survival of breast  
842 cancer patients (**A**, n=89) or treatment-free survival among patients with localized  
843 disease (**B**, n=67). The cohort has been split in netrin-1 High (green) and Low (red) by  
844 the median expression in their tumors. **C.** Netrin-1 mRNA expression level within  
845 tumors segmented in High and Low by the median expression of CD44 mRNA (n=89).  
846 Bars are mean values +/- s.e.m. **D.** Netrin-1 mRNA expression level measured in fresh  
847 human tumors obtained at the diagnosis by q-RT-PCR. Each tumor was split to  
848 analyze the bulk or their corresponding derived mammospheres (Spheres, n=12) after  
849 dissociation. Bars are mean values +/- s.e.m. normalized with the tumor bulk group.  
850 **E-F.** Kaplan-Meier curves representing the percentage of overall survival (**E**) or  
851 treatment free survival (**F**) of high-grade ovarian carcinoma patients (n=51) with netrin-  
852 1 High (green), Low (red) Negative (black) expression in their tumors (Netrin-1 was  
853 measured by immunohistochemistry (IHC)). Median netrin-1 expression was used as  
854 a cutoff between groups. **G.** Boxplot presenting Allred score combining the percentage  
855 of positive cells and signal intensity of netrin-1 measured by IHC in patient's responder  
856 (n=17) vs resistant to platin treatment (n=8). **H.** Netrin-1 mRNA expression level in  
857 tumors segmented on the CD44 mRNA expression from a cohort of High grade serous  
858 ovarian carcinomas (n=31). High and Low have been determined respectively by the  
859 upper and lower values separated by the median of mRNA expression measured by  
860 q-RT-PCR. Bars are mean values +/- s.e.m. In all panels statistics are presented as \*  
861  $p < 0.05$ ; \*\*  $p < 0.01$ .

862

863 **Figure 2: Netrin-1 expression is associated with CSCs in human cell lines and in**  
864 **a PDX model of breast cancer resistance to chemotherapy.**

865 **A.** Netrin-1 mRNA expression level was measured by q-RT-PCR in HS578t (n=3) or  
866 SKBR7 (n=3) cell lines treated 48h with 1  $\mu$ M Adriamycin (Adria). Bars are mean values  
867 +/- s.e.m., normalized with the control group. **B.** Netrin-1 mRNA expression level was

868 measured by q-RT-PCR in BT474 (n=4 vs 5) or Hs578t (n=5) cell lines comparing  
869 standard 2D culture and mammosphere (Sphere) 3D. Bars are mean values +/- s.e.m.,  
870 normalized with the control 2D group. **C.** Netrin-1 mRNA expression level was  
871 measured by q-RT-PCR in CD49<sup>low</sup> and CD49<sup>high</sup> Hs578t (n=4) sorted breast cell  
872 population and in CD44<sup>low</sup> and CD44<sup>high</sup> OV90 ovary cell line (n=6 vs 3). Bars are mean  
873 values +/- s.e.m., normalized with the control Low group. **D.** Tumor volume was  
874 measured along the different phases of the HBC146 PDX breast cancer model<sup>33</sup>  
875 treated with chemotherapy Adriamycin-Cyclophosphamide (AC): Growth, Regression,  
876 Remission and Relapse (n=20). Samples were collected at the different phases (red  
877 circles). **E.** Netrin-1 and CD44 mRNA expression levels were measured by q-RT-PCR  
878 in the different phases (untreated n=8, regression n=8 and relapse n=10). Bars are  
879 mean values +/- s.e.m., normalized with the untreated group. **F.** Percentage of CD44<sup>high</sup>  
880 cells counted by FACS analysis in untreated tumors compared to regressing tumors  
881 (n=9 vs 3). **G.** Number of mammospheres formed by 1000 viable cells isolated from  
882 untreated HBC146 tumors or at the regression phase (n=5 vs 9). Bars are mean values  
883 +/- s.e.m. **H.** Netrin-1 mRNA expression level measured by q-RT-PCR in untreated  
884 bulk tumors and mammospheres (n=7). Bars are mean values +/- SEM, normalized to  
885 the bulk tumor group. **I.** SubG1 cell present in Hs578t cell line treated with siRNA  
886 control (siCtr) or siRNA against Netrin-1 (siNtn-1) (n=3). **J.** Cell viability measured by  
887 WST1 assay in SKBR7 (n=3) cell lines treated with siCtr and siNetrin-1 alone or in  
888 combination with Adriamycin. Bars are mean values +/- SEM, normalized to the siCtr  
889 group (\* are statistics against siCtr and + against siCtr + Adriamycin). **K.** Number of  
890 mammospheres formed by 1000 viable cells of Hs578t (n=5) and OV90 (n=3) cell lines  
891 treated with siCtr and siNetrin-1. Bars are mean values +/- SEM. In all panels statistics  
892 are presented as \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; +++ or \*\*\*  $p < 0.001$ .

893

894 **Figure 3: Netrin-1 blockade delays cancer relapse and impact CSCs.**

895 **A.** Tumor volume was measured along the different phases of the HBC146 PDX  
896 model: Growth, Regression after chemotherapy (Adriamycin-Cyclophosphamide: AC),  
897 Remission and Relapse. At day 21, the mice were randomized into two groups which  
898 were intraperitoneally treated either by vehicle (n=20) or with 10mg/kg of anti-netrin1  
899 antibody weekly (n=20).  $p=0.0002$  by ANOVA on the tumor volume at the regression.

900 **B.** Kaplan-Meier curves representing the percent of mice free of relapse in vehicle

901 (green) and anti-netrin-1 mAb (red) treated groups. The relapse volume threshold has  
902 been determined at 40mm<sup>3</sup>. Median of relapse are respectively 56.0 and 80.5 days  
903 after chemotherapy for the vehicle and anti-netrin-1 groups,  $p=0.0078$  by Gehan-  
904 Breslow-Wilcoxon test. **C.** Number of mammospheres formed by 1000 viable cells (left)  
905 or viable PI negative cells (right) isolated from HBC146 tumors treated with  
906 chemotherapy alone or associated with anti-netrin-1 mAb. Samples were collected in  
907 regressing tumors, below the volume threshold of 40mm<sup>3</sup> (n=5 vs 9). Bars are mean  
908 values +/- s.e.m. **D.** Representative CD44 immunohistochemistry analysis of HBC146  
909 tumors treated with chemotherapy alone or associated with the anti-netrin-1 mAb.  
910 Samples were collected in regressing tumors. Scale bar (represented by a line): 50µm.  
911 **E-F.** Serial transplantation experiments of HBC146 (n=10 per group) and BRE-012 (n=  
912 10 per group) breast PDX. First, tumors were treated with the anti-netrin-1 mAb or with  
913 a control IgG1 and then regrafted in other mice. Secondary recipient were not treated  
914 after the re-engraftment, the percentage of mice free of tumor is presented in both  
915 groups. **G.** Tumor volume was measured in mice bearing the OV21 ovarian PDX model  
916 and treated with Carboplatin/Paclitaxel (CP) chemotherapy and randomly separated in  
917 two groups intraperitoneally treated either with control IgG1 (n=8) or with 10mg/kg of  
918 anti-netrin1 antibody (n=8). ( $p=0.0101$ ; ANOVA test). **H-I.** Serial transplantation  
919 experiments of ovarian cancer cell lines OV90 (n=12) or OVCAR3 (n=8). First, grafted  
920 tumors were treated with the anti-netrin-1 mAb or with a IgG1 control and after  
921 dissociation,  $1 \times 10^6$  living cells were regrafted in new recipient mice Results are  
922 presented as the percentage of mice free of tumor take with respectively. In all panels  
923 statistics are presented as \*  $p<0.05$ ; \*\*  $p<0.01$ ; \*\*\*  $p<0.001$ .

924

925 **Figure 4: Netrin-1 blockade alleviates resistance to immune checkpoint inhibitor**  
926 **anti-CTLA-4.**

927 **A.** Tumor growth quantification of syngeneic EMT6 mammary carcinoma, in mice  
928 treated with control IgG1 or anti-netrin-1 mAb, in combination with anti-CTLA-4 mAb.  
929 **B.** Kaplan-Meier survival analysis of the mice of **A.** after 80 days. **C.** Spider graph  
930 presenting flow cytometry analysis of the immune infiltrate. All components of the  
931 lymphoid panel were detected and quantified after EMT6 tumor dissociation and  
932 isolation of cell population using Lymphocytes specific antibodies. **D.** Analysis of CD8<sup>+</sup>  
933 lymphocytes by flow cytometry infiltrating tumors treated with either anti-CTLA-4 mAb  
934 combined or not with anti-netrin-1 mAb and compared to a IgG1 control (n=17 per

935 group). Results are the mean of 2 different experiments presented in **C.** and are  
936 presented as a ratio on total CD45<sup>+</sup> cells singlets for each tumor. **E.** Analysis of tumor  
937 associated macrophages defined as CD11b<sup>+</sup> LY6C<sup>-</sup> F4/80<sup>+</sup> cells (n=16 per group).  
938 Results where the mean of 2 different experiments and are presented as a ratio on  
939 total CD45<sup>+</sup> cells singlets for each tumor. In all panels statistic are presented as \*  
940  $p < 0.05$ ; \*\*  $p < 0.01$ .

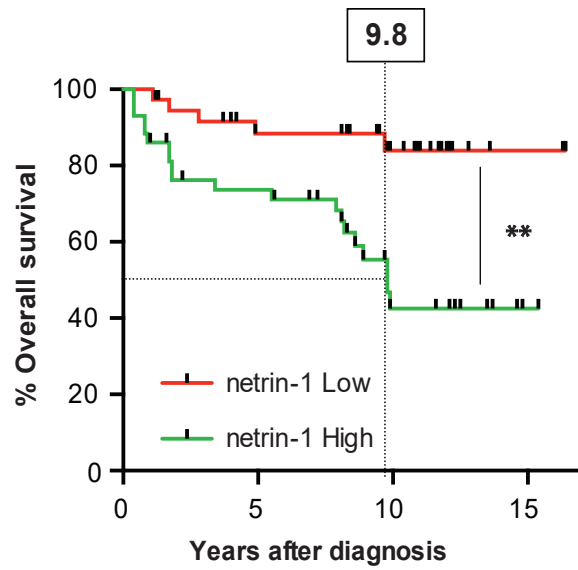
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942 **Figure 5: Netrin-1 blockade efficiently impacts CSCs/TICs untargeted by anti-**  
943 **CTLA-4 mAb.**

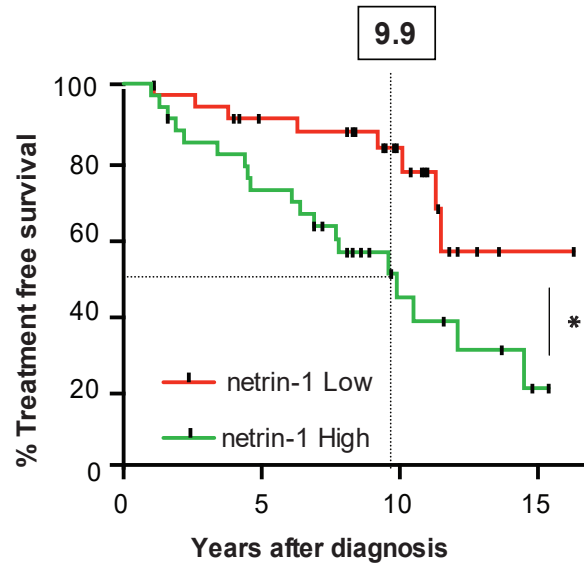
944 **A-B.** Flow cytometry analysis of PMN-MDSC obtained after dissociation of EMT6 (n=9  
945 per group) or MMTV/NeuT (n=16 vs 19) tumors treated with either control isotype IgG1  
946 or anti-netrin-1 mAb. PMN-MDSC are defined as CD11b<sup>+</sup> Singlets<sup>+</sup> cells among the  
947 CD45<sup>+</sup> (CD11b<sup>+</sup> Ly6G<sup>+</sup> Ly6C<sup>int</sup>) population. **C.** EMT-6 tumor implanted in syngeneic  
948 BALBc/J mice were treated with anti-netrin-1 and anti-CTLA4 and injected/not with  
949 PMN-MDSC cells. **D.** Flow cytometry analysis of CD49f<sup>+</sup> EMT6 cells in control parental  
950 cells, or netrin-1 silenced by shRNA (Sh netrin-1) cocultured, or not, with PMN-MDSC  
951 (n=3). **E.** Flow cytometry analysis of EMT6 cancer cells obtained after dissociation of  
952 tumors treated with either three treatments of control IgG1, anti-netrin-1 mAb alone or  
953 in combination with anti-CTLA-4 (respectively n=10, 8, 8 and 10). CSC markers are  
954 defined as CD49f<sup>+</sup> cells among the CD45<sup>neg</sup> (CD45<sup>-</sup>) population. **F.**  
955 Immunohistochemistry analysis and quantification of Ly6G<sup>+</sup> cells in EMT6 obtained  
956 after fixation of tumors treated with either three treatments of control IgG1, anti-netrin-  
957 1 mAb with or without anti-CTLA-4. n=8 field/tumor. **G.** Serial transplantation  
958 experiments with EMT6 cells in syngeneic BALBc/J mice. 5.10<sup>4</sup> living cells, obtained  
959 after the dissociation of tumors from mice treated with either control IgG1 vs. anti-  
960 netrin-1 mAb alone; or in combination with anti-CTLA4 (respectively n=7, 8, 8 and 8)  
961 were grafted into recipient mice. Results are presented as percentage of mice free of  
962 tumor. **H.** Serial transplantation of EMT6 cells were used as an indicator of tumor  
963 initiating potential of CD49f<sup>+/+</sup> populations. Tumors were dissociated from EMT6 tumors  
964 of mice treated with control IgG1 or anti-netrin-1 mAb. After dissociation, cells were  
965 sorted based on CD49f expression and 5.10<sup>4</sup> living cells of each positive or negative  
966 populations were regrafted in new recipient animals (n=10 per group). Results are

967 presented as the percentage of mice free of tumor. In all panels statistics are presented  
968 as \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; \*\*\*  $p < 0.001$ .

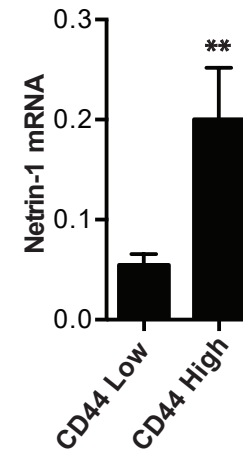
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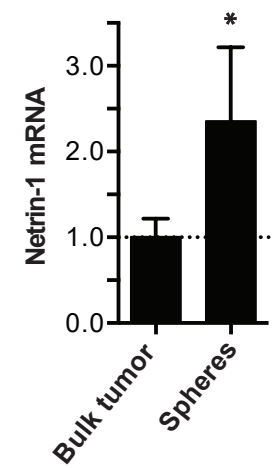
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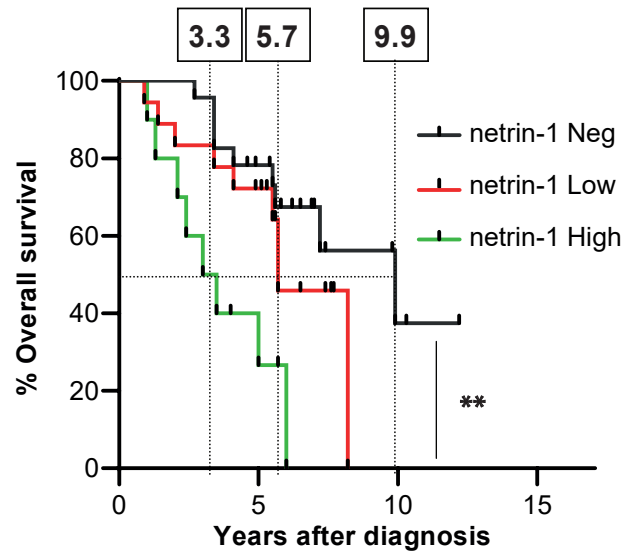
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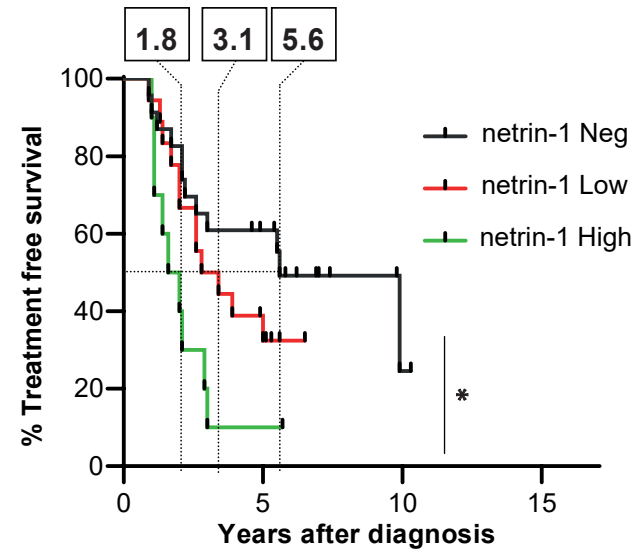
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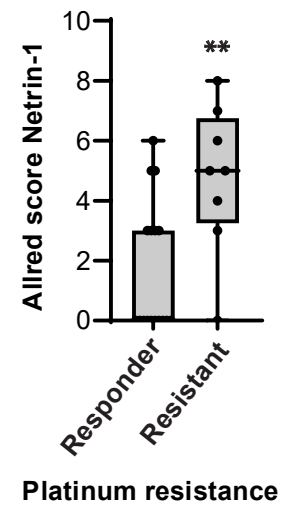
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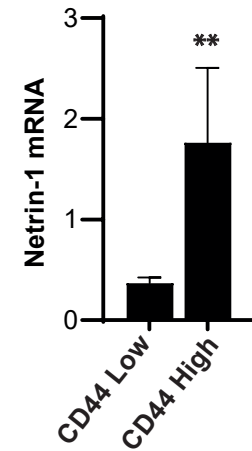
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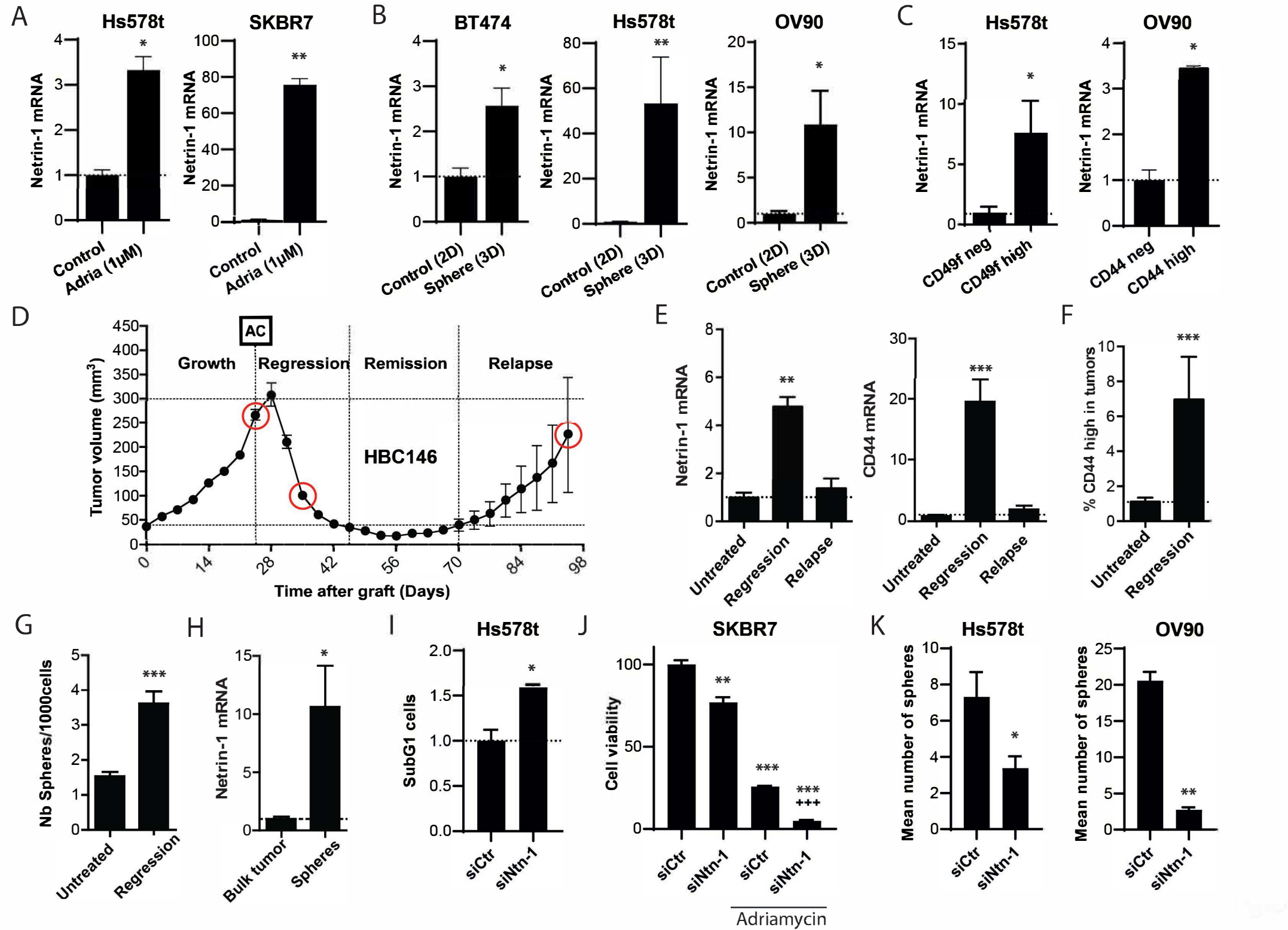


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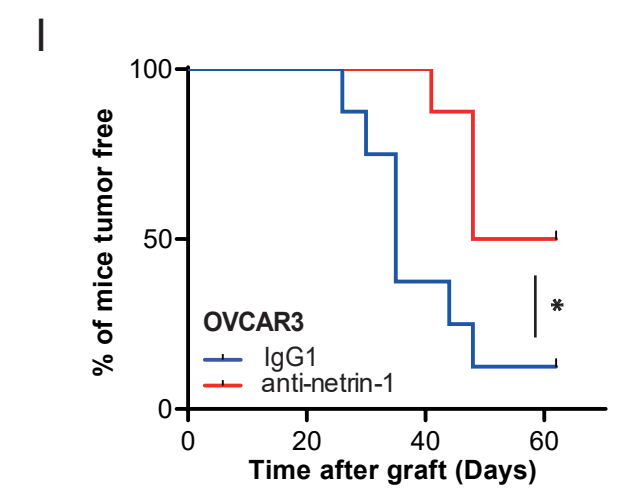
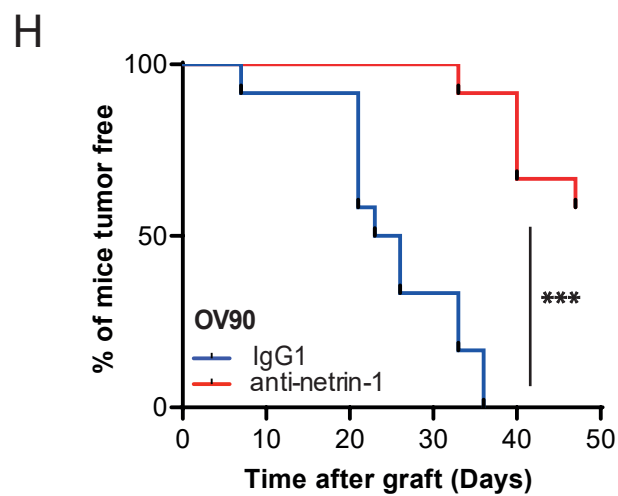
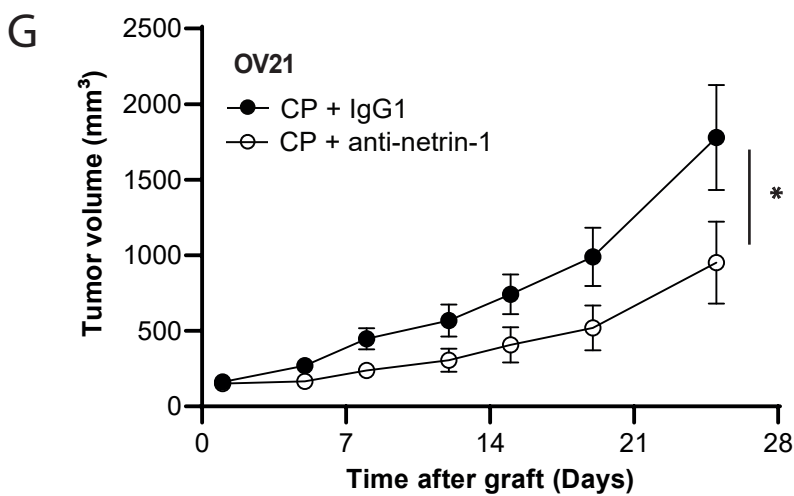
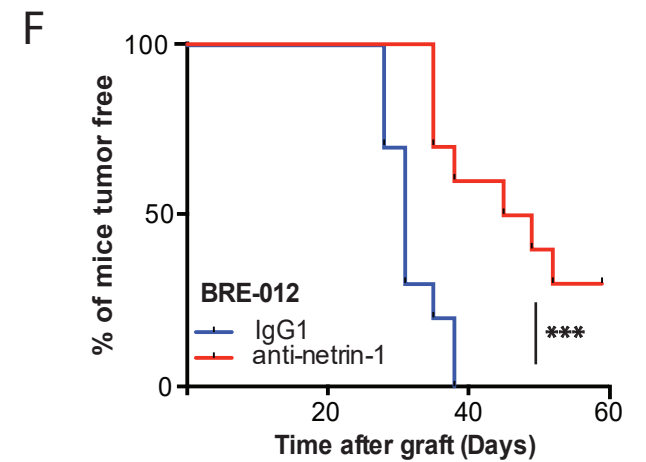
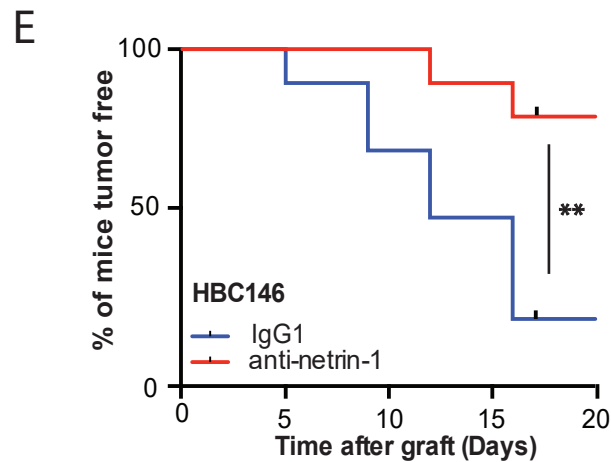
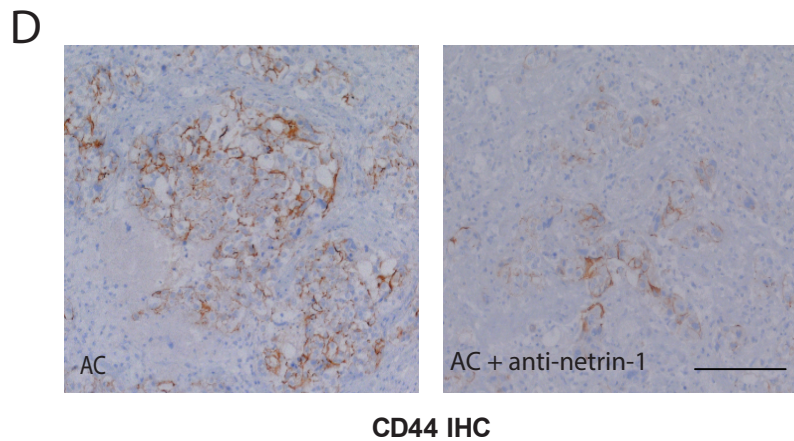
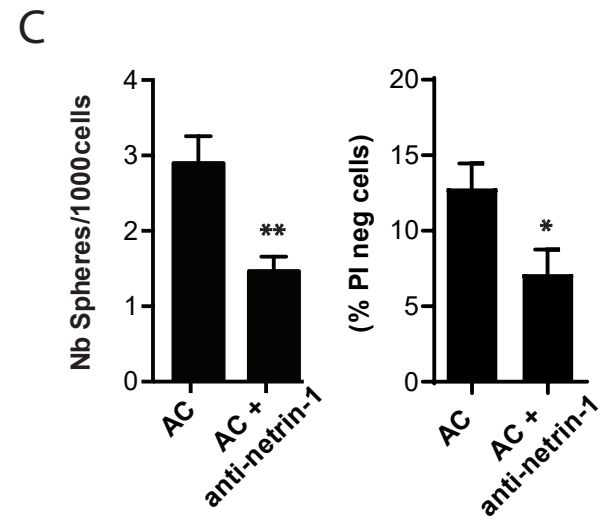
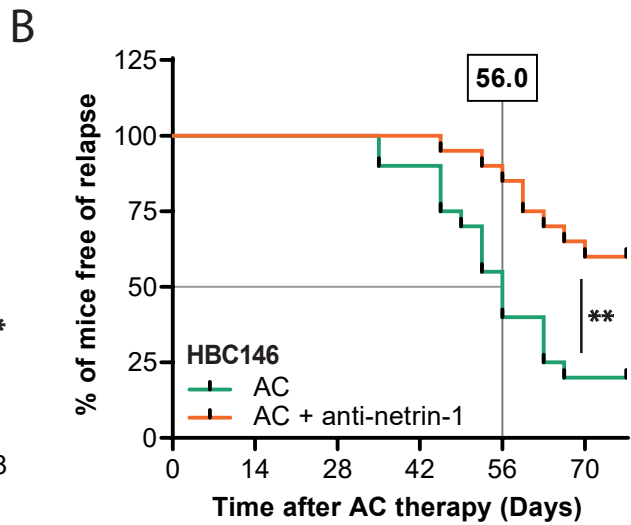
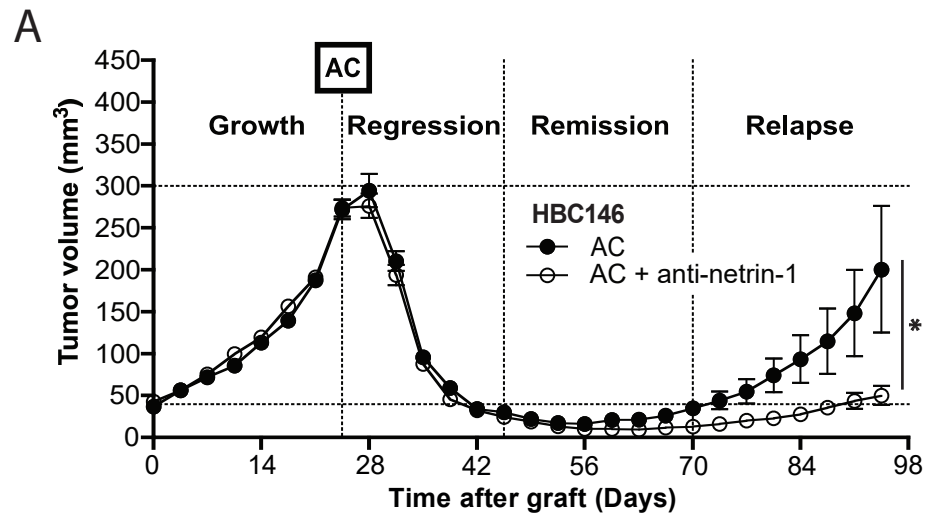


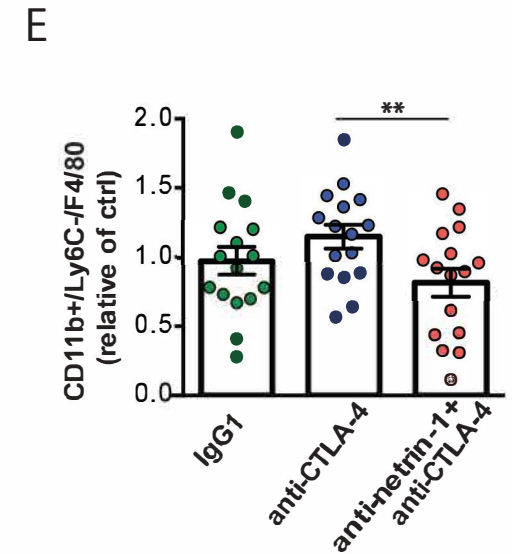
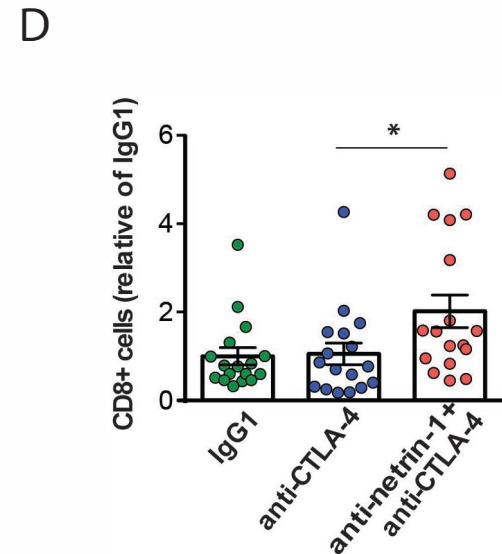
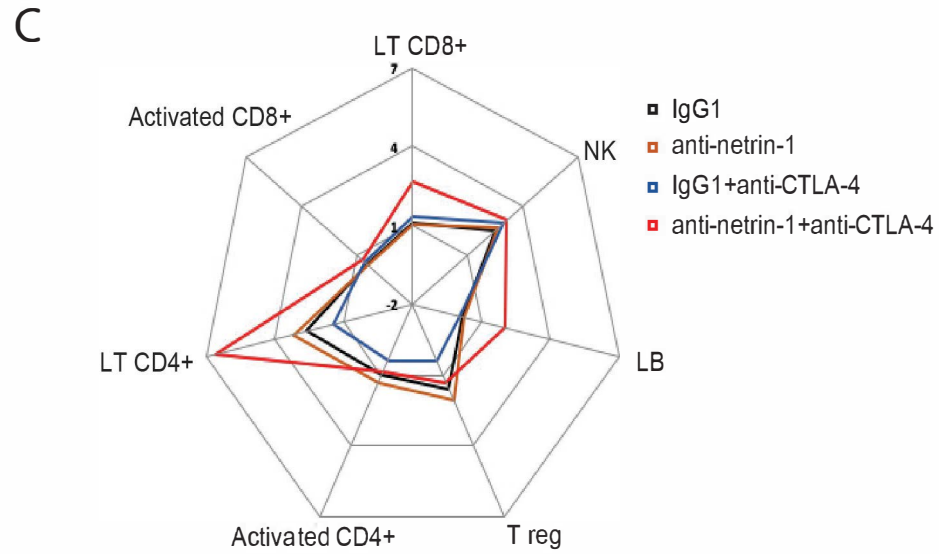
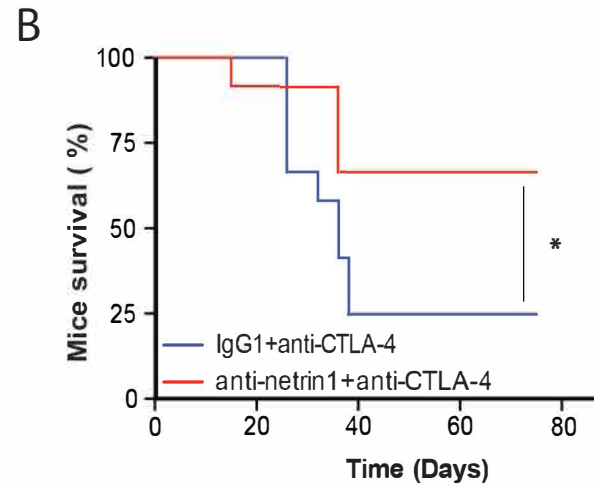
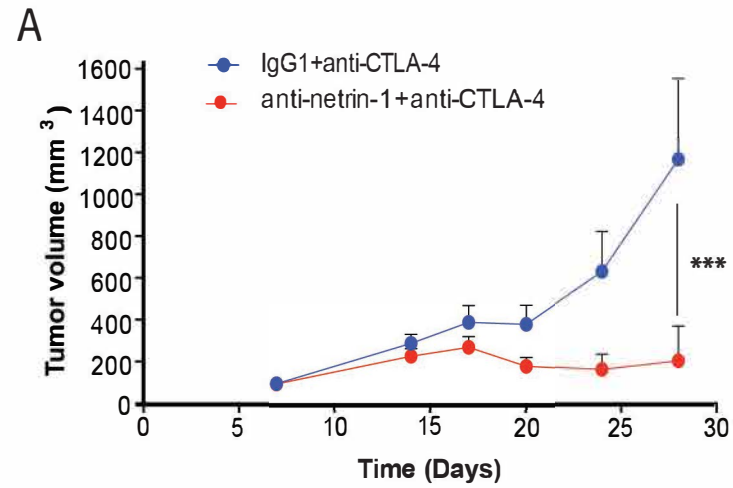
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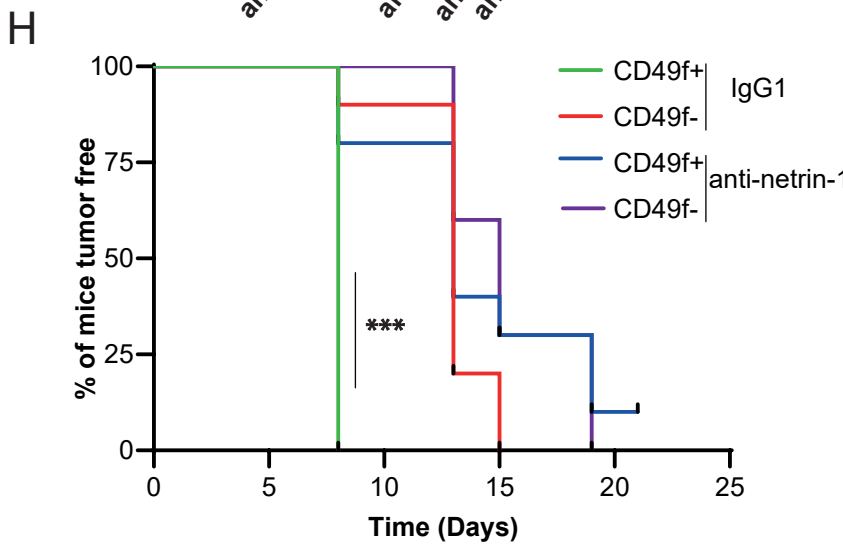
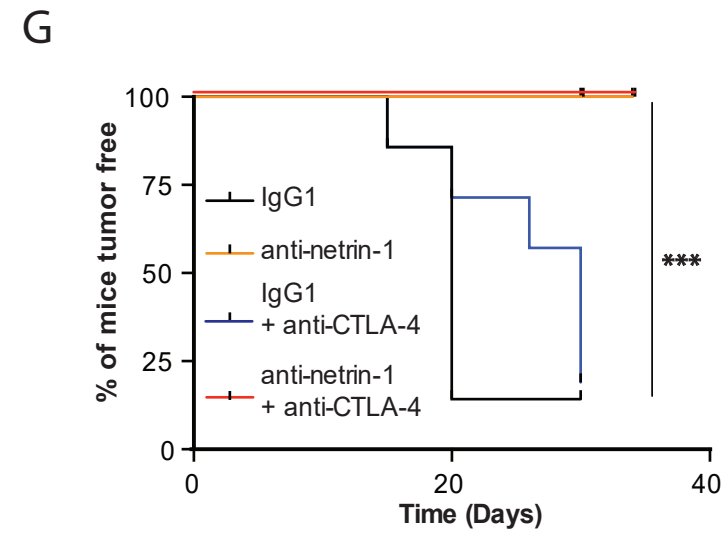
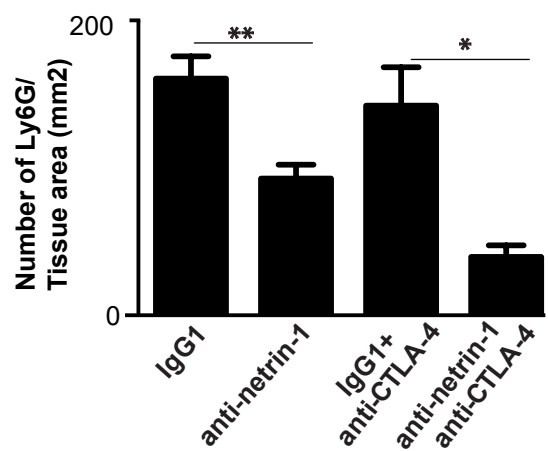
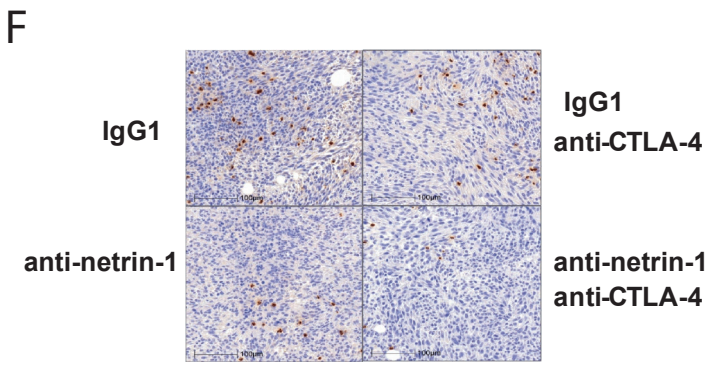
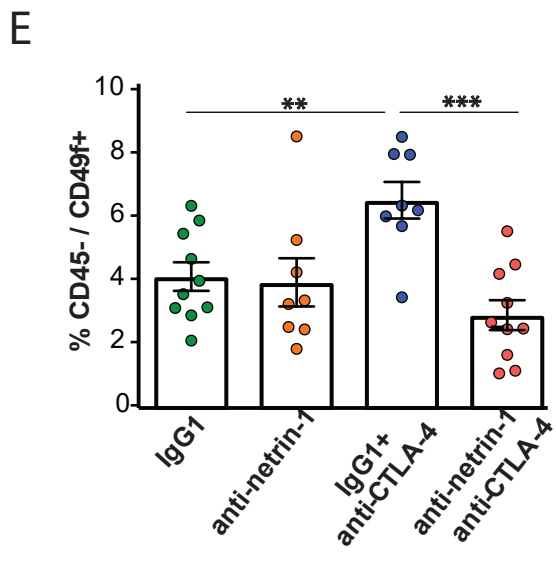
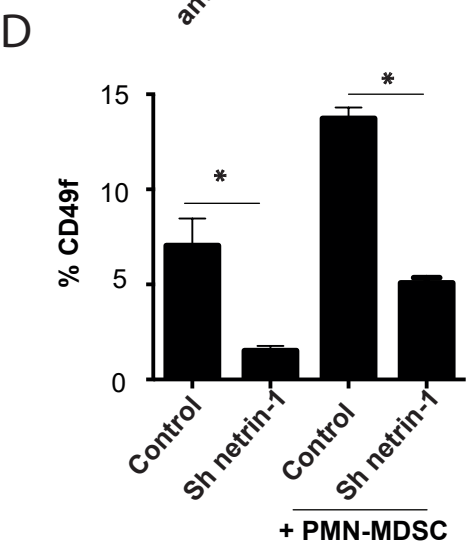
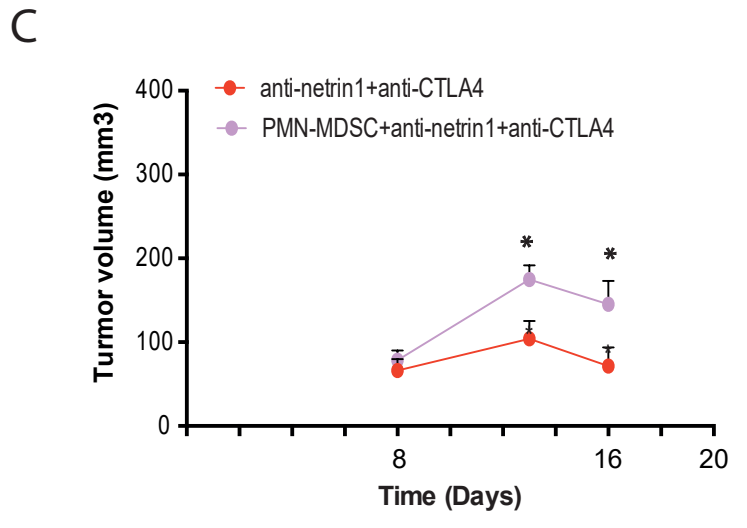
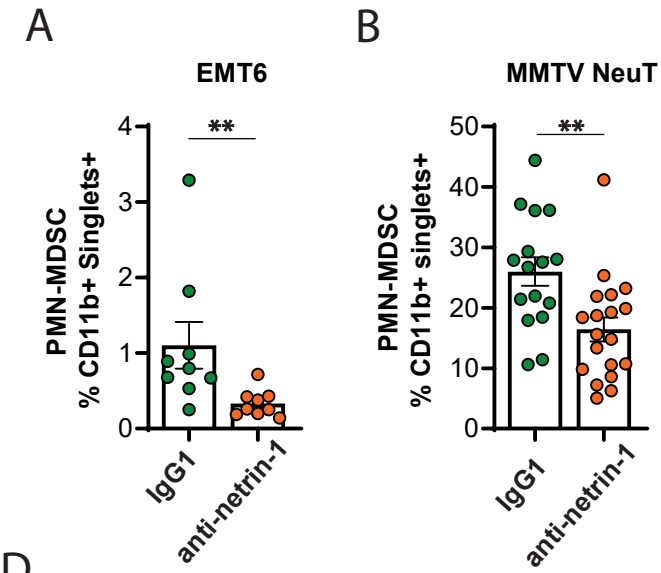


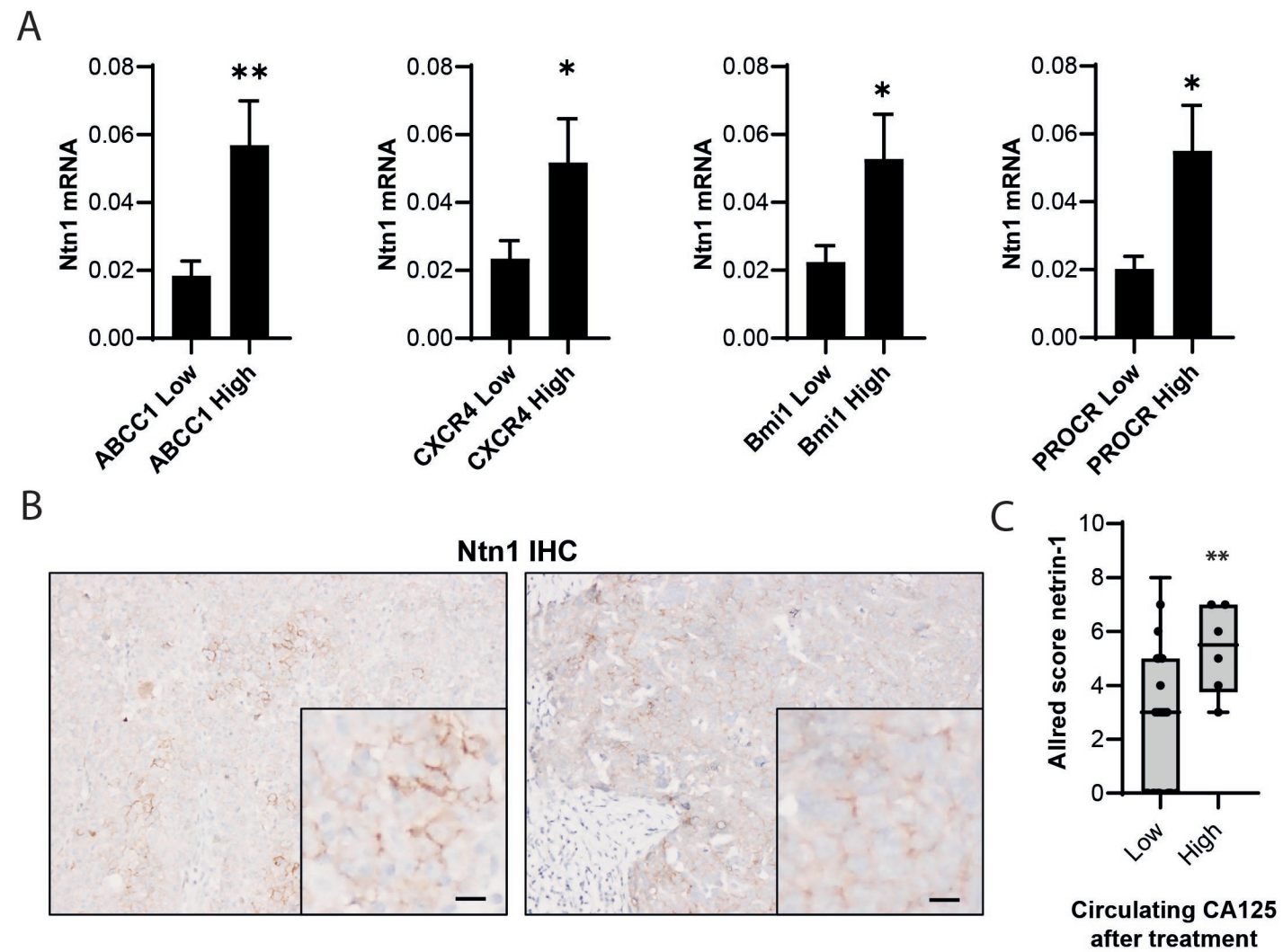


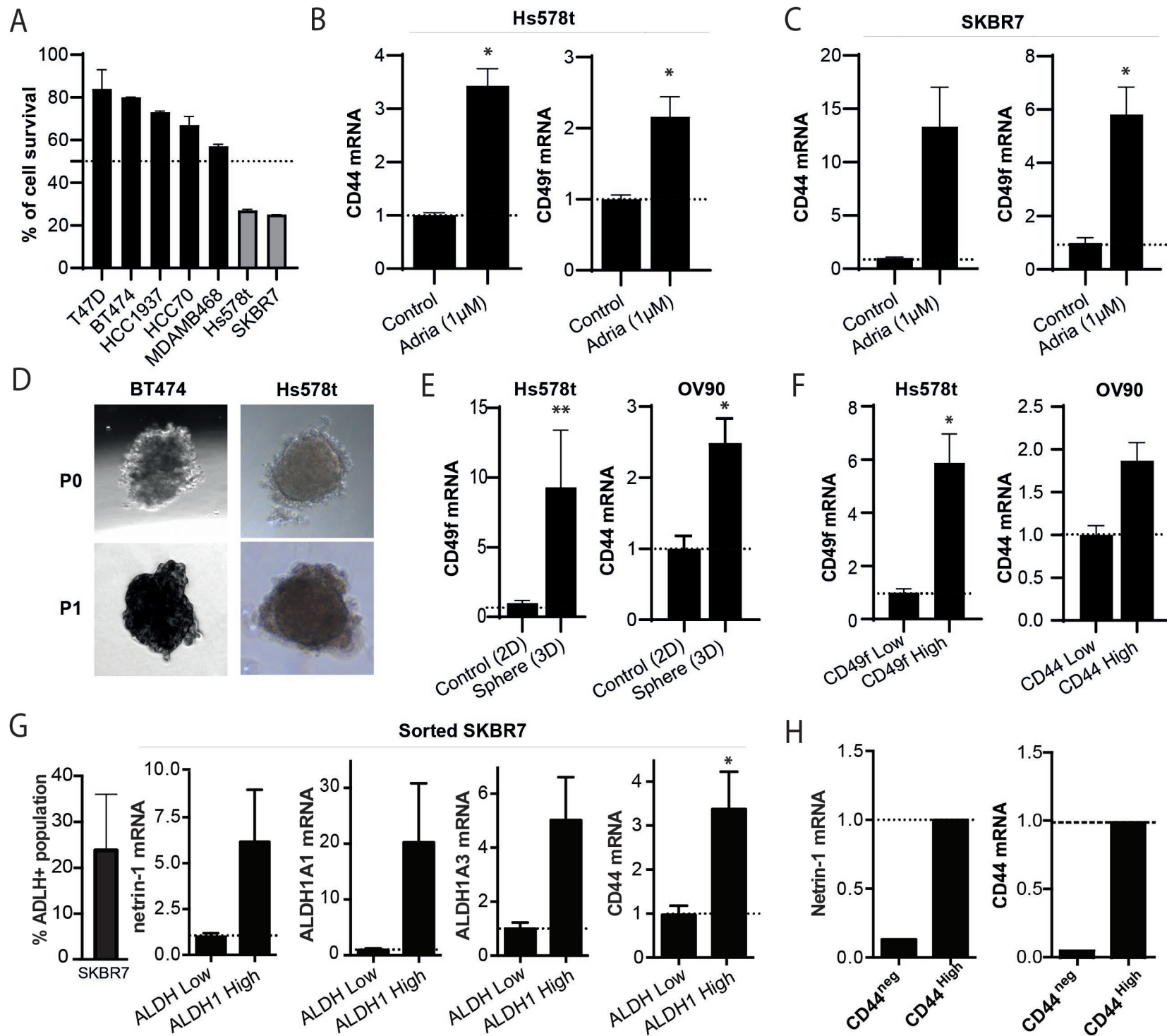




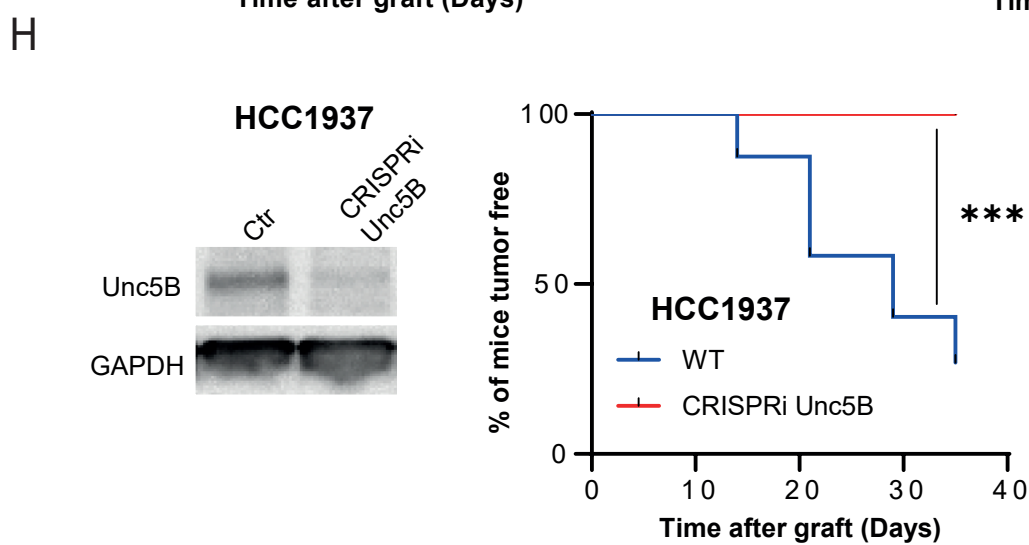
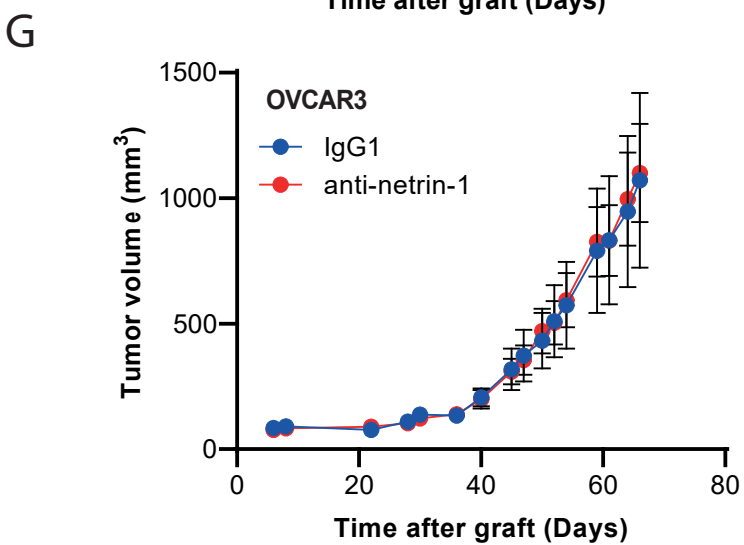
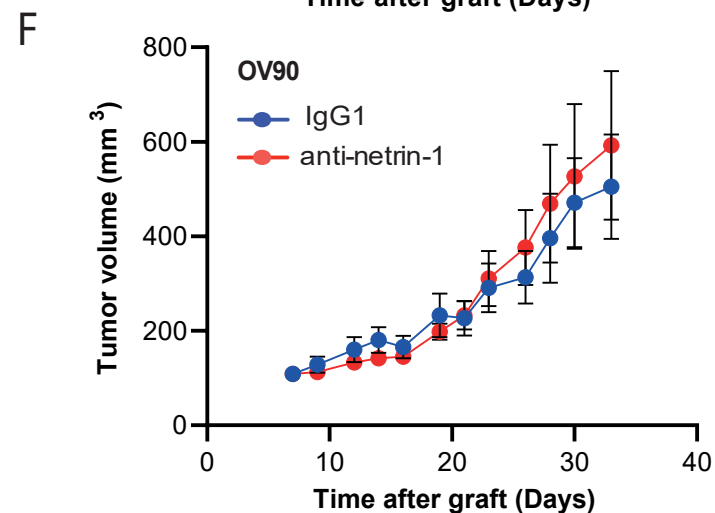
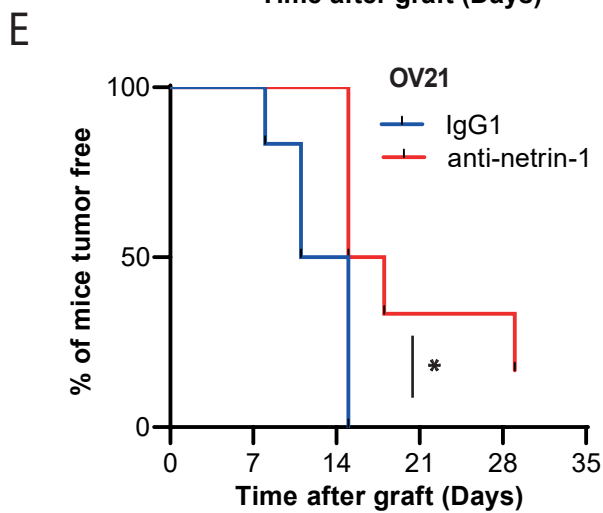
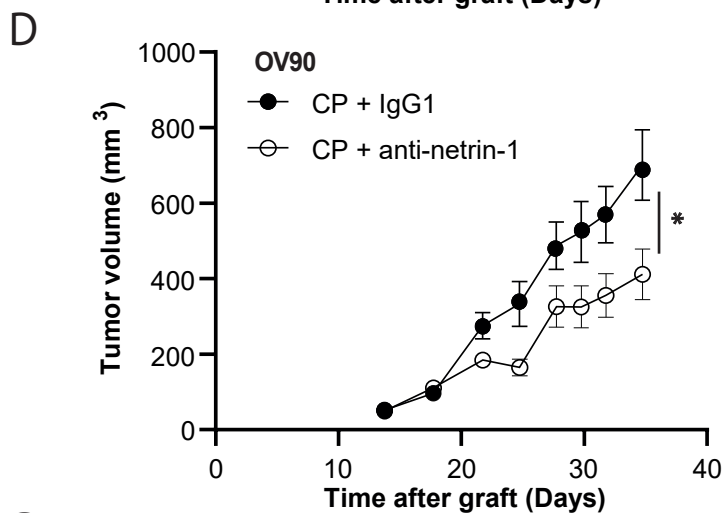
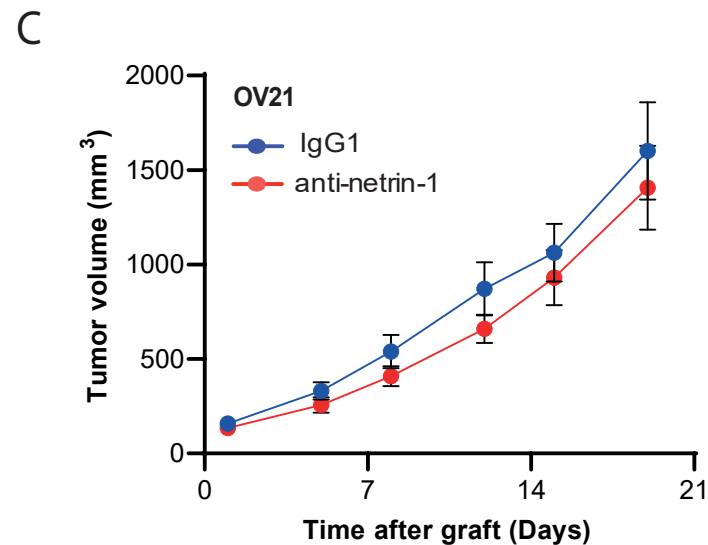
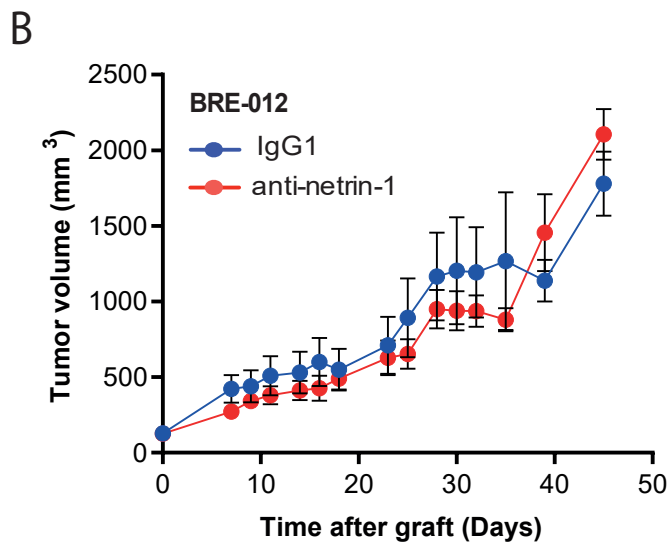
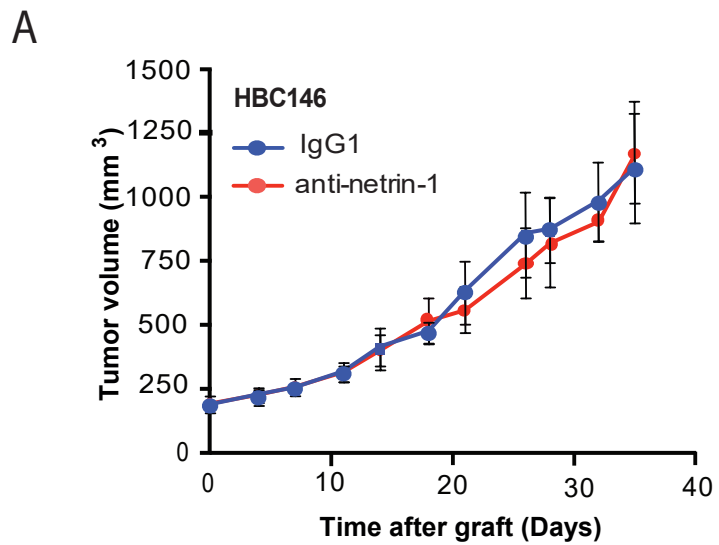




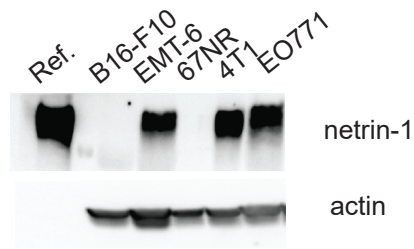




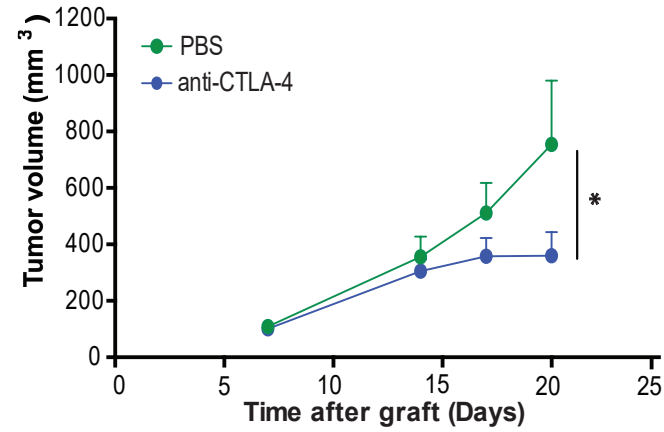




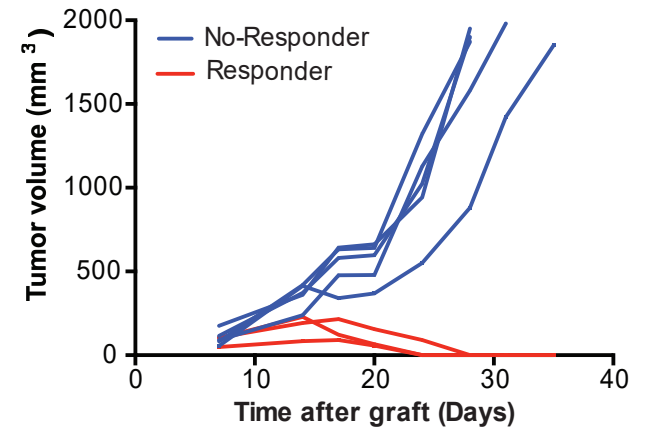
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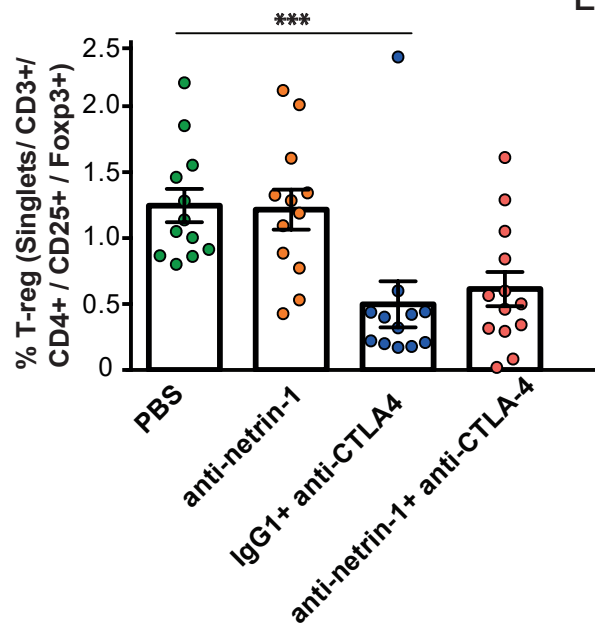
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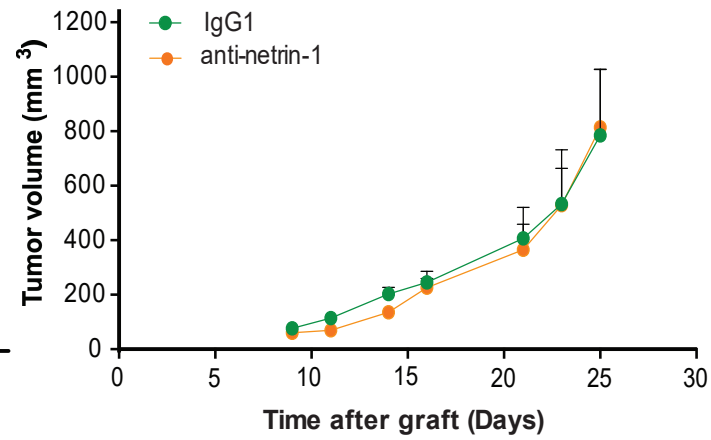
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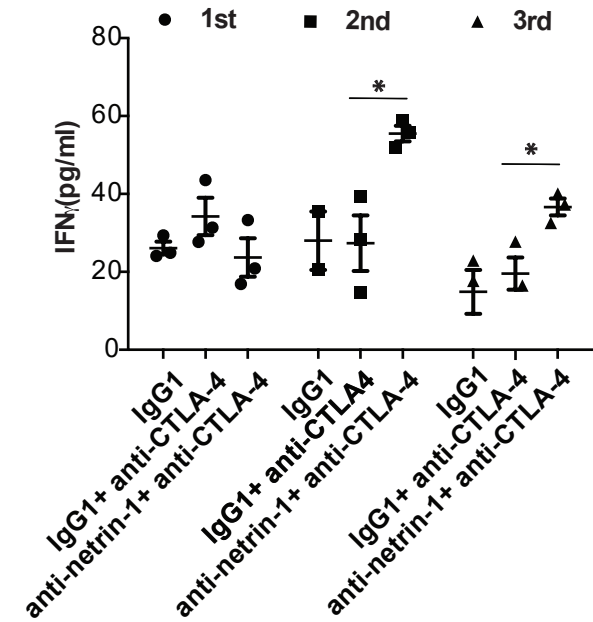
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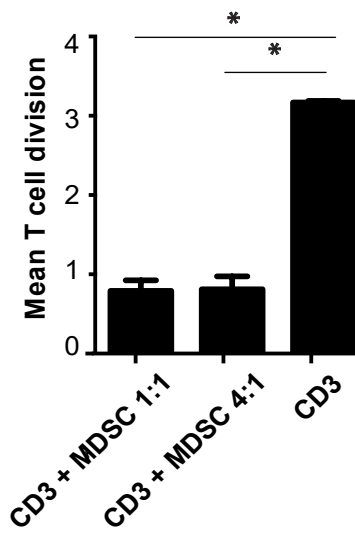
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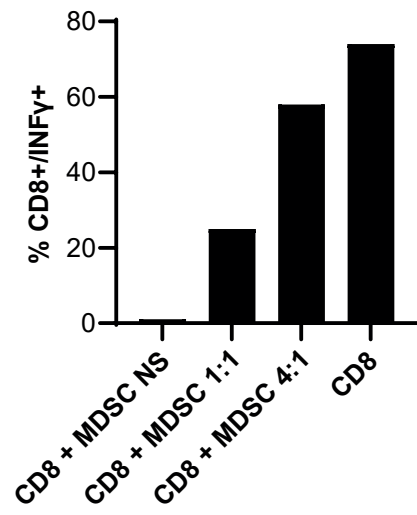
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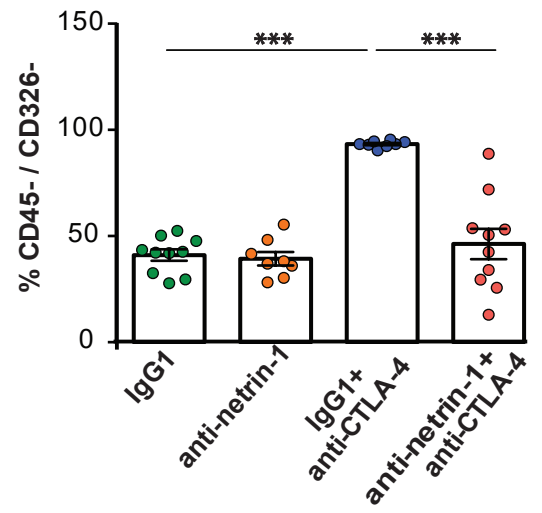
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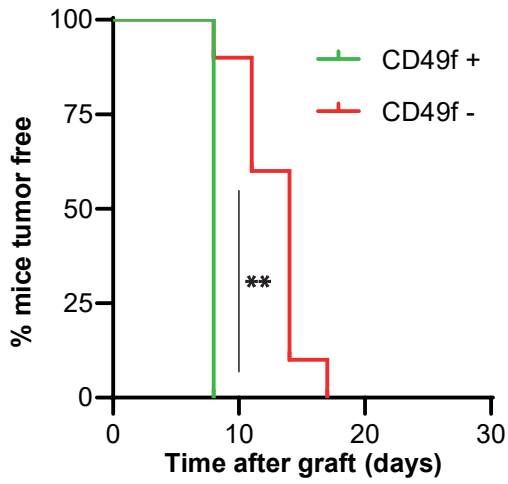
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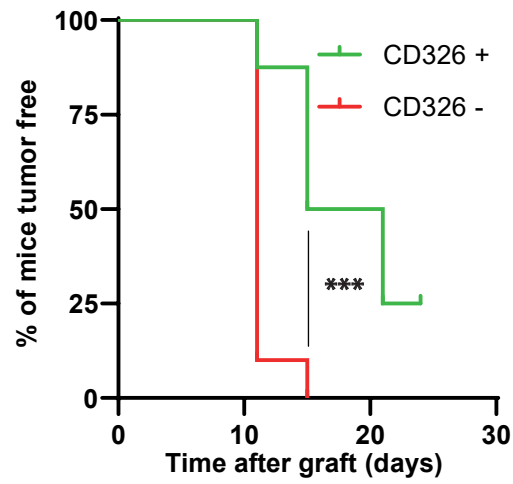
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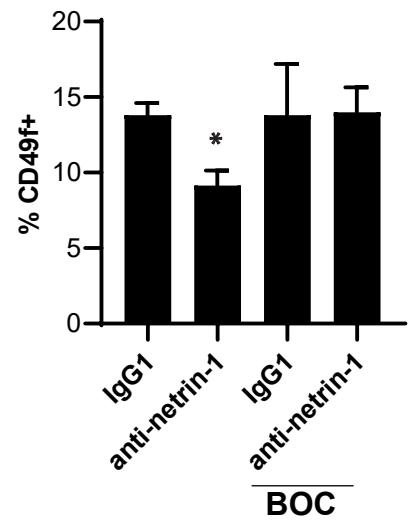
D



E



F



G

