

Immune cell topography predicts response to PD-1 blockade in cutaneous T cell lymphoma

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26 **Keywords**

27 Cutaneous T cell lymphoma (CTCL), anti-PD-1 immunotherapy, tumor microenvironment (TME), CODEX
28 multiplex tissue imaging, RNA sequencing, spatial biomarkers, chemokines

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31 **ABSTRACT**

32 Anti-PD-1 immunotherapies have transformed cancer treatment, yet the determinants of clinical
33 response are largely unknown. We performed CODEX multiplexed tissue imaging and RNA sequencing
34 on 70 tumor regions from 14 advanced cutaneous T cell lymphoma (CTCL) patients enrolled in a clinical
35 trial of pembrolizumab therapy. Clinical response was not associated with the frequency of tumor-
36 infiltrating T cell subsets, but rather with striking differences in the spatial organization and functional
37 immune state of the tumor microenvironment (TME). After treatment, pembrolizumab responders had
38 a localized enrichment of tumor and CD4⁺ T cells, which coincided with immune activation and cytotoxic
39 PD-1⁺ CD4⁺ T cells. In contrast, non-responders had a localized enrichment of Tregs pre- and post-
40 treatment, consistent with a persistently immunosuppressed TME and exhausted PD-1⁺ CD4⁺ T cells.
41 Integrating these findings by computing the physical distances between PD-1⁺ CD4⁺ T cells, tumor cells,
42 and Tregs revealed a spatial biomarker predictive of pembrolizumab response. Finally, the chemokine
43 CXCL13 was upregulated in tumor cells in responders post-treatment, suggesting that chemoattraction
44 of PD-1⁺ CD4⁺ T cells towards tumor cells facilitates a positive outcome. Together, these data show that T
45 cell topography reflects the balance of effector and suppressive activity within the TME and predicts
46 clinical response to PD-1 blockade in CTCL.

47

48 INTRODUCTION

49 Cutaneous T cell lymphomas (CTCL) are a rare, heterogeneous group of T cell malignancies that
50 primarily occur in the skin. The majority of CTCLs are comprised of mycosis fungoides and Sézary
51 syndrome, which originate from mature, skin-tropic CD4⁺ T cells¹. A third of patients present with
52 advanced-stage disease and those meeting high risk criteria have a 5-year survival rate of 28%². With the
53 exception of hematopoietic stem cell transplantation, there are no curative therapies for advanced
54 CTCL, and current treatments typically induce short-lived, partial disease control³. However, immune
55 checkpoint inhibitors, such as antibodies against PD-1, restore T cell effector function at the tumor site^{4,5}
56 and promote robust and durable responses in a number of advanced cancers⁶⁻⁸. In CTCL, PD-1 and its
57 ligands can be simultaneously expressed on tumor cells, making this pathway an attractive therapeutic
58 target for PD-1 blockade⁹⁻¹².

59 A Cancer Immunotherapy Trials Network (CITN) multicenter phase II clinical trial of the anti-PD-1
60 immunotherapy, pembrolizumab, in heavily pre-treated patients with advanced relapsed/refractory
61 CTCL was recently reported (NCT02243579)¹³. In this study, 38% of patients achieved a sustained clinical
62 response, whereas 25% experienced rapid disease progression, likely because inhibition of PD-1 on
63 tumor cells promoted cancer growth^{14,15}. Such outcome discrepancies underscore a need for predictive
64 biomarkers of PD-1 blockade that will allow patients to be stratified into probable responders and non-
65 responders prior to initiating therapy.

66 To date, biomarker studies with immunohistochemistry (IHC), gene expression profiling, and
67 mass cytometry (CyTOF), have not yet provided predictive indices of pembrolizumab responsiveness in
68 CTCL¹³. Similarly, predictive biomarkers remain in-demand for other tumor types and
69 immunotherapies^{16,17}. As the immune system acts via coordinated cell-cell association, it is expected
70 that spatial cellular attributes within the tumor microenvironment (TME) would be predictive of clinical
71 outcomes. As such, organized cellular contexts would be productive whereas incorrectly arranged
72 cellular contexts (i.e., absence or misplacement of certain cell-types) would prognosticate a negative

73 outcome. Indeed, recent studies indicate that immune cells are not randomly distributed within the
74 TME, but are purposefully organized into cellular neighborhoods and niches that facilitate anti- or pro-
75 tumor functions^{18,19}. This raises the question of how PD-1 blockade alters spatial cellular context, and in
76 turn, how such changes in architecture relate to patient responses in CTCL.

77 We combined CO-Detection by indEXing (CODEX) multiplexed tissue imaging^{19,20} with gene
78 expression profiling by RNA-seq to interrogate tumors from 14 patients with advanced CTCL sampled
79 before and after pembrolizumab treatment. Then we computationally correlated spatial protein
80 information at the single cell level with matched global gene expression data to generate insights into
81 the drivers and resistors of PD-1 blockade. Our data revealed a localized enrichment of effector CD4⁺ T
82 cells in responders versus inhibitory Tregs in non-responders. Such topographical differences coincided
83 with variations in the functional immune state of the TME, T cell function, and tumor cell-specific
84 chemokine expression. We integrated these findings into a single *SpatialScore*—a spatial biomarker
85 predictive of pembrolizumab outcome—that can be accurately recapitulated using clinically accessible
86 multiplexed IHC (mIHC) platforms. The results underscore a key determinant of spatial cellular
87 organization, namely a distancing balance of effector and suppressive T cell activity, for predicting anti-
88 PD-1 immunotherapy response in CTCL.

89

90 **RESULTS**

91 **CTCL clinical cohort and multimodal experimental approach**

92 We analyzed pre- and post-treatment biopsies from 14 patients with advanced-stage CTCL, who
93 received pembrolizumab every 3 weeks for up to 2 years as part of the CITN-10 trial (NCT02243579) (**Fig.**
94 **1a.1, Sup. Fig. 1a-b, Sup. Table 1**)¹³. The post-treatment biopsies were collected at several timepoints,
95 which is detailed per patient in **Sup. Fig. 1c** and **Sup. Table 1**. The spatial organization and
96 immunogenomic heterogeneity of the CTCL TME was dissected using a formalin-fixed paraffin-

97 embedded (FFPE) tissue microarray from 70 patient-matched pre- and post-treatment skin tumor
98 samples (**Fig. 1a.2**). The tissue microarray spots were selected from the most infiltrated regions of the
99 skin biopsies. CODEX multiplexed protein imaging identified 117,170 cells in the tissue microarray. These
100 results were integrated with 64 tissue transcriptomes obtained from serial sections using laser capture
101 microdissection and Smart-3Seq²¹ (i.e., RNA-seq) (**Fig. 1a.3**). Integrative analyses, including cellular
102 neighborhood assessment¹⁹ and CIBERSORTx (CSx)²², were then used to profile the molecular dynamics
103 of CTCL and identify predictive biomarkers of anti-PD-1 immunotherapy (**Fig. 1a.4**).

104 Therapeutic response to pembrolizumab was assessed by consensus global response criteria²³.
105 No significant differences were observed at baseline between responders and non-responders for
106 patient demographics, cancer type/stage, and treatment history (**Sup. Fig. 1b**). Clinical outcomes were
107 significantly different between patient groups (**Sup. Fig. 1b**). Responders had a significant improvement
108 in their overall skin response compared to non-responders, as measured by the modified Severity
109 Weighted Assessment Tool (mSWAT)²⁴ (**Sup. Fig. 1b**). Similarly, overall survival was significantly longer in
110 responders than non-responders: Non-responders had a median survival of 109 weeks after treatment
111 initiation, whereas all but one responder was alive at the median follow-up time of 142 weeks (**Fig. 1b**).
112 The expression of key T cell, macrophage and PD-1 signaling markers was assessed by standard IHC for
113 each patient's pre-treatment tumor biopsy: PD-L2 expression was slightly increased in non-responders
114 compared to responders but did not reach statistical significance (**Fig. 1c**). No differences were observed
115 for the other seven markers, as shown for CD4, FoxP3, PD-1, and PD-L1 (**Fig. 1d**).

116

117 **Discrimination of malignant from reactive CD4⁺ T cells in the CTCL TME**

118 Distinguishing malignant from reactive CD4⁺ T cells is a major challenge in CTCL, because no
119 protein marker is 100% specific for tumor cells and both cell-types can be clonal²⁵. Using a 55-marker
120 CODEX panel (**Fig. 1e, Sup. Fig. 1d, Sup. Table 2**), unsupervised machine learning and manual curation
121 based on marker expression, tissue localization, and morphology, we identified and validated 21 unique

122 cell-type clusters (**Fig. 1f, Sup. Fig. 2a-b**), including reactive CD4⁺ T cells and malignant CD4⁺ T cells (i.e.,
123 tumor cells). Comparing the fluorescent stainings of reactive CD4⁺ T cells (blue crosses) and malignant
124 CD4⁺ T cells (red crosses) showed that in tumor cells CD7 was decreased and CD25 and Ki-67 were
125 increased (**Fig. 1g**), consistent with an advanced CTCL phenotype²⁶. Quantifying these expression
126 differences for tumor cells relative to reactive CD4⁺ T cells showed a fold-change of 0.40 for CD7, 1.87
127 for CD25, and 3.47 for Ki-67 (**Fig. 1h**, $p < 0.0001$). Tumor cells were also larger in size than reactive CD4⁺ T
128 cells (**Fig. 1i**), in line with previous reports²⁷. One patient had large cell transformation, but the average
129 size of tumor cells in that patient was not significantly different from the other 13 patients. Additionally,
130 we identified genes predictive of the frequency of tumor cells per tissue microarray spot by fitting an L1-
131 regularized linear model to bulk RNA-seq data. This confirmed that higher expression of several known
132 CTCL marker genes, including *CD27*, *IL-32*, *CXCL13*, *BATF*, and *TIGIT*²⁸⁻³², was associated with spots with
133 higher frequencies of tumor cells (**Fig. 1j**, see yellow highlighted gene names). Notably, CTCL tumor cells
134 can also express FoxP3³³, as was the case for one patient in this clinical trial cohort. Our clustering
135 approach identified this population of malignant FoxP3⁺ CD4⁺ T cells, which differed significantly from
136 Tregs, with lower CD3, CD4, and FoxP3 marker expression and larger cell size (**Sup. Fig. 1e**). Given the
137 heterogeneity of CTCL, the marker expression profile of tumor cells may differ between cohorts,
138 especially those that were heavily pre-treated. However, these findings demonstrate that multiplexed
139 imaging can discriminate malignant from reactive CD4⁺ T cells at the single-cell level, which is supported
140 by cell size measurements and gene expression profiling.

141

142 **Deep profiling of the CTCL TME in response to anti-PD-1 immunotherapy**

143 Anti-PD-1 immunotherapies are premised on the ability to alter the delicate balance of positive
144 and negative immune signaling to promote the recognition and elimination of tumor cells. Tumor cells,
145 however, shift this balance in favor of their own survival and proliferation. We reasoned that delineating

146 the complexities of tumor and immune cell interactions within the TME may reveal the cell specific
147 organizations that determine pembrolizumab response and resistance in CTCL.

148 We first explored how cellular composition contributes to the efficacy of PD-1 blockade in CTCL
149 by cataloging the frequencies of the 21 cell-types identified by CODEX across patient groups. The 21 cell-
150 types included 13 immune cell-types, 6 auxiliary cell-types, and 2 tumor cell-types (**Sup. Fig. 2b**).
151 Markers for T cell subsets (CD4, CD8, FoxP3), macrophages (CD68), dendritic cells (CD11c), tumor cells
152 (CD4), vasculature (CD31), and epithelium (cytokeratin) were clearly visualized in the CODEX fluorescent
153 images (**Fig. 2a-b, upper panels; Sup. Fig. 3a**). The corresponding hematoxylin and eosin (H&E) images
154 (**Fig. 2a-b, inserts; Sup. Fig. 3b**) confirmed accurate staining of structural elements like epithelium and
155 vasculature. Fluorescent staining of immune and tumor cells confirmed the cell-type assignments shown
156 in the corresponding cell-type maps (**Fig. 2a-b, lower panels**).

157 Interestingly, no differences in the content of the 21 identified cell-types were noted between
158 responders and non-responders pre- or post-treatment (**Fig. 2c-d; Sup. Fig. 2c**). The combined
159 frequencies of tumor, immune and auxiliary cell-types each comprised approximately one-third of all
160 cells (**Fig. 2c, upper panel**); the same trend held across patient groups (**Sup. Fig. 2c**). Tumor cell content
161 (averaged across all TMA spots in a patient group) was unchanged in responders or non-responders
162 following pembrolizumab therapy (**Sup. Fig. 2c**), likely because only heavily infiltrated skin tumor biopsy
163 regions were used for this study. Among all immune cells, the ranked cell-type frequencies were 38% for
164 M₁ macrophages, 21% for Tregs, 15% for CD8⁺ T cells, 5% for M₂ macrophages, 5% for CD4⁺ T cells, and
165 <5% for other immune cell-types including B cells, plasma cells, dendritic cells, Langerhans cells, mast
166 cells, and neutrophils (**Fig. 2c, lower panel**). No differences in the mean frequencies of immune cell-
167 types were observed when comparing responders and non-responders pre- and post-treatment; this is
168 highlighted for T cells (CD4⁺ T, CD8⁺ T, Tregs) and macrophages (M1 and M2) (**Fig. 2d**). This finding is
169 consistent with the initial publication on this CTCL cohort and our baseline IHC data (**Fig. 1c-d**), which
170 showed no correlation between pembrolizumab response and the expression of T cell, macrophage or

171 PD-1 signaling markers¹³. However, it contrasts with some solid tumor studies, which have correlated
172 the density of tumor infiltrating lymphocytes with clinical outcome^{34,35} and immunotherapy
173 response^{36,37}.

174 We next focused on differences in immune signaling between responders and non-responders.
175 Immunogenomic analyses were performed to characterize the functional immune state of the TME,
176 which has been shown to be a key determinant of immunotherapeutic activity³⁸. Gene expression
177 signatures that have predicted PD-1/PD-L1 blockade response (e.g., IFN- γ scores³⁹, **Sup. Table 3**) and
178 non-response (e.g., TGF- β scores⁴⁰, **Sup. Table 3**) in solid tumors were applied to our CTCL data. No
179 differences were observed pre-treatment in responders versus non-responders for the IFN- γ (**Fig. 2e**,
180 median R, pre (0.77) versus NR, pre (0.76)) or TGF- β (**Fig. 2f**, median R, pre (0.28) versus NR, pre (-0.32))
181 gene scores; hence, neither signature was predictive of pembrolizumab response in CTCL. Since these
182 predictive gene signatures were derived from single-disease studies, their utilization may be limited to
183 similar solid tumor types⁴¹.

184 Rare cancers like CTCL do not have specifically defined immune gene signatures, making it a
185 challenge to catalogue their tumor immunogenicity. Gene lists of immune activation (e.g., *CD27*, *EOMES*,
186 and *ICOS*) and immunosuppression (e.g., *ENTPD1*, *TGFB1*, and *TIGIT*) molecules were therefore compiled
187 (**Sup. Table 3**) and used to assess how the functional immune state of the CTCL TME was altered in
188 response to pembrolizumab. Notably, genes like *PDCD1* and *TNFRSF18*, which can be both immune
189 activating and suppressive depending on cellular state and microenvironmental context, were excluded
190 from this analysis. The immune activation gene score was significantly increased in responders post-
191 treatment compared with pre-treatment (**Fig. 2g**, median R, pre (-2.54) versus R, post (1.21)), with no
192 significant change in non-responders post- versus pre-treatment. This indicates that only responders
193 develop an immune activated TME phenotype following pembrolizumab therapy. In contrast, the
194 immunosuppression gene scores were significantly increased in non-responders compared to
195 responders both pre- and post-treatment (**Fig. 2h**, median NR, pre (0.59) versus R, pre (-0.83) and NR,

196 post (0.64) versus R, post (-1.12)), implying that non-responders have an immunosuppressed TME
197 phenotype relative to responders. Collectively, these results demonstrate that the functional immune
198 state of the CTCL TME depends on factors beyond raw immune cell counts. Exploring the spatial
199 patterns of immune infiltration is an essential step towards understanding the indicators predictive of
200 immunotherapy outcome.

201

202 **PD-1 blockade induces spatial re-organization of the CTCL TME**

203 It is expected that the functional immune state of the TME will be mediated by its spatial cellular
204 organization. Cellular neighborhood (CN) analysis, applied previously in a study on colorectal cancer¹⁹,
205 was performed to obtain a high level view of CTCL tissue architecture. CNs are analogous to urban
206 neighborhoods—essentially geographically localized areas within a larger city that facilitate social
207 interaction⁴² (**Sup. Fig. 2d**). Likewise, CNs are defined by a localized enrichment of specific cell-types
208 within the tissue that mediate cellular interactions and vital tissue functions (**Sup. Fig. 2e**).

209 Computationally, the CN algorithm extracts quantitative data on the composition and spatial
210 distribution of individual cells to reveal how local cellular niches are organized within tissues (**Fig. 3a**)¹⁹.
211 Specifically, computational parameters like the window size and number of CNs to be computed are
212 manually set (**Fig. 3a.1**). Each cell in the tissue is assigned to a given CN based on the composition of cell-
213 types within the specified window (**Fig. 3a.2**). The windows are then clustered and the correlation of
214 CNs and cell-types are represented as a heatmap (**Fig. 3a.3**). CNs are visualized as Voronoi diagrams and
215 analyzed to better understand cellular spatial behavior (**Fig. 3a.4**).

216 Using a window size of 10 nearest neighbors, we identified 10 distinct CNs that were conserved
217 across this CTCL cohort (**Fig. 3b, Sup. Fig. 3c**). Some CNs recapitulated structural components that are
218 clearly discernable in the corresponding H&E and fluorescent images, such as epithelium (CN-1, green
219 region) and vasculature (CN-3, brown region) (**Fig. 3c**). The other CNs were composed of previously
220 unappreciated sub-structures within the dermal infiltrate that were not apparent in the corresponding

221 H&E or fluorescent images, including immune-infiltrated stroma (CN-2, red region), vascularized stroma
222 (CN-4, gray region), tumor and dendritic cells (CN-5, purple region), lymphatic enriched stroma (CN-6,
223 orange region), tumor and mixed immune cells (CN-7, cyan region), tumor and CD4⁺ T cells (CN-8, yellow
224 region), innate immune cell enriched (CN-9, blue region), and Treg enriched (CN-10, pink region) (**Fig.**
225 **3c**).

226 Representative Voronoi diagrams from a responder (**Fig. 3d**, see CN-5 and CN-8, purple and
227 yellow regions, respectively) and non-responder (**Fig. 3e**, see CN-10, pink region) post-treatment show
228 significant differences in the frequency of CNs between patient groups. CNs enriched in tumor and
229 dendritic cells (CN-5, purple region) (**Fig. 3f**, mean frequency in R, post (21.4%) versus R, pre (3.2%)) and
230 tumor and CD4⁺ T cells (CN-8, yellow region) (**Fig. 3g**, mean frequency in R, post (19.0%) versus R, pre
231 (8.5%)) were present at significantly higher levels in responders post-treatment than in non-responders
232 or either group pre-treatment. In contrast, the Treg enriched CN (CN-10, pink region) was present at a
233 significantly higher frequency in non-responders than responders pre- and post-treatment (**Fig. 3h**,
234 mean frequency in NR, pre (15.5%) versus R, pre (3.3%) and NR, post (22.7%) versus R, post (4.9%)).
235 Notably, these differences in the spatial organization of certain cell-types were observed even though
236 there were no differences in the abundance of CD4⁺ T cells, dendritic cells, Tregs, or tumor cells between
237 patient groups, as previously described (**Fig. 2c-d**).

238 The increased co-localization of dendritic cells, CD4⁺ T cells and tumor cells in responders post-
239 treatment, relative to other patient groups, suggests that the more immune activated TME observed in
240 responders following pembrolizumab therapy (i.e., increased immune activation gene score in **Fig. 2g**)
241 may in part be mediated by CD4⁺ T cell activation by antigen-presenting cells. Indeed, after PD-1
242 blockade, responders had increased frequencies of activated ICOS⁺ CD4⁺ T cells (**Fig. 3i**, mean frequency
243 in R, post (1.9%) versus R, pre (0.7%) and NR, post (0.2%)) and proliferating Ki-67⁺ CD4⁺ T cells (**Fig. 3j**,
244 mean frequency in R, post (0.8%) versus R, pre (0.1%) and NR, post (0.1%)) compared to responders pre-

245 treatment and non-responders post-treatment, suggesting that pembrolizumab therapy activates CD4⁺ T
246 cells through upregulation of immune activating molecules.

247 The increased frequency of the Treg enriched CN in non-responders pre- and post-treatment is
248 consistent with our finding that non-responders have a persistently immunosuppressed TME (i.e.,
249 increased immunosuppression gene score in **Fig. 2h**), which is further supported by the higher frequency
250 of a potently suppressive subset of Tregs expressing ICOS in non-responders (**Fig. 3k**, mean frequency in
251 NR, pre (4.4%) versus R, pre (1.2%) and NR, post (6.2%) versus R, post (1.2%))^{43,44}. Interestingly, despite
252 treatment with PD-1 blockade, no differences were observed in PD-1⁺ subsets of CD4⁺, CD8⁺, Tregs, or
253 tumor cells between groups (**Sup. Fig. 2-i**). Collectively, these data link the spatial re-organization of
254 CD4⁺ T cells, tumor cells, and Tregs with immune activity of the TME, highlighting patterns of immune
255 control and pembrolizumab responsiveness.

256

257 **Spatial signature of PD-1⁺ CD4⁺ T cells, tumor cells, and Tregs predicts pembrolizumab response in** 258 **CTCL**

259 It is reasonable to suspect that specific cellular interactions drive pembrolizumab
260 responsiveness in CTCL. While complex spatial analyses have been used to prognosticate cancer
261 outcomes in select clinical cohorts¹⁹, we hypothesized that a simplified approach based on the distances
262 between specific immune and tumor cells could be employed. Guided by our CN findings, which
263 highlighted the spatial arrangement of CD4⁺ T cells, tumor cells and Tregs, we formalized a
264 computational scoring approach to standardizing a spatial signature. This score, termed *SpatialScore*,
265 calculates the physical distance ratio of each CD4⁺ T cell and its nearest tumor cell (“right” distance)
266 relative to its nearest Treg (“left” distance) (**Fig. 4a**). A lower *SpatialScore* (i.e., CD4⁺ T cells are closer to
267 tumor cells than Tregs) suggests increased T cell effector activity (**Fig. 4a.1**), whereas a higher
268 *SpatialScore* (i.e., CD4⁺ T cells are closer to Tregs than tumor cells) suggests increased T cell suppression

269 (Fig. 4a.2). As such, the *SpatialScore* can be viewed as a proxy of the balance between T cell effector
270 activity and suppression in the TME.

271 The *SpatialScore* is calculated on a per cell basis and the mean value is reported for each patient
272 group. When calculated with all CD4⁺ T cells, the pre-treatment *SpatialScore* was significantly lower in
273 responders than non-responders, with enhancement post-treatment for both groups (Fig. 4b, compare
274 mean *SpatialScore* for R, pre (0.57) versus NR, pre (0.63)). The same trend was observed on a per patient
275 basis (Sup. Fig. 4a). Since the current study trialed PD-1 blockade in CTCL, we next asked how the
276 *SpatialScore* was influenced by the PD-1⁺ CD4⁺ T cell subset. As observed when calculated with all CD4⁺ T
277 cells, when computed with PD-1⁺ CD4⁺ T cells, tumor cells, and Tregs, the *SpatialScore* was lower in
278 responders than non-responders (Fig. 4c, compare mean *SpatialScore* for R, pre (0.41) versus NR, pre
279 (0.62)). The same trend was seen on a per patient basis (Sup. Fig. 4b). Notably, the *SpatialScore* was
280 lower in responders pre-treatment when calculated with PD-1⁺ CD4⁺ T cells versus all CD4⁺ T cells,
281 implying increased effector activity in this T cell subset. These results suggest that PD-1⁺ CD4⁺ T cells in
282 responders are primed for increased anti-tumor activity, which is enhanced in the immune activated
283 TME that results following pembrolizumab therapy.

284 Since the *SpatialScore* approach appears to predict the outcome of PD-1 blockade in CTCL, it
285 stands to reason that there is a deep phenotype of cell-type specific architecture that is driving the
286 *SpatialScore*. Pre-treatment differences were driven by the closer proximity of PD-1⁺ CD4⁺ T cells and
287 Tregs in non-responders (Sup. Fig. 4c, see red arrow), consistent with the increased immunosuppression
288 gene scores in non-responders relative to responders (Fig. 2h). In contrast, post-treatment differences
289 were driven by the closer proximity of PD-1⁺ CD4⁺ T cells and tumor cells in responders (Sup. Fig. 4d, see
290 red arrow), consistent with the increased immune activation gene score in responders relative to non-
291 responders (Fig. 2i). Importantly, no correlation was identified between the abundance of PD-1⁺ CD4⁺ T
292 cells, tumor cells, or Tregs and the *SpatialScore* on a per tissue microarray spot basis (Sup. Fig. 4g-i),
293 implying that the *SpatialScore* is not merely driven by cell-type frequency. Additionally, the mean

294 *SpatialScore* was significantly different from that of a random sample in responders pre- and post-
295 treatment (**Sup. Fig. 4j and l**), but not in non-responders pre- and post-treatment (**Sup. Fig. 4k and m**).
296 This suggests that an active process coordinates the spatial interactions of PD-1⁺ CD4⁺ T cells, tumor cells
297 and Tregs in responders, whereas no such mechanism exists in non-responders. Finally, when the
298 *SpatialScore* was calculated for CD8⁺ T cells (**Sup. Fig. 4e**) or PD-1⁺ CD8⁺ T cells (**Sup. Fig. 4f**), it was not
299 predictive of pembrolizumab response. The increased proximity of CD4⁺ T cells and tumor cells observed
300 in pembrolizumab responders—and lack thereof for CD8⁺ T cells—suggests that CD4⁺ T cells may have a
301 more important effector function in CTCL than previously appreciated.

302

303 **PD-1⁺ CD4⁺ T cells upregulate cytotoxic effector molecules in pembrolizumab responders**

304 We reasoned that the spatial proximity of PD-1⁺ CD4⁺ T cells and tumor cells reflected the
305 enhanced CD4⁺ T cell tumor reactivity seen in responders following pembrolizumab therapy. CODEX and
306 RNA-seq datasets were therefore used to assess the cytotoxic potential of effector PD-1⁺ CD4⁺ T cells. In
307 CTCL, cytotoxic CD4⁺ T cells act through a granzyme-perforin-dependent pathway to achieve tumor cell
308 killing^{45,46}, similar to cytotoxic CD8⁺ T cells⁴⁶⁻⁴⁸. The expression of granzyme B (GZMB) on PD-1⁺ CD4⁺ T
309 cells was increased in responders post- versus pre-treatment (**Fig. 4d**, mean GZMB expression, R, post
310 (26.9) versus NR, post (12.2) and R, pre (12.1)). This increased GZMB expression is confirmed visually in a
311 responder post-treatment with DRAQ5 (nuclear stain), CD4, PD-1, and GZMB fluorescent staining (**Fig.**
312 **4e**, see white arrow) and is consistent with the increased cytotoxicity gene score^{49,50} seen in responders
313 post-treatment compared to pre-treatment (**Fig. 4f**, median R, post (1.21) versus R, pre (-1.77), **Sup.**
314 **Table 3**). No differences were observed between non-responders pre- and post-treatment with regards
315 to GZMB expression on PD-1⁺ CD4⁺ T cells (**Fig. 4d**, mean expression, NR, pre (11.7) versus NR, post
316 (12.2)) or the cytotoxicity gene score (**Fig. 4e**, median NR, pre (0.21) versus NR, post (0.43)).

317 Increased cytotoxicity should coincide with decreased tumor cell aggressiveness and, using
318 genes associated with poor response to therapy⁵¹ and progressive⁵² CTCL (**Sup. Table 3**), it was observed

319 that tumor aggressiveness decreased in 100% of responders following pembrolizumab therapy (**Fig. 4g**)
320 but increased in 71% of non-responders (**Fig. 4h**). This increased tumor aggressiveness in non-
321 responders was further supported by an increased frequency of proliferating (Ki-67⁺) tumor cells in non-
322 responders post- versus pre-treatment and compared to responders post-treatment (**Fig. 4i**, mean
323 frequency, NR, post (17.0%) versus NR, pre (8.9%) and R, post (7.8%))^{26,53}. Collectively, these data show
324 the cytotoxic potential of effector PD-1⁺ CD4⁺ T cells and their association with a favorable response to
325 pembrolizumab therapy in CTCL.

326

327 **Validation of the *SpatialScore* biomarker with a clinically accessible multiplexed IHC platform**

328 While mIHC approaches, such as CODEX, provide the raw data for deep cellular profiling,
329 translating these findings to a clinical arena requires simplifying the predictive signature to a diagnostic
330 platform that can be readily implemented in clinical practice. Vectra, in conjugation with the Phenoptics
331 workflow, is a commercially available, widely adopted clinical mIHC imaging platform, which has been
332 used to identify biomarkers for renal cell carcinoma⁵⁴ and B cell lymphoma⁵⁵. We devised a simplified
333 staining panel (DAPI, CD3, CD4, CD7, CD8, CD25, FoxP3, and PD-1) that captured the PD-1⁺ CD4⁺ T cells,
334 tumor cells, and Tregs used to calculate the *SpatialScore*. Notably, the tumor cell phenotype identified
335 by CODEX (i.e., decreased expression of CD7 and increased expression of CD25 and Ki-67; **Fig. 1h**), was
336 critical for establishing this streamlined staining panel and could be readily transferred to the Vectra
337 platform.

338 Serial sections from the same CTCL TMA used for CODEX and RNA-seq were stained with this
339 simplified panel and imaged with Vectra (**Sup. Fig. 5a**). Across all TMA spots, 126,653 cells were
340 identified, including 2,957 PD-1⁺ CD4⁺ T cells, 6,161 Tregs, and 19,847 tumor cells. The *SpatialScore* was
341 then computed on a per cell basis and the mean was reported for each patient group. Consistent with
342 the CODEX results (**Fig. 4c**), the Vectra-calculated *SpatialScore* was significantly lower in responders than
343 non-responders pre-treatment (**Fig. 4j**, mean *SpatialScore* R, pre (0.35) versus NR, pre (0.76)), with

344 enhancement post-treatment compared to pre-treatment. The Vectra mIHC images (**Fig. 4k**, left) and
345 corresponding spatial maps (**Fig. 4k**, right) provide visual validation at the single-cell level that PD-1⁺
346 CD4⁺ T cells were closer to tumor cells in responders but closer to Tregs in non-responders.
347 Furthermore, on a per patient basis, the pre-treatment *SpatialScore* was nearly 5-times lower in
348 responders than non-responders (**Fig. 4l**, mean *SpatialScore* R, pre (0.31) versus NR, pre (1.52)), with
349 excellent biomarker performance measures at a *SpatialScore* cutoff point of 0.7908 (**Sup. Fig. 5b-e**).
350 Notably, when calculated per patient, the *SpatialScore* extended over a wider range than when
351 calculated for all cells per group; this is due to sample size differences (i.e., n=7 per patient versus
352 n=1000s of cells per group). It is noteworthy that the skin-derived *SpatialScore* is so robust and
353 predictive of pembrolizumab response, since clinical outcome in advanced CTCL is often driven by the
354 burden of disease in the non-skin compartments (i.e., blood or viscera). Collectively, these results show
355 that findings identified by highly multiplexed imaging platforms like CODEX can be translated to more
356 simplified platforms like Vectra, further demonstrating the clinical utility of the *SpatialScore* as a
357 biomarker predictive of PD-1 blockade outcome in CTCL.

358

359 **Tumor cell-specific CXCL13 expression coincides with a favorable response to PD-1 blockade in CTCL**

360 Potential recruitment mechanisms driving the *SpatialScore* were investigated by identifying
361 genes predictive of the spatial interactions between PD-1⁺ CD4⁺ T cells, tumor cells, and Tregs. An L1-
362 regularized linear model was fit to bulk RNA-seq data on a per spot basis, which revealed seven genes
363 predictive of the *SpatialScore* including three chemokines: *CXCL9*, *CCL22* and *CXCL13* (**Fig. 5a**). *CXCL9* and
364 *CCL22* are known to mediate Treg recruitment^{56,57}; they had positive coefficients and were predictive of
365 the higher *SpatialScore* seen in non-responders. *CXCL13* is a chemoattractant expressed on benign
366 lymphocytes and CTCL tumor cells^{28,58}; it had a negative coefficient and was predictive of the lower
367 *SpatialScore* seen in responders. Bulk *CXCL13* gene expression was significantly increased in responders
368 post- versus pre-treatment and compared to non-responders post-treatment (**Fig. 5b**, median

369 normalized *CXCL13* expression, R, post (615.5) versus NR, post (94.7) and R, pre (16.0)). This
370 upregulation of *CXCL13* in responders post-treatment relative to other patient groups was confirmed by
371 *CXCL13* IHC shown quantitatively (**Fig. 5c**, mean *CXCL13* IHC expression, R, post (34.5) versus NR, post
372 (7.8) and R, pre (7.6)) and visually (**Fig. 5d**, see R, post for highest expression of *CXCL13*).

373 We next analyzed a publicly available scRNA-seq dataset of four CTCL skin tumors (Gaydosik et
374 al.)⁵⁹ to identify the main cell-type overexpressing *CXCL13*. Using this dataset, we annotated 10 clusters
375 including tumor cells, CD4⁺ T cells (CD4), Tregs, CD8⁺ T cells (CD8), B & plasma cells (B&PC), macrophages
376 (mac), dendritic cells (DC), stroma, vasculature (vasc), and keratinocytes (KC) (**Sup. Fig. 6a-b**). Examining
377 all cells with a *CXCL13* log_{1p} normalized read count > 0.5 showed that tumor cells had the highest mean
378 expression compared to other cell-types (**Fig. 5e**, median *CXCL13* expression, tumor (3.3) versus less
379 than 2.0 for the other 9 cell-types). Moreover, the frequency of *CXCL13* expressing cells was highest
380 among tumor cells compared to other cell-types (**Fig. 5f**, mean frequency of *CXCL13* positive cells, tumor
381 (0.27) versus less than 0.1 for the other 9 cell-types).

382 CIBERSORTx (CSx) is a computational framework that uses gene expression signatures to enable
383 cell type-specific gene expression to be inferred from bulk RNA-seq data without physical cell isolation²².
384 We used CSx to computationally resolve tumor, stromal, and immune cell subsets in bulk RNA-seq data
385 (**Fig. 5g** and **Sup. Table 4**). Using the 10 annotated clusters shown in **Fig. e-f**, a signature matrix,
386 consisting of cell-type specific marker genes, was generated (**Sup. Fig 6c** and see **Methods**, marker genes
387 are shown as rows). This signature matrix was used to enumerate CSx-resolved cell-type fractions and
388 resolve gene expression profiles from CTCL bulk transcriptomes. Strong correlations were observed
389 between most of the CSx-resolved cell-type frequencies and CODEX-identified cell-type frequencies (**Fig.**
390 **5h**, see correlation coefficients along the diagonal of the heatmap).

391 The CSx-resolved gene expression profiles of tumor cells were then thoroughly characterized
392 and compared across patient groups. Interestingly, *RAB11B*, a RAS superfamily member of small GTP-
393 binding proteins, was the only tumor cell gene that was significantly differentially expressed between

394 responders and non-responders pre-treatment (**Sup. Fig. 6d**). This suggests an absence of tumor cell-
395 intrinsic differences between responders and non-responders at baseline. In contrast, numerous genes,
396 including *CXCL13*, were upregulated in tumor cells of responders post-treatment compared to pre-
397 treatment (**Fig. 5i**, see *CXCL13* in bold). This finding is consistent with an increased susceptibility of
398 responder tumor cells to PD-1 blockade therapy, which is supported by the decreased tumor
399 aggressiveness score observed in responders post-treatment relative to pre-treatment (**Fig. 4g**).
400 Interestingly, only three genes (*BRD3*, *TWF2* and *ZNF365*) were differentially expressed in non-
401 responder tumor cells post-treatment compared to pre-treatment (**Fig. 5j**). This suggests that non-
402 responder tumor cells are resistant to PD-1 blockade, which is in line with the increased tumor
403 aggressiveness score observed in non-responders post-treatment compared to pre-treatment (**Fig. 4h**).
404 Finally, co-staining the tissue section used for the Vectra mIHC experiment with a standard IHC anti-
405 *CXCL13* antibody, followed by co-localization of tumor cells with *CXCL13*-positive cells, provided visual
406 confirmation that tumor cells are the primary expressors of *CXCL13* (**Fig. 5k, Sup. Fig. 6e**).

407 Next, we assessed *CXCL13* expression in tumor cells and its role in recruiting reactive
408 lymphocytes. CSx-resolved tumor cell-specific *CXCL13* expression was significantly increased in
409 responders post-treatment compared with non-responders post-treatment and responders pre-
410 treatment on a per tissue microarray spot basis (**Fig. 5l**, median normalized tumor cell-specific *CXCL13*
411 expression, R, post (12.64) versus NR, post (11.14) and R, pre (10.54)). On a per patient basis, following
412 PD-1 blockade, tumor cell-resolved *CXCL13* expression increased in 100% of responders (**Fig. 5m**) versus
413 29% of non-responders (**Fig. 5n**). *CXCL13* exclusively binds to the chemokine receptor *CXCR5*, which is
414 expressed on B cells, CD4⁺ T cells, CD8⁺ T cells, and skin-derived migratory dendritic cells⁶⁰. Although not
415 significant, *CXCR5* expression was increased in bulk mRNA of responders post-treatment relative to pre-
416 treatment (**Sup. Fig. 6f**). The data lacked significant power to unmix *CXCR5* on CD4⁺ T cells by CSx;
417 however, tumor cell-specific *CXCL13* expression was positively correlated (Spearman correlation

418 coefficient = 0.68) with bulk mRNA *CXCR5* expression (**Fig. 5o**), suggesting a chemoattractant
419 recruitment mechanism.

420 Collectively, these results indicate that PD-1 blockade distinctly alters the CTCL TME of
421 therapeutic responders and non-responders. In responders, pembrolizumab therapy promotes immune
422 activation and upregulates CXCL13 in tumor cells (**Fig. 6**, top panel). CXCL13 overexpression then attracts
423 effector/cytotoxic PD-1⁺ CD4⁺ T cells to tumor cells, promoting CD4⁺ T cell-mediated tumor cell inhibition
424 and killing. As such, the overexpression of CXCL13 in tumor cells provides a mechanism for the sustained
425 clinical activity seen in responders. In contrast, non-responders have a persistently immunosuppressed
426 TME, which mediates the increased interaction between inhibitory Tregs and PD-1⁺ CD4⁺ T cells and
427 maintains this CD4⁺ T cell subset in an exhausted state (**Fig. 6**, bottom panel). In the setting of
428 pembrolizumab resistance, non-responder tumor cells remain active and proliferative following
429 treatment. Thus, underlying differences in the functional immune state of the TME—coupled with
430 alterations in CD4⁺ T cell effector activity versus suppression, and tumor cell-specific CXCL13
431 expression—are associated with distinct spatial cellular patterns that predict the efficacy of PD-1
432 blockade in CTCL.

433

434 **DISCUSSION**

435 For anti-PD-1 immunotherapies to provide maximal benefit to cancer patients, the drivers and
436 resistors of clinical response must be identified. Traditional biomarker studies with IHC, gene expression
437 profiling, and tumor mutational burden assays do not fully account for spatial cellular context and have
438 imperfect correlation with immunotherapy outcomes¹⁶. This was true for our CTCL cohort: No pre-
439 treatment differences were observed in immune composition, expression of PD-1/PD-L1 proteins, or
440 IFN- γ /TGF- β gene signatures between responders and non-responders. We therefore explored
441 alternative approaches, including spatially resolved multiplexed tissue imaging, which has been shown
442 to significantly improve the accuracy of predicting response to PD-1 blockade in several tumor types⁶¹.

443 Multiparameter histologic analysis revealed global prognostic spatial patterns that were
444 predictive of clinical response, including a localized enrichment of tumor and CD4⁺ T cells (CN-8) in
445 responders and of Tregs (CN-10) in non-responders. Profiling the spatial relationships between effector
446 PD-1⁺ CD4⁺ T cells, tumor cells, and immunosuppressive Tregs allowed us to derive the *SpatialScore*—a
447 clinically useful biomarker that correlated strongly with pembrolizumab response in CTCL. Previously
448 identified spatial biomarkers rely on pairwise distances (e.g., PD-1⁺ T cells and PD-L1⁺ tumor cells)^{17,62,63},
449 whereas the *SpatialScore* accounts for the interactions between three functionally distinct cell-types. As
450 such, the *SpatialScore* represents a novel predictive approach and provides insight into three key
451 therapeutic determinants underlying PD-1 blockade in CTCL: 1) functional immune state of the TME, 2) T
452 cell function, and 3) chemoattraction.

453 First, the *SpatialScore* captures differences in the functional immune phenotypes between
454 responders and non-responders. Following pembrolizumab therapy, the TME of responders becomes
455 activated, as evidenced by the increased immune activation gene score, increased frequency of ICOS⁺
456 and Ki-67⁺ CD4⁺ T cells, and a local enrichment of tumor cells with dendritic cells (CN-5) and CD4⁺ T cells
457 (CN-8). These findings suggest that in responders PD-1 blockade specifically activates CD4⁺ T cells
458 through the expression of immunostimulatory molecules and co-stimulation by antigen-presenting cells,
459 resulting in CD4⁺ T cell proliferation^{64,65}. In contrast, non-responders have a persistently
460 immunosuppressed TME. This is supported by the increased frequency of a Treg enriched neighborhood
461 (CN-10) and the expansion of a highly suppressive ICOS⁺ Treg subset, which has been associated with
462 poor clinical outcomes in melanoma⁴³ and liver cancer⁴⁴. These results suggest that the increased
463 suppressive function and spatial organization of Tregs in non-responders account for the strong
464 immunosuppression and lack of pembrolizumab response. We speculate that the absence of this pre-
465 treatment immunosuppression in responders enables priming and activation of CD4⁺ T cells following
466 immunotherapy.

467 Second, the *SpatialScore* reflects differences in T cell function between responders and non-
468 responders: After PD-1 blockade, T cell effector activity is restored in responders, whereas non-
469 responders have a continually exhausted T cell phenotype. Consistent with studies of Hodgkin
470 lymphoma⁶³, bladder cancer⁵⁰, and glioblastoma⁶⁶, our data suggest that CD4⁺ T cells, and particularly
471 the PD-1⁺ CD4⁺ T cell subset, are crucial effectors that influence pembrolizumab efficacy in CTCL. In
472 responders post-treatment, PD-1⁺ CD4⁺ T cells increase GZMB expression and move closer to tumor cells,
473 similar to the granzyme-perforin-dependent tumor killing mechanism used by cytotoxic CD8⁺ T cells⁴⁵⁻⁴⁸.
474 Previous studies have shown that antitumor activity is enhanced by a closer proximity of cytotoxic T cells
475 to tumor cells^{67,68} and by removing inhibitory Tregs from the TME milieu⁵⁰. The *SpatialScore* combines
476 these principles by measuring the physical distances between PD-1⁺ CD4⁺ T cells and tumor cells (i.e.,
477 effector function) as well as PD-1⁺ CD4⁺ T cells and Tregs (i.e., suppressive function). The *SpatialScore*
478 therefore reflects the balance of T cell effector activity versus suppression in the TME and the efficacy of
479 PD-1 blockade in CTCL.

480 Third, pembrolizumab therapy leads to increased RNA and protein expression of CXCL13 in
481 responder tumor cells (or kills cells with lower levels of expression), which may be advantageous in
482 localizing effector PD-1⁺ CD4⁺ T cells within the TME. Overexpression of *CXCL13* is associated with
483 improved clinical outcomes in breast⁶⁹, colon⁷⁰, ovarian⁷¹, lung⁷², and urothelial cancers⁷³ as well as
484 responsiveness to anti-PD-1 immunotherapy^{72,74}. Additionally, upregulation of *CXCL13* can be
485 accompanied by constitutive protein secretion^{69,72}, which strongly attracts CXCR5⁺ CD4⁺ T cells⁷² and PD-
486 1⁺ CD4⁺ T cells⁷⁵ to the tumor site. A recent study also showed that the tumor-specific gene expression
487 program of cytotoxic GZMB⁺ CD4⁺ T cells in bladder cancer treated with anti-PD-L1 therapy was marked
488 by tumor overexpression of *CXCL13*⁵⁰. Furthermore, *CXCL13* null mice with bladder tumors did not
489 respond to anti-PD-1 treatment and had a lower frequency of T cell infiltration compared their wild-type
490 counterparts⁷³. Collectively, these findings support a chemoattractant mechanism for the sustained
491 clinical response to pembrolizumab therapy observed in responders: Upregulation of *CXCL13* in tumor

492 cells attracts effector PD-1⁺ CD4⁺ T cells to them, promoting successful antitumor activity. This aspect of
493 pembrolizumab responsiveness is captured by the lower *SpatialScore* seen in responders and
494 underscores the importance of T cell topography as a spatial biomarker.

495 There are important limitations to this study. Discovery of the *SpatialScore* was based on 14
496 patients. Larger studies are needed to determine if this spatial biomarker translates broadly for
497 prediction of pembrolizumab response in CTCL and to establish a threshold value for the *SpatialScore*
498 that can be used to stratify patients into probabilistic responders and non-responders. Additionally,
499 larger studies might delineate more subtle features of the spatial architecture not detected in this study.
500 Another limitation of this study is that the post-treatment tumor biopsies were collected at various time
501 points, impairing our ability to directly assess modulation of T cell subsets at the precise time of
502 therapeutic success or failure. Finally, our cohort is limited to advanced, heavily pre-treated CTCL
503 patients. Further efforts should examine the therapeutic activity of PD-1 blockade in early stage and
504 treatment naïve patients, since early tumors are generally more responsive to immunotherapy⁷⁶⁻⁸¹ and
505 prior systemic treatment may alter the immune response to therapy. Despite these limitations, the
506 robustness of our findings highlights that spatial organization and immune functionality are conserved in
507 responders and non-responders, which reinforces the importance of these factors for determining
508 pembrolizumab response in CTCL.

509 Future studies with scRNA-seq and paired T cell receptor sequencing are needed to better
510 characterize the heterogeneity of PD-1⁺ CD4⁺ T cells across patient groups and to fully understand the
511 cytotoxic potential of this crucial effector subset. Similarly, investigating the increased suppressive
512 function of Tregs and the ICOS⁺ Treg subset in non-responders may reveal vulnerabilities that could be
513 targeted in combination with PD-1 blockade. Given our findings that non-responders have an increased
514 localization of Tregs and upregulation of *BRD3*, an epigenetic regulator, in their tumor cells pre-
515 treatment, future studies should investigate the therapeutic potential of combining mogamulizumab
516 (which depletes Tregs⁸²) and/or bromodomain inhibitors (which inhibit BRD3 and decrease tumor cell

517 proliferation⁸³) with pembrolizumab therapy for CTCL. Further studies are also required to determine if
518 CXCL13 expression levels—in both skin and blood—can serve as a surrogate of pembrolizumab activity
519 and therefore guide ongoing treatment decisions. Such studies are especially important for advanced
520 CTCL patients, where the burden of disease in the non-skin compartments often dictate clinical
521 outcome.

522 This work provides an important conceptual foundation for improving the efficacy of anti-PD-1
523 immunotherapy in CTCL. We identify effector PD-1⁺ CD4⁺ T cells in responders and inhibitory ICOS⁺ Tregs
524 in non-responders. The *SpatialScore* captures the interactions of these cell-types with tumor cells and
525 reveals how PD-1 blockade manipulates the balance of effector and suppressive T cell function to
526 achieve disease control. We anticipate that the cell-types comprising the *SpatialScore* will depend on the
527 specific cancer and immunotherapy. The *SpatialScore* biomarker approach and the ability to measure it
528 using a clinically accessible mIHC platform should enable its widespread use in cancer immunotherapy
529 studies.

530
531
532

533 **METHODS**

534 **Human subjects and clinical trial study design.** The CITN-10 trial was a multicenter, phase II, single-arm
535 clinical trial that investigated the efficacy of pembrolizumab in 24 patients with two common forms of
536 relapsed/refractory CTCL, mycosis fungoides and Sézary syndrome¹³. Written informed consent was
537 obtained from all enrolled patients. The use of their tissues for research was fully de-personalized and
538 approved by the Stanford University IRB Administrative Panels on Human Subjects in Medical Research
539 (HSR 46894). All patients had a clinicopathologically confirmed diagnosis of mycosis fungoides or Sézary
540 syndrome (clinical stage IB to IV) that had relapsed, was refractory to, or had progressed after at least
541 one standard systemic therapy. Exclusion criteria included central nervous system disease, active
542 autoimmune disease, previous exposure to any anti-PD-1, anti-PD-L1, or anti-PD-L2 therapy, or

543 treatment with radiotherapy or other anti-cancer agents within 15 weeks of the pre-treatment biopsy.
544 Topical medications were not applied to the biopsied areas during treatment or within 8 weeks of the
545 pre-treatment biopsy. Patients were treated with 2 mg/kg pembrolizumab by intravenous infusion every
546 3 weeks for up to 24 months¹³. Response and primary end point (overall response rate) were assessed
547 by consensus global response criteria²³.

548

549 **Sample collection and tissue microarray construction.** Skin biopsy specimens were collected from the
550 primary tumor site and FFPE histology blocks were generated according to standard pathology
551 procedures. Pre-treatment biopsies were obtained prior to the first pembrolizumab infusion and post-
552 treatment biopsies were collected during and at the conclusion of therapy (**Sup. Fig. 1, Sup. Table 1**).
553 H&E-stained sections from all biopsies were reviewed by two board-certified pathologists (C.M.S. and
554 R.H.P.). Fourteen of the 24 biopsy samples had adequate FFPE material to be assembled into a tissue
555 microarray, and two to three cores of 0.6 mm diameter from the most infiltrated regions of each biopsy
556 were digitally annotated and compiled into a tissue microarray. The tissue microarray was sectioned at
557 4- μ m thickness and mounted onto VectabondTM-treated (Vector Labs, #SP-1800) square glass coverslips
558 (22x22 mm, #1 1/2, Electron Microscopy Sciences, #72204-01).

559

560 **Immunohistochemistry.** IHC for CD3 (clone CD3-12; Abd Serotec), CD4 (clone 4B12; Leica), CD8 (clone
561 CD8/144B; Dako), FoxP3 (clone 236A/E7; Abcam), CD163 (clone 10D6; Thermo Fisher Scientific), PD-1
562 (clone NAT105; Cell Marque), PD-L1 (clone 22C3; Merck Research Laboratories), and PD-L2 (clone 3G2;
563 Merck Research Laboratories) was performed as previously described⁸⁴. Images were scored by CITN
564 pathologists, and the positive percentage of the total mononuclear cell infiltrate was reported¹³.

565

566 **Multiplex immunohistochemistry and analysis.** mIHC was performed as previously described⁸⁵. Briefly,
567 4- μ m FFPE tissue sections were baked for 1 h at 60 °C, dewaxed, and stained on a BOND Rx autostainer

568 (Leica) according to Opal Multiplex IHC assay (Akoya Biosciences)s protocol with the following changes:
569 additional high stringency washes were performed after the secondary antibody and Opal fluor
570 applications using high-salt TBST (0.05M Tris, 0.3M NaCl and 0.1% Tween-20, pH 7.2-7.6), and TCT was
571 used as the blocking buffer (0.05M Tris, 0.15M NaCl, 0.25% Casein, 0.1% Tween 20, pH 7.6 +/- 0.1). The
572 antibody panel was stained in the following order, with antibody stripping between positions. Each
573 primary antibody was incubated for 60 min, followed by 10-min incubation with secondary antibody
574 (OPAL polymer HRP mouse plus rabbit, Akoya Biosciences), followed by application of the tertiary TSA-
575 amplification reagent (OPAL Fluor, Akoya Biosciences) for 10 min. Positions were as follows: position 1:
576 CD8 (clone CD8/144B, DAKO #M7103; concentration 0.8 µg/ml; OPAL Fluor 520); position 2: CD25 (clone
577 4C9, Cell Marque #125M-14; concentration 0.17 µg/ml; OPAL Fluor 540); position 3: CD3 (clone SP7,
578 Thermo Fisher Scientific #RM-9107; concentration 0.06 µg/ml; OPAL Fluor 570); position 4: PD-1 (clone
579 EPR4877(2), Abcam #ab137132, concentration 1.0 µg/ml; OPAL Fluor 650); position 5: CD7 (clone MRQ-
580 56; Cell Marque #107M-24; concentration 1.18 µg/ml; OPAL Fluor 690); position 6: CD4 (clone EP204,
581 Epitomics; #AC0173A; concentration 0.08 µg/ml; OPAL Fluor 480); and position 7: FoxP3 (clone 236A/E7;
582 eBioscience #14-4777-82; 5.0 µg/ml; OPAL Fluor 620). Subsequently, slides were stained with Spectral
583 DAPI (Akoya Biosciences) for 5 min, rinsed, and mounted with Prolong Gold Antifade reagent (Thermo
584 Fisher Scientific #P36930). After curing for 24 h at room temperature in the dark, images were acquired
585 on a Vectra Polaris automated quantitative pathology imaging system (Akoya Biosciences). The raw
586 images were spectrally unmixed using the Phenoptics inForm software (Akoya Biosciences) and
587 exported as multi-image TIFF files.

588 After fluorescent imaging, the slides were de-coverslipped, loaded onto the BOND Rx
589 autostainer, stripped of bound antibody, and a post-mIHC staining for CXCL13 (goat polyclonal, R&D
590 Systems #AF801; concentration 0.5 µg/ml; incubation 60 min) was performed. Bound antibody was
591 revealed by anti-goat HRP secondary ImmPress HRP (Vector Labs #MP-7405; incubation 12 min),
592 followed by DAB chromogen using the BOND Polymer Refine Detection kit (Leica) according to the

593 manufacturer's instructions. After counterstaining with hematoxylin, slides were dry-mounted and
594 scanned on an Aperio AT turbo digital slide scanning system (Leica).

595 HALO software (Indica Labs) was used to perform single-cell analysis of mIHC images. Cells were
596 visualized based on nuclear and cytoplasmic stains, and mean pixel fluorescence intensity in the
597 applicable compartments of each cell were measured (i.e., CD4 in the cytoplasmic compartment and
598 FoxP3 in the nuclear compartment). A mean intensity threshold above background was used to
599 determine positivity for each fluorochrome, thereby defining cells as either positive or negative for each
600 marker. The data was then used to define co-localized populations, including PD-1⁺ CD4⁺ T cells, tumor
601 cells, and Tregs. Spatial positions were extracted for each cell, and the spatial distances and ratios
602 between these three cells types were calculated as detailed below. Performance of the *SpatialScore*
603 biomarker was evaluated with the easyROC interface available with the R package shiny⁸⁶. CXCL13 IHC
604 images were scored using a classifier method for the DAB stain based on optical density to obtain the
605 positive percentage of the total mononuclear cell infiltrate per spot.

606

607 **CODEX antibodies.** For CODEX, purified, carrier-free monoclonal and polyclonal anti-human antibodies
608 were purchased from commercial vendors (**Sup. Table 1**). Conjugations to maleimide-modified short
609 DNA oligonucleotides (TriLink) were performed at a 2:1 weight/weight ratio of oligonucleotide to
610 antibody, with at least 100 µg of antibody per reaction, as previously described¹⁹. Conjugated antibodies
611 were validated and titrated under the supervision of a board-certified pathologist (C.M.S.).

612

613 **CODEX multiplex tissue staining and imaging.** The CODEX experiment was performed as previously
614 described¹⁹. Briefly, the coverslip was deparaffinized and rehydrated, and heat-induced epitope retrieval
615 was performed using Dako target retrieval solution, pH 9 (Agilent, #S236784-2) at 97 °C for 10 min. The
616 coverslip was stained with an antibody cocktail (**Sup. Table 2**) to a volume of 100 µl overnight at 4 °C in a
617 sealed humidity chamber on a shaker. After multiple fixation steps using 1.6% paraformaldehyde, 100%

618 methanol, and BS3 (Thermo Fisher Scientific, #21580), the coverslip was mounted onto a custom-made
619 acrylic plate (Bayview Plastic Solutions). Imaging was performed with a Keyence BZ-X710 inverted
620 fluorescence microscope equipped with a CFI Plan Apo λ 20x/0.75 objective (Nikon), an Akoya CODEX
621 microfluidics instrument, and CODEX driver software (Akoya Biosciences). Light exposure times and the
622 arrangement of cycles are outlined in **Sup. Table 2**. At the conclusion of the CODEX multicycle reaction,
623 H&E staining was performed, and images were acquired in brightfield mode.

624

625 **Data processing of CODEX images.** Raw TIFF image files were processed using the CODEX Toolkit as
626 previously described. After processing, the staining quality for each antibody was visually assessed in
627 each tissue microarray spot, and cell segmentation was performed using the DRAQ5 nuclear stain.
628 Marker expression was quantified, and single-cell data were saved as FCS files, which were then
629 imported into CellEngine (<https://cellengine.com>) for cleanup gating. This resulted in a total of 117,170
630 cells across all tissue microarray spots.

631 After cleanup gating, FCS files were exported from CellEngine, imported into Vortex clustering
632 software⁸⁷, and subjected to unsupervised X-shift clustering using an angular distance algorithm.
633 Clustering was based on all antibody markers except CD11b, CD16, CD164, CCR4, CCR6, EGFR, and p53.
634 The optimal cluster number was guided by the elbow point validation tool in Vortex, resulting in 78
635 clusters. Clusters were manually verified and assigned to cell-types based on morphology in H&E and
636 fluorescent CODEX images and on their marker expression profiles. Clusters with similar features were
637 merged, resulting in 21 cell-type clusters. The expression frequencies of ICOS, IDO, Ki-67, and PD-1 were
638 determined for the T cell and tumor cell clusters by manual gating in CellEngine for each tissue
639 microarray spot, with visual comparison to the raw fluorescent image.

640

641 **Cellular neighborhood identification.** CN identification was performed using a custom *k*-nearest
642 neighbors' algorithm in Python¹⁹. For each of the 117,220 cells in this experiment, the window size was

643 set at 10, capturing the center cell and its nine nearest neighboring cells, as measured by the Euclidean
644 distance between X/Y coordinates. To identify 10 CNs, these windows were then clustered by the
645 composition of their microenvironment with respect to the 21 cell-types that were previously identified.
646 This resulted in a vector for each window containing the frequency of each of the 21 cell-types amongst
647 the 10 neighborhoods. These windows were then clustered using Python's *scikit-learn* implementation
648 of MiniBatchKMeans with $k=10$. Each cell was then allocated to the same CN as the window in which it
649 was centered. All CN assignments were validated by overlaying them on the original fluorescent and
650 H&E-stained images.

651

652 **Calculation of spatial distances and ratios between cell-types.** The X/Y coordinates for each cell type
653 were determined during cellular segmentation, as described above. The minimal distance between each
654 cell-type and its nearest other cell-types, and the averages of these minimal distances per tissue spot,
655 were calculated in R. Given our interest in the relationship of cell distances between three cell-types
656 (i.e., effector T cells (CT1), tumor cells (CT2) and Tregs (CT3)), we calculated the ratio of the minimal
657 distances between CT1—CT2 (right distance) versus CT1—CT3 (left distance). This distance ratio
658 represented the *SpatialScore*. To assess whether these ratios were significantly different from those of a
659 random sample, we performed the following analysis per spot: For the number of CT1 cells in each spot,
660 we randomly selected the same number of non-CT1 (nCT1) cells. For each of these nCT1 cells, we
661 calculated the ratio of the minimal distances (nCT1—CT2 / nCT1—CT3) and determined the mean of this
662 sample. We repeated this random sampling 100 times, and the average of all the means was reported.
663 Distribution of the random values was assessed by the quant output variable, which indicates how many
664 of the random means are smaller than the measured means. For instance, a quant of 97 indicates that
665 97% of the random means are smaller than the measured means. Thus, quant values closer to 100 or 0
666 indicate that the measured means are not random (**Sup. Fig. 4j-m**).

667

668 **Laser-capture microdissection (LCM).** Two serial sections of the tissue microarray were taken at 7 μm
669 thickness and mounted onto frame slides with polyethylene naphthalate membranes (Thermo Fisher
670 Scientific, #LCM0521). Slides were immersed for 20 s each in xylene (three times), 100% ethanol (three
671 times), 95% ethanol (two times), 70% ethanol (two times), water, hematoxylin (Dako, #S3309), water,
672 bluing reagent (Thermo Fisher Scientific, #7301), water, 70% ethanol (two times), 95% ethanol (two
673 times), 100% ethanol (three times), and xylene (three times). Immediately after staining, cells were
674 dissected from every tissue microarray spot on an ArcturusXT LCM System (Thermo Fisher Scientific)
675 using the ultraviolet laser to cut out the desired region and the infrared laser to adhere the membrane
676 to a CapSure HS LCM Cap (Thermo Fisher Scientific, #LCM0215). A tissue area containing roughly 1000
677 mononuclear cells was captured from each spot, with cell numbers determined based on density
678 estimates by cell counting in an adjacent H&E-stained section. If a core had more than 1000
679 mononuclear cells, a tissue fragment containing around 1000 mononuclear cells was dissected from that
680 core. If a core had less than 1000 cells, tissue fragments from corresponding cores on the serial section
681 membrane were combined in the same LCM cap to obtain approximately 1000 cells. After
682 microdissection, the caps were sealed using 0.5-ml tubes (Thermo Fisher Scientific, #N8010611) and
683 stored at $-80\text{ }^{\circ}\text{C}$ until cDNA library preparation.

684

685 **Preparation of cDNA libraries and RNA sequencing.** Sequencing libraries were prepared according to
686 the Smart-3Seq protocol for LCM HS caps, as previously described with slight modifications²¹. Briefly, 10
687 μl of lysis mix consisting of 40% (v/v) 5 M trimethylglycine solution (Sigma, #B0300), 20% (v/v) 10 mM
688 nuclease-free dNTP mix (Thermo Fisher Scientific, #R0192), 10% (v/v) 20 μM first-strand primer in TE
689 buffer (1S, /5Biosg/GT GAC TGG AGT TCA GAC GTG TGC TCT TCC GAT CTT TTT TTT TTT TTT TTT TTT
690 TTT TTT TV; Integrated DNA Technologies), 10% (v/v) Triton-X 100 (Sigma #T8787; diluted to 0.5% v/v in
691 molecular biology-grade water), and 20% (v/v) Proteinase K (New England Biolabs, #P8107S; diluted to
692 0.125 mg/ml in in molecular biology-grade water) was added to the center of each LCM cap. Caps were

693 sealed with 0.2 ml low-retention PCR tubes (Corning, #PCR-02-L-C) and incubated on a pre-warmed
694 metal CapSure incubation block (Thermo Fisher Scientific, #LCM0505) at 60 °C in an incubator. Then,
695 tubes were briefly centrifuged, and 10 µl of template-switching reverse-transcription (TS-RT) FFPE LCM
696 mix consisting of 40% (v/v) 5x SMARTScribe first-strand reaction buffer (Clontech, #639537), 20% (v/v)
697 20 mM DTT (Clontech, #639537), 10% (v/v) 20x RNase inhibitor (Thermo Fisher Scientific, #AM2694), 4%
698 (v/v) 50 µM second-strand primer in TE buffer (2S, /5Biosg/CT ACA CGA CGC TCT TCC GAT CTN NNN
699 NrGrG rG; Integrated DNA Technologies), 4% (v/v) 200 mM MgCl₂ (Sigma, #63069), 2% 5 mM proteinase
700 K inhibitor (EMD Millipore, #539470), and 20% (v/v) 100 U/µl SMARTScribe reverse transcriptase
701 (Clontech, #639537) was added. Samples were incubated in a programmable thermal cycler (42 °C for 30
702 min, 70 °C for 10 min, 4 °C hold), and 1.25 µl of a unique P5 primer and 1.25 µl of a universal P7 primer
703 (2 µM in TE buffer each; Integrated DNA Technologies; sequences available upon request) and HiFi
704 HotStart ReadyMix (Kapa, #KK2601) were then added, followed by 22 cycles of PCR amplification (98 °C
705 for 45 s; 22 cycles at 98 °C for 15 s, 60 °C for 30 s, 72 °C for 10 s; then 72 °C for 60 s, and 4 °C hold).
706 Amplified cDNA was next purified with SPRI bead mix (Beckman Coulter, #B23317) and a magnetic
707 separation block (V&P Scientific, #VP772F4). Finally, the samples were washed with 80% ethanol and
708 resuspended in TE buffer to yield the sequencing-ready library.

709 Libraries were profiled for size distribution on an Agilent 2200 TapeStation with High Sensitivity
710 D1000 reagent kits and quantified by qPCR with a dual-labeled probe as previously described⁸⁸. Libraries
711 were excluded if <40% of their transcripts were within a 165-500 bp range. A total of 64 libraries were
712 mixed to equimolarity, according to the qPCR measurements. The RNA libraries were sequenced on an
713 Illumina NextSeq 500 instrument with a High Output v2.5 reagent kit (Illumina, #20024906) to a
714 minimum sequencing depth of 1.5 M reads per sample (mean: 3.7 M) and minimum uniquely aligned
715 reads of 364,468 per sample (mean: 916,607) using read lengths of 76 nucleotides (nt) for read 1 and 8
716 nt for read 2. On average, we obtained reads from 11,166 genes per sample (median: 11,267) and

717 379,615 unique transcripts per sample (median: 336,005), which is comparable to previously published
718 FFPE-based RNA-seq studies of human cancers²¹.

719

720 **Processing of RNA-seq data.** Base calls from the NextSeq were de-multiplexed and converted to FASTQ
721 format with bcl2fastq (Illumina). The five-base unique molecular identifier (UMI) sequence and the G-
722 overhang were extracted from FASTQ data, and A-tails were removed with umi_homopolymer.py
723 (github.com/jwfoley/3SEQtools). Reads were aligned and further processed to remove duplicates using
724 STAR (github.com/alexdobin/STAR). Bulk gene expression profiles were transcript per million (TPM)
725 normalized and log₂ transformed. Differences in *CXCL13* and *CXCR5* expression between groups were
726 modeled with Linear Mixed Effects Models on a per spot basis using the lmer function from package
727 lme4 (v1.1.21)⁸⁹ and taking the patient intercept as a random effect. The pairwise p-values were derived
728 from t-ratio statistics in the contrast analysis using the lmerTest (v3.1.2)⁹⁰ and corrected for multiple
729 hypothesis testing using the Holm Bonferroni method implemented in the modelbased (v0.1.2) package
730 (github.com/easystats/modelbased).

731

732 **Principal component analysis (PCA) immune scores.** PCA scores and principal component 1 (PC1)
733 coefficients were computed for the normalized bulk RNA-seq data on a per spot basis using the prcomp
734 function in base R. The IFN- γ score was calculated using the six gene signature published by Ayers et
735 al.³⁹. The TGF- β score was calculated using the 15 gene signature published by Mariathasan et al.⁴⁰. The
736 immune activation and immunosuppression scores were computed using the genes listed in **Fig. 2g**.
737 Differences in PC1 scores between patient groups were modeled using Linear Mixed Effects Models on a
738 per spot basis using the lmer function from package lme4 (v1.1.21)⁸⁹ and taking the patient intercept as
739 a random effect. Differences between responders and non-responders as well as pre-treatment and
740 post-treatment samples were modeled as fixed effects and tested using Satterthwaite's degrees of
741 freedom method. The pairwise *p* values were derived from t-ratio statistics in the contrast analysis using

742 the ImerTest (v3.1.2)⁹⁰ and corrected for multiple hypothesis testing using the Holm Bonferroni method
743 implemented in the modelbased (v0.1.2) package (github.com/easystats/modelbased).

744

745 **Identifying bulk RNAseq gene signatures associated with tumor cells and the *SpatialScore*.** LASSO

746 regression models were used to find genes predictive of tumor cells and the *SpatialScore*. These models
747 were estimated using the LassoCV object in the scikit-learn python package. Six-fold cross validation was
748 used to select the optimal regularization parameter. Specifically, an L1-regularized linear model was fit
749 to predict the frequency of tumor cells from the gene expression data per tissue microarray spot. For
750 this model, the response variable was the log transformed per spot percentage of tumor cells. A pseudo-
751 count of 1% was added to genes. The features utilized as predictors were the per spot log transformed
752 TPM counts and the log frequency of CD4⁺ T cells. Genes with positive nonzero coefficients were
753 interpreted as positively associated with tumor cells. Similarly, an L1-regularized linear model was fit to
754 predict the *SpatialScore* from the gene expression data on a per spot basis. For this model, the response
755 variable was the log transformed *SpatialScore* distance ratio. The features used as predictors were the
756 per spot log transformed TPM counts. Genes with nonzero coefficients were selected in figures as
757 predictive.

758

759 **CIBERSORTx signature matrix.** To generate a CSx signature matrix, we used a publicly available scRNA-
760 seq dataset from Gaydosik et al.⁵⁹ that was obtained from skin biopsies of five CTCL patients. Datasets
761 were downloaded from the Gene Expression Omnibus (GEO) database (accession code GSE128531), and
762 single-cell profiles were combined and analyzed using the Seurat R package (3.1.4)⁹¹. Cells with between
763 500 and 7500 genes detected and less than 10% mitochondrial transcripts were included in the analysis.

764 Data were log10normalized and clustered with the Louvain method⁹² based on the first 13 PCs
765 and resolution of 1.8. Cell clusters were visualized using Uniform Manifold Approximation and
766 Projection (UMAP)⁹³, with the same PCs. Major cell-types were assigned according to expression of

767 corresponding marker genes (**Sup. Fig. 6a, Sup. Table 4**). Fibroblasts and pericytes were merged into a
768 stromal cluster. The T cell cluster was divided into CD4⁺ T cells, CD8⁺ T cells, Tregs, and tumor cells based
769 on the expression of certain T cell and tumor marker genes (**Sup. Fig 6b, Sup. Table 4**). Tumor cells from
770 patient CTCL-5 were excluded due to extreme heterogeneity. T cell clustering was based on the first 15
771 PCs and resolution of 1.9. The same PCs were used to generate UMAP projections for the T cell clusters.
772 A matrix of single cells and their assigned cell-type identities was used to create a signature matrix using
773 the CSx (v.1.0) website²² (code available from <https://cibersortx.stanford.edu/>). There was good
774 correlation between the CSx and CODEX cell-type clusters (**Sup. Fig 5c**).

775

776 **CIBERSORTx deconvolution.** The signature matrix was used to deconvolve tumor cell gene expression in
777 CSx (v.1.0) with the CSx website (arguments used: rmbatchSmode = T, QN = F)²². Log2 fold changes were
778 computed for every deconvolved gene across patient groups. Differences in gene expression between
779 patient groups were modeled with Linear Mixed Effects Models on a per spot basis using the lmer
780 function from package lme4 (v1.1.21)⁸⁹ and taking the patient intercept as a random effect. The *p*
781 values were derived using Satterthwaite's degrees of freedom method, implemented in the lmerTest
782 (v3.1.2)⁹⁰ package. The *p* values were adjusted with the Benjamini-Hochberg correction using the
783 p.adjust function in R. Volcano plots were generated using the ggplot2 (3.3.0)⁹⁴ and ggrepel (0.8.1)⁹⁵
784 packages in R. Genes with Benjamini-Hochberg-adjusted *p* < 0.1 were considered significant (**Fig. 5i-j,**
785 **Sup. Fig. 5d**).

786 CSx-deconvolved *CXCL13* expression in tumor cells was log2 transformed on a per spot basis.
787 Differences in *CXCL13* expression between patient groups were modeled using Linear Mixed Effects
788 Models on a per spot basis using the lmer function from package lme4 (v1.1.21) and taking the patient
789 intercept as a random effect. The pairwise *p* values were derived from t-ratio statistics in the contrast
790 analysis using the lmerTest (v3.1.2) and corrected for multiple hypothesis testing using the Holm
791 Bonferroni method implemented in the modelbased (v0.1.2) package

792 (github.com/easystats/modelbased). To examine *CXCL13* expression in tumor cells on a per patient
793 basis, the mean from biological replicates was computed before plotting the log2 normalized CSx-
794 deconvolved *CXCL13* expression. The Wilcoxon signed-rank test was used to evaluate whether patient-
795 matched CSx-deconvolved *CXCL13* expression in the tumor cells was different between groups.

796

797 **Statistical analysis.** Statistical analyses were performed with R and Prism v8 (GraphPad Software, Inc).

798 Results with $p < 0.05$ were considered significant, unless otherwise stated. For pre-treatment differences

799 between responders and non-responders, the significance was tested using a two-sided Wilcoxon's

800 rank-sum test. For differences across patient groups (i.e., responders and non-responders pre-

801 treatment, responders pre- and post-treatment, non-responders pre- and post-treatment, and

802 responders and non-responders post-treatment), the significance was tested using a linear mixed-effect

803 model with Bonferroni's corrections for multiple comparisons. Pre- to post-treatment pairwise statistical

804 significance for individual patients was tested using a two-sided Wilcoxon's signed-rank test.

805 Correlations were evaluated with the non-parametric Spearman test. The investigators were not blinded

806 to allocation during experiments and outcome assessment. No sample-size estimates were performed to

807 ensure adequate power to detect a pre-specific effect size. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$,

808 $****p < 0.0001$.

809

810 **DATA AVAILABILITY.** Upon acceptance, raw CODEX data will be deposited in a public repository at The

811 Cancer Imaging Archive (TCIA). RNA-seq data generated in this study have been deposited in the GEO

812 database, with accession code GSE162137 (secure reviewer token: snmnyuqqpfepr).

813

814 **CODE AVAILABILITY.** Cellular neighborhood code:

815 <https://github.com/nolanlab/NeighborhoodCoordination>. Code used to calculate the physical distances

816 between cell-types will be uploaded to the Nolan Lab github page upon acceptance. CIBERSORTx:
817 <https://cibersortx.stanford.edu/>.

818

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844

845 **AUTHOR CONTRIBUTIONS**

846 D.P. and C.M.S. conceived of the study. D.P., M.M., B.R.G., S.S.B., G.L.B., S.J., J.D., K.S.S., Y.G., and C.M.S.
847 performed experiments and analyzed data. R.H.P, S.P.F., N.R., and M.A.C. provided clinical samples and
848 created the tissue microarray. M.S.K. and Y.H.K coordinated trial protocols and provided clinical data.
849 G.P.N. guided experiments and data analysis, with assistance from R.B.W. D.P. and G.P.N. wrote the
850 manuscript, with input from all authors. All authors vouch for the accuracy of the data reported and
851 approve the manuscript.

852

853

854 **COMPETING INTERESTS**

855 G.P.N. and Y.G. are co-founders and stockholders of Akoya Biosciences, Inc. and inventors on patent
856 US9909167. D.P., C.M.S., and G.P.N. are inventors on pending patent US62971722, filed by Stanford
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859

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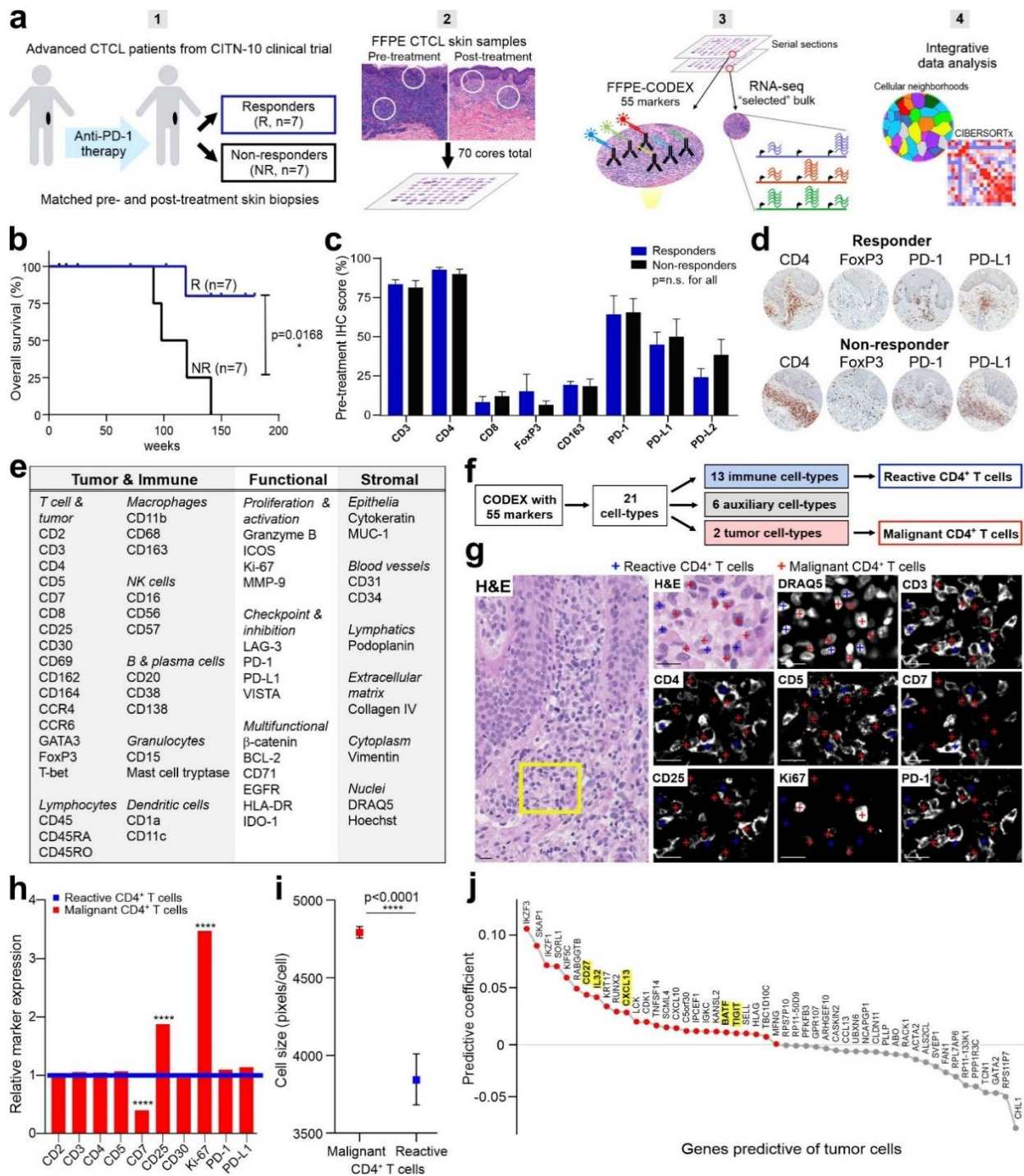
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Fig 1. Discrimination of malignant and reactive CD4⁺ T cells in the CTCL TME. a, Workflow for sample

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preparation, CODEX, RNAseq, and computational analyses. b, Kaplan-Meier overall survival curve,

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comparing responders (R, $n=7$, blue line) and non-responders (NR, $n=7$, black line) (hazard ratio 0.0969

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responder/non-responder; p value calculated by log-rank test). c, IHC protein marker expression in

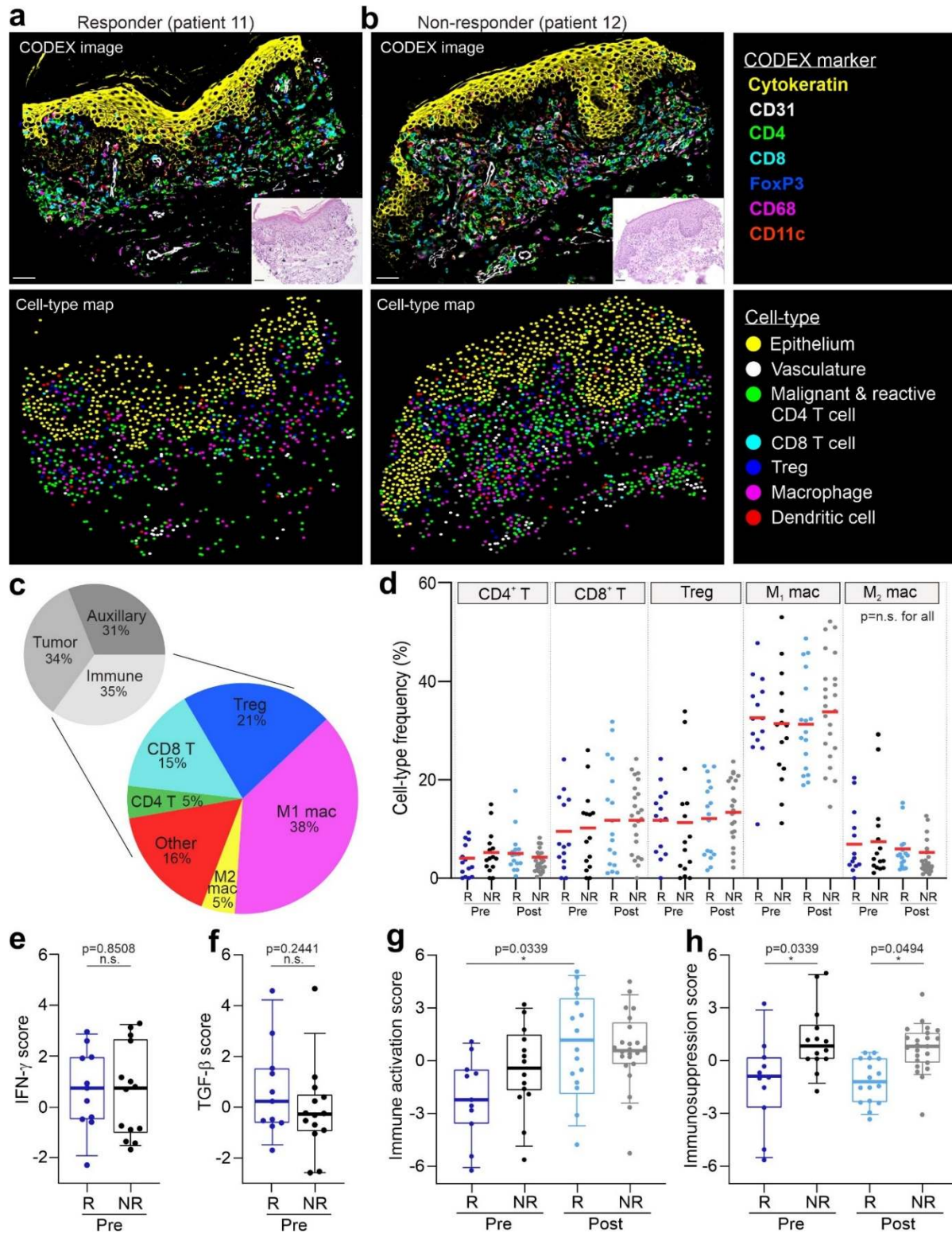
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responders and non-responders pre-treatment (mean \pm s.e.m.). P values were calculated by two-sided

1102 Wilcoxon's rank-sum tests (p = not significant (n.s.) for all comparisons). **d**, Representative pre-
1103 treatment IHC images for select markers from a responder (top) and non-responder (bottom). **e**, CODEX
1104 antibody panel (see also **Sup. Fig. 1d**). **f**, Identification of 21 cell-types by clustering (see also **Sup. Fig.**
1105 **2a-b**). **g**, Visual verification of reactive (blue crosses) versus malignant (red crosses) CD4⁺ T cells in CTCL
1106 tissue. Scale bars, 20 μ m. **h**, Average expression of select markers on malignant (red bars) relative to
1107 reactive (blue line) CD4⁺ T cells. P values were calculated by two-sided Wilcoxon's rank-sum tests (****,
1108 $p < 0.0001$). **i**, Cell size, measured in pixels/cell, of all malignant (red square) versus reactive (blue square)
1109 CD4⁺ T cells (mean \pm s.e.m.). P value was calculated by a two-sided Wilcoxon's rank-sum test. **j**, Ranking
1110 genes most predictive of tumor cells per tissue microarray spot using an L1-regularized linear model.
1111 Red colored genes have positive predictive coefficients (i.e., most likely to represent tumor cells); gray
1112 colored genes have negative predictive coefficients (i.e., less likely to represent tumor cells). Known
1113 CTCL marker genes are highlighted in yellow.

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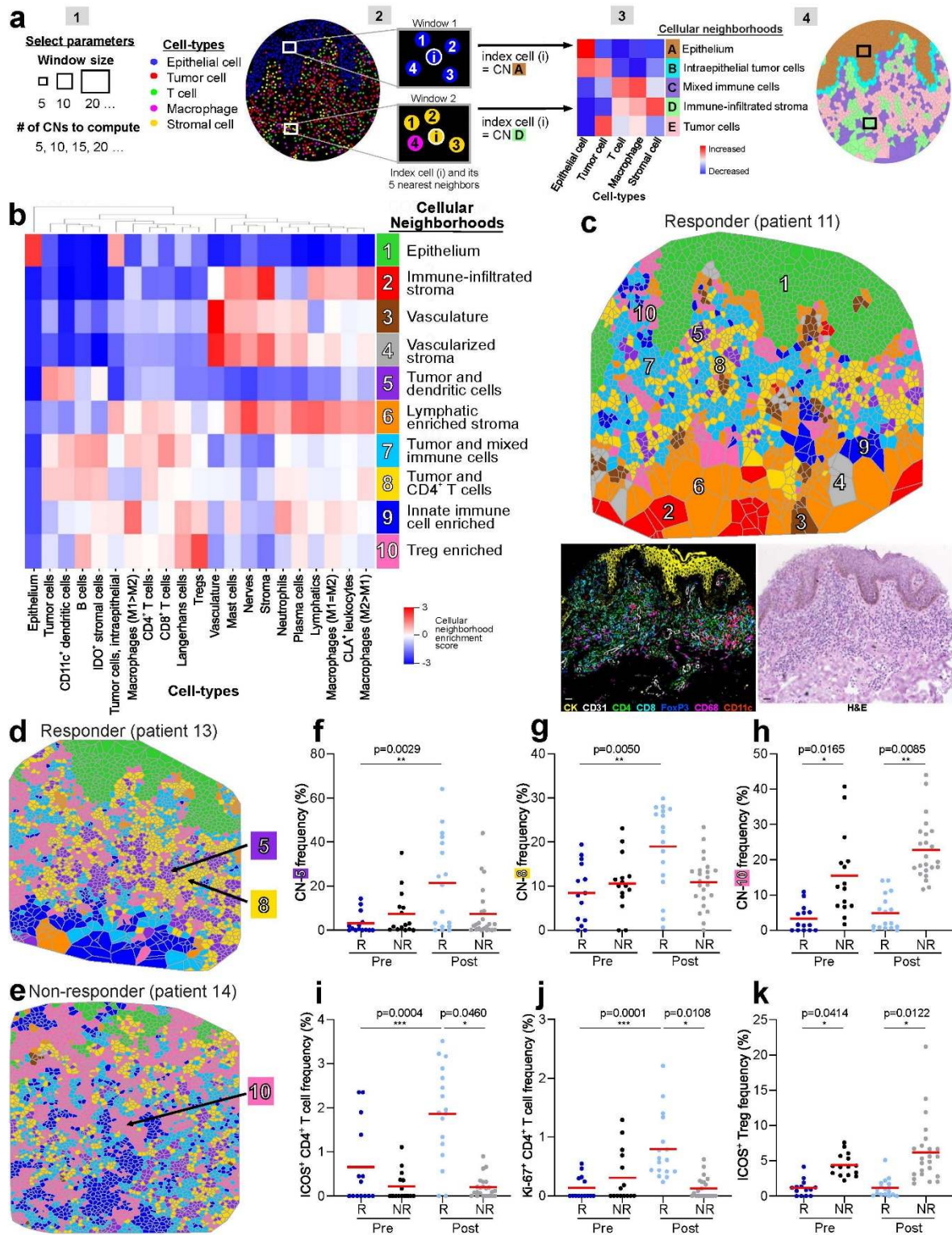
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1117 **Fig 2. Characterization of the CTCL TME pre- and post-pembrolizumab treatment. a-b,** Top panels:

1118 Representative CODEX seven-color overlay images from a responder (left) and non-responder (right)

1119 pre-treatment. Scale bar, 50 μ m. Insets, corresponding H&E images; scale bars, 50 μ m. Bottom panels:

1120 corresponding cell-type maps. **c**, Upper pie chart: overall frequencies of tumor, immune and auxiliary
1121 cell-types. Lower pie chart: overall frequencies of all immune cell-types, including CD4⁺ T cells, CD8⁺ T
1122 cells, Tregs, M1 macrophages, M2 macrophages, and other (B cells, dendritic cells, Langerhans cells,
1123 mast cells, neutrophils, and plasma cells). **d**, Cell-type frequencies of CD4⁺ T cell, CD8⁺ T cell, Treg, M1
1124 macrophage, and M2 macrophage as a percentage of all immune cells per tissue microarray spot in
1125 responders and non-responders pre- and post-treatment (mean, red bar). *P* values were calculated with
1126 a linear mixed-effect model with Bonferroni's corrections for multiple comparisons (*p* = not significant
1127 (n.s.) for all comparisons). **e-f**, IFN- γ (**e**) and TGF- β (**f**) gene scores between responders and non-
1128 responders pre-treatment per spot. Boxes, median \pm interquartile range (IQR); whiskers, 1.5x IQR. *P*
1129 values were calculated with a linear mixed-effect model with Bonferroni's corrections for multiple
1130 comparisons. **g-h**, Immune activation (**g**) and immunosuppression (**h**) gene scores, computed on bulk
1131 RNA-seq data, between responders and non-responders pre- and post-treatment per spot. Boxes,
1132 median \pm IQR; whiskers, 1.5x IQR. *P* values were calculated with a linear mixed-effect model with
1133 Bonferroni's corrections for multiple comparisons.
1134



1139 (five in this schematic). [2] Assignment of an index cell (i, center of window) to a given CN based on the
1140 composition of cell-types within its corresponding window the clustering of windows. [3] Heatmap of
1141 cell-type distribution for each CN and assignment of CN names. [4] Visualization of CNs as a Voronoi
1142 diagram. **b**, Identification of 10 conserved CNs in the CTCL TME using a window size of 10. **c**,
1143 Representative Voronoi diagram of the 10 CNs in a responder post-treatment, with the corresponding
1144 H&E and seven color fluorescent CODEX images. Scale bar, 20 μm . **d-e**, Voronoi diagrams of CNs in a
1145 responder (**d**) and non-responder (**e**) post-treatment, highlighting CN-5 (tumor and dendritic cells), CN-8
1146 (tumor and CD4⁺ T cells) and CN-10 (Treg enriched). **f-h**, Frequencies of CN-5 (**f**), CN-8 (**g**) and CN-10 (**h**)
1147 per tissue microarray spot in responders and non-responders pre- and post-treatment (mean, red bar). *P*
1148 values were calculated with a linear mixed-effect model with Bonferroni's corrections for multiple
1149 comparisons. **i-k**, Frequencies of ICOS⁺ CD4⁺ T cell (**i**), Ki-67⁺ CD4⁺ T cell (**j**) and ICOS⁺ Treg (**k**) as a
1150 percentage of all immune cells per spot in responders and non-responders pre- and post-treatment
1151 (mean, red bar). *P* values were calculated with a linear mixed-effect model with Bonferroni's corrections
1152 for multiple comparisons.

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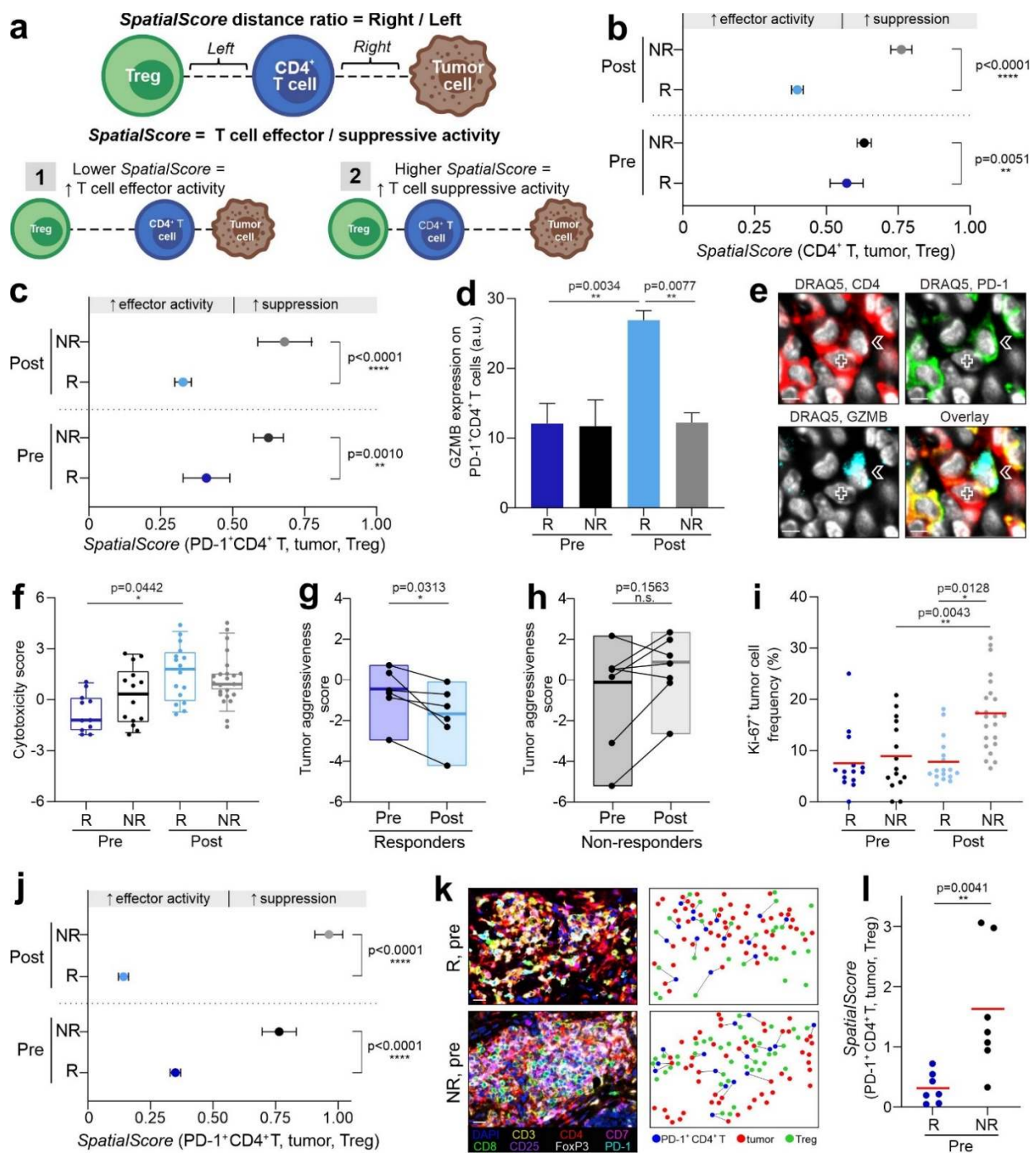
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1160 **Fig 4. Spatial relationship between CD4⁺ T cells, Tregs and tumor cells predicts pembrolizumab**

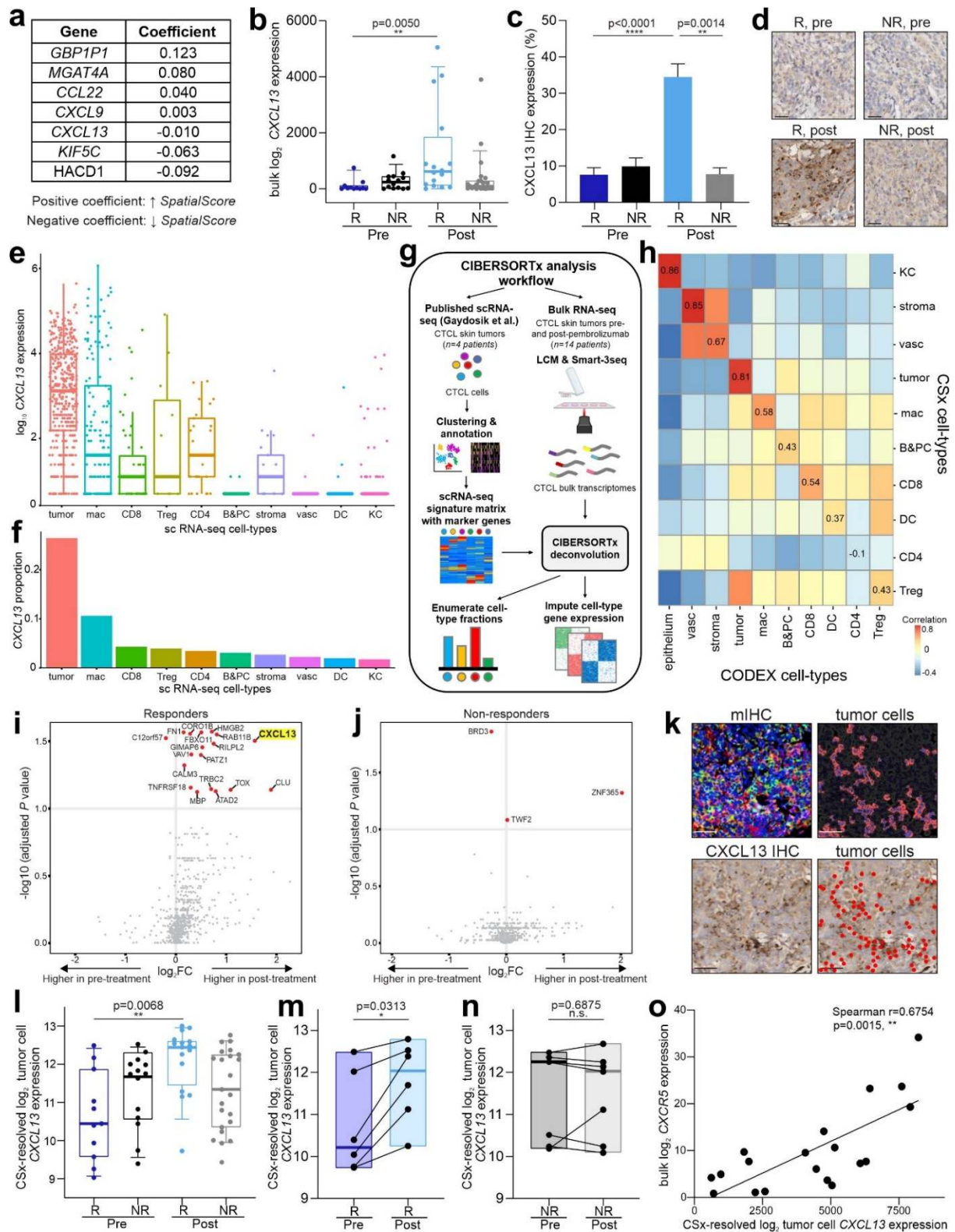
1161 **response in CTCL. a**, $SpatialScore$ schematic. The $SpatialScore$ is calculated by taking the ratio of the

1162 physical distance between each CD4⁺ T cell and its nearest tumor cell (distance “right”) relative to its

1163 nearest Treg (distance “left”). [1] A lower $SpatialScore$ (i.e., CD4⁺ T cells are closer to tumor cells than

1164 Tregs) suggests increased T cell effector activity. [2] A higher spatial score (i.e., CD4⁺ T cells are closer to

1165 Tregs than tumor cells) suggests increased T cell suppression. **b-c**, *SpatialScore* on a per cell basis for
1166 CD4⁺ T cells (**b**) and PD-1⁺ CD4⁺ T cells (**c**) for each patient group (mean \pm s.e.m.). *P* values were
1167 calculated by two-sided Wilcoxon's rank-sum tests, with no adjustments for multiple comparisons. **d**,
1168 GZMB protein expression on PD-1⁺ CD4⁺ T cells by CODEX in responders and non-responders pre- and
1169 post-treatment per tissue microarray spot (mean fluorescence intensity (arbitrary units, a.u.) \pm s.e.m.) *P*
1170 values were calculated with a linear mixed-effect model with Bonferroni's corrections for multiple
1171 comparisons. **e**, CODEX images of DRAQ5 (white), CD4 (red), PD-1 (green), GZMB (cyan), and overlay
1172 showing contact between a tumor cell (marked by cross) and GZMB-expressing PD-1⁺ CD4⁺ T cell
1173 (marked by arrow) in responder patient 13 post-treatment. Scale bars, 10 μ m. **f**, Cytotoxicity gene
1174 scores, computed on bulk RNA-seq data, between responders and non-responders pre- and post-
1175 treatment per spot. Boxes, median \pm IQR; whiskers, 1.5x IQR. *P* values were calculated with a linear
1176 mixed-effect model with Bonferroni's corrections for multiple comparisons. **g-h**, Pre- to post-treatment
1177 changes in tumor aggressiveness gene scores, computed on bulk RNA-seq data, on a per patient basis in
1178 responders (**g**) and non-responders (**h**). Boxes, median \pm IQR; whiskers, 1.5x IQR. *P* values were
1179 calculated by two-sided Wilcoxon's signed-rank tests. **i**, Ki-67⁺ tumor cell frequencies per spot in
1180 responders and non-responders pre- and post-treatment (mean, red bar). *P* values were calculated with
1181 a linear mixed-effect model with Bonferroni's corrections for multiple comparisons. **j**, *SpatialScore* on a
1182 per cell basis calculated from Vectra mIHC data using PD-1⁺CD4⁺ T cells for each patient group (mean \pm
1183 s.e.m.). *P* values were calculated by two-sided Wilcoxon's rank-sum tests, with no adjustments for
1184 multiple comparisons. **k**, Vectra mIHC images (left panels; representative regions of a tissue microarray
1185 spot) in responder patient 13 (R) and non-responder patient 14 (NR) pre-treatment. Scale bars, 20 μ m.
1186 The corresponding spatial plots (right panels) show that PD-1⁺ CD4⁺ T cells (blue dots) are closer to
1187 tumor cells (red dots) than Tregs (green dots) in the responder and vice versa in the non-responder. **l**,
1188 Pre-treatment *SpatialScore* calculated from Vectra mIHC data for responders and non-responders on a
1189 per patient basis (mean, red bar). *P* values was calculated by a two-sided Wilcoxon's rank-sum test.



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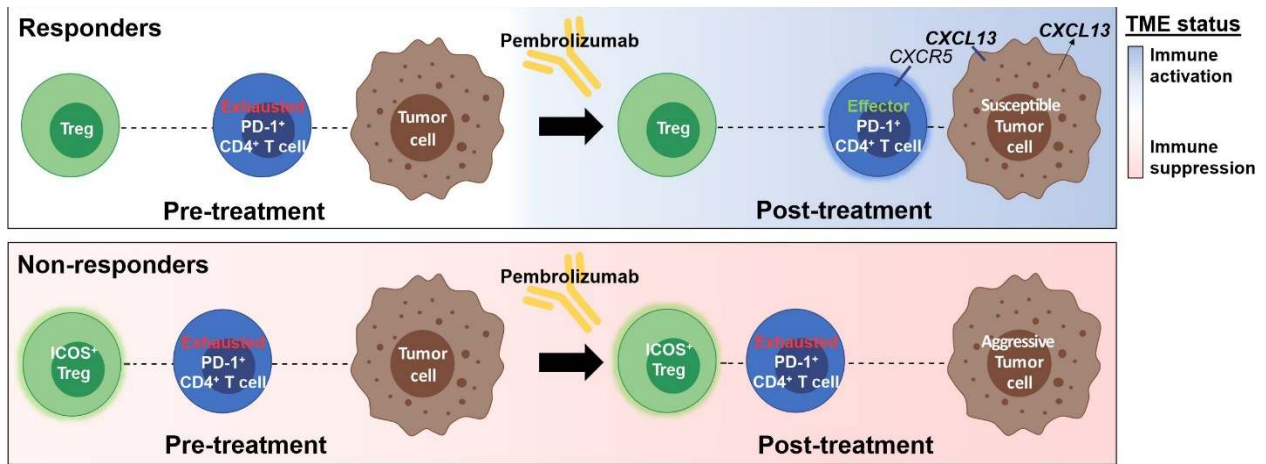
1191 **Fig 5. CXCL13 is a key driver of pembrolizumab response in CTCL.** a, Seven genes predictive of the
1192 *SpatialScore* identified from bulk RNA-seq data. Genes with a positive coefficient are associated with a

1193 higher *SpatialScore* (i.e., a suppressive phenotype), whereas those with a negative coefficient are
1194 associated with a lower *SpatialScore* (i.e., an effector phenotype). **b**, Normalized bulk *CXCL13* gene
1195 expression between responders and non-responders pre- and post-treatment per tissue microarray
1196 spot. Boxes, median \pm IQR; whiskers, 1.5x IQR. *P* values were calculated with a linear mixed-effect
1197 model with Bonferroni's corrections for multiple comparisons. **c**, *CXCL13* protein expression by IHC in
1198 responders and non-responders pre- and post-treatment per spot (mean \pm s.e.m.). *P* values were
1199 calculated with a linear mixed-effect model with Bonferroni's corrections for multiple comparisons. **d**,
1200 Representative *CXCL13* IHC images from responder patient 9 pre-treatment (top left) and post-
1201 treatment (bottom left) as well as non-responder patient 14 pre-treatment (top right) and post-
1202 treatment (bottom right). Scale bars, 20 μ m. **e-f**, Single-cell transcriptomes from a publicly available
1203 scRNA-seq dataset of CTCL skin tumors (Gaydosik et al.)⁵⁹ were analyzed for *CXCL13* expression.
1204 Abbreviations: mac (macrophages), B&PC (B and plasma cells), vasc (vasculature), DC (dendritic cells),
1205 and KC (keratinocytes). **e**, Normalized expression of *CXCL13* in single cells (each dot represents a cell
1206 positive for *CXCL13*). Cells with *CXCL13* log_{1p} normalized read counts less than 0.5 were excluded (see
1207 **Methods**). Boxes, median \pm IQR; whiskers, 1.5x IQR. **f**, Proportion of *CXCL13*-expressing cells per cell-
1208 type. **g**, CIBERSORTx analysis workflow schematic. Single cell transcriptomes from Gaydosik et al.⁵⁹ were
1209 used to generate a CSx deconvolution signature matrix, consisting of cell-type-specific marker genes (left
1210 portion of schematic). This matrix was then applied to CTCL bulk transcriptomes obtained with laser-
1211 capture microdissection (LCM) and Smart-3Seq (right portion of schematic) to enumerate cell-type
1212 fractions and resolve gene expression profiles (see **Methods**). **h**, Heatmap of the correlation between
1213 CSx-resolved cell-type frequencies and CODEX-identified cell-type frequencies. Spearman correlation
1214 coefficients between corresponding cell-types are shown along the diagonal. For this analysis, some of
1215 the original 21 CODEX cell-types were manually merged to match CSx cell-types (e.g., B cells and plasma
1216 cells). **i-j**, Volcano plots of differential gene expression of CSx-resolved tumor cell genes in responders (**j**)
1217 and non-responders (**k**) pre- and post-treatment. *P* values were calculated with a linear mixed-effect

1218 model with Benjamini-Hochberg correction. Significantly different genes ($p < 0.1$) are colored red;
1219 *CXCL13* is highlighted yellow. **k**, Vectra mIHC image of DAPI (blue), CD3 (yellow), CD4 (red), CD7
1220 (magenta), CD8 (green), CD25 (purple), FoxP3 (white), and PD-1 (cyan) (top left), corresponding tumor
1221 cell depiction (top right), corresponding *CXCL13* IHC image (bottom left), and overlaid image showing
1222 that *CXCL13* IHC staining most commonly localized to tumor cells (red circles) (bottom right) in
1223 responder patient 9 post-treatment. Scale bars, 20 μm . **l**, Normalized CSx-resolved *CXCL13* expression in
1224 tumor cells between responders and non-responders pre- and post-treatment per spot. Boxes, median \pm
1225 IQR; whiskers, 1.5x IQR. *P* values were calculated with a linear mixed-effect model with Bonferroni's
1226 corrections for multiple comparisons. **m-n**, Pre- to post-treatment changes in normalized *CXCL13* gene
1227 expression from CSx-resolved tumor genes on a per patient basis in responders (**m**) and non-responders
1228 (**n**). Boxes, median \pm IQR; whiskers, 1.5x IQR. *P* values were calculated by two-sided Wilcoxon's signed-
1229 rank tests. **o**, Correlation of CSx-resolved tumor cell *CXCL13* expression and bulk *CXCR5* expression per
1230 spot. Data was evaluated with the Spearman test and a two-tailed t-distribution with $n-2$ degrees of
1231 freedom.

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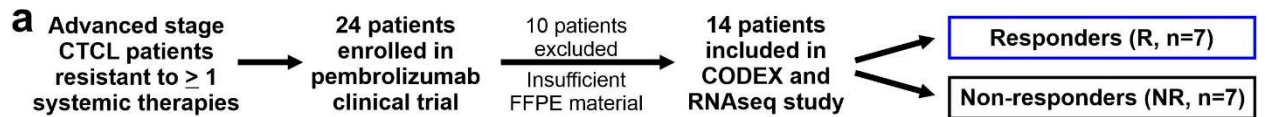
1235

1236 **Fig 6. Proposed mechanisms of pembrolizumab response in CTCL.**

1237 Proposed mechanisms of pembrolizumab response in therapy responders (top panel) and non-
 1238 responders (bottom panel) pre- and post-treatment. The functional immune status of the TME is
 1239 represented by blue shading when activated and pink shading when suppressed. In non-responders, the
 1240 TME is continually immunosuppressed and persistently exhausted PD-1⁺CD4⁺ T cells are in closer
 1241 proximity to potentially suppressive ICOS⁺ Tregs. Due to pembrolizumab resistance, non-responder tumor
 1242 cells become more aggressive following therapy. In contrast, responders have a neutral functional
 1243 immune state pre-treatment, which becomes activated following pembrolizumab therapy, enabling the
 1244 transition from exhausted to effector PD-1⁺ CD4⁺ T cells. Additionally, responder tumor cells are
 1245 susceptible to PD-1 blockade and overexpress CXCL13. This attracts effector PD-1⁺ CD4⁺ T cells toward
 1246 tumor cells, providing a mechanism for the sustained clinical response seen in responders.

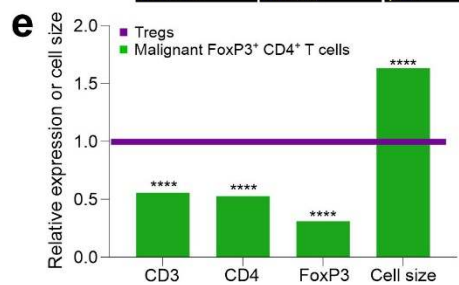
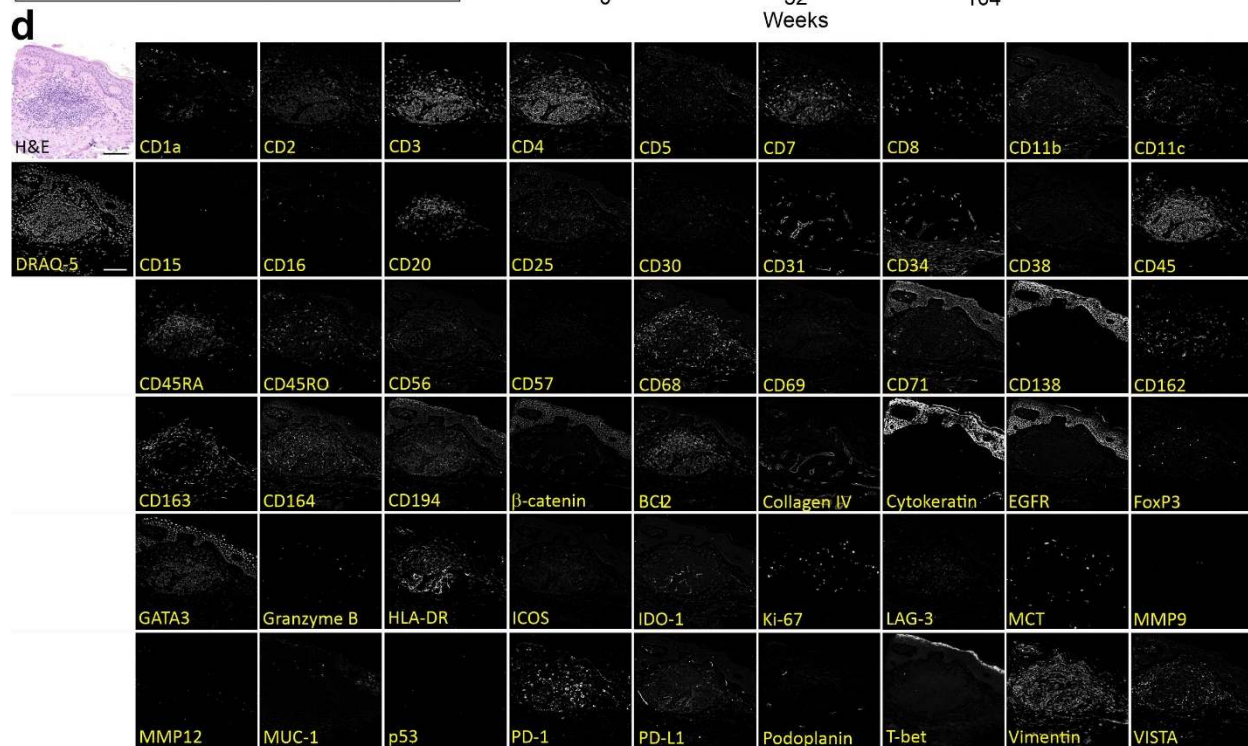
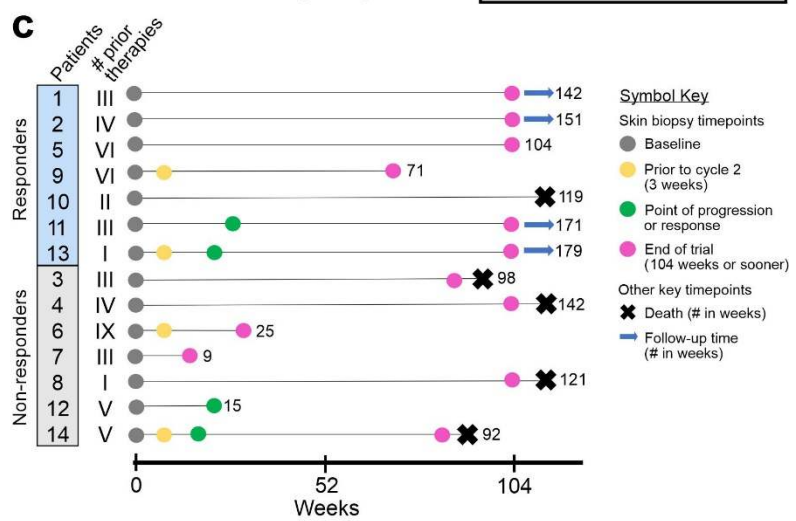
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b

Patient characteristics	Responders	Non-responders
Patients (n)	7	7
Male (n)	5	5
Female (n)	2	2
Age, year (mean \pm SD)	67.5 \pm 12.0	62.1 \pm 14.4
Cancer type (n)		
Mycosis fungoides (MF)	4	3
Sézary syndrome (SS)	3	4
Cancer stage (TNM) (n)		
IB	0	1
IIIB	1	0
IIIA	2	0
IIIB	1	2
IIVA	3	4
Prior systemic therapies		
Number (mean \pm SD)	4 \pm 2	4 \pm 3
Range	1 - 6	1 - 9
Patient outcomes		
mSWAT change (**)	41.5	-90.5
1 year PFS (*)	85.7%	28.6%
Patient deaths (n) (*)	1	4



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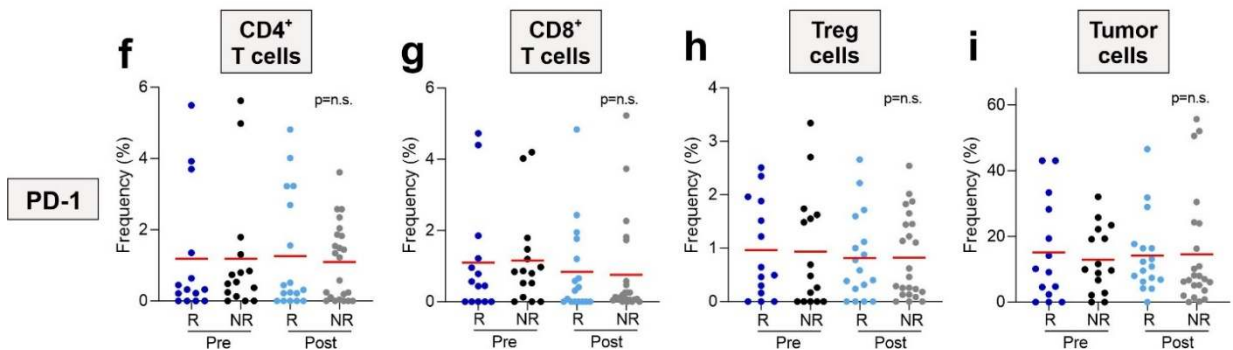
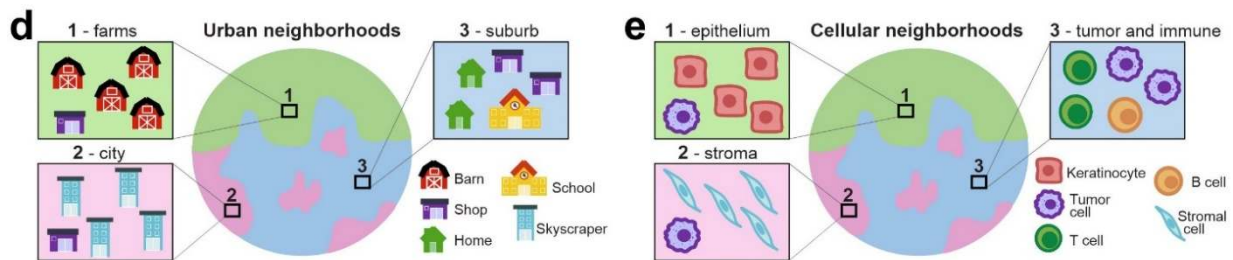
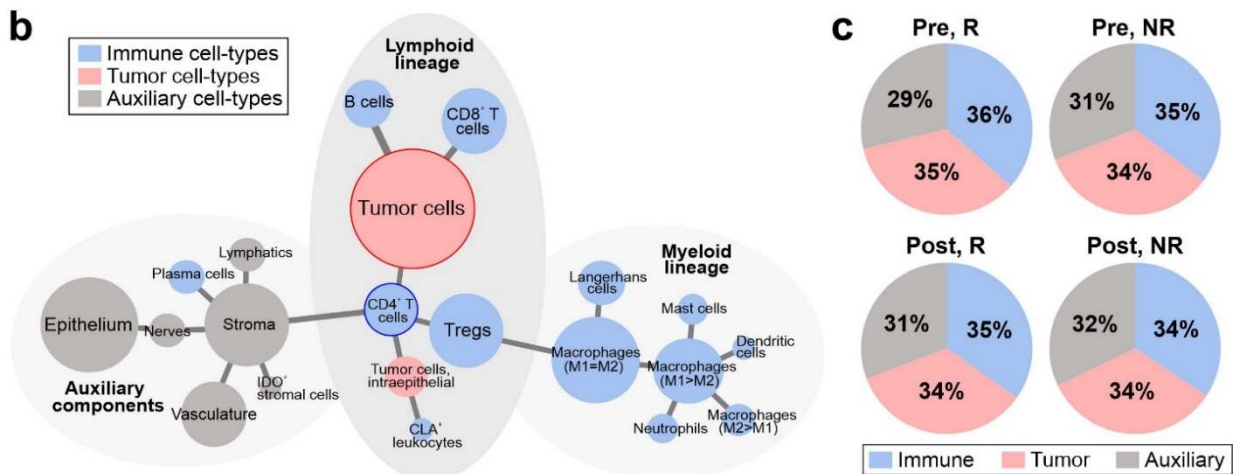
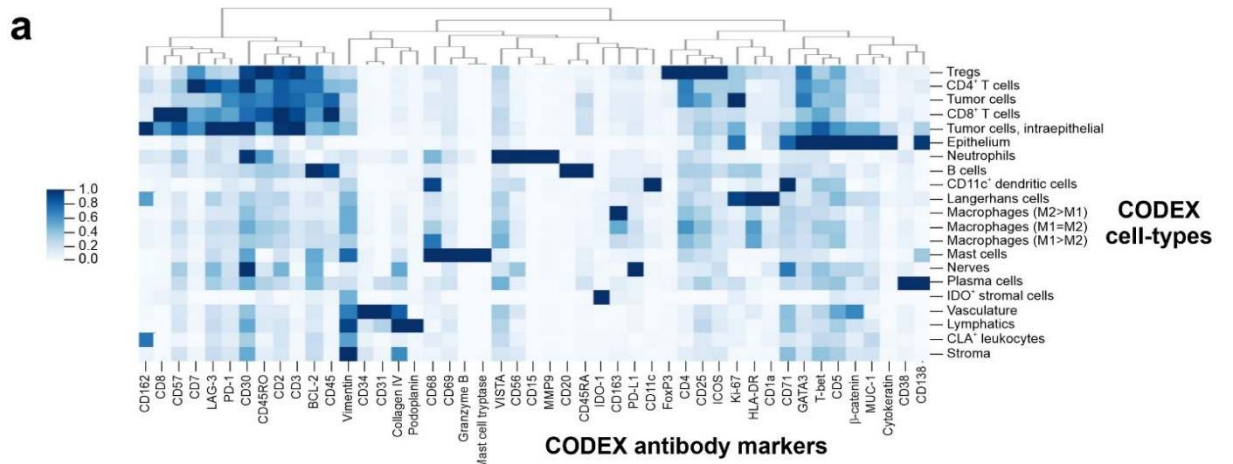
1250 **Sup. Fig. 1. Study design and CODEX experimental validation. a**, Framework for the 14 included CTCL

1251 patient samples used for CODEX tissue imaging, RNA-seq and mIHC imaging. **b**, Patient characteristics

1252 for the 7 responders and 7 non-responders. Significant differences between responders and non-
1253 responders were only noted for patient outcomes: the change from baseline in skin by the modified
1254 Severity Weighted Assessment Tool (mSWAT) (**, $p=0.0019$ by Wilcoxon's rank sum test), the 1 year
1255 progression free survival (PFS) (*, $p=0.0306$ by Wilcoxon's rank sum test), and patients deaths (*,
1256 $p=0.0168$ by log rank test in Kaplan-Meier overall survival curve; see **Fig. 1b**). **c**, Swimmer plot of
1257 individual patients, depicting treatment history and timepoints in weeks for every tumor biopsy used in
1258 this study. Lines represent overall survival in weeks. A rightward arrow indicates that the patient was
1259 still alive following the final tumor collection. A rightward X indicates that the patient died. **d**, Validation
1260 of the 55-marker CODEX antibody panel used to stain the CTCL skin tissue microarray. Images of a single
1261 microarray spot are depicted in false gray color for each marker. H&E staining is also shown. Scale bars,
1262 100 μm . **e**, Average expression of CD3, CD4, FoxP3 and cell size, measured in pixels/cell (green bars),
1263 relative to Tregs (purple line). *P* values were calculated by two-sided Wilcoxon's rank-sum tests (****,
1264 $p<0.0001$).

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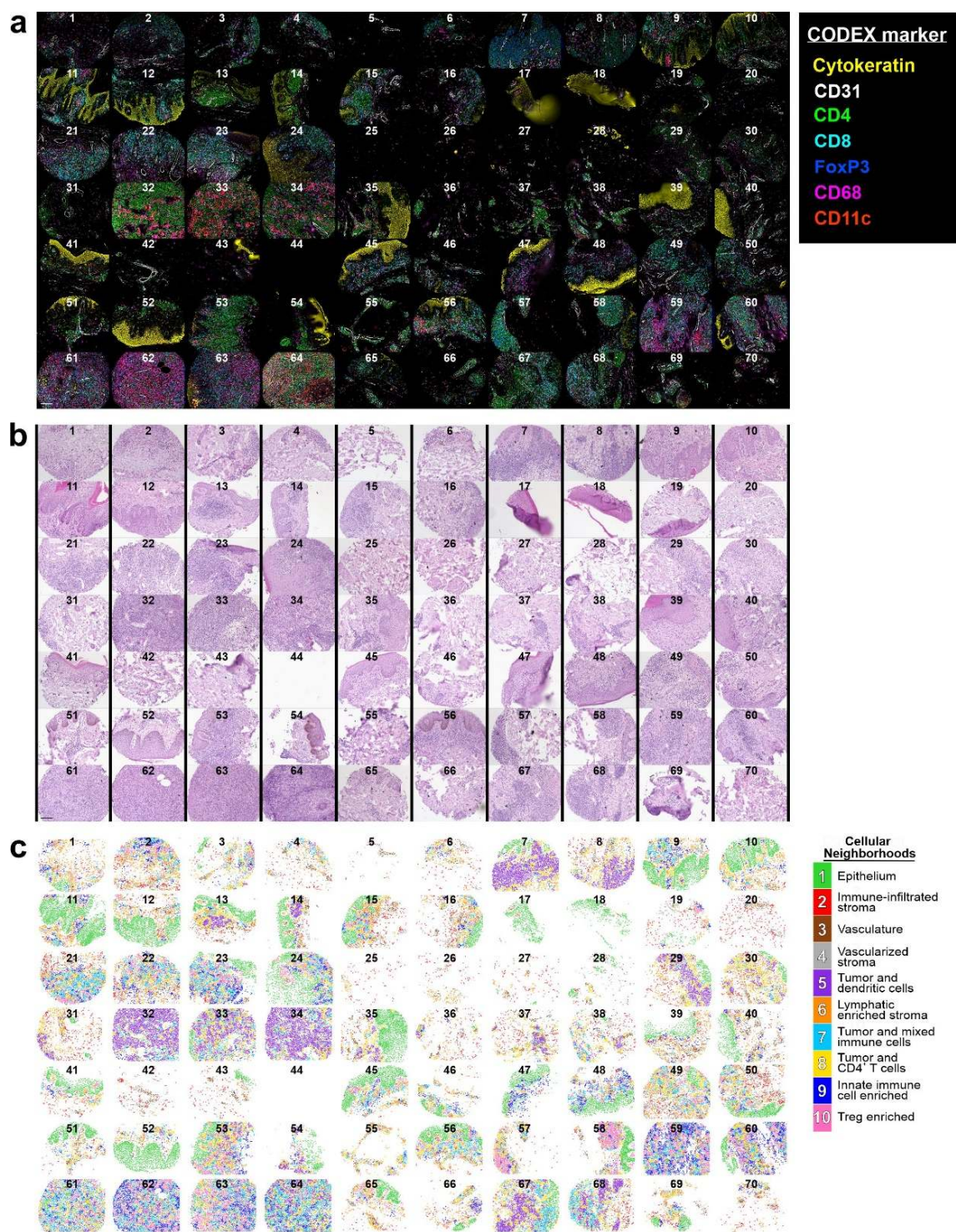
1268 **Sup. Fig. 2. Characterizing CODEX cell-types, the cellular neighborhood concept, and PD-1 positivity. a,**

1269 **Heatmap of CODEX antibody marker expression in each of the 21 identified cell-types. b, Minimal**

1270 spanning tree of the 21 cell-types, which are colored blue (immune cell-types), red (tumor cell-types)
1271 and gray (auxiliary cell-types). **c**, Frequencies of tumor, immune and auxiliary cell-types for each patient
1272 group ($p=n.s.$ for all comparisons). **d-e**, Conceptual neighborhood schematic showing how urban
1273 neighborhoods are determined based on their composition of buildings (**d**) and cellular neighborhoods
1274 based on cell-types (**e**). **f-i**, Frequencies of PD-1⁺ CD4⁺ T cells (**f**), PD-1⁺ CD8⁺ T cells (**g**), PD-1⁺ Tregs (**h**),
1275 and , PD-1⁺ tumor cells (**i**) per tissue microarray spot in responders and non-responders pre- and post-
1276 treatment (mean, red bar). *P* values were calculated with a linear mixed-effect model with Bonferroni's
1277 corrections for multiple comparisons.

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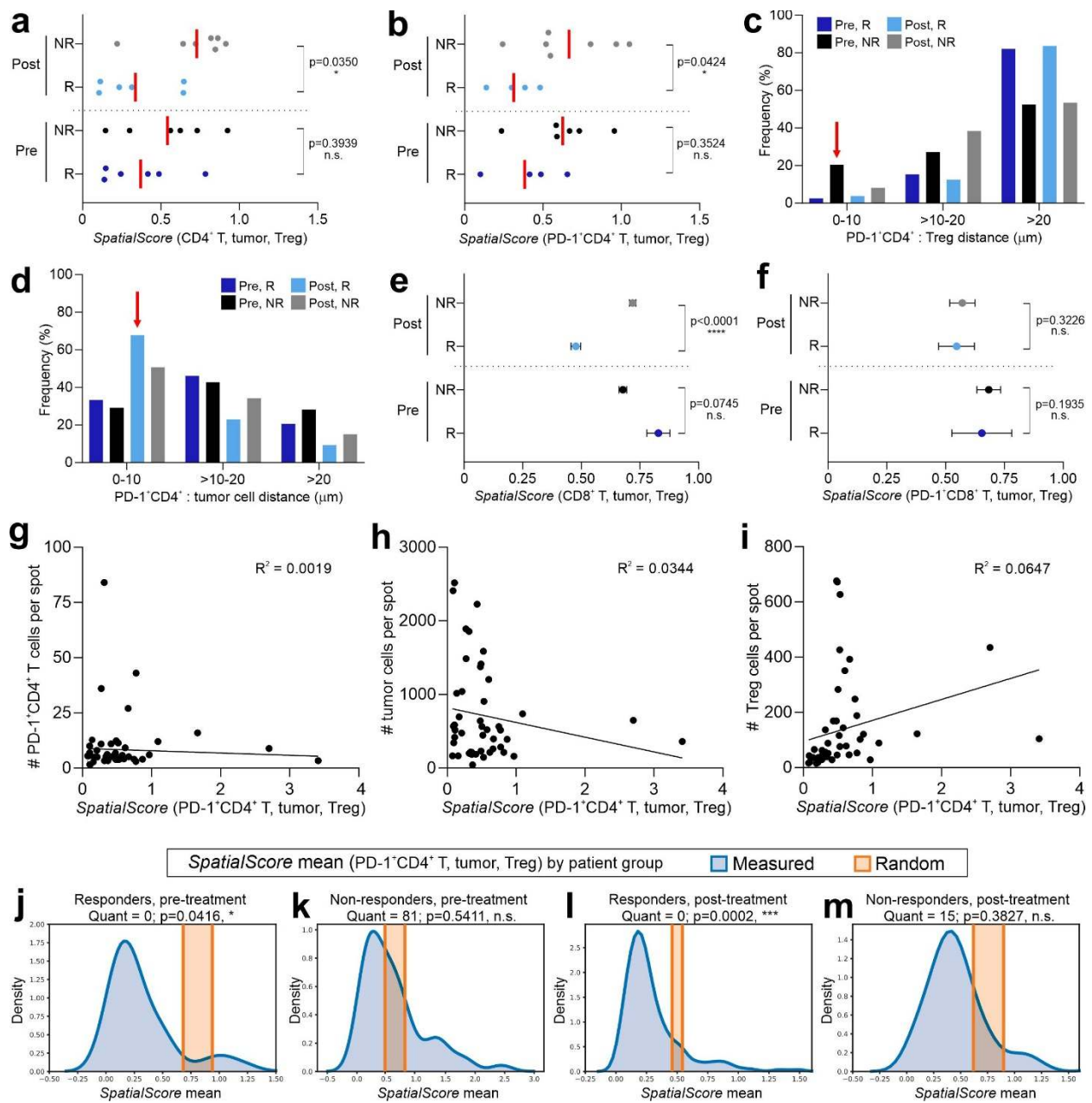
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1281 **Sup. Fig. 3. CODEX, H&E, and cellular neighborhood images for the CTCL tissue microarray. a, CODEX**

1282 **seven-color overlay image of the CTCL tissue microarray. Scale bar, 100 μ m. b, Corresponding H&E**

1283 **image of the CTCL tissue microarray. Scale bar, 100 μ m. c, Dot plot Voronoi diagram of the 10 identified**

1284 **CNs, colored according to the corresponding legend.**



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1287 **Sup. Fig. 4. Detailing the *SpatialScore* in CTCL. a-b, *SpatialScore* based on all CD4⁺ T cells (a) and PD-1⁺**

1288 CD4⁺ T cells (b) on a per patient basis across patient groups (mean, red bar). Patients were excluded

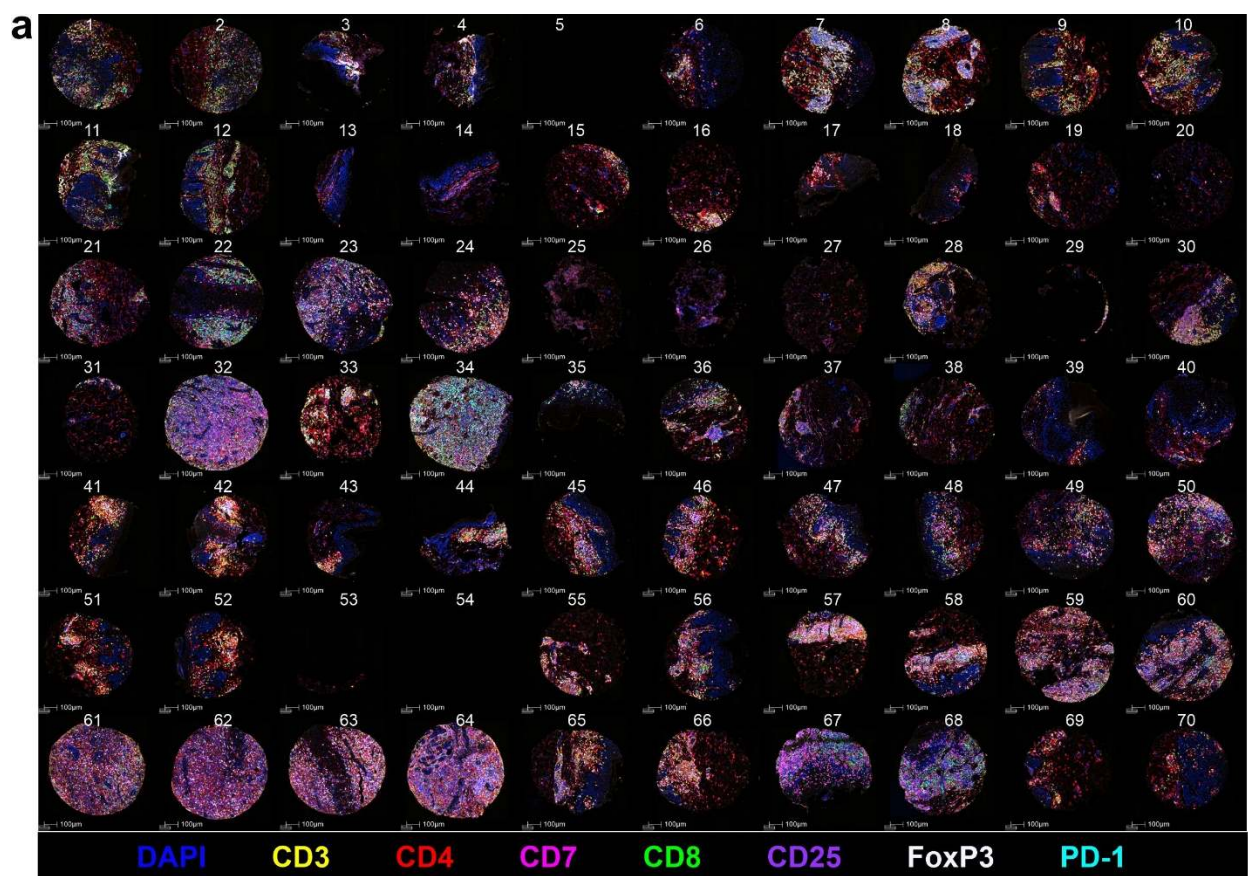
1289 from this analysis if they did not have at least 10 CD4⁺ or PD-1⁺ CD4⁺ T cells. *P* values were calculated by

1290 two-sided Wilcoxon's rank-sum tests, with no adjustments for multiple comparisons. c, Frequency

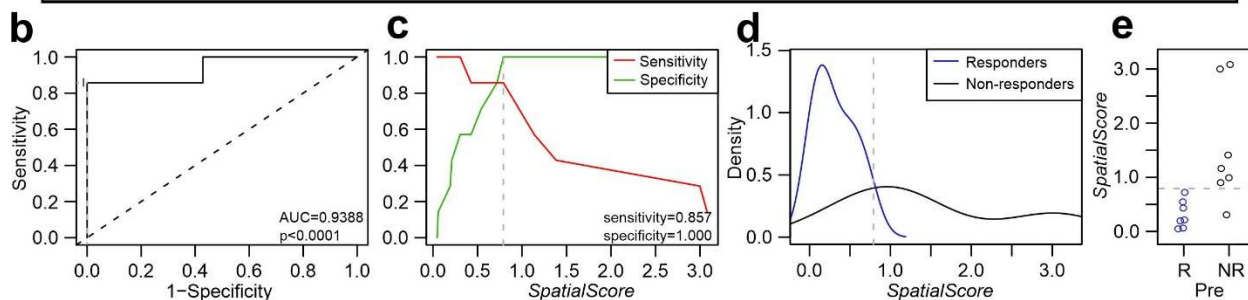
1291 distribution of the physical distances in the tissue between PD-1⁺ CD4⁺ and Tregs by patient group, with

1292 the red arrow showing that these two cell-types are closest in non-responders pre-treatment. d,

1293 Frequency distribution of the distances between PD-1⁺ CD4⁺ and tumor cells by patient group, with the
1294 red arrow showing that these two cell-types are closest in responders post-treatment. **e-f**, *SpatialScore*
1295 on a per cell basis using CD8⁺ T cells (**e**) and PD-1⁺ CD8⁺ T cells (**f**) for each patient group (mean ± s.e.m.).
1296 *P* values were calculated by two-sided Wilcoxon's rank-sum tests, with no adjustments for multiple
1297 comparisons. **g-i**, Correlations of the number of PD-1⁺ CD4⁺ T cells (**g**) tumor cells (**h**), and Tregs (**i**)
1298 relative to the *SpatialScore* per tissue microarray spot. Correlations were evaluated with the Spearman
1299 test. **j-m**, Density plots of the measured mean *SpatialScore* distribution (blue) and corresponding
1300 random mean with its standard deviation (orange) for responders pre-treatment (j) non-responders pre-
1301 treatment (k) responders post-treatment (l), and non-responders post-treatment (m). *P* values were
1302 calculated by two-sided Wilcoxon's rank-sum tests, with no adjustments for multiple comparisons. The
1303 Quant values correspond to the percentage of randomly measured values that are smaller than the
1304 measured value; values closer to 0 or 100 indicate that the measurement is not random.
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Optimal cut-off method: Youden Optimal cut-off point: 0.7908 *SpatialScore* (PD-1⁺ CD4⁺ T, tumor, Treg)



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1309 **Sup. Fig. 5. Validation of the *SpatialScore* biomarker using a clinically accessible mIHC platform. a,**

1310 Vectra mIHC staining of the CTCL tissue microarray with an eight-color overlay image, including DAPI

1311 (blue), CD3 (yellow), CD4 (red), CD7 (magenta), CD8 (green), CD25 (purple), FoxP3 (white), and PD-1

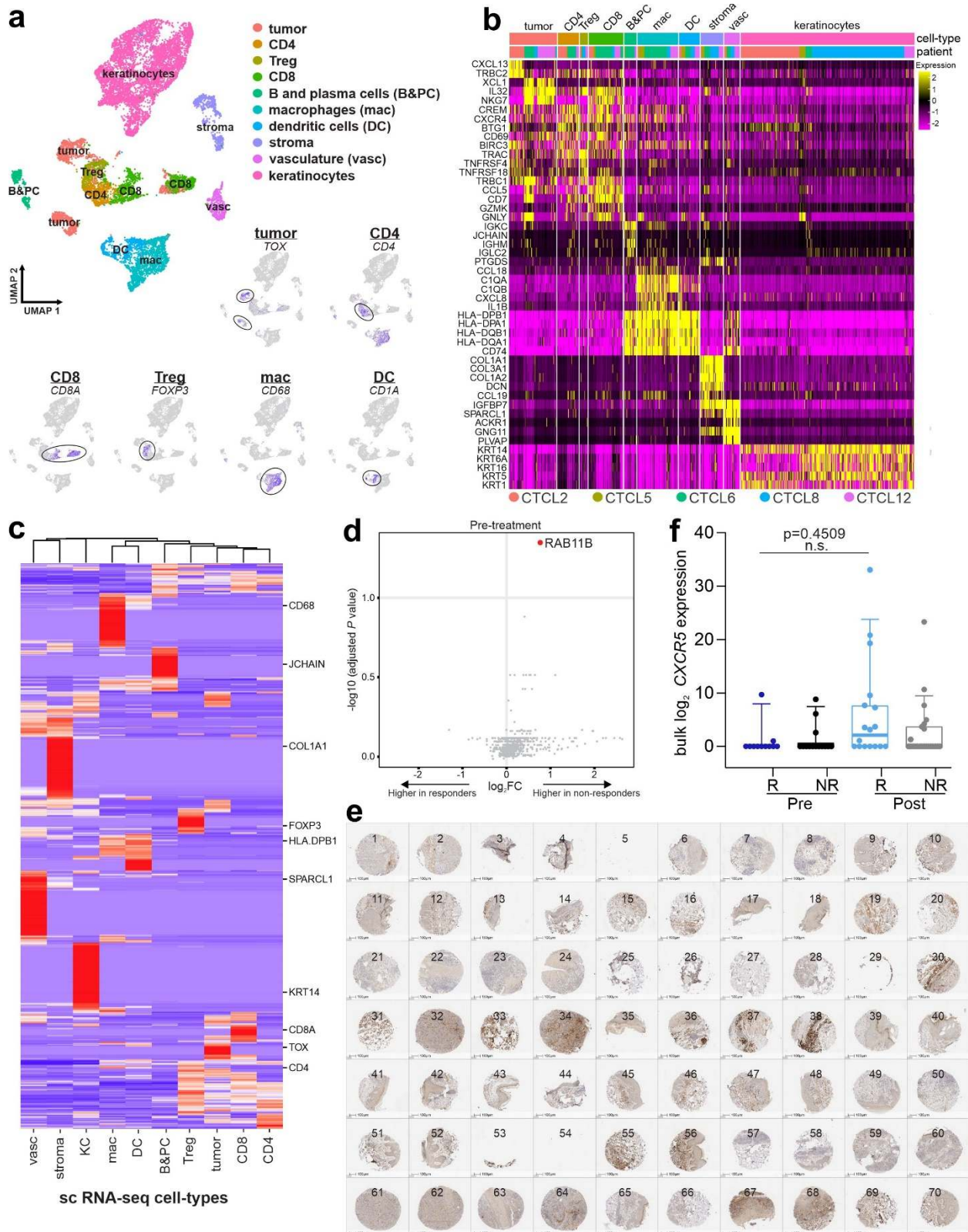
1312 (cyan). Scale bars, 100 μm. **b-e,** Biomarker performance measures, using an optimal cut-off point of

1313 0.7908, for the pre-treatment *SpatialScore* calculated from data obtained with the Vectra mIHC platform

1314 including an ROC curve (b), sensitivity and specificity plots (c), density plots (d), and patient-based scatter

1315 plots (e), whereby 100% of responders were below the *SpatialScore* cut-off point and 85.7% of non-
1316 responders were above the cut-off point).

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1319 **Sup. Fig. 6. Characterizing CIBERSORTx cell-types and CXCL13 expression patterns. a**, UMAP of 10

1320 major cell-types identified in the Gaydosik et al.⁵⁹ CTCL scRNA-seq dataset (see **Methods**), with feature

1321 plots showing expression for a subset of marker genes including *TOX* (tumor cells), *CD4* (CD4⁺ T cells),
1322 *CD8A* (CD8⁺ T cells), *FOXP3* (Tregs), *CD68* (macrophages), and *CD1A* (dendritic cells). **b**, Heatmap of the
1323 top differentially expressed genes (rows) for the 10 cell-types, corresponding to the top color bar. The
1324 other color bar corresponds to patient samples from the Gaydosik et al.⁵⁹ CTCL scRNA-seq dataset
1325 (legend is at the bottom of the heatmap). **c**, Signature matrix, highlighting key marker selected genes
1326 (rows), used to enumerate cell-type fractions and resolve gene expression profiles from CTCL bulk RNA-
1327 seq. **d**, Differential expression of CSx-resolved tumor cell genes in responders versus non-responders
1328 pre-treatment. **e**, CXCL13 IHC staining of the CTCL tissue microarray. Scale bars, 100 μm. **f**, Normalized
1329 bulk *CXCR5* gene expression across patient groups. Boxes, median ± IQR; whiskers, 1.5x IQR. *P* values
1330 were calculated with a linear mixed-effect model with Bonferroni's corrections for multiple
1331 comparisons.

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1336 **Supplementary Table 1. Cohort and tissue microarray layout.**

1337 **Sup. Table 1a. Cohort.**

Patient ID	Age at screening (years)	Gender	Diagnosis	Disease stage	Prior therapies	Response status	Biopsy timepoints	C02 (weeks)	Response / progression time (weeks)	EOT time (weeks)	Death time (weeks)	Follow-up time (weeks)
1	76.14	Male	MF	IIIA	3	Responder	Pre, EOT			104		142
2	60.59	Male	MF	IIIB	4	Responder	Pre, EOT			104		151
3	52.01	Male	SS	IVA	3	Non-responder	Pre, EOT			98	98	98
4	85.14	Female	SS	IVA	4	Non-responder	Pre, EOT			104	142	142
5	75.24	Male	MF	IIB	6	Responder	Pre, EOT			104		103
6	46.74	Male	MF	IVA	9	Non-responder	Pre, C02, EOT	3		25		25
7	46.71	Female	MF	IIIB	3	Non-responder	Pre, EOT			9		9
8	67.44	Male	SS	IIIB	1	Non-responder	Pre, EOT			104	121	121
9	77.65	Female	SS	IVA	6	Responder	Pre, C02, EOT	3		71		71
10	66.40	Male	MF	IIIA	2	Responder	Pre			N/A	119	119
11	72.23	Male	SS	IVA	3	Responder	Pre, Response		22	104		171
12	72.59	Male	SS	IVA	5	Non-responder	Pre, Progression		15	15		15
13	44.10	Female	SS	IVA	1	Responder	Pre, C02, Response, EOT	3	15	104		179
14	63.69	Male	MF	IB	5	Non-responder	Pre, C02, Response, EOT	3	10	92	92	92

MF: mycosis fungoides

SS: Sezary syndrome

Pre: biopsy obtained pre-treatment

C02: biopsy obtained prior to cycle 2

Response / Progression: biopsy obtained at point of response or progression

EOT: end of trial

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1347 **Sup. Table 1b. Tissue microarray layout.**

TMA spot	Patient ID	Biopsy timepoint
1	1	Pre_1
2	1	Pre_2
3	1	EOT_1
4	1	EOT_2
5	2	Pre_1
6	2	Pre_2
7	2	EOT_1
8	2	EOT_2
9	3	Pre_1
10	3	Pre_2
11	3	EOT_1
12	3	EOT_2
13	4	Pre_1
14	4	Pre_2
15	4	EOT_1
16	4	EOT_2
17	5	Pre_1
18	5	Pre_2
19	5	EOT_1
20	5	EOT_2
21	6	Pre_1
22	6	Pre_2
23	6	EOT_1
24	6	EOT_2
25	7	Pre_1
26	7	Pre_2
27	7	EOT_1
28	7	EOT_2
29	8	Pre_1
30	8	Pre_2
31	8	Pre_3
32	8	EOT_1
33	8	EOT_2
34	8	EOT_3
35	9	Pre_1
36	9	Pre_2
37	9	EOT_1
38	9	EOT_2
39	10	Pre_1
40	10	Pre_2
41	11	Pre_1
42	11	Pre_2
43	11	Resp_1
44	11	Resp_2
45	12	Pre_1
46	12	Pre_2
47	12	Resp_1
48	12	Resp_2
49	6	C02_1
50	6	C02_2
51	13	Pre_1
52	13	Pre_2
53	13	Resp_1
54	13	Resp_2
55	13	EOT_1
56	13	EOT_2
57	14	Pre_1
58	14	Pre_2
59	14	Resp_1
60	14	Resp_2
61	14	Resp_3
62	14	EOT_1
63	14	EOT_2
64	14	EOT_3
65	14	C02_1
66	14	C02_2
67	9	C02_1
68	9	C02_2
69	13	C02_1
70	13	C02_2

Pre: pre-treatment biopsy
C02: biopsy obtained prior to cycle 2 (3 weeks)
Response / Progression: biopsy obtained at point of response or progression
EOT: end of trial (104 weeks)

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1350 **Supplementary Table 2. Targets.**

Antibody target	Company	Clone	Oligonucleotide	Fluorophore	Dilution	Exposure time	Reaction cycle	Reaction channel
CD1a	Novus Biologicals	O10+CA1/71	43	Cy5	1:100	1/2s	20	4
CD2	Biologend	RPA-2.10	25	Cy5	1:25	1/2s	7	4
CD3	Cell Marque	MRQ-39	77	Cy5	1:100	1/2s	17	4
CD4	Abcam	EPR6855	20	ATTO550	1:100	1/2s	9	3
CD5	Biologend	UCHT2	75	ATTO550	1:50	1/2s	8	3
CD7	Cell Marque	MRQ-56	63	ATTO550	1:100	1/2s	19	3
CD8	Novus Biologicals	C8/144B	8	Cy5	1:50	1/5s	18	4
CD11b	Abcam	EPR1344	28	Cy5	1:50	1/2s	13	4
CD11c	Abcam	EP1347Y	49	ATTO550	1:50	1/2s	12	3
CD15	BD Biosciences	MMA	14	Alexa488	1:200	1/10s	7	2
CD16	Cell Signaling Technology	D1N9L	26	ATTO550	1:100	1/2s	13	3
CD20	Novus Biologicals	rIGEL/773	48	ATTO550	1:200	1/4s	11	3
CD25	Cell Marque	4C9	24	ATTO550	1:100	1/2s	10	3
CD30	Cell Marque	BerH2	57	ATTO550	1:25	1/2s	7	3
CD31	Novus Biologicals	C31.3+C31.7+C31.10	68	ATTO550	1:200	1/8.5s	25	3
CD34	Novus Biologicals	QBEnd/10	38	ATTO550	1:100	1/4s	23	3
CD38	Abcam	EPR4106	66	ATTO550	1:100	1/2s	24	3
CD45	Novus Biologicals	B11+PD7/26	56	ATTO550	1:400	1/8.5s	20	3
CD45RA	Biologend	HI100	72	Cy5	1:50	1/2s	19	4
CD45RO	Biologend	UCH-L1	2	ATTO550	1:100	1/4s	22	3
CD56	Cell Marque	MRQ-42	29	Cy5	1:50	1/2s	10	4
CD57	Biologend	HCD57	30	ATTO550	1:200	1/4s	21	3
CD68	Biologend	KP-1	70	Cy5	1:100	1/4s	23	4
CD69	R&D Systems	polyclonal	36	ATTO550	1:200	1/2s	18	3
CD71	Cell Marque	MRQ-48	3	Cy5	1:100	1/5s	22	4
CD138	Novus Biologicals	B-A38	76	ATTO550	1:100	1/8.5s	26	3
CD162	Novus Biologicals	HECA-452	46	Cy5	1:200	1/8.5s	12	4
CD163	Novus Biologicals	EDHu-1	45	Cy5	1:200	1/3s	26	4
CD164	BD Biosciences	N6B6	69	Alexa488	1:200	1/2s	3	2
CD194	Biologend	L291H4	55	ATTO550	1:100	1/2s	14	3
Beta-catenin	BD Biosciences	14	51	Cy5	1:50	1/2s	21	4
BCL-2	Novus Biologicals	124	41	ATTO550	1:50	1/2s	17	3
Collagen IV	Abcam	polyclonal	33	Cy5	1:200	1/4s	24	4
Cytokeratin	Biologend	C11	67	Alexa488	1:200	1/5s	6	2
DRAQ5	Cell Signaling Technology	N/A	N/A	Cy5	1:100	1/8.5s	29	4
EGFR	Cell Signaling Technology	D38B1	58	ATTO550	1:25	1/2s	15	3
FoxP3	Abcam	236A/E7	61	ATTO550	1:100	1/4s	3	3
GATA3	Cell Marque	L50-823	60	Cy5	1:100	1/2s	3	4
Granzyme B	Abcam	EPR20129-217	81	Alexa488	1:200	1/8.5s	8	2
HLA-DR	Abcam	EPR2692	65	ATTO550	1:200	1/4s	16	3
ICOS	Cell Signaling Technology	D1K2T	74	Cy5	1:100	1/2s	16	4
IDO-1	Cell Signaling Technology	D5J4E	59	Cy5	1:25	1/2s	14	4
KI-67	BD Biosciences	B56	6	Cy5	1:100	1/5s	6	4
LAG3	Cell Signaling Technology	D2G4O	42	Cy5	1:25	1/2s	9	4
Mast cell tryptase	Abcam	AA1	44	ATTO550	1:200	1/80s	27	3
MMP9	Biologend	L51/82	62	Alexa488	1:400	1/3s	9	2
MMP12	Abcam	polyclonal	80	Cy5	1:100	1/2s	27	4
MUC-1	Novus Biologicals	955	15	Alexa488	1:100	1/2s	4	2
p53	Cell Marque	DO7	52	ATTO550	1:50	1/2s	4	3
PD-1	Cell Signaling Technology	D4W2J	23	Cy5	1:50	1/2s	11	4
PD-L1	Cell Signaling Technology	E1L3N	11	ATTO550	1:50	1/2s	6	3
Podoplanin	Biologend	D2-40	32	Cy5	1:200	1/3s	25	4
T-bet	Cell Signaling Technology	D6N8B	5	ATTO550	1:100	1/2s	5	3
Vimentin	BD Biosciences	RV202	7	Alexa488	1:200	1/4s	5	2
VISTA	Cell Signaling Technology	D1L2G	79	Cy5	1:50	1/2s	15	4

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1354 **Supplementary Table 3. Genes lists; related to Figures 2e-h, 4f-h.**

1355 **Sup. Table 3a. Interferon gamma gene score³⁹.**

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Gene	Coefficient
IFNG	-0.30685
HLA-DRA	-0.35484
IDO1	-0.36499
STAT1	-0.45212
CXCL10	-0.46207
CXCL9	-0.47832

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1358 **Sup. Table 3b. Transforming growth factor beta gene score⁴⁰.**

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Gene	Coefficient
IL6ST	0.48031
PDGFRB	0.40325
TGFBR2	0.39434
TNFRSF1A	0.39151
PDGFA	0.25311
KIT	0.25002
FLT4	0.24409
IFNGR1	0.10497
TNFRSF14	0.03628
ACVR1	-0.03375
TGFB1	-0.04924
TNFRSF10B	-0.15319
LIF	-0.17415
IL4R	-0.20508

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1362 **Sup. Table 3c. Immune activation gene score.**

Gene	Coefficient
CXCL9	0.28056
CCL5	0.27507
GZMH	0.27229
EOMES	0.26916
GZMK	0.26379
CD27	0.25655
TNFRSF9	0.25375
IL2RG	0.24956
PRF1	0.24024
FASLG	0.23683
IL2RA	0.22805
IFNG	0.21895
ICOS	0.20233
CD40	0.18924
CD40LG	0.18919
IL17RA	0.14444
IL12B	0.13478
TNFSF9	0.12999
CD28	0.12862
CX3CR1	0.11662
TNFRSF4	0.11086
GZMB	0.07612
CCR7	0.06002
IL23A	0.05400

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1365 **Sup. Table 3d. Immunosuppression gene score.**

Gene	Coefficient
TGFB1	0.31255
ENTPD1	0.30707
IL24	0.30658
CXCL1	0.30076
IL6	0.29989
TGFBR1	0.27375
IL10	0.24295
HAVCR2	0.20469
PDGFRA	0.20016
HLA-G	0.18822
TGFBRAP1	0.18282
PVT1	0.16990
PDGFRB	0.16723
LINC00473	0.16219
CXCL3	0.16065
TIGIT	0.15326
LIF	0.14962
CXCL12	0.12772
CD274	0.12391
TIAF1	0.11831
LAG3	0.10954
DNM3OS	0.02112
NIFK-AS1	-0.00826
IL4	-0.04139
GNAS-AS1	-0.05070
HOTAIR	-0.06027
PDCD1LG2	-0.08477
GAS5	-0.14413

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1368 **Sup. Table 3e. Cytotoxicity gene score^{49,50}.**

Gene	Coefficient
NKG7	0.39045
PRF1	0.35018
TNF	0.34146
GZMH	0.32965
TBX21	0.31663
GZMK	0.31526
GZMM	0.29080
ID2	0.26046
IFNG	0.25047
GNLY	0.22788
GZMB	0.18149

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1370 **Sup. Table 3f. Tumor aggressiveness^{51,52}.**

Gene	Coefficient
IL2RB	0.30377
BATF	0.29598
IL21R	0.28415
CCND2	0.26500
RGS16	0.25940
MYO7A	0.25910
SLA	0.25436
ANP32E	0.23795
MTHFD2	0.21538
IL10	0.21012
TRIB2	0.20521
RARRES3	0.19264
GNLY	0.19238
DUSP5	0.18767
C1GALT1	0.15656
BCL2	0.15272
EHD1	0.13197
HSPD1	0.13011
DAD1	0.12384
MMP12	0.08265
IL26	0.07073
IL22	0.06240
P4HB	-0.05212
TCN1	-0.07340
SFTPD	-0.12905
TGFBR3	-0.18280

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1372 **Supplementary Table 4. CIBERSORTx marker genes.**

1373 **Sup. Table 4a. Markers of CIBERSORTx cell-types.**

Cell-types	Marker genes	CIBERSORTx cell-types
B cells & plasma cells	<i>MS4A1, LTB, CD79A, CD79B</i>	B and plasma cells
T cells	<i>CD3E, CD3D, CD274</i>	CD4 T, CD8 T, Tregs, tumor cells
Macrophages & dendritic cells	<i>AIF1</i>	Macrophages, dendritic cells
Keratinocytes	<i>KRT1, KRT14</i>	Epithelium
Vasculature	<i>CD43, PECAM1, CLDN5</i>	Vasculature
Fibroblasts	<i>COL1A1, SFRP2</i>	Stroma
Pericytes	<i>RGS5, ACTA2</i>	

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1375 **Sup. Table 4b. T cell and tumor cell markers for CIBERSORTx.**

T cell markers (reactive CD4+, CD8+, Tregs)				Tumor markers			
<i>CD4</i>	<i>FOXP3</i>	<i>IL2</i>	<i>STAT6</i>	<i>ACTG1</i>	<i>CDK1</i>	<i>KIAA0101</i>	<i>PRDX1</i>
<i>CD7</i>	<i>GATA3</i>	<i>IL2RA</i>	<i>TBX21</i>	<i>ANP32B</i>	<i>CDK6</i>	<i>MCTS1</i>	<i>PSMB2</i>
<i>CD8A</i>	<i>GZMA</i>	<i>IL4</i>	<i>TGFB1</i>	<i>ATP5C1</i>	<i>CENPE</i>	<i>MKI67</i>	<i>RAN</i>
<i>CD8B</i>	<i>GZMB</i>	<i>ITGAE</i>	<i>TIGIT</i>	<i>BCL2</i>	<i>CENPM</i>	<i>MYC</i>	<i>RANBP1</i>
<i>CD27</i>	<i>HAVCR2</i>	<i>LAG3</i>	<i>TNF</i>	<i>BCL2L12</i>	<i>DUT</i>	<i>NPM1</i>	<i>SET</i>
<i>CD44</i>	<i>ICOS</i>	<i>PDCD1</i>	<i>TRAC</i>	<i>BIRC3</i>	<i>FOS</i>	<i>NUSAP1</i>	<i>SMC4</i>
<i>CD69</i>	<i>IFNG</i>	<i>PRF1</i>	<i>TRBC1</i>	<i>BIRC5</i>	<i>HMG1</i>	<i>PCNA</i>	<i>STMN1</i>
<i>CD160</i>	<i>IKZF2</i>	<i>RORC</i>	<i>TRBC2</i>	<i>CCNA2</i>	<i>HMMR</i>	<i>PIM2</i>	<i>TOP2A</i>
<i>CTLA4</i>	<i>IL10</i>	<i>RUNX3</i>	<i>TRDC</i>	<i>CCND1</i>	<i>HN1</i>	<i>PLK1</i>	<i>TOX</i>
<i>EOMES</i>	<i>IL17A</i>	<i>STAT3</i>	<i>TRGC1</i>	<i>CDC20</i>	<i>IGF2</i>	<i>PPA1</i>	<i>TSC22</i>
<i>FASLG</i>	<i>IL1B</i>	<i>STAT4</i>	<i>TRGC2</i>	<i>CDCA8</i>	<i>IL2RA</i>	<i>PPIA</i>	

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responder/non-responder; p value calculated by log-rank test). c, IHC protein marker expression in responders and non-responders pre-treatment (mean \pm s.e.m.). P values were calculated by two-sided Wilcoxon's rank-sum tests (p = not significant (n.s.) for all comparisons). d, Representative pre-treatment IHC images for select markers from a responder (top) and non-responder (bottom). e, CODEX antibody panel (see also Sup. Fig. 1d). f, Identification of 21 cell-types by clustering (see also Sup. Fig. 2a-b). g, Visual verification of reactive (blue crosses) versus malignant (red crosses) CD4+ T cells in CTCL tissue. Scale bars, 20 μ m. h, Average expression of select markers on malignant (red bars) relative to reactive (blue line) CD4+ T cells. P values were calculated by two-sided Wilcoxon's rank-sum tests (****, p<0.0001). i, Cell size, measured in pixels/cell, of all malignant (red square) versus reactive (blue square) CD4+ T cells (mean \pm s.e.m.). P value was calculated by a two-sided Wilcoxon's rank-sum test. j, Ranking genes most predictive of tumor cells per tissue microarray spot using an L1-regularized linear model. Red colored genes have positive predictive coefficients (i.e., most likely to represent tumor cells); gray colored genes have negative predictive coefficients (i.e., less likely to represent tumor cells). Known CTCL marker genes are highlighted in yellow.

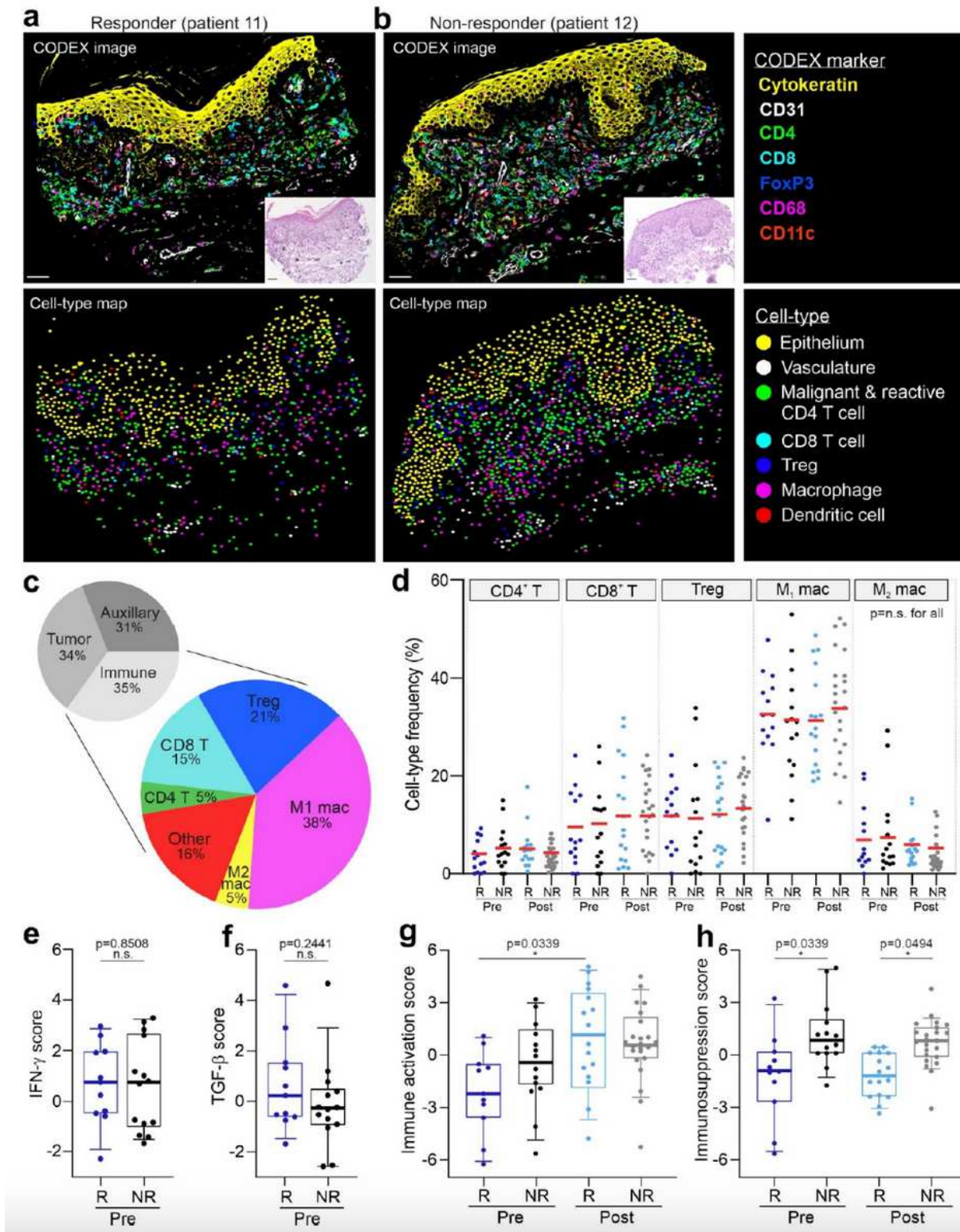


Figure 2

Characterization of the CTCL TME pre- and post-pembrolizumab treatment. a-b, Top panels: Representative CODEX seven-color overlay images from a responder (left) and non-responder (right) pre-treatment. Scale bar, 50 μ m. Insets, corresponding H&E images; scale bars, 50 μ m. Bottom panels: corresponding cell-type maps. c, Upper pie chart: overall frequencies of tumor, immune and auxiliary cell-types. Lower pie chart: overall frequencies of all immune cell-types, including CD4⁺ T cells,

CD8+ T cells, Tregs, M1 macrophages, M2 macrophages, and other (B cells, dendritic cells, Langerhans cells, mast cells, neutrophils, and plasma cells). d, Cell-type frequencies of CD4+ T cell, CD8+ T cell, Treg, M1 macrophage, and M2 macrophage as a percentage of all immune cells per tissue microarray spot in responders and non-responders pre- and post-treatment (mean, red bar). P values were calculated with a linear mixed-effect model with Bonferroni's corrections for multiple comparisons (p = not significant (n.s.) for all comparisons). e-f, IFN- γ (e) and TGF- β (f) gene scores between responders and non-responders pre-treatment per spot. Boxes, median \pm interquartile range (IQR); whiskers, 1.5x IQR. P values were calculated with a linear mixed-effect model with Bonferroni's corrections for multiple comparisons. g-h, Immune activation (g) and immunosuppression (h) gene scores, computed on bulk RNA-seq data, between responders and non-responders pre- and post-treatment per spot. Boxes, median \pm IQR; whiskers, 1.5x IQR. P values were calculated with a linear mixed-effect model with Bonferroni's corrections for multiple comparisons.

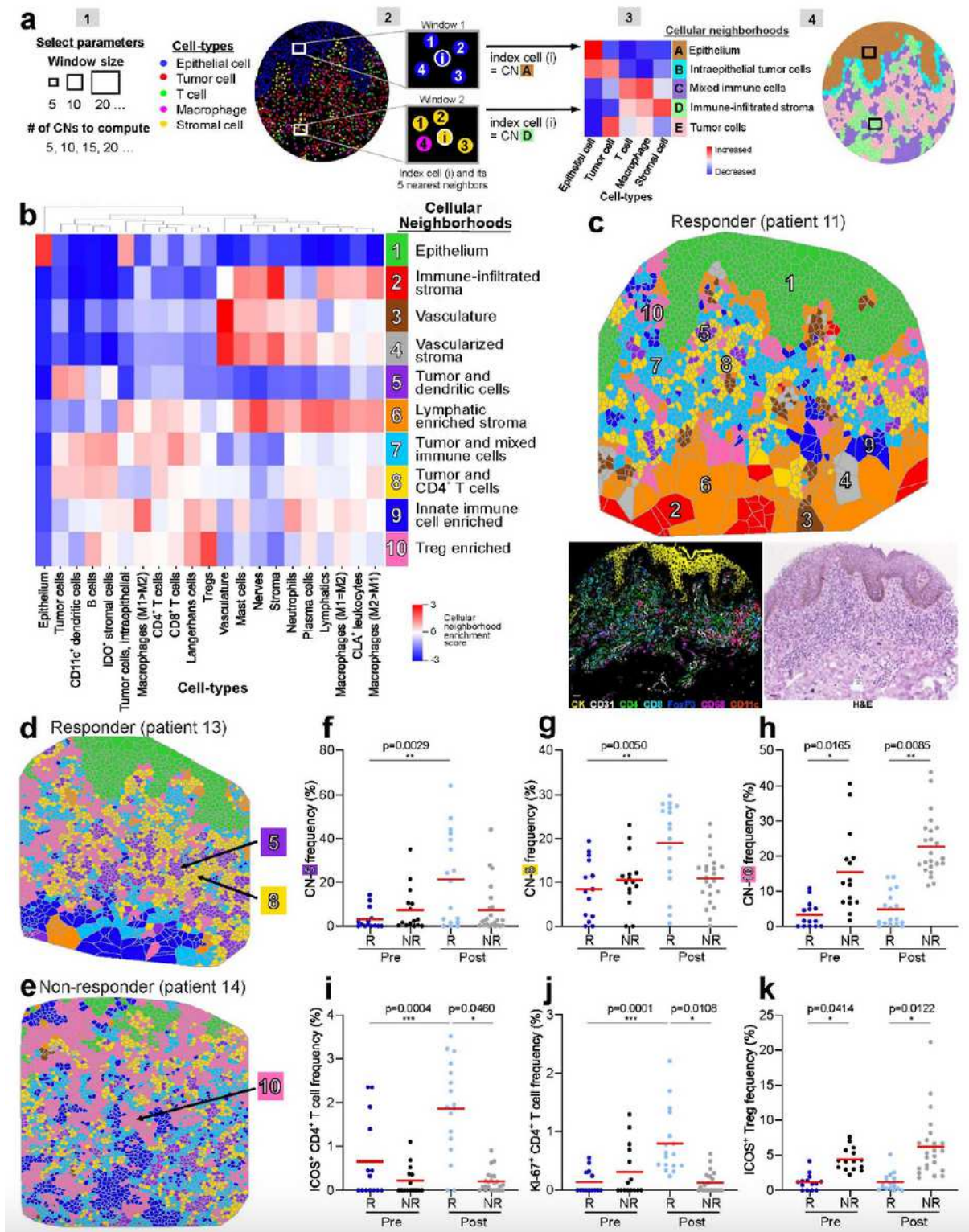


Figure 3

Cellular neighborhoods reveal differences in the spatial TME organization in responders and non-responders. a, Cellular neighborhood (CN) analysis schematic. [1] Selection of computational parameters, including the window size (five in this schematic) and the number of CNs to be computed (five in this schematic). [2] Assignment of an index cell (i, center of window) to a given CN based on the composition of cell-types within its corresponding window the clustering of windows. [3] Heatmap of cell-type

distribution for each CN and assignment of CN names. [4] Visualization of CNs as a Voronoi diagram. b, Identification of 10 conserved CNs in the CTCL TME using a window size of 10. c, Representative Voronoi diagram of the 10 CNs in a responder post-treatment, with the corresponding H&E and seven color fluorescent CODEX images. Scale bar, 20 μm . d-e, Voronoi diagrams of CNs in a responder (d) and non-responder (e) post-treatment, highlighting CN-5 (tumor and dendritic cells), CN-8 (tumor and CD4+ T cells) and CN-10 (Treg enriched). f-h, Frequencies of CN-5 (f), CN-8 (g) and CN-10 (h) per tissue microarray spot in responders and non-responders pre- and post-treatment (mean, red bar). P values were calculated with a linear mixed-effect model with Bonferroni's corrections for multiple comparisons. i-k, Frequencies of ICOS+ CD4+ T cell (i), Ki-67+ CD4+ T cell (j) and ICOS+ Treg (k) as a percentage of all immune cells per spot in responders and non-responders pre- and post-treatment (mean, red bar). P values were calculated with a linear mixed-effect model with Bonferroni's corrections for multiple comparisons.

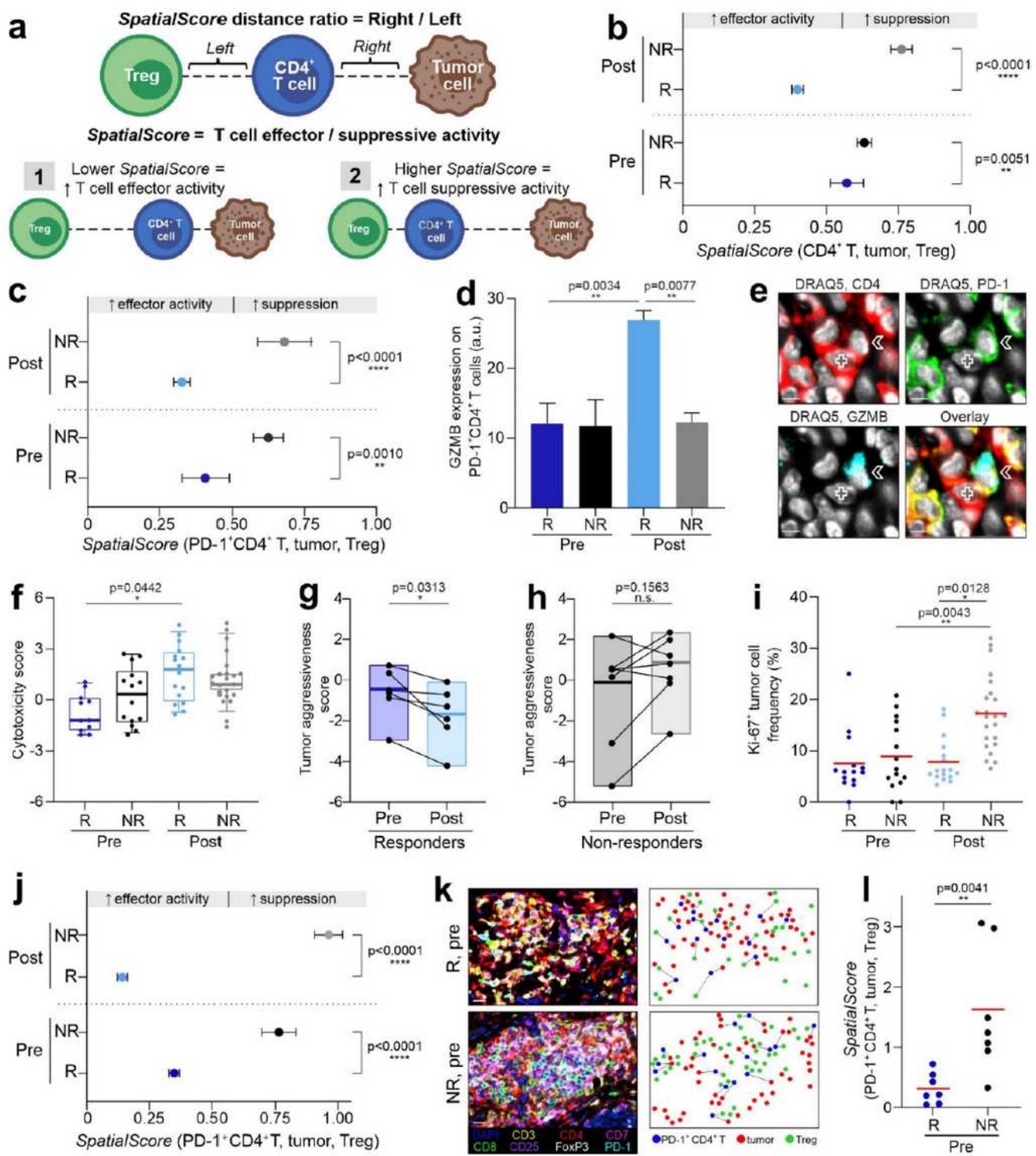


Figure 4

Spatial relationship between CD4⁺ T cells, Tregs and tumor cells predicts pembrolizumab response in CTCL. a, SpatialScore schematic. The SpatialScore is calculated by taking the ratio of the physical distance between each CD4⁺ T cell and its nearest tumor cell (distance “right”) relative to its nearest Treg (distance “left”). [1] A lower SpatialScore (i.e., CD4⁺ T cells are closer to tumor cells than Tregs) suggests increased T cell effector activity. [2] A higher spatial score (i.e., CD4⁺ T cells are closer to Tregs than

tumor cells) suggests increased T cell suppression. b-c, SpatialScore on a per cell basis for CD4+ T cells (b) and PD-1+ CD4+ T cells (c) for each patient group (mean \pm s.e.m.). P values were calculated by two-sided Wilcoxon's rank-sum tests, with no adjustments for multiple comparisons. d, GZMB protein expression on PD-1+ CD4+ T cells by CODEX in responders and non-responders pre- and post-treatment per tissue microarray spot (mean fluorescence intensity (arbitrary units, a.u.) \pm s.e.m.) P values were calculated with a linear mixed-effect model with Bonferroni's corrections for multiple comparisons. e, CODEX images of DRAQ5 (white), CD4 (red), PD-1 (green), GZMB (cyan), and overlay showing contact between a tumor cell (marked by cross) and GZMB-expressing PD-1+ CD4+ T cell (marked by arrow) in responder patient 13 post-treatment. Scale bars, 10 μ m. f, Cytotoxicity gene scores, computed on bulk RNA-seq data, between responders and non-responders pre- and post-treatment per spot. Boxes, median \pm IQR; whiskers, 1.5x IQR. P values were calculated with a linear mixed-effect model with Bonferroni's corrections for multiple comparisons. g-h, Pre- to post-treatment changes in tumor aggressiveness gene scores, computed on bulk RNA-seq data, on a per patient basis in responders (g) and non-responders (h). Boxes, median \pm IQR; whiskers, 1.5x IQR. P values were calculated by two-sided Wilcoxon's signed-rank tests. i, Ki-67+ tumor cell frequencies per spot in responders and non-responders pre- and post-treatment (mean, red bar). P values were calculated with a linear mixed-effect model with Bonferroni's corrections for multiple comparisons. j, SpatialScore on a per cell basis calculated from Vectra mIHC data using PD-1+CD4+ T cells for each patient group (mean \pm s.e.m.). P values were calculated by two-sided Wilcoxon's rank-sum tests, with no adjustments for multiple comparisons. k, Vectra mIHC images (left panels; representative regions of a tissue microarray spot) in responder patient 13 (R) and non-responder patient 14 (NR) pre-treatment. Scale bars, 20 μ m. The corresponding spatial plots (right panels) show that PD-1+ CD4+ T cells (blue dots) are closer to tumor cells (red dots) than Tregs (green dots) in the responder and vice versa in the non-responder. l, Pre-treatment SpatialScore calculated from Vectra mIHC data for responders and non-responders on a per patient basis (mean, red bar). P values was calculated by a two-sided Wilcoxon's rank-sum test.

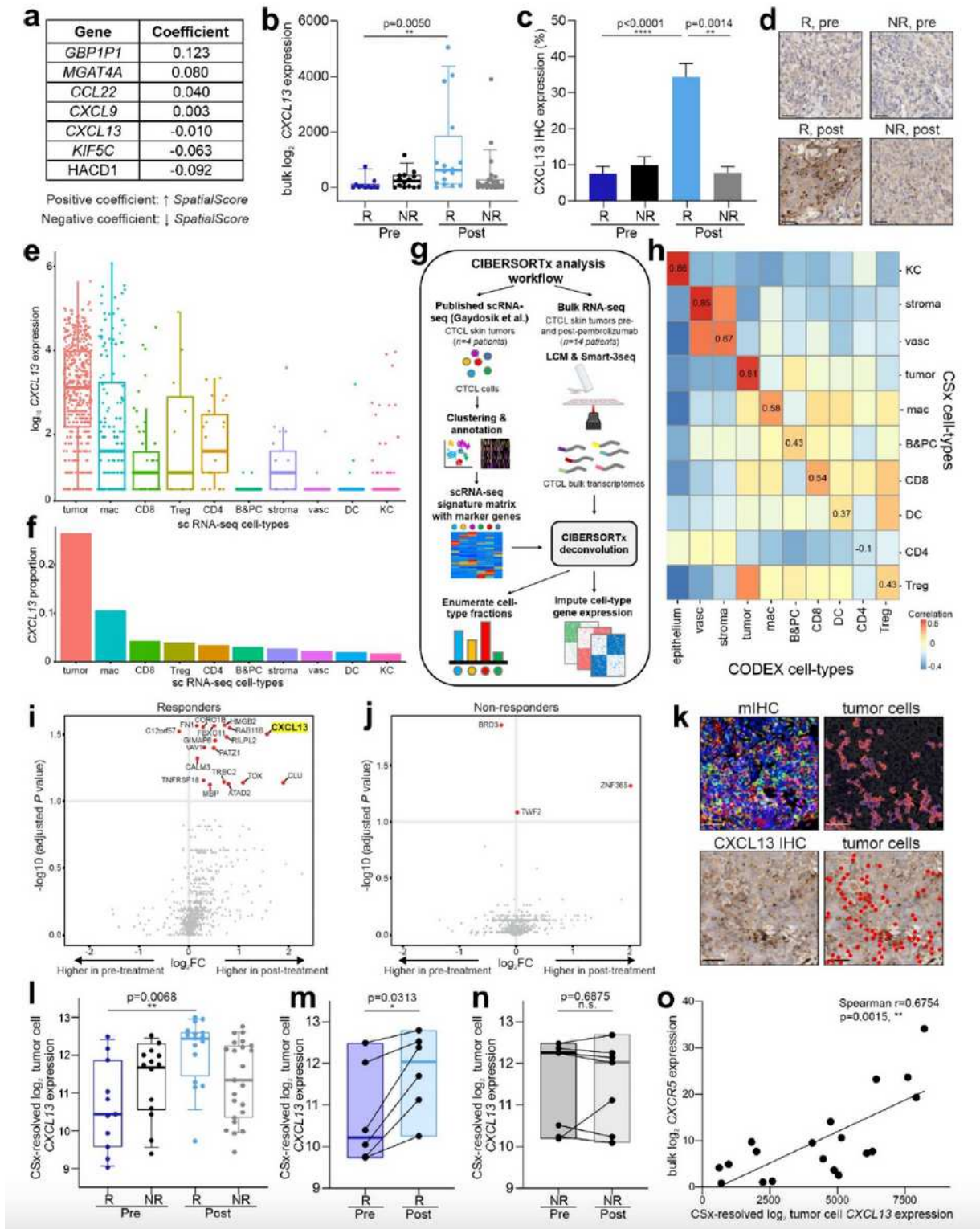


Figure 5

CXCL13 is a key driver of pembrolizumab response in CTCL. a, Seven genes predictive of the *SpatialScore* identified from bulk RNA-seq data. Genes with a positive coefficient are associated with a higher *SpatialScore* (i.e., a suppressive phenotype), whereas those with a negative coefficient are associated with a lower *SpatialScore* (i.e., an effector phenotype). b, Normalized bulk CXCL13 gene expression between responders and non-responders pre- and post-treatment per tissue microarray spot.

Boxes, median \pm IQR; whiskers, 1.5x IQR. P values were calculated with a linear mixed-effect model with Bonferroni's corrections for multiple comparisons. c, CXCL13 protein expression by IHC in responders and non-responders pre- and post-treatment per spot (mean \pm s.e.m.). P values were calculated with a linear mixed-effect model with Bonferroni's corrections for multiple comparisons. d, Representative CXCL13 IHC images from responder patient 9 pre-treatment (top left) and post-treatment (bottom left) as well as non-responder patient 14 pre-treatment (top right) and post-treatment (bottom right). Scale bars, 20 μ m. e-f, Single-cell transcriptomes from a publicly available scRNA-seq dataset of CTCL skin tumors (Gaydosik et al.)⁵⁹ were analyzed for CXCL13 expression. Abbreviations: mac (macrophages), B&PC (B and plasma cells), vasc (vasculature), DC (dendritic cells), and KC (keratinocytes). e, Normalized expression of CXCL13 in single cells (each dot represents a cell positive for CXCL13). Cells with CXCL13 log₁₀ normalized read counts less than 0.5 were excluded (see Methods). Boxes, median \pm IQR; whiskers, 1.5x IQR. f, Proportion of CXCL13-expressing cells per cell-type. g, CIBERSORTx analysis workflow schematic. Single cell transcriptomes from Gaydosik et al.⁵⁹ were used to generate a CSx deconvolution signature matrix, consisting of cell-type-specific marker genes (left portion of schematic). This matrix was then applied to CTCL bulk transcriptomes obtained with laser-capture microdissection (LCM) and Smart-3Seq (right portion of schematic) to enumerate cell-type fractions and resolve gene expression profiles (see Methods). h, Heatmap of the correlation between CSx-resolved cell-type frequencies and CODEX-identified cell-type frequencies. Spearman correlation coefficients between corresponding cell-types are shown along the diagonal. For this analysis, some of the original 21 CODEX cell-types were manually merged to match CSx cell-types (e.g., B cells and plasma cells). i-j, Volcano plots of differential gene expression of CSx-resolved tumor cell genes in responders (j) and non-responders (k) pre- and post-treatment. P values were calculated with a linear mixed-effect model with Benjamini-Hochberg correction. Significantly different genes ($p < 0.1$) are colored red; CXCL13 is highlighted yellow. k, Vectra mIHC image of DAPI (blue), CD3 (yellow), CD4 (red), CD7 (magenta), CD8 (green), CD25 (purple), FoxP3 (white), and PD-1 (cyan) (top left), corresponding tumor cell depiction (top right), corresponding CXCL13 IHC image (bottom left), and overlaid image showing that CXCL13 IHC staining most commonly localized to tumor cells (red circles) (bottom right) in responder patient 9 post-treatment. Scale bars, 20 μ m. l, Normalized CSx-resolved CXCL13 expression in tumor cells between responders and non-responders pre- and post-treatment per spot. Boxes, median \pm IQR; whiskers, 1.5x IQR. P values were calculated with a linear mixed-effect model with Bonferroni's corrections for multiple comparisons. m-n, Pre- to post-treatment changes in normalized CXCL13 gene expression from CSx-resolved tumor genes on a per patient basis in responders (m) and non-responders (n). Boxes, median \pm IQR; whiskers, 1.5x IQR. P values were calculated by two-sided Wilcoxon's signed-rank tests. o, Correlation of CSx-resolved tumor cell CXCL13 expression and bulk CXCR5 expression per spot. Data was evaluated with the Spearman test and a two-tailed t-distribution with $n-2$ degrees of freedom.

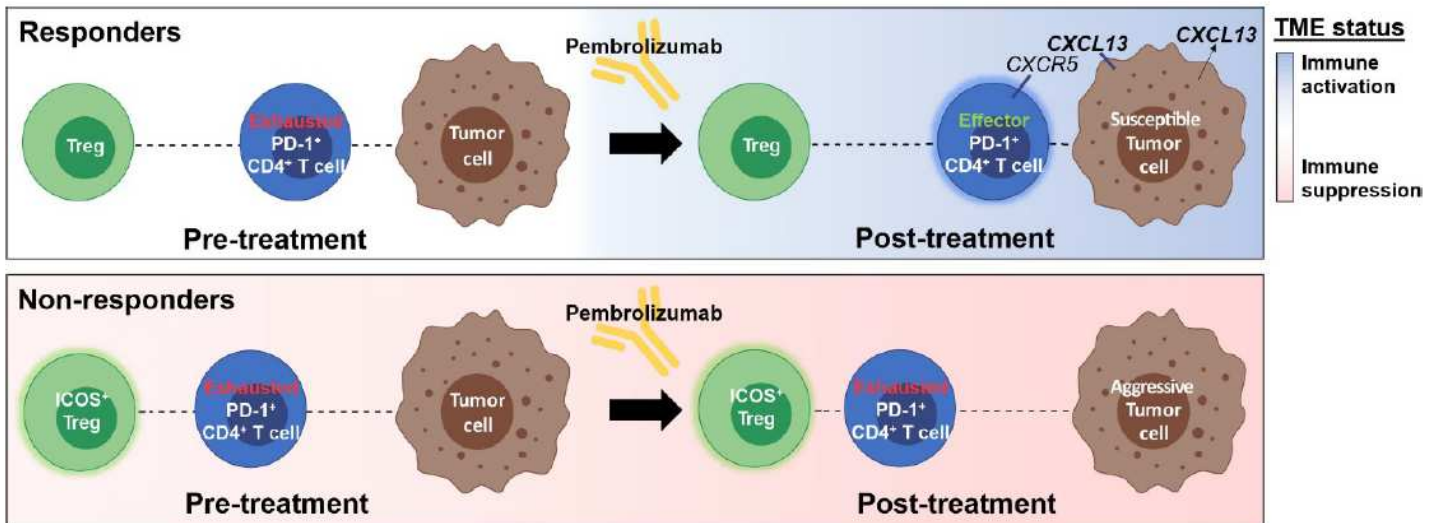


Figure 6

Proposed mechanisms of pembrolizumab response in CTCL. Proposed mechanisms of pembrolizumab response in therapy responders (top panel) and non-responders (bottom panel) pre- and post-treatment. The functional immune status of the TME is represented by blue shading when activated and pink shading when suppressed. In non-responders, the TME is continually immunosuppressed and persistently exhausted PD-1⁺CD4⁺ T cells are in closer proximity to potentially suppressive ICOS⁺ Tregs. Due to pembrolizumab resistance, non-responder tumor cells become more aggressive following therapy. In contrast, responders have a neutral functional immune state pre-treatment, which becomes activated following pembrolizumab therapy, enabling the transition from exhausted to effector PD-1⁺ CD4⁺ T cells. Additionally, responder tumor cells are susceptible to PD-1 blockade and overexpress CXCL13. This attracts effector PD-1⁺ CD4⁺ T cells toward tumor cells, providing a mechanism for the sustained clinical response seen in responders.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementalData.pdf](#)