1   **Title:** Tumor cell heterogeneity drives spatial organization of the intratumoral immune

2   response in squamous cell skin carcinoma

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22   **Keywords:**

23   Intratumoral heterogeneity, tumor immune microenvironment, immune checkpoint blockade,

24   spatial immune heterogeneity

25

26   **Abstract:**

27   Intratumoral heterogeneity (ITH)—defined as genetic and cellular diversity within a tumor—is

28   linked to failure of immunotherapy and an inferior anti-tumor immune response. The

29   underlying mechanism of this association is unknown. To address this question, we modeled

30   heterogeneous tumors comprised of a pro-inflammatory ("hot") and an immunosuppressive

31   ("cold") tumor population, labeled with YFP and RFP tags respectively to enable precise spatial

32   tracking. The resulting mixed-population tumors exhibited distinct regions comprised of YFP^+

33   (hot) cells, RFP^+ (cold) cells, or a mixture. We found that tumor regions occupied by hot tumor

34   cells (YFP^+) harbored more total T cells and a higher frequency of Th1 cells and $IFN\gamma^+$ CD8 T cells

35   compared to regions occupied by cold tumor cells (RFP^+), whereas immunosuppressive

36   macrophages showed the opposite spatial pattern. We identified the chemokine CX3CL1,

37   produced at higher levels by our cold tumors, as a mediator of intratumoral macrophage

38   accumulation, particularly immunosuppressive $CD206^{Hi}$ macrophages. Furthermore, we

39   examined the response of heterogeneous tumors to a therapeutic combination of PD-1

40   blockade and CD40 agonist on a region-by-region basis. While the combination successfully

41   increases Th1 abundance in "cold" tumor regions, it fails to bring overall T cell activity to the

42   same level as seen in "hot" regions. The presence of the "cold" cells thus ultimately leads to a

43   failure of the therapy to induce tumor rejection. Collectively, our results demonstrate that the

44   organization of heterogeneous tumor cells has a profound impact on directing the spatial

45   organization and function of tumor-infiltrating immune cells as well as on responses to

46   immunotherapy.

47

48

49   **Introduction:**

50   Immunotherapies have shown great promise in treating cancer by activating a patient's

51   own immune cells to fight their tumor, but only a fraction of patients respond to current

52   treatment regimens[1–4]. Immune checkpoint blockade (ICB) therapy routinely achieves durable

53   cures in only a limited number of cancer types, and in many tumor types, response rates are as

54   low as 15% with high relapse rates[3,4]. Genetic heterogeneity within a tumor is a significant

55   factor that has been linked to poor ICB response[5–7]. Tumors are often comprised of multiple

56   populations of cancer cells, with each population carrying a distinct set of genetic alterations

57   and phenotypic behaviors[8–10]. Genetic heterogeneity—often given the generalized label

58   "intratumoral heterogeneity" (ITH)—is inherent in all cancer types[6], and studies in multiple

59   cancer types have shown that tumors with high genetic heterogeneity (high ITH) are less likely

60   to respond to ICB regimens[5,7]. Further, in patients, tumors are frequently a patchwork of "hot"

61   and "cold" regions[11–13]—characterized by high and low T cell infiltrates, respectively —

62   indicating that a dysfunctional T cell phenotype can be spatially localized[14]. One study of 85 pre-

63   treatment lung cancers revealed that over two-thirds of tumors investigated contained both

64   hot and cold regions[11]. However, model systems that lend themselves to mechanistically

65   interrogating the impact of tumor heterogeneity on the spatial organization of immune cells

66   within a tumor are limited at present.

67   Heterogeneity-induced impairment of the anti-tumor immune response has also been

68   observed in mouse models[15–17]. A mixture of two mouse pancreatic ductal adenocarcinoma

69   (PDAC) cell lines, one giving rise to immune hot and the other to immune cold tumors on its

70   own, resulted in an overall cold tumor, suggesting that coldness may be a dominant

71   phenotype[15]. Another study in a murine melanoma model showed that high-heterogeneity

72   tumors exhibited an inferior CD8 T cell response compared to low-heterogeneity tumors[16].

73   Building on this, we set out to ask not only how tumor heterogeneity impacts the immune

74   response in the tumor as a whole, but also to interrogate its impact on the spatial organization

75   of the intratumoral immune response.

76   Our studies employed tumor cell lines derived from carcinogen-induced squamous cell

77   skin carcinomas, induced by dimethylbenzanthracene and 12-O-tetradecanoylphorbol-13-

78   acetate (DMBA/TPA)[18,19]. The DMBA/TPA skin carcinogenesis model gives rise to tumors that

79   exhibit a physiologically relevant range of mutational burdens[20,21] and develop in immune-

80   competent hosts, resulting in tumors with neoantigen and immune profiles that mimic those

81   found in human tumors. We have generated cell lines from DMBA/TPA-induced tumors, which,

82   when implanted into mice, give rise to tumors with reproducible immune phenotypes. Here, we

83   develop a novel model of heterogeneous tumors by implanting a mixture of two such cell lines.

84   Each cell line, labeled with a fluorophore, represents a distinct, trackable population within the

85   tumor. The two cell lines we selected share a common oncogenic driver, *Hras* Q61L, but also

86   each contain over 100 distinct nonsynonymous mutations; their combination thus models a

87   genetically heterogeneous tumor.

88   Using this novel model system, we here interrogate the mechanisms by which

89   heterogeneity in tumor cells impacts the immune response, the spatial organization of

90   infiltrating immune cells, and the response to ICB therapy. We find, not surprisingly, that the

91   cold tumor population drives an overall cold immune phenotype at a whole tumor level.

92   Further, we observe that our constituent tumor populations form a patchwork pattern within

93   the tumor. Our study reveals that the spatial organization of the tumor cells themselves creates

94   an architectural blueprint that dictates the spatial localization and function of infiltrating

95   immune cells. We observe T cells preferentially accumulating and exhibiting superior effector

96   function in the neighborhood of hot tumor cells, while cold tumor cells recruit an

97   immunosuppressive microenvironment to their immediate vicinity. The presence of the cold

98   tumor cells further mediates resistance to ICB therapy in mixed-population tumors. However,

99   to our surprise, we observe that immunotherapy successfully improves the quality of the

100   immune response in cold tumor regions—but not to a sufficient degree to induce robust tumor

101   regression. Our model system of heterogeneity thus illuminates how heterogeneity in tumor

102   cells impacts both the endogenous anti-tumor immune response and the efficacy of therapy on

103   a highly localized spatial scale.

104

105   **Results:**

106

107   **Modeling heterogeneous tumors with trackable tumor populations.**

108   We have established a series of squamous cell skin carcinoma cell lines, named the CIT

109   ("carcinogen-induced tumor") lines, derived from DMBA/TPA-induced skin carcinomas that

110   were initiated in *K5-CreER^T2-Confetti* mice on an FVB background[19]. To establish a model system

111   in which to study the impact of tumor heterogeneity on the immune response, we selected and

112   combined two of these cell lines, CIT6 and CIT9, that give rise respectively to tumors exhibiting

113   a high and low frequency of overall T cells, CD4 T cells and CD8 T cells, representing

114   immunologically hot and cold tumors. We introduced fluorescent labels into these tumor cells,

115   taking advantage of CIT lines originating from mice carrying an unactivated *Confetti* cassette.

116   We treated CIT6 and CIT9 with adenoviral Cre recombinase (AdCre), leading to recombination

117   of the *Confetti* allele and stochastic labeling of each cell with one of four fluorescent proteins—

118   yellow, red, cyan and green fluorescent protein (YFP, RFP, CFP and GFP, respectively)[22]. We

119   then sorted YFP+ cells from the CIT6 line and RFP+ cells from the CIT9 line to establish

120   fluorescently-tagged CIT6-YFP and CIT9-RFP cell lines. For all subsequent experiments, these

121   fluorescently labeled cell lines were implanted into syngeneic *Confetti* mice, carrying an

122   unactivated *Confetti* allele, which recapitulated the expected hot and cold immune phenotypes

123   we observed when unlabeled CIT6 and CIT9 tumor cells were implanted into wild-type FVB mice

124   (**Fig. 1A-D**; Supp Fig. 1).

125   To model heterogeneous tumors, we injected mice subcutaneously with an equal

126   mixture of CIT6-YFP and CIT9-RFP cell lines (6.25×10^4 cells each, totaling 1.25×10^5 cells per

127   injection) (**Fig. 1E**). Tumors were assessed for composition by fluorescent stereomicroscope at

128   the time of harvest, when tumors were 1cm in longest diameter (21-28 days). We consistently

129   observed approximately 2/3 of the resulting tumors were comprised of both YFP+ and RFP+ cells

130   with clearly defined YFP+ and RFP+ regions, whereas the remaining 1/3 consisted of only RFP+

131   cells in most cases, or only YFP+ cells in rare cases (**Fig. 1F**, data not shown). The tumors that

132   contained both YFP[+] and RFP^{+} cells, termed "mixed-population tumors" herein, were used for

133   downstream analyses. When the equal mixture of CIT6-YFP and CIT9-RFP cells was implanted

134   into immunodeficient NOD/SCID/IL-2Rɣ null (NSG) mice, which lack T, B and NK cells and innate

135   lymphoid cells (ILCs), we observed a higher penetrance of mixed-population tumors, indicating

136   that immune activity against tumor cells was likely responsible for cases where mixed tumors

137   failed to establish (Supp Fig. 2). Inspection of the mixed-population tumors by cryosectioning

138   and fluorescent imaging showed a patchwork of regions that could be classified as being

139   occupied primarily by YFP^{+} cells, primarily by RFP^{+} cells, or by a mixture of both populations

140   (**Fig. 1G**). The fluorescent tags in these cell lines thus successfully enabled spatial tracking and

141   quantification of each tumor population in the mixed-tumor population tumors and enabled us

142   to establish a novel model to study ITH.

143

144   **An immunosuppressive phenotype dominates the immune microenvironment of mixed-**

145   **population tumors.**

146   To determine how each constituent tumor population affects the overall immune

147   response in mixed-population tumors, we performed a comprehensive flow cytometric analysis

148   of the immune infiltrates (Supp Fig. 3) found in CIT6-YFP + CIT9-RFP mixed-population tumors

149   and compared this to infiltrates found in each CIT6-YFP and CIT9-RFP single-population tumors.

150   Mixed-population tumors have an overall cold immune phenotype, exhibiting CD4 and CD8 T

151   cell infiltration comparable to CIT9-RFP tumors and significantly lower than that found in CIT6-

152   YFP tumors (**Fig. 2A**). MHC-I proteins were expressed on more than 85% of CIT6-YFP and CIT9-

153   RFP tumor cells and expressed at a similar level (**Fig. 2B**), suggesting that the differences in T

154   cell abundance are not due to the differences in MHC-I expression. MHC-II expression was very

155   low on both CIT6-YFP and CIT9-RFP tumor cells (Supp. Fig. 4A), in line with these tumors being

156   of epithelial origin. Although there was no significant difference in the fraction of CD4 T cells

157   that were regulatory T cells (Tregs; CD4$^+$ CD25$^+$ Foxp3$^+$) or in the CD8 T cell-to-Treg ratio

158   between any of the groups, CIT9-RFP and mixed-population tumors showed a lower CD4 Teff

159   (CD4$^+$ CD25$^-$ Foxp3$^-$)-to-Treg cell ratio than CIT6-YFP tumors, suggesting a skewing in CIT9-RFP

160   and mixed tumors toward an immunosuppressive microenvironment (**Fig. 2C**). Other

161   lymphocytes, specifically B cells and NKp46$^+$ lymphocytes (including natural killer (NK) cells and

162   innate lymphoid cells (ILCs)), showed a similar pattern: CIT6-YFP tumors showed significantly

163   higher infiltration by both lymphoid populations than CIT9-RFP tumors, and mixed-population

164   tumors overall resembled CIT9-RFP tumors (although the difference between NKp46$^+$

165   lymphocytes in CIT6-YFP versus mixed tumors was not statistically significant) (**Fig. 2D**). By

166   contrast, CIT9-RFP tumors showed a higher infiltration than CIT6-YFP tumors of F4/80$^+$ CD11b$^+$

167   MHC-II$^+$ macrophages (**Fig. 2E**) and CD11b$^+$ myeloid cells (Supp Fig. 4B), which often harbor

168   immunosuppressive properties in the tumor microenvironment[23,24]. Mixed-population tumors

169   also had an increase in CD11b$^+$ myeloid cells and a trend toward more macrophages compared

170   to CIT6-YFP tumors, again exhibiting more similarity in immune profile to CIT9-RFP tumors (**Fig.**

171   **2E,** Supp Fig. 4B). Ly6G$^+$ Ly6C^{mid} neutrophils, Ly6G$^-$ Ly6C$^+$ monocytes, and CD11b$^+$ CD11c$^+$ MHC-

172   II$^+$ F4/80$^-$ dendritic cells (DCs), which are conventionally anti-tumoral effector cells, all showed a

173   trend toward lower frequency in both CIT9-RFP and mixed tumors compared to CIT6-YFP

174   tumors, albeit not statistically significant differences (Supp Fig. 4C). Collectively, these data

175   support our designation of CIT6-YFP tumors as pro-inflammatory and immune hot, and of CIT9-

176   RFP tumors as immunosuppressive and immune cold, and further reveal that the

177   immunosuppressive phenotype is dominant in mixed-population tumors.

178   Macrophages are conventionally known to facilitate antigen presentation and assist in T

179   cell activation, but in the context of a tumor, evidence suggests that tumor-associated

180   macrophages (TAMs) are more frequently a mediator of immunosuppression[24]. TAMs are the

181   most abundant immune cells in CIT6-YFP and CIT9-RFP tumors, comprising 30-80% of total

182   CD45$^+$ immune cells. Therefore, we sought to determine if F4/80$^+$ CD11b$^+$ MHC-II$^+$ macrophages

183   in our tumors are functionally immunosuppressive. Macrophages were sorted from CIT6-YFP,

184   CIT9-RFP, and mixed-population tumors, and assessed for their capacity to suppress CD8 T cells

185   isolated from naïve, non-tumor bearing syngeneic *Confetti* mice and activated by CD3/CD28

186   beads. In comparison to CD8 T cells alone, the addition of TAMs significantly suppressed CD8 T

187   cell proliferation, measured as the number of cells that had undergone division (as marked by

188   CFSE dilution) (**Fig. 2F**). The expansion of activated T cells, measured as CD69$^+$ CD25$^+$ CD8$^+$ T

189   cells (early activation), CD69$^-$ CD25$^+$ CD8$^+$ T cells (late activation), or PD-1$^+$ CD8$^+$ T cells, showed

190   similar results, with the addition of macrophages decreasing the number of activated T cells

191   (**Fig. 2G**, Supp Fig. 4D). Moreover, the expansion of the CD62L$^-$ CD44$^+$ effector T cell subset and

192   the CD62L$^+$ CD44$^+$ central memory T cell subset was significantly suppressed in the presence of

193   TAMs (**Fig. 2H**, Supp Fig. 4E). Surprisingly, no difference in immunosuppressive capacity was

194   observed between TAMs derived from CIT6-YFP, CIT9-RFP or mixed-population tumors (**Fig. 2F-**

195   **H**). Thus, macrophages in CIT6-YFP, CIT9-RFP and mixed-population tumors are equally

196   immunosuppressive, but we conclude that the increased abundance of these macrophages in

197   CIT9-RFP and mixed-population tumors **(Fig. 2E)** may be responsible for creating a more

198   suppressive immune microenvironment in those tumors.

199

200   **Spatial organization of tumor populations drives the spatial organization of immune cells.**

201   Since the mixed-population tumors were comprised of a patchwork of YFP, mixed, and

202   RFP tumor regions, we next sought to better understand whether the "dominant cold" immune

203   phenotype was uniformly true in all tumor regions. To this end, we first quantified CD3$^+$ T cells

204   by immunofluorescent (IF) staining of 8μm sections. We classified each field of view as

205   predominantly occupied by CIT6-YFP cells ("YFP regions"; >60% of tumor cells are YFP$^+$),

206   predominantly occupied by CIT9-RFP cells ("RFP regions"; >60% of tumor cells are RFP$^+$), or

207   mixed ("mixed regions"; YFP and RFP each represent more than 40% of tumor cells), and then

208   quantified the number of CD3$^+$ T cells in the field (**Fig. 3A**). This analysis revealed a significantly

209   higher average count of CD3$^+$ T cells in YFP regions compared to RFP regions, with mixed

210   regions showing an intermediate level (**Fig. 3B**). Further, we observed a significant positive

211   correlation between the number of T cells and the fraction of YFP tumor cells (R^2=0.22, *p*=4.7 x

212   10^{-35}) in each field, and noticed that the fields of view with the highest number of infiltrating T

213   cells were all comprised of >50% YFP$^+$ cells (**Fig. 3C**). These data suggest that T cells

214   preferentially localize to the regions occupied by CIT6-YFP tumor cells in mixed-population

215   tumors.

216   We next turned to flow cytometry-based analysis of the immune cells in each YFP,

217   mixed, and RFP regions to gain a higher dimensional profile of the immune response in each

218   region. Mixed population tumors were embedded into agarose gel and each tumor was sliced

219 into five to ten 400µm-thick live sections. Each section was manually microdissected into YFP,

220 mixed, and RFP regions using a surgical scalpel under a fluorescent dissecting microscope. All

221 YFP pieces, all mixed pieces, and all RFP pieces from the same tumor were pooled and digested

222 into a single cell suspension for immune profiling by flow cytometry (**Fig. 3D**). Mirroring our

223 findings by IF, we found a higher frequency of total CD3$^+$ T cells in YFP regions than in RFP

224 regions, with mixed regions showing intermediate infiltration **(Fig. 3E)**. This localization pattern

225 was also seen in, and was driven by, CD4 T cells. The abundance of total T cells and CD4 T cells

226 in YFP regions of mixed-population tumors was comparable to that in single-population CIT6-

227 YFP tumors, and similarly, the abundance of total and CD4 T cells in RFP regions of mixed

228 tumors was comparable to that in single-population CIT9-RFP tumors (**Fig. 3F**). By contrast, CD8

229 T cells were at low abundance in all tumor regions, including YFP regions, and were found at

230 levels resembling those in single-population CIT9-RFP tumors (**Fig. 3G**). A more detailed analysis

231 of T cell function showed that nearly one-third of the CD4 T cells in YFP regions were Th1 cells

232 (CD4$^+$ IFNγ$^+$), whereas only ~2% of CD4 T cells in RFP regions were Th1 cells (median frequency

233 of Th1 cells in YFP regions = 28.9%, RFP regions = 1.99%) (**Fig. 3H**). Th2 (CD4$^+$ IL-4$^+$) and Th17

234 (CD4$^+$ IL-17$^+$) cells were equally rare in all regions (**Fig. 3I**). Even more strikingly, despite their

235 low abundance, CD8 T cells showed increased effector function in YFP regions: over 70% of CD8

236 T cells in YFP regions showed capacity to produce IFNγ, whereas less than 10% of CD8 T cells in

237 mixed and RFP regions showed the same (**Fig. 3J**). There was no apparent difference in Treg

238 (Foxp3$^+$ CD25$^+$) cell frequency between regions, but the ratio of Treg:Th1 cells and Treg: IFNγ$^+$

239 CD8 T cells was higher in RFP regions compared to YFP and mixed regions, suggestive of an

240 overall more immunosuppressive microenvironment in RFP regions (**Fig. 3J, K**). In addition,

241   macrophages ($F4/80^+$ $CD11b^+$ MHC-II^+) were significantly enriched in mixed regions compared

242   to YFP regions, and also trended toward higher enrichment in RFP regions (**Fig. 3L**).

243   Collectively, these data show that local tumor cells themselves play a significant role in

244   driving the immune microenvironment in their vicinity. Moreover, the observation that mixed

245   regions look similar to RFP regions—in terms of effector T cell infiltration and macrophage

246   infiltration—suggests that in regions in which both CIT6-YFP and CIT9-RFP tumor cells co-exist,

247   cold CIT9-RFP tumor cells exert dominance over the local immune infiltrate. The "dominant

248   coldness" of CIT9-RFP is also seen in the global suppression of CD8 T cell infiltration in all

249   regions. However, CD8 T cells that managed to infiltrate YFP regions show a highly active

250   phenotype.

251

252   **CD206^{Hi} macrophages and lack of neutrophils and monocytes mediate an immunosuppressive**

253   **TME in RFP regions.**

254   To characterize immune cell spatial patterning more deeply, we employed ZipSeq spatial

255   transcriptomics[25] to CIT6-YFP + CIT9-RFP mixed-population tumors. ZipSeq allows immune cells

256   within user-defined regions of interest in live tissue sections to be tagged with distinct

257   nucleotide sequences (akin to assigning each region of interest a unique zip code), making use

258   of photocaged oligonucleotides and precise light exposure. Using this method, immune cells

259   from YFP, RFP and mixed regions of mixed population tumors (n=2) were tagged with distinct

260   "zip code" barcodes, and sections were subsequently disaggregated and subjected to single cell

261   RNA sequencing (scRNA-seq) (**Fig. 4A**). Immune cells were subjected to UMAP clustering, and

262   cluster identities were assigned based on expression of well-established markers (**Fig. 4B**, Supp

263   Fig. 5A, B). Using ZipSeq barcodes, we mapped each immune cell back to the region it

264   originated from. In line with our flow cytometry analyses, we observed a higher infiltration of T

265   cells in YFP regions compared to RFP regions (**Fig. 4C, D**), and also saw that myeloid cells

266   comprised a majority of the immune cells (**Fig. 4B**). In particular, we observed three large

267   macrophage clusters, CD206^{Hi} macrophages, MHC-II^{Hi} macrophages, and Arg1^{Hi} macrophages

268   (**Fig. 4B,** Supp Table 1). These three clusters were all enriched in RFP regions (**Fig. 4E**). By

269   contrast, neutrophils and monocytes were observed at lower abundance in RFP regions than

270   YFP regions (**Fig. 4F, G**). We carried out ZipSeq single cell spatial transcriptomics on a second

271   mixed-population tumor, and found similar results (Supp Fig. 6). Myeloid cells comprised a

272   majority of the immune cells (Supp Fig. 6A-C) and T cells were enriched in YFP regions (Supp Fig.

273   6D, E). Notably, out of seven macrophage clusters in the second tumor, six of them were

274   enriched in RFP regions compared to YFP regions (Supp Fig. 6F). Moreover, the second tumor

275   showed enrichment of neutrophils and monocytes in YFP regions (Supp Fig. 6G, H). Pathway

276   analysis with EnrichR, using genes enriched in each cluster and the GO Biological Process 2021

277   repository, suggested neutrophils had pro-inflammatory properties, as seen by neutrophil

278   activation-related genes (**Fig. 4F**, Supp Fig. 6G). Monocytes also exhibited evidence of

279   activation, with a particularly clear pro-inflammatory signature in the second tumor (**Fig. 4G**,

280   Supp Fig. 6H). Together, the combination of an enrichment of immunosuppressive

281   macrophages and a de-enrichment of inflammatory monocytes and neutrophils point to a

282   myeloid cell-driven immunosuppressive TME in RFP regions.

283

284   **Chemokine CX3CL1 is a mediator of the CIT9-RFP-driven immunosuppressive TME.**

285   To identify the drivers behind the striking spatial organization of myeloid cell infiltrates

286   in our mixed tumors, we next assessed the protein expression of a total of 44 cytokines and

287   chemokines using the lysates from CIT6-YFP and CIT9-RFP tumors. Cytokines and chemokines

288   are major mediators of immune cell localization and function, thus we hypothesized differences

289   in their expression between CIT6-YFP and CIT9-RFP might explain the differences we saw in

290   myeloid infiltrate. We found the chemokine CX3CL1 was significantly enriched in CIT9-RFP

291   tumors compared to CIT6-YFP tumors (**Fig. 5A**, Supp Fig. 7A). CX3CL1 is a chemokine with

292   reported pro-tumor properties, capable of directly promoting tumor cell proliferation and

293   migration as well as of attracting various immune cell types[26,27]. To test the hypothesis that

294   CX3CL1 plays a role in driving the immunosuppressive microenvironment in our squamous cell

295   carcinomas, we generated a CIT6-YFP cell line overexpressing *Cx3cl1* and analyzed the immune

296   profile of these tumors. The abundance of macrophages was increased in Cx3cl1-

297   overexpressing tumors compared to control tumors (**Fig. 5B**). We next asked if it was a

298   particular macrophage subset(s) that showed increased abundance, and for this analysis

299   divided macrophages into the 3 main clusters we identified in our ZipSeq analysis: CD206^{Hi}

300   macrophages (CD206^{Hi} MHC-II^{Low}), MHC-II^{Hi} macrophages (CD206^{Mid} MHC-II^{Hi} Arg1$^-$) and Arg1^{Hi}

301   macrophages (CD206^{Mid} MHC-II^{Hi} Arg1$^+$) (Supp Fig. 7B). We found that CD206^{Hi} macrophages

302   were increased in frequency in CX3CL1-overexpressing CIT6-YFP tumors compared to control

303   tumors, while the other two macrophage subsets showed no difference in frequency (**Fig. 5C**).

304   In line with this, expression of CX3CR1, a cognate receptor of CX3CL1, was the highest on

305   macrophages among all immune cell types (Supp Fig. 7C), and highest on CD206^{Hi} macrophages

306   among the three macrophage subsets (**Fig. 5D**). Additionally, we observed a lower abundance

307 of neutrophils (Ly6G$^+$) and monocytes (Ly6C$^+$) in CX3CL1-overexpressing CIT6-YFP tumors (**Fig.**

308 **5E**). Other immune cell types including B cells, CD8 T cells and DCs showed no difference in

309 frequency (Supp Fig. 7D).

310 We next asked whether CD206^{Hi} macrophages, as the main macrophage subtype

311 increased by CX3CL1 overexpression, had a greater capacity to suppress T cells than non-

312 CD206^{Hi} macrophages (which include both MHC-II^{Hi} and Arg1^{Hi} macrophages). We isolated

313 CD206^{Hi} and non-CD206^{Hi} macrophages from CIT9-RFP and CIT6-YFP tumors by flow cytometry-

314 based sorting (Supp Fig. 7B) and tested for their ability to suppress CD8 T cell proliferation using

315 an *in vitro* co-culture assay. We found that CD206^{Hi} macrophages could suppress T cell

316 proliferation and activation even at a low macrophage-to-CD8 T cell ratio (1:16), whereas non-

317 CD206^{Hi} macrophages could suppress CD8 T cells only at a higher co-culture ratio (1:4) (**Fig. 5F**,

318 Supp Fig. 7E). Thus, while both CD206^{Hi} and non-CD206^{Hi} macrophages exhibited suppressive

319 capacity to some extent, the CD206^{Hi} macrophage subset in our tumors has a particularly strong

320 ability to suppress T cell proliferation.

321 Because we also observed an enrichment of CD206^{Hi} macrophages and lower

322 abundance of neutrophils and monocytes in RFP regions of mixed-population tumors in our

323 ZipSeq data (**Fig. 4**), we hypothesized that the CX3CL1-CX3CR1 axis plays a role in driving the

324 immunosuppressive microenvironment in these regions. There were few reads of *Cx3cl1* in the

325 ZipSeq data (data not shown) likely due to limitations associated with single cell RNAseq.

326 Therefore, to test this hypothesis, we assessed the correlation between the *Cx3cr1* expression

327 level in immune cells and their abundance in RFP regions. *Cx3cr1* transcript level was high in

328 clusters that were enriched in RFP regions (**Fig. 5G** and **Fig. 4C**; Supp Fig. 8A and Supp Fig. 6D),

329 and was the highest on the CD206^{Hi} macrophage cluster (**Fig. 5H**, Supp Fig. 8B). Moreover,

330 there was a positive correlation across clusters between the abundance of a cluster and its

331 average *Cx3cr1* transcript expression level in RFP regions, but not in YFP or mixed regions (**Fig.**

332 **5I**). Collectively, these data indicate that CX3CL1 plays a role in driving local myeloid cell

333 abundance and enriching immunosuppressive CD206^{Hi} macrophages in RFP regions of mixed-

334 population tumors.

335

336 **CX3CL1 is a mediator of a cold TME in human cancers.**

337 To assess the role of CX3CL1 in shaping the TME and myeloid cell abundance in human cancers,

338 we turned to a previously published pan-cancer human dataset containing transcriptomic

339 profiles of whole tumors and of individual immune compartments (e.g., myeloid compartment)

340 from 364 individual tumors across 12 cancer types[28]. Previous analysis identified 12 recurrent

341 immune infiltration patterns across these 364 tumors, denoted "immune archetypes", spanning

342 a range from immune rich to immune poor TMEs. Looking at T cell abundance, 7 of these

343 archetypes could be classified as immune hot and 5 of them as immune cold, corresponding

344 with high and low T cell infiltrates based on T cell transcriptomic scores **(Fig. 6A).** We assessed

345 whole-tumor *CX3CL1* RNA expression in each archetype, and found that, strikingly, *CX3CL1*

346 levels were highest in 3 of 5 cold archetypes (archetypes 9, 10 and 12) and comparatively low in

347 all hot archetypes **(Fig. 6B)**. Next, we examined the macrophage:monocyte ratio across all

348 human archetypes, using transcriptomic scores for macrophages and monocytes based on

349 RNAseq data from the myeloid compartment of each tumor.[28] We observed a bias in the

350 myeloid compartment toward macrophages (log2 of the macrophage:monocyte ratio > 0) in the

351 same 3 cold human archetypes that showed high *CX3CL1* (archetypes 9, 10, and 12, **Fig. 6C**).

352 Using a gene signature based on the CD206^{hi} cluster in our ZipSeq dataset, we also asked if

353 CD206^{hi} macrophages were enriched in *CX3CL1*-high human immune archetypes. We found

354 that, among cold archetypes, the macrophage compartment showed a high CD206^{hi}

355 macrophage score in 2 archetypes, both of which were *CX3CL1*-high (archetypes 10 and 12, **Fig.**

356 **6D**). Finally, we turned back to our CX3CL1-overexpressing mouse tumors. We saw that CX3CL1

357 overexpression led to an increase in both the macrophage:monocyte ratio and the CD206^{hi}

358 macrophage: non-CD206^{hi} macrophage ratio in CX3CL1-overexpressing CIT6-YFP tumors

359 compared to controls **(Fig. 6E)**. Of note, we also analyzed the 7 hot human archetypes that

360 show low *CX3CL1* expression, and found a range of macrophage:monocyte ratios and CD206^{hi}

361 macrophage scores (Supp. Fig.9), suggesting that additional and likely different factors shape

362 these ratios in hot, CX3CL1-low tumors. Collectively, these data show that CX3CL1 is associated

363 with cold TMEs enriched in CD206^{hi} macrophages not only in our mouse model, but also across

364 multiple human cancers.

365

366 **An immunosuppressive tumor population drives resistance of mixed-population tumors to**

367 **anti-PD-1 blocking and CD40 agonistic antibody combination treatment.**

368 Having shown that CIT9-RFP tumor cells in mixed-population heterogeneous tumors

369 exert a dominant immunosuppressive effect and drive spatial organization within the tumor,

370 we next sought to determine if the presence of CIT9-RFP tumor cells impacts the response of

371 mixed-population tumors to immune checkpoint blockade (ICB) therapy. In particular, as an

372 immunosuppressive tumor microenvironment is linked to resistance to ICB therapy, we asked if

373 CIT9-RFP tumor cells make otherwise therapy-sensitive CIT6-YFP tumor cells resistant. We

374 tested multiple ICB combinations on our tumors, and we selected a regimen combining PD-1

375 blockade and CD40 agonist antibodies, a combination that is currently in clinical trials for

376 multiple tumor types. The combination treatment was administered in two doses at a 3-day

377 interval when the CIT6 and CIT9 tumors reached 5mm in diameter (**Fig. 7A**) and induced 50% of

378 CIT6-YFP tumors (5 out of 10 tumors) to regress completely, with 2 tumors eventually growing

379 back after 5-17 days (**Fig. 7B**). In contrast, only 9% (1 out of 11) of CIT9-RFP tumors showed a

380 decrease in tumor volume after therapy, and none achieved a complete regression (**Fig. 7B**).

381 Furthermore, the increase in the average volume of CIT6-YFP tumors over time was blunted by

382 the combination treatment in comparison to isotype control treatment, whereas CIT9-RFP

383 tumors showed similar growth kinetics in both combination treatment and isotype control arms

384 (**Fig. 7B**). This coincided with prolonged overall survival of the mice harboring CIT6-YFP tumors,

385 but not CIT9-RFP tumors, after treatment with the combination therapy (**Fig. 7C**). Thus, the

386 combination of anti-PD-1 blocking and CD40 agonistic antibodies mimics prototypical patient

387 outcomes to immunotherapy where immune hot tumors respond and immune cold tumors do

388 not. Further, this combination showed the clearest dichotomy in responses between hot and

389 cold tumors among the regimens tested, and so we chose to proceed with using it to

390 investigate how mixed-population tumors would respond.

391 We then assessed the response of tumors derived from a 1:1 mixture of CIT6-YFP and

392 CIT9-RFP cell lines to the anti-PD-1 blocking and CD40 agonist combination treatment. The

393 combination therapy achieved complete regression (CR) in 1 out of 23 mice (4.3%) but the

394 tumor quickly grew back (**Fig. 7D**). The CR rate of mixture-derived tumors was higher than CIT9-

395   RFP single-population tumors (0% CR), but lower than CIT6-YFP tumors (50% CR) (**Fig. 7B, D**).

396   The average tumor volume was slightly smaller in the combination treatment group (**Fig. 7D**),

397   although this did not correlate to an extension of overall survival (**Fig. 7D**), suggesting therapy

398   had only a modest effect in these mixed tumors.

399   Immune profile analysis of tumors at the time of harvest (10mm in diameter) revealed

400   that CD4 and CD8 T cell infiltration was increased in both CIT6-YFP tumors and mixed tumors

401   treated with the anti-PD-1 + anti-CD40 agonist combination, and that this influx was of similar

402   magnitude in both (**Fig. 7E**). By contrast, CIT9-RFP tumors did not show a change in T cell

403   infiltration after treatment (**Fig. 7E**). However, despite the influx of T cells into mixed tumors,

404   this influx did not correspond to the same level of treatment efficacy seen in CIT6-YFP tumors.

405   Collectively, these data suggest that the presence of CIT9-RFP tumor cells dampens the

406   response of mixed tumors to the combination treatment, and also reveals an improved influx of

407   T cells into the tumor after treatment is not necessarily a marker of a successful response in the

408   context of a heterogeneous tumor.

409

410   **Spatial immune infiltration patterns persist in mixed-population tumors following anti-PD-1**

411   **blockade and CD40 agonist combination treatment.**

412   Given our previous data showing that RFP regions (and to a lesser extent, mixed regions)

413   of mixed-population tumors were spatially organized pockets of immune coldness, we sought

414   to test the hypothesis that the immunotherapy combination treatment was acting successfully

415   in YFP regions, but failing to improve the immune response in RFP regions, leading to overall

416   failure. We applied our live tumor slice microdissection technique (**Fig. 3D**) to mixed-population

417   tumors treated with either anti-PD-1 blockade + CD40 agonist or control antibodies. The

418   immune infiltration profiles of YFP, RFP and mixed regions were analyzed 6 days after the initial

419   treatment dose, at which point tumors have not started to shrink yet (**Fig. 7B and D**). We

420   focused our analysis on T cells, as we had observed that T cell infiltration was increased by the

421   combination treatment in mixed population tumors (**Fig. 7E**).

422   At the 6-day timepoint, the frequencies of overall CD4 and CD8 T cells did not differ

423   between the isotype control arm and the combination treatment arm in any of the three

424   regions (Supp Fig. 10). However, the presence of Th1 cells was dramatically increased in RFP

425   and mixed regions by the combination treatment compared to isotype control treatment (**Fig.**

426   **8A**). There was also a trend toward increased Th1 cells in YFP regions, but it did not reach

427   statistical significance. The treatment-induced change in abundance was unique to Th1 cells, as

428   other CD4 T cell subsets including Treg, Th2 and Th17 cells showed a similar frequency between

429   isotype control and combination treatment arms (**Fig. 8B, C**). The changes in Th1 cell

430   abundance resulted in a favorable increase in the Th1:Treg ratio in mixed and RFP regions of

431   the combination therapy-treated tumors (**Fig. 8D**). The functional impact on the CD8 T cell

432   compartment was directionally the same, but much less dramatic. The frequency of IFNγ^+ CD8 T

433   cells was modestly increased by the combination therapy in RFP regions, but not in mixed

434   regions (**Fig. 8E**), and the IFNγ^+ CD8 T cell to Treg ratio showed a trend of improvement in mixed

435   and RFP regions in response to the treatment (**Fig. 8F**). However, because the abundance of

436   CD8 T cells remained low in all regions (as previously observed in mixed-populations tumors,

437   Fig. 3G), these improvements in CD8 T function were of small magnitude on an absolute scale.

438   Collectively, these data show that anti-PD-1 and CD40 agonist antibody treatment was

439   successful at improving the quality of the immune response in cold RFP and mixed tumor

440   regions—contrary to our expectations that we would only observe such improvements in the

441   hot YFP regions. However, despite the therapy-induced boost, the quality of the immune

442   response in RFP regions remained inferior to that in YFP regions, and the absolute number of

443   tumor-infiltrating CD8 T cells remained low. Thus, while the therapy-induced improvement in

444   anti-tumor Th1 CD4 T cell response in RFP and mixed regions likely contributed to the blunted

445   growth of treated tumors—which was also seen in this 6-day cohort (**Fig. 8G**)—it was largely

446   insufficient to mediate tumor rejection.

447

448   **Discussion**

449   Combining fluorescently tagged pro-inflammatory (hot) and immunosuppressive (cold)

450   squamous cell skin carcinoma tumor populations, we have developed a novel model system to

451   interrogate the spatial heterogeneity of tumor cells and intratumoral immune cells. When

452   combined in a 1:1 ratio, we see that an immunosuppressive tumor population has a "dominant

453   negative" suppressive effect, in line with a previous report in a model of pancreatic cancer[15].

454   The overall immune phenotype of mixed-population tumors most closely resembles that of

455   cold, immunosuppressive tumors, and exhibits a poor infiltration of CD8 T cells—which are

456   critical to anti-tumor immunity—in all regions. Moreover, the dominant effect of the

457   immunosuppressive tumor population is also seen locally in "mixed" regions where the two

458   tumor populations co-exist.

459   However, when we look at regions locally dominated by hot tumor cells (YFP[+] regions in

460   our tumors), we find CD4 T cells accumulate specifically in these regions, as do inflammatory

461   monocytes and neutrophils. Both CD4 and CD8 T cells also show better effector phenotypes in

462   the vicinity of hot tumor cells. By contrast, immunosuppressive macrophages preferentially

463   accumulated in the neighborhoods of cold tumor cells. Tumor cells themselves thus set up a

464   blueprint for the spatial architecture of infiltrating immune cells, with different tumor cell

465   populations driving distinct "micro-microenvironments" in their vicinities. Our study is the first,

466   to our knowledge, to demonstrate a clear, reproducible link between spatial organization of

467   infiltrating immune cells and the underlying spatial organization of tumor cells.

468   Tumor cell-driven spatial differences in the intratumoral immune response exist at

469   baseline in the absence of therapy, but also persist during immunotherapy and exert an

470   influence on the response to therapy. Regional analysis of response to anti-PD-1 blockade +

471   anti-CD40 agonist combination immunotherapy showed a more nuanced pattern than we

472   expected: Rather than seeing a simplistic "good" response in hot regions and "poor" response

473   in cold regions, the relative influx of Th1 cells and IFNγ^+ CD8 T cells was strongest in RFP$^+$ cold

474   regions. Nonetheless, cold regions remained more poorly infiltrated than hot regions at an

475   absolute level, even after the therapy-induced boost, indicating that therapy did not entirely

476   overcome the spatial patterning that existed pre-treatment. While the anti-PD-1 plus CD40

477   agonist combination we employed failed to cure our mixed tumors, our model system

478   facilitated detailed region-by-region analysis of the effects and efficacy of therapy. This system

479   thus establishes a platform on which additional drug combinations or dose regimens can be

480   probed to iterate on how best to induce a productive tumor-wide anti-tumor immune response

481   across all tumor regions.

482   We identify CX3CL1 as a mediator of a myeloid-cell-centric immunosuppressive

483   microenvironment, and a candidate driver of the "pockets of coldness" we observe in our

484   heterogeneous tumors. We show that CX3CL1-expressing tumor cells drive enrichment of

485   immunosuppressive CD206[Hi] macrophages, and depletion of inflammatory monocytes and

486   neutrophils. In a pan-cancer analysis of human patients, high levels of CX3CL1 are associated

487   with three specific immune cold TME archetypes, all of which show macrophage-enriched

488   myeloid compartments and 2 of the 3 show a specific enrichment of CD206[Hi] macrophages. The

489   CX3CR1:CX3CL1 axis has also been found to be linked to macrophage accumulation in both skin

490   SCC and breast tumors on a whole-tumor level[29,30]. Further, our findings that CX3CL1 is a

491   candidate mediator of local immunosuppressive myeloid cell organization are in line with

492   previous reports of chemokine-driven spatial organization of immune cells within tumors[31,32].

493   Of note, CX3CL1 has also been described as an "anti-tumor" chemokine, particularly in lung

494   cancer, due to its role interacting with CX3CR1+ T cells,[26,33–35] highlighting that its role in tumor

495   immunity is context-dependent and not yet fully understood. Therefore, careful investigation

496   into CX3CL1 is essential to assess its potential therapeutic application. Of course, CX3CL1 does

497   not fully define the immune microenvironment. Future work, in addition to more deeply

498   investigating the role of CX3CL1, is also expected to uncover additional pathways by which

499   tumor cells shape their local micro-microenvironments and influence responses to

500   immunotherapy on a highly local spatial scale.

501

502   **Figure legends:**

503   **Figure 1. Modeling intratumoral heterogeneity using fluorescently-labeled DMBA/TPA-**

504   **induced squamous cell skin carcinoma cell lines.**

505   **(A-C)** Flow cytometric analysis of T cell infiltration in subcutaneous tumors derived from

506   injection of CIT6-YFP and CIT9-RFP cell lines into mice carrying an unactivated *Confetti* cassette.

507   **(D)** Immunofluorescent staining of $CD3^+$ T cells in 8µm cryosections of subcutaneous CIT6-YFP

508   and CIT9-RFP tumors, with DAPI staining cell nuclei, and quantification of T cell count per field

509   Scale bar = 100µm. **(E)** Schematic of mixed-population tumor generation. A 1:1 mixture of CIT6-

510   YFP and CIT9-RFP cell lines subcutaneously was injected into the right hind flank of mice

511   carrying an unactivated *Confetti* cassette, and the resulting tumors were harvested for

512   downstream analyses when they reached 1cm in diameter at 21-28 days post injection. **(F, G)**

513   Whole-tumor images by fluorescent dissecting microscope (F) and a representative image of a

514   tumor cross-section (8µm cryosection) (G) of tumors that were a 1:1 mixture of CIT6-YFP and

515   CIT9-RFP cell lines. Scale bars in (F) and (G) are 2mm and 1mm respectively. All data are a

516   representation of at least 3 independent experiments.

517

518   **Figure 2. The immunosuppressive tumor population drives an overall immunosuppressive**

519   **phenotype in mixed-population tumors.**

520   **(A-E)** Flow cytometric immune profiling of tumors derived from CIT6-YFP and CIT9-RFP cell lines

521   and mixed-population tumors derived from a 1:1 mixture of the two cell lines, including **(A)** T

522   cells, CD4 T cells and CD8 T cells, **(B)** frequency of tumor cells ($CD45^-$ YFP^- or $CD45^-$ RFP^-)

523   expressing MHC-I molecules ($H2-K^q$ and $H2-L^qD^q$) (left) and median fluorescence intensity (MFI)

524   of the MHC-I molecules on $H2-K^q$ and $H2-L^qD^q$ double positive tumor cells (right), **(C)** Treg

525   (Foxp3$^+$ CD25$^+$) frequency, CD8 T cell-to-Treg ratio, and effector CD4 T cell (Teff, Foxp3$^-$ CD25$^-$)-

526   to-Treg ratio, **(D)** B cells (B220$^+$) and natural killer (NK) or innate lymphoid cell (ILC) (NKp46$^+$)

527   lymphocytes and **(E)** macrophages (F4/80$^+$ CD11b$^+$ MHC-II$^+$). Data in (A)-(E) are a representation

528   of at least two independent experiments with ≥5 mice per experiment. **(F-H)** *In vitro* CD8 T cell

529   suppression by macrophages sorted from the tumors. CFSE-stained and CD3/28 beads-

530   stimulated CD8 T cells isolated from naïve *Confetti* mice were co-cultured with tumor-

531   associated macrophages (TAM) at varying ratios (50K T cells and 12.5K, 25K, or 50K TAMs) and

532   analyzed for proliferation via CFSE signal and activation markers. Data are a representation of 2

533   independent experiments, with 3 biological replicates in each experiment. **(F)** Representative

534   histogram showing the CFSE signal in CD8 T cells, with gates marking divided cells, defined as

535   the cells that have divided more than twice in no TAM condition or 50K T cell + 50K TAM

536   conditions (left) and graph showing the count of divided cells (right). "No TAM", T cell

537   stimulation control without macrophages; "No stim", control with no T cell stimulation. **(G)**

538   Count of activated (CD69$^+$ CD25$^+$) CD8 T cells, **(H)** Count of effector CD8 T cell subsets marked

539   by combinations of CD62L and CD44 expression.

540

541   **Figure 3. Spatial organization of tumor populations drives the spatial organization of immune**

542   **cells.**

543   **(A)** Representative cross-section images showing examples of a YFP, mixed and RFP region of a

544   mixed-population tumor and CD3 stain. **(B)** Quantification of CD3$^+$ T cells per field, plotted for

545   each region. **(C)** Correlation of YFP$^+$ cell fraction and CD3$^+$ T cell frequency in each field. CD3$^+$ T

546   cell count was scaled to DAPI$^+$ cell count in each field to normalize the difference in cell density

547   between fields. **(D)** Schematic of immune cell profiling in YFP, mixed and RFP regions of mixed-

548   population tumors. Each mixed-population tumor was sliced into five to ten 400µm-thick live

549   sections, and each section was manually microdissected into YFP, mixed, and RFP regions using

550   a surgical scalpel under a fluorescent dissecting microscope. All YFP pieces, all mixed pieces,

551   and all RFP pieces from the same tumor were pooled for immune profiling by flow cytometry.

552   White scale bar = 5mm, blue scale bar = 1mm. **(E-L)** Frequency of T cell subsets, ratio of Treg to

553   IFNγ$^+$ CD8 T cells or Th1 cells, and frequency of macrophages in YFP, mixed and RFP regions.

554   Grey dotted lines in panels (E), (F), (G) and (L) separate data of mixed-population tumors from

555   the data of single-population tumors. All data are a representation of two independent

556   experiments, with ≥3 mice per experiment (B,C) or ≥6 mice per experiment (E-L).

557

558   **Figure 4. CD206^{Hi} macrophages and lack of neutrophils and monocytes mediate an**

559   **immunosuppressive TME in RFP regions.**

560   **(A)** Schematic of ZipSeq spatial transcriptomic analysis of mixed-population tumors. Composite

561   stitched image of a 200µm-thick live section marked with YFP regions (encircled with green

562   lines), RFP regions (red lines) and mixed regions (blue lines) is shown. YFP, RFP and mixed

563   regions were illuminated individually to allow for the binding of a unique barcode ("zipcode") to

564   a photocaged oligo-CD45 antibody complex. ZipCode-tagged immune cells were analyzed by

565   scRNAseq. **(B)** Uniform manifold approximation and projection (UMAP) representation of

566   zipcode-labeled cells with cluster overlay. n = 3,238 cells, n_{YFP} = 1,236, n_{RFP} = 1,318, n_{Mixed} = 684.

567   **(C)** UMAP representation of zipcode-labeled cells with zipcode identity overlaid. **(D)** Abundance

568   of cells belonging to the T cell cluster (cluster 9), calculated as percentage of total immune cells

569   in each region. **(E)** Abundance of cells belonging to MHC-II^{Hi}, Arg1^{Hi} and CD206^{Hi} macrophage

570   clusters (clusters 1, 2, and 3 respectively), calculated as percentage of total immune cells in

571   each region. **(F)** Percentage of cells belonging to neutrophil cluster (cluster 7) in each region and

572   pathway analysis showing the gene families enriched in the neutrophil cluster based on the top

573   250 differentially expressed genes. **(G)** Percentage of cells belonging to monocyte cluster

574   (cluster 6) and pathway analysis of genes enriched in the monocyte cluster based on the top 31

575   differentially expressed genes.

576

577   **Figure 5. Chemokine Cx3cl1 mediates the CIT9-RFP-driven immunosuppressive TME.**

578   **(A)** ELISA-based quantification of CX3CL1 using tumor lysates of CIT6-YFP and CIT9-RFP single-

579   population tumors (n=3 per cell line) **(B, C)** Infiltration of total macrophage (B) and macrophage

580   subsets (C) in CIT6-YFP tumors overexpressing CX3CL1 (CIT6-YFP^{Cx3cl1}) and empty vector (CIT6-

581   YFP^{Empty}). **(D)** Expression of CX3CR1 on macrophage subsets in CIT6-YFP^{Cx3cl1} tumors, measured

582   as MFI from flow cytometric analysis. **(E)** Frequency of neutrophils and monocytes in CX3CL1-

583   overexpressing CIT6-YFP tumors. Data in (B)-(E) are representation or combination of two

584   independent experiments with ≥10 mice per experiment. **(F)** CD8 T cell suppression assay using

585   CD206^{Hi} macrophages and a pool of Arg1^{Hi} macrophages and MHC-II^{Hi} macrophages (Non-

586   CD206^{Hi} TAMs) isolated from CIT6-YFP and CIT9-RFP single-population tumors. Representation

587   of two independent experiments with at least 2 mice per arm. **(G)** Feature plot overlaid on

588   UMAP representation for *Cx3cr1* gene from the tumor analyzed by ZipSeq in Fig. 4. **(H)** Violin

589   plot showing *Cx3cr1* distribution based on cluster identity. **(I)** Correlation between an average

590   relative *Cx3cr1* transcript level of each cluster and the percentage of cells belonging to the

591   cluster. Combined analysis of two tumors analyzed in Fig. 4 and Supp Fig. 6.

592

593   **Figure 6. CX3CL1 is a mediator of a cold TME in human cancers.**

594   **(A-D)** Analysis of immune cells and *CX3CL1* expression using a previously published dataset of

595   364 human tumors from 12 cancer types. Computational clustering of tumors using flow

596   cytometry data and transcriptomic data enabled identification of 12 dominant patterns of

597   immune composition across various cancers, referred to as immune archetypes (ref. 28).

598   RNAseq data was generated separately for all viable cells and for sorted mononuclear

599   phagocytes. Prior to RNA sequencing, cells were sorted by flow cytometry from untreated,

600   freshly resected human tumors. **(A)** T cell score of each tumor, based on RNAseq of all viable

601   cells, grouped on the x-axis by immune archetype. **(B)** Heatmap of average *CX3CL1* RNA

602   expression, based on RNAseq of all viable cells, for each immune archetype. **(C)** Ratio of

603   macrophage score and monocyte score, based on RNAseq of mononuclear phagocytes, for

604   tumors in the 5 cold immune archetypes. **(D)** CD206^{Hi} macrophage score, based on RNAseq of

605   mononuclear phagocytes, for tumors in the 5 immune cold archetypes. **(E)** Ratio of

606   macrophages to monocytes (left) and CD206^{Hi} macrophages to non-CD206^{Hi} macrophages

607   (right) detected in mouse CIT6-YFP tumors overexpressing *Cx3cl1* or controls.

608

609   **Figure 7. An immunosuppressive tumor population drives resistance of mixed-population**

610   **tumors to anti-PD-1 blocking and CD40 agonistic antibody combination treatment.**

611   **(A)** Dosing schedule of anti-PD-1 and CD40 agonist treatment. **(B)** Tumor growth curves of CIT6-

612   YFP and CIT9-RFP tumors showing tumor volume of individual tumors and average volume of

613   isotype control and anti-PD-1+CD40 agonist combination treatment arms. Red arrows under

614   the x axis shows the timing of treatments (day 0 and 3), fraction shown on the top right corner

615   of individual tumor volume plots shows the number of mice whose tumors completely

616   regressed post-treatment. **(C)** Kaplan-Meier curves comparing the survival of mice from each

617   treatment arm. Statistical test was performed using log-rank test. **(D)** Tumor growth and

618   survival of mice harboring tumors derived from a 1:1 mixture of CIT6-YFP and CIT9-RFP cell

619   lines. **(E)** Frequency of tumor-infiltrating T cells post-treatment in CIT6-YFP, CIT9-RFP, and 1:1

620   mixture-derived tumors. All experiments were performed with at least 8 mice per treatment

621   arm.

622

623   **Figure 8. Spatial immune infiltration patterns persist in mixed-population tumors following**

624   **anti-PD-1 blockade and CD40 agonist combination treatment.**

625   **(A–F)** Tumors derived from a 1:1 mixture of CIT6-YFP and CIT9-RFP cell lines were treated with

626   α-PD-1 +CD40 agonist combination treatment or isotype control antibodies at day 0 and 3 and

627   analyzed at day 6 for spatial T cell infiltration profile via microdissection and flow-based

628   analysis. **(A)** Th1 cell frequency in each region of isotype control-treated and combination

629   therapy-treated tumors, **(B)** Treg frequency, **(C)** Frequency of Th1, Th2, Th17 and Treg cells, **(D)**

630   Th1-to-Treg ratio, **(E)** Frequency of IFNγ^+ CD8 T cells and **(F)** IFNγ^+ CD8 T cell to Treg ratio. **(G)**

631   Average tumor volume of each treatment arm. Red arrows under the x axis shows the timing of

632   treatments. Experiment was performed with at least 10 mice per treatment/tumor arm.

633

634

635

636   **Materials and methods:**

637   <u>Carcinogenesis and cell line generation</u>

638   To induce tumors, *K5-CreER^{T2}-Confetti FVB/N* mice were treated with 25 mg DMBA followed by

639   TPA (200 µl of a 10^{-4} M solution in acetone) two times a week for 20 weeks, as described[19]. To

640   generate cell lines, carcinomas were resected at a size of >1 cm in longest diameter, finely

641   chopped, digested in DMEM containing Collagenase I and IV and DNase I for 45 minutes at

642   37 °C, washed with PBS, plated in supplemented DMEM (high-glucose DMEM (Gibco, catalog#

643   11995065) plus 10% heat-inactivated FBS (Gibco), 2mM L-glutamine, with 100 units/ml

644   penicillin, 100 µg/ml streptomycin, and 2.5 µg/ml amphotericin B (Gibco)), and passaged until

645   cells stably grow in culture. The established cell lines were cultured in the supplemented DMEM

646   for all experiments.

647

648   <u>Cell line labeling</u>

649   Unlabeled cell lines were treated with adenoviral Cre recombinase (Vector Biolabs catalog

650   #1045) to induce labeling with Confetti fluorescent proteins. YFP- and RFP-expressing cells were

651   sorted via fluorescence-activated cell sorter and further grown in vitro to establish CIT6-YFP and

652   CIT9-RFP cell lines.

653

654   <u>In vivo experiments</u>

655   A total of 1.25×10^5 cells were injected subcutaneously into the dorsal flank of 8 to 16 week-old

656   male and female *Confetti*-homozygous *FVB/N* mice. Approximately 21-28 days later, when

657   tumors measured ~1cm in diameter, tumors were dissected and digested as described above,

658   and subsequently used in flow cytometric analysis. For treatment experiments, when tumors

659   measured approximately 5mm in diameter, mice were injected intraperitoneally with one dose

660   (CD40 agonistic antibody) or two doses (anti-PD-1 antibody) of therapeutic antibodies,

661   administered at a three day interval. Antibodies used were rat anti-mouse PD-1 IgG2a antibody

662   (RMP1-14, BioXCell), rat anti-CD40 agonist IgG2a antibody (FGK4.5, BioXCell), and rat IgG2a

663   isotype control (2A3; BioXCell) at 200µg per antibody per dose. Tumors were resected at 1cm in

664   diameter and used for downstream analyses, except in 6-day post-treatment analyses where

665   tumors were resected 6 days after treatment initiation. All animal experiments were approved

666   by the University of California San Francisco Laboratory Animal Resource Center (IUCAC;

667   AN187679). This work complies with all the relevant ethical regulations regarding animal

668   research.

669

670   Flow cytometric immune profiling and macrophage cell sorting

671   For whole tumor analysis and sorting, 5 million cells were stained with antibodies. For spatial

672   immune profiling, tumors were embedded in 2% agarose gel, sliced into 400µm section by a

673   Leica Vibratome, microdissected into YFP, RFP and mixed regions using surgical scalpel while

674   visualizing each region on MVX10 fluorescent stereoscope. Multiple pieces of each region from

675   a single tumor were pooled for digestion, followed by flow cytometric analysis. Cell sorting was

676   performed on FACS Aria III (BD Biosciences). Antibodies used for flow cytometric analyses and

677   sorting are listed in Table 2.

678

679   Immunofluorescence imaging

680   After half of the tumors were processed for flow cytometric analysis, the remaining halves were

681   fixed in 4% paraformaldehyde for 2 hours, dehydrated in PBS with 30% sucrose overnight at

682   4C°, embedded in Tissue-tek O.C.T. compound (Sakura Finetek USA) and stored at -80C°.

683   Embedded blocks were cryosectioned into 8μm sections using Leica CM3050 S Cryostat (Leica

684   Biosystems) and stained with rabbit anti-mouse CD3 antibody (ab5690, Abcam) overnight,

685   followed by stain with secondary anti-rabbit Alexa Fluor 647 antibody (Invitrogen) for 1 hour at

686   room temperature and DAPI stain for 5 minutes. Fluorescence image was taken on microscope

687   (DMi8, Leica Biosystems) using Leica Application Suite X (Leica Biosystems) imaging software,

688   and fraction of YFP+ and RFP+ cells as well as the number of CD3+ cells in each tile were

689   quantified using CellProfiler ver. 3.1.9.

690

691   Overexpression of cytokines and chemokines

692   Mouse *Cx3cl1* gene was amplified using forward primer 5' -aactcgagatggctccctcgccgctcg -3' and

693   reverse primer 5' -ttccgcggtcacactggcaccaggacgta- 3', cDNAs generated from CIT9 cell line and

694   Q5® High-Fidelity DNA polymerase (NEB). MSCV 2.2-IRES-CFP retroviral vector was generated

695   by replacing GFP of MSCV 2.2-IRES-GFP vector (obtained from James Carlyle lab at University of

696   Toronto) with CFP. Using restriction enzymes XhoI and SacII, *Cx3cl1* coding sequencing was

697   cloned into MSCV 2.2-IRES-GFP vector. Proviral vectors Gag/Pol and VSV-G were co-transfected

698   with Cx3cl1-IRES-GFP MSCV2.2 vector using lipofectamine 3000 (Invitrogen) into HEK293T cells.

699   48 hours later, the viruses were collected, mixed with CIT6-YFP cells and spinfected at 800g for

700   90 minutes. YFP⁺ CFP⁺ transduced cells were sorted 72 hours post-transduction.

701

702   <u>ZipSeq spatial transcriptomics analysis</u>

703   Mixed-population tumors were harvested at 1cm in diameter and sliced into 200μm section in

704   2% agarose using vibratome. Section was incubated with CD45 antibody linked to Cy5-

705   conjugated photocaged oligo for 1 hour at 4C°, followed by patterned illumination of a user-

706   defined region of interest[25] and incubation with a unique ZipCode. The process was repeated

707   for YFP, RFP and mixed regions. The section then was digested in a Collagenase I and IV blend

708   for 30 minutes at room temperature, and sorted for live Cy5⁺ cells using FACS Aria II. washed in

709   PBS + 0.04% BSA and then encapsulated following 10× Genomics specifications for 3' v3

710   chemistry with target cell number of 8,000.

711   Libraries were sequenced with a target number of 30,000 reads per cell for gene

712   expression and 3,000 reads per cell for ZipCode barcodes on a Illumina NovaSeq. Demultiplexed

713   fastqs were aligned using CellRanger to the mm10 Ensembl 93 reference genome. Count

714   matrices were used to generate Seurat objects in R keeping only cells with at least 200 detected

715   genes and genes found in at least 3 cells. Cells were further filtered based on a minimum 500

716   gene cutoff and maximum 15% mitochondrial reads. ZipCode counts were normalized using

717   centered log ratio transform and then assigned a dominant ZipCode identity (as in [25]). Cells

718   were then passed through SCTransform regressing for percent mitochondrial reads. Following

719   PCA analysis on the top variable genes, the first 18 principal components were used as inputs

720   for UMAP dimensional reduction and clustering via the FindNeighbors and FindClusters

721   commands. For pathway analysis, lists of genes with average Log_2 fold change >1 for each

722   cluster were used as input for GO Biological Process 2021 repository on EnrichR

723   (https://maayanlab.cloud/Enrichr/).

724

725   Cytokine array

726   70-150mg of tumor pieces were chopped into small pieces in RIPA buffer containing proteinase

727   inhibitor (Cell BioLabs) and homogenized using OctoMACS (Miltenyi Biotec). The homogenate

728   was centrifuged at 400g for 5 minutes followed by further centrifugation at 10,000g for 10

729   minutes at 4C°. The supernatant was collected and stored at -80C°. Protein concentration was

730   measured using DC protein assay kit (BioRad). The sample concentrations were normalized

731   prior to the submission for the analysis by Eve Technologies DM-44 mouse Discovery Assay

732   panel.

733

734   *In vitro* T cell suppression co-culture assay

735   CD8 T cells were isolated from the lymph node of naïve mice with unactivated Confetti cassette

736   using EasySep™ Mouse CD8$^+$ T Cell Isolation Kit (StemCell Technologies) and macrophages were

737   sorted from the tumors via FACS sorting. CD8 T cells were labeled with CFSE and co-cultured

738   with the macrophages at varying ratios in the presence of Mouse T-Activator CD3/CD28

739   Dynabeads™ (Gibco) and analyzed for division and T cell activation markers.

740

741   Immune cell abundance analysis for human RNAseq dataset

742   For analysis of human tumors, T cell, macrophage and monocyte scores were based on

743   previously calculated scores using an average expression level of genes that are uniquely

744   expressed in human T cells, macrophages and monocytes[28]. The CD206[Hi] macrophage score was

745   calculated by identifying a combination of four genes that are uniquely expressed in the

746   CD206[Hi] macrophage cluster (cluster 3) of our ZipSeq dataset (*Hmox1, Cx3cr1, Folr2 and F13a1*).

747   This score was normalized against the average expression level of four genes (*Ccr2, Mgl2,*

748   *Nr4a2 and Ciita*), which were expressed in other macrophages but were not expressed in the

749   CD206[Hi] macrophage cluster.

750

751   <u>Statistical analysis</u>

752   Data were analyzed using Prism 9 (GraphPad) with either Student's t-test for experiments with

753   two arms, one-way ANOVA analysis for experiments with one variable and more than 3

754   experiment conditions, or two-way ANOVA for experiments with two variables and more than 3

755   experiment conditions. Statistical tests for spatial analyses (Fig. 3 and 8) were done using

756   Student's t-test. For Kaplan-Meier survival analyses, log-rank test was used. Graphs show mean

757   ± SEM or mean ± SD; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

758

765

766   **Author Contributions**

767   M.T., L.L., C.L.-S., and M.Q.R. designed the project and experiments. M.T., L.L. and C.L.-S. led all

768   experiments and data analyses. K.H. and M.T. carried out the ZipSeq single cell transcriptomics

769   experiments and analysis, with support from M.K. A.J.C. and B.S. conducted the analysis of

770   human RNAseq and immune archetype datasets. M.T. and M.Q.R. wrote the manuscript, with

771   input from other co-authors. K.K., D.S., K.N., and Z.A. assisted with experiments. L.F. provided

772   guidance to the studies.

773

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852

Figure 1

A T cells

B CD4 T cells

C CD8 T cells

D CIT6-YFP CIT9-RFP

DAPI CD3

T cell count

E

CIT6-YFP CIT9-RFP

1 : 1

21-28 days

Tumor harvest

F

G

YFP
RFP

Figure 2

Figure 3

Figure 4

A Tagging of immune cells of each region with a unique barcode ("ZipCode")

YFP region
Mixed region
RFP region

Immune cells from YFP region — ZipCode 1
Immune cells from mixed region — ZipCode 2
Immune cells from RFP region — ZipCode 3

Single cell dissociation

scRNA-seq

Analysis
Clustering → Assigning originating region

1: MHC-II^{Hi} macrophage
2: Arg1^{Hi} macrophage
3: CD206^{Hi} macrophage
4: Ctsk⁺ macrophage
5: Prolif. macrophage
6: Inflammatory monocytes
7: Neutrophils
8: DCs
9: T
10: NK/ILC
11: Dying neutrophils

B
1: MHC-II^{Hi} macrophages
2: Arg1^{Hi} macrophages
3: CD206^{Hi} macrophages
4: Ctsk⁺ macrophages
5: Prolif. macrophages
6: Inflammatory monocytes
7: Neutrophils
8: DCs
9: T cells
10: NK cells/ILCs
11: Dying cells

C
Mixed
RFP
YFP

D T cells

E MHC-II^{Hi} mac.   Arg1^{Hi} mac.   CD206^{Hi} mac.

F Neutrophils
neutrophil activation involved in immune response (GO: 0002283)
neutrophil degranulation (GO: 0043312)
neutrophil-mediated immunity (GO: 0002446)
cytokine-mediated signaling pathway (GO: 0019221)
inflammatory response (GO: 0006954)
cellular response to cytokine stimulus (GO: 0071345)
regulation of inflammatory response (GO: 0050727)
response to lipopolysaccharide (GO: 0032496)
granulocyte chemotaxis (GO: 0071621)
neutrophil chemotaxis (GO: 0030593)

G Monocytes
cytokine-mediated signaling pathway (GO: 0019221)
positive regulation of intracellular signal transduction (GO: 1902533)
extracellular structure organization (GO: 0043062)
external encapsulating structure organization (GO: 0045229)
membrane raft assembly (GO: 0001765)
positive regulation of cell migration (GO: 0030335)
cellular response to cytokine stimulus (GO: 0071345)
chemokine-mediated signaling pathway (GO: 0070098)
extracellular matrix organization (GO: 0030198)
cellular response to chemokine (GO: 1990869)

Figure 5

Figure 6

A

Distribution of T cell
in 12 feature archetypes

B

RNA expression from all viable cells

Archetype: 1 2 3 4 5 6 7 8 9 10 11 12

CX3CL1

Immune hot   Immune cold

Z score (TPM) -2 0 2 4

C

Macro/Mono score
in 5 cold tumor archetypes

D

CD206^{Hi} Macro score
in 5 cold tumor archetypes

E

Macro:Mono ratio

CD206^{Hi}:Non-CD206^{Hi}
macrophage ratio

Figure 7

A

-D14 — (~5mm) D0 — D3 — (~1cm) D12-18 → Immune analysis

Injection (125K cells)

α-PD1 + CD40 ago   α-PD1

B

Isotype control   α-PD-1 + CD40ago   Average volume   **C**

CIT6-YFP — Tumor volume (mm³)   0/8   5/10

- Isotype control
- anti-PD-1 + CD40 ago

**

CIT9-RFP — Tumor volume (mm³)   0/9   1/11   n.s.

CIT6-YFP — % Survival
- Isotype control
- α-PD1+CD40ago
*

CIT9-RFP — % Survival   n.s.

D

CIT6-YFP + CIT9-RFP

Isotype control   α-PD-1 + CD40ago   Average volume   Overall survival

0/18   1/23

- Isotype control
- anti-PD-1 + CD40 ago
*

% Survival
- Isotype control
- α-PD1+CD40ago
n.s.

E

CIT6-YFP

T cells * | CD4 T cells ** | CD8 T cells *

%CD45+ cells

Isotype control / α-PD1+CD40ago

CIT9-RFP

T cells | CD4 T cells | CD8 T cells

%CD45+ cells

Isotype control / α-PD1+CD40ago

CIT6-YFP + CIT9-RFP

T cells ** | CD4 T cells * | CD8 T cells **

%CD45+ cells

Isotype control / α-PD1+CD40ago

Figure 8