1   **Integrated single-cell and spatial analysis identifies context-dependent myeloid-T**

2   **cell interactions in head and neck cancer immune checkpoint blockade response.**

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Declarations

Ethics approval and consent to participate

The clinical sample collection and analyses were conducted in accordance with the Declaration of Helsinki and received institutional ethics approval from the University of Wisconsin-Madison (UW18144 with IRB#2018–1510, subproject number 2022-009), under a waiver of informed consent.

Availability of data and materials

All in-house data and source code will be available upon manuscript acceptance.

Competing interests

All authors have confirmed no competing interests.

46   responsibility of the authors and does not necessarily represent the official views of the

47   National Institutes of Health.

48

49   **Authors' contributions**

50   HQD, MBF, PFL, and AEGO conceptualized the project. MBF, RH, and TL identified

51   patient samples and generated tumor microarrays to collect Nanostring CosMX and 10X

52   Visium data. AEGO, PK, and HQD analyzed the data. AEGO and HQD wrote and edited

53   the manuscript, with contributions from all co-authors. HQD, MBF, PFL, and PH acquired

54   funding for the project.

55

56   **Acknowledgments**

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List of abbreviations

HNSCC = Head and neck squamous cell carcinoma; ICB = immune checkpoint blockade therapy; scRNA-seq = single cell RNA-sequencing; TME = tumor microenvironment; CAFs = cancer associated fibroblasts; DCs = dendritic cells; TMA = tumor microarray; Tregs = Regulatory T cells; CCI = cell-cell interaction; LR = ligand-receptor; R = ICB responders; NR = ICB non-responders; DEGs = differentially expressed genes; UMAP = Uniform Manifold Approximation and Projection; FOVs = fields of view

74 **Abstract**

75 **Background**. Approximately 15-20% of head and neck cancer squamous cell carcinoma

76 (HNSCC) patients respond favorably to immune checkpoint blockade (ICB). Previous

77 single-cell RNA-Seq (scRNA-Seq) studies identified immune features, including

78 macrophage subset ratios and T-cell subtypes, in HNSCC ICB response. However, the

79 spatial features of HNSCC-infiltrated immune cells in response to ICB treatment need to

80 be better characterized.

81

82 **Methods**. Here, we perform a systematic evaluation of cell interactions between immune

83 cell types within the tumor microenvironment using spatial omics data using

84 complementary techniques from both 10X Visium spot-based spatial transcriptomics and

85 Nanostring CosMx single-cell spatial omics with RNA gene panel including 435 ligands

86 and receptors. In this study, we used integrated bioinformatics analyses to identify cellular

87 neighborhoods of co-localizing cell types in single-cell spatial transcriptomics and

88 proteomics data. In addition, we used both publicly available scRNA-Seq and in-house

89 spatial RNA-Seq data to identify spatially constrained Ligand-Receptor interactions in

90 Responder patients.

91

92 **Results**. With 522,399 single cells profiled with both RNA and protein from 26 patients,

93 in addition to spot-resolved spatial RNA-Seq from 8 patients treated with ICB together

94 with bioinformatics analysis of publicly available single-cell and bulk RNA-Seq, we have

95 identified a spatial and cell-type specific context-dependency of myeloid and T cell

96 interaction difference between Responders and Non-Responders. We defined further

97   cellular neighborhood and the sources of chemokine CXCL9/10-CXCR3 interactions in

98   Responders, emerging targets in ICB, as well as CXCL16-CXCR6, CCL4/5-CCR5, and

99   other underappreciated and potential markers and targets for ICB response in HNSCC.

100   In addition, we have contributed a rich data resource of cell-cell Ligand Receptor

101   interactions for the immunotherapy and HNSCC research community.

102

103   **Discussion**. Our work provides a comprehensive single-cell and spatial atlas of immune

104   cell interactions that correlate with response to ICB in HNSCC. We showcase how

105   integrating multiple technologies and bioinformatics approaches can provide new insights

106   into potential immune-based biomarkers of ICB response. Our results suggested refining

107   future studies using preclinical animal models in a more context-specific manner to

108   elucidate potential underlying mechanisms that lead to improved ICB responses.

109

110 **What is already known on this topic**

111 Most cancer patients still do not experience clinical benefits from immune checkpoint

112 blockade (ICB), necessitating the development of response biomarkers and new

113 immunotherapeutic targets.

114 **What this study adds**

115 Here, we use integrated high-dimensional omics and bioinformatics approaches to

116 identify immune cell-cell interaction markers associated with ICB response in patients

117 with Head and neck squamous cell carcinoma.

118 **How this study might affect research, practice or policy**

119 We identified spatial and cell-type specificity of Ligand-Receptor interactions between

120 myeloid and T cells in ICB Responder patients that may help inform further mechanistic

121 studies and biomarker development.

Introduction

Metastatic and recurrent head and neck squamous cell carcinoma (HNSCC) shows approximately 15-20% response rate to immune checkpoint blockade (ICB) therapy[1,2]. Understanding of the tumor microenvironment (TME) heterogeneity in HNSCC has rapidly increased due to the advance of high-dimensional single-cell omics data, allowing for the characterization of cellular compartments such as tumor and stromal cells[3,4], immune cells[5], and B cells[6]. Recent studies investigating ICB response in HNSCC have described valuable TME features, including the prognostic association of macrophage[7], T cell[8], and immune cell densities and tumor proximity of tertiary lymphoid structures markers[9]. Spatial omics and imaging technologies have identified HNSCC spatial TME features associated with survival[10], human papillomavirus infection status[11], invasive tumor phenotype[12], and CD8+ T cell infiltration and dysfunction[13]. These insights have provided potential markers of ICB response. However, spatial and single-cell analysis of immune cell-cell interactions, which could potentially identify immunotherapeutic markers and targets for ICB treatment, is still lacking.

A scRNA-Seq analysis of HNSCC TME identified CXCL9:SPP1 macrophage polarization as a new marker for pro- and anti-tumoral tumor-associated macrophages, and interactions between myeloid and T cells, such as CXCL9-CXCR3 and CXCL16-CXCR6 associated with anti-tumoral features[7]. To systematically evaluate immune cell-cell interactions, we have generated spatial multi-omics data using 10X Visium and Nanostring CosMX platforms, together with a reanalysis of publicly available scRNA-seq[8], to identify spatially-dependent immune features, such as cellular neighborhoods of co-

145 localizing immune cell types, and Ligand-Receptor interaction pairs in HNSCC tumors

146 from ICB-treated patients. We identified CD45+ cellular niches associated with ICB

147 response, including higher frequencies of stromal-immune interface enriched for antigen-

148 presenting and inflammatory cancer associated fibroblast (CAFs), dendritic cells (DCs)

149 and CD8+ T cells in Responders to ICB. Furthermore, we defined a spatially aware and

150 cell-type-specific context-dependent Ligand-Receptor pairs in different groups of ICB

151 responses. This work provides a comprehensive single-cell and spatial atlas of immune

152 cell interactions correlating with response to ICB in HNSCC and showcases how

153 integrating multiple technologies and bioinformatics approaches can offer new insights

154 into potential immune-based biomarkers of ICB response.

155

156 **Materials and Methods**

157

158 A full description of the method is supplied in the Online Supplementary Methods section.

159

160 **In Brief**

161 Nanostring CosMX single-cell spatial omics (protein and RNA) profiling was used for

162 three HNSCC TMAs consisting of 68 cores from 24 patients (11 Responders and 13

163 Non-Responders), followed by integrated bioinformatics analysis with 8 10X Visium

164 samples (4 Responders vs. 4 Non-Responders) using Seurat framework. Cellular

165 neighborhood identification was based on a window-based approach[14]. In addition, we

166 reanalyzed scRNA-Seq from treatment-naïve[5] and ICB treatment[8] with unified

167 annotations of myeloid and T-cell subsets that allowed for cell-cell Ligand-Receptor

168 interaction analysis using CellChat[15], which was also utilized for CosMX RNA data.

169 Other computational and statistical analysis were done in Python and R, details in

170 Supplementary Methods.

171

172 **Results**

173 **Multi-omics single-cell and spatial analyses of cellular neighborhood and Ligand-**

174 **Receptor immune cell-cell interactions of HNSCC tumor microenvironment (TME)**

175 **in response to ICB**

176 To evaluate the spatially resolved immune landscape of ICB-treated HNSCCs, we

177 collected Nanostring CosMX single-cell spatial transcriptomics and proteomics data from

178 68 tumor microarray (TMA) cores from 24 ICB-treated HNSCC patients (11 Responders

179 and 13 Non-Responders) (**Fig 1A-B**). With 64 immune-centric protein markers, we

180 identified most of the primary immune cell types, including three T cell subsets (CD4+,

181 CD8+, and regulatory (Tregs) T cells), four myeloid cell types (monocytes, macrophages,

182 dendritic cells - DCs, and neutrophils), together with B, plasma and NK cells with distinct

183 lineage markers (**Fig 1A**) with 39.9% of cells, on average, are immune cells for both

184 Responder and Non-Responder samples (**Fig 1A**). Except for tumor cells (Beta-

185 catenin+PanRAS+EPCAM+), which decreased in Responders (p<0.01, **Fig 1A**), we

186 observed trending changes in cell type frequencies between Responder and Non-

187 Responder (CD8+ T cells higher in Responders, **Suppl Fig 1A**). With a 1000-gene RNA

188 panel, we identified a larger extent of cell type identity, including three cancer-associated

189 fibroblasts (CAF) subsets (myofibroblast, inflammatory, and antigen-presenting CAFs),

190 and different tumor cell types expressing different keratin genes (**Fig 1B, Suppl Fig 1B**).

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191 Among them, we identified a lower abundance of KRT17+ tumor cells in Responders

192 (p=0.02, **Suppl Fig 1C**), as reported previously[16]. In addition, B cell and Plasma

193 frequency trends were higher in Responders (p=0.07 and p = 0.05, **Fig 1B, Suppl Fig**

194 **1C**). We also observed increased CD4+ and CD8+ T cells and fewer Tregs in Responders

195 (p>0.05). Notably, the major cell type frequencies correlate highly in two CosMX assays

196 (**Suppl Fig 1D**), and NK cells were not sufficiently detected (<0.2% from protein assay,

197 Suppl Fig 1A, and none from RNA assay).

198

199 To complement the small size of TMA cores and the limited number of 1000 RNA

200 transcripts captured in CosMX data, we generated whole transcriptome spatial data using

201 spot-based 10X Visium from FFPE-preserved tumor slides of 8 ICB-treated HNSCC

202 patients (4 Responders and 4 Non-Responders) (**Fig 1C**). We observed less than 20%

203 of immune CD45+ spots using computational deconvolution of the three major cellular

204 components (Tumor/Epithelial cells, stromal cells/fibroblasts, and immune cells, **Fig 1C**),

205 likely due to the tissue and Visium variability. Deconvolution of cell type frequency per

206 spot enabled us to better characterize the distribution of immune cells, epithelial/tumor

207 cells, and fibroblasts within the tissue and suggested no significant differences in their

208 proportion between Responders and Non-Responders while remaining generally

209 consistent with cell type frequencies in our single-cell spatial transcriptomics RNA data

210 (**Suppl. Fig 1E**).

211

212 To identify immune cell-cell interactions, with a focus on myeloid-T cells, in response to

213 ICB in HNSCCs, we started with scRNA-seq by reanalyzing multiple scRNA-seq datasets

214 encompassing both treatment-naive and ICB-treated HNSCC patients to look at ligand-

215 receptor (LR) interactions at the single cell level. To determine tumor-specific myeloid cell

216 heterogeneity, we interrogated 26,444 myeloid cells from both scRNA-seq of PBMC and

217 tumors in treatment-naive HNSCC patients[5] (**Fig 1D**). Including matched PBMC and

218 tumors helped identify 7 monocyte subsets, including one subset only in tumors (**Fig 1E**),

219 4 macrophage subsets, and 5 DC subsets. Our reanalysis improved the previous

220 identification of HNSCC tumor-infiltrating myeloid cells (**Fig 1E**)[5]. Next, we used Seurat

221 label transfer to identify these myeloid cell types in publicly available scRNA-seq of 19

222 ICB-treated oral squamous cell carcinoma patients (8 Non-Responders, 11 Responders)

223 (**Methods**). We recovered all treatment-naive myeloid cell types in the ICB-treated

224 samples (**Fig 1F**), with trending differences, including dendritic cell subset elevation in

225 Responders (**Suppl. Fig 1F**). Comparing the originally published annotations from the

226 ICB-treated cohort with our inferred annotations using a Gene Ontology-based method

227 reveals a high correlation between corresponding subsets, especially within the dendritic

228 cell and macrophage compartments (**Suppl. Fig 1G**). We kept the original annotations

229 for T cell subset annotation comparing across ICB-treated patient cohorts (**Suppl. Fig**

230 **1H**). In summary, we provide a comprehensive and diverse single-cell and spatial omics

231 data collection of the HNSCC TME from patients treated with ICB, incorporated with

232 scRNA-seq analysis from previously published ICB-treated tumors in an integrated

233 analysis framework to identify potential immune cell interactions in the form of immune

234 neighborhood hubs or LR expression as markers of ICB responses in HNSCC.

Single-cell spatial omics analysis identifies stroma-immune mixed cellular neighborhoods in Responder patients

Since cellular neighborhoods, defined by the spatial colocalization of different cell types, are associated with ICB response, we used a window-based neighborhood identification method for single-cell spatial data[14] to identify cellular neighborhoods in the HNSCC TME from CosMX data. From protein data, we identified 7 neighborhoods with distinct cell type compositions, derived by merging the original 20 neighborhoods identified based on the similarity of cell type enrichment within the neighborhood (**Fig 2A, Suppl Fig 2A**), including a tumor epithelial-rich region (Tumor_High neighborhood) significantly more abundant in Non-Responders (p<0.01, **Fig 2B**). Overall, neighborhoods with immune cells are more abundant in Responders (**Fig 2B**), except for the one enriched for CD11b+CD15+ neutrophils (Neutrophil_High neighborhood), which is lower in Responders. Visual inspection with representative immune markers (CD19, CD3E, HLA-DRA-CCR7, GZMB for B, T, and DCs) from CosMX data showed agreement with the corresponding cell type annotations from the RNA profiles in an immune-rich Responder and an immune-desert Non-Responder (**Fig 2B**). With a 1000-gene RNA assay, we identified nine neighborhoods with distinct cell-type compositions, derived by merging the original 20 neighborhoods identified based on the similarity of cell-type enrichment within the neighborhood (**Fig 2C, Suppl Fig 2B**). Assessing neighborhood frequency per FOV by ICB response identified a neighborhood enriched for immune cells, primarily CD8+ T cells, monocytes and DCs (ap_iCAF_Immune_Mixed), including VEGFA-expressing inflammatory-like cancer-associated fibroblasts (iCAFs) similar to those previously reported[17], and to lesser extent with antigen-presenting CAFs (apCAFs) more abundant

13

258   in Responders, and a neighborhood enriched for myeloid cell types and KRT17+ tumor

259   cells (Myeloid_K17+_Tumor_Mixed) in Non-Responders   (**Fig 2D**). Comparing ICB

260   Response-specific enrichment of each cell type within these neighborhoods suggests

261   concordance in the cell type frequencies (**Suppl Fig 2C-D**). This neighborhood also

262   included co-localization of conventional antigen presenting T cell activation, like DCs, and

263   the newly defined apCAF[18] in the absence of tumor cells expressed pro-tumoral and ICB-

264   non-responsive KRT17. Looking into this neighborhood, we found higher CD4+ T and

265   Treg cell frequencies in Responders (**Supp. Fig 2C**). Furthermore, differential expression

266   analysis identified higher CXCL10 chemokine expression in DCs from Responders but

267   higher CXCL9 in monocytes from Non-Responders (**Fig 2E**), while its receptor CXCR3

268   was found higher in CD8+ T and Treg cells within the same neighborhood (**Fig 2F**).

269   Recent work showed that CXCL9/10-engineered DCs could enhance ICB response in

270   lung cancer by activating T cell immunity[19]; however, we do not observe increased CXCL9

271   expression in Responder DCs in this neighborhood (**Fig 2E**). Assessing the average

272   distance between myeloid senders, T cell receivers, and tumor cells by Response

273   identified monocytes and Tregs closer to tumor cells in Non-Responders in the

274   Tumor_Immune_Tcell_High neighborhood. We also observe a variable proximity of

275   CXCL10+ DCs and CXCR3+ CD4+ T cells to tumor cells, with half of FOVs being closer,

276   in Responders compared to that in Non-Responders in the apCAF_iCAF_immune_mixed

277   neighborhood (**Suppl. Fig 2E**). Together, single-cell spatial omics data revealed

278   differences in cellular neighborhoods not detected through cell type frequency

279   comparisons. These include the stromal-immune cell neighborhoods containing antigen-

280   presenting cells (monocytes, dendritic cells, and apCAF) associated with CD8+ T cells.

281   These neighborhoods are enriched in Responders showing higher co-expression of the

282   CXCL10-CXCR3 ligand-receptor pair in DC-T cell interactions but lower expression of

283   CXCL9-CXCR3 in monocyte-T cell interactions.

284   **ScRNA-seq analysis of myeloid to T cell ligand-receptor interactions in HNSCC**

285   **response to ICB therapy**

286   To identify myeloid-T cell interactions in HNSCC ICB response, we inferred LR

287   interactions based on the LR CellChat database, with associated biological pathway

288   categorization defined previously[20] (**Methods**). To compare the difference in Responders

289   vs Non-Responders and before and after treatment, we used the number of LR

290   interactions inferred in each patient cohort (**Fig 3A**) from the scRNA-seq dataset[8] with

291   our label-transferred myeloid subset annotations from the treatment-naive samples[5] while

292   keeping and merging original T cell annotations based on GO-based cross-dataset

293   comparisons (**Methods** and **Suppl. Fig 3B**). Various interacting pairs in both Responders

294   and Non-Responders were detected in pre-treatment samples. The primary pre-treatment

295   sender cell types in Responders include IL1B+ macrophages and CD33+ cDC2s, while

296   Non-Responders have various interacting pairs (**Fig 3B**). However, post-treatment

297   interactions include IL1B+ macrophages in Responders and a large increase in significant

298   interactions from PBMC-like CD14+ monocytes and tumor-infiltrating monocytes in Non-

299   Responders to various receiving cell types. Comparing Non-Responders and

300   Responders, fewer interactions were identified in the pre-treatment samples (**Fig 3B**). In

301   Non-Responder patients, we found more interactions in the after-treatment samples,

302   mostly from interactions to all T cell subsets with CD14+ classical monocytes with similar

303   gene signatures to those defined in PBMCs as senders (**Fig 3B**). Notably, of all pre-ICB

15

304   Non-Responder-specific CD14+ monocyte interactions, 76% remained Non-Responder

305   specific post-ICB, 10% became non-significant, and 13.3% shared. Conversely, of all

306   Responder-specific CD14+ monocyte interactions pre-treatment, 52.3% became Non-

307   Responder specific, 38.6% became non-significant, and 9% became shared (**Fig 3C-D**).

308   For example, Non-Responder interactions conserved in pre- and post-treatment and

309   included chemokine (CXCL16-CXCR6), galectin (LGALS9-CD44, LGALS9-PTPRC,

310   LGALS9-P4HB,   LGALS9-HAVCR2),   thrombospondin   (THBS1-CD47),   and

311   costimulatory/coinhibitory (CD86-CTLA4, CD86-CD28) interactions. Lastly, at the per

312   subset level, the one subset where all Non-Responder specific pre-treatment interactions

313   maintain significance and persist as Non-Responder specific post-treatment are the

314   ITGAE+ CD8+ T cells. Narrowing in on enriched sender-receiver pairs at the pathway

315   level showed mregDCs as the top sending cell type post-treatment in both groups in the

316   chemokine pathway, showing little preference per group, which was similarly seen in the

317   whole database analysis (**Suppl Fig 3A).** Within the complement pathway, Responders

318   have an increase in outgoing signaling post-ICB from CXCL9+ Macrophages, whereas

319   most outgoing signaling from this population pre-ICB was found in Non-Responders, but

320   these interactions do not have a big difference as in other pathway categories (**Suppl Fig**

321   **3B).** Lastly, outgoing cDC1 signaling within the Costimulatory/Coinhibitory pathway was

322   enriched in Responders, a trend reflected in the whole database analysis post-ICB, but

323   not pre-ICB through an increased number of receiving cell types interacting with cDC1s

324   post-ICB in the Responders (**Suppl Fig 3C).** These data suggest that interactions

325   enriched in ICB treatment, such as CXCL16-CXCR6, THBS1-CD47, and CD86-CTLA4,

16

326   originating from CD14+ monocytes with a transcriptional signature similar to that of PBMC

327   CD14+ monocytes, are prevalent in post-treatment Non-Responders.

328

329   **Integrated single-cell and spatial omics analysis refined context-specific immune**

330   **cell LR interactions in HNSCC ICB responses**

331   Because we found heterogeneity of ligand expression in different myeloid cell subsets

332   (**Fig 2F**) that are spatially and cell-type dependent, we evaluated LR interactions between

333   myeloid and T cells at the levels of both cell-type-specific and spatial resolutions using

334   scRNA-Seq and CosMX data. First, we divided the LR interactions into four functional

335   pathways: Chemokine, Complement, Interleukin, and Co-stimulatory/Co-inhibitory (**Fig**

336   **4A**). We identified most interactions pre- and post-ICB from the

337   Costimulatory/Coinhibitory pathway, but also with notably higher pre-treatment

338   interactions through the Complement pathway, especially those "sent" from macrophages

339   in Responders and monocytes in Non-Responders (**Suppl Fig 4A-B**). When comparing

340   paired samples from pre- and post-ICB in Responders and Non-Responders by pathway,

341   we observe varied patterns including enriched pre-ICB chemokine signaling from

342   monocytes and post-ICB chemokine signaling from cDC1 DCs in Non-Responders. In

343   contrast, ICB Responders had enriched post-treatment chemokine signaling sent from

344   mregDCs (**Suppl Fig 4C**). pre-ICB complement signaling in Responders was primarily

345   sent from IL1B+ macrophages, whereas CXCL9+ macrophages were the primary

346   complement signaling sender pre-ICB in Non-Responders (Suppl Fig 5D). Costimulatory

347   and Coinhibitory interactions were numerous and varied greatly by ICB response and

348   treatment stage (**Suppl Fig 4E**). Interleukin pathways, specifically IL10-IL10RA/IL10RB,

17

349   were found in pre-treatment Non-Responders and mainly from macrophages (**Fig 4A**).

350   Chemokine interactions were sparse but generally shared between Responders and Non-

351   Responders. Among chemokine interactions enriched in pre-treatment Responder

352   samples, we found CXCL16-CXCR6 between CD33+ cDC2s and CXCL13+ and tissue-

353   resident memory CD8+ T cells (CD103/ITGAE+ cells), and CXCL9-CXCR3 between

354   cDC1 to the same two T cell subsets and GZMK+ CD8+ T cells (**Fig 4B**). In contrast, the

355   top enriched interactions in pre-treatment Non-Responder samples were less than 1.5-

356   fold in their changes, including CCL4-CCL5 signaling from CD33+ cDC2s to GZMK+

357   CD8+ T cells (**Fig 4B**). In contrast, the most enriched interactions in post-treatment

358   Responders were CXCL9/10-CXCR3 from LAMP3+ mregDCs to NR4A2+ CD8+ T cells,

359   indicating a cell-type interaction switch after ICB treatment. Like pre-treatment samples,

360   IL16-CD4 interactions between CD16+ non-classical monocytes or CD1C+ cDC2

361   dendritic cells with CXCL13 T cells, or cDC1 dendritic cell interaction with NR4A2-

362   expressed CD8+ T cells through CXCL16-CXCR6, which were found in different cell type

363   pairs pre-treatment.

364

365   To complement and gain better resolution than provided by the scRNA-seq LR interaction

366   analysis, we adapted the CellChat cell-cell interaction (CCI) analysis (**Methods**) for

367   CD45+ myeloid and T cell-rich neighborhoods identified from a window-based cellular

368   neighborhood analysis (**Methods** & **Fig 2C**). Using CCI LR likelihood probabilities, we

369   defined 7 LR interaction patterns using an unsupervised hierarchical clustering method,

370   reflecting context-dependency of spatial neighborhood and cell types of senders and

371   receivers. Furthermore, we merged into three major groups (**Fig 4C**): (i) Responder-

372 specific LRs including CXCL9/10-CXCR3 and CXCL16-CXCR3; (ii) Non-Responder-

373 specific LRs including IL16, IL11 and CCL19/21 signaling in Tumor_Immune_Cell_High

374 neighborhood as well as CCL3/4-CCR5 and IL1B signaling mainly in the

375 Immune_Macrophage_High and Myeloid_K17+_Tumor_Mixed neighborhoods; and (ii)

376 Mixed Responders and Non-Responders with IL16 and CXCL16-CXCR6 signaling in the

377 myCAF_Immune_Mixed neighborhood. Examples were provided in representative

378 contexts, with CXCL10-expressed DC/macrophages interacting with CXCR3-expressed

379 T cells in Responders, CCL19/21-expressed monocytes interacting with CCR7/CD4-

380 expressed Treg and CD4+ T cells in Non-Responders, and CCL3/4/13-expressed

381 macrophages interacting with CCR5-expressed Treg in Non-Responders (**Fig 4D**).

382 Additionally, in the Immune_Macrophage_High neighborhood, we observed CXCL9 most

383 highly expressed in dendritic cells and CXCL16 in macrophages from Non-Responders,

384 and CXCR3 was not highly expressed in any T cell population from these same patients,

385 suggesting that T cell recruitment in this neighborhood may be limited by a lack of CXCR3

386 in the T cells. In contrast, CXCL10 in DCs and CXCR3 in CD4+ T cells were enriched in

387 ICB Responders (**Suppl Fig 4F**). In the Tumor_Immune_Tcell_High neighborhood, we

388 observed that Responders had enriched CXCL10 in macrophages, CCL5 in DCs, and

389 CXCR3 in CD8+ T cells (**Suppl Fig 4G**). Interestingly, we also observed a group of CCIs,

390 enriched in Responder samples, with CD4+ T cells as receivers mainly from

391 ap_iCAF_Immune_Mixed and MyCAF_Immune_Mixed neighborhoods with few LRs from

392 the three most dominant immune signaling pathways (IL, CCL, CXCL). In summary, our

393 analysis revealed context-dependent myeloid-T cell interactions that correlate with ICB

394 response status in HNSCC ICB treatment. This includes thoroughly examining

395   established interactions (such as CXCL9/10-CXCR3) and several under-appreciated LR

396   pairs that have not been documented in HNSCC and ICB contexts.

397

398   **Spatially-constrained immune cell-cell interaction inference identified potential LR**

399   **interactions enriched in ICB Responders**

400   To further interrogate the spatially-constrained immune-cell interactions enriched in

401   HNSCC ICB response with larger tissues than the TMA cores, we looked at Visium spots

402   from the eight patients' data (**Fig 1C**) that had at least 5% of immune cells inferred by

403   computational deconvolution (**Methods**). We computed a bidirectional co-expression

404   score to identify which co-expressed LR pairs were enriched by ICB response (**Fig 5A**).

405   Our scoring method identified LR pairs enriched in Responders, including HLA-C-CD8A,

406   CXCL16-CXCR6, CXCL9/10-CXCR3, CXCL13-CXCR3 (**Fig 5B**), CXCL11-ACKR3, DLL-

407   NOTCH1, and TNF-TNFRSF1A (**Suppl Fig 5A-B**). In contrast, Non-Responders enriched

408   interactions included CXCL3-ACKR1, CXCL5-ACKR1, AND CCL13-ACKR1 (**Suppl Fig**

409   **5A-C**). The limitation of CD45+ spots and marker expression did not allow performing

410   deconvolution at the resolution of immune subtypes inferred in scRNA-seq; however, we

411   identified CXCL9/10-CXCR3 and CXCL16-CXCR16 for which LR bidirectional scores

412   were enriched in CD45+ spots in tissues (**Fig 5C**). In summary, spot-based Visium data

413   assists in identifying potential candidates for LR pairs, including known markers CXCL9-

414   CXCR3, CXCL16-CXCR6, which were not previously reported, co-expressed in spatially-

415   constrained locations in Responder tissues through a bidirectional co-expression scoring

416   system.

417

Evaluating spatial and cell-type context-dependent Ligand-Receptor immune cell interactions using publicly available and independent ICB-treated HNSCC cohorts

To evaluate LR pairs from the Responder and Non-Responder group (**Fig 4C**), we used a publicly available bulk RNA-Seq dataset of CD68+ and CD45+ cells from Nanostring Digital Spatial Profiling technology for an ICB-treated TMA[21] and a customized panel gene expression of HNSCC patients treated with ICB from a clinical trial[22,23]. Among the LRs defined from single-cell spatial data, we found that the CXCL10 ligand has the highest expression in CD68+ samples (**Fig 6A-B**). Assessing individual LR pairs, we found CXCL10 and CXCL16 show higher expression in CD68+ from Responders (p=0.013, and p=0.025, Wilcox test, respectively) (**Fig 6B**), but their receptors (CXCR3 and CXCR6) show no change in CD45+ samples. A few noticeable differences from LR pairs identified from CosMX data (**Fig 4C**) were observed in this dataset (**Suppl Fig 6**). Additionally, we confirmed that CXCL9 was a better predictor of ICB response than PDL1(CD274) (p =4e-05, p = 0.57, respectively) in a cohort of 107 patients from clinical trials, providing additional support for CXCL9 as an effective predictor of ICB response in large-scale studies, even compared to known clinical markers (**Fig 6C**). In sum, reanalyzing publicly available RNA-Seq data supports CXCL9/10-CXCR3 and CXCL16-CXCR6 myeloid-T cell context-dependent interactions associated with ICB response in HNSCC (**Fig 6D**).

Discussion

HNSCC is the 6th - 7th most common cancer type worldwide. Only 15-20% of HNSCC patients show favorable response to ICB therapy. Improving our understanding of the HNSCC TME may reveal new biomarkers and treatment strategies to augment ICB

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441 response and better select those patients most likely to benefit from ICB treatment

442 approaches. In this study, we present a comprehensive, integrated analysis of spatial

443 and single-cell omics of HNSCC TME to evaluate immune cell-cell interactions, focusing

444 on myeloid and T cells in response to ICB. While we did not find much difference in cell

445 type frequencies, despite observing immunological hot tumor phenotypes in

446 Responders[16], we identified an increase of cellular neighborhoods at the stroma-immune

447 interface in Responders in both protein and RNA single-cell spatial data (**Fig 2**).

448 Interestingly, we identified a decrease in the neutrophil neighborhood in Responders from

449 CosMX protein assay, which was usually underappreciated in spatial RNA assays.

450 Importantly, we defined a spatial and cell-type context-dependent pattern of immune cell-

451 cell Ligand-Receptor interactions in HNSCC response to ICB (**Fig 4C**), including both

452 known and not yet reported interactions in cancer immunotherapy context across different

453 data modalities and profiling resolutions. In addition, we identified an increase of

454 interactions from CD14+ monocytes with T cells in Non-Responders only after ICB

455 treatment from scRNA-Seq (**Fig 6D**).

456 Our analysis identified multiple candidate interactions in ICB Responders, including

457 widely reported CXCL9/10-CXCR3 between myeloid and CD8+ T cells, an emerging

458 marker of ICB response in HNSCC[7,19,24–28]. In breast cancer mouse models, CXCL9/10+

459 macrophages were upregulated following dual PD-1/CTLA-4 blockade via a CXCR3-

460 dependent CD8+ T cell infiltration[41]. Our data showed CXCL9/10, mainly expressed in

461 macrophages and DCs (especially LAMP3+ mregDC[29] subset) and their receptors

462 CXCR3 expressed in CD8+ T cells, associated with ICB response in the absence of

463 CCL4/5-CCR5 interactions between monocytes and CD4+ T cells (including Tregs),

464 highlighting the TME-specificity of immune CCIs. Previous studies have identified both
465 pro- and anti-tumoral roles of CCL4/5-CCR5 in the context of cancer[30]. However, one
466 study in mouse lymphoma and melanoma also revealed pro-tumoral roles of these
467 interactions through the myeloid-T cell axis—identifying CCL4/5 expression in tumor-
468 infiltrating granulocytic and monocytic myeloid-derived suppressor cells (MO-MDSCs),
469 and CCR5 in Tregs. Upon knocking out CCR5, there was a significant decrease in
470 infiltrating Tregs into tumors. In contrast, intratumorally injection of CCL4 or CCL5
471 increased tumor-infiltrating Tregs, reflecting *in vivo* evidence of our *in silico* observations
472 in the present study[31]. However, we showed a context-dependency of these CCIs due to
473 the complexity and heterogeneous nature of HNSCC tissues in our data. Another
474 interaction, CXCL16-CXCR6, was identified in Responders from scRNA-Seq (between
475 DC subsets and CXCL13+ and tissue-resident CD103+ T cells, **Fig 4B**) and in spatial
476 Visium data (**Fig 5**) but also in Non-Responders in specific cellular neighborhoods from
477 single-cell spatial data (**Fig 4C**). CXCR6 plays an important role in cytotoxic T cells[32],
478 CD8+ resident memory T cell recruitment in HNSCC[33]. However, CXCR6 was also found
479 to be involved in exhausted T cell phenotypes by a pan-cancer analysis[34].

480 Overall, we highlighted the complexity and context-dependent nature of immune cell-cell
481 interactions in human HNSCC, as summarized in **Fig 6D**. This would help further *in vivo*
482 work in mouse models to manipulate and induce these LR interactions for anti-tumoral
483 immunity. Our work also showed the importance of integrated multi-omics analysis in
484 human cancer data, which could generate potential immunotherapeutic biomarkers and
485 targets for HNSCC immunotherapy.

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Figure Legends

Figure 1. Overview of spatial and single-cell immune landscape in HNSCC treated with Immune Checkpoint Blockade (ICB)

a. Uniform Manifold Approximation and Projection (UMAP) visualization, average percentage by cell type (star annotated the cell type abundance difference between Responders (R) and Non-Responders (NR), with p<0.05, Wilcox test, also see **Suppl 1A** for detailed comparison), cell type gene signatures defined from differentially expressed gene (DEG) list, of HNSCC R and NR patients treated with ICB identified from Nanostring CosMx single-cell spatial protein profiling data.

b. UMAP visualization, average percentage by cell type (star annotated the cell type abundance difference between R and NR, with p<0.05, Wilcox test, also see **Suppl 1C** for detailed comparison), cell type gene signatures defined from DEG list, of HNSCC R and NR patients treated with ICB identified from Nanostring CosMx single-cell spatial RNA profiling data.

c. Major cell type compartment percentage (using computational deconvolution) by spot for representative NR and R samples sequenced with 10X Visium spatial transcriptomics, and representative cell type markers. Distribution of cell type compartment proportion in 8 R and NR samples from 10X Visium.

d. Gene signatures from DEGs of different myeloid subsets, UMAP visualization from reanalyzed treatment-naïve HNSCC scRNA-seq.

e. UMAP visualization of reanalyzed myeloid subsets split by PBMC and tumor samples.

514   f. Label-transferred defined myeloid cell subsets and established T cell subsets in

515   IBC-treated HNSCC R and NR using computational label transfer.

516   **Figure 2. Single-cell imaging-based spatial omics suggests enrichment of a**

517   **stromal-immune mixed cellular neighborhood in Responders**

518   a. Normalized enrichment score of cell type proportion in cellular neighborhood from

519   protein data identified using a window-based neighborhood identification

520   method[14].

521   b. Representative fields of view (FOVs) from immune-CAF mixed neighborhood

522   inferred from protein data: the left plot shows representative immune and stromal

523   marker fluorescence and the right plot includes fibroblast and immune cell types

524   within the neighborhood (one R FOV representative above, one NR FOV

525   representative below). Cellular neighborhood frequency comparison between R

526   and NR in protein FOVs.

527   c. Normalized enrichment score of cell type proportion within each cellular

528   neighborhood from RNA data identified using a window-based neighborhood

529   identification method[14].

530   d. Representative FOVs from immune-CAF neighborhood inferred from RNA data:

531   the left plot shows representative immune and stromal marker fluorescence, and

532   the right plot includes CAF and immune cell types within the neighborhood (one R

533   FOV representative above, one NR FOV representative below). Cellular

534   neighborhood frequency comparison between R and NR in protein FOVs.

535   e. DEGs of DCs in CD45+ neighborhoods between R and NR.

536   f.   DotPlot of CXCL9/10 ligand and their receptor (CXCR3) in myeloid and T cell

537   subsets between R and NR in Myeloid_K17+_Tumor_Mixed and

538   ap_iCAF_Immune_Mixed neighborhoods, showing opposite frequency

539   differences.

540 **Figure 3. CD14+ monocytes sharing a transcriptional signature with PBMC**

541 **monocytes have increased outgoing signaling in Non-Responders**

542   a.   Schematic of *in silico* cell-cell interaction (CCI) analysis. Comparison analyses

543   were plotted with interaction rank between groups (with

544   R-enriched interactions at the lower end) on the x-axis and log2 fold change of

545   CellChat interaction count on the y-axis. Enriched interactions are visualized with

546   the left box representing senders, and the right box representing receiver cell

547   types.

548   b.   Rank plot of CCIs enriched by Response for pre- and post-ICB.

549   c.   UpSet plots representing the degree of overlap of LR interactions to receiving T

550   cell types sent from PBMC-like CD14 monocytes both pre- and post-ICB.

551   d.   Alluvial plots representing sent signal to T cell subsets from PBMC-like CD14

552   monocytes, pre-ICB, and the specificity and receiving cell type of these same

553   interactions post-ICB. The left strata and links represent pre-ICB receiving cell

554   type, and the right strata represent post-ICB specificity by Response. Grey stratum

555   represents interactions either gained or lost following ICB. Left: post-ICB specificity

556   of NR-specific interactions from pre-ICB. Right: post-ICB specificity of R-specific

557   interactions from pre-ICB.

Figure 4. Spatial and cell-type context-specific identification of myeloid-T cell interactions in HNSCC ICB Response

a. Circos plots representing CCI interaction probability from myeloid to T cell compartments by pathway for pre- and post-treatment using previously published pathways[20]. Chord color represents pathway. Interactions from each sender/receiver pair are categorized by group specificity: specific to Responders (R), Non-Responders (NR), or shared (Sh), and converted to percentage of total probability contribution to all interactions within that pair and group specificity.

b. Rank plot of CCIs within the chemokine pathway pre- and post-ICB. Interactions on the top left are enriched in R, and interactions at the bottom right are enriched in NR. Individual interactions are represented by a 2-segment box, the left box indicating sender cell type, the right box indicating receiver cell type, ligand is written in the left box, and receptor written in the right box.

c. Unsupervised hierarchical clustering identified context-specific myeloid-T LR interaction differences between HNSCC ICB R and NR. (Left) Heatmap represents 7 Ligand-Receptor interaction likelihood probability patterns, merged into 3 major patterns: R-enriched, NR-enriched and Mixed. (Right) LR gene expression in representative neighborhood and cell type specificity identified for R and NR.

d. DotPlot for representative LR gene expression in myeloid and T cells within select immune-rich neighborhoods.

578 **Figure 5. CXCL9/10-CXCR3 and CXCL16-CXCR6 are elevated in immune-rich**

579 **regions of Responder tumors in Visium tissues**

580   a. Schematic of LR bidirectional co-expression score calculation in CD45+ spots (as

581   defined by >5% immune cells per spot from SCDC deconvolution).

582   b. Rank plot of the difference in R vs. NR mean CD45+ spot proportion for a given

583   LR pair. The left side of the plot highlights interactions enriched in R, and the right

584   side highlights interactions enriched in NR. Select chemokine interactions are

585   highlighted in red.

586   c. Top: Spatial feature plot of CXCL9, CXCL10, CXCR3, CXCL16, and CXCR6

587   expression per spot for one example R (top) and NR (bottom) tumor. Bottom:

588   Spatial feature plot and boxplots of select bidirectional co-expression LR scores

589   per spot in CD45+ spots for R and NR tumors. The frequency of LR+ CD45+ spots

590   among CD45+ spots was split by response for CXCL9-CXCR3, CXCL10-CXCR3,

591   and CXCL16-CXCR6.

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594 **Figure 6. Publicly available independent cohort validation of elevated chemokine**

595 **interactions in ICB Responder patients**

596   a. Volcano plots show DEGs of CD68+ and CD45+ RNA-Seq from Nanostring

597   GeoMX for NR vs R, highlighting immune LR pairs inferred from **Fig 4C**.

598   b. Comparison of three top LRs between Responders (n=20) vs. Non-Responders

599   (n=76) with Ligands in CD68+ samples and Receptors in CD45+ samples.

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600 c. Comparison of CXCL9 and CD274(PD-L1) expression by Response for publicly

601   available clinical trial data from ICB-treated HNSCC patients[22,23].

602 d. Summary of main differences between myeloid-T cell interactions between

603   HNSCC Responders vs Non-Responders.

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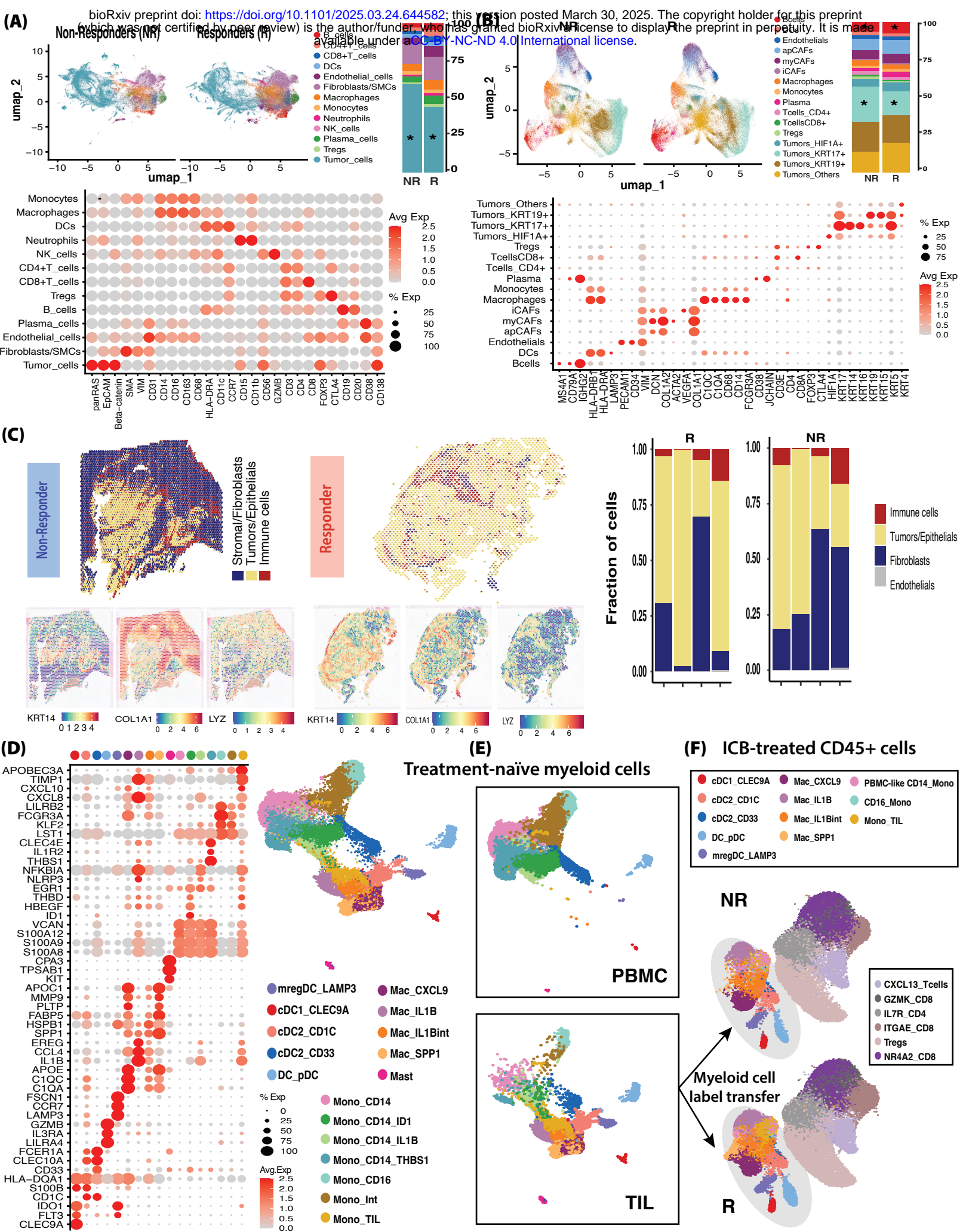
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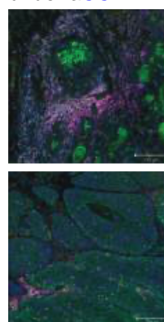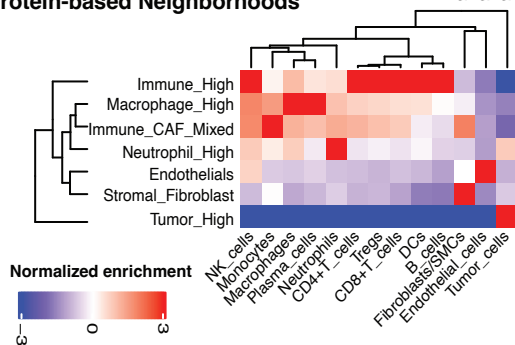
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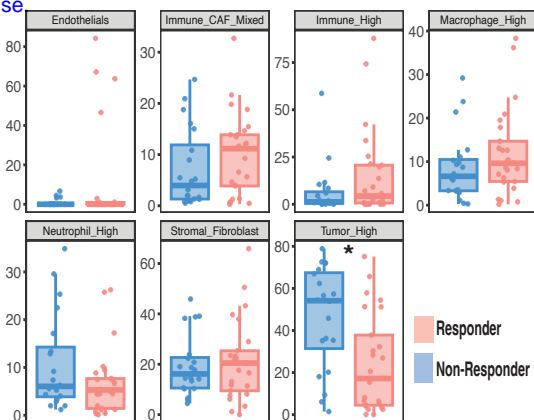
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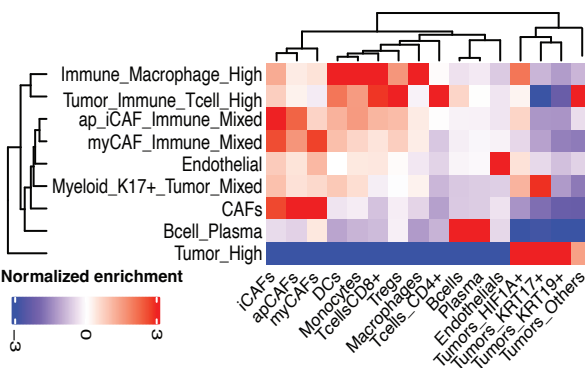
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(E)
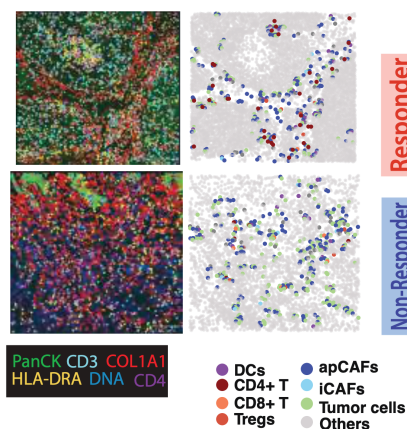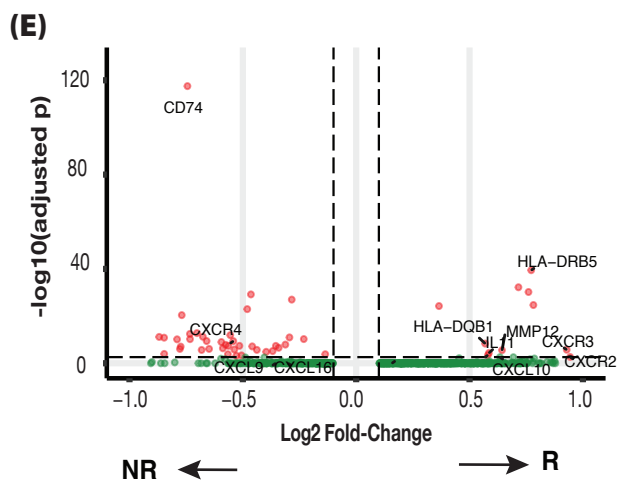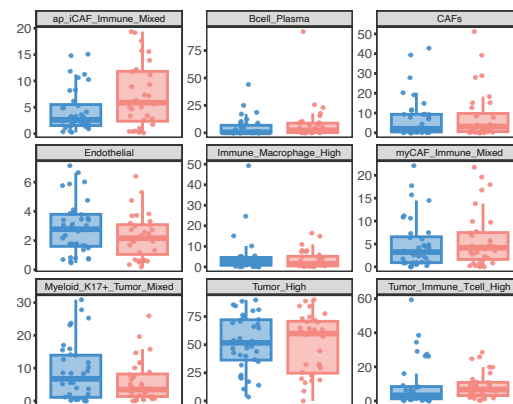
(F) Myeloid_K17+_Tumor_Mixed   ap_iCAF_Immune_Mixed

(A), (B), (C), (D)