Dendritic cell effector mechanisms and tumor immune microenvironment infiltration define TLR8 modulation and PD-1 blockade

1 **Daniel A. Ruiz-Torres[1,2,3+], Jillian Wise[3,4,5,6+], Brian Yinge Zhao[1], Joao Paulo Oliveira-Costa[3,5,7],**
2 **Sara Cavallaro[3,5], Peter Sadow[1,2,4], Jacy Fang[3], Osman Yilmaz[7], Amar Patel[8], Christopher**
3 **Loosbroock[8], Moshe Sade-Feldman[3,5*], Daniel L. Faden[1,2,5*], Shannon L. Stott[3,5,9*]**

4 [1] Department of Otolaryngology-Head and Neck Surgery, Harvard Medical School, Boston, MA
5 02115, USA.

6 [2] Massachusetts Eye and Ear, Boston, MA 02118, USA.

7 [3] Massachusetts General Hospital Cancer Center, Boston, MA 02118, USA.

8 [4] Department of Pathology, Massachusetts General Hospital, Harvard Medical School, Boston,
9 Massachusetts, USA.

10 [5] Broad Institute of MIT and Harvard, Cambridge, MA 02142, USA.

11 [6] Salve Regina University, Newport, RI 02840, USA.

12 [7] Department of Pathology, Beth Israel Deaconess Medical Center, Boston, Massachusetts, USA.

13 [8] Bristol Myers Squibb, Seattle, WA 98109, USA.

14 [9]Center for Engineering in Medicine and BioMEMS Resource Center, Surgical Services,
15 Massachusetts General Hospital, Harvard Medical School, 114 16th Street, Charlestown, MA 02129,
16 USA.

17 [+] These authors contributed equally to this work and share the first authorship.

18 *These authors contributed equally to this work and share the last authorship.

19 **Correspondence:** Shannon L. Stott sstott@mgh.harvard.edu

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Abstract

31 The potent immunostimulatory effects of toll-like receptor 8 (TLR8) agonism in combination with

32 PD-1 blockade have resulted in various preclinical investigations, yet the mechanism of action in

33 humans remains unknown. To decipher the combinatory mode of action of TLR8 agonism and PD-1

34 blockade, we employed a unique, open-label, phase 1b pre-operative window of opportunity clinical

35 trial (NCT03906526) in head and neck squamous cell carcinoma (HNSCC) patients. Matched pre-

36 and post-treatment tumor biopsies from the same lesion were obtained. We used single-cell RNA

37 sequencing and custom multiplex staining to leverage the unique advantage of same-lesion

38 longitudinal sampling. Patients receiving dual TLR8 agonism and anti-PD-1 blockade exhibited

39 marked upregulation of innate immune effector genes and cytokines, highlighted by increased

40 *CLEC9A+* dendritic cell and *CLEC7A/SYK* expression. This was revealed via comparison with a

41 previous cohort from an anti-PD-1 blockade monotherapy single-cell RNA sequencing study.

42 Furthermore, in dual therapy patients, post-treatment mature dendritic cells increased in adjacency to

43 CD8$^+$ T-cells. Increased tumoral cytotoxic T-lymphocyte densities and expanded CXCL13$^+$CD8$^+$ T-

44 cell populations were observed in responders, with increased tertiary lymphoid structures (TLSs)

45 across all three patients. This study provides key insights into the mode of action of TLR8 agonism

46 and anti-PD-1 blockade immune targeting in HNSCC patients.

1. Introduction

48 Head and neck cancers are the sixth most common malignancy worldwide [1], and head and neck

49 squamous cell carcinomas (HNSCC) constitute over 90% of these cases. Approximately 50-60% of

50 patients experience loco-regional recurrence within two years of starting therapy [2, 3], underscoring

51 the urgent need for new therapeutic approaches. Immune checkpoint blockade (ICB) activates T cell

52 antitumor activity by targeting immunoregulatory molecules such as the programmed cell death

53 receptor 1 (PD-1). This approach has become the standard of care for recurrent or metastatic

54 HNSCC, as these tumors often have the highest levels of immune cell infiltration for solid

55 malignancies [4]. Despite this, response rates to single-agent anti-PD-1 are low (<18%) [2], and

56 durable complete responses are scarce. Neoadjuvant ICB has shown significant promise in HNSCC,

57 but overall response rates remain modest [5]. Thus, further exploration into mechanisms of

58 neoadjuvant immune targeting in HNSCC is needed.

59   The activation of Toll-like receptors (TLRs) stimulates innate immune cells, releasing cytokines and

60   enhancing T-cell and dendritic cell (DC) functions. [6]. Dendritic cells are antigen-presenting cells

61   present in the tumor immune microenvironment (TME) of HNSCC [7] and have been widely

62   proposed as promising targets for cancer immunotherapy [8]. TLR agonism and dendritic cell

63   vaccination have shown increased interferon gene expression and prolonged survival in a randomized

64   phase II clinical trial in malignant glioma [9]. Recent work in HNSCC mouse models has shown the

65   ability of DCs to overcome resistance to ICB by enhancing T-cell infiltration after conventional

66   dendritic cell vaccination [10], underscoring the potential role of DCs in ICB success.

67   Within the TLR family, TLR8 recognizes single-stranded RNA molecules and leads to the induction

68   of a type I interferon response, which results in the production and secretion of proinflammatory

69   cytokines and chemokines (e.g., CXCL9, CXCL10). Recently, motolimod (VTX-2337), a selective

70   synthetic TLR8 agonist, has been tested as adjuvant therapy in combination with chemotherapy in

71   HNSCC patients, showing promising results, particularly in human papillomavirus (HPV) positive

72   patients [11]. Motolimod's properties may rely on understudied distinct TMEs, and to the best of our

73   knowledge, its effects on the transcriptional phenotypes of the malignant cells and adjacent TME

74   have never been studied in humans.

75   Prior preclinical work on TLR stimulation has shown that CD11c-expressing dendritic cells are

76   activated to enter an organized non-lymphoid tissue aggregate known as a tertiary lymphoid structure

77   (TLS) [12]. TLR agonism also contributes to the formation of TLSs and the upregulation of CXCL13

78   in myofibroblasts and lung tumors [13,14]. Importantly, TLSs have also been revealed as a surrogate

79   marker of a robust immune response and are associated with improved responses to ICB, yet

80   CXCL13 serum levels are a poor prognostic marker in certain lung cancers [15,16]. Thus, the

81   composition and spatial characterization of TLSs could provide a better understanding of the impact

82   of TLR8 agonism and PD-1 blockade in HNSCC patients.

83   Advancements in single-cell genomics have provided an avenue for capturing the heterogeneity of

84   epithelial tumors and their microenvironment. Single-cell RNA sequencing (scRNAseq) studies have

85   revealed that DCs decrease in the HNSCC tumor microenvironment post-ICB therapy [17].

86   Multiplex-immunofluorescence (mIF) imaging has been proven to be an effective tool for biomarker

87   discovery and to predict responses to ICB in different solid malignancies, including HNSCC [18].

88   Given myeloid cells' importance in driving antitumor activity, the decrease of DCs post- ICB

89   therapy, and the association of TLSs with response to ICB, we set out to determine the effect of

90   TLR8 agonist (motolimod), in combination with anti-PD-1 blockade (nivolumab) on the tumor

91   ecosystem in HNSCC patients. As part of a phase 1b clinical trial (NCT03906526), we used single-

92   cell sequencing alongside multiplex immunofluorescence (mIF) imaging tools to analyze pre- and

93   post-treatment tumor samples taken from the same lesion to investigate this dual mode of action. Our

94   study design allowed us to profile TME changes across treatments, identifying dynamic

95   transcriptional signatures in the DC population that were unique to dual therapy and were not present

96   in a patient cohort treated with anti-PD-1 blockade monotherapy alone.

97

98   **Materials and Methods**

99   **Tissue collection, processing, and response assessment.** Patients with previously untreated,

100   resectable HNSCC were recruited as part of a clinical trial

101   https://clinicaltrials.gov/ct2/show/NCT03906526. Tissue genomic profiling was approved by the

102   Dana-Farber/Harvard Cancer Center Institutional Review Board (DF/HCC Protocol # 21-312).

103   Briefly, fresh tumor tissue biopsies were collected before two doses of anti-PD-1 blockade

104   (nivolumab) and four doses of subcutaneous (SC) toll-like receptor 8 (TLR8) agonist (motolimod).

105   The tumor was then surgically resected (days 23, 30, and 28 for patients 1, 2, and 3, respectively)

106   (**Figure 1A**). Response assessment was evaluated by a head and neck neuroradiologist according to

107   RECISTv1.1 criteria (**Supplementary Table 1**). Patients are designated by their response as

108   responders: Pt1_R and Pt2_R, and non-responder: Pt3_NR.

109

110   **Isolation of tumor and immune cell infiltrates for scRNAseq**. All tumors were dissociated using

111   the Human Tumor Dissociation kit (Miltenyi Biotec; Cat#130-095-929) within 30 minutes after

112   surgery (**Supplementary Methods**) and were prepared as previously described [19]. Briefly, minced

113   fragments were incubated with DMEM, enzyme H, enzyme R, and enzyme A and vortexed for 15

114   minutes at 400 rpm, 37°C. Tissue was filtered (50µm filter, Sysmex; Cat# 04-004-2327), washed,

115   minced with a 1mL syringe plunger, and re-washed. Dissociated cells were filtered and spun again

116   (30µm filter, Sysmex; Cat# 04-004-2326) at 1500 rpm at 4°C for 5 minutes. Next, incubation with

117   ACK buffer (Gibco, Cat#A1049201) was performed, followed by removal of the ACK buffer. In all

118   cases, the viability of dissociated single-cell suspensions was increased to >=90% by using a

119   modified protocol of the EasySep Dead Cell Removal (Annexin V) kit (STEMCELL; Cat#17899).

120   Gene expression (GEX) and TCR libraries were generated using the 10x Genomics Chromium

121   NextGEM Single Cell V(D)J Reagents kits v1.1 (PN-1000165; PN-1000020; PN-1000005; PN-

122   1000120) according to the manufacturer instructions.

123

124   **FFPE tissue specimens.** Serially cut 4-µm thick sections were obtained from Formalin Fixed

125   Paraffin Embedded (FFPE) tumor biopsies. Human tonsils, and discarded HNSCC FFPE tissue

126   blocks were used for conventional immunohistochemistry (IHC), multiplex validation, and assay

127   optimization. Before staining, all tissue slides were deparaffinized and rehydrated by serial passage

128   through graded concentrations of ethanol. Briefly: slides were submerged in xylene (Millipore

129   XX0060-A) during 10 min (x3), quick wash in deionized (DI) water, 100% ethanol x 10 min, 95%

130   ethanol x 10 min, 70% ethanol x 5 min, quick wash in DI water, 10% neutral buffered formalin

131   (Thermo Scientific Cat#5725) x 15 min, followed by 5 min of running water before antigen retrieval

132   in Tris EDTA buffer pH 9.0 (Vector Laboratories H-3301-250) or Citrate-based pH 6.0 (Vector

133   Laboratories H-330-250) buffer (exclusively for anti-CD20 antibody).

134

135   **Multiplex Immunofluorescence (mIF) staining.** After immunohistochemistry validation and

136   spectral library creation (**Supplementary Methods**), we utilized a commercially available manual

137   mIF staining kit (Opal 7-plex, Akoya, Cat# NEL811001KT) and applied an optimized protocol to all

138   mIF staining (**Supplementary Table S2**). Serial manual staining was performed as suggested by the

139   manufacturer (https://www.akoyabio.com/wp-content/uploads/2020/04/Opal-

140   Reagents_Brochure_Opal-Assay-Development-Guide.pdf). Two panels were optimized for staining,

141   Immune Panel (Panel A) and TLS panel (Panel B). Two serial sections were used, each stained with

142   one panel. An additional slide was later used to identify TLR8 expression in tissue. This slide was

143   stained with TLR8 and DAPI, scanned, and subsequently added to the analysis (**Supplementary**

144   **Table 2**). The whole tissue section was scanned using our multiplex immunofluorescence (mIF)

145   imaging platform (Vectra 3, Perkin Elmer). Phenochart was used to select all the regions of interest

146   (ROI) for higher-resolution scanning (20x). Spectral unmixing was done using InForm 2.5.1, and

147   unmixed component mIF images (QPTiff format) were then uploaded to the analysis platform (Halo

148   software; Indica Labs).

149   **Downstream mIF Image Processing.** Serially cut stains were registered, and a synchronous

150   navigation tool was used to check fusing quality (**Supplementary Figure S1**). Tumor-stroma

151   classification with Hematoxylin Eosin (H&E) was performed by a head and neck pathologist (PS),

152   and then mIF slides were classified by a random forest classifier trained with pathologists'

153   annotations. Single-cell phenotyping was done using Indica Labs – HighPlex FL v4.1.3 module

154   (**Supplementary Table S3**). The same thresholds were applied to all the slides, and spatial analysis

155   was performed based on average values. A paired t-test was used to compare pre- and post-treatment

156   values. The peritumoral region was defined as the area extending 100 μm outside the tumor margin,

157   as previously described [20].

158   **Tertiary Lymphoid Structures quantification.** Tertiary Lymphoid Structures were defined as

159   aggregates of lymphoid cells with histologic features resembling follicles in lymphoid tissue. A head

160   and neck pathologist (PS) identified these structures on serially cut H&E slides. Additionally, TLSs

161   were identified on serially cut slides stained with mIF panels using random forest classifiers as

162   described above. At single-cell mIF phenotyping, we included TLSs with more than 50 B cells

163   (CD20+) and more than 5 dendritic cells (LAMP3+). The number of TLSs was normalized by the

164   area analyzed (TLS/mm^2).

165

166   **Processing of GEX and TCR scRNAseq.** Raw sequencing reads were aligned, and samples

167   aggregated without normalization (Cell Ranger v6.1.1). Cells with fewer than 150 genes or 15% of

168   unique molecular identifiers (UMIs) mapped to mitochondrial genes were filtered (Seurat (v3)).

169   Potential doublets were filtered using DoubletFinder (chris-mcginnis-ucsf/DoubletFinder). Filtered

170   TCR alpha and beta, contigs were then integrated by barcode with scRepertoire

171   (ncborcherding/scRepertoire) based on the variable, diversity, and joining (VDJ) genes and CDR3

172   nucleotide sequence. 10X genomics feature matrices from Luoma et.al [17], representing anti-PD-1

173   monotherapy, were accessed from Gene Expression Omnibus Accession (GSE200996) and processed

174   as described above (**Supplementary Methods**).

175

176   **Clustering and annotation of tumor and tumor-infiltrating immune cells.** Principal component

177   analysis used 2000 highly variable genes from scaled and log normalized expression matrices.

178   Clustering utilized the first 40 principal components with a Louvain algorithm. The annotation of the

179   resulting 22 clusters was based on differential gene expression (log fold threshold: 0.25). T cells and

180   myeloid cell clusters gene expression and TCR clonotypes were extracted for further investigation

181   (**Supplementary Methods**). To complete this work, we also used a published dataset from Luoma et

182   al. [17]. Datasets were integrated by ranking 2000 variable features. 40 principal components were

183   used to identify clusters in the integrated dataset. The annotation was performed based on differential

184   gene expression analysis in combination with the integration of previously labeled clusters from

185   Luoma et al. [17].

186   **Copy number calling and phylogenetic tree construction.** InferCNV (InferCNV of the Trinity

187   CTAT project: https://github.com/broadinstitute/InferCNV) was used to predict copy number

188   alterations with CD45+ cell clusters as references. Phylogenetic trees of tumor cells were then

189   constructed using Uphyloplot2 (harbourlab/uphyloplot2; **Supplementary Figure S2-4,**

190   **Supplementary Table S4**).

191   **Pre- and post-treatment dendritic cell differential gene expression analysis.** The gene

192   expressions of extracted myeloid cell clusters from our cohort and the Luoma anti-PD-1

193   monotherapy cohort [17] were separately log-normalized and scaled, and 2000 variable features were

194   defined. After integrating both myeloid cell matrices, the first 8 principal components were used for

195   clustering. Differential gene expression analysis alongside previous labeling from Luoma et al. [17]

196   provided cluster annotations. Differential gene expression for pre- and post- treatment subset data

197   was performed on cell clusters identified as non-plasmacytoid dendritic cells (LAMP3+ DC,

198   CLEC9A+ XRC1+ DC, LAMP3+ CCL17+ DC, and CD1C+ DC) using a hurdle model (MAST) [21]

199   (fold change cutoff: 0.01). Gene set enrichment was performed against MSigDB GO Biological

200   Ontology (c5.go.bp.v2022.1.Hs.symbols.gmt) using the fgsea library in R (v4.2.1).

201   **Data Availability.** Processed scRNAseq data is available in the Gene Expression Omnibus (GEO) as

202   h5 files (GSE273138). The scripts used in this study are available at

203   https://github.com/jfwise/HSNCC_Single_Cell_TLR8.

204

205   **<u>Results</u>**

206 **Single-cell RNA-sequencing and spatial analysis of HNSCC patients treated with TLR8 + anti-**
207 **PD-1 combinatorial therapy**

208   To define the effect of combined TLR8 agonism and anti-PD-1 blockade in humans, HNSCC
209   patients with previously untreated, clinically diagnosed, resectable tumors were recruited for an
210   open-label, phase 1b pre-operative window of opportunity biomarker trial (NCT03906526). From
211   combination therapy arm 4, three patients receiving nivolumab (anti-PD-1 blockade) IV for 2 doses
212   on days 1 and 15, and motolimod (VTX-2337/TLR8 agonist) SC injection weekly for four doses on
213   days 1, 8, 15, and 22, had same lesion tumor biopsies, collected pre- and post-treatment. To
214   determine the immunomodulatory mode of action, we employed scRNAseq with T-cell clonotype
215   identification to leverage high-resolution insight into immune cell population dynamics. However,
216   given the loss of tissue architectural elements, we also applied a customized staining protocol (mIF)
217   for spatial organization details of critical immune cell subsets for three patients with the same lesion-
218   matched pre- and post-treatment biopsies (**Figure 1A**). From the scRNAseq sample collection,
219   41,985 cells were analyzed after applying initial quality controls and doublet removal
220   (**Supplementary Methods**). We partitioned the cells into 22 clusters by their expression states and
221   annotated them using differential gene expression of known markers (**Figure 1B-C; Supplementary**
222   **Figure S2, Supplementary Table-S5**). To further classify epithelial tumor cells, we identified copy
223   number variations using InferCNV and phylogenetic tree reconstruction of subclones, highlighting
224   the diverse heterogeneity of HNSCC (**Supplementary Figure S2-4, Supplementary Table S4**).

225

226 **scRNAseq and mIF identify post-treatment changes in TLR8 expression and TME**
227 **composition.**

228   Cell proportion estimates were calculated by scRNAseq and a serial multi-immunofluorescence
229   (mIF) imaging technique (**Figure 1D-E**; **Supplementary Table S6**). For our patient cohort, two
230   patients responded to therapy (Pt1_R and Pt2_R), while one was identified as a non-responder
231   (Pt3_NR). For all patients, scRNAseq analysis showed a decrease in epithelial tumor proportions
232   post-treatment. However, using mIF, a decrease in epithelial tumor proportions was observed for
233   Pt2_R and Pt3_NR, while an increase was found in Pt1_R (**Figure 1E**). Similar to previous
234   investigations of anti-PD-1 blockade, the total percentage of T-cells increased post-therapy via
235   scRNAseq (**Figure 1E**). Since innate immune cells are the target of TLR8 modulation, we were

236   surprised to see a decrease in the total myeloid population post-treatment, as shown by scRNAseq

237   (**Supplementary Figure S5 A**). However, mIF revealed an increase in dendritic cells (LAMP3+).

238   Importantly, while scRNAseq could not detect TLR8 transcripts, TLR8 was detected at the protein

239   level (**Figure 1D**). We discerned a tendency for higher average TLR8+ cell signal intensity (13, 82

240   and, 67% increase for Pt1-R, Pt2-R, and Pt3-NR respectively) (**Supplementary Figure S5 B, C**),

241   and a higher percentage of cells positive for TLR8 in post-treatment biopsies (Pt1-R pre: 38.3%,

242   post: 74.41%; Pt2-R pre:54%, post: 57%; Pt3-NR pre: 33%, post: 72%);(**Supplementary Figure S5**

243   **D**), mainly co-expressed in CK+ cells (**Supplementary Figure S5 E**). These results highlight the

244   effect of combined TLR8 agonism and anti-PD-1 blockade on the TME in HNSCC.

245

246   **Spatial analysis of macrophages and T cells across therapy.**

247   Macrophages are essential agents in antitumor immunity but can also have immunosuppressive

248   features. Hence, we examined M2 macrophage spatial behavior via the CD163 marker [22].

249   Interestingly, the non-responder (Pt3_NR) showed a higher density of CD163+Ki67+ macrophages

250   post-treatment, alongside increased non-proliferating macrophages in the peritumoral space

251   (**Supplementary Figure S6 A-C**). However, we observed no difference in CD163 expression in M2

252   macrophages via scRNAseq based on response to therapy (**Supplementary Figure S6 D-E**).

253

254   T cells, key in antitumor immunity, activate after antigen recognition by antigen-presenting cells

255   (APCs). Anti-PD-1 blockade is designed to help release restraints on T cell activation. Post-

256   treatment, CD4+ helper cells were in closer proximity to CD8+ cytotoxic T cells in all biopsies,

257   while CD19+ B lymphocytes were closer exclusively in responders (**Supplementary Figure S7**).

258   These results suggest that effective combinatorial therapy may be evidenced by T cell activation

259   supported by helper T cells, and that T-B cell interactions may have an important role in their

260   response. The spatial distribution between T cells and M2 macrophages was inconsistent across all

261   patients (**Supplementary Figure S7**). An increase in peri- and intra-tumoral CD8+ cytotoxic T cells

262   was observed among all patients in post-treatment biopsies, with a higher proportion in responders

263   (**Figure 2A: Supplementary Figure S8 A**). Increased T-cell density in the peritumoral area may

264   result in increased exposure to neoantigens through T-cell receptor (TCR) engagement and

265   subsequent T-cell clonal expansion.

266

267   **CD8 T-cell clones expand in response to treatment**. Given the assumed activation of APCs by

268   TLR8-agonism and increased T-cell density in areas enriched with B cells, we investigated TCR

269   expansion through single-cell TCR-VDJ sequencing. Examination of the frequency and cluster

270   distribution of TCR clonotypes, defined as those TCR sequences identical across more than one cell,

271   revealed an increase in TCR clonotypes post-treatment (**Supplementary Figure S8 B**). Moreover,

272   there was a substantial increase in new TCR clones in post-treatment biopsies (**Figure 2B**). We

273   observed that the majority of expanded TCR clones resided in clusters previously identified as CD8+

274   T-cells (**Supplementary Figure S8 C, D**). Interestingly, in the pre-treatment biopsy of the non-

275   responder, there was an expansion in ILC1-like T-cells (*GZMH+*, *NKG7+*, *GNLY+)*, while the

276   majority of TCR expansion in responders occurred within cycling CD8+CXCL13+ T cells, a

277   chemotactic for B-cells (**Figure 2C**).

278

279   To further refine TCR clonotypes and investigate the contribution of CXCL13+ expression in TCR

280   expansion, we partitioned the seven clusters identified as T cells. We identified 13 clusters (**Figure**

281   **2D**; **Supplementary Table S7**). Two clusters had little CD4 or CD8 expression, representing an

282   NKT cell cluster and the previously described ILC1-like T cells (**Supplementary Figure S8 E**). Four

283   clusters (Cycling:Exhausted TRM-CXCL13+HLA-DRhigh: Exhausted TRM-

284   CXCL13+TRAV36DV7+TRBV20_1+: Exhausted CXCL13+LAIR2+CD4+ T cells) had a

285   differential expression of *CXCL13* including a CD4 positive cluster. Intriguingly, all four clusters

286   differentially expressed exhaustion markers *HAVCR2* and *ENTPD1* (**Supplementary Figure S8 E**).

287   We further investigated *CXCL13* expression across all patients and saw that both, the average

288   expression and the percentage of cells expressing *CXCL13* increased in all patients post-treatment,

289   yet the non-responding patient had overall lower levels of *CXCL13* (**Figure 2E**).

290

291   **Tertiary Lymphoid Structures increase after treatment.** Next, we evaluated TLSs presence and

292   composition in our patient's pre- and post-treatment biopsies. This effort was initiated after observing

293   CD4+ and CD8+ T cells closer to CD19+ B cells post-therapy. Further, the role of *CXCL13* in TLS

294   formation has been linked to improved response to ICB [23, 24]. TLSs were defined using criteria

295   established by others [15], with the additional inclusion of lymphoid follicles with germinal centers

296   (>50 CD20+ B cells, and >5 LAMP3+ dendritic cells). With these criteria in place, random forest

297   classifiers were used for mIF TLSs detection (**Supplementary Figure S9 A, B**). Normalized by

298   tissue size, post-treatment biopsies showed a trend of increased TLS/mm2 via mIF (pre: 0.09

299   TLS/mm2; post: 0.27 TLS/mm2; **Figure 2F**); this trend was confirmed on H&E slides from patients

300   Pt2_R, and Pt3_NR, but not Pt1_R as reviewed by a pathologist (PS) (**Figure 2 G, H;**

301   **Supplementary Figure S9 C).** Interestingly, on average, for all pre-treatment biopsies, helper T cells

302   (CD4+) were the primary cell type in TLSs (41%), followed by CD20+ B cells (32%;

303   **Supplementary Table S8 D**). For all post-treatment biopsies, TLSs were largely comprised of

304   cytotoxic T cells (CD8+) (47%), and PD-1 positivity did not increase, although dendritic cells

305   (LAMP3+) did (**Supplementary Figure S9 D; Supplementary Table S8**). No difference was found

306   in their distance to the tumor from pre- to post-treatment biopsies (**Supplementary Figure S9 E**).

307   Literature on dendritic cell infiltration into TLSs using pre-clinical models, particularly with TLR

308   stimulation, has been contradictory [25], although it is hypothesized that their antigen presentation

309   capabilities enhance T-cell mediated ICB response [26].

310

311   **Unique dendritic cell activation in the context of combinatorial therapy.** We then aimed to define

312   dendritic cell activation by TLR8 agonism plus PD-1 blockade compared to PD-1 blockade alone.

313   Although we did not have access to samples from this arm of the clinical trial, we utilized a publicly

314   available scRNAseq dataset of three pre- and post-anti-PD-1 treated HNSCC patients as controls

315   (**Figure 3A**) [17]. After integrating the two datasets, we defined 11 distinctive myeloid clusters

316   (**Figure 3B**; **Supplementary Tables S9-10),** including *CD14+* and *FCGR3A+* monocytes. Two

317   tissue-associated macrophage populations were defined by *ITGAX* expression, cytokine IL-8

318   producing *CXCL8,* and EMT-related *SPP1* (**Supplementary Figure 10A**). Inflammatory CCL18+

319   M2-like macrophage subset was defined by *CD206*, *CD163*, and *CD209*. One cluster exhibited *CD3*

320   expression, suggesting a myeloid/T-cell interaction or doublet with failed identification by doublet

321   filtering tools. Thus, we did not investigate this cluster further. The remaining clusters represented

322   five dendritic cell (DC) populations, including plasmocytoid DCs, conventional DCs (*CD1C*), two

323   mature LAMP3+ DC subsets containing markers for Th2 chemoattractant, *CCL17*, and cell migration

324 factor *FSNC1*. Prior investigations revealed decreased CD1C+ conventional and LAMP3+ DCs with

325 ICB therapy. Importantly, dual-immune targeting of TLR8 and PD-1 revealed an increase in

326 conventional dendritic cells as well as dendritic cells expressing *CLEC9A+* and *XCR1* (**Figure 3C**).

327 To further explore the effects of TLR8 agonism and PD-1 blockade in DCs, we performed

328 differential gene expression analysis of non-plasmacytoid DC populations. With dual therapy, we

329 revealed multiple significant gene-level changes in DCs but few significant changes with anti-PD-1

330 monotherapy (**Figure 3D**). Gene-set enrichment revealed that up-regulated genes are involved in

331 humoral and immune effector responses, including MHC Class II (**Figure 3E; Supplementary**

332 **Figure S10**), signaling proteins *MYD88*, *SCIMP,* and interferon associated signaling *AXL* and

333 *SAMHD1*. Positive cytokine regulation was also seen with the up-regulation of *CLEC7A* and *SYK*.

334 Moreover, post-treatment, we identified down-regulated genes that have a role in cytokine signaling,

335 including significant decreases in *CCL17* and *CCL22,* suggesting a potential decrease in Th17 and

336 Treg recruitment. A decrease in *CCR7* suggests decreased migration to lymph nodes (**Figure 3F**;

337 **Supplementary Figure S10**). We investigated whether changes in cytokine signaling and MHC

338 Class II activation resulted in shifts in the spatial dynamics of myeloid cells within the TME using

339 mIF. Our analysis revealed a closer proximity between proliferative DCs (LAMP3+Ki67+) and

340 natural killer cells (CD56+CD16-) in the post-treatment biopsies *(*pre: 251.58 µm; post: 137.37 µm;

341 **Supplementary Figure S7**). Additionally, cytotoxic T cells (CD8+; pre: 200.62 µm: post: 127.03

342 µm) were found to be closer to DCs (LAMP3+) in all post-treatment biopsies (**Figure 3G**).

343 Furthermore, a high density of DCs (LAMP3+) was found in the peritumoral area in post-treatment

344 biopsies (paired t-test; p-value 0.0601, **Figure 3H**), indicating a trend toward tumor-specific

345 dendritic cell activation after dual therapy.

346

Discussion

348 In this distinctive window of opportunity phase 1b clinical trial setting, we characterized the effect of

349 TLR8 agonism and anti-PD-1 blockade in the TME of three patients with treatment-naive HNSCC.

350 To accomplish this, we performed single-cell transcriptomic profiling together with high-plex tissue

351 imaging, enabling us to obtain a high-resolution understanding of immune cell responses while

352 preserving the spatial and architectural elements of the TME. Of the patients analyzed, two

353 responded to treatment, while one did not respond. The two responding patients exhibited clonal

354 expansion of *CXCL13*+ exhausted tissue-resident memory CD8+ T cells, suggesting successful

355 reinvigoration of CD8+ T cells and antigen stimulation, as shown by scRNAseq analysis [27].

356 Consequently, a higher density of tertiary lymphoid structures was found in post-treatment biopsies

357 using machine learning and mIF. The ability to identify and characterize an increased number of

358 TLSs highlights the potential for novel digital pathology methods, such as mIF, to assist with the

359 rapid and reliable identification of these complex structures. These findings are aligned with prior

360 conclusions that correlate *CXCL13* and the presence of TLSs with improved anti-PD-1 response in

361 several solid malignancies [23]. Given our finding of increased LAMP3+ dendritic cells, we used

362 scRNAseq to compare a prior study of anti-PD-1 alone [17] regimens with our combinatorial TLR8

363 agonism and revealed an exclusive increase in the percentage of conventional DCs (CD1C+) and

364 CLEC9A+XRC1+DCs, which excel at cross-presentation and cytotoxic T lymphocyte responses

365 [28]. Moreover, we observed a humoral and innate effector immune response uniquely in the DC

366 populations exposed to TLR8 agonism and anti-PD-1 blockade (scRNAseq). Lastly, to further

367 investigate the dendritic cell findings from scRNAseq we identified a closer localization of LAMP3+

368 DC to CD8+ T cells after combinatorial treatment (mIF). Our findings highlight the potential role of

369 activated and mature DCs interaction with CD8+ T cells as critical surrogates of TLR8 agonism and

370 anti-PD-1blockade in a human clinical trial setting, which should be further investigated with

371 increased patient numbers.

372

373 TLR8 expression is thought to be enriched in neutrophils, monocytes, dendritic cells, and

374 macrophages. While we were unable to capture *TLR8* RNA expression, and our protein expression

375 was mainly associated with epithelial cells, we revealed major shifts in innate cell populations in the

376 three patients post-treatment. LAMP3+ cells marking mature dendritic cell populations were found

377 closer in distance to CD163+ cells in the non-responder as opposed to CD8+ T-cells in responders

378 (mIF). Additionally, we did not see an increase in tumor-infiltrating DCs by scRNAseq, however, we

379 were able to identify a distinct DC activation following TLR8 agonism and anti-PD-1 blockade.

380 After comparing our results with an available scRNAseq dataset of HNSCC patients of similar size

381 (n=3) treated with anti-PD-1 monotherapy [17], we saw an increase in dendritic cells associated with

382 T-cell responses and antigen presentation (CD1C+ and CLEC9A+ XCR1+ DCs) in two of three

383 patients. This increase imparted a distinct upregulation of immune effector genes in non-

384 plasmacytoid DCs (scRNAseq) and a clear localization of LAMP3+ DC to CD8+ T cells after

385   treatment (mIF). Prior reports have shown functional localization of T cells around mature DC as a
386   prognostic factor [29]. Indeed, a high peritumoral density of mature DCs has been associated with
387   significantly longer survival in solid malignancies, including esophageal squamous cell carcinoma
388   [30], highlighting DCs as a key component of antitumor activity.

389

390   Despite using scRNAseq and cell-to-cell interactions through an image registration machine-learning
391   algorithm, our study has limitations. Our findings were determined from a small subset of one arm
392   from our clinical trial (n=3), and scRNAseq dendritic cell findings required the additional use of
393   publicly available data (n=3) to analyze our cohort. Moreover, despite being a target of TLR8
394   agonism, we did not see an increase in TLR8 signaling molecules (except *MYD88* in dendritic cell
395   populations) in post-treatment biopsies, possibly because of tumor resection timing (scRNAseq).
396   Considering motolimod half-life of 6 hours [31], it is possible that TLR8 agonism was not enough to
397   generate a sustained myeloid cell intra-tumoral activity, that we were unable to capture the initial
398   signaling pathway, or that the main location for APC activation took place in the lymph node. While
399   analyzing pre-post drug tissue biopsies is a powerful approach, immediate post-treatment local
400   effects remain poorly assessed with such an approach. Negative feedback after stimulation might
401   explain the absence of TLR8 RNA-based gene expression despite the upregulation of downstream
402   targets such as *MYD88* and *BLC6* [32]. Future investigations into early (<24h) and acute (<2 weeks)
403   post-treatment TME states will be of interest. Moreover, due to our sample size, we were unable to
404   decipher whether TLSs formation increased due to TLR8 agonism or simply by anti-PD-1 blockade
405   alone. Lastly, we could not perform statistical correlates with response due to the small cohort size.
406   Despite the limited sample size afforded by this unique clinical trial, we were able to perform a deep
407   and detailed analysis per sample at the single-cell level (RNA and protein), which identified unique
408   dendritic cell molecular and spatial changes after combined therapy with TLR8 agonism and anti-PD-
409   1 blockade and indicate that further investigation with a larger sample size is warranted.

410

422

Author Contributions

424   S.L.S., D.L. F., and M.SF. conceptualized the investigation and provided supervision, and D.L.F. led

425   the funding acquisition. Formal analysis, including visualization, was performed by D.A.RT., J.F.W,

426   B.Y.Z, and P.S, J.F processed the single-cell RNA sequencing samples. S.C., J.P.O.C., and P.S.

427   provided methodology and software support. C.L. and A.P. assisted with study design and sample

428   acquisition. O.Y. provided essential resources. D.A.R.T. and J.F.W. wrote the original draft, and all

429   authors performed critical reviews and revisions.

430

Figure legends:

432   **Figure 1. Overview of TLR8 agonism and anti-PD-1 blockade workflow and cell types**

433   **identified. (A)** Schematic representation of treatment and biopsy collection created with

434   BioRender.com. The tables show the number of cells recovered after scRNAseq processing, patient

435   biopsy site, patient response, and antibodies used for multi-immunofluorescence (mIF) imaging. **(B)**

436   UMAP shows the clustering of all 41,985 cells collected in this study. **(C)** UMAP shows the patient

437   contribution to the twenty-two clusters identified in B. **(D)** Representative image showing the mIF

438   image of post-treatment biopsy of Pt2_R. **(E-F)** Cell proportions found by mIF imaging **(E)** and by

439   scRNAseq **(F)** for all patients pre- and post-treatment.

440   **Figure 2. TLR8 agonism and anti-PD-1 blockade increase TCR clones and Tertiary Lymphoid**

441   **Structures (A)** Representative image showing tumor infiltrating CD8 T cells (green) before and after

442   anti-PD-1 blockade and TLR8 agonism for each patient. **(B)** An alluvial plot depicts the proportion

443   of new TCR clones post-treatment per patient; only the top thirty clones are represented per patient.

444   **(C)** A UMAP of each patient's pre-treatment biopsy with the density of cells with a TCR expansion

445   >10 depicts which cell subsets expanded prior to TLR8 agonism and anti-PD-1 blockade. **(D)** UMAP

446   displays the 13 T cell clusters identified after isolation and sub-setting of prior recognized T and

447   NKT clusters. **(E)** A bar plot with varied colors reveals the average gene expression and percentage

448   of *CXCL13*-expressing cells across T cells. **(F)** Number of TLS/mm^2 identified by mIF. **(G)** Number

449   of TLS/mm^2 identified by the pathologist. **(H)** Representative image of tertiary lymphoid structures

450   in pre- and post-treatment biopsies from Pt2_R.

451   **Figure 3. Dendritic cells effector activation in combinatorial treatment compared to**

452   **monotherapy and spatial dendritic cell proximity to T cells**. **(A)** Schematic representation of data

453   processing and comparison between our (top) and Luoma et al. (bottom) single-cell RNA datasets,

454   created with BioRender.com. **(B)** A UMAP displays the clustering and annotation of selected

455   myeloid cells after merging the two myeloid cells from the two cohorts. **(C)** Percent of mature

456   LAMP3+, conventional CD1C+, and CLEC9+XRC1+ dendritic cells. **(D)** A volcano plot displays

457   the differentially expressed genes of non-plasmacytoid dendritic cells pre- vs. post-treatment in the

458   MGH and Luoma et al. cohorts. **(E)** Gene Set Enrichment Analysis from the gene ontology

459   biological processes of differentially expressed genes in non-plasmacytoid dendritic cells from the

460   MGH cohort in pre- and post-treatment cells was performed, and a dot-plot displays the enrichment

461   score with color representing the direction of expression for the gene sets, and size represents the

462   number of genes involved in the analysis. **(F)** Heatmap display of the relative expression of selected

463   genes from the humoral immune response gene sets for non-plasmacytoid dendritic cells pre- and

464   post-treatment for our cohort. **(G)** Representative image of Pt_2_R showing the spatial behavior of

465   CD8+ T cells (green) and LAMP3+ DC (red) pre-and post-treatment. **(H)** Average distance of CD8+

466   T cells to LAMP3+ dendritic cells (upper plot) and LAMP3+ dendritic cell density in the peritumoral

467   area (lower plot).

468

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Figure 1: Overview of TLR8 agonism and anti-PD-1 blockade workflow and cell types identified

A. Treatment timeline, sample collection, and processing
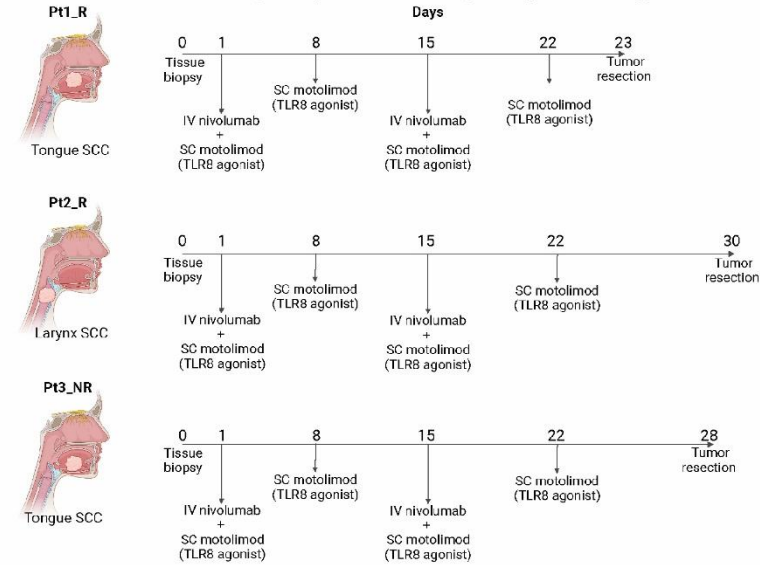
Single Cell Dissociation: 10X Genomics GEX:TCR

Patient	Pre-	Post-	Response
Pt1-R	7561	8587	Responder
Pt2-R	2730	8666	Responder
Pt3-NR	7268	7173	Non-Responder

Multi-immunofluorescence (mIF): Protein markers

Immune panel	CD56	TLS panel	CD20	TLR8 single-plex
CD16	Ki67	PD1	CD4	DAPI
α-SMA	CK	LAMP3	CD163	TLR8
CD19	DAPI	CD8	DAPI	

B. scRNA clustering
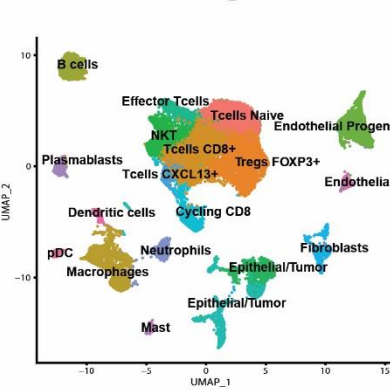
C. scRNA clustering per patient and timepoint
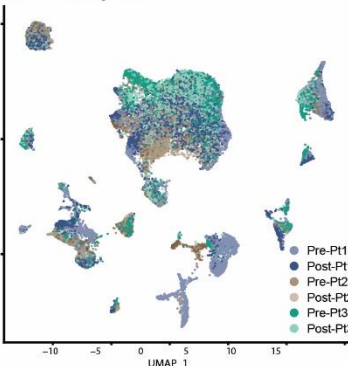
D. Multi-Immunofluorescence (mIF) image
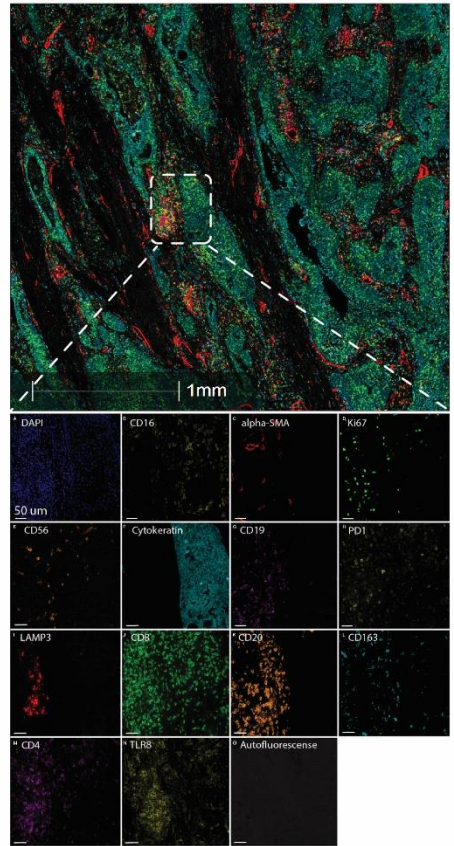
E. Cell Distribution mIF
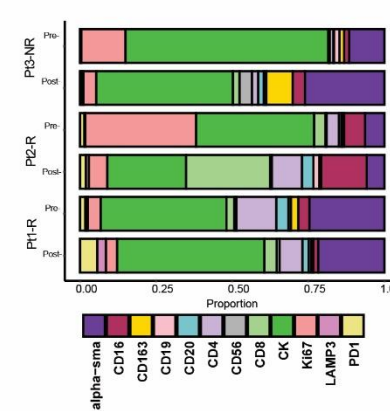
F. Cell Distribution scRNAseq
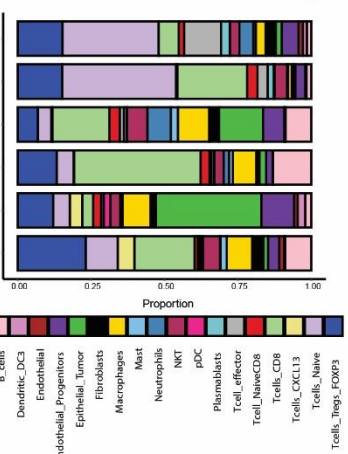
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Figure 2: TLR8 agonism and anti-PD-1 blockade increase TCR clones and Tertiary Lymphoid Structures
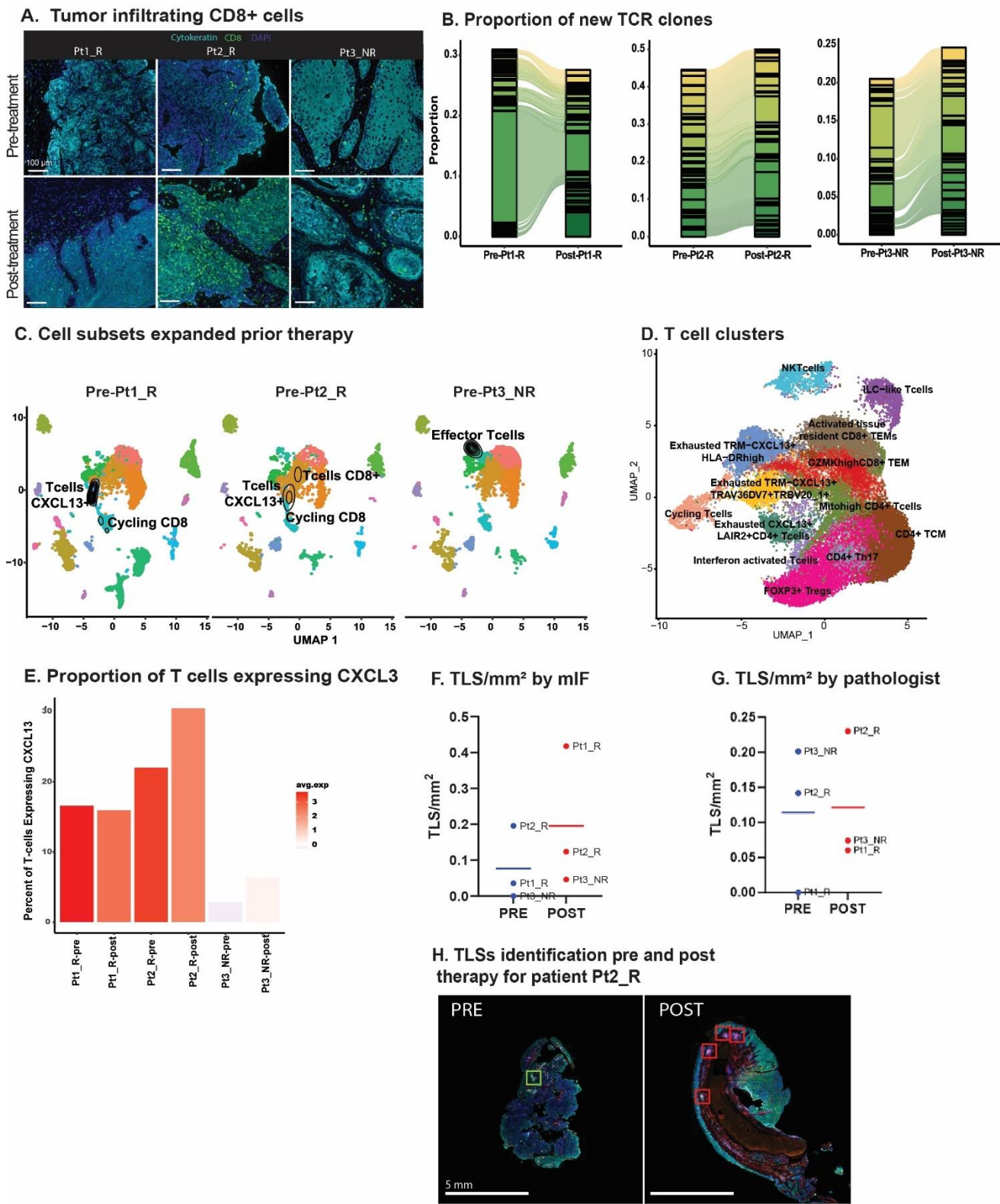
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Figure 3: Dendritic cells effector activation in combinatorial treatment compared to monotherapy and spatial dendritic cell proximity to T cells

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Figure 3: Dendritic cells effector activation in combinatorial treatment compared to monotherapy and spatial dendritic cell proximity to T cells
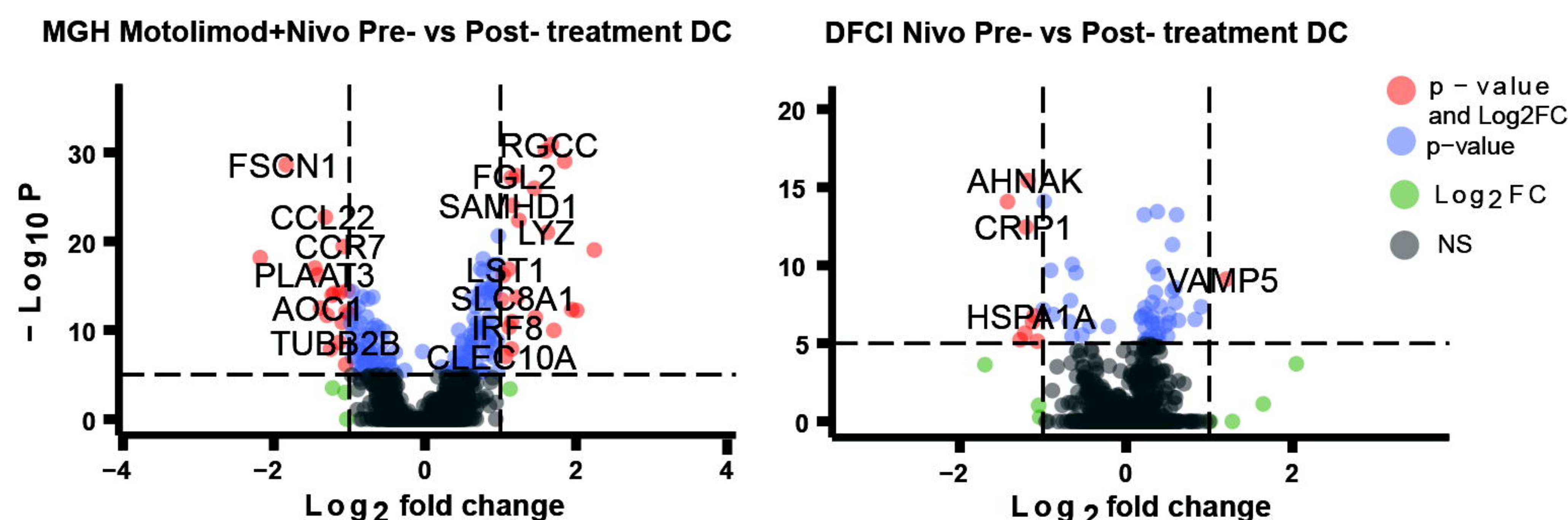
A. Overview of Data

D. DEG Analysis DC

B. Clustering and Annotation
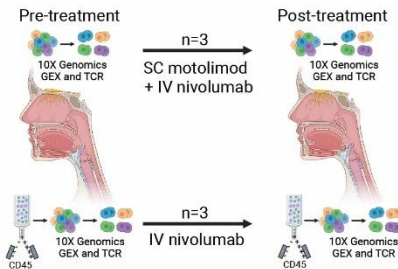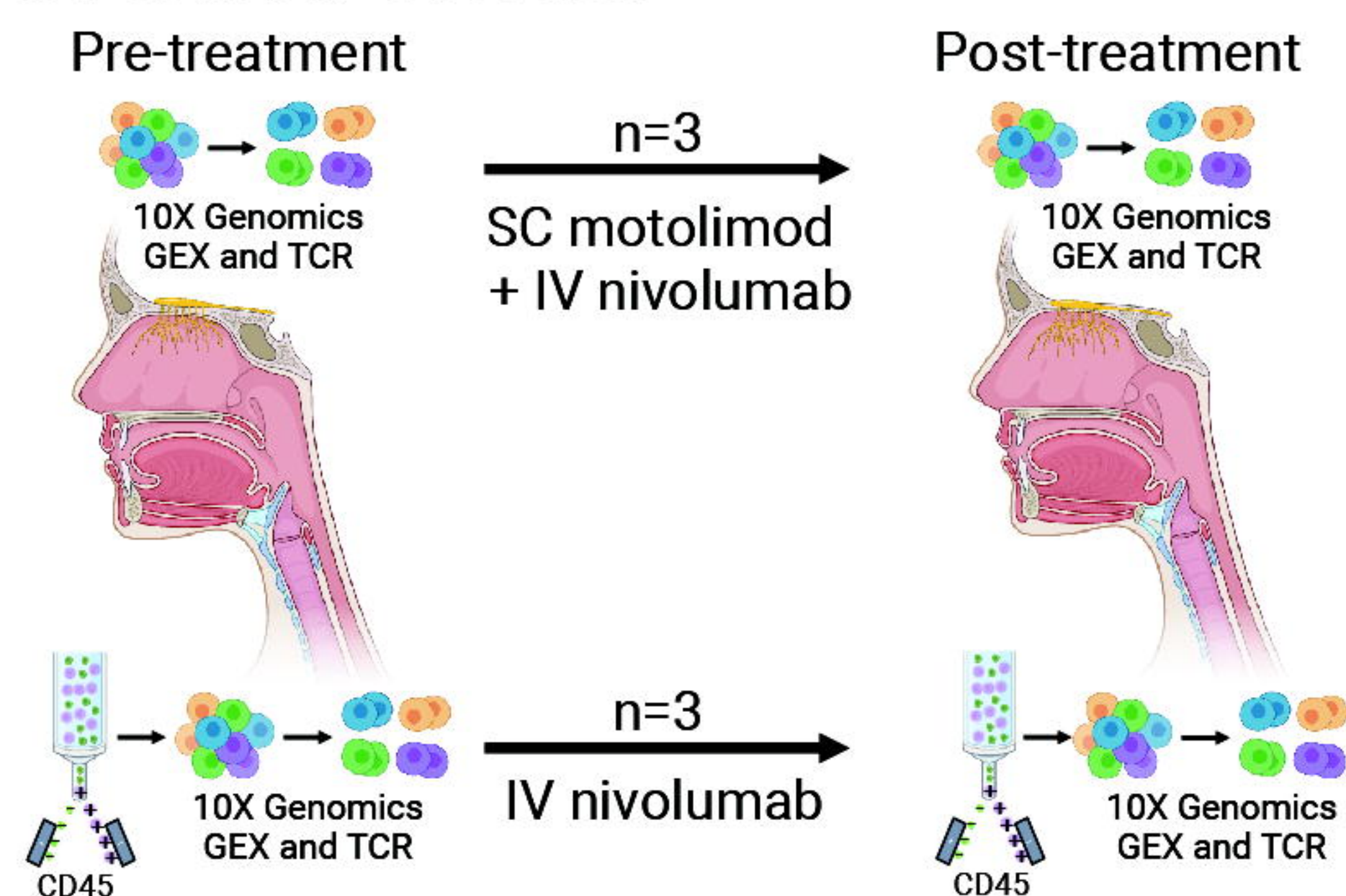
E. GO Biological Processes GSEA in DC
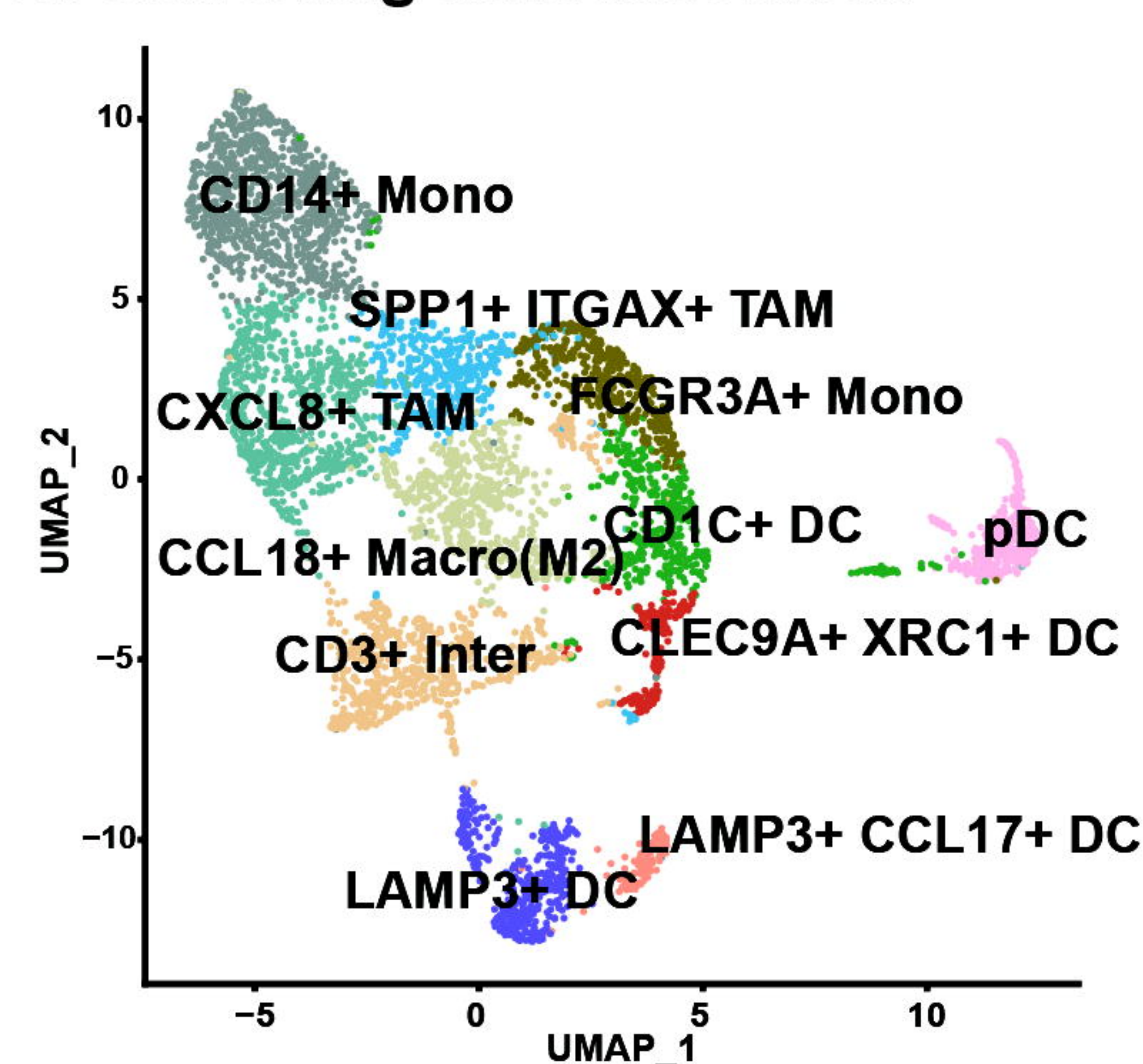
C. Increases in CD1C and CLEC9+ DC

F. DC Gene Expression Immune Effector Processes
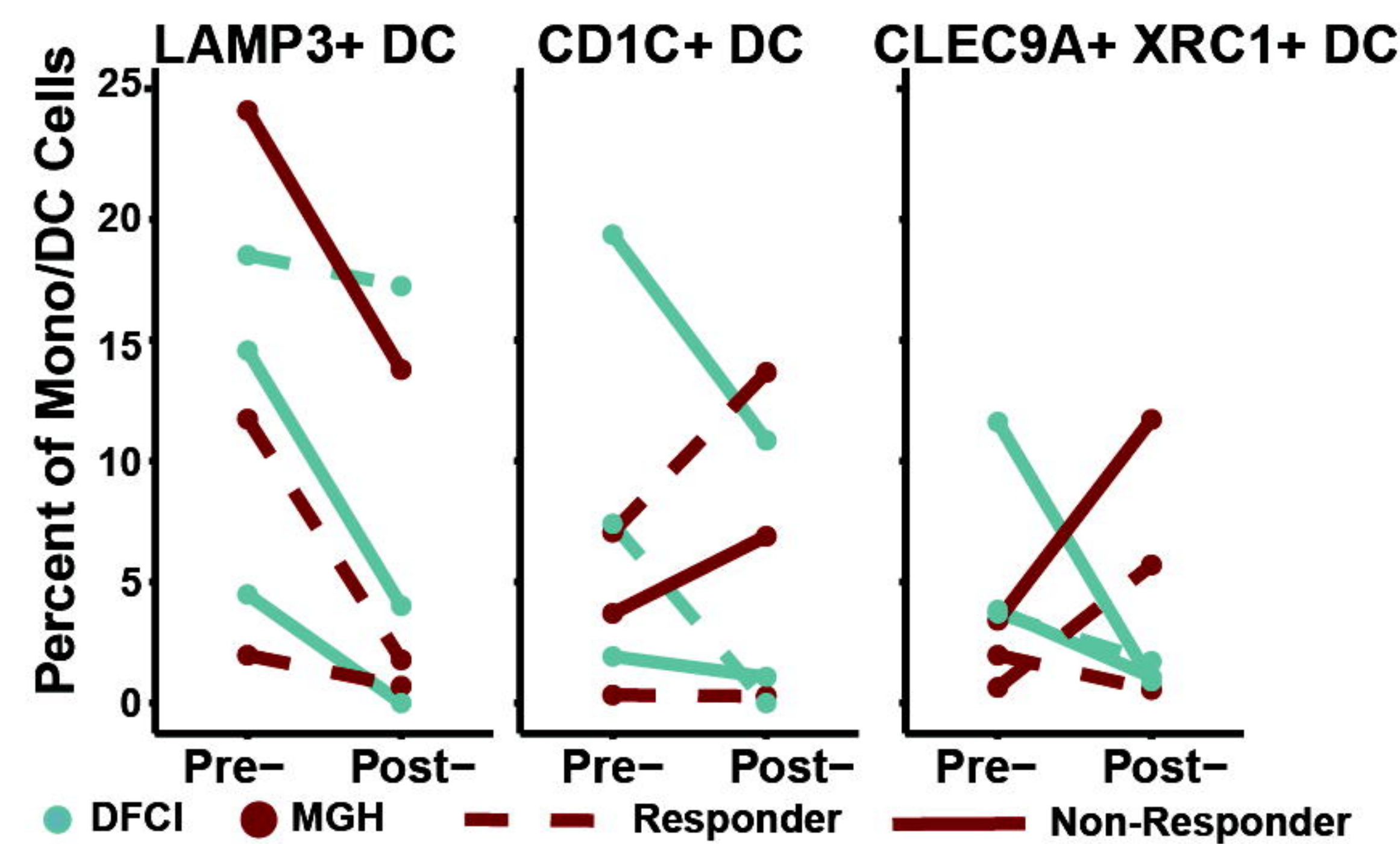
G. Spatial proximity of CD8+ T cells to LAMP3+ dendritic cells via mIF

Yellow dots: CD8+ cells; Blue dots: LAMP3+ cells
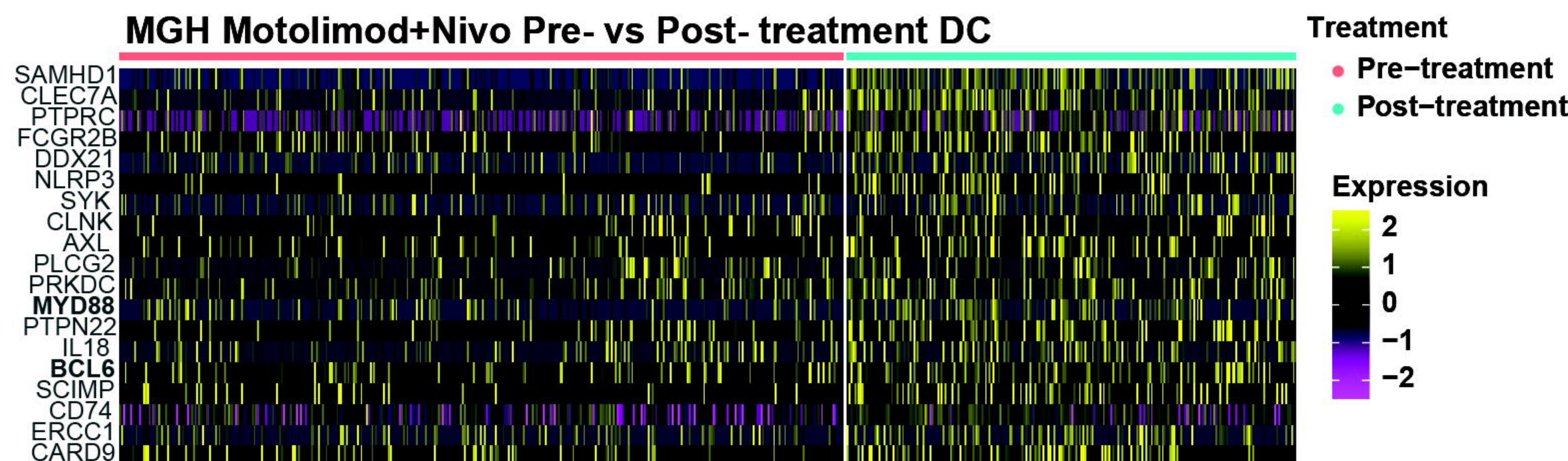
H. Average distance of CD8+ to LAMP3+ cells
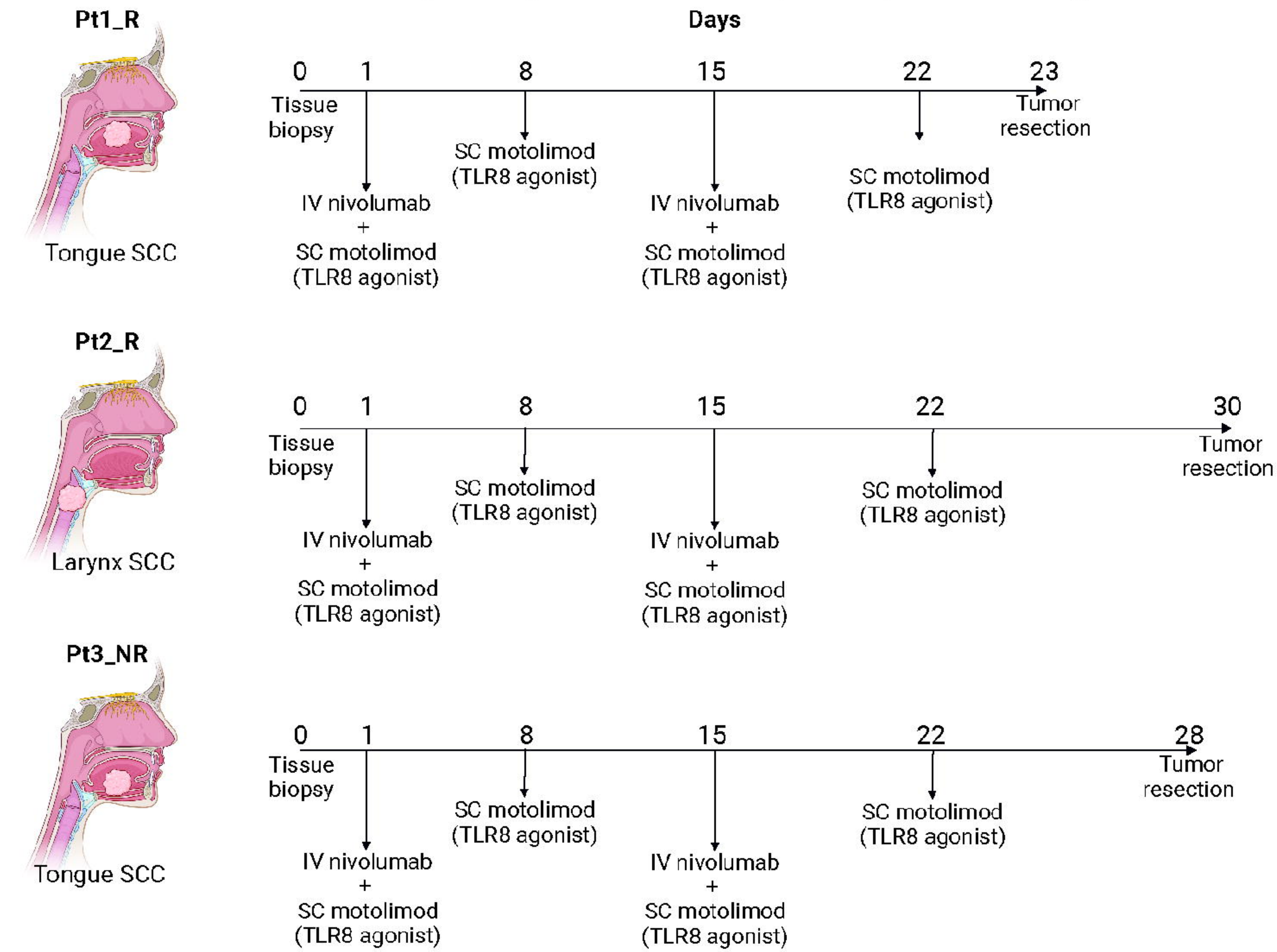
Figure 1: Overview of TLR8 agonism and anti-PD-1 blockade workflow and cell types identified

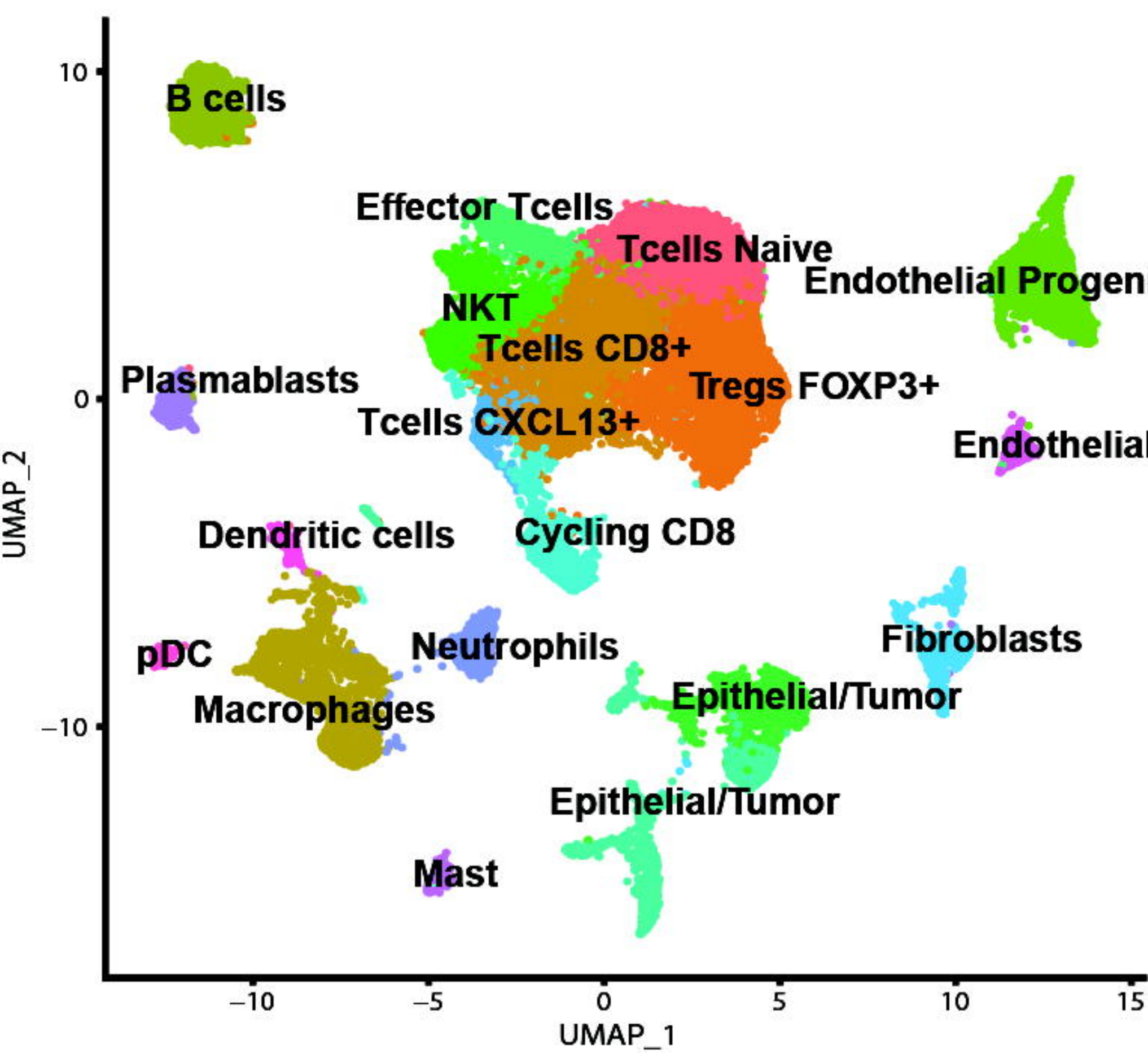## A. Treatment timeline, sample collection, and processing

Pt1_R
Tongue SCC

Days

0 — 1 — 8 — 15 — 22 — 23

Tissue biopsy
IV nivolumab + SC motolimod (TLR8 agonist)
SC motolimod (TLR8 agonist)
IV nivolumab + SC motolimod (TLR8 agonist)
SC motolimod (TLR8 agonist)
Tumor resection

Pt2_R
Larynx SCC

0 — 1 — 8 — 15 — 22 — 30

Tissue biopsy
IV nivolumab + SC motolimod (TLR8 agonist)
SC motolimod (TLR8 agonist)
IV nivolumab + SC motolimod (TLR8 agonist)
SC motolimod (TLR8 agonist)
Tumor resection

Pt3_NR
Tongue SCC

0 — 1 — 8 — 15 — 22 — 28

Tissue biopsy
IV nivolumab + SC motolimod (TLR8 agonist)
SC motolimod (TLR8 agonist)
IV nivolumab + SC motolimod (TLR8 agonist)
SC motolimod (TLR8 agonist)
Tumor resection

Single Cell Dissociation:10X Genomics GEX:TCR

Patient	Pre-	Post-	Response
Pt1-R	7561	8587	Responder
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Multi-immunofluorescence (mIF): Protein markers

Immune panel		TLS panel		TLR8 single-plex
CD56		CD20		
CD16	Ki67	PD1	CD4	DAPI
α-SMA	CK	LAMP3	CD163	TLR8
CD19	DAPI	CD8	DAPI	DAPI

B. scRNA clustering

B cells, Effector Tcells, Tcells Naive, Endothelial Progenitors, NKT, Tcells CD8+, Tregs FOXP3+, Plasmablasts, Tcells CXCL13+, Endothelial, Dendritic cells, Cycling CD8, pDC, Neutrophils, Fibroblasts, Macrophages, Epithelial/Tumor, Epithelial/Tumor, Mast

UMAP_2 / UMAP_1

C. scRNA clustering per patient and timepoint

UMAP_2 / UMAP_1

Pre-Pt1-R, Post-Pt1-R, Pre-Pt2-R, Post-Pt2-R, Pre-Pt3-NR, Post-Pt3-R

D. Multi-Immunofluorescence (mIF) image

1mm

DAPI, CD16, alpha-SMA, Ki67, CD56, Cytokeratin, CD19, PD1, LAMP3, CD8, CD20, CD163, CD4, TLR8, Autofluorescense

50 um

E. Cell Distribution mIF

Pt3-NR (Pre-, Post-), Pt2-R (Pre-, Post-), Pt1-R (Pre-, Post-)

Proportion: 0.00, 0.25, 0.50, 0.75, 1.00

alpha-sma, CD16, CD163, CD19, CD20, CD4, CD56, CD8, CK, Ki67, LAMP3, PD1

F. Cell Distribution scRNAseq

Pt3-NR (Pre-, Post-), Pt2-R (Pre-, Post-), Pt1-R (Pre-, Post-)

Proportion: 0.00, 0.25, 0.50, 0.75, 1.00

B_cells, Dendritic_DC3, Endothelial, Endothelial_Progenitors, Epithelial_Tumor, Fibroblasts, Macrophages, Mast, Neutrophils, NKT, pDC, Plasmablasts, Tcell_effector, Tcell_NaiveCD8, Tcells_CD8, Tcells_CXCL13, Tcells_Naive, Tcells_Tregs_FOXP3
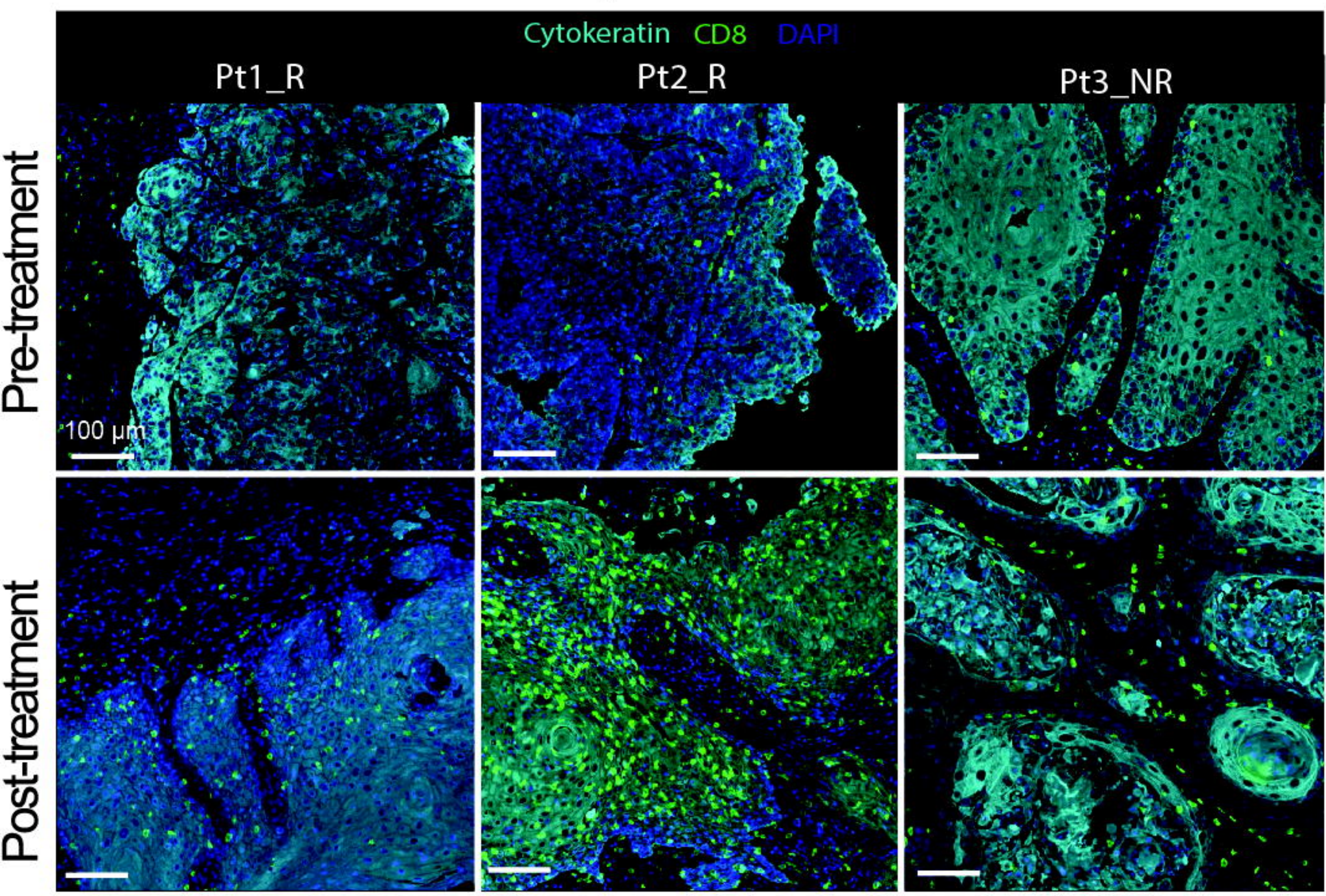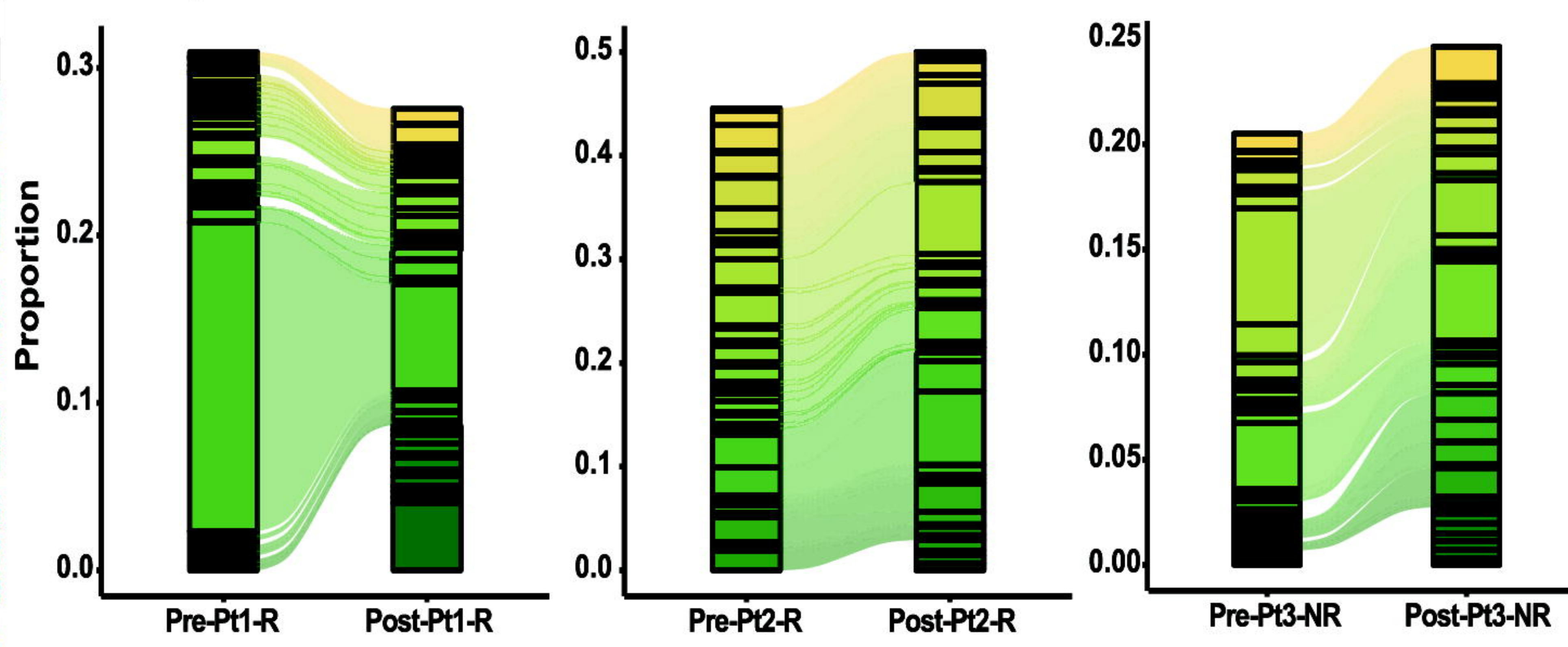
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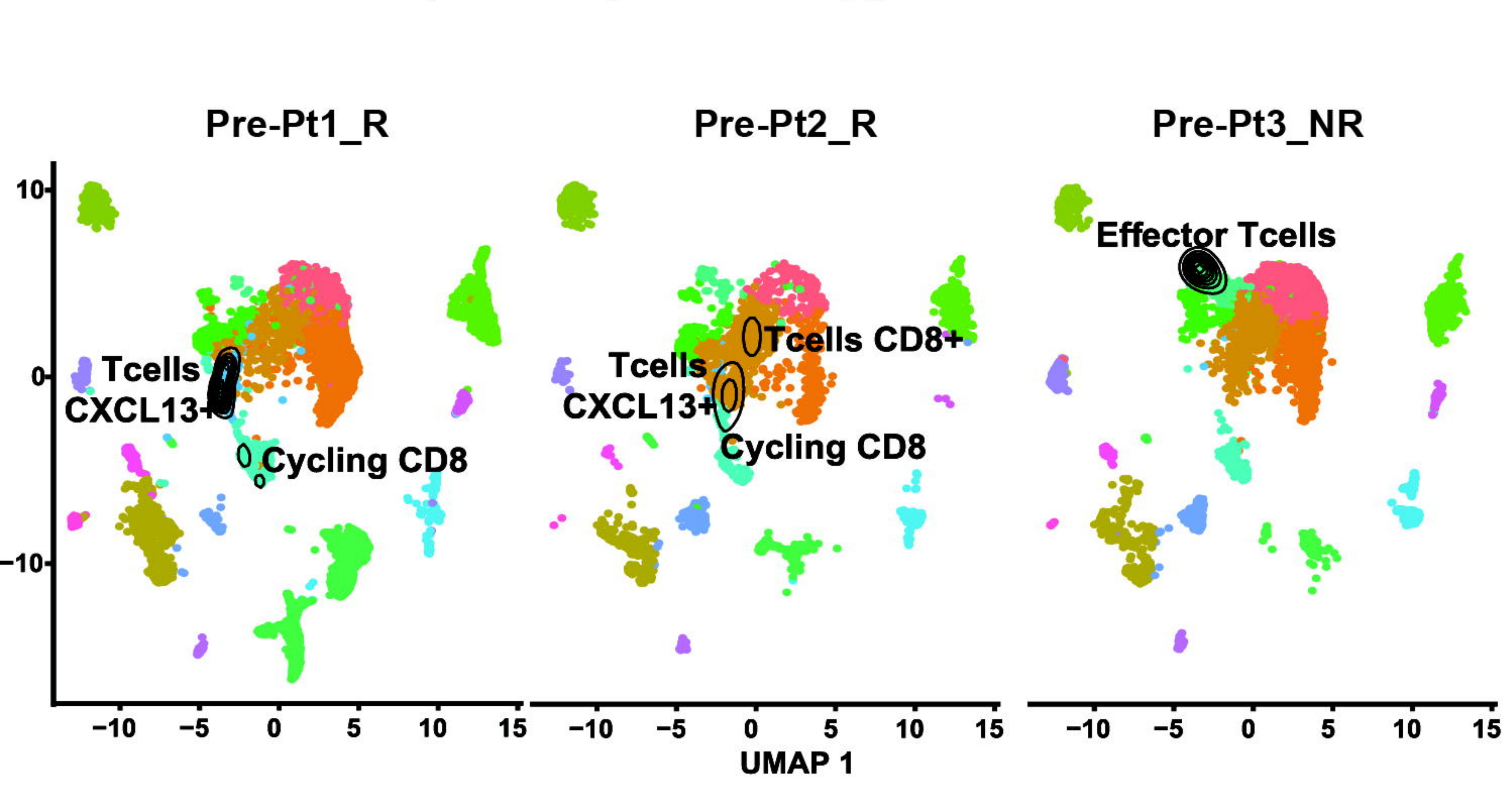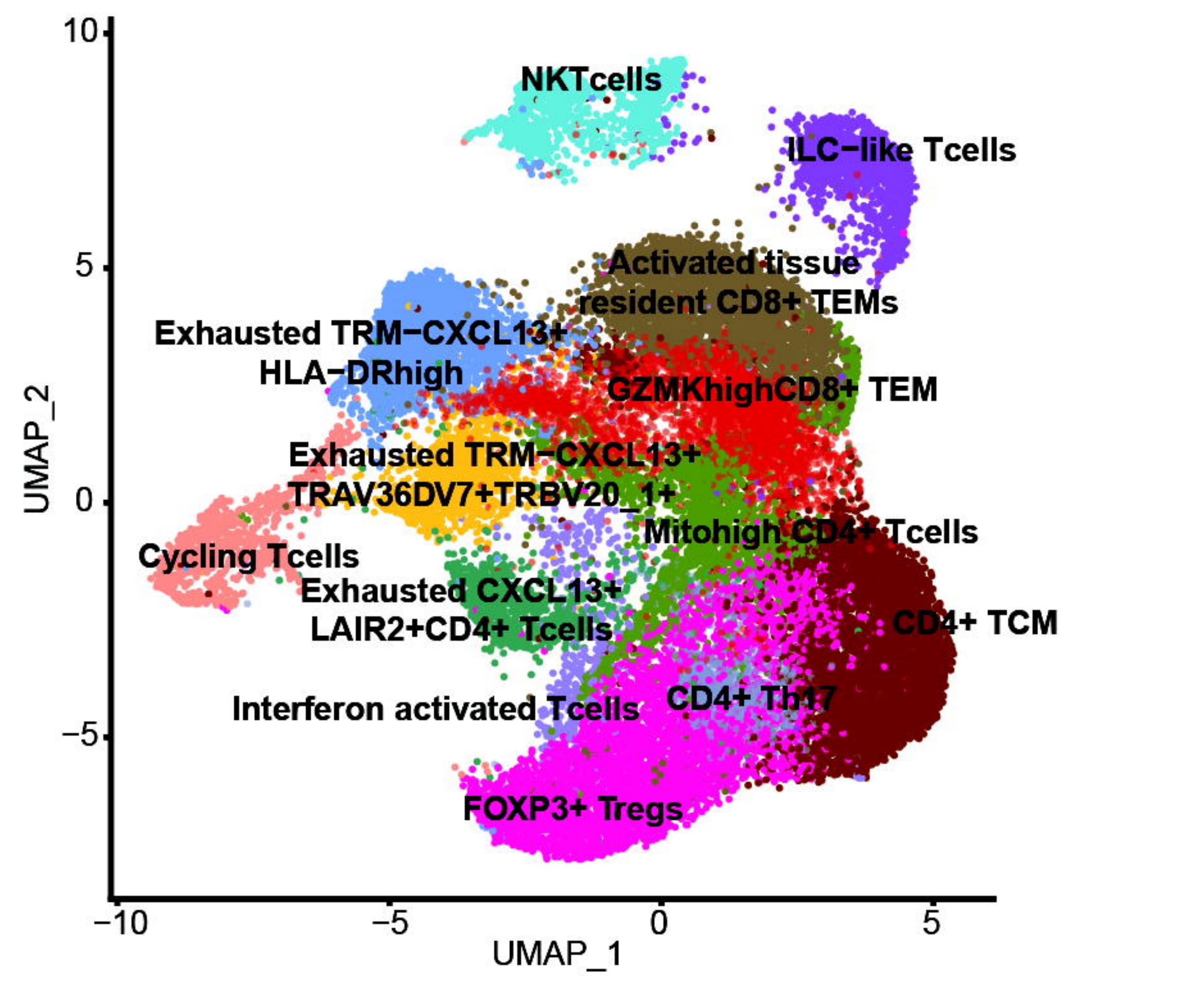
A. Tumor infiltrating CD8+ cells
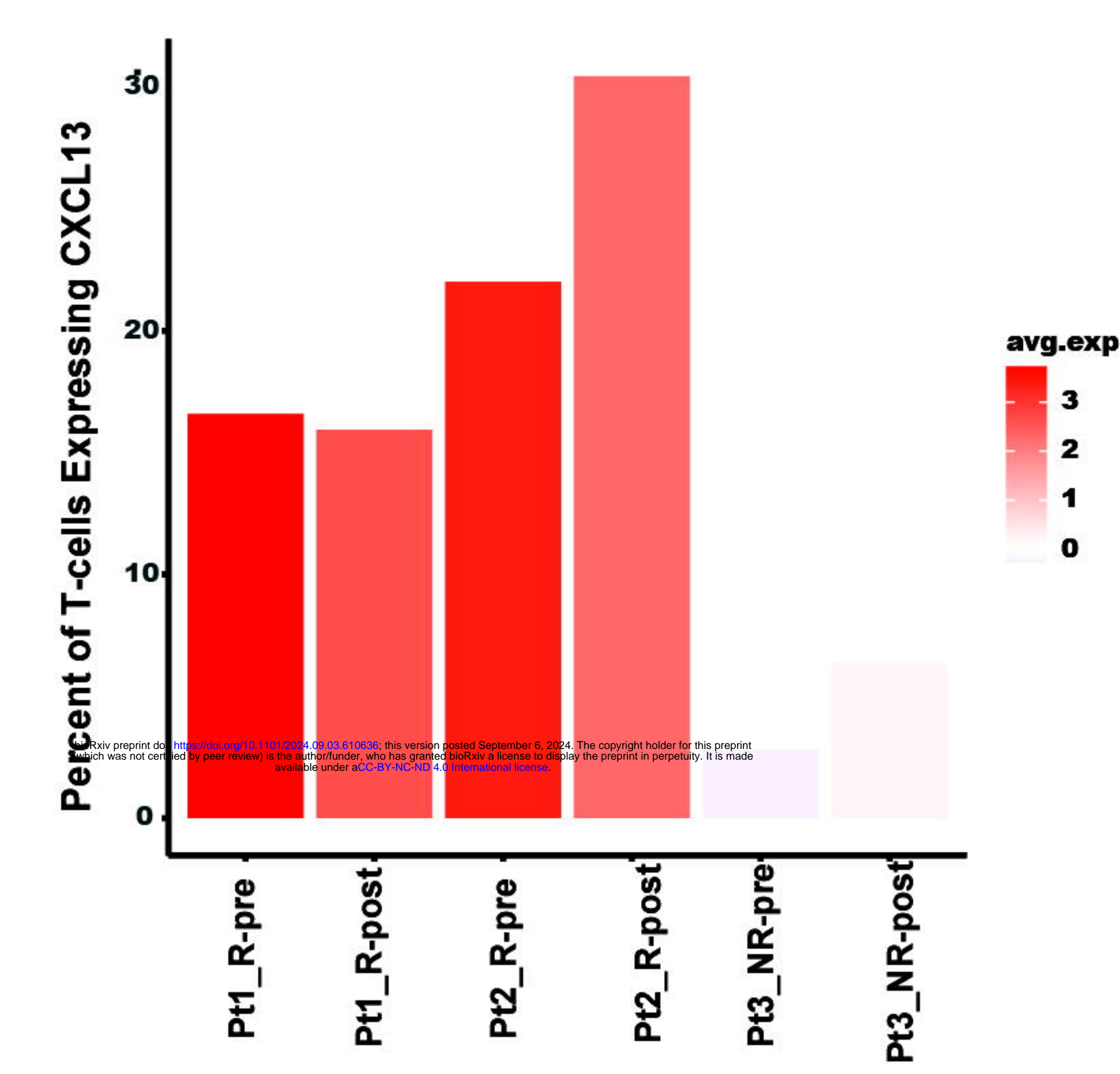

B. Proportion of new TCR clones

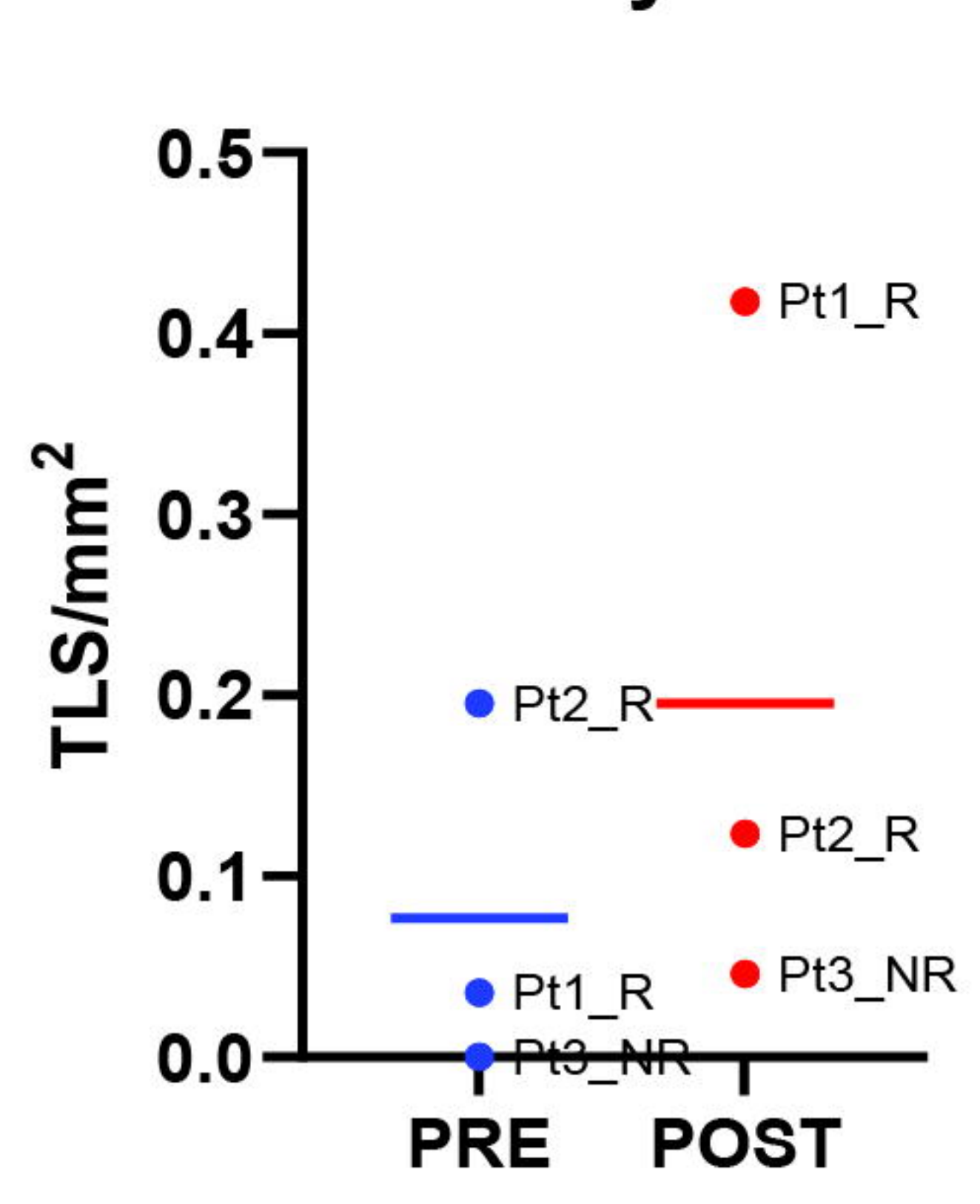

C. Cell subsets expanded prior therapy
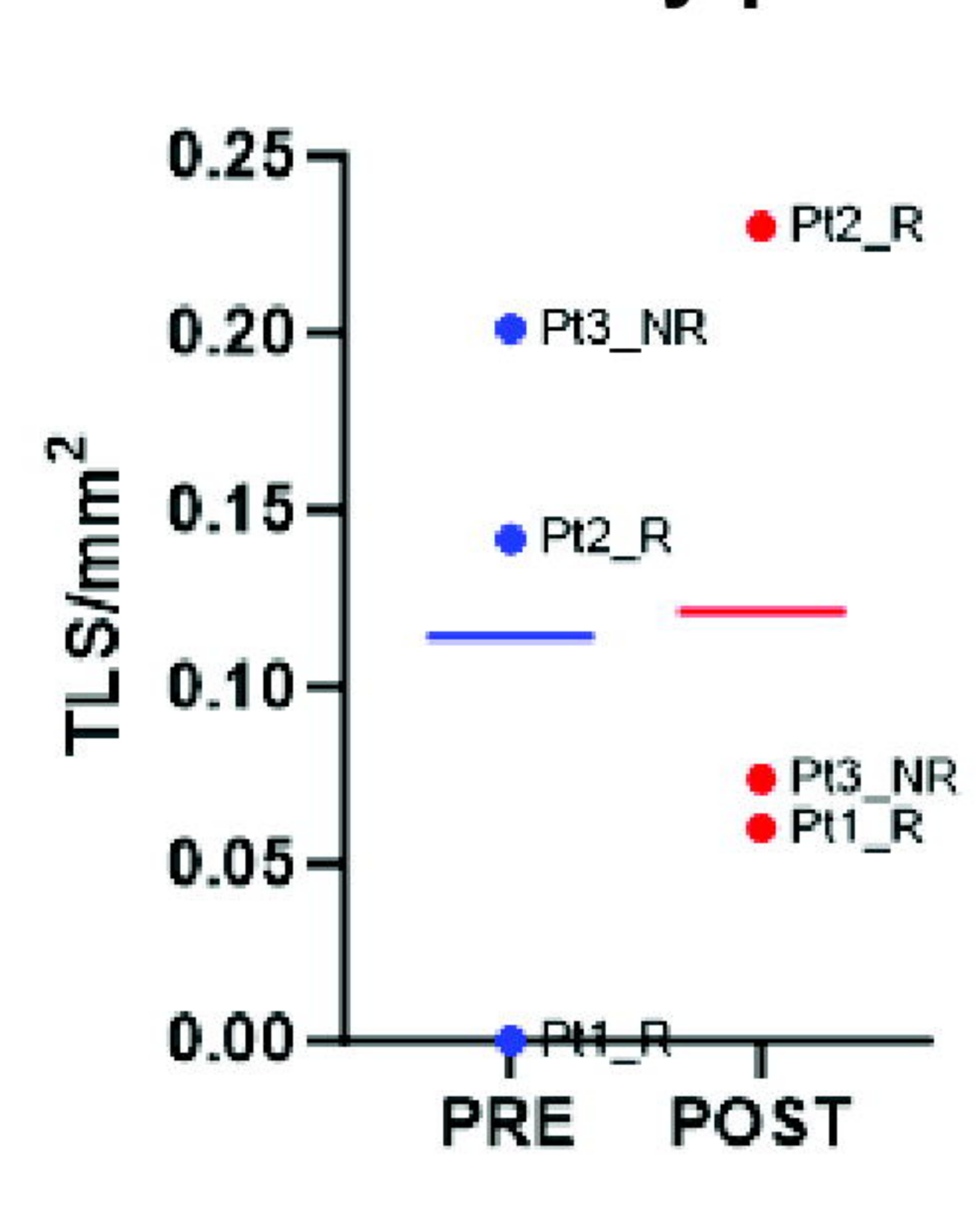

D. T cell clusters

E. Proportion of T cells expressing CXCL3

F. TLS/mm² by mIF

G. TLS/mm² by pathologist

H. TLSs identification pre and post therapy for patient Pt2_R