Critical role of CD206+ macrophages in promoting a cDC1-NK-CD8 T cell anti-tumor immune axis

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Abstract:

Tumor-associated macrophages (TAMs) are frequently categorized as being 'M1' or 'M2' polarized, even as substantial data challenges this binary modeling of macrophage cell state. One molecule consistently referenced as a delineator of a putative immunosuppressive 'M2' state is the surface protein CD206. We thus made a novel conditional CD206 (*Mrc1*) knock-in mouse to specifically visualize and/or deplete CD206+ 'M2-like' TAMs and assess their correspondence with pro-tumoral immunity. Early, but not late depletion of CD206+ macrophages and monocytes (here, 'Mono/Macs') led to an indirect loss of a key anti-tumor network of NK cells, conventional type I dendritic cells (cDC1) and CD8 T cells. Among myeloid cells, we found that the CD206+ TAMs are the primary producers of CXCL9, and able to differentially attract activated CD8 T cells. In contrast, a population of stress-responsive TAMs ("Hypoxic" or *Spp1*+) and immature monocytes, which lack CD206 expression and become prominent following early depletion, expressed markedly diminished levels of CXCL9. Those NK and CD8 T cells which enter CD206-depleted tumors express vastly reduced levels of the corresponding receptor *Cxcr3,* the cDC1-attracting chemokine *Xcl1* and cDC1 growth factor *Flt3l* transcripts. Consistent with the loss of this critical network, early CD206+ TAM depletion decreased tumor control by antigen specific CD8 T cells in mice. Likewise, in humans, the $CD206^{Replete}$, but not the $CD206^{Depleted}$ Mono/Mac gene signature correlated robustly with CD8 T cell, NK cell and stimulatory cDC1 gene signatures and transcriptomic signatures skewed towards $CD206^{Replete}$ Mono/Macs associated with better survival. Together, these findings negate the unqualified classification of CD206+ 'M2-like' macrophages as immunosuppressive by illuminating contexts for their role in organizing a critical tumor-reactive archetype of immunity.

50 **Introduction:**

51 Macrophages have diverse roles in homeostasis and disease and a refined understanding of the
52 direct and indirect effects of targeting them in tumors is an imperative, given the current impetus
53 in developing myeloid targeting therapies for cancer(*1, 2*). A widely used shortcut for describing
54 macrophage function in tumors involves an 'M1' versus 'M2' nomenclature, derived from in vitro
55 skewing with Th1 versus Th2 cytokines, and often equated with pro and anti-inflammatory
56 functions respectively. However, this binary M1/M2 delineation of macrophage phenotype does
57 not capture the heterogeneity at the single cell level (*3-5*). In addition, there is scant evidence of
58 these markers being part of coordinated gene programs in vivo. In wound healing, *Arg1* and
59 *Mrc1*(gene corresponding to the mannose-binding C-type lectin CD206), both purportedly key
60 markers of an M2 state, have distinct expression patterns (*6*). In both mouse and human tumors,
61 there is also a complete lack of correlation among genes characterizing M1 or M2 phenotypes
62 within Mono/Macs (*3*). In fact, M1 and M2 signatures in Mono/Macs often show correlated instead
63 of opposing expression patterns in tumors (*4, 5*). Nonetheless, myeloid cells expressing CD206,
64 sometimes therefore designated as 'M2-like', continue to be used as a marker of an
65 immunosuppressive state. The detrimental effects of tumor-associated macrophages (TAMs) on
66 anti-tumor immunity have indeed been highlighted by a number of critical studies (*7-11*). However,
67 a holistic dissection of the role of CD206-expressing Mono/Macs and the precise effects of
68 targeting them in tumors in vivo is lacking. We therefore developed a conditional knock-in reporter
69 mouse using the *Mrc1* (CD206) allele that allows specific visualization and depletion of those cells
70 to define their true impact on anti-tumor immunity.

71 **Results:**

72 To highlight CD206 surface expression variation across Mono/Mac differentiation in tumors, we
73 identified relevant subsets from previously published single cell transcriptomics in B16F10 tumors
74 (**Fig. 1A**, (*3*)), and applied flow cytometry to gate on those populations in a related B78chOVA
75 ((*11*); B78: an amelanotic clone of B16 to allow imaging of tumors, chOVA: mCherry and
76 Ovalbumin) tumor model. CD206 was most prominently expressed by terminal VCAM-1^{hi}IL-7Rα^{lo}
77 C1q TAMs (*3*) followed by the VCAM-1^{lo}IL-7Rα^{hi} stress-responsive population (associated with
78 enriched glycolysis, increased *Arg1* and *Il7r* expression(*3, 12*) and possibly hypoxic(*13*)), where
79 TAMs were defined as CD45^{+}Lin(CD90.2, Ly6G, B220, NK1.1, Siglec-F)$^{-}$Ly6C^{-}F480^{+}CD24^{-}
80 (**Supplementary Fig. S1A**). In contrast, CD206 was expressed at low levels by other less
81 differentiated VCAM^{-}IL-7Ra^{-} (DN) TAMs (**Fig. 1B, C**). Among monocytes, defined as
82 CD45^{+}Lin(CD90.2, Ly6G, B220, NK1.1, Siglec-F)$^{-}$Ly6C^{+}, the MHCII+ subset was the prominent
83 CD206 expressor as opposed to early (immature, MHCII-) monocytes, albeit at lower levels than
84 Stress and C1q TAMs (**Fig. 1B, C**). Overall, this analysis showed that CD206 is variably
85 expressed across multiple monocyte and macrophage subsets, and generally increases with
86 differentiation. However, when considering the use of this protein and the gene encoding it as a
87 means of eliminating these macrophages and thereby studying their function, we noted that this
88 scavenger receptor is frequently also expressed on other cells including endothelial cells and
89 keratinocytes (*14, 15*) and may further be ectopically produced by other cells in the TME.

90

91 We thus generate a conditional system where a lineage-specific Cre could drive the
92 recombination of a 3' knock-in *Mrc1*$^{\text{LSL-Venus-DTR}}$ allele (Venus : Yellow Fluorescent Protein variant
93 for visualization; DTR: Diphtheria toxin receptor for depletion) (**Fig. 1D**). Then, using a *Csf1r*^{Cre};
94 *Mrc1*$^{\text{LSL-Venus-DTR}}$ cross (DTR), compared to a *Mrc1*$^{\text{LSL-Venus-DTR}}$ (WT) control, we assessed the
95 reporter expression in various immune and CD45- non-immune compartments in the

96 subcutaneous melanoma model B78chOVA (**Fig. 1D, Supplementary Fig. S1A**). As predicted
97 from CD206 expression, nearly 75% of TAMs showed robust Venus expression tightly correlated
98 with surface expression of CD206 protein (**Fig. 1F**). We also found that nearly half of cDC2s,
99 consistent with their monocytic origin as previously described(*16*), expressed Venus in this
100 system. A small subset of CD206+ monocytes expressed the reporter, again consistent with *Csf1r*
101 driven expression. Weak expression was also found in a yet smaller population of neutrophils.
102 When viewed by the level of CD206-driven expression of the Venus marker, macrophages were
103 2-3x brighter than the other populations (**Fig. 1F**). Importantly, no reporter expression was
104 detected in non-immune cells (which include endothelial cells and keratinocytes and where a
105 small fraction is CD206+), lymphocytes and cDC1s (**Fig. 1E, F**). Likewise, in the proximal tumor-
106 draining lymph nodes (tdLN), the same hierarchy of expression patterns was observed, albeit at
107 much lower levels (**Supplementary Fig. S1B**). In addition, some tissue-resident macrophages,
108 such as alveolar macrophages in the lung express high levels of CD206 and therefore the
109 reporter, while interstitial macrophages, monocytes, neutrophils, and non-immune cells showed
110 very modest to no expression (**Supplementary Fig. S1C, D**). Therefore, in *Csf1r*^{Cre}; *Mrc1*$^{LSL\text{-}Venus\text{-}}$
111 ^{DTR} mice, a robust marking of mature CD206+ macrophages in the subcutaneous tumor was
112 observed, along with faithful marking of CD206+ subsets of monocytes, neutrophils and cDC2s,
113 but not cDC1s, lymphocytes and non-immune cells.
114
115 **CD206+ TAM depletion leads to indirect loss of a reactive immune archetype:** To test the
116 impact of CD206+ macrophage targeting on the overall tumor immune microenvironment, we took
117 advantage of the linkage of Venus and DTR expression in this background to deplete those cells.
118 In our setup using subcutaneous B78chOVA tumors as described previously, adoptively
119 transferred ovalbumin-specific OT-I cells allow the tracking of antigen-specific CD8 T cell
120 responses, which nevertheless do not mediate tumor control(*11, 17*). We first confirmed that Cre-
121 mediated induction of reporter expression without diphtheria toxin (DTx) administration did not

122 alter the immune composition of these OVA-expressing tumors with OT-I transfer in the WT
123 (*Mrc1*$^{LSL\text{-}Venus\text{-}DTR}$) vs. DTR mice (*Csf1r*^{Cre}; *Mrc1*$^{LSL\text{-}Venus\text{-}DTR}$) (**Supplementary Fig. S2A**). With this
124 baseline, we administered DTx either 'late/acute', namely in the last 4 days prior to the tumor
125 harvest or 'early/chronic', i.e., every day 2-3 days, starting 2 days post T cell injection until harvest
126 to parse out the role of CD206+ Mono/Macs in the TME (**Figure 2A**). These two modes of
127 depletion represent perturbations at different phases of the establishment of the tumor immune
128 microenvironment.
129
130 In the context of late DTx administration, we found that this regimen specifically depleted the cells
131 of interest and otherwise had little overall effect on other non-targeted cells. Thus, there was a
132 strong reduction in the TAMs (**Figure 2B**) which corresponded to a specific loss of the CD206+
133 population (**Fig. 2P**). We found a compensatory rise in monocytes overall, accompanied by a
134 specific loss of its CD206+ subset (**Figure 2C, 2P**). No significant loss was observed in cDC2s,
135 cDC1s, NK cells, the adoptively transferred OTI, or neutrophils (**2D-H**), although the regimen did
136 select against the CD206+ populations in the case of cDC2s and neutrophils (**Figure 2P**). Thus,
137 direct depletion was reliably restricted to the highest expressors of the construct, namely the
138 CD206+ TAM and monocyte populations.
139
140 When we depleted CD206+ populations with DTx early, i.e., starting 2d after OTI adoption, again
141 we found robust depletion of TAMs (**Fig. 2I**) expectedly with a strong selection against those
142 expressing CD206 (**Fig. 2Q**). As before, the CD206+ subsets of other populations were still
143 depleted—robustly in monocytes and cDC2s and mildly in neutrophils (**Fig. 2K, Q**). A
144 compensatory increase in neutrophils was observed, while overall abundance of monocytes,
145 albeit biased towards CD206-, remained similar, in contrast to acute depletion. (**Fig. 2J, L**).
146 Strikingly, under this early elimination regime, we also observed a decrease in intratumoral cDC1

147 abundance (**Fig. 2M**). Further, NK cells and transferred OT-I abundance in the tumor was also
148 significantly compromised (**Fig. 2N, O**).
149
150 These trends in abundances were similar when expressed as percentage of live cells, indicating
151 numerical changes, in all cases except the increase in monocytes following acute depletion
152 (**Supplementary Fig. S2B-O**), which only trended higher. When we similarly treated non-tumor
153 bearing DTR mice with DTx with six doses akin to the early depletion regimen in tumors, and
154 analyzed the immune compositions in the skin (site of the ectopic tumor
155 injections)(**Supplementary Fig. S2P**), no robust indirect loss of populations were observed, but
156 an increase of neutrophils in an otherwise scarcely immune-populated skin was recorded
157 (**Supplementary Fig. S2Q**). Given the associated increase in neutrophils, we repeated the same
158 early depletion experiment in tumors, now with the addition of anti-Ly6G neutrophil depleting
159 antibody or isotype control (**Supplementary Fig. S2R)** to assess whether the gained neutrophils
160 played a role in the indirect loss of lymphocytes and cDC1s. As expected, both in terms of the
161 total number of cells per gram of tumor (**Supplementary Fig. S2S)** and the percentage of CD45+
162 (immune) cells (**Supplementary Fig. S2T)**, the abundance of immune cell types in WT and DTR
163 mice treated with isotype control mirrored those of the early depletion regimen. With anti-Ly6G
164 treatment in DTR mice, neutrophils were reduced to levels below those of untreated controls,
165 without any concomitant effect on the indirect depletion of cDC1s, NK cells and OT-I T cells
166 (**Supplementary Fig. S2S, T**). Noting that none of these CD8, NK and cDC1 populations express
167 the reporter, we concluded that a direct, targeted ablation of 'M2-like' CD206+ Mono/Macs by
168 early DTx treatment in tumors led to the indirect loss of this key anti-tumor reactive archetype
169 comprising of NK cells, cDC1s and antigen specific CD8 T cells(*18*), (**Fig. 2R**). This suggested
170 that CD206+ Mono/Macs were involved in the recruitment and early establishment of this module
171 in the TME.
172

173 **Early depletion skews Mono/Macs towards immature and hypoxic subsets:**

174 To define the macrophage subtypes associated with reactive immunity and their potential spatially
175 segregated modes of action, we performed spatial transcriptomics of B78chOVA tumors, guided
176 by Venus (CD206 reporter) expression. For this, we first spatially mapped the CD206+
177 macrophage population by Venus expression, using two-photon microscopy of B78chOVA tumor
178 slices with transferred OT-I T cells marked by the CD2dsRed allele (**Fig. 3A**). Doing this revealed
179 three distinct niches of CD206+ macrophage and T cell localization. The 'edge', which is
180 macrophage and collagen-rich with modest T cell presence, 'mid', the interfacial layer with
181 abundant T cell: macrophage interaction zones and 'interior'. The interior is sparser in both
182 immune cell types but represents the bulk of the tumor by volume (**Fig. 3A**). We then performed
183 post-imaging spatial transcriptomics by ZipSeq (*19*) on CD45+ cells in these three zones (*11*) of
184 B78chOVA tumors, with or without early DTx treatment harvested at d12 post T cell injection.
185 UMAP projection of non-linear dimensional reduction and louvain clustering clearly showed the
186 remarkable shift in tumor immune composition among control and DTx treated groups (**Fig. 3B,**
187 **C, Supplementary Fig. S3A**). Notably, previously defined C1q and Stress-responsive (Stress)
188 TAMs, which most robustly express CD206 at the protein level, along with MHCII+ and Interferon-
189 stimulatory gene (ISG) -expressing monocytes were expectedly depleted by direct DTx action
190 (**Fig. 3C**), with the robust depletion of C1q TAMs (VCAM-1^{hi}IL-7Rα^{lo}) and MHCII+ Monocytes
191 verified by flow cytometry (**Fig. 3D**). On the other hand, early monocytes, neutrophils and a *Spp1*,
192 *Hif1α*-expressing subset related to the Stress TAMs by shared expression of *Arg1, Il7r* (i.e.,
193 Stress^{Spp1} TAM), became prominent in the DTx treated condition (**Fig. 3C, Supplementary Fig.**
194 **S3A**). The loss of cDC1:NK:CD8 populations was again evident in the analysis of relative
195 abundance from the scRNASeq data (**Fig. 3C**). Even though the small area of the tumor edge
196 was much denser in the CD206+ Mono/Macs as shown by imaging, the transcriptomic data
197 suggests high CD206-expressing C1q and Stress TAMs were more or equally as abundant in the

198 interior than the edge (**Fig. 3E**). consistent with the trajectory of increasing Mono/Mac
199 differentiation towards the interior of the tumor(*11*). Overall, in this subcutaneous tumor model,
200 the changes in immune subpopulations were not limited to a specific region of the tumor (**Fig.**
201 **3C**), but permeated throughout as a holistic overhaul of the tumor immune microenvironment.
202
203 **CD206+ TAMs attract CXCR3-expressing, cDC1-supportive lymphocytes to the tumor:**
204 A well-established positive functional role of TAMs is the production of CXCL9 and CXCL10,
205 inducing CXCR3-dependent lymphocyte recruitment in tumors(*20*). Given the indirect loss of
206 lymphocytes upon early removal of CD206+ Mono/Macs, we hypothesized that this axis is
207 prominent in CD206 positive myeloid populations. Analyzing the scSeq data in detail, we found
208 that expression of *Cxcl10* (**Supplementary Fig. S3C**) and *Cxcl9* (**Fig. 3G**) in particular were
209 markedly reduced in the DTx treated tumors and this corresponded to substantial expression by
210 the directly depleted subsets (CD206+MHCII+ Mono/Macs) and none of the indirectly increased
211 ones (Early Mono, Stress^{Spp1} TAM and Neutrophils) (**Supplementary Fig. S3B, Fig. 3F**). We
212 confirmed this finding by flow cytometry for intracellular CXCL9 expression in TAMs from WT and
213 DTR mice with early depletion (~50% decline, **Fig. 3H, Supplementary Fig. S3D**). This analysis
214 further revealed a positive association between this chemokine and CD206 expression in
215 B78chOVA TAMs (**Fig. 3I**), resulting in a significant difference (again ~50%) in CXCL9 expression
216 in CD206+ vs. CD206- TAMs (**Fig. 3J**) in the WT mice. This finding was substantiated in another
217 subcutaneously injected tumor model MC38chOVA and the spontaneous breast tumor model
218 PyMTchOVA, both with lower overall CXCL9 positivity in the absence of OT-I adoptive transfer,
219 but a consistent ~50% or more difference between the CD206+ and CD206- groups
220 (**Supplementary Fig. S3E**). CD206+ monocytes also showed higher CXCL9 expression,
221 compared to CD206- counterparts (**Supplementary Fig. S3F**), but CXCL9+ monocytes were only
222 1/4th as abundant as CXCL9+ TAMs in the B78chOVA TME, thus limiting their relative role in
223 myeloid CXCL9 production (**Supplementary Fig. S3G**). We therefore sorted CD206+ vs. CD206-

224 TAMs from B78chOVA tumors (d14 post tumor injection without OT-I treatment) and interrogated
225 their relative effects on in vitro activated CD8 T cell transmigration in a 3h window. Consistent
226 with their CXCL9 expression, the CD206+ but not the CD206- TAMs induced enhanced
227 transmigration over no TAM-added controls. (**Fig. 3K**). Since CD206 TAM-depleted tumors still
228 had small numbers of lymphocytes, we compared their levels of CXCR3 at the transcript level,
229 which reflects receptor-ligand engagement avoiding the confounding effect of receptor
230 internalization(*21*), and found that *Cxcr3* expression was markedly lower in the DTx treated
231 condition in all the lymphocyte subsets (**Fig. 3L, Supplementary Fig. S3H**). Taken together,
232 these data point to the role of CD206+ TAMs in the recruitment of CXCR3-expressing
233 lymphocytes to the TME.
234 Lymphocytes are well-established as key producers of cDC1-formative chemokines FLT3L (*18*)
235 and XCL1(*22*). Therefore, given the loss of cDC1s in concert with lymphocytes with this depletion
236 regime, we probed for these chemokines in the intratumoral lymphocytes in control vs. CD206-
237 depleted dataset. This demonstrated that both *Flt3l* and *Xcl1* (**Fig. 3M, Supplementary Fig. S3I**)
238 transcripts were markedly reduced in the NK cells and CD8 T cells in the DTx-treated condition.
239 These changes on a per cell basis, in addition to the overall decrease in CD8 T cells and NK
240 cells are consistent with the loss of cDC1s in the TME, as a result of the disruption of the
241 CD8:NK:cDC1 module (*18*).
242
243 **Depletion of CD206+ TAMs thwarts CD8 T cell mediated anti-tumor immunity in mice:** Given
244 our finding that CD206+ 'M2-like' macrophages are critical to the organization a key node of anti-
245 tumor immunity, we asked whether they were necessary for successful CD8 T cell mediated tumor
246 regression. To test this, we used a MC38chOVA model where an adoptive transfer of OT-I T cells
247 that results in efficient tumor control(*17*) (**Supplementary Fig. S3J**). We confirmed first that
248 reporter expression in these tumors followed largely the same pattern as the B78chOVA tumors,
249 with substantial expression only in TAMs and cDC2s, albeit at lower levels due to the overall lower

250 CD206+ fraction, and little to no expression in neutrophils and monocytes in this model.
251 (**Supplementary Fig. S3K, L**). Importantly, lymphocytes and cDC1s again showed no reporter
252 expression (**Supplementary Fig. S3K, L**). As with the B78chOVA model, we applied early and
253 late depletion regimens to the MC38chOVA tumors, first without the addition of OT-I T cells, to
254 assess differential effects on early establishment and maintenance of immune cells without the
255 confounding variable of tumor regression (**Fig. 4A**). Some differences were observed compared
256 to the B78chOVA model, including an overall maintenance of TAM abundance in late and only a
257 modest (~25%) decline in early depletion (**Supplementary Fig. S3N, R**) despite robust depletion
258 of the CD206+ populations (**Fig. 4B, E**). Indeed, following reporter expression patterns, the
259 CD206+ subsets were depleted robustly in TAMs and cDC2s and modestly in monocytes and
260 neutrophils in both the late (**Fig. 4I**) and early (**Fig. 4J**) depletion regimens. We also noted other
261 variations namely increased monocyte abundance in early but not late depletion (**Supplementary**
262 **Fig. S3O, S**) and neutrophil enrichment in both regimens (albeit with much lower effect size in
263 late depletion; **Supplementary Fig. S3Q, U**). As in the case of B78chOVA tumors, CD206+
264 cDC2s were depleted (**Fig. 4I, J**) but no change was detected in overall cDC2 abundance
265 (**Supplementary Fig. S3P, T**) with both early and late DTx treatment. Importantly, there was once
266 again a robust indirect loss of cDC1s and CD8 T cells specifically under the early but not the late
267 depletion regimen (**Fig. 4C, D, F, G**). When evaluated further by directly measuring total number
268 of cells per g of tumor, we observed once again the direct depletion of CD206+ TAMs, indirect
269 increase in neutrophils and the indirect loss of cDC1s and CD8 T cells, but not NK cells
270 (**Supplementary Fig. S3U**) in MC38chOVA tumors with early DTx treatment. With confirmation
271 of this key indirect effect of CD206+ TAM depletion in the MC38chOVA model, we treated
272 subcutaneous MC38chOVA tumors in WT and DTR mice with OT-Is and concomitant early DTx
273 administration. With the prediction that depletion of CD206+ TAMs would thwart the tumor control
274 ability of OT-Is, we tracked changes in tumor size and indeed observed significantly reduced OT-
275 I-mediated tumor control of MC38chOVA tumors (**Fig. 4H**) in the DTR group.

276 **CD206+ Mono/Mac signatures associate with anti-tumor immunity in human cancers:** The
277 data presented thus far provided substantial evidence of a context in which CD206+ populations
278 of Mono/Macs were in fact positive contributors to reactive anti-tumor immunity in mice, rather
279 than being simply immunosuppressive. Consistent with this understanding, we found that higher
280 levels of *MRC1* RNA alone correlated with slightly better survival from patient data rather than
281 worse in a large cohort curated from The Cancer Genome Atlas (TCGA) (*23*) (**Fig. 5A**). We also
282 sought to determine whether the revealed relationships between CD206+ TAMs, CXCL9 and the
283 cDC1:CD8:NK module in our study might similarly extended to human disease. To do so, we first
284 applied differential gene expression (DGE) analysis of the Ctrl vs. DTx treated Mono/Mac
285 populations (**Fig. 5B, C**) (excluding neutrophils, cDC1, cDC2 and lymphocyte subsets) from our
286 scRNASeq dataset (**Fig. 3B**). We then used the top 10 DEGs (by average log fold change and
287 having an adjusted p-value <0.01) to create CD206 'Replete' and 'Depleted' gene signatures (**Fig.
288 5D**). The former are DEGs associated with the presence of CD206+ populations and not only
289 included *C1qa, Cxcl9, Apoe,* but also several MHC-II related genes (**Fig. 5D, E**), consistent with
290 flow cytometry data on C1q TAM, CD206, CXCL9 and MHC-II expression described above. The
291 Depleted signature contains genes differentially expressed in macrophages that remain post
292 CD206+ Mono/Mac depletion and included *Il1b*, *S100a8*, along with *Spp1* (**Fig. 5D, E**). Even
293 though we obtained these gene signatures based upon depletion of Mono/Macs using the
294 prominent "M2" marker CD206, both M1 and M2-associated genes were differentially upregulated
295 in Replete signature (**Fig. 5D**), reiterating the lack of coordination among such markers when
296 studied in vivo.
297
298 Using these signatures, we queried a previously described immune compartment-specific bulk
299 RNA-Seq data derived from sorted HLA-DR+ myeloid (to capture Mono/Macs and DCs), T and
300 total live cells from >200 human tumor biopsies(*23*) (**Fig. 5F, G**) belonging to five common solid
301 tumor indications (CRC: Colorectal Cancer, GYN: Gynecological Cancer, HNSC: Head and Neck

302 Squamous Cell Carcinoma, KID: Kidney Cancer; LUNG: Lung Carcinoma). Given our finding that
303 the CD206+ CXCL9-expressing TAMs recruit CXCR3-expressing cDC1-supportive lymphocytes,
304 we predicted that the CD206 "Replete" but not the "Depleted" signature in the myeloid
305 compartment would associate with previously established CD8, NK and stimulatory cDC1s
306 (stimulatory dendritic cell or SDC) gene signatures (*10*) (*23*). Indeed, the Replete signature, but
307 not the Depleted signature correlated significantly with those of each component of the tumor-
308 reactive immune module (**Fig. 5F**) of SDCs (**Fig. 5G**), CD8 T cells (**Fig. 5H**) and NK cells
309 (**Supplementary Fig. 3W**). Given that it is by now well-established that SDCs are associated with
310 survival (*10*), we also queried whether the relative abundance of CD206 Replete Mono/Macs (i.e.,
311 $CD206^{Replete}/CD206^{Depleted}$ ratio) was correlated with better survival in patients. In the TCGA
312 dataset, we observed a large (~20% in 5-year survival) and significant shift in survival for patients
313 biased towards the $CD206^{Replete}$ Mono/Mac signature (**Fig. 5I**). Indeed, patients scoring high on
314 the $CD206^{Replete}$ Mono/Mac signature alone were also found to have significantly better survival
315 but with a reduced effect size (**Fig. 5J**) as compared to the ratio. Among specific indications, the
316 Replete/Depleted signature ratio was associated with overall survival in Lung, Liver, Pancreatic,
317 Bladder, Kidney Cancer and Melanoma (**Supplementary Fig. S3X**). Thus, contrary to the
318 simplistic labeling of CD206 expressing macrophages as immunosuppressive, this data
319 establishes contexts in which these Mono/Macs are a critical organizing fulcrum for the reactive
320 archetype of NK cells, cDC1s and CD8 T cells. Taken together, these data contribute to a nuanced
321 understanding of the context-dependent role of TAMs in the TME, necessary to rationally design
322 next-generation myeloid-targeting immunotherapies in cancer.

323 **Discussion:**

324 cDC1s have been previously linked to FLT3L and XCL1 producing NK cells and activated CD8 T
325 cells(*18, 22*), and this network represents one module of immunity that predisposes to immune
326 checkpoint blockade response (reviewed in(*24*)). The same CD8 T cells in turn may be recruited
327 and expanded by chemokines and antigen-presentation by cDC1s, creating a virtuous feedback
328 loop for anti-tumor immunity. It was however, previously unexplored how specific macrophage
329 subsets support or thwart this anti-tumor archetype. Here, we demonstrate that CD206 expression
330 in macrophages is robustly correlated with their expression of CXCL9 and these macrophages
331 play a critical role in initiating the assembly of the cDC1:NK-CD8 anti-tumor reactive immune
332 archetype in tumors.

333

334 This work is the latest in a series of publications (*3, 5, 12*). that force re-evaluation of the prevalent
335 but insufficient M1/M2 classification of macrophages in tumors. Notably, CD206 expression is
336 still often used to categorize macrophages as immunosuppressive and 'M2-like', even though
337 strong in vivo data supporting this assertion is lacking. Here, we show that CD206 should not be
338 used as an unqualified indicator of immunosuppressive function. Indeed in the context of ongoing
339 anti-tumor responses studied here in the early depletion setting, these TAMs are crucial for
340 effective recruitment of critical immune cells.

341

342 When thinking about these revealed functions of TAMs expressing CD206, we also note that this
343 study specifically found them critical in early T cell recruitment. However, our work and others
344 have shown that some mature TAMs which include those that express CD206 may also be
345 involved in coupling with CD8 T cells and promote T cell exhaustion (*11, 25*). Thus, TAMs may
346 have distinct phenotypes and functions depending on the immunological state of the tumor –
347 perhaps reflected in the early and late depletion conditions shown here. Future studies to
348 understand this balance of pro and anti-tumor effects of TAMs is critical. At present, one should

349 not simplistically take our study to indicate that CD206+ macrophages are universally favorable
350 for anti-tumor immunity. However, the M1/M2 dichotomy—and particularly a version that equates
351 CD206 with pro-tumoral functions—appears to be a misleading lens through which to view
352 macrophage functional heterogeneity.
353
354 Other recent data using *CXCL9* versus *SPP1* gene expression to functionally classify
355 macrophages in human tumors (*12*) as anti- or pro-tumor respectively, are aligned with our
356 findings. In our earlier studies of *SPP1* in macrophages (*3*), these were observed in human
357 tumors, likely embedded within an Arg1 (Stress) TAM subset in mice, and here they only emerged
358 as a distinct cluster due to their disproportionate enrichment post depletion. As we have previously
359 noted(*3*), these non-CD206 expressing 'Stress' macrophages are distinctly glycolytic, express
360 *Hif1*α, and are likely the cells that have previously been defined as hypoxic macrophages (*26, 27*)
361 and now associated with poor patient outcomes.
362
363 Studies prior to ours and using more universal Mono/Mac depletion have also variably reported
364 compensatory neutrophil influx when depleting cells of monocytic origin in tumors (*28-30*). Our
365 data also shows an increase in neutrophils in the early CD206-gated depletion condition, but a
366 lack of similar influx in the late depletion regimen. One interpretation of our data is that a
367 microenvironment-dependent opportunistic filling of the early myeloid niche by neutrophils takes
368 place in the absence of sufficient Mono/Macs and the reactive immune components. While further
369 studies may uncover key nodes of this balance of myeloid populations, our results show that the
370 compensatory neutrophils do not contribute to the reduction of CXCR3-dependent lymphocyte
371 recruitment (*20*), which is the primary driver leading to the loss of the key tumor-reactive
372 archetype.
373

374 One key success of our study is the ability to differentially target subsets of TAMs within the TME.
375 Prior to our work, many questions regarding the specific role of TAMs have remained obscured
376 or unanswered partly owing to the lack of sufficiently specific and penetrant tools to manipulate
377 them in vivo. Commonly used methods, while useful lack sufficient specificity, including the
378 depletion of all monocytes and monocyte-derived dendritic cells (CSF1R blocking antibody;(*31-*
379 *33*)) and the depletion of all phagocytic cells and arrest of neutrophils (Clodronate; (*34*)). In this
380 context, the novel conditional CD206 reporter introduced here—paired with *Csf1r*-Cre to avoid
381 depleting other non-myeloid cells that express CD206—provides a more selective marking and
382 depletion tool for CD206+ TAMs, with a further potential to target various subpopulations by
383 altering the Cre driver alleles.
384
385 Overall, our results indicate that even this subset-dependent depletion of Mono/Mac populations
386 may not be prudent in all contexts. To this extent, while anti-CSF1R antibodies have failed to
387 show benefits in clinical trials (*35*), other strategies using for example, drugs that modulate
388 specific subsets such as those expressing TREM2 or trigger others by engaging TREM1 may
389 prove more surgical(*36*). Systematically dissecting the role of individual TAM subtypes will
390 continue to be crucial to deciphering their context-dependent and complex roles in the TME, with
391 a view towards harnessing them for better immunotherapy outcomes.
392

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Human tumor data curation and analysis: TC, AJC, BS

Writing: AR, MFK

Supervision: MFK

Declaration of Interests:

The authors declare no competing interests

Data and materials availability:

Relevant data will be made publicly available before publication in its final form. Meanwhile, data will be available upon reasonable request, please contact the authors directly.

List of Supplementary Materials:

Materials and Methods

Fig. S1-S3

Figure and Figure Legends:

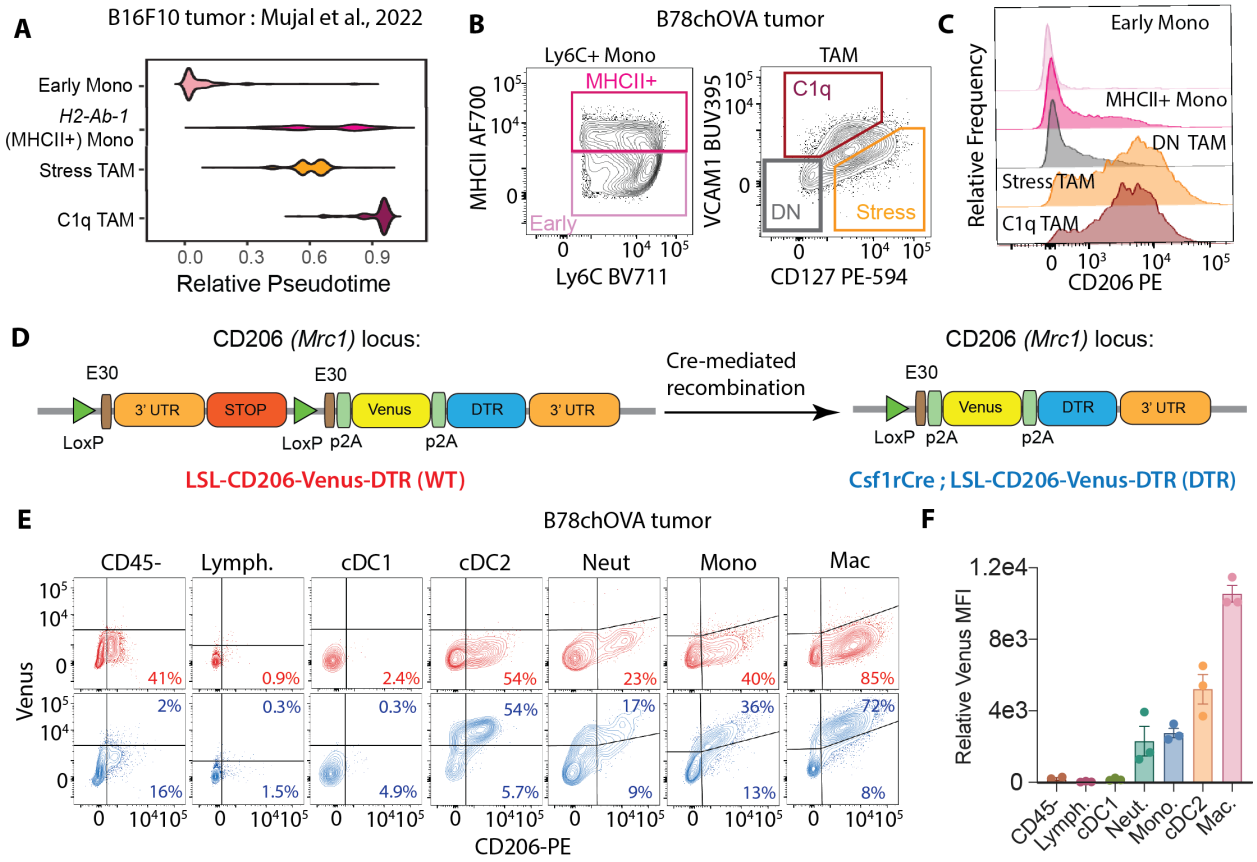

Fig. 1: Genetic Myeloid-specific Labeling of CD206+ Macrophages in Tumors: (A) Pseudotime plots of select Mono/Mac subsets in B16F10 tumors from Mujal et al(*3*); **(B)** Gating on the equivalent subsets in B78chOVA tumors by flow cytometry and **(C)** CD206 expression in each of these subsets; **(D)** Schematic representation of the *Mrc1*$^{\text{LSL-Venus-DTR}}$ knock-in construct before (WT) and after (DTR) Cre-mediated recombination by crossing to the *Csf1r*^{Cre} allele; **(E)** Flow cytometry plots showing reporter (Venus) and CD206 expression in different immune cells in d18 B78chOVA tumors in WT (red) and DTR (blue) mice with **(F)** quantification of relative reporter expression (DTR – WT) in the different subsets. data are mean +/- SEM, from 3 biological replicates, WT levels averaged from 2 biological replicates.

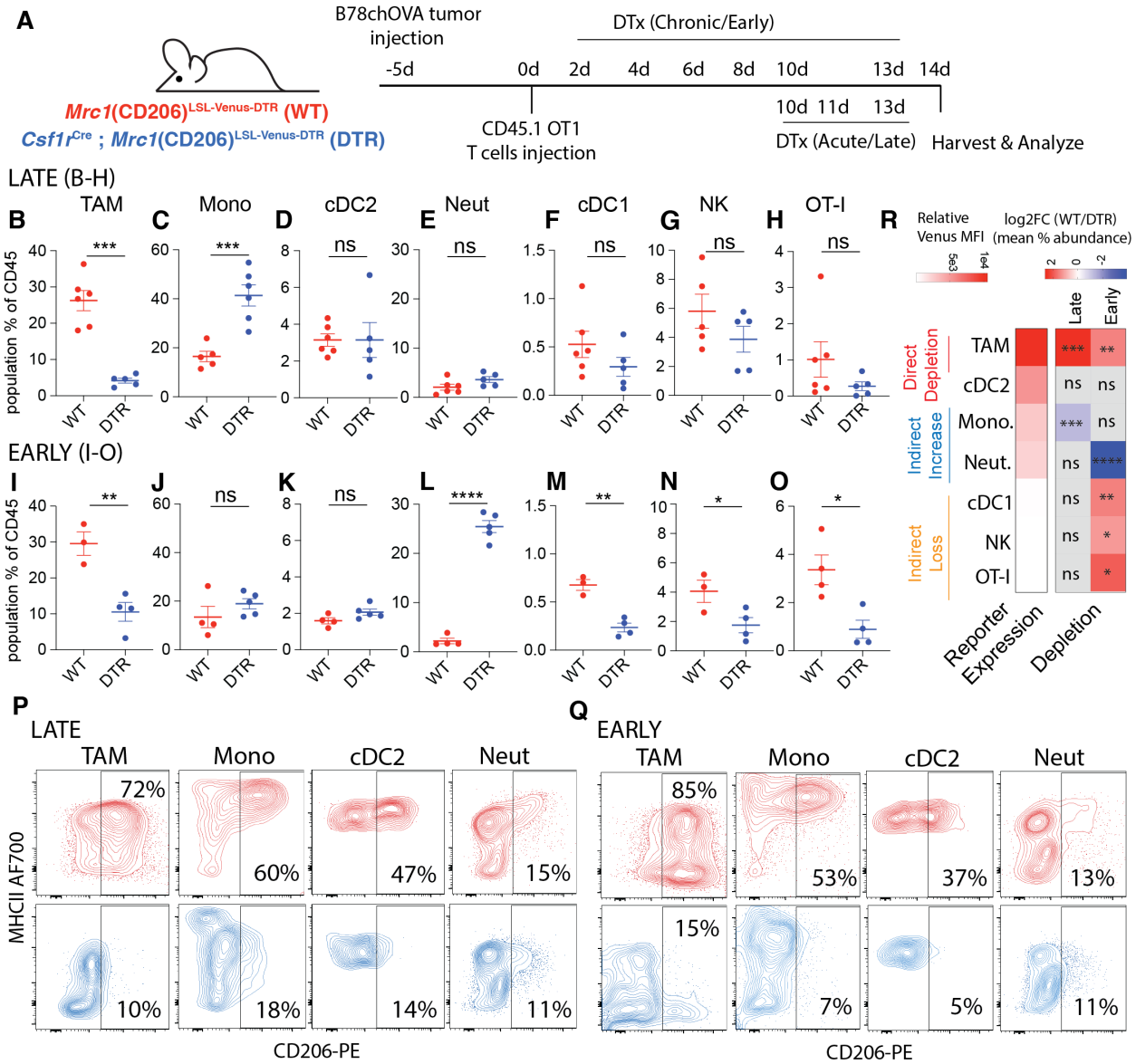

Fig. 2: Early CD206+ TAM depletion leads to a coordinated and indirect loss of NK, cDC1 and CD8 T cells in the TME: (A) Schematic representation of the experimental setup for early and late CD206+ TAM depletion in B78chOVA tumors using *Mrc1*(CD206)$^{\text{LSL-Venus-DTR}}$ (WT) and *Csf1r*^{Cre}; CD206$^{\text{LSL-Venus-DTR}}$ (DTR) mice; Relative abundance of different immune populations as a percentage of CD45+ cells with **(B-H)** late and **(I-O)** early depletion regimens; Representative flow cytometry plots showing CD206 vs. MHCII expression in different myeloid subsets in WT (red) and DTR (blue) mice in the (**P**) late and (**Q**) early depletion regimens. **(R)** heatmap representation of the log fold change of the ratio of mean abundances in WT and DTR mice (data from B-O), alongside the extent of reporter expression (mean relative Venus MFI from Fig. 1F) to indicate direct depletion and indirect loss or enrichment. (statistical significance is indicated on the respective squares; ***p <0.001, **p<0.01, *p <0.05, ns = no significance by Student's t-tests).

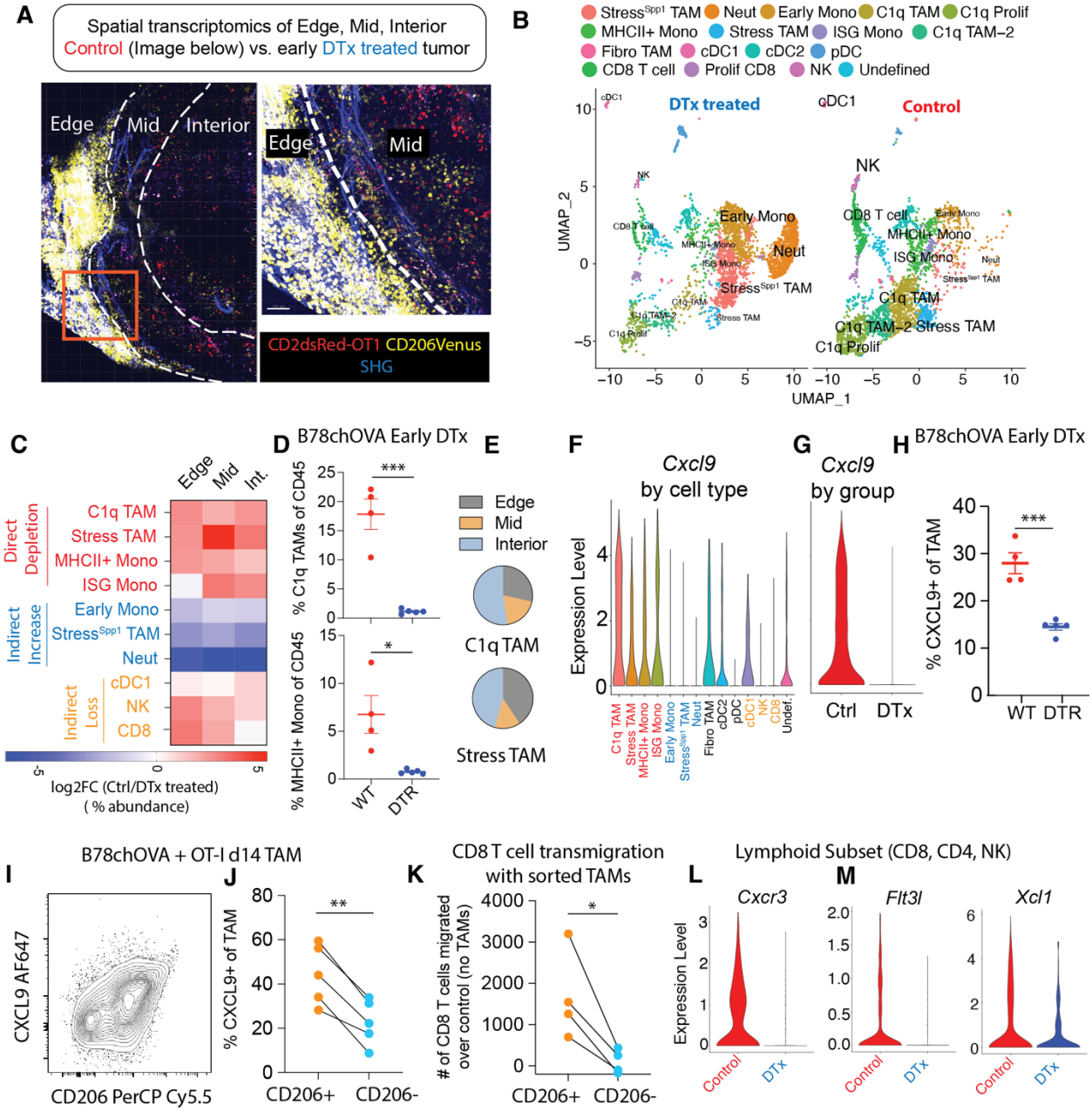

Fig. 3: Loss of CXCL9-positive TAMs and CXCR3-expressing, cDC1 supportive lymphocytes with CD206+ TAM depletion (A) Two-photon imaging of representative (control) B78chOVA tumors d12 post adoptive transfer of CD2dsRed; OT-I CD8 T cells showing three zones of Venus-expressing macrophage and associated CD8 T cell localization – edge, mid and interior (Int.) mapped by spatial transcriptomic barcoding ZipSeq; Boxed region is magnified (*right*) to show corresponding edge-mid interface SHG: Second Harmonic Generation; **(B)** UMAP representation of major immune cell populations obtained from Control and early DTx treated B78chOVA tumors d12 post OT-I injection aggregated across all three regions; **(C)** Summary heatmap showing relative log fold change of the abundance (calculated as the % of the total number of cells recovered within that region) of each major cluster in Ctrl/DTx treated conditions, split by region of tumor; *Cxcl9* expression; **(D)** Flow cytometry data showing abundance of C1q TAMs and MHCII+ Monocytes in Ctrl and DTx treated conditions; (**E**) Distribution of C1q and

Stress-responsive TAMs in the three spatial regions in control B78chOVA tumors; *Cxcl9* expression **(F)** aggregated across treatment conditions by cluster and **(G)** aggregated across clusters by condition; **(I)** Representative flow cytometry plot showing intracellular CXCL9 vs. surface CD206 expression in TAMs in B78chOVA tumors at d14 post OT-I adoptive transfer and (**J**) the same CXCL9 expression split by CD206 positivity; **(K)** in vitro activated CD8 T cell migration at 3h through a 5µm transwell insert in the presence of sorted CD206+ vs. CD206- TAMs from B78chOVA tumors, normalized to migration with no TAMs; **(L)** *Cxcr3,* **(M)** *Flt3l* and *Xcl1* expression in the lymphocyte subset (CD8 T cell, NK cell and CD4 T cell) by treatment group. ***p <0.001, **p<0.01, *p <0.05, ns = no significance by Mann-Whitney test (D), unpaired t-test (H) and ratio paired t-test (J, K).

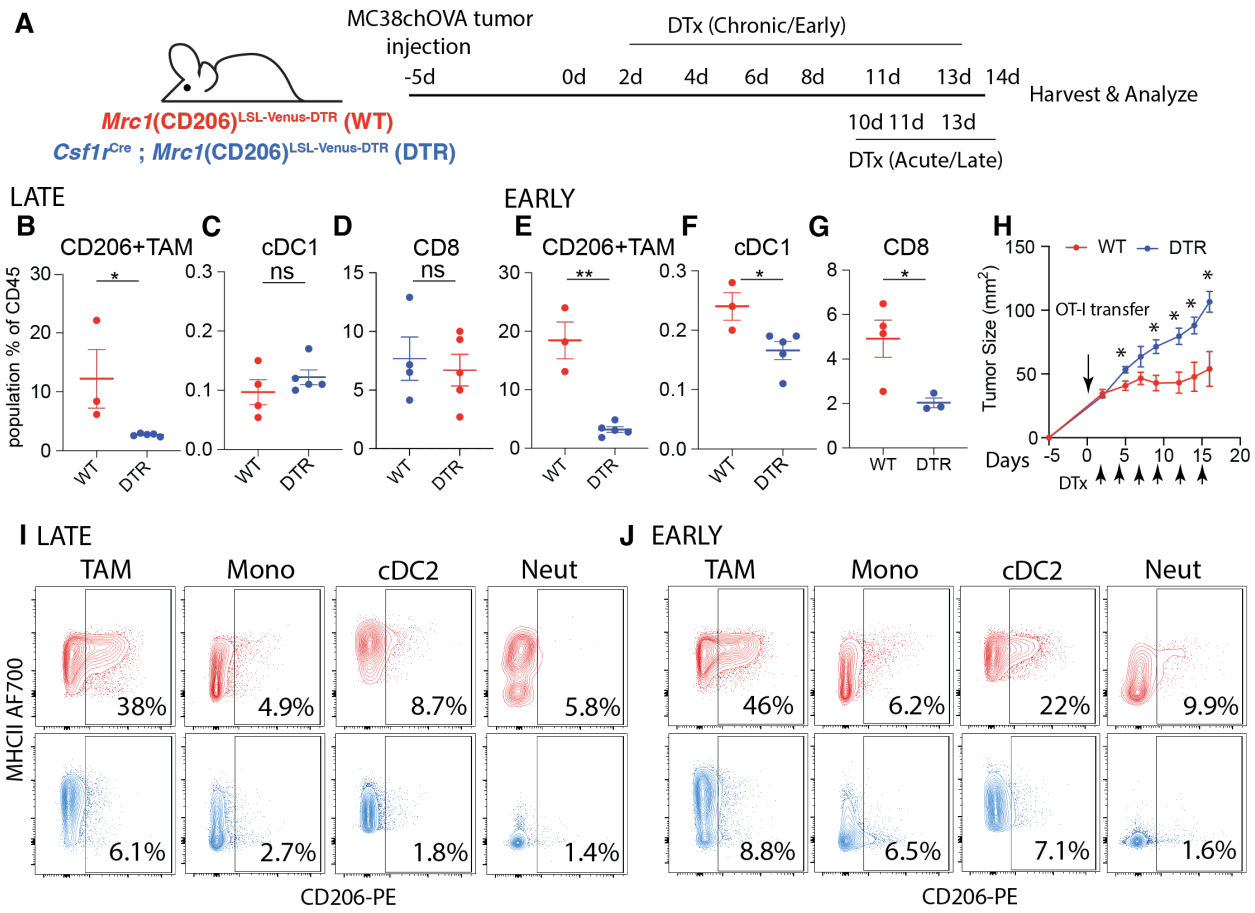

Fig. 4: CD206+ TAM depletion disrupts the cDC1:CD8 module and attenuates T cell-mediated tumor control in an immune-responsive tumor model: (A) Schematic representation of the experimental setup for early and late CD206+ TAM depletion in MC38chOVA tumors using *Csf1r*^{Cre}; *Mrc1*$^{LSL\text{-}Venus\text{-}DTR}$ mice; Relative abundance of **(B, E)** CD206+ TAMs, **(C, F)** cDC1s and **(D, G)** CD8 T cells as a percentage of CD45+ cells with late and early depletion regimens respectively; **(H)** tumor growth kinetics of MC38chOVA tumors in WT and DTR mice with DTx treatment beginning 2d post OT-I adoptive transfer at Day 0; Representative flow cytometry plots showing CD206 vs. MHCII expression in different myeloid subsets in WT (red) and DTR (blue) mice in the (**I**) late and (**J**) early depletion regimens; **p<0.01, *p <0.05, ns = no significance by Mann-Whitney U test t-tests.

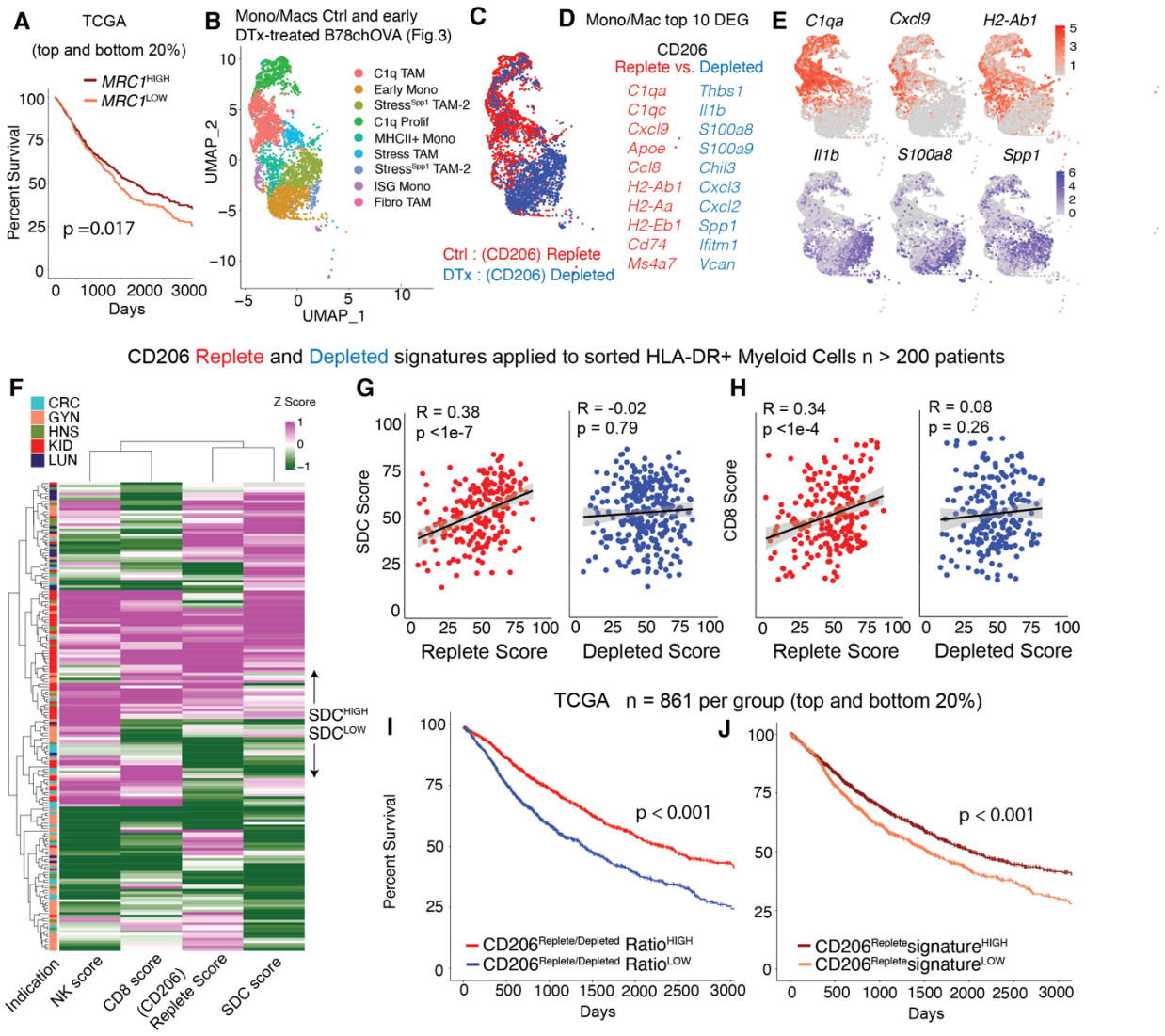

Fig. 5: CD206^{Replete} Mono/Mac signature associates with anti-tumor immunity in human cancers: (A) Kaplan-Meier survival curves of patients in TCGA grouped by the expression of *MRC1* gene; **(B)** UMAP representation of the Mono/Mac subsets and **(C)** overlay of the CD206 Replete (Ctrl) and Depleted (DTx) groups on the UMAP from spatial scSeq described in Fig. 3; **(D)** Top 10 genes from Differential Gene Expression (DGE) of Mono/Macs in the Ctrl vs. DTx treated conditions, which were used to generate CD206 Replete and CD206 Depleted Mono/Mac signature scores **(F)** Heatmap of z-scored CD206^{Replete}, CD8, NK and Stimulatory dendritic cell (SDC) score, calculated from sorted immune compartments, as previously described (*23*); Scatter plots of the Myeloid-specific CD206 Replete and Depleted score per patient with the **(G)** stimulatory dendritic cell (SDC) score and (**H**) CD8 T cell score (Pearson R and p value for the null hypothesis that there is not a correlation are noted); Kaplan-Meier survival curves of patients grouped by the value of the (**I**) CD206^{Replete}: CD206^{Depleted} signature ratio and (**J**) CD206^{Replete} signature in TCGA, p values for the log-rank test are noted in (A, I, J).

Supplementary Materials for

Critical role of CD206+ macrophages in promoting a cDC1-NK-CD8 T cell anti-tumor immune axis

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629 **Materials and Methods:**

630 **Mice:** All mice were treated in accordance with the regulatory standards of the National Institutes
631 of Health and American Association of Laboratory Animal Care and were approved by the UCSF
632 Institution of Animal Care and Use Committee. *Mrc1*(CD206)$^{LSL\text{-}Venus\text{-}DTR}$ mice in the C57BL6/J
633 background were custom-generated from Biocytogen Inc. and then maintained heterozygous
634 (bred to C57BL6/J wild type mice) at the UCSF Animal Barrier facility under specific pathogen-
635 free conditions. C57BL6/J, C57BL6/J CD45.1 (B6.SJL-Ptprc^{a} Pepc^{b}/BoyJ), OT-I (C57BL/6-
636 Tg(TcraTcrb)1100Mjb/J), Csf1r^{Cre} (C57BL/6-Tg(Csf1r-cre)1Mnz/J) mice were purchased for use
637 from Jackson Laboratories and maintained in the same facility in the C57BL6/J background. For
638 adoptive transfer experiments, CD45.1^{het}; OT-I^{het} (denoted simply as CD45.1; OT1) mice were
639 used. Mice of either sex ranging in age from 6 to 14 weeks were used for experimentation.

640

641 **Depletion of select immune cell populations:** For depletion of CD206-expressing
642 macrophages, 500ng (20ng/g body weight, assuming an average 25g weight for each mouse)
643 diphtheria toxin (DTx; List Biological Laboratories) in 100µL 1X PBS was injected intraperitoneally
644 into each mouse – for both *Csf1r*^{Cre}; *Mrc1*(CD206)$^{LSL\text{-}Venus\text{-}DTR}$ (DTR) and *Mrc1*(CD206)$^{LSL\text{-}Venus\text{-}DTR}$
645 (WT) groups - at every time point. For the early depletion regime, injections were started 2 days
646 after adoptive transfer of T cells and continued every 2-3 days till endpoint, while for the late
647 depletion regime, injections began at d10 after T cell injection and continued till endpoint. For
648 testing the effects of DTx in tumor-free tissue, similar dosing of DTx as the early depletion regime
649 was implemented without tumor injection, and the skin (ectopic tumor site) and skin-draining
650 lymph nodes were isolated for analysis. Mice were found to be healthy and without frank health
651 issues with 6 doses of 500ng DTx (early depletion regime), but were monitored nevertheless
652 throughout the experiment, as per IACUC guidelines.

653 For depletion of neutrophils, mice were treated with 200µg/dose of anti-Ly6G antibody (Clone
654 1A8, InvivoMAb) in PBS intraperitoneally every 2-3 days starting one dose after the beginning of

655 DTx treatment and coincident with DTx treatment thereafter. Control mice were similarly treated
656 with the corresponding isotype control antibody (Clone 2A3, InvivoMAb).
657
658 **Mouse tumor digestion and flow cytometry:** Tumors from mice were processed to generate
659 single cell suspensions as described previously(*18*). Briefly, tumors were isolated and
660 mechanically minced on ice using razor blades, followed by enzymatic digestion with 200 µg/mL
661 DNAse (Sigma-Aldrich), 100U/mL Collagenase I (Worthington Biochemical) and 500U/mL
662 Collagenase IV (Worthington Biochemical) for 30 min at 37°C while shaking. Digestion was
663 quenched by adding excess 1X PBS, filtered through a 100µm mesh, spun down and red blood
664 cells were removed by incubating with RBC lysis buffer (155 mM NH_4Cl, 12 mM $NaHCO_3$, 0.1 mM
665 EDTA) at room temperature for 10 mins. The lysis was quenched with excess 1X PBS, spun down
666 and resuspended in FACS buffer (2mM EDTA + 1% FCS in 1X PBS) to obtain single cell
667 suspensions. Similarly, tumor draining lymph nodes (dLN) were isolated and mashed over 100µm
668 filters in PBS to generate single cell suspensions. For counting absolute numbers of cells,
669 CountBright Absolute Counting Beads were added to the cell suspensions prior to staining, while
670 noting the total weight of the tumor and the fraction of the total tumor cell digest used for staining.
671
672 For each sample, 2.5-3 million cells/sample were stained in a total of 50µL of antibody mixture for
673 flow cytometry. Cells were washed with PBS prior to staining with Zombie NIR Fixable live/dead
674 dye (1:500) (Biolegend) for 20 min at 4°C. Cells were washed in FACS buffer followed by surface
675 staining for 30 min at 4°C with directly conjugated antibodies diluted in FACS buffer containing
676 1:100 anti-CD16/32 (Fc block; BioXCell) to block non-specific binding. Antibody dilutions ranged
677 from 1:100-1:400, optimized separately. After surface staining, cells were washed again with
678 FACS buffer. For intracellular staining, cells were fixed for 20 min at 4°C using the IC Fixation
679 Buffer (BD Biosciences) and washed in permeabilization buffer from the FoxP3 Fix/Perm Kit (BD
680 Biosciences). Antibodies against intracellular targets were diluted in permeabilization buffer

681 containing 1:100 Fc Block and cells were incubated for 30 min at 4°C followed by another wash
682 prior to readout on a BD LSRII or Fortessa Cytometer.
683
684 **Processing and flow cytometry analysis of other mouse organs:** To phenotype cells from
685 lymphoid organs, inguinal, axillary and brachial (tumor-draining) lymph nodes were isolated, pried
686 open with tweezers (lymph nodes) or cut into small pieces (spleen) and digested with the same
687 digestion cocktail as above, intermittently pipetting with cut P1000 pipette tips to enhance
688 mechanical digestion. The resulting suspensions were then filtered using 100μm filter, washed
689 with 1X PBS to generate single cell suspensions. For splenic digests, RBC lysis was performed
690 as described above before staining for flow cytometry.
691 For lung digests both lobes were isolated, cut into small pieces with scissors and minced by using
692 gentleMACS dissociator (Miltenyi Biotec) in RPMI. Next, the mixture was spun down and
693 resuspended in the digestion mixture described above and allowed to digest with shaking at 37°C
694 for 20 mins, following which, the remaining tissue was either minced again using the gentleMACS
695 dissociator and/or directly mashed over a 100μm filter in FACS buffer to generate a single cell
696 suspension, ready to be processed for staining and flow cytometry.
697 Skin digestion was done as previously described(*37*). Briefly, mice were shaved and depilated
698 prior to removal of dorsal skin. The skin was then rid of fat, minced with scissors and razor blade
699 in the presence of 1 ml of digest media (2 mg/ml collagenase IV (Roche), 1 mg/ml hyaluronidase
700 (Worthington), 0.1 mg/ml DNase I (Roche) in RPMI-1640 (GIBCO). The minced skin was then
701 moved to a 50 ml conical with 5 ml additional digest solution and incubated at 37°C for 45 min
702 with shaking and intermittent vortexing before being washed and passed through a 70μm strainer
703 prior to staining.
704
705 **Flow cytometry Data Analysis:** Analysis of flow cytometry data was done on FlowJo and later
706 plotted on GraphPad Prism or R. Relative MFI of the Venus reporter was calculated by subtracting

707 the background average MFI of the same channel in WT samples from those in each DTR sample.
708 For analysis of a shift in relative abundance of a population x (Fig. 2), the $\log_2$ (% x of CD45 in
709 WT/ % x of CD45 in DTR) was calculated and plotted as a heatmap, such that positive values
710 indicate depletion and negative values indicate enrichment.
711
712 **Tumor injections and adoptive transfer of CD8 T cells into tumors:** The B78chOVA and
713 MC38chOVA cancer cell lines, as previously described(*11, 18*), were generated by incorporating
714 the same mcherry-OVA construct used to establish the PyMTchOVA spontaneous mouse
715 line(*38*). For tumor injections, the corresponding cells were grown to near confluency (cultured in
716 DMEM with 10% FCS (Benchmark) and 1% PSG (Gibco)) and harvested using 0.05% Trypsin-
717 EDTA (Gibco) and washed 3x with PBS (Gibco). The number of cells to be injected per mouse
718 was resuspended in PBS to a final volume of 50μL per injection. The suspension was injected
719 subcutaneously into the flanks of anesthetized and shaved mice. Tumors were allowed to grow
720 for 14–21 days unless otherwise noted, before tumors and tumor-draining lymph nodes were
721 harvested for analysis. CD8 T cells were isolated from CD45.1;OT-1;Cd69-TFP mice using the
722 EasySep Negative Selection Kit (Stem Cell Bio), resuspended in 1X PBS at 10X concentration
723 100μL was injected into each tumor-bearing mice. For B78chOVA 1 million and for MC38chOVA
724 tumors, 200,000 CD8 T cells were injected retro-orbitally into each mouse either 5d (B78chOVA),
725 7d (MC38chOVA) post tumor injection. Tumor measurements were done by measuring the
726 longest dimension (length) and approximately perpendicular dimension (width) using digital
727 calipers, rounded to one decimal place each. For experiments using the transgenic PyMTchOVA
728 strain, mammary tumor-bearing females in the age range of 15 to 24 weeks were used when mice
729 developed at least 2 palpable tumors.
730
731 **Spatial single cell RNA Sequencing and Analysis:** Spatial scSeq of immune cell populations
732 at the tumor edge, interface and interior zones was performed using ZipSeq, as previously

733 described(*11*), with the additional condition of DTx treatment integrated into the dataset. Briefly,
734 B78chOVA tumors subcutaneously grown in Csf1rCre; CD206$^{LSL\text{-}Venus\text{-}DTR}$ mice d12 post adoptive
735 transfer of 1 million CD2dsRed; OT-I CD8 T cells with (DTx) and without (Control) DTx treatment
736 (early depletion regime) were harvested and sliced into 160µm slices using a Compressotome
737 (Precisionary Instruments VFZ-310-0Z). Imaging, spatial barcoding, subsequent digestion,
738 sorting, encapsulation (10X Genomics) and library construction, CellRanger processing and
739 alignment were performed as described previously(*11, 19*). The two separate sequencing runs
740 (Control and DTx) were assembled and integrated into a single data structure using Harmony(*39*).
741 The final object underwent scaling and then scoring for cell cycle signatures (S and G2M scores
742 as computed using Seurat's built-in CellCycleScoring function. The object then underwent
743 regression for cell cycle effects (S and G2M score as described in the Seurat vignette) and percent
744 mitochondrial reads before PCA.
745
746 Relative abundance from scSeq data was calculated by: log2 (% of each cluster (cell type) within
747 a tumor region (Edge, Mid, Inner) in the Ctrl / (% of the same cluster in the same region in the
748 DTx treated group), thereby yielding positive values for depletion and negative values for
749 enrichment. While abundances were calculated with the broad clusters from the overall object,
750 the lymphoid clusters were isolated to a separate object, re-clustered to further probe for individual
751 gene expression (*Cxcr3, Flt3l, Xcl1*) in the resulting subsets.
752
753 **Transwell Assay of CD8 T cell migration:** For transwell assays, subcutaneously injected
754 B78chOVA tumors grown for 14 days and then harvested, digested, and sorted for CD206+ vs.
755 CD206- TAMs. 3 days before the sort, CD8 T cells from a B6 mouse were harvested and
756 stimulated in vitro with anti-CD3/anti-CD28 Dynabeads (Thermo Fisher) for 24h, taken off the
757 beads and rested in 10U/mL IL-2 for an additional 48h to produce effector-like CD8 T cells. Post-
758 sort, 500,000 activated T cells were plated in 75µL T cell media (RPMI + 10% FCS + 50µM β -

759 marcaptoethanol) on top of a 5μm transwell insert (Corning), allowed to settle for 30mins and
760 subsequently, 10,000 sorted CD206-, CD206+ TAMs or no TAMs were added to the bottom well
761 to induce T cell migration. Cells at the bottom were collected at 3h, mixed with CountBright
762 absolute counting beads, stained and analyzed by flow cytometry to quantify the number of CD8
763 T cells migrated. Total number of CD8 T cells migrated in each condition was normalized to the
764 average number of cells migrated in the no TAM condition.
765
766 **Human tumor samples:** All tumor samples were collected with patient consent after surgical
767 resection under a UCSF IRB approved protocol (UCSF IRB# 20-31740), as described
768 previously(*23*). In brief, freshly resected samples transported in ice-cold DPBS or Leibovitz's L-
769 15 medium before digestion and processing to generate a single-cell suspension. The five most
770 well-represented cancer indications in this collection were included in the cohort: Colorectal
771 cancer (CRC), gynecological cancers (GYN), head and neck cancer (HNSC), kidney cancer
772 (KID), lung cancer (LUNG). Clinical data including survival of patients were obtained through
773 regular clinical follow-up at UCSF.
774
775 **Transcriptomic analysis of human tumors:** All tumor samples were collected under the UCSF
776 Immunoprofiler project as described(*23*). Briefly, tumor samples were thoroughly minced with
777 surgical scissors and transferred to GentleMACS Tubes containing 800 U/ml Collagenase IV and
778 0.1 mg/ml DNase I in L-15/2% FCS per 0.3 g tissue. GentleMACS Tubes were then installed onto
779 the GentleMACs Octo Dissociator (Miltenyi Biotec) and incubated for 20 min (lymph node) or
780 35 min (tumor) according to the manufacturer's instructions. Samples were then quenched with
781 15 mL of sort buffer (PBS/2% FCS/2mM EDTA), filtered through 100μm filters and spun down.
782 Red blood cell lysis was performed with 175 mM ammonium chloride, if needed. Freshly digested
783 tumor samples were sorted by FACS into conventional T cell, Treg, Myeloid, tumor and in some
784 cases, stromal compartments and bulk RNA-seq was performed on sorted cell fractions. mRNA

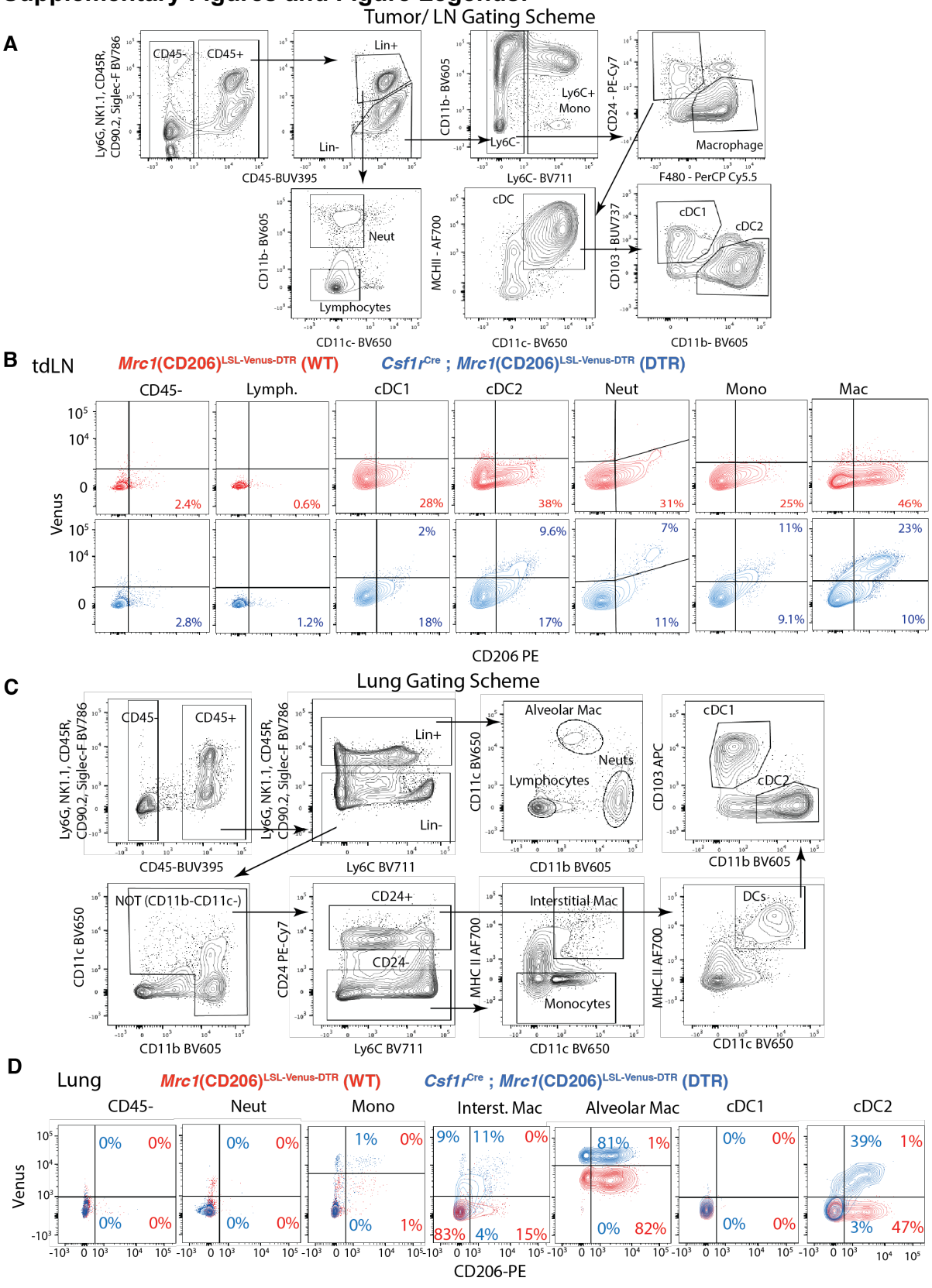
785 was isolated from sorted fractions and libraries were prepared using Illumina Nextera XT DNA
786 Library Prep kit. The libraries were sequenced using 100bp paired end sequencing on HiSeq4000.
787 The sequencing reads we aligned to the Ensembl GRCh38.85 transcriptome build using
788 STAR(*40*) and gene expression was computed using RSEM(*41*). Sequencing quality was
789 evaluated by in-house the EHK score, where each sample was assigned a score of 0 through 10
790 based on the number of EHK genes that were expressed above a precalculated minimum
791 threshold. The threshold was learned from our data by examining the expression distributions of
792 EHK genes and validated using the corresponding distributions in TCGA. A score of 10
793 represented the highest quality data where 10 out of 10 EHK genes are expressed above the
794 minimum threshold. The samples used for survival analysis and other gene expression analyses
795 had an EHK score of greater than 7 to ensure data quality. Ensemble gene signatures scores
796 were calculated by converting the expression of each gene in the signature to a percentile rank
797 among all genes and then determining the mean rank of all the genes in the signature (*17*). The
798 corresponding gene list for obtaining the stimulatory dendritic cell score is as described
799 before(*10*).
800
801 **TCGA analyses**. Survival analyses using the TCGA dataset was performed using the TCGA sub-
802 cohort described in(*23*). Briefly, tumor RNAseq counts and TPM along with curated clinical data
803 for 13 cancer types (BLCA, COAD, GBM, GYN (grouping OV, UCEC and UCS), HNSC, KIRC,
804 LIHC, LUAD, PAAD, SARC and SKCM) was filtered down to include primary solid tumors and
805 metastatic samples only, to parallel the IPI cohort samples. This reduced the TCGA sample set
806 to 4341 tumor samples. $CD206^{Replete}$ gene scores were generated by first normalizing (using
807 percentiles) the expression values of each gene composing the signature across all patients,
808 followed by averaging these normalized values for each patient. The same method was used for
809 deriving $CD206^{Depleted}$ gene scores and we then calculated the ratio of $CD206^{Replete}/CD206^{Depleted}$
810 gene scores by dividing each score value for each patient. For survival analysis, patients were

split into either $CD206^{Replete}$ gene score ^{HIGH} vs ^{LOW} (top/bottom 20% respectively, n=861) or ($CD206^{Replete}$:$CD206^{Depleted}$ gene signature ratio)^{HIGH} vs ($CD206^{Replete}$:$CD206^{Depleted}$ gene signature ratio)^{LOW} (top/bottom 20% respectively, n=861) and analyzed using a log-rank test.

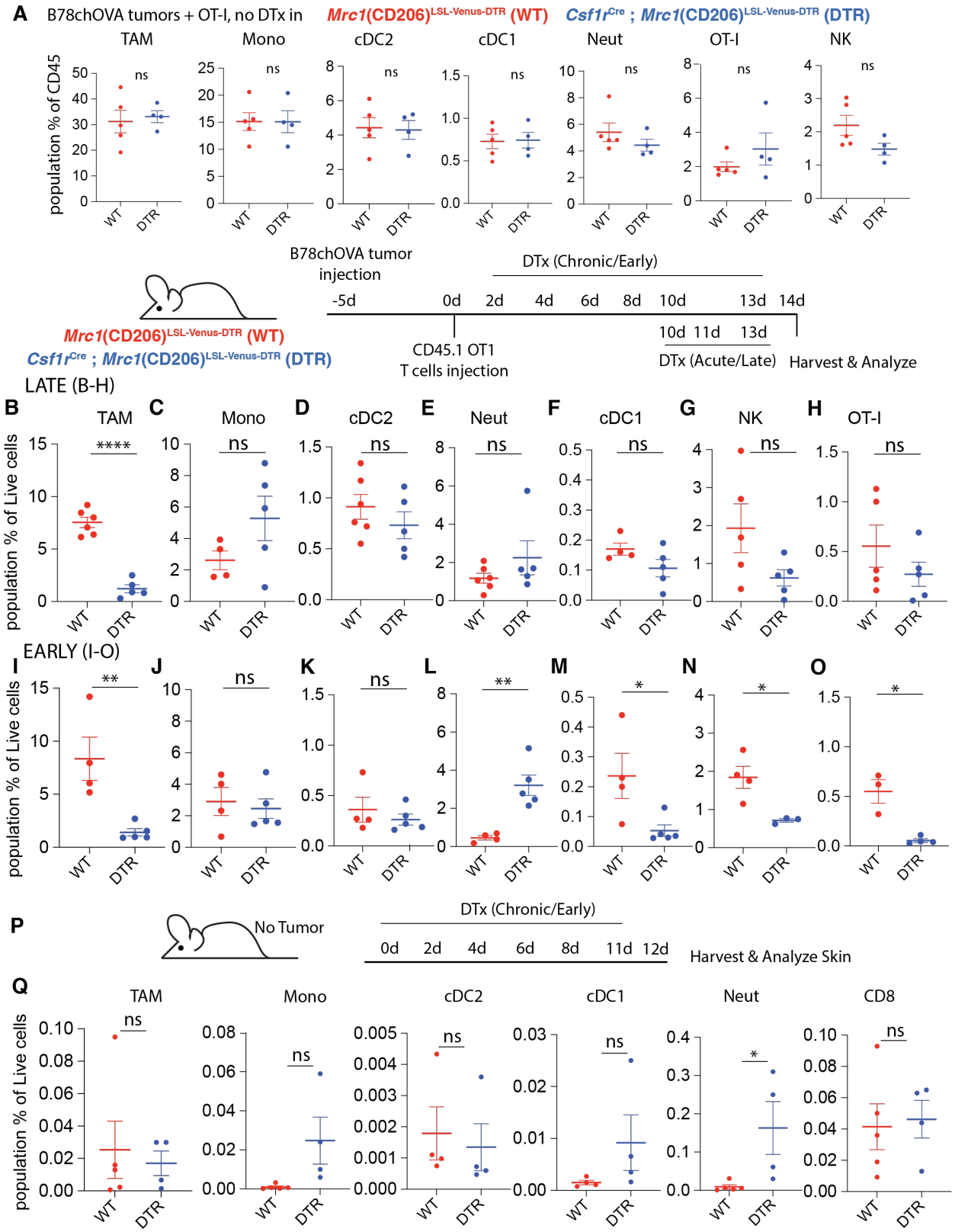
Two-photon imaging of tumor slices: Tumor slices (adjacent to the ones used for spatial barcoding by ZipSeq) were fixed in 2% paraformaldehyde (PFA; Sigma), washed and left overnight in 1X PBS before imaging on a custom-made 2-photon microscope as previously described(*10*) to visualize the Venus reporter and CD2dsRed marked CD8 T cells and fibrous collagen by second harmonic generation (SHG). Dual laser excitations at 800nm and 950nm were used to excite the requisite fluorophores.

Statistical Analysis: Statistical analysis was done in GraphPad Prism or in R. For testing null hypothesis between two groups, either Student's t tests and or the non-parametric Mann-Whitney U tests were used, depending on the number and distribution of data points. Likewise, for testing null hypotheses among 3 or more groups, ANOVA or non-parametric tests were performed, followed by post-hoc test, correcting for false discovery rates (threshold = 0.05) in multiple comparisons. Unless otherwise mentioned, data are representative of at least 2 independent experiments.

Supplementary Figures and Figure Legends:



832 **Fig. S1:** Representative flow cytometry gating scheme to identify myeloid cells and lymphocytes
833 from **(A)** tumor and tdLN and **(C)** lung;Flow cytometry plots showing reporter (Venus) and CD206
834 expression in different immune cells in (**B**) d18 B78chOVA tdLN and (**C**) lung in WT (red; *Mrc1*$^{LSL\text{-}Venus\text{-}DTR}$)
835 and DTR (blue; *Csf1r*^{Cre}; *Mrc1*$^{LSL\text{-}Venus\text{-}DTR}$) mice.


A
B78chOVA tumors + OT-I, no DTx in
Mrc1(CD206)LSL-Venus-DTR (WT)
Csf1rCre ; Mrc1(CD206)LSL-Venus-DTR (DTR)
TAM
Mono
cDC2
cDC1
Neut
OT-I
NK
population % of CD45
ns
WT
DTR
B78chOVA tumor injection
DTx (Chronic/Early)
-5d
0d
2d
4d
6d
8d
10d
13d
14d
10d 11d 13d
CD45.1 OT1 T cells injection
DTx (Acute/Late)
Harvest & Analyze
Mrc1(CD206)LSL-Venus-DTR (WT)
Csf1rCre ; Mrc1(CD206)LSL-Venus-DTR (DTR)
LATE (B-H)
B
C
D
E
F
G
H
population % of Live cells
****
EARLY (I-O)
I
J
K
L
M
N
O
**
*
P
No Tumor
DTx (Chronic/Early)
0d 2d 4d 6d 8d 11d 12d
Harvest & Analyze Skin
Q
CD8

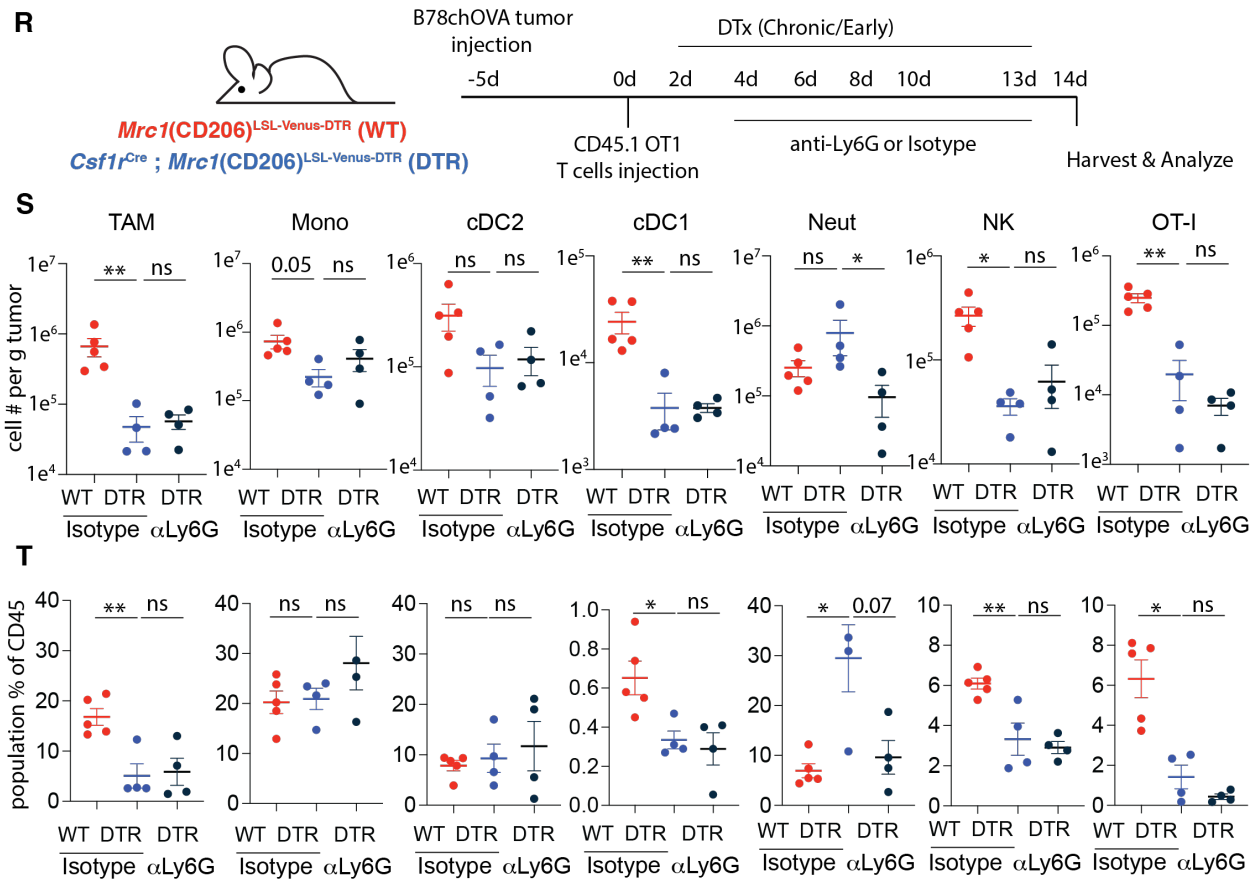
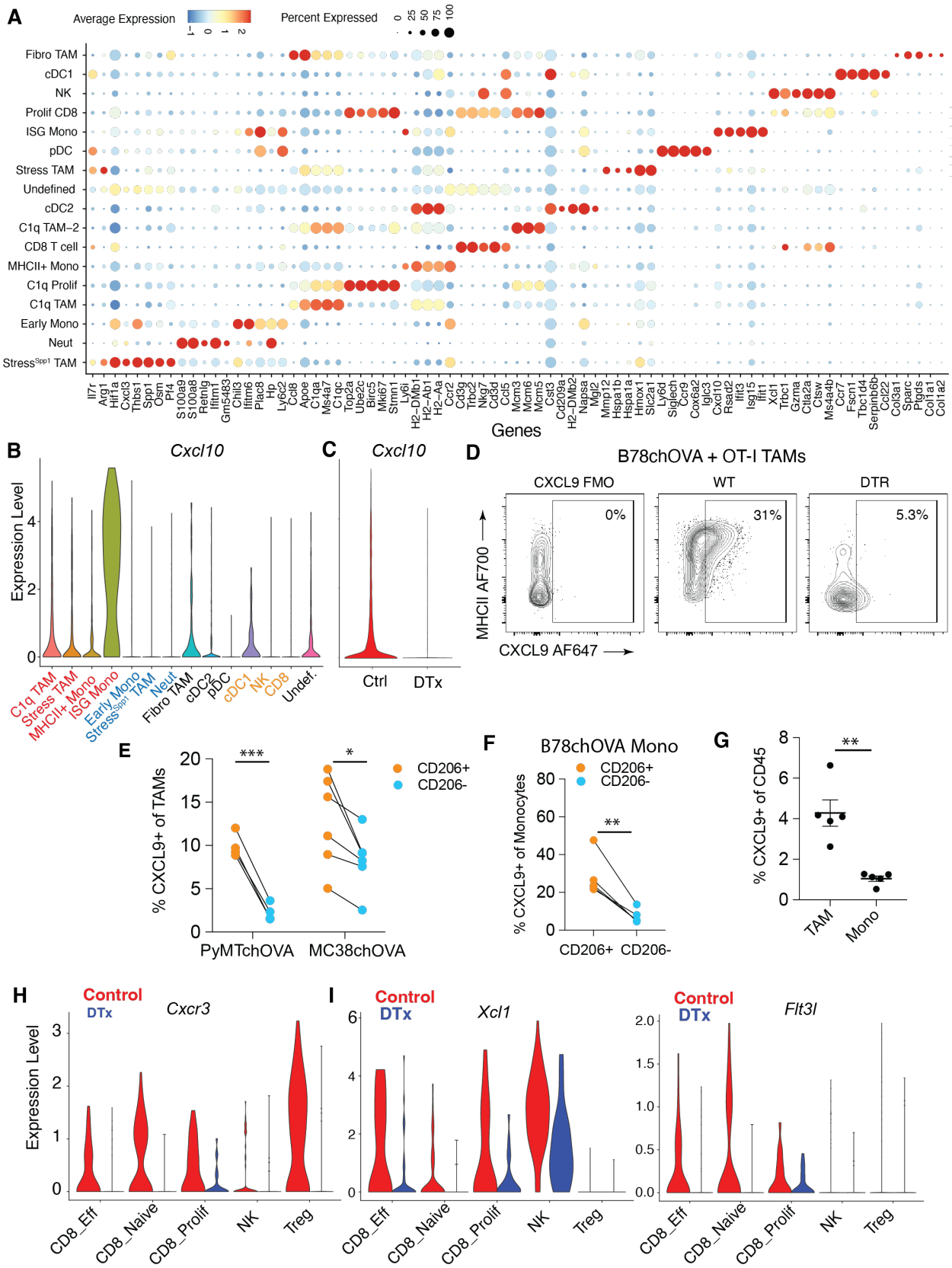
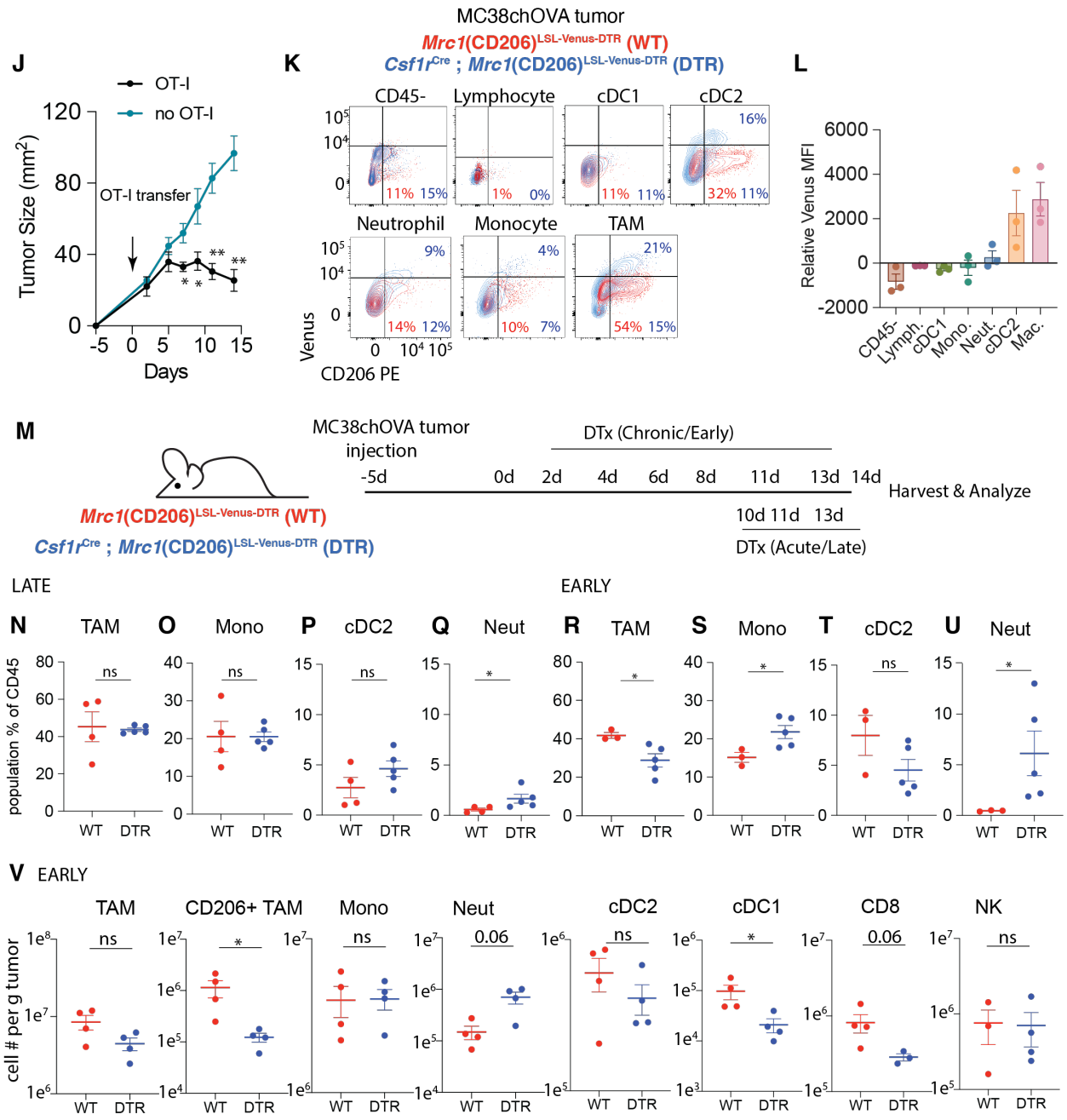


Fig. S2: (A) Relative abundance of different immune populations as a percentage of CD45+ cells in *Mrc1*$^{\text{LSL-Venus-DTR}}$ (WT) and *Csf1r*^{Cre}; CD206$^{\text{LSL-Venus-DTR}}$ (DTR) mice with B78chOVA tumors (day-5), OT-I adoptive transfer (day 0) and harvest at d14 without DTx administration; Schematic representation of the experimental setup for tumor injection, OT-I T cell adoptive transfer, early and late diphtheria toxin administration and analysis; Relative abundance of different immune populations as a percentage of live cells with **(B-H)** late and **(I-O)** early depletion regimens. (**P**) Schematic representation of the experimental setup for analysis of skin in *Mrc1*$^{\text{LSL-Venus-DTR}}$ (WT; red) and *Csf1r*^{Cre}; *Mrc1*$^{\text{LSL-Venus-DTR}}$ (DTR; blue) mice with DTx administration; **(Q)** Relative abundance of different immune populations in the skin as a percentage of live cells; (**R**) Schematic representation of the experimental setup for B78chOVA tumor injection, OT-I T cell adoptive transfer, and early diphtheria toxin administration with either isotype control or anti-Ly6G antibody treatment and analysis; Abundance of different immune populations as (**S**) cells per g of tumor and **(T)** percentage of CD45+ cells in WT and DTR mice. ****p<0.0001, **p<0.01, *p <0.05, ns = no significance by Student's t-tests or Mann-Whitney test, or ANOVA with post-hoc test correcting for false discovery (*alpha < 0.05, ** alpha < 0.01). Bar graph data are shown as mean +/- SEM.


A
Average Expression
Percent Expressed
Fibro TAM
cDC1
NK
Prolif CD8
ISG Mono
pDC
Stress TAM
Undefined
cDC2
C1q TAM−2
CD8 T cell
MHCII+ Mono
C1q Prolif
C1q TAM
Early Mono
Neut
Stress Spp1 TAM
Genes
B
Cxcl10
Expression Level
C1q TAM
Stress TAM
MHCII+ Mono
ISG Mono
Early Mono
Stress Spp1 TAM
Neut
Fibro TAM
cDC2
pDC
cDC1
NK
CD8
Undef.
C
Cxcl10
Ctrl
DTx
D
B78chOVA + OT-I TAMs
CXCL9 FMO
WT
DTR
0%
31%
5.3%
MHCII AF700
CXCL9 AF647
E
% CXCL9+ of TAMs
CD206+
CD206-
PyMTchOVA
MC38chOVA
F
B78chOVA Mono
% CXCL9+ of Monocytes
CD206+
CD206-
G
% CXCL9+ of CD45
TAM
Mono
H
Control
DTx
Cxcr3
Expression Level
CD8_Eff
CD8_Naive
CD8_Prolif
NK
Treg
I
Control
DTx
Xcl1
Flt3l


J
OT-I
no OT-I
Tumor Size (mm2)
OT-I transfer
Days
K
MC38chOVA tumor
Mrc1(CD206)LSL-Venus-DTR (WT)
Csf1rCre ; Mrc1(CD206)LSL-Venus-DTR (DTR)
CD45-
Lymphocyte
cDC1
cDC2
Neutrophil
Monocyte
TAM
Venus
CD206 PE
L
Relative Venus MFI
CD45-
Lymph.
cDC1
Mono.
Neut.
cDC2
Mac.
M
MC38chOVA tumor injection
-5d
0d
2d
4d
6d
8d
11d
13d
14d
DTx (Chronic/Early)
Harvest & Analyze
10d 11d 13d
DTx (Acute/Late)
Mrc1(CD206)LSL-Venus-DTR (WT)
Csf1rCre ; Mrc1(CD206)LSL-Venus-DTR (DTR)
LATE
EARLY
N TAM
O Mono
P cDC2
Q Neut
R TAM
S Mono
T cDC2
U Neut
population % of CD45
WT
DTR
V EARLY
TAM
CD206+ TAM
Mono
Neut
cDC2
cDC1
CD8
NK
cell # per g tumor

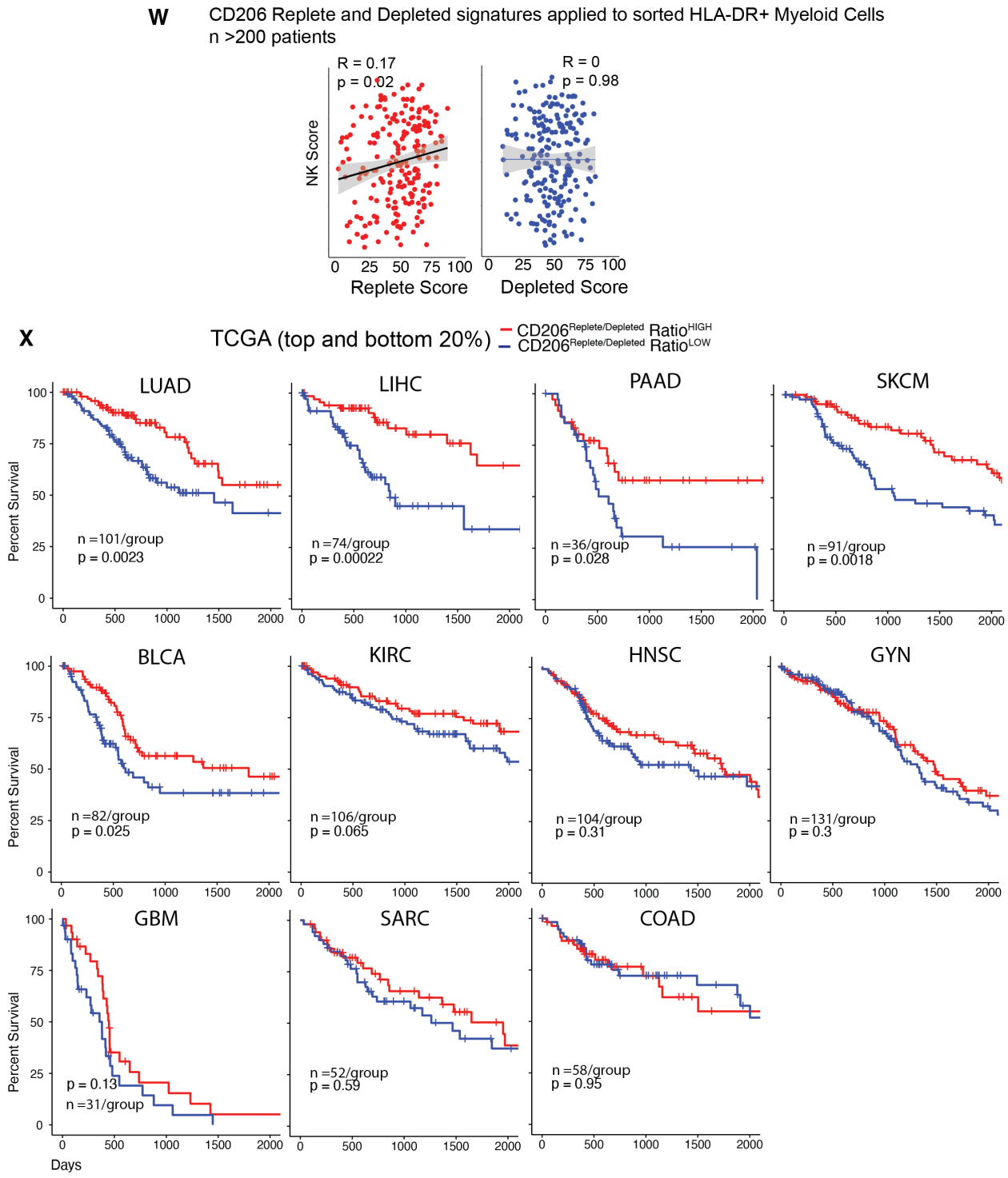


Fig. S3: (A) Dotplot representing top5 differentially expressed genes and select other genes in each immune cell cluster identified from a harmonized dataset of spatially barcoded Control and DTx treated B78chOVA tumors d12 post adoptive transfer of CD2dsRed; OT-I cells; *Cxcl10* expression **(B)** aggregated across treatment conditions by cluster and **(C)** aggregated across clusters by treatment; **(D)** Representative flow cytometry plots showing CXCL9 expression in

B78chOVA TAMs with or without DTx mediated depletion; **(E)** CXCL9 expression in PyMTchOVA and MC38chOVA (both without OT-I adoptive transfer) TAMs split by their CD206 expression; **(F)** CXCL9 expression in OT-I treated B78chOVA (d14 post adoptive transfer) monocytes and **(G)** relative abundance of CXCL9+ TAMs and Monocytes in the same context; Violin plot representing **(H)** *Cxcr3*, **(I)** *Xcl1* and *Flt3l* expression in the lymphoid compartment in Control and DTx treated conditions; **(J)** Representative time course of MC38chOVA tumor size with or without adoptive transfer of OT-I T cells; **(K)** Overlaid flow cytometry plots showing reporter (Venus) and CD206 expression in different immune cells in MC38chOVA tumors in WT (red; *Mrc1*$^{\text{LSL-Venus-DTR}}$) and DTR (blue; *Csf1r*^{Cre}; *Mrc1*$^{\text{LSL-Venus-DTR}}$) mice and **(L)** quantification of relative reporter expression (DTR – WT) in the different subsets. **(M)** Schematic representation of the experimental setup for early and late CD206+ TAM depletion in MC38chOVA tumors using *Mrc1*$^{\text{LSL-Venus-DTR}}$ (WT) and *Csf1r*^{Cre}; *Mrc1*$^{\text{LSL-Venus-DTR}}$ (DTR) mice; Relative abundance of different immune populations as a percentage of CD45+ cells with **(N-Q)** late and **(R-U)** early depletion regimens. **(V)** Abundance of different immune populations as total number of cells per g of MC38chOVA tumor in WT and DTR mice by the early DTx administration regimen; **(W)** Scatter plots of the CD206^{Replete} and CD206^{Depleted} Mono/Mac score per patient with the NK cell score (Pearson R and p value for the null hypothesis that there is not a correlation are noted); **(X)** Kaplan-Meier survival curves of patients grouped by the value of the CD206^{Replete}: CD206^{Depleted} signature ratio (top and bottom 20%) from TCGA split by indications , p values for the log-rank test are noted for each curve in (X) bar graph data are mean +/- SEM, ****$p<0.0001$, **$p<0.01$, *$p<0.05$, ns = no significance by paired ratio t-tests (E, F) or unpaired t-tests or Mann-Whitney test.